LETTERS

PTC124 targets genetic disorders caused by nonsense mutations

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Nonsense mutations promote premature translational termination and cause anywhere from 5–70% of the individual cases of most inherited diseases[1]. Studies on nonsense-mediated cystic fibrosis have indicated that boosting specific protein synthesis from <1% to as little as 5% of normal levels may greatly reduce the severity or eliminate the principal manifestations of disease[2,3]. To address the need for a drug capable of suppressing premature termination, we identified PTC124—a new chemical entity that selectively induces ribosomal readthrough of premature but not normal termination codons. PTC124 activity, optimized using nonsense-containing reporters, promoted dystrophin production in primary muscle cells from humans and *mdx* mice expressing dystrophin nonsense alleles, and rescued striated muscle function in *mdx* mice within 2–8 weeks of drug exposure. PTC124 was well tolerated in animals at plasma exposures substantially in excess of those required for nonsense suppression. The selectivity of PTC124 for premature termination codons, its well characterized activity profile, oral bioavailability and pharmacological properties indicate that this drug may have broad clinical potential for the treatment of a large group of genetic disorders with limited or no therapeutic options.

Nonsense mutations give rise to in-frame UAA, UAG or UGA codons in the messenger RNA coding region, lead to premature translational termination and truncated polypeptide products, and promote mRNA destabilization by nonsense-mediated mRNA decay (NMD)[1]. The NMD pathway depends on a set of three conserved factors that modulate both transcript stability and translation termination efficiency[4,5]. Inactivation of any of these stabilizes nonsense-containing transcripts and promotes nonsense codon readthrough[6–10]. Such observations indicated that a nonsense-containing mRNA might produce significant amounts of functional protein if either its decay rate or extent of premature termination is altered.

High concentrations of aminoglycosides such as gentamicin promote readthrough of premature nonsense codons in mammalian cells[11] and in animal models of nonsense mutation diseases. Gentamicin treatment of the *mdx* mouse—a model of Duchenne muscular dystrophy (DMD)—and of a mouse model of cystic fibrosis led to its evaluation in patients[12–16]. Treatment of patients harbouring nonsense mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) or dystrophin genes promoted production of the respective missing proteins; however, the lack of potency, the potential renal and otic toxicities, and the need for intravenous or intramuscular gentamicin administration have limited the clinical usefulness of this approach. These proof-of-concept experiments, and the effects of the NMD pathway on mRNA turnover and premature translation termination efficiency, strongly indicated that an orally bioavailable, non-toxic, small-molecule drug that promotes selective and specific readthrough of disease-causing premature termination codons might alleviate the pathologies of nonsense-mediated diseases and we therefore sought an alternative approach.

Two high-throughput screens (comprising ~800,000 low molecular weight compounds) were performed to identify compounds that promoted UGA nonsense suppression. Chemical scaffolds were identified and optimized through extensive medicinal chemistry efforts. Minimally toxic compounds demonstrating UGA readthrough activity were intensively characterized (see Supplementary Information). These analyses identified PTC124 (3-[5-(2-fluorophenyl)-[1,2,4]oxadiazol-3-yl]-benzoic acid; $C_{15}H_9FN_2O_3$) as a candidate for further development. PTC124 is a 284.24 Da, achiral, 1,2,4-oxadiazole linked to fluorobenzene and benzoic acid rings (Supplementary Figs 1 and 2). The compound has no structural similarity to aminoglycosides or other clinically developed drugs, and its anhydrous free carboxylic acid form, despite having low aqueous solubility ($<1\,\mu g\,ml^{-1}$), is orally bioavailable when prepared in aqueous suspension.

PTC124 promoted dose-dependent readthrough of all three nonsense codons in stable cell lines harbouring *LUC-190* nonsense alleles. Levels of suppression correlated inversely with established termination efficiencies[11,17–19], with the highest readthrough at UGA, followed by UAG and then UAA (Fig. 1a). Differences in transcript levels do not account for the observed differences in readthrough (Supplementary Fig. 3a). The minimal concentration of PTC124 showing discernable readthrough was 0.01–0.1 μM (2.8–28 $ng\,ml^{-1}$), whereas the concentration promoting maximal activity was approximately 3 μM (852 $ng\,ml^{-1}$). Because gentamicin is only active at much higher concentrations (Fig. 1b, and data not shown), PTC124 is a more potent nonsense-suppressing agent in this system.

Termination efficiencies are also influenced by the nature of the nucleotide following the nonsense codon (the +1 position)[18–20]. Like

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gentamicin[11], PTC124 was most active when a pyrimidine (in particular cytosine, C) was located in the +1 position (data not shown). The UGAG termination context was the only efficiently suppressed exception to this trend. Additional experiments with *LUC-190* constructs established that PTC124 did not suppress multiple proximal nonsense codons and that continuous exposure to PTC124 maximizes and maintains suppression activity (Supplementary Fig. 3c–e).

