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Synthesis and characterization of quinoline-based thiosemicarbazones and correlation of cellular iron-binding efficacy to anti-tumor efficacy

Maciej Serda [a], Danuta S. Kalinowski [b,*], Anna Mrozek-Wilczkiewicz [a,c], Robert Musiol [a], Agnieszka Szurko [c,d], Alicja Ratuszna [c], Namfon Pantarat [b], Zaklina Kovacevic [b], Angelica M. Merlot [b], Des R. Richardson [b,*], Jaroslaw Polanski [a,*]

[a] Department of Organic Chemistry, Institute of Chemistry, University of Silesia, PL-40006 Katowice, Poland
[b] Department of Pathology and Bosch Institute, University of Sydney, Sydney, New South Wales 2006, Australia
[c] Department of Solid State Physics, Institute of Physics, University of Silesia, PL-40007 Katowice, Poland
[d] Department of Experimental and Clinical Radiobiology, Center of Oncology, M. Sklodowska-Curie Memorial Institute, PL-44101 Gliwice, Poland

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ABSTRACT

Iron chelators have emerged as a potential anti-cancer treatment strategy. In this study, a series of novel thiosemicarbazone iron chelators containing a quinoline scaffold were synthesized and characterized. A number of analogs show markedly greater anti-cancer activity than the 'gold-standard' iron chelator, desferrioxamine. The anti-proliferative activity and iron chelation efficacy of several of these ligands (especially compound **1b**), indicates that further investigation of this class of thiosemicarbazones is worthwhile.

Iron (Fe) chelators are commonly used to treat diseases connected with altered iron metabolism, for example, β-thalassaemia major.[1] However, considering the marked anti-proliferative activity of this group of agents, recent investigations have focused on the anti-cancer efficacy of iron chelators.[2,3] In fact, there are many reports of the anti-proliferative activity of desferrioxamine (DFO), Triapine® and other ligands based on the (thio)urea moiety.[2]

The cytotoxic mechanisms of chelators include: (**1**) the inhibition of cellular iron uptake from the iron-binding protein, transferrin (Tf);[4–7] (**2**) mobilization of iron from cells;[4–7] (**3**) the inhibition of the iron-containing enzyme involved in the rate-limiting step of DNA synthesis, ribonucleotide reductase;[8] and (**4**) the formation of redox-active iron complexes that generate reactive oxygen species (ROS).[5,7] The latter mechanism is significant, especially in the context of recent reports demonstrating the role of ROS generation in increasing the anti-proliferative activity of chelators against tumor cells.[5,7,9]

Alterations in the metabolism of iron[10–13] and copper[14,15] are known to occur in cancer cells and may play a role in angiogenesis[16] and metastasis.[17] The rationale behind the potential application of iron chelators for cancer treatment is due to the higher demand for iron in rapidly proliferating tumor cells in comparison to their normal counterparts.[10–12] The greater requirement for iron in tumor cells results in high levels of the transferrin receptor (TfR1) on the cell surface which binds Tf.[13] Furthermore, the expression of ribonucleotide reductase is markedly higher in neoplastic cells relative to their normal counterparts.[2] Hence, this also increases the sensitivity of cancer cells to iron-depletion.

Although DFO was investigated as an anti-cancer agent, interest in this compound was diverted in favor of more effective ligands such as aroylhydrazones that show greater iron chelation efficacy and cellular permeability.[3] Some of these compounds were shown to have moderate anti-tumor activity that was significantly greater than that of DFO for example, 2-hydroxy-1 naphthylaldehyde isonicotinoyl hydrazone (311; Fig. 1).[18,19] Further structural modifications of this series produced the 2-hydroxy-1-naphthylaldehyde thiosemicarbazone (NT)[20] and di-2-pyridyl ketone isonicotinoyl hydrazone (PKIH) series[21] of chelators (Fig. 1), which showed superior activity.

Abbreviations: Bp44mT, 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; BpT, 2-benzoylpyridine thiosemicarbazone; DFO, desferrioxamine; DpT, dipyridyl thiosemicarbazone; Dp44mT, di-2-pyridyl ketone-4,4-dimethyl-3-thiosemicarbazone; NT, 2-hydroxy-1-naphthylaldehyde thiosemicarbazone; PKIH, di-2-pyridyl ketone isonicotinoyl hydrazone; ROS, reactive oxygen species; Tf, transferrin; TfR1, transferrin receptor 1; QCIH, 2-quinolinecarboxaldehyde isonicotinoyl hydrazone; QT, quinoline thiosemicarbazone.

