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Title

Discovery of Novel Small Molecule Inducers of Heme Oxygenase-1 that Protect Human iPSC-derived Cardiomyocytes from Oxidative Stress

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Running Title: Cardioprotectants of Human iPSC-derived Cardiomyocytes

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237 Words in Abstract

726 Words in Introduction

1495 Words in Discussion

CDDO-Me – Bardoxolone methyl, CI - Cell index, CoPP – Cobalt protoporphyrin, EPO - Erythropoietin, HIF - Hypoxia-inducible factor, hiPSC-CMs - Human induced pluripotent stem cell cardiomyocytes, HK2 – Hexokinase 2, HMOX1 - Heme oxygenase-1, HTS - High throughput screening, Keap1- Kelch-like ECH-associated protein 1, LOPAC - Library of Pharmacologically Active Compound , Nrf2 - Nuclear factor erythroid 2 p45-related factor 2, NQO1 - NAD(P)H quinone dehydrogenase, PDK1 - Pyruvate dehydrogenase kinase 1 , PHD - Prolyl hydroxylase domain , qRT-PCR – Quantitative reverse transcriptase – polymerase chain reaction

Section: Cardiovascular

Abstract

Oxidative injury to cardiomyocytes plays a critical role in cardiac pathogenesis following myocardial infarction. Transplantation of stem cell-derived cardiomyocytes has recently progressed as a novel treatment to repair damaged cardiac tissue but its efficacy has been limited by poor survival of transplanted cells due to oxidative stress in the post-transplantation environment. Identification of small molecules that activate cardioprotective pathways to prevent oxidative damage and increase survival of stem cells post-transplantation is therefore of great interest to improve the efficacy of stem cell therapies. This report describes a chemical biology phenotypic screening approach to identify and validate small molecules that protect human induced pluripotent stem cell cardiomyocytes (hiPSC-CMs) from oxidative stress. A luminescence-based high-throughput assay for cell viability was used to screen a diverse collection of 48,640 small molecules for protection of hiPSC-CMs from peroxide-induced cell death. Cardioprotective activity of 'hit' compounds was confirmed using impedance-based detection of cardiomyocyte monolayer integrity and contractile function. Structure-activity relationship studies led to the identification of a potent class of compounds with 4-(pyridine-2-yl)thiazole scaffold. Examination of gene expression in hiPSC-CMs revealed that the hit compound, designated cardioprotectant 312 (CP-312), induces robust upregulation of heme oxygenase-1, a marker of the antioxidant response network that has been strongly correlated with protection of cardiomyocytes from oxidative stress. CP-312 therefore represents a novel chemical scaffold identified by phenotypic high-throughput screening using hiPSC-CMs that activates the antioxidant defense response and may lead to improved pharmacological cardioprotective therapies.

Introduction

Ischemic heart disease is one of the leading causes of death worldwide with projected healthcare costs associated with cardiovascular disease to reach one trillion U.S dollars by 2025 (Moran et al., 2014). In the United States alone, approximately one million myocardial infarctions ("heart attack") occur per year and roughly 40% of patients survive one year after suffering a heart attack.

Recent advances using stem cell-derived cardiomyocytes to repair the cardiac tissue injured during myocardial infarction have been encouraging. Transplantation of stem cell-derived cardiomyocytes have been reported to partially repair cardiac damage in rat, pig, and primate myocardial infarction models (Kawamura et al., 2012; Luo et al., 2014a; Luo et al., 2014b; Higuchi et al., 2015; Wendel et al., 2015; Shiba et al., 2016; Wang et al., 2016a). Therapeutic efficacy of this approach, however, has been limited by the fact that a majority of the exogenous cells delivered to the myocardium die in the first 24 h due to ischemic and oxidative stress in the transplant environment. The loss of these cells may further initiate immune and inflammatory responses in the injured heart. Thus, therapies that provide protection during cardiac oxidative stress post-transplantation are the focus of significant biomedical and clinical research.

Strategies to stimulate stem cell survival in the post-transplantation environment include cellular preconditioning by physical, genetic and pharmacological manipulation (Laflamme et al., 2007; Fischer et al., 2009; Haider and Ashraf, 2010; Mohsin et al., 2011). Ischemic conditioning, the application of one or more cycles of ischemia and reperfusion to the heart, is the most powerful method known for reducing cardiac damage following myocardial infarction (Hausenloy and Yellon, 2016); however, translation to human clinical efficacy is lacking. Other approaches including pretreatment of cardiac progenitor cells with a miRNA pro-survival cocktail (Hu et al., 2011) or genetic modulation of cells for overexpression of growth factors (Askari et al., 2003; Pasha et al., 2008; Haider and Ashraf, 2010) have shown improvement in donor cell survival, engraftment, differentiation and improvement in cardiac function. A drawback of genetic modifications, however, is the risk of inducing oncological disease (Anisimov et al., 2010). Pharmacological preconditioning represents a novel strategy to protect cells under post-engraftment ischemic stress that may be devoid of long term side effects (Afzal et al., 2010; Luo et al., 2014b).

We seek to identify a small molecule agent able to block oxidative stress in cardiomyocytes through a phenotypic chemical biology screening approach. A small molecule that prevents, inhibits or delays the death of cardiomyocytes by priming the cells before transplantation to increase the time window for cells to acclimate and engraft will provide better understanding of mechanisms of cardiomyocyte cell survival during oxidative stress that may lead to improved therapeutics for cardiac cell therapy approaches.

Human pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) provide new models for studying cardiac stem cell survival for drug discovery and safety pharmacology that can be extended to cell therapy approaches. Autologous transplantation of these cells is a particularly attractive approach to cardiac cell therapy and development of small molecules that protect hiPSC-CMs from the acute oxidative stress exposure following transplantation will significantly improve the efficacy of this procedure. In addition, hiPSC-CMs are an ideal screening platform for the study of cardioprotective mechanisms because they are commercially available as cryopreserved products and provide a consistently pure cell population that exhibits normal cardiomyocyte physiology and expression of specific cardiac myocyte biomarkers (Khan et al., 2013; Mordwinkin et al., 2013; Peters et al., 2015).

We executed a high throughput phenotypic screen of nearly 50,000 small molecules from the Sanford Burnham Prebys (SBP) compound collection using hiPSC-CMs targeting protection against hydrogen peroxide-induced cell death quantified by ATP content to identify small molecule protectors of cardiomyocyte oxidative stress. Hydrogen peroxide exposure of hiPSC-CMs models the oxidative stress of the post-transplantation environment. Cardioprotective activity of screening hits was further confirmed using an impedance-based measurement of cell monolayer integrity and contractile function. Our chemical biology approach successfully identified a novel chemical scaffold with low micromolar potency that protected hiPSC-CMs from peroxide-induced cell death. Subsequent structural activity and target identification studies by qRT-PCR indicate that the chemical hit series imparts protection to hiPSC-CMs against peroxide-induced cell death by upregulation of the hypoxia-responsive gene, heme-oxygenase-1 (HMOX1), a potent mediator of the antioxidant defense response.

