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RU 58 668, a New Pure Antiestrogen Inducing a Regression of Human Mammary Carcinoma Implanted in Nude Mice*

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RU 58 668, a new steroidal antiestrogen, has been synthesized. Its *in vitro* and *in vivo* pharmacological activities have been compared to those of tamoxifen and ICI 182,780. *In vitro*, it displayed affinities for human and murine estrogen receptors equivalent to those of 4-hydroxy-tamoxifen, along with moderate affinities for progestin and glucocorticoid receptors. RU 58 668 proved to be a potent antiproliferative agent on MCF-7 cells stimulated by estradiol, or by exogenous or endogenous growth factors (IC_{50}, 0.01 nM). It also inhibited the growth of the insulin-stimulated T47D cell line. *In vivo*, RU 58 668 displayed a total antiuterotrophic activity in mice or rats without exhibiting any agonistic effect. Moreover, RU 58 668 was the only antiestrogenic compound tested so far to be able to induce a long term regression of human mammary MCF-7 tumors implanted in nude mice, suggesting its potential use for the treatment of advanced breast cancer.

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INTRODUCTION

The clinical use of tamoxifen in the treatment of estrogen receptor (ER)-positive breast cancer, both in the advanced disease and as an adjuvant therapy, has demonstrated the utility as well as the limitations of partial antiestrogens in this indication [1]. The emergence of pure antiestrogens, devoid of any estrogenic activity [2–5] raises the hope for increased efficacy at various levels: 1, more rapid and complete tumor inhibition; 2, activity against tamoxifen-resistant ER-positive tumors; 3, increased time to relapse; and 4, decreased potential for tumor flare and induction of endometrial cancer [6, 7].

In our search for pure antiestrogens, we showed that estradiol (E2) derivatives substituted at the 11β position by amidoalkyl [8] or amidoalkoxyphenyl substituents [9] were totally antiestrogenic on the uterotrophy induced by estradiol in mice, as well as in the estrogen-induced proliferation of the MCF-7 human breast cancer cell line. While investigating the incidence of the replacement of the thioacetamido moiety of RU 54 876 (Fig. 1) by various polyfluoroalkylthio groups, we selected RU 58 668 (Fig. 1) for extended evaluations on the basis of its *in vitro* activity on MCF-7 cells.

This paper reports the antihormonal and biological profile of RU 58 668: the binding to steroidal receptors, the antiproliferative activity on MCF-7 and T47D cell lines, the antiuterotrophic activity in mice and rats and the antitumoral activity on MCF-7 tumors implanted in nude mice. It has been compared, on these different models, with tamoxifen and ICI 182,780 (Fig. 1), a new pure antiestrogen in early clinical trials, as reported recently by Wakeling *et al.* [4, 5].

EXPERIMENTAL

Synthesis

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. Scheme 1 shows the synthesis of the following compounds.

*This paper is dedicated to Dr Edouard Sakiz in appreciation for his 40 years of commitment to research in endocrinology.

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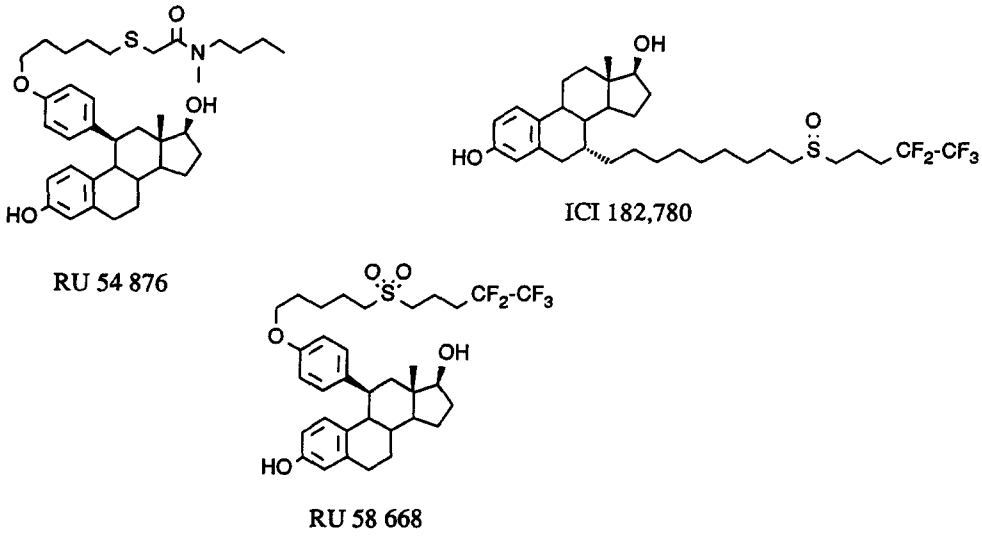
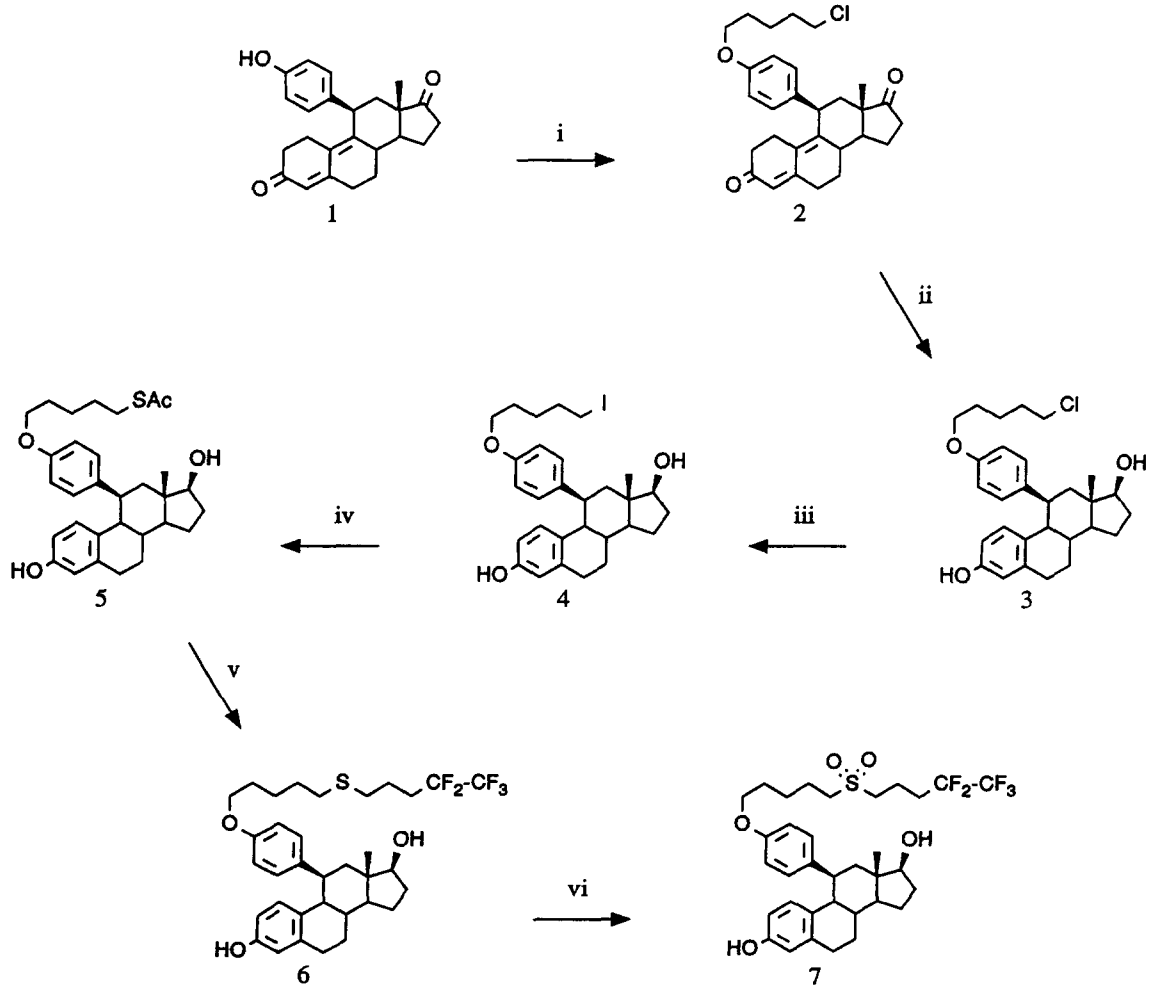


