Original Article

Chronic cardiotoxicity assessment of BMS-986094, a guanosine nucleotide analogue, using human iPS cell-derived cardiomyocytes

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(Received May 7, 2021; Accepted May 23, 2021)

ABSTRACT — Predicting drug-induced side effects in the cardiovascular system is very important because it can lead to the discontinuation of new drugs/candidates or the withdrawal of marketed drugs. Although chronic assessment of cardiac contractility is an important issue in safety pharmacology, an *in vitro* evaluation system has not been fully developed. We previously developed an imaging-based contractility assay system to detect acute cardiotoxicity using human iPS cell-derived cardiomyocytes (hiPSC-CMs). To extend the system to chronic toxicity assessment, we examined the effects of the anti-hepatitis C virus (HCV) drug candidate BMS-986094, a guanosine nucleotide analogue, which was withdrawn from phase 2 clinical trials because of unexpected contractility toxicities. Additionally, we examined sofosbuvir, another nucleotide analogue inhibitor of HCV that has been approved as an anti-HCV drug. Motion imaging analysis revealed the difference in cardiotoxicity between the cardiotoxic BMS-986094 and the less toxic sofosbuvir in hiPSC-CMs, with a minimum of 4 days of treatment. In addition, we found that BMS-986094-induced contractility impairment was mediated by a decrease in calcium transient. These data suggest that chronic treatment improves the predictive power for the cardiotoxicity of anti-HCV drugs. Thus, hiPSC-CMs can be a useful tool to assess drug-induced chronic cardiotoxicity in non-clinical settings.

Key words: Chronic cardiotoxicity, Contractility, iPS cell, Cardiomyocyte, Nucleotide analogue

INTRODUCTION

Evaluation of drug-induced cardiotoxicity is challenging but essential in order to avoid adverse effects, such as lethal arrhythmia, contractility dysfunction, or conduction disturbances, in non-clinical and clinical studies (Cook *et al.*, 2014). Current guidelines focus on acute electrophysiological effects to detect the risk of QT prolongation and cardiac arrhythmias. In addition, drug-induced cardiac contractility changes can lead to cardiovascular side effects. For instance, negative inotropes may induce heart failure associated with pump dysfunction and left ventricular ejection fraction (LVEF) dysfunction (Wallis *et al.*, 2015). Langendorff assays and echocardiography using non-human primate models have been widely used (Guth *et al.*, 2015). However, these models have limited

translatability to humans due to species differences, as well as the high cost and low throughput. Moreover, chronic effects on cardiac contractility have only been investigated at a later stage of drug development using *in vivo* models or clinical studies (Laverty *et al.*, 2011).

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) are expected to serve as an alternative *in vitro* model to assess drug-induced cardiotoxicity. To date, we have successfully performed validation studies to assess drug-induced proarrhythmic risk in non-clinical settings (Ando *et al.*, 2017; Blinova *et al.*, 2018; Gintant *et al.*, 2020; Kanda *et al.*, 2018). In addition to proarrhythmic risk assessment, cardiac contractility changes play a key role in drug development in terms of acute cardiotoxicity and chronic effects (Guth *et al.*, 2019; Ribeiro *et al.*, 2019). Several studies have assessed the

acute effects of drugs on hiPSC-CMs contractions using various platforms (Hayakawa *et al.*, 2014; Scott *et al.*, 2014). In addition, other studies have reported that hiPSC-CMs can be used to assess the drug-induced chronic effects of anticancer drugs on both cardiac contractility and cytotoxicity (Kopljär *et al.*, 2017; Sakamoto *et al.*, 2019).

