Endocrine-Related Cancer (2004) **11** 255–263

REVIEW

Copper deficiency as an anti-cancer strategy

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Abstract

Copper is a tightly regulated trace element. Disruptions of copper homeostasis are rare and they cause serious disorders such as Wilson's disease and Menkes disease. Copper also plays an important role in promoting physiological and malignant angiogenesis. Formation of new blood vessels by a tumor enables tumor growth, invasion and metastasis. The copper chelator tetrathiomolybdate (TM), which quickly and effectively depletes copper stores, is under investigation as an anti-angiogenic agent. Promising results *in vitro*, in pre-clinical animal models and in an early (phase I) clinical trial have led to ongoing phase II evaluation of TM in patients with advanced cancers.

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Copper balance in biological systems

Copper is an essential trace element for most organisms. Critical proteins such as cytochrome oxidase, zinc–copper superoxide dismutase, lysyl oxidase and several transcription factors require copper for activity. Consequently, complex systems for acquiring and regulating copper influx and efflux have evolved. In the yeast *Saccharomyces cerevisiae*, for example, several essential enzymes are copper dependent. *S. cerevisae* has therefore developed an elaborate system to ensure adequate levels of copper. In an environment where copper is scarce, genes are activated to enhance copper uptake (Dancis *et al*. 1994, Hassett & Kosman 1995, Lesuisse *et al*. 1996, Labbe *et al*. 1997, Yamaguchi-Iwai *et al*. 1997, Winge 1999). Two such genes encode the high affinity copper permeases Ctr1p (Dancis *et al*. 1994, Labbe *et al*. 1997) and Ctr3p (Labbe *et al*. 1997), and another codes for Fre1p, an enzyme responsible for iron and copper uptake from the environment (Lesuisse *et al*. 1996). In mammals, copper balance is established by maintaining equilibrium between copper absorption from dietary intake and copper excretion in the stool (Ravestyn 1944).

Based on the tightly controlled homeostasis of copper in primitive organisms, we have developed the hypothesis that copper availability was a primitive growth regulator, and that this regulatory aspect of copper has been maintained throughout evolution (Brewer 2001*a*). For example, the fungus *Podospora anserina* utilizes a copper-dependent transcription factor to maintain normal growth; organisms such as yeast grow more slowly when copper is unavailable, but survive by using fermentation for energy (Osiewacz & Nuber 1996, Borghouts *et al*. 1997, Borghouts & Osiewacz 1998). We hypothesize that primitive organisms required copper but, unlike other essential elements such as zinc and iron, copper may have been much more variable in the environment. Support for this concept comes from studies of metal content in soils of diverse geographical origins, which demonstrate that copper content is much more variable than the content of other trace metals (Bendell-Young *et al*. 1994, Kashin & Ivanoc 1999, Kudashkin 2000, Tebaldi *et al*. 2000). Consistent with this, copper deficiency is a much more frequent occurrence in grazing animals than is deficiency of other metals.

In the human and other mammals, the first observable evidence of copper deficiency is a drop in the level of blood ceruloplasmin (Cp) (Brewer *et al*. 2000). Cp is a copper-containing protein secreted by the liver into the blood. The copper in Cp accounts for about 90% of the

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1351-0088/04/011–255

total plasma copper. As copper availability to the liver decreases, the liver decreases production and secretion of Cp (Linder *et al*. 1979). Thus, plasma Cp is a good surrogate marker of body copper status.

There are no known harmful effects of a decreased level of Cp, or the decreased availability of copper, associated with moderately reduced Cp levels (defined as a reduction of up to 80%). As the Cp becomes very low, however, clinical evidence of copper deficiency emerges. The first sign is bone marrow suppression manifested as anemia, with or without leukopenia (Brewer *et al*. 2000). The next stages of copper deficiency may involve connective tissue defects, and possibly decreasing bone density if copper deficiency persists. These intermediate stages of copper deficiency are not well described in the human. A late stage of copper deficiency, which has been seen only very rarely in patients, involves a severe neuropathy in addition to pancytopenia.

Disorders of copper balance

Free copper is a potent oxidant. Cells therefore rigorously limit the amount of free copper by binding copper to other molecules. Chaperone proteins shuttle copper through the cell to copper-requiring enzymes in different cellular compartments. Copper toxicity can exist either because of the ingestion of excessive amounts of copper or because of genetic defects that interfere with copper homeostasis. Ingestion of toxic amounts of copper leads to acute gastrointestinal symptoms of nausea, vomiting and diarrhea (Commission on Life Sciences 2000). On the other hand, chronic copper toxicity is almost always due to a genetic defect in copper excretion, known in the human as Wilson's disease (Brewer & Yuzbasiyan-Gurkan 1992, Brewer 2001*b*).

Wilson's disease is a rare autosomal recessive disorder caused by mutations in ATP7B, a copper-binding ATPase and a key component of the biliary copper excretory pathway (Bull *et al*. 1993, Tanzi *et al*. 1993, Yamaguchi *et al*. 1993). Copper slowly accumulates in affected patients with subsequent liver damage and, in many cases, brain damage (Brewer & Yuzbasiyan-Gurkan 1992, Brewer 2001*b*). Most patients present in the second and third decades of life. Presentation of liver disease may vary from mild hepatitis or cirrhosis to acute fulminant hepatic failure requiring emergent liver transplantation.

