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Antitumor Activity of Atractylenolide II on Breast Cancer Cells through Regulation of Estrogen Receptor Protein Expression and NF- K b Signaling Pathways

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Abstract

Many women in the world are suffering from breast cancer deeply. Although many efforts are made in the study of breast cancer prevention and treatment, little attention is paid to the molecular mechanism of the disease. In the present study, considerable techniques such as western blotting, quantitative RT-PCR (qRT-PCR), Luciferase Immunohistochemistry, and flow cytometry analysis has been performed to analyze the effect of Atractylenolide II(ATR II) addiction for breast cancer research. As indicated by our research outcomes, ATR II could prohibit the proliferation of Prostate cancer as well as Breast cancer cells, in particular, ATR II induces MDA-MB231 and MCF-7 cells apoptosis, through G2/M -phase cell arrest. Also, cell apoptosis was induced by ATR II mainly associated with extrinsic mitochondrial pathways followed by activation of death receptor (DR4) that regulated activation of caspase-8 through cascade promotes activation of caspase-3, and therefore, drive breast cancer cells line to apoptosis. The apoptosis induced by ATR II is also associated with its ability to regulate the activity of androgens receptors and inhibition of NF-κB signaling pathways. Regarding this finding, ATR II might be promising chemotherapies drugs for breast cancer cells lines.

Keywords: ATR II; Apoptosis; ER/NF-KB; G2/M

Introduction

Breast cancer is understandably one of the most severe heterogeneous diseases for women all over the world, leading to millions of deaths every year [1]. Different efforts are devoted to overcoming this critical issue by paying special attention to its prevention, diagnosis, and treatment. More specifically, breast cancer can be treated through surgically, chemotherapy, or radiotherapy methods which differ in their action [2,3]. Nevertheless, these approaches are impactful only at the earlier stage of tumor activity. These strategies present little and short-term effectiveness because of their high toxicity, off-targeting ability, and inefficiencies for long-term use [4].

Natural drugs have been used as an antitumor agent during century due to their potent anti-inflammatory and antioxidant activities and because of their abilities to regulate the activity of molecular targets as well as their signaling pathways, which were related to cell differentiation [5,6]. Among these sesquiterpene lactones attire more attention on behalf of their ability to possess less side effect [7]. Atractylenolide II is a sesquiterpene derivate of plant Atractylodes Chinensis. ATR II was reported to have promising antitumor activity notably on Gastric, Colorectal, and melanoma cells line, through several signaling pathways [8-11].

Worldwide, more than 80% of breast cancers express estrogen receptor α (ER- α). As one steroid hormone receptor, ER divided into ER alpha (ERα) and ER beta (ERβ) further. Previous studies reported that the ERα-positive cell has a higher content than the ERα-negative cell line to regular breast cancer genes in an estrogen-dependent manner [12]. Studies also showed that an over-expression of ER-β and inhibition of ERα could be an effective anti-tumor strategy against breast cancer [13]. The level of nuclear factor kappa-B (NF-κB) is elevated in ER– human breast cancers, as compared with ER+ cells. Moreover, several studies have reported the elevated ration of the nuclear factor kappa-B (NF-κB) in ER– human breast cancers, as compared with ER+ cells.

The transcription factor NF-κB is the key point in regulating immune system responses associated with disease such as cancer. In its inactive form, NF-κB remains in the cytoplasm by its protein inhibitor (IκBs). In response to a variety of stimuli, such as the binding of tumor necrosis factor (TNF)-α to its membrane receptor, IκBα is phosphorylated at Ser32/Ser36 by IκB kinase (IKK). IKK is a multi-subunit kinase complex, typically composed of ER α and IKKβ, and

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two molecules of IKKγ/NF-κB essential modulator (NEMO) [14]. Phosphorylated IκB is then degraded by the proteasome, which allows NF-κB dimers to translocate to the nucleus, where they stimulate the expression of target genes [15].

Despite its effective biological response against different cancer cells, ATR II has never been employed in the against breast cancer cells before. The possible reason consists of the perplexity and non-availability of breast cancer molecular pathogenesis. This research aimed to utilize the ATR II as an anti-cancer agent to cure breast cancer by finding out the functional mechanism of ATR II against breast cancer. So we hypothesized that ATR II could induce apoptosis in MCF-7 and MDA-MB 231 apoptosis by regulating the ERs expression after the stimulation of the androgen receptor through the regulation of the NF-κB signaling pathway.

Materials and Methods

Ethics/guidelines

It is certified that we followed all ethical system classification and our laboratory guidelines; no miss identified cell lines has been used. MCF-10A, MCF-7, MDA-MB 231, BPH1, LNCaP, DU145 cell line was purchased from ATCC (Shanghai. P. R).

Cell culture

Human cancer cells MCF-7 and MDA-MB 231, LNCaP, DU145, BPH1, cells were cultured and maintained in DMEM medium contain 10% of fetal bovine serum (Gibco, P.R. China).MCF-10A cells were cultured in DMEM/F12 (1:1) media supplemented with 5% horse serum, 1% PS, 0.05% hydrocortisone, 0.1% human insulin, 0.02% epidermal growth factor, and 0.01% cholera toxin. Cells were culture then; cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and allowed for the growth of 70-80% of their confluent.

