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## Inhibition of Acidic Sphingomyelinase Reduces Established Hepatic Fibrosis in Mice

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### Abstract

**Aims**—Liver fibrosis occurs as a result of several chronic liver diseases and leads to portal hypertension, cirrhosis and liver failure, often requiring liver transplantation. Activated hepatic stellate cells (HSC) are known to contribute to liver fibrosis, but currently there are no effective therapies for the treatment of established liver fibrosis. Activation of the acidic sphingomyelinase (ASM) has been shown to be involved in HSC activation. In the present study we investigated whether treatment with the ASM inhibitor, amitriptyline (TCA), could prevent and/or reverse fibrosis induced in mice by carbon tetrachloride (CCl<sub>4</sub>).

**Methods**—Mice were treated with CCl<sub>4</sub> for 8 weeks to induce fibrosis. Concurrently, mice received drinking water with or without 180 mg/L TCA.

**Results**—Mice receiving TCA in the water had decreased hepatic collagen deposition and reduced liver mRNA expression of the fibrogenic mediators, TGF- $\beta$ 1, TIMP-1, collagen, and TNF $\alpha$ . TCA treatment also reduced HSC activation determined by  $\alpha$ -smooth muscle actin staining. In a separate set of experiments, mice were treated with CCl<sub>4</sub> for 5 weeks prior to treatment with TCA, to test whether TCA had any effect on established fibrosis. Remarkably, in mice with established fibrosis, treatment with TCA significantly reduced collagen deposition, HSC activation, and prevented portal hypertension and improved hepatic architecture. Treatment of isolated HSC *in vitro* with TCA completely inhibited TGF- $\beta$ 1-induced collagen expression and PDGF-BB-induced proliferation.

**Conclusions**—The data suggest that ASM is a critical signaling component in HSC for the development of liver fibrosis and represents an important therapeutic target.

## Keywords

liver fibrosis; non-alcoholic steatohepatitis; stellate cells

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## Introduction

Hepatic fibrosis may result from a variety of different etiologies including viral hepatitis, cholestatic hepatitis, alcoholic steatohepatitis and non-alcoholic steatohepatitis. In many patients, hepatic fibrosis will progress to cirrhosis and end stage liver disease, for which liver transplantation is the only life-saving therapy.<sup>1, 2</sup> However, a significant gap exists between the number of liver transplantation candidates and the availability of organs<sup>3, 4</sup> Therefore, developing strategies to limit the development and progression of hepatic fibrosis are of great clinical interest.

It is established that perisinusoidal hepatic stellate cells (HSCs) are the primary cell type responsible of liver fibrosis.<sup>5</sup> The retinoid-storing HSCs are quiescent physiologically, but become progressively activated during liver injury. The activation process is characterized by their transformation into a proliferating, contractile and fibrogenic myofibroblast-like phenotype. Thus, targeting activated HSCs as a therapeutic approach to control or reverse hepatic fibrosis has been intensely investigated.<sup>6, 7</sup> Unfortunately, despite these efforts, there are no effective therapies for the treatment of liver fibrosis at present.

Ceramide is a sphingolipid which has been previously implicated in the development and progression of liver disease. Ceramide is produced by hydrolysis of sphingomyelin catalyzed by acidic sphingomyelinase (ASM). ASM is a lysosomal glycoprotein, which translocates to the cell surface and plays an important role in cellular stress responses.<sup>8</sup> High concentrations of ceramide in the cellular membranes serve to form ceramide-enriched domains, which regroup receptors and signaling molecules.<sup>9</sup> This reorganization of the plasma membrane facilitates and amplifies various signaling processes related to cellular responses to injury, which include cell migration, survival, contraction, proliferation, gene expression and cell-to-cell interaction.<sup>10–13</sup>

ASM activation and resulting ceramide production have been implicated in a variety of human diseases including atherosclerosis, emphysematous lung disease, neurodegenerative disease, insulin resistance and diabetes mellitus, as well as non-alcoholic steatohepatitis.<sup>14–16</sup> As such, the inhibition of ASM is appealing given the many potential therapeutic implications. One ASM inhibitor, amitriptyline, is a tricyclic antidepressant that causes detachment of ASM from the inner lysosomal membrane, resulting in subsequent inactivation by lysosomal proteases.<sup>8, 17</sup> Amitriptyline treatment has been used to influence ceramide producing pathways in both murine *in vitro* and *in vivo* studies, as well as in phase II clinical trials for cystic fibrosis.<sup>18–20</sup>

In the present study, we sought to determine whether therapeutic inhibition of ASM with amitriptyline would reduce hepatic fibrosis induced by carbon tetrachloride (CCl<sub>4</sub>). Previous studies have demonstrated that ASM heterozygous mice are protected from developing hepatic fibrosis in cholestatic and CCl<sub>4</sub> models of liver disease in association with reduced

activation of HSCs<sup>19</sup>, and other studies have shown that treatment with amitriptyline reduces liver pathology in a rat model of Wilson's Disease.<sup>21</sup> However, we tested the ability of amitriptyline to treat established liver fibrosis. Secondly, we investigated the mechanism by which ASM inhibition with amitriptyline in HSCs controls their fibrogenic activity.

