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Tetrathiomolybdate anticopper therapy for Wilson's disease inhibits angiogenesis, fibrosis and inflammation

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Abstract

The need for agents to lower body copper in Wilson's disease, a disease which results from copper toxicity has been the driving force for the development of the effective anticopper drugs penicillamine, trientine, zinc, and now tetrathiomolybdate (TM). Because of its rapid action, potency, and safety, TM is proving to be a very effective drug for initial treatment of acutely ill Wilson's disease patients. Beyond this, TM has antiangiogenic effects, because many proangiogenic cytokines require normal levels of copper. This has led to use of TM in cancer, where it is generally effective in animal tumor models, and has shown efficacy in preliminary clinical studies. Most recently, it has been found that TM has antifibrotic and antiinflammatory effects through inhibition of profibrotic and proinflammatory cytokines.

Keywords: tetrathiomolybdate - copper - angiogenesis - fibrosis - inflammation

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Introduction

Wilson's disease [1-4], which produces liver and brain damage due to copper accumulation and copper toxicity, has been the major stimulus for the identification of clinically useful anticopper drugs. Walshe [5] was the first to discover an effective oral drug, in penicillamine. Penicillamine was soon found to have a large number of toxicities, and Walshe [6] developed trientine as a substitute for use in penicillamine intolerant patients. Trientine shares some of penicillamine's side effects, but appears to be significantly less toxic. Both drugs act as chelators, increasing urinary excretion of copper sufficient to provide a negative copper balance in Wilson's disease patients.

The anticopper effects of zinc for potential use in Wilson's disease were discovered independently by two groups, one in the Netherlands and one in the U.S. Schouwink [7] was the first to use zinc in Wilson's disease, giving it to two patients who then seemed to do well on this therapy. Schouwink's work appeared in his PhD thesis, but was never published. His work was followed up by Hoogenraad and his group in the Netherlands [8,9]. In the U.S., our group observed that zinc therapy was consistently producing copper deficiency in sickle cell anemia patients [10,11]. This led to clinical trials of zinc in Wilson's disease [12,13], ultimately culminating in approval by the U.S. Food and Drug Administration for maintenance therapy in Wilson's disease, in 1997.

While zinc is proving to be almost ideal for the maintenance therapy of Wilson's disease, it is viewed as too slow-acting for initial treatment of acutely ill patients. Penicillamine is fast acting, but we have learned that it carriers a 50% risk of making neurologically-presenting patients worse neurologically [14]. The mechanism seems to be mobilization of some of the hepatic copper into the brain. Of the worsened patients, about 50% (one in four of the original sample) never recover their original function.

To fill this therapeutic void, we have developed tetrathiomolybdate (TM), as a fast acting, very potent, anticopper agent. In the remainder of this review we will discuss the use of TM in Wilson's disease, and then go on to discuss its use as an antiangiogenic, antifibrotic, and antiinflammatory agent.

Early work on the development of tetrathiomolybdate

The discovery of the anticopper effect of TM dates back to observations in New Zealand and Australia, where ruminants, but not non-ruminants, grazing on certain pastures developed a disease later identified as copper deficiency [15-17]. The soil of these pastures had a high concentration of molybdenum. Molybdenum fed to ruminants, but not when fed to non-ruminants, was shown to cause copper deficiency [18-20]. Then it was realized that the sulfur-rich rumen was converting molybdenum to thiomolybdates [21], and these compounds were found to have anticopper activity in all animals, with the tetra-sulfur compound, TM, being the most potent [22-24].

Considerable experimental work with TM was done in animals over the years. TM is a very potent anticopper agent, and is quite toxic. However, all of its side effects in animals are due to copper deficiency, that is, copper supplementation prevents all TM toxicity [24]. TM acts as an anticopper agent by forming a tight tripartite complex with copper and protein[22-25]. Given with food, TM complexes food copper with food protein and prevents copper absorption [26-29]. Given between meals, or parenterally, TM forms a tight complex with available serum copper (often called free copper) and albumin, and renders the copper unavailable for cellular uptake. This complex is metabolized in the liver and the components excreted in the bile [30-32]. TM is life saving when given intravenously to copper poisoned sheep, who usually die from acute liver failure [33-35].

Tetrathiomolybdate for the therapy of Wilson's disease

Prior to our use of TM, it had been used in one or two terminal Wilson's disease patients in Australia, and Walshe had tried it briefly in England for maintenance therapy in two patients who were intolerant of both penicillamine and trientine [36]. Walshe gave up on it when it produced a reversible anemia in one of these patients.

To me, TM seemed ideal for initial therapy of the neurologically-presenting patient, because of its potency, fast action, and low toxicity in animal stud-

ies. Although formal toxicity studies had not been done, based on the rather extensive animal studies which had been carried out, we received FDA approval for use of TM for up to eight weeks in Wilson's patients presenting with neurological disease.

This study involved an eight week admission to the General Clinical Research Center of the University of Michigan, with weekly semi-quantitative neurological and semi-quantitative speech examinations. TM was given both with meals, most often 20 mg three times daily, to block absorption of food copper, and between meals, most often 20 mg three times daily, to allow complexing of free copper, serum albumin and TM. (A note of importance for later cancer and other studies, serum copper is not usable in TM treatment as a marker of copper status, because the blood complex builds up, elevating serum copper). In an open label study now totaling 55 patients [26-29], TM has shown excellent efficacy in protecting neurological function while gaining control over copper toxicity in about two weeks time. Two of the 55 patients did reach our criteria for neurological worsening. We postulate that in an occasional patient, events are in motion leading to additional neurological deterioration irrespective of the anticopper drug treatment. However, it appears that penicillamine produces a drug-catalyzed neurological worsening in about half of the patients. Currently we are in the final stages of a double blind trial comparing TM to trientine for initial therapy of the neurologically-presenting patient.

