Synthesis and Evaluation of Isomeric (17α,20*E*)-11β-Methoxy-21-(trifluoromethylphenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17β-diols as ERα-Hormone Binding Domain Ligands: Effect of the Methoxy Group on Receptor Binding and Uterotrophic Growth

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In this study we have introduced the 11β-methoxy group, a substituent known to increase *in vivo* potency in other steroidal estrogens, into the (17α,20*E*)-21-(trifluoromethylphenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17β-diols: (trifluoromethylphenyl)vinyl estradiols. Receptor binding, using the ERα-HBD, indicated that the 11β-methoxy group had little effect on the relative binding affinity of the target compounds compared to the corresponding 11β-unsubstituted analogs, however, the 11β-methoxy derivatives were significantly more potent in stimulating uterotrophic growth in immature female rats. Molecular modeling studies suggest that while the 11β-methoxy group does not contribute significantly to the overall binding energy of the ligand−ERα-HBD complex, it stabilizes residues associated with the coregulator protein binding site. Such effects would influence the dynamics of subsequent events, such as transcription and biological responses.

Introduction

Breast cancer is the most common cancer diagnosis among women, with an estimated 215 000 new cases reported each year in the United States alone.[1] The majority of the patients are diagnosed with hormone responsive disease, meaning that the tumor contains elevated levels of estrogen receptor (ER[a]) and requires the presence of circulating estrogens to maintain tumor growth.[2] This relationship has stimulated many efforts to develop therapeutic agents that either block the action of estrogens at the receptor level (anti-estrogens)[3−5] or reduce the production of endogenous estrogens (particularly aromatase inhibitors).[6,7] Although the initial development of anti-estrogens exemplified by tamoxifen arose through a drug design program patterned after the triarylethylene agonists, more recent work has been guided by the evaluation of the target receptor. Determining of the amino acid sequence of the nuclear receptors (NRs), including the ER, identified the proteins as members of a homologous family, possessing discrete functional regions.[8−10] These included the DNA binding domain (DBD), hinge region, an N-terminal activation factor-1 (AF-1) binding region, and the C-terminal hormone binding domain (HBD) which contains the activation factor-2 (AF-2) binding region. Subsequent crystallographic studies with the ligand−ER-HBD complexes have provided the best representation of the mode in which the ligands, both agonists and antagonists, bind to the receptor.[11−14] The results of those studies have guided strategies for designing more effective ER-targeted agents. This information is more critical because it is now clear that there are at least two ER subtypes (α and β) that possess different expression patterns and mediate different physiological actions.[15−17] It is also known that the liganded ER subtypes interact with a variety of coactivator and corepressor proteins, also tissue selective, which modulate the intracellular responses.[18−20] Therefore, it is increasingly relevant that specific ER ligands be available to enable investigators to identify the molecular processes that promote receptor affinity, modulate receptor−protein interactions, and effect transcriptional expression.

In addition to our interest in developing new therapeutic agents for the treatment of hormone-responsive breast cancer, we have maintained an ongoing program to generate specific probes for the ER. Many groups have used nonsteroidal estrogens[21−27] or steroids containing modifications on the core steroidal skeleton,[28−30] however, we have focused on the 17α-(X-vinyl)-estradiols, where X = halogen, S/Se-phenyl, or phenyl (Figure 1).[31−33]

Our preliminary data indicated that the ER tolerated these17α-substituents remarkably well, with RBA values exceeding 700 (e.g., X = I). With the availability of the crystal structures of the estradiol−ER-HBD complex, we conducted preliminary ligand docking studies that suggested, assuming that the steroidal portions of our compounds would align with the estradiol structure, the 17α-X-vinyl group would be accommodated within the region bounded by helix-11 and helix-12. Because these two helices are associated with the regulation of agonist and antagonist conformations of the receptor, we hypothesized that the introduction of functional groups would create new interactions with the helices. Such interactions, as evidenced by altered affinity, efficacy, or selectivity, would provide insight into the molecular dynamics of receptor function.

We recently described the synthesis and evaluation of 17α-*E*- and *Z*-(4-substituted phenyl)vinyl estradiols.[34−36] The results indicated that the presence of the 17α-phenylvinyl substituent, in either *E*- or *Z*-configuration, did not convert the ER to an antagonist conformation. The compounds retained significant affinity for the ERα-HBD compared to estradiol and the *E*-and *Z*-(unsubstituted phenyl)vinyl estradiols. Analysis of the two series of compounds with molecular modeling provided binding modes for each in which the 4-substituted phenyl moiety

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[a] Abbreviations: ER, estrogen receptor; ERα, estrogen receptor alpha; NR, nuclear receptor; DBD, DNA binding domain; HBD, hormone binding domain; LBP, ligand binding pocket; RBA, relative binding affinity; ED_{50}, effective dose for a 50% response; NMR, nuclear magnetic resonance; PDB, protein data bank; AF-1, activation factor-1; AF-2, activation factor-2.

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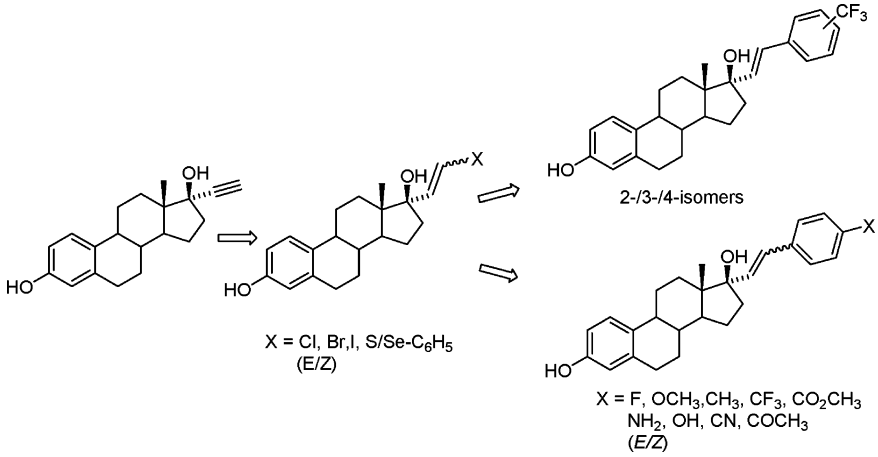


Figure 1. Generation of initial series of 17α-(X-vinyl)estradiols from 17α-ethynyl estradiol.

