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ORIGINAL ARTICLE

Effects of Fulvestrant, an Estrogen Receptor Antagonist, on MMQ Cells and Its Mechanism

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Abstract

OBJECTIVES: Unlike the successful endocrine therapy of breast cancers and other estrogen-dependent diseases, little is known about the effect of anti-estrogen treatment on pituitary tumors. Our objectives were to study the effect of fulvestrant, a new type anti-estrogen devoid of any agonistic activities, on prolactinoma cell line MMQ in vitro and its possible mechanisms. **DESIGN**: In the experiment, the prolactin concentration, proliferation and apoptosis of the MMQ cell were measured to investigate the anti-tumor effect of the fulvestrant. The expression of estrogen receptor (ESR) mRNA and protein and MAPK pathway-related proteins ERK1 and 2, JNK, and p38 were measured to investigate the possible mechanisms. **RESULTS**: Fulvestrant significantly inhibited prolactin secretion (up to 85.5%), decreased proliferation (IC50 = 32.4 nmol/l), and promoted apoptosis of the MMQ cells. **CONCLUSIONS**: The suppression was possibly mediated by inhibition of ESR mRNA expression, down-regulation of ESR expression and activation of MAPK pathway-related proteins. Thus, fulvestrant has suppressive effects on prolactinoma cells and its anti-tumor mechanism appears to be related to the inhibition of ESR and the MAPK pathway.

INTRODUCTION

Studies have shown that the sex hormone estradiol (E2) regulates the secretion and synthesis of all the pituitary hormones and selectively stimulates the proliferation of normal and transformed prolactin cells and gonadotropin cells (Chaidarum *et al.* 1994; Lieberman *et al.* 1980; Shupnik *et al.* 1989). In Fisher 344 rats, long-term estradiol can induce the occurrence of pituitary tumors (Lloyd, 1983). Besides these, many clinical evidences suggest that estrogen plays an important role in the occurrence and development of pituitary tumors. For example, 1) the gender difference in pituitary tumor incidence (female:male = 3:1); 2) high E2 levels during pregnancy account for symptomatic pituitary tumor enlargement in up to 30% of women with macroadenomas (Holmgren *et al.* 1986); 3) the incidence of prolactinoma is also elevated in transgender people taking estrogen (Gooren *et al.* 1988; Serri *et al.* 1996). The effects of E2 are mediated by the nuclear estrogen receptor (ESR), which has two subtypes, ESR1 and ESR2. And all prolactinomas express ESR1 in high level which exert main

Abbreviations:
E2 – Estradiol
ERE – Estrogen Response Elements
ERK – Extracellular-Regulated Kinases
ESR – Estrogen Receptor
Junk – Jun Kinase
MAPK – Mitogen Activated Protein Kinase
PRL – Prolactin

biological function in the tumors. ESR activation promotes transcription of target genes such as the prolactin (PRL) gene and anti-apoptotic (*Bcl2*) gene. In addition, ESR can directly interact with peptide growth factors without E2 to activate the transcription of ESR and ESR regulating genes (Ignar-Trowbridge *et al.* 1996; Newton *et al.* 1994; Weigel and Zhang, 1998). Several serine/threonine protein kinases of the mitogen-activated protein kinase (MAPK) pathway may be involved in the ESR function, including the extracellular-regulated kinases (ERKs), p38 and Jun kinase (Junk) (Aronica *et al.* 1994; English *et al.* 1999; Filardo *et al.* 2000; Kelly *et al.* 1999; Nadal *et al.* 1998; Razandi *et al.* 2003; Nataliya *et al.* 2004). Almost all the interactions between growth factors and ESR rely on MAPK (Bunone *et al.* 1996; Kato *et al.* 1995), and the rapid non-genome effects of E2 are also mediated by the MAPK pathway (Collins and Webb, 1999). Therefore, ESR may mediate proliferation and promote PRL secretion through many intracellular signaling pathways. Theoretically, inactivating ESR can reduce excessive secretion of PRL and control the growth of prolactinomas.

