A Diterpenoid Acanthoic Acid from *Acanthopanax koreanum* Protects against D-Galactosamine/Lipopolysaccharide-Induced Fulminant Hepatic Failure in Mice

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The hepatoprotective effects of a diterpenoid acanthoic acid isolated from *Acanthopanax koreanum* NAKAI were evaluated in a D-galactosamine/lipopolysaccharide-induced fulminant hepatic failure mouse model. Mice were pretreated orally with acanthoic acid 12 and 1 h before intraperitoneal injection of D-galactosamine and lipopolysaccharide. Pretreatment with the compound markedly reduced lethal liver injury in experimental animals. The effects were likely associated with a significant decrease in serum tumor necrosis factor (TNF)-α levels, which are correlated not only with those of alanine aminotransferase and aspartate aminotransferase but also with the reduced number of apoptotic hepatocytes in the liver as confirmed using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling method and DNA fragmentation assay. These results suggest that acanthoic acid protects against D-galactosamine/lipopolysaccharide-induced fulminant liver failure at least in part by a mechanism associated with the down-regulation of TNF-α secretion.

Key words *Acanthopanax koreanum*; acanthoic acid; fulminant hepatitis; apoptosis; tumor necrosis factor-α

D-Galactosamine/lipopolysaccharide-induced fulminant hepatic failure in mice is a useful animal model for elucidating the mechanisms of clinical liver complaints and for evaluating the hepatoprotective activity of various plant components.[1,2] It was found that dysfunction of liver microcirculation, metabolic changes, direct cytotoxicity and macrophage-mediated liver injury occurs in lipopolysaccharide (LPS)-treated mice.[3] A low dose of LPS in combination with D-galactosamine (GalN) has been shown to induce experimental liver injury, which is similar to clinical acute hepatic failure.[4] GalN, a specific hepatotoxic agent, has been known to increase the sensitivity to the lethal effects of endotoxin.[5] It is believed that the lethal hepatic failure induced by GalN/LPS results from an abrupt increase in tumor necrosis factor (TNF)-α levels, which plays a critical role in pathogenesis.[6] TNF-α induces apoptosis in hepatocytes and neutrophil transmigration, a critical step in the necrosis of hepatocytes, which occurs at a later stage in liver injury.[7,8] The hepatic lesions in this model resemble those of human hepatitis in the up-regulation of TNF-α expression and hepatic apoptosis.[9]

Acanthopanax koreanum NAKAI (Araliaceae) is a native plant that grows in Jejudo, Korea. The root and stem bark of *Acanthopanax* species have been used as a tonic and sedative as well as in the treatment of rheumatism and diabetes.[10] Diterpenoids such as acanthoic acid [(−)-pimara-9(11),15-dien-19-oic acid] and kaurenoic acid [(−)-kaur-16-en-19-oic acid] and lignans such as eleutheroside B and E are major metabolites isolated from the root of *A. koreanum*.[11,12]

With the recent renewed interest in the pharmaceutical potential of natural products, we were interested in the hepatoprotective effects of acanthoic acid. For evaluation of the hepatoprotective effects of acanthoic acid on GalN/LPS-induced fulminant hepatic failure, serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and TNF-α and lethality and histopathologic observations with the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method as well as hematoxylin and eosin (H&E) staining and DNA fragmentation were determined. Here, we report that acanthoic acid has a significant protective effect in the GalN/LPS-induced liver failure model.

MATERIALS AND METHODS

Chemicals LPS, GalN, and silymarin were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). The TNF-α ELISA kit was purchased from Genzyme (Cambridge, MA, U.S.A.). All other chemicals used were of analytical grade.

Plant Material *A. koreanum* roots were provided by Susin Ogapi Co., Cheonan, Korea and identified by Dr. Y. H. Kim, College of Pharmacy, Chungnam National University. A voucher specimen (KRIBB96076) was deposited in the Herbarium of the Korea Research Institute of Bioscience and Biotechnology, Korea.