* Corresponding authors. Tel.: +61 2 9036 6547; fax: +61 2 9351 3429 (D.S.K.); tel.: +61 2 9036 6548; fax: +61 2 9351 3429 (D.R.R.); tel./fax: +48 322599978 (J.P.).
E-mail addresses: danuta.kalinowski@sydney.edu.au (D.S. Kalinowski), d.richardson@med.usyd.edu.au (D.R. Richardson), polanski@us.edu.pl (J. Polanski).

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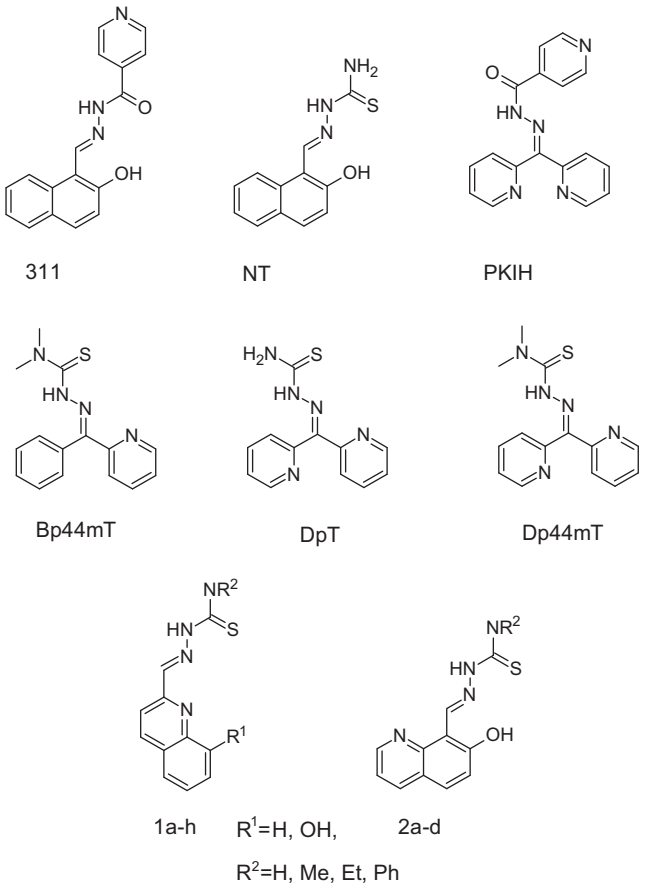
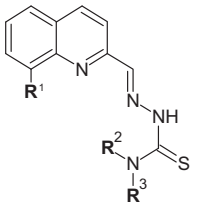
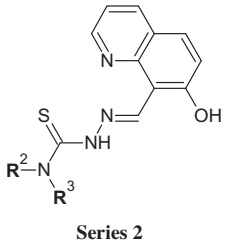


Figure 1. Representative structures of the hydrazones and thiosemicarbazones used to design the quinoline thiosemicarbazone (QT) analogs, **1a**–**h** and **2a**–**d**.

Table 1
The quinoline-based thiosemicarbazones examined in this study





Compd	R^1	R^2	R^3
1a	8-OH	Me	Me
1b	H	Me	Me
1c	8-OH	Me	H
1d	H	Me	H
1e	8-OH	Et	H
1f	H	Et	H
1g	8-OH	Ph	H
1h	H	Ph	H

Compd	R^2	R^3
2a	Me	Me
2b	Et	H
2c	Me	H
2d	Ph	H

The di-2-pyridyl ketone thiosemicarbazone (DpT; Fig. 1) class of chelators are essentially hybrids of these two latter classes of chelators.[7,22] The DpT series, and in particular the chelator, di-2-pyridyl ketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Fig. 1), have shown potent and selective anti-tumor activity both in vitro and in vivo against a variety of murine and human xenografts.[7,22,23] For example, Dp44mT has demonstrated marked activity in vivo, reducing the growth of a murine M109 lung cancer by approximately 50% within 5 days of treatment, while at the same time having little effect on normal hematological indices.[7] In addition to the ability of Dp44mT to effectively induce cellular iron-deprivation in vitro, initial studies revealed that the iron complex was redox-active within cells.[5,7] Hence, it was proposed that the anti-tumor activity of these compounds relates both to their ability to bind intracellular iron and to form redox-active iron complexes that generate cytotoxic ROS.[5,24]

