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INTERACTION OF DP-TAT-59, AN ACTIVE METABOLITE OF NEW TRIPHENYLETHYLENE-DERIVATIVE (TAT-59), WITH ESTROGEN RECEPTORS

TOSHIYUKI TOKO,[1]* KEN-ICHI MATSUO,[1] JIRO SHIBATA,[1] KONSTANTY WIERZBA,[1] MAMORU NUKATSUKA,[1] SETSUO TAKEDA,[1] YUJI YAMADA,[1] TETSUJI ASAO,[1] TAKAHISA HIROSE[2] and BUNZO SATO[2]

[1]Biological Research Laboratories, Taiho Pharmaceutical Co. Ltd, Kawauchi-cho, Tokushima and [2]The Third Department of Internal Medicine, Osaka University Hospital, Fukushima-ku, Osaka, Japan

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Summary—DP-TAT-59, (*Z*)-2-(4-(1-(4-hydroxyphenyl)-2-(4-isopropylphenyl)-1-butenyl) phenoxy)-*N*, *N*-dimethylethylamine, has been reported to inhibit estrogen-stimulated growth of MCF-7 cells as well as rat uterus at lower concentrations than the hydroxymetabolite of tamoxifen (4-OH-TAM). In the present study, the growth of mouse Leydig cell tumor, B-1F cells were also more effectively inhibited by DP-TAT-59 than 4-OH-TAM. Additionally, the expression of estrogen responsive element ligated CAT gene transfected into B-1F cells was also suppressed by DP-TAT-59. Thus, the interaction of DP-TAT-59 with estrogen receptor (ER) was characterized and compared with that of 4-OH-TAM using immature rat and bovine uteri. The dissociation constant of DP-TAT-59 to ER of immature rat uterus was 0.24 nM and was similar to that of 4-OH-TAM ($K_d = 0.20$ nM) and estradiol ($K_d = 0.29$ nM). Using sucrose density gradients, the sedimentation constant of DP-TAT-59 with bovine uterus was 4.9S, which was similar to that of estradiol (5.1S) and 4-OH-TAM (5.3S). However, the elution profile of the DP-TAT-59–ER complex from a DEAE-Sephadex column was different for both estradiol–and 4-OH-TAM–ER complexes. These results suggest that ER forms different complexes with DP-TAT-59 than estradiol or 4-OH-TAM, while the ER binding affinity of these compounds are similar to each other.

INTRODUCTION

Triphenylethylene-derivatives, such as tamoxifen (TAM), are used as an adjuvant therapy in the treatment of hormone-dependent breast cancer [1]. While some investigators reported that the growth inhibitory effect of antiestrogens was related to their affinity to estrogen receptor (ER) [2–4], in this respect the relative binding affinity of TAM to ER ranges from 0.01 to 30% in which the affinity of estradiol is considered 100% [5], the other mechanism of antitumor action of TAM is considered. In fact, up to the concentration range of 10^{-5} to 10^{-7} M, the other type of activity than that mediated through ER, was observed, e.g. calmodulin antagonistic activity [6], binding to nonsteroidal antiestrogen binding sites [7]. However, the action mechanisms of triphenylethylene compounds are far from conclusive. In addition, their limited antitumor effects require us to develop more potent drugs. Therefore, in order to improve the therapeutic efficacy of antiestrogens, we synthesized new triphenylethylene-derivatives with an increased intratumor accumulation. Among these compounds, TAT-59, exhibiting such properties and superior to TAMs antitumor effect, was selected for further clinical studies [8]. The mechanism of action of these antiestrogens is at present unclear, it is assumed that antiestrogenic activity may be mediated through competition with the binding of estrogens to ER [4], followed by the inhibition of estrogen-induced activation of ER [9]. Also, the difference between the conformation of ER complexes with antiestrogen and that with estradiol might contribute to the antiestrogenic activity. Such conformational differences of ER were shown by the different binding ability of antiER antibody [10], different sedimentation constant [11], and an altered elution pattern of ER from DEAE-Sephadex anion exchange column chromatography [12]. Therefore, we assumed that the superior antiestrogenic effect of TAT-59, compared to TAM, may result from such conformational differences.

