Caffeoyl pyrrolidine derivative LY52 inhibits hepatocellular carcinoma invasion via suppressing matrix metalloproteinase-2

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Abstract

Purpose In this study, we examined the effects of LY52, a caffeoyl pyrrolidine derivative designed to fit the S'1 active pocket of gelatinases, on the expressions of matrix metalloproteinases and invasion abilities of hepatocellular carcinoma cells.

Methods The effects of LY52 on the proliferations of HepG2 (hepatitis B virus (HBV) negative) and HepG2.2.15 (HBV-producing) cells were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Gelatin zymography was used to detect the effects of LY52 on matrix metalloproteinases expressions and Western blot was used to detect matrix metalloproteinase-2 expressions. Transwell chamber assay was used to detect the effects of LY52 on invasion of the cells.

Results Gelatin zymography and Western blot showed that matrix metalloproteinase-2 expressions were inhibited by LY52 in a dose-dependent manner, and inhibitory rates

of LY52 on HepG2 cells were higher than on HepG2.2.15 cells. Transwell chamber showed that LY52 could significantly inhibit the invasion of both cells, although the inhibitory effects of LY52 on HepG2.2.15 cells were not as obvious as on HepG2 cells.

Conclusions These results suggested that LY52 might inhibit the invasion of hepatocellular carcinoma cells by suppressing matrix metalloproteinase-2, although the inhibitory effects of LY52 on HBV-negative cells were more obvious than that of HBV-infected cells.

Keywords LY52 · Hepatocellular carcinoma · Matrix metalloproteinases · Invasion

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in Asia. Although recent progress in the diagnosis and treatment modalities has improved the prognosis of patients with HCC, the long-term prognosis remains disappointing because of the frequent recurrence and the development of intrahepatic metastasis in 16–65% of HCC patients [1]. Hepatitis B virus (HBV) is recognized as a human carcinogen and is the cause of 60–80% of HCC worldwide [2]. A number of researches have shown that chronic infection by hepatitis virus, especially HBV, leads to the progression of chronic hepatitis to liver cirrhosis and contributes to HCC [3, 4]. It was also shown that HBV infection increased the invasion potential of HCC, though the role of HBV in the invasion and metastasis of HCC was not elucidated clearly [5].

During the process of tumor invasion, the destruction of the extracellular matrix (ECM), including basement membranes, is the initial step [6]. Gelatinases, including

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matrix metalloproteinase (MMP)-2 and MMP-9, play an important role in the degradation of ECM and basement membranes. Active MMP-2 and MMP-9 were proven to be the major enzymes responsible for the high gelatinolytic activity at the invasive front of HCCs [7, 8]. Therefore, regulation of MMP-2 and MMP-9 is important in the development of novel therapeutic strategies against intra-hepatic metastasis of HCCs. Three-dimensional structural analyses of MMP molecules showed that the S'1 active pocket in MMP-2 and MMP-9 was deeper than that of other types of MMPs, which provides a helpful clue for constructing gelatinase-specific inhibitors via structure-based design strategies [9, 10]. In our previous studies, we had constructed a series of caffeoyl pyrrolidine derivatives, which were designed based on a lead MMP inhibitor, CGS27023A, to fit the deeper S'1 pocket in the gelatinase molecules [11–13]. LY52 is one of these caffeoyl pyrrolidine derivatives that displayed a substantial inhibitory effect on gelatinase activity in vitro and in vivo [14]. However, the effect of LY52 on HCC cells was not investigated.

In this study, we examined the effects of LY52 on the expressions of MMPs and the invasion abilities of human HCC cell lines, HepG2 (HBV-negative cells) and HepG2.2.15 (HBV-infected cells).

Materials and methods

Materials

Caffeoyl pyrrolidine derivative LY52 (Fig. 1), white powder, insoluble in water, was designed and synthesized as described previously [12]. The compound was dissolved in dimethylsulfoxide for in vitro assays. Roswell Park Memorial Institute (RPMI) 1640 medium and high glucose Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen, Carlsbad, CA, USA. Cell proliferation kit I was purchased from Roche Diagnostic, Mannheim, Germany. Rabbit polyclonal anti-MMP-2

antibody was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Mouse monoclonal anti- β -actin antibody was from Sigma, St. Louis, MO, USA. BD Bio-Coat™ Tumor Invasion System was purchased from BD Biosciences (CA, USA). ECL Western Blotting Starter Kit was purchased from GE Healthcare Bio-Sciences, Piscataway, NJ, USA. Diff-Quik kit was purchased from Dade Behring, Newark, DE, USA.

