

# Inhibition of Systemic Hyaluronan Synthesis Exacerbates Murine Hepatic Carcinogenesis

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**Abstract.** *Background/Aim:* Hyaluronan (HA) is used as a biomarker of liver fibrosis, which is a key risk factor for the development of hepatocellular carcinoma (HCC). We examined the effects of prolonged pharmacological inhibition of HA synthesis on liver carcinogenesis. *Materials and Methods:* Liver tumors were induced in mice by administering 0.03% thioacetamide (TAA) in drinking water over a 12-month period. Animals simultaneously received either a diet containing of an inhibitor of HA synthesis [4-methylumbelliferone (4-MU)], or a control diet. *Results:* Addition of 4-MU resulted in a significantly higher number of tumors compared to TAA treatment alone. Moreover, addition of 4-MU resulted in a dose-dependent increase in maximum tumor size. *Conclusion:* While local HA suppression has been shown to have an inhibitory effect on HCC *in vitro* and in tumor cell implantation experiments, the present results indicate that systemic inhibition of HA synthesis by 4-MU supplementation facilitates hepatic carcinogenesis *in vivo*.

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the second leading cause of cancer-related deaths in the world (1). It is estimated that the incidence and mortality of HCC are increasing steadily (2). Therefore, HCC will continue to be a serious social concern for several decades, and it is critical to develop an effective anticancer therapy. The relationship between chronic liver disease,

including cirrhosis, and HCC is clear. Most HCC cases arise in the setting of chronic liver disease or cirrhosis due to chronic viral infection, excessive alcohol intake, and non-alcoholic fatty liver disease (3). In chronic liver disease, the extracellular matrix (ECM) including hyaluronan (HA) and collagen deposits in the liver depend on fibrosis progression, regardless of the etiology (4). In fact, elevation of serum levels of HA and collagen has been found in patients with chronic liver disease, and these can be used as a noninvasive biomarker to assess the progression of liver fibrosis (5).

HA is an anionic glycosaminoglycan consisting of repeating polymeric disaccharides *N*-acetylglucosamine and glucuronic acid. It is a major component of the ECM and exists ubiquitously in human tissues (6). HA has various functions in a normal biological state, such as hydration, lubrication of joints, space filling, and provision of a matrix through which cells can migrate (7). During tissue injury, HA is actively produced for tissue repair with regulation of epithelial cell and fibroblast behavior (8, 9). In regard to cancer, many previous studies have demonstrated that stromal HA may create a permissive extracellular microenvironment for tumor progression and metastasis through cancer cell proliferation, migration, and invasion (10-16). An increase in HA deposits has been correlated with poor clinical prognosis in pancreatic, colorectal, ovarian, and breast cancer (17). Thus, HA signaling is expected to be a target for anticancer therapy (18).

The coumarin derivative 4-methylumbelliferone (4-MU) inhibits HA production *via* depletion of cellular uridine diphosphate glucuronic acid and down-regulation of expression of HA synthase 2 and 3 (19, 20). 4-MU has been clinically used for the treatment of functional and obstructive biliary tract spasms, but recent experimental studies have demonstrated that 4-MU may have other potential therapeutic benefits for treating cancer in various organs such as the pancreas, prostate, skin, breast, and ovaries (21-25). 4-MU also has antitumor effects in HCC that are similar in other cancer types with *in vitro* and tumor cell transplantation models (26, 27). However, the therapeutic effects of 4-MU on liver carcinogenesis have not

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yet been studied. Of particular note is the pathogenesis of HCC that characteristically develops based on chronic liver damage during the course of progression of fibrosis in the liver (3, 4). For this purpose, it is important to clarify the effects of 4-MU on liver carcinogenesis.

In this study, we investigated whether 4-MU supplementation would inhibit murine liver carcinogenesis induced by administration of thioacetamide (TAA), which causes chronic liver damage and fibrosis, as previous studies showed the beneficial effect of 4-MU in *in vitro* and tumor cell implantation models (26, 27).

## Materials and Methods

**Materials.** TAA and 4-MU were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Chow containing 4-MU was pelleted by Oriental Yeast Co., Ltd. (Tokyo, Japan).

**Animals and treatments.** All animals received humane care, with the study being conducted in accordance with Hiroshima University's Guidelines for Animal Experimentation. C57Bl/6J mice were obtained from CLEA Japan (Tokyo, Japan). Animals were housed in cages in a temperature- and humidity-controlled room with a 12-hour light/dark cycle, and they were given free access to food and water.

