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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

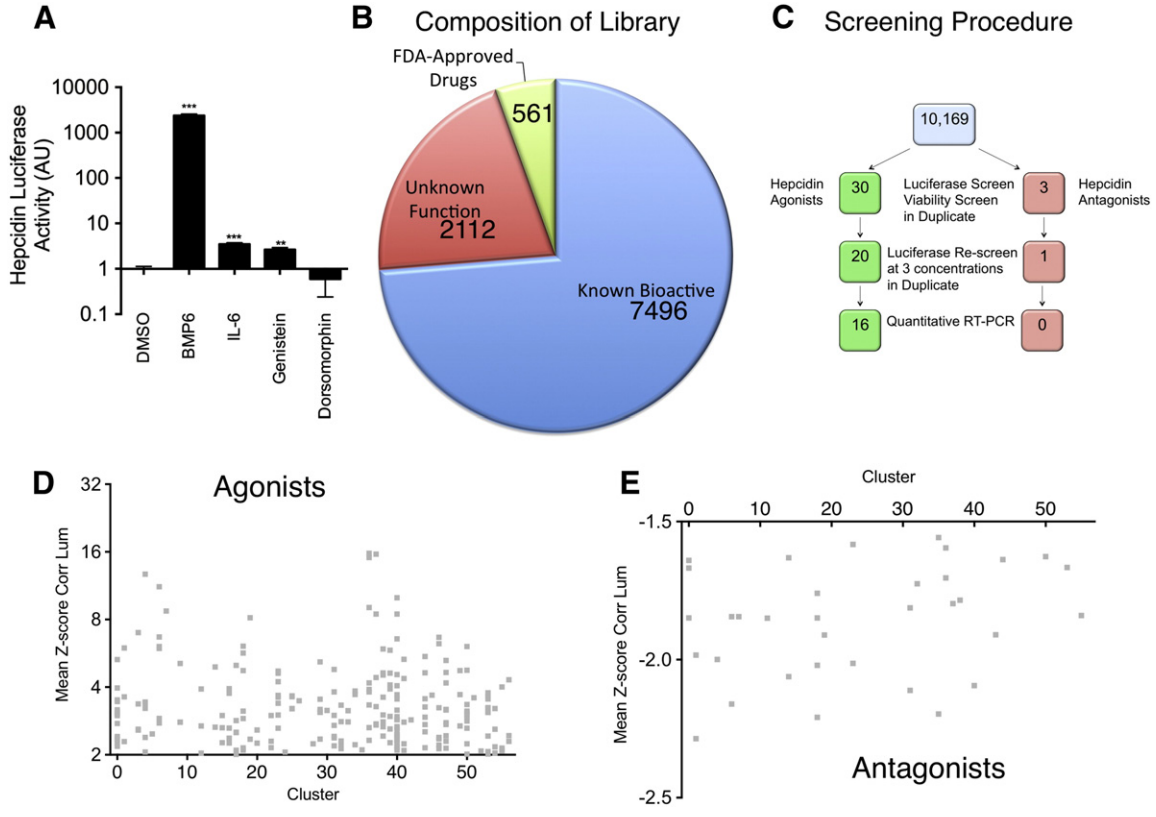

Fig. 1. A. Effect of positive and negative controls on *Hepcidin*-luciferase activity in stably transfected HepG2 cells. After 16 h of serum starvation in α-MEM/0% FBS, HepG2 cells stably transfected with *Hepcidin*-luciferase were treated for 24 h with DMSO 1%, BMP6 50 ng/ml, IL-6 100 ng/ml, Genistein 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.

morphogenic protein 6 (BMP6) (R&D Systems, Minneapolis, MN), interleukin-6 (R&D Systems), and genistein (Sigma-Aldrich, St. Louis, MO) were used as positive controls for *Hepcidin*-luciferase activity, while dorsomorphin (#171260, Calbiochem, Billerica, MA) was used as a negative control. WP1066 (#573097, Calbiochem) was used as an inhibitor of Stat3 signaling. Interleukin-6, EGF, FGF, PDGF, and VEGFA were obtained from R&D Systems.

Chemical screen

The Institute of Chemistry and Cell Biology (ICCB) Screening Facility at Harvard Medical School provided drug libraries. The complete list of chemicals screened and the screening data are provided in Supplementary Table 1. The day before the addition of compounds, HepG2-*Hepcidin* luciferase cells were plated at 5000 cells in 30 μl per well of a 384-well microtiter plate (Nunc 142762) in α-MEM/1% penicillin/streptomycin using a WellMate MicroPlate Dispenser (Thermo Scientific, Rockford, IL). Twenty-four hours later, 100 nl aliquot of chemical was pin-transferred from each well on the chemical library plate to a corresponding well on the screening plate. BMP6 (50 ng/ml) was used as a positive control while vehicle only, DMSO (0.3%), was used as a negative control. After 24 h of treatment, the cell viability and *Hepcidin* promoter activity were measured with the OneGlo + Tox Cell Viability and Luciferase Reporter assay (E7120, Promega, Madison, WI) according to the manufacturer's instructions using an EnVision 2102 Plate Reader (PerkinElmer, Waltham, MA). Fluorescence was measured using an excitation wavelength of 380–400 nm and emission wavelength of 505 nm. The entire screen was performed in duplicate.

The primary readout was the crosstalk-corrected *Hepcidin* luminescence for each well. The secondary readout was cell viability fluorescence for each well. For each readout and each well, a z-score was calculated using the formula: z-score [z = (x − mean of samples on the plate)/standard deviation of samples on the plate] where x = the fluorescence or luminescence intensity for the particular well. The positive and negative controls were excluded from the calculation of the mean and standard deviation for the plate. An agonist compound was considered a hit if the luciferase z-scores for both replicates were >3. An antagonist compound was considered a hit if the luciferase z-score for each replicate was <−1. Any agonist or antagonist with a cell viability fluorescence z-score <−1 on either replicate was excluded from being considered a hit.

After identifying hits in the screening, we re-screened selected regulators at the original and two additional dilutions using the same luciferase and fluorescence assays. We considered a hit to be validated if it increased *Hepcidin* promoter activity at least 2-fold above the vehicle-only control (1% DMSO) at one of the concentrations. Negative regulators were identified as those that produced at least a 50% reduction in *Hepcidin* promoter activity. Supplementary Table 2 provides the sources, functional categories, and chemical structures for the candidate regulators that were characterized further by quantitative realtime RT-PCR and Western blots.

