Cardiovascular (CV) toxicity is a leading contributor to drug attrition. Implementing earlier testing has successfully reduced human Ether-à-go-go-Related Gene-related arrhythmias. However, analogous assays targeting functional CV effects remain elusive. Demand to address this gap is particularly acute for kinase inhibitors (KIs) that suffer frequent CV toxicity. The drug class also presents some particularly challenging requirements for assessing functional CV toxicity. Specifically, an assay must sense a downstream response that integrates diverse kinase signaling pathways. In addition, sufficient throughput is essential for handling inherent KI nonselectivity. A new opportunity has emerged with cellular impedance technology, which detects spontaneous beating cardiomyocytes. Impedance assays sense morphology changes downstream of cardiomyocyte contraction. To evaluate cardiomyocyte impedance assays for KI screening, we investigated two distinct KI classes where CV toxicity was discovered late and target risks remain unresolved. Microtubule-associated protein/microtubule affinity regulating kinase (MARK) inhibitors decrease blood pressure in dogs, whereas checkpoint kinase (Chk) inhibitors (AZD7762, SCH900776) exhibit dose-limiting CV toxicities in clinical trials. These in vivo effects manifested in vitro as cardiomyocyte beat cessation. MARK effects were deemed mechanism associated because beat inhibition potencies correlated with kinase inhibition, and gene knockdown and microtubule-targeting agents suppressed beating. MARK inhibitor impedance and kinase potencies aligned with rat blood pressure effects. Chk inhibitor effects were judged off-target because Chk and beat inhibition potencies did not correlate and knockdowns did not alter beating. Taken together, the data demonstrate that cardiomyocyte impedance assays can address three unmet needs—detecting KI functional cardiotoxicity in vitro, determining mechanism of action, and supporting safety structure-activity relationships.

Key Words: impedance; cardiotoxicity; kinase inhibitors.

Cardiovascular (CV) toxicity contributed to more than a third of all safety-related drug withdrawals between 1990 and 2006 (Shah, 2006), which suggests that the pharmaceutical industry’s approach to preclinical CV safety screening needs additional components to complement the existing arsenal of tools. Historically, preclinical CV testing cascades have been forced to rely on in vivo models to replicate the integrated nature of CV physiology. Although in vivo models offer good translation for positive findings, they suffer from limited capacity. As a consequence, many CV risks are missed entirely or identified in trials that lack the capacity to redress issues. Adding in vitro assays with higher throughput can permit earlier hazard identification and enable medicinal chemists to design-out CV liabilities as has been demonstrated with human Ether-à-go-go-Related Gene (hERG) assays. The introduction of cellular and biochemical hERG assays dramatically reduced hERG-related arrhythmias, and the accumulated structure-activity relationship (SAR) enables in silico models to be applied before compound synthesis (Pollard et al., 2010). Unfortunately, replicating the hERG success story with new in vitro assays predictive for other CV risks has lagged. Given the high rate of CV-related attrition and failure, in vitro correlates for contractility, heart rate (HR), blood pressure (BP), or other CV liabilities are urgently needed (Stevens and Baker, 2009).

The challenges of developing new in vitro screens for functional cardiotoxicity are formidable. This is particularly true for kinase inhibitor (KI)–mediated CV toxicity (Mellor et al., 2011), which is notable for a convergence of technical hurdles. First, from the kinase perspective, the number of kinases associated with CV toxicity has surpassed 20 (Force and Kolaja, 2011) and is likely to expand once new approaches enable systematic kinome-wide scans for CV toxicity. From the small-molecule kinase inhibitor (SMKI) point of view, the issue is further complicated by the inherent lack of selectivity. The majority of SMKIs interact with the ATP-binding site, which is highly conserved across the kinome. From a cell signaling perspective, kinases are notoriously pleiotropic and phosphorylate a plethora of proteins, including many that are involved in the cardiac action potential, calcium flux, or mechanical contraction. Thus, in contrast to a single target screen like hERG, in vitro assays for KI-induced CV toxicity
must detect an integrated response that is downstream of the excitation-contraction coupling cascade.

Several new technology platforms allow monitoring of synchronous, spontaneous beating cardiomyocyte cultures with high resolution and high throughput. Two platforms sensitive to calcium flux introduce accelerated data acquisition speed sufficient to monitor calcium dynamics in beating cardiomyocytes (Cerignoli et al., 2012; Sirenko et al., 2013). In addition, a new cellular impedance platform with accelerated data collection (12.9 ms) allows label-free detection that is sensitive to morphological changes (Xi et al., 2011). Although each of these platforms is likely to have an array of applications, the impedance technology offers label-free detection of minute morphological changes enabling a readout that is downstream of contraction. Therefore, impedance-based detection of beating cardiomyocytes appears particularly well suited to meet the gap in SMKI CV toxicity screening.

To evaluate cardiomyocyte impedance assays for SMKI functional cardiotoxicity testing, we selected KIs with two different primary pharmacologies for which the existing complement of CV assays resulted in late-stage failure due to functional CV toxicity. Inhibitors of microtubule-associated protein (MAP)/microtubule affinity regulating kinase (MARK) developed for Alzheimer’s disease (AD) reached an advanced stage before being found to dramatically reduce mean arterial BP in anesthetized dogs (see patent; Lim et al., 2011). Similarly, inhibitors of checkpoint kinase (Chk) aimed at enhancing the efficacy of both conventional chemotherapy and radiotherapy in the treatment of cancer exhibited cardiac toxicities that limited exploration of efficacy as a monotherapy or combination therapy in clinical trials (Daud et al., 2010; Ho et al., 2011).

