Jpn. J. Cancer Res. 85, 639–644, June 1994

Binding Sites of Droloxifene in the Cytosol of 7,12-Dimethylbenz[*a*]anthracene-induced Rat Mammary Tumor Cells

Ikuo Kawamura,[1,3] Elizabeth Lacey,[1] Yoshio Tanaka,[2] Fusako Nishigaki,[1] Toshitaka Manda[1] and Kyoichi Shimomura[1]

[1]*Pharmacological Research Laboratories,* [2]*Pharmaceutical and Pharmacokinetic Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-ku, Osaka 532*

The binding sites, other than the estrogen receptor (ER), of the antiestrogens droloxifene (DROL, (E)-α-[*p*-[2-(dimethylamino)ethoxy]-phenyl]-α′-ethyl-3-stilbenol) and tamoxifen (TAM), and estradiol-17β (E_2) in the cytosol of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary ER-positive tumor cells were studied using a high-performance liquid chromatography (HPLC) gel filtration assay. The cytosol was incubated with ^{3}H-labeled drug with or without unlabeled drug, and separated by HPLC gel filtration. ^{3}H-E_2 produced two major peaks of radioactivity at fractions No. 40 and No. 70. The peak at fraction No. 70 was identified as the ER in an ER-enzyme-immuno assay. This peak was dose-dependently inhibited by unlabeled DROL or TAM, DROL being a more potent inhibitor than TAM. The peak at fraction No. 40 was also inhibited by co-incubation with unlabeled DROL or TAM. ^{3}H-DROL or ^{3}H-TAM provided only one peak at fraction No. 43. This peak was thought to be an antiestrogen binding site (AEBS), because it was inhibited by unlabeled antiestrogen but not by E_2. The results suggest that the antiestrogens DROL and TAM have a higher affinity for the AEBS than for the ER in the absence of E_2, while in the presence of E_2 both have an affinity for the ER and inhibit E_2 binding to the ER.

Key words: Antiestrogen drug — Droloxifene — Antiestrogen binding site — Estrogen receptor — DMBA-induced rat mammary tumor

The antiestrogen tamoxifen (TAM) has been widely used in the treatment of estrogen receptor (ER)-positive breast cancer and has achieved great success in prolonging the recurrence-free duration and survival time.[1–3] TAM is thought to act by inhibiting the binding of estrogen to the ER in tumors.[4–6] On the other hand, inhibition of protein kinase C and antagonism to calmodulin are also reported to be involved in the antitumor effect.[7,8] Therefore the pharmacological actions of TAM are extremely complex and may not all be explicable in terms of interaction with the ER. Moreover, in addition to the ER, the presence of another specific binding site for TAM, but not for estradiol-17β (E_2), has been demonstrated; it has been termed the antiestrogen binding site (AEBS).[9,10] AEBS are observed in most estrogen target tissues[9,10,11–15] and compounds with a higher affinity for the AEBS have more potent antiproliferative effects on breast cancer cells.[16–19] Thus, some of the antitumor activity of TAM may be mediated by AEBS in the tumors. Therefore it is important to examine the binding of drugs to the AEBS, as well as to the ER.

Droloxifene (DROL, (E)-α-[*p*-[2-(dimethylamino)-ethoxy]-phenyl]-α′-ethyl-3-stilbenol) is a new derivative of TAM with stronger antiestrogenic and weaker estrogenic activities than TAM.[20–23] In a previous study, we showed that DROL inhibited the growth and the initiation of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumors and that the binding affinity of DROL for the ER in the mammary tumors was stronger than that of TAM in the dextran-coated charcoal (DCC) assay.[22] The DCC assay is the most commonly used technique for the determination of the binding affinity of a drug for the ER.[24,25] However, in the DCC assay, it is only possible to determine the competitive binding between antiestrogens and labeled E_2, but not the individual proteins to which each antiestrogen drug binds. Since the purpose of this study was to examine the binding affinity of drugs for the ER and the AEBS, it was necessary to analyze each binding protein separately. Thus, high-performance liquid chromatography (HPLC) gel filtration assay was chosen instead of the DCC assay, because the binding proteins can be examined separately in the HPLC assay[26] and the binding affinity of a drug for each protein can be determined easily by comparing the peak areas.

