Readthrough-Promoting Drugs Gentamicin and PTC124 Fail to Rescue Na\textsubscript{1.5} Function of Human-Induced Pluripotent Stem Cell–Derived Cardiomyocytes Carrying Nonsense Mutations in the Sodium Channel Gene \textit{SCN5A}

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Background—Several compounds have been reported to induce translational readthrough of premature stop codons resulting in the production of full-length protein by interfering with ribosomal proofreading. Here we examined the effect of 2 of these compounds, gentamicin and PTC124, in human-induced pluripotent stem cell (hiPSC)–derived cardiomyocytes bearing nonsense mutations in the sodium channel gene \textit{SCN5A}, which are associated with conduction disease and potential lethal arrhythmias.

Methods and Results—We generated hiPSC from 2 patients carrying the mutations R1638X and W156X. hiPSC-derived cardiomyocytes from both patients recapitulated the expected electrophysiological phenotype, as evidenced by reduced \textit{Na}\textsubscript{1} currents and action potential upstroke velocities compared with hiPSC-derived cardiomyocytes from 2 unrelated control individuals. While we were able to confirm the readthrough efficacy of the 2 drugs in Human Embryonic Kidney 293 cells, we did not observe rescue of the electrophysiological phenotype in hiPSC-derived cardiomyocytes from the patients.

Conclusions—We conclude that these drugs are unlikely to present an effective treatment for patients carrying the loss-of-function \textit{SCN5A} gene mutations examined in this study. (Circ Arrhythm Electrophysiol. 2016;9:e004227. DOI: 10.1161/CIRCEP.116.004227.)

Key Words: induced pluripotent stem cells • mutation • nonsense mutation • readthrough • \textit{SCN5A} • sodium channels

The cardiac voltage-gated sodium channel Na\textsubscript{1.5}, encoded by the gene \textit{SCN5A}, is a key player in cardiac conduction in the human heart.\textsuperscript{1} Channel dysfunction caused by mutations in \textit{SCN5A} or by Na\textsubscript{1.5} channel–blocking drugs impact conduction or repolarization and are associated with sudden cardiac death. Loss-of-function mutations in particular are associated with isolated (progressive) conduction disease, sick sinus syndrome, atrial arrhythmias, and Brugada syndrome, a disease characterized by signature ECG features arising from the right ventricle which is associated with ventricular fibrillation and sudden death.\textsuperscript{2} To date, no cures exist for these diseases. Instead, preventative measures, including pacemakers or cardioverter defibrillator implants, can be taken.

A subset of patients with conduction disease or Brugada syndrome have nonsense mutations in \textit{SCN5A}, which lead to haploinsufficiency by premature termination of translation and production of truncated proteins. Several compounds have been described as inducing translational readthrough of premature stop codons and production of full-length protein by interfering with ribosomal proofreading. Gentamycin, an aminoglycoside antibiotic, for example, has been shown to promote readthrough in several different genetic diseases,\textsuperscript{3,4} but just as with other promoting aminoglycosides, toxicity is a complicating issue.\textsuperscript{5} Recently, however, a non-aminoglycoside drug called PTC124 was described as being effective in suppressing nonsense mutations in vitro and in vivo in different disease models,\textsuperscript{6} whereas this drug was well-tolerated in a phase I clinical trial.\textsuperscript{7}

With respect to nonsense mutations in cardiac ion channel genes, including \textit{SCN5A}, both gentamycin and PTC124...
WHAT IS KNOWN

• Cardiac conduction disease patients carrying mutations in the sodium channel gene SCN5A are at risk of developing bradydcardic or tachyarrhythmic events, however effective treatment is lacking.
• The compounds gentamycin and PTC124 can induce translational readthrough of premature stop codons, thereby providing a putative therapeutic approach for patients carrying SCN5A nonsense mutations.

WHAT THE STUDY ADDS

• For patients carrying the nonsense mutations R1638X and W156X, gentamicin and PTC124 are unlikely to present an effective treatment.

have been shown to restore protein expression significantly, although, to date, this has only been evaluated in heterologous expression systems, in which expression of the SCN5A gene was realized by expressing the cDNA in a noncardiac cell. However, heterologous systems lack the complete architecture and components of native cardiomyocytes. Also expression is not driven by regulatory elements in the genome and lacks, for example, noncoding, functional regions. Furthermore, degradation of mutant RNA by nonsense-mediated mRNA decay (NMD) cannot take place because it depends on exon–junction complexes, which are not present in transfected cDNA. Success of readthrough drugs in the native context depends among others on the rate of NMD.

