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

Y.-F. XIA[1], H.-J. CHU[1], G.-F. KUANG[1], G.-J. JIANG[2], Y.-C. CHE[1]

[1]Department of Gynecology, The Affiliated Hospital of Qingdao University, Qi[illegible]ao, Ch[illegible]
[2]Department of Pharmacy, Tengzhou Central People's Hospital, Zaozhuang[illegible]na

Yufang Xia and *Huijun Chu* contributed equally

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 [illegible] respectively. At the end of the animal [illegible]ment, the weight of tumors was recorded [illegible]al-culate the tumor inhibition rate. The serum [illegible]-mone levels in peripheral blood were de[illegible]mined using ELISA. Hematoxylin and eosin (H[illegible] staining was used to analyze th[illegible]patholog[illegible]ical changes. The expressio[illegible] protein and mRNA were determine[illegible]Weste[illegible]lot and RT-PCR, respectively.

RESULTS: The inhib[illegible]rate[illegible] growth in the high-d[illegible]m[illegible]e, and low-dose groups were [illegible]8%, 17[illegible] and 4.27%, respectively. Co[illegible]ed with c[illegible] and low-dose group, the[illegible]growth rate[illegible]h-dose and middle-d[illegible]gro[illegible]ere decreas[illegible]d signifi-cantly. Histolo[illegible]ically, t[illegible]nors were inhibited in the gro[illegible]rate, the tis[illegible]ructure was bro-ken. Es[illegible]en in all groups [illegible]acridone treat-ment [illegible]eased [illegible]e progesterone in high-dose and [illegible]dle-do[illegible]groups increased remarkably. The [illegible]ess[illegible]of ABC[illegible]2 protein and ABCG2 mRNA [illegible]ed afte[illegible]eatment with acridone. CONC[illegible]NS: [illegible]showed that acridone [illegible]indu[illegible]optosis, inhibited ABCG2 (AT[illegible]nding [illegible]te sub-family G member 2) pro[illegible] and adj[illegible]sted hormone level. The re-su[illegible] that acridone could serve as a [illegible]hoth[illegible]eutic agent for treatment of breast [illegible]ncer *in vivo*.

[illegible]rds:
A[illegible]one, Breast cancer, MCF-7 cells, Anticancer, ABCG2.

Abbrevi[illegible]ns

ER-es[illegible] receptor, [illegible]ogesterone receptor, MD[illegible]rug resistance, A[illegible]-ATP-binding cassette, A[illegible]G2-Breast cancer resistance protein, MCF-human [illegible]st cancer cell li[illegible]NIH-National Institutes of Health, [illegible]EM-Dulbecco[illegible]dified Eagle Medium, FBS-fetal [illegible] serum, EL[illegible]enzyme-linked immuno sorbent as[illegible]E-hema[illegible]in-eosin, RT-PCR-reverse tran-script[illegible]e chain reaction, OD-optical densi-ty, QRT-P[illegible]quantitative Real-time-polymerase chain [illegible]action, BCA-bicinchoninic acid, SDS-PAGE-sodium [illegible]sulphate-polyacrylamide gel electrophoresis, [illegible]yvinylidene fluoride, TBST-Tris-buffered sa-[illegible]ne and Tween 20, ECL-Electrochemiluminescence, GAPDH-Glyceraldehyde 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-

Corresponding Author: Yanci Che, MD; e-mail: cheyanci@126.com

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-d compounds could inset into the DNA s interfere cellular machinery. Those comp could also act as DNA topoisomerases p and G-quadruplexes-DNA stabilizing agents inhibit telomerase activity and in-kinas inhibitors to contribute to th iferation or cytotoxicity in cancer t ment. o, acridone has been identifie the i of ABCG2[18,19]. Therefore, ac serve as the MDR r lato mor chemotherapies. Researc as confirm t acridone is a potential c rapeutic age breast cancer treatm du anti-prolif ation effect on brea ncer ce inhibitory activity to breast er resistance p ABCG2. However, st s on the *in vivo* fu ons of acridone have been r ted. In order to acquire more evi *in* and t provide new insights for br er trea t, we investigated the anc ivity mechanism of acridone usin an breast cancer cell lines (MC 7 xenog d mice model.

