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Novel small molecules potentiate premature termination codon readthrough by aminoglycosides

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ABSTRACT

Nonsense mutations introduce premature termination codons and underlie 11% of genetic disease cases. High concentrations of aminoglycosides can restore gene function by eliciting premature termination codon readthrough but with low efficiency. Using a high-throughput screen, we identified compounds that potentiate readthrough by aminoglycosides at multiple nonsense alleles in yeast. Chemical optimization generated phthalimide derivative CDX5-1 with activity in human cells. Alone, CDX5-1 did not induce readthrough or increase TP53 mRNA levels in HDQ-P1 cancer cells with a homozygous TP53 nonsense mutation. However, in combination with aminoglycoside G418, it enhanced readthrough up to 180-fold over G418 alone. The combination also increased readthrough at all three nonsense codons in cancer cells with other TP53 nonsense mutations, as well as in cells from rare genetic disease patients with nonsense mutations in the CLN2, SMARCAL1 and DMD genes. These findings open up the possibility of treating patients across a spectrum of genetic diseases caused by nonsense mutations.

INTRODUCTION

Nonsense mutations change an amino acid codon to a premature termination codon (PTC). mRNAs bearing PTCs typically show decreased stability or form defective truncated proteins, resulting almost invariably in profound loss of function and severe forms of disease (1). Nonsense mutations have been identified in most of the >5000 human genetic diseases identified to date and account overall for about 11% of the causative mutations (1). In sporadic cancer, nonsense mutations similarly account for ~11% of mutations in tumor suppressor genes such as TP53.

It has been known for decades that genetic and epigenetic alterations can partially restore the function of genes carrying nonsense mutations, a phenomenon termed nonsense suppression (2–4). Nonsense suppression or PTC readthrough can also be produced by exogenous chemicals. High concentrations of certain aminoglycoside antibiotics were found in 1979 to suppress several nonsense alleles in yeast (5,6) and subsequently in a reporter gene introduced into mammalian cells (7).

Aminoglycosides exert their PTC readthrough activity by binding at the decoding center of the eukaryotic ribosome. Binding alters the ability of translation termination factors to accurately recognize a PTC. Consequently, aminoglycosides increase the frequency of pairing of near-cognate aminoacyl-tRNAs to the PTC and enables formation of full-length protein (8,9). In 1997, the aminoglycoside gentamicin was found to induce CFTR protein expression from the CFTR gene harboring nonsense mutations in bronchial epithelial cells from patients with cystic fibrosis (10). This raised hopes of using PTC readthrough drugs to treat diseases caused by nonsense mutations.

The therapeutic potential of gentamicin has been investigated in preclinical models of genetic disease and in patients. Studies in mice bearing the human CFTR G542X transgene demonstrated increased CFTR function upon gentamicin administration (11). Gentamicin treatment also elevated CFTR chloride conductance in patients with CFTR nonsense mutations (12,13). However, improvements were small and patient response was variable (14). PTC readthrough by gentamicin was also demonstrated .

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in mdx mice (15) harboring nonsense mutations in their Dmd gene to model human Duchenne muscular dystrophy (DMD). The first small trial in DMD patients showed no effect and two others showed dystrophin expression in some patients (16) but the level of expression was insufficient for patient improvement. The lack of potency of gentamicin and its recognized nephrotoxicity and ototoxicity at high dose discouraged its further development.

Major efforts have been put into developing aminoglycoside derivatives with reduced toxicity (17,18) and discovering non-aminoglycoside readthrough compounds such as negamycin, tylosin, RTC13, RTC14, GJ71, GJ72 and ataluren (19–23). These compounds increased protein production in several cell culture and animal disease models, but often at the detection limit of western blotting for endogenous protein expression and with variable responses between genes, cell lines, and PTC mutations.

Ataluren is the sole new compound to have advanced through clinical trials. It has an excellent safety profile compared with gentamicin. Ataluren’s PTC readthrough activity has been challenged based on artifactual activity in luciferase reporter assays of the type used for its discovery, and lack of demonstrable readthrough activity in many assays (24–26). Nevertheless, it has shown activity in several animal models, including increased dystrophin expression and muscle function in the mdx mouse (27) and CFTR protein expression and improved chloride conductance in the intestine of the G542X-hCFTR mouse (20). Ataluren has undergone Phase 3 clinical trials (28,29). It has been given conditional approval for DMD treatment by the European Medicines Agency (30) but received a Refuse to File Letter by the Medicines Agency (30) but received a Refuse to File Letter on 21 January 2014. The read alignments in the regions of interesting candidate SNVs were visually inspected with the IGV viewer (33,34). Using standard homologous recombination technique we deleted two major efflux pumps PDR1 and PDR3 from both yeast strains and generated B0133-3B-AB13 ([PSI]+ MATa can1-100 cycl-72 his5-2 leu2-1 lys1-1 met8-1 trp5-48 ura4-1 PDR1Δ::URA4 PDR3Δ::HIS5) and IS110-18A-AB13 ([PSI]− MATa ar07-1 leu2-2 ilv1-2 his4-166 lys2-101 met8-1 trp5-48 ura4-1 PDR1Δ::URA4 PDR3Δ::HIS5) strains. Primers used to amplify PCR products for homologous recombination and primers to confirm deletion of PDR1 and PDR3 are listed in Supplementary Table S3.

**Yeast PTC readthrough screening assay**

Exponentially growing B0133-3B-AB13 cells were seeded in 384-well plates at A600 of 0.05 in Synthetic Complete medium lacking methionine and supplemented with 2.5 μM paromomycin. 157,000 chemicals from Prestwick, Sigma LOPAC, Microsource Spectrum, Biomol, Chembridge DiverSet and Lankenau Chemical Genomics Center were added to the wells using a Platemat Plus automated liquid handler (Matrix Technologies) equipped with a FP3 384 pin tool (0.7 mm pin diameter), at a final concentration of ~7 μM. Plates were incubated at 30°C for 48 h and yeast growth was determined by A600 measurement using a PowerWave HT Microplate Spectrophotometer (BioTeK Instruments). Compounds that induced >2.5-fold increase in cell growth compared with 2.5 μM paromomycin alone were retested at various concentrations in the presence or absence of paromomycin. Fresh samples of the active compounds were acquired from the respective suppliers and tested against various nonsense mutations including met8-1, trp5-48 and lys2-101 in B0133-3B-AB13 and IS110-18A-AB13 strains.

