Cancer Chemother Pharmacol (2000) 45: 133–141 © Springer-Verlag 2000

ORIGINAL ARTICLE

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Estrogen agonistic/antagonistic effects of miproxifene phosphate (TAT-59)

Received: 4 January 1999 / Accepted: 2 July 1999

Abstract *Purpose*: We evaluated miproxifene phosphate (TAT-59) to elucidate its efficacy in antiestrogen therapy for breast cancer patients and to assess its tissue-selective estrogenic/antiestrogenic activity. *Methods*: Using DP-TAT-59, a major and active metabolite of TAT-59, an in vitro cell growth inhibition test was performed. Antitumor activity was determined using TAT-59 against human tumor xenografts of the MCF-7 and the Br-10 cell lines and MCF-7-derived tamoxifen-resistant cell lines, R-27 and FST-1. The antitumor activity of DP-TAT-59 and DM-DP-TAT-59, major metabolites of TAT-59 found in human blood following a TAT-59 dose, was also examined after intravenous administration to experimental animals. The residual estrogenic activity of TAT-59, evaluated in terms of bone and lipid metabolism in ovariectomized rats, was then compared with that of tamoxifen. *Results*: DP-TAT-59 significantly inhibited the proliferation of estrogen receptor-positive MCF-7 and T-47D tumor cells in the presence of 1 n*M* estradiol. TAT-59, given to mice bearing MCF-7 or Br-10 xenografts, at the dose level of 5 mg/kg, exerted a significant growth inhibitory effect that was stronger than that of tamoxifen. Moreover, R-27 and FST-1 tumors, which show a resistance to tamoxifen, responded strongly to TAT-59, suggesting that TAT-59 might be effective against tumors resistant to tamoxifen. The metabolites of TAT-59, DP-TAT-59 and DM-DP-TAT-59, showed similar antitumor activity. Both TAT-59 and tamoxifen suppressed the decrease in bone density and reduced the blood cholesterol levels in ovariectomized rats, suggesting that the estrogenic activity of TAT-59 is comparable to that of tamoxifen. *Conclusions*: On the basis of the above results, one may expect TAT-59 to become an effective drug in patients with tumors less sensitive to tamoxifen, while its estrogenic activity as determined by bone and lipid metabolism is similar to that of tamoxifen.

Key words Miproxifene phosphate (TAT-59) · Tamoxifen · Antitumor activity · Estrogen-antiestrogen

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Introduction

In recent years, studies of the role played by hormones in the proliferation of breast carcinoma cells have produced many interesting results. Endocrine therapy, especially that consisting of triphenylethylene derivatives such as tamoxifen, which is widely used in postoperative adjuvant therapy, is currently used as an effective approach in treating breast cancer [9]. However, the response rate to tamoxifen therapy is reported to be 30% in the general population of breast cancer patients [1], among whom 60% are positive for estrogen receptors (ER). One of the major problems preventing long-term effective endocrine therapy for breast cancer is the development of resistance to therapy, in particular to tamoxifen therapy. In some patients, clinical data indicate that the current endocrine therapies for breast cancer result in a temporary tumor regression or growth stabilization, followed by tumor progression [12]. Therefore, many studies have been performed on the development of new treatments which will avoid the problem of resistance to tamoxifen.

The development of raloxifene for the treatment of osteoporosis introduced the idea of tissue-selective estrogen/antiestrogen therapy [2]. Raloxifene has been shown to exert estrogenic activity in bone tissue [18] and to exhibit antiestrogenic activity, resulting in the suppression of the onset of breast cancer [13], but a limited clinical phase study has shown raloxifene not to be effective as a second-line treatment [3]. Also tamoxifen serves as an

antiestrogen in the mammary glands, while it works as an estrogen in bone tissue and lipid metabolism [7].

We have synthesized about 100 triphenylethylene derivatives and have subsequently focused on one, TAT-59 [20]. In contrast to tamoxifen, the metabolites of TAT-59 detected in humans have an affinity for all ERs, similar to the affinity of estradiol for ERs [22]. Moreover, the complex formed by DP-TAT-59 (a major metabolite of TAT-59) and ER has a different ion-exchange chromatography elution profile from the estradiol-ER complex [21]. TAT-59 exhibits an antitumor activity greater than that of tamoxifen against tumors expressing low levels of ER [20]. In vitro experiments have revealed an increased production of cell growth inhibitory factors by ER-positive breast carcinoma cells exposed to TAT-59 that is more intensive than that induced by tamoxifen, indicating that TAT-59 may also suppress the proliferation of ER-negative breast carcinoma cells [23]. The above-mentioned properties indicate that TAT-59 may have a growth inhibitory effect against breast cancers not responding or resistant to tamoxifen therapy. We therefore conducted the present study to examine whether TAT-59 may improve the efficacy of antiestrogen therapy in breast cancer patients with low sensitivity to tamoxifen and to assess the tissue-selective estrogenic activities of this compound.

Materials and methods

Materials

Tamoxifen citrate and 17β-estradiol were purchased from Sigma Chemicals (St. Louis, Mo.). Toremifene, 4-OH-tamoxifen, raloxifene, TAT-59 and its metabolites were synthesized in our institute. Estrogen and antiestrogens were first dissolved in dimethylsulfoxide (DMSO) to prepare stock solutions which were then diluted with buffer or culture medium for in vitro experiments. For in vivo experiments, a suspension in the vehicle for oral administration and a solution in saline, containing 3.5% DMSO/6.5% Tween 80, for intravenous administration were prepared. MCF-7 cells obtained from the American Type Culture Collection (Rockville, Md.) were routinely grown in RPMI-1640 medium, supplemented with 10^{-9} *M* estradiol and 5% fetal calf serum (FCS).

