Contents lists available at ScienceDirect IF: 0.926

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi: 10.1016/S1995-7645(14)60354-0

HS-4, a highly potent inhibitor of cell proliferation of human cancer cell

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ARTICLE INFO

Article history:
Received 15 February 2015
Received in revised form 20 March 2015
Accepted 15 April 2015
Available online 20 May 2015

Keywords:
HS-4
Antitumor
Orthotopic xenotransplantation
In vivo
BeL-7402 cells

ABSTRACT

Objective: To investigate the antitumor activity of the compound HS-4 and the action mechanism. **Methods:** MTT method was used to test *in vitro* antitumor activity of the compound HS-4. Orthotopic xenotransplantation tumor model of liver cancer was established in nude mice, and, *in vivo* antitumor activity of compound HS-4 was tested with a small animal *in-vivo* imaging system. Sequencing of small RNA library and RNA library was performed in HS-4 treated tumor cell group and control group to investigate the anti-cancer mechanism of HS-4 at level of functional genomics, using high-throughput sequencing technology. **Results:** HS-4 was found to have relatively high *in-vitro* antitumor activity against liver cancer cells, gastric cancer cells, renal cancer cells, lung cancer cells, breast cancer cells and colon cancer cells. The IC_{50} values against SMMC-7721 and Bel-7402 of liver cancer cells were 0.14 and 0.13 nmol/L respectively, while the IC_{50} values against MGC-803 and SGC-7901 of gastric cancer cells were 0.19 and 0.21 nmol/L, respectively. It was demonstrated that HS- 4 possessed a better therapeutic effect in liver cancer. **Conclusions:** A new reliable orthotopic xenotransplantation tumor model of liver cancer in nude mice is established. The new compounds HS-4 was found to possess relatively high *in vivo* and *in vitro* antitumor activity against liver cancer cells.

1. Introduction

HS-4 is a compound isolated from Mangrove Kandelia candel in Dongzhaigang, Hainan province in China. Its molecular weight is 529, and the molecular formula is $C_{25}H_{30}O_{14}$. The structural formula is shown in figure 1. It was demonstrated by the results of in vitro antitumor experiment to have relatively high antitumor activity against liver cancer cells. Primary hepatic carcinoma (PHC) is one of the most deadly cancers[1], and there are 626 000 new cases presented every year in China, more than half a million people are died from liver cancer[2]. China has become the country with highest incidence of liver cancer in the world[3], which accounts for about 55% in the world[3]. The causes of death in patients with liver cancer are primarily related to the high metastasis and high recurrence[1]. At present, chemotherapy is still an important treatment method for liver cancer[3], but issues such as the drug resistance, liver cirrhosis and hepatic hypofunction are often presented in treatment. Therefore, it is becoming increasingly urgent for seeking new antitumor drugs, new prognostic markers and therapeutic targets.

2. Materials and methods

2.1. Material

HS-4 was prepared by our laboratory, the chemical name: 10-(4- hydroxy-3',5'-dimethoxy benzoic acyl)-Geniposidic acid, the molecular weight is 529, the molecular formula is $C_{25}H_{30}O_{14}$. The liver cancer cells (Bel-7402 and SMMC-7721), gastric cancer cells (MGC-803H and SGC-7901), breast cancer cells(MCF-7), renal cancer cells (TE-1) and colon cancer cells (HT-29) were purchased

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Foundation project: This work is supported by National Natural Science Foundation of China (No. 81160408).

from Shanghai Cell Bank of Chinese Academy of Sciences, while the bio-luminescence marking was performed for Bel-7402 by Shanghai Lecheng Biological Technology Co., Ltd ,China. RPMI 1640 culture medium was purchased from G ibco (NewYork, NY, USA). Trypsin-ethylenediamine tetraacetic acid (EDTA), MTT, and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO , USA). Fetal bovine serum (FBS) was from Zhejiang Tian hang Biological Technology Co., Ltd., China (Hangzhou, Zhejiang Province, China). BALB/c nude mice were purchased from the Medical Experimental Animal Center of Guangdong province, China.