Fig. 1. Chemical structures of antiestrogens.



Scheme 1. RU 58 668 synthesis. (i) $Br(CH_2)_5Cl$, NaOH, acetone, 50°C, 83%; (ii) Ac_2O, AcBr, then NaOH, then $NaBH_4$, MeOH, 52%; (iii) NaI, methyl ethyl ketone, reflux, 100%; (iv) AcSK, EtOH, 50°C, 78%; (v) $C_2F_5(CH_2)_3I$, NaOH, MeOH, 50°C, 82%; (vi) MCPBA, CH_2Cl_2, 0°C, 87%.

11β-[4-[(5-chloropentyl)oxy]phenyl]-estra-4,9-dien-3,17-dione (2)

2 N sodium hydroxide (45 ml), followed by 1-bromo-5-chloropentane (Fairfield) (21.65 g, 0.117 mol) were added to a solution of the phenol 1 [9] (28.2 g, 0.078 mol) in acetone (450 ml). The mixture was heated at 50°C for 6 h and the solvent was removed by evaporation. The product was dissolved in methylene chloride, washed with brine, dried over magnesium sulfate and the solvent was evaporated (usual work-up). Recrystallization of the residue from methylene chloride–isopropyl ether afforded pure 2 as colorless crystals (26.3 g, 72%), m.p. 215°C, $[\alpha]_D$ + 223° (c = 0.9% $CHCl_3$), NMR: 0.57 (s, 3H, Me18), 3.57 (t, 2H, CH_2Cl), 3.93 (t, 2H, CH_2O), 4.38 (d, 1H, H11), 5.80 (s, 1H, H4), 6.80 and 7.08 (2d, 4H, aromatics). A second crop of 2 was obtained after chromatography of the mother liquors (eluent: petroleum ether–ethyl acetate, 1:1, v/v) and recrystallization as above (4.2 g, 11%), m.p. 220°C.

11β-[4-[(5-chloropentyl)oxy]phenyl]-estra-1,3,5(10)-triene-3,17β-diol (3)

Acetic anhydride (30 ml) and acetyl bromide (15 ml) were added to an ice-cooled solution of chloride 2 (30 g, 64 mmol) in methylene chloride (300 ml). The mixture was allowed to warm to room temperature and after stirring for 1 h, the solution was cooled to 0°C. Methanol (30 ml) was added, followed by saturated aqueous sodium bicarbonate. The aqueous phase was removed and extracted with methylene chloride. Usual work-up of the organic phase gave the crude phenolic acetate (37 g), which was immediately dissolved in THF (200 ml), methanol (200 ml) and 2 N sodium hydroxide (64 ml). The brownish solution was stirred for 45 min and was acidified with 2 N hydrochloric acid. The crude product (32 g) obtained after extraction with methylene chloride and usual work-up was dissolved in THF (150 ml) and methanol (150 ml). The solution was cooled in an ice bath and sodium borohydride (2.56 g, 64 mmol) was added. The mixture was stirred for 1 h at this temperature, acetone (10 ml) was added and the solvents were evaporated. The residue was partitioned between methylene chloride and 2 N hydrochloric acid. The usual work-up of the organic fraction gave an amorphous solid which was chromatographed (toluene–ethyl acetate, 8:2, v/v) and crystallized from ethyl acetate to yield pure 3, as colorless crystals (15.8 g, 52%), m.p. 165°C, $[\alpha]_D$ −37° (c = 1% $CHCl_3$), NMR: 0.33 (s, 3H, Me18), 3.54 (t, 2H, CH_2Cl), 3.68 (t, 1H, H17), 3.86 (t, 2H, CH_2O), 3.94 (t, 1H, H11), 6.41 (dd, 1H, H2), 6.58 (d, 1H, H4), 6.82 (d, 1H, H1), 6.61 and 6.95 (2d, 4H, other aromatics).

11β-[4-[5-iodopentyl)oxy]phenyl]-estra-1,3,5(10)-triene-3,17β-diol (4)

A solution of the chloro derivative 3 (469 mg, 1 mmol) and sodium iodide (225 mg, 1.5 mmol) in methyl ethyl ketone (5 ml) was stirred at reflux temperature overnight. The solvent was evaporated and the residue was taken up in ethyl acetate. Usual work-up afforded compound 4 (580 mg, 100%), as a yellowish amorphous solid, $[\alpha]_D$ −22° (c = 1% $CHCl_3$), NMR: 0.33 (s, 3H, Me18), 3.19 (t, 2H, CH_2I), 3.68 (t, 1H, H17), 3.85 (t, 2H, CH_2O), 3.93 (t, 1H, H11), 6.40 (dd, 1H, H2), 6.58 (d, 1H, H4), 6.82 (d, 1H, H1), 6.60 and 6.95 (2d, 4H, other aromatics).

S-[5-[4-(3,17β-dihydroxy-estra-1,3,5(10)-trien-11β-yl)-phenyloxy]pentyl]ethane thioate (5)

A suspension of iodo derivative 4 (250 mg, 0.45 mmol) and potassium thioacetate (102 mg, 0.88 mmol) in ethanol (3 ml) was stirred at 50°C for 1 h. The resulting solution was evaporated and the residue was taken up in ethyl acetate. Usual work-up led to an amorphous solid which was chromatographed (petroleum ether–ethyl acetate, 6:4, v/v) to yield pure 5 (175 mg, 78%), as a semi-crystalline white solid, m.p. 95°C, $[\alpha]_D$ −28° (c = 0.8% $CHCl_3$), NMR: 0.33 (s, 3H, Me18), 2.32 (s, 3H, MeCO), 2.87 (t, 2H, CH_2S), 3.68 (m, 1H, H17), 3.84 (t, 2H, CH_2O), 3.93 (t, 1H, H11), 6.41 (dd, 1H, H2), 6.58 (d, 1H, H4), 6.82 (d, 1H, H1), 6.60 and 6.95 (2d, 4H, other aromatics).

