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Small-Molecule Screening Identifies the Selanazal Drug Ebselen as a Potent Inhibitor of DMT1-Mediated Iron Uptake

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Summary

HEK293T cells overexpressing divalent metal transporter-1 (DMT1) were established to screen for small-molecule inhibitors of iron uptake. Using a fluorescence-based assay, we tested 2000 known bioactive compounds to find 3 small molecules that potently block ferrous iron uptake. One of the inhibitors, ebselen, is a seleno compound used in clinical trials as a protective agent against ischemic stroke. Ebselen inhibited Fe(II) uptake (IC_{50} of ~0.22 μM), but did not influence Fe(III) transport or DMT1-mediated manganese uptake. An unrelated antioxidant, pyrrolidine dithiobarbamate (PDTC), also inhibited DMT1 activity (IC_{50} of ~1.54 μM). Both ebselen and PDTC increased cellular levels of reduced glutathione. These observations indicate that Fe(II) transport by DMT1 can be modulated by cellular redox status and suggest that ebselen may act therapeutically to limit iron-catalyzed damage due to transport inhibition.

Introduction

Small molecules can help to define biological pathways by inhibiting protein function to discover the factors involved in dynamic cellular processes. In particular, studies of membrane transport by carriers and channels have been significantly advanced by the use of pharmacological inhibitors to analyze transport mechanisms. Recent developments in the area of iron transport have led to the discovery of several novel membrane transporters and a new understanding of the regulation of iron absorption [1, 2]. Unfortunately, this area of research has been hampered by the lack of pharmacological reagents to probe the underlying molecular mechanisms involved in these processes. To identify small-molecule inhibitors of iron transport, we previously established a cell-based screening assay that takes advantage of iron-induced quenching of calcein fluorescence [3]. Using this approach, we discovered ten inhibitors of nontransferrin bound iron (NTBI) uptake [4]. Two other pathways of iron uptake are known to be mediated by divalent metal transporter-1 (DMT1). DMT1 is the transporter responsible for dietary iron absorption across the apical membrane of intestinal enterocytes [5] and is also involved in the delivery of iron to peripheral tissues by transferrin [6]. Defects in the DMT1 gene cause microcytic anemia in the *mk* mouse, an animal model that displays defective dietary iron absorption [7]. Defective transferrin-mediated iron uptake is also well characterized for a different animal model, the Belgrade rat, which harbors the same genetic defect in DMT1 [6]. Electrophysiological studies have shown that DMT1 not only mediates uptake of ferrous iron, but that it also interacts with other divalent metals, including Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, and Pb^{2+} [8]. In addition, the DMT1 mutation present in the *b* rat and *mk* mouse (G185R) confers Ca^{2+} transport activity to the transporter [9]. DMT1 activity has been characterized to be voltage and pH dependent [8], but despite intense effort to understand the transporter's molecular properties [10], relatively little is known about cellular control of its function. To further our understanding of DMT1-mediated iron uptake, we established a HEK293T cell line that stably overexpresses this transporter, and we adapted the cell-based calcein assay to screen for small-molecule inhibitors of ferrous iron uptake in chemical libraries of known bioactive compounds. Among the inhibitors identified in this chemical genetic screen was ebselen, an antioxidant, anti-inflammatory selenium compound that has been found to be useful in treating patients with ischemic stroke [11, 12] and aneurismal subarachoid hemorrhage [13]. This report characterizes inhibition of DMT1 activity by ebselen and another unrelated antioxidant, pyrrolidine dithiocarbamate (PDTC). Based on these results, we propose that DMT1 activity is inversely regulated by cellular redox status. This study demonstrates the utility of cell-based assays using transporter overexpression as a means of identifying small-molecule inhibitors as well as the usefulness of chemical genetic screening as a tool for determining cellular factors involved in fundamental biological processes like membrane transport.

Results

A Screen for DMT1 Transport Inhibitors

HEK293T cells were transfected with DMT1 cDNA subcloned in the sense (coding) or antisense (noncoding) orientations [14] and selected for stable expression by using puromycin resistance. Figure 1A confirms robust expression of the transporter in cells transfected with sense DMT1 cDNA; DMT1 could not be detected either in nontransfected control cells (data not shown) or HEK293T cells transfected with antisense cDNA. Transport assays to determine the uptake of ^{55}Fe presented in the ferrous form at pH 6.75 indicated that DMT1 activity was ~25-fold greater in the HEK293T(DMT1) cells overexpressing the transporter (Figure 1B). Indirect immunofluorescence microscopy experiments with anti-DMT1 performed to cytolocalize exogenously expressed transporters revealed cell surface as well as punctate intracellular staining (Figure 1C).