We have seen bone marrow suppression by TM in about 11% of patients [26-29]. This is due to copper deficiency in the bone marrow, and responds quickly to dose reduction to half the standard dose of 120 mg/day, or to a brief drug holiday. The other side effect is a mild transaminitis to two or three times baseline levels in 15% of patients [26-29], again responsive to halving the dose. These side effects have caused us to initiate a protocol in which we are examining whether or not efficacy can be retained and side effects minimized by using half the daily dose (60 mg/day) for twice as long.

Tetrathiomolybdate copper-lowering therapy for antiangiogenesis

Folkman has pioneered the concept that tumors require angiogenesis for growth, and that since normal adult tissues do not require angiogenesis except for wound healing and the menstrual cycle, angiogenesis is a potential Achilles heel of cancer [37-39]. In recent years the approach of antiangiogenesis for cancer therapy has seen great activity [40,41]. Angiogenic factors, or their receptors, are often targeted, or angiogenic inhibitors are used. This field remains quite promising, although it is clear that it also has not held up to some of the early expectations [42-44]. The likely problem lies in the very large number of angiogenic promoters. Inhibition of one promoter or its receptor may lead to only temporary effects, because the tumor, particularly if it is advanced, with large numbers of cancer cells, will find ways, such as by mutation, to bypass the blocked system and use other angiogenic factors.

Literature dating back to the 1980s has dealt with the role of copper in angiogenesis. Early work used the rabbit cornea model. Copper, or molecules containing copper such as ceruloplasmin, were shown to be angiogenic in this model [45,46]. Even more interesting, if rabbits were made modestly copper deficient with a combination of low copper diet and penicillamine therapy, a known angiogenic stimulus placed in the eye, such as prostaglandin E_1, became much less angiogenic [47]. This suggested that a normal level of copper was required for active angiogenesis.

Brem and colleagues [48,49] used this copper deficient model to show that tumors implanted in the brains of rabbits or rats grew much more slowly and had no tendency to invade normal tissues in copper deficient animals compared to normal controls. However, there was a lack of survival advantage, apparently due to equivalent brain stem herniation in treated and control animals, and this work was not pursued.

The development of TM for Wilson's disease gave our group an opportunity to enter the antiangiogenesis field with a new, potent, fast-acting, relatively non-toxic, anticopper agent. Initial work was positive, in a mouse model injected with MCA 205 sarcoma cells (Brewer, unpublished results). The tumor grew more slowly in the TM treated animals. This work also led to the successful development of serum ceruloplasmin (Cp) levels as a surrogate marker of copper status when TM is used as therapy. Serum copper can't be used because it builds up in an albumin, copper, TM complex in the

blood. Cp is made by the liver at a rate dependent upon the availability of copper to incorporate into the protein [50]. At that point, I teamed up with an oncologist, Dr. Sofia Merajver, for further work. She designed a study in the HER2/neu transgenic mouse which was strongly positive [51,52]. These mice are genetically programmed to develop breast cancer during the first year of life. At 100 days of life, 15 mice began daily treatment with TM by gavage, while 22 received an equivalent amount of vehicle (water). By 218 days, 15 of 22 control mice had developed grossly visible, often multiple, mammary tumors. None of the 15 TM treated animals had developed visible tumors. At this point the effect was highly significant statistically, and the experiment was stopped [52]. Upon dissection and microscopic examination, TM treated animals were found to have small, avascular, clusters of tumor cells. Therapy was stopped in a few TM treated mice and they all quickly developed obvious tumors.

Positive studies have now been done in other rodent models, including head and neck cancer, both under the tongue and in the flank [53], inflammatory breast cancer [52], lung cancer [54], and prostate cancer [55]. A study has also been done in 13 pet dogs presenting with a variety of advanced and metastatic cancers [56]. Nine of these dogs were evaluable, meaning that the Cp levels were in the target range at least one month before progression caused the dog to be withdrawn. Three of the nine dogs had stable disease for several months, and a fourth had a partial response in reduction of lung metastases size, and elimination of hypertrophic osteopathy that had resulted from the malignancy. This dog, and one other, had rather remarkable disease stabilization compared to what would have been expected without therapy.

Our initial human clinical study involved a phase I/II study in 18 patients with a variety of advanced and metastatic cancers [57]. With the doses of TM we used it takes 3-4 weeks to get the Cp into the target range of 5-15 mg/dl (normal is 20-35, but the values are often quite increased in metastatic cancer, because Cp is an acute phase reactant). We viewed patients as evaluable after the Cp was in the target range for two months. Many patients withdrew before that because of unacceptable progression. In the 6 evaluable patients of the 18 total patients reported, disease stabilization for some period of months was seen in all. The study has since been expanded to 42 patients, of whom 18 were evaluable (Merajver and Brewer, unpublished results). In these 18 patients, freedom from progression averaged 11 months, against an expected of about two months in this type of patient. Results were exceptional in three of these patients. One a renal cell cancer patient, had freedom from progression for 18 months, and a metastatic breast cancer patient went almost 36 months before there was progression in one lymph node. A third patient, with metastatic chondrosarcoma, is out now four years without progression. The quality of life of these patients was deteriorating at a significant pace prior to therapy, and then leveled off during therapy.

