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Report

Differential effects of raloxifene, tamoxifen and fulvestrant on a murine mammary carcinoma

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Summary

The purpose of this study was to evaluate the effect of the selective estrogen receptor modulators raloxifene and tamoxifen and of the pure antiestrogen fulvestrant on tumor growth and progesterone receptor (PR) expression in an experimental model of breast cancer. The effects of these compounds on cell proliferation were studied in primary cultures of a progestin-dependent mammary carcinoma tumor line, in the presence of medroxyprogesterone acetate (MPA) or 17-β-estradiol (E_2). In *in vivo* studies the tumor was inoculated subcutaneously in BALB/c female mice treated with 20 mg MPA depot. Raloxifene (12.5 mg/kg) or tamoxifen (5 mg/kg) were administered in daily doses or E_2 silastic pellets (5 mg) were implanted. When the tumors reached about 25–50 mm^2 MPA was removed in half of the animals. E_2 induced complete tumor regressions, tamoxifen inhibited tumor growth *in vivo* while raloxifene disclosed proliferative effects in animals in which MPA had been removed. *In vitro*, E_2 inhibited cell proliferation at concentrations higher than 10^{-14} M. Raloxifene and fulvestrant, but not tamoxifen, partially reverted E_2-induced inhibition. Fulvestrant and tamoxifen inhibited MPA-induced cell proliferation while raloxifene had a stimulatory effect. Tamoxifen and E_2 increased, raloxifene induced no effect, and fulvestrant significantly decreased PR expression. In this study we provide evidence for differential effects of tamoxifen and raloxifene on experimental mammary tumors. Since raloxifene is under evaluation for use in breast cancer prevention, these results may have important clinical implications.

Introduction

Steroid hormone antagonists such as the antiestrogen tamoxifen or the antiprogestin mifepristone, are synthetic pharmaceutical agents which suppress the activity of natural steroidal agonists such as 17-β-estradiol (E_2), progesterone (Pg), or glucocorticoids [1]. The ability of antagonists to suppress the transcriptional effects of agonists has important clinical value [2]. In some tissues and tumors steroid antagonists can have agonist-like effects [3, 4]. The precise mechanisms by which antagonists inhibit transcription under some conditions, but stimulate it in others, are unknown [1]. All the different estrogen receptor (ER) ligands including tamoxifen, are now been generically named SERMs (Selective Estrogen Receptor Modulator) in order to include substances that, although binding ER, may have agonist or antagonist properties in different tissues, by differentially modulating receptor structure and thus conferring distinct changes in receptor function [2].

Tamoxifen has been for years the endocrine treatment of choice in metastatic breast cancer and it is also used as adjuvant chemotherapy [5, 6]. The rationale for its use is the fact that estrogen is a mitogen in ER-expressing breast cancer cells and its activity can be inhibited by tamoxifen [7, 8]. From the clinical point of view, two features set it apart from other chemotherapeutic agents; it is the only single agent that confers a survival advantage with almost no side

effects except for its uterotrophic actions, and its administration is associated with a diminished incidence of contralateral cancer. Because estrogens are also necessary for the preservation of skeletal integrity it was thought that the administration of tamoxifen to breast cancer patients might also cause osteoporosis. Surprisingly, tamoxifen has shown estrogenic effects in bone, leading to an increase in mineral density [9]. These observations led to the idea that an ideal compound to be developed should associate antiestrogenic actions on both mammary gland and uterus as well as estrogenic effects on bone and cardiovascular systems.

Raloxifene, also known as keoxifene (LY 156758), is a benzothiophene-derived antiestrogen with high affinity for ER (almost three times greater than E_2 in rat uterus) and exerts minimal uterotrophic activity [10]. First conceived as a drug that could maintain bone density and lower circulating levels of cholesterol, it lacks the potentially harmful uterotrophic activity of tamoxifen; it has also been demonstrated that it can inhibit experimental mammary tumor growth [11]. This compound is currently used in the treatment of osteoporosis. A tamoxifen and raloxifene trial (STAR) is currently comparing the ability of these SERMs to reduce breast cancer incidence in high-risk postmenopausal women [2]. In tamoxifen-refractory breast cancer it had no effect [12].

Fulvestrant is a compound with significantly pure estrogen antagonist activity with a relative binding affinity of 0.89 as compared to that of E_2 in rat uterus. This compound has demonstrated *in vitro* and *in vivo* growth-inhibitory effects, a single injection providing antitumor efficacy equivalent to that of daily tamoxifen treatment for at least 4 weeks [13]. At the present time it is being studied versus the non-steroidal aromatase inhibitor anastrazole and tamoxifen in two separate phase III trials, as part of a large clinical program involving postmenopausal women with advanced breast cancer [14].

Tamoxifen also prevents rat mammary carcinogenesis induced by dimethylbenzanthracene, N-nitrosomethylurea and ionizing radiation [15–17], and long-term treatment prevents spontaneous carcinogenesis in C3H/OUJ mice infected with mouse mammary tumor virus [18, 19]. Raloxifene on the other hand, also inhibits dimethylbenzanthracene and N-nitrosomethylurea-induced tumors in rats [11, 20] although it seems to be less effective than tamoxifen because of its shorter biological half-life [10].