2.2. In vitro antitumor experiment

MTT method was used to screen *in vitro* antitumor activity of the compound HS-4 against tumor cells. The tumor cell was cultured in cell culture bottles, and digested with 0.25% Trypsin-EDTA. The cells were collected by centrifugation to prepare cell suspension, and they were added to 96-well plate by 4 000 cells/200 AL/well. Then the cells were put into CO_2 incubator for culture (concentration of CO_2 was 5%, the temperature was 37 ℃). After cultured for 24 hours, 2 μL of compound HS-4 DMSO solution with different concentrations was added into the cells. Then cells were continuously cultured for another 24 hours, before 20 μL (5 mg/mL) MTT solution was added. After the MTT solution addition, the cells were cultured for another 4 hours, the liquid in 96 well-plate was carefully removed, 150 μL DMSO was added to each well. OD value was tested with ELIASA at detection wave length of 492 nm. At the end, the *in-vitro* tumor inhibitory rate of compound HS-4 was calculated with the formula as below:

IR %=(1-ODHS-4/OD contrast) 100% and IC_{50} values.

2.3. Establishment of subcutaneous xenotransplantation model of tumor

Healthy BALB/c nude mice were selected, and 0.2 mL suspension of tumor cells with cell concentration of about 1×10^7 cells/mL was subcutaneously injected at their right axillary fossa. Put the mice back to the cage to breed after injection was completed, and observe growth of tumor cells.

2.4. Therapeutic effect of compound HS-4 in multiple subcutaneously xenotransplanted tumor

We had established subcutaneously xenotransplanted tumor model of liver cancer cell Bel-7402, gastric cancer cell SGC-7901, renal cancer cell ACHN, lung cancer cell A549, breast cancer cell MCF-7 and colon cancer cell HT-29 in nude mice to screen *in vivo* antitumor activity of compound HS-4. The mice were divided into HS-4 group and solvent control group in the experiment, wherein, HS-4 was classified into 3 dose groups based on the dosage provided as 4, 2 and 10 mg/kg, respectively. There were 6 animals in each group, and the route of administration was intragastric medication. Time of drug administration: 14 d.

2.5. Establishment of orthotopic xenotransplanted tumor model of liver cancer

Successfully subcutaneously transplanted solid tumor of liver cancer under normal growth was taken to cut into small pieces with scalpel (2 mm 2 mm 1 mm), and rinse 3 times with proper amount of RPMI-1640 medium containing no fetal bovine serum, then, the tumor mass was placed into sterile, dry culture dish and proper amount of RPMI-1640 culture solution containing no fetal bovine serum was added for spare use. Gas anesthesia was performed in healthy nude mice using isoflurane, supine position was taken, the site of surgical operation was disinfected with Anerdian, the skin was cut open in transverse direction at about 5 mm downward from xiphoid process to expose the liver, the prepared tumor tissue mass was sewed with surgical sutures on the side of left lobe of mouse liver closed to the middle lobe of liver, and the skin was sutured layer by layer, sterilized with Anerdian, the mice were put back to the cage to breed. Test with small animal *in-vivo* imaging system at 7 d after surgical operation was performed.

2.6. Therapeutic effect of compound HS-4 in orthotopic xenotransplanted tumor of liver cancer

After orthotopic xenotransplanted tumor model of liver cancer was established, random assignment was performed in animals according to the data about tumor tested by small animal *in-vivo* imaging system, the mice were divided into HS-4 group and solvent control group in the experiment, wherein, HS-4 was classified into 3 dose groups based on the dosage provided as 4, 2 and 10 mg/kg, respectively. There were 6 animals in each group, and sintragastric administration was provided. Time of drug administration: 14 d.

2.7. Data analysis

SPSS statistical analytical software was used to perform variance analysis in experimental data, and the difference between HS-4 group and solvent control group was compared.

3. Results

3.1. Screening of in vitro antitumor activity of the compound HS-4

MTT method was used to screen *in vitro* antitumor activity of the compound HS-4. Cell lines included human liver cancer cell Bel-7402, human liver cancer cell SMMC-7721, human gastric cancer cell MGC-803, human gastric cancer cell SGC-7901, human breast cancer cell MCF-7, human cervical cancer cell Hela, human

esophageal cancer cell TE-1, human lung cancer cell A549, human lung cancer cell NCI-H446, human colon cancer cell HT-29, human kidney cancer cell ACHN and human leukemia cell HL-60. The results of experiment were shown in Table 1. It was indicated by the results that, compound HS-4 had relatively high tumor inhibitory rates against all above tumor cells. The IC_{50} values against liver cancer cell SMMC-7721and Bel-7402 were 0.14 and 0.13 nmol/L respectively, and the IC_{50} values against gastric cancer cell MGC-803 and SGC-7901 were 0.19 and 0.21 nmol/L respectively.

Table 1
Antitumor activity of HS-4 *in vitro*.

