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11β-Amidoalkoxyphenyl Estradiols, a New Series of Pure Antiestrogens

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11β-Amidoalkoxyphenyl estradiols, a series of new antiestrogens, have been prepared and compared with tamoxifen (TAM) and 4-hydroxytamoxifen (OH-TAM). *In vitro*, these compounds were up to 20 times as active as OH-TAM on estradiol (E2)-stimulated MCF-7 cells. Unlike TAM or OH-TAM which were inactive, they displayed potent growth inhibitory effects on MCF-7 cells stimulated by a cocktail of epidermal growth factor and platelet derived growth factor. One of the most active compounds, 5e, was tested *in vivo* for its antiuterotrophic and antitumoral activities: it proved to be fully antiuterotrophic at 3 mg/kg subcutaneously in mice while being devoid of any uterotrophic activity. It inhibited the E2-induced growth of MCF-7 tumors implanted in nude mice and prevented the partial agonistic activity of TAM on MCF-7 tumor growth in ovariectomized mice. Moreover, on MCF-7 variant tumors, 5e, unlike TAM, did not display any proliferative activity, but inhibited the TAM-induced growth. Overall, these results show that this new series of compounds displays an improved activity profile compared with that of TAM, on tests relevant to human breast cancer treatment.

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INTRODUCTION

Substitution of 19-nor steroids with an 11β-aryl group was shown to yield compounds with high affinities for several hormonal receptors [1], leading to different families of antihormones [2], exemplified by the antiprogestin/antiglucocorticoid RU 38 486 [3] and the antiestrogen RU 39 411 [4] (Fig. 1). Whereas RU 39 411 proved to be a potent antiestrogen retaining partial agonistic activity [4, 5], introduction of various alkylamide side chains at position 7α of estradiol (E2) gave rise to pure antiestrogens [6].

Similarly, introduction of amidoalkyl substituents at position 11β of E2 led to antiproliferative and antitumoral antiestrogens, totally devoid of estrogenic activity [7]. In order to get more insight into the incidence of the nature of the 11β substituent on activity, we synthesized a series of 11β-amido-alkoxyphenyl estradiol derivatives and explored their antiestrogenic and antiproliferative activities in comparison with tamoxifen and/or 4-hydroxytamoxifen (OH-TAM).

This paper presents the synthesis and the biochemical and pharmacological profiles of several derivatives from this series. The most interesting of them, 5e, has been further tested for its antitumoral activity on the classical MCF-7 model as well as on a MCF-7 variant tumor model implanted into nude mice, a model which has been selected for its tamoxifen-resistance.

EXPERIMENTAL

Synthesis: general procedure

Melting points were determined on a Kofler hot stage apparatus and are uncorrected. Optical rotations $[\alpha]_D$ were measured at 20°C on a Roussel-Jouan polarimeter. Silica gel Merck (5–20 μm) was used for all column chromatographies. NMR spectra were recorded in $CDCl_3$ with TMS as the internal standard. All compounds described showed analytical data (microanalyses, u.v., i.r., ^1H NMR) consistent with the assigned structures.

11β-(4-Hydroxyphenyl)-estra-4,9-diene-3,17-dione (2)

Copper$^{(I)}$ chloride (450 mg, 4.5 mmol) was suspended at 0–5°C into a solution of epoxide 1 [8] (10 g, 30 mmol) in dry tetrahydrofuran (THF) (100 ml). A 0.95 M solution of 4-(trimethylsilyloxy) phenyl

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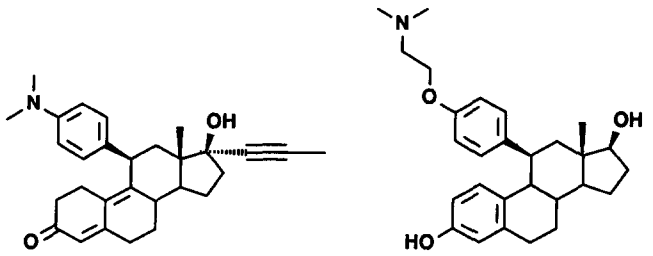
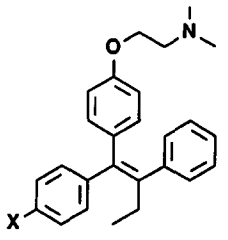


RU 38 486 RU 39 411



X = H tamoxifen (TAM)

X = OH 4-OH-TAM

Fig. 1. Chemical structures of reference compounds.

magnesium bromide [9] (110 ml, 0.1 mol) was slowly added over a 45 min period and the mixture was stirred for 2 h at that temperature. After pouring the reaction mixture into a saturated aqueous ammonium chloride solution, the product was extracted with ethyl acetate, washed with saturated aqueous sodium chloride, dried over magnesium sulfate and the solvent was removed under reduced pressure (usual work-up). To the residue (35 g), dissolved in methanol (350 ml), 2 N aqueous hydrochloric acid (100 ml, 0.2 mol) was added and the mixture was stirred during 2 h leading to a solution 20 min later. After concentration, the product was extracted with methylene chloride. The usual work-up gave an oil (27 g) which was chromatographed (methylene chloride–ethyl acetate, 7:3, v/v) to yield pure **2** (8 g, 73%) and its 5(10), 9(11)-diene isomer (1 g, 9.5%), as yellow solids. Recrystallization of **2** from methylene chloride–isopropyl ether produced light yellow crystals, m.p. 248°C, $[\alpha]_D + 250°$ (c = 0.8% $CHCl_3$), NMR: 0.57 (s, 3H, Me18), 4.36 (d, 1H, H11), 5.80 (s, 1H, H4), 6.75 and 7.02 (2d, 4H, aromatics).