Cell proliferation assay

Cells were culture and maintained in DMEM, or DMEM/F12 (1:1) medium contain 10% of fetal bovine serum (Gibco, PR. China), then cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and allow to growth 70-80% of their confluent. Afterward, cells were harvest and seeded in a 96-well dish to a final concentration of 5×10^3 cells/well and incubated in DMEM medium containing 1% FBS for 24h. Furthermore, cells were treated with the indicated concentrations of ATR II (Baoji Herbest Bio-tech Beijing, P.R China) and incubated for 72h. After that 20μl of MTT (Sigma Chemical St. Louis, MO, USA) solution (5mg/ml) was added in each well and further incubated at 37°C for 4h. Finally, the medium was discarded, and 150 μL of DMSO was added to each well. The plates were read at the wavelength of 570 nm using Varioskan Flash Multimode Reader (Thermo Scientific, USA). Six reduplicate wells were used for each treatment, and experiments were repeated three times, and their inhibition ratio (I %) was calculated based on the following equation

$$I\ \% = A570\ (control) - A570\ (treated)] / A570\ (control) \times 100$$

Where I is the inhibition rate, and A is the absorbance at 570 nm.

Annexin V/PI assays for apoptosis

The apoptotic cells were investigated by Annexin V/PI followed by flow cytometric regarding the manufacture protocol. MCF-7 and MDA-MB 231 cells (5×10^3 cells/well) were cultured in 6 wells plates and treated with different concentrations (0, 35 and 70) μM of ATR II for 48 h. After, cells were washed twice with PBS and then stained with 5 μL of Annexin V-FITC (Beyotime P. R. China) and 10 μL of PI in 500 μL binding buffer for 15 min at room temperature in the dark. The apoptotic cells were determined by flow cytometry (Cytomics FC 500; Beckman Coulter Inc., Miami, FL).

Determination of MCF-7 and MDA-MB 213 cells cycle distribution

The cell cycle distributions in different phases after exposure of ATR II were analyzed by flow cytometry. In brief, MCF-7 and MDA-MB 231 (5×10^3 cells/well plates) were seeded into a 6-well plate and exposure to (0,35 and 70 μM) of ATR II for 48 h. Further, the cells were harvested, washed with PBS twice, and subsequently fixed with 70 % ethanol for two hours. After that, cells were centrifuged and washed with PBS, finally resuspended in 500 μl of buffer containing 10 μl of RNase and 25 μl of PI (Beyotime PR. China), then incubated in the dark at RT for 15 minutes. The distributions of the cell cycle were determined by flow cytometry (Cytomics FC 500; Beckman Coulter Inc., Miami, FL).

Determination of ROS expression in MCF-7 and MDA-MB 231 cells

The generation of ROS was determined with 2,7-dichlorofluorescein diacetate (DCFH-DA) taking the manufacturer protocols. In brief, MCF-7 and MDA-MB 231 cells were cultured (5×10^3 cells/well), then incubated with or without NAC for 1 h. After that culture was treated with (0, 35, and 70) μM of ATR II for 48 h. Further, the cells were collected, centrifuged and washed with PBS, then resuspended in PBS containing 10 μM of DCFH-DA (Beyotime PR.China) and incubated in the dark at RT for 15 minutes. After that, the cells were washing with PBS and measured immediately by using flow cytometry (Cytomics FC 500; Beckman Coulter Inc., Miami, FL) to monitor the formation of the fluorescent-oxidized derivative of DCFH-DA at an emission wavelength of 525 nm and an excitation wavelength of 488 nm.

Determination of Mitochondrial Membrane expression (MMP)

The changes induced by ATR II in Mitochondrial membrane was determinate by Rhodamine 123 staining (Beyotime PR.China) according to the manufacturer protocol. Briefly, MCF-7 and MDA-MB 231 cells (5×10^3 cells/well) were seeded in 6 well plates then treated with or without NAC incubated at 37˚C for 1 h , then cells were treated with or without ATR II (0,35 and 70) μM for 48 h and stained with Rhodamine 123 for 15 min at 37°C. Mitochondrial membrane potential was detected by flow cytometry (Cytomics FC 500; Beckman Coulter Inc., Miami, FL).

Western-blot analysis

The proteins expression regulating by ATR II were analyzed by western blot and followed the protocol as described previously [16], with a small modification. In brief, MCF-7 and MDA-MB 231 cells were treated with (0, 35 and 70) μM of ATR II for 48 h. Then cells were harvested and lysed with RIPA buffer. Afterward, the insoluble protein lysate was removed by centrifugation at 13500 rpm for 15 min at 4°C The protein concentrations were determined using NanoDrop 1000 (Thermo Scientific). An equal amount of protein was loaded on SDS-PAGE electrophoresis gel (10 or 12% according to the protein size). Further, the gel was transferred on polyvinylidene fluoride membrane (PVDF). After necessary transfer time, the membrane was blocked in 5% (w/v) non-fat milk incubated for 2h. The membrane were therefore incubated with appropriate primary antibodies Caspase-3, Caspase-8, Bcl-2, Bax, NF-κB, Cox-2, IKK (1:1000 Cell Signaling Technology, Danvers, MA, USA) and Cyclin A, CDK1, p21, p53,ER-α, ER-β(1:2000

Santa Cruz Biotechnology Dallas, TX, USA) at 4°C for overnight and washed three times with washing with a Tris-buffered saline-Tween solution (TBST). Finally, The blots were incubated with appropriate secondary antibodies anti-rabbit or anti-mouse horseradish peroxidase-conjugated(1:1000 Santa Cruz Biotechnology Dallas, TX, USA) for one hour at room temperature, then wash with TBST for 30 minutes and signals were detected using ECL plus chemiluminescence's kit on X-ray film (Millipore Corporation, Billerica, USA).

