

RESEARCH ARTICLE

A Novel All-trans Retinoid Acid Derivative Induces Apoptosis in MDA-MB-231 Breast Cancer Cells

Bei Wang^{1,2*}, Yun-Wen Yan², Qing Zhou², Shu-Yu Gui³, Fei-Hu Chen⁴, Yuan Wang^{2,3*}

Abstract

Aims: To explore the effect and probable mechanism of a synthetic retinoid 4-amino-2-tri-fluoromethyl-phenyl ester (ATPR) on apoptosis of MDA-MB-231 breast cancer cells. **Materials and Methods:** MTT assays were performed to measure the proliferation of MDA-MB-231 cells treated with different concentrations of all-trans retinoic acid (ATRA) and ATPR. Morphologic changes were observed by microscopy. The apoptosis rates and cell cycling of MDA-MB-231 cells treated with ATRA or ATPR were assessed using flow cytometry analysis. Expression of retinoic acid receptor and phosphorylation of ERK, JNK, p38 proteins were detected by Western blotting. **Results:** Treatment of the cells with the addition of 15 $\mu\text{mol/L}$ ATPR for 48 h clearly demonstrated reduced cell numbers and deformed cells, whereas no changes in the number and morphology were observed after treatment with ATRA. The apoptosis rate was 33.2% after breast cancer MDA-MB-231 cells were treated by ATPR (15 $\mu\text{mol/L}$) whereas ATRA (15 $\mu\text{mol/L}$) had no apoptotic effect. ATPR inhibited the phosphorylation of ERK, JNK, and p38 while ATRA had no significant effect. ATPR inhibited the expression of BiP and increased the expression of Chop at the protein level compared with control groups, ATRA and ATPR both decreased the protein expression of RXR α , ATPR reduced the protein expression of RAR β and RXR β while ATRA did not decrease RAR β or RXR β . **Conclusions:** ATPR could induce apoptosis of breast cancer MDA-MB-231 cells, possible mechanisms being binding to RAR β /RXR β heterodimers, then activation of ER stress involving the MAPK pathway.

Keywords: Breast cancer - ATPR - ER stress - MAPK pathway - retinoic acid receptor

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Introduction

Breast cancer is one of the most common cancer around the world, and remains the second leading cause of cancer-related deaths in women (Ma et al., 2013). Breast cancer incidence and death rates increase with age, approximately 95% of new cases occur in women 40 years of age and older (DeSantis et al., 2011), whereas women at age 40 or younger have a poorer prognosis than their older counterparts and bear a disproportionate degree of physical and psychological morbidity (Andres et al., 2009). Therefore, it is important to find a drug to induce the apoptosis of breast cancer cells. All trans-retinoic acid (ATRA) induces apoptosis and differentiation in solid tumors, including breast cancer, and has become a therapeutic tool in this disease (Marchetti et al., 2011). Apoptosis, the most common form of programmed cell death in vertebrate, defines a type of regulated cell death associated with various morphological features that include cell shrinkage, nuclear/cytoplasmic fragmentation, and formation of dense bodies. Many studies have

indicated that crucial apoptotic modulators are deregulated in metastatic cancer cells and supported the hypothesis that suppression of apoptosis has a vital role during the metastatic process (Korzeniewska et al., 2007). Recently reported that caspase-Mediated Pathway involved in Apoptosis in breast cancer cells (Zhou et al., 2013; Zhang et al., 2014). ER stress-induced apoptosis has been implicated in the pathogenesis of several conformational diseases. Studies performed with a number of cell lines and *in vivo* model have shown that survival and death decisions during UPR are mediated via the proapoptotic CHOP (C/EBP-homologous protein transcription factor also known as GADD 153 growth arrest and DNA damage inducible protein) and the anti-apoptotic BIP (GRP78: a Ca²⁺dependent, ER associated transmembrane chaperone), respectively (Price et al., 2010). The Ras-mitogen-activated protein kinase (MAPK) pathway participates in the control of many important cellular processes including survival, proliferation, apoptosis and differentiation. Recent studies have shown that MAPK pathway could be as an apoptosis enhancer in melanoma (Haydn et al., 2014).

