Tetrathiomolybdate Protects Against Bile Duct Ligation-induced Cholestatic Liver Injury and Fibrosis

Ming Song, Ion V Deaciuc, Zhenyuan Song, Shirish Barve, Jingwen Zhang, Theresa Chen, Marcia Liu, Gavin E Arteel, George J Brewer, and Craig J McClain

Division of Gastroenterology/Hepatology, Department of Internal Medicine (M.S., I.V.D., Z.S., S.B., J.Z., C.J.M.), Department of Pharmacology and Toxicology (S.B., T.C., M.L., G.E.A., C.J.M.), University of Louisville School of Medicine, Louisville, KY 40202; the Veterans Administration Medical Center (C.J.M.), Louisville, KY and the Departments of Human Genetics and Internal Medicine (G.J.B.), University of Michigan, Ann Arbor, Michigan.
Running Title: Copper and Liver fibrosis

Corresponding author:
Craig J. McClain, M.D.
Division of Gastroenterology/Hepatology
Department of Internal Medicine
University of Louisville School of Medicine
550 S. Jackson Street
Louisville, KY 40202, USA
Phone: (502)-852-6189
Fax: 502-852-8927
E-mail: cjmccl01@gwise.louisville.edu

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ABBREVIATIONS: TM, tetrathiomolybdate; BDL, bile duct ligation; α-SMA, smooth muscle α-actin; MDA, malondialdehyde; HAE, hydroxyalkenals; SOD1, copper/zinc superoxide dismutase; TNF, tumor necrosis factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; NF-κB, nuclear factor kappa B; Con A, concanavalin A; ALT, alanine aminotransferase; AST, aspartate
aminotransferase; ALP, alkaline phosphatase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of metalloprotease; MMP, matrix metalloprotease; PAI-1, plasminogen activator inhibitor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF, hypoxia-inducible factor; CTGF, connective tissue growth factor;

**Section:** Gastrointestinal, Hepatic, Pulmonary, and Renal
ABSTRACT

Tetrathiomolybdate (TM), a potent copper-chelating drug, was initially developed for the treatment of Wilson’s disease. Our working hypothesis is that the fibrotic pathway is copper dependent. As biliary excretion is the major pathway for copper elimination, a bile duct ligation (BDL) mouse model was used to test the potential protective effects of TM. TM was given in a daily dose of 0.9 mg per mouse by means of intragastric gavage five days prior to BDL. All the animals were killed five days after surgery. Plasma liver enzymes and total bilirubin were markedly decreased in TM-treated BDL mice. TM also inhibited the increase in plasma levels of tumor necrosis factor-α (TNF-α) and transforming growth factor (TGF)-β1 seen in BDL mice. Cholestatic liver injury was markedly attenuated by TM treatment as shown by histology. Hepatic collagen deposition was significantly decreased and was paralleled by a significant suppression of hepatic smooth muscle α-actin (α-SMA) and fibrogenic gene expression in TM treated BDL mice. Although the endogenous antioxidant ability was enhanced, oxidative stress as shown by malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), hepatic GSH/GSSG ratio, was not attenuated by TM treatment, suggesting the protective mechanism of TM may be independent of oxidative stress. In summary, TM attenuated BDL-induced cholestatic liver injury and fibrosis in mice, in part by inhibiting TNF-α and TGF-β1 secretion. The protective mechanism appears to be independent of oxidative stress. Our data provide further evidence that TM might be a potential therapy for hepatic fibrosis.
Introduction

Tetrathiomolybdate (TM) was first developed as an anticopper drug for the treatment of neurological symptoms in patients with Wilson’s disease (Brewer et al., 1991). Because of its fast action and low toxicity, TM may prove to be a useful drug for the initial treatment of Wilson’s disease. Subsequently, it was found to have anticancer, anti-inflammatory, antifibrotic, and immune mediating effects.