The MCF-7 sublines R-27 (kindly provided by Dr. Abe, Keio University, Japan), LY-2 (a gift from Dr. R. Dickson, Georgetown University, Washington D.C.) and MCF-7/TAM^{R}-1 (a gift from Dr. Lykkesfeldt, Danish Cancer Institute, Copenhagen) resistant to tamoxifen were grown in RPMI-1640 medium supplemented with 10^{-9} *M* estradiol, 5% FCS and 10^{-6} *M* tamoxifen. KPL-1 cells, a spontaneous tamoxifen-resistant cell line, were kindly provided by Dr. Kurebayashi (Kawasaki Medical School, Kurashiki, Japan), Br-10 cells were supplied by the Central Institute for Experimental Animals (Tokyo, Japan) and T-47D cells were obtained from Dainippon Seiyaku (Tokyo, Japan). For in vivo experiments, these cells have been maintained by serial subcutaneous transplantation into the subaxillary region of female athymic nude mice. The FST-1 cells, an MCF-7 variant resistant to tamoxifen, were established in our laboratory by chronic exposure to tamoxifen.

Female Sprague-Dawley (SD) rats and BALB/c (nu/nu) mice were purchased from Japan SLC (Hamamatsu, Japan) and Japan Clea (Tokyo, Japan), respectively. The animals were housed according to institutional guidelines in a protected environment, and maintained under a 12-h lighting cycle at a temperature of 22–25 °C with food and water available ad libitum.

Methods

Antiproliferative activities against human mammary cancer cells

Cells were plated in a 96-well multiplate in the presence of a RPMI-1640 medium including 5% FCS treated with dextran-coated charcoal. After cell attachment, the cells were treated with antiestrogens for 8 days and then the plates were fixed with glutaraldehyde and stained with crystal violet. The percentage change in cell count (T/C%) was determined on the basis of the concentration of dye extracted from stained cells using the formula:

$$\mathrm{T/C}(\%) = (\text{absorption of treated group})/(\text{absorption of control group}) \times 100$$

The ED_{50} was determined by the SAS program as a measure of relative cytotoxicity.

Antitumor activity in an animal model of breast cancer

Estradiol slow-release pellets were prepared by the method of Wieder et al. [25]. A pellet was implanted into the left flank and 2-mm square tumor fragments were implanted into the right flank of each female nude mouse to create a model of human breast carcinoma. When a tumor had reached a diameter of 6 or 7 mm, the mice were divided into experimental groups and treated orally with antiestrogens daily for 6 consecutive days per week for 4 weeks. The size of the tumors was recorded in terms of two perpendicular diameters (in millimeters) and the tumor volume (TV) was calculated from the formula:

$$\mathrm{TV}(\mathrm{mm}^3) = (\text{length}) \times (\text{width})^2/2$$

The relative tumor volume (RTV) was then calculated as the ratio of TV on day *n* to that on day 0. The T/C was then calculated based on the RTV from the formula:

$$\mathrm{T/C}(\%) = (\text{mean RTV of treated group})/(\text{mean RTV of control group}) \times 100$$

The antitumor activities of the metabolites of TAT-59 were evaluated using MCF-7 xenografts. Metabolites were administered intravenously for 9 days and then the RTV was calculated.

Uterotrophic activity

SD rats at 4 weeks of age were ovariectomized under ether anesthesia 1 week before treatment to deplete their internal estrogen supply. The test compounds were administered orally for 3 days. Each rat of the positive control group was given 0.3 μg estradiol subcutaneously in sesame oil. The day after the final administration, the rats were sacrificed and their uterus was removed and weighed. The relative uterine weight was calculated from the formula:

$$\text{Relative uterine weight}(\%) = (\text{antiestrogen treated} - \text{untreated})/(\text{estradiol treated} - \text{untreated}) \times 100$$

Estrogenic activity of antiestrogens in bone and lipid metabolism

SD rats at 9 weeks of age were ovariectomized under ether anesthesia 1 week before treatment. Under the same conditions, a sham operation was carried out in control animals. Ethynyl estradiol (0.1 mg/kg) or antiestrogens (2 mg/kg TAT-59, 2 mg/kg tamoxifen, 6 mg/kg raloxifene) were administered by oral gavage for 28, 55 or 78 days. After the last administration, the rats were fasted overnight and then sacrificed and their left femur, blood and urine were collected. The trabecular density of the left femurs was determined using peripheral quantitative computed tomography (XCT Research, Research SA, Norland, N.Y.). A 3 to 5.5-mm section distal to the growth plate of the femur was analyzed. The levels of pyridinium

crosslinks in urine samples were determined by PYRILINKS (Metra Biosystems, Calif.) and adjusted for the levels of creatinine. Serum alkaline phosphatase levels were determined by the ALP-B test (WAKO Pure Chemicals, Osaka, Japan). Serum cholesterol levels were determined by the cholesterol E test (WAKO Pure Chemicals) for total cholesterol, by the L-type HDL-C test (WAKO Pure Chemicals) for HDL cholesterol and by the Cholestest LDL test (Daiichi Chemicals, Tokyo, Japan) for LDL cholesterol.

Statistical analysis

The data obtained were analyzed using the two-sided Dunnett's *t*-test for continuous data or Student's *t*-test.

