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A boronate prochelator built on a triazole framework for peroxide-triggered tridentate metal binding

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Abstract

Iron chelating agents have the potential to minimize damage associated with oxidative stress in a range of diseases; however, this potential is countered by risks of indiscriminant metal binding or iron depletion in conditions not associated with systemic iron overload. Deferasirox is a chelator used clinically for iron overload, but also is cytotoxic to cells in culture. In order to test whether a prodrug version of deferasirox could minimize its cytotoxicity but retain its protective properties against iron-induced oxidative damage, we synthesized a prochelator that contains a self-immolative boronic ester masking group that is removed upon exposure to hydrogen peroxide to release the bis-hydroxyphenyltriazole ligand deferasirox. We present here the synthesis and characterization of this triazole-based, self-immolative prochelator: TIP (4-(5-(2-((4-boronobenzyl)oxy)phenyl)-3-(2-hydroxyphenyl)-1*H*-1,2,4-triazol-1-yl)benzoic acid). TIP does not coordinate to Fe³⁺ and shows only weak affinity for Cu²⁺ or Zn²⁺, in stark contrast to deferasirox, which avidly binds all three metal ions. TIP converts efficiently *in vitro* upon reaction with hydrogen peroxide to deferasirox. In cell culture, TIP protects retinal pigment epithelial cells from death induced by hydrogen peroxide; however, TIP itself is more cytotoxic than deferasirox in unstressed cells. These results imply that the cytotoxicity of deferasirox may not derive exclusively from its iron withholding properties.

Keywords

Chelation therapy; Iron; Oxidative stress; Fenton chemistry; Reactive oxygen species; Prodrug

1. Introduction

Chelating agents have been used clinically for more than 40 years to reduce the iron overload that comes with chronic blood transfusions of patients with thalassemia major, sickle cell disease, and other rare anemias [1]. The excess iron accumulates particularly in the heart, liver and endocrine organs, which eventually experience significant tissue damage as a result of oxidative stress from iron-catalyzed formation of highly reactive hydroxyl radicals [1]. There are currently three chelating agents used worldwide for the treatment of transfusion-induced iron overload, the gold standard being desferal (desferrioxamine B, DFO), which has been used since the 1970s but must be administered parenterally via diffusion pump [2]. The two oral alternatives to DFO now available are Exjade[®] (deferasirox, ICL670) and Ferriprox[®] (deferiprone, L1) [3]. While there remain drawbacks and side effects of each of these drugs, they have certainly made dramatic improvements in the quality of life and survival of these patients [4].

The success of using iron chelation to minimize tissue damage due to iron overload has sparked an interest in exploring iron chelation for diseases that are unrelated to systemic iron overload but for which iron-promoted oxidative stress contributes to disease. For example, neurodegenerative disorders, ischemia/reperfusion injury, and cardiovascular disease, among others, show signs of localized iron accumulation or misappropriation that may contribute to oxidative damage [5,6]. Recent attention is therefore focusing on expanding the use of approved chelators for new indications, as well as designing novel chelating agents specifically targeting conditions of localized metal imbalance [7–12].

There are many properties of deferasirox that make it an attractive agent for use in other oxidative stress related diseases. Its *N*-substituted bis-hydroxyphenyltriazole core, shown in Fig. 1, positions two phenolate oxygen donors and one triazole nitrogen donor for tridentate metal coordination with adjacent 5-membered chelate rings that favor small cations like Fe^{3+} over larger divalent cations [13]. This construction provides a high affinity binding pocket that has a favorable pFe^{3+} value of 22.5 and negative redox potential that prevents reduction to Fe^{2+} under physiological conditions [13]. The inhibition of redox cycling is particularly attractive for preventing hydroxyl radical formation via Fenton chemistry. With an octanol/water partition coefficient of 3.8, the hydrophobicity of deferasirox predicts that it readily accesses cells and accumulates in tissues. While this property is advantageous for its oral availability and its ability to decrease cardiac iron [14], it may be disadvantageous in diseases not associated with systemic iron overload, as it increases the chances of undesirable metal binding or extraction from key metalloproteins. Indeed, cell culture studies have shown that deferasirox is cytotoxic to several cell lines [12,15,16]. While the mechanism of cytotoxicity has not been firmly established, the removal or withholding of iron from critical iron proteins is speculated [15]. Regardless of its inherent cytotoxicity, deferasirox exerts significant protection of cultured cardiac cells against oxidative stress induced by exposure to catecholamines and *tert*-butyl hydroperoxide [12,16].

The previous results suggest that deferasirox has interesting properties for protecting against oxidative damage via iron chelation, especially if its cytotoxicity can be minimized. Our lab has developed a prodrug, or prochelator, strategy that employs a chemically reactive masking group to block adventitious metal binding [17,18]. By choosing a masking group that is reactive specifically under conditions unique to a pathological site, a chelating agent can in principle be released at the site of damage, thereby minimizing its impact on the metal ion balance of normal cells. We have found that this strategy indeed increases the protective window of a hexadentate chelator that is otherwise cytotoxic [19]. We were therefore interested to test whether a prochelator version of deferasirox could minimize its toxicity while retaining its protective properties against oxidative damage. Herein, we present a prochelator of deferasirox that contains a self-immolative boronic ester masking group that is removed upon exposure to hydrogen peroxide to release deferasirox.

2. Experimental

2.1. Materials and methods

Unless otherwise noted, chemicals were purchased commercially from Sigma Aldrich and used without further purification. Methyl 4-hydrazinylbenzoate (**2**), 2-(2-hydroxyphenyl)-4H-benzotriazole-4-one (**1**) and 4-[3,5-bis(2-hydroxyphenyl)-1,2,4-triazol-1-yl]benzoic acid (deferasirox) were synthesized by following previously published procedures [13,20]. NMR spectra were collected on a Varian Inova 400 or Varian Unity 500 spectrometer with chemical shifts reported in ppm and J values in Hz. Liquid chromatography/mass spectrometry (LC/MS) was performed using an Agilent 1100 Series apparatus with an LC/MSD trap and a Daly conversion dynode detector. A Supelco Ascentis C18 (50 × 1.0 mm) column was used and peaks were detected by UV absorption at 280 nm.

