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Antiestrogenic activity of two 11β-estradiol derivatives on MCF-7 breast cancer cells

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Two 11β-derivatives of estradiol (E_2) were tested for their potential antiestrogenic activity in the MCF-7 breast cancer model: one contained a phenoxydimethylaminoethyl side-chain (RU 39 411), the other a pentafluoropentylsulfinyl side-chain (RU 58 668). The former compound displayed mixed estrogenic/antiestrogenic properties, while the latter indicated only an antiestrogenic activity. Both the compounds produced a growth inhibition of MCF-7 cells at doses related to their binding affinity for the estrogen receptor (ER); E_2 suppressed this inhibition. The compounds also down-regulated the estrogen binding capacity of the cells but failed to reduce ER mRNA levels, indicating that the grafting of their side-chains prevented this antagonistic effect usually observed with steroidal estrogens. Assessment of ER levels by enzyme immunoassay revealed a marked increase with RU 39 411 and a decrease with RU 58 668; different mechanisms of action should, therefore, be considered. Finally, the estrogenic activity of RU 39 411 was demonstrated by its strong ability to induce synthesis of the progesterone receptor; RU 58 668 failed to display this agonistic activity. (Steroids **60**:512–518, 1995)

Keywords: estradiol; breast cancer; MCF-7 cells

Introduction

The therapeutic utility of triphenylethylene antiestrogens in the treatment of breast cancer is well established.[1] All of these compounds contain a dialkylaminoethyl side-chain which is involved in their mode of action.[2] Of these drugs, tamoxifen is now the compound of preference in view of the lack of major cytotoxic effects.[3] Recent investigations carried out at the ICI pharmaceutical division (now Zeneca Pharmaceuticals) revealed that grafting of a functional group at 7α-position of estradiol (E_2) through a long hydrocarbonated side-chain may also result in development of antiestrogenicity.[4] The compounds ICI 164,384[4,5] and ICI 182,780,[6] which bear a grafted carboxamide and pentafluoropentylsulfinyl group, respectively, were found to be of potential clinical interest due to their total lack of residual estrogenic activity, a property almost invariably observed with the therapeutic doses of triphenylethylene antiestrogens.

In order to extend the panel of antiestrogens of potential clinical interest, several synthesis programs were initiated at various drugs companies. The know-how of Roussel UCLAF in the field of steroids led chemists of this company to link various residues or side-chains at position 11 of estradiol-17β in view of the important role of this part of the molecule in estrogen receptor (ER) binding.[7–9] Two compounds of this class were assessed in our laboratory. One contained a phenoxy dimethylaminoethyl side-chain (RU 39 411), the other a pentafluoropentylsulfinyl side-chain (RU 58 668; formulas in Figure 1). It has been speculated that the first of these two steriods may be an analog of *trans*-hydroxytamoxifen,[10] while the second could be a pure antiestrogen like ICI 182,780. Both RU 39 411 and RU 58 668 were analyzed for their potential anti-mammary tumor activity in the MCF-7 breast cancer cell line; induction of progesterone receptor (PgR) synthesis as well as regulation of ER protein and ER mRNA levels were analyzed as additional tests of estrogenicity. Data reported here confirm the following speculations: mixed agonistic/antagonistic activity for RU 39 411 and pure antagonistic activity for RU 58 668.