More recent studies have led to the 2-benzoylpyridine thiosemicarbazone (BpT) series of ligands (e.g., 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone; Bp44mT; Fig. 1) that show selective anti-tumor activity in vitro and in vivo and which are effective via the intravenous or oral routes.[9,25] Additionally, thiosemicarbazones are more stable in plasma and are advantageous over aldehyde-derived aroylhydrazones (such as 311) which undergo hydrolysis of the hydrazone bond.[26]

In the current study, a series of novel thiosemicarbazones were synthesized as potential anti-cancer agents. These compounds were designed to contain a quinoline scaffold (quinoline thiosemicarbazones; QTs; compounds **1a–h** and **2a**–**d**; Fig. 1 and Table 1). In fact, the quinoline moiety is often a fragmental motif in the design of novel anti-cancer agents[27] (e.g., the clinically used camptothecin derivatives, topotecan and irinotecan)[28] and it was of interest to assess its effect on the biological activity of thiosemicarbazones. In series **1**, the four-atom moiety (N=C–C=N), which is found in the DpT and BpT ligands is maintained (Fig. 1). In contrast, in series **2**, a five-atom fragmental construct (N=C–C=C–OH) similar to 311 or NT (Fig. 1) was preserved, while the four-atom moiety (N=C–C=N) of the DpT and BpT series was extended (N=C–C–C=N). Of the two series of QTs described herein, several analogs acted similarly to Dp44mT, markedly preventing cellular iron uptake and promoting iron mobilization.[5,7,22]

All the QTs herein were synthesized as shown in Figure 2 by reacting the respective quinolinecarbaldehyde (**3**) and thiosemicarbazide (**4**) in a microwave reactor (experimental details for all procedures in this study are described in the Supplementary data). We confirmed the iron chelating ability of these QTs by titrating the two most biologically active analogs (see below), **1b** and **2a** with Fe^{3+} to generate their Fe^{3+} complexes in situ at the following ligand to Fe ratios: 2:1, 2.5:1, 3.3:1, 5:1, and 10:1 (Supplementary Fig. 1). This was carried out in comparison to the well characterized iron chelator, Dp44mT.[5] As previously observed, the electronic spectrum of the Fe^{3+} complex of Dp44mT displayed characteristic intense transitions (400 nm) that spanned into the visible region (Supplementary Fig. 1A).[5] Additionally, isosbestic points were observed at 310 and 365 nm (Supplementary Fig. 1A). The Fe^{3+} complexes of **1b** and **2a** exhibited a similar shift into the visible region in comparision to that of the free ligands. The Fe^{3+} complex of compound **1b** displayed an intense band at 430 nm, with an isosbestic point found at 375 nm (Supplementary Fig. 1B). Similarly, the Fe^{3+} complex of **2a** showed a transition at 450 nm, while isosbestic points were observed at 330 and 385 nm (data not shown). The electronic spectra of the Fe^{3+} complexes of **1b** and **2a** display characteristics that are typical of Fe^{3+} thiosemicarbazone complexes[5,9,29] and confirm their ability to act as iron chelators.

The anti-proliferative activity of the QT analogs was assessed against cancer and non-neoplastic cells, including the human SK-N-MC neuroepithelioma, HCT116 colon cancer cell lines, and normal human dermal fibroblast (NHDF) cells by standard methods.[5,9,19] The SK-N-MC cell line was chosen as the effects of iron chelators are well characterized in this cell line.[5,9] Additionally, we examined whether the p53 status of HCT116 cells altered their response to the QT analogs. The protein, p53, is an important tumor

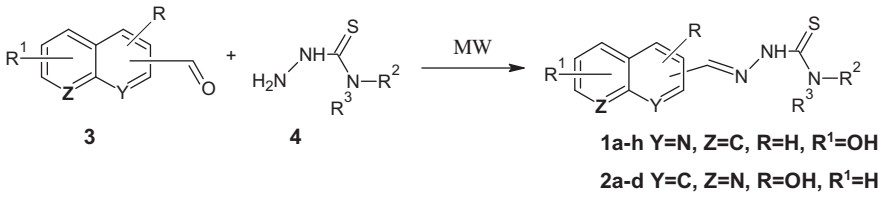


Figure 2. Synthesis of quinoline-based thiosemicarbazones.

