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COPPER STORAGE DISEASES: MENKES, WILSON'S, AND CANCER

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1. ABSTRACT

The trace element copper is vital to the healthy functioning of organisms. Copper is used in a multitude of cellular activities including respiration, angiogenesis, and immune responses. Like other metals, copper homeostasis is a tightly regulated process. Copper is transported from dietary intake through the serum and into cells *via* a variety of transporters. There are a variety of copper chaperones designed to insure that copper is sequestered from interaction with cellular membranes, proteins, or DNA where its properties can result in oxidative damage. However, there are disease states in which copper transporters crucial to homeostasis are impaired resulting in potentially toxic copper accumulation. Wilson's and Menkes diseases are two such cases. Wilson's disease (hepatolenticular degeneration) is an autosomal recessive disorder resulting in extreme accumulation of copper in the liver with deposits elsewhere in the body. Menkes is characterized by a systemic copper deficiency (different from the liver specificity of Wilson's disease) and is the result of an X-linked recessive mutation in a copper transporter. Uptake of copper is impaired due to inability to remove existing copper from cells primarily in the small intestine. Though the causes are dramatically different, cancer also shares a similar diagnostic in the accumulation of copper in effected tissues. Studies have shown greatly elevated levels of copper in cancer tissues, and some diagnostics and treatments from Wilson's and Menkes diseases, such as copper chelation therapy, have been used in the treatment of cancer. Given the commonality of copper accumulation in these diseases and that common therapies exist between them, it may prove beneficial to study all three diseases in light of copper homeostasis. This review will examine the chemical nature and biological roles of copper, Wilson's

and Menkes disease and their therapies, and the use of copper related therapies in cancer.

2. INTRODUCTION

Copper is a trace element essential to all organisms. Copper is ubiquitous in the diet and systemic copper concentrations are highly regulated. Typically, the amount of copper consumed is equal to the amount excreted each day. Chemical reactions involving copper are typically oxidative-reductive in nature. Biologically, copper fulfills a variety of roles including detoxification of reactive oxygen species, mitochondrial respiration, and connective tissue development. When copper regulation fails a variety of disease states can develop. Two examined here are failure of copper elimination, Wilson's Disease, and extreme systemic copper deficiency, Menkes Disease. A hallmark of cancer is the accumulation of copper in effected tissues. By examining the diagnosis and therapy of Wilson's and Menkes diseases, new insight may be gained into mechanisms of angiogenesis in cancer and new approaches may be developed to treat cancer. The accumulation of copper by cancer cells might prove to be a distinguishing characteristic that can be targeted by future chemotherapeutic agents.

3. THE CHEMICAL NATURE OF COPPER

Numerous excellent textbooks and monographs exist that describe the chemistry of copper (1-6). Here we provide a brief synopsis of the chemical nature of copper. The 29th element in the periodic table is copper (Cu). The most abundant form of copper on earth is the mineral chalcopyrite ($CuFeS_2$), which accounts for approximately 80% of the world's copper deposits. Copper can be isolated from ore by oxidative roasting and smelting, or by microbial-assisted acid leaching, followed by electrodeposition from sulfate containing ore solutions. Although there are numerous isotopes of copper, the two most common ones are the stable, non-radioactive isotopes ^{63}Cu and ^{65}Cu with abundances of 69.17% and 30.83%, respectively.

Elemental copper is a reddish metallic solid that is malleable, ductile, and a good conductor of heat and electricity. Copper is an important element of industrial alloys such as brass and bronze and is completely miscible with gold. Lustrous Cu is slowly oxidized in moist air, often giving rise to a green coating of hydroxy carbonate and/or hydroxy sulfate caused by the absorption of CO_2 or SO_2 from air.

Copper catalyzes numerous reactions, particularly in aqueous solution. These reactions often involve oxidation-reduction (redox) systems and typically employ the reductive capacity of the Cu(I)-Cu(II) redox pair frequently using molecular oxygen as the oxidant. Reactions involving the Cu (I)-Cu (II) redox pair are especially important in biochemical reactions in living organisms.

