

Inhibition Effect of Brazilin to Human Bladder Cancer Cell Line T24

Liansheng Ren, Xihua Yang, Guoping Wang, Hong Zhang, Lili Zhao, and Zhenguo Mi

Abstract—The inhibition effect of brazilin to human bladder tumor cell line T24 in vitro and in vivo was studied. The results of the in vitro experiments showed that brazilin has strong inhibition activity on the target cells. The inhibition ratio of 100 µg/mL brazilin and 100 µg/mL mitomycin to the target cells was 90.90 % and 63.24 % respectively, which showed that brazilin has higher inhibition activity than mitomycin under the same concentration. Brazilin could induce cell apoptosis in T24 cells. Significant antitumor activity of brazilin was also showed in the animals experiments. The life extension rate of 200 mg/mL, 300 mg/kg, and 400 mg/kg brazilin intraperitoneally injected into Balb/c-nu nude mice that with human bladder cancer were 51.50 %, 56.90 %, and 58.42 % ($P < 0.05$). Our study showed that brazilin has significant inhibitory effect on human bladder tumor cell.

Keywords—bladder cancer, brazilin, inhibition, T24 cell line

I. INTRODUCTION

SAPPAN Wood (*Caesalpinia sappan L.*) is a kind of traditional Chinese herbal medicine. We found that its aqueous extract has prominent inhibitory activity to human ovarian cancer cell, and could induce apoptosis to human ovarian cell line SKOV3 [1]-[3]. Further research demonstrated that the main effective constituent was brazilin which showed remarkable inhibitory activity to several kinds of tumor cells.

Brazilin is one of the main active constituents in the heartwood of Sappan Wood[4]. Recent research showed that brazilin has kinds of bioactivity. It was reported that brazilin possesses antiinflammatory actions in macrophages and works through a novel mechanism involving the action of HO-1[5] and iNOS gene[6]. The suppressive effect of iNOS gene expression by brazilin might also provide one possible mechanism for its cancer chemopreventive activity[6]. Brazilin is one kind of antioxidant that protects oxidative injury through the expression of heme oxygenase-1 and protects cultured rat hepatocytes from BrCCl₃-induced toxicity[7]. Brazilin may increase glucose transport by recruitment of GLUT4 from intracellular pools to the plasma membrane of adipocytes via the activation of PI3-kinase[8], inhibit the down-stream of

cAMP signaling pathways[9], and inhibits hepatic gluconeogenesis by elevating the F-2, 6-BP level in hepatocytes, possibly by elevating cellular F-6-P/H-6-P levels and PFK-2 activity[10] to enhance insulin receptor function and lower blood sugar. It was reported that brazilin has potent activity against antibiotic-resistant bacteria, notably methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), multi-drug resistant *Burkholderia cepacia* as well as a number of other bacteria[11]. Brazilin could induce vasorelaxation by the increasing intracellular Ca(2+) concentration in endothelial cells of blood vessels, the increasing cGMP content[12], and the relaxing of alpha1-receptor agonist phenylephrine-precontracted aortic rings[13]. The results of the inhibition of phospholipase (PLA2) activity and [Ca²⁺] elevation might be a part of antiplatelet mechanism of brazilin[14]. Brazilin could augment cellular immune responses by increasing IL-2 production[15], by inhibiting the decrease in IL-2 production, by suppressing the elevation of suppressor cell activity[16], and by affecting the function of T cells[17].

To find anticancer agents from higher plants, an ethyl acetate extracts of the heartwood of *Caesalpinia sappan L.* (Leguminosae) was found to be exhibited potent DNA strand-scission activity by Mar W., and further separation and purification approved that the functional component was brazilin[18]. Bae IK regarded that suppressive effect of iNOS gene expression by brazilin might provide one possible mechanism for its cancer chemopreventive activity[6].

The inhibition effect of brazilin to human bladder tumor cell line T24 in vitro and in vivo was reported in this paper.