Using the HEK293T(DMT1) cell line, we adapted a cell-based screening assay for iron uptake based on calcein fluorescence to identify small-molecule inhibitors of DMT1. Briefly, the metal-sensitive fluorophore calcein is used to measure intracellular "labile" or free iron. In

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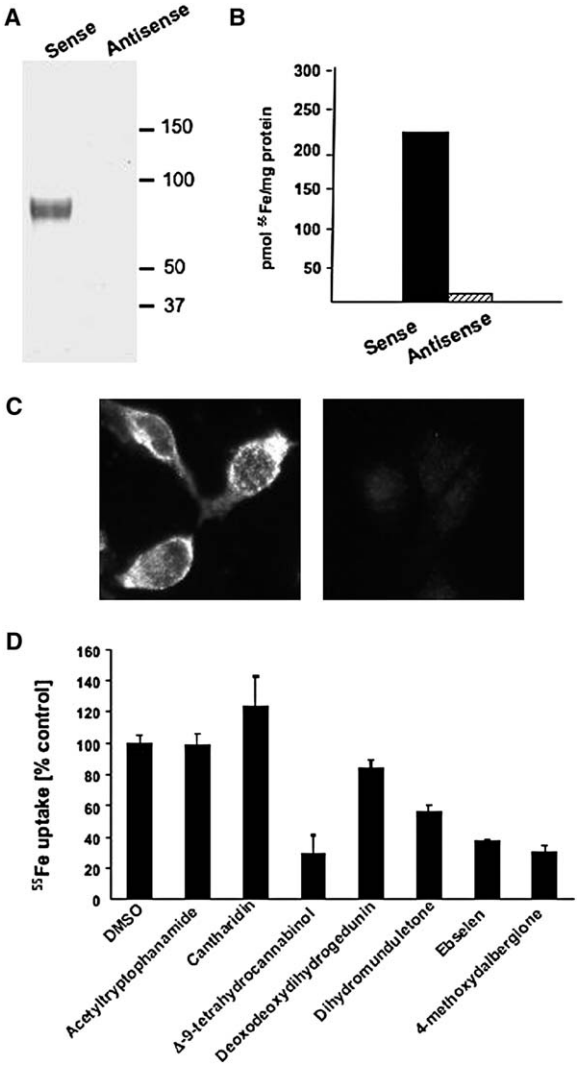

Figure 1. Stable Expression of DMT1 Allows for a Chemical Genetic Screen for Transport Inhibitors

(A) Western blot detecting DMT1 immunoreactivity in HEK293T(DMT1) cells stably transfected with pMT2 containing transporter's cDNA in the sense and antisense (noncoding) orientations. Cell lysates (5 μg) were electrophoresed on a 4%–15% polyacrylamide gel. Proteins were transferred onto a PVDF membrane by using a Tris-glycine transfer buffer without SDS. The membrane was first exposed to a stripping buffer (0.2 M glycine, 0.5 M NaOH [pH 2.8]) for 5 min, and, after blocking (Odessey Blocking Buffer, Li-Cor Biosciences), the membrane was incubated with a 1:2000 dilution of rabbit anti-DMT1 antibodies (a kind gift of Dr. Deborah Trinder, University of Western Australia). After washing, the blot was incubated with a 1:10,000 dilution of goat anti-rabbit antibody conjugated with IR-dye 800 (Rockland Immunochemicals, Inc). After further washing, immunoreactivity was detected by infrared imaging (Li-Cor Odessey Infrared Imaging System). Indicated standards are Precision Plus markers (BioRad).

(B) ^{55}Fe uptake was measured for the cell lines shown in (A). Briefly, cells were incubated at 37°C for 20 min in assay buffer containing 1 μM ^{55}Fe and 50 μM ascorbic acid (pH 6.75). Cells were chilled on ice and washed three times with PBS, and cell-associated radioactivity was determined by scintillation counting. Shown is the average value of duplicate transport assays from a single experiment; similar results were obtained on four separate occasions.

(C) HEK293T cells grown on lysine-coated cover slips were fixed with 4% paraformaldehyde, blocked with 5% nonfat dry milk in PBS, then permeabilized with 0.1% Triton X-100 and incubated with a 1:1000 dilution of anti-DMT1 antisera. After rinsing with PBS, cover slips were incubated with a 1:250 dilution of donkey anti-rabbit IgG labeled with Cy3, then washed, dried, and mounted to detect immunoreactivity by fluorescence microscopy (left, stable HEK293T(DMT1) cells; right, nontransfected control HEK293T cells).

(D) Secondary screen of DMT1 inhibitors by using the ^{55}Fe uptake assay in the presence of 50 μM of the indicated compounds or vehicle alone (0.5% DMSO). Uptake was normalized to % control (DMSO alone), and mean values (± absolute deviation) determined in several different experiments performed on separate occasions (n = 4) are shown. Control values were 210.5 ± 10.73 pmol ^{55}Fe/mg cell protein and 140.5 ± 40.22 pmol/10^6 cells.

the presence of iron or other metals, the fluorescence of calcein is quenched [3]. Calcein does not bind to calcium or magnesium at physiological pH, and since the intracellular concentrations of other metal ions are low, a loss of cell-associated calcein fluorescence in the presence of extracellular iron is indicative of an increase in free intracellular iron levels due to transport. Using a 384-well format, 2000 compounds from the NINDS Custom Collection and SpecPlus Collection (Microsource Discovery Systems), available through the Institute of Chemistry and Cell Biology at Harvard Medical School, were robotically pin transferred and assayed at 50 μM for the ability to block iron uptake by HEK293T(DMT1) cells, as measured by fluorescence quenching upon addition of 1 μM ferrous iron (nominal cutoff for potency $\geq$ 50%).

