Anticopper therapy against cancer and diseases of inflammation and fibrosis

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Anticopper drugs that have been developed to treat Wilson's disease, a disease of copper toxicity, include tetrathiomolybdate, zinc, penicillamine, and trientine. Lowering copper levels by a modest amount in non-Wilson's patients with tetrathiomolybdate inhibits angiogenesis, fibrosis and inflammation while avoiding clinical copper deficiency. Through this mechanism tetrathiomolybdate has proven effective in numerous animal models of cancer, retinopathy, fibrosis, and inflammation. Penicillamine has efficacy in rheumatoid arthritis and trientine has efficacy in diabetic neuropathy and diabetic heart disease. If clinical studies support the animal work, anticopper therapy holds promise for therapy of cancer, fibrotic disease and inflammatory and autoimmune diseases.

Wilson's disease, an inherited disease of copper toxicity [1,2], has fueled the development of the clinically effective anticopper drugs tetrathiomolybdate (TM) [3], zinc [4], penicillamine [5] and trientine [6]. Wilson's disease leads to liver damage and in many cases to brain damage. Treatment with one, or sometimes a combination, of these drugs leads to reversing the accumulation of copper and in most patients a gratifying improvement in the patient's condition. The concepts that angiogenesis was critical to tumor growth [7], and that copper was critical to angiogenesis [8], resulted in successful trials of anticopper drugs, primarily TM, as antiangiogenic, anticancer agents [8–19]. The antiangiogenic mechanism of TM, involving inhibition of angiogenic promoting cytokines, led to the hypothesis that TM would also inhibit cytokines promoting excessive fibrosis and inflammation. TM trials in animal models of excessive fibrosis [20–22] and excessive inflammation [22,23] have all been positive. Clinical trials of TM in cancer are well underway, and are just beginning in fibrotic and inflammatory diseases.

Copper is an essential trace element and is a key component of many important enzymes, such as cytochrome oxidase, superoxide dismutase, and lysyl oxidase. It is also important in cell proliferation in the bone marrow. Thus, copper levels can't be lowered too much, or copper deficiency toxicity will ensue. The earliest manifestation of this is anemia and/or leukopenia. Later signs and symptoms would include delayed wound heading, hair color change, hair loss and peripheral neuropathy.

The aim of copper-lowering therapy is therefore to decrease copper levels (or copper availability) to a midrange, a 'therapeutic window,' where cytokine signaling is reduced but copper deficiency toxicity does not occur. This is best achieved by using a surrogate marker of copper availability, blood ceruloplasmin, and targeting values of 5–15 mg/dl (normally 18–35 mg/dl).

The main thrust of this review is to summarize the data showing how copper lowering therapy with TM has potential efficacy as an antiangiogenic agent (primarily for cancer) and as an antifibrotic and antiinflammatory agent. Work that has been done

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TABLE 1

Some of the endogenous angiogenic stimulators and inhibitors

Angiogenesis stimulators	Angiogenesis inhibitors
Vascular endothelial growth factor	Angiostatin
Fibroblast growth factor (basic and acidic)	Endostatin
Angiogenin	Interferon α
Angiotropin	Tissue inhibitor of metalloprotease
Platelet derived growth factor	Plasminogen activator inhibitor-1
Epidermal growth factor	Platelet factor-4
Hepatocyte growth factor	Thrombospondin
Tumor necrosis factor α	Angiopoietin-2
Granulocyte colony stimulating factor	Interleukin-10
Secreted protein acidic and rich in cysteine	Interleukin-12
Interleukin-1	Angiotensin
Interleukin-6	
Interleukin-8	
Cathepsin	
Urokinase-type plasminogen activator	
Angiopoietin-1	
Nitric oxide synthase	
Prostaglandin E	
Ceruloplasmin	
Heparin	
Gly-His-Lys	
Nuclear factor kappa B (NFκB)	
Transforming growth factor β	

in these areas with the other anticopper drugs will also be reviewed.