Human-induced pluripotent stem cells (hiPSCs), derived from patients carrying nonsense mutations, are a potential in vitro model to evaluate the efficiency of readthrough-promoting drugs. hiPSCs can self-renew but also differentiate into cardiomyocytes (hiPSC-CMs) using a variety of methods based on recapitulating developmental signals. These cardiomyocytes should possess the noncoding genomic information necessary for NMD to occur. hiPSC-CMs have been shown in multiple studies to recapitulate the phenotype of patients with various inherited cardiac disorders, including patients carrying SCN5A mutations, as reflected by their electrophysiological properties. Here, we generated and characterized hiPSC-CMs from individuals carrying the nonsense mutations R1638X and W156X in SCN5A and evaluated the effects of gentamicin and PTC124 in restoring protein function.

Materials and Methods

Generation of Patient-Specific hiPSCs

Skin fibroblasts were isolated from dermal biopsies, which were obtained according to the protocol approved by the medical ethical committee of the Academic Medical Center and Leiden University Medical Center. Skin biopsies were obtained from 2 male mutation carriers (mutation R1638X and W156X in SCN5A) and from unrelated (sex-matched) healthy control individual after informed consent was secured. Reprogramming of the skin fibroblasts was performed as described previously. In brief, skin fibroblasts derived from a donated biopsy were infected with Sendai virus encoding OCT4, SOX2, KLF4, and MYC and cultured on mouse embryonic feeder cells until hiPSC colonies appeared and could be picked for further expansion and characterization for pluripotency in culture and cryopreserved. A second, non-sex matched, control hiPSC line was generated previously and characterized in detail.

hiPSC Culture and Cardiac Differentiation

All hiPSC lines were maintained in mTeSR1 medium (Stem Cell Technologies, Vancouver, Canada) and mechanically passaged on Matrigel- (BD Biosciences, San Jose, CA) coated tissue culture plates according to the suppliers’ protocol. Differentiation was induced in a monolayer culture using timed addition of growth factors BMP4, Activin A, CHIR-99021 (a small molecular activator of Wnt signaling), and Xav939 (inhibitor of Wnt signaling) in BPEL medium as previously described. For electrophysiological recordings and immunofluorescence measurements, the cultures were dissociated to single cells with 10x Tryple Select ( Gibco Life Technologies, Waltham, MA) at day 20 of differentiation and seeded on Matrigel-coated glass coverslips. Electrophysiological properties were measured 8 to 12 days after dissociation from at least 3 independent differentiations.

DNA Sequencing

Genomic DNA was isolated from hiPSCs using the Gentra PureGene Cell Kit (Qiagen, Germantown, MD). An genomic region surrounding each mutation was amplified by polymerase chain reaction (PCR) using SilverStar DNA polymerase (Eurorgenetics, Maastricht, the Netherlands) and specific primer sets (Table I in the Data Supplement). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced.

Human Embryonic Kidney 293 Cell Transfections With H2B-GFP Plasmids

Human embryonic kidney 293 (HEK293) cells, used to confirm readthrough capacity of gentamicin and PTC124, were cultured in medium containing minimal essential medium, supplemented with 10% fetal bovine serum, penicillin, streptomycin, and nonessential amino acids. The H2B-GFP wild-type (WT) and H2B-GFP opal plasmids were transfected into the cells when they had reached 70% confluence in 24-well plates using FuGene HD (Promega, Madison, WI) according to the manufacturer’s protocol. For transfection, 1 μg of DNA and 3 μL of FuGene HD was used for each well.

