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Suppression of HDAC nuclear export and cardiomyocyte hypertrophy by novel irreversible inhibitors of CRM1

Lauren Monovich [a,*], Keith A. Koch [b], Robin Burgis [a], Ekundayo Osimboni [a], Thierry Mann [a], Daniel Wall [a], Jinhai Gao [a], Yan Feng [a], Richard B. Vega [a], Benjamin A. Turner [b], David B. Hood [b], Andy Law [b], Philip J. Papst [b], David Koditek [b], Joseph A. Chapo [b], Brian G. Reid [b], Lawrence S. Melvin [b], Nikos C. Pagratis [b], Timothy A. McKinsey [b]

[a] Novartis Institutes for Biomedical Research, USA
[b] Gilead Colorado, Inc., USA

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ABSTRACT

Histone deacetylase 5 (HDAC5) represses expression of nuclear genes that promote cardiac hypertrophy. Agonism of a variety of G protein coupled receptors (GPCRs) triggers phosphorylation-dependent nuclear export of HDAC5 via the CRM1 nuclear export receptor, resulting in derepression of pro-hypertrophic genes. A cell-based high-throughput screen of a commercial compound collection was employed to identify compounds with the ability to preserve the nuclear fraction of GFP-HDAC5 in primary cardiomyocytes exposed to GPCR agonists. A hit compound potently inhibited agonist-induced GFP-HDAC5 nuclear export in cultured neonatal rat ventricular myocytes (NRVMs). A small set of related compounds was designed and synthesized to evaluate structure-activity relationship (SAR). The results demonstrated that inhibition of HDAC5 nuclear export was a result of compounds irreversibly reacting with a key cysteine residue in CRM1 that is required for its function. CRM1 inhibition by the compounds also resulted in potent suppression of cardiomyocyte hypertrophy. These studies define a novel class of anti-hypertrophic compounds that function through irreversible inhibition of CRM1-dependent nuclear export.

1. Introduction

A common outcome of stress in the heart is cardiomyocyte hypertrophy, a growth response during which individual myocytes increase in size without dividing, assemble additional contractile units, called sarcomeres, to maximize force generation, and reactivate a "fetal" program of gene expression [1]. Prolonged hypertrophy in response to pathological signals is associated with an increase in morbidity and mortality due to heart failure [2].

Emerging evidence suggests roles for histone deacetylases (HDACs) in the control of cardiomyocyte hypertrophy [3]. There are multiple mammalian HDACs that fall into four classes on the basis of similarity to yeast transcriptional repressors [4,5]. Class I HDACs (1, 2, 3, 8) are related to yeast RPD3, class II HDACs (4, 5, 6, 7, 9 and 10) to yeast HDA1, and class III HDACs (SirT1–7) to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDACs 4, 5, 7 and 9) and IIb (HDACs 6 and 10). HDAC11 is the sole member of class IV.

Class IIa HDACs possess highly conserved amino-terminal extensions of about 500 amino acids and serve as endogenous repressors of cardiac hypertrophy [6,7]. Class IIa HDACs are recruited to regulatory elements in pro-hypertrophic genes through sequence-specific DNA binding transcription factors, including myocyte enhancer factor-2 (MEF2). Ectopic overexpression of class IIa HDACs 4, 5 or 9 in cultured neonatal rat ventricular myocytes (NRVMs) coordinately suppresses MEF2-dependent transcription and agonist-dependent cardiac hypertrophy [6–8]. In contrast, mouse knockouts for HDAC5 or HDAC9 develop exaggerated cardiac hypertrophy in response to pressure overload [6,7].

Induction of genes that contribute to pathological cardiac remodeling is dependent on neutralization of the repressive functions of class IIa HDACs [9]. Derepression of class IIa HDAC target genes is accomplished, in part, through nucleo-cytoplasmic shuttling of these transcriptional repressors. The amino-terminal extensions of class IIa HDACs harbor two conserved serine residues that are hypophosphorylated in unstimulated cardiac myocytes. In response to agonists that activate G protein coupled receptors (GPCRs), these serines are phosphorylated, which triggers nucleo-cytoplasmic shuttling of HDACs via the CRM1 nuclear export receptor [10–12]. Inhibition of class IIa HDAC nuclear export through substitution of the phosphoacceptor sites with non-phosphorylatable alanine residues results in suppression of cardiac hypertrophy [6–8,13]. A kinase-independent mechanism for regulation of class IIa HDAC nuclear export in cardiomyocytes was also recently described [14]. Based on the collective results, there has been interest in identifying small molecules that control class IIa HDAC shuttling in cardiomyocytes,

* Corresponding author. Tel.: +1 617 871 7606; fax: +1 617 871 7045.
E-mail address: lauren.monovich@novartis.com (L. Monovich).

