Inhibition effects of acridone on the growth of breast cancer cells *in vivo*

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Abstract. – **OBJECTIVE:** To investigate the anti-tumor effect of acridone against breast cancer *in vivo* and provide a therapeutic agent for treatment of breast cancer.

MATERIALS AND METHODS: The nude mice xenografted tumor model was established by MCF-7 cells. The mice were randomly divided into four groups. The mice in each group (n=6) were intraperitoneally injected with 0.1 mg/kg saline (low-dose), 0.5 mg/kg (middle-dose) and 1.0 mg/kg (high-dose) of acridone for 21 days, respectively. At the end of the animal experiment, the weight of tumors was recorded to calculate the tumor inhibition rate. The serum hormone levels in peripheral blood were determined using ELISA. Hematoxylin and eosin (HE) staining was used to analyze the histopathological changes. The expression of ABCG2 protein and mRNA were determined by Western blot and RT-PCR, respectively.

RESULTS: The inhibition rates of tumor growth in the high-dose, middle-dose, and low-dose groups were 29.18%, 17.21%, and 4.27%, respectively. Compared with control and low-dose group, the tumors growth rate in high-dose and middle-dose groups were decreased significantly. Histologically, the tumors were inhibited in the growth rate, the tissue structure was broken. Estrogen in all groups with acridone treatment decreased, the progesterone in high-dose and middle-dose groups increased remarkably. The expression of ABCG2 protein and ABCG2 mRNA decreased after treatment with acridone.

CONCLUSIONS: We showed that acridone could induce cell apoptosis, inhibited ABCG2 (ATP-binding cassette sub-family G member 2) protein and adjusted hormone level. The results suggested that acridone could serve as a chemotherapeutic agent for treatment of breast cancer *in vivo*.

Key Words:
    Acridone, Breast cancer, MCF-7 cells, Anticancer, ABCG2.

Abbreviations

ER-estrogen receptor, PR-progesterone receptor, MDR-multidrug resistance, ABC-ATP-binding cassette, ABCG2-Breast cancer resistance protein, MCF-human breast cancer cell line, NIH-National Institutes of Health, DMEM-Dulbecco's Modified Eagle Medium, FBS-fetal bovine serum, ELISA-enzyme-linked immuno sorbent assay, HE-hematoxylin-eosin, RT-PCR-reverse transcriptase-polymerase chain reaction, OD-optical density, QRT-PCR-quantitative Real-time-polymerase chain reaction, BCA-bicinchoninic acid, SDS-PAGE-sodium dodecyl sulphate-polyacrylamide gel electrophoresis, PVDF-polyvinylidene fluoride, TBST-Tris-buffered saline and Tween 20, ECL-Electrochemiluminescence, GAPDH-Glyceraldheyde 3-phosphate dehydrogenase, SD-standard deviation.

Introduction

As the most common form of female cancer, breast cancer has become the second leading cause of cancer death in women[1,2]. Meanwhile, an increasing attack rate of male breast cancer has been reported[3]. Researches[4] have shown that there was a positive relationship between the serum estrogen levels and breast cancer. Moreover, with antagonism action to estrogen, an increase of progesterone would reduce breast cancer risk[5]. Estrogen receptor (ER) and progesterone receptor (PR), which have been employed for breast cancer detection and treatment, are two main cancer markers[6-8].

To date, chemotherapy still plays essential roles in the treatment of breast cancer. However, the quick multidrug resistance (MDR) of cancer cells turns into a major obstacle in treatment. MDR can help tumor cells to avoid drug targeting, leading to treatment failure[9,10]. As the drug efflux pump on plasma membrane, the ATP-bind-