Quantitative realtime RT-PCR

In order to evaluate whether or not candidate regulators upregulate *Hepcidin* via the Stat3 pathway and/or the Smad4 pathway, we plated 400,000 wild type HepG2 cells per well of a 12-well tissue culture plate. After 8 h of serum starvation in α-MEM/1% FBS, we added each candidate regulator. After 24 h of treatment, we extracted RNA, and generated cDNA according to the method [18]. We measured the transcript levels of *Hepcidin* and key genes in each of these pathways in quantitative realtime RT-PCR using primers and probes as described (Supplementary Table 3).

Western blots

To test for the effects of the *Hepcidin* regulators on proteins involved with the Smad4 or Stat3 signaling pathways, we plated 400,000 cells in a 12-well tissue culture plate and changed the media to α-MEM/1% FBS for 16 h prior to treating the cells with chemicals for 1 h. The cells were lysed in radio-immunoprecipitation assay buffer (RIPA buffer) with 1× Protease Inhibitor/1× Phosphatase Inhibitor Cocktail (both from Roche). The Pierce BCA Assay (Thermo Scientific) was used to measure the protein concentrations. Twenty micrograms of protein lysate was loaded with 0.5× TruSep SDS Sample Buffer (NuSep Inc., Bogart, GA) in each lane of a Tris-Glycine 4–10% SDS polyacrylamide gel (NuSep Inc.). After the gel was run and transferred to a polyvinylidene difluoride (PVDF) membrane, the membrane was blocked with TBS/0.05% Tween 20/5% bovine serum albumin for antibodies against phosphorylated proteins or Pierce Protein-Free TBS Blocking Buffer (Thermo Scientific) for all other antibodies. The primary antibodies, all rabbit anti-human, were used at the following dilutions: phospho-Smad1, 5, and 8 (#9511S, Cell Signaling Technology, Inc., Danvers, MA) 1:200, phospho-Stat3 (SC-8001-R, Santa Cruz Biotechnology Inc., Dallas, TX) 1:100, Smad1 (#9743S, Cell Signaling Technology Inc.) 1:500, Stat3 (#SC-482, Santa Cruz Biotechnology, Inc.) 1:200, or β-actin (#4967S, Cell Signaling Technology Inc.) 1:1000. The blots were developed with

Table 1
Re-screening data on potential agonists or antagonists identified in the initial chemical screen. Small molecules that were identified as potential regulators in the screen were retested in duplicate at the original concentration screened (Concentration #1) and two dilutions (Concentrations #2 and #3) for their effects on *Hepcidin*-luciferase activity, measured as fold-change over the activity from cells treated with DMSO only. Candidate regulators that increased mean *Hepcidin*-luciferase activity at least 2-fold at the original concentration were considered agonists (shaded in green), while candidate regulators that decreased mean *Hepcidin*-luciferase activity at least 2-fold were considered antagonists (shaded in orange).

Regulator name	Concentration #1 (μM)	Hepcidin-luciferase activity at conc. #1 mean+SE (AU) N=2	Concentration #2 (μM)	Hepcidin-luciferase activity at conc. #2 mean+SE (AU) N=2	Concentration #3 (μM)	Hepcidin-luciferase activity at conc. #3 mean+SE (AU) N=2
Potential agonists						
Vorinostat	33	56.08±35.27	6.6	48.14±6.70	1.3	14.60±4.68
9-Aminoacridine	33	49.76±12.27	6.6	1.14±0.40	1.3	0.68±0.07
Acrisorcin	33	29.85±1.50	6.6	0.99±0.06	1.3	0.77±0.00
Ethacridine lactate	33	15.18±1.13	6.6	1.35±0.24	1.3	1.12±0.18
Doxorubicin	12	11.39±0.49	2.4	0.68±0.06	0.5	0.77±0.02
Daunorubicin	12	11.33±0.07	2.4	0.99±0.05	0.5	0.67±0.05
Leflunomide	33	5.50±0.29	6.6	3.10±0.23	1.3	1.12±0.18
#5322770	66	5.03±0.21	12.2	1.42±0.04	2.6	0.84±0.06
Ipriflavone	33	4.57±0.30	6.6	3.41±0.08	1.3	2.56±0.07
Lansoprazole	33	4.09±0.44	6.6	0.98±0.01	1.3	0.58±0.01
Pterostilbene	33	4.06±0.59	16.5	3.91±0.28	8.25	2.21±0.19
Amlexanox	33	3.84±0.95	6.6	2.65±0.06	1.3	0.67±0.02
Nabumetone	33	3.74±0.19	16.5	3.16±0.30	8.25	3.61±0.95
Topotecan	33	3.63±1.39	6.6	0.57±0.05	1.3	0.73±0.04
Camptothecin	33	3.62±0.62	6.6	1.55±0.34	1.3	0.60±0.05
Chrysin	26	3.53±0.60	5.2	1.09±0.11	1.0	0.76±0.01
GTP-14564	11	3.07±0.60	2.2	1.35±0.07	0.4	1.17±0.15
10058-F4	33	2.92±0.23	6.6	2.17±0.01	1.3	1.25±0.13
SB 204741	33	2.87±0.22	6.6	1.96±0.18	1.3	1.09±0.04
Phenazopyridine	26	2.85±0.04	5.2	1.51±0.08	1.0	0.95±0.12
AS-252424	33	2.84±0.02	6.6	1.40±0.03	1.3	0.73±0.02
AG 1296	11	2.41±0.14	2.2	1.03±0.11	0.4	0.90±0.07
SP 600125	33	1.86±0.00	6.6	1.11±0.02	1.3	0.93±0.09
Fenbendazole	22	1.75±0.08	4.4	1.03±0.20	0.9	0.96±0.12
#5105276	66	1.73±0.04	12.2	0.75±0.13	2.6	0.74±0.02
GW9662	33	1.66±0.08	6.6	1.12±0.05	1.3	1.09±0.01
#5102420	66	1.66±0.06	12.2	0.80±0.11	2.6	0.83±0.16
Imatinib	33	1.24±0.13	16.5	1.18±0.06	8.25	1.31±0.06
Nocodazole	22	1.13±0.02	4.4	0.84±0.11	0.9	0.84±0.01
#5193825	66	1.13±0.14	12.2	0.67±0.01	2.6	0.69±0.06
Potential agonists						
#5215082	66	1.09±0.08	12.2	0.87+0.04	2.6	0.79+0.02
#5146972	66	1.08±0.00	12.2	0.72+0.05	2.6	0.66+0.03
SU-6668	33	0.31±0.02	6.6	0.67+0.09	1.3	0.88+0.01

secondary antibody, mouse anti-rabbit IgG-horseradish peroxidase (#SC-2357, Santa Cruz Biotechnology Inc.) 1:5000, followed by addition of Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the manufacturer's instructions. The blots were exposed to Kodak Biomax light film (Sigma-Aldrich) for 5–30 min at room temperature.

