Functional and Transcriptional Characterization of Histone Deacetylase Inhibitor-Mediated Cardiac Adverse Effects in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

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ABSTRACT
Histone deacetylase (HDAC) inhibitors possess therapeutic potential to reverse aberrant epigenetic changes associated with cancers, neurological diseases, and immune disorders. Unfortunately, clinical studies with some HDAC inhibitors displayed delayed cardiac adverse effects, such as atrial fibrillation and ventricular tachycardia. However, the underlying molecular mechanism(s) of HDAC inhibitor-mediated cardiotoxicity remains poorly understood and is difficult to detect in the early stages of preclinical drug development because of a delayed onset of effects. In the present study, we show for the first time in human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) that HDAC inhibitors (dacinostat, panobinostat, vorinostat, entinostat, and tubastatin-a) induce delayed dose-related cardiac dysfunction at therapeutic concentrations associated with cardiac adverse effects in humans. HDAC inhibitor-mediated delayed effects on the beating properties of hiPS-CMs developed after 12 hours by decreasing the beat rate, shortening the field potential duration, and inducing arrhythmic behavior under form of sustained contractions and fibrillation-like patterns. Transcriptional changes that are common between the cardiotoxic HDAC inhibitors but different from noncardiotoxic treatments identified cardiac-specific genes and pathways related to structural and functional changes in cardiomyocytes. Combining the functional data with epigenetic changes in hiPS-CMs allowed us to identify molecular targets that might explain HDAC inhibitor-mediated cardiac adverse effects in humans. Therefore, hiPS-CMs represent a valuable translational model to assess HDAC inhibitor-mediated cardiotoxicity and support identification of better HDAC inhibitors with an improved benefit-risk profile.

SIGNIFICANCE
Histone deacetylase (HDAC) inhibitors are a promising class of drugs to treat certain cancers, autoimmune, and neurodegenerative diseases. However, treated patients can experience various cardiac adverse events such as hearth rhythm disorders. This study found that human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) can predict cardiac adverse events in patients caused by HDAC inhibitors. Furthermore, transcriptional changes at the level of gene expression supported the effects on the beating properties of hiPS-CMs and highlight targets that might cause these cardiac adverse effects. hiPS-CMs represent a valuable translational model to assess HDAC inhibitor-mediated cardiotoxicity and to support development of safer HDAC inhibitors.

INTRODUCTION
Histone deacetylases (HDACs) are epigenetic regulatory enzymes involved in the remodeling of chromatin structure through deacetylation of N-terminal lysine residues within histones [1]. HDACs equally target nonhistone proteins [2], which can regulate transcription and other cellular processes such as cellular signaling and protein localization/stability [3]. Chromatin remodeling controls the epigenetic regulation of gene expression, whereas aberrant expression or function of specific HDAC isoforms is observed in certain cancers and autoimmune and neurodegenerative diseases [1]. There are 18 known HDAC isoforms classified into four classes (I–IV) according to their structure and function [4, 5]. A substantial effort has been placed in developing both
broad-spectrum and class-specific HDAC inhibitors as anticancer drugs, reflected by more than 490 clinical trials initiated over the last 10 years [6], currently with 4 U.S. Food and Drug Administration (FDA)-approved drugs.

Unfortunately, the therapeutic potential of HDAC inhibitors in clinical studies is hampered by reports of delayed cardiac arrhythmias, including atrial fibrillation and ventricular tachycardia, some of which are associated with QT prolongation [6–8]. However, the molecular mechanism of HDAC inhibitor-mediated cardiotoxicity remains poorly understood. Furthermore, because of the delayed onset, occurring only after several hours up to days, cardiac side effects are difficult to detect in safety pharmacology studies, where most (in vitro) cardiac safety studies are focused on acute effects (≤1 hour).

Human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs) are a promising human-based cellular model to investigate human cardiac diseases and drug-induced cardiac side effects [9]. In this study, we evaluated the potential use of hiPS-CMs as a translational model for HDAC inhibitor-mediated cardiotoxicity in humans by investigating five HDAC inhibitors (dacinostat, panobinostat, vorinostat, entinostat, and tubastatina) with different levels of cardiotoxicity reported in clinical trials. Dacinostat (LAQ824) is a pan-HDAC inhibitor that has been associated with cardiac problems in phase I studies evidenced by dose-related atrial fibrillation [10] and QT prolongation, with a single event of nonsustained Torsade de Pointes (TdPs) [11]. The development of dacinostat has been stopped in favor of panobinostat (LBHS89), another potent pan-HDAC inhibitor in phase II/III trials and recently approved by the FDA as a combined treatment for multiple myeloma. However, an early phase I study with panobinostatin also reported QT prolongations in 33% of patients [12], leading to an adjusted dosing schedule with a lower rate of incidence [13]. Also, vorinostat (suberanilohydroxamic acid) is a pan-HDAC inhibitor that was FDA-approved in 2006 with limited effects on QT interval at therapeutic concentrations [14]. Conversely, entinostat (MS275) is a selective class I (HDAC1-3) inhibitor currently in phase II/III trials with low reports of cardiotoxicity. Tubastatin-a is a selective HDAC6 (class IIb) inhibitor that is in an experimental stage.