From the survey of the 2000 bioactive compounds contained in these 2 libraries, 23 were "cherry picked" and rescreened by using the calcein assay in a 384-well format to validate results. Of these, eight compounds were obtained from outside suppliers and further studied by using a secondary screen for transport inhibition based on cellular uptake of radioactive ^{55}Fe (Figure 1D). From this final analysis, only three compounds were confirmed to be bona fide transport inhibitors: ebselen, Δ^9-tetrahydrocannabinol (THC), and 4-methoxy-dalbergione. Acetyl-tryptophanamide, deoxodeoxydihydrogendurin, and dihydromunduletone were relatively weak inhibitors in the secondary screen, while cantharidin appeared to slightly enhance ^{55}Fe uptake. One other compound, methyl 7-deshydroxypryogallin-4-carboxylate, produced a more profound stimulatory effect (data not shown). ^{55}Fe in the assay mixture was collected onto filters with or without cells; therefore, the effects of this particular compound in the screening assay were most likely due to extracellular interactions with ferrous iron to indirectly block uptake. Such results emphasize the importance of secondary screens with a separate methodological approach in validation studies.

Using the ^{55}Fe tracer assay, ebselen and Δ^9-THC were determined to inhibit DMT1 activity with IC_{50} values of ~0.22 and 0.45 μM, respectively (Figure 2). While 4-methoxy-dalbergione also inhibited uptake, only about 50% of the measured activity was blocked at concentrations above 0.1 μM, suggesting that limited cell permeability may reduce the efficacy of this compound. Because previous animal studies suggested that ebselen might affect tissue iron levels [15], our subsequent experiments focused on characterizing its capacity to inhibit DMT1-mediated iron uptake.

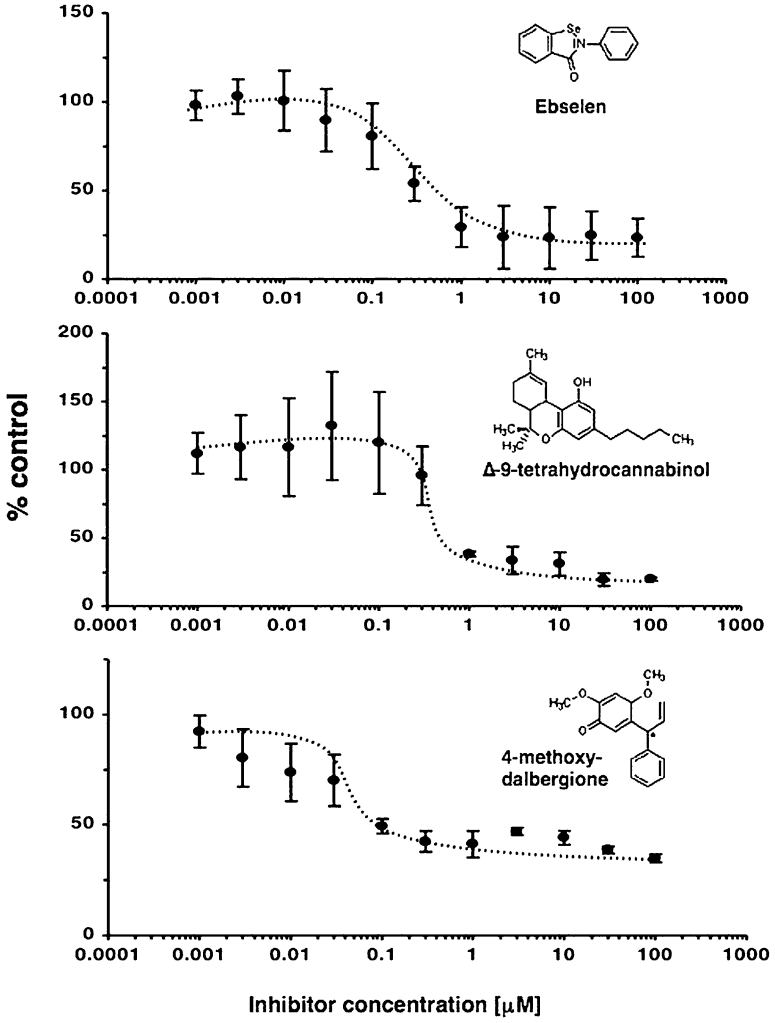

Figure 2. Dose-Response Studies of Potent DMT1 Inhibitors

HEK293T(DMT1) cells were incubated at 37°C for 20 min with 0.001–100 μM of the indicated compounds in the presence of 1 μM ^{55}Fe and 50 μM ascorbic acid (pH 6.75). Cells were chilled on ice, collected on nitrocellulose filters, and washed with PBS, and cell-associated radioactivity was determined and normalized to control (vehicle alone). Shown are results pooled from at least two independent experiments with mean values ± absolute deviation (n = 4). Data were analyzed by using a four-parameter sigmoidal model (r^2 > 0.95). The IC_{50} values and 95% confidence intervals were 0.22 μM (0.15–0.32 μM) for ebselen and 0.45 μM (0.27–0.73 μM) for Δ^9-THC.