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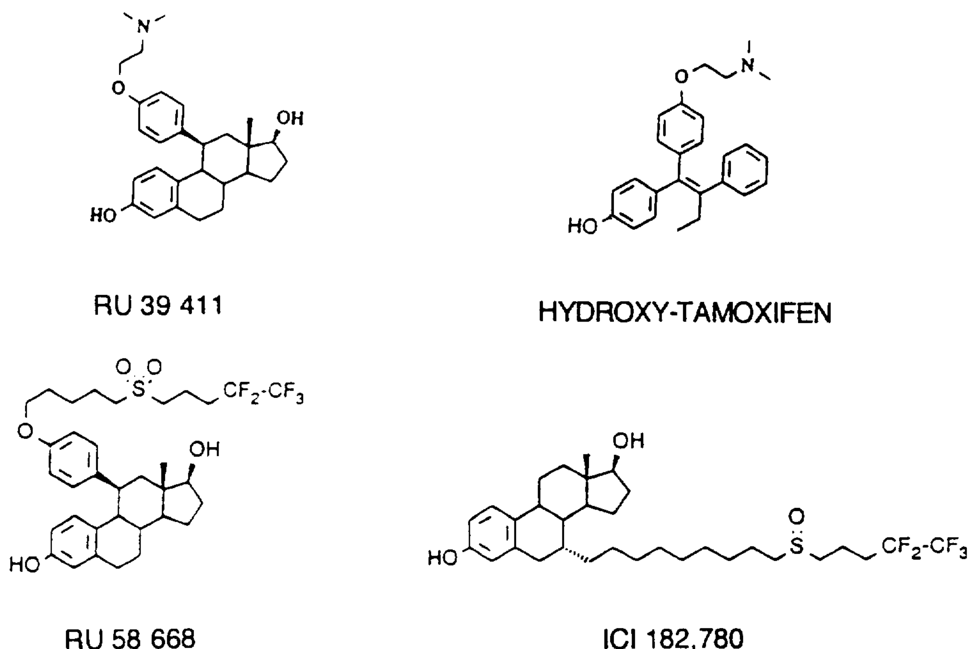

Figure 1 Formulas of RU 39 411 and RU 58 668. The figure shows the analogy between hydroxytamoxifen and RU 39 411 (top), ICI 182,780 and RU 58 668 (bottom).

Experimental

Reagents

$[^3H]E_2$ (±100 Ci/mmol) and $[^3H]$ORG 2058 (±50 Ci/mmol) were purchased from Amersham (UK). Unlabeled E_2 was obtained from Sigma (St. Louis, MO, USA).

RU 39 411 and RU 58 668 (RU compounds) were kindly provided by Dr P. Van de Velde (Roussel UCLAF, Romainville, France). Hydroxytamoxifen was a gift from Zeneca Pharmaceuticals (Macclesfield, UK).

1300 bp Eco RI fragment of pOR3 used as an ER mRNA probe was from the American Type Culture Collection (Rockville, MD, USA); control 28S RNA was obtained from Clontech (Palo Alto, CA, USA).

Cells and culture materials

MCF-7 cells were from the Michigan Center Foundation (Detroit, MI, USA). Since their introduction to our laboratory in 1977, they have been maintained in monolayer culture at 37°C in Earle's based minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine, penicillin, streptomycin, and gentamicin at the usual concentrations (one passage per week).[11]

Growth estimations and receptor assays were performed in MEM containing 10% of serum depleted of endogenous steroids by a dextran-coated charcoal (DCC) treatment.[11]

Binding affinity of RU compounds to ER

Biochemical assay. Aliquots of KCl extracts of MCF-7 cells prepared as described before,[12] were incubated overnight at 0–4°C with 5×10^{-9} M $[^3H]E_2$ in the absence or presence of increasing amounts of unlabeled E_2 or one of the two investigated RU compounds (100 μl extract in a total reaction volume of 200 μL). Unbound compounds were then removed with DCC[13] and the amount of bound $[^3H]E_2$ was measured by scintillation counting with an efficiency of ~40% (scintillation fluid: Ecoscint H, National diagnostic, Atlanta, GA, USA). The relative concentrations of unlabeled E_2 and RU compound required to achieve 50% inhibition of $[^3H]E_2$ binding gave the relative binding affinity (RBA). RBA = $(I_{50})E_2/(I_{50})_{RU} \times 100$. All measurements were performed in triplicate.