To get a better understanding of HDAC inhibitor-related cardiotoxicity, we investigated whether HDAC inhibitors can induce cardiotoxicity in hiPS-CMs. Because HDAC inhibitor-specific cardiac adverse effects develop with a delayed onset of 1–3 days [8], we followed the long-term effects of HDAC inhibitors on the beating properties of hiPS-CMs using an impedance-based functional assay [15]. HDAC inhibitors caused contractile dysfunction and arrhythmic-like events in hiPS-CMs at concentrations comparable to the free (unbound) peak plasma concentrations (Ceff) associated with cardiac adverse effects in humans [10–14, 16, 17]. Combining the functional data with transcriptional changes in hiPS-CMs allowed us to identify molecular targets and pathways that may potentially be involved in HDAC inhibitor-mediated cardiotoxicity.

Materials and Methods

Cell Culture and Reagents

hiPS-CM were purchased from Axio genesis (Cor.4U cardiomyocytes, Ax-B-HC02-4M; Axio genesis, Cologne, Germany, http://axiogenesis.com) as frozen vials or living preplated cells (batch numbers CB63CL, CB77CL, F-KW24CL, F-KW25CL, and F-KW26CL). Cor.4U cardiomyocytes represent a mix of 60% ventricular, 20% atrial, and 20% nodal cells, according to the cell provider. Frozen vials were thawed and cultured in an incubator at 37°C and 5% CO2 according to the manufacturer’s instructions. Briefly, cardiomyocytes were thawed in thawing medium and seeded onto fibronectin-coated “E-plate Cardio 96” (ACEA Biosciences, San Diego, CA, http://www.acebio.com) at ~25,000 cells per well. For multi electrode array (MEA) experiments, cells were seeded on fibronectin-coated six well MEAs (Multi Channel Systems MCS GmbH, Reutlingen, Germany, http://www.multichannelsystems.com) at ~10,000 cells per well and measured by using MEA2100 (MCS). Culturing for all experiments was done by using culture medium, which was changed every 1–2 days. Two days before the start of the experiments, the cells were switched to serum-free BMCC medium. During the culturing and experiments, cells were maintained in a humidified incubator with 5% CO2 at 37°C. Cells were allowed to form a synchronously beating network during 5–7 days before initiating compound treatment for 1 day (MEA) or 4 days (xCelligence), where compound treatments were refreshed every 2 days.

Drug stocks were prepared in 100% dimethylsulfoxide (DMSO) and stored at 4°C. Stock solutions were diluted in serum-free BMCC medium to a final concentration of 0.1% DMSO on the day of initial compound addition. Concentration ranges were based on the Ceff [10, 18–20] or the IC50/EC50 [21–24] values of the drug as obtained from the literature. Chemicals were purchased from the following vendors: Entinostat (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), Tubastatin-A (Sequoia Research Products, Pangbourne, United Kingdom, http://www.seqchem.com), Iverapradine (WuXi AppTec, Shanghai, People’s Republic of China, https://www.wuxiapptec.com), Nitrendipine (Bayer AG, Leverkusen, Germany, http://www.bayer.com), and vorinostat (J&W PharmLab, Levittown, PA, http://www.jwpharmlab.com). Panobinostatin, dacinostat, and doxetilide were synthesized internally. Fibronectin (catalog no. F-1141; Sigma-Aldrich) was dissolved in Dulbecco’s phosphate-buffered saline (catalog no. D8662, Sigma-Aldrich) as a 1 mg/ml stock solution.

Functional Data Analysis

Functional properties of cardiomyocytes were assessed through impedance-measurements by using the xCELLigence Cardio instrument (ACEA Biosciences-Roche Diagnostics) [15]. Impedance signals were recorded at baseline and after compound addition at ≤1-hour intervals during 84 hours. Data were analyzed by the RTCA Cardio Software, which allowed calculation of beating rate and amplitude of the impedance signals. MEA experiments were recorded at baseline and 6 and 24 hours after dose. Data were normalized to baseline. A second normalization incorporated data from time-matched vehicle control to compensate for changes unrelated to drug exposure.

Microarray Setup and Data Analysis

Treated cardiomyocytes were lysed by using RLT buffer (Qiagen, Hilden, Germany, http://www.qiagen.com) and RNA extracted with the RNeasy kit (product no. 74181; Qiagen). All microarray-related steps for target preparation, including the amplification of total RNA and labeling, were carried out as described in the GeneChip 3’ IVT Express Kit User Manual (Affymetrix, Santa Clara, CA, http://www.affymetrix.com). Biotin-labeled target samples were hybridized to a GeneChip Human Genome HG-U219 Array containing probes for approximately 18,000 genes.
Target hybridization was processed on the GeneTitan Instrument according to the instructions provided in the User Guide for Expression Array Plates (product no. 702933). Images were analyzed by using the GeneChip Command Console Software (AGCC) (Affymetrix).

