Paradoxical interactions among estrogen receptors, estrogens and SERMS: mutual annihilation and synergy

A.M. Kaye a,*, M. Spatz b, A. Waisman a, S. Sasson c, S. Tamir d, J. Vaya d, D. Somjen e

a Department of Molecular Genetics, The Weizmann Institute of Science, PO Box 26, Rehovot 76100, Israel
b Pharmos Ltd, Kiryat Weizmann, Rehovot 76326, Israel
c Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University, Jerusalem 91120, Israel
d Migal—Galilee Technological Institute, Kiryat Shmona, Israel
e Institute of Endocrinology, Tel Aviv Sourasky Medical Center and the Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 64239, Israel

Abstract

The phenomenon of mutual annihilation of action between 17β estradiol (E2) and a selective estrogen receptor modulator (SERM), previously described in prepubertal rat diaphysis, epiphysis and uterus, has been investigated in ROS 17/2.8 rat osteoblastic cells and in transiently co-transfected cells in culture. In ROS 17/2.8 cells, the estrogen-induced marker enzyme creatine kinase B (CKB) was stimulated by raloxifene, tamoxifen and tamoxifen methiodide to a specific activity equal to or greater than that induced by 10 nM E2. However, when a fully inhibitory dose of any of these SERMS was given simultaneously with E2, no stimulation of CK activity resulted. Therefore, SERMS can be full agonists when acting alone, but complete antagonists to a super-physiological dose of estrogen. It is expected that excess tamoxifen would prevent the action of a SERM, but that the agonist activity of a SERM is abolished by 1000-fold less estrogen is a phenomenon without obvious explanation by classical pharmacology of competitive inhibition. To probe the mechanism of this interaction further, a ckb-CAT reporter plasmid, plus the human receptor expression plasmid, HEO, was transfected transiently into several cell types. In MCF-7 cells, a 1:10 ratio of E2 to tamoxifen produced mutual annihilation, but the same ratio in ROS 17/2.8 or HeLa cells led to synergistic stimulation. In HeLa cells, co-transfected with the more efficient wild-type estrogen receptor plasmid, HEGO, synergy was demonstrated only at sub-saturation levels of HEGO. We speculate that, in the presence of estradiol and a SERM, not only active homodimers would be formed, but also hetero-dimers of estrogen-liganded and tamoxifen-liganded receptor monomers, depending on the molar ratio of their ligands and their relative affinities. The resulting hetero-dimer conformation would change the specific receptor surface for interactions with the growing number of co-activators and co-repressors, structural changes which could help to explain the mutual annihilation and synergy phenomena and their cell selectivity. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Interactions between estrogens of different potency and between estrogens and selective estrogen receptor modulators (SERMS) [1,2] in cells or tissues have both physiological and therapeutic importance. These interactions occur in diverse areas related to hormonal regulation, including during the third month of pregnancy, when the concentration of estriol (E3) rises markedly [3,4]; in the successful use of tamoxifen against breast cancer [1,2,5], and in the evaluation of the significance of dietary phytoestrogens as a possible contributor to the lower incidence of cancer in certain populations [6,7].

In addition to the large and growing literature on the inhibition of estrogen activity in breast cancer by drugs designed as anti-estrogens, several SERMS, such as...
tamoxifen and raloxifene, have been shown to act as effective estrogen agonists in skeletal and other tissues, in the absence of high concentrations of 17β-estradiol (E2) [1,8]. However, data from experiments in prepubertal rats show that, concomitant with the inhibition of E2 activity by the SERM, estradiol also inhibits the considerable stimulatory activity of the SERM. We have called this phenomenon ‘mutual annihilation’ [9]; examples have also been found in cultured cells [10] and in cell-free transcription experiments [11]. On the other hand, in transient co-transfection experiments, we found synergy between E2 and tamoxifen [12].

The candidate cancer-inhibiting ligand, enterolactone [10] and E2 were found by Mousavi and Adlercreutz [10] to show mutual annihilation in the stimulation of growth of MCF-7 human breast cancer cells in culture. Enterolactone, a diphenol produced by intestinal bacteria from plant precursors, stimulated cell growth, at a concentration of 1 μM, to the same extent as 1 nM E2. However, when enterolactone and E2 were both present, there was no stimulation of growth. Since similar concentrations of enterolactone can be found in vivo [13], this mutual annihilation may have physiological significance.