In both these examples, the late-stage data did not resolve whether the mechanisms were related to the target kinase or secondary pharmacology and thus left potentially high-value targets with undefined yet intractable CV risks. Amending CV screening cascades to include higher throughput in vitro assays sensitive to integrated cardiomyocyte function could open a host of diverse options ranging from earlier identification of direct effects on cardiomyocytes to target-specific risk assessment. Here, we interrogate cardiomyocyte impedance assays for the capacity to (1) detect functional CV effects earlier in drug discovery, (2) enable mechanism of action studies, and (if CV effects are target independent) (3) deliver data with the throughput and robustness needed to support SAR development and guide medicinal chemists to design-out CV liabilities.

**MATERIALS AND METHODS**

All animal procedures were performed with the approval of the internal AstraZeneca Institutional Animal Care and Use Committee (IACUC) in accordance with recommendations of the panel on euthanasia of the American Veterinary Medical Association and the National Institutes of Health publication: “The Guide for the Care and Use of Laboratory Animals” (ILAR, 1996).

**Myocyte isolation and culture.** Rat neonatal cardiomyocytes (RNCM) were isolated from 3-day-old Han Wistar rats (Harlan, Indianapolis, IN) using Neonatal Cardiomyocyte Isolation System (Worthington Biochemical Co., Lakewood, NJ) following the manufacturer’s instructions as described recently (Peters et al., 2012). Isolated cell suspensions were plated in a T150 flask to allow attachment of noncardiomyocyte cells. After 3 h, the media containing enriched cardiomyocytes was centrifuged at 100 × g for 5 min. The pellet was resuspended and cells were counted using a Vi-CELL (Beckman Coulter, Brea, CA) with a 12-µm cutoff to avoid counting red blood cells. Cells were plated in 96-well xCELLigence Cardio E-plates coated with fibronectin (10 µg/ml; Sigma, St Louis, MO) at 30,000 cells well in Dulbecco’s modified Eagle medium (DMEM; Mediatech, Manassas, VA) plus 20% dialyzed fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA) and 100 µg/ml penicillin-streptomycin (Mediatech). Cells were incubated at 5% CO₂ and 37°C. The growth medium was changed 24 h postseeding and again 2 h before to drug addition.

**Drug source, preparation, and addition.** SCH900776 was obtained from ChemieTek (Indianapolis, IN), Colchicine, vinblastine, nocodazole, SB218078, PF477736, PD407824, and NCS109555 dityosine were purchased from Tocris Bioscience (Bristol, United Kingdom). LY2603618 and CHIR-124 were purchased from Selleck Chemicals (Houston, TX), Paclitaxel and UCN-01 were purchased from Sigma Chemical. 2-Anilino-7-[18]-4-hydroxy-2,3-dihydro-1H-inden-1-yl]pyrimidin-6-one (referred to as MK68), (1R,2R)-3,3-difluoro-2-[4-[(7-[4-fluorophenyl)]imidazo[1,2-][1H-pyrimidin-6-yl]pyridazin-3-yl]carbonylamino]cyclohexanaminium trifluoroacetate (referred to as MK8), and AZD7762 (Oza et al., 2012) were synthesized at AstraZeneca. Compound stock solutions and serial dilutions were prepared in dimethyl sulfoxide (DMSO). After being cultured for 48 h, cardiomyocytes were treated with various concentrations of compound in a final concentration of 0.1% DMSO or 0.1% DMSO as a vehicle control. Because temperature decreases reduce myocyte beat rates, liquid handling steps were automated on a BioMek FX in order to minimize the time that cell cultures were out of the incubator to fewer than 90 s.

**Measurement of beat parameters as determined by cellular impedance recordings.** Data were collected with the xCELLigence Cardio System instrument (ACEA Biosciences, San Diego, CA) with three consecutive 58-s reads separated by 2-s intervals. In this study, we focused on beat amplitude because in our experience, amplitude data are preferable for determining potencies of drugs that induce cessation of beating. In these instances, beat amplitude consistently shows graded concentration-dependent decreases, whereas beat rate may initially increase to high rates with high variability before reversing as beating stops (see Supplementary figs. 1–3). In Excel, data were transformed first to percent of baseline for each well and subsequently to percent of time-matched vehicle control. Data were then exported to Prism (GraphPad Software, La Jolla, CA) for graphing, statistical analysis, and calculation of potency values. Data are representative of at least three independent experiments. Drug responses were measured at 20 min, 2 h, and 24 h. For the present studies, the main time-dependent change was a progressive increase in variance. This may be related to the growth of fibroblasts relative to nondividing cells, because in our experience, amplitude data are preferable for determining potencies of drugs that induce cessation of beating. In these instances, beat amplitude consistently shows graded concentration-dependent decreases, whereas beat rate may initially increase to high rates with high variability before reversing as beating stops (see Supplementary figs. 1–3). These supplementary figures include concentration-dependent changes in beat rate, beat amplitude, and cell index for each compound tested to allow direct comparison of drug-induced changes.

**Quantification of cellular ATP levels.** ATP content per well was determined using the CellTiter-Glo luminescent assay (Promega, Madison, WI) according to the manufacturer’s instructions. All but 75 µl of cell culture supernatant was removed from each well and an equal volume of CellTiter-Glo reagent was added. Plates were vigorously shaken for 1 min, incubated for 10 min at ambient temperature, and ~125 µl was transferred to a white-walled 96-well plate (Costar, Corning, NY). Luminescence was recorded on a Victor Light...
Luminescence Counter (PerkinElmer, Santa Clara, CA). Data are representative of at least three independent experiments.

**Cardiac injury marker release assay.** Cell culture supernatant was collected and stored at −20°C until time of analysis. Release of cTnT in cell culture media was determined using the rat-specific Cardiac Injury Panel 2 multiplex immunoassay (Meso Scale Discovery, Gaithersburg, MD) according to manufacturer’s instructions. Briefly, 25 μl of supernatant or standard was added to each well and plate was incubated for 2h with shaking. After 3 washes, 25 μl of detection antibody solution was added per well and plate was incubated for an additional 2h with shaking. Following three washes, 150 μl of read buffer was added per well, and electrochemiluminescence was determined using SECTOR Imager 6000 (Meso Scale Discovery). Concentration of cTnT was calculated from an eight-point standard curve. Data are representative of at least three independent experiments. To account for troponin derived from the serum in culture media, compound effects were assessed relative to DMSO-treated controls.

