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Antiestrogens: Mechanisms and Actions in Target Cells

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Antiestrogens, acting via the estrogen receptor (ER) evoke conformational changes in the ER and inhibit the effects of estrogens as well as exerting anti-growth factor activities. Although the binding of estrogens and antiestrogens is mutually competitive, studies with ER mutants indicate that some of the contact sites of estrogens and antiestrogens are likely different. Some mutations in the hormone-binding domain of the ER and deletions of C-terminal regions result in ligand discrimination mutants, i.e. receptors that are differentially altered in their ability to bind and/or mediate the actions of estrogens vs antiestrogens. Studies in a variety of cell lines and with different promoters indicate marked cell context- and promoter-dependence in the actions of antiestrogens and variant ERs. In several cell systems, estrogens and protein kinase activators such as cAMP synergize to enhance the transcriptional activity of the ER in a promoter-specific manner. In addition, cAMP changes the agonist/antagonist balance of tamoxifen-like antiestrogens, increasing their agonistic activity and reducing their efficacy in reversing estrogen actions. Estrogens, and antiestrogens to a lesser extent, as well as protein kinase activators and growth factors increase phosphorylation of the ER and/or proteins involved in the ER-specific response pathway. These changes in phosphorylation alter the biological effectiveness of the ER. Multiple interactions among different cellular signal transduction systems are involved in the regulation of cell proliferation and gene expression by estrogens and antiestrogens.

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INTRODUCTION: ESTROGEN TARGET TISSUES AND ANTIESTROGEN EFFECTIVENESS

Estrogens influence the growth, differentiation and functioning of many target tissues. These include tissues of the reproductive system such as the mammary gland and uterus, cells in the hypothalamus and pituitary, as well as bone where estrogens play important roles in bone maintenance; and the liver and cardiovascular systems where estrogens influence liver metabolism, the production of plasma lipoproteins, and exert cardioprotective effects [1–3]. Estrogens, in addition to stimulating mammary gland growth and duct development, also increase proliferation and metastatic activity of breast cancer cells [4] and stimulate the proliferation of uterine cells [1]. Antiestrogens, which antagonize the actions of estrogens, therefore have much potential as important therapeutic agents. Our studies have examined the effects of antiestrogens on a variety of target cells including liver [5] and hypothalamus and pituitary [6], but have primarily focused on their effects on breast cancer and uterine cells [7].

The actions of estrogens on breast cancer and uterine cells are antagonized by antiestrogens, which bind to the estrogen receptor (ER) in a manner that is competitive with estrogen but they fail to effectively activate gene transcription [7–9]. Two of the major challenges in studies on antiestrogens are to understand what accounts for their antagonistic effectiveness as well as the partial agonistic effects of some antiestrogens; and to understand how one can achieve tissue selective agonistic/antagonistic effects of these compounds. One of our approaches to addressing these issues has been to try to understand in detail how the ER discriminates between

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estrogen and antiestrogen ligands and between different categories of antiestrogens. This has involved the generation and analysis of variant human ERs with mutations throughout the ER hormone-binding domain and study of the activity of these receptors on different estrogen-responsive genes in several cell backgrounds when liganded with antiestrogen or estrogen. These studies and those of others have provided consistent evidence for the promoter-specific and cell-specific actions of the estrogen-occupied and antiestrogen-occupied ER. In addition, in the studies described below, we have observed that protein kinase activators enhance the transcriptional activity of the ER and alter the agonist/antagonist balance of some antiestrogens, suggesting that changes in cellular phosphorylation state should be important in determining the effectiveness of antiestrogens as estrogen antagonists.

