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Synthesis and biological properties of iron chelators based on a bis-2-(2-hydroxy-phenyl)-thiazole-4-carboxamide or -thiocarboxamide (BHPTC) scaffold

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ABSTRACT

Bis-2-(2-hydroxy-phenyl)-thiazole-4-carboxamides and -thiocarboxamides (BHPTCs) form a family of gemini hexacoordinated bis-tridentate chelating scaffolds. Four molecules were synthesized and shown to chelate iron(III) efficiently with a 1:1 stoichiometry. A dithioamide BHPTC displayed promising antiproliferative activity in several cancerous cell lines, making this molecule an interesting lead compound for the design of new iron-chelating anticancer drugs. Conversely, diamide BHPTCs had significant cytoprotective activity against iron overload in HepaRG cells in vitro, and were as efficient as and less toxic than deferoxamine B (DFO).

1. Introduction

Metals play a key role in the functional dynamics of living systems by promoting interactions and transformations of biomolecules. Iron is undoubtedly one of the most important metals in biological systems. This metal is predominant in the active sites of enzymes catalyzing many essential processes, such as the respiratory chain, metabolic transformations and deoxyribonucleotide biosynthesis. This predominance in such important systems makes iron chelators potentially interesting bioactive agents for use in medicine. The development of new iron chelators for medical purposes[1] is opening up new possibilities for the treatment of severe diseases, such as malaria,[2] AIDS,[3] and neurodegenerative diseases.[4] This list is far from exhaustive. However, two types of pathologies are historically more concerned with applications of iron chelators:iron chelation therapy (ICT) for patients suffering of chronic iron overload and pioneer approaches for the treatment of cancer by iron sequestration. In humans, iron homeostasis is regulated through the effects of hepcidin on intestinal iron absorption and iron storage in the liver.[5] Some genetic diseases lead to iron overload, which may be symptomatic (primary iron overload), as in hemochromatosis.[6] Alternatively, from repeated transfusions may result in secondary iron overload, as in sickle cell anemia[7,8] and thalassemia major.[8,9] Chronic iron overload causes progressive and severe liver damage. Secondary iron overload is mostly treated by iron chelation therapy with suitable chelators protecting sensitive organs and promoting metal excretion by natural routes. Three compounds are used for ICT.[8] The oldest of these compounds, deferoxamine B (DFO or Desferal™), provides effective relief from symptoms in many patients. However, the bioavailability of this compound is low, necessitating overnight administration by perfusion.[8,9] Severe side-effects have also been reported in patients on long-term treatment.[10] A second molecule, 1,2-dimethyl-3-hydroxypyridin-4-one or deferiprone (Feriprox™), is highly effective when administered per os,[11] but is thought to cause several rare but very severe side-effects.[10b,12] More recently, deferasirox (ICL670 or Exjade™), has been approved in several countries.[8,13] This oral chelator is administered daily but has not been in use for long enough to draw any firm conclusions about its toxicity, although some adverse effects are suspected.[14]

Iron chelators have several biological targets in humans, accounting for their high degree of efficacy for treating certain diseases and their toxicity when used over long periods. Cell division, for example, is a complex process involving a large number of metal-dependent enzymes and regulatory proteins.[15,16] Some of the proteins regulating the cell cycle, such as cyclins, are strongly affected by chelator-mediated metal depletion. Iron is also an essential component of the active sites of crucial enzymes involved

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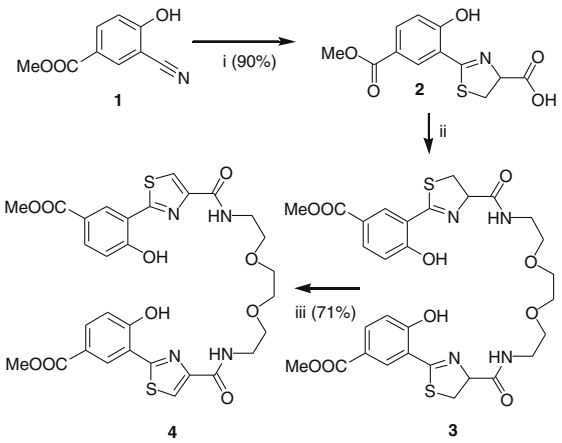
in DNA biosynthesis and repair.[15,16] Iron chelators could therefore be used to decrease DNA synthesis, stop cell division and promote apoptosis. They are therefore promising medicinal tools for containing the proliferation of cancer cells and lowering the risk of metastatic dissemination.[16,17] The first molecules tested in this context were originally developed for ICT.[18,19] However, a new generation of iron chelators specifically developed for cancer chemotherapy, subsequently emerged.[16,19] Triapine[20] (or 3-AP), tachpyridine,[21] di-2-pyridyl thiosemicarbazones like Dp44mT[22] and the pyridoxal isonicotinoyl hydrazone (PIH) family[23] currently appear to be the most promising molecules for this application, although none has yet been approved.