11β-[4-[5-[(4,4,5,5,5-pentafluoropentyl)thio]pentyloxy]-phenyl]-estra-1,3,5(10)-triene-3,17β-diol (6)

Preparation of 5-iodo-1,1,1,2,2-pentafluoropentane. Iodine (269 mg, 1.06 mmol) was added while stirring to an ice-cooled solution of triphenylphosphine (275 mg, 1.05 mmol) and imidazole (71 mg, 1.05 mmol) in methylene chloride (1.5 ml). After 5 min 4,4,5,5,5-pentafluoropentanol (182 mg, 1.02 mmol) was added dropwise. The ice bath was removed after 2.5 h and the formed crystals were removed by filtration.

Condensation. The above crude solution of iodopentafluoro derivative (1.05 mmol) was added, under an argon atmosphere, to a solution of thioacetate 5 (350 mg, 0.69 mmol) in methanol (3.5 ml), followed by 10 N aqueous sodium hydroxyde (0.14 ml). After heating to 50°C for 1 h, the mixture was acidified with 2 N hydrochloric acid and the product was extracted with ethyl acetate. Usual work-up and chromatography (methylene chloride–ethyl acetate, 95:5, v/v) afforded pure 6 (353 mg, 82%), as a white amorphous solid, $[\alpha]_D$ −16° (c = 0.8% $CHCl_3$), NMR: 0.33 (s, 3H, Me18), 2.51 (m, 4H) and 2.58 (t, 2H) ($2CH_2S$ and CH_2CF_2), 3.68 (m, 1H, H17), 3.85 (t, 2H, CH_2O), 3.93 (t, 1H, H11), 6.40 (dd, 1H, H2), 6.58 (d, 1H, H4), 6.81 (d, 1H, H1), 6.60 and 6.95 (2d, 4H, other aromatics).

11β-[4-[5-[(4,4,5,5,5-pentafluoropentyl)sulfonyl]pentyl-oxy]phenyl]-estra-1,3,5(10)-triene-3,17β-diol (7), RU 58 668

m-Chloroperbenzoic acid (containing 80% peracid) (186 mg, 0.86 mmol) was added to an ice-cooled solution of sulfide 6 (225 mg, 0.36 mmol) in methylene chloride (3 ml). After 1 h at that temperature, the solution was washed with 0.5 N aqueous sodium thiosulfate, followed by the usual work-up. The crude product was purified by chromatography (ethyl acetate–petroleum ether, 6:4, v/v) to yield pure 7 (206 mg, 87%) as an amorphous white powder, $[\alpha]_D$ −17.5° (c = 1% $CHCl_3$), NMR: 0.33 (s, 3H, Me18), 3.00 (m, 4H, $2CH_2SO_2$), 3.69 (m, 1H, H17), 3.87 (t, 2H, CH_2O), 3.93 (t, 1H, H11), 6.40 (dd, 1H, H2), 6.59 (d, 1H, H4), 6.81 (d, 1H, H1), 6.59 and 6.95 (2d, 4H, other aromatics).

Compounds

Tamoxifen, E2, progesterone, dexamethasone, insulin, platelet derived growth factor (PDGF), epidermal growth factor (EGF), DNA from calf thymus were obtained from Sigma and sodium pentobarbital from Sanofi. Culture media, sodium bicarbonate, non-essential amino acids, antibiotics and glutamine were purchased from Gibco; fetal calf serum (FCS) was obtained from Boehringer-Mannheim, tritiated hormones from New England Nuclear and diamino benzoic acid (DABA) from Aldrich. All cell lines: MCF-7 (HTB 22),

T47D (HTB 133) and HFL-1 (CCL 153) were purchased from ATCC.

4-Hydroxy-tamoxifen (4-OH-tamoxifen) [10] and ICI 182,780 [11] were prepared according to literature procedures. Unless otherwise stated, percutaneously, orally and subcutaneously administered compounds were respectively dissolved in ethanol or suspended in methyl cellulose or in arachis oil.

Animals

Mice and rats were purchased from Iffa-Credo, Les Oncins, France and rabbits from ESD, Châtillon-Chalaronne, France.

Relative Binding Affinities (RBAs)

Tissues (or the recombinant human ER) were homogenized in a Tris–sucrose buffer pH 7.4 and centrifuged at 0°C for 30 min at 209,000 ***g***. The supernatants were incubated with the relevant ^{3}H-ligand in the presence of the reference or the test compound (t.c.). After incubation, the bound tritiated ligand was measured by the Dextran-coated charcoal adsorption technique described previously [12].

Antiproliferative Activities In Vitro

MCF-7 cells were routinely cultured and subpassaged in minimum essential medium (MEM) with phenol red, supplemented with sodium bicarbonate 2.25 g/l, non-essential amino acids 1%, penicillin 100 U/ml, streptomycin 100 μg/ml, glutamine 2 mM, in the presence of 5% FCS. After subpassaged for 1 week in the same medium with 50 ng/ml insulin and 5% charcoal-stripped FCS, cells were plated 1 extra week in MEM–F12 (v/v) without phenol red in the presence of 500 ng/ml insulin. Two days before the experiments, insulin was removed. Experiments were carried out in the above mentioned medium, in 24-well dishes (40,000 cells per well). 24 h after seeding, the medium was changed and cell growth was stimulated either by 0.1 nM E2 or by 500 ng/ml insulin or by 10 ng/ml EGF and 1 ng/ml PDGF which proved to be, in our conditions, the optimal stimulating concentrations. Every concentration of t.c. was assayed in triplicate.

Moreover we tested a paracrine MCF-7 cell growth stimulation model by a coculture with the human fibroblastic cell line HFL-1: 10,000 HFL-1 cells were seeded in the upper chamber of 24-well Millicell® (Millipore) in the steroid/insulin free medium described previously. In other respects, 50,000 MCF-7 cells were plated in the lower chamber of another Millicell® on day 3 of HFL-1 growth. 24 h later, the upper chambers which contains the HFL-1 were transferred into the MCF-7-containing chambers and t.c. were added.

The inhibition of insulin or E2-stimulated T47D cell growth was carried out in the same experimental conditions as used for the MCF-7 cell line.

