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Effects of CH4893237, a new orally active estrogen receptor downregulator, on breast cancer xenograft models with low serum estrogen levels

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Abstract. We compared the antitumor efficacy and estrogen receptor (ER) degradation of CH4893237, a new orally active selective ER downregulator, with fulvestrant and tamoxifen in human breast cancer xenografts with low levels of serum estrogen (E_2) (50.6, 22.9 and <16.7 pg/ml), equivalent to the ranges in postmenopausal or aromatase inhibitor-treated breast cancer patients. In addition, using proteolysis assays, we tested the conformational changes induced in ERα and ERß by CH4893237, fulvestrant, and 4-OH tamoxifen (4OHT). In ZR-75-1 xenografts with 50.6 pg/ml E_2, CH4893237 (100 and 300 mg/kg/day p.o.) as well as fulvestrant (1 and 3 mg/body/week s.c.) showed complete growth inhibition (>90%) and tamoxifen (30 and 100 mg/kg/day p.o.) showed moderate tamoxifen resistance. The antitumor activity of CH4893237 (300 mg/kg) was the same as that of fulvestrant (3 mg/body) but the rate of ER degradation induced by CH4893237 (300 mg/kg) was significantly stronger than that of fulvestrant (3 mg/body) (94.3 vs. 85.5%, $P<0.01$). In Br-10 xenografts with 22.9 pg/ml E_2, CH4893237 (30 mg/kg) and fulvestrant (1 mg/body) showed potent growth inhibition (>70%) whereas tamoxifen (1, 10 and 100 mg/kg) showed strong tamoxifen resistance. In Br-10 xenografts with ovariectomized-level E_2 (<16.7 pg/ml), tamoxifen (30 mg/kg) increased the tumor volume but CH4893237 (30 mg/kg) showed no agonistic activity. In the ERα and ERß proteolysis assays, the band pattern for CH4893237 was different from fulvestrant. Thus, CH48793237 showed potent antitumor efficacies without agonistic activity and superior ER degradation in human breast cancer xenografts with low serum E_2. Furthermore, the proteolysis studies suggest that CH4893237 induces conformational changes of ER different from those induced by fulvestrant. Therefore, CH4893237 alone or in combination with an aromatase inhibitor may be an efficient treatment for postmenopausal breast cancer patients.

Introduction

About 80% of postmenopausal breast cancer patients express estrogen receptor (ER) (1), and these tumors are largely responsive to antiestrogenic therapies. Currently, three classes of antiestrogenic drugs are available for postmenopausal women with ER-positive advanced breast cancer (2). Selective estrogen receptor modulators (SERMs), such as tamoxifen, compete with estrogen for the ER, and aromatase inhibitors (AIs), such as anastrozole, letrozole, and exemestane, block the conversion of androgen precursors into estrogens. In addition, the novel selective estrogen downregulator (SERD) fulvestrant is an ER antagonist with no agonist effects; it binds to and downregulates ER (3).

Among these antiestrogenic agents, fulvestrant, the first SERD, was shown to be as effective as the AI anastrozole in patients who relapsed during tamoxifen treatment (4,5). Fulvestrant has also shown similar efficacy to tamoxifen for postmenopausal women with ER-positive advanced breast cancer as the first-line treatment (6), and a regime combining fulvestrant with the AI anastrozole is currently being investigated for these patients as a first-line treatment (7,8). Thus, fulvestrant is considered to be an effective therapeutic agent for ER-positive postmenopausal breast cancer patients even though it must be administered intramuscularly in the clinical settings owing to its poor bioavailability.

Hoffmann *et al* introduced an orally available steroidal SERD, ZK-253, which showed antitumor activities against ER-positive human breast cancer xenografts in 2004 (9). Dardes *et al* reported that an orally available nonsteroidal SERD, GW5638, was efficacious against ER-positive human breast cancer and endometrial cancer xenografts in 2002 and 2007 (10,11). We reported in 2005 that a new orally

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active steroidal SERD, CH4893237, reduced the amount of ER and impaired the nuclear accumulation of ER in MCF-7 cells *in vitro*, and indicated oral antitumor activity against the MCF7 xenograft *in vivo* (12). As the next step, we decided to evaluate the clinical potential of CH4893237 in postmenopausal or AI-treated breast cancer patients. To estimate the clinical potential of CH4893237, we needed human breast cancer xenografts with low serum estrogen (E_2) levels that would reflect the conditions of postmenopausal or AI-treated breast cancer patients. Few studies, however, are available on human breast cancer xenografts with low serum E_2 except for long-term E_2-treated MCF-7 xenografts (13,14). In addition, these tumors grow slowly and are small. Therefore, we established new breast cancer xenografts with low serum E_2 levels (50.6 pg/ml and 22.9 pg/ml) and with ovariectomized (OVX)-level E_2 (<16.7 pg/ml) using human breast cancer cells ZR-75-1 or Br-10 and then assessed the clinical potential of CH4893237 by comparing its efficacy with those of fulvestrant and tamoxifen. Moreover, to distinguish the molecular mechanisms underlying the ERα and ERß of CH4893237, fulvestrant, and 4-OH tamoxifen (4OHT), we tested the conformational changes induced in ERα and ERß by these compounds using proteolysis assays.

Materials and methods

Drugs. CH4893237 and fulvestrant (Fig. 1) were synthesized in Chemistry Research Department I, Chugai Pharmaceutical Co., Ltd. Tamoxifen (Fig. 1), 4-OH tamoxifen, and estradiol were purchased from Sigma (St. Louis, MO).