Continual efforts are thus required to develop new chelating molecules with therapeutic profiles, suitable for a specific application in cancer treatment or ICT. Deferoxamine B (DFO), which is still considered the gold standard for ICT, is a siderophore synthesized by *Streptomyces pilosus*.[24] These secondary metabolites excreted by microorganisms to facilitate the acquisition of iron, a crucial nutrient, are highly diverse,[25] providing an inexhaustible source of inspiration for the design of new metal-chelating agents. Using this strategy, Bergeron and coworkers have developed, over the last 20 years, several synthetic chelators derived from desferrithiocin, a siderophore from *Streptomyces antibioticus*.[26] These studies led to the development of deferitrin (GT56-252), one of the most promising iron chelators currently in clinical trials.[27] Previous studies of the physical and chemical properties of compounds related to pyochelin,[28] a siderophore common to several bacterial species, suggested that 2-(2-hydroxyphenyl)-thiazolin-4-carboxamides and 2-(2-hydroxyphenyl)-thiazole-4-carboxamides or -thiocarboxamides would be efficient tridentate iron(III) chelators.[29] The ICT drugs Exjade™ and deferitrin and the anticancer chelators Dm44pT, triapine, tachpyridine and the PIHs are all also tridentate chelators.[30] Thus, iron complexation by a bis-tridentate molecule appears to be a promising avenue of research for the development of the next generation of chelators for treatment purposes. The chelators developed to date[1] includes only few rare examples of extensively studied bis-tridentate chelators for therapeutic purposes.[26a,b] We report here the synthesis and the biological data concerning four molecules of the bis-2-(2-hydroxyl-phenyl)-thiazole-4-carboxamide and -thiocarboxamide (BHPTC) family characterized by a gemini hexacoordinate bis-tridentate chelating scaffold.

2. Results and discussion

2.1. Synthesis

The BHPTC molecular scaffold is based on the chelating properties of two 2-(2-hydroxyphenyl)-thiazole-4-carboxylic acid molecules. The heteroatoms involved in metal coordination are the phenol oxygen, the thiazole nitrogen and the oxygen/sulfur atom from the amide/thioamide group, as suggested by previous results.[29] In a first approach, the aromatic ring was substituted with a methylester function in *para* from phenol. This function may be converted easily into many other organic functions. This strategy make possible, at one and at the same time, to tune the acido-basic/chelating properties of the phenol function and the solubility of our chelators in physiological medium. The two tridentate moieties are connected to each other by a spacer arm with amide/thioamide groups. We initially selected 2,2′-(ethylenedioxy)-bis-ethylamine as the spacer. Molecular modeling showed this spacer to be of an ideal length and flexibility for optimal organization of the two tridentate moieties in the iron(III) coordination shell. Starting from commercially available 4-hydroxy-methylbenzoate, 3-cyano-4-hydroxy-methylbenzoate **1** was prepared in two steps, according to an efficient, published protocol.[31] The nitrile function of compound

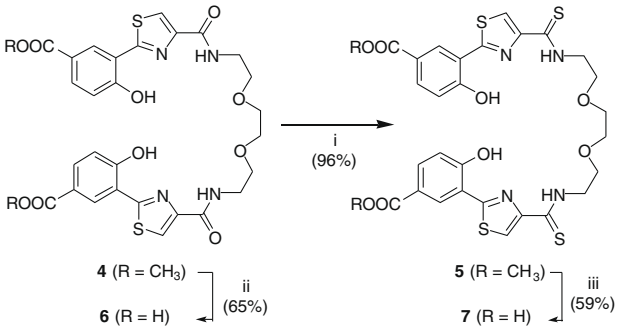


Scheme 1. Synthesis of compound **4**. Reagents and conditions: (i) L-cysteine, MeOH, 0.1 N phosphate buffer pH 6.4, 60 °C (90%); (ii) EDCI, $NH_2C_2H_4O$-$C_2H_4OC_2H_4NH_2$, CH_2Cl_2, 25 °C; (iii) $CBrCl_3$, DBU, CH_2Cl_2, 25 °C (71% for two steps).

1 was condensed with L-cysteine in a buffered hydromethanolic medium.[26a] The crude resulting thiazolin **2** isolated with a 90% yield was coupled with 2,2′-(ethylenedioxy)-bis-ethylamine in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI). The resulting crude mixture of diastereoisomers **3** was then treated with $CBrCl_3$ in the presence of DBU,[32] generating the corresponding dithiazole compound **4** isolated with a 71% overall yield in two combined steps (Scheme 1). This method has been successfully used in the past for the synthesis of thiazole analogs of pyochelin.[33] Other methods tested for the conversion of dithiazoline **3** into the corresponding dithiazole compound **4** resulted in complex mixtures in which unreacted starting material is predominant.

Diamide **4** was then converted into the corresponding dithioamide **5**, using Lawesson's reagent.[34] This reaction appears to be fully regioselective, as we detected no side products resulting from the thionation of one of the other carbonyl groups on the molecule. Further saponification of compounds **4** and **5** converted these two diesters into the corresponding diacids, **6** and **7**, isolated with yields of 65% and 59%, respectively (Scheme 2).