suppressor involved in various mechanisms that prevent tumorigenesis.[30–32] In fact, it is well known that p53 acts as a molecular guardian of the cell cycle and induces a G_1/S arrest upon the induction of DNA damage and results in a repair response that prevents propagation of mutations.[30] Lack of p53 expression in some cancers prevents repair, aids tumor cell progression and promotes resistance against chemotherapeutics.[33] The development of agents that demonstrate activity against tumors with and without p53 is vital,[22] particularly considering the high prevalence of p53 mutations in advanced cancer.[32] Thus, we examined the anti-proliferative activity of the novel QT analogs against HCT116 cell lines with ($p53^{+/+}$) and without the p53 protein ($p53^{-/-}$). The quinoline analogs were compared to a number of chelators with well characterized activity which acted as positive controls, namely DFO,[19] 311[19] and Dp44mT.[5]

The control chelators, DFO (IC_{50}: 12.50 ± 1.00 μM) and 311 (IC_{50}: 1.30 ± 0.19 μM), demonstrated moderate anti-proliferative effects against SK-N-MC cells (Table 2).[19] In contrast, Dp44mT possessed potent anti-cancer activity in the SK-N-MC (IC_{50}: 0.014 ± 0.016 μM), HCT116 $p53^{-/-}$ (IC_{50}: 0.005 ± 0.002 μM) and HCT116 $p53^{+/+}$ (IC_{50}: 0.002 ± 0.001 μM) cell-types. The most effective QT analogs, namely **1b** and **2a–2d**, had moderate anti-proliferative activity (IC_{50}: 0.11–1.63 μM) against SK-N-MC cells. In contrast, the remaining compounds were relatively inactive against this cell line (IC_{50}: 9.69 to >12.50 μM; Table 2). In fact, all the QT series **2** analogs demonstrated moderate anti-cancer activity in SK-N-MC cells, while only one member of series **1** (**1b**) showed appreciable anti-proliferative effects (Table 2). Interestingly, a marked difference in the anti-cancer activity was observed between compound **1b** (IC_{50}: 0.81 ± 0.30 μM; Table 2) and **1d** (IC_{50}: >12.5 μM; Table 2) that differ by a single methyl group at the terminal N4 atom. This is a common structure–activity relationship that is observed in a number of thiosemicarbazone classes, including the DpT and BpT series,[5,9,29] and may be explained by the increased lipophilicity of **1b** in comparison to the more hydrophilic ligand, **1d**. Due to its increased lipophilicity, **1b** may target different intracellular iron pools to that of **1d**, which are critical for cellular proliferation (e.g., iron pools required by RR).[5] However, no clear overall relationship ($r = 0.16$) was found between the $\log P_{calc}$ value of the QT ligands and their anti-proliferative efficacy, suggesting that factors other than lipophilicity influenced their activity.

Table 2
Anti-proliferative activity of the QT analogs in comparison to the positive control chelators, DFO, 311 and Dp44mT, after a 72 h incubation

	SK-N-MC	HCT116 ($p53^{-/-}$)	HCT116 ($p53^{+/+}$)	NHDF
IC_{50} (mean ± SD) (μM)				
DFO	12.50 ± 1.00	—	—	—
311	1.3 ± 0.19	—	—	—
Dp44mT	0.014 ± 0.016	0.005 ± 0.002	0.002 ± 0.001	15.38 ± 5.06
1a	9.69 ± 1.50	0.27 ± 0.01	>15.00	15.84 ± 2.65
1b	0.81 ± 0.30	0.37 ± 0.02	4.86 ± 1.48	11.66 ± 2.63
1c	>12.50	2.17 ± 0.47	1.71 ± 0.34	>25.00
1d	>12.50	>25.00	>25.00	—
1e	>12.50	3.15 ± 0.68	2.62 ± 0.59	>25.00
1f	>12.50	19.56 ± 3.92	23.25 ± 4.02	—
1g	>12.50	20.87 ± 3.22	24.97 ± 4.29	—
1h	>12.50	21.39 ± 5.69	22.34 ± 5.18	—
2a	0.11 ± 0.04	>25.00	20.75 ± 5.34	>25.00
2b	0.19 ± 0.07	>25.00	>25.00	>25.00
2c	0.62 ± 0.09	8.06 ± 1.03	>25.00	>25.00
2d	1.63 ± 0.34	18.62 ± 2.79	16.28 ± 1.69	14.94 ± 1.61

Results are mean ± SD (three experiments).