TM was shown to have anticancer effects in both tumor models (Cox et al., 2003; Pan et al., 2002) and a phase I clinical study (Brewer et al., 2000). One mechanism for the anticancer effects is through inhibition of angiogenesis (Lowndes and Harris, 2005), with copper serving as an important cofactor for angiogenesis. Many proangiogenic cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), IL-6 and IL-8, are copper dependent (Pan et al., 2002). One mechanism of suppression of cytokine signaling is through inhibition of nuclear factor kappa B (NF-κB) (Pan et al., 2002). Based on its antiangiogenic property, TM was determined to be an effective treatment in retinal neovascularization (Elner et al., 2005).

Antifibrotic effects of TM were also observed in bleomycin-induced pulmonary fibrosis (Brewer et al., 2003) and carbon tetrachloride-induced cirrhosis mouse models (Askari et al., 2004). Both studies showed that TM protected against fibrosis by inhibition of TGF-β, the key cytokine in fibrogenesis.

In some other animal experiments, TM was shown to protect against liver injury induced by concanavalin A (Con A) (Askari et al., 2004) and acetaminophen (Ma et al., 2004), and heart injury induced by doxorubicin in mice (Hou et al., 2005). In these
studies, levels of the inflammatory cytokines, TNF-α and IL-1β, were significantly decreased by TM treatment.

TM was also shown to have protective effects in autoimmune disease animal models, including a type I diabetes model in non-obese diabetic mice (Brewer et al., 2006), an autoimmune arthritis model (Omoto et al., 2005; McCubbin et al., 2006) and a Con A autoimmune hepatitis model (Askari et al., 2004).

The mechanism of action of TM involves forming a stable tripartite complex with copper and protein which is unavailable for cellular uptake (Mills et al., 1981). Given with food, TM binds copper in food and endogenously secreted copper with protein in the alimentary tract, and prevents copper absorption. Given away from food, TM is absorbed into the blood and complexes free copper with plasma albumin. This complex is primarily degraded in the liver, with copper excretion in the bile.

Copper is an essential trace element for many biological processes. It serves as a cofactor for a number of enzymes, such as cytochrome oxidase, SOD1, metallothionein and several transcription factors (Linder and Hazegh-Azam, 1996). Generally, copper is taken up into hepatocyte and incorporated into ceruloplasmin in the Golgi apparatus, and then secreted into the serum as holoceruloplasmin, a mature form of ceruloplasmin (Murata et al., 1995). Because the synthesis of ceruloplasmin is directly regulated by the bio-availability of copper to the liver, it is a good surrogate marker of body copper status. The copper in ceruloplasmin accounts for about 90% of the total plasma copper (Goodman et al., 2004). Because 80% of the copper leaving the liver is excreted via the bile, biliary excretion represents the major pathway of copper elimination (Luza and Speisky, 1996). Excessive copper accumulation in the liver secondary to cholestasis has
been well documented in patients with primary biliary cirrhosis (Deering et al., 1977). Copper levels are also elevated in a variety of other clinical and experimental liver diseases, probably due to impaired excretion (Togashi et al., 1992; Ebara et al., 2003).

The present study investigated the potential use of TM in an animal model of hepatic cholestasis. The working hypothesis is that the hepatic fibrotic pathway is modulated by copper (Brewer et al., 2004). Our objective was to test the potential protective effects of TM in a BDL mouse model of hepatic fibrosis.
Methods

Animal Surgery and Experimental Protocol

Male C57BL/6J mice weighing 20-25g were obtained from the Jackson Laboratory (Bar Harbor, ME). They were housed in the animal facilities of University of Louisville Research Resources Center on a 12 hour light/dark cycle and fed food and water *ad libitum* for one week before beginning the experiments. All studies were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. The animals were randomly divided into four groups: five for sham operation alone, five for sham operation plus TM, ten for BDL plus TM, and ten for BDL alone. Tetrathiomolybdate (TM, (NH₄)₂MoS₄, PubChem Substance ID 24859366), as an ammonium salt (kindly provided by Dr. George Brewer, University of Michigan, Ann Arbor, Michigan) was dissolved in deionized water. In TM-treated animals, it was given in a daily dose of 0.9 mg per mouse by means of intragastric gavage, beginning 5 days before BDL. Mice were fed a low copper diet (Harlan Teklad, Madison, WI) with copper content of 2 mg/kg.

