Interactions of synthetic estrogens with human estrogen receptors

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Abstract

Synthetic estrogens have diverse chemical structures and may either positively or negatively affect the estrogenic signaling pathways through interactions with the estrogen receptors (ERs). Modeling studies suggest that 4-(1-adamantyl)phenol (AdP) and 4,4'-(1,3-adamantanediyl)diphenol (AdDP) can bind in the ligand binding site of ER α . We used fluorescence polarization (FP) to compare the binding affinities of AdP, AdDP and 2-(1-adamantyl)-4-methylphenol (AdMP) for human ER α and ER β with the binding affinities of the known ER ligands, diethylstilbestrol (DES) and 4-hydroxytamoxifen (4OHT). Competition binding experiments show that AdDP has greater affinity for both ERs than does AdP, while AdMP does not bind the receptor proteins. The relative binding affinities of AdDP and AdP

are weaker than the affinity of DES or 4OHT for both ERs with the exception of AdDP, which binds ER β with higher affinity than does 4OHT. We also found that AdDP and AdP cause differential conformational changes in ER α and ER β , which result in altered affinities of the ERs for fluorescein-labeled estrogen response elements (EREs) using a direct binding FP assay. The results show that ER β liganded with either AdDP or AdP has greater affinity for human pS2 ERE than the ER β -4OHT complex. The data suggest that synthetic molecules like adamantanes may function as biologically active ligands for human ERs. This demonstrates the importance of considering the potential of novel classes of synthetic compounds as selective ER modulators.

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Introduction

The estrogen receptor (ER) is a ligand-dependent transcriptional factor with domain structure (Kumar *et al.* 1987) and belongs to a supergene family that includes receptors for steroid and thyroid hormones, vitamin D₃ and retinoic acid (Evans 1988). In the absence of a ligand the ER resides in the cell nucleus associated with heat-shock proteins (Joab *et al.* 1984, Sanchez *et al.* 1990). Binding of the hormone estrogen causes the ER to undergo conformational changes leading to dissociation of the heat-shock proteins and formation of stable receptor dimers (Kumar & Chambon 1988). The ligand-occupied receptor dimers interact with estrogen response elements (EREs) located within the regulatory region of target genes, and then are able to interact with other cellular components to either activate or suppress transcription of a target gene in a promoter- and cell-specific manner (Tora *et al.* 1989).

Recently it has been discovered that a second ER (ER β) exists, in addition to the traditional ER, now called ER α . ER α and ER β share common physical and functional properties; they also have high degrees of homology in their ligand binding domains (LBDs) and DNA binding domains (Kuiper *et al.* 1996, Mosselman *et al.* 1996). Both

ERs have similar affinities for 17 β -estradiol (E₂), recognize the same EREs and are expressed in distinct and overlapping tissues (Couse *et al.* 1997).

In addition to the endogenous estrogens, a group of exogenous chemicals called xenoestrogens can display estrogen-like functions in estrogen responsive tissues. The source of xenoestrogens can be dietary in nature including phytoestrogens (Kurzer & Xu 1997) or industrial chemicals (pharmaceuticals, pesticides, pollutants) (Korach *et al.* 1997). The xenoestrogens interact with the ERs and can either induce a response that mimics endogenous estrogen stimulation or produce an inactive receptor–ligand complex that inhibits the transcription of ER regulated genes.

The synthetic xenoestrogens have diverse chemical structures but there are some common structural motifs among these compounds. The most essential structural motif that elicits estrogenic activity is a phenol that is relatively unhindered, attached to a rather bulky hydrophobic structure (Katzenellenbogen 1995). Adlercreutz & Mazur (1997) have noted that although the length and the width of the E₂ molecule fit compactly within the LBD of the ER protein, binding of E₂ within this domain leaves large unoccupied regions opposite the B- and C-rings. Endo *et al.* (1999) have recently described binding of

Figure 1 Structures of the compounds used in this study. (1) E₂; (2) DES; (3) 4OHT; (4) AdDP; (5) AdP; (6) AdMP.

dicarba-closo-dodecaborane and an adamantyl substituted phenol (4-(1-adamantyl)phenol (AdP)) to ER α . They suggested that the carborane cage provides stronger hydrophobic van der Waals' contacts with the interior of the LBD than do rings C and D of the E₂ molecule (Adlercreutz & Mazur 1997).