**Human cells**

GM16485 primary fibroblasts were purchased from the Coriell Biorepository, hTERT immortalized SD123 fibroblasts were provided by Dr Cornelius Boerkoel (University of British Columbia). C25C148 and HSK001 immortalized myoblasts from an unaffected individual and a DMD patient, were generated as previously described (35). HDQ-P1 and ESS-1 cell lines were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). EKVX cells were from the NCI Developmental Therapeutics Program (DTP). MDA-MB-361, Caov-3, SW900, NCI-H1688, Calu-6, SK-MES-1, HCC1937, UACC-893, NCI-H1299 and HCT116 cells were purchased from ATCC. GM16485, SD123, HDQ-P1, Caov-3, SK-MES-1 and HCT116 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% or 15% fetal bovine serum (FBS, Sigma-Aldrich) and 1× antibiotic-antimycotic (Gibco/Thermo Fisher Scientific) at 37°C.

**MATERIALS AND METHODS**

**Yeast cells**

*Saccharomyces cerevisiae* B0133-3B ([PSI]+ MATa can1-100 cycl-72 his5-2 leu2-1 lys1-1 met8-1 trp5-48 ura4-1) and IS110-18A ([PSI]− MATa ar07-1 leu2-2 ilv1-2 his4-166 lys2-101 met8-1 trp5-48 ura4-1) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). EKVX cells were from the NCI Developmental Therapeutics Program (DTP). EKVX cells were from the NCI Developmental Therapeutics Program (DTP). MDA-MB-361, Caov-3, SW900, NCI-H1688, Calu-6, SK-MES-1, HCC1937, UACC-893, NCI-H1299 and HCT116 cells were purchased from ATCC. GM16485, SD123, HDQ-P1, Caov-3, SK-MES-1 and HCT116 cells were cultured in high glucose Dulbecco’s Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% or 15% fetal bovine serum (FBS, Sigma-Aldrich) and 1× antibiotic-antimycotic (Gibco/Thermo Fisher Scientific) at 37°C.
and 5% CO₂. Human myoblasts were cultured in Skeletal Muscle Cell Growth Medium (PromoCell) supplemented with 20% FBS and 1× antibiotic-antimycotic at 37°C and 5% CO₂. Myoblasts were differentiated into myotubes in differentiation medium consisting of DMEM (Gibco/Thermo Fisher Scientific) supplemented with 10 μg/ml insulin (Sigma) and 1× antibiotic-antimycotic. All other cell lines (ESS-1, MDA-MB-361 and UACC-893) were cultured in RPMI-1640 medium supplemented with 10% or 20% FBS and 1× antibiotic–antimycotic at 37°C and 5% CO₂. GM16485 fibroblasts are derived from a patient with a late infantile neuronal ceroid lipofuscinosis harboring compound heterozygous nonsense mutations in the TPP1 gene (NM_000391.3:c.379C>T/c.622C>T; NP_000382.3:p.127X). SD123 fibroblasts are derived from an SIOD patient harboring homozygous nonsense mutations in the SMARCAL1 gene (NM_014140.3:c.49C>T; NP_054892.2:p.R17X). HSK001 fibroblasts are derived from a patient with DMD harboring nonsense mutation in the DMD gene (NM_004006.2:c.6103G>T; NP_003997.1:p.E208X). The HCT116 cell line has wild-type TP53 while other cancer cell lines harbor the following homozygous mutations in their TP53 gene: MDA-MB-361 (NM_000546.5:c.166G>T; NP_000537.3:p.E56X), Calu-3 (NM_000546.5:c.406C>T; NP_000537.3:p.Q136X), SW900 (NM_000546.5:c.499C>T; NP_000537.3:p.Q167X), NCI-H1688 (NM_000546.5:c.574C>T; NP_000537.3:p.Q192X), Calu-6 (NM_000546.5:c.586G>T; NP_000537.3:p.R196X), EKVX (NM_000546.5:c.610G>T; NP_000537.3:p.E204X), HDQ-P1 (NM_000546.5:c.637C>T; NP_000537.3:p.R213X), ESS-1 (NM_000546.5:c.637C>T; NP_000537.3:p.R213X), SK-MES-1 (NM_000546.5:c.892G>T; NP_000537.3:p.E298X), HCC1937 (NM_000546.5:c.916C>T; NP_000537.3:p.R306X), UACC-893 (NM_000546.5:c.1024C>T; NP_000537.3:p.E342X), NCI-H1299 (NM_000546.5:c.1954>AAG; NP_000537.3:p.?)(COSMIC).

Automated p53 immunofluorescence 96-well plate assay

HDQ-P1 cells cultured in DMEM containing 10% FBS and 1× antibiotic-antimycotic were seeded at 4000 cells per well in PerkinElmer View 96-well plates. The next day, the medium was replaced with fresh medium containing compounds to be tested and cells were incubated for 48–96 h. The wells were replenished with fresh medium and compounds every 48 h. The medium was removed by aspiration, and cell monolayers were rinsed with 1 ml ice-cold PBS. Cells were lysed in 80 μl lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (v/v) Triton X100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate) supplemented with fresh 1 mM Na3VO4, 1 mM dithiothreitol and 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals). Lysates were pre-cleared by centrifugation at 18 000 g for 20 min at 4°C. Supernatants were collected, protein was quantitated using the Bradford assay and lysates were adjusted to 0.5–1 mg/ml protein. Capillary electrophoresis western analysis was carried out with manufacturer’s reagents according to the user manual (ProteinSimple WES). Briefly, 5.6 μl cell lysate was mixed with 1.4 μl fluorescent master mix and heated at 95°C for 5 min. The samples, blocking reagent, wash buffer, DO-1 p53 antibody (0.5 μg/ml), mouse α-vinculin antibody (1:800 or 1:2000, clone 728526, R&D Systems), secondary antibody and chemiluminescent substrate were dispensed into the microplate provided by the manufacturer. The electrophoretic separation and immunodetection was performed automatically using default settings. The data was analyzed with built-in Compass software (ProteinSimple). The truncated and full-length p53 peak intensities (area under the curve) were normalized to that of the vinculin peak, used as a loading control. In most figures, electropherograms are represented as pseudo-blots, generated using Compass software. TPPI western analysis was carried out similarly, using the cell lysates prepared for TPPI activity measurements and Abcam ab54685 α-TPP1 antibody (0.8 μg/ml). Dystrophin western analysis was carried out similarly using cell lysates prepared from myotubes and Abcam ab15277 α-dystrophin antibody (1:200).

