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貴財団より助成金を受領して行った研究テーマについて報告いたします。

添付資料：研究報告書

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2．研究テーマ

肝細胞がん肝内転移抑制剤候補化合物としての新規ピロリジン誘導体の評価

3．成果の概要（100字程度）

肝細胞がんの治療において外科的切除は有効的な方法であるが、術後の再発率は6割を超えており、血管侵襲や周囲への増殖などの肝内進展がその最大の原因と考えられている。本研究は、肝がんの肝内転移を抑制する新規ピロリジン誘導体に着目し、肝がんの肝内転移を抑制する化学療法剤の開発を目指して一連の研究を行った。

4．研究組織

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—日中医学協会助成事業—

肝細胞がん肝内転移抑制剤候補化合物としての新規ピロリジン誘導体の評価

——Novel Pyrrolidine Derivatives as Candidates for Inhibitors against Intrahepatic Metastasis of Hepatocellular Carcinoma

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Abstract. LY52 is a caffeoyl pyrrolidine derivative designed to fit S'_1 active pocket of gelatinases that act in tumor invasion and metastasis. Here we examined the effect of LY52 on angiogenesis and the subsequent invasion and metastasis of human hepatocellular carcinoma (HCC). Anti-metastasis ability of LY52 was first evaluated in the pulmonary metastasis of B16F10 murine melanoma cells in C57/BL6 mice. Growth inhibition of LY52 on tumor cells was assayed in the human HCC SMMC-7721 tumor xenografts in nude mice. Vascular endothelial growth factor (VEGF) and vascular endothelial cell marker CD34 expression were also evaluated immunohistochemically in the SMMC-7721 tumor xenografts in nude mice. Tumor microvessel density (MVD) was quantified according to immunohistochemical staining for CD34 in tumor xenografts. Furthermore, effect of LY52 on angiogenesis *in vitro* was tested in the chicken chorioallantoic membrane (CAM) model. LY52 showed an inhibitory effect on pulmonary metastasis of B16F10 murine melanoma cells in C57/BL6 mice without significant toxic effects. The growth of SMMC-7721 cells in nude mice was significantly inhibited after treatment with LY52 for 4 continuous weeks. Immunohistochemical study showed that VEGF and CD34 expression in the tumor xenografts were suppressed in the presence of LY52. Accordingly, MVD was significantly decreased in the LY52-treated group compared with the control group. A dose-dependent inhibition on angiogenesis was demonstrated in the chicken CAM after incubation with LY52 for 72 hour. Thus, LY52, a caffeoyl pyrrolidine derivative, would be a lead compound to suppress intrahepatic invasion and metastasis of hepatocellular carcinoma via inhibiting proteolytic activities of MMP-2 and MMP-9.

Key words: Caffeoyl pyrrolidine derivative, LY52, human hepatocellular carcinoma, invasion, metastasis, SMMC-7721 cell line, angiogenesis, matrix metalloproteinase

Introduction

Hepatocellular carcinoma (HCC) is one of the most common forms of malignant cancer, especially in Asian countries. Recent progress in the diagnosis and treatment modalities has improved the prognosis of patients with HCC. However, the long-term prognosis remains disappointing because of the frequent recurrence and the development of intrahepatic metastasis of HCCs in 16%-65% of patients. The intrahepatic metastasis is mostly caused by the portal vein invasion (PVI) of HCC, which eventually results in the spread of cancer cells into the liver. PVI, the first and the key step of intrahepatic metastasis of HCCs, is initiated by extracellular matrix (ECM) degradation and determines the prognosis of patients with HCCs. As HCC is a highly vascular tumor, the

progression of HCC is greatly related to active neovascularization, which helps in the nutrient and oxygen supply. Angiogenesis, the process of forming new blood vessels from existing ones, requires degradation of the vascular basement membrane and remodeling of the ECM in order to allow endothelial cells to migrate and invade into the surrounding tissue. Thus, degrdation of basement membranes and ECM might closely correlate with angiogenesis and intrahepatic metastasis of HCCs.

Gelatinases, including matrix metalloproteinase (MMP)-2 and MMP-9, play an important role in degradation of basement membrane collagen type IV, which is associated with tumor invasion and metastasis. Active MMP-2 and MMP-9 were proven to be the major enzymes responsible for the high gelatinolytic activiy at the invasive front of HCCs. Therefore, regulation of MMP-2 and MMP-9 is important in the development of novel therapeutic strategies against angiogenesis and intrahepatic metastasis of HCCs.