All data were processed by using the statistical computing R program [R Foundation for Statistical Computing, Vienna, Austria, https://www.r-project.org] and Bioconductor tools [25]. The gene expression values were normalized by using RMA [26]. Grouping of the individual probes into genespecific probe sets was performed based on Entrez Gene by using the metadata package hgu219hsentrezg (version 19.0.0) [27].

Sample similarity is based on log ratio data—that is, per gene the log 2 intensity of each treatment minus the log 2 intensity of the medoid sample of the corresponding vehicles. The sample similarity plot is based on a spectral map analysis [28]. The limma-based differential expression analysis [29] was performed in two steps: (a) for each compound:dose:time combination, the differential expression was tested versus the vehicles (based on the precomputed log fold-change); and (b) the cardiotoxicity and noncardiotoxic compounds, as defined in Results, were compared by using the sample similarity values. Note that genes are named upregulated or downregulated for cardiotoxic treatments relative to noncardiotoxic treatments. Pathway analysis is based on MLP by using the p values of all genes from step b of the differential analysis [30].

Statistical Analysis

All functional data are expressed as means ± SEM. Statistical significance between vehicle and compound were calculated based on their changes from baseline with two-way repeated-measures analysis of variance using nonnormalized values. For all functional experiments, values of p < .05 were considered statistically significant. Microarray analysis is explained in Microarray Setup and Data Analysis.

RESULTS

HDAC Inhibitor-Mediated Contractile Dysfunction in hiPS-CMs

HDAC inhibitor-induced cardiac toxicity identified in the clinic appears to involve both atrial and ventricular effects. Fortunately, hiPS-CMs used in this study represent a mixed population of ventricular, atrial, and nodal cells, allowing an in vitro assessment of HDAC inhibitor-mediated adverse effects encompassing cellular components of the “whole” heart. After seeding, cells formed a synchronously beating network during 5–6 days, after which they were treated with compound for 84 hours. The beating properties of cardiomyocytes were assessed through impedance measurements by using the xCELLigence Cardio platform, which has been shown to predict drug-induced human cardiac arrhythmic risk [31]. To validate our model, several cardiac ion channel drugs (dofetilide, a human ether-a-go-go related gene (HERG) (I_Ks) blocker; nitrendipine, a Ca²⁺ antagonist; isoprenaline, a β-agonist; and ivabradine, an I₁ blocker) were tested for effects on the beating properties of hiPS-CMs (supplemental online Fig. 1). Dofetilide decreased the beating rate, prolonged the contractions, and caused arrhythmic events at concentrations slightly higher than the IC₅₀ value for HERG [21]. Nitrendipine [24] increased the beat rate while decreasing the amplitude of the contractions. These effects are in line with previous studies with nitrendipine or other Ca²⁺ channel antagonists in hiPS-CMs [31, 32]. Isoprenaline strongly increased the beat rate at low nanomolar concentrations according to the EC₅₀ values for β₁ and β₂ [22]. Ivabradine slowed the beat rate and altered the rhythmicity in hiPS-CMs in line with IC₅₀ values for hyperpolarization-activated, cyclic nucleotide-gated channels [23]. Hence, hiPS-CMs responded to the drugs as expected based on target affinity and in line with previous studies [31, 32], supporting their use as an in vitro model to investigate HDAC inhibitor-mediated cardiac adverse effects in humans.

Next, we investigated the effect of HDAC inhibitors on the beating properties of hiPS-CMs. Figure 1 shows impedance tracings of cardiomyocytes at 6, 24, and 72 hours after dose using concentrations around the free (unbound) peak therapeutic plasma concentration (Cₑff) and approximately 10-fold higher. Cₑff values were based on current dosing schedules for panobinostat [19], vorinostat [20], and entinostat [18], whereas for dacinostat, we used the plasma concentration associated with dose-limiting toxicities [10]. Within 6 hours, none of the HDAC inhibitors altered the beating pattern of cardiomyocytes, suggesting no acute effects. In contrast, at 24 and 72 hours, several treatments began to affect cardiomyocyte function by decreasing beat rate and amplitude of the contractions—in some cases even leading to beating arrest.

Detailed time- and concentration-dependent effects on the beating rate of cardiomyocytes (Fig. 2) showed that HDAC inhibitors displayed an onset of cardiotoxicity at approximately 12 hours of exposure and became more pronounced over time. Daciniostat caused a significant decrease in beat rate beginning at a concentration of 0.1 μM, whereas concentrations ≥ 0.5 μM caused beating arrest by 72 hours (Fig. 2A). Panobinostat showed moderate effects on beating rate at a concentration of 10 nM (Fig. 2B), whereas at 0.1 μM it drastically reduced beating rate, causing...
beating arrest in six of seven experiments. Vorinostat displayed moderate effects on beating function of cardiomyocytes at a concentration of 0.3 μM ($C_{\text{eff}}$) (Fig. 2C). Entinostat did not affect the beating rate of hiPS-CMs at concentrations up to 10-fold higher than $C_{\text{eff}}$, indicating a better safety margin compared with the other HDAC inhibitors in this study. Tubastatin-a decreased the beat rate only at the highest concentration (10 μM) 60 hours after compound exposure (Fig. 2E). A similar read-out was observed for all HDAC inhibitors (with the exception of tubastatin-a) on the amplitude of contractions (supplemental online Fig. 2). Hence, HDAC inhibitors induce delayed contractile dysfunction where both beat rate as well as amplitude of the impedance-measured contractions are decreased. Furthermore, an acute decrease in beat rate was observed with high, therapeutically irrelevant concentrations of panobinostat (1–5 μM) and dacinostat (3–10 μM), which corresponds to their respective IC$_{50}$ values for hERG [12, 33].