Our pharmacokinetic study, by oral administration to rats, revealed that TAT-59 was

*To whom correspondence should be addressed.

rapidly metabolized to its active metabolite, DP-TAT-59 and that TAM was highly converted to its hydroxymetabolite, 4-OH-TAM, having similar ER affinity to estradiol [8]. We then examined the interaction of DP-TAT-59 with the ER and characterized the ER complexes formed in the presence of DP-TAT-59 and 4-OH-TAM, to test whether the alteration in the conformation of ER complexes may contribute to their different antitumor potencies.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]estradiol (115 Ci/mmol) and *Z*-4-hydroxy-[*N*-methyl-^{3}H]TAM (85 Ci/mmol) were obtained from Amersham Int. (Amersham, England). [^{3}H]DP-TAT-59 was synthesized by Amersham. 16a-^{125}iode-3,17β-estradiol (2200 Ci/mmol) and D-threo-[dichloroacetyl-1,2-^{14}C] chloramphenicol (60 mCi/mmol) were purchased from New England Nuclear (Boston, MA). 17β-Estradiol was purchased from Sigma Chemicals (St Louis, MO). 4-OH-TAM and DP-TAT-59 were prepared in our laboratory. DEAE-Sephadex A-25 (Pharmacia LKB, Uppsala, Sweden) was used for ion-exhange column chromatography. The B-1F cells were grown in RPMI1640 medium, supplemented with 5% fetal calf serum (FCS; Gibco, NY). Immature rats 3- to 4-week-old (Japan Clea Inc., Tokyo, Japan) and bovine uteri, obtained from our local slaughterhouse, were used in this study.

Growth inhibition test

The B-1F cells were detached with 0.25% trypsin, pelleted, resuspended in Ham's F-12–Dulbecco's modified MEM (1:1) containing 0.1% bovine serum albumin (BSA), 10^{-9} M estradiol and phenol red (8.2 mg/l) and plated in triplicate in 60 mm plastic tissue culture dishes. After cell attachment (20–24 h), antiestrogens were added to the medium. Final concentration of dimethylsulfoxide (DMSO) (0.1%) had no effect on the cell growth. Seven days after the drug application, cells were collected and counted using a hemocytometer.

CAT assay

Using synthetic oligonucleotide containing estrogen responsive element (ERE), the reporter plasmid which had an ERE-thymidine kinase promotor-CAT fusion gene [$(ERE)_3$-tk-CAT] was constructed and transfected into a ER-positive mouse Leydig tumor cell line, B-1F cell, by the electroporation method as described previously [13]. After transfection, the cells were placed in culture dishes with or without antiestrogens in the presence of 10^{-10} M of estradiol. After 48 h, cells were collected and suspended with 120 μl of 0.25 mM Tris–HCl, pH 7.8, and lysed by freezing–thawing. The supernatant (10,000 *g*, 10 min) was used as a cell extract. The extract was incubated with 0.1 μCi of [^{14}C]chloramphenicol and 20 μl of 4 mM acetyl-CoA for 1 h at 37°C to determine CAT enzymic activity. The reaction products were separated by thin-layer chromatography (TLC), and the TLC plate was subjected to autoradiography.

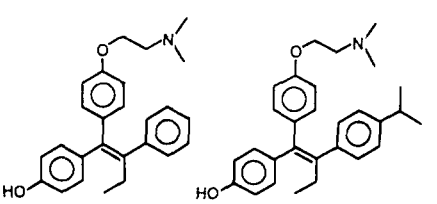
Cytosol preparation

Uterine tissues were stored at −80°C until use. There was no loss in ER content during 1 month storage under this condition. Tissues were thawed, minced and homogenized in 3 vol of buffer A (20 mM Tris, 5% glycerol, 12 mM monothioglycerol, pH 7.5) using a Biotron homogenizer. The homogenate was then centrifuged at 105,000 *g* for 1 h to obtain the cytosol. For sucrose density gradient analysis and DEAE-Sephadex column chromatographic analysis, the cytosol was incubated on ice for 2 h with either [^{3}H]estradiol, [^{3}H]4-OH-TAM or [^{3}H]DP-TAT-59 both in the absence and presence of 100-fold excess of cold estradiol. The labeled cytosol was mixed with a pellet from an equal volume of dextran-coated charcoal (DCC, 1.0% activated charcoal and 0.1% dextran in buffer A) for 15 min and centrifuged at 3000 rpm for 25 min at 4°C.

If it was necessary to avoid the influence of an endogenous estrogen, we used the cytosol of immature rat uteri as the source of ER. In other experiments, the bovine uterine cytosol was used as the source of ER.

Binding studies

Relative binding affinities to ER were determined in the competition experiments (18 h, on ice) using 5 nM tritiated ligand (estradiol, 4-OH-TAM or DP-TAT-59) and increasing



4-OH-Tamoxifen DP-TAT-59

Fig. 1. Chemical structures of 4-OH-TAM and DP-TAT-59.

