Anti-fibrotic Effects of a Methylenedioxybenzene Compound, CW209292 on Dimethylnitrosamine-Induced Hepatic Fibrosis in Rats

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Received November 18, 2008; accepted May 7, 2009; published online May 13, 2009

A series of methylenedioxybenzene compounds were synthesized and found to have hepatoprotective effects in chemical-induced hepatotoxicity models. The purpose of the present study was to investigate the anti-fibrotic effects of a synthetic methylenedioxybenzene compound, CW209292, using the dimethylnitrosamine (DMN)-induced chronic liver injury model in rats. Liver injuries were induced in Sprague Dawley rats by injection of DMN (intraperitoneally, 10 μl/kg) 3 times per week for 4 weeks. The rats were treated with CW209292 (*per os*, 25 or 75 mg/kg/d) for 4 weeks. Treatment of rats with DMN for 4 weeks resulted in significant decreases in serum albumin levels, whereas concomitant treatment with CW209292 prevented these decreases. CW209292 treatment also shortened prothrombin time prolonged by DMN, providing evidence that the agent was active in preserving liver function against DMN insult. DMN treatment caused marked increases in plasma bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), and hyaluronic acid levels; CW209292 treatment reversed these increases. CW209292 also significantly reduced hepatic hydroxyproline content as well as hepatic fibrosis and inflammation in histological examination. Additionally, immunochemically detectable hepatic collagen type IV and α-smooth muscle actin levels were decreased by CW209292 treatment. Proliferation of hepatic stellate cells isolated from DMN-treated rats was inhibited by CW209292. Furthermore, tumor growth factor (TGF)-β1 mRNA expression was increased in DMN-treated rats, whereas CW209292 treatment prevented these increases. These results suggest that CW209292 exhibits anti-fibrotic effects in Sprague Dawley rats with DMN-induced hepatic fibrosis by blocking the mRNA expression of TGF-β1 and subsequent inhibition of the proliferation of hepatic stellate cells.

Key words dimethylnitrosamine; hepatic fibrosis; methylenedioxybenzene; CW209292; rat

Hepatitis, or inflammation of the liver, is induced by a variety of causative agents including viral infection, chronic alcohol consumption, constant cholestasis, nonalcoholic steatohepatitis and drug use. The net result of this process is liver damage and destruction, primarily through substantial hepatocellular necrosis. When the extent of the damage is low, the liver regenerates normal hepatic tissue. In contrast, when liver damage occurs repeatedly, the disease becomes chronic and is characterized by a different healing process with consequent accumulation of extracellular matrix proteins. The harmful outcome of chronic hepatitis is fibrosis, which can further develop into cirrhosis and ultimately to end-stage liver disease, liver failure, or hepatocellular carcinoma.[1—3)]

Activation of hepatic stellate cell is crucial for the development of hepatic fibrosis. Injury to hepatocytes results in the recruitment and stimulation of inflammatory cells, as well as the stimulation of resident inflammatory cells including Kupffer cells. Factors released by these inflammatory cells lead to transformation of hepatic stellate cell into a myofibroblast-like phenotype. Activation of hepatic stellate cells then leads to accumulation of fibrillar extracellular matrix.[4,5)]

No effective treatment for liver fibrosis is available, and extensive research is being conducted to elucidate the mechanisms underlying the development of disease- or toxicity-induced liver fibrosis and to develop anti-fibrotic therapeutics. Naturally occurring or synthetic methylenedioxybenzenes (MDBs) were reported to have antioxidant activity and were effective at preventing CCl_4-induced hepatocellular necrosis *in vivo*.[6,7)] We have synthesized a series of MDBs and evaluated their effects in the CCl_4-induced acute hepatic injury model in mice. Among the synthetic MDBs evaluated, oral treatment of CW209292 (Fig. 1) showed good efficacy in preventing the damage imposed in the chemical-induced hepatic injury models, rats and mice injected with CCl_4, rats treated with D-galactosamine, mice treated with lipopolysaccharide (LPS)/D-galactosamine or thioacetamide to induce hepatic toxicity. Depending on the model, the ED_{50} (mortality as endpoint in LPS/D-galactosamine model and alanine aminotransferase (ALT) as endpoint in other models) of CW209292 in those short-term hepatic toxicity models varied from 1.9—50 mg/kg in mice to 19.4—40.6 mg/kg in rats.

The dimethylnitrosamine (DMN)-induced liver fibrosis model can reproduce most of the features observed during human liver fibrosis.[8)] DMN is a potent hepatotoxin and exposures to repeated, lower doses of this chemical causes subacute and chronic liver injury with varying degree of hepatocyte necrosis, fibrosis and nodular degeneration.[9,10)] This model has benefit over other liver fibrosis model such as bile-duct ligation model, or CCl_4-induced model in terms of its progressive and its remarkable pathological alteration, and a high reproduction rate of fibrosis.[8,11)] In the present study, we investigated the anti-fibrotic effects of CW209292 compound using the DMN-induced chronic liver injury model in rats and also antiproliferative activity for isolated primary hepatic stellate cells.

