The Key to the Antiestrogenic Mechanism of Raloxifene Is Amino Acid 351 (Aspartate) in the Estrogen Receptor

Anait S. Levenson and V. Craig Jordan

Robert H. Lurie Comprehensive Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611

ABSTRACT

The crystallization of the ligand-binding domain (LBD) of the estrogen receptor (ER) with 17β-estradiol and raloxifene [A. M. Brzozowski et al., Nature (Lond.), 389: 753—758, 1997] now provides a molecular basis for the biological activity of complexes as either agonists or antagonists. It is well established that the critical structural feature of antiestrogens is a correctly positioned alkylaminoethoxy side chain. The X-ray crystallography clearly shows that the alkylaminoethoxy side chain of raloxifene causes a specific and inappropriate molecular perturbation of the LBD correctly positioned alkylaminoethoxy side chain. The X-ray crystallography clearly shows that the alkylaminoethoxy side chain of raloxifene correctly positioned alkylaminoethoxy side chain (1). Alterations in the distance between the nitrogen and oxygen atoms (2, 3), restrictions in the movement of the side chain (4), or alterations in the basicity of the nitrogen (5) all cause changes in antiestrogenic activity. Furthermore, removal of the side chain causes a change in the pharmacology of the molecule from an antiestrogen to an estrogen. By contrast, a mutation at AA 400 does not, and the antiestrogenic properties of raloxifene are retained. These data and the fact that the nitrogen in the side chain must specifically interact with aspartate 351 makes this the key to the antiestrogenic activity of raloxifene.

INTRODUCTION

The key structural feature of the nonsteroidal antiestrogens is a correctly positioned alkylaminoethoxy side chain (1). Alterations in the distance between the nitrogen and oxygen atoms (2, 3), restrictions in the movement of the side chain (4), or alterations in the basicity of the nitrogen (5) all cause changes in antiestrogenic activity. Furthermore, removal of the side chain causes a change in the pharmacology of the molecule from an antiestrogen to an estrogen or results in the complete loss of biological activity (6).

Recently, the crystal structure of the LBD3 of the ER was determined with either E2 or the antiestrogen raloxifene in the ligand binding site (Ref. 7; Fig. 1, A and B). As anticipated, the backbone of raloxifene concisely binds to the appropriate AAs that lock E2 into the LBD (7). As predicted (8), the alkylaminoethoxy side chain projects out of the hydrophobic pocket. However, the interesting observation is that the nitrogen in the side chain binds to AA aspartate at position 351 (Fig. 1B).

Clearly proof of the essential role of AA 351 in the antiestrogenic action of raloxifene can be obtained by comparing and contrasting the biology of wild-type ER with mutant forms of the receptor. We have previously identified a natural mutation of the ER in a tamoxifen-stimulated breast tumor grown in athymic mice (9). Using single-stranded conformational polymorphism and sequencing, we identified a single point mutation at AA 351 where aspartate was replaced by tyrosine. Additionally, another mutant is available with an AA substitution in approximately the same region. A cloning error produced a mutant ER, in which a glycine is replaced by valine at position 400 (Ref. 10; Fig. 2).

Our goal in this paper was to test the hypothesis that the AA 351 is the key to the antiestrogenic activity of raloxifene. To accomplish the goal, we have compared and contrasted the pharmacology of raloxifene to block or induce estrogen-stimulated increase of TGF-α mRNA in MDA-MB-231 breast cancer cells stably transfected with cDNA for the wild-type or mutant ERs (Gly400Val and Asp351Tyr; Refs. 11 and 12).

