Original Article

Triptolide inhibits human breast cancer MCF-7 cell growth via downregulation of the ER α -mediated signaling pathway

Han Li^{1, #}, Guo-feng PAN^{2, #}, Zhen-zhou JIANG¹, Jing YANG¹, Li-xin SUN^{1, *}, Lu-yong ZHANG^{1, *}

¹Jiangsu Center for Pharmacodynamics Research and Evaluation, China Pharmaceutical University, Nanjing 210009, China;

²Department of TCM, Beijing Shijitan Hospital affiliated to Capital Medical University, Beijing 100038, China

Aim: To investigate the anticancer mechanisms of triptolide, a diterpenoid isolated from the plant *Tripterygium wilfordii* Hook F, against human breast cancer cells and the involvement of the estrogen receptor- α (ER α)-mediated signaling pathway in particular.

Methods: Human breast cancer ER α -positive MCF-7 cells and ER α -negative MDA-MB-231 cells were tested. PrestoBlue assay was used to evaluate the cell viability. The levels of ER α mRNA and protein were detected with real-time PCR and immunoblotting, respectively. Mouse models of MCF-7 or MDA-MB-231 xenograft tumors were treated with triptolide (0.4 mg·kg⁻¹·d⁻¹, po) or a selective estrogen receptor modulator tamoxifen (mg·kg⁻¹·d⁻¹, po) for 3 weeks, and the tumor weight and volume were measured.

Results: Triptolide (5–200 nmol/L) dose-dependently inhibited the viability of both MCF-7 and MDA-MB-231 cells, with a more potent inhibition on MCF-7 cells. Knockdown of ER α in MCF-7 cells by siRNA significantly attenuated the cytotoxicity of triptolide, whereas overexpression of ER α in MDA-MB-231 cells markedly enhanced the cytotoxicity. Triptolide dose-dependently decreased the expression of ER α in MCF-7 cells and MCF-7 xenograft tumors. Furthermore, treatment of MCF-7 cells with triptolide inhibited the phosphorylation of ERK1/2 in dose- and time-dependent manners. In the mice xenografted with MCF-7 cells, treatment with triptolide or tamoxifen resulted in significant reduction in the tumor weight and volume. Similar effects were not obtained in the mice xenografted with MDA-MB-231 cells.

Conclusion: The anticancer activity of triptolide against ER α -positive human breast cancer is partially mediated by downregulation of the ER α -mediated signaling pathway.

Keywords: triptolide; breast cancer; estrogen receptor- α ; tamoxifen; ERK1/2; MCF-7 cells; MDA-MB-231 cells; xenograft nude mouse model

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Introduction

Breast cancer is the most common malignant disease affecting women worldwide. It has been reported that estrogen receptor- α (ER α) plays a critical role in the initiation and progression of breast cancer because approximately 70% of primary breast cancers are ER α -positive^[1]. ER α is a member of a large superfamily of nuclear receptors and acts as a ligand-activated transcription factor^[2] by binding to 17 β -estradiol (E2) and interacting with specific estrogen response elements (EREs) in the promoters of target genes in the nucleus^[3]. Additionally, non-nuclear ER α rapidly activates downstream

growth factor signals, such as extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and signal transducer and activator of transcription 3 (STAT3)^[4]. For patients with ER α -positive breast cancer, anti-ER therapeutic agents include selective estrogen receptor modulators (SERMs), selective estrogen receptor downregulators (SERDs) and disruptors of estrogen synthesis^[5]. Although SERMs (such as tamoxifen) and SERDs (such as fulvestrant) are effective treatments for ER α -positive breast cancer, these treatments are commonly limited by drug resistance^[6]. The drug resistance that results from long-term anti-ER treatment may be induced by the loss of ER α , an increase in the levels of nonnuclear ER α or growth factor receptor signaling, or the abnormal activities of signal transducers (such as ERK1/ERK2, JNK, and STAT3), cell-cycle regulators or apoptosis regulators. The mitogen-activated protein kinase (MAPK) pathways are involved in cell proliferation and survival. The best characterized MAPK

[#] These two authors contributed equally to this paper.

^{*} To whom correspondence should be addressed.

