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Summary

Liver fibrosis is an excessive accumulation of scar tissue resulting from inflammation and cell death. Thioacetamide (TAA) is a well-known hepatotoxin that induces liver fibrosis. A marker of injured hepatocytes is transforming growth factor-beta 1 (TGF-β1), while alpha-smooth muscle actin (α-SMA) and tissue inhibitor of metalloproteinase 1 (TIMP-1) are markers of activated hepatic stellate cells. Alpha-mangostin, a major xanthone derivative from the mangosteen pericarp, has been shown to have anti-oxidant and anti-inflammatory activities. The objective of this study was to determine whether alpha-mangostin has a protective effect on TAA-induced liver fibrosis in rats. The rats were treated by intraperitoneal injection of compounds for eight weeks. For the control group a mixture of dimethyl sulfoxide and phosphate buffered saline was administered. Two hundred mg/kg BW of TAA was administered three times weekly. Alpha-mangostin was administered at 5 mg/kg BW and silymarin at 100 mg/kg BW, both twice weekly. TAA induced histologically recognizable liver damage and fibrosis, as anticipated. Furthermore, it increased immunohistochemically detectable TGF-β1, α-SMA and TIMP-1. Co-administration of alpha-mangostin or silymarin with TAA prevented or ameliorated the effects of TAA administration alone. The anti-fibrotic effect of alpha-mangostin was stronger than that of silymarin.
Introduction

Liver diseases caused by hepatitis virus B and C, ethanol, metabolic dysfunction, genetic disease, and autoimmune hepatitis are important world-wide health problems (Bataller and Brenner, 2005). Chronic liver diseases result in liver fibrosis. Liver fibrosis can be considered a wound-healing response characterized by the excessive accumulation of extracellular matrix (ECM) due to an imbalance between fibrogenesis and fibrolysis. Excess deposition of ECM proteins disrupts the normal architecture of the liver (Rockey and Friedman, 2006).

Increased oxidative stress due to e.g. overproduction of malondialdehyde (MDA), a marker of lipid peroxidation, is one of the factors that causes damage in hepatocytes (Gawel et al., 2004). The damage to hepatocytes, in turn, activates inflammatory cells to release cytokines, in particular transforming growth factor–beta 1 (TGF-β1). TGF-β1 activates hepatic stellate cells (HSCs) (Dooley and ten Dijke, 2012) and causes them to transform into myofibroblast-like cells. Activated HSCs are characterized by loss of lipid and vitamin A storage, a high expression of alpha-smooth muscle actin (α-SMA), and the production of large amounts of collagen and ECM (Moreira, 2007). Activated HSCs also synthesize matrix metalloproteinase enzymes (MMPs) that regulate the degradation of ECM (Kisseleva and Brenner, 2006; Moreira, 2007) and their specific inhibitors, tissue inhibitors of matrix metalloproteinase (TIMPs). TIMPs play an important role in promoting liver fibrosis by inhibiting the activity of MMPs. The balance between the activity of MMPs and TIMPs determines whether liver fibrosis develops or regresses. Advanced liver fibrosis results in cirrhosis (Schuppan et al., 2001; Puche et al., 2013).

Several chemical substances have been used to induce liver fibrosis in animal models (Tsukamoto et al., 1990; Mitsuhashi et al., 2004). One of the most
commonly used toxins is the organosulfur compound thioacetamide (TAA) (Liedtke et al., 2013). TAA is a hepatotoxic agent that produces hepatic fibrosis through an increase of oxidative stress and the activation of hepatic stellate cells (Akhtar and Sheikh, 2013).