Real-time qPCR analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription was then carried out, as described previously [17]. Briefly, the first-strand cDNA was reverse-transcribed from 1 μg total RNA using the Super-Script First-Strand cDNA System (Invitrogen, Carlsbad, CA, USA) and amplified by Platinum SYBR Green qPCR Super Mix-UDG (Invitrogen). A master mix was prepared for each PCR reaction, which included Platinum SYBR Green qPCR Super Mix-UDG, forward primer, reverse primer and 10 ng of template cDNA. PCR conditions were 10 min at 95°C, followed by 50 cycles at 95°C for 30 s and 60°C for 1 min and 72°C for 30 s. The forward and reverse primer sequences for ERα were 5'-AATTCAGATAATCGACGCCAG-3'(Forward), and 5'- GTGTTTCAACATTCTCCCTCCTC-3'(Reverse), the primer sequences for ERβ were 5'- TAGTGGTCCATCGCCAGTTAT-3'(Forward) and 5'-GGGAGCCAACACTTCACCAT-3'(Reverse). Relative gene expression was obtained after normalization with β-actin were 5'-CACGATGGAGGGGCCGGACTCATC-3(Forward) and 5'-TAAAGACCTCTATGCCAACACAGT-3'(Reverse), the primer sequences for and determination of the difference in threshold cycle (Ct) between treated and untreated cells using the 2-ΔΔCq method [18].

Transient transfection and luciferase report

Luciferase assays were performed as we reported previously [19], with a small modification. Briefly, transient transfections were performed using transfection reagent (Mirus Bio LLC) according to the manufacturer's protocol. Cells were seeded into 48-well plates for 16 h and transfected with NF-κB reporter (SA Biosciences) 100 ng in the presence of Renilla Luciferase control pREP7 vector 25 ng and then treated in the presence or absence of ATR II(0, 35, and 70μM) for 24h. NF-κB mediated gene expression was measured with ONE-Glo luciferase assay kit (Promega, Southampton, UK) according to the manufacturer's instruction using a Polarstar Optima Plate reader.

Statistical analysis

All statistical analyses were performed using Origin Lab software version 8.0 (Origin Lab, Northampton, MA, USA), and statistically significant differences between groups were determined by one-way ANOVA with Bonferroni post-hoc test. $P<0.05$ was considered statistically significant.

Results

Cytotoxicity of ATR II

We first examined the effect of ATR II on the viability of breast, prostate normal, and cancer cells line through the use of the MTT assay. The cells were treated with 0, 6, 12.5, 25, 50, 100, and 200 μM concentrations of ATR II. As shown in Figure 1, ATR II was able to considerably inhibit the proliferation of MCF-7, MDA-MB 231, LNCaP, and DU145 cells, with consequently their half minimum concentration IC50 of, 70, 68 and 100, 98 μM. It can be clarified from these results that ATR stimulated the maximum cytotoxicity against human breast cancer cells with lower drug concentration. Whereas ATR II does not have a significant effect on prostate and breast normal cells line.

ATR II induces apoptosis in MCF-7 and MDA-MB 231 cells

Next to verify wheatear the cytotoxicity induction by ATR II, was associated by apoptosis, we performed flow cytometric of annexin V-FITC and PI double-staining to quantitatively asses the early apoptosis (Q4-2), late apoptosis (Q2-2) and necrosis (Q1-2). As shown in Figure 2A, the (Q4-2) values increased with the rise of the ATR II concentration, respectively, in both MCF-7 and MDA-MB 231 cells.

To further comprehend the molecular mechanism by which ATR II-mediated apoptosis in MCF-7 and MDA-MB 231 cells, we simulated the molecular binding of ATR II to death receptors or DR4 by AUTO-DOCK calculations. We found that ATR II was able to dock to DR4 protein structures with the lowest binding energy of -5.5Kcal/mol (Figure 2B). We might, therefore, suppose that the apoptosis induces by ATR II in breast cancer through extrinsic mitochondrial pathways. Furthermore, western blot was thus conducted to evaluated caspases activity. As illustrated in Figure 2C, ATR II can stimulate the activation of caspase-8, which would lead to activate caspase-3 consequently. These findings verify our hypothesis that ATR II presents the potential to induce apoptosis by through extrinsic mitochondrial pathways.

ATR II promotes MCF-7 and MDA-MB 231 cells apoptosis through induction of ROS and collapse of mitochondrial membrane potential

The reactive oxygen species plays a dual role in cancer cells line [20]. In non-cancerous cells, the rate of ROS production remains low as compared to the cancer cells in which stimuli as such inflammation leads to an overexpression of ROS in cells that conduct the death of cells by apoptosis or autophagy [21]. Herein to underline whether the apoptosis induced by ATR II was associated by induction of ROS in MCF-7 and MDA-MB 231 cells. Cells were treated with 0, 35, and 70 μM of ATR II for 48h followed by the treatment with the fluorescent probes DCF-DA for the detection and assessment of H2O2 by flow cytometry in the presence and absence of N-acetyl-cysteine (NAC). The outcomes showed that the generation of intracellular H2O2 improved considerably with the treatment of ATR II (0, 35, and 70) μM. In the meantime, NAC treatment results in to regulate the ROS expression induced as a result of treatment with different concentration of ATR II (35 and 70 μM) correspondingly, which therefore depicted the role of ATR II in the generation of H2O2 as illustrated in Figure 3A.