A linear gradient from 35% B in A to 95% B in A was run from 2 to 17 min, where A is water/2% MeCN/0.1% formic acid and B is MeCN/2% water/0.1% formic acid. High-resolution mass spectra (HRMS) were recorded on an Agilent G6224 LCMS-TOF system. Thin layer chromatography was performed on TLC sheets (silica gel 60 F₂₅₄) from Merck (Darmstadt, Germany). Column chromatography was performed on silicagel (Silica gel 60) from Sigma Aldrich. HPLC analysis and purification was performed on a Waters 600 system using method 1 (Waters analytical XBridge column, 4.6 × 250 mm, 1 mL/min, with a 25-min gradient from 2–98% B into A, where mobile phase B is acetonitrile and A is H₂O), or method 2 (semipreparative XBridge column, 19 × 250 mm, 10 mL/min 2–98% B into A). UV–Vis spectra were recorded on a Varian Cary 50 spectrophotometer.

2.2. Synthesis

2.2.1. Methyl 4-(3,5-bis(2-hydroxyphenyl)-1H-1,2,4-triazol-1-yl)benzoate (3)—A solution of **2** (760 mg, 4.6 mmol), **1** (1.01 g, 4.2 mmol), and triethylamine (660 µL, 4.3 mmol) in ethanol (70 mL) was heated to 110 °C in a closed reaction vessel for 2 h. The reaction mixture was diluted with water (100 mL) and the volume was reduced to one half on a rotary evaporator. The pH of the remaining solution was adjusted to 4 using glacial acetic acid, and it was extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were dried with sodium sulfate and the solvent was removed in vacuo. The residue was purified by column chromatography on silica (CH₂Cl₂ to 1% MeOH in CH₂Cl₂). The product was obtained as a yellowish solid (1.03 g, 2.7 mmol, 63%). δ_{H} (CDCl₃, 400 MHz): 3.99 (1H, s, OCH₃), 6.66 (1H, ddd, ³J = 6.8, ³J = 7.2, ⁴J = 1.2), 6.92 (1H, dd, ³J = 8.0, ⁴J = 1.6), 7.04 (1H, ddd, ³J = 7.2, ³J = 6.8, ⁴J = 1.2), 7.08 (1H, dd, ³J = 8.4, ⁴J = 0.8), 7.15 (1H, dd, ³J = 8.4, ⁴J = 1.2), 7.35 (1H, m), 7.39 (1H, m), 7.61 (2H, d, ³J = 8.8), 8.13 (1H, dd, ³J = 8.0, ⁴J = 1.6), 8.23 (2H, d, ³J = 8.8), 9.58 (1H, s, OH), 11.32 (1H, s). δ_{C} (CDCl₃, 125 MHz): 52.6, 109.8, 113.1, 117.1, 118.4, 119.0, 119.9, 126.0, 127.6, 127.7, 131.2, 131.6, 131.9, 133.1, 141.5, 152.1, 156.5, 158.0, 159.6, 165.7. HRMS (ES⁺) 388.1290 (C₂₂H₁₈N₃O₄ requires 388.1292) (MH⁺).

2.2.2. Methyl 4-(3-(2-hydroxyphenyl)-5-(2-((4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)phenyl)-1H-1,2,4-triazol-1-yl)benzoate (5)—A mixture of **3** (430 mg, 1.1 mmol), 4-bromomethylphenylboronic acid pinacol ester (**4**) (315 mg, 1.1 mmol), and potassium carbonate (150 mg, 1.1 mmol) in acetonitrile was heated to reflux for 20 h. The solvent was removed in vacuo and the residue was purified by column chromatography on silica (CH₂Cl₂ to 2% MeOH in CH₂Cl₂) to give the product as a yellowish solid (400 mg, 0.66 mmol, 60%). δ_{H} (CDCl₃, 400 MHz): 1.32 (12H, s, CH₃), 3.94 (3H, s, OCH₃), 4.67 (2H, s, CH₂), 6.84 (3H, m), 6.96 (1H, ddd, ³J = 6.8, ³J = 7.2, ⁴J = 1.2), 7.05 (1H, dd, ³J = 8.4, ⁴J = 1.2), 7.13 (1H, ddd, ³J = 7.2, ³J = 7.6, ⁴J = 1.6), 7.32 (1H, m), 7.35 (2H, d, ³J = 8.8), 7.43 (1H, ddd, ³J = 7.2, ³J = 7.6, ⁴J = 1.6), 7.65 (2H, d, ³J = 8.0), 7.67 (1H, dd, ³J = 7.6, ⁴J = 1.6), 7.93 (2H, d, ³J = 8.4), 8.16 (1H, dd, ³J = 8.0, ⁴J = 1.6), 10.82 (1H, s, OH). δ_{C} (CDCl₃, 125 MHz): 24.9, 52.4, 70.2, 83.9, 112.8, 113.9, 117.3, 117.4, 119.5, 121.5, 122.6, 126.1, 127.0, 129.5, 130.5, 131.4, 131.6, 132.7, 134.8, 138.8, 141.8, 151.4, 155.8, 157.1, 161.3, 166.1. HRMS (ES⁺) 604.2621 (C₃₅H₃₅BN₃O₆ requires 604.2619) (MH⁺).

