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Rescue of non-sense mutated p53 tumor suppressor gene by aminoglycosides

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ABSTRACT

Mutation-based treatments are a new development in genetic medicine, in which the nature of the mutation dictates the therapeutic strategy. Interest has recently focused on diseases caused by premature termination codons (PTCs). Drugs inducing the readthrough of these PTCs restore the production of a full-length protein. In this study, we explored the possibility of using aminoglycoside antibiotics to induce the production of a full-length functional p53 protein from a gene carrying a PTC. We identified a human cancer cell line containing a PTC, for which high levels of readthrough were obtained in the presence of aminoglycosides. Using these cells, we demonstrated that aminoglycoside treatment stabilized the mutant mRNA, which would otherwise have been degraded by non-sense-mediated decay, resulting in the production of a functional full-length p53 protein. Finally, we showed that aminoglycoside treatment decreased the viability of cancer cells specifically in the presence of nonsense-mutated p53 gene. These results open possibilities of developing promising treatments of cancers linked with non-sense mutations in tumor suppressor genes. They show that molecules designed to induce stop-codon readthrough can be used to inhibit tumor growth and offer a rational basis for developing new personalized strategies that could diversify the existing arsenal of cancer therapies.

INTRODUCTION

A large number of human diseases are caused by premature termination codons (PTCs) leading to the production of a truncated protein and mRNA degradation by the non-sense mediated mRNA decay (NMD) pathway. Aminoglycoside antibiotics (gentamicin, G418, amikacin) can bind to mammalian ribosomal RNA, promoting PTC readthrough and inducing the partial restoration of full-length protein synthesis. This has been demonstrated both in cultured mammalian cells (1) and in animal models. The considerable potential of this approach was first demonstrated *in vivo* by the work of Barton-Davis *et al.* (2) who reported the restoration of dystrophin levels to 10–20% of normal levels in the skeletal muscle of *mdx* mice, following subcutaneous injections of gentamicin.

Bedwell and coworkers have also shown that G418 and gentamicin can restore the expression of the cystic fibrosis transmembrane conductance regulator (CFTR) in a bronchial cell line carrying a non-sense mutation in the CFTR gene (3). In the last few years, this therapeutic strategy has been studied in hereditary genetic diseases, such as cystic fibrosis (CF) and muscular dystrophies (4,5) and several clinical trials have already been performed (6–10). A recent clinical trial in CF patients showed that the parenteral administration of gentamicin at a dose previously demonstrated to be safe has beneficial clinical effects and that there is a correlation between the level of readthrough level and improvements in the clinical state of the patients (11).

In this study, we investigated the possibility of extending this approach to cancer treatment. Many cancers are linked to the presence of a PTC in a tumor suppressor gene, resulting in the synthesis of a truncated protein unable to inhibit cell proliferation or promote apoptosis. We focused on the p53 tumor suppressor gene, which is mutated in >50% of human cancers, and for which 8% of all the mutations identified to date are non-sense mutations. p53 is the cellular gatekeeper for growth and division. It acts as a transcription factor and triggers cell-cycle arrest and apoptosis in response to diverse cellular stresses, including DNA damage, oncogene activation and hypoxia (12,13). Most of the drugs currently used to treat cancer patients are genotoxic agents that exert their anti-tumor activity at least partly by

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p53-dependent tumor suppression. Poor responses to conventional radiotherapy and chemotherapy are often associated with mutant p53 status (14–16). Moreover, it has been shown that the reintroduction of wild-type p53 into cancer cells lacking p53 actually promotes the repression of cell growth (17,18) or the induction of apoptosis (19). The re-expression of functional p53 in cancer cells may thus lead to a synergistic effect with genotoxic anti-tumor drugs (14). Gene therapy has been studied in preclinical settings, in which impressive anticancer activity has been reported (20,21). However, gene therapy approaches are limited by the lack of efficient and safe delivery systems and by the immune response directed against viral vectors.

Direct targeting and rescue of the endogenous mutated tumor suppressor is an interesting alternative. Promising results have been obtained for mutant p53 carrying amino acid substitutions. In this approach, small molecules or synthetic peptides were used to restore the active conformation and DNA binding, resulting in an inhibition of cell growth (22–25). One of these small molecules, PRIMA-1, when used in combination with adriamycin, strengthened the effect of adriamycin, triggering an apoptotic response in non–small cell lung cancer cells (26). PRIMA-1 also inhibited tumor cell growth *in vivo* in mice carrying endogenous missense mutations in p53 (27). These results strongly suggest that forcing the protein synthesis machinery to read through a PTC present in a mutant p53 mRNA would interfere with cancer development through a direct effect on cell proliferation or apoptosis.

The present study was designed to evaluate this possibility. We first quantified readthrough levels in a dual reporter assay described in a previous study and shown to reflect the readthrough levels obtained *in vivo* (28).Three mutations displaying high levels of induced readthrough level were retained for further analysis. We showed that aminoglycoside treatment strongly and specifically stabilized mutant p53 mRNAs that would otherwise be subject to NMD. Moreover, aminoglycoside treatment resulted in the production of a full-length p53 protein, and this effect was dose-dependent. We further demonstrated the recovery of transactivation activities by the re-expressed proteins. Consistent with these findings, aminoglycoside treatment reduced the viability of cultured cancer cells, specifically in the presence of non-sense-mutated p53 gene.

MATERIALS AND METHODS

Cell lines and cell culture

All cells were cultured in DMEM plus GlutaMAX (Invitrogen), except for H1299 cells, which were cultured in RPMI plus GlutaMAX (Invitrogen). The medium was supplemented with 10% fetal calf serum (FCS, Invitrogen) and 100 U/ml penicillin/streptomycin. Cells were kept in a humidified atmosphere containing 5.5% CO_2, at 37°C. NIH3T3 cells are embryonic mouse fibroblasts. H1299 is p53-null cell line established from a human lung carcinoma (provided by ATCC). HDQ-P1 is homozygous for the non-sense mutation at codon 213 (CGA→TGA) in the p53 gene. This cell line was established from a human primary breast carcinoma (29) and was provided by the German Collection of microorganisms and Cell Cultures, DSMZ. LoVo (WT p53 and APC R1114X and del 1 bp 1430) and DLD-1 (S241F p53) cells are epithelial cells derived from a human colorectal adenocarcinoma.

Readthrough quantification in cell culture

Complementary oligonucleotides corresponding to non-sense mutations embedded in their natural context (sequences in Table 1) were annealed and ligated into the pAC99 dual reporter plasmid, as described earlier (30). This dual reporter allows the quantification of stop-codon readthrough, thanks to the measurement of luciferase and β-galactosidase (internal calibration) activities, as previously described (11). The readthrough levels of non-sense mutations were analyzed in the presence or absence of gentamicin. NIH3T3 cells were electroporated with 20 μg of reporter plasmid and, the following day, cells were rinsed and fresh medium, with or without gentamicin, amikacin or G418 supplementation, was added. In these experiments, no cell toxicity was observed for the doses of antibiotics used.