About half of patients with Wilson's disease develop central nervous system toxicity as the initial clinical manifestation of the disease. The basal ganglia and other parts of the brain co-ordinating movement are affected, producing a movement disorder. Speech, swallowing, co-ordination of fine movement and, later, more coarse movements are incrementally affected (Starosta-Rubinstein *et al*. 1987, Brewer & Yuzbasiyan-Gurkan 1992, Brewer 2001*b*). The neurological syndrome is often accompanied by tremor and patients may have a variety of behavioral abnormalities including depression, personality changes, emotional lability and difficulty in focusing on tasks. If undiagnosed and untreated, the disease is inexorably progressive and ultimately fatal.

Anti-copper drugs

The realization that Wilson's disease was caused by a copper excess spurred the search for anti-copper drugs to treat this disease. The first successful oral drug was D-penicillamine, studied and developed by Walshe (1956). Penicillamine is a reductive chelator; the reduction step decreases the affinity of copper for protein and allows it to be more efficiently chelated. Initiation of penicillamine in patients with Wilson's disease causes massive excretion of copper in the urine, and leads to a strong negative copper balance. Penicillamine is an effective therapy in Wilson's disease except in the setting of neurological disease, where it worsens the patient's neurological symptoms in about half the cases, perhaps by further elevating brain copper during the early copper mobilization process (Brewer *et al*. 1987). Half of the patients who worsen, or 25% of all patients, never recover from this treatment-related functional decline. Another drawback of penicillamine is the side-effect profile. This drug has multiple severe side-effects including hematologic and renal toxicities.

Other agents with efficacy in Wilson's disease include trientine and zinc. Trientine was developed for patients who exhibited penicillamine intolerance (Walshe 1982). Trientine, like penicillamine, has never had a formal toxicity study, but experience indicates that it is effective and safer than penicillamine. It has not been studied with respect to the initial treatment of neurological patients. Zinc produces copper deficiency by inducing synthesis of intestinal metallothionein. This protein has a high affinity for copper and a copper–protein complex is formed (Hall *et al*. 1979, Menard *et al*. 1981, Yuzbasiyan-Gurkan *et al*. 1992). The complexed copper cannot be absorbed into the bloodstream and is excreted in the stool. As zinc is essentially non-toxic, it has become the drug of choice for maintenance therapy in Wilson's disease.

However, a significant therapeutic problem remained in terms of how to treat the acutely ill Wilson's disease patient with neurological manifestations. As discussed above, penicillamine is contraindicated (Brewer *et al*. 1987), and zinc does not act rapidly enough to treat acute neurological disease. Previous work demonstrating that thiomolybdates promote copper deficiency suggested that

tetrathiomolybdate (TM) might be a useful agent in the treatment of Wilson's disease.

TM has dual mechanisms of action (Brewer *et al.* 1991). Given with meals it forms a tripartite complex of TM, copper and food protein, thereby preventing copper absorption. Given between meals, it is absorbed into the blood, and forms a tripartite complex with TM, albumin and the freely available serum copper. The complexed copper is rendered unavailable for cellular uptake, thus the amount of free copper is rapidly reduced.

Although TM had not undergone formal toxicity evaluation, there was enough veterinary literature to allow the Federal Drug Administration (FDA) to approve an 8-week trial of TM as first-line therapy in acute neurological Wilson's disease. TM worked rapidly, titrating the potentially toxic copper of the blood and eliminating further copper toxicity (Brewer *et al.* 1991, 1994, 1996). Over 60 patients were studied in an open trial, and neurologic function was assessed with scored neurologic and speech tests. Two patients experienced deterioration in neurological function. This compares favorably with penicillamine treatment, which, as mentioned above, results in functional decline in 50% of patients. Many of these patients later had functional improvements while receiving zinc maintenance therapy. Toxicity is uncommon with TM use but includes further elevation of transaminase enzymes, and anemia and/or leukopenia related to overtreatment. The bone marrow toxicity is quickly responsive to decreasing the dose of TM treatment.

A comparison of penicillamine, trientine and TM treatment is shown in Table 1.

In an ongoing trial, TM is undergoing a double-blind comparison to evaluate it for superiority relative to trientine in the treatment of the neurological presentation of Wilson's disease. The primary endpoint of the study is the frequency of neurologic deterioration over the first 8 weeks of therapy.

Thus, the remaining needs and questions with respect to anti-copper drugs in Wilson's disease are as follows: (1) to establish the most effective available therapy for initial treatment of the neurological presentation through the double-blind comparison of trientine and TM. If TM proves superior to trientine, to pursue approval of TM through the FDA new drug approval process; (2) to establish the most effective available therapy for initial treatment of the patient presenting with acute liver failure. Currently, if these patients do not require immediate transplant, they are treated in most centers with penicillamine. At our institution, patients with Wilson's disease are treated with a combination of trientine and zinc, which appears to be somewhat superior to penicillamine. Theoretical considerations backed by evidence in one patient suggest that TM may be an even better treatment for these patients, and a study of this hypothesis is planned; and (3) to evaluate and report the final outcomes of ongoing toxicology studies of TM.