Ebselen Does Not Influence DMT1 Activity by Indirect Effects on Fe(II)

To maintain iron in the ferrous state during transport assays, a 50 molar-fold excess of ascorbic acid is added. To determine if ebselen influenced DMT1-mediated transport by altering levels of ascorbate or Fe(II) present in the assay system, the following control experiments were performed. First, the stability of ascorbic acid over time was measured. The presence of 1 μM FeNTA (1:50 complex ratio) led to a loss of ascorbic acid over time, as measured by absorbance at 260 nm (Figure 3A). The half-life of ascorbic acid under the same conditions was nearly doubled by the addition of 50 μM ebselen. In the absence of FeNTA, ascorbic acid levels were relatively stable with or without the addition of ebselen. Likewise, ebselen itself was also stable under the assay conditions with ascorbic acid in the presence or absence of 1 μM FeNTA (Figure 3B). Finally, the amount of ferrous iron, measured spectrophotometrically by using the Fe(II)-specific iron chelator ferrozine ($A_{563\ nm}$), was determined (Figure 3C). Fe(II) levels were stable in the presence of ascorbic acid for 1 hr, and were further stabilized by the presence of ebselen. Since our transport assays to measure ^{55}Fe(II) transport determined uptake within 20 min of the initial mixing of reaction components, these combined results confirmed that uptake of ferrous iron was measured and that ebselen did not affect the redox state of the transport substrate.

Ebselen Does Not Influence Fe(III) or Mn(II) Uptake

To examine whether ebselen interfered with ferric iron transport, both transferrin bound and NTBI uptake were measured in the presence of this inhibitor. NTBI uptake by HeLa cells was determined as previously described [16]. Briefly, cells were incubated at 37°C with or without ebselen in the presence of 1 μM ^{55}FeNTA (1:4 complex) in serum-free DMEM for 1 hr. After quenching reaction components on ice, excess unlabeled FeNTA was added to remove any surface bound radiolabel and cell-associated ^{55}Fe was counted. As shown in Figure 4A, addition of up to 100 μM ebselen did not affect HeLa cell ^{55}Fe uptake compared to control cells incubated with vehicle alone (1% DMSO).

To examine whether ebselen influenced the uptake of transferrin bound iron, apotransferrin was first loaded with ^{55}Fe and then incubated with cells in serum-free medium for 4 hr at 37°C (Figure 4B). Uptake of ^{55}Fe from transferrin was studied by using HeLa and HEK293T cells, including both the parental and HEK293T(DMT1) cell lines, as previously described [4]. The presence of up to 50 μM ebselen did not inhibit transferrin-mediated iron

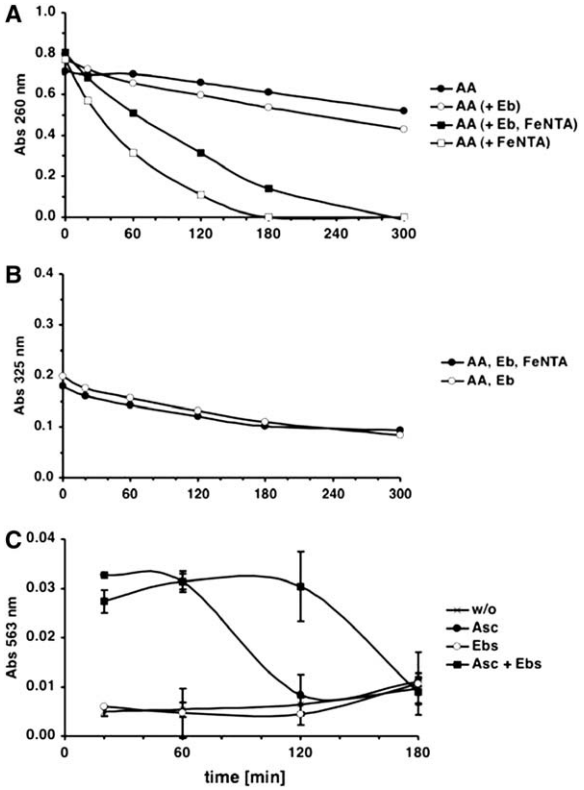

Figure 3. Stability of Assay Components in the Presence of Ebselen

(A) The stability of ascorbic acid (50 μM) in the assay mixture was determined alone (closed circle), in the presence of 50 μM ebselen (open circles), in the presence of 50 μM ebselen and 1 μM FeNTA (closed squares), and in the presence of 1 μM FeNTA alone (open squares) over time by measuring absorbance at 260 nm.

(B) The stability of ebselen in the assay mixture in the presence (closed circles) and absence (open circles) of FeNTA over time was measured ($A_{325\ nm}$).

(C) The amount of ferrous iron in the assay mixture was measured by using bathophenanthroline sulphonate (BPS) without any addition (X) or with the addition of 50 μM ascorbic acid and/or 50 μM ebselen and was determined as indicated: ascorbic acid alone (closed circle), ebselen alone (open circle), and ascorbic acid and ebselen together (closed square).

Results are the mean (±SEM) of triplicate determinations.