The QT analogs showed varying anti-proliferative efficacy when comparing the SK-N-MC and HCT116 cell lines (Table 2). Most of the active QT series (i.e., those with an IC_{50} <12.5 μM) had more potent anti-cancer effects on SK-N-MC cells when compared to the HCT116 cell lines, which is in good agreement with the general sensitivity of these cell-types, as shown in previous studies in our laboratories.[34–36] Compounds **1a** and **1b** were the only analogs that displayed significantly higher anti-proliferative activity against the $p53^{-/-}$ HCT116 cell line than SK-N-MC and $p53^{+/+}$ HCT116 cells. Notably, the activity profile of **2a–2d** appeared to be different in the SK-N-MC and HCT116 cell lines. In particular, compounds **2a** and **2b** that possessed the most potent anti-cancer activity of the QT analogs in SK-N-MC cells, showed poor or very low anti-proliferative activity (IC_{50} >20.75–25 μM) in both the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell-types.

Comparing the activity of the QT analogs in HCT116 $p53^{+/+}$ and $p53^{-/-}$ cells, the anti-proliferative activity was either similar, or the IC_{50} was greater in the $p53^{+/+}$ cell-type (i.e., for **1a**, **1b**, **2a**). Hence, loss of p53 does not lead to a general increase in resistance against the QT analogs, which is similar to previous observations using other chelators.[22] This is an important characteristic of these compounds especially considering that approximately half of all cancers have non-functional p53, which can lead to resistance to some chemotherapeutics.[33]

For the QT series to be considered as anti-tumor agents, they must demonstrate selective anti-proliferative activity againts neoplastic cells and leave non-neoplastic cells unaffected. Thus, a selection of the most potent analogues were screened against NHDF cells (Table 2). Importantly, the anti-proliferative effects of the QT series were generally markedly decreased against NHDF cells. For example, the anti-proliferative effects of the most potent analogs, **1b** and **2a**, were decreased in NHDF cells in comparison to SK-N-MC cells by 14- and 131-fold, respectively. This suggests that an appreciable therapeutic index exists, allowing these analogs to selectively target cancer cells over normal cells, a feature that has been observed with other thiosemicarbazone iron chelators.[7,9,22]

The clonogenicity assay[37] was used to evaluate the survival fraction and was assessed for a representative QT analog (**2c**) and two positive control chelators, namely Dp44mT and Bp44mT (see Supplementary Tables 1 and 2), in HCT116 cells. The latter two chelators are members of the DpT and BpT series, respectively, and possess pronounced anti-tumor efficacy.[7,9,22,25] In these studies, **2c** demonstrated similar activity in both the HCT116 $p53^{+/+}$ and $p53^{-/-}$ cell lines, although the cells without p53 appeared slightly more sensitive to the agent in terms of the survival fraction (Supplementary Table 1). As suggested by their marked anti-tumor activity in vitro and in vivo,[5,24,38] Dp44mT and Bp44mT (Supplementary

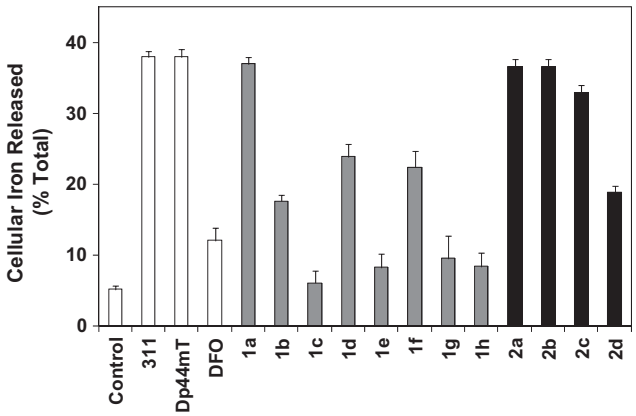


Figure 3. Effect of the chelators on ^{59}Fe mobilization from prelabeled SK-N-MC cells. Cells were incubated for 3 h/37 °C with ^{59}Fe-transferrin (0.75 μM), washed 4 times with ice-cold PBS and then reincubated for 3 h/37 °C in the presence or absence of the chelators (25 μM). Release of ^{59}Fe was then assessed using a γ-scintillation counter. Results are mean ± SD (three experiments).

