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Cardiomyocytes Derived from Human Induced Pluripotent Stem Cells: An *In-Vitro* Model to Predict Cardiac Effects of Drugs

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ABSTRACT

Introduction: Cardiomyocytes derived from human induced pluripotent stem cells (hiPSC-CM) form spontaneously beating syncytia *in-vitro*. We evaluated whether hiPSC-CM are a compelling model of human cardiac pharmacology useful for early drug development. Methods: We measured hiPSC-CM beating frequency using Ca-sensitive dyes and a high-throughput screening system. We quantified the effects of 640 drugs with various structures and pharmacologies. Results: When tested at 1 μM, most drugs without direct effects on heart rhythm or with effects at high concentrations do not change frequency, indicating specificity. In contrast, the preparation detects compounds with direct activity on heart rhythm, demonstrating sensitivity. In particular, β-adrenergic agonists increase frequency and the model differentiates $\beta 2$ from $\beta 1$ agonists, as well as partial from full agonists. Phosphodiesterase inhibitors have subtype-specific actions and PDE4 is particularly important in controlling frequency. The preparation is sensitive to cardiac ion channel blockers: L-type calcium channel blockers, Class-I and Class-III antiarrhythmics change frequency but drugs acting on K_{ATP} channels do not. The assay detects compounds blocking the cardiac rapid delayed-rectifier K channel and is an alternative to the classic "hERG test". Conclusion: hiPSC-CM are a useful *in-vitro* cardiac model in drug development since they respond appropriately to drugs that modify heart rate in humans.

1. INTRODUCTION

Human induced pluripotent stem cells (hiPSC) can differentiate into functional cardiomyocytes [1] and bio-engineering technologies can produce and reliably supply large quantities of isolated cells that are

highly enriched in functional cardiomyocytes, as evidenced by their phenotypic and electrophysiological characteristics, and that develop into spontaneously-beating syncytia *in-vitro* [2-4]. These hiPSC-derived cardiomyocytes (hiPSC-CM) are likely to provide an ideal source of human cells to generate models for drug development, particularly when questions arise regarding cardiac effects that may be seen when a drug is administered to humans.

For hiPSC-CM to be useful in early drug development, the assay methodology must be amenable to high-throughput screening (HTS). The beating rate of hiPSC-CM syncytia can be measured with high definition under physiological conditions using microelectrode arrays (MEA), which measure field potentials, or using impedance-sensing, which measures mechanical movements [4]. These techniques can provide valuable and very detailed information on drug effects in addition to simple effects on beating rate but they are low-throughput and they do not permit testing thousands of compounds within practical time and budget constraints. A faster, more cost-effective system was presented using a fluorescent 384-well plate reader and a Ca-sensitive dye [5]. This HTS system can be integrated into standard automated platforms but it is limited by two practical issues. First, temperature control is suboptimal as evidenced by the beating rates of ~15 bpm compared to the 35 - 55 bpm found in well-controlled environments [4]. Second, the acquisition frequency (8 Hz) is at the lower limit when trying to record accurately beating rates that can reach 120 bpm (2 Hz) under stimulated conditions and it is too low to extract additional information (*e.g.*, slope or duration). To overcome these limitations, we developed a similar method using a faster reader (up to 30 Hz) with improved temperature control (Hamamatsu FDSS7000).

For hiPSC-CM to be compelling as a model of human cardiac pharmacology, they must respond appropriately to known pharmacological agents. In particular, for the pharmaceutical industry, they should react to drugs used in medical practice as predicted from existing clinical data. As it is unpractical to evaluate the effects of the thousands of drugs sold today, we selected a commercially-available subset of 640 compounds that covers a spectrum of chemical structures and pharmacological actions. We tested these drugs for their ability to change the spontaneous beating rate of hiPSC-CM and we evaluated their effects in the light of their known cardiac actions *in-vivo*. Since this study investigates all potential drug effects on cardiac rhythm, it complements the work done under the CiPA initiative (http://cipaproject.org), which aims specifically at improving the assessment of a drug's pro-arrhythmic potential.

2. MATERIALS AND METHODS

2.1. Cells and Reagents

The hiPSC-CM ("iCell-cardiomyocytes", catalog #CMC-100-110-001), the iCell-cardiomyocytes Plating Medium and the iCell-cardiomyocytes Maintenance Medium were from Cellular Dynamics International (Madison, WI). The Calcium-4 kit was from Molecular Devices (Sunnyvale, CA). The commercially-available set of 640 drugs ("FDA-Approved Drug Library", catalog #BML-2841) was from Enzo Life Sciences (Lausen, Switzerland); all compounds were tested at a single identical concentration (1 μM) to mimic the flow of standard HTS. All other chemicals, including those used for follow-up concentration-response curves (CRCs), were from Sigma-Aldrich (Buchs, Switzerland).

2.2. Calcium Fluorescence Assay

On day-0, the hiPSC-CM were thawed, plated in 384-well assay plates (catalog #781091, Greiner-Bio-One, Frickenhausen, Germany) at 10,000 cells per well in 25 μL Plating Medium and maintained at 37˚C in a humidified atmosphere containing 5% CO_2. On day+1, the medium was replaced with 50 μL maintenance medium and the plates were covered with an adhesive gas-permeable membrane (catalog #676051, Greiner-Bio-One). Thereafter, half of the medium was renewed every 2 - 3 days and it was renewed entirely on day+21.

On day+22, the 640 compounds were prepared in two 384-well microplates (catalog #781280, Greiner-Bio-One) from 2-mM DMSO stock solutions by diluting 1 μL in 94 μL Maintenance Medium (1.05%

DMSO). The positive controls, forskolin and N6-cyclopentyl-adenosine,were prepared similarly and added to both plates (4 wells each) while the negative control, DMSO alone, was added to the remaining 56 wells of each plate. The two compound plates were loaded into a FDSS7000 fluorescent plate reader (Hamamatsu Photonics, Massy, France) heated to 37˚C.

The following protocol was repeated on four cell plates to test the two compound plates in duplicate. 20 µL medium was replaced with 30 µL medium containing the Ca-sensitive fluorescent dye Fluo-4 and a quencher (component A from the Calcium-4 kit reconstituted in 50 mL Maintenance Medium) and the cell plate was loaded into the FDSS7000. After 60-min in dye-containing medium, a 2-min segment of Ca waves was recorded to define the baseline beating rate for each well. Then the compounds were added by pipetting well-to-well 3 µL from the compound plate into the cell plate. After addition, the concentration was 1 µM and DMSO was 0.05% (v/v). Thereafter, 2-min segments of Ca waves were recorded starting 5, 15, 30, 45, and 60 min after compound addition. For this study, we used a 10-Hz sampling frequency since we only evaluated changes in beating frequency.

For follow-up CRCs, an identical methodology was used with DMSO stock solutions prepared from dry compound powders such that the final DMSO concentration was also 0.05% (v/v).

2.3. Analysis

Beating frequency was calculated with Igor Pro software (Wavemetrics, Portland, OR) and a custom analysis routine using the Lomb Periodogram function (based on the Lomb-Scargle method of least-squares spectral analysis). For each well of the cell plate, the beating frequency at each time point after compound addition was normalized to baseline and the time-matched mean DMSO effect for the corresponding cell plate was subtracted. Results were averaged for the two cell plates receiving the same compound plate. For compounds that changed frequency (see Results for the definition), a visual examination of the beating pattern evaluated whether the compounds produced arrhythmias.

3. RESULTS

Baseline Activity and Effect of Solvent

After 2 - 3 days in culture, hiPSC-CMs establish spontaneously-beating syncytia with a uniform rhythm that evolves with days in culture, starting slow (~10 bpm), accelerating progressively during 10 - 12 days (to ~40 - 50 bpm), then remaining stable up to 28 days (not shown). We decided to start the experimental procedure mid-way into the window of stability, on day+21 post-thaw. This is also the time when the expression profile of cardiac channels and maturation markers has stabilized [6]. In the fluorescent reader, on day+22, the Ca waves are regular over the baseline sampling period (**Figure 1(a)**) and their shape is well-defined (**Figure 1(b)**). The spectral analysis periodograms are narrow, which allows the determination of beating frequency (**Figure 1(c)**). Over one 384-well plate and across multiple plates, the distribution of beating frequency is narrow (mean ± sd = 40.1 ± 3.5; range = 34 - 54 bpm) and reproducible (**Figure 1(d)**).

