

RESEARCH ARTICLE

Anti-tumor Effects and Apoptosis Induction by Realgar Bioleaching Solution in Sarcoma-180 Cells *in Vitro* and Transplanted Tumors in Mice *in Vivo*

Qin-Jian Xie¹, Xin-Li Cao³, Lu Bai¹, Zheng-Rong Wu¹, Ying-Ping Ma², Hong-Yu Li^{1*}

Abstract

Background: Realgar which contains arsenic components has been used in traditional Chinese medicine (TCM) as an anticancer drug. However, neither Realgar nor its formula are soluble in water. As a result, high dose of Realgar has to be administered to achieve an effective blood medicine concentration, and this is associated with adverse side effects. The objective of the present study was to increase the solubility of a formula using hydrometallurgy technology as well as investigating its effects on *in vitro* and *in vivo* cell proliferation and apoptosis in Sarcoma-180 cell line. **Materials and Methods:** Antiproliferative activity of Realgar Bioleaching Solution (RBS) was evaluated by MTT assay. Further, effects of RBS on cell proliferation and apoptosis were studied using flow cytometry and transmission electron microscopy. Kunming mice were administered RBS *in vivo*, where arsenic specifically targeted solid tumors. **Results:** The results indicated that RBS extract potently inhibited the tumor growth of Sarcoma-180 cell line in a dose-dependent manner. Flow cytometry and transmission electron microscopy further indicated that RBS significantly induced cell apoptosis through the inhibition of cell cycle pathway in a dose-dependent manner. Further, on RBS administration to mice, arsenic was specifically targeted to solid tumors. **Conclusions:** RBS could substitute for traditional Realgar or its formula to work as a potent tool in cancer treatment.

Keywords: Anti-tumor - realgar bioleaching solution - apoptosis - sarcoma - 180(S180).

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Introduction

TCMs Realgar (also named “*Xiong-Huang*” in Chinese) is the ore of sulfide mineral Realgar, which consists of a series of arsenic-containing metal sulfides (AS₄S₄) (Xuemin, 2000; National Pharmacopoeia Committee 2004). Pure realgar is an orange-red crystalline mineral. The spatial structure of realgar is analogous to that of sulfur (S₈), in which 4 arsenic atoms regularly replace 4 of the 8 sulfur atoms to form a similar ring, but the monoclinic crystal structure of realgar is different from the orthorhombic symmetry of sulfur. Realgar is soft and sectile; and usually occurs in granular, compact, or powder form. Considerable documents and clinical observations have established that arsenic-related medication can produce potent anti-cancer effects possibly by inducing cell apoptosis (Daopei et al., 1998; Mengchang et al., 2002; Xiaoou, 2006). Arsenicals have been known as toxins and paradoxically as therapeutic agents. In the 1970s, Chinese physicians revived the medicinal use of arsenicals as anticancer agents. Distinguished success was observed in the treatment of acute promyelocytic leukemia (APL) with arsenic trioxide (ATO). The FDA approved ATO injection in the year 2000 for the treatment

of APL. According to ancient medical records and recent findings in clinical trials, realgar was found as effective as arsenic trioxide (ATO), but with relatively good oral safety profiles even on chronic administration. These give realgar an advantage over ATO in maintenance treatment. Unfortunately, neither Realgar nor its formula is dissolvable in water. As a result, high dose of Realgar has to be administered to achieve the effective blood medicine concentration. Excess dose brings about severe side-effects, which limit the clinical use of this medicine (Peng et al., 1996; Mengchang et al., 2002; Huangbi et al., 2004). The low bioavailability of realgar probably is the reason behind its mild toxicity. Therefore, the key point for Realgar in treatment of cancer is to increase its dissolvability and hence the bioavailability.

