Published in final edited form as:

Science. 2017 May 12; 356(6338): 608–616. doi:10.1126/science.aah3862.

Restored iron transport by a small molecule promotes absorption and hemoglobinization in animals

Anthony S. Grillo¹, Anna M. SantaMaria³, Martin D. Kafina⁴, Alexander G. Cioffi³, Nicholas C. Huston⁴, Murui Han⁷, Young Ah Seo⁸, Yvette Y. Yien⁴, Christopher Nardone³, Archita V. Menon⁷, James Fan¹, Dillon C. Svoboda³, Jacob B. Anderson¹, John D. Hong¹, Bruno G. Nicolau¹, Kiran Subedi¹, Andrew A. Gewirth¹, Marianne Wessling-Resnick⁹, Jonghan Kim⁷, Barry H. Paw^{4,5,6}, and Martin D. Burke^{1,2,3}

¹Department of Chemistry, University of Illinois at Urbana-Champaign

²Carle-Illinois College of Medicine, University of Illinois at Urbana-Champaign

³Department of Biochemistry, University of Illinois at Urbana-Champaign

⁴Department of Medicine, Division of Hematology, Brigham and Women's Hospital, Harvard Medical School

⁵Department of Medicine, Division of Hematology-Oncology, Boston Children's Hospital, Harvard Medical School

⁶Department of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School

⁷Department of Pharmaceutical Sciences, Northeastern University

⁸Department of Nutritional Sciences, University of Michigan School of Public Health

⁹Department of Genetic and Complex Diseases, Harvard TH Chan School of Public Health

Abstract

Multiple human diseases ensue from a hereditary or acquired deficiency of iron-transporting protein function that diminishes transmembrane iron flux in distinct sites and directions. Because other iron-transport proteins remain active, labile iron gradients build up across the corresponding protein-deficient membranes. Here we report that a small molecule natural product, hinokitiol, can harness such gradients to restore iron transport into, within, and/or out of cells. The same compound promotes gut iron absorption in DMT1-deficient rats and ferroportin-deficient mice, as well as hemoglobinization in DMT1- and mitoferrin-deficient zebrafish. These findings illuminate a general mechanistic framework for small molecule-mediated site- and direction-selective

Correspondence to: Marianne Wessling-Resnick; Jonghan Kim; Barry H. Paw; Martin D. Burke.

SUPPLEMENTARY MATERIALS

Materials and Methods

Supplementary Text

Figs. S1 to S31

Tables S1 to S8

Caption for Movie S1

References (53-109)

Movie S1

restoration of iron transport. They also suggest small molecules that partially mimic the function of missing protein transporters of iron, and possibly other ions, may have potential in treating human diseases.

Site- and direction-selective transmembrane ion transport is achieved in most living systems via the concerted functions of active ion-transport proteins that generate localized electrochemical gradients and the passive ion-transport proteins that use them (1). Deficiencies of passive ion-transport proteins cause many human diseases including anemias, cystic fibrosis, arrhythmias, and neurological, skeletal muscle, endocrine, and renal disorders (2-5). Because the corresponding active ion-transport proteins typically remain functional, there may be a build-up of ion gradients upstream of the membranes that normally host these missing proteins. Noting the capacity for these robust networks to achieve ion-selective transport despite the unselective nature of many ion-transport proteins (1, 2), we hypothesized that small molecules capable of autonomously performing ion transport could leverage such gradients to restore transmembrane ion flux in a site- and direction-selective manner (Fig. 1A).

Iron homeostasis, in particular, is maintained by dynamic networks of active and passive iron-transport proteins and their regulators that permit essential use while minimizing toxicity of this redox-active metal (2). No known regulatory mechanisms of iron excretion exist (6), and thus systemic iron levels are primarily controlled through rigorous regulation of dietary iron absorption (2, 6). Deficiencies or dysfunction of proteins involved in iron transport, homeostasis, or metabolism often impede the movement of iron into, within, and/or out of cells (Fig. 1A), and are associated with more than twenty-five Mendelian diseases (table S1) (6-9). We questioned whether a small molecule iron transporter could leverage transmembrane gradients of the labile iron pool (2) that selectively build up in such situations to restore the movement of iron into, within, and/or out of cells, and thereby enable its use in endogenous iron-dependent physiological processes (Fig. 1A).

We specifically chose to study three disease-relevant iron transporter deficiencies that disrupt iron movement in different directions, cellular locations, and tissues (2, 6). Deficiencies of divalent metal transporter 1 (DMT1, aka Nramp2, DCT1, SLC11A2) reduce apical iron uptake into duodenal enterocytes (fig. S1A) and prevent endosomal iron release in red blood cell progenitors (fig. S1B) (2, 6). Mitoferrin (Mfrn1, aka SLC25A37) deficiencies in the inner mitochondrial membrane impair iron import into the mitochondrial matrix (fig. S1C) (10, 11). Ferroportin (FPN1, aka IReg1, MTP1, SLC40A1) deficiencies reduce iron efflux from gut epithelium (fig. S1D) and from reticuloendothelial macrophages (fig. S1E) (12-15).

Previous reports suggest high doses of hydrophilic iron chelators, such as deferiprone and pyridoxal isonicotinoyl hydrazone (PIH), as well as more lipophilic derivatives such as salicylaldehyde isonicotinoyl hydrazone (SIH), may bind and relocate excess iron (16, 17). However, the corresponding complexes of many of these chelators show limited membrane permeation and may require the action of co-localized proteins to achieve iron mobilization (18, 19). We alternatively sought to identify a lipophilic small molecule that can

autonomously perform transmembrane iron transport to promote physiology in cells and animals missing each of the aforementioned proteins.

Small molecule-mediated functional complementation in yeast

To find such a molecule, we designed a modified functional complementation experiment (20), in which candidate compounds known or predicted to bind iron were tested for their capacity to restore growth to a strain of *Saccharomyces cerevisiae* missing the iron transporting complex FetFtr1 (fet3 ftr1⁻, fig. S1F) (21). Deferiprone, PIH, and SIH showed no growth rescue (fig. S2B). In contrast, the natural product hinokitiol (Hino, β -thujaplicin, Fig. 1B), originally isolated by Nozoe from essential oil of the *Chamaecyparis taiwanensis* (*Taiwan Hinoki*) tree (22), was highly effective (Fig. 1C-E and fig. S3A). This natural product has previously been characterized as a potent chelator of iron and other metals (23-26) that exerts a range of other biological activities (25-31). Hinokitiol restored growth to iron transporter-deficient yeast under fermentative and respiratory conditions (Fig. 1D and fig. S3A) and independent of known siderophore transporters (fig. S3B) (21, 32). Hinokitiol sustainably restores growth to wild type levels with similar doubling times (Fig. 1E and fig. S3C-E).

Synthetic removal of the C-2 oxygen atom by hydrogenolysis yielded C2-deoxy hinokitiol (C2deOHino, Fig. 1B and fig. S3F). In contrast to hinokitiol, C2deOHino cannot bind or transport iron and thus served as a negative control (fig. S4A, B). Hinokitiol dose-dependently restores yeast growth whereas C2deOHino does not (Fig. 1F). Hinokitiol, but not C2deOHino, also restores iron influx (Fig. 1G) and hinokitiol-mediated growth is iron-dependent (fig. S3G, H). Growth restoration was similarly observed with other lipophilic α -hydroxy ketones, but not with hydrophilic α -hydroxy ketones nor small molecules that transport other ions (fig. S2A-C and fig. S3I).