SDS-PAGE and immunoblotting

SIOD fibroblasts were seeded, treated and lysed as above, protein was quantified, and western blotting was assayed as previously described (36). In brief, 20 μg protein from each lysate was separated on an 8% polyacrylamide gel, electroblotted onto a nitrocellulose membrane and blocked overnight in 5% (w/v) nonfat milk supplemented with 2% (v/v) goat serum. Membranes were incubated with primary
antibodies, rabbit α-SMARCAL1 (1:2000, gift from Dr Cornelius Boerkoel) and rabbit α-β-actin (1:20 000, Novus Biologicals), washed three times with PBS + 0.1% (v/v) Tween-20 (PBS-T), and incubated with HRP-conjugated goat α-rabbit IgG secondary antibody, washed again with PBS-T and developed using enhanced chemiluminescence substrate (Millipore).

Aminoglycosides
G418 disulfate was purchased from Toku-E, gentamicin sulfate and hygromycin B from MicroCombiChem, netilmicin sulfate from AK Scientific and all other aminoglycosides from Sigma.

Synthesis of CDX5 compounds
CDX5-1: A mixture of 4-aminophthalimide (500 mg, 3.1 mmol; Aldrich) and 2,6-dichlorobenzyl bromide (740 mg, 3.1 mmol; TCI) in N,N-dimethylformamide (0.5 M) was stirred at 60°C for 6 h then at room temperature overnight. The reaction mixture was quenched with saturated NaHCO3 and extracted with ethyl acetate. The combined organic layers were dried over Na2SO4 and concentrated under vacuum. The crude material was purified either by automated flash chromatography (10–50% ethyl acetate in hexanes) or by preparative HPLC to give the desired compound in 30%-50% yield.

CDX5-35: 2-(Naphthalen-1-ylmethyl)isoindoline-1,3-dione. 1H NMR (400 MHz, CDCl3) δ 8.37 (d, J = 8.4 Hz, 1H), 7.88–7.82 (m, 3H), 7.80 (d, J = 8.2 Hz, 1H), 7.74–7.69 (m, 2H), 7.64–7.56 (m, 2H), 7.54–7.47 (m, 1H), 7.43 (dd, J = 8.0, 7.3 Hz, 1H), 5.34 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 168.40 (s), 134.19 (s), 133.90 (s), 132.20 (s), 131.48 (s), 131.31 (s), 128.87 (s), 127.44 (s), 126.67 (s), 125.96 (s), 125.42 (s), 123.53 (s), 39.67 (s).

CDX5-52: 2-(3,4-dichlorobenzyl)isoindoline-1,3-dione. 1H NMR (400 MHz, CDCl3) δ 8.76 (dd, J = 5.4, 3.1 Hz, 2H), 7.73 (dd, J = 5.5, 3.1 Hz, 2H), 7.52 (d, J = 2.0 Hz, 1H), 7.39 (d, J = 8.2 Hz, 1H), 7.28 (dd, J = 8.5, 2.2 Hz, 1H), 4.79 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 167.95 (s), 136.52 (s), 134.37 (s), 132.08 (s), 130.81 (s), 130.76 (s), 128.21 (s), 123.68 (s), 40.65 (s).

CDX5-53: 2-(2,6-Dichlorobenzyl)-5-fluoroisoindoline-1,3-dione. 1H NMR (400 MHz, chloroform-d) δ 8.44 (dd, J = 8.2, 4.5 Hz, 1H), 7.50 (dd, J = 7.0, 2.3 Hz, 1H), 7.44 – 7.33 (m, 3H), 7.22 (dd, J = 8.6, 7.4 Hz, 1H), 5.16 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 167.69, 166.45, 165.14, 136.49, 134.69, 134.59, 130.48, 129.71, 128.50, 127.63, 127.60, 125.75, 125.66, 121.17, 120.93, 113.00, 111.06, 38.47.

CDX5-109: 2-(2-Chloro-5-(trifluoromethyl)benzyl)isoindoline-1,3-dione. 1H NMR (400 MHz,chloroform-d) δ 7.91 (dd, J = 5.3, 3.2 Hz, 2H), 7.78 (dd, J = 4.1, 3.0 Hz, 2H), 7.51 (t, J = 9.4 Hz, 3H), 5.02 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 167.90 (s), 137.10 (s), 134.70 (s), 134.50 (s), 131.97 (s), 130.41 (s), 129.61 (q, J = 33.0 Hz), 125.97 (p, J = 3.8 Hz), 123.82 (s), 39.34 (s).

CDX5-186: 2-(2,6-Dichlorobenzyl)-4,7-difluorooisoindoline-1,3-dione. 1H NMR (400 MHz, chloroform-d) δ 7.89 (t, J = 6.9 Hz, 3H), 7.72 (dd, J = 8.7, 7.4 Hz, 1H), 5.15 (s, 2H). 13C NMR (101 MHz, CDCl3) δ 163.07, 155.00, 154.96, 152.37, 152.33, 152.32, 136.52, 130.01, 129.85, 128.52, 125.06, 124.93, 124.88, 124.75, 38.50.

RNA extraction and RT-PCR
RNA was extracted from HDQ-P1 cells using the RNAsesy Plus Mini Kit (Qiagen). cDNA synthesis was performed using High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific). Quantitative real-time RT-PCR for detection of TP53 mRNA was performed using the ABI StepOnePlus Real-Time PCR system, using the primers listed in Supplementary Table S3.

