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Development of novel steroid sulfatase inhibitors II. TZS-8478 potently inhibits the growth of breast tumors in postmenopausal breast cancer model rats

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Abstract

In postmenopausal breast cancer tissue, steroid sulfatase (STS) activity is high and much estrone sulfate also exists; these facts reveal that estrone sulfate may be involved in the growth of breast cancer as an estrogen source. Steroid sulfatase is an enzyme, which catalyzes hydrolysis from estrone sulfate to estrone, and the development of steroid sulfatase inhibitors is expected as novel therapeutic drugs for postmenopausal breast cancer. We have developed a novel compound 2′,4′-dicyanobiphenyl-4-*O*-sulfamate (TZS-8478), which has potent steroid sulfatase-inhibitory activity and exhibits no estrogenicity in vitro and in vivo. To elucidate its usefulness as a therapeutic drug for postmenopausal breast cancer, we examined the breast cancer cell proliferation- and breast tumor growth-inhibitory activity of TZS-8478 in postmenopausal breast cancer model rats. TZS-8478 dose-dependently suppressed the estrone sulfate-stimulated proliferation of MCF-7 cells. Regarding nitrosomethylurea (NMU)-induced postmenopausal breast cancer models, furthermore, TZS-8478 (0.5 mg/kg per day) markedly inhibited the estrone sulfate-stimulated growth of breast tumors similarly to estrone sulfate-depletion. TZS-8478 completely inhibited steroid sulfatase activity in tumor, uterus and liver, and also markedly lowered plasma concentrations of estrone and estradiol. The above mentioned results suggested that TZS-8478 may be useful as a therapeutic drug for estrogen-dependent postmenopausal breast cancer.

Keywords: TZS-8478; Steroid sulfatase; Breast tumor; MCF-7; Postmenopausal

1. Introduction

Estrogen is an important pathogenic factor for the proliferation and progression of breast cancer. However, estrogen-dependent breast cancer develops also after menopausal when the production of estrogen in ovaries is decreased. Concentrations of estradiol (E_2) in postmenopausal breast cancer tissue are 10–40-fold higher compared with blood concentrations [1,2], and tissue E_2 concentrations are nearly comparable in both postmenopausal and premenopausal breast cancers [2]. These reports have evidenced that postmenopausal breast cancer itself synthesizes estrogen which is required for its proliferation. Currently, it is widely accepted that estrogen in breast cancer tissue is synthesized via two different pathways, i.e., the aromatase pathway from androstenedione [3,4] and the sulfatase pathway from estrone sulfate (E_1S) [5,6].

In postmenopausal women, blood and tissue concentrations of E_1S, a substrate in the sulfatase pathway, are much higher than blood and tissue concentrations of estrone (E_1) or E_2 [7,8], and the half-life of E_1S is much longer than that of these hormones [9]. Therefore, E_1S in blood and tissues of postmenopausal women is considered to exist as a reservoir for active estrogen which is produced due to the action of steroid sulfatase (STS) [10,11]. The production of E_1 in postmenopausal breast cancer tissue has been reported to be higher by 10 or more times via the sulfatase pathway than via the aromatase pathway [12–14]. Furthermore, recently STS activity in tumors stimulates the development of estrogen-dependent breast cancer and is involved in the growth of the tumor [15]. These facts are based to consider that the growth of postmenopausal breast cancer can be inhibited by blocking STS which catalyzes the hydrolysis of E_1S, inactive estrogen, to E_1 and E_2, active estrogen.

To date, many STS inhibitors have been developed and reported [16,17]. Estrone-3-*O*-sulfamate (EMATE) is one of the most potent inhibitors among them [18]. However,

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EMATE cannot be used as a therapeutic drug for postmenopausal breast cancer because the compound itself is converted to E_1 when blocking STS [19]. COUMATE and its derivatives also block STS activity to a less extent than does EMATE [20,21]. Recently, we developed a novel STS inhibitor, 2′,4′-dicyanobiphenyl-4-*O*-sulfamate (TZS-8478). TZS-8478 is a compound which exhibits no estrogenicity, although its STS-inhibitory activity in vivo is nearly comparable to that of EMATE [22]. To evidence the possibility of TZS-8478 as a therapeutic drug for postmenopausal breast cancer, therefore, we examined its inhibitory activity on the proliferation of breast cancer cells and on the growth of breast tumors in experimentally induced breast cancer model rats.

