

The urotension II antagonist SB-710411 arrests fibrosis in CCL₄ cirrhotic rats

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Abstract. Urotensin II (UII) is a relatively novel peptide that functions as a potent vasoactive mediator throughout the human body, and also possesses mitogenic and fibrogenic potential. Recent reports showed increased plasma levels of UII in human cirrhotic populations; these levels were correlated with the severity of the disease. We therefore hypothesized that the blockade of UII signaling would arrest the progression of hepatic fibrosis in a rat model of cirrhosis. Cirrhosis was induced in rats by carbon tetrachloride. SB-710411 was used as the UII antagonist. Treatment lasted 8 weeks. Plasma hyaluronic acid (HA) and laminin (LN) were evaluated by radioimmunoassay, and plasma UII was determined by ELISA for the quantity of hydroxyproline (Hyp) in the liver tissues. Fibrosis was assessed histologically. The activated hepatic stellate cells (HSCs) were assessed by α -smooth muscle actin immunostaining. The relative mRNA expression of UII/G protein-coupled receptor (UT), collagen I, collagen III, transforming growth factor β 1 (TGF- β 1) and tissue inhibitor of metalloproteinase-1 (TIMP-1) in the liver was determined by real-time reverse transcriptase-polymerase chain reaction. Western blot analysis was used to assess liver levels of UT. The mitogenic activity of UII on HSC-T6 cells was also evaluated. Animals with cirrhosis showed increased plasma UII. UII/UT mRNA expression was up-regulated in the liver. Plasma levels of UII were also positively correlated with HA, LN and Hyp. *In vivo*, treatment with SB-710411 significantly reduced fibrosis development and down-regulated the profibrogenic cytokines TGF- β 1 and TIMP-1. *In vitro*, UII induced the proliferation of HSCs. This mitogenic effect was significantly inhibited by 10^{-3} M SB-710411 ($p < 0.05$). These data suggest that the selective blockade of UT has an arresting effect on fibrosis progression *in vivo*, and inhibits UII-mediated HSC proliferation *in vitro*.

Introduction

Portal hypertension occurs in large part through changes in hepatic resistance. In cirrhosis, increased intrahepatic resistance results from both vascular factors and fibrosis (1). Liver fibrosis is characterized by the deposition of extracellular matrix (ECM), mainly fibrillar collagen, and the substitution of normal hepatocytes. The progression to fibrosis induces hepatocellular dysfunction and portal hypertension due to increasing intrahepatic vascular resistance (2-5).

Several vasoactive mediators, such as nitric oxide and endothelin, have been shown to be involved in increased vascular tone in cirrhosis (1). Urotensin II (UII) is also considered a potential mediator of intrahepatic portal hypertension, as plasma levels of UII were found to be increased in patients with cirrhosis and its administration induced the elevation of portal pressure (6,7). UII is a vasoactive cyclic neuropeptide that activates the G protein-coupled receptor (UT) and exerts various cardiovascular effects. UII may influence different pathways depending on the cells and the vascular compartment where the receptor is located (8). Both UII and its receptor are expressed on the endothelial cells of the arteries, veins and bile ducts, as well as on sinusoidal lining cells in the liver (9,10). UT mRNA expression is up-regulated in cirrhotic rats (6). Recently, several physiological roles for UII have been identified, including a role in the pathogenesis and maintenance of high blood pressure, and the stimulation of fibroblast proliferation and collagen synthesis (11). Despite major advances in the treatment of liver cirrhosis, the disease remains a severe public health problem. Therefore, the identification of novel therapeutic targets for liver cirrhosis treatment is critical to the development of more beneficial treatment strategies.

UII receptor antagonist can reduce portal pressure in cirrhotic rats and improve renal function in diabetic rodents and patients (7,12). However, its possible therapeutic potential in the control of fibrosis progression has not been examined. In the current study, the protective effects of a UT antagonist on liver fibrosis in rats were investigated.

Materials and methods

Animals and experimental design. Thirty male Sprague Dawley rats, 11-12 weeks of age and weighing 250-300 g, were used in the experiment. Cirrhosis was induced by the intragastric administration of 50% carbon tetrachloride (CCL₄) dissolved

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in corn oil for 3 days/week according to a previously described method (13). Animals were obtained from the Animal Center (Beijing, P.R. China). Animal care and experimental protocols were in compliance with the Animal Management Rules of China (Documentation no. 55, 2001, Ministry of Health) and the Guide for Care and Use of Laboratory Animals (Capital Medical University Friendship Hospital, P.R. China).

Experimental groups. The rats (n=30) were randomly divided into three experimental groups. The first group was untreated (n=8, normal control group); the second was administered only CCL₄ (Beijing Chemical Factory, P.R. China) (n=11, cirrhosis group); the third was simultaneously administered CCL₄ and 1 µg/kg of body weight/day of the UII receptor antagonist Cpa-D-Cys-Pal-D-Trp-Lys-Val-Cys-Cpa-NH₂ (SB-710411; Phoenix Pharmaceutical Inc., CA, USA) by hypodermic injection (n=11, SB-treated group) (14–16). At the end of the experimental period, the animals were sacrificed under ether anesthesia and terminally bled via cardiac puncture. The liver of each animal was removed. Blood was sampled via cardiac puncture and centrifuged, and the serum was stored at -20°C until use. Liver specimens were immediately snap-frozen and stored in liquid nitrogen for the determination of hydroxyproline (Hyp) and the extraction of total RNA. The remaining tissues were fixed in 10% formalin and embedded in paraffin for histological examination.

Histological analysis. Formalin-fixed sections (5 µm) of the right and left liver lobes were stained with hematoxylin and eosin (H&E), Gordon-Sweet reticulin and Masson trichrome. Fibrosis was assessed histologically as follows: stage 0, no fibrosis; stage 1, short fibrous tissue in central venule (C); stage 2, fibrous C-C septa appearance; stage 3, C-C fibrous septa developed incompletely; stage 4, C-C septa completely connected (pseudo-lobule); stage 5, C-P (Portal Tract) bridging fibrosis, nodular appearance ≤50%; stage 6, nodular appearance >50% (17). Immunohistochemical staining of α-smooth muscle actin (α-SMA) was performed by the avidin-biotin-peroxidase complex method. Semiquantitative analysis of immunopositive cells was carried out with a Olympus DP71 color digital camera (Olympus, Tokyo, Japan) and the Image-pro plus image analyzing system (Medialy Bernetics Inc., USA) in ten intralobular ocular fields (magnification x400) per specimen, excluding the α-SMA-positive cells in the portal area.

Measurement of hepatic hydroxyproline content. Hepatic tissue (200 mg wet weight) was hydrolyzed using a hydroxyproline kit (sample alkaline hydrolysis method) according to the manufacturer's guidelines (Jian Cheng Bioengineering Institute, Nanjing, P.R. China). Absorbance was measured by spectrometry at a wavelength of 550 nm, and the quantitation of Hyp levels was calculated against the standard curve ($Y = A + BX$, $A=0.034$, $B=0.081$, $R=0.996$) of 4-hydroxy-1-proline after the color development reaction. Hydroxyproline levels were expressed as µg of Hyp/g of liver.