N-butyl-2-[5-[4-(3,17-dioxo-estra-4,9-dien-11β-yl) phenoxy]pentylthio]-N-methyl-acetamide (3e)

2 N aqueous sodium hydroxide solution (5.1 ml, 10.2 mmol) was added to a solution of **2** (3.09 g, 8.5 mmol) in acetone (45 ml). This mixture was heated under gentle reflux while 2-[5-(bromopentyl)thio]-*N*-butyl-*N*-methyl-acetamide **6e** (6.2 g, 20 mmol) was added in four fractions over a 2 h period. Reflux was maintained for an additional 1.5 h, then the solution was diluted with a saturated aqueous ammonium chloride solution. Extraction with ethyl acetate followed by the usual work-up gave an oil (10 g), which was chromatographed (petroleum ether–ethyl acetate, 4:6, v/v) to yield pure **3e** (3.4 g, 67%) as an amorphous solid, along with a small fraction of unreacted starting material (0.28 g, 9%). NMR of **3e**: 0.57 (s, 3H, Me18), 0.92 and 0.95 (2t, 3H, butyl Me), 2.92 and 3.04 (2s, 2H, MeN), 3.28 (m, 2H, SCH_2CO), 3.36 (m, 2H, CH_2N), 3.91 (t, 2H, CH_2O), 4.38 (d, 1H, H11), 5.79 (s, 1H, H4), 6.80 and 7.07 (2d, 4H, aromatics).

2-[5-(Bromopentyl)thio]-N-butyl-N-methyl-acetamide (6e)

Butyl methyl amine (26 g, 0.3 mol) dissolved in diethyl ether (120 ml) was slowly added to a cooled (−20°C) solution of 2-bromoacetyl bromide (27.65 g, 0.137 mol), with stirring. The mixture was then allowed to reach room temperature. 30 min later water was added, the organic phase was separated and the aqueous phase was extracted with diethyl ether. The usual work-up gave an oil which was purified by vacuum distillation. Pure 2-bromo-*N*-butyl-*N*-methyl-acetamide was obtained as a colorless liquid (19.38 g, 68%), b.p. = 79–83°C/0.05 mbar.

5-Chloropentanol (10 g, 82 mmol) and potassium thioacetate (9.6 g, 84 mmol) were dissolved in ethanol (100 ml) and heated under reflux for 1 h, in a nitrogen atmosphere. The mixture was cooled and the residue obtained after evaporation of the solvent was diluted with ethyl ether. The insoluble was filtered off and the solvent was removed under reduced pressure to yield *S*-(5-hydroxypentyl)ethanethioate (12.5 g, 94%), as a brown oil.

The compound thus obtained was dissolved in methanol (75 ml) along with 2-bromo-*N*-butyl-*N*-methyl-acetamide (20.8 g, 0.1 mol). An aqueous 10 N sodium hydroxide solution (10 ml, 0.1 mol) was added with stirring in a nitrogen atmosphere, while maintaining the temperature at 20°C with an ice bath. This orange solution was stirred for 2.5 h at room temperature, then concentrated and diluted with ethyl acetate. The crude product obtained by the usual work-up was chromatographed over silica gel (ethyl acetate–cyclohexane, 8:2, v/v) to yield *N*-butyl-2-[(5-hydroxypentyl)thio]-*N*-methyl-acetamide (14.5 g, 76%) as a colorless oil, NMR: 0.93 and 0.95 (2t, 3H, butyl Me), 2.92 and 3.05 (2s, 3H, MeN), 2.65 (m, 2H) and 3.25 to 3.40 (m, 4H, CH_2S, SCH_2CO and CH_2N), 3.62 (t, 2H, CH_2O), i.r. ($CHCl_3$): 1632 cm^{-1}, 3623 cm^{-1}.

A solution of the above alcohol (5.3 g, 21 mmol) and triphenylphosphine (6.9 g, 26 mmol) in methylene chloride (50 ml) was cooled to 0°C and tetrabromomethane (8.7 g, 26 mmol) was added. After 1 h stirring at that temperature the solution obtained was directly chromatographed (cyclohexane–ethyl acetate, 2:1, v/v) to yield 2-[(5-bromopentyl)thio]-*N*-butyl-*N*-methyl-acetamide **6e** (6.2 g, 95%) as a colorless oil which should be stored below −20°C to avoid quick decomposition, i.r. ($CHCl_3$): 1638 cm^{-1}.

N-butyl-2-[5-[4-(3-hydroxy-17-oxo-estra-1,3,5(10)-trien-11β-yl)phenoxy]pentylthio]-N-methylacetamide (4e)

Acetic anhydride (2.6 ml) and acetyl bromide (1.3 ml) were added, while stirring, to an ice-cooled solution of the dienedione **3e** (2.6 g, 4 mmol) in methylene chloride (26 ml). The reaction mixture was allowed to warm to room temperature and stirring was continued for 4 h. A saturated aqueous sodium bicarbonate solution was carefully added to the mixture, after cooling to 0°C. Extraction with methylene chloride, followed by the usual work-up, gave the intermediate phenolic acetate (2.8 g), which was treated by 2 N sodium hydroxide (5 ml) in methanol (30 ml) for 1 h. After acidification with 2 N hydrochloric acid extraction with ethyl acetate, usual work-up and chromatography (cyclohexane–ethyl acetate, 6:4, v/v) the keto-phenol **4e** was obtained as an amorphous solid (1.6 g, 61%), NMR: 0.46 (s, 3H, Me18), 0.91 and 0.94 (2t, 3H, butyl Me), 2.91 and 3.03 (2s, 3H, MeN), 3.26 (s, 2H, SCH_2CO), 3.82 (t, 2H, CH_2O), 3.98 (t, 1H, H11), 6.45 (dd, 1H, H2), 6.62 (d, 1H, H4), 6.78 (d, 1H, H1), 6.59 and 6.95 (2d, 4H, other aromatics).

