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Synthesis and evaluation of 17α-(dimethylphenyl)vinyl estradiols as probes of the estrogen receptor-α ligand binding domain

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ABSTRACT

As part of our program to explore the influence of small structural modifications on the biological response of the estrogen receptor-α (ERα), we prepared and evaluated a series of mono-and di-substituted phenyl vinyl estradiols. The target compounds were prepared in 45–80% yields using the Stille coupling reaction and evaluated using competitive binding analysis with the ERα-ligand binding domain (hERα-LBD) and estrogenic activity (induction of alkaline phosphatase in Ishikawa cells). Results indicated that the 2,4- and 2,5-dimethyl derivatives, **5b** and **5c**, had the highest relative binding affinity (RBA = 20.5 and 37.3%) and relative stimulatory activity (RSA = 101.0% and 12.3%) of the di-methyl series.

1. Introduction

Our research program has focused on the preparation of specifically modified derivatives of estradiol as probes for the ligand binding domain (LBD) of the estrogen receptor (ER). These structural probes would permit enhanced insight into the physicochemical factors that influence receptor affinity, subtype selectivity and efficacy resulting from cofactor recruitment. Our early studies suggested the presence of a region within the LBD that could accommodate phenyl (-X-) vinyl groups at the 17α-position of estradiol [1–4]. Analysis of the E-(4-substituted phenyl)vinyl estradiols indicated that there existed within the LBD significant steric tolerance toward 4-substituent and that some polar influences were present [5]. Comparison with the corresponding Z-(4-substituted phenyl)-vinyl isomers suggested that the two phenyl vinyl moieties accessed different regions of the LBD, leading to different structure–activity relationships [6]. Subsequent examination of the E-(2-,3-, and 4-trifluoromethylphenyl)vinyl estradiols demonstrated significant dependence on substitution patterns, as the 2-isomer was more potent *in vitro* as well as *in vivo* [7]. Introduction of an 11β-methoxy group into the E-(2-,3-, and 4-trifluoromethylphenyl) vinyl estradiols had little effect on the affinity for theER-LBD, however, it provided a significant enhancement of *in vivo* potency [8]. In parallel with biological assays, we used computational and crystallographic methods to evaluate the results. Molecular modeling studies suggested, and crystallographic studies confirmed [9], that the phenyl vinyl group induced the formation of a new pocket on the alpha face of the ligand binding pocket (LBP), similar to one observed for thyroid (TR), glucocorticoid (GR) and progesterone (PgR) receptors [10–12]. The position of functional groups on the phenyl ring and their physicochemical properties produce interactions with specific amino acid side chains, leading to significant differences in affinity (RBA values) and *in vivo* potency profiles. Notably, all of our derivatives evaluated to date exhibited full agonist responses, indicating that the compounds induced ER agonist, not antagonist, conformations. Because of the close interactions between the ligand and the LBP, we hypothesized that the introduction of additional substituents on the phenyl group would impart further changes in the biological responses. For example, small changes at the 11β-position, such as the extension of an alkyl chain by a single methylene group, converted a potent ER agonist to an ER antagonist [13,14]. We initially considered evaluating the series of bis(trifluoromethyl)phenyl vinyl isomers, however, the entire set of requisite isomeric bis(trifluoromethyl)phenyl starting materials was neither commercially available nor readily accessible via synthesis. However, the corresponding isomeric di-methylphenyl iodides (iodoxylenes) were available and, because the differences in RBA values between the methyl and trifluoromethyl substituted phenylvinyl estradiols were relatively small, we undertook this

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study investigating the effect that di-methyl substitution on the phenyl ring would have on ER binding and estrogenic activity. In this paper we report the preparation and evaluation of a series of (mono- and di-substituted phenyl)vinyl estradiols as ligands for the estrogen receptor ligand binding domain.

2. Experimental

2.1. 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 on an Argonaut Flashmaster using prepacked Isolute silica gel columns (Biotage). 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 internal standard for deuterochloroform or deuteroacetone solvent peak. All compounds gave satisfactory elemental analyses, ±0.4%, (Desert Analytics, Tucson, AZ) unless otherwise stated. ^{1}H spectra and ^{13}C spectra and elemental analyses are provided in the Supporting Information.