Materials and Methods

A l 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 C Committee. Thirty female BALB/c (4 weeks old) were from Shangh aboratory Animal Center, Chinese Acad f Sciences (Shanghai, China). The mice we for one week in the quiet specific thogen (SPF) raising condition at 22-2 with good tion, a controlled relati midit f 50%- and free access to fo d wat

In vivo Antitu A gainst MCF-7 Xeno t

MCFl-7 breast ca ls, which were pur ed the Cell Ba k of Chinese Academy f Scie Shanghai, China), were conve ally cultu Dulbecco's Modified Medium (DM) (HyClone, South L n, UT, USA) containing 10% fetal bovine um (FBS) co, Rockville, MD, USA), double anti cs (Gibco, Rockville, MD, U t 37°C aining 5% CO_2. To generate xen e, MCF-7 human breast cancer cells grown orthotopically as subcutaneous xengrafts in the region of thoracic mammary fat pad of mice. 0.3 mL of single-cell susp on (2.0×10^7/mL) in buffer solutions were injected into every mouse. Xenografts were grown until they were 100 mm^3, 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 h toxylin for 5-10 min, followed by washi water to remove the floating reagent. The s were treated with 0.1% hydrochloric acid fo followed by washing with water for 10 min. slides were immersed in eosin n for 8 followed by washing with w ove the floating reagent. The slide e deh ated by passing a graded series o anol co tions (75%, 85%, 90%, 95% a ti for 5 min each. Nex des ashed twice with xylene, 15 mi ch time. l gum was used to seal the efore the co ly dry. The tissues w ob d under a microscope (Nikon, Tokyo, Japan).

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

The issue collected and TRIzol ent (ogen rlsbad, CA, USA) was or RN ion. The concentration and pur of the A sample were checked by me the optical density (OD) value at 260 m using Nanodrop 2000 micro spectrophotometer (Thermo Fisher Scientif- Waltham, MA, USA). The reverse transcripti cedure 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 were set for each reaction and β-act as the endogenous control. The foll g primers were used: ABCG2: Forward: GCCTGG-ACAAAGTAGCAGA-3', Reverse TCCAT-TCCTATGCTTGTCCTT-5' -actin ward: 5'-CGGGAAATCGTGC ACAT-3', 5'-GAAGGAAGGCTC AGAC G-3'. A primers were synthe by S n (Shanghai, China). Reaction co was follo 95°C for 30 s, followe 35 of 95° r 10 s, 60°C for 15 s 72°C fo th 60°C for 15 s, and 4° p the reacti CFX connect TM c tem was use to detect the fluorescen thresh cle (Ct) values for each sampl lting curve is was performed to en specificity. 2 method was used to c ate the relative expression level of the target e mRNA.

Western Blot to Detect the Expression of ABCG2 Protein

Weste ot kit was from Bio-Rad (Hercules, CA, USA). Total protein extraction and ein concentration were measured by ch inic 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 t[illegible]rs were weighed to afford the mean tumor[illegible] of mice from each group. The average [illegible]or weight of mice in low-dose group, middle-[illegible] group, high-dose group and control group [illegible] 1.302 ± 0.097 g, 1.044 ± 0.102 g, [illegible]65 ± 0.08 g and 1.364 ± 0.086 g, respect[illegible]e I). For the tumor growth inhibitio[illegible]es, th[illegible]alues in the high-, middle-, and [illegible]-dose g[illegible]were 29.18%, 17.21%, and 4.27[illegible]m[illegible] mors in both control group a[illegible]dose group, the growth (rate) o[illegible] tumors t[illegible]with high- and middle-dos[illegible]ridone decr[illegible]significantly ($p < 0.0$[illegible]Me[illegible]le, the inhibition effect of high acridone dose w[illegible]ongest. In addition, through t[illegible]comparative s[illegible]n body weight change[illegible] histopathological [illegible]anges of organs amo[illegible]roups, found no evidence that acridone [illegible]s to[illegible]o mice. These xenograft based experiments indicated that, with an appropriate dosage, acridone could inhibit the tumor growth *in vivo*.

Influence of Acridone on Est[illegible]en and Progesterone Levels In[illegible] Tumor Bearing Mice

In order to investigate th[illegible]echan[illegible]acridone in anticancer, we te[illegible]he serum [illegible] levels inside tumor-be[illegible]g mic[illegible] before death. The 3 weeks o[illegible]ministr[illegible]n of acridone to tumor-bearing m[illegible]ro[illegible]d a n[illegible]able change in their s[illegible]n es[illegible]and pr[illegible]terone levels (Figure[illegible] Compar[illegible]ith[illegible] control group, the e[illegible] level of mi[illegible] all groups treated w[illegible]ridone decreased [illegible] some extent ($p < 0.05$), while t[illegible]gesterone level of mice from h[illegible]and middl[illegible] groups treated with ac[illegible]creased remar[illegible]y ($p < 0.05$).

