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Structural plasticity in the oestrogen receptor ligand-binding domain

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The steroid hormone receptors are characterized by binding to relatively rigid, inflexible endogenous steroid ligands. Other members of the nuclear receptor superfamily bind to conformationally flexible lipids and show a corresponding degree of elasticity in the ligand-binding pocket. Here, we report the X-ray crystal structure of the oestrogen receptor α (ERα) bound to an oestradiol derivative with a prosthetic group, ortho- trifluoromethlyphenylvinyl, which binds in a novel extended pocket in the ligand-binding domain. Unlike ER antagonists with bulky side groups, this derivative is enclosed in the ligand-binding pocket, and acts as a potent agonist. This work shows that steroid hormone receptors can interact with a wider array of pharmacophores than previously thought through structural plasticity in the ligand-binding pocket.

Keywords: oestrogen receptor; structure–activity relationships; nuclear receptors; X-ray crystallography

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INTRODUCTION

The physiological effects of oestradiol occur through two receptors—oestrogen receptor (ER)α and ERβ—with distinct tissue distributions and biological roles (Koehler *et al*, 2005). As a member of the nuclear receptor superfamily of transcription factors, ERα contains conserved domains for binding to DNA and small-molecule ligands. Ligand binding facilitates dimerization and DNA binding, and also conformational control of a cofactor-binding site in the ligand-binding domain (LBD), known as AF2. The fundamental mechanism of transcriptional control by nuclear receptors is through ligand-mediated recruitment of transcriptional co-regulator proteins (Rosenfeld *et al*, 2006), which in turn control chromatin structure by post-translational modifications.

The LBD contains a molecular switch helix—helix 12—which regulates the communication between ligand- and coactivator-binding sites (Nettles & Greene, 2005; Shiau *et al*, 1998; Xu *et al*, 2002). In ERα, as in other steroid hormone receptors, the ligand-binding pocket is a compact ellipsoid cavity, closely resembling the surface of the steroid ligands. In agonist ligands, helix 12 is stabilized in a conformation that allows it to form one side of the coactivator-binding site and to encapsulate completely the ligand in the pocket. Antagonist ligands, such as the selective ER modulator (SERM) tamoxifen, resemble agonist ligands, but contain an additional extended group. The bulky side chain on tamoxifen protrudes between helices 3 and 11, and physically obstructs helix 12 from adopting the agonist conformation (Shiau *et al*, 1998), thus preventing coactivator recruitment to the LBD. So far, X-ray crystal structures of ER ligands with bulky side chains have all been shown to protrude from the ligand-binding cavity and act as antagonists by displacement of helix 12 (Brzozowski *et al*, 1997; Shiau *et al*, 1998; Pike *et al*, 2001; Blizzard *et al*, 2005).

In contrast to the steroid receptors, other members of the nuclear receptor superfamily bind to a wide range of natural lipids and synthetic compounds that interact with only a portion of the ligand-binding pocket. For example, peroxisome proliferator activator receptor (PPAR) has a Y-shaped binding pocket, with ligands showing broad diversity in the portion occupied and some ligands showing alternative conformations in different parts of the pocket (Xu *et al*, 1999). The pregnane X receptor (PXR) acts as a xenobiotic receptor. PXR binds to a promiscuous diversity of ligands through flexibility in the ligand-binding pocket (Watkins *et al*, 2001; Chrencik *et al*, 2005).

In this study, we characterized an oestradiol derivative with a bulky side group, and found that it acts as a highly potent and

