Identification of a novel, orally bioavailable estrogen receptor downregulator

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Tamoxifen has been widely used for the treatment of estrogen receptor (ER)-positive breast cancer, but its partial agonist activity is considered to limit the efficacy, and cause tumor flare and endometrial cancer. Fulvestrant, on the other hand, binds and degrades ER, thereby acting as a pure anti-estrogen without partial agonist activity. However, due to its low oral bioavailability, fulvestrant has to be intramuscularly administered to patients, which limits the convenience of the drug, and causes pain and inflammation at the site of injection. In search of a patient-friendly pure anti-estrogen, we screened and identified an ER antagonist, CH4893237, which bound to ER with an IC_{50} value of 1.4 μM and, by oral administration, inhibited estrogen-stimulated uterine growth in ovariectomized mice. CH4893237 reduced the amount of ER at the protein level and impaired the nuclear accumulation of ER, indicating an orally active pure anti-estrogen. Furthermore, CH4893237 inhibited the estrogen-stimulated proliferation of MCF-7, ZR-75-1 and BT-474 cells, and caused a marked growth inhibition of the MCF-7 xenograft *in vivo*. Thus, CH4893237 will provide an additional option for second-line hormone treatment of breast cancer. *Anti-Cancer Drugs* 16:751–756 © 2005 Lippincott Williams & Wilkins.

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Introduction

Approximately 50–80% of breast cancers express estrogen receptor (ER) [1,2] and the growth of these tumors largely depends on estrogen. Tamoxifen, an anti-estrogenic agent, has been widely used for the treatment of ER-positive breast cancer. About half of the patients with ER-positive advanced breast cancer respond to tamoxifen, but tumors eventually relapse even in these patients [3,4].

Tamoxifen is referred to as a partial agonist because, in the breast, tamoxifen acts as an antagonist, whereas in bone, liver and endometrium, it acts predominantly as an agonist [5–7]. It binds to ER, and exerts both anti-estrogenic and estrogenic effects. The estrogenic activity of tamoxifen is thought to limit the efficacy, and to cause tumor flare and endometrial cancer after long-term treatment [8–11].

Recently, a number of anti-estrogens, including selective ER modulators, aromatase inhibitors and estrogen receptor downregulators, have appeared to be effective in the clinical settings [12]. Among them, fulvestrant was found to bind to ER and degrade it in human breast cancer cells, thereby acting as a pure anti-estrogen without agonist activity [13,14]. In phase III clinical trials, fulvestrant was shown to be as effective as the aromatase inhibitor anastrozole in patients who relapsed during tamoxifen treatment [15,16]. Nevertheless, because of its low bioavailability, fulvestrant needs to be intramuscularly administered to patients, which limits the convenience of the drug use, and often causes pain and inflammation at the injection sites. Therefore, there is a strong demand for orally available pure anti-estrogen. Recently, Hoffmann *et al.* reported an orally available ER downregulator, ZK-253, that was efficacious against ER-positive human breast cancer xenografts [17].

Through chemical compound screening, we identified another novel orally active pure anti-estrogen. The compound, designated CH4893237, reduced the amount of ER and impaired the nuclear accumulation of ER. In addition, it inhibited the estrogen-stimulated growth of MCF-7, ZR-75-1 and BT-474 cells; oral administration of CH4893237 caused a marked growth inhibition of the MCF-7 xenograft *in vivo*. Thus, CH4893237 will provide a new therapeutic option for second-line therapy of hormone treatment for breast cancer.

Materials and methods

Drugs

CH4893237 and fulvestrant (Fig. 1) were synthesized in the Chemistry Research Department I, Chugai Pharmaceutical. Tamoxifen, 4OH-tamoxifen, 17β-estradiol (E_2)

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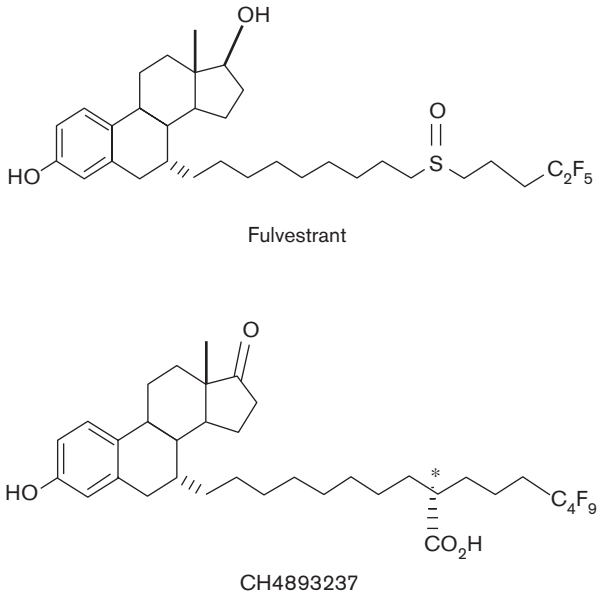
Fig. 1



Chemical structures of fulvestrant and CH4893237.

and 17β-estradiol 3-benzoate (17β-EB) were purchased from Sigma (St Louis, Missouri, USA).