Anticopper action of tetrathiomolybdate

TM was developed to fill a therapeutic niche in Wilson's disease, that of initial treatment of those patients presenting with brain damage from copper toxicity [3]. Penicillamine and trientine have a high risk of causing further neurologic deterioration in this type of patient, and zinc is too slow acting. TM acts by forming a tripartite complex with copper and many types of proteins. Given with meals, it complexes copper with food proteins and thereby prevents the absorption of copper. Both food-derived copper and endogenously secreted copper in saliva and gastric juice are bound in this manner, putting the patient into a negative copper balance. Given between meals, TM is absorbed into the blood, and combines copper, serum albumin and itself to form a complex, from which the copper is unavailable for cellular uptake. This complex is initially formed faster than it is degraded by the liver, and builds up in the blood, reaching a steady state about two weeks after the start of the treatment in Wilson's disease [3] as well as in other types of patients. Because of this complex in the blood, serum copper can't be used as a read-out for the body status of copper in patients or animals on TM therapy. Serum ceruloplasmin (Cp) serves as a good surrogate marker for copper status. Cp is a copper-containing molecule, made by the liver and secreted into the blood depending upon the availability of copper [24]. In animal studies we reduce Cp to 20–50% of baseline, and in patients to 5–15 mg/dl. TM is on track for a New Drug Application (NDA) for initial treatment of Wilson's disease.

Cancer

Antiangiogenesis and cancer

Folkman and his associates [7] are generally credited with the concept that tumor growth is dependent upon angiogenesis. Cancer cells can grow into a small mass 1–2 mm in diameter without a blood supply, but to grow further, the tumor must develop a blood supply to nourish cells not near the surface. Antiangiogenesis is attractive as an anticancer approach because there is little angiogenesis in normal tissue. In the past 15 years there has been a great deal of activity to develop antiangiogenic anticancer drugs. The most successful approach so far has been the use of an antibody to inhibit vascular endothelial growth factor (VEGF), an important angiogenic cytokine. The drug, called Avastin, approved both by the FDA in the USA and by the NHS in the UK, has efficacy in renal [25] and colon cancer [26].

In general, however, the efforts to develop effective antiangiogenic anticancer agents have been disappointing [27]. The probable reason is the large number of proangiogenic molecules available in the body (see Table 1) for the tumor to recruit if one is inhibited.

Copper and angiogenesis

The demonstration that copper is involved in angiogenesis dates back to the 1980s, primarily involving the rabbit cornea model (reviewed in [10] and [11]. Brem *et al.* [8] explanted tumors into the brains of rats and rabbits, and found that a mild penicillamine-induced copper deficiency greatly reduced the growth of the tumors and their invasiveness. Brem *et al.* [28] later extended penicillamine treatment to patients with brain tumor but did not find improvement in survival.

TM and cancer

(a) Preclinical studies

An early study was in Her/2*neu* mice [12], which develop breast cancer during the first year of life. At ~100 days of age, one group of mice began daily treatment with orally administered TM, while untreated animals served as controls. Treatment with TM prevented visible tumors, but by 221 days, most of the control animals had developed large, often multiple, mammary tumors. If TM treatment was stopped, large mammary cancers developed. The breasts of some TM treated animals were examined histologically and small, avascular, clusters of cancer cells could be seen.

TABLE 2

Phase 2 studies of tetrathiomolybdate in advanced cancers

Cancer type	Principal investigator[a]
Renal cell	Bruce Redman
Mesothelioma	Harvey Pass, Wayne State University
Hepatocellular	Jorge Marrero
Adjunctive in colorectal	Elaina Gartner and Mark Zalupski
Prostate	David Smith
Head and neck	Francis Worden
Esophageal	Susan Urba
Multiple myeloma, with bone marrow transplant	Chris Reynolds

[a]All from University of Michigan, unless otherwise noted.