ANALYSIS OF THE ER HORMONE BINDING DOMAIN AND LIGAND DISCRIMINATION

We have examined the interactions of estrogen and antiestrogens with the ER and the modulation of ER activity by phosphorylation and interaction with other proteins which result in changes in ER-mediated responses. Studies by us [10–17] have provided strong documentation that the response of genes to estrogen and antiestrogen depend on four important factors: (1) the nature of the ER, i.e. whether it is wild-type or variant; (2) the promoter; (3) the cell context; and (4) the ligand. The gene response, in addition, can be modulated by cAMP, growth factors, and agents that affect protein kinases and cell phosphorylation [15, 18–21]. These may account for differences in the relative agonism/antagonism of antiestrogens like tamoxifen on different genes and in different target cells such as those in breast cancer cells, versus uterus, versus bone.

Antiestrogens are believed to exert their effects in large measure by blocking the actions of estrogens by competing for binding to the ER and altering ER conformation such that the receptor fails to effectively activate gene transcription. In addition, antiestrogens exert anti-growth factor activities, via a mechanism that requires ER but is still not fully understood [22].

Models of antiestrogen action at the molecular level are beginning to emerge, and recent biological studies as well indicate that antiestrogens fall into two distinct categories: antiestrogens, such as tamoxifen, that are mixed or partial agonists/antagonists (type I), and compounds, such as ICI 164,384, that are complete/pure antagonists (type II). The type I antihormone-ER complexes appear to bind as dimers to estrogen response elements (EREs); there, they block hormone-dependent transcription activation mediated by region E of the