Three-dimensional structure analysis of MMP molecules showed that the S'1 active pocket in MMP-2 and MMP -9 is deeper than that of the other type of MMPs such as MMP-3. This has provided helpful clues when using structure-based design strategies to discover novel MMP inhibitors that selectively block the activity of MMP-2 and MMP -9. We have previously reported that some caffeoyl pyrrolidine derivatives, which were designed based on a lead MMP inhibitor CGS27023A, were potential gelatinase inhibitors and suggested that LY52, one of these caffeoyl pyrrolidine derivatives, might suppress tumor invasion and metastasis via selectively blocking proteolytic activities of MMP-2 and MMP-9. But the effect of LY52 on tumor angiogenesis has not been studied. In the present study, we examined the effect of LY52 on angiogenesis and growth and metastasis of human HCC SMMC-7721 cells.

Materials

Reagents. Caffeoyl pyrrolidine derivative LY52 (**Fig. 1**), white power, insoluble in water, was designed and synthesized as described previously. The compound was dissolved in dimethylsulfoxide for in vitro assay and in 5% amylum for in vivo study. Monoclonal antibody against vascular endothelial growth factor (VEGF), monoclonal antibody against CD34 and peroxidase-conjugated affinipure goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). White chicken eggs were provided by Institute of Animal Sciences, Shandong Academy of Agricultural Sciences (Jinan, China).

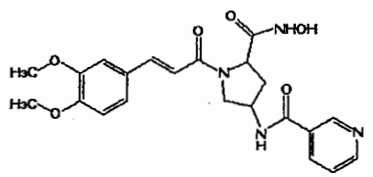

Fig.1 *Chemical structure of LY52.*

Cells and animals. Human HCC SMMC-7721 cell line, which simultaneously expresses both MMP-2 and MMP-9, was provided by Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). B16F10 murine melanoma cell line was purchased from the Institute of Materia Medica, Chinese Academy of Medical Sciences (Beijing, China). Female C57/BL6 mice, 5-6 weeks of age, and female Balb/c athymic (nu+/nu+) mice, 19-22 g, were purchased from the Institute of Experimental Animal, Chinese Academy of Medical Science.

Results

Inhibition of pulmonary metastasis of B16F10 melanoma cells in C57BL/6 mice.

Anti-metastasis ability of LY52 was evaluated in the pulmonary metastasis model of B16F10 murine melanoma cells in C57/BL6 mice. As shown in **Table 1**, LY52 significantly reduced the number of pulmonary metastatic nodules of B16F10 melanoma cells in C57BL/6 mice after continuous treatment for 3 weeks.

Inhibition of the growth of SMMC-7721 cells in nude mice. The nude mice inoculated with SMMC-7721 cells were treated with LY52 for 4 continuous weeks. Tumor weights of each group were shown in **Table 2**. The inhibition rates by 50 and 100 mg/kg of LY52 were 23.6 and 34.1%, respectively.

Inhibition of VEGF expression in SMMC-7721 tumor xenografts. VEGF expression in SMMC-7721 tumor xenografts was immunohistochemically examined. As shown in **Fig. 2A** the inhibition rates by 25 and 100 mg/kg of LY52 were 32.3 and 52.3%, respectively.

Inhibition of MVD-CD34 in SMMC-7721 tumor xenografts. Tumor MVD was evaluated by immunostaining for CD34 expression in SMMC-7721 tumor xenografts. LY52 significantly inhibited the CD34 expression in SMMC-7721 tumor xenografts in nude mice. As shown in **Fig. 2B** at the doses of 0, 50 and 100 mg/kg of LY52, CD34 expression rates were 42.1±6.1, 34.9±2.8* and 28.5±3.1*, respectively (*p<0.05 vs control).

Inhibition of angiogenesis in chicken CAM. As shown in **Fig. 3A**, dramatic vasculature with intact structure was visible in the CAMs of the control group. LY52 significantly inhibited the angiogenesis in the CAMs after incubating for 72 hours. In the doses of 1 and 10 μg/0.5cm2, LY52 slightly inhibited the blood-vessels growth and branching (**Fig. 3B** and **3C**, p>0.05). In the dose of 100 μg/0.5cm2, vasculature density was significantly reduced and vessel structure disordered with the main vessel branches intervening, tortuous and attenuated (**Fig. 3D**). At the doses of 1, 10 and 100μg/0.5cm2 of LY52, VI was 92.8±1.9, 74.2±2.1 and 59.1±1.3% (p<0.05), respectively.

Table 1. Effect of LY52 on pulmonary metastasis of B16F10 melanoma cells in C57BL/6 mice.

Dosage (mg/kg)	Mice (n)	Mice weight (g)	Lung weight (g)	Foci/lung (n)	Inhibition (%)
0	10	22.5±2.8	0.13±0.03	88.6±11.7	-
25	9	23.1±3.1	0.12±0.01	65.0±12.3*	24.4
50	10	21.7±2.1	0.12±0.01	49.1±11.3*	43.5
100	10	21.3±3.6	0.12±0.02	35.3±10.2*	60.2
Carboxylates	7	18.2±4.1	0.13±0.02	68.6±14.4*	22.6

Table 2. Effect of LY 52 on the growth of SMMC-7721 tumor xenografts in nude mice.