Animals. Female 5-week-old athymic nude mice were purchased from Nihon Clea (Tokyo, Japan). Mice were freely provided with mouse chow CE2 and tap water. All the experiments were approved by the Animal Experimentation Ethics Committee of Chugai and carried out according to the guidelines for the Care and Use of Laboratory Animals promulgated by Chugai Pharmaceutical Co., Ltd.

Cells and tumors. The human breast cancer cell line ZR-75-1 was purchased from the American Type Culture Collection (Manassas, VA, USA). For the ZR-75-1 xenograft experiments, ZR-75-1 cells were cultured in DMEM medium supplemented with 10% FBS (Logan, UT, USA).

The human breast cancer Br-10 tumor was purchased from the Central Laboratories for Experimental Animals (Tokyo, Kanagawa). For Br-10 xenograft experiments, Br-10 tumors were maintained by serial subcutaneous transplantation in the subaxillary region of female nude mice.

Antitumor activity and ER degradation of CH4893237 in ZR-75-1 xenografts with 50.6 pg/ml of serum E_2. Ovariectomized (OVX) female nude mice were subcutaneously implanted with E_2 implants (0.2 mm I.D. in a 1.0-cm silastic tube; Dow Corning Corp, Michigan) containing a weight ratio of estradiol (E_2) to cholesterol (C) of 1:99 as previously described (12) 1 week before ZR-75-1 cell transplantation. In preliminary studies, we found that the E_2 implants (E_2:C=1:99) at 2 weeks after implantation produced an average of 50.6 pg/ml of serum E_2 in OVX female nude mice. ZR-75-1 at 1×10^6 cells/mouse was subcutaneously transplanted into the right flank of the nude mice. Drug administration was initiated when the tumor volume reached between 50 and 150 mm^3. CH4893237 (30, 100 and 300 mg/kg) or tamoxifen (30 and 100 mg/kg) were orally administered 5 times per week for 6 weeks. Fulvestrant (1 and 3 mg/body) was subcutaneously administered once a week for 6 weeks. Tumor weights were measured on the next day after the final administration.

At the end of the experiment period, the tumors were collected and frozen at -140˚C. To determine the ER levels, the tumors were homogenized in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM DTT, 10% glycerol, and protease inhibitor (Boehringer Mannheim, Ingelheim, Germany) on ice. After centrifugation at 1750 x g for 10 min at 4˚C, the pellets were suspended in a lysis buffer containing 500 mM NaCl using a vortex mixer and centrifuged at 25000 x g for 30 min at 4˚C. The ER protein in the supernatants was quantified with an ER enzyme immunoassay kit (Abbott, Abbott Park, IL, USA).

Antitumor activity of CH4893237 in Br-10 xenografts with 22.9 pg/ml of serum E_2. OVX female nude mice were subcutaneously implanted with E_2 implants (E_2:C=1:299) 1 week before tumor transplantation, as described above. In preliminary studies, we found that E_2 implants (E_2:C=1:299) produced a mean 22.9 pg/ml of serum E_2 at 2 weeks after implantation in OVX female nude mice. Small pieces (~3x3 mm) of the previously grown Br-10 xenografts were transplanted into the right flanks of nude mice. Drug administration was initiated when the tumor volume had reached between 50 and 150 mm^3. CH4893237 (10 and 30 mg/kg) or tamoxifen (1, 10 and 100 mg/kg) were orally administered 5 times per week for 6 weeks. Fulvestrant (1 mg/body) was subcutaneously administered once a week for 6 weeks. Tumor weights were measured on the day following the final administration.

Effect of CH4893237 on Br-10 xenografts with OVX level (<16.7 pg/ml) of serum E_2. Small pieces (~3x3 mm) of previously grown Br-10 xenografts were transplanted into OVX female nude mice. In preliminary studies, we found that serum E_2 in OVX female nude mice had a mean concentration of <16.9 pg/ml at 2 weeks after ovariectomization. An E_2 solution in EtOH was percutaneously administered at 0.1 mg/head once a week to preserve the transplanted Br-10 tumors. Drug administration was initiated when the tumor volume reached about 100 mm^3. CH4893237 (30 mg/kg) and tamoxifen (30 mg/kg) were orally administered 5 times per week for 25 days. Tumor volumes were measured once a week. Tumor volume (V) was estimated using the equation $V=ab^2/2$, where a and b are tumor length and width, respectively.

ER proteolysis assay. ^{35}S-radiolabeled ERα and ERß proteins were generated by *in vitro* translation using a TNT-coupled transcription-translation system (Promega, Madison, WI)

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to the manufacturer's instructions. Aliquots (25 ml) labeled ER proteins were incubated with E_2, CH4893237, fulvestrant, and 4OHT at a final concentration of 10 μM for 20 min at 25˚C. Aliquots (4 ml) of the drug-treated ER were incubated with or without trypsin at final concentrations of 8, 12 and 16 μg/ml (Sigma). After a 10-min incubation at 25˚C, the digestions were halted with the addition of 2X loading buffer. The samples were analyzed on 10-20% gradient Tris-Glycine gels (Novex, Richmond, BC). Gels were dried under vacuum for 1 h, exposed to X-ray film at -80˚C for 2 h, and then developed.