N-butyl-2-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)phenoxy]pentylthio]-N-methyl-acetamide (5e)

Sodium borohydride (0.06 g, 1.58 mmol) was added to a solution of keto-phenol **4e** (0.6 g, 1.01 mmol) in methanol (6 ml), cooled in an ice bath. After stirring for 1 h at this temperature, the solution was diluted with a saturated aqueous solution of ammonium chloride and extracted with ethyl acetate. The usual work-up followed by chromatography (ethyl acetate–cyclohexane, 7:3, v/v) yielded pure **5e**, as an amorphous solid (0.55 g, 91%), $[\alpha]_D - 32.5°$ (c = 1% $CHCl_3$), NMR: 0.34 (s, 3H, Me18), 0.91 and 0.94 (2t, 3H, butyl Me), 2.91 and 3.03 (2s, 3H, MeN), 3.25 and 3.26 (2s, 2H, SCH_2CO), 3.31 and 3.35 (2t, 2H, CH_2N), 3.68 (t, 1H, H17), 3.83 (t, 2H, CH_2O), 3.90 (t, 1H, H11), 6.42 (dd, 1H, H2), 6.59 (d, 1H, H4), 6.77 (d, 1H, H1), 6.59 and 6.94 (2d, 4H, other aromatics).

The following compounds were prepared according to the same procedure, using the appropriate ω-bromoamide:

7-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)-phenoxy]-*N*-methyl-*N*-(1-methyl ethyl)-heptanamide (**5a**), $[\alpha]_D - 30°$ (c = 0.9% $CHCl_3$);

8-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)-phenoxy]-*N*-methyl-*N*-(1-methyl ethyl)-octanamide (**5b**), $[\alpha]_D - 45°$ (c = 0.7% $CHCl_3$);

N-butyl-8-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)phenoxy]-*N*-methyl-octanamide (**5c**), $[\alpha]_D - 29°$ (c = 1.1% $CHCl_3$);

N-butyl-2-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)phenoxy]pentyloxy]-*N*-methyl-acetamide (**5d**), $[\alpha]_D - 31°$ (c = 1% $CHCl_3$).

N-butyl-2-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)phenoxy]pentylsulfinyl]-N-methyl-acetamide (5f)

A 0.1 M aqueous solution of sodium periodate (4 ml) was added to sulfide **5e** (147 mg, 0.25 mmol) dissolved in methanol (10 ml). The mixture was heated to reflux for 40 min, then cooled and diluted with water. The crude product obtained by extraction with chloroform and usual work-up was chromatographed (ethyl acetate–acetone, 85:15, v/v) to yield pure **5f** as an amorphous solid (150 mg, 99%), $[\alpha]_D - 22.5°$ (c = 0.8% $CHCl_3$), NMR: 0.34 and 0.35 (2s, 3H, Me18), 0.92 and 0.95 (2t, 3H, butyl Me), 2.94 and 3.04 (2s, 3H, MeN), 3.25 and 3.40 (2t, 2H, CH_2N), 3.80 to 4.00 (m, 5H, SCH_2CO, CH_2O and H11), 6.40 (dd, 1H, H2), 6.58 (d, 1H, H4), 6.76 (dd, 1H, H1), 6.59 and 6.94 (2d, 4H, other aromatics).

N-butyl-2-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)phenoxy]pentylsulfonyl]-N-methylacetamide (5g)

70% perphthalic acid (180 mg, 0.7 mmol) was added to a cooled (0°C) solution of sulfide **5e** (209 mg, 0.35 mmol) in methylene chloride (4 ml). After stirring for 1.5 h at that temperature, aqueous sodium thiosulfate was added and the product was extracted with methylene chloride. The organic phase was washed with saturated aqueous sodium bicarbonate, dried over sodium sulfate, evaporated under reduced pressure and purified by chromatography (ethyl acetate) to afford pure **5g** as a colorless amorphous solid (138 mg, 63%), NMR: 0.34 (s, 3H, Me18), 0.93 and 0.96 (2t, 3H, butyl Me), 2.98 and 3.14 (2s, 3H, MeN), 3.26 and 3.41 (2m, 4H, CH_2N and CH_2SO_2), 3.67 (m, 1H, H17), 3.87 (t, 2H, CH_2O), 3.92 (t, 1H, H11), 4.02 (m, 2H, SCH_2CO), 6.41 (dd, 1H, H2), 6.60 (d, 1H, H4), 6.79 (d, 1H, H1), 6.60 and 6.94 (2d, 4H, other aromatics).

Receptor binding

The relative binding affinities (RBAs) of test compounds (t.c.) for the mouse estrogen receptor (ER) were determined using a method previously described by Ojasoo and Raynaud [10]. Briefly, uterine cytosol from 18 to 19-day old Swiss mice (Iffa-Credo, Les Oncins, France) was incubated for 5 h in a Tris–sucrose buffer at 25°C with 2.5 nM of tritiated E2 in the presence of increasing concentrations of cold E2 or t.c. Bound radioactivity was measured by the dextran-coated charcoal adsorption technique. The RBA of E2 was taken arbitrarily as 100.

