

## **Redox cycling metals: Pedaling their roles in metabolism and their use in the development of novel therapeutics**

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**Abbreviations:** AC, adenylyl cyclase; AI, anemia of inflammation; cdks, cyclin-dependent kinases; bFGF, basic fibroblast growth factor; BpT, 2-benzoylpyridine thiosemicarbazone; Bp4eT, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone; Bp44mT, 2-benzoylpyridine 4,4-dimethyl-3-thiosemicarbazone; Cp, ceruloplasmin; CREB, cAMP response element-binding protein; Ctr1, copper transporter 1; Ctr2, copper transporter 2; Cu(II), cupric copper; Cu(I), cuprous copper; DCYTB, duodenal cytochrome *b*; DFO, desferrioxamine; DMT1, divalent metal transporter 1; DOX, doxorubicin; DpT, di-2-pyridylketone thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; EGFR, epidermal growth factor receptor; eIF3a, eukaryotic initiation factor 3a; EMT, epithelial to mesenchymal transition; FACS, fluorescent activated cell sorting; FBXL5, F-box and Leucine-rich Repeat Protein 5; Fe(II), ferrous iron; Fe(III), ferric iron; FPN1, ferroportin 1; G6PD, glucose-6-phosphate dehydrogenase; GTSM, glyoxal-bis(N4-methyl-3-thiosemicarbazone); HCP1, heme-carrier protein 1; HIF-1 $\alpha$ , hypoxia inducible factor-1 $\alpha$ ; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HO-1, heme oxygenase-1; HRE, hypoxia responsive element; IL, interleukin; IRE, iron responsive element; IRP, iron regulatory protein; ISC, iron-sulfur cluster; KTS, kethoxal-bis(thiosemicarbazone); LIP, labile iron pool; LRP1, lipoprotein receptor-related protein 1; NB, neuroblastoma; NDRG1, N-myc downstream regulated gene 1; NF- $\kappa$ B, nuclear factor- $\kappa$ B; O<sub>2</sub><sup>-</sup>, superoxide; 2-OG, 2-oxoglutarate; <sup>•</sup>OH, hydroxyl radical; PIH, pyridoxal isonicotinoyl hydrazone; PKA, cAMP-dependent protein kinase; PCBPs, poly (rC)-binding proteins; PCFT, proton-coupled

folate transporter; Pgp, p-glycoprotein; PI3K, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; pMLC2, phosphorylated myosin light chain 2;  $\alpha_2$ -M,  $\alpha_2$ -macroglobulin; MTD, maximum tolerated dose; NCOA4, nuclear receptor coactivator 4; ROCK1, Rho-associated, coiled-coil containing protein kinase 1; ROS, reactive oxygen species; RGS, regulators of G-protein signaling; RR, ribonucleotide reductase; SOD, superoxide dismutase; STEAP2, six transmembrane epithelial antigen of the prostate 2; Tf, transferrin; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; TGF- $\beta$ , transforming growth factor  $\beta$ ; TGN, trans-Golgi network; TIBC, total iron-binding capacity; TM, tetrathiomolybdate; TNF- $\alpha$ , tumor necrosis factor alpha; Triapine<sup>®</sup>, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Trx1, thioredoxin 1; Trx2, thioredoxin 2; UTR, untranslated region; VEGF, vascular endothelial growth factor; ZIP14, ZRT/IRT-like protein 14.

## **Abstract**

Essential metals, such as iron and copper, play a critical role in a plethora of cellular processes including cell growth and proliferation. However, concomitantly, excess of these metal ions in the body can have deleterious effects due to their ability to generate cytotoxic reactive oxygen species (ROS). Thus, the human body has evolved a very well-orchestrated metabolic system that keeps tight control on the levels of these metal ions. Considering their very high proliferation rate, cancer cells require a high abundance of these metal compared to their normal counterparts. Interestingly, new anti-cancer agents have been developed that take advantage of the sensitivity of cancer cells to metal sequestration and their susceptibility to ROS. These ligands can avidly bind metals ions to form redox active metal complexes, which lead to generation of cytotoxic ROS. Furthermore, these agents also act as potent metastasis suppressors due to their ability to up-regulate the metastasis suppressor gene, *N-myc downstream regulated gene 1*. This review discusses the importance of iron and copper in the metabolism and progression of cancer, how they can be exploited to target tumors and the clinical translation of novel anti-cancer chemotherapeutics.

## **Keywords:**

Iron; copper; cancer; thiosemicarbazones; Dp44mT; DpC; bis(thiosemicarbazones).

## 1. Introduction

The essential transition metals, iron and copper, play an integral role in many important biological functions [1-3]. By acting as a co-factor within active sites, these metals are an essential component required for the activity of many proteins and enzymes that are vital for normal growth and development [2, 4]. For instance, both iron and copper are involved in oxidative phosphorylation, making them essential elements for energy generation [2, 3]. Additionally, iron is also required for the rate-limiting step of DNA synthesis that is catalyzed by ribonucleotide reductase (RR) [5-7]. Copper plays an important role in the detoxification of reactive oxygen species (ROS) *via* superoxide dismutase (SOD) and in the cross-linking of elastin and collagen [3, 8]. Moreover, copper is also a co-factor essential for the formation of new blood vessels, a process termed angiogenesis [9].

The utility of both iron and copper as co-factors originates from their ability to redox cycle [2, 3]. However, this same property that allows for the gain or loss of electrons also results in their cytotoxicity through the donation of electrons to oxygen, leading the generation of ROS, such as superoxide ( $O_2^{\cdot-}$ ) and hydroxyl radicals ( $\cdot OH$ ) [2, 10, 11]. Notably, ROS react rapidly with biological molecules, such as proteins, lipids and DNA [11, 12]. As a consequence, mammalian cells have evolved homeostatic mechanisms to tightly regulate the concentration of redox active metals, allowing their bioavailability for use, but at the same time preventing ROS generation [13-15].

Notably, the dyshomeostasis of iron and copper and the altered expression of proteins involved in their metabolism are often observed in malignancies [16, 17]. Evidence from epidemiological, clinical and experimental studies also suggests a link between altered iron and copper levels and the development of neoplasia [18-20]. This dysregulation of metal homeostasis reveals the potential vulnerability of cancer cells to therapeutic metal chelation.

In fact, the targeting of transition metals, such as iron and copper, has become an attractive strategy in the development of anti-cancer agents due to the higher requirement of neoplastic cells for these essential metals [2, 21].

In this review, developments in the physiological metabolism of iron and copper will be discussed and their roles in the progression of cancer and metastasis will be examined. Finally, the burgeoning field of targeting transition metals, such as iron and copper, as a novel anti-cancer strategy will be considered.

## **2. Physiological Iron Metabolism**

### **2.1 Systemic Iron**

Iron is vital for mammalian cell survival, with cellular iron-depletion causing cell death by apoptosis [22, 23]. Adult humans contain 3-5 grams of iron, more than four fifths of which is found in hemoglobin within erythrocytes, while a further one fifth is stored within mononuclear phagocytes, also known as reticuloendothelial macrophages (*e.g.*, splenic macrophages and hepatic Küpffer cells) and hepatocytes [24, 25]. Cellular iron storage typically occurs within the intracellular iron storage protein, ferritin [26]. The majority of the remaining iron is found within other heme-containing proteins (*e.g.*, cytochromes), iron-sulfur cluster (ISC)-containing proteins (*e.g.*, succinate dehydrogenase of the mitochondrial respiratory chain) [13, 27, 28] and non-heme/non-ISC iron-containing proteins (*e.g.*, iron- and 2-oxoglutarate-dependent dioxygenases) [29, 30]. Critically, ~20 mg of iron is required daily for the synthesis of new hemoglobin, with at least 90% of this iron being recycled on a daily basis from the turnover of old and damaged erythrocytes by splenic macrophages. Approximately 1-2 mg of iron is absorbed in the proximal duodenum to replace iron losses from bleeding, epithelial desquamation, sweating and urinary excretion [25].

## **2.2 Iron Absorption**

Although there is no regulated pathway for systemic iron excretion, dietary iron absorption is very tightly regulated [31, 32]. Iron absorption occurs mainly in the duodenum (and upper jejunum), and this is increased by iron deficiency and decreased by systemic iron loading [25, 33]. Iron is absorbed across the apical membrane of duodenal epithelial enterocytes of the mid and upper villus (Figure 1). These cells can absorb dietary iron, which is present in two major forms: non-heme iron, which abundant in most foods and is the major form of iron in cereals and vegetables; and heme iron, which is predominantly derived from hemoproteins (*e.g.*, hemoglobin and myoglobin) in meat (for comprehensive reviews, see Sharp [34] and Gulec *et al.* [32]; Figure 1).

### **2.2.1 Absorption of Heme Iron**

Notably, the absorption of heme is more efficient than non-heme iron, but the mechanisms remain poorly understood. Heme uptake is known to involve receptor-mediated endocytosis, but the mediators of this process are not known [35, 36]. Heme-carrier protein 1 (HCP1) was identified as a possible low affinity heme transporter, but subsequent studies showed it to act as a high affinity folate transporter (proton-coupled folate transporter (PCFT); Figure 1) [35, 37, 38]. However, there is no convincing evidence that it plays a physiologically important role in heme transport [38]. Once inside enterocytes, heme is broken down into biliverdin and Fe(II) by heme oxygenase (HO), likely HO-1 (Figure 1) [39]. The regulation of intestinal heme iron uptake has recently been reviewed [32].

### **2.2.2 Absorption of Non-Heme Iron**

The uptake of dietary non-heme iron, which is typically in the form of low- $M_r$  chelates of Fe(III), occurs in two phases. First, iron is taken up across the apical membrane of duodenal enterocytes, which requires soluble iron in its reduced form [40]. The reduction of non-heme

iron is thought to be mediated by an apical membrane ferrireductase. A candidate for such a reductase is duodenal cytochrome *b*<sub>561</sub> (DCYTB/CYBRD1/CYB561A2; Figure 1) [41], although other reductases may play a more significant role. DCYTB is a di-heme transmembrane oxidoreductase that utilizes intracellular ascorbate as an electron donor to reduce extracellular Fe(III) [42]. An NAD(P)H-dependent transmembrane oxidoreductase, six transmembrane epithelial antigen of the prostate 2 (STEAP2) is also expressed in the apical membrane of duodenal enterocytes and may be involved in reduction of non-heme iron prior to uptake [43]. Importantly, non-heme iron can also be reduced by endogenous and secreted reductants [40], such as ascorbate [44-46].

Ferrous iron is then imported across the enterocyte's apical membrane *via* the ferrous iron transporter, divalent metal transporter 1 (DMT1/SLC11A2; Figure 1) [47]. Other divalent metal transporters, such as the putative zinc transporter, ZRT/IRT-like protein 14 (ZIP14/SLC39A14), may also be involved in iron transport [48].

### **2.3 Systemic Iron Transport**

Iron that has been imported by enterocytes can be exported to the circulation across the basolateral membrane by ferroportin 1 (FPN1/SLC40A1; Figure 1) [49, 50]. The trans-plasma membrane copper-dependent ferroxidase and ceruloplasmin homologue, hephaestin, associates with FPN1 in the basolateral membrane and, in combination with copper-dependent plasma ceruloplasmin, catalyzes the oxidation of exported Fe(II) to Fe(III) (Figure 1) [51, 52]. During this process, two atoms of Fe(III) are complexed to the major plasma iron transport protein, transferrin (Tf), for transport and uptake by distal tissues (Figure 2). Importantly, Tf-bound iron is virtually the sole source of iron for the erythroid compartment leading to the daily production of 200 billion erythrocytes [24].



## 2.4 Cellular Iron Uptake

Under normal physiological conditions, most non-dietary iron is internalized by the uptake of Tf-bound iron (Figure 2). The Tf-bound iron is internalized by receptor-mediated endocytosis following its binding to Tf receptor 1 (TfR1; Figure 2) [53, 54]. Ferric iron is released from Tf within the endosome after its acidification and is then reduced by an endosomal ferrireductase, STEAP3 (Figure 2) [43, 54], or by a novel mechanism involving cellular ascorbate-dependent endosomal reduction [55-57]. Similarly to non-heme iron uptake by duodenal enterocytes, this ferrireduction is followed by transport of Fe(II) across the endosomal membrane by DMT1 (Figure 2) [58] or ZIP14 [59]. This newly acquired cytosolic iron then becomes part of a poorly-characterized “labile iron pool” (LIP) and can be utilized for metabolism, stored within the iron-storage protein, ferritin, or released back to the extracellular space (Figure 2) [53, 54]. Although TfR1-mediated endocytosis is the major pathway of iron uptake, other processes may be involved, these include: **(1)** a second Tf receptor (TfR2) [60] and **(2)** a quantitatively important second mechanism of iron uptake from Tf that is distinct from that mediated *via* TfR1 and which occurs by a process consistent with fluid phase endocytosis [61-64].

Although the uptake of Tf-Fe prevails under normal conditions, in diseases of iron overload (*e.g.*, hereditary hemochromatosis and  $\beta$ -thalassemia), Tf becomes saturated with iron [53, 65]. Under these conditions, iron that is in excess of Tf-iron-binding capacity occurs as low- $M_r$  iron chelates (*e.g.*, ferric citrate) [24, 66]. These low- $M_r$  complexes of iron and citrate are rapidly and markedly internalized by hepatocytes [67] and other cell-types [68] and are thought to play a major role in the pathogenesis of iron overload disease [53]. The uptake of this form of iron requires an initial ferrireduction event, which may involve one or more cell surface ferrireductases (*e.g.*, DCYTB [42] or STEAPs 2-4 [69]). More recently, it has been shown that the release of cellular ascorbate may contribute to the reduction of at least 50% of

non-Tf-bound iron prior to uptake [45, 46, 55]. As above, the Fe(II) that is formed is imported by divalent metal transporters, such as DMT1 [58], ZIP14 or ZIP8 [59].

## **2.5 Intracellular Iron Trafficking**

Upon release from the endosome, iron is thought to enter the LIP (Figure 2) [70, 71]. The LIP is a poorly-characterized compartment believed originally to be comprised of iron complexed with low molecular-weight ligands, including citrate, ATP, other inorganic ions, proteins and sugars [70]. A recent study has suggested that Fe(II)-glutathione is a major component of this pool [72]. However, this proposal is not supported by studies using the GSH synthesis inhibitor, buthionine sulfoximine, which demonstrated no alteration in the ability of chelators to bind intracellular labile iron pools and induce efflux from cells [73]. Moreover, studies assessing the presence of a labile iron pool in reticulocytes demonstrated that there was no significant low- $M_r$  pool and suggested that the iron was transported directly from protein to protein [74, 75], in analogy with intracellular copper trafficking [76, 77].

Direct organelle-organelle interactions may also be involved in iron transport, where Tf-loaded endosomes transfer iron to the mitochondria through a “kiss-and-run” mechanism [75]. However, the precise molecular mechanisms involved remain unclear. More recently, the human poly (rC)-binding proteins (PCBPs) 1-4 have been proposed to act as chaperones to facilitate the delivery of iron to ferritin for storage [78, 79]. In addition, PCBPs 1 and 2 appear to mediate iron acquisition by several members of a class of iron- and 2-oxoglutarate (2-OG)-dependent dioxygenases, which form a large family of non-heme iron enzymes that depend on the insertion of a single iron atom into their active site [29].

From the enigmatic LIP, iron is distributed to specific compartments within the cell. For instance, within erythroid precursors iron is directly targeted to mitochondria for heme

synthesis in a manner that appears to bypass the LIP [74, 80, 81]. In non-erythroid cells, iron derived from Tf-dependent iron uptake is either: *(i)* utilized by downstream metabolic pathways (*e.g.*, imported into mitochondria for usage in ISC and heme synthesis, and/or incorporated in cytoplasmic iron-containing proteins); *(ii)* released from the cell by FPN1; and/or *(iii)* stored in ferritin (Figure 2).

## 2.6 Intracellular Iron Storage: Ferritin

In nucleated cells, most nascent imported iron is incorporated into ferritin (Figure 2) [26]. Ferritin is a multimeric protein composed of 24 subunits that forms a hollow sphere capable of storing ~4,500 iron atoms as a mineralized ferric, phosphate and hydroxide core [26, 82, 83]. In mammals, cytosolic ferritin is composed of two different subunits: H-ferritin (heavy subunit, encoded by *FTH1*) and L-ferritin (light subunit, encoded by *FTL*), which form hetero-polymers of differing subunit compositions with tissue specific distributions [26, 82, 83]. As Fe(II) enters a ferritin “nano-cage” it is oxidized to Fe(III) by the ferroxidase activity of H-ferritin in an oxygen-dependent manner [26, 82, 83]. Subsequently, iron(III) is then transported to the protein cavity, where core formation commences at the carboxyl or glutamate groups of L-ferritin, which are devoid of ferroxidase activity [26, 82, 83]. This enclosure and sequestration of iron as ferrihydrite prevents the occurrence of cytotoxic redox reactions [26, 82, 83].

Intracellular ferritin in the cytoplasm can be used as a source of bioavailable iron following their targeted autolysosomal proteolysis [84]. Lysosomal proteolysis is the primary degradation pathway for cytosolic ferritin and cytosolic ferritin degradation is necessary for iron exit [85], although proteasomal degradation may occur under specific conditions [12, 26]. The targeting of ferritin for autophagic turnover (*i.e.*, ferritinophagy) involves nuclear receptor coactivator 4 (NCOA4), which binds to ATG8 proteins during the formation of

autophagosomes and recruits ferritin as a cargo molecule [86]. Considering that autophagocytosed macromolecules and organelles, such as ferritin and mitochondria, contain high iron levels, active lysosomal compartments are rich in iron [87].

Apart from cytosolic ferritin, a unique mitochondrial ferritin of the H-type has also been characterized that is found exclusively within the mitochondrion [88, 89]. It is encoded by an intronless gene on chromosome 5q23.1, which, once transcribed, leads to a subunit with a  $M_r$  of 22,000 Da that forms ferritin shells that may sequester potentially toxic iron [89]. Mitochondrial ferritin is highly expressed in the testis, but there are only low levels in iron storage organs. The role of mitochondrial ferritin remains unclear, although its expression markedly increases in sideroblastic anemia where heme synthesis is disturbed and mitochondrial iron-loading occurs [90]. Considering this, over-expression of mitochondrial ferritin in cellular models leads to mitochondrial iron-loading and a cytosolic iron-deficiency that increases transferrin receptor 1 expression [91], as well as the inhibition of tumor xenograft growth *in vivo* [92]. In a mouse model of Friedreich's ataxia, where frataxin is conditionally deleted in the heart, mitochondrial ferritin expression is decreased [93]. This may prevent the appropriate sequestration of mitochondrial iron and play a role in the mitochondrial iron overload, which has characteristics of an inorganic iron crystallite [94]. Mitochondrial ferritin remains probably the most enigmatic protein of the ferritin family and deserves further investigation in terms of its roles in normal physiology and disease states.

## **2.7 Iron Homeostasis**

### **2.7.1 Post-Transcriptional Regulation by Iron Regulatory Proteins**

As the human body is not able to actively excrete iron, a balance between iron uptake, utilization and storage must be maintained to ensure adequate amounts of iron for various cellular processes while preventing excess iron accumulation, which can be deleterious to

cells [12]. Iron homeostasis is achieved through tightly regulated transcriptional and post-transcriptional mechanisms, which are controlled by intracellular iron concentrations [14, 95].

The post-transcriptional control of cellular iron metabolism acts to modulate the rates of translation of key iron metabolism proteins [14, 23]. The iron regulatory protein (IRP)-iron responsive element (IRE) system is predominantly responsible for this post-transcriptional regulation, and allows for rapid alterations in translation of key iron metabolism proteins in response to intracellular iron levels [14, 23, 96]. This system relies on two key mRNA-binding proteins, IRPs 1 and 2, which directly regulate the translation or stability of mRNAs possessing IREs [14, 23, 96]. Specifically, in iron depleted cells, there is increased binding of IRPs to IREs in the 5'- or 3' untranslated regions (UTRs) of key mRNAs, which acts to either suppress translation of the mRNA (*i.e.*, mRNAs in which the IRE is located in the 5' UTR; *e.g.*, *FTH1*, *FTL*, *FPN1*, *etc.*), or enhance stability of the mRNA against nuclease attack (*i.e.*, mRNAs in which the IRE is located in the 3'-UTR; *e.g.*, *TfR1*, *DMT1-I*, *etc.*) [14, 54].

However, under conditions of cellular iron loading, there is a decrease in the level of IRP-IRE binding activity [14, 23]. In the case of IRP1 or IRP2 under conditions of high cellular iron, the mechanisms involved are categorically different. In the case of IRP1, this protein loses its IRE-binding activity by acquiring an ISC (4Fe-4S cluster) [96]. The acquisition of this 4Fe-4S cluster converts IRP1 into a cytosolic aconitase that cannot bind IREs. In the case of IRP2, iron-dependent, proteasomal degradation is the major regulatory mechanism [97]. This occurs by the ubiquitination and targeting of IRP2 by a subunit of the SKP1-Cullin-1-F-box E3 ubiquitin ligase complex, namely F-box and Leucine-rich Repeat Protein 5 (FBXL5). FBXL5 is itself post-translationally regulated by iron and oxygen [98, 99], such that high

cellular iron stabilizes levels of the protein, allowing it to down-regulate IRP2 [98, 99]. Consequently, high iron levels promote loss of IRP2 due to proteasomal turnover [98, 99].

### **2.7.2 Hepcidin and Systemic Iron Homeostasis**

Over the past 15 years, a great deal of iron metabolism research has focused on hepcidin, the “hormone of iron metabolism”, which appears to be a keystone regulator of systemic iron homeostasis [100]. This 25-amino acid peptide is predominantly expressed in the liver with its levels being regulated by iron, inflammation, hypoxia and erythroid activity [100]. Hepcidin is a small (~2.8 kDa) disulfide-rich peptide that is synthesized and secreted by hepatocytes [101, 102], and at lower levels by the kidney and heart [24]. Once secreted into the plasma, the major known biological activity of this peptide is to post-translationally decrease the expression of FPN1 [100]. Hepcidin-dependent down-regulation of FPN1 is most significant in enterocytes, mononuclear phagocytes, and hepatocytes [24]. Hepcidin decreases Fpn1 levels *via* lysosomal degradation [24], which reduces the amount of iron released by the affected cells. Thus, hepcidin acts to decrease circulating iron levels that are bound to Tf. The regulation of hepcidin production and synthesis is complex, and is outside the scope of this review. As such, we refer readers to the following recent review on the topic [31].

The dysregulation of hepcidin expression is etiologically involved in many diseases of iron metabolism. Pathologically low hepcidin levels induces iron overload (*e.g.*, as occurs in hereditary hemochromatosis types 1-3 [24]), while pathologically high hepcidin typically induces iron-limited anemias [24]. Importantly, the anemia of inflammation (AI) is the second most common anemia after iron-deficiency anemia [103]. Indeed, AI is the most common form of anemia in hospitalized patients, and is most common in individuals with a chronically activated immune system. This can result from conditions such as chronic

infections, inflammatory diseases, kidney disease, solid organ transplantation rejection, and malignancies [103, 104].

In AI, the major pathophysiological event is the anemia resulting from iron-limited erythropoiesis that leads to decreased red blood cell production. The iron limitation is primarily due to an increase in circulating levels of the inflammatory cytokine, interleukin-6, that up-regulates expression of hepcidin, which is a type II acute-phase reactant [105, 106]. An increase in circulating hepcidin down-regulates FPN1 resulting in decreased iron absorption and recycling [103, 104]. The retention of iron within the major iron-recycling cells greatly diminishes the body's effective iron supply for erythropoiesis and is a major cause of the restricted iron availability to erythroid precursors [104]. Indeed, it has been demonstrated that the pharmacological blockade of endogenous hepcidin production releases "trapped" iron from splenic macrophages, thereby stimulating erythropoiesis and decreasing the levels of anemia [107].

Interestingly, hepcidin-lowering therapeutics have the potential to play an important role in the treatment of AI [107]. While current strategies aim to target and down-regulate the transcription of the hepcidin gene, other strategies that target hepcidin transport and renal clearance have been proposed.

The standard view of hepcidin transport is that it traverses the circulation as a free peptide. However, emerging evidence indicates that most hepcidin is in fact bound to plasma carrier proteins such as  $\alpha_2$ -macroglobulin ( $\alpha_2$ -M) and albumin [108, 109], and possibly other proteins such as  $\alpha_1$ -antitrypsin [110]. Indeed,  $\alpha_2$ -M was identified as the major specific hepcidin-binding partner in human plasma, accounting for up to 30% of protein-bound hepcidin [109]. This study demonstrated that  $\alpha_2$ -M in its native form possesses two high

affinity binding sites ( $K_d$ :  $177 \pm 27$  nM). Intriguingly, upon the well-described activation of  $\alpha_2$ -M, there is exposure of further high affinity binding sites that bind hepcidin allosterically ( $K_d$ :  $\sim 300$  nM) [109]. Notably, although there is quantitatively more hepcidin bound to albumin, the binding of this peptide to albumin was of a far lower affinity ( $K_d$ :  $\sim 1$  mM) and non-saturable [109]. Moreover, the complexation of hepcidin with  $\alpha_2$ -M led to a significantly greater decrease in FPN1 levels in J744 cells than hepcidin alone or hepcidin in combination with albumin [109]. In fact, the combination of albumin and hepcidin did not enhance FPN1 down-regulation more than with hepcidin alone [109].