At present a series of phase II studies of advanced and metastatic cancers is underway, and it is too early to know any of the findings. Studies of combination therapy of TM with other modalities are just being started or being planned.

The mechanism of TM action is probably inhibition of pro-angiogenic cytokines through various mechanisms. One mechanism is probably copper dependence for activity of various cytokines. Table 1 provides a list of cytokines thought to be dependent upon copper. A second mechanism, also listed in Table 1, involves inhibition of NFκB, which potentially results in reduced transcription of a large number of angiogenic cytokines [52]. These mechanisms, collectively, may result in a somewhat more global inhibition of angiogenesis than that obtained with other therapies currently under trial.

My crystal ball on TM and cancer is that it will have some efficacy as sole therapy for advanced cancer, with an average 10-11 months freedom from progression, with some, perhaps 20% excellent stabilization and occasional, perhaps 5-10%, remarkable remission. I'm basing these guesstimates on both the canine and the human studies. However, the usual course will be that these large tumor masses will find a way to progress. I believe the real potential of TM is exemplified by the HER2/neu mouse study. That is, I think there is great potential for chemoprevention in high risk patients and for inhibiting the growth of micrometastatic disease. A corollary to that would be to use TM to prevent regrowth in conjunction with agents such as chemotherapy, radiation, or surgery that greatly debulk the tumor and make remaining tumor remnants as small as possible.

Table 1 Possible copper-dependent mechanisms for TM antiangiogenesis

Cytokines which may be copper dependent	
Cytokine or Factor	**References**
Vascular endothelial growth factor (VEGF)	[40,58]
Fibroblastic growth factor (FGF)	[59,60]
Angiotropin	[61]
Angiogenin	[62]
Secreted protein acidic and rich in cysteine (SPARC)	[63]
Ceruloplasmin	[64]
Heparin	[64]
Gly-His-Lys	[64]
NFκB Master Switch Inhibition	[52]

The toxicity of TM in cancer patients has been limited to bone marrow suppression in conjunction with overtreatment and too severe copper deficiency [57,65], and occasional complaints of sulfur smelling burps. The bone marrow suppression has been a rather benign side effect, responding quickly to dose reduction or drug holiday. It occurs frequently when the Cp is below 5 mg/dl, fairly often between 5 and 10, uncommonly between 10 and 15, and essentially not at all over 15. The transaminitis we have observed in Wilson's disease has not been seen in cancer patients or in animal studies.

Besides cancer, other diseases of neovascularization include wet-type macular degeneration, diabetic retinopathy, rheumatoid arthritis, and psoriasis [66]. A small clinical trial of TM in 10 patients with wet-type macular degeneration was negative in that the progression of the disease in terms of loss of sight was not arrested, and choroidal neovascularization continued (Vine and Brewer, unpublished results).

However, the choroidal and retinal vasculatures are different, so we have initiated work on a rodent model of retinopathy. There is not an ideal model of diabetic retinopathy, so we have used the retinopathy of prematurity for initial studies [67]. In this model, newborn mice are exposed to 75% oxygen for five days, and then to room air. In the relative hypoxia of room air, they have a large increase of vascular endothelial growth factor (VEGF), the major angiogenic cytokine in the eye, within 24 hours, followed by a retinopathy. TM treatment, begun before exposure to room air, greatly inhibits both the VEGF increase and the neovascularization in the retina (Elner and Brewer, unpublished results). This work lends hope that TM will be an effective therapy for diabetic retinopathy.

Tetrathiomolybdate copper-lowering therapy for antifibrosis and antiinflammation

The pathway of fibrosis involves transforming growth factor beta (TGFβ), which stimulates connective tissue growth factor (CTGF) which activates a number of collagen genes and other genes contributing to fibrosis [68,69]. This pathway is physiologically important, for example in wound

repair. However, it becomes dysregulated and overactive after injury in a large number of different organs [68].

In looking at this pathway, it seemed that some of the cytokines had a good chance of being copper dependent in the same manner as many of the angiogenic cytokines. For example SPARC, one of the copper dependent cytokines listed in Table 1, is one stimulator of TGFβ[70]. CTGF itself is very cysteine rich, suggesting copper dependence.

To test this hypothesis, I collaborated with Sem Phan, a pathologist at the University of Michigan, who had the bleomycin mouse model for pulmonary fibrosis [71] set up and running. In this model, bleomycin placed in the trachea causes lung injury, with a major inflammatory response, primarily stimulated by tumor necrosis factor alpha (TNFα), peaking at seven days, followed by a strong fibrotic response easily measurable at 21 days when the experiment is terminated. Meanwhile the animals lose a great deal of weight as part of the illness.

TM therapy is able to almost completely abrogate these bleomycin induced injuries [72,73]. Fibrosis is measured by an increase in hydroxyproline, a major amino acid constituent of collagen, in the lung. This level doubles in bleomycin control animals, but was not different in TM treated bleomycin animals than in saline controls. Histologically, TM protected against the fibrotic and inflammatory changes induced by bleomycin. TM completely protected against bleomycin induced weight loss. TM could be started after the inflammatory peak, and still protect against much of the fibrosis, indicating it is probably inhibiting both fibrotic and inflammatory responses [72,73].