The ROS was reported to promote and de-regulate mitochondrial membrane depolarization [22]. Targeting mitochondria is a novel

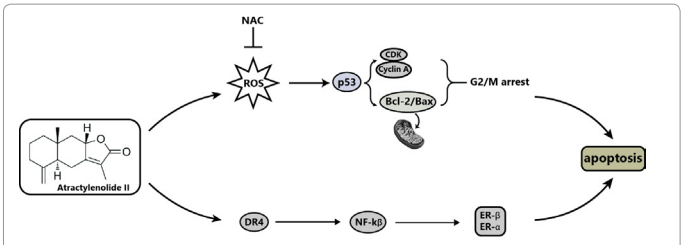

Figure 1: Cytotoxicity of ATR II prostate and breast normal and cancer cells line: Prostate and Breast normal/cancer cells line were treated with 0, 6, 12.5, 25, 50, 100, and 200 μM of ATR II, Proliferation was assessed after 72 h, as described in Materials and Methods. Each bar represents the mean ± standard deviation of three experiments*p<0.05; and **p<0.01 compared with the control.

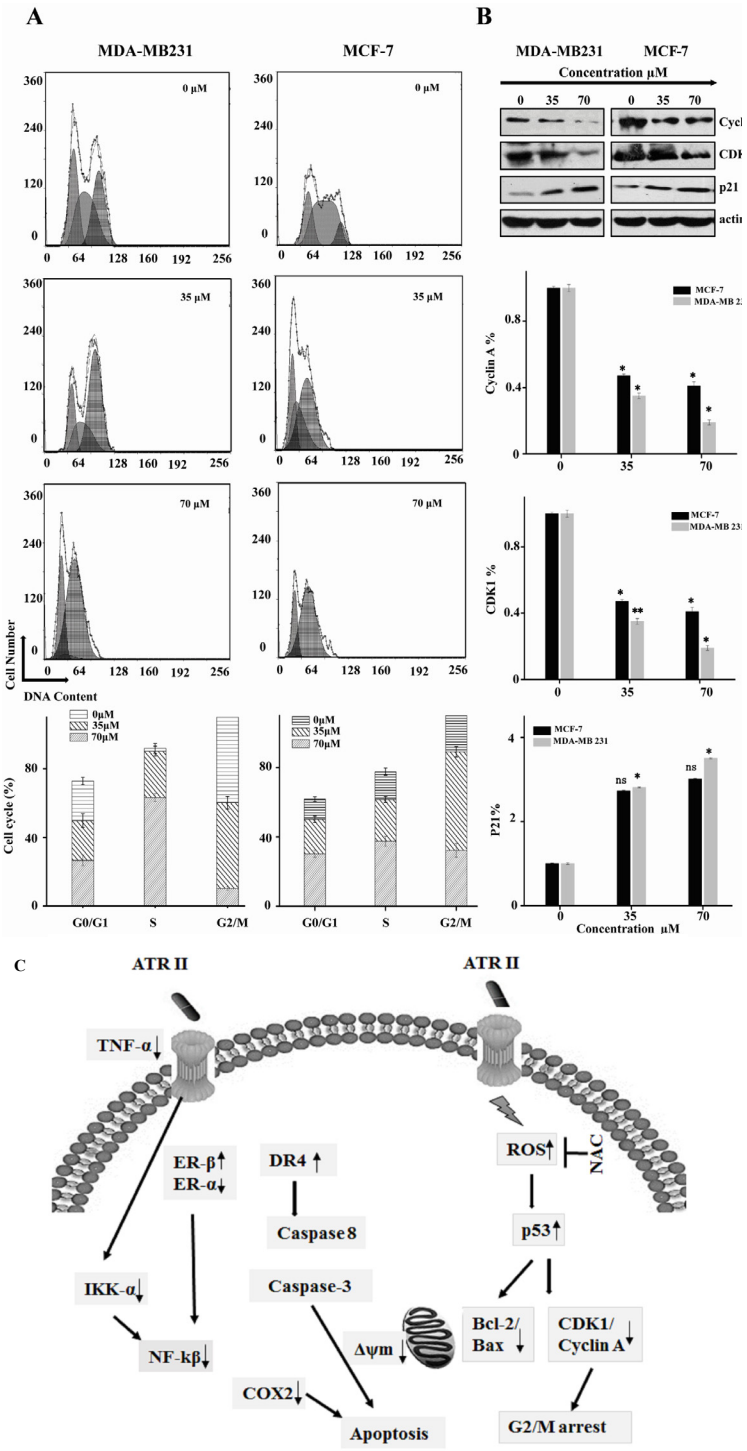

Figure 2: Mechanism of induction of apoptosis by ATR II on MCF-7 and MDA-MB 231 cells. (A) MCF-7 and MDA-MB 231 cells were threaded, with or without ATR II (0,35 and 70 µM) then, stained with annexin V /PI ,The lower right quadrant showed V+/ PI- annexin, the upper right quadrant showed V+/ PI+ annexin cells. Each bar represented the percentage (mean ± SD of triplicate determinations) of both annexin V+/ PI- and V+/ PI+ annexin cells(*p<0.05; and **p<0.01 compared with the control). (B) DR4 binding site as well as binding energy was evaluated through AUTO-DOCK calculations. (C) The cellular proteins were extracted to detect the levels of DR4, Caspase-8, and Caspase-3 as well as beta actin (control) by Western blotting.

approach for cancer therapy because of its involvement in stimulating apoptosis. In this regard, Mitochondrial mediate apoptosis can be highly regulated by counterbalancing between the expression of pro-apoptotic as well as anti-apoptotic proteins in the Bcl-2 proteins. A loss in the mitochondrial transmembrane potential (Δψm) has been observed as a result of the disruption of these counterbalances [23].