Statistical analysis

Graphs were created and statistical analyses were performed using Prism 6.0c (Graphpad, San Diego, CA). We used the Kruskal–Wallis method to generate a global P-value for each experiment. Where the global P-value was <0.05, Student's t-tests were performed. $P < 0.05$ was considered a significant result on the Student's t-test.

Structural analysis of small molecules

To assess patterns of structural similarity, the structures of all the compounds producing an average crosstalk corrected *Hepcidin*-luciferase z-score >3 or <-1, regardless of effects of viability were analyzed. The 405 compound structures were imported into Vortex (Dotmatics, Inc., version 2012.07.15406) and a 1024-bit Dotmatics hex-packed fingerprint was generated. Compounds were clustered on the basis of this fingerprint using Rogers–Tanimoto similarity, leading to 57 structural clusters (378 compounds) plus 27 singleton compounds that were not included in any of the clusters.

Results

In order to evaluate the effects of a broad range of small molecules on *Hepcidin* expression, we screened 10,169 chemicals in a dual *Hepcidin* luciferase assay and viability assay. The screening assays were performed in HepG2 cells stably transfected with a human *Hepcidin* promoter fragment (2.7 kb) upstream of a firefly luciferase reporter. *Hepcidin*-luciferase activity in treated cells was measured as fold-change over controls treated with vehicle only (DMSO $\leq 1\%$). We confirmed that treatment for 24 h with positive controls, BMP6 50 ng/ml, IL-6 100 ng/ml, or genistein 10 μM, significantly increased *Hepcidin*-luciferase activity in the stably transfected cells (2378 ± 185.4, 3.48 ± 0.24, 2.64 ± 0.28, respectively), while dorsomorphin 40 μM decreased it (0.59 ± 0.35) (Fig. 1A).

The composition of the library screened (Fig. 1B) included a diverse range of chemicals with the majority known bioactives (7496), followed by molecules of unknown function (2112), and FDA-approved drugs (561). To each well, 100 nl of a single small molecule was transferred prior to incubation of the cells at 37 °C for 24 h. The entire screen was performed in duplicate. Of the 10,169 chemicals originally screened, 343 agonists and 62 antagonists were initially identified by producing a z-score >3 or <-1 for *Hepcidin* expression, respectively (Fig. 1C). Analysis of these chemicals with the Vortex program separated these chemicals into 57 structural groups. Agonists (Fig. 1D) and antagonists (Fig. 1E) were scattered across the structural groups without a dominant structure. When toxic chemicals were excluded by eliminating compounds that produced a z-score for viability <-1, i.e. <1 standard deviation reduction in cell viability, 30 agonists and 3 antagonists remained. We re-screened these molecules at the original concentration and at 2 dilutions in duplicate. Of these chemicals, 22 agonists and 1 antagonist were confirmed on re-screening (Table 1). We did not evaluate acrisorcin further, because it is a salt of 9-aminoacridine with 4-hexylresorcinol [19], that produced a similar effect to 9-aminoacridine, one of the other *Hepcidin* stimulating agents (Table 1). We also did

