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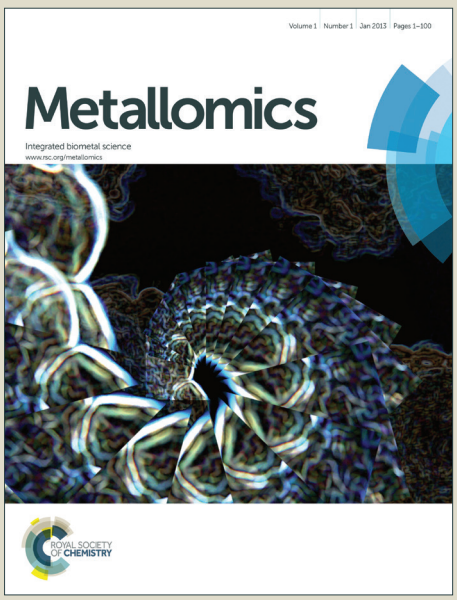

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Targeting copper in cancer therapy: '*Copper That Cancer*'

Delphine Denoyer[1], Shashank Masaldan[1], Sharon La Fontaine[1,2] and Michael A. Cater[1,3]

[1]Centre for Cellular and Molecular Biology, School of Life and Environmental Sciences, Deakin University, Burwood, Victoria, Australia

[2]The Florey Institute of Neuroscience and Mental Health, Victoria 3052, Australia

[3]Department of Pathology, The University of Melbourne, Parkville, Victoria, Australia

Centre for Cellular and Molecular Biology
School of Life and Environmental Sciences
221 Burwood Highway,
Burwood, VIC 3125, Australia
Dr Michael Cater: mcater@deakin.edu.au

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Abstract

Copper is an essential micronutrient involved in fundamental life processes that are conserved throughout all forms of life. The ability of copper to catalyze oxidation-reduction (redox) reactions, which can inadvertently lead to the production of reactive oxygen species (ROS), necessitates the tight homeostatic regulation of copper within the body. Many cancer types exhibit increased intratumoral copper and/or altered systemic copper distribution. The realization that copper serves as a limiting factor for multiple aspects of tumor progression, including growth, angiogenesis and metastasis, has prompted the development of copper-specific chelators as therapies to inhibit these processes. Another therapeutic approach utilizes specific ionophores that deliver copper to cells to increase intracellular copper levels. The therapeutic window between normal and cancerous cells when intracellular copper is forcibly increased, is the premise for the development of copper-ionophores endowed with anticancer properties. Also under investigation is the use of copper to replace platinum in coordination complexes currently used as mainstream chemotherapies. In comparison to platinum-based drugs, these promising copper coordination complexes may be more potent anticancer agents, with reduced toxicity toward normal cells and they may potentially circumvent the chemoresistance associated with recurrent platinum treatment. In addition, cancerous cells can adapt their copper homeostatic mechanisms to acquire resistance to conventional platinum-based drugs and certain copper coordination complexes can re-sensitize cancer cells to these drugs. This review will outline the biological importance of copper and copper homeostasis in mammalian cells, followed by a discussion of our current understanding of copper dysregulation in cancer, and the recent therapeutic advances using copper coordination complexes as anticancer agents.

1. Biological importance of copper

1.1 Copper: an essential element for life

Copper is an essential micronutrient for all organisms. It is required as a catalytic cofactor or as a structural component for proteins, with roles in critical biological functions such as enzyme activity, oxygen transport and cell signaling. Copper is highly redox active, readily donating and accepting electrons to shift between its two valence states ($Cu^{+} \Leftrightarrow Cu^{2+}$). Many critical enzymes harness this activity and hence copper plays important roles in biological oxidation-reduction (redox) reactions. In prokaryotes, over 10 proteins that require copper for their function have been identified. These include cytochrome *c* oxidase (COX), NADH dehydrogenase-2 (ND2), Cu/Zn-superoxide dismutase (SOD1) and tyrosinase, to name a few of the key proteins. Remarkably, no other metal can functionally substitute for copper in these 'cuproproteins'.[1] Likewise, copper is critical for the activity of eukaryotic orthologs of these proteins and in mammals acts as a catalytic cofactor (or allosteric) for numerous proteins involved in multiple facets of our biology, from free radical scavenging, erythropoiesis, iron metabolism, connective tissue synthesis, pigment formation, immunity, cell signaling and neurotransmission.[2-17] The functional role of copper in COX-mediated ATP production illustrates the importance of copper in sustaining life.[18] Examples of cuproproteins in mammalian cells are listed in **Table 1**.

While the redox activity of copper is essential for enzymatic reactions, this property also renders it potentially toxic. Copper can catalyze the production of free radicals and this can be damaging to lipids, proteins, DNA and other biomolecules.[19,8] Copper can also interfere with proteins containing iron-sulfur clusters and can displace other metals such as zinc from metalloproteins inhibiting their activity.[20] Therefore, copper cannot exist free in the cytosol, but must be complexed at all times.[21, 22] All organisms have evolved sophisticated mechanisms to strictly regulate both copper levels and the delivery of copper to copper-requiring proteins, as described below.

1.2 Human copper homeostasis

Any imbalance in copper bioavailability through genetically inherited mutations or altered environmental conditions, invariably leads to deficiency or toxicity and consequently to pathological outcomes. Therefore, copper concentrations in the body are maintained by homeostatic mechanisms that regulate its absorption, excretion and bioavailability. Copper is

absorbed by the intestinal mucosa (enterocytes), and the liver is primarily responsible for regulating the copper status of the body, controlling copper distribution to serum and tissues and excretion of excess copper into the bile. A negligible amount of copper is excreted in the urine.[6] In the body, most bioavailable copper is bound to proteins and free copper is estimated at less than 1 atom per cell.[22] In the general circulation, copper is transported by plasma proteins and not by low molecular weight complexes such as amino acids as previously thought.[23] Plasma cuproproteins include ceruloplasmin, a multicopper ferroxidase that is synthesized and secreted by hepatocytes and binds approximately 70% of the copper in plasma, albumin and the macroglobulin transcuprein.[23] Administration of radioactive copper in animals results in rapid binding of Cu^{2+} to albumin and transcuprein. Most of the radioactive copper is then distributed to the liver before returning to the blood incorporated into ceruloplasmin.[24]

The mechanisms by which copper is taken up by mammalian cells have not been completely elucidated. The current view is that plasma proteins (albumin, transcuprein and ceruloplasmin) deliver Cu^{2+} to transporters located at the plasma membrane and that enzymes (reductases) are responsible for the reduction of copper (Cu^{2+} to Cu^{+}) prior to uptake into cells.[25,26] Potential cupric reductases involved are the metalloreductases from the Steap family.[23] In particular, Steap 3 and Steap 4 are implicated in the reduction of copper in hepatocytes and embryonic fibroblasts, respectively.[27] Ctr1 (SLC31A1) has been established as the major copper import protein.[28-31] However, studies in mouse embryonic fibroblasts lacking Ctr1 and in human kidney, hepatic and mammary cells, have revealed the existence of additional uptake systems for copper. Among these are the divalent metal transporter 1, DMT1 (DCT1, Nramp2)[32, 33] and Ctr2 (SLC31A2).[34] A small fraction of Ctr2 was associated with the plasma membrane and promoted copper accumulation in COS-7 cells.[35] However, other reports suggested that the involvement of Ctr2 is unlikely since the protein mainly localizes in lysosomes and late endosomes and functions as a regulator of intracellular copper, transporting copper from intracellular vesicles to the cytoplasm.[23, 25, 36, 37] Further studies will be necessary to clarify the exact involvement of Ctr2 in copper uptake and to identify additional sources of copper entry into cells.[23]