Compounds **4** and **6** were isolated in respectively 64% yield in three steps and 41% yield in four steps, starting from 3-cyano-4-hydroxy-methylbenzoate **1**, with only one chromatographic purification step. Chromatographic purification should be performed on demetallated silica gel column in order to avoid a contamination by the various metals present in commercial stationary phase. The compounds **5** and **7** were isolated respectively in 40% yield in four steps and 36% yield in five steps from the starting nitrile compound **1**.



Scheme 2. Synthesis of compounds **5** to **7**. Reagents and conditions: (i) Lawesson's reagent, toluene, reflux; (ii) 1 N $NaOH_{aq}$, THF, 70 °C; (iii) 1 N KOH_{aq}, dioxane, 60 °C.

2.2. Iron chelation properties

Compounds **4**, **5**, **6** and **7** were treated with a hydromethanolic solution of iron trichloride. The corresponding dark blue complexes were isolated quantitatively and mass spectrometry showed that ferric chelates with a 1:1 stoichiometry were the only products. The desulfurization products which may be expected by the reaction of compounds **5** and **7** with a Lewis acid such iron trichloride were neither observed nor isolated.

The cellular labile iron pool (LIP) is a pool of chelatable and redox-active iron, which plays a key role as a crossroad of cell iron metabolism. The ability of iron chelators to mobilize this temporary iron pool bound to low-molecular weight and low-iron affinity chelators (citrate, ascorbate, phosphate and adenosine triphosphate) is an essential factor influencing their biological efficiency. So, in parallel, an acellular calcein test was performed to compare the potential ability of compounds **4**, **5**, **6**, **7** and DFO to compete for this chelatable iron pool in a physiological medium. This test uses calcein, a fluoresceinated analog of EDTA that binds both iron(II) and iron(III), with the trivalent cation bound more slowly (K_a = 1024 M^{-1}). This metal-chelating dye, mainly used to estimate cellular iron level was previously shown to be oxidatively degraded by iron(II) in a H_2O_2-dependant pathway.[35] Therefore we checked first that calcein-iron(III) complex, which probably involves also ferric hydroxide and ferric oxide interactions, is not degraded in our experiment conditions (1 h in HEPES buffer at pH 7.3, data not shown). In solution, the fluorescence of this metallosensor dye is quenched during its interaction with iron and restored when iron is removed from the calcein-iron complex by various chelators. The rate and extent of fluorescence recovery (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) depend on chelator concentration, the kinetics and stoichiometry of iron binding and the relative binding affinity. Increases in fluorescence may therefore be correlated directly with iron sequestration following the gradual addition of a competitive iron chelator to ferric calcein. Thus, compounds **4**–**7** were compared with deferoxamine (DFO) in titration experiments using the calcein test. DFO, which is still considered to be the gold standard in the field of iron chelation for treatment purposes, with an IC_{50} of 4.4 μM, has efficiency similar to that of chelator **6**. With IC_{50} values of 0.07 μM and 0.50 μM for dithioamide compounds **7** and **5**, respectively, these compounds are much more efficient than DFO for iron chelation. Conversely, chelator **4** (IC_{50} = 1.0 μM), which only partly restores calcein fluorescence in this range of concentrations, seems to be less efficient that DFO for iron chelation (Fig. 1).

Molecules **4**–**7** were found to be iron chelators of sufficient potency to compete for this metal in physiological medium. We therefore assessed the cytotoxicity of these molecules and their antiproliferative activity in the human hepatoma cell line HepaRG[36] and several other cancer cell lines. In parallel, we evaluated the cytoprotective effects of the chelators **4** and **6** against iron overload.

2.3. Cytotoxicity and antiproliferative activity in HepaRG cells

We evaluated the effects of compounds **4**–**7** and DFO on cell viability in an MTT test assessing succinate dehydrogenase (SDH) activity in proliferating HepaRG cells. Mitochondrial SDH converts 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) into formazan, a compound detectable at 535 nm. The residual SDH activities of the cells after treatment with the new chelators and DFO are expressed as a percentage of the control value. Enzyme activity was assessed in the absence of iron(III) or in the presence of 20 μM iron(III). In absence of iron(III), chelator **5** (IC_{50} = 42 ± 7 μM) decreased cell viability more strongly than DFO (IC_{50} = 50 ± 4 μM), and compounds **4** (IC_{50} = 64 ± 11 μM), **6** (IC_{50} = 120 ± 6 μM) and **7** (IC_{50} = 114 ± 8 μM) were less cytotoxic than DFO (Fig. 2).