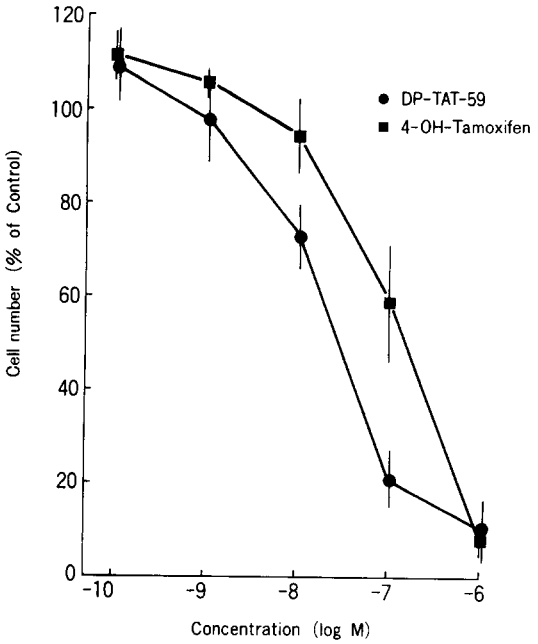


Fig. 2. Inhibitory effect of antiestrogens on the estrogen-stimulated growth of B-1F cells. Each point represents the mean of three experiments. The growth was maintained in the presence of 10^{-9}M estradiol. ●—●, DP-TAT-59; ■—■, 4-OH-TAM.

concentrations of unlabeled estradiol or antiestrogen. To calculate the number of specific binding sites and dissociation constant, the data were analyzed according to Scatchard [14, 15].

High salt sucrose density gradient

The labeled cytosols were adjusted to 0.4 M KCl and layered on 5.0 ml of buffer A containing 0.4 M KCl and a 5–20% (w/v) linear sucrose density gradient. The tubes were centrifuged in a Hitachi model 65P ultracentrifuge equipped with Hitachi RPS50-2 rotor at 50,000 rpm for 16 h. Three drops were collected for each fraction. [^{14}C]methylated gamma-globulin (6.6S) and [^{14}C]methylated ovalbumin (3.7S), obtained from Amersham, were used as internal sedimentation-coefficient markers.

DEAE-Sephadex column chromatography

DEAE-Sephadex A-25 columns (5 ml bed volume) were washed with 50 vol of buffer B (10 mM Tris, 1.5 mM EDTA, 10 mM monothioglycerol, 0.5 nM PMSF, 10 mM molybdate, pH 7.5). The labeled cytosols were loaded onto the columns and were then washed with 25 ml of buffer B. The biological material was eluted from the column with a linear concentration gradient of KCl (0–0.4 M) in buffer B at 1 ml/min. The eluent was collected in 2 ml fractions. The peak fractions were collected and concentrated by ultrafiltration with Centriflo membrane cones (type CF-25, Amicon). After ultrafiltration, the concentrated fractions in membrane cones were rinsed with buffer B to remove the KCl and rechromatographed.

[^{125}I]estradiol exchange assay

The concentrated peak fractions, obtained from DEAE-Sephadex column chromatography of the receptor labeled with [^{3}H]DP-TAT-59, were incubated for 2 h at 20°C in the presence of [^{125}I]estradiol and then treated with DCC to remove free ligands.