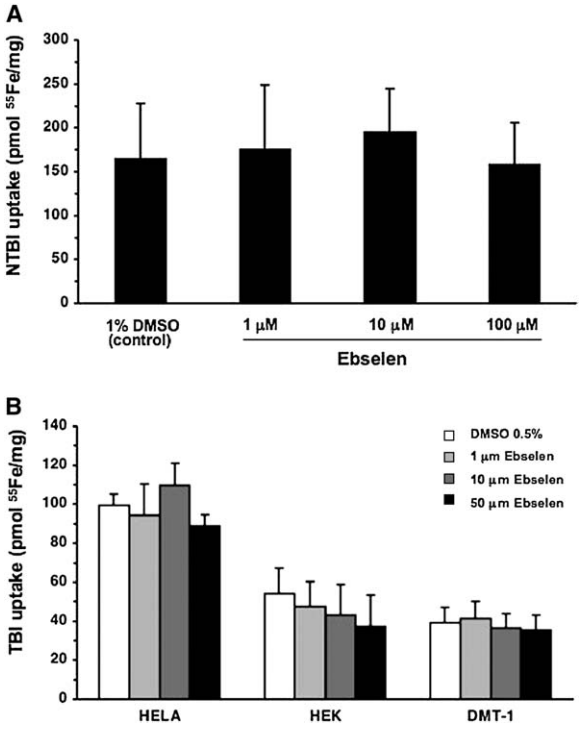

Figure 4. Ebselen Does Not Inhibit Nontransferrin Bound Iron, NTBI, or Transferrin Bound Iron, TBI, Uptake

(A) HeLa cells were plated at 300,000 cells per well. After overnight incubation in DMEM supplemented with FBS, cells were washed with serum-free DMEM and incubated in 1 ml of the same media with 1 μM ^{55}FeNTA (1:4 ratio metal:chelate) with or without the indicated concentrations of ebselen for 1 hr. Cells were chilled on ice, washed in PBS++, and then incubated with quench buffer containing 1 mM FeNTA for 1 hr on ice. After washing with PBS++, cells were lysed, and cell-associated radioactivity was determined by scintillation counting and was normalized to cell protein.

(B) HeLa, control HEK293T, or HEK293T(DMT1) cells were plated as described for (A), except that 800,00 cells per well were used for the latter two cell lines. Uptake assays were performed in serum-free DMEM containing 40 nM ^{55}Fe-Tf in the presence or absence of 1, 10, or 50 μM ebselen. Cells were incubated for 4 hr, then chilled on ice and incubated with a quench buffer containing 1 μM unlabeled Tf for 1 hr. After washing with PBS++, cells were lysed, and cell-associated radioactivity was determined and normalized to cell protein.

Results shown in both panels are the means (± absolute deviation) from at least two independent studies (n = 4).

delivery to any of the cell lines tested. One interesting finding from these experiments was that transferrin-mediated iron uptake was not enhanced in HEK293T(DMT1) cells, suggesting that transferrin receptor number rather than endosomal iron transport is limiting for iron uptake by this pathway. Because DMT1 has a defined role in endosomal transport of iron, the lack of ebselen inhibition was also somewhat surprising. However, unlike the DMT1 transport assays to measure Fe(II) uptake, determination of NTBI and transferrin-mediated uptake was conducted at neutral pH. Previous studies have shown that ebselen interactions with the thioredoxin/thioredoxin reductase system to reduce dehydroascorbate are optimal at pH 6.4 [17], which is closer to the pH range used to detect the proton-coupled DMT1 activity (pH ~6.75). It is possible that a similar pH profile modifies the response of cellular iron uptake to ebselen, or that this inhibitor affects factors specifically involved in mediating ferrous iron import across the cell surface, but not endosomal membrane compartments.

DMT1 is known to transport other divalent cations in addition to Fe(II). Compared to parental control cells, HEK293T(DMT1) cells take up 25-fold greater levels of ^{54}Mn, consistent with DMT1 transport of this metal (Figure 5A). Inhibition studies, however, revealed that ebselen did not inhibit ^{54}Mn uptake at concentrations of up to 50 μM (Figure 5B). This result implies that inhibition of DMT1 by ebselen is specific to the transport substrate Fe(II). This observation is important because ebselen can potentially modify thiols to inactivate enzymatic activity by forming a selenylsulfide [18]. For example, the Na^+, K^+-ATPase is one target for ebselen inhibition, which occurs through the chemical modification of its cysteine residues [19]. Consistent with the idea that ebselen does not chemically modify DMT1 to inhibit its