Site directed mutagenesis
The three TP53 mutations c.637C>T, c.637-639delCGAinsTAG, and c.637-638delICGinsTA resulting in p53 R213X with all three possible stop codons (TGA-C, TAG-C, TAA-C, respectively) were engineered into pDONR223-p53 (hORFeome V8.1) using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and primers listed in Supplementary Table S3. The mutant strand synthesis reaction was performed by PCR using 10x kit reaction buffer, pDONR223-p53 (60ng), forward and reverse primers (125 ng), dNTP mix, Quick Solution reagent and lightning enzyme. Eighteen PCR
reaction cycles were performed with DNA denaturing step at 95°C for 20 s, primer annealing at 60°C for 10 s, and polymerase extension at 68°C for 3.5 min. Following digestion of the parental DNA with Dpn I restriction enzyme, DNA for each sample was chemically transformed into XL10-Gold Ultracompetent cells and spread on spectinomycin-containing (100 μg/ml) LB agar plates. DNA was isolated from spectinomycin resistant clones and the TP53 cDNA was Sanger-sequenced in its entirety to confirm the introduction of the desired nonsense mutations and to ensure that mutations were not introduced elsewhere in the cDNA. Finally, to generate expression clones we used the LR recombination reaction to recombine the mutated cDNA samples from the Entry vectors into the pcDNA-6.2/V5-DEST vector (Thermo Fisher Scientific). Samples were chemically transformed into Top10 competent cells and spread on ampicillin-containing (100 μg/ml) LB agar plates. DNA from resistant clones was isolated and the p53 R213X was confirmed by Sanger sequencing.

**Transient transfection**

H1299 cells were transiently transfected with pcDNA-6.2/V5-DEST vector expressing either WT p53 or one of the three p53 R213X mutants with different stop codons using Lipofectamine 2000 (Thermo Fisher Scientific). Immunofluorescence microscopy using anti-V5 antibody revealed more than 80% transfection efficiency 48 h after transfection. Twenty four hours after transfection, each sample was split into four wells of a six-well plate (10^4 cells/well) and 24 h later either left untreated or treated with 20 μM G418, 10 μM CDX5-1, or 20 μM G418 and 10 μM CDX5-1. After 48 h, the cells were lysed and subjected to automated capillary electrophoresis western analysis (ProteinSimple WES).

**In vitro transcription and translation assay**

pcDNA-6.2/V5-DEST vectors expressing either WT p53 or one of the three p53 R213X mutants were linearized with FastDigest MssI restriction enzyme (Thermo Fisher Scientific). Linear DNA fragments were subjected to RNA synthesis using mMESSAGE mMACHINE T7 ULTRA Transcription Kit (Thermo Fisher Scientific) to generate 5’ capped and poly(A) tailed RNA. The *in vitro* translation assay was carried out using the 1-Step Human Coupled IVT Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Briefly, 500 ng RNA was added to the Hela cell lysate-based protein expression system and incubated at 30°C for 90 min without or with 0.5 μM G418, 1 μM CDX5-1, or a combination of 0.5 μM G418 and 1 μM CDX5-1. Samples were diluted in water and subjected to automated electrophoresis western analysis for p53 detection.

**TPP1 activity assay**

GM16485 primary fibroblasts seeded into six-well plates were exposed to compounds for up to 9 days. The wells were replenished with fresh medium and compounds every 3 days. At the end of the experiment, the cell monolayers were washed with ice-cold PBS and scraped using a rubber policeman into 0.1 ml lysis buffer without Na3VO4. The lysates were clarified by centrifugation at 15 000 × g for 10 min at 4°C. TPP1 enzyme activity was determined as in (37) with modifications. Lysates were diluted 1:5 in 50 mM sodium acetate pH 4.0 and pre-incubated at 37°C for 1 h. After pre-incubation, 20 μg of total protein from GM16485 lysates or 5 μg of total protein from lysates of fibroblasts from unaffected individuals was incubated in 150 μl of 50 mM sodium acetate pH 4.0 containing a final concentration of 62.5 μM Ala-Ala-Phe-7-amido-4-methylcoumarin for 2 h at 37°C. Fluorescence was measured using a TECAN Infinite M200 spectrophotometer with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Assays were carried out under conditions where product formation was linear with respect to protein concentration and time.

**Statistical analysis**

Data are presented as mean ± SD unless otherwise stated. Comparisons were made using the two-tailed Student’s t-test and differences were considered significant at a P-value of <0.05. Combination index (CI) was calculated using the CompuSyn software (38), with very strong synergistic activity defined as CI < 0.1.

**RESULTS**

**Development of a yeast PTC readthrough screening assay**

We wished to design a stringent high-throughput screening assay that reports readthrough of endogenous genes rather than artificial constructs. We developed a yeast cell-based assay that links readthrough of endogenous PTC-containing genes to increased cell growth. Saccharomyces cerevisiae cells carrying a PTC allele of a biosynthetic gene (e.g. *met8*-1) do not grow in the absence of the relevant product metabolite (e.g. methionine). Exposure to a compound that stimulates PTC readthrough allows cells to grow in the absence of the metabolite, i.e. acts as a chemical suppressor of the nonsense mutation. We selected strains B0133-3B and IS110-18A, reported to harbor PTCS in several biosynthetic genes (39,40). The strains were subjected to whole genome sequencing to verify the PTC sequences and identify their location within each gene (Supplementary Table S1). To maximize the likelihood of identifying active chemicals, the pleiotropic drug resistance genes *PDR1* and *PDR3* were deleted in both strains, redesignated *B0133-3B-AB13* and *IS110-18A-AB13*. *B0133-3B-AB13* was selected for the high-throughput screening assay because it carries the [PSF] prion form of translation termination factor erf3p which weakly suppresses nonsense mutations by ~1% (41). The [PSF] strain IS110-18A-AB13 was used for further testing of active compounds.

A number of aminoglycoside antibiotics have been shown to suppress certain nonsense alleles in yeast (5,6). To validate the screening assay we tested 10 different aminoglycosides for suppression of *met8*-1 (TAG-A) and *trp5*-48 (TAA-C) in the [PSF] strain. Paromomycin was the most potent suppressor, enabling growth in the absence of methionine or tryptophan; hygromycin B suppressed *met8*-1 only; and
gentamicin suppressed both, but only at very high concentrations (Figure 1A and B) consistent with previous reports (5,6). The seven other aminoglycosides showed negligible activity (Figure 1A and B).