¹Department of Pathology and Pathophysiology, medical School, Southeast University, Nanjing, Jiangsu, ²Laboratory of Molecular Biology, Dept of Biochemistry, ³Key Laboratory of Gene Resource Utilization for Severe Disease of Anhui Province, ⁴School of Pharmacy, Anhui Medical University, Hefei, Anhui, China *For correspondence: xuewuhenwang@126.com, wangyuan@ahmu.edu.cn

Retinoids regulate various biological activities by binding RARs and RXRs. Ligand binding to the receptor causes conformational changes that modulate receptor complex function. In addition, these receptor complexes have a range of additional co-activators and co-repressors that modulate receptor activity. The composition of receptor complex is complicated by the presence of distinct sub-types and isoforms. Both RAR and RXR have three sub-types α , β and γ , each with different isoforms. RAR α and RAR γ both have two isoforms 1 (RAR α 1, RAR γ 1) and 2 (RAR α 2, RAR γ 2) whereas RAR β have five isoforms (RAR β 1-4, and 11). In the case of RXR, all sub-types have two isoforms 1 and 2 (Das et al., 2014). The RARs bind ATRA, whereas the RXRs bind 9 Cis-RA selectively.

Since 1989, when ATRA was introduced as a targeted therapy against acute promyelocytic leukemia (APL), it has been used extensively as an anti-tumor agent for many types of tumors (Yung et al., 1989). However, as we know, the toxicity of retinoids has limited their general use for cancer prevention. Therefore, the present work is aimed at developing new synthetic retinoids which are less toxic and more effective as chemoprevention agents. ATPR is synthesized by school of Pharmacy, Anhui Medical University and is a novel ATRA derivatives. Recently, a series of studies associated with ATPR about migration of lung cancer were published (Wang et al., 2013; Fang et al., 2014). In a previous study, we have demonstrated that ATPR plays a more important role in inducing the differentiation and inhibiting the proliferation and migration of MDA-MB-231 cells than ATRA at the same dose and treatment time (Wang et al., 2013). So far, whether ATPR could induce apoptosis of MDA-MB-231 cells and the precise mechanism are unknown, although ATPR has anti-tumor effects such as inhibiting the proliferation and arresting the cell cycle in human breast cancer cell line MCF-7 (Wang et al., 2013). Therefore, the present study was undertaken to investigate the effect of ATPR inducing apoptosis of Breast cancer MDA-MB-231 cells and explore their mechanism whether ATPR induces apoptosis via ER stress involving MAPK signal pathway and binding which one of retinoic acid receptors. Finally, the mechanisms underlying anti-neoplastic potential of ATPR in breast cancer could be partly elucidated.

Materials and Methods

Materials

ATRA, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Dimethyl Sulfoxide (DMSO) were obtained from sigma Chemical (USA). Dulbeccos modified Eagles medium (DMEM) medium was obtained from Gibco BRL life Technologies (USA). ATPR was provided by school of Pharmacy, Anhui Medical University (Anhui, China). MDA-MB-231 cells were obtained from the American Type Culture Collection (USA). Bovine serum was purchased from the Zhejiang Tianhang Biological Technology Co (China). Primary antibodies (anti-BiP, anti-p-CHOP, anti-RAR α , anti-RAR β , anti-RAR γ , anti-RXR α , anti-RXR β , anti-RXR γ , anti-p-ERK, anti-ERK, anti-p-JNK, anti-JNK, anti-p-p38,

anti-p38, anti- β -actin) were purchased from Santa Cruz Biotechnology (USA). All secondary antibodies were purchased from MILLIPORE (USA).

Cell lines and cell culture

Breast cancer MDA-MB-231 cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin, 100U/ml streptomycin in humidified 5% CO₂ at 37°C. The media were changed every two or three days.

Cell viability assay

Cell viability was measured using the MTT assay. Breast cancer MDA-MB-231 cells (5x10³ cells/well) were seeded into 96-well plates and cultured. The cells were treated at different times (24hr, 48hr, 72hr) and different concentration of ATRA and ATPR (10, 12.5, 15, 17.5, 20 μ mol/L), equivalent DMSO was added as the control group, then incubation with MTT solution for 4h. Finally, the cells were exposed to an MTT-formazan dissolving solution (DMSO) for 30 minutes. The optical density (OD) was measured using an absorbance microplate reader (Bio-Tek, ELX800) at a wavelength of 490nm. The cell viability was expressed as a percentage of the OD value of the control cultures. The IC₅₀ value was measured using a sigmoidal equilibrium model regression by XLfit version 4.3.2 (ID Business Solutions Ltd), and defined as the concentration of ATPR required for a 50% reduction in growth.