Preparation of Acanthoic Acid Acanthoic acid was isolated essentially as described previously.[11] Briefly, air-dried roots of *A. koreanum* (10 kg) were extracted with MeOH three times under reflux for 5 h to yield 960 g of a dark solid extract. The MeOH extract (950 g) was suspended in H_2O and extracted with CH_2Cl_2. The resulting CH_2Cl_2 solution was concentrated *in vacuo* to obtain the CH_2Cl_2-soluble fraction (470 g). The CH_2Cl_2-soluble fraction (200 g) was repeatedly chromatographed on silica gel column chromatography eluted with a hexane–EtOAc gradient system (20 : 1—1 : 1). Finally, acanthoic acid (0.82% recovery; >95% purity assessed with HPLC) was obtained by preparative HPLC using a J'sphere ODS-H80 column eluted with MeOH (flow rate 7 ml/min) as a white powder.

Animals and Experimental Design Male C57BL/6

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mice (20—22 g) were purchased from Dae Han Laboratory Animal Research and Co. (Chungbuk, Korea), fed with a standard chow diet (Jae Il Chow, Korea), and given tap water *ad libitum*. Animals were housed in plastic cages and maintained at 22±2 °C and 50—60% relative humidity, with 12 h light/dark cycles throughout the experiment. The animals were maintained in these facilities for at least 1 week before the experiment. Acanthoic acid or silymarin was dissolved in 0.5% Tween 80 in saline and administered orally to mice after 12 h fasting 12 and 1 h before GalN/LPS administration. One hour after the second dose of each sample, mice were given an intraperitoneal injection of GalN (700 mg/kg body weight), immediately followed by intraperitoneal injection of LPS (10 μg/kg body weight). The animals were killed and blood was collected from the carotid artery 1 h after LPS injection to measure serum TNF-α levels and 8 h after LPS injection to measure serum ALT and AST levels. Blood samples were allowed to coagulate at 4 °C for 30 min. Serum was then separated by centrifugation at 4 °C, 3000 rpm, for 10 min. Mice livers were removed immediately after blood collection and kept at −70 °C until analysis. There were 8—12 animals in each group. Animal experiments were carried out under the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (U.S.A.) in July 1989 and revised in March 1999. The Animal Care Committee of our institution approved the present study.

Determination of Lethality Acanthoic acid was administered orally at a dose of 100 mg/kg or 200 mg/kg to mice 12 and 1 h before GalN/LPS injection. Control mice were given 0.5% Tween 80 in saline or a dose of 100 mg/kg of silymarin. The survival rate of mice was monitored for 24 h after GalN/LPS injection.

Blood Biochemistry and Serum TNF-α Aassay Blood was collected 1 and 8 h after GalN/LPS administration. Serum levels of AST and ALT 8 h after GalN/LPS injection were quantified using an Autodry Chemistry Analyzer (SPOTCHEM SP4410, Arkray, Japan). Serum TNF-α levels were determined 1 h after GalN/LPS injection with a TNF-α ELISA kit (Genzyme, Cambridge, MA, U.S.A.) according to the manufacturer's protocol.

Detection of Apoptotic Cells and Histopathologic Analysis Liver samples obtained 8 h after the GalN/LPS injection were kept in liquid nitrogen for DNA fragmentation analysis and other samples were immersed in 10% neutral formalin for histologic analysis. Liver samples obtained 8 h after GalN/LPS injection were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections were stained with H&E staining for histopathologic analysis. Apoptotic cells were detected with the TUNEL method using an *in situ* cell detection kit (Roche, Mannheim, Germany) for detection and quantification of apoptosis at the single-cell level. Staining of tissue sections was performed according to the manufacturer's protocol. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated by passing through a graded series of ethanol solutions, ending with phosphate-buffered saline. Sections were permeabilized with proteinase K (20 μg/ml in Tris–HCl 10 mM, pH 7.4—8.0) at 37 °C for 15 min. After washing, sections were stained with fluorescent anti-TdT. Sections were viewed and photographed using standard fluorescent microscopic techniques.

DNA Fragmentation Analysis Liver tissues were obtained 8 h after GalN/LPS injection. Genomic DNA was extracted from liver tissues using a Wizard genomic DNA purification kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol. Extracted DNA was subjected to electrophoresis on 2% agarose gel containing ethidium bromide 0.1 μg/ml.

