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# STUDY OF AN ANTIESTROGENIC EFFECT OF RETINOIC ACID IN MCF-7 CELLS

EDIZ DEMIRPENCE [1], MICHEL PONS [1], PATRICK BALAGUER [1,2] AND DIDIER GAGNE [1,3]

[1]INSERM U 58, 60 rue de Navacelles, 34090 Montpellier, France

[2]LGME-CNRS, INSERM U 184, 11 rue Humann, 67085 Strasbourg, France

[3]Laboratoire de Biochimie II, Faculté de Pharmacie, avenue Charles Flahaut, 34060 Montpellier Cédex 01, France

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**SUMMARY:** We recently developed the cellular model MVLN-15 in which estrogenic action can be detected by bioluminescence. Using this cellular model, we characterized the inhibitory effect of retinoic acid on the estrogen-dependent induction of luciferase transcription. We present evidence that i) the inhibitory effect of retinoic acid is not due to a simple competition between retinoic acid and estradiol for binding to the estrogen receptor, ii) a DNA sequence restricted to an estrogen-responsive element (ERE) was sufficient for the antiestrogenic effect of retinoic acid, and iii) retinoic acid does not act via a cryptic AP-1 binding site associated with this ERE. Therefore, we conclude that the antiestrogenic effect of retinoic acid is due to an inhibition of estrogen receptor activity, for example by altering the amount of estrogen receptor protein bound to the ERE or affecting the transcriptional efficiency of this complex. 

Steroid hormone receptors represent a sub-class in the superfamily of nuclear receptors, which includes those for thyroid hormones, retinoic acid, vitamin D and several "orphan" receptors (1-3), and regulate gene transcription in a ligand-dependent manner. They recognize specific DNA sequences called hormone-responsive elements (HRE) and modulate the transcription of cognate-target genes resulting in a wide variety of developmental and physiological effects.

Previously, we have established a stable estrogen-dependent expression system which allowed us to detect estrogenic activity by assaying the easily measurable reporter enzyme activity of the firefly luciferase (4). This cell system, called MVLN-15 cells, was obtained by transfecting with the pVit-tk-Luc plasmid, the breast cancer cell line MCF-7 which contains high levels of the estrogen receptor (ER). In this reporter gene, the 5' flanking region of the *Xenopus* vitellogenin A2 gene (Vit), which contains an estrogen-responsive element (ERE) linked to the *herpes simplex* virus promoter for thymidine kinase (tk), controls transcription of the firefly luciferase gene

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(Luc) (5). In investigating various aspects of the MVLN-15 model, we found that retinoic acid (RA) was able to inhibit the estrogen-induced transcription (and also, the estrogen-dependent stimulation of cell growth). Here, we report our study aimed at understanding the mechanisms involved in this phenomenon.

## MATERIALS AND METHODS

Chemicals, materials, cell culture: materials for cell culture as well as for obtention of the stable transfectants MVLN 15-C7 cells (called here MVLN-15) have been described elsewhere (4). All the tested cell lines (MVLN-15, MCF-7 and Hela) were cultured with DMEM without phenol red and supplemented as described (4). Experiments were performed in 6-well tissue culture cluster plates with cells cultured in the medium supplemented with 3% dextran-coated charcoal-treated fetal calf serum (DCC-FCS) instead of FCS as in routine culture. DCC-FCS was prepared as previously described (4). 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), staurosporine, all-trans retinoic acid (RA), luciferin and estradiol were purchased from Sigma Chemical, St. Louis. 4-Hydroxy-tamoxifen (OH Tam) was from ICI. Experiments with RA were performed under subdued yellow light.