Application of solvent (DMSO, 0.05%) induces a rapid acceleration (~7% over 5 min, **Figure 2(a)**) followed by a progressive regular acceleration (~0.5%/min over the remaining time). As DMSO does not change frequency measured with a non-invasive impedance device and as very similar changes are observed with H_2O, MeOH, or EtOH (not shown), we suspected that the effect observed here is related to the presence of a Ca-sensitive dye and/or to the lack of CO_2/humidity control in the plate reader, rather than to the actual application of solvent. When we tested the effect of these factors using an impedance device, we found that the presence of the Ca-sensitive dye did not have short-term effects on beating rate whereas the lack of CO_2 control led to an acceleration of beating rate similar to that we observed in the fluorescence reader (**Figure 2(e)**). The effect of solvent on the Ca wave amplitude is more variable in magnitude within a plate but qualitatively similar across plates (**Figure 2(b)**). For each time point, we calculated a "DMSO-corrected effect" (see Methods) for each of the wells where only DMSO was applied, in order to

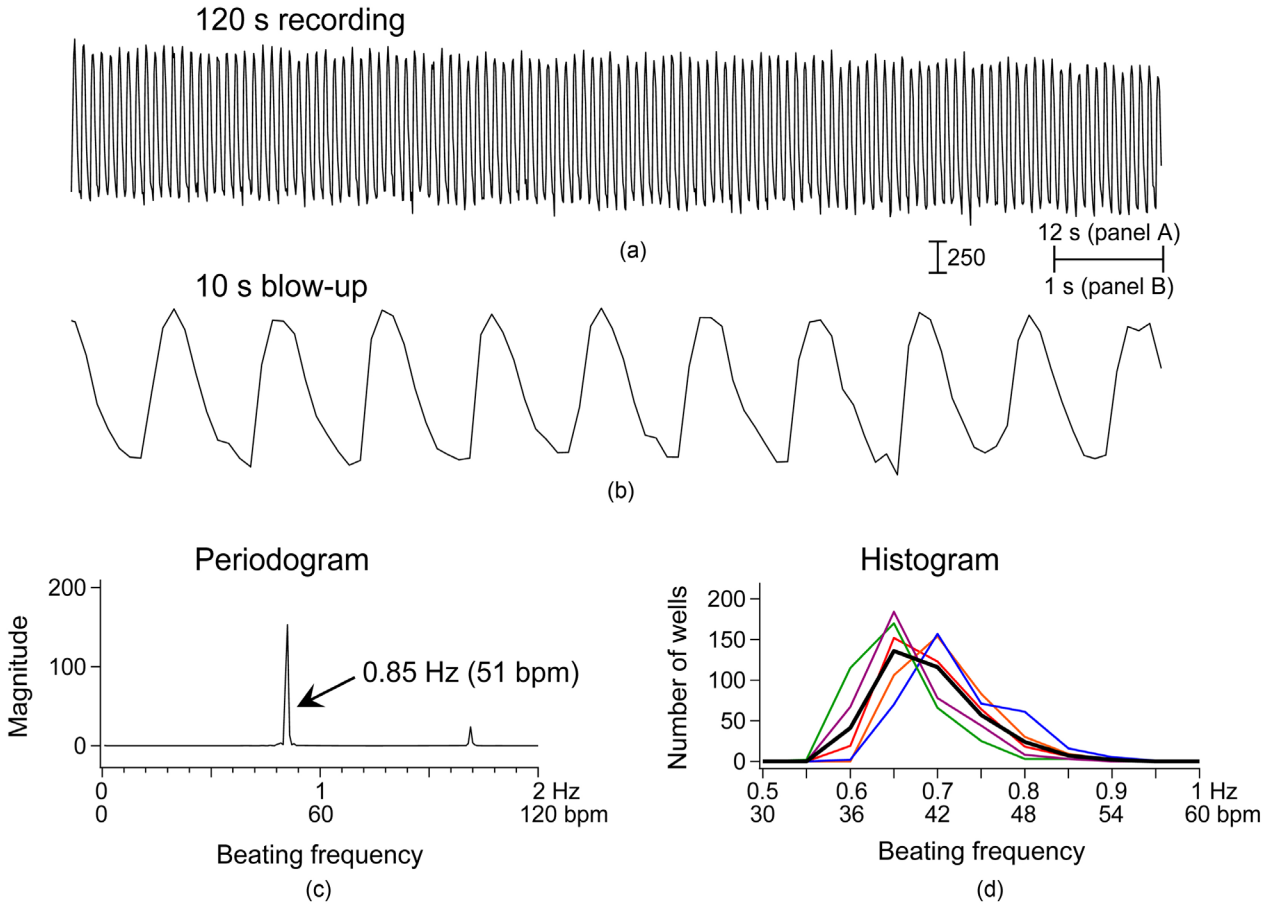

Figure 1. Baseline recording of Ca waves. (a) 2 min segment from one well; (b) 10-s blow-up; (c) periodogram for this segment; the baseline frequency is clearly defined; (d) histograms of the baseline frequencies across five 384-well plates (colored) and of the ensemble (black). Fluorescence intensity and periodograms magnitude are in relative light units derived from the video camera greyscale.

determine the smallest effect that can be defined as compound-related. The variability of the DMSO effect on frequency increases slightly during the experiment but remains below 5% (**Figure 2(c)**). For amplitude, the variability is larger and increases markedly over the experimental duration (**Figure 2(d)**).

Based on this result, we took a frequency change >5% as indicating a compound-related effect (amplitude changes were not further analyzed). In the context of HTS, when comparing single or duplicate measurements of many test samples to positive and negative controls, standard statistical tests are not suitable to determine whether a single compound produces a "real effect" because correcting for multiple comparisons (640 here) would render all effects non-statistically-significant. It is possible, however, to evaluate the assay quality statistically using the Z-factor [7]. With the 4 cell plates, we find Z-factors for DMSO-corrected changes in frequency of 0.72, 0.61, 0.53, 0.43, and 0.32 at 5, 15, 30, 45, and 60 min.

4. DRUGS WITH A THERAPEUTIC TARGET PLAYING A DIRECT ROLE IN HEART RATE REGULATION

4.1. HTS Methodology

Among the 640 drugs, 95 have a therapeutic target that plays a role in heart rate regulation at the car-

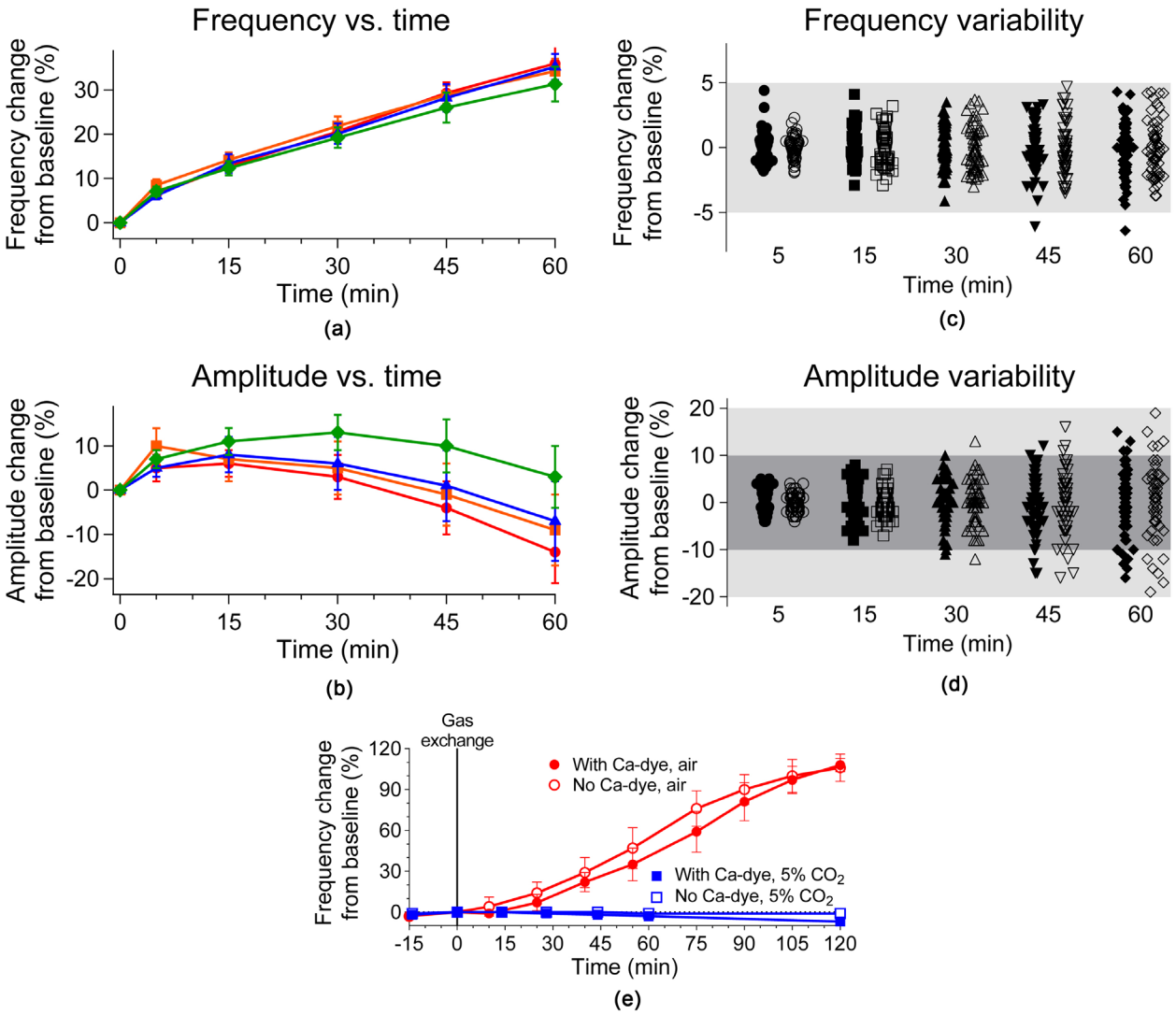

Figure 2. DMSO effect on Ca waves. ((a), (b)) Change in frequency (a) or amplitude (b) after DMSO. Each color represents one cell plate and each point is the average of 56 wells where only DMSO was applied (mean ± std). One-way ANOVA with Tukey's multiple comparisons test identified only the green plate as statistically different from the orange plate, in frequency but not in amplitude; ((c), (d)) individual changes in beating frequency (c) or Ca wave amplitude (d) after DMSO. Filled and open symbols correspond to the two compound plates; (e) changes in beating frequency measured with an impedance device in the presence or absence of the Ca-sensitive dye and with or without CO_2 control.

diomyocyte level. They comprise 13 agonists and 14 antagonists of β-adrenergic receptors, 6 agonists and 8 antagonists of muscarinic receptors, 19 phosphodiesterase (PDE) inhibitors, 1 Na/K pump inhibitor, 6 Class I or Class III antiarrhythmics, 17 Ca channel blockers (CCB). These compounds all produce their expected effect on the beating rate of the hiPSC-CMs.