Table 2) were highly effective, with anti-proliferative activity evident at nanomolar levels.

Considering that thiosemicarbazones avidly bind cellular iron and that this plays a role in their anti-cancer activity,[7,9] the ability of the studied compounds to induce cellular ^{59}Fe mobilization and prevent iron uptake from Tf[2,3] was crucial to assess. Thus, this was examined in the SK-N-MC neuroepithelioma cell line as the effects of other chelators on inducing ^{59}Fe mobilization and preventing ^{59}Fe uptake in these cells have been well characterized.[38–40] The ability of the compounds to affect iron metabolism was compared to that of the control compounds, DFO, 311 and Dp44mT, that have been extensively assessed in this cell line.[7,9,19,40]

As is shown in Figure 3, the highly hydrophilic chelator, DFO, exhibited limited ability to promote ^{59}Fe mobilization, as previously described,[9,38] releasing only 12 ± 2% of intracellular ^{59}Fe. The highly efficient iron chelators, 311[19] and Dp44mT,[7] increased ^{59}Fe mobilization resulting in the release of 38% of total cellular ^{59}Fe (i.e., ^{59}Fe efflux was approximately 8-fold higher than the control).[2,7,41]

In the context of the QT analogs studied in this investigation, compounds **1a** and **2a–2c** were the most effective at inducing

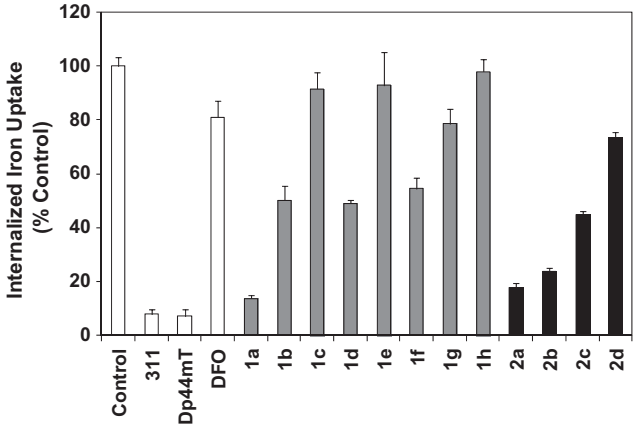


Figure 4. Effect of the chelators on ^{59}Fe uptake from ^{59}Fe-transferrin by SK-N-MC cells. Cells were incubated for 3 h/37 °C with ^{59}Fe-transferrin (0.75 μM) in the presence or absence of the chelators (25 μM). At the end of this incubation, cells were washed 4 times with ice-cold PBS. Internalization of ^{59}Fe was assessed by incubation for 30 min/4 °C with the protease, Pronase (1 mg/mL).[19] Cellular ^{59}Fe was then assessed using a γ-scintillation counter. Results are mean ± SD (three experiments).

^{59}Fe mobilization (Fig. 3). In fact, their activity was similar or slightly less than 311 and Dp44mT, resulting in the release of 33–37% of cellular ^{59}Fe. Of interest, limited correlation (r = 0.40) was identified between the extent of iron release from SK-N-MC cells and moderate anti-proliferative activity (i.e., IC_{50} <12.5 μM) against this cell line (Table 2). For example, compounds **1a**, **2a–2c** that showed marked iron mobilization efficacy (Fig. 3), demonstrated moderate anti-proliferative effects against SK-N-MC cells (IC_{50}: 0.11–9.69 μM; Table 2). In contrast, **1b** and **2d** that displayed significantly (p <0.01) decreased ^{59}Fe mobilization efficacy relative to **1a** and **2a–2c,** had similar or greater anti-proliferative activity (IC_{50}: 0.81–1.63 μM) against SK-N-MC cells. Compounds **1c**–**1h** were significantly (p <0.01) less effective than **1a** and **2a–2c** at inducing cellular ^{59}Fe release (Fig. 3) and had low anti-proliferative effects against SK-N-MC cells (IC_{50} >12.5 μM; Table 2). Notably ligands **1c, 1e**, **1g** and **1h** showed reduced ability to promote cellular ^{59}Fe mobilization (Fig. 3), which may be due to their increased hydrophilicity relative to the other analogs of the QT series. Limited correlation (r = −0.60) was observed between the $\log P_{calc}$ value of the QT ligands and their ability to mobilize cellular ^{59}Fe, suggesting that other factors, apart from lipophilicity, are also important in this process.