Animals

Female 5-week-old ICR mice and female 5-week-old athymic nude mice were purchased from Charles River Japan (Tokyo, Japan) and Nihon Clea (Tokyo, Japan), respectively. Mice were freely fed mice chow CE2 and tap water. All the experiments were approved by the Animal Experimentation Ethics Committee in Chugai, and carried out according to the Guidelines for the Care and Use of Laboratory Animals in Chugai Pharmaceutical.

Determination of anti-estrogenic and estrogenic activities *in vivo*

Estrogenic and anti-estrogenic activities of the drugs were determined as uterotrophic and anti-uterotrophic effects in ICR mice. Mice were ovariectomized (OVX) 2 weeks before the administration of the drugs. To evaluate the anti-estrogenic activity of the drugs, OVX mice were s.c. administrated 0.1 μg of 17β-EB and then orally administered the indicated doses of CH4893237 or fulvestrant once a day for 3 days. To evaluate the estrogenic activity of the drugs, the OVX mice were orally administered the indicated doses of CH4893237 or tamoxifen once a day for 3 days. Uterine weights were determined on the next day of the last administration.

Cells

Human breast cancer cell lines MCF-7, ZR-75-1, BT-474 and COS-7 were purchased from ATCC (Manassas, Virginia, USA). Unless otherwise specified, MCF-7 cells were cultured in MEM medium supplemented with 2 mM L-glutamine, 0.1 mM sodium pyruvate, 10 mM HEPES, 6 μg/l insulin and 5% fetal bovine serum (FBS); ZR-75-1 and BT-474 cells were cultured in RPMI 1640 supplemented with 10% FBS; and COS-7 cells in DMEM medium supplemented with 10% FBS.

Quantification of the cellular ER protein

To determine the effects of the drugs on the cellular levels of ER, 4×10^5 MCF-7 cells were seeded on a 6-cm dish and cultured for 3 days. The indicated drugs were then added to the culture and the cells were further cultured for 2 days. At the end of the culture, the cells were collected and lysed in a lysis buffer containing 20 mM Tris–HCl (pH7.4), 1 mM DTT, 10% glycerol and protease inhibitor (Boehringer Mannheim, Ingelheim, Germany). After centrifugation at 1750 *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 25 000 *g* for 30 min at 4°C. The ER protein in the supernatants was quantified with an ER enzyme immunoassay kit (Abbott, Abbott Park, Illinois USA).

Determination of the subcellular localization of ER

COS-7 cells (1.5×10^5/well) were plated on six-well dishes and cultured in phenol red-free medium supplemented with 3% dextran-coated charcoal FBS (DCC-FBS) for 1 day. Then the cells were transfected with 2 μg of plasmid carrying the human estrogen receptor cDNA (HEG0) using lipofectamine (Promega, Madison, Illinois USA), and further cultured for 3 days in the presence and absence of CH4893237, fulvestrant or 4OH-tamoxifen. The cells were then fixed in EtOH at −20°C for 5 min and were incubated with anti-ER monoclonal antibody (G-20; Santa Cruz Biotechnology, Santa Cruz, California, USA) for 2–3 h, then with the horseradish–peroxidase-conjugated goat anti-rabbit Ig (Invitrogen, Carsbad, California, USA) for 1–2 h, and finally with the color former, which was prepared by mixing equal volume of 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride in 0.1 M Tris–HCl (pH 7.2) and 0.03% H_2O_2. The cellular localization of ER was determined by immunofluorescent microscopy. Plasmid carrying the full-length human estrogen receptor α cDNA (HEG0) was kindly provided by S. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo, Japan).