Characterization of iron binding and transport with hinokitiol

Biophysical experiments were performed to better understand the capacity for hinokitiol to bind and transport ferrous and ferric iron across lipid membranes. This natural product rapidly binds iron to form a hinokitiol:iron complex, as evidenced by an immediate change in color and UV-Vis spectra upon addition of ferric or ferrous iron (Fig. 2A, B and fig. S4A, C). Unlike water-soluble iron chelators (17), hinokitiol:iron complexes predominantly partition into non-polar solvents over water (Fig. 2A and fig. S3I). For example, >95% of hinokitiol:iron complexes partition into octanol over water, whereas deferiprone:iron complexes alternatively exhibit >95% partitioning into water (fig. S3I). This is consistent with quantitative extraction of hinokitiol:iron complexes from the aqueous to the organic layer as determined via ICP-MS analysis (fig. S4D).

Hinokitiol strongly binds ferrous and ferric iron, with a $K_A = 5.1 \times 10^{15}$ for ferrous iron and $K_A = 5.8 \times 10^{25}$ for ferric iron, the latter of which is more than an order of magnitude stronger than deferiprone (fig. S4E-H and table S2). Consistent with its high affinity, hinokitiol removes iron from iron-citrate complexes that compose the labile iron pool (fig. S4A). In buffered solution, competition experiments indicate hinokitiol can also remove iron

from iron-binding proteins transferrin and ferritin, but only when hinokitiol is used in >1,000-fold excess relative to transferrin and >1,000,000-fold excess relative to ferritin (fig. S4I-K). Hinokitiol has a $pK_a = 7.33$ suggesting both the neutral and anionic states are accessible under physiological conditions (fig. S4L). Moreover, ^{56}Fe bound to hinokitiol readily exchanges with ^{55}Fe in solution, with >20% exchange observed within 10 minutes (fig. S4M). Thus, the binding of iron by hinokitiol under physiological conditions is expected to be highly dynamic, which may allow for the facile release of iron from hinokitiol complexes to iron-binding proteins and its subsequent use in iron-related physiological processes.

Hinokitiol autonomously transports both ferrous and ferric iron across model liposomal membranes whereas C2deOHino, deferiprone, and PIH show minimal transport (Fig. 2C, D). Although the transport-active complex remains to be identified, speciation studies are consistent with the predominant formation of a 3:1 Hino:Fe^{III} complex in aqueous buffer (fig. S4N, O). X-ray crystallography of tris(hinacolato) iron (III) revealed a pair of C1-symmetric complexes, each composed of a lipophilic outer shell encasing a hydrophilic and iron-binding central core (Fig. 2E and fig. S4P).

Hinokitiol is a broad spectrum metallophore capable of binding and transporting multiple divalent metals (fig. S5A-I, and table S3). Hinokitiol competitively bound 10-fold more Cu^{II} than Fe^{II} and transported Cu^{II} 80-fold faster than Fe^{II} in liposomes, yet the low accessibility of copper likely leads to high iron selectivity *in vivo*. Specifically, the cytosolic labile copper pool is ten billion times lower than iron (table S3) (33-35). This is attributed to robust networks of transporters, chaperones, storage proteins, and regulators that bind Cu^{II} with exceptional affinities and selectivities (35). For example, the transcriptional activator Mac1, which is essential in regulating yeast copper homeostasis, binds copper with a K_D of 9.7×10^{-20} M (35). Upon treatment of *fet3 ftr1* yeast with hinokitiol, intracellular iron levels increased relative to vehicle-treated controls, whereas levels of manganese, cobalt, nickel, zinc, and copper were unchanged (fig. S5J).

The redox potential of Fe(Hino)₃ in aqueous systems is estimated to be as low as -361 mV, compared to +770 mV for free iron (Fig. 2F, fig. S6A-J, and table S2, 4-6). Consistent with this, in a reducing environment the reduction of iron (III) is slowed in the presence of hinokitiol, but still nearly quantitative in less than two hours (fig. S6K, L). Moreover, the redox potential increases with decreasing pH and decreasing hinokitiol concentrations (fig. S6F, I, J and table S4, 5). Collectively, these data suggest that both ferric and ferrous iron should be readily accessible in the presence of hinokitiol under physiological conditions.

Restored iron transport promotes absorption and hemoglobinization in cells

We thus asked whether hinokitiol could promote iron movement into, within, and/or out of mammalian cells deficient in DMT1, Mfrn1, or FPN1. We first studied iron uptake and transepithelial transport in differentiated DMT1-deficient Caco-2 gut epithelia monolayers (fig. S1A) (36, 37) established through stable shRNA transfection (fig. S7A-C). Relative to wild type controls, DMT1-deficient monolayers showed reduced iron uptake into cells and

reduced transepithelial iron transport to the basolateral fluid after apical addition of $^{55}\text{FeCl}_3$ (Fig. 3A, B). Apical addition of hinokitiol (500 nM) restored uptake and transport (Fig. 3A, B) in a timeframe commensurate with dwell times in the gut (Fig. 3C). Hinokitiol did not disrupt monolayer integrity (fig. S7D), caused no observable toxicity (table S7), and did not affect basal DMT1 expression (fig. S7B, C). Hinokitiol-mediated transport occurs across a range of pHs found throughout the duodenum, and increases with decreasing pH (fig. S7E). Whereas hinokitiol promotes uptake and transport over a wide range of concentrations, C2deOHino and sub-toxic concentrations of iron chelators deferiprone, deferoxamine, PIH, and SIH did not promote both uptake and transport (fig. S7F, G and table S7). High concentrations of these more hydrophilic iron chelators alternatively decreased iron uptake into DMT1-deficient monolayers (fig. S7F).

If DMT1 is missing, depleted, or hypomorphic, intracellular iron (II) efflux from endosomes of erythroid precursors is precluded, thus preventing hemoglobinization (fig. S1B) (2, 6, 38). We tested for DMSO-induced differentiation and hemoglobinization in DS19 murine erythroleukemia (MEL) cells (39), as well as in shRNA-transfected DMT1-deficient MEL cells (fig. S8A-C), in the absence or presence of hinokitiol. Control cells differentiated normally after three days as indicated by the characteristic pink color of hemoglobin in cell pellets (Fig. 3D) and brown staining of hemoglobinized cells with α -dianisidine (fig. S8D, E). Reduced hemoglobinization was observed in DMT1-deficient cells (Fig. 3D-F, and fig. S8D-F). Three days of hinokitiol treatment (1 μM) restored ^{55}Fe uptake (fig. S8F), ^{55}Fe -heme incorporation (Fig. 3F), and hemoglobinization (Fig. 3D and fig. S8D-J) without observable toxicity (fig. S8K and table S7) whereas C2deOHino had no effect (Fig. 3E, F and fig. S8F, I, J). As expected, no differentiation was observed in the absence of DMSO with or without hinokitiol treatment (fig. S8L).