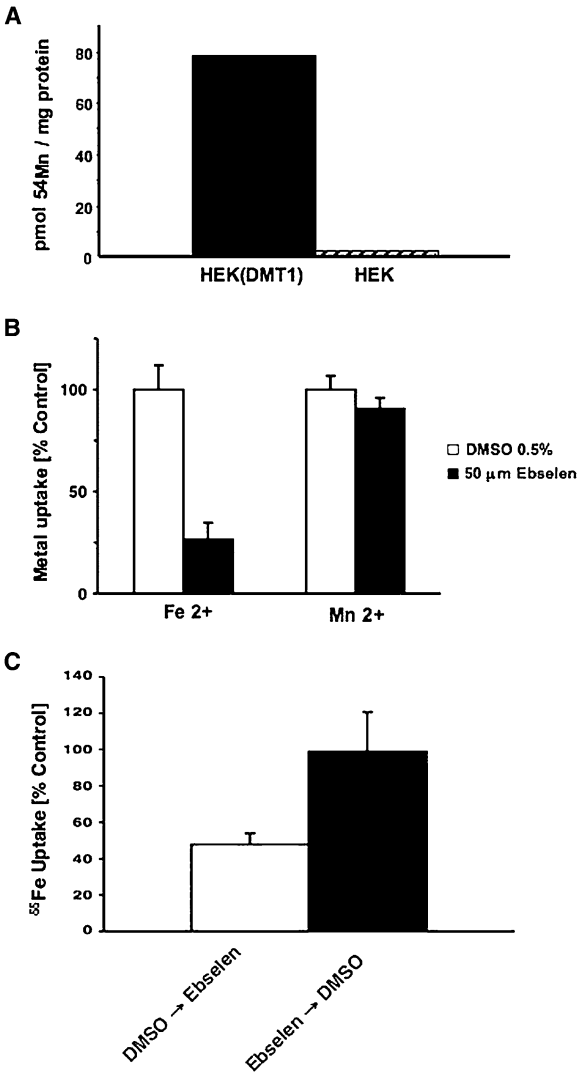

Figure 5. Ebselen Inhibits Fe(II), but Not Mn(II), Uptake and Acts in a Reversible Manner

(A) ^{54}Mn uptake was measured for control HEK293T and HEK293T(DMT1) cells. The average value for uptake calculated as pmol ^{54}Mn/mg protein from a single experiment performed in duplicate is shown; similar results were obtained on a separate occasion.

(B) Uptake studies were performed to compare inhibition of Fe(II) and Mn(II) uptake by ebselen. Assay conditions were the same as in Figure 2, except that 1 μM ^{55}FeNTA or 1 μM $^{54}MnCl_2$ was added to the transport reaction mixtures containing 50 μM ebselen or vehicle alone (0.5% DMSO). Uptake was normalized to control values, which were 128.11 ± 19.32 pmol $^{55}Fe/10^6$ cells and 51.39 ± 21.61 pmol $^{54}Mn/10^6$ cells.

(C) The reversibility of transport inhibition was determined by first incubating HEK293T(DMT1) cells with either 0.01% DMSO or 1 μM ebselen for 20 min in PBS++, then washing the cells three times with PBS++, and allowing a recovery period of 30 min at 37°C in PBS++ containing 5 mM glucose. Subsequently, ^{55}Fe uptake was measured by adding 1 μM ebselen or vehicle back to the reaction mixtures. Uptake was normalized to control cells (pretreated with DMSO and assayed in the presence of DMSO).

Results shown in (B) and (C) are mean values (± absolute deviation) determined in two independent experiments (n = 4).

transport activity, the addition of dithiothreitol (DTT) as a reducing agent did not reverse ebselen's effects on Fe(II) uptake (data not shown). Moreover, inhibition of Fe(II) uptake by ebselen was fully reversed after treatment of cells and subsequent wash out of the drug (Figure 5C).

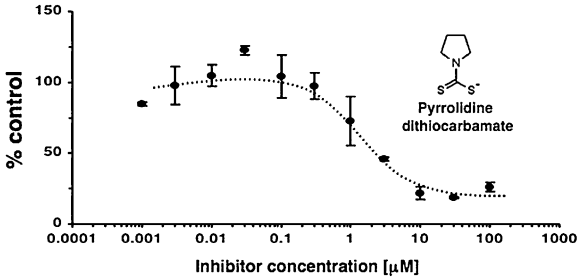

Figure 6. Antioxidant Activity Inhibits DMT1-Mediated Iron Uptake

Inhibition of ^{55}Fe uptake by HEK293T(DMT1) cells was determined in the presence of 0.001–100 μM PDTC, an antioxidant that is structurally unrelated to ebselen. Shown are mean values (± absolute deviation) from data collected in two different experiments (n = 4). Dose response was analyzed by using a four-parameter sigmoidal model (r^2 = 0.94).

PDTC Inhibits DMT1-Mediated Fe(II) Uptake

Because ebselen appeared to target the pathway of Fe(II) uptake rather specifically, we reasoned that the antioxidant effects of this compound might influence transport of this redox-active metal. We therefore studied another antioxidant, PDTC, which is structurally unrelated to ebselen. This antioxidant is commonly used to probe redox-sensitive NF-κB activation in vivo [20–22]. Inhibition studies demonstrated that PDTC inhibited DMT1 ^{55}Fe transport activity (Figure 6). The IC_{50} was determined to be 1.54 μM, although it should be noted that the 95% confidence interval for this value is large (0.68–3.45 μM). To directly compare the effects of both ebselen and PDTC on cellular glutathione levels, the ratio of reduced to oxidized glutathione was measured in HEK293T(DMT1) cells treated with 10 μM ebselen, 50 μM PDTC, or 0.5 % DMSO (vehicle control) for 20 min at 37°C under the transport assay conditions. The GSSG, GHS + GSSG, and GSH/GSSG values determined in these experiments are summarized in Table 1. Both compounds increased cellular levels of reduced GSH, as anticipated based on their antioxidant capacity. A simple interpretation of these combined results is that DMT1-mediated Fe(II) uptake activity is modulated by cellular redox status.