TABLE 3

Pro-angiogenic molecules which may be copper dependent

Molecule	Refs
Fibroblastic growth factor	[12,52]
Angiotropin	[53]
Angiogenin	[54]
Secreted protein acidic and rich in cysteine	[46]
Ceruloplasmin	[55]
Heparin	[55]
Gly-His-Lys	[55]
Vascular endothelial growth factor	[12]
Interleukin-1	[12,23]
Interleukin-6	[12]
Interleukin-8	[12]
Nuclear factor kappa B (NFκB)	[12]
Tumor necrosis factor α	[21]
Transforming growth factor β	[21]

A total of six other rodent tumor models, including prostate [13], head and neck [14], breast [15], inflammatory breast [12], squamous cell [16], and lung cancer [17], have been studied with TM therapy, and all have shown marked inhibition of tumor growth by TM. A study of TM in canine cancer involved pet dogs with a variety of advanced and refractory cancers [18]. Of nine evaluable dogs, three had relatively prolonged periods of disease stability, and a fourth, with osteosarcoma and large pulmonary metastases, had a partial remission and prolonged survival. In all of the preclinical studies, Cp was generally maintained at 20–50% of baseline.

(b) Clinical studies

A Phase I/II study of 42 patients with a variety of advanced and refractory cancers has been done [19]. Eighteen patients were evaluated and showed an average 11 months freedom from progression, whereas only 1–2 months would have been expected. Quality of life stabilized during this period, compared with a previous rapid decline. Some patients had excellent responses, for example one metastatic chondrosarcoma patient has been on TM therapy for five years with apparent disappearance of disease. Eight Phase 2 studies of individual cancers have been initiated (Table 2). Results in renal cancer have been published, with mixed results [29]. However, preliminary results in other cancers, such as mesothelioma and hepatocellular cancer, are encouraging.

The side of effects of TM in cancer trials has been primarily limited to anemia and/or leukopenia (also seen in Wilson's disease) due to bone marrow depletion of copper. The frequency of these relate to whether enough TM is given to drive the Cp to very low levels. Below 10 mg/dl, anemia and leukopenia occur with some frequency, whereas at 10–15 mg/dl they are relatively rare. Recovery follows quickly if a 2–3 day drug holiday is given, or if the dose is reduced.

The following thoughts about the efficacy of TM in cancer are reasonably supported by the data so far:

1) As a sole agent in advanced cancer TM might allow some months of freedom from progression, on average. Results will vary by cancer type, and occasionally striking results can be seen.
2) Preclinical trials with TM as an adjunct to chemotherapy, radiation, and other modalities so far suggest at least additive effects [13,17].
3) TM can give excellent results in micrometastatic disease, as exemplified by the Her/2*neu* mouse study. As an example, breast cancer with positive axillary nodes might be very effectively treated to prevent distant recurrence, such as in lymph nodes, lung, bones and liver.

(c) Molecular mechanisms of TM action in cancer

The action of TM as an antiangiogenic anticancer agent almost certainly involves lowering copper levels, but how does this impact on angiogenesis? It appears that many angiogenic stimulators are copper dependent, and Table 3 provides a list of some of these. Within that list is nuclear factor kappa B (NFκB), a master regulator of many cytokines and other factors. Merajver and her group [30] have asserted that NFκB inhibition by TM is the main mechanism by which TM inhibits angiogenesis and tumor metastases. This conclusion is based on mouse studies where knockouts of NFκB led to similar inhibition of tumor growth and metastases as with TM. TM had no additional antitumor effect in the NFκB-null animals.

Although NFκB is probably a major player in mediating the effect of TM, various cancer types can vary in their angiogenic mechanisms, and as we go from mouse to human, there might be additional differences. Some angiogenic substances might be directly dependent on copper.

