N1-acetyl substituted pyrrolidine derivative CIP-A5: A novel compound that could ameliorate liver cirrhosis through modulation of hepatic stellate cell activity

Xiao-dan Wang a,b,1, Zu-hua Gao c,1, Xia Xue a, Yan-na Cheng a, Pan Yue a, Xu-wen Fang a, Xian-jun Qu a,*

a Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Jinan, Shandong 250012, China
b Department of Pharmacology, School of Pharmaceutical Sciences, Taishan Medical College, Taian, Shandong, China
c Department of Pathology and Laboratory Medicine, University of Calgary and Calgary Laboratory Services, Calgary, Alberta, Canada

1 These authors contributed equally to this work.

Abstract

(2S,4R)-methyl 1-acetyl-4-(N-(4-bromophenyl)sulfamoyloxy)pyrrolidine-2-carboxylate (CIP-A5) is the N1-acetyl substituted pyrrolidine derivative which was designed against the structure of matrix metalloproteinase (MMP-2) and MMP-9. CIP-A5 has been considered as a candidate compound for treatment of liver cirrhosis. In this study, we evaluated the efficacy of CIP-A5 on the activity of hepatic stellate cells. CIP-A5 prevented the transforming growth factor β (TGF-β)-induced proliferation of hepatic stellate HSC-T6 cells as estimated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. CIP-A5 stimulated MMPs activity as evidenced by an increase of degradation of succinylated gelatin. Gelatin zymography analysis showed that CIP-A5 stimulated the secretion and activity of MMP-2 and MMP-9 in HSC-T6 cells. This stimulatory effect on MMPs was verified by the observation of increased expression of MMP-2 and MMP-9 as evaluated by Western blot assay. At the same time, a significant decrease of the expression of tissue inhibitors of matrix metalloproteinases-1 (TIMP-1) was observed, suggesting a modulation of the balance of MMPs/TIMPs in hepatic stellate cells. CIP-A5 treatment also resulted in suppression of the profibrogenic cytokines, such as TGF-β, tumor necrosis factor alpha (TNF-α) and connective tissue growth factor (CTGF) in HSC-T6 cells. CIP-A5 did not have cytotoxicity to human normal hepatic cells. These results implied that CIP-A5 could selectively ameliorate the process of liver cirrhosis through modulation of activated hepatic stellate cell activity, which offers hope for prevention and treatment of this devastating end-stage liver disease.

Keywords:
N1-acetyl substituted pyrrolidine derivative
Matrix metalloproteases
Profibrogenic cytokines
Liver cirrhosis

1. Introduction

Hepatic fibrosis represents the final pathological outcome for the majority of chronic liver insults, due to toxic agents and diverse infectious, immunologic, and metabolic disorders (Rombouts and Marra, 2010; Thompson and Patel, 2010). Regardless of the underlying etiology, reiteration of liver injury results in persisting inflammation and progressive fibrogenesis, with chronic activation of the wound healing response in chronic liver disease, representing a major driving force for progressive accumulation of extracellular matrix (ECM) components, which eventually lead to liver cirrhosis (Wang et al., 2010). Liver fibrogenesis can lead to different patterns of fibrosis and is sustained by myofibroblast-like cells of different origin, with activated hepatic stellate cells (HSCs) being the major cell type involved (Park et al., 2009; Guo and Friedman, 2007). In response to liver injury, HSCs undergo activation and transdifferentiation into myofibroblasts. Moreover, increased synthesis of ECM components usually follows activation of HSCs and probably other matrix producing liver-derived myofibroblast-like cells. Inhibition of collagen degradation during the fibrogenic process also contributes to matrix accumulation in liver (Povero et al., 2010). Major profibrogenic mechanisms also include oxidative stress, as well as derangement of epithelial–mesenchymal interactions and, as recently suggested, the process of epithelial to mesenchymal transition (EMT) (Dechêne et al., 2010; Aoyama et al., 2010; Mehal and Imaeda, 2010).

