African Journal of Pharmacy and Pharmacology Vol. 5(21), pp. 2355-2358, 8 December, 2011
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP11.020
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

BS and BS-contained rat serum's anticancer activities

Chen-Xiaoxi

Basic Medicine College, Zhejiang Chinese Medicine University, Binjiang District, Hangzhou City, Zhejiang Province, P. R. China. E-mail: chenxiaoxi82@gmail.com. Tel: 0086-0571-86613774.

Accepted 25 July, 2011

The aim of the study is to evaluate the anticancer potential of BS. Cytotoxicity assays was carried out to test the activity of BS against mouse model cancer and to test the activity of BS-contained serum against SGC; the human gastric cancer cell. Cytotoxicity assays showed that BS has a wide range of antitumor spectrum. Activity evaluation toward a set of BS-contained-serum that was derived from rat, at different time-intervals, respectively, indicates that when BS existed within rat body, as time was going on, its anticancer activity fluctuated dramatically, with two activity peak that was isolated by 2 to 3 h. It was concluded the BS' potential as anticancer drug is promising.

Key words: Antibiotic, *Bacillus subtilis* (BS), *in vivo*, *in vitro*, anticancer.

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 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., Diso razole A1, doxorubicin, bleomycin, mitomycin, lipopeptide and dactinomicines) (Grever, 2001; Elnakady et al., 2004).

In the process of screening bacteria that can antagonize *Xanthomonas oryzae pv. oryzae* (a pathogen of rice), we happened to obtain a strain of *Bacillus sublilis* (named bacterium BS) that can secret a novel antibiotic (named BS for abbreviation). We found that BS had better *in vitro* anticancer activity, so we were interested in whether BS had antitumor potential *in vivo*. The purpose of the paper was to assay BS's *in vitro* anticancer activity against six cancer cells via SRB (Sulfforhodamine B) method and to evaluate BS's *in vivo* anticancer activity by testing the activity of BS-contained rat serum.

MATERIALS AND METHODS

Culture medium, animal, cell line and microorganism

KMB culture medium

BBI company peptone 20 g, glycerol 15 ml, K_2HPO 1.5 g, MgSO, 0.75 g, volume was adjusted to 1000 ml by distilled water, sterilized at 121℃ for 20 min.

Quartz sand

Immersed in acidic potassium dichromate solution for 24 h to oxidize 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℃ fo r 2 h.

Animal

KM mouse, half male and half female, 6 to 8 weeks old, 20 to 25 g, and SD rat, half male and half female, 6 to 8 weeks old, 300 to 350 g were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch, P. R. China.

Cell (human gastric tumor cell) was kindly given by Medical Molecular institute of Zhejiang Chinese Medicine University, P. R. China. All other cancer cells involved in this paper were from Shanghai Institute of Material Medical, Chinese Academy of Science. BS-producing-bacterium or bacterium 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 Biotechlogical Institute of Zhejiang University, P. R. China.

Fermentation, BS extraction and purification

At 37℃, for ten days, bacterium BS was cultured on surface of the quartz sand that was immersed in KMB culture medium, the surface

Table 1. Cancer cell's growth inhibition by BS-contained rat serum, which was derived at different time intervals from rats that had been i.v. injected with BS or normal saline

Cell line	IC_{50} (µg/ml)
P388	0.021
HL-60	0.030
Molt-4	0.027
U937	0.013
K562	0.088
Raji	0.037

Assay was by SRB; All the cells were human cancer cells except P388.

of the quartz sand not being covered with liquid culture medium (previous work had shown that bacterium BS produced more BS if it was cultured on solid medium). Thereafter, the quartz sand, which absorbed BS secreted by bacterium 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 to 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 to 30℃). In the previously mentioned purifying process, bio-activity was tracked by inhibition zone of a fungus (*R. solani)*. Antitumor activity was finally confirmed by MTT assays or 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (test cell used was SGC). The Bs-contained rat serums were placed in -70℃ refrigerator for late use.

Cytotoxicity assays

The cytotoxicity of BS toward P_{388}, HL-60, Molt-4, U937, K562, Raji, was determined in 96-well microtiter plates by SRB (Sulfforhodamine B) method as described by Skehan and coworkers for measuring the cellular protein content [(5)]. IC_{50} was determined by Logit model.

