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Synthesis and antiproliferating activity of iron chelators of hydroxyamino-1,3,5-triazine family

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ABSTRACT

We synthesized and evaluated new specific tridentate iron(III) chelators of 2,6-bis[hydroxyamino]-1,3,5-triazine (BHT) family for use in iron deprivation cancer therapy. Physical properties of BHT chelators are easily customizable allowing easy penetration through cellular membranes. Antiproliferative activity of new BHT chelators was studied on *MDA-MB-231* and *MiaPaCa* cells and compared to a clinically available new oral iron chelator, deferasirox (DFX). The antiproliferative activity of new chelators was found to correlate with iron(III) chelation ability and some of analogs showed substantially higher antiproliferative activity than DFX.

Iron is essential for cellular metabolism in critical steps of cell progression, growth, and division, including DNA synthesis.[1] Cancer cells as compared to normal cells uptake iron at a substantially greater rate[2] mainly because of expression of ribonucleotide reductase (RR). RR is critical for synthesis of deoxyribonucleotides and cell division. RR is overexpressed in rapidly dividing cancer cells making it an important target of anticancer therapy.[3] Synthesis of RR requires a constant supply of NADPH, oxygen, and iron cations. Deficiency of labile iron cations in the cytoplasm results in inactivation of RR and inhibition of synthesis of deoxyribonucleotides which is the rate limiting step in DNA synthesis. The overall result is the changed expression of cyclins and cdk proteins essential for cell cycle progression causing cell cycle arrest of G1/S phase and apoptosis.[4]

Substantially decreased cancer cell growth in vitro and in vivo has been demonstrated for different strategies based on iron deprivation, including dietary iron restriction,[5] treatment with Ga^{3+} salts,[6] and introduction of anti-transferrin antibodies.[7] These results indicate that controlling iron homeostasis can be a potentially important tool for inhibition of proliferation of cancer cells.

Iron chelators change cellular iron homeostasis by preventing iron acquisition by the cell and/or binding of the labile iron pool inside the cell.[8] A number of in vitro and in vivo studies as well as clinical trials demonstrated efficiency of desferrioxamine (DFO) against several cancer cell lines,[9] especially neuroblastoma[10] and hematologic neoplasms.[11] An important advantage of DFO is a substantially higher therapeutic index compared to normal cells and no significant side effects.[12] Recent studies have demonstrated high efficiency of a combination of aminolevulinic acid photodynamic therapy with DFO.[13] However, DFO suffers a number of disadvantages due to long administration through infusion (12–24 h day, 5–6 times per week), short plasma half-life, and low potency resulting in high doses (150 mg/kg/day) required for treatment.[14]

The antiproliferative activity of DFO stimulated progress in search of iron chelators with higher bioavailability and activity. Hexadentate chelators such as Tachpyr[15] have moderate antiproliferative activity and were found to possess lower selectivity toward iron binding.[16] Substantially higher antiproliferative activity has been achieved with tridentate chelators based on semicarbazone and thiosemicarbazone derivatives such as Triapine.[17] These compounds have a relatively high Fe^{2+}/Fe^{3+} potential and are capable of redox cycling, thus producing reactive oxygen species (ROS). The resultant hydroxyl radicals are capable of directly attacking cellular components resulting in DNA damage, lipid peroxidation, and protein oxidation.[18]

Further improvement of antiproliferative activity of iron chelators necessitates development of new types of iron chelators. We

