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Use of human stem cell derived cardiomyocytes to examine sunitinib mediated cardiotoxicity and electrophysiological alterations

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ABSTRACT

Sunitinib, an oral tyrosine kinase inhibitor approved to treat advanced renal cell carcinoma and gastrointestinal stroma tumor, is associated with clinical cardiac toxicity. Although the precise mechanism of sunitinib cardiotoxicity is not known, both the key metabolic energy regulator, AMP-activated protein kinase (AMPK), and ribosomal S 6 kinase (RSK) have been hypothesized as causative, albeit based on rodent models. To study the mechanism of sunitinib-mediated cardiotoxicity in a human model, induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) having electrophysiological and contractile properties of native cardiac tissue were investigated. Sunitinib was cardiotoxic in a dose-dependent manner with an IC_{50} in the low micromolar range, observed by a loss of cellular ATP, an increase in oxidized glutathione, and induction of apoptosis in iPSC-CMs. Pretreatment of iPSC-CMs with AMPK activators AICAR or metformin, increased the phosphorylation of pAMPK-T172 and pACC-S79, but only marginally attenuated sunitinib mediated cell death. Furthermore, additional inhibitors of AMPK were not directly cytotoxic to iPSC-CMs up to 250 μM concentrations. Inhibition of RSK with a highly specific, irreversible, small molecule inhibitor (RSK-FMK-MEA) did not induce cytotoxicity in iPSC-CMs below 250 μM. Extensive electrophysiological analysis of sunitinib and RSK-FMK-MEA mediated conduction effects were performed. Taken together, these findings suggest that inhibition of AMPK and RSK are not a major component of sunitinib-induced cardiotoxicity. Although the exact mechanism of cardiotoxicity of sunitinib is not known, it is likely due to inhibition of multiple kinases simultaneously. These data highlight the utility of human iPSC-CMs in investigating the potential molecular mechanisms underlying drug-induced cardiotoxicity.

Introduction

Tyrosine kinase-targeted therapies have revolutionized the treatment of a variety of cancers. Targeted inhibitors of kinases have improved antitumor efficacy and have fewer toxic side-effects, compared to traditional chemotherapy, but have been implicated in causing serious adverse cardiac events (Di Lorenzo et al., 2009; Force and Kolaja, 2011; Menna et al., 2008; Motzer et al., 2007; Orphanos et al., 2009). Sunitinib malate (Sutent; Pfizer) is a multitargeted tyrosine kinase inhibitor that inhibits both tumor cell proliferation and angiogenesis and is approved to treat advanced renal cell carcinoma and gastrointestinal stromal tumours. However, clinically observed serious cardiac adverse events associated with sunitinib include a high incidence of hypertension, cardiac left ventricular systolic dysfunction, and congestive heart failure (Chu et al., 2007; Di Lorenzo et al., 2009; Faivre et al., 2007; Telli et al., 2008). The exact mechanism of this cardiotoxicity is not known but has been broadly attributed to the lack of kinase selectivity of sunitinib, which in a competitive binding assay bound 57 of the 317 kinases tested (18%) at the clinically relevant dose of 0.1 μM (Karaman et al., 2008). Similar to other tyrosine kinase inhibitors (TKIs), the lack of selectivity of sunitinib makes it challenging to pinpoint whether there are specific molecular target(s) that are the critical mediators of cardiotoxicity. In addition to off-target kinase inhibition by TKIs, the interaction with non-kinase targets should also be considered. For example, imatinib (Gleevec; Novartis) is a more selective kinase inhibitor than sunitinib, binding only 10 of the 317 kinases tested with K_d values less than 0.1 μM (Karaman et al., 2008), but binds to non-kinase targets including NQO2 and BCRP (Bantscheff et al., 2007; Meissner et al., 2006).

Due to its role in energy homeostasis, AMP-activated protein kinase (AMPK) has been suggested to be a mediator of sunitinib cardiotoxicity (Force et al., 2007). Depletion of intracellular ATP and increased AMP activates AMPK (α, β, γ subunits) which stimulates catabolic pathways and suppresses non-essential ATP-consuming processes (Kudo et al., 1995, 1996). The rhythmic contraction of cardiac tissues requires a constant, stable source of energy, leaving

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a limited reserve of ATP. Thus, inhibition of AMPK could disrupt a cardiomyocyte's ability to adapt to energy demands leading to deleterious consequences (Force et al., 2007).

