Inherited arrhythmia syndromes
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Chapter 5

Readthrough-promoting drugs gentamicin and PTC124 fail to rescue Na\(_v\)1.5 function of human-induced pluripotent stem cell-derived cardiomyocytes carrying nonsense mutations in the sodium channel gene **SCN5A**


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**ABSTRACT**

**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 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 Na⁺ 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 SCN5A gene mutations examined in this study.
INTRODUCTION

The cardiac voltage-gated sodium channel Na\(_{1.5}\), encoded by the gene \textit{SCN5A}, is a key player in cardiac conduction in the human heart.\(^1\) Channel dysfunction caused by mutations in \textit{SCN5A} or by Na\(_{1.5}\) 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 and associated with ventricular fibrillation and sudden death.\(^2\) To date, no cures exist for these diseases. Instead, preventative measures including pacemakers or cardioverter defibrillator (ICD) implants can be taken.

A subset of patients with conduction disease and/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,\(^3,\,4\) but just as with other promoting aminoglycosides, toxicity is a complicating issue.\(^5\) Recently, however, a non-aminoglycoside drug called PTC124 was described as being effective in suppressing nonsense mutations \textit{in vitro} and \textit{in vivo} in different disease models,\(^6\) while this drug was well-tolerated in a phase I clinical trial.\(^7\)

With respect to nonsense mutations in cardiac ion channel genes, including \textit{SCN5A}, both gentamycin and PTC124 have been shown to restore protein expression significantly,\(^8\,\,\,\,\,\,\,\,11\) although to date this has only been evaluated in heterologous expression systems, in which expression of the \textit{SCN5A} gene was realized by expressing the cDNA in a non-cardiac cell. However, heterologous systems lack complete architecture and components of native cardiomyocytes. Also expression is not driven by regulatory elements in the genome and lacks, for example, non-coding, functional regions. Furthermore, degradation of mutant RNA by nonsense mediated mRNA-decay (NMD), cannot take place since it depends on exon-junction complexes which are not present in transfected cDNA.\(^12\) Success of readthrough drugs in the native context depends among others on the rate of NMD.\(^13\)

Human induced pluripotent stem cells (hiPSCs), derived from patients carrying nonsense mutations, are a potential \textit{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.\(^14\) 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,\(^15\) including patients car-
rying 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 gentamycin 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 two male mutation carriers (mutation R1638X and W156X in SCN5A) and from unrelated (gender 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 (MEF) cells until hiPSC colonies appeared and could be “picked” for further expansion and characterization for pluripotency in culture and cryopreserved. A second, non-gender 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 8–12 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). A genomic region surrounding each mutation was amplified by PCR using SilverStar DNA polymerase (Eurogentec, Maastricht, Netherlands) and specific primer sets (supplemental table S1). The PCR products were purified using the QIAquick PCR Purification kit (Qiagen, Germantown, MD) and sequenced.
HEK293 cell transfections with H2B-GFP plasmids

HEK293 cells, used to confirm readthrough capacity of gentamicin and PTC124, were cultured in medium containing minimal essential medium (MEM), supplemented with 10% fetal bovine serum, penicillin, streptomycin and non-essential amino acids. The H2B-GFP wildtype 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 (PBS)/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 TNNI (rabbit polyclonal, Santa Cruz Biotechnology, Dallas, Texas) and α-ACTININ (mouse monoclonal, Sigma-Aldrich, St. Louis, MO) or for 2 hours at 37°C with anti-Na,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, Waltham, MA). Images were acquired using a Leica DMI6000-AF6000 fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