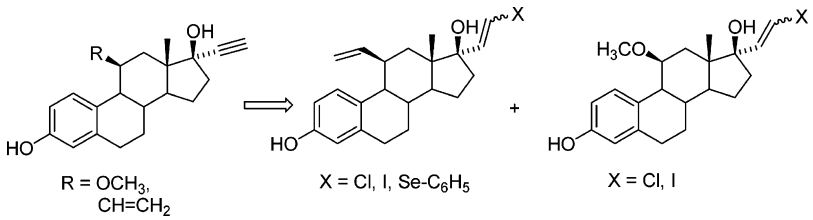
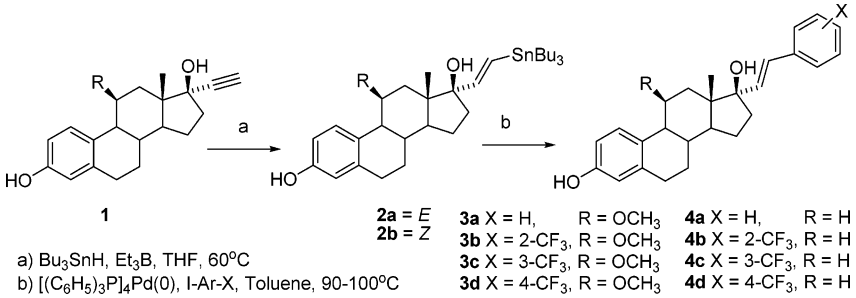


Figure 2. Generation of subsequent series of 11β-substituted 17α-(X-vinyl)estradiols from 11β-substituted 17α-ethynylestradiols.

Scheme 1. Synthesis of 11β-Methoxy-17α-(substituted Phenyl)vinyl Estradiols from Moxestrol **1**[a]



[a] Reagents and conditions: (a) Bu_3SnH, Et_3B, THF, 60 °C; (b) $[(C_6H_5)_3P]_4Pd(0)$, I−Ar−X, toluene, 90−100 °C.

occupied a bounded region located on the alpha face of the estradiol ligand binding pocket (LBP). Determination of calculated binding energies and comparison with observed binding affinities generated graphs with high correlations.

We have reported that the position of functional groups on the terminal phenyl ring also influenced ER-HBD binding affinity as well as *in vivo* uterotrophic growth potency.[37] Ortho-substitution provided the highest affinity for the receptor and the highest potency. *meta*-Substitution was less effective and *para*-substitution was weaker still. These results indicated that subtle structural changes within a small region of the LBP can exert significant differences in biological responses by ligands.

Other studies demonstrated that small substituents at the 11β-position of estradiol confer enhanced receptor binding to the ER and/or enhanced *in vivo* activity.[38−42] We previously reported the preparation of 11β-substituted derivatives of 17α-halovinyl and phenylthiovinyl estradiols (Figure 2).[43,44]

These compounds, which contained both an 11β- and a 17α-group, also expressed high ER affinity, although the influence of each substituent on ER binding was not apparently synergistic.

In this paper we describe the preparation and evaluation of a series of 11β-methoxy-estradiol derivatives bearing the (trifluoromethyl)phenyl vinyl group at the 17α-position. The purpose of the study was to examine the mutual influences of disubstitution on ERα-HBD binding and *in vivo* activity. The results indicate that the effects of substitution on receptor binding correspond to initial receptor binding primarily being influenced by the 17α-subsituted phenylvinyl group, with the 11β-methoxy substituent exerting a major effect on *in vivo* activity. Molecular modeling provides an interpretation of these observations and a strategy for future studies.

Synthesis of Estrogenic Ligands: The target compounds in this series were prepared as part of our larger program to probe the interactions between estrogenic ligands and the ERα-HBD. As a result, we employed the synthetic strategy developed for the preparation of *E*-17α-(substituted-phenyl)vinyl estradiols (Scheme 1). This approach employs the preparation of a common precursor that can undergo ready transformation to a variety of functionalized targets. Moxestrol (11β-methoxy-17α-ethynyl estradiol) **1** was obtained via ethynylation of 11β-methoxy estrone using literature procedures.[38] Hydrostannation of moxestrol with tri-*n*-butyltin hydride gave a mixture of *E*- and *Z*-tri-*n*-butylstannylvinyl estradiols (**2a** and **2b**), with the *E*-isomer **2a** predominating.[44] Stille coupling of the *E*-tri-*n*-butylstannylvinyl-11β-methoxy estradiol with iodobenzene or the isomeric trifluoromethyl phenyl iodides gave the desired 11β-methoxy-17α-arylvinyl estradiols **3a**−**3d** in good overall

Table 1. RBAs of 11β-17α-*E*-(Trifluoromethylphenyl)vinyl Estradiols and their Unsubstituted 11β

cmpd	RBA[a] 25 °C	cmpd	RBA[a] 25 °C
3a	15	**4a**	18
3b	212	**4b**	223
3c	65	**4c**	75
3d	5	**4d**	8

[a] RBA = 100 × [*E*]/[*C*], where [*E*] is the concentration of unlabeled estradiol necessary to reduce the specific binding of tritiated estradiol to the ERα-HBD by 50% and [*C*] is the concentration of the competitive ligand necessary to reduce specific binding by 50%. The RBA of estradiol is 100% at each incubation temperature. Curves for ligand and estradiol had correlation coefficients >95%.

yields after crystallization. Maintenance of the *E*-stereochemistry was determined by ^{1}H NMR spectrometry, which showed a coupling constant (J_d = 18 Hz) for the vinyl protons, consistent with a *trans*-relationship. Chromatographic comparison of the 11β-methoxy products with the corresponding 11-unsubstituted analogs **4a**−**4d**[37] indicated that the new compounds were more polar.