In the [PSF] strain, which also carries a TGA-C PTC (lys2-101), paromomycin suppressed lys2-101, as well as met8-1 and trp5-48 (Supplementary Figure S1). Therefore, the assay provides a sensitive and quantitative high-throughput means of examining PTC readthrough at any of the three termination codons (TGA, TAG and TAA) in different biosynthetic genes and genetic backgrounds. The data also illustrate the earlier observations that aminoglycosides do not uniformly suppress all nonsense mutations (5,6).

Identification of small molecules that potentiate the activity of paromomycin

A first screen of 30,000 compounds tested for suppression of met8-1 in the [PSF] strain yielded no active compound. This disappointing result led us to consider the hypothesis that efficient PTC readthrough might require the concerted action of two compounds. We therefore screened for small molecules that potentiate the PTC readthrough activity of paromomycin by testing 157,000 compounds for suppression of met8-1 in the presence of 2.5 μM paromomycin, a sub-active concentration in this assay (Figure 1A). This screen yielded ten compounds capable of suppressing met8-1 (TAG). Five of these compounds also suppressed trp5-48 (TAA) in the same [PSF] strain in the presence of a low paromomycin concentration (Figure 1C–E).

The five compounds which demonstrated PTC readthrough activity against both met8-1 and trp5-48 were further evaluated at different concentrations, with and without various concentrations of paromomycin for suppression of the three genes with PTC in the [PSF] and the [PSF-] strains. Four showed readthrough activity as single agents on at least one of the three genes (Figure 1D–F). Interestingly, all five compounds showed increased activity in the presence of a sub-active concentration of paromomycin and all combinations suppressed all three genes (Figure 1D–F and Supplementary Figure S1 for complete data). Remarkably, several combinations demonstrated very strong synergistic activity, with combination indices (CI) below 0.1 (42). As a striking example, compound CDX5 showed no activity as a single agent on lys2-101 even at a high concentration of 50 μM and paromomycin showed no activity at 10 μM, but the combination of 1 μM CDX5 and 10 μM paromomycin showed optimal activity, comparable to that of paromomycin alone at 100 μM (Figure 1F). Four of the five active compounds (CDX4, 5, 10 and 11) are closely related members of the same N-substituted phthalimide class while CDX3 is structurally distinct. These findings show that combining two compounds can efficiently suppress different nonsense alleles with all three possible termination codons in yeast.

PTC readthrough in a human cell line

We next examined whether the five active compounds identified in the yeast screen could also induce PTC readthrough in human cells. Mammary carcinoma HDQ-P1 cells homozygous for R213X (TGA-C, where C is the 3′-flanking nucleotide) in exon 6 of the TP53 gene (43) were selected for testing based on convincing evidence of readthrough by the aminoglycoside G418 (44), confirmed in this study (Supplementary Figure S2A).

Nuclear localization sequences and a tetramerization domain located in the C-terminus of p53 contribute to its retention in the nucleus (45,46). p53 truncated at R213 lacks these sequences. To assess p53 R213X readthrough with relatively high throughput, we established an automated 96-well fluorescence microscopy assay to detect and quantitate nuclear p53, reasoning that PTC readthrough would result in the nuclear accumulation of full-length p53. HDQ-P1 cells showed little or no nuclear p53 signal (Figure 2A). Among several aminoglycosides tested at concentrations of 50, 100 and 200 μM only G418 induced a concentration-dependent increase in cells showing nuclear p53, consistent with the formation of full-length p53 due to readthrough. Paromomycin, which is active in yeast, and other approved aminoglycosides displayed no PTC readthrough activity at these concentrations. After 72 h exposure, 50 μM G418 induced nuclear p53 expression in 9% of cells (Figure 2A and B) while 200 μM G418 induced nuclear p53 expression in nearly all cells (Supplementary Figure S2B). None of the CDX compounds showed detectable PTC readthrough activity when used alone and only CDX5 in combination with 50 μM G418 induced a concentration-dependent increase in cells with nuclear p53 (Figure 2A and B).

Western analysis using an automated capillary electrophoresis system was used to measure p53 R213X readthrough quantitatively. HDQ-P1 cells expressed very low but measurable levels of truncated p53 and no detectable full-length p53 (Figure 2C and D). Alone, CDX5 caused no increase in truncated or full-length p53. G418 alone caused a 4-fold increase in both truncated and full-length p53 relative to the amount of truncated p53 present in untreated cells (Figure 2C and D). The combination of CDX5 and G418 caused a 7-fold increase in truncated p53 and a 19-fold increase in full-length p53 (Figure 2C and D). Therefore, CDX5 potentiates the PTC readthrough activity of G418 at p53 R213X in human HDQ-P1 cells.

CDX5 structure-activity relationship (SAR)

To better understand the structural requirements for PTC readthrough potentiation by CDX5 and to identify more potent analogs, structural derivatives were prepared wherein both aromatic rings were systematically functionlized with a wide range of polar and non-polar functional groups. Additionally, template hopping was explored to provide a more diverse family of core structures. Over 170 compounds were prepared and their effect on p53 R213X readthrough in HDQ-P1 cells was analyzed by automated fluorescence microscopy at different concentrations, with and without 50 μM G418. None of these compounds increased the number of cells with nuclear p53 when used alone but eight compounds, when combined with G418, increased nuclear p53 over 2-fold compared to G418 alone (Supplementary Table S2). Western analysis confirmed that the eight compounds did not induce PTC
Chemical suppression of yeast nonsense alleles. (A, B) Suppression of met8-1 and trp5-48 nonsense alleles by aminoglycosides. Yeast cells were grown in 96-well plates without methionine (A) or without tryptophan (B) and with the indicated concentrations of aminoglycosides. Yeast growth (A600) was measured at 40 h. Shown are mean ± S.D. (n = 3). (C–F) Synergistic suppression of met8-1, trp5-48 and lys2-101 nonsense alleles by CDX compounds and paromomycin. (C) Structures of the CDX compounds identified in the high throughput screen. (D and E) yeast cells were grown in 96-well plates without methionine (D) tryptophan (E) or lysine (F) and with different concentrations of the CDX compounds shown above each column, without (colored curves) or with paromomycin (black curves). The selected paromomycin concentration of 2.5 μM (D) or 10 μM (E and F) was subactive for these alleles. The dashed lines labelled P10 or P100 indicate growth with paromomycin alone at 10 or 100 μM, respectively. The +4 nucleotide is indicated for each codon. The asterisks indicate synergistic combinations (CI < 0.1). Shown are mean ± S.D. (n = 3).
readthrough when used alone (Figure 3). When combined with 25 μM G418, CDX5-1, CDX5-4 and CDX5-35 induced more readthrough than the parent compound CDX5 (Figure 3). CDX5-1 was the most potent, inducing PTC readthrough at a lower concentration of 10 μM in combination with G418 (Figure 3). CDX5-1 also potentiated PTC readthrough by high concentrations of gentamicin (Figure 3D). CDX5-1 was therefore chosen for further study.

