Discovery and characterization of a novel non-competitive inhibitor of the divalent metal transporter DMT1/SLC11A2

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Abstract

Divalent metal transporter-1 (SLC11A2/DMT1) uses the H⁺ electrochemical gradient as the driving force to transport divalent metal ions such as Fe²⁺, Mn²⁺ and others metals into mammalian cells. DMT1 is ubiquitously expressed, most notably in proximal duodenum, immature erythrocytes, brain and kidney. This transporter mediates H⁺-coupled transport of ferrous iron across the apical membrane of enterocytes. In addition, in cells such as to erythroid precursors, following transferrin receptor (TfR) mediated endocytosis; it mediates H⁺-coupled exit of ferrous iron from endocytic vesicles into the cytosol. Dysfunction of human DMT1 is associated with several pathologies such as iron deficiency anemia, hemochromatosis, Parkinson’s disease and Alzheimer’s disease, as well as colorectal cancer and esophageal adenocarcinoma, making DMT1 an attractive target for drug discovery. In the present study, we performed a ligand-based virtual screening of the Princeton database (700,000 commercially available compounds) to search for pharmaphore shape analogs of recently reported DMT1 inhibitors. We discovered a new compound, named pyrimidinone 8, which mediates a reversible linear non-competitive inhibition of human DMT1 (hDMT1) transport activity with a Ke of ~20 μM. This compound does not affect hDMT1 cell surface expression and shows no dependence on extracellular pH. To our knowledge, this is the first experimental evidence that hDMT1 can be allosterically modulated by pharmacological agents. Pyrimidinone 8 represents a novel versatile tool compound and it may serve as a lead structure for the development of therapeutic compounds for pre-clinical assessment.

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1. Introduction

Iron is an essential element that participates in many vital functions such as oxidative metabolism, adequate erythropoietic function and cellular immune responses. Iron absorption must be delicately balanced to supply enough iron for the metabolism and to avoid accumulation of excessive, toxic levels. Iron catalyses the conversion of hydrogen peroxide into hydroxyl radicals by the Fenton/Haber–Weiss reaction cycle. Overproduction of free radicals as a consequence of cellular iron-overloading results in oxidative stress, a deleterious process that leads to damage of cell structures, including lipids, proteins and DNA.

Body iron levels depend almost exclusively on its intestinal absorption, since there is no regulated excretory pathway for this metal ion. Divalent metal transporter-1 (SLC11A2/DMT1), also known as DCT1 and Nramp2, uses the H⁺ electrochemical gradient
as the driving force to transport divalent metal ions such as Fe\textsuperscript{2+}, Mn\textsuperscript{2+}, Cd\textsuperscript{2+}, and others metals across the cell membrane [1,2]. DMT1 is a 62-kDa protein that has 12 predicted membrane-spanning domains with intracellular N and C termini and is ubiquitously expressed, most notably in proximal duodenum, immature erythroid cells of the bone marrow, brain and kidney [3]. Enterocytes take up dietary, non-heme-bound iron in the form of Fe\textsuperscript{2+} (ferrous iron) across the apical membrane through DMT1 [1]. This transporter is also involved in the transport of iron from the endocytic vesicles to the cytosol as part of transferrin receptor (TfR)-mediated cellular uptake [4]. Kinetic analysis has shown that DMT1 mediates transport of Fe\textsuperscript{2+} and H\textsuperscript{+} in a coupled way with a stoichiometry of 1:1. DMT1 displays high affinity for Fe\textsuperscript{2+} and H\textsuperscript{+} with apparent affinities of 1–5 μM and 1 μM, respectively [2].

Dysfunction of human DMT1 is associated with iron deficiency anemia [5,6], iron overload disorders [7–9], neurodegenerative diseases (e.g., Parkinson’s [10] and Alzheimer’s disease [11]), as well as cancer (colorectal [12] and esophageal adenocarcinoma [13]). The involvement of DMT1 in these disorders makes this protein a promising target for drug discovery. Moreover, specific modulators of DMT1 transport activity could be used as tools to study the structure and transport mechanism of this transporter as well as its role in iron homeostasis in health and disease. During the past few years, significant efforts have been devoted toward the discovery of new DMT1 modulators. Emerging technologies such as chemical genetic screening have led to the discovery of small-molecule compounds that distinguish between non-transferrin bound iron uptake and transferrin-mediated iron uptake [14]. Moreover, two small-molecule compounds were reported to specifically inhibit DMT1-mediated iron uptake [15]. More recently, a series of benzylisothiourea and pyrazole-derived compounds were shown to act as potent DMT1 inhibitors [16,17]. However there is scarce information available about the mechanisms of action of these small-molecule compounds on DMT1 transport activity.