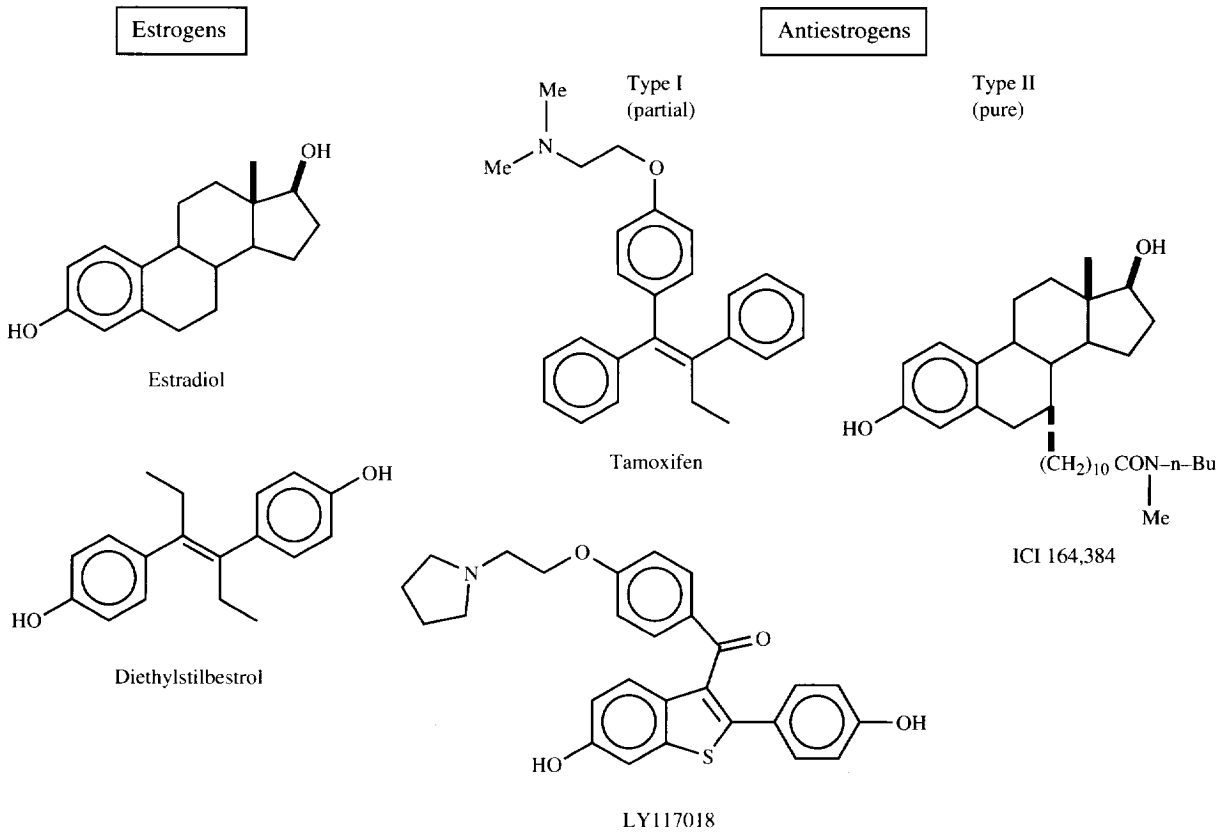

Fig. 1. Structures of several estrogenic and antiestrogenic ligands for the estrogen receptor used in our studies. The antiestrogens include the nonsteroidal compounds tamoxifen and LY117018 that often show partial agonist/antagonist activity (type I antiestrogens) and the steroidal, more pure antiestrogen ICI164,384 (type II antiestrogen).

receptor, but are believed to have little or no effect on the hormone-independent transcription activation function located in region A/B of the receptor [16]. Thus, they are generally partial or mixed agonist/antagonists, and their action must involve some subtle difference in ligand–receptor interaction, very likely associated with the basic or polar side chain that characterizes the antagonist members of this class. In the case of the more complete antagonists, such as ICI 164,384, obstruction of ER binding to DNA and reduction of the ER content of target cells appear to contribute to [23, 24], but may not fully explain, the pure antagonist character of this antiestrogen [25]. The structures of these antiestrogens, which can be both steroidal or non-steroidal in nature, are shown in Fig. 1, along with the structures of the naturally occurring estrogen estradiol, and the non-steroidal synthetic estrogen diethylstilbestrol. Of note, is the fact that antiestrogens typically have a bulky side chain which is basic or polar. This side chain is important for antiestrogenic activity; removal of this side chain results in a compound which is no longer an antiestrogen and, instead, has only estrogenic activity. Therefore we believe that interaction of this side chain with the ER must play an important role in the interpretation of the ligand as an antiestrogen.

In order to examine issues of ligand discrimination by the ER, we have used site-directed and random chemical mutagenesis to generate ERs with selected changes in the hormone binding domain. We have been particularly interested in identifying residues in the hormone binding domain important for the ligand binding and transactivation functions of the receptor, and in elucidating the mechanism by which the ER discriminates between agonistic and antagonistic ligands. Although both estrogens and antiestrogens bind within the HBD, the association must differ because estrogen binding activates a transcriptional enhancement function, whereas antiestrogens fully or partially fail in this role. Our studies have indicated that selective changes near amino acid 380, and amino acids 520–530, and changes at the C-terminus of the ER result in ER ligand discrimination mutants [10, 13, 26]. These data provide evidence that some contact sites of the receptor with estrogen and antiestrogen differ; and that the conformation of the receptor with estrogen and antiestrogen must also be different as a consequence [10, 27 and refs therein]. Our structure–function analysis of the hormone binding domain of the human ER has utilized region-specific mutagenesis of the ER cDNA and phenotypic screening in yeast, followed by the analysis of interesting receptor mutants in mammalian cells [14, 28]. Our observations, as well as very important studies by Malcolm Parker and colleagues [29, 30] have shown a separation of the transactivation and hormone-binding functions of the ER.