The β-agonists except bambuterol increase frequency (non-selective or β1- or β2-selective: albuterol, bambuterol, cimaterol, clenbuterol, procaterol, salmeterol, tulobuterol, xamoterol, dobutamine, ractopamine, epinephrine, norepinephrine, isoproterenol; Figure 3(a)). Bambuterol is a pro-drug of terbutaline

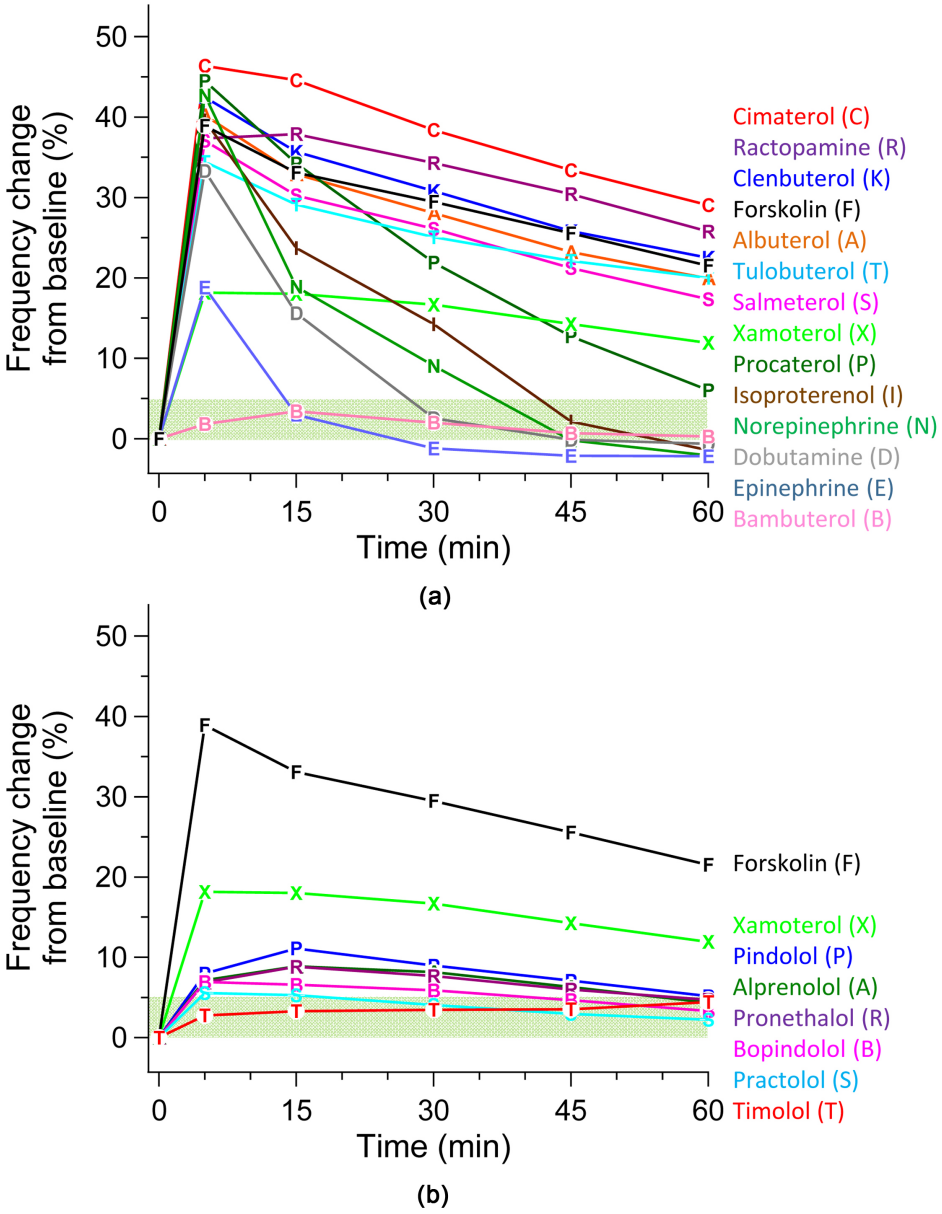

Figure 3. **Effect of β-adrenergic compounds on Ca wave frequency. (a) Agonists; (b) antagonists (only compounds with effect). The green band shows the 5% change selected as indicating a compound-related effect. Each data point represents the average of measurements in 2 wells, the standard deviation was below 5% in all cases except for all time points of dobutamine (5% - 10%), four time points of norepinephrine (5% - 8%), and the first time point of isoproterenol (13%).**

and has little inherent agonistic activity, which explains the absence of effect; indeed, terbutaline produced an effect similar to albuterol when tested in a separate experiment (see next section). All compounds accelerate >20% without arrhythmias; the effect develops within ~5 min then decreases with time. Four classes of compounds emerge when comparing the time course of the frequency increase: 1) for six compounds (albuterol, cimaterol, clenbuterol, salmeterol, tulobuterol, ractopamine), the effect decays slowly with ki-

netics similar to forskolin (<50%/60 min); 2) for four compounds (dobutamine, epinephrine, norepinephrine, isoproterenol), the effect decays rapidly (100%/60 min); 3) for procaterol, we see intermediate kinetics (~90%/60 min); and 4) for xamoterol, the frequency increases to an intermediate level then decays minimally (<20%/60 min). Remarkably, all slowly-decaying agonists are β2-selective whereas all fully-decaying agonists are β1-selective or non-selective. The β2-agonist albuterol has an EC_{50} of ~3 nM in our assay (separate experiment, not shown); therefore activation of β2-adrenergic receptors increases frequency in these cells. Procaterol is particular as the only β2-agonist with an effect that decays faster than forskolin. However, procaterol is unstable in buffered solutions and degradation explains the rapid decay [8]. Finally, the β1-selective partial agonist xamoterol is notable because it increases frequency partially (~18%) and its effect barely decays; this is similar to what is observed in humans [9].

When β-blockers are applied, most produce no effect indicating that there is no basal activation of β-adrenergic receptors (non-selective or β1- or β2-selective: alprenolol, atenolol, betaxolol, bopindolol, carvedilol, esmolol, metoprolol, pindolol, practolol, pronethalol, propranolol, (S)-propranolol, sotalol, timolol). However, six compounds accelerate the beating frequency slightly (alprenolol, bopindolol, pindolol, practolol, pronethalol, timolol; ~5% - 10%) without arrhythmias and with kinetics similar to xamoterol (**Figure 3(b)**). These six β-blockers possess intrinsic sympathomimetic activity (ISA) [10-12], indicating that they are in fact, like xamoterol, partial β-adrenergic agonists. The remaining compounds do not possess ISA [12-15], demonstrating that the preparation is extremely sensitive in distinguishing compounds with or without ISA.

Among muscarinic agonists, the more potent, slightly M2-selective compounds carbachol and oxotremorine transiently decrease beating frequency (6% - 7%) but there is no effect of the weaker acetylcholine and butyrylcholine and the M1/M3-selective bethanechol and pilocarpine. Carbachol has an EC_{50} of ~250 nM and is antagonized by atropine (separate experiment, not shown), consistent with an M2-mediated effect. Among the eight muscarinic antagonists, atropine, butylscopolamine, ipratropium, pirenzepine, scopolamine, telenzepine, tropicamide have no effect but tiotropium accelerates transiently (~7%): no described property of tiotropium can explain this specificity.

Among PDE inhibitors, nine compounds (denbufylline, etazolate, IBMX, ibudilast, pentoxifylline, rolipram, trequinsin, vardenafil, zardaverine) produce a sustained acceleration (6% - 17%) without arrhythmias. The remaining compounds (aminophylline, anagrelide, cilostamide, dipyridamole, doxofylline, irsogladine, milrinone, siguazodan, sildenafil, zaprinast) have no effect. Except vardenafil, which produces a small sustained acceleration (7% - 9%), all active compounds are non-selective PDE inhibitors or PDE4-selective inhibitors. When we repeated the experiment with new compound and higher concentrations, milrinone and sildenafil also accelerated starting at 3 - 10 μM. Thus, PDE4 is of particular importance in controlling hiPSC-CM beating frequency but PDE3 and PDE5 may play a small role.

Following application of the Na/K pump inhibitor ouabain, there is no effect after 5 min, a strong reduction in frequency after 15 min (−40%) and a complete arrest of beating after 30 min. In contrast, the laxative bisacodyl, which was also described as inhibiting the Na/K pump [16], strongly accelerates at 5 min (+30%) but not later, suggesting that it does not inhibit the Na/K pump in this preparation.