Calculation of tumor growth inhibition rate (%) and ER degradation rate (%) of the ZR-75-1 xenografts. For the ZR-75-1 xenograft experiments, the tumor growth inhibition rate (TGI%) was calculated as follows: TGI%=[1-(tumor weight of the treatment group on the next day after the final administration)/(tumor weight of the control group on the next day after the final administration)] x100.

The ER degradation rate (ERD%) was calculated as follows: ERD%=[1-(amount of ER protein of the treatment group on the next day after the final administration)/(amount of ER protein of the control group on the next day after the final administration)] x100.

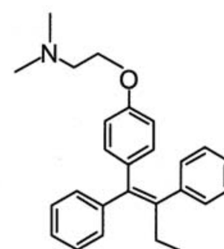
Statistical analysis. Differences in tumor weights or amounts of ER protein were compared using the Student's unpaired t-test.

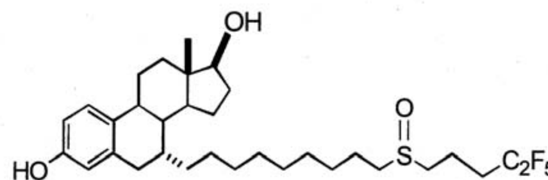
Results

Antitumor activity and ER degradation of CH4893237 in ZR-75-1 xenografts with 50.6 pg/ml of serum E_2. Earlier, we reported that CH4893297 decreased the amount of ER in MCF-7 cells *in vitro* and strongly inhibited tumor growth in tamoxifen-sensitive MCF-7 xenografts *in vivo* (12). In the MCF-7 xenograft experiment, we used E_2 implants (E2:C=1:99) and maintained the level of serum E_2 at 50.6 pg/ml.

In the present study, we established two different human breast cancer models: ZR-75-1 and Br-10 xenografts with respective serum E_2 concentrations of 50.6 and 22.9 pg/ml. The growth rates for the ZR-75-1 xenografts with 50.6 pg/ml of serum E_2 and the Br-10 xenografts with 22.9 pg/ml of serum E_2 were slow compared with the rates for MCF-7 xenografts, but, by using E_2 implants, tumor volumes were greater than 500 mm^3 after 40 days of treatment (Table I).

To evaluate the antitumor activity and ER degradation of CH4893237 against human breast tumors with low serum E_2, we first used the ZR-75-1 xenografts which have been reported to show partial tamoxifen resistance (9). As shown in Fig. 2A and Table II, in ZR-75-1 xenografts with 50.6 pg/ml of serum E_2, tamoxifen at 30 and 100 mg/kg p.o. showed moderate tamoxifen resistance with a maximum TGI% (30 mg/kg p.o.) of 69%. In contrast, CH4893237, administered orally, inhibited tumor growth in a dose-dependent manner and the TGI% of CH4893237 at 100 and 300 mg/kg p.o. was >90%. The antitumor activity of CH4893237 (100 mg/ kg p.o.) was significantly stronger than that of tamoxifen (30 mg/kg p.o.) (P<0.001). Also, the antitumor activity of CH4893237 (100 and 300 mg/kg p.o.)





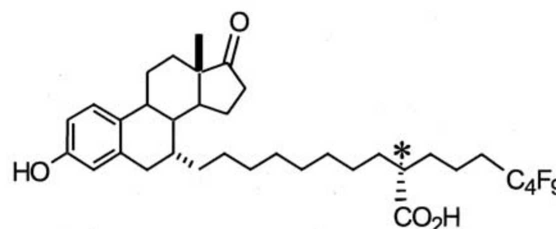


Figure 1. Chemical structures of tamoxifen, fulvestrant, and CH4893237.

was almost the same as that of the OVX control (E2(-) cont.) or fulvestrant (1 and 3 mg/body/week s.c.).

We further analyzed the ER protein levels in the ZR-75-1 xenograft tumors (Fig. 2B, Table II) and found that CH4893237 decreased the level of ER in a dose-dependent manner. Surprisingly, although the antitumor activity of CH4893237 at 300 mg/kg p.o. was almost the same as that of fulvestrant at 3 mg/body/week s.c. (TGI: 91.4 vs. 92.4%), ER degradation induced by CH4893237 at 300 mg/kg p.o. was significantly stronger than that from fulvestrant at 3 mg/body/week s.c. (94.3 vs. 85.5%, P<0.01). ER degradation from tamoxifen at 30 and 100 mg/kg p.o. was very weak, and the maximum ERD% (100 mg/kg p.o.) was only 26.5%.

Antitumor activity of CH4893237 in Br-10 xenografts with 22.9 pg/ml of serum E_2. CH4893237, unlike tamoxifen, caused marked antitumor activity and ER degradation in the ZR-75-1 xenografts with 50.6 pg/ml of serum E_2. These findings raised the possibility that CH4893237 would show efficacy against postmenopausal or AI-treated post-menopausal breast cancer patients having lower serum E_2 levels. To address these questions, we next used the Br-10 xenografts, which had been implanted with the E_2 implants