Elemental Cu has a ground state electronic configuration of $[Ar]3d^{10}4s^1$. The *d* shell is not very effective at shielding the lone outer *s* electron from the positively charged nucleus. As a result, the first ionization energy for copper is higher for copper than for alkali metals, such as potassium. This property is also responsible for its radius, which is smaller than alkali metals (0.93 Å vs. r_{Na^+} = 0.95 Å and r_{K^+} = 1.33 Å) (2, 3). The second and third ionization energies are low with respect to alkali metals, contributing to its transition metal character. The electrons of the *d* shell are also involved in metallic bonding resulting in a high heat of sublimation and high a melting point.

3.1. Oxidation states of copper

Copper can be oxidized to form Cu(I), Cu(II), Cu (III), and Cu (IV). Copper (I) is the favored oxidation state in the gas phase. In crystals and in solution, Cu(II) is generally favored.

3.1.1. Copper (I)

The electronic configuration of Cu(I) is $[Ar]3d^{10}$. Complexes of Cu(I) are diamagnetic and are generally colorless, unless the accompanying anion is paramagnetic or colored. Cu(I) stability is particularly dependent on the solvent in which the Cu(I) ions are dissolved. Aqueous solvents have very low equilibrium concentrations of Cu(I) ($<10^{-2}$ M), because Cu(I) often becomes rapidly oxidized to Cu(II). Generally, Cu(I) complexes can be formed with halides or amines and have a tetrahedral geometry like Zn(II), which is isoelectronic with Cu(I). It is noteworthy that other geometries for Cu(I) complexes have been observed including linear and trigonal planar geometries. Since Cu(I) is a "soft" (*i.e.*, polarizable) ion, it typically prefers ligands that have polarizable electrons like sulfur ligands or aromatic ligands like 2, 2'-bipyridine.

3.1.2. Copper (II)

The electronic configuration of Cu(II) is $[Ar]3d^9$. Complexes of Cu(II) are generally paramagnetic and give rise to a signal in their EPR spectrum. Cu(II) can adopt coordination numbers of four, five, or most commonly, six, though regular octahedral structures are not commonly observed. The geometry of the octahedral complexes distort so that four short Cu-L bonds and two long bonds form to Cu(II). Halide containing complexes of Cu(II) are readily available and are generally very soluble in aqueous or alcoholic solvents.

Multi-dentate ligands complexed to Cu(II) by O or N (*e.g.* amino acids) are quite stable. Whether ligands form monodentate, bidentate, or tridentate complexes with Cu(II) is frequently dependent on the pH of the solution. The strengths of the coordinate covalent bond for complexes with amine ligands are as follows: $NH_3 > RNH_2 > R_2NH > R_3N$. Imidazole containing compounds often react with copper(II) to produce complexes with the formula, $Cu(L)_4X_2$, where L is the ligand and X is a halide. The imidazole ligands coordinate to copper in a coplanar fashion, whereas the halides make up the other vertices of a distorted octahedron. Coordination to Cu(II) (and Cu(I)) of the imidazole from histidine is, of course, important in copper ligation in proteins. Numerous other heterocyclic compounds can coordinate to Cu(II). For example, Cu(II)

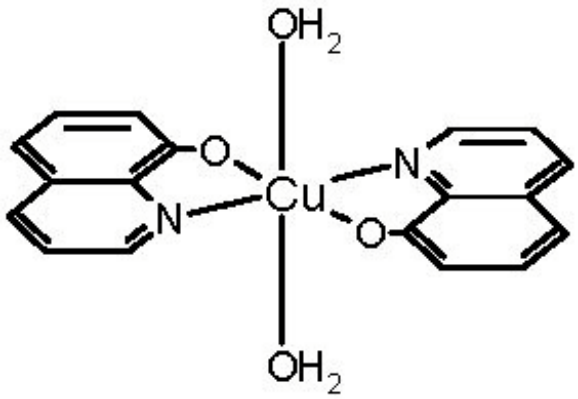

Figure 1. Depiction of the structure of bis-aquo (bis-8-hydroxyquinolinato-N, O) Cu(II) based upon crystallographic data (6).

can form an octahedral complex with 8-hydroxyquinoline in aqueous solution (6) (Figure 1). Copper hydrides have been thought to play a part as reactive intermediates in hydrogenation reactions involving Cu(II), but this has not been confirmed.

