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EVALUATION OF ESTROGEN RECEPTOR, ANTIESTROGEN BINDING SITES AND CALMODULIN FOR ANTIESTROGEN RESISTANCE OF TWO CLONES DERIVED FROM THE MCF-7 BREAST CANCER CELL LINE

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Abstract—Estrogen receptor (ER), antiestrogen binding sites (AEBS) and calmodulin (CaM) are potential targets of antiestrogen (AE) action. To analyse further which of these targets are primarily involved in the antiproliferative activity of these drugs against human breast cancers, two cell clones, namely the RTx6 and LY-2 variants, selected from MCF-7 cells for their resistance to high doses of tamoxifen (TAM) and the Keoxifen (KEO) analog LY 117018, respectively, were studied for their sensitivity to hydroxytamoxifen (OH-TAM) and KEO as well as the strong calmodulin antagonist calmidazolium. The effects of these drugs on both cell growth and progesterone receptor (PgR) concentration were assessed. Binding properties for ER, AEBS and CaM of each compound were also measured. Our results confirmed that basal growth of RTx6 and LY-2 cells was more resistant to OH-TAM and KEO than parent MCF-7 cells, although both displayed a significant inhibition at the highest doses assessed. In regard to calmidazolium inhibition, each variant behaved as did the MCF-7 line indicating that a modification at the CaM level was not responsible for their lower sensitivity to AEs. Nor could the association of CaM to ER which did not differ among all cell lines. Resistance of these variants was not related to AEBS in view of the total lack of such sites in RTx6 cells. However, under estrogenic growth stimulation such sites may play some role, since LY-2 cells in the presence of estradiol displayed a real antiestrogen-resistant pattern while RTx6 cells were more sensitive than MCF-7 cells to OH-TAM. This property was not found in the antagonism against estradiol-induced PgR synthesis which was observed with each variant. Thus the PgR concentration of RTx6 cells was strongly down-regulated by OH-TAM and KEO and reduced in LY-2 cells to the same extent as in MCF-7 cells. All these observations show that AE resistance is not entirely related to ER mediated events and that alterations at the ER and CaM levels are unlikely to account for the lower AE sensitivity of the variants investigated.

Key words: antiestrogen-resistance; antiestrogen binding sites; calmodulin; estrogen receptor; progesterone receptor; breast cancer cells

AEs† belong to a large class of synthetic compounds which includes triarylethylene, triarylpropenone, stilbene and steroid derivatives [1–3]. All these drugs bind to the ER and were, therefore, thought to elicit their antagonistic effects by competition with endogenous estrogens. Now it is clear that "antiestrogen" is certainly not the most appropriate term because most of these drugs act either as full estrogen agonists, partial agonists/partial antagonists or pure antagonists depending on species, tissues and growth conditions [4, 5]. Only a small category of E_2 derivatives classed as "pure AEs" exert exclusively antagonistic activity [3]. Moreover, most of these agents not only block ER but also affect a number of cellular growth mediators. For example, high concentrations of TAM have been reported to antagonize PKC [6], reduce calcium currents [7], affect calmodulin-dependent catalyses [8], bind to cytochrome P450 [9] and modulate the action of various receptors other than ER (i.e. muscarinic [10], dopamine [11] and histamine [12]). The additional discovery of AEBS [13] without affinity for E_2 has also received some attention although there is no consensus as to their function.

AEs—especially TAM—have been extensively used in the treatment of ER-positive breast cancers [14] even if their efficacy disappears after long term administration [15]. One may logically assume that this so-called "antiestrogen resistance" does not necessarily derive from a dysfunction at the ER level in view of the large number of targets involved in the mode of action of these drugs. The aim of this study was to evaluate events mediated by the AEBS

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† Abbreviations: AEBS, antiestrogen binding sites; AE, antiestrogen; CaM, calmodulin; DCC, dextran-coated charcoal; E_2 estradiol; ER, estrogen receptor; FCS, fetal calf serum; KEO, keoxifen; MEM, Minimal Essential Medium; OH-TAM, hydroxytamoxifen; PgR, progesterone receptor; PKC, protein kinase C; PETG, −10 mM phosphate buffer pH 7.4 containing 1.5 mM EDTA, 1 mM monothioglycerol and 10% glycerol; PTG, PETG without EDTA; RBA, relative binding affinity; RPMI, Roswell Memorial Park Institut; TAM, tamoxifen; TPA, 12-*o*-tetradecanoylphorbol-β-acetate; WCA, whole cell assay.

and CaM on AE resistance since the importance of these two targets in the regulation of the antiproliferative activity of such drugs in mammary tumor cells has been stressed. Thus, on the one hand it has been suggested that AEBS concentrations may modulate AE efficacy [16] while on the other CaM, which governs several phosphorylation and dephosphorylation processes involved in signal transduction [17], has been reported to confer E_2 binding ability to ER [18]. The fact that TAM impedes the association of ER to CaM [19] is an important indication of the antagonistic activity of AEs on the latter. However, CaM antagonism does not hold for all AEs since triarylpropenone derivatives, of which KEO is the drug of reference, are devoid of inhibitory activity towards CaM-dependent catalyses [20]. Therefore, investigating the effects of triarylethylene and triarylpropenone derivatives on AE resistant cells appeared to be of major interest. Such a study is described here.

