Antiestrogenic Action of Raloxifene and Tamoxifen: Today and Tomorrow

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On the basis of results from initial studies in the laboratory and extensive clinical testing, raloxifene (Evista®) is now available to physicians as a bone maintenance therapy to prevent osteoporosis in postmenopausal women. Raloxifene (originally named LY156758 and then keoxifene) is, however, a potent antiestrogen in the rodent uterus, exhibiting a high affinity for the estrogen receptor (ER) (1). Although this seems paradoxical, raloxifene is a selective ER modulator. It is partially estrogenic in bone (2) and will lower cholesterol (3), but at the same time, it is antiestrogenic in the mammary gland and will prevent rat mammary carcinogenesis (4,5). Similarly, raloxifene has very low estrogenic activity in the rodent uterus (1) and will inhibit the growth of tamoxifen-stimulated human endometrial cancers in athymic animals (6).

Knowledge about raloxifene builds on the well-known, mixed agonist and antagonist actions observed with tamoxifen in estrogen target tissues. Tamoxifen exhibits estrogen-like effects by maintaining bone density in postmenopausal patients (7) and lowering circulating cholesterol (8). The latter effect may contribute to a reduced risk for coronary heart disease noted in patients during adjuvant therapy for breast cancer (9–11). Most important, tamoxifen is a proven therapy for breast cancer, producing survival advantages in both node-positive and node-negative disease and a reduction in contralateral breast cancer (12). It is fair to say that tamoxifen must now be viewed as a successful example of a first-generation selective ER modulator targeted to treat all stages of breast cancer. Raloxifene, by contrast, has been developed for a much broader application as a bone maintenance therapy to prevent osteoporosis in postmenopausal women. Raloxifene is a selective ER modulator. It is partially estrogenic in bone (2) and will lower cholesterol (3), but at the same time, it is antiestrogenic in the mammary gland and will prevent rat mammary carcinogenesis (4,5). Similarly, raloxifene has very low estrogenic activity in the rodent uterus (1) and will inhibit the growth of tamoxifen-stimulated human endometrial cancers in athymic animals (6).

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Until recently, the molecular mechanism of action of antiestrogens on the ER was vague (16,17). However, the publication (18) of the crystal structure of the ER ligand-binding domain containing either estradiol or raloxifene provides an exciting glimpse at the final shape of the complexes. However, the sterile crystal structure is more than a shape. The crystallographic data now make it possible to tie together seemingly unrelated biologic facts and, thus, to identify the key to antiestrogenic activity and a mechanism of drug resistance to tamoxifen.

Crystal Structure of the ER

The estradiol binding cavity is largely hydrophobic and is completely partitioned from the external environment. The exact protein conformation is made up of a number of helices (H1–H12) that form the ligand-binding domain (Fig. 1). The critical phenolic hydroxyl (O3) of the A ring of estradiol (Fig. 1, A) is positioned between H3 and H6 and forms hydrogen bonds with Glu353, Arg394, and a water molecule. The 17β-hydroxyl (O17) on the D ring of estradiol forms hydrogen bonds with His524 in H11. Once the ligand is tethered, H12 seals the steroid into the pocket. This final conformational maneuver, originally referred to as “transformation” (19), exposes the activating function 2 (AF-2) region [with the critically important amino acids at positions, 545, 542, and 538 (20,21)], which binds additional coactivator proteins to complete the active transcription complex (Fig. 1, C).

Raloxifene is also tethered in the ligand-binding domain by its phenolic hydroxyls, which interact with the same amino acids (Fig. 1, B). However, H12 of the receptor cannot envelop the ligand and has to be repositioned along a groove between H3 and H5 (Fig. 1, D). As a result, the AF-2 region is masked and cannot bind coactivators. These data clearly show that the estrogen antagonist properties of raloxifene are directly related to the inability to form a competent AF-2 region. However, this is the final consequence of raloxifene binding rather than the molecular mechanism that causes the perturbation.

