Journal of Molecular and Cellular Cardiology 97 (2016) 106–113

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Discovery of novel small molecule inhibitors of cardiac hypertrophy using high throughput, high content imaging

Brian G. Reid [a], Matthew S. Stratton [b,c], Samantha Bowers [a], Maria A. Cavasin [b,c], Kimberley M. Demos-Davies [b], Isidro Susano [a], Timothy A. McKinsey [b,c,*]

[a] Department of Pharmaceutical Sciences, University of Colorado Skaggs School of Pharmacy and Pharmaceutical Sciences, Anschutz Medical Campus, Aurora, CO, United States
[b] Department of Medicine, Division of Cardiology, University of Colorado, Anschutz Medical Campus, United States
[c] Department of Medicine, Consortium for Fibrosis Research and Translation, University of Colorado, Anschutz Medical Campus, United States

ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 15 April 2016
Accepted 25 April 2016
Available online 27 April 2016

Keywords:
Cardiomyocyte
Hypertrophy
Small molecule
High throughput
High content
Screening

ABSTRACT

Chronic cardiac hypertrophy is maladaptive and contributes to the pathogenesis of heart failure. The objective of this study was to identify small molecule inhibitors of pathological cardiomyocyte hypertrophy. High content screening was performed with primary neonatal rat ventricular myocytes (NRVMs) cultured on 96-well plates and treated with a library of 3241 distinct small molecules. Non-toxic hit compounds that blocked hypertrophy in response to phenylephrine (PE) and phorbol myristate acetate (PMA) were identified based on their ability to reduce cell size and inhibit expression of atrial natriuretic factor (ANF), which is a biomarker of pathological cardiac hypertrophy. Many of the hit compounds are existing drugs that have not previously been evaluated for benefit in the setting of cardiovascular disease. One such compound, the anti-malarial drug artesunate, blocked left ventricular hypertrophy (LVH) and improved cardiac function in adult mice subjected to transverse aortic constriction (TAC). These findings demonstrate that phenotypic screening with primary cardiomyocytes can be used to discover anti-hypertrophic lead compounds for heart failure drug discovery. Using annotated libraries of compounds with known selectivity profiles, this screening methodology also facilitates chemical biological dissection of signaling networks that control pathological growth of the heart.

1. Introduction

The power of small molecule high throughput screening (HTS) in finding leads for drug discovery is well established [1]. HTS has historically been performed in industry and has largely focused on identifying modulators of distinct biochemical targets using simple in vitro assays or engineered reporter cell lines [2]. The standard approach has been to screen for modulators of a single target. Recently, phenotype-based screening has emerged as a more information-rich alternative to traditional target-based screening [3]. Phenotypic screening attempts to incorporate as much relevant biological information as possible and eliminate toxic hits, or hits with undesirable mechanisms-of-action, at an early stage of the discovery process. As such, phenotype-based screens have the potential to significantly lower the otherwise high attrition rates of lead compounds in the path of optimization and development into new drugs.

Heart failure affects 6 million people in the US alone, with 500,000 new diagnoses annually and a 5-year mortality rate of 42%, exceeding that of many cancers [4]. Cardiac hypertrophy is a hallmark of heart failure. Long-term suppression of cardiac hypertrophy is associated with reduced morbidity and mortality, and thus there is intense interest in developing novel therapeutics to target this growth response [5–7]. Current treatment of heart failure involves the use of drugs that inhibit signaling pathways triggered by cell surface receptors, such as the angiotensin receptor and the β-adrenergic receptor [8]. However, given the multitude of redundant signaling pathways capable of promoting pathological cardiac hypertrophy, it is hypothesized that increased efficacy will be obtained with therapies that target distal nodal points in the hypertrophic response. Phenotypic screening methods provide a unique opportunity to identify and target such nodal points [9].

Here, we describe results of an HTS campaign designed to discover small molecule inhibitors of pathological cardiomyocyte hypertrophy. This phenotypic screen employed primary neonatal rat cardiomyocytes and a high content screening platform, which enabled simultaneous image-based quantification of effects of compounds on cardiomyocyte cell area, biomarker expression, and viability. The results illustrate the power of phenotypic screening as a means to identify leads for heart

* Corresponding author at: Department of Medicine, Division of Cardiology, University of Colorado, Anschutz Medical Campus, United States.
E-mail address: timothy.mckinsey@ucdenver.edu (T.A. McKinsey).

http://dx.doi.org/10.1016/j.yjmcc.2016.04.015
0022-2828/

failure drug discovery, and to yield compounds that can be employed as chemical biological probes to uncover nodal effectors of pathological cardiac hypertrophy.

2. Materials and methods

2.1. Chemical libraries

The NIH Clinical Collections I and II (446 and 281 compounds, respectively) were obtained from Evotec US (South San Francisco, CA). The Spectrum Collection (2320 compounds) was purchased from Microsource (Gaylordsville, CT). The Kinase Inhibitor Library (194 compounds) was obtained from SelleckChem (Houston, TX). All compounds were supplied as 10 mM stock solutions in DMSO.

