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Thiosemicarbazones from the Old to New: Iron Chelators That Are More Than Just Ribonucleotide Reductase Inhibitors

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1. General Introduction

The chemical and biological effects of thiosemicarbazones have received considerable interest from medicinal chemists for many years. This is attributed to, first, their wide pharmacological utility that includes antineoplastic, antibacterial, antiviral, and antifungal activity,[1] and second, their versatility as ligands that allows them to give rise to a great variety of coordination modes.[2]

The antineoplastic activity of thiosemicarbazones has been continually examined since the 1950s, where some compounds of this general class were found to have antileukemic activity.[3] The pronounced antineoplastic efficacy of these ligands has been widely attributed to their inhibition of the mammalian enzyme ribonucleotide reductase (RR[a]).[4,5] The ability to inhibit RR is of particular importance owing to the role of RR in the de novo synthesis of deoxyribonucleotides required for DNA replication and repair.[6] Considering this, these compounds have often been described as RR inhibitors without mention of the property that actually leads to this effect, namely, metal chelation.

Apart from the inhibition of RR, it is also known that thiosemicarbazones are typically excellent chelators of transition metals such as iron (Fe), copper (Cu), and zinc (Zn).[7−9] Such ability for metal chelation is also an attractive strategy in developing anticancer drugs because of the high requirement of neoplastic cells for essential metals needed in growth and proliferation.[10] In fact, thiosemicarbazones were the first class of chelators to be comprehensively assessed as antineoplastic agents against cancer cells[4] in vitro and in clinical trials.[11]

Considering the authors' recent contributions to this field and their new findings regarding the mechanisms of action of these agents, including that they form cytotoxic redox active metal complexes,[12−15] this Perspective will focus on understanding the antineoplastic activity of thiosemicarbazones from the point of view of chelation. To do this effectively, introduction to the roles of metals in cellular proliferation is required in addition to an evaluation of the development of chelators for the treatment of cancer and other diseases.

2. Essential Metals: Their Homeostatic Regulation and Cellular Proliferation

Essential transition metal ions such as $Fe^{II/III}$, $Cu^{I/II}$ and Zn^{II} play vital roles in normal metabolism, being essential for growth and proliferation. These ions are important nutrients for cells, as they are cofactors of numerous molecules that play crucial roles such as oxygen transport and metabolism and DNA synthesis.[16] However, homeostatic control of the cellular levels of these metal ions is essential because an excess can lead to toxicity while a deficiency could result in metabolic impairment or disease.[17] Below, we discuss the metabolism and regulation of the most abundant transition metal ion in mammals, namely, Fe, in order to understand how chelators can more effectively disturb the growth of neoplastic cells relative to their normal counterparts.

2.1. Iron. Iron participates in many essential biological processes,[18] and its utility stems from its redox activity that is generated by the conversion of the metal between its di- or trivalent forms after interaction with cellular oxidants or reductants.[19] Iron plays a pivotal role in a variety of physiological cellular functions such as oxygen transport, energy metabolism, electron transport and modulation of H_2O_2 levels.[20] This same facile property that permits Fe to gain and lose electrons can also make it toxic through the donation of electrons to oxygen, causing the generation of cytotoxic species such as superoxide and hydroxyl radicals.[19] These reactive oxygen species (ROS) readily react with biological molecules, including proteins, lipids, and DNA.[19] Thus, Fe works as a double-edged sword with an excess of the metal being a risk for cancer, presumably via generation of ROS. Indeed, Fe overload evident in the

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[a] Abbreviations: 3-AP, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; ApT, 2-acetylpyridine thiosemicarbazone; DFO, desferrioxamine; BPBH, 2-benzoylpyridine benzoyl hydrazone; BpT, 2-benzoylpyridine thiosemicarbazone; BPTBH, 2-benzoylpyridine thiobenzoyl hydrazone; DpT, di-2-pyridylketone thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; FDA, Food and Drug Administration; HPTC, 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone; IRE, iron-responsive element; IRP, iron-regulatory protein; LIP, labile iron pool; NB, neuroblastoma; NBH, 2-hydroxy-1-naphthaldehyde benzoyl hydrazone; NIH, 2-hydroxy-1-naphthaldehyde isonicotinoyl hydrazone; NDRG1, *N*-myc downstream regulated gene-1; NT, 2-hydroxy-1-naphthylaldehyde-3-thiosemicarbazone; NTBH, 2-hydroxy-1-naphthaldehyde thiobenzoyl hydrazone; PBH, pyridoxal benzoyl hydrazone; PCIH, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone; PIH, pyridoxal isonicotinoyl hydrazone; PKBH, di-2-pyridylketone benzoyl hydrazone; PKIH, di-2-pyridylketone isonicotinoyl hydrazone; PKTBH, di-2-pyridylketone thiobenzoyl hydrazone; PTBH, pyridoxal thiobenzoyl hydrazone; SBH, salicylaldehyde benzoyl hydrazone; STBH, salicylaldehyde thiobenzoyl hydrazone; ROS, reactive oxygen species; RR, ribonucleotide reductase; Tf, transferrin; TfR1, transferrin receptor 1.

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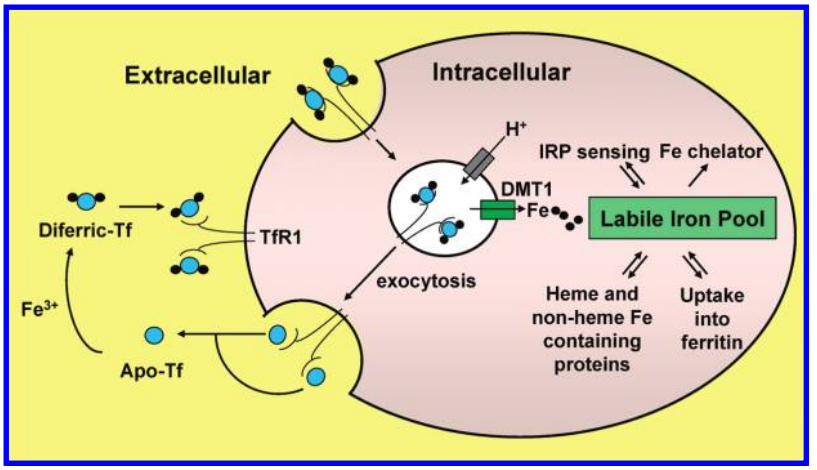


Figure 1. Diagram illustrating Fe uptake from transferrin (Tf) by mammalian cells. The release of Fe from cells into the bloodstream leads to its binding by apo-Tf to form diferric-Tf. The latter binds to the transferrin receptor 1 (TfR1) that is expressed almost ubiquitously on cells, forming the Tf−TfR1 complex that is internalized within an endosome. Within the cell, a decrease in endosomal pH mediates the transport of Fe through the divalent metal ion transporter 1 (DMT1), where it enters the labile iron pool (LIP). Iron in the LIP is then either incorporated into Fe-containing proteins or stored in ferritin. The content of Fe in the LIP is also a rapidly adjustable source of Fe and is thought to be sensed by iron-regulatory proteins (IRPs) that play a role in homeostatically controlling Fe metabolism.

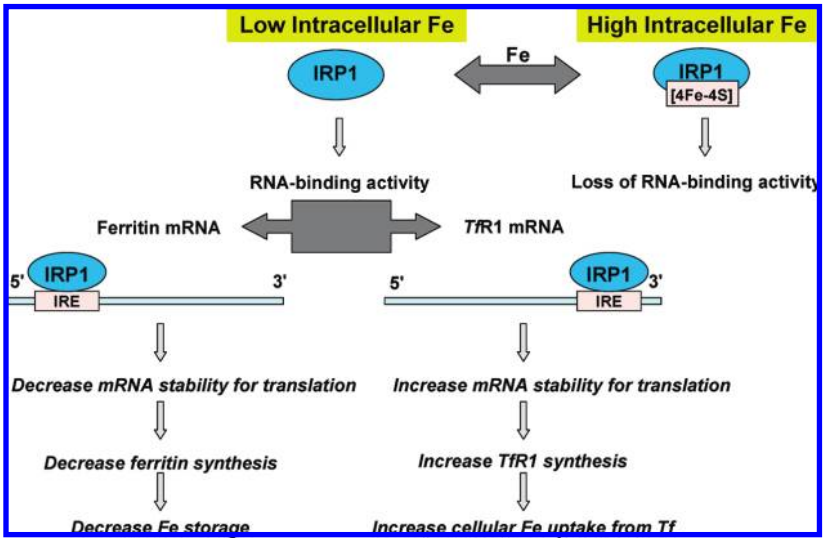


Figure 2. Regulation of Fe homeostasis. Iron homeostasis is post-transcriptionally regulated by IRPs. During low intracellular Fe concentration, IRP1 binds to the 3′-untranslated (UTR) of *TfR1* mRNA, stabilizing it from degradation and hence increasing TfR1 expression. On the other hand, binding of IRP1 to the 5′- UTR of ferritin mRNA under these conditions prevents translation. Under high intracellular Fe levels, a [4Fe-4S] cluster is incorporated into IRP1, preventing its mRNA-binding activity.

common genetic disease hemochromatosis is associated with hepatocellular carcinoma.[21] Consequently, homeostatic mechanisms have evolved to tightly regulate the concentration of Fe so that it is available for use but at the same time preventing Fe overload and the generation of ROS.[19,22]

Iron is transported in the blood bound to the glycoprotein, transferrin (Tf), which binds two ferric ions (Figure 1).[23] Entry of Fe into the cell occurs when two molecules of diferric-Tf form a complex at the cell surface with the transferrin receptor 1 (TfR1).[23] The Tf−TfR1 complex is then internalized within an endosome, and Fe is released from Tf by a decrease in intravesicular pH mediated by a proton pump (Figure 1). Iron is then reduced to its ferrous form and is transported across the endosomal membrane by the divalent metal transporter-1 (DMT1, Figure 1).[24−26]

Once within the cytosol, Fe is available for cellular metabolic processes and becomes part of a very poorly characterized compartment known as the chelatable Fe pool or labile Fe pool (LIP).[27,28] Iron in this pool is used for incorporation into heme or [FeS] clusters or is stored in the multimeric Fe storage protein, ferritin (Figure 1).[23] Iron can also be released from cells by the transplasma membrane protein, ferroportin, that transports Fe from the inside to the outside of the cell.[29]

The LIP has been assumed to provide a rapidly adjustable source of Fe for immediate metabolic utilization and is thought to be sensed by iron-regulatory proteins (IRPs). These are mRNA-binding molecules that post-transcriptionally control the expression of a variety of molecules involved in Fe uptake, utilization, and storage.[27] Systemic regulation of Fe absorption and cellular uptake is possible through both

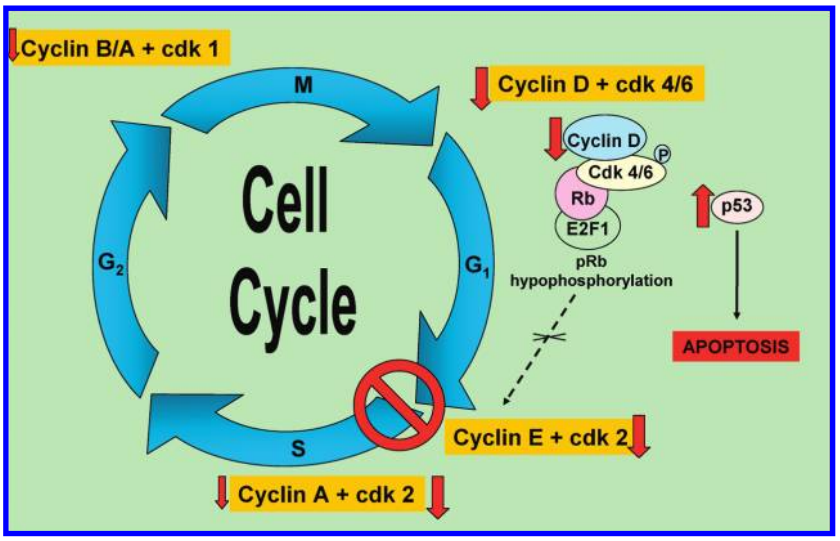


Figure 3. Cell cycle. Fe deprivation results in a G_1/S phase cell cycle arrest. Fe chelation markedly decreases the expression of cyclin D family (cyclins D1, D2, and D3) and cdk2. In the G_1 phase of the cell cycle, cyclin D binds cdk4/6 to form a catalytic complex that phosphorylates the retinoblastoma protein (pRb). This then allows the release of E2F family of transcription factors that promotes the expression of genes important for S-phase entry such as cyclin E. The formation of the cyclin A−cdk2 complex is essential for G_1/S progression. Cyclin B and cdk1 are important for mitosis. Chelation of Fe also increases protein expression and DNA-binding activity of the tumor suppressor, p53, that acts to induce a G_1/S arrest.

transcriptional and post-transcriptional mechanisms.[19] IRP1 and IRP2 are important components of post-transcriptional regulation.[30] The IRPs bind to iron-responsive elements (IREs) present in the 3′ or 5′ untranslated regions of mRNA that encode critical proteins of the Fe absorption and uptake pathways (Figure 2).[19] IRP1 is only active as a mRNA-binding protein when intracellular Fe levels are low, as otherwise it contains a [4Fe-4S] cluster that prevents its binding to IREs (Figure 2).[30] Similarly, IRP2 is not present in Fe-replete cells, as it is degraded under this condition by the proteasome.[30]

As an example of this type of regulation, under conditions of Fe deprivation, IRP1 and IRP2 bind to the 3′-untranslated regions (UTR) of *DMT1* and *TfR1* mRNA[31] and to the 5′ UTR of ferritin[31] and ferroportin (*Fpn1*) mRNA.[29] Binding of IRPs to the 3′ IRE stabilizes the mRNA and prevents it from being degraded, while binding at the 5′ IRE blocks translation of the mRNA. Consequently, as a response to Fe deficiency, there is increased expression of DMT1 and TfR1 protein allowing cells greater Fe uptake capacity.[29,31] Further, the concomitant reduction in Fpn1 and ferritin protein levels due to the inhibition of translation reduces Fe export and storage to maximize the intracellular availability of Fe for metabolism.[29] An opposite response occurs during Fe loading, leading to the homeostatic control of intracellular Fe metabolism. Interestingly, IRPs may respond both to fluctuations in LIP per se and also to secondary signals associated with redox-active species such as nitric oxide.[32,33]