This effect was only partly reversed in the presence of 20 μM exogenous iron(III) for DFO (IC_{50} = 70 ± 10 μM), chelators **4** (IC_{50} = 78 ± 6 μM) and **5** (IC_{50} = 50 ± 8 μM), whereas no significant difference was observed for ligands **6** and **7** (data not shown). These results are consistent with the increase in extracellular lactate dehydrogenase (LDH) activity observed only for concentrations of compounds **4**, **6**, **7** and DFO greater than 100 μM. LDH is a cytoplasmic protein, so this increase in the extracellular LDH activity is a marker of cell membrane disruption correlated to cytotoxicity. Conversely, compound **5** was highly cytotoxic to HepaRG cells at concentrations above 50 μM (data not shown). We therefore assessed the antiproliferative activity of the four BHPTC ligands in a wider range of cancer cell lines. These compounds were tested on 13 human cancer cell lines representative of various tissues or organs. We compared the four chelators tested with DFO. All five compounds were tested at two concentrations—10 μM and 1 μM—in the following cell lines: KB (epidermoid carcinoma),

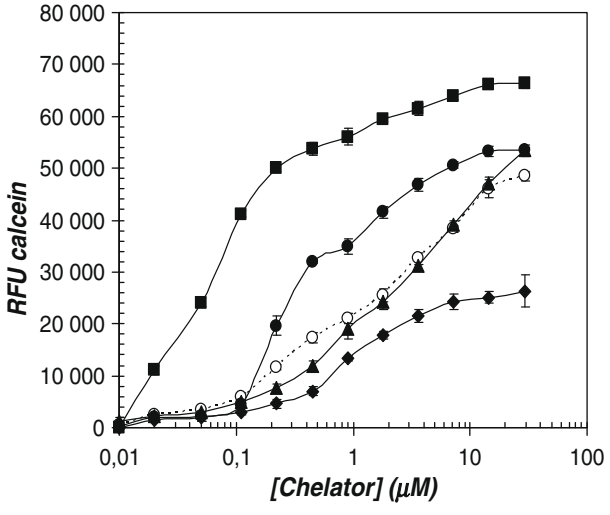


Figure 1. Comparison of the iron-chelating efficiency of compounds **4** (♦), **5** (●), **6** (▲) and **7** (■), with DFO (○), by calcein fluorescence measurements in a cell-free system. Fluorescence of 100 nM calcein (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) in the presence of 1 μM Fe(III) in Hepes buffer (20 mM HEPES, 150 mM NaCl, pH 7.3) was detected in a microplate fluorescence reader (free calcein). Values are means of three independent experiments.

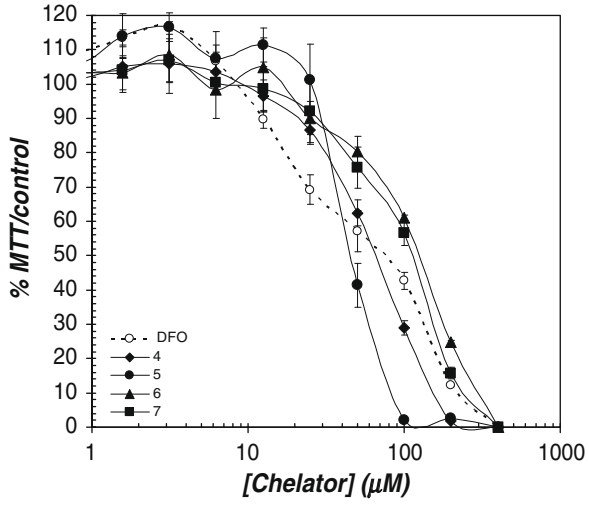


Figure 2. Effect of ligands **4** to **7** and DFO on cell viability (MTT assay) in proliferating HepaRG cell cultures. HepaRG cells at D4 were maintained in culture in the absence of iron(III) for 72 h, with various concentrations of **4** (♦), **5** (●), **6** (▲), **7** (■) and DFO (○).

HCT116, HT29 and HCT15 (colon adenocarcinoma cells), MCF7 (breast adenocarcinoma), MCF7R (doxorubicin-resistant MCF7), SK-OV-3 (ovary adenocarcinoma), HepG2 (hepatocarcinoma), PC-3 (prostate adenocarcinoma), A549 (lung carcinoma), HL60 (promyeocytic leukemia), K562 (chronic myelogenous leukemia) and SF268 (glioblastoma) (Table 1). DFO displayed moderate antiproliferative activity at 10 μM and had only a very slight impact on proliferation at a concentration of 1 μM. Compounds **4**, **6** and **7** were less active than DFO, with little or no antiproliferative activity at either of the concentrations and in any of the cells studied. Consistent with our findings for HepaRG cells, compound **5** appeared to be the most interesting molecule, as it was more active, at a concentration of 10 μM, than DFO for almost all the cell lines tested, and had a particularly strong impact on the proliferation of A549 lung carcinoma cells. However, the effect of this compound was much weaker at a concentration of 1 μM.

Based on the results of the MTT test, diamide compounds **4** and **6**, which were less toxic than DFO, were tested for cytoprotective activity against iron overload.

