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The Influence and Mechanism of Benzodihydropyran Benzene and Dihydro Pyran Derivatives xy2004 Effect on Breast Cancer Cell Proliferation and its Mechanism Ramification

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ABSTRACT

Objective To investigate the effects of xy2004 on the proliferation of MCF-7 cells and its mechanism. Methods Effects of xy2004 on the proliferation of MCF-7 cells was determined by MTT assay. Cell apoptosis was examined by flow cytometry. Apoptosis proteins were examined by western blot Competitive estrogen-receptor binding assay was used to investigate the affinity of xy2004 ER. Results xy2004 induced cell proliferation of MCF-7cells in low concentrations and inhibited cell proliferation in high concentrations. Antiestrogen tamoxifen eliminated the stimulation of proliferation of MCF-7 induced by xy2004. The relative binding affinity of xy9906 for ERα and ERβ showed an IC50 of 7.38×10-5M and 4.12×10-7M respectively. After MCF-7 cells were treated with xy2004 in high concentration, Bcl-2 protein expressions were reduced and Bax protein expressions were increased. Conclusion xy2004 directly stimulates cell proliferation of MCF-7 cells through ligand-receptor binding in low concentrations and inhibited cell proliferation in high concentrations through regulating the apoptosis proteins.

Keywords: *benzodihydropyran; proliferation; ER; MCF-7*

INTRODUCTION*

The estrogen replacement therapy(ERT) was a significant measure to prevent and treat the rarefaction of bones, breast cancer and cardiovascular diseases(CVD) arising after the menopause of women. The protective effects of ERT for the angiocarpy and skeletal systems were affirmed. But long-term usage of ERT may increase the risk of developing the cancers of genital system. Therefore, its clinic application was greatly restricted.Under this circumstances, the selective estrogen receptor modulators(SERMs) arose as the times required. SERMs was the ER agonist or antagonist which closely combined with the estrogen receptor and selectively worked to different target tissues. The selective characteristics for specific target tissues made SERMs have the estrogen-like effects in bones and angiocarpy and the anti-estrogenic-like effects in mammary gland and endometrium[1-6]. Like the tamoxifen(TAM) and raloxifene, SERMs was applied into clinic treatment for the rarefaction of bones and breast cancer after the menopause of women. However, long-term usage of these drugs could cause some untoward effects and increase the morbidity of metrocarcinoma, hectic fever, thrombus and resistance to drugs. In our previous experiments, we found that a series of benzene and dihydro pyran

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derivatives which compounded on the basis of genistein structure played the protective effect in the bones and angiocarpy [4,5,6,15]. Among those derivatives, xy2004(2,3-dihydro; 7-methoxy group;4-hydrogen;1-benzopyran;4-ftivazide, the chemical construction is shown as Figure 1) promoted the proliferation of the osteoblast, influenced the bone metablolism of ovariectomized rats, enhanced the bone mineral density and prevented the rarefaction of bone of ovariectomized rats [7,8]. However, the effects on the breast cancer cells from xy2004 was uncertain. This study discussed the effects on the breast cancer cells from xy2004.

MATERIAL AND METHOD

Material

Xy2004 was compounded by our laboratory with purity of 84%; 17β-estradio(E2),

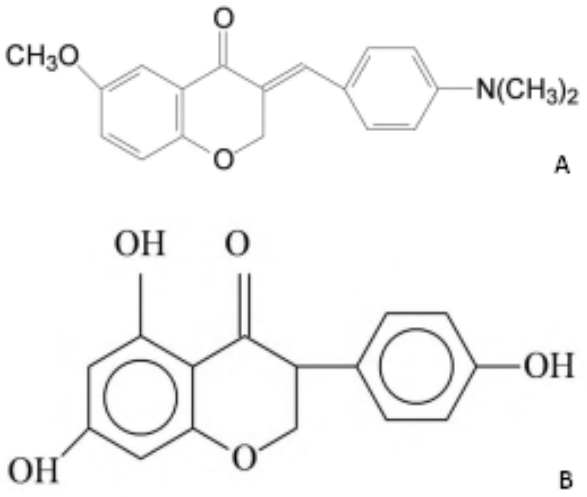

Fig.1: Structure of xy2004(A) and genistein(B).

