Investigation of Therapeutic Effects of α-Mangostin on Thioacetamide-Induced Cirrhosis in Rats

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Objective: *To determine the effects of alpha-mangostin on thioacetamide (TAA)-induced liver cirrhosis in rats.*
Material and Method: *Male Wistar rats were divided into 3 groups and treated with intraperitoneal injections of TAA (200 mg/kg) 3 times per week for per week for 8, 12 and 16 weeks, respectively. One subgroup was left untreated whereas the other two were treated either with 100 mg/kg α-mangostin or vehicle alone (80% DMSO, 20% water), which were administered intraperitoneally 3 times per week for a total of 4 weeks. The incidence of fibrotic nodules on the liver and the serum levels of the liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) were measured. Moreover, the liver cirrhosis-related genes expression and p53 protein level in liver were analyzed by quantitative reverse transcription PCR and Western blot analysis, respectively.*
Results: *Fibrotic nodules on the liver were formed upon treatment with TAA for 12 or 16 weeks. The nodules were then reduced by treatment with α-mangostin as compared to treatment with the vehicle DMSO. Moreover, the serum levels of the liver enzymes AST and ALT after treatment with α-mangostin decreased as compared to DMSO alone. The liver cirrhosis-related genes expression showed no significant differences, whereas the p53 protein level in liver showed that α-mangostin reduced risk of liver fibrosis through the decrease in p53 expression as compared to the TAA_DMSO treatment.*
Conclusion: *The results suggest that α-mangostin has a beneficial therapeutic effect in the TAA liver cirrhosis model. Further investigations on mechanisms of α-mangostin as therapeutic agent should be determined.*

Keywords: *Cirrhosis, α-mangostin, Thioacetamide, Dimethyl sulfoxide*

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Cirrhosis is a major cause of morbidity and mortality worldwide. Infections from hepatitis B and hepatitis C viruses and chronic alcohol abuse are the most common causes of cirrhosis[1,2]. These factors damage the liver tissue and trigger various repair reactions of the organ, leading to the formation of scar tissue and regenerative nodules[3]. Failed tissue repair and the diminishment of normal tissue are followed by the loss of essential functions of the liver and eventually death. While the precursors of cirrhosis, hepatitis and steatosis are reversible, cirrhosis cannot be cured completely. Accordingly, the treatment is aimed at the prevention or slowdown of further progression and the management of disease complications[4]. Many advanced stage cirrhosis patients require liver transplantation.

The most common animal model of cirrhosis is thioacetamide (TAA)-induced liver damage in rats. TAA is a small water-soluble organic compound that contains sulfur (C_2H_5NS). Repeated intraperitoneal administration of TAA in rats induces severe liver damage and ultimately cirrhosis. Various substances have been tested as protectants against the development of TAA-induced cirrhosis in rats[5,6]. However, after initial treatment with TAA, candidate drugs tend to fail in achieving beneficial effects in this model[5].

Alpha (α)-mangostin is a xanthone derivative that can be prepared from the outer layer (pericarp) of fruits and other parts of the mangosteen (*Garcinia mangostana*), a tree that grows in Southeast Asia[7]. The traditional medicine of Thailand and other countries has used mangosteen products for the treatment of wounds and infections. α-mangostin has anti-oxidant, anti-fibrotic, anti-cell proliferative, pro-apoptotic activities in experimental settings[8-12]. In the TAA-

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induced cirrhosis rat model, α-mangostin reduced the number of type-I collagen-positive-hepatocytes when it was administered during the induction phase of the cirrhosis (injections of TAA and α-mangostin on alternating days)[6].

The molecular indicators suggest a cirrhosis condition was used for monitoring the effect of cirrhosis-therapeutic agents. The inflammatory condition which is involved in cirrhosis development including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS) and collagen α1[13-16]. In recent years, correlation between tumor suppressor p53 protein level and liver fibrosis condition in mice was reported[17]. Therefore, the decreased p53 protein level was helpful for screening of cirrhosis-therapeutic agents, which reduced the risk of liver fibrosis in cirrhosis-induced liver.

Here, we have tested the pharmacological activity of α-mangostin in rats that had been pre-treated with TAA to induce cirrhosis.

Material and Method

Animal studies

Male Wistar rats (body weight 120-150 g) were obtained from the National Laboratory Animal Center (NLAC, Mahidol University, Salaya Campus, Nakhon Pathom, Thailand). The animals were housed in a dedicated animal facility maintained at 25°C on a 12 hour light/dark cycle and fed *ad libitum*. The study was approved by the National Research Council (NRC) and by the Faculty of Medicine, Srinakharinwirot University, Institutional Animal Care and Use Committee (IACUC).