In all the experiments, tested compounds (RU 58 668, ICI 182,780 and 4-OH-tamoxifen) were dissolved in ethanol–MEM (10:90, v/v). 10 μl of these solutions were added in multiwell plates containing 1 ml of culture medium (final ethanol concentration: 0.1%). The medium was changed three times a week. On day 8 the DNA content was evaluated with the help of a standard curve of calf thymus DNA by a DABA assay derived from Puzas and Goodman [13]. Briefly, culture medium was removed and cells were fixed with 0.25 ml methanol and allowed to dry at room temperature. 0.2 ml of a 2 M aqueous solution of DABA was added and multiwell dishes were incubated for 1 h at 60°C. At the end of incubation, 2 ml of 1 N hydrochloric acid were added. Moreover, appropriate dilutions of 10 μl calf thymus DNA in 1 N aqueous ammonia were put into 5 ml tubes, allowed to dry at room temperature and treated as the multiwells. The fluorescence was evaluated (excitation 408 nm, emission 510 nm) with a fluorimeter Konton Instruments SFM 25.

Uterotrophic and Antiuterotrophic Activities

Female Swiss mice (18–19 days old) weighing 9–11 g or female Sprague–Dawley rats (18–19 days old) weighing 38–42 g received for 3 days either a daily subcutaneous injection or a daily oral administration of the t.c., alone or with 10 μg/kg E2. 24 h after the last administration the animals were sacrificed by cervical dislocation and the uteri were 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 $[(UW_{E2} - UW_{t.c.})/UW_{E2}] \times 100$; (UWI = uterine weight increase, UW = uterine weight).

Antitumoral Activities in Nude Mice

MCF-7 (5×10^6) cells, cultured as described previously in MEM in the presence of 5% FCS, were subcutaneously injected into the right mammary pad of 4 to 5-week-old female homozygous balb/ca nude mice weighing 19–21 g. The animals received a weekly percutaneous administration of 100 μg E2 in order to obtain tumors with a volume of 250–500 mm^3 (calculated as width^2 × length/2). One of the tumors was removed and cut to 1–2 mm pieces in sterile culture medium. These small tumor fragments were then subcutaneously implanted under anesthesia (sodium pentobarbital 60 mg/kg i.p. in physiological serum) into female nude mice. Tumor growth was stimulated by percutaneous administration of 100 μg E2 in 10 μl ethanol, once a week for 5 weeks, in order to obtain tumors with a volume of 20–200 mm^3 (animals bearing tumors smaller than 20 mm^3 were discarded). Mice were then randomized (week 0) in groups of 10 animals, according to their tumor volume. Then animals received 5 μg E2 percutaneously once a week and either an oral administration of 10 mg/kg t.c. 5 days a week, or a single s.c. injection of 250 mg/kg t.c. on weeks 0 and 5. A weekly individual monitoring of the tumor volume was performed and tumor growth evolution was expressed as: tumor volume on week n/tumor volume on week 0.

Other Hormonal and Antihormonal Activities

Progestomimetic and antiprogestomimetic activities on endometrial transformation

Immature New-Zealand female rabbits (30–35 days old) weighing 900–1100 g were treated subcutaneously for 5 consecutive days with 5 μg/kg E2 (days 0–4) and received a daily s.c. administration of 10 mg/kg RU 58 668, on days 7 to 10, with or without 1 mg/kg progesterone. On day 11 the animals were sacrificed and the uteri were fixed in order to carry out an histological evaluation of the endometrial transformation, as described by Mac Phail [14].

Glucocorticoid and antiglucocorticoid activities

Male Sprague–Dawley adult rats weighing 100–120 g received for 4 days a daily s.c. administration of 30 mg/kg RU 58 668, alone or in combination with 50 μg/kg dexamethasone in ethanol–water (1:99,v/v) by oral route. 24 h after the last administration, the animals were sacrificed, and the thymus,

liver and adrenals removed and weighed. Results were expressed as g/100 g of body wt.

RESULTS

RBAs for steroid receptors

As shown in Table 1, RU 58 668 interacted with murine and human ER. Recorded RBAs (respectively 56 and 17%, E2 = 100%) were equivalent to those of 4-OH-tamoxifen but smaller than those of ICI 182,780 (133 and 32%). Interestingly, unlike 4-OH-tamoxifen and ICI 182,780 which did not bind to the rabbit progestogen receptor (PR), RU 58 668 displayed a moderate affinity for PR (17% of progesterone). It also bound to the rat glucocorticoid receptor (GR) with a stronger affinity than ICI 182,780 (respectively 16 and 2% of dexamethasone), and displayed a weak affinity for rat androgen receptor (AR) (3% of testosterone).

Antiproliferative activities in vitro

Figure 2 exemplifies the comparative antiproliferative activities of RU 58 668, ICI 182,780 and 4-OH-tamoxifen on the MCF-7 cell line stimulated by E2, insulin or EGF/PDGF in one particular experiment. The IC_{50} values for RU 58 668, ICI 182,780 and 4-OH-tamoxifen presented in Table 2 are the mean of at least 3 separate experiments. Whatever the stimulating agent, RU 58 668 was 30 to 50 times more potent than 4-OH-tamoxifen and 2.7 to 4.2 times more potent than ICI 182,780.

When MCF-7 cells were stimulated by E2, the three compounds were complete inhibitors of the cell growth, but when stimulated by growth factors (insulin or a mixture of EGF and PDGF), 4-OH-tamoxifen was only a partial inhibitor of the growth (70 and 50% inhibition, respectively, at 10 nM), whereas, at this concentration, RU 58 668 and ICI 182,780 led to a near complete (90%) inhibition [Fig. 2(a, b and c)].

Moreover, as previously described by Robinson and Jordan, growth of the MCF-7 cell line can be induced by factors secreted by another cell line [15]. Thus, as shown on Fig. 3, MCF-7 growth was efficiently stimulated when the cells were cocultured with the lung fibroblastic non-tumoral cell line HLF-1. RU 58 668 proved to be able to inhibit the HFL-1-stimulated MCF-7 cell growth. In such a paracrine stimulation, RU 58 668 was at least 5 times more potent than ICI 182,780 (respective IC_{50}s = 0.09 and 0.5 nM), whereas 4-OH-tamoxifen displayed only a weak activity.

The effect on the T47D cell line, which presents a higher PR/ER ratio than MCF-7 cell line [16], has also been studied. RU 58 668 and ICI 182,780 inhibited the insulin-stimulated growth of that cell line with IC_{50}s of 0.06 and 0.4 nM, respectively [Fig. 4(a)]. Here again, 4-OH-tamoxifen was only a partial inhibitor. When stimulated by E2, the respective IC_{50}s of the two pure antiestrogens were 0.25 and 0.5 nM. In identical conditions, 4-OH-tamoxifen displayed the same inhibitory potential with an IC_{50} of 4 nM [Fig. 4(b)].

Uterotrophic and antiuterotrophic activities

RU 58 668 and ICI 182,780 displayed a complete antiuterotrophic activity when administered s.c. to mice at 3 mg/kg [Fig. 5(a)], unlike tamoxifen which displayed only a limited effect due to its partial agonistic activity as shown in Table 3. Whatever the dose, the antiuterotrophic activities of RU 58 668 and ICI 182,780 were not significantly different. Administered alone by s.c. route, both compounds showed no uterotrophic activity at doses up to 30 mg/kg. Despite a total antiuterotrophic activity of RU 58 668 and ICI 182,780 at low doses when given subcutaneously, the compounds displayed a total antiuterotrophic effect only at 30 mg/kg orally, suggesting a poor absorption and/or a rapid metabolism by this route (Fig. 6).

