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TAT-59, a New Triphenylethylene Derivative with Antitumor Activity Against Hormone-dependent Tumors

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Abstract—*The antiestrogenic action of TAT-59 {(E)-4-[1-[4-[2-(dimethylamino)ethoxy]-phenyl]-2-(4-isopropyl)phenyl-1-butenyl]phenyl monophosphate} was characterized and compared with that of Tamoxifen (TAM). Its active metabolite, 4-OH-TAT-59, had a high binding affinity to estrogen receptor (ER), present in the cytosol of the uterus of immature rat, similar to estradiol. TAT-59 and 4-OH-TAT-59 inhibited* in vitro *estrogen-stimulated proliferation of MCF-7 cells at a lower concentration than TAM. In the absence of estradiol, TAT-59 and 4-OH-TAT-59 were effective at a lower concentration than that of 4-OH-Tamoxifen (4-OH-TAM), the active metabolite of TAM. In uterine growth inhibition, the effective dose of TAT-59 was about 3–6-fold lower than that of TAM, in various administration schedules. The minimum effective dose of TAT-59 against* in vivo *MCF-7 cells was about 3-fold lower than that of TAM. In DMBA-induced rat mammary tumors, TAT-59 inhibited the growth of existing tumors at about a 10-fold lower dose than TAM. Especially in the tumors with low ER levels (10–20* fmol/mg *protein), TAT-59 showed a significantly stronger inhibitory effect than TAM. These experiments showed that TAT-59 was more effective in lower doses than TAM, even against the tumors with low ER content.*

INTRODUCTION

RECENTLY, endocrine therapy has been recognized as one of the useful treatments of patients with breast cancer. In particular, some antiestrogenic agents, such as Tamoxifen (TAM), have been found to be effective adjuvant therapy of surgical treatment [1].

However, in premenopausal patients with breast cancer, the clinical effect of TAM is not well established. Some clinical investigations indicate that premenopausal patients still have functioning ovaries, which may interfere with TAM activity. Pritchard *et al.* [2] have reported TAM treatment to be effective at the beginning of therapy, and later, it failed to show a beneficial activity, although in the above case the subsequent ovariectomy gave good clinical results. Senoo *et al.* [3] described that TAM was still effective in some cases of recurrent breast cancer, when used at a high dose, which can be accompanied by several side-effects, usually observed at doses higher than 40 mg daily (a commonly used dosage in clinical practice) without significant improvement of its antitumor efficacy [4].

Still the mechanism of action of antiestrogens is not clear; it is supposed that antiestrogen suppresses the estrogenic stimulation of tumor growth competing with the estrogen to its binding site [5, 6]. Since the values of the apparent affinity of TAM to the estrogen receptor (ER) range between 0.01 and 30% of that obtained for estradiol [7], a high concentration of TAM is necessary to exert antiestrogenic effects in cell culture [8, 9]. Increased plasma estradiol levels in premenopausal women receiving TAM have been reported [10, 11], consequently, the elevated estradiol concentration of in the plasma may decrease the competitive binding of TAM to ER, resulting in lower antitumor activity of TAM.

In postmenopausal patients, the clinical efficacy of TAM was established to be approximately 50% in ER positive cases [12]. There is a relationship between clinical efficacy and tumor ER content: in patients with tumor ER levels < 30 fmol/mg pro-

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tein, the recurrence of the tumor was significantly increased in comparison to those with ER levels > 30 fmol/mg protein [13]. Furthermore, the addition of TAM to already well-established chemotherapeutic regimes does not give any benefit to patients with tumors containing a low ER concentration [14]. These clinical observations have been confirmed in laboratory animals with DMBA-induced mammary carcinoma. Tumor regression was correlated with its ER concentration [15]. Therefore, it may be concluded that the antitumor effect of TAM depends on ER content in the tumor, and a low ER level is a bad prognostic factor.

Recently, other mechanisms of antitumor activity than that mediated through the ER have been reported [16–19]. Therefore, if a new antiestrogen can penetrate to the tumor more easily, leading to a greater accumulation in the tumor, such compounds may well show a growth inhibitory effect against the tumor with low ER content and/or without ER.

Approximately 50% of ER positive patients respond to TAM therapy. Also, the ER content in the tumors of about 50% of patients varied from 10 to 60 fmol/mg protein [20], which can be considered as a low level. It may be possible that a new antiestrogen will show a better clinical effect in the group of patients with low ER concentrations.