Despite the progress in understanding the mechanism of liver fibrogenesis, less has been achieved in the medical management of liver cirrhosis. For most of the drugs that have been proposed, the anti-fibrotic effect is quite minor and they lack a specific effect pathway (Ramachandran and Iredale, 2009; Kim et al., 2009). Major profibrogenic mechanisms also include oxidative stress, as well as derangement of epithelial–mesenchymal interactions and, as recently suggested, the process of epithelial to mesenchymal transition (EMT) (Dechêne et al., 2010; Aoyama et al., 2010; Mehal and Imaeda, 2010).

In our previous studies, we constructed a series of N1-acetyl substituted pyrrolidine derivatives, which were designed against the structure of MMP-2 and MMP-9 (Cheng et al., 2008a,b,c). Among these synthesized chemical compounds, (2S,4R)-methyl 1-acetyl-4-(N-(4-bromophenyl)sulfamoyloxy)pyrrolidine-2-carboxylate (CIP-A5) is the N1-acetyl substituted pyrrolidine derivative which was designed against the structure of matrix metalloproteinase (MMP-2) and MMP-9.
1-acetyl-4-(N-(4-bromophenyl)sulfamoyloxy)pyrrolidine-2-carboxylate (No. A5 termed CIP-A5, Fig. 1) displayed stimulatory effects on MMPs secretion and expression in hepatic stellate cell line HSC-T6, and inhibitory effect on HSC-T6 cell proliferation. CIP-A5 has been considered as a candidate compound for treatment of liver cirrhosis. In this study, we evaluated the effect of CIP-A5 on the balance of MMPs/TIMP and on the production of profibrogenic cytokines including transforming growth factor β (TGF-β), tumor necrosis factor alpha (TNF-α), and connective tissue growth factor (CTGF) in HSC-T6 cells. These results offer an objective assessment of the potential therapeutic value of CIP-A5 in the management of liver cirrhosis.

2. Materials and methods

2.1. Chemical

N1-acetyl substituted pyrroline derivative (2S,4R)-methyl 1-acetyl-4-(N-(4-bromophenyl)sulfamoyloxy)pyrroline-2-carboxylate (CIP-A5, Fig. 1) was synthesized from 4-L-hydroxyproline through a sequence reaction including methylation, esterification, condensation, mesylation, S-O reaction upon treatment with sodium azide, hydrogenation over 5% Pd-C/CaCO3, acylation, and ester exchange upon treatment with hydroxylamine (Cheng et al., 2008a,b,c). CIP-A5 was dissolved in dimethylsulfoxide for reader (Perkin–Elmer, USA). The activation of gelatinase was estimated by comparing the OD value in the presence and absence of CIP-A5.

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

HSC-T6 cells (2–5 × 10⁴ per well) seeded in 96-well plates were treated with increasing concentrations of CIP-A5 in the presence or absence of TGF-β (10 ng/ml) for 72 h (Li et al., 2007; Zvibel et al., 2008). The controls were treated with an equal volume of the drug's vehicle DMSO, but the applied concentration did not exhibit a modulating effect on cell growth. The medium was then removed and the wells were washed with PBS. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μl of MTT (5 mg/ml, Sigma–Aldrich, USA) for 4 h. Light absorbance of the solution was measured at 540 nm on a multilabel plate reader (Perkin–Elmer, USA). Triplicate experiments with triplicate samples were performed.

The inhibitory effect of CIP-A5 on proliferation of human normal hepatic HL-7702 cells was also evaluated by the MTT assay as described above.

2.5. SDS–PAGE gelatin zymography

The activities of MMP-2 and MMP-9 were estimated by gelatin zymography analysis as described previously (Chen et al., 2008). HSC-T6 cells were cultured (2 × 10⁵ cells/ml) in 24-well plates and then exposed to increasing concentrations of CIP-A5 in 2 ml of serum-free medium for 24 h at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. Ten microliters of the supernatant was subjected for 20 h at 37°C. The inhibitory effect of CIP-A5 on proliferation of human normal hepatic HL-7702 cells was also evaluated by the MTT assay as described above.