The ‘weak’ or ‘short-acting’ estrogen, E3 [4] interacts with E2 in a cell-free system to produce a situation approaching mutual annihilation of transcriptional activation [11]. E3, at a concentration of 1 μM, plus one tenth of this concentration of E2 (0.1 μM), stimulated cell-free transcriptional activity to less than one fifth of the activity of either 0.1 μM E2 or 1 μM E3 alone. At a higher concentration of E3 (ratio 50:1 to E2) the mutual antagonism was no longer observed. This result is of particular interest because the concentration of E3 is ten times that of E2 in late pregnancy [3,14], which is consistent with the antagonistic effect of E3 playing a role in modulating the agonistic effect of E2 [4]. The mechanism proposed to explain this paradox is the formation of inactive hetero-liganded receptor dimers. When both monomers bind the same ligand, the resulting dimer is active, but the higher the concentration of hetero-liganded dimers that form, greater is the loss of agonist activity of E2. As the concentration of E3 increases, a nadir is reached at the point of 10-fold excess of E3, when the expected agonist action almost completely disappears. A conformationally distorted mixed estradiol–estriol estrogen receptor (ER) dimer was postulated [11], which would be incapable of binding an estrogen response element (ERE); and indeed, no such bound complex was detected by electrophoretic mobility shift assay at high ER concentration [11]. Hetero-liganded dimers have also been considered, for example, for the progesterone receptor interacting with a progestin agonist and an antagonist [15].

We have postulated mixed dimer formation as the unifying concept to explain estrogen receptor paradoxes [9]. As a marker of estrogen stimulation, we used creatine kinase (CK) activity, in vivo and in cell culture [8], and the induction of CAT activity by the 5′ flanking region of CKB in co-transfection experiments [12]. The brain-type (B) isozyme of CK was identified [16] as the estrogen-induced protein (IP) discovered by Notides and Gorski [17]. This response marker gave us the advantage of using the same gene, a gene with a non-consensus ERE, at −550 [18] downstream from a multiple silencer region [19], at the three levels of organization studied.

The aim of the present study is to focus greater attention on the extremes of the interactions between estrogens, namely, mutual annihilation of activity at the negative extreme, and synergy at the positive extreme, relating to the well-studied anti-estrogenic action of SERMS, such as tamoxifen and raloxifene. We surveyed a range of other estrogenic compounds, particularly phytoestrogens, to define the extent of the phenomena of mutual annihilation and synergy. We found that, in addition to cell type, the concentration and affinity of ERα for E2 are crucial factors in determining the result of interaction between E2 and a SERM.

2. Materials and methods

2.1. Reagents

17β-Estradiol, 17α-estradiol, genistein, daidzein, resveratrol and the UV spectrophotometric creatine kinase assay kit were purchased from Sigma Chemical Co., and estriol from Organon, Oss, The Netherlands, Tamoxifen and ICI 182780 were gifts from Dr A Wakeling (Zeneca Ltd, Macclesfield, UK), Tamoxifen methiodide was a gift from Professor G. Fink (Pharmos Ltd, Rehovot, Israel), and raloxifene was a gift from Dr B. Fournier (Ciba-Geigy Ltd, Basel, Switzerland). 14C-chloramphenicol was purchased from Amersham, Bucks., UK.

2.2. Expression plasmids

PAM 117, containing 2.9 kb of the 5′ flanking region of rat ckb, linked to the CAT reporter gene, was kindly provided by Dr Pamela Benfield (Du Pont Merck, Wilmington, DE, USA). Human estrogen receptor expression plasmids, HEO (PSG5) and HEGO were gifts from Professor P. Chambon (IGBMC, Illkirch, Strasbourg, France).

2.3. Cell cultures

Fetal calf serum (FCS) was purchased from Biological Industries (Beit Ha’emek, Israel). Media were pre-
pared by the Biological Services of the Weizmann Institute of Science.

Osteoblast-like ROS 17/2.8 osteosarcoma cells [20] were cultured in 24 well plates, in 1 ml Dulbecco’s modified Eagle’s medium (DMEM) + F12 medium (1:1), in a humidified 7.5% CO₂ atmosphere.