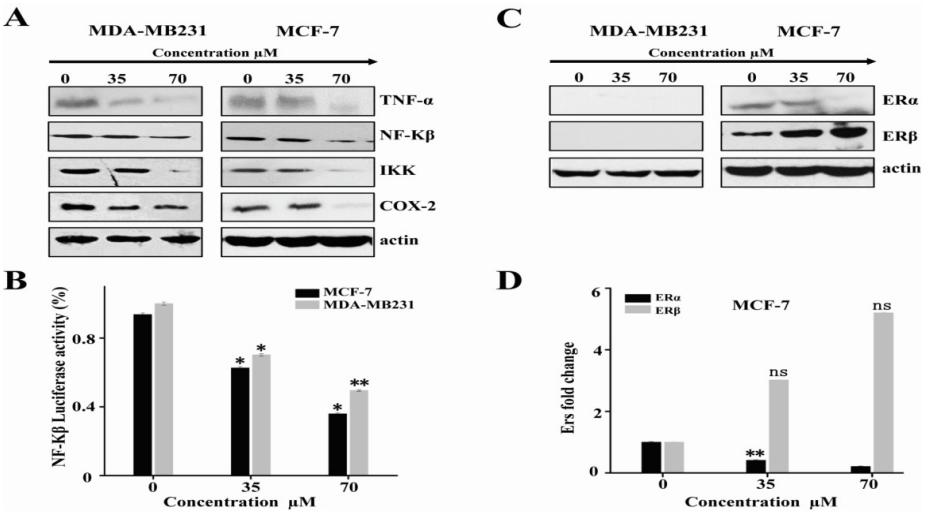

Figure 3: ATR II induces apoptosis in MCF-7 and MDA-MB231 through overexpress oxidative stress (ROS), while collapse Mitochondrial Membrane Potential, (A) MCF-7 and MDA-MB 231 cells were pre-incubated in the absence or presence of NAC 3mM for 30 minutes then treated with ATR II (0, 35 and 70 μM) incubated 48h, then stained with DCFH-DA and analyzed by flow cytometric The data shown are representative of three independent experiments with the similar results. *p<0.05; and **p<0.01 compared with the control. (B) MCF-7 and MDA-MB 231 cells were threaded with ATR II (0, 35, and 70 μM) then stained by Rhodamine 123. The data shown are representative of three independent experiments with the similar results. *p<0.05; and **p<0.01 compared with the control. (C) Relative protein expression controlling the mitochondrial membrane potential: MCF-7 and MDA-MB 231: Cells were treated with or without ATR II (0, 35 and 70 μM) then incubated 48 h. The cellular proteins were extracted to detect the levels Bax and Bcl-2 as well as beta actin (control) protein.

To validate whether the apoptosis induced by ATR II was associated by on mitochondrial depolarization, the investigation was conducted on MCF-7 and MDA-MB 231 cells through the employment of Rhodamine 123 staining flow cytometry. ATR II induces mitochondrial membrane potential collapse in dose depend-manner. However, the pre-treatment of MCF-7 and MDA-MB 231 cells with NAC completely reverse the action of ATR II (Figure 3B), we might, therefore, conclude that the ROS induce by ATR II was mainly associated with the mitochondrial membrane potential collapse.

As shown in Figure 3C, treatment of MCF-7 and MDA-MB 231 cells by ATR II would induce the loss of mitochondrial membrane potential. This fall of mitochondrial membrane potential by ATR II was particularly caused by the capability of ATR II to activate tumor suppressor p53; proteins were shown in Figure 3B. These findings showed the role of ATR II in stimulating the apoptosis through the mitochondrial pathways. ATR II induces apoptosis through phosphorylation of NF-KB signaling pathways.

The transcription factor, NF-κB, is a regulator of cells proliferation as well as cells death. Once it is activated, it will be translocated into the nucleus to induce activation of IκB and inhibition of IκB [24]. We assessed NF-κB signaling pathways through the use of ART II treatment completely, whereas the phosphorylation of IκB and expression of NF-κB transcription would be targeted by TNF-α receptors. Results showed a reduction in the expression of IKK-α, COX-2, and NF-κB as well as TNF-α as illustrated in Figure 4A, these finding shown the role of ATR II can be described as an anti-inflammatory agent as well as an inducer of apoptosis in MCF-7 and MDA-MB 231 cells by inhibiting inflammatory factors

To further make evidence the role of ATR II on transcription factor NF-KB, we conduct transient Transfection and Luciferase tests in MCF-7 and MDA-MB 231 cells, and we find out that ATR II prominently prohibits NF-KB transcription in MCF-7 and MDA-MB 231 cells (Figure 4B).

ATR II induce ER-β Up-regulation and ERα Down-regulation in MCF-7 cells line

Different from ERα, ER-β is rather uncertain in the resistance of breast cancer. In consideration of the discrepancy, this study was planned to analyze the expression of ERα as well as the functions of ER-β on MCF-7 (ER-positive) and MDA-MB231 (ER-negative) via western blot. The research results, as shown in Figure 4C, indicate that ER-β expression was prominently up-regulated and ERα expression was down-regulated together with the growing intensity of ATR II in MCF-cells. In contrast, there was no ER expression in MDA-231 cells (Figure 4C). RT-PCR further testifies the findings mentioned above (Figure 4D). Consequently, ATR II could indeed prohibit ER-positive breast cancer cells through increasing ERβ expression and regulating ERβ pathway. It can be concluded that ATR II could inhibit the breast cancer cells proliferation independent of their ER expression.