## Methods

### Model of Liver Fibrosis

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. The University of Cincinnati Animal Care and Use Committee approved all animal care and procedures performed. To induce fibrosis, mice were treated twice weekly with intraperitoneal injections of 1 mL/kg CCl<sub>4</sub> (Sigma-Aldrich, St. Louis, MO) diluted in three volumes of mineral oil.<sup>22</sup> Age and sex matched control animals were treated twice weekly with similar volumes of mineral oil injected intraperitoneally. To pharmacologically inhibit ASM, mice were provided 180 mg/L of amitriptyline (Sigma) in their drinking water, which was changed every 3 days. This regimen has been shown to effectively inhibit ASM *in vivo*.<sup>18, 23, 24</sup> In one set of experiments, mice were given amitriptyline in the water two weeks prior to treatment with CCl<sub>4</sub> to ensure that ASM was inhibited prior to induction of fibrosis. Mice were sacrificed 2, 4, 6 and 8 weeks after treatment with CCl<sub>4</sub> with or without amitriptyline. In a second set of experiments, mice received amitriptyline in their water beginning 5 weeks after initiation of CCl<sub>4</sub> treatment to determine whether ASM inhibition could arrest and reverse established fibrosis. These mice were sacrificed 9, 12 and 15 weeks after initiation of CCl<sub>4</sub> treatment.

### Blood and Tissue Analysis

Blood was obtained via cardiac puncture for analysis. Liver tissues were fixed in 10% neutral-buffered formalin (Richard Allen Scientific, Kalamazoo, MI), processed and then embedded in paraffin for light microscopy. Serial sections were stained with hematoxylin and eosin for histological examination and sirius red for determination of collagen deposition. Activated HSCs were identified by immunohistochemical staining of alpha-smooth muscle actin ( $\alpha$ -SMA, Abcam, Cambridge, MA). Sections were examined by a blinded hepatopathologist and hepatic fibrosis was assessed using the criteria described by Kleiner et al.<sup>25</sup>

### Isolation and Culture of Hepatic Stellate Cells

Murine HSCs were isolated from male C57BL/6 mice, as previously described.<sup>26, 27</sup> Livers were perfused and digested *in situ* with Hank's balanced salt solution containing collagenase and protease. The liver was excised, minced and strained through a steel mesh sieve. The resulting cell suspension was centrifuged 3 to 4 times at 65 g for 2 minutes to remove hepatocytes and cellular debris. The supernatant was then centrifuged at 650 g for 10 minutes and hepatic stellate cells were clarified from the resulting pellet via Nycodenz gradient centrifugation. HSCs were suspended in Dulbecco's Modified Eagle Medium containing 10% FBS, 10% horse serum, 100 mg/mL penicillin and 100 U/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were seeded at a density of  $1.75 \times 10^6$  cells/mL. Media was renewed on the following day and on alternating days thereafter. Cells were passaged

after becoming approximately 70% confluent and were used during their third and fourth passage. Passaged cells were treated with or without 25  $\mu$ M of amitriptyline in reduced serum media (2% FBS) for 48 hours to inhibit ASM.<sup>19</sup> Cells were then treated with 5 nM of transforming growth factor-beta (TGF- $\beta$ 1, R&D Systems, Minneapolis, MN) or 50 nM platelet derived growth factor-beta, beta (PDGF-BB, Peprotech, Rocky Hill, NJ) with or without the presence of amitriptyline for 24 hours. Genetic expression of collagen was assessed by real-time polymerase chain reaction (RT-PCR) and cellular proliferation was assessed by bromodeoxyuridine incorporation.

### RT-PCR Analysis

Total RNA was extracted from liver tissue and HSCs using Trizol (Invitrogen, Carlsbad, CA). cDNA was generated through the reverse-transcription of 2  $\mu$ g of RNA using random primers and Superscript RNase H-reverse transcriptase (Invitrogen). Samples were incubated at 20°C for 10 minutes, 42°C for 30 minutes, 99°C for 5 minutes to inactivate the reverse transcriptase and then cooled at 5°C for 5 minutes. The amount of mRNA was quantified by real-time reverse transcriptase polymerase chain reaction per the manufacturer's specifications (Eppendorf, Mastercycler Real-Time PCR). Four microliters of diluted cDNA samples (1:10 dilution) were used for quantitative two-step PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Each sample was analyzed in duplicate. Beta-actin ( $\beta$ -actin) was used as a housekeeping control gene. Threshold cycles were automatically calculated by the iCycler iQ Real-Time Detection System. Threshold cycle values were normalized to the housekeeping control ( $\beta$ -actin) to give relative genomic equivalence. Primers utilized are listed in the Table 1.

### Cellular Proliferation

DNA synthesis was estimated using bromodeoxyuridine (BrdU) incorporation and enzyme-linked immunosorbent assay kit (Abcam). Passaged cells were incubated with BrdU for 4 hours. BrdU uptake was then measured according to standard enzyme-linked immunosorbent assay protocol per the manufactures' instructions.

