The Interaction of Raloxifene and the Active Metabolite of the Antiestrogen EM-800 (SC 5705) with the Human Estrogen Receptor 1

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ABSTRACT

A naturally occurring mutation at amino acid 351 (D351Y) in the human estrogen receptor (ER) can change the pharmacology of antiestrogens. Raloxifene is converted from an antiestrogen to an estrogen, whereas the biological properties of the steroidal pure antiestrogen ICI 182,780 are not affected by the D351Y ER (Levenson, A. S., and Jordan, V. C. Cancer Res., 58: 1872–1875, 1998). We propose an assay system that can be used to classify antiestrogens by determining their ability to up-regulate transforming growth factor α (TGF-α) mRNA in MDA-MB-231 cells stably transfected with either wild-type or D351Y ER. The novel compound EM-800 and its active metabolite, EM-652, have been reported to be p.o. active nonsteroidal pure antiestrogens. Using the D351Y cell line, EM-652 is able to up-regulate TGF-α mRNA in a dose-dependent manner and to a similar extent as estradiol, whereas in the wild-type cell line, it acts as an antiestrogen. In addition, the pure antiestrogen ICI 182,780 is capable of inhibiting EM-652-induced TGF-α mRNA expression at the D351Y ER. In MCF-7 cells expressing wild-type ER, it has previously been shown that ICI 182,780 decreases ER only at the protein level. EM-652 treatment does not decrease ER protein levels to a similar extent as ICI 182,780 treatment, and, in addition, EM-652 has no effect on ER mRNA levels. In proliferation assays, EM-652 is as effective as raloxifene in inhibiting cell growth. From these studies, we conclude that the reason the pharmacology of EM-652 is similar to that of raloxifene is because they both fit the ER in the same manner, and their biology depends on an interaction of the antiestrogenic side chain with amino acid 351.

INTRODUCTION

The development of the antiestrogen tamoxifen for the treatment and prevention of breast cancer has established a new therapeutic modality targeting the ER 3 (1–3). However, the successful development of tamoxifen has also presented opportunities to develop new drugs to be used either after tamoxifen failure (4) or to exploit the bone-preserving qualities of tamoxifen (5). Raloxifene, a nonsteroidal antiestrogen (6), is a preventative for osteoporosis (7) but has also been associated with a reduction in the risk of breast cancer (8, 9).

The resolution of the crystal structure of the LBD of the ER with either estradiol or raloxifene (10) has provided an invaluable insight into the final molecular shapes of estrogenic and antiestrogenic complexes. This information has also provided an explanation for the relevance of a naturally occurring mutation in the ER. The D351Y ER originated from a tamoxifen-stimulated tumor grown in athymic mice (11, 12). When stably transfected into ER-negative MDA-MB-231 cells, the D351Y ER can enhance the estrogenic properties of tamoxifen (13, 14), but not the pure antiestrogen ICI 182,780. This assay system is based on the up-regulation of TGF-α mRNA and provides a unique opportunity to study the interactions of novel antiestrogens with the D351Y ER.

The critically important alkylaminothoxy side chain of nonsteroidal antiestrogens such as raloxifene interacts with amino acid 351 to prevent the molecular events necessary to seal an estrogenic ligand into the LBD of the ER (10). Because D351Y ER is capable of forming estrogenic complexes with raloxifene (15), the amino acid at position 351 is critical for the antiestrogenic mechanism of nonsteroidal antiestrogens (8, 16).

The novel nonsteroidal antiestrogen EM-800 (17) has been classified as a p.o. active pure antiestrogen because there are virtually no estrogenic properties in the rodent uterus (18). In contrast, tamoxifen, which is a partial antiestrogen, has estrogen-like properties in the mouse uterus (19, 20) as well as agonist and antagonist properties in the immature rat (21, 22). To date, unlike EM-800, the pure antiestrogens are steroidal compounds with long hydrophobic side chains (23, 24). The drug ICI 182,780 is effective in controlling breast tumor growth after tamoxifen failure in the laboratory (25) and in the clinic (26).