MATERIALS AND METHODS

Cell Culture. The three stable transfectants (S30, ML-α-2H, and BC-2 cell lines) used in this study were constructed from the ER-negative MDA-MB-231 (clone 10A) human breast cancer cells as described previously (11, 12). These cells stably express either the wild-type ER (S30 cells; Ref. 11), the Gly400Val mutant ER (ML-α-2H cells; Ref. 11), or the Asp351Tyr mutant ER (BC-2 cells; Ref. 12). Cells were maintained in phenol red-free MEM with 5% charcoal-dextran treated calf serum, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 6 ng/ml bovine insulin, 100 mM nonessential AAs, and 500 μg/ml G418. All of the tissue culture solutions were from Life Technologies, Inc. (Gaithersburg, MD). E2 was purchased from Sigma Chemical Co. (St. Louis, MO), raloxifene was obtained from Eli Lilly Laboratories (Indianapolis, IN), and ICT 182,780 was a gift from Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, United Kingdom). All compounds used in these experiments were dissolved in 100% ethanol and added to the medium in 1:100 dilution for a final concentration no higher than 0.2%.

Northern Blot Analysis. RNA extraction and Northern blot analyses were performed essentially as described previously (13). Briefly, total RNA was isolated from cells following 24-h treatment with compounds using the Trizol reagent (Life Technologies). Twenty μg of RNA sample were fractionated in a 1.2% agarose-formaldehyde gel and transferred to a nylon membrane (Nybond-N°; Amersham Corp., Arlington Heights, IL). The membranes were hybridized at 42°C with 32P-labeled TGF-α probe (plasmid generously provided by Dr. Rik Derynck, Genentech, Inc., South San Francisco, CA). The membranes were then washed and autoradiographed by exposure to Hyperfilm (Amersham) at —80°C with intensifying screens for 2–3 days, and the expected 4.8 kb transcript was detected in all three cell lines. Subsequently, the blots were stripped and reprobed with β-actin cDNA. The signals were quantitated by PhosphorImager (Molecular Dynamics) analysis (Image Quant software).

RESULTS

Regulation of Endogenous TGF-α mRNA. We have previously demonstrated that there is a concentration-dependent induction of TGF-α mRNA by E2 in MDA-MB-231 cells stably transfected with cDNAs from wild-type and mutant ER (13, 14). In the present study, we compared and contrasted the ability of E2, raloxifene, and the pure antiestrogen ICI 182,780 to regulate TGF-α mRNA in the transfe-
**ANTIESTROGENIC MECHANISM OF RALOXIFENE**

Antiestrogenic

Side Chain

Asp 351

H

V

His 524

\/

//

NH

Glu 353

Arg 394

B

Estradiol

Glu 353

HO'

Water

Arg 394

Fig. 1. The interaction of raloxifene (A) and E2 (B) in the LBD of the ER adapted from Ref. 7.

tants so that we could evaluate our results in the light of the known crystal structure for the ER complex (7). The effects of compounds on the expression of TGF-α mRNA in S30, ML-α-2H, and BC-2 cells were determined by Northern blot analyses 24 h after the addition of compounds alone or in combinations. Although the results from multiple experiments are combined in Fig. 4, the data shown in Fig. 3 illustrate that E2 stimulates accumulation of TGF-α mRNA in all three cell lines. Raloxifene and ICI 182,780 did not cause an increase in TGF-α mRNA levels in either the S30 or ML-α-2H cell lines, and both compounds blocked the action of E2. In contrast, raloxifene did increase TGF-α mRNA in BC-2 cells, and unlike ICI 182,780, raloxifene was not an inhibitor of E2 action. As shown in Fig. 4, raloxifene was an estrogen only when using BC-2 cells and not the ML-α-2H cells. However, the pure antiestrogen ICI 182,780 was an antiestrogen, and blocked the action of raloxifene in all cell lines.