In rats the complete antiuterotrophic effects induced by RU 58 668 and ICI 182,780 were reached at a dose as low as 1 mg/kg s.c. and the effects of the two compounds are similar [Fig. 5(b)].

Antitumoral activities in nude mice

Oral route. The daily administration of 10 mg/kg RU 58 668 and ICI 182,780 on MCF-7 tumors implanted in nude mice led to a low growth inhibitory effect, weaker than that observed with the same dose of tamoxifen (Fig. 7), but at 100 mg/kg/day, RU 58 668 displayed the same effect as 10 mg/kg/day tamoxifen.

Subcutaneous route. In the same model, a single subcutaneous injection of 250 mg/kg RU 58 668 every 5 weeks caused long lasting regression of the tumors (for at least 10 weeks), as measured by their volume evolution. The mean tumor weight of the RU 58 668 treated-group on week 10 was significantly lower than

Table 1. RBAs for the steroid hormonal receptors of RU 58 668, ICI 182,780 and 4-OH-tamoxifen

	Estrogen		Receptors		
	Mouse uterus (5 h, 25°C)	Human (5 h, 25°C)	Progestogen rabbit uterus (24 h, 0°C)	Glucocorticoid rat thymus (24 h, 0°C)	Androgen rat prostate (24 h, 0°C)
Compounds	E2 = 100	E2 = 100	Progesterone = 100	Dexamethasone = 100	Testosterone = 100
RU 58 668	56 ± 7	17 ± 2	16 ± 2	17 ± 2	3.0 ± 0.5
4-OH-tam	40 ± 8	19 ± 4	0	0.04 ± 0.01	0.15 ± 0.01
ICI 182,780	133 ± 16	32 ± 2	0	2.0 ± 0.2	0

The RBAs are expressed as the ratio of the concentration of the reference compound over the concentration of the competitor to inhibit ^{3}H-ligand binding by 50% and multiplied by 100.

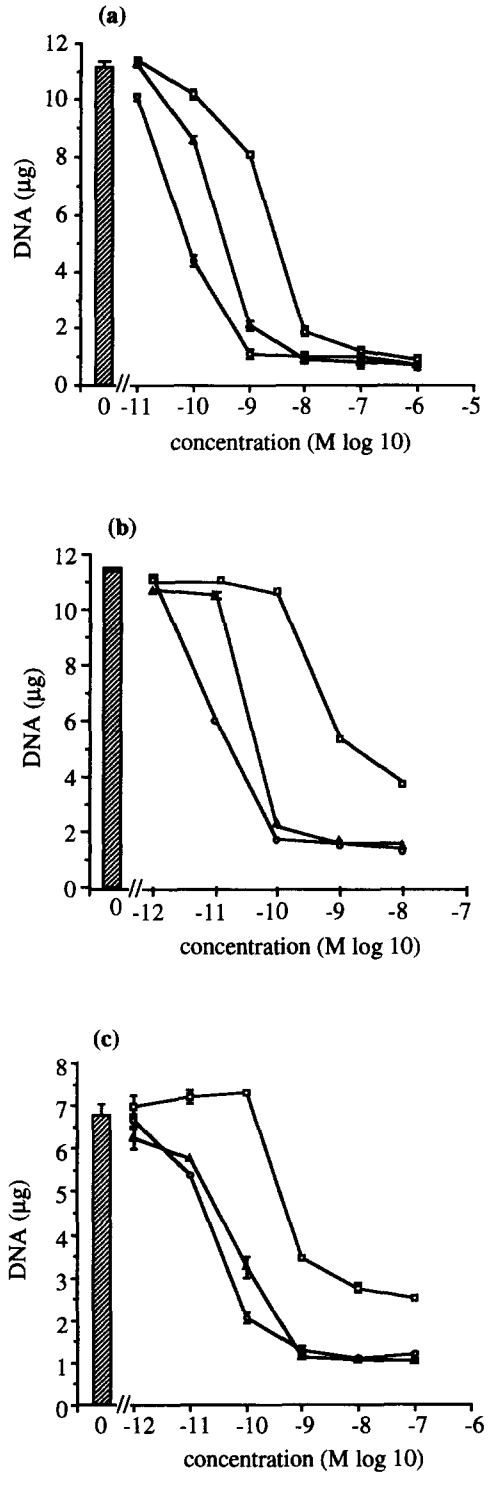


Fig. 2. Effect of RU 58 668 ○, ICI 182,780 ▲ and 4-OH-tam □ on MCF-7 cell growth cultured for 8 days in MEM without phenol red in the presence of 5% charcoal-stripped FCS. Cells were stimulated either by 0.1 nM E2 (a), 500 ng/ml insulin (b), or 1 ng/ml PDGF and 10 ng/ml EGF (c). DNA was assayed as described in Experimental. Points are the mean ± SEM of triplicate observations. Hatched bars are the mean ± SEM of DNA level of stimulated controls (vehicle alone).

the mean tumor weight of a group sacrificed at week 0. These tumors were also lighter than those treated with tamoxifen or ICI 182,780 although the uteri weights were not significantly different in the groups

Table 2. Effect of RU 58 668, ICI 182,780 and 4-OH-tamoxifen on MCF-7 cells cultured in medium without phenol red, in the presence of 5% charcoal-stripped FCS

	IC_{50} (nM) on MCF-7 cells stimulated by:		
	E2	Insulin	EGF/PDGF
RU 58 668	0.133 ± 0.084	0.016 ± 0.004	0.021 ± 0.005
ICI 182,780	0.353 ± 0.074[a]	0.050 ± 0.005[b]	0.089 ± 0.029[b]
4-OH-tamoxifen	3.30 ± 0.40[c]	0.93 ± 0.38[c]	0.87 ± 0.24[c]

The values are the mean ± SEM of at least three experiments in each of which the points used to determine the IC_{50}s were the mean of triplicates. [a]NS, [b]$P < 0.05$ and [c]$P < 0.01$, cf. RU 58 668 (Dunnet test).

treated either by RU 58 668 or by ICI 182,780 (Table 4).

Treatment with tamoxifen led, at first, to a regression of the tumor volumes (for 3–4 weeks), followed by a regrowth period, while ICI 182,780 did not show this biphasic response, but led at best to a stabilization of the tumor volume. No tumoral regression could be observed with this latter compound (Fig. 8).

Other antihormonal activities

Progestomimetic and antiprogestomimetic activities in immature female rabbits. After 5 days of estradiol priming (days 0–4), endometrial transformation was not induced by a 4 day s.c. treatment (days 7–10) with 10 mg/kg RU 58 668, showing that the compound had no progestomimetic activity, whereas 1 mg/kg progesterone induced a complete endometrial transformation. When given along with a daily s.c. injection of 1 mg/kg progesterone (days 7–10), RU 58 668 did not inhibit the progesterone-induced endometrial proliferation, showing that the compound had no antiprogesterone activity at this 10 mg/kg dose (Table 5).