PTC124 promoted suppression of human and mouse nonsense alleles of the dystrophin gene. Drug-treated and untreated primary muscle cell cultures from DMD patients or *mdx* mice were subjected to immunocytochemistry with an antibody recognizing a carboxy-terminal dystrophin epitope. Readthrough of dystrophin mRNA premature nonsense codons was evident in all samples and at all PTC124 concentrations tested. Dystrophin was present at the myofibre membrane, the location critical for maintenance of muscle structural integrity. Consistent with the stable cell line reporter assay (Fig. 1a), the most efficient readthrough was observed in cultures treated with 5 $\mu g\,ml^{-1}$ (17 μM) of PTC124, and there was no further increase at 10 $\mu g\,ml^{-1}$ (Fig. 2). The ratio of dystrophin:myosin obtained at 5 $\mu g\,ml^{-1}$ of PTC124 was 40–60% of normal ($n = 2$; Fig. 2 and data not shown). For the *mdx* samples, the dystrophin:myosin ratio was approximately 35% of normal at 10 $\mu g\,ml^{-1}$ ($n = 8$). Normal myotubes were unaffected by PTC124, and the DMD and *mdx* myotubes had no detectable expression in the absence of drug (Fig. 2).

The ability of PTC124 to promote nonsense suppression was also assessed using *mdx* mice; this analysis required a dosing regimen that would maintain target plasma concentrations of 5–10 $\mu g\,ml^{-1}$. Oral feeding of PTC124 by a liquid diet achieved significantly better blood exposure than when administered by intraperitoneal injection (Supplementary Fig. 4). However, to prolong PTC124 exposure during the day (sleep period), the drug was administered intraperitoneally three times per day in addition to oral ingestion by the liquid diet. Using this combination regimen, plasma levels remained >10 $\mu g\,ml^{-1}$ at nearly all measured time points (Supplementary Fig. 4).

The functional effects of PTC124 were monitored in *mdx* mice treated with oral, intraperitoneal or combined dosing for 2–8 weeks (during which time there was no significant difference in weight gain among the treatment and control groups). As shown in Fig. 3a, 4 weeks of PTC124 treatment by any of the 3 treatment regimens partially rescued the functional strength deficit (decreased force per cross-sectional area) characteristic of the *mdx* mouse. Equivalent results were obtained at 2 and 8 weeks (data not shown).

The major functional deficit in dystrophic muscles of *mdx* mice (and, most likely, in DMD patients) is increased susceptibility to contraction-induced injury, especially in muscle that is simultaneously stretched[20]. This susceptibility results in repeated cycles of degeneration–regeneration, ongoing inflammation and necrosis, with the eventual destruction of muscle. Thus, the best predictor of long-term therapeutic outcome may be protection from contraction-induced injury. The mean percentage drop in force after five eccentric contractions of extensor digitorum longus (EDL) muscles from PTC124-treated or control animals is shown in Fig. 3b. Either intraperitoneal injections or oral dosing alone of PTC124 for 4 weeks resulted in partial protection against contraction-induced injury in the EDL muscles. The effects at 2 and 8 weeks were similar (data not shown). When oral and intraperitoneal dosing were combined (Fig. 3b), a further improvement in protection against contraction-induced injury was observed, such that the decrement in force was not different to that of wild-type (C57) mice.

Elevated levels of creatine kinase are found in the serum of both *mdx* animals and DMD patients. Over 8 weeks, vehicle-treated *mdx* mice showed little change in serum creatine kinase values (data not shown), but those treated with combined oral and intraperitoneal dosing demonstrated significant reductions in serum creatine kinase values by 2 weeks (Fig. 3c), which were maintained for up to 8 weeks. These data corroborate the findings of reduced eccentric contraction injury in the EDLs and suggest that PTC124 leads to decreased muscle fragility.

The functional recovery was associated with dystrophin production, as measured by western blotting. Full-length dystrophin was detected in both C57- and PTC124-treated *mdx* mice (Fig. 3d). *mdx* animals treated with the combined regimen had dystrophin levels approximately 20–25% that of muscles from C57 mice (Fig. 3d). PTC124-treated *mdx* mouse muscles also exhibited increased levels of γ-sarcoglycan (Fig. 3d), consistent with production of dystrophin and stabilization of the dystrophin-associated membrane complex, which is missing in the absence of dystrophin. To confirm the proper membrane localization of dystrophin and associated proteins, striated muscles were subjected to immunohistological analyses. Partial restoration of dystrophin to the membrane was detected in

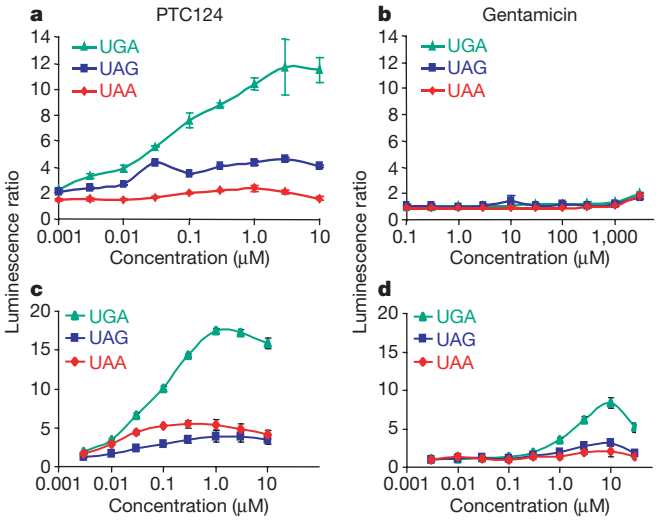


Figure 1 | PTC124 suppresses premature nonsense codons. **a**, **b**, Cultured HEK293 cells harbouring UAA, UAG or UGA *LUC-190* nonsense alleles were treated with increasing concentrations of PTC124 (**a**) or gentamicin (**b**) for 16 h, and assayed for luciferase activity. **c**, **d**, Synthetic *LUC* mRNAs, each harbouring different premature termination codons, were incubated with HeLa cell-free extract supplemented with varying concentrations of PTC124 (**c**) or gentamicin (**d**), and assayed for luciferase activity after 4 h. Luminescence ratio, drug-treated:control; error bars (±s.d.) are derived from three independent experiments.