Biological Studies: The new compounds were evaluated for their relative binding affinities (RBAs) using the ERα-HBD isolated from transfected BL21 cells.[45] RBA values were determined using a competitive radiometric receptor binding assay and were compared to estradiol and the 11-unsubstituted phenylvinyl analogs. The results are summarized in Table 1, where the RBA of estradiol is 100%.

The second component of our biological evaluation process involved assaying the series of compounds for estrogenic efficacy.[37] The immature rat uterotrophic growth assay is well established for demonstrating estrogenic responses mediated through ERα. The 11β-methoxy ligands, their 11-unsubstituted analogs, and estradiol were evaluated over a 5 log dose range (0.001−100 nmol) for their uterotrophic potency (Figure 3).

All compounds in the new series were full agonists and, at the highest tested doses, yielded uterotrophic responses comparable to or greater than 1.0 nmol estradiol.

Molecular Modeling Studies: Docking of the 11β-methoxy-17α-(2-trifluoromethylphenyl) vinyl estradiol and the 11-unsubstituted analog, the most potent ligands of the series, was performed on the ERα-HBD cocrystallized with estradiol and refined for the *E*-17α-phenylvinyl estradiols as previously described.[34,35] Docking involved superimposition of the steroidal components of the ligands and performing simulated annealing to optimize the binding mode and determine binding energies. Evaluation of the docked structures indicated that the overall complexes are almost identical and that the 11β-methoxy-17α-(2-trifluoromethylphenyl) vinyl estradiols can be accommodated within the LBP without significant additional conformational changes by either the ligand or the protein. Comparison with the estradiol−ERα-HBD complex suggests that the steroidal scaffolds are essentially superimposable (Figure 4).

The most significant differences in the structure of the complexes are observed on the α-face of the steroid where the side chains of several amino acids undergo conformational adjustments to accommodate the 2-trifluoromethyl group on the phenylvinyl substituent (Figure 5).

The analysis suggests that the 2-trifluoromethyl group is oriented toward Phe-404 and the Phe-425/Ile-424 side chains (3.46 and 3.92 Å) and the peptide backbone (4.65 Å). The 11β-methoxy group is accommodated within the β-face of the LBP (5 Å radius) without conformational changes by the surrounding amino acids, including Leu-346,-384,-525,-540, Ala-350, and Trp-383. The most significant difference between the 11β-methoxy and 11-unsubstituted compounds is the interaction between the terminal methyl of the methoxy group and the side chain of Leu-540, which are within 4.4 Å.

Results and Discussion

Robust synthetic methods developed in our research program provided the opportunity to evaluate some of the factors that affect estrogen ligand binding and activity. The ability to readily introduce a wide variety of substituents at the 17α-position of estradiol that induce an agonist conformation for the ER-HBD allowed us to explore the influence of substituents at other positions that are more difficult to introduce. While appending functional groups at either the 7α- or 11β-position from readily available starting materials, such as estradiol or estrone, requires multiple synthetic and separation steps, preparation of the target 17α-(substituted phenyl)vinyl estradiols can be achieved in good overall yields in two steps from the corresponding ethynyl estradiols. Therefore, preparation of the appropriate 11β-substituted precursor (moxestrol) provided the entry to diversely functionalized target compounds. In this study, we chose to examine the response of the ERα-HBD to the simultaneous presence of the 11β-methoxy group, known to enhance the stability of ligand−ER complexes and the 17α-(trifluoromethylphenyl)vinyl group, which we reported previously.[37] Because the ERα-HBD undergoes a significant adaptive response to accommodate the 17α-(trifluoromethylphenyl)vinyl group, it was not clear how well the HBD would also accept the additional substituent. A preliminary study suggested that such an adaptive response was permitted,[43,44] however, it had been undertaken prior to publication of crystal studies of estrogen−ER-HBD complexes. Also, no data had been reported on the *in vivo* effects of such disubstituted estradiol derivatives. The results of this study provide new insights into the interaction between estrogenic ligands and the ER-HBD.

The results of the competitive binding assays (Table 1) demonstrated that there was essentially no significant difference in RBA values between the 11β-methoxy-17α-(2-/3-/4-trifluoromethyl-phenyl)vinyl estradiols and their corresponding 11-unsubstituted analogs. The 2-trifluoromethyl isomers **3b** and **4b** had the highest affinity (212 vs 223), followed by the 3-isomers **3c** and **4c** (65 vs 75), with the 4-isomers **3d** and **4d** having the lowest affinity (5 vs 8). Even the unsubstituted phenylvinyl ligands **3a** and **4a** were similar (15 vs 18). Therefore, the introduction of the 11β-methoxy group had no significant effect on the binding of the steroidal ligands to the ERα-HBD.