Cell	Tumor type	IC_{50} (nmol/L)
Bel-7402	Liver	0.13
SMMC-7721	Liver	0.14
MGC-803	Stomach	0.19
SGC-7901	Stomach	0.21
MCF-7	Breast	0.31
Hela	Uterus	0.44
TE-1	Esophgus	0.67
A549	Lung	0.54
NCI-H446	Lung	0.68
HT-29	Colon	0.46
ACHN	Kidney	1.24
HL-60	Leukemia	3.41

3.2. Investigation on therapeutic effect of compound HS-4 in subcutaneously xenotransplanted tumor

Investigations have been performed on therapeutic effect of compound HS-4 in subcutaneously xenotransplanted tumor against 11strains of tumor cells except HL-60. It was demonstrated by the results that, the best therapeutic effect of HS-4 was achieved in treatment of liver cancer, and the result was shown in Table 2. At 3W after intragastric drug administration was performed, tumor inhibitory rates against 11 tumors implanted were all over 65% in dose group of 2 mg/kg, while tumor inhibitory rates in dose group of 10 mg/kg were all over 80%, and the tumor inhibitory rates against the other 7 tumors except breast cancer, lung cancer and kidney cancer in dose group of 0.4 mg/kg were all over 40%.

Table 2
Antitumor activity of HS-4 *in vitro*.

Cell	Tumor type	Initial tumor volume(mm^3)	TCI%		
			0.4 mg/kg	2 mg/kg	10 mg/kg
Bel-7402	Liver	393	53.7*	82.6*	>100*
SMMC-7721	Liver	362	50.9*	80.1*	>100*
MGC-803	Stomach	426	48.8*	76.4*	97.4*
SGC-7901	Stomach	384	41.4*	71.6*	98.6*
MCF-7	Breast	433	32.7	68.8*	86.4*
Hela	Uterus	410	40.3*	70.9*	82.7*
TE-1	Esophgus	326	46.3*	66.2*	82.4*
A549	Lung	462	26.8	70.6*	83.1*
NCI-H446	Lung	428	28.4	68.4*	85.7*
HT-29	Colon	468	50.8*	80.2*	91.4*
ACHN	Kidney	360	33.4	73.4*	94.3*

NOTE: The percentage of tumor-growth inhibition (TGI%) compared with the vehicle-treated group was calculated on the day after the last treatment (day 14), *P<0.05.

3.3. Investigation on therapeutic effect of HS-4 in orthotopic xenotransplanted tumor of liver cancer Bel-7402

According to the investigation on therapeutic effect of HS-4 in subcutaneously xenotransplanted tumor in Table 2, the liver cancer cell Bel-7402 in which the best therapeutic effect was achieved was selected to perform orthotopic xenotransplantation, so as to identify the therapeutic effect of HS-4 in liver cancer. During the experiment, IVIS small animal *in-vivo* imaging system was used to effectively detect the growth of tumor. It could be seen from Figure 1B, the amount of tumor cells in 3 dose groups of HS-4 was significantly lower than that in solvent control group, and significant dose-effect relationship was presented, indicating that the growth of tumor cells were significantly controlled. Growth of tumor tissue in solvent control group and HS-4 group (dosage was 10 mg/kg) was shown in Figure 2. It was shown by the results that, tumor tissue was relatively small in HS-4 group, while the tumor tissue was clearly seen in solvent control group. Comparison of tumor cell quantity before and after drug administration between animals in HS-4 group (dosage was 10 mg/kg) and solvent control group was shown in Figure 3. At 3W after drug administration, the amount of tumor cells in solvent control group was significantly increased, while the amount of tumor cells in HS-4 group was significantly decreased, wherein, live tumor cells could not be detected in animal No.3, while the amount of live tumor cells were hardly detected in animal No.5.

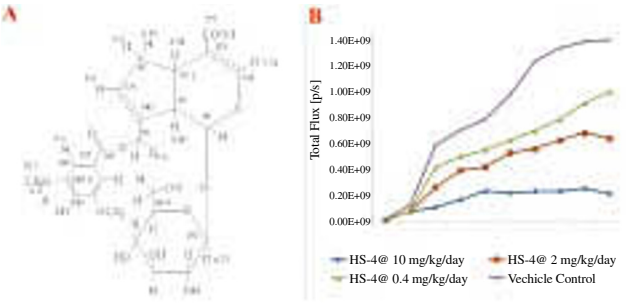

Figure 1. Antitumor activity of HS-4 *in vivo*.
(A) chemical structure ofHS-4.(B) HS-4 inhibits the growth of established Bel-7402 tumor xenografts in athymic mice.

