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Structure-Related Cytotoxicity and Anti-Hepatofibric Effect of Asiatic Acid Derivatives in Rat Hepatic Stellate Cell-Line, HSC-T6

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The structural relationship of 16 asiatic acid (AA) derivatives, including AA and asiaticoside (AS) to cytotoxicity and anti-hepatofibrotic activity in HSC-T6 cells, were investigated. Cytotoxicities of AA derivatives varied from 5.5 μM to over 2000 μM of IC_{50} depending on AA functional group modifications. Substituting the hydroxyl group at the C(2) to N≡C and substituting bulky groups for dihydroxyl groups at (3), (23) of the A-ring increased the cytotoxicity, but keto group at C(11) and benzoyl ester at C(2) were greatly reduced it. Modification of the carboxylic acid group at C28 also reduced the cytotoxicity. The collagen synthesis determined by hydroxyproline content in the cells was inhibited from a maximum of 48% (Zlx-i-85 and 87) to 15% (AS) by AA derivatives. The anti-hepatofibrotic effect of these compounds might be due to the reduced expression of prolyl 4-hydroxylase α and β subunits and TIMP2. However, the inhibition of collagen by asiaticoside derivatives did not show any structural-activity relationship.

Key words: Asiatic acid derivatives, Rat hepatic stellate cell, Cytotoxicity, Anti-hepatofibrotic effect

INTRODUCTION

Liver fibrosis represents a wound-healing process in response to a variety of chronic stimuli. Fibrosis is characterized by an excessive deposition of extracellular matrix proteins, of which type I collagen is predominant. Activation of the hepatic stellate cell (HSC) is responsible for the increased synthesis and deposition of type I collagen in the liver. Following a fibrogenic stimuli, the HSC undergoes a complex activation process in which the cell changes from a quiescent vitamin A-storing cell to an activated myofibroblast-like cell, which proliferates and becomes fibrogenic. Thus, the inhibition of HSC proliferation and collagen synthesis may delay or recover the hepatofibrosis process.

Asiaticoside (AS), a biologically active triterpenoid present in *Centella asicatica* (L.) Urban, has been known to exert a variety of biological effects such as wound-healing, anti-inflammatory, and β-amyloid induced neurotoxicity protection (Maquart *et al.*, 1999; Mook-Jung *et al.*, 1999). Asiatic acid (AA) was reported to induce apoptosis through intracellular Ca^{2+} release and to enhance the expression of p53 in HepG2 human hepatoma cells (Lee *et al.*, 2002). Qi *et al.* (2000) reported that AA could significantly affect the ultrastructure of fibroblast and inhibit its collagen synthesis and proliferation *in vitro* culture. Among the AS, 19α-hydroxyl asiatic acid and 19α-hydroxyl asiatic acid 28-O-β-D-glucoside did not protect against the CCl_4-, acetaminophen-, or Cd-induced hepatotoxicity (Liu *et al.*, 1994). However, Han *et al.* (2003) reported that, among the 150 synthetic AS derivatives, 19α-hydroxyl asiatic acid was the most effective in decreasing chemical-induced liver injury and also had an inhibitory effect in chronic liver cirrhosis, particularly AS6, *in unpublished data*.

In this study, we reported the structural relationship of 16 AA derivatives, including AA and AS (Fig. 1) to cytotoxicity and anti-hepatofibrotic activity.

Structurally, AA has three kinds of functional groups in Fig. 1: three hydroxyl groups at C(2), C(3) and C(23); an olefine group at C(12); a carboxylic acid group at C(28).