Discussion

The use of small molecules to alter cellular function provides new opportunities to determine mechanistic elements involved in complex biological pathways. While molecular genetic approaches have provided new insights into iron transport, such as the identification of the iron transporter DMT1 [7, 8], there is a significant need to develop pharmacological tools to gain further insight into the molecular basis of metal uptake and regulation. To discover small-molecule inhibitors of DMT1-mediated transport activity, we established a phenotypic screen based on calcein fluorescence quenching in a cell-based assay for iron uptake. Use of the stable HEK293T(DMT1) cell line provided the necessary

Table 1. Antioxidant Effects on GSH/GSSG Ratio

	GSSG	GSH + GSSG	GSH/GSSG
0.5% DMSO (n = 13)	0.73 ± 0.15	40.52 ± 4.35	56.42 ± 9.11
10 μM ebselen (n = 10)	0.49 ± 0.15[a]	37.11 ± 3.31	81.65 ± 20.1[a]
50 μM PDTC (n = 13)	0.62 ± 0.05[a]	40.79 ± 5.85	65.14 ± 6.77[a]

GSSG equivalents (nmol/million cells) were determined by using the GSH/GSSG-412 kit as described by the manufacturer (Oxis-Research, Portland, OR). Five million cells were assayed per point, and measurements were made by using a microtiter plate (405 nm). Shown are the mean values ± absolute deviation determined in five independent experiments.

[a] Different from control (0.5% DMSO); $p < 0.05$.

resource to detect DMT1 Fe(II) transport activity in an amplified and sensitive manner. This report demonstrates the utility of this cell-based approach in a screen of 2000 known bioactive compounds. Two potent transport inhibitors were identified: ebselen (IC_{50} of ~0.22 μM) and Δ^9-THC (IC_{50} of ~0.47 μM). Δ^9-THC, the major psychoactive component of the marijuana plant *Cannabis sativa*, is known to produce a number of behavioral and pharmacological effects mediated through interactions with the central nervous system cannabinoid receptor CB_1 and the peripheral receptor CB_2. Endogenous cannabinoids also activate these G protein-coupled receptors to negatively regulate adenylate cyclase activity and positively regulate inward rectifying K^+ channels [23]. It is interesting to note that receptor-independent signal transduction pathways have also been recently reported to negatively regulate a number of ion channels, including T-type Ca^{2+} channels, TASK-1 channels, and Na^+ channels [24]. There is significant interest in identifying signaling targets for cannabinoids since drugs that modify endocannabinoid activity are currently being developed to control obesity (Rimonabant), prevent osteoporosis (HU-308), and treat multiple sclerosis (Sativex). Marinol (pure THC) is still often used in treating AIDS and cancer patients. Thus, future studies must address both the mechanism of DMT1 inhibition by Δ^9-THC as well as the significance of these effects.

Our immediate efforts focused on defining how ebselen affected DMT1-mediated iron uptake. Ebselen (also called PZ51), or 2-phenyl-1,2-benzisoselenazol-3[2H]-one, is thought to exert antioxidant effects as a glutathione peroxidase mimic (reviewed by Schewe [18]). More recent studies have shown that ebselen rapidly oxidizes reduced thioredoxin to interact with the thioredoxin reductase system [25, 26]. Ebselen is also known to directly inhibit several inflammatory enzymes by thiol modification to form a selenosulfide [18]. Interestingly, previous in vivo studies have demonstrated that ebselen treatment is associated with reduced tissue iron in a model of iron overload, suggesting its potential inhibition of iron uptake [15]. Although the antioxidant, anti-inflammatory actions of ebselen provide a mechanistic explanation for its efficacy in clinical trials, many animal studies have shown that iron chelation also successfully limits damage in models of ischemic stroke [27], consistent with the idea that ebselen could act therapeutically by inhibiting tissue iron uptake.