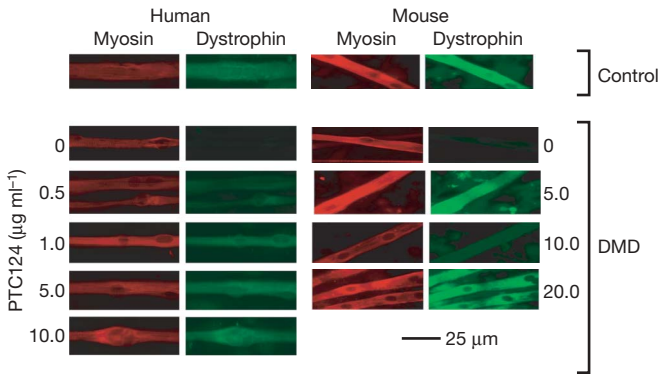


Figure 2 | Full-length dystrophin is produced in PTC124-treated cultured myotubes. Immunohistochemistry of myotubes from primary cell cultures derived from muscle biopsies. Left panel, a non-DMD individual (control) and a patient with a premature UGA codon in exon 28 of the dystrophin gene (*DMD*). Right panel, wild-type C57 mice (control) and *mdx* mice containing a premature UAA codon in exon 23 of the dystrophin gene (*Dmd*). The effect of adding varying amounts of PTC124 on levels of detectable dystrophin (green) is shown. Myosin (red) levels remain unchanged.

all skeletal muscles examined, including tibialis anterior, diaphragm and cardiac muscle (Fig. 3e). The intensity of fluorescence in drug-treated animals, compared with revertant fibres (white arrow, Fig. 3e), is indicative of lower levels of protein than in wild-type animals, consistent with the data of Fig. 3d.

In principle, nonsense suppression could result from an increase in stop codon readthrough, a decrease in mRNA turnover, or both[7–10]. To elucidate PTC124's mechanism of action, we monitored its effects on the translation and stability of nonsense-containing mRNAs *in vitro*. PTC124 promoted readthrough at each of the nonsense codons (Fig. 1c), showing maximal activity with UGA, while having no effect on mRNA levels (data not shown). Unlike the stable cell line assays (Fig. 1a, b), PTC124 did not discriminate significantly between the UAG and UAA mRNAs. PTC124 was a more potent nonsense-suppressing agent than gentamicin (Fig. 1d), and exhibited 4- to 15-fold stimulation of *in vitro* readthrough relative to the controls (Fig. 1c) at levels similar to those in the stable cell reporter assays. These results indicate that PTC124 modulates termination efficiency at premature nonsense codons.