It is notable that the IRP−IRE system is not the only mechanism of regulating intracellular Fe levels, with the hormone hepcidin playing an essential role at the systemic level.[34−36] Hepcidin synthesis is stimulated by high liver Fe levels leading to increased secretion in the blood.[37] Hepcidin is thought to bind to Fpn1 at the cell surface, leading to its down-regulation and preventing Fe release from cells.[38] This prevents Fe absorption from the gut and the release of Fe from macrophages, thereby preventing more Fe uptake into the body and regulating Fe levels.[39]

While Fe uptake from Tf is an important process, the recycling of stored Fe in hemoglobin yields far greater amounts of the metal.[22] In fact, up to 20 times more Fe is supplied through the phagocytosis of aged erythrocytes by macrophages than is absorbed through the diet on a daily basis.[22] This is in line with the knowledge that Fe bound to hemoglobin in erythrocytes accounts for approximately two-thirds of total body Fe.[40] A further 25−30% of body Fe exists bound to the intracellular Fe storage protein ferritin.[41] The remaining small portion is either circulating in the plasma bound to Tf or exists as part of the LIP.[40]

2.1.1. Iron and Cancer. One of the important roles of Fe includes its catalytic activity in RR which bears a di-Fe center at its active (R2) site where it catalyzes the rate-limiting step in DNA synthesis, i.e., the formation of deoxyribonucleotides from their ribonucleotide precursors.[42] In neoplastic cells, the rates of proliferation and DNA synthesis are increased relative to normal cells.[42] This translates to an increased requirement for active RR in cancer cells, reflecting their pronounced rate of DNA synthesis compared to their normal counterparts.[43]

Concurrently, in an effort to meet the increased Fe demand of cancer cells, TfR1 expression is markedly increased relative to normal cells,[44,45] leading to high rates of Fe uptake.[46] Hence, TfR1 has also been proposed as a suitable target for the treatment of cancer using antibodies conjugated to toxic molecules.[47,48] The greater need for Fe leads to cancer cells being more susceptible to the effects of chelation compared to normal cells.

Further to its role in DNA synthesis, Fe is also involved in the progression of cells through the cell cycle by influencing the expression of molecules involved in cell cycle control.[49,50] Deprivation of Fe leads to G_1/S phase cell cycle arrest[51−53] (Figure 3). The result of this is the potential to prevent cancer cell proliferation by controlling availability of Fe to tumors that have significantly higher Fe uptake than their normal counterparts.[54] This has prompted researchers to assess the effect of Fe chelation on the expression of cell cycle control molecules.

A number of studies have found that Fe chelation by desferrioxamine (DFO) or 2-hydroxy-1-naphthylaldehyde isonicotinoyl hydrazone (known as 311 or NIH)[55,56] markedly decrease the expression of cyclin D (D1, D2, and D3) and to a lesser extent cyclin A and B.[54,57,58] In contrast, the expression of cyclin E was increased in neuroepithelioma cells after Fe depletion.[54] However, another report examining mimosine and DFO in breast cancer cells found a decrease in cyclin E kinase-associated activity.[57] The expression of cyclin dependent kinase 2 (cdk2) was also decreased upon Fe chelation.[54] These effects were dependent on Fe deprivation, as the Fe complexes of DFO and NIH were unable to induce such effects.[54] Cyclins D, A and cdks 2, 4, and 6 are involved in progression through the G_1 phase (Figure 3).[49] The formation of the cyclin A−cdk2 complex is essential for G_1/S progression. Cyclin B and cdk1, on the other hand, are important for mitosis.[49] During the G_1 phase, cyclin D binds to cdk4 and cdk2 to phosphorylate the retinoblastoma protein (pRb).[49] This results in the release of molecules such as E2F transcription factor from pRb that promotes the expression of genes for progression into S phase.[59] The decrease in the expression of the cyclins upon Fe chelation caused hypophosphorylation of pRb,[54] which in turn leads to the G_1/S phase arrest observed (Figure 3).

Other molecules that can inhibit growth and metastasis, induce apoptosis and are up-regulated by cellular Fe depletion include p53, the growth arrest and DNA damage (*GADD*) family of genes, and the growth and metastasis suppressor *N*-myc downstream regulated gene-1 (*NDRG1*).[49,60−62] The latter molecule is up-regulated by Fe depletion induced by Fe chelators through hypoxia inducible factor-1α (HIF1α)-dependent and -independent mechanisms.[61] *NDRG1*, has been identified as a growth and metastasis suppressor gene in a number of different neoplasms including prostate, breast, and pancreatic cancer both in vitro and in vivo.[62−65] Moreover, NDRG1 was found to significantly reduce angiogenesis of pancreatic cancer in vivo, leading to less aggressive tumors.[65]

Recent studies have also suggested that NDRG1 is necessary for p53-dependent apoptosis in lung and colon cancer, effectively increasing the efficiency of this important pathway.[66] Although the expression of NDRG1 is often reduced in neoplastic compared to adjacent normal tissue, prostate and breast cancer patients with higher endogenous NDRG1 levels in their tumors had a significantly greater survival rate compared to those with low NDRG1.[63,64] Hence, NDRG1 plays an important role in regulating multiple facets of cancer progression and presents an ideal therapeutic target for the treatment of cancer. An examination of the efficacy of a range of chelators on NDRG1 expression demonstrated that a potent dipyridyl thiosemicarbazone was highly effective at up-regulating this molecule at low concentrations,[61] probably because of its marked Fe chelation efficacy.[12] This up-regulation of NDRG1 may be a part of the effective antitumor activity of the thiosemicarbazones.

Like hypoxia, Fe depletion stabilizes HIF1α through the inhibition of prolyl hydroxylases that act to induce proteasomal degradation of this transcription factor.[49,67] Considering that hypoxia can increase BNIP3 expression via HIF1α,[68,69] the increase in HIF1α transcriptional activity after Fe depletion may, in addition to HIF1α-independent mechanisms, lead to the up-regulation of BNIP3 that can induce apoptosis.[70,71] The ability of Fe chelators to up-regulate key molecules involved in cancer progression and apoptosis such as NDRG1 and BNIP3 makes these agents an increasingly attractive treatment strategy for this belligerent disease.

These factors combined identify Fe as a potential target in cancer therapy either through chelating vital supplies of Fe and subsequently modulating key cell regulatory molecules or through the generation of cytotoxic redox active Fe complexes within the cell.[72]

3. Redox Chemistry of Iron Complexes

Iron is a rare case of a transition element that can be isolated in one of two oxidation states in structurally similar forms. This feature leads to facile redox reactions often unaffected by any changes to the coordination sphere. Interconversion between their di- and trivalent oxidation states results in very small changes in the coordination bond lengths in thiosemicarbazone Fe complexes,[13,14,73] which is the focus of this Perspective. Moreover, rapid and totally reversible $Fe^{III/II}$ couples have been identified in these complexes by cyclic voltammetry.[13,14,73] The ease with which the two forms may be generated is relevant to their biological reactivity. The so-called Fenton reaction was reported more than 100 years ago, and its mechanism is still contentious. However, one certainty is that hydroxyl radicals are generated by breakdown of H_2O_2. The overall reaction may be represented by eq 1.

$$Fe^{II} + H_2O_2 \rightarrow Fe^{III} + \cdot OH + OH^- \quad (1)$$

$$Fe^{III} + red_{cell} \rightarrow Fe^{II} + ox_{cell} \quad (2)$$

The Fenton reaction highlights the potential toxicity of free redox active ferrous ions within the cell where hydrogen peroxide may be present. The generation of hydroxyl radicals may cause lethal damage (via hydroxylation) to vital biomolecules. Equation 1 becomes more critical when the active Fe^{II} ion is regenerated by cellular reductants (represented generally as red_{cell}) in eq 2. Equation 1 is thought to be linked with the toxicity of excessive levels of Fe localizing in vital organs such as the heart and liver.[74]

Fenton chemistry may be possible with complexed Fe (assuming an outer sphere process) behaving similarly as a catalyst for the decomposition of H_2O_2 to $\cdot OH$ if the redox potentials are in the appropriate range, that is, if the $Fe^{III/II}$ redox potential is not so high that oxidation by H_2O_2 is impractical and not too low that the ferrous complex cannot survive in an oxygenated solution. This hypothesis suggests an optimal $Fe^{III/II}$ potential window outside which biological activity is poor because of the oxidative or reductive half reactions (eqs 1 and 2) of the catalytic cycle becoming unfavorable. The production of hydroxyl radicals through Fenton chemistry of thiosemicarbazone Fe complexes has been shown to be an important factor that mediates their cytotoxicity, making them suitable as anticancer agents.[72]

4. Iron Chelators as Antitumor Agents

Although Fe chelators were initially designed and implemented for the treatment of Fe overload diseases, such as β-thalassemia, more recent developments have highlighted their potential to act as potent antitumor agents.[10,75,76] This is illustrated by the entrance of the Fe chelator, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP), into clinical trials.[77−79]

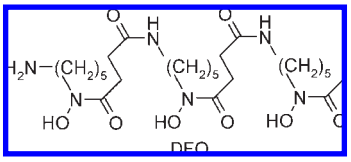


Figure 4. Structure of DFO.

A vast array of Fe chelators have been developed in the quest to create more potent and selective anticancer agents. From these studies, a number of lead compounds have been identified that show potential. Moreover, these studies have furthered our understanding of the structure−activity relationships of Fe chelators as potent antineoplastic agents. Herein, we discuss the development of Fe chelators in terms of their structure−activity relationships from initial lead compounds through to the generation of efficient ligands with marked antiproliferative activity.

4.1. Desferrioxamine (DFO). DFO (Figure 4) is a hexadentate siderophore secreted by *Streptomyces pilosus* that is used to sequester and take up Fe in a soluble form[10] and currently the "gold-standard" chelator used for the treatment of β-thalassemia major.[80] The use of this ligand has validated Fe chelation therapy as an effective approach for the treatment of Fe overload disease with improved patient survival and well-being.[81,82] DFO has high affinity for Fe^{III}, forming a 1:1 complex that prevents the formation of ROS, making it suitable for the treatment of Fe overload disease.[10]

In addition to its role in the treatment of Fe overload, DFO has been investigated as a potential antitumor agent in a number of in vitro studies and in vivo investigations in animals and clinical trials.[83−90] One in vitro study demonstrated a greater than 80% reduction in the viability of neuroblastoma (NB) cell lines upon incubation with DFO (60 μM) for 72 h.[85] DFO was also shown to inhibit DNA synthesis in a NB cell line after 4 h.[90] Importantly, the antiproliferative activity of DFO was shown to be inhibited upon the addition of Fe or Fe-saturated DFO,[85] indicating that Fe depletion is central to its mechanism of action. NB cells were also found to have a 10-fold higher sensitivity to Fe depletion after DFO treatment than normal bone marrow cells.[83]

A number of clinical trials have demonstrated mixed results with the use of DFO as an anticancer agent. For example, a 50% reduction in bone marrow infiltration in 7 out of 9 NB patients was observed, with one patient experiencing a 48% decrease in tumor size after DFO treatment at 150 mg/kg/5 days.[87] When used at this dosage level, no significant side effects were observed.[87] A combination study of DFO with other anticancer agents, including thio-TEPA, carboplatin, etoposide, and cyclophosphamide, in 57 NB patients resulted in 24 complete responses, 26 partial responses, 3 minor responses, and 4 nonresponses in patients.[86] Another case study of an infant acute leukemia patient showed no increase in peripheral blood blast cell numbers and a rise in normal hematopoietic progenitor cells after an iv dose of DFO.[88] On the other hand, treatment of some cancer patients with DFO has shown little response. For instance, 10 children with recurrent NB failed to produce a response after DFO administration,[84] while DFO also failed to inhibit the growth of human tumor xenografts in mice.[89]

The moderate antiproliferative activity of DFO demonstrates its limitations that stem from its high hydrophilicity, including its poor membrane permeability, short half-life,

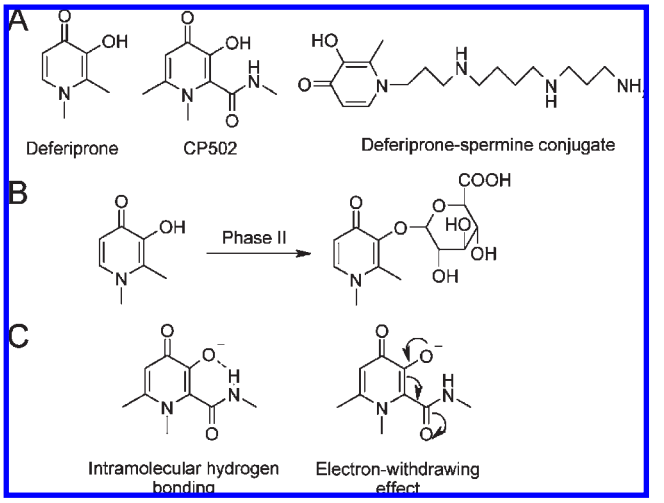


Figure 5. (A) Chemical structures of deferiprone, a deferiprone analogue, and the deferiprone−spermine conjugate. (B) Phase II metabolism of deferiprone to form the glucuronide. (C) Stabilization of the anionic species of the deferiprone analogue in (A) by the amido moiety through intramolecular hydrogen bonding and the electron-withdrawing effect.

and oral inactivity.[82] Thus, DFO must be administered over long periods via subcutaneous infusion.[81] Because of the high cost pain and swelling experienced by this cumbersome route of administration, patient compliance is poor.[82,91] Hence, many alternatives to DFO have been developed in the search for ever increasingly active, selective and specific Fe chelators for the treatment of cancer. These alternatives are discussed below.