**Figure 2.** Histone deacetylase (HDAC) inhibitors decrease the beat rate and cause arrhythmic events in human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs). (A–E): Concentration-dependent effect of HDAC inhibitors on the normalized beat rate is shown as a function of time. A concentration range based on the effective concentration ($C_{\text{eff}}$) was compared with the vehicle treatment. Note that the red line represents the $C_{\text{eff}}$. Data are presented as mean ± SEM ($n = 7–11$), $p < .05$ (differences between treatments and vehicles). (F): Examples of arrhythmic-like events representing prolonged/sustained contractions and fibrillation-like pattern on hiPS-CMs. Abbreviation: Norm., normalized.

**HDAC Inhibitors Evoke Arrhythmic Events in hiPS-CMs**

Besides the observed contractile dysfunction, we noticed with some treatments occasional arrhythmic events in the form of sustained/prolonged contractions, a fibrillation-like pattern, and beating arrest. Prolonged contractions—represented by an extremely prolonged relaxation time or by at least three additional contractions starting from a precontracted state (before the complete relaxation of the first beat) (Fig. 2F; supplemental online Table 1)—were occasionally observed with dacinostat at concentrations of 0.1–0.5 μM. Furthermore, 0.5 μM dacinostat caused a fibrillation-like pattern in four of eight experiments (50%) and beating arrest in eight of eight experiments (100%). For panobinostat, sustained contractions were observed in one of seven (14%) and two of seven (29%) experiments at concentrations of 10 nM and 0.1 μM, respectively. Beating arrest occurred in a concentration-dependent manner in one of seven (14%) and six of seven (86%) experiments at concentrations of 10 nM and 0.1 μM, respectively. Conversely, such arrhythmic-like events were not observed with either vorinostat or entinostat at their $C_{\text{eff}}$. Tubastatin-a only showed a single incidence (13%) of sustained contractions at a concentration of 10 μM.

**Delayed Effects of HDAC Inhibitors on Field Potential Duration in hiPS-CMs**

To understand the contractile dysfunction from an electrophysiological point of view, we measured the effect of HDAC inhibitors on the external, potential of hiPS-CMs (Fig. 3A) using a multielectrode array platform. Field potential duration (FPD), which provides an estimation of action potential duration, was not affected at 6 hours of exposure to HDAC inhibitor treatments compared with the vehicle (Fig. 3B, 3C). However, after 24 hours of compound exposure we observed a shortening of baseline-normalized FPD with dacinostat at a concentration of 0.1 μM (0.44 ± 0.12; $n = 5$), panobinostat at 10 nM (0.62 ± 0.16; $n = 4$), and, to lesser extent, with
Histone deacetylase (HDAC) inhibitors shorten the field potential duration in hiPS-CMs. (A): MEA recordings representing an external potential signal of human induced pluripotent stem cell-derived cardiomyocytes (hiPS-CMs). FPD is determined from the depolarization spike to the repolarization peak. Note that the repolarization peak can be either negative or positive. (B): Some HDAC inhibitors shorten the FPD shown by a faster manifestation of the repolarization peak (arrow) at 24 hours. The level of FPD shortening is HDAC inhibitor-dependent, that is, more pronounced for dacinostat and panobinostat than for entinostat and vorinostat. (C, D): Bar plots showing the baseline-normalized FPD and beat rate for vehicle (n = 9), 0.1 μM dacinostat (n = 5), 10 nM panobinostat (n = 4), 0.3 μM entinostat (n = 5), and 0.1 μM vorinostat (n = 5) treatments at 6 and 24 hours after dose. Data are represented as mean ± SEM. *p < .05 (differences between treatments and vehicles). Abbreviations: Daci, dacinostat; Pano, panobinostat; Vori, vorinostat; Enti, entinostat; FPD, field potential duration; norm., normalized; Pano, panobinostat; Vori, vorinostat.

Vorinostat at 0.1 μM (0.85 ± 0.11; n = 5), whereas entinostat at a concentration of 0.3 μM (0.92 ± 0.07; n = 5) did not significantly change baseline-normalized FPD compared with the vehicle (1.03 ± 0.03; n = 9). Dacinostat (0.43 ± 0.14; p < .001; n = 5) strongly decreased beat rate of hiPS-CMs using MEA (Fig. 3D), which was also observed with the impedance-based experiments (Fig. 2A). Panobinostat (0.81 ± 0.02; p = .01; n = 4) and entinostat (0.67 ± 0.11; p < .001; n = 5) showed a modest effect on beat rate (Fig. 3D), whereas vorinostat (0.84 ± 0.05; n = 5) showed a minor, but not significant, effect on the beat rate compared with vehicle (0.95 ± 0.02; n = 9) in the MEA system (Fig. 3D).