Materials and Methods

Cell Preparation. Cryopreserved hiPSC-CMs (CMC-100-010-001) derived from a female donor were purchased from Cellular Dynamics International (CDI, Madison, WI). H9c2 and Hep3b cells were purchased from ATCC (CRL-1446 and CRL-2695, respectively). DMEM (Mediatech, Manassas UT) was obtained from Thermo Fisher (Waltham, MA). Fetal Bovine serum (FBS) was from Hyclone (Logan, UT). L-glutamine, Penicillin/Streptomycin solution, and Trypsin were from Invitrogen (Carlsbad, CA).

Human iPSC-CMs were thawed and maintained in CellBind T-25 vented tissue culture flasks (Corning, NY) in proprietary plating media (CDI) at 37°C and 5% CO_2 according to manufacturer's instructions. Thawing media was replaced with maintenance media after 48 h and was changed every 48 h for a minimum of 7 d before harvesting. On the day of the assay, hiPSC-CMs were harvested with 0.5% trypsin, washed with PBS, and suspended in assay media (DMEM without phenol red, 2% FBS, 10mM galactose, pH7.3) at 1.25 x 10^5 cells/ml.

Cellular Screening Assays. Control compounds (±)-Chloro-APB hydrobromide, SKF 83959 hydrobromide, R(-)-Propylnorapomorphine hydrochloride, and ciclopirox were purchased (Sigma-Aldrich, St. Louis, MO). 20 nl test compounds in DMSO (0.5% final concentration) were transferred to 1536-well white tissue culture treated HiBase assay plates (Aurora Biotechnologies, Carlsbad, CA) using acoustic dispensing by an Echo 555 (Labcyte, San Jose, CA). Cells were suspended in assay media containing 1 mM sodium pyruvate for HTS assays to maintain cell viability prior to peroxide treatment. Assay media without pyruvate was used for all other cell viability assays. Cells were added to compound plates in 4 µl suspension volume using a BioRAPTR (Beckman Coulter, Pasadena, CA) dispenser at a density of 500 cells per well. Plates were spun at 500 rpm and incubated with Kalypsys metal lids in 37°C and 5% CO_2

incubator for 2 h. Following pre-incubation, cells were treated with 1 µl 5X hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) diluted fresh in assay media without sodium pyruvate and incubated for an additional 30 min at 37°C and 5% CO_2. Assay plates were equilibrated to room temperature for 10 min followed by addition of 3 µl ATPLite 1-step luminescence reagent (Perkin Elmer, Waltham, MA). Plates were spun at 2000 rpm. After an additional 10 min at room temperature in the dark, plates were read for luminescent signal on an EnVision microplate reader (Perkin Elmer). Results were normalized to positive controls treated with 0.5% DMSO only and negative controls treated with DMSO and peroxide concentration resulting in 80-90% cell death (300 µM for HTS assay with pyruvate; 60 µM without pyruvate).

The effect of compounds on H9c2 cell viability in the presence of peroxide was screened under the same conditions as hiPSC-CMs except assay media without pyruvate contained 4.5 g/l glucose instead of galactose. Amplex Red hydrogen peroxide assay kit was purchased from Thermo Fisher and the assay was performed according to manufacturer instructions. 30 µM H_2O_2 prepared in assay media without pyruvate was determined to be in the linear range of detection. Test compounds were incubated for 30 min with 30 µM H_2O_2 in assay media followed by peroxide detection. Glutathione was titrated as a standard control for reactive oxygen species (ROS) scavenger activity and exhibited expected activity for elimination of H_2O_2 (EC_{50} = 0.13 mM).

Cellular Impedance. hiPSC-CMs were thawed in plating media (CDI) and $2x10^4$ cells/well plated directly onto 96-well E-Plate Cardio (ACEA Biosciences, San Diego, CA) pre-coated with 0.01 mg/mL fibronectin in PBS for 3h at 37°C. Cells were cultured for 14 d at 37ºC, 5% CO_2 and maintenance media (CDI) was changed every 2 d after plating and 24 h prior to dosing using a Viaflo 96 channel pipettor (INTEGRA Biosciences, Hudson, NH) placed in the tissue culture hood. Viability and contractility of cardiomyocytes were monitored by impedance using the xCELLingence RTCA (real-time cell analyzer) Cardio system (ACEA Biosciences, San Diego, CA). Impedance was measured for 60-s sweeps (recorded at a sampling rate of 12.9 ms) at selected time points and reported as cell index (CI). Prior to compound treatment, a baseline was recorded to ensure the cells established a regular beat rate of 40-60 beats/min. Test compounds were prepared at 10 mM stock concentration and serial diluted in 100% DMSO for concentration response, then further diluted in assay media in a separate 96-well plate at 6x target concentration. Compound plates were equilibrated to 37ºC prior to diluting 1:6 into the E-plate using the Viaflo 96 channel pipet to give a final medium volume of 150 µl/well in 0.1% DMSO. After pre-incubation with compounds for indicated time, hydrogen peroxide was prepared at 7x target concentration in assay media and 25 µl/well added using the Viaflo 96 channel pipet. Cell index values were normalized to the time point just prior to treatment with compounds and/or peroxide using the ACEA xCELLigence Cardio software platform. Contractile impedance traces were obtained by a high speed scan for 1 min at 12.9 msec intervals. EC_{50} values for concentration-response curves were calculated from two separate experiments performed in triplicate by nonlinear regression analysis (four parameters) and least squares fit using GraphPad Prism version 7.0 for Windows (GraphPad, La Jolla, CA, USA).

HIF reporter assays. HIF transcriptional response was examined in HIF1-specific MIAPaCa-2 and HIF2-specific PANC luciferase reporter cell lines as previously described (PubChem AID 651581). Briefly, cells were grown overnight in 1536-well white plates, treated with test compounds for 24 h and luciferase activity was measured using One-Glo reagent (Promega, Madison, WI) on an EnVision microplate reader (Perkin Elmer, Waltham, MA). Test compounds were compared with known HIF inducers, FG-4592 (Selleck Chemicals, Houston, TX) and BAY 85-3934 (BioVision, Milpitas, CA).

Iron Chelation. Chelation of Fe^{2+} was measured in solution by fluorescent assay using calcein (Esposito et al., 2002). Test compounds were mixed in 384-well black non-binding plates (Greiner, Kremünster, Austria) with 10 µl 1.2 µM ammonium iron(II) sulfate (Sigma-Aldrich) prepared fresh in deionized water and incubated for 10 min at room temperature. 10 µl 0.6 µM calcein (Sigma-Aldrich) prepared in 40 mM HEPES; 150 mM NaCl was added and incubated for 30 min at room temperature in the dark. Fluorescence was measured (ex = 485 nm; em = 535 nm) on an EnVision microplate reader (Perkin Elmer, Waltham, MA) and results were normalized to the mean of wells treated with DMSO vehicle with or without iron. Chelation activity of test compounds was compared with the known chelator, deferoxamine (Sigma-Aldrich).