The cardiotoxicity of anti-hepatitis C virus (HCV) agents has been a key issue during drug development (Ucciferri *et al.*, 2018). BMS-986094, which was developed as a guanosine nucleotide analogue inhibitor of HCV non-structural (NS) 5b, showed good clinical tolerance during the treatment of HCV-infected patients. However, a phase II study was terminated by the unexpected cardiotoxicity in a patient who showed progressive heart failure characterized by decreased LVEF after treatment for 4 weeks (Vernachio *et al.*, 2011; Ahmad *et al.*, 2015). Other cases of unexpected, adverse cardiac events were also observed later. Although clinical dosing was considered safe from non-clinical studies, *in vitro* and *in vivo* studies were conducted to further characterize the potential mechanisms associated with BMS-986094-induced cardiotoxicity. As a result, BMS-986094 was found to decrease LVEF following long-term treatment in monkeys (Gill *et al.*, 2017; Baumgart *et al.*, 2016). Thus, it remains to be determined whether *in vitro* models predict the chronic effect of BMS-986094 on cardiac contractility. In contrast, sofosbuvir, another nucleotide analogue inhibitor of HCV NS5b, has been approved for the treatment of patients with HCV. A recent clinical study showed that sofosbuvir had little effect on cardiac function in patients with HCV (Ibrahim *et al.*, 2020).

In this study, we examined the chronic effects of BMS-986094 and sofosbuvir on cardiac contractility using motion analysis with hiPSC-CMs. Furthermore, we conducted calcium imaging analysis to understand the mechanism by which BMS-986094 induces contraction dysfunction. Our data suggest that hiPSC-CMs are useful for assessing the chronic effects of drugs on contraction.

MATERIALS AND METHODS

Chemicals

BMS-986094 was purchased from R&D Systems (Minneapolis, MN, USA). Fibronectin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sofosbuvir was purchased from Selleck Chemicals (Houston, TX, USA). All other reagents were of analytical grade and obtained from commercial sources. BMS-986094 was prepared in dimethyl sulfoxide (DMSO). The drug stock solution was diluted in culture medium to a final concen-

tration of 0.1% DMSO.

Cell culture

hiPSC-CMs were purchased from FUJIFILM Cellular Dynamics International (CDI; iCell Cardiomyocytes 2.0, Madison, WI, USA). The central area of 24-well plates was coated with 2 μ L fibronectin solution and incubated at 37°C for at least 1 hr. Thereafter, the cells were thawed and suspended in iCell Cardiomyocytes Plating Medium (CDI) and plated onto 24-well plates at a density of 3.5×10^4 cells in 2 μ L of plating medium. The cells were incubated at 37°C in 5% CO₂ for 3–4 hr prior to filling each well with 1 mL of iCell Cardiomyocyte Maintenance Medium (CDI, containing 10% fetal bovine serum), which was used as the culture medium. The medium was changed every 2–3 days, and the cells were cultured in well plates for 5–6 days to obtain a sheet of cardiomyocytes with spontaneous and synchronous electrical activity. hiPSC-CMs were treated with the drugs for six days. To introduce the drugs, half of the medium was replaced with media containing the drug at a double final concentration. Half of the medium was then replaced daily with media containing the drug at a final concentration.

Video microscopy for motion vector analysis

Movie images of beating hiPSC-CMs were recorded as sequential phase-contrast images with a $10 \times$ objective at a frame rate of 150 fps, resolution of $2,048 \times 2,048$ pixels, and depth of 8 bits using the SI8000 cell motion imaging system (Sony Corporation, Tokyo, Japan), as previously reported (Kanda *et al.*, 2021). The focus of the movie images was auto-adjusted or manually adjusted, if necessary. Cell culture plates were placed in a stage-top incubation system and incubated at 37°C in 5% CO₂ for at least 30 min for stabilization. The recordings were performed for six consecutive days.

Data analysis for motion vector

A motion waveform was obtained from the movie images using the SI8000C Analyzer software after each recording of beating hiPSC-CMs. This software system analyzes the contractile movements of hiPSC-CM sheets using a block-matching algorithm (Hayakawa *et al.*, 2014). A series of contractile parameters, which are contraction velocity (CV), relaxation velocity (RV), contraction-relaxation deformation distance (CRDD), and beating area, were automatically obtained from the average of the motion waveforms recorded during 10 sec measurements.