ATR II induces G2/M phase cell cycle arrest in MCF-7 and MDA-MB 231 cells

Similar to apoptosis, cell death might occur by cell cycle arrest. To evaluate whether the cytotoxicity of ATR II might affect their cell cycle arrest, flow cytometry is conducted to establish the specification perturbation in the distribution of MCF-7 and MDA-MB 231 cells cycle arrest stimulated by ATR II (Figure 5A). It is clear that in MCF-7 and MDA-MB 231 cells, the G2/M phases are growing. Conversely, G0/G1 and S phases declined radically facing the increase of the ATR II concentration. To make sense of the anticancer effect of ATR II, we performed a western plot to examine its roles in regulating the MCF-7 and MDA-MB 231 cycle proteins about the G2/M arrest.

It is well known that the human MDM2 is a ubiquitin ligase controlling the p53. The overexpression of MDM2 can result in the inactivation of the p53 pathway and inhibits p53-mediated cell cycle arrest [25,26]. Moreover, a recent study [27], indicated that MCF-7

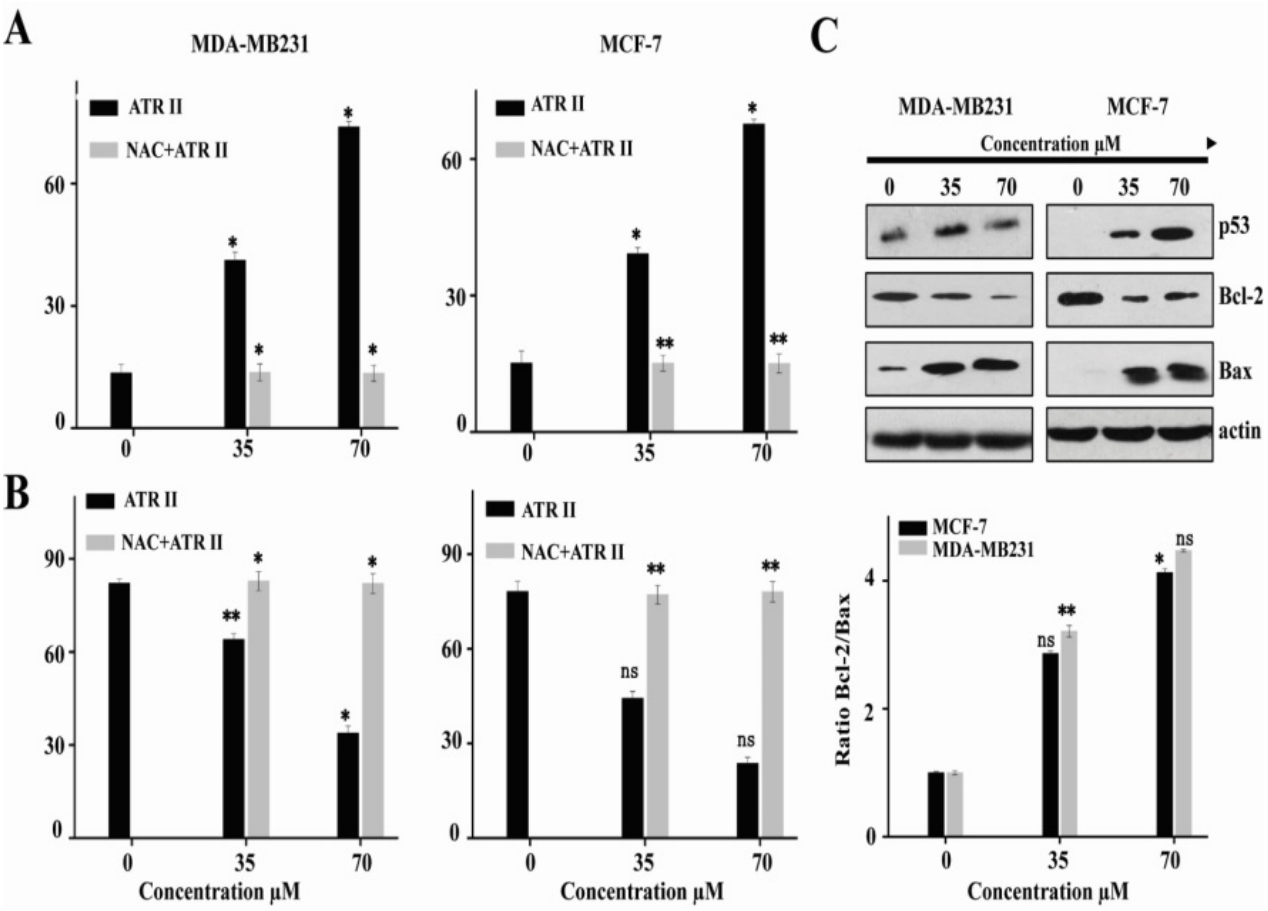

Figure 4: ATR II induces MCF-7 and MDA-MB 231 cells apoptosis through the regulation of NF-κB/ER pathways. (A) Cells were treated with or without ATR II (0, 35 and 70 μM) for 48 h. The cellular proteins were extracted to detect the levels of NF-κB, IKK-α and COX-2 as well as beta actin (control) by Western blotting. (B) MCF-7 and MDA-MB 231 cells were transiently transfected NF-κB luciferase reporter plasmid. Cells were treated with ATR II (0, 35 and 70 μM) respectively for 24 h and then harvested for Luciferase assays. (C) MCF-7cells were treated with or without ATR II (0, 35 and 70 μM) for 48 h. The cellular proteins were extracted to detect the levels of ERα and ERβ as well as beta actin (control) by Western blotting. (D) Real-time RT-PCR was used to quantify the ERα and ERβ mRNA levels in MCF-7 cells following 48 h treatment of ATR II (0, 35 and 70 μM). Fold decrease of gene expression was calculated by dividing the normalized gene expression activity by that of the untreated control. The data shown are representative of three independent experiments with the similar results. *p<0.05; and **p<0.01 compared with the control.