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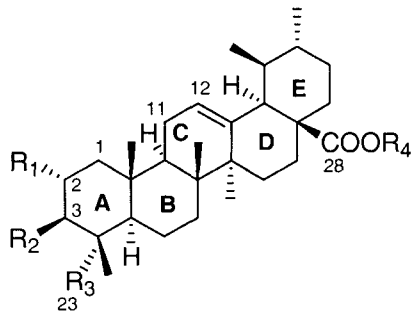
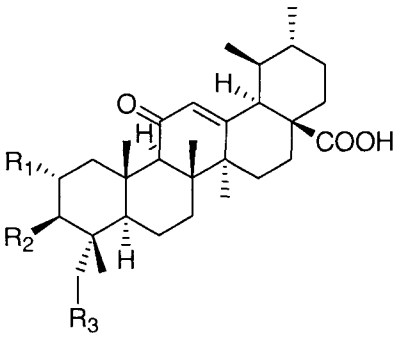
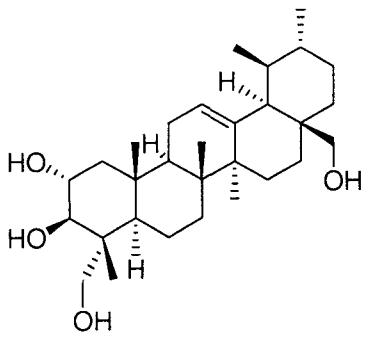


	R_1	R_2	R_3	R_4
AA	OH	OH	CH_2OH	H
AS	OH	OH	CH_2OH	Glu-Glu-Rhm
Zlx-i-69	OAc	OAc	CH_2OAc	CH_3
Zlx-i-70	OAc	OAc	CH_2OAc	H
Zlx-i-85	OCH_3	OAc	CH_2OAc	CH_3
Zlx-i-87	=O	OAc	CH_2OAc	H
Zlx-i-89	H	OH	$CH_2OCOC(CH_3)_3$	H
Zlx-i-92	OCH_3	=O	CHO	CH_3
Zlx-i-100	OCH_3	OCH_3	OCH_3	CH_3
Zlx-i-111	CN			CH_3
Zlx-i-112	CN	OH	CH_2OH	CH_3
Zlx-i-113	COOH	OH	CH_2OH	CH_3
Zlx-i-132	OBn	OAc	CH_2OAc	CH_3



Zlx-i-102 R_1= OAc R_2= OAc R_3= OAc
Zlx-i-103 R_1= OH R_2= OH R_3= OH



Zlx-i-123

Fig. 1. Asiaticoside derivatives tested in the present study.

Among these functional groups, the unusual triol and C(28)-carboxylic acid were modified, keto group was introduced at C(11) and cytotoxicity and anti-hepatofibrotic activity were observed in rat stellate cell-line, HSC-T6.

MATERIALS AND METHODS

Preparation of asiaticoside derivatives

Fig. 1 shows the chemical structures of 16 AA derivatives that were examined in this study. The AA derivatives were synthesized by chemical modifications of AA, and details of the chemical process will be reported elsewhere.

Cell culture

HSC-T6 cell, an immortalized rat hepatic stellate cell-line, which had the stable phenotype and biochemical characters, was kindly provided by Dr. Friedman SL (Mount Sinai School of Medicine, NY). The cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco-BRL), penicillin (100 units/mL) and streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air.

Cytotoxicity

HSC-T6 cells were plated in 96-well microplate at an initial concentration of 2×10^4 cells per well. Approximately 24 h after cell plating, cells were cultured with the serum-free DMEM containing various concentrations of AS derivatives, penicillin (100 units/mL) and streptomycin (100 mg/mL) for 24 h and followed to add MTS solution (CellTiter 96 non-radioactive cell proliferation assay kit, Promega). The plate was incubated under 5% CO_2 at 37 °C for 90 min, and then the absorbance was measured on a Molecular Dynamics Plate Reader at 490 nm.

Hydroxyproline content

The hydroxyproline content in HSC-T6 cell was determined to assess the collagen changes. Approximately 24 h after cell plating, HSC-T6 cells were treated with or without AS derivatives for 24 h. The cells were then scrapped and sonicated for 20 sec three times. 800 ml of this solution was mixed with an equal volume of 12 N HCl, finally to be 6 N HCl concentration and hydrolysis at 110 °C for 18 h in oil bath. The solution was dried after finishing the acid hydrolysis, and then 1.2 mL of 50% isopropanol and 0.2 mL of 5.6 mM chloramine T solution were added. After 10 min of incubation at room tempera-

ture, 1 mL of Enrlichs reagent was added to the solution, and incubated at 60 °C for 30 min. Then the absorbance was determined at 558 nm.

