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ORIGINAL ARTICLE

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Clinically relevant aminoglycosides can suppress disease-associated premature stop mutations in the *IDUA* and *P53* cDNAs in a mammalian translation system

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Abstract Recent studies have suggested that the use of aminoglycosides to suppress disease-causing nonsense mutations may be a promising new therapy for a large number of genetic diseases. However, gentamicin is currently the only clinically relevant aminoglycoside shown to suppress premature stop mutations in a mammalian system. We compared the ability of the clinically approved aminoglycosides gentamicin, tobramycin, and amikacin to suppress premature stop mutations. Using readthrough reporter constructs as well as mammalian cDNAs containing naturally occurring premature stop mutations, we found that each of these aminoglycosides can suppress many premature stop mutations in a context-dependent manner in a mammalian translation system. Our results indicate that the tetranucleotide termination signal (the stop codon and the nucleotide 3′ of the stop codon) is the primary determinant for aminoglycoside-mediated suppression. The levels of termination suppression achieved by tobramycin were substantially lower than those observed with gentamicin. In contrast, amikacin stimulated suppression in a manner that was generally similar to gentamicin. Amikacin produced higher levels of readthrough than gentamicin at some contexts, demonstrating a unique pattern of context dependence. Experiments with mammalian cDNAs confirmed these results and demonstrated that these aminoglycosides can also suppress disease-associated premature stop mutations previously identified in the *IDUA* gene (responsible for the lysosomal storage disease mucopolysaccharidosis I) and the *P53* gene (associated with many forms of cancer). Taken together, these results suggest that amikacin represents an alternative to gentamicin for suppression therapy in certain contexts, thus providing a means of optimizing the efficacy of aminoglycoside-mediated suppression of premature stop mutations.

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Abbreviations *LTR*: Long terminal repeat · *MPS*: Mucopolysaccharidosis · *REF*: Rat embryo fibroblast

Introduction

Translational fidelity is mediated by recognition of the codon-anticodon complex within the ribosomal aminoac-

yl-tRNA acceptor site (A site). This recognition occurs through contacts between a region of the 16S rRNA called the decoding site and the mRNA [1]. Aminoglycoside antibiotics bind strongly to the prokaryotic decoding site. At high concentrations these compounds inhibit protein synthesis, while at low concentrations they induce translational misreading at both sense and nonsense codons [2]. From kinetic studies comparing cognate and near-cognate aminoacyl-tRNA interactions within the A site, Pape et al. [3] hypothesized that an induced fit normally occurs within the decoding site during aminoacyl-tRNA selection such that GTPase activation and A site accommodation of cognate aminoacyl-tRNAs is more rapid than that of noncognate aminoacyl-tRNAs. They suggested that aminoglycoside binding at the decoding site induces a conformational change that alters the kinetics of cognate and noncognate selection. This is thought to lead to a reduction in proofreading, resulting in an increase in the misincorporation of near-cognate aminoacyl-tRNAs [4]. Nuclear magnetic resonance structures of the aminoglycosides paromomycin and gentamicin bound to a model decoding site RNA indicate that these compounds bind in the major groove of a stem-loop structure within the decoding site through specific hydrogen bonds to both the nucleotide bases and the phosphate backbone [5, 6, 7, 8]. Formation of non-Watson-Crick hydrogen bonds between certain nucleotides within the decoding site creates a pocket for aminoglycoside accommodation, which is further stabilized upon aminoglycoside binding. The prokaryotic A1408 nucleotide is critical to the formation of the aminoglycoside-binding pocket.

The decoding site located in the eukaryotic 18S rRNA is similar to the prokaryotic decoding site, with the main exception being that the prokaryotic A1408 nucleotide is replaced by a G residue. This difference is thought to reduce the affinity of aminoglycoside binding to the eukaryotic decoding site. This idea is supported by studies in *Escherichia coli* where an A1408→G nucleotide change significantly increased aminoglycoside resistance [9]. Dimethylsulfate protection assays also indicated that the aminoglycosides did not bind as effectively to the prokaryotic decoding site containing the G1408 nucleotide. A recent nuclear magnetic resonance structure of paromomycin bound to a model 18S rRNA decoding site suggested that the G1408 nucleotide, as well as differences in the RNA sequence in the lower portion of the decoding site, allow only a shallow pocket for aminoglycoside binding to the eukaryotic decoding site [10]. This is thought to reduce the contacts between the aminoglycoside and the RNA, resulting in a more limited conformational change within the decoding site. The reduced affinity of aminoglycosides for the eukaryotic decoding site has allowed them to be used therapeutically as antibiotics. However, several studies have shown that aminoglycosides can induce low levels of translational misreading in eukaryotic systems [11, 12, 13, 14, 15, 16, 17].

The context surrounding a stop codon can have a strong influence on the efficiency of translation termination. In particular, a significant bias toward a purine nucleotide at the position immediately 3′ of natural stop codons has been shown to occur [18, 19]. Consistent with this observation, several studies have shown that stop codons followed by a purine nucleotide usually promote translation termination more efficiently than stop codons followed by a pyrimidine [20, 21, 22]. These observations have led to the hypothesis that the release factor eRF1 recognizes at least a tetranucleotide termination signal. More recently, studies have shown that the sequence context also influences the ability of aminoglycosides to suppress termination in a mammalian translation system [13, 17]. In those studies several aminoglycosides, including gentamicin, were shown to have distinct differences in their ability to suppress termination as a function of the termination codon and surrounding context.

The suppression of eukaryotic translation termination by aminoglycosides occurs through the insertion of a near-cognate amino acid at the stop codon [23]. Translation can continue in the correct open reading frame once an amino acid is inserted at the stop codon, resulting in the synthesis of a full-length protein. This ability to suppress stop mutations has led to several studies investigating whether aminoglycosides can suppress disease-causing premature stop mutations in human mRNAs. It has been shown that aminoglycosides can suppress premature stop mutations in cell-based models of cystic fibrosis and Hurler syndrome, resulting in the partial restoration of functional protein [24, 25, 26]. In addition, a recent study using a mouse model of Duchenne muscular dystrophy found that aminoglycoside treatment restored a sufficient amount of functional protein to reduce the severity of the disease phenotype [27]. Human trials testing this therapeutic strategy have thus far produced mixed results. Two clinical trials have shown that the administration of gentamicin to cystic fibrosis patients carrying nonsense mutations in the *CFTR* gene can produce a partial restoration of cystic fibrosis transmembrane conductance regulator protein and activity in nasal epithelia [28, 29]. However, another trial carried out with Duchenne and Becker muscular dystrophy patients was unable to document a significant increase in full-length dystrophin in muscle biopsy specimens taken from patients following gentamicin treatment [30].