With this positive lung data in hand, with Fred Askari, a hepatologist at the University of Michigan, we have moved to testing TM protective effects in liver injury [74]. Intravenous injection of concanavalin A (con A) in mice produces a serum transaminase response. TM therapy completely prevented an elevation of amino leucine transaminase (ALT) from con A injection. After four weeks of con A injections initiation of TM therapy returned previously elevated ALT levels to normal in spite of continued con A injections. After 10 weeks of con A injections control mice showed inflammatory changes and early fibrosis in their livers, while TM treated mice were histologically normal [74].

How and why copper-lowering therapy might work

As discussed earlier, it appears that the antiangiogenic effect of TM is through inhibition of multiple angiogenic cytokines, probably through multiple mechanisms. Similarly, the antifibrotic and antiinflammatory effects of copper-lowering by TM probably results from inhibition of key cytokines, such as TGFβ, TNFα, and interleukin 1β (IL1β). SPARC, which activates TGFβ in the fibrotic pathway, is known to be copper dependent.

Thus it seems likely that a large number of angiogenic, growth promoting, inflammatory, and fibrotic cytokines are in some manner inhibited by copper-lowering therapy with TM. A reasonable question to ask is, what is the physiological rationale for this dependence on copper levels for so much cytokine signaling? Copper is an essential trace element, but so are many other metals such as zinc, iron, calcium, magnesium, manganese, and so forth, yet these are not used in this general manner for signaling or modulating cytokine activity. We have previously speculated that the answer may be in environmental variability in copper bioavailability [65,75]. There is evidence for this variability in soils themselves, as well as the susceptibility of grazing animals to frequent problems with copper deficiency [75]. Our hypothesis is that primitive organisms developed signaling mechanisms for growth in the presence of adequate copper, and that these signals were shut off when copper was scarce, allowing survival but not growth. We postulate that these copper dependent signals have been retained in the evolution of higher organisms [75].

Potential future outlook and what remains to be done

My current views on where TM will eventually fit in cancer therapy is as follows. Used as sole therapy, it has the potential to be very good as a prophylactic agent in high risk patients for cancer, as long as the safety profile remains as good as it appears to be currently. It also should be excellent for prevention of growth of micrometastatic disease, as modeled by the HER2/neu transgenic mouse mammary cancer study. For example, if a primary tumor is removed by surgery, but there is likely to be micrometastatic dis-

ease elsewhere, TM may be excellent at preventing growth of these small pockets of disease. In more advanced and macroscopically metastatic cancer, TM's role will probably best fit as an adjunctive therapy, in combination with other modalities. For example, if the tumor masses can be greatly reduced by another therapy, TM may help in preventing regrowth. In some types of tumors, it may be very effective as sole therapy.

Based on one pilot clinical trial, it seems unlikely that TM will be effective in macular degeneration. On the other hand, a positive retinopathy animal model study predicts TM may be helpful therapy in diabetic retinopathy. We have no basis for prediction whether TM will be useful in other diseases where neovascularization is a factor, such as psoriasis and rheumatoid arthritis. However, I'll comment further on rheumatoid arthritis below.

My current views on where TM will eventually fit in antifibrotic and antiinflammatory therapy are very speculative at present because so far we only have limited animal data. This animal data is very positive, involves multiple organs, and includes evidence of inhibition of key cytokines, such as TNFα and TGFβ. Since the inflammatory and fibrotic dysregulations that lead to inflammatory and fibrotic diseases generally use the same cytokine systems, it seems possible that TM will be effective against these diseases in most organs. Similarly, since these systems are common between rodents and humans, it is quite possible that strong therapeutic effects in a large variety of diseases will also be seen in patients.

Thus, a rosy scenario could be suggested (or fantasized!) that fibrotic diseases such as idiopathic pulmonary fibrosis, cirrhosis, renal fibrosis, scleroderma, graft rejection, restenosis, etc could be effectively treated and progression halted, possibly even with reversal. As part of that, for now fantasy, it is conceivable that dysregulated inflammation could be better and more safely treated than with steroids. On the other hand, TM may be ineffective for fibrosis and inflammation in the human. We'll have to wait and see.

Which brings us to the last topic, what do we have to do now? I believe the foregoing paragraphs in this section lay this out rather clearly. In cancer, we need to evaluate clinical effectiveness as sole therapy in a wide variety of tumor types, to see if there are some which are very susceptible. We need to see if TM is effective in preventing growth of micrometastases. If this is positive, and the toxicity profile remains low, chemoprevention trials in high-risk cancer patients should be started. In situations of likely remaining micrometastatic disease, for example after surgical removal of a primary, the capability of TM to prevent growth of these metastases needs to be tested. Finally, combination therapies with TM where part of the combination, such as chemotherapy, strongly "debulks" the tumor need to be evaluated for TM's ability to stabilize the remaining tumors' growth. On a related topic, it would be useful to explore the effects of TM in lymphoma and leukemia. While these are not solid tumors of course, and thus are not dependent upon angiogenesis, they may very well be under the positive influence of some of the growth factors we know TM inhibits, and TM therapy might very well have efficacy.

In other diseases of neovascularization, such as diabetic retinopathy, psoriasis, and rheumatoid arthritis, it would seem worthwhile to do pilot type TM clinical trials.