qRT-PCR. Erythropoietin (EPO) expression was measured in Hep3b cells grown to confluence in 96-well plates and treated with test compounds for 5 h. Expression of heme oxygenase 1 (HMOX1), pyruvate dehydrogenase kinase 1 (PDK1), hexokinase 2 (HK2) and NAD(P)H quinone dehydrogenase (NQO1) was assessed in hiPSC-CMs grown for 7-10 d in fibronectin-coated 96-well plates and treated with test compounds for 3-24 h. Prior to compound treatment, media was changed to DMEM containing 2% FBS; 10 mM galactose and cells were equilibrated for 1 h. Cells were then treated with compounds or DMSO vehicle, incubated for indicated duration, washed twice with PBS, and RNA was isolated using RNeasy Mini kit (Qiagen, Germantown, MD). Bardoxolone methyl (CDDO-Me) and cobalt propotphyrin (CoPP) were obtained from Sigma-Aldrich. RNA was quantitated using a NanoDrop spectrophotometer and cDNA was produced using Qiagen's Reverse Transcriptase kit. Taqman primer-probe sets for human *HPRT1* (HS_02800695), *PDK1* (HS_00176853), *HK2* (HS_00606086), *HMOX1* (HS_01110250), *NQO1* (HS_01045993) and *EPO* (HS_01071097) were from Applied Biosystems (Foster City, CA). PCR reactions were performed in 384-well format on a Roche LightCycler 480 II using Taqman Gene Expression Master Mix (Applied Biosystems). Target gene expression was normalized to *HPRT1* and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). P values were determined by two-tailed student t-test.

Medicinal chemistry. CP-312 and its analogs were typically prepared via a four-step synthetic protocol as outlined below. All building blocks, reagents, and necessary solvents were purchased from Sigma-Aldrich. Nuclear Magnetic Resonance characterization data was recorded on Bruker BioSpin instrument operating at 500 MHz proton frequency.

General Procedure. CP-312 was prepared starting from 1-(pyridin-2-yl)ethan-1-one. Bromination at the methyl group was performed at 65°C for 1 hour via treatment with bromine in concentrated hydrobromic acid. The reaction was quenched with ice and the product, an α-bromo ketone, was isolated via lyophilization and used 'as is' for the next step. The α-bromo ketone was dissolved in ethanol and treated with thiourea at room temperature. After 1 hour of reaction time the solvent was evaporated and the resulting mass was dissolved in water, washed with dichloromethane, and basified to form the final product, 4-(pyridin-2-yl)thiazol-2-amine, as a precipitate (58% yield over two steps). Treatment of thus prepared amine in dimethylformamide (DMF) and pyridine with chloroacetyl chloride at 0°C produced the last intermediate in 63% yield. Substitution of the chloride with the corresponding 4-chlorothiophenol was achieved in DMF in the presence of sodium hydride. The final product, CP-312, was purified via high-performance liquid chromatography and isolated in 45% yield (16%

overall). ^{1}H NMR (500 MHz, Chloroform-*d*), δ: 10.31 (br s, 1H), 8.66 (s, 1H), 7.97 (d, *J* = 8.0 Hz, 1H), 7.89 (t, *J* = 7.7 Hz, 1H), 7.80 (s, 1H), 7.38 – 7.27 (m, 5H), 3.86 (s, 2H).

LCMS analysis was completed on a Waters Autopurification system, which consists of a 2767 sample manager, a 2545 binary gradient module, a system fluidics organizer, a 2489 UV/vis detector, and a 3100 mass detector, all controlled with MassLynx software. A Sunfire Analytical C18 5 µm column (4.6 × 50 mm) and stepwise gradient {10% [(MeCN + 0.1% TFA) in (water + 0.1% TFA)] to 98% [(MeCN + 0.1% TFA) in (water + 0.1% TFA)] for 9 min} was used for analytical LCMS of test samples. Any effect of H_2O_2 on the structural integrity and oxidation of CP-312 following exposure to a 100-fold excess of peroxide in the relevant cell culture medium was also evaluated. No chemical change in the structure of the compound after 40 min of incubation was observed.

Results

As a model of oxidative stress, we exposed hiPSC-CMs to hydrogen peroxide (H_2O_2) at a concentration that decreased cell viability by 80-90% in 30 min and tested compounds for their ability to protect cells from cell death under acute conditions as measured by the amount of remaining ATP present in cells pre-treated with compound as compared to untreated cells. Human iPSC-CMs are grown in media containing sodium pyruvate and galactose. Although pyruvate is a scavenger of peroxide, cells were maintained in media containing 1 mM sodium pyruvate for high throughput screening (HTS) to provide robust and stable cell viability readouts over several hours in the absence of peroxide. Secondary cell viability assays were performed in the absence of pyruvate. In this case, the concentration of peroxide was reduced from 300 µM to 60 µM peroxide to maintain an equivalent decrease in cell viability as determined by titration with peroxide in the presence or absence of pyruvate, respectively. The concentration-response curve for H_2O_2-induced cell death in the presence of 1 mM pyruvate is shown in figure 1A.

A pilot screen of the Library of Pharmacologically Active Compound (LOPAC) collection and US Food and Drug Administration (FDA) Drug Collection identified the following three dopamine receptor agonists: (±)-Chloro-APB hydrobromide, SKF 83959 hydrobromide, and R(-)-Propylnorapomorphine hydrochloride as well as ciclopirox, an antifungal agent from the FDA collection, as protective with EC_{50} values of 9.8, 5.7, 6.6, and 6.3 µM, respectively. These pharmaceutical agents are documented in the literature as cardioprotective and non-scavengers of H_2O_2 (Choi et al., 2002; Lee et al., 2005; Khaliulin et al., 2006; Gero et al., 2007). We screened an additional 48,640 small molecules from SBP’s internal collection in 1536-well format resulting in a Z’ for the screen of 0.63 ± 0.07 and a signal-to-background of 9.0 ± 3.5 when cells were exposed to 300 µM hydrogen peroxide compared to DMSO-treated cells (Fig 1B).

Employing ≥ 35% activity as cutoff criteria, we identified 220 hits (Fig 1C and D). These compounds were retested in triplicate under the same conditions as the HTS and 112 compounds were confirmed, representing a confirmation rate of 52%. The confirmation rate employing cutoff criteria of ≥ 50% was 74%; however, we chose a lower confirmation rate because even a 35% improvement in cardiomyocyte viability is significant.

We describe CP-312, representing a distinct chemical scaffold that emerged as a hit from our screen of the SBP internal library and was confirmed in concentration-response (Fig. 2). To determine whether CP-312 acted as a direct scavenger of reactive oxygen species, we measured the amount of peroxide remaining in media containing the compound without cells using a colorimetric assay and determined that the compound had no effect on peroxide level at any concentration tested (Fig. 2B). CP-312 exhibited good potency in protecting hiPSC-CMs from acute peroxide-induced cell death in our 1536-well suspension assay using an ATP endpoint readout with EC_{50} = 6.7 µM (pEC_{50} = -5.19 ± 0.05); protection was confirmed in the absence of pyruvate and presence of 60 µM peroxide with similar potency (EC_{50} = 4.6 µM; pEC_{50} = -5.3 ± 0.1) as compared in figure 2C. Cells co-treated with CP-312 and peroxide exhibited decreased potency (EC_{50} > 10 µM) and efficacy (E_{max} < 50%) relative to cells pretreated with CP-312 prior to peroxide exposure (Fig. 2C), suggesting that the protection we observe by compound pretreatment is dependent on a cellular adaptive response. To determine the extent of protection provided by CP-312, we titrated peroxide in the presence and absence of the compound. 2 h pretreatment of hiPSC-CMs with 10 µM CP-312 decreased the peroxide toxicity by an order of magnitude (Fig. 2D).