Determination of anti-proliferative activity

To determine the anti-proliferative activity against MCF-7 cells, 2500 cells/well of MCF-7 were plated on 96-well plates and cultured in phenol-red-free medium supplemented with 3% DCC-FBS for 7 days in the presence and absence of the indicated drugs and 0.1 nM E_2. To determine the anti-proliferative activity against ZR-75-1 cells, 5000 cells/well of ZR-75-1 were plated on 96-well plates and cultured in phenol-red-free RPMI 1640

medium supplemented with 3% DCC-FBS for 7 days in the presence and absence of the indicated drugs and 0.1 nM E_2. To determine the anti-proliferative activity against BT-474 cells, 5000 cells/well of BT-474 were plated on 96-well plates and cultured in phenol-red-free RPMI 1640 medium supplemented with 3% DCC-FBS for 7 days in the presence and absence of the indicated drugs and 0.1 nM E_2 for 7 days. The number of cells was determined using a Cell Titer 96 Aqueous One solution cell proliferation assay kit (Promega) for MCF-7 and ZR-75-1and with an SF-kit (Nacalai Tesque, Kyoto, Japan) for BT-474.

Determination of anti-tumor activity

OVX female nude mice were s.c. implanted with estrogen implants (estradiol:cholesterol = 1:99, silastic tube) within 1 week before tumor transplantation as described [18]. Small pieces (approximately 3 × 3 mm) of the MCF-7 xenograft grown in female nude mice were s.c. transplanted into the right flank of nude mice. Drug administration was initiated when tumor volume reached 50–150 mm^3. CH4893237 (30, 100 and 300 mg/kg) and tamoxifen (30 and 100 mg/kg) were orally administered 5 times per week for 6 weeks. Fulvestrant (1 and 3 mg/body) was s.c. administered once a week for 6 weeks. Tumor weights were measured on the next day of the final administration.

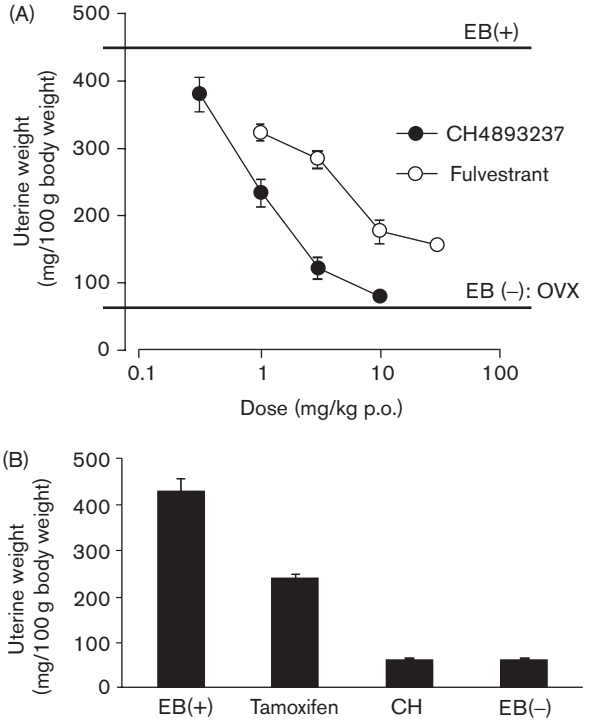
Results and discussion

In vivo anti-estrogenic effects of CH4893237 after oral administration

In an attempt to identify an orally active pure anti-estrogen, we synthesized a number of steroidal compounds and tested their ability to exert anti-estrogenic effects *in vivo* by oral administration. One of the compounds, CH4893237, inhibited the binding of estrogen to ER with an IC_{50} value of 1.4 μM and the ER-dependent transcription in the luciferase reporter gene assay with an IC_{50} value of 49.8 nM. To assess the anti-estrogenic activity of CH4893237 after oral administration, we examined the effects on the estrogen-stimulated uterine growth in OVX mice. Uterus weights in the OVX mice were about 60 mg/100 g body weight, whereas those of the OVX mice given 17β-EB increased to about 430 mg/100 g body weight. Oral administration of CH4893237 to the OVX mice that had received 17β-EB decreased the uterus weight in a dose-dependent manner, and 10 mg/kg CH4893237 lowered the uterus weight to the level of the OVX mice. Fulvestrant also caused anti-estrogenic effects even when it was administered orally, but its effect was much weaker than CH4893237; even at 30 mg/kg, fulvestrant did not reduce the uterus weights to the level of the OVX mice (Fig. 2A).