Statistical Analysis All values are expressed as mean±S.D. A comparison of the results was performed with one-way ANOVA and Tukey's multiple-comparison tests. Statistically significant differences between groups were defined as p values of less than 0.05. Calculations were performed using the GraphPad Prism program (GraphPad Software, Inc., San Diego, CA, U.S.A.).

RESULTS

Acanthoic Acid Suppressed Lethality in Mice with GalN/LPS-Induced Fulminant Hepatitis To determine the protective effects of acanthoic acid against GalN/LPS-induced lethal toxicity, the compound or silymarin was dissolved in 0.5% Tween 80 in saline and after 12 h fasting administered orally to mice 12 and 1 h before GalN/LPS administration. Mice treated with the vehicle control began to die within 8 h after GalN/LPS injection, and the lethality rate was 100% at 24 h. However, pretreatment with acanthoic acid at doses of 100 mg/kg or 200 mg/kg reduced the lethality rate to 33.3% and 25% (survival of 8 and 9 of 12 mice), respectively. Silymarin at dose of 100 mg/kg showed a significantly lower level of protection (survival of 2 of 8 mice) than acanthoic acid (Fig. 1).

Effects of Acanthoic Acid on Serum AST and ALT Levels The administration of GalN/LPS induced marked increases in serum AST and ALT levels, which reached 3654±345 IU/l and 2456±456 IU/l, respectively, while vehicle-treated mice had levels of 136±20 IU/l and 57±13 IU/l, respectively (Fig. 2). Consistent with the effects against GalN/LPS-induced lethal toxicity, oral pretreatment with 100 mg/kg or 200 mg/kg of the compound reduced serum AST levels to 23% ($p<0.001$) and 16% ($p<0.001$), respectively, of those in GalN/LPS-treated mice 8 h after GalN/LPS administration (Fig. 2A). ALT levels in the 100 mg/kg or 200 mg/kg compound groups also significantly decreased by ap-

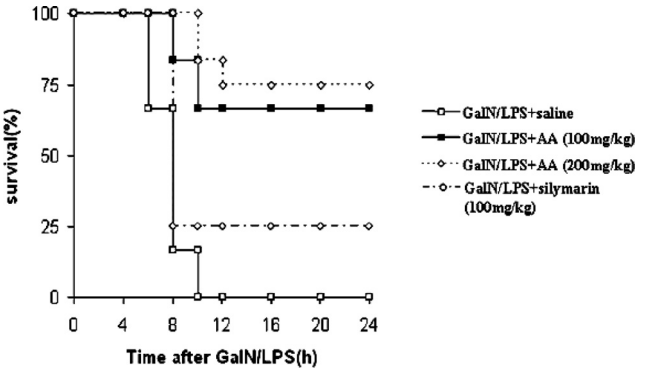

Fig. 1. Effects of Acanthoic Acid on the Survival of Mice after GalN/LPS Injection

Mice were intraperitoneally injected with GalN (700 mg/kg body weight)/LPS (10 μg/kg body weight). Acanthoic acid (AA) (100 mg/kg or 200 mg/kg body weight) or silymarin (100 mg/kg body weight) were orally administered 12 and 1 h before GalN/LPS injection. The GalN/LPS-treated mice were pretreated with 0.5% Tween 80 in saline as a vehicle. Each group consisted of 8—12 mice.

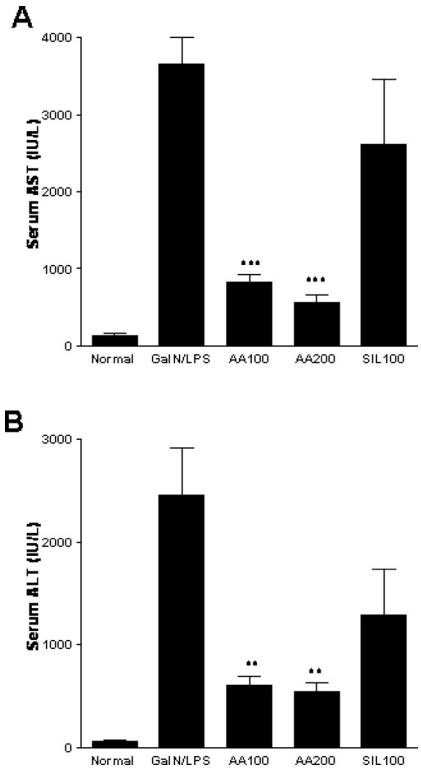

Fig. 2. Effects of Acanthoic Acid on Serum ALT and AST Levels in Mice after GalN/LPS Injection