Cardioprotective activity of CP-312 in hiPSC-CMs was further confirmed using an adherent real-time label-free assay measured on the ACEA XCELLigence RTCA Cardio system. Average impedance measurements reported as cell index provide real-time, continuous monitoring of cell viability and monolayer integrity. In addition, microscale measurement of impedance changes with a 12.9 ms resolution provide detailed monitoring of cell morphology, allowing examination of cardiomyocyte contraction and relaxation. We monitored the change in cell index and contractility for hiPSC-CMs after 14 d in culture followed by peroxide exposure in the presence and absence of CP-312. Decreased cell index, reflective of cell death and disruption of the cardiomyocyte monolayer was observed over 1 h following treatment with increasing concentrations of peroxide (Fig. 3A); whereas normalized cell index was maintained over time in cells pretreated with 10 µM of CP-312 (Fig. 3B). Cells pretreated with varying CP-312 concentrations demonstrate a concentration response in the protection provided after 1 h exposure to 40 µM peroxide with EC_{50} = 1.6 µM ($pEC_{50} = -5.79 \pm 0.02$) (Fig. 3C).

Contractile function was maintained following peroxide exposure in hiPSC-CMs pretreated with CP-312 (Fig. 3D). Human iPSC-CMs exhibit consistent synchronized beating as shown by impedance traces of vehicle-treated control cells. Following exposure to 40 µM peroxide, cardiomyocyte contractility ceased after 1 h. Cells pretreated with CP-312, however, continued beating with no observed change in beat pattern at 1 h and contractile function was maintained for 24 h after peroxide exposure, although beat amplitude was diminished (Fig. 3D).

The 4-(pyridin-2-yl)thiazole class of compounds (CP-312 and congeners) was advanced as a potent hiPSC-CM cardioprotecting scaffold. More than sixty analogs of CP-312 were synthesized with a subset presented in **Table 1**. SAR analysis revealed the significance of the biaryl core and the connectivity between the pyridine and the thiazole rings. The substituent R_1 affected the protection of the scaffold favoring relatively electron-withdrawing group such as CF_3 (entry 1) versus electron donating groups (entry 6, OMe causes 5-fold decrease in activity).

The placement of R_1 on the phenyl ring was also found to be important with *para* position being the most favorable and *ortho* the least (data not shown). The heteroatom X in the linker X-Y was essential perhaps due to its role in positioning the right-hand side of the molecule precisely in the binding pocket of the target. When NH and SO_2 were introduced in place of S the activity and E_{max} diminished substantially (entries 7 and 8, respectively). The substituent Y, however, influenced the activity to a lesser extent (entries 9 and 10) suggesting that this moiety could further be explored and expanded in subsequent lead optimization efforts. An introduction of a substituent either on the thiazole (entries 11 and 12) or on the pyridine (entries 13 and 14) rings was expected to bring torsion in the thiazole-pyridine plane leading to a change in the overall conformation of the molecule and subsequent binding affinity. Small alkyl group on the thiazole (Me, entry 11) was well tolerated while a bulkier isopropyl (entry 12) was not favorable. Similarly, methyl substituent on the pyridine (entry 13) did not yield substantial change in cardioprotective properties. However, the electron-withdrawing ester moiety caused full obliteration of activity.

Simultaneously with structure-activity studies, we investigated potential target pathways of CP-312 and other hit scaffolds. A backup scaffold in our screening hit set shared structural similarity to a 8-hydroxyquinoline class of compounds that are known iron chelators and have been previously reported to inhibit iron-containing prolyl hydroxylase domain (PHD) enzymes that regulate hypoxia inducible factor (HIF) and its target genes (i.e. EPO, HK2, and PDK1) (Hong et al., 2014). Therapeutic inhibition of PHDs activates ischemic preconditioning pathways promoting multiple protective responses (Eckle et al., 2008; Ong et al., 2014; Martin-Puig et al., 2015; Vogler et al., 2015). We therefore investigated whether a potential mechanism of action for our screening hits to protect hiPSC-CMs from oxidative stress was via targeting the HIF-

PHD pathway. We examined the expression on HIF-regulated target genes, hexokinase 2 (HK2) and pyruvate dehydrogenase 1 (PDK1), in hiPSC-CMs by qRT-PCR and observed no difference following treatment with CP-312 for 3 h (Fig. 4A). We further examined the effect of CP-312 in HIF1-specific MIAPaCa-2 and HIF2-specific PANC luciferase reporter cell lines (Fig. 4B and C) and determined the effect of CP-312 on HIF-inducible expression of erythropoietin in Hep3b cells by qRT-PCR (Fig 4D). Our results revealed that our screening 'hits' including CP-312 and the 8-hydroxyquinolines (not shown) had no significant effect on HIF transcriptional activity as compared to known PHD inhibitors FG4592, FG2216, and BAY85-3934 (Fig. 4B-D).

We also tested CP-312 for the compound's ability to chelate Fe^{2+} as determined by calcein fluorescence. CP-312 showed significantly less potency and efficacy (EC_{50} = 1.27 µM; pEC_{50} = -5.9 ± 0.1, E_{max} = 60%) as compared to deferoxamine (EC_{50} = 0.24 µM; pEC_{50} = -6.62 ± 0.04, E_{max} = 97%), a potent chelator (Fig. 4D). Deferoxamine did not provide protection in viability in iPSC-CMs when tested under the same conditions as our primary assay, nor did FG4592 and BAY85-3934 (Fig. 4E). These results taken together indicate that the mechanism of action of our screening hit to protect hiPSC-CMs from oxidative stress is not specific to the HIF-PHD pathway and not due to chelation alone.

We continued to investigate the hypoxia-responsive antioxidant response network in cells by examining the nuclear factor erythroid 2 p45-related factor 2 (Nrf2) signaling pathway. Nrf2 acts as a sensor of oxidative stress and through its inhibitory binding protein kelch-like ECH-associated protein 1 (keap1) regulates the expression of antioxidant and detoxifying genes such as heme oxygenase-1 (HMOX1). We therefore examined gene expression of *HMOX1* following treatment with CP-312 to determine whether upregulation by CP-312 contributes to the protection of hiPSC-CMs from peroxide-induced cell death. We observed a robust induction of

HMOX1 expression, ranging from 80 to 600-fold over baseline, from RNA isolated from adherent hiPSC-CMs treated for 3 h with CP-312 (Fig. 5A and B). CP-131, a close analog of CP-312 that was inactive in providing protection following peroxide exposure (Table 1, Entry 14), did not have a significant effect on *HMOX1* expression (Fig. 5A).

