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Short Communication

In vitro and *in vivo* anticancer activities of a novel antibiotic

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IC_{50} for nine cancer cell lines was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays. In KM mice bearing sarcoma S180 that was treated with subcutaneous (s.c.) injection of antibiotic BS, marked cures were obtained. In terms of tumor weight, the treated/control = 0.43. The conclusion is that BS merit further investigation as a potential anti-cancer candidate drug.

Key word: Antibiotic, *Bacillus subtilis*, *in vivo, in vitro*, antitumor.

INTRODUCTION

Global cancer rates could increase by 50% to 15 million by 2020 (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 has found many applications in cancer chemotherapy. Over 70% of the anticancer compounds are either natural products or natural product-derived substances (Karikas, 2010) and the therapeutic application of microbial metabolites provides the opportunity for the discovery of anticancer agent (for example, Diso razole A1, doxorubicin, bleomycin, mitomycin, lipopeptide and dactinomicines) (Grever, 2001; Elnakady et al., 2004).

In the process of screening the bacteria that can antagonize *Xanthomonas oryzae* pv. oryzae (a pathogen of rice), we happened to obtain a strain of *Bacillus sublilis* (named bacteria BS) that could secret a novel antibiotic (named BS). We found that BS had better *in vitro* anticancer activity, so we were interested whether or not BS had antitumor potential *in vivo*. The purpose of the paper is to assay the BS's *in vitro* anticancer activity via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method and to evaluate the BS's *in vivo* anticancer activity via sarcoma S180 mouse model.

Abbreviations: MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; **s.c,** subcutaneous.

MATERIALS AND METHODS

Culture medium, animal, cell line and microorganism

KMB culture medium

The volume of BBI company peptone (20 g), glycerol (15 ml), K_2HPO (1.5 g) and $MgSO_2$ (0.75 g), was adjusted to 1000 ml by distilled water, sterilized at 121°C for 20 min. Quartz sand was immersed in acidic potassium dichromate solution for 24 h to oxidize the organic substance (the acidic potassium dichromate solution: $K_2Cr_2O_7$ (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 mice (half male and half female, 6 to 8 weeks old, 20 to 25 g) were purchased from the National Rodent Laboratory Animal Resources, Shanghai Branch, People's Republic of China.

Cell lines

The cells of mouse sarcoma S180 were generously provided by the Pharmaceutical Department of Zhejiang Chinese Medicine University, P. R. China. BS-producing-bacterium or bacteria BS (a strain of *Bacillus subtilis*) was isolated from the egg plant leaf derived from the suburb of Hangzhou city, Zhejiang province, P. R China. The fungus (*Rhizoctonia solani)* was generously given by the

Table 1. Cell growth inhibition by BS against 15 cancer cell lines.

Cell line	IC_{50} (μg/ml)
S 180	0.021
A549	0.039
KB	24.7
SGC-7901	0.0079
HCT-116	0.050
Hela	0.012
BEL-7402	0.0076
PC-3	0.16
Skov3	0.084

The results are expressed as mean from three independent experiments; all cell lines were human cancer cells except mouse sarcoma S180.

Biotechnological Institute of Zhejiang University, P. R. China.

Fermentation, BS extraction and purification

At 37℃, for ten days, bacteria BS was cultured on the surface of the quartz sand that was immersed in KMB culture medium, while the surface of the quartz sand is not covered with the liquid culture medium (previous work had shown that bacteria BS could produce more BS if it was cultured on the solid medium). Thereafter, the quartz sand, which had absorbed BS secreted by bacteria BS, was immersed in the 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℃) 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℃). In the aforementioned purifying process, the bio-activity was tracked by an inhibition zone of a fungus (*R. solani*). The activity was finally confirmed by the MTT method (the test cell used was mouse sarcoma S180).

Cytotoxicity assays

The inhibitory effect of BS on nine cancer cell lines was assessed in a 96-well microtiter plates by measuring MTT dye as described by Scudiero DA and Shoemaker RH (Scudiero et al., 1988). However, IC_{50} was determined by the Logit model.