Since the basic or polar side chain is essential for antiestrogenic activity, and our previous studies identified cysteine 530 as the amino acid covalently labeled by affinity labeling ligands [31], we introduced by site directed mutagenesis of the ER cDNA changes of specific charged residues close to C530 [10]. Interestingly, two mutants in which lysines at position 529 and 531 where changed to glutamines, so that the local charge was changed, resulted in receptors with an approx. 30-fold increased potency of antiestrogen in suppressing estradiol-stimulated reporter gene activity. Interestingly, these mutants receptors showed a reduced binding affinity for estrogens, but retained unaltered binding affinity for antiestrogen. These findings suggest that we are able to differentially alter estrogen and antiestrogen effectiveness by rather modest changes in the ER, and that the region near C530 is a critical one for sensing the fit of the side chain of the estrogen antagonist. Studies from the Parker Laboratory [27] have shown that nearby residues (i.e. G525 and M521 and/or S522 in the mouse ER) are also importantly involved in conferring differential sensitivity to these two categories of ligands.

We have also shown that if C530 is mutated, the covalent ligand tamoxifen aziridine binds to C381 instead, another cysteine in the hormone binding domain [32]. One interpretation of this result is that the 530 and 380 regions of the hormone-binding domain are close to one another in the three-dimensional ligand binding pocket of the ER, such that the ligand can label either site by alternative positioning of the reactive side chain [32]. We therefore investigated charged amino acids in the N-terminal portion of the hormone binding domain and showed the region around amino acid 380 to be important in transcriptional activity of the receptor [13]. As opposed to what was observed with charge changes in the region near C530, we observed that change of the charged residue E380 to E380Q resulted in a receptor more sensitive to estrogen, but less sensitive than wild-type ER to antiestrogen for suppression of transcriptional activity. Although estrogen and antiestrogen showed no alteration of their binding affinity for the wild-type or E380Q mutant, the E380 mutant showed greater transcriptional activity and enhanced binding to estrogen response element DNA, resulting in its increased sensitivity to estrogen. Our findings suggest that this region is important in influencing DNA binding and protein–protein interaction of the receptor that modulates transcriptional activity and provide additional evidence, suggesting that the conformation of the receptor with estrogen and antiestrogen results in differential transactivation activity. Our recent data [26] has also shown that tamoxifen-like antiestrogens are more pure antiestrogens with the ER missing the C-terminal F domain, approx. the last 40 amino acids of the receptor. The basis for the difference in the estrogenic activity of tamoxifen-like estrogens with wild-type ER versus ER missing this F domain is under investigation and should provide important information regarding the differential agonistic/antagonistic effects of this category of antiestrogens.

ALTERATION IN THE AGONIST/ANTAGONIST BALANCE OF ANTIESTROGENS BY ACTIVATION OF PROTEIN KINASE A SIGNALING PATHWAYS: ANTIESTROGEN SELECTIVITY AND PROMOTER DEPENDENCE

There is increasing evidence for ER interaction with other cell signaling pathways. We became interested in this cross-talk between cell signaling pathways in our studies of estrogen regulation of the progesterone-receptor and estrogen responsive promoter-reporter gene constructs in cells. These studies showed stimulation by growth factors (IGF-1, EGF) as well as stimulation by cAMP and estrogen. The observation that the stimulation by these agents could be suppressed by antiestrogens or protein kinase inhibitors implied the involvement of the ER and phosphorylation pathways in these responses [18–21, 33]. We therefore have undertaken studies to examine directly whether activators of protein kinases can modulate transcriptional activity of the ER.

We find that activators of protein kinase A and protein kinase C markedly synergize with estradiol in ER-mediated transcriptional activation and that this transcriptional synergism shows cell- and promoter-specificity [15, 21, 34]. The synergistic stimulation of ER-mediated transcription by estradiol and protein kinase activators did not appear to result from changes in ER content or in the binding affinity of ER for ligand or the ERE DNA, but, rather, may be a consequence of a stabilization or facilitation of interaction of target components of the transcriptional machinery, possibly either through changes in phosphorylation of ER or other proteins important in ER-mediated transcriptional activation [34].