### ASM Activity

ASM activity was measured by incubating immunoprecipitated ASM with [<sup>14</sup>C]sphingomyelin and quantifying the release of [<sup>14</sup>C]choline as described.<sup>28</sup> The liver was removed, snap-frozen in liquid nitrogen, and lysed in 250 mM sodium acetate (pH 5.0), 1% NP40, and 1.3 mM EDTA for 15 min. The tissues were then homogenized with a tip sonicator. Aliquots of the lysates were diluted to 250 mM sodium acetate (pH 5.0), 0.1% NP40, and 1.3 mM EDTA and incubated with 50 nCi per sample [<sup>14</sup>C]sphingomyelin for 30 min at 37 °C. The substrate was dried prior to the assay, resuspended in 250 mM sodium acetate (pH 5.0), 0.1% NP40, and 1.3 mM EDTA and bath-sonicated for 10 min to obtain micelles. The enzyme reaction was terminated by the addition of 800  $\mu$ L chloroform/methanol (2:1, v/v), phases were separated by centrifugation and radioactivity of the aqueous phase was measured by using liquid scintillation counting to determine the release of [<sup>14</sup>C]phosphorylcholine from [<sup>14</sup>C]sphingomyelin as a measure of ASM activity.

## Statistical Analysis

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Data were analyzed with a one-way analysis of variance with subsequent Student Newman Keuls test. Differences were considered significant when  $p < 0.05$ .

## Results

### Inhibition of ASM reduces hepatic fibrogenesis by suppressing activation of HSCs

Using a murine model of liver fibrosis induced by CCl<sub>4</sub>, animals received amitriptyline in their drinking water to inhibit ASM throughout an 8-week course of CCl<sub>4</sub> administration. Hepatic ASM activity was determined after 8 weeks of treatment. While vehicle- and CCl<sub>4</sub>-treated animals had similar levels of liver ASM activity, amitriptyline treatment significantly decreased ASM activity compared to both vehicle and CCl<sub>4</sub>-treated animals (Figure 1). Over the course of 8 weeks of treatment with CCl<sub>4</sub>, mice developed increasing hepatic fibrosis, as determined by collagen deposition using sirius red staining as well as pathological scoring (Figure 2). However, inhibition of ASM with amitriptyline significantly reduced the degree of fibrosis (Figure 2). To investigate if inhibition of ASM with amitriptyline decreased HSC activation as a mechanism for the reduced hepatic fibrosis observed, we performed RT-PCR on liver samples prior to induction of fulminant hepatic fibrosis. Liver samples were obtained after two weeks of treatment. As shown in Figure 3A, inhibition of ASM significantly decreased CCl<sub>4</sub>-induced expression of TGF- $\beta$ 1, TIMP-1, collagen, and TNF $\alpha$ , all markers associated with HSC activation and liver fibrosis (Figure 3A). This effect persisted throughout the duration of treatment (data not shown). Supporting these findings, immunohistochemical staining for  $\alpha$ -SMA revealed decreased activation of HSCs in animals treated with amitriptyline at all time points relative to animals treated with CCl<sub>4</sub> alone (Figure 3B).

### Inhibition of ASM reduces established hepatic fibrosis and portal hypertension

In order to determine if inhibition of ASM could serve as a potential therapeutic modality for those with established hepatic fibrosis, mice were subjected to 5 weeks of CCl<sub>4</sub> treatment to induce hepatic fibrosis, with some mice receiving amitriptyline in their drinking water along with continued CCl<sub>4</sub> treatment until week 15. Mice treated with CCl<sub>4</sub> alone developed increasing amounts of bridging fibrosis throughout the time course of treatment (Figure 4). However, mice treated with amitriptyline showed resolution of fibrosis at weeks 9 and 12 despite the continued profibrogenic stimulus. Interestingly, by 15 weeks, mice treated with amitriptyline did develop bridging fibrosis (Figure 4). However, liver architecture in these mice was preserved compared to those mice receiving only CCl<sub>4</sub>, which showed increased central fibrosis and hepatocellular loss (Figure 4). Immunohistochemical staining for  $\alpha$ -SMA revealed decreased HSC activation in the mice treated with amitriptyline (Figure 5A). Furthermore, by week 15, CCl<sub>4</sub>-induced liver fibrosis was associated with splenomegaly, a putative marker of portal hypertension in the context of liver fibrosis<sup>29, 30</sup> (Figure 5B). Treatment with amitriptyline, abrogated this pathology (Figure 5B).