In this study we investigated the binding sites, other than ER, of DROL, TAM and E_2 in the cytosol of DMBA-induced rat mammary tumor cells using HPLC gel filtration assay, and established that the antiestrogens DROL and TAM have a binding affinity for different proteins depending on the presence or absence of E_2.

[3] To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals DROL and TAM were provided by Klinge Pharma GmbH (Munich, FRG). Both drugs were used as the citrate salt form. DMBA was purchased from Tokyo Kasei Kogyo Co. (Tokyo) and E_2, from Sigma Chemical Co. (St. Louis, MO, USA). The gel filtration size marker proteins, blue dextran 2000, catalase, bovine serum albumin and ribonuclease A from Pharmacia (Uppsala, Sweden). [6,7-^{3}H]Estradiol-17β (1576.2 GBq/mmol, ^{3}H-E_2) and [N-methyl-^{3}H]tamoxifen (3059.9 GBq/mmol, ^{3}H-TAM) were from New England Nuclear Research Products (Boston, MA, USA). [N-methyl-^{3}H]Droloxifene (3120 GBq/mmol, ^{3}H-DROL) was prepared by Amersham Japan (Tokyo). DROL, TAM and E_2 were prepared at 10 m*M* concentrations in dimethyl sulfoxide and diluted with the mobile phase buffer. DMBA was dissolved at a concentration of 20 mg/ml in sesame oil.

Animals and mammary tumors Female Sprague-Dawley rats were purchased from Japan SLC, Inc. (Shizuoka). DMBA (20 mg) was given p.o. to rats (7 weeks old). About 100 days after the administration of DMBA, rats with mammary tumors were ovariectomized. Four days later they were killed and their tumors were removed. The tumors were proven to have a high ER content by receptor assay, as reported previously.[22]

Preparation of cytosol of DMBA-induced mammary tumors The tumors were homogenized in Tris-EDTA-dithiothreitol (TED) buffer (10 m*M* Tris/HCl, 1.5 m*M* EDTA, 0.5 m*M* dithiothreitol, 10% glycerol, pH 7.4), and then centrifuged at 105,000*g* for 1 h to obtain the cytosol as the supernatant.

Analysis of binding sites of DROL, TAM and E_2 in DMBA-induced mammary tumors One hundred and fifty μl of the cytosol (protein content: 9.8 mg/ml) of DMBA-induced mammary tumors was incubated with 10 n*M* ^{3}H-labeled drug (75 μl) in the presence or absence of unlabeled drug (75 μl) at 4°C for 18 h and then subjected to HPLC gel filtration. The HPLC system consisted of a pump (Hitachi 655 liquid chromatograph), a detector (Toyo Soda U.V. 8000) and a Hitachi 833A data processor. The column was reverse-phase TSK gel G3000 SW_{XL} (Tosoh, Tokyo) with a 4 cm SW_{XL} guard column packed with the same material. The mobile phase