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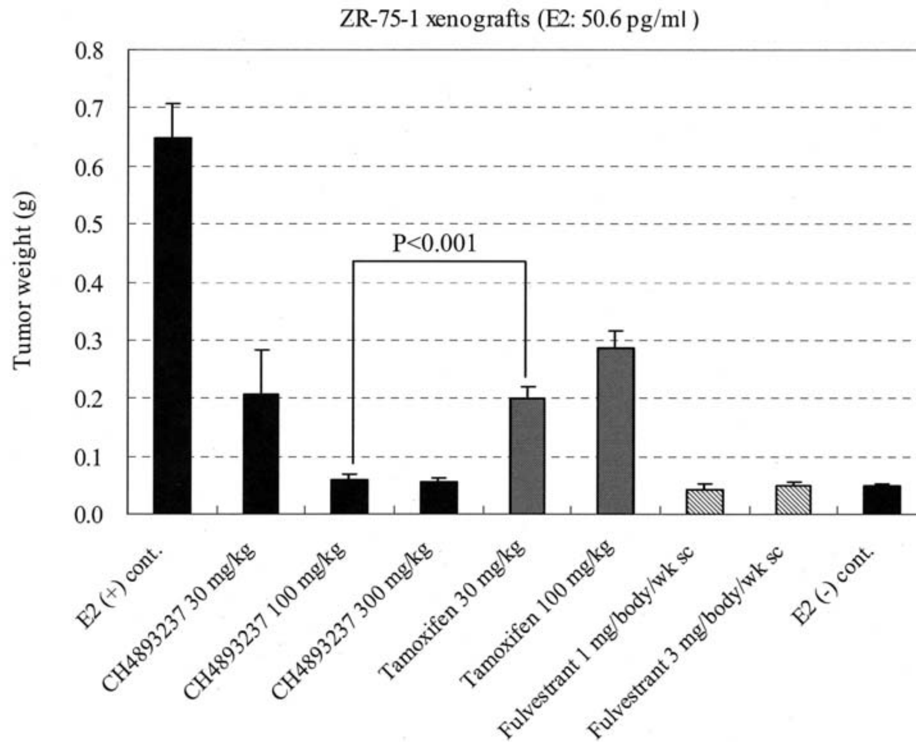

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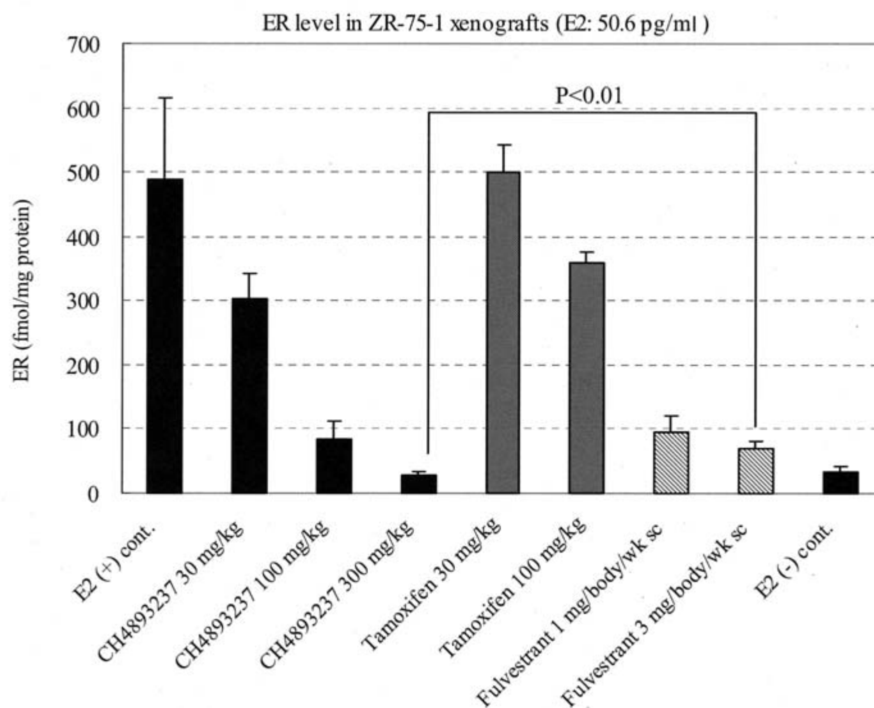

Figure 2. Antitumor activities of tamoxifen, fulvestrant, and CH4893237 in ZR-75-1 xenografts with 50.6 pg/ml of serum E_2 (A) and degradation of ER in the ZR-75-1 tumor by tamoxifen, fulvestrant, and CH4893237 (B). OVX nude mice carrying the E_2 implant (E_2:C=1:99) were inoculated with ZR-75-1 cells ($1x10^6$ cells/head). Drug administration was initiated when the tumor volume reached between 50 and 150 mm^3. (A) The mice were orally administered 30, 100 or 300 mg/kg of CH4893237 or 30 or 100 mg/kg of tamoxifen 5 times per week for 6 weeks, or they were administered s.c. 1 or 3 mg/body of fulvestrant once a week for 6 weeks. Control mice received only vehicle. Tumor weights were determined on the next day after the final administration. (B) At the end of the study, the tumors were collected, frozen (-140˚C), and the ER content quantified by ER-EIA. Each group consisted of six to seven tumors and the data is the mean value of the six to seven tumors with standard errors. $P<0.01$, $P<0.001$ (unpaired t-test). E_2(+) cont, Control OVX mice that received estrogen; E_2(-) cont, Control OVX mice that did not receive estrogen.

(E_2:C=1:299) yielding serum E_2 levels of 22.9 pg/ml, similar to those in postmenopausal women (15).

As shown in Fig. 3, in the Br-10 xenografts with 22.9 pg/ml of serum E_2, interestingly, tamoxifen (1, 10, and 100 mg/kg p.o.) showed strong tamoxifen resistance, and the maximum TGI% (10 mg/kg p.o.) was only 29.5%. In contrast, CH4893237 at 10 and 30 mg/kg p.o. inhibited the tumor growth in a dose-dependent manner, and the TGI% of

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Human breast cancer xenograft models with low-serum E_2 levels.