Our modeling studies suggested that along with AdP, adamantyl diphenol, 4,4'-(1,3-adamantanediyl)diphenol (AdDP), can also be effectively bound within the ligand site of ER α .

We have employed fluorescence polarization (FP) competition and direct binding experiments (Checovich *et al.* 1995, Nikov *et al.* 2000) to test the prediction of our modeling studies and determine the ability of AdP and AdDP (Fig. 1) to interact with both ER α and ER β and also to determine the effectiveness of adamantyl phenol ligands on the formation of complexes between ER α or ER β and *Xenopus vit* A2 or human pS2 EREs. 2-(1-Adamantyl)-4-methylphenol (AdMP), which is not a *para* substituted phenol and which modeling studies suggest would not bind effectively to ER α , was also tested as a ligand for ER α and ER β in these studies. FP competition and direct binding experiments were also carried out with the planar aromatic estrogens diethylstilbestrol (DES) and 4-hydroxytamoxifen (4OHT) in an attempt to detect differences between the binding behavior of planar hydrophobic molecules and molecules possessing a distinctly three dimensional (adamantyl) hydrophobic group.

Materials and Methods

Materials

The steroids E₂, DES and 4OHT were obtained from Sigma Chemical Co. (St Louis, MO, USA). AdP, AdDP and AdMP were from Aldrich (Milwaukee, WI, USA).

Human recombinant ER α and ER β , and fluorescein-labeled E₂ (ES2) were purchased from Pan Vera Corporation (Madison, WI, USA).

Fluorescein end-labeled *Xenopus vit* A2, human pS2 EREs and glucocorticoid response element (GRE) were custom synthesized by Oligos Etc. (Wilsonville, OR, USA).

Molecular modeling

Molecular modeling studies were done using SYBYL molecular modeling software (SYBYL release 6.6.2; Tripos, St Louis, MO, USA). All molecules were drawn using SYBYL sketcher and minimized with the Powell method using simplex initial optimization and Tripos force field.

ES2-ER direct binding experiments

Recombinant human ER α and ER β were serially diluted from 256 nM to 0.5 nM in screening buffer (100 mM potassium phosphate, pH 7.5; 100 μ g/ml bovine gamma globulin; 0.02% sodium azide) to a final volume of 100 μ l in borosilicate test tubes. ES2 was added to each test tube to a final concentration of 1 nM and incubated for 60 min at room temperature. The FP of each tube was measured on a Beacon 2000 Fluorescence Polarization Instrument (Pan Vera Corporation) with a 490 nm excitation filter and 530 nm emission filter (Jameson & Sawyer 1995). FP values were plotted vs ER concentration.

K_d was calculated using a nonlinear least-square curve fitting program (Prizm; Graphpad Inc., San Diego, CA, USA) as the concentration of ER at which half of the ligand is bound.

Competitive binding experiments

DES, 4OHT, AdP, AdDP and AdMP were tested for their ability to displace the ES2 molecule from ER α -ES2 and ER β -ES2 complexes.

Serial dilutions of each competing compound were prepared from an 8 mM ethanol stock solution in screening buffer. Preincubated ER α or ER β (13 nM) and ES2 (1 nM) were added to produce a final volume of 100 μ l. After 60 min incubation at room temperature, the polarization values at each competitor's concentration were measured using the Beacon 2000 FP system with a 490 nm excitation filter and 530 nm emission filter. The polarization values were converted to percent inhibition using the equation $I_{\%} = (P_0 - P) / (P_0 - P_{100}) \times 100$, where P_0 is the

Table 1 Double stranded DNA sequences containing EREs and GRE. The underlined sequences represent the limits of the 13 bp reverse repeat of the EREs and the GRE, containing 5 bp arms and a 3 bp spacer region