RNA preparation and Reverse Transcription-Polymerase Chain Reaction

Levels of mRNA for hepatic fibrosis-related proteins were analyzed by the reverse transcription polymerase chain reaction (RT-PCR) method. Total RNA was isolated using Easyblue solution (Intron Co., Korea) from the cells according to the manufactures procedure. The first-strand synthesis was performed at 42 °C for 1 h using the oligo-dT primer, M-MLV Reverse Transcriptase (Promega) and 2 mg of total RNA as a template. PCR employing primers for the β-actin confirmed the integrity of the resultant cDNA . Table I lists the primers used and the conditions of PCR. The resultant band was quantitatively analyzed using an analyzing software (TINA 2.0, Bio-Imager) and was normalized with that of β-actin.

Table I. Primer sequences used in PCR for determining levels of hepatic fibrosis-related protein expression

Gene	Primers	product
β-Actin	(F) 5- CAG AGC AAG AGA GGC ATC CT (R) 5- TTA ATG TCA CGC ACG ATT TCC	463 bp
Procollagen 1	(F) 5-AACGATGGTGCCAAGGGT (R) 5-AGCACCATCTCTTCCAGG	974 bp
TGFβ-1	(F) 5-GCTTTGTACAACAGCACC (R) 5-CTGCTCCACCTTGGGCTT	882 bp
TIMP-1	(F) 5' ACA GCT TTC TGC AAC TCG 3' (R) 5' CTA TAG GTC TTT ACG AAG GCC 3'	334 bp
TIMP-2	(F) 5' ATT TAT CTA CAC GGC CCC 3' (R) 5' CAA GAA CCA TCA CTT CTC TTG 3'	341 bp
Prolyl 4-hydroxylase α	(F) 5' GAA GT T AGA CCG GCT AAC AA 3' (R) 5' GAG ACT TTG TCT ACA GTA GAC 3'	434bp
Prolyl 4-hydroxylase β	(F) 5' CAG CAG TAT GGT GTC CGT G 3' (R) 5 TT GCC ATC GTA GTC AGA CAC 3'	538 bp

RESULTS AND DISCUSSION

The structural effect of asiaticoside derivatives on cytotoxicity

The study of Structure-activity relationship (SAR) to cytotoxicity was performed with semi-synthetic derivatives of AA in rat hepatic stellate cell-line, HSC-T6 (Fig. 2). The cells were cultured for 24 h in serum-free media containing various concentrations of AA derivatives. The cytotoxicities of 16 AA derivatives varied, depending on the modifications of AA functional groups; IC_{50} values in HSC-T6 cells ranged from 5.5 to more than 2000 μM in HSC-T6 cells (Table II). Several structural patterns versus cytotoxicity are described in Table II. The free C(28)-COOH derivative