We have developed an experimental mammary tumor model in which the continuous administration of medroxyprogesterone acetate (MPA) to female BALB/c mice leads to the development of ductal mammary metastatic carcinomas which express ER and progesterone receptors (PR) [21, 22]. These tumors are maintained by syngeneic transplants in progestin-treated female mice (progestin-dependent tumor lines) or in untreated mice (progestin-independent tumor lines) [23]. In previous papers we have demonstrated that estrogen [24] or antiprogestin [25] administration induced tumor regression in both types of tumors. The object of this paper is to evaluate the role of two different SERMs (tamoxifen and raloxifene) and a pure antiestrogen (fulvestrant) in our experimental mammary tumor model.

Materials and methods

In vitro *studies*

Hormones. MPA, E_2 and tamoxifen were obtained from Sigma Chem. Co., St Louis MO, USA; tamoxifen citrate and raloxifene from Gador Laboratory and Rontag Laboratory, Buenos Aires, respectively and fulvestrant was a kind gift from Zeneca Pharmaceuticals, England. Hormones and antihormones were dissolved in absolute ethanol at 10^{-3} M (stock solution). Fresh working solutions were prepared before each experiment.

Steroid-stripped FCS (ssFCS). Activated charcoal (Mallinckrodt Chemical Works, New York, NY, USA) was added to fetal calf serum (FCS) (Life Technologies Inc., Gaithersburg, MD, USA or Gen Sociedad Anónima, Buenos Aires, Argentina) to a final concentration of 0.05 g/ml. The steroid extraction was carried out at 4°C overnight. Charcoal was removed by five consecutive centrifugations at 10,000 rpm for 15 min. The procedure was repeated twice, the second time for 3 h, to increase the efficiency of the stripping.

Culture media. DMEM/F12 (Dulbecco's modified Eagle's medium: Ham's F12, 1:1, without phenol red, Sigma Chem. Co.), 100 U/ml penicillin and 100 μg/ml streptomycin.

Washing medium: DMEM/F12 + 5% FCS.

Standard medium: DMEM/F12 + 5% ssFCS.

Primary cultures. C4-HD tumors are ductal mammary carcinomas expressing ER and PR which are routinely maintained by syngeneic transplantation in

MPA-treated mice [26, 27]. Hormone dependency is checked for each passage. Tumors were aseptically removed, minced, washed with DMEM/F12, suspended in 5 ml of enzymatic solution (trypsin [Life Technologies Inc.]: 2.5 mg/ml, albumin [Life Technologies Inc.]: 5 mg/ml and collagenase type II [Life Technologies Inc.]: 850 U/ml in phosphate buffered saline) and incubated at 37°C for 20 min under continuous stirring. The liquid phase of the suspension was then removed and the undigested tissue was incubated for an additional 20 min with fresh enzymatic solution. Enzyme action was interrupted by adding washing medium. Epithelial and fibroblastic cells were separated as previously described [28]. Briefly, the cells were resuspended in 20 ml of washing medium and allowed to sediment for 20 min. The upper 15 ml were discarded, the sedimented cells were resuspended in other 20 ml of washing medium and allowed to sediment for 20 min; the procedure was repeated 10 times. The cells were plated in culture flasks with standard medium and allowed to attach for 24–48 h. The medium was then removed and replaced by fresh medium with 10^{-8} M MPA and was thereafter changed every 2–3 days. At confluence, or when cell clusters looked overcrowded, the cells were detached with 0.25% trypsin, washed, resuspended in fresh standard medium, and plated again.

Cell proliferation

^{3}H-Thymidine uptake assay. In a Corning 96-well microplate, 0.1 ml/well of a cell suspension were seeded in standard medium at a concentration of 10^5 cell/ml. After attachment (24 h), the cells were incubated for 48 h with the experimental solutions to be tested (in 2.5% ssFCS). Fifty percent of the medium was replaced with fresh medium every 24 h. The cells were incubated with 0.4 μCi of ^{3}H-thymidine (NEN, Boston, MA) for 24 h, trypsinized and harvested in a cell harvester. Filters were counted in a liquid scintillation counter. The assays were performed in octuplicates. Mean and standard deviation were calculated for each solution tested.

Cell counting. To corroborate that decreases in ^{3}H-thymidine uptake correlated with decreases in cell number, C4-HD epithelial cells were seeded in 24-well microplates and incubated in DMEM/F12 without phenol red, in the presence of 2.5% ssFCS plus MPA 10 nM and E_2 or the antihormones for 7 days. Cell number was assessed with a Neubauer chamber slide. The assay was performed in quadruplicates.

In vivo *experiments*

All experiments were carried out using 2-month-old, non-ovariectomized virgin female BALB/c mice (National Academy of Medicine, Buenos Aires, Argentina), housed six per cage in air-conditioned rooms at 20 ± 2°C, kept under an automatic 12 h light/12 h darkness schedule and given pellets and tap water *ad libitum*. Animal care was in accordance with institutional guidelines. Mice (6 per group) treated with 20 mg MPA depot (Medrosterona, Laboratorios Gador, Buenos Aires, Argentina) subcutaneously (sc), were inoculated by trocar with C4-HD tumors in the contralateral flank. C4-HD is a progestin-dependent tumor line which does not grow in ovariectomized animals. When tumors reached a size of about 25–50 mm^2 treatments were started. Daily doses of raloxifene (12.5 mg/kg) or tamoxifen (5 mg/kg) were administered sc in aqueous solution. In the experiments in which we wished to evaluate tumor growth in the absence of MPA, the hormone depot was removed and 5 days later the daily inoculations of tamoxifen and raloxifene treatments were started. Tumors were measured with a Vernier caliper three times a week. Animals were sacrificed when tumor sizes were higher than 400 mm^2. The experiments were repeated three times.