Antiproliferative activities in vitro

The MCF-7 cell line from ATCC was routinely subpassaged in minimal essential medium (MEM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM), sodium bicarbonate (2.25 g/l), non-essential amino acids (1%) in the

presence of 5% fetal calf serum (Boehringer-Mannheim). Cells were maintained in a 5% CO_2-enriched humidified air atmosphere. One week before use, the cells were subpassaged in the same medium without phenol red and with 50 ng/ml insulin in the presence of 2.5% charcoal stripped serum. 24 h prior to seeding, the medium was changed to the same medium without insulin. Cells were seeded in triplicate in 24-well tissue culture dishes at an initial density of 50,000 cells/ml/well and allowed to attach for 24 h in the presence of 0.1 nM E2 or 50 ng/ml insulin, or 1 ng/ml platelet derived growth factor (PDGF) plus 10 ng/ml epidermal growth factor (EGF). These concentrations proved to be optimal, in our experimental conditions, leading to stimulations of about 100 to 200% for E2 and insulin and 30 to 100% for PDGF/EGF, after a 7 day-culture. 10 μl of the appropriate concentrations of the t.c., dissolved in ethanol–MEM (10:90) were added at the same time. The medium was renewed on days 2 and 5 and on day 7 the cell growth was evaluated by a DABA DNA assay derived from Puzas and Goodman [11]. The results were expressed as the concentration which inhibited cell growth by 50% (IC_{50}). Unless otherwise stated all culture media were obtain from Gibco.

Uterotrophic and antiuterotrophic activities

Swiss female mice (18- to 19-day-old) weighing 9–11 g or Sprague–Dawley female rats weighing 38–42 g (Iffa-Credo, Les Oncins, France) received the t.c. daily, by subcutaneous route, in solution in sesame oil containing 5% benzyl alcohol, for 3 days, either alone or in combination with 10 μg/kg of E2. 24 h after the last administration the animals were sacrificed and the uteri removed and weighed. The estrogenic activity was calculated according to the formula: $(UWI_{t.c.}/UWI_{E2}) \times 100$. The antiestrogenic activity was calculated as $[(UW_{E2} - UW_{t.c.})/UWI_{E2}] \times 100$ (UWI, uterine weight increase; UW, uterine weight).

Antitumoral activities in nude mice

For the standard model, pieces of 1–2 mm of MCF-7 tumor were implanted subcutaneously at the level of the first right mammary pad in 4- to 5-week-old female nude mice weighing 19–21 g (Balb/ca, Clea, Japan). The tumour growth was stimulated by 100 μg