2.2.3. 4-(5-(2-((4-boronobenzyl)oxy)phenyl)-3-(2-hydroxyphenyl)-1H-1,2,4-triazol-1-yl)benzoic acid (TIP, 6)—A mixture of **5** (150 mg, 0.25 mmol) and potassium hydroxide (25 mg, 0.45 mmol) in water (10 mL) and MeOH (5 mL) was stirred at r.t. for 48 h. The pH of the solution was adjusted to 4 by addition of 1 M HCl. The solvent was removed in vacuo and the residue was purified by semipreparative HPLC using method 2. The product was obtained as a white solid (80 mg, 0.16 mmol, 64%). δ_{H} (CDCl₃, 500 MHz): 4.59 (2H, s, CH₂), 6.80 (3H, m), 6.87 (1H, ddd, ³J = 7.5, ³J = 7.5, ⁴J = 1.0), 6.9 (1H, dd, ³J =

8.5, $^4J = 1.0$), 7.59 (1H, ddd, $^3J = 7.5$, $^3J = 7.5$, $^4J = 1.0$), 7.23 (3H, m), 7.38 (1H, ddd, $^3J = 6.5$, $^3J = 6.5$, $^4J = 1.5$), 7.58 (1H, dd, $^3J = 7.5$, $^4J = 2$), 7.62 (2H, d, $^3J = 8.0$), 7.86 (2H, d, $^3J = 8.5$), 8.06 (1H, dd, $^3J = 7.5$, $^4J = 2.0$), 10.76 (1H, s, OH). δ_C (CDCl₃, 100 MHz): 70.0, 112.7, 113.7, 116.9, 117.0, 119.3, 121.2, 122.3, 126.0, 126.8, 130.4, 131.1, 131.3, 132.5, 134.5, 137.5, 141.2, 151.2, 155.7, 156.8, 160.7, 167.2. HRMS (ES⁺) 508.1684 (C₂₈H₂₃BN₃O₆ requires 508.1680) (MH⁺).

2.2.4. 4-(3-(2-hydroxyphenyl)-5-(2-((4-(3a,5,5-trimethylhexahydro-4,6-methanobenzo[d][1,3,2]dioxaborol-2-yl)benzyl)oxy)phenyl)-1H-1,2,4-triazol-1-yl)benzoic acid (TIP-pd, 8)—A portion of (1R, 2R, 3S, 5R)-(-)-pinanediol (90 mg, 0.53 mmol) was added to a solution of unpurified compound **6** dissolved in DMF (10 ml) and toluene (90 ml). The mixture was stirred under reflux in an apparatus equipped with Dean–Stark adaptor for 24 h. The solvent was removed in vacuo and the residue was purified by semipreparative HPLC using method 2 to yield the product as a white solid (50 mg, 0.08 mmol, 31%). δ_H (CDCl₃, 500 MHz): 0.90 (3H, s, CH₃), 1.23 (1H, d, $^3J = 8.0$), 1.33 (3H, s, CH₃), 1.50 (3H, s, CH₃), 1.97 (2H, m), 2.17 (1H, t, $^3J = 5.0$), 2.26 (1H, m), 2.42 (1H, m), 4.46 (1H, m), 4.73 (2H, s, CH₂), 6.94 (3H, m), 7.02 (1H, m), 7.10 (1H, d, $^3J = 8.0$), 7.20 (1H, m), 7.38 (1H, m), 7.42 (2H, m), 7.52 (1H, m), 7.72 (2H, d, $^3J = 7.5$), 7.75 (1H, m), 8.03 (2H, d, $^3J = 9.0$), 8.24 (1H, m). δ_C (CDCl₃, 125 MHz): 24.0, 26.5, 27.1, 28.7, 35.5, 35.8, 38.2, 39.5, 51.4, 70.3, 78.3, 86.4, 112.9, 113.4, 117.4, 119.7, 121.7, 122.7, 126.4, 127.2, 128.7, 131.1, 131.7, 133.0, 134.0, 138.5, 142.4, 151.3, 155.8, 157.1, 160.9, 169.8. HRMS (ES⁺) 642.2779 (C₃₈H₃₇BN₃O₆ requires 642.2775) (MH⁺).

2.2.5. 4-(5-(2-(benzyloxy)phenyl)-3-(2-hydroxyphenyl)-1H-1,2,4-triazol-1-yl)benzoic acid (TIP-ctrl, 11)—A mixture of **3** (150 mg, 0.43 mmol), benzyl bromide (75 mg, 0.44 mmol), and potassium carbonate (160 mg, 1.2 mmol) in DMF was heated to 110 °C. The solvent was removed in vacuo and the residue was purified by column chromatography on silica (CH₂Cl₂) to yield the intermediate methyl 4-(3-(2-(benzyloxy)phenyl)-5-(2-hydroxyphenyl)-1H-1,2,4-triazol-1-yl)benzoate (**10**), which was treated with KOH (35 mg, 0.63 mmol) in water (10 ml) and MeOH (5 ml) and stirred at r.t. for 48 h. The pH of the solution was adjusted to 4 by addition of 1 M HCl. The solvent was removed in vacuo and the residue was purified by semipreparative HPLC using method 2. The product was obtained as a white solid (30 mg, 0.06 mmol, 15%). δ_H (CDCl₃, 400 MHz): 4.69 (1H, s, CH₂), 6.88 (2H, m), 6.93 (1H, d, $^3J = 8.4$), 6.98 (1H, m), 7.07 (1H, d, $^3J = 8.4$), 7.18 (1H, m), 7.25 (3H, m), 7.35 (3H, m), 7.50 (1H, ddd, $^3J = 6.8$, $^3J = 7.2$, $^4J = 1.2$), 7.72 (1H, dd, $^3J = 7.6$, $^4J = 1.6$), 7.98 (2H, d, $^3J = 7.6$), 8.18 (1H, dd, $^3J = 8.0$, $^4J = 1.6$). δ_C (CDCl₃, 100 MHz): 70.3, 112.8, 113.7, 117.2, 117.3, 119.5, 121.5, 122.5, 127.0, 127.1, 128.1, 128.3, 128.4, 131.0, 131.4, 131.6, 132.7, 135.5, 142.6, 151.4, 155.8, 157.1, 181.3, 170.4. HRMS (ES⁺) 464.1600 (C₂₈H₂₂N₃O₄ requires 464.1610) (MH⁺).

