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Application of a high-throughput fluorescent acetyltransferase assay to identify inhibitors of homocitrate synthase

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ABSTRACT

Homocitrate synthase (HCS) catalyzes the first step of L-lysine biosynthesis in fungi by condensing acetyl-coenzyme A and 2-oxoglutarate to form 3*R*-homocitrate and coenzyme A. Due to its conservation in pathogenic fungi, HCS has been proposed as a candidate for antifungal drug design. Here we report the development and validation of a robust fluorescent assay for HCS that is amenable to high-throughput screening for inhibitors in vitro. Using this assay, *Schizosaccharomyces pombe* HCS was screened against a diverse library of approximately 41,000 small molecules. Following confirmation, counter screens, and dose–response analysis, we prioritized more than 100 compounds for further in vitro and in vivo analysis. This assay can be readily adapted to screen for small molecule modulators of other acyl-CoA-dependent acyltransferases or enzymes that generate a product with a free sulfhydryl group, including histone acetyltransferases, aminoglycoside *N*-acetyltransferases, thioesterases, and enzymes involved in lipid metabolism.

Invasive fungal infections represent an escalating threat to human health. Although these types of infections generally pose a low risk to healthy individuals, they occur frequently and have a high rate of morbidity and mortality in immunocompromised individuals such as burn patients, transplant recipients, cancer patients undergoing chemotherapy, and individuals suffering from HIV/AIDS or other immunodeficiency syndromes [1–6]. Although progress has been made in devising new therapies to treat fungal infections, there remains a pressing need to develop novel antifungal agents. Several classes of antifungal drugs are used clinically to treat invasive fungal infections, including polyenes, azoles, and echinocandins, which target components of the cell membrane or cell wall [7–10]. However, many of these drugs are associated with severe cytotoxic side effects or can interfere with other medications. Furthermore, there has been an alarming rise in drug-resistant fungal strains that do not respond to conventional therapies [11]. For example, treatment of invasive aspergillosis with a single drug has a cure rate of only 50% [10], underscoring the need to develop new drugs and combinatorial therapies to combat fungal pathogens.

Several metabolic pathways have been proposed as targets for novel antifungal drug design. The α-aminoadipate (AAA)[1] pathway represents one such target. This pathway is responsible for L-lysine biosynthesis in certain archaebacteria and fungi, including pathogens from the genera *Candida, Cryptococcus,* and *Aspergillus* [12]. In fungi, the AAA pathway consists of eight steps catalyzed by seven enzymes. The divalent metal-dependent enzyme homocitrate synthase (HCS) catalyzes the first reaction in this pathway by condensing 2-oxoglutarate (2-OG) and acetyl-coenzyme A (AcCoA) to yield 3*R*-homocitrate and coenzyme A (CoA) [13] (see Fig. S1 in Supplementary material). In the second reaction, homoaconitase has been proposed to catalyze a two-step isomerization of 3*R*-homocitrate to form 2*R*,3*S*-homoisocitrate, which is subsequently oxidized by homoisocitrate dehydrogenase to produce 2-oxoadipate. The enzymes 2-aminoadipate aminotransferase, 2-aminoadipate reductase, saccharopine reductase, and saccharopine dehydrogenase catalyze the remaining four reactions in which 2-oxoadipate is converted to L-lysine (Fig. S1). Homologs of these latter four enzymes catalyze lysine catabolism in mammals rendering them unfavorable candidates for drug design [14].

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[1] *Abbreviations used:* AAA, α-aminoadipate; HCS, homocitrate synthase; 2-OG, 2-oxoglutarate; AcCoA, acetyl-coenzyme A; CoA, coenzyme A; HTS, high-throughput screening; HAT, histone acetyltransferase; MMBC, 10-(2,5-dihydro-2,5-di-oxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-, methyl ester 3*H*-naphthol(2,1-b) pyran-*S*-carboxylic acid, also known as ThioGlo 1; SpHCS, *S. pombe* HCS; DMSO, dimethyl sulfoxide; CV, coefficient of variation; S/N ratio, signal-to-noise ratio; SD, standard deviation.

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Of the enzymes in the AAA pathway, HCS represents a primary target for inhibition for several reasons. Notably, HCS catalyzes the first and committed reaction in the AAA pathway and, thus, has a key role in regulating lysine homeostasis in fungi. HCS homologs from several fungi are feedback inhibited by L-lysine, which has an essential role in modulating the metabolic flux through the AAA pathway [15–18]. Recent structural studies have illustrated that L-lysine is a competitive inhibitor of the substrate 2-OG, highlighting the potential for small molecules to regulate HCS activity [19,20]. In addition to feedback inhibition, genetic studies of a *Schizosaccharomyces pombe* mutant lacking Cu/Zn superoxidase dismutase have revealed that the activity and protein level of HCS within cells is diminished under conditions of oxidative stress, suggesting that the enzyme is also subject to redox regulation [21]. At the transcriptional level, HCS expression in *Saccharomyces cerevisiae* is regulated through general control of amino acid biosynthesis [22,23] as well as by the transcription factor Lys14, which is activated in the presence of the AAA pathway intermediate 2-aminoadipate semialdehyde [24–26]. Finally, a very recent study by Schobel and coworkers reported that the deletion of the HCS gene in the pathogen *Aspergillus fumigatus* virtually abolished virulence in a mouse model for bronchopulmonary aspergillosis, whereas the virulence of the knockout strain was unaffected in a disseminated model for invasive aspergillosis [27]. Because the major route of *A. fumigatus* infections is through inhalation into the lungs, these findings imply that HCS inhibitors may find clinical applications in treating allergic bronchopulmonary aspergillosis, aspergilloma, and chromic pulmonary aspergillosis.