**Quantitative RT-PCR.** Quantitative RT-PCR (qRT-PCR) was performed using the Ambion Gene Expression Cells-to-CT kit from Life Technologies (Carlsbad, CA) according to manufacturer’s instructions. Cells were lysed and complementary DNA was synthesized using the provided reverse transcriptase. Quantitative PCR was performed using an Applied Biosystems 7900HT SDS instrument (Life Technologies) with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s alternating with 60°C for 1 min. PCR reactions contained TaqMan Universal PCR Master Mix, nuclease free water, VIC-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) TaqMan Gene Expression Assay (assay ID Rn01775673_g1) as endogenous control, and FAM-labeled TaqMan Gene Expression Assays specific to MARK1 (assay ID Rn00588487_m1), MARK2 (assay ID Rn00433039_m1), MARK3 (assay ID Rn00590679_m1), MARK4 (assay ID Rn01545798_m1), Chk1 (assay ID Rn00589669_m1), or Chk2 (assay ID Rn00586616_m1). For each sample, gene-specific product was normalized to GAPDH and quantified using the comparative ΔΔCt method. Data are representative of at least three independent experiments.

**siRNA-mediated gene knockdown.** RNCM (30,000 cells/well of an E-plate) were cultured for 24h, then transiently transfected with a 25nM pool of two siRNA oligonucleotides (oligos) targeting the specific gene of interest or 25nM of nontargeting siRNA oligos (QIAGEN, Valencia, CA) using DharmaFECT 1 transfection reagent (0.4 μl/well; Thermo Fisher Scientific). For each transfection, a siRNA oligo stock and a Dharmafect 1 stock were prepared in OptiMEM (Life Technologies) and incubated separately for 5 min. The two stocks were then mixed and incubated for 20 min. DMEM plus 10% FBS without antibiotics was added to the mixture and 100 μl was added to each well. Cells were incubated for 1 h and then 50 μl of DMEM plus 20% FBS without antibiotics was added to each well. Following overnight incubation, transfection medium was removed and replaced with complete growth medium containing antibiotics. Data were transformed to percent of baseline for each well and subsequently to percent of the time-matched nontargeting siControl group. The best amplitude of the siControl-transfected group was not significantly different from control. Data are representative of at least three independent experiments.

**Chk in vitro kinase assays.** The Kinase Profiler screening service (Millipore, Billerica, MA) using radioisotope-based assays was used to determine the inhibitory effect of compounds against human Chk1 and Chk2. A nine-point concentration response curve was generated to calculate IC50 values. The protocol can be found on http://www.millipore.com/techpublications/tech1/pf3036.

**MARK4 phosphorylation cell assay.** 3T3 cells containing plasmids encoding 4-repeat tau and inducible MARK4 were incubated in 96-well plates overnight with doxycycline for MARK4 expression induction and then treated with compounds for 2h. Cells were fixed using 4% paraformaldehyde and phosphorylated tau was stained with an antibody toward pSer262Tau. Hoechst dye was used for visualization of cell nuclei. Well images are captured using the ImageXpress® system and further analyzed with MetaXpress software using Multi Wavelength Cell Scoring Analysis. The average cytoplasm intensity parameter was measured in perinuclear cell area and used to determine MARK4 activity.

**MARK in vitro kinase assays.** Compounds were added at 4x concentration in kinase buffer containing 50μM Tris-HCl pH 7.5, 1μM ethylene glycol tetraacetic acid, 10μM MgCl2, 0.01% Tween 20, 5% glycerol, and 4mM dithiothreitol, then challenged with the appropriate MARK enzyme (MARK1–3), Ulight-PLK substrate, and ATP mix. Mixture was incubated for 1 h at room temperature; the reaction was then stopped by adding a mixture of EDTA and detection antibody and incubated for 1 h at room temperature. The samples were read using TR-FRET in a PheraStar from BMG Labtech (Cary, NC) at an excitation wavelength of 320/330 nm and an emission wavelength of 665/615 nm. IC50 values were calculated using Excel fit software.

**Surgical implantation of radio telemetry and catheters.** Male Han Wistar rats were surgically implanted with PhysioTel multiplus radio transmitters (model TL11MZ-C50-PXT) at Charles River Laboratories (Raleigh, NC) as previously described (Kamendi et al., 2010). Rats arrived at AstraZeneca 7 days before the study to allow for acclimation to the environment.

**Radio telemetry data acquisition.** Two days before study, the rats were moved to the automated blood sampling and telemetry system (ABST), an integrated pharmacology testing platform consisting of the BASi Culex automated blood sampling apparatus (BASI, West Lafayette, IN) and DSI radio telemetry system (DSI, St Paul, MN) (Kamendi et al., 2010) to allow for simultaneous evaluation of functional radiotelemetry signals detected with the modified PhysioTel receiver adapted for the ABST (Litwin et al., 2011) and drug pharmacokinetic parameters. Sampling frequency for BP and temperature were set at 500 and 100 Hz, respectively. One day prior to dosing, the following study parameters were monitored: BP, HR, and temperature. On the day of dosing, animals were randomly assigned to treatment groups. At time t = 0, 240, and 480 min, animals were treated with MK8, MK68, or vehicle (40% PEG-200) iv in an ascending dose paradigm (0.1, 1, and 10mg/kg). BP, HR, and temperature were continuously monitored to study end. In addition, automated timed (min: 0, 3, 8, 15, 30, 60, 120, and 240) blood samples were collected in tubes containing 10 μl EDTA following each dose and maintained at 4°C until the end of the experiment.