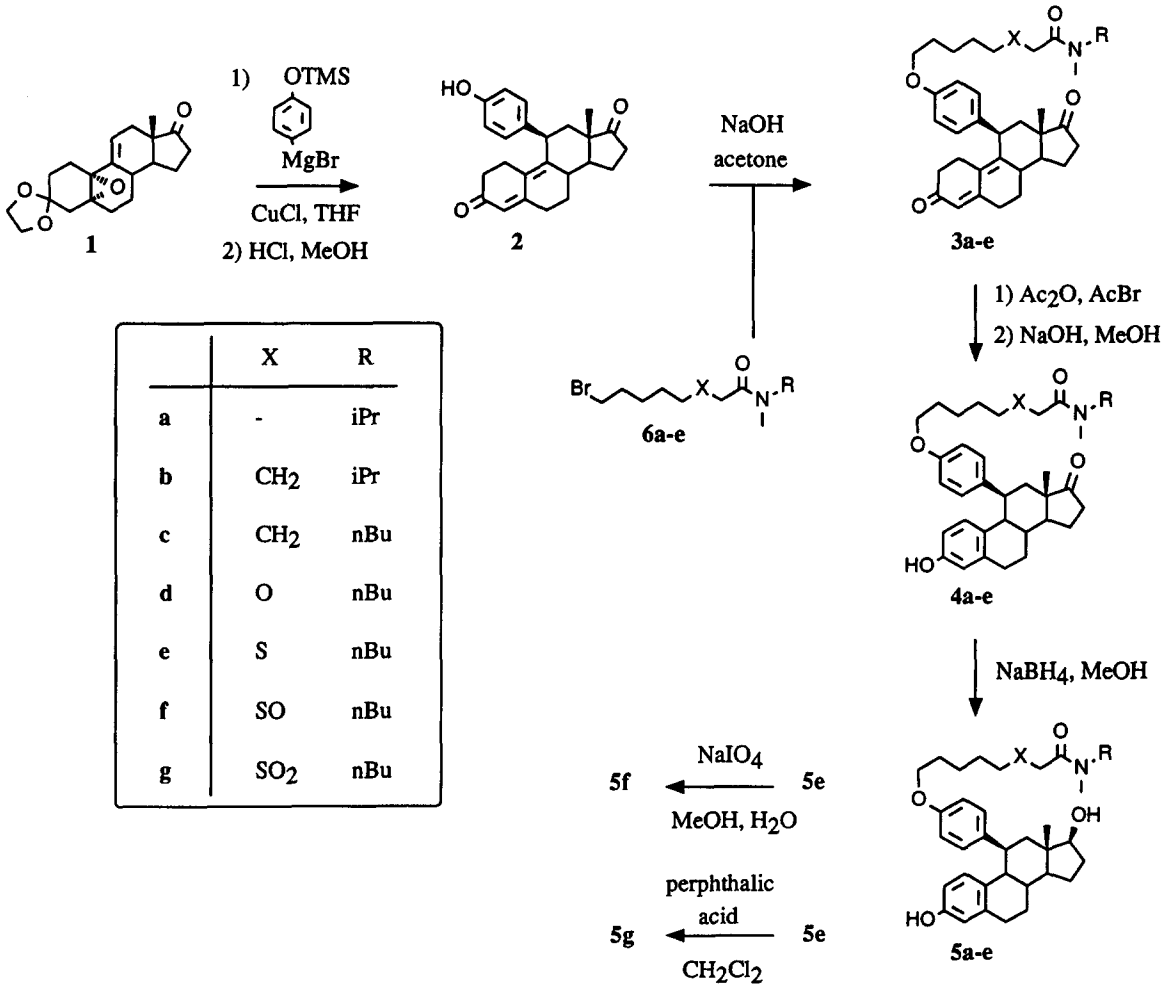


Fig. 2. Synthesis of antiestrogens.

Table 1. Pharmacological profile of antiestrogens

	RBA ER:mice 5h, 25° (E2 = 100)	IC_{50} MCF-7 mean ± SEM (nM) E2-stimulated (0.1 nM)	Insulin stimulated	EGF PDGF stimulated	Antiuterotrophic activity:mice (% of decrease at 3 mg/kg)	Uterotrophic activity:mice (% of increase at 30 mg/kg)
5a	38 ± 13	10 ± 7	2.5 ± 1.4	0.54 ± 0.35	77 ± 5	32 ± 8
5b	32 ± 2	0.82 ± 0.48	0.15 ± 0.09	0.21 ± 0.10	94 ± 6	0
5c	24 ± 3	0.53 ± 0.08	0.11 ± 0.10	0.15 ± 0.12	120 ± 7	0
5d	30 ± 4	0.28 ± 0.07	0.020 ± 0.015	0.012 ± 0.009	116 ± 9	0
5e	15 ± 2	0.43 ± 0.20	0.053 ± 0.036	0.023 ± 0.011	93 ± 5	0
5f	10 ± 1	0.42 ± 0.14	0.068 ± 0.036	0.021 ± 0.006	99 ± 3	0
5g	9.6 ± 0.8	0.32 ± 0.14	0.020 ± 0.018	0.005 ± 0.004	99 ± 4	ND
Tamoxifen	0.6 ± 0.1	316 ± 21	470 ± 230	580 ± 240	36 ± 4	63 ± 8[a]
4-OH-TAM	40 ± 8	7.4 ± 3.4	4.2 ± 2.3	0.66 ± 0.11	ND	ND

[a]3 mg/kg.
ND: not determined.

(5 mg/kg) of E2 in 10 μl of ethanol given by percutaneous route (p.c.) once a week for 5 weeks. The animals were then randomized according to their tumor volume (week 0) and received 5 μg (0.25 mg/kg) of E2 topically once a week with or without 10 mg/kg of the t.c. either percutaneously (three times a week) in 10 μl of ethanol or subcutaneously (three times a week) in 100 μl of sesame oil, or orally (five times a week) in 200 μl of methyl cellulose, for 5 further weeks. The tumor sizes were measured each week and the volumes calculated according to the formula: length × width^2/2. The results are expressed as the ratio: tumor volume on week *n*/tumor volume on week 0.

MCF-7 variant model

We observed that after long term treatment (6 months) with tamoxifen (0.1 mg once a week p.c.) of ovariectomized (Ovx) nude mice bearing MCF-7 tumors, some of the tumors suddenly began to regrow quickly, as previously described by Gottardis and Jordan [12]. Two subpassages of these tumors in Ovx nude mice receiving tamoxifen treatment allowed us to obtain nearly 100% of reproducibly growing tumors. We now possess stable MCF-7 variant tumors that we can implant, every 2 months, in Ovx nude mice and the growth of which is stimulated by TAM or E2. Experiments have been carried out on these tumors using the same protocol as described for the wild MCF-7 tumors, except that during the initial 5 weeks, the growth stimulation was induced with 5 mg/kg tamoxifen p.c. once a week instead of E2.

RESULTS

As shown in Fig. 2, the synthesis of all the compounds started with the copper(I) catalyzed regiospecific addition [1] of the Grignard reagent derived from 4-bromophenol, protected as its trimethylsilyl ether, on the allylic epoxide **1** [8]. Aqueous acidic treatment of the crude 11β-aryl-5-hydroxy-estr-9-ene thus obtained yielded the estradienedione **2** (73%), along with a small amount (9.5%) of its 5(10), 9(11)-diene isomer, by simultaneous deprotection of the phenol, deketalization of the 3-keto group and elimination of the 5-hydroxyl. Alkylation of the phenolic hydroxyl group of **2** was performed using the appropriate bromoamide **6a–e** with aqueous sodium hydroxide in acetone. The A rings of the resulting compounds **3a–e** were aromatized by treatment with a mixture of acetyl bromide and acetic anhydride in dichloromethane [1]. The intermediary phenolic acetates were saponified to give the phenols **4a–e**. Reduction of the 17-keto group of compounds **4a–e** with sodium borohydride in ethanol led to the estratrienediols **5a–e**. Oxidation of the sulfide **5e** with sodium periodate gave the sulfoxide **5f** while oxidation with perphthalic acid produced the sulfone **5g**.