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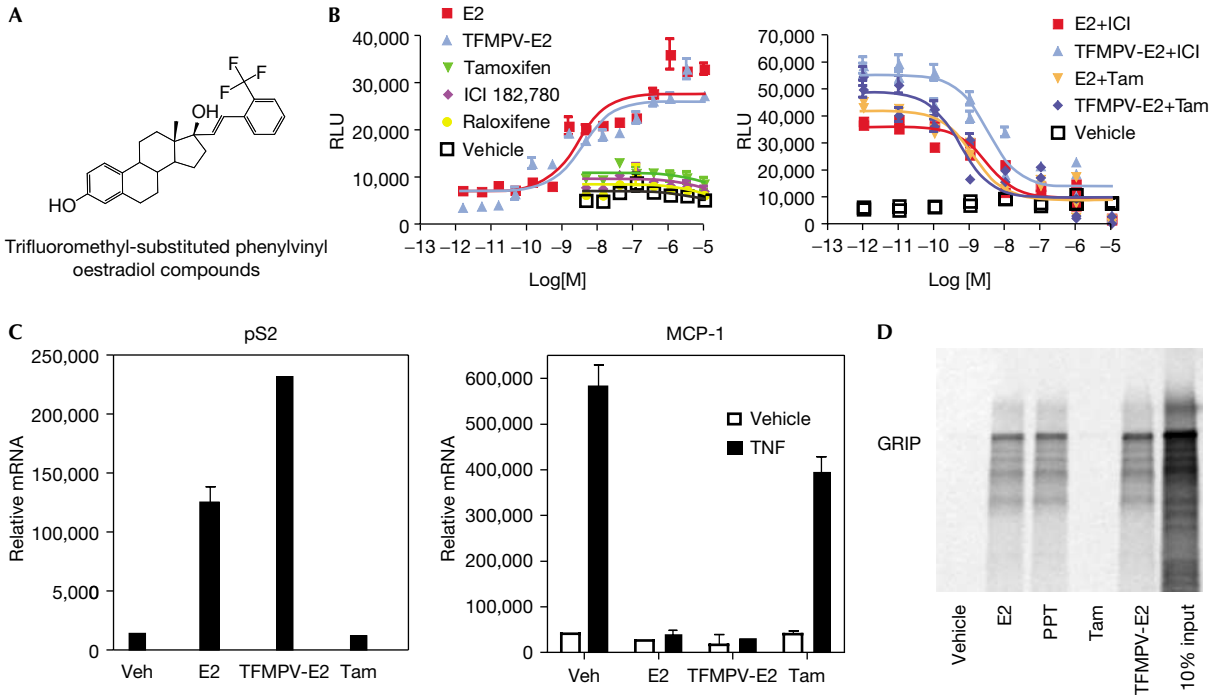

Fig 1 | Characterization of TFMPV-E2 as an agonist. (**A**) Chemical structure of TFMPV-E2. (**B**) MCF-7 cells were transfected with a 3 × ERE-luciferase reporter and expression vector for β-gal. Compounds were added the next day at the doses indicated and incubated overnight before processing for luciferase activity. Each data point represents mean + s.d., and each experiment was repeated 3–5 times. For the inhibition assays, cells were treated with 10 nm E2 or TFMPV-E2 and the indicated doses of antagonists. (**C**) MCF-7 cells were treated with the indicated ligands at 100 nM for 2 h and then processed for quantitative PCR analysis of gene expression, as described in Methods. TNFα was administered at 10 ng/ml. Results are from duplicate experiments + s.d. (**D**) Ligand-induced interaction of GRIP1 with ERα LBD. GST–ERα LBD was incubated with [^{35}S]GRIP in the presence of 1 μM E2, PPT, OHT, TFMPV-E2 or vehicle (0.1% EtOH). Bound proteins were eluted with sample buffer, separated by using SDS–polyacrylamide gel electrophoresis, and [^{35}S]GRIP was detected by autoradiography. ERα, oestrogen receptor α; GRIP, glutamate receptor interacting protein; GST, glutathione-*S*-transferase; LBD, ligand-binding domain; PPT, propyl-pyrazole-triol; TFMPV-E2, trifluoromethyl-substituted phenylvinyl oestradiol compounds; TNF, tumour necrosis factor.

efficacious agonist ligand. This prompted us to use X-ray crystallography to characterize how this unusual agonist pharmacophore is accommodated in the ERα ligand-binding pocket. Although the oestradiol moiety binds in a conserved manner, the phenylvinyl substitution increases the volume of the pocket by 40% through remodelling of helix 7 into an extended loop. These results identify a previously unknown structural plasticity in the ERα LBD.

