Bioorganic & Medicinal Chemistry Letters 22 (2012) 5108–5113

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Discovery of benzylisothioureas as potent divalent metal transporter 1 (DMT1) inhibitors

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ARTICLE INFO

Article history:
Received 5 March 2012
Revised 19 May 2012
Accepted 29 May 2012
Available online 15 June 2012

Keywords:
Hemochromatosis
DMT1
Iron overload
Diarylether
Diarythioether
Dibenzofuran
Dibenzothiophene

ABSTRACT

Inhibition of intestinal brush border DMT1 offers a novel therapeutic approach to the prevention and treatment of disorders of iron overload. Several series of diaryl and tricyclic benzylisothiourea compounds as novel and potent DMT1 inhibitors were discovered from the original hit compound **1**. These compounds demonstrated in vitro potency against DMT1, desirable cell permeability properties and a dose-dependent inhibition of iron uptake in an acute rat model of iron hyperabsorption. Tricyclic compounds increased the in vitro potency by up to 16-fold versus the original hit. Diaryl compounds **6b** and **14a** demonstrated significant iron absorption inhibition in vivo with both 25 and 50 mg/kg doses. The diaryl and tricyclic compounds described in this report represent promising structural templates for further optimization.

Iron is an essential nutrient that is toxic in excess. Iron levels in humans are primarily regulated at the level of intestinal iron absorption. Divalent metal transporter 1 (DMT1) (also known as natural resistance-associated macrophage protein-2 (NRAMP2), divalent cation transporter-1 (DCT1) and solute carrier family 11, member 2 (SLC11A2)) is responsible for the transport of several divalent metal ions across the cell membrane.[1–5] DMT1 is particularly important for iron absorption in the duodenum, where it is localized in the villus enterocytes and mediates the influx of dietary non-heme iron from the intestinal lumen.[6] Once dietary iron is absorbed across the intestinal wall, there is no physiologic mechanism for its excretion. Thus, excess absorbed iron is largely retained in the body and accumulates in the heart, liver and other vital organs.[7,8] Excess tissue iron can lead to significant tissue damage[8] and has been associated with a number of disease states including cardiomyopathy, cirrhosis, arthritis, hepatocellular carcinoma and others.[9–12] Hereditary hemochromatosis (HH) is characterized by excessive intestinal iron absorption resulting in tissue iron overload and damage. There is now compelling evidence associating inappropriate excess DMT1 activity with HH[13,14] and related conditions. Currently, the standard treatment for HH is repeat phlebotomy. However, poor patient compliance is a significant issue with this invasive treatment.[15] Furthermore, phlebotomy itself can exacerbate the underlying causal iron hyperabsorption as the body responds to the loss of red blood cells by increasing erythropoiesis and iron uptake *via* intestinal DMT1.[16]

Another potential indication for DMT1 inhibitor therapy is the common hemoglobinopathy thalassemia major, in which patients exhibit a severe anemia requiring chronic blood transfusions. The iron overload (and ensuing complications) resulting from the transfused erythrocytes is further compounded by the underlying anemia, which in turn stimulates absorption of dietary iron through DMT1. Patients with thalassemia intermedia typically do not require significant blood transfusions, but nevertheless, due to the anemia have DMT1 upregulation and intestinal iron hyperabsorption resulting in iron overload. Phlebotomy is not an option for patients with thalassemia (as they are anemic), and the present standard of care for transfusional iron overload is chelation therapy. However, these agents either have poor oral bioavailability and/or suffer from serious dose-limiting side effects.[17–19] Direct blockade of DMT1-mediated iron influx by an orally-administered

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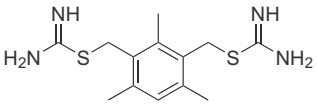
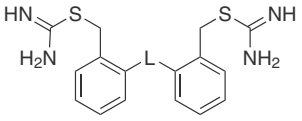
http://dx.doi.org/10.1016/j.bmcl.2012.05.129



1

IC_{50} = 0.29 μM

Figure 1. Structure of the HTS hit **1**.



L = O, S, C=O, NCH_3

Figure 2. Structures of diaryl isothioureas.