Anti-copper therapy in the treatment of cancer

The essential role that angiogenesis plays in tumor development was initially hypothesized by Dr Judah Folkman over 30 years ago. In the absence of new blood vessel formation, solid tumors must receive necessary oxygen and nutrients by diffusion, restricting growth to 1–2 mm (Folkman 1971). Dr Folkman further postulated that tumor cells elaborate a growth factor termed 'tumor angiogenesis factor' (TAF), which would behave as an angiogenic switch (Folkman 1974). Once activated, TAF would promote vessel formation, allowing tumor growth, invasion and metastasis. Thus, blockade of angiogenesis via TAF inhibition might serve as a novel anti-neoplastic strategy.

In the ensuing years, it has been discovered that TAF activity is controlled by not one but several pro-angiogenic mediators including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) (Scappaticci 2002). These pro-angiogenic factors are counterbalanced by inhibitors of angiogenesis such as thrombospondin, angiostatin and endostatin. Tumors exploit an imbalance between the pro-angiogenic and anti-angiogenic factors to allow growth and metastasis (Hanahan & Folkman 1996, Iruela-Arispe & Dvorak 1997).

Table 1 A comparison of penicillamine, trientine and TM treatment in Wilson's disease

Drug	Mechanism of action	Major usage in Wilson's disease	Major toxicities in Wilson's disease
Penicillamine	Chelator	Liver disease	Renal/nephrotic syndrome, autoimmune, cytopenias, worsening of neurologic disease
Trientine	Chelator	Penicillamine intolerance	Anemia
TM	Forms complex with copper and protein	Neurologic involvement	Transaminase elevation, cytopenias

The clinical relevance of angiogenesis in cancer is illustrated by research data that correlate tumor expression of angiogenic growth factors with prognosis. In patients with renal cell carcinoma, for instance, increased basic FGF expression has been correlated with worsened survival (Nanus *et al*. 1993). Additionally, expression of VEGF in breast cancer correlates with a decrease in relapse-free survival (Toi *et al*. 1994).

Following these discoveries, the concept of anti-angiogenic therapy as an approach to treat malignancies has gained popularity, and many anti-angiogenic agents have been developed and are in clinical trials. This review will focus on the role of copper in neovascularization and copper depletion as an anti-angiogenic stategy.

The role of copper in angiogenesis was first appreciated in rabbit cornea models. Copper has been shown to be concentrated in the rabbit cornea during neovascularization induced by prostaglandin E1 (Ziche *et al*. 1982). Addition of copper in the rabbit cornea is sufficient to induce new vessel formation (Raju *et al*. 1982, Parke *et al*. 1988). Furthermore, copper-deficient rabbits were unable to mount an angiogenic response (Raju *et al*. 1982, Ziche *et al*. 1982).

Endothelial cell migration is an essential early step in angiogenesis. Copper has been shown to induce migration of bovine aorta endothelial cells (McAuslan *et al*. 1983). Furthermore, a heparin–copper complex has been shown to stimulate capillary migration *in vitro* as well as angiogenesis *in vivo* (Alessandri *et al*. 1984).

Although the angiogenic-promoting function of copper has been recognized for nearly two decades, the mechanism whereby copper exerts these effects is unknown. Recent work has been aimed at understanding the role of copper in promoting angiogenesis. It has become clear that copper interacts with several angiogenic factors; however, the functional significance of these interactions is in some cases unclear.

Among the angiogenic growth factors known to bind copper *in vitro* is angiogenin. Angiogenin is a protein with ribonucleolytic activity initially isolated from the conditioned media of the human colonic adenocarcinoma cell line, HT-29 (Fett *et al*. 1985). Angiogenin is secreted by vascular endothelial cells and aortic smooth muscle cells in addtion to fibroblasts and tumor cells. It has been shown to induce new vessel formation in the chick chorioallantoic membrane (Rybak *et al*. 1987). An inhibitory antibody to angiogenin inhibits tumor growth of xenografted HT-29 cells (Olson *et al*. 1994) and breast cancer (Piccoli *et al*. 1998) in nude mice. Furthermore, angiogenin antisense oligonucleotides prevent establishment of primary human prostate tumors and metastases in athymic mice (Olson *et al*. 2001).

A functional interaction between angiogenin and copper has been hypothesized on the basis of *in vitro* data. Binding of radioiodine-labeled angiogenin to calf pulmonary artery endothelial cells is increased fourfold by the addition of copper (Badet *et al*. 1989). Using metal ion affinity chromatography, angiogenin was subsequently shown to bind copper (Soncin *et al*. 1997). A similar effect was seen with zinc but not nickel, cobalt or lithium. As copper is known to participate in angiogenesis, the authors postulate that copper binding to angiogenin results in an increased affinity between angiogenin and endothelial cells, thus promoting new vessel formation.

Recent work in an animal model of post-angioplasty restenosis may shed some light on the effects of copper on two pro-angiogenic factors, FGF1 and interleukin (IL)-1α. Both these factors have been implicated in the vascular response to injury (Mandinov 2003). Human acidic FGF1 has been demonstrated to bind copper using a copper-affinity HPLC column (Watanabe *et al*. 1990). FGF1 is an angiogenic factor which requires secretion into the extracellular compartment for activity. As this protein contains no signal sequence for endoplasmic reticulum (ER)–Golgi-mediated secretion, the mechanism of FGF secretion has been investigated. These studies have demonstrated that copper plays a role in the formation of a multi-protein complex implicated in the release of FGF1 in response to heat shock (Landriscina *et al*. 2001). Similarly, IL-1α undergoes copper-dependent secretion into the extracellular compartment. In a rat model of balloon-mediated vascular injury, animals treated with the copper-lowering drug TM developed significantly less intimal thickening than did control rats. TM-treated rats also demonstrate a significant reduction in FGF1 and IL-1α as measured by immunohistochemistry (Mandinov 2003). Taken together, these data suggest that copper chelation may attenuate the restenotic response via inhibition of FGF1 and/or IL-1α release into the extracellular compartment.