2.2. Synthesis of substituted phenylvinyl estradiols

2.2.1. General procedure for the Stille coupling with 17α-E-tri-n-butylstannylvinyl estradiol and the substituted phenyl/xylyl iodides. Method A

To a reaction tube containing (17α-20E)-21-(tri-*n*-butylstannyl)-19-norpregna-1,3,5(10)20-tetraene-3,17β-diol, **2a**, were added a few crystals of 2,6 di-tert-butyl-4-methylphenol and the substituted phenyl/xylyl iodide. The tube was dried under vacuum for 24 h, then exchanged with argon at least four times. Tetrakis(triphenylphosphine) palladium (0) (0.024 g, 0.02 mmol) and dried, degassed toluene (5 mL) were added and the reaction was heated at 110 °C for 6–18 h. After cooling to room temperature, the reactuib mixture was transferred to a flask with ethyl acetate (50 mL), activated charcoal was added, the mixture heated to boiling, and then filtered through a Celite pad. To the filtrate containing the substituted phenyl vinyl estradiol derivative, fluorsil (4–8 g) was added and then mixture was evaporated to dryness. Hexane was then added to the slurry and the mixture was again evaporated to dryness. The substituted phenyl vinyl estradiol was isolated using flash chromatography and characterized by ^{1}H and ^{13}C NMR, elemental analysis.

2.2.2. General procedure for the Stille coupling with 17-E-tri-n-butylstannylvinyl estradiol 3 acetate and the selected substituted phenyl iodide. Method B

The procedure for coupling 17α-E-tri-*n*-butylstannylvinyl estradiol-3-acetate **2b** was the same as given above. The substituted phenyl vinyl estradiol-3-acetate was isolated, characterized and hydrolyzed using the method given below.

2.2.3. Hydrolysis of the acetate

To a methanolic solution of 17α-(substituted phenyl)vinyl estradiol acetate, 10 N sodium hydroxide (0.1 mL) was added and the reaction solution was stirred for 5–10 min at room temperature. The reaction solution was neutralized with 50 mL of ammonium acetate (10%), extracted with ethyl acetate (3 × 100 mL), dried over anhydrous magnesium sulfate, filtered and concentrated to give the crude estradiol derivative. The substituted phenyl vinyl estradiol derivative was purified using flash chromatography and characterized by ^{1}H NMR and ^{13}C NMR, and elemental analysis.

2.2.4. (17α,20E)-21-(phenyl)-19-norpregna-1,3,5(10),20-tetraene-3,17-diol **3**

Method A was used and the reaction conditions were performed on a 0.68 mmol scale. Yield = 176 mg, 69%. Mp 163–165 °C, $C_{26}H_{30}O_2$ 2·H_2O, Anal: C, 76.06; H, 8.35, Found: C, 75.96; H, 8.28. ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.5. (17α,20E)-21-[2-methylphenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17-diol **4a**

Method B was used and the reaction conditions were performed on a 0.626 mmol scale. Yield = 205 mg, 74%, of the acetate. Hydrolysis at the 0.247 mM scale gave the product. Yield 92 mg, 96% yield. Mp 193–195 °C, $C_{27}H_{32}O_2$ 0.25·H_2O, Anal: C, 82.51; H, 8.33, Found: C, 82.32; H, 8.46 ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.6. (17α,20E)-21-[3-methylphenyl]-19-norpregna-1,3,5(10), 20-tetraene-3,17-diol **4b**

Method B was used and the reaction conditions were performed on a 0.50 mmol scale. Yield = 132 mg, 64.4%. Mp 177–178 °C. $C_{27}H_{32}O_2$ 0.75·H_2O, Anal: C, 80.66; H, 8.40, Found: C, 80.93; H, 8.26 ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.7. (17α,20E)-21-[4-methylphenyl]-19-norpregna-1,3,5(10), 20-tetraene-3,17-diol **4c**

Method A was used and the reaction conditions were performed on a 0.34 mmol scale. Yield = 104 mg, 79%. Mp 195–197 °C. $C_{27}H_{32}O_2$ 1.5·H_2O, Anal: C, 78.04; H, 8.49, Found: C, 78.23; H, 8.56. ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.8. (17α,20E)-21-[2,3-dimethylphenyl]-19-norpregna-1,3,5(10), 20-tetraene-3,17-diol **5a**