Immunofluorescence Analysis

Single-cell hiPSC-CMs were fixed in 4% paraformaldehyde, permeabilized with phosphate buffer saline/0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO), and blocked with 10% FCS (Life Technologies, Waltham, MA). The samples were incubated overnight at 4°C with primary antibodies specific for troponin I (immunoglobulin G [IgG]), rabbit polyclonal; Santa Cruz Biotechnology, Dallas, TX) and α-actinin (mouse monoclonal; Sigma-Aldrich) or for 2 hours at 37°C with anti-Naq, 1.5 (rabbit polyclonal; Alomone labs, Jerusalem, Israel). Primary antibodies were detected with either Alexa-Fluor 488 or Cy3-conjugated antibodies. Nuclei were visualized with DAPI (Invitrogen, Waltham, MA) and F-actin with Phalloidin-Alexa-Fluor 594-conjugate (Invitrogen). Images were acquired using a Leica DMi6000-AG6000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

hiPSCs were also fixed and treated as above but incubated overnight at 4°C with primary antibodies for NANO (rabbit polyclonal; Peprotech, London, UK), SSEA4 (mouse monoclonal; Biolegend, San Diego, CA), TRA-1–81 (mouse polyclonal; Biolegend), and OCT-3/4 (mouse monoclonal; Santa Cruz, Dallas, TX) and detected with Alexa-Fluor 488, Alexa 568, or Cy3-conjugated antibodies.

HEK293 cells were fixed and permeabilized as above, blocked with 0.1 mol/L Tris-HCl, 0.15 mol/L NaCl containing 0.5% blocking powder (Roche, Basel, Switzerland), pH 7.5. The cells were incubated overnight at 4°C with a primary antibody for GFP (sc-5384, goat polyclonal; Santa Cruz; dilution 1:100) and then secondary antibody conjugated with Alexa-Fluor 488 for 2 hours. Nuclei were stained with Sytox Orange (Thermo Fisher, Waltham, MA).
Gene Expression Analysis

Total RNA was isolated from hiPSCs and hiPSC-CMs using the RNaseasy Mini (Qiagen). RNA was reverse transcribed using the iScrypt-cDNA Synthesis kit (Bio-Rad, Hercules, CA). Transcription abundance of selected genes was determined by quantitative PCR using the iQ Universal SYBR Green Supermix (Bio-Rad). Gene expression was normalized to GAPDH, and results were analyzed using the ΔΔCt method. The GENE-E analysis platform (http://www.broadinstitute.org) was used to illustrate the results as a heat map. The primer sets used are listed in Table I in the Data Supplement.

Electrophysiology

Sodium Current Measurements

The sodium current (I_Na) was measured with the ruptured patch clamp technique using an Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA). Voltage control, data acquisition, and analysis were realized with custom software. Data were low-pass filtered and digitized at 5 and 20 kHz, respectively. The liquid junction potential, calculated using Clampex 10.2 (Axon Instruments, Sunnyvale, CA), was 2.5 mV and corrected accordingly. For proper voltage control, data were acquired and filtered at 5 and 40 kHz, respectively, and the potentials were corrected by dividing the measured current by the cell capacitance (Cf). Cf was determined using a −5 mV hyperpolarizing step from −50 mV and equals the decay time constant of the capacitative transient, divided by access resistance. To determine the voltage dependence of activation, current-voltage curves were corrected for the driving force. Voltage dependence of inactivation curves were created by normalization of the currents to the maximum peak current and plotted against the test potential. Curves were fitted with a Boltzmann equation ([I/I_peak] = 1/[1+exp((Vth-V)/k)]) to determine the Vth (membrane potential for the half-maximal (in)activation) and the slope factor k.

Action Potential Measurements

Action potentials (APs) were measured at 37°C with the perforated patch clamp technique using an Axopatch 200B amplifier and pClamp10.2 software (Axon Instruments). Analysis of the APs was performed with custom-made software. APs were filtered and digitized at 5 and 40 kHz, respectively, and the potentials were corrected for the calculated liquid junction potential of 15 mV.

Bath contained (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1.0 MgCl2, 5.5 glucose, 5.0 HEPES; pH 7.4 (NaOH). The pipette solution contained (in mM) 125 K-glucurate, 20 KCl, 5 NaCl, 0.22 amphotericin B, 10 HEPES; pH 7.2 (KOH). APs were elicited at the stimulation frequency of 1 Hz by 3 ms, 1.2× threshold current pulses through the patch pipette. Maximal diastolic potential, maximal upstroke velocity (Vmax), AP amplitude, and AP duration (APD) at 50% and 90% repolarization (APD50 and APD90, respectively) were analyzed. Data from 10 consecutive APs were averaged.