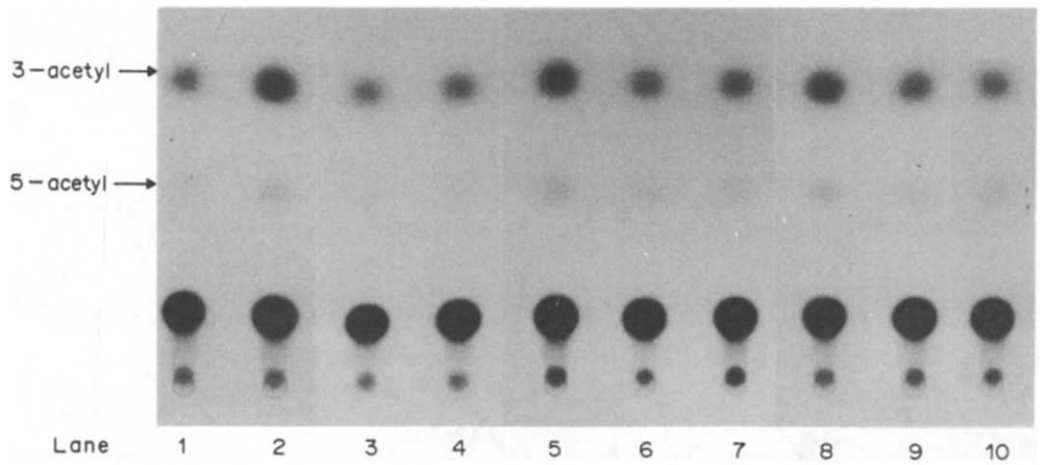


Fig. 3. Suppression of estrogen-induced CAT activity in the ER-positive Leydig tumor cell line (B-1F cells) transfected with reporter plasmid [$(ERE)_3$-tk-CAT] by DP-TAT-59 or 4-OH-TAM. CAT activity in extract from untreated cells (lane 1); cells treated with 10^{-10} M estradiol (lane 2); cells treated with 10^{-10} M estradiol in the presence of 10^{-8} M DP-TAT-59 and 10^{-8} M 4-OH-TAM (lanes 3 and 4, respectively); cells treated with 10^{-8} M estradiol in the presence of 10^{-10}, 10^{-9} and 10^{-8} M DP-TAT-59 (lanes 5, 6, and 7, respectively); cells treated with 10^{-8}M estradiol in the presence of 10^{-10}, 10^{-9} and 10^{-8} M 4-OH-TAM (lanes 8, 9 and 10, respectively).

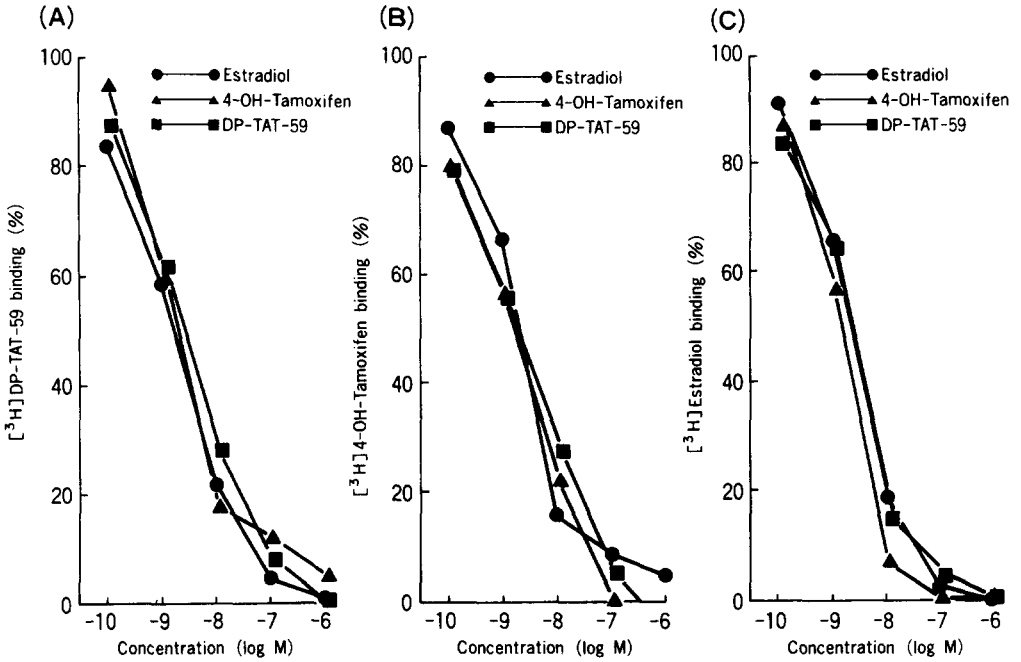


Fig. 4. Competitive binding of 5×10^{-9} M [^{3}H]DP-TAT-59 (panel A), [^{3}H]4-OH-TAM (panel B) or [^{3}H]estradiol (panel C) in the presence of different concentrations of an interacting compound.

RESULTS

Inhibition of estrogen-stimulated growth of B-1F cells

Nishizawa *et al.* [16] reported that the proliferation rate of B-1F cells was stimulated by the addition of estradiol and that antiestrogen 4-OH-TAM blocked the estrogen-induced proliferation of B-1F cells. Therefore, using these B-1F cells, we tried to estimate the antiestrogenic activity of DP-TAT-59. As a result, both DP-TAT-59 and 4-OH-TAM showed a growth inhibitory effect on the estrogen-stimulated growth of B-1F cells (Fig. 2). In the presence