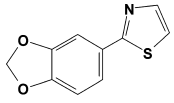

Fig. 1. Chemical Structure of CW209292 (2-Benzo[1,3]dioxol-5-yl-thiazole)

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MATERIALS AND METHODS

Reagents, Test Compound, and Animals DMN was purchased from TCI (Tokyo, Japan). All other chemicals used were analytical grade. CW209292 was synthesized by Choongwae Pharma Corp. (Hwaseong, Korea) and its purity was >99.0% by HPLC analysis. Specific pathogen free young male or female Sprague-Dawley rats were purchased from Charles River Laboratories Japan Inc. and acclimated for 1 week before use. The animals were maintained under 12 h light/12 h dark cycles in an air-conditioned animal room with commercial rat food (Purina, Korea) and water available *ad libitum*. Rats were housed in polypropylene cages with bedding. Animal studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication 86-23, National Academic Press, Washington, DC, U.S.A., 1996).

Induction of Hepatic Fibrosis Hepatic fibrosis was induced in 8 week old female rats by intraperitoneal injections of 1% DMN diluted in sterile saline, at a dose of 10 μl DMN/kg body weight. The injections were given 3 times per week on alternating days for a period of 4 weeks. Control non-treated animals received an equivalent volume of saline. Animals were sacrificed on day 28 of the experiment. All animals were anesthetized with CO_2 before sacrifice. Animals were opened along the abdominal midline and blood was obtained from the *vena cava* with a heparinized syringe. A portion of the blood was treated with sodium citrate for conducting anticoagulation tests. Livers were rapidly removed and rinsed in cold saline; right lobes were stored frozen for hydroxyproline determination and left lateral lobes were fixed in neutralized formalin for histology.[12—14)]

Test Compounds Treatment The test compounds, CW209292 and diphenyl dimethyl bicarboxylate (DDB), were administered orally once a day for 28 d. CW209292 and DDB were suspended in 0.5% carboxymethyl cellulose in saline solution. On DMN treatment days, the test compounds were administered immediately after DMN injection. The vehicle control group (DMN) received only 0.5% carboxymethyl cellulose solution without the test compound.

Blood Chemistry The collected blood was centrifuged at 3000 rpm for 10 min, and plasma was stored at −20 °C until analysis. Plasma levels of total bilirubin, albumin, aspartate aminotransferase (AST), ALT and total protein were analyzed using an automatic blood chemistry analyzer (Selectra II, Merck).

Prothrombin Time The blood was collected using sodium citrate as an anticoagulant. The collected blood was centrifuged at 3000 rpm for 10 min, and the plasma was stored frozen until analysis. The prothrombin time was determined using "Thromboplastin-XS with Calcium (Sigma Diagnostics)" with a coagulometer (Module S14NV4, H. Amelung).

Hyaluronic Acid Assay Plasma concentrations of hyaluronic acid were assayed by an hyaluronic acid assay kit (Chugai Diagnostics Science Ltd., Japan). The kit uses hyaluronic acid binding protein and is a type of sandwich binding protein assay. The hyaluronic acid assay was performed according to the manufacturer's protocol.

Hydroxyproline Determination Portions of the frozen right lobe of the liver were used. Liver tissue was homogenized with 10 volumes of dilution buffer. Hydrolysis was performed by adding 3 ml 10 N HCl to 2 ml liver homogenate in screw capped glass tubes followed by incubation at 110 °C for 16 h. After cooling, the hydrolysate was filtered through a 0.45 μm filter (Millipore). Each tube and filter was rinsed with 2 ml 6 N HCl, and the rinse solution was added to the first filtrate. The hydrolysates were stored at −20 °C until analysis. Hydroxyproline determination was performed as previously described with some modifications.[15,16)] Briefly, a 1 ml sample of the hydrolysate was added to a mixture of 1 ml of 6 N NaOH and 1 ml of citrate buffer. One milliliter 0.05 M chloramine-T in citrate–acetate buffer (pH 6.0—6.5) in the presence of *n*-propanol (1.32 M) was added to this mixture. The solution was mixed well and incubated for 20 min at room temperature. Incubations were stopped by adding 1 ml perchloric acid/*p*-dimethylaminobenzaldehyde solution in *n*-propanol (70% perchloric acid 32.5 ml, dimethylaminobenzaldehyde 18.75 g, *n*-propanol 75 ml). The color was developed by incubation for 20 min at 60 °C. After cooling, the absorbance was determined at 550 nm with a UV–visible spectrophotometer (CARY 3-E, Varian technology) within 2 h. Hydroxyproline content was calculated from standard curves using pure hydroxyproline as a standard.