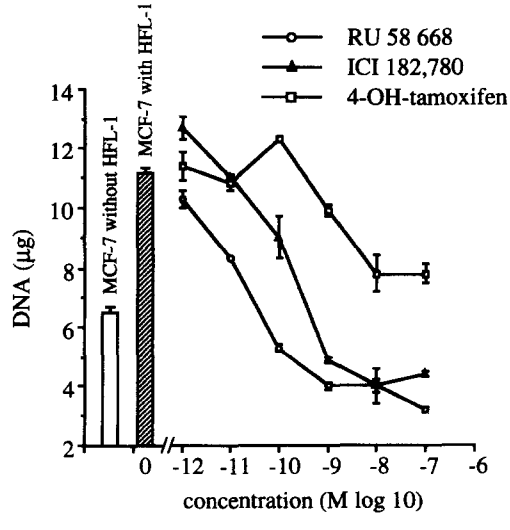


Fig. 3. 50,000 MCF-7 cells were plated in the lower part and 10,000 HFL-1 in the upper part of 24-well dishes (Millicell®) as described in Experimental. The cells were allowed to grow for 7 days in the presence of increasing concentrations of t.c. At the end of the experiment DNA of the lower part was assayed. Each value is the mean ± SEM DNA levels in triplicate wells.

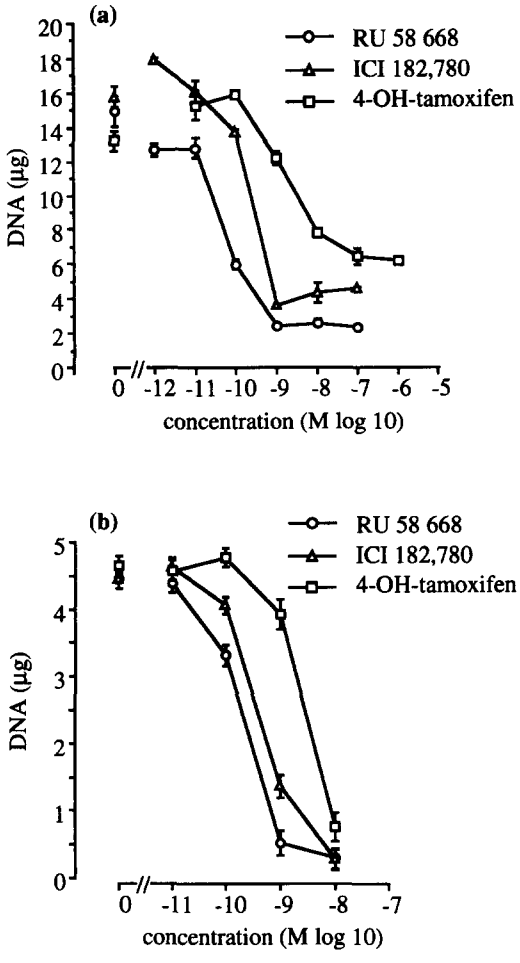


Fig. 4. The growth of the human mammary tumor T47D cell line, cultured in MEM without phenol red in the presence of 5% CSS, was stimulated by 500 ng/ml insulin (a) or 0.1 nM E2 (b). The cells were treated as indicated by RU 58 668, ICI 182,780 or 4-OH-tamoxifen for 8 days and the DNA assayed as described in Experimental. Points are the mean ± SEM DNA levels in triplicate wells.

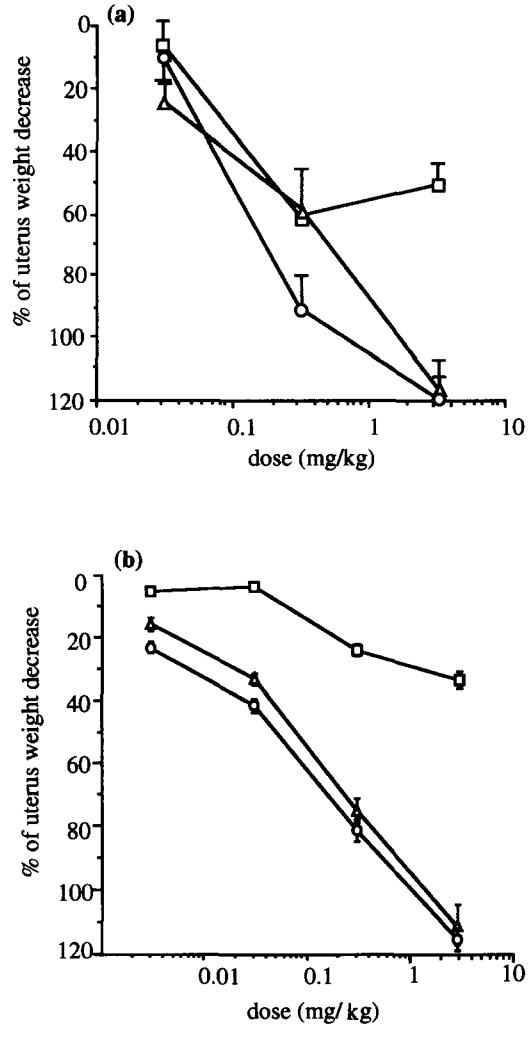


Fig. 5. Antiuterotrophic activities in mice or rats: groups of 5 immature mice (a) or rats (b) received for 3 days a daily s.c. injection of 10 µg/kg E2 alone or with the indicated doses of RU 58 668 ○, ICI 182,780 △ or tamoxifen □. The uteri were weighed 24 h after the last administration.

Thymolysis in rats. As shown in Table 6, 24 h after 4 s.c. administrations of 30 mg/kg RU 58 668, alone or in combination with 50 µg/kg dexamethasone p.o. in male adult rats, RU 58 668 displayed neither glucocorticoid/antiglucocorticoid activity on the thymus weight, nor any effect on the weight of liver or adrenals.

DISCUSSION

It has been suggested that pure antiestrogens could be beneficial in the treatment of advanced ER-positive breast cancer [6]. At the present time, estradiol derivatives substituted at the 7α position by a polar group (amide or sulfoxide), spaced from the steroid nucleus by an alkyl chain have been reported to be fully antiuterotrophic in rodents [2–5]. On the other hand, 11β-substitution of the estradiol molecule by amide groups was also able to provide pure antiestrogens, the spacer being either an alkyl [8] or a phenoxyalkyl chain [9]. In an effort to explore the incidence of the nature of the 11β side chain on the activity profile of 11β-phenoxyalkyl estradiols, we introduced several polyfluoroalkylthio groups. Among the compounds synthesized, RU 58 668 was selected for further evaluation on the basis of its potent inhibition of MCF-7 cell growth in culture.