Once inside the cell, copper is bound and trafficked by cytosolic metallochaperones (e.g. ATOX1, CCS) for delivery to specific cellular destinations. It has been speculated that these chaperones acquire copper from Ctr1 and earlier *in vitro* studies demonstrated that copper could be exchanged between yeast Cu^{+}-Atx1 and the cytoplasmic C-terminal fragment of yeast Ctr1.[38] However, copper acquisition directly from Ctr1 has not been

demonstrated for any of the mammalian chaperones, and recent evidence does not support a direct interaction between the mammalian chaperones (e.g. ATOX1, CCS) and Ctr1.[39] GSH is an abundant intracellular tri-peptide and at millimolar concentrations can buffer free Cu_{aq}^{+} concentrations towards femtomolar levels.[40] Evidence is building in support of the idea that upon copper entry into cells, GSH may serve as an initial copper acceptor. Early studies of the kinetics of ^{67}Cu uptake suggested that GSH bound ^{67}Cu before it was complexed with metallothioneins.[41, 42] Subsequent studies supported a role for GSH as a potential physiological Cu^{+} carrier, showing that metallothioneins could acquire copper from Cu^{+}-GSH[43], and that GSH played a role in copper delivery to SOD1 in the absence of CCS, the copper chaperone for SOD1.[44] Based on *in vitro* copper binding affinities, more recent studies proposed a model whereby copper is transported along an affinity gradient from GSH (millimolar concentrations with low copper affinity) to copper chaperones (micromolar concentrations with higher copper affinity) and then to target proteins with the highest copper affinities.[39, 40, 45] CCS is the chaperone that delivers copper to Cu/Zn-SOD1. COX17 mediates copper transfer within the mitochondrial intermembrane space to SCO1/COX11 for metallation and assembly of cytochrome *c* oxidase. ATOX1 (HAH1) directly exchanges copper with the ion (copper) transporting P_{1B}-Type ATPases (copper-ATPases), ATP7A and ATP7B, for delivery to the secretory pathway and for efflux of excess copper from the cell (reviewed in[46]). ATOX1 was originally identified in the mid-1990s as an antioxidant molecule[47], but this function was overshadowed by the discovery of its role as a copper delivery molecule.[48, 49] There is renewed attention to the interplay between these dual roles of Atox1 and its expanding range of functions, which include copper-dependent nuclear localization, DNA binding and transcriptional activation of secreted SOD3 and cyclin D1**,** the latter promoting cell proliferation (reviewed in[48]). Several lines of evidence suggest that ATOX1 is not absolutely required for copper delivery to copper-ATPases[48], so that other copper carriers may supplement ATOX1 function. Recent evidence that the antioxidant molecule glutaredoxin 1 (GRX1) binds Cu^{+} with high affinity and regulates the redox sulphur chemistry of ATOX1[50], supports a potential copper-chaperone function for this protein.

The copper-ATPases ATP7A and ATP7B are critical components of cellular copper transport and of physiological copper regulation.[46, 51] ATP7A and ATP7B are closely related in structure and function, with approximately 60% amino acid sequence identity. They are large polytopic transmembrane proteins with eight transmembrane domains, highly conserved catalytic domains and large cytoplasmic N-termini containing six metal-binding domains (MBD). They undergo ATP-dependent cycles of phosphorylation and dephosphorylation to

catalyze the translocation of copper across cellular membranes for the metallation of many essential cuproenzymes, as well as for the removal of excess cellular copper to prevent toxicity. An important functional aspect of the copper-ATPases is their copper-responsive trafficking between the *trans*-Golgi network (TGN) and the cell periphery, a key mechanism by which cellular copper levels are regulated. Copper-binding together with other N- and C-terminal signals regulate their activity, intracellular location and copper-induced intracellular trafficking. Their structure, biochemistry, regulation and copper-responsive trafficking have been thoroughly reviewed.[28, 46, 51, 52] ATP7A and ATP7B have a dual role in cells; a biosynthetic role delivering copper to the secretory pathway for metallation of cuproenzymes, and a homeostatic role that involves exporting excess copper from the cell. Under normal physiological conditions, ATP7A and ATP7B reside at the TGN supplying copper to copper-dependent enzymes synthesized within the secretory pathway. For ATP7A, these include enzymes such as peptidylglycine α-amidating monooxygenase (PAM)[53, 54] tyrosinase[55, 56] extracellular SOD3[57], dopamine-β-hydroxylase (DBH)[58] and lysyl oxidase.[59-62] Copper delivery to apo-ceruloplasmin in hepatocytes[63] and mouse cerebellum [64] is mediated by ATP7B, and by ATP7A in macrophages in response to hypoxia-mediated increased copper uptake.[65] The trafficking of ATP7A and ATP7B in response to elevated copper has been described in a wide range of non-polarized and polarized cell types.[51] In the latter, there is vectorial transport of copper across the cell. For instance, in intestinal enterocytes ATP7A traffics from the TGN to a rapidly recycling pool of basolateral vesicles, in order to transport copper across this surface and into the general circulation.[66, 67] Conversely, ATP7B traffics to vesicles near the apical surface of hepatocytes, which constitutes the biliary canalicular membrane, to mediate the secretion of excess copper into the bile.[68-72] When copper levels return to normal ATP7A and ATP7B recycle back to the TGN.[67, 68, 73]

Recently, Ctr2 was shown to regulate Ctr1 function. In particular, the absence of Ctr2 induced the accumulation of copper in endosomal compartments, whereas the presence of Ctr2 increased the biogenesis of a truncated form of Ctr1 (tCtr1) that lacked the metal-binding ecto-domain. This truncated form of Ctr1 was involved in the mobilization of copper from endosomal compartments, thereby decreasing intracellular accumulation of copper.[74] The mode of uptake, distribution and removal of copper in mammalian cells is summarized in **Figure 1**.

1.3 Copper deficiency and clinical manifestations

Copper ingested daily in the diet is estimated to be between 0.6 and 1.6 mg.[24] Despite some reports of copper deficiency in babies as a result of severe malnutrition and in geriatric and pediatric cases due to various medical conditions[75], severe dietary copper deficiency in humans is very rare.[76] In some cases, acquired copper deficiency resulted in myelopathy with patients presenting with spastic gait and prominent sensory ataxia. This was associated with excessive zinc ingestion, gastric surgery or malabsorption.[77] In the case of excess zinc ingestion, zinc interferes with copper absorption in the intestine via induction of MTs. MTs preferentially bind copper which is subsequently lost when enterocytes are shed.[78]

The most severe case of copper deficiency is due to Menkes disease (MD), the genetically inherited X-linked recessive disorder that results from mutation of the *ATP7A* gene.[79] This disease presents in males within the first few months of life, and in severe cases is fatal in early childhood. Reduced or loss of function of the ATP7A protein is responsible for impaired intestinal copper absorption leading to intestinal copper accumulation and systemic copper deficiency. The consequential reduced activity of critical copper-dependent enzymes leads to a clinical presentation that can vary in severity, but commonly includes abnormal neurodevelopment, seizures associated with cerebral atrophy and demyelination, a range of connective tissue and vascular abnormalities, fragile bones, an unusual kinky hair structure (pili torti), hair and skin pigmentation defects and failure to thrive (reviewed in[79, 80]). The neurological symptoms have been attributed to impaired ATP7A-mediated copper transport across the blood-brain barrier (BBB) leading to deficiencies of enzymes such as cytochrome *c* oxidase, SOD1, BDH, PAM, lysyl oxidase and tyrosinase, some of which require ATP7A for metallation in the TGN (reviewed in[81]). Treatment with various copper complexes including copper histidine has been met with variable clinical outcomes, and depends heavily on early diagnosis and treatment.[80] In addition, the clinical phenotype of MD patients and the response to copper-replacement therapy seems to be also determined by the effect of the *ATP7A* mutation on the amount of protein produced, the level of activity of the protein, its correct location in the cell, and its ability to traffic in response to copper (reviewed in[51, 80]). To better understand the reasons for the treatment failure, a clinical trial investigating the correlation of specific molecular defects with response to copper replacement therapy is in progress (clinicaltrials.gov id# NCT00001262).

Occipital horn syndrome is a milder disease also caused by mutations in *ATP7A*, with primarily connective tissue defects and moderate neurological symptoms.[82, 83] Causative mutations are often splice site mutations that result in reduced levels of normal *ATP7A*

mRNA.[80] The milder phenotype suggests that sufficient residual ATP7A is produced that is functional, but the prominent connective tissue defects indicate that copper delivery to lysyl oxidase is severely disrupted.[80]

A third clinical phenotype, distinct from MD but associated with *ATP7A* missense mutations was recently described as a form of distal hereditary motor neuropathy.[84, 85] The phenotype of this ATP7A-related motor neuropathy includes a variable age of onset that ranges from the first to the sixth decade of life, with no overt abnormalities of copper metabolism, and typically distal muscle weakness and atrophy of the lower extremities leading to hand and foot deformities.[80] The causative mutations lie outside of the conserved ATP7A functional domains and cause abnormal ATP7A trafficking, affecting specifically motor neuron function.[84-86]