	Sequence
<i>Xenopus vit</i> A2 ERE	5' XGTC CAA AGT CA <u>GGTCA CAG TGACC</u> TGA TCA AAG TT 3' 3' CAG GTT TCA GT <u>CCAGT GTC ACTGG</u> ACT AGT TTC AA 5'
Human pS2 ERE	5' XGT CCA AAG TCA <u>GGTCA CGG TGGCC</u> TG ATC AAA GTT 3' 3' CA GGT TTC AGT <u>CCAGT GCC ACCGG</u> AC TAG TTT CAA 5'
Consensus GRE	5' XGT CCA AAG TCA <u>GAACA CAG TGTC</u> TGATC AAA GTT 3' 3' CA GGT TTC AGT <u>CTTGT GTC ACAAG</u> ACTAG TTT CAA 5'

polarization value at 0% inhibition, P_{100} is the polarization value at 100% inhibition, and P is the observed FP at each concentration point. Free ES2 (100% inhibition) was used as a positive control and ER–ES2 complex (0% inhibition) was used as a negative control. Polarization values were transformed into percent inhibition in order to normalize the differences at 0% inhibition for each run. The percent inhibition vs competitor concentration curves were analyzed by nonlinear least-squares curve fitting and yielded IC_{50} values (the concentration of competitor needed to displace half of the bound ligand). To compare the binding affinities of the tested compounds, IC_{50} values were converted to relative binding affinities (RBAs) using E_2 as a standard. The E_2 RBA was set equal to 100, and the RBA value for each of the phytoestrogens was calculated using the formula $RBA = (IC_{50} E_2 / IC_{50} \text{ competitor}) \times 100$.

Preparation of EREs

EREs from *Xenopus vit* A2 gene, ERE from human pS2 gene and consensus GRE were tested to bind ER α and ER β (Table 1). The sense DNA strands (Oligos Etc.) containing EREs and GRE were labeled with fluorescein attached via a six-carbon spacer at the 5' terminus. The 35 bp double stranded oligonucleotides were prepared by annealing equimolar concentrations of the sense and anti-sense strands in 10 mM Tris–HCl, pH 7.8 and 150 mM NaCl. This mixture was heated to 95 °C for 10 min and slowly cooled (30 min) to room temperature. To remove any hairpin formations the double stranded DNA was purified by 12% polyacrylamide (1:19 bisacrylamide:acrylamide) gel electrophoresis containing 89 mM Tris–borate, 2.5 mM EDTA, pH 8.3 and 10% ammonium persulfate.

ER–ERE direct binding studies

To further investigate the estrogenic properties of the xenoestrogens we performed direct binding experiments and measured the abilities of ER α and ER β to associate with *Xenopus vit* A2 ERE and human pS2 ERE in the

presence of synthetic ligands. ER α and ER β were serially diluted from 450 nM to 0.8 nM in DNA binding buffer (10 mM potassium phosphate, pH 7.8; 0.1 mM EDTA; 50 μ M magnesium chloride; 10% glycerol). The ERs were incubated for 30 min with concentrations of each of the xenoestrogens required to saturate ER α and ER β as determined by competitive binding experiments, and then for 10 min with poly(dI–dC) (1 μ g/5 μ g protein) at room temperature. The binding, initiated by adding fluorescein-labeled synthetic oligonucleotide EREs (final concentration 0.5 nM), was allowed to proceed at room temperature for 60 min. The polarization values of each ER concentration were then measured on the Beacon 2000 instrument with 490 nm excitation and 530 nm emission maximums. The binding isotherm was constructed by plotting percent saturation vs ER concentration using the formula $S\% = (P - P_0) / (P_{100} - P_0) \times 100$, where P_0 is the polarization value of E_2 at 0% saturation, P_{100} is the polarization value of E_2 at 100% saturation, and P is the observed FP at each concentration point. K_d was calculated from the binding curves using a nonlinear least-squares curve fitting program. The binding affinities of ER α and ER β (liganded with synthetic estrogens) for EREs were also calculated in terms of RBA ($RBA = (K_d E_2 / K_d \text{ competitor}) \times 100$).

In order to prove the reliability and specificity of the method we compared the binding affinities of ER α and ER β (liganded with E_2) for fluorescein-labeled *Xenopus vit* A2 ERE and fluorescein-labeled GRE. At the concentration range tested no ER–GRE complexes were formed as opposed to the high affinity binding of both ERs to the consensus ERE (Nikov *et al.* 2000).