2.6. Western blot analysis

HSC-T6 cells (1 × 10⁵ per well) seeded in 6-well plates were incubated with various concentrations of CIP-A5 for 24 h. The medium was removed and the cells were washed with PBS. Cells were then lysed in 100 μl of lysis buffer through three freeze–thaw cycles between −80 and 37 °C. Total protein was determined using the Bradford method (Okutucu et al., 2007). Cell lysates (30 μg of protein per lane) were separated by 10% SDS–PAGE and then electro-transferred onto nitrocellulose membranes. After blocking with TBST buffer (20 mM Tris-buffered saline and 0.1% Tween-20) containing 5% (w/v) nonfat dry milk for 1 h at room temperature, the membranes were incubated with primary antibodies for 2 h, followed by three washes and exposure to HRP-conjugated secondary antibodies (Santa Cruz, USA) for 1 h at room temperature. The primary antibodies included monoclonal anti-MMP-2 (sc-10736), polyclonal anti-MMP-9 (sc-6840), polyclonal anti-TIMP-1 (natural tissue inhibitors of matrix metalloproteinases-1 (sc-5538), monoclonal anti-TGF-β (transforming growth factor β) (sc-52893), polyclonal anti-TNF-α (tumor necrosis factor alpha) (sc-1351), polyclonal anti-CTGF (connective tissue growth factor) (sc-34772, Santa Cruz, USA) and anti-β-actin (ab2676, Abcam) in dilution

![Fig. 1](image-url). The chemical structure of N1-acetyl substituted pyrroline derivative CIP-A5, (2S,4R)-methyl 1-acetyl-4-(N-(4-bromophenyl)sulfamoyloxy)pyrroline-2-carboxylate.
buffer [2% (w/v) BSA in TBS] for 2 h. The bound antibodies were visualized using an ECL system (Amersham Pharmacia Biotech, USA) and quantified by densitometry using a Bio-profile Bio ID image analyzer (Dang et al., 2010).

2.7. Statistical analysis

Data was expressed as mean ± S.D. for three different determinations. Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple range tests. *P < 0.05 was considered as statistically significant. Statistical analysis was performed using the SPSS/Win13.0 software (SPSS, Inc., Chicago, Illinois, USA).

3. Results

3.1. Activation of gelatinase activity

The effect of CIP-A5 on activation of gelatinase was evaluated by means of succinylated gelatin assay. CIP-A5 possesses a high stimulatory effect on the activity of gelatinase in a cell-free system. As shown in Fig. 2, the degradation of succinylated gelatin by gelatinase was significantly increased by CIP-A5 in a dose-dependent manner (r = 0.99). In the concentration range of 5–30 ng/ml of CIP-A5, the activation of gelatinase (% over control) were increased from 28.4% to a maximum increase of 126.7% (Fig. 2, **P < 0.01 vs. vehicle control).

3.2. Inhibition of TGF-β-induced HSC-T6 proliferation

We evaluated the inhibitory effect of CIP-A5 on the proliferation of HSC-T6 cells in the presence or absence of TGF-β stimulation. HSC-T6 cells were treated with CIP-A5 (1–200 μg/ml) for up to 72 h and then the proliferation of HSC-T6 cells was evaluated based on the viable cell number as estimated by MTT assay. As shown in Fig. 3A, CIP-A5 at the concentration range of 1–100 μg/ml did not have a significant inhibition on HSC-T6 cell proliferation as evidenced by the similar OD value of growth (P > 0.05, vs. untreated control or vehicle control). But, the anti-proliferative effect was observed in high dose of exposure (200 μg/ml, *P < 0.05 vs. untreated control or vehicle control). The concentration range of 1–100 μg/ml was therefore used in the following experiments of MMPs activity and the measurement of pro-fibrogenic cytokines production in HSC-T6 cells.

Fig. 2. Activation of gelatinase by CIP-A5. Proteolysis of succinylated gelatin by gelatinases was evaluated in the presence of increasing concentrations of CIP-A5 for 30 min. After enzyme reaction, 0.03% trinitrobenzene sulfonic acid was added to each sample, which was allowed to stand for 20 min at room temperature, and then absorbance at 450 nm was measured. The activation of gelatinase was estimated by comparing the OD value in the presence and absence (vehicle control) of CIP-A5. The bars were expressed as means ± S.D. of three independent experiments. **P < 0.01 vs. vehicle control.