E-mail sixcpu@126.com (Li-xin SUN);

lyzhang@cpu.edu.cn (Lu-yong ZHANG)

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is ERK, the activation of which is known to inhibit apoptosis and induce cell proliferation in several human malignancies^[7].

Triptolide is a diterpenoid isolated from the plant *Tripterygium wilfordii* Hook F, which is a member of the Celastraceae family. Triptolide has been shown to display a unique bioactive spectrum of immunosuppressive, anti-fertility and anti-cystogenesis activities^[8]. Recent studies of triptolide have revealed many properties relevant to its anti-inflammatory and anticancer activities^[9–11]. Many *in vitro* and *in vivo* studies have aimed to elucidate the mechanism underlying the anticancer activity of triptolide; however, the findings of these studies have been inconsistent. Previously, we have found that triptolide is much more sensitive to ER α -positive MCF-7 cells than that to ER α -negative MDA-MB-231 cells^[12]. In this study, we further characterized the anticancer effects of triptolide in breast cancer cell lines and in an *in vivo* mouse model and further investigated the signaling mechanisms underlying the triptolide-induced inhibition of breast cancer growth. We found that triptolide inhibited breast cancer growth by blocking both the ER α and ERK1/ERK2 pathways. These findings suggest that triptolide represents a promising drug candidate for breast cancer intervention.

Materials and methods

Materials

Reagents were obtained as follows. PrestoBlue® Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA) was purchased from Life Technologies (Grand Island, NY, USA), and Cell Viability Reagent and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). The rabbit polyclonal anti-ER α , anti-ERK1, and anti-ERK2 and mouse monoclonal anti-p-ERK and anti-actin antibodies were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA); ICI 182780 was purchased from Apex Bio Corporation (Boston, MA, USA). Tamoxifen was purchased from Sigma (St Louis, MO, USA). Triptolide (purity >98%) was a gift from the Dermatologic Disease Research Institute of the Chinese Academy of Medical Science (Nanjing, China).

Cell culture and triptolide treatment

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), and MDA-MB-231 cells were maintained in RPMI-1640 medium. All cells were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 10 mg/mL streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were starved for 24 h in medium containing 0.1% FBS before treatment with triptolide. The concentration of DMSO in the cell cultures was less than 0.1%.

Cell viability assay

Exponentially growing cells were seeded in a 96-well plate. Twenty-four hours after seeding, the cells were incubated in the absence or presence of triptolide for the indicated duration. Cell viability was evaluated using a PrestoBlue® Cell Viability Reagent (Invitrogen, Carlsbad, CA, USA).

Plasmids and transient transfection

Transient transfection was performed in a manner similar to that in a previous study^[13]. Briefly, MDA-MB-231 cells were seeded at a density of 1×10^4 cells per well in a 24-well plate and then transfected with an ER α -GFP construct (AddGene) for 24 h using the transfection reagent Lipofectamine 2000 (Invitrogen). Following incubation for 6 h, the transfection medium was replaced with fresh medium, followed by an additional 24 h of incubation to allow for gene expression. The transfection efficiency was monitored based on the intensity of GFP fluorescence. The ER α expression levels were determined via Western blot analysis.

Small interfering RNA (siRNA) transfection

siRNA duplexes against ER- α (Santa Cruz Biotechnology, Santa Cruz, CA, USA; accession No. sc-29305) were used for the targeted knockdown of ER- α protein expression. Non-targeting (NT) scrambled siRNA (Santa Cruz Biotechnology; accession No. sc-37007) was used as a control. MCF-7 cells were seeded in 6-cm dishes and grown in culture medium. At 50%–60% confluence, the cells were transfected with 10 nmol/L ER α siRNA or NT siRNA using HiPerFect Transfection Reagent (QIAGEN, Valencia, CA, USA) in 1 mL of transfection medium (Santa Cruz Biotechnology). After 24 h, the medium was replaced with fresh medium, and the cells were cultured for an additional 24 h before Western blot analysis of ER α expression. In another set of experiments, the cells were treated with triptolide and an ER antagonist, ICI182780, followed by cell viability assays.