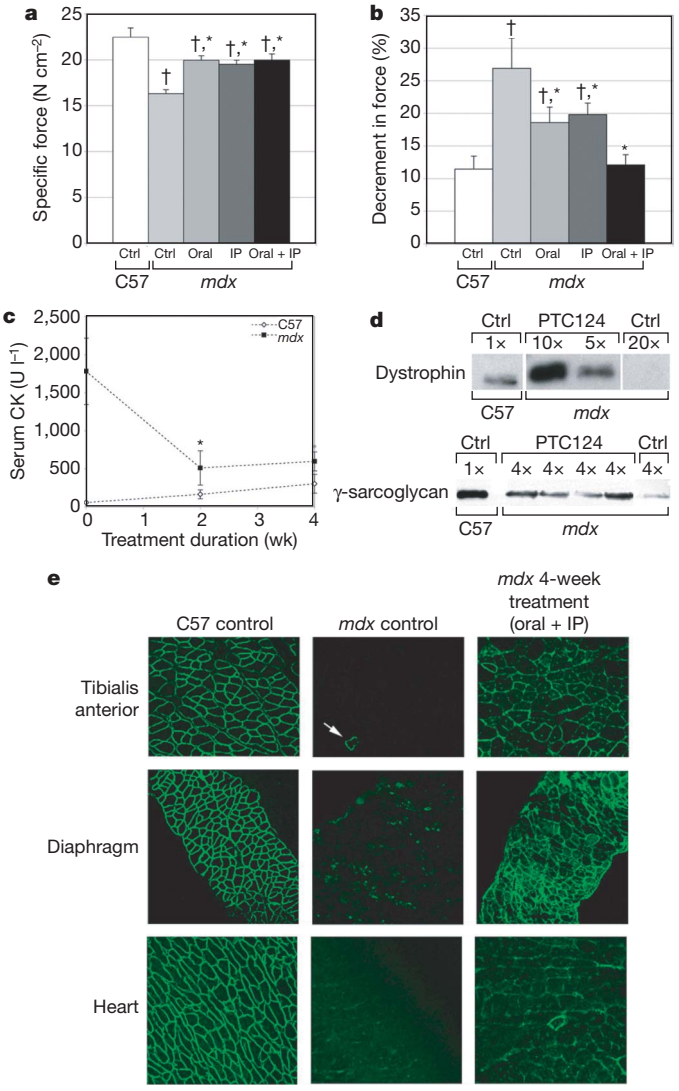
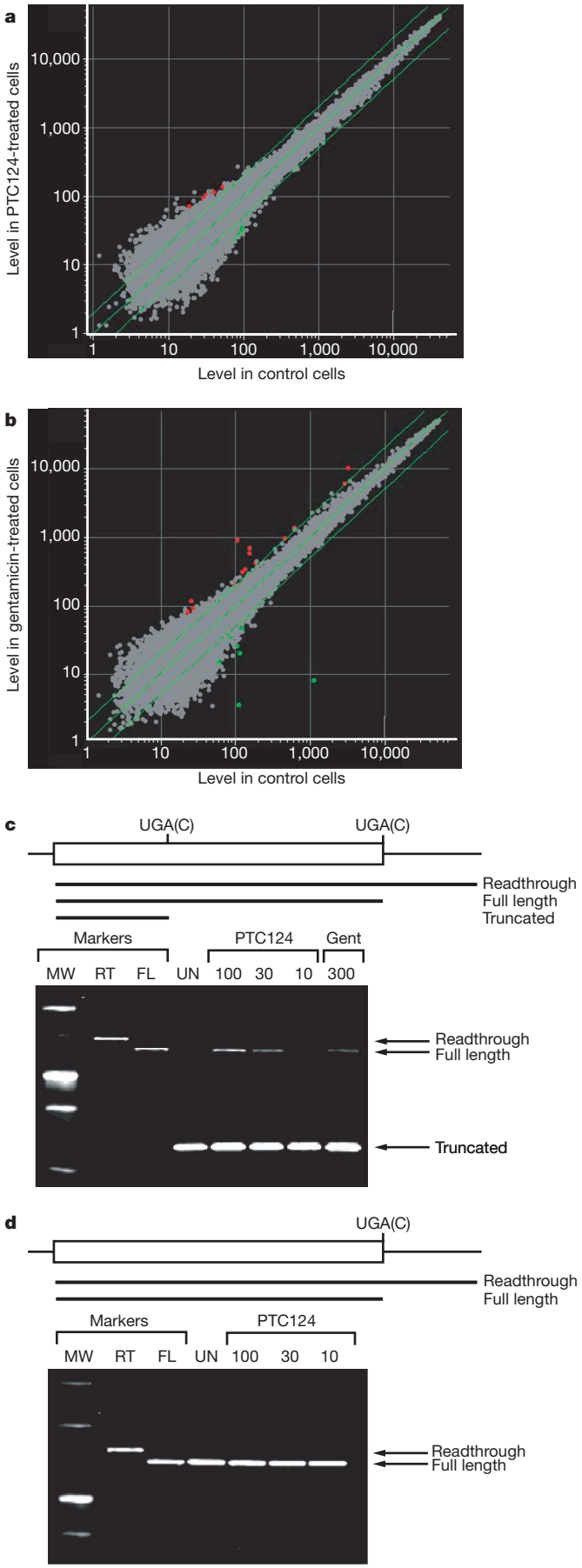


Figure 3 | Rescue of the dystrophic phenotype in muscles of the *mdx* mouse. **a**, The effects of oral dosing, intraperitoneal (IP) injections or combined dosing of PTC124 on the force per cross-sectional area (specific force) of the EDL muscle after 4 weeks of drug treatment. **b**, The effects of 4 weeks of oral dosing, IP injections or combined dosing of PTC124 on preventing loss-of-force production in the EDL muscle following five successive eccentric contractions. **c**, Serum creatine kinase (CK) changes in *mdx* mice after PTC124 dosing. †$P < 0.05$ for comparisons to untreated C57 mice; *$P < 0.05$ for comparisons to untreated *mdx* mice. **d**, Western blot analysis of quadriceps and tibialis anterior muscles for dystrophin and γ-sarcoglycan. Protein load of each lane is indicated relative to the load of C57 wild-type (1×). Ctrl, control. **e**, Immunohistochemistry of indicated muscle cross-sections to visualize dystrophin in the tibialis anterior, diaphragm and heart. The white arrow in the tibialis anterior negative control designates a revertant fibre. **a–c**, Data expressed as means ± s.e.m.; $n = 8$.

LUC-190 transcripts were also monitored by quantitative PCR with reverse transcription (RT–PCR) in cells treated with PTC124 or cycloheximide, a well characterized translation inhibitor that promotes mRNA stabilization[21]. These experiments showed that nonsense-containing *LUC* mRNA levels increased 11-fold in cycloheximide-treated cells, but were unaffected in PTC124-treated cells (data not shown). These observations were extended to a larger pool of mRNAs—including known NMD substrates[22]—by using oligonucleotide microarrays to analyse mRNA expression profiles in HEK293 cells treated for 48 h with or without PTC124 or gentamicin. The averages of six independent comparisons of >54,000-probe sets suggested that very few transcripts deviated from equivalent expression in PTC124-treated versus control cells (Fig. 4a); only 12 transcripts with increased levels and ten with decreased levels were identified in PTC124-treated cells (Supplementary Table 1). None of these are known NMD substrates[22] and all 22 were among the lowest expressing cellular mRNAs; that is, they were normally subject to the largest experimental error. No observed differences were detectable by subsequent northern analysis (data not shown). In contrast, six independent comparisons of cells treated or not treated with gentamicin indicated increases in the abundance of 20 transcripts and decreases in the abundance of 11 additional transcripts, including several for which the fold-change was substantial (Fig. 4b; Supplementary Table 2; Supplementary Fig. 5). There was no overlap of transcripts with altered levels in PTC124-treated cells versus gentamicin-treated cells (Supplementary Tables 1 and 2). These results indicate that: (a) the synthesis and stability of few, if any, cellular mRNAs are altered in response to levels of PTC124 that promote nonsense suppression; (b) PTC124 demonstrates little off-target activity at the level of transcription or mRNA stability; and (c) PTC124 and gentamicin have distinct effects on gene expression. Consistent with the latter conclusion, PTC124 does not manifest antibacterial activity (Supplementary Table 3).