A previous study of DMT1 activity with *Xenopus* oocytes suggested that oxidative agents could inhibit transporter function by direct modification of protein thiols [28]. Both zinc and iron uptake were blocked by treatment with H_2O_2 and Hg^{2+}, and DMT1-mediated transport activity was restored by the addition of DTT. We find that ebselen's influence on DMT1 function is unlikely to be mediated by such direct effects on this transporter since the compound failed to block uptake of DMT1-mediated manganese and DTT failed to reverse inhibition of DMT1-mediated iron uptake. There are at least two possible explanations for how changes in cellular redox might promote inhibition of ferrous iron in a specific manner. First, the reduced cellular environment promoted by ebselen and PDTC could allow an expansion in the "labile" or free iron pool under our assay conditions. The total concentration of cytosolic free iron is balanced between Fe(II) and Fe(III), and the ratio of these forms is known to be determined by the cellular redox capacity [29, 30]. The antioxidants also may influence the activity of other factors known to modulate intracellular levels of free divalent iron, for example, ferric reductases [31]. Mathematical modeling of the mechanisms of DMT1-mediated transport [32] suggests that carrier-mediated uptake of Fe(II) into the cell could become limiting when intracellular concentrations of the transport substrate increased. Alternatively, the antioxidants could possibly influence the activity of specific factors involved in the intracellular targeting of Fe(II) to fulfill specific metabolic functions, for example, transfer to mitochondria for heme synthesis or iron-sulfur cluster formation [33]. This scenario is based on analogy to the family of copper chaperones that mediate movement of this metal throughout the cell to achieve metabolic targeting after import [34]. The discovery of ebselen's inhibitory effects sheds new light on how the cellular redox environment can influence iron uptake. Use of antioxidants like ebselen and PDTC as pharmacological inhibitors of DMT1-mediated iron uptake should help to provide further molecular insights into the pathway's cellular factors involved in this process.

Significance

There is a significant need to develop pharmacological tools to gain further insight into the molecular basis for iron uptake and its regulation. To develop a cell-based screen for inhibitors, a stable HEK293T(DMT1) cell line was established to detect DMT1 Fe(II) transport activity in an amplified and sensitive manner. Using calcein fluorescence quenching to assay ferrous iron transport inhibition, 2000 known bioactive compounds were screened and 3 potent transport inhibitors were identified. This report further characterizes the activity of one of these inhibitors, ebselen. This seleno compound is a protective agent against ischemic stroke. Ebselen inhibited Fe(II) uptake (IC_{50} of ~0.22 μM), but did not influence Fe(III) or DMT1-mediated manganese uptake. Studies with an unrelated antioxidant, PDTC, confirmed that the cellular redox environment influences DMT1 iron uptake activity. Future use of antioxidants like ebselen and PDTC as pharmacological inhibitors of DMT1-mediated iron uptake should help to provide further molecular insights into the pathway's cellular factors involved in this process.

Experimental Procedures

Generation of a Stable DMT1 Cell Line

HEK293T cells were grown to 60% confluency in Dulbecco's modified Eagle's medium (DMEM) containing 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 10% fetal bovine serum. The cells were cotransfected with pMT2-Nramp2 in a coding (sense) or noncoding (antisense) orientation (a kind gift from Dr. Nancy Andrews) and pBABEPuro (carrying a puromycin-resistance gene) at a 20:1 ratio by using Effectine (QIAGEN) according to the manufacturer's instructions. After allowing 48 hr for gene expression, selection was performed by adding 2 μg/ml puromycin to the culture medium. After stable selection under these restrictive growth conditions, cells were subsequently grown in α minimal essential medium (αMEM) supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum.

Small-Molecule Library Screen

HEK293T(DMT1) cells were plated in 384-well poly-D-lysine-coated plates (Becton Dickinson) in columns 1–23 (15,000 cells/well) by using a Wellmate liquid dispensing apparatus (Matrix Technologies Corp.). Column 24 was left empty. After overnight incubation, the plates were washed three times with 65 μl serum- and phenol red-free αMEM by using a Bio-Tek ELx405 plate washer. Calcein-AM was added to a final concentration of 0.25 μM. After a 1 hr incubation at 37°C, the plates were washed ten times with 65 μl phosphate-buffered saline containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS++) with 5 mM glucose added. A final volume of 40 μl of this same buffer was added to each well, and a baseline fluorescence reading was measured by using an Analyst plate reader (485 nm excitation; 535 nm emission; Molecular Devices Corp.). Using a robotic pin-transfer apparatus, ~250 nl of each compound was transferred from master plates to duplicate HEK293T(DMT1) plates, which were then incubated at 37°C for 30 min, after which time a second fluorescence reading was taken. To each well in column 1, 10 μl aliquots of 200 mM HEPES, 200 mM Tris (pH 6.0), and 250 μM ascorbic acid were added. To each well in columns 2–24, 10 μl of the same assay mix with 5 μM FeNTA (1:50 metal:chelate ratio) was added. Columns 1 and 2 were used as controls, and no compounds were transferred to these wells. The plates were incubated at 37°C for an additional 20 min, and a final fluorescence reading was taken.

Inhibition Studies with Radioisotopic Tracer

HEK293T(DMT1) cells were incubated at 37°C for 20 min in assay buffer (25 mM Tris, 25 mM MES, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose [pH 6.75]) containing 1 μM ^{55}Fe or ^{54}Mn and 50 μM ascorbic acid (pH 6.75) (05–1.5 × 10^6 cells/transport reaction). Inhibitors were added at the concentrations noted in the figure legends immediately prior to the start of uptake. Cells were chilled on ice and either collected on nitrocellulose filters or by centrifugation at 2040 ×g for 5 min, then washed three times with PBS to remove any unbound ^{55}Fe or ^{54}Mn. Cell-associated radioactivity was determined by scintillation counting and was normalized to control (vehicle alone) or adjusted to cell protein measured in lysates by using the Bradford assay [35]. Values reported in figures and the text are means ± absolute deviation unless otherwise noted.

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