RESULTS AND DISCUSSION

We prepared a series of trifluoromethyl-substituted phenylvinyl oestradiol compounds (TFMPV-E2), and found that the ortho substitution (Fig 1A) binds to ERα more tightly than oestradiol, with an affinity (K_d) of approximately 50 pM (Hanson *et al*, 2003a,b). This compound showed significant uterotrophic activity (Hanson *et al*, 2003a), which is found with both agonist ligands and antagonists of the SERM class, including tamoxifen. The SERMs are uniformly antagonistic in breast cancer patients and cell lines, but differ in oestrogen-like agonist activity in other tissues, including bone and uterus. These differences in agonist activity in the uterus are associated with differential recruitment of transcriptional coactivators and corepressors (Shang & Brown, 2002). The agonist activity of SERMs requires the recruitment of coactivators to the amino-terminal activation function (AF)1 region of ER (McInerney & Katzenellenbogen, 1996; Webb *et al*, 1998). Although the SERMs uniformly block coactivator recruitment to the LBD, differential recruitment of corepressors to the AF2 surface might in turn block the interaction of AF1 with coactivators (Webb *et al*, 2003; Nettles & Greene, 2005).

Compounds with bulky substitutions on an agonist core commonly behave as antagonists (supplementary Fig 1 online). For example, the full antagonist, ICI 182,780 (Faslodex), contains a long side chain that extends from the 7α position of the oestradiol core. The crystal structure of ER bound to ICI 182,780 shows that the side chain exits the ligand-binding pocket and displaces helix 12 (Pike *et al*, 2001). The SERMs tamoxifen and raloxifene also show structural similarity to full agonist compounds, with the addition of extended side chains. In the crystal structures of both tamoxifen- and raloxifene-bound ER, the pendant group again exits the ligand-binding pocket and forces the relocation of helix 12 from the agonist position to the coactivator-binding site formed by helices 3–5 (Brzozowski *et al*, 1997; Shiau *et al*, 1998). An analysis of substituted oestradiol compounds showed that the addition of four or fewer non-hydrogen atoms could be tolerated to produce some agonist