Method A was used and the reaction conditions were performed on a 0.34 mmol scale. Yield = 75 mg, 54%. Mp 185–187 °C. $C_{28}H_{34}O_2$ 2·H_2O, Anal: C, 76.68; H, 8.73, Found: C, 76.04; H, 8.28 ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.9. (17α,20E)-21-[2,4-dimethylphenyl]-19-norpregna-1,3,5(10), 20-tetraene-3,17β-diol **5b**

Method B was used and the reaction conditions were performed on a 0.64 mmol scale. Yield = 129 mg, yield 45%, of the acetate. Hydrolysis of acetate (0.176 mM) gave 66 mg, Yield 94%, Mp 183–184 °C. $C_{28}H_{34}O_2$ 2·H_2O, Anal: C, 81.71; H, 8.57, Found: C, 81.54; H, 8.76, ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.10. (17α,20E)-21-[2,5-dimethylphenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol **5c**

Method A was used and the reaction conditions were performed on a 0.34 mmol scale. Yield = 98 mg, 72%. Mp 193–194 °C. $C_{28}H_{34}O_2$ 2·H_2O, Anal: C, 76.68; H, 8.76, Found: C, 75.85; H, 8.06 ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.11. (17α,20E)-21-[2,6-dimethylphenyl]-19-norpregna-1,3,5(10), 20-tetraene-3,17β-diol **5d**

Method B was used and the reaction conditions were performed on a 0.64 mmol scale. Yield = 135 mg, 52%, of the acetate. Hydrolysis of the acetate (0.265 mM) gave 56 mg, 52% yield. Mp 163–165 °C. $C_{28}H_{34}O_2$ 0.75·H_2O, Anal: C, 80.83; H, 8.60, Found: C, 80.93; H, 8.30 ^{1}H NMR (d_6-acetone)

2.2.12. (17α,20E)-21-[3,4-dimethylphenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol ***5e***

Method B was used and the reaction conditions were performed on a 0.55 mmol scale. Yield = 125 mg, 50%. Mp 135–138 °C. $C_{28}H_{34}O_2$ $2{\cdot}H_2O$, Anal: C, 81.71; H, 8.57, Found: C, 81.54; H, 8.76 ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.2.13. (17α,20E)-21-[3,5-dimethylphenyl]-19-norpregna-1,3,5(10),20-tetraene-3,17β-diol ***5f***

Method B was used and the reaction conditions were performed on a 0.55 mmol scale. Yield = 185 mg, 80%. Mp 180–182 °C. $C_{28}H_{34}O_2$. $0.5{\cdot}H_2O$; Anal: C, 81.71; H, 8.57; Found: C, 81.49; H, 8.84, ^{1}H NMR (d_6-acetone); ^{13}C NMR (d_6-acetone).

2.3. Competitive binding to human LBD-ERα

Binding affinities of the mono- and di-methylphenyl vinyl estradiol derivatives relative to E_2 were performed in incubations with the LBD of ERα in lysates of *Escherichia coli* in which the LBD of human ERα (M_{250}–V_{595}) [16] is expressed as described [17] The assay was performed overnight in phosphate buffered saline + 1 mM EDTA at room temperature. The competition for binding of [^{3}H] E_2 to the LBD of the E_2-derivatives in comparison to E_2, relative binding affinity (RBA) was determined over a range of concentrations from 10^{-12} to 10^{-6} M. After incubation, the media is aspirated, the plates are washed three times and the receptor bound radioactivity absorbed to the plates is extracted with methanol and counted. The results, as RBAs compared to E_2, of all receptor studies are from three experiments performed in duplicate. RBAs represent the ratio of the EC_{50} of E_2 to that of the steroid analog × 100 using the curve fitting program Prism to determine the EC_{50}.

2.4. Estrogenic potency in Ishikawa cells

The estrogenic potency of the E_2-analogs was determined in an estrogen bioassay, the induction of AlkP in human endometrial adenocarcinoma cells (Ishikawa) grown in 96-well microtiter plates as we have previously described [18]. The cells are grown in phenol red free medium with estrogen depleted (charcoal stripped) bovine serum in the presence or absence of varying amounts of the steroids, across a dose range of at least six orders of magnitude. After 3 days, the cells are washed, frozen and thawed, and then incubated with 5 mM *p*-nitrophenyl phosphate, a chromogenic substrate for the AlkP enzyme, at pH 9.8. To ensure linear enzymatic analysis, the plates are monitored kinetically for the production of *p*-nitrophenol at 405 nm. Each compound was analyzed in at least three separate experiments performed in duplicate. The RSA (RSA = ratio of $1/EC_{50}$ of the steroid analog to that of E_2 × 100) was determined using the curve fitting program Prism.