2.3. Crystal structure determination

Single crystals of compound **5** were obtained by slow evaporation from a solution of diethyl ether. The sample was mounted on a Mitegen polyimide micromount with a small amount of Paratone N oil. X-ray measurements were made on a Bruker-Nonius Kappa Axis X8 Apex2 diffractometer at a temperature of 110 K. The unit cell dimensions were determined from a symmetry constrained fit of 9098 reflections with $4.78^\circ < 2\theta < 61.5^\circ$. The data collection strategy was a number of ω and ϕ scans which collected data up to 72.78° (2θ). The frame integration was performed using SAINT [21]. The resulting raw data was scaled and absorption corrected using a multi-scan averaging of symmetry equivalent data using SADABS [22]. The structure was solved by direct methods using the SIR92 program [23]. All non-hydrogen atoms were obtained from the initial solution. The hydrogen atoms were introduced at idealized positions and were allowed to ride on the parent atom. The molecule

exhibited a conformational disorder in the BO_2C_2 ring and the attached methyl groups. Distinct alternate positions were found for O6 (designated as O6'), C444, C445, and C446, which were designated as C544, C545, and C546 respectively. The occupancy for this disorder refined to a value of 0.848(6) for the predominant conformer. The structural model was fit to the data using full matrix least-squares based on F^2 . The calculated structure factors included corrections for anomalous dispersion from the usual tabulation. The structure was refined using the XL program from SHELXTL [24], graphic plots were produced using the NRCVAX crystallographic program suite [25]. Crystallographic data of compound **5** has been deposited in the Cambridge Crystallographic Data center (CCDC ID: 880777).

2.4. Metal binding studies

Metal salt solutions of Cu^{2+} (as CuSO_4) and Zn^{2+} (as ZnSO_4) were freshly prepared as 30 mM stock solutions in nanopure water, while 2 mM Fe^{3+} stock solutions were freshly prepared from FeCl_3 in nanopure water or ferric ammonium citrate in PBS buffer, pH 7.4. Stock solutions of deferasirox (30.8 mM), TIP (10 mM), and TIP-ctrl (10 mM) were freshly prepared in DMSO and further diluted in PBS buffer with 1% DMSO, pH 7.4 to reach a final concentration of 20 μM . The solutions were equilibrated overnight with 0.5 equiv (10 μM) of an indicated metal ion before UV–Vis spectra were recorded.

2.5. Reaction with hydrogen peroxide

Stock solutions of TIP (10 mM) in DMSO-d_6 or DMSO were prepared for ^1H NMR and LC/MS investigations of the TIP reaction with H_2O_2 , respectively. For NMR detection, 250 μM TIP in D_2O with 2.5% DMSO-d_6 were prepared from the stock solutions. The oxidation of the prochelator TIP was triggered by addition of concentrated H_2O_2 to give a final concentration of 50 mM. The species of the reaction mixtures were then identified by 500 MHz NMR spectrometer after 24 h of equilibration. For LC/MS analysis, 100 μM TIP in PBS buffer, pH 7.4 with 1% DMSO was prepared from the stock solution followed by addition of H_2O_2 to get a 10 mM final concentration. The products of the reaction were analyzed by LC/MS.

The kinetics of TIP (20 μM) deprotection initiated by H_2O_2 were also investigated by UV–Vis spectrophotometry under pseudo-first-order conditions with 10- to 100-fold excess H_2O_2 . The pseudo-first-order rate constant (k_{obs}) at each H_2O_2 concentration (0.5, 1, 2, 4 and 6 mM) was determined in triplicate from plots of Abs at 273 nm versus time. SigmaPlot software was used to fit the data to Eq. (1), where A is Abs_{273} , k is k_{obs} , A_1 is the difference of the initial minus A_{inf} , and A_{inf} is the absorbance at 273 at infinite reaction time. A plot of k_{obs} versus $[\text{H}_2\text{O}_2]$ provides a linear plot where the slope is the second-order rate constant, k .

$$A = A_1 e^{-kt} + A_{\text{inf}} \quad (1)$$

2.6. Cell culture

All cell culture reagents, including minimal essential medium (MEM), Dulbecco's modified eagle medium (DMEM), F12 Ham's nutrient mix (F12), fetal bovine serum (FBS), penicillin–streptomycin (pen–strep), L–glutamine, and trypsin–EDTA were purchased from Gibco. The spontaneously immortalized human retinal pigment epithelial cell line ARPE-19 was purchased from American Type Culture Collection. The cells were grown in 1:1 DMEM and F12 medium supplemented with FBS (10%), pen–strep (1%), and glutamine (1%). Cells were cultured until confluent in 24-well Falcon plates. CellTiter-Blue Cell

Viability Assay was obtained from Promega. Cell viability assays were performed on a Perkin Elmer Victor³ 1420 plate reader.

For cytotoxicity assays, the growth medium was removed when cells reached confluence. Cells were then washed three times with MEM and treated with deferasirox, TIP, TIP-pd, or TIP-ctrl at final concentrations ranging from 0–100 μM in MEM with up to 1% DMSO. After 24 h of incubation, cell viability was determined by the CellTiter-Blue assay. For cytoprotection assays, washed cells were pre-treated for 5 h with deferasirox, TIP, or TIP-ctrl at the indicated concentrations prior to addition of H_2O_2 (200 μM final concentration). The cells were further incubated for 19 h before determination of viability. The conditions compared in each experiment were: positive control (cells treated only with MEM), negative control (cells treated with MEM containing H_2O_2), and cells treated with H_2O_2 and a range of deferasirox/TIP/TIP-pd/TIP-ctrl concentrations. Each condition was run in triplicate and variability was determined as the standard deviation of the results.