II. MATERIALS AND METHODS

A. Brazilin

Sappan Wood (Produced by Anhui Wansheng Biological Slices Limited Company . Product batch: 001206.) was extracted with boiling distilledwater for 90 min, degreased with ligarine, extracted with ethyl acetate, purified by silica gel column chromatography repeatedly, and pure brazilin was obtained. The brazilin obtained was contrasted with standard brazilin (Chengdu Must Biological Technology Limited Company. Brazilin content: 99.10 %) by HPLC (U.S. Waters Company), FT-IR (U.S. NICOLET Company), NMR (Germany Bruker Company) The result showed that the content of pure brazilin we obtained was 98.60 %, the content of brazilin in acetic ester extraction was above 52 %. The molecular formula of brazilin is C₁₆H₁₄O₅, and the molecular weight is 286.28. Pure brazilin was used in the study of cells test and acetic ester extraction was used in animal research.

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B. Cell strain

Human bladder T24 cell strain was bought from Shanghai institutes for biological science, CAS. The cells above were cultured at 37 °C and 5 % CO₂ in RPMI 1640 (U.S. Gibco Company) supplemented with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, 800 mg/L NaHCO₃ and 3.6 g HEPES. The cells were harvested by treatment with 0.25 % trypsin supplemented with 0.02 % EDTA. Viability was determined using the trypan-blue (U.S. Sigma Company) exclusion method. The cells were suspended in prepared PBS, with the concentration of 1×10^7 /mL.

C. Animal

SPF Balb/C-nu-nu mice, female, 6-7 weeks old, 16-19 g, bought from Vital River Laboratories (VRL). Animal produce license number: SCXK (Jing) 2006-0009.

The animal were bred in the clean grade barrier environment animal laboratory with license number of SYXK (Jin) 2007-0002. The temperature was kept at 26 ± 1.5 °C, and the humidity was kept between 40 % - 60 %. 12 h -12 h light-dark condition.

The drinking water was sterilized at high pressure. The animal was fed with sterile complete nutrition feedstuff.

D. Cell growth inhibition rate measurement

1 mL T24 cell suspension were inoculated in 24-well flat-bottomed plates at a concentration of 1×10^5 /mL for 24 h. Supernatants were discarded and brazilin mixed with medium was added. The final concentration of brazilin were 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL respectively. Negative control group didn't add any drug and positive control group was added with mitomycin (Zhejiang Hisun Pharmaceutical Co. Ltd.) at a final concentration of 100 µg/mL. Each group possesses 4 wells, and cells were collected in 4 h, 8 h, 24 h, 48 h after the addition of the drugs. The number of cells killed by the drugs was determined by using 0.4 % trypan-blue. 200 cells were counted randomly under the optical microscope. Living cells (unstained cells) and dead cells (stained cells) were counted separately.

E. Determination of TC₅₀ of brazilin to T24

100 µL T24 cell suspension were inoculated in 96-well flat-bottomed plates at a concentration of 1×10^5 /mL. Normal control group was added with medium only. Supernatants were discarded after 24 h and brazilin mixed with medium was added. The final concentration of brazilin were 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL respectively. Positive control group was added with mitomycin at a final concentration of 100 µg/mL. Normal control group and negative control group was added with same amount of medium. 30 µL luciferase (U.S. Promega Co.) was added into each well after being cultivated 16 h. Oscillated for two minutes, and cultivate for 8 min at room temperature. The luminescence (RLU) was detected by Fluoroskan Ascent FL(U.S. Promega Co.). Each group possesses 4 wells.

F. Affect to apoptin Caspase-3 and Caspase-9

100 µL T24 cell suspension were inoculated in 96-well flat-bottomed plates at a concentration of 1×10^5 /mL. Normal control group was added with medium only. Supernatants were discarded after 24 h and brazilin mixed with medium was added. The final concentration of brazilin were 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL respectively. Positive control group was added with mitomycin at a final concentration of 100 µg/mL. Normal control group and negative control group was added with same amount of medium. Caspase-Glo[®] 3/7 and Caspase-Glo[®] 9 Assay (U.S. Promega Co.) were added into each well after being cultivated 16 h. Oscillated for two minutes, and cultivate for 1 h at room temperature. The luminescence (RLU) was detected by Fluoroskan Ascent FL(U.S. Promega Co.). Each group possesses 4 wells.