Whole cell assay[14]**.** Cells were plated in 6-multiwell dishes (~1.5×10^5 cells/well). After 4 days of culture, medium was removed and replaced by a serum-free medium containing 10^{-9} M $[^3H]E_2$ with or without increasing amounts of one of the two investigated RU compounds. After 1 h of incubation, the medium was again removed and the monolayer washed three times with ice-cold buffered saline solution (PBS). Bound $[^3H]E_2$ was then extracted from the monolayer by a final incubation with 1 mL ethanol at room temperature for 20 min. The radioactivity of these extracts (200 μL) was then measured by scintillation counting. Specifically incorporated radioactivity in presence of unlabeled E_2 and RU compounds gave the RBA values of the latter for ER. All measurements were performed in triplicate.

Capacity of RU compounds to down-regulate ER ("processing")[15]

Cells were plated in 6 multiwell dishes (~1.5×10^5 cells/well). After 3 days of culture, the medium was removed and replaced by fresh medium with or without increasing amounts of unlabeled E_2 or one of the two investigated RU compounds. After overnight incubation, the medium was removed and the monolayer washed three times with PBS. Cells were then incubated in serum-free medium for 1 h with 10^{-9} M $[^3H]E_2$ in the absence or presence of 500-fold excess of unlabeled E_2 for assessment of non-specific binding. Specifically incorporated $[^3H]E_2$ was finally measured as described above to determine the relative processing capacity (RPC) of each RU compound; RPC = $(B_{50})_{E2}/(B_{50})_{RU} \times 100$ where $(B_{50})_{E2}$ and $(B_{50})_{RU}$ are the concentrations of unlabeled E_2 and RU compound, respectively, producing 50% reduction of cellular incorporation of $[^3H]E_2$. All measurements were performed in triplicate.

Effect of RU compounds on cell growth

Growth was assessed by measuring the DNA content of treated and untreated (control) cells after 120 h of culture.[11] Cells were plated in Ø 35 mm Petri dishes (3 × 10^4 cells/dish). After 24 h of culture, increasing amounts (none, 10^{-11} to 10^{-6} M) of one of the two investigated RU compounds were added to the medium, in the presence or absence of 10^{-8} M E_2; 48 h later, the medium was replaced by fresh medium containing the same amount of RU compound and E_2. Cells were harvested after 72 h of additional culture and their total DNA content measured by the diphenylamine method.[16] Each culture was performed in quadruplicate.

Effect of RU compounds on ER and PgR levels

The effect of each RU compound was assessed by measuring cytosolic and nuclear receptors levels after 3 days of culture in its presence or absence (control). Cytosol and nuclear extracts from treated and control cells were prepared as previously described.[12]

Cytosolic and nuclear ER levels were assessed by multipoint DCC assay using $[^3H]E_2$ as the labeling agent[13] and by Abbott enzyme immunoassay (ER-EIA) according to manufacturer's recommendations. As reported in other studies,[17-19] we found that enzyme immunoassays always exhibited higher values than DCC assays; inappropriate calibration of the ER-EIA kits could be one of the reasons for this discrepancy.[20] Therefore, to facilitate the analysis of the data, ER values measured in the presence of an RU compound were always given as the percentage of the receptor concentration established with untreated (control) cells.

Cytosolic PgR levels were assessed by either multipoint DCC assay using $[^3H]$ORG 2058 as the labeling agent[13] or by Abbott enzyme immunoassay (PgR-EIA).