Table 1. p53 non-sense mutations

WT codon	Mutation[a]	Sequence 5′→3′[b]	Mutation frequency[c], %
TGG	W53X	GAT ATT GAA CAA **TAG** TTC ACT GAA	0.6
CAG	Q144X	ACC TGC CCT GTG **TAG** CTG TGG GTT	2.4
TGG	W146X	CCT GTG CAG CTG **TGA** GTT GAT TCC	4.3
CAG	Q192X	CTG GCC CCT CCT **TAG** CAT CTT ATC	4.1
CGA	R196X	CAG CAT CTT ATC **TGA** GTG GAA GGA	11.8
CGA	R213X	AGA AAC ACT TTT **TGA** CAT AGT GTG	14.5
GAG	E298X	GAG CCT CAC CAC **TAG** CTG CCC CCA	3.3
CGA	R306X	GGG AGC ACT AAG **TGA** GCA CTG CCC	7.7
CAG	Q317X	AGC TCC TCT CCC **TAG** CCA AAG AAG	1.3
TAT	Y327X	CTG GAT GGA GAA **TAA** TTC ACC CTT	0.1
CGA	R342X	TTC GAG ATG TTC **TGA** GAG CTG AAT	3.8
All mutations studied			49.6

[a]Mutations are named by the position and the nature of the wild-type amino acid in p53 protein sequence.
[b]These non-sense mutation sequences were inserted into the dual reporter vector in order to determine readthrough level.
[c]Frequencies were given relative to total non-sense mutations listed for p53 gene.

Twenty-four hours later, cells were harvested and lysed with trypsin–EDTA (Invitrogen). β-Galactosidase and luciferase activities were assayed as previously described (30). The readthrough efficiency was estimated by calculating the ratio of luciferase to β-galactosidase activity obtained with the test construct and normalizing it with respect to the ratio obtained with an in-frame control construct. For each construct, at least five independent transfection experiments were performed. For readthrough quantification in H1299 cells, the same protocol was used, except that the Jet Pei method was used for transfection. For each construct, at least three independent transfection experiments were performed.

RNA extraction and quantitative PCR analysis

For the analysis of mRNA for p53 and its transcriptional target genes, ASNS, Bax and p21, and APC, total RNA was extracted from cells that had or had not been treated with gentamicin (800 μg/ml) or G418 (50 or 200 μg/ml) for 72 h (RNeasy Mini Kit, Qiagen) and subjected to DNAse I digestion (RNase-free Dnase). RNA was quantified in a Nanodrop apparatus (ThermoScientific) and the absence of RNA degradation was confirmed by agarose gel electrophoresis. The first-strand cDNA was synthesized from 2 μg of total RNA, with random primers and the SuperScript II Reverse Transcriptase (Invitrogen), as recommended by the manufacturer. Quantitative PCR was then carried out on equal amounts of the various cDNAs, with the thermoCycler CFX96 (Biorad), and the accumulation of products was monitored with the intercalating dye, FastStart Universal SYBRGreen Master (ROX) reagent (Roche). We quantified mRNA levels relative to three reference mRNAs: RPL32, Hprt1 and HMBS. In each experiment, results are expressed relative to untreated cells, for which the value obtained was taken as 1. Relative levels of gene expression were calculated using conditions at the early stages of PCR, when amplification was logarithmic and could therefore be correlated with the initial number of copies of the gene transcripts. The specificity of quantitative PCR was checked by agarose gel electrophoresis, which showed that a single product of the desired length was produced for each gene. A melting curve analysis was also performed. Single product-specific melting temperatures were identified for each gene. Oligonucleotides pairs used for amplification were described in Supplementary Data. For the quantification of each mRNA, three independent experiments (from biological replicates) were performed in triplicate.

Western-blot analysis

HDQ-P1 cells (R213X) were treated with G418 (50, 100 and 200 μg/ml) or gentamicin (800 μg/ml) for 72 h. The medium was replaced and fresh antibiotics were added each day. H1299 cells (p53-null) were transfected with pCMVp53 wild-type, Q192X, R213X or E298X (30 μg) by the Jet Pei method (Ozyme). Each DNA precipitate was dispensed into two 10 mm plates, one of which was left untreated and the other of which was immediately treated with G418 (200 μg/ml) for 48 h. Cells were harvested by treatment with trypsin–EDTA (Invitrogen), lysed in 350 mM NaCl, 50 mM Tris–HCl pH 7.5, 1% NP-40, 1 mM orthovanadate, 1 mM NaF, 1 mM PMSF and protease inhibitor cocktail (Roche) and sonicated. Total proteins were quantified with Bradford reagent (Biorad) and extracts were denatured by incubation in Laemmli buffer for 5 min at 90°C. We subjected 20 μg of total protein for HDQ-1 cells and 2–4 μg of total protein for H1299 cells to SDS–PAGE in NuPAGE Novex 4/12% Bis–Tris pre-cast gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes, as recommended by the manufacturer (Invitrogen). Membranes were saturated by incubation for 1 h in TBS containing 5% non-fat milk powder, and incubated with the primary monoclonal antibody, DO-1 (N-terminal epitope mapping between amino acid residues 11–25 of p53; Santa Cruz Biotechnologies, 1/400) or a monoclonal antibody against mouse actin (Millipore, 1/2000). After three washes in TBS containing 1% non-fat milk powder, the membranes were incubated with the secondary antibody [horseradish peroxidase-conjugated anti-mouse IgG (1/ 2500) or alkaline phosphatase-conjugated anti-mouse IgG (1/7000) Promega] for 45 min. Six washes were performed and chemiluminescence was detected with ECL Western Blotting Detection Reagents (Amersham, GE Healthcare) or NBT/BCIP Tablets (Roche). The signal was quantified with ImageQuant software.

Protein activity assays

We investigated the transcriptional activity of the p53 protein, using the p53BS-luc reporter plasmid containing the firefly luciferase gene downstream from seven p53 binding sites.

For HDQ-P1 cells, which carry the endogenous p53 R213X non-sense mutation, antibiotics (G418 25, 50, 100 and 200 μg/ml; gentamicin 800 μg/ml; amikacin 2 mg/ml) were added the day before transfection and on each subsequent day. HDQ-P1 cells were cotransfected, by the Jet Pei method, with p53BS-luc (2 μg) and pCMVLacZ (2 μg). For each assay, DNA precipitates were dispensed into two 6-well plates, one of which was treated with antibiotics and the other of which was not. Protein extracts were prepared 72 h after transfection, and enzymatic activities were measured. Transfection with pCMVLacZ was used to normalize transfection efficiency, cell viability and protein extraction.

H1299 cells were cotransfected in the presence of the Jet Pei reagent (Ozyme), as recommended by the manufacturer, with pCMVp53 WT or mutant constructs (3 μg), p53BS-luc (2 μg) and pCMVLacZ (0.6 μg). Antibiotics (800 μg/ml gentamicin, 50, 100 and 200 μg/ml G418) were added immediately after transfection. Protein extracts were prepared 24 h after transfection and enzymatic activities were measured.

For each set of conditions, at least five independent transfection experiments were performed.

siRNA transfections

The siRNAs used in this study double-stranded chemically synthesized oligonucleotides provided by Thermo

Scientific. The siRNA targeting p53 is referred to as 3329-14-0020 and the non-targeting siRNA is referred to as 1810-01-05. Transfection protocols were described in Supplementary Data.