Following acclimation, 5-week-old male C57Bl/6J mice received TAA at a concentration of 0.03% in drinking water to induce hepatic tumorigenesis. The control mice were fed a standard diet without TAA administration, whereas TAA-treated mice were simultaneously fed either a diet containing 4-MU (0.01, 0.1, 1.0, or 5.0%; n=4-5/group) or a standard diet. After 12 months, the mice were sacrificed. Liver, spleen, and blood samples were collected for analyses. Liver body weight index and spleen weight index were calculated as liver weight and spleen weight in proportion to body weight, respectively.

**Serum biochemical analyses.** Serum alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and total bilirubin (T-bil) were measured with an automated analyzer (Spotchem EZ SP-4430; Arkrey Inc., Kyoto, Japan).

**Liver tumor analysis.** The number of visible tumor nodules on the liver surface was counted macroscopically. The maximum tumor size was determined by measuring the diameter of the largest tumor nodule on the liver surface for each liver.

**Statistical analysis.** Quantitative values are expressed as the mean±standard error of the mean (SEM). Statistical evaluations were performed using two-tailed Student's *t*-test. Differences were considered to be significant with *p*-values of less than 0.05.

## Results

**Survival and weight measurements.** While lower 4-MU concentrations (0.01-1.0%) did not affect survival, all mice that received TAA died within one week with addition of 5% 4-MU. Significantly lower body weight was seen in TAA-treated mice, while the addition of 4-MU did not significantly affect body

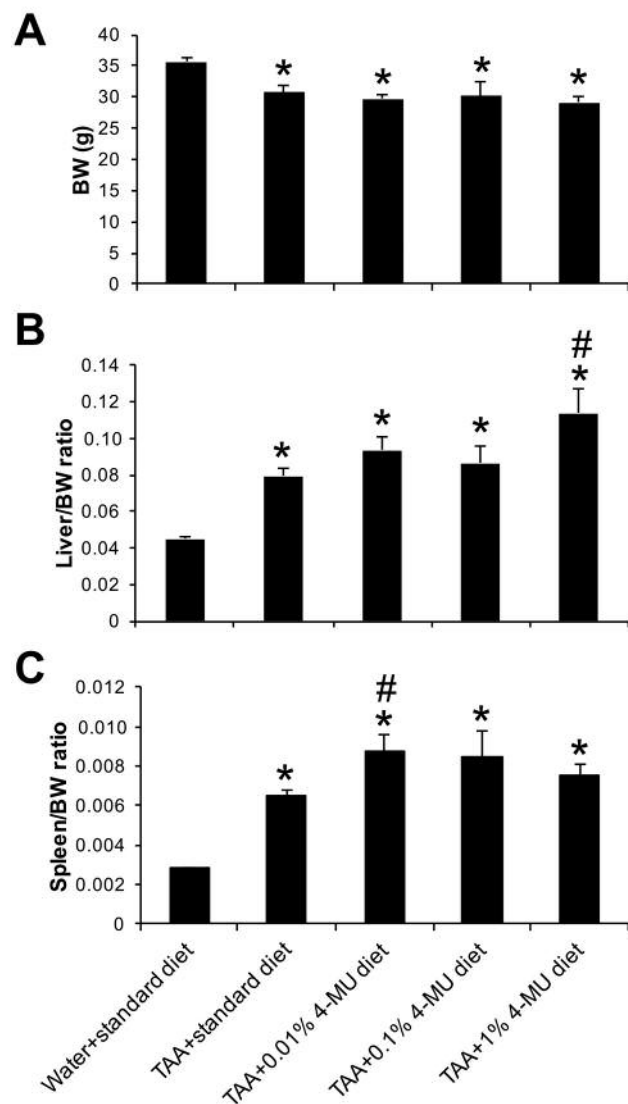


Figure 1. Systemic inhibition of hyaluronan (HA) synthesis induces hepatomegaly and splenomegaly in mice treated with the hepatic carcinogenic thioacetamide (TAA). Body weight (BW) (A), liver weight to body weight ratio (B), and spleen weight to body weight ratio (C) were determined in mice treated with TAA for 12 months with or without 4-MU supplementation. Student's *t*-test: significantly different at  $p < 0.05$  compared to \*water + standard diet, and #TAA + standard diet, n=4-5.

weight (Figure 1A). TAA treatment induced hepatomegaly (increased liver weight to body weight ratio) and splenomegaly (increased spleen weight to body weight ratio), and both were exacerbated by addition of 4-MU (Figure 1B and C).