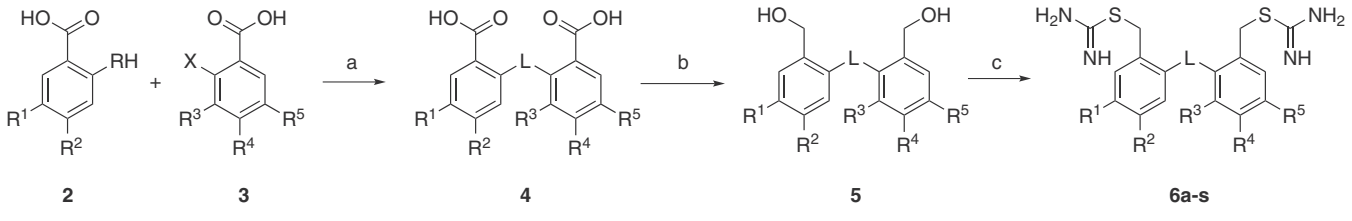
small molecule inhibitor could be expected to lead to an improved therapeutic approach for the treatment of iron overload. However, DMT1 is also expressed in erythroid cells, macrophages and hepatocytes and inhibitors potentially compromise iron metabolism. Therefore, limiting the exposure of a compound to the gut (*i.e.*, minimizing systemic bioavailability) could be advantageous from a safety perspective.

To date, there have been limited reports of small-molecule compounds that specifically modulate or inhibit DMT1.[20,21] We previously reported a series of potent and efficacious pyrazole DMT1 blockers with significant Caco-2 permeability.[22,23] In order to identify potent compounds with a higher potential for gut restriction, a high throughput screen (HTS) using a calcein fluorescence assay was carried out as previously described.[22,24,25] This effort led to the identification of the hit compound **1** (Fig. 1) with an IC_{50} of 0.29 μM against DMT1 and modest Caco-2 permeability ($P_{app} = 0.9 \times 10^{-6}$ cm/s (a→b), $1.2 \rightarrow 10^{-6}$ cm/s (b→a)). Our initial SAR effort was focused on the replacement of the isothiourea head groups present in **1** with other moieties (e.g., amide, reverse amide, amidine, guanidine, cyanoguanidine, sulfonamide and carbamate), but this endeavor proved to be futile as all of these modifications resulted in a dramatic decrease in inhibitory activity on DMT1. Modifications to the chain length between the phenyl ring and the terminal isothiourea groups of the two arms, the introduction of substituents on the benzylic carbons and replacement of the phenyl ring with pyridine or saturated hydrocarbon rings also proved to be detrimental to target potency. Although we were not able to generate improvements over the original hit compound **1** from these early investigations, we gained knowledge that the terminal isothiourea groups and the distance between the two isothiourea head groups were critical to retain the inhibitory activity against DMT1. Therefore, our efforts were then focused on analogues containing the isothiourea head groups.

When the phenyl anchor ring was replaced with a diaryl template (Fig. 2), this transformation (which also afforded novelty) led to the retention of inhibitory activity against DMT1. Based on this development, optimization of the diaryl template was carried out.

Syntheses of the diaryl compounds are outlined in Schemes 1 and 2. The commercially-available starting materials **2** (Scheme 1) were coupled with acids **3** under Ullmann conditions in the presence of Cu powder and K_2CO_3 at 130–140 °C in a sealed tube for 3 h. The di-acids **4** obtained were reduced to the di-alcohols **5** with borane/THF complex at room temperature overnight. The di-alcohol **5** was treated with HBr and thiourea to afford the final diaryl compounds **6a–s** as their corresponding HBr salts.