A recent *in vivo* study showed that hepcidin bound by either  $\alpha_2$ -M or  $\alpha_2$ -M-MA had a greater ability to lower serum iron than an equimolar concentration of hepcidin alone [108]. Interestingly, the ability of hepcidin bound to  $\alpha_2$ -M or  $\alpha_2$ -M-MA to down-regulate FPN1 levels was independent of the  $\alpha_2$ -M receptor, lipoprotein receptor-related protein 1 (LRP1) [108]. This suggests that endocytic uptake of the  $\alpha_2$ -M-hepcidin complex is not required for hepcidin to mediate its effect, which is a distinct mode of action to other  $\alpha_2$ -M peptide cargoes [111].

Importantly, the complexation of hepcidin with  $\alpha_2$ -M led to a significant decrease in the appearance of hepcidin in the urine [108]. As hepcidin is a small low- $M_r$  peptide ( $\sim 2.8$  kDa) that would be expected to be readily cleared by the kidney, the binding of this small peptide to a large protein such as  $\alpha_2$ -M ( $\sim 725$  kDa) would be expected to decrease the peptide's renal clearance, as was observed [108]. Taken together, these results suggest the binding of hepcidin to  $\alpha_2$ -M increased its efficacy in down regulating FPN1, and that this activity may have occurred due to decreased renal clearance of this low  $M_r$  peptide (*i.e.*, an increase in its pharmacokinetic half-life). Such results suggest that targeting the binding of hepcidin by  $\alpha_2$ -M, (*e.g.*, by using a competitive inhibitor) may prove to be an effective "hepcidin lowering"



therapeutic strategy in hepcidin-dependent pathologic hypoferremia (*e.g.*, AI). That is, decreasing the binding of hepcidin to  $\alpha_2$ -M would facilitate renal excretion of this low- $M_r$  peptide.

### **3. Physiological Copper Metabolism**

Copper is an essential micronutrient that is tightly regulated *in vivo* to avoid deleterious effects, including ROS generation [112]. Copper metabolism is controlled by a well-orchestrated system, involving absorption, transport and excretion that will be discussed in detail below.

#### **3.1 Copper Absorption**

Copper is largely absorbed in the small intestine, principally at the duodenum, through a complicated process dependent on various factors and dietary components [113, 114]. There is limited knowledge of the exact mechanisms by which copper crosses the cells of the intestinal mucosa to enter the interstitial fluid [115]. However, studies have indicated that the uptake of copper across the brush border can vary depending on the concentration of copper. At low copper concentrations the uptake was shown to be mediated by a non-energy-dependent saturable carrier, while at higher concentrations it was shown to occur by diffusion [115]. Recent studies have implicated the copper transporter 1 (Ctr1, Figure 3) as being the primary protein responsible for the import of dietary copper across the brush border microvilli [116, 117]. Moreover, some studies have also indicated the role of DMT1 and copper transporter 2 (Ctr2) in copper uptake [118-120].

Ctr1 is a member of a larger family of high affinity copper transporters, including Ctr2, which has been shown to be involved in intracellular copper mobilization [121]. Notably, Ctr1 is an integral membrane protein with three 3 transmembrane domains forming a

homotrimeric pore that is highly specific for copper in the cuprous (Cu(I)) state [122]. Dietary copper exists as Cu(II), and therefore, must be reduced from the oxidized form to Cu(I). The reduction of copper is potentially mediated through metalloreductases from the Steap family (*e.g.*, Steap 3 and Steap 4; Figure 3) [123], or possibly by dietary reductants [117]. The presumed Fe(III) reductase enzyme, duodenal cytochrome *b* (Dcytb), is another potential metalloreductase, which is also able to reduce Cu(II) [124]. The imported Cu(I) is then transported into the secretory compartment for loading onto Cu-dependent enzymes, or out from the basolateral membrane by the Cu(I)-transporting P-type ATPase, ATP7A (Figure 3) [125].

### **3.2 Copper Trafficking**

Once absorbed from the diet, copper is transported into the portal circulation where it is delivered to the liver, a central organ for copper homeostasis, bound primarily to serum albumin, and to a lesser extent, transcuprein [126]. Copper is imported into cells by Ctr1 and rapidly binds to ubiquitously expressed intracellular copper chaperones (Figure 4) [8]. These chaperones transport and deliver copper directly to their specific intracellular sites or enzymes [127]. In the liver, copper is incorporated into the secreted protein, ceruloplasmin (Cp). Cp is the major copper carrying protein in human plasma, containing approximately 70% of total plasma copper [123]. Interestingly, Cp is well known to possess ferroxidase activity, which catalyzes the oxidation of Fe(II) and stimulates iron release from cells [128], thereby facilitating its loading onto Tf for distribution [129]. Notably, mice lacking Cp have no apparent defect in copper absorption or distribution [130]. Therefore, other undefined copper-binding proteins or ligands must participate in peripheral copper distribution.

Intracellular transport of copper is performed by either copper chaperones, such as Atox 1, Cox 17 and CCS, or bound to glutathione as a Cu(I)-glutathione complex (Figure 4) [121].

These copper chaperones shuttle copper to specific compartments inside the cell [126]. For example, Atox1 docks with either the copper-transporting ATPase, ATP7B, in the liver, or ATP7A in other cells [131]. ATP7B is primarily involved in liver copper homeostasis and directs copper to ceruloplasmin or for biliary excretion (Figure 4). On the other hand, ATP7A directs copper for the biosynthesis of various secreted metalloenzymes within the trans-Golgi network (Figure 4) [116]. It is also involved in the basolateral efflux of copper in the intestine and other cells [132].

The copper chaperone, CCS, directs copper to Cu/Zn-SOD that protects cells against ROS-induced damage (Figure 4) [133]. Cox17 delivers copper to the mitochondria for cytochrome *c* oxidase *via* the chaperones Cox11, Sco1, and Sco2 [134]. In contrast, the Cu(I)-glutathione complex serves as a vehicle for delivering copper to a family of proteins involved in intracellular metal detoxification, known as the metallothioneins [121]. Some studies have also suggested the presence of a labile intracellular copper pool, predominantly localized in the mitochondria and the Golgi apparatus (Figure 4) [135]. Such a pool can also be speculated to exist, at least transiently, in the lysosome or autophagolysosome [136] (Figure 4) after the breakdown of copper containing proteins during the process of autophagy. This may be the source of “free” copper that is bound by lysosomotropic chelators that enter the lysosome [137, 138].

### **3.3 Copper Storage**

The mechanism by which eukaryotic cells store copper and mobilize this metal during times of deficiency is still elusive. Intriguingly, copper levels in the mitochondrial matrix are greater than what is thought to be necessary for the activation of copper-dependent enzymes, suggesting that the mitochondrion may act as a copper storage organelle [139]. Recent studies with yeast have demonstrated that the vacuole, analogous to the lysosome, serves in

both copper storage and mobilization [140]. Interestingly, Ctr2 was found to be localized to vacuoles and is suggested to mobilize vacuolar copper to the cytosol [140]. However, the mechanisms involved in the storage and mobilization of copper from these vacuoles remains elusive. A Ctr2-like protein has been identified in humans, but its function is currently not well understood [141].

### **3.4 Copper Homeostasis**

Copper homeostasis is maintained by complex processes involving copper absorption and excretion [125]. Bile is the major pathway for copper excretion (~80%) and it is vital in the homeostasis of liver copper levels [8]. The majority of fecal copper results from biliary excretion, with the rest derived from unabsorbed copper and copper in desquamated mucosal cells [125].

The main gene products related to copper homeostasis are the two homologous cation transporting P-type ATPases, ATP7A and ATP7B [142]. Patients without functional ATP7A develop symptoms comparable to copper deficiency [143]. This is due to the role of ATP7A in the transport of copper into the trans-Golgi network (TGN) for the synthesis of metallo-enzymes and for the efflux of copper in the intestine [144]. Although ATP7B has a similar structure to ATP7A, it is primarily involved in liver copper homeostasis [144] and is also important in the brain and kidneys [145]. Mutations in ATP7B result in retention of copper by the liver and results in brain copper toxicity, leading to Wilson's disease [142]. In addition, ATP7B is also essential in the TGN for the donation of copper to ceruloplasmin [144]. In the presence of excess copper, ATP7B translocates to a vesicular compartment to allow for biliary copper efflux [142]. Secretion of copper and copper-dependent proteins requires the copper chaperone, Atox1, which transfers Cu(I) to ATP7A or ATP7B [131]. This occurs *via* a series of exchanges, leading to the movement of copper across the basolateral

membrane of intestinal cells (ATP7A) or into the bile (ATP7B) [142]. However, despite the roles of ATP7A and ATP7B in copper homeostasis, little evidence suggests that their mRNA or protein levels are dependent on copper levels [133].

#### **4. Essential Metals and Cancer**

Hanahan and Weinberg proposed that all cancers share essential characteristics that collectively dictate malignant growth [146]. These essential hallmarks include: **(1)** self-sufficiency in growth signals; **(2)** insensitivity to inhibitory signals; **(3)** evasion of apoptosis; **(4)** unlimited proliferation; **(5)** sustained angiogenesis; and **(6)** tissue invasion and metastasis (Figure 5) [146]. Given the central importance played by iron and copper in vital cellular processes, such as oxidative phosphorylation, DNA synthesis, detoxification of ROS and angiogenesis, it is not surprising that iron and copper homeostasis is altered in neoplastic cells, contributing to key cancer hallmarks [16, 17, 21]. In addition, the ability of iron and copper to engage in electron exchange reactions (redox reactions), the very attribute that is responsible for their biological utility, also enables them to participate in deleterious free radical generating reactions [147]. The following sections will discuss how metal trafficking and homeostasis is perturbed in cancers, and the potential deleterious consequences of poor regulation of their inherent redox activity.

##### **4.1 Redox Activity of Essential Metals**

As already alluded to, iron and copper are critical for growth and proliferation due to their essential roles in multiple cellular processes [3, 95, 148]. Organisms are dependent on these transition metals due to their ability to act as cofactors within the active sites of various key enzymes, including RR, cytochrome *c* oxidase, SOD and many others [2, 3, 6, 7].

In the case of iron, its catalytic utility lies in its ability to cycle between the ferrous (Fe(II)) and ferric (Fe(III)) states, allowing it to act as an electron donor or acceptor [2]. This feature results in facile redox reactions that are responsible for its biological reactivity [11]. Similarly, copper also exists in two oxidation states, namely the cupric (Cu(II)) and cuprous (Cu(I)) states. However, it is found primarily in the cupric (Cu(II)) state as it is readily oxidized in the presence of oxygen [147].

The Fenton reaction describes the generation of the hydroxyl radical ( $\cdot\text{OH}$ ) from the reaction of ferrous iron with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) [149] and highlights the potential toxicity of free redox active Fe(II). Similarly, cuprous copper is also thought to react in an analogous manner with  $\text{H}_2\text{O}_2$  to that of ferrous iron to generate the hydroxyl radical ( $\cdot\text{OH}$ ) [150]. The products of these reactions can induce cell death by initiating reactions with biomolecules to result in damage to mitochondria, lipid membranes and DNA [2, 151]. For example, oxidative damage to DNA can include DNA base modification or DNA strand breaks [152]. Moreover, excess iron can produce alkoxy and peroxy radicals upon reaction with unsaturated lipids [148] and metal-catalyzed radicals can mediate the oxidation of amino acids, resulting in inactivated proteins [153]. Ultimately, these deleterious reactions lead to the impairment of multiple cellular processes and organ damage, which is evident in iron-loading (*e.g.*,  $\beta$ -thalassemia and Friedreich's ataxia [154-156]) and copper-loading diseases (*e.g.*, Wilson's and Menkes disease [3, 142, 157]). Notably, such harmful oxidative reactions, especially in terms of targeting DNA, can lead to mutagenic alterations and implicates the role of metals in cancer development and progression [18-20].

## **4.2 Iron and Cancer Development**

Numerous clinical and population based studies as well as animal experiments have implicated iron in the development of a variety of human cancers [158-162]. Although it is

well established that iron can cause harmful effects through the Fenton reaction, and that enhanced ROS levels are observed in tumor cells [21, 147], a clear cause-and-effect relationship has proven difficult to establish. This is likely due to the complexity of carcinogenesis, involving various causative factors, including diet, lifestyle and genetics, and the inherent difficulties of making inferences from these types of studies [21].

Epidemiological studies and investigations in rodents have assessed the association between dietary iron intake and cancer risk. This research has demonstrated a link between higher iron intake and an increased risk of developing colorectal [161, 163] and lung cancer [160]. High consumption of red and processed meats is also associated with an increased risk of developing colorectal cancer [164]. However, it is currently unclear whether this link is related to the intake of excess iron, producing ROS that damage the colonic mucosa, or confounding issues, such as fat content or other dietary factors [164].

Studies examining the association between iron stores and cancer have demonstrated that patients who subsequently developed cancer had higher serum iron, total iron-binding capacity (TIBC) and Tf saturation at enrolment [160, 165]. Conversely, the reduction of body iron stores with regular blood donation was associated with a decreased risk of several cancers, which are thought to be promoted by iron overload, including liver, lung, colon, stomach, and oesophageal cancer [166]. This finding should be interpreted cautiously, as the cohort of blood donors is unlikely to reflect the general population.

Genetically-induced iron overload, such as hereditary hemochromatosis, is linked with liver cirrhosis and hepatocellular carcinoma [167]. In hereditary hemochromatosis, excessive intestinal absorption of dietary iron results in a pathological increase in total body iron stores [167]. Liver cirrhosis with hepatocellular carcinoma accounts for ~20–30% of deaths in

untreated or poorly treated patients and represents a 20-200-fold increased risk of hepatocellular carcinoma in comparison to the general population [168, 169]. As further evidence of the role of iron in carcinogenesis, treatment of hepatitis C positive patients with regular phlebotomy and a low-iron diet significantly lowered the risk of hepatocellular carcinoma over a 5 and 10 year period [170].

### **4.3 Iron Metabolism in Cancer**

Unraveling the complex relationship between the homeostasis of iron and other metal ions and tumor initiation and growth remains an active area of investigation [17, 171]. Several proteins that play a role in normal iron metabolism have now been implicated to contribute to malignancy, as their levels or activity are altered [17, 172]. The net result of these cancer-specific alterations is often an increase in intracellular iron levels, promoting proliferation [17]. Nevertheless, cancer cells possess heterogeneity, and hence, different molecular mechanisms inducing alterations of iron-regulated proteins are likely to exist in different neoplastic cells. Below is a discussion of common modifications in iron regulation observed in cancer.

#### **4.3.1 Iron Trafficking**

Important changes in iron uptake have been identified in cancer cells, with TfR1 over-expression observed in bladder cancer, breast cancer, lung cancer, leukemia, lymphoma and others [173]. Consequently, TfR1 antibodies have been used to both inhibit cancer growth and to deliver anti-cancer agents to neoplastic cells [173]. A correlation between increased TfR1 expression and tumor grade and stage have been observed in a number of studies, with higher expression associated with more advanced cancers [174, 175]. Some members of the STEAP family of metalloreductases involved in iron uptake have also been found to be over-expressed in bladder, breast, colon, lung and prostate cancer [176].



Neoplastic cells increase bioavailable iron not only by increasing iron uptake, but also by decreasing iron efflux [21, 177]. Hepcidin is a key regulator of systemic iron levels that binds to Fpn1, inducing its internalization and degradation [178]. As Fpn1 is an important mechanism for iron export, increased hepcidin levels lead to intracellular iron accumulation and reduced serum iron [179]. In fact, mutations that disrupt this pathway lead to iron accumulation and hereditary hemochromatosis [180]. Fpn1 was found to be down-regulated in breast cancer cell lines and in human breast cancer samples [181]. Moreover, decreased *Fpn1* gene expression was significantly associated with poor prognosis of breast cancer patients [181]. High hepcidin levels were associated with decreased expression of both Fpn1 and ferritin in cultured breast cancer cells [181]. Thus, neoplastic cells may possess increased bioavailable iron to stimulate proliferation or cancer promoting ROS.

#### **4.3.2 Iron Storage**

Iron storage is often altered in cancer cells, with ferritin expression observed to be up-regulated in breast cancer [182], esophageal adenocarcinoma [174] and hepatocellular carcinoma [183]. Survival rates have been shown to be lower in primary lung cancer patients with high serum ferritin levels [184] and stage IIIC colorectal cancer patients with either low or high serum ferritin levels [185]. Additionally, high *ferritin* mRNA levels in tissue from breast cancer patients were shown to correlate with the presence of metastasis [186]. In contrast, a reduction in ferritin has also been demonstrated in colon cancer through the direct action of the proto-oncogene, *c-MYC*, on TfR1 and DMT expression *via* inducing IRP2 expression [187, 188]. This is thought to lead to increased bioavailability of intracellular iron for metabolism and proliferation. In contrast, the tumor suppressor, p53, which is mutated in more than half of cancers, is believed to exert the opposite effect, inducing ISCU expression and ferritin expression, while decreasing TfR1 expression [189]. Moreover, *in vitro* and

human prostate cancer xenograft studies have demonstrated that lower ferritin levels promoted angiogenesis through the inhibition of high molecular weight kininogen, an endogenous angiogenesis inhibitor [190]. Thus, both down-regulation and up-regulation of ferritin may contribute to carcinogenesis.

#### **4.3.3 DNA Synthesis and the Cell Cycle**

The depletion of iron typically results in G<sub>1</sub>/S arrest or apoptosis, demonstrating that iron is required for cell cycle progression [172, 191, 192]. Iron is required for the catalytic activity of the rate-limiting step of DNA synthesis that is catalyzed by RR, making DNA synthesis dependent on iron [193]. In fact, RR is up-regulated in cancer cells, facilitating DNA replication and tumor cell division [194]. Additionally, iron can regulate the expression of multiple proteins and modulate signal transduction pathways involved in cellular proliferation, including the cyclins, cyclin-dependent kinases (cdks), p21 and p53 [192, 195, 196]. Iron depletion has been shown to down-regulate cyclin D1, cyclin D2 and cyclin D3 expression and inhibit phosphorylation of the retinoblastoma protein [192]. Collectively, this evidence illustrates that intracellular iron affects the expression of critical cell cycle components, and therefore, iron levels modulate cell cycle progression [192].

#### **4.3.4 The Iron Regulated Metastasis Suppressor, NDRG1**

Apart from its effects on proteins involved in cell proliferation, iron was also found to modulate the expression of a metastasis suppressor gene, namely N-myc downstream regulated gene 1 (NDRG1) [197]. Studies utilizing iron chelators demonstrated that these agents markedly up-regulated NDRG1 expression in a plethora of cancer cells, with this effect being dependent on iron depletion [197]. In fact, supplementation with iron salts prevented the iron chelator-mediated increase in NDRG1 levels, further demonstrating the iron-mediated regulation of this molecule [197]. This study also demonstrated that the

increase in NDRG1 by iron chelators occurred, at least in part, *via* the transcription factor hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ; Figure 6) [197]. Indeed, iron chelation increases levels of HIF-1 $\alpha$ , which is up-regulated under hypoxic conditions and functions to directly increase transcription of multiple down-stream targets by binding to the hypoxia responsive element (HRE) in the promoter region [197]. Notably, NDRG1 has a HRE in its promoter region, accounting for its transcriptional up-regulation in response to HIF-1 $\alpha$  [198]. Interestingly, iron chelators can also up-regulate NDRG1 independently of HIF-1 $\alpha$  [197].

A potential alternate pathway of iron-mediated regulation of NDRG1 could involve the eukaryotic initiation factor 3a (eIF3a; Figure 6), which was found to promote NDRG1 expression during iron depletion in breast cancer cells [199]. In fact, silencing eIF3a diminished the ability of iron chelators to up-regulate NDRG1 expression [199], suggesting that this molecule plays an important role in the iron-mediated regulation of NDRG1.

Another interesting hypothesis that has emerged very recently is the potential involvement of the adenylyl cyclase (AC), cAMP-dependent protein kinase (PKA) and cAMP response element-binding protein (CREB) signaling cascade in the iron-mediated regulation of NDRG1 (Figure 6) [200]. Iron depletion was found to activate AC and its down-stream targets PKA and CREB by modulating G-protein signaling (Figure 6) [200]. Interestingly, regulators of G-protein signaling (RGS) proteins, namely RGS19, were found to play a key role in sensing cellular iron *via* their iron-sulfur binding motif [200]. Hence, under conditions of low iron, RGS19 is stabilized, activating the AC/PKA/CREB signaling cascade (Figure 6) [200]. Interestingly, CREB has been reported to bind to the HRE [201] and, considering that NDRG1 has a HRE in its promoter [198], CREB may be directly involved in regulating NDRG1 expression in response to iron depletion.

The function of NDRG1 in cancer cells has resulted in significant interest in recent years, with the expression of this molecule being correlated with less aggressive cancers and increased patient survival for prostate [202], breast [203], colon [204], glioma [205], neuroblastoma [206], squamous cell carcinoma [207] and pancreatic cancers [208-210]. Conversely, NDRG1 expression was found to promote cancer progression and metastasis in hepatocellular carcinoma [211], although the mechanisms behind this paradoxical effect remain unclear and important to elucidate. Notably, NDRG1 interacts with multiple signaling pathways in cancer cells [212] and it is likely that its function can be altered by mutations and alterations in these pathways.

Recent studies examining the down-stream targets of NDRG1 in cancer cells have discovered that this metastasis suppressor negatively regulates the RAS, phosphoinositide 3-kinase (PI3K), nuclear factor- $\kappa$ B (NF- $\kappa$ B), WNT, SRC and transforming growth factor  $\beta$  (TGF- $\beta$ ) pathways, all of which contribute to cancer progression and metastasis (Figure 6) [213-218]. In particular, NDRG1 inhibited the epithelial to mesenchymal transition (EMT; Figure 6), which is the first step towards metastasis [217]. This latter effect was mediated by the increased membrane levels of E-cadherin and  $\beta$ -catenin in response to NDRG1, which promotes formation of the adherens junctions [215, 217]. Moreover, NDRG1 also inhibited a number of crucial molecules involved in cell motility and migration, namely Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) and its down-stream target phosphorylated myosin light chain 2 (pMLC2), leading to inhibition of stress fiber formation and cell locomotion [219].

The ability of NDRG1 to interact with and modulate such a vast array of signaling pathways indicated that it may affect a crucial up-stream regulator of cell signaling. Indeed, a recent study has demonstrated that NDRG1 markedly inhibits activation of a number of receptor

tyrosine kinases, including the epidermal growth factor receptor (EGFR; Figure 6) as well as its family members, HER2 and HER3, all of which interact with and activate numerous down-stream pathways to promote cell proliferation, migration and metastasis [220].

Considering the important role of iron in regulating NDRG1 expression in cancer cells (Figure 6), and the myriad of oncogenic signaling pathways affected by this metastasis suppressor, it is clear that iron metabolism is integrally involved in cancer progression and metastasis. Clearly, agents that can up-regulate this molecule and its potent anti-metastatic activity could be important therapeutics.

#### **4.4 Copper and Cancer Development**

Excess copper is known to be a potent catalyst of ROS generation [150]. However, the role of copper in the growth of tumors and its profoundly altered distribution and metabolism in cancer was only recently demonstrated [221]. Both serum and tumor copper levels have been found to be significantly elevated in cancer patients [221]. Studies in breast cancer patients demonstrated that serum and tissue copper concentrations were increased compared to age matched samples, with higher values associated with advanced disease and malignancy [18].

Elevated copper levels have been documented in breast, cervical, ovarian, lung, prostate, stomach, and leukemia [222]. Serum copper concentration has also been found to correlate with tumor incidence and burden, malignant progression in Hodgkin's lymphoma, leukemia, sarcoma, brain, breast, cervical, liver and lung cancer [223, 224]. Interestingly, serum copper levels have also been observed to be linked to drug resistance in cancer [225]. Indeed, cancer patients that did not respond to drug treatment demonstrated 130-160% higher levels of copper in their serum than treatment responsive patients [225].

Moreover, expression of Cp has also been reported to be elevated in a variety of cancers, including breast, bowel, lung and stomach cancer [226]. Indeed, Cp levels were reported to be increased during cancer progression and returned to relatively normal levels after tumor regression [226]. In fact, serum Cp has been evaluated as a diagnostic marker of cancer [227].

Copper was also found to play an important role in the metastatic progression of breast cancer [228]. In fact, the metal-binding enzyme, Memo, which requires Cu(II) for its oxidase activity, was found to be essential for breast cancer cell motility in response to receptor tyrosine kinases [228]. Memo was found to affect ROS production, cell migration and *in vivo* metastasis, being correlated with poor prognostic outcomes of breast cancer patients [228].

#### **4.5 Copper and Angiogenesis**

Copper plays an important role in angiogenesis, and thus, it is critical in cancer progression [229]. The dependence of cancer on angiogenesis is based on the observation that tumor cells are unable to grow more than 1–2 mm in diameter without a supply of oxygen and nutrients [230]. In fact, angiogenesis is important for both tumor growth as well as metastasis [231]. The role of copper in angiogenesis was first discovered when it was noted that copper salts could stimulate the proliferation and migration of endothelial cells [232].

The vascular endothelial growth factor (VEGF) family are angiogenic proteins essential in both vasculogenesis and hypoxia-induced angiogenesis, and recent evidence suggests that copper may be a required cofactor of VEGF-mediated angiogenesis [233]. In addition to VEGF, numerous angiogenic factors such as basic fibroblast growth factor (bFGF), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL) 1, IL-6, IL-8 and fibronectin has been reported to be activated by copper [234]. Copper has also been linked with the activation of HIF-1 $\alpha$ ,

by stabilizing nuclear HIF-1 $\alpha$  under normoxic conditions, resulting in HRE-dependent reporter gene expression [235]. As HIF-1  $\alpha$  regulates the expression of the pro-angiogenic molecules, such as VEGF-1, this represents another mechanism by which copper regulates angiogenesis [236].