Key to Antiestrogenic Activity

The alkylaminoethoxy side chain is the essential structural feature of the nonsteroidal antiestrogens (22). The distance between the nitrogen and the oxygen must be optimal (23), the conformations available to the side chain must not be restricted (24), and the basicity of the nitrogen must be correct (25). Removal of the side chain results in loss of all activity or exclusive estrogenic properties (26). The side chain was originally predicted (16,17) to bind to an “antiestrogenic region” in

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the ligand-binding domain of the ER. Simply stated, the antiestrogen was perceived to act like a stick to prevent the jaws of the ER from closing around the ligand. An estrogenic complex would only be created by the protein enveloping the ligand. Resolution of the crystal structure of the ER shows that the alkylaminoethoxy side chain of raloxifene binds to Asp351 (Fig. 1, B). Therefore, this is the key to the antiestrogenic properties of raloxifene. This single critical interaction forces all the other changes to occur. On the basis of this knowledge, it is now possible to confirm the biologic relevance of the crystal structure by tying together the cancer research literature.

**Molecular Mechanism of Drug Resistance**

We, and others (27,28), have found that long term tamoxifen treatment of athymic animals implanted with MCF-7 breast cancer cells eventually leads to tumor growth in response to tamoxifen. The tumors remain ER positive and will grow in response to either estradiol or tamoxifen (28). This laboratory model parallels clinical experience (29,30). One hypothesis to explain tamoxifen-stimulated growth is that a critical receptor mutation has converted the antiestrogen–receptor complex into an “estrogen-receptor complex.” This change in biology would complete the signal transduction pathway and provoke growth. As a result, tamoxifen-stimulated tumors would develop. Although numerous mutant and aberrant messenger RNAs (mRNAs) for ER have been isolated from breast cancer samples and cell lines, these mRNAs were not considered to be relevant (31)—until now.

A number of years ago, we screened tamoxifen-stimulated breast tumors [and subsequently an endometrial tumor (32)] by single-stranded conformational polymorphism analysis and se-

![Interaction of estradiol (A) and raloxifene (B) with specific amino acids in the ligand-binding domain of the estrogen receptor (ER). Note that the hydroxyls of estradiol and raloxifene interact with the same amino acids, supporting the observation that estradiol and raloxifene have a high affinity for the ER. However, the piperazine ring of the aminoethoxy side chain extends away to interact with Asp351. Also shown is the proposed final conformation of the estradiol–ER complex (C) and the raloxifene–ER complex (D). Helix 12 (H12) traps the steroid in the ligand-binding pocket, thereby exposing the transcriptional activating function 2 (AF-2) region. The key amino acids at positions 538, 542, and 545 subsequently bind coactivator molecules. By contrast, when raloxifene occupies the ligand-binding pocket, H12 is reoriented, thereby masking the AF-2 site. (A–D) Reproduced with permission from Nature and the authors [1997; 389:753–8; copyright Macmillan Magazine Limited (18)].
quencing of reverse transcription–polymerase chain reaction products, and we found one tumor with a high proportion of mutated ERs (33). The levels of mutant ER mRNA were in excess of those for wild-type ER mRNA, but the site of the mutations seemed unusual: an aspartate residue was changed to a tyrosine at amino acid 351 (Asp351Tyr), which is far away (200 amino acids) from the biologically relevant AF-2 site. What was worse, since mutagenesis in the AF-2 region can lead to increased estrogenic activity of antiestrogen–receptor complexes, this region was thought to be the biologically relevant site for natural mutations (34,35). However, no mutations were found in the AF-2 region when the ER from tamoxifen-stimulated tumors was sequenced (32).

We decided not to evaluate the biology of the mutant ER by use of transient transfection into animal or yeast cells containing nonhuman (vitellogenin) estrogen-response elements to activate reporter genes. We were concerned about inappropriate transcription factors and artificial targets, and, most important, we felt that it was essential to study the receptor in a breast cancer context. As a result, we developed a model by stably tranflecting complementary DNAs for ERs into the ER-negative breast cancer cell line MDA-MB 231 to determine whether the ER could re-exert control of cell growth (36). Our rationale was that human receptors would operate optimally in a breast cancer cell replete with human coactivators. We subsequently discovered (37) that estradiol would increase the transcription of transforming growth factor-α (TGF-α) mRNA in the transfectants and chose to use this as an appropriate gene target in situ to study estrogen and antiestrogen action. When the wild-type and mutant (Asp351Tyr) ER transfectants were compared, the mutant ER exhibited increased estrogenic activity with tamoxifen (40), whereas estradiol and raloxifene are antiestrogens (41).