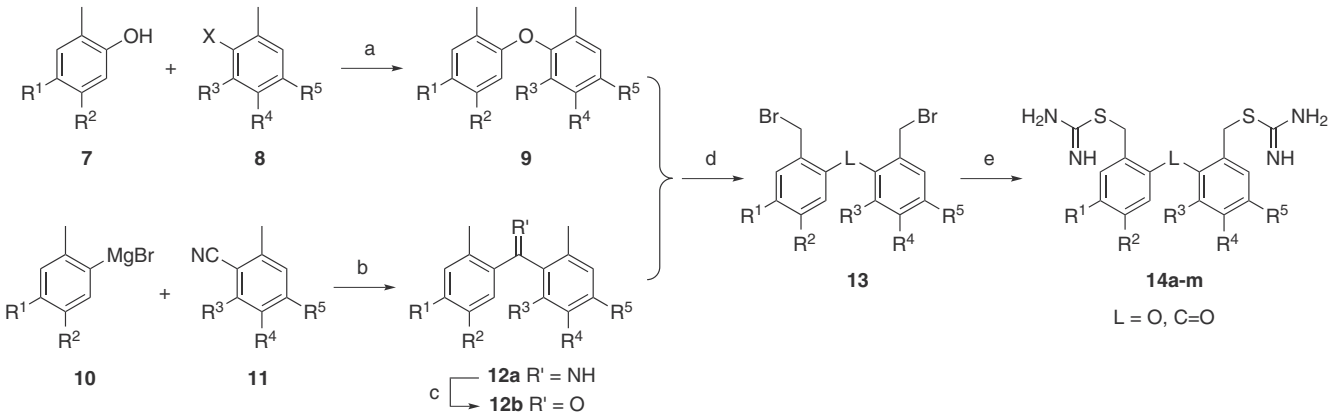
Alternatively, the commercially-available phenols **7** (Scheme 2) were coupled with halides **8** under Ullmann coupling conditions in the presence of CuI and Cs_2CO_3 at 120 °C in a sealed tube overnight



2 **3** **4** **5** **6a-s**

L = S, NCH_3

Scheme 1. Reagents and conditions: (a) Cu powder, K_2CO_3, H_2O, 130–140 °C, 3 h; (b) BH_3, THF, rt, 16 h; (c) thiourea, 48% aqueous HBr, 80 °C, 3 h.



7 **8** **9** **10** **11** **13** **14a-m**

L = O, C=O

Scheme 2. Reagents and conditions: (a) CuI, *N,N*-dimethylglycine hydrochloride, Cs_2CO_3, dioxane, 120 °C, 16 h; (b) 0–80 °C, under nitrogen atmosphere, 16 h; (c) 1-propanol, 6 N hydrochloric acid, rt, 16 h; (d) *N*-bromosuccinimide, benzoyl peroxide, CCl_4, reflux, 12 h; (e) thiourea, ethanol, 80 °C, 16 h.

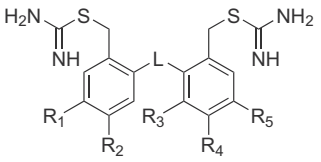
to afford diarylethers **9**. The diaryl ketone **12b** was obtained via an imine intermediate after the reaction between the Grignard reagent **10** and the nitrile **11**, followed by treatment with acid. Both methyl groups of compounds **9** and **12b** were brominated under free radical conditions using NBS and benzoyl peroxide under reflux for 12 h in CCl_4 to generate the di-bromo compounds **13**. The bromo groups of **13** were replaced with thiourea to generate diarylethers or diaryl ketones **14a–m** as their corresponding HBr salts.

The inhibitory activity of these analogues was assessed by calcein fluorescence assay as previously reported[22] and is summarized in Table 1. The modification of the linker between the two aryl groups seemed to have little effect on DMT1 inhibitory activity as shown by compounds **6a** (S), **6s** (N-CH_3), **14a** (O) and **14i** (C=O). All of these linker-modified compounds had IC_{50} values in the calcein quench assay between 0.4 and 0.8 μM, about 2- to 3-fold less potent than the original hit **1**. The most potent compound was **6b** which was about threefold more potent than compound **1**. Substituents at the R_2, R_3 and R_4 positions seemed to have more profound effects on activity and resulted in dramatic decreases in potency as demonstrated by compounds **6o**, **6q**, **6r**, **14h** and **14m**. The simultaneous modification of substituents on both phenyl rings did not afford a substantial increases in inhibitory activity. Most of the compounds tested had very poor cell permeability as demonstrated by the results obtained using a Caco-2 cell permeability assay (Table 1), consistent with the desired gut-restricted profile.

