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A chemical screen identifies small molecules that regulate hepcidin expression

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ABSTRACT

Hepcidin, a peptide hormone produced in the liver, decreases intestinal iron absorption and macrophage iron release via effects on ferroportin. Bone morphogenic protein and Stat3 signaling regulate *Hepcidin*'s transcription. Hepcidin is a potential drug target for patients with iron overload syndromes because its levels are inappropriately low in these individuals. To generate a tool for identifying small molecules that modulate *Hepcidin* expression, we stably transfected human hepatocytes (HepG2) cells with a reporter construct containing 2.7 kb of the human *Hepcidin* promoter upstream of a firefly reporter gene. We used high throughput methods to screen 10,169 chemicals in duplicate for their effect on *Hepcidin* expression and cell viability. Regulators were identified as chemicals that caused a change >3 standard deviations above or >1 standard deviation below the mean of the other chemicals (z-score >3 or <1), while not adversely affecting cell viability, quantified by fluorescence assay. Following validation assays, we identified 16 chemicals in a broad range of functional classes that promote *Hepcidin* expression. All of the chemicals identified increased expression of bone morphogenic protein-dependent and/or Stat3-dependent genes, however none of them strongly increased phosphorylation of Smad1,5,8 or Stat3.

Introduction

Hepcidin is a cysteine-rich peptide hormone that regulates the absorption and distribution of iron in humans and other animals [1]. Hepcidin production is transcriptionally regulated in the liver in response to body iron stores and inflammation [2]. Increases in plasma iron levels result in enhanced signaling via bone morphogenic proteins [3] and phosphorylation of Smad1,5, and 8, which facilitates Smad4 binding to the *Hepcidin* promoter and greater *Hepcidin* transcription [4]. The inflammatory cytokine, interleukin-6, IL-6, can also upregulate *Hepcidin* by activating Stat3 and enhancing Stat3 binding to the *Hepcidin* promoter [5]. Hepcidin binds ferroportin1, the only known vertebrate iron exporter, resulting in internalization and degradation of both proteins [6]. Degradation of ferroportin1 decreases intestinal iron absorption [6] and prevents the release of iron from macrophage iron stores to developing erythrocytes in the bone marrow [7].

Clinical studies have demonstrated that Hepcidin levels are inappropriately low in patients with hereditary diseases associated with iron overload, such as thalassemia, congenital dyserythropoietic anemia, and hereditary hemochromatosis [8]. Iron overload is the major cause of death in patients with thalassemia major [9] and an important cause of morbidity in transfusion-dependent patients, such as bone marrow transplant recipients [10]. Current therapies for iron overload are restricted to chelation or removing blood, phlebotomy [11]. These therapies are not well tolerated or completely effective in many patients [12]. Intriguingly, transgenic over-expression of *Hepcidin* in mouse models of hereditary hemochromatosis [13] or β-thalassemia [14] reduces iron overload. Thus, pharmacologically increasing Hepcidin levels may help patients with iron overload by decreasing intestinal iron absorption. Hepcidin agonists under development include Hepcidin mimics, such as rationally designed peptides (minihepcidins), and Hepcidin stimulators, such as anti-sense oligonucleotides directed against inhibitors of *Hepcidin* expression, bone morphogenic protein 6 (BMP6) and small molecules therapies that activate the Stat and/or Smad pathways [12].

Chemical screens are unbiased approaches to identifying small molecules that affect biological processes. They have been useful in identifying antagonists of specific pathways. For instance the bone

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morphogenic protein receptor 1 antagonist, dorsomorphin, was identified in a chemical screen for small molecules that affect zebrafish embryonic development [15]. Chemical screens identifying small molecules that impact specific biological processes have improved our understanding of these processes and led to clinical trials. For instance, prostaglandin E2, was shown to be important in hematopoietic stem cell proliferation [16] and is now being evaluated in human trials to improve the efficiency of umbilical cord hematopoietic stem cell transplants [17].

In a preliminary chemical screen evaluating the effect of isoflavones and related compounds in zebrafish embryos and human hepatocytes, we identified the small molecule genistein, a phytoestrogen that is one of the major components of soybeans, as a stimulator of *Hepcidin* expression that activated Stat3 and Smad signaling [18]. In order to identify additional small molecules that act via different mechanisms and may have greater potency, we undertook a high throughput chemical screen for small molecules that increase *Hepcidin* expression in human hepatocytes. To achieve this, we generated a line of human hepatoma cells, HepG2 *Hepcidin*-luciferase, that express 2.7 kb of the human *Hepcidin* promoter upstream of a firefly luciferase reporter. We screened a total of 10,169 small molecules in duplicate for their ability to increase or decrease *Hepcidin* expression without impairing cell viability. We validated our hits with quantitative realtime RT-PCR assays for *Hepcidin* expression and characterized them by their effects on genes regulated by BMP's or Stat3, as well as Western blots to detect phosphorylation of Smad1,5,8 or Stat3. We confirmed 16 small molecule *Hepcidin* stimulating agents in a broad range of functional classes. All of the chemicals identified increased expression of bone morphogenic protein-dependent and/or Stat3-dependent genes, however none of them strongly increased phosphorylation of Smad1,5,8 or Stat3. Several of the *Hepcidin* stimulatory chemicals inhibit growth factor receptor dependent signaling (AG1296, GTP 14564, AS252424, 10058-F, SU6668, and pterostilbene), decrease inflammation (leflunomide, amlexanox), or impair DNA repair and promote apoptosis (daunorubicin, 9-aminocridine, ethacridine), while the small molecules, vorinostat and SB 204741, inhibit histone deacetylase and serotonin receptor 2B, respectively. Two of the molecules, ipriflavone and vorinostat, were active at concentrations that were 10-fold below those required for genistein's effect and thus appear to be intriguing candidates for further development.