Tamoxifen，MTT, hydro-xylapatite(HAP) were bought from America. DMEM was bought from GIBCO. MCF-7 was kept in our lab. The new-born calf serum was the product of Hangzhou Sijiqing Biological Products Company. Mouse Anti-Bcl-2 Monoclonal Antibody and Bax monoclonal antibody were both from Santa Cruz [9]. Annexin V/PI kit was produced from Jiamei Biology Company. [2,4,6,7-3H]estradiol ([3H]E2) was bought from Amersham with 3.03 TBq/mmol of radiochemical intensity. ERα and ERβ proteins were bought from PanVera (Madison, WI).

The preparation of the calf serum without sterol(CS-FCS)

Activated carbons was put in the centrifuge tube to roast for 1h under 180 □, then FCS was added into the centrifuge. The concentration of activated carbons finally was controlled to 4%~5%(v/v). After intensively mixing, the mixture was placed for 1h. Then the mixture of 3000g was centrifuged for 20 minutes. The filtrate was CS-FCS which was filtered from the supernatant with 0.22 filter membrane.

Cell culture

MCF-7 cells: The cells were inoculated into the culture flask by using of the DMEM without phenolphthalein involving FCS of 100 ml/L (which contained penicillin of 1×105 U/L and streptomycin of 100 mg/L) under the conditions of 37□, 50 ml/L CO_2 and saturation humidity. After 24h, the liquid was replaced. Afterwards, the nutrient solution was replaced every 2-3 days. When the cells overgrew the culture flask, the pancreatin of 2.5 g/L was added to digest them and then the transfer of culture was proceeded.

Measuring for cell proliferation(MTT experiment)

The cell density was adjusted to 2×103 holes. The cells were ininoculated into 96 holes, 100 μl for each hole. The nutrient solution was replaced after 24h by another nutrient solution containing CS-FCS. Meanwhile, xy2004 of 10-8、10-7、10-6、10-5 molL-1 was added, too. The control group used DMEM nutrient solution containing CS-FCS. Each group was prepared for 8 repeating holes. The incubation time was 72h. MTT of (5g/L)20μl was added before the end of cell culture, then the incubation was lasted for another 4h. DMSO of 200μl was added to each hole of the supernate of the suction hole. Then the nutrient solution

was vibrated for 10 minutes and the absorbancy of each hole(OD value) was measured in the 490 nm wave length [12].

The proliferation and antagonism experiment of cells

For the purpose of verificating whether the proliferation of MCF-7 cell worked by means of the estrogen receptor, every group was added estrogen receptor tamoxifen of the final concentration of 10-6 mol/L at the same time. The cultivation and measure methods were as same as above mentioned.

The cell cycle distribution

The cell density was adjusted to 3×104/m. The cells were ininoculated into the culture flask. After 24h' cultivation in DMEM containing FCS of 15%, the nutrient solution was replaced by FCS of 2% for another 24h' hunger cultivation to meet the synchronization. Then CS-FCS of 5% and xy2004 with different concentrations were added to the DMEM for 48h. After that, the cells were started to collect. The cells of 2×105~1×106/ml were suspend in combining buffer solution of 200 μl. Then 10 μl Annexin V-FITC and 5μl PI were added into the solution. After mixing them and placing them quietly for 15 minutes, the next procedure was analyzeing them by flow cytometer [14].

Competitive radioactive ligand binding tests

Double tube was used to this experiment, the total reacting volume was 500 μl. [3H]E2 0.5 nmol/L, killer tube was added for multiple dilute xy2004 of 100 μl and E2 of 10-5 mol/ Lcombined nonspecificly. Then each tube was added for ERa and ERb(0.5 pmol) of 50 μl. The reaction liquid was buffer solution with Ph index of 7.4((contained 25 mmol/L Tris-Cl, 1.5 mmol/L EDTA，10% glycerinum，1 mg/ ml Bovine serum albumin). Then they were incubated for 13h under 4 □. Then the HAP of 500 μl 50%($^{v}/_{v}$) was added to terminate the reaction. Next, each 600 g of them weas centrifuged for 20 minutes under 4 □. Next, the supemate was wiped off, and ethyl alcohol was added into the sediment. Then the sediment was shifted into the liquid flash cup. The liquid flash counter was used to count the volume and the IC50 value was evaluated by the software Prism. The recipe of scintillation solution contained 5 g PPO , 0.5 g POPOP , 1000 ml Methylbenzene [8,10].

