Stimulatory Effects of 4-Hydroxytamoxifen on Proliferation of Human Endometrial Adenocarcinoma Cells (Ishikawa Line)[1]

Yuzuru Anzai, Christian F. Holinka, Hiroyuki Kuramoto, and Erlio Gurpide[2]

Department of Obstetrics, Gynecology and Reproductive Science, Mount Sinai School of Medicine (CUNY), New York, New York 10029 [Y. A., C. F. H., E. G.], and Department of Obstetrics and Gynecology, Kitasato University, Kanagawa-ken, Japan [H. K.]

ABSTRACT

The effects of *trans*-4-hydroxytamoxifen (OHTam) on proliferation of cells of the Ishikawa human endometrial adenocarcinoma line were studied under serum-free, phenol red-free conditions and compared to those of estradiol. The addition of OHTam (1 μM) to basal medium (BM), consisting of equal parts of Dulbecco's modified Eagle's medium and Ham's F-12 with additional glutamine and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, resulted in significant increases in cell numbers relative to controls. These effects were even greater than those obtained with estradiol (10 nM- 1 μM) or 1% charcoal-treated fetal bovine serum (ctFBS). Addition of 1% ctFBS to BM containing 1 μM OHTam further increased cell numbers whereas addition of estradiol (10 nM) did not do so. The stimulation of growth was positively correlated with OHTam concentrations in the range of 10 nM to 1 μM. Dissociation of estradiol and OHTam proliferative effects was observed in a variant of Ishikawa cells in which estradiol did not increase proliferation while OHTam had a strong stimulatory effect.

The growth-promoting effects of OHTam were also observed in BM containing 5% or 15% ctFBS. In contrast, in parallel experiments in which BM was replaced by minimal essential medium (Eagle's) with Earle's salts, OHTam (1 μM) did not stimulate proliferation under these conditions and acted as an antiestrogen, inhibiting the proliferative effects of estradiol. These results illustrate marked effects of medium composition on proliferation and antiestrogenic actions of OHTam.

Alkaline phosphatase activity was strongly stimulated by estradiol (10 nM) but only very weakly affected by OHTam (1 μM); at these concentrations, OHTam inhibited the effect of estradiol, both in serum-free BM and in minimal essential medium plus 15% ctFBS, demonstrating dissociation in its actions on proliferation and on enzymatic activity.

These findings suggest that OHTam may stimulate the proliferation of particular clones of endometrial cancer cells in human tumors. They also suggest that OHTam can exert effects not mediated by the estrogen receptor system, or form OHTam-estrogen receptor agonistic complexes unlike those resulting from estradiol-estrogen receptor interactions. Clearly, Ishikawa cells provide a useful model to investigate mechanisms of action of antiestrogens.

INTRODUCTION

The antiproliferative effects of Tam,[3] the nonsteroidal antiestrogen widely used in the treatment of breast cancer, has been demonstrated by several investigators using human mammary cancer cell lines (1–3). However, the mechanism of its action is not well understood.

Progestins are the first choice for hormonal therapy in endometrial carcinoma, but Tam has also been proposed as a therapeutic agent for this disease in postmenopausal patients (4, 5). Previous studies have shown that its active 4-hydroxyl-ated metabolite (OHTam) can antagonize the proliferative effect of estradiol on human adenocarcinoma cells of the Ishikawa line (6). This cell line, established by Nishida *et al.* (7), was shown to respond to estrogens by increasing progesterone receptor levels (6) as well as AlkP and DNA polymerase α activities (8, 9), effects that could also be suppressed by OHTam. Cells used for these previously reported studies were maintained and tested in MEM containing 15% ctFBS and phenol red, a pH indicator with weak estrogenic activity (10). In other studies carried out in this laboratory,[4] it was shown that Ishikawa cells proliferate and respond to estrogens in serum-free, phenol red-free BM, consisting of equal parts of Dulbecco's modified Eagle's medium and Ham's F-12, with additional 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and L-glutamine. Unexpectedly, when the effects of OHTam on Ishikawa cell proliferation were tested under these conditions, a stimulation of proliferation even greater than that of estradiol was noted. This observation led to the study that we are now reporting.