Calcium imaging

Calcium imaging was performed using Fluo-8/AM (AAT Bioquest, Sunnyvale, CA, USA) according to the manufacturer's instructions with slight modifications. Briefly, hiPSC-CMs were loaded with 5 μ M Fluo-8/AM in Hank's balanced salt solution (Sigma-Aldrich) (0.05% Pluronic F-127, 1.25 mM Probenecid, and 10 mM Hepes, pH = 7.4). After 20 min of incubation, the medium was changed to phenol red-free DMEM. Cell culture plates were placed in a stage-top incubation system and incubated at 37°C in 5% CO₂ for 30 min for stabilization. Ca²⁺ fluorescence images were captured with a CMOS camera (Hamamatsu, Shizuoka, Japan) at a resolution of 2,304 × 2,304 pixels and 50 fps using a 10 × objective and recorded using NIS-Elements AR ver5.30 (NIKON Corporation, Tokyo, Japan). After the data were quantified using NIS-Elements AR ver5.30, calcium parameters, such as calcium amplitude and Ca²⁺ transient duration 50 (CTD50), were obtained during the last 10 sec of measurements.

Quantitative PCR assays

Gene expression analysis was performed as previously reported (Yamada and Kanda, 2019). Briefly, total RNA was isolated from iCell Cardiomyocytes 2.0 with drug treatment using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and was treated with recombinant DNase I (RNase-free) (Takara Bio Inc., Shiga, Japan) to remove residual genomic DNA. Quantitative real-time reverse transcription (qRT)-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA, USA) on a QuantStudio 7 Flex real-time PCR system (Applied Biosystems, Foster City, CA, USA). Relative transcriptional changes were normalized to the mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The sequences of primers used for real-time PCR were listed in Table S1.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test to determine the level of significance. Statistical significance was set at $P < 0.05$. These analyses were performed using GraphPad Prism 5.00 (GraphPad Software, La Jolla, CA, USA).

RESULTS

The chronic effect of HCV NS5b inhibitor on cardiac contractile motion of hiPSC-CMs

To analyze the chronic effect of HCV NS5b inhibitors on the contractile function of hiPSC-CMs, we per-

formed motion vector analysis and obtained three contraction parameters: contraction velocity (CV), relaxation velocity (RV), and contraction-relaxation deformation distance (CRDD) (Fig. 1A). Our previous study found that the contractile parameters (CV and RV) correspond to the intracellular Ca²⁺ status, and CRDD corresponds to the traction force in hiPSC-CMs (Hayakawa *et al.*, 2014). The baseline values were 9.22 ± 0.124 μ m/sec for CV and 5.10 ± 0.075 μ m/sec for RV (mean $\pm$ S.E., $n = 15$). Fig. 1B shows representative motion waveforms of hiPSC-CMs in the presence of BMS-986094. As shown in Fig. 1C, BMS-986094 decreased CV, RV, and CRDD in a dose- and time-dependent manner. We did not observe any acute effects of BMS-986094 on contractility in hiPSC-CMs (data not shown). BMS-986094 had little effect on these parameters after chronic exposure for 48 hr, and CV began to decrease at 1 μ M for 96 hr, RV at 1 μ M for 72 hr, and CRDD at 0.3 μ M for 96 hr.

We next examined the effects of sofosbuvir, another HCV NS5b inhibitor (Fig. 2). In contrast to BMS-986094, sofosbuvir had little effect on CV, RV, and CRDD up to 144 hr (Fig. 2B). These results suggest that BMS-986094 induces contractility impairment in hiPSC-CMs during long-term treatments, while sofosbuvir had little effect.

The chronic effect of HCV NS5b inhibitor on calcium handling in hiPSC-CMs

To assess the chronic effects of HCV NS5b inhibitors on calcium handling, we performed calcium transient assays using the fluorescent indicator Fluo-8 in hiPSC-CMs. Fig. 3A shows representative fluorescent waveforms from after BMS-986094 treatment (Fig. 3A). BMS-986094 had little effect on calcium amplitude and Ca²⁺ transient duration at 50% (CTD50) at 48 hr (Fig. 3B, and C). Consistent with contractility impairment, we found that BMS-986094 decreased calcium amplitude at 96 hr (Fig. 3B). Moreover, BMS-986094 prolonged CTD50 at 1 μ M, 3 μ M, 96 hr (Fig. 3C). In contrast, sofosbuvir had little effect on the calcium amplitude and CTD50 (Fig. 4B, and C). These data suggest that BMS-986094 induces contractility impairment due to the impairment of calcium handling in hiPSC-CMs.