Western blot analysis

Whole-cell lysates were prepared using lysis buffer containing 150 mmol/L NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris, pH 8.0, and protease and phosphatase inhibitors. The proteins were resolved on 10% SDS-PAGE gels under reducing conditions and then electrophoretically transferred to nitrocellulose membranes. The membranes were incubated in primary antibodies against ER α , t-ERK1/2, ERK1, ERK2, or β -actin at 4°C overnight. Immunoreactive bands were detected using HRP-conjugated secondary antibodies and the Western Lightning Chemiluminescence Plus reagent.

RNA extraction and real-time PCR

Total cellular RNA was isolated from tumor xenografts using TRIzol reagent. Total RNA (2.5 μ g) was used for first-strand cDNA synthesis using a RevertAid First Strand cDNA Synthesis Kit (Takara Bio Inc, Shiga, Japan). ER α mRNA in the tumor xenografts was quantified using the following primers: forward primer 5'-ATCCTGATGATTGGTCTCGTCT-3' and reverse primer 5'-GGATATGGTCCTTCTCTTCCAG-3'. SsoFast™ EvaGreen® Supermix (Bio-Rad) was used as a fluorescent dye to detect double-stranded DNA. The mRNA level of each gene was normalized to that of the internal control β -actin using the following primers: forward primer

5'-CCTGGCACCCAGCACAAT-3' and reverse primer 5'-GCCGATCCACACGGAGTACT-3'. The ratio of the normalized mean value for each treatment group to that for the vehicle DMSO control group was calculated.

Antitumor activity *in vivo*

Female, homozygous, 5- to 6-week-old athymic, inbred BALB/c-nu⁺/nu⁺ mice were supplied by the Laboratory Animal Center of the General Hospital of Nanjing Military Region (Nanjing, China), where they were bred and maintained in vinyl chambers under germ-free conditions. The animals were housed in a pathogen-free environment under controlled light and humidity and received food and water *ad libitum*. After the mice had been in quarantine for 1 week, they were subcutaneously (sc) injected with 5×10⁶ MCF-7 or MDA-MB-231 cells (0.15 mL). To evaluate antitumor activity, the mice were stratified into three groups according to their tumor volume once the mean tumor volume had reached approximately 100–140 mm³ (d 0). Thereafter, triptolide (0.4 mg·kg⁻¹·d⁻¹) and a selective estrogen receptor modulator, tamoxifen (5 mg·kg⁻¹·d⁻¹), were administered via gavage daily to both the MCF-7 and MDA-MB-231 groups. After three weeks, the mice were euthanized, and the xenografted tumors were removed and weighed. All procedures of animal experiments were conducted in compliance with standard ethical guidelines and with the approval of the faculty ethical committee. The relative tumor volume (RTV) was calculated using the following formula: RTV=tumor volume on measured day/tumor volume on d 0.

Histopathology

For histological observation, the tumor xenografts were removed and blotted dry before weighing. The tissues were placed in 4% formalin for 2 d, embedded in paraffin, serially sectioned at 5 μm, and stained in hematoxylin and eosin for microscopic observation. The histological sections were used for qualitative evaluation of the tumor xenografts.

Immunohistochemistry

After endogenous peroxidase activity had been blocked, deparaffinized sections (5-μm thick) of the tumor xenografts were pretreated in 10 mmol/L citrate buffer (pH 6.0) by microwaving (500 W at full power) for 15 min. After cooling for 60 min, the sections were incubated in a primary antibody overnight at 4°C in a moist chamber. Then, the sections were incubated in a biotinylated secondary antibody for 10 min, followed by incubation in an HRP-conjugated streptavidin complex (Beyotime, Haimen, China) for 10 min. To visualize immunoreactivity, diaminobenzidine tetrachloride (1 mg/mL) containing 0.1% hydrogen peroxide (30% *w/v*) was used. The immunostained slides were evaluated and photographed under a microscope (FV-1000; Olympus, Japan). The intensity of immunostaining was semiquantitatively designated as strong, weak, or none, and the ratio of highly stained cells was calculated.

Statistical analysis

The data from the inhibition assays were analyzed by non-linear curve fitting of the delta F% value versus the inhibitor concentration using PRISM version 5.0 software (Graph-Pad Software Inc, CA, USA). The data are expressed as the mean±SEM. The significance of the differences between the groups was evaluated via one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post-test. The differences were considered to be significant at *P*<0.05.