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ing cassette (ABC) transporters are closely associated with MDR, which could pump drugs out of the cells and consequently the intracellular concentrations of drugs are limited[9]. Breast cancer resistance protein (ABCG2) was shown to be involved in MDR phenotype in breast cancer[11,12]. Since isolation of ABCG2 from the doxorubicin resistant MCF-7 human breast cancer cells, considerable efforts have been made to discover their inhibitors to reduce the risk of drug resistance[13], but only a few inhibitors of ABCG2 were reported[14,15]. To study the effect of inhibitors to ABCG2 adequately is significance for therapy of breast cancer. Acridone, which is a type of naturally occurring alkaloids, is considered to be aza-analog of xanthones[16,17]. At the beginning, acridone was mainly used as antiparasite and antibacterial agents. In the early 19th century, researchers realized the anti-cancer potential of acridones. Acridone derivatives have been applied in clinics as anti-tumor chemotherapeutics since 1970's. The cytotoxicity of acridone was not only from their direct binding to DNA but also from the interactions with biological targets. With the planar aromatic structure, the acridone-derived compounds could inset into the DNA strand to interfere cellular machinery. Those compounds could also act as DNA topoisomerases poison and G-quadruplexes-DNA stabilizing agents to inhibit telomerase activity and protein-kinases inhibitors to contribute to their antiproliferation or cytotoxicity in cancer treatment. Also, acridone has been identified as the inhibitors of ABCG2[18,19]. Therefore, acridone can potentially serve as the MDR modulators in tumor chemotherapies. Research[20] has confirmed that acridone is a potential chemotherapeutic agent for breast cancer treatment due to its anti-proliferation effect on breast cancer cells and inhibitory activity to breast cancer resistance protein ABCG2. However, studies on the *in vivo* functions of acridone have not been reported. In order to acquire more evidence *in vivo* and to provide new insights for breast cancer treatment, we investigated the anti-cancer activity and mechanism of acridone *in vivo* using the human breast cancer cell lines (MCF)-7 xenografted mice model.

Materials and Methods

Animal Feeding

All animal experiments are conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory animals and were approved by the Ethics Committee and the Institutional Animal Care Use Committee. Thirty female BALB/c-nude mice (4 weeks old) were from Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). The mice were kept for one week in the quiet specific-pathogen-free (SPF) raising condition at 22-24°C with good ventilation, a controlled relative humidity of 50%-60%, and free access to food and water.

In vivo Antitumor Assay Against MCF-7 Xenograft

MCFl-7 human breast cancer cells, which were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), were conventionally cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 1% double antibiotics (Gibco, Rockville, MD, USA) at 37°C containing 5% CO_2. To generate xenografts in mice, MCF-7 human breast cancer cells were grown orthotopically as subcutaneous xengrafts in the region of thoracic mammary fat pad of mice. 0.3 mL of single-cell suspension (2.0×10^7/mL) in buffer solutions were injected into every mouse. Xenografts were grown until they were 100 mm³, and then all mice implanted with MCF-xenografts were randomized within four groups, which include a control group and three experimental groups (high-dose, middle-dose, and low-dose groups) treated by acridones (Sinopharm. Chemical Reagent Co., Ltd., Beijing, China). With the dosage of 1 mg/kg, 0.5 mg/kg and 0.1 mg/kg, respectively, the drug solutions were injected into the abdominal cavity of every mouse in each experimental group once a day. The mice of the control group were treated with the same volume of saline. After continuous administration for 21 days, the peripheral blood of each mouse was collected by removed eyeball. The blood was centrifuged (3500 rpm, 15 min) to collect the upper serum carefully, and stored at -80°C. Subsequently, mice were sacrificed and dissected to acquire the intact tumor tissues. The tumors were weighed to calculate the tumor inhibition rate using the following equation. Tumor growth inhibition rate (%) = (the mean tumor weight of control group-the mean tumor weight of experimental group)/the mean tumor weight of control group ×100%.

Measurement of Estrogen and Progesterone Levels in Serum

The content of estrogen and progesterone inside mice of all groups was determined in the collected and stored serum above using the enzyme-linked immuno sorbent assay (ELISA) kit (Sigma-Aldrich, St., Louis, MO, USA) according to the manufacturer's instructions.

