ANTITUMOR EFFECT OF BRM

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ABSTRACT

Objective: To evaluate the antitumor effect of BRM, a Chinese traditional medical compound herb, and its mechanism. Methods: Tumor inhibition was tested *in vivo* on mice with subcutaneous transplanted tumor cells *in vitro* with and MTT method and tumor cell apoptosis test including cell electric morphology, agarose gel electrophoresis and flow cytometry. Results: It was found that BRM could the resistance to the attack of 5×10^6 of mouse pancreascarcinoma (MPC-83), Ehrlich ascite tumor (EAC), mouse sarcoma (S-180), and mouse glioblastoma (G422) cells, with the inhibition rate of tumor weight of 66.2%, 50.9%, 47.3%, and 38.7% respectively. The inhibition rate of Suzhou human glioma (SHG-44), human breast carcinoma (MCF-7), and human pancreas carcinoma (PANC1) *in vitro* were 92.2% (BRM 1:10), 85.1% (BRM 1:10), 48.3% (BRM 1:10), respectively. Meanwhile, BRM was able to induce apoptosis in several human tumor cell lines such as SHG-44 and MCF-7 cells. Morphological changes including cell shrinkage and condensation of chromosomes were observed with electric microscope. Agarose gel electrophoresis of DNA from SHG-44 and MCF-7 cells treated with BRM extracts (1:80 to 1:20) for 24 h, 40 h revealed typical DNA "Ladder" pattern. The early stage and middle-late stage apoptosis increased in SHG-44 and MCF-7 cells treated with BRM extracts (BRM 1:160 to 1:40) from 14 h to 48 h by Annexin-V/PI flow cytometry one analysis. Conclusion: BRM has obvious antitumor bioactivity *in vivo* to MPC-83, EAC, S-180, and G422 mouse cell strains, and *in vitro* to SHG-44, MCF-7 human cell lines. Water extracts of BRM could induce apoptosis.

Key words: BRM, Apoptosis, SHG-44 cells, MCF-7 cells

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Apoptosis or programmed cell death plays an important role in antitumor drug action. Malfunctions of apoptosis have been implicated in many forms of human disease. It is well known that antitumor agents induce apoptosis through various manner in tumor cells[1].

BRM is a traditional medicinal compound herb in china. In recent years BRM was shown to exhibit antitumor activity in clinical practice. In this article, we proved that BRM and its water extracts could induce apoptosis in Suzhou human glioma and human breast carcinoma and showed obvious antitumor effect *in vitro* and *in vivo*.

MATERIALS AND METHODS

Drugs and Reagents

MTT, RPMI-1640 medium were purchased from Sigma Co. Annexin-V/PI cell apoptosis detection kits was purchased from Beijing University. Suzhou human glioma cell line (SHG-44), Human breast carcinoma cell line (MCF-7), mouse tumor MPC-83, EAC S-180, and G422 are conventionally preserved by the lab.

MTT Assay

SHG-44, MCF-7 and PANC1 cells were seeded to 96-well plates respectively and BRM was diluted then added to the wells. After 48 h exposure to BRM, 10 μl of MTT (5 g/L) was added to every well and incubated at

37°C for 4 h. Growth inhibitory rates of tumor cells were judged by comparing the percentage of absorbance to control wells[2].

Animals

Male or female Kunming strain of mice, 6-8 weeks of age, purchased from Beijing Animal Institute of Chinese Academy of Medical Science, China, were housed for at least 3 days in a specific pathogen-free state before experiment.

Experimental Therapy in *vivo*

The homotransplantation of MPC-83, EAC, S-180 or G422, was carried out in the Kunming mice. The solid tumor was suspended in steriol normal saline, and the tumor cells were suspended at final concentration of 2.5×10^7 cells/ml. Each mice received 0.2 ml the mixture of tumor suspension subcutaneously on right lateral of front leg armpit. One day later, CGC (Chinese Materia Medica Capsulae Goddess Composita) or BRM were administrated PO at the concentration of 3 g, 2 g, 1 g, 0.5 g/kg body weight, respectively, once daily for 10 days. Then the mice were sacrificed to quantitate the antitumoral effect.

Flow Cytometric Analysis

SHG-44 and MCF-7 cells (1×10^6 c) were incubated with cisplatin (DDP) and BRM extracts of different concentration for 14 h to 48 h. Then the cells were harvested and washed twice with PBS and centrifugated at 1000 rpm for 10 min. The sediment was incubated with 200 μl Binding Buffer, Annexin-V-FITC 10 μl (20 μg/ml) and PI 5 μl (50 μg/ml) at room temperature for 30 min in the dark. Then 400 μl of PBS was added and the samples were immediately applied for FAC analysis.