Having observed hinokitiol-mediated transport of iron into and within DMT1-deficient cells, we asked whether the same small molecule could also substitute for other iron-transport proteins. Mfrn1 in the inner mitochondrial membrane imports iron into the mitochondrial matrix for hemoglobinization (fig. S1C) (2, 10). Mfrn1-deficient MEL cells developed through CRISPR-Cas9-mediated knockout (fig. S9A) exhibited reduced hemoglobinization by α -dianisidine staining (Fig. 3G), ^{55}Fe uptake (fig. S9B), and ^{55}Fe -heme incorporation (fig. S9C) after DMSO induction. Hinokitiol (1 μM) restored hemoglobinization whereas C2deOHino showed no effect (Fig. 3G and fig. S9B, C), suggesting hinokitiol-mediated mitochondrial delivery of iron. As expected, hinokitiol did not promote hemoglobinization to MEL cells alternatively deficient in a protein involved in porphyrin biosynthesis (TMEM14C) (fig. S9D-F)

FPN1 deficiencies reduce iron efflux across the basolateral membrane of gut epithelia (fig. S1D) and from reticuloendothelial macrophages (fig. S1E) that recycle iron from senescent erythrocytes (13, 14). Quercetin (40) and hepcidin (41) were used to transiently decrease FPN1 levels in differentiated Caco-2 epithelia monolayers and J774 macrophages (41), respectively (fig. S9G-J). Hinokitiol (1 μM) restored transepithelial iron transport in FPN1-deficient Caco-2 monolayers (Fig. 3H and fig. S9K) without affecting iron uptake (Fig. 3I) nor disrupting monolayer integrity (fig. S9L). Hinokitiol also time- and dose-dependently

restored iron release from FPN1-deficient J774 macrophages without observable toxicity (Fig. 3J, K, fig. S9M, and table S7).

Site- and direction-selective build-up and release of iron gradients

We next probed the mechanistic hypothesis that hinokitiol promotes site- and direction-selective iron movement by harnessing built-up transmembrane iron gradients in transporter-deficient systems (Fig. 1A). We first visualized compartmentalized iron in DMT1-deficient MEL cells with fluorescent dyes (fig. S10A-C) (42, 43). An oxyburst green-BSA conjugate localized to endosomes fluoresces upon iron-mediated oxidation (fig. S10C), and fluorescence emissions from the turn-off probes calcein green (fig. S10A) and RPA (fig. S10B) in the cytosol and mitochondria, respectively, are quenched upon iron binding. Relatively low endosomal, high cytosolic, and high mitochondrial iron levels were observed in induced shControl MEL cells (Fig. 4A and fig. S11A, B, E, H). We observed 2-fold increases in iron-promoted oxyburst green fluorescence in DMT1-deficient MEL cells (Fig. 4A and fig. S11A, B) along with reduced cytosolic and mitochondrial iron (Fig. 4A and fig. S11A, E, H). Hinokitiol treatment decreased oxyburst green fluorescence 2.1-fold and concomitantly quenched calcein green and RPA fluorescence (Fig. 4A and fig. S11A-J). These data support hinokitiol-mediated release of built-up pools of endosomal iron into the cytosol and subsequent mitochondrial uptake.

Calcein green and ^{55}Fe studies also revealed a build-up of labile iron in FPN1-deficient J774 macrophages relative to wild type cells (Fig. 4B-D). Hinokitiol direction-selectively promotes both iron influx (Fig. 4E) and efflux (Fig. 3J and fig. S9M) from J774 macrophages depending on the presence of high extracellular or intracellular iron, respectively. Further, hinokitiol-mediated iron (II) and iron (III) efflux from liposomes and iron (III) uptake into J774 macrophages is directly proportional to the transmembrane iron gradients (Fig. 4E-G and fig. S12A-D). Finally, we loaded iron into J774 macrophages, rinsed the cells to remove extracellular iron, and stained with calcein green (fig. S12E). Hinokitiol addition ($t = 5$ min) rapidly increased calcein green fluorescence whereas vehicle and C2deOHino had no effect (Fig. 4H, I, fig. S13A-C, and Movie S1). We then reversed the gradient in these same cells via external addition of FeCl_3 ($t = 12$ min) (fig. S12E). DMSO or C2deOHino treated cells had no effect (Fig. 4I and fig. S13A, C), whereas quenching of calcein green fluorescence was observed with hinokitiol treatment (Fig. 4H, I, fig. S13B, and Movie S1). These results are consistent with initial hinokitiol-mediated release of iron from J774 macrophages when intracellular iron levels are high, followed by hinokitiol-mediated uptake of iron into these macrophages when this transmembrane gradient is reversed by addition of extracellular iron (fig. S12E).

Mechanisms for maintaining iron homeostasis

We next asked whether endogenous networks of other ion-transport proteins and regulators (2) in iron transporter-deficient cells can collaborate with the small molecule, hinokitiol, to help promote restoration of site- and direction-selective iron transport while still maintaining iron homeostasis. In yeast, the intracellular movement and storage of iron is dependent on a proton gradient known as the proton motive force, which is generated by the ATP-dependent

active ion-transport proteins Pma1 and V-ATPase in the plasma and vacuolar membranes, respectively (21, 35). Consistent with the dependence of hinokitiol-mediated iron transport on this proton motive force, hinokitiol-rescued *fet3 ftr1* yeast are exceptionally sensitive to chemical inhibition of Pma1 and V-ATPase, but not to off-pathway inhibitors (fig. S14A-C).

In intestinal epithelia, iron-transport proteins are transcriptionally and translationally regulated to maintain systemic iron levels while avoiding overload (2, 44). Specifically, levels of the apical H^+/Fe^{2+} symporter DMT1, heavy (FTH1) and light (FTL1) chains of ferritin responsible for sequestering excess iron, basolateral efflux protein FPN1, and transferrin receptor 1 (TfR1) are translationally regulated through short hairpin iron response elements (IREs) located at the 5' - and 3' -untranslated regions of the corresponding mRNA transcripts (fig. S15A) (2). Iron-sensing iron response proteins (IRP1 and IRP2) bind to these IREs to block translation (5' -IRE, *Fth1*, *Ftl1*, *Fpn1*) or stabilize mRNA (3' -IRE, *Dmt1*, *TfR1*) under iron starvation (fig. S15A). Upon iron stimulation and binding, IRPs dissociate from the mRNAs, reversing their described effects. Transcriptional regulation is achieved through the transcriptional activator, hypoxia-inducible factor 2-alpha (Hif2 α), which is degraded after O₂ and iron-mediated proline hydroxylation (fig. S15B) (2). Hif2 α activates transcription of *Fpn1* to evade IRE-mediated translational repression under iron deprivation (2).

Consistent with these homeostatic mechanisms, an anemic state (45) is initially observed in DMT1-deficient Caco-2 monolayers, with decreased levels of ferritin and increased levels of FPN1 (Fig. 5A and fig. S14D-L), thus providing a favorable cellular environment for small molecule-mediated iron transport. Providing support for functional collaboration with these endogenous proteins, hinokitiol-mediated iron uptake and transport across DMT1-deficient Caco-2 monolayers is unidirectional (Fig. 5B and fig. S14M). Apical treatment with this low dose of hinokitiol (500 nM) allows for ⁵⁵Fe incorporation into ferritin (Fig. 5C), possibly mediated by the high affinity iron chaperone Poly (rC)-binding protein 1 (PCBP1) (2). Finally, quercetin-mediated knockdown of FPN1 (40) antagonizes hinokitiol-mediated transmembrane transport without affecting apical uptake (Fig. 5D and fig. S14N-P).