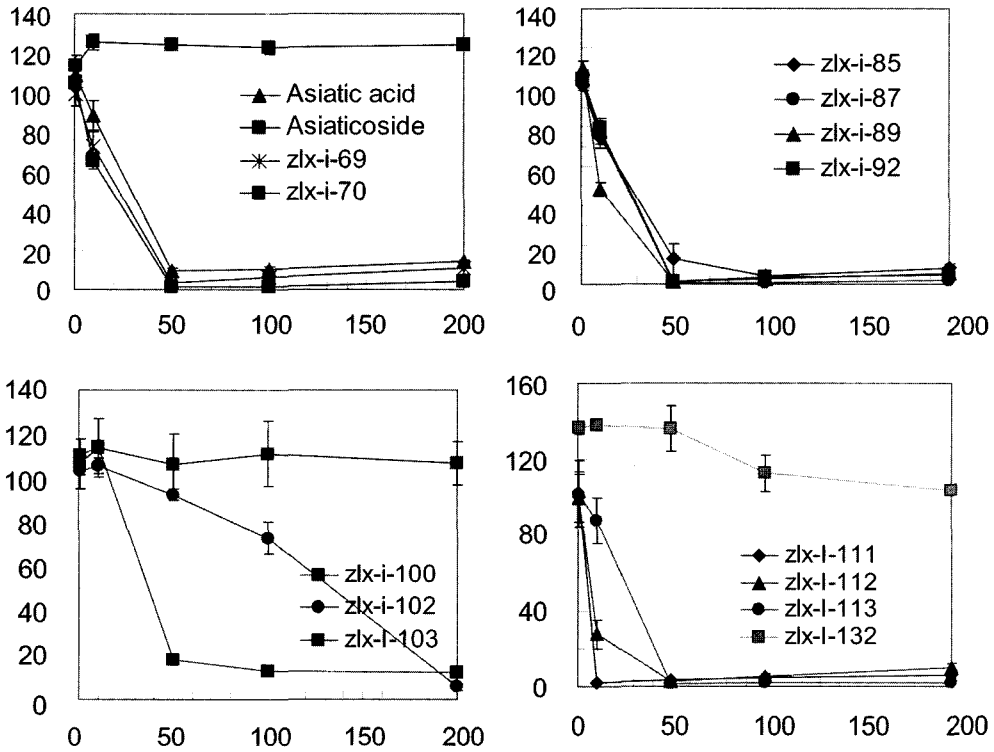


Fig. 2. Cytotoxicity of asiaticoside derivatives in HSC-T6 cells. Cells (1×10^4/0.1 mL) in the serum-free DMEM were treated for 24 h with various concentrations of the asidaticoside derivative and the relative cell viability was determined by the MTS method as described in materials and methods. The data represent the mean ± S.D. (n=4).

showed higher cytotoxicity than the methyl (Zlx-i-69 vs 70) and glycosyl ester derivatives (AA vs AS), suggesting that free carboxyl groups are critical in exerting toxicity. The triol group (R_1-R_3) substitutions at the A-ring are also important for cytotoxicity. The R_1 to CN (Zlx-i-112 vs Zlx-i-113) modification greatly increased the cytotoxicity. However, cytotoxicity was greatly reduced when the benzoyl ester was replaced at R1 (Zlx-i-132 vs Zlx-i-69). Substituting R_2 and R_3 to bulky groups, such as $CH_2OCO(CH_3)_3$ (Zlx-i-89), OAc (Zlx-i-89 vs AA), C(3)-, C(23)-acetonide (Zlx-i-111 vs Zlx-i-112) increased the cytotoxicity. The oxidation of AA at C11 to ketone group was greatly reduced the cytotoxicity (Zlx-i-102 and Zlx-i-103). Based on these results, the SAR of AA to cytotoxicity is depicted in Fig. 3.

The effect of asiaticoside derivatives on the collagen content in HSC-T6 cells

Based on the cytotoxicity of AA derivatives, the appropriate concentrations of the compounds were chosen and added to the HSC-T6 cell culture to evaluate the effects on collagen synthesis. Exposure of HSC-T6 cells to the AA derivatives for 24 h reduced the collagen synthesis to 14%~48%, depending on the structure (Table II). Zlx-i-85, Zlx-i-87, Zlx-i-89 and Zlx-i-92 strongly inhibited collagen synthesis. However, no correlation between the inhibitory activity and the functional groups of R_1 to R_4 were noted. Jew *et al.* (2000) investigated the protective effects of the 11 AA derivatives that contained AA, AS and madecassic acid against β-amyloid-induced neurotoxicity. They reported several patterns in structure versus activity: the free C(28)-CO_2H derivative showed a higher protective activity against β-amyloid-induced neurotoxicity than methyl, octyloxymethyl and glycosyl ester derivatives; the triol groups are very important for biological activity, especially C(3)-OH and C(23)-OH. In this study, most of the AA derivatives were found to reduce collagen synthesis more effectively than AA or AS, but no patterns in structure versus activity could be observed.