Rats were divided into 3 groups: liver cirrhosis was induced by intraperitoneal administration of TAA at 200 mg/kg, 3 times per week for 8, 12 and 16 weeks, respectively. After the treatment with TAA, the rats were then divided into 3 subgroups, each comprising 3-5 animals. One subgroup was left untreated whereas the two others were treated either with α-mangostin at a dose of 100 mg/kg or vehicle alone (80% DMSO, 20% water), which were administered intraperitoneally 3 times per week for a total of 4 weeks. There after, the rats were euthanized and sacrificed by decapitation. The livers were carefully dissected, removed through a midline abdominal wall incision and photographed immediately.

Analysis of liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT) in serum

Activity of AST and ALT in serum samples of each rat was determined by Aspartate Aminotransferase (AST) Activity Assay Kit (Catalog Number MAK055: Sigma-Aldrich, St. Louis, MO, USA) and Alanin Aminotransferase (ALT) Activity Assay Kit (Catalog Number MAK052: Sigma-Aldrich, St. Louis, MO, USA), respectively. Serum sample preparation and both liver enzyme activity assays were manipulated according to the manufacturer's instructions.

Analysis of liver cirrhosis-related gene expression by quantitative reverse transcription PCR

RNA was prepared from freshly isolated rat livers according to a standard protocol. The RNA was reverse-transcribed and investigated by real-time polymerase chain reaction (PCR) using the SYBR Premix Ex Taq (Takara, Siga, Japan). The following primers were used for the specific amplification of the indicated genes:

Interleukin (IL)-1β;

Forward: 5′-ATGGCAACTGTTCCTGAACT CAACT-3′

Reverse: 5′-CAGGACAGGTATAGATTCTTT CCTTT-3′

Tumor necrosis factor (TNF)-α;

Forward: 5′-TTCTGTCTACTGAACTTGG GGGTGATCGGTCC-3′

Reverse: 5′-GTATGAGATAGCAAATCGG CTGACGGTGTGGG-3′

Inducible nitric oxide synthase (iNOS);

Forward: 5′-AGCGACTCCATGACTCTC-3′

Reverse: 5′-CGGAGCATCTCCTGCATT-3′

Collagen α1;

Forward: 5′-CGACTAAGTTGGAGGGAACG GTC-3′

Reverse: 5′-TGGCATGTTGCTAGGCACG AC-3′

Western blot analysis of tumor suppressor p53 protein

Antibodies against p53 and beta-actin antibodies were purchased from Cell Signaling. Liver samples were incubated with RIPA lysis buffer containing a protease inhibitor cocktail (Roche) according to the manufacturer's instructions. Proteins were separated by SDS polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (Biorad) according to a previously published protocol[12]. The membrane was incubated with primary antibodies against p53 and beta-actin (Cell Signaling) followed by incubation with goat-anti-rabbit immunoglobulin conjugated to horse raddish peroxidase. Binding of antibodies was detected with

enhanced chemoluminescence (ECL) solution.

Statistical analysis

Significant differences between groups were determined by the t-test. The results are shown as mean ± standard deviation.

Results

Gene expression in liver cirrhosis treated with α-mangostin

Rats were initially treated with TAA to induce cirrhosis before they were treated with α-mangostin in DMSO or with equal volumes of DMSO vehicle alone. Another group of rats was treated with TAA without subsequent treatment. The expression levels of TNF-α, IL-1β, collagen type I and iNOS were determined by quantitative real-time PCR. The amounts of these mRNA showed high inter-individual variation, and no significant differences between treatment groups could be detected (data not shown).

Protein p53 expression in liver cirrhosis treated with α-mangostin

The expression of p53, a protein involved in the control of DNA repair and programmed cell death, was determined by Western blot analysis. The abundance of p53 did not differ between TAA-treated rats without subsequent treatment and TAA-treated rats that were subsequently treated with α-mangostin (Fig. 1). However, TAA-treated rats that were subsequently treated with the vehicle DMSO had slightly higher levels of p53 as compared to the two other groups. This indicated that, in our experimental setting, DMSO may influence the development of TAA-treated liver and that α-mangostin (diluted in DMSO) may counteract this influence.