The *in vivo* assay provided a very different situation (Figure 3). Importantly for the subsequent molecular modeling studies, all of the new compounds were full ER agonists. A number of trends in estrogenic activity were observed in this study. First, only the 11β-methoxy-(2-trifluoromethyl-phenyl)vinyl estradiol **3b** was more potent than estradiol under these conditions. Second, the 11β-methoxy derivatives **3b**−**d** are 1−2 orders of magnitude more potent than the corresponding 11-unsubstituted (trifluoromethylphenyl)vinyl analogs **4b**−**d**. Third, introduction of the trifluoromethyl substituent onto meta and para positions of the phenyl ring reduced potency for the 11β-methoxy estradiols. A similar comparison for the 11-unsubstituted analogs was not possible because the unsubstituted phenylvinyl estradiol **4a** does not produce a uterotrophic response in immature rats. Fourth, the order of potency for both the 11-unsubstituted and the 11β-methoxy estrogens corresponded to the RBA values, but the magnitude between individual compounds did not. For example, the RBA values for the *ortho*- and *meta*-trifluoromethylphenyl derivatives differ by only a factor of 3 (ΔLog =

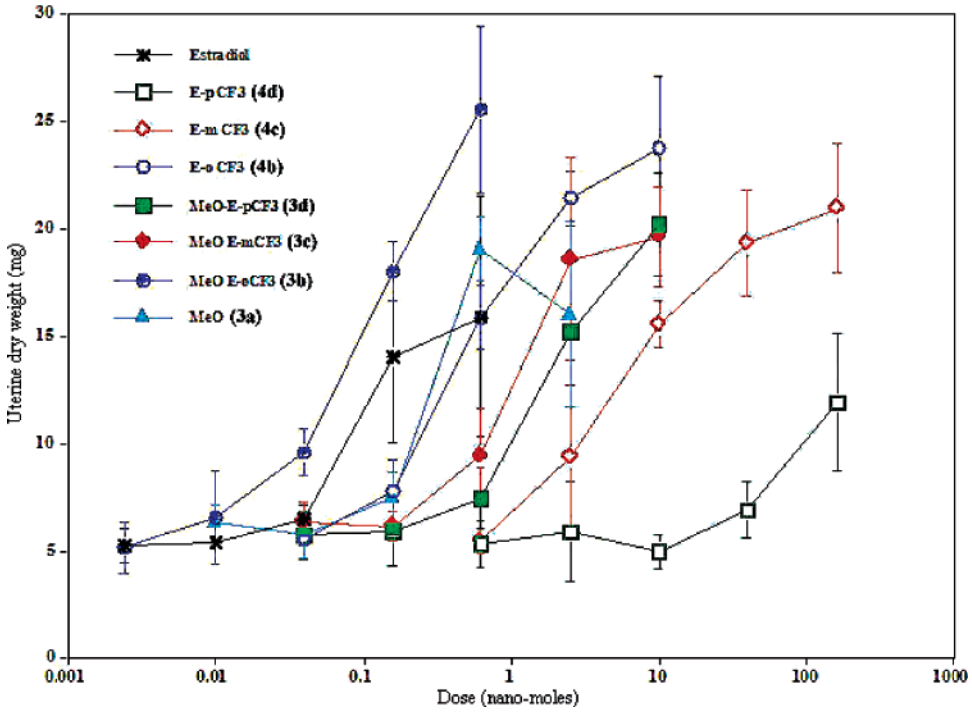


Figure 3. Dose−response curves for the uterotrophic effect in the immature female rat of 11β-methoxy-17α-(trifluoromethylphenyl)vinyl estradiols **3a**−**d**, estradiol, and the corresponding 11β-unsubstituted 17α-(trifluoromethylphenyl)vinyl estradiols **4b**−**d**. The 17α-(phenyl)vinyl estradiol **4a** demonstrated no significant uterotrophic effect in this assay.[37]

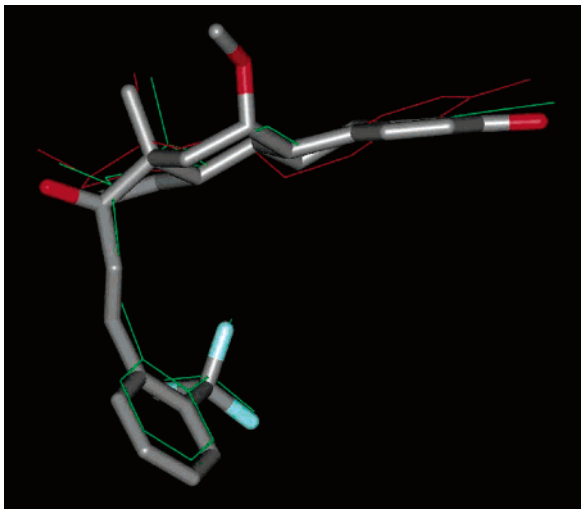


Figure 4. Superimposition of primary binding modes of estradiol (red), 17α-(trifluoromethylphenyl)vinylestradiol **4b** (green), and 11β-methoxy-17α(trifluoromethylphenyl)vinyl estradiol **3b** (gray) in the LBP of ERα-HBD. Protein residues have been removed.

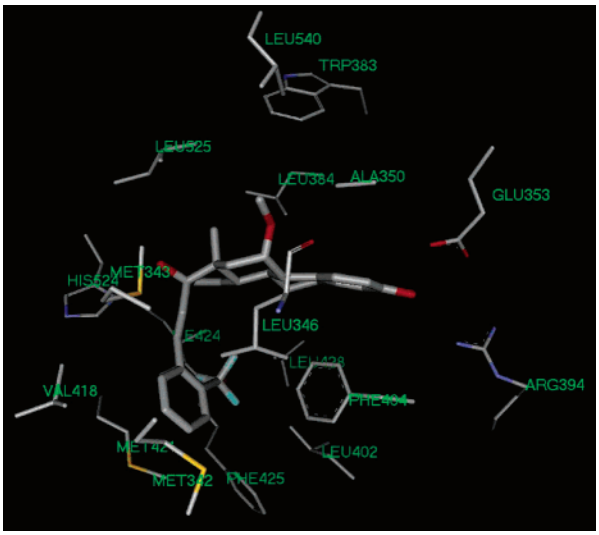


Figure 5. Proposed binding mode of 11β-methoxy-17α-(trifluoromethylphenyl)vinyl estradiol **3b** in ERα-HBD showing association with adjacent protein residues. The terminal 2-trifluoromethylphenyl moiety is bounded by Leu-346, Phe-425 and -404, and Met-342, -343, and -421. The β-methoxy group is associated with Ala-350, Leu-384, -525, and -540, and Trp-383. Interactions with Ala-350, Trp-383, and Leu-540 influence helix-12 orientation and subsequent co-regulator protein binding.