Table 3. Uterotrophic effect of RU 58 668, ICI 182,780 and tamoxifen in mice

	Uterine weight (mg)
Controls	21.5 ± 2.8
RU 58 668 (30 mg/kg)	9.7 ± 1.1[a]
ICI 182,780 (30 mg/kg)	9.0 ± 1.6[a]
Tamoxifen (3 mg/kg)	53.6 ± 10.8[b]
E2 (10 µg/kg)	65.4 ± 5.6[b]

[a] $P < 0.05$, [b] $P < 0.01$, cf. controls (Dunnet test). Groups of 5 immature mice received three s.c. administrations of the studied compounds or the vehicle alone and were sacrificed 24 h after the last administration. Values are the mean ± SEM of the uteri weights.

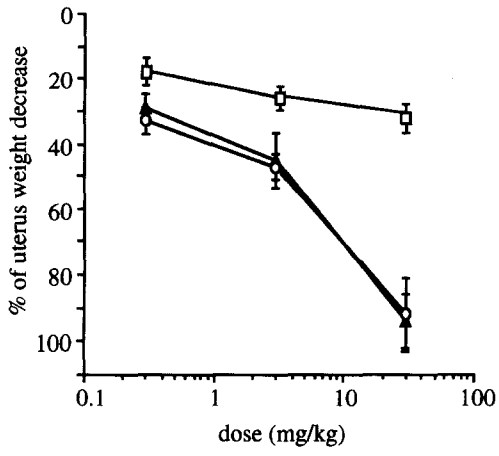


Fig. 6. Antiuterotrophic activity in mice by oral route: groups of 5 immature mice received for 3 days a daily s.c. injection of 10 μg/kg E2 alone or with the indicated doses of RU 58 668 ○, ICI 182,780 ▲ or tamoxifen □ by oral route. The uteri were weighed 24 h after the last administration.

Table 4. After 5 previous weeks of tumor growth the animals were randomized in groups of 10, one group was sacrificed as week 0 control and the other groups were treated as described in Fig. 6 for a further 10 weeks.

	Weights (mg)	
	Tumors	Uteri
Week 0	43 ± 12	115.1 ± 5.1
Week 10		
E2	380 ± 94	117.9 ± 13.4
E2 + tamoxifen	78 ± 21[a]	99.0 ± 18.6
E2 + ICI 182,780	81 ± 17[a]	41.1 ± 4.3[a]
E2 + RU 58 668	23 ± 9[a,b,c]	35.6 ± 1.8[a]

Values are mean ± SEM tumor and uterine weights. Mann and Whitney tests: [a]$P < 0.01$, cf. E2, [b]$P < 0.01$, cf. tamoxifen, [c]$P < 0.05$, cf. week 0 ($n = 10$).

In receptor binding studies (Table 1), RU 58 668 displayed the same RBAs for the ER as 4-OH-tamoxifen, approximately half of that of ICI 182,780. RBAs of the three tested compounds for the human ER were 2 to 3 times lower than those for the mouse ER, reflecting probably the species difference in the sequence of the hormone binding domains [17]. Contrary to ICI 182,780 and 4-OH-tamoxifen which were both selective for ER, RU 58 668 displayed significant affinities for the PR and GR. This result seems to be linked to the presence in RU 58 668 of the 11β-aryl group, as was already observed in structurally related compounds [18 and unpublished observations]. The fact that these affinities do not correlate with any biological activity *in vivo*—absence of progestational (or anti) activity on the female rabbit uterus at doses up to 10 mg/kg (Table 5), and absence of glucocorticoid (or anti) activity demonstrated by the lack of effect on the rat thymus or adrenal weight at doses up to 30 mg/kg s.c. (Table 6)—remains however unexplained, but could possibly be related to the rather modest values of RBAs (as a comparison, the antiprogestin RU 38 486 displays RBAs = 530% of progesterone and 300% of dexamethasone, respectively for PR and GR [18]). Owing to the fact that RU 58 668 is completely antiuterotrophic in rodents at a dose smaller than 3 mg/kg by s.c. route [Fig. 5(a and b)], without any uterotrophic activity at doses up to 30 mg/kg (Table 3), one can consider it as a **pure** antiestrogen, as opposed to a partial agonist. To distinguish a compound which binds only to one receptor, we propose the term **specific** or **selective**. Thus, both ICI 182,780 and RU 58 668 are pure antiestrogens, but the latter compound appears to be less selective than the former regarding the binding profile on steroidal receptors.

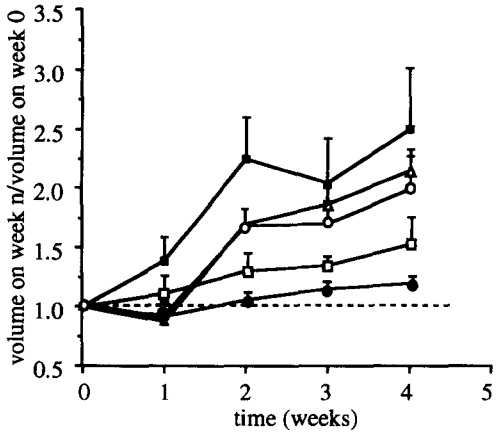


Fig. 7. After 5 weeks of tumor growth stimulation from week −5 to 0, mice were randomized in groups of 10 animals according to their tumor volume. The animals received then a weekly administration of 5 μg E2 by p.c. route alone ■ or along with 5 oral administrations a week of 10 mg/kg tamoxifen □ 10 mg/kg ICI 182,780 △, 10 ○ or 100 ● mg/kg RU 58 668.

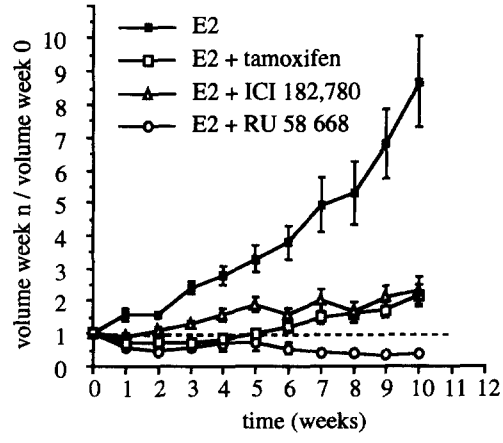


Fig. 8. After 5 weeks of tumor growth stimulation from week −5 to 0, mice were randomized in groups of 10 animals according to their tumor volume. One group was sacrificed in order to evaluate the tumor weight on week 0 and the other groups received on weeks 0 and 5, 250 mg/kg of the studied compounds, or the vehicle alone, along with a weekly administration of 5 μg E2 by p.c. route. The tumor volume was checked every week. At the end of the experiment the tumors and uteri were removed and weighed (c.f. Table 3).