Transient transfections and plasmids used: all plasmids were constructed according to standard protocols for recombinant DNA technology (6). The pVit-tk-Luc and pRSV-Luc plasmids have been previously described (4, 5). The p17M-ERE-βG-Luc was made from pSV40LAΔ5' (5) and p17M-ERE-βG-CAT (7), the $p(17M)_5$-βG-Luc contains 5 repeats of the 17M sequence. The enhancer sequences 17M-ERE and $(17M)_5$ were placed in front of the β-Globine (βG) promoter upstream of the luciferase (Luc) gene. A plasmid pGal-ER (pGal-ER 147-282 (8)) coding for a chimæric Gal-4-estrogen receptor was used to induce the transcription of the Luc gene through the 17M sequence. In the $p(TRE)_3$-tk-Luc plasmid, the firefly luciferase gene is driven by 3 repeats of the AP-1 binding site (9) in front of the *herpes simplex* virus promoter of thymidine kinase (tk). Transfection experiments were performed in MCF-7 and HeLa cells according to the calcium phosphate procedure as described (6).

Luciferase assay: at the end of incubation of cells with various compounds, the culture medium is removed and the cells were extensively washed with 2 x 5 ml of cold luminescence buffer (15 mM potassium phosphate, 8 mM $MgCl_2$, pH 7.4). One ml of the same buffer containing 2 mM ATP was then added and the cells were harvested by scraping, and then disrupted by sonication. The corresponding luciferase activity was determined on an aliquot fraction (0.7-0.9 ml) by evaluation of the luminescence peak (for an integration time of 15 sec), following the injection of 0.1 ml of 1 mM luciferin. Results were expressed as arbitrary units per mg of proteins. Protein assays were performed according to Lowry's method on an aliquot fraction (0.05-0.1 ml) of the cellular homogenate (10).

Binding experiment in intact cells: after incubation of MVLN-15 cells with 1 μM RA for 24 or 48 hrs (80-100 x $10^6$ cells for each time), cells were pooled, washed in phosphate-buffered saline (PBS) and then resuspended in the culture medium (without serum). As previously reported (11), the binding experiment was performed by incubating intact cells at 37°C for 1 hr with various concentrations of [$^3$H]-estradiol (2.5 TBq/mmol, final concentration: 0.1, 0.3 and 1 nM) in the presence or absence of a 100-fold excess of [$^1$H]-estradiol. At the end of the incubation, free hormone was removed by extensive washings of the intact cells before disruption by sonication. The radioactivity corresponding to the bound hormone was assayed by liquid scintillation and results were treated by Scatchard analysis (12). The cell number was evaluated by assaying the protein concentration in the samples (10).

## RESULTS AND DISCUSSION

### Retinoic acid inhibits estrogen-induced transcription in MCF-7 cells.

As previously described (4), MVLN-15 cells respond to estrogen by expressing luciferase with a dose response independent of either the incubation time with estradiol ($E_2$) (24 or 48 hrs) or the experimental conditions used to measure luciferase activity (in cellular extracts or in intact

cells). The maximal response (approx. 22500 molecules of luciferase per cell) was ca. 4-5 times above the basal level. The $EC_{50}$ was ca. 20-30 pM $E_2$ and is in agreement with natural estrogenic responses previously described in MCF-7 cells such as cell growth or induction of progesterone receptor (13). As expected, the antiestrogen 4-hydroxy-tamoxifen inhibited the estradiol-induced luciferase (4).

An incubation of 48 hrs of MVLN-15 cells with retinoic acid (RA) led to a dose-dependent inhibition of the luciferase induced by various $E_2$ concentrations (figure 1A). An even more dramatic effect was observed in MCF-7 cells transiently transfected with pVit-tk-Luc (figure 1B). Thus, we conclude that the RA effect is not dependent on the site of integration of the pVit-tk-Luc plasmid in MVLN-15 cells. The antiestrogenic effect was not reversed by high concentrations of $E_2$ (10 nM) (figure 1A) and RA further increased the inhibitory effect of 4-hydroxy-tamoxifen (data not shown). Interestingly, we found the antiestrogenic effect was dependent on the incubation time with RA (34% and 65% inhibition were obtained for 24 and 48 hour incubations respectively) but not on the incubation time with $E_2$. Taken together, these results suggest that the inhibitory effect of RA on estradiol-induced luciferase is not mediated, as for a classical antiestrogen, by a simple competition of RA with estradiol for binding to the ER. The RA concentrations required to exert the described antiestrogenic effects were high in view of the reported $K_D$ for RAR ($3\ 10^{-10}$ M, (14, 15)). This could be in part due to the presence of extra- and intra-cellular RA-binding proteins (16-18). Alternatively, RXRs which have a much lower affinity for RA than do RARs (19), may be involved in this non-classical antiestrogenic activity.