HeLa human cervical carcinoma cells, MCF-7 human breast cancer cells and HepG-2 human hepatoblastoma cells were grown in 60 or 100 mm culture dishes in DMEM. Media were supplemented with 10% FCS, and cells were grown at 37°C in a humidified 7.5% CO₂ atmosphere.

2.4. CK extraction

Cells were scraped from culture dishes and homogenized by freezing and thawing three times in cold isotonic extraction medium [21]. Supernatant extracts were obtained by centrifugation of the homogenates at 14000 × g for 5 min at 4°C in an Eppendorf microcentrifuge. Rat organs were collected in cold isotonic extraction buffer, homogenized in a Polytron homogenizer (Kinematica AG, Littau, Switzerland), and enzyme extracts were obtained as for the cells (see above). CK activity was measured in a Kontron Model 922 Uvicon spectrophotometer at 340 nm, using a Sigma coupled assay kit. Protein was assayed by Coomassie brilliant blue dye binding, using BSA as the standard [22].

2.5. Transient co-transfection

Cells were seeded 1 day before transfection at 10⁶ cells per 10 cm culture dish, or 4 × 10⁵ cells per 6 cm culture dish, into appropriate medium (see Section 2.3), supplemented with 10% dextran-coated, charcoal-stripped FCS. Cells in 10-cm plates were co-transfected according to Gorman [22], with 4 μg PAM 117, 4 μg HEO, 2 μg luciferase expression plasmid as an internal reference for transfection efficiency, and Bluescript DNA, to maintain a constant DNA concentration (as indicated in the figure legends). For cells in 6-cm plates, 2.4 μg HEO and 1.2 μg luciferase expression plasmids were used. Each experiment consisted of a minimum of duplicate independent calcium phosphate precipitations.

After 40–44 h incubation, cells were washed in ice-cold phosphate-buffered saline (PBS), and harvested into 1 ml extraction buffer (0.1 M KPO₄, 1 mM DTT), precipitated by centrifugation and re-suspended in 60 μl extraction buffer. Cells were lysed by three freeze–thaw cycles and centrifuged at 14000 × g for 20 min at 4°C. The supernatant solutions were then assayed for luciferase activity [24], using a luminometer (Lumac 13M biocounter M 2010A, Ilpen). Samples of equal luciferase activity (≤ 50 μl) were assayed for CAT activity [23], using phase extraction [25,26].

2.6. Statistical analysis

The significance of differences between experimental and control means was evaluated using Student’s t-test.

3. Results and discussion

3.1. Mutual annihilation in ROS 17/2.8 cells

In the light of our finding of mutual annihilation of the stimulation of CK activity [8] in prepubertal rat organs (summarized in Fig. 1), we used ROS 17/2.8 cells in culture as a model for the osteoblasts in diaphyseal bone [27], which contain estrogen-responsive CKB [28]. The in vivo study had revealed that, in all cases in which estradiol (10 nM) or a SERM (1 μM) stimulated
ROS 17/2.8 osteoblastic osteosarcoma cells in culture are faithful models of osteoblasts in vivo [20], and show stimulation of CK activity in response to E2 [31,32] and other estrogens and SERMS [8,28]. Their response to E2 reaches a plateau in the range 10–300 nM (Fig. 2); hence 10 nM was chosen as the standard E2 concentration for comparison. All the three SERMS tested showed dose-dependent stimulation of CK activity when tested alone, reaching an activity equal to, or greater than, that of E2 at concentrations 10–30 times higher (Fig. 2). In the presence of 10 μM E2, all the three SERMS showed dose-dependent inhibition of E2 stimulation, which became significant at 10–30 times the concentration of E2. Mutual annihilation of stimulation, equal to the vehicle control value, was seen at 100–300 times the E2 concentration. These high concentrations of SERMS, which are so effectively antagonized by 10 nM E2, showed no reversal of mutual inhibition at the highest concentration tested, as had occurred previously in the case of E3 [11]. Therefore, we compared the interaction of E2 and E3, in the cell culture model, with a range of other estrogens with a lesser affinity for ERα which can be compared with E3 (Fig. 3).