Two "antiestrogen resistant" variants derived from the MCF-7 breast cancer cell line (RTx6 and LY-2 clones) without any detectable alteration in ER but differing in AEBS contents were used to conduct our experiments. The RTx6 clone, which was isolated by treating MCF-7 cells with high doses of TAM, differs from the latter cells by a total absence of AEBS [21] while the LY-2 clone, selected with high doses of KEO analog LY 11708 [22], contains higher levels of such sites [16]. Both clones were cultured in the presence of KEO, OH-TAM and the strong CaM antagonist calmidazolium to assess their own sensitivity to these classes of drug. The effects of these compounds on both growth and PgR concentrations in the absence of estrogen as well as under estrogenic stimulation were investigated. Binding affinities for ER, AEBS and CaM were also measured to detect possible alterations at the level of these targets. Potential interference with the ER-CaM association was also analysed.

MATERIALS AND METHODS

Reagents

[^{3}H]E_2 (±100 Ci/mmol), [*N*-methyl-^{3}H]TAM (82 Ci/mmol), and [^{3}H]ORG2058 (±50 Ci/mmol) were purchased from Amersham (Amersham, U.K.). Unlabeled E_2 was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). TAM and OH-TAM were kindly provided by ZENECA (Macclesfield, U.K.) and KEO by Eli-Lilly (Indianapolis, IN, U.K.). Calmidazolium was obtained from Boehringer (Mannheim, Germany) and CaM-Sepharose from Pharmacia (Uppsala, Sweden).

Cells and culture materials

MCF-7 cells were from the Michigan Cancer Foundation (Detroit, MI, U.S.A.). LY-2 cells were obtained from Dr R. B. Dickson (Lombardi Cancer Center, Washington DC, U.S.A.) and RTx6 from Dr J. C. Faye (INSERM U 168, Toulouse, France). Two former lines were maintained in monolayer culture at 37° in Earle's based MEM containing 10% heat inactivated FCS (56°, 1 hr), L-glutamine, penicillin, streptomycin and gentamycin at the usual concentrations (all materials from Gibco, Ghent, Belgium). Experiments for investigating target contents (i.e. ER and AEBS) as well as the binding affinity of compounds for such targets were performed in phenol red-free MEM supplemented with 10% dextran-coated charcoal treated FCS (DCC-FCS MEM) [23]. RTx6 was maintained under the same conditions in RPMI in the presence of 1 μM TAM which was removed from the medium 1 week before each experiment.

Growth experiments

The effect of compounds on the growth of MCF-7, RTx6 and LY-2 cells was assessed after 120 hr of culture according to a previously described protocol [24]. Briefly, cells were plated in 35 mm petri dishes (MCF-7=2×10^4 cells/mL; RTx6 and LY-2=4×10^4 cells/mL) in DCC-FCS MEM or RPMI. After 24 hr compounds were added to the medium and 48 hr later replaced by fresh medium containing the compounds. Cells were harvested 72 hr later and their growth evaluated by measuring their DNA content by the diphenylamine method [25].

ER assays

Whole cell assays. ER contents were measured by WCA on cells cultured in DCC-FCS MEM or RPMI as described previously [26]. Briefly, cells were incubated for 45 min at 37° with 1 mL of serum-free medium containing [^{3}H]E_2 at concentrations ranging from 0.2 to 2 nM. Additional dishes were filled with the same concentrations of [^{3}H]E_2 and a 200-fold excess of unlabeled E_2 for non-specific binding measurement. After incubation, the medium was removed and the monolayer washed three times with ice-cold saline solution. Bound [^{3}H]E_2 was extracted from the monolayer by a final incubation of 20 min in 1 mL ethanol at room temperature and aliquots of 200 μL of such ethanolic extracts were transferred to scintillation vials containing 3.8 mL scintillator Ecoscint H (National diagnostic, Atlanta, GA, U.S.A.) for radioactivity counting. All measurements were performed in triplicate. Binding data were analysed according to Scatchard [27]. In each experiment an additional 6-well dish was run in parallel for DNA measurement and ER concentration expressed in fmol/μg DNA.

RBA of OH-TAM, KEO and calmidazolium for ER were determined by competitive binding assays using 1 nM [^{3}H]E_2 and increasing amounts (0.1 nM to 10 μM) of unlabeled E_2 and AEs. The relative concentrations of AEs and unlabeled E_2 required to reduce the specific binding of [^{3}H]E_2 by 50% gave the RBA of each compound [RBA = (I_{50} AE/I_{50} E_2) × 100].

Cytosolic assays. Cells were detached from the T-175 flasks with 1 mM EDTA in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} and harvested by 10 min centrifugation at 300 *g*. Cells were washed twice with HBSS and once with 10 mM phosphate buffer pH 7.4 containing 1.5 mM EDTA, 1 mM monothioglycerol and 10% glycerol (PETG) before homogenization in this buffer by means of a teflon–glass homogenizer. Cytosolic ER and PgR were assessed on 1 hr 100,000 *g* supernatant fraction of ultracentrifugation by multipoint DCC assay