DNA Fragmentation[3, 4]

The cultured SHG-44 and MCF-7 cells (1×10^7c) were exposed to 5-Fu 7.5-15 μg/ml for 40 h, BRM 1:80 and 1:40 for 24 h, 40 h, and ADM 0.3-0.6 μg/ml for 40 h and 48 h respectively. Then cells were harvested, washed once with PBS and suspended in 100 μl lysis buffer (25 mmol/L Tris-Hcl, pH 8.0, 50 mmol/L EDTA, 25 mmol/L Nacl, 0.5% SDS, 500 μg/ml proteinase K). After incubation at 55°C-60°C for 4 h, DNA was extracted and loaded on 1.8% agarose gel for electrophoresis. The results were visualized by staining with ethidium bromide.

Transmission Electron Microscope Observation[5]

The SHG-44 and MCF-7 cells were cultured with DDP 3 μg/ml, BRM 1:40 for 40 h and DDP 7 μg/ml, BRM 1:20 for 48 h respectively and collected and fixed in 2.5% glutaraldehyde at 0°C for 4 h. The fixed cells were washed once with PBS, fixed in osmic, and prepared for TEM observation.

RESULTS

Cytotoxic Activity of BRM Extracts on Human Tumor Cells Strain

The effect of BRM water extracts on the growth of SHG-44, MCF-7, PANCl cells was evaluated by MTT assay. The inhibition rate of tumor cells was increased in a dose-dependent manner (Table 1, 2, 3).

Table 1. Cytotoxic activity of BRM on SHG-44 cells

Name / Dose	BRM $\bar{x}\pm s$	BRM Inhibition of rate (%)	Name / Dose (μg/ml)	CDDP $\bar{x}\pm s$	CDDP Inhibition of rate (%)
Blank	0.102±0.005	-	Blank	0.102±0.005	-
Control	1.260±0.059	-	Control	1.26±0.059	-
1:10	0.192±0.015	92.2	25.00	0.103±0.001	99.9
1:20	0.298±0.003	82.9	12.50	0.108±0.006	99.5
1:40	0.436±0.024	71.2	6.25	0.183±0.019	93.0
1:80	0.469±0.031	68.3	3.12	0.438±0.145	71.0
1:160	0.489±0.068	66.6	1.56	0.481±0.017	67.3
1:320	0.642±0.004	53.4	0.78	0.523±0.072	63.7
1:640	0.772±0.080	42.2			
1:1280	0.941±0.061	27.6			

Table 2. Cytotoxic of BRM on MCF-7 cells

Name / Dose	BRM $\bar{x}\pm s$	BRM Inhibition of rate (%)	Name / Dose (μg/ml)	CDDP $\bar{x}\pm s$	CDDP Inhibition of rate (%)
Blank	0.094±0.002	-	Blank	0.094±0.002	-
Control	0.560±0.008	-	Control	0.560±0.008	-
1:10	0.163±0.020	85.1	25.00	0.127±0.004	92.9
1:20	0.212±0.016	74.5	12.50	0.127±0.003	92.5
1:40	0.358±0.034	43.6	6.25	0.130±0.006	92.3
1:80	0.426±0.049	31.2	3.12	0.308±0.011	54.3
1:160	0.425±0.056	29.3	1.56	0.431±0.016	28.0
1:320	0.548±0.109	3.0	0.78	0.562±0.008	0
1:640	0.475±0.043	18.6			
1:1280	0.518±0.017	9.4			

Table 3. Cytotoxic activity of BRM on PANC1 cells

Name / Dose	BRM $\bar{x}\pm s$	BRM Inhibition of rate (%)	Name / Dose (μg/ml)	CDDP $\bar{x}\pm s$	CDDP Inhibition of rate (%)
Blank	0.094±0.003	-	Blank	0.094±0.003	-
Control	1.071±0.027	-	Control	1.071±0.027	-
1:10	0.597±0.015	48.3	100.00	0.192±0.004	90.0
1:20	0.704±0.036	37.4	50.00	0.160±0.018	93.2
1:40	1.006±0.070	6.7	25.00	0.269±0.068	82.1
1:80	1.079±0.005	0.0	12.50	0.331±0.080	75.7
1:160	0.425±0.056	0.0	6.25	0.569±0.106	51.4
1:320	0.548±0.109	0.0	3.12	0.855±0.072	22.1
1:640	0.475±0.043	0.0			
1:1280	0.518±0.017				

Experiment Treatment with BRM on Mice Transplantation *in vivo*

Table 4 to 7 are the results of inhibitory effect of brm on transplanted tumor in mice. The antitumoral effect was determined by examining the tumor weight. The inhibition of tumor growth was correlated with the dose of BRM.