Hematoxylin Eosin (HE) Staining of Tumor Tissues

The tumors in each group were fixed with 10% neutral formaldehyde solution. After that, the samples were dehydrated by passing a graded series of ethanol concentrations (60%, 70% and 80%) for 4 h. The specimens were further dehydrated by butanol for 8 h. After embedding with paraffin, the samples were sliced into 5 μM thin sections. Slices were transferred to glass slides and treated with xylene for 3 times, 30 min for each time. The slides were treated with a graded series of ethanol concentrations (100%, 95%, 90%, 85%, 80% and 75%) for 5 min each. The slides were immersed in water for 5-10 min. After that, the slides were immersed in the hematoxylin for 5-10 min, followed by washing with water to remove the floating reagent. The slides were treated with 0.1% hydrochloric acid for 5 s, followed by washing with water for 10 min. The slides were immersed in eosin solution for 8 s, followed by washing with water to remove the floating reagent. The slides were dehydrated by passing a graded series of ethanol concentrations (75%, 85%, 90%, 95% and two times of 100%) for 5 min each. Next, slides were washed twice with xylene, 15 min each time. Neutral gum was used to seal the slide before the completely dry. The tissues were observed under a microscope (Nikon, Tokyo, Japan).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to Detect the Expression of ABCG2 mRNA

The tumor tissue was collected and TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA extraction. The concentration and purity of the RNA sample were checked by measuring the optical density (OD) value at 260 nm and 280 nm using Nanodrop 2000 micro UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The reverse transcription procedure was performed according to the instructions of the kit (TaKaRa, Otsu, Shiga, Japan). Quantitative Real-time-polymerase chain reaction (QRT-PCR) amplification was performed using the SYBR Green kit (Roche Molecular Biochemicals, Basel, Switzerland). Two replicates were set for each reaction and β-actin was used as the endogenous control. The following primers were used: ABCG2: Forward: 5'-GGCCTGG-ACAAAGTAGCAGA-3', Reverse: 3'-CTCCAT-TCCTATGCTTGTCCTT-5'; β-actin: Forward: 5'-CGGGAAATCGTGCGTGACAT-3', Reverse: 5'-GAAGGAAGGCTGGAAGAGTG-3'. All the primers were synthesized by Sangon (Shanghai, China). Reaction conditions were as follow: 95°C for 30 s, followed by 35 cycles of 95°C for 10 s, 60°C for 15 s and 72°C for 15 s, then 60°C for 15 s, and 4°C to stop the reaction. The CFX connect TMqRT-PCR system was used to detect the fluorescence threshold cycle (Ct) values for each sample. Melting curve analysis was performed to ensure PCR specificity. $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of the target gene mRNA.

Western Blot to Detect the Expression of ABCG2 Protein

Western blot kit was from Bio-Rad (Hercules, CA, USA). Total protein extraction and the protein concentration were measured by bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotech, Shanghai, China). 40 μg total protein were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transmembrane to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% skim for 1 h. After that, the primary ABCG2 polyclonal antibody (Abcam, Cambridge, MA, USA), which was diluted (1:1000) using 50 g/L bovine serum albumin (BSA) solution, was used to incubate with the membrane overnight at 4°C. The membrane was washed with Tris-buffered saline-tween (TBST, containing 1ml/L Tween-20) for 3 times, 5 min each. After that, the secondary antibody goat anti-rabbit (1:10000, Abcam, Cambridge, MA, USA) was used to incubate with the membrane at room temperature for 2 h. The membrane was washed 3 times with TBST, 10 min each time. After that, enhanced chemiluminescence (ECL) solution was added and the reaction was kept in dark room. Image J 2.1 software (GE Healthcare, Piscataway, NJ, USA) was used to scan and quantify the gray value. The expression level of ABCG2 protein was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Statistical Analysis

SPSS19.0 statistical software (IBM, Armonk, NY USA) was used to analyze the monitoring data. All the data were expressed as mean ± standard deviation (mean ± SD). The comparisons between group were performed by *t*-test, and $p < 0.05$ was considered to be statistically significant.