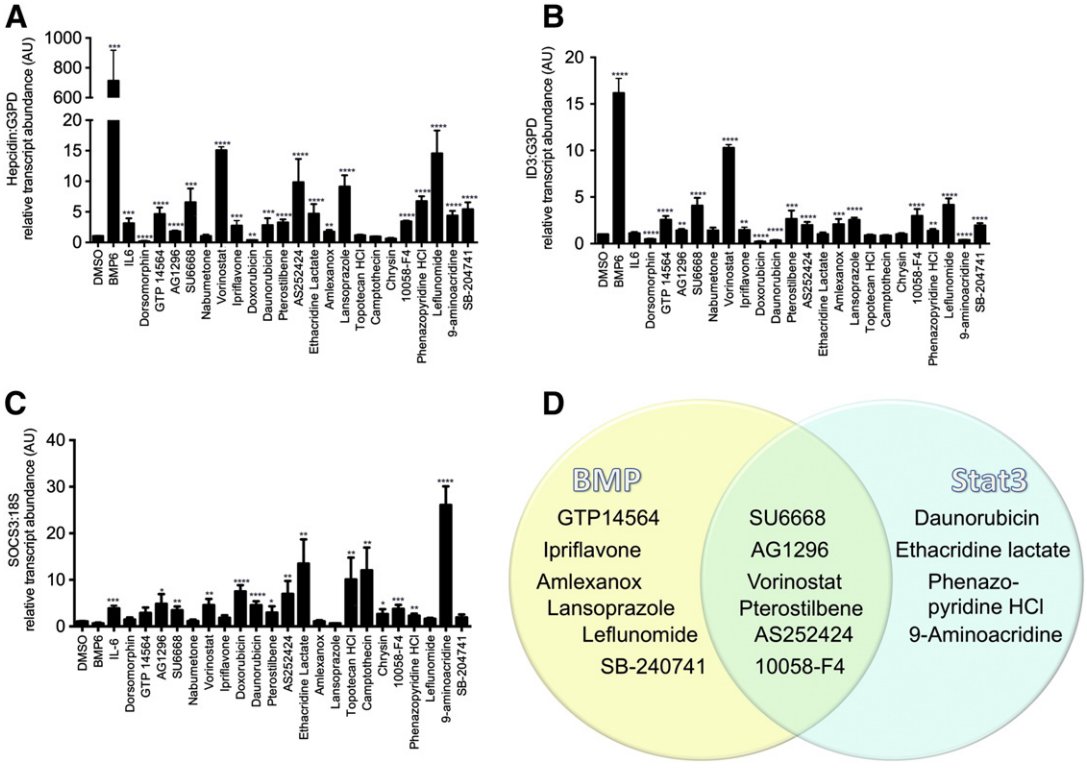

Fig. 2. Quantitative realtime RT-PCR for A. *Hepcidin*, B. *ID3*, and C. *SOCS3*. Following 8 h of serum starvation in α-MEM/1%FBS, HepG2 cells were treated for 24 h in α-MEM/1%FBS with DMSO 1%, BMP6 100 ng/ml, IL-6 100 ng/ml, dorsomorphin (an inhibitor of BMP signaling) 10 μM, GTP 14564 5 μM, AG1296 5 μM, SU6668 10 μM, nabumetone 5 μM, vorinostat 1 μM, ipriflavone 1 μM, doxorubicin 10 μM, daunorubicin 10 μM, pterostilbene 10 μM, AS252424 33 μM, ethacridine lactate 33 μM, amlexanox 33 μM, lansoprazole 33 μM, topotecan HCl 33 μM, camptothecin 33 μM, chrysin 33 μM, 10058-F4 33 μM, phenazopyridine 33 μM, leflunomide 33 μM, 9-aminoacridine 33 μM, and SB-204741 33 μM. RNA was then extracted for quantitative realtime RT-PCR. Data shown are means ± SE's. N = 3–6 biological replicates per treatment. The global P-value generated using the Kruskal–Wallis test was <0.0001 for each experiment. Unpaired Student's t-tests were performed compared to DMSO alone. * denotes $0.009 \leq P < 0.05$, ** denotes $0.0009 \leq P < 0.009$, *** denotes $0.0001 \leq P < 0.0009$, ****$P < 0.0001$. D. Venn diagram illustrating which chemicals appear to increase RNA transcript levels of *Hepcidin* and the BMP-dependent transcript, *ID3*, and/or the Stat3-dependent transcript, *SOCS3*.

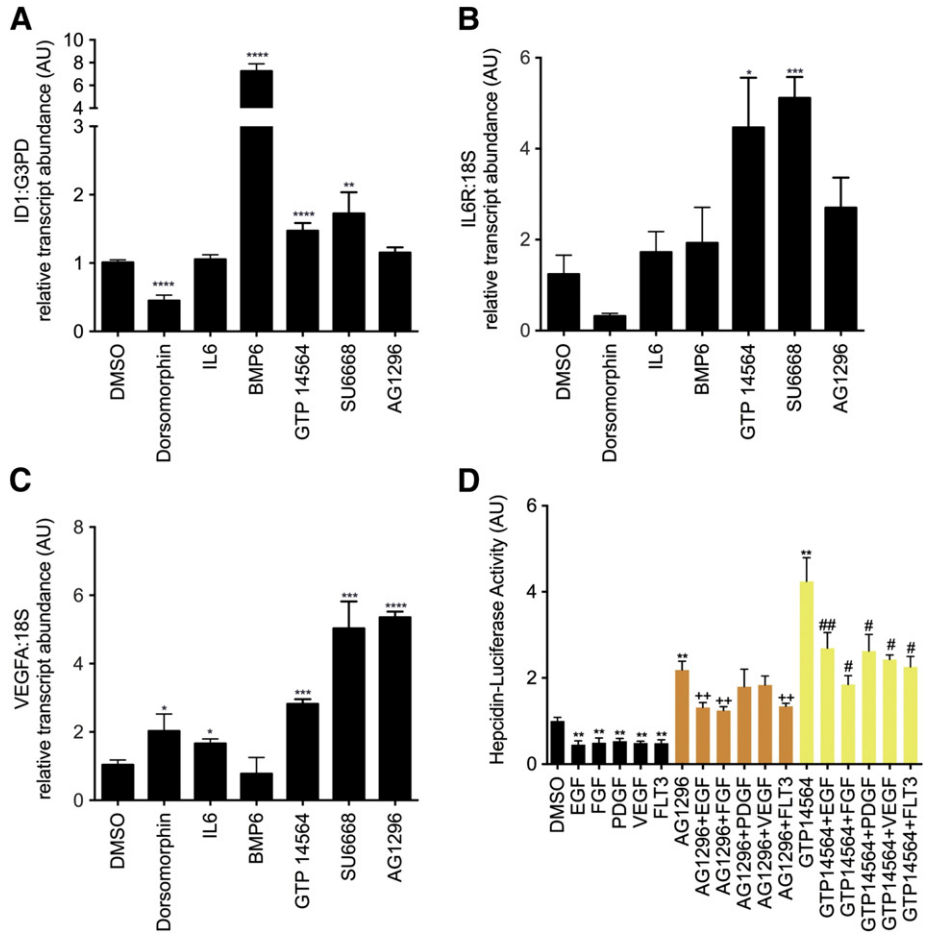

Fig. 3. A–C. Effect of AG1296 and GTP 14564 on transcript levels of *ID1* (A), *IL6 receptor* (B), and *VEGFA* (C). HepG2 cells were treated with chemicals at the same doses and with the same conditions as described in Fig. 2. Data shown are means ± SE's. N = 3–6 biological replicates per treatment. The global P-value generated using the Kruskal–Wallis test was <0.0001 for *ID1* and =0.01 for *IL6R* and *VEGFA*. Unpaired Student's t-tests were performed compared to DMSO alone. * denotes 0.009 ≤ P < 0.05, ** denotes 0.0009 ≤ P < 0.009, *** denotes 0.0001 ≤ P < 0.0009, ****P < 0.0001. D. Effect of AG1296 or GTP 14564 on *Hepcidin*-Luciferase Activity in the presence or absence of growth factors or FLT3. After 16 h of serum starvation in α-MEM/0% FBS, HepG2 cells stably transfected with *Hepcidin*-luciferase were treated for 24 h with AG1296 (5 μM) or GTP 14564 (5 μM) in the presence or absence of EGF 150 ng/ml, FGF 200 ng/ml, PDGF 50 ng/ml, VEGF 150 ng/ml, or FLT3 150 ng/ml. *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. ** denotes 0.0009 ≤ P < 0.009, compared to DMSO-treated control. ++ denotes 0.0009 ≤ P < 0.009, compared to AG1296-treated cells. # denotes 0.009 ≤ P < 0.05, compared to GTP 14564-treated cells. ## denotes 0.0009 ≤ P < 0.009, compared to GTP 14564-treated cells. N = 5–9 biological replicates for each condition.

not evaluate #532270 further because it was only moderately active (5.03 ± 0.21) at 66 μM and weakly active (1.42 ± 0.04) at 12 μM.