EM-800 is a prodrug that is converted to the active metabolite, EM-652, which binds to the ER to block estrogen action (17). EM-652 reportedly has the highest affinity of any known compound for the ER, including estradiol, 4-OHT, and ICI 182,780 (18). In addition, this new drug is now in clinical trials being tested as a second-line therapy in breast cancer patients that have failed tamoxifen treatment.

In the past, the structure of EM-652 has been depicted by analogy with the pure antiestrogen ICI 182,780 (Fig. 1). However, we noticed that when the structure of raloxifene is re-oriented to its position in the ER LBD-raloxifene complex (10), the position of the alkylaminothoxy side chain is virtually identical to the depiction of the structure of the drug EM-652. There is also a striking similarity in the ring structures that becomes apparent when the orientation of raloxifene is realigned.

There are at least two tests that can be applied to a novel compound to classify it as a pure antiestrogen: (a) we have devised an in vitro assay that compares and contrasts the activation of the TGF-α gene in situ with the wild-type (S30) and D351Y (BC-2) ERs stably transfected into MDA-MB-231 breast cancer cells. In this system, compounds can be classified into four categories: estrogen; pure antiestrogen (ICI 182,780); tamoxifen-like compounds; and raloxifene-like compounds (16); and (b) pure antiestrogens cause a rapid destruction of the ER protein by increasing turnover, which can be measured after pure antiestrogen treatment (27, 28).

We have compared and contrasted the activity of EM-652 with other well-characterized antiestrogens to ensure an accurate molecular classification for the drug at the ER. Our predictions will have important implications for the future clinical application of EM-800 or EM-652.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. ER-negative MDA-MB-231 cells were obtained originally from the American Type Culture Collection (Manassas, VA). S30 (MDA-MB-231 stably transfected with wild-type ER; Ref. 29) and
BC-2 cells (MDA-MB-231 stably transfected with D351Y ER; Ref. 13) were grown in phenol red-free minimal essential medium supplemented with 5% dextran-coated charcoal-treated calf serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 units/ml penicillin, 100 µg/ml streptomycin, and nonessential amino acids. MCF-7 cells were maintained in phenol red containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 6 ng/ml bovine insulin, 100 units/ml penicillin, 100 µg/ml streptomycin, and 1× nonessential amino acids. MCF-7 cells were maintained in phenol red-free RPMI 1640 48 h before each experiment. Cells were passaged twice per week with 0.5% trypsin (1:10 dilution). Cells were grown in a 37°C incubator with 5% carbon dioxide.

**Northern Blot Analysis.** Analysis of TGF-α mRNA expression was assessed by Northern blots as described previously (15). Briefly, total RNA was isolated from S30 and BC-2 cells using the TRIZOL reagent (Life Technologies, Inc.) after treatment with a range of concentrations of estradiol, 4-OHT, raloxifene, ICI 182,780, and EM 652 for 24 h. To determine ER and TGF-α expression in MCF-7 cells, mRNA was prepared using the PolyATtract mRNA isolation system (Promega). The human ER cDNA probe was derived from EcoRI digestion of the ER purified plasmid pSG5-HEGO (a generous gift from Dr. P. Chambon; Ref. 30). A human TGF-α cDNA probe derived by EcoRI digestion of a TGF-α-containing plasmid was a generous gift from Dr. R. Derynck, (Genentech, San Francisco, CA).