Cells and cell culture conditions

Human HCC cell lines, HepG2 and HepG2.2.15, were purchased from the Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China. HepG2.2.15 is a cell line derived from HepG2 cells transfected with HBV DNA and can secrete surface antigen of HBV (HBsAg), core particles and virions [15, 16]. HepG2 cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS), and HepG2.2.15 cells in RPMI 1640 containing 10% heat-inactivated FBS supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), 2 mM glutamine and sodium bicarbonate (2.2 g/l) at 37°C in a humid 5% CO₂–air atmosphere.

Cell proliferation assay

The effects of LY52 on the proliferations of the two HCC cell lines were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [17]. Briefly, the cells were plated in 96-well culture plates at a density of 5×10^3 cells/well in 100 μ l culture medium containing different concentrations of LY52 (0, 0.1, 1, 10, 100, 200 and 1,000 μ g/ml). At the time points of 24, 48 and 72 h, 10 μ l of MTT labeling reagent was added to each well and the cells were incubated for 4 h in a humidified atmosphere with 5% CO₂ in air at 37°C. Then, 100 μ l of the solubilization solution was added into each well. Finally, optical densities (ODs) were monitored at 550 nm with 750 nm as a reference wavelength using an ELISA plate reader.

Gelatin zymography

The effects of LY52 on the supernatant MMP-2 and -9 expressions in the two cell lines were measured by gelatin zymography. Cells were cultured (2×10^6 cells/ml) in 24-well plates in the presence of various concentrations of LY52 for 24 h at 37°C in a humidified 5% CO₂ atmosphere. The supernatant (10 μ l) was subjected to electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel containing 1 mg/ml gelatin as a substrate. After electrophoresis, the gel was washed with 2% Triton X-100 solution for 1 h and then incubated in a developing buffer (50 mM Tris–HCl, pH 7.4,

Fig. 1 Structure of LY52

containing 5 mM CaCl_2 and 0.5 μM ZnCl_2) for 20 h at 37°C. Gels were stained with 0.05% Coomassie brilliant blue R-250 for 30 min and then destained in 10% acetic acid. Unstained bands corresponding to MMP-2 were quantified by densitometry using an electrophoresis image analysis system (FR980, Shanghai Furi Science and Technology, Shanghai, China).

Western blot analysis

The effects of LY52 on the MMP-2 expressions in the two cell lines were analyzed by Western blot. Briefly, cells (2×10^6 cells/ml) were seeded in a six-well plate and then treated with various concentrations of LY52 for 24 h. Then the cells were lysed with lysis buffer for 30 min at 4°C and the debris was removed by centrifugation at $20,000 \times g$ for 10 min. Equal amounts of protein (20 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel and then transferred to polyvinylidene difluoride membranes. The membranes were first incubated in blocking solution (5% skim milk) overnight at 4°C and then incubated with the first antibodies: anti-MMP-2 antibody (1:1,000-dilution) or anti- β -actin antibody (1:1,000-dilution) for 1 h at room temperature. After washing with TBST (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% Tween-20 and pH 8.0) three times, the membranes were incubated with HRP-labeled secondary antibodies (1:2,000-dilution) for 1 h. Finally, the reactivity was visualized by enhanced chemiluminescence using ECL Western Blotting Starter Kit.

Invasion assays

Cell invasion was evaluated using 24-well BD BioCoat Matrigel invasion chambers according to previous studies [18]. A suspension of cells (2×10^4 cells/100 μl) in non-serum medium was placed in the matrigel-coated filters. The wells below were filled with 0.75 ml medium supplemented with 5% FBS. After 22 h incubation in a humidified atmosphere with 5% CO_2 in air at 37°C, the non-migrated cells were removed by cotton swab and the migrated cells on the lower side of the filters were fixed and stained with Diff-Quick staining kit. Invaded cells were quantified by counting the stained cells in five random fields per membrane at $\times 200$ magnification.

Statistical analysis

All data are presented as mean (SD). Statistical significance was evaluated by SPSS 11.0 for Windows. Statistical significance was determined by Student's two-tailed *t* test. The limit of statistical significance was $P < 0.05$.