Mice were intraperitoneally injected with GalN (700 mg/kg body weight)/LPS (10 μg/kg body weight). Acanthoic acid (AA) (100 mg/kg or 200 mg/kg body weight), silymarin (SIL) (100 mg/kg body weight), or 0.5% Tween 80 in saline was orally administered 12 and 1 h before GalN/LPS injection. Control animals received 0.5% Tween 80 in saline alone. Serum ALT and AST levels were measured 8 h after GalN/LPS injection. Each value is expressed as mean±S.D. (n=8—12). Asterisks denote significant differences from the control: ** $p<0.01$, *** $p<0.001$.

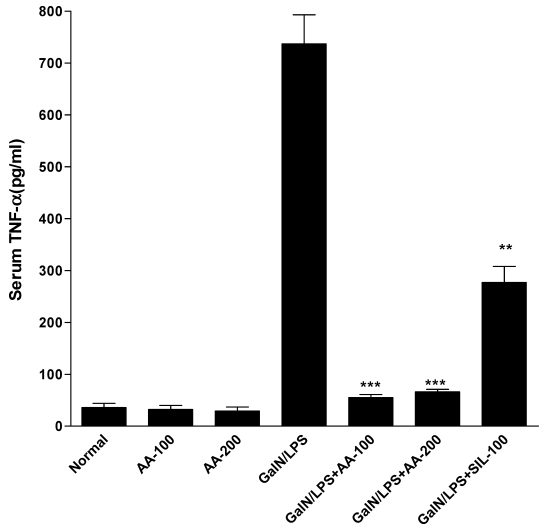

Fig. 3. Effects of Acanthoic Acid on Serum TNF-α Levels in Mice after GalN/LPS Injection

Mice were intraperitoneally injected with GalN (700 mg/kg body weight)/LPS (10 μg/kg body weight). Acanthoic acid (AA) (100 mg/kg or 200 mg/kg body weight), silymarin (SIL) (100 mg/kg body weight), or 0.5% Tween 80 in saline was orally administered 12 and 1 h before GalN/LPS injection. Control animals received 0.5% Tween 80 in saline alone. Serum TNF-α levels were measured 1 h after GalN/LPS injection. Each value is expressed as mean±S.D. (n=8—12). Asterisks denote significant differences from the control: ** $p<0.01$, *** $p<0.001$.

proximately 25% ($p<0.01$) and 22% ($p<0.01$), respectively, of those in GalN/LPS-treated mice 8 h after GalN/LPS administration (Fig. 2B). However, mice that received silymarin 100 mg/kg showed a significantly higher ALT (53% of that in GalN/LPS-treated mice) and AST (72% of that in GalN/LPS-treated mice) levels than mice that received acanthoic acid. There were no significant differences in the serum AST and ALT levels in mice treated with acanthoic acid 100 mg/kg or 200 mg/kg only when compared with those in vehicle treated normal mice (data not shown).

Effects of Acanthoic Acid on Serum TNF-α Levels It is known that the TNF-α is an important cytokine involved in hepatocyte apoptosis in mice with GalN/LPS-induced fulminant hepatitis. Therefore the effects of acanthoic acid on GalN/LPS-induced TNF-α levels were investigated. The administration of GalN/LPS induced fulminant hepatitis with marked increases in serum TNF-α levels, which reached 737±100 pg/ml, while vehicle-treated mice had a mean level of 36±8 pg/ml. Oral pretreatment with 100 mg/kg or 200 mg/kg of the compound reduced serum TNF-α levels to 7.5% ($p<0.001$) and 9.0% ($p<0.001$), respectively, of those in GalN/LPS-treated mice 1 h after GalN/LPS administration. However, mice that received silymarin 100 mg/kg had significantly higher TNF-α (38% of that in GalN/LPS-treated mice) levels than those that received acanthoic acid. There were no significant differences in the serum TNF-α levels in mice treated with acanthoic acid (100 mg/kg or 200 mg/kg) alone when compared with untreated normal mice (Fig. 3).