Results

Inhibition of Tumor Growth in Human Breast MCF-7 Xenografted Nude Mice

To confirm the antitumor activity of acridone against human breast cancer, an *in vivo* experiment was conducted using a human breast MCF-7 xenografted animal model. Female nude mice (BALB/c-nu) were inoculated with MCF-7 cells orthotopically in the region of thoracic mammary fat pad and treated with different dosage of acridone (1 mg/kg for high-dose group, 0.5 mg/kg for middle-dose group, and 0.1 mg/kg for low-dose group) by intraperitoneal injection once daily for 21 days. At the end of the experiment, the tumors were weighed to afford the mean tumor weight of mice from each group. The average tumor weight of mice in low-dose group, middle-dose group, high-dose group and control group was 1.302 ± 0.097 g, 1.044 ± 0.102 g, 0.965 ± 0.08 g, and 1.364 ± 0.086 g, respectively (Table I). For the tumor growth inhibition rates, the values in the high-, middle-, and low-dose groups were 29.18%, 17.21%, and 4.27%. Compared with tumors in both control group and low-dose group, the growth (rate) of the tumors treated with high- and middle-dosage of acridone decreased significantly ($p < 0.05$). Meanwhile, the inhibition effect of high acridone dose was strongest. In addition, through the comparative study on body weight change and histopathological changes of organs among groups, we found no evidence that acridone was toxic to mice. These xenograft based experiments indicated that, with an appropriate dosage, acridone could inhibit the tumor growth *in vivo*.

Influence of Acridone on Estrogen and Progesterone Levels Inside Tumor Bearing Mice

In order to investigate the mechanism of acridone in anticancer, we tested the serum hormone levels inside tumor-bearing mice before their death. The 3 weeks of administration of acridone to tumor-bearing mice produced a noticeable change in their serum estrogen and progesterone levels (Figure 1). Comparing with the control group, the estrogen level of mice from all groups treated with acridone decreased to some extent ($p < 0.05$), while the progesterone level of mice from high- and middle-dose groups treated with acridone increased remarkably ($p < 0.05$).

Growth Inhibition of MCF-7 Cells in HE Staining Xenografts

The xenograft tumor tissues from control groups formed solid structure, and some cancer cells with large and stained deeply nuclei arranged in flake or in nest (Figure 2A). Especially, pathologic division of nucleus, heteromorphic cells and cancer nests, could be visible. Comparatively, the structures of tumor tissues in xenografts treated with acridone were significantly destroyed, and the cancer cells enlarged slightly (Figure 2B-D). Meanwhile, there were less heteromorphic cells and more fibrous tissue in the treated tumors, and some necrosis area exhibited marked lymphatic and leukocytosis permeation as well. HE staining results showed that the growth of MCF-7 cancer cells was inhibited when treated with acridone.

Level of ABCG2 Expression in Untreated and Acridone Treated Tumor-Bearing Mice

The expression of ABCG2 mRNA in different groups was determined by RT-PCR method.

Table I. Results of anticancer assay of acridone in MCF-7 xenografted nude mice.

Groups	Mean weight of tumors (g) (mean ± SD)	Tumor growth inhibition rate (%, mean ± SD)
Control	1.364 ± 0.086	–
Low-dose of acridone (0.1mg/kg)	1.302 ± 0.097	4.27 ± 0 .62
Middle-dose of acridone (0.5 mg/kg)	1.044 ± 0.102*	17.21 ± 3.17[#]
High-dose of acridone (1 mg/kg)	0.965 ± 0.08*	29.18 ± 5.13[#]