In rodent models of drug-induced cardiotoxicity, conflicting evidence for the role of AMPK in mediating sunitinib induced cardiotoxicity have been reported. In neonatal rat ventricular myocytes (NRVM), sunitinib induced apoptosis, depleted ATP, and led to the release of lactate dehydrogenase (LDH) (Hasinoff et al., 2008; Kerkela et al., 2009). Pretreatment with either AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR) (Kerkela et al., 2009) or metformin (Hasinoff et al., 2008) in NRVMs failed to attenuate sunitinib cardiotoxicity, and did not reverse the inhibition of p-ACC by sunitinib (AICAR pretreatment) (Kerkela et al., 2009); however overexpression of a constitutively active mutant form of AMPK reduced sunitinib-mediated apoptosis. In intact rodents, sunitinib induced mitochondrial abnormalities and hypertrophy in cardiac myocytes (Kerkela et al., 2009). However, the use of NRVM to study consequences and causes of adult human cardiac injury may be misleading for a number of reasons including possible differences between human and rodent cardiac biology as well as energy homeostasis mechanisms (Brouillette et al., 2004; Force and Kolaja, 2011; Gussak et al., 2000). Thus, using new, more relevant human *in vitro* models, such as stem cell derived cardiomyocytes will provide novel learning about human drug-induced injury.

Recent advances in stem cell culture have led to the directed differentiation of pluripotent cells into maturing cells that include cardiomyocytes (Kattman et al., 2011). Unlike primary culture models of human cardiomyocytes that proliferate and lose their ability to beat, human iPSC-CMs are post-mitotic, express cardiac contractile proteins, and have the entire complement of functional electrophysical channels to allow the physical contraction of the myocytes (Anson et al., 2011). Characterization of terminally differentiated iPSC-CMs revealed puntated fibrous striations of cardiac specific α/β-myosin heavy chains, cardiac troponin T, actin-related proteins; and ion channels (Na+, L-type Ca 2+, and hERG) expressed at the intermembrane surfaces (Guo et al., 2011). Moreover, successful myocyte formation of iPSC-CMs was further characterized by measuring physical contraction of the myocytes, with synchronous oscillations of 40 beats per minute, and measuring functional electrophysical properties by use of multi-electrode array (Guo et al., 2011). It is reasonable to assume that these functional human cardiomyocytes might possess metabolic mechanisms that are more similar to those in the intact human heart as compared with either primary cell cultures or non-human based *in vitro* models.

In the present study, the involvement of AMPK in sunitinib-mediated cardiotoxicity in human iPSC-CMs was explored. Two known AMPK activators, AICAR and metformin (Corton et al., 1995; Towler and Hardie, 2007), were unable to attenuate substantial sunitinib-mediated toxicity. Furthermore, additional inhibitors of AMPK (RO-3857, RO-9568, RO-1652) were found to be non-cardiotoxic, which led us to reexamine the potential off-target kinase inhibition profile of sunitinib. Similar to AMPK, Ribosomal S 6 kinase (RSK) also had a dissociation constant (Kd) within the therapeutic plasma range of sunitinib (0.1 μM) and was predicted to be a critical off-target kinase mediating-sunitinib cardiotoxicity (Fabian et al., 2005; Force et al., 2007). RSK kinases carry out various cellular functions, such as involvement in proliferation, growth, motility, and survival (Anjum and Blenis, 2008). An irreversible inhibitor of RSK1/2/4 kinases did not induce cytotoxicity. This study highlights the complexity of dissecting sunitinib-mediated cardiotoxicity from adverse conduction effects, as an extensive electrophysiological analysis of sunitinib-mediated inhibition of the sodium and hERG channels and examination of field potential changes were performed. Utilization of a novel *in vitro* human cardiomyocyte model allowed for a unique analysis of sunitinib-mediated electrophysiological effects, which may shed some light on the mechanism resulting in sunitinib-mediated adverse conduction events.