Currently gentamicin is the only clinically relevant aminoglycoside shown to have the ability to suppress nonsense mutations in patients. The aim of this study was to determine whether other aminoglycosides approved for clinical use can also suppress premature stop mutations in a mammalian translation system. Since it has been demonstrated that the ability of aminoglycosides to suppress termination is influenced by the tetranucleotide termination signal, we examined the ability of these aminoglycosides to suppress termination in all 12 possible tetranucleotide termination contexts. In addition, premature stop mutations in the mammalian *IDUA* gene (responsible for the lysosomal storage disease mucopolysaccharidosis I (MPS-I) and the *P53* gene (associ-

ated with many forms of cancer) were also tested to determine the effectiveness of these aminoglycosides in suppressing premature stop mutations in natural contexts. Our results indicate that gentamicin, tobramycin, and amikacin are each capable of suppressing stop codons in a mammalian translation system in a context-dependent manner. However, tobramycin was much less effective in its ability to suppress stop mutations than the other two compounds tested.

Materials and methods

Construction of readthrough plasmids

The QX(N) and SXA readthrough reporter plasmids were previously constructed by Manuvakhova et al. [13]. The murine *P53* cDNA was provided by Dr. Arnold Levine, Rockefeller University. The *P53* cDNA was subcloned into the *Bam*HI site of the pSP64 expression plasmid (Promega). The following mutations were selected from the human *P53* mutation database [31] and introduced into the corresponding position in the murine *P53* cDNA through site-directed mutagenesis: R210X (UGA); W143X (UGA; UAG; UAA); and Q133X (UAG). The human *IDUA* cDNA was provided by Dr. John Hopwood, Women's and Children's Hospital, North Adelaide, Australia. The *IDUA* cDNA was subcloned into the *Hin*DIII and *Eco*RI sites of pSP64 (Promega). The Hurler syndrome-associated W402X UAG premature stop mutation was introduced into the *IDUA* cDNA using site-directed mutagenesis.

In vitro coupled transcription/translation

To determine the optimal concentrations of the aminoglycosides to use in the mammalian translation system, increasing amounts of each aminoglycoside were added to a rabbit reticulocyte translation system while expressing the QX(N) (UGAC) construct as described previously [13]. The effect of aminoglycoside addition on total protein synthesis was monitored by comparing the total amount of proteins synthesized in the presence and absence of aminoglycosides. A range of aminoglycoside concentrations was then determined that allowed a maximal amount of termination suppression without inhibiting total protein synthesis more than two- to threefold. The optimal concentration determined for each aminoglycoside was: 5 µg/ml for gentamicin, 20 µg/ml for tobramycin, and 40 µg/ml for amikacin. In vitro translation reactions were carried out using the TNT coupled transcription/translation system (Promega). Each reaction was carried out in a total volume of 12.625 µl and contained: 6.25 µl reticulocyte lysate, 0.625 µl 20X TNT reaction buffer, 2.5 µl 20 mM rNTPs, 0.25 µl 1 mM amino acids (minus methionine), 1.0 µl [^{35}S]methionine (11 µCi/µl; NEN-Dupont), 0.25-µl 40 U/µl RNAsin inhibitor (Promega), 0.25-µl 80 U/µl SP6 RNA polymerase (Promega), 1.0 µl 1 mg/ml DNA template, 0.5 µl H_2O, tobramycin, amikacin (Calbiochem), or gentamicin (Gibco). The mixture was incubated for 2 h at 30°C, 12.6 µl of sodium dodecyl sulfate sample buffer was added to each reaction, and the samples were boiled. Then 1–2 µl of each sample was loaded onto a 12.5% sodium dodecyl sulfate polyacrylamide gel. The gel was dried and subjected to PhosphorImager analysis in order to quantitate the amount of truncated and full-length protein species. The percentage of readthrough was then calculated as the amount of full-length protein/(truncated+full-length species) ×100.

Foci assay

Rat embryo fibroblast (REF) cells were obtained from American Type Culture Collection (#CRL-1764) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C with 5% CO_2. An H-*ras* construct containing the dominant-negative mutation G12V was provided by Dr. Michael Cole, Princeton University. A 6.6-kb *Bam*HI fragment containing the H-*ras* cDNA was subcloned into pcDNA3.1 Zeo (Invitrogen) under cytomegalovirus promoter control. The murine genomic *P53* construct, long terminal repeat (LTR)–*P53*val, contained an A135V mutation and was expressed under LTR promoter control. This construct was generously provided by Dr. Michael Ruppert, University of Alabama at Birmingham. The R210X mutation was introduced into LTR-*P53*val by site-directed mutagenesis. The REF cells were transfected at 60–70% confluency in a six well (35 mm^2 per well) culture dish with the indicated plasmids using 2 µg total DNA and 8 µl lipofectamine (Gibco-BRL) for 4 h in the presence of sera. Aminoglycosides were added 24 h after the transfection. Foci began to form approximately 2–3 days after the REF cells reached confluency and the number of foci in each 35 mm^2 well was determined in a blinded manner. A *lacZ* gene was subcloned into the pcDNA3.1 Zeo plasmid and used as a transfection control with β-galactosidase staining.

Results

Readthrough reporter systems

In a recent study we used readthrough reporter plasmids to demonstrate that the nucleotide immediately 3′ of the stop codon has a significant effect on the ability of several aminoglycosides to suppress termination in a mammalian translation system [13]. To examine whether the clinically relevant aminoglycosides tobramycin and amikacin can also suppress translation termination, we again utilized a series of readthrough reporter constructs called the QX(N) and SXA plasmids (Fig. 1).

Both the QX(N) and SXA sets of reporter plasmids contain a 25-kDa open reading frame followed by an exchangeable readthrough cassette. This cassette is followed by another open reading frame encoding an additional 10 kDa of protein. This difference in size between the terminated and readthrough products allowed us to quantitate the level of suppression of each termination signal. In the QX(N) series of plasmids each cassette contains one of the 12 possible tetranucleotide termination signals. The SXA constructs were similar, except that one codon on either side of the stop codon in the readthrough cassette was deleted. The use of these different series of readthrough reporter constructs allowed us to examine the effects of different upstream and downstream sequence contexts on aminoglycoside-mediated suppression of translation termination. In both sets of constructs efficient termination at the tetranucleotide termination signal resulted in the synthesis of a 25-kDa protein, while suppression of the stop codon in the readthrough cassette resulted in a 35-kDa polypeptide.