Fulvestrant is a new ESR antagonist without any agonist activities, which can combine with, block, and degrade ESR and has been successfully used to treat patients with tamoxifen-insensitive breast cancer . In this study, different concentrations of fulvestrant were added to cultured MMQ cells (a rat prolactinoma cell line). The effects of fulvestrant on MMQ were studied using the MTS assay and cell growth curve analysis to measure proliferation and flow cytometry (FCM) analysis to measure apoptosis. The molecular mechanism of the anti-tumor effect of fulvestrant was studied through its effects on ESR and the MAPK pathway.

MATERIALS AND METHODS

Materials

The MMQ cell line was purchased from the Cell Center of the School of Basic Medicine, Peking Union Medical College. Trypan blue, estradiol, fulvestrant, and Tween-80 were purchased from Sigma Company (St. Louis, MO, USA). Fetal bovine serum, DMEM, and trypsin were purchased from Gibco (Grand Island, NY, USA). CellTiter 96® Aqueous One Solution Cell Proliferation Assay and Horseradish peroxidase labeled second antibody was purchased from Promega Corporation (Madison, WI, USA). PRL RIA kit was purchased from Adlitteram Diagnostic Laboratories (San Diego, CA, USA), propidium iodide (PI) and annexin V were purchased from BD Pharmingen (San Diego, CA, USA), Trizol was purchased from Invitrogen (Carlsbad, CA, USA), and ESR and housekeeping gene βACTIN primer were designed and synthesized by the TAKARA Biotechnology (Dalian, China). The reverse transcription kit, high-capacity cDNA archive kit, and Power SYBR Green PCR Master Mix kit were purchased from ABI (Foster City, CA, USA). The ABI 7500 Real-Time PCR System was used for quantitative PCR. ESR monoclonal antibody, ERK1 and 2, p-ERK1 and 2; JNK, p-JNK; p38, p-p38 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

MMQ cells were cultured in F12 culture medium containing 2.5% fetal bovine serum (FBS), 7.5% horse serum, penicillin (100 U/mL), and streptomycin (100 μg/mL), and incubated at 37°C in a humidified, 5% CO_2 incubator for 48 h. Then the medium was replaced with F12 culture medium with 10% charcoal stripped serum and Pen/Strep. After 24 h, cells were treated with different concentration of fulvestrant in aboved medium.

Measurement of MMQ cell survival rate using the MTS method

Log-phase MMQ cells were collected, counted in a hemocytometer, found to be > 99 % viable by trypan blue exclusion, and adjusted to 1×10^4/mL with complete culture medium. Cells (1×10^3/well) were plated into 96-well plates and cultured for 24 h. Then 50 μl fulvestrant solution (final concentration [in nMol/L]: 0, 0.008, 0.04, 0.2, 1, 5, 25, 125, and 625) or 50 μL of complete culture medium with the corresponding solvent as control was added. After 72 h of incubation, addition of 40 μL of MTS solution to each well, and incubation (4 h). The absorbance at 490 nm of each well was measured using an ELISA reader. The following formula was used to calculate the inhibition rate of fulvestrant on MMQ cells. The 50% inhibitory concentration (IC_{50}) was determined by linear regression of the Log drug concentration *vs.* the growth inhibition rate, and interpolation of the x-value at y=50. (See formula 1)

Plotting of cell growth curve

Log-phase cells were inoculated into 6-well culture plates (1×10^4 cells/well). After 24 h, drug was added to the test wells, an equal volume of solvent was added to control wells, and the plates were incubated at 37°C in a humidified 5% CO_2 incubator. Every day after the

$$\text{Inhibition rate (\%)} = \frac{\text{OD value of the control group} - \text{OD value of the drug administration group}}{\text{OD value of the control group}} = \times 100\% \qquad \textit{(form. 1)}$$

drug administration, 3 wells were trypsinized and the viability and cell number in the resulting cell suspension were determined under a light microscope after staining with trypan blue.

Measurement of the PRL concentration using ELISA

Log-phase MMQ cells (> 99% viable; 1×10^4/mL) were plated in 96-well plates (1×10^3 cells /well) and cultured for 24 h. The addition 100 μL of each of a series of fulvestrant concentrations to test wells and 100 μL of complete culture medium with corresponding solvent to control wells was followed by culture for 72 h and centrifugation to collect the supernatant. Then following the direction of the PRL-ELISA Kit manual, the concentrations of PRL were estimated from a standard curve of known PRL concentrations.