Effect of RU compounds on ER mRNA levels (Northern blotting)

Cells were cultured for 24 h without (control) or with an RU compound at 10^{-6} M; additional cells were cultured with 10^{-8} M E_2. After removal of the medium, total RNA was extracted from each culture with RNAzol according to the instructions of the manufacturer (Cinna/Biotech, Houston, TX, USA). The RNA was dissolved in RNase-free water and quantified by spectrophotometry at 260–280 nM. Aliquots of 20 μg were electrophoresed through a 1% agarose formaldehyde gel, capillary transferred to a Hybond-N membrane (Amersham, UK), and treated according to the manufacturer's instructions. Blots were hybridized sequentially with a ^{32}P-labeled ER cDNA probe[21] (10^9 cpm/μg cDNA, produced by random priming; Boehringer, Mannheim, Germany) as well as a 28S RNA control oligonucleotide probe. Prehybridization (4 h) and hybridization (18 h) were performed at 42°C for ER mRNA and at room temperature for 28S RNA in an usual buffer [50% formamide, 5 × SSPE (20 × SSPE = 0.2 M phosphate buffer pH 7.4, 2.98 M NaCl, 0.02 M EDTA), 0.1% each polyvinylpyrrolidine, Ficoll, BSA, and SDS, 5% dextran sulfate and 100 μg/mL sheared DNA]. The membranes were then washed with sodium citrate solutions (SSC) of increasing stringency, the last wash being performed in 0.3 × SSC containing 0.1% SDS. Blots were visualized by exposure of the membranes for 1 day to Kodak XAR-5 film in an autoradiography cassette with an intensifying screen.

Results

Interaction of ER and RU compounds

Competition analysis of the binding of $[^3H]E_2$ to MCF-7 cell extract (biochemical assay) revealed the ability of RU 39 411 and RU 58 668 to strongly associate with ER (~5-fold higher RBA value for RU 39 411; 15% versus 3% relative to E_2, n = 2). Assessment of the specific cellular incorporation of $[^3H]E_2$ in MCF-7 cells (whole-cell assay) established the capacity of these two steroids to bind to ER under conditions dependent on various physiological factors such as cell membrane permeability, interaction with other cellular lipophilic molecules, and metabolism (Figure 2, upper panel). Thus, both compounds impede the incorporation of $[^3H]E_2$ with an efficiency only slightly lower than unlabeled E_2; RBA values established from these data amounted to approximately 40% and 4% for RU 39 411 and RU 58 668, respectively, confirming the higher binding affinity for the former compound (the higher RBA value in the whole-cell assay could be ascribed to differences in experimental conditions). As expected,[15] overnight incubation of the cells with each steroid produced a dose-related loss of their capacity to specifically incorporate $[^3H]E_2$ ("ER processing"; Figure 2, lower panel). This phenomenon occurred at lower concentrations than needed in the competition assay as already reported for other estrogens and antiestrogens.[15] Interestingly, RU 39 411 did not appear more efficient than RU 58 668 in ER processing despite its higher binding affinity.

Assay of ER in cell extracts revealed different modes of action for RU 39 411 and RU 58 668 (Figure 3 represents