Acanthoic Acid Reduced Histologic Changes in the Liver Induced by GalN/LPS To confirm the protective effects of acanthoic acid on GalN/LPS-induced liver tissue damage, histologic examinations were performed in liver tissue obtained 8 h after GalN/LPS administration and compared with tissue from vehicle controls. Histopathologic examination of liver sections stained with H&E showed that no degenerative hepatocytes were present in the livers of GalN/LPS-treated mice 1 and 2 h after GalN/LPS administration, and only a few degenerative hepatocytes were present in the livers of GalN/LPS-treated mice 6 h after GalN/LPS administration (data not shown). However, numerous apoptotic hepatocytes and massive necrosis with intralobular hemorrhage appeared in the livers of GalN/LPS-treated mice 8 h after GalN/LPS administration. In contrast, only spotty necrotic hepatocytes were visible in the livers of the acanthoic acid-treated mice 8 h after GalN/LPS administration (Fig. 4).

Acanthoic Acid Reduced Hepatocyte Apoptosis in Mice Treated with GalN/LPS Next, apoptotic hepatocytes were detected using TUNEL staining. Numerous TUNEL-positive hepatocytes were seen in the livers of GalN/LPS-treated mice 8 h after the injection. However, few TUNEL-positive hepatocytes were found in livers from animals pretreated with acanthoic acid (Fig. 4).

To confirm the suppressive effect of acanthoic acid on hepatocyte apoptosis, genomic DNA fragmentation was assayed. Genomic DNA fragmentation was observed in the livers of mice treated with GalN/LPS alone 8 h after GalN/LPS injection, while very little DNA fragmentation was observed in the livers of mice pretreated with 100 mg/kg or 200 mg/kg of the compound (Fig. 5).

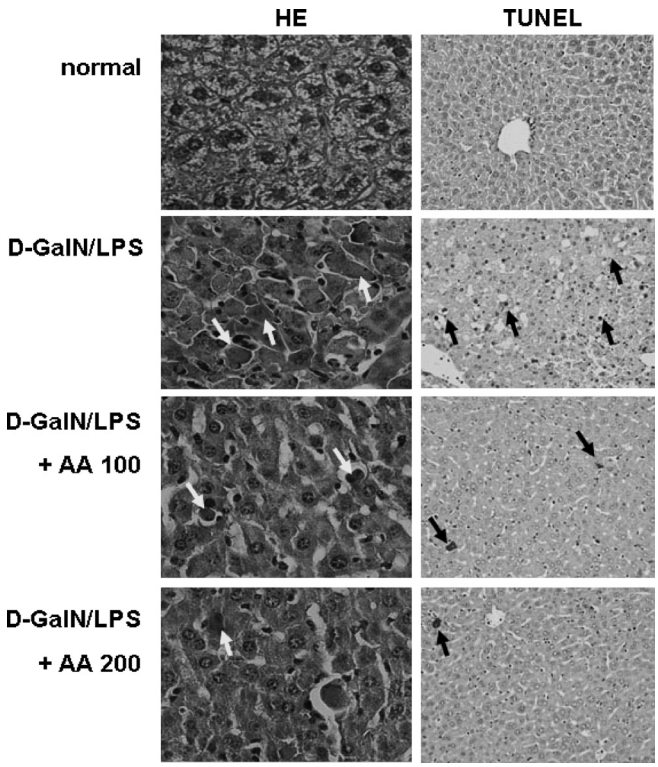

Fig. 4. Liver Histopathology and TUNEL Assay of Apoptotic Hepatocytes after GalN/LPS Injection with Acanthoic Acid or Vehicle Treatment in Mice

Representative pictures are for 8 h after GalN/LPS injection and liver histology at the time of death (around 8 h) in vehicle-treated mice. (H&E staining: left panels, magnification 400×; TUNEL assay: right panels, magnification 200×). White arrows indicate apoptotic hepatocytes and massive necrosis with intralobular hemorrhage (left panels) and black arrows indicate TUNEL-positive hepatocytes (right panels).