Hormone receptor studies

Reagents. [^{3}H]-E_2 and diethylstilbestrol (DES) were purchased from NEN, Boston MA; dithiothreitol, EDTA, dihydrotestosterone and Na_2MoO_4 were purchased from Sigma, Chem. Co. Tris was from Gibco, BRL, New York and glycerol from Raudo, Argentina.

Preparation of total cellular extracts. Tissues and tumors were homogenized in a polytron at setting 50 with three bursts of 5 s in a 1 (tissue):4 TEDG proportion. The buffer was 20 mM Tris–HCl pH 7.4, 1.5 mM EDTA, 0.25 mM dithiothreitol, 20 mM Na_2MoO_4, 10% glycerol. The homogenate was centrifuged at 12,000 rpm, 30 min at 4°C. The supernatant was immediately used in the ligand binding assays. Protein concentration was determined according to Lowry [29].

ER binding assays. Binding of ER to [^{3}H]-E_2 was performed on the total cellular extract. ER were

evaluated by incubating duplicate aliquots of the extracts with 3 nM [^{3}H]-E_2 and displaced with DES, tamoxifen, raloxifene or fulvestrant in concentrations ranging from 20,000 to 0.23 nM. The reaction proceeded for 18 h. A 100-fold excess of unlabeled dihydrotestosterone was used to block unspecific binding. Free and bound hormones were separated by the charcoal–dextran method: charcoal–dextran solution was added (TEDG, 1% charcoal activated, 0.1% dextran) and after 10 min, the samples were centrifuged at 3,500 rpm, 10 min, at 4°C. The supernatant was counted in a β-counter. Ki was calculated according to the Cheng and Prusoff equation using Graph Pad Prism 3.0 (GraphPad Software, Inc, San Diego, CA). The results are expressed as cpm specifically bound as compared with untreated controls.

PR binding, whole cell assay. 10^5 cells were plated in 24 well plates with complete medium. After 3 days with the experimental solutions, whole cell PR assays were performed as previously described [28]. Briefly, a total of 300,000 cpm of 17-α-methyl ^{3}H-R5020 (NEN, Boston, MA, specific activity: 85 Ci/mmol) were added together with a 100-fold excess of R5020 or ethanol. After 2 h of incubation, the cells were washed, trypsinized and counted in a liquid scintillation counter. A significant difference between the experimental groups, those incubated only with radioactive hormone, and those incubated with radioactive plus unlabeled hormone, yields the total cpm bound to the receptors.

Statistical analysis

ANOVA followed by Tukey *t* test was used to analyze the differences between control and experimental groups in ^{3}H-thymidine uptake assays, in receptor studies and in tumor sizes. Tumor growth curves were also compared using linear regression analysis.

Results

Effects of E_2, tamoxifen, raloxifene and fulvestrant on primary cultures of C4-HD epithelial cells in the absence of MPA or Pg

C4-HD cells were cultured in the presence of 2.5% ss-FCS and increasing concentrations of the compounds for 48 h. As previously reported [28], E_2 was able to induce a significant inhibition of ^{3}H-thymidine uptake ($p < 0.001$) starting at 10^{-14} M with a maximum at nM concentrations. Under similar experimental conditions, raloxifene ($p < 0.001$) and tamoxifen ($p < 0.01$) significantly inhibited in concentrations starting at 10^{-8} M and fulvestrant at 10^{-9} M ($p < 0.001$) (Figure 1).

Effects of E_2, tamoxifen, raloxifene and fulvestrant on primary cultures of C4-HD epithelial cells in the presence of MPA or Pg

Pg or MPA at 10 nM were used in these experiments because this concentration is near the PR's Kd [25] and it is within physiological ranges. E_2 inhibited progestin-induced ^{3}H-thymidine uptake even at very low concentrations (10^{-13} M), and fulvestrant had similar effects at 10^{-8} M. Tamoxifen inhibited ^{3}H-thymidine uptake starting at 10^{-9} M but this inhibition was not as strong as that induced by E_2 (tamoxifen 100 nM: $22.61 \pm 5.4\%$; E_2 100 nM: $64.77 \pm 31.3\%$, $p < 0.05$, $n = 3$). Raloxifene, on the other hand, was stimulatory over MPA control values, even at nM concentrations ($p < 0.001$) (Figure 2). Similar results were obtained when Pg was used instead of MPA (not shown). Variations in progestin stimulation represent the heterogeneity typical of primary cultures.

^{3}H-thymidine uptake results were correlated with cell numbers in experiments in which MPA-treated cells were incubated with the addition of E_2 (10 nM), fulvestrant (100 nM), tamoxifen (100 nM) and raloxifene (10 nM) for 7 days. E_2 and fulvestrant significantly decreased cell number (Figure 3). The inhibitory effect of tamoxifen was further confirmed in other experiments: MPA (10 nM): $15.10 \pm 1.56 \times 10^4$ cells, MPA (10 nM) + tamoxifen (100 nM): $7.00 \pm 1.45 \times 10^4$ cells ($p < 0.05$). Raloxifene, on the other hand, significantly increased cell number over MPA control ($p < 0.01$) (Figure 3).