Results

Effects of LY52 on proliferations of the HepG2 and HepG2.2.15 cells

The effects of LY52 on the proliferations of HepG2 and HepG2.2.15 cells were analyzed by MTT assays. As shown in Fig. 2a, we did not find dose-dependent correlation between LY52 concentrations and proliferation of HepG2 in the incubation periods of 24 and 48 h, respectively. In the incubation periods of 72 h, LY52 can inhibit the proliferations of HepG2 cells in a dose-dependent manner. This inhibition of cell growth reached a plateau at a concentration of 200 $\mu\text{g}/\text{ml}$ and time period of 72 h. However, the inhibitory effects of LY52 in HepG2.2.15 cells were found in a dose- and time-dependent manner as shown in Fig. 2b. The cytotoxicity effects of LY52 on both cell lines also increased when the concentrations ($>1,000 \mu\text{g}/\text{ml}$) and incubation time (>48 h) increased, which was verified by Trypan blue staining (data not shown). To investigate the effects of LY52 on the MMPs' expressions and invasion abilities of HepG2 and HepG2.2.15 cells, we used concentrations of 0.1, 1, 10, 100 and 200 $\mu\text{g}/\text{ml}$ and an incubation period of 24 h in the following experiment.

Fig. 2 Effects of LY52 on the proliferations of **a** HepG2 and **b** HepG2.2.15 cells. Cells were treated with different concentrations of LY52 (0.1, 1, 10, 100, 200 and 1,000 $\mu\text{g}/\text{ml}$) for 24, 48 and 72 h

Effects of LY52 on MMP expressions

To analyze the effects of LY52 on MMP expressions in HepG2 and HepG2.2.15 cells, gelatin zymography was used to detect the MMP levels in the supernatants of HepG2 and HepG2.2.15 cells. Although zymography is not a strictly quantitative method, both MMP-2 and MMP-9 can be detected. Our results showed that pro- and active MMP-9 can only be detected in HepG2.2.15 cells, but not in HepG2 cells (Fig. 3). Pro-MMP-2, but not active MMP-2, can be detected in both HepG2 and HepG2.2.15 cells. The expression levels of MMP-9 and MMP-2 in HepG2.2.15 cells were much higher than that in HepG2 cells. Bar graphs were made using electrophoresis image analysis system. The effects of LY52 on MMP-9 expressions in HepG2.2.15 cells were not obvious (data not shown). The inhibition rates of 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$

Fig. 3 Effects of LY52 on MMP expressions of **a** HepG2 and **b** HepG2.2.15 cells by gelatin zymography. Cells were treated with different concentrations of LY52 (0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) for 24 h. The bar graphs show the inhibition rates of different concentrations of LY52 on MMP-2 expressions

of LY52 on pro-MMP-2 levels of HepG2 cells were 48.2, 32.0, 40.4, 78.5 and 82.1%, respectively, compared with the control group (100%). The inhibition rates of 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$ of LY52 on pro-MMP-2 levels of HepG2.2.15 cells were 39.0, 14.4, 18.5, 40.8 and 43.4%, respectively.

Effects of LY52 on MMP-2 expression

Since LY52 might selectively inhibit MMP-2 expression according to the gelatin zymography result, we determined MMP-2 expression in the two cell lines by Western blot. As shown in Fig. 4, the total MMP-2 expressions (pro- and active MMP-2) were inhibited by LY52 in a dose-

Fig. 4 Effects of LY52 on MMP-2 expressions of **a** HepG2 and **b** HepG2.2.15 cells by Western blot. Cells were treated with different concentrations of LY52 (0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$) for 24 h. The bar graphs show the inhibition rates of different concentrations of LY52 on total MMP-2 (pro- and active MMP-2) expressions

dependent manner in both cell lines, which were concordant with the zymography result. The bar graphs were also made using electrophoresis image analysis system. The inhibition rates of 0.1, 1, 10, 100 and 200 $\mu\text{g/ml}$ of LY52 on total MMP-2 levels of HepG2 cells were 19.2, 24.5, 41.2, 58.6 and 78.5%, respectively, while that of HepG2.2.15 cells were 7.7, 8.2, 17.6, 26.4 and 61.2%, respectively, compared with the corresponding control groups (100%).