4.2. Hydroxypyridinones. 4.2.1. Deferiprone. The first orally active chelator for the treatment of Fe overload disease was the hydroxypyridinone chelator, deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one, L1; Figure 5A). This bidentate chelator uses two adjacent oxygen atoms that selectively coordinate tribasic metal cations.[10] Although deferiprone is available in Europe and several other countries, it never gained Food and Drug Administration (FDA) approval and is not available for clinical use in the U.S. This failure may stem from conflicting clinical trial results that highlighted issues about the safety and efficacy of this drug.[92] For example, a study monitoring the long-term safety of deferiprone demonstrated an increase in liver fibrosis in thalassemia patients.[93] However, this conclusion has been seriously questioned because of the inclusion of patients with hepatitis C, which is known to induce liver fibrosis.[91] In fact, other long-term trials have found no deleterious liver fibrosis after deferiprone treatment.[94,95] One clinical trial using a small number of patients reported that deferiprone was more efficient than DFO in removing myocardial Fe.[96] On the other hand, in a relatively large clinical trial, no difference in cardiac Fe levels was evident between DFO or deferiprone.[94]

These contrasting toxicity results have led to speculation that the deferiprone−Fe complex is redox-active and thus, a host of studies have examined this. Speciation plots of the deferiprone−Fe complex have demonstrated that the incomplete bis-complex accounts for almost 40% of the Fe^{III} at micromolar levels at pH 7.[97] This potentially suggests that the incomplete coordination of Fe would enable access of reductants and oxidants to the metal center in a biological system, potentially leading to the generation of ROS.[98]

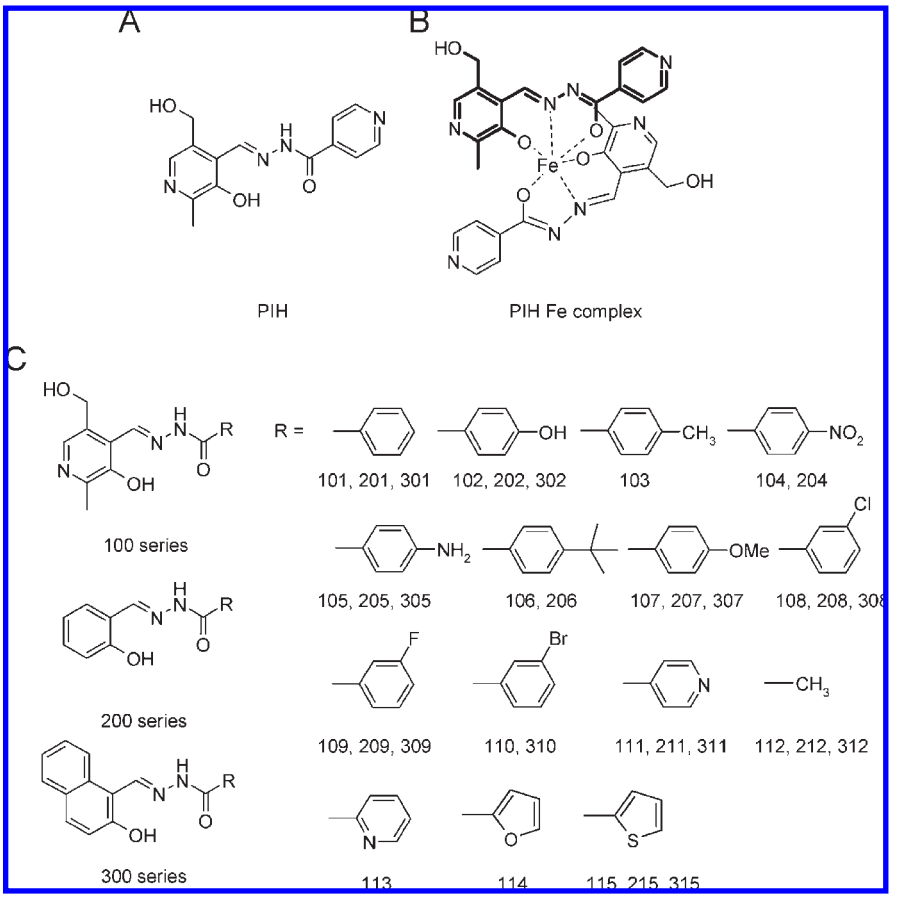


Figure 6. Chemical structure of (A) PIH, (B) the PIH−Fe complex, and (C) the 100, 200, and 300 series of chelators depicting their various R groups.

To achieve a negative Fe balance in Fe overload patients, deferiprone must be administered at a relatively high dose of 75 (mg/kg)/d.[99] This is mainly due to limited efficacy of the drug, as extensive phase II drug metabolism occurs in the liver. During this process the hydroxyl group, necessary for Fe chelation, becomes blocked by glucuronidation (Figure 5B). In fact, greater than 85% of deferiprone administered in humans is converted to the nonchelating 3-*O*-glucuronide form.[100,101] The extensive drug deactivation of deferiprone has initiated the development of other hydroxypyridinone derivatives as alternatives in chelation therapy. A wide variety of deferiprone analogues has been investigated, and one of the most promising is discussed below.

4.2.2. Deferiprone Analogues. To reduce the kinetic lability and drug toxicity and to improve chelation efficacy of deferiprone Fe complexes, a number of analogues with high pFe^{III} values have been investigated. Chelators with high pFe^{III} values are able to scavenge Fe at lower levels and dissociate less readily.[102] An example of a high pFe^{III} chelator is the deferiprone analogue CP502 (Figure 5A), which has a higher pFe^{III} value of 21.7 in comparison to that of deferiprone at 19.4.[103] This deferiprone analogue was found to enhance Fe clearing efficacy in a ^{59}Fe-ferritin-labeled rat model, more so than deferiprone.[104] Additionally, the analogue demonstrated only a minute amount of phase II glucuronidation in a cannulated rat study.[104] This is an important factor in the increased chelation efficacy of this analogue in comparison to deferiprone, prolonging the activity of the ligand.

Because of the improved metabolic profile and complex stability of this high pFe^{III} chelator, Apotex Inc. is further evaluating deferiprone analogues as potential Fe chelators for clinical use.[105]

4.2.3. Deferiprone Conjugates. Deferiprone conjugates have also been designed and prepared to assess their in vitro and in vivo activity. For example, the generation of deferiprone conjugated to polyamines, such as spermine (Figure 5A), has been examined in an effort to increase the membrane permeability of deferiprone.[106] The effect of introducing the polyamine moiety was assessed by examining the ability of this deferiprone conjugate to inhibit the proliferation of L1210 murine leukemia cells.[106] Interestingly, the deferiprone−spermine conjugate (Figure 5A) demonstrated significantly greater antiproliferative activity than deferiprone, with IC_{50} values of 0.2 and 55 μM, respectively.[106] These results suggested that the spermine moiety may enhance the ability of deferiprone to enter cells. However, further studies are required before the validity of this approach can be ascertained.

4.3. Aroylhydrazones. 4.3.1. Pyridoxal Isonicotinoyl Hydrazone (PIH). Pyridoxal isonicotinoyl hydrazone (PIH, Figure 6A) is an orally active Fe chelator belonging to the aroylhydrazone family of ligands.[107,108] This tridentate chelator avidly binds Fe through phenolic and carbonyl oxygens and imine nitrogen donor atoms (Figure 6B). PIH can be easily synthesized through the Schiff base condensation reaction of pyridoxal and isonicotinic acid hydrazide,[107,108] and its tridentate binding mode was demonstrated by the generation of the PIH−Fe complex in a ligand−Fe ratio of 2:1.[107]

Examination of the metal-binding affinity of PIH utilizing potentiometric titrations has demonstrated that this ligand

has a high affinity for Fe^{III} that is comparable to that of DFO, while it has lower affinity for Fe^{II}.[109] This can be expected, as PIH uses predominantly "hard" oxygen donor atoms that prefer Fe^{III}. Interestingly, the ferrous PIH complex was shown to be sensitive to oxidation by oxygen, and this may be an important factor in its ability to protect plasmid pUC-18 DNA against hydroxyl radical-mediated strand breaks.[110,111] This may be due to its ability to scavenge Fe^{II}, limiting the levels of free Fe^{II} that are available to catalyze Fenton chemistry and ROS generation and enhancing its auto-oxidation rate.[111]

The ability of PIH to complex other divalent metals, such as Ca^{II}, Mg^{II} and Zn^{II}, has also been examined.[112] Although PIH was able to bind Zn^{II} with a much higher affinity than Ca^{II} and Mg^{II}, the affinity for these divalent metal ions was far less than that of Fe^{III}.[112] This suggests that the chelation of Fe^{III} and to a lesser extent Zn^{II} can be expected in biological systems.[112]

The ability of PIH to mobilize Fe was initially studied in reticulocytes in which heme synthesis in the mitochondrion was inhibited, leading to Fe accumulation in this organelle.[107] This study demonstrated the marked efficacy of PIH to mobilize Fe from the mitochondrion, confirming the ability of PIH to pass the plasma and mitochondrial membranes and effectively chelate Fe, suggesting its potential usefulness in the treatment of the mitochondrial Fe overload disease, Friedreich's ataxia.[107] In fact, when combined with DFO, PIH has some beneficial effects in reducing the mitochondrial Fe overload and the cardiomyopathy observed in the MCK mouse model of this latter disease.[113]

PIH has also been shown to be as efficient as DFO in preventing the uptake of ^{59}Fe from Tf in hepatocyte cell cultures, reducing ^{59}Fe uptake to approximately half that observed in the control.[114] Additionally, the ability of PIH to reduce ^{59}Fe uptake from Tf was also analyzed in the human SK-N-MC neuroepithelioma cell line.[55] This study showed that PIH was able to effectively reduce ^{59}Fe uptake to a far greater extent than DFO.[55] Interestingly, while DFO showed lower Fe chelation efficacy in terms of mobilizing Fe from cells and preventing Fe uptake from Tf, it was found to be more effective than PIH at inhibiting cellular proliferation.[115] This highlights the suitability of PIH as an agent for the treatment of Fe overload disease and that high Fe chelation efficacy does not necessarily lead to marked inhibition of tumor cell growth.[55]

The very low antiproliferative activity of PIH has been shown to be blocked upon the addition of a soluble Fe source, demonstrating that its ability to bind Fe is central to its very mild cytotoxic effects.[55] The suitability of PIH for the treatment of Fe overload disease was also demonstrated by the ability of its Fe complex to support hemoglobin synthesis[116] and cellular proliferation.[117]

In addition to these in vitro studies, a number of in vivo trials have also been performed highlighting the ability of PIH to induce Fe mobilization when administered orally in animals[118,119] and humans.[120] When administered orally in rodents at doses between 25 and 100 mg/kg, PIH led to the fecal excretion of Fe of up to 8 times greater than basal levels.[121] Intraperitoneal or orally administered PIH was shown to increase biliary ^{59}Fe excretion from labeled rats after only 15 min of PIH administration.[122] Further, a decrease in ^{59}Fe levels was evident in the liver and kidneys of rats after repeated PIH doses.[122] The Fe mobilizing activity of PIH was also examined in a human phase I clinical trial using both normal and Fe-overloaded patients.[120] Interestingly, PIH doses at 30 mg/kg, three times a day for a period of 3 weeks, led to a negative Fe balance in nontransfused patients.[120] Unfortunately, this dosage level would not be sufficient to achieve a negative Fe balance in transfused subjects. However, it is important to note that the doses used were far from optimal.[120]

Speciation studies have been conducted to assess the ionization characteristics of the chelator that could provide more information regarding the mechanism of action of PIH.[123] This study found that the majority of this ligand (~80%) was in the neutral state at physiological pH (7.4), with only a small proportion present as its singly charged anionic species.[123] These results, in combination with this ligand's lipophilicity, suggest that at physiological pH, PIH is readily able to permeate cell membranes and is taken up in the small intestine where the basic pH means that the majority of the chelator will be neutral.[123] Indeed, this is shown by studies examining the ability of the chelator to mobilize ^{59}Fe from prelabeled cells and the much greater efficacy of PIH in comparison to DFO.[124]

In order to further study the structure−activity relationships of PIH, 45 analogues of this compound were developed. These analogues can be divided into three series known as the 100, 200, and 300 groups of ligands. Despite relatively minor structural differences, the PIH analogues were designed to maintain their tridentate coordination with the conservation of the carbonyl oxygen, imine nitrogen, and phenolic oxygen donors (Figure 6C; for review, see ref 125).

4.3.2. 100 Series. The 100 series consist of PIH itself (also known as "111") and its various analogues (Figure 6C), each differing slightly at the hydrazide moiety. In vitro analysis of PIH and the other 14 analogues in a SK-N-MC neuroepithelioma cell line demonstrated that they have low antiproliferative activity.[55] Most of these ligands displayed poorer antiproliferative activity than DFO except for pyridoxal *p-tert*-butylbenzoyl hydrazone (106), pyridoxal *m*-fluorobenzoyl hydrazone (109) and pyridoxal 2-pyridyl hydrazone (113).[55]

Analysis of the relationship between the lipophilicity of the 100 series and their antiproliferative activity demonstrated a weak linear relationship.[55] Although the 100 series shows low antiproliferative activity, most of the analogues were more effective than DFO in preventing ^{59}Fe uptake from Tf and promoting intracellular ^{59}Fe release.[55] Hence, it was suggested that most of the 100 series analogues were suitable for the treatment of Fe overload diseases.

4.3.3. 200 Series. In order to further understand structure−activity relationships of the aroylhydrazone Fe chelators, the 200 series was created by replacing the pyridoxal moiety with the more hydrophobic salicylaldehyde group (Figure 6C). Analysis of the biological activity of 200 series analogues demonstrated higher antiproliferative efficacy than the 100 series.[55,126] Similar to the 100 series, the most pronounced antiproliferative activity was observed with salicylaldehyde *p-tert*-butylbenzoyl hydrazone (analogue 206, Figure 6C). In fact, its efficacy at inhibiting tumor cell growth in culture was similar to the clinically used cytotoxic agents doxorubicin, cisplatin, and bleomycin.[127]

In contrast to the antiproliferative activity of the 100 series, the 200 series analogues demonstrated reduced capacity to mobilize intracellular ^{59}Fe from cells, although they were still more effective than DFO.[55] This emphasizes the fact that chelators with marked antiproliferative activity

work via various mechanisms. The increased antiproliferative activity of the 200 series, which are more lipophilic in comparison with the 100 class, again highlighted the importance of lipophilicity as a factor in determining high antitumor activity.[55] This relationship was further emphasized in the 300 series of chelators discussed below.