Antitumor activity of rat-derived serum that contained BS or metabolite of BS

Eleven rats was anesthetized using coelio-injection of mebumal sodium (45 mg/kg). Among them, nine rats were respectively injected with BS (3 mg/kg) via tail vein; the other two rats were injected with the same volume of physiological saline (control). The time that BS or physiological saline was injected was set to be 0.0 h. Carotid artery was revealed respectively by ophthalmology scissors. For each rat, 0.8 ml blood was drawn by 1 ml syringe at different time interval (0.5, 1.0, 1.5, 2.0, 2.5, 3, 3.5, 4.0, 5.5 h, etc.) respectively, through carotid artery. In order to prevent hematopexis, certain amount of haemostatic (disebrin) was added into syringe/carotid artery. After all blood samples (eleven sets) were prepared, they were centrifuged at 3000 rpm for 5 min, and eleven sets of serum (supernatant) was thus produced. Complement system, which can cause apoptosis and cell death, was inactivated by incubating the serum at 56℃ for 30 min. The eleven sets of serum were tested activity against SGC cell by MTT.

In order to further test BS-derived metabolite's antitumor activity, another independent experiment was conducted as follows: BS was *in vivo* metabolized in five rat body for six hours, with BS administration regimen and serum preparation being the same as described previously. The five sets of serum then combined together and was tested activity against six lines of cells, among which, two were normal cells(as control), and the other were four tumor cells.

RESULTS

In vitro cytotoxicity

Susceptibility evaluations were conducted for BS in five human cancer cell lines, as well as in P_{388} (mouse leukemia cell). All tumor cells, was susceptible to BS, with K562 having the greatest IC50 or 0.088 µg/ml (Table 1).

The anticancer activity of BS-contained rat serum that contained metabolite of BS

Eleven rats were used, therefore, eleven sets of data was obtained as shown in Figure 1. From Figure 1, it can be seen that BS's *in vivo* activity fluctuated dramatically as time goes on. There appeared two *in vivo* activity peaks which were separated by 2 to 3 h. Only the serum derived from the two periods (0.5 to 2 and 4 to 6 h, respectively after BS was administrated) could completely preclude tumor cell's growth; serum derived from other time could not reduce cancer cell growth speed or only reduce cell's growth speed to a certain degree (Figure 1). In Figure 1, in order to emphasize two periods during which cancer cell's growth was 100% prevented, the 100% inhibitory rate was expressed as " —", other inhibitory rate less than 100% were all donoted as +.

Another result should not be omitted. If BS was injected into rat tail vein with dose of 15 mg/kg rather than with dose of 3 mg/kg, the serum samples that were respectively derived from 1.0, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 5.5, 6 and 7hr, respectively, could 100% inhibit tumor cell growth, with exception that serum derived from 0.5 h failed to prevent tumor cell from growth. In this instance, only one rat was used, so only one set of data was obtained.

An independent experiment showed that the serum that contained BS's 6 h *in vivo* metabolite had a potent cytotoxicity toward four lines of cancer cells, as well as two normal cells. Interestingly, if such serum was ten-fold diluted, the four cancer cells were more inhibited by the BS-contained rat serum than the two normal human cells, which was an indication that the BS's 6 h *in vivo* metabolite had selective cytotoxicity (Table 2).

DISCUSSION

BS's *in vitro* antitumor activity against six cancer cell lines was determined by SRB (Sulfforhodamine B) and BS's *in*