MATERIALS AND METHODS

Ishikawa cells were maintained in MEM plus 15% FBS and, in order to remove estrogenic components, the medium was changed to phenol red-free MEM plus 15% charcoal-treated FBS for at least 1 week before using cells for experiments. Treatment of FBS with dextran-coated activated charcoal to remove steroid hormones was carried out as described elsewhere (6). Cells were routinely tested for *Mycoplasma* with the Gen-Probe *Mycoplasma* T.C.II Rapid Detection System (Gen-Probe, San Diego, CA) and found to be negative. For growth studies, cells were suspended in BM or BM plus 1% ctFBS (no differences were found in attachment efficiency) and were equally distributed in several dishes at a density of 0.5 million/dish (6-cm diameter). Basal medium was prepared by adding to a phenol red-free mixture of Ham's F-12 and Dulbecco's modified Eagle's medium, 1:1 (specially prepared by Flow Laboratories, McLean, VA), 10 mM L-glutamine, 1% antibiotic-antimycotic mixture (GIBCO), and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. One day after seeding, the medium in each dish was replaced by BM containing test compounds, ctFBS or, to serve as control, 0.1% vehicle (ethanol) and thereafter changed every other day. For each condition, cells from 3 dishes were counted in a Neubauer-Levy hemacytometer under an inverted light microscope. For studies of cell proliferation in MEM with 15% ctFBS, cells were seeded at a density of 0.25 million/dish in the presence of test compounds or vehicle.

Alkaline phosphatase activity was determined by the method involving the measurement of *p*-nitrophenol formed by hydrolysis of *p*-nitrophenyl phosphate. *p*-Nitrophenol levels were measured spectrophotometrically at 400 nm, pH 10.4, as described in detail elsewhere (11).

trans-4-Hydroxytamoxifen was a gift from Stuart Pharmaceuticals, Division of Imperial Chemical Industries, Wilmington, DE.

RESULTS

Effects of OHTam, Estradiol, and ctFBS on Cell Numbers. Fig. 1 shows a growth curve of Ishikawa cells in serum-free BM

---

[3] The abbreviations used are: Tam, tamoxifen; OHTam, *trans*-4-hydroxytamoxifen; AlkP, alkaline phosphatase; MEM, minimum essential medium (Eagle's) with Earle's salts; ctFBS, charcoal-treated fetal bovine serum; BM, basal medium; DMEM, Dulbecco's modified Eagle's medium; ER, estrogen receptor.
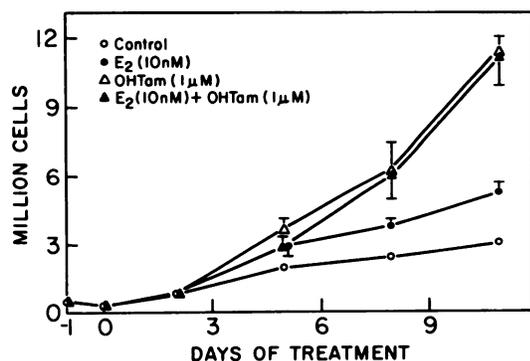
[4] C. F. Holinka, Y. Anzai, H. Hata, N. Kimmel, H. Kuramoto, and E. Gurpide. Proliferation and responsiveness to estrogen of human endometrial cancer cells under serum-free culture conditions, accepted for publication, *Cancer Research*, 1989.