Xenograft model (E_2 implant)[a]	Serum E_2[b]	Duration of treatment (days)	Tumor volume in E_2(+) group (mm^3)	Tumor volume in E_2(-) group (mm^3)
MCF-7 (E_2:C=1:99)	50.6 pg/ml	40	1,007.9±97.4	84.7±9.5
ZR-75-1 (E_2:C=1:99)	50.6 pg/ml	40	535.6±8.4	38.4±6.6
Br-10 (E_2:C=1:299)	22.9 pg/ml	40	888.6±82.5	249.9±43.6

Results for tumor volumes indicate the mean values of six to seven animals with standard errors. [a]OVX female nude mice were subcutaneously implanted with E_2 implants of estradiol (E_2) to cholesterol (C) of 1:99 or 1:299) within 1 week before transplantation with MCF-7, ZR-75-1, or Br-10 breast cancer cells. [b]In preliminary studies, the concentrations of serum E_2 in the E_2 implanted (E_2:C = 1:99 or 1:299) OVX female nude mice were measured 2 weeks after the E_2 implantation. E_2(+) group: OVX mice that received the E_2 implants. E_2(-) group: OVX mice that did not receive the E_2.

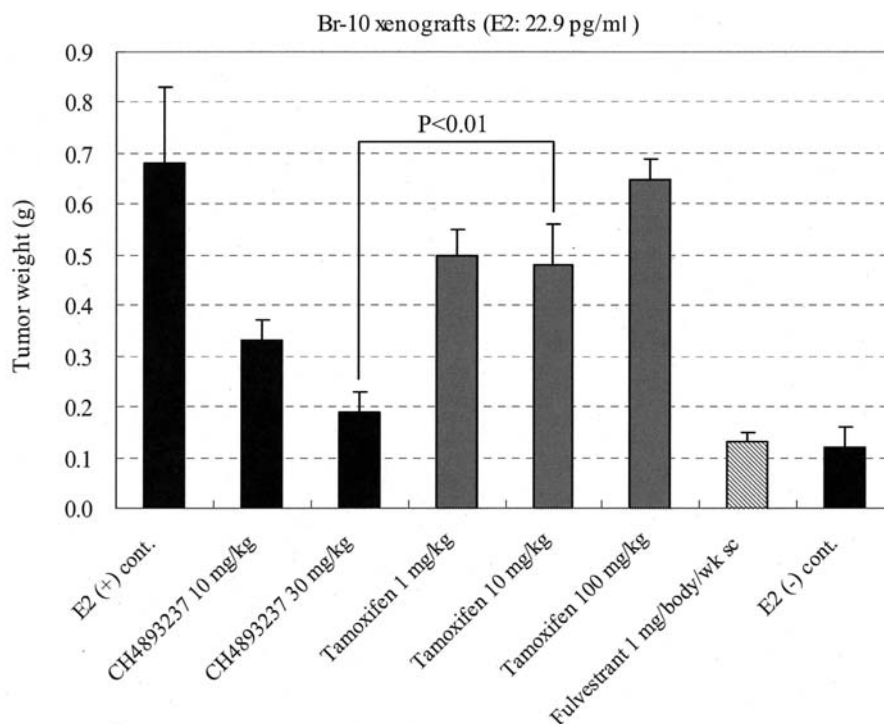

Figure 3. Antitumor activities of tamoxifen, fulvestrant, and CH4893237 in Br-10 xenografts with 22.9 pg/ml of serum E_2. OVX nude mice carrying the E_2 implant (E_2:C=1:299) were inoculated with Br-10 tumors (small pieces ~3x3 mm). Drug administration was initiated when the tumor volume reached between 50 and 150 mm^3. The mice were orally administered 10 or 30 mg/kg CH4893237 or 1, 10 or 100 mg/kg tamoxifen 5 times per week for 6 weeks, or they were administered s.c. 1 mg/body fulvestrant once a week for 6 weeks. Control mice received only vehicle. Tumor weights were determined on the next day after the final administration. Each group consisted of seven animals and the data are the mean values of seven animals with standard errors. P<0.01 (unpaired t-test). E_2(+) cont, Control OVX mice that received estrogen; E_2(-) cont, Control OVX mice that did not receive estrogen.

CH4893237 at 30 mg/kg p.o. was >70%. The antitumor activity of CH4893237 at 30 mg/kg p.o. was significantly stronger than that of tamoxifen at 10 mg/kg p.o. (P<0.01). In addition, the antitumor activity of CH4893237 (30 mg/kg p.o.) was comparable to that of the OVX control [E_2(-) cont.] and fulvestrant (1 mg/body/week s.c.).