Treatment of hiPSC-CMs with other known *HMOX1* inducers, bardoxolone methyl (CDDO-Me) or cobalt protoporphyrin (CoPP), increased *HMOX1* expression to a much lesser extent at 3 h than CP-312 (Fig. 5B) and did not provide protection to peroxide exposure (Fig. 5D). After 24 h, however, cells treated with CDDO-Me or CoPP exhibited greater *HMOX1* expression levels (Fig. 5C) and increased viability following peroxide exposure (Fig. 5D), while cells treated with CP-312 for 24 h maintained both elevated *HMOX1* expression levels and protection from peroxide exposure (Fig. 5C and D). Interestingly, the expression of NAD(P)H quinone dehydrogenase (NQO1), another gene regulated by Nrf2, was unaltered at 3 h by treatment with any of the cardioprotective agents (Fig. 5B) yet was significantly upregulated after 24 h treatment with either CDDO-Me or CoPP, but not CP-312 (Fig. 5C). Dose-response testing indicated that lower concentrations of CDDO-Me, CoPP or CP-312 were less effective inducers of *HMOX1* and *NQO1* gene expression and did not provide significant protection of hiPSC-CMs from peroxide exposure (Supplemental Fig. S1). Compound protection therefore appears to correlate with the induction of genes associated with the antioxidant response network including *HMOX1* yet the transcriptional response differs between CP-312 and the known Nrf2 activators, CDDO-Me and CoPP.

Discussion

Pharmacological manipulation of stem cells has shown improvement in cell survival post-transplantation and represents a viable treatment modality for ischemic disease. Preconditioning mesenchymal stem cells with diazoxide enhanced cell survival after transplantation *in vivo* via NFkB regulation (Afzal et al., 2010). In addition, human embryonic-derived cardiomyocytes pretreated with the heme oxygenase inducer, CoPP, and delivered to injured rat myocardium improved post-infarct ventricular function (Luo et al., 2014b).

The autologous transplantation of a patient's iPSC-CM provides a personalized approach to cardiac cell therapy without the immunosuppressive and oncological complications of donor stem cell based therapies (Anisimov et al., 2010). Thus, small molecules that can reversibly inhibit the death of iPSC-CMs and prime the cells for engraftment without the long-term side effect of carcinogenesis inherent in gene therapy approaches hold promise for improving clinical outcomes.

A phenotypic chemical biology screening approach in hiPSC-CMs is a powerful strategy to identify relevant molecular probes of novel pathways involved in cardiac myocyte survival mechanisms associated with oxidative stress. The goal of this project was to model oxidative damage in hiPSC-CMs for high throughput screening to identify small molecule cardioprotectors of oxidant-induced hiPSC-CM cell death. These probes will shed light on mechanisms of stem cell cardiomyocyte survival and may be used to enhance survival of iPSC-CMs during transplantation.

We report a robust 1536-well HTS screening format (Z' = 0.63) which employs a suspension assay that measures cell viability by ATP in hiPSC-CMs following 2 h pretreatment with compounds and exposure to a lethal concentration of H_2O_2. HTS of 48,640 compounds representative of the SBP internal library and confirmation of hits through concentration-response assays measuring cellular viability followed by monitoring real-time impedance morphology in adherent hiPSC-CMs identified CP-312 with EC_{50} = 4.6 and 1.6 μM, respectively. CP-312 also preserved cardiomyocyte contractility over 24 h.

We previously screened for inhibitors of cell death using a similar approach in H9c2 embryonic rat heart-derived myoblasts and reported lead compounds (Kane et al., 2010). These compounds, however, did not exhibit any protective activity when tested in hiPSC-CMs. Furthermore, CP-312 provided little protection to H9c2 cells indicating a critical difference between these cell lineages. Compared to H9c2 cell line, hiPSC-CMs have increased fatty acid oxidation capacity and reduced glycolytic capacity (CDI reported data). Furthermore, hiPSC-CMs were challenged in our assay with oxidative stress in media containing galactose to maximize the cells use of oxidative phosphorylation to generate ATP, while H9c2 cells were challenged in media containing high glucose as these cells do not maintain viability in media containing only galactose. *HMOX1* expression has previously been reported to be dependent on cellular metabolic state with expression significantly reduced in H9c2 cells treated under high glucose conditions (Li et al., 2015; Gao et al., 2016). Diabetic hyperglycemia has also been shown to block the cardioprotective effects of anesthetic preconditioning *in vitro* and *in vivo* by impairing the Nrf2 signaling response (Li et al., 2015; Canfield et al., 2016; Gao et al., 2016; Wang et al., 2016b). Differences in metabolism between hiPSC-CMs and H9c2 cells likely account for the observed differences in protection between the cell types for the hit compounds and demonstrates the relevance of the cell model and culture conditions for hit identification.

Having identified CP-312 as promoting iPSC-derived cardiomyocyte cell survival under oxidative stress, we probed for potential target identification focusing on the hypoxia-responsive signaling pathways. Activation of the HIF-1 pathway has been associated with cardioprotection following ischemic preconditioning; however, the mechanism by which HIF pathway induction imparts cardioprotection remains poorly understood. PHD2 and PHD3 isoforms are reported to be highly regulated by hypoxia in cardiomyocytes and pharmacological inhibition has been shown to provide protection from ischemic injury (Cioffi et al., 2003; Vogler et al., 2015; Xie et al., 2015). We did not observe any change in HIF-regulated expression of *HK2* and *PDK1* in iPSC-CM treated with CP-312. Our results comparing the response to CP-312 with known PHD inhibitors using HIF-1 and HIF-2 reporter assays in MIAPaCa-2 and PANC-1 cells as well as erythropoietin expression in Hep3b cells also showed that CP-312 is not directly targeting PHD enzyme inhibition or activation of HIF. However, HIF activation may act as an upstream signal triggering paracrine factors that elicit protective responses in the cardiac tissue. This prompted us to investigate downstream target effectors particularly the Nrf2/HMOX1 axis as part of HIF protective anti-oxidant response network.

Induction of heme oxygenase-1 is a critical component of the protective antioxidant response network and recent advances have shown that pharmacologic induction of *HMOX1* following 24 h treatment of stem cell cardiomyocytes with CoPP prior to transplantation significantly improved myocyte graft survival and post-infarct ventricular function (Luo et al., 2014a; Luo et al., 2014b). Increased expression of *HMOX1* by cardiac-specific transgenic overexpression, gene therapy or pharmacological approaches have also proven to significantly mitigate myocardial damage following an ischemic event (Yet et al., 2001; Melo et al., 2002; Vulapalli et al., 2002; Liu et al., 2007; Li et al., 2011). Our gene expression analysis in hiPSC-CMs showed > 100-fold increase in *HMOX1* expression compared to baseline after 3 h treatment with CP-312 that was maintained at elevated levels for 24 h. An inactive analog of CP-312, CP-131, did not induce

HMOX1 expression suggesting a correlation of *HMOX1* expression with protection from peroxide-induced cell death.

The observed increase in *HMOX1* expression induced by CP-312 was greater and more rapid as compared to CDDO-Me or CoPP, which induce *HMOX1* by activating Nrf2 pathway via inhibition of keap1 interaction with Nrf2. In addition to *HMOX1*, *NQO1* is a prototypical target gene of Nrf2 (Kumar et al., 2011; Kim et al., 2017). Differences in the dynamics of the *HMOX1* response and the lack of *NQO1* upregulation in hiPSC-CMs treated with CP-312 lead us to speculate that CP-312 may be regulating *HMOX1* expression in a Nrf2-independent manner. Previous studies in Nrf2 null mice, Nrf2-deficient MEF cells, and HEK293 cells treated with Nrf2-specific shRNA demonstrate *HMOX1* induction by Nrf2-independent mechanisms that are uncoupled from regulation of other antioxidant response genes, including *NQO1* (Wright et al., 2009; Kang et al., 2014). Further studies utilizing CP-312 will elucidate the underlying mechanisms regulating *HMOX1* expression.