Measurement of apoptosis using Annexin V and PI double-staining flow cytometry

After treatment with different concentrations of fulvestrant for 72 h, the cells were digested by trypsin to make a single-cell suspension, rinsed with PBS 3 times, resuspended in buffer, and adjusted to a density of 1×10^6 cells/mL. To 100 μL of cell suspension in each tube was added 15 μL of fluorescein-isothiocyanate-conjugated ANNEXIN-V and 10 μL of PI (20 μg/mL). The samples were stained at 4°C for 20 min, and flow cytometry was used to examine cell apoptosis. Each sample collected fluorescent signal of 10^4 cells.

Measurement of ESR mRNA expression using real-time PCR

After 72 h treatment, the cells were collected and total RNA was extracted using the Trizol method and reverse transcribed into cDNA. The reaction conditions were optimized (as described in the ABI SYBR-Green manual) for primer pairs of the target gene ESR1 (NM012689, forward: TGTTACGAAGTGGGCATGATGAA; reverse: GCCAAAGGTTGGCAGCTCTC) and housekeeping gene βACTIN (NM031144, forward: TGACAGGATGCAGAAGGAGA; reverse: TAGAGCCACCAATCCACACA). The treated samples were examined using real-time PCR after specificity of the primer was verified using melt peak analysis. The reaction system was 2 × Master Mix (12.5 μL), Primer F / R (0.5 μL), sample cDNA (1 μL), DEPC H_2O (11 μL) in a total final volume of 25 μL. The amplification conditions were 95 °C 15 s and 60 °C 1 min for 40 cycles. The relative expression of the ESR gene was calculated from the cycle threshold (Ct) value using the ddCt method for quantification.

Measurement of protein expression using Western blot analysis

From each group 3×10^6 cells were harvested 72 h after treatment, washed twice with ice-cold PBS, and lysed with cell lysis buffer. The protein concentration was assayed using the Bradford method. The samples were adjusted to the same protein concentration using cell lysis buffer and analyzed using SDS-polyacrylamide gel electrophoresis (PAGE). The proteins from the SDS-PAGE gel were blotted using the semi-dry technique onto a nitrocellulose membrane. Non-specific binding sites were blocked with BSA and incubated with the first and second antibody. After rinsing, the membrane was developed using the ultra-sensitive chemiluminescence protein dyeing Detection System (ECL Plus; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and subjected to gray-scale scanning and semiquantitative analysis using Image J software.

Data analysis

For MTS assays, data were calculated as % of control and expressed as a mean ± S.E.M. of multiple experiments, with each experiment including four determinations. ELISA test for PRL and cell growth curve plotting were performed in quadruplicate 3 times. All the other tests were performed in triplicate 3 times. Statistical analysis was performed using Student *t*-test and ANOVA and a p value ≤ 0.05 was considered as significant.

RESULTS

Inhibition of proliferation

The results of MTS assay after 72 h of fulvestrant treatment is shown in Figure 1. Fulvestrant concentrations greater than 1 nM were found to dose-dependently inhibit cell proliferation. At the highest concentration, fulvestrant inhibited proliferation by 81.6 ± 3.6% and treatment with 5 nMol/L produced statistically significant inhibition. When fulvestrant concentration ≥ 1nM, there were linear negative relation between the % of control OD and the Log drug concentration(r= –0.97, $p < 0.05$).The IC_{50} was 32.4 ± 7.8 nMol/L, 95% confidence interval was 15.1-85.4 nMol/L.

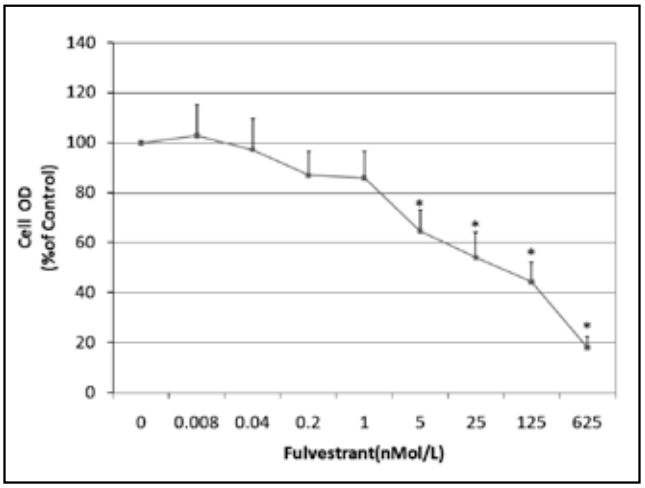

Figure 1. MMQ cell proliferation was measured using the MTS assay after 72 h of fulvestrant treatment at different concentrations.
* indicates statistical significance.