Moreover, increased rates of transepithelial transport in DMT1-deficient monolayers were observed with increased concentrations of apical FeCl₃, but these effects level off at higher concentrations of iron (Fig. 5E). Further, a similar leveling of transmembrane transport is observed when the same monolayers are treated with a persistent iron gradient (25 μ M FeCl₃) and increasing concentrations of hinokitiol (Fig. 5F). This phenomenon was observed over a wide range of hinokitiol and iron concentrations (fig. S16). We thus asked how the endogenous system responds to the hinokitiol-mediated changes in cellular iron status in the presence of a persistent iron gradient. Consistent with IRP-mediated translational regulation, decreased IRP2, increased ferritin subunits (5' -IREs), and decreased TfR1 (3' -IRE) protein levels were observed as a function of hinokitiol concentrations up to 5 μ M in the presence of a persistent iron gradient (Fig. 5G and fig. S17A-E). The transcription factors Hif1 α and Hif2 α similarly decreased along with decreased *Fpn1* mRNA and protein levels (Fig. 5G and fig. S17F-I). As expected, IRE-independent expression of the cytosolic iron chaperone PCBP1 and Hif2 α -independent *Fth1* mRNA levels did not change, and no changes in FPN1

were observed upon the addition of hinokitiol in the absence of iron (Fig. 5G and fig. S17K-N). A modest reversal of these effects was observed with higher concentrations of hinokitiol (Fig. 5G and fig. S17A-J). Visualization of cytosolic iron with calcein green indicated that incubation of DMT1-deficient Caco-2 monolayers with increasing hinokitiol led to increased labile iron up to 5 μ M (Fig. 5H-J). Further increases in hinokitiol prevented fluorescence quenching, possibly due to competitive intracellular chelation of labile iron with high doses of this strongly binding metallophore (Fig. 5H-J). These results collectively support the conclusion that the endogenous homeostatic networks can collaborate with the small molecule hinokitiol to help promote iron transport while maintaining its homeostasis and preventing ferritotoxicity.

Based on this mechanistic framework, we hypothesized hinokitiol would have relatively minimal effects in wild type cells. We tested the capacity for the same concentrations of hinokitiol to perturb transepithelial iron transport, hemoglobinization, and iron release in normal Caco-2 monolayers, MEL cells, and J774 cells, respectively (fig. S18A-F). In contrast to hinokitiol-promoted increases in transepithelial iron transport (Fig. 3B), hemoglobinization (Fig. 3G), and iron release (Fig. 3J) observed in the corresponding protein-deficient systems, negligible effects were observed in hinokitiol-treated wild type systems under identical conditions (fig. S18A-F). Collectively, these results are consistent with hinokitiol restoring site- and direction-selective iron transport by harnessing gradients that selectively build up across lipid membranes missing specific iron transporter proteins.

Restored gut iron absorption and peripheral hemoglobinization in animals

We were thus encouraged to ask whether hinokitiol could restore gut iron absorption and hemoglobinization in animal models of these iron transporter deficiencies. DMT1- and FPN1-deficiencies in duodenal enterocytes reduce rates of iron absorption in the gut by disrupting apical iron uptake into cells and basolateral efflux into the blood, respectively (2, 6, 12-15). We tested gut iron absorption in DMT1-deficient Belgrade (*b/b*) rats (6) and FPN1-deficient Flatiron (*ffe/+*) mice (14, 15) upon administration of a single dose of ^{59}Fe and 1.5 mg/kg hinokitiol via oral gavage. Higher doses of hinokitiol are reported to be non-toxic in rats upon chronic oral administration for two years (46). Similar to the reduced iron absorption previously reported in *b/b* rats (47), a 2-fold reduction in ^{59}Fe absorption was observed in C2deOHino-treated *b/b* rats relative to sibling controls (*+/+* or *+/b*) (Fig. 6A and fig. S19A). Treatment of *b/b* rats with hinokitiol increased ^{59}Fe absorption back to control levels after one hour (Fig. 6A and fig. S19A). Consistent with our previous results (15), *ffe/+* mice also absorbed iron at low rates (Fig. 6B). Hinokitiol increased ^{59}Fe absorption in *ffe/+* mice after both one and two hours (Fig. 6B and fig. S19B). A statistically significant increase in the rate of ^{59}Fe absorption was observed in hinokitiol-treated wild type mice after one hour, but not after two hours (fig. S19C).

We have previously shown restoration of hemoglobinization in *Mfrn1*-deficient zebrafish, via ectopic expression of *Mfrn1* protein with complementary RNA (10). *Danio rerio* is well established as a powerful model organism in the study of hematopoiesis (48), and was employed to alternatively test whether chronic treatment with a small molecule iron transporter could restore hemoglobinization in DMT1- and *Mfrn1*-deficiencies (10, 49). We

first performed morpholino-mediated transient knockdown of DMT1 in a *Tg(globinLCR:eGFP)* zebrafish strain expressing GFP-tagged erythrocytes (50). Injection of a designed anti-sense morpholino targeting the exon 4/intron 4 junction of premature *Dmt1* mRNA reduced steady-state *Dmt1* levels (fig. S19D) and decreased the number of GFP-positive erythroid cells by FACS analysis (Fig. 6C). Addition of hinokitiol to the water twenty-four hours post fertilization (hpf) and incubation for an additional two days promoted hemoglobinization in these DMT1-deficient morphant zebrafish without observable toxicity, whereas C2deOHino had no effect (Fig. 6C). We further tested whether hinokitiol could similarly restore hemoglobinization in genetically mutated *Chardonnay* (*cdy^{te216}*) zebrafish, which contain a nonsense mutation leading to truncated DMT1 and thereby exhibit severe hypochromic, microcytic anemia (49). A heterozygous cross of *+/-cdy* fish led to a Mendelian distribution of ~75% healthy (*+/+* and *+/-cdy*) and ~25% anemic (*cdy/cdy*) embryos in each clutch after α -dianisidine staining 72 hpf (Fig. 6D). Hinokitiol treatment for two days increased the number of fish exhibiting high hemoglobin levels, whereas C2deOHino had no effect (Fig. 6D).

We also tested hemoglobinization in *Mfrn1*-deficient morphant *Tg(globinLCR:eGFP)* zebrafish (10, 50). Forty-eight hours of hinokitiol treatment again restored hemoglobinization and the number of GFP-positive erythrocytes in these morphants (Fig. 6E). Finally, we tested whether hinokitiol could restore hemoglobinization in genetically mutated *Frascati* (*frs^{tq223}*) zebrafish, which contain a missense mutation leading to an inactive *Mfrn1* mitochondrial protein and profound anemia during embryogenesis (10, 11). Hinokitiol treatment of embryos collected from a heterozygous cross of *+/-frs* fish rescued the anemic phenotype (Fig. 6F). Genotyped (Fig. 6G) healthy larvae (*+/+* and *+/-frs*) exhibit brown staining with α -dianisidine, whereas untreated *frs/frs* fish do not (Fig. 6H). Hinokitiol treatment restored brown staining to *frs/frs* fish (Fig. 6H). As expected, hinokitiol did not rescue *sauternes* (*saut^{tb223}*) zebrafish (51) deficient in the initial enzyme involved in porphyrin biosynthesis (*Alas2*) (fig. S19E), indicating the specificity of rescue to defects in iron transport.