Results

Inhibition of the growth of human breast carcinoma cells

The effects of the major metabolites of TAT-59, previously detected in cancer patients, on the proliferation of human breast carcinoma cell lines were investigated in the presence of 1 n*M* estradiol. The ED_{50} value of each drug, determined from the dose-response curves, showed that DP-TAT-59 and DM-DP-TAT-59 were 30 times more potent than tamoxifen in inhibiting the growth of MCF-7 and T-47D cells (Table 1). Thus, the major metabolites of TAT-59 inhibited the proliferation of tumor cells more strongly than tamoxifen, but their growth inhibitory effect was close to that of 4-OH-tamoxifen, an active metabolite of tamoxifen, but hardly detected in blood samples from cancer patients.

Inhibition of the proliferation of tamoxifen-resistant breast carcinoma cells

The effects of DP-TAT-59 on the proliferation of tamoxifen-resistant breast carcinoma cell lines are shown in Fig. 1. The ED_{50} of tamoxifen against MCF-7 cells was 1.403 μ*M*. The other tumor cell lines, FST-1, MCF-7/TAM^R-1, LY2 and KPL-1, showed no or only low sensitivity to tamoxifen, with the ED_{50} being more than five times higher than that for MCF-7. The tumor cell lines other than MCF-7 were thus rated as having low sensitivity to tamoxifen. DP-TAT-59 inhibited the proliferation of these cell lines, with corresponding ED_{50} values of 0.028, 1.027, 0.096 and 0.205 μ*M* for FST-1, MCF-7/TAM^R-1, LY2 and KPL-1, respectively.

Antitumor activity in an animal model of human breast cancer

Nude mice, supplemented with estradiol, were implanted with human carcinoma (MCF-7 or Br-10) cells to create a model of human breast carcinoma. The antitumor activity of TAT-59 examined in these models is shown in Fig. 2. Following treatment with tamoxifen or toremifene the observed T/C ratios were over 50% both in the MCF-7 and the Br-10 group at the administered doses. These two drugs therefore showed a weak effect. The growth of MCF-7 and Br-10 tumor cells was significantly inhibited by TAT-59 at both the dose levels examined (5 and 10 mg/kg). The T/C ratios at the time of the final evaluation of the animals treated with TAT-59 (10 mg/kg) was 23.8% ($P < 0.01$) and 39.6% ($P < 0.01$) in the MCF-7 and Br-10 groups, respectively. Low-dose TAT-59 (5 mg/kg) also appeared to be effective: the T/C ratio was 46.8% ($P < 0.01$) and 38.3% ($P < 0.01$) in the MCF-7 and Br-10 groups, respectively (Table 2).

Table 1 ED_{50} values of antiestrogens

Compound	ED_{50} (μ*M*)	
	MCF-7	T-47D
DP-TAT-59	0.009	0.023
DM-DP-TAT-59	0.055	0.021
Tamoxifen	2.534	1.439
4-OH-Tamoxifen	0.011[a]	NT
Toremifene	4.956	2.939

[a] Tested in a separate experiment
NT not tested

Antitumor activity against tamoxifen-resistant tumors

The antitumor activity of TAT-59 was tested in nude mice implanted with tamoxifen-resistant tumor cells, R-27 or FST-1 (Fig. 3). Tamoxifen, administered at a dose level of 10 mg/kg, weakly inhibited the growth of these tumor cells, confirming that these tumor cell lines have low sensitivity to tamoxifen. TAT-59, used at a dose level of 10 mg/kg, exhibited significant antitumor activity as compared to the control group supplemented with estrogen. The T/C ratio at the time of the final evaluation of the animals treated with TAT-59 at 10 mg/kg was 40.2% ($P < 0.01$) and 42.2% ($P < 0.01$) in the FST-1 and R-27 groups, respectively. The T/C ratio of the animals treated with TAT-59 at 5 mg/kg was 47.3% ($P < 0.01$) in the FST-1 group, confirming the effectiveness of TAT-59 therapy even at a low dose level (Table 2). The T/C ratio for animals that did not receive either estrogen or antitumor drug following implantation of R-27 cells was 62.4%, probably reflecting tumor proliferation induced by endogenous estrogen. Thus, tumor growth was inhibited more strongly by TAT-59 treatment than by cessation of exogenous estrogen supplementation. Toremifene showed little antitumor activity in R-27-implanted animals even at a dose of 10 mg/kg. The T/C ratio of animals treated with toremifene at 30 mg/kg was 51.8% in FST-1-implanted animals (Table 2).

Antitumor activity of TAT-59 metabolites

To establish the contribution of DP-TAT-59 and DM-DP-TAT-59, major metabolites of TAT-59 found in human blood, to the antitumor activity of TAT-59, both