2. Materials and methods

2.1. Reagents

TZS-8478 (2′,4′-dicyanobiphenyl-4-*O*-sulfamate; 99.5% purity) was synthesized in Organic Chemistry Research Department, Teikoku Hormone Mfg. Co., Ltd. (Fig. 1). Estrone-3-*O*-sulfamate, nitrosomethylurea (NMU), E_1S, and MTT (3-[4,5-dimethythiazol-2-yl] 2,5-diphenyltetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). [6,7-^{3}H] Estrone-3-sulfate ammonium salt (specific activity: 1609.1 GBq/mmol) ([6,7-^{3}H] E_1S) and [4-^{14}C] estrone (specific activity: 1898.1 MBq/mmol) ([^{14}C] E_1) were purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA, USA).

2.2. MCF-7 cells

Human breast cancer MCF-7 cells were obtained from Cell Resource Center for Biomedical Research (Tohoku University). MCF-7 cells were routinely cultured in the presence of 5% CO_2, using the phenol red-free RPMI 1640 medium containing 10% fetal bovine serum (FBS).

2.3. Animals

Female Sprague-Dawley rats were purchased from Charles River Japan. Rats were housed under the following environmental conditions: 23 ± 2 °C in temperature; 55 ± 5% in humidity; and 12 h light:12 h dark cycle in lightening. Rats were fed commercially rat chow and sterilized water ad libitum. All the rats were acclimatized for 7 days prior to experiments. All the experiments were approved by our "Animal Experimentation Ethics Committee" and were conducted according to the guide for the Care and Use of Laboratory Animals by the National Institute of Health.

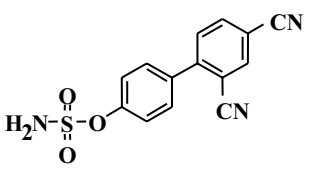

Fig. 1. Chemical structure of TZS-8478.

2.4. Proliferation of breast cancer cells

MCF-7 cells were cultured for 4 days in the phenol red-free RPMI 1640 medium containing 10% FBS which was treated with dextran-coated charcoal (DCC). MCF-7 cells were seeded into the 96-well culture dish to obtain 2×10^3 cells per well and were then cultured for 24 h in the phenol red-free RPMI 1640 medium containing 10% DCC-FBS. The culture medium was replaced with one which contained or did not contain E_1S (1 μM). Furthermore, TZS-8478 or EMATE, previously dissolved in DMSO, was added into the medium to obtain different concentrations. The medium which did not contain any compound was added into the control group. The culture medium was daily replaced with one of same composition, and culture was conduced for 4 days. The number of cells was determined according to the MTT method [35], and absorbance in the control group was expressed as 100%.

2.5. Growth of NMU-induced breast tumors

NMU dissolved in saline was administered (50 mg/kg) intravenously via the tail vein of rats aged 7 weeks. Furthermore, NMU was similarly administered twice every 2 weeks. The development of breast tumors was to be observed. Diameters (minimum axis and maximum axis of the tumors were measured using an electric caliper, and recorded. Rats were ovariectomized at the time when the minimum diameter of the tumors became 10–15 mm, and tumor volume was calculated based on the size of the measured tumors according to the following formula by assuming that the tumor was an ellipse: tumor volume (mm^3) = $4/3 \times \pi \times$ (maximum axis/2) $\times$ (minimum axis/2)^2. Rats, which had shown a reduction in tumor volume during 2-week observation after ovariectomy, were selected, and E_1S (0.02 mg/kg per day) dissolved in saline was administered subcutaneously for 2 weeks. A tumor, which showed an increase in tumor volume due to the administration of E_1S, was considered estrogen-dependent breast tumor. Rats with such tumor were selected. Selected rats were allotted into the following three groups: group 1 ($n = 7$) in which the subcutaneous administration of E_1S (0.02 mg/kg per day) was continued; group 2 ($n = 7$) in which TZS-8478 (0.5 mg/kg per day) suspended in 0.5% Tween 80 solution was administered orally in addition to E_1S; group 3 ($n = 6$) in which the administration of E_1S was discontinued. The vehicle (0.5% Tween 80 solution) was administered orally in groups 1 and 3. During the 4-week administration period, diameters of tumor were measured once a week to calculate tumor volume. Results were expressed as tumor growth (the ratio of initial (0 week) tumor