**Time course of p53 PTC readthrough and mRNA accumulation**

To gain a better understanding of readthrough potentiation by CDX5-1, we measured the accumulation of full-length p53 and truncated p53 over the course of 96 h exposure of HDQ-P1 cells to the compounds (Figure 4). G418 at a low concentration of 25 μM induced a very small amount of full-length p53, first detectable at 48 h and increasing to 2.3-fold at 96 h (Figure 4A and B). 50 μM CDX5-1 induced no full-length p53 at any time tested (Figure 4A and B). When G418 was combined with CDX5-1, full-length p53 levels increased dramatically to nearly 400 times the level of p53 present in untreated cells (Figure 4A and B). Furthermore, full-length p53 became first detectable after only 4 h exposure to the combination and increased steeply from 30- to 361-fold from 24 to 72 h, after which it reached a plateau. This time course illustrates the magnitude by which CDX5-1 potentiates G418 PTC readthrough activity.

We also measured TP53 mRNA during the time course experiment. The level of TP53 mRNA in untreated HDQ-P1 cells was ~12% of that found in HCT116, a cancer cell
line with WT TP53, consistent with its recognition as a PTC-containing mRNA followed by degradation by non-sense mediated RNA decay (NMD). HDQ-P1 cells exposed to G418 showed a ~2-fold increase in TP53 mRNA at 72 and 96 h (Figure 4C). CDX5-1 caused no increase in TP53 mRNA at any time point. Exposure to both G418 and CDX5-1 resulted in a much larger, time-dependent increase in TP53 mRNA (Figure 4C); TP53 mRNA increased ~3-fold by 4 h, coinciding with the first detection of readthrough by western analysis, and it increased further to a maximum of 9-fold at 72 h. Therefore, CDX5-1 also potentiates the stabilization of TP53 mRNA by G418. The lack of increase in TP53 mRNA during exposure to CDX5-1 alone shows that it does not transcriptionally activate the TP53 gene. This result also indicates that exposure of HDQ-P1 cells to CDX5-1 alone does not inhibit TP53 mRNA degradation and that it does not potentiate the PTC readthrough activity of G418 by inhibiting NMD. CDX5-1 and G418 alone did not change the expression of p53 downstream target genes P21, PUMA, and miRNA34a but the combination of CDX5-1 and G418 significantly increased their expression (Supplementary Figure S3), indicating that p53 produced by PTC readthrough is active as a transcription factor.
Potentiation of readthrough at all three termination codons

Previous work showed that basal and gentamicin-induced PTC readthrough efficiency differs for the three termination sequences, being highest for TGA, intermediate for TAG and lowest for TAA (47). To determine whether the identity of the termination codon influences readthrough by the compound combination, we generated four TP53 cDNA expression plasmids containing the WT arginine codon CGA or the termination codons TGA, TAG or TAA at position 213. Following transient transfection of p53-null H1299 cells with each construct, the cells were exposed to CDX5-1, G418, or both for 48 h. Untreated cells transfected with WT TP53 cDNA expressed only full-length p53, as expected, and exposure to the compounds did not affect WT p53 levels (Figure 5A). Untreated cells transfected with p53 R213X (TGA, TAG or TAA) constructs all expressed a prominent truncated p53 band and only p53 R213X (TGA) showed a small amount of full-length p53 indicating a low level of spontaneous readthrough at this PTC (Figure 5A). G418 induced full-length p53 production more efficiently at TGA than TAG, and no detectable readthrough at TAA. CDX5-1 alone at 10 μM did not increase PTC readthrough in any of the cells. However, the combination of CDX5-1 and G418 increased full-length p53 production at the TGA, TAG and TAA codons compared to G418 alone (Figure 5A). These findings indicate that the combination of CDX5-1 and G418 is able to induce full-length p53 from all three different PTC sequences at position R213, with highest efficiency for TGA, followed by TAG and TAA codons.

PTC readthrough in vitro

Aminoglycosides, which bind to ribosomes and directly target the translational machinery, can induce PTC readthrough in cell-free in vitro translation assays (48–51). To gain insight into the mechanism by which CDX5-1 potentiates readthrough by G418, we examined its activity in a HeLa cell-free translation assay. TP53 mRNA bearing the R213X (UGA-C) PTC was generated by in vitro transcription and further 5′-capping and 3′ poly(A)-tail addition. This mRNA was translated efficiently in vitro, form-
Figure 5. PTC readthrough at p53 R213X (TGA, TAG and TAA) sequences in cells and in vitro. (A) H1299 cells transiently transfected with the indicated constructs were exposed for 48 h to CDX5-1 and G418, alone or in combination and full-length and truncated p53 was quantified relative to the amount of p53 in untreated cells. p53-WT: R213; Mock: transfection reagents only. (B) PTC readthrough in a cell-free translation extract. The indicated 5′ capped and 3′ poly(A) tailed TP53 mRNAs were subjected to in vitro translation in the presence of the indicated compounds. Full-length p53 and truncated p53 were quantified relative to full-length p53 (in WT) or truncated p53 (in mutants) in untreated reactions.