Drug Treatment

PTC124 (Selleckchem, Houston, TX) and gentamicin (Sigma, St. Louis, MO) were dissolved as 17 mmol/L and 20 mmol/L stock solutions in dimethyl sulfoxide and water, respectively, and were diluted accordingly. During incubation, medium containing PTC124 or gentamicin was replaced daily. In HEK293 cells, readthrough was evaluated by immunofluorescence after 48 hours of drug treatment. In hiPSC-CMs, drug efficiency was assessed by measuring peak I_Na.

To take into account any variation between different differentiation experiments, drug-treated cells were compared with untreated or vehicle-treated cells measured on the same day.

NMD Evaluation

To assess the presence of NMD, we calculated the relative amount of WT and mutant SCN5A transcripts by means of a cloning approach as follows. We first isolated RNA from the mutant hiPSC-CMs lines (R1638X and W156X) and reversed transcribed it to cDNA as described earlier. Using primer sets that flank each mutation (listed in Table I in the Data Supplement), we generated PCR products that were subsequently cloned into the TOPO vector using the TOPO TA cloning sequencing kit (Invitrogen). The ligation products were used to transform One Shot TOP10 Chemically Competent Escherichia coli (Invitrogen) from which we subsequently isolated plasmids using the PureLink Quick Plasmid Miniprep Kit (Invitrogen). The plasmids, thus, isolated were sequenced by conventional Sanger sequencing to assess how many were WT and how many were mutant.

Statistical Analysis

Results are expressed as mean±standard error of the mean (SEM). Normal distribution of the data was confirmed with the Shapiro–Wilk test. Comparisons of AP parameters, I_Na, voltage dependence of (in)activation, and drug treatment were performed with 1-way ANOVA, followed by post hoc Bonferroni test, whereas I_Na density was compared with 2-way repeated measures ANOVA. Comparisons of WT-mRNA/Mut-mRNA ratios (WT-mRNA, mRNA that does not carry the mutation; Mut-mRNA, mRNA that does carry the mutation) between lines were performed with the Chi-square test. P<0.05 was considered statistically significant.

Results

Patient Characteristics

Two patients with nonsense SCN5A mutations were selected for inclusion in this study. The first was a 34-year-old male Brugada syndrome patient, who carried the c.4912C>T mutation, which leads to substitution of an arginine with a TGA stop codon at a location (R1638X) close to the C terminus of the channel (Figure 1A). He presented with a typical type I Brugada ECG and displayed ventricular tachycardias during electrophysiological investigations. The second patient was a 52-year-old male who carried the c.468G>A mutation, leading to the change of a tryptophan at a position close to the N terminus (W156X; Figure 1A). His phenotype was relatively mild and consisted of first-degree AV block and slight QRS prolongation.

Generation of hiPSC Lines From Individuals With SCN5A Nonsense Mutations

Dermal fibroblasts were grown and expanded from skin biopsies from the 2 patients and a sex-matched unrelated control. A Sendai virus carrying the transcription factors OCT4, SOX2, KLF4, and MYC1 was used to generate the hiPSC lines (hereafter referred to as hiPSCR1638X, hiPSCW156X, and hiPSCWT1).