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

HEK 293 cells were fixed and permeabilized as above, blocked with 0.1 M Tris-HCl, 0.15 M 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, Dallas, Texas, dilution 1:100) 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 RNeasy Mini (Qiagen, Germantown, MD). RNA was reverse transcribed using the iScript-cDNA Synthesis kit (Bio-Rad, Hercules, CA). Transcript abundance of selected genes was determined by qPCR using the iQ Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Gene expression was normalized to GAPDH and results were analysed 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 supplemental table S1.
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, USA). 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, $I_{Na}$ was recorded at room temperature, using a reduced extracellular sodium concentration, and series resistance compensation by ≥ 80%. Bath solution contained (in mM): 20 NaCl, 120 CsCl, 1.8 CaCl$_2$, 1.2 MgCl$_2$, 11.0 glucose, 5.0 HEPES; nifedipine 0.01; pH 7.4 (CsOH). Borosilicate pipettes (2–2.5 MΩ) were filled with (in mM): 3.0 NaCl, 133 CsCl, 2.0 MgCl$_2$, 2.0 Na$_2$ATP, 2.0 TEACl, 10 EGTA, 5.0 HEPES; pH 7.2 (CsOH). $I_{Na}$ was characterized by voltage clamp protocols as depicted in figure 3b with cycle lengths of 5 seconds. $I_{Na}$ was measured as the difference between peak current and steady-state current during a test pulse. Current densities were calculated by dividing the measured current by the cell capacitance ($C_m$). $C_m$ 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 ($I/I_{max}=A/\{1.0+exp[(V_{1/2}-V)/k]\}$), to determine the $V_{1/2}$ (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, Sunnyvale, CA). 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 liquid junction potential of 15 mV. Bath contained (in mM): 140 NaCl, 5.4 KCl, 1.8 CaCl$_2$, 1.0 MgCl$_2$, 5.5 glucose, 5.0 HEPES; pH 7.4 (NaOH). The pipette solution contained (in mM): 125 K-gluconate, 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, 1.2x threshold current pulses through the patch pipette. Maximal diastolic potential (MDP), maximal upstroke velocity ($V_{max}$), AP amplitude (APA), and AP duration (APD) at 50% and 90% repolarization (APD$_{50}$ and APD$_{90}$, respectively) were analysed. Data from 10 consecutive APs were averaged.
**Drug treatment**
PTC124 (Selleckchem, Houston TX) and gentamicin (Sigma, St. Louis, MO) were dissolved as 17 mM and 20 mM stock solutions in DMSO 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 wild-type 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 above. Using primer sets that flanked each mutation (listed in supplemental table S1), we generated PCR products that were subsequently cloned into the TOPO vector using the TOPO® TA cloning sequencing kit (Invitrogen, Waltham, MA). The ligation products were used to transform One Shot® TOP10 Chemically Competent E. coli (Invitrogen, Waltham, MA) from which we subsequently isolated plasmids using the PureLink® Quick Plasmid Miniprep Kit (Invitrogen, Waltham, MA). The plasmids thus isolated were sequenced by conventional Sanger sequencing to assess how many were wild-type and how many were mutant.

**Statistical analysis**
Results are expressed as mean ± standard error of the mean (s.e.m.) 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 One-Way ANOVA, followed by posthoc Bonferroni test, while $I_{Na}$ density was compared with Two-Way Repeated measures ANOVA. Comparisons of WT-mRNA/Mut-mRNA ratios 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 two patients and a gender matched unrelated control. A Sendai virus carrying the transcription factors OCT4, SOX2, KLF4, and MYC was used to generate the hiPSC lines (hereafter referred to as hiPSC$^{R1638X}$, hiPSC$^{W156X}$ and hiPSC$^{WT1}$). qPCR 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 to cell derivatives of all three primary germ layers and had a normal karyotype (supplemental figure S1-S2). RNA was isolated from the undifferentiated hiPSC lines, reverse transcribed and the cDNA was subjected to a microarray. Gene expression patterns were analyzed with the PluriTest algorithm resulting in a pluripotency score, indicating the degree of pluripotency after comparison of the cell line to a reference panel of normal PSCs lines. All three lines ranked high in the pluripotency score of PluriTest (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 hiPSC$^{WT2}$, 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 α-subunit Na$_v$1.5 that was present over the whole cell surface (Figure 2B). qPCR analysis revealed that hiPSC-CMs from all lines expressed key cardiac genes that encode various ion channels including KCNH2, KCNQ1, SCN5A, HCN4 and CACNA1C while pluripotent genes such as NANOG and OCT4 were no longer expressed in these cells (Figure 2C).
Readthrough-promoting drugs fail to rescue Na\textsubscript{v}1.5 function