Table 1. Flow cytometry analysis of MMQ cell apoptosis after 72 h of fulvestrant treatment.

Fulvestrant (nMol/l)	Control	0.04	1	25	625
Early-stage apoptosis %	2.14±0.41	2.54±1.26	16.75±5.96*	30.54±6.64*	50.41±12.21*
Late-stage apoptosis %	0.92±0.29	1.54±0.34	6.34±2.03*	21.76±5.17*	33.8±6.45*

* Indicates statistical significance at the $p \leq 0.05$ level.

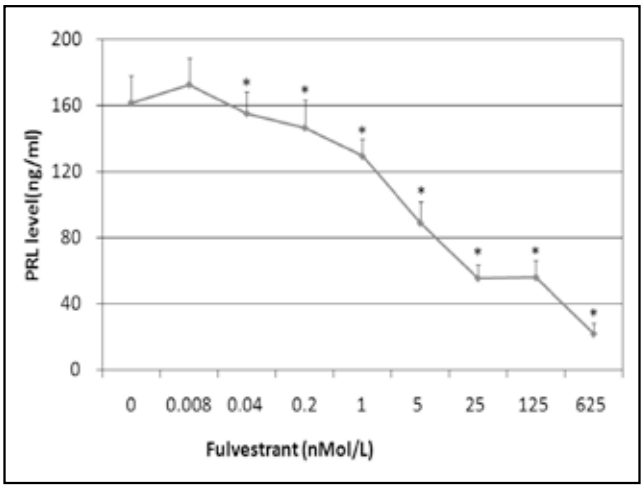

Figure 2. PRL secretion of MMQ cells (measured using ELISA) after 72 h of treatment with different concentrations of fulvestrant. * indicates statistical significance.

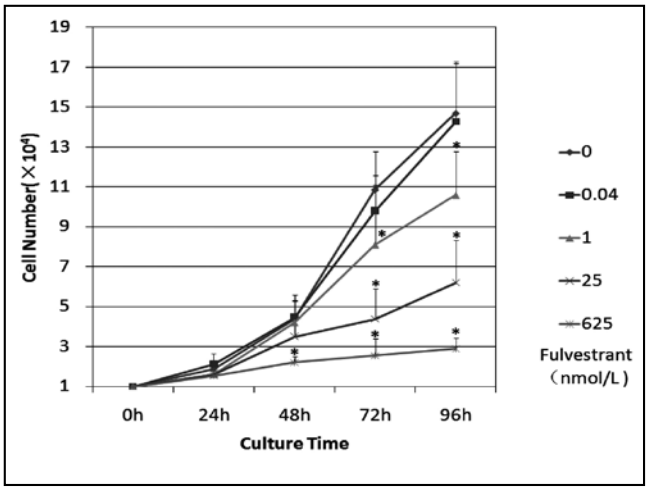

Figure 3. The growth of MMQ cells, as monitored by counting viable (trypan blue excluding) cells in a hemocytometer. * indicates statistical significance.