Bile duct ligation was performed using a standard technique (Uchinami et al., 2006). Briefly, mice were anesthetized with ketamine and xylazine. After midline laparotomy, the common bile duct was exposed and twice ligated with 6-0 silk suture. Sham operation was performed by gently touching the bile duct. The abdomen was closed in layers, and the animals were allowed to recover on a heat pad. All the animals were killed five days after surgery, and blood and liver samples were harvested.

Copper Status
Ceruloplasmin was used as a surrogate marker of copper status because the liver secretes ceruloplasmin into the blood in an amount that depends on copper availability (Hou et al., 2005), and was measured on the basis of its oxidase activity (Schosinsky et al., 1974) in blood from retro-orbital sinus bleeding.

**Liver Enzyme Assay**

Plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), \( \gamma \)-glutamyl transpeptidase (\( \gamma \)-GTP), and total bilirubin assays were performed using commercially available kits (Infinity, Thermo Electron Corp., Melbourne, Australia) based on a colorimetric method.

**Cytokine Assay**

Plasma TNF-\( \alpha \) and TGF-\( \beta 1 \) levels were determined using commercial ELISA kits (BioSource, Camarillo, CA) according to manufacturer’s instructions.

**Histology and Immunohistochemistry**

Formalin-fixed, paraffin-embedded liver sections were cut at 3 \( \mu \)m thickness using a routine procedure. Liver injury was determined by staining with Masson’s trichrome. Extracellular matrix accumulation in liver sections was determined by staining with Sirius red-fast green (Lopez-De Leon and Rojkind, 1985). The area of positive sirius red staining of liver section was quantified using MetaMorph software (Universal Imaging Corporation, Downingtown, PA). Specifically, a Molecular Devices (Sunnyvale, CA) Image-1/AT image acquisition and analysis system incorporating an Axioskop 50 microscope (Carl Zeiss Inc., Thornwood, NY) was used to capture and analyze eight nonoverlapping fields per section at 400\( \times \)magnification. Data from each section were
pooled to determined means. Image analysis was performed using techniques described previously (Bergheim et al., 2006).

For immunohistochemical analysis, sections were incubated with anti-α-SMA (1/1000, DAKO, Carpenteria, CA), for 30 min. Staining was visualized using the horseradish peroxidase-conjugated DAKO staining system (DAKO InVision, Carpenteria, CA).

**Isolation of RNA and Real Time RT-PCR**

Total RNA was extracted from liver tissues using TRIZOL (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. For real-time RT-PCR, the first-strand cDNA was synthesized using TaqMan Reverse transcription reagents (Applied Biosystems, Foster City, CA). The reverse transcription was carried out using 1×Taqman RT buffer, 5.5 mM MgCl₂, 500 mM of each dNTP, 2.5 mM random hexamer, 8 U of RNase inhibitor and 25 U of Multiscribe Reverse Transcriptase with 200 ng of total RNA. The RT conditions were 10 minutes at 25°C, 30 minutes at 48°C and 5 minutes at 95°C. Reactions in which the enzyme or RNA were omitted were used as negative controls. Real-time PCR was performed with an ABI prism 7500 sequence detection system and SYBR green I dye reagents. Primers were designed by Primer Express Software Version 3.0 (Applied Biosystems, Foster City, CA) (Table 1). The parameter Ct (threshold cycle) was defined as the fraction cycle number at which the fluorescence passed the threshold. The relative gene expression was analyzed using \(2^{-\Delta\Delta Ct}\) method (Livak and Schmittgen, 2001) by normalizing with GAPDH gene expression in all the experiments.

**Western Blot**
Western blot analysis was carried out in liver homogenates. Equal amounts of protein were loaded and resolved on 10% SDS-polyacrylamide gels, transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was blocked and probed with primary antibody (Santa Cruz Biotechnology, INC., Santa Cruz, CA) for SOD1 (dilution 1:5,000) overnight at 4°C and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Protein signals were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Band intensities were quantified using Image J software.