3. Results and discussion

3.1. Synthesis

As outlined in Scheme 1, the synthesis of the triazole prochelators was achieved by following a modification of a published synthetic procedure for deferasirox [13]. The methyl ester of 4-hydrazino benzoic acid was used in our procedure instead of the previously used benzoic acid to afford the methyl ester of deferasirox (**3**). This route was chosen to avoid alkylation of the free carboxylic acid of deferasirox in later stages of the prochelator synthesis. The synthesis of the prochelators was then achieved by alkylating the deferasirox methyl ester (**3**) with 4-bromomethylphenylboronic acid pinacol ester (**4**) to give the ester-protected prochelator **5**. Basic hydrolysis of both the carboxylic and boronate esters of **5** provides the triazole self-immolative prochelator **TIP** (**6**). We chose to use the self-immolative boronic acid protecting group because of synthetic ease and its favorable masking and reaction properties, as we and others have observed previously [26,27]. The free boronic acid of TIP was esterified with pinanediol to afford a protected prochelator **TIP-pd** (**8**). A control compound that masks one of the phenol groups but does not contain a peroxide-reactive boronate group, **TIP-ctrl** (**11**), was synthesized by alkylating the methyl ester version of deferasirox (**3**) with benzyl bromide and hydrolyzing the methyl ester in a subsequent step. Remarkably, the alkylation steps in all cases proceeded regioselectively on the phenol attached at position 5 of the triazole ring. The regioselectivity of this reaction was confirmed by X-ray crystallography, as described below.

3.2. Structure of prochelator **5**

Initial investigation of ^1H NMR spectra of the TIP precursor **5** provided evidence for the regioselective formation of a mono-alkylated product, as the spectra contained a single species, and confirmed that the alkylation proceeded on a phenolic oxygen. X-ray crystallography was used to further elucidate the structure of this molecule. Suitable crystals were grown by slow evaporation from a solution of diethyl ether. A summary of crystal data is provided in Table 1, with full details provided in cif format in the Supplemental information. The structure in Fig. 2 shows that the boron containing self-immolative group is attached to the phenolic ring connected to position 5 (C1) of the triazole ring. The unmasked phenol maintains a hydrogen bond with N1 of the triazole, with an O1–N1 distance of 2.653(1) Å and an O1–H $\cdots$ N1 hydrogen bond angle of 148.2°. Intramolecular hydrogen bonds between the phenols and triazole nitrogens to form 6-membered rings are well documented for ligands of the bis-hydroxyphenyltriazole family [13,28]. In the existing structures of such ligands, 2 of the 3 aromatic rings are roughly coplanar with the central triazole ring. In the case of **5**, only 1 of the 3 aromatic rings is roughly coplanar, with the

torsion angle between the phenolic ring and the triazole ring being 6.3° . In contrast, the torsion angle between the masked phenol ring and the triazole is 43.9° while the methyl ester containing ring off of N3 has a torsion angle of 32.9° with respect to the triazole. Some of this torsion is likely due to an offset π -stacking interaction between the masking aromatic ring (defined by C41–C46) and the methyl ester-containing ring (defined by C31–C36), where the distance from C41 and C35 to the center of the corresponding opposite ring is 3.4 Å.

3.3. Differential metal binding of TIP versus deferasirox

An effective prochelator should have a significantly diminished affinity for metal ions in comparison to its unmasked chelator. We therefore compared the interaction of deferasirox and our triazole prochelator TIP with biologically important transition metal ions Fe^{3+} , Cu^{2+} , and Zn^{2+} . The limited aqueous solubility of the pinanediol-modified prochelator TIP-pd precluded similar studies of this compound.

As shown in the UV–Vis spectra in Fig. 3, no significant spectral change occurs after addition of Fe^{3+} to a buffered aqueous solution of TIP. Conversely, addition of Fe^{3+} to a solution of deferasirox induces a significant change in spectrum indicating formation of the 1:2 $[\text{Fe}(\text{deferasirox})_2]^{3-}$ complex [13].

Similar to what was observed for Fe^{3+} , addition of Zn^{2+} to a 20 μM buffered aqueous solution of TIP results in virtually no spectral change, whereas significant change is observed when Zn^{2+} is added to solutions of deferasirox (Fig. 4). We note that addition of ZnSO_4 to higher concentrations of TIP results in visible precipitation, indicating that Zn^{2+} likely does complex with TIP to form insoluble species, similar to what has been noted for deferasirox [29].

In contrast to the results for zinc and iron, addition of Cu^{2+} to solutions of either TIP or deferasirox results in spectral changes in both cases, not just the deferasirox case (Figs. 5 and 6). The change in spectrum of TIP upon the addition of Cu^{2+} suggests that even in its prochelator form, TIP retains binding capacity for cupric ions. Notable also is a weak band centered ~ 650 nm that can be observed at higher concentrations of TIP plus Cu^{2+} (not shown), which is very similar to the d-d band observed for Cu^{2+} complexed to deferasirox [30].

In order to gauge the relative strength of the TIP–Cu interaction, we used nitrilotriacetic acid (NTA) as a competitive chelator that has overall stability constants for Cu^{2+} of 12.7 and 17.4 for $\log \beta_1$ and $\log \beta_2$, respectively [31]. As shown in Fig. 5, the addition of one equivalent of NTA to a Cu–TIP solution at pH 7.4 results in full restoration of the original TIP spectrum, indicating that NTA readily competes with TIP for complete extraction of Cu^{2+} . In contrast, one equivalent of NTA does not change the spectrum of a solution of Cu–deferasirox at all, indicating that deferasirox is significantly stronger than NTA for Cu^{2+} binding (Fig. 6), which is consistent with the known stability constants of deferasirox for Cu^{2+} of 18.8 and 23.9 for $\log \beta_1$ and $\log \beta_2$, respectively [30]. Additionally, we tested the strength of binding of deferasirox to copper using EDTA, which has a higher affinity for Cu^{2+} than NTA, with a $\log \beta_1$ value of 18.78 [31]. Addition of one equivalent of EDTA weakens the signal of the Cu–deferasirox spectrum but does not fully restore the free deferasirox spectrum, indicating comparative binding strength.