The remaining twenty potential *Hepcidin* agonists and one antagonist were subsequently evaluated by quantitative realtime RT-PCR for *Hepcidin* expression at the same concentrations that were effective in the *Hepcidin*-luciferase assay. BMP6 and dorsomorphin, used as positive and negative controls, respectively, produced the expected effects on *Hepcidin* expression (Fig. 2A). Sixteen of the 20 putative agonists significantly increased *Hepcidin* transcript levels, however, the putative agonists, topotecan, campthothecin, nabumetone, and chrysin, failed to increase *Hepcidin* transcript levels, despite increasing *Hepcidin*-luciferase activity, while the putative antagonist, SU6668, increased *Hepcidin* transcript levels, despite decreasing *Hepcidin*-luciferase activity.

In previous RNA sequencing and quantitative RT-PCR experiments [18], we had identified the BMP-regulated transcript, *ID3* [20–22], and the Stat3-regulated transcript *SOCS3* [23], as genes whose expression increased significantly in HepG2 cells following treatment with BMP6 or IL-6, respectively. Thus, we evaluated the effects of the chemicals on *ID3* (Fig. 2B) and *SOCS3* (Fig. 2C) transcript levels, as readouts for bone morphogenic protein signaling and Stat3 signaling [18]. As expected, BMP6 produced a significant increase in *ID3* expression over DMSO alone (16.17 ± 1.57, P < 0.0001) that was not observed with IL-6 treatment, while IL-6 increased *SOCS3* expression (3.88 ± 0.59, P = 0.0002), but BMP6 did not. The BMP receptor antagonist, dorsomorphin, used as a negative control, inhibited *ID3* expression (0.48 ± 0.16, P < 0.0001).

The HDAC inhibitor, vorinostat, which was one of the most potent *Hepcidin* stimulating chemicals identified in the screen, produced a particularly strong increase in *Hepcidin* (15.09 ± 0.55, p < 0.0001) and ID3 expression (10.3 ± 0.33, P < 0.0001). The majority of chemicals that significantly upregulated *Hepcidin* transcript levels significantly upregulated *ID3*, with the exception of daunorubicin, ethacridine, and 9-aminoacridine, which either decreased or did not affect *ID3* expression (Fig. 2B). Interestingly, the *Hepcidin* agonists that did not upregulate *ID3*, did upregulate *SOCS3*, consistent with Stat3 pathway activation (Fig. 2C). Thus the *Hepcidin* agonists can be divided into three classes: (1) upregulators of BMP signaling only, (2) upregulators of Stat3 signaling only, and (3) upregulators of both pathways (Fig. 2D).

We were particularly interested in the kinase inhibitors, GTP 14564, AG1296, and SU6668, since they each affect growth factor dependent signaling, which has previously been shown to affect *Hepcidin* expression [24]. GTP 14564 inhibits FLT3, c-Fms, c-Kit, and PDGFRβ [25], while AG1296 impairs signaling by both PDGF-α and β receptors and by c-Kit [26]. SU6668 has broad effects against receptor tyrosine kinases

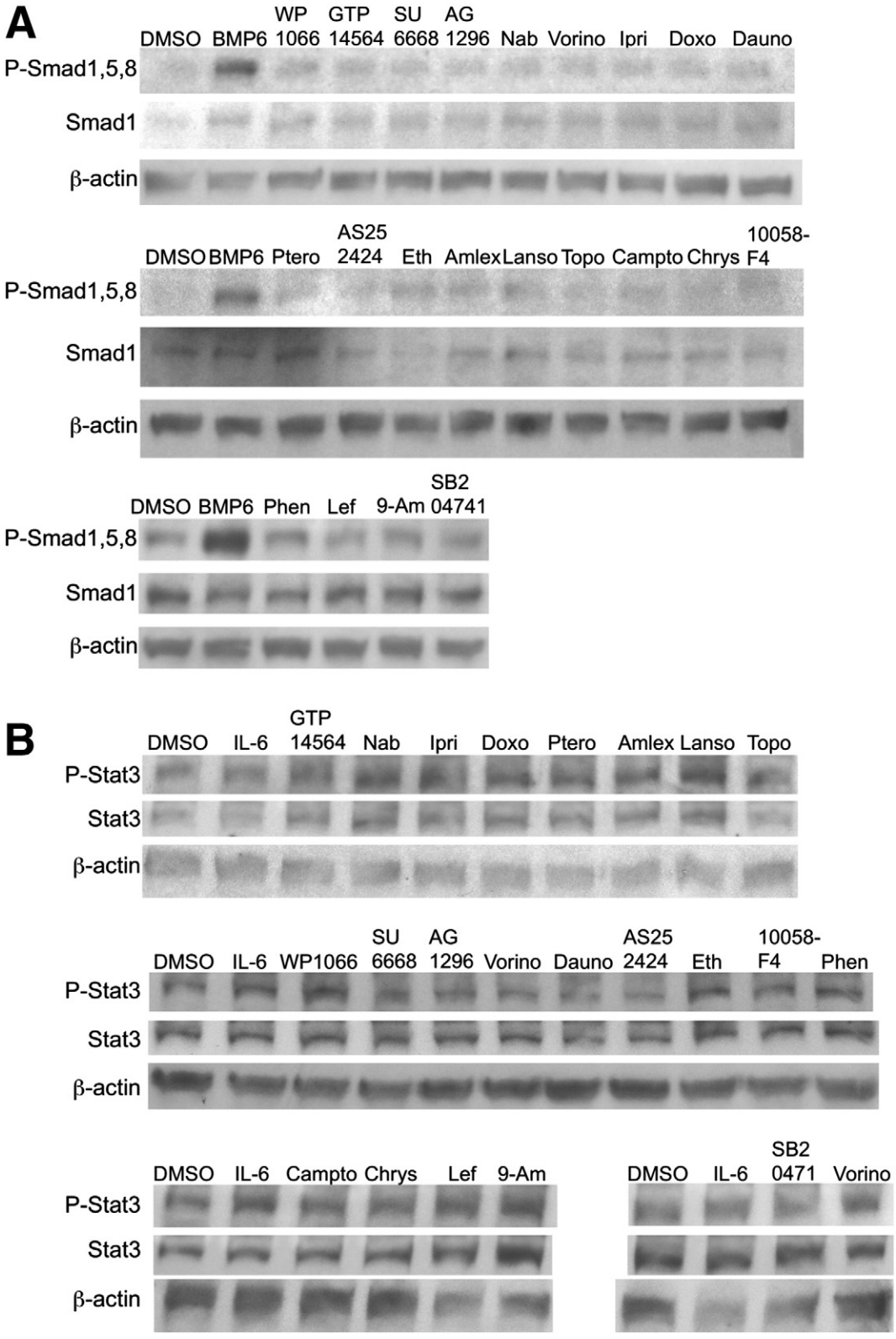

Fig. 4. Western blots. Following 16 h of serum starvation in α-MEM/1%FBS, HepG2 cells were treated for 1 h in α-MEM/1%FBS with the chemicals at the same concentrations as given in Fig. 2. Protein was extracted from the cells, separated by SDS-PAGE, and blotted for incubation with antibodies against anti-P-Smad1,5,8 (A) or P-Stat3 (B). Following immunoblotting, the membranes were stripped and re-probed with either antibody against anti-Smad1,5,8 (A) or Stat3 (B). The blots were then stripped again and probed for β-actin as a loading control. Each chemical was evaluated in two or three biological replicates.