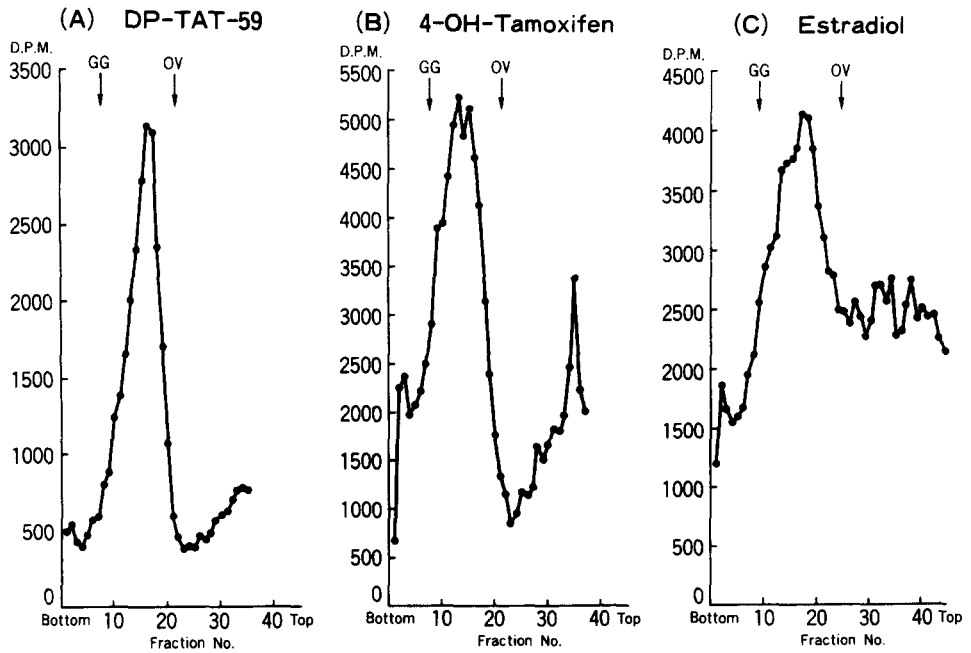


Fig. 5. High salt sucrose gradient analysis of cytoplasmic receptor complexes with [^{3}H]DP-TAT-59 (panel A), [^{3}H]4-OH-TAM (panel B) or [^{3}H]estradiol (panel C). OV, ovalbumin; GG, gamma-globulin.

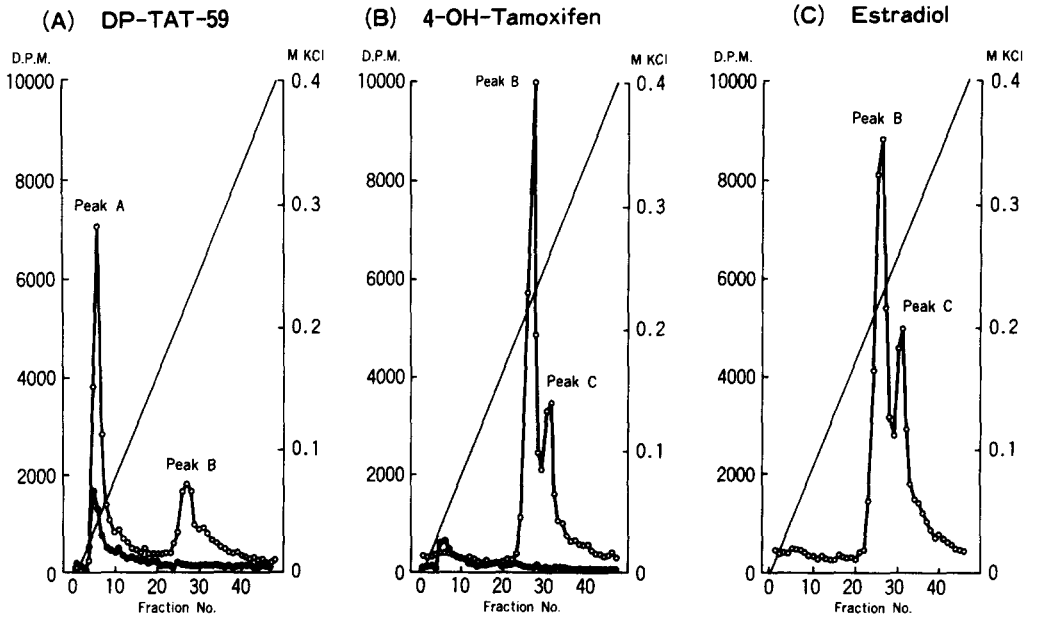


Fig. 6. DEAE-Sephadex chromatography of cytoplasmic receptor complexes with [^{3}H]DP-TAT-59 (panel A), [^{3}H]4-OH-TAM (panel B), or [^{3}H]estradiol (panel C). ●—●; in the presence of 100-fold excess of unlabeled estradiol.

of 10^{-9} M estradiol, the concentration needed to achieve a 50% inhibition of growth of the B-1F cell was 47 and 125 nM for DP-TAT-59 and 4-OH-TAM, respectively. There is a 10-fold difference in the concentration of these two antiestrogens needed to obtain 50% inhibition.