We also examined the effect of the compounds on in vitro translation of TP53 mRNA bearing a UAG or a UAA instead of UGA at codon 213. Little or no spontaneous readthrough was observed with these nonsense codons (Figure 5B). G418 induced readthrough less efficiently at UAG compared to G418 alone (Figure 5B). Additionally, the compounds, alone or in combination, had no effect on the translation of WT TP53 mRNA (Figure 5B). Therefore, CDX5-1 increased readthrough by G418 in vitro, but the enhancement observed was less pronounced than in cells.

PTC readthrough at different positions in the TP53 gene

To determine whether the compound combination can induce p53 PTC readthrough at positions other than R213X and in cancer cell lines from different tissue origins we selected ten human cancer cell lines with homozygous TP53 nonsense mutations at different positions in the TP53 coding region (Figure 6). These cell lines originate from breast, lung, ovary and endometrium and all contain a different spectrum of somatic alterations according to the COSMIC Cell Line Project. Exposure to 50 μM CDX5-1 alone did not induce p53 PTC readthrough in any of the cell lines, as was the case for HDQ-P1 cells. 25 μM G418 induced measurable PTC readthrough in only two of the cell lines, ESS-1 (R213X, TGA) and NCI-H1688 (Q192X, TAG). The combination of 25 μM G418 and 50 μM CDX5-1 induced mea-
mozygous nonsense mutation (R17X/\textit{SMARCA1}) that encodes dystrophin, and the SIOD cells have a homoygous nonsense mutation (E2035X) in the lysosomal enzyme tripeptidyl peptidase 1. The DMD myocytes have compound heterozygous nonsense mutations (R208X) in the \textit{DMD} gene. To exemplify whether the combination of CDX5-1 and G418 is effective against a considerably wider range of \textit{TP53} nonsense mutations than G418 alone.

PTC readthrough in rare genetic disease models

Compounds that induce PTC readthrough could in principle be used to treat inherited diseases caused by nonsense mutations. To exemplify whether the combination of compounds identified in this study can also induce readthrough of PTCs causing genetic diseases, primary fibroblasts from a late infantile neuronal ceroid lipofuscinosis (CLN2) patient, a myoblast line established from a patient with DMD, and a fibroblast line derived from a patient with Schimke immuno-osseous dysplasia (SIOD) were selected. The CLN2 cells have compound heterozygous nonsense mutations (R127X, R213X, TGA-C) in the \textit{TP53} gene. The cell lines (mutations shown in parentheses), were exposed for 96 h to CDX5-1 and G418, alone or in combination, and full-length p53 (red arrowhead) and truncated p53 (black arrowhead) was quantified relative to truncated p53 found in untreated cells, or to truncated p53 found in treated cells if no p53 signal was detected in untreated cells. The +4 nucleotide is indicated for each codon.

![Figure 6](http://nar.oxfordjournals.org/)

Figure 6. PTC readthrough in different human cancer cell lines with homozygous nonsense mutations at different positions in the \textit{TP53} gene. The cell lines (mutations shown in parentheses), were exposed for 96 h to CDX5-1 and G418, alone or in combination, and full-length p53 (red arrowhead) and truncated p53 (black arrowhead) was quantified relative to truncated p53 found in untreated cells, or to truncated p53 found in treated cells if no p53 signal was detected in untreated cells. The +4 nucleotide is indicated for each codon.

![Table](http://nar.oxfordjournals.org/)

| Cell Line | CDX5-1 (μM) | G418 (μM) | TR-p53 | FL-p53 |
|-----------|-------------|-----------|--------|--------|
| MDA-MB-361 (E56X, TAA-G) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |
| Caov-3 (Q136X, TAA-C) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |
| SW900 (Q187X, TAG-C) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |
| NCi-H1688 (Q192X, TAG-C) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |
| Calu-6 (R196X, TGA-G) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |
| EK/1X (E204X, TAG-T) | 0 50 0 50 | 0 0 25 25 | 0 0 0 0 | 0 0 0 0 |

Nonsurivable readthrough in 7 of 8 cell lines with TGA (Calu-6, ESS1, HCC1937, UACC-893) or TAG (SW900, NCi-H1688, SK-MES-1) PTCs (Figure 6), and very weakly in one of the two cell lines with a TAA termination codon, MDA-MB-361 (E56X). Neither compound alone nor in combination induced p53 production in H1299 p53-null cells or increased p53 levels in HCT116 cells with WT \textit{TP53}. These findings show that the combination of CDX5-1 and G418 is effective against a considerably wider range of \textit{TP53} nonsense mutations than G418 alone.

9 days. TPP1 enzyme activity was measured using a fluorometric assay and expression of full-length TPP1, dystrophin and SMARCAL1 proteins were assessed by immunoblotting. Untreated CLN2 cells displayed no detectable TPP1 enzyme activity (Figure 7A). Exposure to CDX5-1 alone induced no enzyme activity. G418 induced a time-dependent increase in activity first detectable at day 3. The combination induced greater TPP1 activity at all time points tested (Figure 7A). The maximum TPP1 activity observed was 17% of the average activity obtained from fibroblasts of two unaffected individuals. The extracts used for enzyme activity measurement were also subjected to western analysis to examine formation of full-length TPP1. Fibroblasts from unaffected individuals showed two immunoreactive bands corresponding to the proenzyme and mature form of TPP1 (37) and patient fibroblasts showed no detectable bands (Figure 7B). As expected from the TPP1 enzyme activity results, CDX5-1 alone induced no TPP1 protein. G418 alone caused a time-dependent increase in both TPP1 proenzyme and mature form and the combination of G418 and CDX5-1 induced more TPP1 protein than G418 alone at all time points tested (Figure 7B). Similarly, untreated or CDX5-1-treated cells from DMD and SIOD patients did not show any detectable full-length dystrophin or SMARCAL1, respectively. Treatment with G418 alone induced a small amount of full-length dystrophin in DMD cells and some full-length SMARCAL1 in SD123. The combination of G418 and CDX5-1 induced more full-length dystrophin and SMARCAL1 in DMD and SIOD cells, respectively (Figure 7C and D).
We also tested the effect of G418 and CDX5-1, alone and in combination, on the viability of HDQ-P1, HCT116, fibroblast and myoblast cell lines (Supplementary Figure S5). During three-day exposure to 25 μM CDX5-1, a concentration that effectively potentiated readthrough in rare disease cells, the rate of cell proliferation decreased and proliferation resumed when the compound was washed away (Supplementary Figure S5). 50 μM CDX5-1, which maximally potentiated p53 readthrough in HDQ-P1 cells, caused a more pronounced slowing of cell proliferation, which was also reversible upon removal of the compound. G418 at 25 or 50 μM had little or no effect on cell proliferation. Combining G418 and CDX5-1 had little additional effect on proliferation compared with CDX5-1 alone, except for fibroblasts, which appeared to become stably arrested.