Results

Modeling studies

Figure 2 illustrates how AdDP may be accommodated within the LBD of ER α with the two *para* phenol groups of the AdDP molecule positioned similarly to the C–3

Figure 2 Typical distances between the two hydroxyl groups in (a) E_2 (11.18 Å), (b) DES (12.17 Å) and (c) AdDP (11.82 Å). All the compounds were drawn and minimized as described in Materials and Methods.

phenolic hydroxyl group and the 17β -hydroxyl group of an E_2 ligand. We have also modeled the polar interactions between ER α and AdDP (Fig. 3).

Ligand binding specificity of ER α and ER β

To determine the affinity of the adamantyl phenols for ER α and ER β we first measured the ability of ES2 to associate with the receptor proteins (Fig. 4). One nano-

Figure 4 The binding isotherms of human recombinant ER α and ER β and ES2. The fluorescence of ES2 by itself produces low polarization values. Upon binding ER molecules, the rotational freedom of the complex decreases and higher polarization values are measured. When all ES2 molecules are bound a further increase in ER concentration does not affect the fluorescence polarization (FP) of the system. The data points represent the mean polarization values $\pm$ S.E.M. from two different experiments. mP, millipolarization.

molar of the labeled ligand was titrated with increasing concentrations of the ERs to produce the binding isotherms. K_d values for both receptors were calculated from the saturation curves; for ER α the K_d was 25 nM and for ER β it was 10 nM. The affinity of the labeled ES2 ligand was approximately 2-fold higher for ER β than for ER α .

We then performed competition binding of DES, 4OHT, AdP, AdDP and AdMP with the ER–ES2 complex. The binding affinities (IC_{50} values) of the tested compounds were determined from the competition curves (Fig. 5, Table 2).

Figure 3 Illustration of the possible polar interactions involved between ER and AdDP (hydrogen bonding interactions are shown as broken lines).

Figure 5 Competition binding curves of E_2 and various synthetic compounds for $ER\alpha$ -ES2 and $ER\beta$ -ES2 complexes. The initial ER -ES2 complexes have high polarization values. When the complex is titrated with competitors, ES2 molecules are displaced from the ER and a gradual decrease in the polarization values is observed. The data points represent the mean percent inhibition values $\pm$ S.E.M. from two different experiments.

The planar aromatic compound DES is a potent synthetic estrogen agonist and binds with high affinity to both ERs. The binding affinity of DES for $ER\alpha$ and $ER\beta$ we measured is about 1.3-fold higher than the affinity of E_2 (Table 2), which is in agreement with the previously reported affinity of DES for ER (Bolger *et al.* 1998).

We found that 4OHT has a 7-fold lower affinity for $ER\alpha$ than does E_2 and a 10-fold lower affinity for $ER\beta$. The binding affinity of 4OHT for $ER\alpha$ is approximately 1.5-fold higher than for $ER\beta$ (Table 2).

AdDP binds with 15-fold and 7-fold lower affinity to $ER\alpha$ and $ER\beta$ than does E_2 respectively. AdP binds with lower affinity to both ERs than does AdDP. The affinity of AdP for $ER\alpha$ is 77-fold lower than that of E_2 , and the affinity of AdP for $ER\beta$ is 17-fold lower. Both AdP and AdDP have higher affinities for $ER\beta$ than for $ER\alpha$ (Table 2). AdMP does not displace the labeled ES2 ligand from

either $ER\alpha$ or $ER\beta$ in the concentration range tested (Fig. 5).

ER-xenoestrogen complex binding to human pS2 and Xenopus vit A2 EREs

The $ER\beta$ -DES complex binds to *Xenopus vit A2* ERE with affinity similar to that of the $ER\beta$ - E_2 complex but the $ER\beta$ -DES complex binds to human pS2 ERE with greater affinity than the $ER\beta$ - E_2 complex. The $ER\alpha$ -DES complex binds to both EREs with about 1.3-fold less affinity than does the $ER\alpha$ - E_2 complex (Fig. 6, Table 3).

Among the compounds tested here, only the 4OHT- $ER\alpha$ complex manifested higher affinity for the EREs than did the $ER\beta$ -xenoestrogen complexes. The $ER\alpha$ -4OHT complex has about 1.6-fold higher affinity for *Xenopus vit A2* and human pS2 EREs than the $ER\alpha$ - E_2 complex. There is also a significant difference in the binding affinities of $ER\alpha$ -4OHT and $ER\beta$ -4OHT for each of the EREs (Fig. 6, Table 3).