**Biochemistry.** 4-MU supplementation induced significant exacerbation of liver injury in TAA-treated mice, as indicated by an increase in serum ALT, ALP, and LDH levels (Figure 2A-C). No significant change was observed in serum

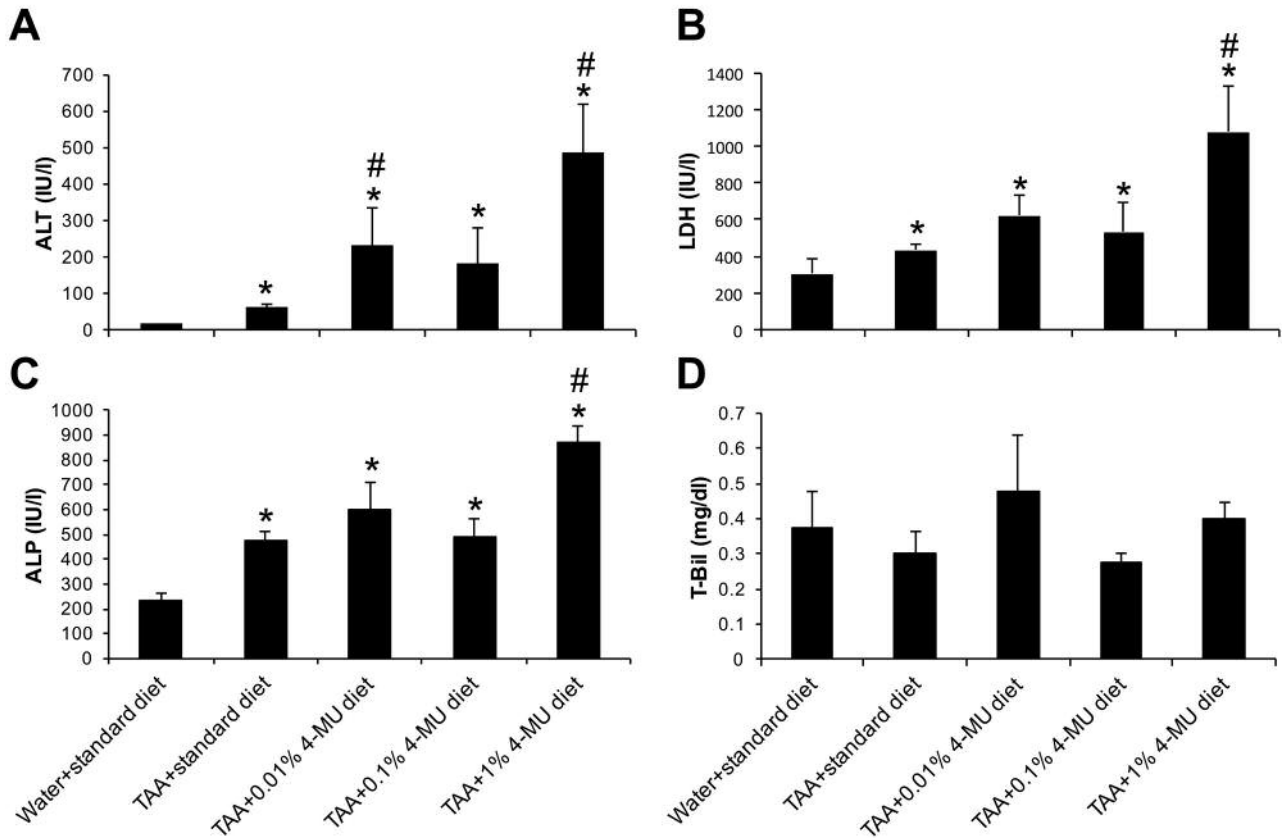


Figure 2. Systemic inhibition of hyaluronan (HA) synthesis worsens liver injury in mice treated with the hepatic carcinogenic thioacetamide (TAA). Serum alanine transaminase (ALT) (A), lactate dehydrogenase (LDH) (B), alkaline phosphatase (ALP) (C) and total bilirubin (T-Bil) (D) levels were measured in mice treated with TAA for 12 months with or without 4-MU supplementation. Student *t*-test: significantly different at  $p < 0.05$  compared to \*water + standard diet, and #TAA + standard diet,  $n = 4-5$ .