Other disease of excessive angiogenesis

Tetrathiomolybdate therapy

Other diseases where excessive angiogenesis is believed to be part of the pathogenesis include retinopathy, such as diabetic retinopathy and retinopathy of prematurity,

wet type macular degeneration, rheumatoid arthritis and psoriasis.

A ten-patient open study of TM in macular degeneration found no apparent efficacy [31]. However, TM has shown positive results in a mouse model of retinopathy of prematurity, inhibiting both neovascularization and VEGF expression [32].

Therapy with other anticopper drugs

None of the other anticopper drugs have been used in the diseases mentioned above with the specific intent of inhibiting angiogenesis. However, it should be noted that penicillamine has long been used as a therapy in rheumatoid arthritis, with some efficacy. Rheumatoid arthritis is a disease of possible pathogenic angiogenesis, as the pannus that grows into the joint is very vascular. However, it is also an inflammatory disease, and TM has potential efficacy in inflammatory diseases, as we will see in the next section. Thus, the mechanism of efficacy of penicillamine in rheumatoid arthritis is unknown. This topic will be discussed in a later section.

Diseases of inflammation and fibrosis

TM and the treatment of diseases of inflammation and fibrosis

(a) Preclinical studies

It has been hypothesized [33,34] that the pathway of fibrosis (Figure 1) might be copper dependent, based first on the known copper dependence of secreted protein acidic and rich in cysteine (SPARC), and second, on the high cysteine content of connective tissue growth factor (CTGF), often a predictor of copper binding. To test this hypothesis, TM therapy was first tried in the bleomycin mouse model of pulmonary fibrosis. (In all of the animal studies described the dose of oral TM was such that Cp was maintained at 20–50% of baseline.)

(i) Bleomycin mouse model of pulmonary fibrosis

In this model, bleomycin is placed in the trachea, and produces an explosive pulmonary inflammation, peaking at 7 days and primarily dependent upon the inflammatory cytokine, tumor necrosis factor α (TNFα) [35]. Severe pulmonary fibrosis follows, evident at 21 days by weight loss, behavioral changes in the mice, and high levels of lung hydroxyproline, a major amino acid constituent of collagen. (Hydroxyproline is measured by a spectrophotometric method utilizing Ehrlich's reagent [36]). TM therapy started several days before bleomycin challenge completely prevented all these changes [20,21]. The TM-treated, bleomycin-challenged, animals gained weight at the same rate as untreated controls, looked healthy, and at sacrifice had essentially normal lungs and pulmonary hydroxyproline levels. This effect was dose dependent. Transforming growth factor β (TGFβ) protein levels in the lungs of bleomycin treated animals were markedly increased at 21 days, and this response was strongly inhibited by TM treatment [21]. Unexpectedly, lung TNFα mRNA levels at day 7, markedly increased in bleomycin-challenged animals, was also strongly inhibited by TM treatment [21]. If TM was started well after bleomycin was given, such that copper wasn't lowered until after the peak inflammatory episode, TGFβ inhibition and the therapeutic effect on fibrosis was still present, indicating the TGFβ and antifibrotic effects were independent of TNFα and inflammation inhibition [20,21]. This experiment also helps in ruling out one explanation of TM's mechanism of action, that of a direct interaction between bleomycin and TM.

(ii) Carbon tetrachloride mouse model of cirrhosis

Carbon tetrachloride (CT) is activated by cytochrome p450 in the liver to produce trichloromethyl free radical, resulting in oxidative stress and altered cellular redox status [37]. It produces liver cell damage, an inflammatory response (hepatitis), and after 12 weeks of continued administration, significant cirrhosis.

TM therapy in this model partially protected against the hepatitis as manifested by significantly lower levels of serum amino leucine transaminase (ALT) in TM treated animals then in CT treated control animals [22]. TM almost completely prevented the cirrhosis at 12 weeks, both as seen histologically and as measured by liver hydroxyproline levels [22]. Serum TGFβ levels were elevated by CT challenge, and these increased levels were strongly and significantly inhibited by TM.