Our aim has been to devise an antiestrogen with the characteristics of a higher affinity to ER, a raised intratumor concentration and an action other than that mediated through the ER, thereby improving its antitumor action compared to TAM. In order to develop such a compound, we synthesized about 100 new triphenylethylene derivatives. Among these compounds, TAT-59 exhibited such a properties and was selected for further preclinical studies. In this paper, we present the results of preclinical studies of TAT-59 (Fig. 1).

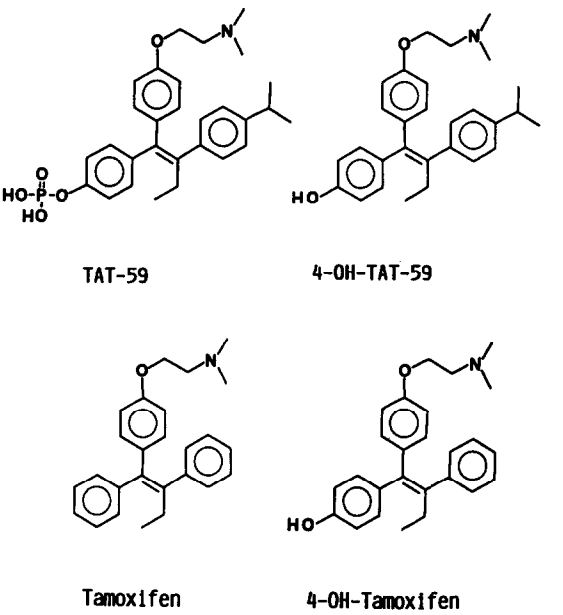

Fig. 1. Chemical structures of TAT-59, Tamoxifen and their hydroxy metabolites.

MATERIALS AND METHODS

Materials

Tamoxifen citrate and 17β-estradiol were purchased from Sigma Chemicals. [2,4,6,7-^{3}H]Estradiol (105 Ci/mmol) was obtained from New England Nuclear. 7,12-Dimethylbenzanthracene (DMBA) was obtained from Tokyo Chemicals Co. 4-OH-TAM, TAT-59 and 4-OH-TAT-59 were synthesized in our institute. Estrogen and antiestrogens were first dissolved in dimethylsulfoxide (DMSO) to prepare stock solutions which were diluted with buffer or culture media for *in vitro* experiments or in 0.5% carboxymethylcellose containing 0.5% Tween 80 for *in vivo* experiments. MCF-7 cells (kindly provided by Dr Abe, Keio University, Japan) were grown in RPMI1640 medium, supplemented with insulin (10 μg/ml; Sigma) and 10% fetal calf serum (FCS; Gibco). Female Sprague–Dawley (SD) rats and BALB/c (nu/nu) mice were purchased from Shizuoka Laboratory Animal Center and Japan Clea Inc.

Methods

Binding studies. Uteri and tumors from female rats were removed as soon as possible after decapitation, and immediately transferred and stored at −80°C until use. Tissues were homogenized in buffer (20 mM Tris, 1.5 mM EDTA, 5% glycerol, 12 μM monothioglycerol, pH 7.8) using a Biotron homogenizer. The homogenate was then centrifuged at 105,000 ***g*** for 1 h, and supernatant (cytosol) was used for ER binding assay. The number of [^{3}H]estradiol-binding sites were determined using the dextran-coated charcoal (DCC) method [21]. The binding was corrected for nonspecific binding using a 100-fold excess of nonradioactive estradiol. Relative binding affinities to ER of uterine cytosol were determined in competition experiments (18 h at 4°C) using 5 nM [^{3}H]estradiol and increasing concentrations of antiestrogens or unlabeled estradiol.

Growth inhibition test. Seven days before the start of experiment, the culture medium was replaced with the same medium containing 5% FCS treated with DCC (DCC-FCS) to remove endogenous estrogens. Then cells were detached with 0.25% trypsin and 1 mM EDTA, pelleted, resuspended in the medium containing 5% DCC-FCS, and plated in triplicate in 60 mm plastic tissue culture dishes. After cell attachment (20–24 h), the drugs were added to the medium. Final concentration of DMSO (0.1%) had no effect on the cell growth. Ten days after the treatment, cells were collected and counted using hemocytometer.

