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貴財団より助成金を受領して行った調査・共同研究について報告いたします。

添付資料：**研究報告書**

受給者氏名：　　許　煥麗　　㊞
所属機関名：東京大学大学院医学系研究科
所属部署名：肝胆膵外科　　職名：客員研究員
所在地：〒113-8655 東京都文京区本郷7-3-1
東京大学医学部附属病院
電　　話：03-5800-8654　　内線：33321

1．助成金額：　1,000,000　円

2．研究テーマ

先進的スクリーニング技術を導入したウイルス性肝炎に対する新規治療薬の開発

3．成果の概要

本研究はウイルス性肝炎の発症及び関連疾患への進行を抑制する化学療法剤を開発することを目的とし、新規に合成された化合物も含めて、基礎生命科学的手法を用いた in vitro 研究及び in vivo 研究を行うことにより、有効性の高い化合物をライブラリから抽出し、有効性が評価された化合物による疾患制御のメカニズムを基礎医学的な視点から解析することを展開した。

4．研究組織

日本側研究者氏名：許　煥麗	職名：客員研究員
所属機関名：東京大学大学院医学系研究科	部署名：肝胆膵外科
中国側研究者氏名：杜　冠華	職名：副所長・教授
所属機関名：中国医学科学院・協和医科大学	部署名：薬物研究所

肝癌・ウイルス性肝炎に対する新規治療薬の開発と評価

Evaluation of new chemotherapeutic agents for hepatocellular carcinoma with hepatitis B virus infection

研究代表者名：許　煥麗
日本研究機関名：東京大学医学部附属病院・肝胆膵外科学
中国側共同研究代表者名：杜　冠華
中国所属機関名：中国医学科学院・協和医科大学・薬物研究所
中国側共同研究者名：趙　新（解放軍第302医院・医），崔 暁燕（川大・医），徐 文方（山大・薬）
日本側共同研究者名：稲垣 善則(東大・医)，唐　偉(東大・医)

Abstract

To find effective chemotherapeutic agents for hepatocellular carcinoma (HCC) with hepatitis B virus (HBV) infection, we evaluated the effects of newly-synthesized compounds 24F and LY52 on the metastasis of a HBV infected HCC cell line and the anti-HBV activities of cinobufacini and its active components bufalin and cinobufagin in this cell line. The effects of these drugs on the cell proliferations were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide assays. The effect of 24F on aminopeptidase (APN) activity was measured by a spectrophotometric method using L-leucine-*p*-nitroanilide as a substrate of APN. The effects of LY52 on the MMPs expressions were measured by gelatin zymography. The effects of 24F and LY52 on cell invasion were performed by 24-well invasion chambers. Effects of Cinobufacini, Bufalin, and cinobufagin on HBV antigens and DNA were quantified with a HBV DNA quantitative kit. The APN activity was inhibited in the presence of 24F in dose-dependent manner. The effects of LY52 on MMP-9 expressions in HepG2.2.15 cells were not obvious. The inhibition rates of LY52 on pro-MMP-2 levels of HepG2.2.15 cells were in a dose-dependent manner. 24F could inhibit the invasion of HepG2.2.15 cells, which displayed 56% of inhibition rate in the concentration of 100 μg/mL. LY52 could also effectively inhibit the invasion of HepG2.2.15 cells, although a dose dependent manner was not found. The effect of cinobufacini on secretion of HBsAg, HBeAg, and HBcrAg was promoted in a time-dependent manner. It was more effective than its components bufalin and cinobufagin in inhibiting the secretion of HBV antigens. These result showed that 24F and LY52 might be effective anti-metastasis regents for HCCs, even when the HCC cells were infected by HBV. Cinobufacini may serve as an anti-viral therapeutic agent for the management of HBV infection, which warrants further investigation.