Table 5. Immature female rabbits received 5 s.c. injections of 5 μg/kg E2 (days 0–4), and were treated for 4 days (7–10) by vehicle alone, progesterone 1 mg/kg, RU 58 668 10 mg/kg or both compounds and were sacrificed on day 11

	Uterus weight (mg/g)	Mac Phail index (mini 0, maxi 4)
Controls	0.847 ± 0.061	0
Progesterone	2.891 ± 0.159^a	3.9 ± 0.1^a
RU 58 668	0.769 ± 0.049	0
Progesterone + RU 58 668	2.784 ± 0.140^b	3.7 ± 0.1^b

The uteri were weighed and an histological examination was carried out in order to evaluate the endometrial proliferation, quantified by the Mac Phail index. $^aP < 0.01$, cf. controls, ^{b}NS, cf. progesterone (Dunnet test).

Despite a lower affinity for human (and murine) ER, RU 58 668 was 2.5 times more potent than ICI 182,780 to inhibit the E2-stimulated growth of the MCF-7 cell line, while being devoid of growth stimulatory activity [Fig. 2(a) and Table 2]. We have no explanation for the discrepancy between the affinities and the *in vitro* activities on MCF-7 cells. Possible hypotheses could include differential diffusion into cells or differential conformation of the antihormone–receptor complexes.

In the absence of E2, the MCF-7 or T47D cell proliferation stimulated either by growth factors or by paracrine secretion from a non-tumoral cell line was inhibited by RU 58 668 or ICI 182,780 [Figs 2(b and c), 3 and 4(a)]. Under these conditions, 4-OH-tamoxifen is only a poor growth inhibitor as reported previously by others [5]. This indicates that the pure antiestrogens have intrinsic antiproliferative activities, which are not linked to the presence of E2 and which might be related to the speculative cross-talk between nuclear and membrane receptors [19]. Convincing evidence has been provided recently by Rochefort *et al.* on a model involving induction of pS2 and cathepsin-D mRNA by growth factors [20]. Such models, which could mimic a situation frequently observed in the clinic [21], underline the potential interest of these compounds in the treatment of autocrine or paracrine-stimulated human breast cancer.

The ratio of the IC_{50}s between ICI 182,780 and RU 58 668 was in the range 3 to 10 when the cells, MCF-7 or T47D, were stimulated by growth factors whereas it was only 2 when the same cells were stimulated by E2. In these latter stimulation conditions, the compounds have to compete with E2 at the receptor level so that the higher affinity of ICI 182,780 for the ER, compared to RU 58 668, could possibly explain its relatively higher antiproliferative activity on E2-stimulated cells as compared to a growth factor-stimulated cells. Alternatively a component of RU 58 668 activity mediated by a hypothetical interference with the AP-1 system [22] cannot be excluded at this time. These results could represent further evidence for a possible dissociation between antiestrogenic and antiproliferative activities, as suggested previously [23]. At this time, the comparison of the *in vitro* activities of ICI 182,780 and RU 58 668 on MCF-7 and T47D cell lines gave no evidence about the implication of RU 58 668 binding to PR and GR in its antiproliferative activity.

In order to test its antitumoral activity, RU 58 668 was administered to athymic nude mice bearing established xenografts of MCF-7 tumors. In a first experiment, RU 58 668, ICI 182,780 and tamoxifen were given daily by oral route while the tumoral growth was continuously sustained by weekly applications of E2. RU 58 668 and ICI 182,780 were only able to slow down the tumor evolution whereas tamoxifen inhibited the tumoral growth at 10 mg/kg but, as reported by others [4, 24], no regression was observed. On the other hand, RU 58 668 given orally at 100 mg/kg/day led to a tumoral growth inhibition not significantly different from that induced by 10 mg/kg tamoxifen (Fig. 7). Owing to the suspected poor oral bioavailability of RU 58 668, as demonstrated by the order of magnitude lower potency between the p.o. and s.c. routes of administration in the antiuterotrophic activity, it was administered as the "oil depot" formulation described previously by Wakeling [4]. In these conditions, injections of 250 mg/kg RU 58 668 every 5 weeks caused a long lasting involution of the tumor volumes whereas ICI 182,780 only slowed down the E2-induced tumoral growth, without regression (Fig. 8). Identical treatment with tamoxifen led to an inhibition of the tumoral growth for 4–5 weeks, followed by a regrowth period, as reported by others [25].

The weight of the tumors correlated well with their volumes (Table 4) which confirm the difference in antitumoral activities between the tested compounds. Nevertheless, in RU 58 668 and ICI 182,780-treated animals, the uterine weights were equivalent (Table 4). That shows that the global antiestrogenic activities of both compounds are the same. Therefore the higher antitumoral effect of RU 58 668 compared to ICI

Table 6. Groups of 6 male adult rats received 4 oral administrations of 50 μg/kg dexamethasone, 4 s.c. administrations of 30 mg/kg RU 58 668 or the association

	Body wt day 0 (g)	Body wt day 4 (g)	Thymus wt (g/100 g)	Liver wt (g/100 g)	Adrenal wt (g/100 g)
Controls	99 ± 2	129 ± 3	0.39 ± 0.02	5.03 ± 0.12	18.3 ± 0.7
Dexa	96 ± 1	112 ± 2^a	$0.13 = 0.01^a$	4.73 ± 0.13^b	13.5 ± 0.9^a
RU 58 668	97 ± 2	123 ± 4^b	0.42 ± 0.04^b	5.01 ± 0.16^b	17.1 ± 0.4^b
Dexa + RU 58 668	99 ± 1	115 ± 2^c	0.14 ± 0.02^c	5.06 ± 0.18^c	12.9 ± 0.8^c

24 h after the last administrations, the animals were sacrificed and above mentioned organs weighed. $^aP < 0.01$, cf. controls, ^{b}NS, cf. controls, ^{c}NS, cf. dexamethasone (Dunnet test).

182,780 cannot be explained by a higher antiestrogenic activity. Before concluding that this result lends support to the hypothesis of dissociation between the antiproliferative/antitumoral and the antiestrogenic activities, it should be checked if the difference does not arise from an inequal tissue distribution.

The escape of the tumors observed with tamoxifen, could be explained, at least, by its partial estrogenic activity, but could also result from an evolution of the tumors whose growth could become less dependent from E2 or by the fact that tamoxifen is poorly effective when tumoral progression is due to autocrine or paracrine stimuli.

In summary RU 58 668 is a pure, non-specific antiestrogen without agonistic activity *in vivo*. It displays potent antiproliferative activity on the MCF-7 human mammary cancer cell line stimulated either by exogenous growth factors or by paracrine interaction with non tumoral cells, models in which tamoxifen is poorly active. It also inhibits the growth of the PR-rich T47D cell line. *In vivo*, RU 58 668 is the only antiestrogenic compound known so far to induce long term regression of human mammary tumors implanted in nude mice. Unlike tamoxifen, no escape from the treatment was observed for at least 10 weeks, suggesting that RU 58 668 is a good candidate for the treatment of advanced breast cancer, especially in the case of autocrine or paracrine stimulation.

Further studies are under way in order to understand the difference in antitumoral activities observed between RU 58 668 and ICI 182,780 and to investigate the efficacy of RU 58 668 over a longer period of time, and its efficiency on the escape from tamoxifen treatment or on models of tamoxifen-resistant tumors *in vivo*.

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