Another important factor to consider was the ability of the chelators to prevent ^{59}Fe uptake from ^{59}Fe-Tf[19,21] (Fig. 4). As previously demonstrated,[9,19,39] the positive controls, Dp44mT and 311, effectively prevented ^{59}Fe uptake, reducing it to <10% of the control. In contrast, DFO only slightly influenced ^{59}Fe uptake, reducing it to 81% of the control.[9,39] As found in ^{59}Fe mobilization experiments (Fig. 3), compounds **1a**, **2a–2c** were the four most active amongst the QT analogs, decreasing ^{59}Fe uptake to 14%, 18%, 24% and 45% of the control, respectively. Other compounds of the QT series had variable effects on the inhibition of ^{59}Fe uptake from ^{59}Fe-Tf, reducing it to 49–98% of the control. Of interest, **1c**, **1e** and **1h** did not have greater activity than DFO at reducing ^{59}Fe uptake (Fig. 4). Importantly, we previously demonstrated that a number of series of thiosemicarbazones, including the DpT and BpT series, cannot directly remove ^{59}Fe from ^{59}Fe-Tf.[29] Hence, it is likely that these structurally related thiosemicarbazones prevent ^{59}Fe uptake by directly chelating ^{59}Fe intracellularly after its release from ^{59}Fe-Tf. As was evident from ^{59}Fe mobilization experiments (Fig. 3), limited correlation (r = 0.58) was observed between the $\log P_{calc}$ of the compounds and their ability to inhibit ^{59}Fe uptake from ^{59}Fe-Tf in the SK-N-MC cell line.

Considering structure activity relationships, in general, the QT series were less efficient as anti-proliferative agents than their precursors, namely the BpT and DpT series.[7,9,22,25] Interestingly, the anti-proliferative activity of these analogs is dependent on the particular cell line examined (Table 2).

Interestingly, in series **1**, where the quinoline nitrogen acts as a donor atom, only **1a** and **1b** demonstrated moderate anti-proliferative activity against the SK-N-MC cell line, respectively. These results are consistent with our previous studies examining the 2-quinolinecarboxaldehyde isonicotinoyl hydrazone (QCIH) series of aroylhydrazone ligands.[21] Importantly, the QCIH series were found to have poor anti-proliferative activity and iron mobilizing efficacy in the SK-N-MC cell line.[21] This suggests that the quinoline motif, where the quinoline nitrogen acts as a donor atom, confers low iron chelation efficacy.

The activity of series **2** was much more pronounced than that of series **1** (Table 2), which reveals the importance of the quinoline OH group. As mentioned previously, in the QT series, the five-atom fragmental construct (N=C–C=C–OH) that is similar to 311[18] or the NT analogs[20] was preserved, while the four-atom moiety (N=C–C=N) of the DpT series was extended (N=C–C–C=N). This indicates that the oxygen of the quinoline OH (in alpha position) group acts as a donor atom for chelation, as observed for 311

and the NT series, leading to increased potency. It is of interest that in series **1** and **2**, the *N*,*N*-dimethyl derivatives, in general, had more potent anti-proliferative activity and iron mobilizing efficacy than their parent or Et-substituted analogs, in accordance with previous findings.[42]

The anti-tumor efficacy of thiosemicarbazones has been shown to involve iron chelation.[5,7,9] In previous investigations, we tested a number of thiosemicarbazones and quinoline analogs as potential anti-cancer drugs.[7,22] In this study, a series of novel thiosemicarbazones were designed to contain a quinoline scaffold in order to investigate their effect on iron uptake, iron mobilization and anti-proliferative activity against tumor cell lines. Several QT analogs demonstrated marked chelation efficiency in terms of mobilizing cellular iron and preventing iron uptake from Tf. The anti-proliferative effect of this series of agents showed limited correlation with their ability to promote cellular iron efflux or inhibit iron uptake. Thus, their iron chelating ability only partially explains their anti-proliferative efficacy and suggests that other properties such as their redox activity may be important. The anti-proliferative activity and iron chelation efficacy of several of these agents, in particular **1b**, indicates that further investigation of this class of thiosemicarbazones is certainly worthwhile.

Acknowledgments

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Supplementary data

Supplementary data (the details of experimental procedures and biological activity data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.07.030.

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