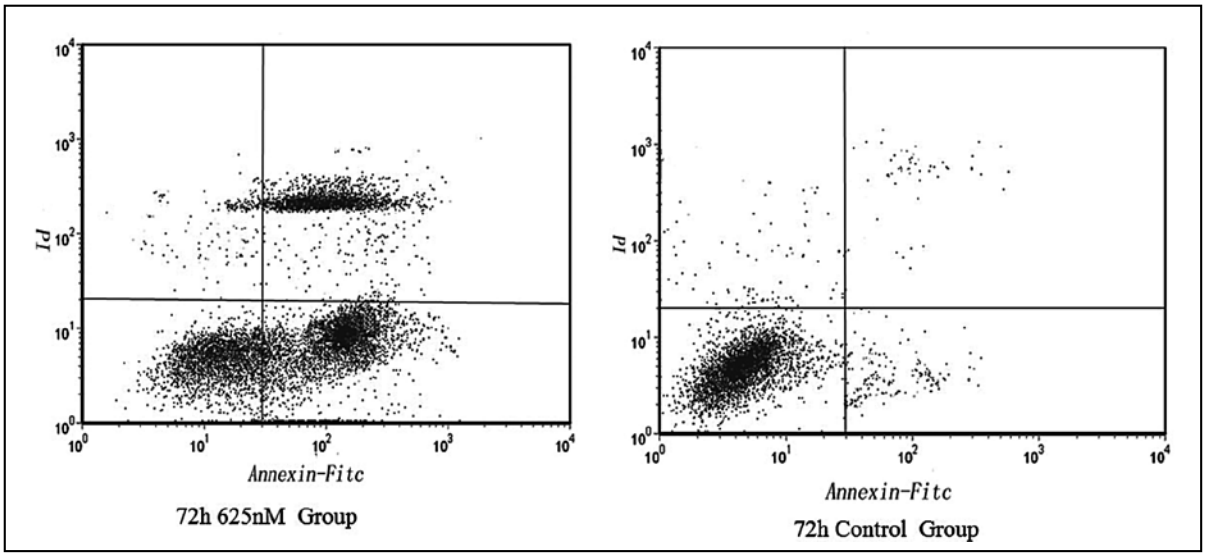

Figure 4. Flow cytometric analysis showing an apoptotic effect of fulvestrant after 72 h of treatment.

Inhibition of PRL secretion

PRL levels in the supernatant after 72 h of fulvestrant treatment are shown in Figure 2. Fulvestrant dose-dependently inhibited PRL secretion of MMQ cells, and maximal inhibition (85.2 ± 5.47%) was achieved at maximal dosage.

Growth suppression

The growth of MMQ cells at different drug concentrations and different time points is shown in Figure 3. Growth suppression was evident at fulvestrant concentrations greater than 1 nM.

Enhancement of apoptosis

The results of flow cytometry used to examine apoptosis after 72 h of treatment with 0, 0.04, 1, 25, and 625 nMol/L fulvestrant is shown in Table1 and Figure 4. Compared with the control (no fulvestrant), increasing doses resulted in increasing numbers of apoptotic cells.

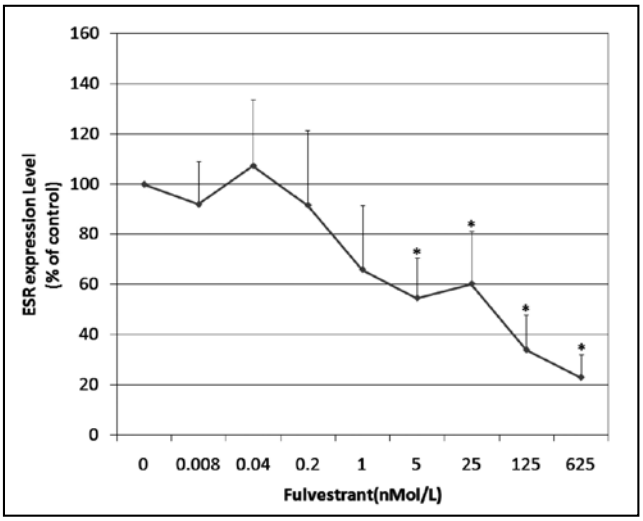

Figure 5. ESR mRNA expression using real-time PCR. The β-ACTIN gene was used as the internal standard, and the ddCT method was used for the relative quantification. * indicates statistical significance.