**DISCUSSION**

Currently available PTC readthrough compounds show only weak activity *in vitro* and in animals, and those tested in clinical trials show response only in a fraction of patients (52), which restricts their usefulness as therapies. Our identification of small molecules that strongly potentiate PTC readthrough by paromomycin at multiple nonsense alleles in yeast was encouraging because it raised the possibility that combining an aminoglycoside with a potentiator might also induce more efficient and broad PTC readthrough in human cells. The phthalimide class of small molecules represented by CDX5 exerted similar effects on TP53 R213X in human cells similar to those observed for alleles like lys2-101 in yeast; CDX5 was inactive as a single agent but, in combination with paromomycin in yeast or G418 in human cells, it strongly potentiated PTC readthrough.

A range of modifications to the CDX5 benzyl ring and phthalimide rings, including polar and non-polar substitutions, heteroaromatics and altered ring size, mostly caused loss of activity and yielded no compounds with higher potency than the parent screening hit. A template hopping approach produced CDX5-1 as the single more potent compound. The extent to which CDX5-1 potentiated the readthrough activity of G418 was remarkable, as illustrated in Figure 3 and particularly in Figure 4B, where CDX5-1 increased G418-induced readthrough at p53 R213X up to 180-fold compared to G418 alone. Equally striking was that CDX5-1 accelerated the earliest detection of readthrough from 48 h for G418 alone to 4 h for the combination.

Studies using reporter constructs *in vitro* and in cell culture have shown that the relative level of basal or aminoglycoside-induced PTC readthrough is
Our results show that CDX5-1 can potentiate the readthrough activity of G418 at all three PTCs and that it does not alter this sequence preference. The presence of a uracil just before and of a cytosine just after a PTC in mRNA increases readthrough by gentamicin (47). In HDQP-1 cells TP53 R213X is flanked by these two nucleotides, likely providing an optimal sequence context for G418-induced readthrough. Substitution nonsense mutations account for 8% of TP53 mutations in cancer. R213X is the most common, affecting 1.3% of patients (COSMIC), but there are several others, most of which are not in an optimal sequence context. Our analysis of ten additional human cancer cell lines with homozygous TP53 nonsense mutations at ten different positions provides a comprehensive description of PTC readthrough in an endogenous gene. In two of these two cell lines the PTC was flanked by 5’T and 3’C, one with the same R213X mutation as HDQ-P1, and the other with Q192X. Both showed readthrough with G418 alone and increased readthrough with the combination. The eight others had either one or none of these flanking nucleotides and showed no readthrough with G418 alone. Six had some level of response with the combination while two showed no response. Therefore, combining G418 with CDX5-1 can elicit PTC readthrough even in non-optimal sequence contexts.

The time course of accumulation of full-length p53 protein and of mRNA during exposure to G418 and CDX5-1 is also informative. During the first 12 h, full-length p53 protein rose slowly, followed by a period of rapid full-length p53 formation that reached a half-maximal point after 45 h (Figure 4). The p53 mRNA increased slowly and by less than 3-fold during exposure to G418 alone but it increased more rapidly and to nearly 9-fold with the combination before reaching a plateau. The sigmoidal curve of protein accumulation is compatible with a self-amplification mechanism whereby TP53 mRNA is normally kept at a very low level because translation of any PTC-containing mRNA molecule leads to its recognition and degradation by NMD. Stalling of a translating ribosome at a PTC and inefficient termination of translation are believed to be triggers for NMD (59,60). The combination of G418 and CDX5-1 may facilitate rapid pairing of a near-cognate aminoacyl-tRNA at the PTC-bound ribosome and incorporation of an amino acid residue, leading not only to formation of full-length p53 but also to escape from recognition by NMD in a fraction of these mRNAs. Further translation of the PTC-containing mRNAs that escaped degradation would then lead to cycles of additional readthrough and further mRNA stabilization. In this model, TP53 mRNA initially increases as a consequence of readthrough but the increased mRNA levels in turn enable additional readthrough events. Such a self-amplification loop could explain the sigmoidal increase in full-length p53 over time.

The combination of CDX5-1 and G418 appears to induce PTC readthrough less efficiently at TP53 nonsense mutations situated closer to the 3’ end of the transcript and showing high levels of truncated p53 (Figure 6). It is likely that translation termination is more efficient at these 3’-proximal PTCs and consequently that the ribosomes do not stall for as long as they do at R213X, thus lowering the probability that a near-cognate aminoacyl-tRNA will pair before translation termination, and consequently reducing readthrough. Therefore, more efficient drug-induced readthrough would occur at PTCs with favorable flanking nucleotides and positions that do not permit efficient translation termination.

Our analysis of ten additional human cancer cell lines with nonsense mutations at ten different positions provides a comprehensive description of PTC readthrough in an endogenous gene. In two of these two cell lines the PTC was flanked by 5’T and 3’C, one with the same R213X mutation as HDQ-P1, and the other with Q192X. Both showed readthrough with G418 alone and increased readthrough with the combination. The eight others had either one or none of these flanking nucleotides and showed no readthrough with G418 alone. Six had some level of response with the combination while two showed no response. Therefore, combining G418 with CDX5-1 can elicit PTC readthrough even in non-optimal sequence contexts.
ification or whether they are mechanistically linked and not separable. It will be important to further optimize the compounds and test them in animal models to determine whether therapeutically relevant PTC readthrough can be achieved with a good therapeutic window in vivo.

Overall, these findings raise hope that combinations of an aminoglycoside and a potentiator might enable use of lower aminoglycoside doses to minimize adverse effects such as nephrotoxicity and ototoxicity, as well as induce PTC readthrough more broadly than current drugs to benefit a larger number of patients.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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