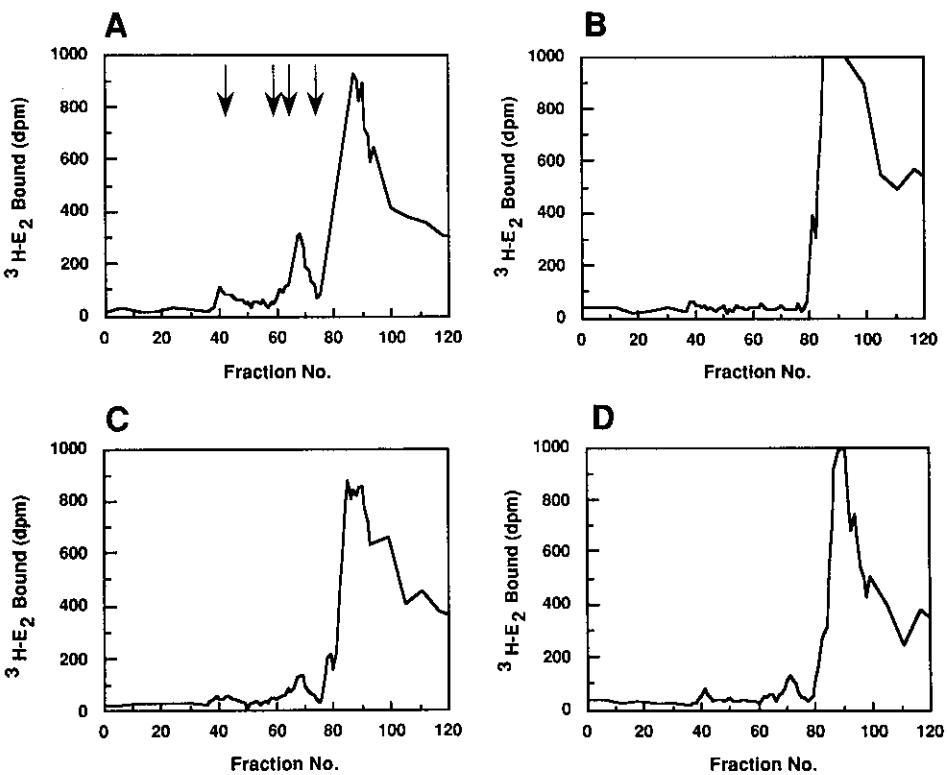

Fig. 1. Binding of ^{3}H-E_2 in the cytosol of DMBA tumors (A) and the competition of E_2 (B), DROL (C) and TAM (D). The cytosol of DMBA-induced mammary tumors was incubated with 10 n*M* ^{3}H-E_2 in the presence or absence of unlabeled drug at 4°C for 18 h, then analyzed by HPLC. Arrows show the elution positions of marker proteins; blue dextran 2000, catalase, bovine serum albumin and ribonuclease A (left to right).

consisted of 0.05% sodium azide and 0.1 *M* sodium sulfite in 0.1 *M* phosphate buffer, pH 6.7; flow rate 0.9 ml/min. One hundred and fifty μl of the sample was injected into the HPLC system and the eluate was automatically collected every 10 s using an Atta mini collector (SJ1410NC). The radioactivity of the eluates collected was measured in a Packard Tri-Carb TR1900CA liquid scintillation analyzer. Marker proteins were separated in the same way and the absorbance of each eluate was determined at 280 nm.

Measurement of ER level The cytosol of DMBA-induced mammary tumors was separated and fractionated in the same way as above and the ER content of each fraction was measured using an ER-EIA kit (Abbott Laboratories).[27,28] Briefly, the fraction (100 μl) was incubated with polystyrene beads coated with an antibody (rat anti-human ER, D547). The beads were next incubated with the second antibody (rat anti-human ER, H222) conjugated with horseradish peroxidase. Following incubation and subsequent washing steps the concentration of horseradish peroxidase was determined using diaminobenzidine and H_2O_2. The ER content in each fraction was calculated from a reference standard curve.

RESULTS

Binding of ^{3}H-E$_2$ Following incubation of the cytosol with ^{3}H-E$_2$ in the presence or absence of unlabeled drug, the complex was separated by HPLC gel filtration assay, and the radioactivity of each eluate was counted. As shown in Fig. 1A, the incubation of the cytosol with 10 n*M* ^{3}H-E$_2$ alone resulted in two major peaks at fractions No. 40 and 70. The unbound radioactive drug formed a large peak that was eluted after fraction No. 80. This was confirmed by HPLC gel filtration of ^{3}H-E$_2$ alone, without the cytosol. Both peaks at fractions No. 40 and 70 of bound ^{3}H-E$_2$ were suppressed by co-incubation with unlabeled 1 μM E$_2$ (Fig. 1B). Co-incubation with unlabeled 1 μM DROL (Fig. 1C) or 10 μM TAM (Fig. 1D) resulted in a reduction in the peaks at fractions No. 40 and 70. Next the inhibitory effects of DROL and TAM on the peak at fraction No. 70 were examined by measuring the peak areas (calculated from the height and width