**DISCUSSION**

We have used a specific bioassay in situ, the estrogen regulation of the TGF-α gene to test the hypothesis that a precise mutation at AA 351 of ER can affect the pharmacology of the receptor-raloxifene complex. The crystal structure of the ER-raloxifene complex shows that there is a key interaction of the alkylaminoethoxy side chain with AA 351 (7). Our results demonstrate that the mutation at AA 351 that replaces aspartate by tyrosine specifically changes the pharmacology of raloxifene from an antiestrogen to an estrogen (Fig. 5). By contrast, a mutation at AA 400 does not, and the antiestrogenic properties of raloxifene are retained. The results from this bioassay now provide important evidence about the key pharmacological role of AA 351. The side chain creates an inappropriate perturbation of the LBD of the wild-type ER so that the whole orientation of the complex prevents gene transcription. The X-ray crystallography of E2 with the ER LBD (7) demonstrates that the steroid is enveloped in a pocket that closed by helix 12. Apparently, this is critical for the recruitment of coactivators to the AF-2 site and the initiation of RNA polymerase activity. The antiestrogen raloxifene causes a change in the orientation of helix 12, so that it is repositioned and now cannot recruit coactivators. During the past few years, there has been a considerable effort to describe the biological relevance of the AF-2 region through site-directed mutagenesis (15-17). The AAs 538, 542, and 545 are known to be essential for AF-2 activity and gene transcription (Fig. 2; Refs. 18 and 19). However, antiestrogen can become more estrogenic if there are mutations at straddling AAs [e.g., L543A/L544A or M547A/L548A (20); see Fig. 2]. It would seem that the mutations permit the recruitment of some coactivators even at the surface of the inappropriately positioned helix 12. Be that as it may, it is clear that the critical AA that provokes all of the other changes is Asp351. A mutation at AA 351 prevents the specific interaction with the side chain and does not allow the conformational change associated with antiestrogen action.

These data not only define the mechanism of action of raloxifene but also illustrate, for the first time, a specific mechanism of drug resistance to antiestrogens. Tamoxifen is effective as a long-term adjuvant therapy to provide survival advantages in node-positive and node-negative breast cancer (22). However, the clinical data point to the eventual emergence of tamoxifen-stimulated tumor growth (23, 24). In the laboratory, tamoxifen-stimulated tumors have been developed by the long-term treatment of athymic mice inoculated with MCF-7 breast cancer cells (25, 26). The tumors retain ER and will grow in response to either tamoxifen or E2 (26). The class of drugs known as pure antiestrogens inhibit tumor growth (27), and preliminary clinical evidence suggests that the pure antiestrogen ICI 182,780...
The TGF-α gene is known to be estrogen regulated, and we discovered that estrogen increases the transcription of TGF-α mRNA in our transfectants (14).

The present results confirm and extend our previous study (13) and determine that a specific mutation in a critical region of the ER from a tamoxifen-stimulated breast tumor can enhance the estrogenic properties of an antiestrogen. The cells with a mutant receptor would have a growth advantage.

For the future, the crystal structure of the 4-OHT-ER complex is required to compare and contrast antiestrogens. On the basis of data we have recently published with our transfectants (32), we predict that the shape of the 4-OHT-ER complex will be significantly different from the raloxifene-ER complex. We have noted that 4-OHT can increase TGF-α mRNA in both wild-type and mutant transfectants, although the mutant Asp351Tyr ER complex appears to be more efficacious (32). The wild-type ER-4-OHT complex is clearly more promiscuous than the raloxifene-ER complex, so the helix 12 containing the AF-2 AAs must be available for ER-protein interactions in a context replete with coactivators. This simple molecular model would explain the reduced uterine estrogenicity of antiestrogens of the raloxifene class and their ability to block the uterotrophic properties of tamoxifen (33, 34). Additionally, and perhaps more importantly, it points to an explanation for the primary mechanism of drug resistance to tamoxifen. Tamoxifen-stimulated growth may be driven by selection of cells that have high levels of coactivators. The selection process could be enhanced with an ER with a mutation in the AA 351 region, but this would not be obligatory for tamoxifen-stimulated growth. The model would then predict that raloxifene would not, in
general, be completely cross-resistant with tamoxifen and could potentially be used on tamoxifen failure.

ACKNOWLEDGMENTS

We thank Henry Muenzner for preparing schematic figures.

REFERENCES

The Key to the Antiestrogenic Mechanism of Raloxifene Is Amino Acid 351 (Aspartate) in the Estrogen Receptor

Anait S. Levenson and V. Craig Jordan


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/58/9/1872

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.