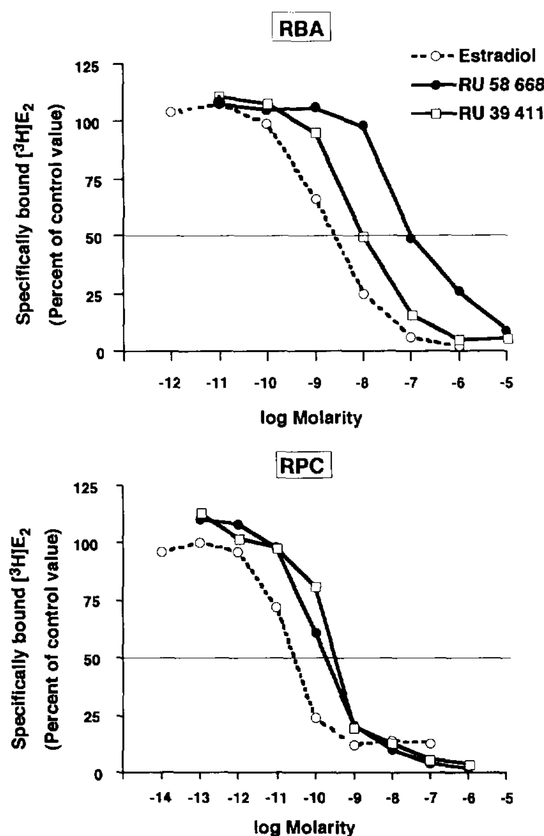

Figure 2 Relative binding affinity (RBA) and relative processing capacity (RPC) of RU 39 411 and RU 58 668. In both cases, MCF-7 cells were incubated with increasing amounts of E_2 (control) and RU compounds. Residual $[^3H]E_2$ binding capacity of ER is given on each graph (15 fmol/μg DNA = 100%).

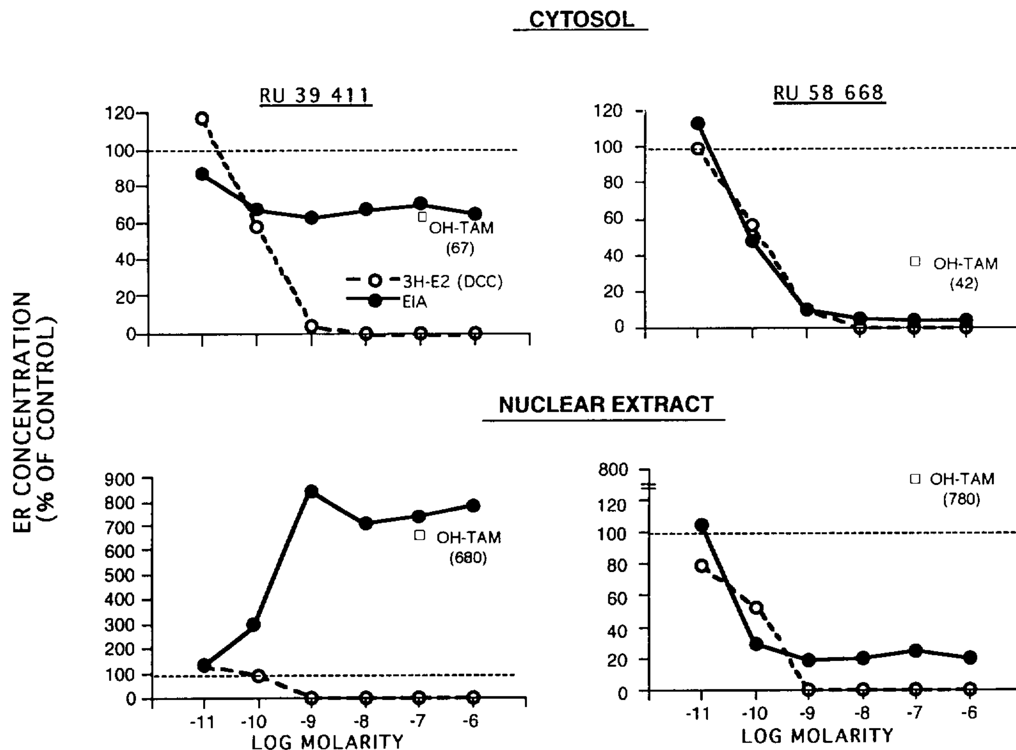

Figure 3 Effect of RU 39 411 and RU 58 668 on ER levels. MCF-7 cells were incubated for 3 days with increasing amounts of each compound; their cytosolic and nuclear ER contents were subsequently measured by either $[^3H]E_2$ binding assay (DCC) or enzyme immunoassay (EIA). Data are expressed as a percentage of untreated (control) cells cultured in parallel; ER levels (fmol/mg protein) of the controls assessed by EIA or DCC assay respectively: for RU 39 411, cytosol: 569 and 363, nuclear extract: 1011 and 198; for RU 58 668, cytosol: 785 and 454, nuclear extract: 974 and 383. ER levels of hydroxytamoxifen-treated cells (10^{-7} M, □) are provided for comparison.