In general, AdP and AdDP produce higher binding affinities of the $ER\beta$ -ligand complex for the EREs. The complexes of $ER\beta$ with AdDP and AdP have approximately 1.2-fold lower affinity for *Xenopus vit A2* ERE, and 1.5-fold less affinity for human pS2 ERE as compared with the affinity of the $ER\beta$ - E_2 complex for these ERE. The affinity of $ER\alpha$ -AdDP and $ER\alpha$ -AdP complexes for human pS2 ERE are about 3-fold lower than the affinity of the $ER\alpha$ - E_2 complexes. The affinities of $ER\alpha$ -AdDP and $ER\alpha$ -AdP complexes for *Xenopus vit A2* ERE are 1.5-fold and 2.6-fold less than the affinity of $ER\alpha$ - E_2 complex respectively. AdP triggers differential binding of $ER\alpha$ and $ER\beta$ to both EREs, and AdDP differentially affects the binding of $ER\alpha$ and $ER\beta$ to human pS2 ERE

Table 2 IC_{50} constants and RBAs of tested synthetic estrogens for human $ER\alpha$ and $ER\beta$ from competition experiments. RBA of each competitor was calculated as a ratio of the IC_{50} values of each competitor and E_2 . RBA value of E_2 was arbitrarily set at 100. The data represents the mean IC_{50} values $\pm$ S.E.M. from two different experiments

Compound	$ER\alpha$		$ER\beta$	
	IC_{50}	RBA	IC_{50}	RBA
E_2	13 ± 0.7 nM	100	12 ± 0.5 nM	100
DES	10 ± 0.5 nM	130	9 ± 0.7 nM	133
4OHT	96 ± 0.8 nM	14	115 ± 0.7 nM	10
AdDP	200 ± 1 nM	6.5	80 ± 0.5 nM	15
AdP	1 ± 1 μ M	1.3	200 ± 1 nM	6.0
AdMP	—	—	—	—

Figure 6 Binding of human recombinant ER α and ER β (saturated with E₂ and various xenoestrogens) to (A) fluorescein-labeled *Xenopus vit A2* ERE, and (B) fluorescein-labeled human pS2 ERE. The data points represent the mean percent saturation values $\pm$ S.E.M. from two different experiments.

(Fig. 6, Table 3). AdMP does not affect the binding of the ligand free ERs to *Xenopus vit A2* and human pS2 EREs (data not shown). For both of the EREs the affinity of ER α –xenoestrogen complex decreases in the order: 4OHT>DES>AdDP>AdP; and for ER β –xenoestrogen complex: DES>AdDP>AdP>4OHT.

There are also differences in the polarization values of ER–ERE complexes at saturation depending on the ligand present (Fig. 6). In the case of *Xenopus vit A2* ERE (Fig. 6A), binding of DES to the ER α –ERE complex gives the highest polarization value followed by E₂. When ER β is complexed with *Xenopus vit A2* ERE, binding of either DES or E₂ gives the same (highest) polarization value

Table 3 K_d constants (nM) and RBAs of ER α and ER β (saturated with xenoestrogens) for *Xenopus vit A2* and human pS2 EREs. RBA of each ER saturated with competitor was calculated as a ratio of the K_d values of each competitor and E₂. RBA values of the ERs saturated with E₂ were arbitrarily set at 100

Compound	<i>Xenopus vit A2</i> ERE				Human pS2 ERE			
	ER α		ER β		ER α		ER β	
	K_d	RBA	K_d	RBA	K_d	RBA	K_d	RBA
E ₂	37	100	60	100	39	100	61	100
DES	46	81	64	94	46	83	54	113
4OHT	25	150	84	71	22	175	99	61
AdDP	57	66	69	87	128	30	94	65
AdP	97	38	76	79	139	27	98	62

while the rest of the compounds tested show lower polarization values at saturation. When ER α and ER β are complexed with human pS2 ERE (Fig. 6B), the polarization values at saturation of the ER–ERE complexes in the presence of all compounds tested are lower than those of ERE–ER–E₂ except ER α –AdDP, which is greater than ER α –E₂.