0.48), but the *in vivo* potencies (ED_{50}) differ by over 30 (ΔLog = 1.5). In the 11-unsubstituted series, the *meta*-isomer **4c** was more potent than the *para*-isomer **4d** by almost 2 orders of magnitude, whereas in the 11β-methoxy series this difference is much less than an order of magnitude. Neither situation corresponded to the observed RBA differences.

Part of the difference between the *in vitro* and *in vivo* results may be due to the inherent properties of the proteins involved in the assays. The initial competitive binding assay employs the truncated hERα-HBD overexpressed in and isolated from *E. coli*, whereas the uterotrophic assay uses the full length rat ERα in the presence of coregulatory proteins. The temperatures at which these assays were run also vary: 25 °C for the competitive binding assay and 37 °C for the *in vivo* assay. Studies have shown that the AF-1 domain in the amino terminus also can bind coregulator proteins, which subsequently affects ER ligand binding.[46−48] Such effects would not be observed with the truncated ER-HBD protein. Other studies suggest that in the absence of coregulator proteins, the binding affinities of ER agonists for human and rat ERα and ERα-HBD preparations are very similar, suggesting the sequence differences exist between the two receptors and they have minimal influence on the initial interaction with the agonist ligands.[49] Therefore, in the absence of coregulatory proteins, one can make correlations from hERα-HBD to full length hERα and from hERα to rERα with reasonable confidence. The extension of correlations from

hERα-HBD to rERα in the intact animal require more cautious consideration because of the effect of coregulatory protein binding at either of the AF-1 or AF-2 binding sites. Nevertheless, binding within the LBP of the HBD is the initial event which triggers the subsequent recruitment of those proteins leading to the biological response. The discrepancies between binding affinities and *in vivo* potency prompted our use of molecular modeling to evaluate the binding characteristics of the ligands.

Molecular Modeling of Ligand−ERα-HBD Complexes. Analysis of the binding environment within 5 Å of the ligands indicated that the two substituents interacted with several additional amino acids compared to estradiol itself. These included the previously listed residues on the α-face that involve the 17α-substituted phenyl vinyl group as well as several residues on the β-face that are specifically associated with 11β-methoxy interactions. Both sets of ligands were readily accommodated within the agonist conformation of the receptor as demonstrated by the *in vivo* results. The key observation in this study is that the 11β-methoxy-substituted ligands have essentially identical RBA values as the 11-unsubstituted analogs, yet the *in vivo* potencies differ dramatically. Part of the explanation, derived from an analysis of the RBA values and a comparison of the protein energies for the 2-trifluoromethyl complexes, is illustrative. The 11β-methoxy isomer **3b** has a calculated protein energy of −437.47 kcal/mol, while the value for the 11-unsubstituted isomer **4b** is −447.50 kcal/mol. This strain energy of 10 kcal/mol comes from the adjustments in protein structure needed to accommodate the 11β-methoxy side chain. This is in contrast to the calculated relative binding energies of **3b** (−44.06 kcal/mol) compared to **4b** (−41.52 kcal/mol), a difference of −2.54 kcal/mol, the result of additional interactions. An analysis of the interactions suggested that generating the lowest energy conformation for the complex involved relieving detrimental steric or electrostatic contacts. If these were significant or "hard", the protein energy would be high (70−100% contribution) or, if they were relatively minor, the protein energy term would be low (0−20% contribution). A survey of the correlations indicated that when the relative binding energies are combined with a 30% contribution of protein energies, the sums for the two compounds are essentially equal. This process holds for each of the ligand pairs and is similar to what had been observed previously for the binding of *Z*-17α-(4-substituted phenyl)vinyl estradiols.[35] The relationship between the 11β-methoxy side chain and Leu-540 appears to have special importance because it is the only strong interaction not also present with the 11-unsubstituted analog (Figure 6).

One key feature of Leu-540 is that it is located on helix-12, the region whose orientation is associated with agonist or antagonist conformation. The amide nitrogen of Leu-540 is involved in hydrogen bonding to Asp-351, an interaction often considered vital in the agonist folded conformation of helix-12. The calculated binding energy between Leu-540 and the 11β-methoxy is −0.44 kcal/mol, which is almost 5-fold greater than for the 11-unsubstituted analog (−0.09 kcal/mol). While the absolute magnitude of the binding energy may not be great, the hydrophobic interaction may serve to stabilize the hydrogen bond between Leu-540 and Asp-351, thereby shifting the equilibrium even further in favor of the agonist conformation. A second interaction that may play a role is that between the 11β-methoxy group and Trp-383. The calculated binding energy for the 11-substituted ligand is −0.46 kcal/mol compared to −0.09 kcal/mol for the unsubstituted analog. The significance of this finding is that Trp-383 directly interacts with Met-543 on helix-12. Met-543 is part of the NR-box cavity and generates hydrophobic interactions with Leu-696 of the co-activator protein upon formation of the ERα-co-activator complexes. Also Met-543 is located adjacent to Glu-542, which constitutes one-half of the charge clamp of the NR-box. The overall effect of these interactions may be the stabilization of the ERα-co-activator complexes through stabilization of the NR-box surface (Met-543) and anchoring of Glu-542 in a more favorable position.