In the present study, we extended the search for new small-molecule compounds that specifically inhibit human DMT1-mediated iron uptake. We found a new compound, pyrimidinone 8, which mediates a reversible linear non-competitive inhibition of hDMT1 transport activity. This compound does not affect hDMT1 cell surface expression and show no dependence with the extracellular pH. To our knowledge, this is the first experimental evidence that hDMT1 can be allosterically regulated by pharmacological agents.

2. Materials and methods

2.1. Materials

HEK293 cells were obtained from American Type Culture Collection (ATCC). Lipofectamine 2000 and genetin were obtained from Life Technologies (Basel, Switzerland). Cell culture medium, fetal bovine serum and cell culture supplements were from Invitrogen. DC Protein Assay was purchased from Bio-Rad Laboratories (Cressier, Switzerland). All other materials were obtained from Sigma–Aldrich Chemie GmbH (Buchs, Switzerland).

2.2. Cell culture and generation of a DMT1 stably transfected cell line

The DsRED-hDMT1 expressing HEK 293 cell clones were established as described previously [18].

2.3. Ligand-based virtual screening and compound testing

A ligand-based virtual screening (LBVS) was performed using the Princeton database (700,000 commercially available compounds [19]) to search for pharmacophore shape analogs of recently reported DMT1 inhibitors as references. The selected references were three pyridino-pyrazoles (compounds 1–3 [16]) and three aromatic bis-thioamidines (compounds 4–6 [17]) as shown in Fig. 1A. LBVS was performed using pharmacophore and shape similarity scoring functions recently developed in our laboratory [20–23]. A total 140 compounds were purchased as 1 mg solid sample from Princeton Biomolecular Research (Monmouth Junction, NJ, USA) and conditioned as 10 mM stocks in DMSO. Final compound solutions (40 μM) were prepared by dissolving the appropriate amount of the compound stock solution in Krebs–Ringer buffer (140 mM NaCl, 4.8 mM KCl, 1 mM MgCl\textsubscript{2}, 1.2 mM CaCl\textsubscript{2}, 10 mM D-glucose, 5 mM HEPES, 5 mM MES, pH = 6.5 and osmolarity = 290 ± 10 mOsm).

Compound screening was performed using the FLIPR Tetra high-throughput, fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) as reported before [18]. Stably transfected clones of hDMT1-expressing HEK293 cells were seeded on poly-d-lysine coated 96-well black walled, clear bottom plates at a cell density of 40,000 cells/well. 24 h later cells were loaded with Calcium-5 dye in Krebs–Ringer buffer accordingly to the manufacturer’s protocol. Briefly, the growth media was removed and 100 μl dye loading buffer was added to each well and cells were incubated for 1 h at 37°C. After that, cells were placed on the FLIPR Tetra and excited using a 470–495 nm LED module, and the emitted fluorescence signal was filtered using a 515–575 nm emission filter for detection. Fluorescence signals were analyzed using the FLIPR Tetra software (ScreenWorks 3.1.2.002). A stable baseline was monitored for 50 s. Then, 50 μl aliquots of Krebs–Ringer buffer containing 10 μM of different test compounds were added to the wells and changes in fluorescence intensities were monitored for 300 s. Thereafter, a second addition of 50 μl of Krebs–Ringer buffer containing 20 μM Cd\textsuperscript{2+} was added and changes in fluorescence intensities were followed for another 300 s. Compound screening was performed by duplicate. Negative and positive controls were present in each plate tested as reported before [18]. Compounds showing 50% or more inhibition of the fluorescence intensity were considered as a hit and were subsequently characterized. Hit compounds were repurchased in higher amount (25 mg) and further purified by RP-HPLC (Gradient 100% A to 100% D in 40 min) to achieve purity higher than 95%.