Antiuterotrophic test. Female SD rats (4 weeks old, seven rats/group) were used to assay the antiuterotrophic activities of the compounds. In order to estimate the value of inhibition without endogenous estrogen, the animals were ovariectomized under ether anesthesia. The compounds were administered orally for 3 weeks at various administration schedules. Then the animals were killed and the wet weight of the uterus was determined. Inhibition % of uterine growth was calculated based on wet weight of uterus using the following equation:

$$\text{Inhibition } \% = \frac{(\text{control} - \text{tested})}{(\text{control} - \text{ovariectomized})} \times 100.$$

The value of ED_{50} was calculated from linear regression lines of the logit–log plot.

Antitumor test. 1×10^7 of MCF-7 cells were inoculated s.c. in female athymic mice (BALB/c, nu/nu, 10 weeks old). Drug administration was started when a tumor had reached a diameter of about 5–10 mm. Antiestrogens were administered daily for 4 weeks (six times/week). 17β-Estradiol (0.1 μg/body) was injected i.m. once a week in 0.05 ml of sesame oil.

Mammary carcinomas were induced in 49-day-old female SD rats by a single p.o. dose of 20 mg DMBA in 1.0 ml of sesame oil. Drug treatment was started when the tumors had reached a diameter of about 10 mm. Antiestrogens were administered p.o., daily for 4 weeks (six times/week). Animals were palpated once a week. The sizes of the tumors were recorded following palpation, using two perpendicular diameters. The volume of the tumor was estimated as follows:

$$\text{Tumor volume } (mm^3) = (\text{width})^2 \times (\text{length})/2.$$

The percentage change of the tumor volume was calculated on the initial and the final tumor volume.

The numbers of ER in DMBA-induced tumors were determined in small biopsy samples taken 10 days before antiestrogen treatment. The DMBA-induced tumors, due to their heterogeneity, were classified as follows: (1) progressing tumors with volume changed by more than 150% of the initial value; (2) stable tumors with volume change ranging from 50 to 150% of initial value; (3) regressing tumors with volume decreased to less than 50% of the original volume.

Student's *t* test and the χ^2-test was used in statistical analysis.

Preliminary pharmacokinetic study. DMBA-induced mammary tumor-bearing rats were given orally 0.3 mg/kg of antiestrogens. Plasma and tumor tissues were collected at the indicated time periods after treatment. Tissue and plasma concentrations of the administered compounds and their metabolites were determined by HPLC as described by Brown *et al.* [22].

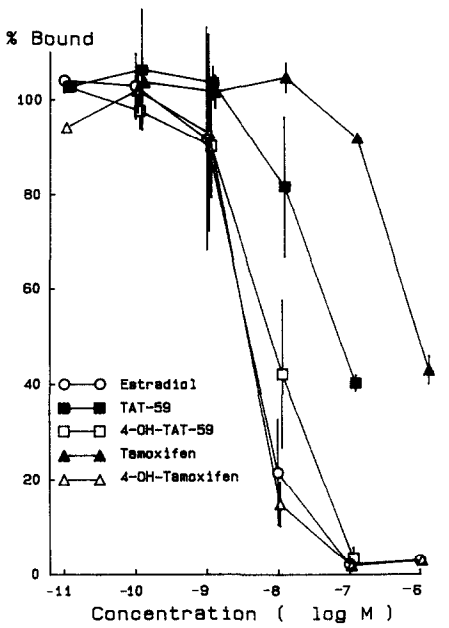

Fig. 2. Competitive binding between TAT-59, 4-OH-TAT-59, Tamoxifen, 4-OH-Tamoxifen and estradiol with 5×10^{-9} M [^{3}H]*estradiol. Points and bars represent the mean and S.D.* (n ≧ 3).

RESULTS

The results of the competitive assay are shown in Fig. 2. The specific binding of [^{3}H]estradiol to rat uterine ER was inhibited by the active metabolite of both TAT-59 and TAM. The concentration necessary to achieve a 50% inhibition of the specific binding of 5×10^{-9} M estradiol, was 5.37×10^{-9} and 3.63×10^{-9} M for 4-OH-TAT-59 and 4-OH-Tamoxifen, respectively. 10^{-7} M of TAT-59 seems to be able to inhibit the binding of estradiol. However, we observed that about 10% of TAT-59 was converted to 4-OH-TAT-59 in the presence of rat uterine cytosol during the incubation for 18 h even at 4°C. Therefore, the specific binding of TAT-59 to ER may be overestimated.