3.1.3. Copper (III)

Copper(III) complexes are prepared by the oxidation of Cu(I) or Cu(II) by using various oxidizing agents such as chlorine or fluorine gases, aqueous hypochlorite (ClO^-), or unusual oxyanions. The electronic configuration of Cu(III) is [Ar]3d^8, so the *d* electrons may be arranged to give two unpaired electrons (high spin, paramagnetic) or all paired electrons (low spin, diamagnetic). However, the diamagnetic case is more common. The formation of Cu(III) complexes typically involves oxygen, nitrogen, and sulfur ligands. The crystal structure of a tripeptide complex, α-aminoisobutyryl-α-aminoisobutyryl-α-aminoisobutyric acid (H_2Aib_3) exhibits a square planar center with the Cu-N and Cu-O bond lengths being shorter than in similar Cu(II) complexes.

3.1.4. Copper (IV)

Copper (IV) complexes are very rare and have only been prepared with F^- and O^{2-}, which are anions of the two most electronegative elements. The best-characterized Cu(IV) complex, $[CuF_6]^{2-}$ is probably paramagnetic and is thought to be octahedral.

For further details on copper chemistry please see references (1-6).

4. THE BIOLOGICAL ROLE OF COPPER

Copper (Cu) is found in all living organisms and is a crucial trace element in redox chemistry, growth, and development (7, 8). A variety of copper-containing metalloenzymes are found in both plants and animals in which copper ions are typically bound to nitrogen or sulfur containing ligands. These metalloenzymes are important in electron transfer, oxygen transport and oxygenation reactions, and can contain Cu(I), Cu(II), or Cu(III) species. Daily intake of copper ranges from 0.6 to 1.6 mg / day with the main sources of copper being seeds, grains, nuts, beans, shellfish, and liver (7, 9). Copper toxicity comes about from its abilities to produce reactive oxygen species, displace other metal ions, peroxidize lipids, and directly cleave DNA and RNA (10, 11). Copper homeostasis is very tightly regulated with excretion being the main factor in maintaining copper levels (7, 8).

4.1. Copper absorption

Copper absorption takes place primarily in the small intestine and excretion occurs primarily through the bile (about 1 mg / day for the adult) (7, 8, 12). Excess copper in cells is bound to glutathione and subsequently metallothionein (13). Metallothioneins are used for metal storage rather than uptake, with copper having a much higher degree of affinity for these proteins and the capability to displace other ions from metallothioneins (13, 14). There are three proteins primarily responsible for delivery of copper throughout the organism. Ceruloplasmin is the principal carrier molecule of copper in the serum carrying 65-70% total available copper to the tissues (8, 15). Next albumin and transcuprein carry 12-18 and 9% respectively (8).

Once absorbed across the brush-border, copper is transported to the trans-Golgi-network via the metallochaperone (ATOX-1) in mice or human atox-1 homolog (HAH1) in humans, and both proteins deliver copper to the P-type ATPases located there (16, 17). For enterocytes, the P-type ATPase is ATP7A (MNK), the protein involved with Menkes disease, and in the liver, the P-type ATPase is ATP7B, the Wilson's disease protein (12, 18). For Cu/Zn super oxide dismutase, the carrier is copper chaperone CCS, and for the mitochondria, COX17 delivers copper for use by cytochrome *c* oxidase (19-23).

The below are some examples of copper-binding/using proteins/enzymes (sections 4.2-4.6).

4.2. Ceruloplasmin

Ceruloplasmin, the primary copper carrier in serum, is also a potent ferroxidase capable of oxidizing Fe(II) to Fe(III) (24). Oxidation of iron is important in its binding to transferrin (24). During copper deficiency iron remains in the liver and upon addition of copper containing ceruloplasmin, iron is immediately released from the liver into the blood (25, 26).

4.3. Cytochrome *c* oxidase

COX17 delivers copper to the mitochondria for use by cytochrome *c* oxidase (12). This protein has a variety of metal binding sites including two copper specific sites (27). This cytochrome is responsible for conversion of O_2 to water and the pumping of protons into the intra-mitochondrial membrane space producing the proton gradient used in ATP synthesis (8).

4.4. Cu/Zn superoxide dismutase

Cu/Zn-SOD (SOD1) is a member of the SOD enzyme family with three known enzymes Cu/Zn-SOD, Mn-SOD, and EV-SOD, which convert superoxide anions

to peroxide (28). SOD1 is a homodimer that is almost exclusively located in intracellular cytoplasmic spaces (28). Typically, this enzyme is constitutively expressed. If there is a shortage of available copper this enzyme is one of the first to lose its activity even in hyperoxia situations (8). A report by Marikovsky, *et al.*, suggests that SOD-1 plays an important role in inflammation (29).