In diseases of fibrosis and inflammation, clinical trials should be carried out where animal model work, or other types of data, suggest a possible positive result. For example, given the positive bleomycin animal work, we are planning a clinical trial of TM in idiopathic pulmonary fibrosis. As another example, as additional animal model liver work accumulates indicating protection of the liver from inflammation and fibrosis, we will consider a clinical trial in a human disease of liver injury progressing to cirrhosis. Rheumatoid arthritis deserves special consideration because it is both a disease of angiogenesis [39] and of inflammation, with TNFα playing a major role in the latter [76]. Thus, a clinical trial in this disease should certainly go forward.

Besides these clinical protocols evaluating the range of efficacy of TM there is need for work to define how to use TM in the most effective manner, and what some of the long range safety issues are. In terms of the use of TM, we lack information on how low the ceruloplasmin need be in patients for optimal results in terms of efficacy against various types of cancer, versus the risks of overtreatment toxicity. Similarly, we need information on the relative effectiveness of with meal dosing vs. away from food dosing in terms of effect on copper levels. Perhaps once-a-day dosing, as we do in animals, would be just as effective as the more complicated current human dose regimen. Obviously, more information on the mecha-

nism of antiangiogenesis would be useful. Knowing the total pattern of proangiogenic suppression by TM would allow plans to combine TM with other antiangiogenic agents that suppress factors not affected by TM.

A formal toxicity study on TM is being carried out by the National Cancer Institute RAID program. The study is almost done, and there have been no unexpected toxicities. However, some answers about long term side effects will probably come only from patients, and planning should begin for getting these answers. For example, there is no evidence of a molybdenum storage disease, but it would be useful to do molybdenum balance studies to show that during long-term TM therapy, molybdenum is in neutral balance. Since copper deficiency has a known effect on bone [77,78], particularly early in life, it would be useful to do bone density studies during long-term treatment. Copper deficiency has been implicated in lowering certain immune functions [79-81], and it would be good to study various immune functions during long-term treatment. On occasion copper deficiency has been claimed to affect the heart [79]. We have not seen this in our patients but perhaps a larger, more through study needs doing. Low copper levels are part of a hypothesis by Klevay [82] that claims that a high zinc/copper ratio is atherogenic by virtue of elevating total cholesterol. We have not seen such a result in our patients so far, but perhaps it deserves further study in patients on long term TM therapy.

The effects of lowering copper levels on wound healing needs more detailed evaluation. We know that lysyl oxidase and other cellular enzymes are not affected by the level of copper deficiency we induce (unpublished results), but major wounds presumably require some of the angiogenic and growth factor cytokine signaling we know we are inhibiting with TM. Therefore, currently we stop TM therapy prior to major surgery. However, we know minor surgery, tooth extractions, minor cuts, etc all heal quite nicely while TM therapy is continued. It would be useful to determine under what conditions TM therapy should be temporarily stopped.

We know that copper is required for growth, therefore we have not used TM therapy in children (other than for Wilson's disease). However, children have cancers that might be very susceptible to copper lowering therapy. The conditions under which TM can be used in children, with minimal effects on growth, should be explored.

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References

1. **Brewer G.J.**, Recognition, diagnosis and management of Wilson's disease, *PSEBM,* **223:** 39-49, 2000
2. **Scheinberg I.H., Sternlieb I.**, Wilson's Disease. In: Smith LH Jr, ed. Major Problems in Internal medicine. Vol. 23. Philidelphia: W.B. Saunders Company, 1984
3. **Brewer, G.J.**, Wilson's disease: a clinician's guide to recognition, diagnosis and management. Kluwer Academic Publishers, Boston. 2001
4. **Brewer, G.J.**, Wilson's Disease for the Patient and Family: A Patients Guide to Wilson's disease and Frequently asked Questions about Copper. Xlibris, Philadelphia. 2001
5. **Walshe J.M.**, Penicillamine. A new oral therapy for Wilson's disease, *Am. J. Med.,* **21:** 487-95, 1956
6. **Walshe J.M.**, Treatment of Wilson's disease with trientine (triethylene tetramine) dichloride, *Lancet.,* **1:** 643-647, 1982
7. **Schouwink, G.**, De hepatocerebrale degeneratie, met een onderzoek naar de zonkstrofwisseling**.** MD thesis (with a summary in English, French, and German), University of Amsterdam; 1961
8. **Hoogenraad T.U., Van den Hamer C.J.A., Koevoet R., De Ruyter Korver E.G.W.M.**, Oral zinc in Wilson's disease, *Lancet.,* **2:** 1262-1263, 1978
9. **Hoogenraad T.U., Van Hattum J., Van den Hamer C.J.A.**, Management of Wilson's disease with zinc sulfate. Experience in a series of 27 patients. *J. Neurol. Sci.,* **77:** 137-146, 1987
10. **Brewer G.J., Schoomaker E.B., Leichtman D.A., Kruckleberg W.C., Brewer L.F., Myers N.**, The uses of pharmacologic doses of zinc in the treatment of sickle cell anemia. In: Brewer GJ, and Prasad AS, eds. Zinc Metabolism: Current Aspects in Health and Disease, New York: Allan R. Liss, Inc.; 241-258, 1977
11. **Prasad A.S., Brewer G.J., Schoomaker E.B., Rabbini P.**, Hypocupremia induced by zinc therapy in adults, *JAMA*, **240:** 2166-2168, 1978
12. **Brewer G.J., Hill G.M., Prasad A.S., Cossack Z.T., Rabbani P.,** Oral Zinc Therapy for Wilson's disease, *Ann. Intern. Med.,*. **99:** 314-320, 1983