The chronic cytotoxic effect of HCV NS5b inhibitor in hiPSC-CMs

To evaluate the cytotoxicity, we analyzed the effect of BMS-986094 on beating area, which exhibited the ratio of beating cell area above the equivalent recording area, and expression levels of cardiac structural genes (Kopljär *et al.*, 2017). Treatment with BMS-986094 at 3 μ M had little effect on the beating area until 96 hr and signifi-

Fig. 1. Chronic effect of BMS-986094 on motion waveforms in hiPSC-CMs. (A) Schematic of a motion waveform in hiPSC-CMs. Contractile parameters were obtained as follows: a: contraction velocity (CV), b: relaxation velocity (RV), c: contraction-relaxation deformation distance (CRDD). (B) Representative motion waveforms in hiPSC-CMs after treatment with BMS-986094 at 0.3 μ M and 3 μ M (pre, 48 hr, 96 hr, 144 hr). (C) Effects of BMS-986094 on CV, RV, and CRDD measured by motion vector analysis. Data are expressed as mean $\pm$ S.E. (n = 3). *P < 0.05, difference between drug treatments and time-matched control.

cantly decreased the beating area by 12.7% at 120 hr and 78.7% at 144 hr in hiPSC-CMs (Fig. 5A). In addition, sofosbuvir had little effect on the beating area up to 144 hr in hiPSC-CMs (Fig. 5A). Furthermore, we found

that BMS-986094 decreased expression levels of cardiac structural genes, *cTnI* and *MYH7*, at 24, 48, 96, and 144 hr, while sofosbuvir had little effect (Fig. 5B). These data suggest chronic treatment with BMS-986094

Fig. 2. Chronic effect of sofosbuvir on motion waveforms in hiPSC-CMs. (A) Representative motion waveforms in hiPSC-CMs after treatment with sofosbuvir at 1 μM or 10 μM (pre, 48 hr, 96 hr, 144 hr). (B) Effects of sofosbuvir on CV, RV, and CRDD measured by motion vector analysis. Data are expressed as mean $\pm$ S.E. ($n = 3$).

induced cytotoxicity in hiPSC-CMs.

Drug-induced changes in calcium handling-related genes transcription

To further examine the effect of BMS-986094 on calcium handling, we analyzed the changes in calcium handling-related gene transcription in hiPSC-CMs. We found that BMS-986094 decreased the expression levels of genes, *CACNA1C*, *RYR2*, and *NCX1*, at 24, 48, 96 and

144 hr, while sofosbuvir had little effect (Fig. 6). These data suggest that BMS-986094 induced cardiac contractility dysfunction by inhibiting the gene expression of calcium handling-related genes.

DISCUSSION

In this study, we assessed the chronic effects of the HCV NS5b inhibitors BMS-986094 and sofosbuvir on

Fig. 3. Chronic effects of BMS-986094 on Ca^{2+} transient in hiPSC-CMs. (A) Representative Ca^{2+} transient in hiPSC-CMs after treatment with BMS-986094 for 48 and 96 hr. (B) Calcium amplitude (%) after treatment with BMS-986094 for 48 or 96 hr. (C) Ca^{2+} transient duration 50 (CTD50) after treatment with BMS-986094 for 48 or 96 hr. Data are expressed as the mean $\pm$ S.E. ($n = 3$). * $P < 0.05$, difference between treatments and time-matched controls.

cardiac contractility using motion analysis with hiPSC-CMs. Consistent with clinical studies, we found that long-term treatments with BMS-986094 induced the contraction dysfunction in hiPSC-CMs at 0.3–3 μ M, while sofosbuvir had little effect. Moreover, BMS-986094 decreased the calcium transient and inhibited the expression of calcium handling-related genes.