In an effort to elucidate the mechanism of copper-mediated angiogenesis, Hu (1988) studied the effect of copper on proliferation of human endothelial cells. Addition of $CuSO_4$ to human umbilical vein endothelial cell culture in a serum- and growth factor-free environment induced cell proliferation and [^{3}H]thymidine incorporation. This effect was specific to endothelial cells, as human fibroblasts and smooth muscle cells did not proliferate under the same conditions. Zinc and iron did not induce endothelial proliferation to a significant extent.

In the same publication, Hu (1998) also studied the effect of antibody-mediated growth factor inhibition on copper-induced cell proliferation. Interestingly, the growth stimulation induced by copper was not blocked by the presence of antibodies to angiogenin, acid

fibroblast growth factor (aFGF), basic FGF, epidermal growth factor, tumor-necrosis factor-α, TGF-β, platelet-derived growth factor, macrophage monocyte chemotactic and activating factor (MCAF) or macrophage inflammatory protein 1α (MIP-1α). Hu (1998) therefore concluded that the proliferative activity of copper on endothelial cells was not mediated through any of these growth factors. Further, he demonstrated that the effects of copper and angiogenin on endothelial cell growth were additive, apparently functioning through discrete mechanisms. Notably, copper-induced proliferation was not studied in the presence of antibodies to VEGF.

VEGFs are a family of angiogenic proteins which are essential in vasculogenesis and hypoxia-induced angiogenesis (Bikfalvi & Bicknell 2002). Recent data suggest that copper may be a required cofactor of VEGF-mediated angiogenesis. Copper sulfate has been shown to induce VEGF expression in primary as well as transformed human keratinocytes at physiologically relevant concentrations (Sen *et al*. 2002). This effect was abrogated by addition of a copper-chelating agent. Furthermore, topical copper sulfate treatment resulted in accelerated wound contraction and closure in BalbC mice with full thickness excisional wounds. Immunohistochemistry of the wound site demonstrated increased VEGF in the copper-treated mice compared with saline-treated controls.

Clearly, copper plays an important role in angiogenesis. The data suggest several mechanisms through which copper may exert this effect: (1) copper may act through binding of angiogenic growth factors and increasing their affinity for endothelial cells, as seen with angiogenin; (2) copper may control the secretion of angiogenic cytokines, as demonstrated with FGF1 and IL-1α; and (3) copper may induce expression of angiogenic growth factors such as VEGF. Thus, therapy aimed at depleting copper may be a successful anti-neoplastic strategy which may target multiple angiogenic growth factors. In fact, as outlined below, inhibiting tumor growth via copper depletion has been a successful strategy in animal models.

In a rat xenograft model, copper depletion prevents invasive spread of gliosarcoma (Brem *et al*. 1990*a*). Fischer 344 rats were injected with 9L gliosarcoma cells. Half were then treated with a low copper diet and penicillamine. Control rats all developed a diffuse invasive pattern of tumor growth whereas 70% of the copper-depleted rats did not. Tumors from treated rats did not develop cytoplasmic extensions and pseudopodia, markers of invasiveness. A follow-up study had similar findings in a rabbit brain tumor model of VX2 carcinomas (Brem *et al*. 1990*b*). Copper-depleted animals developed small, relatively avascular tumors compared with controls. This was correlated with a decrease in serum copper in the treated animals.

The copper-chelating agent trientine has demonstrated anti-tumor activity in a murine model of hepatocellular carcinoma (HCC) (Yoshii *et al*. 2001). In mice treated with trientine as well as a copper-deficient diet, HCC development was nearly abolished. In addition, copper depletion inhibited neovascularization and led to an increase in apoptosis of malignant cells.

Our laboratory has been investigating TM as a novel anti-angiogenic agent. In a murine model of head and neck cancer, TM has shown efficacy in suppressing tumor growth (Cox *et al*. 2001). Mice were injected in the floor of the mouth with 1.5×10^5 SCC VII/SF cells, a squamous cell carcinoma cell line. After achieving measurable tumor growth, mice were treated with fresh water with or without TM added. Copper stores were measured by serum Cp levels, which reflect the pool of bioavailable copper. Cp levels were reduced by 28% in the treated animals. After 7 days of treatment, tumor volume in the control group was 4.7 times that of the TM-treated mice. This was accompanied by a reduction in microvessel density in TM-treated mice versus control.

We have demonstrated that TM-induced copper deficiency suppresses tumor growth in two animal models of breast cancer (Pan *et al*. 2002). First, TM was studied in a mouse xenograft model of inflammatory breast carcinoma. Inflammatory breast cancer is a highly aggressive subgroup of locally advanced breast cancer. These tumors are termed 'inflammatory' because they cause skin thickening, warmth and erythema of the breast, which is mediated by dermal lymphatic invasion and obstruction; interestingly, inflammatory cells are absent. The highly angiogenic and invasive nature of these tumors makes them an ideal model for studying anti-angiogenic strategies. TM inhibited tumor growth of injected SUM 149 cells, a cell line derived from a patient with inflammatory breast cancer. Ten-week-old female athymic mice received intra-mammary injections of 1×10^6 cells, and were subsequently gavaged with water or TM beginning on the day of xenograft transplantation. Cp levels were monitored weekly, and maintained at approximately 20% of baseline. TM treatment resulted in a 69% reduction in the size of primary breast tumors, as well as decreased tumor vascularity as measured by immunohistochemical staining for CD31 an endothelial cell marker.