and inhibits VEGF, FGF, and PDGF receptors [27]. To validate the initial classification of these compounds by *ID3* and *SOCS3* expression (Figs. 2B–D), we evaluated these chemicals for their effects on transcription of an additional BMP-dependent gene, *ID1* [20–22], and additional Stat3-dependent genes [23,18], *IL6 receptor* (*IL6R*) and *VEGFA*. We found that GTP 14564 and SU6668 each significantly

upregulated expression of *ID1*, as well as *IL6R* and *VEGFA* (Figs. 3A–C). Although AG1296 did not significantly increase expression of *ID1*, it did significantly increase transcript levels of BMP and Stat3-dependent genes, including *ID3* (Fig. 2B), *SOCS3* (Fig. 2C), and *VEGFA* (Fig. 3C). Thus it appears that these growth factor receptor tyrosine kinase inhibitors upregulate both the BMP and Stat3 signaling pathways.

To assess the effects of growth factors on *Hepcidin* promoter activity, we treated the *Hepcidin*-Luciferase HepG2 cells with EGF 150 ng/ml, FGF 200 ng/ml, PDGF 50 ng/ml, VEGF 150 ng/ml, or FLT3 150 ng/ml for 24 h in the presence or absence of the tyrosine kinase inhibitors, AG1296 (5 μM) or GTP 14564 (5 μM) (Fig. 3D). We found that each of these proteins significantly reduced baseline *Hepcidin* promoter activity. In combination with GTP 14564 each of the tested proteins significantly impaired GTP 14564's stimulation of *Hepcidin* promoter activity, while in combination with AG1296, only EGF, FGF, or FLT3 significantly blocked AG1296's induction of *Hepcidin* promoter activity. Thus growth factor- or FLT3-dependent signaling appears to inhibit *Hepcidin* promoter activity and to impair the stimulatory effects of AG1296 and GTP 14564, but we did not observe a phenomenon that was limited to one particular growth factor or ligand.

We had hypothesized that the *Hepcidin* stimulatory molecules identified in the screen would increase phosphorylation of Smad1,5, and 8 and/or phosphorylation of Stat3. To evaluate this hypothesis, we performed Western blots to evaluate the ratio of P-Smad1,5,8 to Smad1 (Fig. 4A) and P-Stat3 to Stat3 (Fig. 4B). As expected, BMP6 treatment increased the intensity of P-Smad1,5,8 relative to Smad1 after 1 h of treatment, however, none of the small molecules significantly increased the intensity of P-Smad1,5,8 relative to Smad1, as assessed by densitometry. Furthermore, in the one hour time frame, neither IL-6 nor any of the small molecules tested increased the intensity of P-Stat3 relative to Stat3. WP1066, a known inhibitor of Jak2 and Stat3 phosphorylation [28] for Jak/Stat signaling, did not decrease P-Stat3 to Stat3, however WP1066 is reported to be more effective after 24–48 h of incubation [28]. After 24 h of treatment, we observed a significant increase in Stat3 protein levels relative to DMSO-treated controls in the hepatocytes treated with lansoprazole or vorinostat (2.34 ± 0.96, $P = 0.047$ and 1.88 ± 0.43, $P = 0.03$, respectively, Supplementary Fig. 1), but no significant change in phosphorylation of Stat3 relative to Stat3 levels.

Discussion

In this study, we have demonstrated a high throughput screening method to identify small molecules that regulate *Hepcidin* gene expression using a *Hepcidin*-luciferase reporter cell line. Our study was the first large-scale screen for small molecules upregulating *Hepcidin* transcript levels. Using a screening approach that includes toxicity evaluation, we have identified the largest number of non-toxic small molecules that stimulate *Hepcidin*, which will facilitate future preclinical studies in iron overload syndromes. Several of the *Hepcidin* stimulating agents that we identified are drugs that are orally bioavailable or have been approved by the United States Food and Drug Administration (FDA) for other indications. These factors will facilitate their testing in preclinical models. The FDA-approved drugs that we identified include amlexanox, lansoprazole, leflunomide, vorinostat, and phenazopyridine, while pterostilbene and isoflavone are already commercially available as nutritional supplements. Small scale screening efforts previously identified genistein [18] and three kinase inhibitors [24] as small molecules that stimulate *Hepcidin* expression. Peptide analogs of hepcidin, minihepcidins, have also been injected into *Hepcidin*-deficient mice to prevent iron overload [29], but are not orally available. High throughput screening has previously been used to identify small molecules that function as Hepcidin antagonists, but not agonists [30]. Other antagonists to Hepcidin that have been developed include an antibody to Hepcidin [31], soluble hemojuvelin [32], and the bone morphogenic protein receptor antagonists, dorsomorphin and LDN-193189 [32].

Having screened 10,169 molecules, we identified 33 potential hits, which were reduced to 21 after re-screening with the same assay. Further characterization with quantitative realtime RT-PCR for *Hepcidin* transcript level reduced the number of hits to 16 agonists and no antagonists. Of the publically available small molecule screens in PubChem, 20% rely on bioluminescent assays, such as ours [33]. A recent study of 360,864 compounds in the NIH Molecular Libraries Small Molecule Repository revealed that 12% of the library inhibits firefly luciferase [34]. Interestingly, some of these inhibitors can prolong the half-life of the firefly luciferase enzyme causing an increase in bioluminescence, which can be misinterpreted as increased transcriptional activation of the gene [35–37]. Another possibility, is that the discrepancies between findings in the *Hepcidin* luciferase assay and the *Hepcidin* quantitative realtime RT-PCR assay are caused by the absence of distal elements in the 2.7 kb fragment of the *Hepcidin* promoter-Luciferase construct that are present in the endogenous *Hepcidin* promoter. For these reasons, we believe that it is not surprising that 24% of the 21 hits that we identified did not produce the anticipated effect on *Hepcidin* transcript levels in the quantitative RT-PCR assay.