We also examined the estrogenic activity of CH4893237. CH4893237 did not affect uterus weight of the OVX mice even at 50 mg/kg, whereas 50 mg/kg tamoxifen significantly increased it (Fig. 2B), indicating that CH4893237 exerted anti-estrogenic activity without any estrogenic activity *in vivo*. Furthermore, the action of CH4893237 is considered to be reasonably selective, because CH4893237 neither bound to nor affected transcriptional activities of progesterone receptor (PR), androgen receptor (AR) or glucocortid receptor (GR) (data not shown).

Fig. 2

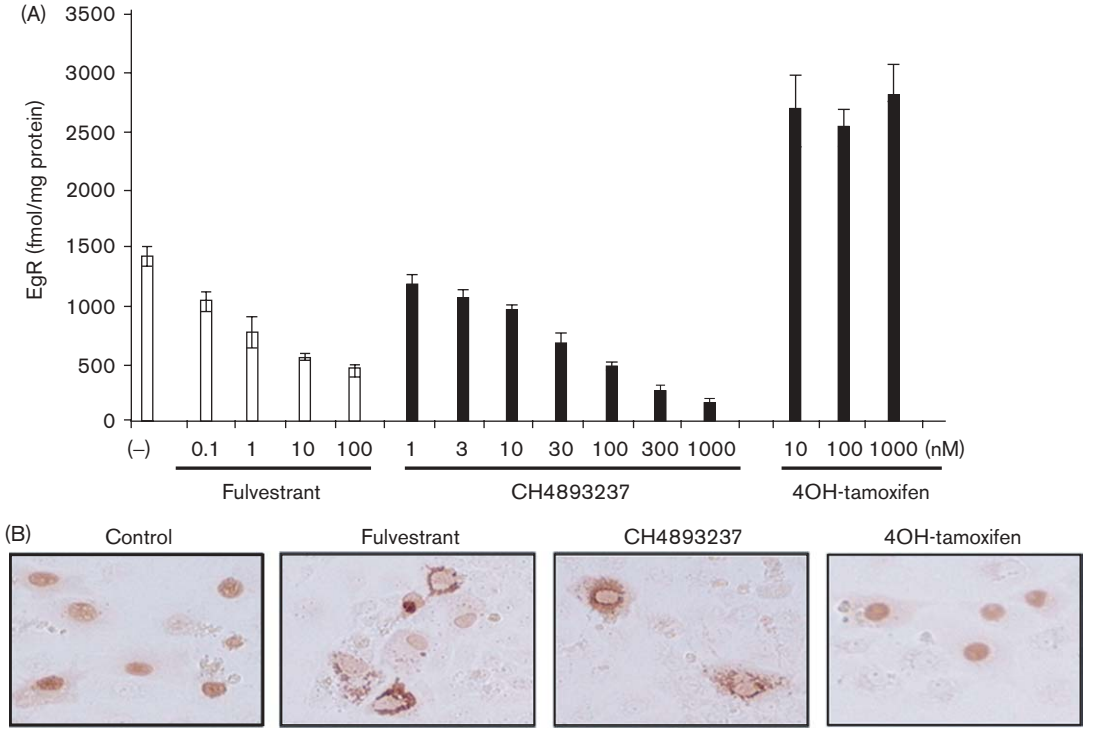


Anti-estrogenic and estrogenic effects of CH4893237. Anti-estrogenic (A) and estrogenic (B) activities of CH4893237 were determined in OVX mice. (A) OVX mice that had received 0.1 μg 17β-(EB(+)) were orally administered 1, 3, 10 or 30 mg/kg fluvestrant, or 0.3, 1, 3 or 10 mg/kg CH4893237 once a day for 3 days. Control mice(EB(−)) received only vehicle. Uterus weights of the OVX mice with or without the administration of 17β-EB are indicated by the vertical lines. (B) OVX mice were administered 0.1 μg 17β-(EB(+)), 50 mg/kg tamoxifen or 50 mg/kg CH4893237(CH) once a day for 3 days. Control OVX mice(EB(−)) received only vehicle. Mice were ovariectomized 2 weeks before drug administration; each group consisted of four animals. Uterus weights were measured on the next day of the final administration. Results indicate the mean value of four animals with standard errors.