TIP-ctrl shows the same trends in metal binding as observed for TIP. As shown in Fig. 7, TIP-ctrl does not interact with Fe^{3+} or Zn^{2+} under these conditions, but does show an obvious interaction with Cu^{2+} . The spectrum of TIP-ctrl in the presence of Cu^{2+} shown in Fig. 7 is notably very similar to that of TIP–Cu in Fig. 5. Addition of NTA to a Cu–(TIP-ctrl)

mixture results in competitive removal of Cu^{2+} from this complex (Fig. 7), again reminiscent of the result found for TIP. These combined results indicate that the bidentate chelating moiety formed from the phenol oxygen and triazole nitrogen, which is preserved in both TIP and TIP-ctrl, is capable of binding to copper, even though relatively weakly.

3.4. Hydrogen peroxide triggered conversion of TIP to deferasirox

The unmasking reaction of TIP with H_2O_2 is anticipated to occur as shown in Scheme 2, where oxidation of the boronic acid by H_2O_2 gives a phenol intermediate that undergoes spontaneous 1,6 elimination to release deferasirox and quinone methide, which converts to 4-hydroxybenzyl alcohol in water [27].

Fig. 8 shows the aromatic region of a ^1H NMR spectrum of a 250- μM sample of TIP in D_2O after reaction with excess H_2O_2 . The expected products deferasirox and 4-hydroxybenzyl alcohol are clearly identified. The clean conversion of TIP to deferasirox was also observed by LC/MS, as shown by the chromatograms in Fig. 9.

The rate of the unmasking reaction was assessed by UV–Vis spectrophotometry. As can be seen in Fig. 10, the UV–Vis spectrum of 20 μM TIP in PBS buffer pH 7.4 changes upon exposure to H_2O_2 . The increase in absorbance at 273 nm versus time for solutions of TIP under pseudo first-order conditions of excess of H_2O_2 was used to obtain observed rate constants k_{obs} . The second-order rate constant for this reaction was then obtained from the data shown in Fig. 10 to be $1.4 \text{ M}^{-1}\text{s}^{-1}$, a value that is similar to that found for prochelator BHAPI, which also contains a self-immolative boronate protecting group [27].

3.5. Cell experiments

Cell culture experiments to test the toxicity and protective effect of the new triazole prochelators were conducted on retinal pigment epithelial ARPE-19 cells because of their implication in macular degeneration. Prior studies have shown iron chelation to be effective at reducing oxidative stress in both cell and animal models of macular degeneration [9,17,32]. The cytotoxicity data in Fig. 11 shows that deferasirox is cytotoxic to this cell line, especially at higher doses, where a 100 μM dose leaves only ~50% viable cells after a 24 h treatment. This result is consistent with other data showing deferasirox toxicity in myocardial and CHO cells in culture [12,15,16].

One of the motivators for creating the current triazole prochelators was to test the hypothesis that masking the iron chelation site of deferasirox might minimize its inherent cytotoxicity. However, the data in Fig. 11 show that the prochelator TIP is actually more cytotoxic than deferasirox itself. We speculated that part of the reason for enhanced cytotoxicity of TIP could be the ability of boronic acids to inhibit proteolytic enzymes. To test this hypothesis, we synthesized the pinanediol-protected prochelator, TIP-pd. In our experience, we have found that pinanediol capping groups retain their boronic ester forms even in aqueous solution, whereas the pinacol ester versions (such as the one in **5**) readily hydrolyze to their boronic acid derivatives [27]. TIP-pd is not readily soluble and was therefore added to cells as a suspension. As shown in Fig. 11, TIP-pd was the most cytotoxic of the molecules tested, suggesting that the boronic acid group is not likely the source of toxicity.

To further explore the structure–activity relationship among these molecules and their cytotoxicity, we tested the benzyl-containing control compound TIP-ctrl, which does not have a boronate functionality, does not have a reactive handle so is unlikely to convert to deferasirox intracellularly, and lacks a strong tridentate iron-binding pocket. As shown in Fig 11, the cytotoxicity of TIP-ctrl tracks very closely with TIP and shows a dose-dependent increase in cytotoxicity.

While deferasirox itself can be cytotoxic at elevated doses, others have found that it retains a protective effect against oxidative insult [12,16]. In order to test whether the prochelators might also show a protective effect against oxidative stress, we pre-treated cells with the compounds then exposed them to a toxic 200- μ M bolus of H_2O_2 . The results in Fig. 12 show that cells are protected from death by both deferasirox and TIP, but not by the non-chelating control TIP-ctrl. While deferasirox is more cytoprotective than TIP, being maximally effective at concentrations of 5 versus 25 μ M, respectively, it should be noted that the protective effect of TIP is observed well below the concentration of added H_2O_2 . These results imply that metal chelation is the probable mode of protective action for both TIP and deferasirox, the consumption of H_2O_2 in reaction with TIP being unlikely to provide much supplementary protection. The decreased protection for deferasirox and its prochelators at higher concentrations is likely the result of the inherent cytotoxicity of these compounds, as observed in the cytotoxicity studies of Fig. 11.