Ion channel modulators are also compounds expected to affect cardiac rhythm. Ten of the eleven dihydropyridine L-type CCBs (amlodipine[1], cilnidipine, felodipine, manidipine, nicardipine, nifedipine, niguldipine, nimodipine, nisoldipine, nitrendipine) accelerate strongly (150% - 250%) with arrhythmias while lacidipine stops the beating immediately; such arrest is observed later with niguldipine and nisoldi-

[1]Amlodipine and captopril were found to be switched in the commercial plate. Before testing the 640 compounds, they had been submitted to high-resolution mass spectrometry to confirm identity and purity. We found that the "captopril" stock contained a compound of the molecular weight of amlodipine whereas the "amlodipine" stock contained a compound of the molecular weight of captopril disulfide. We also measured the Ca channel blocking potency of the compounds on $Ca_V1.2$ channels expressed in HEK293 cells and we found that "amlodipine" produced no block of Ca fluxes whereas "captopril" reduced them with potency similar to nifedipine. The corrected identities of the compounds are used in the text.

pine. The other CCBs produce diverse effects: verapamil provokes a rapid arrest, diltiazem a sustained acceleration (~100%) without arrhythmias, lomerizine and flunarizine a transient acceleration (13% - 25%), whereas gabapentin and bepridil have no effect. This can be expected of the neuronal Ca channel-selective gabapentin but it is more surprising with bepridil. However, bepridil is a weaker CCB than the dihydropyridines and it has much stronger plasma protein binding (99.8%) than verapamil (91%) or diltiazem (82%). Since our assay is performed in culture medium containing serum (~10%), the free fraction of bepridil in a 1-μM solution may be too low to block Ca channels: we repeated the experiment with new compound and no effect was seen again at 1 μM whereas complete arrest was observed at 10 μM (also with lomerizine and flunarizine).

The actions of Class I and Class III antiarrhythmics are predictable. Dofetilide causes immediate arrest, with minimal recovery after 30 min; nifekalant does not stop beating but slows frequency (15% - 50%) with arrhythmias; and amiodarone produces a small acceleration (~12%) without arrhythmias. Although amiodarone blocks the cardiac rapid delayed-rectifier K current (IKr) potently, it is strongly bound to plasma proteins (>99.9%), which may explain its relatively weak effect. The Class Ic antiarrhythmic flecainide slows the rhythm (~13%) whereas propafenone or the Class Ia procainamide have no effect. Since Class I antiarrhythmics are generally not very potent drugs [17], we retested Na channel blockers at higher concentrations and we consistently observed a reduction in beating rate (see next section).

4.2. Concentration-Response Curves

In the previous section, compounds were tested following a HTS methodology, using one single identical concentration. Results are therefore somewhat qualitative as they do not provide a threshold effect concentration or a defined maximal effect. In order to better qualify some of the mechanisms, we performed CRCs with a few characteristic compounds; in the category "Drugs with a therapeutic target playing a direct role in heart rate regulation", we selected the β-agonist terbutaline, the PDE inhibitors rolipram (PDE4) and milrinone (PDE3), the CCBs amlodipine and lomerizine, the K channel blocker E-4031, and the Na channel blocker tetrodotoxin (TTX).

Figure 4 shows the corresponding CRCs for all the measurement times. Terbutaline (**Figure 4(a)**) accelerates the rhythm concentration-dependently but efficacy decreases with time of application as already observed for other β-agonists (**Figure 3**); however, potency does not seem affected. Acceleration starts around 2 - 10 nM and can be well-fit using standard concentration-response curves with a Hill slope of 1 and EC_{50} of 9.1 nM (5 min) or 25 nM (60 min); this is consistent with potent activation of adrenergic receptors and significant receptor reserve as observed in bronchial smooth muscle [18]. Rolipram and milrinone (**Figure 4(b)**) also accelerate the rhythm concentration-dependently but with different concentration- and time-dependencies. Rolipram, a PDE4 inhibitor, is already active at 2 nM and its effect changes little during the 60-min application; its EC_{50} of ~5 nM is consistent with very potent inhibition of PDE4 and increased intracellular cAMP. In contrast, milrinone, a PDE3 inhibitor, is only active initially at ≥3 μM but it becomes more potent and less efficacious during the 60-min application; the slow appearance of an effect may result from cAMP diffusion from subcellular compartments dependent on PDE3 (see Discussion).

Amlodipine and lomerizine (**Figure 4(c)**) produce very similar patterns: they accelerate the rhythm concentration-dependently, up to a maximal concentration; then the rhythm stops suddenly (dashed lines). Acceleration becomes significant at 14 nM (amlodipine) or 140 nM (lomerizine), consistent with potent block of cardiac voltage-gated Ca channels. It is also apparent that drug activity increases during the 1-hour application: acceleration becomes more pronounced with time and arrest occurs at lower concentrations; this could conceivably result from positive feedback through the well-described use- or frequency-dependent block of Ca channels by these CCBs (block⇨acceleration⇨more block⇨more acceleration…). E-4031 and TTX (**Figure 4(d)**) slow the rhythm concentration-dependently but with different concentration- and time-dependencies. The IKr blocker E-4031 is already active at 10 - 50 nM and its effect changes little during the 60-min application; its EC_{50} of 400 - 600 nM is consistent with very potent block of cardiac voltage-gated K channels. In contrast, the INa blocker TTX is only active at ≥1 μM

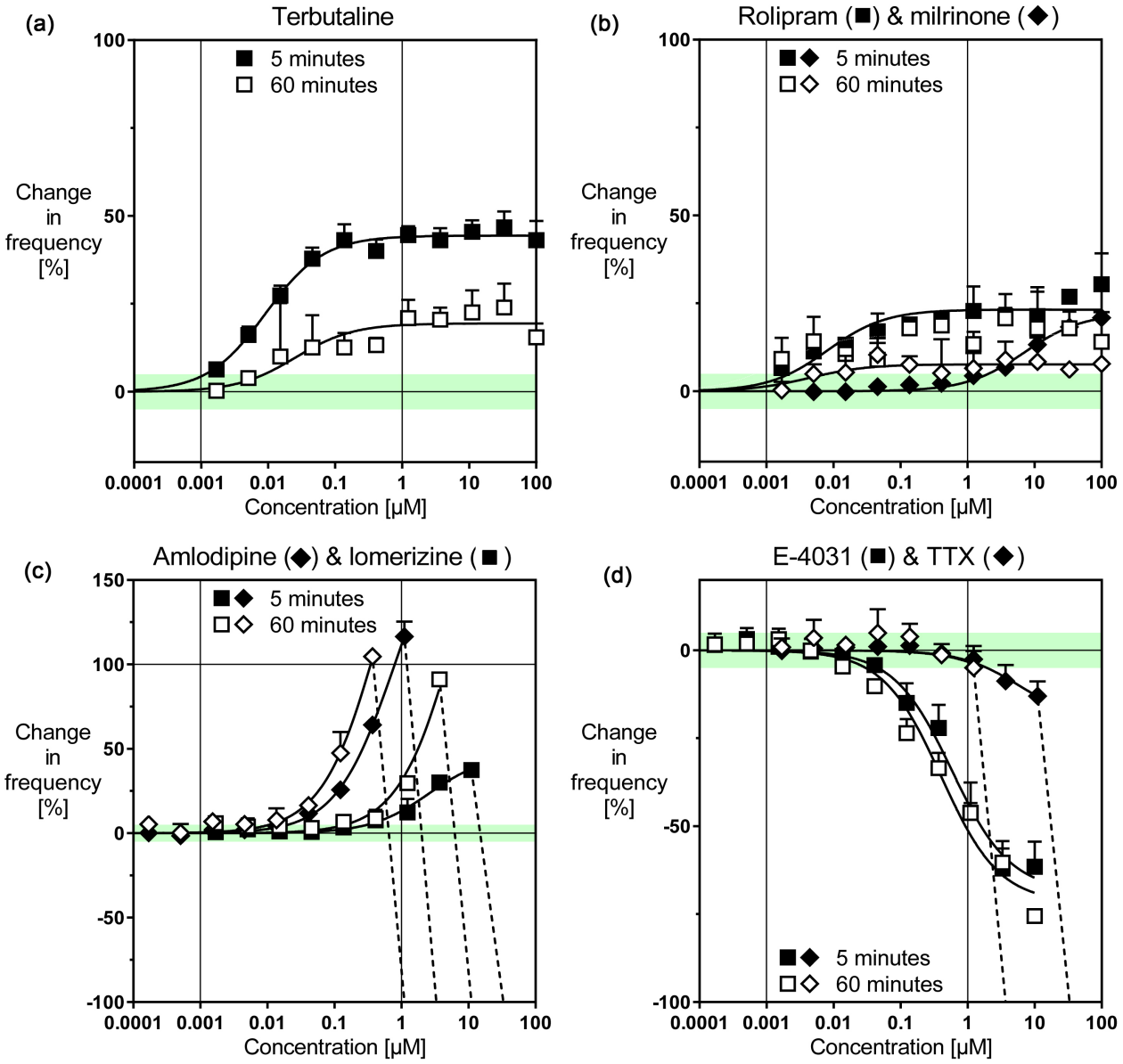

Figure 4. Concentration-response curves with characteristic compounds in the category "Drugs with a therapeutic target playing a direct role in heart rate regulation": the β-agonist terbutaline (a), the PDE inhibitors rolipram and milrinone (b), the CCBs amlodipine and lomerizine (c), the IKr blocker E-4031 and the Na channel blocker TTX (d).

consistent with weaker block of cardiac voltage-gated Na channels. In addition, TTX tends to produce an all-or-none response as it slows beating rate minimally (≤15%) up to a concentration when it suddenly stops beating completely (dashed lines); this limit concentration decreases during the 60-min application. Aside from this last aspect, we observed a very similar pattern with other Na channel blockers and the limit concentration was clearly related to Na channel block potency (50 µM lidocaine & disopyramide; 10 µM mexiletine; 2 µM quinidine, bepridil, flecainide & propafenone; not shown).