Fig. 3. Inhibitory effects of CIP-A5 on the proliferation of hepatic stellate cells. HSC-T6 (A and B) and human hepatic cells HL-7702 (C). (A and B) HSC-T6 cells (2–5 x 10⁵ per well) seeded in 96-well plates were treated with increasing concentrations of CIP-A5 in the presence or absence of TGF-β (10 ng/ml) for 72 h. HSC-T6 cell proliferation was evaluated by the MTT assay and demonstrated by OD value at 540 nm. (A) OD value of HSC-T6 cell growth in the absence of TGF-β stimulation. The control of vehicle was treated with an equal volume of the drug’s vehicle DMSO. (B) OD value of HSC-T6 cell growth in the presence of TGF-β stimulation. The first column was the control without TGF-β or CIP-A5 exposure. The OD values of other columns were measured in the presence of TGF-β stimulation (second column, # indicates mean that is significantly different when compared with first column of the untreated control with P value of less than 0.05). The proliferation of TGF-β-stimulated HSC-T6 cells was significantly suppressed in the presence of CIP-A5. *P < 0.05; **P < 0.01 vs. control of second column. (C) OD value of human hepatic HL-7702 cell growth in the presence or absence of CIP-A5 as estimated by MTT assay. The control of vehicle was treated with an equal volume of the drug’s vehicle DMSO. The bars indicate means ± S.D. (n = 3). *P < 0.05 vs. untreated control or vehicle control.
We then examined the inhibitory effect of CIP-A5 on HSC-T6 cell proliferation in the presence of TGF-β stimulation. TGF-β (10 ng/ml) stimulated the proliferation of HSC-T6 cells by 36.5% increase after 72 h incubation. As shown in Fig. 3B, the stimulation of cell proliferation was demonstrated by the increase of OD value at 540 nm (Fig. 3B, second column, # indicates mean that is significantly different when compared with first column of the untreated control with P value of less than 0.05). The TGF-β-stimulated HSC-T6 cell proliferation was significantly suppressed in the presence of CIP-A5. As shown in Fig. 3B, in the concentration range of 1–200 μg/ml of CIP-A5, the OD values of cell growth were decreased dose-dependently (r = 0.99, * and ** indicate means that are significantly different when compared with the second column with P value of less than 0.05 and 0.01, respectively). In the concentration range of 1–200 μg/ml of CIP-A5, the percentages of inhibition on TGF-β-stimulated HSC-T6 cell proliferation were increased from 8.0% to maximum inhibition of 55.8% for 72 h exposure.

3.3. CIP-A5 did not significantly inhibit HL-7702 cell proliferation

We also examined whether CIP-A5 affect the proliferation of human normal hepatic cells. The immortalized human hepatic cell line HL-7702 was employed and the viable cell number was estimated by MTT assay. As shown in Fig. 3C, CIP-A5 in the range of 1–100 μg/ml did not significantly inhibit HL-7702 cell proliferation as demonstrated by the similar OD values of cell growth (P > 0.05 vs. untreated control or vehicle control). But, the significant inhibition was observed in high dose of exposure (Fig. 3C, 200 μg/ml, *P < 0.05 vs. untreated control or vehicle control). This result indicated that CIP-A5 in the concentration range of 1–100 μg/ml did not have cytotoxicity to human hepatic cells.

3.4. Stimulation of MMPs secretion and activity

Gelatin zymography analysis was performed to evaluate the secretion and activity of MMP-2 and MMP-9 in HSC-T6 cells upon exposure to CIP-A5. CIP-A5 increased the secretion and activity of MMP-2 and MMP-9 both in the presence and absence of TGF-β stimulation. As shown in Fig. 4A, left, the activities of MMP-2 and MMP-9 in supernatants of the cultured HSC-T6 cells were significantly increased in a dose dependent manner in the presence of CIP-A5. At the concentration range of 1–100 μg/ml of CIP-A5, the percentages of increase were increased from 38.6% to maximum increase of 190.5% for MMP-2 (r = 0.97) and from 22.4% to maximum increase of 170.0% for MMP-9 (r = 0.99), respectively (Fig. 4A, right, *P < 0.05; **P < 0.01 vs. untreated control). The activation of MMP-2 and MMP-9 by CIP-A5 was also detected in the presence of TGF-β stimulation (Fig. 4B, left). The percentages of increase were increased from 30.0% to maximum increase of 169.5% for MMP-2 (r = 0.99) and from 21.4% to maximum increase of 170.0% for MMP-9 (r = 0.98), respectively (Fig. 4B, right, *P < 0.05; **P < 0.01 vs. untreated control).