data of an experiment performed 3 times). The former compound led to a large increase of ER peptides (EIA) in the nucleus, while reducing the total $[^3H]E_2$ binding capacity of the cells (DCC assay; note that the cells treated in parallel with hydroxytamoxifen behaved similarly, as already described).[12] On the contrary, RU 58 668 down-regulated the binding capacity as well as ER peptide concentration. Yet, assessment of both steriods with respect to ER mRNA expression did not reveal any decrease as found with E_2 (Figure 4). Thus, it could be concluded that the disappearance of estrogen binding capacity and/or ER peptides induced by these two compounds was mainly post-transcriptional in nature.

Growth and PgR regulation by RU compounds

Figure 5 shows that RU 39 411 and RU 58 668 inhibit growth at low concentrations (less than 10^{-9} M; experiments performed 3 times to ensure reproducibility). An equimolar concentration of E_2 completely suppressed this inhibition, indicating that both compounds operate through ER binding. As derived from RBA values, RU 39 411 appeared slightly more resistant to the suppressive effect of E_2 as compared to RU 58 668 (the inhibition of suppressive effect of E_2 by RU 39 411 was only partial at 10^{-7} M while it was complete with RU 58 668). Interestingly, the inhibition by RU 39 411 never exceeded 50% of the control value, sharply differing from that offered by RU 58 668, which displayed a strong growth inhibition (~20% of the control) at similar concentrations. This relatively weak effectiveness of RU 39 411 is reminiscent of the behavior of hydroxytamoxifen and analogs.[22–23] The identical partial growth inhibition produced by these triphenylethylene antiestrogens was ascribed to a weak estrogenic activity able

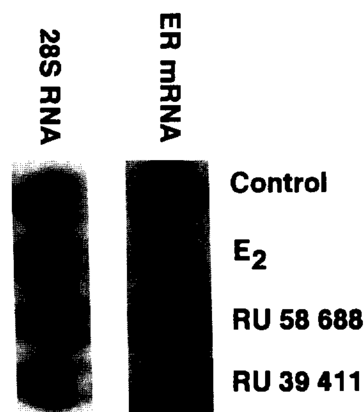

Figure 4 Absence of an effect of RU 39 411 and RU 58 668 on ER mRNA levels. MCF-7 cells were cultured for 24 h with each compound at 10^{-6} M; untreated (control) and E_2 (10^{-8} M) treated cells were incubated in parallel. ER mRNA contents were then evaluated by Northern blot. 28S levels are shown as internal controls.

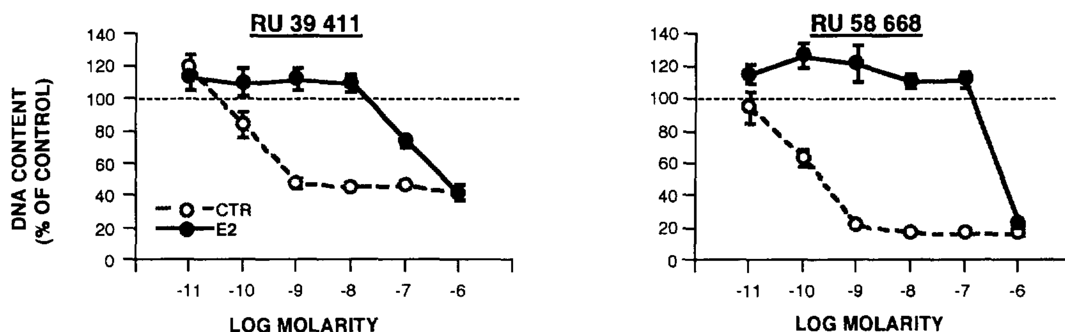

Figure 5 Growth inhibition of MCF-7 cells by RU 39 411 and RU 58 668. MCF-7 cells were incubated for 120 h in the absence or presence of increasing amounts of each RU compound; E_2 at 10^{-8} M was added to additional cultures. Growth was assessed by measuring the DNA content of harvested cells (range of controls; 18–24 μg DNA/well). Values (mean ± SD) refer to 3 experiments run in quadruplicate, student's *t*-test established growth inhibition ($P < 0.001$) at the minimal concentration of 10^{-9} M (RU 39 411) and 10^{-10} M (RU 58 668), respectively.

to partially antagonize their inhibition potency at high concentration. Hence, RU 39 411 could retain an estrogenic activity.