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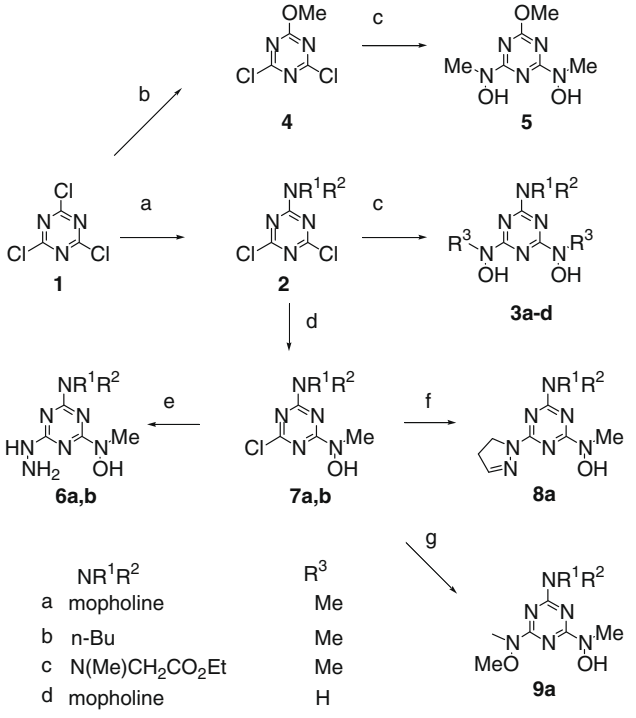
have previously reported on iron chelators of the 2,6-bis[hydroxy-(methyl) amino]-1,3,5-triazine (BHT) family.[19,20] The built-in features of these chelators, (i) strong binding of iron(III) along with high Fe^{3+}/Fe^{2+} selectivity resulting in very low redox potential of the formed complex which precludes redox cycling and uncontrolled formation of reactive oxygen species in normal cells, (ii) rigidity of the ligand resulting in size-selectivity towards iron binding, and (iii) balanced hydrophobicity of the ligand allowing it to be soluble in aqueous media on the one hand and membrane permeable on the other hand, make this family of ligands very promising candidates for iron depletion anticancer therapy agents. Herein we report on antiproliferative activity of tridentate chelators of the BHT family and other tridentate chelators possessing the hydroamino-1,3,5-triazine moiety (Scheme 1).

Syntheses of iron chelators **3a–d** were done from 2,4,6-trichloro-1,3,5-triazine (**1**) through monosubstitution of a chlorine atom with a alkylamino/dialkylamino- or an alkoxy/phenoxy group.[21] Resultant dichlorotriazines of type **2** were treated with an excess of hydroxylamine or *N*-methylhydroxylamine providing corresponding derivatives **3a–d**.[22] Iron chelator **5** was prepared through monosubstitution of a chlorine atom in **1** with a methoxy group followed by the treatment with an excess of *N*-methylhydroxylamine.[23]

Alternatively, treatment of dichlorotriazines of type **2** with 2 equiv of *N*-methylhydroxylamine provided substitution of one chlorine atom giving monochlorotriazines **7a**,**b**. Substitution of the remaining chlorine atom with hydrazine or 1*H*-4,5-dihydropyrazole provided compounds **6a, b** and **8a** possessing two different chelating groups attached to the 1,3,5-triazine ring.[24]

The in vitro cytotoxic activity of a range of BHT analogs with different peripheral functional groups was determined in two different human tumor cell lines *MDA-MB-231* (human breast cancer) and *MiaPaCa* (human pancreatic cancer) using the colorimetric MTT assay. In this study, we used the human breast (MDA-MB231) and human pancreas (MiaPaCa) cancer cell lines because they are highly tumorigenic and are among the well-established human cancer cell lines that are widely accepted in cancer research, including the preclinical evaluation of the antitumor activity of new agents both in vitro and in vivo. The additional reason for using these two cell lines was to avoid potential effects produced by BHT chelators in a tissue type-specific manner.



Scheme 1. Reagents and conditions: (a) NHR^1R^2; (b) MeOH, 2,6-lutidine; (c) MeNHOH, excess; (d) MeNHOH, 2 equiv; (e) N_2H_4, excess; (f) 1*H*-4,5-dihydropyrazole, excess; (g) MeNHOMe, excess.

Results of cytotoxicity assays suggest that the substitution on the nitrogen or oxygen atoms of hydroxyamino groups is essential for modulating the cytotoxic activity of BHT analogs on human cancer cells. Replacement of an *N*-hydroxy group with *N*-methoxy group results in remarkable decrease in their cytotoxicity against both cell lines with IC_{50} values from 1–9 μM (**3a–c**) to 40–150 μM (**9a**). Replacement of one of hydroxy(methyl)amino groups in compounds with sp^2 nitrogen-based function in **6a,b** and **8a** dramatically decreased their ability to iron binding[25] as well as cytotoxicity.