(iii) Concanavalin A mouse model of hepatitis

Concanavalin A (Con A) injections in mice produce an acute hepatitis. Con A is a lectin from the jack bean and is well known to be a T lymphocyte antigen *in vitro* [38]. When injected it preferentially binds in the liver and produces liver damage as manifested by an elevation of serum ALT, due to activated T cells, as per the model in Figure 2. TM therapy almost completely prevented the ALT elevations from Con A, even if TM therapy was started several weeks after Con A injections were begun [22]. This study demonstrates that TM can inhibit injury initiated by an immunological process. Similarly, marked protective effects of TM were seen in a mouse model of hepatitis induced by acetaminophen [23].

(iv) Other preclinical observations

Preliminary data indicate that lymphadenopathy produced in the lupus (mutant gene *lpr*) mouse model of immune disease is markedly inhibited by TM therapy [McCubbin, M., and Brewer, G., pers. commun.]. Preliminary data also indicate that transplant rejection in a mouse model can be inhibited [Brewer, unpublished].

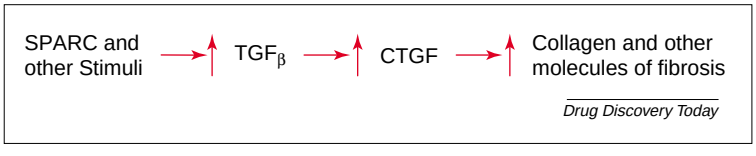

FIGURE 1

Pathway of fibrosis used for normal tissue building and repair, but dysregulated and overactive in many diseases of excessive fibrosis.

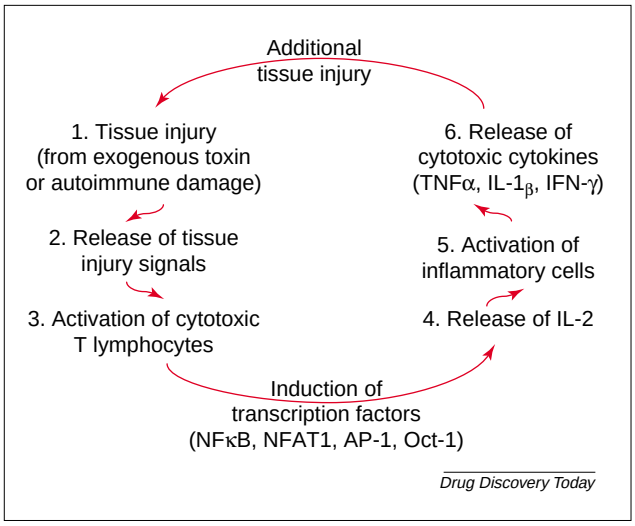

FIGURE 2

Pathway indicating one mechanism by which tissue injury might be enhanced by activation of cytotoxic T lymphocytes culminating in additional tissue injury by release of cytotoxic cytokines.

(b) Clinical studies

A clinical trial of TM therapy in idiopathic pulmonary fibrosis, a progressive, untreatable, autoimmune disease of the lung, fatal in four or five years, is underway. A clinical trial in primary biliary cirrhosis, an autoimmune disease of gradual destruction and loss of the bile ducts, leading to cirrhosis and liver failure, is in the planning stages.

Other anticopper drugs in the treatment of diseases of inflammation and fibrosis

(a) Penicillamine

Since 1950, Penicillamine has been used for the therapy of rheumatoid arthritis [40]. Its mechanism of action is not known and body copper status has not been evaluated.

A meta-type analysis/review [40] examined studies in rheumatoid arthritis in which penicillamine was used in low dose (<500 mg/day), moderate dose (500 to <1000 mg/day) or high dose (1000 mg/day or higher). It was found that penicillamine was effective at all doses in reducing disease activity. There was no detectable dose response.