Flow cytometry analysis

H1299 cells were used to seed 6-well plates at a density of 1.4×10^5 cells per well and were then incubated for 48 h. The cells were transfected, in the presence of Lipofectamine 2000 (Invitrogen), with pCMV p53 WT, R213X or Q192X or pCMV empty (4 μg), according to the manufacturer's instructions. G418 (200 or 400 μg/ml) was added immediately after transfection and the medium was replaced the following day. We determined the number of viable cells 48 hours after transfection, by flow cytometry (Guava Easycyte, Millipore) with the ViaCount reagent (Millipore), as recommended by the manufacturer. In this assay, a nuclear dye stained only nucleated cells, whereas propidium iodide also stained dying cells. The proportion of apoptotic cells was determined with the Nexin reagent, which contains two staining agents, Annexin V-PE and 7-AAD (Millipore), as recommended by manufacturer. For each experiment, at least four independent transfection experiments were performed.

For each experiment, the percentage of cells expressing p53 was estimated as described in Supplementary Data.

RESULTS

Identification of p53 non-sense mutations responsive to aminoglycoside treatment

We chose to study 11 non-sense mutations found in 50% of cancer cells carrying a non-sense mutation in the p53 gene (UMD Database, all curated, October 2008). For each non-sense mutation, the stop codon and the surrounding nucleotide context, shown in Table 1, were inserted into the dual reporter vector pAC99 (11,30). Readthrough levels were quantified in NIH3T3 cells transiently transfected with the dual reporter vector, in the presence or absence of gentamicin (Figure 1A). Readthrough rates ranged from 0.03% (p53 Y327X) to 0.5% (p53 R213X) for basal readthrough, and from 0.15% (p53 Y327X) to 2.8% (p53 R213X) in the presence of 800 μg/ml gentamicin. We also evaluated the readthrough levels for each non-sense mutation in

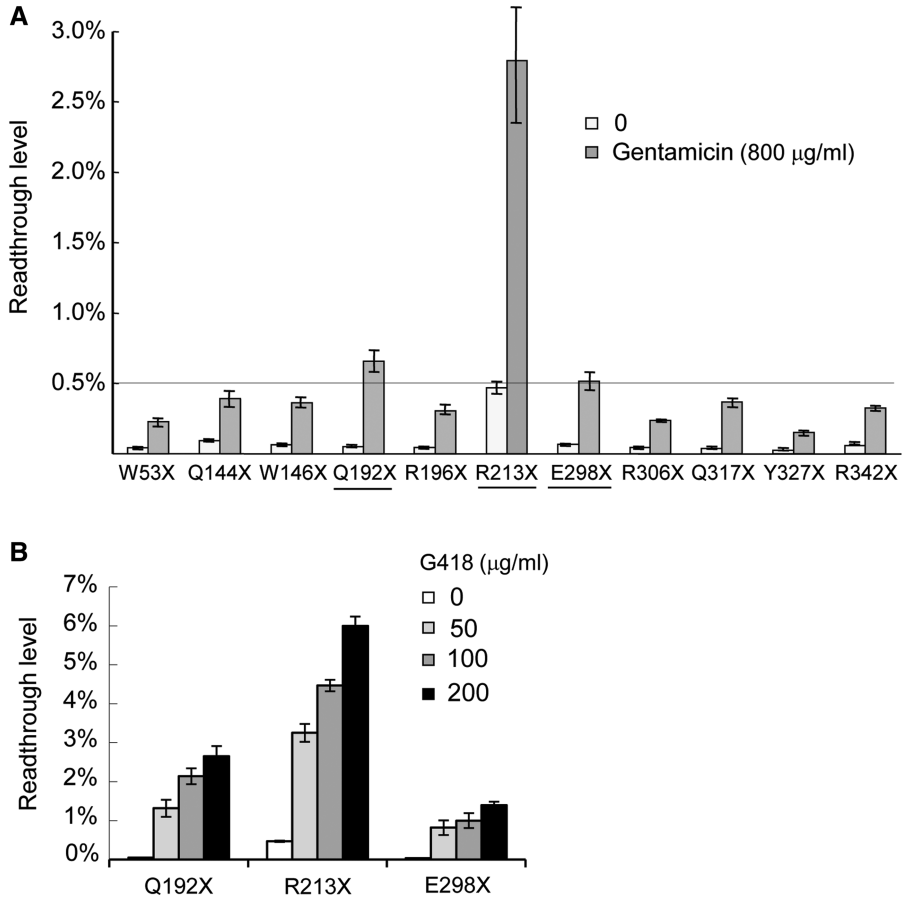

Figure 1. Identification of p53 nonsense mutations responsive to aminoglycoside treatment. (**A**) Readthrough efficiencies of 11 non-sense mutations in the p53 gene were measured in NIH3T3 cells with and without gentamicin (800 μg/ml) treatment for 24 h. Three non-sense mutations (Q192X, R213X and E298X) displayed levels of gentamicin-induced readthrough exceeding 0.5%. Mean values are presented together with the standard error of the mean (SEM) ($n = 5$). (**B**) Readthrough levels were measured for the nonsense mutations p53 Q192X, R213X and E298X in H1299 human cancer cells in the presence of G418 (geneticin). Mean values are presented together with the standard error of the mean (SEM) ($n = 3$).

the presence of the aminoglycoside amikacin, and the results obtained were similar to or lower than those obtained with gentamicin (data not shown). Similar variations in basal readthrough levels and responsiveness to aminoglycosides were reported in several previous studies (28,31).

For further characterization, we focused on the R213X, Q192X and E298X non-sense mutations, for which we obtained the highest readthrough levels in the presence of aminoglycosides. The R213X mutation is the most frequent non-sense mutation in the p53 gene (14.5% of reported non-sense mutations). For the three selected non-sense mutations, we quantified the readthrough levels obtained in the presence of G418 (geneticin), because this antibiotic is the most potent readthrough inducer among the aminoglycosides tested. All three non-sense mutations displayed a dose-dependent response to G418, resulting in readthrough levels greater than those observed for gentamicin, reaching 6% for p53 R213X, the most responsive mutation (Figure 1B).

Aminoglycosides stabilize mutant p53 mRNA in cancer cells carrying the endogenous non-sense mutation R213X

For further characterization, we focused on the human HDQ-P1 cell line, which was established from a human primary breast carcinoma (29). HDQ-P1 cells are homozygous for the p53 R213X non-sense mutation, which yielded the highest readthrough levels in the presence of the various antibiotics tested. This mutation is located in exon 6 and leads to the replacement of an arginine residue by a premature UGA stop codon more than 50 nt upstream from the last exon–exon junction. The mRNA molecule generated is thus entirely suitable for degradation by the NMD pathway. Indeed, inhibition of the NMD pathway by emetine, a potent and specific inhibitor of NMD, increased the level of the mutant p53 R213X transcript by a factor of 11 in HDQP-1 cells (Figure 2A).

The ability of readthrough event to antagonize NMD in mammalian cells has been reported in several studies (3,32,33). We therefore performed quantitative PCR on HDQ-P1 cells with and without gentamicin or G418 treatment. The treatment of HDQ-P1 cells with gentamicin increased p53 R213X transcript levels by a factor of four, whereas treatment with G418 led to an increase in these levels by a factor of eight (Figure 3A). Higher concentrations of G418 stimulate readthrough and increase the accumulation of mutant mRNA. In the LoVo cancer cell line, which carries a wild-type p53 gene, aminoglycoside treatment did not affect p53 transcript level (Figure 2B), demonstrating the dependence of this effect on the presence of the non-sense mutation.