Quantitative PCR confirmed that after several passages, the Sendai vectors had been extruded from the cells. All lines generated expressed the pluripotency associated markers NANOG, SSEA4, TRA-1–81, and OCT-3/4 by immunocytochemistry (Figure 1B). Each line could spontaneously differentiate into cell derivatives of all 3 primary germ layers and had a normal karyotype (Figure I and II in the Data Supplement). RNA was isolated from the undifferentiated hiPSC lines and reverse transcribed, and the cDNA was subjected to a microarray. Gene expression patterns were analyzed with the PluriTest algorithm, resulting in a pluripotency score,
Figure 1. Basic characterization of hiPSC lines. A, The corresponding positions of W156X and R1638X mutations on the protein structure of Na1.5. B, Typical examples of an immunofluorescent analysis of NANOG (red), SSEA4 (green), Dapi (blue) on the first row and TRA-1–81 (red), OCT-3/4 (green), Dapi (blue) on the second row in hiPSCWT1, hiPSCR1638X, and hiPSCW156X lines. C, Pluripotency scores of hiPSCWT1, hiPSCR1638X, and hiPSCW156X lines based on the PluriTest algorithm. D, Genotyping of hiPSCR1638X and hiPSCW156X lines focusing on the loci around each mutation (lower row). The corresponding region of the hiPSCWT1 line is shown as a reference (upper row). hiPSC indicates human-induced pluripotent stem cells; and WT, wild-type.
Figure 2. Differentiation of hiPSC lines into the cardiac lineage. A, Typical examples of an immunofluorescent analysis of tnni3 (green), α-actinin (red), and dapi (blue) in hiPSC-CMWT1, hiPSC-CMR1638X, and hiPSC-CMW156X. Lower row illustrates magnified images of regions of interest of each corresponding cell in the first row. Scale bar is 10 μm. B, Typical examples of an immunofluorescent (Continued)
indicating the degree of pluripotency after comparison of the cell line to a reference panel of normal PSCs lines. All 3 lines ranked high in the pluripotency score of PluripTest (Figure 1C). Sanger sequencing of SCN5A at the mutation sites confirmed that each hiPSC line carried the nucleotide changes of its corresponding progenitor (Figure 1D). All previous results ensured that the lines generated were indeed pluripotent, integration-free, and carried the genotype of the individual from which they were originated. An additional control line, previously characterized in detail for pluripotency, karyotype, and ability to undergo cardiomyogenesis and hereafter referred to as hiPSCWT2, was also included in the analysis.

Cardiac Differentiation

Directed differentiation was used to generate hiPSC-CMs from all lines. Immunocytochemical fluorescence staining indicated that the cells expressed the cardiac sarcomeric proteins α-actinin and troponin I (type 3; Figure 2A). These cells exhibited the typical striation pattern of sarcomeres expected for cardiomyocytes. The cells also stained positively for the sodium channel −subunit Nav1.5 that was present over the α for cardiomyocytes. The cells also stained positively for the α subunit Na1.5 that was present over the α for cardiomyocytes.

Baseline Electrophysiological Characterization of hiPSC-CMs Carrying Mutations R1638X and W156X

To assess and compare the functional phenotype of hiPSC-CMs Carrying Mutations R1638X and W156X, we measured APs and INa in single hiPSC-CMs derived from the 2 WT, R1638X, and W156X hiPSC lines (annotated as hiPSC-CMWT1, hiPSC-CMWT2, hiPSC-CMR1638X, and hiPSC-CMW156X, respectively).

Potential, AP amplitude, APD50, and APD90, did not differ significantly between these cells (Figure 2C).

Nonsense-Mediated Decay in hiPSC-CMs

The presence of NMD was evaluated in hiPSC-CM WT1 and hiPSC-CM WT2 (Figure 3A). We generated cDNA from RNA of both samples and amplified the region surrounding the mutation by PCR. The PCR product from each sample was cloned into a TOPO vector, which was transformed to chemically competent E.coli cells. Transformed bacterial colonies were screened and their plasmids isolated and sequenced. By sequencing a large number of clones, we were able to establish the WT-mRNA Mut-mRNA ratio in each sample. This allowed us to estimate the relative amount of Mut-mRNA that was being degraded by NMD in hiPSC-CM W156X and hiPSC-CM R1638X.

For hiPSC-CM R1638X, 61% of the total clones analyzed (n=33) contained the mutation, whereas for hiPSC-CM W156X, the mutation was present in 19% of the clones (n=42; P<0.05, Chi-square test), confirming the occurrence of NMD in cells harboring the mutation W156X (Figure 4). To validate our results, we treated hiPSC-CMs W156X with puromycin, an NMD inhibitor, at a concentration of 300 μg/mL for 8 hours. After treatment, the WT-mRNA Mut-mRNA ratio reached close to 1:1 with 51% clones (n=63) carrying the mutation, indicating that puromycin had indeed blocked the NMD mechanism.