2.4. Cytoprotective activity

We assessed the cytoprotective effect against iron overload toxicity of compounds **4** and **6** and DFO on differentiating HepaRG cells cultured in the presence of ferric nitriloacetate (FeNTA), as an in vitro model of the hepatocytes of a patient presenting iron overload. The cytotoxicity of iron overload was evaluated by measuring extracellular lactate dehydrogenase (LDH) activity. As mentioned before, this enzyme is a cytoplasmic protein, an increase in the extracellular activity of LDH reflect cell membrane disruption correlated with the cytotoxicity. The addition of 50 μM FeNTA to the culture medium increased extracellular LDH activity by a factor of 6.5 times (643 ± 18%) over that recorded in the control experiment (100%), demonstrating the toxicity of iron overload in hepatocytes. In the presence of deferoxamine (DFO), the impact of iron overload was greatly decreased, with extracellular LDH activity only 1.6 times higher (162 ± 30%) than in the control experiment. With increases of LDH activity of 148 ± 55% and 179 ± 20%, respectively, chelators **4** and **6** proved to be as efficient as DFO for protecting hepatic cells from iron overload. The differences in iron sequestration from calcein observed between **4** and **6** in an acellular test seem to have been equaled in the hepatoma cell cytoprotection test.

Iron chelators are highly effective drugs, but have the downside of being cytotoxic, often due to a lack of selectivity for the iron pools targeted. This aspect is particularly crucial for chelators developed for ICT, whereas a high level of cytotoxicity/antiproliferative activity and large capacity to penetrate membranes is required for chelators for anticancer treatments. Compounds **4**, **5**, **6** and **7** have a similar molecular architecture but differ in lipophilicity and in the heteroatoms involved in the iron coordination shell (amides vs thioamides). These slight chemical differences strongly affected the biological properties of our chelators. The diamide compounds **4** and **6** protected cells against iron overload as efficiently as DFO. However, the hydrosoluble diamide chelator **6** exhibits in vitro a lower cytotoxicity, independent from iron(III), than the reference ICT drug. Our results suggest that hydrophilic BHPTCs bearing amide groups are interesting lead compounds for the development of a new generation of safer chelators for ICT. By contrast, the dithioamide chelator **5** had higher levels of cytotoxic/antiproliferative activity than both chelator **4** and DFO, whereas compounds **6** and **7** were totally ineffective, probably because they were not lipophilic enough to cross the cell membrane and compete for the intracellular iron pool.[37] Thus, liphophilic BHPTC dithioamide derivatives may be considered a promising synthetic platform for the design of new iron chelators for anticancer chemotherapy.

Table 1
In vitro antiproliferative activity in 13 cancer cell lines after 72 h of exposure to compounds **4**–**7** and DFO at concentrations of 10 μM and 1 μM

Cell lines	**4**[a,b]	**5**[a,b]	**6**[a,b]	**7**[a,b]	DFO[a,b]
KB	28/0	58/5	2/0	0/0	16/0
HCT116	13/0	69/6	0/0	0/0	20/0
HT29	4/0	3/0	1/0	0/0	42/0
HCT15	14/11	42/1	0/0	8/0	46/0
MCF7	4/0	10/0	0/0	0/0	0/0
MCF7R	19/0	64/0	2/0	1/0	9/0
A549	11/0	98/8	0/1	7/0	50/2
PC3	37/8	54/10	8/0	0/0	44/1
SF268	19/0	62/0	0/0	5/0	33/0
SK-OV-3	22/10	45/10	7/4	2/3	52/5
HL60	0/0	52/0	0/0	0/0	0/0
K562	10/2	53/1	9/0	2/0	0/0
HepG2	26/0	43/0	0/0	0/0	0/0

[a] Percentage inhibition of cell proliferation at 10^{-5} M/10^{-6} M.
[b] Values are means of triplicate experiments.

3. Conclusion

We have described the synthesis of a new family of gemini bistridentate hexacoordinated iron chelators, BHPTCs—bis-hydroxyphenyl-thiazole-carboxamides or -thiocarboxamides. BHPTCs **4**–**7** were efficiently synthesized and formed one-to-one complexes with iron(III). These chelators were also able to sequester iron in physiological media. Chelators **4** and **6** significantly protected hepatocytes against iron overload and were as efficient as deferoxamine B (DFO). Three of these four compounds (**4**, **6** and **7**) were not more cytotoxic than DFO, the gold standard for iron chelation therapy (ICT). By contrast, chelator **5** had higher levels of cytotoxic/antiproliferative activity than DFO, demonstrating the considerable potential of the BHPTC scaffold for the development of therapeutic iron chelators.[38] We are currently synthesizing new chelating molecules for cancer treatment or ICT, based on the results reported here.

4. Experimental

4.1. Chemistry

All the reactions were carried out under an inert argon atmosphere. Analytical grade solvents were used. Reactions were monitored by thin-layer chromatography (TLC), using *Merck* precoated silica gel $60F^{254}$ (0.25 mm). Column chromatographies were performed with demetallated *Merck kieselgel* 60 (63–200 μm).[39] Melting points were determined with a *Stuart Scientific Bibby* SMP3 apparatus. NMR spectra were recorded on a *Bruker* Avance 300 (300 MHz for ^{1}H and 75 MHz for ^{13}C). Mass spectra were recorded in the *Service Commun d'Analyse (SCA) de la Faculté de Pharmacie de l'Université de Strasbourg* and were measured after calibration in ES-TOF experiments on a *Bruker Daltonic* MicroTOF mass spectrometer.