Histology The left lateral liver lobes were sliced (3 slices per rat) and tissue slices were fixed by immersion in 10% buffered neutral formalin for 6 h. The fixed liver tissue slices were processed and embedded in paraffin according to routine procedures (Automatic Tissue Processor, Shandon, U.K.). Paraffin sections of 4 μm thickness were stained with hematoxylin and eosin. A modified van Gieson staining method was applied for the identification of collagen fibers.[17,18)] The degrees of fibrosis, inflammation and hemorrhage were graded semi-quantitatively as follows: 0=absent, 1=trace, 2=mild, 3=moderate, 4=severe.

Immunohistochemistry Paraffin tissue blocks were prepared as for light microscopy. The 4 μm thick paraffin sections were mounted on organosilane coated slides (DAKO, CA, U.S.A.) and deparaffinized. The antigenic determinants were enhanced by 0.4% pepsin (Sigma) solubilized in a 0.1 M HCl solution (37 °C, 30 min). The sections were exposed to a 0.3% solution of hydrogen peroxide in absolute methanol to inactivate the endogenous peroxidases for 30 min at room temperature. The rabbit polyclonal anti-human placental type IV collagen antibody (1 : 50 dilution, ICN Biomedicals, Inc., Ohio, U.S.A.) or rabbit polyclonal anti-human α-smooth muscle actin (sma) antibody (1 : 100 dilution, Abcam, Cambridge, U.K.) was added for 1 h at room temperature. The sections were successively treated with biotinylated anti-rabbit immunoglobulin (DAKO) for 30 min at room temperature, and peroxidase-conjugated streptavidin (DAKO) for 25 min at room temperature. The peroxidase reaction was performed using diaminobenzidine tetrahydrochloride (DAKO) as a substrate. Between each incubation, slides were thoroughly washed three times in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.09% Tween 20. Negative controls were processed similarly to ensure the accuracy of results, except that primary antiserum or secondary antibodies were omitted. The immunoreactivity of type IV collagen and alpha-SMA were scored semi-quantitatively as follows: 0=absent, 1=slight, 2=moderate, 3=distinct, 4=severe.

Hepatic Stellate Cell Isolation and Proliferation Assay

Hepatic stellate cells were isolated from male Sprague Dawley rats (300—400 g) as previously described with slight modifications.[19,20] DMN (1% in saline, 10 μl/kg) was given by intraperitoneal injections on Day 1 and Day 3. On Day 7, liver was perfused with Ca^{++} and Mg^{++} free Hanks Balanced Salt Solution for 5 min and then with solution containing 0.05% collagenase, 0.05% pronase and 0.001% DNase in Dulbecco's modified Eagle's medium (DMEM) for 30 min. The liver tissue was chopped with aseptic scissors in solution containing 0.05% collagenase, 0.05% pronase and 0.001% DNase in DMEM and kept 20 min in shaking incubator. After the digested livers were dispersed into DMEM, the resultant cell suspension was purified by a single-step density gradient using Nycodenz (final concentration=9.2%). After centrifugation, hepatic stellate cells were obtained from the upper, whitish layer, and resuspended in DMEM containing 10% fetal bovine serum (Gibco BRL, Grand Island, NY, U.S.A.). Cell viability was determined with Trypan blue staining. Cell proliferation was determined by [^{3}H]thymidine incorporation into DNA. Briefly, isolated hepatic stellate cells were added into 96-well tissue plates (5×10^3 cells/well/100 μl) and cultured in CO_2 incubator at 37 °C. After 1 d in culture, the cells were exposed to the test article or vehicle (0.1% dimethylsulfoxide) in DMEM medium for 48 h and were labeled with 1 μCi/well of methyl-^{3}[H]thymidine for the last 22 h of incubation. Radioactivity incorporated into trichloroacetic acid-insoluble material was recovered and measured by scintillation counter (Wallac, Finland).

Tumor Growth Factor (TGF)-β1 mRNA Contents in the Liver Six-week old Sprague Dawley rats received 100 mg/kg of CW209292 by oral gavage 2 h prior to the intraperitoneal injection of DMN (1% in saline, 10 μl/kg). Rats were sacrificed 24 h after DMN injection and total mRNA was prepared from the liver as previously reported.[21] Reverse transcription-polymerase chain reaction (RT-PCR) was carried out to investigate the TGF-β1 mRNA contents of the liver. cDNAs were amplified by PCR for 38 cycles. One round of amplification consisted of 0.5 min at 94 °C, 0.5 min at 49 °C, and 2 min at 72 °C. The PCR products were resolved and visualized by electrophoresis in a 1% gel, stained with ethidium bromide. The following TGF-β1 PCR primers were used, (sense primer: CTTCAGCTCCACAGAGAAGAACTGC, antisense primer: CACGATCATGTTGGACAACT GCTCC) and primer pairs for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were (sense: TCGTGGAGTCTACTGGCGT, antisense: GCCTGCTTCACCACCTTCT). The relative expression level of TGF-β1 against GADPH was determined using ImageJ software (Ver 1.42 g, NIH, U.S.A.).