Materials and methods

Chemicals. Sunitinib was purchased from LGM Pharmaceuticals (Boca Raton, FL). AICAR and metformin were purchased from Sigma Aldrich (St. Louis, MO). RO-3857, RO-9568, and RO-1652 compounds were synthesized at Roche. The RSK1/2/4 inhibitor, fluoromethylketone methoxyethylamine (RSK FMK-MEA), was synthesized by the same procedure used to synthesize fluoromethylketone propargylamine (Cohen et al., 2007), with the exception that methoxyethylamine was used instead of propargylamine.

Cell culture. Human induced pluripotent stem cell-derived human cardiomyocytes (iCells) from Cellular Dynamics International (CDI, http://www.cellulardynamics.com/products/cardiomyocytes.html) were thawed in Plating Media (CDI) and plated as single cells onto collagen-coated 96-well plates (BD Biosciences) at a density of 50×10^3 viable-adherent cells per well (percent adherence documented on iCell data sheet). Additionally, iCells cultured for Micro-electrode Array (MEA) experimentation were thawed in Plating Media and plated onto 0.1% Gelatin (Sigma)-coated 6-well tissue-culture plates at a density of 2.2–2.7×10^6 cells per well. Cells were cultured for 5–7 days, changing the media with Maintenance Media (CDI) every two days after thawing.

Western blot analysis. iCell cardiomyocytes were lysed with Cell Lysis Buffer 1× (Cell Signaling Technology) containing 1 mM Pefabloc SC (Roche Applied Science), 1× Protease Inhibitor Tablet (Roche Applied Science), and Phosphatase Inhibitor Cocktail (Sigma Aldrich). Protein concentration was determined with the Qubit Protein Assay (Invitrogen). Protein was subjected to 10 or 12% SDS-PAGE and proteins were electrophoretically transferred to PVDF membranes (Bio-Rad trans-blot system). Membranes were blocked in ODYSSEY Blocking Buffer (LI-COR Biosciences, Lincoln, NE). Primary antibodies pACC-S79, ACC, pAMPK-T172, AMPK, and RSK1 (Cell Signaling Technologies), and GAPDH (Sigma Aldrich) were incubated, in 5% BSA in 1X TBS-0.1% Tween 20, overnight at 4 °C with gentle shaking. The secondary-immunoglobulin was conjugated with donkey-anti-mouse IR Dye 800 CW or donkey-anti-rabbit IR Dye 680 (LI-COR Biosciences) at a 1:15000 dilution. The blots were visualized Odyssey Infrared Imaging System (LI-COR Biosciences).

Cell viability assays. Following treatment of iCells for 24 h, release of lactate dehydrogenase (LDH), ATP content, Caspase 3/7 cleavage, and GSH were monitored. LDH release by damaged cells into the supernatant was determined by the Cytotoxicity Detection Kit (LDH) (Roche Applied Science). ATP present in metabolically active cells was quantified using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Cellular apoptosis was measured using the Caspase-Glo 3/7 Assay (Promega). Cellular glutathione (GSH) was measured using the GSH-Glo Glutathione Assay (Promega). All assays were performed according to manufacturer's recommendations. Signals were quantified using an Envision 2104 Multilabel Reader (Perkin Elmer) at 490 nm for LDH and luminescent signal for ATP, Caspase-3/7, and GSH.

Kinase inhibition. Sunitinib, RO-3857, RO-9568, and RO-1652 were sent to Ambit Biosciences (San Diego, CA) for kinase selectivity analysis against 227 to 353 kinases using KINOMEscan assays. In this cell free binding assay, ligand-bound kinase quantities are measured in the presence and absence of the compound. The values reported are in terms of percent inhibition (%) for each compound against each of the kinases.

MEA experimentation. MEAs were prepared according to manufacturer guidelines. Briefly, microelectrodes in 6-well MEA dishes were coated with 2 μL Fibronectin (Sigma) diluted 1:20 and incubated at 37 °C for 3 h. iCells were reseeded at the target density of 3×10^4 cells in a 2 μL delivery to microelectrodes and incubated at 37 °C, 7% CO_2 for 3 h