Effect of CH4893237 on Br-10 xenografts with OVX level (<16.7 pg/ml) of serum E_2. We next examined the efficacies of CH4893237 in the OVX Br-10 xenograft with serum E_2 levels similar to those seen in AI-treated postmenopausal breast cancer patients (Fig. 4). In the OVX xenografts, surprisingly, tumor volume was increased by tamoxifen at

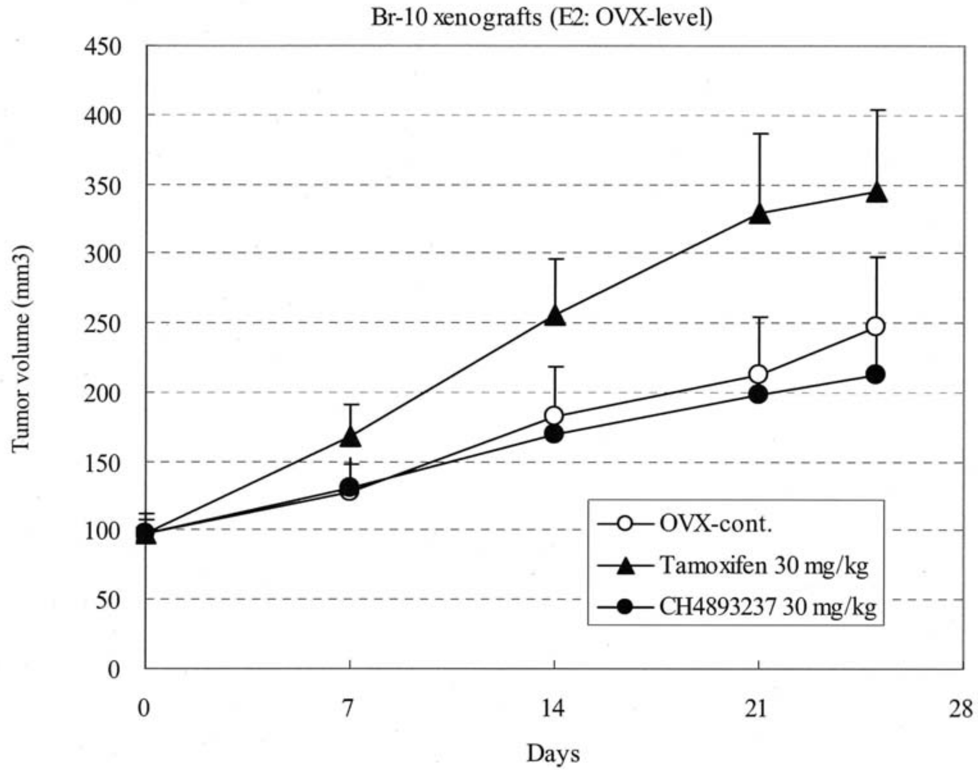

Figure 4. Effects of tamoxifen and CH4893237 in Br-10 xenografts with OVX-level of serum E_2. OVX nude mice were inoculated with Br-10 tumor (small pieces ~3x3 mm). Estradiol solution in EtOH was percutaneously administered at 0.1 mg/head once a week to preserve the transplanted tumors. Drug administration was initiated when tumor volume reached ~100 mm^3. CH4893237 (30 mg/kg) and tamoxifen (30 mg/kg) were orally administered 5 times per week for 25 days. Tumor volumes were measured once a week. Each group consisted of five to six animals and the data are the mean values with standard errors. OVX-cont, Control OVX mice that did not receive estrogen.

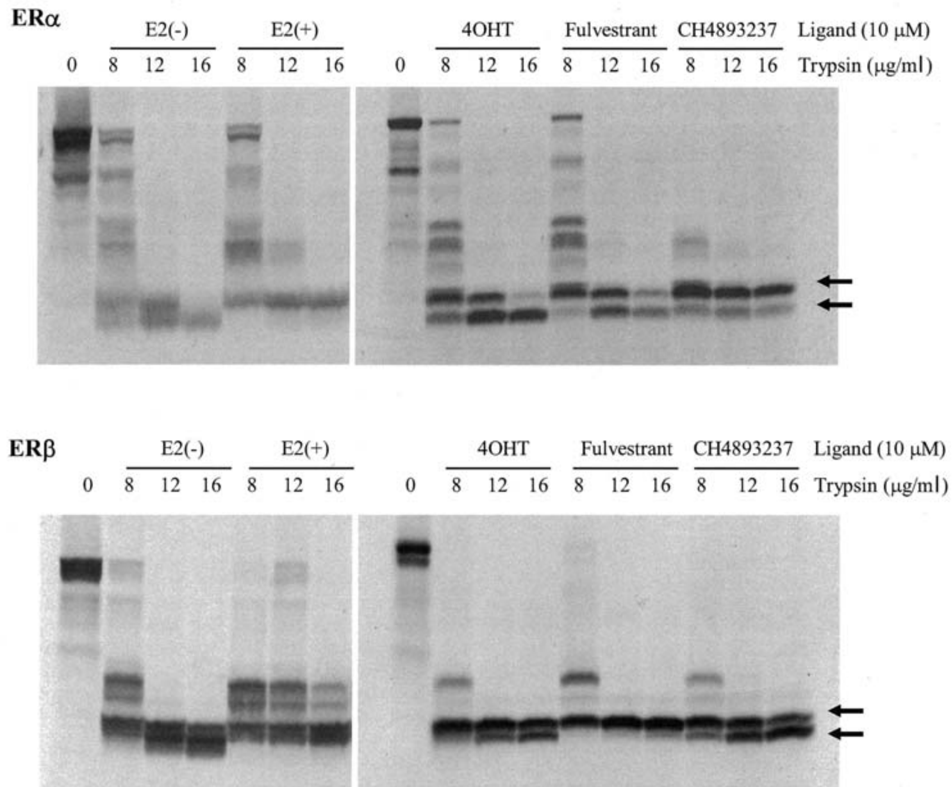

Figure 5. Proteolytic digestion patterns of radiolabeled ERα and ERß. Radiolabeled ERα and ERß were incubated in the presence [E_2(+)] or absence [E_2(-)] of E_2 (10 μM), 4OHT (10 μM), fulvestrant (10 μM), CH4893237 (10 μM) for 20 min at 25˚C and then digested with 8, 12 16 μg/ml trypsin. Digestion products were electrophoresed on 10-20% gradient Tris-Glycine gels. Arrows indicate distinct bands.