2.4. Radiolabeled iron uptake assay

Stably transfected clones of hDMT1-expressing HEK293 cells were plated on poly-d-lysine-coated, white 96-well plates at a cell density of 40,000 cells/well. After 24 h, the growth medium was aspirated and cells were washed with the Krebs–Ringer buffer. To measure iron uptake the Krebs–Ringer buffer was supplemented with 1 mM ascorbic acid and 0.5 μCi radioactive \[^{55}\text{Fe}\] iron (American Radiolabeled Chemicals, St. Louis, MO, USA). Ferrous stock solution (10 mM) was prepared immediately before use by dissolving ferrous iron chloride tetrahydrate in 0.5 mM HCl. A 10 μl aliquot from this stock solution was added to 10 ml of the supplemented Krebs–Ringer buffer. Thereafter, the pH of the solution was adjusted to the desired value with 1 N NaOH. To obtain solutions with the desired iron concentrations (0–50 μM) serial dilutions of the 100 μM solution were made in the supplemented Krebs–Ringer buffer. The assay was terminated after 15 min by washing the Plates 4 times with ice cold Krebs–Ringer buffer (pH = 7.4) using a ELX405 microplate washer (BioTek Instruments, Luzern, Switzerland). Subsequently, 100 μl of Microscint 20 (PerkinElmer, Basel, Switzerland) was dispensed into each well and incubated at RT for 1 h under constant agitation. Radioactive \[^{55}\text{Fe}\] iron uptake was measured using a TopCount Microplate Scintillation and Luminescence Counter (PerkinElmer).
2.5. Cell surface biotinylation and Western blotting

Surface biotinylation experiments to study whether hit compounds affects hDMT1 cell surface density were conducted as described before [24]. Briefly, non-transfected and stably transfected clones of hDMT1-expressing HEK293 cells were seeded on a 6-well plate and maintained in supplemented DMEM as described above. Three different treatments where performed on hDMT1 expressing HEK 293 cell clones before cell surface biotinylation. Cells were incubated with 100 μM hit compound for 15 min at room temperature in presence or absence of 1 μM Fe⁡²⁺. As control, cells were treated with vehicle alone. After the corresponding experimental treatment, cells were washed 3 times with PBS and surface proteins were biotinylated by incubating cells with 1.5 mg/ml sulfo-NHS-LC-biotin (Socochim, Lausanne, Switzerland) in 10 mM triethanolamine (pH 7.4), 1 mM MgCl₂, 2 mM CaCl₂, and 150 mM NaCl for 90 min with horizontal motion at 4 °C. After labeling, plates were washed with quenching buffer (PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂, and 100 mM glycine) for 20 min at 4 °C, and then rinsed once with PBS. Cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, 1% triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) and lysates were cleared by centrifugation. Cell lysates of equivalent amounts of protein were equilibrated overnight with streptavidin-agarose beads at 4 °C. Beads were washed sequentially with solutions A (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 5 mM EDTA) three times, B (50 mM Tris-HCl (pH 7.4) and 500 mM NaCl) two times, and C (50 mM Tris-HCl, pH 7.4) once. Biotinylated proteins were then released by heating to 95 °C for 5 min in 2.5× Laemmli buffer. Samples were separated using 8% SDS gel and proteins were transferred to a PVDF blot membrane. After blocking, the blot was incubated with the primary antibody (mouse monoclonal human DMT1 1:4000; Abnova, Luzern, Switzerland) overnight at 4 °C. Goat HRP-conjugated anti-mouse (1:4000; Bio-Rad) was used as the secondary antibody. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) method (GE Healthcare, Glattburg, Switzerland). As a loading control, all biotinylated proteins were visualized with HRP-conjugated streptavidin (Bio-Rad). To verify membrane fraction purity the blot was probed with anti-actin (rabbit polyclonal 1:1000; Santa Cruz Biotechnologie, Heidelberg, Germany) and goat HRP-conjugated anti-rabbit (Bio-Rad 1:20,000) was used as a secondary antibody.

2.6. Data analysis

Dose–response curves were analyzed by a 4-parameter Logistic model:

\[ E(D) = E_{\infty} + \frac{E_{0} - E_{\infty}}{1 + \left(\frac{D}{I_{C50}}\right)^{n_{h}}} \]  

where \( E(D) \) is the measured response parameter, \( D \) is drug concentration, \( I_{C50} \) is the concentration at half-maximal effect, \( E_{0} \) and \( E_{\infty} \) are the top and bottom asymptotes of the response curve, respectively.