1.4 Hypercupremia and copper toxicity

Wilson disease (WD) is an autosomal recessive copper overload disorder that manifests primarily in the liver and brain. Mutations that inactivate ATP7B lead to impaired biliary copper excretion[87], and consequently cause hepatic copper overload, apoptotic cell death, liver damage, and spillage of copper into the plasma and CSF.[88-91] Hence, copper also accumulates in extrahepatic tissues, notably the brain, kidneys and cornea.[79, 92,93] Approximately 60% of WD cases present with neurological symptoms and typically have a later onset than those with the liver disease.[94, 95] Clinical variability is also a feature of Wilson disease (WD) and genotype/phenotype correlations are complicated by the fact that many WD patients are compound heterozygotes.[95, 96] Defects in the copper transport activity, localization and/or trafficking of ATP7B variants may explain some of the biochemical features of the disease, but the clinical severity of WD may also be affected by environmental factors such as copper intake and allelic variants of modifying genes such as the metallothioneins.[51, 95, 96] The current treatments include the use of chelators to eliminate excess copper from the body, or the administration of dietary zinc to prevent the absorption of copper from enterocytes.[78] Patients are usually treated with chelators as first-line treatment. Copper binds to the chelator and is excreted in the urine. D-penicillamine (D-pen) is commonly used for the treatment of Wilson disease, but its serious side effects prompted the development and use of alternative chelators such as trientine hydrochloride and tetrathiomolybdate (TM), which have milder adverse reactions. Once copper levels are under control, zinc acetate is given to maintain stable copper levels in the body.[97,78]

2. Elevated copper in cancer

The involvement of copper in cancer has been studied for several decades and there have been numerous reports on copper levels being aberrant in cancerous tissues of tumor-bearing mice and in cancer patients.[98-102] In 1975, Schwartz reviewed the role of trace elements including copper, in the context of cancer, underlining their potential roles as carcinogens and as diagnostic/prognostic markers.[103] More recently, Gupte and Mumper (2009) provided an updated review on copper dysregulation in cancer.[104] High serum copper concentrations are associated with a variety of cancers including lymphoma, reticulum cell sarcoma, bronchogenic and laryngeal squamous cell carcinomas, cervical, breast, stomach and lung cancers.[103,104] Strikingly, elevated serum copper correlated with the stage of the disease and its progression in colorectal and breast cancers.[105,101] In a clinical study on patients with hematological malignancies, including chronic lymphoid leukemia, non-Hodgkin's lymphoma, multiple myeloma and Hodgkin's lymphoma, the level of serum copper decreased during periods of remission, sometimes reaching normal levels, then it rebounded to pre-therapy levels during relapses.[106] In patients with advanced breast, lung or colon cancer, in those treated with various chemotherapeutics (e.g. doxorubicin, etoposide or 5-fluorouracil) as single agents or in combination, serum copper levels were clearly linked to drug resistance.[107] Non-responders had approximately 130-160% more copper in their serum.[107] The mechanism(s) that cause copper concentrations to increase in the serum of cancer patients is not known. In a mouse model of carcinoma, the occurrence of elevated serum copper was found to be concomitant with a decrease in copper within the liver.[108] This suggests that copper distribution around the body, which is mediated by the liver, may be fundamentally altered by cancer. Collectively, these observations led to the hypothesis that serum copper level may provide a biomarker of cancer recurrence and may be measured to monitor treatment efficacy. Interestingly, unlike copper, the levels of zinc, iron and selenium are often lower in the serum of cancer patients.[99, 101, 102, 105] In fact, the Cu/Zn, Cu/Fe and Cu/Se ratios all appear to be better indicators of the presence of cancer than Cu, Zn, Fe or Se levels alone.[99]

As described by Gupte and Mumper (2009), elevated copper in malignant tissues has also been established in a range of cancer types, including breast, ovarian, cervical, lung, stomach and leukemia.[104] Surprisingly, leukemic and breast cancer cells can have up to four-fold and three-fold more copper, respectively.[99, 109] We recently demonstrated that only a small subset of patients with prostate cancer harbor elevated intratumoral copper despite previous reports of a more general occurrence.[104, 110] While there are clear demonstrations of

elevated copper in several cancer types (e.g. leukemia, breast and colorectal cancers), larger scale studies are needed to validate many other reports on other cancer types.[104] Despite numerous reports dating back to the 1970s and '80s demonstrating that certain malignant tissues harbor elevated copper, there is still no information on whether cellular transformation to malignancy can drive copper accumulation, or on the mechanisms by which cells adapt to tolerate the ensuing oxidative (redox) pressure.

Copper concentrations have also been reported to increase in nails and/or hair of patients with acute lymphoblastic leukemia[111], prostate[112], breast[113] or cervical cancers.[114] However, in other studies looking at the same cancer types, copper levels are lower[115,116] or do not change[116] in nails and/or hair. The variability of copper levels in nails and hair is likely due to different dietary habits and occupational activities and as such, precludes utilization as a biomarker for cancer diagnosis. Intriguingly, ocular deposition of copper is associated with lung adenocarcinoma[117], multiple myeloma[118] and chronic lymphocytic leukemia.[119] Ocular copper depositions occur in patients with hypercupremia (see **Section 1.4**). As shown in the patients with leukemia, cancerous cells can secrete elevated IgG and consequently copper binds erroneously to IgG and accumulates in eyes rather than being eliminated by the liver.[119]

Is the metal dyshomeostasis seen in cancer patients a cause or a consequence of cancer? Copper is a redox active metal that can enhance the production of ROS, which subsequently can damage most biomolecules.[93] Oxidative stress and chronic inflammation are intrinsically linked to malignant transformation of cells.[120] Therefore, it has been proposed that elevated copper in tissues or serum may be a risk factor for carcinogenesis.[112, 121, 122] Nevertheless, no clear association between copper level and cancer incidence has been found to corroborate this hypothesis. Exposure of wild type mice to 20 μM copper ($CuSO_4$) in drinking water for up to 2 years did not increase the incidence of cancer, suggesting that copper is not carcinogenic.[123] However, this copper concentration in drinking water is unlikely to increase systemic copper levels in mice, as they are proficient at eliminating surplus copper.[124, 125] Controlled studies that actually measure serum copper levels achieved through supplementation are required to properly ascertain whether copper can be carcinogenic.

3. Copper importance in cancer development and progression

Copper is a key component of many cellular functions and increasing evidence places copper as a central modulator of cellular signaling (reviewed in[126]). Not surprisingly, copper is

involved in cancer development and progression and can facilitate cancer growth, angiogenesis and metastasis.

3.1. Cancer growth and copper

Studies investigating the influence of copper on the growth of cancers in mice have yielded discordant results. Over a century ago, it was demonstrated that copper (0.75 mg) administered daily as a colloidal solution over 10 days is able to retard cancer growth in a mouse model of carcinoma.[127] Additionally, cupric acetate (2 mg/kg/week) administered by subcutaneous injection for 26 weeks significantly reduced the initiation of liver carcinogenesis caused by chemical (dimethylnitrosamine) induction in rats.[128] These findings contrast starkly with a more recent study, where copper ($CuSO_4$) administered daily by oral gavage (42.6 mg/kg for 14 weeks) increased cancer growth in a rat model of chemically induced (7,12-dimethylbenz[a]anthraces [DMBA]) mammary tumourigenesis.[129] Likewise, adding 20 μM copper ($CuSO_4$) to the drinking water of mice genetically engineered to develop pancreatic islet cell carcinoma (RIP1-Tag2 model), accelerated cancer growth.[123] Mice bearing $BRAF^{V600E}$-driven lung cancer also had accelerated cancer growth when supplied drinking water supplemented with high levels of copper (125μM $CuSO_4$.).[130] As previously mentioned, elevated copper (20 μM $CuSO_4$) in drinking water did not increase the incidence of cancer in wild type mice.[123] Unfortunately, in all of these studies the level of serum copper achieved was not measured, neither was the effect of copper supplementation on the uptake of other metals considered. The quantity and formulation of copper given to the mice, the cancer type investigated, and whether copper supplementation preceded or succeeded cancer initiation, might all be responsible for the discordant results.

Intriguingly, one group demonstrated that a low copper diet increased cancer incidence and cancer burden in a transgenic mouse model of spontaneous multiple intestinal neoplasia.[131] One of the first physiological signs of severe copper deficiency is bone marrow suppression and anemia.[132,133] Therefore, copper deficiency likely affects the immune system, which plays a central role in preventing cancer development. Similarly, cell-mediated immunity against leukemic cells is impaired in mice severely copper deficient[134], outlining another way copper can affect immunological clearance of malignant cells.