Urotensin II, hyaluronic acid and laminin plasma levels. UII plasma levels were determined using an enzyme immunoassay kit (Phoenix Biotech Co., Ltd., Beijing, P.R. China) based on the principle of a 'competitive' enzyme immunoassay, according to the manufacturer's guidelines (18). Serum hyaluronic acid

(HA) and laminin (LN) were calculated with a clinical radio-immunoanalyzer (Gamby10cr DPC, USA) according to the manufacturer's protocol (Haiyan, Shanghai, P.R. China).

RNA extraction and complementary DNA synthesis. Snap-frozen liver biopsy tissues stored at -80°C were homogenized, and total RNA was extracted using the Trizol Reagent kit (Invitrogen Life Technologies). The amount of RNA recovered was quantitated, and its quality was verified by ultraviolet absorbance spectrophotometric examination at 260 and 280 nm. Total RNA (4 µg) was mixed with 2.5 µl (5.125 µM) of random hexamer primers (Takara Biotechnology, Japan) in 9 µl of water and incubated for 5 min at 70°C. After cooling on ice for 2 min, 10 µl M-MLV 5X reaction buffer, 10 µl of 10 nM of dNT, 2 µl of 40 IU/µl recombinant RNasin Ribonuclease inhibitor and 2 µl of 200 IU/µl of M-MLV RT (Promega Corp., WI, USA) were consecutively added. Nuclease-free water was added to a final volume of 50 µl. Reactions were conducted at 42°C for 60 min and 95°C for 5 min, and terminated at 4°C for 5 min before chilling on ice. The cDNA were stored at -20°C for future use. Parallel reactions for each RNA sample were run in the absence of M-MLV RT (genomic control) to assess the degree of any contaminating genomic DNA.

Real-time polymerase chain reaction. Gene expression of UII, UT, collagen I, collagen III, TIMP-1 and TGF-β1 was quantified by means of real-time PCR. The sequences of these primers are shown in Table I (4). GAPDH, a housekeeping gene, was used as an internal control. Primers for target genes and GAPDH were obtained from SBS Genentech (Beijing, P.R. China). The expression of candidate gene mRNA was measured by SYBR Green real-time PCR using an ABI 7300 instrument (Applied Biosystems, Forster, CA, USA) according to the manufacturer's instructions. PCR was performed in 20 µl buffer containing 1 µl cDNA, 1 µl of each primer and power SYBR Green PCR master mix 10 µl (Applied Biosystems). Comparative cycle threshold (Ct) calculations were all relative to the control group. GAPDH Ct values were subtracted from gene Ct values to yield a Ct value. $\Delta\Delta Ct$ values were achieved by subtracting the average control ΔCt value, and the expression of UII, UT, collagen I, collagen III, TIMP-1 and TGF-β1 relative to the control was derived using the equation $2^{-\Delta\Delta Ct}$ (19). The derived normalized values were the average of three runs.

Western blot analysis for urotensin. Protein was extracted (Protein Extractor IV; DBI, P.R. China) from liver samples, homogenized and assayed using the bicinchoninic acid method (Pierce BCA Protein Assay kit; Thermo Fisher Scientific Inc., IL, USA). The 100-µg protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (80 V for 15 min on a 5% acrylamide stacking gel and 120 V for 1 h on a 12% running gel), and then transferred (390 MA, 45 min) to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were soaked in Tris-buffered saline (10 mmol/l Tris-HCl and 250 mol/l NaCl) containing 5% nonfat powdered milk and 0.1% Tween-20 for 2 h to block non-specific sites, then incubated with primary antibody overnight at 4°C in blocking solution. The resulting blots were washed and incubated with secondary

Table I. Primer sequences used to detect collagen I, collagen III, TGF- β 1, TIMP-1 and UII/UT.

Gene	Primer sequence	Product size (bp)	Accession no.
GADPH	F: CCT GCC AAG TAT GAT GAC ATC AAG A R: GTA GCC CAG GAT GCC CTT TAG T	75	BC_059110
UII	F: CAG AAG CAG AGG GAA GCC TA R: CAA GCT TCC CCT TGG AGT G	68	NM_019160
UT	F: CAT TGG GCT GCT CTA TGT CC R: AAA GAA GCT TGC TGA GAT AGC C	60	NM_020537
Collagen I	F: CAT GTT CAG CTT TGT GGA CCT R: GCA GCT GAC TTC AGG GGA TGT	94	NM_053304
Collagen III	F: GGG ATC CAA TGA GGG AGA AT R: GCT CCA TTC ACC AGT GTG TTT	97	NM_032085
TGF- β 1	F: AGA AGT CAC CCG CGT GCT AA R: TCC CGA ATG TCT GAC GTA TTG	107	NM_021578
TIMP-1	F: TCC TCT TGT TGC TAT CAT TGA TAG CTT R: CGC TGG TAT AAG GTG GTC TCG AT	148	NM_053819

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TGF- β 1, transforming growth factor- β 1; TIMP-1, tissue inhibitor of metalloproteinase-1; UII, urotensin II; UT, urotensin II receptor or GPR14.

Table II. Effect of SB-710411 on the fibrosis change score of CCL₄-induced cirrhosis.

Treatment	Fibrosis score						
	0	1	2	3	4	5	6
Control rats (no.)	8	0	0	0	0	0	0
CCL ₄ ^a -treated rats (no.)	0	0	0	0	2	4	2
SB-treated ^b rats (no.)	0	0	3	3	2	0	0

Rats with stages 0, I, II, III, IV, V and VI received a point score of 0, 1, 2, 3, 4, 5 or 6. Average scores were calculated by multiplying the number of rats by their individual point score and dividing the sum score by the number of animals in each treatment group (n=8/group; ^ap<0.01 compared to the normal control group; ^bp<0.01 compared to the normal control and cirrhosis groups).

antibody for 2 h at room temperature. Immunoreactivity was visualized using an enhanced chemiluminescence kit (Thermo). Films were scanned using a Bio-Rad imaging system (Bio-Rad Laboratories Ltd., CA, USA). Antibody dilutions were as follows: goat polyclonal anti-UT antibody (1:200; Santa Cruz Co., CA, USA) and HRP-linked sheep anti-goat IgG (1:2000; Immunology Consultants Laboratory Inc., OR, USA).

Rat liver hepatic stellate cell culture and proliferation assay. The effect of SB-710411 on UII-stimulated rat liver hepatic stellate cell (HSC) proliferation was investigated in rat HSC-T6 donated by Professor Friedman (Mount Sinai Medical Center). Initially, the T6 cells were plated at an equal density of 5×10^3 cells/well in a 96-well plate in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine serum medium (50 U/ml penicillin and 50 mg/ml streptomycin) for 48 h (37.0°C, 5% CO₂), followed by serum starvation in DMEM/0.01% bovine serum albumin (BSA) (with 50 U/ml penicillin and 50 mg/ml streptomycin) for 48 h. Subsequently, cells were

incubated with DMEM/10% serum media, DMEM/0.01% BSA media or DMEM/0.01% BSA media with 10^{-10} to 10^{-4} M rat UII (Phoenix Biotech), or with DMEM/0.01% BSA media with 10^{-4} M UII + 10^{-6} to 10^{-3} M SB-710411 for 48 h. The number of live cells was measured using the thiazolyl blue (MTT) cell proliferation assay (Sigma) by reading the absorbance at 450 nm according to the manufacturer's instructions.