T-Bil level in TAA-treated mice with or without 4-MU supplementation (Figure 2D).

**Tumor measurement.** Administration of TAA induced liver tumors (TAA alone, number of tumors:  $6.2 \pm 1.5$ ), while mice that did not receive TAA had no tumors. It is important to note that the number of tumors increased significantly with the addition of 4-MU compared to mice that received TAA alone (TAA+0.01% 4-MU:  $19.0 \pm 3.5$ ; TAA+0.1% 4-MU:  $10.5 \pm 4.0$ ; TAA+1% 4-MU:  $22.2 \pm 6.5$ ;  $p < 0.05$ ). Moreover, a dose-dependent increase in maximum tumor size was found with addition of 4-MU. Taken together, these findings indicate that systemic inhibition of HA exacerbates hepatic carcinogenesis in TAA-treated mice.

## Discussion

Here, we showed a HCC development in a hepatic carcinogenic mouse model. Previous reports have shown the

antitumor effectiveness of HA inhibition by 4-MU in HCC (20, 21). However, those studies were conducted with an *in vitro* or tumor cell implantation model. In the present study, however, we tested an experimental HCC carcinogenesis model accompanied by chronic liver damage induced by TAA to mimic human hepatic carcinogenesis. The reason is that HCC clearly develops based on chronic liver injury due to causes such as hepatitis virus infection, alcohol, and non-alcoholic steatohepatitis (3).

The tumor microenvironment plays a critical role in cancer development and progression with the interaction between tumor cells and ECM molecules (28). As an integral component of the ECM, HA influences the behavior of tumor and stromal cells in proliferation, motility, invasion, and stemness through the activation of phosphatidylinositol-3 kinase (PI3K)/AKT and extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathways (29, 30). HA accumulation in tumor tissues indicates tumor progression and poor prognosis in patients with cancer (31-34). Thus,