Overall Transcriptional Response of hiPS-CMs to HDAC Inhibitors

Based on our functional data and the epigenetic effects of HDAC inhibitors, we examined the changes in the transcriptome of hiPS-CMs in response to HDAC inhibitors. We classified treatments into two different groups according to the observed functional cardiotoxicity (Fig. 2; supplemental online Fig. 2): (a) a cardiotoxic group containing treatments causing severe (0.5 μM dacinostat, 0.1 μM panobinostat, and 5 μM vorinostat) and intermediate (0.01 μM panobinostat and 0.3 μM vorinostat) effects and (b) a “relative” noncardiotoxic group (0.3 μM entinostat, 1 μM tubastatin-a, and 0.1 μM vorinostat). Because of the delayed onset of cardiotoxicity and the potential earlier effects on the transcriptome, we selected time points before (2 and 4 hours) and during (12 and 24 hours) HDAC inhibitor-mediated functional changes for expression profiling of hiPS-CMs.

All treatments modified biological pathways related to HDACs, chromatin regulation, and protein acetylation, indicating that the HDAC inhibitors affect their molecular targets (data not shown). When comparing differential expression as represented by the number of differentially expressed genes versus vehicles, an increase in the number of significant genes over time was observed for almost all (and, in particular, for cardiotoxic) treatments (Fig. 4A). The data-driven sample similarity plot based on all measured transcripts illustrates that the main source of overall transcriptional variability between samples (principal component 1 [PC1] = 50%) was linked to time and cardiotoxicity (Fig. 4B). The left side of the spectral map contains intermediate and severe cardiotoxic treatments from the late (12- and 24-hour) time points. Both intermediate and severe cardiotoxic treatments showed a common overall time-dependent path mainly over the x-axis. This justifies the grouping of the intermediate and severe treatments together to identify common transcriptional responses in hiPS-CMs that may be related to cardiotoxicity.

Transcriptional Response of hiPS-CMs Related to Cardiotoxicity

Comparing the differential expression induced by the cardiotoxic treatments with the other treatments, pathways related to HDACs are among the top 5 affected gene sets after 2 and 4 hours of treatment (Fig. 5). Adrenergic receptor activity was also a top-ranked pathway, which is crucial for cardiomyocyte function and linked to cardiomyopathic conditions [34]. Onset of functional side effects (at 12 hours) was accompanied by changes in pathways related to microtubule- and cytoskeleton-based transport, cardiac muscle cell contraction, and Z-disc and unfolded protein binding. Hence, the contractile dysfunction of hiPS-CMs at the functional level could also be observed at the gene expression level.

Volcano plots (Fig. 6), illustrating the significance and magnitude of differential expression for all measured genes, show a time-dependent increase in the number of significantly regulated genes, whereas the ratio of upregulated and downregulated genes seemed to be relatively balanced. Top genes based on significance and differential expression (cardiotoxic versus noncardiotoxic) are highlighted in black across all time points (Fig. 6; supplemental online Table 2). Whereas most are related to on-target effects...
and cancers, several of these genes (focusing on 2, 4, and 12 hours) are also implicated in cardiac pathology (supplemental online Fig. 3). At earlier time points (2 and 4 hours) Timp3 [35], ADRB2 [36], GADD45B [37], DUSP6 [38], CEBPB [39], JARID2 [40], and STC1 [41] are specific genes possibly related to heart failure and hypertrophy. At 12 hours, the top genes are associated with the cytoskeleton (TUBB2B) and Z-disc (MURC, NEXN, and RRAD).

As mentioned, most of the top genes are related to oncology. This is expected because HDAC inhibitors are known for strongly regulating the global transcriptome of (cancer) cell lines. However, because of observed contractile and electrophysiological changes in hiPS-CMs, we highlighted certain genes known to be involved in cardiac contractility and functioning (supplemental online Table 3) in Figure 6 (red labels). Interestingly, several cardiac genes were already differentially expressed before the onset of functional cardiotoxicity. At 4 hours, the differential expression of ADRB2, ADRB1, KCNJ2, KCNJ12, and CALM1 was significantly higher for the cardiotoxic treatments as compared with the noncardioxic ones, whereas GJA5 was relatively downregulated. When functional cardiotoxicity began to surface (12 hours), we observed a further significant downregulation of GJA5 together with an increasing number of cardiac genes being regulated (mostly downregulated) (supplemental online Fig. 3). This trend was further strengthened at 24 hours when some of the HDAC inhibitor treatments caused functional beating arrest of the hiPS-CM cultures. However, the increased number of regulated genes was also observed overall and is not necessarily cardio-specific. Nonetheless, cardiotoxic HDAC inhibitor treatments seem to modulate several cardiac genes compared with nontoxic treatments at multiple time points. However, at 12 and 24 hours, it is challenging to differentiate whether the expression changes are a direct cause of cardiac side effects or a feedback mechanism secondary to the cardiotoxicity. Interestingly, the cardiac genes modulated at the earlier (precardiotoxic) time points highlight potential drivers or markers for HDAC inhibitor-mediated cardiotoxicity.