Our results strongly suggest that CP-312 is a novel scaffold that protects hiPSC-CM viability and function by targeting the anti-oxidant response network through induction of *HMOX1* expression. Following the *in vivo* approach of injecting hiPSC-CMs into the peri-infarct myocardium of rats with acute myocardium infarction, it is our hypothesis that pretreatment of hiPSC-CMs with CP-312 or improved derivatives prior to transplantation will enhance cell survival, attenuate infarct size and improve cardiac function compared to hiPSC-CMs injected alone, similar to previous studies using pharmacological preconditioning strategies (Afzal et al., 2010; Luo et al., 2014b). Furthermore, combining CP-312 with CoPP or other compounds that affect complementary pathways may provide the optimal kinetics of stem cell protection for successful engraftment. Advanced imaging technologies may be applied to track the pharmacologically modified cells *in vivo* and correlate stem cell survival kinetics with functional cardiac changes in the animal to aid drug development (Nguyen et al., 2011).

Initial SAR studies around CP-312 resulted in CP-724 with improved primary activity (entry 2, **Table 1**). The pyridyl-thiazole moiety and the connectivity between these two aryls appeared to be imperative for the bioactivity. Substitution on the ring system favored small alkyl moieties while bulkier alkyl and electron-withdrawing groups hindered activity. The linker tolerated substituents of different sizes, however, sulfur(II) was preferred over other heteroatoms or oxidation level. Ultimately, the *para* substitution of the phenyl ring on the right-hand side of the scaffold was found to be capable of fine attenuation of the cardioprotective properties of the molecule. It can be postulated that similarly to CP-312, CP-724 and in addition, CP-315, CP-127, and CP-723 (entries 4, 10, 11, **Table 1**), which have improved protective ability, will induce *HMOX1* expression to an equal or greater extent. Further SAR studies will be governed by the *HMOX1* expression level and will explore areas of the molecules marked with R_{1-3}, X, Y (Table 1). Overall, the SAR studies successfully recognized key areas in the scaffold for further lead optimization of the chemical series surrounding CP-312 that may result in agents with increased potency and efficacy able to block oxidative stress in stem cell cardiomyocytes.

The post-transplantation environment is complex and cells death occurs by multiple, interconnected pathways. The advantage of our phenotypic approach is the identification of compounds with activities specifically targeting hiPSC-CM survival pathways. Our goal is to protect stem cell viability until the delivered cells have successfully engrafted. CP-312 appears to protect hiPSC-CMs through induction of *HMOX1* by a mechanism distinct from other known *HMOX1* inducers, providing a novel tool compound to better understand the regulation of antioxidant response pathways and a chemical series for development of targeted

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pharmacological agents that may lead to improvement in stem cell survival for cardiac cell therapies.

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Author Contributions

Participated in research design: Kirby, Divlianska, Koo, Maloney, Peddibhotla, Sessions, Hershberger, Malany

Conducted experiments: Kirby, Divlianska, Maloney, Peddibhotla, Sessions, Hershberger, Bryan, Whig, Morfa, Koo

Contributed new reagents or analytic tools: Kirby, Divlianska, Maloney, Peddibhotla, Sessions, Hershberger

Performed data analysis: Kirby, Divlianska, Bryan, Whig, Morfa, Koo

Wrote or contributed to the writing of the manuscript: Kirby, Divlianska, Malany, Smith

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Footnotes:

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Figure Legends

Fig. 1. 1536-well HTS identified hits protective of peroxide-induced hiPSC-CMs cell death. (A) Cell viability of hiPSC-CMs determined by ATP content following 30 min exposure to increasing concentrations of hydrogen peroxide. Data are mean ± S.D. (*n* = 3). (B) HTS assay conditions provided a robust response window between positive (0.5% DMSO) and negative controls (0.5% DMSO, 300 µM peroxide) displayed per screening test plate. (C) Cutoff criteria for HTS was set at ≥ 35% response (dashed line). (D) 220 hits were identified based on the cutoff criteria defined in C.

Fig. 2. CP-312 provides dose-dependent protection of hiPSC-CM viability in the presence of peroxide. (A) Chemical structure of CP-312. (B) Peroxide concentration remaining in hiPSC assay media determined by colorimetric assay following incubation of 30 µM peroxide with CP-312 for 30 min. (C) Concentration response of protection provided by CP-312 against peroxide-induced cell death. Cells were either pretreated with compounds for 2 h in assay media containing 1 mM pyruvate equivalent to HTS conditions followed by addition of 300 µM peroxide (open circles), pretreated in media without pyruvate followed by addition of 60 µM peroxide (closed circles), or co-treated with CP-312 at the time of peroxide addition (closed squares). Cell viability was determined by ATPLite luminescence 30 min following peroxide exposure. (D) Cell viability of hiPSC-CMs following 2 h pretreatment with 10 µM CP-312 or 0.1% DMSO in media without pyruvate and 30 min exposure to varying concentrations of peroxide. Greater than 50% viability was maintained in hiPSC-CMs pretreated with SBI-312 at peroxide concentrations up to 240 µM. Data are mean ± S.D. (*n* = 3).

Fig. 3. hiPSC-CM cell integrity and contractility measured by label-free cell impedance is maintained following 2 h pretreatment with 10 µM CP-312 and 1 h exposure to H_2O_2.

Normalized average cell index values for hiPSC-CMs cell integrity exposed to various peroxide concentrations in (A) the absence or (B) the presence of 10 µM CP-312. (C) Concentration response curve for protection of hiPSC-CMs imparted by 2 h pretreatment with 10 µM CP-312 followed by 1 h exposure to 40 µM H_2O_2. Data are mean ± S.D. (*n* = 3). (D) hiPSC-CMs contractility is maintained in the presence of 10 µM CP-312 (312 + H_2O_2) as compared to DMSO (control) and peroxide (H_2O_2) treated cells.

Fig. 4. Effect of CP-312 on HIF-induced transcriptional response. (A) mRNA expression of hexokinase 2 (HK2) and pyruvate dehydrogenase kinase 1 (PDK1) in hiPSC-CMs following 3 h treatment with 0.1% DMSO vehicle control or 10 µM CP-312. (B,C) Luciferase reporter activity following treatment with CP-312 or known HIF inducers, FG-4592 and BAY 85-3934, in (B) HIF-1 MIAPaCa-2 and (C) HIF-2-specific PANC-1 luciferase reporter cell lines. (D) Erythropoietin (EPO) mRNA expression in Hep3b cells after 5 h exposure to 20 µM FG-2216, BAY 85-3934, or CP-312. Data are analyzed by two-tailed t-test. * $p < 0.01$ compared with DMSO control. (E) Iron chelation activity tested by fluorescent assay and compared with known chelator, deferoxamine. (F) FG-4592, BAY 85-3934 and deferoxamine were tested alongside CP-312 for protection of hiPSC-CMs from H_2O_2-induced cell death after compound pretreatment for 2 h followed by 30 min exposure to 60 µM peroxide in assay media without pyruvate. All data are mean ± S.D. (*n* = 3).