Assessment of the effect of RU 39 411 on PgR levels confirmed its agonistic/antagonistic activity (Figure 6, representative data of an experiment performed 2 times). At doses producing growth inhibition, this compound strongly increased the capacity of the cytosol to bind [^{3}H]ORG 2058 with a maximal efficiency at 10^{-9} M. This increase was due to an induction of the PgR peptide as demonstrated by a 5.4-fold increase of the receptor when assessed by enzyme immunoassay in a complementary experiment. On the contrary, RU 58 668 always down-regulated PgR levels (by [^{3}H]ORG 2058 binding capacity as well as by enzyme immunoassay), suggesting its classification as a "pure antiestrogen."[4–6] Interestingly, this steroid also antagonized the PgR increase induced by RU 39 411 (induction was almost completely abolished in the presence of a 10-fold excess of the compound; data not shown).

Discussion

Data reported here largely confirm the speculation on the foremost endocrinological properties of the presently studied 11β-derivatives of E_2: RU 39 411 displayed a mixed antiestrogenic/estrogenic activity, as tamoxifen and derivatives, while RU 58 668 behaved as a pure antiestrogen at least in regard to growth and PgR regulation, as ICI 164,384 or ICI 182,780. Hence, grafting of an appropriate side-chain in the 11β-position of E_2 may partially or totally reverse its biological activity due to an upsurge of antagonistic potency.

Our data concerning ER regulation reveal that these RU compounds mainly act at the post-transcriptional level. They failed to down-regulate ER mRNA, indicating that the grafting of their side-chains suppressed this characteristic property of estrogenic steroids in breast cancer cells.[24–26] While these RU compounds failed to operate at the ER mRNA level, they decreased the sensitivity of the cells to the trophic effect of E_2, although through different mechanisms. RU 39 411, similar to triphenylethylenic antiestrogens, led to the emergence of ER in a form unable to bind E_2 at least under conventional exchange assays,[12,27,28] while RU 58 668 reduced the amount of ER peptides, analogous to the pure antiestrogen ICI 164,384.[29,30]

E_2 concentrations needed for the saturation of ERs in the whole-cell assay are higher than those producing the disappearance of the estrogen binding capacity,[15] suggesting that