P450 2E1 Assay Effect of CW209292 on P450 2E1 was investigated in liver microsome fraction of rat and human. To assay the activity of P450 2E1, chlorzoxazone 6-hydroxylase was measured after incubation of CW209292 with liver microsome. Briefly, liver microsome (20 μl) and CW209292 were mixed with chlorzoxazone (0.5 mM) in 50 mM potassium phosphate buffer (pH 7.4) and incubated for 3 min at 37 °C. Reaction was initiated by adding 0.2 mg/ml NADPH and incubated for 20 min at 37 °C. Activity of chlozoxazone 6-hydroxylase was assayed by a HPLC method described elsewhere,[22] using metabolite of chlorzoxazone, 6-hydroxychlorzoxazone (RBI, Notick, MA, U.S.A.) as the standard.

Statistical Analysis Data are expressed as means±S.D. One-way analysis of variance (ANOVA) was used for comparison of biochemical parameters. A non-parametric method (the Dunn procedure under the Kruskal–Wallis test) was used for multiple pairwise comparisons between groups for the histological scores. Statistical significance was set at $p<0.05$.

RESULTS

Survival and Body Weight During the 28-d monitoring period, the non-treated control group showed a gradual increase in body weight, whereas the DMN-treated group experienced body weight decreases of up to 15% (D25). Rats treated with CW209292 at 25 mg/kg or 75 mg/kg also showed body weight decreases, but maximal body weight losses were less than for DMN group, at 8% (D4) and 10% (D4) respectively, but it was not statistically different (Fig. 2A). And the final body weight was 90.7±13.8% in DMN group, 101.0±9.2% and 101.0±11.7% in 25 mg/kg and 75 mg/kg of CW209292 groups, respectively. DDB is a well known hepatopreotectant and reported to have anti-fibrotic effect.[23,24] So, we used DDB as a positive reference drug in this study. The body weight loss was generally more severe

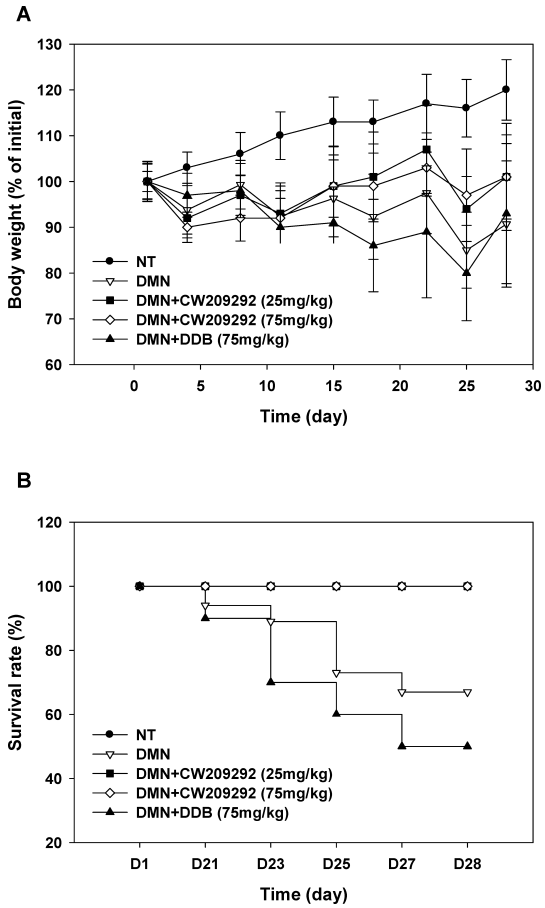

Fig. 2. Body Weight Changes (A) and Survival Rate (B) of Animals during the Experiment

Rats received intraperitoneal injections of DMN at a dose of 10 μl/kg on 3 d a week for 4 weeks to each group except control group (NT). CW209292 and DDB were administered once daily for 28 d by oral gavage. NT; non-treated control (n=10), DMN; DMN and vehicle-treated rats (n=18 at D1 and n=12 at D28), CW209292-25 mg/kg (n=10), CW209292-75 mg/kg (n=10), DDB (n=10 at D1 and n=5 at D28).

Table 1. Blood Chemistry Analysis (Mean±S.D.)