Table 4. Inhibitive effect of BRM on mice inoculated with MPC-83

Groups	Dose (g/kg)	Number of mice (n)	Mode of administration	Body weigh ($\bar{x}\pm s$, g) Before exp.	Body weigh ($\bar{x}\pm s$, g) After exp.	Tumor weight ($\bar{x}\pm s$, g)	Inhibition of rate (%)	*P* value
Control	Distilled water	10	PO×8	20.8±0.6	27.0±1.8	2.69±0.86		
CGC	3	10	PO×8	21.3±0.8	26.5±1.1	1.55±0.05	42.3	<0.01*
BRM	2	10	PO×8	20.9±0.7	19.9±2.1	0.91±0.72	66.2	<0.001
BRM	1	10	PO×8	21.0±0.7	25.5±2.7	1.59±0.89	40.9	<0.01
BRM	0.5	10	PO×8	20.9±0.9	26.5±1.1	2.01±1.08	25.3	>0.05

Table 5. Inhibitive effect of BRM on mice inoculated with EAC

Groups	Dose (g/kg)	Number of mice (n)	Mode of administration	Body weigh ($\bar{x}\pm s$, g)		Tumor weight ($\bar{x}\pm s$, g)	Inhibition of rate (%)	*P* value
				Before exp.	After exp.			
Control	Distilled water	9	PO×9	17.8±0.7	24.9±1.4	1.12±0.15		
CGC	3	9	PO×9	17.8±0.6	23.8±1.2	0.80±0.12	28.6	<0.05*
BRM	2	9	PO×9	17.9±1.0	19.0±0.7	0.55±0.23	50.9	<0.01
BRM	1	9	PO×9	18.0±1.1	21.6±1.8	0.72±0.23	35.7	<0.05
BRM	0.5	9	PO×9	17.7±1.1	22.8±2.0	1.05±0.18	6.25	>0.05

Table 6. Inhibitive effect of BRM on mice inoculated with S-180

Groups	Dose (g/kg)	Number of mice (n)	Mode of administration	Body weigh ($\bar{x}\pm s$, g)		Tumor weight ($\bar{x}\pm s$, g)	Inhibition of rate (%)	*P* value
				Before exp.	After exp.			
Control	Distilled water	8	PO×9	19.0±0.9	27.4±2.7	1.69±0.70		
CGC	3	8	PO×9	19.4±0.9	27.4±1.8	1.10±0.28	34.9	<0.05*
BRM	2	8	PO×9	19.7±1.4	26.5±1.2	0.89±0.40	47.3	<0.01
BRM	1	8	PO×9	19.5±1.2	25.5±2.1	1.52±0.30	10.1	>0.05
BRM	0.5	8	PO×9	19.7±1.4	26.5±3.1	1.58±0.45	6.25	>0.05

Table 7. Inhibitive effect of BRM on mice inoculated with G422

Groups	Dose (g/kg)	Number of mice (n)	Mode of administration	Body weigh ($\bar{x}\pm s$, g)		Tumor weight ($\bar{x}\pm s$, g)	Inhibition of rate (%)	*P* value
				Before exp.	After exp.			
Control	Distilled water	9	PO×9	21.0±1.0	25.8±2.4	1.99±0.28		
CGC	3	9	PO×9	19.2±1.0	20.5±0.8	1.15±0.09	42.2	<0.01*
BRM	2	9	PO×9	20.0±1.0	20.5±2.5	1.22±0.50	38.7	<0.05
BRM	1	9	PO×9	20.3±1.1	21.6±1.9	1.73±0.37	13.1	>0.05
BRM	0.5	9	PO×9	20.8±0.8	22.7±1.3	1.90±0.38	4.5	>0.05

Flow Cytometry Analysis

Induction of apoptosis was correlated with exposure time and concentration of BRM water extracts. Table 8 showed that when SHG-44 cells were exposed to BRM at 1:160 to 1:40 for 14 h to 48 h the apoptosis rate of SHG-44 cells gradually increased. MCF-7 cells also exhibited BRM induced apoptosis dependent on the dose and incubation time of BRM (Table 9).