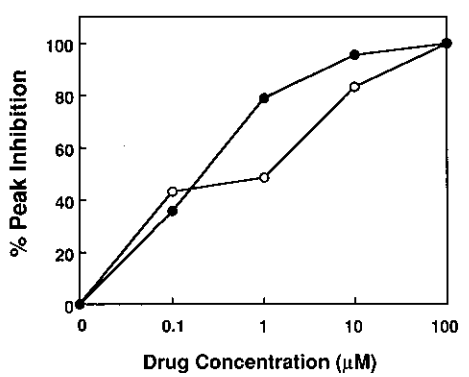

Fig. 2. Inhibitory effects of DROL (●) and TAM (○) on the peak at fraction No. 70 of bound ^{3}H-E$_2$. The cytosol was incubated with 10 n*M* ^{3}H-E$_2$ in the presence of 10- to 10,000-fold excess of unlabeled drug and analyzed. The inhibition of the peak at fraction No. 70 was compared by measuring the peak areas. Each point represents the mean of 2 experiments.

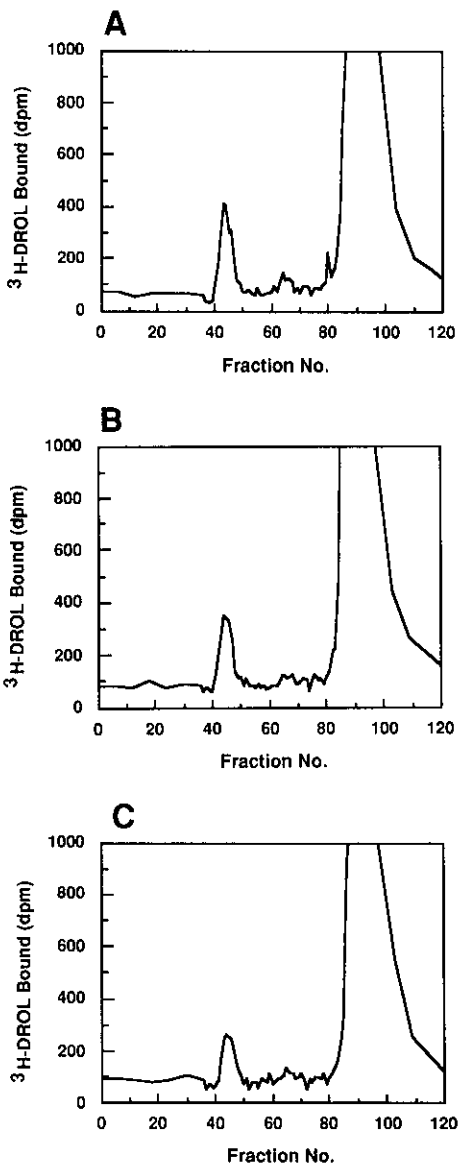

Fig. 3. Binding of ^{3}H-DROL in the cytosol (A) and the competition of E$_2$ (B) and TAM (C). The cytosol was incubated with 10 n*M* ^{3}H-DROL in the presence or absence of unlabeled drug, and then analyzed by HPLC.

of the peak). As shown in Fig. 2, DROL and TAM dose-dependently inhibited this peak, the effect of DROL being stronger than that of TAM.

Binding of ^{3}H-DROL Next the binding of ^{3}H-DROL was examined. As shown in Fig. 3A, the incubation with 10 nM ^{3}H-DROL resulted in one sharp peak at fraction No. 43 and no peak at fraction No. 70. This peak at fraction No. 43 was completely or partially inhibited by co-incubation with unlabeled 1 μM DROL (data not shown) or 10 μM TAM (Fig. 3C), but was unaffected by co-incubation with unlabeled 1 μM E_2 (Fig. 3B).

Binding of ^{3}H-TAM The binding of ^{3}H-TAM was also investigated. As shown in Fig. 4A, incubation with 10 nM ^{3}H-TAM also gave a peak at fraction No. 43, but the peak was low and rounded. This peak was completely eliminated by co-incubation with unlabeled 1 μM TAM (data not shown) and 1 μM DROL (Fig. 4C). Co-incubation with unlabeled 1 μM E_2 did not affect the peak (Fig. 4B).