The extract and the print of proteins

After the MCF-7 cells were managed by compounds, the nutrient solution was poured away. After 2 times' rinse by PBS, the lysate of 150 μl was added and then the liquid was blowed and beated for times. About 20 minutes later, the lysate was collected and centrifuged in speed of 12 000 r/min for 5 minutes. Then the supermatant was extracted to measure the protein level. Through 5 minutes' water bath of 100 □, the lysate came into being the degeneration and then the proteins in it were measured. Then the electrophoresis of 120 V and constant voltage transfer film of 80 V were proceeded. The skim milk of 50 g/L was added into the primary antibodies(contained β-actin、Bcl-2 and Bax) of 1:1000, then they were closed for one night. Then PBST was applied to wash them for 3 times and 3 minutes for once. Then they were added into the second antibodies of 1:5000 and incubated for 2h under the indoor temperature. After the washing of PBST for another 3 times, the developing solution was added to exposure. And then, the purpose stripes could be measured.

Data Analysis

The strength indexes of estrogen-like activity of compound were evaluated by the relative proliferative effect(RPE). The absorbancy during the maximal cell proliferation in test group divided the absorbancy in estradiol group and multiply 100% would count the RPE. All the experimental data were showed by the mean(±standard deviation). All the experiments had to be repeated for 3 times at least. During the experiments, the statistical

software SPSS was used to analyze the variance.

RESULTS

xy2004 cell proliferation promotion effect

Large dose of xy2004(10-5 mol/L) could significantly inhibit MCF-7 cell proliferation, and had statistical significance compared to control group, p<0.05. Small dose of xy2004 with 10-7~10-6 mol/L concentration could promote MCF-7 cell proliferation, and had statistical significance compared to control group, p<<0.01, p<0.05. Its bidirectional effect on breast cancer cells were similar with genistein, as seen in figure 2.

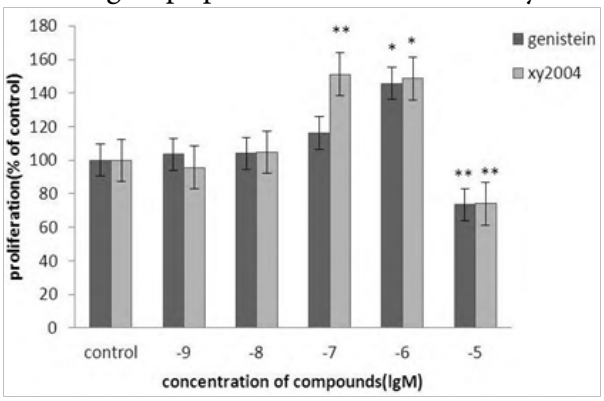

Fig. 2: Effects of xy2004 and genistein on the proliferation of MCF-7cells. *p<0.05,**p<0.01 vs. control(n=3).

Effect of estrogen receptor antagonist tamoxifen on cell proliferation

Large dose of xy2004(10-5 mol/L) could inhibit MCF-7 cell proliferation. The relative proliferated effect (RPE) was the 24.0% of E2, and had significant difference compared to control group, p<0.05. Small dose of xy2004 (with 10-7~10-6 mol/L concentration) could promote MCF-7 cell proliferation, and its cell proliferation promotion effect increased with dose-dependence. The RPE were respectively 49.9% (p<0.01) and 49.1% (p<0.05) of E2, compared to control group. It indicated that xy2004 had bidirectional effect on breast cancer cells. Tamoxifen was the estrogen receptor antagonism, could block a serial of estrogen receptor induced biological effect, including biological effect. Experimental results confirmed that the MCF-7 cell proliferation effect of small dose xy004 induced were completely antagonistic acted by 10-6 mol/Ltamoxifen. The MCF-7 cell inhibition effect of large dose xy2004 was not affected by tamoxifen, as seen in table 1.

Effect of xy2004 on MCF-7 cell apoptosis

The cell apoptosis did not happen on the untreated cells, 10-5 mol/L xy2004 and genistein significantly enhanced the cell apoptosis rate. The results was coincident with the results of

Table 1: Cell proliferation of MCF-7 cells affected by different compounds

Compounds	Concentration (mol/L)	RPE (%)	RPE with 10^{-6} mol/L TAM (%)
Control		33.0±3.2	32.4±2.2
E_2	10^{-9}	100±19.2**	32.6±7.87
xy2004	10^{-5}	24.0±3.5*	22.1±5.22
	10^{-6}	49.1±12.6*	34.1±6.17
	10^{-7}	49.9±11.6**	34.1±6.17
	10^{-8}	34.6±7.12	36.9±6.65
Genistein	10^{-6}	48.1±12.5*	30.9±6.2
	10^{-5}	24.1±2.9*	26.8±7.33

Results were expressed as percentage of cell proliferation. Incubation with E2(10-9mol/L) alone was performed as 100%, significantly different from control. (*:p<0.05, **:p<0.01, respectively, n=3).