DMT1-mediated Fe⁡²⁺ transport initial rate (\( v \)) data were fit to the Hill equation:

\[ v = \frac{V_{\text{max}}[S]^{n_{h}}}{K_{m}^{n_{h}} + [S]^{n_{h}}} \]  

where \( V_{\text{max}} \) is the limiting rate, \( K_{m} \) is the substrate concentration for half-saturation, [S] is substrate concentration and \( n_{h} \) is the Hill coefficient.

The nature of pyrimidinone 8 inhibitory effects on DMT1-mediated Fe⁡²⁺ uptake was analyzed by testing the effect of different inhibitor concentrations on the kinetic parameters of DMT1-mediated Fe⁡²⁺ transport. Kinetic parameters obtained by

![Fig. 1. Structures of the reference compounds used for the LBVS and the newly identified DMT1 inhibitors. (A) Chemical structure of known DMT1 inhibitors used as references for LBVS. (B) Chemical structure of the two DMT1 inhibitors, found during the screening campaign: isothioura 7 and pyrimidinone 8.](image-url)
fitting the Hill equation to the experimental radiolabeled iron uptake results were further analyzed using the Lineweaver–Burk, Dixon and Cornish–Bowden plots. The reciprocal velocity (1/v) was plotted against the inverse of the substrate concentration 1/[Fe2+] or the inhibitor concentration [I] (Lineweaver–Burk and Dixon plots, respectively) and [S]/v ratio against [I] (Cornish–Bowden plot) according to the following equations, respectively:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \frac{1}{[S]} \tag{3}
\]

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[S]}\right) + \frac{1}{V_{\text{max}}} \left(1 + \frac{K_m}{[I]}\right) \tag{4}
\]

\[
\frac{[S]}{v} = \frac{K_m}{V_{\text{max}}} \left(1 + \frac{[I]}{K_i}\right) + \frac{[S]}{V_{\text{max}}} \left(1 + \frac{[I]}{K_i}\right) \tag{5}
\]

The inhibition constants \(K_i\) and \(K_{ii}\) (see the kinetic scheme of Fig. 7) were determined by calculating the x-intercept from the equation generated by the linear regression of the Dixon and Cornish–Bowden plots. To analyze whether inhibition is linear, the reciprocal limiting rate (1/Vmax) and \(K_m/V_{\text{max}}\) ratio were plotted against [I] according to the following equations:

\[
\frac{K_m}{V_{\text{max}}} = \frac{K_m}{V_{\text{max}}} \frac{1}{[I]} + \frac{K_m}{V_{\text{max}}} \tag{6}
\]

\[
\frac{1}{V_{\text{max}}} = \frac{1}{V_{\text{max}}} \frac{[I]}{K_i} + \frac{1}{V_{\text{max}}} \tag{7}
\]

Curve fitting and parameter estimation were carried out using SigmaPlot 12.0 (Systat Software, Chicago, IL, USA). Experimental results were fitted using linear regression (solid lines). The equation generated by the linear regression of the experimental results was used to obtain the x-intercept. A line representing this equation from \(x = 0\) to the x-intercept was added to each plot (dashed line).

2.7. Statistics

Data is presented as mean ± S.D. of a given number of independent experiments. In a given independent experiment, each condition was tested by triplicate. The normality and equality of the standard deviation of the data were tested and based on these results a parametric or a nonparametric test was selected. Significance between two groups was determined by unpaired Student’s t tests. Multiple comparisons between control and different treatment groups were performed using one-way ANOVA analysis followed by Bonferroni test. A P value of <0.05 was considered statistically significant. Statistical comparisons were performed with SigmaPlot 12.0.

3. Results

3.1. Compound screening for DMT1 inhibitors

During the screening campaign two hit compounds, isothiourea 7 and pyrimidinone 8 (Fig. 1B), out of 140 selected from the virtual screening were found to inhibit ~50% of DMT1-mediated Cd2+ transport activity (data not shown). The compound isothiourea 7 was a direct derivative of known inhibitor 6 and this scaffold was not further investigated. Instead, the newly identified DMT1-inhibitor pyrimidinone 8 was further characterized using radioactive [55Fe] iron uptake in HEK cells stably expressing hDMT1.