Suppression of stop codons in the QX(N) plasmids

It was previously shown that the UGAC tetranucleotide termination signal was suppressed efficiently by a variety of aminoglycosides [13]. To initially test the ability of clinically relevant aminoglycosides to suppress termination signals, we utilized the QX(N) construct contain-

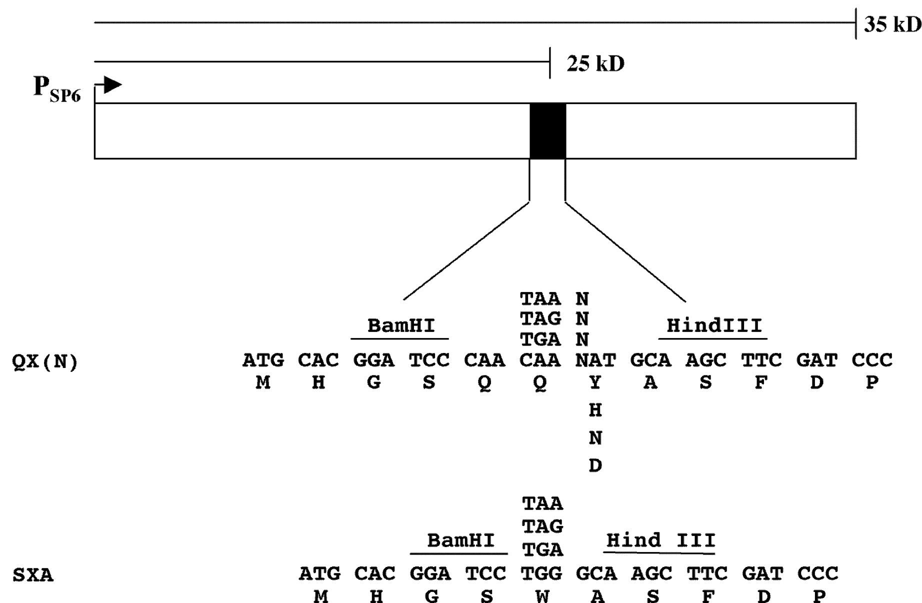

Fig. 1 Readthrough reporter plasmids used in this study

cDNA constructs

Construct													
							TAG						
IDUA W402X	CTG	GAT	GAG	GAG	CAG	CTC	TGG	GCC	GAA	GTG	TCG	CAG	GCC
	L	D	E	E	Q	L	W	A	E	V	S	Q	A
							TGA						
p53 R210X	GAA	GAC	AGG	CAG	ACT	TTT	CGC	CAC	AGC	GTG	GTG	GTA	CCT
	E	D	R	Q	T	F	R	H	S	V	V	V	P
							TAA TAG TGA						
p53 W143X	ACG	TGC	CCT	GTG	CAG	TTG	TGG	GTC	AGC	GCC	ACA	CCT	CCA
	T	C	P	V	Q	L	W	V	S	A	T	P	P
							TAG						
p53 Q133X	CTC	AAT	AAG	CTA	TTC	TGC	CAG	CTG	GCG	AAG	ACG	TGC	CCT
	L	N	K	L	F	C	Q	L	A	K	T	C	P

Table 1 Comparison of aminoglycoside-mediated suppression in the QX(N) context: percentage readthrough (fold-stimulation above background). The values shown are from representative experiments. Measurements were carried out at least three times with each set of tetranucleotide constructs with similar results

Context	No drug	Gentamicin (5 µg/ml)	Tobramycin (20 µg/ml)	Amikacin (40 µg/ml)
UAAA	0.2%	3.0% (15×)	1.6% (6.5×)	2.3% (11.5×)
UAAC	0.7%	5.6% (8×)	2.0% (2.9×)	3.2% (4.6×)
UAAG	0.4%	2.0% (5×)	1.1% (2.8×)	2.7% (6.8×)
UAAU	0.6%	3.0% (5×)	2.5% (4.2×)	3.4% (5.7×)
UAGA	0.4%	7.1% (17.8×)	3.4% (8.5×)	7.5% (18.8×)
UAGC	1.2%	10.5% (8.8×)	3.7% (3.1×)	3.9% (3.3×)
UAGG	0.5%	11.2% (22.4×)	5.1% (10.2×)	15.0% (30×)
UAGU	1.3%	22.7% (17.5×)	2.0% (1.5×)	6.8% (5.2×)
UGAA	2.1%	14.2% (6.8×)	2.5% (1.2×)	14–6% (7×)
UGAC	3.4%	26.5% (7.8×)	3.6% (1.1×)	20.9% (6.1×)
UGAG	1.1%	17.3% (15.7×)	10.6% (9.6×)	14.0% (12.7×)
UGAU	2.0%	12.9% (6.5×)	5.7% (2.9×)	15.2% (7.6×)

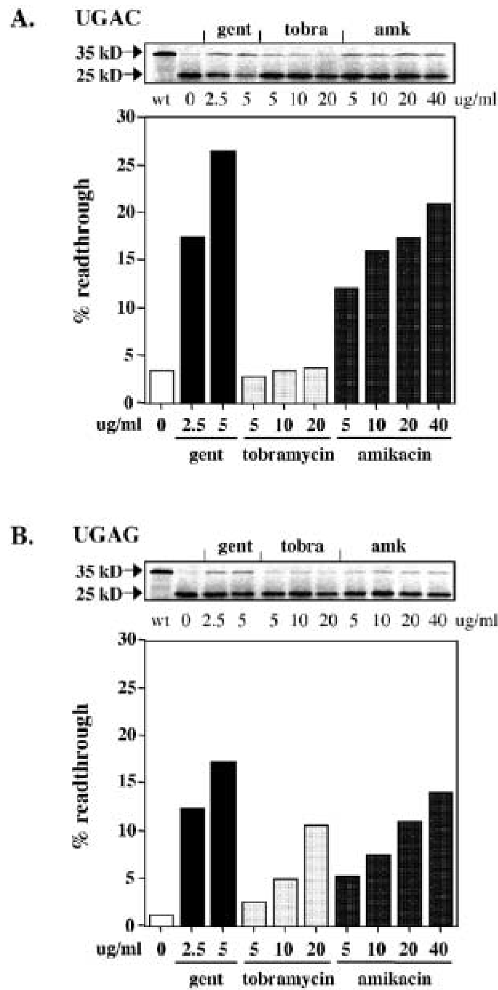

Fig. 2 Aminoglycoside-mediated suppression using the QX(N) plasmid with the UGAC (**A**) or the UGAG termination signal (**B**) in a reticulocyte translation system. *Gent* Gentamicin; *tobra* tobramycin; *amk* amikacin

ing the UGAC tetranucleotide. We found that gentamicin induced suppression to a level of 26.5%, while amikacin stimulated readthrough at a level of 20.9% (Fig. 2A). Surprisingly, tobramycin was unable to suppress the UGAC termination signal. When the QX(N) constructs containing other tetranucleotide termination signals were expressed in the presence of tobramycin, we found that each was suppressed to some degree with the exception of the UGAA and UGAC tetranucleotides (Table 1).