Treatment of liver fibrosis is still a problem. Plant extracts used in traditional medicine are a promising source of potentially anti-fibrotic agents. Mangosteen (Garcinia mangostana Linn.), a tropical evergreen fruit from South-East Asia and known as “the queen of fruits” because of its taste (Obolskiy et al., 2009), has been used for many years as a traditional medicine to treat skin infection, abdominal pain, dysentery and wounds (Ibrahim et al., 2016). The pericarp of mangosteen contains high amounts of alpha-mangostin, which has been reported to exert anti-oxidant (Sampath and Vijayaraghavan, 2007; Pedraza-Chaverri et al., 2008; Tsai et al., 2016) and anti-inflammatory (Tewtrakul et al., 2009; Liu et al., 2012) activities. Previous studies have also shown that alpha-mangostin can reduce the damage in hepatocytes that is caused by reactive oxygen species originating from TAA (Poonkhum et al., 2012) or another hepatotoxin, carbon tetrachloride (CCl₄) (Lee et al., 2013). These data suggest that alpha-mangostin also has anti-fibrotic activity. The present study aims to determine whether alpha-mangostin can prevent TAA-induced liver fibrosis in rats.
Materials and methods

Alpha-mangostin preparation and characterization

Alpha-mangostin, 96% pure as assessed by HPLC, was kindly made available by Assoc. Prof. Primchanien Moongkarndi, Faculty of Pharmacy (Mahidol University, Thailand) (Moongkarndi et al., 2014).

Experimental design

Male Wistar rats weighing between 180-220 g were obtained from the National Laboratory Animal Center at Mahidol University, Thailand. The study was performed in accordance to the Thai guidelines for the handling of experimental animals and was approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University under license No. 2/2558. The rats were housed at 22 °C with a 12 h light - dark cycle and had free access to standard pelleted chow and water. The rats were divided into five groups. All groups were treated for 8 consecutive weeks. Group 1 animals were treated twice a week with 0.8 mL/kg BW dimethyl sulfoxide (DMSO) mixed with 0.2 mL/kg BW phosphate-buffered saline (PBS). Group 2 animals received 200 mg/kg BW TAA dissolved in sterile water 3 times weekly (Mitsuhashi et al., 2004). Group 3 and group 4 animals were injected with 200 mg/kg BW TAA 3 times weekly, and with alpha-mangostin (5 mg/kg BW; group 3) or silymarin (100 mg/kg BW; group 4; 2 times weekly) (Rasool et al., 2014). Group 5 rats only received alpha-mangostin (5 mg/kg BW; 2 times weekly). Silymarin and alpha-mangostin were dissolved in a mixture of 80% DMSO and 20% PBS. All compounds were administered by intraperitoneal injection.
Liver enzyme markers

Blood samples that were obtained by cardiac puncture were assayed in a standard clinical lab (BRIA Lab) for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activity.

Histopathology

The liver specimens were fixed in freshly prepared 4% formaldehyde dissolved in phosphate-buffered saline (pH 7.4) for 48 h at 4 °C and then dehydrated in a graded alcohol series. The specimens were embedded in paraffin and sectioned at 5-7 microns. The sections were stained with hematoxylin and eosin (H&E) for general morphology or with Sirius red to visualize collagen. Histological evaluation was performed by pathologists. The Sirius red-stained sections were better visualized with Image J software using image thresholding (available from https://imagej.nih.gov/ij/). The Ishak scoring system, ranging from 0 to 6, was used to determine the degree of fibrosis (Standish, 2006).

Semi-thin sections

The liver specimens were fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH = 7.2) at 4 °C for 2 h, followed by washing in 0.05 M sodium cacodylate buffer. After that the samples were fixed for another 2 h at 4 °C in 1% osmium tetroxide dissolved in 0.1 M sodium cacodylate buffer. The samples were washed in 0.05 M sodium cacodylate buffer, dehydrated in a graded series of ethanol, infiltrated with 100% propylene oxide and 50% propylene oxide / 50% plastic (dodecenyl succinic anhydride + Araldite (1:1)), and embedded in plastic. The tissue blocks were cut at 0.5-1 micron with an ultramicrotome (RMC, Boeckeler...
Instruments, USA), using a glass knife equipped with a liquid-filled boat. The sections were stained with methylene blue and covered with Permount® (Tasci, 2008).