3.2. Angiogenesis and copper

Angiogenesis involves the migration, proliferation and differentiation of endothelial cells to form new blood vessels. Angiogenesis is controlled by angiogenic stimulating factors (e.g.,

angiogenin, vascular endothelial growth factor [VEGF], basic fibroblast growth factor [bFGF] and transforming growth factor β [TGFβ]) and cytokines (interleukin [IL]-1, 6 and 8) as well as through inhibitors (e.g., angiostatin and endostatin) (reviewed in[135, 136]). The inability of cancers to grow larger than 1-2 millimeters in diameter without angiogenesis, illustrates the importance of new blood vessel formation in cancer progression, and accordingly, this knowledge has led to the development of anti-angiogenic agents for cancer therapy.[137] The pro-angiogenic properties of copper was first reported by McAuslan and Reilly (1979), who established that copper salts, and copper extracted from tumors, both induced migration of endothelial cells, an early step of angiogenesis.[138,139] Strikingly, adding copper to the cornea of rabbits induced the formation of new blood vessels[140] and copper enhanced proliferation of human endothelial cells in the absence of serum and growth factors.[141] In contrast, copper had little impact on the proliferation of both human fibroblasts and arterial smooth muscle cells.[141] Furthermore, zinc or iron used at the same concentration as copper decreased endothelial cell growth. These findings unquestionably place copper as a potent inducer of the angiogenic process.[141]

The molecular pathways that copper influences to induce a pro-angiogenic response are varied. Copper can directly bind to the angiogenic growth factor angiogenin and enhance its affinity for endothelial cells.[142, 143] Copper can also regulate the secretion of angiogenic molecules, such as FGF and IL-1α.[144,145] FGF-1 and IL-1α are secreted only following copper-dependent formation of a multi-protein complex.[144,145] FGF-1 and IL-1α lack the signal sequence for endoplasmic reticulum (ER)-Golgi mediated secretion. Finally, copper is required for the expression of certain angiogenic factors. For instance, copper deficiency inhibits the activity of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn decreases expression of five pro-angiogenic mediators (VEGF, bFGF, IL-1α, IL-6 and IL-8).[146] Copper is also transported into the nucleus of cells by the copper chaperone CCS, where it can regulate formation of the hypoxia-inducible factor 1 (HIF-1) transcriptional complex and thus regulate expression of VEGF, a potent angiogenic factor.[147,148] Likewise, ATOX1 can enter the nucleus of cells to serve as a copper-dependent transcription factor[149] and has been shown to regulate platelet-derived growth factor (PDGF) signally and thus potentially malignant angiogenesis and vascular remodeling.[150] Indeed, compelling evidence that copper is essential for malignant angiogenesis comes from studies demonstrating that copper chelation can impede cancer growth and progression *in vivo*. This is discussed in **Section 4.1**.

3.3. Metastasis and copper

An obvious role for copper in metastasis is through regulating angiogenesis, which is a fundamental process required for metastatic potential. However, there is growing evidence that copper also directly influences the ability of cancerous cells to invade and metastasize. Copper is essential for the activities of both lysyl oxidase (LOX) and lysyl oxidase-like (LOXL) proteins, which are involved in the crosslinking of collagen and elastin.[3, 4] Cancer cells secrete LOX to remodel the extracellular matrix and by doing so create a pre-metastatic niche where bone marrow-derived cells are recruited prior to the development of metastases.[151] The expression of LOXL2 is elevated in highly invasive cancers (reviewed in[152]) and correlates with metastasis and poor survival in estrogen receptor negative breast cancer patients.[153] One proposed mechanism is that LOXL2 induces epithelial-mesenchymal transition (EMT). EMT is an early step of cancer cell invasion and is partly activated through down-regulation of E-cadherin; a protein involved in tight junctions. LOXL2 interacts with a repressor of E-cadherin called Snail, increasing the stability of Snail to inhibit E-cadherin expression.[154] Based on these studies, blocking the activity of LOX and LOXL is an attractive therapeutic approach to inhibit cancer metastases.

More recently, a copper-dependent redox protein called Memo has also been shown to play a role in breast cancer cell migration and metastasis, by increasing intracellular ROS levels.[155] More aggressive breast cancers express elevated levels of Memo and Memo appears to be a reliable prognostic marker of early distant metastases.[155]

4. Copper as a target for cancer therapy

Elevated copper in malignant tissues coupled with the realization that copper promotes angiogenesis, cancer growth and metastasis, has led to the development of copper-coordination compounds for anticancer therapies. Copper chelating to decrease copper bioavailability has been and continues to be investigated in clinical studies as a strategy to inhibit angiogenesis for multiple cancer types (e.g. clinicaltrials.gov id# NCT00003751, NCT00176800, NCT01837329, NCT02068079, NCT00405574). By definition, copper chelators remove copper ions from the body, and as such their therapeutic premise involves impeding the copper-dependent malignant processes to limit cancer progression. Copper-ionophores that raise intracellular copper levels, and other copper complexes that exert direct cytotoxic effects, are also the focus of intense research and clinical trials (e.g. clinicaltrials.gov id# NCT00742911, NCT01907165, NCT01777919). These compounds are mechanistically distinct from copper chelators and rather than removing copper instead

elevate and/or redistribute intracellular copper levels. How these biological properties deliver anticancer activity is discussed below. Herein, we give an overview of the latest advances in the field with a particular focus on how copper coordination compounds alter cancer cell biology and their potential use in the clinic. The better-known anticancer activities of several classes of copper coordination compounds are summarized in **Figure 2**. For structural information on the different copper coordination compounds the reader is referred to the following excellent reviews.[156-162]

4.1. Impeding cancer growth with copper chelators

Historically, copper chelating agents were developed to treat Wilson disease, an autosomal recessive genetic disorder that causes copper accumulation primarily in the liver (see **Section 1.4**).[97] The same agents were later investigated for their capacity to control angiogenesis and thus by inference, to impair cancer growth and metastasis. The depletion of bioavailable copper with D-pen, trientine, or TM, delayed the spread of cancers by inhibiting vascularization of lesions in various animal models including, among others, a rat gliosarcoma[163], a rabbit brain tumor model of VX2 carcinomas[164], a mouse model of hepatocellular carcinoma[165] and of head and neck squamous cell carcinoma.[166] One identified anti-metastatic activity of copper chelators is that they prevent the recruitment of bone marrow-derived endothelial progenitor cells (EPC), which are essential for the angiogenic switch that occurs prior to the development of macroscopic metastases.[167,168] Consistently, copper depletion in a breast cancer mouse model (HER2/neu) inhibited the progression of microscopic to macroscopic tumors.[146,169] Furthermore, administration of TM (1 mg) daily for 3 weeks to a transgenic mouse model of pancreatic neuroendocrine tumor (RIP1-Tag2 mice) also delayed the angiogenic switch observed in premalignant lesions and reduced late-stage tumor growth.[123] Likewise, in a mouse model of mesothelioma tumor, lowering bioavailable copper by using D-pen, TM or trientine, also reduced tumor growth and impeded tumor blood vessel formation.[170] TM-induced copper deficiency is also thought to inhibit angiogenesis through activation of the transcription factor NF-κB, in turn decreasing secretion of angiogenic factors (VEGF, FGF2) and interleukins (IL-1α, IL-6, IL-8), as demonstrated *in vivo* using a human inflammatory breast carcinoma cell line (SUM 149 xenograft).[171] Similarly, trientine has been shown to reduce IL-8 production in hepatocellular carcinoma.[172] Inhibition of lysyl oxidase activity by D-pen, impaired collagen crosslinking and reduced VEGF expression, resulting in delayed progression of glioblastoma multiforme *in vivo.*[173] However, it is important to note that these studies collectively highlighted the

cytostatic rather than cytotoxic properties possessed by copper chelators on both tumor and endothelial cells.[123, 146, 169, 170]

Certain copper chelators have also been reported to possess direct anticancer activities. In murine models of cancer (fibrosarcoma and hepatocellular carcinoma), trientine induced apoptosis through the generation of ROS, attributed to the interaction of the drug with redox active copper.[165, 174] As previously mentioned, copper not only regulates enzymes critical for angiogenesis, but also modulates the activity of cell metabolic and proliferative enzymes such as cytochrome *c* oxidase and MEK1/2 kinase. Therefore, is not surprising that lowering intracellular copper, and thus lowering cuproenymatic activity, could alter tumor biology. In a mouse model of pancreatic islet cell carcinoma, the anti-proliferative effect of TM observed was believed to be mediated by cytochrome *c* oxidase inhibition, and thus by decreasing ATP production.[123] Lowering copper levels with TM impacts on MEK1/2 kinase activity and *BRAF*-driven tumorigenesis thus decreases tumor (xenograft) growth of $BRAF^{V600E}$ transformed cells.[130] Consistent with a cytostatic effect, tumors rapidly develop after the copper chelation treatment ceases.[130]