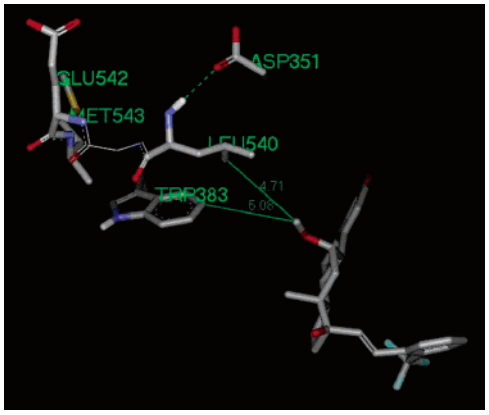


Figure 6. Proposed binding mode of 11β-methoxy-17α-(trifluoromethylphenyl)vinyl estradiol **3b** in ERα-HBD showing involvement with residues adjacent to the 11β-methoxy group. The methoxy group is within 4.71 Å of the indole ring of Trp-383. These residues influence Glu-542 and Met-543 in helix-12.

These calculations provide additional insight into the roles of binding energies, protein energies, RBA values, and the biological response. It appears that an increase in relative binding energy does not necessarily translate into an increased RBA, as seen in this series of ligands. That increase may be partially compensated by strain energy in the protein induced by the ligand. It is the spatial location of those new interactions that may exert significant, but predictable, effects on the biological response. When a gain in binding energy is analyzed, residue by residue, it shows that an interaction, which has a relatively small contribution to the overall energy, may impart a significant effect on the resultant biological response. In this case, the interaction with of the 11β-methoxy group with Leu-540 would be the most significant. Initial studies by Hochberg et al.[45] have indicated that small variations in chain length at the 11β-position of estradiol lead to major alterations in efficacy. It is likely that the interactions identified in our study play a role in the effects observed there as well.

The results of this study provide several observations regarding the design and evaluation of steroidal ligands for the ER. As previously discussed, correlations between *in vitro* RBA values, determined with truncated, bacterially expressed protein, and *in vivo* estrogenic (uterotrophic) potencies, using full length, wild type receptors, require cautious interpretation. While RBA values may predict orders of potency within a given series, they may not predict the magnitude of the activity. In our case, the RBA values correctly predicted the ortho > meta > para order, however, the degree of *in vivo* potency did not correspond to the difference in RBA values. In addition, *in vitro* binding assays did not predict the dramatic difference in potency between the 11β-methoxy estrogens and the 11-unsubstituted analogs. Molecular modeling of the ligand−receptor complexes and analysis of the interactions provided valuable insight into the role that specific residues may play in the estrogenic response. From our modeling studies, it is apparent that the ERα-HBD can readily accommodate estradiol derivatives that are substituted at both

the 11β- and 17α-positions and retain high affinity and efficacy. While the 17α-phenylvinyl group may provide the initial influence on ligand−receptor conformation, the 11β-group apparently influences the subsequent recruitment/stabilization of the coactivator protein. The implications of these results are that we should be able to use the 17α-(2-trifluoromethylphenyl)-vinyl group to stabilize one region of the receptor, then modify the 11β-group to an antagonist-inducing moiety and generate novel steroidal antagonists. Studies to examine this effect are in progress.

Experimental Section

General Methods. All reagents and solvents were purchased from Aldrich or Fisher Scientific. THF and toluene were distilled from sodium/benzophenone. Reactions were monitored by TLC, performed on 0.2 mm silica gel plastic backed sheets containing F-254 indicator. Visualization on TLC was achieved using UV light, iodine vapor, and/or phosphomolybdic acid reagent. Column chromatography was performed with 32−63 μm silica gel packing. Melting points were determined using an Electrotherm capillary melting point apparatus and are uncorrected. NMR spectra chemical shifts are reported in parts per million downfield from TMS and referenced either to TMS or internal standard for deuterochloroform or deuteroacetone solvent peak or external $CFCl_3$ for ^{19}F NMR. All compounds gave satisfactory elemental analyses, ±0.4% (Desert Analytics, Tucson, AZ), unless otherwise stated. ^1H-, ^{13}C-, and ^{19}F-spectra and elemental analyses are provided in the Supporting Information.

Synthesis of Substituted Phenylvinyl Estradiols: General Procedure. To 10 mL of warm toluene (50 °C) were added sequentially the 11β-methoxy-17α-tri-*n*-butylstannylvinyl estradiol (0.11−0.12 g; 0.16−0.20 mmol), tetrakis(triphenylphosphine) palladium(0) (25 mg), butylated hydroxytoluene (2.5 mg), and the iodoarene (2.0−3.0 mmol). The reaction mixture was heated to 90−95 °C and stirred under argon for 16 h. Thin layer chromatography indicated complete consumption of starting stannylvinyl estradiol. The reaction mixture was evaporated to dryness, and the residue was applied to a silica gel column (40 g) and eluted with 1% methanol in chloroform initially, increasing to 3% methanol in chloroform following the elution of unreacted iodoarene. Fractions containing the product were combined and evaporated to dryness, and the crude product was recrystallized from hexane−acetone.

(17α,20*E*)-11β-Methoxy-21-phenyl-19-norpregna-1,3,5(10),-20-tetraene-3,17β-diol, 3a: The general reaction conditions were performed on the 0.15 mmol scale.

Yield = 51 mg, 75%; mp 212−214 °C. ^1H NMR (300 MHz, acetone-d_6): δ 7.83 (1H, s, phenolic-OH), 7.47−7.44 (2H, m), 7.33−7.28 (2H, m), 7.22−7.17 (1H, m), 6.99 (1H, d, J = 8.7 Hz), 6.64 (2H, s), 6.57 (1H, dd, J = 8.7 Hz, 3.3 Hz), 6.49 (1H, d, J = 3.3 Hz), 4.13 (1H, q, J = 3.0 Hz), 3.71 (1H, s, −OH), 3.21 (3H, s), 2.82−1.28 (13H, m, steroid-envelope), 1.19 (3H, s). ^{13}C NMR (75 MHz, acetone-d_6): δ 156.34, 139.83, 139.54, 138.31, 130.12 (2C), 129.90, 128.59, 128.51, 128.20, 127.97 (2C), 116.59, 114.49, 85.20, 78.55, 57.04, 51.84, 50.90, 49.26, 38.36, 36.75, 34.29, 30.60, 29.14, 24.81, 16.90.