Significant increases in CK specific activity were caused by 1 μM doses of E3, resveratrol [33] (the phytoestrogen found in high concentration in the skin of red grapes, and proposed as a cardio-protective factor contributing to the ‘French Paradox’), the phytoestrogens genistein and daidzein [34] and quercetin [35], which produced the highest specific activity of all the ligands tested, despite acting as a pure anti-estrogen in MCF-7 cells [35]. The triphenyl ethylene derivative, ICI 182780, designed to be a ‘pure’ anti-estrogen, indeed showed no significant stimulation of CK activity, and inhibited the agonistic action of E2. None of the other compounds showed any significant effect on E2 stimulation of CK activity, except for daidzein, which caused higher CK activity when in combination with E2 (Fig. 3). Thus, none of the compounds tested (Fig. 3) exhibited mutual annihilation of activity when present at 100 times the E2 concentration of 10 nM.

This comparison indicates the high specificity of the system in which the phenomenon of mutual annihilation is found, in terms of estrogenic compounds and in terms of other parameters of a system, such as the level of organization and the parameter tested. This is exemplified by E3, which exhibited mutual annihilation with E2 in a cell-free transcription system [11], but not in ROS 17/2.8 cells at an E3:E2 ratio of 100:1 (Fig. 3), nor at any ratio in the range of 0.3:1 to 1000:1 (not shown).

Another example of differences in interactions in diverse systems involved glabridine, an isoflavone...
derived from licorice root [36] which is estrogenic in MCF-7 cells. At a concentration of 1 µM, it also significantly stimulated CK activity in ROS 17/2.8 cells, without any significant augmentation of stimulation by E2 (not shown). However, in vivo, glabridine exhibited mutual annihilation with raloxifene in prepubertal rat diaphysis and epiphysis, showing that mutual annihilation of estrogenic activity is not restricted to pairs of ligands that include E2.

### 3.2. Specificities in mutual annihilation and synergy between estrogens in co-transfection systems

In order to exert some control over the components of the test system for mutual annihilation, we used cell lines co-transfected with an estrogen receptor and an estrogen-responsive marker plasmid [12], aware of the limitations of any transient transfection system. MCF-7 human breast cancer cells contain estrogen receptor α (ERα), low levels of ER-β [37], and respond to estrogen by increased proliferation. When co-transfected with the human estrogen receptor expression plasmid, HEO, and the CK reporter plasmid PAM 117, 30 nM E2 stimulated a several-fold increase in CAT activity, as did 1 µM tamoxifen (Fig. 4). Yet, the simultaneous presence of these concentrations of E2 and the SERM, tamoxifen, gave rise to no stimulation whatsoever, demonstrating mutual annihilation in an additional model system. However, when a parallel experiment was conducted in ROS 17/2.8 rat osteosarcoma cells, in which mutual annihilation was demonstrated (Fig. 2), most unexpectedly the opposite reaction — synergy between E2 and tamoxifen — was revealed (Fig. 4).

In HeLa human cervical cancer cells, in which the stimulation of the CK-CAT reporter by 10 nM E2 can exceed 100-fold, the same synergy between E2 and tamoxifen occurred (Fig. 5). The full-length human ERα, given the name HEO, used in the above experiment, contains a mutation at amino acid 400, in the ligand-binding domain, in which a valine replaced glycine [38]. This mutant, converted to the wild type and named HEGO, exhibited maximal activity at 10–100 times lower concentration than HEO [38]. In the parallel co-transfection assay of HEGO with the CK reporter (Fig. 5, lower row), the CAT activity stimulated by 10 nM E2 was inhibited by 1 µM tamoxifen, indicating that the availability (concentration × affinity for E2) of the ER is a factor in determining the interactions among ER, E2 and a SERM. The properties of the SERM were also critical, since, in the presence of either HEO or HEGO, raloxifene and tamoxifen methiodide — which themselves showed several-fold induction of the CK-CAT reporter, PAM 117 — inhibited E2 activity in all the cases (Fig. 5). The subtlety of the structural changes which determine the nature of the interaction can be seen by comparing the ability of tamoxifen, in the presence of HEO, to exhibit synergy with E2, while the conversion of its amine group to a charged quartenary amine, to form tamoxifen methiodide, prevents its synergy with E2 in this co-transfection system, but not its mutual annihilation of CK activity in the presence of E2 in rat diaphysis or epiphysis (Fig. 1), or in ROS 17/2.8 cells (Fig. 2).