## **5. Targeting Metals in Cancer Therapy**

The high iron and copper requirements, as well as dysregulated homeostasis of these metals in cancer cells, has led to the discovery that compounds that can bind and sequester essential metal ions, namely chelators, have anti-cancer activity [237-240]. In fact, these agents belong to a group of chemotherapeutics known as metabolic inhibitors, which represent an important and broad class of anti-cancer agents. A prominent example of these drugs is the highly successful folate antagonists (*e.g.*, methotrexate), which inhibit the use of the nutrient folate which is critical for DNA synthesis [241]. Just as folate is an essential nutrient for DNA synthesis, iron is also crucial for DNA synthesis and both of these nutrients are required in higher amounts by tumor cells, making each appropriate therapeutic targets [2, 237, 238].

For many decades, chelators have been used to treat conditions of iron or copper overload, such as in  $\beta$ -thalassemia or Wilson's disease, respectively [242, 243]. However, it is only more recently that their roles in other diseases, such as neurodegeneration and cancer, have been considered [2, 239, 244]. By coordinating with intracellular and extracellular iron or copper, some of these ligands promote the excretion from the body and their subsequent depletion from biological systems. In contrast, other ligands can be trapped within cells in lysosomes [137, 138] and lead to concentration of metals (as the ligand-metal complexes) within the tumor, which can play a role in their anti-tumor efficacy [244].

Metal chelators can be isolated from natural sources or synthesized. Siderophores are an example of naturally derived ligands excreted by microbes and fungi to sequester iron in an utilizable form [2]. Their iron chelation efficiency has led many to become lead compounds in the search for more effective iron chelators [245]. Currently, the siderophore, desferrioxamine (DFO; Figure 7A), which is produced by the bacterium, *Streptomyces pilosus*, is clinically used for the treatment of the iron overload disease,  $\beta$ -thalassemia major [65]. Moreover, DFO has also demonstrated modest anti-proliferative activity against neuroblastoma and leukemia cells *in vitro* and in clinical trials [1, 239, 246, 247].

The realization that Wilson's disease was caused by an excess of copper [248] stimulated further research for copper chelating drugs. The ligand, D-penicillamine, was the first successful treatment for Wilson's disease [242]. Initiation of therapy in patients with Wilson's disease resulted in urinary copper excretion, leading to a large negative copper balance [242]. While being an effective therapy, D-penicillamine has multiple severe side effects, which include neurological effects and hematologic and renal toxicity [249]. In the search for alternatives with fewer side effects, other clinically effective anti-copper agents have been developed, including zinc and tetrathiomolybdate (TM) [250]. Treatment with one, or a combination, of these drugs leads to a reversal of copper accumulation, and in most patients, a clinical improvement [240]. Importantly, TM has proven particularly useful for the treatment of acutely ill Wilson's disease patients with neurological manifestations [251]. Due to its low toxicity and rapid action, TM has been evaluated in a number of clinical trials as a first line therapy against acute neurological Wilson's disease [251].

The finding that angiogenesis is largely dependent on copper availability has led to successful trials of a number of anti-copper drugs, including D-penicillamine, trientine and TM, as anti-angiogenic and anti-cancer agents [252, 253]. In a phase II trial of advanced kidney cancer,



TM was well tolerated and effectively induced copper depletion, leading to stabilization of the disease [254].

The development of metal chelators as anti-cancer agents has followed two broad strategies, namely: (1) depleting cancer cells of iron or copper, and (2) generating cytotoxic ROS within cancer cells by facilitating iron and copper redox cycling [17]. The following section introduces chelators as therapeutic agents and describes their development from agents for the treatment of iron and copper overload diseases to current thiosemicarbazone chelators, whose mechanism of action includes metal sequestration and ROS generation.

## **6. Genesis of Chelators for Cancer Therapy**

### **6.1 Desferrioxamine**

The siderophore, DFO (Figure 7A) is a hexadentate chelator secreted by *Streptomyces pilosus* [255]. As the current “gold-standard” chelator for the treatment of  $\beta$ -thalassemia major and related conditions [256], this ligand has validated iron chelation as an effective therapeutic strategy [257]. DFO binds ferric iron with high affinity *via* its hydroxamic acid groups [246] to form a stable 1:1 complex, which inhibits the generation of ROS, making it a suitable ligand for the treatment of iron overload disease [2].

Additionally, the ability of DFO to act as potential anti-cancer agent has been investigated in a number of *in vitro* and animal studies and also in clinical trials [258-260]. For example, in *in vitro* investigations, DFO was able to markedly reduce in neuroblastoma (NB) cell viability after a 72 h incubation [261] and the inhibition of DNA synthesis in NB cells after a 4 h incubation [262]. Importantly, iron depletion was found to be central to the mechanism of action of DFO as its anti-proliferative activity was inhibited upon the addition of iron [262].

A number of clinical trials investigating the potential of DFO as an anti-cancer agent have demonstrated mixed results. For instance, a phase II trial demonstrated a 50% reduction in bone marrow infiltration in 7 out of 9 NB patients, with one patient exhibiting a 48% decrease in tumor size after DFO treatment (150 mg/kg/5 days) [246]. In contrast, a study of 10 patients with recurrent NB did not respond to DFO administration [247].

The poor results obtained with DFO reflect its limitations that stem from its high hydrophilicity, short half-life and its oral inactivity [65]. As a result, ongoing research has been conducted to develop far more active, selective and specific chelators for the treatment of iron overload, but also cancer. For further details regarding the development of chelators for the treatment of iron overload, readers are referred to the following review [2]. The sections below focus on the development of chelators for the treatment of cancer, particularly well-developed agents which have, or will, enter clinical trials.

## **6.2 Thiosemicarbazones**

Tridentate thiosemicarbazones were amongst the first ligands to be comprehensively studied as anti-cancer agents [263-265]. These ligands show high affinity for iron, but also bind Cu(II), Zn(II) and other transition metals [266]. Due to the abundance of literature regarding thiosemicarbazones, this section will focus on research concerning the  $\alpha$ -pyridyl thiosemicarbazones (Figure 7B) as anti-tumor agents. Specifically,  $\alpha$ -pyridyl thiosemicarbazones bind iron utilizing the *N,N,S* coordination system to form octahedral iron complexes in a 2:1 ligand to iron ratio [264].

### **6.2.1 Triapine<sup>®</sup>**

Triapine<sup>®</sup> (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; Figure 7C) is an  $\alpha$ -pyridyl thiosemicarbazone that potently inhibits RR activity [265] and modestly suppresses cancer

cell growth [267, 268]. Human RR is a tetramer consisting of two non-identical homodimers, R1 and either R2 or p53R2 [269]. R2 is involved in DNA synthesis for housekeeping, whereas p53R2 is a p53-targeted gene that is transactivated by DNA damage [270]. Examination of structural homology revealed that both R2 and p53R2 possess an iron-binding site important for enzymatic function [269], and thus, could potentially be inactivated by iron chelators [172]. In contrast to the clinically used RR inhibitor, hydroxyurea, Triapine<sup>®</sup> was found to equally inhibit both R2 and p53R2, whereas hydroxyurea was relatively ineffective at inhibiting the p53R2 subunit [269]. Interestingly, Triapine<sup>®</sup> did not remove Fe from the active site of R2 or p53R2, but instead formed redox active Fe(III) complexes [267]. Notably, the subsequent reduction of the Triapine<sup>®</sup>-Fe(III) complex generated ROS and quenched the tyrosyl radical of RR, leading to the inactivation of the enzyme and prevention of DNA synthesis and repair [267].

These above results were consistent with prior observations demonstrating the redox activity of the Triapine<sup>®</sup>-Fe complex [268]. Additionally, a subsequent investigation by Shao *et al.* (2006) demonstrated that the Triapine<sup>®</sup>-Fe(II) complex reduces O<sub>2</sub> to produce ROS [267]. Moreover, Triapine<sup>®</sup> mediated the pronounced oxidation of mitochondrial thioredoxin 2 (Trx2) [271]. Cytosolic (Trx1) and mitochondrial (Trx2) thioredoxins are at the core of cellular thiol redox control and antioxidant defense, making them essential for cellular survival [272]. Thus, the ability of Triapine<sup>®</sup> to increase Trx2 oxidation suggests the contributing role of redox cycling and ROS generation in anti-cancer activity [271]. Triapine<sup>®</sup> also could increase iron release from cells, prevent Fe uptake from transferrin, and increase IRP-RNA-binding activity like other chelators such as DFO, demonstrating that it was effective at chelating iron present in intracellular iron pools [268].

*In vitro* studies demonstrated the ability of Triapine<sup>®</sup> to inhibit the growth of both HU-sensitive and -resistant cancer cells, including L1210 leukemia and KB nasopharyngeal carcinoma [273, 274]. Additionally, Triapine<sup>®</sup> demonstrated pronounced anti-cancer activity without marked toxicity in L1210 leukemia, M109 lung carcinoma and A2780 ovarian carcinoma xenografts in mice [265]. Triapine<sup>®</sup> was also found to act in synergy with other chemotherapeutic agents, such as cisplatin, doxorubicin (DOX) and etoposide, to cause tumor regression in mouse models [265].

Following the success of Triapine<sup>®</sup> in cellular and animal models, this agent began evaluation in >20 phase I and II clinical trials against a number of cancers [275, 276] (for review see [21]). In a phase I trial, Triapine<sup>®</sup>, administered as a single agent, decreased serum tumor markers associated with stable disease in 4 of 21 patients [277]. Triapine<sup>®</sup> was well tolerated at a fortnightly dose of 120 mg/m<sup>2</sup>/d, whereas a dose of 160 mg/m<sup>2</sup>/d resulted in dose-limiting toxicities in three of the six patients [277].

Toxicity after administration of Triapine<sup>®</sup> was primarily hematological, with patients suffering from anemia, methemoglobinemia, leucopenia and thrombocytopenia [277]. Moreover, patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency developed severe methemoglobinemia and hemolysis upon treatment [278]. This observation is likely due to the redox activity of the iron complexes of Triapine<sup>®</sup> [268, 278, 279] and the inability of patients with G6PD deficiency to effectively reduce methemoglobin to hemoglobin [278]. Additional phase I studies examining Triapine<sup>®</sup> in combination with gemcitabine demonstrated anti-tumor activity against a range of metastatic or advanced cancer types [280]. In fact, 3 out of 22 patients showed an objective partial response, while one patient showed evidence of tumor regression [280].

Following these promising results, Triapine<sup>®</sup> progressed to Phase II trials [21]. In a phase II study, the administration of Triapine<sup>®</sup> and gemcitabine in combination resulted in an objective response in 3 out of 23 patients with advanced adenocarcinoma of the gall bladder or biliary tract [281]. Conversely, another study using Triapine<sup>®</sup> as a single agent only reported slight activity against recurrent or metastatic head and neck squamous cell carcinoma [282]. A phase II trial in patients with metastatic renal cell carcinoma highlighted some of the deleterious side effects of Triapine<sup>®</sup> upon administration at 96 mg/m<sup>2</sup> during a 2 h infusion repeated daily for a 4 day period repeated fortnightly [275]. Some of the adverse effects included fatigue, nausea, neutropenia, methemoglobinemia, hypoxia and hypotension [275]. As a result, the study was terminated early due to the limited efficacy of the treatment [275]. Thus, these conflicting clinical trial results have prompted others to further develop and refine the selectivity of potent thiosemicarbazones as anti-cancer agents.

### **6.2.2 Di-2-Pyridylketone Thiosemicarbazones**

More recently, a more effective series of tridentate chelators, namely the di-2-pyridylketone thiosemicarbazone (DpT) series were synthesized (Figure 7D) [283] (Whitnall et al. 2006). These ligands are similar to Triapine<sup>®</sup>, but with the 3-aminopyridine-2-carboxaldehyde moiety replaced with the di-2-pyridylketone group. The generation of the DpT series was the result of 20 years of structure-activity relationship studies that originally began with analogues of the tridentate ligand, pyridoxal isonicotinoyl hydrazone (PIH) [284, 285]. These agents were characterized in terms of their activity [191, 192, 286], refined and eventually led to hybrid ligands of aroylhydrazones and thiosemicarbazones [287, 288], and then finally the DpT series.

In initial *in vitro* studies, many DpT analogues demonstrated high anti-proliferative activity against SK-N-MC neuroepithelioma cells, MCF-7 breast cancer cells and SK-Mel-28

melanoma cells [283]. Of these, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT; Figure 7D) was found to be the most potent in SK-N-MC cells and a more promising candidate for further evaluation [283].

#### **6.2.2.1 Dp44mT**

Dp44mT was subsequently shown to demonstrate selective and potent anti-proliferative activity against over 28 cancer cell lines [244], including leukemia, myeloma [289] and breast cancer cells [290] compared to normal peripheral blood mononuclear cells [289], fibroblasts [244] and mammary epithelial cells [290]. Over these 28 cancer cell-types, Dp44mT was shown to be on average 21 times more effective than the chemotherapeutic agent, DOX, and 47-fold more effective than Triapine<sup>®</sup> [244]. With the emergence of multi-drug resistant cancer cells being a significant clinical problem, it is important to note that Dp44mT was found to be equally or more effective in preventing the proliferation of etoposide-resistant breast cancer cells (MCF-7/VP) and vinblastine-resistant epidermoid carcinoma (KB-V1) when compared to their wild type chemo-sensitive cells [244].

Studies have demonstrated that the cellular uptake of Dp44mT occurs *via* a saturable carrier/receptor-mediated process [291]. Moreover, the abundant serum protein, human serum albumin, enhances the cellular uptake of Dp44mT, potentiating its anti-proliferative and apoptotic activity [292]. Collectively, these findings promote the development of strategies to improve drug delivery of this agent *via* the implementation of albumin-based drug carriers [291-293].

Studies investigating the molecular effects of Dp44mT and other  $\alpha$ -pyridyl thiosemicarbazones have demonstrated their ability to induce apoptosis [283, 289] even though the exact signaling pathways that lead to this event were not completely understood.

Studies performed to examine the pathways involved in the induction of apoptosis by Dp44mT revealed efflux of holo-cytochrome *c*, increased expression of caspase-3, 8 and 9 proteins [283] and a decrease in the mitochondrial trans-membrane potential [289] upon incubation with Dp44mT. Moreover, Dp44mT resulted in decreased expression of the anti-apoptotic Bcl-2 protein, increased expression of the pro-apoptotic Bax protein [283] and induced G<sub>1</sub> cell cycle arrest in leukemia and breast cancer cells [216, 283, 289]. In addition to the above pro-apoptotic effects, Dp44mT was also demonstrated to induce DNA double-strand breaks, activate DNA damage and cell cycle checkpoints and inhibit DNA topoisomerase IIa in p53 mutant MDA-MB-231 breast cancer cells [290].

Additionally, another important target of Dp44mT is the metastasis suppressor, NDRG1 [197, 210, 294, 295]. In fact, NDRG1 was found to be crucial for the anti-metastatic effects of Dp44mT in breast cancer *in vivo*, as knocking-down NDRG1 markedly blocked Dp44mT-induced metastasis suppression [295]. Considering the multiple down-stream effects of NDRG1 (see Section 4.3.4 above), it is notable that Dp44mT was found to have similar down-stream effects, being able to inhibit TGF- $\beta$ , ROCK/pMLC2, SRC, PI3K, RAS and WNT signaling pathways [212, 214, 216-219, 295]. Moreover, Dp44mT also inhibited the EMT, restoring E-cadherin and  $\beta$ -catenin levels at the cell membrane and promoting the formation of adherens junctions [215, 217].

Due to the ability of Dp44mT to up-regulate NDRG1, this agent also was able to inhibit the levels and activation of the crucial ErbB family of receptor tyrosine kinases, namely EGFR, HER2 and HER3 both *in vitro* and *in vivo* in pancreatic cancer cells [220]. Collectively, the ability of the DpT class of thiosemicarbazone chelators to up-regulate NDRG1 expression leads to multiple down-stream effects which result in general inhibition of cancer progression and metastasis.

Several studies have examined the detailed molecular and cellular mechanisms responsible for the anti-tumor efficacy of Dp44mT. Yuan et al. (2004) suggested that ROS partially mediated the cytotoxic effects of the DpT ligands after observing that the Dp44mT-iron complex was redox-active [283]. Furthermore, the redox potentials of the DpT-iron complexes were within the range of cellular reductants and fully reversible, a feature attributed to the thioamide moiety [296]. This assertion was confirmed in later studies where the cytotoxic activity of Dp44mT was shown to be dependent on the generation of cytotoxic ROS [138].

In addition to iron complexation as a source of ROS generation, the copper complexes of Dp44mT have been shown to be potently redox-active and able to induce hydroxyl radical formation [138]. Notably, the copper complex of Dp44mT exhibited greater *in vitro* efficacy against SK-N-MC and MCF-7 tumor cells than either the ligand alone or the iron complex [138]. This suggests that copper binding and the redox activity of the Cu-Dp44mT complex may be a key component of the anti-cancer efficacy of Dp44mT. Moreover, the lysosomotropic properties of Dp44mT lead to it becoming charged at the pH of the lysosome, resulting in its accumulation within this organelle [138]. Once inside the lysosome, Dp44mT forms redox-active complexes with chelatable copper that then induces oxidative stress, resulting in lysosomal membrane permeabilization (LMP) [138]. This subsequently leads to release of cathepsins from the lysosomes into the cytosol, and through downstream effector activation of the mitochondrial apoptotic machinery [138].

Interestingly, the increase in thiosemicarbazone toxicity towards cells over-expressing P-glycoprotein (Pgp), a drug efflux pump that confers drug resistance, was also found to be linked to lysosomal targeting [137, 297]. In many cancers, Pgp expression has been shown to



markedly reduce the toxicity of classical chemotherapeutic treatments (*e.g.*, DOX), due to its ability to efflux these compounds from the cell [298]. More recently, it has also been shown that Pgp is present on lysosomes and can lead to the sequestration of drugs such as DOX within lysosomal “safe houses” (Figure 8A) [299]. Hence, together with active Pgp-mediated efflux from cells, the safe storage intracellularly of DOX in lysosomes by lysosomal Pgp pumps can result in resistance to this cytotoxic agent (Figure 8A) [299, 300].

Notably, Dp44mT due to its very unique properties can overcome multidrug resistance *via* “hijacking” lysosomal Pgp to increase its accumulation in this organelle, leading to enhanced LMP (Figure 8B) [137]. The ability of Dp44mT to overcome multidrug resistance has been attributed to a number of important characteristics of this agent, namely: **(1)** the agent must act as a Pgp substrate; **(2)** the agent must become charged at acidic lysosomal pH, leading to lysosomal accumulation; and **(3)** the drug must cause marked redox stress within the acidic lysosome, leading to LMP [137]. Thus, while DOX which fulfills criteria **(1)** and **(2)**, it does not satisfy criterion **(3)**, as it does not possess the potent redox activity of the Dp44mT-copper complex which markedly induces LMP [137]. Hence, thiosemicarbazones, such as Dp44mT, may prove valuable agents for patients with drug resistant tumors [301].

In addition to the very promising properties above, Dp44mT markedly inhibited the growth of human lung carcinoma, neuroepithelioma and melanoma tumor xenografts in nude mice [244]. Following 7 weeks of treatment with Dp44mT (0.4 mg/kg per day) *via* intravenous administration, SK-Mel-28 melanoma xenografts were 92% smaller than control tumors treated with vehicle [244]. These studies also unfortunately revealed that Dp44mT induced cardiac fibrosis in a dose-dependent manner at high non-optimal doses >0.75 mg/kg/d [244]. Superficially, this dose-limiting toxicity could be said to appear somewhat similar to that observed with the clinically used chemotherapeutic agent, DOX [302]. However, it is notable

that the cardiotoxic effects of DOX and Dp44mT are different anatomically and histologically, suggesting a different cytotoxic mechanism for each of these agents (Richardson, D.R. unpublished data).

#### **6.2.2.1 DpC**

In an effort to develop more highly potent and selective DpT analogues, the structure of Dp44mT was modified to generate a new series of second generation DpT analogues, with di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC; Figure 7D) being the current lead compound [294, 303]. Importantly, DpC has shown to be significantly more effective and better tolerated than Dp44mT both *in vitro* and *in vivo* [294, 303]. Moreover, unlike Dp44mT, DpC has demonstrated *in vivo* anti-tumor activity by both the intravenous and oral routes [294, 303]. In fact, this is a significant advantage of DpC, as Dp44mT is toxic when given orally [304]. DpC has also demonstrated marked activity at the molecular level, with the agent increasing the expression of the potent metastasis suppressor, NDRG1, and the cyclin-dependent kinase inhibitor, p21<sup>CIP1/WAF1</sup>, while decreasing cyclin D1 levels [294]. Furthermore, DpC demonstrated greater activity than the standard chemotherapeutic treatment against pancreatic cancer, namely gemcitabine, with studies showing complete inhibition of pancreatic tumor xenograft growth, and unlike Dp44mT, demonstrated no evidence of cardiac fibrosis [220, 294].

Importantly, similar to Dp44mT, DpC also has been shown to overcome resistance to standard chemotherapy by “hijacking” lysosomal Pgp to increase targeting of lysosomes and inducing LMP (Figure 8B) [137, 297]. Moreover, tumor microenvironment stress induced by extremely low or high glucose concentrations rapidly increased Pgp expression in lysosomes, leading to enhanced cytotoxicity and LMP by Dp44mT and DpC [297]. Hence, both Dp44mT and DpC display great potential to target multidrug resistant tumors where a stressful micro-

environment exists that amplifies their anti-tumor activity and ability to overcome resistance [297].

While the anti-tumor activity of both Dp44mT and DpC is related to their activity in terms of binding metals and inducing ROS and LMP [297, 303], a potential toxic side effect is the ability to induce oxidation of oxyhemoglobin to methemoglobin, which cannot transport oxygen [279]. This problem has been reported for Triapine<sup>®</sup> previously [275, 305, 306] and is potentially due to its redox activity [268]. Similarly to Triapine<sup>®</sup>, Dp44mT induced oxidation of oxyhemoglobin *in vitro* using intact erythrocytes and erythrocyte lysates, and also *in vivo* in mice [279]. This marked activity was due to the formation of a redox active iron complex, but not the copper complex. The results from these studies supported a hypothesis that oxidation of oxyhemoglobin by thiosemicarbazone-iron complexes follow an outer-sphere mechanism occurring over the exposed heme edge, as reported for other iron(III) complexes [307]. Further studies suggested that the terminal –NH<sub>2</sub> groups of both the DpT ligand and Triapine<sup>®</sup> may H-bond to the propionate groups of the heme moiety facilitating electron transfer [308].

In contrast to both Triapine<sup>®</sup> and Dp44mT, DpC demonstrated markedly lower activity at oxidizing oxyhemoglobin in intact erythrocytes and erythrocyte lysates, and did not increase the formation of oxyhemoglobin *in vivo* using mice [279]. In this case, the reduced oxidation may relate to an steric inability of DpC to approach the exposed heme edge due to incorporation of the bulky cyclohexyl substituent, inhibiting H-bonding to the propionate moieties of the heme prosthetic group [308]. Clearly, the markedly reduced oxidation of oxyhemoglobin by DpC in mice is a critical advantage over both Triapine<sup>®</sup> and Dp44mT [279].

Another important property that leads to the greater anti-tumor efficacy of DpC relative to Dp44mT has been shown by recent pharmacokinetic studies in rats [309]. In contrast to DpC, Dp44mT undergoes pronounced demethylation *in vivo*, which may be related to its quicker elimination ( $t_{1/2} = 1.7$  h for Dp44mT *vs.* 10.7 h for DpC) and lower exposure [309]. Significantly, the *in vivo* metabolism of Dp44mT resulted in decreased anti-cancer activity of the agent. Hence, DpC possesses highly favorable pharmacokinetics that demonstrate a marked improvement relative to Dp44mT. Considering the many favorable pharmacological characteristics of DpC, this agent has been commercialized and will undergo clinical trials in humans in 2016 [301].

### 6.2.3 2-Benzoylpyridine Thiosemicarbazones

The 2-benzoylpyridine thiosemicarbazone (BpT) series were developed through modification of the DpT series *via* the substitution of the non-coordinating pyridyl group with a phenyl ring (Figure 7E) [310]. The rationale for this alteration lay in enhancing the lipophilicity of the ligand, while simultaneously lowering the Fe(II)/Fe(III) one electron redox potential by replacing the pyridyl group with phenyl moiety [310].

*In vitro* studies using SK-N-MC neuroepithelioma cells demonstrated the potent and selective anti-proliferative activity of the BpT series [310]. The most potent BpT compounds exhibited greater anti-proliferative activity than DFO, Triapine<sup>®</sup> and most analogs of the DpT class, with the exception of Dp44mT [310]. The most active BpT analog was 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT; Figure 7E), which demonstrated comparable anti-proliferative activity to Dp44mT [310], despite its higher cellular uptake [291, 311]. These ligands, in addition to demonstrating selectivity against neoplastic cells [310], were also found to possess higher anti-proliferative activity against vinblastine-resistant KB-V1

epidermoid cells relative to non-resistant KB3-1 cells, suggesting their ability to overcome drug resistance [304].

Examination of the ability of the BpT analogs to mobilize iron revealed their ability to remove iron from pre-labeled cells and prevent iron uptake from Tf, but they were less effective than their DpT counterparts [312]. Cell-free and *in vitro* studies examining the redox activity of these chelators demonstrated that the iron complexes of the BpT series generally exhibited greater redox activity in comparison to the complexes of DpT-iron complexed counterparts [310, 312]. It was proposed that this latter property contributed to the generally greater observed anti-proliferative activity of the BpT series compared to their DpT counterparts [310]. Further investigation with the lead compound, Bp4eT, revealed that, similarly to Dp44mT, Bp4eT also inhibited RR activity [304]. Yu *et al.* (2011) suggested that the ability of the BpT analogs to inhibit RR could occur *via* iron deprivation and the modulation of cellular thiol-related anti-oxidant systems [313].