Table 1 shows that all the compounds of this series displayed affinities for the mouse ER ranging from 10 to 40% relative to E2. This is comparable to the affinity of OH-TAM (40% of E2). With the same amide substituent, a change in the chain length from $(CH_2)_6$ to $(CH_2)_7$ did not significantly affect the RBA for ER (**5a** vs **5b**). Unlike that observed in the 11β-amidoalkyl series [7], no significant difference in the RBA to ER was observed when the nitrogen amide substituent was changed from an isopropyl to a butyl (**5b** vs **5c**). Replacement of the methylene group β to the amide carbonyl by an oxygen did not change the RBA (**5d** vs **5c**) while replacement by a sulfur atom (optionally oxidized) (**5e** to **5g**) led to a clear decrease in the RBA.

Compounds **5b** to **5g** displayed a full antiuterotrophic effect when administered subcutaneously in mice at 3 mg/kg, while being devoid of uterotrophic activity at 30 mg/kg. On the contrary, **5a**, the compound bearing the shorter side chain, showed a partial agonist effect.

Compounds **5a** to **5f** all showed strong antiproliferative activities on the MCF-7 cell line under various stimulation conditions. Compared to OH-TAM compounds **5b** to **5g** were 10 to 25 times more active with E2 stimulation and 30 to 200 times more active when

the cells were stimulated with insulin. The short-chain derivative, **5a**, displayed a lower activity. Moreover, **5d**, **5e**, **5f** and **5g** showed strong antiproliferative activities with IC_{50}s in the picomolar range when MCF-7 cells were stimulated by growth factors, contrary to OH-TAM which was poorly active (the maximal inhibitory effect of this compound was usually <50%). The best activities were recorded for compounds **5d**, **5e**, **5f** and **5g**, bearing a heteroatom in their side chain.

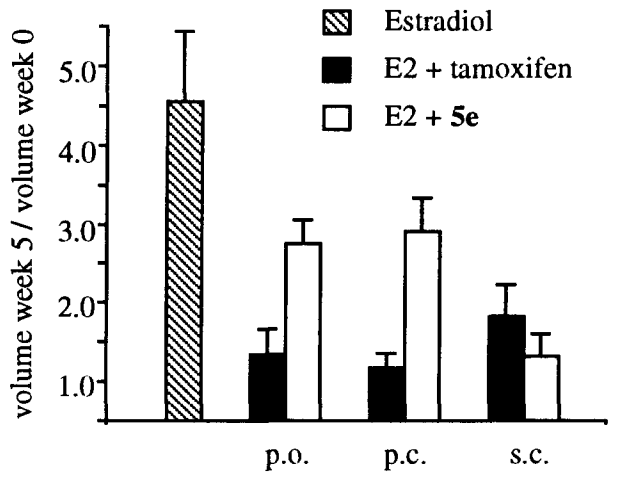


Fig. 3a. Antitumoral activities of 5e and tamoxifen on MCF-7 tumors implanted in nude mice: after 5 weeks of E2-stimulated tumor growth, animals received for a further 5 weeks a weekly administration of 5 μg E2 percutaneously (p.c.) alone or along with 10 mg/kg t.c. by oral route (p.o., 5 times a week), or by p.c. or subcutaneous (s.c.) routes (3 times a week). E2 + tamoxifen: $P < 0.05$ vs E2; E2 + 5e: $P < 0.05$ vs E2 by s.c. route, non-significant vs E2 p.o. and p.c. (Dunnet test).

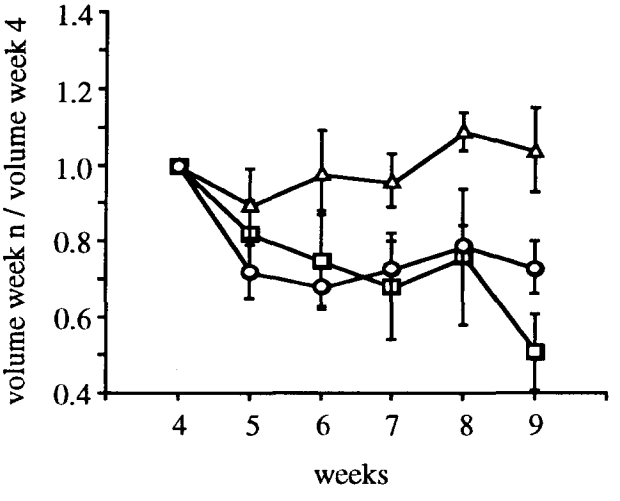


Fig. 3b. After 5 weeks of E2-stimulated tumor growth, mice were ovariectomized and received a percutaneous (p.c.) treatment with 5 mg/kg tamoxifen twice a week by p.c. route for 4 weeks. Such a treatment with tamoxifen was maintained, △, or associated with 50 mg/kg 5e, ○, for a further 5 weeks (weeks 4–9). A control group received vehicle alone, □.

Compound **5e**, showing one of the best antiproliferative profiles, has been further tested for its activity on several models of MCF-7 tumors implanted in nude mice.

Antitumoral activity on MCF-7 tumors

When administered orally or percutaneously to nude mice implanted with MCF-7 tumors and continously treated with E2, **5e** displayed, respectively, 51 and 46% tumor growth inhibition, whereas tamoxifen was a nearly complete inhibitor (90 and 96% inhibition). When given subcutaneously at the same dose, **5e** was at least as active as tamoxifen, respectively, 91 and 77% inhibition (Fig. 3a).