Group	ALT (unit/l)	AST (unit/l)	ALB (g/l)	Bili (unit/l)	PT (s)	HA (ng/ml)
NT	35±6	67±8	3.4±0.2	0.6±0.3	14.0±0.8	28±11
DMN	190±160	314±211	2.3±0.3	2.0±1.1	21.0±3.6	454±159
+CW209292-25 mg/kg	156±54	272±84	3.0±0.0**	0.9±0.3**	20.0±2.9	372±81
+CW209292-75 mg/kg	121±46	211±105	3.4±0.5**	0.7±0.3**	16.4±1.8*	159±117**
+DDB-75 mg/kg	124±53	284±175	2.7±0.8	1.5±1.7	20.3±10.0	345±149

Rats received intraperitoneal injections of DMN at a dose of 10 μl/kg on 3 d a week for 4 weeks to each group except control group (NT). CW209292 and DDB were treated once daily for 28 d by oral gavage. NT; non-treated control, DMN; DMN and vehicle-treated rats. ALT, alanine transaminase; AST, aspartate transaminase; ALB, albumin; Bili, bilirubin; PT, prothrombin time, HA, hyaluronic acid; NT, non-treated control, DMN, dimethylnitrosamine. *, ** Significantly different from DMN group at $p<0.05$ and $p<0.01$, respectively.

in the DDB-treated group than DMN group. In the DMN-treated group, the first animal death occurred on Day 21, and more deaths occurred thereafter. At the termination of the experiment, 33% (6/18) of the animals had died in the DMN-treated group compared to 50% (5/10) in the DDB-treated group. In contrast, no animal deaths occurred in the CW209292-treated groups (Fig. 2B).

Blood Chemistry, Prothrombin Time and HA Level DMN-treated rats developed hepatic injury as evidenced by significantly higher plasma concentrations of AST, ALT, and total bilirubin as compared with normal control rats. CW209292 treatment ameliorated the increase of AST and ALT levels and significantly decreased total bilirubin levels (Table 1). Prothrombin time was significantly prolonged in DMN rats as compared with normal rats, and this was improved by treatment with 75 mg/kg CW209292. Plasma albumin levels were significantly decreased in DMN rats, suggesting malfunction of hepatic protein synthesis. Albumin levels were significantly restored after treatment with CW209292 at both low and high doses. The decreases in the ALT and the bilirubin levels of DDB-treated rats were not statistically significant and there was no effect on AST, prothrombin time and the albumin levels in these animals (Table 1). Plasma levels of hyaluronic acid were markedly increased in DMN-treated rats (454±159 ng/ml) as compared with normal rats (28±11 ng/ml), and hyaluronic acid levels were significantly decreased by high dose treatment with CW209292 (159±117 ng/ml, $p<0.01$ *vs.* DMN). Although the DDB-treated group showed decrease in hyaluronic acid levels by 26% (345±149 ng/ml), but the decrease was not statistically significant (Table 1).

Liver Hydroxyproline Content Hydroxyproline, a product of collagen metabolism, was higher in DMN-treated rats (846±280 μg/g liver) than in normal, unmanipulated controls (305±39 μg/g liver), indicating that liver fibrosis had occurred. Treatment with CW209292 significantly decreased the levels of hydroxyproline at both the 25 mg/kg dose (624±155 μg/g, $p<0.05$) and the 75 mg/kg dose (488±64 μg/g, $p<0.001$) as compared with DMN group. DDB-treated group showed decrease in hydroxyproline (495±53 μg/g), but it was not significant (Fig. 3).

Histology and Immunohistochemistry Livers from DMN-treated rats revealed multifocal centrilobular necrosis of hepatocytes and focal degeneration of hepatocytes compared with livers from non-treated control rats. Distinctive increased fibrous septa with extensive accumulation of collagen were also noted in DMN rats. Treatment with 75 mg/kg of CW209292 significantly reduced the degree of hepatitis, hepatocellular necrosis, and fibrosis, as shown in Table 2.

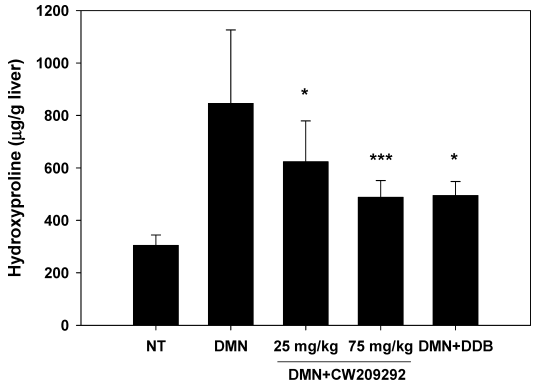

Fig. 3. Hydroxyproline Contents in Liver Tissues

Rats received intraperitoneal injections of DMN at a dose of 10 μl/kg on 3 d a week for 4 weeks to each group except control group (NT). CW209292 and DDB were administered once daily for 28 d by oral gavage. Data represents mean±S.D. of each group. NT; non-treated control ($n=10$), DMN; DMN and vehicle-treated rats ($n=12$), CW209292-25 mg/kg ($n=10$), CW209292-75 mg/kg ($n=10$), DDB ($n=5$). *, *** $p<0.05$ and $p<0.001$ *vs.* DMN group, respectively.