Microscopy observation of Breast cancer MDA-MB-231 cells

Breast cancer cells were seeded into 6-well plates and cultured, then cells were treated by ATRA and ATPR (15 μ mol/L) for 48 h. DMSO was added in the control group. Images of cells were captured using the microscope .

Assessment of cell cycle distribution

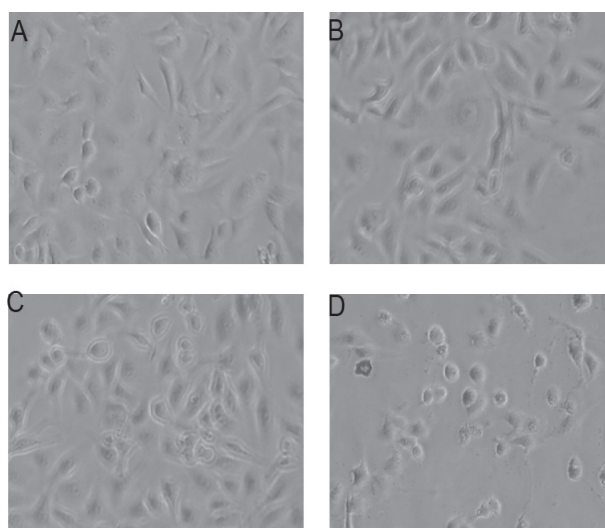
After MDA-MB-231 cells were treated with ATPR and ATRA, the cell cycle analysis and cell apoptotic rate were determined by flow cytometry analysis. MDA-MB-231 were incubated in decanter at a density of 1x10⁵. After 48 h, cells were treated with ATRA and ATPR at the same concentration of 15 μ mol/L. Equivalent DMSO was added as the control group. After treatment, Cells were harvested by trypsinization and washed twice with ice-cold PBS, fixed with 70% alcohol overnight, and stained with PI (1mg/ml) in the presence of 1% RNase A for at least 30 minutes before analysis by flow cytometry (Becton Dickinson, USA). GO/G1, S, and G2/M cells were gated out as appropriate. Data were analyzed with Modfit software.

Western blot analysis

After treatment by ATRA and ATPR (10, 15 μ mol/L), Breast cancer MDA-MB-231 cells were washed with ice-cold PBS for 3 times and lysed in lysis buffer (50mmol/L Tris-HCl, pH 7.14, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton, 0.1% SDS, 5mg/ml Leupeptin, 1mmol/L PMSF). The protein concentrations were measured with a BCA kit (Beyotime, China). The cell lysates were

Table 1. Effects of ATRA and ATPR on the Proliferation of MDA-MB-231 Cells Detected by MTT

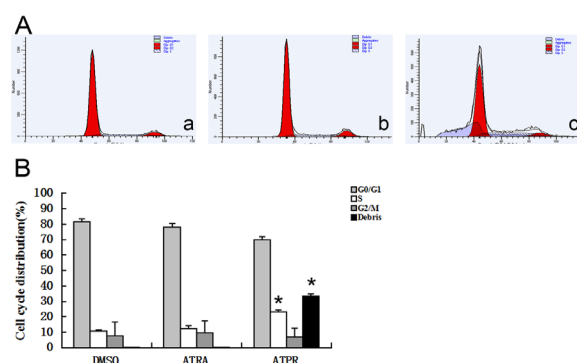
Group	Concentration (umol/L)	24h (OD)	Inhibitor rate	48h (OD)	Inhibitor rate	72h (OD)	Inhibitor rate
Control		0.602±0.00491	-	0.838±0.014	-	1.328±0.022	-
DMSO		0.592±0.00341	0.0157	0.798±0.011	0.048	1.304±0.025	0.018
ATRA	10	0.610±0.0056	-0.0128*	0.786±0.011	0.041*	1.258±0.023	0.053*
	12.5	0.613±0.0061	-0.0178*	0.804±0.003	0.040*	1.205±0.027	0.092*
	15	0.609±0.0056	-0.0120*	0.803±0.009	0.042*	1.243±0.032	0.065*
	17.5	0.629±0.0032	-0.0456*	0.855±0.007	-0.020*	1.162±0.018	0.125*
	20	0.615±0.0166	-0.0216	0.844±0.019	-0.007	1.214±0.053	0.086
ATPR	10	0.661±0.0048	-0.0971*	0.855±0.007	-0.020*	1.013±0.092	0.237*
	12.5	0.624±0.0137	-0.0357*	0.691±0.032	0.176*	0.848±0.039	0.361*
	15	0.489±0.0188	0.1880*	0.351±0.016	0.582*	0.361±0.027	0.728*
	17.5	0.355±0.009	0.4114*	0.199±0.003	0.763*	0.204±0.156	0.847*
	20	0.299±0.0088	0.5043*	0.187±0.002	0.777*	0.208±0.159	0.843*