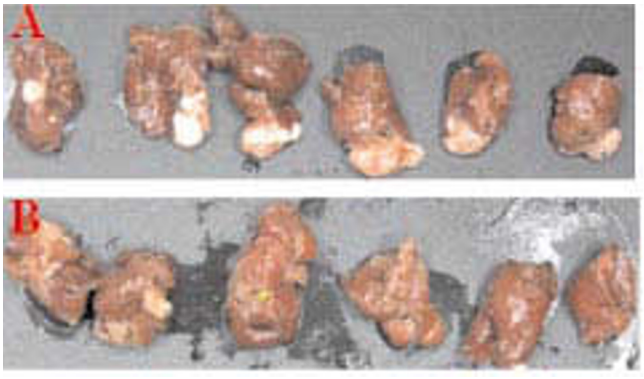

Figure 2. Effect of HS - 4 for liver cancer Bel - 7402 nude mouse orthotopic transplantation tumor therapy.
(A) Vehicle, (B) Glup of HS-4,10 mg/kg for 21days.

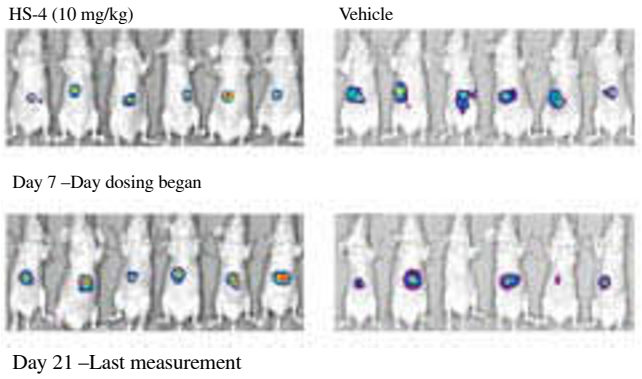

Figure 3. Photon emission from individual mice was visualized before administration of the first dose of HS-4 or vehicle (day 7) and afterthe last measurement (day 21).

Red color indicates more fluorescence indicative of a greater number of live tumor cells. Daily oral administration of HS-4 at 10 mg/kg/day was initiated when the tumors reached an average size.

4. Discussion

HS-4 is a compound isolated from Mangrove Kandelia candel. It was demonstrated by relevant studies that, the primary structure of HS-4-geniposidic acid could be used in protecting against cancer caused by radiation[4]. It was indicated by the study performed by Tang that, compound 6,6'-bis (2,3-dimethoxybenzoyl)-a, a-D-trehalose (DMBT) had relatively high anti tumor invasion and metastasis activity, of which the anti invasion activity against colon cancer cell 26-L5 was even higher than that of the natural product-Brartemicin, where the IC_{50} was 0.15 μM (0.10 μg/mL) [5]. This proved the fact effectively from another perspective that, HS-4 [10-(4'-hydroxy-3',5' –dimethoxybenzoyl)-geniposidic acid] had relatively high antitumor activity. It was proved by extensive screening of *in vivo* antitumor activity against *in vitro* or subscutaneously xenotransplanted tumor and orthotopic xenotransplanted tumor and analysis in small RNA of tumor cells that, HS-4 had relatively excellent therapeutic effect in tumor with significant difference compared to that in solvent control group.

During the process of therapeutic experiment in subscutaneously xenotransplanted tumor, excellent antitumor activity was presented in HS-4 which achieved powerful therapeutic effect in xenotransplanted solid tumors of human tumor cells in nude mice, such as Bel-7402, SMMC-7721, MGC-803, SGC-7901, MCF-7, Hela, TE-1, A549, NCI-H446, HT-29, ACHN, etc, having very highly positive prospect of development.

Primary liver cancer is one of the common malignant tumors in the world. As for the method of orthotopic xenotransplantation of liver cancer in nude mice[6–8], traditionally, a blunt object is applied to make a hole in the liver of mouse, hemostasis is performed by compression after tumor tissue mass is put inside, or the tumor tissue mass is just sealed inside with biocolloid. But the method will result in relatively serious damage to the liver of mouse as well as massive bleeding likely. Therefore, a new method for orthotopic xenotransplantation have been established: that the tumor tissue mass was sewed on the side of left lobe of mouse liver closed to the middle lobe of liver, which can effectively help avoid adhesion of tumor tissue with other tissues except liver. In addition, be careful to wash the likely drop-off cells at the surface of tumor tissue mass with culture solution so as to avoid extensive implantation of tumor in abdominal cavity.

Conflict of interest statement

We declare that we have no conflict of interest.

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