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Activity of a Tamoxifen–Raloxifene Hybrid Ligand for Estrogen Receptors at an AP-1 Site

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Abstract—To test the effect of ligand flexibility on the selective transcriptional activities of ERα and ERβ from an AP-1 site, an analogue of raloxifene was made that removed the ketone functionality and made the ligand more planar and conformationally more similar to 4-hydroxytamoxifen. Desketoraloxifene was found to be a much stronger activator at an AP-1 site with ERα than with ERβ, mimicking 4-hydroxytamoxifen more than raloxifene. © 2001 Elsevier Science Ltd. All rights reserved.

The estrogen receptors alpha and beta (ERα and ERβ) are members of a large family of nuclear receptors that regulate gene transcription in response to binding small molecules.[1] Due to the validated therapeutic importance of these receptors in diseases such as osteoporosis and breast cancer, a number of drugs has been developed that target the ER.[2] Many of these selective estrogen receptor modulators (SERMs) show different activities in different tissues.[3] For example, the breast cancer drug 4-hydroxytamoxifen (Fig. 1, **2**) functions as an antiestrogen in breast tissue, but mimics the activity of the physiological hormone, estradiol (**1**) in the uterus and bone. In contrast, the osteoporosis drug raloxifene (**3**) acts as an antiestrogen in both breast and uterine tissue while being estrogenic in bone.

One explanation for the different tissue effects of these drugs is that a ligand may elicit different responses when the receptor binds to different effector sites in the upstream promoter regions of different genes.[4] Through receptor interactions with these different response elements, the same ligand can cause activation or repression of different sets of genes. It has been shown that the response to both estrogens and antiestrogens at one of these effector sites, the AP-1 site, depends on the subtype of the receptor; estradiol elicits transcriptional activation with ERα, but transcriptional repression with ERβ.[5] The two ER subtypes also respond differently to raloxifene at an AP-1 site; ERβ shows much stronger activation than ERα in response to raloxifene.

The structures of 4-hydroxytamoxifen and raloxifene (**2** and **3**, respectively, Fig. 1) are very similar with a notable exception — a ketone hinge in raloxifene allows for a larger degree of flexibility between the amine-containing side chain and the olefin than is present in 4-hydroxytamoxifen. Removal of that ketone results in an analogue, desketoraloxifene (**4**) that has stimulatory activity in rat uterine tissue similar to 4-hydroxytamoxifen and different from raloxifene.[6] Here, in an effort to better understand the effect of ligand flexibility on SERM activity at different estrogen receptor effector sites, we describe the ERα-selective activity of desketoraloxifene at an AP-1 site. This work provides another link between ERα activity at an AP-1 site with uterine hypertrophy in vivo.[7–9]

Desketoraloxifene was synthesized according to the published procedure[6] and then tested for its ability to antagonize estradiol action of a luciferase reporter gene under the control of a consensus estrogen response element (ERE). The most studied consensus ERE is the palindromic core sequence GGTCANNNTGACC, which is recognized by homodimeric liganded ERs.[10,11] Estradiol activates transcription at this ERE and 4-hydroxytamoxifen and raloxifene antagonize this action. Desketoraloxifene also antagonized estradiol activation in HeLa cells transiently transfected with the ERE-driven luciferase reporter plasmid and either an ERα or ERβ expression plasmid (Fig. 2). Inhibition of activation of ERα was identical to that of ERβ, suggesting that desketoraloxifene binds to the two receptor subtypes with equal affinity.

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Desketoraloxifene was then tested for its regulatory effects on transcription from an AP-1 estrogen response site. In transiently transfected HeLa cells, desketoraloxifene has a profile more similar to 4-hydroxytamoxifen than raloxifene. At saturating doses for the estrogen receptor, desketoraloxifene is similar to 4-hydroxytamoxifen in significantly activating transcription above the level of the hormone independent response at an AP-1 site with wild-type ERα while raloxifene does not (Fig. 3A). In contrast, desketoraloxifene and 4-hydroxytamoxifen are not as effective as raloxifene at activating transcription with ERβ at the AP-1 site (Fig. 3B). The relative level of activation with ERα is similar to previous reports except for the difference in the hormone independent response, which is much higher with wild-type ERα than with a point mutant of ERα used in previous reports[5,7] (see below).