Due to the poor aqueous solubility of Bp4eT, it could not be administered at doses appropriate for potent anti-tumor efficacy during *in vivo* studies [304]. In contrast, the BpT counterpart, 2-benzoylpyridine 4,4-dimethyl-3-thiosemicarbazone (Bp44mT; Figure 7E) was successful at inhibiting DMS-53 tumor growth *via* both the oral and intravenous routes [304]. Toxicological examination of Bp44mT-treated mice revealed mild anemia, decreased hepatic and splenic iron levels, decreased serum albumin levels and an increase in serum liver enzymes [304]. Notably, several of the toxicological changes observed to be reversed upon cessation of Bp44mT treatment. Additionally, in contrast to Dp44mT, administration of Bp44mT did not lead to significant weight loss or cardiac fibrosis [304].

The *in vivo* investigations of Bp44mT against tumor xenografts revealed that tumor iron levels were not significantly changed following Bp44mT administration [244, 304], in line with previous findings for Dp44mT [244, 304]. Upon examination of the tumor xenografts, a significant decrease in TfR1 expression and an increase in ferritin heavy chain expression were observed, indicating augmentation of tumor iron pools by Bp44mT [304]. In contrast, tumor xenograft copper and zinc levels remained unchanged [304]. Collectively, this *in vivo* study demonstrated the potent anti-tumor efficacy of Bp44mT upon oral administration, a property important for improving patient convenience and compliance.

#### **6.2.4 Bis(thiosemicarbazones)**

A related class of agents that have anti-tumor activity are the bis(thiosemicarbazones) [314, 315]. The anti-cancer activity of glyoxal-bis(thiosemicarbazones) were first investigated in a 1958 study against Sarcoma 180-bearing Swiss mice [315]. In this study, the highest anti-cancer activity was evident in compounds administered orally at doses ranging from 50–150 mg/kg for a period of 7 days [315]. Moreover, treatment with glyoxal-bis(N4-methyl-3-thiosemicarbazone) (GTSM, Figure 9) led to a 45% reduction in tumor weight, when compared with untreated controls [316]. Intriguingly, bis(thiosemicarbazones) derived from 2,3-butanedione, including ATSM (Figure 9), were inactive in this model [316].

Investigations regarding the bis(thiosemicarbazone) derivative, kethoxal-bis(thiosemicarbazone) (KTS, Figure 9), demonstrated that this agent possessed excellent anti-tumor activity against rodent tumor xenografts [317]. The mechanism of action of KTS was unknown, but its anti-tumor efficacy was observed to be dependent on nutritional intake and, in particular, dietary Cu(II) intake [318]. The importance of Cu(II) in the activity of bis(thiosemicarbazones) was subsequently underlined by studies which combined Cu(II) and KTS to form Cu(KTS) (Figure 9) [319]. This complex resulted in inhibition of DNA

synthesis in sarcoma 180 ascites cells [319], and importantly, possessed anti-tumor activity against Walker 256 carcinoma in rats [320].

Notably, complexation of bis(thiosemicarbazones) with Cu(II) involves the double deprotonation of the ligand to yield a neutral bis(thiosemicarbazonato)Cu(II) complex (Figure 9). This resulting complex is highly lipophilic and able to rapidly traverse cell membranes [321]. Once inside cells, complexes such as Cu(II)(KTS) are believed to dissociate in a process involving reduction to release Cu(I). This process is possibly assisted by intracellular thiols [322]. Upon dissociation, Cu(I) is then distributed within the cell [321]. Evaluation of a series of substituted bis(thiosemicarbazonato)Cu(II) complexes demonstrated a correlation existed between their cytotoxicity and their reactivity towards thiol groups, suggesting that their activity was dependent on the intracellular release of Cu following the reduction of the complex [322].

Intriguingly, subtle changes to the ligand backbone can dramatically alter the biological properties of bis(thiosemicarbazones) [323]. This particular property has been exploited in investigations evaluating their suitability as radiopharmaceuticals for the imaging of hypoxic tissue [324, 325]. In contrast to Cu(KTS), where Cu is released intracellularly regardless of oxygen levels, the Cu(ATSM) complex enters cells and only releases Cu under hypoxic conditions [321, 324, 326]. This results from the different reduction potentials of Cu(ATSM) and Cu(KTS), and could account for the lack of biological activity of Cu(ATSM) in the above-described *in vivo* studies against Sarcoma 180 [315].

It is currently unknown as to how the Cu is subsequently metabolized once released from the ligand. On binding to intracellular proteins, Cu may be sequestered to particular subcellular compartments with a range of downstream effects. Recently, it was reported that Cu(GTSM),

but not Cu(ATSM), activated PI3K-dependent signaling, leading to downstream regulation of Alzheimer's amyloid peptide accumulation [327]. Subsequent *in vivo* investigations demonstrated that Cu(GTSM) led to cognitive improvement in an Alzheimer's disease mouse model, which was associated with altered metabolism of amyloid processing and microtubule tau protein phosphorylation [328]. In contrast, Cu(ATSM) did not mediate this effect in the same Alzheimer's disease model [327].

An investigation of the anti-cancer activity of Cu(GTSM) against human NB cells demonstrated cell cycle arrest as assessed by fluorescent activated cell sorting (FACS) analysis [329]. Protein array analysis revealed the ability of Cu(GTSM) to mediate cell cycle arrest *via* the potent reduction of cyclin D1 expression and an increase in Kip2 expression [329].

More recently, the activity of both Cu(GTSM) and Cu(ATSM) was assessed in the transgenic adenocarcinoma of mouse prostate (TRAMP) model [314]. In this investigation, Cu(GTSM) was shown to reduce prostate cancer burden and severity, while Cu(ATSM) was ineffective [314]. Interestingly, similarly to observations using Dp44mT and DpC [137, 138, 297, 330], studies have demonstrated that GTSM could mediate the induction of LMP [331]. Both copper chelation and ROS generation were shown to be crucial for this effect, and consequently, its anti-cancer activity [331]. Collectively, these studies demonstrate the potential of bis(thiosemicarbazones) as potent anti-tumor agents and again the role of metals and redox cycling in their mechanism of activity.



## **Conclusions**

A very fine balance is required for the maintenance of the precise levels of iron and copper that are necessary for the physiological functioning of cells. Alterations in metal homeostasis can have deleterious effects, resulting in cancer development and progression. Novel chemotherapeutic strategies have been developed to exploit the dual nature of metal ions in cancer cells.

Notably, agents that can bind iron and copper, such as the DpT series of metal chelators, have shown great promise as potent and selective anti-cancer agents. Importantly, apart from their ability to bind metal ions and form cytotoxic redox active complexes, these agents also affect an array of molecules that play crucial roles in cancer cell proliferation, migration and invasion. As a result of this polypharmacology, the DpT compounds are able to markedly inhibit both tumor growth, as well as metastasis, further contributing to their potent and selective anti-cancer effects.

Another important facet of the DpT compounds is their ability to over-come resistance to currently used chemotherapeutics. Notably, one of these agents, namely DpC, is entering clinical trials in 2016, demonstrating the potential of exploiting redox cycling metal ions as an important target for novel anti-cancer chemotherapeutics.

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## References

- [1] J.L. Buss, B.T. Greene, J. Turner, F.M. Torti, S.V. Torti, Iron chelators in cancer chemotherapy, *Curr. Top. Med. Chem.* 4 (2004) 1623-1635.
- [2] D.S. Kalinowski, D.R. Richardson, The evolution of iron chelators for the treatment of iron overload disease and cancer, *Pharmacol. Rev.* 57 (2005) 547-583.
- [3] D. Denoyer, S. Masaldan, S. La Fontaine, M.A. Cater, Targeting copper in cancer therapy: 'Copper That Cancer', *Metallomics* 7 (2015) 1459-1476.
- [4] L. Cai, X.K. Li, Y. Song, M.G. Cherian, Essentiality, toxicology and chelation therapy of zinc and copper, *Curr. Med. Chem.* 12 (2005) 2753-2763.
- [5] S. Nyholm, G.J. Mann, A.G. Johansson, R.J. Bergeron, A. Graslund, L. Thelander, Role of ribonucleotide reductase in inhibition of mammalian cell growth by potent iron chelators, *J. Biol. Chem.* 268 (1993) 26200-26205.
- [6] L. Thelander, A. Graslund, M. Thelander, Continual presence of oxygen and iron required for mammalian ribonucleotide reduction: possible regulation mechanism, *Biochem. Biophys. Res. Commun.* 110 (1983) 859-865.
- [7] L. Thelander, P. Reichard, Reduction of ribonucleotides, *Annu. Rev. Biochem.* 48 (1979) 133-158.
- [8] H. Tapiero, D.M. Townsend, K.D. Tew, Trace elements in human physiology and pathology: Copper, *Biomed. Pharmacother.* 57 (2003) 386-398.
- [9] L. Finney, S. Mandava, L. Ursos, W. Zhang, D. Rodi, S. Vogt, D. Legnini, J. Maser, F. Ikpatt, O.I. Olopade, D. Glesne, X-ray fluorescence microscopy reveals large-scale relocalization and extracellular translocation of cellular copper during angiogenesis, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 2247-2252.
- [10] K. Jomova, M. Valko, Advances in metal-induced oxidative stress and human disease, *Toxicology* 283 (2011) 65-87.

- [11] Y. Yu, D.S. Kalinowski, Z. Kovacevic, A.R. Siafakas, P.J. Jansson, C. Stefani, D.B. Lovejoy, P.C. Sharpe, P.V. Bernhardt, D.R. Richardson, Thiosemicarbazones from the old to new: Iron chelators that are more than just ribonucleotide reductase inhibitors, *J. Med. Chem.* 52 (2009) 5271-5294.
- [12] I. De Domenico, D. McVey Ward, J. Kaplan, Regulation of iron acquisition and storage: consequences for iron-linked disorders, *Nat. Rev. Mol. Cell Biol.* 9 (2008) 72-81.
- [13] D.J. Lane, A.M. Merlot, M.L. Huang, D.H. Bae, P.J. Jansson, S. Sahni, D.S. Kalinowski, D.R. Richardson, Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease, *Biochim. Biophys. Acta* 1853 (2015) 1130-1144.
- [14] M.W. Hentze, L.C. Kuhn, Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 8175-8182.
- [15] A. Grubman, A.R. White, Copper as a key regulator of cell signalling pathways, *Expert Rev. Mol. Med.* 16 (2014) e11.
- [16] M.L. Turski, D.J. Thiele, New roles for copper metabolism in cell proliferation, signaling, and disease, *J. Biol. Chem.* 284 (2009) 717-721.
- [17] S.V. Torti, F.M. Torti, Iron and cancer: more ore to be mined, *Nat. Rev. Cancer* 13 (2013) 342-355.
- [18] H.W. Kuo, S.F. Chen, C.C. Wu, D.R. Chen, J.H. Lee, Serum and tissue trace elements in patients with breast cancer in Taiwan, *Biol. Trace Elem. Res.* 89 (2002) 1-11.
- [19] S. Toyokuni, Role of iron in carcinogenesis: cancer as a ferrototoxic disease, *Cancer Sci.* 100 (2009) 9-16.
- [20] E.J. Margalioth, J.G. Schenker, M. Chevion, Copper and zinc levels in normal and malignant tissues, *Cancer* 52 (1983) 868-872.

- [21] A.M. Merlot, D.S. Kalinowski, D.R. Richardson, Novel chelators for cancer treatment: where are we now?, *Antioxid. Redox Signal.* 18 (2013) 973-1006.
- [22] L.L. Dunn, Y. Suryo Rahmanto, D.R. Richardson, Iron uptake and metabolism in the new millennium, *Trends Cell Biol.* 17 (2007) 93-100.
- [23] M.U. Muckenthaler, B. Galy, M.W. Hentze, Systemic iron homeostasis and the iron-responsive element/iron-regulatory protein (IRE/IRP) regulatory network, *Annu. Rev. Nutr.* 28 (2008) 197-213.
- [24] A. Lawen, D.J. Lane, Mammalian iron homeostasis in health and disease: uptake, storage, transport, and molecular mechanisms of action, *Antioxid. Redox Signal.* 18 (2013) 2473-2507.
- [25] A.U. Steinbicker, M.U. Muckenthaler, Out of balance--systemic iron homeostasis in iron-related disorders, *Nutrients* 5 (2013) 3034-3061.
- [26] P. Arosio, S. Levi, Cytosolic and mitochondrial ferritins in the regulation of cellular iron homeostasis and oxidative damage, *Biochim. Biophys. Acta* 1800 (2010) 783-792.
- [27] R. Lill, Function and biogenesis of iron-sulphur proteins, *Nature* 460 (2009) 831-838.
- [28] T.A. Rouault, Biogenesis of iron-sulfur clusters in mammalian cells: new insights and relevance to human disease, *Dis. Model. Mech.* 5 (2012) 155-164.
- [29] C.C. Philpott, M.S. Ryu, Special delivery: distributing iron in the cytosol of mammalian cells, *Front. Pharmacol.* 5 (2014) 173.
- [30] D.R. Richardson, D.J. Lane, E.M. Becker, M.L. Huang, M. Whitnall, Y. Suryo Rahmanto, A.D. Sheftel, P. Ponka, Mitochondrial iron trafficking and the integration of iron metabolism between the mitochondrion and cytosol, *Proc. Natl. Acad. Sci. U. S. A.* 107 (2010) 10775-10782.
- [31] B. Silva, P. Faustino, An overview of molecular basis of iron metabolism regulation and the associated pathologies, *Biochim. Biophys. Acta* 1852 (2015) 1347-1359.

- [32] S. Gulec, G.J. Anderson, J.F. Collins, Mechanistic and regulatory aspects of intestinal iron absorption, *Am. J. Physiol. Gastrointest. Liver Physiol.* 307 (2014) G397-409.
- [33] G.J. Anderson, D. Darshan, S.J. Wilkins, D.M. Frazer, Regulation of systemic iron homeostasis: how the body responds to changes in iron demand, *Biometals* 20 (2007) 665-674.
- [34] P.A. Sharp, Intestinal iron absorption: regulation by dietary & systemic factors, *Int. J. Vitam. Nutr. Res.* 80 (2010) 231-242.
- [35] A.H. Laftah, G.O. Latunde-Dada, S. Fakih, R.C. Hider, R.J. Simpson, A.T. McKie, Haem and folate transport by proton-coupled folate transporter/haem carrier protein 1 (SLC46A1), *Br. J. Nutr.* 101 (2009) 1150-1156.
- [36] M.T. Worthington, S.M. Cohn, S.K. Miller, R.Q. Luo, C.L. Berg, Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines, *Am. J. Physiol. Gastrointest. Liver Physiol.* 280 (2001) G1172-1177.
- [37] K. Inoue, Y. Nakai, S. Ueda, S. Kamigaso, K.Y. Ohta, M. Hatakeyama, Y. Hayashi, M. Otagiri, H. Yuasa, Functional characterization of PCFT/HCP1 as the molecular entity of the carrier-mediated intestinal folate transport system in the rat model, *Am. J. Physiol. Gastrointest. Liver Physiol.* 294 (2008) G660-668.
- [38] G. Wolf, Identification of proton-coupled high-affinity human intestinal folate transporter mutated in human hereditary familial folate malabsorption, *Nutr. Rev.* 65 (2007) 554-557.
- [39] R.B. Dawson, S. Rafal, L.R. Weintraub, Absorption of hemoglobin iron: the role of xanthine oxidase in the intestinal heme-splitting reaction, *Blood* 35 (1970) 94-103.
- [40] S. Pollack, R. Kaufman, W.H. Crosby, J.E. Butkiewicz, Reducing agents and absorption of iron, *Nature* 199 (1963) 384.
- [41] A.T. McKie, D. Barrow, G.O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, C. Richardson, D. Barlow, A. Bomford, T.J. Peters, K.B. Raja, S. Shirali,

- M.A. Hediger, F. Farzaneh, R.J. Simpson, An iron-regulated ferric reductase associated with the absorption of dietary iron, *Science* 291 (2001) 1755-1759.
- [42] D.J. Lane, D.H. Bae, A.M. Merlot, S. Sahni, D.R. Richardson, Duodenal cytochrome b (DCYTB) in iron metabolism: an update on function and regulation, *Nutrients* 7 (2015) 2274-2296.
- [43] R.S. Ohgami, D.R. Campagna, E.L. Greer, B. Antiochos, A. McDonald, J. Chen, J.J. Sharp, Y. Fujiwara, J.E. Barker, M.D. Fleming, Identification of a ferrireductase required for efficient transferrin-dependent iron uptake in erythroid cells, *Nat. Genet.* 37 (2005) 1264-1269.
- [44] J.M. May, Z.C. Qu, S. Mendiratta, Role of ascorbic acid in transferrin-independent reduction and uptake of iron by U-937 cells, *Biochem. Pharmacol.* 57 (1999) 1275-1282.
- [45] D.J. Lane, A. Lawen, Non-transferrin iron reduction and uptake are regulated by transmembrane ascorbate cycling in K562 cells, *J. Biol. Chem.* 283 (2008) 12701-12708.
- [46] D.J. Lane, S.R. Robinson, H. Czerwinska, G.M. Bishop, A. Lawen, Two routes of iron accumulation in astrocytes: ascorbate-dependent ferrous iron uptake via the divalent metal transporter (DMT1) plus an independent route for ferric iron, *Biochem. J.* 432 (2010) 123-132.
- [47] M.D. Fleming, C.C. Trenor, 3rd, M.A. Su, D. Foerzler, D.R. Beier, W.F. Dietrich, N.C. Andrews, Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene, *Nat. Genet.* 16 (1997) 383-386.
- [48] J.P. Liuzzi, F. Aydemir, H. Nam, M.D. Knutson, R.J. Cousins, Zip14 (Slc39a14) mediates non-transferrin-bound iron uptake into cells, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 13612-13617.

- [49] A. Donovan, C.A. Lima, J.L. Pinkus, G.S. Pinkus, L.I. Zon, S. Robine, N.C. Andrews, The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis, *Cell Metab.* 1 (2005) 191-200.
- [50] A.T. McKie, P. Marciani, A. Rolfs, K. Brennan, K. Wehr, D. Barrow, S. Miret, A. Bomford, T.J. Peters, F. Farzaneh, M.A. Hediger, M.W. Hentze, R.J. Simpson, A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation, *Mol. Cell* 5 (2000) 299-309.
- [51] H. Chen, Z.K. Attieh, T. Su, B.A. Syed, H. Gao, R.M. Alaeddine, T.C. Fox, J. Usta, C.E. Naylor, R.W. Evans, A.T. McKie, G.J. Anderson, C.D. Vulpe, Hephaestin is a ferroxidase that maintains partial activity in sex-linked anemia mice, *Blood* 103 (2004) 3933-3939.
- [52] C.D. Vulpe, Y.M. Kuo, T.L. Murphy, L. Cowley, C. Askwith, N. Libina, J. Gitschier, G.J. Anderson, Hephaestin, a ceruloplasmin homologue implicated in intestinal iron transport, is defective in the sla mouse, *Nat. Genet.* 21 (1999) 195-199.
- [53] D.R. Richardson, P. Ponka, The molecular mechanisms of the metabolism and transport of iron in normal and neoplastic cells, *Biochim. Biophys. Acta* 1331 (1997) 1-40.
- [54] M.W. Hentze, M.U. Muckenthaler, B. Galy, C. Camaschella, Two to tango: regulation of mammalian iron metabolism, *Cell* 142 (2010) 24-38.
- [55] D.J. Lane, D.R. Richardson, Chaperone turns gatekeeper: PCBP2 and DMT1 form an iron-transport pipeline, *Biochem. J.* 462 (2014) e1-3.
- [56] D.J. Lane, S. Chikhani, V. Richardson, D.R. Richardson, Transferrin iron uptake is stimulated by ascorbate via an intracellular reductive mechanism, *Biochim. Biophys. Acta* 1833 (2013) 1527-1541.
- [57] A. Escobar, V. Gaete, M.T. Nunez, Effect of ascorbate in the reduction of transferrin-associated iron in endocytic vesicles, *J. Bioenerg. Biomembr.* 24 (1992) 227-233.



- [58] H. Gunshin, B. Mackenzie, U.V. Berger, Y. Gunshin, M.F. Romero, W.F. Boron, S. Nussberger, J.L. Gollan, M.A. Hediger, Cloning and characterization of a mammalian proton-coupled metal-ion transporter, *Nature* 388 (1997) 482-488.
- [59] S. Jenkitkasemwong, C.Y. Wang, B. Mackenzie, M.D. Knutson, Physiologic implications of metal-ion transport by ZIP14 and ZIP8, *Biometals* 25 (2012) 643-655.
- [60] D. Trinder, E. Baker, Transferrin receptor 2: a new molecule in iron metabolism, *Int. J. Biochem. Cell Biol.* 35 (2003) 292-296.
- [61] D.R. Richardson, E. Baker, The uptake of iron and transferrin by the human malignant melanoma cell, *Biochim. Biophys. Acta* 1053 (1990) 1-12.
- [62] D. Trinder, O. Zak, P. Aisen, Transferrin receptor-independent uptake of different transferrin by human hepatoma cells with antisense inhibition of receptor expression, *Hepatology* 23 (1996) 1512-1520.
- [63] D.R. Richardson, E. Baker, Two saturable mechanisms of iron uptake from transferrin in human melanoma cells: the effect of transferrin concentration, chelators, and metabolic probes on transferrin and iron uptake, *J. Cell Physiol.* 161 (1994) 160-168.
- [64] D. Trinder, E. Morgan, E. Baker, The mechanisms of iron uptake by fetal rat hepatocytes in culture, *Hepatology* 6 (1986) 852-858.
- [65] N.F. Olivieri, G.M. Brittenham, Iron-chelating therapy and the treatment of thalassemia, *Blood* 89 (1997) 739-761.
- [66] M. Grootveld, J.D. Bell, B. Halliwell, O.I. Aruoma, A. Bomford, P.J. Sadler, Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis. Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy, *J. Biol. Chem.* 264 (1989) 4417-4422.
- [67] D.R. Richardson, A.C. Chua, E. Baker, Activation of an iron uptake mechanism from transferrin in hepatocytes by small-molecular-weight iron complexes: implications for the pathogenesis of iron-overload disease, *J. Lab. Clin. Med.* 133 (1999) 144-151.

- [68] D.R. Richardson, P. Ponka, Identification of a mechanism of iron uptake by cells which is stimulated by hydroxyl radicals generated via the iron-catalysed Haber-Weiss reaction, *Biochim. Biophys. Acta* 1269 (1995) 105-114.
- [69] R.S. Ohgami, D.R. Campagna, A. McDonald, M.D. Fleming, The Steap proteins are metalloreductases, *Blood* 108 (2006) 1388-1394.
- [70] A. Jacobs, Low molecular weight intracellular iron transport compounds, *Blood* 50 (1977) 433-439.
- [71] M. Kruszewski, Labile iron pool: the main determinant of cellular response to oxidative stress, *Mutat. Res.* 531 (2003) 81-92.
- [72] R.C. Hider, X.L. Kong, Glutathione: a key component of the cytoplasmic labile iron pool, *Biometals* 24 (2011) 1179-1187.
- [73] R.N. Watts, D.R. Richardson, Nitrogen monoxide (NO) and glucose: unexpected links between energy metabolism and no-mediated iron mobilization from cells, *J. Biol. Chem.* 276 (2001) 4724-4732.
- [74] D.R. Richardson, P. Ponka, D. Vyoral, Distribution of iron in reticulocytes after inhibition of heme synthesis with succinylacetone: examination of the intermediates involved in iron metabolism, *Blood* 87 (1996) 3477-3488.
- [75] A.D. Sheftel, A.S. Zhang, C. Brown, O.S. Shirihai, P. Ponka, Direct interorganellar transfer of iron from endosome to mitochondrion, *Blood* 110 (2007) 125-132.
- [76] S. Lutsenko, Human copper homeostasis: a network of interconnected pathways, *Curr. Opin. Chem. Biol.* 14 (2010) 211-217.
- [77] D.R. Richardson, Y. Suryo Rahmanto, Differential regulation of the Menkes and Wilson disease copper transporters by hormones: an integrated model of metal transport in the placenta, *Biochem. J.* 402 (2007) e1-3.

- [78] A. Nandal, J.C. Ruiz, P. Subramanian, S. Ghimire-Rijal, R.A. Sinnamon, T.L. Stemmler, R.K. Bruick, C.C. Philpott, Activation of the HIF prolyl hydroxylase by the iron chaperones PCBP1 and PCBP2, *Cell Metab.* 14 (2011) 647-657.
- [79] S. Leidgens, K.Z. Bullough, H. Shi, F. Li, M. Shakoury-Elizeh, T. Yabe, P. Subramanian, E. Hsu, N. Natarajan, A. Nandal, T.L. Stemmler, C.C. Philpott, Each member of the poly-r(C)-binding protein 1 (PCBP) family exhibits iron chaperone activity toward ferritin, *J. Biol. Chem.* 288 (2013) 17791-17802.
- [80] A.S. Zhang, A.D. Sheftel, P. Ponka, Intracellular kinetics of iron in reticulocytes: evidence for endosome involvement in iron targeting to mitochondria, *Blood* 105 (2005) 368-375.
- [81] P. Ponka, Tissue-specific regulation of iron metabolism and heme synthesis: distinct control mechanisms in erythroid cells, *Blood* 89 (1997) 1-25.
- [82] N.D. Chasteen, P.M. Harrison, Mineralization in ferritin: an efficient means of iron storage, *J. Struct. Biol.* 126 (1999) 182-194.
- [83] P.M. Harrison, P. Arosio, The ferritins: molecular properties, iron storage function and cellular regulation, *Biochim. Biophys. Acta* 1275 (1996) 161-203.
- [84] T. Kurz, A. Terman, U.T. Brunk, Autophagy, ageing and apoptosis: the role of oxidative stress and lysosomal iron, *Arch. Biochem. Biophys.* 462 (2007) 220-230.
- [85] Y. Zhang, M. Mikhael, D. Xu, Y. Li, S. Soe-Lin, B. Ning, W. Li, G. Nie, Y. Zhao, P. Ponka, Lysosomal proteolysis is the primary degradation pathway for cytosolic ferritin and cytosolic ferritin degradation is necessary for iron exit, *Antioxid. Redox Signal.* 13 (2010) 999-1009.
- [86] J.D. Mancias, X. Wang, S.P. Gygi, J.W. Harper, A.C. Kimmelman, Quantitative proteomics identifies NCOA4 as the cargo receptor mediating ferritinophagy, *Nature* 509 (2014) 105-109.