3.2. Non-competitive inhibition of hDMT1 by pyrimidinone 8

Following identification of pyrimidinone 8 as an inhibitor of Cd2+ uptake by hDMT1, we determined the inhibition mechanism of the compound. The inhibitory effect of pyrimidinone 8 on DMT1 was evaluated by the uptake of radiolabeled iron in the presence of increasing concentrations of pyrimidinone 8 (0–500 μM). As shown in Fig. 2A, pyrimidinone 8 inhibited radioactive [55Fe] iron uptake in a concentration-dependent manner in stable hDMT1 expressing HEK 293 cell clones with an IC50 value of 13.8 ± 2.9 μM (n = 6). The compound exhibited an 80 ± 2% inhibition at the maximal tested concentration (500 μM). For all analyses, the coefficient of determination (\(r^2\)) was 0.997.

Fig. 2B shows the progress curves of radiolabeled iron uptake in hDMT1 expressing HEK 293 cell clones and control HEK 293 cells. All the reactions were run at pH 5.5, an optimum pH to study DMT1-mediated cellular Fe2+ uptake [2]. Under these conditions, the curves were linear for at least 30 min. To analyze the mode of inhibition of pyrimidinone 8, the concentration dependence of hDMT1-mediated radiolabeled [55Fe] iron uptake in response to extracellular Fe2+ was measured in the absence and presence of 10, 25 and 50 μM pyrimidinone 8. The initial rate of hDMT1-mediated cellular Fe2+ uptake as a function of extracellular Fe2+ followed a typical hyperbola (Fig. 2C). Taken together, these results suggest that the DMT1-mediated cellular Fe2+ uptake followed a typical steady state kinetic mechanism. The kinetic constants were calculated by fitting the initial rates of Fe2+ uptake to the Hill equation (Eq. 2). The \(K_m\) and \(N_h\) values in absence of inhibitor (1.20 ± 0.02 μM and 0.88 ± 0.06 respectively) are comparable to the previous values reported in the literature [1,2]. When the concentration dependence of hDMT1-mediated radiolabeled [55Fe] iron uptake in response to extracellular Fe2+ was tested in the presence of increasing concentrations of pyrimidinone 8, a significant reduction in the limiting rate was observed as compared with control experiments (i.e. radiolabeled [55Fe] iron uptake in the absence of inhibitor). This reduction in \(V_{\text{max}}\) was not accompanied by a change in \(K_m\) value or the Hill coefficient, suggesting that pyrimidinone 8 acts as a non-competitive inhibitor of hDMT1-mediated cellular Fe2+ uptake (Fig. 2C and E). The Lineweaver–Burk plot for the inhibition of hDMT1-mediated Fe2+ uptake by pyrimidinone 8 again strongly indicates a non-competitive inhibition (Fig. 2D). In this plot, a change in the slope and y-intercept of the curve was observed in the presence of pyrimidinone 8, but not on the x-intercept. The values kinetic parameters obtained by fitting the initial rates of hDMT1-mediated cellular Fe2+ uptake to the Hill equation in the absence and presence of 10, 25 and 50 μM of the DMT1 inhibitor pyrimidinone 8 are summarized in Fig. 2E.

3.3. Determination of pyrimidinone 8 inhibition constants on DMT1-mediated Fe2+ uptake

To further investigate the mechanism of the pyrimidinone 8-mediated DMT1 inhibition, we examined in greater detail the effect of inhibitor concentration on the kinetic parameters of hDMT1-mediated Fe2+ transport. As shown by the Dixon plot (Eq. 4) a change in the slope and y-intercept of the curve was observed in the presence of inhibitor but the x-intercept remained unchanged (Fig. 3A). From this graph we calculated the value of 20 ± 3 μM for the \(K_i\), the inhibition constant for inhibitor binding to the free enzyme. A similar result was obtained when the results of DMT1-mediated Fe2+ uptake in the presence of inhibitor concentrations were used to obtain the Cornish–Bowden plot (Fig. 3B). A value of 18 ± 1 μM was calculated for the \(K_i\), the inhibition constant for inhibitor binding to the enzyme/substrate complex, from the x-intercept of the linear regressions of this plot. For all analyses, the coefficient of determination (\(r^2\)) was ≥0.995. The similar values of
both inhibition constants obtained in Dixon and Cornish–Bowden plots suggest that compound pyrimidinone 8 acts as a non-competitive inhibitor of hDMT1.

Two secondary plots were used to determine whether pyrimidinone 8 display a linear non-competitive inhibition. A linear dependency was observed when $1/V_{\text{max}}$ was plotted against the inhibitor concentration $[I]$ ($r^2 = 0.994$, Fig. 3C). A value of $20 \pm 1 \mu M$ for the inhibition constant $K_I$ was calculated from the x-intercept. Similar results were obtained when $K_m/V_{\text{max}}$ was plotted against $[I]$ (Fig. 3D). In this case, a linear dependency was also observed and a value of $17 \pm 2 \mu M$ ($r^2 = 0.985$) was calculated from the x-intercept for the inhibition constant $K_I$. Taken together, these results show that pyrimidinone 8 mediates linear, non-competitive inhibition of hDMT1.