4.1.1. 2-(2-Hydroxy-5-methoxycarbonyl-phenyl)-4,5-dihydrothiazole-4-carboxylic acid (2)

A solution of 3-cyano-4-hydroxy-methylbenzoate **1**[31] (1 g, 5.65 mmol) and L-cysteine (1.59 g, 13.11 mmol) in a mixture of MeOH (32 mL) and phosphate buffer (pH 6.4, 0.1 N, 20 mL) was heated at 60 °C, with stirring, for 16 h. The mixture was then cooled down to room temperature before being evaporated to dryness under reduced pressure. The resulting yellow foamy residue was dissolved in water (100 mL) and washed with a 2:1 mixture

of cyclohexane and Et_2O (75 mL). The pH of the aqueous phase was adjusted to 2.0–3.0 by addition of solid citric acid. The solution was then extracted with CH_2Cl_2 (3 × 75 mL). The organic layers were collected and dried over Na_2SO_4 before filtering and evaporation under reduced pressure. The expected thazoline compound **2** (1.43 g, 5.10 mmol, yield: 90%) was isolated as a yellow crystalline powder and was used in this form for subsequent steps. Mp: 184–186 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.69–3.81 (m, 1H), 3.84 (s, 3H), 5.51 (dd, J = 7.5, 9.6 Hz, 1H), 7.10 (d, J = 9.3 Hz, 1H), 7.98–8.02 (m, 2H). ^{13}C NMR (75Mz, DMSO-d_6) δ 33.72, 52.07, 76.10, 115.46, 117.48, 120.58, 131.69, 134.26, 162.16, 165.08, 170.99, 171.98.

4.1.2. Benzoic acid, 3,3′-[(1,12-dioxo-5,8-dioxa-2,11-diazadodecane-1,12-diyl)-bis(4,2-thiazolediyl)]bis-[4-hydroxy], 1,1′-dimethyl ester (4)

A solution of 2,2′-(ethylenedioxy)-bisethylamine (134 mg, 0.93 mmol) in CH_2Cl_2 (20 mL) was added dropwise at room temperature (25–27 °C) to a solution of 2-(2-hydroxy-5-methoxycarbonyl-phenyl)-4,5-dihydro-thiazole-4-carboxylic acid **2** (565 mg, 2.01 mmol) and EDCI (463 mg, 2.41 mmol) in CH_2Cl_2 (30 mL). The mixture was then stirred for 16 h at 25 °C and washed with 0.5 M aqueous HCl solution (50 mL). The organic phase was collected, dried over Na_2SO_4, filtered and the solvent was evaporated under reduced pressure. The crude mixture was dissolved in CH_2Cl_2 (50 mL) and DBU (546 μL, 555 mg, 3.65 mmol) was added, followed by $CBrCl_3$ (315 μL, 634 mg, 3.20 mmol). The resulting solution gradually turned brown and was stirred for 14 h at 25 °C. The mixture was then adsorbed and purified onto demetallated silica gel (30 g of SiO_2, CH_2Cl_2 then CH_2Cl_2/EtOH: 95/5). The pale yellow solid obtained was washed with boiling ethanol, filtered and dried under reduced pressure. Chelator **4** (444 mg, 0.66 mmol, overall yield over two steps: 71%) was isolated as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.44–3.61 (m, 12H), 3.83 (s, 6H), 7.12 (d, J = 8.6 Hz, 2H), 7.90 (dd, J = 2.3, 8.6 Hz, 2H), 8.28 (s, 2H), 8.50 (t, J = 5.7 Hz, 2H), 8.88 (d, J = 2.3 Hz, 2H). ^{13}C NMR (75 MHz DMSO-d_6) δ 38.35, 51.95, 68.99, 69.61, 116.61, 119.25, 121.00, 124.56, 129.54, 131.95, 149.00, 158.97, 160.75, 161.50, 165.80. MS m/z 671 (M+H$^+$), 693 (M+Na$^+$), 709 (M+K$^+$). HRMS m/z found: 671.1359 (M+H$^+$), m/z calcd for $C_{30}H_{31}N_4O_{10}S_2$: 671.1476.

4.1.3. Benzoic acid, 3,3′-[(1,12-dithioxo-5,8-dioxa-2,11-diazadodecane-1,12-diyl)bis(4,2-thiazolediyl)]bis-[4-hydroxy], 1,1′-dimethyl ester (5)