- [87] T. Kurz, A. Terman, B. Gustafsson, U.T. Brunk, Lysosomes in iron metabolism, ageing and apoptosis, *Histochem. Cell Biol.* 129 (2008) 389-406.
- [88] J. Drysdale, P. Arosio, R. Invernizzi, M. Cazzola, A. Volz, B. Corsi, G. Biasiotto, S. Levi, Mitochondrial ferritin: a new player in iron metabolism, *Blood Cells Mol. Dis.* 29 (2002) 376-383.
- [89] S. Levi, B. Corsi, M. Bosisio, R. Invernizzi, A. Volz, D. Sanford, P. Arosio, J. Drysdale, A human mitochondrial ferritin encoded by an intronless gene, *J. Biol. Chem.* 276 (2001) 24437-24440.
- [90] M. Cazzola, R. Invernizzi, G. Bergamaschi, S. Levi, B. Corsi, E. Travaglino, V. Rolandi, G. Biasiotto, J. Drysdale, P. Arosio, Mitochondrial ferritin expression in erythroid cells from patients with sideroblastic anemia, *Blood* 101 (2003) 1996-2000.
- [91] B. Corsi, A. Cozzi, P. Arosio, J. Drysdale, P. Santambrogio, A. Campanella, G. Biasiotto, A. Albertini, S. Levi, Human mitochondrial ferritin expressed in HeLa cells incorporates iron and affects cellular iron metabolism, *J. Biol. Chem.* 277 (2002) 22430-22437.
- [92] G. Nie, G. Chen, A.D. Sheftel, K. Pantopoulos, P. Ponka, In vivo tumor growth is inhibited by cytosolic iron deprivation caused by the expression of mitochondrial ferritin, *Blood* 108 (2006) 2428-2434.
- [93] M.L. Huang, E.M. Becker, M. Whitnall, Y. Suryo Rahmanto, P. Ponka, D.R. Richardson, Elucidation of the mechanism of mitochondrial iron loading in Friedreich's ataxia by analysis of a mouse mutant, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 16381-16386.
- [94] M. Whitnall, Y. Suryo Rahmanto, M.L. Huang, F. Saletta, H.C. Lok, L. Gutierrez, F.J. Lazaro, A.J. Fleming, T.G. St Pierre, M.R. Mikhael, P. Ponka, D.R. Richardson, Identification of nonferritin mitochondrial iron deposits in a mouse model of Friedreich ataxia, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 20590-20595.

- [95] M.W. Hentze, M.U. Muckenthaler, N.C. Andrews, Balancing acts: molecular control of mammalian iron metabolism, *Cell* 117 (2004) 285-297.
- [96] T.A. Rouault, The role of iron regulatory proteins in mammalian iron homeostasis and disease, *Nat. Chem. Biol.* 2 (2006) 406-414.
- [97] B. Guo, J.D. Phillips, Y. Yu, E.A. Leibold, Iron regulates the intracellular degradation of iron regulatory protein 2 by the proteasome, *J. Biol. Chem.* 270 (1995) 21645-21651.
- [98] A.A. Vashisht, K.B. Zumbrennen, X. Huang, D.N. Powers, A. Durazo, D. Sun, N. Bhaskaran, A. Persson, M. Uhlen, O. Sangfelt, C. Spruck, E.A. Leibold, J.A. Wohlschlegel, Control of iron homeostasis by an iron-regulated ubiquitin ligase, *Science* 326 (2009) 718-721.
- [99] A.A. Salahudeen, J.W. Thompson, J.C. Ruiz, H.W. Ma, L.N. Kinch, Q. Li, N.V. Grishin, R.K. Bruick, An E3 ligase possessing an iron-responsive hemerythrin domain is a regulator of iron homeostasis, *Science* 326 (2009) 722-726.
- [100] T. Ganz, Heparin and iron regulation, 10 years later, *Blood* 117 (2011) 4425-4433.
- [101] C.H. Park, E.V. Valore, A.J. Waring, T. Ganz, Heparin, a urinary antimicrobial peptide synthesized in the liver, *J. Biol. Chem.* 276 (2001) 7806-7810.
- [102] A. Krause, S. Neitz, H.J. Magert, A. Schulz, W.G. Forssmann, P. Schulz-Knappe, K. Adermann, LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity, *FEBS Lett.* 480 (2000) 147-150.
- [103] T. Ganz, Molecular pathogenesis of anemia of chronic disease, *Pediatr. Blood Cancer* 46 (2006) 554-557.
- [104] G. Weiss, Iron metabolism in the anemia of chronic disease, *Biochim. Biophys. Acta* 1790 (2009) 682-693.

- [105] E. Nemeth, S. Rivera, V. Gabayan, C. Keller, S. Taudorf, B.K. Pedersen, T. Ganz, IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin, *J. Clin. Invest.* 113 (2004) 1271-1276.
- [106] E. Nemeth, E.V. Valore, M. Territo, G. Schiller, A. Lichtenstein, T. Ganz, Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein, *Blood* 101 (2003) 2461-2463.
- [107] I. Theurl, A. Schroll, T. Sonnweber, M. Nairz, M. Theurl, W. Willenbacher, K. Eller, D. Wolf, M. Seifert, C.C. Sun, J.L. Babitt, C.C. Hong, T. Menhall, P. Gearing, H.Y. Lin, G. Weiss, Pharmacologic inhibition of hepcidin expression reverses anemia of chronic inflammation in rats, *Blood* 118 (2011) 4977-4984.
- [108] M.L. Huang, C.J. Austin, M.A. Sari, Y.S. Rahmanto, P. Ponka, D. Vyoral, D.R. Richardson, Hepcidin bound to alpha2-macroglobulin reduces ferroportin-1 expression and enhances its activity at reducing serum iron levels, *J. Biol. Chem.* 288 (2013) 25450-25465.
- [109] G. Peslova, J. Petrak, K. Kuzelova, I. Hrdy, P. Halada, P.W. Kuchel, S. Soe-Lin, P. Ponka, R. Sutak, E. Becker, M.L. Huang, Y. Suryo Rahmanto, D.R. Richardson, D. Vyoral, Hepcidin, the hormone of iron metabolism, is bound specifically to alpha-2-macroglobulin in blood, *Blood* 113 (2009) 6225-6236.
- [110] E. Pandur, J. Nagy, V.S. Poor, A. Sarnyai, A. Huszar, A. Miseta, K. Sipos, Alpha-1 antitrypsin binds preprohepcidin intracellularly and prohepcidin in the serum, *FEBS J.* 276 (2009) 2012-2021.
- [111] W. Borth, Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics, *FASEB J.* 6 (1992) 3345-3353.
- [112] S. La Fontaine, M.L. Ackland, J.F. Mercer, Mammalian copper-transporting P-type ATPases, ATP7A and ATP7B: emerging roles, *Int. J. Biochem. Cell. Biol.* 42 (2010) 206-209.

- [113] R.F. Crampton, D.M. Matthews, R. Poisner, Observations on the mechanism of absorption of copper by the small intestine, *J. Physiol.* 178 (1965) 111-126.
- [114] B. Lonnerdal, Bioavailability of copper, *Am. J. Clin. Nutr.* 63 (1996) 821S-829S.
- [115] M.C. Linder, M. Hazegh-Azam, Copper biochemistry and molecular biology, *Am. J. Clin. Nutr.* 63 (1996) 797S-811S.
- [116] E.D. Harris, Cellular copper transport and metabolism, *Annu. Rev. Nutr.* 20 (2000) 291-310.
- [117] J. Lee, M.J. Petris, D.J. Thiele, Characterization of mouse embryonic cells deficient in the *ctr1* high affinity copper transporter. Identification of a *Ctr1*-independent copper transport system, *J. Biol. Chem.* 277 (2002) 40253-40259.
- [118] M. Arredondo, P. Munoz, C.V. Mura, M.T. Nunez, DMT1, a physiologically relevant apical  $\text{Cu}^{1+}$  transporter of intestinal cells, *Am. J. Physiol. Cell Physiol.* 284 (2003) C1525-1530.
- [119] M. Arredondo, M.J. Mendiburo, S. Flores, S.T. Singleton, M.D. Garrick, Mouse divalent metal transporter 1 is a copper transporter in HEK293 cells, *Biometals* 27 (2014) 115-123.
- [120] B.G. Blair, C.A. Larson, R. Safaei, S.B. Howell, Copper transporter 2 regulates the cellular accumulation and cytotoxicity of Cisplatin and Carboplatin, *Clin. Cancer Res.* 15 (2009) 4312-4321.
- [121] T. Wang, Z. Guo, Copper in medicine: Homeostasis, chelation therapy and antitumor drug design, *Curr. Med. Chem.* 13 (2006) 525-537.
- [122] N.K. Wee, D.C. Weinstein, S.T. Fraser, S.J. Assinder, The mammalian copper transporters *CTR1* and *CTR2* and their roles in development and disease, *Int. J. Biochem. Cell Biol.* 45 (2013) 960-963.

- [123] T.Z. Kidane, R. Farhad, K.J. Lee, A. Santos, E. Russo, M.C. Linder, Uptake of copper from plasma proteins in cells where expression of CTR1 has been modulated, *Biometals* 25 (2012) 697-709.
- [124] M.D. Knutson, Steap proteins: implications for iron and copper metabolism, *Nutr. Rev.* 65 (2007) 335-340.
- [125] B.E. Kim, T. Nevitt, D.J. Thiele, Mechanisms for copper acquisition, distribution and regulation, *Nat. Chem. Biol.* 4 (2008) 176-185.
- [126] M. Valko, H. Morris, M.T. Cronin, Metals, toxicity and oxidative stress, *Curr. Med. Chem.* 12 (2005) 1161-1208.
- [127] K.A. Markossian, B.I. Kurganov, Copper chaperones, intracellular copper trafficking proteins. Function, structure, and mechanism of action, *Biochem.* 68 (2003) 827-837.
- [128] D.R. Richardson, Role of ceruloplasmin and ascorbate in cellular iron release, *J. Lab. Clin. Med.* 134 (1999) 454-465.
- [129] M. Arredondo, M.T. Nunez, Iron and copper metabolism, *Mol. Aspects Med.* 26 (2005) 313-327.
- [130] N.E. Hellman, J.D. Gitlin, Ceruloplasmin metabolism and function, *Annu. Rev. Nutr.* 22 (2002) 439-458.
- [131] S. Lutsenko, N.L. Barnes, M.Y. Bartee, O.Y. Dmitriev, Function and regulation of human copper-transporting ATPases, *Physiol. Rev.* 87 (2007) 1011-1046.
- [132] L. Nyasae, R. Bustos, L. Braiterman, B. Eipper, A. Hubbard, Dynamics of endogenous ATP7A (Menkes protein) in intestinal epithelial cells: copper-dependent redistribution between two intracellular sites, *Am. J. Physiol. Gastrointest. Liver Physiol.* 292 (2007) G1181-1194.
- [133] J.R. Prohaska, Role of copper transporters in copper homeostasis, *Am. J. Clin. Nutr.* 88 (2008) 826S-829S.



- [134] S.C. Leary, B.A. Kaufman, G. Pellicchia, G.H. Guercin, A. Mattman, M. Jaksch, E.A. Shoubridge, Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase, *Hum. Mol. Genet.* 13 (2004) 1839-1848.
- [135] L. Yang, R. McRae, M.M. Henary, R. Patel, B. Lai, S. Vogt, C.J. Fahrni, Imaging of the intracellular topography of copper with a fluorescent sensor and by synchrotron x-ray fluorescence microscopy, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 11179-11184.
- [136] P.V. van den Berghe, D.E. Folmer, H.E. Malingre, E. van Beurden, A.E. Klomp, B. van de Sluis, M. Merckx, R. Berger, L.W. Klomp, Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake, *Biochem. J.* 407 (2007) 49-59.
- [137] P.J. Jansson, T. Yamagishi, A. Arvind, N. Seebacher, E. Gutierrez, A. Stacy, S. Maleki, D. Sharp, S. Sahni, D.R. Richardson, Di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) overcomes multidrug resistance by a novel mechanism involving the hijacking of lysosomal P-glycoprotein (Pgp), *J. Biol. Chem.* 290 (2015) 9588-9603.
- [138] D.B. Lovejoy, P.J. Jansson, U.T. Brunk, J. Wong, P. Ponka, D.R. Richardson, Antitumor activity of metal-chelating compound Dp44mT is mediated by formation of a redox-active copper complex that accumulates in lysosomes, *Cancer Res.* 71 (2011) 5871-5880.
- [139] P.A. Cobine, L.D. Ojeda, K.M. Rigby, D.R. Winge, Yeast contain a non-proteinaceous pool of copper in the mitochondrial matrix, *J. Biol. Chem.* 279 (2004) 14447-14455.
- [140] E.M. Rees, D.J. Thiele, Identification of a vacuole-associated metalloreductase and its role in Ctr2-mediated intracellular copper mobilization, *J. Biol. Chem.* 282 (2007) 21629-21638.

- [141] L.B. Moller, C. Petersen, C. Lund, N. Horn, Characterization of the hCTR1 gene: genomic organization, functional expression, and identification of a highly homologous processed gene, *Gene* 257 (2000) 13-22.
- [142] M. Lenartowicz, W. Krzeptowski, Structure and function of ATP7A and ATP7B proteins-Cu-transporting ATPases, *Postepy Biochem.* 56 (2010) 317-327.
- [143] S.G. Kaler, ATP7A-related copper transport diseases-emerging concepts and future trends, *Nat. Rev. Neurol.* 7 (2011) 15-29.
- [144] S. La Fontaine, J.F. Mercer, Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis, *Arch. Biochem. Biophys.* 463 (2007) 149-167.
- [145] J. Telianidis, Y.H. Hung, S. Materia, S.L. Fontaine, Role of the P-Type ATPases, ATP7A and ATP7B in brain copper homeostasis, *Front. Aging Neurosci.* 5 (2013) 44.
- [146] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, *Cell* 100 (2000) 57-70.
- [147] U. Jungwirth, C.R. Kowol, B.K. Keppler, C.G. Hartinger, W. Berger, P. Heffeter, Anticancer activity of metal complexes: involvement of redox processes, *Antioxid. Redox Signal.* 15 (2011) 1085-1127.
- [148] P.T. Lieu, M. Heiskala, P.A. Peterson, Y. Yang, The roles of iron in health and disease, *Mol. Aspects Med.* 22 (2001) 1-87.
- [149] W.H. Koppenol, The Haber-Weiss cycle-70 years later, *Redox Rep.* 6 (2001) 229-234.
- [150] T. Theophanides, J. Anastassopoulou, Copper and carcinogenesis, *Crit. Rev. Oncol. Hematol.* 42 (2002) 57-64.
- [151] K.J. Barnham, C.L. Masters, A.I. Bush, Neurodegenerative diseases and oxidative stress, *Nat. Rev. Drug Discov.* 3 (2004) 205-214.
- [152] M. Dizdaroglu, P. Jaruga, Mechanisms of free radical-induced damage to DNA, *Free Radic. Res.* 46 (2012) 382-419.

- [153] E.R. Stadtman, Metal ion-catalyzed oxidation of proteins: biochemical mechanism and biological consequences, *Free Radic. Biol. Med.* 9 (1990) 315-325.
- [154] G. Alper, V. Narayanan, Friedreich's ataxia, *Pediatr. Neurol.* 28 (2003) 335-341.
- [155] S.L. Schrier, F. Centis, M. Verneris, L. Ma, E. Angelucci, The role of oxidant injury in the pathophysiology of human thalassemias, *Redox Rep.* 8 (2003) 241-245.
- [156] A. Wong, J. Yang, P. Cavadini, C. Gellera, B. Lonnerdal, F. Taroni, G. Cortopassi, The Friedreich's ataxia mutation confers cellular sensitivity to oxidant stress which is rescued by chelators of iron and calcium and inhibitors of apoptosis, *Hum. Mol. Genet.* 8 (1999) 425-430.
- [157] P.C. Bull, D.W. Cox, Wilson disease and Menkes disease: new handles on heavy-metal transport, *Trends Genet.* 10 (1994) 246-252.
- [158] L. Jiang, S. Akatsuka, H. Nagai, S.H. Chew, H. Ohara, Y. Okazaki, Y. Yamashita, Y. Yoshikawa, H. Yasui, K. Ikuta, K. Sasaki, Y. Kohgo, S. Hirano, Y. Shinohara, N. Kohyama, T. Takahashi, S. Toyokuni, Iron overload signature in chrysotile-induced malignant mesothelioma, *J. Pathol.* 228 (2012) 366-377.
- [159] T. Wu, C.T. Sempos, J.L. Freudenheim, P. Muti, E. Smit, Serum iron, copper and zinc concentrations and risk of cancer mortality in US adults, *Ann. Epidemiol.* 14 (2004) 195-201.
- [160] P. Knekt, A. Reunanen, H. Takkunen, A. Aromaa, M. Heliovaara, T. Hakulinen, Body iron stores and risk of cancer, *Int. J. Cancer* 56 (1994) 379-382.
- [161] R.L. Nelson, Iron and colorectal cancer risk: human studies, *Nutr. Rev.* 59 (2001) 140-148.
- [162] S. Akatsuka, Y. Yamashita, H. Ohara, Y.T. Liu, M. Izumiya, K. Abe, M. Ochiai, L. Jiang, H. Nagai, Y. Okazaki, H. Murakami, Y. Sekido, E. Arai, Y. Kanai, O. Hino, T. Takahashi, H. Nakagama, S. Toyokuni, Fenton reaction induced cancer in wild type

- rats recapitulates genomic alterations observed in human cancer, *PloS One* 7 (2012) e43403.
- [163] A.C. Chua, B.R. Klopčič, D.S. Ho, S.K. Fu, C.H. Forrest, K.D. Croft, J.K. Olynyk, I.C. Lawrance, D. Trinder, Dietary iron enhances colonic inflammation and IL-6/IL-11-Stat3 signaling promoting colonic tumor development in mice, *PloS One* 8 (2013) e78850.
- [164] T. Norat, A. Lukanova, P. Ferrari, E. Riboli, Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies, *Int. J. Cancer* 98 (2002) 241-256.
- [165] I.A. van Asperen, E.J. Feskens, C.H. Bowles, D. Kromhout, Body iron stores and mortality due to cancer and ischaemic heart disease: a 17-year follow-up study of elderly men and women, *Int. J. Epidemiol.* 24 (1995) 665-670.
- [166] G. Edgren, M. Reilly, H. Hjalgrim, T.N. Tran, K. Rostgaard, J. Adami, K. Titlestad, A. Shanwell, M. Melbye, O. Nyren, Donation frequency, iron loss, and risk of cancer among blood donors, *J. Natl. Cancer Inst.* 100 (2008) 572-579.
- [167] H.Y. Rienhoff, Jr., Iron-overload-related disease in HFE hereditary hemochromatosis, *N. Engl. J. Med.* 358 (2008) 2294; author reply 2294-2295.
- [168] R.A. Bradbear, C. Bain, V. Siskind, F.D. Schofield, S. Webb, E.M. Axelsen, J.W. Halliday, M.L. Bassett, L.W. Powell, Cohort study of internal malignancy in genetic hemochromatosis and other chronic nonalcoholic liver diseases, *J. Natl. Cancer Inst.* 75 (1985) 81-84.
- [169] M. Elmberg, R. Hultcrantz, A. Ekbom, L. Brandt, S. Olsson, R. Olsson, S. Lindgren, L. Loof, P. Stal, S. Wallerstedt, S. Almer, H. Sandberg-Gertzen, J. Askling, Cancer risk in patients with hereditary hemochromatosis and in their first-degree relatives, *Gastroenterology* 125 (2003) 1733-1741.

- [170] J. Kato, K. Miyanishi, M. Kobune, T. Nakamura, K. Takada, R. Takimoto, Y. Kawano, S. Takahashi, M. Takahashi, Y. Sato, T. Takayama, Y. Niitsu, Long-term phlebotomy with low-iron diet therapy lowers risk of development of hepatocellular carcinoma from chronic hepatitis C, *J. Gastroenterol.* 42 (2007) 830-836.
- [171] J.C. Kwok, D.R. Richardson, The iron metabolism of neoplastic cells: alterations that facilitate proliferation?, *Crit. Rev. Oncol. Hematol.* 42 (2002) 65-78.
- [172] N.T. Le, D.R. Richardson, The role of iron in cell cycle progression and the proliferation of neoplastic cells, *Biochim. Biophys. Acta* 1603 (2002) 31-46.
- [173] T.R. Daniels, E. Bernabeu, J.A. Rodriguez, S. Patel, M. Kozman, D.A. Chiappetta, E. Holler, J.Y. Ljubimova, G. Helguera, M.L. Penichet, The transferrin receptor and the targeted delivery of therapeutic agents against cancer, *Biochim. Biophys. Acta* 1820 (2012) 291-317.
- [174] J. Boulton, K. Roberts, M.J. Brookes, S. Hughes, J.P. Bury, S.S. Cross, G.J. Anderson, R. Spychal, T. Iqbal, C. Tselepis, Overexpression of cellular iron import proteins is associated with malignant progression of esophageal adenocarcinoma, *Clin. Cancer Res.* 14 (2008) 379-387.
- [175] M. Prutki, M. Poljak-Blazi, M. Jakopovic, D. Tomas, I. Stipancic, N. Zarkovic, Altered iron metabolism, transferrin receptor 1 and ferritin in patients with colon cancer, *Cancer Lett.* 238 (2006) 188-196.
- [176] I.M. Gomes, C.J. Maia, C.R. Santos, STEAP proteins: from structure to applications in cancer therapy, *Mol. Cancer Res.* 10 (2012) 573-587.
- [177] S. Zhang, Y. Chen, W. Guo, L. Yuan, D. Zhang, Y. Xu, E. Nemeth, T. Ganz, S. Liu, Disordered hepcidin-ferroportin signaling promotes breast cancer growth, *Cell Signal.* 26 (2014) 2539-2550.

- [178] E. Nemeth, M.S. Tuttle, J. Powelson, M.B. Vaughn, A. Donovan, D.M. Ward, T. Ganz, J. Kaplan, Heparin regulates cellular iron efflux by binding to ferroportin and inducing its internalization, *Science* 306 (2004) 2090-2093.
- [179] S. Rivera, L. Liu, E. Nemeth, V. Gabayan, O.E. Sorensen, T. Ganz, Heparin excess induces the sequestration of iron and exacerbates tumor-associated anemia, *Blood* 105 (2005) 1797-1802.
- [180] A.C. Chua, R.M. Graham, D. Trinder, J.K. Olynyk, The regulation of cellular iron metabolism, *Crit. Rev. Clin. Lab. Sci.* 44 (2007) 413-459.
- [181] Z.K. Pinnix, L.D. Miller, W. Wang, R. D'Agostino, Jr., T. Kute, M.C. Willingham, H. Hatcher, L. Tesfay, G. Sui, X. Di, S.V. Torti, F.M. Torti, Ferroportin and iron regulation in breast cancer progression and prognosis, *Sci. Transl. Med.* 2 (2010) 43ra56.
- [182] S.I. Shpileva, V.P. Tryndyak, O. Kovalchuk, A. Starlard-Davenport, V.F. Chekhun, F.A. Beland, I.P. Pogribny, Role of ferritin alterations in human breast cancer cells, *Breast Cancer Res. Treat.* 126 (2011) 63-71.
- [183] M.G. Tan, M.P. Kumarasinghe, S.M. Wang, L.L. Ooi, S.E. Aw, K.M. Hui, Modulation of iron-regulatory genes in human hepatocellular carcinoma and its physiological consequences, *Exp. Biol. Med.* 234 (2009) 693-702.
- [184] N. Milman, L.M. Pedersen, The serum ferritin concentration is a significant prognostic indicator of survival in primary lung cancer, *Oncol. Rep.* 9 (2002) 193-198.
- [185] M. Lorenzi, B. Lorenzi, R. Vernillo, Serum ferritin in colorectal cancer patients and its prognostic evaluation, *Int. J. Biol. Markers* 21 (2006) 235-241.
- [186] D.C. Yang, F. Wang, R.L. Elliott, J.F. Head, Expression of transferrin receptor and ferritin H-chain mRNA are associated with clinical and histopathological prognostic indicators in breast cancer, *Anticancer Res.* 21 (2001) 541-549.