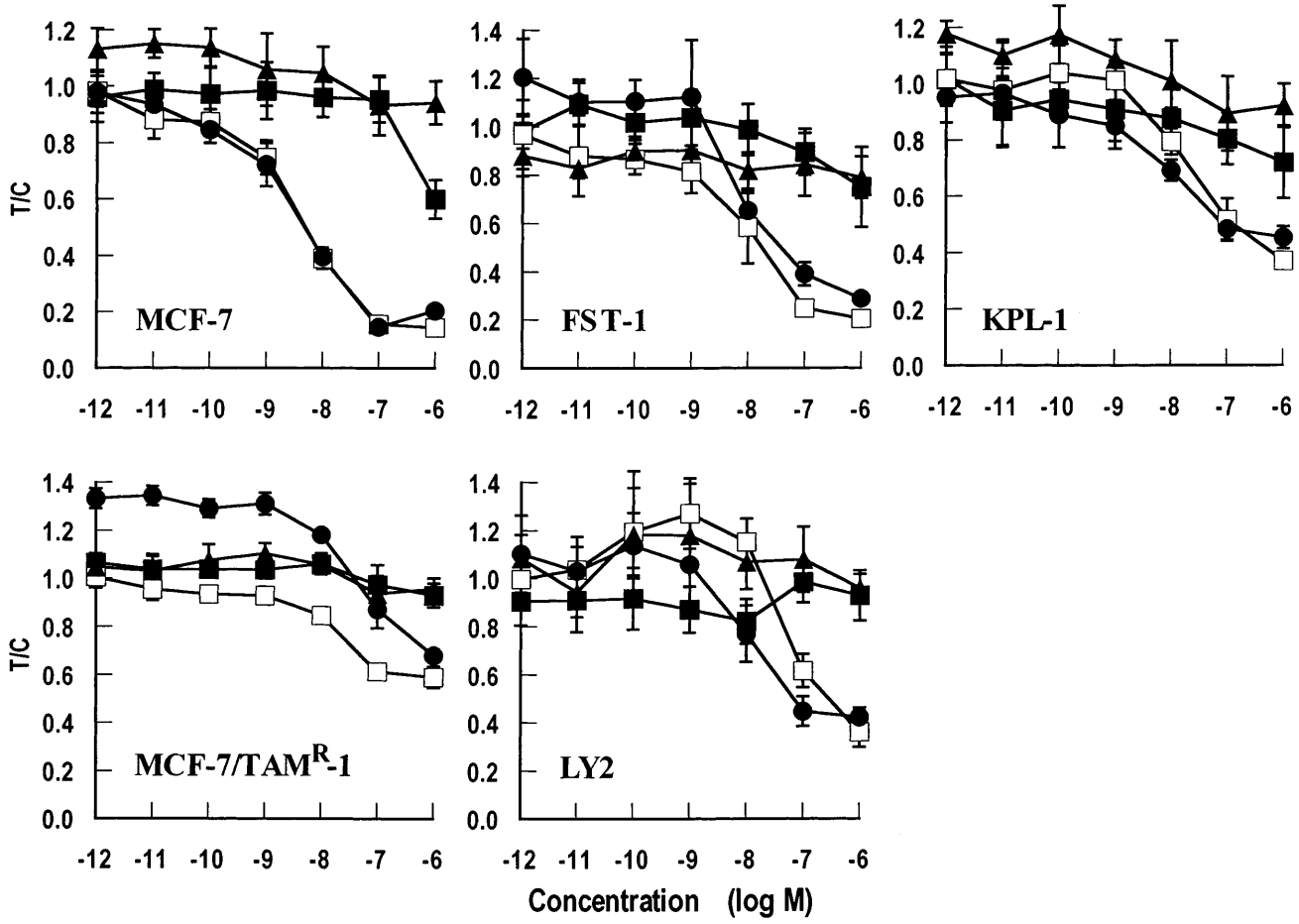

Fig. 1 Antiproliferative activities of antiestrogens against tamoxifen-resistant breast cancer cells (■ tamoxifen, □ 4-OH-tamoxifen, ▲ toremifene, ● DP-TAT-59). The values shown are the means and SD

compounds were administered intravenously to experimental animals (Table 3). Both metabolites significantly inhibited the growth of MCF-7 xenografts in a dose-dependent manner. When either of the two metabolites was used at a dose level of 5.0 mg/kg, the growth of the tumor from day 14 onwards was suppressed by more than 50% as compared to the estradiol-supplemented control group. At the dose levels of 1.0 or 0.2 mg/kg, both metabolites manifested a similar degree of tumor growth inhibition. The ratio of the blood levels of DP-TAT-59 to those of DM-DP-TAT-59 following TAT-59 administration ranged from 1:2 to 1:1 [11], suggesting that both metabolites contribute to a similar degree to the antitumor activity of TAT-59.

Estrogenic activity on uterus

The estrogenic activity of TAT-59 was investigated in ovariectomized immature rats, using uterine weight gain as an indicator. Both TAT-59- and tamoxifen-treated ovariectomized rats showed a similar dose-response in terms of uterine weight gain. However, TAT-59 exhibited its estrogenic activity at lower dose levels than tamoxifen. This result suggests that TAT-59 and tamoxifen have similar estrogenic activities (Fig. 4).

Estrogenic activity on bone and lipids

The estrogenic activity of TAT-59 was examined in ovariectomized rats in terms of its effect on bone and lipid metabolism. The trabecular density of the growth plate of the femur was significantly lower in the untreated ovariectomized group than in the untreated sham-operation group. The trabecular density in rats that received 2 mg/kg of TAT-59, 6 mg/kg raloxifene or 2 mg/kg tamoxifen following ovariectomy was significantly higher than that in the untreated ovariectomized group and was close to that in the untreated sham-operation group (Fig. 5). Urinary levels of pyridinoline, a marker of bone resorption, tended to be lower in the antiestrogen treatment group (Fig. 6a). On the other hand, serum alkaline phosphatase, a marker of ossification, showed an increase in activity in the antiestrogen treatment group (Fig. 6b). When the effects on lipid metabolism were examined, all parameters including total cholesterol, HDL cholesterol and LDL cholesterol, were lower in the TAT-59-treated than in the untreated