volume). On the next day after final administration, rats were weighed; under ether anesthesia, blood was collected and the animals were sacrificed. Plasma was obtained from the blood collected according to the routine procedures. Furthermore, breast tumor, as well as the uterus and liver were isolated to determine wet weight. Each of the isolated tissues was frozen in liquid nitrogen without delay and was then stored at −70 °C until the determination of STS activity.

2.6. Determination of STS activity

Isolated and stored tissues, into which phosphate buffer saline containing 250 mM sucrose had been added, were homogenized and centrifuged (2000 × *g*, 20 min, 4 °C) to remove the nucleus and cell debris. With the centrifuged supernatant containing the microsomal fraction as the STS enzyme source, the concentration of the protein was assayed according to the Bradford method using bovine serum albumin as standard.

[6,7-^{3}H] E_1S and nonlabeled E_1S were added into the microsomal fraction (protein content: 0.3–3 mg/ml) of each tissue to obtain the E_1S final concentration of 20 μM, allowing a reaction at 37 °C for 30 min. The reaction was stopped by cooling with ice water, and [^{14}C] E_1 was added to monitor extraction efficiency. Furthermore, toluene was added before vigorous mixing for 30 s, and a scintillation counter was used to determine radioactivity in toluene phase after centrifugation (2000 × *g*, 10 min, 4 °C). The amount of hydrolyzed E_1S was calculated based on the content of radioactivity for [^{3}H] E_1S and on extraction efficiency for [^{14}C] E_1.

2.7. Determination of plasma estrogen concentrations

The determination of plasma concentrations of E_1S, E_1, and E_2 were commended to Sumika Chemical Analysis Service (Osaka, Japan). The volume of plasma used for determination of E_1S, E_1, and E_2 concentration was 1, 0.5, and 0.5 ml, respectively. These plasma estrogen concentrations were determined using high-performance liquid chromatography and mass spectrometry using a API-4000 tandem mass spectrometer equipped with a turbo ionspray source. Intra- and inter-assay precision (%CV) did not exceed 10.0% for these assays. The lower limit of quantification for E_1S, E_1, and E_2 was 0.63 ng/ml, 1.0 and 1.0 pg/ml, respectively.

2.8. Statistical analysis

Regarding statistical analysis, Student's *t*-test was conducted in a comparison between two groups.

3. Results

3.1. Proliferation of MCF-7 breast cancer cells

The addition of 1 μM E_1S stimulated the proliferation of MCF-7 cells, and the number of cells increased by about eight times in 4-day culture. TZS-8478 dose-dependently inhibited this E_1S-stimulated proliferation of the cells; 10^{-8} M TZS-8478 nearly completely inhibited the proliferation. TZS-8478, when added alone, did not affect the proliferation of the cells at all (Fig. 2A). On the other hand, EMATE failed to completely inhibit the E_1S-stimulated proliferation of MCF-7 cells. On the contrary, EMATE, when added at 10^{-8} M or higher concentrations, accelerated the proliferation of the cells as did E_1S. Furthermore, EMATE, when added alone, dose-dependently stimulated the proliferation of the cells (Fig. 2B).