CAT assay

Since the B-1F cell showed different sensitivity to antiestrogens, in order to examine whether the receptor complex formed with these antiestrogens is able to inhibit the estrogenic stimulation mediated through the acceptor site on the DNA, the ERE plus CAT gene was

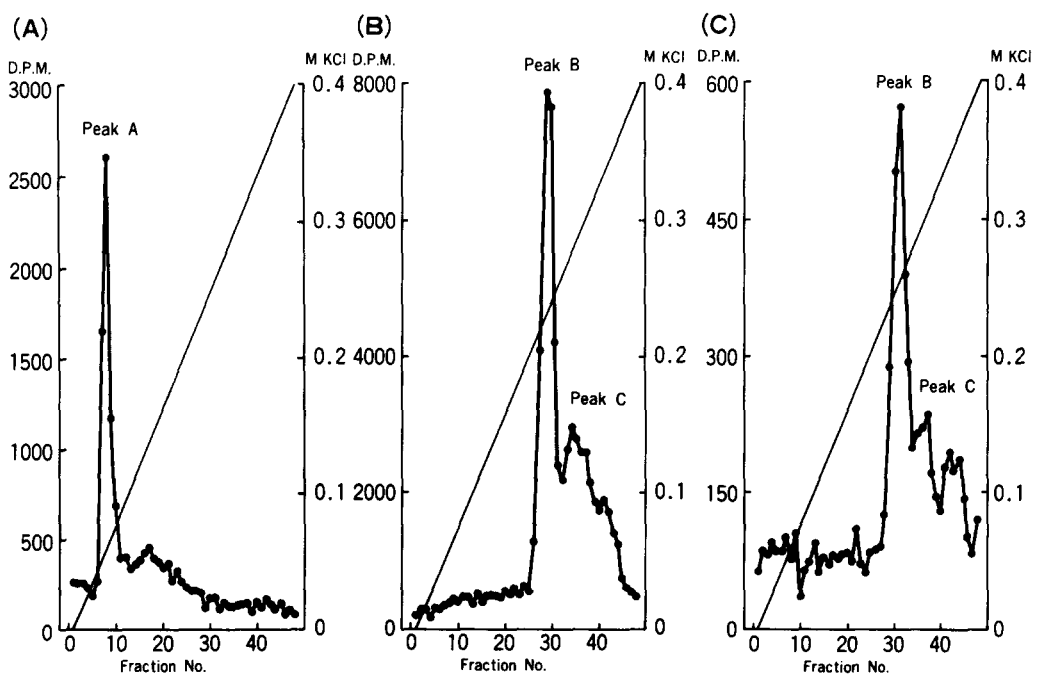


Fig. 7. Rechromatography of peak fractions obtained from DEAE-Sephadex chromatography of receptor complex with [^{3}H]DP-TAT-59. Panel A, peak A fraction; panel B, peak B fraction; panel C, peak C fraction.

constructed and transfected into ER-positive B-1F cells [13]. As shown in Fig. 3, CAT gene activity was enhanced by the addition of 10^{-10} M of estradiol and this estrogen-induced augmentation of CAT activity was suppressed to the control level in the presence of either 4-OH-TAM (10^{-8} M) or DP-TAT-59 (10^{-8} M). Both DP-TAT-59 and 4-OH-TAM did not show any estrogenic activity even a concentration of 10^{-8} M.

Competitive binding of antiestrogens

The binding of 5×10^{-9} M tritiated ligands to immature rat uterine cytosols in the presence of varying concentrations of nonradioactive ligands was determined to compare the binding affinity of antiestrogens. As shown in Fig. 4(A), the binding of [^{3}H]DP-TAT-59 to the cytoplasmic receptor was inhibited by both estradiol and 4-OH-TAM. A similar pattern was observed in the case of [^{3}H]4-OH-TAM [Fig. 4(B)] and estradiol [Fig. 4(C)]. The concentrations of these three compounds necessary to achieve 50% inhibition of the binding of a tritiated ligand to cytoplasmic receptor, were similar to each other. Based on these results, it was suggested that these three compounds were able to bind to the same cytoplasmic receptor with similar affinity, as confirmed by the analysis of corresponding Scatchard-plots. Using immature rat uterine cytosol, the dissociation constants (K_d) for cytosolic receptor were 2.4×10^{-10}, 2.0×10^{-10} and 2.9×10^{-10} M for DP-TAT-59, 4-OH-TAM and estradiol, respectively. In separate experiments, the range of K_d values of these three compounds varied from 1.6×10^{-10} to 6.7×10^{-10} M and overlapped each other, although, the same number of binding sites was obtained for each compound.