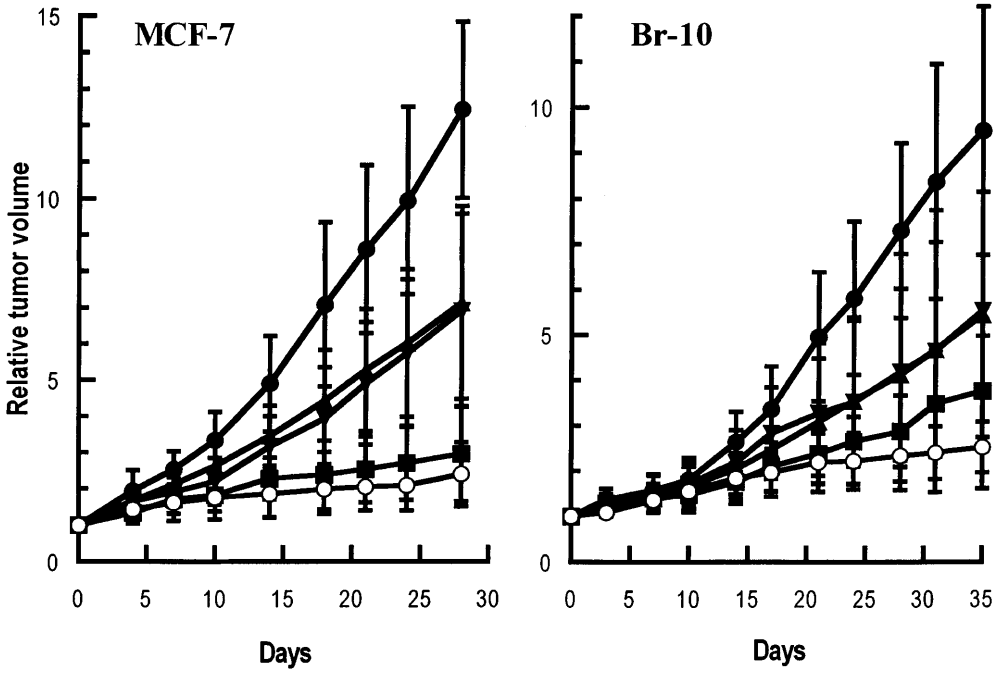

Fig. 2 Antitumor activity of antiestrogens against human mammary carcinoma MCF-7 and Br-10 xenografts (● control with estradiol, ■ TAT-59 10 mg/kg, ▲ tamoxifen 10 mg/kg, ▼ toremifene 10 mg/kg, ○ control without estradiol). The values shown are the means and SD

ovariectomized group (Fig. 7). Thus all animals treated with TAT-59, raloxifene or tamoxifen showed a similar response in bone and lipid metabolism. We may therefore conclude that the estrogenic activity of TAT-59, as revealed by bone and lipid metabolism parameters, is similar to that of tamoxifen.

Discussion

Tamoxifen has been shown to be effective against breast cancer not only in postmenopausal women but also in premenopausal women, but the therapeutic efficacy observed in middle-aged and elderly women is not observed in young women [6]. Although the exact mechanism by which antiestrogens exert their effects has not yet been clarified, they are thought to suppress the proliferation of estrogen-dependent breast cancer by binding to the ERs in breast carcinoma cells, thereby antagonizing estrogen [16, 19]. Since the affinity of tamoxifen for the ER is lower than that of estradiol (0.01–30% of the affinity of estradiol) [8], much higher levels of tamoxifen are needed to exert antiestrogenic activity [10, 17].

After oral administration TAT-59 is immediately metabolized in the digestive tract to its active form DP-TAT-59 which has a high affinity for ER [22]. In previous studies, the ratio of the blood levels of DP-TAT-59 to those of DM-DP-TAT-59 in humans have been shown to range from 1:2 to 1:1 [11], and the affinity of these metabolites for the ER is comparable to that of estradiol [22]. The present study showed that DP-TAT-59 suppresses the proliferation of human breast carcinoma cells even at concentrations lower than 1/30th of the level required for tamoxifen to exhibit this action (Table 1). Moreover, DP-TAT-59 showed antiproliferative activity against MCF-7 cells similar to that exerted by 4-OH-tamoxifen, a potent metabolite of tamoxifen [22]. Since the rate of formation of 4-OH-tamoxifen from tamoxifen is relatively low and mediated by CYP2D6 cytochrome, as revealed in in vitro studies using human liver microsomes [5], one may conclude

Table 2 Antitumor activity of TAT-59 and other estrogens against human mammary carcinoma MCF-7 xenografts

Drug	Dose (mg/kg/day)	T/C (%)			
		MCF-7 (day 28)	Br-10 (day 35)	R-27 (day 25)	FST-1 (day 27)
TAT-59	10.0	23.8**	39.6**	40.2**	42.2**
	5.0	46.8**	38.3**	63.0*	47.3**
Tamoxifen	10.0	57.5**	57.4*	76.3	67.1
	5.0	68.8*	72.2	75.7	64.2*
Toremifene	30.0	–	–	–	51.8**
	10.0	55.7**	58.5	93.9	–
	5.0	–	86.3	97.0	–
Control[a]		19.3**	26.6**	62.4*	–

* $P < 0.05$, ** $P < 0.01$, vs controls with estradiol supplementation, Dunnett's t-test
[a] Without estradiol supplementation