3.4. Pyrimidinone 8 does not modulate hDMT1 surface expression

We performed surface biotinylation experiments to study whether pyrimidinone 8 affects hDMT1 cell surface density. As shown in Fig. 4, pyrimidinone 8 treatment did not affect hDMT1 cell surface expression. No differences in plasma membrane hDMT1 expression were observed when comparing vehicle-treated hDMT1-expressing HEK293 cells with cells incubated with pyrimidinone 8 in presence or absence of $1 \mu M$ Fe$^{2+}$ ($n = 3$ independent experiments). Absence of actin indicates the purity of the plasma membrane fraction. No statistically difference between the different groups was observed when all biotinylated proteins were visualized with HRP-streptavidin as loading controls. No expression of hDMT1 was detected after similar treatment in non-transfected cells. These data thus indicate that pyrimidinone 8 does not significantly influence hDMT1 abundance in the plasma membrane of the cell.

3.5. Pyrimidinone 8 is a reversible inhibitor of hDMT1

To study whether pyrimidinone 8 is a reversible non-competitive inhibitor, an hDMT1-expressing HEK293 cells where incubated for 5 or 10 min in the presence or absence of $50 \mu M$ inhibitor. After preincubation with the inhibitor, cells were rapidly washed 3 times with 200 $\mu L$ Krebs–Ringer buffer at room temperature and...
radiolabeled $^{55}$Fe iron uptake assay was performed as described above. Positive and negative control experiments of pyrimidinone 8-mediated hDMT1 inhibition were performed in every set of experiments. In these cases, the washing step was omitted and the radiolabeled $^{55}$Fe iron uptake assay was performed in the presence or absence of 50 µM pyrimidinone 8. As shown in Fig. 5, the inhibitory effect of pyrimidinone 8 on hDMT1-mediated Fe$^{2+}$ uptake was not observed when the cells were washed before the Fe$^{2+}$ uptake was performed. No significant differences were observed on the $K_m$ and $V_{max}$ values when cells preincubated with 50 µM pyrimidinone 8 for 5 or 10 min and then washed were compared with control experiments in the absence of inhibitor (Fig. 5A). In contrast, a 78% reduction in the limiting rate of hDMT1 transport activity was observed when pyrimidinone 8 was present in the medium (i.e. no washing step was performed; Fig. 5A). The table of Fig. 5B summarizes the kinetic parameters values obtained by fitting the initial rates of hDMT1-mediated cellular Fe$^{2+}$ uptake to the Hill equation for these set of experiments.

3.6. Extracellular pH does not affect hDMT1 inhibition by pyrimidinone 8

To test whether there is any effect of extracellular pH on the pyrimidinone 8-mediated hDMT1 inhibition, the concentration dependence of DMT1-mediated radiolabeled $^{55}$Fe iron uptake in response to extracellular H$^+$ was measured in the absence and presence of 10 µM and 50 µM pyrimidinone 8 at saturating Fe$^{2+}$ conditions (i.e. 20 µM). In presence of pyrimidinone 8, a reduction in $V_{max}$, but not in $K_m$, was observed, when comparing with control experiments (Fig. 6A). A detailed kinetic analysis for the inhibition of hDMT1-mediated Fe$^{2+}$ uptake by pyrimidinone 8 at different extracellular H$^+$ concentrations indicates once again a non-competitive inhibition mode (Fig. 6B and C). In this case, the calculated the values of $K_i$ and $K_m$ were 18 ± 8 µM and 18 ± 9 µM, respectively. The values kinetic parameters obtained by fitting the initial rates of hDMT1-mediated cellular Fe$^{2+}$ uptake to the Hill equation in the absence and presence of 10 and 50 µM of the DMT1 inhibitor pyrimidinone 8 are summarized in Fig. 6F.