- [187] S. Radulescu, M.J. Brookes, P. Salgueiro, R.A. Ridgway, E. McGhee, K. Anderson, S.J. Ford, D.H. Stones, T.H. Iqbal, C. Tselepis, O.J. Sansom, Luminal iron levels govern intestinal tumorigenesis after Apc loss in vivo, *Cell Rep.* 2 (2012) 270-282.
- [188] K.J. Wu, A. Polack, R. Dalla-Favera, Coordinated regulation of iron-controlling genes, H-ferritin and IRP2, by c-MYC, *Science* 283 (1999) 676-679.
- [189] Y. Funouchi, C. Tanikawa, P.H. Yi Lo, J. Mori, Y. Daigo, A. Takano, Y. Miyagi, A. Okawa, Y. Nakamura, K. Matsuda, Regulation of iron homeostasis by the p53-ISCU pathway, *Sci. Rep.* 5 (2015) 16497.
- [190] L.G. Coffman, D. Parsonage, R. D'Agostino, Jr., F.M. Torti, S.V. Torti, Regulatory effects of ferritin on angiogenesis, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 570-575.
- [191] D.R. Richardson, K. Milnes, The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents II: The mechanism of action of ligands derived from salicylaldehyde benzoyl hydrazone and 2-hydroxy-1-naphthylaldehyde benzoyl hydrazone, *Blood* 89 (1997) 3025-3038.
- [192] J. Gao, D.R. Richardson, The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents, IV: The mechanisms involved in inhibiting cell-cycle progression, *Blood* 98 (2001) 842-850.
- [193] M. Kolberg, K.R. Strand, P. Graff, K.K. Andersson, Structure, function, and mechanism of ribonucleotide reductases, *Biochim. Biophys. Acta* 1699 (2004) 1-34.
- [194] H.L. Elford, M. Freese, E. Passamani, H.P. Morris, Ribonucleotide reductase and cell proliferation. I. Variations of ribonucleotide reductase activity with tumor growth rate in a series of rat hepatomas, *J. Biol. Chem.* 245 (1970) 5228-5233.
- [195] S.X. Liang, D.R. Richardson, The effect of potent iron chelators on the regulation of p53: examination of the expression, localization and DNA-binding activity of p53 and the transactivation of WAF1, *Carcinogenesis* 24 (2003) 1601-1614.

- [196] Y. Yu, Z. Kovacevic, D.R. Richardson, Tuning cell cycle regulation with an iron key, *Cell Cycle* 6 (2007) 1982-1994.
- [197] N.T. Le, D.R. Richardson, Iron chelators with high antiproliferative activity up-regulate the expression of a growth inhibitory and metastasis suppressor gene: A link between iron metabolism and proliferation, *Blood* 104 (2004) 2967-2975.
- [198] K. Masuda, M. Ono, M. Okamoto, W. Morikawa, M. Otsubo, T. Migita, M. Tsuneyoshi, H. Okuda, T. Shuin, S. Naito, M. Kuwano, Downregulation of Cap43 gene by von Hippel-Lindau tumor suppressor protein in human renal cancer cells, *Int. J. Cancer* 105 (2003) 803-810.
- [199] D.J. Lane, F. Saletta, Y. Suryo Rahmanto, Z. Kovacevic, D.R. Richardson, N-myc downstream regulated 1 (NDRG1) is regulated by eukaryotic initiation factor 3a (eIF3a) during cellular stress caused by iron depletion, *PLoS One* 8 (2013) e57273.
- [200] J. Hwang, H.S. Kim, B.S. Kang, D.H. Kim, Z.Y. Ryoo, S.U. Choi, S. Lee, RGS19 converts iron deprivation stress into a growth-inhibitory signal, *Biochem. Biophys. Res. Commun.* 464 (2015) 168-175.
- [201] I. Kvietikova, R.H. Wenger, H.H. Marti, M. Gassmann, The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site, *Nucleic Acids Res.* 23 (1995) 4542-4550.
- [202] S. Bandyopadhyay, S.K. Pai, S.C. Gross, S. Hirota, S. Hosobe, K. Miura, K. Saito, T. Commes, S. Hayashi, M. Watabe, K. Watabe, The Drg-1 gene suppresses tumor metastasis in prostate cancer, *Cancer Res.* 63 (2003) 1731-1736.
- [203] S. Bandyopadhyay, S.K. Pai, S. Hirota, S. Hosobe, Y. Takano, K. Saito, D. Piquemal, T. Commes, M. Watabe, S.C. Gross, Y. Wang, S. Ran, K. Watabe, Role of the putative tumor metastasis suppressor gene Drg-1 in breast cancer progression, *Oncogene* 23 (2004) 5675-5681.



- [204] M.A. Shah, N. Kemeny, A. Hummer, M. Drobnjak, M. Motwani, C. Cordon-Cardo, M. Gonen, G.K. Schwartz, Drg1 expression in 131 colorectal liver metastases: correlation with clinical variables and patient outcomes, *Clin. Cancer Res.* 11 (2005) 3296-3302.
- [205] J. Blaes, M. Weiler, F. Sahm, B. Hentschel, M. Osswald, M. Czabanka, C.M. Thome, M.G. Schliesser, S. Pusch, S. Luger, F. Winkler, A. Radbruch, M. Jugold, M. Simon, J.P. Steinbach, G. Schackert, M. Tatagiba, M. Westphal, J.C. Tonn, D. Gramatzki, T. Pietsch, C. Hartmann, H. Glimm, P. Vajkoczy, A. von Deimling, M. Platten, M. Weller, W. Wick, NDRG1 prognosticates the natural course of disease in WHO grade II glioma, *J. Neurooncol.* 117 (2014) 25-32.
- [206] K. Matsushita, K. Uchida, S. Saigusa, S. Ide, K. Hashimoto, Y. Koike, K. Otake, M. Inoue, K. Tanaka, M. Kusunoki, Low NDRG1 mRNA expression predicts a poor prognosis in neuroblastoma patients, *Pediatr. Surg. Int.* 29 (2013) 363-368.
- [207] M. Dos Santos, A.M. da Cunha Mercante, F.D. Nunes, A.M. Leopoldino, M.B. de Carvalho, D. Gazito, R.V. Lopez, P.B. Chiappini, P.B. de Carvalho Neto, E.E. Fukuyama, E.H. Tajara, I.D. Louro, A.M. da Silva, Prognostic significance of NDRG1 expression in oral and oropharyngeal squamous cell carcinoma, *Mol. Biol. Rep.* 39 (2012) 10157-10165.
- [208] F. Hosoi, H. Izumi, A. Kawahara, Y. Murakami, H. Kinoshita, M. Kage, K. Nishio, K. Kohno, M. Kuwano, M. Ono, N-myc downstream regulated gene 1/Cap43 suppresses tumor growth and angiogenesis of pancreatic cancer through attenuation of inhibitor of kappaB kinase beta expression, *Cancer Res.* 69 (2009) 4983-4991.
- [209] X. Wangpu, X. Yang, J. Zhao, J. Lu, S. Guan, Z. Kovacevic, W. Liu, L. Mi, R. Jin, J. Sun, F. Yue, J. Ma, A. Lu, D.R. Richardson, L. Wang, M. Zheng, The metastasis suppressor, NDRG1, inhibits "stemness" of colorectal cancer via down-regulation of nuclear beta-catenin and CD44, *Oncotarget* 6 (2015) 33893-33911.

- [210] B.A. Fang, Z. Kovacevic, K.C. Park, D.S. Kalinowski, P.J. Jansson, D.J. Lane, S. Sahni, D.R. Richardson, Molecular functions of the iron-regulated metastasis suppressor, NDRG1, and its potential as a molecular target for cancer therapy, *Biochim. Biophys. Acta* 1845 (2014) 1-19.
- [211] W.J. Lu, M.S. Chua, W. Wei, S.K. So, NDRG1 promotes growth of hepatocellular carcinoma cells by directly interacting with GSK-3beta and Nur77 to prevent beta-catenin degradation, *Oncotarget* 6 (2015) 29847-29859.
- [212] G.Y. Lui, Z. Kovacevic, V. Richardson, A.M. Merlot, D.S. Kalinowski, D.R. Richardson, Targeting cancer by binding iron: Dissecting cellular signaling pathways, *Oncotarget* 6 (2015) 18748-18779.
- [213] Y. Murakami, F. Hosoi, H. Izumi, Y. Maruyama, H. Ureshino, K. Watari, K. Kohno, M. Kuwano, M. Ono, Identification of sites subjected to serine/threonine phosphorylation by SGK1 affecting N-myc downstream-regulated gene 1 (NDRG1)/Cap43-dependent suppression of angiogenic CXC chemokine expression in human pancreatic cancer cells, *Biochem. Biophys. Res. Commun.* 396 (2010) 376-381.
- [214] W. Liu, F. Yue, M. Zheng, A. Merlot, D.H. Bae, M. Huang, D. Lane, P. Jansson, G.Y. Lui, V. Richardson, S. Sahni, D. Kalinowski, Z. Kovacevic, D.R. Richardson, The proto-oncogene c-Src and its downstream signaling pathways are inhibited by the metastasis suppressor, NDRG1, *Oncotarget* 6 (2015) 8851-8874.
- [215] R. Jin, W. Liu, S. Menezes, F. Yue, M. Zheng, Z. Kovacevic, D.R. Richardson, The metastasis suppressor NDRG1 modulates the phosphorylation and nuclear translocation of beta-catenin through mechanisms involving FRAT1 and PAK4, *J. Cell Sci.* 127 (2014) 3116-3130.
- [216] K.M. Dixon, G.Y. Lui, Z. Kovacevic, D. Zhang, M. Yao, Z. Chen, Q. Dong, S.J. Assinder, D.R. Richardson, Dp44mT targets the AKT, TGF-beta and ERK pathways

- via the metastasis suppressor NDRG1 in normal prostate epithelial cells and prostate cancer cells, *Br. J. Cancer* 108 (2013) 409-419.
- [217] Z. Chen, D. Zhang, F. Yue, M. Zheng, Z. Kovacevic, D.R. Richardson, The iron chelators Dp44mT and DFO inhibit TGF-beta-induced epithelial-mesenchymal transition via up-regulation of N-Myc downstream-regulated gene 1 (NDRG1), *J. Biol. Chem.* 287 (2012) 17016-17028.
- [218] Z. Kovacevic, S. Chikhani, G.Y. Lui, S. Sivagurunathan, D.R. Richardson, The iron-regulated metastasis suppressor NDRG1 targets NEDD4L, PTEN, and SMAD4 and inhibits the PI3K and Ras signaling pathways, *Antioxid. Redox Signal.* 18 (2013) 874-887.
- [219] J. Sun, D. Zhang, Y. Zheng, Q. Zhao, M. Zheng, Z. Kovacevic, D.R. Richardson, Targeting the metastasis suppressor, NDRG1, using novel iron chelators: regulation of stress fiber-mediated tumor cell migration via modulation of the ROCK1/pMLC2 signaling pathway, *Mol. Pharmacol.* 83 (2013) 454-469.
- [220] Z. Kovacevic, S.V. Menezes, S. Sahni, D.S. Kalinowski, D.H. Bae, D.J. Lane, D.R. Richardson, The metastasis suppressor, N-myc downstream regulated gene-1 (NDRG1), down-regulates the ErbB family of receptors to inhibit downstream oncogenic signaling pathways, *J. Biol. Chem.* In press (2015) PMID: 26534963, DOI: 26534910.26531074/jbc.M26534115.26689653.
- [221] V.L. Goodman, G.J. Brewer, S.D. Merajver, Copper deficiency as an anti-cancer strategy, *Endocr. Relat. Cancer* 11 (2004) 255-263.
- [222] A. Gupte, R.J. Mumper, Elevated copper and oxidative stress in cancer cells as a target for cancer treatment, *Cancer Treat. Rev.* 35 (2009) 32-46.
- [223] G.D. Kaiafa, Z. Saouli, M.D. Diamantidis, Z. Kontoninas, V. Voulgaridou, M. Raptaki, S. Arampatzi, M. Chatzidimitriou, V. Perifanis, Copper levels in patients with hematological malignancies, *Eur. J. Intern. Med.* 23 (2012) 738-741.

- [224] A. Nasulewicz, A. Mazur, A. Opolski, Role of copper in tumour angiogenesis--clinical implications, *J. Trace Elem. Med. Biol.* 18 (2004) 1-8.
- [225] S. Majumder, S. Chatterjee, S. Pal, J. Biswas, T. Efferth, S.K. Choudhuri, The role of copper in drug-resistant murine and human tumors, *Biometals* 22 (2009) 377-384.
- [226] M.C. Linder, J.R. Moor, K. Wright, Ceruloplasmin assays in diagnosis and treatment of human lung, breast, and gastrointestinal cancers, *J. Natl. Cancer Inst.* 67 (1981) 263-275.
- [227] A. Senra Varela, J.J. Lopez Saez, D. Quintela Senra, Serum ceruloplasmin as a diagnostic marker of cancer, *Cancer Lett.* 121 (1997) 139-145.
- [228] G. MacDonald, I. Nalvarte, T. Smirnova, M. Vecchi, N. Aceto, A. Dolemeyer, A. Frei, S. Lienhard, J. Wyckoff, D. Hess, J. Seebacher, J.J. Keusch, H. Gut, D. Salaun, G. Mazzarol, D. Disalvatore, M. Bentires-Alj, P.P. Di Fiore, A. Badache, N.E. Hynes, Memo is a copper-dependent redox protein with an essential role in migration and metastasis, *Sci. Signal.* 7 (2014) ra56.
- [229] E.D. Harris, A requirement for copper in angiogenesis, *Nutr. Rev.* 62 (2004) 60-64.
- [230] J. Folkman, M. Klagsbrun, Angiogenic factors, *Science* 235 (1987) 442-447.
- [231] N. Weidner, P.R. Carroll, J. Flax, W. Blumenfeld, J. Folkman, Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma, *Am. J. Pathol.* 143 (1993) 401-409.
- [232] S.A. Lowndes, A.L. Harris, The role of copper in tumour angiogenesis, *J. Mam. Gland Biol. Neopl.* 10 (2005) 299-310.
- [233] C.K. Sen, S. Khanna, M. Venojarvi, P. Trikha, E.C. Ellison, T.K. Hunt, S. Roy, Copper-induced vascular endothelial growth factor expression and wound healing, *Am. J. Physiol. Heart Circ. Physiol.* 282 (2002) H1821-1827.
- [234] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, *Nature* 407 (2000) 249-257.

- [235] F. Martin, T. Linden, D.M. Katschinski, F. Oehme, I. Flamme, C.K. Mukhopadhyay, K. Eckhardt, J. Troger, S. Barth, G. Camenisch, R.H. Wenger, Copper-dependent activation of hypoxia-inducible factor (HIF)-1: implications for ceruloplasmin regulation, *Blood* 105 (2005) 4613-4619.
- [236] J.A. Forsythe, B.H. Jiang, N.V. Iyer, F. Agani, S.W. Leung, R.D. Koos, G.L. Semenza, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, *Mol. Cell Biol.* 16 (1996) 4604-4613.
- [237] D.R. Richardson, Iron chelators as therapeutic agents for the treatment of cancer, *Crit. Rev. Oncol. Hematol.* 42 (2002) 267-281.
- [238] D.R. Richardson, Potential of iron chelators as effective antiproliferative agents, *Can. J. Physiol. Pharmacol.* 75 (1997) 1164-1180.
- [239] J.L. Buss, F.M. Torti, S.V. Torti, The role of iron chelation in cancer therapy, *Curr. Med. Chem.* 10 (2003) 1021-1034.
- [240] G.J. Brewer, Anticopper therapy against cancer and diseases of inflammation and fibrosis, *Drug Discov. Today* 10 (2005) 1103-1109.
- [241] J.J. McGuire, Anticancer antifolates: current status and future directions, *Curr. Pharm. Des.* 9 (2003) 2593-2613.
- [242] J.M. Walshe, Penicillamine, a new oral therapy for Wilson's disease, *Am. J. Med.* 21 (1956) 487-495.
- [243] C. Wong, D.R. Richardson, Beta-thalassaemia: emergence of new and improved iron chelators for treatment, *Int. J. Biochem. Cell Biol.* 35 (2003) 1144-1149.
- [244] M. Whitnall, J. Howard, P. Ponka, D.R. Richardson, A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics, *Proc. Natl. Acad. Sci. U.S.A.* 103 (2006) 14901-14906.

- [245] D.R. Richardson, P. Ponka, Development of iron chelators to treat iron overload disease and their use as experimental tools to probe intracellular iron metabolism, *Am. J. Hematol.* 58 (1998) 299-305.
- [246] A. Donfrancesco, G. Deb, C. Dominici, D. Pileggi, M.A. Castello, L. Helson, Effects of a single course of deferoxamine in neuroblastoma patients, *Cancer Res.* 50 (1990) 4929-4930.
- [247] J. Blatt, Deferoxamine in children with recurrent neuroblastoma, *Anticancer Res.* 14 (1994) 2109-2112.
- [248] H. Porter, Copper excretion in the urine of normal individuals and of patients with hepatolenticular degeneration (Wilson's disease), *Arch. Biochem. Biophys.* 31 (1951) 262-265.
- [249] G.J. Brewer, V. Yuzbasiyan-Gurkan, A.B. Young, Treatment of Wilson's disease, *Semin. Neurol.* 7 (1987) 209-220.
- [250] G.J. Brewer, Zinc and tetrathiomolybdate for the treatment of Wilson's disease and the potential efficacy of anticopper therapy in a wide variety of diseases, *Metallomics* 1 (2009) 199-206.
- [251] G.J. Brewer, R.D. Dick, V. Johnson, Y. Wang, V. Yuzbasiyan-Gurkan, K. Kluin, J.K. Fink, A. Aisen, Treatment of Wilson's disease with ammonium tetrathiomolybdate. I. Initial therapy in 17 neurologically affected patients, *Arch. Neurol.* 51 (1994) 545-554.
- [252] Q. Pan, C.G. Kleer, K.L. van Golen, J. Irani, K.M. Bottema, C. Bias, M. De Carvalho, E.A. Mesri, D.M. Robins, R.D. Dick, G.J. Brewer, S.D. Merajver, Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis, *Cancer Res.* 62 (2002) 4854-4859.
- [253] S.S. Brem, D. Zagzag, A.M. Tsanaclis, S. Gately, M.P. Elkouby, S.E. Brien, Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial

- cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor, *Am. J. Pathol.* 137 (1990) 1121-1142.
- [254] B.G. Redman, P. Esper, Q. Pan, R.L. Dunn, H.K. Hussain, T. Chenevert, G.J. Brewer, S.D. Merajver, Phase II trial of tetrathiomolybdate in patients with advanced kidney cancer, *Clin. Cancer Res.* 9 (2003) 1666-1672.
- [255] A. Ceci, M. Felisi, V. De Sanctis, D. De Mattia, Pharmacotherapy of iron overload in thalassaemic patients, *Expert Opin. Pharmacother.* 4 (2003) 1763-1774.
- [256] G.M. Brittenham, Iron chelators and iron toxicity, *Alcohol* 30 (2003) 151-158.
- [257] C. Hershko, A. Abrahamov, A.M. Konijn, W. Breuer, I.Z. Cabantchik, P. Pootrakul, G. Link, Objectives and methods of iron chelation therapy, *Bioinorg. Chem. Appl.* (2003) 151-168.
- [258] R.A. Selig, L. White, C. Gramacho, K. Sterling-Levis, I.W. Fraser, D. Naidoo, Failure of iron chelators to reduce tumor growth in human neuroblastoma xenografts, *Cancer Res.* 58 (1998) 473-478.
- [259] A. Donfrancesco, B. De Bernardi, M. Carli, A. Mancini, M. Nigro, L. De Sio, F. Casale, S. Bagnulo, L. Helson, G. Deb, Deferoxamine, cyclophosphamide, etoposide, carboplatin, and thiotepa (D-CECaT): A new cytoreductive chelation-chemotherapy regimen in patients with advanced neuroblastoma, *Eur. J. Cancer* 31A (1995) 612-615.
- [260] Z. Estrov, A. Tawa, X.H. Wang, I.D. Dube, H. Sulh, A. Cohen, E.W. Gelfand, M.H. Freedman, In vitro and in vivo effects of deferoxamine in neonatal acute leukemia, *Blood* 69 (1987) 757-761.
- [261] J. Blatt, S.R. Taylor, S. Stitely, Mechanism of antineuroblastoma activity of deferoxamine in vitro, *J. Lab. Clin. Med.* 112 (1988) 433-436.
- [262] J. Blatt, S. Stitely, Antineuroblastoma activity of desferoxamine in human cell lines, *Cancer Res.* 47 (1987) 1749-1750.

- [263] A.C. Sartorelli, B.A. Booth, Inhibition of the growth of sarcoma 180 ascites cells by combinations of inhibitors of nucleic acid biosynthesis and the cupric chelate of kethoxal bis-(thiosemicarbazone), *Cancer Res.* 27 (1967) 1614-1619.
- [264] W. Antholine, J. Knight, H. Whelan, D.H. Petering, Studies of the reaction of 2-formylpyridine thiosemicarbazone and its iron and copper complexes with biological systems, *Mol. Pharmacol.* 13 (1977) 89-98.
- [265] R.A. Finch, M.C. Liu, S.P. Grill, W.C. Rose, R. Loomis, K.M. Vasquez, Y.C. Cheng, A.C. Sartorelli, Triapine (3-aminopyridine-2-carboxaldehyde-thiosemicarbazone): A potent inhibitor of ribonucleotide reductase activity with broad spectrum antitumor activity, *Biochem. Pharmacol.* 59 (2000) 983-991.
- [266] A.C. Sartorelli, K.C. Agrawal, A.S. Tsiftoglou, E.C. Moore, Characterization of the biochemical mechanism of action of alpha-(N)-heterocyclic carboxaldehyde thiosemicarbazones, *Adv. Enzyme Regul.* 15 (1976) 117-139.
- [267] J. Shao, B. Zhou, A.J. Di Bilio, L. Zhu, T. Wang, C. Qi, J. Shih, Y. Yen, A ferrous-triapine complex mediates formation of reactive oxygen species that inactivate human ribonucleotide reductase, *Mol. Cancer Ther.* 5 (2006) 586-592.
- [268] T.B. Chaston, D.B. Lovejoy, R.N. Watts, D.R. Richardson, Examination of the antiproliferative activity of iron chelators: Multiple cellular targets and the different mechanism of action of triapine compared with desferrioxamine and the potent pyridoxal isonicotinoyl hydrazone analogue 311, *Clin. Cancer Res.* 9 (2003) 402-414.
- [269] J. Shao, B. Zhou, L. Zhu, W. Qiu, Y.C. Yuan, B. Xi, Y. Yen, In vitro characterization of enzymatic properties and inhibition of the p53R2 subunit of human ribonucleotide reductase, *Cancer Res.* 64 (2004) 1-6.
- [270] H. Tanaka, H. Arakawa, T. Yamaguchi, K. Shiraishi, S. Fukuda, K. Matsui, Y. Takei, Y. Nakamura, A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage, *Nature* 404 (2000) 42-49.



- [271] J.M. Myers, W.E. Antholine, J. Zielonka, C.R. Myers, The iron-chelating drug triapine causes pronounced mitochondrial thiol redox stress, *Toxicol. Lett.* 201 (2011) 130-136.
- [272] J. Nordberg, E.S. Arner, Reactive oxygen species, antioxidants, and the mammalian thioredoxin system, *Free Radic. Biol. Med.* 31 (2001) 1287-1312.
- [273] J.G. Cory, A.H. Cory, G. Rappa, A. Lorico, M.C. Liu, T.S. Lin, A.C. Sartorelli, Inhibitors of ribonucleotide reductase. Comparative effects of amino- and hydroxy-substituted pyridine-2-carboxaldehyde thiosemicarbazones, *Biochem. Pharmacol.* 48 (1994) 335-344.
- [274] R.A. Finch, M.C. Liu, A.H. Cory, J.G. Cory, A.C. Sartorelli, Triapine (3-aminopyridine-2-carboxaldehyde thiosemicarbazone; 3-AP): an inhibitor of ribonucleotide reductase with antineoplastic activity, *Adv. Enzyme Regul.* 39 (1999) 3-12.
- [275] J.J. Knox, S.J. Hotte, C. Kollmannsberger, E. Winquist, B. Fisher, E.A. Eisenhauer, Phase II study of Triapine in patients with metastatic renal cell carcinoma: A trial of the national cancer institute of canada clinical trials group (NCIC IND.161), *Invest. New Drugs* 25 (2007) 471-477.
- [276] B. Ma, B.C. Goh, E.H. Tan, K.C. Lam, R. Soo, S.S. Leong, L.Z. Wang, F. Mo, A.T. Chan, B. Zee, T. Mok, A multicenter phase II trial of 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (3-AP, Triapine) and gemcitabine in advanced non-small-cell lung cancer with pharmacokinetic evaluation using peripheral blood mononuclear cells, *Invest. New Drugs* 26 (2008) 169-173.
- [277] S. Wadler, D. Makower, C. Clairmont, P. Lambert, K. Fehn, M. Sznol, Phase I and pharmacokinetic study of the ribonucleotide reductase inhibitor, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone, administered by 96-hour intravenous continuous infusion, *J. Clin. Oncol.* 22 (2004) 1553-1563.