**Western Blot Analysis.** Western blotting was performed as described previously (32). Briefly, cells were seeded into T-75 cm² tissue culture flasks. At the summation of the experiment, cells were trypsinized, pelleted, and washed twice in PBS. The pellet was resuspended in protein extraction buffer [0.45 M NaCl, 1 mM MgCl₂, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 25% glycerol, and 20 mM HEPES (pH 7.7)]. Samples were incubated on ice with intermittent vortexing for 30 min and then pelleted. Supernatant was then collected and stored at −80°C. Protein concentration was measured using the Bio-Rad Protein Assay kit, and equal amounts of protein were run in a standard Western blot protocol as described previously (32). The ER primary antibody used was AER311 (Neomarkers, Fremont, CA), and β-actin antibody AC-15 (Sigma) was used to standardize loading. The appropriate secondary antibody conjugated with a horseradish peroxidase kit (Amersham, Arlington Heights, IL) was used to visualize bands using an enhanced chemiluminescence visualization kit (Amersham). The membrane was wrapped in plastic wrap and exposed to Kodak X-OMAT film for a time period ranging from 10 s to 1 h.

**RESULTS**

**EM-652 Increases TGF-α mRNA with the D351Y ER.** The naturally occurring D351Y mutation in the ER changes the pharmacology of specific antiestrogens as determined by their ability to
up-regulate TGF-α mRNA in MDA-MB-231 cells stably transfected with wild-type (S30) or D351Y mutant (BC-2) ER.

It has been reported previously that in BC-2 cells, the D351Y mutation in the ER changes raloxifene from an antiestrogen to an estrogen, and, in addition, 4-OHT becomes more estrogenic (16). The pure antiestrogen ICI 182,780 remains a complete antagonist in both S30 and BC-2 cells. S30 and BC-2 cells were treated for 24 h with either estradiol, 4-OHT, raloxifene, EM-652, or ICI 182,780 and evaluated by Northern blot analysis. In this system, when S30 and BC-2 cells were treated with EM-652, TGF-α mRNA was not induced in S30 cells. However, in BC-2 cells, EM-652 induced TGF-α to the same extent as raloxifene at identical concentrations (Fig. 2). These results suggest that EM-652 can act as an agonist in this system, as predicted.

To further investigate the estrogenic response of EM-652 in S30 and BC-2 cells, we measured TGF-α mRNA induction by Northern blotting and compared increasing concentrations of EM-652 with estradiol and specific antiestrogens. A representative Northern blot comparing increasing doses of EM-652 in combination with a single dose of estradiol showed that in S30 cells, EM-652 acted as an antiestrogen, whereas in BC-2 cells, at similar concentrations, EM-652 acted as an estrogen (Fig. 3a). A separate experiment compared the Northern blots of TGF-α mRNA induction in BC-2 cells with increasing concentrations of estradiol, 4-OHT, raloxifene, ICI 182,780, and EM-652. The results were quantified densitometrically to establish the concentration-response curves to characterize EM-652 (Fig. 3b). As reported previously (14), 4-OHT was the most effective at producing a superinduction of TGF-α, whereas EM-652 was equivalent to estradiol. Raloxifene was a partial agonist.

As reported previously, the pure antiestrogen ICI 182,780 inhibited estradiol-, 4-OHT-, and raloxifene-induced increases in TGF-α mRNA in BC-2 cells. After EM-652 and ICI 182,780 coinoculation, the pure antiestrogen ICI 182,780 is able to inhibit EM-652-induced TGF-α expression (Fig. 4).

Antiestrogenic Activity of EM-652 on Proliferation. To assess the biological relevance of the selected antiestrogen concentrations used in our Western and Northern blotting experiments, we compared the effects of estradiol, 4-OHT, raloxifene, EM-652, and ICI 182,780 on MCF-7 cell growth. It has recently been shown that EM-652 inhibits proliferation in T47D, ZR-75-1, and MCF-7 cells (33). Therefore, it was important to confirm that our system correlates with the previously published report. To address this issue, we performed proliferation assays using MCF-7 cells and screened a number of different antiestrogens including EM-652 (Fig. 5). We found that in...
MCF-7 cells expressing wild-type ER, EM-652 did not act as an estrogen when used alone and acted as an antiestrogen by inhibiting cell growth when used in combination with estradiol. The concentrations used in Fig. 5 were used to determine the effects on ER expression.