If the ER-negative transfectants are replete with coactivators, then an excess of transcription factors must be able to exploit any change in ER shape when 4-hydroxytamoxifen, rather than raloxifene, is the binding ligand. It follows, therefore, that the AF-2 domain in ER H12 must be located in a different place to allow coactivator binding when 4-hydroxytamoxifen occupies the ligand-binding domain. The possibility that different antiestrogens form complexes with different shapes is not unreasonable, as McDonnell et al. (42) have proposed the same hypothesis. It is interesting to note that the pure antiestrogen ICI 182,780 (43) remains an antiestrogen with wild-type and mutant receptors.

Tamoxifen and Raloxifene Are Different

Tamoxifen is more estrogenic in the rodent uterus than raloxifene, and raloxifene analogues can block the uterotrophic properties of tamoxifen (40). Similarly, tamoxifen, or rather the high-affinity metabolite 4-hydroxytamoxifen, and raloxifene differ in their ability to initiate TGF-α mRNA synthesis in stable transfectants. Whereas raloxifene is an antiestrogen with wild-type ERs and only becomes estrogenic with the Asp351Tyr mutant ER (Fig. 2), 4-hydroxytamoxifen is estrogen with wild-type and mutant receptors (41). Indeed, estradiol– and 4-hydroxytamoxifen–ER complexes have equivalent efficacy (Fig. 3), but the mutation enhances TGF-α mRNA synthesis further (41). The shape of the 4-hydroxytamoxifen–wild-type ER complex must be different from the raloxifene–ER complex to allow the promiscuous activation of the TGF-α gene target.

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Asp351Tyr mutant ERs (Fig. 2). This observation is consistent with the fact that the drug has efficacy in treating breast cancer after tamoxifen failure (44). Clearly, it will be important to examine crystals of several different antiestrogens complexed with the ER to build a picture of conformational possibilities and compare the shapes with efficacy. In the meantime, this is perhaps a good opportunity to make three clinical predictions on the basis of developing knowledge in the laboratory.

Implications for Breast Cancer

Drug resistance to tamoxifen may evolve through loss of wild-type ER or through the clonal selection of ER-positive breast cancer cells that are replete with coactivator molecules. The resulting cells will have a growth advantage during tamoxifen treatment and form a tamoxifen-dependent (stimulated) tumor (Fig. 4). The growth advantage will be further amplified by a strategically located mutation at a key site that neutralizes the pharmacologic function of the alkyl aminoethoxy side chain. Although there is no evidence on the prevalence of strategically located point mutations in clinical samples, the principle is illustrated in the laboratory by comparing and contrasting gene activation by estradiol and 4-hydroxytamoxifen with wild-type and mutant ERs in a co-activator-rich breast cancer cell line (Fig. 3). By contrast, raloxifene forms a rigorously antiestrogenic complex with wild-type ER even in a co-activator-rich environment (Fig. 2) and the Asp351Tyr mutant ER increases the estrogenic activity of the complex (Fig. 2). On the basis of this evidence, one could therefore predict that raloxifene and tamoxifen will not be completely cross-resistant. Raloxifene treatment to prevent osteoporosis could be considered after the appropriate course of adjuvant tamoxifen treatment for node-negative breast cancer. However, only clinical trials will confirm the veracity of this hypothesis, which is based only on a molecular mechanism.

Perhaps of greater significance are the prospects for the prevention of breast cancer. Although it is possible that primary breast cancer will remain sensitive to the inhibitory effects of tamoxifen treatment beyond the duration of genetically unstable micrometastatic disease, the fact that raloxifene is not as promiscuous as tamoxifen in activating genes in breast cancer cells implies that clonal selection of raloxifene-dependent tumors will take longer than with tamoxifen. This difference will clearly prove to be beneficial because the prevention of osteoporosis and of coronary heart and breast disease requires a commitment to a prolonged treatment regimen.

Finally, raloxifene may prove to be more effective than tamoxifen in slowing the clonal selection of occult endometrial cancers. Again, only a close clinical evaluation of large patient populations will prove both breast and endometrial safety. As a result, though, raloxifene may fulfill its promise to be the first prevention maintenance therapy for the 21st century.

References


Notes

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