TM also prevents tumor growth in a transgenic murine model (Pan *et al*. 2002). HER2/neu transgenic mice overexpress a protein associated with poor prognosis in patients with breast cancer. Female mice expressing this gene develop intra-mammary adenocarcinomas which metastasize to the lungs. Mice were treated with TM

beginning at 100 days of age so that they would be copper deficient during the key period of tumor growth. Cp levels were maintained at 10–30% in treated mice, who appeared to tolerate this level of copper deficiency well. Although 50% of control mice developed clinically overt tumors by 218 days, none of the TM-treated mice did, as long as their Cp levels were maintained at $\leq$30% of baseline. Upon release from therapy, TM-treated mice developed measurable tumors in 13 ± 5 days.

To investigate the mechanism of the anti-angiogenic effects of TM, SUM 149 cells were grown in culture and treated with TM (Pan *et al*. 2002). Decreased secretion of pro-angiogenic mediators including VEGF, FGF2, IL-1α, IL-6 and IL-8 was seen in treated cells. Furthermore, conditioned media derived from treated cell cultures had impaired ability to promote new vessel formation in the rat aortic ring assay. TM-treated SUM 149 cells also showed reduced activity of the transcription factor nerve factor-κB (NF-κB), a protein known to be involved in tumor invasion, angiogenesis and metastasis. This effect was reversed by the addition of copper, suggesting that induction of copper deficiency is responsible for reduced NF-κB activity. Because VEGF, IL-6 and IL-8 are NF-κB-regulated genes, we postulate that reduced secretion of these proteins is a direct consequence of the inhibition of NF-κB by TM.

TM may also potentiate the effect of cytotoxic therapies. Cultured SUM 149 cells were treated with TM in combination with doxorubicin. The combination therapy was shown to induce apoptosis in a synergistic fashion (Pan *et al*. 2003). Following these promising results, the combination was utilized in a mouse xenograft model of breast cancer. Female nude mice were transplanted with SUM 149 cells, and tumors were allowed to grow to 0.5 cm^3. Mice were then assigned to receive no treatment, single agent doxorubicin, single agent TM or the combination. Mice receiving the combination regimen had stabilization of tumor volume to a greater extent than mice treated with either single agent with no additional toxicity.

TM has also been combined with external beam radiation therapy in a mouse model of lung cancer, the Lewis lung high metastatic carcinoma model. Combination therapy demonstrated an additive effect on reduction of tumor volume, again with no added toxicity over radiation alone (Khan *et al*. 2002). The addition of TM may also provide a modest survival benefit in these animals.

Given the genetic complexity of human solid tumors, it is not surprising that anti-angiogenic strategies that target a single angiogenic growth factor have failed to inhibit tumor growth in clinical trials. Blockade of one pathway of endothelial proliferation and migration may be overcome by exploitation of redundant pathways to promote development of new blood vessels. In fact, tumors have been shown to express multiple angiogenic factors (Relf *et al*. 1997, Bunn 2002). Effective targeting of malignant neovascularization may thus require development of a global inhibitor of angiogenesis rather than an inhibitor of one angiogenic cytokine.

As outlined above, copper is an essential angiogenic cofactor which appears to exert its effects through several pro-angiogenic mediators. In animal models, copper depletion via TM is a useful anti-neoplastic therapy which may be combined with traditional cytotoxic therapy with additive effects and little or no additional toxicity. Furthermore, TM has a demonstrated safety record in humans where it has been utilized for the treatment of Wilson's disease. Induction of copper deficiency can be easily monitored via measurement of serum Cp. For these reasons, TM is currently being evaluated in clinical trials.

A phase I trial of TM in metastatic cancer has been completed (Brewer *et al*. 2000). Eighteen patients with a variety of tumor types (including breast, colon, lung and prostate) and measurable disease were enrolled. Patients were required to have progressive disease at the time of enrollment. The target Cp level was $\leq$20% of baseline; 14 of 18 patients reached the target Cp level. Eight of these patients were copper deficient for less than 90 days; the majority (seven of eight) of these patients had progressive disease. These patients likely did not achieve stable copper deficiency long enough to have a clinical effect. However, among the six patients achieving adequate Cp levels for $>$ 90 days, four maintained stable disease, one had stable disease with partial regression of lung lesions, and one had disease progression at a single site, with stable disease elsewhere. Toxicity was minimal. Mild anemia was noted in four patients whose Cp reached 10–20% of baseline. Anemia was reversible upon discontinuation of the drug. Several patients reported sulfurous belching as the only other reported toxicity.

Results of a phase II trial of TM in advanced kidney cancer have recently been published (Redman *et al*. 2003). Fifteen patients with metastatic kidney cancer who had not responded to IL-2 or were not eligible for IL-2 were treated with TM. All patients were able to achieve the target Cp level of 5–15 mg/dl. Of the 13 patients evaluable for response, four (31%) had stable disease for at least 6 months; none had a partial or complete response. Grade 3 or 4 granulocytopenia developed in eight patients without episodes of febrile neutropenia. Neutropenia improved with temporary cessation of the drug in all cases. Serum levels of VEGF, basic FGF, IL-6 and IL-8 measured at the onset of copper deficiency were significantly reduced

compared with pretreatment levels; by 3 months, however, only IL-6 remained significantly depressed.