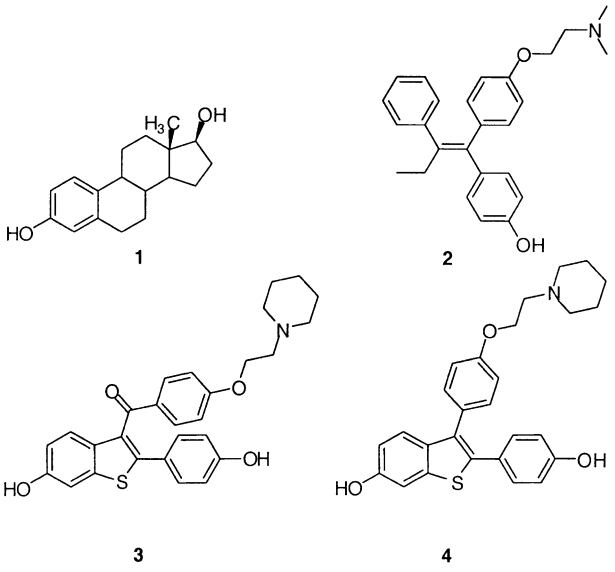

Figure 1. Structures of estradiol (**1**), 4-hydroxytamoxifen (**2**), raloxifene (**3**), and desketoraloxifene (**4**).

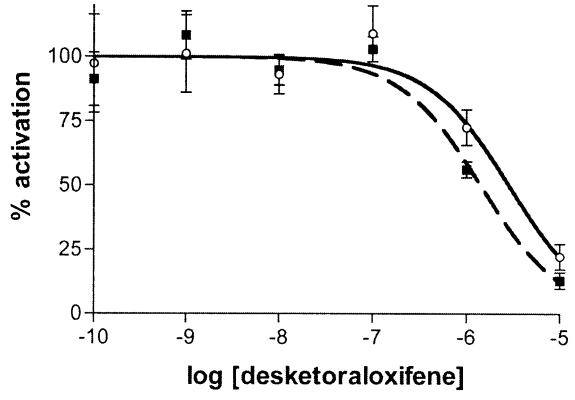

Figure 2. Competition of desketoraloxifene versus 20 nM estradiol in transient transfection assay of HeLa cells with wild-type ERα (open circles) and ERβ (black squares) and the vitellogenin A2 ERE-tk driven luciferase reporter gene. Experiments performed as described previously.[7] The curve represents the best fit to a single-site competition binding model. 100% activation represents the activation with 20 nM estradiol alone.

A comparison of the activation profiles for each ligand at an AP-1 site with ERα or ERβ shows that the compounds have the same potency of activation for both estrogen receptor subtypes, but the level of maximal activation is different. Due to the high hormone independent response at the AP-1 site with ERα, a point mutant of ERα with lower hormone independent activation was used for dose–response experiments.[5] Thus far, ligand activation at AP-1 sites using this mutant has mirrored the trends seen with the wild-type ER, although the degree of activation is higher providing a clearer dose–response curve.[12] Desketoraloxifene and 4-hydroxytamoxifen activate more strongly with the ERα mutant at an AP-1 site than with ERβ (Figs. 4A and B). In contrast, raloxifene activates more strongly with ERβ than with ERα at the AP-1 site (Fig. 4C).

The similarities between desketoraloxifene and 4-hydroxytamoxifen activity at an AP-1 site provides information about the effect of ligand conformation on ER signaling at nonclassical estrogen response sites. Raloxifene and 4-hydroxytamoxifen share many structural features and the structures of these two ligands bound to the ligand binding domain of ERα are very similar.[13,14] The only significant difference between the

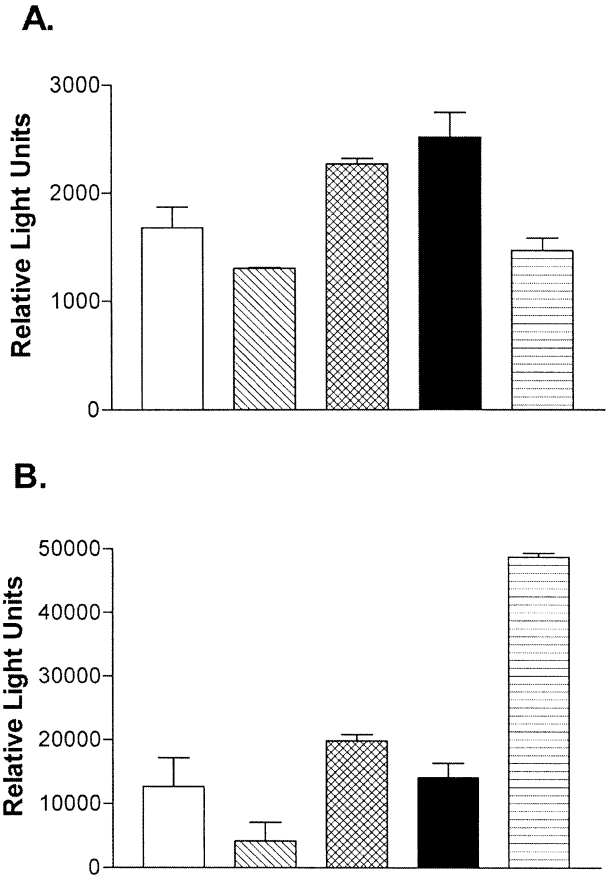

Figure 3. Ligand activation of transcription in transient transfection assay of HeLa cells with (A) wild-type ERα and (B) ERβ and the AP-1 driven luciferase reporter gene. The hormone independent response is shown with white bars. The ligands tested were estradiol (single hatched bar), 4-hydroxytamoxifen (double hatched bar), desketoraloxifene (black bar), and raloxifene (horizontally striped bar). Dose concentration for all ligands was 1 μM. Experiments performed in triplicate as described previously.[7]