2.2. Cardiomyocyte cell culture and compound treatment

Neonatal rat ventricular myocytes (NRVMs) were isolated from the hearts of 1–3 day-old Sprague Dawley rats (Charles River), as previously described [10]. Cell counting and viability was assayed using a Vi-Cell Cell Viability Analyzer (Beckman Coulter). Cells were dispensed using a 5 μL cassette in a MultiFlo dispenser (BioTek) onto 96-well clear-bottom plates (Greiner) coated with 0.2% gelatin (Sigma; G9391) in DMEM with 10% calf serum, 2 mM L-glutamine, and penicillin-streptomycin; each well received 10,000 cells in 100 μL of medium. The following morning, cells were washed with serum-free medium and maintained in DMEM supplemented with L-glutamine, penicillin-streptomycin and Neutridoma-SP (0.1%; Roche Applied Science). Cells were treated with PE (10 μM) or PMA (50 nM) for 48 h prior to fixation. Immediately after addition of hypertrophic agonist, compounds and controls were added to plates. A single column of negative control (DMSO, 8 wells) and a single column of positive control (Trichostatin-A (TSA), 200 nM, 8 wells) were included on all plates. Using a 96-tip MDT head on a Janus automated liquid handler (Perkin Elmer), 0.5 μL of each compound was delivered to each well at a final concentration of 10 μM (1 μM for the Kinase Inhibitor Library), yielding a residual DMSO concentration of 0.5%.

2.3. Cardiomyocyte staining and imaging

All plate washing steps were performed using the ELx 405 plate washer (BioTek). All dispense steps were done using a 5 μL cassette on the MultiFlo (BioTek). Cells were washed with Hank's Balanced Salt Solution (HBSS, Gibco) and fixed for 20 min with paraformaldehyde (3.2%, Electron Microscopy Sciences) in HBSS. Fixed cells were washed three times with HBSS and permeabilized with 0.1% IGEPAL with 3% BSA in HBSS for 20 min. Primary antibodies were added as a cocktail (1:1000 anti-ANF, Phoenix Pharmaceuticals, H-005-24; 1:750 *anti*-α-actinin, Sigma A-7811) in permeabilization buffer and incubated for 1–2 h at room temperature. Cells were washed with HBSS and incubated with a cocktail of secondary antibodies (donkey anti-mouse FITC, Jackson ImmunoResearch; goat anti-rabbit Cy3, Jackson ImmunoResearch) in permeabilization buffer and incubated for 1 h in the dark. Cells were washed and incubated with Hoechst 33342, 10 μM, in HBSS, for 10 min. Finally, plates were washed with HBSS and stored on sealed plates in HBSS until imaged. All imaging was performed on an Operetta (Perkin Elmer). Three fluorescence channels (Hoechst, FITC, Cy3) were measured for each field using a 20× objective. Twenty fields were typically imaged in each well. Image collection was focused on the perimeter of each round well. Imaged cell counts ranged from 0 cells for toxic compounds to 1200 cells, typically, for nontoxic compounds. The NIH Clinical Collection #1 was run in duplicate as a step in the assay validation; all subsequent compound libraries were run as n = 1.

2.4. High content analysis

A multistep algorithm was developed to quantitatively analyze myocyte morphology and biomarker expression using Harmony software (Perkin Elmer). Images were first segmented based on the Hoechst fluorescence. Cytoplasm was mapped around each nucleus based on the FITC channel (*anti*-α-actinin). A perinuclear mask was created for each cell that went from the outer edge of each nucleus to a nuclear radius out from the edge of the nucleus, forming a roughly donut-shaped mask. All partial objects (nuclei and/or cytoplasm) were filtered out of the final analysis. Quantitative parameters that were used for hit selection were the total cell area in μm^2 (mean average value per well, α-actinin), and the sum of Cy3 fluorescence under the perinuclear masks (mean sum value per well, ANF).

2.5. Determination of hit compounds

All well-based data from the high content analysis were exported as text files and imported into an HTS database for secondary analysis, quality assurance, and hit selection (BioAssay HTS, CambridgeSoft). Statistical analysis was performed on a plate-by-plate basis. Z-primes were determined based upon positive and negative controls in plate columns one and twelve, respectively. Negative controls were vehicle control (0.5% DMSO) and positive controls were TSA (200 nM with 0.5% DMSO).

2.6. Experimental animals

Animal experiments were done in accordance with the Institutional Animal Care and Use Committee at the University of Colorado Denver. Ten week-old male C57BL/6 mice (Jackson Laboratories) were used for transverse aortic constriction (TAC). TAC and sham surgeries were performed as previously described, using a 27-gauge needle to guide suture constriction [11]. Artesunate (Sigma) was delivered daily via intraperitoneal injection at a concentration of 50 mg/kg in a 1:4 DMSO:10% (2-hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich, 389145) vehicle, beginning one day post-TAC surgery. Control animals received vehicle alone. Treatment groups were weight-matched prior to the start of the study.

2.7. Hemodynamic analysis

Echocardiographic analyses were performed the day the animals were euthanized using a Vevo770 System equipped with a 30 mHz frequency mechanical transducer (VisualSonics). Hearts were imaged in the two-dimensional parasternal short axis. M-mode images were recorded to measure LV wall dimensions and internal diameter at the level of the papillary muscles. For analyses, animals were anesthetized using 2% isoflurane and their body temperature was maintained at 37 °C. For data from all in vivo studies, GraphPad Prism software was used to generate graphs and analyze data. One-way ANOVA with Newman-Keuls post hoc test ($P < 0.05$) was used to determine statistical differences between groups.

2.8. Determination of ventricular myocyte cross-sectional area

Myocyte cross-sectional area was quantified using latitudinal mid-sections of the LV treated with neuraminidase type V (Sigma), and stained with fluorescein-labeled peanut agglutinin (10 mg/mL; Vector Laboratories). Images were captured with an AxioVert 200 inverted microscope using an AxioCam MRc digital camera, and analyzed with AxioVision Release 4.8.1 imaging software (Zeiss, Germany). Approximately 100–125 myocytes were analyzed and averaged for each animal. Analysis focused on the epicardium and endocardium, where the best cross sections of myocytes were present. Data was analyzed using GraphPad Prism software, with a one-way ANOVA with Newman-