**DISCUSSION**

**HDAC Inhibitors Alter the Electrophysiological and Beating Properties of hiPS-CMs**

Delayed cardiac adverse events (mostly atrial fibrillation and ventricular tachycardia) have been reported for several HDAC inhibitors in clinical studies, but the underlying mechanism(s) of HDAC inhibitor-mediated cardiotoxicity is poorly understood. In this study, we show that HDAC inhibitors induce delayed cardiotoxicity in hiPS-CMs at clinically relevant concentrations. The beating rhythm of cardiomyocytes was unaltered up to 6 hours after dose, indicating no acute effects on the electrophysiology and/or contractility of hiPS-CMs. HDAC inhibitors showed a delayed onset of decreased beat rate and amplitude of impedance-measured contractions starting at 12 hours after dose. Furthermore, pan-inhibitors panobinostat and dacarboxinostat triggered delayed “arrhythmic” events in the form of sustained and/or prolonged contractions and fibrillations at concentrations similar to their C50. These events may be potentially linked to long QT, incidence of TdPs, and atrial fibrillation in the clinic. Mechanistic effects were accompanied at the electrophysiological level by a shortening of the FPD at 24 hours. Noticeably, there seemed to be no direct correlation between shortening of the FPD and decreased beat rate (Fig. 3C, 3D), suggesting mediation by different molecular targets or mechanisms of action rather than inhibition of a specific ion channel such as hERG. Although pan-inhibitors seemed to cause stronger effects on the beating of hiPS-CMs, selective inhibitors entinostat (class I selective) and tubastatin-a (HDAC6 isom form selective) evoked similar adverse effects only at their highest concentrations. Although this may be because of loss of selectivity and thus inhibition of additional HDAC isoforms [6], HDAC inhibitors target HDAC complexes and non-HDAC targets [42], making assumptions regarding the role of individual HDAC isoforms challenging. Nevertheless, our results indicate that more specific HDAC inhibitors might have a better cardiac safety profile compared with pan-inhibitors.
Translation of HDAC Inhibitor-Mediated Cardiotoxicity in hiPS-CMs to Dose-Related Cardiac Events in the Clinic

Good translation of experimental data in vitro (hiPS-CMs) to clinical data is crucial for understanding HDAC inhibitor-mediated cardiotoxicity in humans as well as application of hiPS-CMs in drug discovery for selection of safer HDAC inhibition with reduced cardiotoxicity potential. We compared the concentration-dependent effects of HDAC inhibitors in hiPS-CMs with cardiac events reported in clinical trials at concentration values. Dacinostat caused effects on beating, including arrhythmic-like effects (sustained contractions and fibrillations), at concentrations of 0.1–0.5 mM, which is similar to the therapeutic plasma concentrations associated with cardiac events in phase I studies that included dose-related QT prolongation, atrial fibrillation [10], and a single report of nonsustained TdPs [11]. Sensitivity of hiPS-CMs to panobinostat (Fig. 2) correlated with the dose-related cardiac events reported in the clinic. Phase I studies with i.v. administration (C_{eff} approximately 70–100 nM) resulted in a high percentage of cardiac events in the form of QT prolongation and T-wave and ST-segment changes [12, 13, 16]. Furthermore, cardiac toxicities leading to study discontinuation included a patient with sinus bradycardia and another patient with TdPs [16]. In contrast, phase I studies with oral administration and adjusted dosing schedules (C_{eff} approximately 5–10 nM) displayed a lower incidence of QT prolongation and cardiac events in general [13]. However, a phase Ia/II study with a larger population of patients (C_{eff} of 10 nM for cardiac events) did report dose-limiting toxicities consisting of atrial fibrillation (one patient), congestive cardiac failure (one patient), and QT prolongation (three patients). Vorinostat displayed moderate effects on hiPS-CMs at a concentration of 0.3 μM (C_{eff}), which might reflect the relatively low incidence of cardiac adverse events for vorinostat in the clinic [14]. Entinostat did not affect hiPS-CMs up to 10-fold higher values than C_{eff}, indicating a better safety profile compared with the pan-HDAC inhibitors in this study. These findings are in line with phase I studies of entinostat, in which there were no cardiac events or electrocardiographic changes with alternate-week dosing schedules [18]. However, the initial daily-dosing schedule in phase I showed dose-limiting supraventricular tachycardia in one patient (of two), leading to an adjustment of the dosing schedule [17]. Despite its apparently better safety profile, entinostat nonetheless has a relatively narrow safety margin, which is in alignment with our results in hiPS-CMs. Taken together, the HDAC inhibitors evaluated in this study appear to show a good correlation in (concentration-based) sensitivity between hiPS-CMs and the level/incidence of cardiac adverse effects (based on C_{eff}) in the clinic. These findings support hiPS-CMs as a potential translational model to detect delayed/chronic cardiac adverse effects of HDAC inhibitors in drug discovery and early preclinical development, to guide compounds with potential for an improved benefit-risk profile.