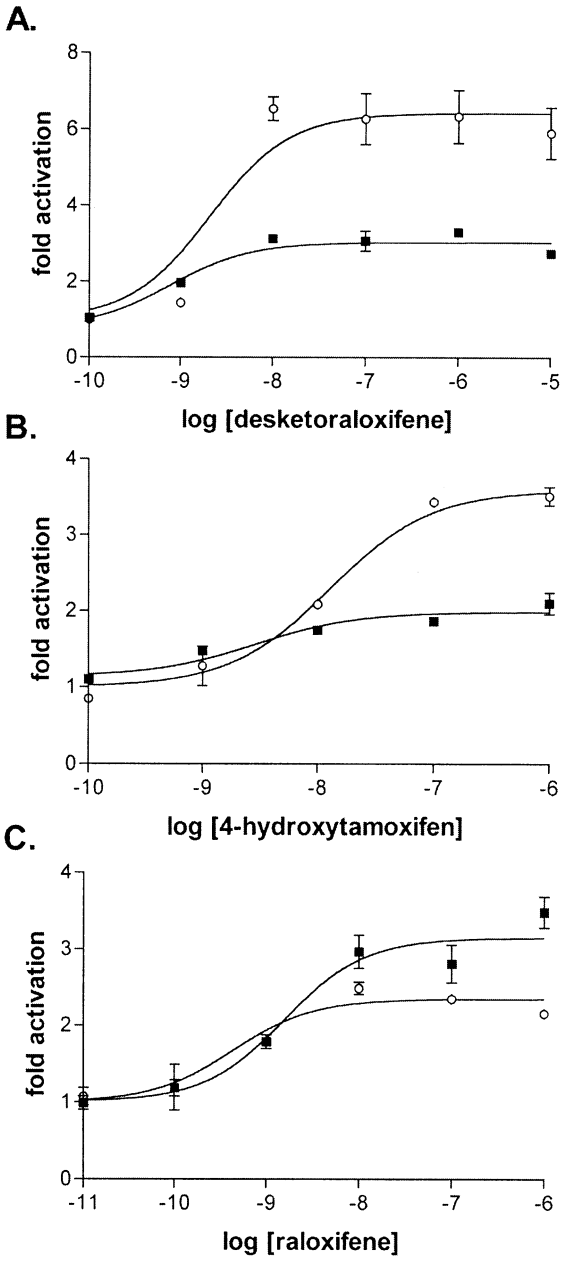

Figure 4. Dose–response curves for transient transfections with the AP-1 driven luciferase reporter gene in HeLa cells using (A) desketoraloxifene, (B) 4-hydroxytamoxifen, and (C) raloxifene. Data for transfections with the ERα point mutant HE0 shown with open circles and data for ERβ shown with black squares. Experiments performed as described previously.[7] Fold activation determined relative to the response with no hormone present. Curve represents best fit of data using a single-site sigmoidal dose response model. The EC_{50} for the desketoraloxifene response is 2.1 nM with HE0 and 0.8 nM with ERβ. The error for each EC_{50} is approximately 4-fold.

two ligands is the presence of a ketone functionality in raloxifene which allows the ligand to adopt a more orthogonal relationship between the hydrophobic core scaffold and the amine-containing side chain. Removing this moiety in desketoraloxifene results in AP-1 activity similar to 4-hydroxytamoxifen, emphasizing the importance of ligand flexibility in attenuating relative levels of transcriptional activation at the AP-1 site.

The correlation between desketoraloxifene and 4-hydroxytamoxifen at the AP-1 site is also significant because of the physiological effects of those ligands. Desketoraloxifene was similar to 4-hydroxytamoxifen in causing uterine weight gain in rats.[6] This uterotrophic activity is not seen with raloxifene. Higher relative levels of activation by ERα at an AP-1 site have been seen with other ER ligands. A uterine-stimulating carboxamide derivative of the SERM GW-5638[15,16] was also shown to have much higher transcriptional activation at an AP-1 site with ERα than with ERβ.[7] In contrast, GW-5638 causes no uterine stimulation and shows higher activation with ERβ at an AP-1 site than with ERα. This suggests that genes with a promoter region containing a site like the AP-1 site might be responsible for proliferative effects of some SERMs in uterine tissue and the AP-1 response site could be a useful test for some of the tissue-dependent effects of new SERMs.

Acknowledgements

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