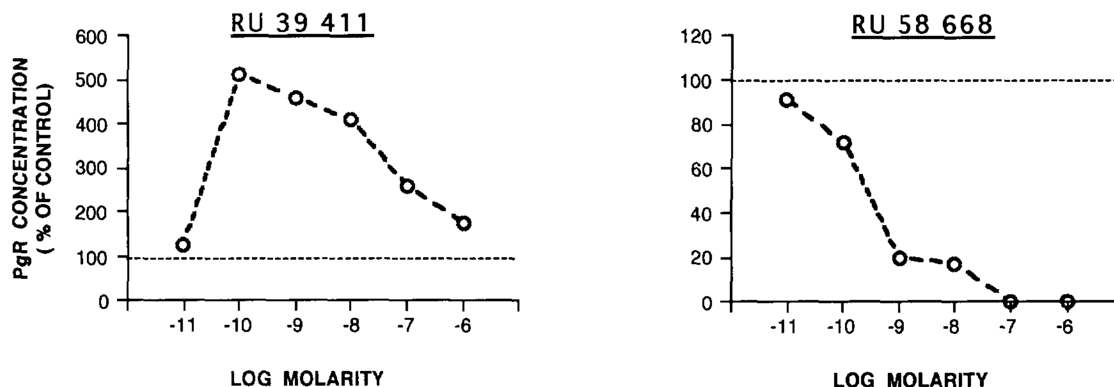

Figure 6 Effect of RU 39 411 and RU 58 668 on PgR levels. MCF-7 cells were incubated for 3 days with increasing amounts of each RU compound; cytosolic PgR levels were subsequently measured by [^{3}H]ORG 2058 binding assay. Data are expressed as a percentage of the binding capacity of untreated (control) cells cultured in parallel; PgR levels of the controls (fmol/mg protein); for RU 39 411: 154; for RU 58 668: 137.

only a part of the total receptor population should be activated to decrease the estrogen sensitivity of the cells in a subsequent step.[26] RU 39 411 appeared less effective than RU 58 668 and E_2 in this regard. This property of RU 39 411, already reported for some triphenylethylene antiestrogens,[15] may result from the ability of these molecules to up-regulate ER peptides. It would be of great interest to analyze the relationship of such an accumulation phenomenon to a prolongation of the half-life of the receptor. A weak but significant reduction of half-life has been detected in the presence of E_2[31] as well as pure antiestrogen ICI 164,384,[30] both of them being able to down-regulate ERs.

The mechanisms by which RU compounds operate are unknown. In this regard, it would be interesting to analyze whether or not RU 58 668 impedes the formation of stable ER dimers as proposed for ICI 164,384.[32] The mode of action of RU 39 411 is obviously different from that of these two pure antiestrogens. Its ability to up-regulate ER in a form unable to bind E_2 suggests the involvement of another target which modulates the estrogen binding activity of the receptor. Such a hypothesis is under study in our laboratory. In this regard, we recently demonstrated that RU 39 411,[33] as well as tamoxifen,[34] both of which lead to the appearance of such a peculiar ER form, also impede the association between ER and calmodulin (CaM). This antagonism on ER-CaM association may influence the activity of a CaM-dependent kinase reported to confer the estrogen-binding ability to the receptor.[35] However, additional data contradicted such a hypothesis: a tamoxifen derivative with strong anti-CaM activity as well as calmidazolium—a specific anti-CaM agent—failed to up-regulate ER.[36] Hence, the origin of the non-binding ER form demands further investigation.

RU 39 411 increased PgR levels at concentrations producing a strong growth inhibition (i.e., 10^{-9} to 10^{-8} M). The fact that this increase was less marked at higher concentrations which blocked a complete inhibition (estrogenic activity) is reminiscent of E_2 action: high E_2 concentrations which suppress the growth inhibition of antiestrogens are less efficient in PgR induction as compared to lower concentrations.[28,37,38] Hence, the estrogenic growth stimulation may not be necessary associated with PgR induction. On the other hand, comparison of the endocrinological profiles of RU 39 411 and conventional triphenylethylene antiestrogens (e.g., hydroxytamoxifen) may suggest a higher therapeutic potency of the former compound: while all of them may induce PgR at low concentration,[37,38] RU 39 411 solely produced a significant growth inhibition at a comparable concentration.

The strong growth inhibition by the presently investigated RU antiestrogens (in the MCF-7 cell model) supports the rational for *in vivo* studies, further leading to a possibility of clinical trials. At the ER level, RU 39 411 most probably acts like tamoxifen; however, metabolic profiles and pharmacokinetic properties of these two compounds may significantly differ with, perhaps, a different efficacy after prolonged administration. RU 56 668 may be effective as its 7α-counterparts produced by ICI. In this regard, it should be stressed that the first paper on RU 58 668,[39] published during the writing of the present report, describes a higher potency of this compound as compared to ICI 182,780 on MCF-7 tumors implanted in nude mice. Though the nature of studies is quite different than the present, both of them point toward "pure antiestrogenic" properties of RU 58 668. Further investigations on this new drug seem warranted.

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