**Hepatic Lipid Peroxidation and GSH/GSSG Assay**

Lipid peroxidation was assessed by measuring MDA and 4-HAE using commercial kits (Oxford Biomedical Research, Oxford, MI). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined by HPLC as described previously (Richie and Lang, 1987).

**Statistical Analysis**

Results are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey’s multiple comparison test. $P < 0.05$ was considered statistically significant.

**Results**

**Copper Status**

The plasma ceruloplasmin level was significantly elevated (approximately five fold) in the mice five days after BDL. The mean ceruloplasmin level in TM-treated BDL animals was markedly lower than that of animals with BDL alone (about 20% of BDL). (Fig. 1)
TM Attenuated Cholestatic Liver Injury Induced by BDL

Liver injury was assessed by plasma levels of liver enzymes (ALT, AST, γ-GT and ALP), total bilirubin, liver/body weight ratio and histology. As expected, after five days, BDL significantly increased plasma levels of these enzymes and total bilirubin compared with sham-operated animals (Fig.2). These parameters were within normal ranges in both sham-operation and sham-operation plus TM-treated mice. The increase in plasma ALT, γ-GT, ALP and total bilirubin caused by BDL was significantly reduced by 30%-34% in TM-treated BDL mice. Liver/body weight ratio in TM-treated BDL mice was also significantly lower than that of BDL mice, suggesting that tissue remodeling was more effective in TM-treated mice compared to untreated BDL mice (Fig.2).

Masson’s Trichrome staining showed extensive bile infarcts, which are confluent foci of hepatocyte feathery degeneration due to bile acid cytotoxicity, bile duct proliferation and bridging fibrosis in untreated BDL mice (Fig.3C). All these lesions were markedly attenuated in TM-treated BDL mice (Fig.3D). No pathological changes were observed in liver tissues from mice with sham operation and sham operation plus TM (Fig.3A, B).

TM Attenuated Increased Plasma TNF-α and TGF-β1 Levels Induced by BDL

Plasma inflammatory and fibrogenic cytokines, TNF-α and TGF-β1, which play important roles in the activation of hepatic stellate cells, were significantly increased by 4-fold and 2-fold after five days BDL (Fig.4). However, both TNF-α and TGF-β1 production were significantly blunted in TM-treated BDL mice compared with untreated BDL mice. TNF-α was decreased by 35% in TM-treated BDL mice. TGF-β1 production was almost completely blocked compared to control level, suggesting that TM may
protect against cholestatic liver injury and fibrogenesis by inhibiting increases in inflammatory and fibrogenic cytokines.

**TM Attenuated Hepatic Fibrosis Induced by BDL**

Collagen content was assessed by morphometrical analysis of Sirius red staining of liver sections. Five days post BDL, the accumulation of collagen was discernible in the liver sections stained with Sirius red. In sham-operated mice livers, only normal staining around vessels was observed (Fig.5A, B), while mild bridging fibrosis was seen in livers from BDL mice (Fig.5C), which was markedly reduced in TM-treated BDL mice liver (Fig.5D). Quantification of Sirius red staining by image analysis showed that collagen content was significantly increased by 2-fold in the livers of BDL mice compared to sham and sham plus TM-treated mice, and it was significantly decreased with TM treatment in BDL mice liver (Fig.5 E).

Immunohistochemical staining for α-SMA, a marker of hepatic stellate cell activation, showed that its expression was significantly increased in the livers of mice with BDL (Fig. 6C, D) compared to that in sham operated mice (Fig. 6A, B). However, it was markedly diminished in the livers of TM-treated BDL mice (Fig. 6D) compared to BDL mice (Fig. 6C). We further evaluated the gene expression implicated in fibrogenesis (Fig. 7). Real time RT-PCR data showed that the mRNA expression of TIMP-1, which inhibits collagen degradation by MMPs and protects hepatic stellate cells from apoptosis (Yoshiji et al., 2002), was significantly up-regulated 40-fold in the livers of BDL mice, and it was decreased to 43% in TM-treated BDL mice. MMP-9, one of the members of MMP family, also called gelatinase B, is known to regulate cell matrix composition by degrading components of the extracellular matrix (Roderfeld et al., 2006). The level of
MMP-9 mRNA expression was significantly up-regulated 13-fold in the livers of BDL mice, and it was decreased to 37% in TM-treated BDL mice. Procollagen I α1 mRNA expression, which encodes the major collagen type in fibrosis, increased about 11-fold in the livers of BDL mice compared to sham-operated mice, and this increase was reduced by 46% in TM-treated BDL mice, which was paralleled by a significant attenuation of liver collagen content, as assessed by Sirius red staining. PAI-1, a key regulator of fibrinolysis by plasmin (Bergheim et al., 2006), showed a 40-fold mRNA level increase in BDL mice liver, which decreased to 62% with TM treatment.