2.5. Molecular modeling and dynamics

We initially evaluated the conformations of our ligands using the Builder module from Insight II [19]. 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) and compared to solution conformations determined by NMR. The ERα-LBD used in our study was obtained from our crystal structure of E-(2-trifluoromethylphenyl)vinyl estradiol [9] 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). All ligands used in this study were constructed using the Builder module from Insight II. Potentials for each atom were assigned automatically or manually when necessary. 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 semi-empirical method (calculation method:PM3; calculation type:optimization; optimizer type: native).

The Affinity program within the Docking module in InsightII 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 angstroms 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 in order 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 and 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 $\Delta E_{binding}$ were calculated as the difference between the potential energy of the complex ($E_{complex}$) and the potential energy of the ligand (E_{ligand}) and receptor ($E_{receptor}$) [20,21]. Binding energy calculations were performed using the Energy Analysis macro within the Discover_3 module.

3. Results and discussion

The target compounds selected for this study were three E-mono- and six di-methylphenyl vinyl estradiol derivatives **4b–d** and **5a–f,** as well as one mono-trifluoromethyl and two

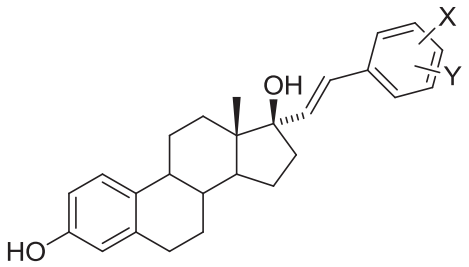
bis(trifluoromethyl)phenyl estradiol derivatives **4d** and **5g,h**. The Stille coupling procedure, developed in our previous studies, was used (Scheme 1). Hydrostannation of ethynyl estradiol or ethynyl estradiol 3-acetate gave the E-tri-*n*-butylstannyl intermediates **2a,b** as the major products (70% isolated yield), readily separable from the Z-isomers (20% isolated yield), with separation easier with the acetate **2b** derivative than with the free phenol **2a**. Coupling with the appropriate iodotoluene or iodoxylene isomer (Method A or B), followed by flash chromatography on silica gel, gave the products in good (45–80%) overall yields. The coupling reactions were generally complete within 2 h, except with the 2,6-dimethyl derivative **5d** for which 16 h was necessary. A change from triphenylphosphine to tri-tert-butylphosphine as the ligand improved the coupling reaction [15]. Synthesis of the trifluoromethyl analogs **4d**, **5g** and **5h** proceeded in a similar manner. The E-stereochemistry was established by ^{1}H NMR where the coupling constant for the vinylic protons was J = 16–18 Hz.

The compounds were evaluated for their relative binding affinity (RBA) using the human ERα-LBD [16,17]. Estrogenic potency was determined using relative stimulatory activity (RSA) of an estrogen responsive gene, alkaline phosphatase, in the human endometrial adenocarcinoma (Ishikawa) cell line [18]. The ERα-LBD binding reflects the initial interaction with the ER while the alkaline phosphatase assay permits us to compare the relationship between binding and efficacy (ER responsiveness). The results for the binding and stimulation assays are shown in Table 1. All of the new compounds demonstrated significant binding to the ERα-LBD and were agonists in the AlkP assay system. Introduction of a single methyl group onto the phenylvinyl moiety generated derivatives with higher (**4a**, RBA = 14.0%) as well as lower (**4b**, RBA = 6.3%; **4c,** RBA = 7.3%) affinity than the parent compound **3** (RBA = 10.3%). This trend is similar to that previously observed for the mono-trifluoromethylated series [7]. Introduction of a second methyl group onto the phenylvinyl moiety expanded the range of RBA values for the series and identified the influence of the second substituent. For example, 2-(ortho)-substitution was still optimal but the highest affinity was observed for those compounds in which the second methyl group was either in the 5-(meta)- (RBA = 37) or 4-(para)- position (RBA = 20.5). 2,3- and 2,6-Di-methyl substitution gave RBA values approximately equal to that of the unsubstituted parent compound (RBAs = 10.5 and 9.3 vs 10.3) while 3,4- and 3,5- di-methyl substitution gave products that were comparable to the 3- and 4-mono-methyl derivatives.