Tumor growth inhibition and ER degradation of tamoxifen, fulvestrant and CH4893237 in ZR-75-1 xenografts with ...l of serum E_2.

Compound	Dose	TGI (%)		ERD (%)	
Tamoxifen	30 mg/kg/day p.o.	69.0		-2.5	
	100 mg/kg/day p.o.	55.8		26.5	
			P<0.001		
CH4893237	30 mg/kg/day p.o.	67.6		37.8	
	100 mg/kg/day p.o.	90.9		82.9	
	300 mg/kg/day p.o.	91.4		94.3	
					P<0.01
Fulvestrant	1 mg/body/week s.c.	93.5		80.6	
	3 mg/body/week s.c.	92.4		85.5	

Results for tumor growth inhibition rate (TGI%) and ER degradation rate (ERD%) of the ZR-75-1 xenografts indicate the mean values of six to seven animals. P<0.01, P<0.001 (unpaired t-test).

30 mg/kg p.o. but not by CH4893237 at 30 mg/kg p.o. Tumor volumes after CH4893237 (30 mg/kg p.o.) treatment were almost the same as observed in the OVX control.

Conformational changes of ERα and ERß induced by CH4893237, fulvestrant, and 4OHT in proteolysis analysis. As mentioned above, the rate of ER degradation in ZR-75-1 xenografts induced by CH4893237 at 300 mg/kg p.o. was significantly stronger than that by fulvestrant at 3 mg/body/week s.c. (ERD%: 94.3 vs. 85.5%, P<0.01) whereas the tumor growth inhibition from CH4893237 at 300 mg/kg was almost the same as that from fulvestrant at 3 mg/body/week s.c. (TGI%: 91.4 vs. 92.4%) (Fig. 2A, Table II). The finding that the ER degradation activity of CH4893237 is superior to that of fulvestrant raised the possibility that CH4893237 and fulvestrant have different mechanisms of ER degradation. Therefore, we used proteolysis assays to investigate the conformational changes induced in ERα and ERß proteins by CH4893237, fulvestrant, and 4OHT (Fig. 5).

For the ERα and ERß proteolysis assays, ^{35}S-labeled ERα and ERß were digested for 10 min using increasing amounts of trypsin. In the absence of E_2, both ERα and ERß were sensitive to trypsin and gave a proteolytic digestion pattern in which the fragment size decreased as concentrations of trypsin increased. In contrast, ERα and ERß in the presence of E_2, only the upper bands were stabilized. That ERα and ERß are more sensitive to trypsin without E_2 than with it are similar to the findings reported by Kraichely *et al* (16).

In the ERα proteolysis assay, the band patterns for CH4893237 were different from those for 4OHT and fulvestrant. Namely, for CH4893237, all the upper bands at 8, 12 and 16 μg/ml trypsin were thicker than the lower bands, but the upper band at 16 μg/ml trypsin for 4OHT was thinner than the lower one. In the case of fulvestrant at 12 and 16 μg/ml trypsin, the thicknesses of both upper and lower bands were similar. In the ERß proteolysis assay, the band patterns were similar for CH4893237 and 4OHT but not for fulvestrant. In the case of CH4893237 and 4OHT, the thicknesses of the upper and lower bands at 12 and 16 μg/ml trypsin were similar. However, in the case of fulvestrant, all of the upper bands at 8, 12 and 16 μg/ml trypsin were thicker than the lower bands.

Discussion

From the results with xenograft models, fulvestrant, the first SERD, was expected to show a higher response rate in the clinical settings, especially for tamoxifen-resistant tumors (17). In phase III, however, the objective response (OR) rate of fulvestrant (250 mg/month i.m.) was only ~20% (4,5). A possible reason for this low response rate was that intramuscularly administrated fulvestrant results in a lower plasma concentration in patients than can be achieved in cell cultures (17). If the reasoning is correct, then an orally available SERD, such as CH4893237 efficiently absorbed to reach blood concentrations sufficient for inhibiting tumor growth, would be expected to exhibit better efficacy in a clinical setting.

In the ZR-75-1 xenografts with 50.6 pg/ml of serum E_2 showing partial tamoxifen resistance, we demonstrated that CH4893237 at 100 mg/kg/day p.o. strongly inhibited tumor growth (>90%) and its antitumor activity at 100 mg/kg/day p.o. was almost the same as that of fulvestrant at 1 mg/body/week s.c. (Fig. 2A, Table II). In preliminary pharmacokinetic investigations with ICR mice, we found that the AUC of fulvestrant in mice one week after 1 mg s.c. administration (1 mg/body/week s.c.) was 230.9 ng·day/ml, which is higher than its therapeutic AUC (79.4-131.6 ng·day/ml) one month after a 250 mg i.m. administration (250 mg/month i.m.) in human subjects (18). Our results demonstrated that the antitumor activity of CH4893237 (100 mg/kg/day p.o.) is nearly equal to that of fulvestrant (1 mg/body/week s.c.) in the xenografts and that the AUC of fulvestrant (1 mg/body/week s.c.) in mice is higher than its therapeutic AUC (250 mg/month i.m.) in humans. These findings raise the possibility that the efficacy of CH4893237 would be equal to or better than that of fulvestrant in a clinical setting.