Readthrough-Promoting Drugs Do Not Exert an Increase in INa in hiPSC-CM R1638X and hiPSC-CM W156X

To assess readthrough efficiency of gentamicin and PTC124, we first confirmed the potency of these drugs in HEK293T cells transfected with a GFP plasmid (H2B-GFP opal) harboring a nonsense mutation (W172X), as reported previously. Cells were transfected and incubated with gentamicin (0.5 and 1 μm/mL) and PTC124 (17 and 34 μM) for 48 hours. Immunofluorescence demonstrated the presence of GFP in gentamicin- and PTC124-treated cells transfected with H2B-GFP opal, whereas untreated cells did not demonstrate any signal, indicating the potency of these drugs in evoking readthrough (Figure 5A). To demonstrate that gentamicin was actually present in the nucleus of hiPSC-CMs, genes known to be upregulated after gentamicin exposure were examined by quantitative PCR. GPR94, CHOP, and BIP expressions were upregulated in gentamicin-treated hiPSC-CM R1638X and hiPSC-CM W156X compared with untreated cells from the same line (Figure 5B).
Figure 3. Action potential (AP) and sodium current ($I_{Na}$) characteristics from hiPSC-CM$^{WT1}$, hiPSC-CM$^{WT2}$, hiPSC-CM$^{R1638X}$, and hiPSC-CM$^{W156X}$. **A**, Top, Representative APs and the first derivatives of the AP upstroke velocities. **Bottom**, Average AP parameters for cells stimulated at 1 Hz, revealing a reduction of maximum upstroke velocity ($V_{\text{max}}$) in hiPSC-CM$^{R1638X}$ and hiPSC-CM$^{W156X}$ compared (Continued)
Moreover, we confirmed expression of the gene LRP2 (Ct values: 24–25), which encodes megalin, the proposed cytoplasmic membrane receptor of gentamicin. Megalin expression did not change under gentamicin treatment (Figure III in the Data Supplement). Because the gene response after PTC124 treatment is presently unknown, an equivalent experiment for this compound could not be performed. However, given the lipophilic nature of the drug, it would be expected to enter the cell through membrane diffusion.

To test the potential readthrough-promoting effects of gentamicin and PTC124 in hiPSC-CMs, we measured $I_{\text{Na}}$ in hiPSC-CM R1638X and hiPSC-CM W156X after incubation in the presence of different concentrations of drugs. On the same day that drug-treated cells were measured, untreated or dimethyl sulfoxide–treated cells were included and used for direct comparison. Neither gentamicin at a concentration of 0.5 mmol/L or 1 mmol/L for 2 days, nor PTC124 at a concentration of 17 μM for 4 days were able to induce a significant effect on $I_{\text{Na}}$ density compared with baseline or dimethyl sulfoxide only, respectively, in any of the groups studied (Figure 6). To rule out detrimental effects that could possibly mask the readthrough-promoting effects, we also examined $I_{\text{Na}}$ density and APs in hiPSC-CM WT on drug exposure. This did not reveal significant effects of gentamicin and PTC124 on any of the studied parameters (Figure IV in the Data Supplement).

Discussion

After the discovery of the readthrough-promoting effects of certain aminoglycosides antibiotics, several studies showed beneficial effects for different types of diseases, providing proof-of-principle for eventual therapeutic application of these drugs in conditions caused by nonsense mutations. More reports followed after the development of the drug PTC124, which is therapeutically more interesting because of the lack of side effects that complicate the use of aminoglycoside antibiotics. Here, we tested the efficacy of the aminoglycoside gentamicin and nonaminoglycoside PTC124 in hiPSC-CMs generated from patients heterozygous for the SCN5A mutations R1638X and W156X. Mutant hiPSC-CMs from a Brugada syndrome patient and a patient with mild conduction disorder exhibited clear electrophysiological phenotypes expected for loss of function, demonstrating the validity of hiPSC-CMs as a model system for these sodium channel–related cardiac disorders. However, neither gentamicin nor PTC124 treatment were capable of increasing $I_{\text{Na}}$ in the mutant hiPSC-CMs.