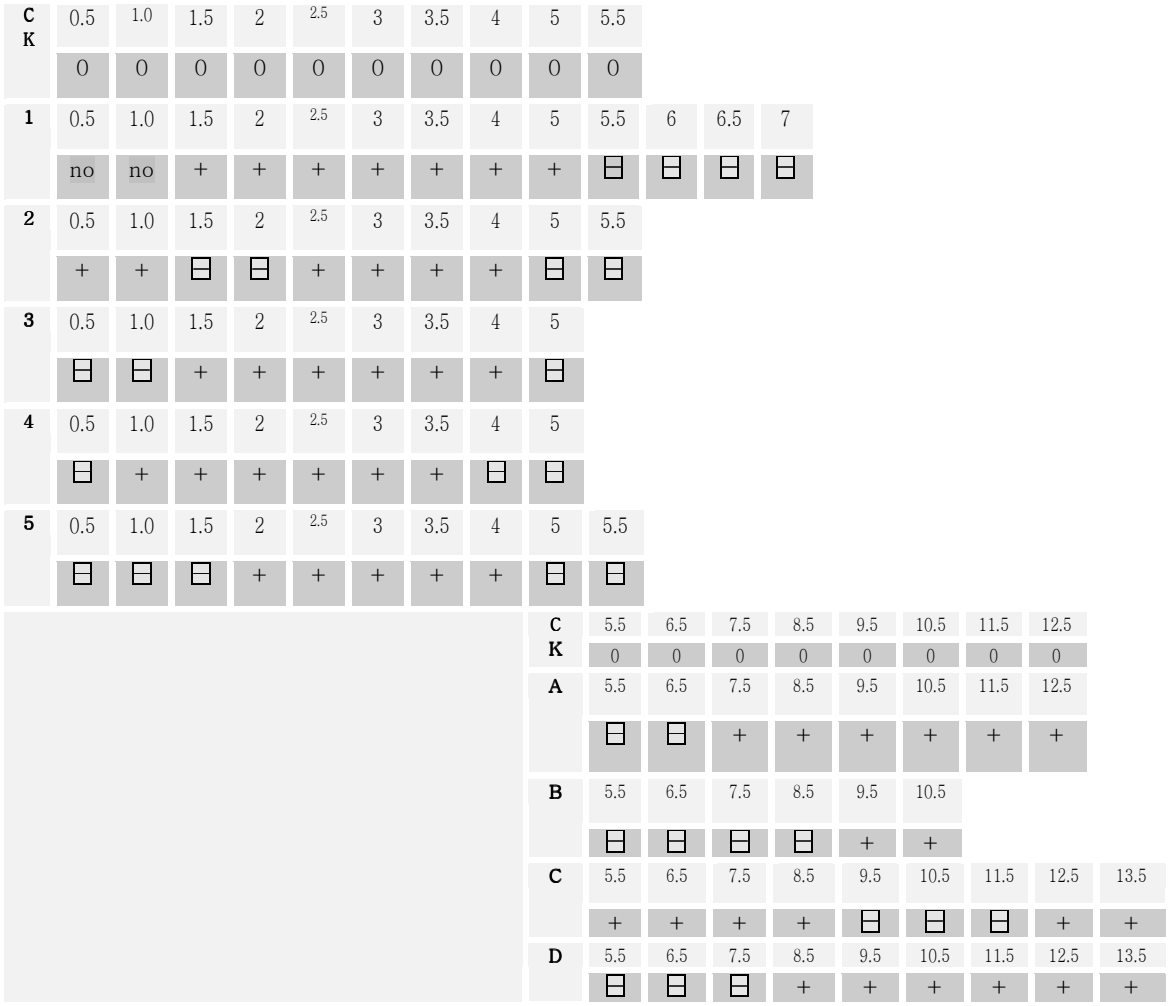

Figure 1. Cancer cell's growth inhibition by BS-contained rat serum, which was derived at different time intervals from rats that had been intraveniously injected with BS or normal saline. Assay was by MTT. Test cell is SGC or human gastric cancer cell. 0.5, 1.0, 1.5, 2.0, 3.0, 3.5 etc. means time (h) to draw blood from carotid artery. The time that BS was injected was set to 0.0 h; "0" mean was results from control serum (that is, BS-free-serum) and "0" means tumor cell could be freely living in control serum; "+", means tumor cell's growth was inhibited to a certain degree by BS-contained-serum; "—" means tumor cell's growth was completely inhibited by BS-contained-serum. CK were serial codes, which were assigned to control rats, that is, rats that were injected with physiological saline; 1, 2, 3, 4 and 5, as well as A, B, C and D were also serial codes which were respectively assigned to different individual rat that was injected with BS. "no" means datum was available because of some reasons.

vivo antitumor activity against SGC or human gastric cancer cell was confirmed by BS-contained rat serum's activity. Therefore, BS merits further investigation as a potential anti-cancer candidate drug.

Our original goal to test the activity of drug-contained serum was to estimate BS's *in vivo* half-time. Due to great fluctuation in BS's *in vivo* activity, it is difficult to obtain BS's *in vivo* half-time. However, this effort led to an interesting finding, that is, when BS existed within rat body, its antitumor activity fluctuated dramatically as time was going on. More accurately speaking, BS's *in vivo* antitumor activity in rat body peaked during 0.5 to 2 h and during 4 and 8 h, respectively after it was administrated.

As we know, there is no anticancer substance or anti-cancer drug whose *in vivo* activity fluctuated so greatly like BS. Undoubtedly, it is BS's metabolite rather than prototype BS that was responsible for second *in vivo* activity peak (during 4 to 8 h). On the other hand, it is very likely that prototype BS was mainly responsible for the first *in vivo* activity peak (during 0.5 to 2 h). We do not exclude the possibility that during 0.5 to 2 h, some prototype BS molecules were metabolized to other forms of molecules with more or less antitumor activity. We also do not rule out the possibility that during 4 to 8 h, there were several different BS derived metabolites that have more or less antitumor activity. The data reported in this

Table 2. Cancer cell's growth inhibition by rat serum that contained BS's *in vivo* metabolite after 6 h of injection into the rat body.