A representative group of diaryl compounds (**6a**, **6b** and **14a**) were then evaluated in an acute model of iron hyperabsorption in anemic (hemoglobin <6 mg/dL, serum iron <50 μg/dL), conscious rats.[22,26] Serum iron was determined 1 h following an oral iron challenge of 1 mg/kg Fe^{2+} *po* (as ferrous sulfate) which, in turn, was administered 1 h post *po* dosing of the test article (formulated in 5% DMSO, 10% EtOH, 30% PEG400, 35% propylene glycol, 20% water). All compounds tested clearly demonstrated a dose-dependent inhibition of iron absorption in vivo (Fig. 3) and the inhibitory effects of all three compounds were statistically significant (p <0.001) at the 50 mg/kg dose level. Diaryl compounds **6b** and **14a** also demonstrated significant inhibition of iron absorption in this model at the 25 mg/kg dose level. Diaryl compound **14a** was the most efficacious among the compounds tested with an eight-fold decrease in iron absorption after oral iron challenge at 50 mg/kg when compared to its vehicle control group. All compounds were well-tolerated with no clinical observations noted during these studies.

In an attempt to further improve in vitro activity against DMT1 by eliminating rotation about the aryl–aryl bond, the diaryl ether compound **14a** was rigidified, leading to the dibenzofuran **18a**. Interestingly, this transformation indeed generated a very potent

Table 1
SAR of diaryl compounds



Compound	L	R^1	R^2	R^3	R^4	R^5	Calcein IC_{50}[a] (μM)	Cell permeability ($\times 10^{-6}$ cm/s)[b]
6a	S	H	H	H	H	H	0.18	0.1/1.4
6b	S	F	H	H	H	NO_2	0.08	0.1/0.2
6c	S	F	H	H	H	H	0.15	0.3/0.2
6d	S	F	H	H	H	F	0.15	0.4/0.4
6e	S	SO_2CH_3	H	H	H	F	0.29	<0.1/0.1
6f	S	F	H	H	H	OCH_3	0.53	2.4/2.4
6g	S	F	F	H	H	H	0.32	1.3/1.4
6h	S	CH_3	H	H	H	H	0.49	1.5/1.4
6i	S	H	H	H	F	H	0.51	0.3/0.2
6j	S	F	F	H	H	F	0.60	<0.1/0.2
6k	S	F	H	H	H	CF_3	0.69	0.1/3.8
6l	S	NH_2	H	H	H	H	0.91	ND[c]
6m	S	$SO_2N(CH_3)_2$	H	H	H	F	0.95	<0.1/0.1
6n	S	OCH_3	H	H	H	H	1.44	ND
6o	S	F	H	H	CH_3	H	1.45	ND
6p	S	Cl	H	H	H	Cl	3.02	ND
6q	S	H	H	CH_3	H	H	3.77	ND
6r	S	F	H	NO_2	H	H	4.15	ND
6s	N-CH_3	H	H	H	H	H	0.73	ND
14a	O	H	H	H	H	H	0.34	0.1/0.2
14b	O	F	H	H	H	NO_2	0.16	ND
14c	O	F	H	H	H	F	0.32	<0.1/0.2
14d	O	H	Cl	H	H	F	0.34	0.2/0.9
14e	O	H	F	H	H	F	0.84	<0.1/0.4
14f	O	H	H	H	H	Cl	1.13	ND
14g	O	Cl	H	H	H	NO_2	2.89	ND
14h	O	H	Cl	H	H	H	15.0	ND
14i	C=O	H	H	H	NO_2	H	0.37	0.1/0.7
14j	C=O	F	H	H	H	H	0.58	ND
14k	C=O	H	F	H	H	H	2.02	ND
14l	C=O	Cl	H	H	H	H	2.92	ND
14m	C=O	H	Cl	H	H	H	5.20	ND

[a] IC_{50}s are an average of at least two independent determinations.
[b] Permeability determined using Caco-2 cells. Data expressed as $P_{app}(a\rightarrow b)/P_{app}(b\rightarrow a)$.
[c] ND: not determined.