Phenotype of hiPSC-CMs Carrying Mutations R1638X and W156X

In accordance with the genotype of the patients, hiPSC-CMs carrying the mutations R1638X and W156X displayed reduced peak $I_{\text{Na}}$ density and maximal upstroke velocity of the AP, compared with the control cell line. The maximal diastolic potential values are similar in all hiPSC lines, and therefore, the (voltage-dependent) availability of sodium channels in mutated hiPSC-CMs and control hiPSC-CMs does not play a role in the reduced $V_{\text{max}}$. This is key to interpreting data using hiPSC-CMs as cardiac disease models; although hiPSC-CMs, in general, have immature electrophysiological features compared with their adult counterparts, there could be a degree of variation in the functional status between cardiomyocytes derived from different hiPSC lines. Although this was not observed in the hiPSC-CMs generated using our cardiac differentiation protocol, the different genetic backgrounds of the control and the mutated hiPSC lines are limitations of the study. Isogenically matched controls produced by genetic repair would be better comparators.

Nevertheless, for nonsense mutations, it would be expected that translation leads to nonfunctional truncated proteins, resulting in haploinsufficiency, as we indeed observed in this study. Interestingly, although the clinical phenotype of the 2 subjects differed remarkably (one being diagnosed with Brugada syndrome, while the other exhibiting mild conduction disorder), the extent of loss of function in hiPSC-CMs was similar across the 2 groups. This

![Image](http://circep.ahajournals.org/)

**Figure 4.** NMD occurrence in patient-derived hiPSC-CMs. Percentage of clones resulting from a TOPO cloning approach that carry the WT-mRNA or the mutant-mRNA transcripts in hiPSC-CM R1638X (n=33), hiPSC-CM W156X (n=42), and hiPSC-CM W156X treated with puromycin (300 μg/mL for 8 h; n=63). The differences in WT-mRNA/mutant-mRNA ratio between each line are attributed to the presence or absence of NMD for each particular mutation. CM indicates cardiomyocyte; hiPSC, human-induced pluripotent stem cells; NMD, nonsense-mediated mRNA decay; and WT, wild-type.
indicated that other factors may be of crucial importance in the development of the phenotype. The development of conduction slowing and Brugada syndrome, besides sodium channel function, may also be influenced by cardiomyocyte coupling (through gap junctions) and collagen deposition, which may, in turn, be modulated by the inheritance of other genetic factors.\(^{32,33}\). In this respect, the role of oligogenic inheritance in susceptibility to these disorders is becoming increasingly clear.\(^{34}\) It could also be speculated that the R1638X truncated channel is associated with a trafficking defect and fails to bind with interacting partners at the cardiomyocyte membrane, which could affect interactions of the sodium channel macromolecular complex (eg, connexins), as opposed to the W156X, which is not expressed entirely because of NMD. It should be noted, however, that important components contributing to sodium channel function or expression might be lacking or are different in hiPSC-CMs compared with human adult cardiomyocytes. Studies aimed at elucidating the complete interactome of the cardiac sodium channel in hiPSC-CMs are, therefore, warranted.

**Lack of Efficiency of Gentamicin and PTC124 for Mutations R1638X and W156X in SCN5A**

Our study showed no obvious beneficial effects of gentamicin and PTC124 in restoring protein function in hiPSC-CMs harboring the mutations R1638X and W156X. Several factors are known to be of importance in determining the efficiency of.
readthrough-promoting drugs. First, the nucleotide sequence that results in a premature stop codon affects the potency of the drugs. For both gentamicin and PTC124, a UGA sequence exhibits the highest probability of being subjected to readthrough, followed by the sequences UAG and UAA, respectively.12 Both mutations here resulted in a premature termination codon based on the most optimum sequence UGA. Apart from this, the downstream nucleotide identity has an impact on readthrough efficiency: C>U>A=G.36 The cell lines studied, however, did not possess the most optimal sequence (G and A for lines hiPSC-R1638X and hiPSC-W156X, respectively). Finally, available transcript levels of the mutant allele increase the chance of success.13 Transcript levels are determined in part by the process of NMD. Here, we evaluated 2 mutations, one expected to be subjected to NMD (W156X) and the other not because of its presence in the last exon of the gene (R1638X).12 We demonstrated the presence of NMD in hiPSC-CM-W156X as evidenced by the low ratio of mutant:WT transcripts. This low ratio was close to one on treatment with the NMD inhibitor puromycin. Nevertheless, cardiomyocytes from neither mutated cell lines where responsive to treatment with the readthrough-promoting drugs. The efficiencies of gentamicin and PTC124 have been demonstrated in vitro and in vivo for different types of diseases, including Duchenne’s muscular dystrophy, Usher syndrome, and cystic fibrosis. Of note, all these diseases are based on (near)-complete loss of protein because they are characterized by X-linked or homozygous inheritance patterns. Also, the in vitro studies that have demonstrated the efficiency of gentamicin or PTC124 on mutations related to other types of disease, including SCN5A mutation associated phenotypes,11 make use of heterologous expression systems in which the mutation is homoygously expressed. In all these studies, a small percentage of restored protein can easily be observed. In the present study, effects of gentamicin and PTC124 were studied in a heterozygous system because homozygous or compound heterozygous nonsense SCN5A mutation carriers are extremely rare. Small effects are possibly masked by the amount of variation we observe in the system. However, it is doubtful whether such small effects of the readthrough-promoting drugs would be of clinically relevant value.