Presence of a methyl group at the nitrogen atom of hydroxylamine also plays a critical role in mediating the cytotoxic effects of BHT analogs in cancer cells, since BHT analogs lacking *N*-methyl group substituents on hydroxyamino groups (**3d**) has very low cytotoxicity in MDA-MB231 cells (IC_{50} > 150 μM) and is moderately cytotoxic in MiaPaCa cells (IC_{50} = 40 μM). This difference can be tentatively attributed either to a much lower lipophilicity of **3d**, which should hinder penetration through the cellular membrane, or to a possible fast metabolic degradation of the hydroxyamino group that does not take place for *N*-methyl derivatives such as **3a–c**.

The obtained results indicate a correlation between stability of iron complexes of chelators **3a–c**, **5, 6a, b, 8a, 9a** and their antiproliferative activity. Chelators **3a–c**, **5** possessing two hydroxy(methyl)amino substituents are characterized by pFe^{3+} in the range 22–24. These compounds were highly cytotoxic in the range of 0.6–5.0 μM for MiaPaCa cells and 4–20 μM for MDA-MB231 cells. Chelators **6a, b, 8a** and **9a** possessed pFe^{3+} below 20 and were weakly cytotoxic. The critical role of iron binding for cytotoxicity is further confirmed by low cytotoxicity of the 2:1 **3a**–iron(III) complex,[26] while the chelator **3a** itself is highly active. The low cytotoxicity of the 2:1 **3a**–iron(III) complex could be explained by low 2:1 BHT–iron(III) complex penetration through the cell membrane while the iron complex of **3a** possess relatively high lipophilicity (Log *P* 2.45)[20] and low molecular weight (*M* = 564) suggesting intrinsically low toxicity of iron(III) complex. In contrast to cytotoxic iron complexes of iron chelators, such as Triapine, that generate reactive oxygen species due to redox cycling, iron(III)-BHT complexes have been reported to possess very low redox potential ranging from −0.79 to −0.97 V[20] effectively precluding reduction of Fe^{3+}.

Ideally, for an effective chemotherapeutic strategy, the agent should be able to impart apoptotic and/or antiproliferative effects specifically in cancer cells without affecting normal cells. Thus, we compared the antiproliferative effect of BHT chelators on a normal foreskin fibroblast cell line (CCD-1064SK) with two human cancer cell lines MDA-MD231 and MiaPaCa. As shown in Table 1, this normal cell line, was more resistant to our chelators since their IC_{50} values were at least 5–20-fold higher than MDA-MB231 and MiaPaCa cells, respectively. This result indicates that BHT chelators specifically and markedly inhibit the growth of human cancer cells with less effect on the growth of normal fibroblast cells.

In conclusion, we prepared new selective iron(III) chelators of the hydroxyamino-1,3,5-triazine family and studied their antiproliferative activities using *MDA-MB-231* and *MiaPaCa* cancer cell lines. Compounds possessing two hydroxy(methyl)amino substituents (BHT chelators) were found to display antiproliferative activity in low micromolar to submicromolar range. The antiproliferative activity of chelators possessing only one hydroxy(methyl)amino group was found to be more than ten times lower than in BHT compounds which correlates with lower stability of their

Table 1
Antiproliferative activity for compounds **3a–d**, **5, 6a**,**b**, and **8a** on cancer and normal cell lines[a,b]