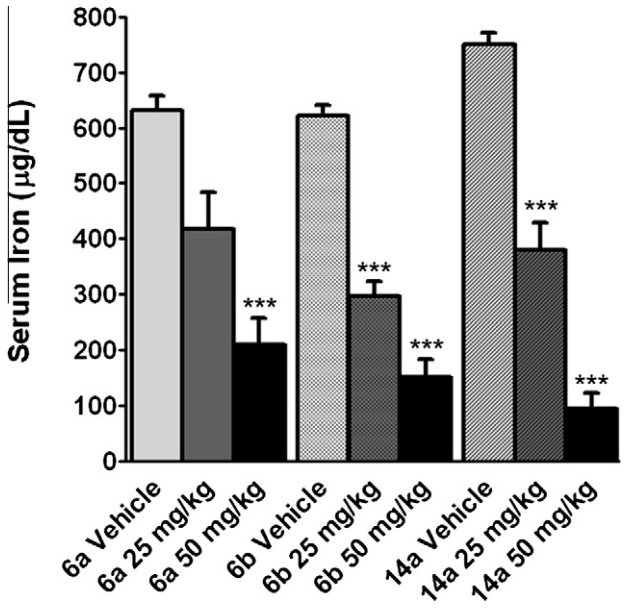
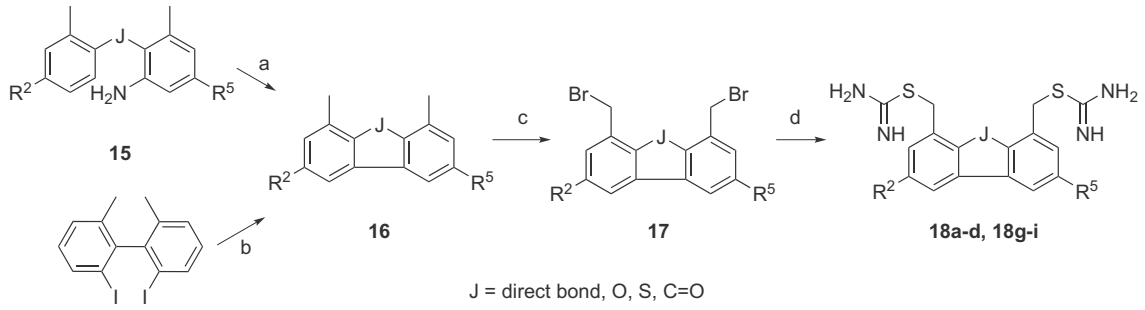


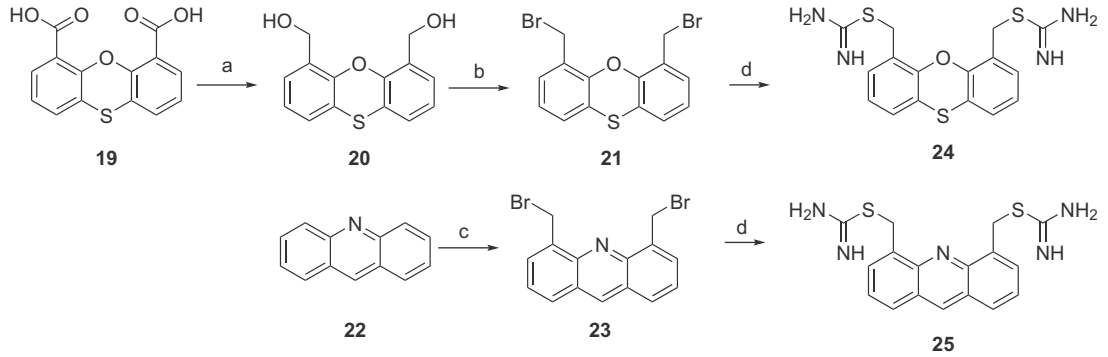
Figure 3. Effect of DMT1 inhibitors on iron absorption in an acute rat model of iron hyperabsorption. Each bar represents the average of 8 animals and the error bars represent standard errors of the mean. ***: one-way ANOVA analysis with Dunnett's multiple comparison tests when compared to its corresponding vehicle group (p <0.001).

