

Acridone suppresses the proliferation of human breast cancer cells *in vitro* via ATP-binding cassette subfamily G member 2

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Abstract. In the past decades, the tricyclic acridone ring system has become a focus of major research by medicinal chemists due to the biological significance of this moiety in drug design and discovery. Acridone has substantial bio-potential since it performs crucial functions, including antibacterial, antimalarial, antiviral and anti-neoplastic activities. However, the anticancer effect and the underlying mechanisms of acridone on breast cancer cells remains unclear. In the present study, the anti-tumor function and the underlying mechanisms of acridone were evaluated *in vitro*. Firstly, an MTT assay was used to evaluate the inhibitory effect of acridone. Subsequently, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to investigate whether ATP binding cassette subfamily G member 2 (ABCG2) was associated with the function of acridone. Finally, western blotting was used to confirm the results of RT-qPCR. The present study demonstrated that acridone may decrease the proliferation of MDA-MB-231 cells dose-dependently. Further experiments revealed that acridone may downregulate the mRNA and protein expression levels of ABCG2, supporting the potential application of acridone in breast cancer treatment. These findings suggested that acridone is a potential agent in the treatment of human breast cancer.

Introduction

Breast cancer is the most common malignant tumor in women and the second leading cause of cancer-associated mortality amongst women worldwide (2009-2010) (1,2). In 2008, the World Health Organization estimated that >1.2 million people were diagnosed with breast cancer globally (3). Breast

cancer may be classified into different subgroups depending on the expression of estrogen receptor (ER), progesterone receptor or human epidermal growth factor receptor 2. These subgroups present with distinct molecular backgrounds and exhibit diverse clinical behavior and treatment responses (4,5). Among all types of breast cancer, tumors with negative expression of ER, which accounts for 25-30% of all types of breast cancer (6), are known for their aggressive nature and high metastatic potential (7).

At present, therapeutic strategies for breast cancer include surgery, radiation and chemotherapy (8). However, despite advances in multimodal treatments, no effective systemic therapy has been established. Drug resistance is considered to be one of the most important factors influencing the clinical outcomes of patients (9). Therefore, the discovery of novel therapeutic approaches is required to advance the treatment outcomes of patients with ER-negative breast cancer. Numerous studies have illustrated that the acridone nucleus, in addition to its derivatives, possess the property of a potent anticancer effect (10,11). However, the efficacy of acridone against human breast cancer is yet to be reported.

In the present study, the cytotoxic effects of acridone on the MDA-MB-231 human ER-negative breast cancer cell line was investigated, in addition to its underlying mechanisms *in vitro*. To the best of our knowledge, the present study was the first to demonstrate that acridone inhibited the proliferation of MDA-MB-231 cells. It was also revealed that acridone significantly inhibited the mRNA and protein expression level of ATP binding cassette subfamily G member 2 (ABCG2). These findings provide evidence that acridone may possess the potential to be used for the clinical treatment of human breast cancer.

Materials and methods

Cell lines and reagents. The MDA-MB-231 cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). MTT was supplied by Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Protease inhibitor cocktail tablets and phosphatase

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inhibitor cocktail tablets were purchased from Roche Applied Science (Mannheim, Germany). Trypsin and TRIzol were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Mouse anti-ABCG2 antibody was purchased from Abcam (Cambridge, MA, USA). Mouse anti- β -actin antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS and 100 μ g/ml penicillin-100 U/ml streptomycin at 37°C in a humidified incubator containing 5% CO₂.

Cell viability assays. MDA-MB-231 cells were seeded in 96-well plates at a density of 2.5×10^3 cells/ml in 200 μ l DMEM medium and cultured at 37°C overnight. Subsequently, cells were treated at 37°C with multiple concentrations of acridone (0.1, 0.5 and 1.0 μ M) for 48 h. Cells treated with medium with 0.1% DMSO were regarded as the negative control. Next, 20 μ l MTT (5 mg/ml in PBS) was added to each well and incubated at 37°C for a further 4 h. Subsequently, the medium was discarded and 200 μ l DMSO was added to each well to dissolve the purple formazan crystals. The optical density (OD) at 570 nm was detected using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). MDA-MB-231 cells were treated with multiple concentrations of acridone (0.1, 0.5 and 1.0 μ M) at 37°C for 48 h. In brief, total cellular RNA was isolated using TRIzol reagent (Sigma-Aldrich; Merck KGaA) according to the manufacturer's instructions. First strand cDNA was prepared using the SYBR Green master kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following primers were used: 5'-TATAGCTCAGATCATTGTTCACAGT C-3' (sense) and 5'-GTTGGTCGTCAGGAAGAAGAG-3' (antisense) for ABCG2. The PCR assay was performed using the SYBR Premix Ex Taq system (Takara Biotechnology Co., Ltd., Dalian, China), and the thermocycling conditions were as follows: 95°C for 15 min, 40 cycles of 95°C for 30 sec, 55°C for 30 min and 72°C for 30 sec. β -actin was used as a loading control. Relative gene expression was obtained following normalization with endogenous β -actin and determination of the difference in threshold cycle (Cq) between treated and untreated cells using the $2^{-\Delta\Delta Cq}$ method (12).