13. **Brewer G.J., Dick R.D., Johnson V., Brunberg J.A., Kluin K.J., Fink J.K.**, Treatment of Wilson's disease with zinc: XV. Long-term follow-up studies, *J. Lab. Clin. Med.,* **132:**264-278, 1998
14. **Brewer G.J., Terry C.A., Aisen A.M., Hill G.M.**, Worsening of neurologic syndrome in patients with Wilson's disease with initial penicillamine therapy, *Arch. Neurol.,* **44:** 490-493, 1987
15. **Ferguson W.S., Lewis A.L., Waterson S.J.**, The teart pastures of Somerset. I. The cause and cure of teartness, *J. Agri. Sci.,* **33:** 44, 1943
16. **Dick A.T., Bull L.B.**, Some preliminary observations of the effect of molybdenum on copper metabolism in herbivorous animals, *Aust. Vet. J.,* **21:** 70, 1945
17. **Miller R.F., Engel R.W.**, Interrelations of copper, molybdenum and sulfate sulfur in nutrition, *Fed. Proc.,* **19:**666, 1960
18. **Macilese Ammerman C.B., Valsecchi R.M., Dunavant B.G., Davis G.K.**, Effect of dietary molybdenum and sulfate upon copper metabolism in sheep, *J. Nutr.,* **99:** 177, 1969
19. **Mills C.F., Monty K.J., Ichihara A., Pearson P.B.**, Metabolic effects of molybendum toxicity in the rat, *J. Nutr.,* **65:** 129, 1958
20. **Cox D.H., Davis G.K., Shirley R.L., Jack F.H.**, Influence of excess dietary molybdenum on rat and calf liver and heart enzymes, *J. Nutr.,* **70:**63, 1960
21. **Dick A.T., Dewey D.W., Gawthorne J.M.**, Thiomolybdates and the copper-molybdenum-sulfur interaction in ruminant nutrition, *J. Agr. Sci.,* **85:** 567, 1975
22. **Mills C.F., El-Gallad T.T., Bremner I.**, Effects of molybdate, sulfide, and tetrathiomolybdate on copper metabolism in rats, *J. Inorgan. Biochem.,* **14:** 189, 1981
23. **Bremner I., Mills C.F., Young B.W.**, Copper metabolism in rats given di- or trithiomolybdates, *J. Inorg. Biochem.,* **16:** 109, 1982
24. **Mills C.F., El-Gallad T.T., Bremner I., Wenham G.**, Copper and molybdenum absorption by rats given ammonium tetrathiomolybdate, *J. Inor. Biochem.,* **14:** 163, 1981
25. **Gooneratne S.R., Howell J.M., Gawthorne J.M.**, An investigation of the effects of intravenous administration of thiomolybdate on copper metabolism in chronic Cu-poisoned sheep, *Br. J. Nutr.,* **46:** 469, 1981
26. **Brewer G.J., Dick R.D., Yuzbasiyan-Gurkan V., Tanakow R., Young A.B, Kluin K.J.**, Initial therapy of patients with Wilson's disease with tetrathiomolybdate, *Arch. Neurol.,* **48:** 42-47, 1991
27. **Brewer G.J., Dick R.D., Johnson V., Wang Y, Yuzbasiyan-Gurkan V, Kluin K.J., Fink J.K., Aisen A.**, Treatment of Wilson's disease with ammonium tetrathiomolybdate: I. Initial therapy in 17 neurologically affected patients, *Arch. Neurol.,* **51:** 545-554, 1994
28. **Brewer G.J., Johnson V., Dick R.D., Wang Y., Kluin K.J, Fink J.K., Brunberg JA.**, Treatment of Wilson disease with ammonium tetrathiomolybdate: II. Initial therapy in 33 neurologically affected patients and follow-up with zinc therapy, *Arch. Neurol.,* **53:** 1017-1025, 1996
29. **Brewer G.J., Hedera P., Kluin K.J., Carlson M., Askari F., Dick R.B., Sitterly J., Fink J.K.**, Treatment of Wilson disease with tetrathiomolybdate: III. Initial therapy in a total of 55 neurologically affected patients and follow-up with zinc therapy, *Arch. Neurol.,* (in press)