Figure 2 shows a model indicating how we think the protein kinase–ER transcriptional synergism may occur. Agents influencing protein kinase pathways may enhance intracellular protein phosphorylation resulting in either phosphorylation of the ER itself or the phosphorylation of nuclear factors with which the receptor interacts in mediating transcription. Likewise, there is evidence that the steroid hormone itself can alter

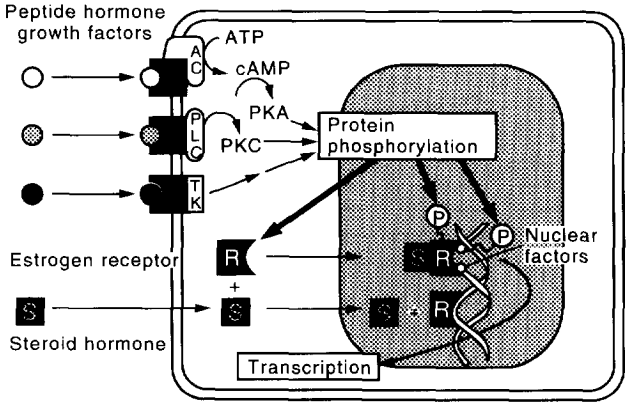

Fig. 2. Model depicting protein kinase–ER transcriptional synergism. See text for description.

Table 1. Levels of ligand-stimulated and protein kinase activator-stimulated phosphorylation of the human ER

Treatments	Phosphorylation level mean ± SE	*n*
Control	1	
10^{-9} M estradiol (E_2)	2.8 ± 0.3	3
10^{-8} M estradiol (E_2)	4.3 ± 0.7	6
10^{-8} M transhydroxytamoxifen (TOT)	2.9 ± 0.1	2
10^{-7} M ICI 164,384	3.6 ± 0.6	3
1 μg/ml cholera toxin (CT) + 10^{-4} M isobutylmethylxanthine (IBMX)	1.9 ± 0.3	3
10^{-7} M TPA	2.6 ± 0.3	3

Human ER was expressed in COS-1 cells and transfected cells were incubated for 4 h with [^{32}P]orthophosphate in the presence of the indicated treatment. ER was immunoprecipitated with anti-receptor antibodies, resolved by SDS–PAGE and transferred to nitrocellulose. ER protein levels were determined by immunoblot and ER phosphorylation by autoradiography as described [35]. The levels of phosphorylation of the different samples were standardized according to ER protein levels and standard errors (SE) were calculated. 1 represents the basal level of phosphorylation (vehicle alone) in each experiment. *n* represents the number of experiments. (From Le Goff *et al.* ref. [35]).

receptor conformation increasing its susceptibility to serve as a substrate for protein kinases [19, 35–38 and Table 1]. Therefore, agents which increase the phosphorylation may, either through phosphorylation of the ER itself, or through phosphorylation of nuclear factors required for ER transcription, result in synergistic activation of ER-mediated transcription.

As shown in Fig. 3, we have compared the effects of cAMP on the transcriptional activity of the estradiol-liganded and antiestrogen-liganded ER complexes. We find that increasing the intracellular concentration of cAMP, or of protein kinase. A catalytic subunit of transfection [15], activates and/or enhances the transcriptional activity of type I but not type II antiestrogen-occupied ER complexes and reduces the estrogen antagonist activity of the type I transhydroxytamoxifen (TOT) antiestrogen. In Fig. 3(A and B), we have determined, in MCF-7 human breast cancer cells, the effect of cAMP on the activity of TOT, ICI 164,384 and E_2 on a simple TATA promoter with one consensus ERE upstream of the CAT gene and on the more complex pS2 gene promoter and 5′-flanking region (−3000 to +10) containing an imperfect ERE. The endogenous pS2 gene is regulated by E_2 in MCF-7 breast cancer cells. Estradiol increased the transcription of both of these gene constructs, and treatment with IBMX/CT and E_2 evoked a synergistic increase in transcription, with activity being ca 2.5 times that of E_2 alone. Both antiestrogens (TOT and ICI) failed to stimulate transactivation of these reporter gene constructs, but in the presence of IBMX/CT, TOT gave significant stimulation of transcription (85 or 60% that of E_2 alone). ICI failed to stimulate transactivation even in the presence of IBMX/CT, and ICI fully blocked E_2 stimulation in the presence or absence of cAMP. By contrast, treatment with IBMX/CT reduced the ability