The inability of tobramycin to suppress the UGAA and UGAC contexts above background levels is unlike any other aminoglycoside previously tested in this mammalian translation system. In fact, the UGAA and UGAC tetranucleotides are generally among the termination signals most susceptible to aminoglycoside suppression. Tobramycin was found to suppress the UGAG and UGAU contexts to levels of 10.6% and 5.7%, respectively. The highest suppression induced by tobramycin among all the contexts examined occurred at the UGAG tetranucleotide (Fig. 2B). Suppression of the QX(N) UAG tetranucleotides by tobramycin ranged from 2% to 5.1%. The order of susceptibility as a function of the 3′ nucleotide was UAGG >UAGC~UAGA >UAGU. Readthrough of the QX(N) UAA tetranucleotides mediated by tobramycin ranged from 1.1% to 2.5%, where the pattern of suppression based upon the 3′ nucleotide was UAAU >UAAC >UAAA >UAAG. Overall, these results indicate that tobramycin is capable of suppressing stop codons in a mammalian translation system, but it is less efficient than many other aminoglycosides previously examined [13].

Amikacin was found to suppress all 12 tetranucleotide contexts in the QX(N) constructs to varying extents. The highest level of suppression was detected at the UGA tetranucleotide termination signals, which ranged from 14.0% to 20.9% (Fig. 2, Table 1). The pattern of suppression based upon the 3′ nucleotide context was UGAC >UGAU~UGAA~UGAG. Amikacin was also able to suppress the UAG tetranucleotides at levels that were comparable to those produced by gentamicin. The greatest level of suppression within the UAG constructs was observed with the UAGG construct, where 15% readthrough occurred (a 30-fold increase above background). The other UAG tetranucleotides had levels of suppression ranging from 3.9% to 7.5%, in the order UAGA~UAGU >UAGC. This is a distinctly different pattern of context dependent suppression than was observed with gentamicin, where the greatest amount of suppression was observed at the UAGU termination signal. The suppression levels induced by amikacin at the QX(N) UAA tetranucleotides ranged from 2.3% to 3.4%. These levels were higher than those observed with most other aminoglycosides at the UAA termination signals [13]. The tetranucleotide pattern of suppression observed with amikacin was UAAU~UAAC~UAAG~UAAA.

Suppression of stop codons in the SXA plasmids

In addition to the nucleotide following the stop codon, the context both 5′ and 3′ of the tetranucleotide signal has also been shown to influence the efficiency of translation termination. In order to test the effectiveness of these clinically relevant aminoglycosides in suppressing termination in the presence of different contexts 5′ and 3′ of the tetranucleotide termination signal, another set of readthrough reporter plasmids called the SXA constructs were used (Fig. 1). The SXA constructs are similar to the QX(N) readthrough constructs, except a single codon on either side of the stop codon was removed from the QX(N) construct such that new 5′ and 3′ contexts surround each of the stop codons. As a result, a G residue is located in the fourth position of each tetranucleotide signal in the SXA constructs.

The ability of tobramycin to suppress termination of the UGAG tetranucleotide within the SXA construct was reduced by approximately twofold compared to the same UGAG tetranucleotide within the QX(N) construct (Table 2), while suppression of the UAGG and UAAG tetra-

Table 2 Comparison of aminoglycoside-mediated suppression in various contexts: percentage readthrough (fold-stimulation above background). The values shown are from representative experiments. Measurements were carried out at least three times with each cDNA with similar results

Construct	Context	No drug	Gentamicin (5 μg/ml)	Tobramycin (20 μg/ml)	Amikacin (40 μg/ml)
SXA	UAAG	0.42%	2.7% (6.4×)	2.3% (5.5×)	4.2% (10×)
	UAGG	1.20%	7.4% (6.2×)	5.3% (4.4×)	13.4%(11.2×)
	UGAG	1.80%	11.5% (6.4×)	7.7% (4.3×)	8.0% (4.4×)
IDUA-W402X	UAGG	0.71%	4.7% (6.6×)	2.6% (3.7×)	4.9% (6.9×)
P53-R210X	UGAC	4.30%	20.5% (4.8×)	3.7% (1×)	14.1%(3.2×)
P53-W143X	UAAG	0.50%	4.9% (9.8×)	2.2% (4.4×)	5.2% (10.4×)
	UAGG	0.71%	8.0%(11.3×)	4.6% (6.5×)	6.2% (8.7×)
	UGAG	0.89%	15.1% (17×)	5.8% (6.5×)	14.1% (15.8×)
P53-Q133X	UAGC	1.0%	6.5% (6.5×)	1.7%(1.7×)	2.7% (2.7×)

nucleotides by tobramycin was the same or slightly increased. The suppression induced by amikacin at the UGAG tetranucleotide of the SXA constructs was also about twofold lower than previously observed in the QX(N) constructs, while amikacin-mediated suppression at the UAGG construct was similar to that observed in the QX(N) context. Finally, amikacin suppressed the UAAG tetranucleotide in the SXA context at a level that was 1.6-fold higher than the same tetranucleotide in the QX(N) context (Table 2). These relatively minor differences between the QX(N) and SXA reporter constructs containing the same tetranucleotide termination signals suggest that the tetranucleotide signal itself, rather than more distal sequences, plays the major role in determining the susceptibility of a stop codon to aminoglycoside-mediated suppression.