In conclusion, "drugs with a therapeutic target playing a direct role in heart rate regulation" produce a well-defined response on the beating rate of the hiPSC-CMs, which can be associated with their primary (*i.e.*, most potent) mode-of-action.

4.3. Drugs Blocking the Cardiac Rapid Delayed-Rectifier K Channels

The actions of dofetilide, nifekalant, and E-4031 suggest that this assay detects IKr blockers as shown previously with a slightly different technology [5]. To evaluate this possibility, we collated published data on IKr-blocking potency and plasma-protein-binding for the 640 compounds. As could be expected for older drugs, such information does not exist for all but IKr-blocking IC_{50}s (K_{50}) could be found for 138 compounds and plasma-protein-binding percentages (PPB) for 365 compounds. Furthermore, for 119 compounds (36 with published K_{50} and 83 without), we evaluated IKr block experimentally by measuring block of K_V11.1 (hERG) channels expressed in CHO cells, using planar patch-clamp (**Table 1**). Finally, for 39 compounds with K_{50} but no published PPB, we measured it in the test medium (MPB) and, since this medium contains only 10% serum, we calculated PPB as 100% − 10%/((MPB/(100% − MPB))+10%). This corrected value is reported in **Table 1** along with published PPB values.

For the 36 drugs with published K_{50}, the correlation with measured values is very good for 30 (within a factor of 3) and good for 2 (factor of 10); however, flunarizine is notably weaker in our measurements whereas nifedipine, nitrendipine and estradiol are more potent. Among 83 compounds without published K_{50}, 11 block IKr with potency between haloperidol and clozapine (published K_{50} of 27 nM & 320 nM [19]; azaperone, loperamide, methiothepin, naftopidil, naltriben, naltrindole, nicergoline, nifekalant, spiperone, trifluperidol, triprolidine). Thirty-six other drugs block with lower potency between clozapine and propranolol (published K_{50} of 10,000 nM [20]; alprenolol, benzamil, benzydamine, bopindolol, butaclamol, cilnidipine, cirazoline, cyclosporine, cyproheptadine, dilazep, disulfiram, dorzolamide, felodipine, gestrinone, harmine, hydroxytacrine, imiquimod, lacidipine, levallorphan, lofexidine, lomerizine, manidipine, naloxone, naltrexone, nicardipine, niguldipine, nimodipine, nisoxetine, phenamil, phentolamine, quinacrine, strychnine, tacrine, telmisartan, tropisetron, vinorelbine).

In order to correct for drug bound to proteins in the test medium, we calculated a "corrected IC_{50}" as $corrK_{50} = K_{50}/(1 - MPB)$, where MPB is calculated from PPB by reversing the above equation. We then compared the effect of compounds on hiPSC-CM rhythm and their $corrK_{50}$ (**Figure 5**). Interesting rules emerge from this figure: first, compounds that stop rhythm are all strong IKr blockers and/or CCBs, with the exception of ouabain. Second, IKr blockers with $corrK_{50} < 1$ μM (the test concentration) stop rhythm or slow it markedly (>20%) with few exceptions: the dopamine agonist pergolide accelerates (12% - 20%) whereas the histamine antagonist terfenadine, the potassium-sparing diuretic benzamil and the opioid antagonist naltrindole slow <20%. The action of pergolide is surprising but, in the published study where it blocked IKr with K_{50}~120 nM, pergolide shortened rather than lengthened action potential duration in canine Purkinje fibers [70]. This could indicate that pergolide is a CCB although we did not observe block of expressed Ca_V1.2 channels (not shown). The weaker effects of terfenadine, benzamil and naltrindole also suggest activities additional to IKr block. Conversely, 3 other compounds slow beating strongly but are not potent IKr blockers (the adrenergic agonist cirazoline, the MAO inhibitor harmine and the microtubule destabilizing agent vinorelbine). Like ouabain, the effect of vinorelbine developed after 5 min, suggesting a cytotoxic mechanism. On the other hand, the effects of cirazoline and harmine developed before 5 min then remained stable.

4.4. Drugs with a Cardiovascular Target without a Direct Role in Heart Rate Regulation

Forty-five drugs have a cardiovascular therapeutic target that is not expected to play a role in heart rate regulation at the cardiomyocyte level. They comprise 9 α-adrenergic agonists and 12 antagonists, 7 angiotensin-converting-enzyme inhibitors (ACEI), 5 angiotensin-1 receptor antagonists (ARA), 1 endothelin receptor antagonist (ERA), 4 activators and 7 blockers of K_{ATP} channels. Most of these compounds produce no effect on hiPSC-CM beating.

None of the 8 selective α_2-adrenergic agonists (clonidine, guanabenz, guanfacine, lofexidine, methyldopa, naphazoline, tizanidine, xylazine) changes frequency but the α_1-selective agonist cirazoline decreases it markedly (21% without arrhythmias). Among the 12 α-adrenergic antagonists (non-selective or α_1- or

Table 1. IKr-blocking potency (K_{50} in nM) and plasma-protein-binding percentages (PPB) for a subset of the test compound library. For the first three columns of compounds, published K_{50} were found; for the last three columns of compounds, K_{50} were measured in our laboratories using a planar patch-clamp system (QPatch, Sophion, Denmark). Strong compounds have K_{50} below 320 nM; moderate compounds have K_{50} below 10,000 nM; weak compounds have K_{50} below 100,000 nM; no-block compounds have K_{50} above 100,000 nM (see text). PPB are either from published data (plain text) or measured in our laboratories (bold italics).

	Published IKr data only						Published & QPatch IKr data				QPatch IKr data only					
Strong	Amiodarone	15[a]	***99.99%***	Pimozide	1[a]	99.0%	Astemizole	1[a]	1	97%	Azaperone	100	90%	Spiperone	47	90%
	Bepridil	26[b]	99.8%	Risperidone	226[a]	90%	Clozapine	320[h]	670	95%	Loperamide	38	97%	Trifluperidol	20	90%
	Clemastine	12[c]	96%	Tamoxifen	111[xx]	98%	Flunarizine	6[i]	120	***99.97%***	Methiothepin	140	***99.2%***	Triprolidine	340	90%
	Dofetilide	5[z]	28%	Thioridazine	96[a]	99.0%	Fluspirilene	3[a]	1	***99.87%***	Naftopidil	190	***99.1%***			
	Domperidone	103[a]	92%	Trifluoperazine	234[a]	99.0%	Haloperidol	27[h]	25	92%	Naltriben	150	***71%***			
	Ifenprodil	88[e]	93%	Verapamil	136[a]	***86%***	Ketanserin	107[a]	120	95%	Naltrindole	220	***76%***			
	Lobeline	340[f]	***76%***	Vinpocetine	32[a]	66%	Mesoridazine	320[h]	490	95%	Nicergoline	56	95%			
	Pergolide	120[a]	***98%***				Terfenadine	11[b]	8	97%	Nifekalant	140	86%			
Moderate	Amoxapine	1700[j]	90%	Metoclopramide	5400[m]	40%	Amlodipine	7800[s]	3100	99.5%	Alprenolol	6700	82%	Levallorphan	4900	35%
	Chloroquine	2500[a]	57%	Mianserin	3200[n]	95%	Aripiprazole	1100[j]	470	99.0%	Benzamil	710	***56%***	Lofexidine	8500	85%
	Chlorpromazine	370[yy]	94%	Miconazole	2100[o]	99.0%	Carvedilol	510[d]	540	98%	Benzydamine	1500	***81%***	Lomerizine	600	***99.65%***
	Citalopram	950[k]	80%	Olanzapine	6000[g]	93%	Dextrome-thorphan	5100[u]	5700	55%	Bopindolol	1600	65%	Manidipine	940	99.0%
	Desloratadine	6300[l]	85%	Prazosin	1600[a]	94%	Diltiazem	9100[a]	1100	82%	Butaclamol	490	***98%***	Naloxone	1800	46%
	Diphenhydra-mine	2600[a]	81%	Promethazine	1500[p]	91%	Dolasetron	6000[v]	4100	72%	Cilnidipine	3100	***99.88%***	Naltrexone	6700	21%
	Fluoxetine	460[a]	94%	Quetiapine	5800[g]	83%	Doxazosin	600[a]	390	98%	Cirazoline	6000	24%	Nicardipine	670	99.0%
	Fluphenazine	997[j]	90%	Raloxifene	1100[q]	99.4%	Escitalopram	2600[oo]	1100	***46%***	Cyclosporine	6700	93%	Niguldipine	710	99.6%
	Imipramine	1900[a]	93%	Ranolazine	14,600[a]	***0%***	Flecainide	3900[w]	1600	48%	Cyproheptadine	1500	96%	Nimodipine	2100	98%
	Ketoconazole	1900[a]	99.0%	Remoxipride	2300[j]	84%	Granisetron	3700[v]	6700	65%	Dilazep	600	10%	Nisoxetine	850	97%
	Loratadine	7900[l]	97%	Sibutramine	2500[r]	97%	Maprotiline	3100[a]	1500	89%	Disulfiram	2800	96%	Phenamil	1200	***67%***
							Mepyramine	6000[x]	910	70%	Dorzolamide	7500	33%	Phentolamine	850	***58%***
							Ondansetron	810[y]	850	73%	Felodipine	1800	99.6%	Quinacrine	1200	87%
							Primaquine	2000[y]	6000	65%	Gestrinone	6000	97%	Strychnine	1200	60%
							Propafenone	440[w]	370	96%	Harmine	1500	89%	Tacrine	910	75%
							Propranolol	3900[d]	7500	87%	Hydroxytacrine	5700	53%	Telmisartan	2100	99.6%
											Imiquimod	850	***72%***	Tropisetron	1800	71%
											Lacidipine	1700	93%	Vinorelbine	6000	13%
Weak	Bupivacaine	20,000[aa]	94%	Propofol	36,000[gg]	98%	Capsaicin	17,450[ee]	41,000	***97%***	Betaxolol	15,000	60%	Zonisamide	18,000	
	Chlorpheniramine	13,000[x]	30%	Roxithromycin	37,000[bb]	15%	Nelfinavir	11,000[ii]	7500	98%	Denbufylline	13,000	***52%***			
	Clarithromycin	33,000[bb]	77%	Sildenafil	33,000[hh]	96%	Nifedipine	135,000[z]	4700	96%	Diazoxide	14,000	94%			
	Fluconazole	48,000[cc]	11%	Sparfloxacin	30,000[a]	45%	Nitrendipine	18,100[s]	1600	98%	Nalbuphine	16,000	50%			
	Glimepiride	74,000[dd]	99.5%	Spironolactone	23,000[a]	98%					Physostigmine	15,000	46%			
	Imatinib	20,000[e]	95%	Terazosin	17,000[a]	92%	Estradiol	>100,000[kk]	8000	98%	Pronethalol	13,000	***73%***			
	Lovastatin	12,500[a]	95%	Vardenafil	13,000[hh]	95%					Quinpirole	15,000				
	Nisoldipine	23,000[ff]	99.7%	Venlafaxine	28,000[k]	27%					Tramadol	16,000	20%			