G. Apoptosis detection

1 mL T24 cell suspension were inoculated in 24-well flat-bottomed plates at a concentration of 1×10^5 /mL for 24 h. Supernatants were discarded and brazilin mixed with medium was added. The final concentration of brazilin were 7.5 µg/mL, 15 µg/mL, 30 µg/mL and 60 µg/mL respectively. Positive control group was added with mitomycin at a final concentration of 30 µg/mL and negative control group was added with same amount of medium. Each group possesses 4 wells, and cells were collected after 16 h. The cells were washed by PBS (0.8 % NaCl, 0.024 % KCl, 0.364 % Na₂HPO₄·12H₂O, 0.02 % KH₂PO₄) for twice and added with 500 µL Bindin Buffer to make the cells suspend, 5 µL Anntxi V-FITC and 5 µL PI dye liquor , m. b, cultivated away from light for 1 h at room temperature. Apoptosis was detected by Flow Cytometry (U.S. BD Co.) . The cell suspension was also dropped to glass slide, covered with cover glass, Apoptosis was detected by laser confocal microscopy(Germany LEICA. TCS. SPS Co.).

H. Life extension effect to tumor-bearing mice

T24 cell suspension was adjusted to 1×10^7 /mL by PBS. Live cell rate was detected above 97 % by trypan bule exclusion test. 0.2 mL cell suspension was injected into each nude mice to establish tumor-bearing animal model. 24 h after the injection of tumor cells, the mice were randomly divided into control group, high dose group of brazilin, middle dose group of brazilin, low dose group of brazilin, and mitomycin group. The control group was injected with 0.2 mL normal saline. Groups of brazilin was injected with 400 mg/kg, 300 mg/kg, and 200 mg/kg respectively. Mitomycin group was injected with 1 mg/kg mitomycin on 1st and 6th day. The administration time in control group and each brazilin group were continued for 6 days. Animal survival time in 60 d was recorded. The extending lifetime rate was calculated as follows:

I. Statistic analysis

Statistical calculations were carried out with the SPSS 17.0 for Windows software package. Results are expressed as the mean \pm S.E.M. ANOVA and LSD method was used for statistical analyses; P values > 0.05 were considered to be significant.

III. RESULTS

A. Cell growth inhibition effect to T24

The inhibition effect of brazilin to T24 cells increased significantly with the increase of concentration of brazilin and time. It reflected little effect to target cells in 4 hours at the concentration of 25 $\mu\text{g/mL}$. When the concentration raised above 50 $\mu\text{g/mL}$, significant inhibitory effect demonstrated to target cells ($P < 0.05$). The inhibition effect of every brazilin group and mitomycin group to the target cells increased in a time-dependent manner after being cultivated for 8 hours. And the effect of brazilin was superior to mitomycin at the same concentration of 100 $\mu\text{g/mL}$ which showed good antitumor activity (Fig. 1).

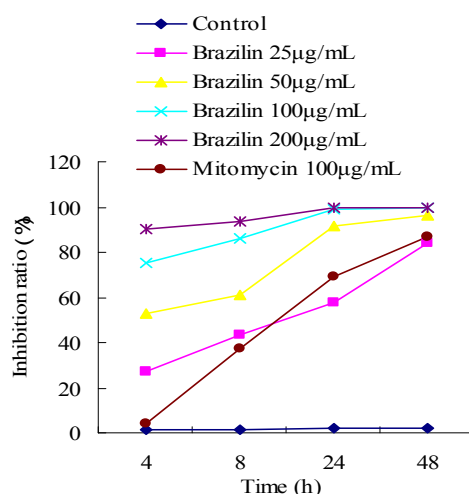


Fig. 1 Inhibition ratio of brazilin to T24(trypsin blue exclusion assay)

T_{50} of brazilin to T24 cells in 16 h was 25.81 $\mu\text{g/mL}$, that was 90 $\mu\text{mol/L}$ which was much lower than 300

$\mu\text{mol/L}$ that was reported safe concentration to normal cells by Hu.CM [5]. This showed clearly that brazilin has high sensitivity and high specificity to bladder tumor cell line T24 (Fig. 2).