[illegible]owth Inhib[illegible]n of MCF-7 Cells in [illegible] Staining X[illegible]ografts

[illegible] xenogr[illegible]umor tissues from control grou[illegible]id structure, and some cancer cells wit[illegible]e and stained deeply nuclei arranged [illegible]flake or in nest (Figure 2A). Especially, patho-[illegible]ision of nucleus, heteromorphic cells and [illegible]ests, 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.

[illegible] Resu[illegible]cer assay of acridone in MCF-7 xenografted nude mice.

[illegible]roups	Mean weight of tumors (g) (mean ± SD)	Tumor growth inhibition rate (%, mean ± SD)
[illegible]ontrol	1.364 ± 0.086	–
[illegible]w-dose of acridone (0.1mg/kg)	1.302 ± 0.097	4.27 ± 0 .62
[illegible]le-dose of acridone (0.5 mg/kg)	1.044 ± 0.102*	17.21 ± 3.17#
[illegible]ose 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, #$p < 0.05$.

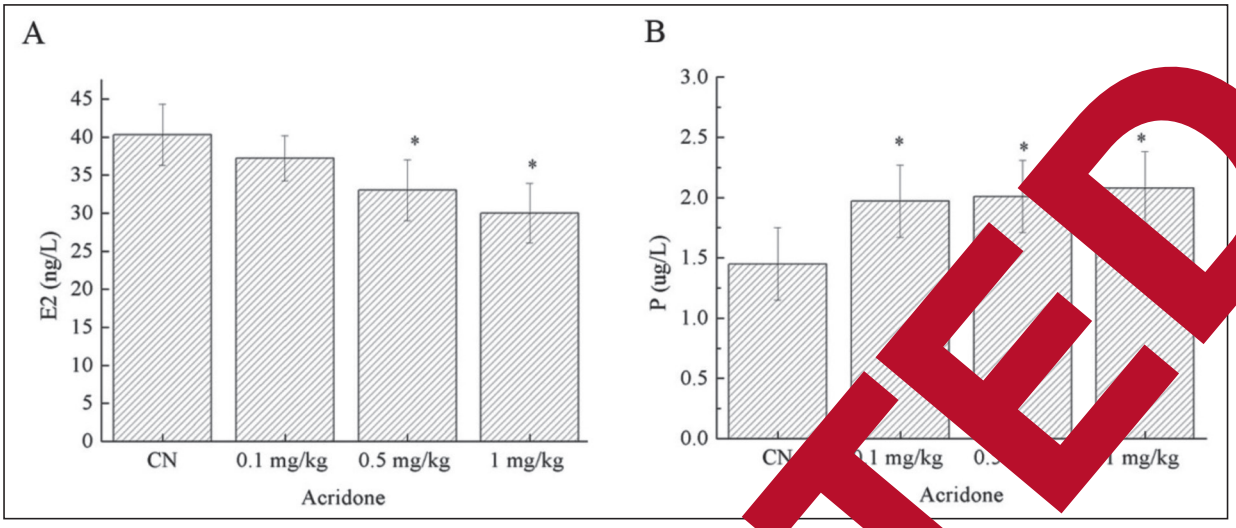

Figure 1. The acridones on estrogen and progesterone levels of each group. Compared with [illegible] group, *$p < 0.05$.

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 [illegible] ted with 1 [illegible] g acridone was decreased to [illegible] which was [illegible] nificantly lower that the rel- [illegible] xpressio[illegible] el of ABCG2 mRNA in the gro[illegible] th 0.1 mg/kg acridone and 0.5 mg/kg [illegible] (0.89 and 0.92, respectively).