Effects of tamoxifen, raloxifene and fulvestrant on primary cultures of C4-HD epithelial cells in the presence of E_2

C4-HD cells were cultured in the presence of 1 nM E_2 and increasing concentrations of tamoxifen, fulvestrant and raloxifene to evaluate the ability of these compounds to revert the estrogenic inhibitory effect. Tamoxifen had no effect at any of the concentrations tested. Fulvestrant ($p < 0.001$) and raloxifene ($p < 0.01$) partially reverted E_2-induced inhibition at

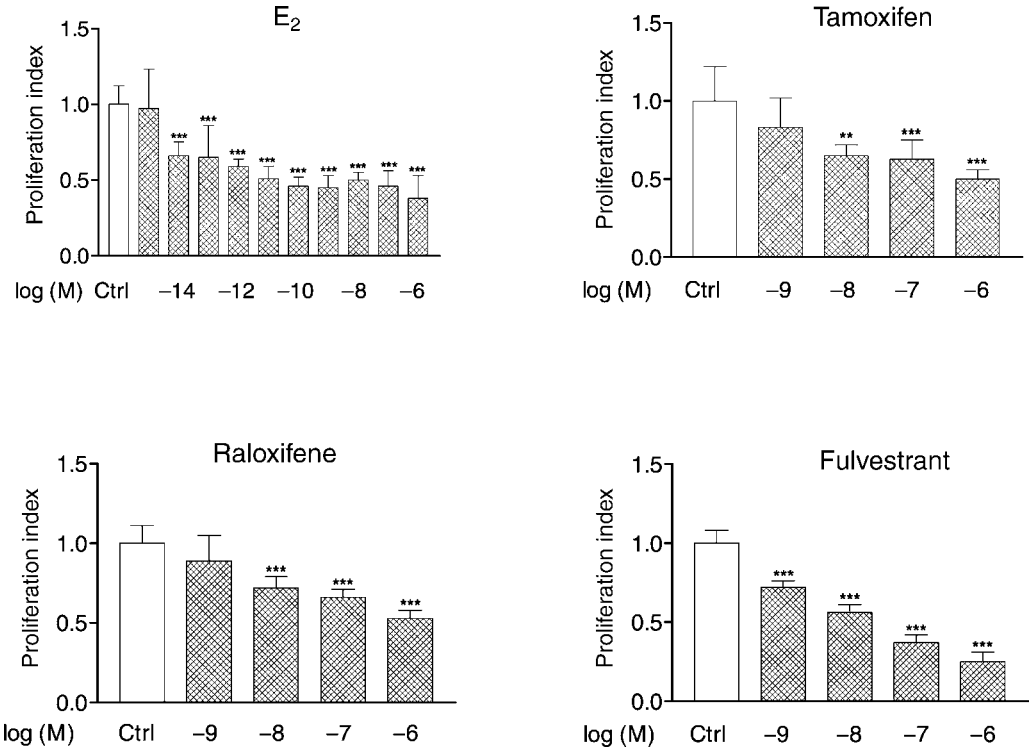

Figure 1. Effect of E_2, tamoxifen, raloxifene and fulvestrant on ^{3}H-thymidine uptake. C4-HD epithelial cells were incubated in DMEM/F12 without phenol red, in the presence of 2.5% ssFCS and E_2, tamoxifen, raloxifene or fulvestrant for 48 h. ^{3}H-thymidine was added in the last 18 h. These are representative experiments of at least three, with each value corresponding to the mean ± SD cpm of octuplicates. ($^{**}p < 0.01$; $^{***}p < 0.001$ between experimental and control groups). Proliferation index: experimental cpm/ control cpm. Ctrl.: control.

10 nM, and beyond E_2 control values at higher concentrations (Figure 4). A concentration of E_2 1 nM was used because (a) it is near the ER's Kd [22], (b) it is within physiological ranges [30] and (c) it exerts the same inhibitory effect as higher concentrations.

Effects of E_2, tamoxifen, raloxifene and fulvestrant and PR expression

Since E_2, in addition to regulating cell proliferation, induces the synthesis of PR, we evaluated the effects of the three compounds on PR induction. Both E_2 (1 nM) and tamoxifen (100 nM) increased PR binding ($p < 0.001$), the former at higher values. Fulvestrant (100 nM) strongly decreased PR ($p < 0.001$) whereas raloxifene (100 nM) exerted no significant effect (Figure 5).

The experiments were repeated using E_2 10^{-12} M and tamoxifen 10^{-10} M. Even at this concentration E_2 increased PR expression (57% beyond control values; $p < 0.01$) while tamoxifen had no effect (data not shown).

Effects of estrogen, tamoxifen and raloxifene on in vivo *tumor growth*

Tumors from tamoxifen-treated animals were significantly smaller in size and weight than those of controls or of raloxifene-treated mice, in both MPA-treated animals and in those in which MPA had been removed (Figure 6). Linear regression curve analysis also revealed significantly different tumor growth rates when comparing these groups ($p < 0.001$). In raloxifene-treated mice the increases in tumor size or weight in animals in which MPA had been removed was not statistically significant. However, comparison between growth slopes in control and raloxifene-treated animals, showed that raloxifene increased tumor growth rate ($p < 0.01$). In previous experiments, E_2 and antiprogestins had been able to induce complete tumor regressions [24].

A group of animals was treated with E_2, 5 mg sc pellet, to confirm tumor sensitivity for this passage.