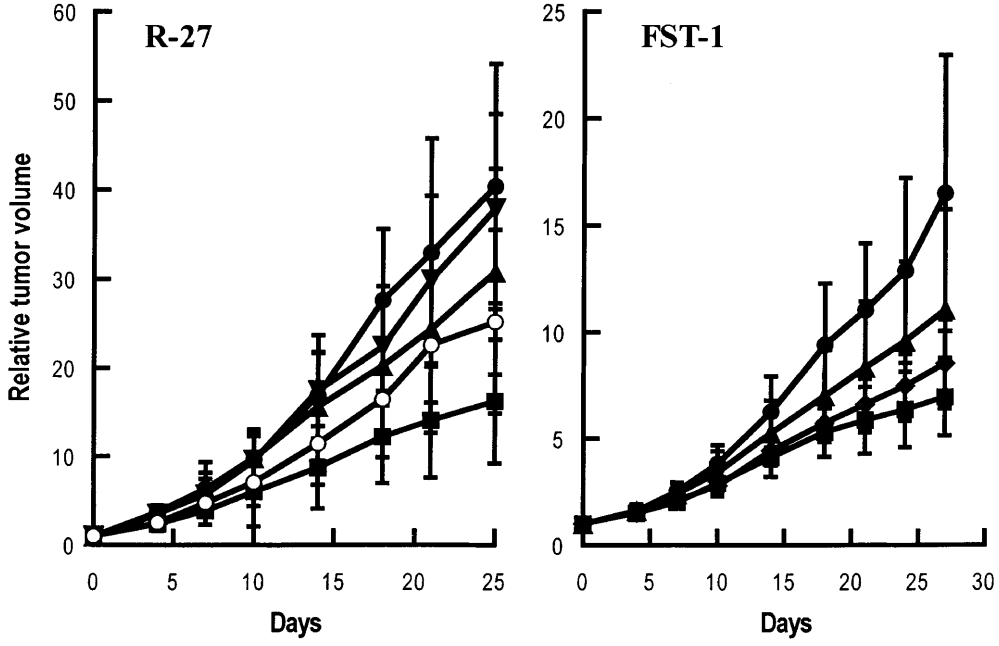

Fig. 3 Antitumor activity of antiestrogens against tamoxifen-resistant human mammary carcinoma R-27 and FST-1 xenografts (● control with estradiol, ■ TAT-59 10 mg/kg, ▲ tamoxifen 10 mg/kg, ◆ toremifene 30 mg/kg, ▼ toremifene 10 mg/kg, ○ control without estradiol). The values shown are the means and SD

that TAT-59 has an advantage by providing a significant amount of biologically active metabolites.

In our in vitro study, DP-TAT-59 suppressed the estrogen-stimulated proliferation of tamoxifen-resistant cell lines LY2, FST-1, and KPL-1 at a concentration of about 10^{-7} *M* (Fig. 1) corresponding to its concentration in human blood after administration of TAT-59. Moreover, DM-DP-TAT-59 inhibits the proliferation of MCF-7/TAM^{R}-1 (personal communication from Dr. Lykkesfeldt) and TAT-59 analogues such as DP-TAT-59 inhibit the proliferation of RTx6 cells, an MCF-7 variant resistant to tamoxifen (personal communication from Dr. Leclercq). Since 4-OH-tamoxifen, whose affinity for the ER is higher than that of other metabolites of tamoxifen, is detected at extremely low levels in human blood following tamoxifen administration, one may expect that such low levels are not sufficient to block cell proliferation of certain breast cancers [4]. On the other hand, DP-TAT-59 and DM-DP-TAT-59, active metabolites of TAT-59 with a high affinity for the ER, are detected at concentrations (about 10^{-7} *M*) in human blood after TAT-59 administration high enough to inhibit the proliferation of breast carcinoma cells weakly or not responding to tamoxifen treatment [11].

The results derived from the studies on FST-1, LY2, RTx6, MCF-7/TAM^{R}-1, and KPL-1 breast carcinoma cell lines confirm the usefulness of TAT-59 treatment. Furthermore, the effects of antiestrogens were examined in vivo by oral administration at a dose of 5 mg/kg (equivalent to a clinical dose of 20 mg/animal after conversion for mouse body surface area) in the presence of estradiol, and TAT-59 was found to be the most effective against MCF-7 and Br-10 of the compounds tested (Fig. 2, Table 2). We showed that DP-TAT-59 and DM-DP-TAT-59 inhibited the growth of MCF-7 xenografts in a dose-dependent manner and that their antitumor activity is comparable to that of TAT-59 (Table 3).

The finding that tamoxifen is less effective in breast cancer patients younger than 50 years old is probably associated with age-related changes in the endocrine environment and with low affinity of tamoxifen for the

Table 3 Antitumor activity of metabolites of TAT-59 against human mammary carcinoma MCF-7 xenografts

Drug	Dose (mg/kg/day)	Schedule	T/C (%)							
			Day 3	Day 7	Day 10	Day 14	Day 17	Day 21	Day 24	Day 28
DP-TAT-59	5	Days 1–9	86.7	68.8**	54.4**	45.7**	43.6**	38.2**	37.8**	35.3**
	1	Days 1–9	94.0	80.1	66.3**	64.3**	66.2**	63.8**	65.6**	62.9**
	0.2	Days 1–9	94.5	77.7	72.5*	67.8**	75.2*	73.1*	66.6**	68.4**
DM-DP-TAT-59	5	Days 1–9	87.3	60.8**	52.4**	46.3**	43.7**	37.6**	35.0**	34.1**
	1	Days 1–9	94.8	75.0*	64.4**	65.7**	61.4**	56.5**	54.9**	57.7**
	0.2	Days 1–9	87.1	70.9**	66.1**	65.9**	62.3**	59.1**	61.7**	61.2**
Control[a]			84.7*	57.5**	44.5**	32.2**	28.1**	22.6**	18.5**	14.0**

*$P < 0.05$, **$P < 0.01$, vs controls with estradiol supplementation, Dunnett's *t*-test
[a] Without estradiol supplementation