The effects of TAT-59 and TAM on the growth of MCF-7 are shown in Fig. 3. In the presence of 10^{-9} M estradiol (Fig. 3A), TAT-59, 4-OH-TAT-59 and 4-OH-TAM were able to inhibit the estrogen stimulated growth of MCF-7 cells in the same dose-dependent manner, whereas a 10-fold higher concentration of TAM was necessary to achieve the same effect. In the absence of estradiol (Fig. 3B), TAT-59 and 4-OH-TAT-59 were able to suppress the growth of MCF-7 cells, at concentrations below 10^{-10} M, while 4-OH-TAM was not effective at this range of concentration.

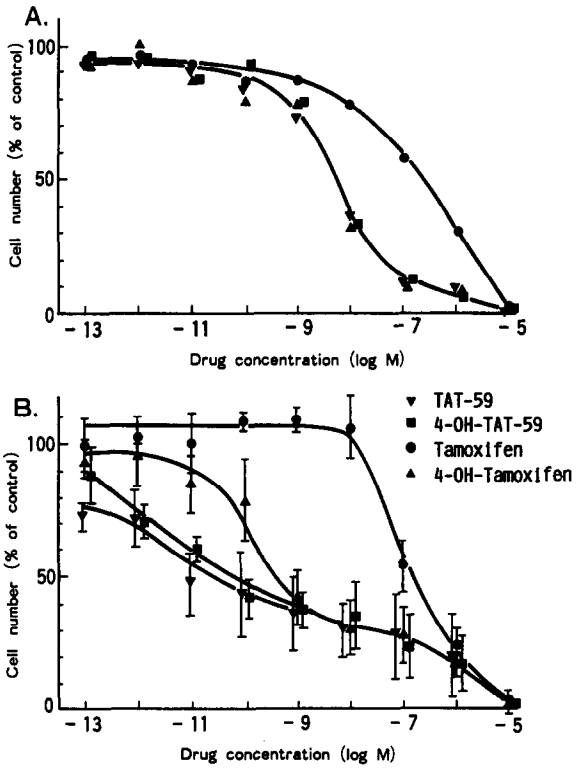

Fig. 3. Effect of TAT-59, 4-OH-TAT-59, Tamoxifen and 4-OH-Tamoxifen on the growth of MCF-7 cells. Each point represents the mean of three experiments. The growth in the presence of 10^{-9} M estradiol (panel A) and without estradiol (Panel B).

Table 1. Comparison of antiestrogenic activity of TAT-59 and Tamoxifen on immature rat uterus

	ED_{50} (mg/kg)*		ED_{50} ratio
Schedule	TAT-59	Tamoxifen	Tamoxifen/TAT-59
q7d	2.95	10.81	3.66
q3d	0.57	2.18	3.82
qd	0.40	2.22	5.55

*Dose required for 50% inhibition of uterine growth at a given administration schedule.

Assuming that antitumor and biological effects may be related to the accumulation of the drug in the target organ, the potency of TAT-59 and TAM was studied using an antiuterotrophic test. The results of this test (Table 1) indicate a much higher potency of TAT-59 administered at various schedules. The dose of TAT-59 required to inhibit the growth of the uterus in immature rats was 2.9–5.5-fold lower than that of TAM, suggesting a higher accumulation of TAT-59 in tumor tissues.

The inhibitory effects of TAT-59 on the growth of MCF-7 cells, transplanted to nude mice, are shown in Table 2. At a dose of 0.3 mg/kg/day, the suppressive effect of TAT-59 was similar to that of TAM. However, at a dose of 0.1 mg/kg/day, TAT-59 still suppressed tumor growth, while TAM had no inhibitory effect. These treatment groups are significantly different ($P < 0.01$).