ER assay In order to determine the point at which the ER was eluted, the fractions separated by HPLC were assayed using an ER-EIA kit. An ER concentration of more than 10 fmol/ml was accepted here as being reliable, since the detection limit of the ER was about 10 fmol/ml with the kit used in this study. As shown in Fig. 5, the ER was detected only around fraction No. 70.

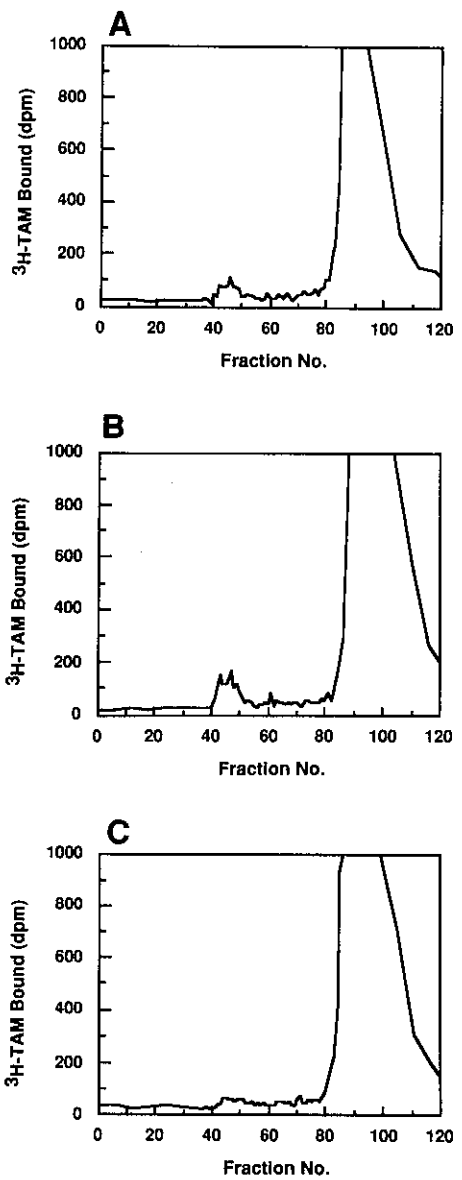

Fig. 4. Binding of ^{3}H-TAM in the cytosol (A) and the competition of E_2 (B) and DROL (C). The cytosol was incubated with 10 nM ^{3}H-TAM in the presence or absence of unlabeled drug, and then analyzed by HPLC.

DISCUSSION

In this study we investigated the binding sites of DROL, TAM and E_2 in the cytosol of DMBA-induced rat mammary ER-positive tumor cells using HPLC gel filtration. The incubation of the cytosol with ^{3}H-E_2 produced two major peaks of bound ^{3}H-E_2 at fractions No. 40 and 70 (Fig. 1A). These two peaks were suppressed by co-incubation with 100-fold excess of unlabeled E_2. The results suggest that these proteins are the binding sites of E_2.

The peak at fraction No. 70 of bound ^{3}H-E_2 was found to be the ER in an ER-EIA assay. DROL and TAM dose-dependently inhibited this peak, the effect of DROL being stronger than that of TAM (Fig. 2). This result suggests that DROL and TAM bind to the ER in the cytosol and that the binding affinity of DROL is stronger than that of TAM. Previously, using the DCC assay, we

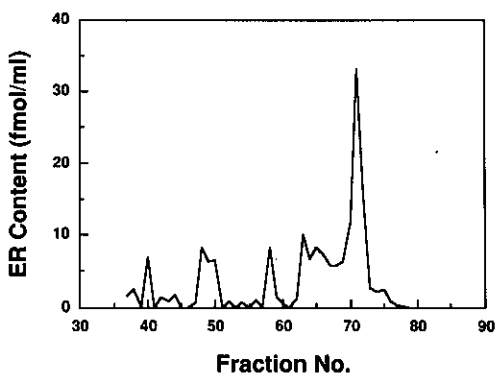

Fig. 5. ER content of the HPLC eluted fractions. The cytosol was separated by HPLC and the ER content of each fraction was measured using an ER-EIA kit.

showed that DROL has a higher affinity than TAM for the ER in DMBA-induced rat mammary tumor cells.[22] The DCC assay allowed us to examine the inhibitory effect of DROL or TAM on the overall E_2 binding to both the ER and other proteins. However, using the HPLC assay we could examine their inhibitory effects on E_2 binding to the ER alone and we confirmed that DROL has a stronger binding affinity than TAM.