We then determined whether the effect of aminoglycosides on the accumulation of a PTC-containing mRNA was specific to transcripts targeted to the NMD pathway. We used the LoVo cell line, which carries the R1114X non-sense mutation in the Adenomatous Polyposis Coli (APC) tumor suppressor gene. This non-sense mutation is in the last exon and is therefore unfavorable for mRNA degradation by the NMD pathway. The quantification of APC R1114X mRNA in LoVo cells demonstrated that neither gentamicin nor G418 affected the amount of APC mRNA (Figure 2C). Thus, aminoglycosides stabilize only mRNAs with a PTC targeting them to the NMD pathway.

Aminoglycosides restore the production of full-length p53 protein from the endogenous non-sense mutation R213X

We investigated whether aminoglycosides could restore the production of a full-length p53 protein from an endogenous mutated gene, by treating the HDQ-P1 cell line with gentamicin or G418. In the absence of treatment, we detected a faint 30 kDa band corresponding to the expected truncated protein (Figure 3B). The weakness of this signal was correlated with the low level of p53 mRNAs targeted to the NMD pathway (see above). After G418 treatment, a 53 kDa band corresponding to the full-length protein was detected, the intensity of which depended on the dose used. The amounts of full-length protein were normalized with respect to those in a cell line with WT p53 (LoVo cells). Full-length p53 levels reached 28, 56 and 85% those in LoVo cells with 50, 100 and 200 μg/ml G418, respectively. Treatment with gentamicin also led to the production of smaller, but

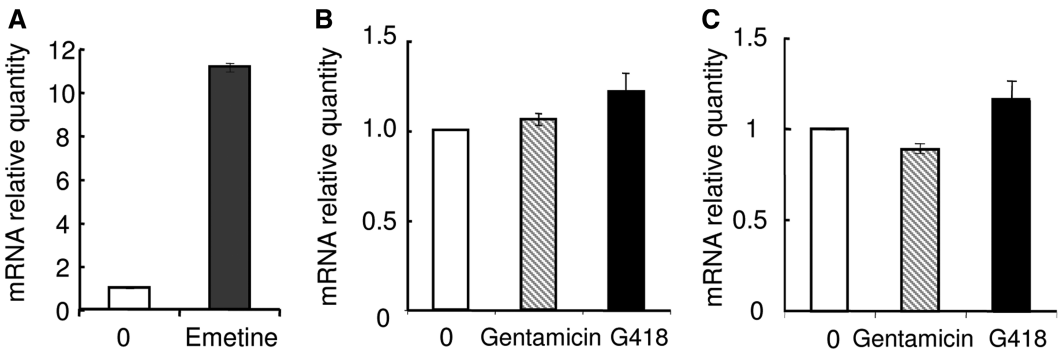

Figure 2. Readthrough of a nonsense mutation in an mRNA not targeted to NMD did not stabilize this transcript. Results are expressed relative to the amount of mRNA in the absence of treatment. Quantitative PCR was used to determine mRNA levels. Mean values are presented together with the standard error of the mean (SEM) ($n = 3$). (**A**) Emetine treatment (25 μg/ml for 10 h) stabilizes non-sense-mutated p53 R213X mRNA in HDQ-P1 cells. (**B**) Gentamicin (800 μg/ml) or G418 (200 μg/ml) does not stabilize a p53 WT mRNA in LoVo cells. (**C**) Gentamicin (800 μg/ml) or G418 (200 μg/ml) does not stabilize the APC R1114X mRNA mRNA, which is not targeted to the NMD pathway in LoVo cells.

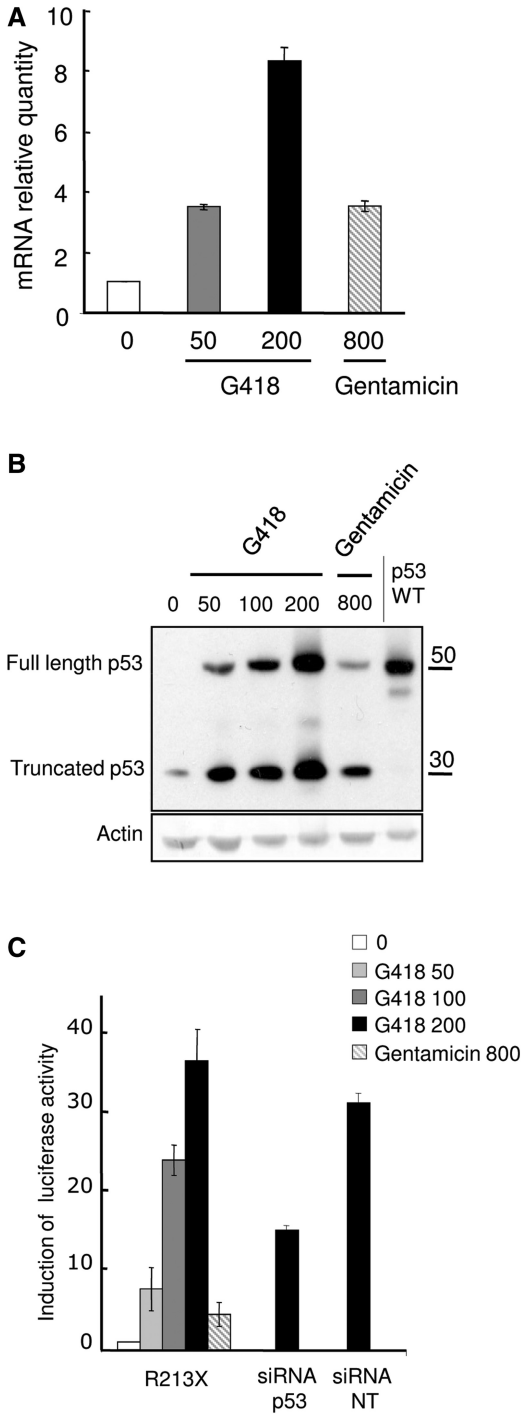

Figure 3. Effects of aminoglycoside treatment on the endogenous non-sense mutation p53 R213X. (**A**) G418 and gentamicin stabilize mutant p53 mRNA in HDQ-P1cells. HDQ-P1 cells carrying the endogenous nonsense mutation R213X were treated with G418 (50 and 200 μg/ml) or gentamicin (800 μg/ml) for 72 h and levels of mutant p53 mRNA were determined by quantitative PCR. The results of each experiment are expressed relative to the quantity of mRNA in the absence of treatment. Mean values are presented, together with the SEM ($n = 3$). (**B**) The full-length p53 protein is re-expressed after aminoglycoside treatment. HDQ-P1 cells were left untreated (−) or were treated with G418 (50, 100 and 200 μg/ml) or gentamicin (800 μg/ml) for 72 h. Western-blot membranes were probed with the DO-1 antibody directed against the N-terminus of p53 and a loading control was performed with an anti-actin antibody. An extract from LoVo cells (p53WT) was used as a control. (**C**) p53 transactivation capacity is restored by the treatment of HDQ-P1 cells with aminoglycosides. We assessed the transactivation capacity of the full-length p53 proteins produced after aminoglycoside treatment, using a reporter plasmid containing seven p53 binding sites upstream from the firefly luciferase gene (p53BS-luc). We measured p53-dependent luciferase activity in the presence or absence of G418 (50, 100 and 200 μg/ml) or gentamicin (800 μg/ml) for 96 h and the factor of increase of this activity is presented. As a control, HDQ-P1 cells were cotransfected with the p53BS-luc reporter plasmid and either the p53-targeting siRNA (siRNA p53) or a non-targeting siRNA (siRNA NT) and treated with G418 (200 μg/ml). Mean values are presented together with the SEM ($n = 5$). See Supplementary Data for statistical analysis.