Figure 1. Basic characterization of hiPSC lines. A. The corresponding positions of W156X and R1638X mutations on the protein structure of Na\textsubscript{v}1.5. 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 hiPSC\textsuperscript{WT}, hiPSC\textsuperscript{R1638X} and hiPSC\textsuperscript{W156X} lines. C. Pluripotency scores of hiPSC\textsuperscript{WT}, hiPSC\textsuperscript{R1638X} and hiPSC\textsuperscript{W156X} lines based on the Pluritest algorithm. D. Genotyping of hiPSC\textsuperscript{R1638X} and hiPSC\textsuperscript{W156X} lines focusing on the loci around each mutation (lower row). The corresponding region of the hiPSC\textsuperscript{WT} line is shown as a reference (upper row).
Figure 2. Differentiation of hiPSC lines towards 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 analysis of F-actin (green), Nav1.5 (red) and Dapi (blue) in hiPSC-CMWT1, hiPSC-CMR1638X and hiPSC-CMW156X. Lower row images illustrate the regions of interest in higher magnification. C. Analysis of qPCR data from 3 independent experiments on various cardiac and pluripotency genes in hiPSC-CMWT1, hiPSC-CMR1638X and hiPSC-CMW156X lines and in the cardiomyocytes resulting from them. The fold change expression in each sample is relative to the hiPSCWT1 sample for each gene.
Readthrough-promoting drugs fail to rescue Na_{1.5} function

Figure 3. Action potential (AP) and sodium current (I_{Na}) characteristics from hiPSC-CM^{WT}, hiPSC-CM^{WT2}, hiPSC-CM^{R1638X} and hiPSC-CM^{W156X}. A. Top panels, representative APs and the first derivatives of the AP upstrokes velocities.
Baseline electrophysiological characterization of hiPSC-CMs carrying mutations R1638X and W156X

In order to assess and compare the functional phenotype of hiPSC-CMs generated from each line, we measured APs and $I_{Na}$ in single hiPSC-CMs derived from the two wildtype, R1638X and W156X hiPSC-lines (annotated as hiPSC-CM WT1, hiPSC-CM WT2, hiPSC-CM R1638X and hiPSC-CM W156X, respectively). Sodium channel function has a direct impact on the fast depolarization phase (phase 0) of the AP and subsequently on the quantifiable parameter characterizing this phase, the maximal upstroke velocity ($V_{max}$). Our results showed that the $V_{max}$ of hiPSC-CM R1638X and hiPSC-CM W156X were significantly reduced when compared to the $V_{max}$ of the two controls hiPSC-CM WT1 and hiPSC-CM WT2 (Figure 3A) suggesting that both mutations reduce the function of the sodium channel in this model. Other AP parameters such as MDP APA, APD50 and APD90 did not differ significantly between the four hiPSC-CMs lines (Figure 3A). In accordance with the $V_{max}$ measurements, both hiPSC-CM R1638X and hiPSC-CM W156X displayed reduced $I_{Na}$ densities compared to hiPSC-CM WT1 and hiPSC-CM WT2 ($p < 0.01$) (Figure 3B-C). On average, $I_{Na}$ densities of the mutant channels were ≈25–30% of the hiPSC-CM WT (Figure 3C, right panel). $V_{1/2}$ of activation was shifted 4 mV in a positive direction in hiPSC-CM W156X and hiPSC-CM WT2 compared to the two other groups ($p = 0.01$), while k did not differ significantly (Figure 3D). Voltage dependence of inactivation did not differ between all groups (Figure 3E).