Figure 5. Gene Ontology (GO)-based enrichment analysis of transcriptional changes related to histone deacetylase inhibitor-mediated cardiotoxicity. For each time point, the five most strongly affected gene sets are shown, as based on three levels: biological processes (GOBP), molecular functions (GOMF), and cellular compartments (GOCC) as defined by GO. The significance determined using the permutation procedure (Materials and Methods) is represented on the y-axis and used to rank the pathways. Pathways are colored by gene set name within the same database. Abbreviations: GOBP, Gene Ontology Biological Process; GOCC, Gene Ontology Cellular Component; GOMF, Gene Ontology Molecular Function.
Cardiotoxic HDAC Inhibitor Treatments Share
Transcriptional Changes in hiPS-CMs Related to Cardiac
Contractility and Structural Cell Organization

To get a better understanding of the observed functional effects, we identified changes in transcriptional expression in hiPS-CMs specific for cardiotoxic HDAC inhibitor treatments. Common pathways between cardiotoxic HDAC inhibitor treatments included “adrenergic receptor activity,” “regulation of cardiac cell contraction,” and “Z-disc,” which are most likely linked to the observed contractile dysfunction of hiPS-CMs. Several pathways and genes related to the structural cell organization (e.g., cytoskeletal- and microtubule-based transport) were top-ranked as well. TUBB2B encodes a βII-tubulin isotype, which is a cytoskeleton protein associated with the mitochondria and might regulate their function in adult rat cardiomyocytes [43]. Z-disc-related genes MURC and NEXN were strongly downregulated at later stages (12 and 24 hours) and linked to cardiomyopathies [44, 45]. These findings point toward structural effects of HDAC inhibitors that might explain the contractile dysfunction in hiPS-CMs and potential congestive cardiac failure in some patients in the clinic.

Transcriptional and Functional Changes in hiPS-CMs Possibly Related to HDAC Inhibitor-Related Cardiotoxicity

Among the reported cardiac adverse effects in the clinic, atrial fibrillation has been the most commonly noted arrhythmia in patients treated with HDAC inhibitors [7]. Electrocardiographic changes in the form of ST depression, T-wave flattening, QT prolongation, and ventricular tachycardia were also frequently observed, whereas life-threatening Torsade de Pointes occurred sporadically in some cases. Hence, it seems these cardiac adverse effects are both atrial and ventricular in nature and most likely involve a myriad of molecular targets/mechanisms. Approximately 80% of atrial fibrillation and 24% of ventricular arrhythmias are documented in inheritable short QT syndromes [46], whereas ventricular fibrillation is also observed in acquired drug-induced short QT syndromes [47]. Functional (shortened FPD and fibrillating-behavior) and transcriptional (early-regulated ↓GJA5 and ↑KCNJ2 expression) effects in hiPS-CMs may reflect mechanisms underlying atrial and ventricular fibrillation.

GJA5 encodes connexin 40 (Cx40) and forms gap junctions in atrial cells, whereas GJA1 encodes for Cx43 and is the most common isoform in ventricular cardiomyocytes. Gap junctions mediate electrical cell-to-cell coupling, and altered expression, function, or localization of Cx40 and Cx43 have been associated with atrial (and ventricular) arrhythmias [48, 49]. In mice, HDAC inhibitors determine Cx43 hyperacetylation, dissociation from gap junctions, and lateralization along the long axis of ventricular cardiomyocytes [50]. In primary neonatal mouse ventricular cardiomyocytes, vorinostat reduced Cx43 mRNA and protein abundance and altered the phosphorylation state of Cx43, leading to a reduced electrical coupling through gap junctions [51]. Whereas these studies focused on Cx43 in ventricular myocytes, our data in hiPS-CMs (containing both atrial and ventricular types) suggest that mainly Cx40 and, to a lesser extent Cx43 (GJA1 in supplemental online Fig. 3), are modulated at the transcriptional level in hiPS-CMs. Furthermore, the “cell-cell contact zone” gene set was identified as one of the top Gene Ontology Cellular Component pathways at 12 and 24 hours. All together, our findings strengthen the hypothesis that modulation of gap junctions might play an important role in atrial fibrillation and HDAC inhibitor-mediated cardiotoxicity.

Relative upregulation of KCNJ2, and to a lesser extent of KCNJ12, was already observed at 4 hours. KCNJ2 encodes Kir2.1, the main isoform that mediates the inward-rectifying current I\textsubscript{K12}, which is responsible for setting and maintaining the cardiac resting membrane potential, as well as contributing to late cardiac repolarization. Upregulation of KCNJ2 might drive the
shortening of field potential duration that we observed with the cardiotoxic HDAC inhibitor treatments in hiPS-CMs (Fig. 3). Indeed, “gain of function” of Kir2.1 may cause shortening of action potential duration, resulting in a short-QT interval [46]. Furthermore, gain of function mutations in KCNQ1 have been linked to an increased propensity to develop atrial fibrillation and ventricular proarrhythmia [52–54].