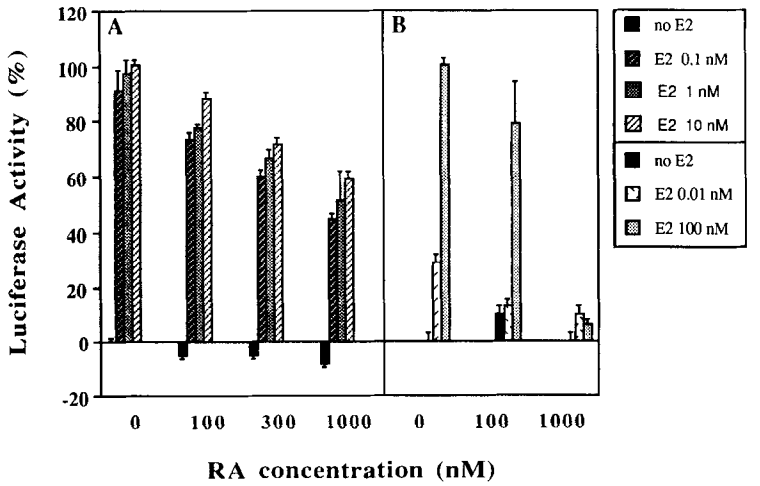


**Fig. 1. Inhibitory effect of retinoic acid on the estradiol-induced luciferase in MVLN-15 and MCF-7 cells.** Cells were incubated for 48 hrs with various concentrations of E2 in the absence or presence of various concentrations of RA. Control cells received the carrier alone (0.3% ethanol). At the end of the incubation, the luciferase activity was determined as described in materials and methods. The luciferase activity is expressed as percentage (mean ± SEM of triplicates) of the mean luminescence value (luciferase quantity / mg protein) induced by 10 nM E2. The left side of the figure corresponds to experiments done with MVLN-15 cells (A), while the right side corresponds to MCF-7 cells transiently transfected with pVit-tk-Luc (B).

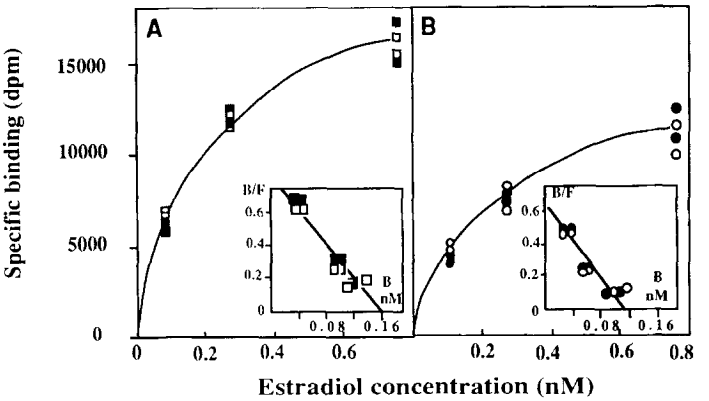


**Fig. 2. Specific [$^3$H] estradiol binding to the estrogen receptor in intact MVLN-15 cells.** The insets represent the Scatchard plots of control cells (A) and of cells treated with 1 μM RA (B), and incubated for 24 hrs (open symbols) or 48 hrs (closed symbols).