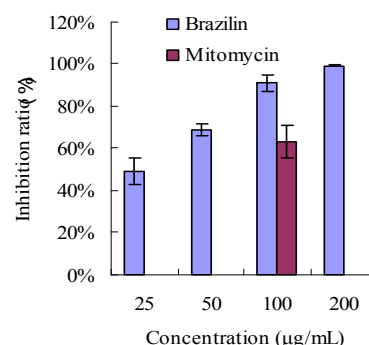


Fig. 2 Inhibition ratio of brazilin to T24(fluorescence staining).

B. Affect to Apoptin Caspase-3 and Caspase-9

Being treated by brazilin for 16 h, except for that the activity of Caspase-3 and Caspase-9 increased slightly in the 25 $\mu\text{g/mL}$ group, other dose of brazilin groups were remarkably lower than the normal control group ($P < 0.05$). And the activity of Caspase-3, Caspase-9 in the mitomycin group increased significantly ($P < 0.05$). This showed that the mechanism of apoptosis induced by brazilin to T24 was not through caspase path, but mitomycin could induce apoptosis through raising related apoptin of caspase (Fig. 3).

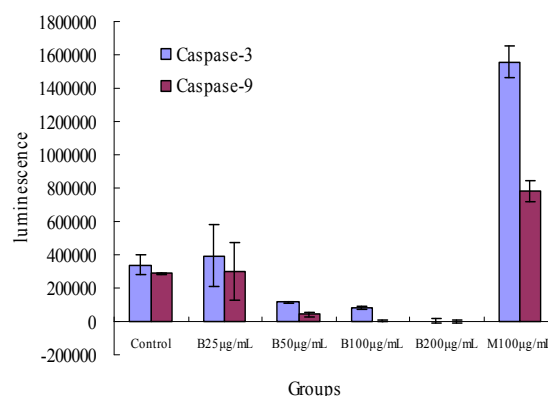


Fig. 3 Effect of brazilin to activity of apoptin Caspase-3, Caspase-9 in T24 after being treated with 16 h.

C. Cell apoptosis

After being treated for 16 h, apoptosis of target cells raised with the increasing of the concentration of brazilin, especially increased significantly in the late stage and live cell rate decreased significantly. It showed that brazilin could induce apoptosis of target cells at a low dose and could kill the target cells directly at a high dose. A majority of apoptotic cells could be seen green fluoresce under the microscope in mitomycin group, which showed that there were mainly early stage apoptosis cells and died cells in mitomycin group (Fig. 4, Fig. 5).