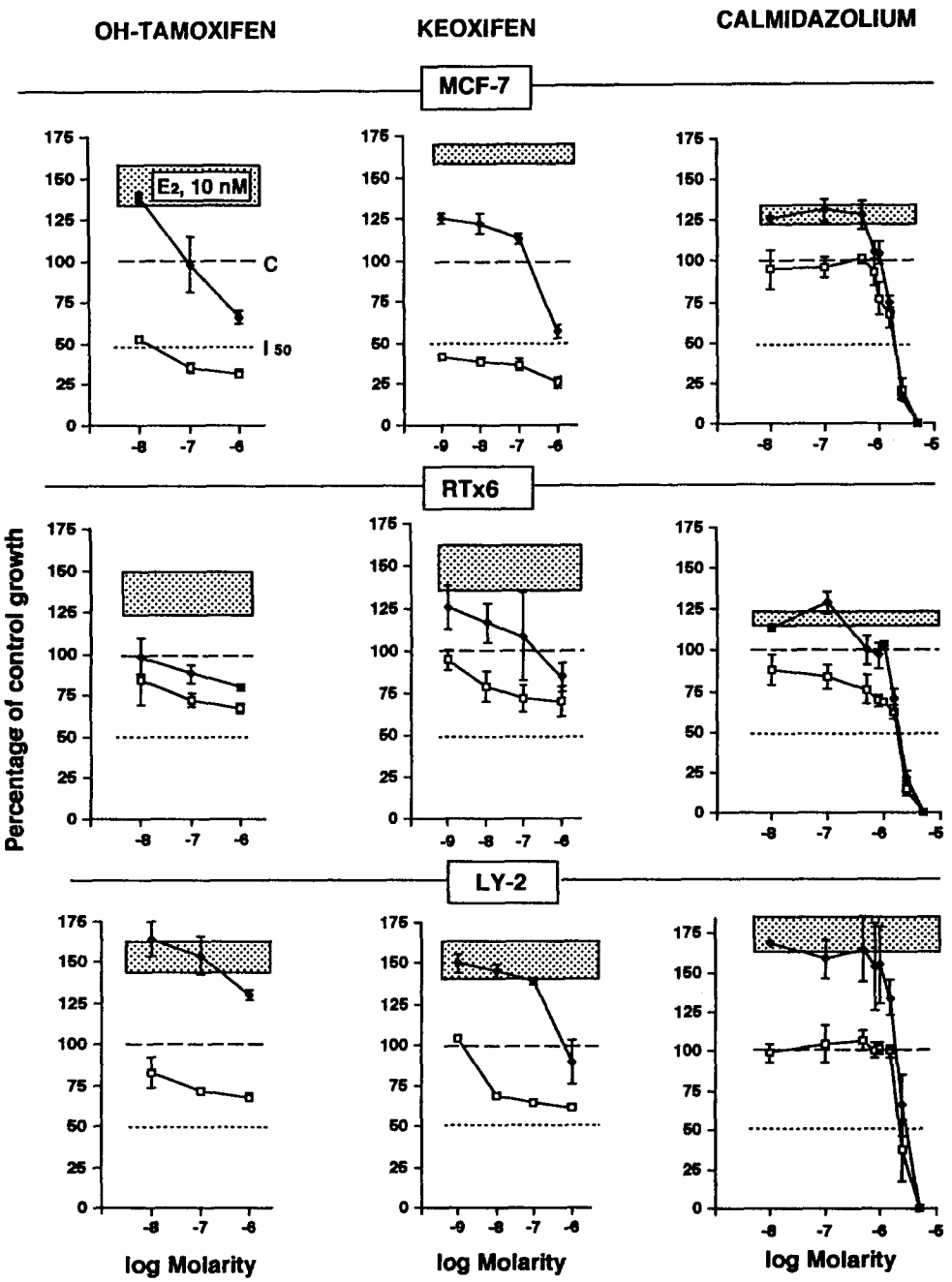

Fig. 1. Growth responses of MCF-7 (top), RTx6 (middle) and LY-2 (bottom) to increasing concentrations of OH-TAM (left), KEO (middle) and calmidazolium (right) in the absence (—□—) or presence (—◆—) of 10 nM E_2. Cells were harvested after 120 hr of culture and their growth evaluated by measuring their DNA content. Each value represents the mean ±SEM of three different experiments (each experiment in quadruplicate in μg DNA/well). E_2-stimulated cell growth (10 nM) is represented as a horizontal hatched strip ±SEM. Control (C) value is taken as 100%. The lower dotted line on the graphs represents 50% of inhibition (I_{50}).

according to EORTC recommendations [28] using [^{3}H]E_2 and [^{3}H]ORG 2058 as labeling ligands. Receptor concentrations assessed by Scatchard plot analysis were expressed in fmol/mg protein, the latter being measured using the Bio-Rad reagent (Bio-Rad, Richmond, CA, U.S.A.). When the effect of E_2 or AEs on PgR was studied, compounds were added 3 days before receptor assay.

AEBS assay. Cells were detached from T-175 flasks and homogenized as described above. AEBS