Key words: Hepatitis B virus, hepatocellular carcinoma, Aminopeptidase N inhibitor, matrix metalloproteinases inhibitor, Cinobufacini

Introduction

Hepatitis B virus (HBV) is recognized as the leading cause of chronic hepatitis and the cause of 60-80% of hepatocellular carcinoma (HCC) worldwide. 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 contribute to HCC. It was also showed 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. Thus, the discovery and development of novel antiviral drugs for HCC is urgently needed. Aminopeptidase N (APN/CD13) inhibitor 24F and matrix metalloproteinases (MMPs) inhibitor LY52 were newly-synthesized compounds by our research group. Our previous studies showed that 24F could inhibit the activity of the targeted enzyme APN and suppress the invasive capacity of HCC cells, and LY52 could inhibit the

invasion and metastasis of HCCs via blocking the proteolytic activities of MMPs. In this study, we evaluated the effects of 24F and LY52 on the invasion ability of a HBV infected HCC cell line, HepG2.2.15.

Cinobufacini (Huachansu), a Chinese medicine prepared from this toad skin, has been extensively used in clinics to treat a number of diseases, such as malignant tumors, chronic hepatitis B. In this study, we also evaluated the anti-hepatitis B virus activities of cinobufacini and its active components bufalin and cinobufagin in HepG2.2.15 cells.

Materials and Methods

Reagents. The hydroxamic acid derivatives 24F was synthesized as one of series of cyclic-imide peptidomimetics with free amino group by using 3D-QSAR model[1]. Caffeoyl pyrrolidine derivative LY52, was designed and synthesized as described previously[2]. LY52 was dissolved in dimethylsulfoxide and 24F was dissolved in phosphate-buffered saline for *in vitro* studies. Cinobufacini was obtained from Anhui Jinchan Biochemical Co., Ltd., China. Bufalin and cinobufagin were purchased from Sigma.

Cell culture and treatment. The human HBV-transfected cell line HepG2.2.15 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 200 μg/mL G418 at 37 °C in a humidified incubator with 5% CO_2. The effects of these drugs on the cell proliferations were detected by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assays. The effect of 24F on APN activity was measured by a spectrophotometric method using L-leucine-*p*-nitroanilide as a substrate of APN. The effects of LY52 on the MMP-2 and -9 expressions were measured by gelatin zymography. The effects of 24F and LY52 on cell invasion was performed by 24-well BD BioCoat Matrigel invasion chambers. Effects of Cinobufacini, Bufalin, and cinobufagin on HBV antigens and DNA were quantified with a HBV DNA quantitative kit.

Results

Effects on proliferations of HepG2.2.15 cells

The HepG2.2.15 cell growth could be inhibited by 24F, but there was no significant difference in the inhibition rate among 1-200 μg/mL of 24F. No acute cytotoxic effect was observed by trypan blue staining. The inhibitory effects of LY52 on HepG2.2.15 cells proliferations increased as the concentrations and incubation periods increased. No obvious inhibitory effects of LY52 were found in lower concentrations (<200 μg/mL) and in shorter incubation period (24 h), which was also verified by trypan blue staining. Cinobufacini at the concentrations below 20 μg/mL, bufalin at the concentrations below 10^{-2} μM, and cinobufagin at the concentrations below 10^{-1} μM were non-toxic to HepG2.2.15 cells. Cinobufacini, bufalin, and cinobufagin could significantly inhibit the growth of HepG2.2.15 cells at the concentrations above 20 μg/mL, 10^{-2} μM, and 10^{-1} μM, respectively.

Effects of 24F on aminopeptidase activity

The aminopeptidase activity was inhibited in the presence of 24F in dose-dependent manner and the inhibition rate of ΔA/min under 0.27 mM (100 μg/mL) of 24F was around 25% compared with the negative control. In this analysis, IC_{50} of 24F (the volume of 24F that displayed 50% inhibition of enzyme activity) was calculated 1.88 mM.