3.2. Growth of NMU-induced breast tumors

Of NMU-induced breast tumors in rats, rats whose tumor had been reduced by ovariectomy and had grown due to the administration of E_1S (0.02 mg/kg per day), were selected as estrogen-dependent postmenopausal breast cancer model rats. The continued administration of E_1S to rats increased tumor volume in these rats, while the discontinuation

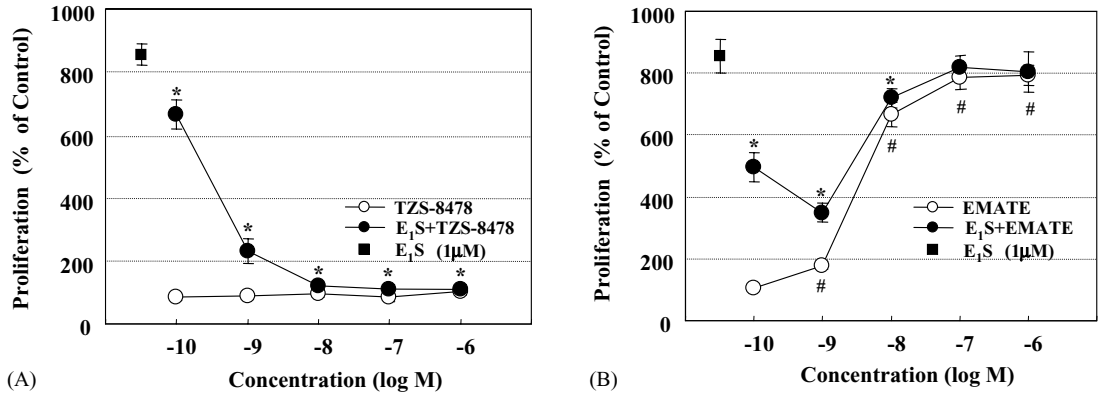

Fig. 2. Effects of TZS-8478 and EMATE on the proliferation of MCF-cells. Various concentrations of TZS-8478 (A) or EMATE (B) were added into a culture medium in the presence or absence of E_1S (1 μM), and MCF-7 cells were cultured for 4 days. Proliferation was assayed according to the MTT method. Results are expressed as means ± S.D. ($n = 4$). $^*P < 0.001$ (Student's *t*-test) vs. E_1S (1 μM), $^\#P < 0.001$ (Student's *t*-test) vs. control.

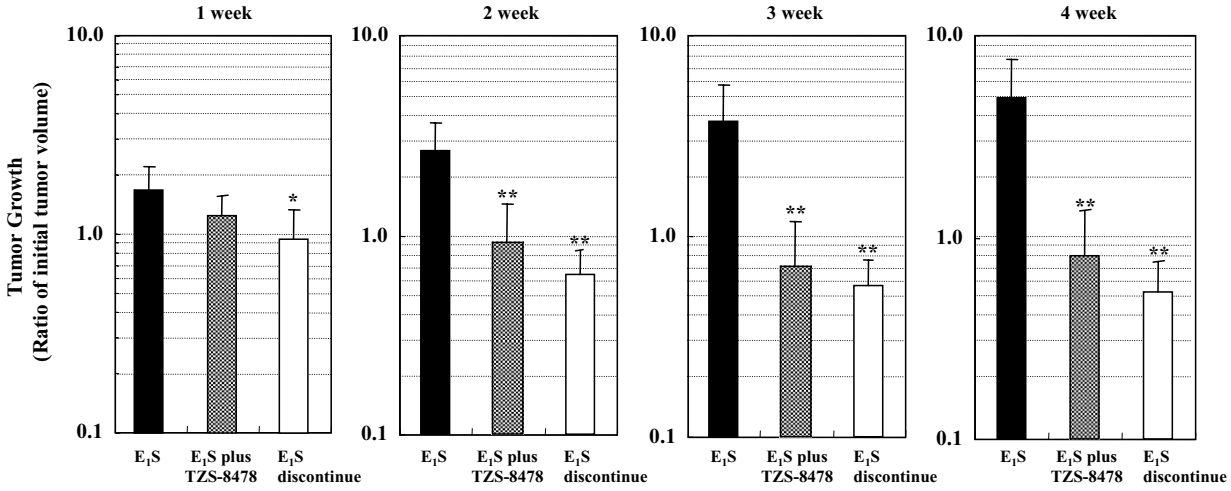

Fig. 3. Effects of TZS-8478 on the growth of NMU-induced breast tumors in postmenopausal breast cancer model rats. E_1S (0.02 mg/kg per day) and TZS-8478 (0.5 mg/kg per day) were administered to NMU-induced postmenopausal breast cancer model rats for 4 weeks, and breast volume was calculated. Results are expressed as means ± S.D. ($n = 6$–7). $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test) vs. the E_1S group.