Fig. 1. Effects of estradiol (E_2) and OHTam on proliferation of Ishikawa cells in serum-free BM. Cells (0.5 million/6-cm dish) were seeded in BM in the presence of 1% ctFBS. On the following day, the medium was replaced by BM containing the indicated experimental compounds. Estradiol (10 nM) or OHTam (1 μM) were added in ethanolic solution (final alcohol concentration in the media was 0.1%); control cultures received ethanol only. Medium was renewed every 2 days. On the indicated days, cells from 3 dishes for each experimental group were collected and counted in a hemacytometer under an inverted light microscope. *Bars*, SD. Addition of OHTam (1 μM) increased cell numbers to a greater extent than did estradiol (10 nM). The effects of OHTam and estradiol were not additive.
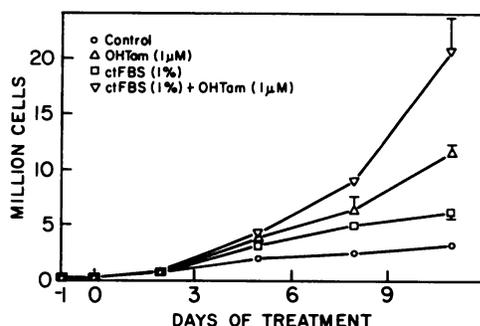

Fig. 2. Effects of OHTam and ctFBS on proliferation of Ishikawa cells in BM. Cells were plated and maintained as described in the legend to Fig. 1. The effect of OHTam (1 μM) was greater than that of 1% ctFBS, and a mixture of OHTam and 1% ctFBS further increased cell numbers.

and illustrates the effects of OHTam and estradiol on cell numbers. As reported in more detail separately,[4] estradiol (10 nM) stimulates growth in the absence of serum. Surprisingly, OHTam (1 μM) increased cell numbers to levels higher than those obtained in BM with 10 nM estradiol or 1% ctFBS (Figs. 1 and 2). A similar stimulation of growth by OHTam was observed in all of 10 additional experiments (Figs. 3–6; Table 1). Evaluation by the Student t test indicated that the effects of both estradiol and OHTam were significant as early as 5 days after addition of the compounds. Cell numbers obtained with mixtures of estradiol (10 nM) and OHTam (1 μM) were not

significantly different from those resulting from exposure to OHTam alone (Fig. 1).

As indicated in Fig. 2, the addition of 1% ctFBS also stimulated cell proliferation, but the effect was smaller than that obtained with 1 μM OHTam. In contrast to the lack of additivity of estradiol and OHTam action, the effects of mixtures of ctFBS (1%) and OHTam (1 μM) were significantly greater than their individual effect (Fig. 2).

Fig. 3 shows concentration dependence in the action of OHTam. Cell numbers in cultures containing 100 nM OHTam were significantly higher than those in control dishes beginning 8 days after addition of the drug; in the presence of 1 μM OHTam cell numbers were significantly higher than controls on day 2 and thereafter. The results from parallel experiments carried out with estradiol and OHTam at various concentrations, shown in Fig. 4, indicate that at high concentration of OHTam (1 μM), the effect was greater than the maximal stimulation obtained with estradiol.

In order to evaluate the influence of medium composition on OHTam actions, we also examined the effects of 1 μM OHTam and 10 nM estradiol in phenol red-free MEM at different concentrations of ctFBS. As shown in Fig. 5, OHTam in BM plus 5% ctFBS stimulated cell proliferation whereas in MEM plus 5% ctFBS it failed to elevate cell numbers and inhibited the stimulatory effects of estradiol. The medium dependence of OHTam effects on cell proliferation was also observed at 15% ctFBS levels (Table 1, Experiments 8 and 9). The antiestrogenic action of OHTam on Ishikawa cell proliferation observed in MEM containing 15% ctFBS agrees with our previously reported findings (6).

Dissociation of Estradiol and OHTam Effects on Cell Proliferation. A culture of Ishikawa cells maintained in phenol red-free MEM plus 15% ctFBS for 3 months lost the proliferative response to estradiol but, as shown in Fig. 6, the cells retained responsiveness to OHTam, revealing dissociation of the agonistic effects of estradiol and the triphenylethylene compound.

Effects of OHTam and Estradiol on AlkP Activity. The effects of OHTam (1 μM) and estradiol (10 nM) on AlkP activity were tested under culture conditions in which the drug was shown either to increase proliferation (serum-free BM) or be antiestrogenic (MEM plus 15% ctFBS). As shown in Fig. 7, OHTam only minimally stimulated AlkP activity but markedly antagonized the effect of estradiol under either culture condition. These results were confirmed in another experiment.