In the past few years, our laboratory has made much breakthrough in fixing this problem by performing a new hydrometallurgy technology, a process which utilizes the catalytic effect of some microorganisms to dissolve the metal sulfides in acid water. We selected *Acidithiobacillus ferrooxidans* BY-3 (At. f BY-3) to bioleach Realgar (Devasia et al., 1996; Emati et al., 1997), and successfully obtained its extract, the bioleaching solution of Realgar (RBS) and we have proved that RBS could significantly

¹Gansu Key Laboratory of Biomonitoring and Bioremediation for Environmental Pollution, School of Life Science, Lanzhou University, ²The Second Hospital of Lanzhou University, ³Gansu Corps Hospital of CAPF, Lanzhou, China *For correspondence: hongyuli777@gmail.com

reduce the dose of Realgar and toxicity, improve its anti-cancer activities (Zhang et al., 2007). However, the effect on sarcoma-180 (S₁₈₀) cells is still unknown and therefore this study was designed to test the effects of RBS on cell proliferation and apoptosis of sarcoma-180 (S₁₈₀) cell line. We found that RBS, at a low dose, produced potent inhibition on tumor growth by inducing cell apoptosis in a dose-dependent manner. The new technology used in the present study to manufacture Realgar significantly enhanced the anti-cancer efficiency of this medicine, and more importantly, decreased the toxicity of Realgar, a particular issue of concern for clinical treatment. We propose that the complete dissolution of Realgar, which solved the preparation problems, makes it possible to develop the multi-type Realgar dosage forms. Our data suggested that RBS could substitute the traditional Realgar or its formula to work as a potent tool in the treatment of cancer.

Materials and Methods

Materials

Realgar was obtained from Shimen County, Hunan Province, China, and purified through traditional methods. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and (Propidium Iodide) PI were purchased from Sigma (St. Louis, MO). Culture medium RPMI 1640 was obtained from Gibco Corporation. Newborn cow blood serum was purchased from Limited Liability Corporation of Sijiqing Bio-engineering Material in Hangzhou. Arsenic trioxide (As₂O₃) for injection was purchased from the Yida Medicinal Ltd. (Harbin, China), and the arsenic concentration was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES; Jobin-Yvon Ultimate 2R) as 0.84 mg/ml.

Animals and cell lines

Kunming mice, weighed 20.0±2.0 g, were purchased from the Specific Pathogen Free (SPF) Laboratory Animal Center of Gansu College of Traditional Chinese Medicine. The animals were housed ten per plastic cage with wood chip bedding in an animal room with a 12h light and 12h dark cycle at room temperature of 20±2°C.

S₁₈₀ cell lines were purchased from the Institute of Cancer Research of Gansu province in China. All the cells were grown in a humidified 5% CO₂ atmosphere at 37 °C in an incubator, and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated newborn calf serum, 100IU/ml penicillin and 100µg/ml streptomycin.

Preparation for RBS

Bioleaching experiments were carried out in 250 ml flasks, each with 100 ml of the medium (with 1.0g of sterile sulfur powder per liter) containing 1.0 g of realgar (1.0 g of powder sample) with an initial pH of 1.8. Each flask was inoculated with *A. ferrooxidans* suspension at 10% (v/v) for the pure culture. For the culture of *A. ferrooxidans* each was incubated with 15% (v/v) inoculums. The flasks were incubated at 150 rpm, 30°C, with a pH of 1.80. The pH value of leaching solution of Realgar was periodically analyzed with a pH probe. Each experiment was conducted

at least in triplicate. After a short period to precipitate solid particles, 5 ml of the supernatant was extracted from each flask. Concentrations of ferrous iron and arsenic in the samples were measured by chemical analyses. The extracted liquid was compensated by the addition of 5 ml of water. The experiments lasted for 25 days.

Preparation and transplantation of the tumor cell

The frozen S₁₈₀ cell lines were incubated with water at 37°C temperature for 15 min and then added proper asepsis normal saline. Then we injected S₁₈₀ cell suspension of 0.3 ml into the mouse abdominal cavity. After 7 days, we extracted cell suspension from the abdominal cavity to transfer to next generation. In the end, the cell lines were first centrifuged at 1000 r. min⁻¹ for 5 min to remove blank liquid and added proper complete medium (RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, and 100 U/ml penicillin and streptomycin), counting under the light microscope, matching 1×10⁶ ml⁻¹ cells suspension per milliliter. We then extracted 0.2 ml cell suspension with the asepsis injector and injected cell suspension into the right armpit of mouse. The entire operation was only carried out on the ultra-asepsis work table.