In an effort to discover small molecule inhibitors of HCS that may prove to be useful in characterizing its functions in vitro and in vivo, we have developed and validated an in vitro assay for HCS that is amenable to high-throughput screening (HTS). This method was adapted from a fluorescent assay for histone acetyltransferases (HATs) [28] and detects the formation of CoA produced through reaction of its free sulfhydryl group with the sulfhydryl-sensitive fluorophore MMBC [10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-, methyl ester 3*H*-naphthol(2,1-b) pyran-*S*-carboxylic acid, also known as ThioGlo 1] in a 384-well plate format. The utility of this assay was demonstrated by screening a diverse chemical library composed of approximately 41,000 compounds to identify inhibitors of *S. pombe* HCS (SpHCS), with dose–response studies identifying several potent inhibitors. This HTS assay not only will aid in discovering novel inhibitors of HCS but also is broadly applicable to other acyl-CoA-dependent acyltransferases that are potential drug targets.

Materials and methods

Reagents and protein purification

All reagents used were of the highest grade commercially available. The disodium salt of 2-OG, trilithium salts of AcCoA and CoA, and Hepes were purchased from Sigma. AcCoA was treated with acetic anhydride (Fluka) to acetylate trace amounts of free CoA as described previously [28] and was quenched with 100 mM Hepes (pH 7.5). AcCoA was diluted 1:2 in 1 M Hepes (pH 7.5) to bring the pH to 5.0 prior to using in assays. The fluorophore MMBC was purchased from Berry & Associates (Ann Arbor, MI, USA) and was prepared by dissolution in anhydrous dimethyl sulfoxide (DMSO). The dye concentration was determined by measuring absorbance at 381 nm ($\varepsilon = 15{,}100\ M^{-1}\ cm^{-1}$, Covalent Associates). $MnCl_2$-tetrahydrate was purchased from Acros Organics. Costar black 384-well polypropylene plates (Corning Life Sciences) and anhydrous DMSO (Fluka or Acros) were used in all assays. Full-length SpHCS was recombinantly expressed in *Escherichia coli* Rosetta 2 DE3 cells (EMD Biosciences) and purified using a Zn(II)-charged immobilized metal affinity Sepharose column (GE Healthcare) followed by gel filtration chromatography as described previously [29].

Small molecule libraries

In the primary screen, approximately 41,000 compounds were tested at the Center for Chemical Genomics (CCG) in the Life Sciences Institute at the University of Michigan. This library comprises several commercially available compound collections, including the Maybridge Hit-finder Chemical Collection, a diversity collection from Chembridge, the MicroSource Spectrum 2000 Library, the National Institutes of Health (NIH) Clinical Compound set, and a diversity set from ChemDiv.

HCS HTS assay

Primary screening was performed at room temperature by adding 100 mM Hepes (pH 7.5) with 160 μM 2-OG (20 μl) to the 384-well microplates using a Multidrop 384 (Thermo Scientific). Inhibitor compounds (0.2 μl of 1.2- to 2.0-mM stocks, $n = 1$) or DMSO (0.2 μl for negative and positive controls for inhibition, $n = 32$/plate) was added using the pin tool application on a Biomek FX liquid handling robot (Beckman). A mixture of 100 mM Hepes (pH 7.5) and 10.7 μM AcCoA (20 μl) was added to the positive controls for inhibition ($n = 16$/plate). A solution of 100 mM Hepes (pH 7.5), 10 nM SpHCS, and 10.7 μM AcCoA (20 μl) was added to the remaining wells with the Multidrop 384 to initiate the assay, yielding final concentrations of 100 mM Hepes (pH 7.5), 80 μM 2-OG, 5.35 μM AcCoA, and 5 nM SpHCS. Plates were incubated at room temperature for 20 min, and the reactions were terminated with the addition of the detection reagent (40 μL of 25 μM MMBC in DMSO). The plates were covered, and after a 10-min incubation the fluorescence of the MMBC–CoA adduct was measured at 470 nm using an excitation wavelength of 380 nm with a PHERAstar plate reader (BMG LabTech).

Data analysis

To validate the HCS assay, the Z' factor (Eq. 1) [30], the coefficient of variation (CV, Eq. 2), and the signal-to-noise (S/N) ratio were calculated from a single 384-well plate containing the negative controls for inhibition (assay solution in the absence of inhibitors, $n = 192$) and the positive controls for inhibition (assay solution without SpHCS, $n = 191$ with one outlier removed):

$$Z' = 1 - [(3SD_{negative} + 3SD_{positive})/(mean_{negative} - mean_{positive})] \quad (1)$$

$$CV = SD_{negative}/mean_{negative}, \quad (2)$$

where SD is standard deviation.

Compounds were considered as initial hits if they (i) exhibited ≥30.0% inhibition by plate, where 0% inhibition is defined as the average of the negative controls for inhibition (inhibitor omitted) and 100% inhibition is defined as the average of the positive controls for inhibition (SpHCS omitted) or (ii) had an inhibition value that exceeded an SD of 3 by plate (calculated from the negative controls and samples). Additional triage removed compounds with unfavorable properties, including compounds flagged for cytotoxicity, promiscuous compounds (>3 SD in 10 or more previous screens), and molecules with maleimide groups that could react with the free sulfhydryl group of CoA. Potential hits meeting these criteria (1399 molecules) were confirmed by rescreening in triplicate at the same concentration used in primary screening with a Mosquito X1 liquid handling robot (TTP LabTech). Compounds with ≥30.0% inhibition by plate were considered as confirmed hits.