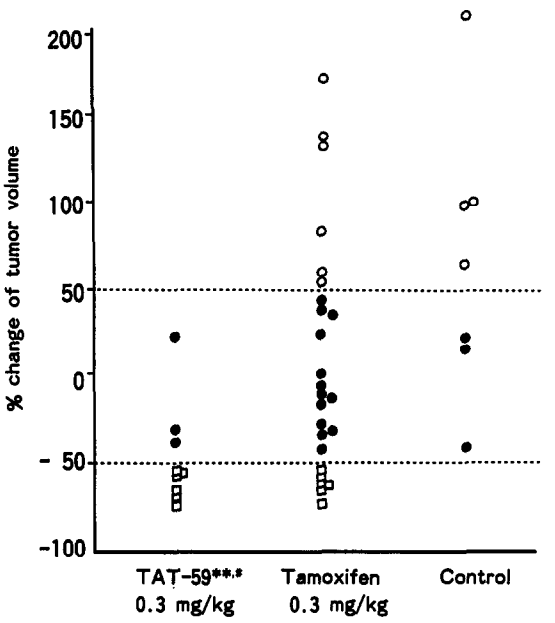

*Fig. 4. Effect of TAT-59 and Tamoxifen on the DMBA-induced mammary tumor with ER, varied from 10 to 20 fmol/mg protein: progressing tumor (○), stable tumor (●), regressing tumor (□). The details of classification are presented in methods section. **,# Significantly different from the control and Tamoxifen-treated group at $P < 0.01$ and $P < 0.05$, respectively.*

The effect of TAT-59 on DMBA-induced mammary tumors is shown in Table 3. At a dose of 0.03 mg/kg/day, TAT-59 was able to suppress tumor growth, whereas more than 0.3 mg/kg/day of TAM was necessary to achieve the same effect. The dose to achieve a 50% inhibition of the tumor growth was 0.04 and 0.38 mg/kg/day for TAT-59 and TAM, respectively. Thus there is a 10-fold difference in the dosage of these two compounds needed to obtain 50% inhibition.

TAT-59 showed a superior activity against DMBA-induced tumors with low ER content, as based on the final percentage change of tumor volume (Fig. 4). The ratio of progressing:stable:regressing tumor was 0:3:6 for TAT-59 treatment and 6:13:6 for TAM treatment. There were no progressing tumors following TAT-59 treatment. In this respect, TAT-59 treatment appeared to be more effective than TAM treatment ($P < 0.05$).

The results on pharmacokinetic studies (Fig. 5) showed that TAT-59 was rapidly metabolized to its active metabolite, 4-OH-TAT-59, and both the plasma and intratumor concentrations were higher than that of 4-OH-TAM after oral administration of TAM. The peak levels of 4-OH-TAT-59 were 7.5 ng/ml and 31.1 ng/g of tumor tissue and it remained at 1.04 ng/ml and at 8.85 ng/g in the blood and the tumor tissue, respectively, 24 h after administration (Fig. 5A). On the other hand, the

Table 2. Comparison of growth inhibitory effect of TAT-59 and Tamoxifen against MCF-7 human mammary carcinoma transplanted to nude mice

Exp. No.	Group	Dose (mg/kg/day)	No. of animals	Percentage change of tumor volume (mean ± S.D.)	Percentage inhibition
1	Control		8	306.1 ± 115.3	—
	TAT-59	1.0	7	190.4 ± 43.2*	37.8
	Tamoxifen	1.0	7	187.2 ± 76.4*	38.8
2	Control		8	251.2 ± 77.7	—
	TAT-59	0.3	7	134.7 ± 29.3**	46.4
		0.1	7	161.1 ± 62.5*	35.9
	Tamoxifen	0.3	7	144.9 ± 29.3**	42.3
		0.1	7	292.0 ± 95.0	−16.5
3	Control		11	217.9 ± 69.3	—
	TAT-59	0.1	11	143.5 ± 41.6**	34.1
	Tamoxifen	0.1	11	236.2 ± 89.1	−8.4
4	Control		12	299.6 ± 104.7	—
	TAT-59	0.03	10	311.8 ± 80.4	−1.9
	Tamoxifen	0.03	10	305.3 ± 92.2	−4.1

*,**Significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively.

Table 3. Antitumor effect of TAT-59 and Tamoxifen against DMBA-induced rat mammary carcinoma

Exp. No.	Group	Dose (mg/kg/day)	No. of animals	Percentage change of tumor volume (mean ± S.D.)	Percentage inhibition
1	Control		20	157.2 ± 70.6	
	TAT-59	0.3	19	53.8 ± 45.0*	65.8
	Tamoxifen	0.3	20	82.2 ± 49.6	47.7
2	Control		20	251.0 ± 225.9	
	TAT-59	0.1	16	112.5 ± 101.8*	55.2
		0.03	15	104.4 ± 71.2*	58.4
	Tamoxifen	0.1	16	213.7 ± 212.7	14.9
		0.03	18	263.0 ± 246.5	−4.8
3	Control		30	291.1 ± 164.7	
	TAT-59	0.03	30	178.3 ± 183.9*	38.7
		0.01	28	357.3 ± 318.8	−22.7
	Tamoxifen	0.03	30	235.2 ± 113.4	19.2
		0.01	30	284.0 ± 262.4	2.4