The activation of MMPs in HSC-T6 cells by CIP-A5 was then evidenced by Western blot analysis. As shown in Fig. 4C, left, pro and active MMP-2 were detected in the gel at molecular weights of 72 and 63 kDa. CIP-A5 treatment of HSC-T6 cells resulted in a significantly increased expression of MMP-2 and remarkable in active MMP-2 more than in pro MMP-2 (Fig. 4C). The increasing expression rates for pro MMP-2 were up to 50% increase and 160.8% increase for active MMP-2 by CIP-A5 treatment (Fig. 4C, right, *P < 0.05; **P < 0.01 vs. untreated control). MMP-9 was measured around position at molecular weight of 92 kDa in the gel (Fig. 4D, left). The expression levels were increased from 30.0% to maximum increase of 105.9% for the same concentrations as described above (Fig. 4D, right, *P < 0.05; **P < 0.01 vs. untreated control).

3.5. Decrease expression of TIMP-1

The expression levels of TIMP-1 in HSC-T6 cells were examined for evaluation the MMPs/TIMPs balance following CIP-A5 treatment (Fig. 4E, left). Treatment of the hepatic stellate cells with CIP-A5 resulted in a significantly decrease of TIMP-1 expression in a dose-dependent manner. As shown in Fig. 4E, right, at the concentration range of 1–100 μg/ml of CIP-A5, the percentages of inhibition were 10.6%, 32.8%, 51.7%, 67.3% and 79.5%, respectively, after 72 h exposure (r = 0.99, *P < 0.05; **P < 0.01 vs. untreated control).

3.6. Decrease expressions of TGF-β, TNF- and CTGF

We measured the levels of liver cirrhosis related profibrogenic cytokines in the transmembrane including TGF-β, TNF-α and CTGF in HSC-T6 cells using Western blot analysis. A dose-dependent decrease of TGF-β and TNF-α was observed within the concentrations (1–100 μg/ml) after 72 h incubation with CIP-A5 (Fig. 5). The percentages of inhibition were measured from 15.7% to 50% decrease for TGF-β (Fig. 5A, right, r = 0.99) and from 32.8% to 160% decrease for TNF-α (Fig. 5B, right, r = 0.99) by CIP-A5 treatment (Fig. 5A and B, * and ** indicate means that are significantly different when compared with the untreated control with P value of less than 0.05 and 0.01, respectively). Because CTGF is the downstream mediator of the fibrogenic cytokine in the pathogenesis of hepatic fibrosis, we measured the expression levels of CTGF after CIP-A5 treatment (Fig. 5C, left). CIP-A5 decreased the expression of CTGF dose-dependently. The percentages of decreasing were 15.3%, 26.8%, 41.5%, 68.6% and 79.1%, respectively, after 72 h exposure (Fig. 5C, right, r = 0.99, * and ** indicate means that are significantly different when compared with the untreated control with P value of less than 0.05 and 0.01, respectively).