These studies raise several interesting points. First, TM is likely to have a cytostatic, rather than cytotoxic, effect in bulky cancers and is therefore most likely to result in disease stabilization rather than reduction of disease burden. Furthermore, achieving adequate levels of copper deficiency may require several months. Thus, TM monotherapy would probably not be beneficial in patients with a large burden of disease and/or rapidly progressive disease. In the metastatic setting, TM may be most beneficial for patients with minimal disease burden (such as after induction of a complete or partial remission), or concurrently with cytotoxic agents or modalities such as chemotherapy or radiation. Combination therapy has been well tolerated in animals, as noted above. Additionally, TM may be useful in an adjuvant setting or as chemoprevention in high-risk patients. Ongoing phase II studies as well as future trials will attempt to exploit this knowledge to define the role of TM in cancer treatment.

Acknowledgements

This work was supported by the Tempting Tables Organization, NIH grant RO1-CA77612, and Department of Defence Breast Cancer Research Program grants DAMD17-02-1-0492 and DAMD 17-00-1-14 0345, and the Susan G Komen Breast Cancer Research Foundation. Disclosure of conflict of interest: SDM and GJB are consultants to and have financial interest in Attenuon Limited Liability Corporation (10130 Sorrento Valley Road, Suite D, San Diego, CA 92121, USA), which has licensed TM for anti-angiogenic applications from the University of Michigan.

References

Alessandri G, Raju K & Gullino PM 1984 Angiogenesis *in vivo* and selective mobilization of capillary endothelium *in vitro* by heparin-copper complex. *Microcirculation, Endothelium and Lymphatics* **1** 329–346.

Badet J, Soncin F, Guitton J, Lamare O, Cartwright T & Barritault D 1989 Specific binding of angiogenin to calf pulmonary artery endothelial cells. *PNAS* **86** 8427–8431.

Bendell-Young L, Chouinard J & Pick FR 1994 Metal concentrations in chironomids in relation to peatland geochemistry. *Archives of Environmental Contamination and Toxicology* **27** 186–194.

Bikfalvi A & Bicknell R 2002 Recent advances in angiogenesis, anti-angiogenesis and vascular targeting. *Trends in Pharmacological Sciences* **23** 576–582.

Borghouts C & Osiewacz HD 1998 GRISEA, a copper-modulated transcription factor from *Podospora anserina* involved in senescence and morphogenesis, is an ortholog of MAC1 in *Saccharomyces cerevisiae*. *Molecular Genes and Genetics* **260** 492–502.

Borghouts C, Kimpel E & Osiewacz HD 1997 Mitochondrial DNA rearrangements of *Podospora anserina* are under the control of the nuclear gene grisea. *PNAS* **94** 10768–10773.

Brem S, Tsanaclis AM & Zagzag D 1990*a* Anticopper treatment inhibits pseudopodial protrusion and invasive spread of 9L gliosarcoma cells in the rat brain. *Neurosurgery* **26** 391–396.

Brem SS, Zagzag D, Tsanaclis AM, Gately S, Elkouby MP & Brien SE 1990*b* Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor. *American Journal of Pathology* **137** 1121–1142.

Brewer GJ 2001*a* Copper control as an antiangiogenic anticancer therapy: lessons from treating Wilson's disease. *Experimental Biology and Medicine* **226** 665–673.

Brewer GJ 2001*b* The Clinician's Challenge: Recognising Wilson's Disease. In *Wilson's Disease: A Clinician's Guide to Recognition, Diagnosis and Management* pp 10–16. Boston: Kluwer Academic Publishers.

Brewer GJ & Yuzbasiyan-Gurkan V 1992 Wilson disease. *Medicine* **71** 139–164.

Brewer GJ, Terry CA, Aisen AM & Hill GM 1987 Worsening of neurologic syndrome in patients with Wilson's disease with initial penicillamine therapy. *Archives of Neurology* **44** 490–493.

Brewer GJ, Dick RD, Yuzbasiyan-Gurkin V, Tankanow R, Young AB & Kluin KJ 1991 Initial therapy of patients with Wilson's disease with tetrathiomolybdate. *Archives of Neurology* **48** 42–47.

Brewer GJ, Dick RD, Johnson V, Wang Y, Yuzbasiyan-Gurkan V, Kluin K, Fink JK & Aisen A 1994 Treatment of Wilson's disease with ammonium tetrathiomolybdate. I. Initial therapy in 17 neurologically affected patients. *Archives of Neurology* **51** 545–554.

Brewer GJ, Johnson V, Dick RD, Kluin KJ, Fink JK & Brunberg JA 1996 Treatment of Wilson's disease with ammonium tetrathiomolybdate: II. Initial therapy in 33 neurologically affected patients and follow-up with zinc therapy. *Archives of Neurology* **53** 1017–1025.

Brewer GJ, Dick RD, Grover DK, LeClaire V, Tseng M, Wicha M, Pienta K, Redman BG, Jahan T, Sondak VK, Strawderman M, LeCarpentier G & Merajver SD 2000 Treatment of metastatic cancer with tetrathiomolybdate, an anticopper, antiangiogenic agent: Phase I study. *Clinical Cancer Research* **6** 1–10.