**Immunohistochemistry**

Paraffin sections were mounted on coated glass slides. Specimens were deparaffinized and rehydrated in a graded series of ethanol. Antigen retrieval was performed by boiling the sections in sodium citrate buffer (pH 6.0) for 10 min at 120 °C. After cooling down at room temperature and washing in PBS, the specimens were incubated in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween20; pH = 8.0), containing 10% normal goat serum, for 30 min in a humidified chamber. The sections were then incubated with mouse anti-TGF-β1 (R&D system) at 1:25, mouse anti-α-SMA (Sigma-Aldrich, St. Louis, MO) at 1:1000, or mouse anti-TIMP-1 monoclonal antibody (R&D system) at 1:250 overnight at room temperature in a humidified chamber. After washing with PBS, the sections were further incubated in alkaline phosphatase–conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, MO) at 1:100 for 2 h at room temperature. After washing in PBS, the sections were incubated in substrate containing nitroblue tetra
dium chloride/5-bromo-4-chloro-3-indolyl phosphate (toluidine salt; Dako Inc. Glostrup, Denmark) diluted in 100 mM Tris (pH = 9.5), 100 mM NaCl, and 50 mM MgCl₂ at room temperature for 30-120 min to visualize the immunopositive regions in the tissue. After stopping color development with double distilled water, the sections were dehydrated quickly, cleared in xylene and covered with Permount® before being examined and photographed under a light microscope (Olympus, Tokyo, Japan). Immunoreactivity was semiquantitatively scored with Olympus cellSens Dimension software, version 17.1 (Olympus) (Pradidarcheep et al., 2008; Poonkhum et al., 2015).
Statistical analysis

All values were represented as means ± SD. The data were analyzed using one-way analysis of variance (ANOVA) with Tukey’s post hoc test to compare between groups. The significance levels were set at $p$ values less than 0.05.

Results

Liver enzyme makers

The serum levels of ALT, AST, and ALP increased significantly in rats treated with TAA compared to the control group ($p<0.05$). In rats treated with TAA and alpha-mangostin group, the levels of ALT, AST, and ALP rose significantly less than in rats treated with TAA only ($p<0.05$) and was similar to that in the control and alpha-mangostin only-treated rats (Table 1).

Histology and collagen fibers

Administration of TAA thrice weekly for 8 weeks induced extensive liver fibrosis in rats. The liver sections of the TAA group showed histopathological changes that are characteristics for centrilobular necrosis (hepatocyte swelling and hydropic degeneration, infiltration of mononuclear cells; Fig. 1B). Enlargement of nuclei, and cloudy and clumpy cytoplasm were visible in semi-thin sections of hepatocytes (Fig. 2B). In the TAA-treated rats, an extensive amount of collagen fibers had aggregated into thick fibrotic septa that invaded the hepatic parenchyma and changed normal lobular architecture into that typical of fibrosis (Fig. 3B). The average fibrotic score was 4.0±0.0, which was significantly higher ($p<0.05$) than that of the control group (Table 2).
Histopathological examination of the liver of rats treated with TAA and alpha-mangostin showed preservation of the normal hepatic architecture, less necrosis and less infiltration of mononuclear cells (Fig. 1C). Semi-thin sections showed normal hepatocytes with binucleated, round nuclei and homogeneously granular cytoplasm (Fig. 2C). Collagen fibers had aggregated around the larger vessels and into few, short septa (Fig. 3C). In agreement, the fibrosis scores in the TAA plus alpha-mangostin group (1.3±0.5) were significantly lower than those in the TAA-only group (Table 2).

In rats treated with TAA and silymarin, the liver showed the diffuse pathological alterations of fibrosis with giant, hydropic degeneration and necrosis of hepatocytes, but few abnormal hepatocytes (Fig. 1D). Addition of silymarin to TAA treatment resulted in a better histological score than treatment with TAA alone, but cloudy hepatocytes with enlarged nuclei were found in semi-thin sections (Fig. 2D). However, liver fibrosis was less severe. Furthermore, the collagen fibers were relatively thin and formed only short fibrous septa (Fig. 3D). The fibrosis scores of the TAA plus silymarin group was, with 1.7±0.8, significantly lower (p<0.05) than that of the TAA-only group (Table 2).