DNA fragmentation

Typical apoptosis DNA ladder on agarose gel was clearly demonstrated in SHG-44 and MCF-7 cells exposed to BRM at 1:80 to 1:40 for 24 h to 40 h or BRM at 1:40 to 1:20 for 40 h to 48 h (Figure 1, 2). DNA ladder was most clear at 48 h, while hardly seen at 24 h, (BRM 1:80, SHG-44 cells), indicating that induction of apoptosis by BRM water extracts was time-dependent.

Transmission Electron Microscope Examination of cell Sections Treated by BRM Water Extracts

Morphological examination demonstrated the occurrence of apoptosis of the SHG-44 and MCF-7 cells treated with BRM water extracts at 40 h and 48 h respectively (Figure 3, 4). The apoptotic cells showed condensation of heterochromatin and nuclear fragmentation. The typical apoptosis was induced by BRM 1:40 (SHG-44 cells) and 1:20 (MCF-7 cells). There could be seen a lot of blebs chromatin condensation and crescentic nuclear.

Table 8. BRM induced apoptosis of SHG-44 cells (Annexin-V/PI)

Group	Dose (μ/mg)	Time (h)	Normal (%)	Necrosis (%)	Early apo. (%)	Middle-late apo. (%)	Early+middle-late apo. (%)
Negative	-	14	89.7	1.0	3.8	5.6	9.4
Positive (CDDP)	3	14	89.6	0.9	3.2	6.3	9.5
BRM	1:40	14	87.1	5.4	1.0	6.6	7.6
BRM	1:80	14	89.2	5.0	0.6	5.2	5.8
BRM	1:160	14	89.8	4.8	0.8	4.6	5.4
Negative	-	24	99.1	0.1	0.9	0.0	0.9
Positive (CDDP)	3	24	68.6	0.2	25.7	5.6	31.3
BRM	1:40	24	56.4	0.1	33.9	9.6	43.5
BRM	1:80	24	84.0	3.4	9.3	3.4	12.7
BRM	1:160	24	91.6	1.9	5.0	1.4	6.4
Negative	-	38	87.8	1.4	4.5	6.3	10.8
Positive (CDDP)	3	38	15.6	0.2	35.2	49.0	84.2
BRM	1:40	38	81.3	0.1	16.4	2.2	18.6
BRM	1:80	38	74.9	0.2	19.4	5.5	24.9
BRM	1:160	38	76.3	0.2	17.3	6.3	23.6
Negative	-	48	74.9	9.6	7.1	8.4	15.5
Positive (CDDP)	3	48	53.1	2.3	35.6	9.0	44.2
BRM	1:40	48	52.3	13.2	16.2	18.4	34.6
BRM	1:80	48	56.2	8.3	16.3	19.3	35.6
BRM	1:160	48	45.7	9.3	17.4	27.6	45.0

Table 9. BRM induced apoptosis of MCF-7 cells (Annexin-V/PI)

Group	Dose (μ/mg)	Time (h)	Normal (%)	Necrosis (%)	Early apo. (%)	Middle-late apo. (%)	Early+middle-late apo. (%)
Negative	-	14	96.2	2.5	0.5	0.9	1.4
Positive (CDDP)	7	14	96.7	1.4	0.5	1.4	1.9
BRM	1:20	14	90.7	4.6	2.2	2.5	4.7
BRM	1:40	14	88.2	6.4	1.0	4.5	5.5
BRM	1:80	14	95.7	2.6	0.7	1.1	1.8
Negative	-	24	95.0	3.6	0.4	1.1	1.5
Positive (CDDP)	7	24	82.7	9.8	5.7	1.7	7.4
BRM	1:20	24	87.8	5.6	3.1	3.6	6.7
BRM	1:40	24	89.4	3.5	3.2	3.9	7.1
BRM	1:80	24	93.5	2.8	1.1	2.6	3.7
Negative	-	38	95.1	2.8	0.7	1.4	2.1
Positive (CDDP)	7	38	12.4	1.9	35.5	50.2	82.6
BRM	1:20	38	62.9	1.2	18.5	17.4	35.9
BRM	1:40	38	54.0	1.3	20.9	23.9	44.8
BRM	1:80	38	85.9	2.9	4.7	6.5	11.2

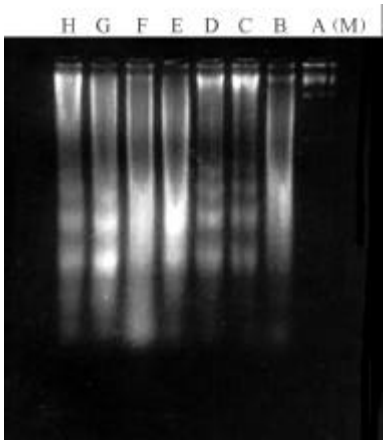

Fig. 1. Apoptotic DNA fragmentation induced in SHG-44 cells by BRM for 24 h, and 40 h, respectively.
A: Marker B: Normal C: 5-Fu 7.5 μg/ml 40 h
D: 5-Fu 15 μg/ml 40 h E: BRM 1:40 24 h
F: BRM 1:80 24 h G: BRM 1:40 40 h
H: BRM 1:80 40 h