Downregulation of ER by CH4983237

The fact that CH4893237 caused anti-estrogenic effects without estrogenic effects *in vivo* prompted us to examine the possibility that, like fulvestrant, CH4893237 has the ability to downregulate ER. To address this question, we quantified the cellular ER levels by EIA. The MCF-7 cells were treated with CH4893237 at doses between 1 nM and 1 μM, with fulvestrant at doses between 0.1 and 100 nM, and with 4OH-tamoxifen at doses between

Fig. 3



Downregulation and prevention of nuclear accumulation of ER by CH4893237. Effects of CH4893237 on the amounts (A) and nuclear accumulation (B) of the ER protein were determined by EIA and immunofluorescent microscopy, respectively. (A) MCF-7 cells were cultured for 2 days in the presence and absence of the indicated concentrations of fulvestrant, CH4893237 and tamoxifen. The amounts of ER were quantified by ER EIA. Results are indicated as mean values of four independent experiments with standard errors. (B) COS-7 cells were transfected with the expression plasmid carrying the human ERα cDNA, and cultured in the presence and absence of 0.1 μM fulvestrant, 1 μM CH4893237 or 1 μM 4OH-tamoxifen for 3 days. The ER protein visualized by immunofluorescent microscopy after fixation and hybridization with an anti-ER monoclonal antibody is shown.

10 nM and 1 μM. CH4893237 and fulvestrant decreased the amounts of ER protein in a concentration-dependent manner with IC_{50} values of 24 and 1.8 nM, respectively, whereas 4OH-tamoxifen increased ER levels (Fig. 3A).

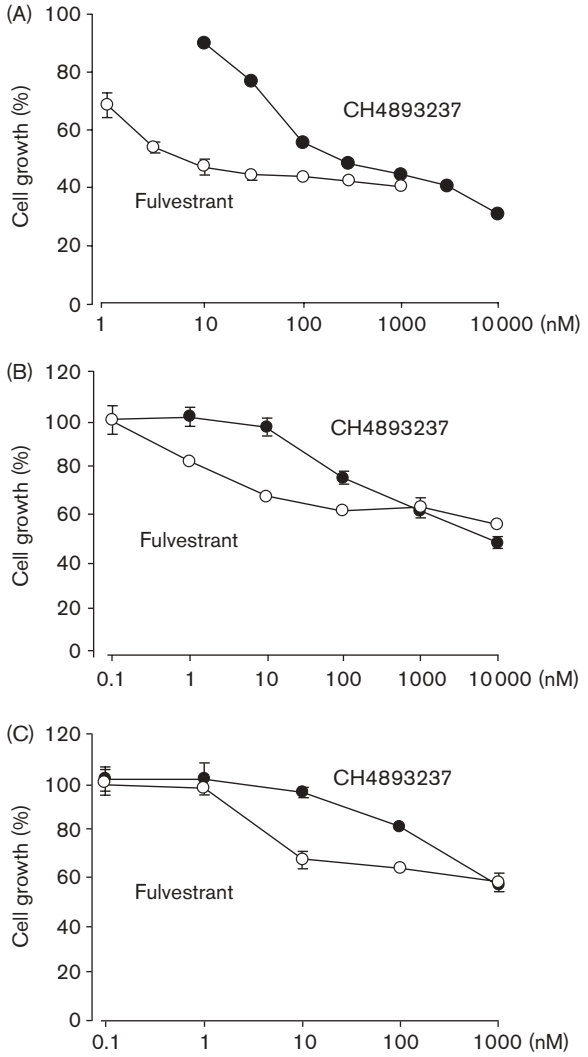
ER predominately locates in the nucleus and so we also asked if CH4893237 impaired the nuclear accumulation of ER. In COS7 cells expressing human ER, most of the fluorescence derived from the human ER was detected in the nuclei, but was in the cytoplasm of the cells treated with 1.0 μM CH4893237 or 0.1 μM fulvestrant. Prevention of nuclear accumulation of ER was a drug-specific effect; 4OH-tamoxifen did not inhibit the nuclear accumulation of ER (Fig. 3B). Thus, it appears that, like fulvestrant, CH4893237 is a pure anti-estrogen with the ability to downregulate ER and to prevent nuclear accumulation of ER.

Anti-tumor activity of CH4893237

Next, we examined the effects of CH4893237 on the estradiol-stimulated proliferation of MCF-7, ZR-75-1 and BT-474 cells. At concentrations higher than 100 nM, CH4893237 inhibited the proliferation of MCF-7 cells in the presence of 0.1 nM estradiol to the level attained by fluvestrant (Fig. 4A). CH4893237 also inhibited the proliferation of ZR-75-1 and BT-474, but the anti-proliferative effect on BT-474 was weaker than that on MCF-7; 1 μM or higher concentrations of CH4893238 were necessary to inhibit the proliferation of ZR-75-1 and BT-474 cells to the levels attained by fluvestrant (Fig. 4B and C).