Suppression of disease-causing premature stop mutations

We next tested whether our readthrough reporter plasmids were accurately representing the susceptibility of different termination signals to aminoglycoside-mediated suppression. To do this we examined the ability of these clinically relevant aminoglycosides to suppress premature stop mutations that have previously been shown to be associated with human diseases. The first example we chose was the human *IDUA* gene, which encodes the lysosomal enzyme α-L-iduronidase. This enzyme cleaves O-glycosidic linkages between specific carbohydrate moieties in glycosaminoglycan (GAG) polysaccharide chains. Without functional α-L-iduronidase, dermatan and heparan sulfate accumulate within the lysosome, resulting in the onset of the lysosomal storage disease MPS-I [32, 33]. The most severe form of MPS-I, Hurler syndrome, is frequently caused by the *IDUA*-W402X mutation, which results in a UAGG premature termination signal. Since we recently demonstrated that the *IDUA*-W402X mutation can be suppressed by gentamicin in primary fibroblasts from a Hurler syndrome patient [26], we next examined the ability of tobramycin and amikacin to suppress this termination signal. We found that tobramycin suppressed the *IDUA*-W402X mutation at a level of 2.6%, approximately twofold lower than the suppression mediated by gentamicin (4.7%) or amikacin (4.9%) (Table 2). These results are similar to those obtained using the QX(N) and SXA constructs, demonstrating that these reporter plasmids provide reasonable estimates of aminoglycoside-mediated readthrough that occurs in authentic mammalian mRNAs.

We next examined the ability of aminoglycosides to suppress mutations within the *P53* tumor suppressor gene. The *P53* gene encodes an important regulator of the G_1 and G_2 cell cycle checkpoints, and is the most commonly mutated gene in many types of cancer. It has been reported that nearly 50% of all tumors contain *P53* mutations [34, 35]. The murine *P53* cDNA was introduced into our in vitro expression plasmid, and several *P53* nonsense mutations previously identified in human tumors were introduced. These mutations included the R210X, Q133X, and W143X mutations, which result in the UGAC, UAGC, and UAGG tetranucleotide termination signals, respectively. For direct comparison of each of the stop codons in an otherwise identical context, the UGAG and UAAG termination signals were also introduced at the W143 position (Fig. 1). These cDNA constructs were expressed in the reticulocyte translation system and tested for aminoglycoside suppression of the stop codons.

We initially examined the ability of tobramycin to suppress the *P53*-R210X UGAC termination signal. As was previously observed with the QX(N) UGAC construct, tobramycin was unable to induce a detectable level of suppression of the *P53*-R210X mutation with the UGAC termination signal (Table 2). The suppression induced by tobramycin at the *P53*-Q133X mutation with the UAGC termination signal was also weak (approx. 1.7%). The *P53*-W143X plasmids containing the UAAG, UAGG, or UGAG termination signals were suppressed by tobramycin at levels of 2.2%, 4.6%, and 5.8%, respectively. As was observed with the QX(N) and SXA plasmids, the level of suppression of each of the *P53* stop mutations by tobramycin was less than was observed with either gentamicin or amikacin.

We found that amikacin was capable of more efficiently suppressing each of the *P53* mutations examined (Table 2). A 14.1% level of amikacin-mediated suppression was observed for the *P53*-R210X mutation containing a UGAC termination signal, compared to a level of 20.5% produced by gentamicin (Table 2). Amikacin was

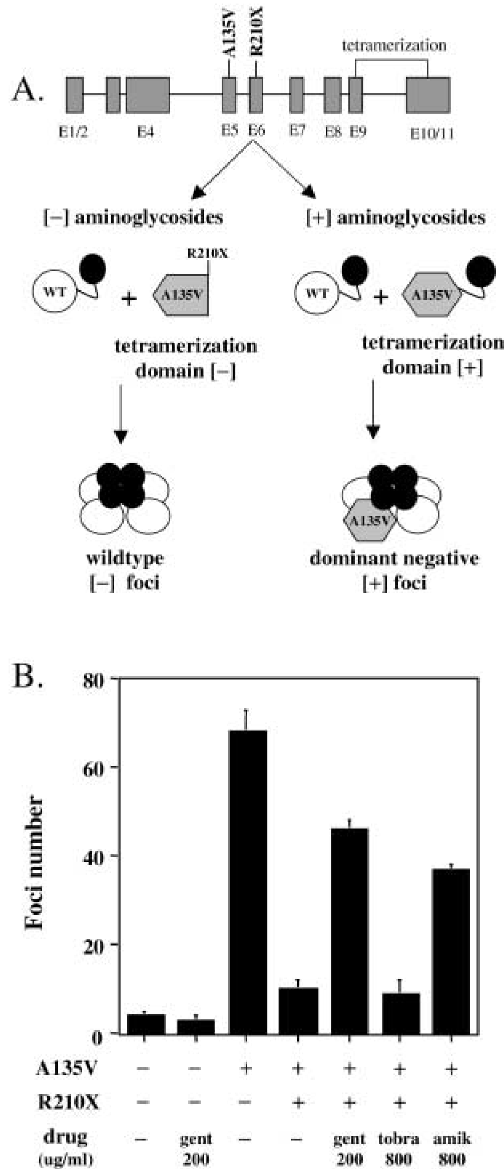

Fig. 3A, B Bioassay to monitor the ability of aminoglycosides to restore *P53* expression in rat embryo fibroblast cells. **A** Schematic of experimental strategy. Rat embryo fibroblasts cotransfected with an activated H-*ras* oncogene and a *P53* gene containing the dominant-negative mutation A135V form foci in culture. In order to test the effectiveness of aminoglycosides in suppressing a *P53* premature stop mutation, the R210X mutation was introduced into the *P53*-A135V construct. The p53 protein functions as a tetramer and subunit assembly occurs via a C-terminus tetramerization domain. Without the tetramerization domain, the *P53*-A135V dominant-negative phenotype is not observed. However, suppression of the R210X mutation will allow the expression of full-length p53 protein, resulting in foci formation. **B** Rat embryo fibroblasts were transfected with the H-*ras* plasmid. Cells were also transfected with either the *P53*-A135V plasmid or the *P53*-A135V/R210X plasmid as indicated. The fibroblasts were grown in the absence or presence of gentamicin, tobramycin, or amikacin and assayed for foci formation. The data are expressed as the average foci number ±standard deviation

able to induce only a 2.7% level of suppression at the Q133X mutation with a UAGC termination signal, while a level of 6.5% was observed in the presence of gentamicin. Amikacin induced a level of suppression very similar to gentamicin (14.1% versus 15.1%, respectively) with the *P53*-W143X mutation containing a UGAG termination signal. The *P53*-W143X mutation with the UAGG termination signal was suppressed at a 6.2% level with amikacin and an 8% level with gentamicin. Amikacin and gentamicin also mediated suppression of the *P53*-W143X mutation containing the UAAG termination signal at similar levels (5.2% and 4.9%, respectively). These results indicate that both gentamicin and amikacin can suppress disease-causing stop mutations in the *P53* gene.