**Retinoic acid does not alter the binding parameters of estradiol for the estrogen receptor.**

We investigated whether the binding parameters of $E_2$ for ER could be altered after RA exposure.To maintain cellular integrity, the binding was measured in intact cells. Figure 2 shows saturation curves and Scatchard plots obtained by incubation of MCF-7 cells with 1μM RA for 24 and 48 hours. This treatment does not significantly affect the affinity of ER to bind its cognate ligand since a similar $K_D$ was obtained whatever cells were previously treated or not with RA ($K_D$ = 0.2 nM). As reflected by the $B_0$ value, a decrease of 15% in the cellular estrogen binding activity can be evaluated after correction for the amount of cells. However, this decrease can not explain the antioestrogenic effect of RA since the remaining ER (85 %) is sufficient to induce the luciferase expression to a level higher than 95%.

**The antiestrogenic effect of retinoic acid is specifically associated with the estrogen receptor activity.**

As shown in table 1, 1μM RA inhibited estrogen-induced luciferase activity only in MCF-7 cells which were transfected with a reporter plasmid containing an ERE (compare line 1, 2, 4 ($ERE^+$) and line 3, 5 ($ERE^-$)). This is most obvious from a comparison of the two reporter genes, p17M-ERE-βG-Luc (line 4) or p(17M)$_5$-βG-Luc (line 5). While ER-E2-induced activity of p17M-ERE-βG-Luc could be inhibited by RA, no such inhibition was observed when the C-terminal part of ER, containing the HBD (hormone binding domain) and a transcriptional activation function (7, 8, 20), linked to the heterologous Gal 4 DBD (DNA binding domain), was used as the activator of the cognate p(17M)$_5$-βG-Luc reporter gene (line 5). This shows together with the absence of any post-transcriptional effect of RA on the estrogenic luciferase expression, that the C-terminal TAF-2 is not the target of the RA induced suppression of transcription (as it was suggested for hydroxy-tamoxifen (7, 8, 20)).

**There is no cryptic AP-1 binding site in the ERE of Vit part.**

Schüle *et al.* recently observed that RA can act as a negative regulator of AP-1-responsive genes (21), and suggested that this could occur via the formation of a nonproductive complex between RAR and c-Jun. Since the ERE could be structurally related to an AP-1 binding site (22),

**Table 1. Effect of 1 μM retinoic acid (RA) on various systems of luciferase expressions placed or not under estrogenic control.** Except for line 2, all experiments were performed using transient transfection of MCF-7 cells with one or two plasmids as indicated (0.5 μg of the reporter plasmid ± 0.15 μg of the pGal-ER per 0.5 $10^6$ cells). Luminescence levels (Luciferase), expressed as a percentage, are the mean of at least 3 values ; the 100% value corresponds to at least 1000 luminescence arbitrary units (or 100 fold the background value of the luminometer). Induction factor (Ind. Fact.) is the ratio of the luminescence in the presence of 10 nM estradiol to the basal level in the absence of the hormone.

| line | plasmid | Cotransfected plasmid | Induction conditions | RA - Luciferase | RA - Ind. Fact. | RA + Luciferase |
|---|---|---|---|---|---|---|
| 1 | pVit-tk-Luc (transient) | | Estradiol | 100 ± 3 | 5.2 | 15 ± 3 |
| 2 | pVit-tk-Luc (stable) | | Estradiol | 100 ± 15 | 5.4 | 50 ± 5 |
| 3 | pRSV-Luc | | no | 100 ± 14 | - | 96 ± 16 |
| 4 | p17M-ERE-βG-Luc | | Estradiol | 100 ± 12 | 4.5 | 55 ± 2 |
| 5 | p(17M)5-βG-Luc | pGal-ER | Estradiol | 100 ± 10 | 100 | 91± 10 |

an hypothesis on the mechanism of RA inhibition could result from this homology of sequence. Therefore, we investigated the possibility of luciferase induction from the Vit-tk-Luc gene by TPA (12-*O*-tetradecanoyl-phorbol 13-acetate), a classical activator of the AP-1 system via the protein kinase C (PKC). However, in MVLN-15 cells the luciferase activity was not stimulated either by 5 nM or by 50 nM TPA (figure 3). In agreement with this result, the presence of 50 nM staurosporine (an inhibitor of PKC) did not inhibit the estradiol induced luciferase, showing that the ERE did not function as an AP-1 binding site. As a control, TPA (at 5 and 50 nM) efficiently induced luciferase activity in MCF-7 cells transiently transfected with p(TRE)3-tk-Luc, a TPA-