Effects of LY52 on the invasion abilities of HepG2 and HepG2.2.15 cells

Effects of LY52 on the invasion abilities of the cells were examined in 24-well transwell chambers. As shown in Fig. 5, invasive HepG2.2.15 cells per field were significantly reduced in a dose-dependent manner after 1, 10, 100 and 200 $\mu\text{g/ml}$ of LY52 treatment. However, invasive HepG2.2.15 cells per field were not reduced in a dose-dependent manner after LY52 treatment. Invasive HepG2.2.15 cells per field were significantly reduced in 0.1, 1, 10 and 100 $\mu\text{g/ml}$ of LY52. Interestingly, at a concentration of 200 $\mu\text{g/ml}$, the inhibitory effect of LY52 on HepG2.2.15 cells was relatively weak, which might need further investigation. Additionally, the inhibition rates of LY52 on HepG2 cells were higher than that of HepG2.2.15 cells at the corresponding concentrations (data not shown).

Discussion

Caffeoyl pyrrolidine derivative LY52 was designed based on the analog reference drug CGS27023A [19, 20], a broad-spectrum MMP inhibitor that inhibits the activity and expressions of MMP-1, 2, 3 and 9. Since the S'1 pocket of gelatinase is deeper than that of MMP-3, we designed a backbone of hydroxyproline linked with the longer and flexible caffeoyl group to extend into the deeper S'1 pocket

[12, 13]. Hydroxyproline is known to be a specific amino acid of collagens, which are the substrate of gelatinases. The *p*-(methylphenyl) sulfonyl group can extend into the S'1 active pocket of MMP-2 and -9, which may increase the flexibility of the chemical structure to combine with the active site. Carboxylic acid, which is a metabolized product of carbomethoxy derivatives, might chelate zinc ions, the essential ions for enzyme activity site [12, 13]. These structures in LY52 molecule might improve its selectivity against gelatinases, such as MMP-2 and MMP-9.

Our previous studies showed that LY52 could effectively inhibit the invasion and metastasis of breast cancer cells by suppressing the activity of MMPs. Enhanced expression of MMPs, especial MMP-2 and MMP-9, were frequently found in HCC tissues and cell lines, and implicated as playing an important role in the invasion and metastasis process of HCC [5, 21, 22]. In this study, we assessed the effects of LY52 on MMP expressions and invasion properties of HCC cells. Previous studies showed that the HBV was a major cause of HCC in which the prognosis is very poor due to its frequent invasion and metastasis [23, 24]. It was suggested that HBV infection may facilitate tumor cell invasion by upregulation of MMPs and subsequent destruction of the ECM [5, 25, 26]. In this study, we chose two cell lines, HepG2 and HepG2.2.15, which originated from the same clone of HCC. The difference between the two cell lines is the HBV infection.

The inhibition effects of LY52 on MMP-2 and MMP-9 expressions were determined by gelatin zymography. Our results implicated that the MMP expression pattern is significantly different in the two cell lines. MMP-2 levels in HepG2.2.15 cells were obviously higher than that in HepG2 cells, and MMP-9 can only be detected in HepG2.2.15 cells (Fig. 3), which might be induced by HBV infection in the HepG2.2.15 cells. LY52 could effectively suppress MMP-2 expressions in both HepG2 and HepG2.2.15 cells, in concentrations that the compound did not display a significant anti-proliferation effect or cytotoxicity. Western blot also showed that LY52 could effectively suppress MMP-2 expressions in both HepG2 and HepG2.2.15 cells, and the inhibition effect of LY52 on MMP-2 expressions was more obvious in HepG2 cells than in HepG2.2.15 cells (Fig. 4). Furthermore, LY52 could effectively inhibit the invasion of both HepG2 and HepG2.2.15 cells, and the inhibition effects of LY52 on HepG2 cells were more obvious than that of HepG2.2.15 cells in the corresponding concentrations. These results suggested that LY52 could effectively inhibit the invasion of HCC cells via suppressing MMP-2 activity, although the inhibitory effects of LY52 on HBV-infected HCC cells was not as obvious as on HCC cells without HBV infection. Since HBV infection may facilitate tumor cell invasion by

Fig. 5 Effects of LY52 on the invasive HepG2 and HepG2.2.15 cells per field by Transwell assays

upregulation of MMPs [5, 24, 25], the concentrations of LY52 we used might be not high enough to inhibit MMP expressions by the cells. Considering the cytotoxicity of the drug, higher concentrations of LY52 have not been used in this study. This result reminds us that the anti-cancer effects of some anti-HCC drugs might be different in HBV-infected HCC cells.

In summary, our results showed the disparities in the expression of MMP-2 and MMP-9 in the HCC cells with or without HBV infection. LY52 might inhibit HCC cell invasion by suppressing MMP-2 activity, although the inhibitory effects of LY52 on HBV-infected HCC cells were not as obvious as HCC cells without HBV infection.

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