30. **Mason J., Lamand M., Hynes M.J.**, 99Mo metabolism in sheep after the intravenous injection of 99Mo thiomolybdates, *J. Inorg. Biochem.,* **19:** 153, 1983
31. **Hynes M., Lamand M., Montel G., Mason J.**, Some studies on the metabolism and the effects of 99Mo- and 35S-lablled thiomolybdates after tnravenous infusion in sheep, *Br. J. Nutr.,* **52:** 149, 1984
32. **Jones H.B., Gooneratne S.R., Howel J.M.**, X-ray microanalysis of liver and kidney in copper loaded sheep with and without thiomolybdate administration, *Res. Vet. Sci.,* **37:** 273, 1984
33. **Gooneratne S.R., Howell J.M., Gawthorne J.M.**, Intravenous administration of thiomolybdate for the prevention and treatment of chronic copper poisoning in sheep, *Br. J. Nutr.,* **46:** 457, 1981
34. **Humphries W.R., Mills C.F., Greig A., Roberts L., Inlis D, Halliday G.J.**, Use of ammonium tetrathiomolybdate in the treatment of copper poisoning in sheep, *Vet. Record,* **119:** 596, 1986
35. **Humphries W.R., Morrice P.C., Bremner I.**, A convenient method for the treatment of chronic copper poisoning in sheep using subcutaneous ammonium tetrathiomolybdate, *Vet. Record,* **123:** 51-53, 1988
36. **Harper P.I., Walshe J.M.**, Reversible pancytopenia secondary to treatment with tetrathiomolybdate, *Br. J. Haematol*., **64:** 851-853, 1986
37. **Folkman J.**, Tumor angiogenesis: therapeutic implications, *N. Eng. J. Med.,* **285:** 1182-1186, 1971
38. **Folkman J., Klagsburn M.**, Angiogenic factors, *Science* **235:** 442-447, 1987
39. **Folkman J.**, Angiogenesis in cancer, vascular, rheumatoid, and other diseases, *Nat. Med.* **1:** 27-31, 1995
40. **Brem S.**, Angiogenesis and cancer control: from concept to therapeutic trial, *Canc. Control,* **6:**436-458, 1999
41. **Beckner M.E.**, Factors promoting tumor angiogenesis, *Canc. Invest.* **17:** 594-623, 1999
42. **Susman E.**, Consensus panel: Antiangiogenesis drugs unlikely as single agents, *Oncology Times*, June 2001
43. **Susman E.**, San Antonio Breast cancer symposium. Prediction: Shutting down cancer blood vessels still years away, *Oncology Times*, April 2002
44. **Dotts T.**, Is angiogenesis inhibition the future of cancer treatment?, *Hem./Onc. Today*, March 2002
45. **Parke A., Battacherjee P., Palmer R.M., Lazarus N.R.**, Characterization and quantification of copper sulfate-induced vascularization of the rabbit cornea, *Am. J. Clin. Path.,* **137:** 1121-1142, 1988
46. **Raju K.S., Alesandrii G, Zinche M., Gullino PM.**, Ceruloplasmin, copper ions, and angiogenesis, *J. Natl. Cancer Inst.,* **69:** 1183-1188, 1982
47. **Ziche M., Jones J, Gullino P.M.**, Role of prostaglandin E1 and copper in angiogenesis, *J. Natl. Cancer Inst.,* **69:** 475-48, 1982
48. **Brem S.S., Zagzag D., Tsanaclis A.M.C., Gatley S., Elkouby M.P., Brein S.E.**, Inhibition of angiogenesis and tumor growth in the brain. Suppression of endothelial cell turnover by penicillamine and the depletion of copper, an angiogenic cofactor, *Am. J. Path.,* **137:** 1121-1147, 1990
49. **Brem S., Tsanaclis A.M., Zagzag D.**, Anticopper treatment inhibits pseudopdial protrusion and invasive spread of 9L gliosarcoma cells in the rat brain, *Neurosurgery,* **26:** 391-396, 1990