Nonsense-suppressing drugs could theoretically promote non-specific readthrough of normal termination codons, but available evidence suggests that normal and premature termination differ mechanistically[9,10,23], thereby implying a basis for selectivity. To test directly for the selectivity of PTC124-promoted readthrough, we monitored the accumulation of truncated, full-length and readthrough luciferase polypeptides in drug-treated HEK293 cells, and also assayed for the presence of specific readthrough polypeptides in humans, rats and dogs. Cells harbouring the *LUC-190-CD40* construct (Fig. 4c, d) were treated with PTC124, and luciferase immunoprecipitated from cell lysates was analysed by western blotting. In the absence of drug, translation of the *LUC-190-CD40* mRNA yielded only a 25 kD amino-terminal truncated luciferase fragment, but treatment of cells with PTC124 led to a dose-dependent accumulation of full-length luciferase (Fig. 4c). Importantly, no luciferase was detected that corresponded in size to the product expected from readthrough of the normal terminator (Fig. 4c). The absence of a detectable readthrough product was unlikely to be a consequence of its instability because the control readthrough protein, generated by replacing the termination codon with CGA, was efficiently expressed in the same cells (Fig. 4c, d). Similar analyses also failed to detect polypeptides corresponding to putative readthrough products in multiple tissues isolated from PTC124-treated human subjects, rats and dogs (Supplementary Fig. 6). For example, western blotting analyses of pooled peripheral blood mononuclear cells from subjects treated with 200 mg kg^{-1} of PTC124 elicited only wild-type β2 microglobulin and no additional, longer polypeptides that would



have originated from termination codon readthrough (Supplementary Fig. 6c). These results indicate that PTC124 promotes readthrough of premature termination without affecting normal termination, even at drug exposure levels substantially greater than the values achieving maximal activity. Consistent with this conclusion, two-dimensional gel analyses of HEK293 cells incubated with or without PTC124 showed no significant differences in the relative amounts or shapes of the respective spots representing several hundred polypeptides (Supplementary Fig. 7).

Systemic delivery of PTC124 was achieved without any obvious toxicity, consistent with the findings of preclinical safety pharmacology and toxicology studies in rats and dogs[24]. Given the significance of NMD as a genomic surveillance mechanism[25], PTC124's lack of effect on this pathway may be an important component of its safety. The safety of PTC124 may also be related to the observation that its readthrough activity is specific for premature stop codons. Such selectivity might be expected from the apparent mechanistic differences between premature and normal termination[23], but may also be enhanced by the inability of PTC124 to efficiently promote readthrough of the multiple stop codons normally present in mRNA 3′-UTRs[26], and by the specific mRNA decay mechanism known to be activated when translation extends into the 3′-UTR[27,28].

Clinical trials of PTC124 have been initiated and their successful completion may ultimately allow therapy of subsets of patients in a large and diverse group of genetic disorders for which the primary disease defect is the presence of a nonsense mutation. As such, this approach is among the first to test the model of personalized medicine, in which the focus shifts from treatment of a disease to treatment of a specific genetic defect.

METHODS SUMMARY

PTC124 was identified by a combination of high-throughput screening of a small-molecule chemical library and subsequent lead optimization of compounds exhibiting significant nonsense suppression activity and low toxicity. Optimization protocols selected for high oral bioavailability, lack of *in vitro* off-target activity, *in vivo* safety and suitability for pharmaceutical formulation. Nonsense suppression analyses in the screening and early characterization steps monitored the production of luciferase from *LUC* nonsense-containing mRNAs expressed in HEK293 cells or incubated as synthetic transcripts in cell-free lysates. Suppression of dystrophin nonsense alleles, in patients and in *mdx* mice, was assayed by established procedures, including those monitoring dystrophin accumulation *in vivo* and in cultured myotubes, and those analysing dystrophin function as inferred from the mechanics of isolated mouse EDL muscles. Levels of cellular mRNAs were quantified by high-density microarray analysis and northern blotting, and readthrough of normal termination codons was assessed by western blotting using antibodies targeted to either full-length polypeptides or to putative readthrough-dependent C-terminal extension peptides. Detailed protocols for all assays can be found in Methods.

Figure 4 | PTC124 activity is selective for readthrough of premature translation termination codons. a, **b**, Microarray analyses of mRNA levels in HEK293 cells treated with 5 μM of PTC124 (**a**) or ~300 μM of gentamicin (**b**). The relative level of each transcript in the drug-treated samples was normalized to that in the untreated samples and the resulting expression ratios from 5/6 independent replicates were averaged and plotted on a logarithmic scale for pair-wise comparisons. The centre line indicates the line of equivalence and the outer lines indicate a twofold difference in expression. **c**, **d**, PTC124 does not promote readthrough of normal termination codons. Shown are a cartoon depicting *LUC* reporter constructs, and western blot analysis of *LUC* readthrough protein. Cells harboured *LUC* nonsense (UGAC) or wild-type alleles with 6×-histidine and Xpress epitope tags, inserted in frame with the *LUC* coding region, as well as a CD40 3′-UTR. Cultures were treated with PTC124 (100 μM (28.4 μg ml^{-1}), lane 5; 10 μM (2.8 μg ml^{-1}), lane 6; and 1.0 μM (0.28 μg ml^{-1}), lane 7) for 72 h, and luciferase protein was purified and analysed by western blotting. Readthrough marker protein was derived by mutating the normal termination codon to CGA. M_r, relative molecular mass marker; RT, readthrough protein marker; FL, full-length protein marker; UN, untreated.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