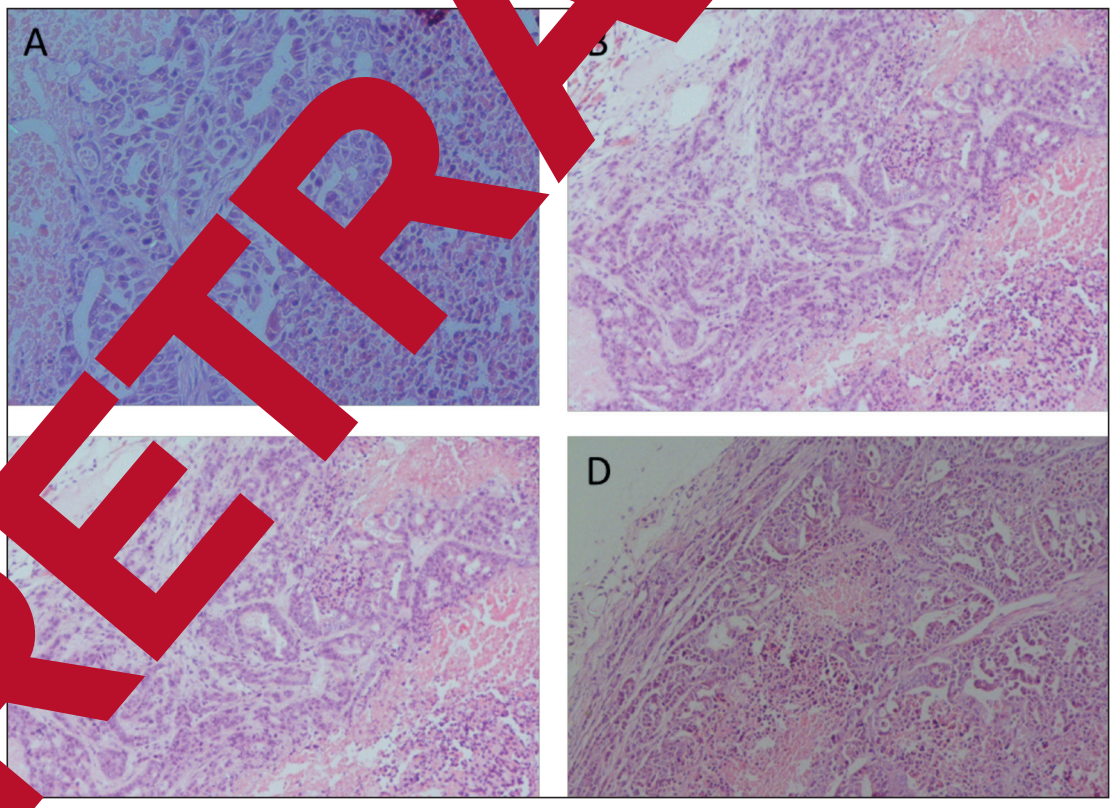

Figure 2. Histological analysis of xenograft tissue stained by HE (×100). ***(A)*** Control group, ***(B)*** Low-dose group, ***(C)*** Middle-dose group, ***(D)*** High-dose group.

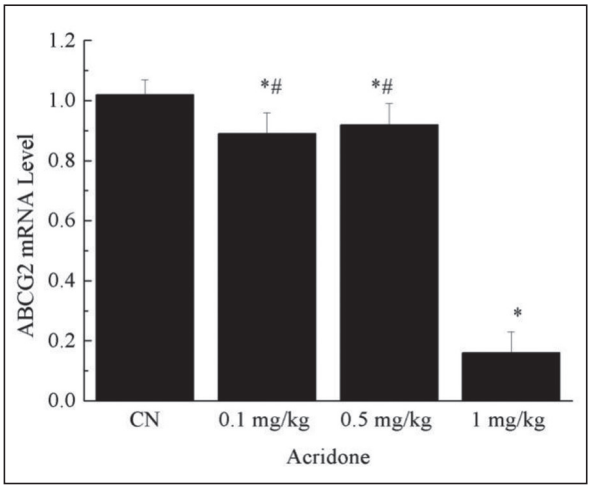

Figure 3. The relative expression levels of ABCG2mRNA in each group. *Notes*: Compared with control group, $*p < 0.05$; compared with high dose acridone group, $^{\#}p < 0.05$.

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). A... the three experimental groups treated with ... dosage of acridone resulted in down-regula... of ABCG2 protein. The high-dose of drugs in... ed the protein expression more significantly t... the low-dose and middle-dose of ... ($p < 0.05$

Discus...n

Breast cancer has ...me ...ne most important killers to h... health. A...gh chemotherapy is playi... nificant role ...atment and preventi... cancer, th... obligate chemothera...ic agents ... limited by the development ... MDR in tu... sue during the applica... of drugs. Therefore, ...e discovery and devel...ent of ... inhibitors against MDR and bre...ncer ... definit...y provide new ideas for chem... f brea...ncer.