Diamide **4** (350 mg, 0.52 mmol) and Lawesson's reagent (422 mg, 1.04 mmol) were suspended in anhydrous toluene (20 mL). The resulting suspension was refluxed (110 °C) for 2 h. The mixture was cooled to room temperature and the solvent was evaporated under reduced pressure. The oily residue was dissolved in CH_2Cl_2 (50 mL) and this organic phase was then washed successively with water (30 mL), a saturated aqueous solution of $NaHCO_3$ (30 mL) and finally brine (30 mL). The organic phase was then dried over Na_2SO_4, filtered and the solvent removed by evaporation under reduced pressure. Chromatography on a demetallated silica gel column (30 g of SiO_2, MeOH/CH_2Cl_2: 1/99) resulted in the purification of the expected dithioamide **5** (351 mg, 0,50 mmol, yield: 96%) isolated as a bright yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.68–3.97 (m, 12H), 3.83 (s, 6H), 7.12 (d, J = 8.6 Hz, 2H), 7.90 (dd, J = 2.2; 8.6 Hz, 2H), 8.45 (s, 2H), 8.90 (d, J = 2.2 Hz, 2H), 10.36 (t, J = 5.7 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 44.57, 51.94, 67.30, 69.70, 116.55, 119.23, 121.03, 127.16, 129.55, 132.32, 152.68, 159.11, 161.14, 165.78, 186.14. MS m/z 703 (M+H$^+$), 725 (M+Na$^+$), 741 (M+K$^+$). HRMS m/z found: 703.1013 (M+H$^+$), m/z calcd for $C_{30}H_{31}N_4O_8S_4$: 703.1019. Anal. Calcd for $C_{30}H_{30}N_4O_8S_4$: C, 51.27; H, 4.30; N, 7.97. Found: C, 51.05; H, 4.55; N, 7.68.

4.1.4. Benzoic acid, 3,3′-[(1,12-dioxo-5,8-dioxa-2,11-diazadodecane-1,12-diyl)bis(4,2-thiazolediyl)]bis-[4-hydroxy] (6)

Diester **4** (257 mg, 0.38 mmol) was suspended in a mixture of THF (30 mL) and an aqueous 1 N solution of NaOH (30 mL). The suspension was heated at 70 °C for 4 h. The crude mixture was cooled to room temperature, diluted in water (50 mL) and Et_2O (100 mL) and vigorously stirred for few minutes. The aqueous phase was then acidified to pH 1 by adding aqueous HCl 1 N. The resulting precipitate was filtered off, washed with ice-cold milliQ water and dried under reduced pressure. The expected diacid **6** (160 mg, 0.25 mmol, yield: 65%) was isolated as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.45–3.60 (m, 12H), 7.12 (d, J = 8.4 Hz, 2H), 7.90 (dd, J = 2.1, 8.4 Hz, 2H), 8.27 (s, 2H), 8.50 (t, J = 5.7 Hz, 2H), 8.88 (d, J = 2.1 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 38.20, 68.65, 69.26, 116.07, 118.97, 122.02, 124.37, 129.67, 132.32, 148.89, 158.58, 160.66, 161.66, 166.82. MS: m/z 643 (M+H$^+$), 665 (M+Na$^+$), 681 (M+K$^+$). HRMS m/z found: 665.099 (M+Na$^+$), m/z calcd for $C_{28}H_{26}N_4NaO_{10}S_2$: 665.071.

4.1.5. Benzoic acid, 3,3′-[(1,12-dithioxo-5,8-dioxa-2,11-diazadodecane-1,12-diyl)bis(4,2-thiazolediyl)]bis[4-hydroxy] (7)

Diester **5** (63 mg, 89 μmol) was suspended in a mixture of dioxane (5 mL) and an aqueous 1 N solution of KOH (5 mL). The suspension was heated at 60 °C for 2 h. The crude mixture was cooled to room temperature, diluted with water (50 mL) and CH_2Cl_2 (30 mL) and vigorously stirred for a few minutes. The aqueous phase was then acidified to pH 1 by adding aqueous HCl 1 N. The resulting precipitate was filtered off, washed with ice-cold water and dried under reduced pressure. The expected diacid **7** (35 mg, 52 μmol, yield: 59%) was isolated as a pale yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.67 (s, 4H), 3.73 (t, J = 5.8 Hz, 4H), 3.94 (q, J = 5.8 Hz, 4H), 7.12 (d, J = 8.7 Hz, 2H), 7.91 (dd, J = 2.1, 8.7 Hz, 2H), 8.45 (s, 2H), 8.91 (d, J = 2.1 Hz, 2H); 10.38 (t, J = 5.7 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 44.44, 67.23, 69.68, 116.34, 118.98, 122.03, 127.00, 129.72, 132.43, 152.61, 158.86, 161.33, 166.87, 186.13. HRMS m/z found 675.070 ([M+H]$^+$), calcd for $C_{28}H_{27}N_4O_8S_4$: 675.071.

4.1.6. Ferric complexes of chelators (4), (5), (6) and (7)

To solutions of compounds **4**, **5**, **6** and **7** (7.11 μmol) in MeOH (2.5 mL), we added 0.22 M $FeCl_3$ (prepared by dilution of a commercial aqueous 2.2 M solution, 32 μL, 7.11 μmol). A dark blue suspension was obtained, which was stirred for 16 h at 20 °C. The solvent was evaporated and the residue suspended in water and sonicated for two minutes. Water was eliminated by freeze-drying, to obtain the corresponding ferric complexes in quantitative yield. Ferric complex of chelator **4**: MS m/z found 724.05 ([M(**4**)−2H+Fe]$^+$). HRMS m/z found 724.0595 ([M(**4**)−2H+Fe]$^+$), calcd for $C_{30}H_{28}FeN_4O_{10}S_2$: 724.0591. Ferric complex of chelator **5**: MS m/z found 756.0 ([M(**5**)−2H+Fe]$^+$). Ferric complex of chelator **6**: MS m/z found 696.0 ([M(**6**)−2H+Fe]$^+$). Ferric complex of chelator **7** MS m/z found 727.9 ([M(**7**)−2H+Fe]$^+$).