### Inhibition of ASM prevents PDGF-BB and TGF- $\beta$ 1 stimulation of hepatic stellate cells

Because our *in vivo* data suggest that the beneficial effects of ASM inhibition are related to decreased HSC activation, we next evaluated the direct effects of amitriptyline on activated (passaged) HSCs *in vitro*. Isolated HSCs were passaged 3–4 times to establish a fully activated phenotype and then were stimulated *in vitro* with PDGF-BB or TGF- $\beta$ 1. Treatment with amitriptyline (25  $\mu$ M) decreased collagen and alpha-smooth muscle actin ( $\alpha$ -SMA) expression nearly 4-fold (Figure 6A). Furthermore, stimulation of HSCs with exogenous PDGF-BB (50 nM) or TGF- $\beta$ 1 (5 nM), could not overcome the inhibitory effects of amitriptyline. An even greater effect of amitriptyline was observed on PDGF-BB-induced proliferation. Treatment of HSCs with PDGF-BB (50 nM) resulted in an 8-fold increase in proliferation (Figure 6B). However, treatment with amitriptyline completely abrogated PDGF-BB-induced proliferation (Figure 6B).

### Discussion

In this study, we examined the impact of the pharmacologic inhibition of ASM with amitriptyline in a murine model of CCl<sub>4</sub> induced hepatic fibrosis. In this model, we found that treatment with amitriptyline decreases hepatic ASM activity and limits CCl<sub>4</sub> induced hepatic fibrosis. When given in a therapeutic fashion, i.e., in the setting of established fibrosis and activated HSCs, amitriptyline impaired the progression of hepatic fibrosis and abrogated the development of portal hypertension. Additionally, we found in our *in vitro* experiments that inhibition of ASM in HSCs significantly reduces collagen production and abrogates cell proliferation. Our data provide new information regarding the therapeutic utility of targeting ASM in reducing existing liver fibrosis and also provides new mechanistic details regarding the role of ASM in HSC activation and proliferation.

ASM has previously been implicated in both liver fibrogenesis and HSC activation. One study, using heterozygous ASM mice, showed that reduced ASM activity was protective in both cholestatic and CCl<sub>4</sub> models of hepatic fibrosis through the regulation of HSC activation and proliferation via cathepsin B and D processing.<sup>19</sup> However, this work did not determine if targeting ASM after established fibrosis could be an effective therapeutic strategy. Furthermore, the study focused on the function of ASM in regulating the expression of cathepsin B and the role of this mediator in the activation of HSCs. In contrast, our study demonstrates, for the first time, that therapeutic inhibition of ASM can reverse established liver fibrosis and prevent portal hypertension, and that these effects are likely linked to disruption of signaling events in HSCs that are essential for pro-fibrotic and cell proliferative signaling. Our data provide strong evidence that inhibition of ASM in HSCs results in reduced TGF- $\beta$ 1- and PDGF- $\beta$ -mediated effects.

Another study showed, in a rat model of Wilson's disease, that inhibition of ASM with daily intraperitoneal injections of amitriptyline, limited the development of hepatocyte injury and liver fibrosis.<sup>21</sup> However, this study did not investigate the mechanisms by which ASM inhibition reduced liver fibrosis. In this regard, the study was limited in scope and examined ASM inhibition only in a prophylactic setting.



PDGF-BB is the most potent mitogen of HSCs and PDGF receptor-beta subunit (PDGFR- $\beta$ ) is required for PDGF induced proliferation of HSCs.<sup>31, 32</sup> *In vivo* genetic silencing of PDGFR- $\beta$  with a HSC-specific promoter demonstrated reduced liver injury and hepatic fibrosis in both a bile duct ligation and CCl<sub>4</sub> model of hepatic fibrosis, suggestive of HSC-specific RNA interference as a treatment of hepatic fibrogenesis.<sup>32</sup> Here we propose that inhibition of the ASM/ceramide axis with amitriptyline may represent an alternative, and more plausible, treatment with a similar mechanism of action.

It is plausible that inhibition of ASM by amitriptyline alters PDGFR- $\beta$  availability. Conversion of sphingomyelin to ceramide by ASM occurs within discrete plasma membrane microdomains enriched in sphingolipids and cholesterol termed caveolae.<sup>33, 34</sup> These localized high levels of ceramide help to form a more stabilized lipid matrix, regrouping receptors and signaling molecules, providing ordered support for receptor-mediated signaling events.<sup>9, 35</sup> PDGFR- $\beta$  has been localized within caveolae and PDGFR- $\beta$  cofractionates with ASM from these ceramide enriched microdomains.<sup>35, 36</sup> More importantly, these microdomains have been shown to be the major site of PDGF stimulated signal transduction.<sup>36</sup> Our *in vitro* studies demonstrate that the inhibition of ASM with amitriptyline limits activated HSC proliferation, even in the presence of PDGF-BB stimulation. Inhibition of ASM may result in decreased stability of plasma membrane microdomains through the reduction in ceramide levels with ensuing loss of support for caveolae based receptor mediated signaling, namely through the PDGFR- $\beta$ .