*Significantly different from the control at $P < 0.05$.

peak levels of TAM and its active metabolite 4-OH-TAM were 3.0 and 0.28 ng/ml in the blood, 12.0 and 2.94 ng/g in the tumor, respectively. Twenty-four hours after administration, there was no detectable amount of either compound in the blood, while in the tumor there were 6.92 and 2.94 ng/g of TAM and 4-OH-TAM, respectively (Fig. 5B). The comparison of the peak levels in the tumor showed that the active metabolite of TAT-59 (4-OH-TAT-59) was able to reach the target tumor tissue at an 11-fold higher concentration than that of 4-OH-TAM.

DISCUSSION

TAT-59 was able to bind to ER and especially its active metabolite 4-OH-TAT-59 showed a binding affinity similar to that of estradiol. TAT-59, administered orally, showed a strong inhibitory effect on the growth of hormone-dependent tumors, such as MCF-7 and DMBA-induced rat mammary tumors. TAT-59 had a 3–10-fold higher activity than TAM. The effect of TAT-59 against DMBA-induced rat mammary tumor with low ER levels was significantly stronger than that of TAM. Based on clinical studies, it is supposed that TAM is not very effective against the ER positive tumors, especially in tumors with low ER levels [13, 14], and that its activity is not promising in premenopausal women with breast cancer [2]. To try to overcome these weak points, we have developed a new antiestrogen possessing not only a higher affinity to ER and an increased uptake by the target organs but also with an anti-

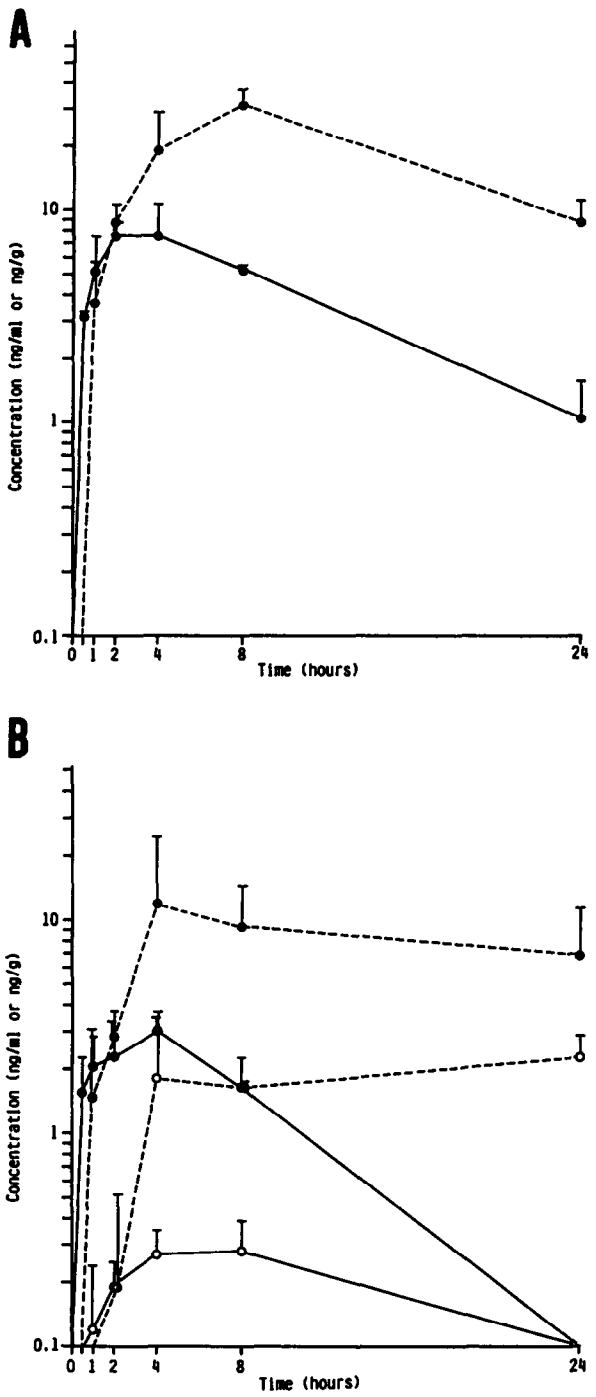

Fig. 5. Plasma and tumor tissue level of antiestrogens and their metabolites after oral administration to tumor-bearing rats (n = 3). *Points and bars represent the mean and S.D., respectively.* (——) *in plasma,* (-----) *in tumor. A: the concentration of 4-OH-TAT-59 (TAT-59 was not detected). B: the concentration of Tamoxifen* (●) *and 4-OH-Tamoxifen* (○).