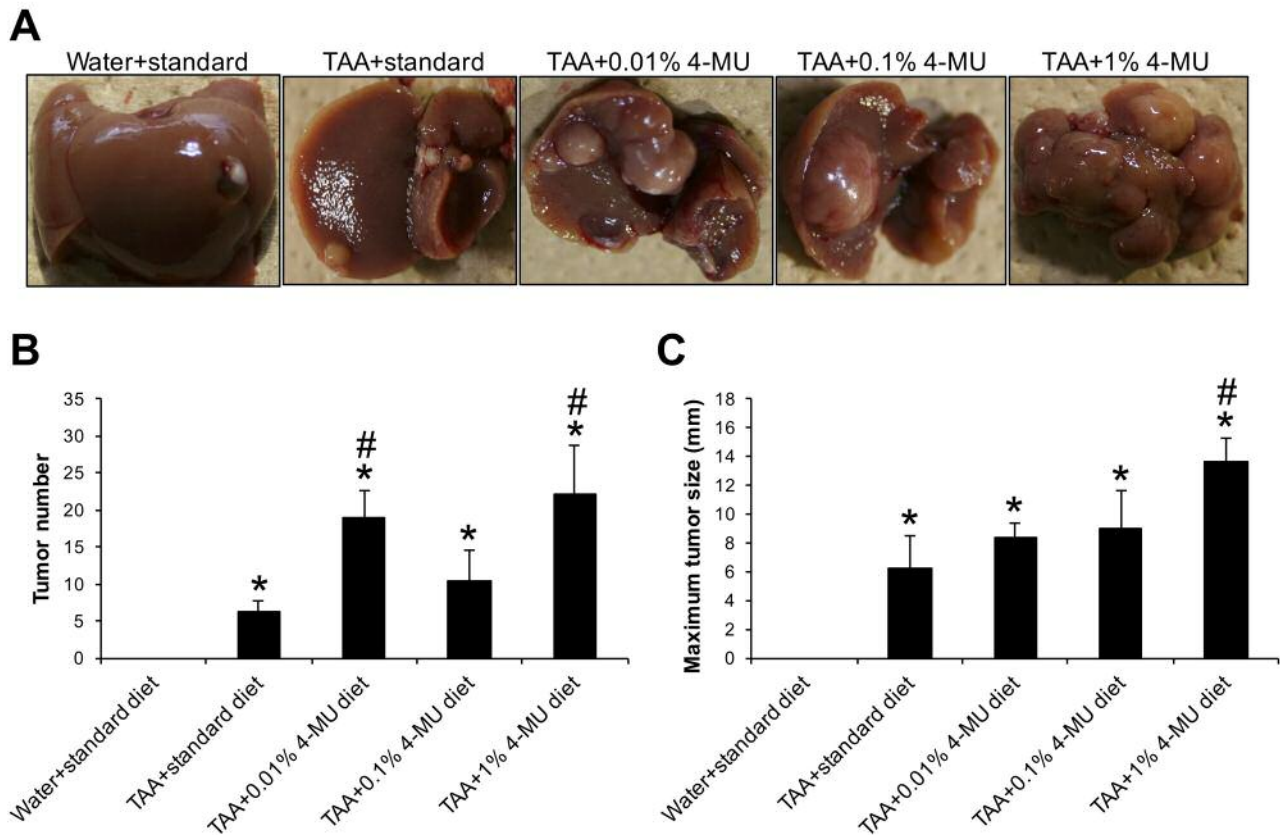


Figure 3. Systemic inhibition of hyaluronan (HA) synthesis aggravates liver tumor progression in mice treated with the hepatic carcinogenic thioacetamide (TAA). A: Representative macroscopic images of livers in mice treated with TAA for 12 months with or without 4-MU supplementation. Number of tumors (B) and maximum tumor size (C) in liver from indicated groups. Student's t-test: significantly different at  $p < 0.05$  compared to \*water + standard diet, and #TAA + standard diet,  $n = 4-5$ .

many researchers have focused on HA signaling as a target for cancer therapy.

Many recent experimental studies of 4-MU have shown it to have antitumor activity against various types of cancer by inhibiting HA synthesis (21-25). Almost all of those studies were implemented in cell culture or an implantation model of a cancer cell line. HA modulates tumor cell behaviors such as proliferation, motility, and metastatic spread through the tumor microenvironment. Therefore, it is speculated that the results of these experiments with 4-MU in various cancer cells show its effectiveness in terms of antitumor activity through the tumor microenvironment. However, in the process of carcinogenesis, there are many factors that affect the development of cancer, not only those in the local tumor microenvironment but also systemic factors such as the immune system, cytokines, and nutrition (35-37).

Furthermore, HCC develops based on chronic liver disease, which involves progression of liver fibrosis and cirrhosis, with accumulation of ECM including HA (4).

Therefore, it is expected that the development of HCC is closely related to HA signaling. In fact, a high preoperative serum HA level was found to correlate with a poor prognosis in patients with HCC (31). Indeed, overexpression of HA promoted progression of HCC cell lines (38). Conversely, suppression of HA expression by using 4-MU inhibited progression of HCC cell lines (26, 27). These previous reports were conducted using *in vitro* and tumor cell implantation models with HCC cell lines. To broaden the scope to translational research, we examined the effect of systemic inhibition of HA synthesis in a TAA-induced hepatic carcinogenesis model. Contrary to our expectations, however, our results showed that the systemic inhibition of HA synthesis actually promoted the development of liver tumors. Although the precise mechanisms for why the systemic inhibition of HA synthesis promoted HCC are unclear, considering both the previous reports and our results, the local inhibition of HA synthesis in the tumor microenvironment undoubtedly is a critical factor for the

decline of HCC. In order to develop a new therapeutic approach for HCC with regulation of HA signaling, further studies are needed to clarify the effects of HA inhibition not only in the liver but also in the whole body.

In conclusion, we found that systemic inhibition of HA synthesis by oral 4-MU administration promoted the development of liver tumors in TAA-treated mice. Despite the fact that many studies have shown that the inhibition of HA synthesis by 4-MU suppresses the progression of cancer in various organs, including the liver, our findings showed the completely opposite effect in liver tumors. In light of the focus on development of a new cancer therapy with regulation of HA signaling, our findings suggest that further studies are needed to reveal the effects of HA inhibition not only in the tumor microenvironment but also in systemic reactions. On the other hand, since local inhibition of HA synthesis is obviously effective in various cancer cell lines including HCC, it is also important to explore new procedures to control the local HA signaling in the tumor microenvironment. Regulation of HA signaling is a promising strategy for cancer therapy; thus, further studies are required to establish an effective therapeutic approach against HCC.

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## Disclosure

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