Table 2. Histopathological Evaluation of Liver Tissues (Mean±S.D.)

Group	Hepatitis	Hepatic fibrosis	Type IV collagen	Alpha-sma
NT	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
DMN	2.8±0.9	3.3±0.6	3.4±0.4	3.0±0.8
+CW209292-25 mg/kg	2.7±0.5	2.7±0.5	2.7±0.3	3.0±0.8
+CW209292-75 mg/kg	1.7±0.9*	1.6±0.7*	2.2±0.7*	1.6±0.9*
+DDB-75 mg/kg	2.0±0.0	2.4±1.1	2.9±0.7	2.6±0.5

Rats received intraperitoneal injections of DMN at a dose of 10 μl/kg on 3 d a week for 4 weeks to each group except control group (NT). CW209292 and DDB were treated once daily for 28 d by oral gavage. NT; non-treated control, DMN; DMN and vehicle-treated rats. * Significantly different from DMN group at $p<0.05$.

CW209292 given at 25 mg/kg and DDB given at 75 mg/kg slightly decreased the amounts of hepatitis and hepatic fibrosis but these differences were not significant (Table 2).

Immunohistochemical examination revealed that hepatic collagen type IV contents and α-sma positive cells were significantly increased in DMN-treated rats as compared with rats from the non-treated control group, demonstrating that DMN treatment increased the number of activated hepatic stellate cells and resultant collagen production and deposition. Hepatic type IV collagen contents and α-sma positive cells in DMN-treated rats were significantly reduced by 35% and 47%, respectively, with high-dose CW209292 (Table 2, Fig. 4).

Hepatic Stellate Cell Proliferation Hepatic stellate cells were isolated from livers of DMN-treated rats and it

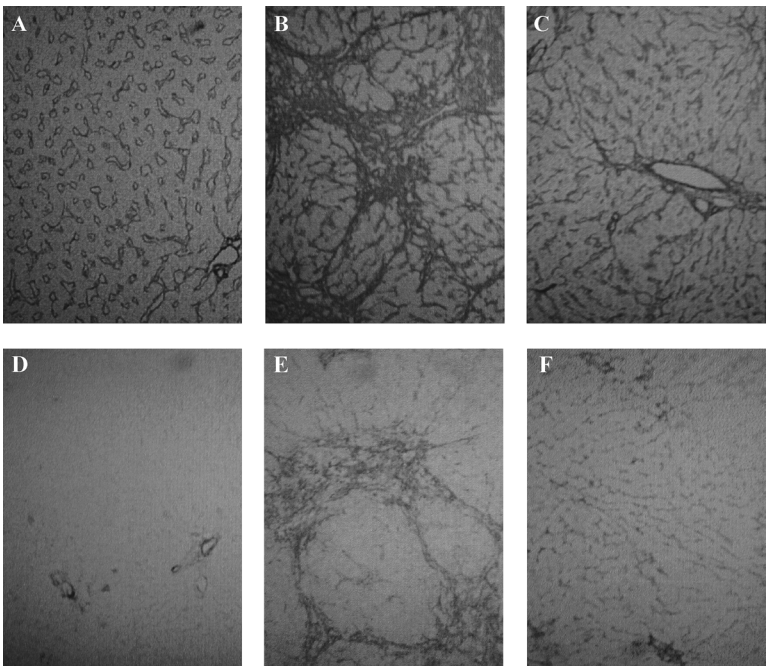

Fig. 4. Immunohistochemical Examination of Liver Sections

Representative photomicrographs of immunostaining for Type IV collagen and alpha-sma in livers. A—C; Type IV collagen, D—E; alpha-sma, A, D; non-treated control, B, E; DMN and vehicle-treated rats, C, F; DMN and CW209292, 75 mg/kg treated.