4.3.4. 300 Series. The 300 series (Figure 6C) of PIH chelators are more lipophilic than both the 100 and 200 series because of the substitution of the pyridoxal or salicylaldehyde moieties in PIH with the highly hydrophobic group 2-hydroxy-1-naphthaldehyde. The 300 series demonstrate the highest antiproliferative activity out of the three series of PIH analogues.[128] As the 300 series are the most lipophilic and demonstrated marked antiproliferative effects, this further illustrated the importance of lipophilicity in the antitumor activity of chelators. Considering the link between chelator hydrophobicity and proliferation, it was speculated that more lipophilic ligands can gain access to certain intracellular Fe pools necessary for proliferation (e.g., those required by RR), while more hydrophilic chelators cannot.[55]

All analogues of the 300 series were far more efficient than DFO at preventing proliferation ($IC_{50} = 1-7\ \mu M$), the most cytotoxic being 2-hydroxy-1-naphthylaldehyde *m*-chlorobenzoyl hydrazone (308), 2-hydroxy-1-naphthylaldehyde *m*-fluorobenzoyl hydrazone (309), NIH and 2-hydroxy-1-naphthylaldehyde 2-thiophenecarboxyl hydrazone (315).[55] Analysis of their Fe mobilization efficacy found that NIH was the most effective of the analogues, mobilizing 44% of intracellular ^{59}Fe compared to 15% with DFO.[55] Precomplexation of NIH with Fe prior to cellular treatment resulted in the inhibition of antiproliferative activity. This demonstrated that the ability of NIH to bind intracellular Fe is necessary for the ligand's antiproliferative activity.[56] Subsequent studies examined the transport and release of NIH from tumor cells to understand its high efficacy. These investigations showed that, in contrast to the parent compound PIH,[129] NIH was shown to be released from cells by an energy-independent mechanism consistent with diffusion or passive transport.[130] Additionally, NIH was found to have antitumor activity in a range of tumor cell types in culture, including CCRF-CEM leukemia cells, breast cancer cells, bladder carcinoma, as well as head and neck cancer cell lines.[18]

A number of studies have consequently investigated the chemistry of NIH to understand its marked antitumor activity. For example, X-ray crystallography studies have demonstrated that NIH was a tridentate ligand, using the phenolic and carbonyl oxygens and imine nitrogen donor atoms to bind Fe in a meridional manner.[56] In fact, the ligands form a distorted octahedral complex with high spin Fe^{III}.[56] The planarity of NIH prearranges the donor atoms in a geometry ready for Fe binding.

Considering that redox activity of some clinically used anticancer agents plays a role in their cytotoxic effects (e.g., anthracyclines),[131,132] studies were initiated to examine if the binding of Fe by NIH resulted in the generation of cytotoxic radical species. These investigations found that NIH was able to inhibit the oxidation of ascorbate and hydroxylation of benzoate, which indicated that the Fe complex was not significantly redox active.[133] Additionally, cyclic voltammetry studies examining NIH and its Fe complex demonstrated that redox cycling does not play a role in its antiproliferative activity.[56]

Other studies showed the ability of NIH to permeate cells, bind Fe, and alter the expression of proteins involved in Fe metabolism. In fact, NIH was found to increase the RNA-binding activity of the IRPs to a greater extent than DFO, leading to increased levels in *TfR1* mRNA and protein.[133] In addition, NIH and DFO increased the mRNA expression of genes that play crucial roles in regulation of the cell cycle, namely, *GADD45*,[134] the growth and metastasis suppressor gene *NDRG1*,[61] and the cyclin-dependent kinase inhibitor, $p21^{CIP1/WAF1}$ (wild-type p53 activating fragment 1 gene).[134,135] This increase in gene expression was due to Fe depletion, and NIH was markedly more active as a function of dose than DFO because of its high permeability and Fe chelation efficacy.[134] As found previously for other Fe chelators,[136−138] NIH was shown to reduce the generation of the tyrosyl radical within RR[18] and inhibit the activity of this enzyme.[133] The inhibitory effects of NIH on [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine incorporation further confirmed the antiproliferative effects this chelator.[23]

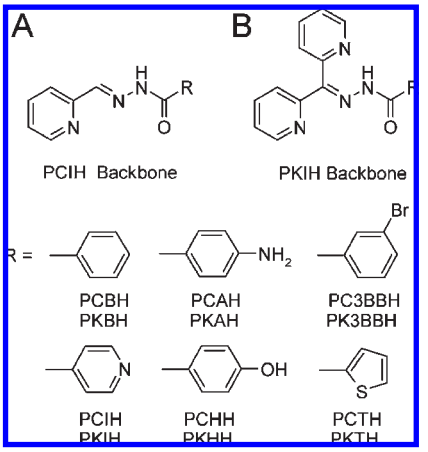


Figure 7. Chemical structures of (A) the PCIH ligands and (B) the PKIH series.

4.3.5. 2-Pyridylcarboxaldehyde Isonicotinoyl Hydrazone (PCIH) Series. In an attempt to synthesize ligands with high antiproliferative activity, novel aroylhydrazone chelators were designed by condensing 2-pyridylcarboxaldehyde with a range of acid hydrazides previously used to synthesize the PIH analogues.[139] The 2-pyridylcarboxaldehyde moiety was chosen because it was previously shown to have potent RR inhibitory and antiproliferative activity.[140,141] This led to the generation of the 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH, Figure 7A) series of ligands.[139,142] These analogues are tridentate with the same hydrazone bridge as the 100, 200, and 300 series and bind Fe through the imine nitrogen, pyridyl nitrogen, and carbonyl oxygen donor atoms.[143]

Unfortunately, in vitro analysis of the PCIH analogues showed low antiproliferative activity, making them suitable as agents for the treatment of Fe overload.[139] A number of members of the PCIH series were able to mobilize intracellular Fe to a greater extent than DFO.[139] They also have far less effect on [^{3}H]thymidine, [^{3}H]leucine, and [^{3}H]uridine incorporation than NIH.[139] Investigations on the redox activity of PCIH Fe complexes showed that they were redox inactive.[133] These results suggested that PCIH and its analogues were more suitable candidates for the treatment of Fe overload disease.

X-ray crystallography studies showed that PCIH undergoes oxidation to isonicotinoyl picolinoyl hydrazine when complexed with Fe^{III}.[143] This oxidation product showed little Fe chelation activity because of its ionized state at physiological pH. These studies illustrate the importance of

charge on the ability of the ligands to cross biological membranes and to access intracellular Fe pools,[143] providing important structure−activity relationship information for the development of new compounds.

4.3.6. Di-2-pyridylketone Isonicotinoyl Hydrazone (PKIH) Series. In order to develop chelators as potent antitumor agents, another series of novel ligands based on PCIH were prepared. In this series, the 2-pyridylcarboxaldehyde moiety was replaced with the more lipophilic di-2-pyridylketone group (Figure 7B). This was done considering our studies showing that lipophilicity was an important criterion of antiproliferative activity in Fe chelators.[55] This generated a new group of chelators known as the di-2-pyridylketone isonicotinoyl hydrazone (PKIH, Figure 7B) series. The PKIH ligands have an additional pyridyl ring, which increased the lipophilicity of these compounds. These ligands use the same donor atoms as PCIH, namely, the imine nitrogen, pyridyl nitrogen, and carbonyl oxygen.[144] Importantly, the PKIH series are predominantly in their neutral form at physiological pH, allowing access across cells membranes.[144]

The in vitro biological activity of the PKIH series in the human SK-N-MC human neuroepithelioma cell line demonstrated that they were more cytotoxic than their corresponding PCIH analogues.[145] In terms of Fe chelation efficacy, nearly all PKIH analogues showed similar or greater activity in mobilizing ^{59}Fe and preventing ^{59}Fe uptake from Tf than the corresponding PCIH series compounds.[145] The chelators PKIH, 2-pyridylketone thiophenecarboxyl hydrazone (PKTH), 2-pyridylketone benzoyl hydrazone (PKBH), and 2-pyridylketone *m*-bromobenzoyl hydrazone (PKBBH) showed high antiproliferative activity, being as potent as NIH ($IC_{50} = 1-3\ \mu M$).[145] Importantly, while PKIH analogues demonstrated marked inhibition of proliferation against human SK-N-MC cells, their activity was less pronounced toward normal MRC-5 human fibroblasts, suggesting that they possess selectivity against tumor cells.[145]

Studies on the chemistry of the PKIH series demonstrated that they formed distorted octahedral complexes with Fe^{II}, with two tridentate ligands arranged in meridional fashion around the metal center.[144] The Fe complexes are oxidized to their Fe^{III} forms at relatively high potential, and this oxidation is coupled to a rapid reaction with water to form a hydrated (carbinolamine) derivative.[144] Further studies on the redox activity and solution chemistry of the PKIH−Fe complexes suggested that they could stimulate benzoate hydroxylation in the presence of Fe^{II} and H_2O_2.[146] The Fe^{II} complexes caused marked DNA degradation in the presence of H_2O_2, confirming that the PKIH−Fe complexes can mediate Fenton chemistry.[144] In addition, PKIH markedly increased intracellular ROS that was inhibited by catalase, an enzyme that can also decrease the antiproliferative effects of these chelators.[144] Hence, it was suggested that the antiproliferative activity of these ligands was mediated by stimulation of Fe-mediated free radical generation via redox cycling of the Fe complexes.[144,146]

Although there was no strong correlation between the log *P* values and the antiproliferative activity of the PKIH series, it was observed that analogues containing electron-donating substituents such as di-2-pyridylketone *p*-aminobenzoyl hydrazone (PKAH) and di-2-pyridylketone *p*-hydroxybenzoyl hydrazone (PKHH) showed low antiproliferative activity ($IC_{50} > 23\ \mu M$).[145] In contrast, analogues bearing electron-withdrawing substituents such as PKIH showed high antiproliferative activity, despite being the most hydrophilic ligand of this series.[145] Therefore, it was proposed that electron-withdrawing substituents can anodically shift the $Fe^{III/II}$ redox potential, while electron-donating substituents result in complexes with lower metal-centered redox couples. Consequently, electron-donating substituents can act to stabilize the trivalent Fe state and reduce their antiproliferative activity.[144] In fact, the PKAH−Fe complex was a poor oxidant of ascorbate, while the PKIH−Fe complex demonstrated the highest ability to catalyze ascorbate oxidation.[144] Hence, the increased anticancer activity of the PKIH series, relative to the PCIH analogues, can be partly attributed to their redox activity.

4.4. Thiohydrazones. The previously discussed aroylhydrazone Fe chelators of the PKIH class use the N,N,O donor set of atoms to bind Fe and demonstrate moderate antiproliferative activity.[145] Their closely related thiosemicarbazones counterparts discussed below, the (di-2-pyridylketone thiosemicarbazone) DpT series, utilize the N,N,S set of donor atoms but display very marked antitumor activity.[13] Thus, in an attempt to elucidate the effect of donor atom identity on anticancer activity, a novel range of intermediate ligands, the thiohydrazones (Figure 8B), were synthesized.[147] The design

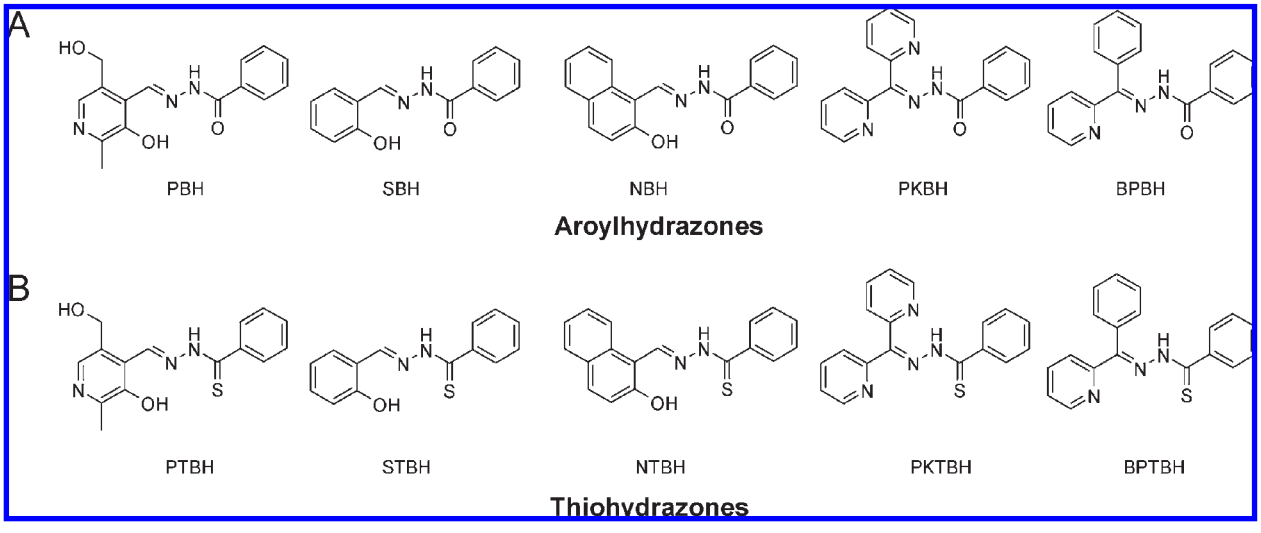


Figure 8. Chemical structures of (A) the aroylhydrazone ligands in comparison to their (B) thiohydrazone counterparts.

of these ligands was based on a number of aroylhydrazone parent compounds (Figure 8A).[147] This novel range of ligands incorporated the hydrazone backbone but replaced the carbonyl oxygen with a sulfur atom.