In vivo anti-tumor effect on S₁₈₀ solid tumor

Six-day-old S₁₈₀ ascites tumor cells (about 5×10⁶ cells per 0.2 ml) were inoculated subcutaneously to mice at the axillary region to establish S₁₈₀ solid tumor model. The mice by S₁₈₀ solid tumor model born were randomly grouped (50 mice were divide into 5 groups, 10 mice per group). After inoculation for 48 hours, 0.2 ml of RBS with different dose levels (1.5, 3.0, 6.0 mg/kg arsenic) was administered into the mice once a day by intraperitoneal injection for consecutive 7 days. Negative (0.2 ml Normal Saline) controls were used for comparisons. Commercially available As₂O₃ for injection was used as the positive control (with a dose of 3.0 mg/kg arsenic). Eventually we took out the tumor, weighed the tumor weight and calculated tumor-weight inhibitory rate by the following formula.

Inhibitory rate (%)=(Tumor-weight of control-Tumor-weight of test)/Tumor-weight of control) ×100%

Pathological morphology of tumor

Tumor tissues were respectively cut out from treated or untreated bearing mice. Samples were sliced at 1mm thickness, and prefixed at 4°C in 2.5% glutaraldehyde in PBS for 2 h. Then washed twice in cold PBS for 10min, and post-fixed in cold 1% osmium tetroxide in PBS for 2 h. Specimens were then washed three times in cold distilled water, dehydrated in a series of alcohols, and embedded in epoxy resin. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. Samples were observed in a Hitachi H-500 transmission electronic microscope with 100 kW voltage.

Determination of inhibitory effect on cell proliferation

The MTT assay was used to assess the cell proliferation inhibitory effects of SRB on S₁₈₀ cells. In brief, S₁₈₀ cells were harvested, washed and resuspended in complete medium at a concentration of 2×10⁴ cells per milliliter.

The cell suspension was added to wells of a 96-well microtiter plate with the addition of 0.1 ml of complete medium (control) or various concentrations of RBS (diluted in complete medium), meanwhile 3 parallel wells of a 96-well micro-titer plate were made. After 24 and 48h of incubation, the plates were centrifuged to remove the supernatants from the culture, and 20 μ l of MTT at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) were added into each well. The plates were incubated for 4h at 37°C to allow for the formation of a colored formazan. The formazan was solubilized by lysing the cells with 0.15 ml of lyses buffer containing 20% dodecylsulfate (w/v) and 50% N, N-dimethyl formamide, PH 4.7. Absorbance of the formazan was measured at 570 nm using a V max plate reader (Molecular Devices, Sunnyvale, CA). The viability of the cells was calculated by the formula.

Inhibitory rate (%)=[1-(mean OD value of control cells/mean OD value of test cells)] \times 100

OD= optical density

Examination of the cell cycle and apoptosis

Logarithmically growing cells were respectively cultured in 5 cell bottles respectively. Each cell bottle was filled with 10 ml S_{180} cells (5×10^5 /ml) suspension. 2.0 μ g. ml^{-1} , 1.0 μ g. ml^{-1} , 0.25 μ g. ml^{-1} RBS and 1.0 μ g. ml^{-1} As_2O_3 were added into the 5 bottles respectively, All the cell bottles were incubated in CO_2 (5% CO_2 at 37°C) incubator for 24h, Then the cells were harvested, washed, fixed in 70% ethanol at 4°C for 24h and then stained with a PI staining solution (PBS containing PI 50 μ g/ml; RNase A 100 U/ml; and 0.1 mM EDTA) for 30 min at background room temperature before FACS analysis (10,000 events/sample). Eventually the stained cells were analyzed by flow cytometry (Becton Dickinson, San Jose, CA) at excitation wavelength of 488 nm. Data were acquired with Cell Quest acquisition software (Becton Dickinson, San Jose, CA).