DISCUSSION

The results obtained in this study demonstrate that OHTam can stimulate cell proliferation in a human endometrial cancer

Table 1 *Effects of estradiol and OHTam on Ishikawa cell density*[a]

Experiment	Days of exposure to test compounds	Medium	Cell no. (million/6-cm dish; mean ± SD; $n = 3$)			
			Control	OHTam (1 μM)	Estradiol (10 nM)	Estradiol (10 nM) + OHTam (1 μM)
1	10	BM	6.5 ± 0.65	11 ± 1.3***[b]		
2	9	BM	6.8 ± 1.0	13 ± 1.4***	8.7 ± 0.68*	
3	10	BM	8.2 ± 1.2	16 ± 1.4***	12 ± 1.4*	
4	10	BM	2.6 ± 0.38		4.0 ± 0.24***	10 ± 0.40***
		BM + 1% S[c]	3.6 ± 1.1		10 ± 1.1***	20 ± 3.8***
5	7	BM + 1% S	6.8 ± 1.2	13 ± 2.4**	10 ± 0.70***	
6	12	BM + 1% S	6.1 ± 0.79	31 ± 0.49***	8.3 ± 0.45**	31 ± 0.85***
7	14	MEM + 1% S	3.2 ± 0.23	2.2 ± 0.40	2.9 ± 0.10	2.5 ± 0.63
8	9	BM + 15% S	13 ± 1.6	21 ± 3.6**	18 ± 2.3**	22 ± 2.0***
9	10	MEM + 15% S	4.6 ± 0.95	4.9 ± 0.41	9.0 ± 1.2***	5.1 ± 0.68

[a] Data not shown in figures.
[b] Significantly different from control values (*, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$) as determined by Student's t test.
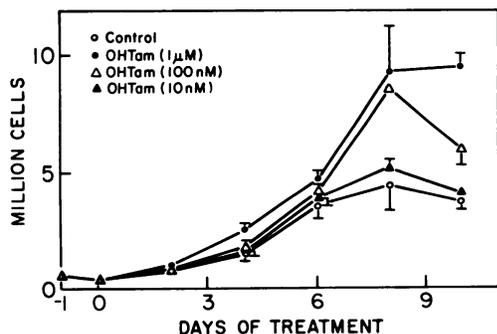[c] S, charcoal-treated fetal bovine serum.

Fig. 3. Concentration dependence of the effects of OHTam on proliferation of Ishikawa cells in BM. Cells were plated and maintained as described in the legend to Fig. 1. Cell densities achieved in the presence of 1 μM OHTam were significantly greater than controls on day 2 and thereafter ($P < 0.05$). Cell numbers in the presence of 100 nM OHTam were significantly greater than controls on days 8 and 10 ($P < 0.05$).

Fig. 6. Effects of estradiol (E_2) and OHTam on proliferation of an estradiol-unresponsive variant of Ishikawa cells in BM. Cells were plated in BM and maintained as described in the legend to Fig. 1. OHTam increased cell numbers in the estradiol-unresponsive cells. The effects of OHTam were significant on days 4, 7, and 10 ($P < 0.05$). The differences between control and estradiol values were not significant.
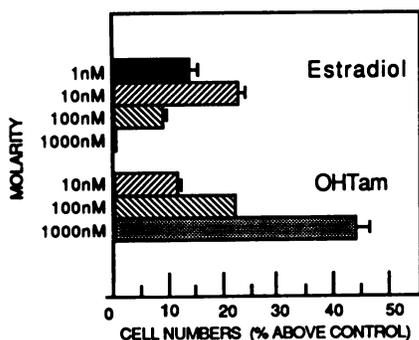

Fig. 4. Dose response to OHTam and estradiol. Cells were seeded and maintained as described in the legend to Fig. 1. The effects on cell numbers of OHTam and estradiol at concentrations ranging from 1 to 1000 nM in serum-free BM were compared after 7 days of treatment in parallel experiments. Although at a 10 nM concentration estradiol was more potent than OHTam, the opposite was true at 100 and 1000 nM concentrations. It is noteworthy that the effect of OHTam at 1 μM was twice the maximal effect obtained with estradiol. Bars, SD.