Interestingly, thiohydrazones utilizing the O,N,S donor atoms, such as pyridoxal thiobenzoyl hydrazone (PTBH), salicylaldehyde thiobenzoyl hydrazone (STBH), and 2-hydroxy-1-naphthaldehyde thiobenzoyl hydrazone (NTBH), demonstrated poorer antiproliferative activity than that of their parent O,N,O aroylhydrazones.[147] Additionally, redox studies demonstrated that the Fe complexes of these O,N,S thiohydrazones showed reduced redox activity.[147]

In contrast to the O,N,S thiohydrazones, the N,N,S thiohydrazones, including di-2-pyridylketone thiobenzoyl hydrazone (PKTBH) and 2-benzoylpyridine thiobenzoyl hydrazone (BPTBH), exhibited more potent and selective antiproliferative activity than those of their N,N,O aroylhydrazone counterparts (Figure 8A).[147] Importantly, these N,N,S thiohydrazone chelators demonstrated anticancer activity that was comparable to the closely related thiosemicarbazones, the DpT and 2-benzoylpyridine thiosemicarbazone (BpT) series.[147]

Intriguingly, the N,N,S thiohydrazone Fe complexes showed completely reversible electrochemistry at potentials accessible to both oxidants and reductants, suggesting that their antiproliferative activity, at least in part, was derived from their ability to produce intracellular ROS.[147] This was supported by the fact that their Fe complexes were able to catalyze both the oxidation of ascorbate and hydroxylation of benzoate.[147]

Collectively, the studies performed with the thiohydrazone ligands confirmed the importance of the N,N,S donor set of atoms for potent antiproliferative and redox activity.[147] These investigations have provided a crucial set of structure−activity relationships necessary for the design of future ligands for cancer treatment.

5. Thiosemicarbazones: Old and New

It has long been established that compounds of the thiosemicarbazones class exhibit strong antiproliferative activity.[4,148−150] Their ability to chelate metal ions depends on the presence of a N,N,S tridentate "soft donor" coordination system (Figure 9). This allows the thiosemicarbazone ligands to bind not only Fe[4,148] but also Cu, Zn, and other transition metals.[150] Although their ability to chelate metal ions is known, the implication it has on the anticancer activity of these compounds was not clear and is discussed below, particularly in relation to their redox activity.

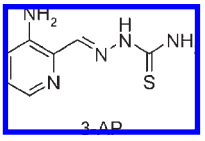


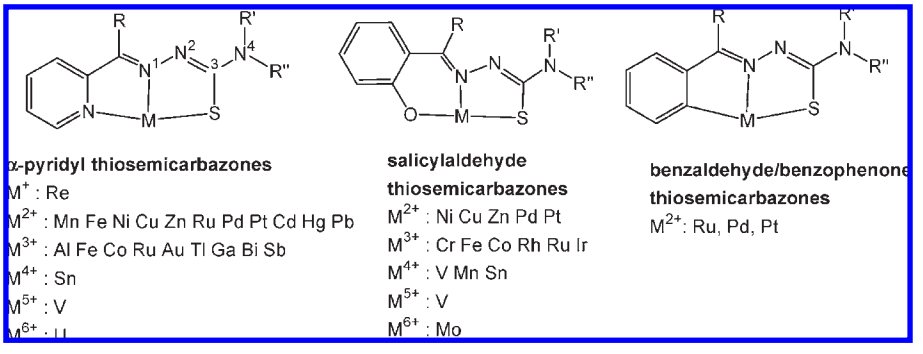
Figure 9. Chemical structure of 3-AP.

5.1. Chemical Properties of Thiosemicarbazones and Their Complexes. Compounds bearing the α-pyridyl thiosemicarbazone backbone (including 2-pyridylcarbaldehyde, 2-pyridylketone, and 2-pyridylformamide thiosemicarbazones) abound in the literature. More than 500 crystallographically characterized examples are reported in the Cambridge Structural Database comprising either free ligands or coordination compounds. The vast range of metal ions complexed by α-pyridyl thiosemicarbazones in an N,N,S configuration is illustrated in Scheme 1. The conventional numbering scheme (from N^1 to N^4) is shown. Other well represented groups based on different parent aldehydes or ketones are the salicylaldehyde thiosemicarbazones and their analogues (∼300 structurally characterized examples) where a phenolate O-donor binds in addition to N^1- and the S-donor. Carbocyclic (organometallic) thiosemicarbazones based on benzaldehyde and relatives are also known, although the range of complexed metal ions is more restrictive.

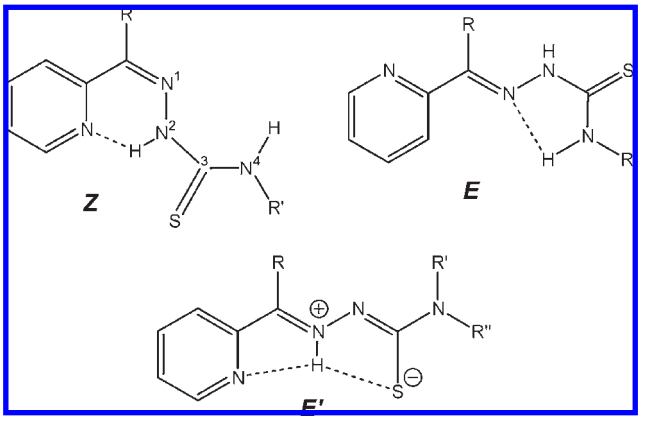
The breadth of thiosemicarbazone chemistry demands a more focused survey, and this section will concentrate on the α-pyridyl thiosemicarbazones specifically with known biological activity. For earlier work, the 1993 review of West et al. on the coordination and biological chemistry of α-heterocyclic thiosemicarbazones (specifically copper complexes) is important to note.[151]

5.1.1. α-Pyridyl Thiosemicarbazone Free Ligands: Structure and Solution Properties. In their metal free form, α-pyridyl thiosemicarbazones have been identified in three isomeric forms, *Z*, *E*, and *E*′, depending on the disposition of substituents about the C=N bond and thioamide (NHC=S) group (Scheme 2). The isomeric distribution in solution is affected by solvent, and two or more forms may be present in solution and discernible by NMR.[15] The chemical shift of the thioamide proton on N^2 is diagnostic of the isomeric form. Intramolecular H-bonding is an important structural determinant, and either N^2 or N^4 is involved in H-bond donation in the *Z* or *E* isomer, respectively (Scheme 2). In the special case where N^4 bears no H-atoms, the ligand converts to the *E*′ tautomeric form (Scheme 2) where the labile proton resides on N^1 (rather than N^2) where it is H-bonded to the pyridyl N-atom and S atom (Scheme 2).

Scheme 1. Structures of the α-Pyridyl Thiosemicarbazone, Salicylaldehyde Thiosemicarbazone, and Benzaldehyde/Benzophenone Thiosemicarbazone Metal Complexes



Scheme 2. Different Isomeric Forms of Pyridine Thiosemicarbazones Showing the *Z*, *E* (anti, anti) and *E′* (syn, syn) Isomeric Forms



A favorable pharmacological property of α-pyridyl thiosemicarbazones is their lipophilicity (commonly quantified by their octanol/water partition coefficient, log *P*). Variations of the substituents R, R′, and R″ enable the lipophilicity to be readily tuned, and this is strongly correlated with biological activity.[13,15,147] In this regard, it is apparent that the solubility properties of the compound will also be affected. The zwitterionic *E′* form should be more hydrophilic than either the *E* or *Z* form because of its polar structure. In fact, we have shown this to be true in a number of cases.[13,15,147] For example, the structurally characterized compounds Ap44mT (*E′* isomer)[152] and Ap4mT (*E* isomer)[153] bear similar substituents, but the latter compound has a partition coefficient that is more than 1 log unit greater than the former.

The pK_a of the N^2H group (see Scheme 2) in α-pyridyl thiosemicarbazones is in the range 9.5−11, while the pyridyl pK_a is around 4; i.e., the compounds are always charge neutral at physiological pH 7.4.[13] The anion itself is not stable in the absence of metal coordination, and thus, there are no structurally characterized examples of pyridine thiosemibarbazone anions unstabilized by metal coordination. The ligands appear more stable in acidic solution, and we have reported[13] the structure of the protonated (pyridinium) derivative Dp4eT·$HClO_4$ compound as its perchorate salt (Figure 10).

The charge neutrality of the ligands at physiological pH (7.4) assists their passage across the cell membrane. However, very high lipophilicity can also be a problem as aqueous solubility and bioavailability become poor. One attempt to improve this has been the addition of solubilizing groups to the well studied 3-AP to give aqueous-soluble prodrugs (Figure 11).[154,155] These alterations led to an improvement in biological efficacy.

5.1.2. α-Pyridyl Thiosemicarbazone Coordination Chemistry. To bind a single metal center in a tridentate fashion, the three possible donor atoms (N_{pyr}, N^1, S) must adopt the *E′* conformation, where all three donor atoms are *syn* with respect to each other (Scheme 2). Deprotonation and coordination bring about a change in the electronic structure where the ensuing negative charge resides mostly on the S-donor atom on the basis of the significant lengthening of the C−S bond and shortening of the adjacent $C-N^2$ bond, which approaches that of a double bond (Scheme 3). Once in this configuration the ligand offers an N,N,S donor set

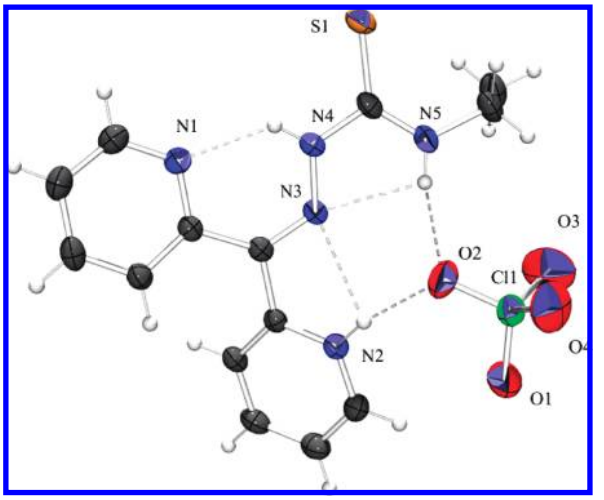


Figure 10. Crystal structure of the hydrogen perchlorate salt Dp4eT·$HClO_4$ showing intramolecular and intermolecular H-bonding. Note that N2 is the site of protonation.

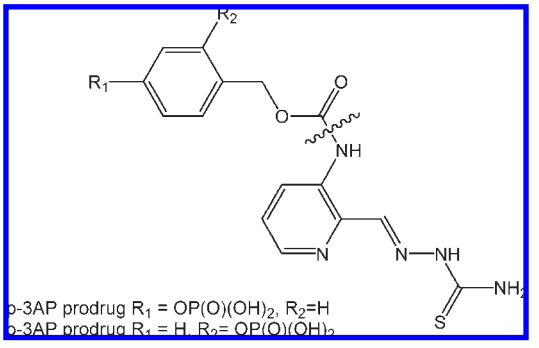


Figure 11. Prodrug derivatives of 3-AP.

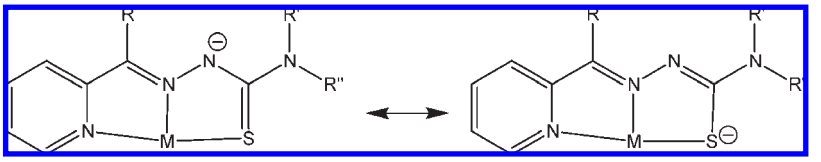
that coordinates in an obligatory meridional fashion because of the rigidity of the ligand. Additionally, the negative charge on the S-donor enhances its binding strength and the ability of the ligand to stabilize metals in higher oxidation states. This is in contrast to hydrazone analogues derived from similar heterocyclic aldehydes and ketones where low oxidation states, i.e., Fe^{II} but not Fe^{III}, are stabilized.[144,156,157]

The fact that the ligands readily lose a proton upon coordination also is a key factor in the biological activity of their complexes. Combination of two ligands with a divalent metal ion preferring 6-coordination (a common example) results in a charge neutral complex (Scheme 4).[73] This charge neutrality means that the complexes themselves may also be able to cross the cell membrane, and this provides a novel method of delivery of the thiosemicarbazone to the cell in the form of a labile divalent complex which may then dissociate within the cell.[73]

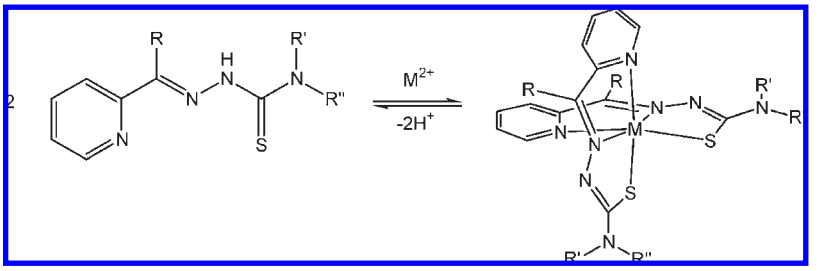
In light of the instability of thiosemicarbazones in strongly acidic or basic solutions, potentiometric determination of complex formation constants are fraught with difficulties. To avoid such complications, spectrophotometric titrations at pH 7.4 have established the formation constants for the divalent first row transition metal ions Mn, Ni, Cu, and Zn.[73] The same approach is not viable for determination of the Fe^{II} stability constants under anaerobic conditions, as the low spin Fe complexes are particularly stable and kinetically inert.

Recently, several Ga^{III} complexes of thiosemicarbazones have been prepared and their anticancer properties

Scheme 3. Resonance Structures of Complexes of Pyridine Thiosemicarbazones in Their Deprotonated Form



Scheme 4. Deprotonation upon Formation of the Pyridine Thiosemicarbazones Metal Complexes



assessed.[158,159] In most cases, the Ga complex is more active than either the free ligand or the corresponding Fe complex. It is possible that the Ga complexes undergo transmetalation in vivo and the effects observed are due to formation of the Fe thiosemicarbazone complex within cells and also delivery of Ga^{III} to intracellular targets. These can interfere with normal metabolism by substitution for Fe and hindering iron-dependent processes, such as ribonucleotide reduction.[158] The facile solution interconversion of [Ga(Ap44mT)$_2$][$GaCl_4$] to give a 1:2 equilibrium mixture of the original complex and [Ga(Ap44mT)Cl_2] suggests that transmetalation in vivo is quite likely.[158]

Several Ru^{III} and Ru^{II} complexes have also been investigated.[152,160] In the case of these inert Ru complexes, ligand exchange seems unlikely. Indeed, in one study it was found that incorporation of a 2-benzoylpyridinethiosemicarbazone ligand into heteroleptic Ru complexes resulted in less activity compared to the precursor complex with more readily displaceable ligands.[161] In fact, the Ru complexes were likely to be acting as DNA-binding agents and their cytotoxicity may be mediated through this mechanism.