Bull PC, Thomas GR, Rommens JM, Forbes JR & Cox DW 1993 The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nature Genetics* **5** 327–337.

Bunn PA Jr 2002 Molecular biology and early diagnosis in lung cancer. *Lung Cancer* **38** S5–S8.

Commission on Life Science 2000, Health Effects of Excess Copper. In *Copper in Drinking Water*, pp 78–127. Washington DC: National Academies Press.

Cox C, Teknos TN, Barrios M, Brewer GJ, Dick RD & Merajver SD 2001 The role of copper suppression as an antiangiogenic strategy in head and neck squamous cell carcinoma. *Laryngoscope* **111** 696–701.

Dancis A, Haile D, Yuan DS & Klausner RD 1994 The *Saccharomyces cerevisiae* copper transport protein (Ctr1p). Biochemical characterization, regulation by copper, and physiologic role in copper uptake. *Journal of Biological Chemistry* **269** 25660-25667.

Fett JW, Strydom DJ, Lobb RR, Alderman EM, Bethune JL, Riordan JF & Vallee BL 1985 Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry* **24** 5480–5486.

Folkman J 1971 Tumor angiogenesis: therapeutic implications. *New England Journal of Medicine* **285** 1182–1186.

Folkman J 1974 Tumor angiogenesis factor. *Cancer Research* **34** 2109–2113.

Hall AC, Young BW & Bremner I 1979 Intestinal metallothionein and the mutual antagonism between copper and zinc in the rat. *Journal of Inorganic Biochemistry* **11** 57–66.

Hanahan D & Folkman J 1996 Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86** 353–364.

Hassett R & Kosman DJ 1995 Evidence for Cu(II) reduction as a component of copper uptake by *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* 128–134.

Hu GF 1998 Copper stimulates proliferation of human endothelial cells under culture. *Journal of Cellular Biochemistry* **69** 326–335.

Iruela-Arispe ML & Dvorak HF 1997 Angiogenesis: a dynamic balance of stimulators and inhibitors. *Thrombosis and Haemostasis* **78** 672–677.

Kashin VK & Ivanoc GM 1999 Copper in Trasbaikal flora. *Agrokhimiya* **10** 52–57.

Khan MK, Miller MW, Taylor J, Gill NK, Dick RD, Van Golen K, Brewer GJ & Merajver SD 2002 Radiotherapy and antiangiogenic TM in lung cancer. *Neoplasia* **4** 164–170.

Kudashkin MI 2000 The content of different copper compounds in gray forest soils of Mordovia and effectiveness of preplanting treatment of barley seeds with copper sulfate. *Agrokhimiya* **3** 16–24.

Labbe S, Zhu Z & Thiele DJ 1997 Copper-specific transcriptional repression of yeast genes encoding critical components in the copper transport pathway. *Journal of Biological Chemistry* **272** 15951–15958.

Landriscina M, Bagala C, Mandinova A, Soldi R, Micucci I, Bellum S, Prudovsky I & Maciag T 2001 Copper induces the assembly of a multiprotein aggregate implicated in the release of fibroblast growth factor 1 in response to stress. *Journal of Biological Chemistry* **276** 25549–25557.

Lesuisse E, Casteras-Simon M & Labbe P 1996 Evidence for the *Saccharomyces cerevisiae* ferrireductase system being a multicomponent electron transport chain. *Journal of Biological Chemistry* **271** 13578–13583.

Linder MC, Houle PA, Isaacs E, Moor JR & Scott LE 1979 Copper regulation of ceruloplasmin in copper-deficient rats. *Enzyme* **24** 23–35.

McAuslan BR, Reilly WG, Hannan GN & Gole GA 1983 Angiogenic factors and their assay: activity of formyl methionyl leucyl phenylalanine, adenosine diphosphate, heparin, copper, and bovine endothelium stimulating factor. *Microvascular Research* **26** 323–338.

Mandinov L 2003 Copper chelation represses the vascular response to injury. *PNAS* **100** 6700–6705.

Menard MP, McCormick CC & Cousins RJ 1981 Regulation of intestinal metallothionein biosynthesis in rats by dietary zinc. *Journal of Nutrition* 1351–1361.

Nanus DM, Schmitz-Drager BJ, Motzer RJ, Lee AC, Vlamis V, Cordon-Cardo C, Albino AP & Reuter VE 1993 Expression of basic fibroblast growth factor in primary human renal tumors: correlation with poor survival. *Journal of the National Cancer Institute* **85** 1597–1599.

Olson KA, French TC, Vallee BL & Fett JW 1994 A monoclonal antibody to human angiogenin suppresses tumor growth in athymic mice. *Cancer Research* **54** 4576–4579.

Olson KA, Byers HR, Key ME & Fett JW 2001 Prevention of human prostate tumor metastasis in athymic mice by antisense targeting of human angiogenin. *Clinical Cancer Research* **7** 3598–3605.

Osiewacz HD & Nuber U 1996 GRISEA, a putative copper-activated transcription factor from *Podospora anserina* involved in differentiation and senescence. *Molecular Genes and Genetics* **252** 115–124.

Pan Q, Kleer CG, van Golen KL, Irani J, Bottema KM, Bias C, De Carvalho M, Mesri EA, Robins DM, Dick RD, Brewer GJ & Merajver SD 2002 Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis. *Cancer Research* **62** 4854–4859.