Fig. 7. Effects of estradiol (E_2) and OHTam on AlkP activity in Ishikawa cells. Cells (2–3 $\times$ 10^6) were plated in 10-cm dishes using either MEM plus 15% ctFBS or serum-free BM containing the compounds indicated. Cells were incubated for 4 days and media were replaced 2 days after seeding. Under both conditions, estradiol markedly stimulated AlkP activity and OHTam antagonized the stimulatory effect of estradiol.

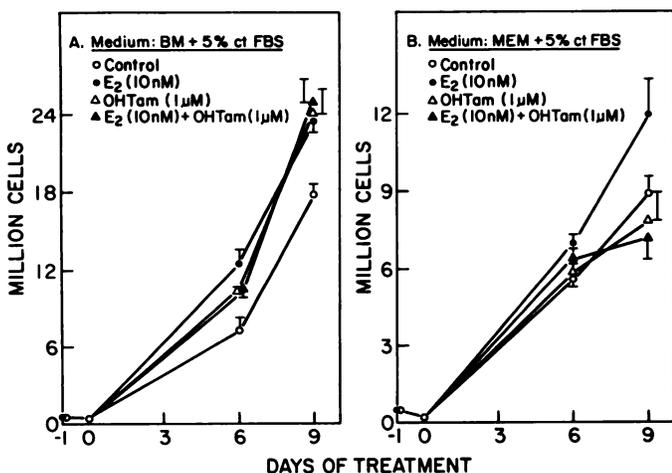
Fig. 5. Influence of medium on the effects of OHTam and estradiol (E_2). Cells were seeded in MEM or BM containing 5% ctFBS and maintained as described in the legend to Fig. 1. The effects on cell numbers of estradiol (10 nM) and OHTam (1 μM) in these two media in the presence of 5% ctFBS were compared in parallel experiments. When BM was used (A) the proliferative effect of OHTam was evident. In contrast, in MEM (B), OHTam was not significantly stimulatory and acted as an antiestrogen when mixed with estradiol.

line (Ishikawa) and that, under some culture conditions, this effect can be significantly greater than that obtained with estradiol. Although partial agonistic effects of triphenylethylene derivatives have been observed in human estrogen-responsive systems and significant estrogenic effects of these drugs have
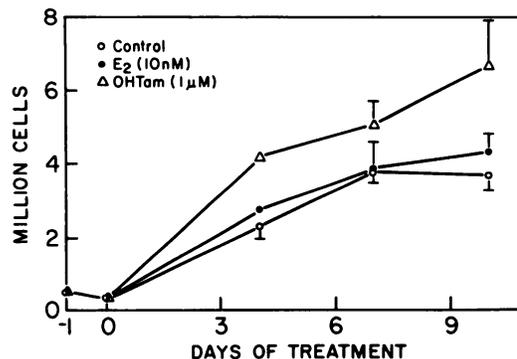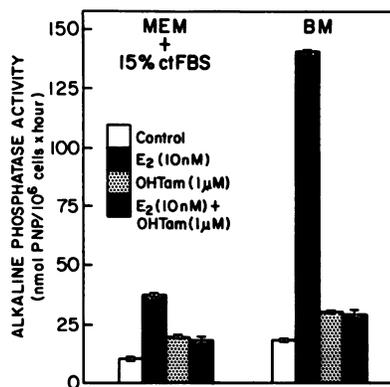
been shown on the proliferation of human breast cancer cells in culture (12, 13) or on the growth of human endometrial tumors transplanted into nude mice (14, 15), these effects were still weaker than those of estradiol. Furthermore, OHTam in those studies acted as an antiestrogen when mixed with estradiol. Species and end point differences resulting in either estrogenic or antiestrogenic effects of Tam and OHTam have been described (16, 17).