Analysis of receptor complexes by high salt sucrose density gradient

Sedimentation profiles of cytosolic receptor complexes with [^{3}H]estradiol, [^{3}H]4-OH-TAM or [^{3}H]DP-TAT-59 are shown in Fig. 5. The major peak of ER complexes with [^{3}H]DP-TAT-59, [^{3}H]4-OH-TAM or [^{3}H]estradiol appeared at 4.9, 5.3 or 5.1S, respectively. The sedimentation constant of receptor complexes labeled with [^{3}H]DP-TAT-59 or [^{3}H]4-OH-TAM were similar to that of [^{3}H]estradiol. In the presence of a 100-fold excess of unlabeled estradiol, the binding of tritiated ligand to cytosolic receptor was completely inhibited.

Analysis of receptor complexes by DEAE-Sephadex column chromatography

Although there were no significant differences in sedimentation profiles indicating a similar size of receptor complexes, the difference among receptor complexes can be detected by another analytical method, such as surface ion charge. To investigate this aspect, the receptor complexes were chromatographed on DEAE-Sephadex. As shown in Fig. 6, two peaks were eluted at approx. 0.22 and 0.26 M KCl concentration (peaks B and C) in the elution profile of the receptor complexes labeled with [^{3}H]estradiol. However, column chromatography of the receptor complexes labeled with [^{3}H]DP-TAT-59 yielded an additional peak at 0.05M KCl (peak A) which was not present when estradiol was used. As already described by Ruh and Ruh [17], we also used molybdate, a phosphorylase inhibitor, as a stabilizer of ER and PMSF to protect ER against enzymic digestion. Under this condition, ER should retain its conformation intact during the chromatography.

To check the binding capacity of cytosol for each ligand, we used both the same cytosol and the same amount of each ligand to form a

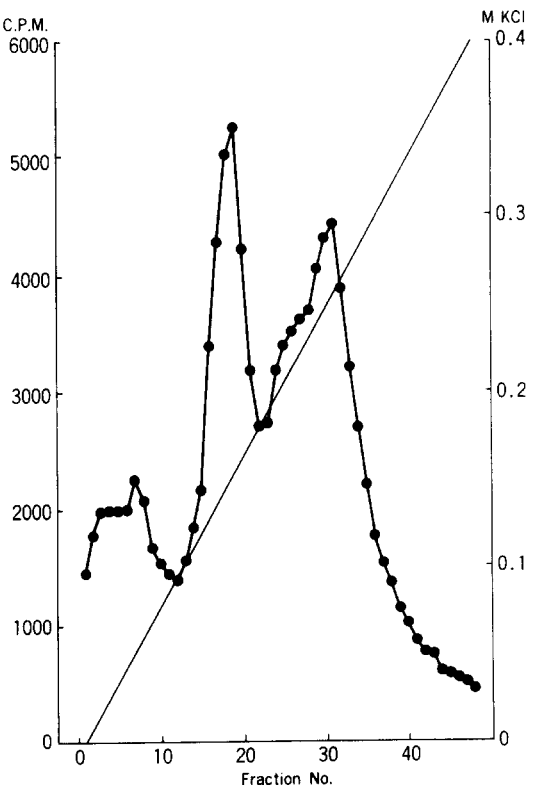


Fig. 8. DEAE-Sephadex chromatography of recovered ER complex from peak A, monitored with [^{125}I]estradiol.

complex with the receptor. As shown in Fig. 6, total radio-activities of peaks obtained from the chromatography of each ligand–receptor complex were similar to each other. In the presence of a 100-fold molar excess of estradiol during the incubation period of cytosol with each tritiated ligand, all peaks disappeared due to displacement of the radio-labeled ligand from its cytosolic receptor complex.

In a separate experiment, the receptor complexes labeled with [^{3}H]4-OH-TAM yielded a peak at 0.05M KCl but it was only found at a minor level. Then, one may think that peak A was formed from ER by binding with antiestrogen. These results suggest that DP-TAT-59 formed a different receptor complex from estradiol at a higher level than 4-OH-TAM did.