p*<0.05 vs the DMSO controlFigure 1. Effect of ATPR on the Morphology of MDA-MB-231 Cells.** (A) Cell control (B) DMSO Control (C) ATRA 15 µmol/L (D) ATPR 15 µmol/L

solubilized in SDS sample buffer, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membranes. The membrane was blocked with blocking buffer (Tween 20 (TBST)/ 5% nonfat dry milk) overnight at 4°C, then, the membrane was incubated with the indicated primary antibody with BiP (1:1000), CHOP (1:500), RAR α (1:1000), RAR β , (1:1000) RAR γ (1:1000), RXR α (1:1000), RXR β (1:1000), RXR γ (1:1000), *p*-ERK (1:500), *p*-JNK (1:500), *p*-p38 (1:500), ERK (1:1000), JNK (1:1000), p38 (1:1000), β -actin (1:1000) respectively and followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody with BiP (1:500), CHOP (1:500), RAR α (1:500), RAR β , (1:500) RAR γ (1:500), RXR α (1:500), RXR β (1:500), RXR γ (1:500), *p*-ERK (1:500), *p*-JNK (1:500), *p*-p38 (1:500), ERK (1:1000), JNK (1:1000), p38 (1:1000), β -actin (1:1000) respectively and visualized with enhanced chemiluminescence (ECL, Beyotime, China) using hydrogen peroxide and luminol as substrate with Kodak X-AR film. Negatives were scanned using a ScanPrisa1240OUT (Acer, China).

Statistical analysis

Three or more separate experiments were performed

**Figure 2. Effect of ATPR on Cell Cycle in MDA-MB-231 cells.** (A) PI-stained cells analyzed using flow cytometry: a: DMSO Control b: ATRA 15 µmol/L c: ATPR 15 µmol/L (B) Percentage (%) of cell cycle distribution. Compared with DMSO control: **P*<0.05

independently for each experiment. Statistical analysis was performed by Student's test or ANOVA. Data are presented as means±standard deviation. Significance was noted at *p*<0.05

Results

Effect of ATPR on proliferation of MDA-MB-231 cells

In previous published article (Wang et al., 2013), we have already chosen a concentration of ATRA and ATPR (15µmol/l) as a treated concentration (Table 1).

Effect of ATPR on the morphology of MDA-MB-231 cells

Breast cancer MDA-MB-231 cells were treated with ATRA (15 µmol/L) and ATPR (15 µmol/L) and DMSO (1.5%), the morphology of breast cancer MDA-MB-231 treated by ATPR changed from spindle to round, and the number of cell reduced. (Figure 1) Bright field images were taken at the indicated time points. Magnification, 100×

Apoptosis analysis by flow cytometry

The percentage of cells with the sub-G1 DNA content reflects the apoptotic rate of the cell population. The ratio of apoptosis was 33.23% after breast cancer MDA-MB-231 cells were treated by ATPR (15 µmol/L) whereas ATRA (15 µmol/L) had no apoptotic effect on MDA-MB-231 cells. Figure 2 also showed that the cell cycles