Arsenic distribution in bearing mice

In this study, we selected the sarcoma S_{180} bearing mice treated by different concentrations of RBS, the normal saline control group and the As_2O_3 inoculation fluid group as toxicity study models. After administration by intraperitoneal injection for consecutive 7 days, All the S_{180} solid tumor-born mice were dissected and each tested organ was excised, like heart, liver, spleen, lung, kidney as well as solid tumor of the As_2O_3 inoculation fluid group (0.5mg/ml arsenic concentration) and RBS low dosage group (0.5 mg/ml arsenic concentration). Organ samples

were washed, cut down, accurately weighed, digested with 10 ml of nitric acid per 0.5g tissue overnight at room temperature and boiled for 2 h. The digestions were diluted into 25 ml ion-free H_2O and the arsenic concentrations were measured by ICP-AES. Arsenic accumulating rates were calculated by the following formula.

Arsenic accumulating rates (%)=(Arsenic accumulation for treatment-Arsenic accumulation for control/ Arsenic accumulation for control) \times 100%

Results

Inhibitory effects of RBS administration on sarcoma S_{180} tumor growth

Tumor-weight inhibitory rates (TWIR) were calculated relative to the normal saline group and the results are shown in Table 1. The RBS significantly inhibited the growth of mouse sarcoma S_{180} in mice and the inhibitory effect was concentration-dependent. Compared to the negative control group, differences of both tumor weight gain and inhibitory rate showed high statistical significance ($p < 0.01$). At the same arsenic concentration, inhibitory rate for the RBS group was apparently higher than that of As_2O_3 . Showing higher efficacy of RBS over As_2O_3 .

Results of transmission Electron Microscope (SEM) observation

Tumor cell apoptosis is mostly observed by Scanning Electron Microscopy (SEM). Our SEM results are mentioned as follows: *i*) The normal cells (normal saline) showed untreated cells with intact nuclear membrane, huge and circular nuclei, more chromatin and ribosome, big binuclear, abundant mitochondria and endoplasmic reticulum with good morphous in the even and transparent cytoplasm (Figure 1A). *ii*) The low dosage group in RBS showed that the chromatin accumulated inside the nuclear membrane was lumped, and some of it appeared in marginal state. Besides that, intact nuclear membrane and massive expanded mitochondria in the nuclei were found (Figure 1B). *iii*) The medium dosage group in RBS (Figure 1C) ($1.62 \text{ mg}\cdot\text{kg}^{-1}$) showed that intercellular space got increased, the cytoplasm was concentrated, partial nuclear membranes were disrupted and nuclei were broken up and dead *iv*). The high dosage group in RBS (Figure 1D) showed that the cells were shrunken, became smaller, the nuclear membrane was disrupted completely, the nuclei were broken up and even the small apoptotic bodies were formed. *v*) The As_2O_3 group (Figure 1E) showed that the chromatin was accumulated inside the nuclear membrane,

Table 1. Bioleaching Solution of Realgar to Mice S_{180} Solid Tumor Inhibitory Action ($\bar{x} \pm s$; n=2)

Group	Arsenic concentration (mg. kg^{-1})	n	Body weight gain (g)		Tumor weight (g)	TWIR (%)
			Before	After		
Normal Saline	-	10	20.49 \pm 1.45	23.59 \pm 1.76	1.71 \pm 0.12	-
Bioleaching solution of realgar	0.81	10	21.27 \pm 1.26 ^a	23.41 \pm 1.05 ^a	1.26 \pm 0.41 ^a	26.6a
	1.62	10	21.21 \pm 1.42 ^a	23.97 \pm 1.67 ^a	1.25 \pm 0.32 ^a	27.0 a,b
	3.24	10	21.19 \pm 1.85 ^a	23.08 \pm 1.77 ^a	1.16 \pm 0.43 ^a	32.2 a
As_2O_3	1.62	10	21.1 \pm 1.43	24.11 \pm 1.54	1.32 \pm 0.4	22.7

*n stands for total number of mice; The bioleaching solution of Realgar group compares to the negative control group, ^a $p < 0.01$, the equal concentration bioleaching solution of Realgar group compared with As_2O_3 inoculation fluid group, ^b $p < 0.01$

partial nuclear membranes were disrupted and the nuclei were broken up and dead.