Dosage (mg/kg)	Mice (n)	Mice weight (g)	Tumor weight (g)	Inhibition (%)
0	6	23.1±3.2	2.34±0.63	-
50	6	23.1±3.1	1.76±0.81*	23.6
100	6	21.7±2.1	1.45±0.96*	34.1
Carboxylates	6	23.2±4.1	1.53±1.12*	22.6

Carboxylates, 100 mg/kg. *$p<0.05$ *vs* control group

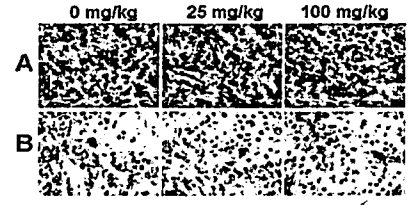

Fig. 2. *Immunohistochemical staining of VEGF (Fig. 2A) in the SMMC-7721 tumor xenografts in nude mice and CD34(Fig. 2B) in the vascular endothelial cells of the SMMC-7721 tumor xenografts (Original magnification: ×400) with LY52 treatment at 0, 25 mg/kg, and 100 mg/kg.*

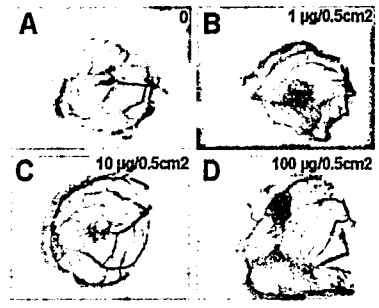

Fig. 3 *Effect of LY52 on the angiogenesis in chicken CAMs. CAMs were exposed to 0.5 cm^2 of filter papers containing 10 μl of different doses of LY52. Eggs were sealed and incubated under constant humidity for 72 h incubation. CAMs were collected and the vessels were counted as described in the method.*

Discussion

HCC cells possessed gelatinolytic activity, which was significantly and closely associated with cancer invasion to the capsule and also to the portal veins. Gelatinases especially MMP-2 and MMP-9 play an important role in regulating processes of tumor angiogenesis, invasion and metastasis. LY52 has an inhibitory effect on the proteolytic activity of MMP-2 and MMP-9 in carcinoma cells. Thus, the present study focused on effects of LY52 on angiogenesis, growth and metastasis of HCCs. First,

examination for anti-metastatic ability of LY52 in vivo was performed with pulmonary metastatic model of B16F10 melanoma cells in C57BL/6 mice. Oral administration of LY52 significantly prevented the pulmonary metastasis of B16F10 cells in mice devoid of toxic effects (**Table 1**). Then, Human HCC MMC-7721 cells, simutaneously express MMP-2 and MMP-9, were selected to inoculate to nude mice to form tumor xenografts, and the effect of LY52 on the growth and angiogenesis in tumor xenografts was examined. LY52 significantly inhibited the growth of MMC-7721 tumor xenografts (**Table 2**). The angiogenetic activity is reflected in the development of novel microvessels in tumor tissue that is quantified by the intratumoral MVD. Previous studies showed that high tumor MVD-CD34 was a significant predictor of tumor recurrence after resection of small HCCs < 5 cm. VEGF is one of the most extensively investigated angiogenetic factors. Increased expression of both VEGF and CD34 enhanced the adhesion of tumor cells to basement membrane and the release of MMP-2 and MMP-9. Thus, VEGF expression and MVD-CD34 were used as predictive factors of the response to anti-angiogenetic drugs in our study. Immunohistochemical studies showed that LY52 significantly inhibited VEGF expression and MVD-CD34 in SMMC-7721 tumor xenografts (**Fig. 2**). LY52 was also found to directly inhibit angiogenesis in the chicken CAM assay (**Fig. 3**). These results showed that LY52 inhibited angiogenesis both in vivo and in vitro, as well as growth and metastasis of HCCs.

LY52 was designed to specifically block the activity of MMP-2 and MMP-9. Thus, our studies suggested that LY52 might inhibit angiogenesis in invasion and metastasis of HCCs via blocking the proteolytic activities of MMP-2 and MMP-9. The metastasis of tumor cells is a multistep and extremely complex process in which many factors participate. LY52 can not be involved in the full process. However, our preliminary results suggested that LY52 might be a better candidate compound for anti-invasion and anti-metastasis of HCCs at present.

Parts of this study were presented as follows:

1. Qu X, Yuan Y, Tian Z, Xu W, Chen M, Cui S, Guo Q, Gai R, Makuuchi M, Nakata M, Tang W: Using caffeoyl pyrrolidine derivative LY52, a potential inhibitor of matrix metalloproteinase-2, to suppress tumor invasion and metastasis. *Int J Mol Med. 2006; 18 (4), 609-614.*
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