Cancer Letters 346 (2014) 94–103

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

A novel anti-cancer agent, acetyltanshinone IIA, inhibits oestrogen receptor positive breast cancer cell growth by down-regulating the oestrogen receptor

Ting Yu [a], Zhicai Zhou [a], Yuguang Mu [b], Gilberto de Lima Lopes [c], Kathy Qian Luo [a,*]

[a] Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore
[b] School of Biological Sciences, Nanyang Technological University, Singapore
[c] Medical Oncology, Johns Hopkins Singapore International Medical Centre, Singapore

ARTICLE INFO

Article history:
Received 8 March 2013
Received in revised form 27 November 2013
Accepted 10 December 2013

Keywords:
Anti-breast cancer agent
Oestrogen receptor
Acetyltanshinone IIA
Tamoxifen
Fulvestrant

ABSTRACT

In this paper we show that acetyltanshinone IIA (ATA), a novel anti-cancer agent, preferentially inhibits cell growth of oestrogen receptor positive (ER+) breast cancer cells and that it is more potent than the commonly used anti-breast cancer agent, tamoxifen. The metabolic product of ATA, hydroquinone tanshinone IIA (HTA) binds to the ERα and causes its degradation mainly in the nucleus via an ubiquitin-mediated proteasome-dependent pathway. In addition, ATA also reduced the mRNA levels of the ERα encoding gene, ESR1, distinguishing ATA from another anti-breast cancer drug, fulvestrant. Finally, ATA reduced the transcription of an ER-responsive gene, GREB1.

1. Introduction

Breast cancer is the second most common type of cancer in women and caused approximately 460,000 deaths worldwide in 2008 [1]. Breast cancer can be classified into three subtypes based on the expression of the following hormone receptors: oestrogen receptor (ER), progesterone receptor (PR), and cell surface receptor of human epidermal growth receptor 2 (HER2). The expression of these receptors is the most commonly used predictive factor in therapy selection for breast cancer [2,3]. Over 70% of breast cancer patients express ER and are thus candidates for hormonal therapy [2,4,5].

The ER is a transcriptional activator that belongs to the nuclear receptor superfamily and is mainly localised in the nucleus in an inactive state [6–10]. Two types of oestrogen receptors, ERα and ERβ, are found in humans [11–13]. ERα plays major roles in the uterus, breasts and ovaries, whereas ERβ plays significant roles in the central nervous, cardiovascular, and immune systems, as well as the bones, kidneys, and lungs [14]. The ER can be activated by oestrogen, a steroidal hormone. Oestrogen can bind to the ER, thereby changing its conformation and triggering the release of the receptor from heat shock protein (HSP), including HSP90, and then form dimers [10,15–17]. The dimerised ERs recruit their co-activators and bind to oestrogen response elements in promoter regions, thereby activating the transcription of ER-responsive genes. The translation of ER-responsive genes can promote cell division, resulting in breast cancer cell proliferation and tumour growth [18–20].

Consequently, targeting oestrogen and its receptors has become an important strategy in the treatment of breast cancer. Commonly used drugs include (a) tamoxifen, which competitively binds to the ER and therefore prevents its activation by oestrogen [21], and (b) fulvestrant, which causes ER degradation [22]. Although these anti-cancer drugs are routinely used in cancer treatment, they have limitations. Tamoxifen, for instance, is not effective in approximately 30% of breast cancer patients and resistance has been observed in 80% of patients or more after 15 months of treatment for metastatic disease [23,24]. Moreover, its partial agonist activity has been associated with detrimental effects, particularly endometrial cancer in a significant minority of patients. The major limitation of fulvestrant, which is often used as a second-line therapy in the treatment of women with advanced ER+ breast cancer, is its formulation. Because fulvestrant has low bioavailability and pre-systemic metabolism, it is administered as a painful intramuscular injection [25].

As such, there is a clear need to continue to develop new treatments that target ER. We have developed a small molecular weight compound, acetyltanshinone IIA (ATA) that inhibits growth

* Corresponding author. Address: Division of Bioengineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457, Singapore. Tel.: +65 6790 4257; fax: +65 6791 1761.
E-mail address: kluo@ntu.edu.sg (K.Q. Luo).

0304-3835/$ - see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canlet.2013.12.023

of ER+ breast cancer cells. Our group chemically modified ATA from tanshinone IIA (TIIA), a major compound that was isolated from a medicinal plant, *Salvia miltiorrhiza* (also known as Danshen in Traditional Chinese Medicine). In our previous study, we showed that ATA inhibited the growth of xenografted human tumours in nude mice [26]. In the present study, we investigated the molecular mechanisms by which ATA inhibits the growth of ER+ breast cancer cells.