Inhibitory effects of RBS on S_{180} cell proliferation

The drug concentration causing a 50% inhibition in cell proliferation (IC_{50}) was calculated after 24, 48 and 72 hours according to the inhibitory rate and expressed as the mean \pm SD as shown in Table 2. In the range of 0.625-0.15625 $\mu\text{g}\cdot\text{mol}\cdot\text{ml}^{-1}$ arsenic concentration, RBS could inhibit the proliferation of S_{180} cells in vitro, the values were also significantly different from that of the normal saline group during 72h ($p < 0.05$).

Cell cycle distribution and induction of apoptosis by RBS

Cell cycle analysis was carried out by the flow cytometric analysis system using a FACS Caliber. The results are presented as follows: In the range of 1~5

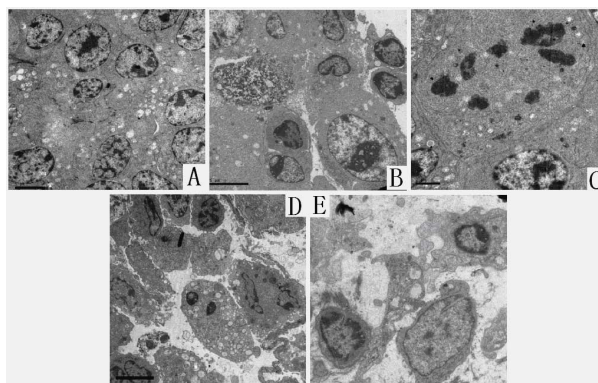


Figure 1. RBS Induced Cell Apoptosis Assayed by Electron Microscope. A) normal saline; B): RBS (0.5 mg/ml arsenic concentration), C): RBS (1.0 mg/ml arsenic concentration), D): RBS (2.0 mg/ml arsenic concentration), E): As_2O_3 (1.0 mg/ml arsenic concentration)

Table 2. Inhibitory Rate of Bioleaching Solution of Realgar at Different Concentration on S_{180} Cell Growth ($\bar{x} \pm s$; n=3)

	Arsenic concentration		OD _{570nm}			Inhibitory rate/%		
	$\mu\text{g}\cdot\text{mol}\cdot\text{ml}^{-1}$	n	24h	48h	72h	24h	48h	72h
bioleaching solution of Realgar	20	3	0.041 \pm 0.025	0.064 \pm 0.056	0.067 \pm 0.012	61.9	84.8	83
	10	3	0.046 \pm 0.033	0.07 \pm 0.032	0.071 \pm 0.037	58.3	83	82
	5	3	0.054 \pm 0.035	0.074 \pm 0.026	0.073 \pm 0.021	55.9	80.1	81.5
	2.5	3	0.074 \pm 0.019	0.087 \pm 0.026	0.087 \pm 0.028	48.2	72.6	77.9
	1.25	3	0.091 \pm 0.081	0.091 \pm 0.023	0.103 \pm 0.015	45.8	66.4	73.9
	0.625	3	0.102 \pm 0.013	0.119 \pm 0.07	0.106 \pm 0.084	29.1 a	62.3 a	73.1 ^a
	0.3125	3	0.15 \pm 0.01	0.139 \pm 0.13	0.227 \pm 0.077	17.2 a	44.6 a	42.5 ^a
	0.15625	2	0.175 \pm 0.037	0.143 \pm 0.038	0.287 \pm 0.067	14.8 a	35.4 a	27.3 ^a
	-	2	0.271 \pm 0.25	0.168 \pm 0.005	0.395 \pm 0.082	-	-	-