Acrido... a nat... alkaloids, which attracts ...ttenti... anti-tumor activity against brea... cancer. P...ious studies have shown that the ... application of acridone can significant ...feration of cancer cells and inhibit ... expression of to ABCG2. We evidenced that ... hemotherapeutic agent, acridone displayed e...t antitumor activity against breast cancer *in vi...*. 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 exten... hibition effect of the low-dose of ad... was confined, which indicated e...gh dosage of acridone should be utilized i... to inhibit breast tumor growth. In order to ...r detect the effects of acridone on ...ast tu... sues, we observed the histologi...tructures o... xenografts using the H...taining ...ethod. result, acridone was ...d to h...le to destroy the structure of MC... o...ues by ...venting the enlargem... of c... ells, re...ng cell atypia, and in...sing the ... ...crosis of cancer tissu...

At pre..., t...ticancer ac...vity of acridone aga...st brea...ncer *in vivo* has been verifi...sing the ...ssfully established br...F-7 xenograf... mice model. How ...his type of compounds work in mice to ...t against b...t cancer? In the following ...riment, we ...und evidence to illustrate t...chanism... the actions of acridone in inhi... tumor.

Since ...one had been reported to be able ... serve as an inhibitor of ABCG2, we mea... levels of ABCG2 protein and mRNA

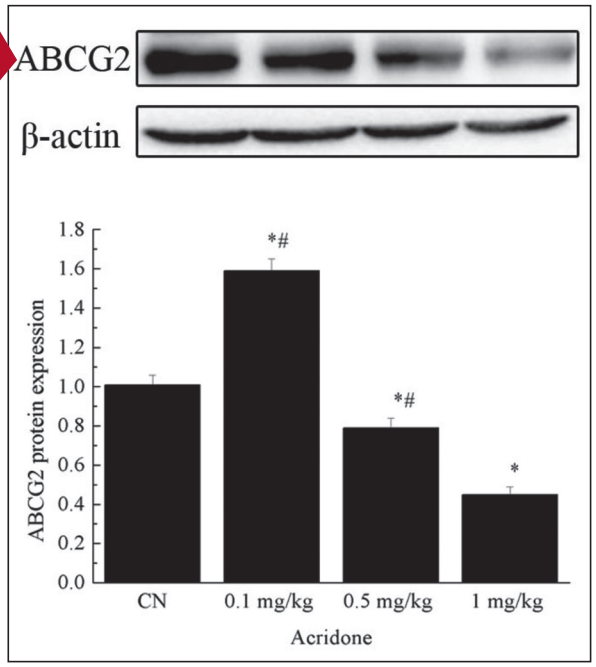

Figure 4. Western blot analysis for ABCG2 expression in tumor bearing mice. *Notes*: Compared with control group, $*p < 0.05$; compared with high dose acridone group, $^{\#}p < 0.05$.

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 proges[illegible] levels, acridone could also work well in tr[illegible] of breast cancer *in vivo*. We showed that ac[illegible]e can serve as a chemotherapeutic agent to [illegible] ticipate in the chemoprevention of solid bre[illegible] MCF-7 cancer in nude mice. Ac[illegible]e was ab[illegible] to inhibit the tumor growth an[illegible]e tumo[illegible] structure histologically th[illegible]n pre[illegible]ing the enlargement of cancer ce[illegible]educin[illegible]tunia and increasing the de[illegible]cr[illegible] tissue. Further invest[illegible]ons [illegible]olved in the studies of anti-can[illegible]mechanis[illegible]e anti-proliferation of acri[illegible]gainst breas[illegible]er cells *in vivo* was ve[illegible]d u[illegible]its multiple actions on inducing of ce[illegible] apopto[illegible]hibition of ABCG2 pathway, [illegible]nregulation [illegible] expression of ABCG[illegible]NA, and decreased [illegible]strogen level and incre[illegible] prog[illegible]one level. It is notable that ac[illegible] cou[illegible] used [illegible]an ABCG2 inhibitor *in vivo* t[illegible]e the [illegible]R occurring in chemo-[illegible]apy o[illegible]st ca[illegible] *in vivo*.

Conclusions

We showed that acridone could induce cell [illegible]tosis, inhibits ABCG2 protein and adjusts h[illegible]e level. The results suggested that acridone could serve as a chemotherapeutic agent for treatment of breast cancer *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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