- [278] L.M. Foltz, B.I. Dalal, L.D. Wadsworth, R. Broady, K. Chi, E. Eisenhauer, K. Kobayashi, C. Kollmannsburger, Recognition and management of methemoglobinemia and hemolysis in a G6PD-deficient patient on experimental anticancer drug Triapine, *Am. J. Hematol.* 81 (2006) 210-211.
- [279] P. Quach, E. Gutierrez, M.T. Basha, D.S. Kalinowski, P.C. Sharpe, D.B. Lovejoy, P.V. Bernhardt, P.J. Jansson, D.R. Richardson, Methemoglobin formation by 3-AP, Dp44mT and other anti-cancer thiosemicarbazones: Identification of novel thiosemicarbazones and therapeutics that prevent this effect, *Mol. Pharmacol.* 82 (2012) 105-114.
- [280] Y. Yen, K. Margolin, J. Doroshow, M. Fishman, B. Johnson, C. Clairmont, D. Sullivan, M. Sznol, A Phase I Trial of 3-Aminopyridine-2-carboxaldehyde Thiosemicarbazone in Combination with Gemcitabine for Patients with Advanced Cancer, *Cancer Chemother. Pharmacol.* 54 (2004) 331-342.
- [281] A.J. Ocean, P. Christos, J.A. Sparano, D. Matulich, A. Kaubish, A. Siegel, M. Sung, M.M. Ward, N. Hamel, I. Espinoza-Delgado, Y. Yen, M.E. Lane, Phase II trial of the ribonucleotide reductase inhibitor 3-aminopyridine-2-carboxaldehydethiosemicarbazone plus gemcitabine in patients with advanced biliary tract cancer, *Cancer Chemother. Pharmacol.* 68 (2010) 379-388.
- [282] C.M. Nutting, C.M. van Herpen, A.B. Miah, S.A. Bhide, J.P. Machiels, J. Buter, C. Kelly, D. de Raucourt, K.J. Harrington, Phase II study of 3-AP Triapine in patients with recurrent or metastatic head and neck squamous cell carcinoma, *Ann. Oncol.* 20 (2009) 1275-1279.
- [283] J. Yuan, D.B. Lovejoy, D.R. Richardson, Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: In vitro and in vivo assessment, *Blood* 104 (2004) 1450-1458.

- [284] D.R. Richardson, E.H. Tran, P. Ponka, The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents, *Blood* 86 (1995) 4295-4306.
- [285] E. Baker, D. Richardson, S. Gross, P. Ponka, Evaluation of the iron chelation potential of hydrazones of pyridoxal, salicylaldehyde and 2-hydroxy-1-naphthylaldehyde using the hepatocyte in culture, *Hepatology* 15 (1992) 492-501.
- [286] G. Darnell, D.R. Richardson, The potential of iron chelators of the pyridoxal isonicotinoyl hydrazone class as effective antiproliferative agents III: The effect of the ligands on molecular targets involved in proliferation, *Blood* 94 (1999) 781-792.
- [287] E.M. Becker, D.B. Lovejoy, J.M. Greer, R. Watts, D.R. Richardson, Identification of the di-pyridyl ketone isonicotinoyl hydrazone (PKIH) analogues as potent iron chelators and anti-tumour agents, *Br. J. Pharmacol.* 138 (2003) 819-830.
- [288] D.B. Lovejoy, D.R. Richardson, Novel "hybrid" iron chelators derived from aroylhydrazones and thiosemicarbazones demonstrate selective antiproliferative activity against tumor cells, *Blood* 100 (2002) 666-676.
- [289] E. Nulsri, D.R. Richardson, S. Lerdwana, S. Fucharoen, T. Yamagishi, D.S. Kalinowski, K. Pattanapanyasat, Antitumor activity and mechanism of action of the iron chelator, Dp44mT, against leukemic cells, *Am. J. Hematol.* 84 (2009) 170-176.
- [290] V.A. Rao, S.R. Klein, K.K. Agama, E. Toyoda, N. Adachi, Y. Pommier, E.B. Shacter, The iron chelator Dp44mT causes DNA damage and selective inhibition of topoisomerase IIalpha in breast cancer cells, *Cancer Res.* 69 (2009) 948-957.
- [291] A.M. Merlot, N. Pantarat, S.V. Menezes, S. Sahni, D.R. Richardson, D.S. Kalinowski, Cellular uptake of the antitumor agent Dp44mT occurs via a carrier/receptor-mediated mechanism, *Mol. Pharmacol.* 84 (2013) 911-924.
- [292] A.M. Merlot, S. Sahni, D.J. Lane, A.M. Fordham, N. Pantarat, D.E. Hibbs, V. Richardson, M.R. Doddareddy, J.A. Ong, M.L. Huang, D.R. Richardson, D.S.

- Kalinowski, Potentiating the cellular targeting and anti-tumor activity of Dp44mT via binding to human serum albumin: two saturable mechanisms of Dp44mT uptake by cells, *Oncotarget* 6 (2015) 10374-10398.
- [293] A.M. Merlot, D.S. Kalinowski, Z. Kovacevic, P.J. Jansson, D.J. Lane, M.L. Huang, S. Sahni, D.R. Richardson, Making a case for albumin - a highly promising drug-delivery system, *Future Med. Chem.* 7 (2015) 553-556.
- [294] Z. Kovacevic, S. Chikhani, D.B. Lovejoy, D.R. Richardson, Novel thiosemicarbazone iron chelators induce up-regulation and phosphorylation of the metastasis suppressor N-myc down-stream regulated gene 1: a new strategy for the treatment of pancreatic cancer, *Mol. Pharmacol.* 80 (2011) 598-609.
- [295] W. Liu, F. Xing, M. Iizumi-Gairani, H. Okuda, M. Watabe, S.K. Pai, P.R. Pandey, S. Hirota, A. Kobayashi, Y.Y. Mo, K. Fukuda, Y. Li, K. Watabe, N-myc downstream regulated gene 1 modulates Wnt-beta-catenin signalling and pleiotropically suppresses metastasis, *EMBO Mol. Med.* 4 (2012) 93-108.
- [296] D.R. Richardson, P.C. Sharpe, D.B. Lovejoy, D. Senaratne, D.S. Kalinowski, M. Islam, P.V. Bernhardt, Dipyriddy thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity, *J. Med. Chem.* 49 (2006) 6510-6521.
- [297] N.A. Seebacher, D.J. Lane, P.J. Jansson, D.R. Richardson, Glucose modulation induces lysosome formation and increases lysosomotropic drug sequestration via the P-glycoprotein drug transporter, *J. Biol. Chem.* In press (2015) PMID: 26601947, DOI: 26601910.26601074/jbc.M26601115.26682450.
- [298] D.J. Booser, G.N. Hortobagyi, Anthracycline antibiotics in cancer therapy. Focus on drug resistance, *Drugs* 47 (1994) 223-258.

- [299] T. Yamagishi, S. Sahni, D.M. Sharp, A. Arvind, P.J. Jansson, D.R. Richardson, P-glycoprotein mediates drug resistance via a novel mechanism involving lysosomal sequestration, *J. Biol. Chem.* 288 (2013) 31761-31771.
- [300] J.M. Zamora, W.T. Beck, Chloroquine enhancement of anticancer drug cytotoxicity in multiple drug resistant human leukemic cells, *Biochem. Pharmacol.* 35 (1986) 4303-4310.
- [301] P.J. Jansson, D.S. Kalinowski, D.J. Lane, Z. Kovacevic, N.A. Seebacher, L. Fouani, S. Sahni, A.M. Merlot, D.R. Richardson, The renaissance of polypharmacology in the development of anti-cancer therapeutics: Inhibition of the "Triad of Death" in cancer by Di-2-pyridylketone thiosemicarbazones, *Pharmacol. Res.* 100 (2015) 255-260.
- [302] T. Simunek, M. Sterba, O. Popelova, M. Adamcova, R. Hrdina, V. Gersl, Anthracycline-induced cardiotoxicity: overview of studies examining the roles of oxidative stress and free cellular iron, *Pharmacol. Rep.* 61 (2009) 154-171.
- [303] D.B. Lovejoy, D.M. Sharp, N. Seebacher, P. Obeidy, T. Prichard, C. Stefani, M.T. Basha, P.C. Sharpe, P.J. Jansson, D.S. Kalinowski, P.V. Bernhardt, D.R. Richardson, Novel second-generation di-2-pyridylketone thiosemicarbazones show synergism with standard chemotherapeutics and demonstrate potent activity against lung cancer xenografts after oral and intravenous administration in vivo, *J. Med. Chem.* 55 (2012) 7230-7244.
- [304] Y. Yu, Suryo Rahmanto, Y., Obeidy, P. and Richardson, D.R. , Bp44mT: An orally active iron chelator of the thiosemicarbazone class with potent anti-tumour efficacy, *Br. J. Pharmacol.* 165 (2012) 148-166.
- [305] O.M. Odenike, R.A. Larson, D. Gajria, M.E. Dolan, S.M. Delaney, T.G. Karrison, M.J. Ratain, W. Stock, Phase I study of the ribonucleotide reductase inhibitor 3-aminopyridine-2-carboxaldehyde-thiosemicarbazone (3-AP) in combination with high

- dose cytarabine in patients with advanced myeloid leukemia, *Invest. New Drugs* 26 (2008) 233-239.
- [306] A.M. Traynor, J.W. Lee, G.K. Bayer, J.M. Tate, S.P. Thomas, M. Mazurczak, D.L. Graham, J.M. Kolesar, J.H. Schiller, A phase II trial of triapine (NSC# 663249) and gemcitabine as second line treatment of advanced non-small cell lung cancer: Eastern cooperative oncology group study 1503, *Invest. New Drugs* 28 (2010) 91-97.
- [307] J.P. Harrington, R.L. Hicks, Spectral analysis of Fe(III)-complex reduction by hemoglobin: possible mechanisms of interaction, *Int. J. Biochem.* 26 (1994) 1111-1117.
- [308] M.T. Basha, C. Rodriguez, D.R. Richardson, M. Martinez, P.V. Bernhardt, Kinetic studies on the oxidation of oxyhemoglobin by biologically active iron thiosemicarbazone complexes: relevance to iron-chelator-induced methemoglobinemia, *J. Biol. Inorg. Chem.* 19 (2015) 349-357.
- [309] V. Sestak, J. Stariat, J. Cermanova, E. Potuckova, J. Chladek, J. Roh, J. Bures, H. Jansova, P. Prusa, M. Sterba, S. Micuda, T. Simunek, D.S. Kalinowski, D.R. Richardson, P. Kovarikova, Novel and potent anti-tumor and anti-metastatic di-2-pyridylketone thiosemicarbazones demonstrate marked differences in pharmacology between the first and second generation lead agents, *Oncotarget* In Press (2015) PMID: 26623727; DOI: 26623710.26618632/oncotarget.26626389.
- [310] D.S. Kalinowski, Y. Yu, P.C. Sharpe, M. Islam, Y.T. Liao, D.B. Lovejoy, N. Kumar, P.V. Bernhardt, D.R. Richardson, Design, synthesis, and characterization of novel iron chelators: Structure-activity relationships of the 2-benzoylpyridine thiosemicarbazone series and their 3-nitrobenzoyl analogues as potent antitumor agents, *J. Med. Chem.* 50 (2007) 3716-3729.

- [311] A.M. Merlot, N. Pantarat, D.B. Lovejoy, D.S. Kalinowski, D.R. Richardson, Membrane transport and intracellular sequestration of novel thiosemicarbazone chelators for the treatment of cancer, *Mol. Pharmacol.* 78 (2010) 675-684.
- [312] D.S. Kalinowski, P.C. Sharpe, P.V. Bernhardt, D.R. Richardson, Design, synthesis, and characterization of new iron chelators with anti-proliferative activity: Structure-activity relationships of novel thiohydrazone analogues, *J. Med. Chem.* 50 (2007) 6212-6225.
- [313] Y. Yu, Y. Suryo Rahmanto, C.L. Hawkins, D.R. Richardson, The potent and novel thiosemicarbazone chelators di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone and 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone affect crucial thiol systems required for ribonucleotide reductase activity, *Mol. Pharmacol.* 79 (2011) 921-931.
- [314] M.A. Cater, H.B. Pearson, K. Wolyniec, P. Klaver, M. Bilandzic, B.M. Paterson, A.I. Bush, P.O. Humbert, S. La Fontaine, P.S. Donnelly, Y. Haupt, Increasing intracellular bioavailable copper selectively targets prostate cancer cells, *ACS Chem. Biol.* 8 (2013) 1621-1631.
- [315] F.A. French, B.L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives. I. 3-Ethoxy-2-ketobutyraldehyde and related compounds, *Cancer Res.* 18 (1958) 172-175.
- [316] F.A. French, B.L. Freedlander, Carcinostatic action of polycarbonyl compounds and their derivatives .4. Glyoxal bis(thiosemicarbazone) and derivatives, *Cancer Res.* 18 (1958) 1290-1300.
- [317] H.G. Petering, H.H. Buskirk, G.E. Underwood, The anti-tumor activity of 2-keto-3-ethoxybutyraldehyde bis(thiosemicarbazone) and related compounds, *Cancer Res.* 24 (1964) 367-372.

- [318] H.G. Petering, H.H. Buskirk, J.A. Crim, The effect of dietary mineral supplements of the rat on the antitumor activity of 3-ethoxy-2-oxobutylaldehyde bis(thiosemicarbazone), *Cancer Res.* 27 (1967) 1115-1121.
- [319] B.A. Booth, A.C. Sartorelli, Synergistic interaction of kethoxal bis(thiosemicarbazone) and cupric ions in sarcoma 180, *Nature* 210 (1966) 104-105.
- [320] J.A. Crim, H.G. Petering, The antitumor activity of Cu(II)KTS, the copper (II) chelate of 3-ethoxy-2-oxobutylaldehyde bis(thiosemicarbazone), *Cancer Res.* 27 (1967) 1278-1285.
- [321] J.L. Dearling, J.S. Lewis, G.E. Mullen, M.J. Welch, P.J. Blower, Copper bis(thiosemicarbazone) complexes as hypoxia imaging agents: Structure-activity relationships, *J. Biol. Inorg. Chem.* 7 (2002) 249-259.
- [322] D.T. Minkel, L.A. Saryan, D.H. Petering, Structure-function correlations in the reaction of bis(thiosemicarbazonato) copper(II) complexes with Ehrlich ascites tumor cells, *Cancer Res.* 38 (1978) 124-129.
- [323] P.J. Blower, T.C. Castle, A.R. Cowley, J.R. Dilworth, P.S. Donnelly, E. Labisbal, F.E. Sowrey, S.J. Teat, M.J. Went, Structural trends in copper(II) bis(thiosemicarbazone) radiopharmaceuticals, *Dalton Trans.* (2003) 4416-4425.
- [324] Y. Fujibayashi, H. Taniuchi, Y. Yonekura, H. Ohtani, J. Konishi, A. Yokoyama, Copper-62-ATSM: a new hypoxia imaging agent with high membrane permeability and low redox potential, *J. Nucl. Med.* 38 (1997) 1155-1160.
- [325] P.J. Blower, J.R. Dilworth, R.I. Maurer, G.D. Mullen, C.A. Reynolds, Y. Zheng, Towards new transition metal-based hypoxic selective agents for therapy and imaging, *J. Inorg. Biochem.* 85 (2001) 15-22.
- [326] R.I. Maurer, P.J. Blower, J.R. Dilworth, C.A. Reynolds, Y. Zheng, G.E. Mullen, Studies on the mechanism of hypoxic selectivity in copper bis(thiosemicarbazone) radiopharmaceuticals, *J. Med. Chem.* 45 (2002) 1420-1431.



- [327] P.S. Donnelly, A. Caragounis, T. Du, K.M. Laughton, I. Volitakis, R.A. Cherny, R.A. Sharples, A.F. Hill, Q.X. Li, C.L. Masters, K.J. Barnham, A.R. White, Selective intracellular release of copper and zinc ions from bis(thiosemicarbazonato) complexes reduces levels of Alzheimer disease amyloid-beta peptide, *J. Biol. Chem.* 283 (2008) 4568-4577.
- [328] P.J. Crouch, L.W. Hung, P.A. Adlard, M. Cortes, V. Lal, G. Filiz, K.A. Perez, M. Nurjono, A. Caragounis, T. Du, K. Laughton, I. Volitakis, A.I. Bush, Q.X. Li, C.L. Masters, R. Cappai, R.A. Cherny, P.S. Donnelly, A.R. White, K.J. Barnham, Increasing Cu bioavailability inhibits Abeta oligomers and tau phosphorylation, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 381-386.
- [329] L. Bica, J. Meyerowitz, S.J. Parker, A. Caragounis, T. Du, B.M. Paterson, K.J. Barnham, P.J. Crouch, A.R. White, P.S. Donnelly, Cell cycle arrest in cultured neuroblastoma cells exposed to a bis(thiosemicarbazonato) metal complex, *Biometals* 24 (2011) 117-133.
- [330] E. Gutierrez, D.R. Richardson, P.J. Jansson, The anticancer agent di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) overcomes prosurvival autophagy by two mechanisms: Persistent induction of autophagosome synthesis and impairment of lysosomal integrity, *J. Biol. Chem.* 289 (2014) 33568-33589.
- [331] C. Stefani, Z. Al-Eisawi, P.J. Jansson, D.S. Kalinowski, D.R. Richardson, Identification of differential anti-neoplastic activity of copper bis(thiosemicarbazones) that is mediated by intracellular reactive oxygen species generation and lysosomal membrane permeabilization, *J. Inorg. Biochem.* 152 (2015) 20-37.

## **Figure Legends**

**Figure 1.** The cellular uptake of iron by enterocytes. Dietary iron, including heme and inorganic iron, is absorbed from the apical membrane of duodenal enterocytes. Ferric iron is reduced to its ferrous form by duodenal cytochrome b (Dcytb), allowing for its transport across the apical border by the divalent metal transporter 1 (DMT1). Heme iron is potentially absorbed *via* the heme carrier protein 1 (HCP1 or PCFT) into enterocytes, where it is subsequently degraded by heme oxygenase (HO-1) to release iron in the ferrous state. Iron (likely as iron(II)) is then effluxed at the basolateral membrane through the iron efflux pump, ferroportin1 (Fpn1), whereby hephaestin and ceruloplasmin act to oxidize iron(II) to iron(III).

**Figure 2.** Cellular iron homeostasis. Iron circulates throughout the body bound to transferrin (Tf). This Tf-iron(III) complex then binds to transferrin receptor 1 (TfR1) present on the plasma membrane of cells and is subsequently endocytosed by receptor-mediated endocytosis. The acidic environment of the endosome results in the disassociation of iron(III) from Tf. This released iron(III) is then reduced by the ferrireductase enzyme (STEAP3) to iron(II) and is then transported out of the endosome by divalent metal transporter 1 (DMT1). Upon entering the cytosol, iron joins the labile iron pool (LIP). Iron can then be utilized for storage in ferritin, for protein synthesis, for heme or Fe sulfur-cluster synthesis in the mitochondria, and/or for export *via* ferroportin 1 (Fpn1).

**Figure 3.** Copper absorption and distribution. Dietary copper (Cu(II)) is absorbed by brush border microvilli at the apical surface. An unidentified brush border metalloreductase reduces Cu(II) to Cu(I), allowing for its transport across the membrane by the high-affinity Cu(I) transporter Ctr1. Cu(I) can then be utilized for incorporation into Cu-dependent enzymes or be shuttled by Atox1 to be pumped out into the blood stream by the Cu(I)-transporting P-type ATPase, ATP7A.

**Figure 4.** Copper uptake, trafficking and excretion. Circulating Cu(I) is imported at the plasma membrane of hepatocytes by Ctr1 and directed to either Cu/Zn SOD1 by the chaperone, CCS. On the other hand, copper can be excreted into the bile duct by ATP7B after the donation of Cu to this protein by Atox1. Copper may also form complexes with unidentified ligands (L) or enter a labile intracellular copper pool, predominantly localized in the mitochondria, Golgi apparatus and possibly the lysosome or autophagolysosome [136].

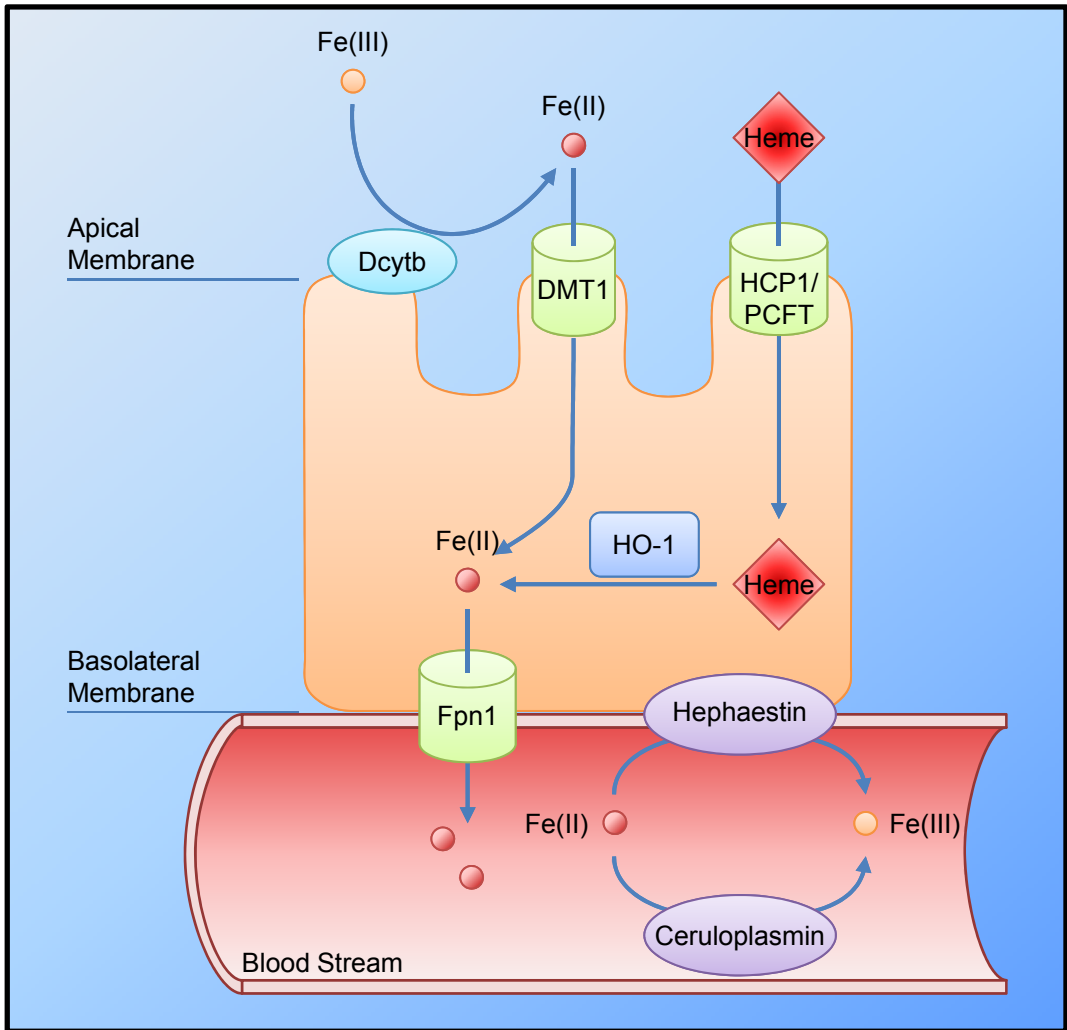
**Figure 5.** The six essential characteristics of cancer include the ability to sustain proliferative signaling, an insensitivity to inhibitory growth signals, resisting cell death, unlimited proliferation, sustained angiogenesis and the invasion of tissue and metastasis.

**Figure 6.** The expression of the metastasis suppressor protein, N-myc downstream-regulated gene 1 (NDRG1), can be regulated by iron levels *via* the hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), eukaryotic initiation factor 3a (eIF3a) and/or the cAMP response element-binding protein (CREB) signaling cascade. Notably, this metastasis suppressor negatively regulates the RAS, phosphoinositide 3-kinase (PI3K), nuclear factor- $\kappa$ B (NF- $\kappa$ B), transforming growth factor  $\beta$  (TGF- $\beta$ ), WNT and the Rho-associated, coiled-coil containing protein kinase 1 (ROCK1)/phosphorylated myosin light chain 2 (pMLC2) pathways, which is potentially mediated by its effects on inhibiting the epidermal growth factor receptor (EGFR), leading to the subsequent inhibition of the epithelial to mesenchymal transition (EMT), cell proliferation, migration and motility.

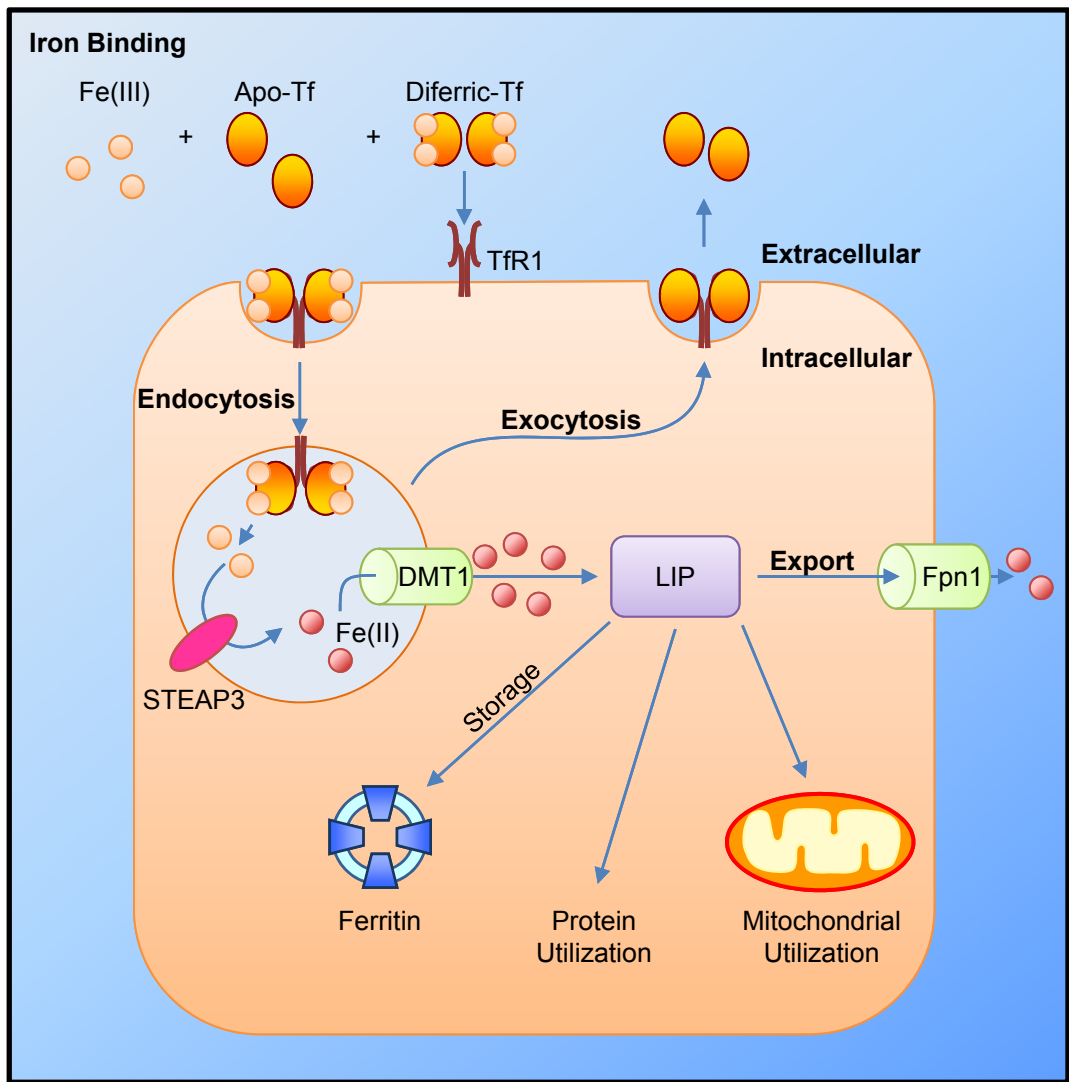
**Figure 7.** Line drawings of the chemical structures of: (A) desferrioxamine (DFO); (B)  $\alpha$ -pyridyl thiosemicarbazone; (C) Triapine<sup>®</sup>; (D) di-2-pyridylketone thiosemicarbazone (DpT); and (E) 2-benzoylpyridine thiosemicarbazone (BpT).