A least a dozen clinical trials investigating the anticancer activities of D-pen and TM have been conducted. These copper coordination complexes have been prescribed to Wilson disease patients for decades and thus their toxicity profiles are quite well known (see **Section 1.4**). In the context of treating cancer patients, TM was found to be well tolerated, however, anemia and neutropenia have been reported but are reversible with cessation of the drug.[132,175,176] In contrast, D-pen produced severe adverse effects including hematologic and renal toxicities in some studies.[133] In a phase II clinical trial, D-pen did not improve survival of patients with brain tumor, specifically glioblastoma multiforme, despite producing a marked reduction in the level of bioavailable copper in the serum of patients.[177] The lack of clinical activity was somewhat surprising given the very encouraging preclinical results obtained in a rabbit model of brain tumor.[164] These authors suggest that preclinical success was due to pretreatment with D-pen before tumor cell implantation, and thus lowering serum copper levels may be more effective before the onset of the angiogenic switch.[163, 164, 177] This is consistent with other studies demonstrating that chelating copper perturbs the angiogenic switch and may be ineffective on late stage vascularized tumors.[170,123] Indeed most studies indicate chelating copper is best used as a strategy to inhibit the progression of micrometastases to macroscopic nodules. This suggestion has been tested in a recent clinical trial on breast cancer patients with high risk of relapse and no sign of disease at enrollment.[175] The investigators of this trial concluded that TM-induced copper deficiency decreased

circulating EPCs and might prevent recurrence by promoting tumor dormancy.[175] However, a larger cohort of breast cancer patients is needed to validate these findings. Consistently, another trial demonstrated that TM stabilizes tumors in patients with various metastatic cancers, including breast, colon, lung and prostate cancers and melanoma.[132] Furthermore, the investigators of this trial provided valuable parameters on TM treatment, including the dose therapeutic window and the level of copper deficiency required in patients' serum for efficacy. Ceruloplasmin activity served as a surrogate measure of serum copper status and was used to adjust TM dose during treatment.[132] TM is seemingly not toxic providing ceruloplasmin levels are reduced to no lower than 15-20%, which represents a mild stage of copper deficiency. These investigators also reported that there were no combined drug toxicities when TM was used in combination with radiotherapy, trastuzumab or IFN-α, opening new avenues for combination therapies.[132] More recent phase II clinical trials showed that TM used as a single agent did not provide significant survival benefit for patients with kidney cancers[176], hormone refractory prostate cancer[178] or malignant mesothelioma[179], but might be more effective if used in combination with standard therapies or other antiangiogenic therapies. TM analogs (e.g. ATN-224) are currently being trialed in patients with prostate cancer (clinicaltrials.gov id# NCT00405574).

There is compelling evidence that copper chelation alone is insufficient to kill malignant cells, necessitating its use in combination with other agents to be an effective therapeutic option.[180-182] Supporting this approach, TM and doxorubicin together are more potent at delaying SUM149 breast carcinoma xenograft growth and at inducing apoptosis, than either treatment administered alone.[182] Similarly, treating mice bearing head and neck squamous cell carcinoma[180] or lung cancer[181] with TM in combination with radiation therapy improves tumor growth inhibition. The use of standard antiangiogenic therapies in combination with radiation therapy has also shown promise in clinical trials (reviewed in[183]). However, current standard antiangiogenic drugs often target only one component of the angiogenic process (e.g. VEGF) leading to the emergence of drug resistance. TM targets multiple angiogenic factors, making this drug potentially more effective in long-term treatment regimes. Beyond its mode in inhibiting angiogenesis, TM also impairs mitochondrial energy metabolism and decreases ATP levels as explained above.[123] This is accompanied by increased glycolysis, presumably in an attempt to compensate for the lack of energy production quashed by TM. Combining TM with an inhibitor of glycolysis and thereby blocking the two major ATP production pathways, provided greater tumor growth inhibition than with TM alone.[123] Additionally, copper chelators in combination with BRAF

inhibitors have potential for the treatment of cancers harboring the *BRAF*^{V600E} mutation, given that copper is required for oncogenic BRAF signaling and BRAF-driven tumourigenesis.[130] Nevertheless, clinical trials will be necessary to validate these promising preclinical findings with combination therapies involving TM (or analogs thereof).

4.2. Targeting cancer with copper ionophores

Another therapeutic approach involves the use of copper-specific ionophores. Distinct from the sequestering nature of a chelator, an ionophore transports specific metal(s) into cells, often allowing them to become bioavailable.[157] Three structurally different compounds, Cu^{2+}gtsm) [*bis*(thiosemicarbazone) analog], clioquinol (hydroxyquinoline analog) and disulfiram (dithiocarbamate analog), all commonly release coordinated copper under the reductive intracellular environment[184], and display anticancer activity *in vitro* and in mouse models.[184-193] The therapeutic efficacy of clioquinol and disulfiram has been studied in numerous clinical trials.[185, 194-197] While these compounds transport copper into mammalian cells and display selective toxicity towards cancer cells, the basis for this selectivity has not been elucidated. Elevated copper in malignant cells may predispose them to ionophoric-copper toxicity, but this has not been confirmed. Ionophoric-copper can also be toxic due to redox activity (ROS production) and by displacing other metals from binding sites within critical proteins.

Both clioquinol and disulfiram reduced tumor growth in preclinical models of breast and prostate cancer.[192, 193, 198] Amongst the myriad of biological activities ascribed to these compounds, clioquinol, disulfiram and Cu^{2+}(gtsm) inhibit proteasomal chymotrypsin-like activity[186, 199, 200], a feature we established as being common to copper-ionophores that increase intracellular bioavailable copper.[184] The anticancer activities of these three ionophores are completely dependent on copper as the ligands alone (metal-free compounds) display negligible activity.[184] We have also previously shown that clioquinol induces nuclear translocation of the X-linked inhibitor of apoptosis protein (XIAP), a modulator of caspase activity, thereby allowing caspase-dependent apoptosis of hyperplastic and carcinoma prostate cell lines.[186] In this study, the anticancer activity of clioquinol increased concomitantly with the level of copper in the extracellular medium and could be abrogated by removing bioavailable copper through copper chelator (TM).[186] Accordingly, disulfiram is only active against prostate cancer xenografts when co-administered with copper.[192] The treatment of human breast cancers both *in vitro* and *in vivo* with disulfiram and copper, decreased PTEN expression and activated AKT signaling, providing a strong rational to

combine copper ionophore treatment with PI3K-AKT inhibitors in future clinical trials.[193] The disulfiram-copper complex has also been shown to inhibit aldehyde dehydrogenase (ALDH), displaying cytotoxicity toward ALDH expressing cancer stem cells (CSCs).[201] ALDH has emerged as a target for anticancer therapy and inhibiting ALDH has the potential to sensitize CSCs to standard chemotherapeutic drugs.[201]

The first clinical evidence that disulfiram possesses anticancer activity goes back to the late 1970s. Ditiocarb, a metabolite of disulfiram which forms the copper complex in the body[194], cured a patient with bone metastases from breast cancer.[195] More recently, a patient with liver metastases from ocular melanoma was successfully treated with disulfiram.[185] Disulfiram used in combination with cisplatin and vinorelbine increased survival in patients newly diagnosed with non-small cell lung cancer and appeared to be well tolerated when administered at a dose of 40mg three times daily.[202] In contrast, disulfiram did not show clinical benefit in patients with non-metastatic recurrent prostate cancer.[197] Additionally, disulfiram was found to be extremely toxic in these patients and the authors advised that further development of disulfiram should not be continued for patients with non-metastatic prostate cancer after local therapy.[197] However, it should be noted that patients received either 250 mg or 500 mg of disulfiram daily, doses well above that administered in previous trials. Other clinical trials in cancer patients evaluating disulfiram as a single agent or in combination with other drugs are near completion and results should be available soon to the public (clinicaltrials.gov id# NCT00742911, NCT01907165, NCT01777919).