Effects of LY52 on MMPs activities

The effects of LY52 on MMP-9 expressions in HepG2.2.15 cells were not obvious. The inhibition rates of 0.1, 1, 10, 100, and 200 μg/ml of LY52 on pro-MMP-2 levels of HepG2.2.15 cells were 4.0%, 9.4%, 11.5%, 15.8%, and 34.4%, respectively, compared with the control group (100%).

Effects of 24F and LY52 on the invasion of HepG2.2.15 cells

The number of invading cells was significantly decreased in the presence of 100 μg/mL of 24F compared with that in non-treated cells. This result suggested that 24F has an ability to inhibit the invasion of HepG2.2.15 cells, which displayed 56% of inhibition rate in the sample incubated with 100 μg/mL of 24F. LY52 could also effectively inhibit

the invasion of HepG2.2.15 cells, although a dose dependent manner was not found. The inhibition rates of 0.1, 1, 10, 100, and 200 μg/mL of LY52 on invasion abilities of the HepG2.2.15 cells were 21.8%, 26.4%, 43.3%, 50.2%, and 19.7%, respectively.

Effects of cinobufacini, bufalin, and cinobufagin on the HBV antigens and DNA

The data clearly showed that the inhibitory effect of cinobufacini on secretion of three HBV antigens (HBsAg, HBeAg, and HBcrAg) was promoted in a time-dependent manner. On day 6 cinobufacini at the concentration of 1 μg/mL significantly reduced the secretion of HBsAg, HBeAg, and HBcrAg, which was more potent than the positive control 3TC (100 μg/mL) in inhibiting HBV antigen secretion. On day 6, bufalin at the concentration of 10^{-4} μM significantly inhibited secretion of HBeAg and HBcrAg at the rates of 11.36 and 19.58%, respectively. In this concentration of bufalin was more potent than the positive control 3TC in inhibiting HBcrAg secretion. The data for bufalin at 10^{-4} μM on day 3 and 6 showed that the inhibitory effects of bufalin on secretion of two HBV antigens (HBeAg and HBcrAg) were time-dependent. After incubation with cinobufagin for 3 days or 6 days, secretion of HBeAg and HBcrAg in the culture medium was slightly less than that with the control. Moreover, cinobufagin at a concentration of 10^{-3} μM on day 3 exhibited the same potent activity as the positive control 3TC (100 μg/mL) in terms of the inhibition of HBeAg secretion.

Discussion

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 HCCs patients. Since APN functions to degrade extracellular matrix and thereby promote the cancer cell invasion and metastasis, inhibition of APN function would have a significant role in the development of cancer chemotherapeutic agents. This study showed that our newly-developed APN/CD13 inhibitor 24F can inhibit the activity of the targeted enzyme APN and suppress the invasive capacity of HCC cell. It was suggested that HBV infection may facilitate tumor cell invasion by upregulation of MMPs and subsequent destruction of the extracellular matrix. In this study, we assessed the effects of MMPs inhibitor LY52 on MMPs expressions in HepG2.2.15 cells, which is originated from the same clone of HCC. It was suggested that LY52 could effectively inhibit invasion of HCC cells by suppressing MMP-2 expressions. These result showed that 24F and LY 52 might be effective anti-metastasis regents for HCCs, even when the HCC cells were infected by HBV.

We also evaluated the anti-hepatitis B virus activities of cinobufacini and its active components bufalin and cinobufagin in HepG2.2.15 cells. It was demonstrated that the effects of cinobufacini on secretion of HBsAg, HBeAg, and HBcrAg was promoted in a time-dependent manner. It was more effective than its components bufalin and cinobufagin in inhibiting the secretion of HBV antigens. The present findings suggested cinobufacini may serve as an anti-viral therapeutic agent for the management of HBV infection, which warrants further investigation.

参考文献

1 Cui SX, Qu XJ, Gao ZH, et al. Targeting aminopeptidase N (APN/CD13) with cyclic-imide peptidomimetics derivative CIP-13F inhibits the growth of human ovarian carcinoma cells. Cancer Lett 2009.

2 Qu X, Yuan Y, Tian Z, et al. 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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