4. Discussion

Liver cirrhosis is a major cause of morbidity and mortality worldwide and has very limited therapeutic options (Zaton´ ski et al., 2010). The cell responsible for hepatic fibrosis appears to be the activated myofibroblasts, which may be derived from hepatic stellate cells, transformed epithelial cells through epithelial to mesenchymal transition, or circulating bone marrow precursors. Among which, the activated hepatic stellate cells are the primary cells responsible for the development of liver fibrosis (Choi et al., 2010; Brenner, 2009; Mann and Mann, 2009). Mounting evidences suggest that the activation of hepatic stellate cells is strongly associated with many profibrogenic cytokines, such as TGF-β, TNF-α, CTGF, and TIMP-1 (Lim et al., 2010; Ruelh et al., 2009; Wallace et al., 2008). Following liver injury, hepatic stellate cells undergo a complex process of activation followed by increased synthesis and deposition of ECM components (Guo and Friedman, 2007; Hui and Friedman, 2003). Moreover, the activation of hepatic stellate cells is mediated by the release of various cytokines and growth factors to form a typical autocrine loop of TGF-β1 autoinduction, making hepatic stellate cells a central target for antifibrotic strategies (Saile and Ramadori, 2007; Kuriyama et al., 2007; Breitkopf et al., 2005). Therefore, the reversal of fibrosis depends upon the elimination of the fibrogenic cytokine and the activated myofibroblast. HSC-T6 cell, an immortalized rat hepatic stellate cell line, has a phenotype and biological characteristics of the myofibroblasts in liver (Lim et al., 2010). Many studies revealed that the activated HSC-T6 cells expressed high levels of TGF-β, TNF-α, CTGF and many other profibrogenic cytokines (Saile and Ramadori, 2007; Kuriyama et al., 2007; Breitkopf et al., 2005). Therefore, HSC-T6 cell line has been considered as an appropriate model for investigating the mechanism of liver cirrhosis and for evaluation the
Fig. 4. The activation of MMP-2 and MMP-9 in HSC-T6 cells evaluated by gelatin zymography assay (A and B) and Western blot analysis (C and D). (A and B) Proteolytic activity of MMP-2 and MMP-9 estimated by SDS–PAGE gelatin zymography. (A) Bands of MMP-9 and MMP-2 in the absence of TGF-β stimulation (left). \( P < 0.05; \quad ** P < 0.01 \) vs. the untreated control (right). (B) Bands of MMP-9 and MMP-2 in the presence of TGF-β stimulation (left). \( P < 0.05; \quad ** P < 0.01 \) vs. the untreated control (right). (C) The bands of pro and active MMP-2 in HSC-T6 cells (left). \( P < 0.05; \quad ** P < 0.01 \) vs. the untreated control (right). (D) Activation of MMP-9 in HSC-T6 cells (left). \( P < 0.05; \quad ** P < 0.01 \) vs. the untreated control (right). (E) Decrease of TIMP-1 in HSC-T6 cells by CIP-A5. HSC-T6 cells were incubated with various concentrations of CIP-A5 for 24 h and then subjected to Western blot assay (left). \( P < 0.05; \quad ** P < 0.01 \) vs. the untreated control (right). Triplicate experiments with triplicate samples were performed.
drugs targeting hepatic stellate cells (Ping et al., 2011; Cheng et al., 2009). In this study, we evaluated the efficacy of CIP-A5, a novel compound of the N1-acetyl substituted pyrrolidine derivative, on the activity of hepatic stellate HSC-T6 cells and the molecular mechanism that underlies the therapeutic action of CIP-A5. We examined the inhibitory effect of CIP-A5 on proliferation of HSC-T6 cells in the presence or absence of TGF-β stimulation. We initially showed that CIP-A5 at the concentration range of 1–100 µg/ml was unable to inhibit the proliferation of HSC-T6 cells in absence of TGF-β stimulation. This result implied that the inhibitory effect of CIP-A5 on hepatic stellate cell proliferation was not based on the cytotoxicity of the compound. However, the cytotoxicity of CIP-A5 on HSC-T6 cells was observed in high dose of exposure (200 µg/ml). We then demonstrated the inhibitory effect of CIP-A5 on the proliferation of hepatic stellate HSC-T6 cells in the presence of TGF-β stimulation. After incubation with TGF-β, the proliferation of HSC-T6 cells was significantly increased, implying that HSC-T6 cells were activated. Further examination showed that the proliferation of the activated hepatic stellate cells was suppressed by CIP-A5, indicating that the compound could block the fibrogenic cytokines such as TGF-β induced activation of hepatic stellate cells. In addition, we examined whether CIP-A5 has cytotoxicity to human normal hepatic cells. Human hepatic cell line HL-7702 was employed and the experiment was carried out in the same condition as described above. CIP-A5 did not significantly affect the proliferation of human hepatocytes, indicating that the compound could selectively suppress the proliferation of the activated hepatic stellate cells. These results implied that CIP-A5 could ameliorate liver cirrhosis through modulation of the activated hepatic stellate cell activity without affecting hepatocyte regeneration.