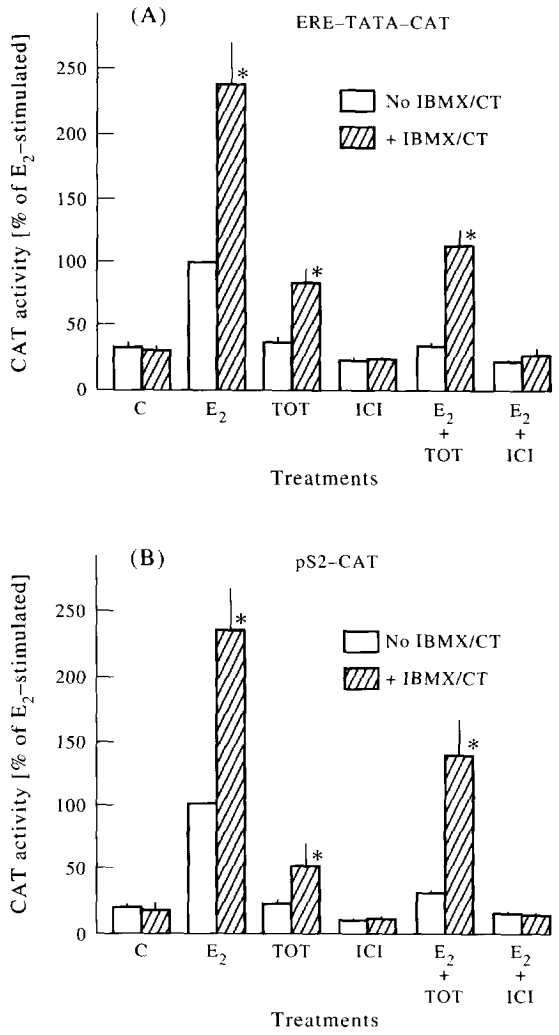

Fig. 3. Effect of IBMX/CT on the ability of E_2 and antiestrogens to stimulate transactivation of ERE-TATA-CAT (panel A) and pS2-CAT (panel B), and on the ability of antiestrogens to suppress E_2-stimulated transactivation. MCF-7 cells were transfected with the indicated reporter plasmid and an internal control plasmid that expresses β-galactosidase and were treated with the agents indicated for 24 h. Each bar represents the mean ± SEM ($n = 3$ experiments). * Indicates significant difference from the no IBMX/CT cells ($P < 0.05$). C, control ethanol vehicle; E_2, 10^{-9} M; TOT (hydroxytamoxifen), 10^{-6} M; ICI (ICI 164,384), 10^{-6} M; IBMX (3-isobutyl-1-methyl-xanthine), 10^{-4} M; and CT (cholera toxin), 1 μg/ml. (From Fujimoto and Katzenellenbogen, ref. [15]).

of TOT to inhibit E_2 transactivation. While TOT returned E_2 stimulation down to that of the control in the absence of IBMX/CT (compare open bars E_2 vs E_2 + TOT), TOT only partially suppressed the E_2 stimulation in the presence of IBMX/CT (compare stippled bars E_2 vs E_2 + TOT).