Materials and methods

Cell culture and reagents

The human hepatocarcinoma cell line, HepG2, (American Type Culture Collection, Manassas, VA) was maintained in α-Minimum Essential Medium (α-MEM)/10% certified endotoxin-free fetal bovine serum (FBS)/1% penicillin–streptomycin (Life Technologies, Grand Island, NY) at 37 °C, 5% CO_2. To generate a *Hepcidin* reporter cell line, HepG2 cells, were transfected using SuperFect (Qiagen, Valencia, CA) transfection reagent and a reporter construct including a 2.7 kb fragment of the human *Hepcidin* promoter upstream of a firefly luciferase promoter (gift of Drs. Ganz and Nemeth). Transfected clones were selected for resistance to G418 (Life Technologies) and subsequently maintained in the conditions described above with the addition of G418 1 mg/ml. Bone

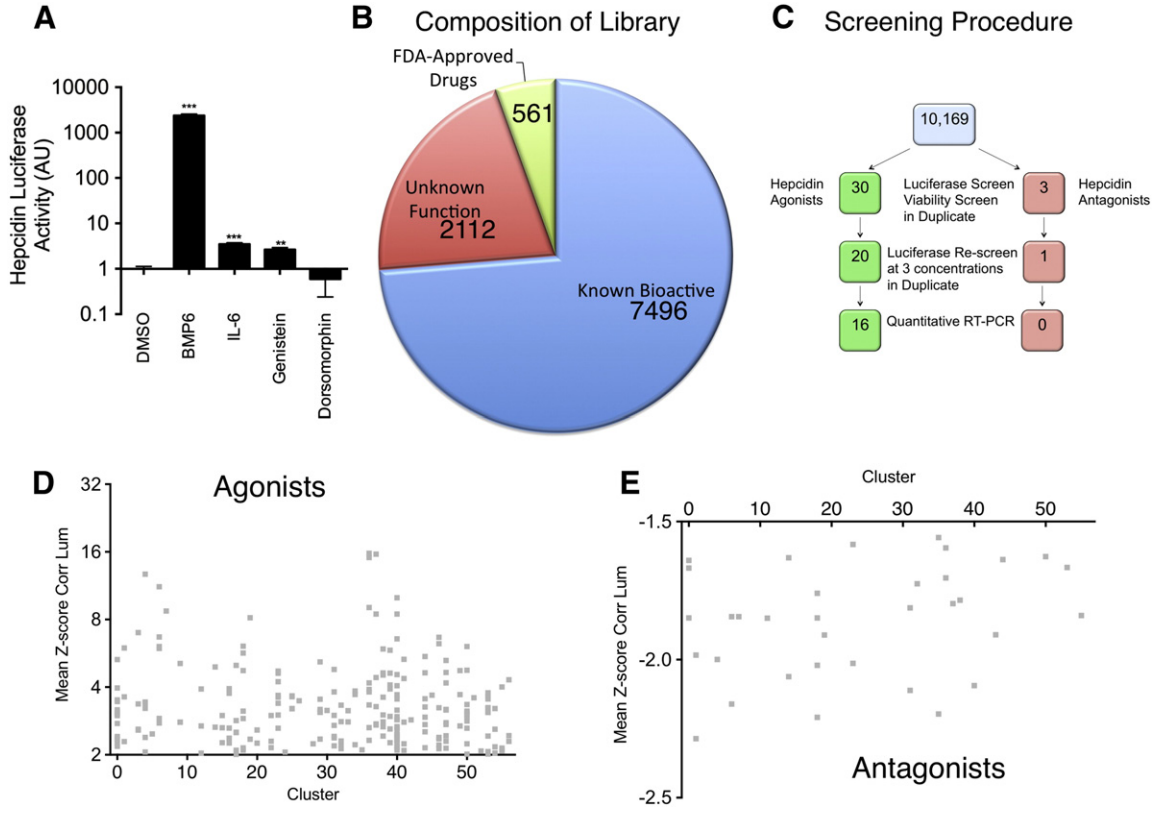

Fig. 1. A. Effect of positive and negative controls on *Hepcidin*-luciferase activity in stably transfected HepG2 cells. After 16 h of serum starvation in α-MEM/0% FBS, HepG2 cells stably transfected with *Hepcidin*-luciferase were treated for 24 h with DMSO 1%, BMP6 50 ng/ml, IL-6 100 ng/ml, Genistein 10 μM, or Dorsomorphin 40 μM. *Hepcidin*-luciferase activity was measured using the OneGlo Assay (Promega) and is shown as mean fold-change over DMSO-treated control. The global P-value generated using the Kruskal–Wallis test was <0.0001. Unpaired Student's t-tests were performed compared to DMSO alone. *** denotes $0.0001 \leq P < 0.0009$ and ** denotes $0.0009 \leq P < 0.009$. $N = 3$ biological replicates per condition. B. Library Composition. The screening library included known bioactive molecules, molecules of unknown function, and FDA-approved drugs. C. Screening Method. 10,169 chemicals were evaluated for *Hepcidin*-luciferase activity and viability in HepG2 cells stably transfected with a *Hepcidin*-luciferase promoter construct. The hits were then re-evaluated in the same assay at three concentrations and in a quantitative realtime RT-PCR assay. D,E. Scatter-plot of structural cluster vs. mean z-score for *Hepcidin*-luciferase corrected luminescence activity for 343 molecules found to increase (D) or 62 molecules found to decrease (E) *Hepcidin*-luciferase activity.