(17α,20*E*)-11β-Methoxy-21-(2-trifluoromethylphenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol, 3b: The general reaction conditions were performed on the 0.20 mmol scale. Yield = 51 mg, 55%; mp 265−268 °C. ^1H NMR (500 MHz, acetone-d_6): δ 8.02 (1H, s, phenolic-OH), 7.80 (1H, d, J = 8.0 Hz), 7.68 (1H, d, J = 8.0 Hz), 7.59 (1H, t, J = 7.9 Hz), 7.42 (1H, t, J = 7.9 Hz), 7.01 (1H, qd, J = 2.5 Hz, 15 Hz), 6.98 (1H, d, J = 9.0 Hz), 6.64 (1H, d, J = 15 Hz), 6.57 (1H, dd, J = 2.7 Hz, 9.0 Hz), 6.50 (1H, d, J = 2.7 Hz), 4.13 (1H, q, J = 3.0 Hz), 3.99 (1H, s, −OH), 3.21 (3H, s), 2.93−1.26 (13H, m, steroid-envelope), 1.20 (3H, s). ^{13}C (75 MHz, acetone-d_6): δ 156.36, 139.82 (2C), 136.38 (q, $J_{(CF_3)}$ = 348.7 Hz), 129.85 (2C), 129.47, 128.66, 128.57, 128.21 (q, $J_{(C-C-F)}$ = 29.2 Hz), 127.18 (q, $J_{(C-C-C-F)}$ = 5.6 Hz), 124.62, 124.21, 116.61, 114.51, 85.39, 78.50, 57.05, 51.78, 50.88, 49.40, 38.42, 36.70, 34.24, 30.60, 29.16, 24.81, 16.88. ^{19}F (282 MHz, acetone-d_6, relative to external $CFCl_3$ = 0 ppm): δ −60.

(17α,20*E*)-11β-Methoxy-21-(3-trifluoromethylphenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol, 3c: The general reaction conditions were performed on the 0.20 mmol scale. Yield = 72 mg, 78%; mp 236−239 °C. ^1H NMR (300 MHz, acetone-d_6): δ 7.87 (1H, s, phenolic-OH), 7.78−7.75 (2H, m), 7.55−7.53 (2H, m), 6.99 (1H, d, J = 8.7 Hz), 6.85 (1H, d, J = 15.9 Hz), 6.76 (1H, d, J = 15.9 Hz), 6.57 (1H, dd, J = 2.7 Hz, 8.7 Hz), 6.50 (1H, d, J = 2.7 Hz), 4.13 (1H, q, J = 3.0 Hz), 3.82 (1H, s, −OH), 3.21 (3H, s), 2.85−1.27 (m, 13H, steroid envelope), 1.20 (3H, s). ^{13}C (75 MHz, acetone-d_6): δ 156.37, 140.82, 140.74, 139.83, 133.53, 133.40, 131.34 (q, $J_{(C-C-F)}$ = 44 Hz), 129.87, 128.94 (q, $J_{(CF_3)}$ = 315.8 Hz), 128.58, 128.00, 124.92, 124.40, 116.61, 114.51, 85.33, 78.54, 57.06, 51.85, 50.83, 49.37, 38.47, 36.76, 34.36, 29.83, 29.14, 24.85, 16.92. ^{19}F (282 MHz, acetone-d_6, relative to external $CFCl_3$ = 0 ppm): δ −63.8.

(17α,20*E*)-11β-Methoxy-21-(4-trifluoromethylphenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol, 3d: The general reaction conditions were performed on the 0.16 mmol scale. Yield = 45 mg, 62%; mp 226−228 °C. ^1H NMR (500 MHz, acetone-d_6): δ 7.90 (1H, s, phenolic-OH), 7.69 (2H, d, J = 9.0 Hz), 7.64 (2H, d, J = 9.0 Hz), 6.99 (1H, d, J = 8.6 Hz), 6.86 (1H, d, J = 15.7 Hz), 6.77 (1H, d, J = 15.7 Hz), 6.59 (1H, dd, J = 2.8 Hz, 8.6 Hz), 6.51 (1H, d, J = 2.8 Hz), 4.13 (1H, q, J = 3.0 Hz), 3.87 (1H, s, −OH), 3.22 (3H, s), 2.84−1.28 (13H, m, steroid envelope), 1.21 (3H, s). ^{13}C (75 MHz, acetone-d_6): δ 156.37, 143.61, 141.71, 139.81, 133.52, 133.39, 130.10 (q, $J_{(C-C-F)}$ = 29.8 Hz), 129.83, 128.56, 128.47 (q, $J_{(CF_3)}$ = 250.8 Hz), 127.01(q, $J_{(C-C-C-F)}$ = 3.7 Hz), 116.61, 114.51, 85.33, 78.52, 57.04, 51.93, 50.83, 49.41, 38.49, 38.75, 34.36, 30.67, 29.14, 24.85, 16.91. ^{19}F (282 MHz, acetone-d_6, relative to external $CFCl_3$ = 0 ppm): δ 73.4.