**Effects of EM-652 on ER Expression.** To further characterize the biological activity of EM-652 as an antiestrogen, we determined how EM-652 treatment affects ER protein expression in MCF-7 cells. It has been reported previously that estrogen decreases the level of ER in MCF-7 cells at the protein and mRNA levels (32). Interestingly, 4-OHT increases ER in MCF-7 cells, but only at the protein level. In addition, it is a widely accepted fact that the pure antiestrogen ICI 182,780 is able to degrade ER protein while having no effect on ER mRNA. Because EM-800 has been classified as a pure antiestrogen, we tested the effects of EM-652 on ER protein and mRNA expression in MCF-7 cells. The concentrations used were based on our findings in the antiestrogenic experiments with MCF-7 cells (Fig. 5).

![Fig. 4. Effects of the pure antiestrogen ICI 182,780 on TGF-α mRNA expression in combination with either estradiol or antiestrogen treatment.](image)

![Fig. 5. Effect of EM-652 on the proliferation of MCF-7 cells. MCF-7 cells were treated for 7 days with either vehicle (Control), 10^{-9} M estradiol (E2), 10^{-7} M 4-OHT, 10^{-7} M raloxifene (Ral), 10^{-7} M ICI 182,780 (ICI), 10^{-7} M EM-652, or combinations thereof for 24 h before RNA isolation. Northern blot analysis was performed using TGF-α cDNA probe. β-Actin mRNA was measured to ensure even loading.]() (image)

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![Fig. 6. a, Western blot analysis of ER protein expression in MCF-7 cells grown in estrogen-free media and treated with estradiol and antiestrogens. ER protein was measured by Western blot after 24 h of treatment with estrogen-containing media (Whole Serum), vehicle, 10^{-9} M estradiol (E2), 10^{-7} M 4-OHT, 10^{-7} M raloxifene (Ral), 10^{-7} M ICI 182,780 (ICI), or 10^{-7} M EM-652. β-Actin protein was measured to ensure even loading. The blot shown is representative of four independent experiments that yielded similar results. b and c, Northern blot analysis using polyadenylated mRNA of ER and TGF-α mRNA in MCF-7 cells treated with estrogen and different antiestrogens. ER mRNA (b) and TGF-α mRNA (c) were measured in estrogen-stripped MCF-7 cells after treatment with vehicle, 10^{-9} M estradiol (E2), 10^{-7} M 4-OHT, 10^{-7} M raloxifene (Ral), 10^{-7} M ICI 182,780 (ICI), or 10^{-7} M EM-652. β-Actin mRNA was measured to ensure even loading.]()
These compounds affect ER protein levels as predicted. In estrogen-containing whole serum, ER is down-regulated to a similar extent as estradiol. Treatment with 4-OHT caused an increase in ER protein, whereas ICI 182,780, which causes degradation of ER protein (32), resulted in low expression levels. Lastly, vehicle, raloxifene, and EM-652 had comparable effects on ER protein.

To determine their effects on ER mRNA expression, we performed Northern blots on antiestrogen-treated MCF-7 cells (Fig. 6b). As we and others have reported previously (32), estradiol reduced the levels of ER mRNA, whereas the other compounds, including EM-652, had no effect on ER mRNA. We also noted that only estradiol is capable of up-regulating TGF-α mRNA in MCF-7 cells that express wild-type ER (Fig. 6c). From these results and the previous data, we conclude that EM-652 is not a pure antiestrogen and is, in fact, a close structural relative of raloxifene.

**DISCUSSION**

We have demonstrated that the novel antiestrogen EM-652 is pharmacologically and structurally related to raloxifene rather than the pure antiestrogen ICI 182,780. This conclusion is based on the following observations: EM-652, the active metabolite of EM-800, forms an estrogenic complex with the D351Y ER. We have previously noted that raloxifene (15), but not ICI 182,780, is estrogen-like when complexed with the D351Y ER. We report that EM-652 produces the same effect as raloxifene when complexed with D351Y ER. This is compelling evidence that EM-652 binds to the ER in a manner similar to raloxifene. Consequently, based on our mRNA bioassay results alone, EM-800 cannot be classified as either a tamoxifen-like antiestrogen or a pure antiestrogen but could be classified as a raloxifene-like compound.