***LUC* constructs.** All DNA manipulations used standard procedures[29] and were confirmed by sequence analysis. Primary sequences of oligonucleotides used for cloning and mutagenesis are listed in Supplementary Table 7. *LUC-190* constructs were initiated by isolating a DNA fragment containing the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) from pBS-E-CAT, using *Apa*I and *Nco*I, and a fragment containing a *β-globin* intron from pmCMV-G1-Norm (kindly provided by L. Maquat), using *Nco*I and *Bam*HI. The two fragments were inserted into *Apa*I/*Bam*HI-digested pBS(SK), the resulting plasmid was digested with *Bam*HI and *Not*I, and the firefly *LUC* gene was then inserted into it as a *Bam*HI–*Not*I fragment from p2Luci[30]. The DNA of interest was transferred to pcDNA3 (-) hygro (Clonetech) using the *Apa*I and *Not*I sites (pPTC29). Site-directed mutagenesis, using the QuikChange Kit (Stratagene) and oligonucleotides 1–6, was used to introduce TAA, TGA and TAG nonsense mutations at position 190 of the *LUC* coding region (pPTC30, pPTC31 and pPTC32, respectively). To monitor nonsense codon context, the +1 nucleotide was changed to C, T or G by site-directed mutagenesis using oligonucleotides 39–56. To monitor the effect of two or three tandem nonsense codons, *LUC-190* was mutagenized using oligonucloetides 15–18. *LUC-190-CD40* constructs were derived by cloning *LUC190* genes in-frame and downstream of the His6 and Xpress epitopes using plasmid pcDNA4-hygro (Clonetech). The CD40 3′-UTR (harbouring 5′-*Not*I and 3′-*Apa*I restriction sites prepared using olignonucleotides 7–10) was inserted downstream of the *LUC* coding region and site-directed mutagenesis (using oligonucleotides 11–14) was used to make two mutations creating sense codons in the remaining vector sequence before the CD40 3′-UTR. Translation into the CD40 3′UTR produces an 84 amino acid (9 kDa) extension of the luciferase protein. To change the termination codon to UGAC, oligonucleotides 57 and 58 were used for site-directed mutagenesis. To prepare plasmid T7-LUC-190, used for the preparation of synthetic mRNA, the pCEL plasmid harbouring the luciferase cDNA[31] was digested with *Hpa*I and *Xho*I to remove the EMCV-Luciferase complementary DNA fragment and the latter DNA was inserted into pBluescriptKS+. This plasmid was digested with *Xho*I for transcription from the T7 promoter. Mutations were introduced using the same oligonucleotides and methods used to construct the stable luciferase reporter cell line constructs.

Assays for readthrough of *LUC* premature termination codons. HEK293 cells growing in medium containing fetal bovine serum (FBS) were stably transfected with *LUC* genes containing premature terminators at codon 190 (and +1 contextual variants thereof) and treated with the indicated concentrations of PTC124, gentamicin or vehicle. Luminescence of each culture relative to the control was quantified by standard procedures. Cell-free translation assays used synthetic *LUC* mRNAs harbouring an EMCV IRES[32] and codon 190 premature terminators (with a +1A) prepared using the MegaScript *in vitro* transcription kit (Ambion). The reaction mixtures (20 μl) contained 16.5 mM HEPES (KOH), pH 7.4, 85 mM potassium acetate, 1.48 mM magnesium acetate, 0.56 mM ATP, 0.075 mM GTP, 18.75 mM creatine phosphate (di-Tris), 1.275 mM dithiothreitol (DTT), amino acids, creatine kinase, HeLa cell cytoplasmic extract (60% of the reaction volume), 100 ng RNA and 0.25% DMSO. Increasing concentrations of PTC124 and gentamicin were added to the *in vitro* reactions, and the amount of luminescence produced was determined after approximately 4 h. Stock solutions of PTC124 (6 mM) were prepared in 100% DMSO (Sigma); gentamicin (sulphate salt, Sigma) stock solutions (1 mM) were prepared in water. Luminescence was determined using a Viewlux CCD imager (Perkin-Elmer). Luminescence data were normalized to that produced with solvent alone (DMSO or water), and the fold suppression over background was calculated as ($PTC124_{light\ units}/DMSO_{light\ units}$) or ($gentamicin_{light\ units}/water_{light\ units}$).

Chemistry. Melting points were determined on an Electrothermal Mel-temp 1201D or Thomas Hoover melting point apparatus, and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian MVX 300 MHz instrument. Mass spectral data of final products were collected from a Waters Micromass ZQ mass spectrometer with an electrospray detector, coupled to a Waters 2795 high-performance liquid chromatograph (LC/MS). Detected were positive ions (ES+) and negative ions (ES−); relative ion intensities are given in per cent. Elemental analyses were obtained by Quantitative Technologies. HPLC methods are as follows: method A—Hewlett-Packard, 254 nm detector; method B—Hewlett-Packard, 280 nm detector; method C—Waters 2795, 254 nm detector.