4.2. Calcein fluorescence measurements

The fluorescence (λ_{Exc} = 485 nm, λ_{Em} = 520 nm) of calcein (100 mM) in Hepes buffer (20 mM HEPES, 150 mM NaCl, pH 7.3) was measured as a function of time, at room temperature (20 °C), in a microplate fluorescence reader (Packard, Fusion™), equipped with an orbital stirrer. Iron(III) (1 μM) reacted slowly with calcein and maximal fluorescence quenching was observed after 6 h. The kinetics of fluorescence recovery was monitored over a period of one hour in the presence of various chelator concentrations, and the initial rate of dequenching was deduced from the kinetics.

4.3. HepaRG cell cultures

HepaRG cells were obtained from a liver tumor (hepatocarcinoma) from a female patient.[36] Small pieces of the tumor were digested with 0.025% collagenase D diluted in Hepes buffer supplemented with 0.075% $CaCl_2$. The cell population was suspended in William's E medium supplemented with 10% fetal calf serum (FCS), 5 μg/mL insulin and 5×10^{-7} M hydrocortisone hemisuccinate and dispensed into several dishes. The cell populations most closely resembling hepatocytes were selected and passaged by gentle trypsin treatment. After three passages, cell aliquots were preserved by freezing. The cells from a frozen aliquot were defrosted and used to seed, at low density ($2.5 \times 10^4/cm^2$), a growth medium consisting of William's E medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin, 100 μg/mL streptomycin, 5 μg/mL insulin, 2 mM glutamine and 5×10^{-5} M hydrocortisone hemisuccinate. After two weeks of culture, the cells reached confluence and began to differentiate. A higher state of differentiation was attained by shifting the cells to the same culture medium supplemented with 1.5% DMSO and incubating for two weeks.

4.4. Lactate dehydrogenase (LDH) activity

The cytotoxicity associated with iron overload or iron chelators was evaluated by assessing extracellular lactate dehydrogenase (LDH) activity (cytotoxicity detection kit—LDH, Roche, Penzberg, Germany). Extracellular LDH activity was measured with the manufacturer's protocol, on a 20 μL aliquot of cell-free medium obtained by centrifugation (2500 rpm, 5 min). Intracellular LDH activity was evaluated after the lysis of hepatocytes in phosphate-buffered saline, by sonication for 15 s, and centrifugation as described above. LDH activity was assessed by reading absorbance at 485 nm. A standard curve for LDH (0–4000 mU/mL) was used to quantify enzyme activity (L-lactate dehydrogenase, Sigma, St Louis, MO). Experimental values are expressed in terms of LDH release into the medium as a percentage of the total activity of the culture.

4.5. MTT test

Cell viability was evaluated by measuring mitochondrial succinate dehydrogenase activity (SDH) in proliferating HepaRG human hepatoma cells, by the tetrazolium colorimetric assay (MTT, Sigma, St Louis, MO). SDH activity was detected after 3 h of incubation in 100 μL serum-free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/L). Formazan salts were solubilized with 200 μL DMSO and absorbance was read at 535 nm. SDH activity is expressed as a percentage of the control value.

4.6. Test for antiproliferative activity

The antiproliferative activity of 13 cancer cell lines was evaluated at the *Ciblothèque Cellulaire de l'Institut de Chimie des Substances Naturelles* at Gif-sur-Yvette (France). The human cell lines KB (mouth epidermoid carcinoma) and HepG2 (hepatocarcinoma) were obtained from ECACC (Salisbury, UK) and grown in DMEM medium supplemented with 10% FCS, in the presence of penicillin, streptomycin and fungizone, in a 75 cm^2 flask, under an atmosphere containing 5% CO_2. By contrast, HCT116, HT29 and HCT15 (colon adenocarcinoma), MCF7, MCF7R (breast adenocarcinoma), SK-OV3 (ovary adenocarcinoma from NCI), PC-3 (prostate adenocarcinoma), A549 (lung carcinoma), HL60 (promyeocytic leukemia), K562 (chronic myelogenous leukemia) and SF268 (glioblastoma from NCI) cells were grown in RPMI medium. Cells were plated in 200 μL of medium, in 96-well tissue culture microplates and were treated 24 h later with compounds dissolved in DMSO at concentrations of 1 and 10 μM, using a Biomek 3000 (Beckman-Coulter). Controls received the same volume of DMSO (1% final volume). After 72 h of incubation, MTS reagent (Promega) was added and the plates incubated for 3 h at 37 °C. Absorbance was monitored at 490 nm and the results are expressed as the inhibition of cell proliferation, calculated as the ratio $[(1 - (OD_{490}\,\text{treated}/OD_{490}\,\text{control})) \times 100]$ in triplicate experiments.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.11.057.

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