In vitro and in vivo inhibitory action of a novel antibiotic on mouse hepatoma H$_{22}$

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IC$_{50}$ for a novel antibiotic against mouse hepatoma H$_{22}$ was determined to be 0.019 µg/mL by MTT assays. In KM mice bearing this kind of tumor that was treated by s.c. injection of antibiotic BS, marked cures were obtained. In terms of tumor weight, the treated/control=0.35. The conclusion is that BS merit further investigation as a potential anti-cancer candidate drug.

Key word: Bacillus subtilis, antibiotic, in vivo, in vitro, antitumor.

INTRODUCTION

Global cancer rates could increase by 50% to 15 million by 2020 and new drugs will not necessarily eradicate tumors (WHO, 2010). Chemotherapy is one of the potent treatments for prolonging the patient’s life (Sirinet, 2010). Natural products have afforded a rich source of compounds that have found many applications in cancer chemotherapy. Over 70% of anticancer compounds are either natural products or natural product-derived substances (Karikas, 2010) and the therapeutic application of microbial metabolites provided the opportunity for the discovery of anticancer agent (e.g., Disorazole A1, doxorubicin, bleomycin, mitomycin, lipopeptide and dactinomines) (Grever, 2001; Elnakady et al., 2004).

In the process of screening bacteria that can antagonize Xanthomonas oryzae ps. oryzae (a pathogen of rice), we happened to obtain a strain of Bacillus subtilis (named bacteria BS) that can secret an novel antibiotic (named BS). We found that BS had better ability to inhibit the growth of mouse hepatoma H$_{22}$ cell in vitro, so we were interested in whether BS had antitumor potential in vivo.

The purpose of the paper was to valuate the anticancer potential of BS by using mouse hepatoma H22 as cancer model.

MATERIALS AND METHODS

Culture medium, animal, cell line and microorganism

KMB culture medium

BBI company peptone 20 g, glycerol 15 ml, K$_2$HPO 1.5 g, MgSO$_4$ 0.75 g, volume was adjusted to 1000 ml by distilled water, sterilized at 121°C for 20 min. Quartz Sand: immersed in acidic potassium dichromate solution for 24 h to oxidize organic substance (the acidic potassium dichromate solution: K$_2$Cr$_2$O$_7$ 37 g plus 300 ml was heated and stirred until potassium dichromate was dissolved. After it was cooled, 300 ml 98% sulphuric acid was gradually added). The Quartz Sand was eluted with distilled water for 10 h to remove metallic ion and oxidizing substances. Then, it was sterilized at 180°C for 2 h.

Animal

KM mouse, half male and half female, 6 to 8 weeks old, 20 to 25 g, were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch, P.R.China.

Cell lines

The cells of mouse hepatoma H$_{22}$ were generously provided by Pharmaceutical Department of Zhejiang Chinese Medicine
Table 1. Toxicity of BS in normal KM mice. (Dose that resulted in death and the day that died).

<table>
<thead>
<tr>
<th>Group</th>
<th>Day1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Mice died/ n</th>
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<td>control</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0/6</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1/1</td>
</tr>
<tr>
<td>BS◆◆</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2/2</td>
</tr>
<tr>
<td>BS◆◆◆</td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>4/4</td>
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<tr>
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<td>1</td>
<td></td>
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<tr>
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<td>0/6</td>
</tr>
</tbody>
</table>

◆100 mg/kg, s.c., one time /per day x1day, ◆◆50 mg/kg, s.c., one time /per day x1day, ◆◆◆25 mg/kg, s.c., 0, 20, 40, 60, 80, 100, 120 min/per day, x1, 2, 3, 5, 6, 7day, ◆◆◆◆3 mg/kg, s.c., one time /per day x1day, **50 mg/kg, s.c., one time /per day x1day, ***50 mg/kg, s.c., 0, 20, 40, 60, 80, 100, 120 min /per day, x1, 2, 3, 5, 6, 7day, ****6 mg/kg, s.c., 0, 20, 40, 60, 80, 100, 120 min/per day, x1, 2, 3, 5, 6, 7day.

University, P. R. China. BS-producing-bacteria or bacteria BS (a strain of Bacillus subtilis) was isolated from the eggplant leaf derived from the suburb of hangzhou city, Zhejiang province, P. R. China. Fungus (Rhizoctonia solani) was generously given by Biotechnical Institute of Zhejiang University, P. R. China.