TAA-induced macronodular cirrhosis is modulated by α-mangostin

The liver of rats was prepared immediately after sacrificing the animals. The gross morphology was evaluated by visual inspection and the numbers of fibrotic nodules on the surface of the liver were determined. Without TAA treatment, the liver of rats did not develop any nodules (Fig. 2A and B). TAA treatment for 12 and 16 weeks and leaving the rats for the following 4 weeks without treatment caused the development of fibrotic nodules (Fig. 2C and D). When TAA treatment was followed by injections of DMSO over 4 weeks, the incidence of fibrotic nodules increased (Fig. 2E and F). This apparent effect of DMSO treatment

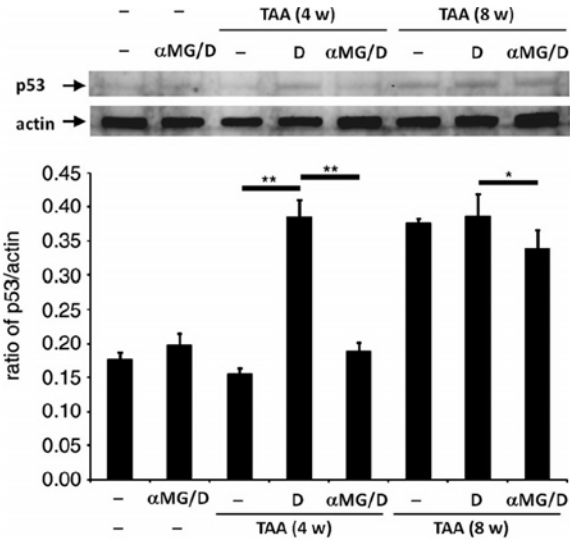

Fig. 1 Western blot of p53 in the liver of rats treated initially with TAA for 4 or 8 weeks (w) and subsequently left untreated or treated with DMSO (D) or α-mangostin (α MG/D) for 4 weeks. The results were observed from five wistar rat (n = 5). Bar graph was expressed as mean ± SD. *p<0.05 was considered statistically significance.

was reverted by α-mangostin (Fig. 2G and H). The difference between DMSO alone and DMSO plus α-mangostin was evident both at the levels of gross morphology and detailed inspection of the liver surface (Fig. 2I and J). Quantification of fibrotic nodules showed that there was a significant difference between 16 weeks TAA treatment followed by 4 weeks of DMSO treatment versus 16 weeks TAA treatment followed by 4 weeks of DMSO plus α-mangostin treatment (Fig. 3B). In contrast, the differences between subgroups of rats treated with TAA for 12 weeks (Fig. 3A) or less did not reach statistical significance.

Effect of α-mangostin on liver enzyme AST and ALT in serum of TAA-induced cirrhosis

The high level of enzyme AST and ALT in serum indicated liver damage, one of the cirrhosis characters. The results showed decreased liver enzymes AST and ALT levels in serum after treatment with α-mangostin as compared to TAA induced cirrhosis, both with and without DMSO (Table 1).

Discussion

This study used TAA to induce cirrhosis in rats and tested α-mangostin as a potential therapeutic agent that was applied after the end of TAA

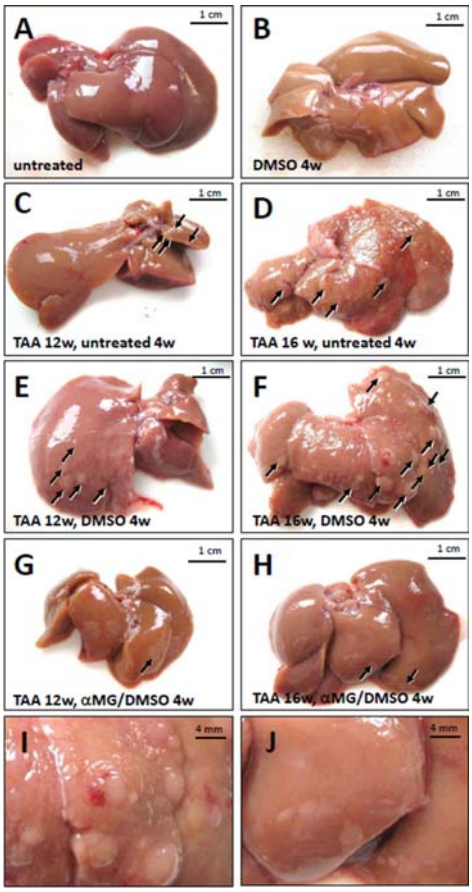

Fig. 2 Macronodular cirrhosis is induced by TAA and ameliorated by α-mangostin. (A) untreated, (B) treated with DMSO for 4 weeks, (C) treated with TAA for 12 weeks followed by no treatment for 4 weeks, (D) treated with TAA for 16 weeks followed by no treatment for 4 weeks, (E) treated with TAA for 12 weeks followed by DMSO for 4 weeks, (F) treated with TAA for 16 weeks followed by DMSO for 4 weeks, (G) treated with TAA for 12 weeks followed by α-mangostin (αMG)/DMSO for 4 weeks, (H) treated with TAA for 16 weeks followed by α-mangostin (αMG)/DMSO for 4 weeks, (I) detail of F, (J) detail of H.