and MBA-MD 231cells, cell cycle arrest in the G2/M phase is regulated by the complex CDK1/Cyclin A and its downstream target p21. Therefore, MDM2, p53, p21, Cyclin A, and Cdk1 protein expression levels were examined by the western blot analysis. ATR II involved in down-regulation of Cyclin A, CDK1, while p21 and p53 expression were accordingly up-regulated (Figure 5B). Put together; these findings confirmed the flow cytometric analysis, which thus implies the G2/M phase development arrest by the down-regulation of complex Cyclin A/Cdk1. Moreover, go parallel with our apoptosis results that ATR II-induced same phase cell cycle arrest independent of their ER expression.

Discussion and Conclusion

In this study, ATR II was used to observe the signal transduction of NF-κB and the inhibitory effect of estrogen receptor (ER) in breast cancer cells. The rationale for targeting NF-κB ER pathway as a therapeutic target on account of the decreased level of activated NF-κB observed in many human breast tumors.

The interactive correlation between estrogen and ER-α is found to help in Breast cancer therapy [28]. On the contrary, although in vivo studies insist that ER-β could prohibit cell proliferation and cell cycle arrest based on its cancer suppression attributes, the relationship between estrogen and ER-β remains to be uncertain as usual. Therefore, this study predicts that ER-β expression in breast cancer cells according to the ATR II-induced anti-proliferation activity. For ER-positive cells (MCF-7), it is feasible to add ATR II to improve ERβ concentration. Alternatively, otherwise, despite that is high, ERβ expression in ER-negative MDA-231 cells will never be intensified. At this moment, it can be concluded that ATR II could trigger ERβ expression and prohibit cell proliferation. It undoubtedly affirms the relationship between phytoestrogen and ERβ in breast cancer cells.

Considerably, previous studies showed that ATR II retains antioxidant properties [29,30]. In order to elucidate whether ATR II can stimulate the apoptosis of human breast cancer cells, Flow cytometry of ROS was performed through the generation of hydrogen peroxide regulation in MCF-7 and MDA-MB 231 cells. Our study showed that