In rats treated only with alpha-mangostin the architecture of the liver was normal (Fig. 1E) and could not be distinguished from that in control rats. The hepatocytes were polygonal in shape, had prominent nuclei, clear nucleoli and a uniform cytoplasm (Fig. 2E). The collagen fibers showed the same distribution as that in the control group (Fig. 3E).

**Immunohistochemistry**

TGF-β1, α-SMA and TIMP-1 are key molecules in the development of hepatic fibrosis (Rockey and Friedman, 2006). The presence of TGF-β1, α-SMA and
TIMP-1 in the rat livers is summarized in Table 3. The immunoreactivity for TGF-β1, α-SMA and TIMP-1 was weak in control rats (Fig. 4-6A) and most pronounced in TAA-treated rats (Fig. 4-6B). TGF-β1 staining was visible as a diffuse cytoplasmic staining and a more intense nuclear staining. The latter staining was probably due to its high (basic) isoelectric point and, hence, positive charge of TGF-β1. α-SMA was found in the walls of the hepatic arteries and portal veins, and in the cells populating the fibrous septa of the fibrotic livers. TIMP-1, finally, was seen in the cytosol, but not in the nuclei of the parenchymal cells. Its expression was higher in the pericentral than the periportal hepatocytes. The diffuse spots seen at random locations in the sections were interpreted as non-specific precipitations of the staining solution during the incubation (some spots were also seen in panels B and C of the TGF-β1 staining).

Co-administration of alpha-mangostin and TAA markedly reduced the tissue content of TGF-β1, α-SMA, and TIMP-1 (Fig. 4-6C). Co-administration of silymarin also reduced the tissue content of TGF-β1, α-SMA, and TIMP-1 (Fig. 4-6D) to a similar or slightly smaller extent. Alpha-mangostin treatment alone had no effect on hepatic TGF-β1 and α-SMA content (Fig. 4,5E), but increased TIMP-1 content in the pericentral hepatocytes (Fig. 6E; also Fig. 6C). The semi-quantification of TGF-β1-, α-SMA- and TIMP-1-positive areas of the TAA-treated rats that were co-treated with alpha-mangostin or silymarin revealed significantly decreased relative to the TAA-treated group (p<0.05; Table 3).

**Discussion**

As expected TAA induced liver fibrosis. Cytochrome P450 (CYP450) enzymes in the microsomes convert TAA to TAA-S-oxide and TAA-S,S-dioxide, which are the bioactive compounds. TAA-S-oxide can cause mitochondrial
dysfunction by increasing the intracellular concentration of Ca$^{2+}$, which increases mitochondrial permeability. TAA-S,S-dioxide is a reactive compound that binds to proteins and nucleic acids (Akhtar and Sheikh, 2013). CYP450 enzymes are localized mainly in the downstream, pericentral hepatocytes and, therefore, localize with the hepatocellular toxicity seen after TAA treatment (Jungermann and Keitzmann, 1996; Gebhardt and Matz-Soja, 2014).

The present study showed that administration of TAA three times weekly for eight weeks increased plasma ALT, AST and ALP levels, implying liver injury, and induced severe fibrosis in rat liver. Standard histology, collagen staining, and immunohistochemistry of key molecules in fibrogenesis support this conclusion. These observations concur with preexisting studies which reported that TAA can induce the elevation of ALT, AST and ALP in serum (Amin et al., 2012; Kono et al., 2012; Kadir et al., 2013) and induce centrilobular necrosis, followed by repair and (bridging) fibrosis in the liver (Chilakapati et al., 2007; Liedtke et al., 2013; Chen et al., 2014). Importantly, the histological features of TAA-induced liver damage were similar to that seen in cirrhotic human livers (Guerra et al., 2010).