of E_1S administration markedly reduced tumor volume on a time-course basis. The TZS-8478 administration group showed time-course reductions in tumor volume to an extent comparable to the E_1S discontinued administration group (Fig. 3). On the other hand, uterine weight at the time of necropsy markedly reduced in the E_1S discontinued administration group. TZS-8478 strongly inhibited E_1S-stimulated uterine weight to the level of the E_1S discontinued administration group (Fig. 4).

3.3. STS activity

In any tissue examined, STS activity in the liver, uterus, and tumor of postmenopausal breast cancer model rats was so markedly inhibited by the administration of TZS-8478 as to become undetectable (Fig. 5A–C).

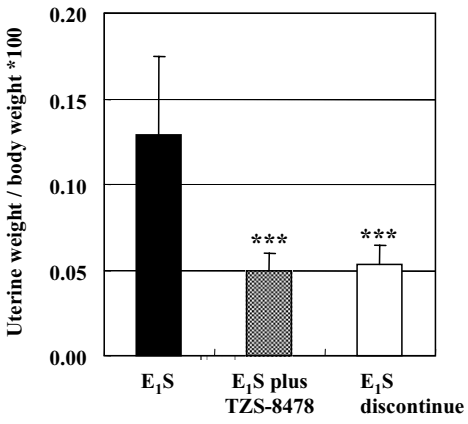

Fig. 4. Effects of TZS-8478 on uterine weight in postmenopausal breast cancer model rats. E_1S (0.02 mg/kg per day) and TZS-8478 (0.5 mg/kg per day) were administered to NMU-induced postmenopausal breast cancer model rats for 4 weeks, and uterine weight was measured. Results are expressed as means ± S.D. ($n = 6$–7). $^{***}P < 0.001$ (Student's *t*-test) vs. the E_1S group.

3.4. Plasma estrogen concentrations

E_1 and E_2, in addition to E_1S, were detected in plasma in the E_1S administration group and TZS-8478 administration group. Plasma E_1S concentrations increased, however, plasma E_1 and E_2 concentrations markedly decreased in the TZS-8478 administration group as compared with the E_1S administration group (Fig. 6A–C).

4. Discussion

STS activity is high and much E_1S also exists in postmenopausal breast cancer tissue. Therefore, inhibitors of STS which catalyzes the conversion of E_1S to E_1 are expected as therapeutic drugs. To elucidate the usefulness of a newly developed STS inhibitor, TZS-8478, as a therapeutic drug for postmenopausal breast cancer, we examined its inhibitory activity on the proliferation of breast cancer cells and its inhibitory activity on the growth of breast tumors in postmenopausal breast cancer model rats.

TZS-8478 dose-dependently inhibited the E_1S-induced proliferation of MCF-7 cells; the inhibitory activity of TZS-8478 was more potent than that of EMATE. On the other hand, TZS-8478, when administered alone, did not affect the proliferation of MCF-7 cells at all. However, EMATE, when administered alone, dose-dependently stimulated the proliferation of MCF-7 cells. This finding allows us to presume that TZS-8478, when administered alone, did not show cell proliferation-stimulating activity because it has no estrogenicity [22]. On the other hand, we considered that EMATE, which has potent STS inhibitory activity,