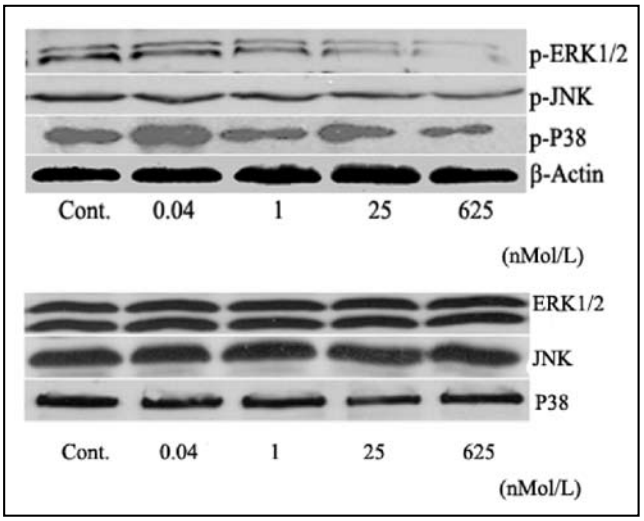

Figure 7. Western blots showing that fulvestrant inhibits the activation of MAPK pathway related proteins.

Figure 6. Western Blot showing that fulvestrant inhibits ESR protein expression.

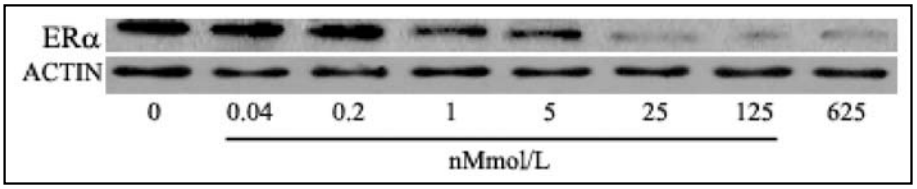

Table 2. Semi-Quantitative Measurement of ESR Proteins by gray scale value.

Fulvestrant (nMol/L)	Control	0.008	0.04	0.2	1	5	25	125	625
ESR/β-actin	1.12±0.12	1.13±0.02	0.94±0.03	0.87±0.04*	0.65±0.02*	0.53±0.03*	0.42±0.05*	0.24±0.02*	0.11±0.01*

* Indicates statistical significance at the $p \leq 0.05$ level.

Table 3. Semi-Quantitative Measurement of activated MAPK related proteins by gray scale value.

Protein \ Fulvestrant (nMol/L)	control	0.04	1	25	625
p-ERK1/2/β-actin	0.81±0.05	0.78±0.11	0.63±0.03*	0.42±0.02*	0.14±0.02*
p-JNK/β-actin	0.92±0.07	0.85±0.03	0.64±0.12*	0.43±0.05*	0.26±0.01*
p-P-38/β-actin	0.9±0.05	0.91±0.08	0.72±0.04*	0.59±0.04*	0.31±0.07*

* Indicates statistical significance at the $p \leq 0.05$ level.

When drug concentration ≥ 1 nM, the inhibition was significant.

The molecular mechanism of the effects of fulvestrant: ESR mRNA and protein expression

The mRNA expression of ESR in MMQ cells after 72 h of fulvestrant treatment is shown in Figure 5. Compared with the control (no fulvestrant), concentrations greater than 1 nM decreased ESR mRNA expression. Also the protein expression is decreased shown in Figure 6 and Table 2 when the drug concentration ≥ 0.2 nM.

The molecular mechanism of the effects of fulvestrant: Expression of MAPK pathway related proteins

The expression of MAPK pathway related proteins (ERK1/2, JNK and p38 protein) after 72 h of fulvestrant treatment is shown in Figures 7 and Table 3. Compared with the control (no fulvestrant), fulvestrant concentrations greater than 1 nM decreased the expressions of activated but not non-activated ERK1/ 2, JNK, and p38 protein.

DISCUSSION

Estrogen promotes the proliferation and reduces the apoptosis of a variety of target cells including uterine epithelial cells (Pollard *et al.* 1987), breast cells (Kyprianou *et al.* 1991), and neurons (Sawada *et al.* 2000; Singer *et al.* 1996). And there is ample information on the effects of anti-estrogens on breast cancers and other estrogen-dependent diseases, but we don't know much about that on pituitary tumors. In this study, we found that treatment with fulvestrant (1 nM or greater) for 72 h dose-dependently inhibits the proliferation of MMQ cells and induce cell apoptosis and necrosis which was confirmed by flow cytometry.