**Determination of MK8 and MK68 in plasma.** Plasma samples of 50 μl were mixed with 250 μl of acetonitrile containing two internal standards, glyburide and clozapine, to precipitate proteins and centrifuged at 2500 × g for 5 min. The supernatants were dried under nitrogen and then reconstituted with 250 μl of 80:20 10mM ammonium formate:acetonitrile + 0.1% (vol/vol) formic acid prior to liquid chromatography-mass spectrometry (LC-MS) analysis. Standard solutions of MK8 and MK68 were prepared by dissolving the compounds in DMSO at 2mg/ml each and diluted to various concentrations with blank rat plasma to generate a calibration curve. Sample separation was carried out using an ACE C8 2.1×20mm, 3 μm particle size LC column (MAC-MOD Analytical Inc., Chadds Ford, PA) held at 45°C, connected to a Shimadzu HPLC system with LC10AD VP pump, CBM20A controller (Shimadzu Scientific Instruments, Columbia, MD), and CTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) held at 10°C and performing 5 μl injections. Samples were eluted at 800 μl/min with a 2.3-min gradient elution method. Mobile phase A consisted of 10mM ammonium formate + 0.1% (vol/vol) formic acid. Mobile phase B consisted of 0.1% (vol/vol) formic acid in acetonitrile. The elution method was as follows: 5% B for 0.5 min, then a linear increase from 5 to 95% in 1 min, hold at 95% B for 0.5 min, then a return to 5% B for 0.3 min. MS measurements were made using a Sciex API4000 mass spectrometer (Applied Biosystems, Framingham, MA) fitted with an atmospheric pressure chemical ionization sample sprayer operated in positive ion mode with selected reaction monitoring of m/z 387.1–255.0 with a collision energy of 15V (MK68), m/z 472.2–322.0 with a collision energy of 25V (MK8), m/z 494.2–169.1 with a collision energy of 34V (glyburide), and m/z 372.0–192.1 with a collision energy of 36V (clozapine), using 75-ms dwell time for each analyte. Data were collected and analyzed using Analyst software v1.4.1 (Applied Biosystems). Area under the curve and half-life were
determined by noncompartmental analysis with intravascular input (Phoenix WinNonlin, version 6.1).

**Statistical analysis.** Graphing and statistical analyses were performed using Prism (GraphPad Software). Data in text and figures are represented as mean ± SEM. Significance was judged as \( p \leq 0.05 \), two tailed, determined by two-way repeated measures ANOVA test (in vivo data only) or one-way ANOVA with Tukey’s post hoc test. The relationship between enzyme inhibition and beat amplitude inhibition was determined by Pearson correlation analysis.

**RESULTS**

**Effect of MARK inhibition on RNCM beat amplitude**

MARKs phosphorylate the MAP tau, and hyperphosphorylated tau has been implicated as a toxic agent in AD. Thus, MARK inhibition is a compelling drug target for AD, with the potential to provide a much needed agent for modifying disease progression (Yu *et al.*, 2012). One advanced drug discovery program targeting MARK developed potent and highly selective MARK inhibitors (Altman *et al.*, 2010; Haidle *et al.*, 2009; Lim *et al.*, 2011). *In vivo* data were reported for two of these compounds (herein termed MK4 and MK9) and both significantly reduced BP within 5 min of each successively higher dose in anesthetized dogs. At the highest dose, MK9 reduced BP by 85% and was fatal although no structural toxic evaluation was reported (see patent; Lim *et al.*, 2011).

Because there are no MARK-selective inhibitors commercially available, we synthesized two compounds from separate patents, herein referred to as MK8 and MK68. The compounds were selected to share high MARK selectivity while representing different chemical series and differing in MARK potency by 10-fold (Table 1) (Altman *et al.*, 2010; Haidle *et al.*, 2009).

The effects of MK8 and MK68 on cardiomyocyte beat amplitude were evaluated. In the RNCM impedance assay, MK8 and MK68 caused a concentration-dependent decrease in RNCM beat amplitude (Fig. 1A). At high concentrations, both compounds stopped cardiomyocyte beating. This cessation of beating was not a consequence of cytotoxicity because cellular ATP levels were unchanged and cTnT, a marker of cardiac injury, was not released into the culture media (Figs. 1B and 1C). The relative potencies of MK8 and MK68 in the beat assay (IC\(_{50}\) = 0.55 and 4.3 \( \mu M \)) aligned with their \( \sim 10\)-fold difference in enzyme inhibition potency against all four MARK isoforms (Table 1).

As an independent approach to evaluate the role of MARKs in cardiomyocyte beating, the expression of each isoform was targeted using siRNA. Each gene was effectively knocked down in

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Assay</th>
<th>Compound (nM)</th>
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<tbody>
<tr>
<td>MARK1</td>
<td>TR-FRET</td>
<td>0.1</td>
</tr>
<tr>
<td>MARK2</td>
<td>TR-FRET</td>
<td>0.1</td>
</tr>
<tr>
<td>MARK3</td>
<td>TR-FRET</td>
<td>0.2</td>
</tr>
<tr>
<td>MARK4</td>
<td>3T3 phospho cell imaging</td>
<td>2.4</td>
</tr>
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</table>