Likewise, a similar argument could be made for TGF- $\beta$ 1 receptor, which also has been found to colocalize and cofractionate with caveolae.<sup>37</sup> We note that exogenously added TGF- $\beta$ 1 did not stimulate collagen synthesis over the basal value in activated HSCs indicating that the system is already fully activated. Indeed, constitutively increased nuclear Smad3/Smad4, which is coupled to TGF- $\beta$ 1-induced collagen production by activated HSCs<sup>38</sup>, was observed in a cell line developed from HSCs from the cirrhotic liver, and exogenous treatment with TGF- $\beta$ 1 did not affect the increased collagen expression in them.<sup>39</sup> Thus, a strong amitriptyline-induced inhibition of collagen synthesis by HSCs both in the absence and presence of exogenous TGF- $\beta$ 1 suggests that the inhibitor may interfere with constitutively activated intracellular signaling responsible for collagen synthesis.

In summary, the current study demonstrates that inhibition of ASM can limit the development of liver fibrosis and more importantly can reverse established liver fibrosis. These beneficial effects are mediated by inhibition of PDGF-BB and TGF- $\beta$ 1 signaling, possibly via decreased ceramide in caveolin-enriched domains in HSCs resulting in dissociation of PDGF- $\beta$  receptors from these microdomains or by interfering with the signaling coupled to profibrogenic activity. These findings suggest that the inhibition of ASM may be an effective therapeutic modality to improve liver fibrosis.

## Acknowledgments

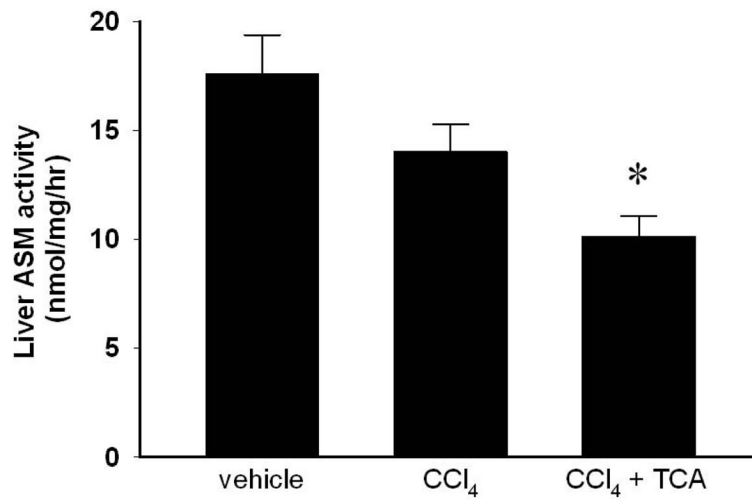
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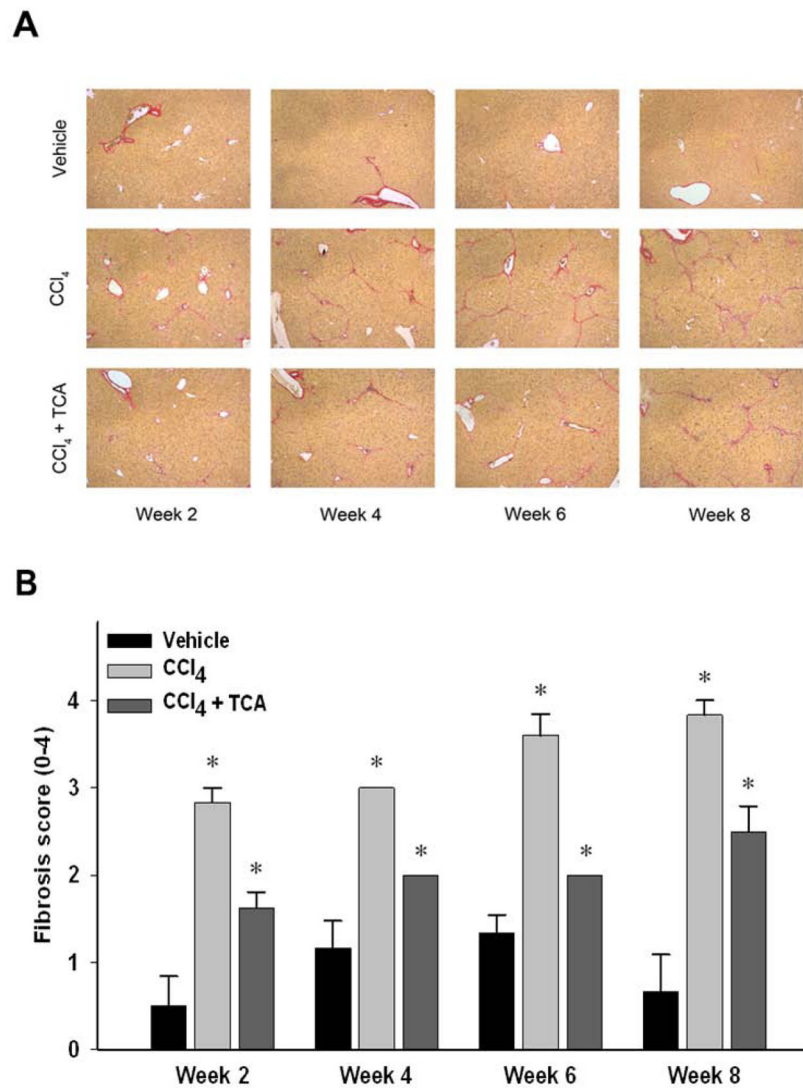