The agonist effect of tamoxifen could clearly be demonstrated in the following experiment: nude mice bearing MCF-7 tumors (established as usual with E2) were castrated and treated percutaneously for 4 weeks with 5 mg/kg tamoxifen alone, which did not induce any change in the tumor volume, then the animals were divided in 4 groups. In the first one, in which the tamoxifen treatment was maintained for 5 further weeks, the tumoral volume remained stable (Fig. 3b). A significant decrease of the tumor volume occured in the second group receiving no treatment. In the third group, in which the animals received **5e** percutaneously plus tamoxifen percutaneously, a decrease of the tumor volumes was also noticed, equivalent to that observed with ovariectomy alone. The same result was obtained when **5e** was administered alone to the last group (data not shown).

Antitumoral activity on MCF-7 variant tumors

Unlike the wild MCF-7 tumors, the MCF-7 variant tumors implanted in nude mice could be stimulated either by E2 or by tamoxifen (Fig. 4a). On this kind of tumor, administration of **5e** alone did not demonstrate any agonistic activity on growth, whereas the tamoxifen-induced growth could be entirely inhibited by **5e** (Fig. 4b).

The same type of inhibition was observed in the case of E2-stimulation of the MCF-7 variant tumor growth, tamoxifen being inactive under these conditions (data not shown).

DISCUSSION

E2 derivatives substituted at the 7α position by a polar function (amide or sulfoxide) have been described by Wakeling and co-workers [6, 13] as completely antiuterotrophic in rodents (pure antiestrogens). On the other hand, it has been shown in these laboratories that substitution of the E2 molecule at the 11β position by either a dialkylaminoethoxyphenyl-type side chain or aliphatic long-chain amides yielded respectively partial [4] and total [7] estrogen antagonists. The much higher ER affinity of 11β-aryl antiestrogens such as RU 39 411 (~130% of E2) [4] as compared to the

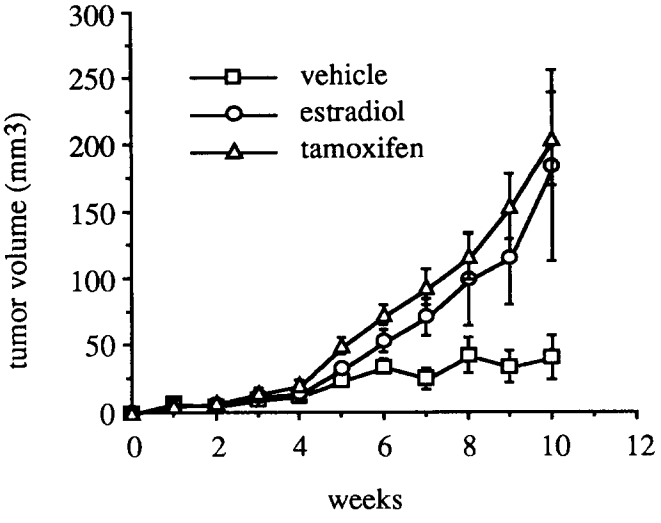


Fig. 4a. Growth of MCF-7 variant tumors implanted in nude mice: 1–2 mm pieces of MCF-7 variant tumors were implanted subcutaneously into Ovx nude mice. The animals received a weekly percutaneous administration of 100 μg (5 mg/kg) E2, tamoxifen or vehicle alone.

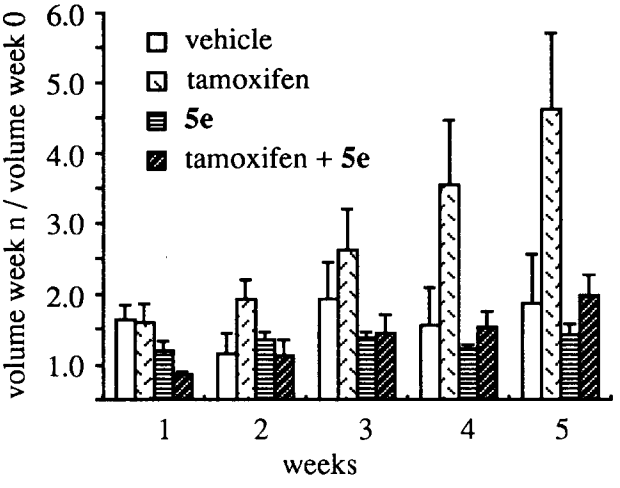


Fig. 4b. Effect of tamoxifen, 5e, and their association on the growth of MCF-7 variant tumors in Ovx nude mice: after 5 weeks of tumor growth stimulation with 5 mg/kg tamoxifen weekly by percutaneous route, the animals received for a further 5 weeks the same dose of tamoxifen or 50 mg/kg 5e twice a week or the association of the two compounds. A control group received vehicle alone. After 5 weeks treatment: tamoxifen + 5e: $P < 0.05$ vs tamoxifen; 5e: non-significant vs vehicle (Dunnet test).

amidoalkyl analogs (15–50% of E2) [7] prompted us to investigate compounds bearing 11β-substituents which integrate both features. The results obtained for compounds **5a–g** show that the overall range of affinities for the mouse ER is very similar to the 11β-amidoalkyl series, suggesting that in RU 39 411, it is not the aromatic ring which is responsible for the high affinity, but rather the dimethylamino moiety, also present in OH-TAM. Closer examination of the structure–affinity relationships indicates that replacement of the methylene group β to the amide carbonyl by oxygen (**5d**) leaves affinity nearly unchanged, whereas a sulfur atom at this position, whether oxidised or not (**5e–g**), leads to a decrease in affinity.