The dissociation of proliferative effects of estradiol and OHTam in an estradiol-unresponsive variant of Ishikawa cells cultured in serum-free BM (Fig. 6) appears to contradict the common assumption that OHTam acts by competition with estradiol for a common ER. Some variants of MCF-7 cells also show discrepancies between estradiol and Tam effects, such as Tam resistance associated with responsiveness to estradiol (18) or inhibition of proliferation by Tam in estradiol-unresponsive cells (12). These observations suggest direct effects of Tam and OHTam on cell proliferation by mechanisms which may not involve the estrogen receptor system. The weak stimulation by OHTam of AlkP activity, a sensitive marker for estrogenic activity (8), and its antagonistic effect on the action of estradiol on the activity of the enzyme in BM (Fig. 7) also indicate that the effect of OHTam on proliferation occurs by a different mechanism than its action on AlkP activity and may not involve the estrogen receptor.

Another puzzling finding in these studies relates to the influence of the medium on proliferative responses. Namely,

OHTam stimulated cell proliferation in BM whereas in MEM, at the same concentration of ctFBS, it did not do so. These results indicate that medium components influence the action of OHTam.

The possibility that the effects of Tam and OHTam are mediated by interaction with specific antiestrogen binding sites, showing high affinity for Tam and OHTam but not for estradiol (19), remains unproved. It should be considered, however, that Tam or OHTam at 1 μM concentrations interact with other receptors, e.g., histaminergic (20) or dopaminergic (21) receptors, influence calmodulin action (22) and may regulate phosphatidylinositide hydrolysis, consequently affecting intracellular levels and distribution of Ca^{2+}, known to modify protein kinase C activity either directly or by altering diacylglycerol levels (23). If OHTam does not exert its action on Ishikawa cell proliferation by interacting with the ER, its mechanism of action remains unidentified. Estradiol and OHTam may even exert different changes on ER due to differences in their interaction with the receptor (24). The activation of receptors, usually considered to involve conformational changes, may be influenced by the medium.

A mediation of transforming growth factor β on the actions of OHTam, suggested by the results obtained with MCF-7 cells (25), is unlikely in our system since OHTam enhances Ishikawa cell proliferation while transforming growth factor β inhibits it.[5]

The relevance of our findings to a suggested treatment of endometrial cancer with Tam is unclear since the results are influenced by the composition of the culture medium. It is also questionable whether the concentrations of OHTam to which the cells were exposed in these experiments (10 nM–1 μM) are reached during administration of Tam to patients. However, Ishikawa cells may be representative of clones present in endometrial tumors which could proliferate more rapidly in the presence of Tam or OHTam in vivo. Certainly Ishikawa cells provide a useful model to study mechanisms of action of estrogens and antiestrogens.

REFERENCES

1. Lippman, M., Bolan, G., and Huff, K. The effects of estrogens and antiestrogens on hormone-responsive human breast cancer in long-term tissue culture. Cancer Res., 36: 4595–4601, 1976.
2. Allegra, J. C., and Lippman, M. E. Growth of a human breast cancer cell line in serum-free hormone-supplemented medium. Cancer Res., 38: 3823–3829, 1978.
3. Briand, P., and Lykkesfeldt, A. E. Effect of estrogen and antiestrogen on the human breast cancer cell line MCF-7 adapted to growth at low serum

[5] Y. Anzai, L. Murphy, C. Holinka, and E. Gurpide. Effects of EGF and TGF on proliferation of endometrial cancer cells, manuscript in preparation.