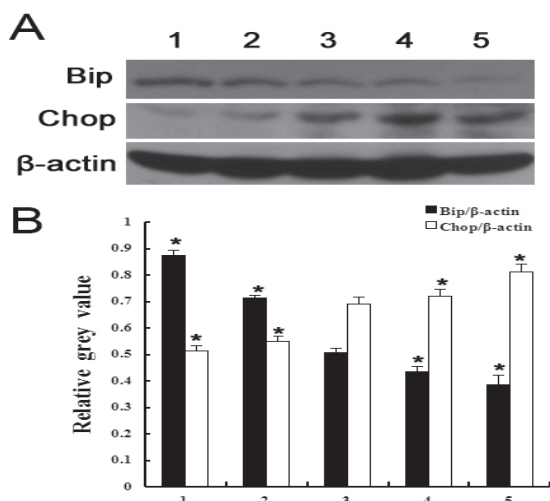


Figure 3. Effect of ATPR on ER Stress in MDA-MB-231 Cells. (A) 1:ATRA 15 μ mol/L 2:ATRA 10 μ mol/L 3:DMSO control 4:ATPR 10 μ mol/L 5:ATPR 15 μ mol/L (B) Analysis of Grey value Compared with DMSO control: * P <0.05

in different group detected by flow cytometry. ATPR (15 μ mol/L) treatment could result in S-phase cell cycle arrest of MDA-MB-231 cells. The percentage of cells in S-phase increased from 10.66% to 23.16%.

Effect of ATPR on ER stress in MDA-MB-231 cells

To catch out the effect of ATPR on ER stress, the expression of BiP and CHOP were detected at the protein level by Western blot analysis as BiP and CHOP were the markers of ER stress. The expression of BiP decreased while the expression of CHOP increased after the breast cancer MDA-MB-231 cells were treated by ATPR (Figure 3).

Effect of ATPR on MAPK signal pathway in MDA-MB-231 cells

To find out the whether MAPK signal pathway involved in apoptosis of breast cancer MDA-MB-231 cells, western blot was used to investigate the expression of phosphorylation of ERK, JNK and p38. Interesting to find the expression of phosphorylation of ERK had a significant decrease when the breast cancer MDA-MB-231 cells were treated by ATPR. In contrast, no significant decreases can be observed, to our surprise, minor increases can be found in comparison to control (Figure 4).

Effect of ATPR on retinoid receptor in MDA-MB-231 cells

To check which one of all retinoid-receptors binds to ATPR in breast cancer MDA-MB-231 cells, the expression of RXR and RAR were detected by western bolt. ATRA and ATPR both reduced the expression of RXR α . In compared with ATRA, data revealed that ATPR significantly decreased the expression of RAR β and RXR β . By comparison with DMSO group, the protein level of RAR γ and RXR γ exhibited no significant decrease (Figure 5).

Discussion

In this research, we studied the anti-tumor effect of

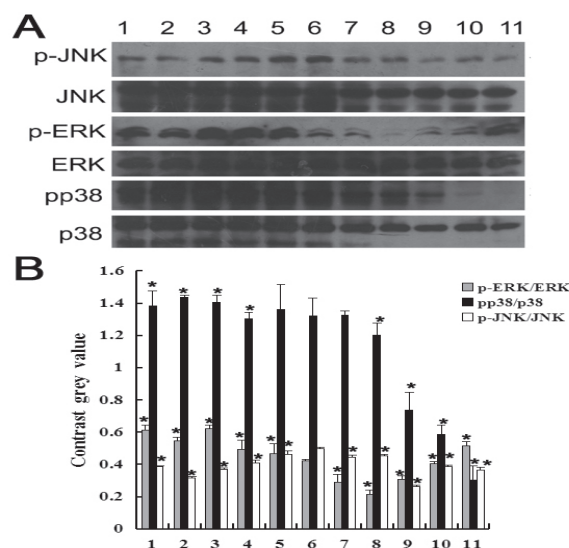


Figure 4. Effect of ATPR on MAPK Signal Pathway in MDA-MB-231 Cells. (A) 1:ATRA 15 μ mol/L 48h 2: ATRA 15 μ mol/L 24h 3: ATRA 15 μ mol/L 12h 4: ATRA 15 μ mol/L 2h 5:ATRA 15 μ mol/L 30min 6: DMSO control 7:ATPR 15 μ mol/L 30min 8: ATPR 15 μ mol/L 2h 9: ATPR 15 μ mol/L 12h 10: ATPR 15 μ mol/L 24h 11: ATPR 15 μ mol/L 48h (B) Analysis of Grey value Compared with DMSO control: * P <0.05