**TM Protects against Cholestatic Hepatic Injury and Fibrosis Induced by BDL May be Independent of Oxidative Stress**

To evaluate the possible effects of TM on the state of oxidative stress, measurements of lipid peroxidation products, GSH/GSSG ratio, and SOD1 were carried out in liver homogenates. MDA and 4-HAE are the end products of lipid peroxidation, and can serve as markers of lipid peroxidation (Esterbauer et al., 1991). As shown in Fig.8, MDA and 4-HAE increased about 2-fold in the livers of BDL mice compared with sham operation mice. However, TM pretreatment did not prevent the increase of hepatic MDA and 4-HAE. GSH/GSSG ratio, which is an indicator of oxidative stress (Baron and Muriel, 1999), significantly increased in BDL mice compared with those with sham operation, and there was no significant change with TM treatment. SOD1, one of the three eukaryotic SOD enzymes, which plays an important role in the antioxidant defense system in the liver by eliminating superoxide anion radicals (Zelko et al., 2002), was significantly decreased in BDL mice liver compared to sham operated mice, which have abundant expression as shown by Western blot analysis (Fig. 8 bottom panel).
decrease in BDL mice liver was abolished by TM treatment. Despite enhanced endogenous antioxidant capacity in terms of SOD1, oxidative stress as assessed by lipid peroxidation and GSH/GSSG ratio was not affected by TM treatment in BDL mice. These data suggest that TM protection against hepatic injury and fibrosis induced by BDL may be independent of oxidative stress.

**Discussion**

TM was first introduced as an anticopper drug for the initial treatment of patients with Wilson’s disease. Later, it was found to be effective in murine models of carbon tetrachloride-induced liver fibrosis and bleomycin-induced pulmonary fibrosis. Currently, there is no ideal drug therapy for hepatic fibrosis. We postulated that copper may be important in the development/evolution of hepatic fibrosis. Our objective was to test the potential protective effects of TM in BDL mouse model. We showed that TM effectively protected against liver injury as assessed by histologic examination (Fig. 3), and attenuated fibrosis as evaluated by Sirius red staining (Fig. 5), α-SMA (Fig. 6) and fibrogenic gene expression, such as TIMP-1, MMP-9, Procollagen I α1 and PAI-1 (Fig. 7). The increase in the plasma inflammatory cytokine and fibrogenic cytokine, TNF-α and TGF-β1, was markedly blocked by TM pretreatment in BDL mice, suggesting that TM can exert both anti-inflammatory and antifibrotic effects (Fig. 4). This is consistent with previous reports in other models (Brewer et al., 2004; Askari et al., 2004). Because TM appears to have both anti-inflammatory and antifibrotic effects, it was important to study TM in a system such as the BDL model which induces rapid hepatic fibrosis with only a modest inflammatory response. TM previously has been shown to be protective in models of liver injury which involve acute inflammation/necrosis/apoptosis such as that
caused by acetaminophen, Con A, and carbon tetrachloride (Askari et al., 2004; Ma et al., 2004;). If the major therapeutic target for TM is hepatic fibrosis, then evaluating a model such as BDL which causes reproducible fibrosis was critical.