A linear dependence was observed when $1/V_{max}$ and $K_m/V_{max}$ was plotted against the inhibitor concentration [I] (Fig. 6D and E). Values of 19 ± 7 µM and 22 ± 13 µM were calculated from these plots for the inhibition constants $K_i$ and $K_m$, respectively. For all analyses, the coefficient of determination ($r^2$) was >0.963. Taken altogether, these results indicate that extracellular pH tested does not affect the pyrimidinone 8 mediated non-competitive inhibition of Fe$^{2+}$ uptake by hDMT1.

4. Discussion

The main goal of the present study was to identify and characterize novel inhibitors of hDMT1, a membrane transporter that plays a major role in whole body and cellular iron metabolism.
Two novel hDMT1-inhibitors were discovered by performing a LBVS on the chemically diverse Princeton database (700,000 commercially available compounds). These two hits were obtained from a total of 140 tested compounds during the screening campaign, implying a hit rate of ~1.5%. This approach shows at least a 10-fold higher hit rate compared to classical approaches using random compound libraries [14,15]. The compound isothiourea 7, is structurally related to a benzylisothiourea (compound 6 in Fig. 1A) that has been shown to act as a DMT1 inhibitor [17]. Hence, our results suggest that substitution of the aromatic ring of compound 6 (Fig. 1A) with methyl groups is not required for its inhibitory activity on DMT1. Isothiourea 7 acts as a reversible competitive inhibitor and block hDMT1 transport activity with a $K_i$ of 1.9 ± 0.7 μM (data not shown). A previous report has shown that hDMT1 can be inhibited in a competitive fashion by pharmacological agents. The compound NCS06711, a polyaromatic sulfonate dye, is a competitive inhibitor of hDMT1 with a $K_i$ value of 7 μM [15]. In contrast, the compound pyrimidinone 8, a 2-pyrazolyl 4(3H)-pyrimidinone derivative, acts as a non-competitive inhibitor of hDMT1 and is structurally unrelated to previous known DMT1 inhibitors, representing a novel candidate as a basis for lead optimization to be used for preclinical studies.

We tested the concentration dependence of DMT1-mediated iron uptake in response to extracellular Fe$^{2+}$ in absence or presence of different concentrations of pyrimidinone 8. In these experiments we found that increasing concentrations of pyrimidinone 8 decreased the limiting rate for hDMT1-mediated Fe$^{2+}$ transport without affecting the $K_m$ value. We observed a change in the slope and on the y-intercept, but not on the x-intercept, in both Lineweaver–Burk- and Dixon-type plots for the inhibition of hDMT1-mediated Fe$^{2+}$ uptake by pyrimidinone 8. These two plots are widely used to distinguish between competitive and non-competitive enzyme inhibition. However, when used individually to estimate kinetic parameters these plots present some limitations [25–27]. In this study, we use a combination of different graphical analyses to unambiguously distinguish the type of inhibition displayed by pyrimidinone 8 on hDMT1. Similarly to the Dixon plot, a change in the slope and on the y-intercept, but not on the x-intercept, was observed in the Cornish–Bowden plot. Importantly, no statistically significant differences were observed between the calculated inhibition constants ($K_i$ and $K_m$, respectively). The two secondary plots show that $1/V_{\text{max}}$ and $K_m/V_{\text{max}}$ display a linear relationship with $I$, indicating that the inhibitor binds at a single allosteric binding site [28,29]. Taken together, our results strongly suggest that pyrimidinone 8 acts as a simple non-competitive inhibitor of hDMT1. The simplest interpretation of the linear non-competitive inhibition of hDMT1 by pyrimidinone 8 is that this inhibitor binds to a non-catalytic allosteric site of the transporter, which can be adequately described by the simple kinetic scheme showed in Fig. 7. Indirect evidence that hDMT1 can be allosterically modulated has been presented in a previous study [30], showing that alkaline-earth metals that are not transported by hDMT1 act as low-affinity non-competitive inhibitors of this transporter. In this study, we provide the first experimental evidence that hDMT1 can be allosterically regulated by pharmacological agents.