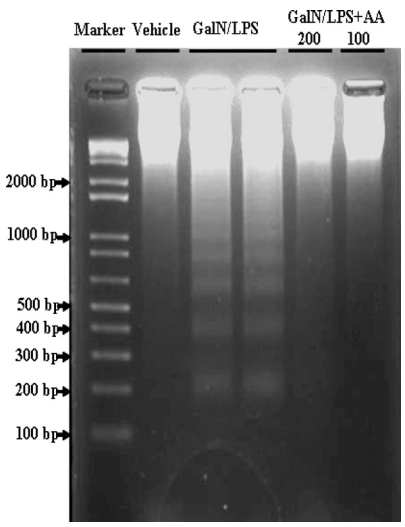

Fig. 5. Protective Effect of Acanthoic Acid on DNA Fragmentation of the Liver in GalN/LPS-Induced Liver Injury

Acanthoic acid (100 mg/kg or 200 mg/kg) or vehicle was orally administered 12 and 1 h before GalN/LPS injection. DNA was extracted from liver tissues of mice 8 h after GalN/LPS injection. DNA 2 μg was loaded on 2% agarose gel and separated by electrophoresis. The gels were stained with ethidium bromide to visualize DNA fragmentation.

DISCUSSION

A. koreanum has been used as a traditional herbal medicine for the treatment of various chronic inflammatory diseases in Korea.[11] Acanthoic acid, a major constituent in the plant, has been reported to reduce the writhing syndrome induced by acetic acid and the production of prostaglandin E_2 in mice, to suppress interleukin (IL)-1 and TNF-α production in human monocytes/macrophages, and to suppress granuloma formation and fibrosis in experimental liver fibrosis induced by carbon tetrachloride or silica.[13,14] Moreover, the inhibitory effect of acanthoic acid on IL-8 production *via* nuclear factor-kappa B (NF-κB) in TNF-α-stimulated human colon epithelial cells was also demonstrated.[15]

We previously reported that intraperitoneal injection of a water extract of either the roots or the stems of *A. koreanum*, which did not contain acanthoic acid, markedly protected experimental mice from GalN/LPS-induced fulminant hepatic failure, although no report on the protective effects of acanthoic acid in the same animal model.[16] In our present study, we investigated the effects of acanthoic acid on the mice liver injury induced by GalN/LPS. Pretreatment of mice with the compound at doses of 100 mg/kg or 200 mg/kg reduced the GalN/LPS-induced necrotic liver damage as well as hepatic apoptosis, as determined based on the serum biochemical parameters AST and ALT (Fig. 2). Microscopic observations of H&E- and TUNEL-stained liver sections demonstrated that acanthoic acid significantly decreased hepatocyte necrosis, fibrotic area, hepatocyte apoptosis, and DNA fragmentation to levels comparable with those in normal liver (Figs. 4, 5). Importantly, the serum TNF-α level was markedly decreased with acanthoic acid treatment (Fig. 3). This result is consistent with the previous report that acanthoic acid suppresses TNF-α production in human monocytes and macrophages.[13] TNF-α is a pleiotropic proinflammatory cytokine mainly produced by activated macrophages and monocytes and is involved in many different biological and pathologic processes including inflammation, septic shock, autoimmune diseases, and cancer. The production of most proinflammatory cytokines is regulated by NF-κBs, a family of transcription factors, which are central mediators in the regulation of immune and inflammatory responses.[17] Acanthoic acid has been suggested to interfere with the NF-κB activation pathway by inhibiting IL-1β, TNF-α, or IL-8 production. The inhibitory effects of a new synthetic analogue of acanthoic acid against IκBα phosphorylation and degradation were also reported.[13,15,18] It will be necessary to investigate the possible regulation of NF-κB activity by acanthoic acid to obtain better insight into the potential molecular mechanism by which it protects against GalN/LPS-induced hepatotoxicity. It is also worthwhile to note that acanthoic acid showed much better liver protection than silymarin, a mixture of polyphenolic flavonoids from the fruit of the milk thistle (*Silybum marianum* L.), which is used for oral treatment of toxic liver damage induced by alcohol, drugs, or environmental toxins and for supportive therapy in chronic inflammatory liver disease and in liver cirrhosis.[19] Taken together, our results indicate that the acanthoic acid effectively protects against liver injury in a mouse model and suggest that acanthoic acid could be a valuable candidate for further development for the treatment of inflammatory diseases of the liver.

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