**A** **D**RB1 and **A** **D**RB2 genes, which encode β1- and β2-adrenergic receptors, were strongly upregulated at early (2 and 4 hour) time points, whereas at later time points these effects decreased. β2-adrenergic receptors control myocardial function through intracellular signaling pathways and are linked to various pathological conditions [34]. Alteration of β-adrenergic receptors could contribute to contractile dysfunction in hiPS-CMs, but the window of transcriptional regulation (beginning at 2 hours) does not correlate with the onset of functional side effects (≥12 hours). Nevertheless, HDAC inhibitors can modulate ADRB8 gene expression, which might influence downstream signaling pathways. Interestingly, β-adrenergic receptors are able to regulate the expression and function of cardiac Cx43 and Cx40 [55], as well as upregulate the expression of Kir2.1 channels through adrenergic-based PKA signaling [56]. Hence, the early-regulated cardiac genes (ADRB1, ADRB2, KCNJ2, and GJA5) might be functionally connected and should be further investigated to clarify their role in HDAC inhibitor-driven effects in hiPS-CMs.

Although we did not observe HDAC inhibitor-mediated prolongation of the FPD, we did observe arrhythmic-like events in hiPS-CMs in the form of sustained contractions and prolonged depolarizations (Fig. 2E) that could be related to QT prolongations and ventricular proarrhythmic events (e.g., TdPs). Although the mechanism behind this could be a downregulation of KCNH2, several arguments might also implicate calcium-related mechanisms. Proteins encoded by the HDAC cardiotoxicity-related top cardiac genes CALM1, STC1, and RRAD (Fig. 6) have been reported to modulate L-type Ca2+ channels [41, 57, 58], which can contribute to ventricular arrhythmia or cardiomyopathies. Together with the functional effects (sustained contractions and FPD shortening), transcriptional regulation of these genes suggests a potential role for calcium-based mechanisms in HDAC inhibitor-mediated ventricular adverse effects.

Although HDAC inhibitors can cause cardiotoxicity in clinical studies, in recent years there is growing evidence of their beneficial effects, albeit in preclinical models of cardiac hypertrophy and heart failure [59, 60]. Hence, HDAC inhibitors are considered to display opposite effects in “healthy” hearts and certain animal models for cardiac hypertrophy. In our study, several of the top genes related to cardiotoxic HDAC inhibitor treatments (Fig. 6; supplemental online Table 2) are also associated with hypertension and heart failure (TIMP3, ADRB2, GADD45B, DUSP6, CEBPB, JARID2, and STC1) [37–41]. However, pathway analysis (grouping multiple genes) did not show strongly affected pathways related to hypertrophy or heart failure in hiPS-CMs (Fig. 5). Therefore, the specific role of these top genes can be either pathological in hiPS-CMs or might be associated with beneficial effects of HDAC inhibitors shown in cardiac hypertrophy and heart failure [59, 60]. The differing effects of HDAC inhibitors in healthy versus diseased heart emphasizes the importance of HDAC/histone acetyltransferase balance in cardiomyocytes and suggest a prominent role of HDAC inhibitors in the regulation of cardiac plasticity.

**Limitations and Further Perspectives**

Although our study shows novel insights into HDAC inhibitor-related cardiotoxicity, one needs to keep in mind that hiPS-CMs still remain a simplified in vitro model of the human heart lacking other cell types (e.g., fibroblasts) and normal mechanical stressors and autonomic and neuro-hormonal influences. Therefore, the varied and poorly understood nature of cardiac effects linked to HDAC inhibitors in the clinic might not be manifested and completely explained through this in vitro model. Furthermore, HDAC inhibitors are strong epigenetic modulators developing more pronounced effects in vitro as compared with in vivo. Nonetheless, despite these limitations, the delayed arrhythmic abnormalities, good Curr-based correlation with clinical cardiac events, and novel mechanistic associations strongly support the value of hiPS-CMs as a model for HDAC inhibitor-mediated cardiotoxicity. Additional experimental work is needed to elucidate the role of the highlighted genes and pathways at a proteomic and functional level. Furthermore, electrophysiological characterization of single cardiomyocytes (e.g., patch clamp) might enhance our understanding at the level of specific ionic currents or cell types (atrial versus ventricular). Finally, despite potential species differences, studies in in vivo animal models that account for other important physiological influences might provide additional understanding of cardiotoxicity linked to HDAC inhibitors in the clinic.

**SUMMARY**

We have demonstrated that hiPS-CMs are a useful platform to detect and investigate functional delayed cardiotoxicity and to support preclinical identification of new HDAC inhibitors and other therapeutic drug classes with reduced potential for cardiotoxicity.

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**AUTHOR CONTRIBUTIONS**

I.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; D.J.G.: conception and design, final approval of manuscript; A.D.B.: conception and design, data analysis and interpretation, manuscript writing; L.C.: data analysis and interpretation, manuscript writing; E.V. and I.V.d.W.: collection and/or assembly of data, data analysis and interpretation; H.R.L.: conception and design, data analysis and interpretation, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

I.K., D.J.G., A.D.B., E.V., I.V.d.W., and H.R.L. are employees of Janssen Pharmaceutica NV. The other author indicated no potential conflicts of interest.
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