Nonsense mediated decay in hiPSC-CMs

The presence of NMD was evaluated in hiPSC-CMs R1638X and in hiPSC-CMs W156X. In hiPSC-CMs R1638X the premature termination sequence is located more than 50 nucleotides upstream of the last exon junction point of the mRNA. Because of its location, it is predicted that NMD does not occur. In the case of hiPSC-CMs W156X it is predicted that the NMD mechanism leads the mRNA carrying the premature stop codon to its degradation. We generated cDNA from RNA of both samples and amplified the region.
Readthrough-promoting drugs fail to rescue Nav1.5 function 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 (WT-mRNA: mRNA that does not carry the mutation, Mut-mRNA: mRNA that does carry the mutation). This allowed us to estimate the relative amount of Mut-mRNA that was being degraded by NMD in hiPSC-CMs<sub>W156X</sub> and hiPSC-CMs<sub>R1638X</sub>. For hiPSC-CM<sub>R1638X</sub>, 61% of the total clones analysed (n = 33) contained the mutation while for hiPSC-CM<sub>W156X</sub> the mutation was present in 19% of the clones (n = 42) (p < 0.05, Chi-Square test), confirming the occurrence of NMD in cells harbouring the mutation W156X (Figure 4). To validate our results, we treated hiPSC-CMs<sub>W156X</sub> with puromycin, a NMD inhibitor<sup>24</sup> at a concentration of 300 μg/ml for 8h. 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.

![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<sub>R1638X</sub> (n = 33), hiPSC-CM<sub>W156X</sub> (n = 42) and hiPSC-CM<sub>W156X</sub> treated with puromycin (300 μg/ml for 8 hours) (n = 63).](image)

Readthrough promoting drugs do not exert an increase in \(I_{\text{Na}}\) in hiPSC-CM<sub>R1638X</sub> and hiPSC-CM<sub>W156X</sub>

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) harbouring a nonsense mutation (W172X), as reported previously.<sup>20</sup> Cells were transfected and incubated with gentamicin (0.5 mM and 1 mM) and PTC124 (17 μM and 34 μM) for 48 hours. Immunofluorescence demonstrated the presence of GFP in gentamicin- and PTC124-treated cells transfected with H2B-GFP opal, while untreated cells did not demonstrate any signal, indicating the potency of these drugs in evoking readthrough
To demonstrate that gentamicin was actually present in the nucleus of hiPSC-CMs, genes known to be upregulated following gentamicin exposure\textsuperscript{25} were examined by qPCR. GRP94, CHOP and BiP expressions were upregulated in gentamicin-treated hiPSC-CM\textsuperscript{R1638X} and hiPSC-CM\textsuperscript{W156X} compared to untreated cells from the same line (Figure 5B). Moreover, we confirmed expression of the gene LRP2 (Ct values:24–25), which encodes megalin, the proposed cytoplasmic membrane receptor of gentamicin.\textsuperscript{26} Megalin expression did not change under gentamicin treatment (supplemental figure S3). Since the gene response following PTC124 treatment is presently unknown, an equivalent experiment for this compound could not be carried out. However, given the lipophilic nature of the drug, it would be expected to enter the cell through membrane diffusion.

![Image of immunofluorescent analysis](image)

**Figure 5. Potency of gentamicin and PTC124 compounds.** A. Immunofluorescent analysis of GFP in HEK293T cells transfected with H2B-GFP opal in the following conditions: untreated, treated with gentamicin 0.5 mM-1 mM and treated with PTC124 17 μM-34 μM for 48h. The positive control corresponds to HEK293T cells transfected with the H2B-GFP wildtype plasmid. B. Analysis of qPCR data from 3 independent experiments on the gentamicin response genes of GRP94, CHOP and BiP in hiPSC-CM\textsuperscript{R1638X} and hiPSC-CM\textsuperscript{W156X} after gentamicin treatment (0.5 mM-1 mM for 24h-48h).
To test the potential readthrough promoting effects of gentamicin and PTC124 in hiPSC-CMs, we measured $I_{Na}$ 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 DMSO-treated cells were included and used for direct comparison. Neither gentamicin at a concentration of 0.5 mM or 1 mM for 2 days, nor PTC124 at a concentration of 17 μM for 4 days were able to induce a significant effect on maximal peak $I_{Na}$ density compared to baseline or DMSO only, respectively, in any of the groups studied (Figure 6). To rule out detrimental effects which could possibly mask the readthrough promoting effects, we also examined $I_{Na}$ density and APs in hiPSC-CM$^{WT2}$ upon drug exposure. This did not reveal significant effects of gentamicin and PTC124 on any of the studied parameters (supplemental figure S4).