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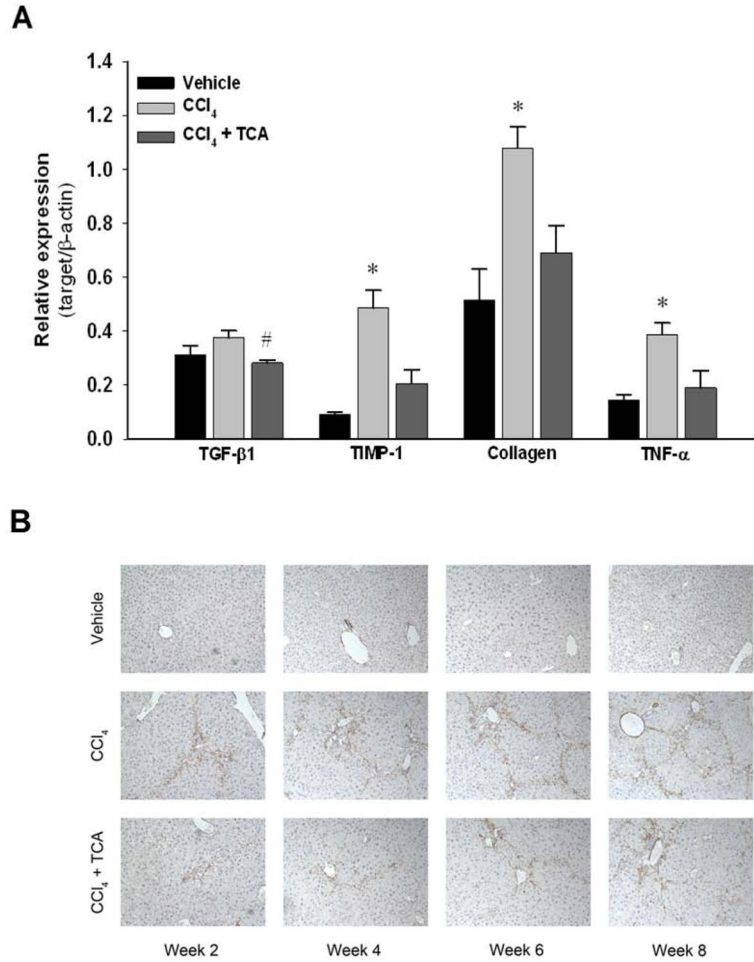


**Figure 1.**

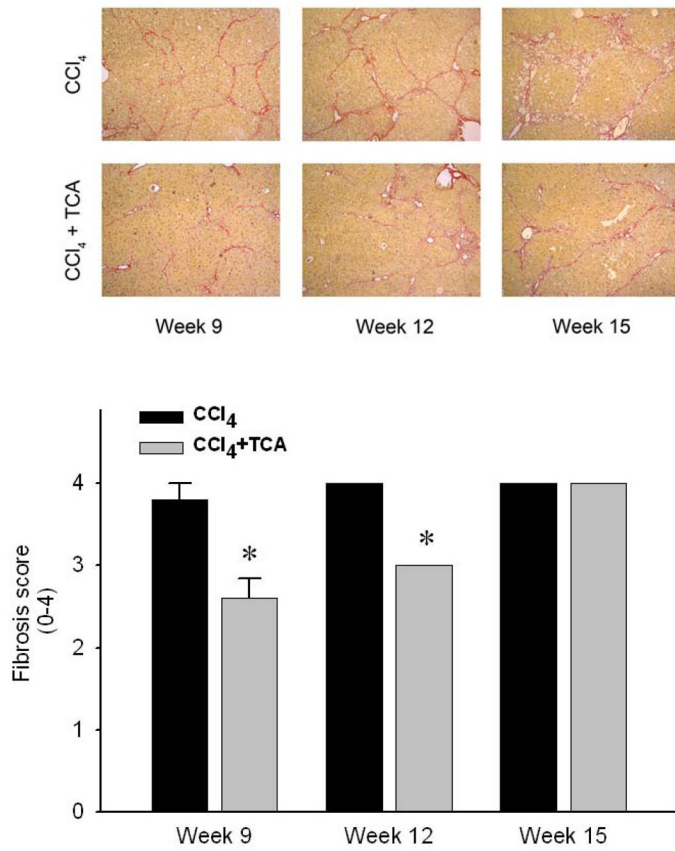
Amitriptyline (TCA) treatment decreases hepatic acidic sphingomyelinase (ASM) activity. Hepatic ASM activity was determined after 8 weeks of treatment. TCA treatment decreased ASM activity compared to vehicle and CCl<sub>4</sub> treatment. n=4–6 per group. Data are expressed as mean and SEM. \*p<0.05 compared to all other groups.