A bioassay for suppression of the P53-R210X mutation

Our results indicate that aminoglycosides can suppress a variety of *P53* stop mutations in the reticulocyte translation system. In order to determine whether aminoglycosides can suppress a naturally occurring *P53* premature stop mutation in cultured mammalian cells a foci assay was developed. Normally p53 functions as a homo-oligomeric protein where association of the subunits occurs through a tetramerization domain located in the C-terminal portion of the p53 protein [36, 37]. Several dominant-negative mutations in the *P53* gene have been reported, including the murine A135V mutation [38, 39, 40]. When a murine *P53* cDNA containing the A135V dominant-negative mutation is expressed in REF cells in the presence of an activated H-*ras* oncogene, foci form within the confluent cell monolayer [41, 42, 43]. We utilized this observation to test whether aminoglycosides can suppress the *P53*-R210X premature stop mutation in REF cells (Fig. 3A). The R210X mutation was introduced into a genomic copy of the murine *P53* gene containing the A135V dominant-negative mutation in exon 5. The R210X mutation lies downstream of the A135V mutation within exon 6, and well upstream of the C-terminal tetramerization domain within exons 9 and 10. Therefore foci formation should occur only if the tetramerization domain is translated as a result of read-through of the R210X mutation.

REF cells were transfected with a plasmid carrying an activated H-*ras* oncogene in conjunction with the indicated *P53* constructs and then grown in the presence or absence of the aminoglycosides gentamicin, tobramycin, or amikacin. REF cells transfected with only the H-*ras* oncogene showed little foci formation both when grown with (3±1) or without (4±1) aminoglycosides (Fig. 3B). Significant foci formation did occur when the REFs were transfected with both the activated H-*ras* oncogene and the *P53* construct containing only the A135V dominant negative mutation (68±5). The REF cells transfected with the H-*ras* construct and a *P53* plasmid containing both the A135V and R210X mutations showed a slight increase in foci formation (10±2), possibly due to endog-

enous readthrough of the R210X mutation or the interaction of the truncated form of the p53 protein with other cellular factors. When the REF cells transfected with these same constructs were incubated in the presence of gentamicin or amikacin, a significant increase in foci formation was observed (46±2 and 37±1, respectively), indicating that these aminoglycosides can also suppress *P53* premature stop mutations in mammalian cells. However, no increase in foci formation was observed in the presence of tobramycin (9±3). These foci assay results are well correlated with the readthrough of the *P53*-R210X mutation observed in the in vitro translation system, since amikacin and gentamicin were both able to suppress the R210X mutation at moderate levels, while tobramycin was ineffective in suppressing the R210X mutation due to the presence of the UGAC termination signal. These results provide the first evidence that these clinically approved aminoglycosides are effective in suppressing premature stop mutations within the *P53* gene.

Discussion

The results of this study indicate that both amikacin and gentamicin, and to a lesser extent tobramycin, have the ability to suppress premature stop mutations in distinct ways as a function of the stop codon and surrounding context in a mammalian translation system. While tobramycin also exhibited weak activity, its ability to suppress stop mutations was significantly less than the other two compounds tested. These findings are consistent with a previous report that multiple factors are responsible for determining the efficiency of aminoglycoside-mediated termination suppression [13]. While the absolute levels of aminoglycoside-induced suppression at the same tetranucleotide termination signals differed among the QX(N), SXA, and mammalian cDNAs by approximately twofold in the current study, the pattern of context-dependent suppression based on the fourth nucleotide of the tetranucleotide termination signal was generally found to be similar for the various contexts tested with each aminoglycoside. A comparison of the QX(N) and SXA reporter constructs containing the UAGG termination signal with the *IDUA*-W402X mutant cDNA carrying the same UAGG termination signal allowed us to examine the contribution of more distal contexts in greater detail. The QX(N) reporter is similar to the *IDUA*-W402X cDNA only at the UAGG tetranucleotide signal, while the stop signal in the SXA construct containing the UAGG termination signal is identical to the *IDUA*-W402X cDNA from the first nucleotide 5′ of the stop codon to the second nucleotide 3′ of the stop codon (a span of six nucleotides). We found that suppression with the *IDUA*-W402X cDNA was reduced approximately twofold with gentamicin, tobramycin, or amikacin, relative to the suppression of the SXA reporter (and reduced two- to threefold compared to the suppression observed with the QX(N) reporter). Based upon previous findings that an extended sequence context can affect the efficiency of translation termination in bacterial, viral, and yeast systems [20, 44, 45, 46, 47], it is likely that an extended context 5′ and 3′ of the stop codon are responsible for some of the differences we observed in the relative level of suppression. However, our results clearly indicate that the tetranucleotide termination signal plays the predominant role in determining the efficiency of aminoglycoside-mediated termination.

The mRNA and aminoacyl-tRNA in the ribosomal A site have been shown to contact many of the same nucleotides within the prokaryotic decoding site as the aminoglycosides [2]. As a result, the mRNA context surrounding the codon within the A site may affect the ability of aminoglycosides to bind to the decoding site directly, or it may limit the conformational change induced in the decoding site by aminoglycosides. It has also been proposed that aminoglycosides may form hydrogen bonds directly with the mRNA molecule in the A site [48]. Thus, the complexity of the context dependence observed in aminoglycoside-mediated suppression may be due to the formation of different hydrogen bonding configurations between the aminoglycoside, the mRNA, and the decoding site RNA, thus influencing conformational transitions within the decoding site RNA. While aminoglycoside binding has been shown to interfere with ribosomal proofreading during aminoacyl-tRNA recognition by stimulating the transition from a “binding” state to a “productive” state [3, 4], it is possible that the bound aminoglycoside may also reduce the efficiency of release factor recognition of the termination signal.