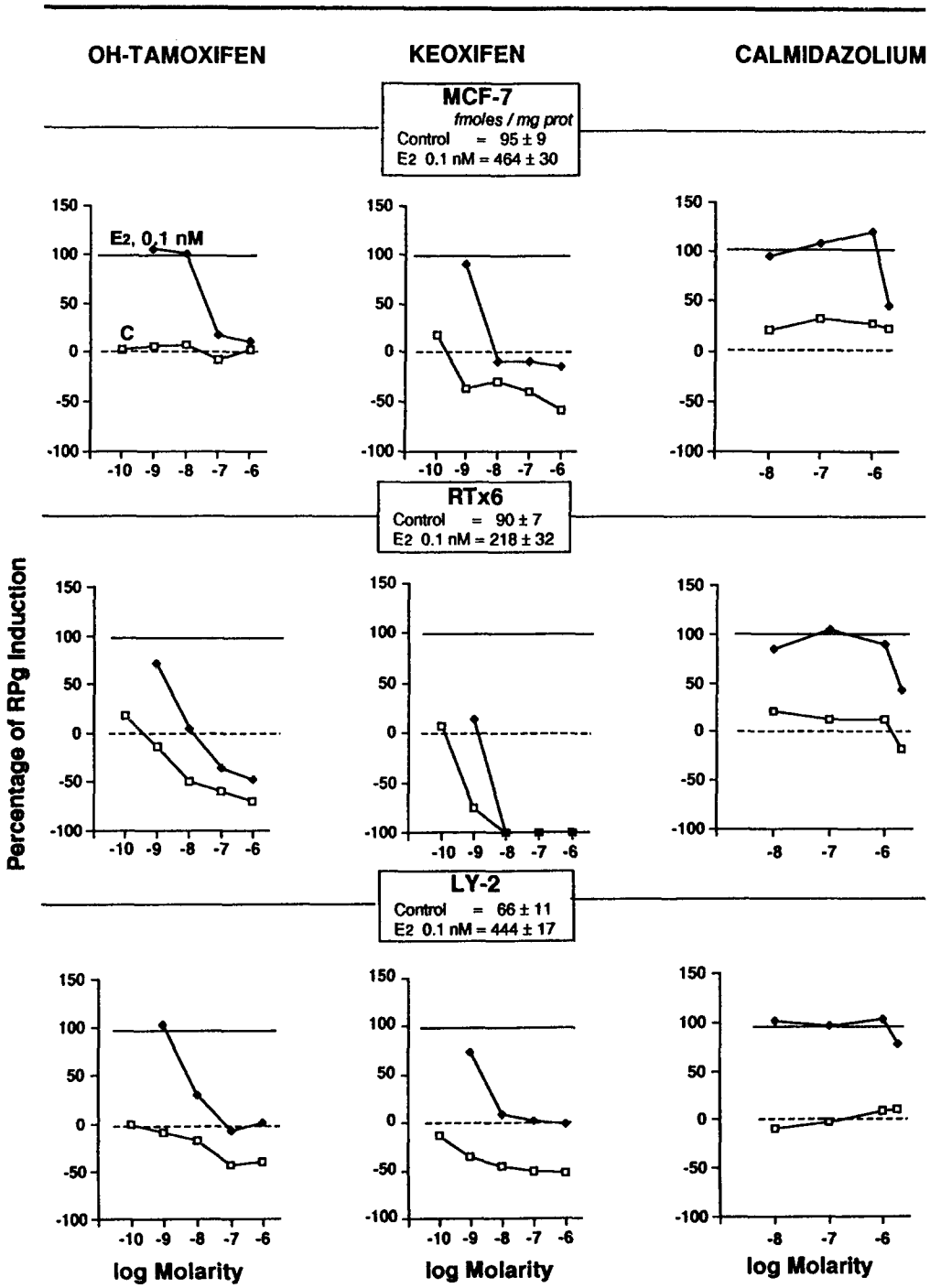

Fig. 2. Variation of PgR concentrations of MCF-7 (top), RTx6 (middle) and LY-2 (bottom) cells by OH-TAM, KEO and calmidazolium. Cells were incubated for 72 hr in the absence or presence of 0.1 nM E_2 with or without increasing concentrations of a given compound and their cytosolic PgR levels measured by multipoint DCC assay. Data were expressed as a percentage of the optimal PgR induction (100%) produced by 0.1 nM E_2 alone (—◆—). Basal PgR levels (C) measured in the absence of any compound were taken as 0% of induction (—□—). Absolute values of PgR of control and E_2-stimulated cells are given in front of the data for each cell line; they represent the means ±SEM of three different experiments.

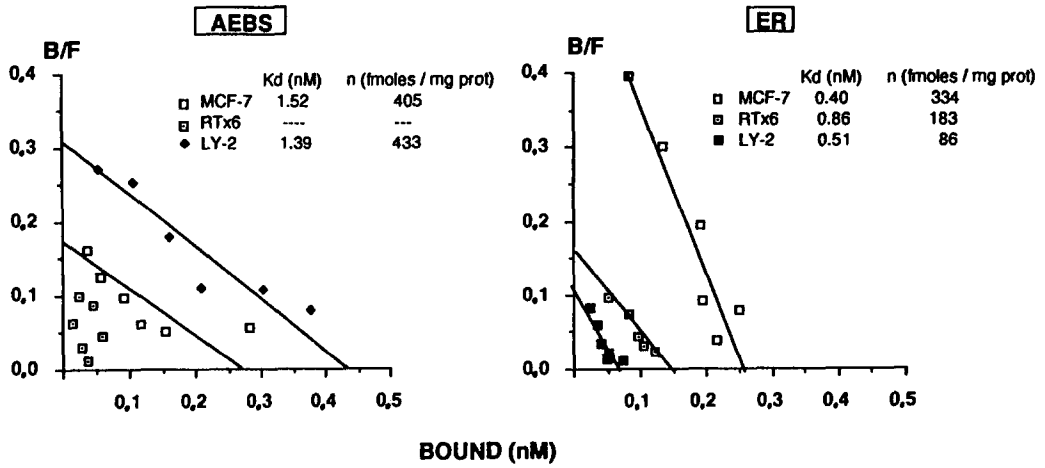

Fig. 3. Levels of AEBS and ER in 12,000 *g* supernatant of cellular extracts from MCF-7, RTx6 and LY-2 cells. AEBS and ER were measured by multipoint DCC assay. Values were expressed as fmol/mg supernatant protein. Data were analysed by Scatchard plot analysis.

were measured in the 12,000 *g* supernatant fraction after 30 min centrifugation [29] by multipoint DCC assay with increasing concentrations of [*N*-methyl-[^{3}H]TAM (0.25–5 nM) as labeling ligand in the absence or presence of a 200-fold excess of TAM; the potential interference of ER in the assay was avoided by a 30 min pretreatment of the fraction with 1 μM E_2 to fully occupy the receptor. Binding data were analysed according to Scatchard.