Fig. 5. mRNA expression of (A-C) heme oxygenase-1 (HMOX1) and (B,C) NAD(P)H quinone dehydrogenase (NQO1) in hiPSC-CMs following (A,B) 3 h or (C) 24 h treatment with 0.1% DMSO vehicle control, 10 µM CP-312, 10 µM CP-131, 1 µM CDDO-Me, or 20 µM CoPP. Relative gene expression was determined by qRT-PCR using TaqMan primer-probe sets

normalized to expression of HPRT1 and illustrated as fold-change relative to DMSO vehicle control. Data are mean ± S.E.M. from two independent experiments (n = 4-5) analyzed by two-tailed t-test.* $p < 0.05$; ** $p < 0.005$ compared with DMSO vehicle control. (D) Normalized cell index values measured by cell impedance for hiPSC-CMs pretreated for 3 h or 24 h with 0.1% DMSO vehicle control,10 µM CP-312, 1 µM CDDO-Me, or 20 µM CoPP followed by 1 h exposure to 40 µM H_2O_2. Data are the mean ± S.E.M. from two independent experiments (n = 6-11) analyzed by two-tailed t-test. ** $p < 0.005$; *** $p < 0.0001$ compared with DMSO vehicle control.

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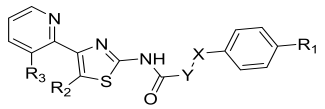
Table 1

Structure-activity relationship of CP-312



Entry	CP-	R_1	R_2	R_3	X	Y	EC_{50} (μM)	E_{max}, %
1	312	Cl	H	H	S	CH_2	4.60	82
2	724	CF_3	H	H	S	CH_2	1.67	79
3	317	F	H	H	S	CH_2	4.70	80
4	315	H	H	H	S	CH_2	3.53	90
5	311	Me	H	H	S	CH_2	4.23	78
6	796	OMe	H	H	S	CH_2	7.40	118
7	139	Cl	H	H	NH	CH_2	11.84	63
8	465	H	H	H	SO_2	CH_2	13.89	45
9	124	Cl	H	H	S	CHMe	4.79	92
10	127	Cl	H	H	S	CHPh	3.67	63
11	723	F	Me	H	S	CH_2	2.85	82
12	133	F	$CHMe_2$	H	S	CH_2	10.68	82
13	129	CF_3	H	Me	S	CH_2	6.34	79
14	131	Cl	H	CO_2Et	S	CH_2	>100	n/a