and novel compound. As a result, several tricyclic analogues were then prepared (Schemes 3–5). The starting materials **15** (Scheme 3) were obtained following the procedures as outlined in Scheme 1 or Scheme 2. Compounds **15** were then treated with sodium nitrite at 0 °C in the presence of tetrafluoroboric acid. The intramolecular cyclization of the diazonium salt in the presence of a catalytic amount of palladium(II) acetate afforded the tricyclic intermediates **16**. Alternatively, intermediates **16** were obtained *via* intramolecular cyclization of 2,2′-diiodo-6,6′-dimethyl-1,1′-biphenyl by heating at 250–270 °C in the presence of Cu for 2 h. Bromination of intermediates **16** with *N*-bromosuccinimide generated di-bromo compounds **17** and subsequent displacement of the bromo groups with thiourea afforded compounds **18a–d** and **18g–i** as their corresponding HBr salts. Compounds **18e** and **18f** were obtained similarly following the bromination of the corresponding di-methyl intermediates, which were synthesized according to literature procedures.[27,28] The di-acid **19** (Scheme 4) was reduced to di-alcohol **20** with borane-THF complex. Bromination of **20** with PBr_3 followed by treatment with thiourea afforded compound **24**. Compound **25** was prepared by treatment of acridine **22** with bromo(methoxy)methane in concentrated sulfuric acid followed by replacement of the bromo groups of dibromide **23** with thiourea. Compound **26** (Scheme 5) was treated with bromine in acetic acid at room temperature to generate either di- or mono-substituted bromo compounds **27** followed by treatment with *n*-butyl lithium and subsequently either *N*-fluorobenzene-sulfonimide or hexachloroethane to afford the fluoro or chloro compounds **28**. Bromination of the methyl groups with NBS generated compound **29**. The displacement of the bromo groups with thiourea provided compounds **30a–e** as their corresponding HBr salts.

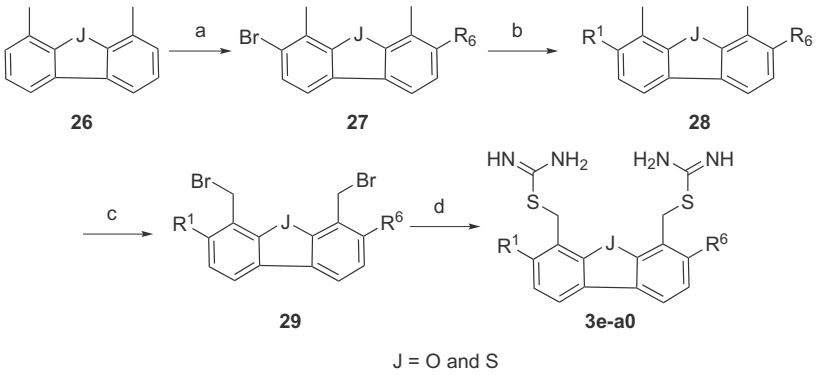
The inhibitory activities of these tricyclic compounds are summarized in Tables 2 and 3. Dibenzofuran compound **18a** was the most potent compound with an IC_{50} of 51 nM. The biphenylene compound **18b** was also very potent (IC_{50} = 97 nM). Both dibenzothiophene **18c** and fluorenone **18d** were less potent compared to **18a** and **18b**, but they were nevertheless comparable to compound **1**. Interestingly, compound **18e** was about 20-fold less potent than compound **18c** and compound **18f** was not potent while compound



Scheme 3. Reagents and conditions: (a) 48% aqueous tetrafluoroboric acid, $NaNO_2$, THF/H_2O, $Pd(OAc)_2$, 0–60 °C to rt; (b) Cu, 250–270 °C, 2 h; (c) NBS, benzoyl peroxide, CCl_4, reflux, 12 h; (d) thiourea, ethanol, 80 °C, 16 h.

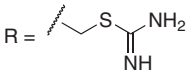


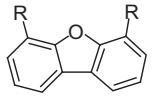
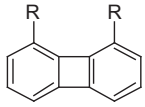
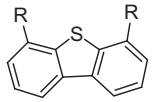
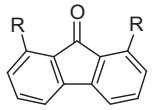
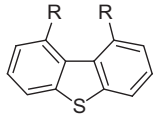
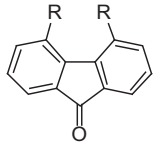
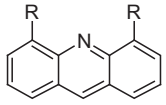
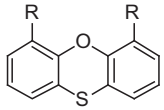
Scheme 4. Reagents and conditions: (a) BH_3, THF, rt, 16 h; (b) PBr_3, anhydrous diethyl ether, rt, 16 h; (c) bromo(methoxy)methane, concd H_2SO_4; (d) thiourea, ethanol, 80 °C, 16 h.



Scheme 5. Reagents and conditions: (a) bromine, acetic acid, rt, 16 h; (b) *n*-butyllithium, *N*-fluorobenzenesulfonimide or hexachloroethane, THF, −78 °C, 5 h; (c) NBS, benzoyl peroxide, CCl_4, reflux, 12 h; (d) thiourea, ethanol, 80 °C, 16 h.