**TABLE 1**

Potencies of MK8 and MK68 Against MARK1–4

**FIG. 1.** MARK inhibitors cause a dose-dependent decrease in RNCM beat amplitude at concentrations that do not affect cell viability. (A) Concentration-response curves for inhibitory effects of MK8 (●) and MK68 (○) on beat amplitude determined by impedance measurements in RNCM treated with drug for 20 min. (B) Cellular ATP levels expressed as percent control of RNCM treated with the highest dose of compounds tested in (A) for 2h. (C) Release of cardiac injury marker cTnT into cell culture media of RNCM treated with the highest dose of compounds tested in (A) for 2h. All data points are plotted with error bars expressed as mean ± SEM of three independent experiments (three wells/experiment).
an isoform-specific fashion as determined by qRT-PCR (Fig. 2A). MARKs 2 and 4 were the predominate isoforms expressed in RNCM (2.4±0.1 and 1.6±0.2% of the GAPDH level, respectively), whereas MARKs 1 and 3 mRNA were present at lower levels (0.5±0.03 and 0.04±0.004% of GAPDH, respectively). Consistent with the balance in relative expression levels, siRNA-mediated suppression of MARK 1 or 3 did not influence cardiomyocyte beat amplitude (Fig. 2B), whereas both MARK 2 and 4 siRNA resulted in a significant decrease in beat amplitude at 72-h post-transfection (Fig. 2B). Knockdown of all four MARKs in RNCM had an additive effect on beat inhibition. Importantly, in pan-MARK knockdown experiments, the ATP levels were 95.7±1.7% of control, indicating that knockdown effects on beating were not a consequence of cytotoxicity (72-h post-siRNA transfection; data not shown). Collectively, these data indicate that the decrease in beat amplitude observed in RNCM is mediated, at least in part, by MARK 2 and MARK 4, the two MARK isoforms with the highest expression levels in these cultures.

The canonical cellular role for MARKs is regulation of microtubule (MT) dynamics through phosphorylation of MAPs. As an independent approach to investigate the impact of altering MT dynamics on RNCM cardiomyocyte beating, well-established MT targeting drugs including paclitaxel, vinblastine, nocodazole, and colchicine were tested. Paclitaxel, classified as a MT-stabilizing drug, and vinblastine, nocodazole, and colchicine, known as MT-destabilizing agents all have an effect on cellular function by suppressing MT polymerization dynamics (Jordan and Wilson, 2004). Vinblastine, nocodazole, and colchicine did not stop cardiomyocyte beating but reduced beat amplitude after a 20-min treatment period, with IC50 values of 57nM, 480nM, and 2.1µM, respectively (Fig. 3A). For all three drugs, no significant changes in cellular ATP levels or release of cTnT were detected after a 2-h treatment period at the highest concentration tested (Figs. 3B and 3C), indicating that the decrease in beat amplitude observed after 20 min was not attributable to a decrease in cell viability. In contrast to the other MT targeting drugs, paclitaxel treatment did not have an effect on beat amplitude after a 20-min exposure (Fig. 3A), however, after 24-h beat amplitude was substantially decreased even at the lowest concentration tested (1nM; data not shown). As with the other MT-binding agents, the decrease in beat amplitude was not associated with cytotoxicity as judged by cellular ATP levels and cTnT release (24-h treatment; data not shown).

To explore the MK8 and MK68 in vitro findings for translation to in vivo, their effects on BP were examined using a three-dose escalation scheme designed to match the published studies with MK4 and MK9 (Fig. 4; 0.1, 1, and 10mg/kg successively dosed every 4h; n = 3 rats/group). With all three doses, area under the curve (AUC) and half-life of plasma concentration measured (680nM) exceeded the IC50 calculated for inhibition of cardiomyocyte beat amplitude (550nM). No effect on BP was seen in rats dosed with 10mg/kg of MK68. However, with this compound, the maximum plasma concentration (1.44µM) did not reach the IC50 value calculated for inhibition of cardiomyocyte amplitude (4.3µM). These in vivo data add valuable corroborative evidence to the published findings of MK4 and MK9 in dogs. The apparent relationship between in vitro potency and in vivo activity suggests good correlation, although additional exploration will be needed to define in vitro-in vivo translation for this assay.

**Chk KI Functional Cardiotoxicity**

Clinical trials for the Chk1/2 KI AZD7762 were stopped in the monotherapy dose escalation phase due to cardiac dose-limiting toxicities, particularly ventricular dysfunction (Ho et al., 2011). The Chk1 inhibitor SCH900776 exhibited supraventricular tachycardia as the lead dose-limiting toxicity when administered in combination with Gemcitabine (Daud et al., 2010). These data lead us to posit three criteria as prerequisites for advancing another Chk inhibitor into the
Impedance assays for cardiotoxicity

Clinic to test this target for efficacy: (1) An assay is needed that reproduces functional CV effects for AZD7762 and SCH900776. (2) Evidence that these CV effects are not mediated by Chk inhibition must be established. (3) Finally, new Chk inhibitors must lack similar CV activity. Heretofore, none of these features have been addressed.

Testing AZD7762 and SCH900776 in cardiomyocyte impedance assays revealed concentration-dependent decreases in beat amplitude with potencies of $0.83 \pm 0.12$ and $5.67 \pm 2.03\mu M$, respectively (Table 3 and Fig. 5A). The effects on beating were not associated with cytotoxicity (Figs. 5B and 5C). The potent effect on beating indicates that cardiomyocyte impedance assays are appropriate to investigate functional changes associated with these two Chk inhibitors.

To extend the investigation of Chk inhibition effects on RNCM beat amplitude, additional commercially available

<table>
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<th>TABLE 2</th>
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<tr>
<td>Exposure of MARK Inhibitors</td>
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<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
</tr>
<tr>
<td>AUC (min µg/ml)</td>
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inhibitors were tested. The effects of nine Chk inhibitors on cardiomyocyte beat amplitude varied in potency and efficacy (Table 3 and Fig. 5A). NSC109555 and SB218078 did not influence cardiomyocyte beat amplitude at the highest soluble concentrations (10 and 100µM, respectively). The remaining seven Chk inhibitors reduced beating, but with potencies that varied by 450-fold (198nM–89.3µM; Table 3). Cytotoxicity did not appear to be a common contributor to effects on beating. Only PF477736 significantly decreased cellular ATP content (41.0±9.1%) and increased release of cTnT (290%) at 31.6h, the highest concentration tested (beat amplitude IC<sub>50</sub> = 12.7µM) (Figs. 5B and 5C). Interestingly, SCH900776 decreased beat amplitude (Fig. 5A), but increased cellular ATP (Fig. 5B), an effect not associated with cTnT release (Fig. 5C).