In our previous work, we identified genistein as a small molecule that increases *Hepcidin* expression in human hepatocytes and zebrafish embryos by activating both bone morphogenic protein and Stat3 signaling pathways [18]. Genistein strongly upregulated transcript levels of *ID3* and *SOCS3* [18], BMP- and Stat3-dependent genes, respectively, thus we assayed for effects on expression of these genes as a shorthand for BMP and Stat3-dependent gene expression associated with treatment by the hits identified in the screen. We found that all the hits that increased *Hepcidin* expression in the screen upregulated one or both of these genes (Figs. 2A–C). Thus we were able to classify the hits by their association with BMP or Stat3 signaling pathways (Fig. 2D).

Interestingly, none of the chemicals tested caused enhanced phosphorylation of Smad1,5,8 or Stat3. While Western blots for P-Smad1,5,8 appeared to be highly sensitive, indicating a clear increase in P-Smad1,5,8 signal to Smad1 for hepatocytes treated with BMP6 (Fig. 4A), Western blots for PStat3 to Stat3 (Fig. 4B) were less sensitive and unable to detect the 3-fold increase in PStat3 to Stat3 that we had previously observed with an ELISA assay [18] performed on HepG2 cells treated with IL-6 for at the same concentration and conditions used in these experiments. Thus it is possible that the chemicals activated Stat3 at a level that was below the limit of detection for this assay. We did observe, however, that Stat3 protein levels significantly increased in hepatocytes after 24 h of treatment with lansoprazole or vorinostat (Supplementary Fig. 1). It appears likely that the chemicals either potentiated or stabilized Smad or Stat3 binding to the *Hepcidin* promoter without increasing phosphorylation of the proteins, caused phosphorylation at a later time point, which would most likely be an indirect effect after other signal transduction cascades were activated, or acted via other pathways.

Potent agonists

The two most potent agonists, ipriflavone and vorinostat, active at 1 μM concentrations, were 10-fold more potent than genistein [18]. Interestingly, ipriflavone, like genistein, is an isoflavone with estrogenic properties [38]. Ipriflavone is used to treat osteoporosis based on its ability to inhibit osteoclast activity, promote mineralization of osteoblasts [39], and increase bone mineral density in postmenopausal women [40]. However, our previous work indicated that estradiol does not increase *Hepcidin* expression and that blockade of the estrogen receptor fails to inhibit genistein's effect on *Hepcidin* expression [18], thus we think it is unlikely that ipriflavone is promoting *Hepcidin* expression in an estrogenic manner. Similar to our observation of genistein [18], ipriflavone increased expression of the BMP-dependent gene, *ID3* (Fig. 2B), however, unlike genistein, ipriflavone did not

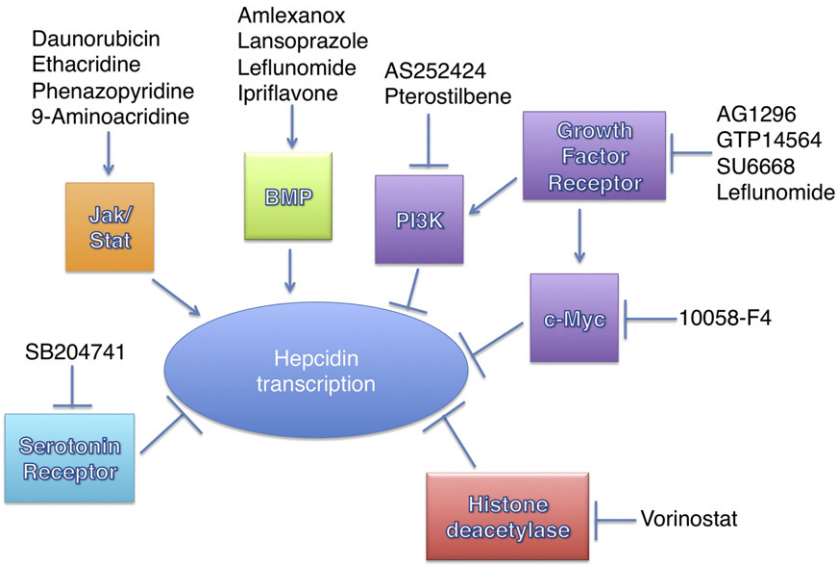

Fig. 5. Schematic illustrating the pathways implicated in transcriptional regulation of *Hepcidin* by the small molecules identified as *Hepcidin* stimulators in the screen. → indicates a stimulatory effect, while ⊣ indicates an inhibitory effect.

increase expression of the Stat3-dependent gene, SOCS3 (Fig. 2C), or increase Stat3 phosphorylation (Fig. 4B).

Tyrosine kinase inhibitors

Several of the hits that increased *Hepcidin* transcript levels were tyrosine kinase inhibitors affecting growth factor signaling (Fig. 5), including SU6668, GTP 14564, and AG1296. SU6668 inhibits VEGF, FGF, and PDGF receptors [27]. We found that SU6668 exhibited the paradoxical effect of inhibiting *Hepcidin*-luciferase activity, but increasing *Hepcidin* transcript levels in the quantitative realtime RT-PCR experiments. GTP 14564 and AG1296, however, both increased *Hepcidin*-luciferase activity and *Hepcidin* transcript levels in quantitative realtime RT-PCR assays. GTP 14564 is a potent inhibitor of FLT3, c-Fms, c-Kit, and PDGFRβ [25], while AG1296 inhibits signaling by both PDGF-α and β receptors and by c-Kit, without affecting VEGF receptor signaling [26]. We demonstrated that AG1296 or GTP 14564's stimulatory effects on the *Hepcidin* promoter can be significantly impaired by co-treating with EGF, FGF, or FLT3 (for AG1296 or GTP 14564) or PDGF or VEGF (for GTP 14564). Both PDGF-α and β receptors signal via PI3 Kinase, among other pathways, and can activate Src leading to transcription of *c-Myc* [41]. Two of the other *Hepcidin* stimulating agents that we identified in the screen, AS252424 and 10058-F4, affect pathways that can act downstream of PDGF receptor. AS252424 inhibits PI3 Kinase isoform γ [42], while 10058-F4 blocks c-Myc's activity [43,44]. Interestingly, pterostilbene, another *Hepcidin* agonist that we identified in the screen, is a naturally occurring polyphenolic antioxidant compound that has been shown to inhibit human growth factor signaling via PI3 Kinase in breast cancer cells and PDGF-driven proliferation in vascular smooth muscle cells [45].