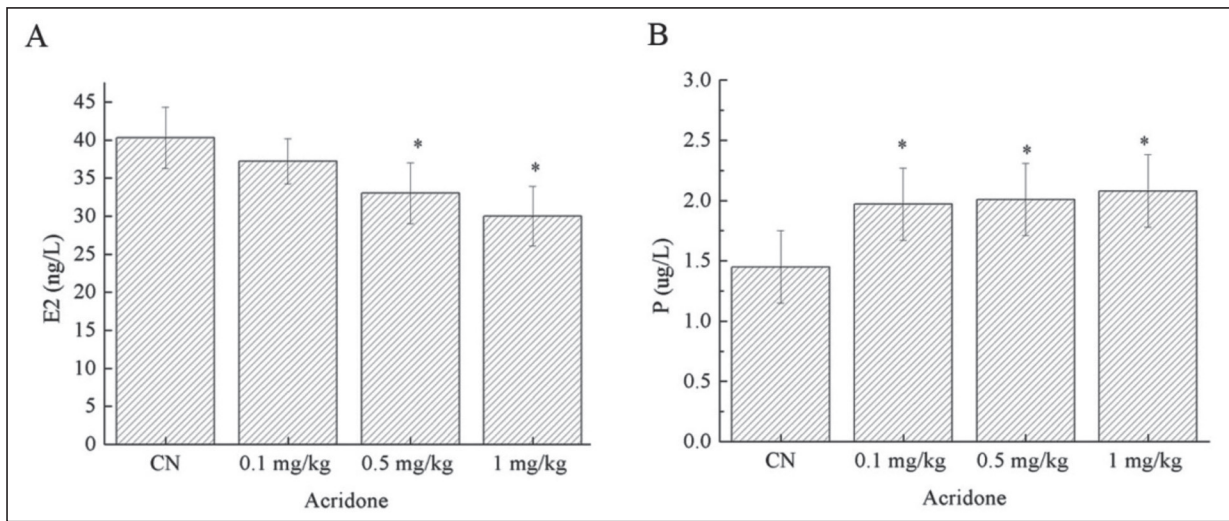Compared with control group, *$p < 0.05$; compared with high dose acridone group, [#]$p < 0.05$.

Figure 1. The acridones on estrogen and progesterone levels of each group. Compared with control group, *$p < 0.05$.

Compared with the control group, the relative expression level of ABCG2 mRNA in acridone groups was significantly decreased ($p < 0.05$) (Figure 3). The relative expression level of ABCG2 mRNA in breast cancer tissue of the group treated with 1 mg/kg acridone was decreased to 0.16, which was significantly lower that the relative expression level of ABCG2 mRNA in the groups treated with 0.1 mg/kg acridone and 0.5 mg/kg acridone (0.89 and 0.92, respectively).
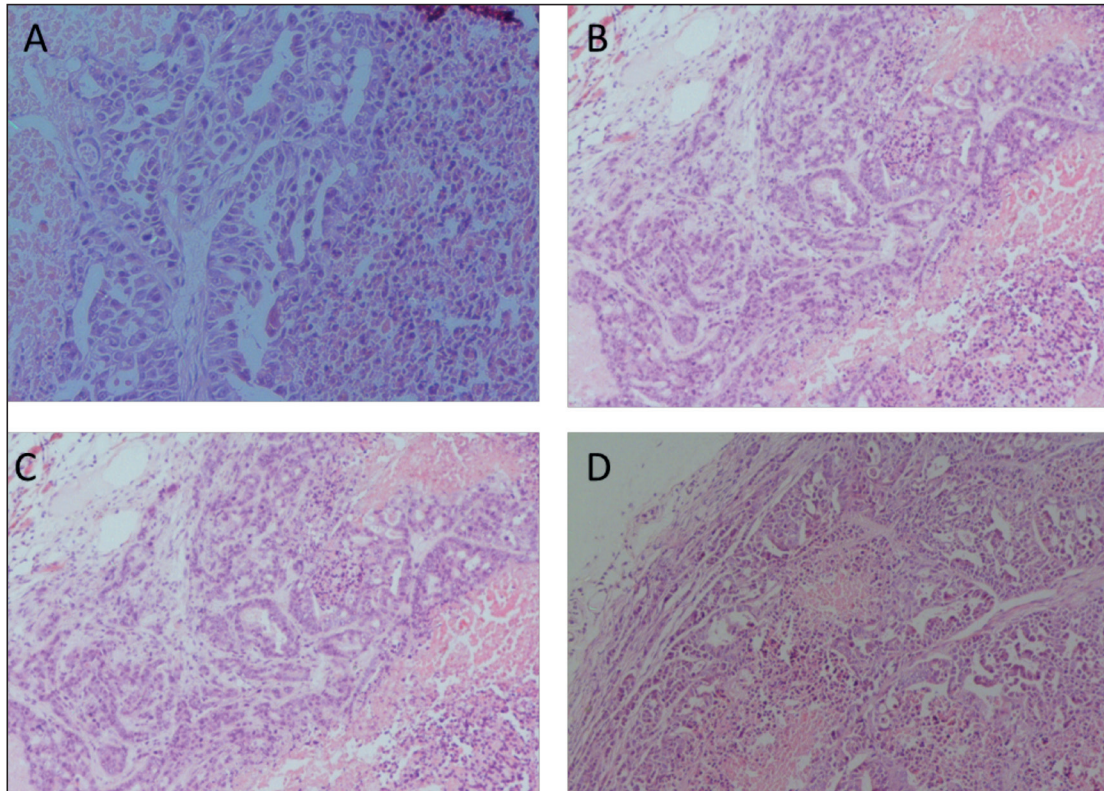