4.5. Metallothioneins

The primary purpose of this group of proteins is to sequester metal ions when they are present in excess (30). Metallothioneins can bind Cu, Zn, Cd, Hg, Ag, or Ni (30). However, Cu has the highest apparent affinity and can displace other metal ions (30). It has been reported that metallothioneins with bound copper possess some SOD activity (31).

4.6. Lysyl oxidase

Protein-6-lysine oxidase plays an important role in development of connective tissue as it is required for cross-linking collagen and elastin (8, 32). In Menkes disease, connective tissue development is altered due to failure of protein-6-lysine oxidase although copper may play a structural role rather than a catalytic role in this enzyme (33, 34). Due to reduced functionality of lysyl oxidase, patients show hyperelastic skin, hernias, and aortic aneurysms among other connective tissue related abnormalities (32, 35).

4.7. Copper uptake

The principal copper uptake enzymes are members of the copper transporter (Ctr) family. These high affinity transporters are composed of 3 transmembrane domains and are believed to form trimers that create channels through which copper migrates (36). The transporters conduct uptake with a *Km* of 1-5 μM and demonstrate a preference for Cu(I) (36). Since there is some correlation between K^+ uptake and Cu(I) uptake, it has been suggested that these two ions are taken up together, though whether this is true and whether this occurs through the same enzyme remains unknown at this time.

4.8. Other copper associated proteins and enzymes

Copper can be found in a variety of crucial enzymes where it plays a role ranging from structural support to a redox active center. Other enzymes include, but are not limited to: tyrosinase, dopamine-β-monooxygenase, α-amidating enzyme, diamine oxidase, angiogenin, some matrix metalloproteinases such as secreted protein acidic and rich in cysteine (SPARC), and blood clotting factors V and VIII (8, 37). Disruption of copper homeostasis can have effects on all of these enzymes and their activities as well as the development of toxic levels of the metal and reactive oxygen species. In both Wilson's and Menkes diseases, there is a failure of copper transportation. As a result, copper uptake is impaired and copper becomes deposited in various tissues (esp. liver and eye in Wilson's disease). Likewise, cancer tissues have reportedly high levels of copper. Though neurodegeneration and liver disease are not the result, the excess copper may be involved in angiogenesis, through the activity of matrix metalloproteinases, induction and release of vasoendothelial growth factor (VEGF), and in direct damage through creation of reactive oxygen species, although the role of copper in angiogenesis remains unclear. So while Wilson's and Menkes disease differ significantly from cancer, it is possible that certain therapies and diagnostic tools may be applicable to cancer since the therapies involve monitoring and control of copper.

5. MENKES DISEASE

The *ATP7A* gene (chromosome location Xq12-q13, OMIM 309400) codes for a P-type ATPase that is responsible for excretion of copper from cells and delivery of copper to enzymes in the trans-golgi network (32, 38-43). Menkes disease is an X-linked disease that affects 1 in 200,000 births due to mutations in the *ATP7A* gene leading to an inactive enzyme. This results in accumulation of copper systemically (32, 39, 40). This is a fatal disorder that results in neuronal degeneration and connective tissue abnormalities and is typically lethal by 3-4 years of age (32, 38-41, 43).

Loss of MNK (the gene product of *ATP7A*) forces copper to remain sequestered in the cell (especially cells of the intestinal tract) and creates a systemic copper deficiency since MNK is primarily responsible for delivery of copper to enzymes (such as lysyl oxidase) and efflux of copper when the ion is present in excess (38, 39). While presentation of clinical features of Menkes disease can vary, there are some commonalities. Primarily is the "kinky hair" first described as "steely wool" in sheep leading to the first descriptor of the disease as Kinky Hair Disease (42). The classic disease, presented in the Menkes Family, showed early growth retardation, cerebral and cerebeller degeneration, and death in boys before the age of two (44). Other biochemical markers include low serum copper and ceruloplasmin levels (45).

Menkes disease can be diagnosed by genetic screening to ascertain mutations in the *ATP7A* gene. However, detection is more commonly found *via* cultured cells that accumulate copper and have significantly reduced copper efflux (46, 47). Unfortunately, parenteral therapy with copper salts to correct the copper deficiency has not been successful in dealing with the neurodegeneration that accompanies Menkes disease (39). Prenatal diagnosis of Menkes disease is critical due to the fact that genetic screening of mothers may not always reveal *ATP7A* mutations (48).