In this study, MCF-7 cells were used to confirm the antiproliferative action of EM-652 (33) so that appropriate concentrations of antiestrogens would be used to evaluate drug action on ER. Dauvois et al. (28, 34) used MCF-7 cells to establish that steroidoid pure antiestrogens would cause the destruction of the estrogen receptor. We subsequently confirmed these findings using ICI 182,780 (32) and noted that ER mRNA is maintained, although protein is lost. By contrast, an estrogen, such as estradiol, down-regulates both ER mRNA and protein (32). These findings are replicated in Fig. 6, and we conclude that EM-652 is neither an estrogen nor a pure antiestrogen.

On the basis of our assay system that compares and contrasts the efficacy of antiestrogen-ER complexes for wild-type and D351Y ER, we conclude that EM-652 is neither tamoxifen-like nor ICI 182,780-like. Raloxifene and EM-652 act as antiestrogens with zero efficacy with wild-type ER, but 4-OHT acts as an estrogen-like compound. The structures of the raloxifene (10) and 4-OHT (35) ER LBDs have been shown to be different, and we suggest that the raloxifene-ER complex is less likely to bind coactivators than the 4-OHT-ER complex in the context of a MDA-MB-231 cell. We believe that it is possible that MDA-MB-231 cells have an excess of suitable coactivator molecules to aid transcription. 4-OHT is a partial agonist that is known to silence only AF-2 in the LBD of the ER (36). The fact that 4-OHT does not induce TGF-α in MCF-7 cells at 10^{-8} M suggests that there are few available coactivators that will permit the formation of a transcription complex. Although antiestrogen receptor complexes are known to bind corepressor molecules (37), this is unlikely to be the explanation for the context-specific estrogenicity of the 4-OHT-ER complex. If MDA-MB-231 cells contain no corepressors, and corepressors were essential for antiestrogenic action, then it would be unlikely that raloxifene and EM-652 could form antagonist complexes with wild-type ER in transfecants. We hypothesize that raloxifene and, by analogy, EM-652 silence AF-2 and AF-1 at ERα in our model system.

Based on the structure, the results of the mutant ER bioassay, and the inability to down-regulate ER completely, we conclude that EM-800 is not a pure antiestrogen but a raloxifene-like compound. The reclassification of EM-800 as a selective ER modulator has important implications for the clinical application of the drug and will be helpful to guide a rapid targeted clinical development. Conversely, clinical findings from the evaluation of EM-800 as an antitumor agent after tamoxifen treatment failure might also apply to raloxifene. Interestingly enough, a chroman very closely related to raloxifene and EM-652 has very similar effects to raloxifene in the rat uterus (38). The compound is a potent antiestrogen with virtually no estrogen-like properties in the uterus. However, high doses of the novel raloxifene-like chroman have bone-sparing properties in the rat (38).

Most importantly, based on the analogy with raloxifene, we would predict that EM-800 should be effective as a bone-preserving drug with low stimulatory activity in uterus. An early evaluation of these end points will allow a much broader application of EM-800 in general medicine than the original application as a second-line breast cancer drug.

In summary, we have developed a new bioassay system that can classify novel compounds based on an interaction with amino acid 351 in the LBD of the ER. The assay can classify antiestrogens as pure antiestrogens (ICI 182,780), tamoxifen-like compounds, or raloxifene-like compounds. The knowledge of the importance of amino acid 351 for drug interaction now provides an important new system to identify the orientation of compounds in the LBD of the ER. Using this assay, we have reclassified EM-800 as a raloxifene-like compound based on its molecular pharmacology rather than the previous classification as a pure antiestrogen based on uterine actions and a structural similarity to ICI 182,780.

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