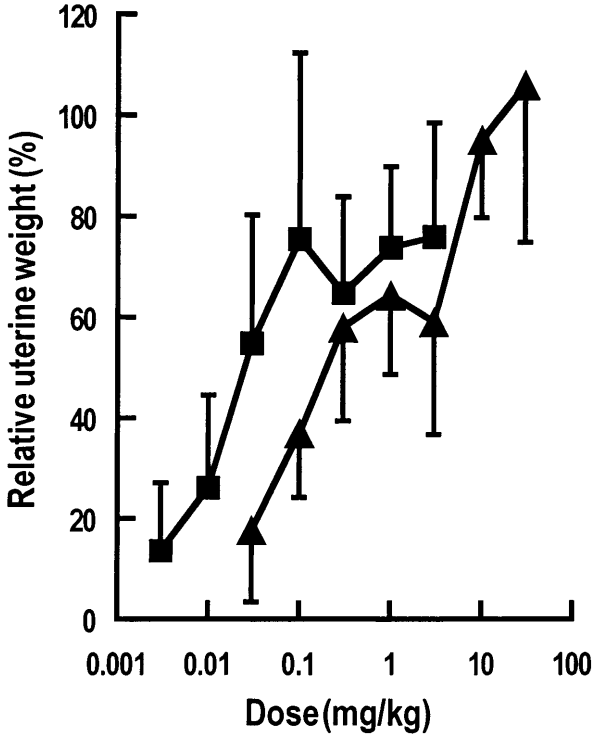

Fig. 4 Estrogenic effect of TAT-59 and tamoxifen in the ovariectomized (*OVX*) rat model (■ TAT-59, ▲ tamoxifen). The values shown are the means and SD

ER. It seems possible that TAT-59, which has a high affinity for the ER [22] and is easily transferred to tumor tissue in high concentrations [22], will be highly effective against breast cancer in patients whose tumors have a low sensitivity to tamoxifen. As shown in our experiments, human breast carcinoma R-27, which is resistant to tamoxifen, responded well to TAT-59 therapy but not to treatment with 10 mg/kg tamoxifen or toremifene. The other human breast carcinoma cell line FST-1, established in our laboratory, responded to tamoxifen, but TAT-59 appeared to be more effective even at a dose level as low as 5 mg/kg (Fig. 3, Table 2).

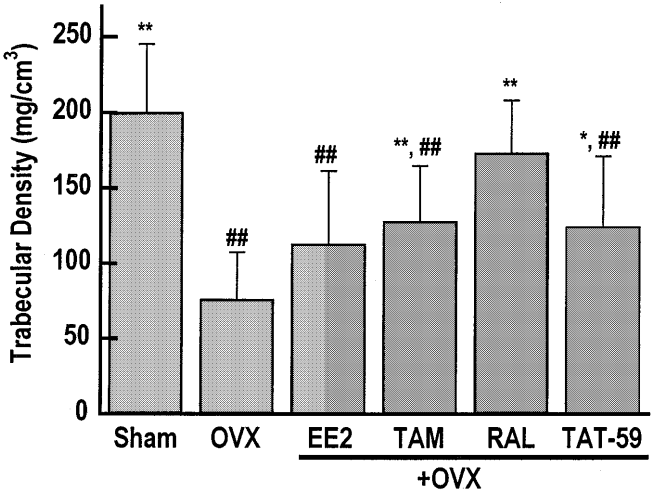

Fig. 5 Effects of antiestrogens on the trabecular density in ovariectomized (*OVX*) rats after 28 days of treatment. OVX rats were treated with ethynyl estradiol (*EE2*), tamoxifen (*TAM*), raloxifene (*RAL*) and TAT-59. The values shown are the means and SD. $*P < 0.05$, $**P < 0.01$, vs ovariectomized controls; $\#P < 0.05$, $\#\#P < 0.01$, vs sham-operated controls

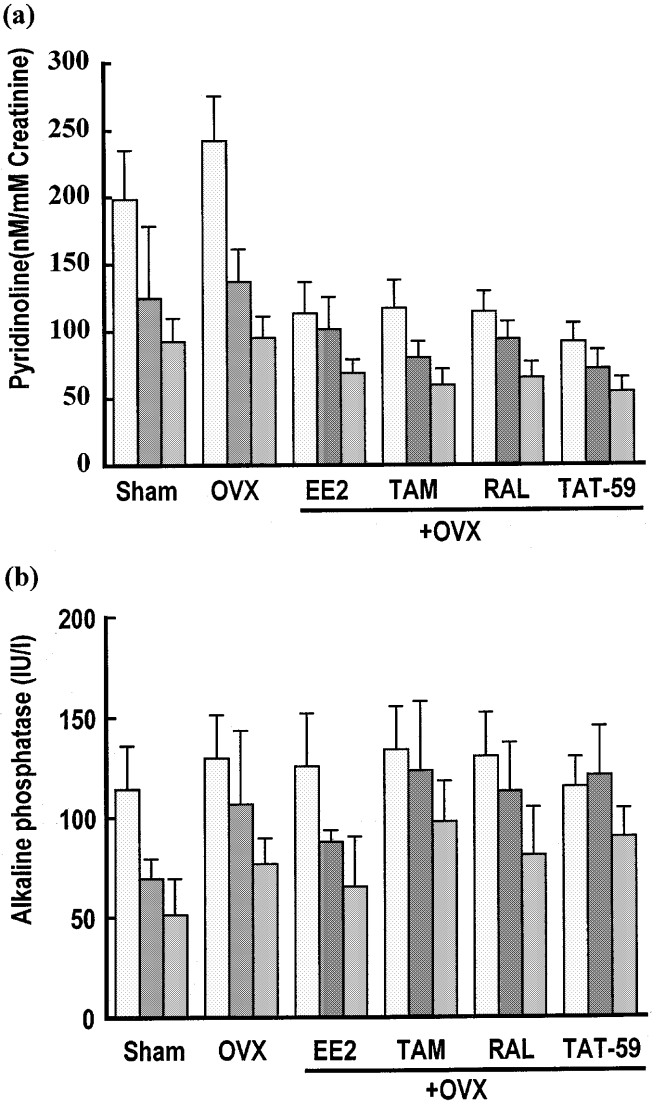

Fig. 6a,b Effects of antiestrogens on pyridinoline (**a**) and alkaline phosphatase (**b**) in ovariectomized (*OVX*) rats after 28, 55 and 78 days of treatment. The values shown are the means and SD (□ 28 days treatment, ▨ 55 days treatment, ▤ 78 days treatment)