Of note, the metal ion can, in some cases, promote decomposition of the coordinated thiosemicarbazone. Apart from hydrolysis of the Schiff base, some novel metal-catalyzed decomposition processes of thiosemicarbazone ligands have been identified. The extent to which these processes may occur in vivo is currently unknown but may be relevant to the fate of these compounds in vivo and warrants further investigation. For example, Cu^{II} complexes of pyridine-2-carbaldehyde thiosemicarbazone undergo base-catalyzed loss of sulfur, ultimately leading to a nitrile, as shown in Scheme 5.[162] Desulfurization was also detected in solutions at physiological pH at 40 °C. It is of interest that the insolubility of CuS may be a key factor in this reaction.

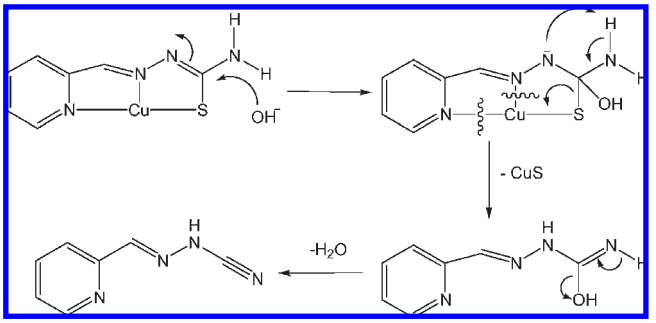
5.1.3. Iron Coordination Chemistry: A Key Aspect of Thiosemicarbazone Biological Activity. Although thiosemicarbazones and their complexes show a broad spectrum of biological activity, the link between their anticancer properties and Fe chelation has only recently emerged. The most intensively investigated thiosemicarbazone is 3-AP, which is currently in phase II trials as a drug against a number of cancers.[78,155,163−166] Little has been reported about the coordination chemistry of 3-AP, but it appears that Fe chelation is strongly linked with its biological activity. Our own studies with the DpT, BpT, and 2-acetylpyridine thiosemicarbazone (ApT) analogues have shown that the structural, spectroscopic, and redox properties of α-pyridyl thiosemicarbazones are very similar. The little data that have been published on the Fe coordination chemistry of 3-AP mirror that of our own work on other related thiosemicarbazones.[13,15,147]

As Fe shows a distinct preference for octahedral coordination geometries in both its di- and trivalent oxidation states, complexes of α-pyridyl thiosemicarbazones are typically of the form $[Fe^{II}L_2]$ or $[Fe^{III}L_2]^+$ where L^- is the monoanionic, N,N,S-coordinated thiosemicarbazone (Figure 12). An extra consideration is the electronic ground state of the metal in each of its oxidation states, i.e., whether it is high or low spin. All α-pyridyl thiosemicarbazones form low-spin Fe^{III} complexes of the type $[Fe^{III}L_2]^+$, as shown by both X-ray crystallography and EPR spectroscopy. The Fe−N_{pyr} bond lengths (∼1.99 Å) are much longer than the central Fe−N_{imine} bonds (to atoms N2a and N2b in Figure 12), which are around 1.91 Å. The Fe−S coordinate bonds (∼2.22 Å) are the longest, as expected from the respective bonding radii of the donor atoms. Most importantly, these bond lengths are much shorter than seen in high spin ferric complexes of related hydrazones.[56,110]

The Fe^{II} α-pyridyl thiosemicarbazone complexes are usually slowly oxidized in aerated solutions to their ferric form, so there are few structurally characterized examples of complexes in the divalent state. One of these, [Fe(NBp4eT)$_2$], is stabilized in its divalent form by the electron-withdrawing nitro groups.[147] The second comprises the Fe^{II} complex of a neutral bis-pyridineformamide N^4-phenyl thiosemicarbazone (where the proton on N^2 remains).[167] The higher positive charge of the complex brings about a similar stabilization of the ferrous oxidation state against oxidation, although in neutral aqueous solution these protons will almost certainly be lost. When compared to analogous low spin Fe^{III} complexes, there is little change in bond lengths, and this is relevant to their electrochemical properties (see below).

The α-pyridyl thiosemicarbazones are potent ligands for Fe. We have shown that ferrioxamine B (or Fe(DFO), the ferric complex of desferrioxamine) reacts with excess acetylpyridine thiosemicarbazones to afford the ferric thiosemicarbazone complex over a period of days.[73] Although this

Scheme 5. Formation of the Nitrile Product after Complexation of Cu^{II}



process is very slow, it does highlight the competitiveness of the α-pyridyl thiosemicarbazones for Fe. The formation constant of Fe(DFO) is very high, and DFO has been used for many years as the chelator of choice for the treatment of Fe overload.[74] Hence, any ligand that can exhibit a similarly high affinity for Fe as well as being able to gain access to intracellular Fe pools will also be an effective Fe chelator. Indeed, an important finding was that the Mn, Ni, Cu, and Zn complexes of the DpT analogues exhibit the same antiproliferative activity as their free ligands, which strongly suggests that the complexes dissociate, probably within the cell.[73] The Fe complexes are less active, although still active in their own right, and evidently survive intact upon entry into the cell. In fact, it appears that Fe is a target of these ligands and that the unique redox chemistry of the Fe complexes is linked to their antiproliferative activity.

5.2. 5-Hydroxypyridine-2-carboxaldehyde Thiosemicarbazone (HPCT) and Older Generation Thiosemicarbazones. Some of the earlier thiosemicarbazones include 5-hydroxypyridine-2-carboxaldehyde thiosemicarbazone (HPCT), pyrazinecarboxaldehyde thiosemicarbazone (PCT), sodium pyrazinecarboxaldehyde dithiocarbazonate (SPD), and pyrazinecarboxaldehyde 2′-pyrazinylhydrazone (PPH). Of these ligands, PCT, SPD, and PPH were found to possess considerable Fe chelation efficacy and reduced liver, spleen, and transferrin Fe levels in Fe-overloaded mice.[149] Moreover, the ability of these chelators to bind and increase Fe excretion was demonstrated in studies using an Fe-overloaded animal model[149] and in clinical trials.[9]

HPCT was the first thiosemicarbazone to be studied in patients for its toxicity and pharmacological disposition.[9] After administration, characteristic dark-green urine was observed, presumably resulting from the excretion of the Fe^{II}−HPCT complex (2−11 mg/24 h). Plasma levels of HPCT decayed in a biphasic mode with an initial half-life of 2.5−10.5 min. Of the administered dose, 47−75% was excreted within 24 h and the major (50−74%) metabolites were glucuronide conjugates.[9] Transient decreases in blast counts were observed in three of five patients with acute leukemia, although no remission or antitumor effects were noted in eight patients with solid tumors.[9] Administration of larger doses was limited by gastrointestinal toxicity. Mild myelosuppressant effects and hemolysis were noted in five patients treated with a 5-day course of the drug. Although toxicity appeared to limit the usefulness of this compound as an antineoplastic agent, antileukemic activity was shown.[9]

5.3. 3-AP and Ribonucleotide Reductase Inhibition. After it was recognized that the antitumor activity of early thiosemicarbazones was at least partly due to an interaction with

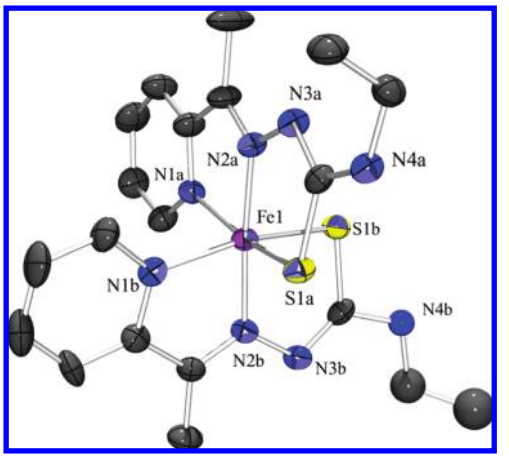


Figure 12. Crystal structure of $[Fe(Ap4eT)_2]ClO_4$ (H-atoms and perchlorate anion omitted for clarity).

RR, a potent RR inhibitor, 3-aminopyridine carboxaldehyde thiosemicarbazone (3-AP) (Figure 9B), was synthesized and later entered clinical trials.[4,148,163,164] 3-AP exhibited more potent inhibition of L1210 leukemia cells both in vitro[163] and in vivo[164] when compared to hydroxyurea (HU), another clinically used RR inhibitor. A further advantage was that 3-AP is active against HU-resistant cells, suggesting a different mechanism of action than HU itself.[163]

While 3-AP was shown to be a potent RR inhibitor, it was not clear if this was due to the compound directly removing Fe from the protein or if the 3-AP−Fe complex inhibited RR activity via a redox mechanism, similar to other thiosemicarbazones.[137] Subsequent studies by our group demonstrated that the 3-AP−Fe complex was redox active, leading to ascorbate oxidation, hydroxylation of benzoate, plasmid DNA degradation, as well as glutathione depletion and DNA degradation in cultured tumor cells.[133]A later study by Shao et al.[168] suggested that the Fe^{II}−3-AP complex was a more effective RR inhibitor than the free ligand, in agreement with our previous studies.[133] Shao and colleagues showed that the cytotoxic effect of 3-AP was the result of both ROS-mediated quenching of the tyrosyl radical in the R2 subunit of RR, rendering the enzyme inactive, as well as the toxic effects of ROS on proteins.[168] Earlier studies also showed that the Fe complex of similar monothiosemicarbazones inhibit RR more effectively than the free ligand.[169] Similar results were reported for the cupric complex of 2-formylpyridine monothiosemicarbazone.[170]

5.4. 3-AP: Clinical Trials. Phase I clinical trials utilizing 3-AP revealed concern over the toxicity of the compound.[165] After a 2 h iv dose of 96 mg/m^2 for 5 days every other week, myelosuppression indicative of RR inhibition was noted.[165] Although this dose was deemed safe, it was recommended for further clinical studies on the condition that it would be given every 4 days to reduce the incidence of grade 4 leukopenia which was noted in 13 of 14 patients.[165] Further, a phase II trial of 3-AP in combination with the anticancer agent, gemcitabine, given to patients with non-small-cell lung cancer was terminated after no objective response was observed in two patients after two treatment cycles.[166] Accompanying this was the development of hypoxia in three patients and methemoglobinemia in four patients.[166] Clearly, neither is desirable in patients already compromised with pulmonary conditions.[166]

The activity of 3-AP has also been assessed in a phase II trial of patients with metastatic renal cell carcinoma

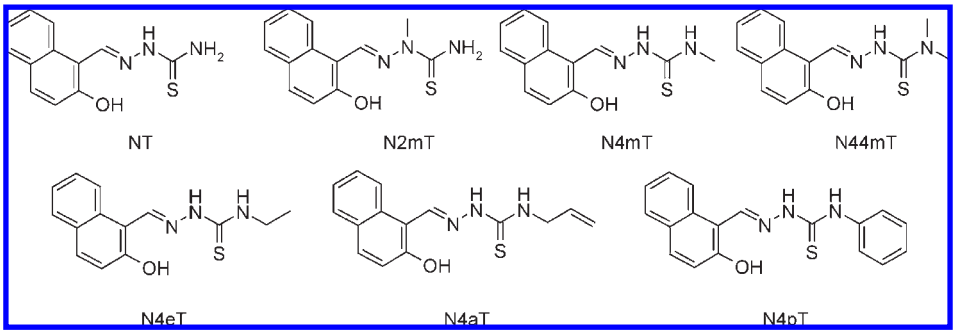


Figure 13. Chemical structures of the hybrid chelator NT series.

(RCC).[78] Nineteen patients were given four courses of 3-AP at the doses recommended by Murren et al. (2003).[165] Again, acute hypoxia and methemoglobinemia were noted, as well as grade 3/4 neutropenia in 79% of patients.[78] This resulted in only 47% of patients receiving more than 90% of the initially planned dose, as the treatment dosage was reduced to minimize adverse effects.[78] The study was terminated before completion, with only one patient exhibiting a partial response.[78]

A number of other clinical trials with 3-AP have also been recently reported. These include studies examining pancreatic carcinoma, non-small-cell lung cancer (NSCLC), leukemia, and myeloproliferative disorders. A phase II clinical trial with 3-AP in both chemotherapy-naive and gemcitabine-refractory (GR) patients with advanced pancreatic cancer was conducted.[171] Fifteen patients were enrolled, including one chemotherapy-naive individual and 14 that were GR. The chemotherapy-naive patient progressed during cycle 1 with grades 3 and 4 toxicities. Of 14 GR patients, seven received two cycles, six received one cycle, and one received eight cycles. Progression precluded further treatment in 11 GR patients. Additionally, one patient died in cycle 1, and this death was considered to be related to treatment and two stopped treatment because of toxicity. Five GR patients had grade 4 toxicities possibly related to 3-AP, and six GR patients had grade 3 fatigue.[171] Toxicities and lack of meaningful clinical benefit prompted early closure of the study. The 4-month survival in GR patients was 21%, and it was concluded that this regimen appeared inactive against predominantly GR pancreatic cancer.

Another phase II study of 3-AP in combination with gemcitabine was conducted in advanced pancreatic carcinoma patients who had not received prior chemotherapy.[79] These patients were treated with 3-AP at 105 mg/m^2 given over 2 h.[79] Four hours after the 3-AP infusion, gemcitabine, 1000 mg/m^2, was given over 30 min. Both drugs were given on days 1, 8, and 15 of a 28-day cycle. At this dose and schedule, no objective responses were observed. The combination resulted in moderate toxicities in these patients.[79]

A phase II trial with gemcitabine in advanced non-small-cell lung cancer (NSCLC) was also evaluated.[166] Twelve patients were treated with a median of two treatment cycles without objective response. Hence, the study was terminated at interim analysis. Four patients had stable disease, and the median time to progression was 3 months. Grade 3 toxicities included neutropenia (two patients), hypoxia (three patients), and dyspnea (one patient).[166] Four patients developed reversible symptomatic methemoglobinemia. 3-AP did not enhance clinical response to gemcitabine in this cohort of patients with prior exposure to gemcitabine for advanced NSCLC. The further development of 3-AP in lung cancer is challenged by its potential of causing methemoglobinemia and hypoxia, which could be problematic in patients with compromised pulmonary reserves.