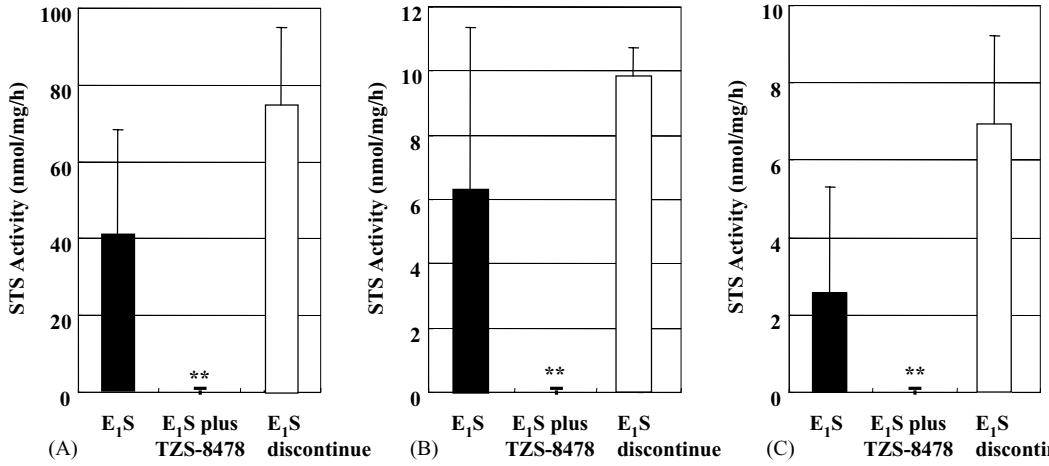

Fig. 5. Effects of TZS-8478 on the STS activity in postmenopausal breast cancer model rats. E_1S (0.02 mg/kg per day) and TZS-8478 (0.5 mg/kg per day) were administered to NMU-induced postmenopausal breast cancer model rats for 4 weeks, and STS activity in the liver (A), uterine (B), and tumor (C) was assayed. Results are expressed as means ± S.D. ($n = 6$–7). $^{**}P < 0.01$ (Student's t-test) vs. the E_1S group.

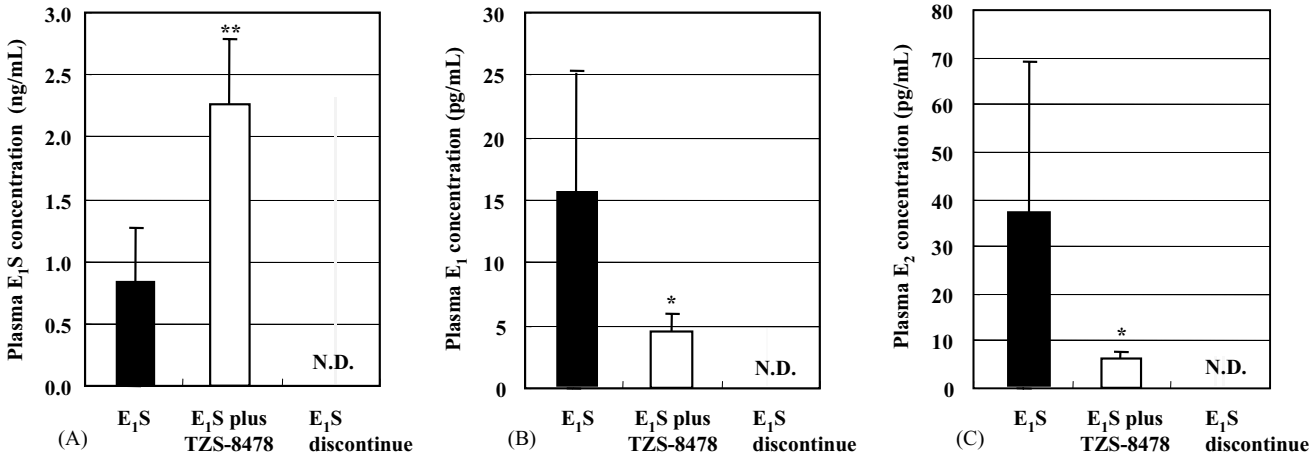

Fig. 6. Effects of TZS-8478 on plasma estrogen concentrations in postmenopausal breast cancer model rats. E_1S (0.02 mg/kg per day) and TZS-8478 (0.5 mg/kg per day) were administered to NMU-induced postmenopausal breast cancer model rats for 4 weeks, and plasma concentrations of E_1S (A), E_1 (B), and E_2 (C) were determined. Results are expressed as means ± S.D. ($n = 6$–7). $^{*}P < 0.05$ (Student's t-test) vs. the E_1S group; N.D.: not detected.

exerted marked estrogenicity in a low concentration range inclusive because STS decomposes EMATE into E_1 [19]. TZS-8478 was thus evidenced to completely inhibit the E_1S-induced proliferation of breast cancer cells at a relatively low concentration (10^{-8} M), which suggested its potential to become a therapeutic drug for postmenopausal breast cancer.