concentration. Cancer Res., 44: 1114–1119, 1984.
4. Swenerton, K. D. Treatment of advanced endometrial adenocarcinoma with tamoxifen. Cancer Treat. Rep., 64: 805–811, 1980.
5. Swenerton, K. D., Chruma, K., Paterson, A. H., and Jackson, G. C. Efficacy of tamoxifen in endometrial cancer. Prog. Cancer Res. Ther., 31: 417–424, 1984.
6. Holinka, C. F., Hata, H., Kuramoto, H., and Gurpide, E. Responses to estradiol in a human endometrial adenocarcinoma cell line (Ishikawa). J. Steroid Biochem., 24: 85–89, 1986.
7. Nishida, M., Kasahara, K., Kaneko, M., and Iwasaki. H. Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors. Acta Obstet. Gynaecol. Jpn., 37: 1103–1111, 1985.
8. Holinka, C. F., Hata, H., Kuramoto, H., and Gurpide, E. Effects of steroid hormones and antisteroids on alkaline phosphatase activity in human endometrial cancer cells (Ishikawa line). Cancer Res., 46: 2771–2774, 1986.
9. Gravanis, A., and Gurpide, E. Effects of estradiol on deoxyribonucleic acid polymerase α activity in the Ishikawa human endometrial adenocarcinoma cell line. J. Clin. Endocrinol. Metab., 63: 356–359, 1986.
10. Berthois, Y., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc. Natl. Acad. Sci. USA, 83: 2496–2500, 1986.
11. Holinka, C. F., and Finch, C. E. Age-related changes in the decidual response of the C57BL/6J mouse uterus. Biol. Reprod., 16: 385–393, 1977.
12. Katzenellenbogen, B. S., Kendra, K. L., Norman, M. J., and Berthois, Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res., 47: 4355–4360, 1987.
13. Katzenellenbogen, B. S., Norman, M. J., Eckert, R. L., Peltz, S. W., and Mangel, W. F. Bioactivities, estrogen receptor interactions and plasminogen activator-inducing activities of tamoxifen and hydroxytamoxifen isomers in MCF-7 human breast cancer cells. Cancer Res., 44: 112–119, 1984.
14. Satyaswaroop, P. G., Zaino, R. J., and Mortel, R. Estrogen-like effects of tamoxifen on human endometrial carcinoma transplanted into nude mice. Cancer Res., 44: 4006–4010, 1984.
15. Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G., and Jordan, V. C. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res., 48: 812–815, 1988.
16. Pasqualini, J. R., Gulino, A., Sumida, C., and Screpanti, I. Antiestrogens in fetal and newborn target tissues. J. Steroid Biochem., 20: 121–128, 1984.
17. Pasqualini, J. R., Giambiagi, N., Sumida, C., Nguyen, B-L., Gelly, C., Mayrand, C., and Lecerf, F. Biological responses to tamoxifen in the fetal and newborn vagina and uterus of the guinea pig and in the R-27 mammary cancer cell line. J. Steroid Biochem., 24: 99–108, 1986.
18. Nawata, H., Bronzert, D., and Lippman, M. E. Isolation and characterization of a tamoxifen-resistant cell line derived from MCF-7 human breast cancer cells. J. Biol. Chem., 256: 5016–5021, 1981.
19. Sutherland, R. L., Murphy, L. C., Foo, M. S., Green, M. D., and Whybourne, A. M. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. Nature (Lond.), 288: 273–275, 1980.
20. Brandes, L. J., MacDonald, L. M., and Bogdanovic, R. P. Evidence that the antiestrogen binding site is a histamine or histamine-like receptor. Biochem. Biophys. Res. Commun., 126: 905–910, 1985.
21. Hiemke, C., and Ghraf, R. Interaction of non-steroidal antiestrogens with dopamine receptor binding. J. Steroid Biochem., 21: 663–667, 1984.
22. Lam, H-Y. P. Tamoxifen is a calmodulin antagonist in the activation of cAMP phosphodiesterase. Biochem. Biophys. Res. Commun., 18: 27–32, 1984.
23. O'Brian, C. A., Liskamp, R. M., Solomon, D. H., and Weinstein, I. B. Inhibition of protein kinase C by tamoxifen. Cancer Res., 45: 2462–2465, 1985.
24. Sasson, S., and Notides, A. C. Mechanism of the estrogen receptor interaction with 4-hydroxytamoxifen. Mol. Endocrinol., 2: 307–312, 1988.
25. Knabbe, C., Lippman, M. E., Wakefield, L. M., Flanders, K. C., Kasid, A., Derynck, R., and Dickson, B. Evidence that transforming growth factor β is a hormonally regulated negative growth factor in human breast cancer cells. Cell, 48: 417–428, 1987.