The more interesting point to discuss however, is the fact that there seems to be no correlation between affinity and activity: this is obvious if we recall that both agonists and antagonists have to bind to the receptor in order to express activity. Thus, by definition, E2 will be fully uterotrophic in mice by binding to ER, whereas pure antiestrogens like compounds **5b** to **5g** will be totally antiuterotrophic by binding to the same receptor. Tamoxifen, which is active via its metabolite OH-TAM, will display both agonist and antagonist activities. As already stated by Teutsch [2] for glucocorticoid ligands, the quality of the response (agonistic vs antagonistic) is not directly related to receptor affinity, but rather to the chemical structure of the ligand. At best, affinity–activity correlations could be expected only from compounds with very close structural similarities. A pure antihormone may be envisaged as a compound which stabilizes exclusively the inactive conformation of the receptor whereas a mixed antagonist would be able to stabilize both the active and the inactive conformers to various extents. The example of compound **5a** which lacks only one methylene group relative to **5b**, is demonstrative of this fact: although the affinities of the two compounds are nearly identical, the first one is a partial agonist, while the second one is a pure antagonist, as judged by uterotrophic versus antiuterotrophic activities. This partial agonistic component of the molecules bearing the shortest chains was also reported for 7α-substituted steroidal antiestrogens [6]. This could be an explanation of the partial agonistic activity of RU 39 411 [4] or of tamoxifen.

For the same reason, no correlation between *in vitro* antiproliferative activity and receptor affinity can be expected. Incidentally, it should also be remembered that MCF-7 cells contain human ER, which is not identical to the mouse ER. The most interesting feature of the *in vitro* studies was the potent antiproliferative activities displayed by the 11β-substituted compounds (particularly the oxa and thia derivatives **5d**, **5e**, **5f** and **5g**), when MCF-7 cell growth was stimulated by growth factors. Based on various reports on the effects of antiestrogens on growth factor stimulated cells [14–16] and on the role of growth factors on cell proliferation [17–21], it was established in this laboratory that a mixture of EGF and PDGF could promote the growth of the MCF-7 cell line, in the total absence of estrogen, though the intensity of stimulation may vary. Pure antiestrogens were able to strongly inhibit this growth, whatever the stimulation intensity. On the contrary, compounds with partial estrogenic activity (like OH-TAM) were only poor inhibitors. For these latter compounds, the greater was the achieved

stimulation, the weaker was the antagonist effect; alternatively, only when the growth factors led to a weak stimulation of the cell growth, could the inhibitory effect of OH-TAM exceed 50%. Under these circumstances the IC_{50} was recorded at about 1 nM.

In order to test the antitumoral activity of the most representative compound from this new series, **5e** was administered to athymic nude mice bearing established xenografts of MCF-7 tumors. In a first experiment designed to compare the different ways of administration, 10 mg/kg of **5e** were given orally, percutaneously or subcutaneously to the animals, in comparison to the same dose of tamoxifen (Fig. 3a). By the two former routes of administration, **5e** only slowed down the tumoral growth, weekly sustained by applications of E2, while tamoxifen was a nearly complete inhibitor of the growth for 5 weeks. When **5e** was injected subcutaneously, it was at least as potent as tamoxifen to inhibit the tumoral growth. This difference in activity between the different routes of administration may be related to differences in absorption or metabolism of the compound as previously shown by Wakeling and Bowler [13] for 7α derivatives of E2. Incidentally this experiment indicates that for TAM the subcutaneous route of administration is less efficient than the percutaneous or oral routes.

In a second experiment, the agonist effect of mixed antiestrogens like tamoxifen could clearly be demonstrated in nude mice bearing MCF-7 tumors, after ovariectomy: when the animals were castrated, a significant decrease of the tumor volume occured. In the group treated percutaneously with tamoxifen alone after the ovariectomy, this effect was prevented (Fig. 3b). **5e** added to tamoxifen entirely counteracted the action of the latter compound. This experiment shows that **5e** impedes the partial agonist activity of tamoxifen on tumors, in the absence of E2, without promoting cancerous cell growth.

Moreover, we were able to obtain MCF-7 "variant" tumours whose growth could be promoted either by E2 or by tamoxifen (Fig. 4a), as described by Gottardis and Jordan [12]. In nude mice bearing this kind of tumors, a slight spontaneous growth could be noticed and **5e** given alone percutaneously had no stimulatory effect (Fig. 4b). More importantly, **5e** was able to completely inhibit the tamoxifen-induced growth, for at least 5 weeks. This model of tamoxifen-stimulated tumors may be relevant to the treatment of the escape from tamoxifen treatment, currently observed in the clinic [22].

CONCLUSION

The results described above show that **5e** is a highly potent antiproliferative compound on the estrogen positive MCF-7 cell line in different culture conditions; it is of particular interest when the cells are stimulated by growth factors. This advantage over tamoxifen is also confirmed *in vivo* on the inhibition of the MCF-7 variant tumor growth, a model which may be relevant to relapse under tamoxifen-treatment in human breast cancer. Nevertheless, in the studied models, **5e** was unable to induce a tumor regression. Beyond these promising antiproliferative and antitumoral pharmacological activities, **5e** displayed neither tumoral growth stimulatory effects *in vitro* or *in vivo*, nor agonistic effects on mouse uterus weight. Owing to the interesting properties displayed by the 11β aryl substituted E2 derivatives, in the amide series, several other compounds bearing this structural feature are now under investigation.

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