The peak at fraction No. 40 of bound ^{3}H-E_2 was inhibited by co-incubation with DROL or TAM but was not detected as ER in an ER-EIA assay. The ER-EIA kit we used in this study contains two antibodies to the ER, H222, which recognizes a site close to the estrogen-binding domain, and D547, which recognizes a site near the DNA binding domain.[29, 30] It is necessary for both antigenic determinants to be present in order to detect the ER using this kit. Therefore, there is a possibility that this protein at fraction No. 40 may be a complex of the ER and another component, with only the estrogen-binding domain being expressed, but further investigation is needed.

Next we examined the binding sites of DROL and TAM in the cytosol of DMBA-induced rat mammary tumors. The incubation with ^{3}H-DROL resulted in no peak near fraction No. 70 but a sharp peak at fraction No. 43 (Fig. 3A). ^{3}H-TAM also produced only one peak at fraction No. 43, but the peak was low and rounded (Fig. 4A). The peak at fraction No. 43 of bound ^{3}H-DROL or ^{3}H-TAM was inhibited by co-incubation with unlabeled TAM or DROL, respectively, but the peaks were not affected by unlabeled E_2. These findings imply that the protein at fraction No. 43 is different from that at fraction No. 40 obtained with bound ^{3}H-E_2 and may be an AEBS, because it fulfills the criteria of an AEBS proposed by Sutherland *et al.*[9, 10]; that is, it binds antiestrogens but not E_2. The existence of AEBS in the cytosol or nuclear fractions from rat uteri, rat liver and human breast cancer cells has already been reported.[9, 11-15] In this study, we showed that AEBS, in addition to the ER, may be present in DMBA-induced rat mammary tumors and that DROL has a binding affinity for the AEBS in these tumors. Our previous study showed that DROL inhibited the growth and the initiation of DMBA-induced rat mammary tumors.[22] We have no evidence that the AEBS mediates the antitumor effect of DROL on these tumors, and also it was recently shown that AEBS is related to biological actions (e.g., resistance to antiestrogens) other than antitumor effects.[31] Thus, further studies are needed to clarify the relationship between the binding affinity of DROL for the AEBS and the antitumor effect of DROL.

In this study an interesting interaction of DROL or TAM with the ER was observed. From the data shown in Fig. 1A, Fig. 3A and Fig. 4A, the binding ratio of each labeled drug to each receptor in HPLC gel filtration assay was calculated. The binding ratios of ^{3}H-E_2, ^{3}H-DROL and ^{3}H-TAM to the ER and AEBS were 1.5% and 0, 0.1% and 3.2%, <0.1% and 1.0%, respectively. These results suggest that DROL and TAM preferentially bound to the AEBS in the absence of E_2. However, both drugs competed with E_2 for the ER in the presence of E_2 (Fig. 1C, Fig. 1D and Fig. 2). We could not confirm these results in a binding assay other than HPLC gel filtration, because in our preliminary experiment unbound ^{3}H-DROL was not sedimented by the usual charcoal method and it was impossible to separate unbound DROL from DROL bound to the ER or AEBS. These results suggest that DROL and TAM show a binding affinity for the ER only in the presence of E_2. A binding study of 4-hydroxytamoxifen and E_2 to the ER has suggested that 4-hydroxytamoxifen in the presence of E_2 will produce a change in the ER conformation that can reduce the receptor's affinity for E_2.[32] However, we speculate that E_2 may cause a conformational change in the ER and this change may allow binding of DROL to the ER, resulting in a reduction in the affinity of E_2 for the ER. Thus, when E_2 and antiestrogen are present with the ER, there exists not only competition between E_2 and antiestrogens for the ER, but also more complex interactions, which require further examination.

In summary, DROL and TAM have a higher affinity for the AEBS than the ER in the absence of E_2, while in the presence of E_2 both have an affinity for the ER and inhibit the E_2 binding, thus showing an antitumor effect.