Despite promising preclinical data, clioquinol failed to elicit pharmacodynamic or clinical activity in a recent clinical trial in patients with advanced hematologic malignancies.[196] The investigators in this trial suggested that insufficient concentrations of clioquinol reached the general circulation[196], consistent with a previous report demonstrating that most ingested clioquinol transits through the gut.[203] Elesclomol (formely STA-4783) is another promising copper ionophore with a unique mechanism of action.[204] This compound forms a complex with Cu^{2+} that is subsequently transported to the mitochondria, where Cu^{2+} is reduced to Cu^{+}, which can result in oxidative stress and subsequent cell death.[205] Following intra-mitochondrial dissociation from copper, elesclomol can diffuse out of the cell and transports more extracellular copper into the cell, amplifying the generation of ROS within the mitochondria. Initially, elesclomol enhanced paclitaxel therapeutic efficacy in patients with refractory solid tumors and in stage IV metastatic melanoma.[206,207] Unfortunately, subsequent results published from a phase III clinical trial in patients with advanced melanoma, demonstrated that combining elesclomol with paclitaxel did not significantly

improve progression-free survival.[208] The lack of encouraging results in this particular trial might be explained by inadequate selection criteria for enrolling patients, since higher lactate dehydrogenase levels were systematically found in non-responders.[208]

Many other classes of copper ionophores have been synthesized and tested for their potential as anticancer drugs, including copper *bis*(thiosemicarbazone) complexes.[184, 189, 190, 209-211] The first *bis*(thiosemicarbazone) analogs tested demonstrated potent therapeutic effects in preclinical studies and their activity was dependent on coordinated copper or zinc.[184, 190, 209] However, these compounds displayed severe hepatic toxicity in mouse models.[212] Considerable effort is now underway to synthesize and characterize new copper *bis*(thiosemicarbazone) analogs with similar biological activity and reduced toxicity. Interestingly, some of these copper *bis*(thiosemicarbazone) analogs retain their coordinated metal under the reductive intracellular environment [e.g. Cu^{2+}(atsm)].[184, 213] These copper coordination complexes are discussed in the next section, as we focus here on ionophores that increase intracellular bioavailable copper. Analogous to elesclomol, some copper *bis*(thiosemicarbazone) analogs, such as Cu^{2+}(gtsm), dissociate their coordinated copper intracellularly and the ligand (H_2gtsm) can recycle out and back into cells with more re-coordinated copper.[184] This property explains how increasing extracellular copper significantly enhances Cu^{2+}(gtsm) anticancer activity[184] and may be applicable for the clinical setting where many patients with cancer have elevated serum copper levels. Some copper *bis*(thiosemicarbazone) complexes also inhibit mitochondrial respiration by specifically targeting Complex I in the mitochondrial electron transport chain.[214] This biological activity seems to be independent of increasing intracellular bioavailable copper and rather is due to the binding of the compound to the site of ubiquinone binding in Complex I.[214] Recently, we have shown that Cu^{2+}(gtsm) selectively destroys cancerous prostate cells *in vitro* and significantly reduced prostate cancer burden in an orthotopic mouse model.[184] However, like most other copper *bis*(thiosemicarbazone) analogs, Cu^{2+}(gtsm) produced acute side effects in mice, specifically renal toxicity.

As mentioned above, Cu^{2+}(gtsm), clioquinol and disulfiram inhibit proteasomal chymotrypsin-like activity[186, 199, 200], a feature common to copper-ionophores that increase intracellular bioavailable copper.[184] Several conventional proteasome inhibitors, such as Bortezomib, are approved for the treatment of multiple myeloma and others are currently in clinical trials (reviewed in[215]). However, due to their limited activity in solid tumors, these agents are currently restricted to hematological malignancies. Copper ionophores may offer both enhanced selectivity towards cancer cells and activity against a broader range of cancer

types. However, most often encouraging preclinical results are coupled to subsequent disappointing results obtained in patient clinical trials. This likely reflects the need for a better understanding of both the mechanism-of-action and the pharmacokinetics of these compounds before commencing efficacy studies in patients. Furthermore, experience from the elesclomol trial[208] highlights the importance of selecting patients that would be more likely to benefit from treatments with copper-ionophores. However, there are currently no reliable biomarkers to accurately predict or assess treatment efficacy. One possibility is to use positron emission tomography (PET) radiopharmaceuticals to allow for noninvasive visualization of cellular functions, which may prove particularly useful for the development and validation of biomarkers, as well as for the assessment of tumor response to copper-based therapies.[216-219]

4.3. Targeting cancer with other copper complexes

The success of platinum-based therapeutics, such as cisplatin and carboplatin, as treatments for various cancer types has prompted the development of further metal coordination compounds to target DNA, with the aim of reducing side effects and overcoming chemoresistance. To this end, many classes of copper coordination compounds have been designed and characterized *in vitro*, but only a few have been evaluated in preclinical animal models.[160,161] Platinum-based therapeutics exert their action by binding to nitrogens on adjacent DNA bases, which interferes with the binding of essential proteins for transcription. Recently, a group of researchers have developed new complexes containing two copper centers, which target two neighboring phosphates on the DNA backbone that provide active sites for metalloenzymes such as nucleases.[220] These copper containing compounds inhibit DNA synthesis and induce the cell death of multiple cancer cell types with much higher potency than cisplatin. These results are promising and we look forward to the *in vivo* studies.

Endeavoring to overcome the chemoresistance observed with cisplatin, Pivetta and colleagues (2015) evaluated the effect of three copper coordination compounds containing either one or two 1,10-phenathroline molecules used in binary combination with cisplatin.[221] A clear synergistic effect was observed with the combination therapy, even against cells identified as being resistant to cisplatin. Encouraging also was the fact that when administered in combination each drug could be used at a reduced dose in comparison to when utilized as single agents. Lowering administered drug concentrations reduces side effects. While the mechanism of action was not established, these authors suggested that the

formation of mixed copper-platinum complexes may be responsible for the synergistic antiproliferative effects seen in both cisplatin-sensitive and -resistant cell lines.[221]

As mentioned above, many copper *bis*(thiosemicarbazone) analogs have demonstrated encouraging anticancer properties. Some of these compounds retain their coordinated metal under the reductive intracellular environment [e.g. Cu^{2+}(atsm)].[184, 213] Palanimuthu and colleagues (2013) selected two analogs, Cu(gtsc) and Cu(gtscHCl), for their strong cytotoxic effect that was similar to the potency of the mainstream chemotherapeutic drug Adriamycin.[210] These compounds were shown to inhibit DNA synthesis and to induce apoptotic cells death in various human cancer cell lines.[210] In addition, Cu(gtscHCl) was able to cleave DNA and inhibit topoisomerase II.[210] In mice, Cu(gtsc) significantly delayed the growth of colorectal carcinoma xenografts.[210] Another copper *bis*(thiosemicarbazone) compound, Cu(atsm), displays anticancer activity and is selectively accumulated in hypoxic cells.[184, 213] This interesting property led to the radiolabeled synthesis of ^{64}Cu(atsm), which can be used both for targeted radionuclide therapy and for diagnosis.[216,218] The principle of radionuclide therapy is to deliver cytotoxic radiation specifically to cancer cells and by doing so limit inadvertent toxicity to normal tissues/organs. As such, ^{64}Cu(atsm) showed significant anticancer activity in an explant model of human colon cancer in hamsters.[222] Since ^{64}Cu has decay characteristics that allow PET imaging, ^{64}Cu(atsm) also has diagnostic applications, allowing the selection of patients that are likely to benefit from ^{64}Cu(atsm) therapy. In addition, to increase selectivity towards cancer cells while reducing toxicity to normal cells, copper *bis*(thiosemicarbazone) compounds have been conjugated to specific peptides for targeting delivery. This includes bombesin, which has cell surface receptors highly expressed in cancers.[223]

In recent years, heterometallic complexes containing copper and tin (Sn) have gained much attention. These compounds combine the potential of both copper and tin coordination compounds as anticancer agents.[224,225] One such heterometallic complex, $CuSn_2$(Trp), induces apoptotic cells death in various cancer cell lines *in vitro*.[224,225] In rat, the maximum tolerated dose for $CuSn_2$(Trp) is 8 times higher than for cisplatin.[225] At equivalent doses, $CuSn_2$(Trp) shows less toxic side effects than cisplatin, with no signs of kidney, liver or brain toxicity and thus $CuSn_2$(Trp) is being investigated as a promising alternative to cisplatin.[225]

5. Copper transporters and resistance to platinum-based cancer treatments

The primary reason standard chemotherapeutic treatments fail is due to cancer cells acquiring resistance. A number of molecular mechanisms exist, but there is amassing evidence that

copper transporters play a central role in drug resistance, in particular towards platinum-based therapeutics. A significant overlap exists between cellular copper homeostatic mechanisms and those involved in the uptake and detoxification of platinum-based compounds. Changes in the expression, activity, or the cellular localization of the copper transporters have been linked to cancer cells, in particular ovarian and non-small cell lung cancers, developing resistance to platinum drugs such as cisplatin. A better understanding of the interplay between the copper transporters and acquired chemoresistance is essential for the identification of new biomarkers of resistance and for the prediction of therapeutic efficacy (reviewed in[226,227]).