On the other hand, combination of 3-AP with the nucleoside analogue, fludarabine, in patients with refractory acute leukemias and aggressive myeloproliferative disorders showed that 3-AP at 105 mg/m^2 followed by fludarabine at 30 mg/m^2 administered daily for 5 days is active, warranting further studies.[172] A phase I study in combination with high dose cytarabine in patients with advanced myeloid leukemia also concluded that the combination is feasible in advanced myeloid leukemia, although methemoglobinemia is a common toxicity that requires close monitoring.[173]

5.5. Hybrid Chelators. 5.5.1. 2-Hydroxy-1-naphthylaldehyde-3-thiosemicarbazone (NT). The failure of 3-AP and other thiosemicarbazones in the clinics dictates the need for the development of more effective and selective thiosemicarbazones. The PCIH series of chelators as mentioned above (section 4.3.5), were designed on the basis of the strong antiproliferative activity observed with 2-pyridylcarboxaldehye-derived thiosemicarbazones.[139] However, subsequent analysis showed that PCIH chelators possess low antiproliferative activity.[139] This led to the observation that the antitumor activity may be mostly conferred by the thiosemicarbazide moiety instead of 2-pyridylcarboxaldehyde.[139] Hence, a new class of chelators known as 2-hydroxy-1-naphthylaldehyde-3-thiosemicarbazone (NT) series (Figure 13) were prepared by condensing the 2-hydroxy-1-naphthylaldehyde moiety, associated with high antiproliferative activity in the 300 series (section 4.3.4)[55,127] and a number of thiosemicarbazides.[174] These studies were also performed in order to gain further insight into the effect of lipophilicity on the antiproliferative activity of these chelators. As such, the NT series chelators were synthesized with increasingly lipophilic thiosemicarbazides.[174]

The investigation assessing the antiproliferative activity of the NT series demonstrated that the chelators N4mT and N44mT (Figure 13) had significant antiproliferative activity against SK-N-MC neuroepithelioma cells ($IC_{50} = 0.5-1.5\,\mu M$). These chelators were clearly more cytotoxic than DFO ($IC_{50} = 22\,\mu M$) and comparable in activity to NIH ($IC_{50} = 0.3\,\mu M$). In addition, the NT series demonstrated greater antiproliferative activity against tumor cells such as neuroepithelioma cells ($IC_{50} = 0.5\,\mu M$) than normal fibroblasts ($IC_{50} > 25\,\mu M$), demonstrating some selectivity.[174]

In order to determine the role of Fe chelation in the antitumor efficacy of the NT series, the ability of the ligands to promote ^{59}Fe mobilization from cells and to prevent uptake of ^{59}Fe from Tf was assessed.[174] The results showed that some

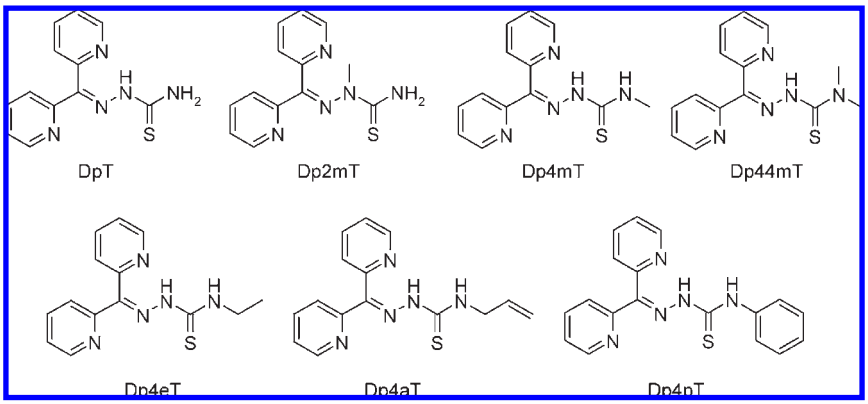


Figure 14. Chemical structures of the DpT series.

of the chelators, namely, NT and N2mT, displayed low Fe chelation efficacy whereas the rest of the remaining analogues, especially N44mT, had high Fe chelation efficacy that was similar to that of NIH.[174] The other two analogues with antiproliferative activity, NT and N4mT, did not show marked Fe chelation efficacy. Other NT members with lower antiproliferative activity such as N4pT had higher Fe chelation efficacy than NT and N4mT, suggesting that depletion of cellular Fe pools was not crucial for their antiproliferative activity.[174]

Analysis of the relationship between lipophilicity and antiproliferative activity of the NT series suggested an interesting trend. This showed a loss of antiproliferative activity as the substituents on the terminal nitrogen atom of the thiosemicarbazide became increasingly lipophilic.[174] This was an important structure−activity relationship that was considered in future studies.

5.5.2. Di-2-pyridyl Thiosemicarbazones. Building on the promise of the aroylhydrazones and early thiosemicarbazones classes of chelators as well as the success of the PKIH series of compounds,[145,174] seven novel chelators were synthesized and termed the DpT series (Figure 14).[12] The chelators Dp44mT, Dp4eT, Dp4aT, and Dp4pT all showed very high antiproliferative activity in vitro against SK-N-MC neuroepithelioma cells.[12] Dp44mT was the most active, with an IC_{50} value of 0.03 μM, being more effective than DFO, NIH, and 3-AP, with IC_{50} values of 5.0, 0.3, 0.26 μM, respectively. This made Dp44mT the most effective antiproliferative chelator prepared in the Richardson laboratory to that date.[12] Similar results were obtained when these compounds were tested against SK-Mel-28 melanoma cells and MCF-7 breast cancer cells, with Dp44mT again proving to be the most active.[12]

The possibility of selective antitumor activity of these compounds in vitro was shown by the finding that all compounds were largely ineffective at inhibiting the proliferation of MRC-5 fibroblasts, a normal cell line.[12] Assessment of the antiproliferative activity of the most promising DpT analogue, Dp44mT, continued in vivo through administration to mice bearing the aggressive murine M109 lung carcinoma.[12] Tumor weights were reduced by 47% compared to controls in mice treated with the maximum tolerated dose (MTD) of Dp44mT (0.4 mg/kg) after only 5 days, indicating marked antitumor efficacy.[12] In the same series of studies, 3-AP at its MTD (6 mg/kg) was more effective than Dp44mT (0.4 mg/kg), with tumor weights being reduced to 10% compared to the controls.[12] However, this dose of 3-AP appeared toxic to mice, as it caused a significant decrease in animal weight, hemoglobin level, hematocrit and also erythrocyte and leukocyte cell count.[12] This was not observed in animals treated with Dp44mT.[12]

Further studies investigating the antitumor activity of the DpT series have been conducted by Whitnall et al.[175] These compounds were tested against 28 cell lines in vitro with Dp44mT having the most pronounced and broadest antitumor effects.[175] The average IC_{50} value of Dp44mT (0.03 ± 0.01 μM) was significantly lower than those of 3-AP (1.41 ± 0.37 μM) and DOX (0.62 ± 0.35 μM) across all cell lines.[175] With the development of resistance of tumors to chemotherapeutic drugs being an important issue, it was of interest to note that Dp44mT retained its antiproliferative efficacy against etoposide-resistant cells while being more effective against vinblastine-resistant cells than -sensitive cells.[175]

Whitnall et al.[175] also used a variety of human tumor xenografts in mice to assess the antitumor potential of Dp44mT. It was found that Dp44mT at 0.4 (mg/kg)/d was well tolerated and that by use of nude mice with SK-Mel-28 melanoma xenografts, tumor growth over 7 weeks was reduced to only 8% of that found in animals treated with vehicle only.[175] At this dose there were no significant differences in animal weight and hematological indices between the treated and control groups.[175] However, it is notable that higher nonoptimal doses of Dp44mT (0.75 mg/kg) administered over 2 weeks led to cardiac fibrosis in nude mice.[175]

In an attempt to elucidate the mechanism of action of the DpT series of compounds, studies examined various molecular markers obtained from both in vitro and in vivo studies.[12,175] It was obvious that the ability of the DpT ligands to bind metal ions was essential to their mechanism of action.[12] Dp44mT, the analogue with the greatest antiproliferative effect, facilitated the release of 47% of ^{59}Fe from SK-N-MC cells and also limited ^{59}Fe uptake to 9% of the control, confirming similar efficacy to NIH.[12] The importance of Fe binding in the activity of these compounds was reinforced through studies showing that Dp2mT (Figure 14), a structurally similar analogue that cannot bind Fe, also exhibited poor antiproliferative activity.[12]

The inhibition of cellular proliferation by the DpT chelators was shown to be induced by not only the ability to bind Fe but also their ability to cause oxidative stress, a mechanism of action described as the "double-punch effect".[13,72] Yuan et al.[12] first suggested the possibility of ROS mediating, in part, the cytotoxic effects of the DpT ligands after observing that the Dp44mT−Fe complex was redox-active. This concept was supported by the finding of pronounced ascorbate oxidation and benzoate hydroxylation by

Dp44mT.[13] Indeed, the lower redox potentials of the DpT series in comparison to the PKIH chelators is the most likely reason they exhibit greater antiproliferative activity.[13] Furthermore, the redox reactions of the DpT−Fe complexes were reversible, unlike the PKIH series, a feature attributable to the addition of the thioamide moiety.[13] In light of this, it was clear that this structure−activity relationship may be central to the design of future chelators, with redox activity being an important criterion for optimal efficacy.[13]

Additional studies by other investigators at the U.S. FDA and National Cancer Institute (U.S.) confirmed the pronounced and selective antitumor efficacy of Dp44mT.[176] These in vitro studies examined the cytotoxicity of Dp44mT in breast cancer cells, both as a single agent and in combination with DOX. Dp44mT alone induced selective cell killing in the breast cancer cell line MDA-MB-231 when compared with healthy mammary epithelial cells (MCF-12A).[176] It induced G_1 cell cycle arrest and reduced cancer cell clonogenic growth at nanomolar concentrations. Interestingly, Dp44mT, but not DFO, induced DNA double-strand breaks in MDA-MB-231 cells. The chelator also caused selective poisoning of DNA topoisomerase IIα (top2α).[176] Studies using heterozygous Nalm-6 top2α knockout cells and top2α and top2β small interfering RNA knockdown in HeLa cells showed that Dp44mT is cytotoxic to breast cancer cells, at least in part, because of selective inhibition of top2α.[176]

By use of acute leukemia cells, the effect of Dp44mT on apoptosis, cell cycle, caspase-3 activation and mitochondrial transmembrane potential has also been examined by flow cytometry.[53] Dp44mT acted to induce a G_1/S arrest in NB4 promyelocytic leukemia cells at low concentrations (0.5−2.5 μM).[53] Moreover, Dp44mT induced apoptosis of NB4 cells in a dose- and time-dependent manner with markedly less effect on nonproliferating peripheral blood leukocytes.[53] Furthermore, this study also showed that Dp44mT had broad activity, inducing apoptosis through reduction in the mitochondrial transmembrane potential and caspase-3 activation in several types of acute leukemia and multiple myeloma cell lines.

Considering all these studies collectively, it is clear that the DpT group of ligands possesses potential as effective cytotoxic agents for the treatment of a range of cancers.[53] The compounds are protected by a suite of national phase patents, and toxicological evaluation is now ongoing in the authors' laboratory. Clearly, patent protection is a vital aspect for commercialization and provides the basis for further development and clinical trials of the agents.

5.5.3. 2-Benzoylpyridine Thiosemicarbazones. A legitimate concern from the investigations with Dp44mT was that these compounds showed that high nonoptimal doses of the chelator led to cardiac fibrosis in nude mice.[175] Considering this, the next step in the development of chelators as antineoplastic agents involved the structural modification of DpT analogues to create the BpT class of chelators (Figure 15).[147] This structural modification involved the addition of a phenyl ring in place of the non-coordinating 2-pyridyl group.[147] The rationale for this alteration was that it would increase the lipophilicity and that the removal of the electron-withdrawing pyridyl ring could lead to lowered $Fe^{III/II}$ potentials and elevated redox cycling of the Fe complex.[14]

Examination of this series through in vitro assays revealed the BpT analogues to have greater antiproliferative activity than the corresponding DpT analogues.[147] Treatment of SK-N-MC neuroepithelioma cells identified Bp4eT as having the

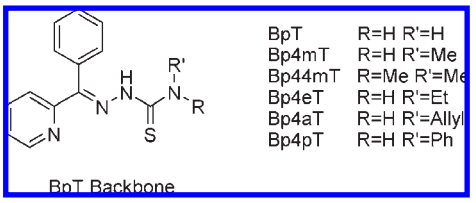


Figure 15. Chemical structures of the BpT series.

greatest antiproliferative activity within this series, surpassing Dp44mT as the most effective chelator to date (IC_{50} = 0.002 μM).[147] These compounds were also selective for cancer cells over normal cells, as IC_{50} values in normal MRC-5 fibroblasts were 1500−3000 times higher than that observed in SK-N-MC cells.[147]

The investigators also examined the redox activity of the BpT chelators in vitro, as this was considered a probable explanation for their enhanced antiproliferative activity.[147] The results from ascorbate oxidation and hydroxylation assays highlighted the enhanced redox activity of the BpT series of chelators.[147] The BpT−Fe complexes, especially [Fe(Bp4eT)$_2$], were significantly more effective at oxidizing ascorbate compared to the DpT Fe complexes, which was consistent with the finding of increased benzoate hydroxylation with these complexes.[147] It was thought that the presence of the unsubstituted phenyl moiety in the BpT series could be the major factor contributing to the lowered redox potentials of the BpT−Fe complexes.[147] This results in the complexes being more redox active and so exerting a greater antiproliferative effect.[147]

Considering the potential of the BpT and DpT series of ligands as promising drugs, it is useful to assess their chemical properties relative to the "Lipinski rule of five" that predicts the qualitative concept of "druglikeness."[177] It primarily predicts permeability, solubility, and the absorption of drugs from the gut. This general "rule of thumb" indicates that the compounds should have no more than 5 hydrogen bond donors (nitrogen or oxygen atoms with one or more hydrogen atoms), not more than 10 hydrogen bond acceptors (nitrogen or oxygen atoms), a molecular weight under 500 Da, and an octanol−water partition coefficient (log P) of less than 5. Analysis of the BpT and DpT series of compounds demonstrates that they comply to this rule (Table 1). Considering these data in conjunction with the marked activity of the Dp44mT against a range of human tumor xenografts in vivo,[12,175] these drugs appear to be good candidates for drug development.