The second component of the study involved the ability of the ligands to stimulate the induction of alkaline phosphatase in the Ishikawa cell line. The parent unsubstituted phenylvinyl estradiol **3** possessed an RSA value essentially identical to its RBA value (9.4% vs 10.3%), suggesting that the relationship between receptor binding and biological potency of the ligand–receptor complex is parallel to that formed with estradiol. This relationship between RBA and RSA values held with some but not all of the compounds evaluated in this study. In particular, the 2-methylphenylvinyl estradiol **4a** demonstrated a more potent stimulatory effect (RSA = 31.8) than binding affinity (RBA = 14.0). This relationship was reversed for the 2,4-, the 2,5- and 2,6-dimethyl derivatives for which the RSA values were 1/3 to 1/2 those of the RBA values.

Although a complete series of trifluoromethylphenyl vinyl estradiols was not prepared, we synthesized and evaluated three

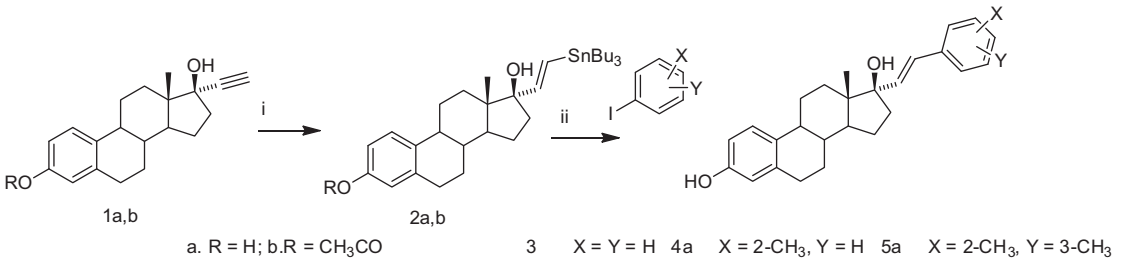
Table 1
Relative binding and stimulatory activity for estradiol and derivatives **3**, **4a–d**, **5a–h**.



Compound	Substitution	ERα-LBD (RBA)[a]	Ishikawa AlkP (RSA)[b]
Estradiol	None	100	100
3	X = H, Y = H	10.3 ± 2.9	9.4 ± 2.5
4a	X = 2-CH_3, Y = H	14.0 ± 0.8	31.8 ± 7
4b	X = 3-CH_3, Y = H	6.3 ± 1.5	5.0 ± 1.4
4c	X = 4-CH_3, Y = H	7.3 ± 0.5	5.5 ± 0.7
4d	X = 2-CF3, Y = H	80.2 ± 16.5	100 ± 9
5a	X = 2-CH_3, Y = 3-CH_3	10.5 ± 1.7	8.4 ± 4
5b	X = 2-CH_3, Y = 4-CH_3	20.5 ± 1.9	11.0 ± 3.7
5c	X = 2-CH_3, Y = 5-CH_3	37.3 ± 4.3	12.3 ± 4.3
5d	X = 2-CH_3, Y = 6-CH_3	9.3 ± 0.5	2.5 ± 0.7
5e	X = 3-CH_3, Y = 4-CH_3	6.0 ± 0.8	1.5 ± 2
5f	X = 3-CH_3, Y = 5-CH_3	5.0 ± 0.8	3.5 ± 0.7
5g	X = 2-CF_3, Y = 5CF_3	26.3 ± 1.5	29.7 ± 3.4
5h	X = 3-CF_3, Y = 5CF_3	9.0 ± 1.8	3.5 ± 0.7

[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 25 °C. Curves for ligand and estradiol had correlation coefficients >95%.

[b] RSA, relative stimulatory activity compared to E_2 = 100%, in stimulation of alkaline phosphatase (AlkP) in the Ishikawa cell line. EC_{50} for E_2 = 0.9 ± 0.2 nM.