Rechromatography and [^{125}I]estradiol exchange assay of a receptor complex formed with [^{3}H]DP-TAT-59

To compare the properties of peaks, identified during the DEAE-Sephadex chromatography of the receptor complex formed with [^{3}H]DP-TAT-59, rechromatography of each peak and exchange assays were performed. The peak fractions were collected, concentrated 10-fold by ultrafiltration and rinsed with buffer B on the membrane cones and rechromatographed. As shown in Fig. 7, peak A was eluted at the same position on rechromatography, whereas rechromatography of either peak B or C revealed the presence of two peaks [Fig. 7(B and C)]. In order to characterize peak A, the ligand of the receptor complex which eluded at this position was exchanged with [^{125}I]estradiol and then the complex was loaded onto a DEAE-Sephadex column. After exchanging the ligand, no residual radio-activity due to ^{3}H was observed. As shown in Fig. 8, chromatography of the receptor complex yielded similar peaks to that shown in Fig. 6(C), whereas the detailed profile was different. Therefore, it was concluded that the [^{3}H]DP-TAT-59 binding component eluted at peak A is the ER.

DISCUSSION

Comparison of the new antiestrogen DP-TAT-59 with an active metabolite of known antiestrogen 4-OH-TAM revealed different characteristics of their ER complexes (Fig. 6), despite a similarity in binding affinities (Fig. 4). In principle, studies on the mechanism of antagonistic action of the antiestrogens are complicated by the fact that triphenylethylene-derivatives are not only antagonist but also partially agonist [5, 18, 19]. A minimal agonistic activity was also observed in the case of DP-TAT-59, its behavior was similar to that of estradiol in terms of binding kinetics and sedimentation profile. Despite that fact, the elution pattern of the ER complex formed with DP-TAT-59 from a DEAE-Sephadex column showed a different pattern from that formed with estradiol, while the profile obtained from the elution of the ER complex bound with estradiol was similar to that reported by Ruh and Ruh [17]. Therefore, based on our experiment, we might conclude that the difference in the elution profile of the ER complex formed with DP-TAT-59 with estradiol was due to the different characteristics of these two compounds.

Substances interacting with DNA are positively charged on their surface. Parchman and Litwack [20] reported that the activation of glucocorticoid receptor, possessing an acceptor on the molecule of DNA, altered the affinity to DEAE-Sephadex from high to low, due to an increase of their basicity. Similar properties can be induced in ER following activation either by heat, high ionic strength, calcium ion, etc [21], the ER may be conformationally changed to express a positive charge on the surface of the receptor complex, such as glucocorticoid receptor [22]. Through such interaction, the estradiol–ER complex can activate the estrogen-related gene [23]. Although the suppressive ability of DP-TAT-59 against the expression of the CAT gene, transfected into ER-positive B-1F cells, mediated through ERE was similar to that of 4-OH-TAM (Fig. 3), DP-TAT-59 showed a stronger inhibitory effect on the estrogen-induced proliferation of B-1F cells than 4-OH-TAM (Fig. 2). The apparent difference of DP-TAT-59 potencies between estradiol mediated gene expression and cell proliferation may be due to the duration required for those assays (2 days for CAT assay and 6 days for cell growth assay). Nonetheless, DP-TAT-59 exerts the profound actions to ER-dependent events and also formed a different receptor complex from estradiol at a higher level than 4-OH-TAM did (Fig. 6). Therefore, one may suspect that a quantitative difference of fraction of the ER complexes eluted at a lower KCl concentration may contribute to the reactivity of the antiestrogen–ER complex with the other acceptor

site(s) on the DNA molecule than ERE, while the suppressive ability against the response mediated through ERE, which was used in this assay was similar to each other.

As shown in our previous report [8], DP-TAT-59 exhibited superior growth inhibitory activity against MCF-7 cells *in vitro* than 4-OH-TAM, despite sharing a similar chemical structure and competitive activity to estradiol on ER. No differences in their ability to penetrate cells or to bind to cystosolic proteins have been found to account for their different activities (unpublished observations). Since H1285, CI628M and 4-OH-TAM were reported to form a different complex with ER than that of ER and estradiol [9, 12, 24], it was supposed that the different hormonal activity of these antiestrogens on ER was caused by such conformational changes in the ER complex induced by antiestrogens. Although both DP-TAT-59 and 4-OH-TAM possess a similar positive charge on their molecule, the complexes formed with the ER are showing a pronounced quantitative difference between these two compounds on the ion-exchange chromatography. The exact differences between these two complexes are still unclear, however they may contribute to a stronger antagonistic activity of DP-TAT-59 against estrogen-dependent tumors.

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