Compound	Cytotoxicity, IC_{50} (μM)		
	MiaPaCa	*MDA-MB-231*	*Foreskin fibroblasts*
3a	1.0 ± 0.3	9.0 ± 3.0	200 ± 20
3b	0.8 ± 0.2	4.0 ± 0.5	16.5 ± 2.5
3c	4.0 ± 1.0	20 ± 3.0	200 ± 10
3d	40 ± 10	250 ± 50	350 ± 30
5	0.6 ± 0.2	5.0 ± 2.0	100 ± 10
6a	150 ± 30	150 ± 50	400 ± 20
6b	50 ± 5.0	75 ± 25	300 ± 20
8a	50 ± 5.0	75 ± 25	250 ± 10
2:1 Complex of **3a** with iron	25 ± 5.0	20 ± 5.0	100 ± 10
9a	40 + 10	150 ± 50	250 ± 20

[a] Both MDA-MB231 and MiaPaCa cells were treated with various concentrations of compounds for 96 h. Cell viability was measured by MTT assay. Results represent mean ± S.D. of three experiments.
[b] Cytotoxicity for Deferasirox (DFX) in the same experiments were 4 ± 1.0 μM for *MiaPaCa,* 10 ± 2.5 μM in *MDA-MB-231* cells, and 100 ± 10 in foreskin fibroblasts.

iron(III) complexes. Simplicity of the synthesis of BHT chelators, easy modification of their physical and chemical properties, and their high antiproliferating activity make BHT chelators promising candidates for further study as cancer chemotherapeutics.

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21. Chelators **3a**,**c** and their iron complexes have been reported in the Ref. 21.
22. *General procedure for preparation of* **3a–d**: To a solution of **1** (18.4 g, 100 mmol) in ethyl acetate (300 mL) a solution of a corresponding amine R^1R^2NH (200 mmol) in THF (100 mL) was added dropwise at −2 °C under vigorous stirring. The reaction was continued 15 min after completing the addition and filtered. The filtrate was evaporated to afford 2,6-dichloro-4-*R*1,*R*2-amino-1,3,5-triazines of type **2** that are purified by recrystallization from isopropanol. To a suspension **2** (30 mmol) in dioxane at 0 °C (90 mL) was added a solution of R^3NHOH hydrochloride (120 mmol) and NaOH (5.4 g, 108 mmol) in water (20 mL). The reaction mixture was stirred for 14 h, diluted with water and the precipitated **3a–d** were collected by filtration and recrystallized from isopropanol.
23. A solution of 2,4,6-trichlorotriazine (50 mmol) in acetone (100 mL) at −2 °C under vigorous stirring was added dropwise a solution of MeOH (50 mmol) and 2,6-lutidine (50 mmol) in acetone (50 mL). The reaction mixture was stirred for 30 min. after completing the addition and then allowed to warm to room temperature and stirred overnight. The reaction mixture was filtered, the filtrate is poured into crushed ice and filtered. The precipitate of **4** is dried and recrystallized from isopropanol. To a suspension of **4** (30 mmol) in dioxane at 0 °C (90 mL) was added a solution of MeNHOH hydrochloride (120 mmol) and NaOH (5.4 g, 108 mmol) in water (20 mL). The reaction mixture was stirred for 14 h, evaporated to dryness, and triturated with water to give **5**. ^{1}H NMR (400 MHz).
24. All newly synthesized compounds has adequate spectral data, for example, **3d** (400 MHz, $CDCl_3$) 3.70 (m, 4H), 3.83 (m, 4H); **5** (400 MHz, D_2O) 3.24 (s, 6H), 3.95 (s, 3H); **6a** (400 MHz, D_2O) 3.21 (s, 3H), 3.64 (br s, 8H); **6b** (400 MHz, $CDCl_3$) 0.96 (t, J = 7 Hz, 3H), 1.38 (m, 2H), 1.54 (m, 2H), 3.37 (br s, 5H); **8a** (400 MHz, $CDCl_3$) 2.94 (t, J = 9.6 Hz, 2H), 3.36 (s, 3H), 3.6–3.8 (m, 8H), 3.93 (t, J = 9.6 Hz, 2H); **9a** (400 MHz, $CDCl_3$) 3.31 (s, 3H), 3.34 (s, 3H), 3.70–3.78 (m, 8H) 3.76 (s, 3H).
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26. The 2:1 **3a**–iron complex was prepared according to the procedure described in Ref. 20.