Continued

No block	Albuterol[a], amiloride[a], ampicillin[a], aspirin[a], carbamazepine[nn], cimetidine[a], ciprofloxacin[a], clindamycin[a], clonidine[a], doxycycline[a], dipyridamole[ll], doxorubicin[z], enalapril[a], famotidine[a], furosemide[a], gabapentin[pp], galantamine[qq], gatifloxacin[rr], glyburide[a], guaifenesin[a], ibuprofen[a], indapamide[a], indomethacin[ss], isoproterenol[z], itopride[tt], lamotrigine[pp], levofloxacin[rr], lidocaine[a], lomefloxacin[uu], milrinone[ll], minoxidil[a], naproxen[a], nicorandil[vv], norfloxacin[uu], ofloxacin[a], penicillin[a], phenytoin[nn], pinacidil[a], pentamidine[a], procainamide[s], progesterone[ww], ranitidine[a], sulindac[a], sulpiride[j], thalidomide[a], trimethoprim[a], tizanidine[mm], troleandomycin[a], zaprinast[ll]	Atenolol, azithromycin, captopril disulfide, metoprolol, ouabain, rolipram, sotalol	Bisacodyl, bumetanide, candesartan, carbachol, eprosartan, esmolol, fenoldopam, fulvestrant, idazoxan, itraconazole, metoprolol, IBMX, ibudilast, losartan, miltefosine, naloxonazine, neostigmine, olopatadine, ouabain, oxotremorine, pindolol, practolol, rivastigmine, timolol, vincristine, zalcitabine, zardaverine

a: [21]; b: [22]; c: [23]; d: [24, 25]; e: [26]; f: [27]; g: [28]; h: [19]; i: [29]; j: [30]; k: [31]; l: [32]; m: [33]; n: [34]; o: [35]; p: [36]; q: [37]; r: [38]; s: [39]; t: [40]; u: [41]; v: [42]; w: [43]; x: [44]; y: [45]; z: [46]; aa: [47]; bb: [48]; cc: [49]; dd: [50]; ee: [51]; ff: [52]; gg: [53]; hh: [54]; ii: [55]; jj: [56]; kk: [57]; ll: [58]; mm: [59]; nn: [60]; oo: [61]; pp: [62]; qq: [63]; rr: [64]; ss: [65]; tt: [66]; uu: [67]; vv: [68]; ww: [69]; xx: [84]; yy: [85].

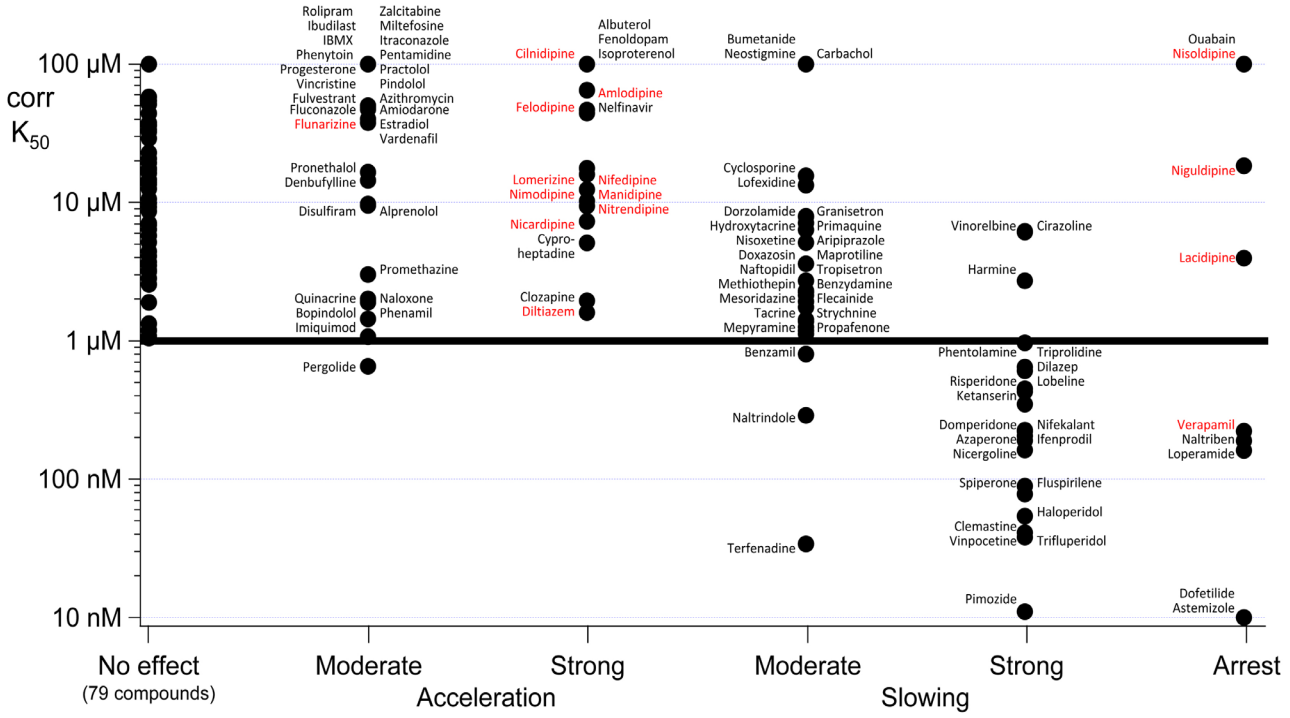

Figure 5. Correlation of IKr-blocking potency and effect on frequency. Strong slowing or acceleration are defined as >20%. The corrK_{50} value for IKr-blocking potency is calculated as indicated in the text. Red text for the CCBs.

α_2-selective: doxazosin, idazoxan, efaroxan, naftopidil, nicergoline, oxymetazoline, phenoxybenzamine, phentolamine, prazosin, tamsulosin, terazosin, yohimbine), only nicergoline and phentolamine decrease frequency markedly (>40% with arrhythmias) whereas doxazosin and naftopidil decrease transiently (−12% and −7%). These actions were all confirmed with new compound and major effects were seen at 10 µM and above (frequency reduction of >20% up to arrest). This is consistent with the observation that nicergoline and phentolamine block cardiac IKr with corrK_{50} below 1 µM, while doxazosin and naftopidil block have corrK_{50} below 10 µM (Figure 5). The particular case of cirazoline may indicate that α_1-selective agonism can directly slow beating in these cells. This would need to be verified with additional selective agonists.

The 7 ACEIs (captopril-disulfide[1], enalapril, enalaprilat, fosinopril, lisinopril, quinapril, ramipril), the 5 ARAs (candesartan, eprosartan, losartan, olmesartan, telmisartan), the ERA bosentan, the 4 activators and 7 blockers of K_{ATP} channels (diazoxide, minoxidil, nicorandil, pinacidil and gliclazide, glimepiride, glipizide, glyburide, nateglinide, tolazamide, tolbutamide) have no effect on the beating rate of the hiPSC-CM.

4.5. Drugs with a Non-Cardiovascular Target

In addition to the compounds discussed above, 511 drugs have a therapeutic target that should not play a role in heart rate regulation at the cardiomyocyte level. Among these, 417 compounds have no effect at 1 μM on the rhythm of the hiPSC-CM[2]. Among the 94 compounds that affect rhythm, 23 are strong IKr blockers ($corrK_{50} \leq 1$ μM, **Figure 5**) while two weaker blockers slow beating strongly (>20%: harmine, vinorelbine). There are no published properties of the chemotherapeutic alkaloid vinorelbine that explain its strong actions whereas the MAO inhibitor harmine depresses the automaticity of sinoatrial myocytes with an unknown mechanism [71].