2. Materials and methods

2.1. Reagents

MTT, oestrogen, fulvestrant, tamoxifen and 4-hydroxytamoxifen were purchased from Sigma–Aldrich (St. Louis, MO). ERα, ERβ, ubiquitin antibodies and protein A/G-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). β-Tubulin, Lamin A, HSP90 and GAPDH antibodies were obtained from Cell Signalling Technology (Danvers, MA). HRP-conjugated goat anti-rabbit IgG was purchased from Bio-Rad (Hercules, CA). HRP-conjugated goat anti-mouse IgG was obtained from Pierce (Rockford, IL). FITC-conjugated goat anti-mouse IgG was obtained from Calbiochem (La Jolla, CA). The proteasome inhibitor MG132 was obtained from Calbiochem (San Diego, CA). Culture media, foetal bovine serum (FBS), and all antibiotics were purchased from Invitrogen (Carlsbad, CA).

2.2. Cell culture

MCF-7 cells were cultured in minimum essential medium (MEM). MDA-MB-231, IMR-90, and C2C12 cells were cultured in DMEM, and T-47D cells were cultured in RPMI 1640. MCF-7, IMR-90, and C2C12 cells were obtained from the ATCC (Manassas, VA). T-47D and MDA-MB-231 cells were provided by Prof. Xiaofeng Le from the Department of Experimental Therapeutics at the University of Texas M.D. Anderson Cancer Centre, Houston, TX, USA. To reduce hormone interference during certain experiments, the cells were maintained in phenol red free MEM containing 5% charcoal-stripped serum for 4 days prior to use.

2.3. MTT assay to determine the IC_{50} value and growth inhibition rate

To determine the growth inhibition rate, the cells were seeded into 96 well plates at 2500 cells/well for MCF-7 and 231 cells, and at 5000 cells/well for T-47D cells. After 24 h culturing, the test agents were added into each well. After 72 h of treatment, cell numbers were determined using the MTT assay according to a previously described protocol, with some modifications [26]. First, MTT powder was dissolved in PBS at a concentration of 5 mg/ml. For the MTT assay, 10 μl of MTT solution was added into each well of a 96 well plate. After a 2 h incubation at 37 °C, 100 μl of a 10% SDS solution containing 10 mM HCl was added to dissolve the purple crystals. After a 24 h incubation, the optical density (OD) of the control group at 0 h (C_0) and 72 h (C_{72}), and the compound-treated groups at 72 h (T_{72}), were measured at 595 nm using a plate reader. The 72 h cell growth inhibition rate was calculated using the following definition: $[1 - (T_{72} - C_0)/(C_{72} - C_0)] \times 100\%$. The IC_{50} is the concentration of a compound that inhibits 50% of the cell growth at 72 h.

2.4. LC–MS

MCF-7 cells were treated with 10 μM ATA for 2 h. The cells were collected and lysed in ultrapure water containing the reducing agent, DTT, at a concentration of 1 mM to prevent the oxidation of the putative metabolic product of ATA, hydroquinone TIIA (HTA). The cell lysates were extracted with two volumes of cold acetonitrile containing 1 mM DTT and analysed using LC–MS under the following conditions: An A line mobile phase of 0.1% acetic acid, a B line mobile phase of increasing concentrations of acetonitrile (70% at 0 min, 80% from 30 min to 40 min, and 90% at 50 min), and a flow rate of 0.5 ml/min. Extracts that were obtained from cells that were not treated with ATA were used as negative controls.

2.5. Total cell lysate preparation

The cells were collected with a disposable cell scraper, washed once with cold PBS and centrifuged at 1500 rpm for 3 min. The cell pellets were resuspended in RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl at a pH of 7.4, 2 mM EDTA and protease inhibitors (1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin, and 10 μM aprotinin). After lysing the cells for 20 min on ice, the cell lysates were sonicated 3 times with 40% AMP for 5 s, with a 10 s interval between each sonication. The cell lysates were then centrifuged at 13,000*g* for 10 min at 4 °C. The protein concentrations of the supernatants were measured using the Bradford reagent from Bio-Rad.