Statistical analysis. Results are the means \pm SD. Comparisons were made using the unpaired Student's t-test and one-way ANOVA, following by the Student Newman-Keuls test. Linear regression analysis was performed by the method of least squares, with the aid of the statistical program SPSS 17.0. A p-value <0.05 was considered statistically significant.

Results

Pathological examination. Three rats from the cirrhosis and SB-treated groups died during the experiment. No deaths

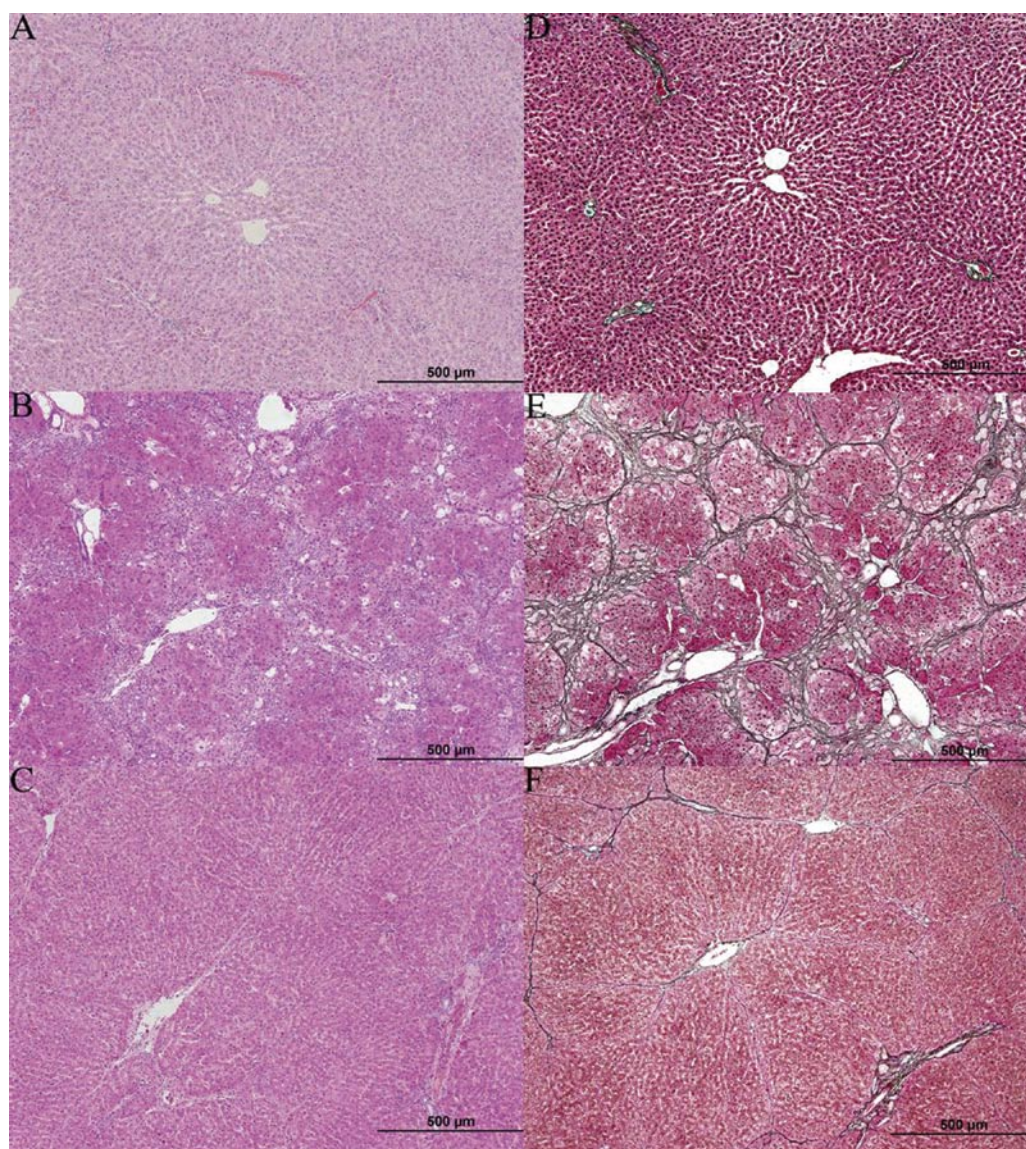


Figure 1. Histologic findings: comparison of liver architecture of normal control, cirrhosis and SB-710411-treated rats. (A) Normal control rats (H&E); (B) cirrhotic rats (H&E); (C) SB-710411-treated rats (H&E); (D) normal rats (Gordon-Sweet reticulum and Masson trichrome); (E) cirrhotic rats (Gordon-Sweet reticulum and Masson trichrome); (F) SB-710411-treated rats (Gordon-Sweet reticulum and Masson-trichrome) (original magnification x50). (E) In the cirrhosis group, extensive portal-portal and portal-central fibrous linkage, distortion of liver architecture and marked regeneration nodules were observed. (F) SB-710411 treatment markedly reduced liver fibrosis as compared with the cirrhosis group.

occurred among the control rats. Due to the small number of animals in this study, the mortality rate was not analyzed. Histopathologic findings of rat liver tissues are shown in Fig. 1 and Table II. The liver of CCL₄-injured rats showed massive fatty changes, gross necrosis and broad infiltration of lymphocytes (Fig. 1B). The severity of hepatic fibrosis was more pronounced, with thick and often completed fibrotic centro-central septa (Fig. 1F). SB treatment significantly reduced the mean score of fibrosis as compared with the cirrhosis group (Fig. 1G, Table II). To clarify the mechanism by which SB-710411 attenuates the development of liver fibrosis induced by CCL₄, we examined the α -SMA-positive cells. CCL₄ injections over eight weeks led to a progressive increase in the number of α -SMA-positive cells in the pericentral zone and fibrotic septa, infiltrating the lobules from the edges of the septa and at the junction between the thick fibrotic band and the parenchyma (Fig. 2B). Morphometric analysis (Fig. 2D)

confirmed that SB-710411 treatment significantly reduced the area occupied by α -SMA-positive cells as compared with the CCL₄-induced cirrhotic rats.

Urotensin II, hyaluronic acid and laminin plasma levels. UII levels were significantly higher in the plasma of cirrhotic rats than in that of normal control rats. Treatment with SB-710411 induced a significant decrease in plasma UII levels (Fig. 3A). The level of serum fibrosis indexes, such as HA and LN, increased more significantly in cirrhotic rats than in control rats (Fig. 4A and B). In SB-710411-treated rats, the levels of plasma HA and LN were decreased (Fig. 4A and B).