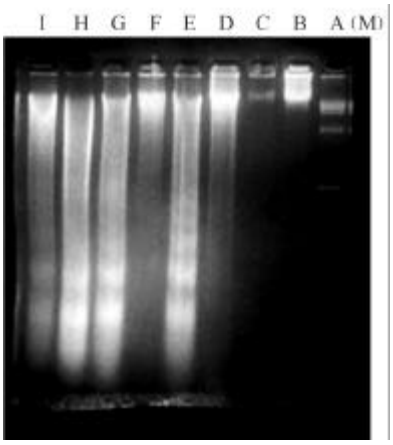

Fig. 3. Apoptotic DNA fragmentation induced in MCF-7 cells by BRM for 40 h, and 48 h, respectively.
A: Marker B: Normal C: ADM 0.3 μg/ml 40 h
D: ADM 0.6 μg/ml 40 h E: ADM 0.6 μg/ml 48 h
F: BRM 1:40 40 h G: BRM 1:40 48 h
H: BRM 1:20 40 h I: BRM 1:20 48 h

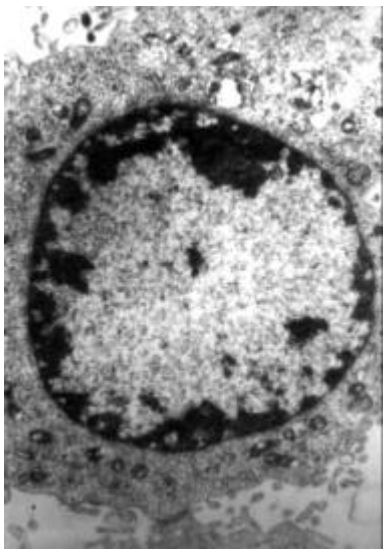

Fig. 2. Typical morphological changes of apoptosis in SHG-44 cells, treated with 1:40 of BRM for 48 h. The chromatin of apoptotic cells condensed and formed crescentic nucleus. ×4400

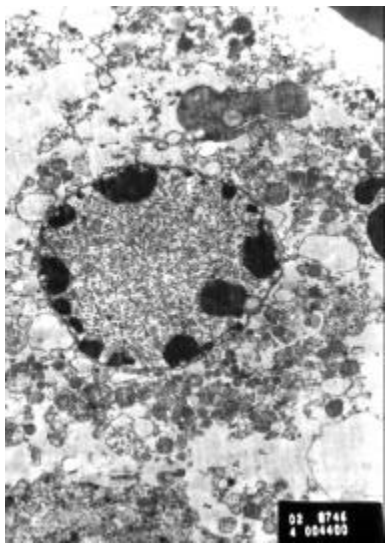

Fig. 4. Typical morphological changes of apoptosis in MCF-7 cells, treated with 1:40 of BRM for 40 h. The chromatin of apoptotic cells condensed and formed crescentic nucleus, the MCF-7 cell undergoing necrosis when treated with BRM 1:40 for 40 h. ×4400

DISCUSSION

Apoptosis is a mode of cell death that occurs under normal physiological conditions. In recent years, many chemotherapeutic drugs function by inducing apoptosis[6–8], such as Ara-C, 5-Fu, DDP, etc. BRM is a Chinese traditional herb. Our research showed that BRM water extracts could induce apoptosis of SHG-44 and MCF-7 cells. Cells undergoing apoptosis showed characteristic morphological and biochemical features. These features include chromatin aggregation, nuclear and cytoplasmic condensation, etc. In the *in vivo* experiment, we selected several rapid proliferating tumor cells line such as MPC-83, EAC, S-180, G422, etc. These rapid proliferating tumors were implanted into mice subcutaneously. The results showed that the inhibitory rate of tumor weight was dose-dependent. BRM also exhibited antitumor activity *in vitro*.

DNA from BRM treated SHG-44 and MCF-7 cells showed typical ladder on the agarose gel. Annexin V/PI FACS showed early and middle-late apoptosis cells in SHG-44 and MCF-7 cells. Induction of apoptosis by BRM extracts displayed time-dependent and dose-dependent manner. These results confirmed that BRM is a potential anticancer herbal medicine extracts.

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