To investigate the correlation between Chk inhibition and cardiomyocyte effects, each compound was assessed for Chk1 and Chk2 enzymatic inhibition potencies using radioisotope-binding assays under common assay conditions. All of the molecules tested except NSC109555 were similarly potent Chk1 inhibitors (IC<sub>50</sub> = 3–7nM); however, the beat amplitude IC<sub>50</sub> values varied markedly (0.2 to > 100µM; Fig. 6A). There was a similar lack of correlation between Chk 2 enzyme inhibition and beat amplitude effects (Fig. 6B). For instance, UCN-01 and NSC109555 had similar potencies for Chk2 enzymatic inhibition (897 and 685nM, respectively) but varied greatly in beat amplitude potencies (0.2µM vs. >10µM, respectively). Further correlation analysis for inhibition of either Chk or dual inhibition of both Chk1 and 2 did not reveal a direct relationship between Chk pharmacology and beat inhibition (not shown).

The effect of Chk kinase inhibition on RNCM beat amplitude was also evaluated using siRNA-mediated knockdown of Chk1, Chk2, and Chk1/2. The mRNA transcript levels for Chk1 and Chk2 in RNCM were similar as measured by qRT-PCR (0.21±0.03 and 0.29±0.08% of GAPDH, respectively). Specificity of each Chk siRNA knockdown was confirmed by qRT-PCR (Fig. 7A). Suppression of Chk1, Chk2, or Chk1/2 did not influence cardiomyocyte beat amplitude for time points up to 72-h post-siRNA transfection (Fig. 7B). Consistent with pharmacological inhibition, the genetic knockdown results suggest inhibition of Chk kinases have a minimal effect on beat amplitude in RNCM and do not account for the beat effects of AZD7762 or SCH900776.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RNCM amplitude IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Chk1 enzyme IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Chk2 enzyme IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<td>AZD7762</td>
<td>0.8±0.1</td>
<td>0.006±0.0004</td>
<td>0.001±0.00005</td>
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<tr>
<td>SCH900776</td>
<td>5.7±2.0</td>
<td>0.006±0.0003</td>
<td>6.259±0.536</td>
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<td>UCN-01</td>
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<td>0.003±0.0001</td>
<td>0.897±0.132</td>
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<tr>
<td>SB218078</td>
<td>&gt; 100</td>
<td>0.006±0.0003</td>
<td>0.244±0.028</td>
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<tr>
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<td>0.002±0.0002</td>
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<td>LY2603618</td>
<td>89.3±35.0</td>
<td>0.006±0.0003</td>
<td>7.921±1.524</td>
</tr>
<tr>
<td>PD407824</td>
<td>17.8±2.6</td>
<td>0.007±0.0004</td>
<td>0.046±0.0016</td>
</tr>
<tr>
<td>NSC109555</td>
<td>&gt; 10</td>
<td>48.26±7.521</td>
<td>0.685±0.116</td>
</tr>
</tbody>
</table>

FIG. 5. Differential effects of Chk inhibitors on RNCM beat amplitude and viability. (A) Concentration-response curves for inhibitory effects of SCH900776 (gray ●), AZD7762 (gray ○), CHIR-124 (●), LY2603618 (●), PF477736 (♦), NSC109555 (▲), and UCN-01 (▼) on beat amplitude determined by impedance measurements in RNCM treated for 20min. (B) Cellular ATP levels expressed as percent control of RNCM treated with the highest dose of compounds tested in (A) for 2h. (C) Release of cardiac injury marker cTnT into cell culture media of RNCM treated with the highest dose of compounds tested in (A) for 2h. All data are points are plotted with error bars expressed as mean ± SEM of three independent experiments (three wells/experiment). Asterisks indicate significant differences relative to siControl-transfected cells or DMSO-treated controls (**p < 0.01; ***p < 0.001).
Impedance assays for cardiotoxicity

The fundamental features of the cardiomyocyte impedance assay suggest broad potential for improving CV toxicity screening. Cultured cardiomyocytes replicate an essential and integrated CV function—beating—in a cellular format. Impedance technology is exquisitely sensitive to contraction-induced morphology change (Giaever and Keese, 1991; Guo et al., 2011), which is downstream and therefore responsive to changes in the cardiac action potential, calcium flux, and mechanical contraction. Finally, the assay can employ cardiomyocytes derived from human stem cells (Guo et al., 2011; Jonsson et al., 2011), which adds important capability for translating to clinical data. These essential features coupled with higher throughput offer the opportunity to address the gap in frontline screens in CV safety testing cascades.

Herein, we challenged the proposed utility of cardiomyocyte impedance assays using MARK and Chk KIs. KIs were chosen based on the combination of multiple technical hurdles that this class presents for in vitro evaluation of CV toxicity. The specific examples of MARK and Chk were selected because traditional CV cascades allowed unsafe CV activity to progress into late-stage discovery or human clinical trials. Evaluation of impedance

**FIG. 6.** Lack of correlation between Chk enzyme inhibition and inhibitor-mediated decrease in RNCM beat amplitude for Chk inhibitors. pIC<sub>50</sub> values calculated for inhibition of Chk1 (A) or Chk2 (B) are plotted versus pIC<sub>50</sub> values for inhibition of RNCM beat amplitude. Error bars represent SEM from three independent experiments. The highest concentrations of NSC109555 and SB218078 tested did not have an effect on RNCM beat amplitude, and therefore data are represented by ▼ to indicate “greater than.” The correlation of enzymatic inhibition and inhibition of cardiomyocyte beat amplitude was not judged to be significant for Chk1 (Pearson r<sup>2</sup> = 0.15 and p = 0.70) or Chk2 (Pearson r<sup>2</sup> = 0.10 and p = 0.79).