Results

Triptolide suppresses breast cancer cell growth

To assess the effects of triptolide on breast cancer cell growth, breast cancer (MDA-MB-231 and MCF-7) cells were treated with triptolide at varying concentrations or for different durations. Cell viability was measured using the PrestoBlue cell proliferation assay. The MCF-7 cells positively expressed ERα, whereas the MDA-MB-231 cells did not^[14, 15]. Triptolide decreased the viabilities of both types of cancer cells and exerted a greater suppressive effect on the growth of the MCF-7 cells than on that of the MDA-MB-231 cells (Figure 1A). Triptolide dose-dependently (5–200 nmol/L) decreased the survival of the MCF-7 cells at 24, 48, and 72 h (Figure 1B). To explore the effects of triptolide in combination with estradiol, MCF-7 cells were exposed to increasing concentrations of triptolide in the presence of estradiol (10 nmol/L) (Figure 1C). In contrast with the stimulatory effects of estradiol alone, the presence of triptolide at 10, 25, or 50 nmol/L suppressed estradiol-stimulated cell growth by 9%, 20%, and 55% (*P*<0.05), respectively.

The ERα signaling pathway partially mediates the triptolide-induced suppression of breast cancer cell growth

To investigate the effect of triptolide on ERα expression, real-time PCR and an immunoblotting assay were used to examine mRNA and protein expression, respectively, after MCF-7 cells were treated with triptolide for 24 h. Triptolide decreased ERα expression at both the mRNA and protein levels in a dose-dependent manner in the MCF-7 cells (Figure 2A, 2B). To determine the effects of triptolide on the ERα signaling pathway *in vivo*, we used an MCF-7 xenograft mouse tumor model and immunostained for ERα at the endpoint. The ERα expression levels were significantly reduced in the tumor-bearing mice treated with triptolide in a dose-dependent manner (Figure 2C, 2D).

Knockdown of ERα expression using siRNA in MCF-7 cells significantly attenuated the inhibitory effects of triptolide on cell proliferation (Figure 3B). The IC₅₀ value of triptolide in the MCF-7 cells transfected with siRNA targeting ERα was 90.14 nmol/L, whereas that in the cells transfected with control siRNA was 254.6 nmol/L. The pattern of proliferation of the MCF-7 cells treated with the estrogen receptor inhibitor ICI 182780 was similar to that of the cells treated with triptolide. In addition, the knockdown of ERα using siRNA in MCF-7

Figure 1. Triptolide reduces the proliferation of human breast cancer cells. (A) The IC₅₀ values of MCF-7 and MDA-MB-231 cells treated with triptolide. GraphPad Prism 5 (GraphPad Software) was used for the calculations. (B) MCF-7 cells were treated with triptolide (from 0 to 200 nmol/L). Cell viability was measured using PrestoBlue following treatment for 24, 48, or 72 h. (C) The proliferation of MCF-7 cells in the presence of triptolide or E2 *in vitro*. The cells were cultured under hormone-free conditions or in E2, and cell growth was evaluated. All data are presented as the mean±SD of 3 repeated experiments. ^b*P*<0.05, ^c*P*<0.01.

Figure 2. Triptolide decreases ERα expression in MCF-7 cells and MCF-7 solid tumors. (A) Western blot analysis showing that triptolide reduced the ERα protein levels in MCF-7 cells. (B) Triptolide reduced the ERα mRNA levels as determined by RT-PCR. (C) Immunohistochemical staining for ERα in MCF-7 solid tumors treated with triptolide. (D) Sections immunostained for ERα were classified into three groups (highly stained, moderately stained, and not stained). The values indicate the mean±SD of the proportions of highly stained cells (*n*=6). ^b*P*<0.05, ^c*P*<0.01 compared with the control as determined by ANOVA.