RNA analysis. Quantitative real-time PCR was performed using primers for *LUC* and dystrophin mRNAs, with the levels of 18S rRNA and GAPDH mRNA serving as normalization factors for the amount of starting material. Northern blot analysis was performed using probes specific to *LUC* mRNA, with the level of GAPDH mRNA serving as a control for loading. High density oligonucleotide arrays (Affymetrix $U133^{+}$, 2.0), containing 54,675 probe sets/chip) were used to analyse expression profiles of PTC124-treated, gentamicin-treated and untreated HEK293 cells. cRNA sample preparation, array hybridization and scanning followed protocols recommended by the manufacturer (http://www.affymetrix.com/index.affx). The hybridization intensity (signal) of each transcript was determined using the Affymetrix Microarray Suite 5.0 software package. Intensity values were scaled such that the overall fluorescence intensity of each array was equivalent. Scaling factors for each experimental set varied by less than 20%. Transcript levels specifically enhanced or reduced by drug treatment were identified by applying several stringent standards to the raw data, including: (a) the signal values of a transcript in the treated and untreated samples had to have a minimal level of at least 26 units and a relative change of at least twofold; (b) these changes had to be reproducible in five of the six independent replicate experiments; and (c) these changes had to demonstrate statistically significant P-values ≤ 0.05 in a group t-test. The GeneSpring (version 7; http://www.silicongenetics.com) and GENECLUSTER (version 1.0; http://www-genome.wi.mit.edu) software packages were used to identify transcripts that were differentially expressed. Primary data can be found at: http://jacobsonlab.umassmed.edu/cgi-bin/pubcontents.cgi?pubcontents = 2006-PTC.

Normal luciferase termination codon in stable cell lines. Cells were treated with PTC124 (up to 100 μM) for 72 h, harvested and lysed, and luciferase was purified using Ni-NTA magnetic agarose beads (Qiagen). Purified proteins were fractionated by PAGE (12.5%) and detected by western blotting, using anti-Xpress antibody (Invitrogen).

Normal termination codons in animals. Sprague-Dawley rats were treated for 14 days with vehicle or PTC124 at doses of 200 to 1,800 mg kg^{-1} day^{-1} and male beagles were treated with vehicle or PTC124 at 500 to 1,500 mg $kg^{-1}day^{-1}$ (Charles River Laboratories). A 14-day recovery period after the last dose was included. Tissues (heart, kidney, liver, lung, small intestine (jejunum and/or ileum), mandibular salivary glands, thymus and peripheral blood mononuclear cells) were collected between 2 and 8 h after the last dose (that is at the time of expected maximal plasma drug concentration) and again at the end of the recovery period. Tissue pieces (5 mm × 5 mm) were snap frozen in liquid nitrogen and stored at −70 °C. Frozen tissues were suspended (1:1) with tissue protein extraction reagent (T-PER; Pierce) containing protease inhibitor cocktail (Sigma) diluted 1:100, and homogenized on ice using a Fisher PowerGen 25 homogenizer equipped with an Omni Tip probe. A 100 μl aliquot was added to 400 μl of sample buffer[29], incubated for 8 min at 100 °C, then transferred to wet ice. Peripheral blood mononuclear cells samples were mixed with 200 μl of sample buffer, heated to 100 °C for 8 min, and transferred to ice. Homogenized samples (50 μl aliquots) were pooled by dose level and sex, and fractionated by SDS–PAGE. Western analyses used the ECL Plus chemiluminescent detection kit (Amersham). Primary mouse monoclonal antibodies for α- and β-actin (Oncogene Research Products), cofilin (BD Biosciences), GAPDH (Abcam), U1 snRNP A (Acris) and vimentin (BD Pharmingen), and a goat anti-mouse IgG horseradish peroxidase (HRP) conjugate as secondary antibody (Promega) were used.

Positive controls for animal studies. The readthrough controls for rat vimentin (Mammalian Gene Collection accession number, BC061847), cofilin (BC086533) or U1 snRNP A (BC086331) were amplified using a rat fetal liver cDNA (Marathon, BD Biosciences, Clonetech), *Pfu* Turbo DNA Polymerase (Stratagene) and oligonucleotide pairs 19 and 20, 27 and 28, and 23 and 24, respectively. PCR products were purified, digested with *Not*I and *Bam*HI, and cloned into pcDNA3.1-hygro (Invitrogen). Mutagenesis of the cloned DNAs, using oligonucleotide pairs 21 and 22, 29 and 30, and 25 and 26 converted the TAA stop codon of vimentin to TAC, the TAG stop codon of cofilin to TGG, and the TGA stop codon of snRNP A to TGG. Dog cofilin (XM_533231) and U1 snRNP A (XM_533663) constructs were prepared using dog kidney cDNA (BioChain) and oesophageal cDNA (BioChain). Oligonucleotide pairs for cloning the cofilin cDNA and then mutagenizing its TGA stop codon to TGG were 35 and 36, and 37 and 38, respectively. For dog U1 snRNP A, PCR amplification used oligonucleotides 31 and 32; mutagenesis to convert the TAG stop codon to TGG used oligonucleotides 33 and 34.

Normal termination codons in healthy volunteers. Peripheral blood mononuclear cells and plasma was obtained from untreated subjects ($n = 3$ at Day 1, Time 0) and at several time points ($n = 3$ per timepoint) relative to the last dose in subjects receiving a single 200 mg kg^{-1}dose of PTC124. Samples were pooled and primary anti-wild-type-protein antibody was used to probe for wild-type protein and anti-readthrough-protein antibody was used to probe for the readthrough protein (Supplementary Fig. 6c). The antibody to the β2 microglobulin readthrough protein was produced in rabbits using a peptide corresponding to the predicted translation product (AASWRFEDAAFGLDEFQILLACFLILICLYTYTLCTKCRVIIMLTWT).