It is important to understand how aminoglycosides differ in their ability to suppress stop codons as a function of the sequence context in order to choose the most efficient aminoglycoside to suppress specific disease-causing mutations. For example, tobramycin was unable to suppress any constructs containing the UGAA or UGAC tetranucleotide signal. In contrast, the UGAA and UGAC tetranucleotides were both suppressed at relatively high levels by amikacin and gentamicin. In the UAA(N) reporter constructs, amikacin suppressed the UAAU termination signal at the highest levels, while gentamicin suppressed the UAAC signal with the greatest efficiency. Another clear difference in the context dependence of aminoglycoside suppression was observed in the relative levels of suppression as a function of the fourth base of the UAG(N) tetranucleotide signals. Tobramycin and amikacin suppressed the UAGG tetranucleotide with the highest efficiency, while gentamicin suppressed the UAGU and UAGC termination signals most efficiently. The pattern of suppression we observed with gentamicin in the UAG(N) series of tetranucleotide termination signals differed from a study conducted by Howard et al. [17], who used a luciferase-based assay to examine aminoglycoside-mediated readthrough in HEK293 cells. Those investigators found that gentamicin suppressed termination at the UAG(N) tetranucleotide sequence in the relative order UAGC>UAGU>UAGA=UAGG. It is likely that these differences are attributable to sequence divergence upstream and down-

stream of the tetranucleotide termination signal in the respective reporter systems.

Even though the structures of the aminoglycosides used in this study are similar, subtle changes appear to strongly influence their ability to suppress translation termination. For example, gentamicin and amikacin were clearly much more efficient at suppressing termination than tobramycin at all contexts studied. This suggests that gentamicin and amikacin are more appropriate for general use in clinical suppression therapy. Our results also indicate that a higher concentration of amikacin is required to achieve a maximal level of readthrough than gentamicin in the mammalian translation system. This higher dose may be feasible for suppression therapy, since higher concentrations of amikacin can be safely administered to humans [49]. At a concentration of 20 μg/ml within the translation system, amikacin was able to suppress most tetranucleotide contexts as effectively, and in some contexts, more effectively than 5 μg/ml gentamicin (Fig. 2 and data not shown). Although a direct clinical comparison of the nephrotoxic effects induced by amikacin and gentamicin has not been made, a study comparing gentamicin- and amikacin-induced nephrotoxicity in rats found that amikacin is less nephrotoxic than gentamicin [50, 51]. In addition, alternative ways of delivering either of these compounds, such as encapsulation within unilammelar liposomes, may also provide a means of further reducing nephrotoxicity while increasing their ability to suppress termination [52, 53]. This study provides a first step in identifying alternative compounds that will allow us to optimally suppress premature stop mutations as a function of the specific stop codon and surrounding context within an mRNA molecule.

Previous studies have shown that aminoglycosides can effectively suppress mutations associated with cystic fibrosis [24, 25, 28, 29], Duchenne muscular dystrophy [27], and Hurler syndrome [26]. The results of this study using the *IDUA* cDNA further support the results of the Hurler syndrome study and also provide the first evidence that aminoglycosides can suppress cancer-associated premature stop mutations in the *P53* tumor suppressor gene. A significant fraction of cancer-associated mutations found in many tumor suppressor genes are nonsense mutations, including 27% of *APC* mutations, 18% of *BRCA1* mutations, 12% of *BRCA2* mutations, 16% of *ATM* mutations, and 10% of *P53* mutations [31, 54]. This suggests that suppression therapy may also be useful in suppressing premature stop mutations in many forms of cancer. Since over 200 different genetic disorders have been reported to result from nonsense mutations [55], a therapeutic approach based on the suppression of stop mutations may also be applicable to the treatment of many other genetic diseases.

References

1. Yoshizawa S, Fourmy D, Puglisi JD (1999) Recognition of the codon-anticodon helix by ribosomal RNA. Science 285:1722–1725
2. Purohit P, Stern S (1994) Interactions of a small RNA with antibiotic and RNA ligands of the 30 S subunit. Nature 370: 659–662
3. Pape T, Wintermeyer W, Rodnina M (1999) Induced fit in initial selection and proofreading of aminoacyl-tRNA on the ribosome. EMBO J 18:3800–3807
4. Pape T, Wintermeyer W, Rodnina MV (2000) Conformational switch in the decoding region of 16S rRNA during aminoacyl-tRNA selection on the ribosome. Nat Struct Biol 7:104–107
5. Yoshizawa S, Fourmy D, Puglisi JD (1998) Structural origins of gentamicin antibiotic action. EMBO J 17:6437–6448
6. Fourmy D, Recht MI, Blanchard SC, Puglisi JD (1996) Structure of the A site of Escherichia coli 16 S ribosomal RNA complexed with an aminoglycoside antibiotic. Science 274: 1367–1371
7. Fourmy D, Recht MI, Puglisi JD (1998) Binding of neomycin-class aminoglycoside antibiotics to the A-site of 16 S rRNA. J Mol Biol 277:347–362
8. Fourmy D, Yoshizawa S, Puglisi JD (1998) Paromomycin binding induces a local conformational change in the A-site of 16 S rRNA. J Mol Biol 277:333–345
9. Recht MI, Douthwaite S, Puglisi JD (1999) Basis for prokaryotic specificity of action of aminoglycoside antibiotics. EMBO J 18:3133–3138
10. Lynch SR, Puglisi JD (2001) Structural origins of aminoglycoside specificity for prokaryotic ribosomes. J Mol Biol 306: 1037–1058
11. Wilhelm JM, Jessop JJ, Pettitt SE (1978) Aminoglycoside antibiotics and eukaryotic protein synthesis: stimulation of errors in the translation of natural messengers in extracts of cultured human cells. Biochemistry 17:1149–1153
12. Palmer E, Wilhelm JM (1978) Mistranslation in a eucaryotic organism. Cell 13:329–334
13. Manuvakhova M, Keeling K, Bedwell DM (2000) Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. RNA 6:1044–1055
14. Phillips-Jones MK, Hill LS, Atkinson J, Martin R (1995) Context effects on misreading and suppression at UAG codons in human cells. Mol Cell Biol 15:6593–6600
15. Singh A, Ursic D, Davies J (1979) Phenotypic suppression and misreading Saccharomyces cerevisiae. Nature 277:146–148
16. Palmer E, Wilhelm JM, Sherman F (1979) Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. Nature 277:148–150
17. Howard MT, Shirts BH, Petros LM, Flanigan KM, Gesteland RF, Atkins JF (2000) Sequence specificity of aminoglycoside-induced stop condon readthrough: potential implications for treatment of Duchenne muscular dystrophy. Ann Neurol 48:164–169
18. Brown CM, Stockwell PA, Trotman CN, Tate WP (1990) Sequence analysis suggests that tetra-nucleotides signal the termination of protein synthesis in eukaryotes. Nucleic Acids Res 18:6339–6345
19. Brown CM, Dalphin ME, Stockwell PA, Tate WP (1993) The translational termination signal database. Nucleic Acids Res 21:3119–3123
20. Bonetti B, Fu L, Moon J, Bedwell DM (1995) The efficiency of translation termination is determined by a synergistic interplay between upstream and downstream sequences in Saccharomyces cerevisiae. J Mol Biol 251:334–345
21. McCaughan KK, Brown CM, Dalphin ME, Berry MJ, Tate WP (1995) Translational termination efficiency in mammals is influenced by the base following the stop codon. Proc Natl Acad Sci U S A 92:5431–5435
22. Tate WP, Poole ES, Horsfield JA, Mannering SA, Brown CM, Moffat JG, Dalphin ME, McCaughan KK, Major LL, Wilson