Pan Q, Bao LW, Kleer CG, Brewer GJ & Merajver SD 2003 Antiangiogenic tetrathiomolybdate enhances the efficacy of doxorubicin against breast carcinoma. *Molecular Cancer Therapeutics* **2** 617–622.

Parke A, Bhattacherjee P, Palmer RM & Lazarus NR 1988 Characterization and quantification of copper sulfate-induced vascularization of the rabbit cornea. *American Journal of Pathology* **130** 173–178.

Piccoli R, Olson KA, Vallee BL & Fett JW 1998 Chimeric anti-angiogenin antibody cAb 26-2F inhibits the formation of human breast cancer xenografts in athymic mice. *PNAS* **95** 4579–4583.

Raju KS, Alessandri G, Zinche M & Gullino PM 1982 Ceruloplasmin, copper ions, and angiogenesis. *Journal of the National Cancer Institute* **69** 1183–1188.

Ravestyn AH 1944 Metabolism of copper in man. *Acta Medica Scandinavica* **118** 163–196.

Redman BG, Esper P, Dunn RL, Pan Q, Merajver SD, Hussain HK, Chenevert T & Brewer GJ 2003 Phase II trial of tetrathiomolybdate in patients with advanced kidney cancer. *Clinical Cancer Research* **9** 1666–1672.

Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, Moghaddam A, Whitehouse R, Bicknell R & Harris AL 1997 Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Research* **57** 963–969.

Rybak SM, Fett JW, Yao QZ & Vallee BL 1987 Angiogenin mRNA in human tumor and normal cells. *Biochemical and Biophysical Research Communications* **146** 1240–1248.

Scappaticci FA 2002 Mechanisms and future directions for angiogenesis-based cancer therapies. *Journal of Clinical Oncology* **20** 3906–3927.

Sen CK, Khanna S, Venojarvi M, Trikha P, Ellison EC, Hunt TK & Roy S 2002 Copper-induced vascular endothelial growth factor expression and wound healing. *American Journal of Physiology. Heart Circulation Physiology* **282** H1821–H1827.

Soncin F, Guitton J, Cartwright T & Badet J 1997 Interaction of human angiogenin with copper modulates angiogenin binding to endothelial cells. *Biochemical and Biophysical Research Communications* **236** 604–610.

Starosta-Rubinstein S, Young AB, Kluin K, Hill G, Aisen AM, Gabrielsen T & Brewer GJ 1987 Clinical assessment of 31 patients with Wilson's disease. Correlations with structural changes on magnetic resonance imaging. *Archives of Neurology* **44** 365–370.

Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W & Ross B 1993 The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nature Genetics* **4** 44–50.

Tebaldi FLH, da Silva JFC, Vasques HM & Thiebaut JTL 2000 Mineral composition of pastures in the North and Northwest regions of Rio de Janeiro State. 2. Manganese, iron, zinc, copper, cobalt, molybdenum and lead. *Revista Brasileira de Zootecnia* **29** 616–629.

Toi M, Hoshina S, Takayanagi T & Tominaga T 1994 Association of vascular endothelial growth factor expression with tumor angiogenesis and with early relapse in primary breast cancer. *Japanese Journal of Cancer Research* **85** 1045–1049.

Walshe JM 1956 Penicillamine: a new oral therapy for Wilson's disease. *American Journal of Medicine* 487–495.

Walshe JM 1982 Treatment of Wilson's disease with trientine (triethylene tetramine) dihydrochloride. *Lancet* **i** 643–647.

Watanabe T, Seno M, Sasada R & Igarashi K 1990 Molecular characterization of recombinant human acidic fibroblast growth factor produced in *E. coli*: comparative studies with human basic fibroblast growth factor. *Molecular Endocrinology* **4** 869–879.

Winge DR 1999 Copper-regulatory domain involved in gene expression. In *Copper Transport and its Disorders: Molecular and Cellular Aspects*, Eds A Leone & JFB Mercer. New York: Kluwer Academic Publishers.

Yamaguchi Y, Heiny ME & Gitlin JD 1993 Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. *Biochemical and Biophysical Research Communications* **197** 271–277.

Yamaguchi-Iwai Y, Serpe M, Haile D, Yang W, Kosman DJ, Klausner RD & Dancis A 1997 Homeostatic regulation of copper uptake in yeast via direct binding of MAC1 protein to upstream regulatory sequences of FRE1 and CTR1. *Journal of Biological Chemistry* **272** 17711–17718.

Yoshii J, Yoshiji H, Kuriyama S, Ikenaka Y, Noguchi R, Okuda H, Tsujinoue H, Nakatani T, Kishida H, Nakae D, Gomez DE, De Lorenzo MS, Tejera AM & Fukui H 2001 The copper-chelating agent, trientine, suppresses tumor development and angiogenesis in the murine hepatocellular carcinoma cells. *International Journal of Cancer* **94** 768–773.

Yuzbasiyan-Gurkan V, Grider A, Nostrant T, Cousins RJ & Brewer GJ 1992 Treatment of Wilson's disease with zinc: X. Intestinal metallothionein induction. *Journal of Laboratory and Clinical Medicine* **120** 380–386.

Ziche M, Jones J & Gullino PM 1982 Role of prostaglandin E_1 and copper in angiogenesis. *Journal of the National Cancer Institute* **69** 475–482.