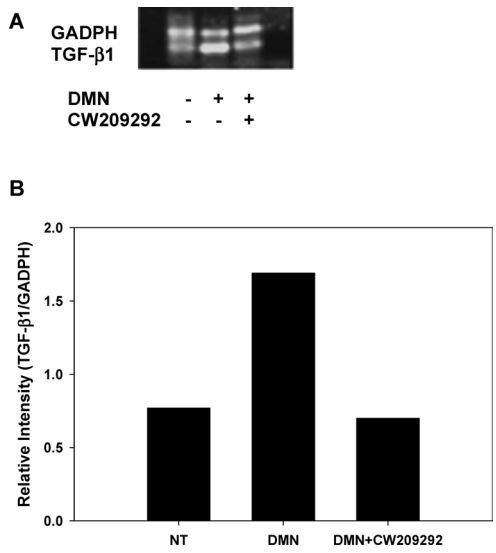

Fig. 5. Effects of CW209292 on TGF-β1 mRNA Expression Levels in the Liver

Rats received CW209292 (oral, 100 mg/kg) 2 h prior to the treatment of DMN (i.p., 10 μl/kg). Livers were sampled at 24 h after DMN treatment and TGF-β1 mRNA levels were determined by RT-PCR. Three rats were investigated for each condition and representative images from each group were shown. A; scanned image of the gel. B; quantified relative expression level of each condition in "A" using an image analysis software (ImageJ from NIH). NT, non-treated rat; DMN, DMN and vehicle-treated rat; CW209292, DMN and CW209292, 100 mg/kg-treated rat.

Table 3. Effect of CW209292 on Cytochrome P450 2E1 Activity

Microsome	Activity of chlorzoxazone 6-hydroxylase (% of control)				
	0 μg/ml	5 μg/ml	10 μg/ml	50 μg/ml	100 μg/ml
Rat	100.0±6.6	156.0±21.3	122.1±8.7	156.1±37.7	140.8±29.9
Human	100.0±21.5	177.0±55.0	127.2±1.4	104.7±3.7	106.3±8.0

Rat and human liver microsome was incubated with CW209292 and P450 2E1 activity was assayed 20 min after incubation by measuring metabolite of chlorzoxazone which is a substrate of chlorzoxazone 6-hydroxylase by HPLC method. The data represent the mean±S.E. for duplicate determinations.

was subjected to the experiment only when its viability was >85% after isolation. Hepatic stellate cell proliferation was inhibited after CW209292 treatment in a dose-dependent manner and with IC_{50} of 19.0 μM.

TGF-β1 mRNA Contents in the Liver TGF-β1 is a key cytokine that promotes hepatic stellate cell activation.[1,2] To further elucidate the action mechanism of CW209292, effect of CW209292 on mRNA expression level of TGF-β1 was determined with RT-PCR technique. Single injection of DMN markedly increased the mRNA expression levels of TGF-β1 in the liver when compared to those levels in naïve control. This enhanced expression of TGF-β1 induced by DMN was completely blocked by CW209292 treatment. Three rats were used for each condition (naïve, DMN, and DMN+CW209292) and the same tendency was observed cross the groups. Figure 5 shows representative images and the results of quantification using an image analysis software.

Effect of CW209292 on P450 2E1 Activity CW209292 did not significantly change the level of 6-hydroxychlorzoxazone up to 100 μg/ml in rat and human liver microsome, suggesting CW209292 do not affect the activity of P450 2E1 in these species (Table 3).

DISCUSSION

Liver fibrosis is the final pathological outcome for the majority of chronic liver insults, and progresses to liver cirrhosis and hepatic carcinoma if not reversed. Extensive accumulation of extracellular matrix during liver fibrosis reflects imbalances in the production and degradation of matrix proteins. Our current knowledge indicates that the central medi-

ator of liver fibrosis is the hepatic stellate cell population. During fibrotic injury, these retinoid-rich perisinusoidal cells proliferate and undergo a phenotypic transformation into myofibroblast-like cells, a process termed activation. Activated hepatic stellate cells proliferate and synthesize extracellular matrix, and TGF-β1 is the one of the strongest factors to induce the activation of hepatic stellate cells.[25] In the present study, we observed that a synthetic methylenedioxybenzene compound, CW209292 inhibited the proliferation of hepatic stellate cells isolated from rat liver as well as number of α-sma-positive cells and TGF-β1 mRNA transcript in the liver tissue *in vivo*.

Liver fibrosis in DMN model is associated with altered immune function, similar to the mechanism involved in human liver fibrosis, and iron deposition reflects pathological changes.[26] Thus, the DMN-induced liver fibrosis model is an excellent choice for screening drug candidates for the treatment of hepatic fibrosis in humans. Additionally, the importance of hepatic stellate cells in the pathogenesis of DMN-induced liver fibrosis has been well documented.[14,27,28]

To determine the appropriate dose for the current study, we performed preliminary study to evaluate the safety of CW209292 in rats. The LD_{50} of CW209292 by single oral administration was 1031 mg/kg and 1122 mg/kg in naïve male and female rats, respectively. In a 14-d consecutive oral dosing study (once daily), 250 mg/kg of CW209292 was tolerable with no mortality or any adverse effects. Based on the efficacious doses in short-term hepatotoxicity models in mouse and rat and this preliminary toxicity study, 25 mg/kg and 75 mg/kg were selected for the current study.