Western blotting. MDA-MB-231 cells (2×10^6 cells/dish) were seeded in 100 mm culture dishes and cultured in DMEM at 37°C overnight, followed by treatment with multiple concentrations of acridone (0.1, 0.5 and 1 μ M) at 37°C for 48 h. Following trypsinization, cells were transferred to centrifuge tubes and centrifuged at 4°C, 800 x g for 3 min. The supernatant was discarded and the cell pellets were washed using ice-cold PBS 2-3 times. RIPA buffer [250 mmol/l NaCl, 50 mmol/l HEPES, 5 mmol/l egtazic acid, 20 mmol/l ethylenediaminetetraacetic acid (pH 8.0), 0.1% Triton X-100, 2 mg/ml leupeptin, 2 mg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride] containing protease inhibitor cocktail tablets and phosphatase inhibitor (Roche Applied Science) was used to dissolve the pellets

Table I. Effects of acridone on the proliferation of MDA-MB-231 cells.

Acridone concentration (μ M)	Optical density value
0.0	0.836 \pm 0.009
0.1	0.714 \pm 0.013
0.5	0.498 \pm 0.005 ^a
1.0	0.156 \pm 0.011 ^b

Data are presented as the mean \pm standard deviation of three independent experiments. ^aP<0.05 vs. the 0.1 μ M group; ^bP<0.05 vs. the 0.5 μ M group.

for 30 min at 4°C. Once the cell lysate was centrifuged at 12,000 x g at 4°C for 15 min, supernatant was collected and stored at -80°C. Using the BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's instructions, protein concentration was determined. Cell lysates (30 μ g/lane) were separated using 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes, blocked with 5% fat-free dried milk for 2 h at room temperature and probed with ABCG2 antibody (1:500; cat. no. ab207732; Abcam, Cambridge, UK) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:1,000; cat. no. ab6702; Abcam). Immunoreactive bands were visualized using enhanced chemiluminescence system (ImageQuant LAS 4000; General Electric Company, Fairfield, CT, USA) with LabImage version 2.7.1 (Kapelan Bio-Imaging, Leipzig, Germany).

Statistical analysis. Each experiment was performed at least three times and analyzed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The data are presented as the mean \pm standard deviation and analyzed using one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Anti-proliferation effect of acridone on MDA-MB-231 cells in vitro. Acridone demonstrated an anti-proliferation effect on MDA-MB-231 cells, which was determined using an MTT assay (Table I; Fig. 1). Compared with control group, acridone significantly decreased the proliferation of MDA-MB-231 cells in a dose-dependent manner (P<0.05). At 48 h, the OD values of 0.0, 0.1, 0.5 and 1.0 μ M acridone were 0.836 \pm 0.009, 0.714 \pm 0.013, 0.498 \pm 0.005 and 0.156 \pm 0.011, respectively. The results of the present study indicated that acridone may inhibit the proliferation of MDA-MB-231 cells.

Effect of acridone on ABCG2 mRNA expression levels in MDA-MB-231 cells. To further study the mechanism underlying the anti-proliferation function of acridone, RT-qPCR was performed to examine the mRNA expression levels of ABCG2. As presented in Fig. 2, 0.5 and 1.0 μ M acridone

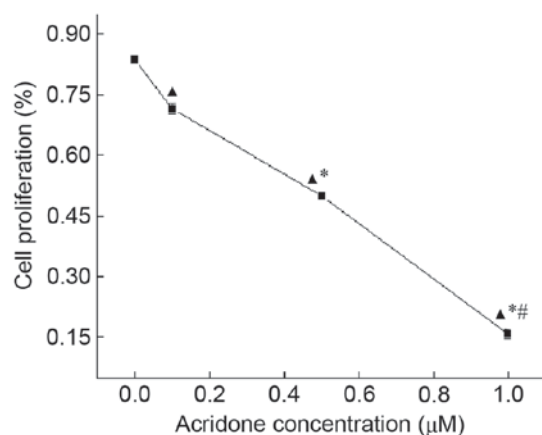


Figure 1. Effects of acridone on the proliferation of MDA-MB-231 cells. Proliferation of MDA-MB-231 cells was determined using an MTT assay. *P<0.05 vs. the 0.1 μM group; #P<0.05 vs. the 0.5 μM group; ΔP<0.05 vs. the control group.