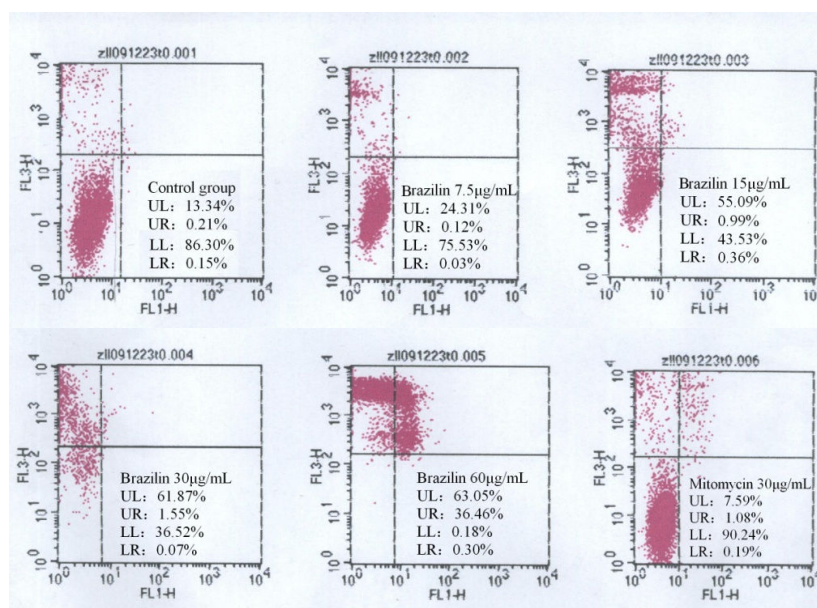


Fig. 4 Fluorescence micrographs of apoptosis that induced by brazilin to T24.

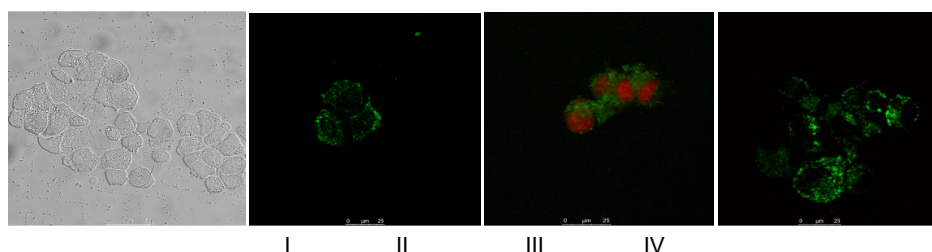


Fig. 5 Apoptosis figures of T24 affected by brazilin observed by laser confocal microscopy. I, control group; II, brazilin group (30 µg/mL); III, brazilin group (60 µg/mL); IV, mitomycin group (30 µg/mL)

A. Life extension effect to tumor-bearing mice

Intraperitoneal inoculated with T24 cells, the mean survival time of mice in normal control group was 21.67 ± 4.80 d. The mean lifetime in high, middle and low dosage groups of brazilin were all prolonged. The extending lifetime rate were 58.42 %, 56.90 % and 51.50 % respectively, among which high and middle group had statistical significance as compared with the

control group ($P < 0.05$). The extending lifetime rate was 63.04 % ($P < 0.05$) in the mitomycin group. It showed good antitumor activity in of brazilin intraperitoneally injected into mice and obvious inhibitory effect of brazilin was showed to human bladder tumor cell line T24 (TABLE. I).

TABLE I

LIFE EXTENSION EFFECT OF BRAZILIN TO NUDE MICE THAT WERE TRANSPLANTED WITH T24 CELLS INTRAPERITONEALLY ($\bar{x} \pm s$)

| Groups | n | | Dosage $\text{Mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ | Time d | Average survival time d | Life extension rate % |
|-------------------------------|-------|-----|--|-----------|-------------------------------|-----------------------------|
| | Begin | End | | | | |
| Control group | 9 | 0 | - | - | 21.67 ± 4.89 | - |
| High dose of brazilin group | 9 | 0 | 400 | 6 | $32.83 \pm 3.06^*$ | 58.42 |
| Middle dose of brazilin group | 9 | 0 | 300 | 6 | $34.00 \pm 4.47^*$ | 56.90 |
| Low dose of brazilin group | 9 | 0 | 200 | 6 | $34.33 \pm 2.34^*$ | 51.50 |
| Mitomycin group | 9 | 0 | 1 | 2 | $35.33 \pm 2.73^*$ | 63.04 |

Note: * stands for significant difference compared with control group ($P < 0.05$)

IV. CONCLUSION

Our study suggested that brazilin has the effect of inhibiting and inducing apoptosis to human bladder cancer cell T24. It showed significant inhibition to the growth of T24 and could prolong the lifetime of tumor-bearing mice. But the mechanism of brazilin to target cells and the applying of brazilin to bladder cancer need further investigation.

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