(Received December 13, 1993/Accepted February 18, 1994)

REFERENCES

1) Mouridsen, H. T. and Paridaens, R. Advanced breast cancer — new approaches to treatment: workshop report. *Eur. J. Cancer Clin. Oncol.*, **24**, 99–105 (1988).
2) Baum, M., Brinkley, D. M., Dosset, J. A., McPherson, K., Jackson, I. M., Rubens, R. D., Smiddy, F. G., Stoll, B. A., Wilson, A. J., Birch, I. H. and Palmer, M. K. Controlled trial of tamoxifen as a single adjuvant agent in management of early breast cancer. *Br. J. Cancer*, **57**, 608–611 (1988).
3) Early Breast Cancer Trialists' Collaborative Group. Effects of adjuvant tamoxifen and of cytotoxic therapy on mortality in early breast cancer. *N. Engl. J. Med.*, **319**, 1681–1692 (1988).
4) Katzenellenbogen, B. S., Miller, M. A., Eckert, R. L. and Sudo, K. Antiestrogen pharmacology and mechanism of action. *J. Steroid Biochem.*, **19**, 59–68 (1983).

5) Rochefort, H., Borgna, J. L. and Evans, E. Cellular and molecular mechanism of action of antiestrogens. *J. Steroid Biochem.*, **19**, 69–74 (1983).
6) Jordan, V. C. Biochemical pharmacology of antiestrogen action. *Pharmacol. Rev.*, **36**, 245–276 (1984).
7) 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).
8) Rowlands, M. G., Parr, I. B., McCague, R., Jarman, M. and Goddard, P. M. Variation of the inhibition of calmodulin dependent cyclic AMP phosphodiesterase amongst analogues of tamoxifen; correlations with cytotoxicity. *Biochem. Pharmacol.*, **40**, 283–289 (1990).
9) Sutherland, R. L. and Foo, M. S. Differential binding of antiestrogens by rat uterine and chick oviduct cytosol. *Biochem. Biophys. Res. Commun.*, **91**, 183–191 (1979).
10) Sutherland, R. L., Murphy, L. C., Foo, M. S., Green, M. D., Whybourne, A. M. and Krozowski, Z. S. High-affinity anti-oestrogen binding site distinct from the oestrogen receptor. *Nature*, **288**, 273–275 (1980).
11) Kon, O. L. An antiestrogen-binding protein in human tissues. *J. Biol. Chem.*, **258**, 3173–3177 (1983).
12) Sudo, K., Monsma, F. J. and Katzenellenbogen, B. S. Antiestrogen-binding sites distinct from the estrogen receptor: subcellular localization, ligand specificity, and distribution in tissues of the rat. *Endocrinology*, **112**, 425–434 (1983).
13) Winneker, R. C. and Clark, J. H. Estrogenic stimulation of the antiestrogen specific binding site in rat uterus and liver. *Endocrinology*, **112**, 1910–1915 (1983).
14) Kon, O. L. Characterization of an antiestrogen-binding protein in high salt extracts of human breast cancer tissue. *J. Steroid Biochem.*, **22**, 177–186 (1985).
15) Watts, C. K. W. and Sutherland, R. L. Microsomal binding sites for antiestrogens in rat liver; properties and detergent solubilization. *Biochem. J.*, **236**, 903–911 (1986).
16) Murphy, L. C. and Sutherland, R. L. Antitumor activity of clomiphene analogs in vitro: relationship to affinity for the estrogen receptor and another high affinity anti-estrogen-binding site. *J. Clin. Endocrinol. Metab.*, **57**, 373–379 (1983).
17) Reddel, R. R., Murphy, L. C., Hall, R. E. and Sutherland, R. L. Differential sensitivity of human breast cancer cell lines to the growth-inhibitory effects of tamoxifen. *Cancer Res.*, **45**, 1525–1531 (1985).
18) Murphy, L. C. and Sutherland, R. L. Differential effects of tamoxifen and analogs with nonbasic side chains on cell proliferation in vitro. *Endocrinology*, **116**, 1071–1078 (1985).