One of the key diagnostic features of Menkes disease is the capacity for primary culture cells from patients to accumulate copper (46, 47). Furthermore, early diagnosis and treatment is critical for Menkes disease. Use of labeled copper to monitor copper accumulation in cultured amniocytes (from the fetus) or even skin fibroblasts from patients can diagnose the presence of Menkes disease (48). Therefore, it is possible that inductively coupled plasma optical emission spectroscopy

(ICP-OES) could be used to determine subtler changes in copper concentrations.

The current treatment of Menkes disease is administration of copper-histidine to the patient (39). Copper-histidine represents the main copper-amino acid complex found in serum (39). Four case studies of boys provided with copper-histidine as a therapy all showed stabilized or dramatically improved disease conditions (39). Though one child died before the age of 11 years and another was 20 years old at the time of the report on the efficacy of therapy (39).

Menkes disease is a fatal X-linked genetic defect in the MNK protein (product of the *ATP7A* gene). Much like cancer, successful therapy requires early detection. Only early diagnosis (prior to or just after birth) can prevent the neurodegeneration and development of the child can proceed normally otherwise those afflicted die before the age of three (48). However, the diagnostic tool of genetic analysis is not 100% reliable. Though in cases where Menkes disease is suspected, analysis of copper accumulation by host cells will provide a clear indication of the presence of the disease. In this case, cellular copper retention proves to be an excellent marker for the presence of the disease.

6. WILSON'S DISEASE

Similar to Menkes disease, the rare autosomal recessive Wilson's disease (chromosomal location 13q14.3-q21.1 OMIM: 277900) is caused by a defect in the *ATP7B* gene that codes for a copper transport gene required for copper excretion via the bile (39, 49). In Wilson's disease, liver copper levels rise and serum ceruloplasmin levels decrease. This decrease is believed to be partially due to the role ATP7B plays in ceruloplasmin formation (39, 49, 50). Wilson's disease is more common than Menkes disease (about 1 in 40,000 people as opposed to 1 in 200,000 for Menkes disease), and if detected before permanent damage takes place, it is readily treatable through copper chelation and zinc loading therapies (39, 49, 51).

The clinical presentation of Wilson's disease can involve hepatic symptoms and neurological symptoms. These symptoms include liver failure (mild or severe), tremors, slurred speech, and other neurological impairments (49). Initially the disease results in accumulation of copper in the liver leading to cirrhosis and hemolysis. Later stages result in copper deposition in the brain and the eyes, in the form of the Kayaer-Fleischer ring. These deposits (esp. Kayaer-Fleischer rings) can serve as a diagnostic marker of Wilson's disease (39, 49, 50).

Traditionally the therapy for Wilson's disease is copper chelation and excretion via the urine (39, 49, 51). The consequences of the disease are due to the toxicity of the copper load in the liver (slightly different from Menkes disease which arises from copper deficiency) and subsequent deposition in non-hepatic tissues (39, 49-51). In 1948 Mandelbrote, *et al.*, determined that copper chelation / elimination would be therapeutic in the treatment of Wilson's disease and found that the chelating agent British anti-Lewisite served this purpose effectively (18). However, British anti-Lewisite requires intramuscular injection of about 3 mL of peanut oil carrying the drug and furthermore the drug itself is a mercaptan resulting in a foul odor associated with it (51). These factors led to the development of orally administered chelators: penicillamine (*D*-PA), trientine, tetrathiomolybdate (TM), and zinc (39, 49, 51). Aside from zinc therapy, which is not an ideal therapy for patients with acute symptomology, only tetrathiomolybdate has minor and reversible side effects (49). Penicillamine therapy is discouraged due to the possibility of permanent neurological damage the drug can cause (39, 49, 51). Trientine has only limited application and a limited toxicity profile, and is likewise discouraged for use (49). Zinc is an inducer of metallothionein, which is a high affinity copper binding protein. However, compared to TM treatment, zinc induction of metallothionein is a slow treatment and not ideal for patients with acute symptomology. Since TM can be toxic (though mildly and reversibly so), after initial hematologic or neurological symptoms have abated and when copper levels have been appropriately reduced, zinc becomes the preferred treatment for Wilson's disease (39, 49, 51). For details on each chelator, please see section 6 below.