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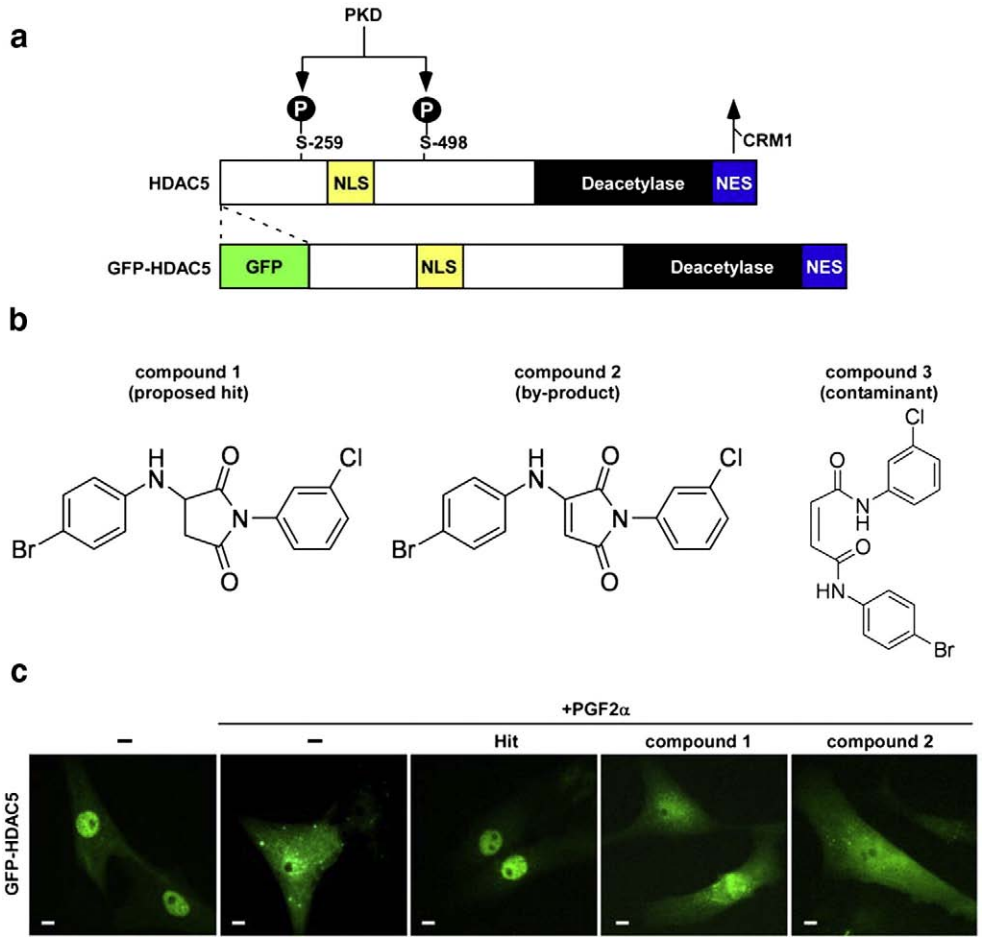

Fig. 1. Identification of small molecule inhibitors of HDAC5 nuclear export. (a) HDAC5 domain structure. Screening was performed with an amino-terminal GFP-tagged version of HDAC5. HDAC5 contains a nuclear localization signal (NLS) that is flanked by serines that are phosphorylated by protein kinase D (PKD). Upon phosphorylation, these sites are bound by the intracellular chaperone protein 14-3-3, resulting in activation of a cryptic nuclear export sequence (NES). HDAC5 also contains a conserved deacetylase domain. (b) Proposed structure of the hit molecule from the vendor. Compound 1 was re-synthesized based on this structure. Compound 2 is a by-product of the re-synthesis. Compound 3 is the structure of the contaminant identified in the sample obtained from the vendor. (c) Neonatal rat ventricular myocytes (NRVMs) expressing GFP-HDAC5 were treated with compounds (1 μM) or DMSO vehicle (−; 0.1%) for 30 min as indicated, and stimulated with PGF2α (10 μM) for 1 h. PGF2α caused redistribution of GFP-HDAC5 that was blocked by the hit compound but not compound 1 or 2. Scale bar = 10 μm.

with the notion that it may be possible to manipulate cardiac hypertrophy with such compounds.

Here, we describe a subset of results from a cell-based high-throughput screen for small molecule regulators of class IIa HDAC nuclear export in cardiac myocytes. We report the discovery of a novel series of CRM1 inhibitors that serve as general repressors of nuclear export with potent anti-hypertrophic activity.

2. Experimental procedures

2.1. NRVM preparation

Hearts were dissected from 1 to 3 day-old Sprague–Dawley rats, minced, and digested with collagenase (Worthington; 600 μg ml^{-1}) and pancreatin (Sigma; 1× activity equivalent) in 1× Ads buffer (NaCl [116 mM], HEPES [20 mM; pH 7.4], NaH_2PO_4 [4.8 mM], KCl [5 mM], $MgSO_4$ [400 μM], and glucose [5.5 mM]). Cells were centrifuged through a step gradient of Percoll (Pharmacia) to separate myocytes from fibroblasts, and the myocyte pool was further enriched by pre-plating for 2 h to remove adherent fibroblasts from the cell population.

2.2. Adenovirus production

Complementary DNA (cDNA) for full-length human HDAC5 (encoding 1122 amino acids) was fused to sequences encoding enhanced green fluorescent protein (EGFP; Clontech) in $pcDNA3.1^+$ (Invitrogen). The resultant construct encodes GFP fused in-frame to the amino-terminus of HDAC5. For adenovirus production, GFP-HDAC5 cDNAs were subcloned into pAC-CMV [12] and constructs cotransfected into 293 cells with pJM17 employing Fugene 6 (Roche). Primary lysates were used to re-infect 293 cells and viral plaques obtained with the agar overlay method. Clonal populations of adenovirus were amplified upon re-infection of 293 cells. Complementary DNA for full-length HDAC4 was fused in-frame with a carboxy-terminal GFP tag. Adenovirus for

Table 1
EC_{50} values (nM) for compounds in nuclear export assays.

Compound	HDAC5 export inhibition EC_{50} (nM)	Rev export inhibition EC_{50} (nM)
1	>8000	>8000
2	>8000	>8000
3	2.2	3.5
4	7.0	88
5	100	970
6	>8000	>8000
7	>8000	>8000

Dose–response analysis of the indicated compounds was performed using NRVMs and the quantitative nuclear export assays for HDAC5 and Rev, as described in the Experimental procedures section.