It currently appears that a goal of TM therapy is to maintain ceruloplasmin at midrange, which is between 20%-70% of baseline. Reducing copper to this level can inhibit some copper-containing angiogenic promoters, such as VEGF and FGF which require higher levels of copper to be active, and yet meet the basic cellular needs for copper (Brewer et al., 2003). In this study, the copper level in BDL mice treated with TM was maintained at about 30% of that normal mouse with standard diet (ceruloplasmin 10-11mg/dL, unpublished data). Maintaining ceruloplasmin at this level was effective at attenuating liver injury and fibrosis induced by BDL. In sham-operated mice treated with TM, copper level dropped to about 15% of baseline, and weight loss was observed compared with sham-operated mice without TM therapy. However, there was no further weight loss in BDL mice treated with TM compared to untreated BDL mice.

Evidence of oxidative stress has been reported in cholestatic liver disease such as in primary biliary cirrhosis patients (Kawamura et al., 2000) and in BDL animal models (Uchinami et al., 2006; Baron et al., 1999), and is often associated with decreased antioxidant defenses. We observed enhanced lipid peroxidation five days after BDL (Fig. 8). GSH/GSSG ratio, an indicator of antioxidant defenses, was significantly increased in BDL mice livers (Fig. 8). This increase may be a compensatory response to enhanced lipid oxidation. TM treatment did not influence this glutathione response. However, SOD1, one of the oxygen radical scavenging enzymes in liver, which is copper related, was markedly suppressed after BDL, and it was partially rescued by anticopper treatment
(Fig. 8 bottom panel). Collectively, despite some improvement in antioxidant defenses, enhanced lipid peroxidation was not attenuated by TM treatment, suggesting the protective mechanism involving anticopper therapy for BDL mice may be independent of oxidative stress. Interestingly, while lipid peroxidation can be attenuated by antioxidants such as Vitamin E and N-acetylcysteine, liver injury or fibrosis generally are not prevented (Baron et al., 1999; Tahan et al., 2007). A recent study by Zhong and co-workers (Zhong et al., 2002) showed that gene delivery of mitochondrial Mn-SOD (SOD2) blocked formation of oxygen radicals and TNF-α and TGF-β synthesis, thereby attenuating liver injury caused by cholestasis. However, those effects can not be attained by gene delivery of cytosolic SOD1, suggesting mitochondrial oxidative stress may be playing a role in cholestasis-induced liver injury and fibrosis.

The mechanism(s) of anticopper therapy for fibrosis remain to be elucidated. It is already known that the fibrogenic cytokines, connective tissue growth factor (CTGF) and TGF-β are copper dependent (Brewer et al., 2006). However, how copper regulates fibrotic pathway is still unknown. It has been reported that hypoxia induced activation of hepatic stellate cells occurs through the TGF-β signaling pathway, and hypoxia-inducible factor (HIF)-1α gene expression was significantly up-regulated in cultured stellate cells in response to hypoxia (Shi et al., 2007). HIF-1 is an ubiquitously expressed transcriptional master regulator of many genes involved in mammalian oxygen homeostasis. It was originally identified as a regulatory factor for the erythropoietin gene. Other target genes of HIF-1 are involved in iron metabolism, angiogenesis, control of blood flow, glucose uptake and glycolysis. HIF-1 also is a metal responsive transcription factor and may play an important role in metal induced carcinogenesis. HIF-1 is a αβ₁ heterodimer with the α
subunit the regulatory component which is unique to the hypoxic response (Martin et al., 2005). In cultured human cardiomyocytes, copper stimulated VEGF expression and angiogenesis is mediated via activating HIF-1α (Jiang et al., 2007). Copper also has been shown to modulate HIF-1 transcriptional activity in hepatoma cells by stabilizing nuclear HIF-1α under normoxic condition (Martin et al., 2005). In the present study, copper/ceruloplasmin levels and plasma TGF-β1 were markedly increased after BDL. Whether high copper can induce HIF-1α expression and whether HIF-1α induces TGF-β expression and activation of hepatic stellate cells in cholestatic liver diseases require further investigation.