Outlook

Thus, a small molecule can restore site- and direction-selective iron transport in different cells deficient in three distinct iron-transport proteins, and the same compound can promote dietary gut iron absorption or peripheral hemoglobinization in corresponding animal models. Mechanistic studies support the role of transmembrane ion gradients that build up in the setting of missing iron transporters, enabling hinokitiol to restore site- and direction-selective transmembrane iron transport. Further, endogenous protein-based homeostatic mechanisms interface with this imperfect small molecule to promote iron-related physiological processes without disrupting other cellular processes.

Like hinokitiol, many ion-transport proteins are imperfectly selective. However, the relative abundance of different ions contributes to increased selectivity in living systems. For example, protein chloride channels are largely unselective for chloride versus bromide and iodide, but the low natural abundance of the latter halogens favors chloride selectivity *in vivo* (1, 52). Differential ion accessibility further enhances the *in vivo* selectivity observed

for many imperfect ion-transport proteins (33-35). Like hinokitiol, DMT1 and FPN1 transport Co^{2+} , Mn^{2+} , Zn^{2+} , and/or Cu^{2+} (6, 13, 15). However, high affinity metalloproteins markedly decrease the labile pool of these other metals, leading to higher accessibility and thus selective binding and transport of iron *in vivo* (33-35).

These findings also provide a conceptual framework and proof-of-concept demonstration to support the pursuit of small molecule surrogates for missing or dysfunctional iron-transport proteins that underlie many human diseases. It has recently been recognized that acquired deficiencies of FPN1 underlie the anemia of chronic inflammation that frequently occurs in patients suffering from many common diseases, including rheumatoid arthritis, systemic lupus erythematosus, and inflammatory bowel disease (9). Further, this approach may have potential in promoting the rapid excretion of excess iron that builds up in tissues (e.g. liver or brain) in many diverse iron overload disorders.

These results also reveal frontier opportunities to probe biology using small molecules that autonomously perform protein-like functions. For example, our results demonstrate that in both cells and zebrafish, hinokitiol-mediated rescue of physiology can distinguish between defects in porphyrin biosynthesis and iron transport, which can be challenging to differentiate using genetic and biochemical techniques.

Because networks of active and passive ion-transport proteins similarly underlie the directional movement of many other ions in most living systems, including humans, these findings may also have even broader scientific and therapeutic implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge A. Ringel for assistance with zebrafish experiments and A. Li for assistance with octanol/water partition coefficients. We thank J. Hou, K. Muraglia, and R. Chorghade for independently repeating the results shown in Fig. 1C, fig. S2A-C, Fig. 3A, B, and fig. S9M. We further acknowledge D. Kosman and C. Philpott for providing yeast strains, L. Zon for the *Tg(globinLCR:Egfp)* transgenic zebrafish line, W. Boulanger for the generous gift of comenic acid, and J. Katzenellenbogen for use of liquid scintillation counter. We thank M. Garrick, L. Garrick, and M. Fleming for helpful discussions. We thank D. Gray and the George L. Clark X-Ray Facility for x-ray analysis, M. Sivaguru and the Carl R. Woese Institute for assistance with confocal microscopy, B. Pilas and the Roy J. Carver Biotechnology Center for help with flow cytometry, and the SCS microanalysis facility for ICP-MS/OES. M.D.B. thanks the NIH (GM118185) and HHMI for funding. A.S.G was an NSF Graduate Fellow. J.B.A. was supported by HHMI EXROP. Y.Y.Y. and B.H.P. were supported by NIH grants K01 DK106156 and F32 DK098866 (to Y.Y.Y.) and R01 DK070838 and P01 HL032262 (to B.H.P.). Y.A.S. was supported by NIH grant K99ES024340. M.W.-R. was supported by NIH grants R01ES0146380 and R01DK064750. JK was supported by NIH grant R21EB023025. The University of Illinois has filed a patent application (62/101,706) based on the findings described in this paper. Additional data reported in this paper are available in the Supplementary Materials. Crystallographic data for $\text{Fe}(\text{Hino})_3$ is available free of charge from the Cambridge Crystallographic Data Centre under reference CCDC 1536565.

References and Notes

1. Gouaux E, MacKinnon R. Science. 2005; 310:1461–1465. [PubMed: 16322449]
2. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Cell. 2010; 142:24–38. [PubMed: 20603012]
3. Shah V, et al. Science. 2016; 351:503–507. [PubMed: 26823428]