Many different drugs slow rhythm moderately or accelerate it but, in most cases, there is insufficient published information to propose a mechanism. The frequency is slowed 11% - 20% by 6 com-

[2]Inactive compounds (n = 417) ordered alphabetically:. 10-hydroxy camptothecin, 17-hydroxy progesterone, abamectin, aceclofenac, acipimox, aclarubicin, acyclovir, alendronate, alfacalcidol, allopurinol, alprostadil, altretamine, ambroxol, amifostine, amiloride, aminoglutethimide, aminosalicylic acid, amorolfine, amoxapine, ampicillin, ampiroxicam, amprenavir, anastrozole, anethole-trithione, aniracetam, apomorphine, apramycin, aprepitant, argatroban, artemisinin, artesunate, aspirin, atazanavir, atovaquone, atracurium, auranofin, aztreonam, benserazide, betamethasone, bexarotene, bezafibrate, bicalutamide, bifonazole, bleomycin, bortezomib, bromebric acid, bromhexine, bupivacaine, bupropion, buspirone, butaclamol, butenafine, calcifediol, calcipotriene, camptothecin, canthaxanthin, capsaicin, carbadox, carbamazepine, carbidopa, carboplatin, cefepime, cefoperazone, cefotaxime, ceftazidime, celecoxib, cerivastatin, chlorambucil, chloramphenicol, chlormadinone acetate, chloroquine, chlorpheniramine, chlorpromazine, cilastatin, cimetidine, cinanserin, ciprofloxacin, citalopram, clarithromycin, climbazole, clinafloxacin, clindamycin, clindamycin palmitate, clindamycin phosphate, clobetasol, clodronate, clofarabine, clofibrate, clomiphene, clopamide, clopidogrel, clopidol, closantel, clothiapine, conduritol b epoxide, corticosterone, cromolyn, crotamiton, cyclocytidine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, danazol, daunorubicin, debrisoquin, decamethonium, dehydroepiandrosterone, delavirdine, deprenyl, desloratadine, dexamethasone, dexketoprofen, dextromethorphan, dibenzepin, diclazuril, diclofenac, didanosine, diethylstilbestrol, diflunisal, dihydroergocristine, dihydroergotamine, dinoprost, dinoprostone, diphenhydramine, docetaxel, dolasetron, doxifluridine, doxorubicin, doxycycline, efavirenz, emtricitabine, enoxacin, enrofloxacin, entacapone, ergotamine, ergothioneine, erlotinib, escitalopram, esomeprazole, estriol, ethacrynic acid, ethisterone, etidronate, etoposide, etoricoxib, etretinate, famciclovir, famotidine, fenbufen, fenoprofen, fenretinide, fentiazac, finasteride, fleroxacin, florfenicol, floxuridine, flumazenil, fluocinolone, fluorouracil, fluoxetine, fluperlapine, fluphenazine, flurbiprofen, flutamide, fluvastatin, formestane, ftorafur, fumagillin, furafylline, furosemide, galantamine, gallamine, ganciclovir, gatifloxacin, gefitinib, gemcitabine, gemfibrozil, gentamicin, ginkgolide a, goserelin, guaiacol, guaifenesin, hexestrol, hydrocortisone, hydrocortisone 21-acetate, ibandronate, ibuprofen, idarubicin, idebenone, idoxuridine, ifosfamide, iloprost, imatinib, imipenem, imipramine, indapamide, indomethacin, iproniazid, isoniazid, itopride, ivermectin, kasugamycin, ketoconazole, ketoprofen, ketotifen, lamotrigine, lapatinib, latanoprost, leflunomide, letrozole, levallorphan, levamisole, levetiracetam, levocabastine, levodopa, levofloxacin, levonorgestrel, levothyroxine, lidocaine, lincomycin, linezolid, lomefloxacin, lomofungin, lomustine, loratadine, lorglumide, lovastatin, mebendazole, mecamylamine, medroxyprogesterone, mefenamic acid, megestrol acetate, meglumine, melatonin, melengestrol acetate, meloxicam, melphalan, memantine, mephenytoin, meropenem, mesalamine, mesulergine, metformin, methimazole, methylprednisolone, methylsalicylate, methysergide, metoclopramide, mevastatin, mianserin, miconazole, miglustat, misoprostol, mitomycin, mitoxantrone, molsidomine, montelukast, moroxydine, myclobutanil, mycophenolate mofetil, mycophenolic acid, nabumetone, nadifloxacin, nalbuphine, naloxonazine, naltrexone, naproxen, nedaplatin, nefazodone, neomycin, nialamide, nimesulide, norfloxacin, novobiocin, nystatin, octreotide, ofloxacin, olanzapine, oltipraz, omeprazole, ondansetron, oseltamivir, oxacillin, oxaliplatin, oxatomide, oxcarbazepine, oxiconazole, ozagrel, pamidronate, pantoprazole, pantothenic acid, paroxetine, pazufloxacin, pefloxacin, penciclovir, penicillin, phenylacetate, phenylbutazone, phenylbutyrate, phenylpropanolamine, phloridzin, physostigmine, picotamide, pioglitazone, piperacillin, piribedil, piroxicam, plicamycin, pranlukast, pranoprofen, pravadoline, pravastatin, praziquantel, prednisolone, prednisone, pregnenolone, procarbazine, propofol, puromycin, pyrantel, quetiapine, quinine, racecadotril, raclopride, raloxifene, ranitidine, ranolazine, rebamipide, remoxipride, ribavirin, ricobendazole , rifampin, rifamycin sv, rilmenidine, riluzole, rimantadine, risedronate, rivastigmine, rocuronium, rofecoxib, roxatidine, roxithromycin, rufloxacin, sarafloxacin, secnidazole, selegiline, sertaconazole, shikonin, sibutramine, simvastatin, sirolimus, sparfloxacin, spectinomycin, spironolactone, spiroxatrine, stanozolol, streptomycin, succinylcholine, sulbactam, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfasalazine, sulindac, sulpiride, tamoxifen, tanshinone iia, taurocholic acid, taxol, temozolomide, tenatoprazole, tenoxicam, terbinafine, thalidomide, thiamphenicol, thioridazine, tibolone, ticlopidine, tinidazole, tioconazole, tiotidine, tobramycin, tolcapone, tolfenamic acid, tolmetin, toltrazuril, topotecan, toremifene, tramadol, tranilast, trans-anethole, tranylcypromine, tretinoin, triamcinolone, trichlormethiazide, trifluoperazine, trimethoprim, triptorelin, troglitazone, troleandomycin, tubocurarine, valacyclovir, valproic acid, vatalanib, vecuronium, venlafaxine, vidarabine, vinblastine, vindesine, vitamin a, zafirlukast, zidovudine, zileuton, zoledronic, zolmitriptan, zonisamide.

pounds[3] and 6% - 10% by 10 compounds[4] whereas it is accelerated 11% - 42% by 11 compounds[5] and 6% - 10% by 40 compounds[6].

Among antibacterial agents, only azithromycin and tylosin produce a notable effect (acceleration of 10% - 11%, confirmed with new compound and exceeding 50% at 10 μM). The cardiac safety of azithromycin has been under question for many years and this compound was recently shown to increase the risk of cardiovascular death [72]. Similarly, the veterinary antibiotic tylosin increases ventricular arrhythmic risk in dogs [73]. None of the quinolone antibiotics (ciprofloxacin, clinafloxacin, enoxacin, enrofloxacin, fleroxacin, gatifloxacin, levofloxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, rufloxacin, sarafloxacin, sparfloxacin) affect beating at 1 μM. It is likely that this concentration is too low and, when we retested some quinolones at higher concentrations, moxifloxacin and sparfloxacin slowed beating (>20% at 100 μM) whereas ciprofloxacin, enrofloxacin, fleroxacin, levofloxacin, lomefloxacin, norfloxacin, ofloxacin, and pefloxacin do not. This is consistent with a PPB of 20% - 50% for quinolones and with their published K_{50}s (~18 μM for sparfloxacin, 129 μM for moxifloxacin, 915 μM for levofloxacin, 966 μM for ciprofloxacin, 1420 μM for ofloxacin) [64].

Four histamine antagonists decrease frequency (astemizole, clemastine, terfenadine, triprolidine); this is likely due to IKr block as discussed above. The weaker IKr blocker mepyramine [44] slows beating transiently (~8% for <15 min); whether this results from IKr block is not certain. It is worth noting that hiPSC-CM respond to histamine with a very strong acceleration (EC_{50} ~100 nM), which is prevented by the H_2-selective antagonist famotidine and not by the H_1-selective antagonist pyrilamine (separate experiments, not shown); however, these antagonists have no effect in the absence of histamine, indicating that there is no constitutive activation of histamine receptors or paracrine secretion of histamine. Finally, the weak IKr blockers cyproheptadine, olopatadine and promethazine accelerate rhythm (~25% for 60 min for cyproheptadine, minimally for the others). Cyproheptadine has high plasma protein binding (96%) and strongly increases frequency, suggesting that it acts through amechanism distinct from IKr block.

Many hormone receptor agonists and antagonists produce sustained or transient acceleration (estradiol, estriol, estrone, ethisterone, fulvestrant, gestrinone, mifepristone, norethindrone, progesterone, raloxifene) but no pattern is obvious. Diethylstilbestrol and many progesterone receptor agonists produce no effect, suggesting that activation of ER*α*, ER*β*, or PR receptors does not accelerate beating. Estradiol, estriol, estrone, fulvestrant, and raloxifene are agonists of the G-protein-coupled estrogen receptor, which may play a role in their effect, but such activity has not been reported for ethisterone, gestrinone, mifepristone, norethindrone, or progesterone.

5. DISCUSSION

The results of our study indicate that recording of Ca transients in syncytia of cardiomyocytes derived from human induced pluripotent stem cells represents a useful *in-vitro* cardiac model for early drug development. The non-dividing cells must be kept in standard culture conditions for 2 - 3 weeks before

[3]Compounds decreasing frequency 11% - 20% (n = 6) ordered alphabetically: benzydamine, dorzolamide, maprotiline, strychnine, tacrine, tropisetron.