We have previously reported that the conditioned medium of MCF-7 cultures treated with DP-TAT-59 inhibits proliferation of even ER-negative cells, and that this activity is partially attributable to TGF-β [23]. The induction of TGF-β, observed after DP-TAT-59 treatment, is stronger than that after treatment with tamoxifen or 4-OH-tamoxifen [23]. Thus, the pronounced induction by DP-TAT-59 of TGF-β, a factor inhibiting epithelial and cancer cell proliferation and

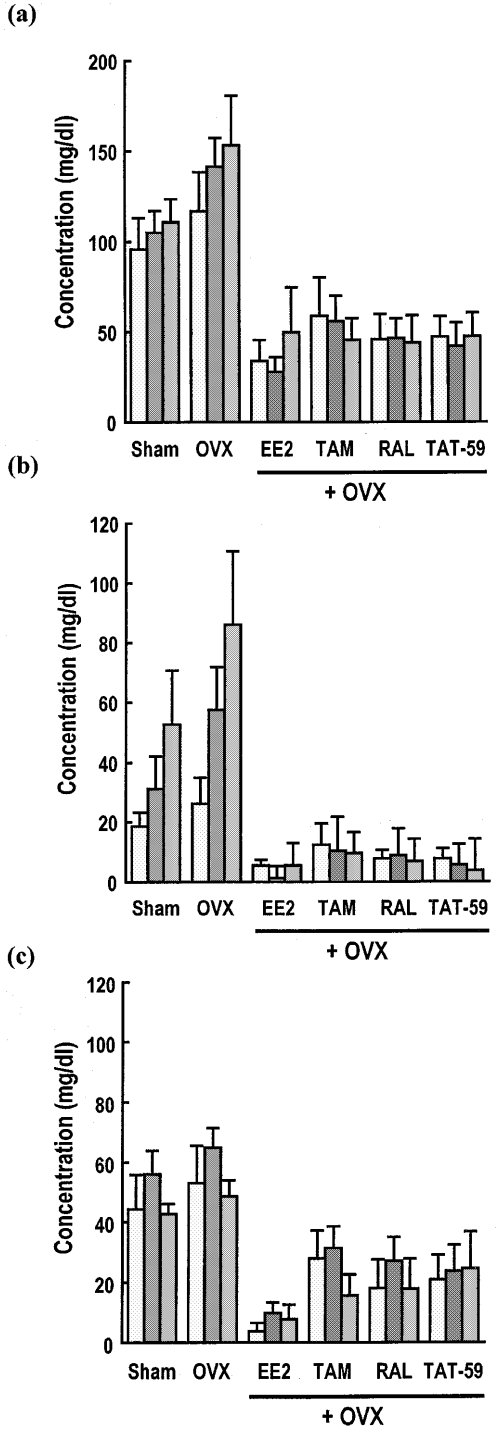

Fig. 7a–c Effects of antiestrogens on the cholesterol levels in ovariectomized (*OVX*) rats after 28, 55 and 78 days of treatment (**a** total cholesterol, **b** LDL cholesterol, **c** HDL cholesterol). The values shown are the means and SD. (□ 28 days treatment, ▨ 55 days treatment, ▤ 78 days treatment)

acting in an autocrine and paracrine manner [15], may provide an additional opportunity to affect cancer cells not responding well to tamoxifen.

TAT-59 exhibits estrogenic activity in the uterus, in a manner similar to that of tamoxifen (Fig. 4). Tamoxifen is often used clinically as the drug of first choice for the treatment of breast cancer in postmenopausal women. The incidence of endometrial cancer in such women treated with tamoxifen is said to be several times higher than in women not receiving tamoxifen. However, the incidence of endometrial cancer among women receiving tamoxifen does not exceed 1% [24]. Tamoxifen has also been reported to reduce blood LDL cholesterol levels in women before and after the menopause [14, 18] and to contribute to the prevention of bone fractures due to its estrogen agonistic activity [18]. Thus, it seems likely that tamoxifen contributes to the reduction in the incidence of cardiovascular disease and osteoporosis in elderly women. Although we cannot rule out that treatment with TAT-59 may elevate the risk of endometrial cancer in breast cancer patients, as is the case with tamoxifen treatment, TAT-59 is expected to contribute to the prevention of cardiovascular disease and osteoporosis, since it suppressed the reduction in bone mineral density and elevation of LDL cholesterol level in ovariectomized rats to an extent similar to that induced by raloxifene or tamoxifen.

Based on these findings, one may expect that TAT-59 will show good therapeutic effects in breast cancer patients not responding to tamoxifen therapy. This reasoning is supported by the fact that TAT-59 is able to deliver a sufficient amount of DP-TAT-59 and DM-DP-TAT-59, its active metabolites with a high affinity for the ER, to estrogen-dependent tumors with a low sensitivity to tamoxifen. In addition, more potent induction of TGF-β by TAT-59-related biotransformation products may complement good antitumor activity of TAT-59.

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