We found that BMS-986094 caused delayed the effect on contractile function in hiPSC-CMs. Consistent with our observations, clinical trials with BMS-986094 reported that some patients showed LVEF dysfunction after treatment for between 1 and 6 weeks (Ahmad *et al.*,

2015). BMS-986094 at 15 mg/kg/day decreased LVEF in cynomolgus monkeys, which C_{max} , maximum blood concentration, is estimated about 3 μ M (Li *et al.*, 2017; Gill *et al.*, 2017). In the case of our *in vitro* data, BMS-986094 at 1 μ M decreased RV on day 3 and CV on day 4. The concentrations that cause chronic cardiotoxicity in cynomolgus monkeys are equivalent to those in hiPSC-CMs. In the case of sofosbuvir, consistent with our data, clinical studies reported that sofosbuvir had little effects on cardiac function in patients with HCV infection (Ibrahim *et al.*, 2020). Our data showed that sofosbuvir had little effect on cardiac contractility at 10 μ M, which

Fig. 4. Chronic effect of sofosbuvir on the Ca^{2+} transient in hiPSC-CMs. (A) Representative Ca^{2+} transient in hiPSC-CMs after treatment with sofosbuvir for 48 and 96 hr. (B) Calcium amplitude (%) after treatment with sofosbuvir for 48 or 96 hr. (C) Ca^{2+} transient duration 50 (CTD50) after treatment with sofosbuvir for 48 or 96 hr. Data are expressed as mean $\pm$ S.E. ($n = 3$).

is 10-fold higher than the C_{max} (approximately 1 μM) in patients (Kirby *et al.*, 2015) for 144 hr in hiPSC-CMs. Although it is necessary to change the culture medium containing the drug to evaluate the chronic effects, we did not determine the actual concentrations of the medium used for the hiPSC-CMs. In addition, it is not easy to estimate the number of days for which they should be monitored to assess chronic cardiotoxicity *in vitro* before starting a non-clinical evaluation. Further investigation should be performed to determine the suitable parameters to pre-

dict contractility impairment by chronic treatment in hiPSC-CMs.

In addition to functional cardiotoxicity, the detection of structural cardiotoxicity with hiPSC-CMs is expected to provide comprehensive cardiac liabilities (Yang and Papoian, 2018). Treatment with BMS-986094 for 24 hr decreased gene expression levels of *MYH7* and *cTnI*, suggesting BMS-986094 induced cytotoxicity. In contrast, BMS-986094 at 3 μM did not affect beating area up to 96 hr. Thus, expression of cardiac structural genes is a

Fig. 5. Chronic cytotoxic effects of HCV NS5b inhibitors in hiPSC-CMs. (A) After treatment with BMS-986094 and sofosbuvir, beating area was measured by motion vector analysis. (B) After treatment with BMS-986094 (1 μ M) and sofosbuvir (3 μ M) for 24, 48, 96, and 144 hr, the expression levels of cardiac structural genes, *cTnI* and *MYH7*, were measured using real-time PCR. Data are expressed as the mean $\pm$ S.E. (n = 3). *P < 0.05, differences between drug treatments and time-matched controls.

more sensitive marker than beating area in hiPSC-CMs. Consistent with our observations using hiPSC-CMs, a previous study using cynomolgus monkeys showed that BMS-986094 induced structural cardiotoxicity at 30 mg/kg/day for 1-month treatments (Gill *et al.*, 2017). Cardiac troponins are widely used as serum biomarkers to assess cardiotoxicity by doxorubicin, which is known to cause structural cardiotoxicity, in patients (Zamorano *et al.*, 2017). Furthermore, a previous study reported that doxorubicin decreased the gene expression of *cTnI* in hiPSC-CMs (Kopljär *et al.*, 2017). Taken together, expression levels of *cTnI* in hiPSC-CMs might have translational implication for evaluation of structural cardiotoxicity.