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Figure 1

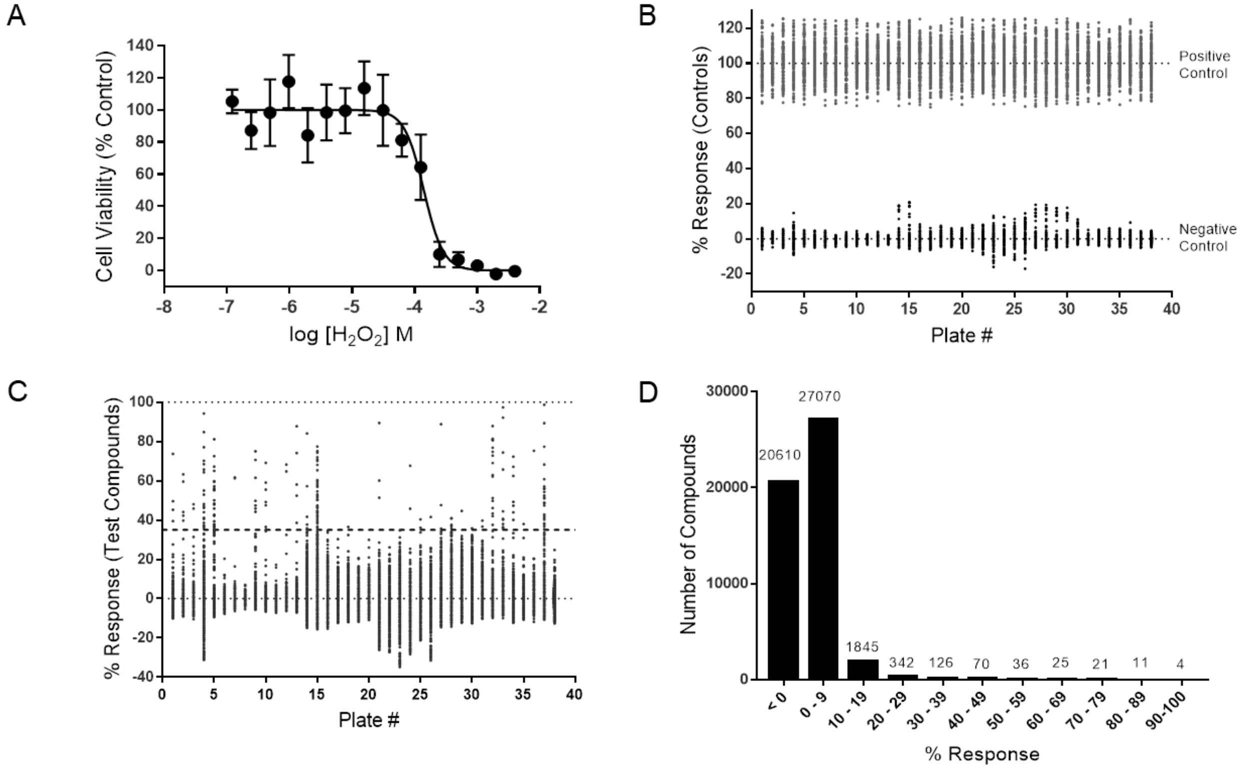

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Figure 2

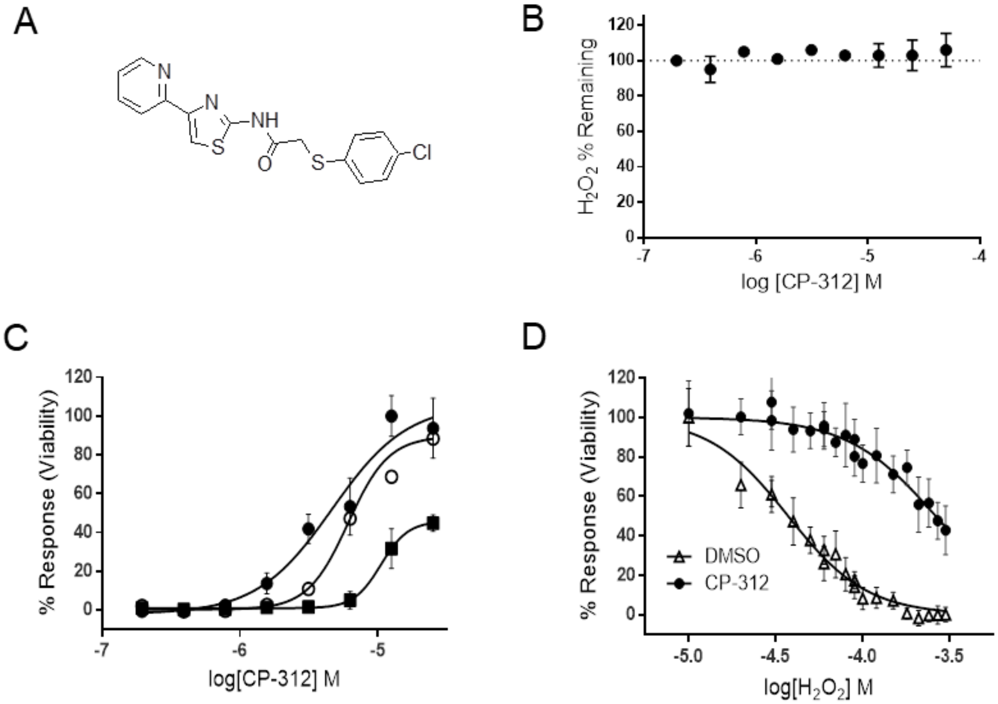

Figure 3

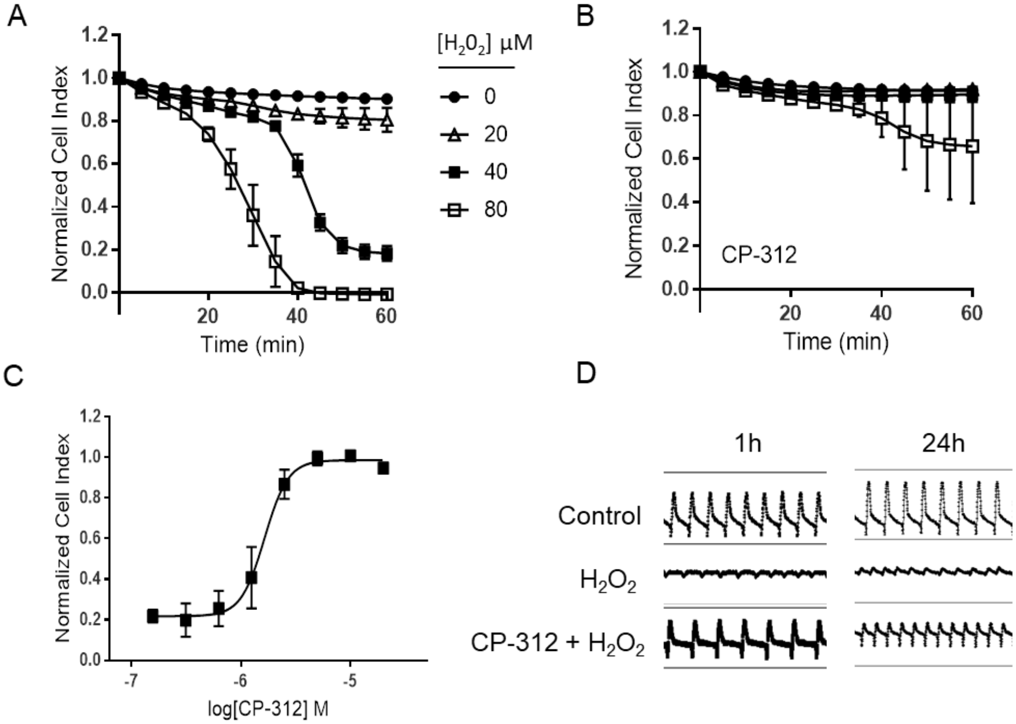
A
Normalized Cell Index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
20
40
60
Time (min)
[H$_2$0$_2$] µM
0
20
40
80
B
Normalized Cell Index
CP-312
Time (min)
C
Normalized Cell Index
-7
-6
-5
log[CP-312] M
D
1h
24h
Control
H$_2$O$_2$
CP-312 + H$_2$O$_2$

Figure 4

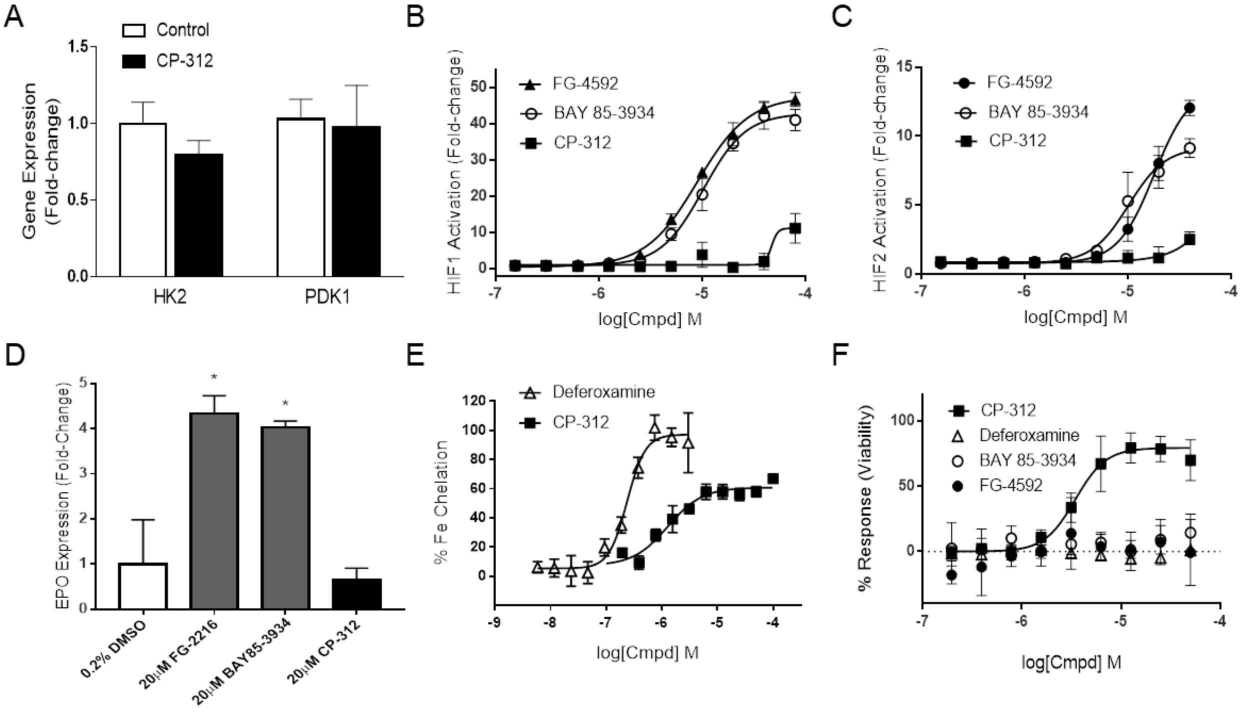
A
Control
CP-312
Gene Expression (Fold-change)
1.5
1.0
0.5
0.0
HK2
PDK1
B
HIF1 Activation (Fold-change)
FG-4592
BAY 85-3934
CP-312
50
40
30
20
10
0
-7
-6
-5
-4
log[Cmpd] M
C
HIF2 Activation (Fold-change)
FG-4592
BAY 85-3934
CP-312
15
10
5
0
-7
-6
-5
-4
log[Cmpd] M
D
EPO Expression (Fold-Change)
5
4
3
2
1
0
*
*
0.2% DMSO
20μM FG-2216
20μM BAY85-3934
20μM CP-312
E
% Fe Chelation
Deferoxamine
CP-312
120
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
log[Cmpd] M
F
% Response (Viability)
CP-312
Deferoxamine
BAY 85-3934
FG-4592
100
50
0
-7
-6
-5
-4
log[Cmpd] M

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Figure 5