In summary, our data provide evidence that TM is effective at attenuating BDL induced cholestatic liver injury and fibrosis, in part by reducing TNF-α and TGF-β1. The protection may be independent of oxidative stress. The molecular mechanism(s) involving copper modulation of fibrotic pathways is an important area for future investigation and represents a potential therapeutic target for hepatic fibrosis.
Acknowledgments

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References


Footnotes

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Dr. Brewer receives research support from Pipex Therapeutics, Inc. The University of Michigan has recently licensed the antifibrotic and anti-inflammatory uses of TM to Pipex Therapeutics, Inc, Ann Arbor, MI. Dr. Brewer has equity in and is a paid consultant to Pipex Therapeutics, Inc.

This manuscript was presented as a poster presentation at DDW (Digestive Disease Week) in Washington DC on May 19-24, 2007.
Legends for Figures

Fig.1. Plasma ceruloplasmin levels after 5 days bile duct ligation. Bile duct ligation (BDL) or sham surgery (Sham) was performed in male C57BL/6J mice as described under Materials and Methods. Some mice were pretreated with tetrathiomolybdate 0.9mg/mouse/day by intragastric gavage 5 days prior to BDL until 5 days after BDL (BDL+TM), and some mice received the same amount of tetrathiomolybdate from the day of sham surgery (sham+TM). Ceruloplasmin levels were determined in plasma samples. Data represent means ± SEM (n=5). *, significantly different from sham group. †, significantly different from BDL group.

Fig.2. Effect of TM on plasma liver enzymes, total bilirubin and liver/body weight ratio on sham-operated and BDL mice. The animals were subjected to the same treatment protocol as described in Fig. 1. ALT, AST, γ-GTP, ALP and total bilirubin were determined in plasma samples by colorimetric assay. Data represent means ± SEM (n=5). *, significantly different from sham group; †, significantly different from BDL group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; ALP, alkaline phosphatase.

Fig.3. BDL-induced histopathological changes in the livers 5 days after surgery. Representative photomicrographs of liver sections processed for Masson’s Trichrome staining: 3A, sham; 3B, sham+TM; 3C, BDL; 3D, BDL+TM. Extensive bile infarcts, bile duct proliferation and bridging fibrosis in untreated BDL mice was shown in Fig. 3C. All these lesions were markedly attenuated in TM-treated BDL mice (Fig.3D). No pathological changes were observed in liver tissues with sham operation and sham+TM (Fig.3A, B). Original magnification: ×100.
Fig.4. Plasma TNF-α and TGF-β1 levels after 5 days BDL or sham surgery. Plasma TNF-α and TGF-β1 levels were determined by ELISA. There is no significant difference between sham and sham+TM group in both TNF-α and TGF-β1 levels. Plasma TGF-β1 in BDL+TM group is significantly decreased compared to BDL group. There is no significant difference among BDL+TM, sham and sham+TM group. Data represent means ± SEM (n=5). *, significantly different from sham group; †, significantly different from BDL group.

Fig.5. Hepatic collagen accumulation after 5 days BDL. Representative photomicrographs of liver sections processed for Sirius Red staining: 5A, sham; 5B, sham+TM; 5C, BDL; 5D, BDL+TM. In sham-operated and sham-operated plus TM mice livers (Fig.5A, B), only normal staining around vessels was observed, while mild bridging fibrosis was seen in livers from BDL mice (Fig.5C), and it was markedly reduced in TM-treated BDL mice liver (Fig.5D). Original magnification: ×100. Quantification of Sirius red positive staining showed that collagen content in BDL+TM group is significantly decreased compared to BDL group (Fig.5 E). There is no significant difference among BDL+TM, sham and sham+TM group. Data represent means ± SEM (n=5). *, significantly different from sham group; †, significantly different from BDL group.

Fig.6. Hepatic smooth muscle α-actin (α-SMA) expression after 5 days BDL. Representative photomicrographs of immunohistochemistry staining for liver smooth muscle α-actin (α-SMA): 6A, sham; 6B, sham+TM; 6C, BDL; 6D, BDL+TM. α-SMA expression was significantly increased in the liver of mice with BDL (Fig.6C) compared
to that in sham operated mice (Fig.6A, B), and it was markedly diminished in the liver of BDL mice treated with TM (Fig.6D). Original magnification: ×100.