cancer activity other than that mediated through ER. TAT-59, given orally, was rapidly metabolized to 4-OH-TAT-59 which has a similar affinity to ER as estradiol. However, based on the clinical analysis of TAM biotransformation, it is supposed that the antitumor activity of TAM may be mediated by TAM itself and its main metabolite demethylated TAM (Des-TAM) [23]. Both TAM and Des-TAM were reported to have a similar affinity to ER [24], however this affinity was equivalent only to 0.55% of estradiol and approximately 0.5% of that of 4-OH-TAT-59.

The uptake of TAT-59, given orally, by the tumor tissues was better than that of TAM (Fig. 5). It was reported that both 4-OH-TAM and estradiol had similar affinity for ER [24, 25] and the same affinity was observed for the main metabolite of TAT-59. One may expect that the higher growth inhibitory activity of TAT-59 is due to its increased penetration to the tumor tissues while its affinity to the target substructures, e.g. ER, is conserved. In human beings, TAM is metabolized to 4-OH-TAM to a lesser extent than in the rat [26], and, because of its high ER affinity, it might be suggested that the antitumor activity of TAM was related to 4-OH-TAM production. As shown in Fig. 4, the peak concentration of 4-OH-TAT-59 in the tumor was 13-fold higher than that of 4-OH-TAM. One of the advantages of TAM already reported was its prolonged retention in the tumor [21, 27]. The antiuterotrophic test also showed a prolonged biological activity of TAT-59. The ED_{50} of TAM when administered every third day or daily was lower than that following every 7th day administration. TAT-59 showed a similar effect, except that the ED_{50} for every 7th day administration of TAT-59 was similar to the ED_{50} of TAM administered every 3rd day or even daily, suggesting a longer retention of its biological activity than in case of TAM. Therefore, we conclude that the antitumor activity of TAT-59 depends not only on its high ER affinity and a high uptake by the tumor but also on its prolonged retention in the tumor tissue.

It was reported that antiestrogens, which had a high ER affinity *in vitro*, like 4-OH-TAM or Keoxifen, were not enough effective *in vivo* [21, 28]. At present the exact reason of such ineffectiveness is unknown, it was supposed to be related to the shorter biological half-life [21] or the lack of influence on the hormone levels, such as prolactin [28], suggesting that the *in vivo* antitumor effect was not related only to the high concentration of the high ER affinity antiestrogen. The growth inhibitory effect of triphenylethylene antiestrogens have been reported to be related to their calmodulin antagonistic activity [16], the inhibition of lipoxygenase activity [19] or an interaction with the antiestrogen binding sites [17, 18]. The growth inhibitory activity of TAT-59, but not 4-OH-TAM, against estradiol depleted cells did not reflect the ability to bind to ER (Fig. 3); therefore, we supposed that TAT-59 might be more effective than TAM based on these other mechanisms. The ER are not uniformly distributed inside the tumor [29]. Hence, it may be possible that an ER negative cell is stimulated to proliferate by a paracrine growth factors secreted from ER positive cells receiving estro-

genic stimulation [30, 31]. Knabbe *et al.* reported that antiestrogens can stimulate the secretion of growth inhibitory factor from the ER positive cells [32]. Because TAT-59 can deliver more of an active metabolite with a high ER affinity than TAM, it is supposed that TAT-59 may suppress such a paracrine growth stimulation, or may stimulate more secretion of growth inhibitory factor than TAM, eventually showing anticancer activity against the tumors with low ER levels.

The precise mode of action of TAT-59 antitumor activity is still unclear. It is supposed that TAT-59 may show a clinical effect even against the ER negative tumors. Due to the ability of TAT-59 to suppress the growth of hormone-dependent tumors at about a 10 times lower dose than TAM, both *in vivo* and *in vitro*, and its growth suppressing activity of tumors with low ER contents is stronger than TAM, we hope that TAT-59 will show a sufficient antiestrogenic effect in premenopausal women with breast cancer and will show stronger effects in postmenopausal women with tumors with a low ER content.

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