5.1. Copper transporters in acquired resistance against chemotherapeutics

The high-affinity copper transporter Ctr1 can mediate the cellular uptake of platinum-based therapeutics, including cisplatin.[228] Many cell types when lacking Ctr1 expression accumulate less platinum-based drugs and therefore are more resistant to these drugs[228, 229,230] Additionally, the organic cation transporter 2 (OCT2) can mediate the cellular uptake of cisplatin and tissues where OCT2 is expressed are sites of severe side effects in patients, such as oto- (ear) and nephrotoxicity.[231, 232] In kidney cells where OCT2 expression is high Ctr1 is not required for the cellular uptake of cisplatin.[232, 233] In human ovarian cancers, high levels of *Ctr1* mRNA are associated with sensitivity to platinum-based therapy and correlates with increased disease-free survival following treatment.[234] Similarly, in patients with non-small cell lung cancers, tumor response is reduced in patients with no detectable Ctr1 expression in comparison to patients with Ctr1 at any level.[235] However, for both ovarian and non-small cell lung cancers, the level of cellular uptake of platinum-based drug does not usually correlate well with the level of Ctr1 expression.[235, 236] One explanation is that cisplatin treatment may rapidly down-regulate the activity of Ctr1 in certain cells types, as previously described in human ovarian carcinoma cells.[236] This could mimic how copper regulates Ctr1 expression and thus its own uptake in certain cells, such as kidney epithelial cells.[237] In other cell types such as hepatocytes copper does not regulate Ctr1 expression.[232, 237] Therefore, for certain cancer types internalization of Ctr1 from the plasma membrane by macropinocytosis and its subsequent proteasomal degradation may have important clinical implications for the success of platinum-based therapies.[236, 237]

The copper transporter Ctr2 has also been implicated in cancer cells acquiring resistance to platinum-based therapeutics (reviewed in[227]). In contrast to the correlation with Ctr1, loss of Ctr2 expression increased the accumulation of either cisplatin or carboplatin in

human ovarian cancer cell lines and conferred sensitivity to these drugs.[34] Moreover, low levels of Ctr2 expression increased the success rate of platinum treatment in patients with ovarian cancer.[238,239] One way that Ctr2 may exert its effect on drug sensitivity is through modulating the activity of Ctr1.[74] Ctr2 can increase the generation of a truncated form of Ctr1 that lacks the copper and cisplatin-binding ecto-domain[74], thus the more Ctr2 expressed the less cisplatin will enter the cell. Conceivably, Ctr2 expression in conjunction with Ctr1 should be examined, since patients with a Ctr2/Ctr1 ratio greater than 1 have a poorer prognosis.[239] Adding another level of complexity, Elijack and colleagues recently proposed a non-protein mediated solubility-diffusion mechanism for cisplatin transfer across the plasma membrane.[240] Using unilamellar lipid vesicle preparations, cisplatin when holding a neutral charge (e.g. in high chloride concentration) could passively traverse the lipid bilayer. Note that high chloride ion concentrations present in blood may promote the persistence of the electroneutral complex. These authors did not rule out active pathways for cisplatin internalization[240] and future studies are required to ascertain the contributions of Ctr1, Ctr2, OCT2, and passive diffusion in the cellular uptake of cisplatin (and other platinum-based drugs); in a cell specific manner as expression of each transporter varies considerably between cell types.

The copper efflux transporters, ATP7A and ATP7B, are also reported to be involved in certain cancer types acquiring chemoresistance. ATP7B expression is associated with poor overall survival in oral squamous cell carcinoma patients[241] and could predict recurrence in patients where ovarian carcinoma was treated with platinum-based therapy.[242] *In vitro*, ATP7B has been shown to modulate cisplatin resistance in human epidermoid carcinoma and prostate cancer cell lines.[243] A functional interaction between cisplatin and up-regulated ATP7B results in the active transport of cisplatin into exocytic vesicles, however, there have also been suggestions that ATP7B can mediate active efflux directly across the membrane.[244, 245] Platinum-based drugs can directly bind to the six N-terminal metal-binding domains of ATP7B.[246] Analogous to ATP7B, ATP7A can mediate resistance to platinum-based drugs in ovarian, colon and non-small cell lung cancer cells.[247-250] In patients, ATP7A expression correlates with a poorer survival in non-small cell lung cancers.[248] In addition to platinum-based therapeutics, ATP7A confers cellular resistance to SN-38, taxol, mitoxantron, doxorubicin, etoposide and vincristin in an *ex vivo* assay using human patient colon cancer samples.[249] ATP7A induces the compartmentalization of cisplatin, doxorubicin and SN-38 in the Golgi apparatus[249,250] preventing the drugs from reaching their nuclear target, DNA. ATP7A also enhances the efflux rates of doxorubicin and SN-38 by a mechanism dependent

on the vesicle transport system.[249] Supporting the notion that cisplatin efflux occurs via vesicle trafficking, fluorescein-labeled cisplatin is sequestered into lysosomes, the Golgi apparatus and vesicles of the secretory pathway.[251]

5.2. Overcoming acquired resistance with copper modulating agents

The strong link described above between copper transporters and resistance to chemotherapeutic drugs provides a rationale for moderating this association during cancer treatment. One possibility might be to prevent Ctr1 degradation, and thus increase the uptake of platinum-based drugs into applicable cancer cells to enhance their efficacy. Ctr1 expression is regulated by copper availability at both the transcriptional and post-translational levels. When the bioavailable (exchangeable) pool of copper is low, the transcription factor Sp1 binds to the Ctr1 promotor and up-regulates Ctr1 expression.[252] When copper is high, Ctr1 is internalized and degraded in certain cell types.[236] Based on these observations, a clinical trial was performed on 5 patients with platinum-resistant high-grade epithelial ovarian cancers using a combination of trientine, a copper lowering agent (chelator), and carboplatin, a second-generation platinum drug.[253] One patient had partial remission, three had stable disease and one had progressive disease after two cycles of therapy.[253] The greater response to therapy was observed in patients where low serum copper levels were achieved, as measured by ceruloplasmin. An exploratory phase I clinical trial was then performed on a larger cohort of patients (n = 55, including 45 patients with tumors resistant to platinum-based agents) with various advanced malignancies including head and neck, non-small cell lung and epithelial ovarian cancers.[254] The combination of carboplatin and trientine was well tolerated and had improved anticancer activity when compared to carboplatin used alone, but again only in a subset of patients who achieved a significant decrease in serum copper level.[254] A separate study using xenografts of human ovarian cancers in mice, provided additional evidence that platinum-based drugs (cisplatin) and copper chelation (D-pen) together decreased tumor growth more effectively than either treatment alone.[255] Furthermore, D-pen treatment was found to be more effective on cisplatin resistant cells, where it up-regulated Ctr1 expression by 20-fold in comparison to 2-fold in cisplatin sensitive cells.[255] Therefore, it is possible that patients with low Ctr1 expression associated with cisplatin resistance might benefit more from the combination therapy with a platinum-based therapeutic and a chelator. Similarly, the copper chelator TM enhanced cisplatin treatment efficacy in a mouse model of cervical cancer, by increasing cisplatin-DNA adduct levels and by impairing angiogenesis.[234] However, Ctr1 expression and localization did not

change in cervical cancers treated with TM and cisplatin, indicating different mechanisms for Ctr1 regulation in cervical cancer. Another proposed way to modulate Ctr1 expression is to increase intracellular GSH levels.[256] Sequestration of intracellular copper by GSH has been suggested to lower the bioavailable copper pool, in turn up-regulating Ctr1 expression and increasing cisplatin sensitivity.[256] A potential problem with this approach is that GSH already exists in millimolar concentrations, far exceeding the concentration of intracellular copper.[39] However, it is important to note that enhancing the toxicity of platinum-based therapeutics in any manner, requires specificity towards cancer cells, as such therapies are already extremely toxic systemically.

6. Concluding remarks

Preclinical and clinical studies have marshaled enough evidence to merit the thorough investigation of copper coordination compounds as anticancer therapies, both as single agents and in combination with other treatments. Efforts are now clearly underway to better categorize the different types of copper coordination compounds and to define the biological features ideal for their anticancer activity. However, also essential is the need to better understand the role copper plays in cancer etiology and pathogenesis, and to delineate which cancer types are appropriate for treatments that target or utilize copper. Also required is the development of accurate biomarkers for both personalize treatment strategies and for evaluating clinical activity. Therefore, the future success of copper coordination compounds in the clinic necessitates close collaborations between biomedical scientists, chemists and clinicians.