Our results show that co-treatment with alpha-mangostin prevented TAA from inducing increased levels of liver enzymes (ALT, AST, and ALP) in plasma and the development of fibrotic changes in liver. Earlier studies have shown that a high dose of alpha-mangostin (200 mg/kg rat) induces an elevation of plasma transaminases after intraperitoneal (Sornprasit et al., 1987) or oral administration (Hutadilok-Towatana et al., 2010; Chivapat et al., 2011). However, at doses less than 200 mg/kg BW alpha-mangostin is not toxic for rat livers. Alpha-mangostin is a xanthone derivative with anti-oxidant and anti-inflammatory properties (Obolskiy et al., 2009). Previous studies showed that alpha-mangostin decreased the activity of
antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), and the tissue concentration of glutathione (GSH) in infarcted myocardium of rats (Sampath and Vijayaraghavan, 2007). It also decreased mitochondrial ROS levels in steatotic rat livers (Tsai et al., 2016). Moreover, alpha-mangostin inhibited the production of tumor necrosis factor - alpha (TNF-α) and of interleukin - 4 (IL-4) in macrophage cell lines (Tewtrakul et al., 2009; Liu et al., 2012). TNF-α and IL-4 are pro-inflammatory, fibrogenic cytokines (Bataller and Brenner, 2005). TGF-β1 is a proinflammatory cytokine produced by injured hepatocytes and activated Kupffer cells, and plays a central role in fibrogenesis by activating HSCs from their quiescent state (Dooley and ten Dijke, 2012). Activated HSCs, in turn, are transformed into myofibroblast-like cells that express α-SMA (Kisseleva and Brenner, 2006; Moreira, 2007) and induce the secretion of TIMP-1. TIMP-1 decreases extracellular matrix degradation through inhibition of MMP (Puche et al., 2013). In the present study alpha-mangostin attenuated the production of TGF-β1, α-SMA, and TIMP-1, suggesting that it exerts an anti-inflammatory effect. Alpha-mangostin increased the production of TIMP-1 in pericentral hepatocytes. The significance of this finding is unclear at present, but appears necessary for hepatocyte health, because TIMP-1 deficiency exacerbates CCl₄-induced pericentral necrosis (Wang et al., 2011). Alpha-mangostin may improve mitochondrial function in steatotic livers (Tsai et al., 2016). Furthermore, alpha-mangostin can reduce α-SMA production in HSC-T6 cells and collagen formation in cirrhotic rat (Poonkhum et al., 2012; Lee et al., 2013). In aggregate, alpha-mangostin appears to have anti-fibrotic properties, which it may exert by inhibiting HSCs activation and/or promoting apoptosis of activated HSCs (Lee et al., 2013; Rahmaniah et al., 2018).
Silymarin is a mixture of flavonoids with anti-oxidant properties that is well-known used as a hepatoprotective drug (Vargas-Mendoza, 2014). These beneficial effects of silymarin were observed in animals treated with TAA (Rasool et al., 2014) or CCl₄ (Tsai et al., 2008; Gupta et al., 2014), and prevented increases in expression of TGF-β1 (Das and Mukherjee, 2012; Abhilash et al., 2013), α-SMA (Tsai et al., 2008; Abhilash et al., 2013), and TIMP-1 in rats (Jia et al., 2001). Silymarin may inhibit the activity or effect of CYP450 enzymes and Kupffer cell migration (Fraschini et al., 2002; Abhilash et al., 2013). However, silymarin also increased, at the dose used, plasma AST levels. Judging from the lower rise of liver enzyme levels in plasma, alpha-mangostin was clearly more effective than silymarin in preventing liver damage due to TAA treatment.