**DISCUSSION**

The fundamental features of the cardiomyocyte impedance assay suggest broad potential for improving CV toxicity screening. Cultured cardiomyocytes replicate an essential and integrated CV function—beating—in a cellular format. Impedance technology is exquisitely sensitive to contraction-induced morphology change (Giaever and Keese, 1991; Guo et al., 2011), which is downstream and therefore responsive to changes in the cardiac action potential, calcium flux, and mechanical contraction. Finally, the assay can employ cardiomyocytes derived from human stem cells (Guo et al., 2011; Jonsson et al., 2011), which adds important capability for translating to clinical data. These essential features coupled with higher throughput offer the opportunity to address the gap in frontline screens in CV safety testing cascades.

**FIG. 7.** siRNA-mediated Chk1 and/or Chk2 knockdown does not influence RNCM beat amplitude. RNCM were transfected with either nonsilencing control siRNA (siControl) or a pair of siRNA targeted against Chk1, Chk2, or both Chk1 and Chk2. (A) Chk mRNA transcripts in RNCM 48-h post-siRNA transfection quantified by qRT-PCR and expressed as percent siControl. (B) Cardiomyocyte beat amplitude determined by impedance measurements in RNCM 72-h post-siRNA transfection expressed as percent siControl. All data are expressed as mean ± SEM of three independent experiments (≥ 12 wells/experiment). Asterisks indicate significant differences relative to siControl-transfected cells (***p < 0.001).
assays with these compounds/targets demonstrates the ability of impedance assays to impact three core unmet needs: (1) Functional CV effects can be detected earlier (in vitro) by monitoring direct actions on cardiomyocyte beating. (2) CV activity can be evaluated for mechanism of action with tailored combinations of pharmacological analysis, genetic knockdown, and pathway-specific tools. (3) CV safety–related SAR studies can be supported with robust concentration-response data in instances where CV activity is mediated by off-target activity. Each one of these advances would likely lead to a substantial cost benefit for drug discovery.

**MARK Inhibitors**

The MARK family of kinases phosphorylates tau, and hyperphosphorylated tau is linked to the pathogenesis of AD (Castillo-Carranza et al., 2013; Kopelikina et al., 2012). Hence, MARK inhibition is a highly compelling drug target with enormous societal potential due to the absence of disease-arresting treatments for AD. Despite the pathway validation and therapeutic demand, a recent patent uncovered CV risks, thereby raising concerns about this target (Lim et al., 2011). This single publically disclosed MARK inhibitor drug discovery program revealed effects on BP for two advanced compounds with good MARK potency and selectivity. In anesthetized dogs, one compound (MK4; MARK3 IC$_{50} = 27$nM) resulted in a ~25% decrease in BP following a 5 mg/kg dose ($n = 3$). The second more potent compound (MK9; MARK3 IC$_{50} = 3$nM) resulted in a ~50% decrease in BP following a 2 mg/kg dose and an 89% decrease in BP following a 10 mg/kg dose ($n = 1$) (Lim et al., 2011). Although it is conceivable that the CV effects stem from secondary pharmacology and could be avoided with new chemical scaffolds, testing this hypothesis has heretofore required a full campaign to find new leads capable of in vivo testing. Thus, MARKs embody the CV toxicity dilemma for KIs and offer an appropriate test for impedance assays as a novel approach.

Here, we extended the evaluation MARK CV risks by selecting two additional inhibitors, MK8 and MK68 from distinct chemical scaffolds. In impedance assays, both compounds stopped cardiomyocyte beating (Fig. 1A and Supplementary fig. 1). Beat inhibition was concentration dependent and the relative potencies closely aligned with the MARK IC$_{50}$s in biochemical kinase inhibition assays (Table 1). When administered to rats at 10 mg/kg, the $C_{max}$ for MK8 (678nM) was greater than the beat inhibition potency (550nM) and significantly reduced BP (Fig. 4). By contrast, the $C_{max}$ for MK68 administered at 10 mg/kg (1.4μM) was below the beat inhibition potency (4.3μM), and no significant change in BP was observed (Fig. 4). These exposure data are consistent with MARK inhibitor BP effects occurring when a plasma concentration is achieved that inhibits cardiomyocyte beat amplitude in vitro.

siRNA inhibition of MARK expression provided an independent link between MARK inhibition and cardiomyocyte beat effects (Fig. 2). Consistent with previous studies (Klein, 1983; Lampidis et al., 1986; Mery et al., 1994), the direct targeting of MT dynamics with colchicine, nocodazole, and vinblastine also altered beating without cytotoxicity (Fig. 3 and Supplementary fig. 2). The effect of paclitaxel on cardiomyocyte beat amplitude was less robust at 20 min but was detectable at 24 h, a finding that is in agreement with an earlier report (Lampidis et al., 1992). Cytotoxicity was not observed with MT agents, siRNA MARK inhibition, or small-molecule inhibition as measured by ATP reduction, troponin release, or reduced cell index (Figs. 1 and 3 and Supplementary figs. 1 and 2). The combined data suggest that MARK inhibitors induce CV toxicity that is functional and includes target-mediated inhibition of cardiomyocyte beating in vitro and reduced BP in vivo.