Receptor Binding Studies: *In Vitro* Competitive Binding Assay. The compounds were screened for their affinity for the ERα-HBD isolated from BL 21 cells that overexpressed the 33kDa PER-23d ERG vector.[45,50] The cells were induced with 0.6 mM isopropyl-β-thiogalactopyranoside for 3 h at rt, pelleted by centrifugation, frozen, and stored at −75 °C. The cells were thawed and lysed by sonication (4 × 20 s) in four volumes of lysis buffer (50 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 M urea, pH 7.4 several times. Clarified fractions, obtained at 30 000 × g for 30 min were pooled, assayed for receptor binding, and diluted to 50 nM in ER, and 100 μL aliquots were frozen and stored at −75 °C until ready for use. Then 80 μL of the ERα-HBD-containing extract was incubated with 10 μL of 10 nM 6,7-[H-3]-estradiol (specific activity = 51 Ci/mmole) and 10 μL of either buffer, unlabeled estradiol or test ligand in 100 μL total volume. The final concentrations were 1 nM 6,7-[H-3]- estradiol, 2 nM unlabeled estradiol, (using 200 nM estradiol to define specific binding), and 0.5−5000 nM of the test ligand. In all cases, 10 μL of each incubation solution was removed for assay of the actual initial concentration of [H-3]-estradiol, and the remainder was incubated at 2 °C or 25 °C for 18 h. After incubation, 100 μL of dextran-coated charcoal suspension (fines removed) was added to adsorb the unbound [H-3]-estradiol, incubated for 10 min, and centrifuged, and 100 μL samples were taken from the supernatant fraction for assay of radioactivity. The results were calculated and plotted as % specific binding as a function of log of competitor concentration using the best fit equation for the binding inhibition to define 50% inhibition level. The RBA was calculated as 100 times [*E*]/[*C*], where [*E*] was the concentration of unlabeled estradiol needed to reduce the specific binding of [H-3]-estradiol by 50% and [*C*] was the concentration of test ligand needed to reduce the specific binding by 50%.

Immature Rat Uterotrophic Growth Assay. Test ligands were evaluated using the uterotrophic growth assay.[37] Groups of immature female rats (at least five per group) were injected subcutaneously starting with either peanut oil vehicle (control) or part or all of the range of 0.04, 0.156, 0.625, 2.5, 10, 40, 160, or 640 nmoles of test ligand in 0.1 mL peanut oil (with less than 5% ethanol), and the uterine weights were compared to that of rats receiving estradiol

for three days. Animals were sacrificed 24 h after the last injection, uteri were removed, stripped free of fat and connective tissue, weighed wet, dried in vacuo, and weighed to dry weight. Curves of uterine weight (wet and dry) versus amount of compound injected were compared to assess the potency of the test compound versus the estradiol. The relative estrogenicity of the test ligands to that of estradiol was assessed by determining the dose at which the compound or estradiol gave a uterine growth response equal to 50% of that of 10 nmoles of estradiol.

Molecular Modeling and Dynamics

We initially evaluated the conformations of our ligands **3a**−**d** using the Builder module from Insight II.[51] Potentials for each atom were assigned automatically or manually, when necessary. Low energy conformations were generated using the molecular mechanics method (Discover program, 100 steps, 0.001 final convergence). The ERα-HBD used in our study was obtained from the Protein Data Bank (PDB ID 1G50, wild type ERα-HBD cocrystallized with estradiol). From the three available monomers, monomer A for the A/C homodimer was selected for the docking and molecular dynamics studies. All water molecules present in the crystal structure were deleted. The monomer contains all the amino acid residues between ASN 304 and HIS 550. All manipulations were performed using the Builder module in Insight II. The complex of ERα-HBD monomer and estradiol bound within the binding cavity was minimized using the molecular mechanics method with restraints applied to the backbone atoms of the protein (Discover_3 module, CVFF force field, dielectric constant 2.0, conjugate gradient minimization 10 000 steps or until 0.001 final convergence). Each ligand was optimized using the molecular mechanics method as done with the receptor. Partial charges for each atom were calculated using the Mopac program from the Ampac/Mopac module in the Insight II package. In addition, ligands were further optimized using semiemperical method (calculation method, PM3; calculation type, optimization; optimizer type, native).

The Affinity program within the Docking module in Insight II was used to perform the docking studies of the ligands with the ERα-HBD. This module includes elements from Monte Carlo, simulated annealing, and minimization for automatically docking and finding the best structures of the ligand complexed to the receptor based on the energy of the ligand−receptor complex. The ligand was superimposed on the estradiol molecule (A-ring over A-ring), and the estradiol was then deleted. The complex was subjected to energy minimization to obtain a starting structure in which bad steric contacts are removed and internal energies are relieved. During the docking procedure both the ligand and the protein residues within the ligand binding cavity (amino acids within 15 Å of the ligand as well as all amino acids in helix-12, loops 11−12, 1−3, 6−7) were allowed to flex, while the backbone atoms and the rest of the protein were restrained in their original positions. In addition, the phenylvinyl side chain of the ligand was rotated with maximum of 180° increments to more fully explore the potential binding modes of the conformational choices of the ligand. After each docking procedure, structures within 10 kcal/mol of the lowest energy structure and rms distance of more than 0.125 Å were selected and used in simulated annealing studies. At the beginning of each run, the ligand−receptor complex was minimized over 5000 steps or until 0.001 final convergence. Then each structure was heated from 300 to 500 K over 5000 fs and allowed to equilibrate for an additional 5000 fs. Each structure was allowed to cool to 300 K in 20 stages with 10 K decrements for each stage and 100 fs long equilibration periods for each stage. The structure at the end of the final stage was recorded in an archive file and further minimized 200 steps. Each of the dynamics and simulated annealing cycles was repeated 10 times. During these calculations, additional restraints were applied to amino acids facing the outer surface of the protein. All calculations involving docking and refinement of generated structures were performed with a dielectric constant = 2.0.

Results of the docking studies were analyzed using a combination of modules: Analysis, Discover_3, Docking, and Viewer. Each structure generated during the docking, simulated annealing, and dynamics runs was analyzed in terms of binding energy, ligand energy, and protein energy. Values of the binding energy ΔE binding were calculated as the difference between the potential energy of the complex (Ecomplex) and the potential energy of the ligand (Eligand) and receptor (Ereceptor).[48,52−53] Binding energy calculations were performed using the Energy Analysis macro within the Discover_3 module.

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Supporting Information Available: ^{1}H, ^{13}C, ^{19}F, and elemental analysis of compounds **3a**, **3b**, **3c**, and **3d**. This material is available free of charge via the Internet at http://pubs.acs.org.

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