Figure 2. Histological analysis of xenograft tissue stained by HE (×100). **(A)** Control group, **(B)** Low-dose group, **(C)** Middle-dose group, **(D)** High-dose group.
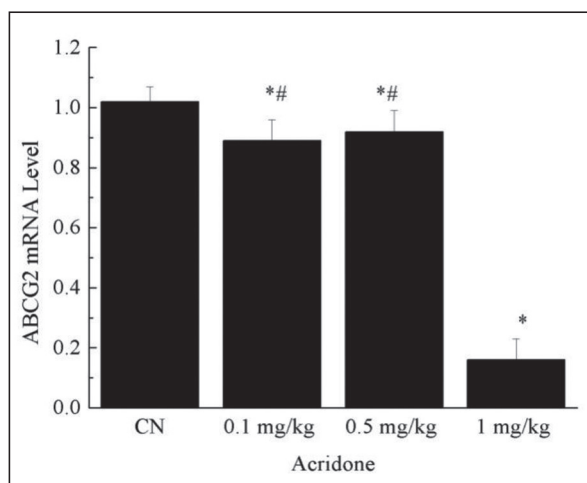
Figure 3. The relative expression levels of ABCG2mRNA in each group. *Notes*: Compared with control group, *$p < 0.05$; compared with high dose acridone group, #$p < 0.05$.

The expression of ABCG2 protein and β-actin were detected by Western blot. The ABCG2 protein was highly expressed in the control group, which formed significant difference with three treatment groups ($p < 0.05$) (Figure 4). All the three experimental groups treated with different dosage of acridone resulted in down-regulation of ABCG2 protein. The high-dose of drugs inhibited the protein expression more significantly than the low-dose and middle-dose of drugs ($p < 0.05$).

Discussion

Breast cancer has become one of the most important killers to human health. Although chemotherapy is playing a significant role in treatment and prevention of breast cancer, the obligate chemotherapeutic agents were limited by the development of MDR in tumor tissue during the application of drugs. Therefore, the discovery and development of dual inhibitors against MDR and breast cancer will definitely provide new ideas for chemotherapy of breast cancer.

Acridone is a natural alkaloids, which attracts much attention for its anti-tumor activity against breast cancer. Previous studies have shown that the *in vitro* application of acridone can significant reduce the proliferation of cancer cells and inhibit the expression of to ABCG2. We evidenced that as chemotherapeutic agent, acridone displayed excellent antitumor activity against breast cancer *in vivo*. In the MCF-7 xenografted animal experiment, it was quite explicit that the administration

of acridone in high-dose (1 mg/kg) and middle-dose (0.5 mg/kg) both inhibited the growth of tumor in living mice to a large extent. The inhibition effect of the low-dose of administration was confined, which indicated enough dosage of acridone should be utilized *in vivo* to inhibit breast tumor growth. In order to further detect the effects of acridone on breast tumor tissues, we observed the histological structures of MCF-7 xenografts using the HE staining method. As a result, acridone was found to be able to destroy the structure of MCF-7 tumor tissues by preventing the enlargement of cancer cells, reducing cell atypia, and increasing the degree of necrosis of cancer tissue.