### 3.3. Synergy between E2 and tamoxifen is favored by non-saturating concentrations of ER

When comparison was made in Hep G2 cells of the interactions between E2 and tamoxifen in the presence of HEO and HEGO, neither mutual annihilation nor synergy was found (Fig. 6). However, the differences in

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**Fig. 4.** Diverse responses in co-transfection assays to a combination of 17β-estradiol (E2) and tamoxifen (Tam) by ROS 17/2.8 and MCF-7 cells. ROS 17/2.8 cells in 10-cm dishes were co-transfected as described in Section 2, with 4 µg of an estrogen responsive reporter plasmid (PAM 117) containing 2.9 kb of the 5' flanking sequence of the rat ckb gene linked to the CAT reporter gene, 4 µg HEO human estrogen receptor expression plasmid and 2 µg luciferase expression plasmid. ROS 17/2.8 cells were treated for 40 h with 10 nM E2, 1 µM Tam or both ligands. Relative CAT activity is expressed as the percent conversion of [14C] chloramphenicol, normalized to the internal standard, luciferase. MCF-7 cells were treated for 40 h with 30 nM E2, 3 µM Tam or both ligands. CAT activity was determined as described in Section 2, and expressed relative to vehicle-treated cells after normalization according to luciferase activity.
the ratios of agonistic and antagonistic activities of tamoxifen to the induction of PAM 117 by E2 were striking. Since all the previous comparisons had been made with saturating amounts of co-transfected ER (2.4 µg HEO or 1.0 µg HEGO), we asked whether synergistic interactions of estrogen would be found if HEGO were reduced to sub-saturating levels, to bring its transcriptional efficiency closer to that of HEO.

To determine an appropriate sub-saturating amount of HEGO for co-transfection into HeLa cells, E2 dose–response curves were obtained at three levels of HEGO (Fig. 7). In cultures with 0.4 µg HEGO used for co-transfection, a suitable level of response was found, with a maximum at 10 nM E2 (Fig. 7). This is in contrast to the plateau of nearly three times greater response, shown with 0.6 µg HEGO at E2 concentrations from 0.01 to 1 nM. The shift to lower concentrations of hormone at higher levels of receptor, needed to achieve maximal response, has been studied previously, e.g. in the glucocorticoid system [39]. In this case (Fig. 8), the use of 0.4 µg HEGO permitted the demonstration of synergy between E2 and tamoxifen.

While 1 µM or 100 pM tamoxifen plus 10 nM E2 produced additive stimulation of PAM 117 activity in HeLa cells co-transfected with a limiting amount of wild-type ERα, when the saturating E2 concentration of 10 nM was lowered to 10 or even 1 pM, there was no loss in synergy (Fig. 8). Thus, the combination of 1 pM E2 plus 100 pM tamoxifen showed eight times more stimulation than 100 pM tamoxifen, five times more than 1 pM E2, and 1.4 times more than 10 nM E2 (Fig. 8), the optimal E2 concentration at this co-transfection level of HEGO (Fig. 7).

3.4. An hypothesis: ER co-modulators determine the nature of ER ligand interactions in which hetero-liganded dimers are formed

If hetero-liganded dimers are part of the explanation for mutual annihilation, there must be an involvement of additional components of the final active complex of

Fig. 5. Co-transfection assays of the combination of 17β-estradiol (E2) and SERMS in HeLa cells, in the presence of the wild-type estrogen receptor HEGO or the mutant receptor HEO. Cells in 6-cm dishes were co-transfected as described in Section 2, with 2.4 µg PAM 117, 1.2 µg luciferase plasmid and either 1.0 µg HEGO or 2.4 µg HEO, and treated for 40 h with E2 (10 nM), 1 µM SERM or with both. CAT activity was determined as described in Section 2 and expressed relative to vehicle-treated cells after normalization according to the luciferase internal standard.

Fig. 6. Co-transfection assays of a combination of 17β-estradiol (E2) and tamoxifen (Tam) in Hep G-2 cells in the presence of HEO or HEGO. Cells were co-transfected as described in Section 2 and Fig. 5, with either 1.0 µg HEGO or 2.4 µg HEO, and treated for 40 h with 10 nM E2, with 1 µM Tam or with both ligands. CAT activity was determined as described in Section 2 and expressed as in Fig. 5.
Fig. 7. Shift in optimum estrogen concentration in a co-transfection assay for induction of the ckb promoter, depending on concentration of wild-type estrogen receptor HEGO. HeLa cells were co-transfected as described in Section 2 and Fig. 5, with either 0.6, 0.4 or 0.2 \( \mu \)g HEGO, plus Bluescript DNA, to reach the same total DNA concentration in each dish, and treated for 40 h with E2. CAT activity was determined as described in Section 2 and expressed as in Fig. 5.