4. Yi F, et al. *Science*. 2016; 352:aaf2669. [PubMed: 26966193]
5. Imbrici P, et al. *Front Pharmacol*. 2016; 7:eCollection.
6. Andrews NC. *Nat Rev Genet*. 2000; 1:208–217. [PubMed: 11252750]
7. Andrews NC. *N Engl J Med*. 1999; 341:1986–1995. [PubMed: 10607817]
8. Lieu PT, Heiskala M, Peterson PA, Yang Y. *Mol Aspects Med*. 2001; 22:1–87. [PubMed: 11207374]
9. Weiss G, Goodnough LT. *N Engl J Med*. 2005; 352:1011–1023. [PubMed: 15758012]
10. Shaw GC, et al. *Nature*. 2006; 440:96–100. [PubMed: 16511496]
11. Chung J, et al. *J Biol Chem*. 2014; 289:7835–7843. [PubMed: 24509859]
12. Donovan A, et al. *Nature*. 2000; 403:776–781. [PubMed: 10693807]
13. McKie AT, et al. *Mol Cell*. 2000; 5:299–309. [PubMed: 10882071]
14. Zohn IE, et al. *Blood*. 2007; 109:4174–4180. [PubMed: 17289807]
15. Seo YA, Wessling-Resnick M. *FASEB J*. 2015; 29:2726–2733. [PubMed: 25782988]
16. Sohn YS, Breuer W, Munnich A, Cabantchik ZI. *Blood*. 2008; 111:1690–1699. [PubMed: 17975016]
17. Hatcher HC, Singh RN, Torti FM, Torti SV. *Future Med Chem*. 2009; 1:1643–1670. [PubMed: 21425984]
18. Huang XP, Spino M, Thiessen JJ. *Pharm Res*. 2006; 23:280–290. [PubMed: 16388408]
19. Buss JL, Hermes-Lima M, Ponka P. *Adv Exp Med Biol*. 2002; 509:205–229. [PubMed: 12572996]
20. Cioffi AG, Hou J, Grillo AS, Diaz KA, Burke MD. *J Am Chem Soc*. 2015; 137:10096–10099. [PubMed: 26230309]
21. Bleackley MR, MacGillivray RTA. *Biometals*. 2011; 24:785–809. [PubMed: 21479832]
22. Nozoe T. *Bull Chem Soc Japan*. 1936; 11:295–298.
23. Bryant BE, Fernelius WC. *J Am Chem Soc*. 2002; 76:1696–1697.
24. Barret MC, Mahon MF, Molloy KC, Steed JW, Wright P. *Inorg Chem*. 2001; 40:4384–4388. [PubMed: 11487346]
25. Nomiya K, et al. *J Inorg Biochem*. 2004; 98:46–60. [PubMed: 14659632]
26. Nomiya K, et al. *Inorg Chim Acta*. 2009; 362:43–55.
27. Meck C, D'Erasmo MP, Hirsch DR, Murelli RP. *MedChemComm*. 2014; 5:842–852. [PubMed: 25089179]
28. Ido Y, et al. *Cell Prolif*. 1999; 32:63–73. [PubMed: 10371304]
29. Murakami K, Ohara Y, Haneda M, Tsubouchi R, Yoshino M. *Basic Clin Pharmacol Toxicol*. 2005; 97:392–394. [PubMed: 16364055]
30. Lee MJ, Kim JW, Yang EG. *Biochem Biophys Res Commun*. 2010; 396:370–375. [PubMed: 20416277]
31. Bohme G, Schonfeld P, Kuster U, Kunz W, Lyr H. *Acta Biol Med Ger*. 1980; 39:1153–1163. [PubMed: 6787824]
32. Ghssein G, et al. *Science*. 2016; 352:1105–1109. [PubMed: 27230378]
33. Finney LA, O'Halloran TV. *Science*. 2003; 300:931–936. [PubMed: 12738850]
34. Ba LA, Doering M, Burkholz T, Jacob C. *Metallomics*. 2009; 1:292–311. [PubMed: 21305127]
35. Cyert MS, Philpott CC. *Genetics*. 2013; 193:677–713. [PubMed: 23463800]
36. Espinoza A, et al. *Biol Trace Elem Res*. 2012; 146:281–286. [PubMed: 22068728]
37. Hubatsch I, Ragnarsson EGE, Artursson P. *Nat Protoc*. 2007; 2:2111–2119. [PubMed: 17853866]
38. Tabuchi M, Yoshiomori T, Yamaguchi K, Yoshida T, Kishi F. *J Biol Chem*. 2000; 275:22220–22228. [PubMed: 10751401]
39. Friend C, Scher W, Holland JG, Sato T. *Proc Natl Acad Sci U S A*. 1971; 68:378–382. [PubMed: 5277089]
40. Lesjak M, et al. *PLoS One*. 2014; 9:e102900. [PubMed: 25058155]
41. Knutson MD, Oukka M, Koss LM, Aydemir F, Wessling-Resnick M. *Proc Natl Acad Sci U S A*. 2005; 102:1324–1328. [PubMed: 15665091]

42. Espósito BP, Breuer W, Cabantchik ZI. *Biochem Soc Trans.* 2002; 30:729–732. [PubMed: 12196179]
43. Sohn YS, et al. *Haematologica.* 2012; 97:670–678. [PubMed: 22180428]
44. Arredondo M, Orellana A, Garate MA, Nunez MT. *Am J Physiol.* 1997; 273:G275–280. [PubMed: 9277404]
45. Zhang DL, Hughes RM, Ollivierre-Wilson H, Ghosh MC, Rouault TA. *Cell Metab.* 2009; 9:461–473. [PubMed: 19416716]
46. Imai N, et al. *J Toxicol Sci.* 2006; 31:357–370. [PubMed: 17077589]
47. Veuthey T, Wessiling-Resnick M. *Front Pharmacol.* 2014; 5:1–82. [PubMed: 24478702]
48. Avagyan S, Zon LI. *Hum Gene Ther.* 2016; 27:287–294. [PubMed: 27018965]
49. Donovan A, et al. *Blood.* 2002; 100:4655–4659. [PubMed: 12393445]
50. Ganis JJ, et al. *Dev Biol.* 2012; 366:185–194. [PubMed: 22537494]
51. Brownlie A, et al. *Nat Genet.* 1998; 20:244–250. [PubMed: 9806542]
52. Miller C. *Nature.* 2006; 440:484–489. [PubMed: 16554809]
53. Kwok EY, Severance S, Kosman DJ. *Biochemistry.* 2006; 45:6317–6327. [PubMed: 16700543]
54. Yun CW, Tiedeman JS, Moore RE, Philpott CC. *J Biol Chem.* 2000; 275:16354–16359. [PubMed: 10748025]
55. Seo YA, Elkhader JA, Wessling-Resnick M. *Biometals.* 2016; 29:147–155. [PubMed: 26693922]
56. Severance S, Chakraborty S, Kosman DJ. *Biochem J.* 2004; 380:487–496. [PubMed: 14992688]
57. Dearden JC, Bresnen GM. *Quantitative structure-activity relationships.* 2016; 7:133–144.
58. Andres A, et al. *Eur J Pharm Sci.* 2015; 76:181–191. [PubMed: 25968358]
59. Park SR, et al. *Nat Commun.* 2016; 7:1–11.
60. Gentry LE, Thacker MA, Doughty R, Timkovich R, Busenlehner LS. *Biochemistry.* 2013; 52:6085–6096. [PubMed: 23909240]
61. Katoh H, Hagino N, Ogawa T. *Plant Cell Physiol.* 2001; 42:823–827. [PubMed: 11522907]
62. White GP, Jacobs A, Grady RW, Cerami A. *Blood.* 1976; 48:923–929. [PubMed: 1000084]
63. Vyoral D, Petrak J. *Biochim Biophys Acta.* 1998; 1403:179–188. [PubMed: 9630620]
64. Li CY, Watkins JA, Glass J. *J Biol Chem.* 1994; 269:10242–10246. [PubMed: 8144605]
65. Davis SA, et al. *J Am Chem Soc.* 2015; 137:15102–15104. [PubMed: 26580003]
66. Berlett BS, Levine RL, Chock PB, Chevion M, Stadtman ER. *Proc Nat Acad Sci, U S A.* 2001; 98:451–456.
67. Illing AC, Shawki A, Cunningham CL, Mackenzie B. *J Biol Chem.* 2012; 287:30485–30496. [PubMed: 22736759]
68. Ehrnstorfer IA, Geertsma ER, Pardon E, Steyaert J, Dutzler R. *Nat Struct Mol Biol.* 2014; 21:990–996. [PubMed: 25326704]
69. Lovenberg W, Buchanan BB, Rabinowitz JC. *J Biol Chem.* 1963; 238:3899–3913. [PubMed: 14086723]
70. Barton ZJ, Rodríguez-López J. *Anal Chem.* 2014; 86:10660–10667. [PubMed: 25310056]
71. Diouf PN, et al. *Appl Environ Microbiol.* 2002; 68:4377–4382. [PubMed: 12200290]
72. Chung J, et al. *Sci Signal.* 2015; 8:ra34. [PubMed: 25872869]
73. Canver MC, et al. *J Biol Chem.* 2014; 289:21312–21324. [PubMed: 24907273]
74. Yien YY, et al. *J Clin Invest.* 2014; 124:4294–4304. [PubMed: 25157825]
75. Hildick-Smith G, et al. *Am J Hum Genet.* 2013; 93:906–914. [PubMed: 24119684]
76. Wilcock BC, Endo MM, Uno BE, Burke MD. *J Am Chem Soc.* 2013; 135:8488–8491. [PubMed: 23718627]
77. Foresti M, Paoletti I, Mele F, Geraci G. *Mutat Res.* 1997; 374:269–275. [PubMed: 9100850]
78. Cooney JD, et al. *Dev Biol.* 2013; 373:431–441. [PubMed: 22960038]
79. Amigo JD, et al. *Blood.* 2009; 114:4654–4663. [PubMed: 19729519]
80. Nilsson R, et al. *Cell Metab.* 2009; 10:119–130. [PubMed: 19656490]
81. Kardon JR, et al. *Cell.* 2015; 161:858–867. [PubMed: 25957689]