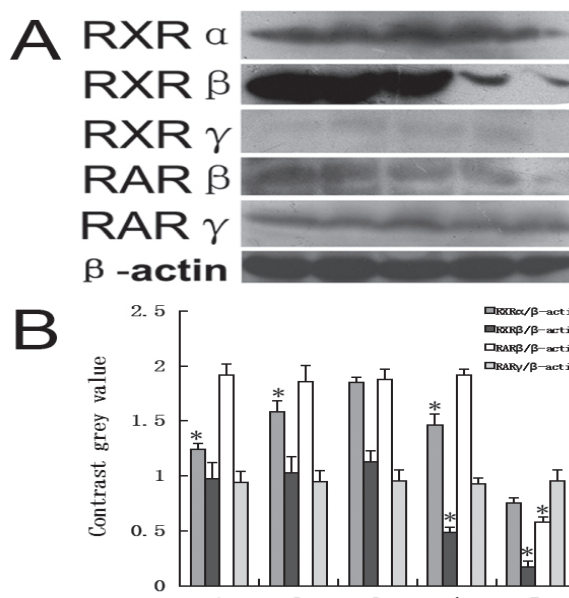


Figure 5. Effect of ATPR on Retinoid Receptor. (A) 1:ATRA 15 μ mol/L 2:ATRA 10 μ mol/L 3:DMSO control 4:ATPR 10 μ mol/L 5:ATPR 15 μ mol/L (B) Analysis of Grey value Compared with DMSO control: * P <0.05

ATPR on the breast cancer MDA-MB-231 cells, and the possible mechanisms of apoptosis of breast cancer MDA-MB-231 cells. We demonstrate for the first time that ATPR down-regulates the expression of BiP and up-regulates the expression of CHOP. The expression levels of BiP and CHOP are markers of endoplasmic reticulum (ER) stress. Protein folding in the ER is impaired under various physiological and pathological conditions, collectively referred to as “ER stress” (Kaufman et al., 1999). ER stress is generally regarded as a protective cellular response against apoptosis. Cells respond to ER stress via activation of the unfolded protein response (UPR) pathway, which

emanates from the ER. Proteins must be folded into proper conformations, so as to realize their cellular functions. Unfolded or misfolded proteins are extremely harmful to cells because cell survival can be threatened (Kopito et al., 2000). The UPR enables cells to reduce unfolded protein folding, secretion and degradation (Zhao et al., 2006). In many tumors, BiP is highly expressed and is crucial for tumor cells survival (Dudek et al., 2009). BiP, a chaperone predominantly in the ER lumen interacts with unfolded proteins through its C-terminal substrate-binding domain, which is tightly regulated by a conformational change depending on ATP occupation of its NBD (nucleotide-binding domain) (Mayer et al., 2005). As a negative regulator of UPR, BiP sequesters PERK, ATF6, and IRE1 on the ER membrane via binding to their luminal domains. BiP dissociating from PERK, ATF6, and IRE1 leads to downstream UPR signaling. ATF6 is synthesized as an inactive precursor coding for a bZIP transcription factor in the cytoplasmic domain. Under ER stress, ATF6 traffics to Golgi apparatus where S1P and S2P proteases cleave the cytosolic and transmembrane domains, releasing a fragment named ATF6f (Haze et al., 1999). ATF6 translocates to the nucleus regulating the transcription of genes involved in ER homeostasis, such as ER chaperones and ERAD components (Asada et al., 2011). PERK is a transmembrane protein kinase that under ER stress conditions dimerizes and autophosphorylates, favoring the phosphorylation of eIF2 α (eukaryotic translation initiation factor 2 α). Phosphorylated eIF2 α causes a global translational arrest as a fast adaptive reaction (Harding et al., 2000) and favors the selective translation of ATF4 (activating transcription factor 4) (Vattem et al., 2004), regulating the expression genes involved in folding oxidative stress and amino acid metabolism (Harding et al., 2003).

The loss of BIP, which means the lack of a key player in ER stress, triggered by DNA damage could accelerate cell death (Takayanagi et al., 2013). As mentioned above, BiP expression decreased after MDA-MB-231 cells were treated by ATPR leading to unfolded proteins accumulate, as a result of activating ER stress. At the same time, the expression of CHOP increased. CHOP is a 29KDa protein with 169 (human) or 168 (rodents) amino-acid residues. CHOP protein was first identified to be a member of the CCAAT/enhancer binding proteins (C/EBPs) that serves as a dominant negative inhibitor of C/EBPs. CHOP is also known as growth arrest-and DNA damage-inducible gene 153 (GADD153) and DNA-damage-inducible transcript 3 (DDIT3) (Oyadomari et al., 2003). In general, clearance of misfolded ER proteins, along with proapoptotic outputs such as CHOP production.