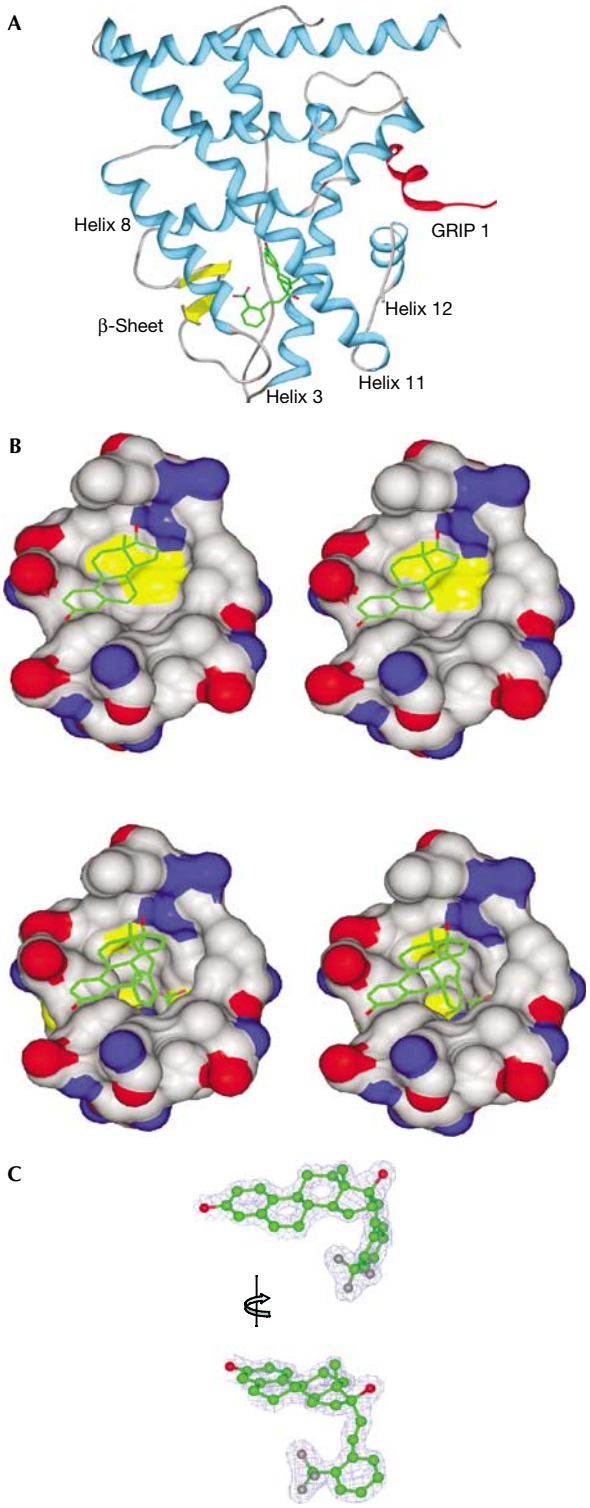

Fig 2 | Structure of TFMPV-E2/ERα ligand-binding domain. (**A**) The structure of one monomer of ERα is shown as a ribbon diagram, with the bound GRIP1 coactivator peptide coloured red. The compound is shown in a stick representation, with carbon atoms coloured green, oxygen atoms coloured red and fluorine atoms coloured pink. (**B**) A stereo view of part of the ligand-binding pocket bound to oestradiol (top panels; Protein Data Bank code 1ERE), or TFMPV-E2 (bottom panels). The ligands are coloured green and are shown in a stick representation. (**C**) TFMPV-E2 is shown in a $2F_o–F_c$ electron density map, contoured to 1.5σ. ERα, oestrogen receptor α; GRIP, glutamate receptor interacting protein; TFMPV-E2, trifluoromethyl-substituted phenylvinyl oestradiol compounds.

activity, whereas larger substitutions resulted in antagonists in cell-based assays (Zhang *et al*, 2005). These observations lead to the question of whether TFMPV-E2 is a SERM, with cell-type-selective agonist activity, or represents a novel class of agonist ligands for the ER.

To characterize TFMPV-E2 as an agonist or a SERM, we carried out cell-based luciferase assays. We initially compared the compound with oestradiol in COS-7 cells co-transfected with an oestrogen-responsive luciferase reporter and an ERα expression vector. TFMPV-E2 proved to be a potent and efficacious agonist over a wide range of concentrations (supplementary Fig 2A online). In these cells, several SERMs showed no agonist activity. Similar results were seen in 293 T cells (supplementary Fig 2B online), and in the ER + MCF-7 breast cancer cell line (Fig 1B), in which SERMs act as antagonists (Shang & Brown, 2002), consistent with their biological activity in inhibiting breast tumour growth. The agonist activity of TFMPV-E2 was antagonized by co-treatment with increasing amounts of tamoxifen or the full antagonist ICI 184,162, in both the MCF-7 (Fig 2B) and 293 T cell lines (supplementary Fig 2C online).

The pharmacological profile of TFMPV-E2 was also compared with E2 and tamoxifen in controlling native gene expression in the MCF-7 cell line, using quantitative PCR. The *pS2* gene is well characterized in terms of its regulation by E2 through an oestrogen response element DNA sequence. Both TFMPV-E2 and E2 induced robust expression of the *pS2* gene (Fig 1C), whereas tamoxifen did not. We also examined the monocyte chemo-attractant protein 1 gene (*MCP1*), which is regulated by nuclear factor κB (NF-κB) on treatment of MCF-7 cells with tumour necrosis factor (TNFα). Co-treatment of cells with either E2 or TFMPV-E2 led to a suppression of TNFα-induced mRNA expression, whereas tamoxifen was ineffective in suppressing the *MCP1* gene (Fig 1C). Thus, TFMPV-E2 acts as a full agonist in breast cancer cells as well as other cell lines in which SERMs behave as antagonists, suggesting that the compound allows full recruitment of coactivators to the LBD. We also examined a subclone of the MCF-7 cell line that was incubated in steroid-free media to develop an oestrogen-independent growth phenotype. These cells showed an identical pattern of gene expression responses to TFMPV-E2 (supplementary Fig 2D online).