82. Pangborn AB, Giardello MA, Grubbs RH, Rosen RK, Timmers FJ. *Organometallics*. 1996; 15:1518–1520.
83. Provencher PA, Love JA. *J Org Chem*. 2015; 80:9603–9609. [PubMed: 26393809]
84. Gadsby DC. *Nat Rev Mol Cell Biol*. 2009; 10:344–352. [PubMed: 19339978]
85. Harris, D., Swain, E., Robey, C., Aiello, E. *Electrochemical Methods: Fundamentals and Applications*. 2nd. Bard, AJ., Faulkner, LR., editors; John Wiley & Sons, Inc; New York, NY: 2001.
86. Iolascon A, et al. *J Pediatr*. 2008; 152:136–139. [PubMed: 18154916]
87. Want Y, et al. *Exp Hematol*. 2011; 39:784–793. [PubMed: 21627978]
88. Pietrangelo A. *Blood Cells Mol Dis*. 2004; 32:131–138. [PubMed: 14757427]
89. Brissot P, Bardou-Jacquet E, Jouanolle AM, Loréal O. *Trends Mol Med*. 2011; 17:707–713. [PubMed: 21862411]
90. Roy CN, Andrews NC. *Hum Mol Genet*. 2001; 10:2181–2186. [PubMed: 11673399]
91. Sheth S, Brittenham GM. *Annu Rev Med*. 2000; 51:443–464. [PubMed: 10774476]
92. Gouya L, et al. *Blood*. 1999; 93:2015–2110.
93. Gregory, A., Hayflick, S. *Neurodegeneration with Brain Iron Accumulation Disorders Overview*. University of Washington; Seattle, WA: 2014.
94. Papanikolaou G, Pantopoulos K. *Toxicol Appl Pharmacol*. 2005; 202:199–211. [PubMed: 15629195]
95. Grandchamp B, et al. *Blood*. 2011; 118:6660–6666. [PubMed: 22031863]
96. Kato J, et al. *Am J Hum Genet*. 2001; 69:191–197. [PubMed: 11389486]
97. Mendler MH, et al. *Gastroenterology*. 1999; 117:1155–1163. [PubMed: 10535879]
98. Gardenghi S, et al. *Blood*. 2007; 109:5027–5035. [PubMed: 17299088]
99. Ye H, et al. *J Clin Invest*. 2010; 120:1749–1761. [PubMed: 20364084]
100. Gajowiak A, Sty A, Starzyński RR, Staro R, Lipiński P. *Postepy Hig Med Dosw*. 2016; 70:709–721.
101. Guernsey DL, et al. *Nat Genet*. 2009; 41:651–653. [PubMed: 19412178]
102. Raghupathy R, Manwani D, Little JA. *Adv Hematol*. 2010; 2010:1–9.
103. Shenoy N, Vallumsetla N, Rachmilewitz E, Verma A, Ginzburg Y. *Blood*. 2014; 124:873–881. [PubMed: 24923296]
104. Hider, RC., Ma, Y. *Metal Chelation in Medicine*. Crichton, R, Ward, RJ., Hider, RC., editors. Vol. Chapter 2. The Royal Society of Chemistry; Cambridge, UK: 2017. p. 24-55.
105. Burgess J, Rangel M. *Adv Inorg Chem*. 2008; 60:167–243.
106. Franza, T., Expert, D. *Iron Uptake and Homeostasis in Microorganisms*. Cornelis, P., Andrews, SC., editors. Vol. Chapter 6. Caister Academic Press; Norfolk, UK: 2010. p. 101-116.
107. Merkofer M, Kissner R, Hider RC, Koppenol WH. *Helv Chim Acta*. 2004; 87:3021–3034.
108. Kazmi SA, Amin S. *J Chem Soc Pak*. 2008; 30:824–828.
109. Wen YH, et al. *J Electrochem Soc*. 2006; 153:A929–A934.

Fig. 1. Restoring physiology to iron transporter-deficient organisms

(A) A small molecule that autonomously performs transmembrane iron transport is hypothesized to harness local ion gradients of the labile iron pool that selectively accumulate in the setting of missing protein iron transporters. Brown spheres represent labile iron, which includes both ionic iron and iron weakly bound to small molecules such as citrate. (B) Structures of hinokitiol (Hino) and the transport-inactive derivative C2-deoxy hinokitiol (C2deOHino). (C) Disc diffusion with hinokitiol of *fet3 ftr1* cells streaked on a low iron SD-agar plate containing 10 μM FeCl₃ restored yeast cell growth at intermediate concentrations of small molecule. (D) In the absence of hinokitiol, reduced *fet3 ftr1* yeast cell growth was observed on low iron SD-agar plates containing 10 μM FeCl₃ by serial 10-fold dilution plating (from OD₆₀₀ = 1.0). Under identical conditions, restored cell growth was observed on the same low iron SD-agar plates containing 10 μM hinokitiol. (E) Yeast cell growth in liquid SD media containing 10 μM FeCl₃ in the absence or presence of 10 μM hinokitiol. *N* = 3. (F) Hinokitiol restored growth of *fet3 ftr1* yeast while C2deOHino did not. *N* = 3. (G) Hinokitiol increases ⁵⁵Fe influx into *fet3 ftr1* yeast while C2deOHino does not. *N* = 3. (E-G) NS, not significant; **** *P* < 0.0001; Graphs depict means ± SEM.

Fig. 2. Physical characteristics of hinokitiol binding and transport

(A) Opposite to water soluble chelators, such as deferiprone, the hinokitiol-iron complex partitions into non-polar solvents. (B) UV-Vis titration study of hinokitiol with increasing FeCl₃ indicates hinokitiol binds iron. Arrows indicate changes in UV spectrum with increasing iron from 0:1 Fe:Hino to 6:1 Fe:Hino. (C and D) In contrast to water soluble iron chelators and C2deOHino, hinokitiol autonomously promotes the efflux of (C) ferrous and (D) ferric iron from model POPC liposomes. *N* = 3. (E) X-ray crystal structure of a C1-symmetric Fe(Hino)₃ complex. (F) Cyclic voltammogram of the iron-hinokitiol complex in 0.1 M Tris buffer in 1:1 MeOH:H₂O at pH=7.2 using 500 μM Hino and 100 μM Fe(NO₃)₃ with a 100 mV/s scan rate. (C, D) Graphs depict representative runs of three independent experiments. (F) Graph depicts a representative run of four independent experiments.