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 due to the lack of side effects that complicate the use of aminoglycoside antibiotics. Here, we tested the efficacy of the aminoglycoside gentamicin and non-aminoglycoside 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 disease, 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 the $I_{Na}$ of 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 $I_{Na}$ density and maximal upstroke velocity of the AP, compared with the control cell line. The MDP 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 $V_{max}$. This is key to interpreting data using hiPSC-CMs as cardiac disease models; although hiPSC-CMs in general have immature electrophysiological features compared to their adult counterparts,30 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 a limitation of the study. Isogenically matched controls produced by genetic repair would be better comparators.17, 31 Nevertheless, for nonsense mutations, it would be expected that translation leads to non-functional truncated proteins, resulting in haploinsufficiency, as we indeed observed in this study. Interestingly, while the clinical phenotype of the two subjects differed remarkably (one being diagnosed with BrS while the other exhibiting mild conduction disorder), the extent of loss-of-function in hiPSC-CMs was similar across the two groups. This 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 (e.g. connexins), as opposed to the W156X which is not expressed entirely due to 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 to 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 harbouring 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.35 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 two mutations, one expected to be subjected to NMD (W156X), the other not due to 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:wildtype transcripts. This low ratio was close to one upon treatment with the NMD-inhibitor puromycin. Nevertheless, cardiomyocytes from neither mutated cell lines where responsive to treatment with the readthrough promoting drugs. The efficiency of gentamicin and PTC124 has 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, as they are characterized by X-linked or homozygous inheritance patterns. Also the in vitro studies which 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 “homozygously” 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, as 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 which is usually different from the wildtype protein. In the case of SCN5A, this may have deleterious consequences, as 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 respon-
sible for the slow delayed rectifier potassium current) showed changes in the kinetics of the corresponding channel in response to readthrough,\(^6\) 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.

**Acknowledgements**

The authors thank Prof. Aldo Di Leonardo (STEBICEF Department, University of Palermo, Italy) for providing the H2B-GFP plasmids, Elisa Giacomelli (Anatomy and Embryology Department, Leiden Medical University Centre) for differentiating the hiPSC\(^{WT2}\) line into cardiomyocytes and the iPS-core facility of Leiden Medical University Centre.
Readthrough-promoting drugs fail to rescue Na,1.5 function

REFERENCES


## SUPPLEMENTARY MATERIALS

### Supplementary Tables

**Table S1.** List of the applied primers used for PCR per gene.

<table>
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<tr>
<th>Gene</th>
<th>Fwd Primer (5’-3’)</th>
<th>Rev Primer (5’-3’)</th>
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<td><strong>BIP</strong></td>
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Supplementary Figures

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{WT}, hiPSC\textsuperscript{R1038X} and hiPSC\textsuperscript{W156X} lines.
Figure S2. Karyotype analysis of hiPSC lines. Combined binary ratio (COBRA) of fluorescence in situ hybridization (FISH) labelling chromosome arms of hiPSC\textsuperscript{WT1}, hiPSC\textsuperscript{R1638X} and hiPSC\textsuperscript{W156X} lines.
Figure S3. Analysis of qPCR data from 3 independent experiments on megalin (LRP2) expression in hiPSC-CM<sup>R1638X</sup> and hiPSC-CM<sup>W156X</sup> after gentamicin treatment (0.5 mM-1 mM for 24h-48h).
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<sup>WT2</sup>. 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<sub>50</sub>, APD<sub>90</sub>, action potential duration at 20 and 50% of repolarization, respectively.