4. Conclusion

The new boronate-masked compounds presented here fulfill many of the design properties that are desirable for a prochelator, specifically: a significantly decreased affinity for metal ions in the masked form, and a reactive handle that enables conversion from the prochelator to the chelator under specific conditions. Indeed, the prochelator TIP has weak to no affinity for Fe^{3+} , Zn^{2+} or Cu^{2+} , while its chelator version deferasirox has well-established metal binding properties. Furthermore, H_2O_2 activates the boronate reactive handle, which results in clean and efficient conversion to deferasirox. Unfortunately, masking one of the phenol groups of deferasirox does *not* alleviate the cytotoxicity of this family of compounds. While TIP shows similar ability as deferasirox to protect cells from oxidative stress caused by H_2O_2 exposure, all of the phenol-masked compounds tested were more cytotoxic than deferasirox. The trends in cytotoxicity observed for this small set of triazole compounds suggest that the source of cytotoxicity of deferasirox is likely not due to adventitious iron chelation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Ward R. Hematology. 2010; 15:311. [PubMed: 20863426]
2. Bernhardt PV. Dalton Trans. 2007:3214. [PubMed: 17893764]
3. Vichinsky E. Am J Hematol. 2008; 83:398. [PubMed: 18058997]
4. Neufeld EJ. ASH Education Program Book. 2010; 2010:451.
5. Kell D. BMC Med Genet. 2009; 2:2.
6. Crichton RR, Dexter DT, Ward RJ. Coord Chem Rev. 2008; 252:1189.
7. Boddaert N, Sang K, Rotig A, Leroy-Willig A, Gallet S, Brunelle F, Sidi D, Thalabard JC, Munnich A, Cabantchik ZI. Blood. 2007; 110:401. [PubMed: 17379741]
8. Zhou, T.; Ma, Y.; Kong, X.; Hider, RC. Dalton Trans. 2012. <http://dx.doi.org/10.1039/c2dt12159j>

9. Hadziahmetovic M, Song Y, Wolkow N, Iacovelli J, Grieco S, Lee J, Lyubarsky A, Pratico D, Connelly J, Spino M, Harris ZL, Dunaief JL. *Invest Ophthalmol Vis Sci*. 2011; 52:959. [PubMed: 21051716]
10. Perez LR, Franz KJ. *Dalton Trans*. 2010; 39:2177. [PubMed: 20162187]
11. Kalinowski DS, Richardson DR. *Pharmacol Rev*. 2005; 57:547. [PubMed: 16382108]
12. Bendova P, Mackova E, Haskova P, Vavrova A, Jirkovsky E, Sterba M, Popelova O, Kalinowski DS, Kovarikova P, Vavrova K, Richardson DR, Simunek T. *Chem Res Toxicol*. 2010; 23:1105. [PubMed: 20521781]
13. Steinhäuser S, Heinz U, Bartholoma M, Weyhermüller T, Nick H, Hegetschweiler K. *Eur J Inorg Chem*. 2004;4177.
14. Al-Rousan RM, Paturi S, Laurino JP, Kakarla SK, Gutta AK, Walker EM, Blough ER. *Am J Hematol*. 2009; 84:565. [PubMed: 19650117]
15. Hasinoff BB, Patel D, Wu X. *Free Radical Biol Med*. 2003; 35:1469. [PubMed: 14642395]
16. Haskova P, Koubkova L, Vavrova A, Mackova E, Hruskova K, Kovarikova P, Vavrova K, Simunek T. *Toxicology*. 2011; 289:122. [PubMed: 21864640]
17. Charkoudian LK, Dentchev T, Lukinova N, Wolkow N, Dunaief JL, Franz KJ. *J Inorg Biochem*. 2008; 102:2130. [PubMed: 18835041]
18. Charkoudian LK, Pham DM, Franz KJ. *J Am Chem Soc*. 2006; 128:12424. [PubMed: 16984186]
19. Leed MGD, Wolkow N, Pham DM, Daniel CL, Dunaief JL, Franz KJ. *J Inorg Biochem*. 2011; 105:1161. [PubMed: 21708101]
20. Stieber F, Grether U, Waldmann H. *Chem Eur J*. 2003; 9:3270. [PubMed: 12866071]
21. Bruker-Nonius SAINT version 2009.9. Madison, WI 53711, USA: 2009.
22. Bruker-Nonius SADABS version 2009.9. Madison, WI 53711, USA: 2009.
23. Altomare A, Cascarano G, Giacovazzo C, Guagliardi A, Burla MC, Polidori G, Camalli M. *J Appl Crystallogr*. 1994; 27:435.
24. Bruker-AXS XL version 2009.9. Madison, WI 53711, USA: 2009.
25. Gabe EJ, Le Page Y, Charland JP, Lee FL, White PS. *J Appl Crystallogr*. 1989; 22:384.
26. Major Jourden J, Cohen S. *Angew Chem, Int Ed*. 2010; 49:6795.
27. Kielar, F.; Helsel, ME.; Wang, Q.; Franz, KJ. *Metallomics*. 2012. <http://dx.doi.org/10.1039/C1032MT20069D>
28. Heinz U, Sander J, Hegetschweiler K. *Z Kristallogr New Crystallogr Struct*. 2001; 216:113.
29. Heinz U, Hegetschweiler K, Acklin P, Faller B, Lattmann R, Schnebli HP. *Angew Chem, Int Ed*. 1999; 38:2568.
30. Steinhäuser S, Heinz U, Sander J, Hegetschweiler K, Anorg Z. *Allg Chem*. 2004; 630:1829.
31. Martell, AE.; Smith, RM. *NIST Critically Selected Stability Constants of Metal Complexes*. In: Motekaitis, RJ., editor. *NIST Standard Reference Database*. Vol. 46. NIST; Gaithersburg, MD: 2001.
32. Lukinova N, Iacovelli J, Dentchev T, Wolkow N, Hunter A, Amado D, Ying GS, Sparrow JR, Dunaief JL. *Invest Ophthalmol Vis Sci*. 2009; 50:1440. [PubMed: 19182262]

Appendix A. Supplementary material

CCDC 880777 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ica.2012.06.011>.

deferasirox
(ICL670, Exjade)

Fig. 1.
Structure of the metal-chelating agent deferasirox.