To measure directly coactivator recruitment to the AF2 surface, we assayed the recruitment of *in vitro*-translated glutamate receptor interacting protein 1 (GRIP1) coactivator to a fusion protein of the GST–ERα LBD. The TFMPV-E2 compound induced robust binding of GRIP1 to the ERα LBD, which is comparable with the effects of E2 or the ERα-selective agonist propyl-pyrazole-triol (PPT; Fig 1D). By contrast, tamoxifen did not promote binding of GRIP1 to ERα. Thus, the combination of cell-based and *in vitro* assays indicates that TFMPV-E2 shows the pharmacological profile of an agonist rather than a SERM. These observations indicate that the bulky TFMPV-E2 compound is accommodated within the

Table 1 | Summary of crystallographic statistics

Data collection	
Beam line	APS-19ID
Space group	*P*1211
Unit cell (Å)	56.04 × 84.22 × 58.69
Resolution (Å)	28.8–1.94
Unique reflections	36,913
Average redundancy	4.7/3.7
Completeness (%)	97/74.4
R_{merge}	0.08/0.33
I/σ	17.3/2.6
Refinement	
Resolution (Å)	28.8–1.94
R_{free}/R	21.0/15.7
Total number of atoms	4,617
Water	461
Average B factor	20.40
Protein	18.47
Ligand	18.14
Water	37.78
R.m.s. deviations	
Bonds	0.010
Angles	1.21
Ramachandran analysis	
Disallowed	0

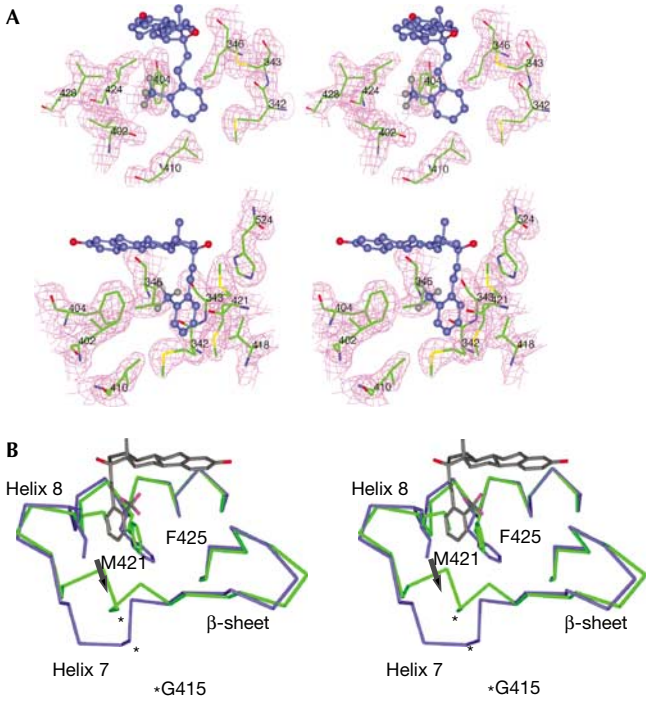

Fig 3 | Details of TFMPV-E2 binding. (**A**) Stereo views of the ERα amino acids that contact the trifluorophenylvinyl group. The ligand is coloured blue, with oxygen atoms in red and fluorine atoms coloured grey. The electron density is from a $2F_o–F_c$ map, contoured to 1.0σ. (**B**) The interaction of TFMPV-E2 (coloured grey) is shown with a c-α trace of helix 7, helix 8 and the β-sheet, coloured blue. The green trace represents the E2–ERα structure, PDB code 1ERE. The red trace is from the tamoxifen–ERα structure, PDB code 3ERT. The asterisks show the disruption of Gly 415 by Met 421, and the associated relocation of helix 7 into an extended conformation (black arrow). ERα, oestrogen receptor α; PDB, Protein Data Bank; TFMPV-E2, trifluoromethyl-substituted phenylvinyl oestradiol compounds.

ligand-binding pocket without disrupting the AF2 coactivator-binding site.