TZS-8478 (0.5 mg/kg per day) nearly completely inhibited the STS activity in the liver or uterus of normal rats [22]. Therefore, we examined the effects of TZS-8478 on postmenopausal breast tumor growth at this dose level. The breast tumors in postmenopausal breast cancer model rats were further stimulated by the administration of E_1S, and tumor growth reduced on a time-course basis due to the discontinuation of E_1S administration. In this model rats, TZS-8478 showed not only inhibitory activity on the E_1S-induced growth of tumor but also atrophying activity on the tumor. This tumor-atrophying activity of TZS-8478 was comparable to the discontinuation of E_1S, i.e., estrogen depletion. In the breast cancer model which we used in the present study, extrinsically administered E_1S was used to stimulate tumor growth. Therefore, it is also possible that E_1 or E_2, which is required for tumor growth, is produced and supplied not only in the tumor but also in the liver where STS activity is high. Nevertheless, TZS-8478 nearly completely inhibited the STS activity in tissues and markedly lowered plasma concentrations of E_1 and E_2 to be produced from E_1S due to the action of STS. TZS-8478 was suggested to inhibit the E_1 production not only in the liver but also in the tumor and inhibited the conversion from E_1S to E_1, i.e., the production of active estrogen, due to its potent STS-blocking activity. TZS-8478 thus markedly inhibited the E_1S-induced tumor growth.

We have not examined the mode of inhibition of STS activity by TZS-8478. However, the complete sulfatase inhibition was observed in the diluted microsomal fractions prepared from tissues which administered TZS-8478. This result suggested that TZS-8478 is probably not a reversible inhibitor which repeats binding to and dissociations from the STS enzyme but a suicide inhibitor which never shows dissociations once bound to the enzyme.

A number of steroidal and nonsteroidal compounds have been synthesized as STS inhibitors. However, most of them have not yet been examined with respect to their usefulness as therapeutic drugs. Among them, 667 COUMATE—a compound with potent STS-inhibitory activity—inhibited the E1S-stimulated growth of tumors at a dose level of 2 mg/kg per day, and its possibility as a therapeutic drug for postmenopausal breast cancer has been reported [21]. We did not make any direct comparison between the two compounds. However, TZS-8478 is expected to have more potent actions than 667 COUMATE because the former nearly completely inhibits tumor growth at a dose level of 0.5 mg/kg per day. The above results suggested the usefulness of TZS-8478 as a therapeutic drug for postmenopausal breast cancer.

Very high concentrations of dehydroepiandrosterone sulfate (DHEAS) exist in blood and breast cancer tissue in postmenopausal women [23]. Dehydroepiandrosterone (DHEA) which is produced from DHEAS due to the action of STS [24] and its metabolites—androstenedione and androstenediol—[25] stimulate the proliferation of breast cancer cells [26–28] and dimethylbenzanthracene-induced rat breast tumor cells [29–31]. Therefore, DHEAS is also considered one of the estrogen sources which stimulate postmenopausal breast cancer. Aromatase inhibitors have been developed as therapeutic drugs for postmenopausal breast cancer, and their favorable clinical results have been reported [32,33]. However, androstenediol has binding affinity for the estrogen receptor [26] and possesses direct estrogenicity which is not affected by aromatase inhibitors. Furthermore, the proliferation of breast cancer cells which is stimulated by DHEAS has been reported not to be affected by aromatase inhibitors but to be inhibited by STS inhibitors [27,34]. Since TZS-8478 inhibits not only the hydrolysis from E_1S to E_1 but also the hydrolysis from DHEAS to DHEA (results not shown), the compound is considered to block even the estrogen production system in which aromatase is not involved. In conclusion, TZS-8478 is conjectured to have potential to be an effective therapeutic drug for postmenopausal breast cancer.

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