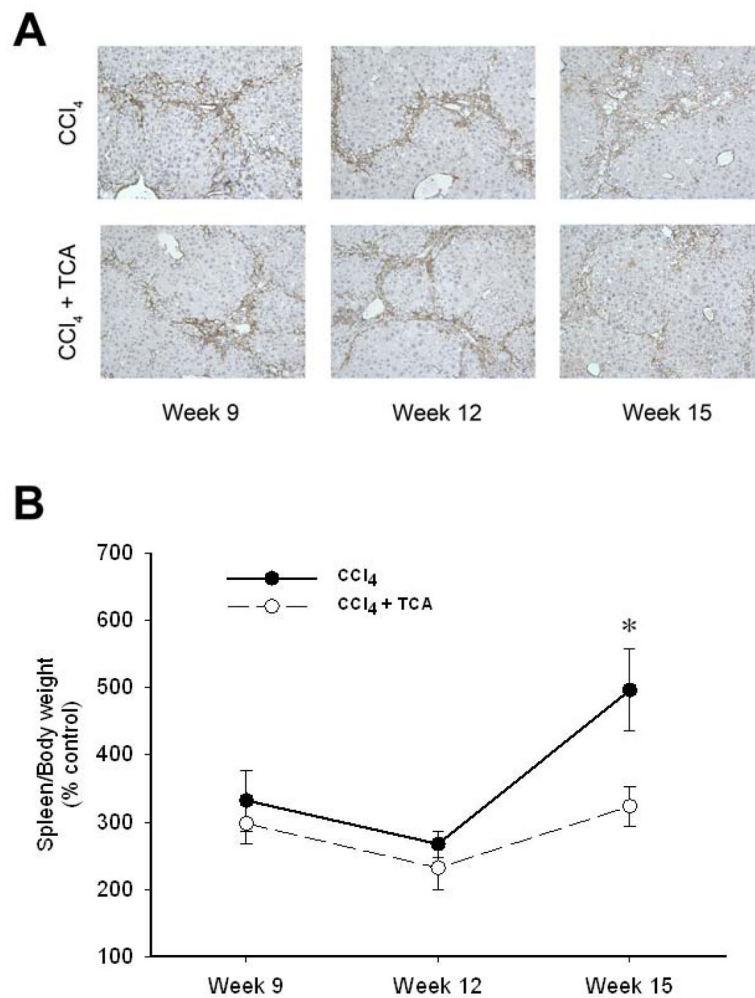
**Figure 2.** Inhibition of ASM activity limits CCl<sub>4</sub>-induced hepatic fibrosis. (A) Sirius red staining of liver sections (magnification 50X) reveals increased collagen deposition over time in animals treated with CCl<sub>4</sub> compared to animals treated with CCl<sub>4</sub> and amitriptyline (TCA). (B) Quantitation of hepatic fibrosis using pathological scoring. Decreased fibrosis in animals treated with CCl<sub>4</sub> and TCA vs. CCl<sub>4</sub> alone was confirmed via quantification of liver sections by a blinded pathologist. n=4–8 per group. Data are expressed as mean and SEM. \*p<0.05 compared to all other groups. #p<0.05 compared to CCl<sub>4</sub> group.



**Figure 3.** Inhibition of ASM activity reduces hepatic stellate cell activity *in vivo*. (A) RT-PCR analysis of whole liver tissue after two weeks of treatment with CCl<sub>4</sub> demonstrated that treatment with amitriptyline (TCA) decreased levels of transforming growth factor beta one (TGF- $\beta$ 1), tissue inhibitor of metalloprotease one (TIMP-1), collagen and tumor necrosis factor alpha (TNF- $\alpha$ ) mRNA compared to CCl<sub>4</sub> alone. n=6–8 per group. Data are expressed as mean and SEM. \*p<0.05 compared to all other groups. (B) Hepatic stellate cell staining of  $\alpha$ -SMA. Immunohistochemical staining of liver tissues for  $\alpha$ -SMA reveals decreased activated HSC burden at each time point in animals treated with CCl<sub>4</sub> and TCA relative to both vehicle and CCl<sub>4</sub> treatment alone (magnification x 50). Data are representative of 4 separate histological analyses per group/time point. Original magnification was 50X.