Fermentation, BS extraction and purification

At 37°C, for ten days, bacteria BS was cultured on surface of the Quartz Sand that was immersed in KMB culture medium, the surface of the Quartz Sand not being covered with liquid culture medium (previous work had shown that bacteria BS produced more BS if it was cultured on solid medium). Thereafter, the Quartz Sand, which absorbed BS secreted by bacteria BS, was immersed in water to be distilled. The condensed water was collected and passed through active carbon chromatographic column, which was then eluted with ether. The eluted ether was left at room temperature (25~30°C) overnight to evaporate ether. The remainder was chromatographed on silica gel column which was eluted with ether. Fraction with the greatest activity was further chromatographed on silica gel column and then eluted with normal pentane: ether = 1:4. The normal pentane and ether were both evaporated at room temperature (25~30°C). In above purifying process, bio-activity was tracked by inhibition zone of a fungus (Rhizoctonia solani). Activity was finally confirmed by MTT assays (test cell used was mouse hepatoma H22).

Cytotoxicity assays

Inhibitory effect of BS on hepatoma H22 was assessed in 96-well microtiter plates by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye as described by Scudiero et al. (1988). IC50 was determined by Logit model.

In vivo antitumor effects

In vivo antitumor work was performed at Animal Center of Zhejiang Chinese university, P. R. China. Hepatoma H22 cells, 1 x 107/ml, 200 µl per mouse, was implanted s.c. respectively on day 0. Treatments were given on days 1, 2, 3, 5, 6 and 7. BS, as well as 5-FU, was respectively s.c. injected at intervals, that is, injected at 0, 20, 40, 60, 80, 100 and 120 min. For each s.c. injection, the dose was 3 mg/kg for SB or 6 mg/kg for 5-FU. Tumor volume was measured every other day that is on day 2, 4, 6, 8, 10, 12 and 14 with vernier. Tumor volume was calculated by V = 0.5 x a x b^2 (a = length, b = width) (Sawaoka, 1999). After treatment was completed on day 14, all tumors were respectively isolated and measured for tumor weight.

RESULTS

In vitro cytotoxicity

By using MTT assay, IC50 for BS against hepatoma H22 was determined to be 0.019 µg/ml.

In vivo antitumor activities

The safe dosage and administration of BS and 5-FU

Before engaging in treatment, dose that was safe for mice was explored. Results are summarized in Table 1. From Table 1, it can be seen that, for BS, s.c. injected at intervals (0, 20, 40, 60, 80, 100 and 120 min /per day, x1, 2, 3, 5, 6, 7 days, each injection dosage=3 mg/kg was safe. As for 5-FU, s.c. injected at intervals (0, 20, 40, 60, 80, 100 and 120 min /per day, x1, 2, 3, 5, 6, 7 days, each injection dosage=6 mg/kg was safe (Table 1). Why drug was given several times per day rather than one time per day as most documents described? The reason was that preliminary experiments had suggested that the efficiency of BS was time-dependent.

Activity against mouse hepatoma H22

KM mice bearing hepatoma H22 were treated with BS and
5-FU respectively, according to the dosage and administration regimen as described above. Both treatments yielded significant therapeutic effects (Figures 1 and 2) and no mouse had died. In the case of BS, in terms of tumor size on day 14, treated/control=0.38; in terms of tumor weight on the day 14, treated/control = 0.35. In the case of 5-FU, in terms of the tumor size on day 14, treated/control=0.41, in terms of tumor weight on the day 14, treated/control=0.39. The value of tumor size/body weight, as well as the value of tumor weight, was less for BS than for 5-FU.

**DISCUSSION**

In this paper, BS’s antitumor potential was confirmed by mouse hepatoma H\textsubscript{22} in vitro and in vivo. All mice had survived the effective dosage of BS. Also, in terms of tumor weight, the value of treated/control of BS was less than that of 5-FU. Furthermore, the value of tumor size/body weight was less for BS than for 5-FU. All above was the indication that BS’s therapeutic index (the ratio of toxic dose VS the therapeutic dose) was good and therefore BS merit further investigation as a potential anti-cancer candidate drug.

BS was a metabolite secreted by a newly isolated *B. subtilis*. *B. subtilis* is the best-characterized member of the Gram-positive bacteria (Kunst, 1997) and some of this strain could produce bioactive substance (U.S. environmental protection agency, 1997), among which, some are anticancer substance. (i.g., lipopeptides, Surfactin, Glutamines) (Mazza 1994; Kim 2007; Pasupuleti 2009; Liao et al., 2009). However, it has not been documented that *B. subtilis*-derived anticancer substance has been developed as a clinical drug. Obviously, before *B. subtilis*-derived anticancer substances are developed as clinical anticancer drugs, as many effective anticancer substances as possible should be collected from *B. subtilis* and more basic research should be done. This paper's work constitutes part of such efforts.
Figure 2. Antitumor efficiency of BS, 5-FU as comparison (tumor’s volume) (1) the left figure was about tumor size change, and the right figure was about body weight change. (2) on day 14, The tumor size of treated/control=0.38 for BS, while the tumor size of treated/control=0.41 for 5-FU.

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