Furthermore, the analysis of ER degradation in the ZR-75-1 xenograft, confirmed that the rate of ER degradation from CH4893237 at 300 mg/kg p.o. was significantly stronger than from fulvestrant at 3 mg/body/week s.c. (94.3 vs. 85.5%, P<0.01) (Fig. 2B, Table II), even though the tumor growth inhibition rate from CH4893237 at 300 mg/kg p.o. was almost equal to that from fulvestrant at 3 mg/ body/week s.c. (91.4 vs. 92.4%) (Fig. 2A, Table II). A similar result indicating the superiority of CH4893237 over fulvestrant was obtained in our previously reported MCF-7 *in vitro* cell growth inhibition and ER degradation experiments (12). In these *in vitro* MCF-7 experiments, the rate of ER degradation induced by CH4893237 at 300 nM was significantly stronger than that from fulvestrant at 10 nM (82.3 vs. 61.3%, P<0.001), though the cell growth inhibition rate exhibited by CH4893237 at 300 nM was almost equal to that of fulvestrant at 10 nM (51.9 vs. 51.2%).

To date, a correlation between ER degradation and clinical efficacy has not been clarified. Some evidence suggests, however, that a greater rate of ER degradation correlates with better efficacy in clinic. In primary breast cancer patients, fulvestrant decreased both ER and Ki67, a proliferation-associated antigen, in a dose-dependent manner (19), and a further reduction in ER level after 6 weeks of tamoxifen treatment was associated with a significantly better quality of response (20). Thus, increased ER degradation may correlate with better efficacy. In the ZR-75-1 xenografts, CH4893237 decreased the ER level in a dose-dependent manner, with a superior rate of ER degradation compared with fulvestrant. In a phase II study of fulvestrant, the ER degradation rate of fulvestrant at a clinical dosage (250 mg/month i.m.) was only 60% (19), lower than that of CH4893237 at 100 and 300 mg/kg/day p.o. (100 mg/kg: 82.9%, 300 mg/kg: 94.3%) in the ZR-75-1 xenografts (Fig. 2B, Table II). Therefore, CH4893237 may lead to a better clinical response than fulvestrant.

In the Br-10 xenografts with serum E_2 levels of 22.9 pg/ml and within the range of postmenopausal women (15), tamoxifen at 1, 10 and 100 mg/kg/day p.o. resulted in strong tamoxifen resistance. On the other hand, CH4893237 at 30 mg/kg/day p.o. showed tumor growth inhibition comparable to that of the OVX control (E_2(-) cont.) or fulvestrant at 1 mg/body/s.c. (Fig. 3). In the OVX Br-10 xenografts with serum E_2 levels of <16.7 pg/ml and within the range of AI-treated postmenopausal patients, tamoxifen at 30 mg/kg/day p.o. lead to an increase in tumor volume. On the other hand, tumor volume was not increased by CH4893237 at 30 mg/kg/day p.o. (Fig. 4). Thus, CH4893237, unlike tamoxifen, demonstrated potent efficacies without agonistic activity in Br-10 xenografts with lower serum E_2 levels such as are found in postmenopausal or AI-treated postmenopausal breast cancer patients, suggesting that CH4893237 has clinical benefits for postmenopausal or AI-treated postmenopausal breast cancer patients.

On the other hand, CH4893237, a steroidal SERD, is expected to function in a manner similar to fulvestrant because of their similar chemical structures (Fig. 1). From certain experiments, we have confirmed that CH4893237, like fulvestrant, demonstrated ER-downregulating properties. Namely, CH4893237 inhibited the ERα-dependent transcription and the estrogen response element-DNA binding to ERα. Moreover, CH4893237 as well as fulvestrant inhibited the recruitment of two SRC/p160 coactivator protein family members (SRC1a, GRIP-1) to ERα (data not shown). In the ER degradation experiments, however, CH4893237 induced superior ER degradation as mentioned above; therefore, we surmised that CH4893237 induces distinct conformational changes in ERα and ERß from fulvestrant. In the ER proteolysis assays, CH4893237 showed band patterns of ERα and ERß different from those of fulvestrant (Fig. 5), suggesting that CH4893237 induces distinct conformational changes of ERα and ERß. The superior ER degradation from CH4893237 may derive from the distinct conformational changes induced by CH4893237 in ERα and ERß.

In summary, our data indicated that oral treatment with CH4893237 showed potent efficacies without agonistic activity against human breast cancer xenografts with serum E_2 levels (50.6, 22.9, <16.7 pg/ml) equivalent to the ranges in postmenopausal or AI-treated breast cancer patients. In addition, CH4893237 demonstrated enhanced ER degradation compared with fulvestrant. The superior ER degradation from CH4893237 may be a result of conformational changes in ERα and ERß distinctly induced by CH4893237, but not by fulvestrant. The bioavailability of CH4893237 in rat, mouse, and monkey was 32, 40 and 3.1%, respectively (12,21). Although we need to clarify the bioavailability of CH4893237 in humans in clinical settings, the present study suggests that CH4893237, unlike fulvestrant, is a new orally bioavailable receptor downregulator. CH4893237 alone or in combination with AIs, therefore, may have clinical benefits for postmenopausal breast cancer patients.

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