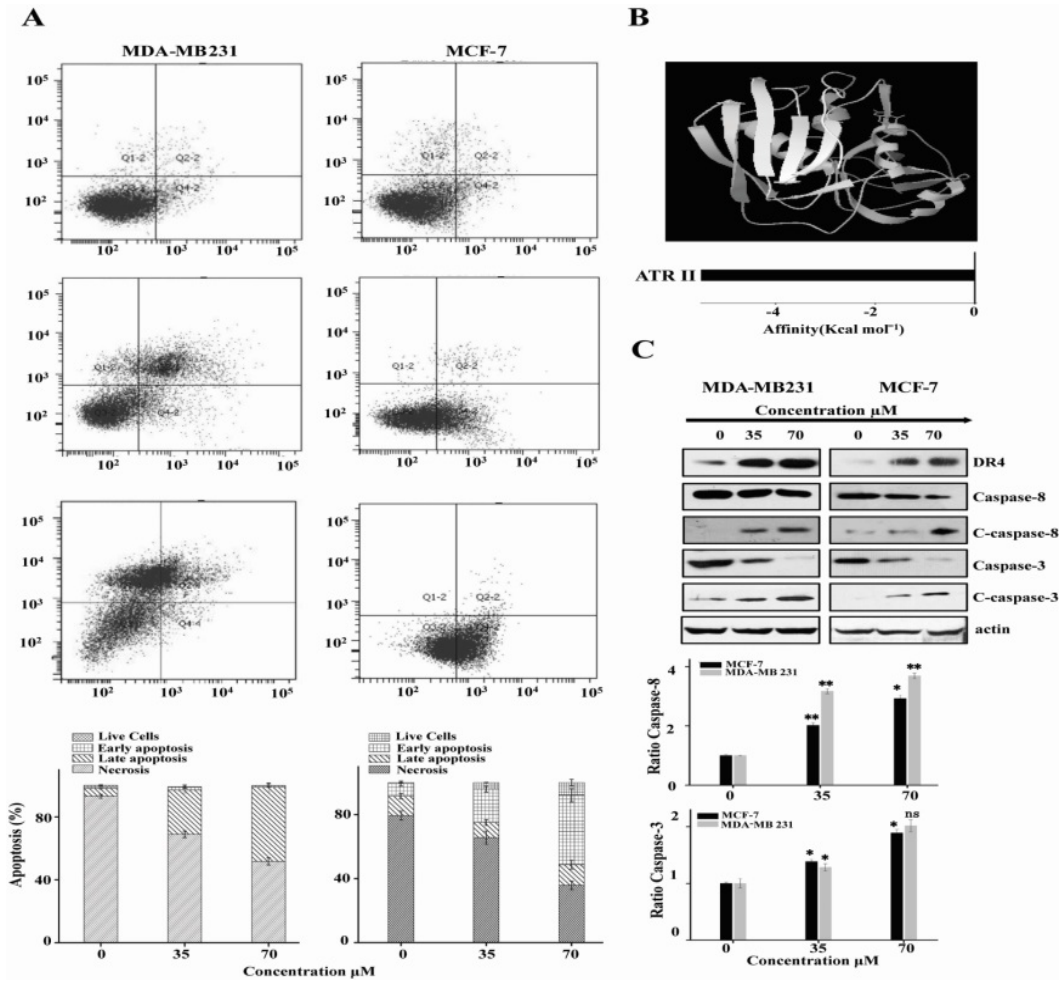

Figure 5: ATR II induces MCF-7 and MDA-MB 231 cells cycle arrest at G2/M phase arrest. (A) MCF-7 and MDA-MB 231 cells were treated with or without ATR II (0, 35 and 70 μM) for 48 h, and then they were stained with PI for flow cytometric analysis. Histograms show number of cells/channel (y-axis) vs. DNA content (x-axis). The values indicate the percentage of cells in the indicated phases of the cell cycle. The data shown are representative of three independent experiments with the similar results. *p<0.05; and **p<0.01 compared with the control. (B) MCF-7 and MDA-MB 231 Cells were treated with ATR II (0, 35 and 70 μM) for 48h. The cellular proteins were extracted to detect the levels of p21, Cyclin A, CDK1, as well as beta actin (control) by Western blotting.

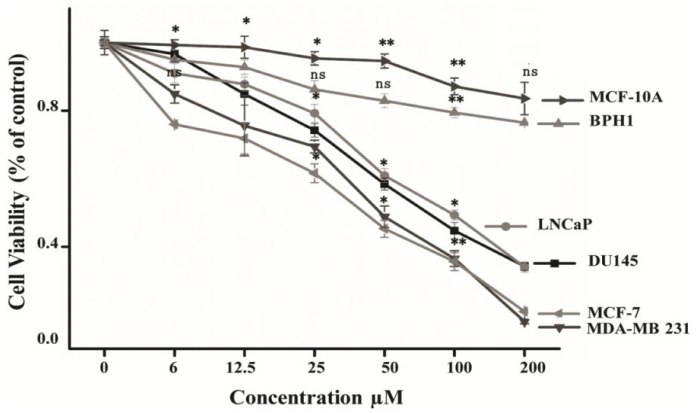

Figure 6: Hypothetical mechanism of cytotoxicity meditated by ATR II on Breast cancer cells: ATR induces apoptosis in by activated death receptor DR4, that result in the apoptosis of breast cancer through extrinsic pathways, Moreover, ATR II also induce oxidative stress which might induces mitochondrial membrane collapse, and promote in MCF-7 and MDA-MB 231 cells apoptosis and cell cycles arrest at G2/M phase. By the other hand ATR also might induces Breast cancer cell apoptosis, by inhibiting inflammatory factors such TNF-α, Cox2 as well as NF-KB that result in the inhibition of ER-α with overexpression of ER-β, consequently drives the cells to their death by apoptosis.

ART II could produce H_2O_2, which can be reversed in the presence of NAC. However, studies have already proven that the generation of ROS is interrelated to the improved cells apoptosis. These results affirmed the antioxidant property of ATR II against human breast cancer MCF-7 as well as MDA-MB 231 cells.

Nuclear factor NF-κB plays an essential role in the development of the sensitivity of breast cancer cells against chemotherapeutics and radiotherapy. NF-κB also plays a significant role in the inflammatory action of mediated cells death by apoptosis. For instance, the human breast cancer cells MCF-7 cells could not be growing in the case of reserving the NF-κB pathway [31,32]. NF-κB remains in the cytoplasm in an inactive form through the regulation of IKK. The phosphorylation of IKK by TNF-α induces ubiquitination and degradation of IκB that result in the translocation of NF-κB in the nucleus and subsequently initiated cell death [33]. In our studies, we observed that ATR II inhibited the inflammatory factors, COX-2, TNF-α, and NF-κB. We may thus propose that the ATR II treatment may result in inhibition of NF-κB in MCF-7 as well as MDA-MB 231 cells, independent of their ER expression, might be due to the phosphorylation of COX-2.

Furthermore, the cytotoxicity of ATR II is significantly expressed through chemical barrier proliferation. Cell proliferation is automatically regulated by the cell cycle, which is regulated by numerous complex cyclin and CDK proteins [34,35]. Among various cell cycle checkpoint proteins, a complex between CDK1 and cyclin A or CDK1 and cyclin B participates in the process of G2/M transition. The activity of the CDK/cyclin complex is negatively regulated to induce cell cycle arrest by the p53-dependent or p53-independent pathway. In the case of the p53-dependent pathway, the DNA-induced CDK inhibitor p21 is increased in DNA-damaged cells for cell cycle arrest, and then non-repaired cells may be eliminated by apoptosis via inducing Bax and repressing Bcl-2 activity. Our data show that ATR II-induced MCF-7 and MDA-MB 231 cell accumulation in the G2/M phase. These results suggest that ATR II-mediated cell cycle arrest is followed by apoptosis. Western blot analysis showed that ATR II increased the expression of p53 and p21, a CDK inhibitor, leading to the G2/M arrest. These results suggest that ATR II-mediated G2/M arrest occurs by inhibition of CDK1/cyclin A complex via p53-dependent p21 induction.

In a word (Figure 6), this manuscript predominantly explains the mitochondrial anti-proliferation role and pro-apoptosis role of ATR II in breast cancer cells. ATR II is found for the first time to trigger oxidative stress which might induce mitochondrial membrane collapse, and promote in MCF-7 and MDA-MB 231 cells apoptosis, by the other hand ATR II drive activation of DR4 and promote extrinsic apoptosis signaling. Furthermore, ATR II-mediated mainly cells breast cancer cells apoptosis by an inhibited inflammatory factor that lead to the regulation of the estrogen receptor. At the same time, ATR II causes Breast cancer cells proliferation arrest at G2/M through inhibition of the complex CDK1/Cyclin A and overexpression of the complex p53/p21.

Conflict of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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