The key question is whether or not copper status was affected enough to decrease serum Cp levels to a midrange, which would indicate that penicillamine efficacy in rheumatoid arthritis employs a similar mechanism of action as TM. Based on experience with penicillamine in Wilson's disease, we do not believe that a dose of <500 mg/day would affect copper balance enough to lower Cp significantly. Based on the TM dose response study in the bleomycin mouse model [20], it appears that sufficient copper deficiency to lower Cp significantly is required for the therapeutic effect of TM. As low doses of penicillamine in rheumatoid arthritis seem to have as much efficacy as higher doses, we suspect that penicillamine's effect in rheumatoid arthritis, although probably involving copper chelation, involves a different mechanism than the one involved in TM protection in inflammation and fibrosis, reviewed above.

(b) Penicillamine and scleroderma

A well designed, multi-institutional, trial of low-dose (120 mg every other day) and high-dose (mean of 822 mg/day) penicillamine was carried out in scleroderma [41]. Overall, no clinical efficacy was seen with either dose, but toxicity was greater with the higher dose. Although no information is available on copper status, it seems unlikely that even the higher dose would affect copper balance enough to produce partial copper deficiency in the length of time the drug was given.

(c) Trientine and diabetic heart disease

Trientine given to rats with diabetes and heart failure after streptozotocin administration alleviated heart failure without lowering blood glucose [42]. Cardiac myocyte structure was improved, and elevations in left ventricular collagen and β_1 integrin were reversed.

In a clinical study, six months of trientine therapy at 1.2 g/day in diabetic patients caused elevated left ventricular mass that had resulted from diabetes to decline significantly [42]. Copper status was evaluated in these patients and did not differ from controls, indicating that the trientine treated patients were not significantly copper depleted. This suggests that copper chelation of freely exchangeable copper, without causing copper deficiency detectable by a change in serum copper, helps the heart in diabetes. There are similar suggestions that treatment with trientine is helpful in the neuropathy of diabetes [43,44]. We assume that both the efficacy of penicillamine in rheumatoid arthritis and the efficacy of trientine in diabetes are due to copper chelation, as both drugs are copper chelators. Serum copper or Cp levels would not be sensitive enough to identify a change in available copper with only mild chelation of copper. Of course, these effects could also be due to some effects of the drugs other than on copper. In any case, we believe they are due to a different mechanism than the TM effects reviewed, which work at significant levels of copper depletion.

Possible mechanisms of TM action for antifibrosis and antiinflammation

(a) Fibrosis

The normal pathway underlying fibrosis is shown in Figure 1. A key cytokine in this path is TGFβ. TGFβ is probably activated by many upstream effectors, one of them being SPARC [45], a copper dependent molecule [46]. Thus, a possible mechanism of inhibition of fibrosis by TM is inhibition of the TGFβ pathway through inhibition of SPARC.

A significant problem in modern medicine is the inexorable progression of fibrosis, gradually reducing organ function [47], for which there is no effective treatment. As we have shown and will discuss further below, TM is capable of inhibiting the inflammatory process. But

in many diseases of fibrosis, the inflammatory process is already well along before it is discovered, fibrosis is already well initiated, and there is no treatment to prevent the progression of fibrosis. It is encouraging that TM seems to be able to halt and prevent fibrosis in animal models, even in situations where the fibrosis has already been initiated.

(b) Inflammation

As we have reviewed, TM is able to interfere with the excessive inflammatory response to exogenous toxins or autoimmunity. Here we will consider the possible mechanisms of this effect.

One of the events that occurs in the inflammatory response is activation of T lymphocytes (Figure 2). Cytotoxic T lymphocytes are activated by signals from dying and stressed cells. This activation includes the induction of many transcription factors, four of which are listed in Figure 2, because they are involved in the transcription and regulation of the gene encoding interleukin-2 (*IL-2*) [39].