Dystrophin expression in primary mouse and human muscle cells. Primary muscle cell cultures of either wild-type (C57) or dystrophin-deficient (*mdx*) mice were obtained as previously described[33]. Primary human myoblasts were

derived from muscle biopsies. Two biopsies were obtained from the extensor digitorum brevis (EDB) muscles of DMD patients with premature stop codons in dystrophin exons 28 (UGA) and 42 (UAG), respectively. An additional biopsy was obtained form an unaffected adult human male. Biopsies were rinsed in Ham's F10 medium and minced. Explants were then subjected to a low speed centrifugation, and the pellet was resuspended in medium and transferred to culture dishes with F-10 medium and 20% fetal bovine serum (FBS). On migration of satellite cells from the explants and onto the dish, the cells were harvested and frozen in 95% FBS and 5% DMSO.

To form myotubes, an aliquot of cells was thawed and expanded in growth medium containing 20% FBS, F-10 medium and penicillin–streptomycin. To induce myotube differentiation, the cells were switched to medium containing DMEM, insulin, apo-transferrin and penicillin–streptomycin, and then plated on collagen-coated dishes with Matri-gel[34].

At the switch to differentiation medium, PTC 124 was added to normal and DMD cultures in the following doses: 0, 0.5, 1, 5 and 10 $\mu g\,ml^{-1}$ of differentiation medium (0, 1.7, 3.4, 17 and 34 μM, respectively). Cultures were allowed to form myotubes and harvested 12 days after switching to differentiation medium. After fixation in 2% formaldehyde, the fixed myotubes were incubated with polyclonal anti-dystrophin antibodies (targeted to the C-terminal region; Abcam 15277) and monoclonal anti-sarcomeric myosin primary antibodies (a gift from Howard Holtzer). The secondary antibodies (Molecular Probes) were conjugated with Alexa 488 for dystrophin and Alexa 586 for myosin. Imaging was performed on a Leica confocal microscope and the ratio of the dystrophin fluorescence intensity to the myosin intensity was obtained to allow estimation of dystrophin levels induced in the DMD and normal cultures. Treated and untreated myotubes appeared healthy, as confirmed by robust myosin staining and the expected presence of striations (Fig. 2).

Assessment of phenotypic rescue of the *mdx* mouse. Isolated whole-muscle mechanics were performed on EDL muscles from drug-treated and control animals using a previously described apparatus[8]. After determining optimum length (L_O) with a series of twitch stimulations at supramaximal voltage, maximum isometric force was measured with 120 Hz pulses delivered for 500 ms. Protection against mechanical injury, induced by a series of 5 eccentric tetanic contractions with stretches of 10% L_O, was evaluated. Damage was determined as the loss in force between the first and last eccentric contraction. At the end of the mechanical measurements, muscles were blotted and weighed, mounted in embedding compound, then rapidly frozen in melting isopentane. Specific force was calculated from maximum tetanic tension and cross-sectional area of the EDL muscles. Muscle cross-sectional area was determined using previously published methods[33].

An estimate of restoration of the dystrophin glycoprotein complex to the sarcolemma was performed by immunoblotting of muscle homogenates. Samples were partially enriched for the dystrophin-associated glycoprotein complex using wheat germ agglutinin. Antibodies to both dystrophin (C-terminal antibody, Dys2 (Novacastra); rod antibody, Sigma8168 (Sigma-Aldrich)) and gamma-sarcoglycan (NCL-gSarc, Novacastra) were used for these measurements.

The presence and membrane localization of dystrophin was observed by immunohistochemistry on 10-mm frozen sections of striated muscles. The primary antibody for dystrophin was a rabbit polyclonal antibody generated against a synthetic peptide corresponding to the C-terminal of human dystrophin (abcam 15277, Abcam), and diluted 1:1000 in 10% bovine serum albumin, and the secondary antibody (Alexa Fluor 488-conjugated anti-rabbit, Molecular Probes, Invitrogen) was diluted 1:750. Sections were covered with aqueous mounting media containing 4,6-diamidino-2-phenylindole (DAPI; Vectashield, Vector Laboratories) to visualize myonuclei. Stained sections were visualized on a Leica TCS SL confocal microscope (Leica Microsystems), and captured with a digital camera using associated image analysis software (Version 2.61).

All data were expressed as means ± s.e. A one-way ANOVA was used to compare all variables among groups. Serum CK comparisons used repeated measures test. Significance was indicated if $P < 0.05$ for all comparisons.

Two-dimensional electrophoresis. Duplicate samples of HEK293 cells harbouring *LUC-190* (UGA) were incubated in the presence of 5 μM PTC124 (treated) or 1% DMSO (untreated) for 20 h. The cells were collected, washed twice in phosphate buffered saline (PBS), resuspended in sample buffer (Bio-Rad) and shipped on dry ice to Kendrick Laboratories for two-dimensional electrophoretic analysis[35] Isoelectric focusing (pH 3.5–10) was carried out in glass tubes for 20,000 V-hours. One μg of a tropomyosin internal standard was added to each sample. Second dimension SDS slab gel electrophoresis was carried out for approximately 6 h at 25 mA per gel. After electrophoresis, gels were transferred to PVDF paper. Computerized analysis of spot mobility used Phoretix software.

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