5.6. 2-Acetylpyridine Thiosemicarbazones. As mentioned above, the DpT and BpT series of Fe chelators illustrate potent antiproliferative activity through redox cycling.[13,14] In an attempt to examine the effect of substituents at the imine carbon on antitumor and redox activity, the ApT (Figure 16) series of ligands were developed.[15] Through this strategy, the electron-withdrawing pyridine ring of the DpT series was replaced by an inductively donating methyl group of the ApT chelators. These compounds were prepared by a Schiff-base condensation of 2-acetylpyridine with the various thiosemicarbazides used to generate the DpT and BpT series.[15]

Four of the six ApT chelators demonstrated potent antiproliferative activity in the human SK-N-MC neuroepithelioma cell line (IC_{50} = 0.001−0.002 μM) and demonstrated Fe chelation efficacy similar to that of the most effective BpT and DpT ligands.[15] The parent analogue,

Table 1. DpT and BpT Ligands Satisfy the "Lipinski Rule of Five", Indicating Their Potential Utility as Bioavailable, Membrane-Permeable Drugs

DpT Chelators	Structure	Not more than 5 hydrogen bond donors	Not more than 10 hydrogen bond acceptors	A molecular weight under 500 daltons	Octanol-water partition coefficient[a] (logP) less than 5
DpT	R', R'' = H	√ (3)	√ (4)	√ (257.32)	√ (0.78)
Dp4mT	R' = Me, R'' = H	√ (2)	√ (4)	√ (271.35)	√ (3.18)
Dp44mT	R', R'' = Me	√ (1)	√ (4)	√ (285.37)	√ (2.19)
Dp4eT	R' = Et, R'' = H	√ (2)	√ (4)	√ (285.37)	√ (1.23)
Dp4aT	R' = Allyl, R'' = H	√ (2)	√ (4)	√ (297.38)	√ (1.68)
Dp4pT	R' = Ph, R'' = H	√ (2)	√ (4)	√ (333.42)	√ (1.96)
Dp44eT	R', R'' = Et	√ (1)	√ (4)	√ (313.43)	√ (2.91, calc.[§])

BpT Chelators	Structure	Not more than 5 hydrogen bond donors	Not more than 10 hydrogen bond acceptors	A molecular weight under 500 daltons	^Calculated Octanol-water partition coefficient (logP) less than 5
BpT	R', R'' = H	√ (3)	√ (3)	√ (256.33)	√ (2.25)
Bp4mT	R' = Me, R'' = H	√ (2)	√ (3)	√ (270.36)	√ (2.77)
Bp44mT	R', R'' = Me	√ (1)	√ (3)	√ (284.38)	√ (3.14)
Bp4eT	R' = Et, R'' = H	√ (2)	√ (3)	√ (284.38)	√ (3.11)
Bp4aT	R' = Allyl, R'' = H	√ (2)	√ (3)	√ (296.39)	√ (3.60)
Bp4pT	R' = Ph, R'' = H	√ (2)	√ (3)	√ (332.43)	√ (4.43)

[a] Octanol−water partition coefficients from ref 13. [b] Octanol−water partition coefficient from ref 14. [§] Calculated using ChemDraw Ultra 11.0 using Crippen's fragmentation.[186]

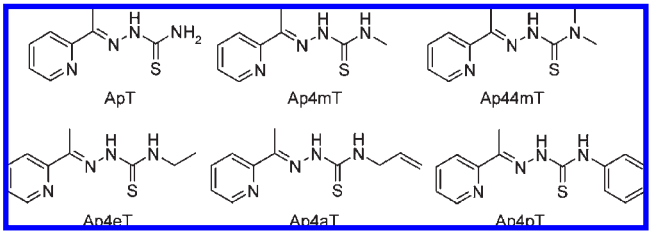


Figure 16. Chemical structures of the ApT series.

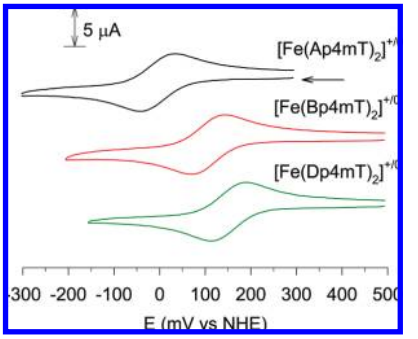


Figure 17. Cyclic voltammograms of $[Fe(Ap4mT)_2]^+$ (black), $[Fe(Bp4mT)_2]^+$ (red), and $[Fe(Dp4mT)_2]^+$ (green). Experimental conditions were 1 mM concentrations of complex in $MeCN/H_2O$ (70:30) and 0.1 M Bu_4NClO_4 using a sweep rate 100 mV s^{-1}. All sweeps were initiated in the direction of the arrow. Studies could not be performed in aqueous solution alone because of the insolubility of the Fe complexes at the concentration required.

ApT, was the least effective of the series, having an IC_{50} of 3.53 μM. This could be related to the fact that this analogue represents the most hydrophilic compound of the series. The ApT−Fe complexes had the lowest $Fe^{III/II}$ redox potentials of any thiosemicarbazone series generated by our group (Figure 17) and maintained the ability to oxidize ascorbate, having activity comparable to the DpT series but less than the BpT analogues.[15] The cathodic shift in the $Fe^{III/II}$ redox potentials of the ApT−Fe complexes was attributed to the inductively donating imine methyl group present close to the metal center, and the potentials lie within a range accessible to both cellular oxidants and reductants. These results suggested that redox cycling and the generation of ROS may play a role in the antiproliferative activity of the ApT series.[15] More importantly, the high Fe chelation efficacy of the ApT series to mobilize

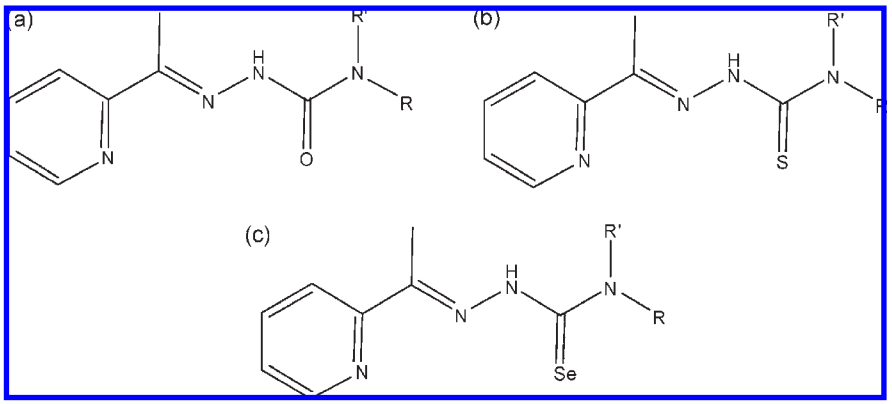


Figure 18. Chemical structures of chalcogen semicarbazone iron chelators: (a) 2-acetylpyridine semicarbazone; (b) 2-acetylpyridine thiosemicarbazone; (c) 2-acetylpyridine selenosemicarbazone.

intracellular ^{59}Fe and inhibit ^{59}Fe uptake from Tf may play a crucial part in their anticancer effects.[15] This study demonstrated the significance of substituents positioned at the imine carbon and the dramatic effects of this substitution on antiproliferative activity and $Fe^{III/II}$ redox potentials.

The biological activity of the general class of 2-acetylpyridine thiosemicarbazones has been known for many years. Indeed, earlier reports suggested this group of compounds may bind heavy metals[178] and have activity against viruses,[179] parasites,[180] and bacteria.[181] Hence, this group of compounds could have multiple medicinal applications and deserves further investigation.

5.7. Thiosemicarbazone Derivatives: Selenosemicarbazone Iron Chelators. Selenosemicarbazones represent a class of semicarbazones in which the chalcogen donor atom is selenium (Figure 18). These compounds represent an evolution from the thiosemicarbazone class of Fe chelators which have been demonstrated to possess a wide spectrum of pharmacological activity, including antibacterial, antimalarial, antifungal, antiviral, and antitumor activity.[10] The antimalarial properties of 2-acetylpyridine thiosemicarbazone were first reported in 1979 against *Plasmodium berghei* in mice.[182]

Following this latter discovery, extensive investigations were undertaken by the same group to ascertain the molecular features essential for activity.[182] Among these structural moieties, the nature of the chalcogen donor atom (O, S, Se) was found to be crucial for antimalarial activity.[183] Replacement of the thiocarbonyl group with a carbonyl group produced a compound devoid of antimalarial activity, indicating the importance of the sulfur atom for activity. This observation was further examined with the substitution of the sulfur atom with selenium, an electronically similar element.

Biological screening of the most active antimalarial thiosemicarbazones in comparison to their selenium analogues revealed that the selenium compounds had comparable or slightly lower activity than their corresponding thiosemicarbazones.[183] However, they showed markedly lower toxicity. The most potent selenosemicarbazones produced cures at the 20 mg/kg dosage level in mice against *Plasmodium berghei*. On average, the selenium analogues were found to exhibit toxicities at twice the dosage levels of the parent thiosemicarbazone.[183] This finding is pertinent given the observed toxicities of some of the most potent thiosemicarbazones, namely, the *N*,*N*′-dialkyl thiosemicarbazones, which were found to be toxic at the lowest dose administered (40 mg/kg) against *Plasmodium berghei* in mice.[182]

The discovery of the antitumor activity of chalcogen semicarbazones in the past few decades has led to extensive structure−activity studies aimed at examining the molecular features required for optimal antineoplastic activity. Evaluation of the impact of the identity of the chalcogen donor atom on the cytotoxicity of 2-acetylpyridine *N*,*N*′-dimethyl-(chalcogen)semicarbazones in 41M and SK-BR-3 cancer cell lines revealed very pronounced effects.[152] An atomic size dependent increase in cytotoxicity was observed for the oxygen, sulfur, and selenium chalcogen atoms evaluated. The selenosemicarbazones were found to be about 13- to 22-fold more potent than their thiosemicarbazone analogues.[152] In contrast, the semicarbazones were between 3 and 5 orders of magnitude less potent than their corresponding thiosemicarbazone analogues. Complexation of the above chalcogen semicarbazones with gallium led to a further enhancement in cytotoxicity following a similar trend to the ligand alone.[152] Hence, the antiproliferative activity of gallium complexed semicarbazones appears primarily determined by that of the corresponding chalcogen semicarbazone ligand.[184]

The observed antineoplastic activity of chalcogen semicarbazones is believed to occur via two mechanisms. The pronounced Fe chelating properties of these compounds enables either the sequestering of Fe from the R2 subunit of RR or prior formation of the complex inhibiting the enzyme. The second is through Fe-dependent free radical damage of RR and other cellular targets. Traditionally, free radical damage is believed to occur through redox cycling of Fe between the ferrous and ferric state leading to the generation of ROS. Recently, ligand-centered electron transfer has been proposed as an additional mechanism for the observed cytotoxicity of this class of compounds. This is thought to occur via electron transfer at the C=N Schiff base double bond, leading to the formation of reactive species capable of attacking cellular targets.[184]

The influence of the chalcogen donor atom (O, S, Se) on ligand-centered redox potentials was investigated with a series of Ga and Fe complexed 2-acetylpyridine 4,4′-dimethyl-(chalcogen)semicarbazones.[184] The semicarbazones displayed the lowest redox potentials at −1.03 and −1.34 V vs NHE for the first two ligand-centered redox waves, whereas the thio- and selenosemicarbazones displayed similar redox potentials at −0.93 to −0.94 V and −1.22 V vs NHE for the corresponding redox waves.[184] Metal-free thiosemicarbazone ligands exhibited an irreversible reduction response at −1.01 to −1.47 V vs NHE.[184] The identity of the complexed metal ion was also found to strongly influence the ligand-centered redox

potentials. Gallium complexes were found to be reduced at ~0.3−0.8 V more positive redox potentials than the uncoordinated ligands. The Fe^{II} complexes instead displayed ~0.0−0.4 V more negative redox potentials than the uncoordinated ligand.[184] These results suggest the possibility that the observed cytotoxicity of Ga complexed chalcogen semicarbazones may, in part, be due to ligand-centered as well as metal-centered redox activity. The cytotoxicity of $Fe^{III/II}$ complexed chalcogen semicarbazones appears less likely to involve ligand-centered reduction, due to the relative biological inaccessibility of the redox potentials of these complexes.

Structure−activity studies undertaken in our laboratory point to the identity of the chalcogen donor atom as crucial for both the iron chelation efficacy and antiproliferative activity of iron chelators. Substitution of the coordinating carbonyl moiety with a thiocarbonyl moiety in PKIH, forming DpT, resulted in compounds with potent antiproliferative activity.[14,185] In contrast to the PKIH series, the electrochemistry of the Fe complexes of the DpT series exhibited facile redox cycling between Fe^{II} and Fe^{III} at biologically relevant conditions.[14] This suggested that the enhanced antiproliferative activity of the DpT series was due to both their ability to bind intracellular Fe and redox cycling, inducing oxidative cellular damage.[175] The establishment of the coordinating carbonyl moiety being vital to the antiproliferative activity of Fe chelators suggested the evaluation of softer donor atoms as potential targets in Fe chelator design. Softer donor atoms are expected to enhance redox cycling ability at biological conditions, leading to improved antiproliferative activity through the generation of ROS.

6. Summary

Iron chelators have potential therapeutic use for the treatment of cancer. Studies with a range of chelators indicate that Fe chelation alone is not enough to generate compounds with pronounced antitumor efficacy. Structure−activity relationships demonstrated that chelators containing hard electron donors such as oxygen typically lead to ligands that bind Fe^{III} with high affinity that do not have pronounced antitumor efficacy. Such compounds are more suitable for the treatment of iron-overload disease, e.g., β-thalassemia major.

In contrast, ligands with soft donors such as sulfur and nitrogen lead to compounds that can redox cycle and induce a "double punch", namely, marked chelation and redox activity. Such compounds include the thiosemicarbazone chelators such as 3-AP and the ApT, BpT, and DpT series.

Detailed investigations of the thiosemicarbazone group of ligands have demonstrated that they are highly effective chelators that, besides RR, also target a range of other molecules including NDRG1 and top2α, all of which contribute to their anticancer effects.

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