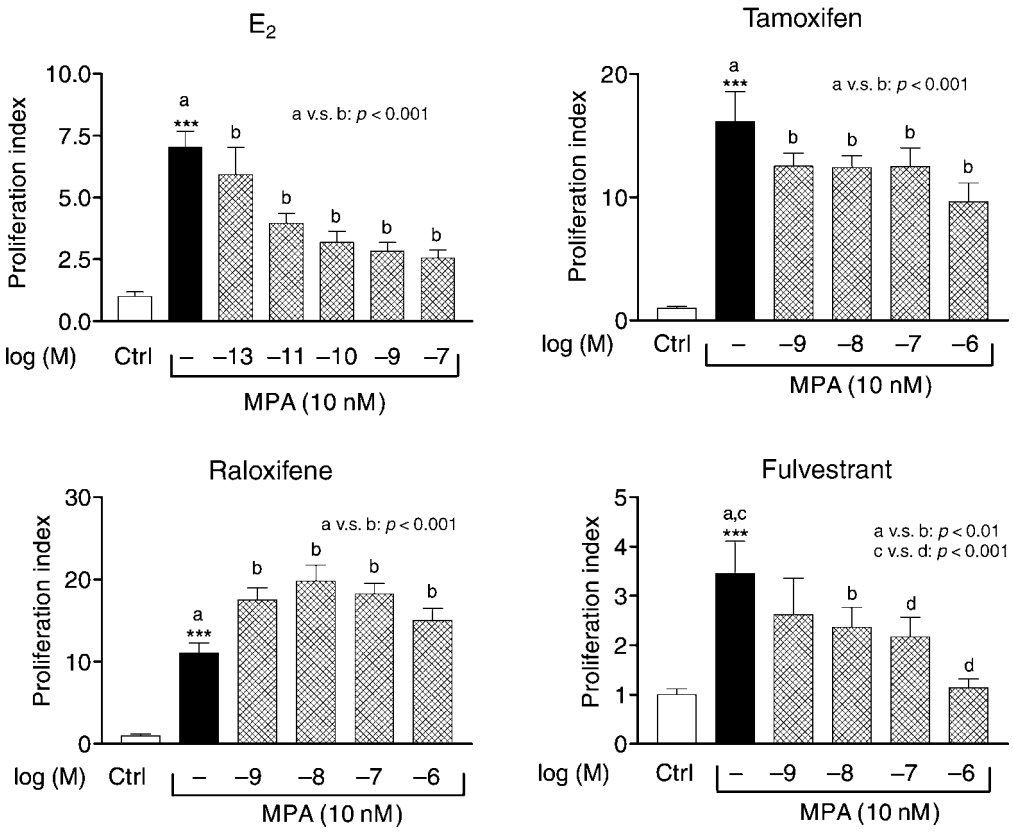

Figure 2. Effect of E_2, tamoxifen, raloxifene and fulvestrant on MPA-induced ^{3}H-thymidine uptake. C4-HD epithelial cells were incubated in DMEM/F12 without phenol red, in the presence of 2.5% ssFCS plus MPA 10 nM and E_2 or the antihormones for 48 h. ^{3}H-thymidine was added in the last 18 h. These are representative experiments of at least three, with each value corresponding to the mean ± SD cpm of octuplicates. (***$p < 0.001$ between MPA-treated and control group). Proliferation index: experimental cpm/control cpm. Ctrl.: control.

Raloxifene and tamoxifen in competitive binding assays

Aliquots of total C4-HD cell extract were incubated with 4 nM [^{3}H]-E_2 for 3 h either alone or with 0.01 to 20,000 nM concentrations of DES, raloxifene and tamoxifen. In all cases a 100 fold excess of dihydrotestosterone was added to block non-specific binding. Tamoxifen showed much less affinity for ER than raloxifene: the relative binding affinity, defined as Ki of SERM/Ki of DES, was 0.51 ± 0.32 ($n = 3$) for raloxifene and 41.92 ± 10.87 ($n = 3$) for tamoxifen (data not shown).

Discussion

In this paper we report on the differential effects of two currently used SERMs, tamoxifen and raloxifene, and a pure antiestrogen, fulvestrant, on experimental mammary tumors, in terms of agonism/antagonism. These metastatic ductal carcinomas originated in MPA-treated mice [21]. They are ER(+), PR(+) [22] and are maintained by serial syngeneic transplantation. The fact that their growth is stimulated by progestins and inhibited by estrogens [24] makes this model specially suited to evaluate the effect of SERMs under different experimental conditions. Current dogma is that estrogens drive proliferation in human breast cancer although most of the evidence is indirect. However, there is increasing evidence that Pg may play a proliferative role not only in animal models but also in humans. Going et al. [31] demonstrated that in normal mammary epithelia the higher mitotic index is observed during the luteal phase. Direct evidence that progestins may actually be harmful in terms of breast cancer risk was first suggested by Bergkvist et al. [32] in a cohort study of Swedish women receiving a combination hormone replacement therapy for more than 6 years yielding a 4.4 fold increase of breast cancer.