Changes in hepatic hydroxyproline concentration. Changes in hepatic collagen were measured by means of Hyp quantification. The cirrhosis group had an increased liver Hyp concentration compared with the normal control group ($p < 0.01$), while the

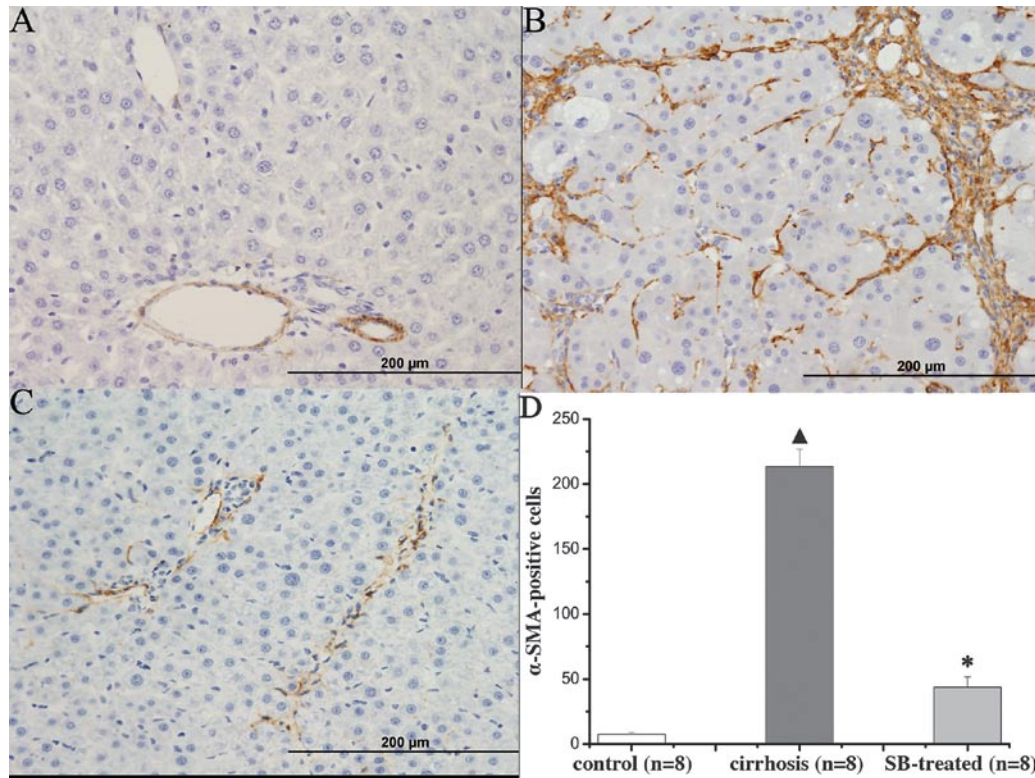


Figure 2. Effects of SB-710411 treatment on the distribution and quantification of α -smooth muscle actin (α -SMA)-positive hepatic cells during the progression of carbon tetrachloride (CCL_4). Hepatic fibrosis was induced (original magnification x400). Representative photomicrographs of α -SMA-immunostained liver sections from (A) normal, (B) cirrhotic and (C) SB-treated rats. (D) Quantification of the number of α -SMA-positive cells in normal (n=8), cirrhotic (n=8) and SB-710411-treated (n=8) rats (* $p < 0.01$ compared with the 8-week-treated CCL_4 livers).

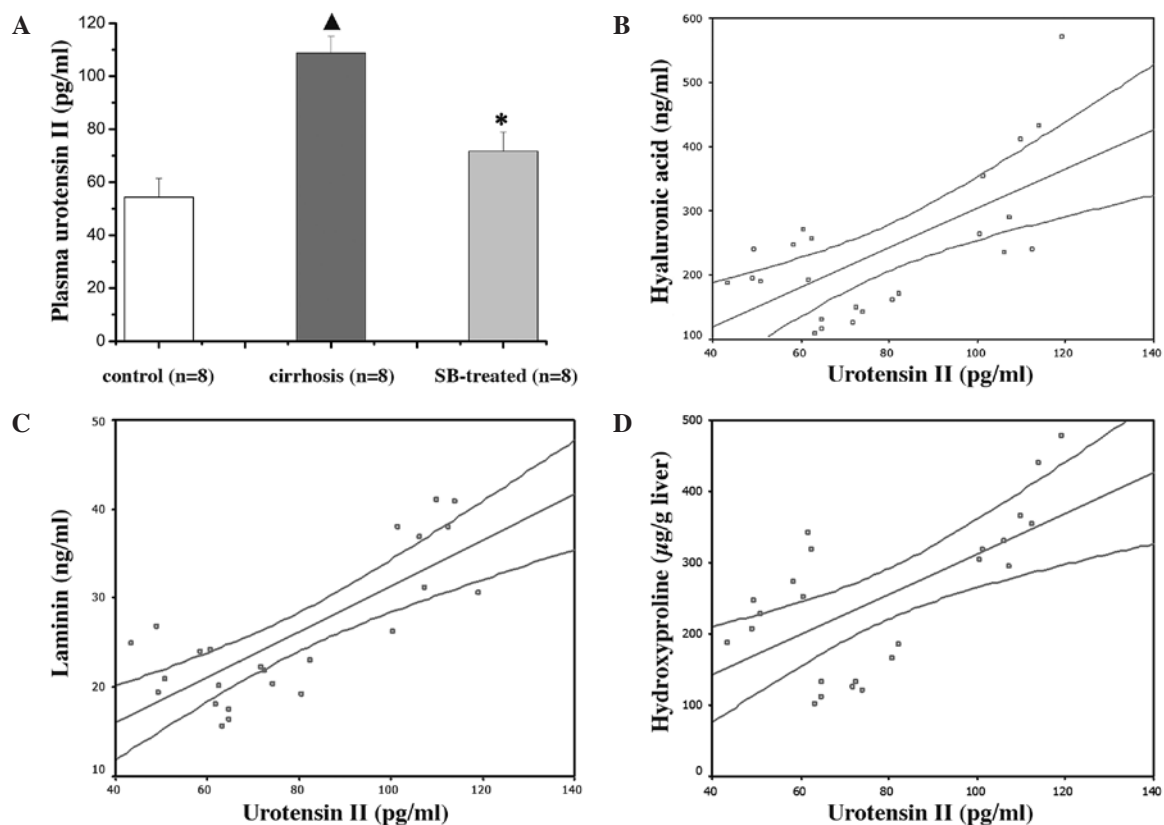


Figure 3. Correlation between plasma urotensin II (UII) levels and plasma hyaluronic acid (HA) and laminin (LN) levels. (A) Plasma UII levels were determined using an enzyme immunoassay (normal, n=8; cirrhotic, n=8; SB-treated, n=8) (* $p < 0.05$ compared to the normal control and cirrhosis groups; * $p < 0.05$ compared to the normal control group). Plasma UII levels were correlated with (B) plasma HA, (C) LN and (D) hydroxyproline. (B) $R = 0.660$, $p < 0.001$; (C) $R = 0.775$, $p < 0.001$; (D) $R = 0.471$, $p < 0.05$. R, linear regression correlation; p, pearson correlation coefficient. Values are the means \pm SD.