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Figure Legends

Figure 1. **Copper homeostasis in mammalian cells**. After reduction to its Cu^{+} form, copper enters the cell via the copper importer Ctr1. Copper is then passed on to the chaperones CCS, COX17 and ATOX1, which deliver copper to cytosolic SOD1, COX in the mitochondria and to ATP7A/B at the trans-Golgi network (TGN), respectively. Additionally, binding of copper to MTs and GSH, two cellular antioxidants, helps prevent free copper catalyzing the formation of reactive oxygen species. At the TGN, copper is incorporated into copper-dependent enzymes such as ceruloplasmin, which migrate through the secretory pathway. When intracellular copper is elevated (↑ Cu), Ctr1 is internalized and is subsequently degraded, whereas ATP7A and ATP7B traffic from the TGN to the plasma membrane to facilitate copper excretion. Ctr2 can increase the generation of a truncated form of Ctr1 (tCtr1), which transports endosomal copper to the cytoplasm resulting in decreased intracellular copper accumulation. ATOX1 = antioxidant protein, ATP7A/B = copper transporting ATPase A/B, COX = cytochrome c oxidase, CCS = copper chaperone for SOD1, COX17 = cytochrome c oxidase copper chaperone, Ctr1/2 = copper transporter 1/2, Cu = copper, GSH = glutathione, MT = metallothioneins, SOD1 = Cu/Zn-superoxide dismutase, tCtr1 = truncated Ctr1.

Figure 2. Copper coordination compounds targeting cancer cells. Chelators sequester copper making it unavailable for tumor growth, angiogenesis and metastasis. In contrast, ionophores facilitate copper entry into cells, often providing bioavailable intracellular copper. Amongst the different copper coordination compounds there have been a myriad of anticancer activities ascribed, including but not limited to, proteasome inhibition, ROS production, DNA interactions, topoisomerase inhibition, paraptosis and apoptosis. COX = cytochrome c oxidase, Ctr1 = copper transporter 1, FGF2 = fibroblast growth factor 2, IL-1α, -6, -8 = interleukin-1α, -6, -8, LOX = lysyl oxidase, MEK1/2 = mitogen-activated protein kinase/ERK (extracellular-signal-regulated-kinase) kinase 1/2, NF-κB= nuclear factor-kappa B, UPR = unfolded protein response, VEGF = vascular endothelial growth factor, XIAP = X-linked inhibitor of apoptosis

Table 1 Prominent cuproenzymes in mammals

Common Name	Major Localization	Enzymatic Function
Ceruloplasmin	Plasma	Oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+})
Lysyl Oxidase	Extracellular fluid, cartilage, bone and blood	Connective tissue synthesis (cross-linking of collagen and elastin)
Tyrosinase	Melanocytes of eye and skin	Pigment (melanin) synthesis
Dopamine-β-hydroxylase	Catecholamine storage vesicles in neuron	Neurotransmitter synthesis, conversion of dopamine to acetylcholine (noradrenaline)
Cu/Zn superoxide dismutase (SOD)	Cytoplasm and mitochondria	Free radical detoxification, dismutation of superoxide radicals
Cytochrome oxidase	Inner mitochondrial membrane	Electron-transport enzyme
Methionine synthase	Cytoplasm	Catalyzes the conversion of homocysteine to methionine
Vascular Adhesion Protein 1 (VAP-1) Aka, Semicarbazide Sensitive Amine Oxidase (SSAO)	Cell surface, expressed in endothelial cells, smooth muscle cells and adipocytes	Oxidative conversion of amine to aldehydes Adhesion of leukocytes to endothelial cells

Figure 1

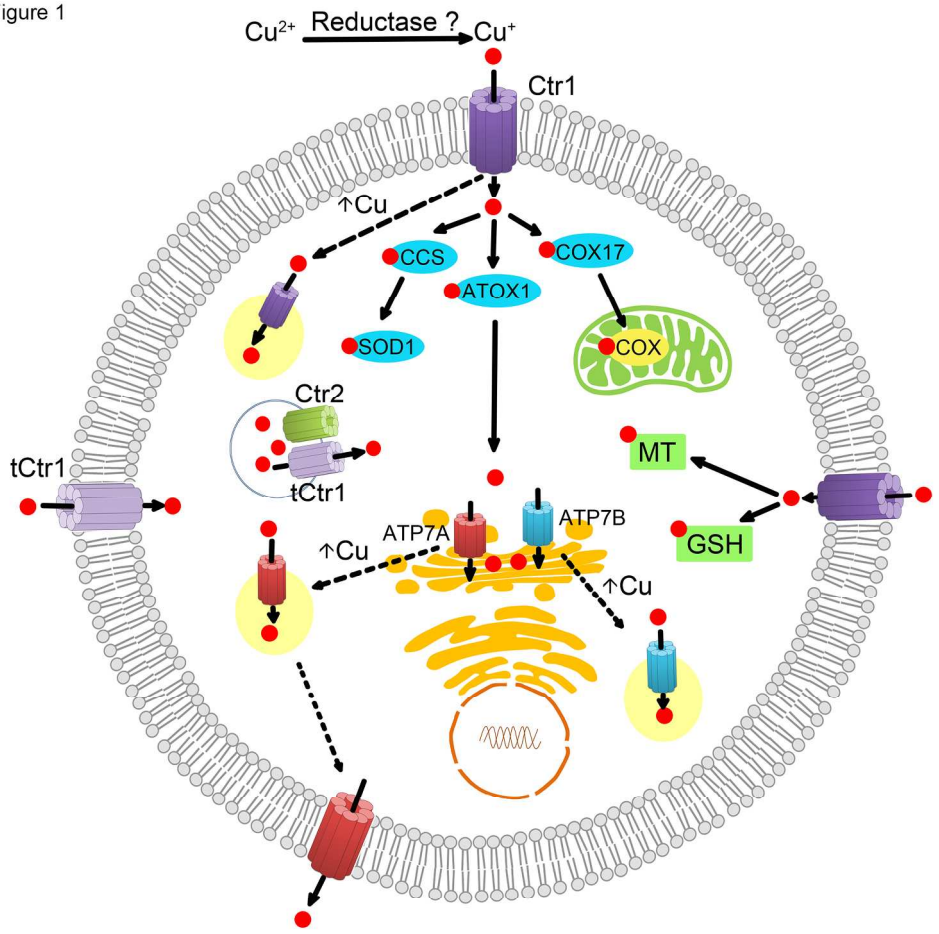

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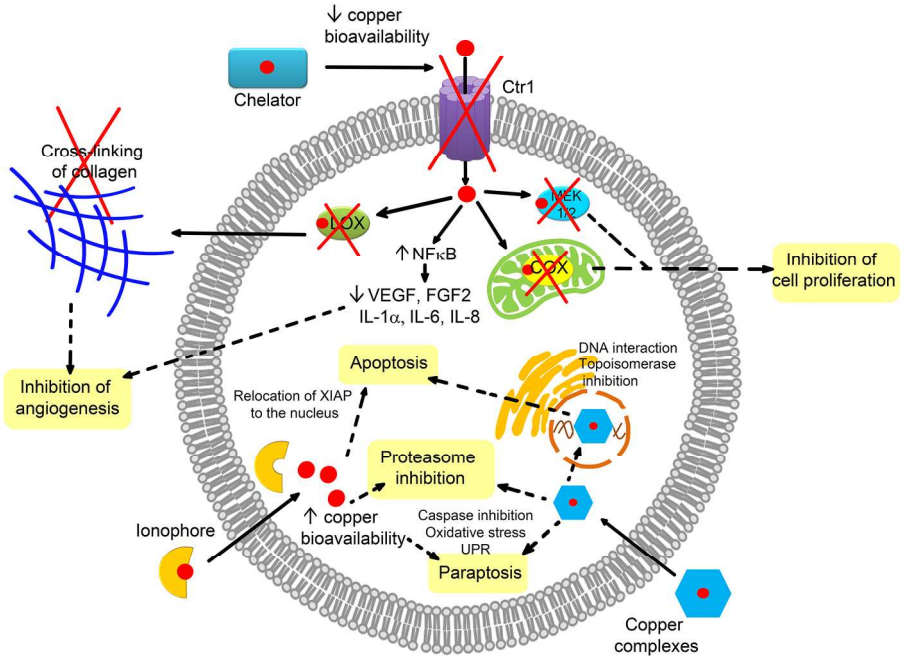
Figure 2



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