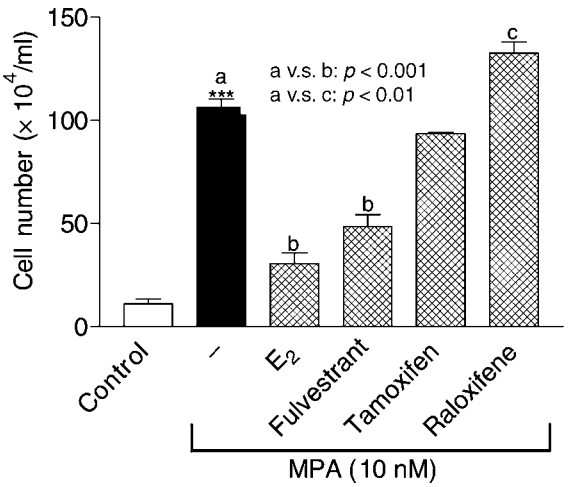

Figure 3. Effect of E_2, tamoxifen, raloxifene and fulvestrant on MPA-induced cell proliferation. C4-HD epithelial cells were incubated in DMEM/F12 without phenol red, in the presence of 2.5% ssFCS plus MPA 10 nM and E_2 or the antihormones for 7 days. Cell number was assessed with a Neubauer chamber slide. This is a representative experiment of two, with each value corresponding to the mean ± SE cell number of quadruplicates. (***$p < 0.001$ between MPA-treated and control group).

More recently, in a case-control study based on interviews of 2653 breast cancer patients and 2429 control subjects, it was shown that while progestins added to hormone replacement therapy successfully decreased the incidence of endometrial cancer, they increased that of breast cancer [33].

Estrogens, on the other hand, have also been associated with inhibitory effects in human breast cancer. They have been used as successfully as tamoxifen in the treatment of breast cancer but they were replaced due to their higher side effects [34]. It has been reported that human breast tumors transplanted in nude mice [35] and human breast cell lines overexpressing PKCα [36] may regress in the presence of E_2. Thus, our experimental model may be representing a particular subset of human breast cancers.

In vitro, E_2 elicited a significant growth inhibitory effect, even at concentrations much lower than the Kd of the ER for these tumors [5×10^{-9} M [25]],

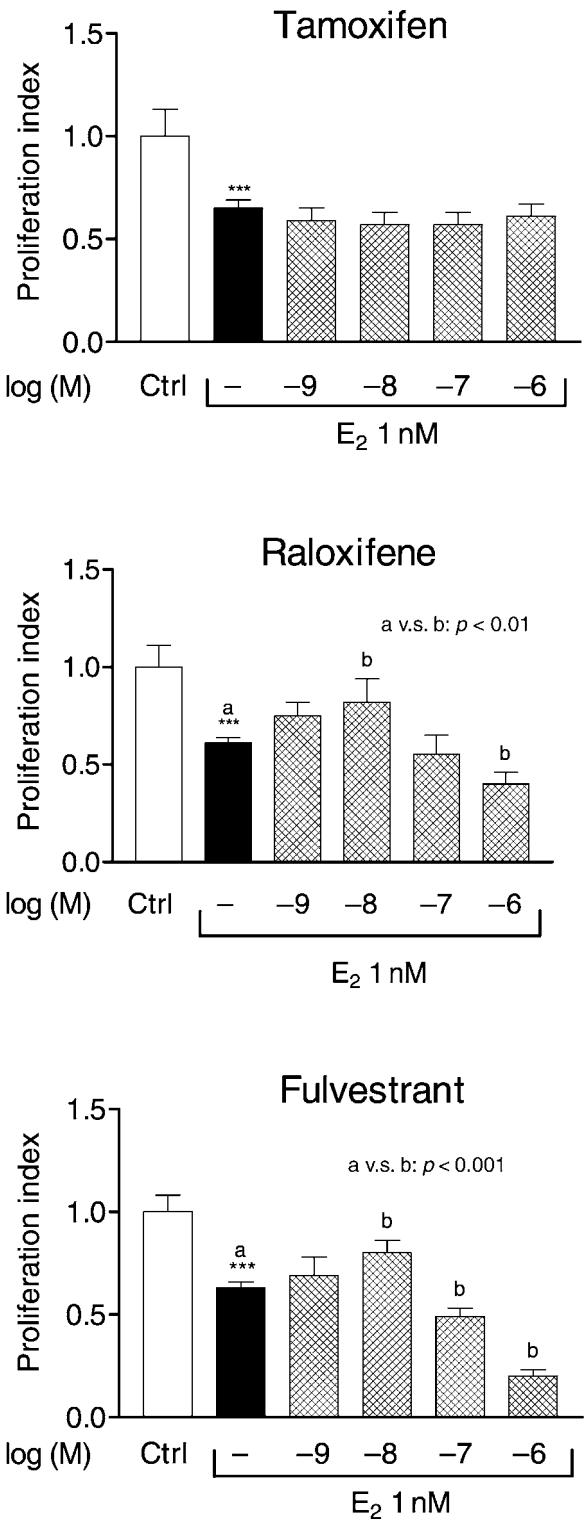

Figure 4. Ability of tamoxifen, raloxifene and fulvestrant to revert E_2-induced cell inhibition. C4-HD epithelial cells were incubated in DMEM/F12 without phenol red, in the presence of 2.5% ssFCS and E_2 1 nM and the different antihormones for 48 h. ^{3}H-thymidine was added in the last 18 h. These are representative experiments of at least three, with each value corresponding to the mean ± SD cpm of octuplicates. (***$p < 0.001$ between E_2-treated and control group). Proliferation index: experimental cpm/control cpm. Ctrl.: control.