nonetheless significant levels of full-length protein (18%). Thus, aminoglycoside treatment restores the production of a full-length p53 protein from an endogenous mutated gene, in a dose-dependent manner, this effect being proportional to the effect of the drug on readthrough level. The additional band around 45 kDa observed in the WT lane might correspond to a degradation product or to one of the several isoforms that have been described for human p53 protein (34). Aminoglycoside treatment also triggered accumulation of the truncated protein (Figure 3B). However, the difference in the increases achieved with 50 and 200 μg/ml G418 were greater for the full-length protein (factor of 3) than for the truncated form (factor of 1.6). This reflects the dual effect of aminoglycoside treatment: inducing readthrough (full-length protein) and stabilizing the transcript (truncated and full-length proteins).

Aminoglycosides restore the production of full-length p53 proteins from two other non-sense mutants

No human cancer cell lines harboring the Q192X and E298X p53 non-sense mutations, selected on the basis of the high levels of readthrough achieved in the presence of antibiotics, were available. We therefore investigated whether aminoglycoside treatment led to the production of a detectable full-length p53 protein from mutant cDNA. We used the p53-null H1299 cell line, which was derived from a non–small cell lung carcinoma and is homozygous for a partial deletion of the p53 gene. Western blot analysis were performed on protein extracts from H1299 cells transiently transfected with wild-type or mutant p53 expression vectors and left untreated or treated with G418 (Figure 4A). As a reference, we also included the p53 R213X cDNA in these transient transfection experiments. In cells transfected with the Q192X, R213X or E298X construct, the bands detected corresponded to the expected sizes of the truncated proteins: ~28, ~30 and 40 kDa, respectively.

For each mutation, G418 treatment also restored the production of a full-length p53 protein. The amounts of full-length protein detected after G418 treatment were 5.8, 44.2 and 5.4% those of the corresponding truncated form, for p53 mutants Q192X, R213X and E298X, respectively. Steady-state levels of full-length p53 were higher for the R213X mutation, with which we obtained the highest level of G418-induced readthrough. For this mutant, a 53 kDa full-length protein was detected even in the absence of

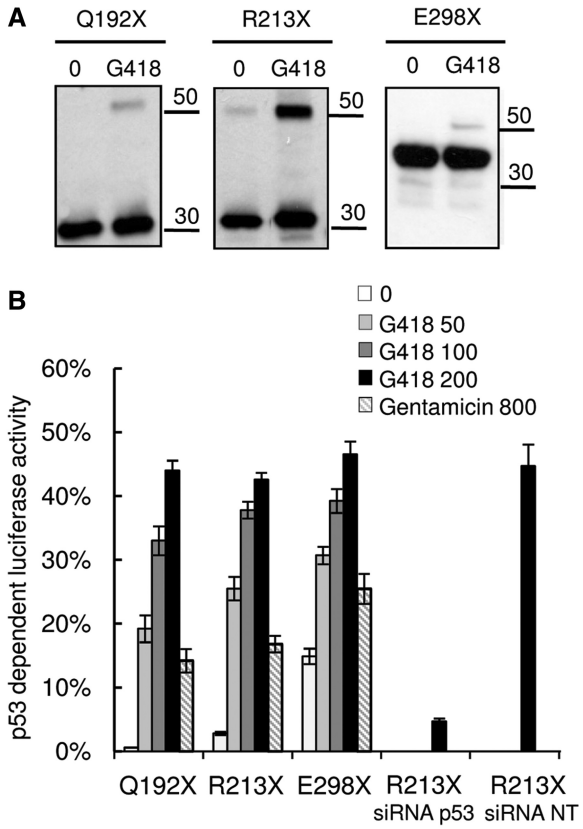

Figure 4. Aminoglycosides restore the production and function of full-length p53 proteins from p53 cDNA Q192X, R213X and E298X. (**A**) Re-expression of full-length p53 proteins after 48 h of G418 treatment (200 μg/ml) in H1299 cells transiently transfected with mutant p53 cDNA Q192X, R213X or E298X. Western-blot analysis was performed with the DO-1 antibody directed against the N-terminus of p53. (**B**) Aminoglycoside treatment restored p53 activity. The experiment was carried out as in Figure 3C, except that luciferase activity is expressed as a percentage of the activity obtained with the wild-type p53 cDNA. Mean values are presented together with the SEM ($n = 5$). See Supplementary Data for statistical analysis.

antibiotic treatment (at levels ~5.6% those for the truncated form), probably due to a combination of high basal readthrough levels and high levels of expression.

Endogenous re-expressed p53 proteins recover their transcriptional activity

The transactivation function of normal p53 protein underlies its function as a tumor suppressor gene. We investigated whether the full-length p53 protein produced after aminoglycoside treatment was an active transcription factor, by transfecting HDQ-P1 cells (p53 R213X) with a reporter plasmid containing seven p53 binding sites upstream from the luciferase firefly gene (p53BS-luc) and treating them with G418 or gentamicin. In this context, an active p53 protein is required to induce luciferase reporter gene expression. G418 treatment strongly induced firefly luciferase expression, in a dose-dependent manner, by a factor of up to 35 with respect to basal levels. Treatment with gentamicin or amikacin also increased p53-dependent firefly luciferase expression, by a factor of up to five (Figure 3C and data not shown). We checked that active p53 protein was indeed responsible for the increase in firefly luciferase expression, using a siRNA specifically targeting p53 mRNA. We first checked the efficacy of this siRNA, by assessing its ability to decrease the levels of p53 mRNA generated from a wild-type p53 expression vector in H1299 cells (Supplementary Figure S1). We cotransfected cultures of HDQ-P1 cells with the p53BS-luc reporter plasmid and either the p53-targeting siRNA or a siRNA not targeting the p53 mRNA and designed to have a minimal impact on known human genes. In the presence of the p53-targeting siRNA, the induction of luciferase expression by G418 was decreased by a factor of 2.3, whereas the non-targeting siRNA had no significant effect on the induction of luciferase expression (Figure 3C). Thus, the induction of luciferase activity by G418 is actually mediated by p53.

Aminoglycosides also restore the transcriptional activity of full-length p53 proteins produced from two other non-sense mutants

We assessed the transcriptional activity of p53 proteins re-expressed from the two other mutants, Q192X and E298X, by cotransfecting H1299 cells with a pCMV expression vector containing either the wild-type or a mutant p53 cDNA, together with p53BS-luc (Figure 4B). We included the R213X mutation in this experiment, for comparison of the results obtained with those reported above for the HDQ-P1 cell line. Transfection with the wild-type p53 construct led to a high level of luciferase activity, about 250 times higher than that observed after transfection with the insert-less control vector. This level was taken as 100% transactivation potential. In the absence of treatment, luciferase activity induction for the p53 mutants Q192X and R213X reached 0.6 and 2.5%, respectively. This difference is consistent with the basal readthrough level for the two mutations: 0.05 and 0.5%, respectively (Figure 1A). Surprisingly, E298X gave a residual luciferase activity of 18.5%. For the Q192X, R213X and E298X mutants, G418 treatment induced transcriptional activity in a dose-dependent manner, reaching 47, 43 and 42%, respectively. Treatment with gentamicin also induced p53 transcriptional activity, to a lesser, but nonetheless significant level, reaching 14, 17 and 25%, respectively. For confirmation that the enhancement of luciferase expression was mediated by p53 protein, we used the same siRNA strategy as described above. In the presence of p53-targeting siRNA, the level of induction of luciferase expression by G418 was 90% lower than that in the absence of this siRNA, whereas a siRNA not targeting p53 had no significant effect on luciferase expression (Figure 4B).