19) Sutherland, R. L., Watts, C. K. W. and Ruenitz, P. C. Definition of two distinct mechanisms of action of antiestrogens on human breast cancer cell proliferation using hydroxytriphenylethylenes with high affinity for the estrogen receptor. *Biochem. Biophys. Res. Commun.*, **140**, 523–529 (1986).
20) Löser, R., Seibel, K., Roos, W. and Eppenberger, U. *In vivo* and *in vitro* antiestrogenic action of 3-hydroxytamoxifen, tamoxifen and 4-hydroxytamoxifen. *Eur. J. Cancer Clin. Oncol.*, **21**, 985–990 (1985).
21) Kawamura, I., Mizota, T., Mukumoto, S., Manda, T., Masuda, K., Nakamura, T., Kubota, H., Matsumoto, S., Nishigaki, F., Shimomura, K., Mori, J., Masugi, T. and Shibayama, F. Antiestrogenic and antitumor effects of droloxifene in experimental breast carcinoma. *Arzneim. Forsch. Drug Res.*, **39**, 889–893 (1989).
22) Kawamura, I., Mizota, T., Kondo, N., Shimomura, K. and Kohsaka, M. Antitumor effects of droloxifene, a new antiestrogen drug, against 7,12-dimethylbenz(a)-anthracene-induced mammary tumors in rats. *Jpn. J. Pharmacol.*, **57**, 215–224 (1991).
23) Kawamura, I., Mizota, T., Lacey, E., Tanaka, Y., Manda, T., Shimomura, K. and Kohsaka, M. The estrogenic and antiestrogenic activities of droloxifene in human breast cancers. *Jpn. J. Pharmacol.*, **63**, 27–34 (1993).
24) Korenman, S. G. and Dukes, B. A. Specific estrogen binding by the cytoplasm of human breast carcinoma. *J. Clin. Endocrinol. Metab.*, **30**, 639–645 (1970).
25) Thorpe, S. M. Steroid receptors in breast cancer: sources of inter-laboratory variation in dextran-charcoal assays. *Breast Cancer Res. Treat.*, **9**, 175–189 (1987).
26) Pavlik, E. J., van Nagell, J. R., Muncey, M., Donaldson, E. S., Hanson, M., Kenady, D., Ress, E. D. and Talwalkar, V. R. Rapid analysis of estrogen and progesterone receptors using gel-exclusion high-performance liquid chromatography. *Biochemistry*, **21**, 139–145 (1982).
27) Nicholson, R. I., Colin, P., Francis, A. B., Keshra, R., Finlay, P., Williams, M., Elston, C. W., Blamey, R. W. and Griffiths, K. Evaluation of an enzyme immunoassay for oestrogen receptors in human breast cancers. *Cancer Res.*, **46**, 4299–4302 (1986).
28) Walker, K. J., Bouzubar, N., Robertson, J., Ellis, I. O., Elston, C. W., Blamey, R. W., Wilson, D. W., Griffiths, K. and Nicholson, R. I. Immunocytochemical localization of estrogen receptor in human breast tissue. *Cancer Res.*, **48**, 6517–6522 (1988).
29) Greene, G. L., Sobel, N. B., King, W. J. and Jensen, E. V. Immunochemical studies of estrogen receptors. *J. Steroid Biochem.*, **20**, 51–56 (1984).
30) Robertson, J. F. R., Bates, K., Pearson, D., Blamey, R. W. and Nicholson, R. I. Comparison of two oestrogen receptor assays in the prediction of the clinical course of patients with advanced breast cancer. *Br. J. Cancer*, **65**, 727–730 (1992).
31) Pavlik, E. D., Nelson, K., Srinivasan, S., Powell, D. E., Kenady, D. E., Depriest, P. D., Gallion, H. H. and van Nagell, J. R. Resistance to tamoxifen with persisting sensitivity to estrogen: possible mediation by excessive antiestrogen binding site activity. *Cancer Res.*, **52**, 4106–4112 (1992).
32) Sasson, S. and Notides, A. C. Mechanism of the estrogen receptor interaction with 4-hydroxytamoxifen. *Mol. Endocrinol.*, **2**, 307–312 (1988).