*Differences obtained at levels of $p < 0.05$ were considered significant

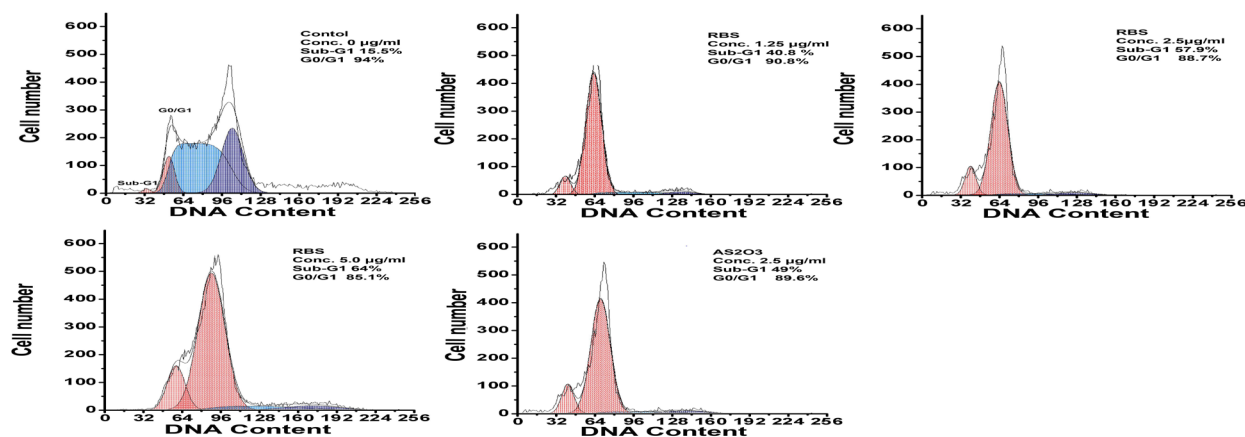


Figure 2. Apoptosis Induction in S_{180} Cells Regulated by RBS. A) normal saline, B) RBS (1.25 $\mu\text{g}\cdot\text{ml}^{-1}$ arsenic concentration), C) RBS (2.50 $\mu\text{g}\cdot\text{ml}^{-1}$ arsenic concentration), D) RBS (5.00 $\mu\text{g}\cdot\text{ml}^{-1}$ arsenic concentration), E) As_2O_3 (2.50 $\mu\text{g}\cdot\text{ml}^{-1}$ arsenic concentration)

Table 3. Accumulation of Arsenic in S_{180} Bearing Mice

	normal saline group		As_2O_3		bioleaching solution of Realgar1		Rates ^a of accumulation/%	Rates ^b of accumulation/%
	weight/g	cont/ μg	weight/g	cont/ μg	weight/g	cont/ μg		
heart	0.1105	1.4175	0.1426	1.6925	0.1515	1.595	19.4	12.5
liver	0.2624	1.315	0.3908	1.78	0.3268	1.625	35.4	23.6
spleen	0.1098	1.0175	0.0912	1.1525	0.182	1.25	13.5	25
lung	0.2473	1.18	0.109	1.875	0.1363	1.8825	58.9 ^c	59.53 ^{cd}
kidney	0.1499	1.425	0.1632	1.58	0.2303	1.6575	10.8	16.3
tumor	0.2777	1.052	0.2302	1.4375	0.2262	1.805	36.6 ^c	71.5 ^{cd}

*Rates^a of accumulation were obtained from As_2O_3 inoculation fluid group; rates^b of accumulation were obtained from RBS group. The RBS group compares to the negative control group, $p < 0.01$, the equal concentration RBS group compared with As_2O_3 inoculation fluid group, $p < 0.01$

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ug.ml⁻¹, typical apoptotic peaks appeared after treatment with RBS for 24h. Number of apoptotic cells increased with increase in the drug concentration used, and as compared with the control group, RBS group showed statistical significance ($p < 0.05$). (Figure 2).

Accumulation of RBS in Sarcoma 180 bearing mice

Analysis results in solid tumor mice group are shown in Table 3. We observed that there was no significant difference in arsenic distribution in different tissues from the two groups, except the distribution in liver, lung and tumor. Every rate of arsenic accumulation was in 10%~20%. However, the arsenic's accumulation in liver, lung and tumor, could reach 23.6%, 59.53% and 71.5% respectively, which were more than those in other tissues. Thus we may suppose that RBS might be more effective for the sarcoma S₁₈₀ in liver, lung and tumor.