DN (1995) Translational termination efficiency in both bacteria and mammals is regulated by the base following the stop codon. Biochem Cell Biol 73:1095–1103
23. Fearon K, McClendon V, Bonetti B, Bedwell DM (1994) Premature translation termination mutations are efficiently suppressed in a highly conserved region of yeast Ste6p, a member of the ATP-binding cassette (ABC) transporter family. J Biol Chem 269:17802–17808
24. Bedwell DM, Kaenjak A, Benos DJ, Bebok Z, Bubien JK, Hong J, Tousson A, Clancy JP, Sorscher EJ (1997) Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. Nat Med 3:1280–1284
25. Howard M, Frizzell RA, Bedwell DM (1996) Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations. Nat Med 2:467–469
26. Keeling KM, Brooks DA, Hopwood JJ, Li P, Thompson JN, Bedwell DM (2001) 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 10:291–299
27. Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL (1999) Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice. J Clin Invest 104:375–381
28. Clancy JP, Ruiz F, Bebok Z, King C, Greer H, Hong J, Wing L, Macaluso M, Lyrene R, Sorscher EJ, Bedwell D (2001) A pilot study of gentamicin treatment to suppress premature stop mutations in patients with cystic fibrosis. Am J Respir Crit Care Med 163:1683–1692
29. Wilschanski M, Famini C, Blau H, Rivlin J, Augarten A, Avital A, Kerem B, Kerem E (2000) A pilot study of the effect of gentamicin on nasal potential difference measurements in cystic fibrosis patients carrying stop mutations. Am J Respir Crit Care Med 161:860–865
30. Wagner KR, Hamed S, Hadley DW, Gropman AL, Burstein AH, Escolar DM, Hoffman EP, Fischbeck KH (2001) Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. Ann Neurol 49:706–711
31. Beroud C, Verdier F, Soussi T (1996) p53 gene mutation: software and database. Nucleic Acids Res 24:147–150
32. Matalon R, Dorfman A (1972) Hurler's syndrome, an – L-iduronidase deficiency. Biochem Biophys Res Commun 47:959–964
33. McKusick V, Neufeld EF, Kelly T (1978) The polysaccharide storage diseases. In: Stanbury J, Wyngaarden J, Fredrickson D (eds) Metabolic basis of inherited disease. McGraw-Hill, New York, p 1282–1307
34. Levine AJ (1997) p53, the cellular gatekeeper for growth and division. Cell 88:323–331
35. Agarwal ML, Taylor WR, Chernov MV, Chernova OB, Stark GR (1998) The p53 network. J Biol Chem 273:1–4
36. McLure KG, Lee PW (1998) How p53 binds DNA as a tetramer. EMBO J 17:3342–3350
37. Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E, Gronenborn AM (1994) High-resolution structure of the oligomerization domain of p53 by multidimensional NMR. Science 265:386–391
38. Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C, Levine AJ (1993) Gain of function mutations in p53. Nat Genet 4:42–46
39. Chene P (1998) In vitro analysis of the dominant negative effect of p53 mutants. J Mol Biol 281:205–209
40. Brachmann RK, Vidal M, Boeke JD (1996) Dominant-negative p53 mutations selected in yeast hit cancer hot spots. Proc Natl Acad Sci U S A 93:4091–4095
41. Parada LF, Land H, Weinberg RA, Wolf D, Rotter V (1984) Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature 312:649–651
42. Hinds P, Finlay C, Levine AJ (1989) Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol 63:739–746
43. Eliyahu D, Raz A, Gruss P, Givol D, Oren M (1984) Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature 312:646–649
44. Zhang S, Ryden-Aulin M, Isaksson LA (1996) Functional interaction between release factor one and P-site peptidyl-tRNA on the ribosome. J Mol Biol 261:98–107
45. Mottagui-Tabar S, Tuite MF, Isaksson LA (1998) The influence of 5′ codon context on translation termination in Saccharomyces cerevisiae. Eur J Biochem 257:249–254
46. Namy O, Hatin I, Rousset JP (2001) Impact of the six nucleotides downstream of the stop codon on translation termination. EMBO Rep 2:787–793
47. Skuzeski JM, Nichols LM, Gesteland RF, Atkins JF (1991) The signal for a leaky UAG stop codon in several plant viruses includes the two downstream codons. J Mol Biol 218:365–373
48. VanLoock MS, Easterwood TR, Harvey SC (1999) Major groove binding of the tRNA/mRNA complex to the 16 S ribosomal RNA decoding site. J Mol Biol 285:2069–2078
49. Arky R (1996) Physicians' desk reference. Medical Economics, Montvale
50. Laurent G, Carlier MB, Rollman B, Van Hoof F, Tulkens P (1982) Mechanism of aminoglycoside-induced lysosomal phospholipidosis: in vitro and in vivo studies with gentamicin and amikacin. Biochem Pharmacol 31:3861–3870
51. El Mouedden M, Laurent G, Mingeot-Leclercq MP, Taper HS, Cumps J, Tulkens PM (2000) Apoptosis in renal proximal tubules of rats treated with low doses of aminoglycosides. Antimicrob Agents Chemother 44:665–675
52. Fielding RM, Lewis RO, Moon-McDermott L (1998) Altered tissue distribution and elimination of amikacin encapsulated in unilamellar, low-clearance liposomes (MiKasome). Pharm Res 15:1775–1781
53. Fielding RM, Moon-McDermott L, Lewis RO, Horner MJ (1999) Pharmacokinetics and urinary excretion of amikacin in low-clearance unilamellar liposomes after a single or repeated intravenous administration in the rhesus monkey. Antimicrob Agents Chemother 43:503–509
54. Krawczak M, Ball EV, Fenton I, Stenson PD, Abeysinghe S, Thomas N, Cooper DN (2000) Human gene mutation database-a biomedical information and research resource. Hum Mutat 15:45–51
55. McKusick V (1998) Mendelian inheritance in man. John Hopkins University Press, Baltimore