To understand the mechanism by which BMS-986094 caused contractility impairment, we examined calcium signaling in hiPSC-CMs. We found that BMS-986094 decreased calcium amplitude in a concentration- and time-dependent manner in hiPSC-CMs. In addition, BMS-986094 inhibited expression levels of calcium handling-related genes, including *CACNA1C*, *RYR2*, and *NCX1*,

suggesting that BMS-986094 induced contractility dysfunction via calcium handling gene expression. Because BMS-986094 decreased the expression of cardiac structural genes at 24 hr, it is not clear whether contractility impairment is due to the cytotoxicity or calcium handling. Future studies should be performed to understand how various types of drugs cause structural toxicity and functional toxicity in hiPSC-CMs.

Mitochondrial toxicity has been recognized to monitor the long-term effects of toxicity (Meyer *et al.*, 2018). A previous study reported that BMS-986094-induced cardiotoxicity was induced by mitochondrial dysfunction associated with the incorporation of mitochondrial RNA polymerase (POLRMT), correlating with the reduction of protein expression of mitochondrial genes and reduction in mitochondrial respiration in PC-3 prostate cancer cells (Feng *et al.*, 2016). Moreover, another study observed that treatment with BMS-986094 for 6 and 10 days reduced mitochondrial respiration in hiPSC-CMs at ≥ 0.1 μ M (Baumgart *et al.*, 2016). However, an *in vivo*

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Fig. 6. Chronic effects of HCV NS5b inhibitors on gene expression in hiPSC-CMs. After treatment with BMS-986094 (1 μ M) and sofosbuvir (3 μ M) for 24, 48, 96 and 144 hr each, the expression levels of calcium-related genes, *CACNA1C*, *RYR2*, and *NCX1*, were measured using real-time PCR. Data are expressed as mean $\pm$ S.E. (n = 3). *P < 0.05, differences between drug treatments and time-matched controls.

study did not demonstrate mitochondrial toxicity in cardiomyocytes (Gill *et al.*, 2017). Thus, it remains to be investigated whether BMS-986094 causes mitochondrial cardiotoxicity.

As discussed above, the cardiotoxicity of anti-HCV agent candidates has been a key issue during drug development (Ucciferri *et al.*, 2018). In addition to BMS-986094, BILN-2061, which was developed as an anti-HCV drug candidate by inhibiting HCV NS3/NS4A serine protease, was terminated during clinical trials, owing to cardiotoxicity, resulting in LVEF dysfunction and myocardial vacuolation related to mitochondrial swelling in Rhesus monkeys (Stoltz *et al.*, 2011). Since our data showed that hiPSC-CMs can distinguish cardiotoxicity between the cardiotoxic BMS-986094 and less toxic sofosbuvir, it would be interesting to see the effects of BILN-2061 in hiPSC-CMs. Moreover, a combination of sofosbuvir and amiodarone has been shown to cause severe symptomatic bradycardia (Ucciferri *et al.*, 2018). This cardiotoxicity is considered to be caused by impaired intracellular calcium handling and excitation-contraction coupling (Millard *et al.*, 2016). Notably, hiPSC-CMs identified bradycardia after the combination treatment with sofosbuvir and amiodarone in hiPSC-CMs (Yu *et al.*, 2018). Thus, hiPSC-

CMs might improve cardiac hazard identification in early drug safety de-risking for anti-HCV agents as well as other types of drugs.

In conclusion, the imaging-based contractility assay with chronic exposure revealed a difference in cardiotoxicity between the cardiotoxic BMS-986094 and the less toxic sofosbuvir in hiPSC-CMs. Furthermore, BMS-986094 caused contractility impairments via a decrease in Ca^{2+} signaling in hiPSC-CMs, while sofosbuvir had little effect. Therefore, hiPSC-CMs can be a useful tool to predict drug-induced chronic cardiotoxicity in non-clinical settings.

ACKNOWLEDGMENTS

This study was supported by the Research on Regulatory Science of Pharmaceuticals and Medical Devices of the Japan Agency for Medical Research and Development (AMED) (JP21mk0101189), JSPS KAKENHI Grant (21H02634), and a grant from the Smoking Research Foundation.

Conflict of interest---- The authors declare that there is no conflict of interest.

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