CaM–Sepharose chromatography

Cells were detached from T-175 flasks and homogenized in 10 mM phosphate buffer pH 7.4 containing 1 mM monothioglycerol and 10% glycerol (PTG) as described above. Cytosol was subsequently obtained by ultracentrifugation of the homogenate at 100,000 *g* for 1 hr. Total extracts were obtained in the same way except that harvested cells were stored at −50° in presence of PTG buffer containing 500 mM KCl for 1–2 hr before homogenization.

Cytosol and total extracts were incubated for 2 hr at 0–4° with 1 nM [^{3}H]E_2 either in the absence or presence of a 200-fold excess of unlabeled E_2; samples were then treated with DCC to remove unbound steroids. Specifically bound [^{3}H]E_2 was calculated from the difference in bound radioactivity between both samples.

CaM–Sepharose was equilibrated with PTG buffer containing 1 μM $CaCl_2$ ($PTGCa^{2+}$) in a 1 × 10 cm column. [^{3}H]E_2 labeled cytosolic and total extracts were incubated overnight at 4° with this gel (1 mL of ER preparation per 1 mL of gel); unbound material was released from the column with 6 mL of $PTGCa^{2+}$ and adsorbed ER was eluted with PTG buffer containing 10 mM EDTA [19]. ER concentration of all elution fractions was determined either by measuring their radioactivity ([^{3}H]E_2 labeled samples) or by Abbott enzyme immunoassay (EIA) according to the manufacturer's instructions. AEs were added to the samples 30 min before mixing with the CaM–Sepharose in order to assess their ability to impede the association of ER to CaM.

RESULTS

Estrogenic and antiestrogenic sensitivity of MCF-7, RTx6 and LY-2 cells

Cell growth. Proliferation of MCF-7, RTx6 and LY-2 cell lines in response to OH-TAM, KEO and calmidazolium either in the absence or presence of 10 nM E_2 are shown in Fig. 1 (left, middle and right columns, respectively). While OH-TAM and KEO markedly inhibited basal growth of MCF-7 cells (more than 50%), they only slightly affected RTx6 and LY-2 cells (±25%) indicating a partial resistance to these compounds under our culture conditions. E_2 increased the growth rate of these three cell lines to a similar extent (±50% stimulation). As expected, OH-TAM and KEO inhibited the E_2-induced growth stimulation of MCF-7 cells in a concentration-related manner, KEO being the more powerful compound in this regard. Interestingly, OH-TAM abolished the growth stimulation of RTx6 cells even at the lowest concentration assessed (10 nM), whereas KEO was effective as it was in MCF-7 cells indicating that this cell variant did not display any resistance to these AEs under estrogenic stimulation. Such behavior was not observed with LY-2 cells in which the E_2-stimulated growth was almost not abrogated by OH-TAM and KEO, the latter compound producing a weak inhibition only at the highest concentration tested (1 μM).

Calmidazolium at concentrations above 0.8 μM produced a significant growth inhibition of MCF-7 and RTx6 cells whereas concentrations higher than 1.5 μM were required to affect LY-2 cells. This phenomenon also occurred in the presence of E_2 indicating its independence of the estrogen-sensitivity of the cells. It is noteworthy that the same rates of inhibition were observed in anchorage-independent growth experiments run in agar (data not shown), thereby discrediting the hypothesis of an apparent growth suppression due to a loss of cell adherence.

PgR regulation. Measurement of PgR contents revealed higher basal levels in MCF-7 and RTx6 cells than LY-2 cells although the difference was not

Table 1. ER binding characteristics of MCF-7, LY-2 and RTx6 cells

Cell line	DCC (Cytosol)		WCA	
	Receptor content (fmol/mg protein*)	Binding affinity K_d nM†	Receptor content (fmol/μg DNA)	Binding affinity K_d nM
MCF-7	485 ± 43	0.13 ± 0.06	15.5	0.10
RTx6	285 ± 33‡§	0.08 ± 0.07 NS	7.3	0.17
LY-2	150 ± 15‖	0.13 ± 0.09 NS	3.4	0.13

* Mean of three different experiments ± SEM.
† K_d = Dissociation constant of the binding reaction.
‡ Statistical significance: § $P < 0.05$; ‖ $P < 0.01$; NS = not significant (Student's *t*-test).

significant (Fig. 2, front of the data of each cell line). The highest rate of PgR induction after 3 days of E_2 stimulation at 0.1 nM was detected in LY-2 cells (E_2/control = fold increase:MCF-7 = 4.9; RTx6 = 2.4; LY-2 = 6.7).

In MCF-7 cells, KEO (Fig. 2, middle column) produced a strong reduction in basal PgR levels while OH-TAM (Fig. 2, left column) was ineffective. Both AEs down-regulated PgR contents of RTx6 and LY-2 cells; this effect was particularly marked in RTx6 cells in which KEO produced a total receptor disappearance. PgR reduction was only partly suppressed by E_2.