Table II. EC_{50} values of cytotoxicity of asiaticoside derivatives and hydroxyproline contents after treatment of asiaticosides derivatives in HSC-T6 cells

	EC_{50} of cytotoxicity (μg/mL)	Hydroxyproline	
		Treatment (μg/mL)	% of control
Asiatic acid	29.80	10	81.9
Asiaticoside	>2000	100	86.1
zlx-i-69	24.90	5	83.5
zlx-i-70	16.20	5	60.1
zlx-i-85	23.10	5	52.7
zlx-i-87	25.20	5	52.8
zlx-i-89	9.10	5	55.1
zlx-i-92	25.90	10	54.4
zlx-i-100	42.40	10	56.8
zlx-i-102	140.60	25	66.5
zlx-i-103	>2000	100	71.0
zlx-i-111	5.56	2	73.7
zlx-i-112	7.57	2	71.8
zlx-i-113	26.70	10	62.8
zlx-i-123	31.50	20	84.9
zlx-i-132	620.10	100	69.1

The effect of asiaticoside derivatives on mRNA expression of hepatic fibrosis-related proteins

HSC-T6 cells in the presence of 10% fetal bovine serum expressed high basal levels of hepatic fibrosis-related protein mRNAs, such as α1 (I) procollagen, TGFβ1, TIMP1, TIMP2, and prolyl 4-hydroxylase α and β subunits. 24 h treatments of AA derivatives to cells did not significantly change the levels of cell expression, except α and β subunits of prolyl 4-hydroxylase and TIMP2 (Fig. 4). The inhibition of collagen synthesis by AA derivatives did not correlate with gene expression related to hepatic fibrosis, such as α1 (I) procollagen, TGFβ1 and TIMP1. These results indicate that the inhibition of collagen synthesis by most of AA deriveatives might be due to decrease the expression of TIMP2 and prolyl 4-hydroxylase which plays an important role in cells in collagen processing at the post-translational level. However, although zlx-1-69 and zlx-1-123 show relatively high hydroxyproline values, mRNA levels for α and β subunits of prolyl 4-hydroxylase were less expressed in comparison to other AA derivatives (Table II and Fig. 4). Thus, AA derivatives might be reduced the collagen content in HSC-T6 cells with several other mechanisms.

In summary, AA derivatives showed SAR to cytotoxicity (Fig. 3) but not to collagen synthesis in HSC-T6 cells. Because HSC play a central role in liver fibrosis, the inhibitory effect of them on HSC might reduce the pool of

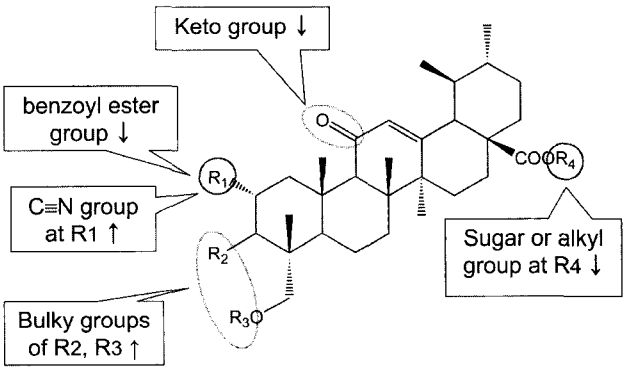


Fig. 3. The structural relationship of asiaticoside derivatives to cytotoxicity in HSC-T6 cells.