Cell line	Inhabiting rate (%)	
	Original rat serum	Ten-fold diluted rat serum
PC-3	94.0	50.5
DU-140	96.4	91.8
MBA-MD-231	92.9	88.1
SMMC-7721	91.1	85.7
HMEC*	93.9	1.3
WI-38*	82.4	36.9

Assay was by SRB. The results are expressed as mean from three independent experiments; *HMEC = normal cell or human mammary epithelial cell and *WI-38 = normal cell or fetal cell Line characteristics for WI-38.

paper suggest that BS's metabolite or metabolites (during 4 to 8 h) has good performance in selective toxicity. It is well known that some clinical drugs are developed from *in vivo* metabolite because in efficiency, the *in vivo* metabolite is superior to prototype drug, and prontosil is an example (Dunn, 2008). Therefore, BS-derived *in vivo* metabolite merit further investigation. According to our experience, in order to get sufficient BS's metabolite for further research (e.g., for purifying BS-derived metabolite), a large amount of purified BS should be consumed. But we now face a challenge in getting enough purified BS, which had hindered our efforts to further research BS-derived metabolite.

BS was 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. Environmental Protection Agency, 1997), among which, some are anticancer substance (e.g. lipopeptides, Surfactin, 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, 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.

ACKNOWLEDGEMENTS

The author thank Zhou Wei-ming for his valuable technical help in collecting BS-contained rat serum, Dr. Ding Zhi-san for providing SGC cell line. Dr. Chen Wei-liang for giving the fungus *R. solani, and* Ms. Xu Cheng-hui for her assistance with conducting *in vivo* cytotoxicity assays and IC_{50} analysis. This work was supported by Natural Science Fund Project of Zhejiang province, P. R. China (Project code number: Y2101176); (2) Medical Science Research Fund Project of Health Bureau of Zhejiang Province P. R. China (Project code number: 2004A062, and Medical Science Research Fund Project of Health Bureau of Zhejiang Province, P. R. China (Project code number: 2002A070).

REFERENCES

Elnakady YA, Sasse F, Lunsdorf H, Reichenbach H (2004). Disorazol A1, a highly effective antimitotic agent acting on tubulin polymerization and inducing apoptosis in mammalian cells. Biochem. Pharmacol., 67: 927-935..

Grever MCB (2001). Cancer drug discovery and development. In: the VITA, V.H.S. and rosenbrg, S.A., eds. Cancer: Principles and practice of oncology. Philadelphia, Lippincott Raven, pp. 328-339.

Karikas GA (2010). Anticancer and chemopreventing natural products: some biochemical and therapeuticaspects. J BUON., 15(4): 627-38.

Kim SY, Kim JY, Kim SH, Bae HJ, Yi H, Yoon SH, Koo BS, Kwon M, Cho JY, Lee CE, Hong S (2007). Surfactin from Bacillus subtilis displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression. FEBS Lett., 581: 865-71.

Kunst F, Ogasawara N, Moszer I, Albertini AM, Alloni G, Azevedo V, Bertero MG, Bessieres P, Bolotin A, Borchert S, Borriss R, Boursier L, Brans A, Braun M, Brignell SC, Bron S, Brouillet S, Bruschi CV, Caldwell B, Capuano V, Carter NM, Choi SK, Codani JJ, Connerton IF, Danchin A (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis* Nature. 20: 249-56.

Mazza P (1994). Anticancer and Immunostimulatory effects of Nucleoprotein Fraction of *Bacillus subtilis*". Experimental Oncology. 25: 119–123.

Pasuleti V (2009). Glutaminase: An anticancer enzyme from Microbe.http://www.pharmainfo.net/venkatpasupuleti/glutaminase-anticancer-enzyme-microbe.

Sirinet P (2010). Anticancer and apoptosis-inducing activities of microbial metabolites. DOI: 10.2225/vol13-issue5-fulltext-7.

U.S. Environmental Protection Agency (1997). *Bacillus subtilis*- Final Risk Assessment.http://www.epa.gov/opt?pubs/fra/fra009.htm.

WHO (2010). http://www.who.int/mediacentre/news/releases/2003/pr27/en/.