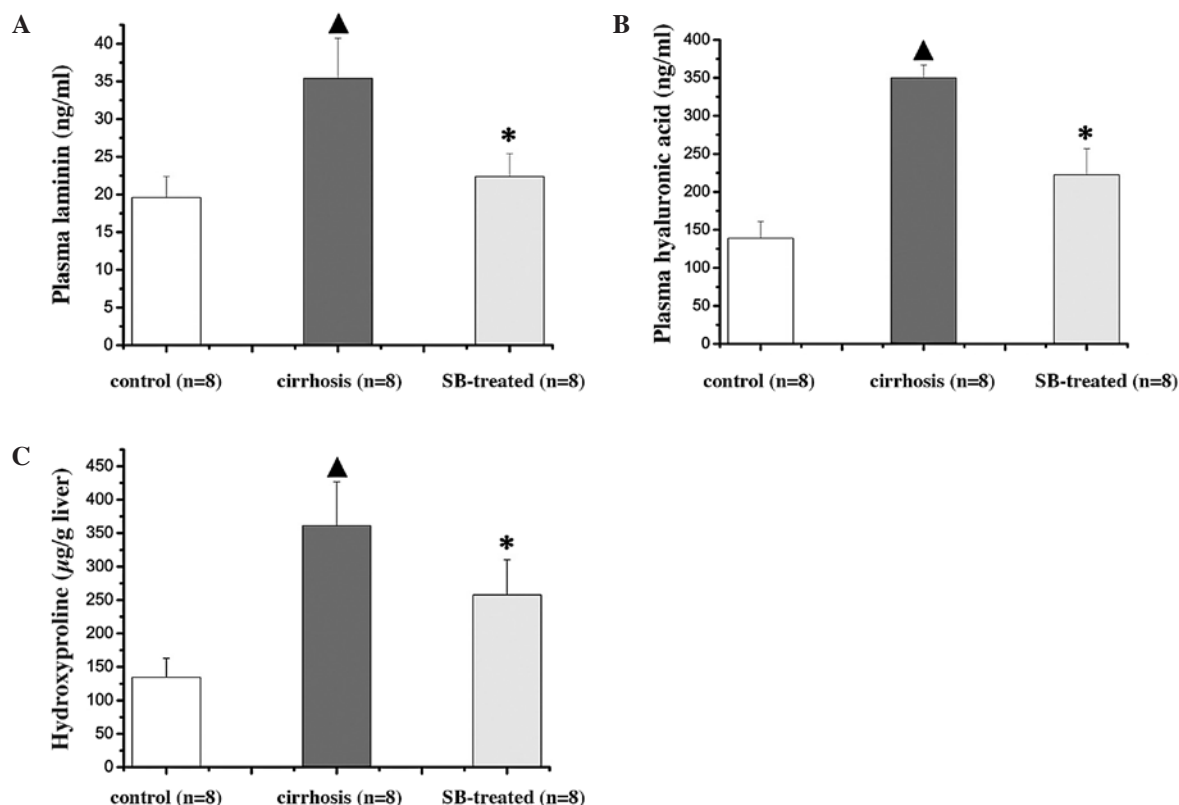


Figure 4. (A) Plasma hyaluronic acid (HA) and (B) plasma laminin (LN) levels were calculated using a clinical radioimmunoanalyzer. (C) Hepatic hydroxyproline concentrations in the three experimental groups were analyzed by the sample alkaline hydrolysis method (normal, n=8; cirrhotic, n=8; SB-treated, n=8) (* $p < 0.05$ compared to the normal control and cirrhosis groups; [▲] $p < 0.05$ compared to the normal control group).

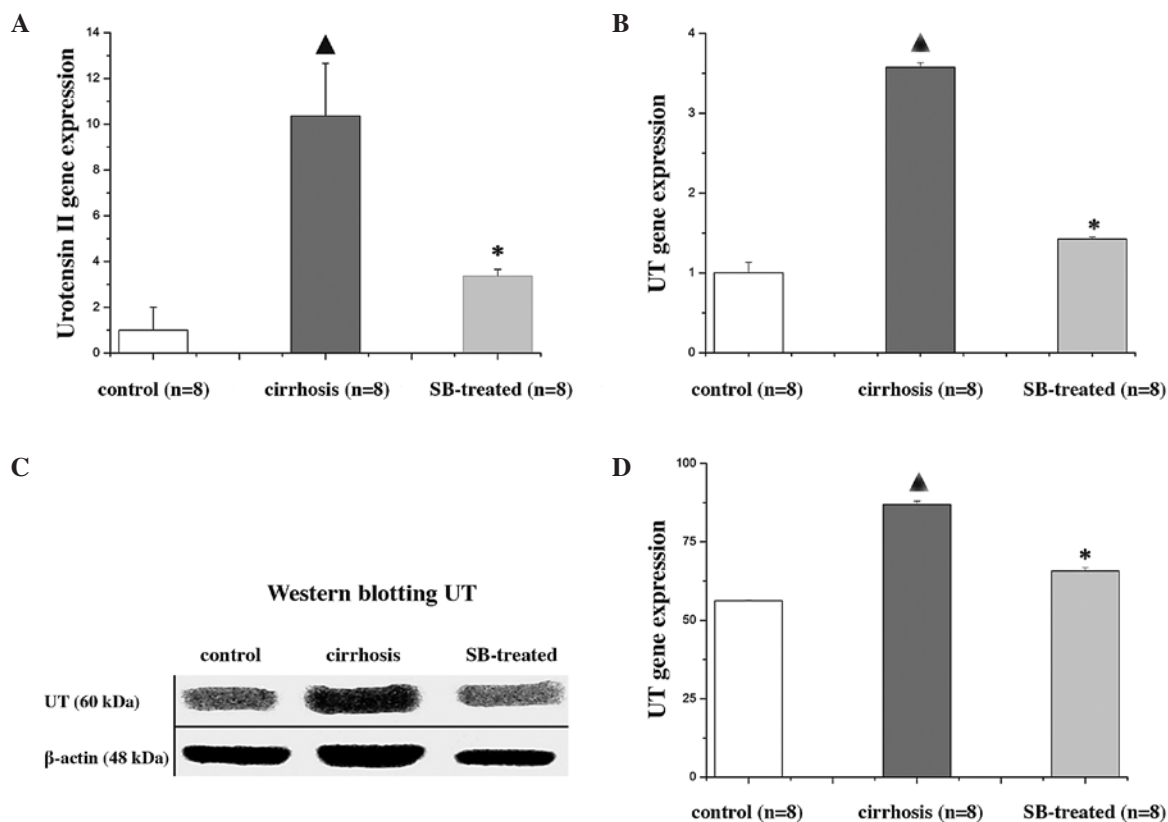


Figure 5. (A) Urotensin II (UII) and (B) G protein-coupled receptor (UT) mRNA in rat liver tissue in the three experimental groups. Results are expressed as the threshold cycle for the target gene standardized to the threshold cycle for the housekeeping gene β -actin. Values are expressed as the mean (95% confidence interval) relative to the controls, which were arbitrarily assigned a value of 1. [▲] $p < 0.05$ compared to the control group; ^{*} $p < 0.05$ compared to the cirrhosis group. (C) Expression of hepatic UT detected by Western blot analysis. A 60-kDa band, indicative of UT expression, was observed for the liver. A significant increase in UT protein expression in the liver of cirrhotic rats compared with the controls was observed. (C and D) SB-710411 decreased UT protein expression.