These results prompted us to undertake crystallization trials of the compound bound to ERα. The structure of TFMPV-E2 bound to the ERα LBD was solved using molecular replacement, with data to 1.94 Å resolution (Table 1). The overall fold resembles other agonist structures that have helix 12 folded in the agonist position and the nuclear receptor box II peptide from the GRIP1 coactivator bound in the coactivator-binding site (Fig 2A). The 17α-trifluoromethylphenyvinyl moiety occupies a novel extended binding pocket located between helix 8 and the β-sheet, as discussed further below. Fig 2B shows a surface rendering of the portion of the pocket that is altered in comparison with the oestradiol-bound receptor (top panel), illustrating the extensive remodelling induced by TFMPV-E2 (bottom panel). The overall secondary structure is highly similar to the structure of oestradiol bound to ERα, with 12 α-helices and a β-sheet. Each monomer of the TFMPV-E2 structure was superimposed on the A chain ERα structure bound to two full agonists, oestradiol and diethylstilbesterol. The r.m.s. deviations of each monomer to the diethylstilbesterol structure (0.58 Å and 0.64 Å) and to an E2–ERα complex (0.83 Å and 0.85 Å) are similar, comparable with the r.m.s. deviation for the two monomers of the TFMPV-E2 structure (0.45 Å). Similar to other agonist structures, the TFMPV-E2 is encapsulated within the ligand-binding pocket. Thus, the TFMPV-E2–ERα structure resembles the other full agonist structures, despite the atypical bulky prosthetic group.

The remarkably high affinity of the ligand for ERα, in the picomolar range, is associated with a marked increase in the number of atomic contacts between ligand and receptor, compared with the oestradiol–ERα complex. The ligand could be clearly placed in the electron density (Fig 2C), and the side chains in contact with the pendant group were similarly well ordered (Fig 3A); this allowed an accurate assessment of ligand contacts. There were no apparent clashes, and the core oestradiol scaffold bound in an identical manner as seen in the published oestradiol–ERα structures. The atomic contacts were calculated by using the 'WhatIF' crystallographic web-server, comparing oestradiol–ERα (Protein Data Bank code 1GWR) with the phenylvinyl oestradiol structure. As shown in Table 2, the trifluoromethylphenylvinyl oestradiol makes approximately twice as many contacts as oestradiol with the receptor. An illustration of all ligand contacts for the oestradiol- and TFMPV-E2-bound ER

Table 2 | Number of atomic contacts between the ligand and oestrogen receptor α

	E2	TFMPV-E2	% Change
0.25 Å*	13	34	161
1.0 Å*	97	188	93

*Atoms that were within this distance of the combined van der Waals radii were counted.

is provided in supplementary Fig 3A online. Several amino acids that contact the oestradiol core make further contacts with the extended side group, including Met 342, Phe 404, Ile 424 and Leu 436, as shown in Fig 3A. There are other novel contacts with Met 343, Leu 402, Val 418, Met 421, Phe 425, Leu 428 and His 524. Thus, the high affinity of the compound is associated with a marked increase in the number of hydrophobic contacts compared with oestradiol.

The accommodation of the bulky trifluoromethylphenylvinyl group is through the unique remodelling of helix 7 into an extended conformation. An overall comparison of the receptor bound to oestradiol, TFMPV-E2 or tamoxifen is provided in supplementary Fig 3B online, showing the high degree of similarity among the superimposed molecules. The remodelling of helix 7 in the TFMPV-E2-bound structure is shown in Fig 3B. Helix 7 is a two-turn helix located between the β-sheet and helix 8, and is conserved in the steroid receptor LBD (Williams & Sigler, 1998; Poujol *et al*, 2000; Bledsoe *et al*, 2002). In the oestradiol–ERα, and all other published agonist structures, this novel extended pocket is filled by Met 421 and Phe 425 in helix 8. The trifluoromethylphenylvinyl group repositions both of these side chains to make the novel extended binding pocket. Notably, Met 421 is relocated so as to produce a clash with Gly 415 in helix 7. Among the steroid receptors, ER is unique in that it contains a glycine residue in the middle of helix 7, whereas the others, including the receptors for androgen, progesterone and glucocorticoids, have a methionine at this position. Progesterone has also been shown to tolerate substitutions at the 17α position of the steroid. The smaller substitutions that are tolerated for progesterone, such as ethynyl or furoate (Madauss *et al*, 2004), might reflect differences in the flexibility of helix 7 between ER and progesterone. Glycine is known as a 'helix breaker,' and probably provides ER with a unique ability to remodel this helix into a loop structure.