Calmidazolium had almost no effect on PgR levels whether the cells were stimulated with E_2 or not (Fig. 2, right column). Only a slight inhibition was observed at 2 μM probably due to the toxicity of this compound towards the cells. As reported for growth experiments, LY-2 cells appeared slightly more resistant in this regard.

ER and AEBS levels of MCF-7, RTx6 and LY-2 cells

Cytosolic DCC and whole cell assays revealed an ER binding affinity for [^{3}H]E_2 in the same order of magnitude in MCF-7, RTx6 and LY2 cells, indicating no alteration at this level in the latter two variants (Table 1). Cytosolic ER contents in MCF-7 cells were approximately twice as high as in RTx6 cells and about three to four times higher than in LY-2 cells. The same ratios were found by whole cell assay.

AEBS measurement in the 12,000 *g* supernatant of MCF-7 and LY-2 cells showed a practically identical binding affinity for [^{3}H]TAM ($K_d \approx$ 1.5 nM); contents of such sites were slightly higher in LY-2 cells (Fig. 3, left panel). As expected, AEBS were not detected in RTx6 cells. ER contents of the three cell lines in this AEBS-containing fraction were in the same order of magnitude as those measured in the cytosol and whole cells (Fig. 3, right panel). Thus, the ratio of AEBS:ER was four times greater for LY-2 cells than for MCF-7 cells (~5 vs 1.2).

Binding characteristics of OH-TAM, KEO and Calmidazolium for ER and AEBS

In all cell lines, the binding affinity of OH-TAM and KEO for ER was approximately 10 times higher for the latter compound (Table 2); as expected [30] these binding affinities were largely lower than for E_2 because they were established by WCA. The relative binding affinity for AEBS of OH-TAM and KEO in MCF-7 and LY-2 cells indicated an affinity approx. 50 times lower for the latter AE confirming previously reported data [31]. E_2 did not compete at all for binding to these sites (Fig. 4).

Table 2. RBA for ER of 4-OH-TAM and KEO

Cell line	E_2 = 100%* OH-TAM	KEO
MCF-7	0.25	2.51
RTx6	0.16	1.21
LY-2	0.27	1.90

* RBA was assessed by whole cell assay.

As presumed, calmidazolium had no binding affinity for either ER or AEBS (data not shown).

Association of ER with CaM

[^{3}H]E_2 labeled extracts from MCF-7, RTx6 and LY2 cells were eluted on CaM–Sepharose. A large part of the radioactivity of each preparation adsorbed to the column suggesting a strong binding of their ER contents to CaM (Fig. 5, left panels); binding was confirmed by immunoenzymatic measurement (ER-EIA, Abott) of the main elution fractions (Fig. 5, right panels).

Addition of calmidazolium or OH-TAM to the [^{3}H]E_2 labeled extracts from MCF-7 cells strongly reduced the ER adsorption to CaM–Sepharose (Fig. 6). KEO appeared totally ineffective in agreement with its lack of antagonistic activity in CaM-dependent catalyses [20].

DISCUSSION

Since LY-2 and RTx6 cells were selected from MCF-7 cells for their "resistance" to TAM and the KEO analog LY117018, respectively, we originally thought that they had largely lost their sensitivity to such compounds. Our data refute this assumption: while both cell lines displayed a significant loss of

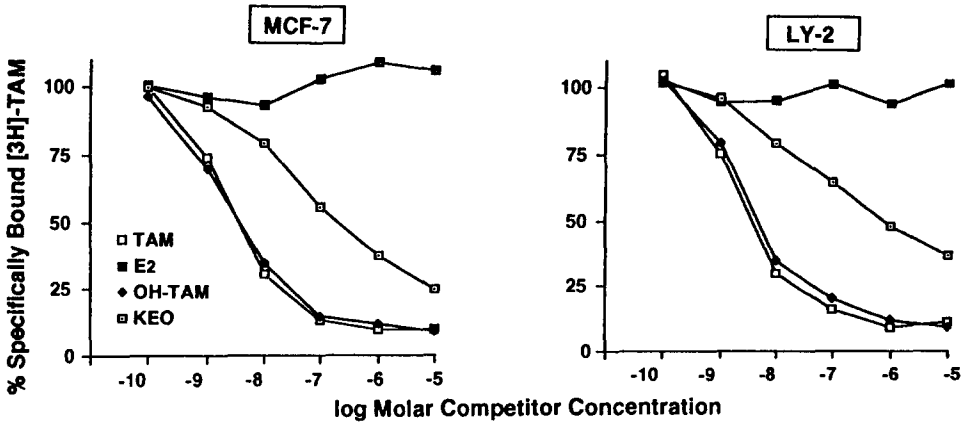

Fig. 4. Relative binding affinity of E_2, TAM, OH-TAM and KEO for AEBS from MCF-7 and LY-2 cells. Assays were run on the 12,000 *g* supernatant fraction added with 1 μM E_2 (30 min at 0° to fill ER) and incubated with 1 nM [^{3}H]TAM in the absence or presence of a 200-fold excess of TAM. Supernatants were incubated at 4° for 18 hr with increasing concentrations of the tested competitors. Values are expressed as a percentage of specifically bound [^{3}H]TAM in the absence of any competitor.