Aminoglycosides also rescued the transcriptional activity of these three non-sense-mutated p53 proteins in another type of human cancer cell, DLD-1 (colorectal adenocarcinoma) cells, demonstrating that this effect was not cell line-specific (Supplementary Figure S2).

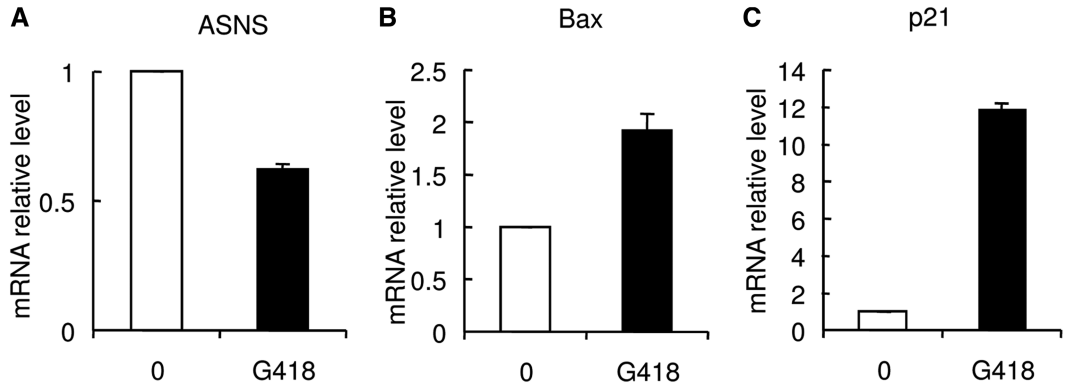

Figure 5. Full-length p53 proteins re-expressed after G418 treatment regulate cellular target genes. Quantitative PCR analysis was used to assess differences in the levels of (**A**) ASNS, (**B**) Bax and (**C**) p21 mRNAs between HDQ-P1 (p53 R213X) cells with and without G418 (200 μg/ml) treatment for 72 h. The results of each experiment are expressed relative to the amount of mRNA in the absence of treatment. Mean values are presented with the SEM ($n = 3$). See Supplementary Data for statistical analysis.

The W53X mutant, which had a low rate of gentamicin induced-readthrough (0.2%), failed to trigger a significant level of luciferase activity, although this activity increased with G418 treatment (Supplementary Figure S3). Thus, aminoglycoside treatment specifically rescues non-sense p53 mutants with significant induced readthrough levels.

Endogenous re-expressed full-length p53 proteins recover their ability to regulate cellular target genes

We investigated whether the full-length p53 protein produced after aminoglycoside treatments regulated endogenous cellular target genes, by carrying out quantitative PCR to assess differences in the expression of the ASNS, Bax and p21 genes between HDQ-P1 cells with and without G418 (200 μg/ml) treatment. ASNS, an asparagine synthetase, is known to be down-regulated by p53 (35) The Bax gene is a well characterized pro-apoptotic gene and p21 is a cyclin-dependent kinase inhibitor playing a crucial role in cell cycle control. Both are up-regulated by p53. In each experiment, the results obtained are expressed relative to untreated cells (normalized to 1). For ASNS, transcript levels were 1.7 times lower in treated than in untreated cells (Figure 5A). Levels of Bax and p21 mRNA in HDQ-P1 cells treated with G418 were 1.9 and 12 times higher, respectively, than those in untreated cells (Figure 5B and C). In these experiments, none of the three references genes displayed a change in mRNA level in response to G418 treatment. Thus, the full-length p53 protein produced by G418-induced readthrough was able to regulate the transcription of three of its principally cellular gene targets, with correct up- and down-regulation observed.

Re-expressed p53 full-length proteins recover their ability to induce apoptosis

As p53 induces cell cycle arrest and initiates apoptosis, we investigated whether the re-expression of an active, full-length p53 protein following G418 treatment affected these pathways in cultured human cancer cells. We previously observed that HDQ-P1 cells, which carry the endogenous p53 R213X non-sense mutation, were not sensitive to the re-introduction of WT p53 (data not shown). We therefore could not use this cell line to investigate the effects of G418 treatment on cell proliferation. We thus assessed the effects of G418 on the induction of p53-dependent growth inhibition in H1299 ($p53^{-/-}$) human cancer cells, which have been reported to be sensitive to the re-expression of WT p53 (19). H1299 cells were transfected with p53 WT, Q192X, R213X or empty vector constructs and exposed to G418. Viable cells were then counted by flow cytometry, with the Viacount reagent. Transfection with p53 WT resulted in numbers of viable cells half those obtained after transfection with an empty vector. However, viable cell number was not affected by G418 treatment. For both non-sense p53 mutants, G418 treatment was associated with a decrease in the number of viable cells from 9.5×10^5 cell/well without treatment to 6×10^5 cells/well for the highest dose of G418 (400 μg/ml) (Figure 6A and Table 2). By contrast, when H1299 cells were transfected with empty vector, G418 treatment had no effect on cell number. An analysis of the cell cycle gated on non-sense p53 mutant-transfected cells showed no effect of G418 treatment on the cell cycle.

We then investigated whether G418-induced full-length p53 protein promoted apoptosis in H1299 cells. For cells transfected with the R213X or Q192X construct, percentages of cells in early and late apoptosis were assayed by flow cytometry, with double staining (Nexin reagent, containing 7-AAD and Annexin V-PE) (Table 2). In the presence of the empty vector, the percentages of early and late apoptotic cells were ~15 and 9%, respectively, reflecting the toxic effect of the transfection reagent itself. Similar percentages were obtained for transfection with the R213X and Q192X mutant p53 constructs, in the absence of treatment. After G418 treatment, an increase was observed in the percentages of both early (~22%) and late (~20%) apoptotic cells, for both mutants, as shown in Table 2. As a reference, transfection with the p53 WT construct resulted in 22% of cells in early and 25% of cells in late apoptosis. Thus, following G418 treatment, re-expressed p53 proteins induced overall levels of