It should also be noted that the conformation of TFMPV-E2 in the crystal structure differs significantly from both the liquid and gas-phase lowest energy conformations (Sebag *et al*, 2000). Therefore, generation of the observed ligand–receptor complex involves adaptive responses by both partners in this process. This observation has significant implications for drug design, in which one tends to sample ligand conformations against relatively rigid proteins, or selected ligand conformations against flexible receptors.

The combination of cell-based assays and structural biology shows that TFMPV-E2 is a potent full agonist that interacts with the ERα LBD in an unusual manner. Unlike the SERMs, the bulky 17α-extended group is tolerated in the ligand-binding pocket, stabilizing helix 12 in the agonist conformation. The picomolar affinity derives from a tremendous increase in the number of contacts with the ligand-binding pocket, compared with oestradiol-bound ERα. In addition, the mechanism of accommodation involves a unique remodelling of helix 7 into an extended formation, showing a degree of plasticity in the secondary structure that is atypical in the steroid hormone receptors.

METHODS

Protein purification, crystallization and structure determination. The ERα LBD Y537S (aa 298–554) was mutated with the Stratagene (La Jolla, CA, USA) Quickchange Mutagenesis kit and cloned into a modified pET vector (Novagen, Madison, WI, USA) with a ligation-independent cloning site, 6× His tag and TEV protease site. This surface mutation increases the solubility of the compound without affecting ligand binding. The protein was induced in BL21 (DE3) cells, and purified with nickel chromatography, with slight modification from previously published procedures, as detailed in the supplementary information online. Data were collected at the SBC (Structural Biology Center) beamline at advanced photon source (APS) and scaled with HKL2000. The structure was solved with molecular replacement using Molrep/CCP4, and the structure of DES/ERα as a search model (PDB code 3ERD). Refinement and rebuilding were carried out with CCP4 and Coot. The addition of riding hydrogens during refinement reduced R/R_{free} by approximately 1%. The beneficial effects of TLS (translation, libration, screw) refinement on R/R_{free} plateaued with ten groups per ERα molecule, yielding an improvement of approximately 2%. The coordinates and structure-factor amplitudes have been deposited in the PDB with access code 2P15.

Cell culture and transient transfections. MCF-7 cells were maintained in phenol red-free DMEM with 10% FBS charcoal/dextran treated (Hyclone, Logan, UT, USA). The cells were transfected using Fugene HD (Roche, Indianapolis, IN, USA) with a 3× ERE-TATA-luciferase reporter. After 6 h, the cells were incubated and transferred into 384-well plates using a WellMate Microplate Dispenser (Matrix, Hudson, NH, USA). The next day, ligands were added and allowed to incubate overnight before processing for luciferase activity. An equal volume of Britelite (PerkinElmer, Waltham, MA, USA) was dispensed by a WellMate Microplate Dispenser and the luminescence was measured by ViewLux ultraHTS Microplate Image (PerkinElmer).

RNA isolation and quantitative PCR. Total RNA was isolated from MCF-7 cells using RNeasy (Qiagen, Valencia, CA, USA), which was used to generate complementary DNA. PCR analysis was carried out on an ABI PRISM 7900HT. Values are normalized with GAPDH content. Further details are provided in the supplementary information online.

Glutathione-*S*-transferase pull-down. The GST–ERα LBD was used to pull down *in vitro*-translated GRIP1 as described previously (Shiau *et al*, 1998). Proteins were eluted by boiling the beads for 10 min in sample buffer. Bound ^{35}S-GRIP1 was visualized by autoradiography after SDS–polyacrylamide gel electrophoresis.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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