Although alteration in the agonist and antagonist activity of TOT was observed with promoter-reporter-constructs containing a simple TATA promoter and a more complex, pS2 promoter, elevation of cAMP did not enhance the transcription by either TOT or estradiol of the reporter plasmid ERE-thymidine kinase-CAT [15]. Thus, this phenomenon is promoter-specific. Of note, cAMP and protein kinase A catalytic subunit transfection failed to evoke transcription by the more pure antiestrogen ICI 164,384 with any of the promoter-reporter constructs tested. These findings, which document that stimulation of the protein kinase A signaling pathway activates the agonist activity of tamoxifen-like antiestrogens, may in part explain the development of tamoxifen resistance by some ER-containing breast cancers. They also suggest that the use of antiestrogens like ICI 164,384, that fail to activate ER transcription in the presence of cAMP, may prove more effective for long-term antiestrogen therapy in breast cancer.

PHOSPHORYLATION OF THE ESTROGEN RECEPTOR

Since our data suggested that estrogens, and agents that activate protein kinases, might influence ER transcription by altering the state of phosphorylation of the ER and/or other factors required for ER regulation of transcription, we undertook studies to examine directly the effects of these agents on ER phosphorylation. In addition, we compared the effects of the type I and type II antiestrogens on phosphorylation of the ER (Table 1). Estradiol, each of the two antiestrogens, as well as protein kinase A and C activators enhanced overall ER phosphorylation, and in all cases, this phorphorylation occurred exclusively on serine residues [35]. Tryptic phosphopeptide patterns of wild-type and domain A/B-deleted receptors and site-directed mutagenesis of several serines involved in known protein kinase consensus sequences allowed us to identify serine 104 and/or serine 106 and serine 118, all three being part of a serine–proline motif, as major ER phosphorylation sites. Mutation of these serines to alanines so as to eliminate the possibility of their phosphorylation, resulted in an approx. 40% reduction in transactivation activity in response to estradiol while mutation of only one of these serines showed an approx. 15% decrease in activation [35]. Of note, estradiol and antiestrogen-occupied ERs showed virtually identical two-dimensional phosphopeptide patterns suggesting similar sites of phosphorylation. In contrast, the cAMP-stimulated phosphorylation likely occurs on different phosphorylation sites as indicated by some of our mutational studies [35] and this aspect remains under investigation in our laboratory.

cAMP-DEPENDENT SIGNALING PATHWAY INVOLVEMENT IN ACTIVATION OF THE TRANSCRIPTIONAL ACTIVITY OF ERs OCCUPIED BY TAMOXIFEN-LIKE BUT NOT ICI 164,384-LIKE ANTIESTROGENS

Our data provide strong evidence for the involvement of cAMP-dependent signaling pathways in the agonist actions of tamoxifen-like estrogen antagonists. The promoter-specificity of the transcriptional enhancement

phenomenon suggests that factors in addition to ER are probably being modulated by protein kinase A pathway stimulation. The findings imply that changes in the cAMP content of cells, which can result in activation of the agonist activity of tamoxifen-like antiestrogens, might account, at least in part, for the resistance to antiestrogen therapy that is observed in some breast cancer patients. Of interest, MCF-7 cells transplanted into nude mice fail to grow with tamoxifen treatment initially, but some hormone-resistant cells grow out into tumors after several months of tamoxifen exposure [8, 39, 40]. Studies have shown that this resistance to tamoxifen is, more correctly, a reflection of tamoxifen stimulation of proliferation, representing a change in the interpretation of the tamoxifen–ER complex and its agonist/antagonist balance. It is of interest that we found the pS2 gene, which is under estrogen and antiestrogen regulation in breast cancer [41], to be activated by tamoxifen in the presence of elevated cAMP. By contrast, however, antiestrogens such as ICI, shown in many systems to be more complete estrogen antagonists, are not changed in their agonist/antagonist balance by increasing intracellular concentrations of cAMP. Therefore, ICI-like compounds may prove to be more efficacious and less likely to result in antiestrogen-stimulated growth.

The transcriptional enhancement we have observed between protein kinase A activators and ER occupied by tamoxifen-like antiestrogens and estradiol provides further evidence for cross-talk between the ER and signal transduction pathways regulated by cAMP that are important in ER-dependent responses.

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