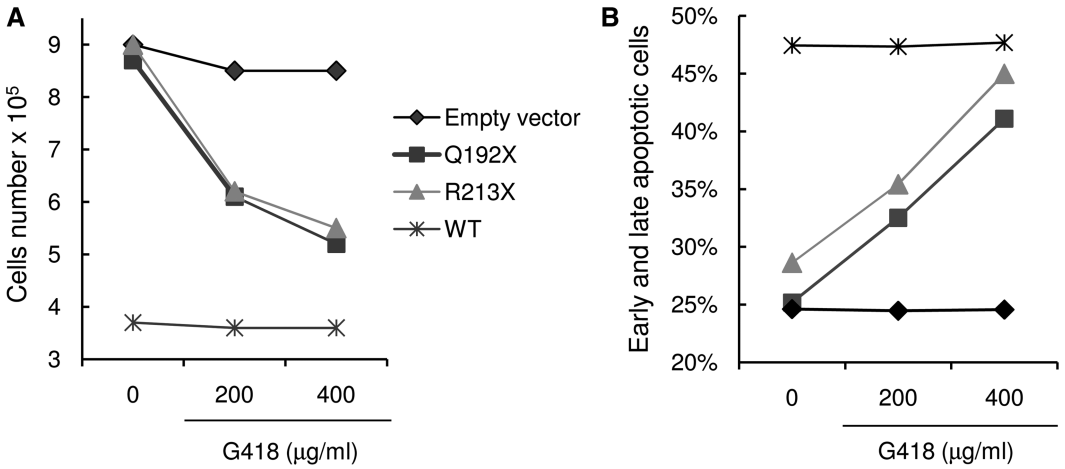

Figure 6. G418 induces apoptosis in a p53-dependent manner. H1299 cells transiently transfected with p53 Q192X, R213X, WT or empty vector were left untreated or treated with G418 (200 and 400 µg/ml) for 48 h. (**A**) Cell numbers were determined by flow cytometry with the Viacount reagent. Mean values are indicated with the SEM ($n = 5$) in Table 2. (**B**) Percentages of cells in early and late apoptosis were estimated by flow cytometry with Annexin-PE/7-AAD double-staining (Nexin reagent). The values shown are the percentages of cells in early and late apoptosis (overall apoptosis). The percentages of cells in each category (early and late) with the SEM ($n = 4$) are indicated in Table 2. See Supplementary Data for statistical analysis.

Table 2. Number of viables cells and percentages of early and late apoptotic cells

	G418 (µg/ml)	Number of viable cells (10^5)	Early apoptotic cells (%)	Late apoptotic cells (%)	Overall apoptotic cells (%)
WT	0	3.65 ± 0.19	21.7 ± 1.6	25.7 ± 2.7	47.5 ± 4.2
	200	3.63 ± 0.33	21.3 ± 3.5	26.1 ± 3.6	47.4 ± 5.4
	400	3.59 ± 0.25	22.7 ± 3.1	25.0 ± 4.3	47.7 ± 5.1
Empty vector	0	9.00 ± 0.25	15.7 ± 1.1	8.9 ± 0.6	24.6 ± 1.0
	200	8.47 ± 0.66	16.4 ± 1.4	8.1 ± 1.3	24.5 ± 1.0
	400	8.48 ± 0.65	15.6 ± 1.1	9.0 ± 1.0	24.6 ± 0.8
Q192X	0	8.67 ± 0.27	15.0 ± 1.0	10.1 ± 1.0	25.2 ± 1.7
	200	6.12 ± 0.19	19.6 ± 1.4	13.2 ± 1.1	32.8 ± 1.9
	400	5.17 ± 0.26	22.2 ± 1.3	19.0 ± 0.2	41.1 ± 0.6
R213X	0	9.05 ± 0.47	17.8 ± 0.9	11.1 ± 0,8	28.9 ± 1.6
	200	6.22 ± 0.39	20.7 ± 0.9	14.7 ± 1.0	35.5 ± 1.8
	400	5.45 ± 0.57	24.1 ± 1.3	21.4 ± 2.3	45.5 ± 3.1

apoptosis similar to those obtained with p53 WT (Figure 6B). In these experiments, the percentage of cells expressing p53, as assessed with the DO-1 antibody and a secondary FITC-conjugated antibody, was between 25 and 50%. Consequently, effects observed here are likely to have been underestimated.

Thus, the treatment of cancer cells specifically expressing p53 Q192X or R213X with G418 decreased the number of viable cells and increased the percentage of cells undergoing apoptosis.

DISCUSSION

Several recent studies have demonstrated the potency of readthrough-stimulating molecules for inducing the production of full-length proteins from genes carrying a PTC [reviewed in ref. (4,36)]. This approach has been applied to several cell culture models of different diseases, demonstrating the potential value of this strategy for treating patients with genetic disorders linked to the presence of a non-sense mutation. In parallel, a few clinical trials have been performed on DMD and CF patients, and some of these trials have generated promising results (6–9). In this study, we investigated whether aminoglycosides, a family of readthrough-stimulating drugs, could be used to rescue tumor suppressor genes altered by a non-sense mutation.

Only a few other studies have focused on this topic. Keeling and Bedwell (37) have shown that aminoglycosides can suppress non-sense mutations within the p53 gene. However, although the results of this study were encouraging, only one mutation was tested in cell culture and the functionality of the re-expressed protein was not assayed. More recently, Zilberberg *et al.* (38) showed that antibiotics improved clinical symptoms of tumorigenesis in a mouse model carrying a non-sense mutation in the APC gene and in xenografts of human cancer cells. However, the relationship between the lower level of tumor development and PTC readthrough was not established.

In this study, we carried out a comprehensive analysis of the effect of aminoglycoside on an endogenous non-sense mutation present in a human cancer cell line and on two other mutations frequently observed in cancers found in patients. We were able to decipher the impact of aminoglycoside treatment on the whole gene expression process including readthrough level, mRNA stability, protein production and function and cell proliferation. Our results strongly suggest that treating cancers involving a PTC in a tumor suppressor gene is a realistic new therapeutic approach.

Only a subset of p53 non-sense mutations is responsive to readthrough inducers

Several factors determine the efficacy of readthrough-promoting molecules. The various stop codons are suppressed with different efficiencies (UGA≥UAG >UAA), and the efficacy of suppression depends in part on the identity of the +4 nucleotide immediately downstream from the stop codon (C > U > A > G) (28,39,40). We show here that, as for other genes, only a few p53 non-sense mutations are sensitive to antibiotic treatment. Only three of the 11 PTC naturally occurring in the p53 gene display rates of gentamicin-induced readthrough >0.5% (Figure 1A). This threshold was selected on the basis of its being higher than the highest basal readthrough level (p53 R213X). For these three mutations, the +4 nt is a C. However, other PTCs with a C in this position present gentamicin-induced readthrough rates below 0.5% (Q144X, Q317X).

Even moderate readthrough promotes the strong stabilization of mutant mRNA

The amount of the mutant mRNA is another significant factor when using treatments to restore the production of a full-length protein. Here, we observed that both G418 and gentamicin stabilized p53 mutant R213X mRNA in cultured human HDQ-P1 cells (Figure 3A). We also showed that mRNA stabilization was directly proportional to the level of readthrough induced (Figure 1B). These observations are very encouraging, because even moderate levels of readthrough (2.8% for gentamicin and 6% for G418) were sufficient to counteract the induction of mRNA degradation by the NMD pathway. These findings are consistent with our previous data showing that aminoglycoside induced readthrough, even if modest, is able to antagonize the NMD pathway (37). More generally, the capacity of readthrough-promoting drugs to inhibit NMD has already been supported by several reports (3,33,41). The mechanism by which suppression events can interfere with NMD remains unclear. Several studies have suggested that, in mammals, NMD is triggered during the early round of translation and is enhanced by the proteins of the EJC, located at each exon–exon junction [reviewed in refs (42–44)]. One can expect that if readthrough occurs at this early stage, then the EJC protein complex is stripped off the mutant transcripts, which are no longer targeted for NMD. We cannot exclude a direct effect of aminoglycosides on mRNA stabilization not mediated by PTC readthrough. However, it has been shown in yeast that ura3 mRNA stability altered by amber non-sense mutations is restored when a UAG suppressor tRNA is added (45). Keeling and his coworkers (46) also demonstrated, in yeast, that readthrough above a threshold value of ~0.5% antagonizes NMD in the absence of any treatment. Moreover several data indicate that for some selenocystein mRNAs, an efficient incorporation of selenocystein by the Sec tRNA at UGA codons is required to antagonize NMD (47,48). All these results are in favor of a direct role of readthrough in the stabilization of the mRNA by aminoglycoside treatment.