***In vivo* antitumor effects**

In vivo antitumor work was performed at the Animal Center of Zhejiang Chinese Medicine University, P. R. China. Cells of sarcoma S180, 1×10^7/mL, 200 μl per mouse, were implanted by subcutaneously (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 injected at intervals by s.c., 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, and it was calculated by: $V = 0.5 \times a \times b^2$ (a = length, b = width) (Sawaoka et al., 1999). After treatment was completed on day 14, all tumors were respectively isolated and measured for tumor weight.

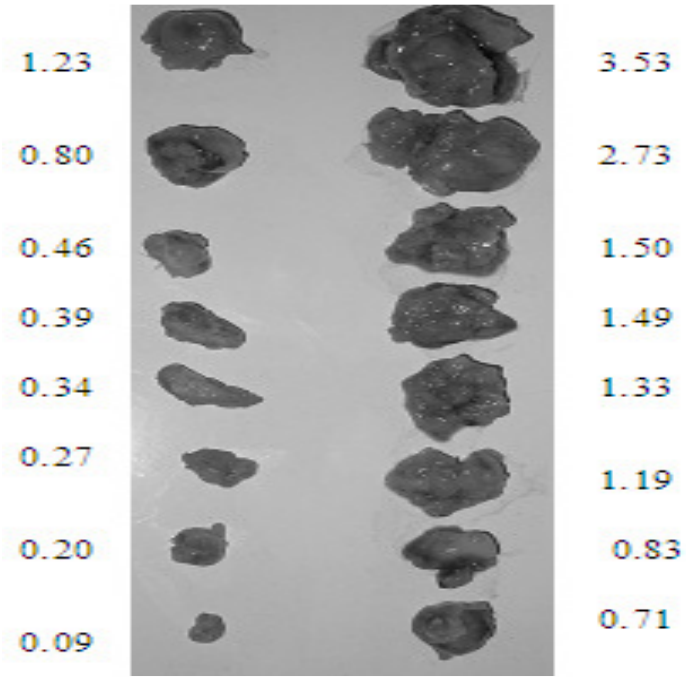

Figure 1. The BS's anticancer efficacy (tumor weight of treated/control).

RESULTS

In vitro cytotoxicity

By MTT assay, IC_{50} for BS against nine cancer cell lines H_{22} was determined, with KB human cancer cell having the largest IC_{50} (24.7) and BEL-7402 human cancer cell having the smallest IC_{50} (0.0076) (Table 1). Thus, the difference of IC_{50} between these two cancer cells was more than 3000 times. This meant that different cancer cells had different sensitivity to BS.

In vivo antitumor activity against sarcoma S180

KM mice bearing sarcoma S180 were treated with BS and 5-FU, respectively. Both treatments yielded significant therapeutic effects and no mouse died. In the case of BS, in terms of tumor weight on day 14, treated/control=0.43 (Figure 1), while in the case of 5-FU, in terms of tumor weight on day 14, treated/control = 0.35. After the treatment with BS, tumor weight of treated/control = 0.43 while after the treatment with 5-FU, tumor weight of treated/control = 0.35 (the figure for 5-FU was not shown).

DISCUSSION

The BS's *in vitro* antitumor activity against nine cancer cell lines had been determined by MTT method, while the

BS's *in vivo* antitumor activity against sarcoma S180 had been confirmed by mouse cancer model. However, all mice had survived the effective dosage of BS. These indicated that the BS merited further investigation as a potential anti-cancer candidate drug.

BS is a metabolite secreted by a newly isolated *B. subtilis*. *B. subtilis* is the best-characterized member of the gram-positive bacteria (Kunst et al., 1997) and some of this strain can produce bioactive substance (U.S. environ-mental protection agency, 1997), among which, some are anticancer substances (for example, lipopeptides, surfactin and glutamines) (Mazza, 1994; Kim et al., 2007; Pasupuleti, 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, many effective anticancer substances should be collected, as much as possible, from *B. subtilis* and more basic research should be done. However, the work of this paper constitutes part of such efforts.

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