To facilitate high throughput screening, we performed our screen entirely in human hepatoma cells (HepG2). As hepatocellular carcinoma cells exhibit increased signaling by FGF, PDGF, and VEGF, this may have biased our results to identify antagonists of growth factor signaling. Others, however, have used small scale screening of kinase inhibitors to demonstrate that hepatocyte growth factor (HGF) and epidermal growth factor (EGF) reduce BMP-stimulated *Hepcidin* expression in a mitogen-activated ERK kinase/extracellular signal-related kinase (MEK/ERK) dependent manner in primary mouse hepatocytes [24]. HGF's inhibitory activity on *Hepcidin* expression can be suppressed by pre-treatment with small molecule inhibitors of Met (PHA665752), MEK1/2 (U0126), or PI3Kinase (LY2940021) [24]. Furthermore intraperitoneal injection of EGF in wild type mice reduces the induction of *Hepcidin* expression in response to iron loading [24]. Given these findings, we propose that SU6668, GTP 14564, AG1296, AS252424, 10058-F, and pterostilbene enhance *Hepcidin* transcript levels in HepG2 cells by inhibiting growth-factor dependent signaling.

HDAC inhibitors

One of the most interesting findings in the screen is that the nonselective histone deacetylase (HDAC) inhibitor, vorinostat, is a potent stimulant of *Hepcidin* expression. These data are consistent with the finding that histone acetylation increases *Hepcidin* expression [4,46,47]. In particular, post-translational modification of Histone H3, one of the core proteins of the nucleosome, regulates transcription and chromatin condensation [48]. Transfection of Smad4 into Smad4-null hepatocytes increases binding of Histone 3 acetylated at lysine 9 (H3K9) to the *Hepcidin* promoter [4]. Histone acetylation also appears to affect Stat3 binding to the *Hepcidin* promoter. Hepatitis C viral infection of cultured hepatoma cells causes hypoacetylation of histones and decreased *Hepcidin* expression, while treatment with the pan-HDAC inhibitor, trichostatin A, increases Stat3 binding to the *Hepcidin* promoter [46] and enhances *Hepcidin* expression [46,47]. Vorinostat has been approved for the treatment of refractory cutaneous T-cell lymphoma [49] and thus may be amenable to clinical investigation in patients with iron overload syndromes who produce inappropriately low levels of Hepcidin.

Drugs that increase ID3 expression

The anti-inflammatory drugs, amlexanox, lansoprazole, and leflunomide each increased *Hepcidin* expression and *ID3* expression in the screen. Amlexanox is an anti-allergic drug that binds the cytoskeletal protein S100A13 and inhibits heat shock-induced release of FGF1 [50]. Lansoprazole, a drug commonly used to treat stomach ulcers, inhibits the H+/K+-adenosine triphosphatase in gastric parietal cells, but it also has been shown to have anti-inflammatory properties, in the esophagus, intestine, and lung and can stimulate heme oxygenase-

1 expression [51]. Leflunomide, used for the treatment of rheumatoid arthritis, yields an active metabolite, A771726, which potently blocks pyrimidine synthesis by inhibiting dihydroorotate synthase. At higher concentrations, however, such as those used in this study, leflunomide inhibits phosphorylation of PDGF receptor or EGF receptor (IC_{50} 30–55 and 150–200 μM, respectively) [52].

The chemical screen described here also demonstrated a potential role for serotonin receptor 2B in regulating *Hepcidin* expression. We found that SB 204741, a serotonin receptor 2B (5-HT2B) antagonist, increased *Hepcidin* expression and *ID3* expression. Serotonin stimulates proliferation of hepatocellular carcinoma cells [53], but represses liver regeneration via effects on hepatocyte stellate cells [54]. Animal studies indicate that the 5-HT2B inhibitor, SB 204741, confers the converse effect, decreasing growth of human hepatocellular carcinoma xenografts in mice [53], but enhancing liver regeneration following partial hepatectomy in animal models [54].

Drugs that increase SOCS3 expression

Daunorubicin, ethacridine lactate, phenazopyridine, and 9-aminoacridine each increased *Hepcidin* transcript levels and expression of the Stat3-dependent gene, *SOCS3*. As Stat3 is critically involved in liver injury and regeneration [55], it may be that these drugs stimulate *Hepcidin* expression by facilitating cell injury. Daunorubicin is an anticancer drug and DNA intercalator that inhibits Topoisomerase II resulting in breaks in double stranded DNA and increased apoptosis [56]. Daunorubicin has also been shown to increase expression of Stat3-dependent genes, such as *SOCS3* [57]. Ethacridine lactate provokes uterine contractions and histamine release [58], but also inhibits poly(ADP-ribose) glycohydrolase [59], the major enzyme that catabolizes poly(ADP-ribose). Inhibition of poly(ADP-ribose) glycohydrolase has been shown to promote apoptosis and impair DNA repair in cells damaged by oxidative stress [60]. Phenazopyridine can cause liver injury [61,62], while 9-aminoacridine is a DNA intercalator and experimental mutagen [63].

Conclusions

As a result of our screen, we have identified 16 small molecules that increase *Hepcidin* transcript levels in human HepG2 cells. Several of these chemicals affect growth factor receptor signaling, have anti-inflammatory properties, or impact DNA repair and apoptosis. The identification of multiple inhibitors of growth factor receptors and their downstream targets (Fig. 5) indicates the importance of this pathway in regulating *Hepcidin* expression, while the discovery of inhibitors of histone deacetylase and serotonin receptor as *Hepcidin* stimulating agents indicates new avenues for further study. While each of these candidate molecules was associated with increases in transcript levels of other BMP and/or Stat3 associated genes, none of them exhibited a strong effect on Smad1,5,8 or Stat3 phosphorylation. Further studies will be needed to determine how each of these different molecules functions to increase *Hepcidin* transcript levels. We also plan experiments to determine if these chemicals are effective in raising *Hepcidin* levels in vivo. In the future, we would like to test these candidate Hepcidin stimulatory chemicals in animal models of iron overload to determine if they could be adapted into therapeutic agents for patients with iron overload syndromes.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bcmd.2014.06.002.

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