The anti-tumor effects of fulvestrant rely on the strong inhibition of ESR activity. Compared with tamoxifen, fulvestrant is a more powerful inhibitor of ESR activity. Frasor found that fulvestrant can block 95 percent of the expression of E2-up-regulated genes and block 91 percent of the expression of E2-down-regulated genes, but tamoxifen can only block 47% and 26%, respectively (Frasor *et al.* 2004). Fulvestrant inhibits the effects of estrogen in several ways: 1. It competes with estradiol for the ESR and its binding capacity is approximately 89% of that of estradiol (Wakeling & Bowler, 1987). Binding to the ESR causes a conformational change that inhibits dimerization of the ESR and energy-dependent nucleocytoplasmic shuttling, and thereby the nuclear localization of the ESR and the genome signaling pathway of the ESR (Fawell *et al.* 1990; Dauvois *et al.* 1993). Fulvestrant also can inhibit the activation of the ESR induced by other signaling factors such as EGFR and IGFR (Ignar-Trowbridge *et al.* 1993; Smith *et al.* 1993). 2. It can decrease ESR expression, promote ESR degradation, and decrease ESR half-life (Wijayaratne *et al.* 1999). (This study confirmed that fulvestrant decreases ESR expression at the mRNA level and protein level.) 3. It also affects the non-genomic signaling pathway of estrogen such as blocking the estradiol-induced activation of the MAPK pathway (Improta-Brears *et al.* 1999).

Abnormal activation of the MAPK pathway play an important role in the proliferation-promoting and anti-apoptotic effects of estrogen. Abnormal phosphorylation of the MAPK pathway is found in many human tumor tissues (Hoshino *et al.* 1999). The activation of the ERK-MAPK pathway increases the cell death threshold, and the activated p38 and JNK-MAPK signal cascade can increase anti-apoptotic activity within cells. Fulvestrant can promote the activation of adenylyl cyclase mediated by GPR-30 and increase the cAMP levels, thereby inhibiting ERK-1/2 and further inhibiting the EGFR-MAPK pathway (Filardo *et al.* 2000; Filardo *et al.* 2002). Otherwise, 2 estrogen response elements (EREs) in the promoter region of the *Bcl2* gene (Perillo *et al.* 2000) react with the ESR and directly up-regulate the expression of Bc12 (Dubal *et al.* 1999; El Etreby *et al.* 1998) and exerting anti-apoptosis effects. Fulvestrant can reverse these effects by antagonizing the ESR activity. This study confirmed that fulvestrant can inhibit the phosphorylation of three kinases in the MAPK pathway through down-regulating ESR expression, thereby inhibiting cell proliferation and promoting cell apoptosis. But it does not affect the expression of MAPK protein.

However, inhibition of PRL secretion did not uniformly parallel inhibition of proliferation. Even at 0.04 nM, fulvestrant can inhibit the PRL releasing, but only when the concentration was greater than 1 nM, it could suppress the cell proliferation and enhance the apoptosis. The lack of a tight correlation between PRL secretion and inhibition of cell proliferation suggests that the two effects have different mechanisms. Previous studies have concluded similarly but the mechanisms remain unclear (Chun *et al.* 1998). In short, besides directly enhance apoptosis of the tumor cells to inhibit the PRL secretion, fulvestrant antagonize the ESR to down-regulates the transcription and translation of PRL gene of the survival cells. The PRL promoter contains an estrogen response element with a weak transcription initiation site. That element, in concert with Pit-1, activates the transcription of *PRL* gene. Thus fulvestrant can directly antagonize the activity of the ESR to decrease the expression of PRL. In addition, Watters *et al.* found that the PRL secretion relies on the MAPK pathway (Watters *et al.* 2000), and the inhibition of the MAPK pathway by fulvestrant can also inhibit the secretion of PRL. In our study, we observed the ESR expression and the activated MAPK pathway related protein decreased caused by fulvestrant, which possibly play an important role in the inhibition of PRL releasing. But the definite mechanisms need further research.

In conclusion, fulvestrant can inhibit the proliferation of the MMQ cell, promote its apoptosis and necrosis, and inhibit its secretion of PRL. The functional mechanism of fulvestrant is related to the inhibition of the ESR and the suppression of the MAPK pathway.

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