In the treatment of Wilson's disease, management of extremely high amounts of liver copper is of primary importance. Initial treatment for patients presenting Wilson's disease with liver damage or neurological impairments centers on the elimination of excess copper usually through copper chelation. Copper chelators prove extremely potent at reducing these highly elevated copper levels to nontoxic levels where zinc maintenance becomes the ideal therapy.

7. CANCER, COPPER, AND ANTICOPPER DRUGS

In has not been reported that cancer is associated with a failure of a copper transporter or with copper toxicity or deficiency. However, it is interesting to note that mislocalization of the ATP7B (Wilson's) protein has been found in hepatocarcinoma cells (52) and that overexpression of ATP7B is associated with cisplatin resistance (53). It is also known that cancer tissues contain extremely elevated levels of copper (54-57). The reasons for this elevation are unclear but one possible result is increased angiogenesis (58-60). Tumor growth and metastasis depend upon angiogenesis, the neovascularization process (61, 62) that requires growth factors, proteases, and the trace element copper (58-60). Copper, but not other transition metals, is a co-factor essential for the tumor angiogenesis processes (58-60). Consistently, high levels of copper have been found in many types of human cancers (54-57). Copper stimulates proliferation and migration of human endothelial cells (63, 64). A specific amount of local copper appears to be required for angiogenesis to occur. It has been shown that three anti-copper drugs used in the treatment of Wilson's Disease, TM, trientine, *D*-PA, have antiangiogenic effects in murine cancer models (65-67). Based on this

information, several studies and some clinical trials (in the case of TM) have been performed to evaluate the antiangiogenic effects of these anti-copper drugs on solid tumors (58).

7.1. Penicillamine

Prior to the advent of TM, treatment with *D*-PA was the most commonly used therapeutic agent for Wilson's Disease. This compound is capable of binding to copper in proteins and stabilizing the complex for copper elimination (66). When used in conjunction with a copper restricted diet, *D*-PA blocked angiogenesis and gliosarcoma in F-344 rats (67). However, this same treatment failed to block lung metastasis or vascularization and tumor growth of VX2 tumors in thigh muscle (67). The failure of the treatment was unknown but possibly due to differences in copper deposition (67). As with many cancers, hepatocellular carcinoma (HCC) cells have been shown to have significantly elevated copper loads (66). Yoshii, *et al.*, found that high-dose *D*-PA (3,000 ppm) administered to rats bearing HCC xenografts blocked tumor development and especially so when in conjunction with a copper deficient diet (66). However, *D*-PA was not as potent as trientine, and *D*-PA treatment can result in significant side effects including auto-immune disease (66). For these reasons and others, *D*-PA has not been further pursued since other copper chelating agents are available.

7.2. Trientine

The development of trientine came about in response to the severe side effects seen in some patients using penicillamine treatment. Trientine is generally more potent than penicillamine at binding copper, and binds free copper, as well as having more potent anticancer effects even without the addition of a copper deficient diet (66). In a previous study, SH-SY5Y neuroblastoma cells were cultured in the presence of trientine (68). As expected, cellular copper was depleted. An early response included overexpression of p53 and p21 as well as activation of caspase-9 and caspase-3 (68). In this case, copper elimination resulted in apoptosis induction *via* the mitochondrial pathway (68). In another murine study, 1500 ppm of trientine was provided to Long-Evans cinnamon rats (a murine model for Wilson's disease) (69). Compared to untreated rats, those receiving trientine possessed 33% smaller HCCs and significantly lowered numbers of HCCs (0.6 average in treated animals compared to 4.1 for untreated), suggesting a strong tumor preventative effect by trientine without toxicity (69).

7.3. Tetrathiomolybdate

Of the compounds for Wilson's therapy, the one most frequently used, currently, is TM. This is due to the toxicity profile of penicillamine and to the limited toxicity profile of trientine (see section 5). However, minimal toxicity has been observed with TM treatment and this effect is rapidly reversible. TM has been used in pre-clinical mouse studies and clinical trials. Earlier studies examined cancer prone Her2/*neu* transgenic mice and athymic nude mice with breast tumor xenografts which were given TM treatment (65). TM treatment (0.7 mg/day) resulted in a nearly 70% reduction in tumor volume and only minimal neovascularization in nude mice with xenografts (65). Furthermore, treatment with 0.75 mg/day TM was capable of preventing development of tumors in Her2/*neu* mice (65). While there was histological evidence of full transformed breast cells in these mice, there was a complete absence of any invasive activity or angiogenesis (65). In another study, male C3H/HeJ mice served as an orthotopic model of head and neck squamous cell carcinoma (70). Treated mice received 50 mg of TM per day in drinking water, which was well tolerated (70). Control mice developed tumors 4.7 times greater in volume than TM treated mice, and control mice also had 50% increased development of microvasculature (70). These results further supported the potential role of TM as an anticancer drug that acts through prevention of angiogenesis.