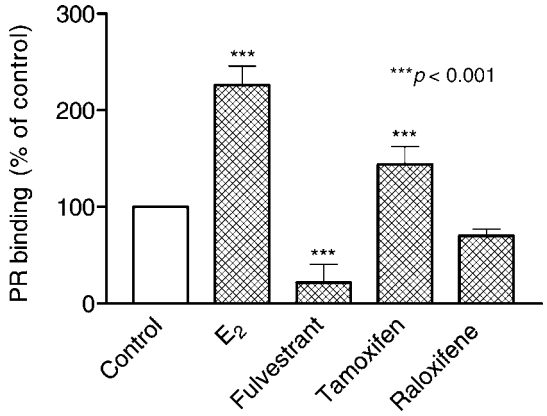

Figure 5. PR binding was evaluated using the whole cell binding method in primary cultures of C4-HD incubated with E_2 (1 nM), fulvestrant (100 nM), tamoxifen (100 nM) or raloxifene (100 nM). Briefly, cells were incubated in the presence of ^{3}H-R5020 (300,000 cpm/well) and ethanol or 100X of unlabelled R5020, washed and counted in a scintillation β-counter. The difference between the total binding and the non-specific binding for control group was considered as 100%. A representative experiment of three is shown.

suggesting that mechanisms other than an interaction with the classical ER may be responsible [37]. Hypersensitivity to E_2 have already been described in different experimental settings in which cells respond to concentrations as low as 10^{-15} M [38–40]. Alternatively, the low growth rate of certain carcinomas in postmenopausal women could be explained by the presence of low estrogen concentrations. The superfamily of the nuclear/steroid receptors seems to operate across a wide range of ligand concentrations, successively triggering effects related with proliferation, differentiation and hormone metabolism [39, 41].

The effect of estrogens entail binding to ER and genomic actions through ERE (estrogen responsive element) or AP1 sites [42]. However, ER activation through pathways unrelated to the classical ligand/nuclear receptor interaction have also been described, and interpreted as evidence of the involvement of a putative membrane ER, the nature of which remains still unclear. Some authors suggest that these are the same or similar nuclear receptors [37] while others point towards different membrane receptors [43]. We have explored a wide range of E_2 concentrations, in relation with proliferation and PR expression (differentiation). Both effects were induced at the same low concentration.

Tamoxifen had an estrogenic agonistic effect in cell proliferation and also in PR induction, as previously demonstrated by others [44], but it is less efficient than E_2 in inhibiting cell proliferation. Raloxifene behaved as an ER antagonist, reverting E_2-induced inhibition, although at higher concentrations it exerted an inhibitory effect. It also slightly potentiated the stimulatory effect of progestins as measured by ^{3}H-thymidine uptake and cell proliferation but it did not significantly modify PR expression. Fulvestrant partially reverted E_2 inhibitory effect, and it consistently downregulated PR expression. Since in this experimental model, PR are the key proteins regulating hormone-dependent and independent cell proliferation [45], the inhibitory effect of fulvestrant on MPA-induced proliferation may be explained by the absence of appropriate amounts of PR. Another possible explanation is that fulvestrant is acting as an antiprogestin, as previously suggested by Hyder [46].

ER affinity in ours and other mouse models are within the 1–5 nM range [25, 47]. While growth inhibitory effects of E_2 may be difficult to interpret, since 1 per 1000 of occupancy are enough to obtain the maximal effect, the actions of raloxifene, tamoxifen or fulvestrant were evident at concentrations close to the ER Kd and similar to those described by others [10, 13, 48]. The results obtained with raloxifene or fulvestrant in the presence of E_2 are in agreement with what would be expected for the classical estrogen/nuclear receptor interaction. In the phenomenon of 'mutual annihilation' of action, as described by Kaye et al. [49], concomitantly with the inhibitory effects of E_2 by a SERM, E_2 also inhibits the stimulatory activity of the SERM. Both compounds may interact forming not only active homodimers, but also heterodimers of estrogen and tamoxifen-linked receptor monomers, depending on the molar ratio of the ligands and their relative affinities. The resulting heterodimer conformation may fail to develop the proper specific interactions with the different co-activators and co-repressors, and these changes may explain the mutual annihilation, agonistic phenomena and their cell selectivity. In our experimental setting, in the presence of E_2, E_2-SERM heterodimers would block E_2-mediated effects. Increasing concentrations of SERMs would favor the formation of homodimers SERM–SERM that would eventually induce the same effect as in experiments in which the compound is added in the absence of E_2.

In our *in vivo* studies, tamoxifen showed a clear inhibitory effect, although it did not induce complete tumor regressions such as those observed with E_2 or with antiprogestins [25] within the study time frame.