growth sensitivity to OH-TAM and KEO they were still inhibited by each compound. This relative lack of resistance was reflected on basal PgR concentration which was reduced to the same extent as their parent MCF-7 cells. The additional observation that all cell lines were similarly killed by high doses of calmidazolium refutes the hypothesis of a defect at the CaM level in RTx6 and LY-2 cells. The lack of antagonism of KEO towards CaM [20] supports this view. Although the three cell lines investigated differed substantially in regard to ER concentrations, our data do not suggest any defect at this level as to the origin of the lower growth sensitivity of LY-2 and RTx6 cells to OH-TAM and KEO. Thus, all cells responded to E_2 by a significant increase in growth rate and PgR concentration and their ER binding affinities for both AEs were also in the same range. A difference in AEBS contents should also be rejected in view of the fact that RTx6 cells were totally devoid of such sites. However, AEBS concentration or more precisely the AEBS:ER ratio may modulate the growth sensitivity of the cells to AEs under estrogenic stimulation as suggested by the different behavior of RTx6 and LY-2 cells in the presence of E_2. In this regard, LY-2 cells appear more "antiestrogen resistant" than RTx6 cells since they display only a marginal growth inhibition in the presence of OH-TAM as well as KEO, the latter being effective at the highest concentration assessed in agreement with its lower binding affinity for AEBS [31]. Thus, as already suggested [16], high AEBS contents may reduce the impact of AEs on growth; this property would not hold for PgR regulation in view of the similar behavior of LY-2 and RTx6 cells.

Direct correlation between growth responsiveness to AEs and PgR regulation was not found, indicating that both events are independently modulated by AEs. This absence of relationship is not surprising in view of the fact that the regulation of cathepsin-D and pS2, two other estrogen-regulated proteins, was also shown not to play a key role in the antiestrogen-resistant growth of LY2 cells [32]. Expression of the genes coding for these proteins may be differently regulated by AEs in antiestrogen-resistant variants and wild type cells [33, 34].

Of the two AEs tested on the three cell lines, KEO appears the stronger antagonist on both growth and PgR synthesis due most probably to its higher binding affinity for ER (on the basis of RBA values, KEO binds to ER with a 5–10-fold higher affinity than OH-TAM) [35]. The implication of ER in the mode of action of these drugs seems, therefore, of prime importance, at least in the range of concentrations used in our experiments.

The ER-independent inhibitory effects of triarylethylene AEs have been attributed, among other hypotheses, to their CaM antagonistic activity. Furthermore, such compounds have been shown to impede the association between ER and CaM [18] which regulates ER binding activity [19]. The data reported here show identical characteristics of the ER from the three cell lines in regard to CaM, indicating that neither this association nor the inhibition of CaM-mediated intracellular signal-transduction are responsible for the OH-TAM resistance of RTx6 and LY2 cells.

Mammary tumors are characterized by a gradient in estrogen sensitivity dependent both on ER levels and various intra- and extracellular factors [36]. If we assume that such a property also holds for AEs, a total lack of sensitivity to such drugs should be a relatively rare event. The conditions required for such behavior were actually not present here; we can obviously not exclude a higher degree of resistance under other experimental conditions. Investigating the sensitivity of RTx6 and LY-2 cells to AEs in the presence of various growth modulators [36, 37] might be an attractive avenue to pursue. Among such modulators, TGFβ seems a growth factor of choice since it displays growth inhibitory properties and its secretion has been shown to