Figure 3. ER α signaling is essential for the triptolide-mediated suppression of breast cancer cell growth. (A) MCF-7 cells were transfected with siRNA duplexes against ER α or NT scrambled siRNA for 24 h. The cell lysates were subjected to Western blot analysis using anti-ER α antibodies. The cells were transfected with siRNA followed by treatment with triptolide (B) or ICI182780 (C) at the indicated concentration for 24 h. The MDA-MB-231 cells were transfected with an empty vector or the ER α -GFP construct for 24 h. The cell lysates were subjected to Western blot analysis using anti-ER α antibodies. The MDA-MB-231 cells were transfected with an ER α -GFP construct followed by treatment with triptolide (E) or ICI182780 (F) at the indicated concentration for 24 h. Cell viability was assessed via the PrestoBlue assay. The data are expressed as the mean $\pm$ SD. ^b P <0.05, ^c P <0.01 compared with the cells transfected with an empty vector according to ANOVA.

cells attenuated the inhibitory effects of ICI 182780 on cell proliferation (Figure 3C). In contrast, triptolide exerted a strikingly greater inhibitory effect on the proliferation of MDA-MB-231 cells overexpressing ER α (Figure 3E) than on the proliferation of empty vector-treated control cells (IC_{50} =40.57 and 216.96 nmol/L, respectively).

To further investigate the effect of triptolide on the ER α signaling pathway, we examined the estrogen-induced activation of ERK in MCF-7 cells. Treatment with 10 nmol/L E2 resulted in an increase in ERK1/2 phosphorylation in the MCF-7 cells, which was attenuated by triptolide treatment (Figure 4A). We also compared the effect of triptolide on ERK1/2 phosphorylation between MCF-7 and MDA-MB-231 cells. As shown in Figure 4B, treatment with triptolide significantly decreased ERK1/2 phosphorylation in the MCF-7 cells. Because triptolide-induced growth inhibition was observed in the MDA-

MB-231 cells after triptolide treatment, we investigated the effect of this agent on ERK1/2 phosphorylation in these cells. As shown by immunoblotting, the levels of phosphorylated ERK1/2 proteins did not significantly change after triptolide treatment.

Triptolide inhibits the growth of MCF-7 cell xenografts in a mouse model

To determine the effects of triptolide on breast cancer *in vivo*, we compared its effects on tumor growth in the MCF-7 and MDA-MB-231 xenograft mouse tumor models. Triptolide treatment was initiated when the tumors were palpable and was continued for 21 d. As shown in Figure 5, the growth of MCF-7 tumors was significantly inhibited by triptolide administration for 21 consecutive days. At the end of this period, the MCF-7 tumor volume and weight in the triptolide-treated

Figure 4. Effects of triptolide on the p-ERK and ERK protein levels in MCF-7 and MDA-MB-231 cells. (A) Time-dependent effects of triptolide on the phosphorylation of the ERK1/2 proteins in MCF-7 cells. ERK1/2 protein phosphorylation was measured by immunoblotting and was normalized to the expression of the control. (B) The MCF-7 and MDA-MB-231 cells were treated with different concentrations of triptolide for 12 h. The cells were analyzed via Western blot using anti-phospho-ERK or anti-ERK antibodies.

Figure 5. The breast tumor growth of MCF-7 cells is more sensitive to triptolide than that of MDA-MB-231 cells in mice. Triptolide and a selective estrogen receptor modulator, tamoxifen, were administered on d 0. The tumor volume was measured twice a week until d 21. (A) The tumor volume changes in the MDA-MB-231 cells *in vivo*. (B) The tumor volume changes in the MCF-7 cells *in vivo*. (C and D) The changes in the tumor weight in nude mice xenografted with MCF-7 and MDA-MB-231 cells. The tumor weights were measured on d 21. The values indicate the mean±SD of the tumor weights (n=6). ^aP<0.05, ^cP<0.01 compared with the control as determined by ANOVA.

mice were reduced by 41% and 67%, respectively, compared with those in the control mice ($P < 0.01$). The tumor weight and volume were also reduced in the mice treated with triptolide compared with the vehicle-treated control mice in the MDA-MB-231 group. However, the ability of triptolide to inhibit tumor growth in the MDA-MB-231 group was not as significant as that in the MCF-7 group. The MDA-MB-231 tumor volume and weight in the triptolide-treated mice were reduced by 11% ($P > 0.05$) and 24% ($P < 0.05$), respectively, compared with those in the control mice.