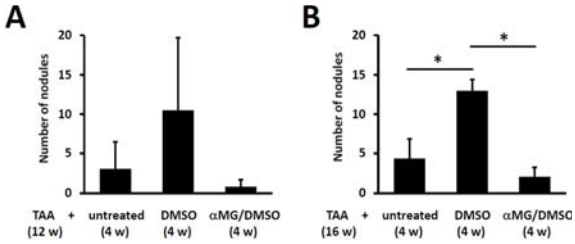

Fig. 3 Quantitative analysis of macronodular cirrhosis. Rats were treated with TAA for 12 (A) and 16 (B) weeks (w). Subsequently, the rats were lefted untreated or were treated with DMSO alone or with α-mangostin (αMG) in DMSO. The number of fibrotic nodules on the liver was counted. Asterisks indicate significant differences (p<0.05).

administrations. Surprisingly, our results suggest that TAA-induced cirrhosis is aggravated by subsequent treatment with DMSO. The latter was used as a vehicle for the application of α-mangostin, but it was also tested alone. The mechanism by which DMSO increased the effect of prior TAA treatment in this model remains to be determined. However, the liver enzymes AST and ALT were reduced in α-mangostin treatment when compared to TAA induced cirrhosis, both with and without DMSO (Table 1), which correlated with the decrease of the nodules in α-mangostin treatment as compared to TAA induced cirrhosis, both with and without DMSO (Fig. 2 and 3). These indicated that α-mangostin treatment in TAA-induced cirrhosis condition able to rescue the liver function and recover anatomical architecture of liver in cirrhosis[18]. Importantly, comparison of DMSO plus α-mangostin versus DMSO alone showed that α-mangostin reduced the development of fibrotic nodules.

α-mangostin has many effects on cells and tissues; the mechanism of action in the TAA-induced cirrhosis model needs further investigations. The expression of p53 in the liver was slightly decreased by α-mangostin (Fig. 1) and large inter-individual variation in mRNA levels of inflammatory cytokines and type-I collagen did not allow reliable conclusions on gene expression changes in our study. However, increased p53 expression resulting in liver fibrosis in mice was reported[17] supporting our study that α-mangostin reduced risk of liver fibrosis through the decrease in p53 expression as compared to cirrhosis induction by TAA treatment-subsequently treated with DMSO. α-mangostin also acts as an antioxidant, suppresses cell proliferation and is able to induce apoptosis[8,10-12,19]. Recently, the cellular regeneration program of autophagy[20-22] was reported to modulate liver fibrogenesis[23], autophagy thus being affected by α-mangostin[24].

The results of this study suggested that the effects of α-mangostin on the progression of pre-initiated cirrhosis should be further investigated. In the present study, no more than 5 animals were used per treatment subgroup, and the variation in disease severity between individual rats was high. For future studies, higher number of animals should be used.

Table 1. The serum levels of the liver enzymes aspartate transaminase (AST) and alanine transaminase (ALT)

Induction of cirrhosis	Treatment (4 weeks)	AST (units/L) (mean ± SD)	ALT (units/L) (mean ± SD)
TAA (12 weeks)	None	129.4±44.4	54.3±32.9
	DMSO	162.7±41.9*	49.8±6.6
	α-mangostin/DMSO	115.3±15.6**	34.1±3.4*,**
TAA (16 weeks)	None	148.3±22.1	46.8±3.6
	DMSO	142.6±55.1	54.1±12.1
	α-mangostin/DMSO	123.3±14.5*,**	32.0±8.8*,**

The $p<0.05$ was considered statistically significance. Statistical analysis, label * represents comparing with TAA treatment without subsequently treatment and label ** represents comparing with TAA treatment with subsequently DMSO treatment in the similar time of TAA induction

Together with the results of a previous study in which the anti-fibrotic role of α-mangostin was tested using a different treatment protocol[6], the new data provide the basis for the design of the follow-up studies. Mangosteen xanthones may have suppressive effects both on viral infections causing cirrhosis[25] and on the further course of the disease. More investigations need to be performed to evaluate the multifunctional activities of α-mangostin in the treatment of cirrhosis.

What is already known on this topic ?

Cirrhosis is a major cause of morbidity and mortality worldwide.

TAA-induced liver damage in rats is the most common animal model of cirrhosis.