Vitamin A has been demonstrated to inhibit the induction and retard the growth of experimental tumors. The effect of vitamin A varies in different animals and is also dependent on the site of application (Alizadeh et al., 2014). Isomerization of all-trans RA was done under experimental and physiological conditions. Different isomers activate different receptors, which lead to different biological effects. Retinoids that specifically bind to RXR are called rexinoids and have been effective in cancer treatment. Retinoids are comprised of three units: a bulky

hydrophobic region, a linker unit and a polar terminus, which is usually a carboxylic acid. Modification of each unit has generated many more compounds. Active retinoid-receptors consist of RAR/RXR heterodimers, which bind to Retinoic-acid-responsive-elements (RAREs) in retinoid-responsive genes (Baumrucker et al., 2006). In the present study, after cells were treated by ATPR, ATPR and ATRA both reduced the expression of RXR α and the expression of RAR β and RXR β both decreased while other Retinoic-acid receptors had no significant decrease. Therefore, we suppose that ATPR induced the apoptosis of MDA-MB-231 cells through binding RAR β and RXR β , and RAR β forms heterodimers with RXR β . Because of antibody of RAR α , the protein expression of RAR α was not detected.

MAPK are a group of protein serine/threonine kinases, which are activated in response to various extra-cellular stimuli including the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 MAPK kinases (Zhang et al., 2014). MAPK super-family consists of three serine/threonine kinase cascades. ERKs responded to growth factors or other external mitogenic signals by promoting cell proliferation and opposing cell death signal. The other two pathway-p38 MAPK and JNK pathways are typically described as stress-activated kinase that promote inflammation, or, in certain cases, programmed cell death (Olson et al., 2004). We found that the ATPR inhibited phosphorylation of JNK, p38, and ERK within 48 h. We previously reported that ATPR could inhibit phosphorylation of p38, JNK and ERK within 2 h. We further extended the time in order to determine which time of ATPR could inhibit phosphorylation of MAPK signal pathway significantly. As a result, ATPR inhibited phosphorylation of p38 after cells were treated by ATPR for 2h. The level of phosphorylation of ERK and JNK reduced continuously within 48h. These data indicated that ATPR induced apoptosis of breast cancer MDA-MB-231 cells involving MAPK signal pathway. In the following work, inhibitors of various MAPK signal pathway would be required to use in treating MDA-MB-231, for instance, SB203580, an inhibitor of p38, PD98059, an inhibitor of ERK and SP600125, an inhibitor of JNK. These inhibitors alone and in combination can further explore the mechanism of MAPK signal pathway in inducing the apoptosis of MDA-MB-231 cells treated by ATPR.

It is not clear whether MAPKs and ER stress are involved in ATPR induced cell death of Breast cancer MDA-MB-231 cells and Little is known about the relevance between ATPR and RARs and RXRs in inducing the apoptosis of breast cancer MDA-MB-231 cells. In the present study, we demonstrated, for the first time, ATPR could induce apoptosis of MDA-MB-231 cell through ER stress and associated with MAPK signal through binding RAR β /RXR β heterodimer. These data qualify ATPR as a potent anti-tumor drug to better understand the mechanisms that induces the apoptosis of breast cancer cells. So far, we only did the preliminary research, and future further investigation are needed to clearly define the mechanism of apoptosis of ATPR on breast cancer MDA-MB-231. For example, to further confirm the relation about ATPR and ER stress, MAPK signal pathway and

retinoic acid receptor, inhibitors of MAPK signal pathway need to be added to the MDA-MB-231 cells. In addition, ER-stress sensors IRE1, PERK, and ATF6 needed to be detected in order to further confirm that ATPR induced apoptosis involving ER stress.

In summary, newly synthesized retinoic acid derivative ATPR could inhibit the growth and induce the apoptosis of MDA-MB-231 cells. The possible mechanism of apoptosis of MDA-MB-231 cells treated by ATPR could be through binding to RAR β /RXR β heterodimers, then activated ER stress involving MAPK pathway. The mechanisms of apoptosis of breast cancer MDA-MB-231 cells induced by ATPR need to be further studied. These findings support that the potential application of ATPR in the treatment of breast cancer.

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