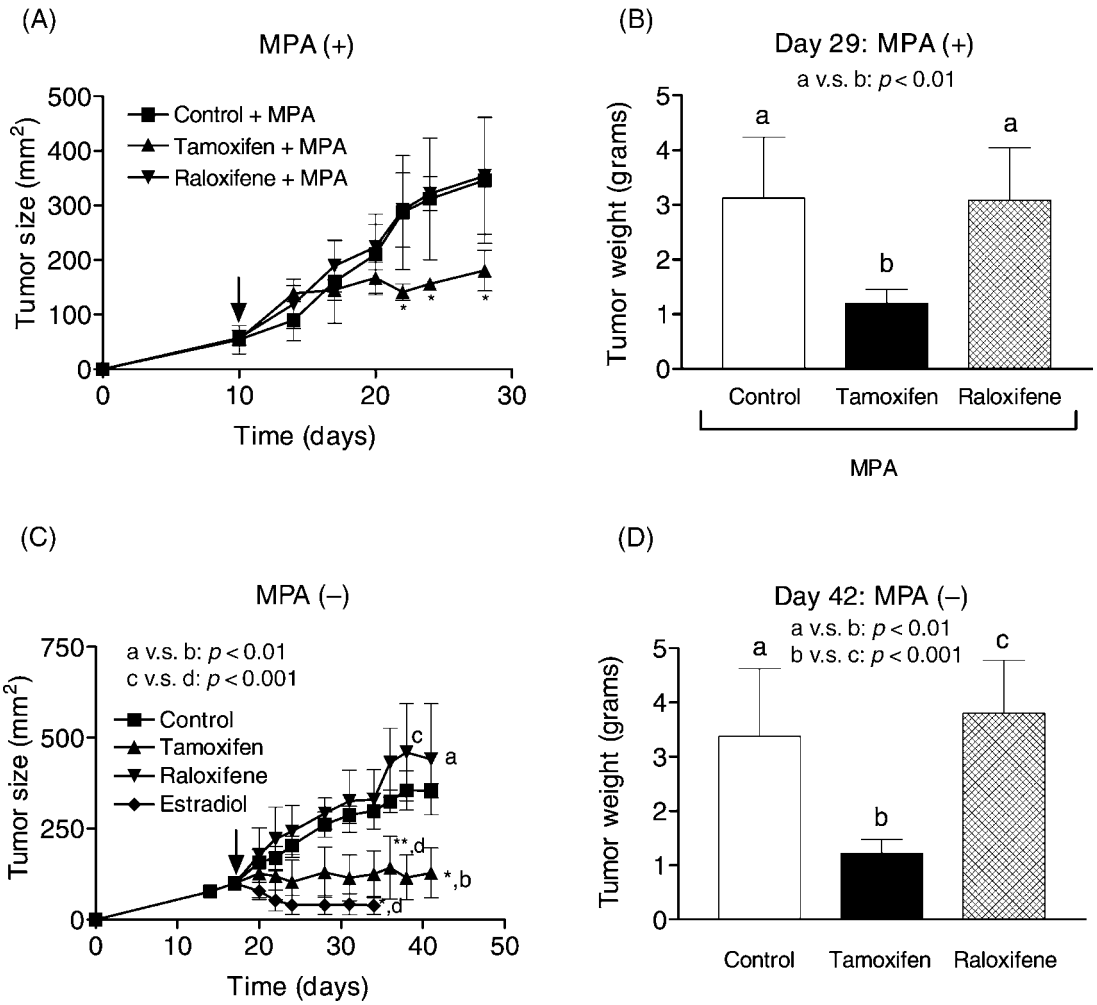

Figure 6. Effects of tamoxifen or raloxifene on *in vivo* growth of C4-HD. Tumors were transplanted into virgin female BALB/c mice treated with MPA. When tumors reached approximately 50 mm^2 MPA was removed in half of the animals. After 1 week the mice were administered daily sc injections of tamoxifen (5 mg/kg), raloxifene (12.5 mg/kg) or saline, for approximately 20 days. At the end of the experiment tumors were excised and weighed (B and D). ($^*p < 0.05$, $^{**}p < 0.01$, treated v.s. control). (A) Linear regression analysis: slope(s) control + MPA: 13.78 ± 1.42; raloxifene + MPA: 13.43 ± 1.078; tamoxifen + MPA: 6.63 ± 0.51 ($p < 0.001$ v.s. control + MPA). (C) Linear regression analysis: slope(s) control: 9.58 ± 0.40; raloxifene: 12.26 ± 0.86 ($p < 0.01$ v.s. control); tamoxifen: 2.78 ± 0.61 ($p < 0.001$ v.s. control); E_2: -3.44 ± 0.92 ($p < 0.001$ v.s. control).

Raloxifene, on the other hand, induced a slight increase in growth rate in experiments in which MPA had been withdrawn. Curiously, *in vitro* the stimulating effects of raloxifene were evident in the presence of MPA 10 nM or Pg and *in vivo* the effect becomes evident in the absence of exogenous MPA. It is conceivable that the MPA serum concentrations present in the MPA-treated animals are higher than the 10 nM used *in vitro*, thus interfering with the effect of raloxifene at the concentration used.

In analyzing these results, it becomes evident that the ability of E_2 and tamoxifen to modulate the expression of PR is unrelated to its growth inhibitory effect. In this progestin-dependent tumor line, basal PR levels are higher than 100 fmol/mg protein [50]. Both tamoxifen and E_2 are able to increase PR and at the same time inhibit tumor growth. Since progestins are the stimulatory hormones in this model, a further increase in PR levels would either have no effect or it would further stimulate cell proliferation. Raloxifene, on the other hand, did not modify PR levels, but it further stimulated the effect of MPA. These results show a consistent lack of correlation between the effect of SERMs on cell proliferation and PR expression,

suggesting that if the activation of PR is an essential pathway for proliferation [25], ER may be modulating it through interference with events up or downstream of PR, regardless, within certain limits, of the amount of PR expressed.

In this paper we have provided experimental evidence for a differential growth effect of clinically used SERMs under experimental conditions. We have demonstrated that (a) raloxifene can stimulate cell proliferation in certain experimental conditions, (b) tamoxifen exerts an inhibitory effect both *in vivo* and *in vitro*, (c) fulvestrant also exerts an inhibitory growth effect, probably through the inhibition of PR expression and (d) the effects of tamoxifen, raloxifene and E_2 on cell proliferation seem to be unrelated to their effect on PR induction. Since there is great expectation concerning the possible use of raloxifene in breast cancer patients, these results suggest that more experimental data is required in order to assess the benefits of raloxifene in the management of breast cancer.

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