Fig. 3. Hinokitiol restores mammalian cell physiology

(A) ^{55}Fe uptake into DMT1-deficient Caco-2 monolayers and (B) transepithelial transport (apical to basolateral) indicated hinokitiol (500 nM) restored normal iron absorption. $N = 3$. (C) Hinokitiol-promoted ^{55}Fe transport occurs on times commensurate with dwell times in the gut. $N = 3$. (D) Cell pellets from shControl and hinokitiol-treated (1 μM) DMT1-deficient MEL cells appear pink, characteristic of hemoglobin, while DMT1-deficient cell pellets do not. (E) ImageJ quantification of MEL cells stained brown with α -dianisidine. Dotted line represents shControl levels. $N = 6-48$. (F) ^{55}Fe incorporation into heme in hinokitiol-rescued DMT1-deficient MEL cells. Dotted line represents shControl levels. $N = 3-23$. (G) Hinokitiol increases the number of α -dianisidine stained Mfrn1-deficient MEL cells. Dotted line represents DS19 levels. $N = 21-48$. (H) Hinokitiol (1 μM) restores ^{55}Fe transepithelial transport across FPN1-deficient Caco-2 monolayers (I) without affecting iron uptake. $N = 12$. (J) Hinokitiol (5 μM) promotes the release of ^{55}Fe from hepcidin-treated FPN1-deficient J774 macrophages ($t = 2$ hours). $N = 6-20$. (K) Time-dependent release of ^{55}Fe from wild type and FPN1-deficient J774 macrophages treated with or without hinokitiol and C2deOHino. $N = 6-20$. (A, B, E-J) NS, not significant; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; Graphs depict means $\pm$ SEM.

Fig. 4. Hinokitiol leverages built-up iron gradients

(A) Representative fluorescence images of differentiated shControl and DMT1-deficient MEL cells in the absence or presence of hinokitiol (1 μM) using oxyburst green, calcein green, and RPA to detect relative endosomal, cytosolic, and mitochondrial iron levels, respectively. A build-up of labile iron was observed in endosomes of DMT1-deficient cells, which was released after hinokitiol treatment. (B and C) A build-up of intracellular labile iron is observed in FPN1-deficient J774 macrophages treated with 200 μM FeSO_4 by quenching of calcein green fluorescence. $N = 3$. (D) Iron (III) uptake into J774 macrophages with 50 μM FeCl_3 similarly revealed a build-up of total intracellular iron in FPN1-deficient cells after 4 hours using ^{55}Fe as a radiotracer. $N = 8$. (E) Increased extracellular iron (III) levels increased rates of iron uptake into J774 macrophages when treated with hinokitiol (1 μM) using ^{55}Fe as a radiotracer. $N = 3$. (F and G) Increased intraliposomal (F) ferrous iron and (G) ferric iron leads to increased rates of iron efflux in the presence of hinokitiol (10 μM). No efflux was observed in the absence of hinokitiol. $N = 3$. (H) Fluorescence imaging of cytosolic iron with calcein green using artificially created iron gradients in J774 macrophages in opposite directions. Cells were loaded with FeSO_4 (200 μM), rinsed, then hinokitiol (100 μM) was added at $t = 5$ min. An increase in fluorescence was observed, consistent with decreased intracellular labile iron. The gradient was then reversed in these same cells by addition of 100 μM FeCl_3 to the media at $t = 12$ min. Fluorescence quenching was observed, consistent with iron uptake. (I) Representative ImageJ quantification of calcein green fluorescence in iron-loaded J774 cells with addition of DMSO, hinokitiol, or

C2deOHino at $t = 5$ min and FeCl_3 at $t = 12$ minutes. Scale bar = $10\ \mu\text{m}$ (**A**), $20\ \mu\text{m}$ (**B**, **H**). (**C-E**) ** $P < 0.01$; **** $P < 0.0001$; Graphs depict means $\pm$ SEM. (**F**, **G**) Graphs depict means of three independent experiments. (**I**) Representative graph from six independent experiments.

Fig. 5. The endogenous network is involved in hinokitiol-mediated CacO-2 transport
(A) Representative western blot images of proteins involved in iron absorption and regulation indicate an anemic state is observed in shDMT1 CacO-2 monolayers to promote maximal iron absorption. **(B)** Unidirectional hinokitiol-mediated transport in shDMT1 CacO-2 monolayers by apical or basolateral addition of hinokitiol (500 nM) and ^{55}Fe radiotracer. $N=3$. **(C)** Determination of ^{55}Fe levels in immunoprecipitated ferritin in CacO-2 monolayers. $N=3$. **(D)** Knockdown of FPN1 in shDMT1 CacO-2 monolayers with quercetin abrogates hinokitiol-mediated transport. $N=3$. **(E)** Rates of CacO-2 transport with varying concentrations of iron treated with DMSO or hinokitiol (500 nM) after 4 hours. The rates of transport level off with increasing iron concentrations. $N=3$. **(F)** Increased doses of hinokitiol increase uptake into shDMT1 CacO-2 monolayers apically treated with 25 μM FeCl_3 ; however, a bimodal effect is observed in transepithelial iron transport at 5 μM hinokitiol. $N=3$. **(G)** Representative western blot images of proteins involved in iron absorption and regulation after treatment with increasing hinokitiol and 25 μM FeCl_3 . Bimodal effects were similarly observed in protein levels involved in iron absorption and regulation. **(H)** Intermediate concentrations of hinokitiol lead to significant calcein green quenching in shDMT1 monolayers treated with 25 μM FeCl_3 after 1 hour, consistent with increased labile iron. This effect was reversed at high doses of hinokitiol. **(I and J)** ImageJ quantification of calcein green fluorescence in these monolayers. $N=3-6$. Scale bar = 20 μm **(H)**. **(B-F, I, J)** NS, not significant; **** $P=0.0001$; Graphs depict means $\pm$ SEM.

Fig. 6. Hinokitiol restores physiology in iron transporter-deficient animals

(A and B) Oral gavage of 1.5 mg/kg hinokitiol promotes the gut absorption of ^{59}Fe into (A) DMT1-deficient Belgrade (b/b) rats and (B) FPN1-deficient Flatiron ($ffl+/+$) mice after 1 hour. $N = 4-7$. (C) Hinokitiol treatment (1 μM) to the water to embryos at 24 hpf and incubation for an additional forty-eight hours increases the number of GFP-positive erythroids by FACS analysis in DMT1-deficient morphant zebrafish using a transgenic fish containing GFP-tagged erythroids. $N = 7-17$. (D) Hinokitiol decreases the number of anemic fish from a heterozygous cross of $+/-cdy$ fish as determined by *o*-dianisidine staining, while C2deOHino does not. (E) Hinokitiol (1 μM) increases the number of GFP-positive erythrocytes in Mfrn1-deficient morphant zebrafish. $N = 3-13$. (F) Hinokitiol increases the number of non-anemic embryos from a heterozygous cross of $+/-frs$ fish. (G) Embryos from a heterozygous cross of $+/-frs$ fish were genotyped by restriction enzyme digestion with *BsrI*. Lanes 4 and 5 correspond to frs/frs fish treated with hinokitiol for forty-eight hours. (H) Hinokitiol-treated frs/frs fish stain brown with *o*-dianisidine while anemic frs/frs fish do not, indicating increased hemoglobin levels after hinokitiol treatment. (A-F) NS, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Graphs depict (A-C, E) means $\pm$ SEM or (D, F) weighted means $\pm$ SEM.