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Fig. 1 Photomicrographs of hematoxylin and eosin-stained liver sections of the control group (A), TAA-treated group (B), TAA+alpha-mangostin-treated group (C), TAA+silymarin-treated group (D) and the alpha-mangostin-only group (E). Central veins (CV) and portal triads (PT) were recognized in all animals. Panels A and E show a normal architecture of liver, whereas panel B reveals bridging fibrosis (arrowhead) and hydropic degeneration areas (star). Panels C and, to a lesser extent, D show attenuation of fibrosis due to the additional treatment with alpha-mangostin or silymarin, respectively. Panel D shows some hydropic degeneration areas (stars).

Fig. 2 Photomicrographs of semi-thin methylene blue-stained liver sections of the control group (A), TAA-treated group (B), TAA+alpha-mangostin-treated group (C), TAA+silymarin-treated group (D) and the alpha-mangostin-only group (E). Panels A, C and E show normal hepatocytes, whereas panels B and D show cloudy hepatocytes (black arrowhead), hydropic degeneration (arrow) and enlargement of nuclei (white arrowhead).

Fig. 3 Photomicrographs of Sirius red-stained liver sections of the control group (A), TAA-treated group (B), TAA+alpha-mangostin-treated group (C), TAA+silymarin-treated group (D) and the alpha-mangostin-only group (E). Panels A and E show only collagen fibers around the central vein (CV) and in the portal triads (PT). Panel B shows extensive accumulation of collagen fibers with bridging fibrosis (arrowhead). The livers in panels C and D have developed mild collagen deposits which appear as short septa (arrowhead). Collagen fibers stain red.
Fig. 4 Immunohistochemical staining of TGF-β1 in rat livers from control (A), TAA-treated (B), TAA+alpha-mangostin-treated (C), TAA+silymarin-treated (D) and alpha-mangostin only groups (E). The immunoreactivity in panels A and E was similarly weak, that in panel B was strongest, whereas immunoreactivity was moderate in panels C and D. Abbreviations: CV: central vein; PT: portal triad.

Fig. 5 Immunohistochemical staining of α-SMA in rat liver from control (A), TAA-treated (B), TAA+alpha-mangostin-treated (C), TAA+silymarin-treated (D) and alpha-mangostin-only groups (E). In panels A and E, staining is, as expected, confined to the perivascular cuff of smooth muscle. The staining is strongest in panel B, especially in the fibrous septa. Panels C and D show intermediate staining densities. Abbreviations: CV: central vein; PT: portal triad.

Fig. 6 Immunohistochemical staining of TIMP-1 in rat livers from the control (A), TAA-treated (B), TAA+alpha-mangostin-treated (C), TAA+silymarin-treated (D) and alpha-mangostin-only groups (E). Weakest staining was seen in panel A and strongest in panel B, with the other groups showing intermediate staining densities. Note staining of TIMP-1 in pericentral hepatocytes in panels A, C and a bit stronger in E. Abbreviations: CV: central vein; PT: portal triad.

Fig. 7 Proposed mechanism of the protective effect of alpha-mangostin against liver fibrosis. Alpha-mangostin may have an anti-inflammatory effect mechanism.
Table 1. Comparison of the liver function tests.

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<tr>
<td>AST (U/L)</td>
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</tr>
<tr>
<td>ALT (U/L)</td>
<td>40 ± 6</td>
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<tr>
<td>ALP (U/L)</td>
<td>73 ± 28</td>
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</table>

Data are shown as the Mean±Standard Deviation. (* p<0.05 vs control group, # p<0.05 vs. TAA group)

Table 2. Ishak scoring in the liver tissue of different groups of rats.

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<tr>
<td>Ishak score</td>
<td>0.0 ± 0.0</td>
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</table>

Data are shown as the Mean±Standard Deviation. (* p<0.05 vs control group, # p<0.05 vs. TAA group)
Table 3. Positive areas in liver tissue of the respective groups of rats.

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<tr>
<td>α-SMA</td>
<td>1.0 ± 0.0</td>
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<tr>
<td>TIMP-1</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

Data are shown as the Mean±Standard Deviation. (* p<0.05 vs control group, # p<0.05 vs TAA group)