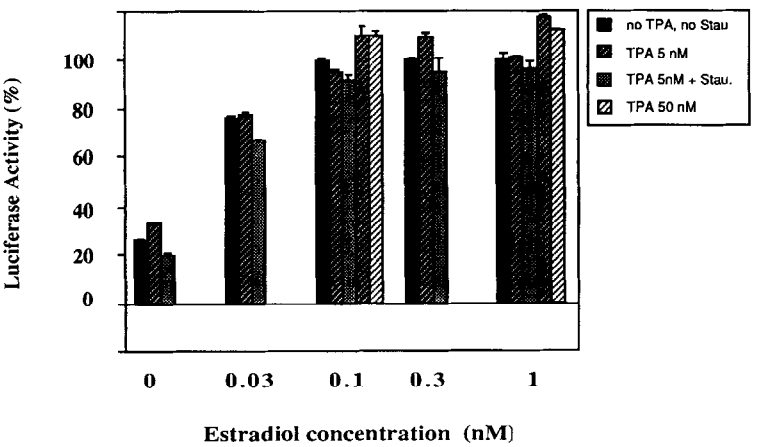


**Fig. 3. Examination of potential AP-1 binding site in the pVit-tk-Luc plasmid.** MVLN-15 cells were incubated for 24 hrs with different estradiol concentrations (0, 0.03, 0.1, 0.3 and 1 nM) alone or in presence of TPA (5, 50 nM) with or without 50 nM staurosporine (Stau) ; control cells received the carrier (0.15% ethanol + 0.15% DMSO). At the end of the incubation, the luciferase activity was determined as described in materials and methods. The luciferase activity was finally expressed as percentage (mean ± SEM of triplicates) of the mean luminescence value (luciferase quantity / mg protein) obtained with 1 nM E2 while the 0% was the mean luminescence value (luciferase quantity / mg protein) obtained with the luminescence buffer.

responsive plasmid, and this induction could be inhibited by 50 nM staurosporine (data not shown). This result was confirmed by transtient transfection of the ER-negative HeLa cells known to contain endogenous RAR and Jun protein by pVit-tk-Luc: no luciferase induction was observed after addition of 5 or 50 nM TPA (data not shown).

Taken all together, these data suggested that the antiestrogenic effect of RA is due to an inhibition of estrogen receptor activity, for example by altering the amount of estrogen receptor protein bound to the ERE or affecting the transcriptional efficiency of this complex. This conclusion is supported by previous studies concerning the functional interrelations between nuclear receptors of the ER/TR subfamily. Thus, retinoic acid receptor (RAR) was shown to be an efficient inducer of transcription from a vitamin D responsive element (VDRE) (23) and from two distinct thyroid-hormone responsive elements (TRE) (24, 25). Interactions between RAR and thyroid hormone receptor (TR) may lead to positive or negative effect depending upon the nature or organization of the TRE sequences (25-27). ER was also reported as an efficient activator of the palindromic TRE and this induction can be repressed by TR in the absence of its ligand (28). A competition between TR and ER for the same ERE was also proposed to explain a negative regulation by thyroid hormones (29) and this led us to hypothesize the existence of a competition between RARs (or RXRs) and ER for the ERE as it was recently suggested (30, 31). Work is now in progress in our lab to determine which retinoic acid receptor (RARs, RXRs) is involved in the antiestrogenic effect presented in this paper and by which mechanism (32).

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