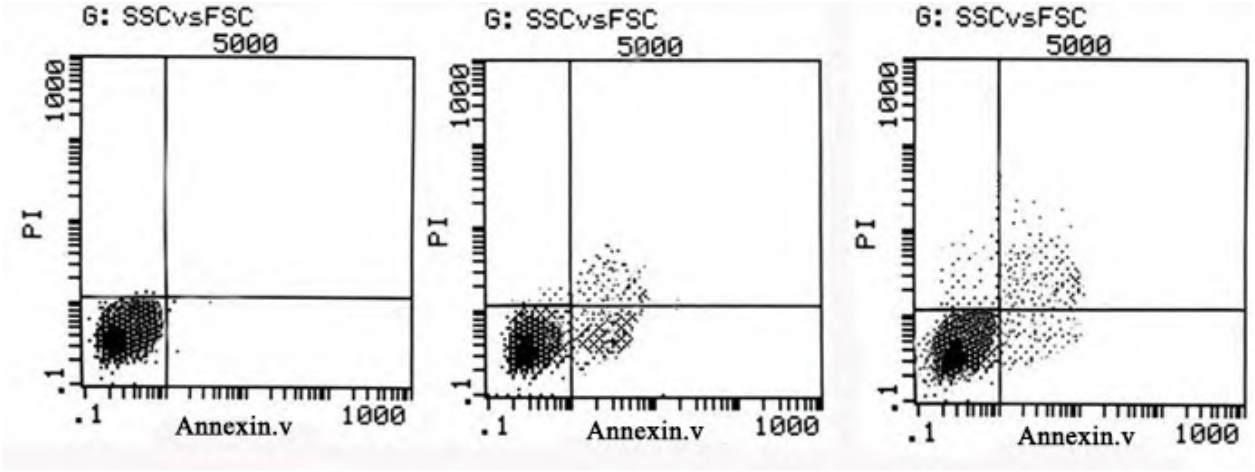

Fig.3: Effects of xy2004 on cell apoptosis detected by flow cytometry using Annexin V/PI staining. (A:control group, B:xy2004 group, C:genistein group).

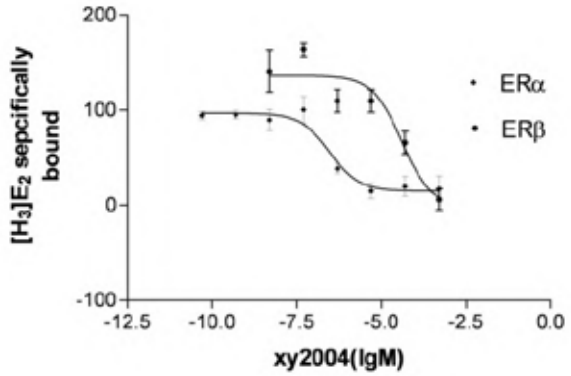

Fig.4: The competitive inhibition curve for the affinity of xy2004 and estrogen receptor

Table 2: affinity difference of different compounds with estrogen receptor (n=3)

Compounds	ERα IC50 (M)	ERβ IC50 (M)
E_2	$(1.35\pm0.27)\times10^{-9}$	$(1.67\pm0.45)\times10^{-9}$
Genistein	$(3.51\pm0.76)\times10^{-5}$	$(2.12\pm0.31)\times10^{-7}$
xy2004	$(4.12\pm0.22)\times10^{-7}$	$(7.38\pm0.17)\times10^{-5}$

cell proliferation experiment.