Table 2
SAR of tricyclic compounds

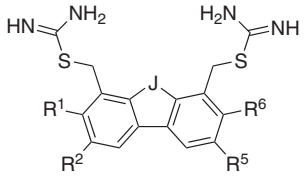
R = 

Compound	Structure	Calcein IC_{50}[a] (μM)	Cell permeability ($\times 10^{-6}$ cm/s)[b]
18a		0.05	<0.1/0.2
18b		0.10	<0.1/0.4
18c		0.26	<0.1/<0.1
18d		0.58	0.4/0.3
18e		5.04	ND[c]
18f		>15	ND
24		0.15	ND
25		0.29	0.2/0.5

[a] IC_{50}s are an average of at least two independent determinations.
[b] Permeability determined using Caco-2 cells. Data expressed as $P_{app}(a\rightarrow b)/P_{app}(b\rightarrow a)$.
[c] ND: not determined.

18d had sub-micromolar activity. The six-membered middle ring analogues **24** and **25** retained activity against DMT1. Introduction of electron-withdrawing groups to the *meta*-positions (R_2 and R_5) of the phenyl rings increased the potency by about 2- to 3-fold (compounds **18g**, **18h**, and **18i** when compared to their un-substituted counterparts **18a** and **18c,** respectively). The introduction of

Table 3
SAR of tricyclic compounds bearing further aryl ring substituents



Compound	J	R^1	R^2	R^5	R^6	Calcein IC_{50}[a] (μM)	Cell permeability ($\times 10^{-6}$ cm/s)[b]
18g	O	H	F	H	H	0.02	ND[c]
18h	S	H	F	Cl	H	0.04	0.5/0.2
18i	S	H	F	H	H	0.09	ND
30a	O	F	H	H	F	0.09	5.0/1.0
30b	O	Br	H	H	Br	0.11	0.2/0.1
30c	O	Cl	H	H	Cl	0.27	<0.1/0.4
30d	S	Br	H	H	H	0.75	1.3/0.2
30e	S	Br	H	H	BR	0.86	4.0/2.0

[a] IC_{50}s are an average of at least two independent determinations.
[b] Permeability determined using Caco-2 cells. Data expressed as $P_{app}(a\rightarrow b)/P_{app}(b\rightarrow a)$.
[c] ND: not determined.

fluoro groups to the *ortho*-positions (R_1 and R_6) of the phenyl rings did not have much impact on activity (compound **30a**). However, the introduction of other halides at these same positions decreased the activity by at least 10-fold (compounds **30b–e**). None of these tricyclic compounds demonstrated any significant cell permeability. It should be noted that all compounds listed in Tables 1–3 were found to be inactive (>10 μM) against the closely related transport protein NRAMP1 (as determined in a variant of the calcein quench assay using NRAMP1-transfected CHO cells).

In summary, we discovered diaryl and tricyclic benzylisothiourea compounds as novel and potent DMT1 inhibitors from the original hit compound **1**. Both diaryl and tricyclic compounds demonstrated in vitro potency against DMT1, desirable cell permeability properties and good selectivity against the closely related transporter protein NRAMP1. Several analogs reported herein are the most potent DMT1 inhibitors reported to date (e.g., **18g** with threefold greater potency than compound **8a** of Ref. 22). The diaryl compounds also demonstrated a dose-dependent inhibition of iron absorption in an acute rat model of iron hyperabsorption. The tricyclic compounds showed increases in in vitro potency of up to 16-fold with respect to **1**. Diaryl compounds **6b** and **14a** demonstrated significant iron absorption inhibition in vivo with both 25 and 50 mg/kg doses. The diaryl and tricyclic compounds described in this report represent promising structural templates for further optimization, the results of which will be reported in due course.

Acknowledgments

The authors are grateful to Genome British Columbia and to the Natural Sciences and Engineering Research Council of Canada for financial support and to Monica Mork for technical assistance.

Supplementary data

Supplementary data (A detailed description of the conditions employed for the calcein quench assay as well as representative IC_{50} curves generated by this assay) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.05.129.

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25. As previously reported (Ref. 22), a cell-free competition variant of the calcein quench assay was developed in order to rule out chelation of ferrous iron as the primary source of the observed activity. All compounds reported herein had responses <20% of that of the known chelator desferoxamine in this assay.
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