Fig. 8. Synergy between 17\beta-estradiol (E2) and tamoxifen (Tam) in stimulation of the ckb promoter. HeLa cells were co-transfected as described in Section 2 and in Fig. 5, with 0.4 \( \mu \)g HEGO, and treated for 40 h with E2, Tam or both. CAT activity was determined as described in Section 2 and expressed as in Fig. 5.

receptor, E2 and SERM, which differ between cell types, such as MCF-7, which shows mutual annihilation (Fig. 4), and HeLa, in which synergy can be demonstrated (Fig. 8).

Natural candidates for such additional interactive proteins and even RNA [40] are the large and growing variety of co-modulators (co-activators and co-repressors) which have been shown to stimulate or repress E2-dependent transcription (see recent review by Klinge [41]). The balance between positive and negative binding factors has been considered a major regulator of transcription and of the ‘residual agonist activity of antagonists’ [42]. At least a dozen different co-activators and three co-repressors have been found for ER\( \alpha \) [41]. Although there are as yet only limited indications of ER selective co-modulators [43,44], and most co-modulators have been found in many tissues, differences in the types and concentrations of co-modulators among cell types will be decisive. In the specific case of ckb, an example of positive and negative regulation of the 5' flanking region contained in PAM 117 was found in co-transfection experiments parallel to those described above. The inhibition of induction by a multiple silencer region, upstream of ERE at – 451 in ckb, was reversed, in a dose-dependent manner, by the addition to the co-transfection system of an expression vector for the co-activator TIF2.

Since the structure of ER\( \alpha \) (most dramatically helix 12) depends on whether the interacting ligand is E2 or raloxifene [45], it may be that each ER ligand induces its characteristic conformation. The dimerization and the resulting co-modulator interacting surface(s) of hetero-liganded ER would vary widely, supporting the full spectrum of interactions. In the extreme negative case, a given pair of estrogens could cause complete mutual inhibition of transcriptional stimulation. Through a broad range of ratios of E2 and SERMS, in the intermediate situation, there would be dose-dependent antagonistic activity of a SERM. The positive extreme of the spectrum, at a favorable concentration ratio of ligands, would result in synergy.

The list of variables which can be involved in the interaction between two estrogens and thereby determine the nature of the resulting activity include:

1. The ligand: its availability within the cell, its metabolism (which determines its half-life) and its structure and resulting affinity for ER.

2. The nature and concentration of the receptors present. Both E2 and SERMS may modulate ER concentrations. Although, initially, it was natural to consider hetero-dimerization of ER\( \alpha \) and ER\( \beta \) as part of a mechanism for providing inactive hetero-liganded dimers [9], the mutual annihilation found in MCF-7 cells which apparently have a low concentration of ER\( \beta \) [37], makes it possible that the ER\( \alpha \) ratio can modulate the ER hetero-dimer structure in cell types in which ER\( \beta \) has a significant concentration. However, reports that ER\( \beta \) can act as an inhibitor of ER\( \alpha \) [46], and that it binds raloxifene and genistein in a different manner [47], make it possible that ER\( \beta \) can participate in the phenomenon in cell types in which it has a significant concentration.

3. The spectrum of co-modulators and their relative concentrations, which in turn depends on cell type, differentiation state and hormonal milieu.
4. The gene: all the elements of gene regulation could play a part in determining whether a particular gene would be subject to mutual annihilation and/or synergy, including the sequence of its estrogen response element and its context, its silencer sequences and its upstream regulatory elements.

While this study has concentrated on transcriptional mechanisms to provide explanations, non-nuclear and/or post-transcriptional mechanisms are certainly possible participants. Hopefully, the information required to solve these estrogen receptor paradoxes of mutual annihilation and synergy will contribute to both the basic understanding of how estrogen acts and to the eventual design and clinical utilization of SERMs.

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