Fig. 2. ORTEP drawing of 5 showing naming and numbering scheme. Ellipsoids are at the 50% probability level and the hydroxyl hydrogen atom was drawn with an arbitrary radius while all other hydrogen atoms were omitted for clarity. The disordered portion of the molecule is depicted by “hollow” bonds and atoms.

Fig. 3. UV-Vis spectra of TIP and deferasirox (20 μ M in PBS with 1% DMSO at pH 7.4) before and after addition of Fe³⁺ (10 μ M).

Fig. 4. UV-Vis spectra of TIP and deferasirox (20 μM in PBS with 1% DMSO at pH 7.4) before and after addition of ZnSO_4 (10 μM).

Fig. 5. UV-Vis spectra showing interaction of TIP (20 μM) with CuSO_4 (10 μM) and the effect of NTA (20 μM) in PBS with 1% DMSO at pH 7.4.

Fig. 6. UV-Vis spectra showing interaction of deferasirox (20 μM) with CuSO_4 (10 μM) and the effect of NTA (20 μM) and EDTA (20 μM) in PBS with 1% DMSO at pH 7.4.

Fig. 7.

UV-Vis spectra of TIP-ctrl (20 μ M in PBS with 1% DMSO at pH 7.4) before and after addition of FeCl_3 , ZnSO_4 and CuSO_4 (10 μ M). The orange dash line indicates the effect of NTA (20 μ M) on the interaction of TIP-ctrl with Cu^{2+} .

Fig. 8. The aromatic regions of ¹H-NMR spectra of (a) TIP, (b) the reaction mixture of TIP with 50 mM H₂O₂ after 24 h, and (c) deferasirox at the concentration of 250 μM in D₂O with 2.5% DMSO-d₆. The arrows indicate the peaks corresponding to the aromatic protons of 4-hydroxybenzyl alcohol.

Fig. 9.

LC trace of (a) TIP (100 μ M in PBS with 1% DMSO), and (b) reaction mixture of TIP with H₂O₂ (10 mM) after 12 h. The peak eluting at 9 min in (a) has a corresponding m/z value of 508, consistent with intact TIP, while the peak eluting at 8.5 min in (b) has a corresponding m/z value of 374, matching that of authentic deferasirox.

Fig. 10. UV-Vis spectra of TIP (50 μM in PBS with 1% DMSO at pH 7.4) upon reaction with H₂O₂ (5 mM) for indicated time intervals. Inset shows a plot of k_{obs} at 273 nm versus H₂O₂ concentration for its reaction with TIP (20 μM).

Fig. 11. Viability of ARPE-19 cells exposed to various concentrations of deferasirox, TIP, TIP-ctrl and TIP-pd for 24 h.

Fig. 12.

Viability of ARPE-19 cells stressed with 200 μM H_2O_2 in the presence of deferasirox, TIP and TIP-ctrl. Cell viability was measured 19 h after peroxide treatment.

Scheme 1.

Synthetic scheme for triazole prochelators. The two prochelators TIP and TIP-pd along with the control compound TIP-ctrl that are used in cell culture studies are highlighted in boxes.

Scheme 2.

Reaction of TIP with hydrogen peroxide generates deferasirox and 4-hydroxybenzyl alcohol.

Table 1Summary of crystal data for **5**.

Formula	$C_{35}H_{34}BN_3O_6$
Formula weight (g/mol)	603.46
Crystal dimensions (mm)	$0.38 \times 0.33 \times 0.09$
Crystal color and habit	colorless prism
Crystal system	triclinic
Space group	$P\bar{1}$
T (K)	110
a (Å)	8.704(3)
b (Å)	13.004(5)
c (Å)	14.806(4)
α (°)	70.487(11)
β (°)	77.924(8)
γ (°)	85.695(15)
V (Å ³)	1544.6(9)
Number of reflections to determine final unit cell	9098
Minimum and Maximum 2θ for cell determination (°)	4.78, 61.5
Z	2
$F(000)$	636
ρ (g/cm ³)	1.297
λ (Å), (Mo K α)	0.71073
μ (cm ⁻¹)	0.089
Diffraction type	Bruker-Nonius Kappa Axis X8
	Apex2
Scan type(s)	ω and ϕ scans
Maximum 2θ for data collection (°)	72.78
Measured fraction of data	0.986
Number of reflections measured	79918
Unique reflections measured	13015
R_{merge}	0.0394
Number of reflections included in refinement	13015
Cut off threshold expression	$>2\sigma(I)$
Structure refined using	full matrix least-squares using F^2
Weighting scheme	calc ^a
Number of parameters in least-squares	442
R_1^b	0.0552
wR_2^c	0.1328
R_1 (all data)	0.1065
wR^2 (all data)	0.1532
Goodness-of-fit (GOF) ^d	1.046

Formula	C ₃₅ H ₃₄ BN ₃ O ₆
Maximum shift/error	0.001
Minimum and Maximum peak heights on final δF Map ($e^-/\text{\AA}$)	-0.274, 0.831

$$^a w = 1/[\sigma^2(F_0^2) + (0.0676P)^2 + 0.2020P] \text{ where } P = (F_0^2 + 2F_c^2)/3.$$

$$^b R_1 = \Sigma(|F_0| - |F_c|)/\Sigma F_0.$$

$$^c wR_2 = [\Sigma(w(F_0^2 - F_c^2)^2)/\Sigma(wF_0^4)]^{1/2}.$$

$$^d \text{GOF} = [\Sigma(w(F_0^2 - F_c^2)^2)/\text{No. of reflections} - \text{No. of parameters}]^{1/2}.$$