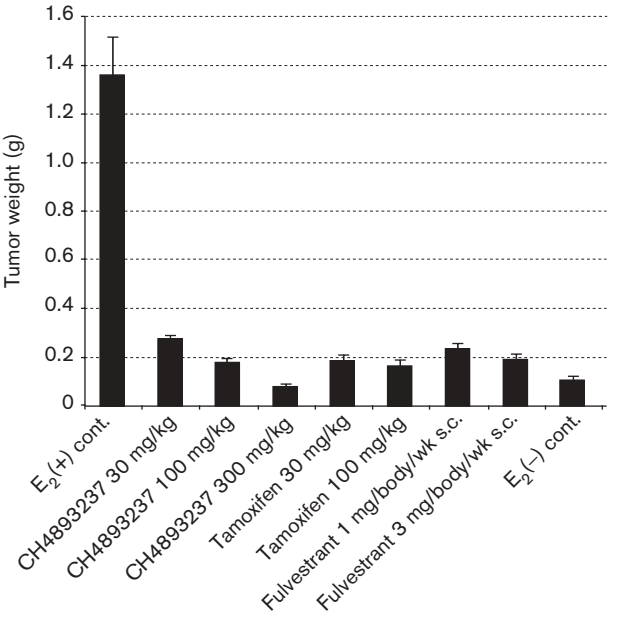
At lower concentrations, the anti-proliferative activity of CH4893237 was weaker than that of fulvestrant. Nevertheless, CH4893237 would exhibit strong anti-tumor activity *in vivo* if efficiently absorbed to reach blood concentrations sufficient for tumor growth inhibition. In fact, the bioavailability of CH4893237 was 40.1% in mice and 32.2% in rats. Therefore, we examined the anti-tumor activity of CH4893237 in the MCF-7 xenograft model. The OVX mice that had received estradiol were transplanted with MCF-7 and then orally administered CH4893237, fulvestrant or tamoxifen. As shown in Fig. 5, CH4893237 inhibited the tumor growth in a

Fig. 4



Anti-proliferative activities of CH4893237 and fulvestrant. MCF-7 (A), ZR-75-1 (B) and BT-474 (C) cells were cultured for 7 days in the presence and absence of 0.1 nM estradiol, CH4893237 or fulvestrant. CH4893237 and fulvestrant were added to the cultures at final concentrations of 10, 100, 1000 and 10000 nM for CH4893237; 1, 10, 100 and 1000 nM for fulvestrant in MCF-7; 0.1, 1, 10, 100, 1000 and 10000 nM for ZR-75-1, and 0.1, 1, 10, 100, 1000 nM for BT-474. Percentages of the viable cells cultured in the presence of the indicated drugs as compared to those cultured in the absence of the drugs are shown. Results indicate the mean value of three (for MCF-7 and BT-474) or four (for ZR-75-1) independent experiments with standard errors.

Fig. 5



Anti-tumor activity of CH4893237 against MCF-7 xenografts. OVX mice carrying the estrogen implant were inoculated with MCF-7 tumor. When the tumor volume reached between 50 and 150 mm^3, the mice were orally administered 30, 100 or 300 mg/kg CH4893237, or 30 or 100 mg/kg tamoxifen 5 times per week for 6 weeks, or they were s.c. administered 1 or 3 mg fulvestrant once a week for 6 weeks. Control mice received only vehicle. Tumor weights were determined on the next day of the final administration. Each group consisted of seven animals; results indicate the mean value of seven animals with standard errors. $E_2(+)$ cont: control OVX mice that received estrogen; $E_2(-)$ cont: control OVX mice that did not receive estrogen.

dose-dependent manner, as revealed by tumor weights, and it completely inhibited the tumor growth at 300 mg/kg; the tumor weights of the mice administered 300 mg/kg CH4893237 were nearly the same as those of the OVX mice that did not receive estradiol. Oral administration of fluvestrant and tamoxifen also caused anti-tumor effects, but the tumor growth inhibition achieved by 3 mg/kg fulvestrant or 100 mg/kg tamoxifen was weaker than that by 300 mg/kg CH4893237 (Fig. 5). Further increase in the doses of fulvestrant and tamoxifen did not increase the anti-tumor activity.

Although the bioavailability of CH4893237 in human needs clarification in the clinical settings, the results of this study demonstrate that CH4893237 is as an orally active pure anti-estrogen. CH4893237, therefore, will offer an additional option for second-line hormone treatment of breast cancer.

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