2.6. Co-immunoprecipitation

This method was modified from a previously published protocol [27]. After the different treatments, the MCF-7 cells were collected and resuspended in a lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1.0% IGEPAL® CA-630, 0.5% Triton X-100 and protease inhibitors. The whole cell lysates were incubated with either 2 μg of an ERα antibody or IgG at 4 °C for 3 h and then incubated with 20 μl of protein A/G-agarose beads for 2 h. The agarose beads were then centrifuged at 1000*g* for 5 min, washed 3 times with lysis buffer containing protease inhibitors, and then resuspended in 60 μl of SDS–PAGE loading buffer for use in Western blot analyses.

2.7. Western blot analysis

Cell lysates containing 50–100 μg of protein were separated using 10% or 12% SDS–PAGE and transferred onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare, Waukesha, WI). After blocking the membranes with 5% non-fat milk, the membranes were incubated with primary antibodies at 1:1000 dilutions overnight at 4 °C. The membranes were then incubated with HRP-conjugated secondary antibodies at 1:3000 dilutions at room temperature for 1 h and developed using an Amersham ECL™ Western-blotting Analysis System (GE Healthcare).

2.8. Nuclear and cytosolic fractionations

The nuclear and cytosolic fractions were prepared using the method previously described by us, with some modifications [26]. Briefly, MCF-7 cells were collected following various treatment conditions and resuspended in Cytosol Lysis Buffer containing 0.01% digitonin, 2 mM EDTA and protease inhibitors in PBS at a pH of 7.4. The cell lysates were incubated at 4 °C for 5 min and then centrifuged at 13,000*g* for 10 min. The obtained supernatants were used as the cytosolic fractions. The remaining cell pellets were further resuspended in the RIPA buffer that was described above, sonicated using an ultrasonicator, and centrifuged at 13,000*g* for 10 min at 4 °C. The obtained supernatants were used as the nuclear fractions. The protein concentrations of both the nuclear and cytosolic fractions were determined using the Bradford reagent.

2.9. Immunofluorescent staining

The cells were grown on cover slips and treated with different agents. Afterwards, the cells were fixed for 15 min using 4% paraformaldehyde that had been dissolved in PBS and then permeabilised for 15 min using 0.2% Triton X-100 that had been prepared in PBS. The cells were blocked using 3% BSA plus 0.2% Triton X-100 for 1 h at room temperature. Following blocking, the cells were stained with an anti-ERα antibody at a 1:100 dilution in 3% BSA plus 0.2% Triton X-100 at 4 °C overnight. The cells were then stained with a FITC-conjugated secondary antibody at a 1:100 dilution for 1 h. The cover slips were finally mounted onto a clean glass slide using Mowiol® 4–88 (Calbiochem, San Diego, CA). The cell images were obtained using an inverted fluorescence microscope (Zeiss Axiovert S100) and recorded using a SPOT CCD camera (Diagnostic Instruments). The scale bar was added using SPOT Advanced software.

2.10. Real-time PCR

The cells were lysed using TRIzol and total RNA was extracted. The extracted RNA samples were reverse-transcribed into cDNA using an ImProm-II™ Reverse Transcription System (Promega, Madison, WI, A3800). The acquired cDNA was then quantitatively amplified using real-time PCR with reagents from the iQ™ SYBR® - Green Supermix kit (Bio-Rad, 170-8880). For each amplified gene, one forward and one reverse primer were designed and used for the real-time PCR experiments. The DNA sequences of all six primers are shown below:

(1) ESR1: forward: 5′-gcaccctgaagtctctggaa-3′, and reverse: 5′-gatgtgggagaggatgagga-3′,
(2) GREB1: forward: 5′-caccacagccacctcttctt-3′, and reverse: 5′-cccactgaacagcaagtcc-3′,
(3) GAPDH: forward: 5′-gtcagtggtggacctgacct-3′, and reverse: 5′-aaaggtggaggagtgggtgt-3′.

The following program was used to conduct the PCR experiments: Step 1 consisted of 95 °C for 30 s, step 2 consisted of 56 °C for 30 s, and step 3 consisted of 72 °C for 30 s. These steps were repeated for 40 cycles. The amplified DNA can be quantitatively determined after each cycle of PCR, and the threshold cycle of each sample can indicate the relative cDNA template concentration. Because the cDNA was reverse-transcribed from the mRNA, the relative levels of mRNA expressed from the target gene can be calculated based on the threshold cycle of each sample in a real-time manner. The mRNA levels of a non-ERα responsive housekeeping gene, GAPDH, were measured and used as an internal reference.