Scheme 1. Synthesis of mono- and di-substituted phenylvinyl estradiols.

analogs, the 2-trifluoromethyl, 2,5- and 3,5-bis(trifluoromethyl) phenyl vinyl estradiols **4d,5g,5h** for comparison purposes. In the binding assays, the mono-trifluoromethylated derivative **4d** had a higher RBA value than **4a** (80.2 vs 14.0) while the 2,5-bis(trifluoromethyl) derivative **5g** had a lower RBA values than the corresponding analog **5c** (26.3 vs 37.3). The 3,5-bis(trifluoromethyl) derivative **5h** also had a slightly higher affinity than the corresponding analog **5f** (9.0 vs 5.0). Disparities were also observed in the Ishikawa assay. The 2-trifluoromethyl derivative was very potent (RSA = 100) and more active than the methyl analog (RSA = 31.8) while the 2,5-bis(trifluoromethyl) derivative **5g** was somewhat weaker (RSA = 29.7), but still more potent than the 2,5-di-methyl analog (RSA = 12.3). There was no significant difference between the 3,5-di-methyl and 3,5-bis(trifluoromethyl) analogs (RSA = 3.5 for each).

Although there may be alternate explanations for the observed differences between ER binding and biological activity, for example, cell penetration or intracellular metabolism, it appears more likely that the presence of the second substituent affects the ability of the ligand–receptor complex to readily assume the agonist conformation, thereby leading to altered *in vitro* potency. The presence of the second substituent clearly does not prevent the complex from forming an agonist conformation as evidenced by the observation that all of the compounds were full agonists in this assay, however, it may influence how easily this conformation is obtained. Using the crystal structure of 17α-E-(2-trifluoromethylphenyl)vinyl estradiol **4d** complexed with ERα-LBD [9] we evaluated the low energy conformations of the corresponding di-methylphenyl vinyl estradiol complexes. The binding modes for the compounds, superimposed on each other, are shown in Fig. 1.

As can be seen, the general conformation of the ligand within the binding pocket is the essentially the same for each compound, with minor torsional differences around the C_{20}–C_{21} bond resulting from the need to accommodate the additional methyl group within the receptor. Subtle movements of the surrounding peptide side chains are required to provide adequate space for the methyl groups, resulting in perturbations of the conformations of the amino acids associated with ligand binding. Enhanced affinity for the 2-trifluoromethyl compound **4d** compared to the 2-methyl derivative **4a** is likely due to enhanced dipole–dipole interactions between the fluoro-group and the complementary peptide backbone [9].

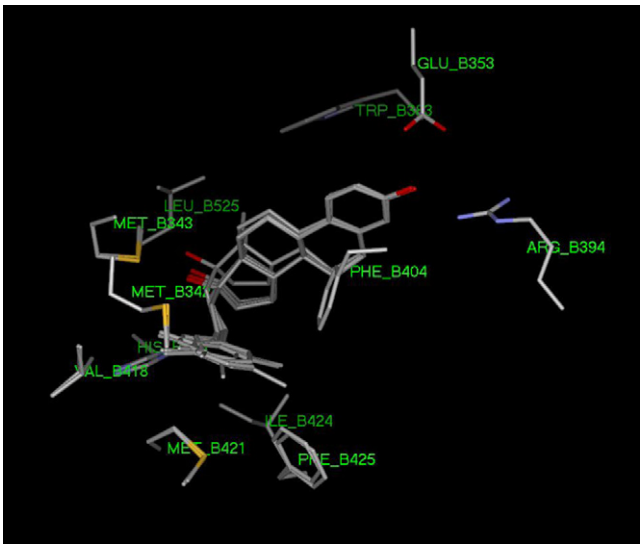


Fig. 1. Superimposition of 17α-(di-methylphenyl)vinyl estradiol isomers **5a–f** within ligand binding pocket (LBP) of ERα. As with the mono-substituted phenyl vinyl estradiols, the terminal phenyl ring is bounded by three methionines and one phenylalanine. Only slight side chain or steroidal adaptations are required to accommodate the ligands within the LBP.

Based upon predictions that ligand–protein remodeling may be involved with the biological response, we undertook to correlate measured RBA and RSA values with calculated binding energies, including total, ligand and protein energy components (see Supporting Information). Given the small number of compounds involved in this study it may not be possible to generate definitive conclusions, however, distinct trends could be noted. When RBA values were initially plotted against the total binding energy for the complexes, there was a poor correlation for the di-methyl substituted ligands, however, inclusion of a term for protein energy (0.9 protein energy), reflecting the energy price paid for remodeling of the receptor, yielded a plot with a clear correlation (R^2 = 0.804). Plotting the RSA values, which reflect the generation of a physiologically competent complex, against the protein energy also gave very clear correlation (R^2 = 0.811).