Where premature termination is suppressed, the translational machinery inserts an amino acid that is usually different from the WT protein. In the case of SCN5A, this may have deleterious consequences because the change in amino acid may induce a gain of function of the protein, resulting in an increase in the sustained component of \( I_{Na} \). Increased sustained \( I_{Na} \) can cause Long QT syndrome, which is characterized by life-threatening arrhythmia and sudden cardiac death. Indeed, previous studies on readthrough of nonsense mutations in the gene KCNQ1 (encoding the channel responsible for the slow delayed rectifier potassium current) showed changes in the kinetics of the corresponding channel in response to readthrough,10 highlighting this potential risk.

In conclusion, in this study, the readthrough-promoting agents PTC124 and gentamycin failed to suppress translational termination by premature stop codons in hiPSC-CMs carrying the mutations R1638X and W156X in SCN5A. Although effects might be different for other mutations, therapeutic application of these agents in this patient group is, based on our experiments, predicted not to have a beneficial effect.

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Disclosures
None.

References
Kosmidis et al. Readthrough Drugs for SCNSA Nonsense Mutations


Readthrough-Promoting Drugs Gentamicin and PTC124 Fail to Rescue Na\textsubscript{v}1.5 Function of Human-Induced Pluripotent Stem Cell–Derived Cardiomyocytes Carrying Nonsense Mutations in the Sodium Channel Gene SCN5A
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### Supplemental Table S1

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<th>Gene</th>
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<th>Rev Primer (5”-3”)</th>
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<td>SCN5A (genotype</td>
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Supplemental Figure S1:

Spontaneous differentiation of hiPSC lines to cell lineages of all 3 primary germ layers. Typical examples of an immunofluorescent analysis of AFP (green), CD31 (red), and β3-tubulin (red), endoderm, mesoderm and ectoderm markers respectively, in cell derivatives of hiPSC\textsuperscript{WT1}, hiPSC\textsuperscript{R1638X} and hiPSC\textsuperscript{W156X} lines.
Supplemental Figure S2:

Karyotype analysis of hiPSC lines. Combined binary ratio (COBRA) of fluorescence in situ hybridization (FISH) labelling chromosome arms of hiPSC^{WT1}, hiPSC^{R1638X} and hiPSC^{W156X} lines.
Supplemental Figure S3:

Analysis of qPCR data from 3 independent experiments on megalin (LRP2) expression in hiPSC-CM$^{R1638X}$ and hiPSC-CM$^{W156X}$ after gentamicin treatment (0.5 mM-1 mM for 24h-48h).
Supplemental Figure S4:

Effects of 1 mM gentamicin (incubated 2 days, panel A,C) and 17 µM PTC124 (incubated 4 days, panel B,D) on peak sodium current ($I_{Na}$, panel A,B) and action potential parameters (panel C,D) on hiPSC-CM$^{WT2}$. Comparisons were made with no treatment and DMSO for gentamicin and PTC124, respectively. No significant effects were observed. $V_{max}$, maximal upstroke velocity; MDP, maximal diastolic potential; APA, action potential amplitude; APD$_{50}$, APD$_{90}$, action potential duration at 20 and 50% of repolarization, respectively.