50. **Linder M.C., Houle P.A., Isaacs E., Moor J.R., Scott L.E.**, Copper regulation of ceruloplasmin in copper-deficient rats, *Enzyme,* **24:**23-35, 1979
51. **Merajver S.D, Irani J., van Golen K., Brewer G.**, Copper depletion as an anti-angiogenic strategy in HER2-neu transgenic mice, Proc AACR Special Conference on Angiogenesis and Cancer, B11, 1998
52. **Pan Q., Kleer C., van Golen K., Irani J., Bottema K., Bias C., De Carvalho M., Mesri E., Robins D., Dick R., Brewer G., and Merajver S.**, Copper deficiency induced by tetrathiomolybdate suppresses tumor growth and angiogenesis, *Cancer Research,* **62:** 4854-4859, 2002
53. **Cox C.D., Teknos T.N., Barrios M., Brewer G.J., Dick R.D., Merajver S.D.**, The role of copper suppression as an antiangiogenic strategy in head and neck squamous cell carcinoma, *Laryngoscope,* **111:** 696-701, 2001
54. **Khan M.K., Miller M.W., Taylor J., Navkiranjit K.G., Dick R.D., Val Golen K., Brewer G.J., Merajver S.D.**, Radiotherapy and antiangiogenic TM in lung cancer, *Neoplasia,* **4:**1-7, 2002
55. **van Golen K., Bao L., Brewer G., Pienta K., Karadt J, Livant D., Merajver S.**, Suppression of tumor recurrence and metastasis by a combination of the PHSCN sequence and the antiangiogenic compound tetrathiomolybdate in prostate carcinoma, *Neoplasia*, **4:** 373-379, 2002
56. **Kent M.S., Madewell B.R., Dank G., Dick R., Merajver S.D., Brewer G.J.**, An anticopper antiangiogenic approach for advanced cancer in spontaneously occurring tumors, using tetrathiomolybdate: a pilot study in a canine animal model, (submitted for publication)
57. **Brewer G.J., Dick R.D., Grover D.K., LeClaire V., Tseng M., Wicha M., Pienta K., Redman B.G., Thierry J., Sondak V.K., Strawderman M., LeCarpentier G., Merajver S.D.**, Treatment of metastatic cancer with tetrathiomolybdate, an anticopper, antiangiogenic agent: I. Phase I study, *Clinical Cancer Research,* **6:** 1-10, 2000
58. **Connolly D.T., Olander J.V., Heuvelman D., Nelson R., Monsell R., Siegel N., Haymore B.L., Leimgruber R., Feder J.**, Human vascular permeability factor isolation from U937 cells, *J. Biol. Chem.,* **264:** 20017-20024, 1989
59. **Engleka K.A., Maciag T.**, Inactivation of human fibroblast growth factor-1 (FGF-1) activity by interaction with copper ions involves FGF-1 dimer formation induced by copper-catalyzed oxidation, *J. Biol. Chem.* **267:** 11307-11315, 1992
60. **Tabata Y., Matsui Y, Ikada Y.**, Growth factor release from amylopectin hydrogel based on copper coordination, *J. Controlled Release,* **56:** 135-148, 1998
61. **Höckel M.A., Sasse J., Wissler J.H.**, Purified monocyte-derived angiogenic substance (angiotropin) stimulates migration, phenotypic changes, and "tube formation" but not proliferation of capillary endothelial cells *in vitro*, *J. Cell. Phys.,* **133:** 1-13, 1987
62. **Soncin F., Guitton J.D., Cartwright T, Badet J.**, Interaction of human angiogenin with copper modulates angiogenin binding to endothelial cells, *Biochem. Biophys. Res. Com.,* **136:** 604-610, 1997
63. **Lane T.F., Iruela-Arispe M.L., Johnson R.S., Sage E.H.**, SPARC is a source of copper-binding peptides that stimulate angiogenesis, *J. Cell. Bio.,* **125:** 929-943, 1994
64. **Folkman J., Shing Y.**, Angiogenesis, *J. Biol. Chem.,* **267:**10931-10934, 1992
65. **Brewer G.J., Merajver S.**, Cancer therapy with tetrathiomolybdate: antiangiogenesis by lowering body copper – A Review, *Integrative Cancer Therapies* (in press)
66. **Folkman, J.**, Angiogenesis in cancer, vascular, rheumatoid, and other diseases, *Nat. Med.* **1:** 27-31, 1995
67. **Yoshida A., Yoshida, S., Ishibashi T., Inomata H.**, Intraocular neovascularization, *Histol. Histopathol.,* **14:** 1287-1294, 1999
68. **Border W.A., Noble N.A.**, Transforming growth factor beta in tissue fibrosis, *N. Engl. J. Med.* **331:** 1286-1292, 1995
69. **Duncan M.R., Frazier K.S., Abramson S., Williams S., Klapper H., Huang X.,** Connective tissue growth factor mediates transforming growth factor α-induced collagen synthesis: down-regulation by cAMP, *FASEB J.*, **13:** 1774-1786, 1999
70. **Francki A., Bradshaw A.D., Bassuk J.A., Howe C.C., Couse W.G. Sage E.H.,** SPARC regulates the expression of collagen type I and transforming growth factor-â1 in mesangial cells, *J. of Bio. Chem.,* **274:**32145-32152, 1999
71. **Phan S.H., Kunkel S.L.**, Lung cytokine production in bleomycin-induced pulmonary fibrosis. *Exp. Lung. Res.,* **18:**29-43, 1992
72. **Brewer G., Phan S.**, Tetrathiomolybdate anticopper therapy protects against bleomycin-pulmonary fibrosis in mice, *J. Invest. Med.*, **50:** 227A, 2002
73. **Brewer G.J., Ullenbruch M.R., Dick R., Olivarez L. Phan S.H.**, Tetrathiomolybdate therapy protects against bleomycin-pulmonary fibrosis in mice, *J. Lab. Clin. Med.,* (in press)
74. **Askari F., Brewer G.**, Tetrathiomolybdate anticopper therapy protects against concanavalin A liver damage in mice, *J. Invest. Med.*, **50:** 220A, 2002
75. **Brewer G.J.**, Copper control as an antiangiogenic anticancer therapy: lessons from treating Wilson's disease, *EBM* **226:** 665-673, 2001
76. **Criscione L.G., St Clair E.W.**, Tumor necrosis factor-alpha antagonists for the treatment of rheumatic diseases, *Cur. Opin. Rheu.*, **14:** 204-211, 2002
77. **Hurley L.S., Keen C.L.,** Teratogenic effects of copper. in Nriagu J.O. ed., Copper in the Environment. Pat II: Health Effects, John Wiley & Sons, New York, 1979, pp. 33-56
78. **Rucker R.B., Kosonen T., Clegg M.S., Mitchell A.E., Rucker B.R., Uriu-Hare J.Y., Keen C.L.**, Copper, lysyl oxidase, and extracellular matrix protein cross-linking. *Am. J. Clin. Nutr.*, **67**(Suppl.)**:** 996S-1002S, 1998
79. **Danks D.M.**, Copper defiency in humans. *Annu. Rev. Nutr.*, **8:** 235-257 1988
80. **Percival, S.S.,** Copper and immunity, *Am. J. Clin. Nutr.*, **67**(Suppl.)**:** 1064S-1068S 1998
81. **Bala S., Failla M.L.**, Copper deficiency reversibly impairs DNA synthesis in activated T lymphocytes by limiting interleukin 2 activity, *Proc. Natl. Acad. Sci.,* **89:** 6794-6797, 1992
82. **Klevay L.M.**, Lack of a recommended dietary allowance for copper may be hazardous to your health*, J. Am. Coll. Nutr.*, **17:** 322-326, 1998