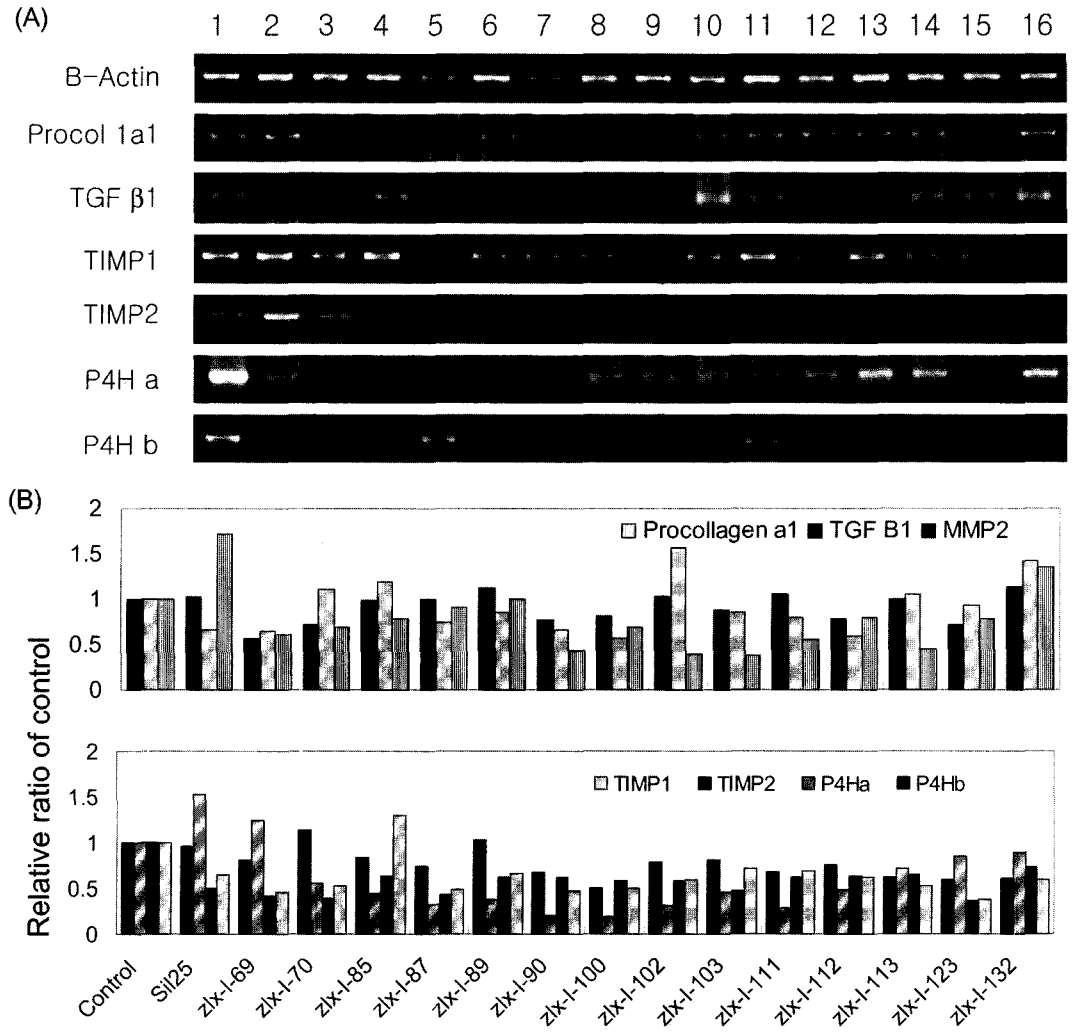


Fig. 4. Effects of asiaticoside derivatives on A) the gene expression related to hepatic fibrosis in HSC-T6 cells by RT-PCR and B) the analysis of PCR bands. Cells (3×10^4/mL) in the serum-free DMEM were treated for 24 h with non-cytotoxic doses of the asiaticoside derivatives. Total RNA was extracted using the Easyblue solution (Intron) from the cells following the manufactures procedure. The first-strand synthesis using 2 mg of total RNA as a template was performed at 42 °C for 1 h, using the oligo-dT primer and M-MLV reverse transcriptase (Promega). The integrity of the resultant cDNA was confirmed by PCR employing primers for β-actin. PCR bands were analyzed quantitatively using an analyzing software (TINA 2.0, Bio-Imager) and was normalized with that of β-actin. Lanes 1 : Control, 2 : Silibinin, 3 : zlx-l-69 (10 μg/mL), 4 : zlx-l-70 (10 μg/mL), 5 : zlx-l-85 (10 μg/mL), 6 : zlx-l-87 (10 μg/mL), 7 : zlx-l-89 (5μg/mL), 8 : zlx-l-92 (10 μg/mL), 9 : zlx-l-100 (20 μg/mL), 10 : zlx-l-102 (50 μg/mL), 11 : zlx-l-103(100 μg/mL), 12 : zlx-l-111 (2 μg/mL), 13 : zlx-l-112 (2 μg/mL), 14 : zlx-l-113 (10 μg/mL), 15 : zlx-l-123 (20 μg/mL), 16 : zlx-l-132 (100 μg/mL).

HSC and deposit of extracellular matrix, such as collagen within the liver. Their therapeutic potential and mechanism should be evaluated further.

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