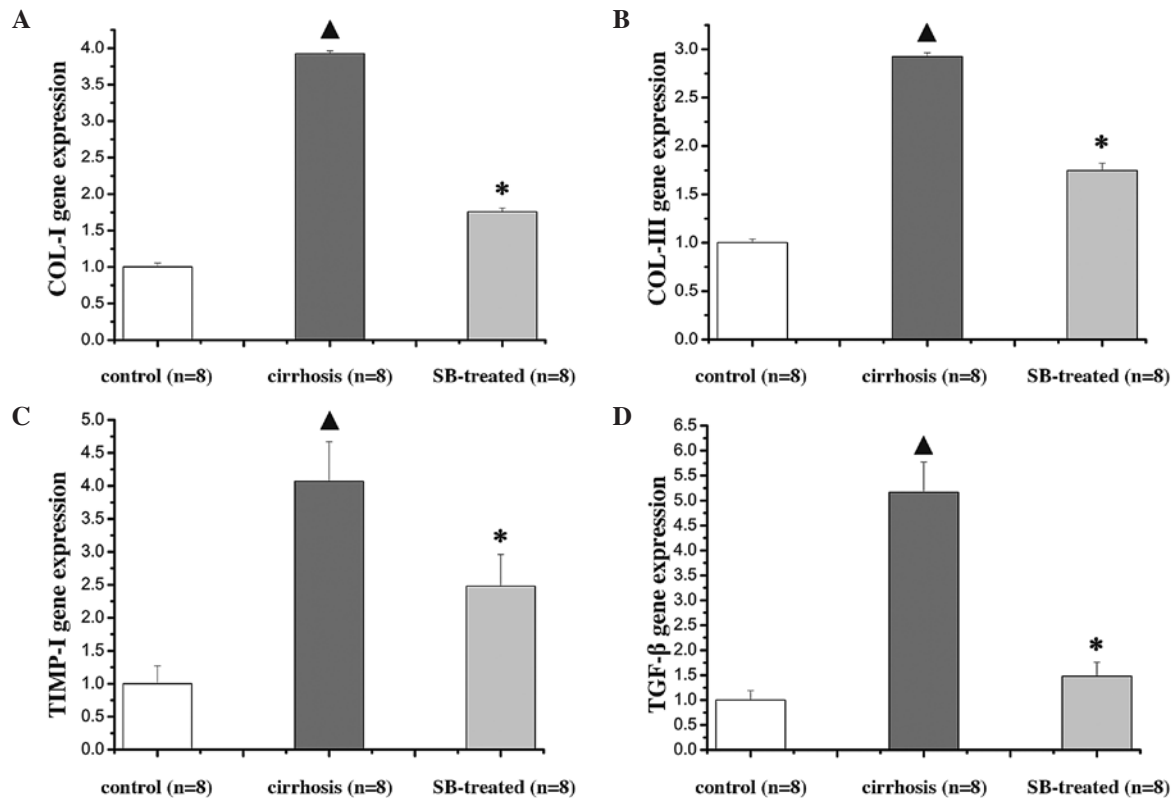


Figure 6. (A) COL-I, (B) COL-III, (C) TIMP-1 and (D) TGF-β1 mRNA in rat liver tissue in the three experimental groups. mRNA was quantified using real-time reverse-transcriptase PCR. Results are expressed as the threshold cycle for the target gene standardized to the threshold cycle for the housekeeping gene β-actin. Values are expressed as the mean (95% confidence interval) relative to controls, which were arbitrarily assigned a value of 1. * $p < 0.05$ compared to the control group; * $p < 0.05$ compared to the cirrhosis group.

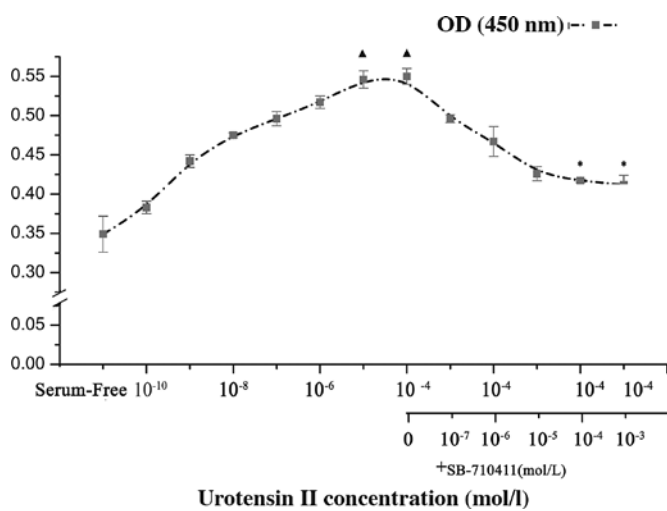


Figure 7. Dose-response graph demonstrating that UUI-induced rat fibroblast proliferation is inhibited by SB-710411. UUI acted as a potent mitogen for rat HSCs, and significantly increased proliferation at 10⁻¹⁰ and 10⁻⁴ M. Antagonism by 10⁻³ M SB-710411 inhibited this effect at the highest UUI concentration. (* $p < 0.05$ compared to the serum-free media group; * $p < 0.01$ compared to the 10⁻⁴ M UUI group).

liver Hyp concentration in the SB-treated group was decreased compared with the cirrhosis groups ($p < 0.01$) (Fig. 4C).

Changes in hepatic UUI, UT, collagen I, collagen III, TIMP-1 and TGF-β1 mRNA expression. Real-time PCR demonstrated

a significant increase in UUI/UT mRNA expression in the hepatic tissue of the CCL₄-treated rats compared with the controls (Fig. 5A and B; $p < 0.01$). UUI/UT mRNA expression in the hepatic tissue of the SB-treated rats had a tendency to be decreased compared with the cirrhotic rats. The mRNA levels of collagen I (COL-I) and collagen III (COL-III) were significantly increased in cirrhotic rats compared with the controls. SB-710411 treatment significantly attenuated the increase in mRNA levels of COL-I and COL-III (Fig. 6A and B). To clarify the mechanism by which SB-710411 attenuates the development of liver fibrosis induced by CCL₄, we examined hepatic tissue inhibitor levels of metalloproteinase-1 (TIMP-1) mRNA and transforming growth factor β1 (TGF-β1). The mRNA levels of TIMP-1 and TGF-β1 were significantly increased in cirrhotic rats compared with the controls. SB-710411 treatment tended to attenuate the increase in mRNA levels of TIMP-1 and TGF-β1 (Fig. 6C and D).

Western blot analysis of G protein-coupled receptor. A 60-kDa band, indicative of UT expression, was observed for the liver. Western blot analysis demonstrated a significant increase in UT protein expression in the liver of cirrhotic rats compared with the controls. SB-710411 decreased UT protein expression (Fig. 5C and D), similar to UT gene expression (Fig. 5B).

Hepatic stellate cell proliferation assay. UUI demonstrated potential low efficacy mitogenic effects on rat liver HSCs, as it significantly induced the proliferation of these cells at 10⁻¹⁰ M and 10⁻⁴ M following incubation for 48 h ($p < 0.05$) (Fig. 7).

Treatment with the UT antagonist SB-710411 at a concentration of 10^{-3} M almost completely inhibited 10^{-4} M UII-induced HSC proliferation ($p < 0.01$).