The mechanism of non-competitive inhibition of hDMT1 exerted by pyrimidinone 8 was examined in further detail. A decrease in the rate limiting $V_{\text{max}}$ may reflect a reduction in the cell surface expression of the transporter. Internalization and transporter trafficking may be one mechanism that permits the regulation of DMT1 transport activity [31]. Biotinylation experiments showed that the inhibitor pyrimidinone 8 did not affect plasma membrane hDMT1 expression. These results indicate that the decrease observed in the hDMT1 rate limiting in the presence of pyrimidinone 8 is not related to reduction of hDMT1 abundance in the plasma membrane of the cell. We tested whether pyrimidinone 8 is a reversible non-competitive inhibitor. No significant differences were observed on the $K_m$ and $V_{\text{max}}$ values

**Fig. 4.** Pyrimidinone 8 does not modulate hDMT1 surface expression. (A) A typical experiment representative of three independent experiments is shown. Control HEK 293 cells and hDMT1-expressing HEK293 cell clones were treated with the vehicle alone (DMSO), 100 mM pyrimidinone 8 (Pyr 8), and with 100 mM pyrimidinone 8 in the presence of 1 mM Fe$^{2+}$ (Pyr 8 + Fe$^{2+}$). Absence of actin indicates the purity of the plasma membrane fraction. (B) Quantification of hDMT1 plasma membrane expression.

**Fig. 5.** Pyrimidinone 8 is a reversible inhibitor of hDMT1. (A) The hDMT1-expressing HEK 293 cell clones were preincubated with 50 μM pyrimidinone 8 for 5 (gray triangle up) or 10 (gray diamond) minutes. Positive (presence of inhibitor, black circle) and negative (absence of inhibitor, black square) control experiments of pyrimidinone 8-mediated hDMT1 inhibition were included. Experimental results are presented as the mean ± S.D. of 5 independent experiments for each curve. Experimental results were fitted to the Hill equation (black and gray lines). (B) Summary of kinetic parameters values obtained by fitting the initial rates of hDMT1-mediated cellular Fe$^{2+}$ uptake to the Hill equation for these set of experiments.
Fig. 6. Extracellular pH does not affect hDMT1 inhibition by pyrimidinone 8. (A) The concentration dependence of DMT1-mediated radiolabeled $^{59}$Fe iron uptake in response to extracellular H$^+$ was measured in the absence or presence of pyrimidinone 8. Experimental results were fitted to the Hill equation (black line). (B) Dixon plot. (C) Cornish–Bowden plot. (D) Linear dependence of the reciprocal limiting rate ($1/V_{\text{max}}$) on inhibitor concentration [I]. (E) The ratio between substrate concentration for half-saturation and the limiting rate ($K_m/V_{\text{max}}$) shows a linear dependence on the inhibitor concentration [I]. In all the cases, experimental results are presented as the mean ± S.D. of 4–5 independent experiments for each curve. (F) Summary of kinetic properties for inhibition of hDMT1-mediated Fe$^{2+}$ uptake by pyrimidinone 8 at different extracellular H$^+$ concentrations.

when a washing step was performed after incubating the hDMT1-expressing HEK293 cell clones with 50 μM pyrimidinone 8. These results indicate that pyrimidinone 8 is a reversible non-competitive inhibitor of hDMT1. Finally, we tested the dependence of pyrimidinone 8-mediated hDMT1 inhibition on extracellular pH. In a previous study we showed that H$^+$ and Fe$^{2+}$ are translocated within the same transport cycle with an ordered binding of first H$^+$ and then Fe$^{2+}$ [2]. To facilitate the analysis, our experiments to test the dependence of DMT1-mediated radiolabeled $^{59}$Fe iron uptake in response to extracellular Fe$^{2+}$ in the presence of pyrimidinone 8 were performed at low extracellular pH. When performing a series of experiments at different extracellular H$^+$ concentration in saturating Fe$^{2+}$ conditions, we found similar values for inhibition constants $K_i$ and $K_{i0}$. These results indicate that the non-competitive inhibition of hDMT1-mediated Fe$^{2+}$ transport by pyrimidinone 8 did not exhibit any dependence on extracellular pH at the pH values measured.

In summary, we found that the compound pyrimidinone 8 mediates a linear non-competitive inhibition of hDMT1-mediated Fe$^{2+}$ transport activity. This inhibition is reversible, does not affect the cell surface expression of the transporter and show no dependence on extracellular pH. The discovery of pyrimidinone 8 not only represents a breakthrough in pharmaceutical discovery but also may help unravel how this transporter works and how it can be pharmacologically modulated. Recently, the three-dimensional structure of a close prokaryotic homolog of hDMT1 has been elucidated [32] which may lead to structure-based drug design. Further efforts are needed to identify the precise binding site residues on hDMT1 that are critical for the non-catalytic allosteric regulation of the transporter so that a rational, structurally based approach may be applied to chemically modify and develop inhibitors with optimal inhibitory properties.

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