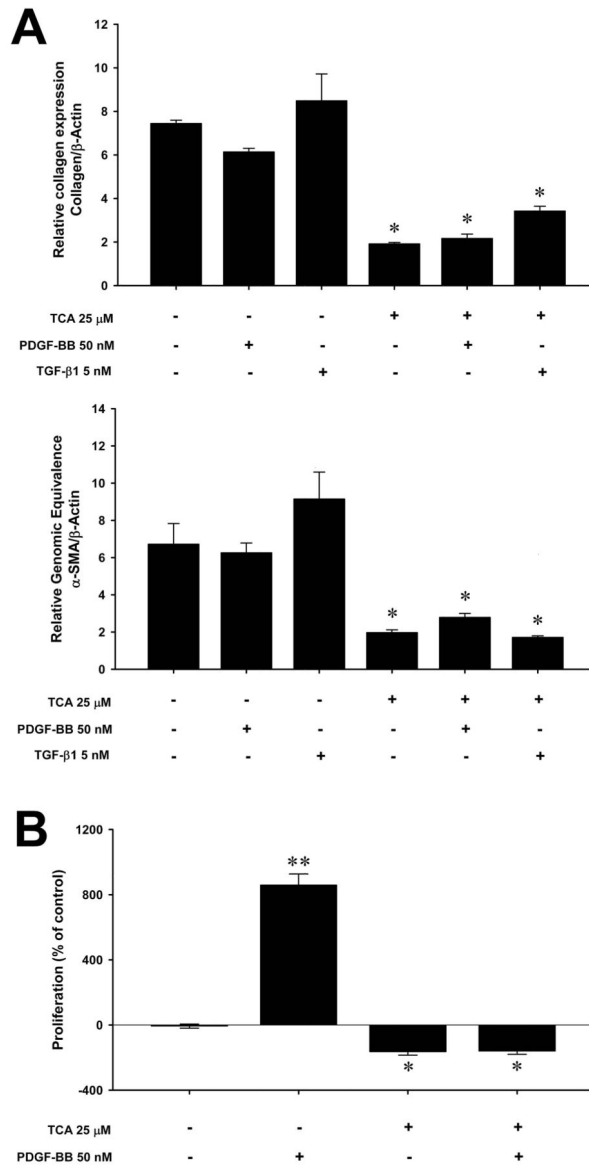


**Figure 4.** Delayed treatment with amitriptyline (TCA) reduces CCl<sub>4</sub>-induced hepatic fibrosis. Sirius red staining of liver sections reveals progressive bridging fibrosis in animals treated with CCl<sub>4</sub>. In contrast, animals treated with CCl<sub>4</sub> + TCA after establishment of fibrosis (TCA started after 5 weeks of CCl<sub>4</sub> treatment) had significantly reduced hepatic fibrosis and slowed progression. (original magnification 50X) Quantitation of hepatic fibrosis using pathological scoring. Decreased fibrosis in animals treated with CCl<sub>4</sub> and TCA in a delayed fashion vs. CCl<sub>4</sub> alone was confirmed via quantification of liver sections by a blinded hepatopathologist. n=4–6 per group. Data are expressed as mean and SEM. \*p<0.05 compared to all other groups, #p<0.05 compared to CCl<sub>4</sub> alone group.



**Figure 5.** Delayed treatment with amitriptyline reduces activated hepatic stellate cells and reduces portal hypertension after established hepatic fibrosis. (A) Representative samples of  $\alpha$ -SMA immunohistochemical staining (magnification 50X) reveals decreased activated HSC burden by week 15 in animals that treated with amitriptyline (TCA) in a delayed fashion (after 5 weeks of treatment with CCl<sub>4</sub>) vs. animals treated with CCl<sub>4</sub> alone. (B) Animals treated with CCl<sub>4</sub> alone had more significant splenomegaly, reflective of their progressive liver disease, relative to the animals treated with CCl<sub>4</sub> and TCA in a delayed fashion. n=4–6 per group. Data are expressed as mean and SEM. \*p<0.05 compared to CCl<sub>4</sub> alone group.





**Figure 6.** Amitriptyline (TCA) inhibits collagen expression and proliferation of activated hepatic stellate cells *in vitro*. (A) Treatment with 25 $\mu$ M TCA caused a marked reduction in collagen and alpha-smooth muscle actin ( $\alpha$ -SMA) expression by activated HSCs, which was maintained despite stimulation with 5nM of transforming growth factor beta type one (TGF- $\beta$ 1) or 50 nM platelet-derived growth factor-beta, beta (PDGF-BB). \* $p$ <0.05 compared to untreated, TGF- $\beta$ 1, PDGF-BB alone groups. (B) Treatment with 25 $\mu$ M TCA caused a marked reduction in proliferation by activated HSCs as measured by BrdU incorporation. Stimulation of HSCs with 50nM of PDGF-BB markedly increased proliferation. Concomitant treatment with TCA completely abrogated this effect. Data are expressed as mean and SEM. \* $p$ <0.05 compared to untreated PDGF-BB alone groups; \*\* $p$ <0.05 compared to all other groups.

**Table 1**

Primers used for RT-PCR.

Gene	Sequence	
Collagen 1 $\alpha$ 1	Sense	GAGCGGAGAGTACTGGATCG
	Antisense	GCTTCTTTTCCTTGGGGTTC
$\alpha$ -SMA	Sense	GTCCAGACATCAGGGAGTAA
	Antisense	TCGGATACTTCAGCGTCAGGA
TIMP-1	Sense	CCTTGCAAACCTGGAGAGTGACA
	Antisense	AAGCAAAGTGACGGCTCTGGT
TGF- $\beta$ 1	Sense	TTGCCCTCTACAACCACAA
	Antisense	GGCTTGCGACCCACGTAGTA
TNF $\alpha$	Sense	TCGTAGCAAACCACCAAGTG
	Antisense	AGATAGCAAATCGGCTGACG
GAPDH	Sense	TGTTGAAGTCACAGGAGACAACCT
	Antisense	AACCTGCCAAGTATGATGACATCA