Discussion

Several studies have shown that urotensin II (UII) plasma levels are increased in patients with liver cirrhosis and portal hypertension (6,7). These UII plasma levels were negatively correlated with mean arterial pressure and positively correlated with portal pressure and the severity of the liver disease, as assessed using the Child-Pugh score (6,7). Recently, several physiological roles for UII have been identified, including a role in the pathogenesis and maintenance of high blood pressure, and in the stimulation of fibroblast proliferation and collagen synthesis (11). In the present study, UII mRNA in liver and the plasma levels of the vasoactive peptide UII were up-regulated in cirrhotic rats. The plasma UII was positively correlated with hyaluronic acid (HA) and laminin (LN) levels (Fig. 5C and D). Additionally, the results of real-time PCR and Western blot analysis showed that UII receptors were markedly well expressed in the cirrhotic rat liver. Furthermore, UII was shown to have a mitogenic effect on hepatic stellate cells (HSCs), which may contribute to liver fibrosis. These findings demonstrate that UII is crucially involved in liver cirrhosis, and suggest a potential therapeutic role for selective UT antagonism in the treatment of liver fibrosis. Based on these findings, the efficacy of the blockade of UII by the UT antagonist SB-710411 was evaluated in CCL₄ cirrhotic rats. This blockade of UII by SB-710411 led to a significant decrease in fibrosis, as well as a reduction in collagen deposition.

Hepatic fibrosis is a long-term consequence of chronic hepatic injury caused by alcohol abuse or hepatitis virus infection, and is characterized by extensive fibrous scarring of the liver and hepatic dysfunction (2-5). During liver fibrogenesis, activated HSCs synthesize a large amount of connective tissue components, composed mainly of extracellular matrix (ECM), HA, LN, and fibril-forming collagen types I and III (20-23). Evidence has recently been presented regarding the impressive capacity of the liver to remodel, even in cases of advanced fibrotic change (24,25). Furthermore, it has been suggested that ECM degradation and HSC apoptosis are the most important factors affecting reversibility. It may be that the resolution of hepatic fibrosis requires the elimination of causative agents and suitable reductions in pro-inflammatory cytokine levels. Nevertheless, the role of HSCs in this reversal is likely to be of central importance. Moreover, activated HSCs produce ECM and secrete pro-inflammatory cytokines, contributing to portal hypertension by contracting the intra-hepatic vasculature (26,27). Therefore, the prevention of HSC activation or apoptotic induction by controlling these processes might normalize hepatic fibrosis and prevent the development of cirrhosis or portal hypertension.

Immunohistochemical staining also showed significant reductions in α -SMA-positive cells in the animals administered a UII blockade (SB-treated group), which is indicative of HSC activation. Thus, the results provide evidence that a UII blockade can inhibit HSC activation via the suppression of activated UII and UT expression in the liver. In order to determine whether UII possessed mitogenic activity in the

HSCs, it was demonstrated that UII induced HSC proliferation. These findings were complemented by the observation that UII-induced proliferation in rat HSCs was inhibited by the use of the UT antagonist SB-710411.

TIMP-1 is capable of inhibiting matrix metalloproteinase activity, and therefore plays a role in regulating collagen degradation (28). In the SB-710411-treated cirrhosis group, SB-710411 limited the progression of fibrosis and the up-regulation of collagen I and TIMP-1 mRNA, two genes chiefly expressed by HSCs during fibrogenesis (28). These changes indicate that SB-710411, directly or indirectly, reduced the number and/or activation of matrix-producing cells, thereby blocking fibrosis progression. In addition, it is worth noting that UII is positively correlated with HA and LN, which are closely associated with the pathogenesis of hepatic fibrosis (29-33). UII may therefore be a useful non-invasive serum marker for estimating the degree of liver fibrosis.

TGF- β 1, derived from both paracrine and autocrine sources, remains the classic fibrogenic cytokine (34). UII significantly increased COL-I and COL-III mRNA expression and 3H-proline incorporation. Cell culture studies have demonstrated that TGF- β 1 is up-regulated in cardiac fibroblasts by UII via UT, and that it modulates the profibrotic effects of UII (35). Furthermore, Wang *et al*, utilizing an endothelial cell culture, found that UII increased the expression of COL-I mRNA and protein, and conversely decreased the expression of MMP-1 mRNA and protein as well as the activity of MMP-1 (36). In the present study, the expression of TGF- β 1 mRNA and collagen was decreased in the UII-blocked groups, which suggests that UII also plays an key role in hepatic fibrosis by inducing the expression of potent cytokines.

The comparison of SB-710411 with other receptor antagonists in similar models indicates a large therapeutic potential for this antagonist. Of note, when administered chronically, a congener of bosentan, one of the endothelin receptors, had no haemodynamic effect but worsened fibrosis, since the endothelin system may participate in the autocrine loop that counteracts the development of liver fibrogenesis (37). On the other hand, the use of angiotensin-converting enzyme inhibitors was reported to markedly reduce portal pressure (38). However, these results were not reproduced in subsequent studies, in which significant reduction in arterial pressure, glomerular filtration rate and almost negligible effects on the hepatic vein pressure gradient were observed (39). Therefore, the use of these drugs may not be advisable in patients with cirrhosis and portal hypertension (40).

A recent study by Trebicka *et al* using cirrhotic bile duct ligation rats showed that palosuran, another UT antagonist, reduced portal pressure without changing mean arterial pressure (9). Oral treatment with palosuran induced a further significant increase in plasma UII levels. In contrast to Trebicka's work, we found UII levels to be significantly lower in SB-710411-treated cirrhotic rats compared to cirrhotic rats without SB-710411 treatment. It is possible that a chronic feedback mechanism affected UII synthesis in our study. However, the existence of marked differences between the prior investigation and the current study regarding the antagonist used (SB-710411 vs. palosuran), the experimental model (CCL₄ vs. bile duct ligation rats) and the experimental design (preventive effect for 8 weeks vs. therapeutic effect for 3 days) makes it difficult to identify the reason for this discrepancy. Moreover,

responses to palosuran cannot be replicated with alternate UT antagonists, such as SB-710411 (41). Although drug efficacy cannot be directly compared because of variations in study parameters, it is evident that SB-710411 attenuated fibrosis in a fashion similar to other therapeutic modalities, including angiotensin-converting enzyme inhibitors.

In conclusion, the present study provides novel evidence that UT antagonists exert an arresting effect on fibrosis progression through the inhibition of the proliferation of HSCs. These findings suggest an important therapeutic role for UT antagonists in liver fibrosis.