**Fig.7. Hepatic fibrogenic gene expression after 5 days BDL.** Real time RT-PCR was performed as described under *Materials and Methods* to determine hepatic TIMP-1, MMP-9, Procollagen I α1 and PAI-1 mRNA expression. The expression was normalized as a ratio using GAPDH as housekeeping gene. A value of 1 for this ratio was arbitrarily assigned to the data obtained from sham-operated mice. Data represent means ± SEM (*n*=5). *, significantly different from BDL group. TIMP-1, tissue inhibitor of metalloprotease-1; MMP-9, matrix metalloprotease-9; PAI-1, plasminogen activator inhibitor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig.8. Oxidative Stress after 5 days BDL.** Lipid peroxidation was assessed by MDA+4-HAE in liver homogenate. Liver GSH/GSSG ratio was determined by HPLC. Copper/Zinc SOD (SOD1) expression was examined by Western blot analysis using whole liver extract, and optical density of band was quantified by Image J software. A value of 1 was arbitrarily assigned to the data obtained from sham-operated mice. MDA+4-HAE and liver GSH/GSSG ratio are significantly increased in both BDL and BDL+TM group compared to sham and sham+TM group. There is no significant difference between BDL and BDL+TM group. Data represent means ± SEM (*n*=5). *, significantly different from sham group.
Table 1

Primers used for real-time RT-PCR detection of gene expression

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TIMP-1, tissue inhibitor of metalloprotease-1; MMP-9, matrix metalloprotease-9; PAI-1, plasminogen activator inhibitor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Fig. 1

![Graph showing plasma ceruloplasmin levels for different groups (Sham, Sham+TM, BDL, BDL+TM). The graph indicates significantly higher ceruloplasmin levels in the BDL group compared to the Sham and Sham+TM groups, with a trend towards higher levels in the BDL+TM group.](image-url)
Fig. 2

Plasma Activity (U/L)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>![Graph](Plasma Activity (U/L) - ALT)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AST</td>
<td>![Graph](Plasma Activity (U/L) - AST)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ-GTP</td>
<td>![Graph](Plasma Activity (U/L) - γ-GTP)</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasma ALP (U/L)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>![Graph](Plasma ALP (U/L))</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Plasma Total Bilirubin (mg/dL)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>![Graph](Plasma Total Bilirubin)</td>
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</table>

Liver/Body Weight Ratio

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>![Graph](Liver/Body Weight Ratio)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 4

Plasma TNF-α (pg/mL)

Plasma TGF-β1 (ng/mL)

Sham Sham+TM BDL BDL+TM

Sham Sham+TM BDL BDL+TM

* †
Fig. 5

5A

5B

5C

5D

5E

Sirius Red Positive Staining (% of microscope field)

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>*</td>
<td>†</td>
</tr>
</tbody>
</table>

* Significant difference
† Very significant difference
Fig. 7

TIMP-1 mRNA (fold change)

Procollagen I α I mRNA (fold change)

MMP-9 mRNA (fold change)

PAI-1 mRNA (fold change)
Fig. 8

**MDA+4-HAE (nM/mg)**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
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</thead>
<tbody>
<tr>
<td>Mean</td>
<td>11.2</td>
<td>13.4</td>
<td>18.5</td>
<td>16.8</td>
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<tr>
<td>SD</td>
<td>1.2</td>
<td>1.4</td>
<td>2.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**Liver GSH/GSSG Ratio**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>22.5</td>
<td>22.8</td>
<td>20.5</td>
<td>22.8</td>
</tr>
<tr>
<td>SD</td>
<td>1.2</td>
<td>1.4</td>
<td>2.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

**CuZn-SOD**

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham+TM</th>
<th>BDL</th>
<th>BDL+TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold change</td>
<td>1 ± 0.034</td>
<td>1.036 ± 0.223</td>
<td>0.568 ± 0.083</td>
<td>0.801 ± 0.26</td>
</tr>
</tbody>
</table>

* denotes statistically significant differences.