[4]Compounds decreasing frequency 6% - 10% (n = 10) ordered alphabetically: aripiprazole, bumetanide, cyclosporine, granisetron, hydroxytacrine, mepyramine, mesoridazine, methiothepin, neostigmine, primaquine.

[5]Compounds increasing frequency 11% - 42% (n = 11) ordered alphabetically: bisacodyl, clozapine, cyproheptadine, disulfiram, estradiol, fenoldopam, fulvestrant, gestrinone, imiquimod, nelfinavir, phenamil, quinacrine, quinpirole.

[6]Compounds increasing frequency 6% - 10% (n = 40) ordered alphabetically: acemetacin, albendazole, azithromycin, bromocriptine, cabergoline, calcitriol, canrenone, docebenone, estrone, fasudil, fenbendazole, fenofibrate, flubendazole, fluconazole, flufenamic acid, itraconazole, metronidazole, mifepristone, miltefosine, minocycline, naloxone, niflumic acid, norethindrone, olopatadine, oxfendazole, oxibendazole, pancuronium, pentamidine, phenytoin, pramipexole, progesterone, promethazine, prothionamide, sumatriptan, suramin, tetracycline, tosufloxacin, tylosin, vincristine, zalcitabine.

usage but they require no particular maintenance. At that time, they provide a reproducible and stable test system where many compounds can be evaluated simultaneously using ordinary Ca imaging technologies. The regular pattern of the Ca transients is amenable to simple analysis methods although the current software of commercial fluorescence imaging devices does not provide routines of sufficient quality and customized add-ons are required. In this paper, we focus on changes in beating frequency but additional information is clearly contained in the Ca transients (*e.g.*, duration, amplitude, slope; see **Figure 1**). This needs further evaluation.

After the cells are treated with the fluorescent dye, a 60 - 90 min window remains to evaluate compound effects. During this period, application of solvent alone produces a time-dependent acceleration of the rhythm, independent of the solvent used (DMSO, EtOH, MeOH, or H_2O). This acceleration is marked (30% over 60 min) but it is highly reproducible from one recording to the next and it can be easily corrected for. It is not seen with non-invasive impedance recordings performed in a CO_2-controlled incubator, with or without pre-incubation with the Ca-sensitive dye, but a similar tachycardia is observed when impedance recordings are performed in an incubator where CO_2 control is disabled. Therefore, the rate increase is unlikely to result from mechanical stress to the syncytia due to compound injection or from a direct effect of solvent, or from cell toxicity due to the Ca-sensitive fluorescent dye; it is more likely to result from the slow drift in extracellular pH due to the lack of control of ambient CO_2. In addition, the acceleration does not appear to interfere with the measurement of drug effects since even small drug-induced rhythm changes are essentially identical whether they are measured using this technique or impedance recordings. After 60 - 90 min, recording quality deteriorates such that it is only possible to evaluate short-term effects with our procedure; however, a different protocol could be developed in which cells are pre-treated with compounds in the absence of dye: we have not evaluated this method, which would probably require larger replicates since single syncytia could not be used as their own pre-treatment controls.

The hiPSC-CM respond appropriately to known pharmacology. When tested at 1 μM, most drugs without direct effect on cardiac rhythm or with effects only at high concentrations do not change the beating frequency indicating that the test has good specificity. In particular, ARAs, ERAs, and ACEis do not change frequency. In contrast, the test detects correctly most compounds with a direct activity on cardiac rhythm, demonstrating high sensitivity. In particular, β-adrenergic agonists (except the pro-drug bambuterol) accelerate frequency and, based on different kinetics of action, the model can distinguish β_2-selective from β_1-selective or non-selective agonists as well as partial from full agonists.

PDE inhibitors are markedly subtype-specific: PDE4 inhibitors (selective or not) accelerate rhythm whereas other subtype inhibitors (1, 2, 3, 5, 6) are inactive, except for high concentrations of PDE3 or PDE5 inhibitors. This indicates that PDE4 is of particular importance in controlling hiPSC-CM beating frequency. A critical role for PDE4 has also been suggested in human heart based on the observations that inhibition of PDE4 in atrial myocytes increases Ca spark frequency and initiates spontaneous Ca waves [74], and that the PDE4D isoform immunoprecipitates with the cardiac RyR2 channel in heart extracts [75]. In the "intracellular Ca clock" model of cardiac pacemaking, such tight relationship between PDE4, RyR2, and Ca spark frequency would ensure a crucial role for PDE4 in controlling heart rate [76]. Nevertheless, it is surprising that PDE3 inhibitors do not produce more effect [77], but it is possible that the subcellular compartmentalization of cAMP pools is sufficient to insulate PDE3 inhibition from pacemaker mechanisms [78]; the observation that the more prolonged inhibition of PDE3 can lead to small increases in beating frequency would support the compartmentalization idea. It may also be that the contribution of PDE subtypes to cardiac pacemaking is special in hiPSC-CM since this contribution changes with postnatal development [79].

hiPSC-CMs are extremely sensitive to cardiac ion channel blockers. L-type CCBs produce marked changes in frequency whereas gabapentin, a neuronal CCB, has no effect. Dihydropyridines, verapamil, diltiazem, lomerizine, flunarizine, and bepridil (the last three at 10 μM not at 1 μM) accelerate frequency markedly then stop spontaneous activity entirely or induce arrhythmias, except for diltiazem. The fact that CCBs accelerate rhythm provides some clues as to the basis of spontaneous activity in the hiPSC-CM syn-

cytia. Direct measurements and mathematical models have shown that reducing L-type Ca currents produces opposite effects on the cycle length of action potentials in the periphery and center of the rabbit sinoatrial node: cycle length is lengthened if tissue is from the center but shortened if it is from the periphery [80, 81]. hiPSC-CM syncytia are a mixture of cells with ventricular, atrial, and nodal phenotypes, the latter likely being responsible for the spontaneous activity of the tissue [2]: the action of the CCBs suggests that, although myocytes with a central-nodal phenotype may be present, it is myocytes with a peripheral-nodal phenotype that are mostly responsible for spontaneous rhythm.

Class I and Class III antiarrhythmics also modify beating frequency: K channel blockers produce strong bradycardia, arrest, or marked arrhythmias, while Na channel blockers slow the rhythm moderately at low concentrations then produce complete arrest at higher drug levels. Only amiodarone, which is very highly protein-bound, has little effect on frequency. Finally, drugs acting on KATP channels have no effect, indicating that KATP channels play little or no role in controlling rhythm in these cells.

As suggested by the actions of the Class III antiarrhythmics, all compounds blocking IKr markedly affect rhythm, if they are sufficiently potent to block >50% of the current under the assay conditions: after correcting IKr-blocking potency for PPB, most drugs blocking with potency ≤1 μM strongly reduce rhythm when they are tested at 1 μM. On the other hand, most drugs blocking with potency >1 μM have no effect, slow the rhythm minimally, or accelerate it. Therefore, the assay reliably separates drugs blocking or not blocking IKr and it may represent an alternative to the classic "hERG test" where compound potency is measured on recombinant $K_V11.1$ channels. It has the advantage of taking into account the "plasma shift" inherent to compounds acting on the heart of humans or animals and it can detect many ancillary pharmacologies such as Ca or Na channel block, PDE inhibition, and activation of adrenergic or muscarinic receptors.

However, like other *in-vitro* assays, this model may detect effects that are not physiologically-relevant. The stability of rhythm over 60 min and the reproducibility of vehicle controls make it possible to determine that small increases or decreases in frequency are compound-related (±5%) but it is not certain that such changes are necessarily physiologically-relevant. Many partial β-adrenergic agonists increase frequency 6% - 10% and some M2-selective agonists reduce it by a similar amount, indicating that small alterations can reflect a pharmacologically-relevant action. Yet, 71 non-cardiovascular compounds also produce changes of similar magnitude but published data regarding their cardiac effects is insufficient to determine if comparable effects are seen in cardiac tissues or animals and whether they represent a risk for patients. It should also be reemphasized that although the hiPSC-CM show gene expression profiles that resemble adult cardiomyocytes [6], they tend to exhibit immature functional and structural characteristics [82]: it is conceivable that some of the effects reported here may not be seen in cardiac tissue from adult patients. The use of biophysically-based computational models may help reconcile measurements performed in hiPSC-CM with predictions formulated for adult human ventricular cardiomyocytes [83]. Finally, with our method, the hiPSC-CM syncytia are not paced and they are left producing their own spontaneous rhythm. This "physiology-like" situation limits the possibilities to perform mechanistic evaluations of drug actions, such as examining precisely the interactions with heart rhythm of drugs with voltage- and/or use-dependent actions. It would be useful to design a means to examine compound effects at different beating rates, particularly under a simulated vagus nerve input known to favor pro-arrhythmic drug effects, but cholinergic agonists display a rapid desensitization in our hands. Finally, the assay is not designed to explain definitely how a given compound affects beating rate but it can call attention to potential drug cardiac effects and it can provide some initial hints into further avenues of investigation.

6. CONCLUSION

Cardiomyocytes derived from human induced pluripotent stem cells are a very useful *in-vitro* system to predict cardiac effects that may be seen when a drug is administered to humans. The preparation is relatively easy to put in place and it reacts to drugs used in human medicine as predicted from existing clinical data. Prospective studies should be designed to strengthen the value of this model.

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CONFLICT OF INTEREST AND FUNDING SOURCES

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