Interleukin-2 is a key cytokine in activating the inflammatory response [48,49] and in mobilizing and activating macrophages, neutrophils, monocytes, and other lymphocytes. These cells release large quantities of cytotoxic cytokines, such as TNFα, IL-1β, and interferon gamma (IFN-γ), which can cause severe tissue damage (Figure 2).

The concept that excessive inflammation causes damage and should be controlled is widely accepted in medicine. Steroids, non-steroidal antiinflammatory agents, and chemotherapeutic drugs are used to control excessive inflammation in a variety of diseases. The key role of TNFα in causing much of the excessive inflammation and tissue injury has been shown by the successful use of TNFα antibody approaches in a series of diseases, such as rheumatoid arthritis, psoriasis, and Crohn's disease (reviewed in references [33] and [34]). Antibodies to IL-1β have also proved efficacious in some inflammatory diseases.

Thus, it would appear that the suppression of TNFα and IL-1β levels by TM, shown in several of the animal model studies reviewed here, is likely at least partially responsible for TM's beneficial effects. But what is the mechanism of suppression of TNFα and IL-1β? Neither are known to be copper dependent. Our working hypothesis is that the suppression of IL-2 by TM is an important key. As modeled in Figure 2, IL-2 release from T lymphocytes is required for activation of inflammatory cells and their release of cytotoxic cytokines, such as TNFα and IL-1β. The mechanism of IL-2 suppression by TM is probably due to inhibition of NFκB, required for IL-2 transcription.

However, because TM inhibits NFκB activity, TM's protective effects against organ damage could be multifaceted, as NFκB has a very large numbers of biological effects. Another possibility is inhibition of apoptotic injury by TM, as under some circumstances NFκB activity can promote apoptosis [50,51].

Much of modern medicine is a struggle with a very large number of inflammatory diseases, many of which are autoimmune in etiology. Although medicine has made some progress in developing therapies for these diseases, we still have a long way to go before we are able to reliably halt the progression and/or reverse the disease process without undue side effects in these conditions.

Conclusions and outlook

We have presented evidence that TM, through a copper-lowering effect, has efficacy in cancer, in diseases of fibrosis and in diseases of inflammation. The mechanism in cancer is through antiangiogenesis. Inhibition of tumour growth by TM is probably mediated by blocking angiogenesis. TM's mechanism of antiangiogenesis appears to involve NFκB inhibition, although in addition, certain angiogenic promoters appear to be directly copper dependent. TM's antiinflammatory mechanism may also involve NFκB inhibition. NFκB is required for IL-2 transcription by T lymphocytes, and in the absence of increased IL-2, inflammatory cells do not release pro-inflammatory cytokines such as TNFα and IL-1β that cause more damage and inflammation. Additional mechanisms, such as inhibition of apoptosis, are also possible. Our working hypothesis for protection against fibrosis is inhibition of the TGFβ pathway due to inhibition of SPARC, an activator of TGFβ, which is known to be copper dependent.

In cancer, we believe the best efficacy for TM, at least as sole therapy, will be in protecting against tumor growth in micrometastatic disease. A trial to test this hypothesis is underway. TM may also be very useful in situations where the tumor mass can be greatly debulked, as in mesothelioma, and as an adjunct to limit regrowth in combination with other therapies.

In fibrotic and inflammatory diseases, protection against organ damage in animal models is very broad [20–23]. It is now important to see if these broad indications of efficacy in animal models can be supported in clinical trials. If they are, the anticopper approach using TM may offer a new tool to the therapeutic approach of a wide array of autoimmune, inflammatory and fibrotic disease. The drug can be given orally, and has a very good safety profile. Of course, the hope of such general efficacy depends on actual clinical trials, which are just beginning.

Conflict of interest acknowledgement

The University of Michigan has recently licensed the antiangiogenic uses of TM to Attenuon LLC, San Diego, CA. and Brewer has equity in and is a paid consultant to Attenuon LLC.

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