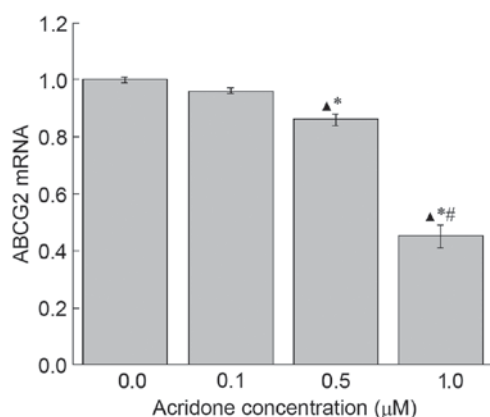


Figure 2. Effects of acridone on ABCG2 mRNA expression levels of MDA-MB-231 cells. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the 0.1 μM group; #P<0.05 vs. the 0.5 μM group; ΔP<0.05 vs. the control group. ABCG2, ATP binding cassette subfamily G member 2.

treatment significantly decreased ABCG2 mRNA expression levels compared with the control group (P<0.05). However, there was no significant difference (P>0.05) identified between the 0.1 μM acridone treatment group and the control group in the mRNA expression levels of ABCG2. The data revealed that, compared with control group, acridone may decrease ABCG2 mRNA expression at 0.5 and 1.0 μM doses.

Effects of acridone on the expression levels of ABCG2 protein in MDA-MB-231 cells. Expression levels of the ABCG2 protein in MDA-MB-231 cells treated with acridone were detected using western blotting. Compared with control group, the expression level of ABCG2 was significantly down-regulated in a dose-dependent manner following acridone treatment (P<0.05; Fig. 3). The protein levels of ABCG2 in the control group were significantly increased compared with those in the 0.1, 0.5 and 1.0 μM acridone treatment groups (P<0.05; Fig. 3). The results of the present study revealed that, compared with control group, acridone may

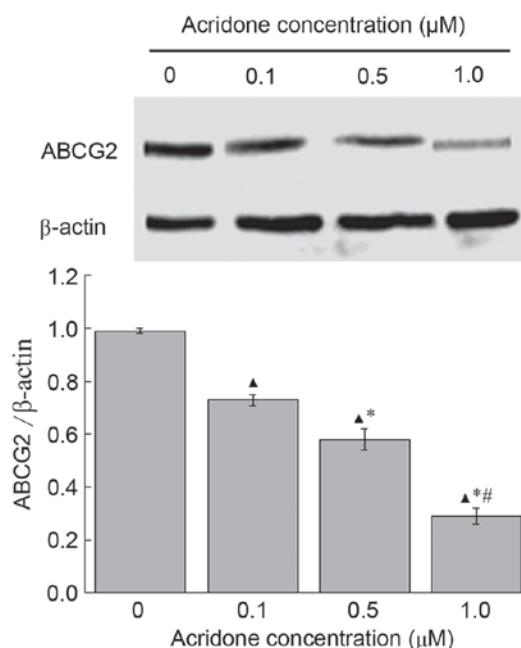


Figure 3. Effects of acridone on ABCG2 protein expression levels in MDA-MB-231 cells (β-actin was used as a control). Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. the 0.1 μM group; #P<0.05 vs. the 0.5 μM group; ΔP<0.05 vs. the control group. ABCG2, ATP binding cassette subfamily G member 2.

decrease ABCG2 protein expression at 0.1, 0.5 and 1.0 μM doses.

Discussion

There are numerous functions of the acridone nucleus in addition to its derivatives that have been reported in previous studies, including anticancer (13-16), anti-herpes (17), anti-malarial (18,19), antiviral (20), anti-allergy (21) and anti-leishmanial (22) activities. These features are attributed to the semi planar heterocyclic structure, which interacts with different biomolecular targets.

However, the antitumor function of acridone against human breast cancer and its mechanism of action remain to be elucidated. Therefore, the purpose of the present study was to examine the effect of acridone on human breast cancer and to elucidate the underlying molecular mechanisms. The present study revealed, to the best of our knowledge for the first time, that acridone inhibited the proliferation of MDA-MB-231 cells *in vitro*. In addition, the molecular mechanisms by which acridone affects MDA-MB-231 cells were revealed.

Membrane transporters serve a critical function in regulating drug resistance. In the last decade, the ATP-binding cassette protein superfamily has received notable attention, particularly the protein ABCG2 (23). This ATP-binding cassette transporter serves a crucial function in mediating drug efflux and is associated with the primary or acquired drug resistance presented in clinical settings. A number of studies have attempted to develop specific inhibitors targeting ABCG2 (24,25). In consideration of the potent inhibitory effect of acridone, the present study further explored whether ABCG2 is associated with the underlying mechanism of the inhibitory effect of acridone. Compared with control group, the mRNA

and protein expression levels of ABCG2 were decreased in acridone-treated MDA-MB-231 cells, indicating that acridone treatment may be associated with ABCG2-dependent cell proliferation inhibition.

To conclude, the present study demonstrated that acridone may inhibit the proliferation of MDA-MB-231 cells, in addition to downregulating the mRNA and protein expression levels of ABCG2. These results revealed details regarding the mechanisms behind the effect of acridone on MDA-MB-231 and suggest that acridone may be a potential candidate for the development of novel treatment strategies for human breast cancer. However, the specific molecular targets of acridone and the signaling pathways affected *in vitro* require further study.

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