In the current study, liver fibrosis was successfully induced, as shown by biochemical and histological changes, in response to repeated intraperitoneal injections of DMN. As shown in Fig. 3, DMN treatment resulted in animal deaths from D21 onward. These deaths were attributed to impairment of liver function by DMN intoxication; upon gross observation during autopsy, granularity of the liver surface and abundant ascites were detected. In contrast, no deaths occurred in CW209292-treated animals, suggesting alleviation of DMN intoxication by this compound.

Collagen is an important component of fibrotic liver tissue. In liver fibrosis, the total liver collagen content increases up to 6—8 fold, and all collagen types are affected.[29] In the DMN model, an early increase in mRNA for type IV collagen has been reported, suggesting enhanced production of basement membrane collagen.[30] In the present study, immunoreactivity to type IV collagen antibody of DMN rats was markedly increased compared with naïve control animals. However, its expression was substantially reduced in CW209292-treated animals. Alpha-sma is typically used as a marker of hepatic stellate cell activation.[31] Alpha-sma expression increased remarkably in the rat livers injured by DMN administration, and decreased in response to CW209292 administration. Hydroxyproline is a major constituent of collagen, and in our study we also assessed the activation of hepatic stellate cell indirectly through determination of the hydroxyproline content. Compared with the non-treated control group, the hydroxyproline content in the DMN group increased markedly. In response to CW209292 treatment, hydroxyproline content was diminished relative to that seen in the DMN group. Rats in the DMN group displayed increased ALT and AST activities, indicative of hepatic injury, and these were reduced by treatment with CW209292. Plasma albumin level, which is indicative of overall liver biosynthetic function, was decreased in DMN rats compared with naïve rats and significantly restored by CW209292 treatment. Total plasma bilirubin levels were markedly increased in the DMN group, but were close to normal in the test compound treated groups. Hyaluronic acid is an acidic glycosaminoglycan with high molecular weight that is widely distributed in connective tissues. In the liver, hyaluronic acid is synthesized by hepatic stellate cells; it accumulates in fibrotic livers and serum hyaluronic acid levels elevate during various liver diseases.[32,33] In the DMN group, hyaluronic acid levels were significantly increased (16 fold) compared with naïve rats, but were reduced in CW209292-treated rats, demonstrating the anti-fibrotic activity of the test compound.

TGF-β1 is one of the best-characterized cytokine in the pathogenesis of liver fibrosis. A large number of studies identified TGF-β1 as an important cytokine in promoting hepatic stellate cell activation and upregulation of extracellular matrix expression in these activated hepatic stellate cells. It has widely been shown that persistent stimulation of activated hepatic stellate cells by TGF-β1 is the key profibrogenic response in liver fibrosis.[1,2,25,34] In this study, we demonstrated that CW209292 treatment blocked TGF-β1 mRNA expression induced by DMN injection in the liver. Taken together, these data suggest that CW209292 inhibits proliferation of hepatic stellate cells by blocking TGF-β1 expression which leads to the prevention of liver fibrosis induced by multiple DMN treatments.

It has been shown that the DMN-induced toxicity is mediated by its reactive metabolites produced mainly by P450 2E1.[35,36] Methylenedioxybenzene compounds have also been reported to modulate P450 activity, both inhibit or induce in a compound specific manner.[37,38] To elucidate that the mechanism underlying the suppression of fibrosis by CW209292 reported in this study was not due to possible modulation of P450 2E1 activity, we assayed activity of CW209292 on P450 2E1. The hydroxylation of chlorzoxazone, a substrate considered to be selective for P450 2E1, were not altered by CW209292 up to 100 μg/ml relatively high concentration of the compound compared to the effective concentration for with its *in vitro* activity. Furthermore, maximum concentration of CW209292 in the plasma after oral administration (75 mg/kg) in rat was 10.82 ± 3.21 μg/ml ($n=3$, data not shown), demonstrating possibility of inhibition of P450 2E1 activity *in vivo* by CW209292 is low. Thus, the antifibrotic effect of CW209292 may not be ascribed to the inhibition of DMN activation by P450 2E1.

DDB is a hepatoprotectant used in the treatment of chronic viral hepatitis patients and various liver disease patients in some countries. DDB is also known to have hepatoprotective and anti-fibrotic effects in animal models.[23,24] We used DDB (75 mg/kg, daily oral) as a reference drug in this study, but it did not show significant anti-fibrotic efficacy in this study.

In conclusion, our results suggest that the novel synthetic methylenedioxybenzene compound, CW209292, exerted anti-fibrotic effects in DMN-induced fibrotic rats by inhibiting proliferation of hepatic stellate cells, perhaps through blocking the expression of TGF-β1. The more intensive

study on the mechanism of the anti-fibrotic effects of CW209292, as well as its efficacy in other liver fibrosis models and safety profiles, should be explored so that this compound can be developed as a preventive or fibrosis-attenuating agent for hepatic fibrosis.

Acknowledgement The authors thank Dr. Katayoon H. Emami for kind review of the manuscript.

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