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References

- Langer DA and Shah VH: Nitric oxide and portal hypertension: Interface of vasoreactivity and angiogenesis. *J Hepatol* 44: 209-216, 2006.
- Jonsson JR, Clouston AD, Ando Y, *et al*: Angiotensin-converting enzyme inhibition attenuates the progression of rat hepatic fibrosis. *Gastroenterology* 121: 148-155, 2001.
- Ohishi T, Saito H, Tsusaka K, *et al*: Anti-fibrogenic effect of an angiotensin converting enzyme inhibitor on chronic carbon tetrachloride-induced hepatic fibrosis in rats. *Hepatol Res* 21: 147-158, 2001.
- Knittel T, Kobold D, Saile B, Grundmann A, Neubauer K, Piscaglia F and Ramadori G: Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential. *Gastroenterology* 117: 1205-1221, 1999.
- Bataller R, Ginès P, Nicolás JM, *et al*: Angiotensin II induces contraction and proliferation of human hepatic stellate cells. *Gastroenterology* 118: 1149-1156, 2000.
- Heller J, Schepke M, Neef M, Woitas R, Rabe C and Sauerbruch T: Increased urotensin II plasma levels in patients with cirrhosis and portal hypertension. *J Hepatol* 37: 767-772, 2002.
- Kemp W, Krum H, Colman J, Bailey M, Yandle T, Richards M and Roberts S: Urotensin II: a novel vasoactive mediator linked to chronic liver disease and portal hypertension. *Liver Int* 27: 1232-1239, 2007.
- Ames RS, Sarau HM, Chambers JK, *et al*: Human urotensin II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature* 401: 282-286, 1999.
- Trebicka J, Leifeld L, Hennenberg M, *et al*: Hemodynamic effects of urotensin II and its specific receptor antagonist palosuran in cirrhotic rats. *Hepatology* 47: 1264-1276, 2008.
- Maguire JJ, Kuc RE, Klein MJ and Davenport AP: Immunocytochemical localization of the urotensin-II receptor, UT, to rat and human tissues: relevance to function. *Peptides* 29: 735-742, 2008.
- Papadopoulos P, Boussette N and Giaid A: Urotensin-II and cardiovascular remodeling. *Peptides* 29: 764-769, 2008.
- Clozel M, Hess P, Qiu C, Ding SS and Rey M: The urotensin-II receptor antagonist palosuran improves pancreatic and renal function in diabetic rats. *J Pharmacol Exp Ther* 316: 1115-1121, 2006.
- Sogni P, Moreau R, Gomola A, *et al*: Beneficial hemodynamic effects of bosentan, a mixed ET(A) and ET(B) receptor antagonist, in portal hypertensive rats. *Hepatology* 28: 655-659, 1998.
- Behm DJ, Herold CL, Ohlstein EH, Knight SD, Dhanak D and Douglas SA: Pharmacological characterization of SB-710411 (Cpa-c [D-Cys-Pal-D-Trp-Lys-Val-Cys]-Cpa-amide), a novel peptidic urotensin-II receptor antagonist. *Br J Pharmacol* 137: 449-458, 2002.
- Behm DJ, Herold CL, Camarda V, Aiyar NV and Douglas SA: Differential agonistic and antagonistic effects of the urotensin-II ligand SB-710411 at rodent and primate UT receptors. *Eur J Pharmacol* 492: 113-116, 2004.
- Huitron-Resendiz S, Kristensen MP, Sánchez-Alavez M, *et al*: Urotensin II modulates rapid eye movement sleep through activation of brainstem cholinergic neurons. *J Neurosci* 25: 5465-5474, 2005.
- Zhao XY, Wang BE, Li XM and Wang TL: Newly proposed fibrosis staging criterion for assessing carbon tetrachloride- and albumin complex-induced liver fibrosis in rodents. *Pathol Int* 58: 580-588, 2008.
- Porstmann T and Kiessig ST: Enzyme immunoassay techniques. An overview. *J Immunol Methods* 150: 5-21, 1992.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Pinzani M: Novel insights into the biology and physiology of the Ito cell. *Pharmacol Ther* 66: 387-412, 1995.
- Niki T, Pekny M, Hellemans K, *et al*: Class IV intermediate filament protein nestin is induced during activation of rat hepatic stellate cells. *Hepatology* 29: 520-527, 1999.
- Levy MT, McCaughan GW, Abbott CA, Park JE, Cunningham AM, Müller E, Rettig WJ and Gorrell MD: Fibroblast activation protein: a cell surface dipeptidyl peptidase and gelatinase expressed by stellate cells at the tissue remodeling interface in human cirrhosis. *Hepatology* 29: 1768-1778, 1999.
- Friedman SL: Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis. Mechanisms and treatment strategies. *N Engl J Med* 328: 1828-1835, 1993.
- Issa R, Zhou X, Constantinou CM, *et al*: Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking. *Gastroenterology* 126: 1795-1808, 2004.
- Iredale JP and Arthur MJ: Hepatocyte-matrix interactions. *Gut* 5: 729-732, 1994.
- Reynaert H, Urbain D and Geerts A: Regulation of sinusoidal perfusion in portal hypertension. *Anat Rec (Hoboken)* 291: 693-698, 2008.
- Moreira RK: Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 131: 1728-1734, 2007.
- Benyon RC and Arthur MJ: Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 21: 373-384, 2001.
- McHutchison JG, Blatt LM, De Medina M, Craig JR, Conrad A, Schiff ER and Tong MJ: Measurement of serum hyaluronic acid in patients with chronic hepatitis C and its relationship to liver histology. Consensus Interferon Study Group. *J Gastroenterol Hepatol* 15: 945-951, 2000.
- Guéchet J, Laudat A, Loria A, Serfaty L, Poupon R and Giboudeau J: Diagnostic accuracy of hyaluronan and type III procollagen amino-terminal peptide serum assays as markers of liver fibrosis in chronic viral hepatitis C evaluated by ROC curve analysis. *Clin Chem* 42: 558-563, 1996.
- Murawaki Y, Ikuta Y, Okamoto K, Koda M and Kawasaki H: Diagnostic value of serum markers of connective tissue turnover for predicting histological staging and grading in patients with chronic hepatitis C. *J Gastroenterol* 36: 399-406, 2001.
- Parés A, Deulofeu R, Giménez A, *et al*: Serum hyaluronate reflects hepatic fibrogenesis in alcoholic liver disease and is useful as a marker of fibrosis. *Hepatology* 24: 1399-1403, 1996.
- Matsumoto S, Yamamoto K, Nagano T, Okamoto R, Ibuki N, Tagashira M and Tsuji T: Immunohistochemical study on phenotypical changes of hepatocytes in liver disease with reference to extracellular matrix composition. *Liver* 19: 32-38, 1999.
- Friedman SL: Mechanisms of hepatic fibrogenesis. *Gastroenterology* 134: 1655-1669, 2008.
- Dai HY, Kang WQ, Wang X, *et al*: The involvement of transforming growth factor-beta1 secretion in urotensin II-induced collagen synthesis in neonatal cardiac fibroblasts. *Regul Pept* 140: 88-93, 2007.
- Wang H, Mehta JL, Chen K, Zhang X and Li D: Human urotensin II modulates collagen synthesis and the expression of MMP-1 in human endothelial cells. *J Cardiovasc Pharmacol* 44: 577-581, 2004.
- Poo JL, Jiménez W, María Muñoz R, *et al*: Chronic blockade of endothelin receptors in cirrhotic rats: hepatic and hemodynamic effects. *Gastroenterology* 116: 161-167, 1999.
- Schneider AW, Friedrich J and Klein CP: Effect of losartan, an angiotensin II receptor antagonist, on portal pressure in cirrhosis. *Hepatology* 29: 334-339, 1999.
- González-Abraldes J, Albillos A, Bañares R, *et al*: Randomized comparison of long-term losartan versus propranolol in lowering portal pressure in cirrhosis. *Gastroenterology* 121: 382-388, 2001.
- Heim MH, Jacob L and Beglinger C: The angiotensin II receptor antagonist candesartan is not effective in reducing portal hypertension in patients with cirrhosis. *Digestion* 75: 122-123, 2007.
- Behm DJ, McAtee JJ, Dodson JW, *et al*: Palosuran inhibits binding to primate UT receptors in cell membranes but demonstrates differential activity in intact cells and vascular tissues. *Br J Pharmacol* 155: 374-386, 2008.