At present, the anticancer activity of acridone against breast cancer *in vivo* has been verified using the successfully established breast MCF-7 xenografted mice model. How did this type of compounds work in mice to fight against breast cancer? In the following experiment, we found evidence to illustrate the mechanism of the actions of acridone in inhibiting breast tumor.

Since acridone had been reported to be able to serve as an inhibitor of ABCG2, we measured the levels of ABCG2 protein and mRNA
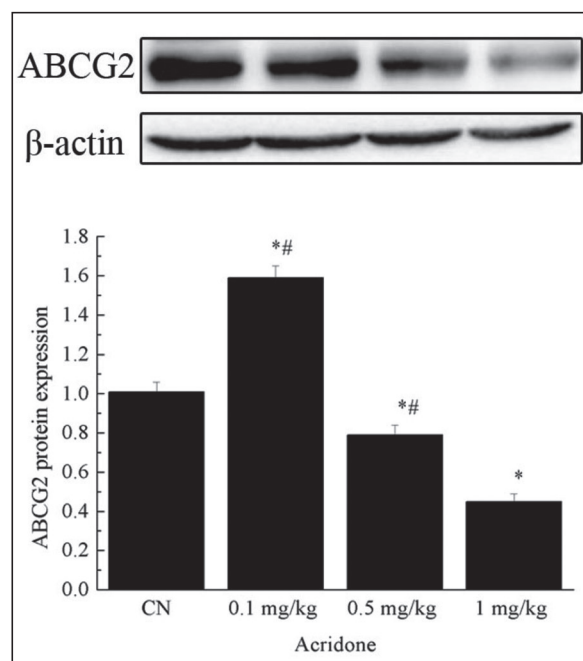

Figure 4. Western blot analysis for ABCG2 expression in tumor bearing mice. *Notes*: Compared with control group, *$p < 0.05$; compared with high dose acridone group, #$p < 0.05$.

in acridone treated and untreated tumor-bearing mice via Western blot and the RT-PCR assay. The mice from treatment groups displayed lower levels of ABCG2 protein expression and mRNA content. In the meanwhile, the level of ABCG2 declined at the extreme in the treatment group with high dose of acridone, which indicated that abundant drugs were necessary to inhibit the expression of ABCG2 protein. ABCG2 protein is an important member of ATP-binding cassette transporters that was closely associated with MDR. So as the inhibitor of ABCG2, acridone could overcome effectively through limiting drugs be pumped out of the cells and increasing intracellular concentrations of drugs.

In addition, the levels of serum hormone were also determined. Acridone may induce the decrease of serum estrogen level and increase serum progesterone level. It is considered that estrogen content is positively related to breast cancer risk, while progesterone plays an opposite role to estrogen, which can inhibit the growth and development of breast tumor. Hence, by virtue of the adjustment of estrogen and progesterone levels, acridone could also work well in treatment of breast cancer *in vivo*. We showed that acridone can serve as a chemotherapeutic agent to participate in the chemoprevention of solid breast MCF-7 cancer in nude mice. Acridone was able to inhibit the tumor growth and destroy the tumor structure histologically through preventing the enlargement of cancer cells, reducing cell atypia and increasing the degree of necrosis of cancer tissue. Further investigations were involved in the studies of anti-cancer mechanism. The anti-proliferation of acridone against breast cancer cells *in vivo* was verified due to its multiple actions on inducing of cell apoptosis, inhibition of ABCG2 pathway, downregulation of the expression of ABCG mRNA, and decreased estrogen level and increased progesterone level. It is notable that acridone could be used as an ABCG2 inhibitor *in vivo* to overcome the MDR occurring in chemotherapy of breast cancer *in vivo*.

Conclusions

We showed that acridone could induce cell apoptosis, inhibits ABCG2 protein and adjusts hormone level. The results suggested that acridone could serve as a chemotherapeutic agent for treatment of breast cancer *in vivo*.

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