The conclusions from our direct evaluation of MARK inhibition are consistent with several independent lines of inquiry. Hasinoff and Patel (2010) assessed the structural/cytotoxic effects on cardiomyocytes of 18 SMKIs by determining the concentrations that would induce a 5% increase in lactate dehydrogenase (LDH) release and compared these values with affinity profiles across a panel of 290 kinases. Lack of selectivity for the intended kinase significantly correlated with cardiomyocyte cytotoxicity and 12 off-target kinases, including the MARKs, were implicated as contributors. Furthermore, clinical data are consistent with our findings translating to humans. Approximately one third of patients administered paclitaxel, used to treat a broad range of cancers, display cardiac dysfunction including hypotension, arrhythmias, and chronic heart failure (Floyd et al., 2005; Rowinsky and Donehower, 1995). Vinblastine, also used to treat several cancers, has been shown to cause cardiac ischemia (Senkus and Jassim, 2011) and hypotension when administered in combination with other chemotherapeutics (Pectasides et al., 1998; Schiller, 1996). Colchicine, used as an immunosuppressant and for the treatment of gout, has been shown to cause cardiac dysfunction including hypotension and arrhythmias (Finkelstein et al., 2010; Sussman et al., 2004). Taken together, the published findings and the present impedance data strongly suggest that MARK inhibitors for treatment of AD will require strategies to limit CV risks.

**Chk Inhibitors**

The cellular response to DNA damage includes cell cycle arrest and is mediated in part by activating Chks (Dent et al., 2011). Inhibition of the Chk response could potentiate the DNA damage induced by cancer therapies such as radio- and chemotherapy (Anderson et al., 2011; Borst et al., 2013; Gutierrez-Gonzalez et al., 2013; Walton et al., 2012). The putative potentiation of treatments used in a wide variety of tumors across different tissues and cell types makes Chk inhibitors an attractive target for drug discovery (Ma et al., 2011). Unfortunately, clinical trials with AZD7762, a Chk1/2 inhibitor, and SCH900776, a Chk 1 inhibitor, revealed dose-limiting CV toxicities as leading adverse events (Daud et al., 2010; Ho et al., 2011). This clinical data raise the possibility that Chk kinase activity may play an essential role in CV function and its inhibition may be congruent with safe human pharmaceutical treatment.
In cardiomyocyte impedance assays, both AZD7762 and SCH900776 stopped spontaneous beating without cytotoxicity. By contrast, seven other Chk inhibitors had a range of effects on beating including some that were inactive at the highest soluble concentrations (Fig. 5 and Supplementary fig. 3). For those compounds that did reduce beating, the potencies of beat inhibition did not correlate with potencies at Chk1, Chk2, dual inhibition of Chk1 and 2, or inhibition of either Chk singularly (Fig. 6). Similarly, inhibition of cardiomyocyte beat amplitude did not correlate with measures of cytotoxicity, including reduced cellular ATP levels, troponin release, or decreased cell index (Fig. 5 and Supplementary fig. 3). siRNA inhibition of Chk1, Chk2, or pan-Chk expression had no detectable effect on cardiomyocyte beating (Fig. 7). We are unaware of any literature reporting mechanistic links between Chks and cardiotoxicity. Thus, we have identified positive evidence for both AZD7762 and SCH900776 having direct effects on cardiomyocyte function; however, this activity does not appear to be linked to Chk inhibition. The data suggest that Chk inhibitors that lack effects on cardiomyocytes may be safe, but this hypothesis will need to be tested with new candidate drugs.

In addition to dissociating Chk activity from direct cardiomyocyte effects, the impedance assay offers two practical approaches to advancing safer Chk SMKIs. New or related inhibitors could be screened for Chk inhibition and counterscreened for effects on cardiomyocyte beating. Alternatively, the secondary pharmacology profiles of AZD7762 and SCH900776 could be mined for lists of off-target kinase(s) or other targets that are candidates for contributing to beat inhibition. Each of the putative off-target contributors could be successively evaluated in follow-up impedance experiments with appropriate small-molecule inhibitors, siRNA, and pathway-specific agents. After determining the off-target culprit(s), counter-screening could be shifted to biochemical or cellular assays that are more narrowly focused on the particular molecular off-target concern.

Future Directions

In combination with a series of recent advances, cardiomyocyte impedance assays may contribute to a new approach capable of unraveling the molecular constituents of KI CV toxicity. First, impedance assays introduce the throughput needed for screening KI libraries on beating cardiomyocytes. Second, nearly kinome-wide selectivity panels now allow one to build sets of SMKIs with selectivity profiles that complement each other to collectively span the kinome (Davis et al., 2011). Importantly, the kinase selectivity data are based on human kinases, which can now be matched with stem cell–derived human cardiomyocytes. Finally, with impedance-based detection of beating, a sufficiently downstream endpoint is achieved to capture diverse kinase activities. The concept of combining functional in vitro data with kinome selectivity data follows a precedent set by Olaharski et al. (2010) who successfully tested a collection of 48 SMKIs to develop an in silico model predictive of kinase-mediated bone marrow toxicity. Deconvoluting KI cardiotoxicity into either individual or polypharmacology profiles holds promise for developing a similar predictive model and thus would allow drug discovery programs to build-in CV safety.

Beyond kinases, adding cardiomyocyte beat detection to the arsenal of CV assays is likely to develop a broad impact. The present findings demonstrate successful application to selected high-value drug targets previously abandoned due to either poorly defined risk mechanism or difficulty prioritizing/optimizing compounds. Next steps are likely to include evaluation of impedance assays for detecting cardiac contractility, which has been difficult to assess in vitro but appears suitable for use with beating cardiomyocytes. As experience is gained, one might expect a preference to emerge for specific in vitro parameters (e.g., rate, amplitude, rhythmicity, or cell index) with set analysis metrics (e.g., IC₅₀ as used herein or 20% change threshold; Guo et al., 2011) that combine to enhance translation for particular in vivo endpoints (e.g., HR, BP, and rhythmicity). Generally, impedance assays appear well suited for filling the gap as a primary screen when paired with subsequent mechanistic testing. Ultimately, amending CV testing cascades to include routine frontline screening with an integrated functional response has the potential to shift the paradigm from screening-out CV toxicity to designing-in safety.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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REFERENCES


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