Alpha-mangostin is used for the treatment of wounds and infections in the traditional medicine, and inclines use in cirrhosis treatment potentially.

What this study adds ?

Alpha-mangostin reduced risk of liver fibrosis through the decrease in p53 expression.

Alpha-mangostin treatment was able to rescue the liver function and recover the anatomical architecture of liver in cirrhosis as determined by decreased liver enzymes AST and ALT in serum, and decrease fibrotic nodules in liver architecture, respectively.

Acknowledgement

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Potential conflicts of interest

None.

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การศึกษาผลการรักษาด้วยแอลฟาแมงโกสตินต่อการเกิดภาวะตับแข็งในหนูแรทที่ถูกชักนำด้วยสารไธโออะเซตาไมด์

สุภาวดี สุขเสรี, ธเนศ โสภณนิธิประเสริฐ, วิสุทธิ์ ประดิษฐ์อาชีพ, สมนึก นิลบุหงา, สิรินันท์ นิลวรางกูร, รมิดา วัฒนโภคาสิน

วัตถุประสงค์: เพื่อศึกษาผลของแอลฟาแมงโกสตินต่อการเกิดภาวะตับแข็งในหนูแรทที่ถูกชักนำด้วยสารไธโออะเซตาไมด์

วัสดุและวิธีการ: หนูวิสตาร์แรทถูกแบ่งเป็น 3 กลุ่ม และชักนำด้วยสารไธโออะเซตะไมด์ขนาด 200 mg/kg ทาง intraperitoneal 3 ครั้งต่อสัปดาห์ เป็นเวลา 8, 12 และ 16 สัปดาห์ตามลำดับ หลังจากนั้นกลุ่มควบคุมไม่ได้รับสารแอลฟาแมงโกสติน และอีก 2 กลุ่มได้รับสารแอลฟาแมงโกสติน ขนาด 100 mg/kg และ 80% DMSO จำนวน 3 ครั้งต่อสัปดาห์ เป็นเวลา 4 สัปดาห์ เมื่อครบกำหนดเวลาในแต่ละกลุ่ม ทำการเก็บตัวอย่างชิ้นเนื้อตับและตัวอย่างเลือดจากหนูเพื่อนำไปตรวจระดับค่าดัชนีชี้วัดการทำงานของตับซึ่งได้แก่เอนไซม์ aspartate transaminase (AST) และ alanine transaminase (ALT) นอกจากนั้น การแสดงออกของจีนกลุ่มที่มีความสัมพันธ์กับภาวะตับแข็งและระดับของโปรตีน p53 ในชิ้นเนื้อตับได้ทำการวิเคราะห์ด้วยวิธี quantitative reverse transcription PCR และ Western blot ตามลำดับ

ผลการศึกษา: การให้สารไธโออะเซตาไมด์เป็นเวลา 12 หรือ 16 สัปดาห์ชักนำให้เกิดก้อนพังผืดบนตับหนู ภายหลังการเกิดก้อนพังผืดบนตับหนู ผลของสารแอลฟาแมงโกสตินสามารถทำให้จำนวนก้อนพังผืดลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ได้รับ DMSO เท่านั้น นอกจากนี้ระดับเอนไซม์ AST และ ALT ในซีรัมของหนูกลุ่มที่ให้แอลฟาแมงโกสตินมีระดับลดลงเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ได้รับ DMSO เท่านั้นผลการแสดงออกของจีนกลุ่มที่มีความสัมพันธ์กับภาวะตับแข็งมีความแปรผันภายในกลุ่มตัวอย่างสูง และไม่มีความแตกต่างอย่างมีนัยสำคัญระหว่างหนูในแต่ละกลุ่ม ระดับโปรตีน p53 ในชิ้นเนื้อตับแสดงให้เห็นว่าสารแอลฟาแมงโกสตินสามารถลดอัตราเสี่ยงของการเกิดภาวะ fibrosis ในตับผ่านทางการลดการแสดงออกของโปรตีน p53 ได้เมื่อเปรียบเทียบกับกลุ่มหนูที่ชักนำให้เกิดภาวะตับแข็งด้วยสารไธโออะเซตะไมด์ก่อนให้รับ DMSO

สรุป: การศึกษานี้แสดงให้เห็นว่าสารแอลฟาแมงโกสตินมีประโยชน์ในการรักษาการเกิดภาวะตับแข็งในหนูแรทที่ถูกชักนำด้วยสารไธโออะเซตาไมด์ และควรมีการศึกษากลไกการทำงานของสารแอลฟาแมงโกสตินต่อไป