**Figure 8.** (A) During endocytosis, the invagination of the plasma membrane results in the topology of Pgp to be inverted in the early endosomes formed. Upon the maturation of endosomes into lysosomes, the functional Pgp present in lysosomal membranes leads to the accumulation of Pgp substrates, such as doxorubicin, in the lysosomal “safe house”. Due to the acidic pH of the lysosome, doxorubicin becomes protonated and trapped in this organelle without leading to lysosomal membrane permeabilization (LMP). This results in decreased levels of free doxorubicin to interact with its intracellular target (*e.g.*, the nucleus) and confers drug resistance. (B) The Pgp substrates, Dp44mT or DpC, are also pumped into the lysosome where they become protonated and trapped. However, in contrast to doxorubicin, Dp44mT or DpC form redox active iron and copper complexes in this organelle, leading to pronounced ROS generation and marked LMP. This leads to the initiation of apoptosis, and collectively, the ability to overcome drug resistance due to death of the cancer cell.

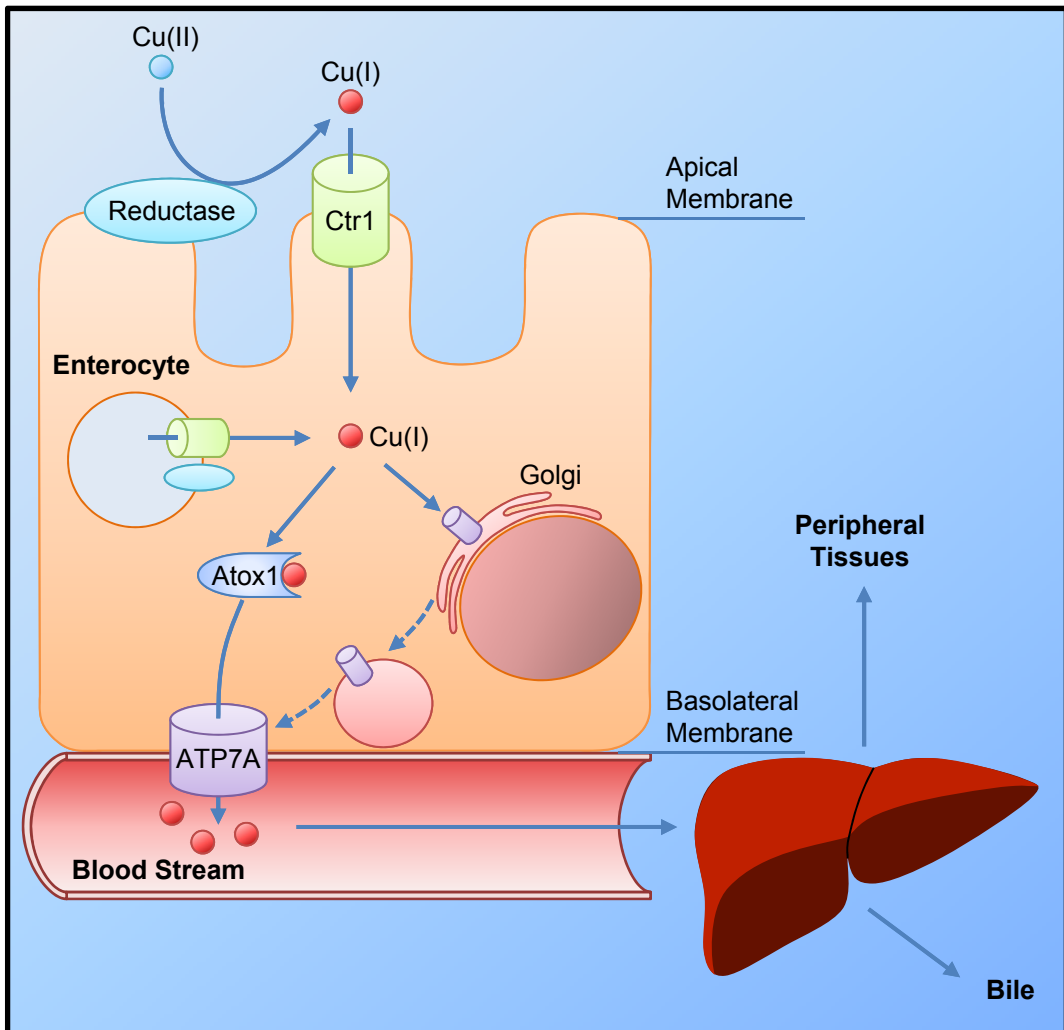
**Figure 9.** Line drawings of the chemical structures of 1,2-bis(thiosemicarbazones), including GTMS, ATSM and KTS, and their Cu complexes.



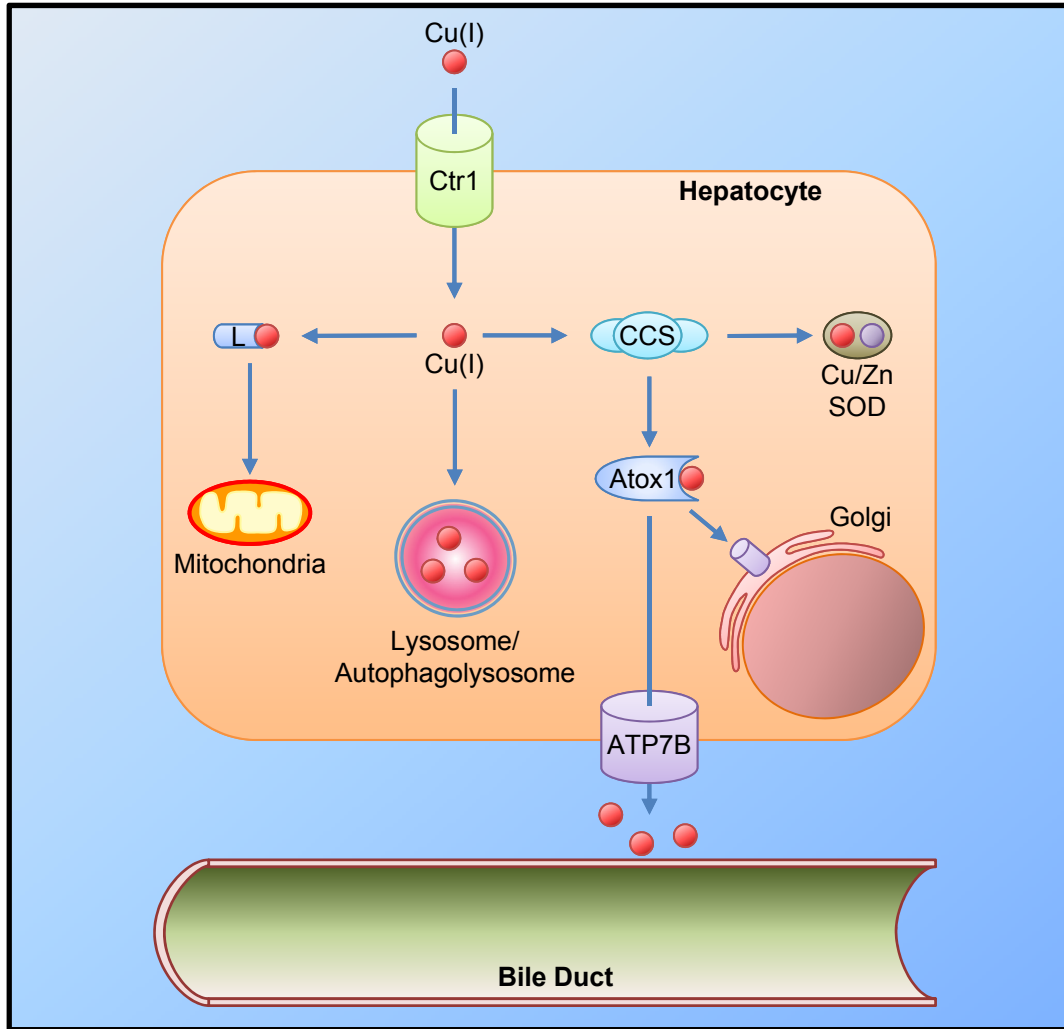
**Figure 1**



**Figure 2**

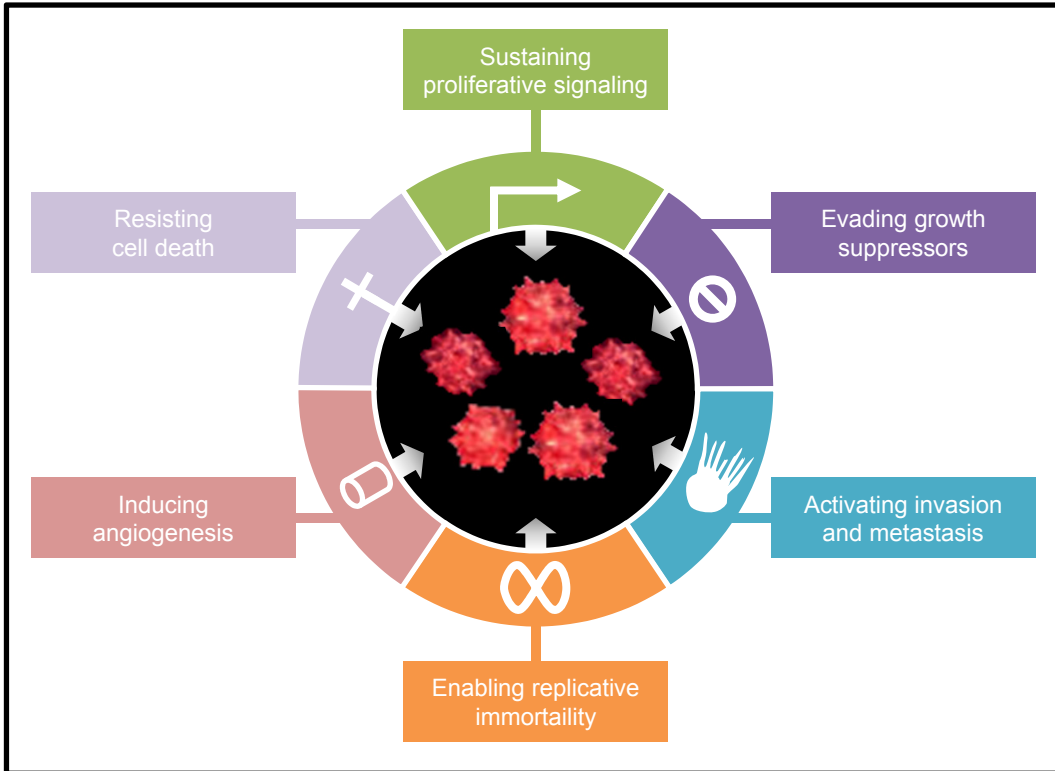


**Figure 3**

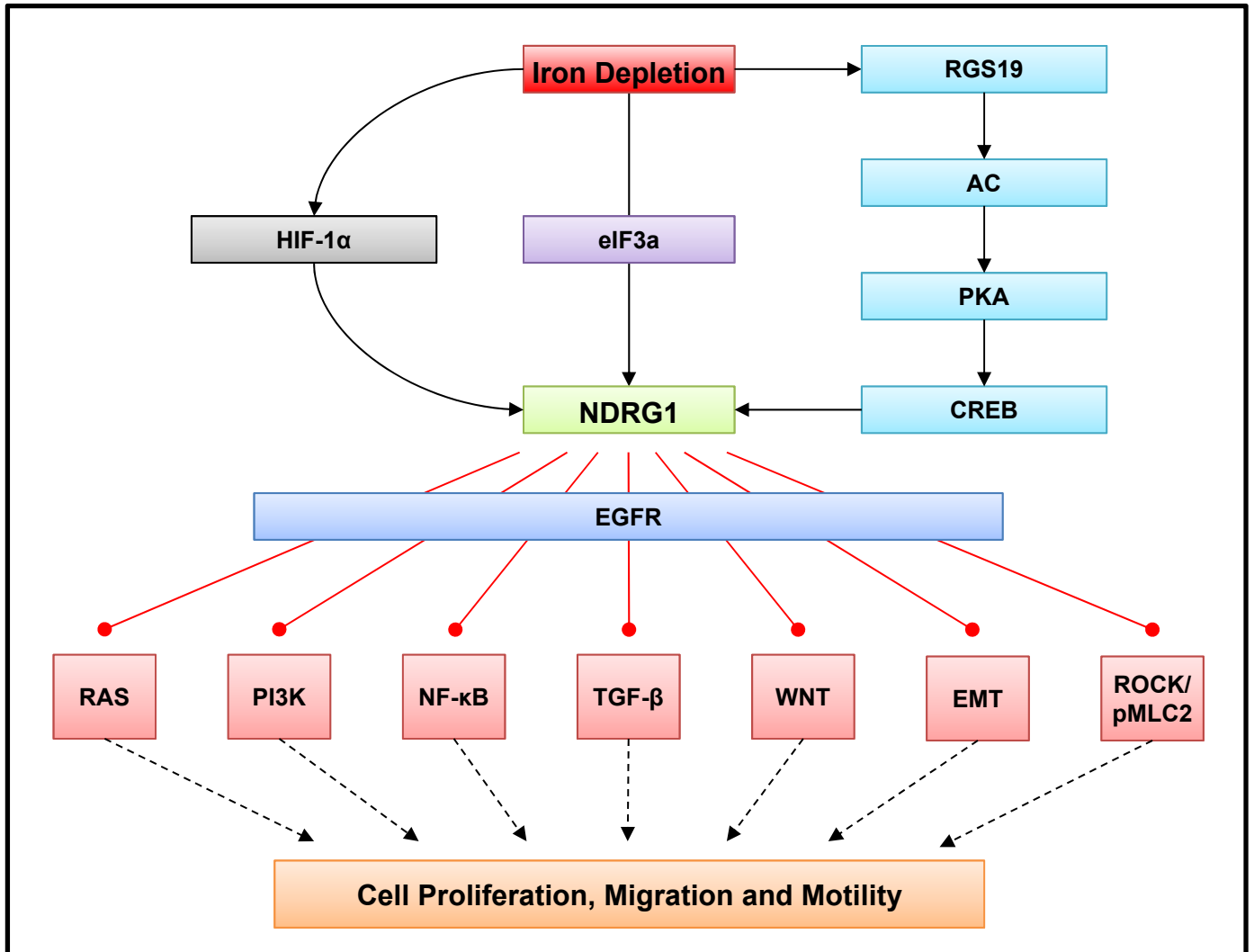


**Figure 4**

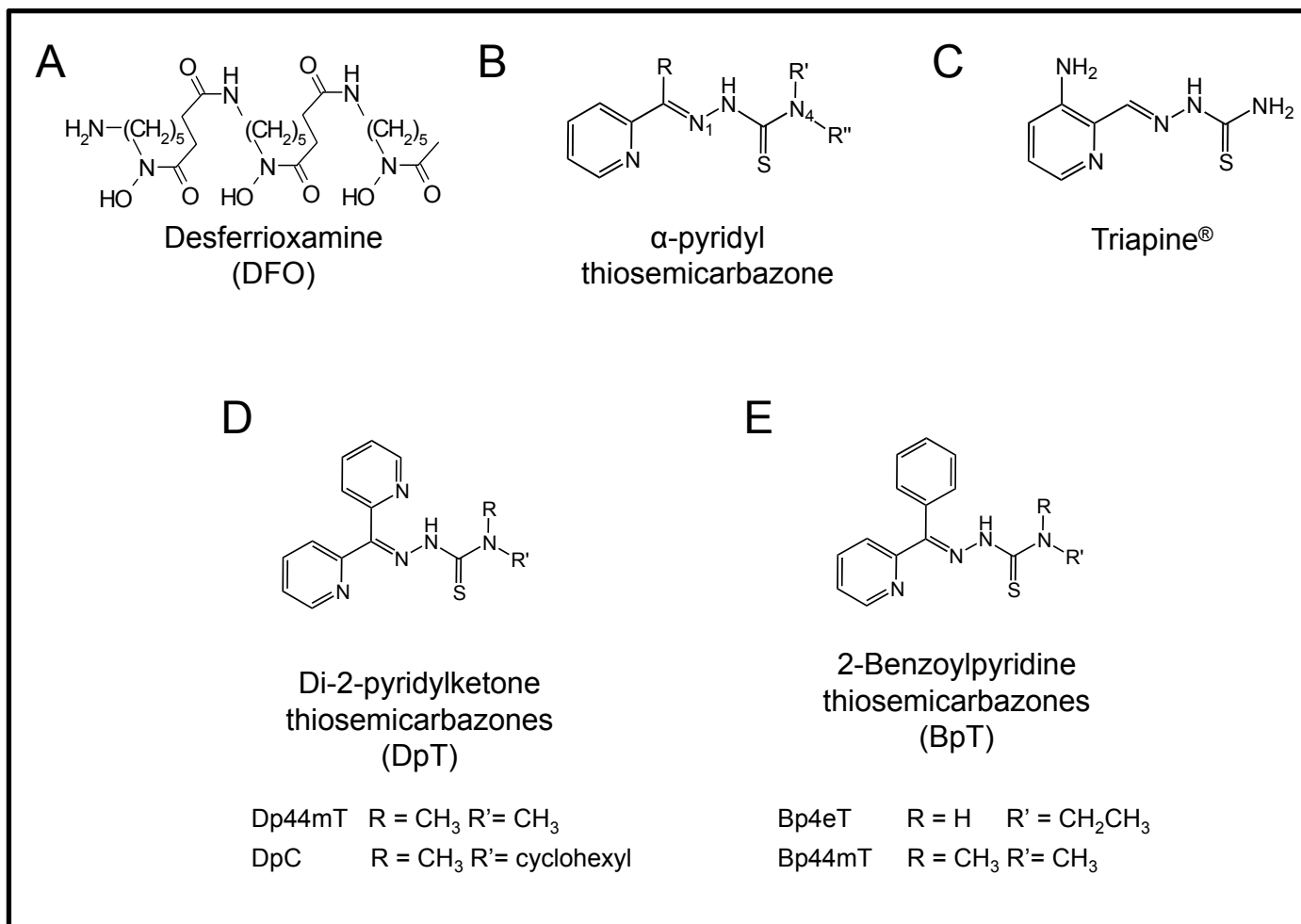




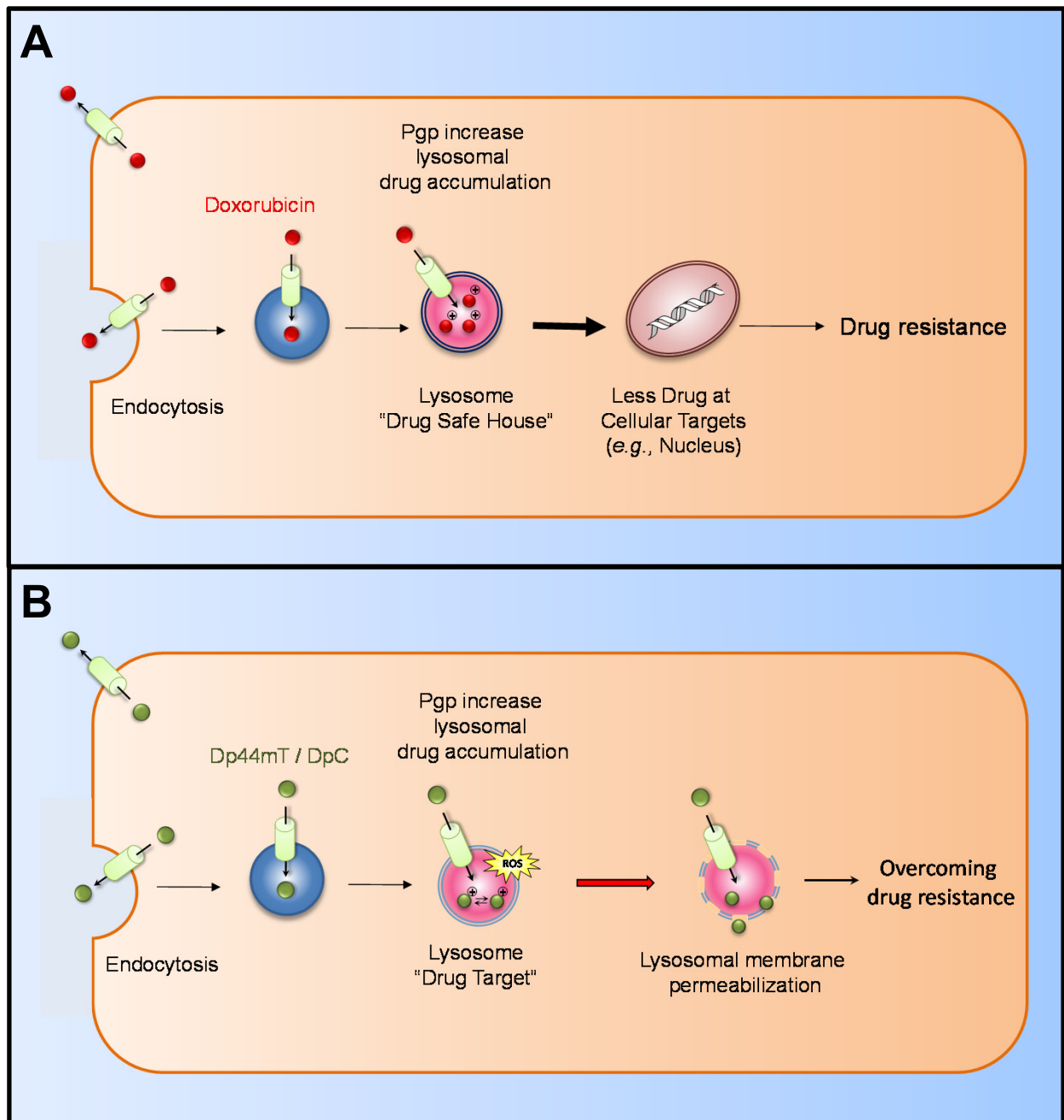
**Figure 5**



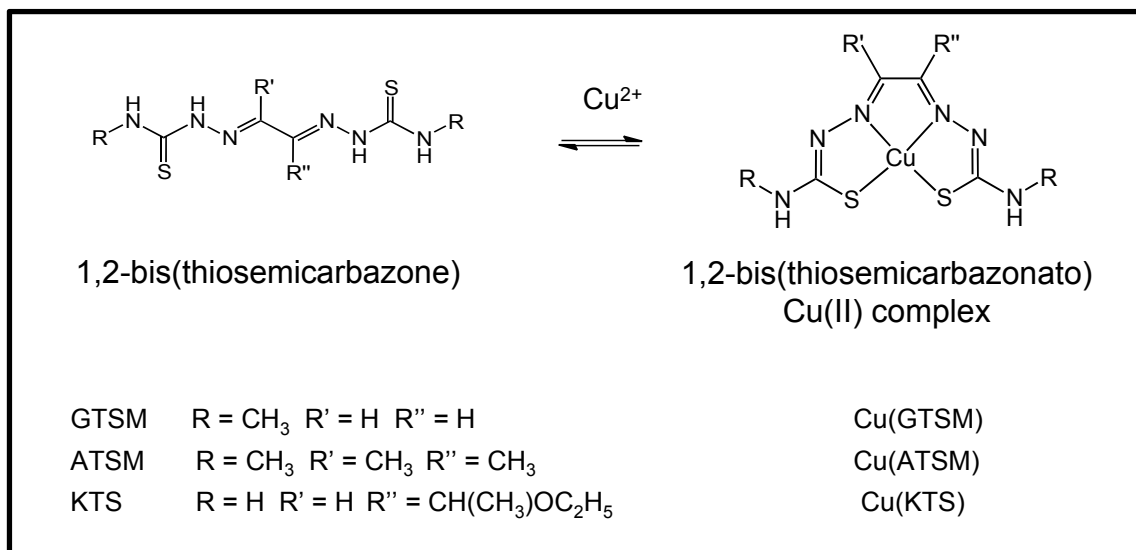
**Figure 6**



**Figure 7**



**Figure 8**



**Figure 9**