Phase I clinical trials were conducted during 1998 and 1999 to measure the pharmacodynamics of TM in patients with metastatic cancer (71). The trial was conducted with 18 patients receiving 90 - 120 mg/day TM treatment. No toxicity was found when ceruloplasmin (Cp, a marker of systemic copper load) was reduced to 15 - 20% of baseline (71). However, reversible anemia was found when Cp levels dropped below that threshold (71). While this was not an efficacy trial, *per se*, it should be noted that 5 of 6 patients with TM induced copper deficiency displayed stable disease and reduced angiogenesis within tumor masses (71). These results strongly support the idea that copper binding compounds and their resulting complexes can have very mild and reversible toxicity, a necessary characteristic for chemotherapeutic agents. In 2003, the results from a phase II clinical trial with kidney cancer patients were reported (72). The treatment consisted of 40 mg of TM three times a day with 60 mg prior to sleep (72). While no patients demonstrated complete or partial response, the disease stabilized in four patients during the six-month treatment (72). Overall, TM was well tolerated with mild toxicities (72). It should be noted that this was a small study with patients bearing advanced kidney cancer and this factor may have contributed to the relatively minimal effect of TM on the disease.

Wilson's and Menkes diseases both offer insights into cancer therapy. Menkes disease diagnostics in which cells are assayed for copper accumulation (46, 47) could be adapted for analysis of copper accumulation in potentially cancerous tissues. Therapies for Wilson's disease have already been used in cancer in an antiangiogenic modality that includes both animal studies and, in the case of TM, clinical trials. Thus demonstrating that copper control can serve as a chemotherapeutic strategy for cancer.

8. THE PROTEASOME AND ORGANIC COPPER

The proteasome is a massive multicatalytic protease responsible for degrading a large number of cellular proteins. These target proteins are first tagged with ubiquitin in order to be targeted for degradation by the proteasome. Several regulatory proteins involved in cell cycle and apoptosis processes, such as cyclins, bcl-2 family members, and p53, are degraded by the ubiquitin-

proteasome pathway (73, 74). The eukaryotic proteasome possesses at least three distinct activities: chymotrypsin-like (cleavage after hydrophobic residues), trypsin-like (cleavage after basic residues), and caspase-like (cleavage after acidic residues) (75). In a broad range of cell culture models, proteasome inhibitors rapidly induce tumor cell apoptosis, selectively activating the cell death program in oncogene-transformed, but not normal or untransformed cells, and are able to trigger apoptotic death in human cancer cells that are resistant to various anticancer agents (73, 74, 76-78). Inhibition of the chymotrypsin-like, but not the trypsin-like, activity has been found to be associated with induction of tumor cell apoptosis (79, 80).

8.1. Current Proteasome Inhibitors

The antitumor activity of proteasome inhibitors has been confirmed by the results of Phase I and II trials using MLN-341 (PS-341), a potent and selective dipeptidyl boronic acid proteasome inhibitor that recently gained FDA approval (79, 81, 82). Phase III clinical trials for myeloma were conducted in spring 2003 (83). However, some associated side effects were observed in the PS-341 trials (81, 84), suggesting that there is a need to discover novel proteasome inhibitors with no, or much less, toxicity.

Lactacystin, an antibiotic derived proteasome inhibitor, and MG132 (benzyloxycarbonyl-Leu-Leu-leucinal), a peptide proteasome inhibitor, have been found to induce apoptosis in human leukemia cells (85-88). The novel proteasome inhibitor, CEP1612, was found to induce p53-independent apoptosis in human leukemia, prostate, breast, brain, and tongue tumor cells (79). Proteasome inhibition has been shown to be a very effective and potentially non-toxic chemotherapeutic strategy (73, 74, 76-78, 89). Recently we have shown that there is a link between organic copper and inhibition of proteasome activity (89). This leads to the possibility that cancer could be treated with copper binding compounds to prevent angiogenesis, inhibit the proteasome, and induce cancer cell apoptosis thereby enhancing the activity of either traditional copper chelation or proteasome inhibition alone.