Interestingly, CIP-A5 was found to stimulate the activity of MMP-2 and MMP-9 in HSC-T6 cells. Thus a question may be raised as to whether the increase in MMPs expression with CIP-A5 treatment can be pro-fibrogenic in terms of stellate cell activation. There is mounting evidence that the increased and disordered

**Fig. 5.** Western blot analysis of TGF-β, TNF-α and CTGF in HSC-T6 cells. HSC-T6 cells were exposed to various concentrations of CIP-A5 for 24 h. Cells were lysed and subjected to Western blot analysis (left). The percentages of inhibition on TGF-β, TNF-α and CTGF were estimated by comparing the densitometry in the presence and absence of CIP-A5 (right). (A) Decrease of TGF-β; (B) inhibition of TNF-α; and (C) inhibition of CTGF. *P < 0.05; **P < 0.01 vs. the untreated control (right). Triplicate experiments with triplicate samples were performed.
deviation of ECM components in fibrotic liver is due to unbalanced excessive production and reduced degradation of ECM (Wang et al., 2009; Das and Vasudevan, 2008). Degradation of ECM by MMPs fails to keep pace with increased synthesis, in part due to sustained expression of MMPs inhibitors (e.g., tissue inhibitors of metalloproteinases). The activated hepatic stellate cells produce both TGF-β and tissue inhibitors of metalloproteinases (TIMPs), which lead to enhanced extracellular matrix synthesis and decreased matrix degradation, respectively (Olaso et al., 2001; Reif et al., 2004; Bruck et al., 2001). Therefore, enhancing the production of MMPs may be a possible strategy to promote tissue remodeling and minimize the net accumulation of ECM components in liver fibrosis (Olaso et al., 2001; Reif et al., 2004; Bruck et al., 2001). The implication of this hypothesis is that if the MMPs/TIMPs balance could be altered then fibrosis might prove to be reversible and degradation of the excess matrix possible. To gain further insights into the inhibitory effect of CIP-A5 on activation of hepatic stellate cells, we then examined the expression level of TIMP-1 in HSC-T6 cells following CIP-A5 treatment. CIP-A5 suppressed the expression of TIMP-1 in the activated hepatic stellate cells. These observations led us to the hypothesis that CIP-A5 might have the ability to modulate the balance of the TIMPs/TIMPs in the activated hepatic stellate cells.

It is well known that connective tissue growth factor (CTGF) is the downstream mediator of the fibrogenic master cytokine TGF-β in the pathogenesis of hepatic fibrosis (Gressner et al., 2007; Suk et al., 2009). CTGF is a highly profibrogenic molecule and plays a crucial role in the pathogenesis of hepatic fibrosis. The activated hepatic stellate cells produced high level of CTGF and the production of ECM is consequently increased (George and Tsutsumi, 2007; Rachfal and Brigstock, 2003; Li et al., 2006). In this study, CIP-A5 inhibited the production of TGF-β and prevented the high expression of CTGF in the activated hepatic stellate cells, indicating that the downstream mediator of the fibrogenic stimulators was abrogated by CIP-A5 treatment.

In conclusion, CIP-A5 is a novel N-acetyl substituted pyrrolidine derivative compound that could ameliorate the process of liver cirrhosis through modulation of the activated hepatic stellate cell activity, CIP-A5 did not have cytotoxicity to human hepatic stellate cells. These observations led us to the hypothesis that CIP-A5 might have the ability to modulate the balance of the TIMPs/TIMPs in the activated hepatic stellate cells.

Conflicts of interest
None declared.

Acknowledgements
This project was supported by the Natural Science Foundation of China (30973550, 30901833), Shandong Provincial Foundation for Natural Science (2009ZR201798) and the Doctoral Science Foundation of the Ministry of Education of China (200911110063). This work was also supported by Department of Pathology and Laboratory Medicine, University of Calgary and Calgary Laboratory Services, Canada.

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