Discussion

Transcriptional activation mediated by ERs involves binding of a ligand to the receptor and association of the ER–ligand complex to an ERE located within the regulatory region of target genes. The conformation of the ER–ligand complex is crucial for the activation of the transcriptional machinery and the chemical structure of the ligand plays an important role in triggering agonistic or antagonistic effects.

The crystal structure of the ER α LBD complexed with E₂ provides important information about the orientation and the structure of putative ER ligands (Brzozowski *et al.* 1997, Tanenbaum *et al.* 1998). The E₂ molecule is oriented by hydrogen bonding and hydrophobic van der Waals' contacts and it fits well in the LBD. The phenolic hydroxyl group of the A-ring of E₂ lies between α -helices 3 and 6, and makes several direct hydrogen bonds. The polar residue Glu353 (helix H3) has been identified as a putative hydrogen bond partner for the steroidal hydroxyl group at C-3 position of E₂. At the other end of the molecule the 17 β -hydroxyl group of the D-ring makes a single hydrogen bond with the δ -nitrogen of His524 in helix H11. Additional stability of this hydrogen-bonding network is provided via salt bridge interactions between Glu353 and Arg394 (helix H5), and Glu419 and Lys531/Asn532 (Tanenbaum *et al.* 1998, Wurtz *et al.* 1998). Arg394 is also involved in the orientation of the hormone in the LBD; the side chain of this residue is braced by a hydrogen bond to the carbonyl group of the residue preceding Phe404, which is fixed by its van der Waals'

contact to ring A of the E₂. Thus, the recognition of the 3-hydroxy function of the hormone is coordinated with a van der Waals' contact to its hydrophobic rings (Tanenbaum *et al.* 1998). The length and the width of the E₂ skeleton are matched by the receptor's LBD, but there are large unoccupied spaces opposite the B- and C-rings of E₂.

The E₂ agonist DES fits in the LBD of ER α in a manner that resembles that of E₂; one of the phenolic rings of DES lies in the same position as the E₂ A-ring near helices 3 and 6, and the other phenolic ring of DES rests in a position close to the location of the E₂ D-ring (Shiau *et al.* 1998). Our competition binding experiments show that DES binds to both ER α and ER β with affinity even greater than that of E₂ (Fig. 5, Table 2). This can be attributed to the structure of DES; the ethyl groups of DES project perpendicularly from the plane of the phenolic rings and fit into the spaces adjacent to the B- and C-rings of E₂ in the LBD (Shiau *et al.* 1998). The affinity of DES for the ERs determined by us is similar to that observed by Bolger *et al.* (1998) using the same FP method, but weaker than the affinity of this ligand measured by Kuiper *et al.* (1997) using a traditional radiolabeled E₂ binding assay.

Using direct binding experiments we studied the effect of DES on the binding of human ERs to *Xenopus vit A2* and human pS2 EREs. The data indicate that the conformational changes in the ERs caused by DES do not differ much from those evoked by E₂ and the ER–DES complexes bind to both EREs with affinity similar to that of ER–E₂ complexes (Fig. 6, Table 3). These results are in agreement with the elucidated crystal structure of the ER–DES complex.

Studies of the crystal structure of the LBD of ER α in a complex with 4OHT show that this compound is bound within the same pocket that recognizes E₂ and DES. The bulky side chain of 4OHT changes the orientation of some of the helices in the LBD and as a result 4OHT binding promotes conformation of the LBD that is distinct from that stabilized by either DES or E₂ binding (Shiau *et al.* 1998).

The competition binding experiments show that the antiestrogen 4OHT binds with less affinity to the ERs than does DES. The RBAs of 4OHT are much lower compared with the RBAs of E_2 for ER α and ER β , which differ from the findings of Kuiper *et al.* (1997).

The direct binding FP assay reveals that the conformational changes in the ERs caused by 4OHT result in different binding affinities of the ER α –4OHT and ER β –4OHT complexes for the EREs, relative to the affinities of ER– E_2 complexes. ER α –4OHT complex has the highest affinity for both EREs among the compounds tested here, while ER β –4OHT has the lowest affinity (Fig. 6, Table 3).

In this study we also investigated the ability of AdP, AdDP and AdMP to associate with human ERs and their effect on the interactions of ER α and ER β with two different EREs.