Whatever the case, the results presented here indicate that a treatment protocol combining a readthrough inducer and an NMD inhibitor would strongly potentiate the production of a full-length protein.

Aminoglycosides induce the re-expression of full-length active proteins

This treatment requires sufficient amounts of mRNA to be produced if it is to be effective. However, PTC readthrough corresponds to the incorporation of a near-cognate aminoacyl tRNA (complementary to two of the three nucleotides of a stop codon), potentially resulting in the replacement of the normal residue by an amino acid incompatible with the stability or activity of the full-length protein. In this study, the treatment of HDQ-P1 cells carrying the genomic R213X p53 mutation with G418 or gentamicin led to the detection of the full-length protein (Figure 3B). The amount of re-expressed p53 protein was proportional to the readthrough level. For the highest dose of G418, full-length protein levels were similar to those in a cell line producing wild-type p53.

We next showed, with a reporter plasmid carrying p53 binding sites upstream from a luciferase gene, that the p53 protein re-expressed in HDQ-P1 cells had recovered its transcriptional activation properties (Figure 3C). Moreover, this full-length p53 protein was able to up- and down-regulate three of its known cellular target genes (Figure 5). The up-regulated Bax gene is involved in the apoptosis pathway, the up-regulated p21 gene encodes a cyclin-dependent kinase inhibitor playing a crucial role in cell cycle control and ASNS encodes an asparagine synthetase that is down-regulated by p53. Thus, both the quantity and the quality of the full-length p53 protein induced by G418 treatment were compatible with the appropriate regulation of targeted genes.

We also tested other p53 mutants for which no mutant cell lines were available, in a transient transfection assay with cDNA. These experiments also showed that the re-expressed p53 protein recovered its transactivation activity in a dose-dependent manner upon G418 treatment (Figure 4B). This activity reached ~45% that of the wild-type p53. However, Q192X had the same transactivation potential as R213X despite having only one-fifth its readthrough efficiency. This suggests that the amino acids incorporated at the stop codon may have different effects on protein activity. In the case of p53 Q192X, the amino acids incorporated after aminoglycoside treatment result

in full p53 activity, whereas, for p53 R213X, at least some of the incorporated amino acids may result in lower levels of activity.

We have recently obtained preliminary results showing that aminoglycoside treatment can also rescue the activity of APC tumor suppressor gene carrying a PTC (C. Floquet, J.-P. Rousset and L. Bidou, unpublished data). Thus, this approach may be applicable to a large proportion of cancers.

Recovering tumor suppressor function

We then analyzed the effect of re-expressed p53 on cell cycle progression and apoptosis in H1299 cancer cells transiently expressing p53 Q192X or R213X. We choose this cell model because it is sensitive to the reintroduction of p53 WT. We first demonstrated that, in presence of G418, cultures re-expressing p53 contain only 50% as many viable cells as untreated cells (Figure 6A). This effect was very evident and pronounced despite only a proportion of the cells being transfected. We therefore might expect this effect to be amplified in a population in which all the cells carried an endogenous PTC. We then determined the respective roles of cell cycle arrest and apoptosis in this diminution. Indeed, induction of the p53 tumor suppressor protein in deficient cells may lead to either cell cycle arrest or apoptosis, but the mechanisms leading to one or other of these responses being elicited in a given cell remain unclear. Cell type appears to be one of the factors involved in this process, together with p53 levels, with low levels resulting in cell cycle arrest whereas high levels seem to direct apoptosis (19).

We observed no significant disturbance in cell cycle, but there was a clear impact on the number of apoptotic cells, which were identified on the basis of their ability to bind Annexin V, a ligand of Phosphatidyl Serine specifically externalized early in the apoptosis pathway (Figure 6B). These data may reflect a preferential triggering of the apoptotic pathway due to the high level of G418-induced p53 expression in the population of transfected cells. However, we cannot rule out the possibility that p53 also induced cell cycle arrest in a manner not detectable in the model used here. Whatever the actual situation, these results demonstrate that readthrough inducers can be used to limit cancer cell growth.

Therapeutic use of readthrough inducers

Gentamicin, commonly used to treat bacterial infections in clinical care, displays nephrotoxicity and ototoxicity that might restrict its long-term use (49,50). This could be a serious problem if we need to apply this type of PTC therapy to classical genetic disorders, which would require lifetime treatment. However a six-month course of gentamicin treatment in DMD patients was recently completed safely, with no impairment of renal or hearing function (10). As previously reported, G418 stimulates readthrough more efficiently than gentamicin, but high doses are toxic in cultured cells. The toxicity of this drug to humans is unknown, but its structural similarity to gentamicin suggests that similar restrictions may apply to chronic treatment. However, this molecule has been shown to be very potent in a mouse model of non-sense mutation bypass, at doses inducing no adverse effects (51). A certain level of toxicity is acceptable for anticancer drugs, which are used for relatively short periods of time and may significantly improve what would otherwise be a severe, untreatable disease. Furthermore, several strategies have recently emerged for preventing gentamicin toxicity by targeting megalin, the receptor for these compounds on the cell (52,53). Moreover, new derivatives of geneticin with enhanced stop suppression ability and lower cell toxicity are currently being developed (54). With the exception of aminoglycosides, only a few compounds are known to promote readthrough. This is the case for the antibiotic negamycin and the recently identified compound PTC124. PTC124 has been described to induce re-expression of full length protein in mouse model of Duchenne muscular dystrophy (55) and CF (56). This compound is thought to act in a fashion that is distinct from aminoglycoside and to present fewer side effects than gentamicin. PTC124 is currently being evaluated in clinical trials for the treatment of hemophilia and CF [(57,58), and information obtained from *ClinicalTrials.gov*], and its effects on the re-expression of tumor suppressor genes may also be evaluated.

It should be noted that readthrough-inducing treatments may also trigger the accumulation of a truncated form of the protein, as illustrated here (Figure 3B). This results from the stabilization of the mRNA, allowing the production of both the truncated and full-length proteins. Therapeutic approaches based on promoting readthrough should thus take into account the potential deleterious effect of truncated proteins, which may have a dominant-negative effect. However, truncated forms of p53 would be likely to lack the nuclear localization signal and the tetramerization domain located at the C-terminus of the protein, and would therefore be unable to interact with the re-expressed full-length p53.

In summary, these results provide evidence that the treatment of cancer cells carrying a PTC in the p53 gene with aminoglycosides results in the re-expression of sufficient functional protein to affect cell viability. These findings may lead to the development of new strategies for treating any type of cancer involving the presence of a PTC in a tumor suppressor gene, and will also help to broaden the existing range of treatments for cancer.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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