Discussion

Breast cancer is a common cancer that displays a high mortality rate among women. Endocrine therapy has become the most effective treatment option for women with ER-positive breast cancer. Nevertheless, many breast cancer patients with tumors expressing high levels of ER are unresponsive to endocrine therapy, and all patients with advanced disease ultimately develop resistance to this therapy. The potential mechanisms underlying this intrinsic or acquired endocrine resistance involve ER-co-regulatory proteins and cross-talk between the ER pathway and other growth factor signaling networks^[16]. The cross-talk between receptor tyrosine kinase (RTK) and ER signal transduction pathways may contribute to the acquisition of endocrine resistance by sensitizing breast tumor cells to estrogens or by circumventing the need for hormones. Based on this evidence, one strategy for improving the efficacy of current endocrine agents and for delaying the onset of resistance is to simultaneously target the ER and RTK signaling pathways. Several studies have proposed a role for the ERK signaling pathway in the initiation and pathogenesis of breast cancer^[17]. The activation of the ERK cascade modulates the phosphorylation, and thus, the activity of several nuclear transcription factors, which in turn regulate genes involved in cell proliferation and survival. ER α is functionally regulated via phosphorylation by several protein kinases^[18]. The phosphorylation of Ser118 of ER α is mediated by CDK7 in response to E2, and this residue is phosphorylated by pERK1/ERK2 in a ligand-independent manner^[19]. In contrast, Ser167 is a target of AKT and p90RSK that is activated by pERK1/ERK2. Therefore, increased ERK1/ERK2 activity may result in endocrine resistance. Laboratory studies have demonstrated that the activation of the EGF receptor family and the ERK/MAPK pathway promotes estrogen-independent growth, which has led to several clinical trials using signal transduction inhibitors to enhance endocrine sensitivity^[20, 21]. There is currently increased interest in the application of endocrine agents in combination with signal transduction inhibitors that selectively block the activities of growth factor receptors and their downstream partners, such as ERK, AKT, mTOR and farnesyltransferase, and several clinical trials are underway^[22].

Triptolide has been shown to exert potent anticancer effects via multiple molecular targets and signaling pathways. In the present study, the inhibitory effects of triptolide were investigated in several breast cancer cell lines, specifically the ER α -positive MCF-7, ER α -negative MDA-MB-231 and

HER2-positive BT-474 cell lines^[23]. The molecular targets and signaling pathways include apoptosis-related genes (such as caspase-7 and -9), focal adhesion kinase (FAK), Wnt/ β catenin and tumor suppressors (such as p53) were investigated^[23–25]. In particular, MCF-7 is an estrogen receptor-positive cell line that lacks caspase-3^[26], thus representing a cell model displaying compromised apoptotic machinery activity, and this deficiency might influence the cellular response to anti-cancer drug treatments. In this study, we demonstrated that triptolide inhibited the proliferation of ER α -positive breast cancer cells partially via ER α -related pathways *in vitro* and *in vivo*. Our conclusion was based on the following observations: first, triptolide more potently reduced the growth of the ER α -positive breast cancer cells (MCF-7) than that of the ER α -negative cells (MDA-MB-231). In addition, triptolide exerted suppressive effects on estrogen-stimulated MCF-7 cell proliferation and gene expression. The knockdown of ER α via siRNA transfection in MCF-7 cells attenuated these effects of triptolide. In contrast, the overexpression of ER α in MDA-MB-231 cells enhanced these effects. Similarly, significant reductions in tumor weight and volume were observed in the mice treated with triptolide compared with the vehicle-treated control mice in the MCF-7 group. However, the ability of triptolide to inhibit tumor growth in the MDA-MB-231 group was not as significant as that in the MCF-7 group. In summary, we characterized the anticancer properties of triptolide in both breast cancer cells and *in vivo* in tumor-bearing mice. The inhibitory effects of triptolide on estrogen receptor-positive breast cancer cells might occur via the ER α pathway.

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Author contribution

Han LI, Guo-feng PAN, Li-xin SUN, and Lu-yong ZHANG designed the study; Jing YANG, Zhen-zhou JIANG, and Han LI performed the research; Han LI and Li-xin SUN wrote the manuscript.

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