The three-dimensional structure of AdDP is similar to that of DES and could exploit hydrogen bonding as well as van der Waals' interactions with the LBD of the ER (Figs 2 and 3). As suggested by our modeling studies AdDP was found to be a more potent ligand for both ERs than AdP (Fig. 5, Table 2). The ER α –AdDP and ER β –AdDP complexes bind with higher affinities to both *Xenopus vit* A2 and human pS2 ERE than do the ER–ADP complexes (Fig. 6, Table 3). These results can be explained by the fact that the two phenolic hydroxyl groups of AdDP are situated to function as both the phenolic hydroxyl group and the 17 β -hydroxyl group of E_2 ligand. The importance of the phenolic hydroxyl groups and their orientation is also illustrated by the observation that AdMP, which lacks *para* phenolic function, neither binds to the ERs nor affects the binding of ER α and ER β to the EREs (Table 2).

The direct binding experiments also show that when ER β is bound to either AdDP or AdP the resulting complexes have greater affinity for both EREs than does the ER β –4OHT complex. These results suggest that, compared with 4OHT, the conformational changes in ER β caused by binding of the three-dimensional AdDP or AdP molecules favor the formation of the ER β –ERE complexes. These data may be helpful in regard to previous findings that binding affinity is not always directly related to transcriptional activation with certain ER ligands (Sun *et al.* 1999).

The bulky adamantyl moiety of AdDP and AdP may fill spaces in the LBD that are unoccupied when planar molecules like E_2 or DES are bound. These relatively novel structural features of AdDP and AdP may account for their high affinity for ER α and ER β as compared with other known synthetic estrogens (Bolger *et al.* 1998, Kuiper *et al.* 1998). The additional van der Waals' contacts in the hydrophobic cavity of the LBD when adamantyl phenol ligands are bound may also be responsible for an enhanced binding of the ER β –ligand complexes to the EREs relative to the ER α –ligand complexes.

Figure 7 Speed of rotation (represented as polarization values) of the labeled human pS2 ERE in the presence of human recombinant ER α liganded with E_2 , AdDP and AdP. The speed of rotation of the ERE complexed with ER α – E_2 was arbitrarily set at 100. The data represents the mean FP values $\pm$ S.E.M. from two different experiments. mP, millipolarization.

As we previously noted (Nikov *et al.* 2000), ER ligands differentially affect the effective molecular volume of ER–ERE complexes, which results in altered speed of rotation of these complexes. The measured polarization values indicate that the *Xenopus vit* A2 ER α –DES complex has a higher effective molecular volume (higher polarization value, decreased speed of rotation of the fluorescein label) than the *Xenopus vit* A2 ER α – E_2 complex (lower polarization value, increased speed of rotation of the fluorescein label). In contrast, when complexed with *Xenopus vit* A2, the ER β –DES and ER β – E_2 complexes have comparable molecular volumes (Fig. 6A). We also observed differential effects of the binding of the ER α –AdP and ER α –AdDP complexes to human pS2 ERE. The presence of a second phenolic group in the molecule of AdDP affects the molecular volume of human pS2 ERE–ER α complex. The effective molecular volume of human pS2 ERE complexed with ER α –AdDP (higher polarization value, decreased speed of rotation) is about 1.8-fold higher than the effective molecular volume of human pS2 ERE–ER α –AdP complex (lower polarization value, increased speed of rotation) (Fig. 7). The data demonstrate that AdP and AdDP affect differentially the geometry (bending of the DNA or loosening the end of the DNA that is labeled) of the ER α –human pS2 ERE complex.

Routledge *et al.* (2000) observed that xenoestrogens might induce conformational changes in the tertiary structure of the ERs that differ from that of E_2 and thus affect the ability of ER α and ER β to recruit coactivator proteins. In this context the conformational changes in the ERs caused by AdDP and AdP may affect the transcriptional activation of ER regulated genes.

We conclude that adamantyl phenols with a distinct three-dimensional structure may function as effective

ligands for human ERs. AdDP and AdP represent a novel class of synthetic molecules, not based upon the steroidal structure or the planar DES and tamoxifen structures, which may affect the ER signaling pathways. AdDP and AdP should possess biological activity based on their ability to bind the ERs and might be potentially useful for the design of new selective ER modulators.

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