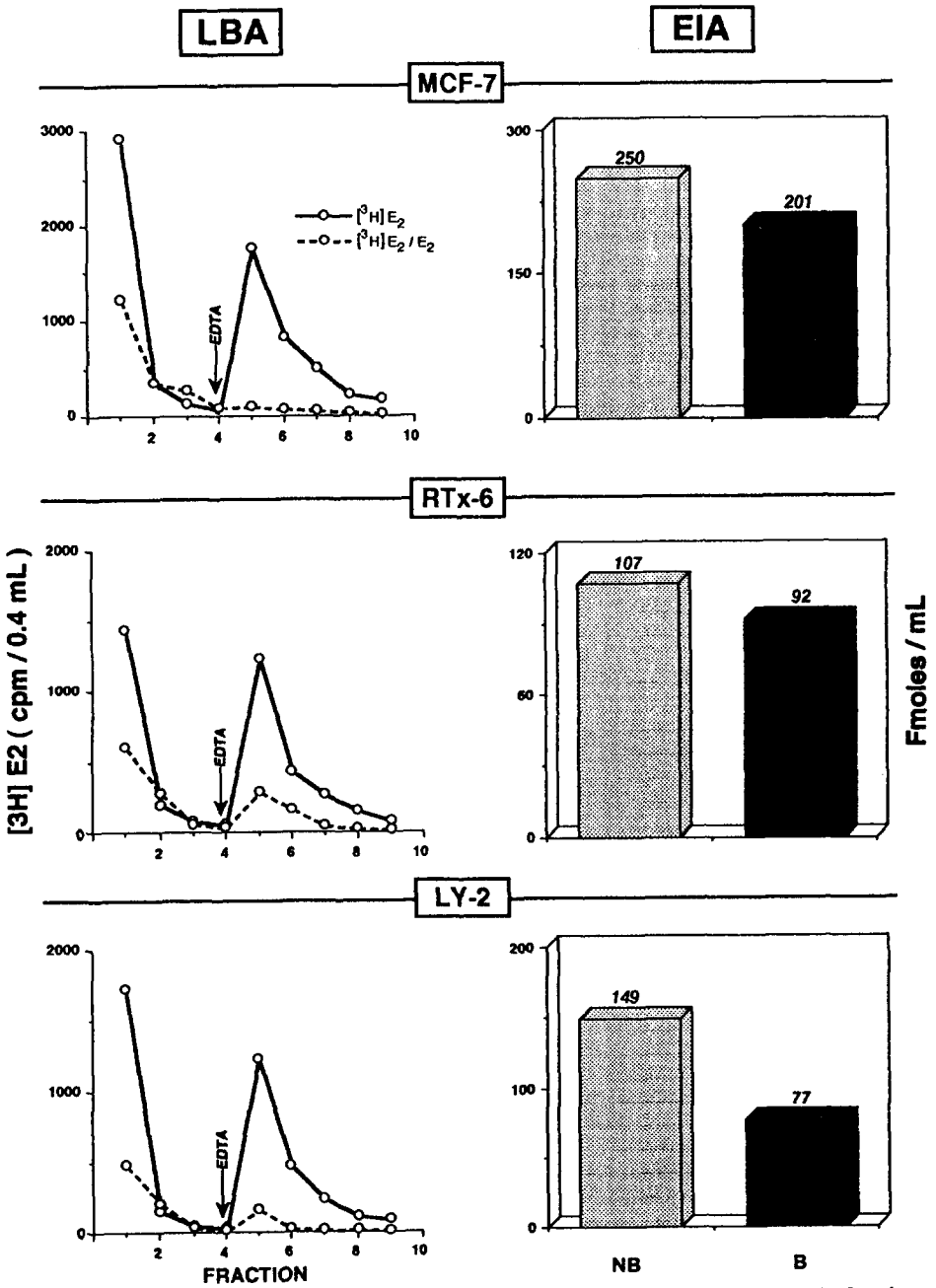

Fig. 5. Adsorption of [^{3}H]E_2 labeled ER to CaM–Sepharose of MCF-7, RTx6 and LY-2. Graphs on the left refer to the radioactivity of each elution fraction [Ligand Binding Assay (LBA)]; graphs on the right to the enzyme immunoassay (EIA) of main elution fractions. Samples of labeled extract of each cell line were adsorbed on CaM–Sepharose and eluted with EDTA (arrow); ER contents of the elution fractions are given by the difference between the radioactivity of the samples labeled with [^{3}H]E_2 alone (full line) and the excess of unlabeled E_2 (dotted line). The EIA column refers to both not bound (NB) and bound (B) ER contents released with EDTA from CaM–Sepharose.

increase under AE administration in MCF-7 cells while this effect was not observed in LY-2 cells [38].

In summary, our results confirm that RTx6 and LY-2 cells are more resistant than their parent MCF-7 to conventional AEs, although their degree of resistance is relatively low. They reject the hypothesis of an alteration of ER and CaM leading to a strong decrease in affinity for these compounds. Moreover, they suggest that AEBS contents may regulate an inhibitory effect of such AEs on estrogenic growth stimulation without affecting their antagonistic activity on the induction of PgR. Finally our data indicate that the assessment of cell growth by itself may lead to incorrect interpretation with regard to

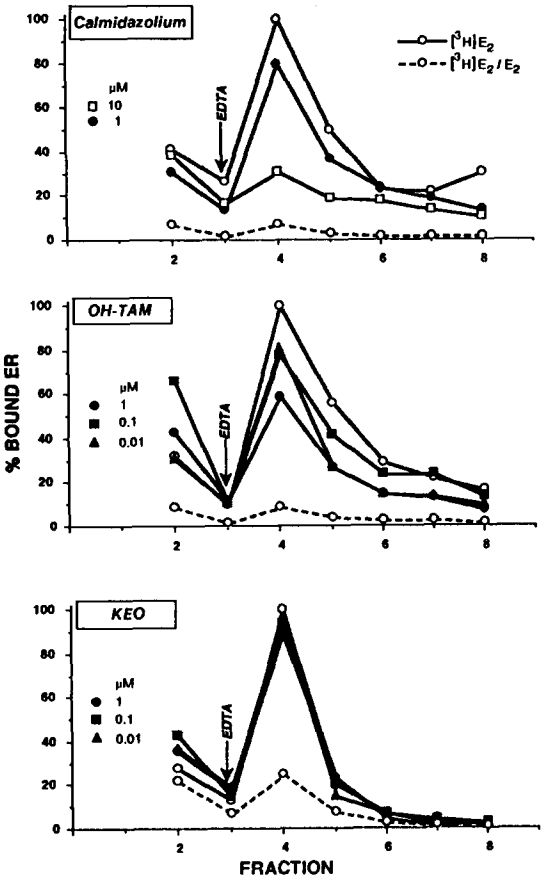

Fig. 6. Antagonism of calmidazolium, OH-TAM and KEO on the adsorption of $[^3H]E_2$ labeled ER from MCF-7 cells to CaM–Sepharose. Each compound was added to the $[^3H]E_2$ labeled cytosol sample before chromatography at the concentrations indicated on the graph. Experimental conditions and analysis of the data are the same as in Fig. 5. Data are expressed as a percentage of the highest level of radioactivity released with EDTA (control values). The figure shows that both calmidazolium and OH-TAM impede the adsorption of ER to CaM–Sepharose while KEO is totally ineffective in this regard.

AE resistance; a study of other markers of estrogen sensitivity such as PgR, pS2 and cathepsin-D is required to define the degree of resistance to such drugs and help to understand the underlying mechanisms.

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