8.2. Copper-based Proteasome Inhibition

Our laboratory has determined that certain types of copper compounds can function as potent inhibitors of proteasome activity and are capable of inducing apoptosis within transformed, but not non-transformed, cell lines (89). Since the compounds effective in Wilson's disease (TM, Trientine, and *D*-PA) are copper binding agents, we examined whether or not these compounds, when complexed with copper, also functioned as proteasome inhibitors. As may be suspected, these compounds possessed no proteasome inhibitory activity (89). However, we were able to find a copper complex from the NCI Diversity Set (NCI-109268) that is a proteasome inhibitor (89). We noticed that NCI-109268 is structurally similar to 8-hydroxyquinoline. A variety of 8-hydroxyquinoline copper complexes were then synthesized and tested for proteasome inhibition. Most were found to be potent, and transient, proteasome inhibitors capable of inducing apoptosis in human leukemia cells (89). Perhaps even more significantly, when human prostate cancer cells, PC-3, were grown under copper-enriched conditions resulting in elevated copper levels within the cells, treatment with the copper binding compound, 8-hydroxyquinoline, resulted in proteasome inhibition and apoptosis (89). These results suggest that tumor cellular copper can be bound by certain types of organic ligands (such as 8-hydroxyquinoline, but not tetrathiomolybdate), which results in formation of a proteasome inhibitor inside the tumor cell. Cells that lacked elevated copper levels were not sensitive to treatment with the copper binding compound alone (89). This approach provides a distinct means to targeting tumor cells over normal cells and uses formation of a compound that does not effect the normal cell population. However, further studies including animal studies, still need to be performed to provide proof of concept information.

It appears that particular types of compounds could serve a dual role in the treatment of cancer by targeting copper. First, such compounds might eliminate copper making this pro-angiogenic factor unavailable for angiogenesis (the TM modality). Second, by using the proper type of ligand the final complex could become a potent proteasome inhibitor (the proteasome inhibition modality). Our analyses show that complex formation between a copper ligand and copper can occur in cells with elevated copper, and the complex itself was non-toxic to non-transformed cells (89). Normal tissues could be protected by several potential checkpoints. First, the ligand used should be a non-toxic compound. Second, the ligand should bind primarily to cellular copper and copper is abundant in cancer cells and vanishingly scarce in normal cells. Third, even though some complex formation could occur away from cancer sites, normal cells are more resistant to proteasome inhibition. Fourth, the proteasome inhibition by copper complexes appears to be transient, suggesting that the complex is degraded or metabolized. Even so this does not save cancer cells from apoptosis, which may act as a further safeguard against toxicity to normal cells. In the final analysis, all these checks against toxicity should promote the development of novel copper binding compounds. Moreover, these two key approaches to cancer control (proteasome inhibition and anti-angiogenesis) should provide the impetus to create nontoxic chemotherapeutic agents by targeting a unique feature of cancer cells: copper content.

9. FUTURE DIRECTIONS

Wilson's and Menkes diseases are currently well-characterized and possess effective non-toxic therapies. Both diseases offer information that may prove useful in the battle against cancer. Menkes disease shows that cellular and tissue accumulation of copper can serve as a marker even in the absence of detected mutations in the causative gene. Likewise, it might be possible for those individuals with a family history of cancer to have tissue samples analyzed for copper accumulation by tracking accumulation of labeled copper in pre-cancerous tissues, similar to analysis of ceruloplasmin levels which has been previously reported. This may serve as a very early

indication of the potential of angiogenesis that helps drive tumor formation and growth. Wilson's disease therapy (which has already been used as an antiangiogenic strategy) demonstrates that copper levels can be managed with very mild and reversible toxicity. Furthermore with the correct compounds (*i.e.* TM), nephrotoxicity and other serious side effects can be avoided. Our own research takes this information and the studies surrounding the anticancer effects of proteasome inhibitors and applies this to other copper chelation agents. These results show that active copper complex proteasome inhibitors could not only block angiogenesis, but also induce cancer apoptosis. Thus, this may prove to be a very potent, specific strategy for the development of non-toxic chemotherapies for cancer.

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