Discussion

Apoptosis is a form of cellular suicide that is essential for the development and homeostasis of all multicellular organisms. The main mediators of apoptosis are cysteine proteases belonging to the family of caspases. Two main pathways for the induction of apoptosis have been described, comprising of induction via a complex signalling sequence and through the activation of caspases. Targetting mitochondrial apoptotic proteins in different ways can affect mitochondrial function. The apoptotic proteins can pass through the mitochondrial membrane pores, causing mitochondrial swelling, or the permeability of the mitochondrial membrane causing increase or leakage of the apoptotic effectors. Apoptotic cells show alterations in morphological features from viable cells. This can be differentiated from a normal cell under the light and fluorescence microscope (Ratana et al., 2013; Gupta et al., 2013; ; Adisak et al., 2014; Guan et al., 2014)

Previous studies have indicated that superfine-diameter particles of Realgar exhibited stronger efficacy in inhibiting cell proliferation of SMMC7721 cell lines than larger-sized ones, suggesting that the reduction in Realgar granularity contributed to enhanced Realgar serum concentration and improved its bioavailability (Lu et al., 1999; Xiuqin et al., 2006). In agreement with this, the present study showed that RBS, which directly dissolved Realgar in water, significantly inhibited the growth of tumor S₁₈₀ in a dose-dependent manner. Comparison of the inhibitory rates between RBS and diverse Realgar formula indicated that RBS markedly decreased the dose of Realgar (Peng et al., 1996). Our data convincingly demonstrated that RBS, at the concentration of 0.2125 mg.kg⁻¹ (equivalent to raw Realgar 2.5 mg.kg⁻¹), displayed the similar inhibition rate (26.6%) on tumor S₁₈₀ growth as did 100 mg.kg⁻¹ of raw Realgar, 50 mg.kg⁻¹ of the nanometer Realgar, and 8.3 mg.kg⁻¹ of As₂O₃ injection (3). Thus, the new technology used in the present study to manufacture Realgar significantly enhanced the anti-cancer efficiency of this medicine, and more importantly, decreased the toxicity of Realgar, a particular issue of concern for clinical treatment. We propose that the complete dissolution of Realgar, which solved the

preparation problems, makes it possible to develop the multi-type Realgar dosage forms.

Our research illustrated that RBS induced tumor S₁₈₀ cell apoptosis dose-dependently. The electron microscope assay showed that typical apoptosis appeared at a high dose of RBS. When medium-dose RBS was used, we found that the cells became shrunken, with the cell volume smaller, the mitochondrion expanding accompanied with partial plasmatorrhesis. At a smaller dose, RBS induced endochylema, lumped the chromatin inside the nuclear membrane. These results are consistent with anti-tumor pharmacodynamic rule of anti-bare mouse lung cancer with As₂O₃ (Guangzhou et al., 2006). Some published studies reported that Realgar formula interfered with DNA and RNA synthesis in S₁₈₀ cells. However, it still requires further investigation whether the inhibitory effects of RBS on mouse sarcoma S₁₈₀ are related to the nucleic acid metabolism (Tingdong 1984; Lu et al., 1999; Huibi 2000; Bingli et al., 2004).

Interestingly, our data convincingly showed that administration of RBS resulted in more arsenic accumulation in the solid tumor when compared with control or As₂O₃ alone at the same dose. We propose that arsenic in RBS existed possibly as the arsenic methylamine metabolite, which resulted in relative tumor targeting. It is likely that the formation of arsenic methylamine metabolite is correlated with the complicated metabolism process of microorganism which changed the existent form of arsenic in RBS in order to reduce the arsenic toxicity to itself or facilitate the energy supply for growth (Schipper et al., 1996; Nagpal 1997; Schipper et al., 1999; Ronald et al., 2003).

In summary, the successful extraction of RBS in the present study through hydrometallurgy technology provided not only a potent anti-cancer medication for clinical use, but also a good model for study of other mineral drugs, such as Spanish red and native copper. At the same time, it will furnish further impetus for re-development of mineral drugs and other traditional Chinese medicines containing mineral drugs such as Spanish red, native copper and so on.

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