The affinity of xy2004 on ER

Xy2004 competed with estrogen E2 for the same receptor, which suggested that it had affinity on both ERα and ERβ, as seen in figure 4 and table 2. Prism software was utilized to calculate IC50. The IC50 of xy2004 on ERα and ERβ were respectively 4.12×10-7 mol/L and 7.38×10-5 mol/L. The IC50 of genistein on ERα and ERβ were respectively 3.51×10-5 mol/L and 2.12×10-7 mol/L, as seen in figure 3. The

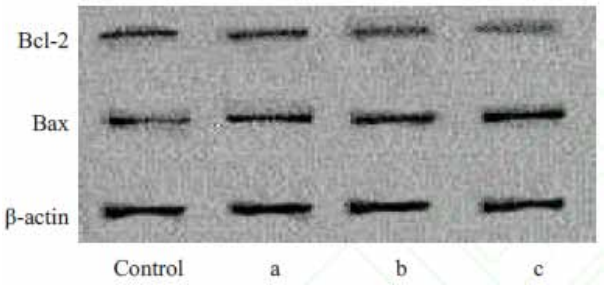

Fig.5: Western blot analysis for Bcl-2 and Bax protein expression in MCF-7 cells incubated with different concentrations of xy2004 for 24 h. Lane a:10-5 mol/L xy2004, Lane b: 10-4 mol/Lxy2004, Lane c: 10-3 mol/L xy2004.

xxy2004 had stronger affinity on ERα than ERβ. The difference were 100 times, which indicated that xy2004 had selectivity on ERα.

Cell proliferation of xy2004 on MCF-7 cells

The xy2004 with 10-5~10-3 mol/L concentration enhanced the expression of MCF-7 cell Bax protein, and reduced the expression of Bcl-2 protein, as seen in figure 5.

DISCUSSION

In our previous experiments, we found that xy2004 benzene and dihydro pyran derivatives promoted the proliferation of the osteoblast, influenced the bone metablolism of ovariectomized rats, enhanced the bone mineral density and prevented the rarefaction of bone of ovariectomized rats. Its effect was

better than genistein. This study discussed the effect to breast cancer cellcaused by xy2004.

MCF-7 cells , the ERα positive breast carcinoma cell lines of human, could specificly come up effects with estrogen or estrogen-like substances and then proliferate. Considering that the estrogen proliferation test of MCF-7 cells was extremely sensitive and easy to handle, it was generally applied to rapidly cull and evaluate the environmental estrogen and phytoestrogen[11,12]. The estrogen receptor had two subtypes, ERα and ERβ, which distributed in many human tissues and cells. ERα mainly distributed in urethral canals and mammary gland and ERβ mainly embodied in skeleton, cardiovascular, immune system and central nervous system. When selective estrogen receptor modulator(SERM) combined with ERα and ERβ in different tissues or under different physiological conditions, their interactions could produce estrogen-like effect of antiestrogen-like effect[12-13]. In this experiment, the radioligand receptor binding assays indicated that xy2004 had affinity with ERα and ERβ. Small dose of xy2004 could promote the cell proliferation of MCF-7. But obviously its effect was weaker than E2 since its maximum rate of proliferation was only 49.9% of E2. The estrogen receptor blocking pharmacon Tamoxifen could completely eliminate the promoting effect of xy2004 for MCF-7 cells proliferation, which indicateds that the effect was related to the exciting of ERα receptor.

Higher dose of xy2004 could restrain the MCF-7 cells proliferation and induce the MCF-7 cells apoptosis. The western blot hinted xy2004 to lower Bcl-2 proteins and increase Bax proteins. Bcl-2 proteins were for restraining the cells apoptosis and Bax proteins were for promoting cells apoptosis. When the expression of Bax proteins increased and came into being the homodimer, it would promote the cells apotosis; When the expression of Bcl-2 proteins increased and came into being heterodimer with Bax, it would restrain the cells apoptosis. When the ratio of Bax/Bcl-2 increased, it would form the homodimer on mitochondrial membrane, change the membrane permeability, and cause the release of cytochromec in mitochondria. Their combination with other factors would then activate Caspase-3, induce the cascade reaction of Caspase and promote the cells apoptosis[14,15]. Our research showed that the expression of Bax and Bcl-2 proteins was in low level and there was no apoptosis of MCF-7 cells; After xy2004 managed MCF-7 cells, the expression of Bax proteins increased, the expression of Bcl-2 proteins discreased, the ratio of Bax/Bcl-2 rose and the cells started to undergo apoptosis, which indicated that xy2004 was related to the anti-proliferation effect on MCF-7 cells, the activationg effect on apoptosis factors and the inducing effect onr cells apoptosis.

CONCLUSION

In conclusion, this research found that xy2004 of different concentrations plays a different part in MCF-7 cells. Small dose can promote the proliferation of MCF-7 cells, but large dose may restrain the proliferation of MCF-7 cells and promote the cells apoptosis. And it remains to be further experiments to research if the inducing effect of xy2004 on MCF-7 cells apoptosis could be applied to resist cancers.

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