4. Conclusions

The results of this study suggest that relatively small changes in structure of the steroidal estrogens produce significant variations in the response of the target ERα-LBD. The introduction of the second methyl group provides a generally consistent change in physicochemical properties, which may affect pharmacokinetic parameters such as rates of metabolism and clearance. However, at the molecular level, where pharmacodynamic properties are generated, small changes in protein architecture can influence the orientation of functional groups involved with recruitment of activation factors or conformational equilibria. In order to accommodate the second methyl substituent on the phenyl ring, the amino acids within the 17α-region of the ER ligand binding pocket must undergo differential remolding [9]. This process can lead to conformations which possess higher affinity for the ligand, but not necessarily more favorable orientations for subsequent coregulatory peptide recruitment (efficacy). Our results suggest that substituents at the 2 and/or 5-positions of the phenylvinyl group lead to compounds that retain high binding affinity yet do not stimulate the receptor as effectively. Such compounds may function as "impeded estrogens" and studies to explore that possibility are in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2012.01.003.

References

[1] Napolitano E, Fiaschi R, Herman LW, Hanson RN. Steroids 1996;61:384.
[2] Hanson RN, Herman LW, Fiaschi R, Napolitano E. Steroids 1996;61:718.
[3] Hanson RN, Napolitano E, Fiaschi R. Steroids 1998;63:479.
[4] Hanson R, Napolitano E, Fiaschi R. J Med Chem 1998;41:4686.

[5] Hanson RN, Lee CY, Friel CJ, Dilis R, Hughes A, DeSombre ERJ. Med Chem 2003;46:2865.
[6] Hanson RN, Friel CJ, Dilis R, Hughes A, DeSombre ERJ. Med Chem 2005;48:4300.
[7] Hanson RN, Lee CY, Friel C, Hughes A, DeSombre ER. Steroids 2003;68:143.
[8] Hanson RN, Dilis R, Tongcharoensirikul P, Hughes A, DeSombre ERJ. Med Chem 2007;50:472.
[9] Nettles KW, Bruning JB, Gil G, O'Neill EE, Nowak J, Hughes A, Kim Y, DeSombre ER, Dilis R, Hanson RN. EMBO Rep 2007;8:563.
[10] Borngraeber S, Budny M-J, Chiellini C, Cunha-lima ST, Togashi M, Webb P, Baxter JD, Scanlan TS, Fletterick RJ. Proc Natl Acad Sci U S A 2003;100: 15358.
[11] Barker M, Clackers M, Demaine DA, Humphreys D, Johnston MJ, Jones HT, Pacquet F, Pritchard JM, Salter M, Shanahan SE, Skone PA, Vinader VM, Uings I, McLay IM, Macdonald SJFJ. Med Chem 2005;48:4507.
[12] Madauss KP, Deng S-J, Austin RJH, Lambert MH, McLay I, Pritchard J, Short SA, Stewart EL, Uings I, Williams SPJ. Med Chem 2004;47:3381.
[13] Zhang J, Labaree DC, Mor G, Hochberg RB. J Clin Endocrinol Metab 2004;89:3527.
[14] Zhang JX, Labaree DC, Hochberg RB. J Med Chem 2005;48:1428.
[15] Menzel K, Fu GCJ. Am Chem Soc 2003;125:3718.
[16] Green S, Walter P, Kumar V, Krust A, Bornert JM, Argos P, Chambon P. Nature 1986;320:134.
[17] Harris HA, Bapat AR, Gonder DS, Frail DE. Steroids 2002;67:379.
[18] Littlefield BA, Gurpide E, Markiewicz L, McKinley B, Hochberg RB. Endocrinology 1990;127:2757.
[19] Insight II, 97.2/Discover 2.9.7 (MSI Inc. San Diego) 1997.
[20] Nair AC, Miertus S, Tossi A, Romeo DA. Biochem Biophys Res Commun 1998;242:545.
[21] DeLisle RK, Yu S-J, Nair AC, Welsh WJJ. Mol Graph Mod 2001;20:155.