DOI: 10.1002/cmdc.200600183

Protein-Structure-Based Prediction of Animal Model Suitability for Pharmacodynamic Studies of Subtype-Selective Estrogens

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Subtype-selective estrogens are of increasing importance as tools used to unravel physiological roles of the estrogen receptors, ERα and ERβ, in various species. Although human ERα and ERβ differ by only two amino acids within the binding pockets, we and others recently succeeded in generating subtype-selective agonists. We have proposed that the selectivity of the steroidal compounds 16α-lactone-estradiol (16α-LE$_2$, hERα selective) and 8β-vinyl-estradiol (8β-VE$_2$, hERβ selective) is based on the interaction of certain substituents of these compounds with essentially one amino acid in the respective ER binding pockets. For in vitro and ex vivo pharmacological experiments with these compounds we intended to use bovine tissues available from slaughterhouses in larger quantities. Using homology modeling techniques we determined that the amino acid conferring high hERβ-selectivity to 8β-VE$_2$ is not exchanged between human and bovine ERα and bovine ERβ. Thus, we predicted our steroidal hERβ-selective compound to exhibit only weak agonistic activity at bERβ and that bovine tissue is therefore not suited for investigation of ERβ functions. The situation is presumably identical for pig, sheep, and the common marmoset, whereas rats, mice, and rhesus macaques are appropriate animal models to study pharmacological effects of 8β-VE$_2$ in vivo. This prediction was confirmed in transactivation studies assessing estradiol (E$_2$) and the two subtype-selective ligands on bovine ERβ and on a series of hERα and hERβ with mutations in their respective ligand-binding pockets. We have shown that the detailed understanding of the interactions of a compound with its target protein enables the identification of relevant species for pharmacological studies.

Introduction

In drug discovery, biologically active compounds are routinely identified and characterized using biochemical and cellular assays, which directly interrogate the target protein. Subsequently, the pharmacological effects of potent compounds are monitored in animal models. Ultimately, selected candidates reach clinical trials in humans. In spite of the high sequence identity of the target protein in different mammalian species the compound's pharmacological profile could considerably differ between animals and humans. Therefore, the choice of a suitable species for in vivo experiments is a crucial step in the drug discovery process. It is generally accepted that species chosen for in vivo experiments should be easy to handle (for example, small) and possess the desired target protein with a high degree of overall sequence identity to the human orthologue. In this study we show that the overall or domain sequence identity is actually not the best predictor for the suitability of animal models. Knowledge of the 3D-structure of the target proteins in humans and animals and the interactions of small molecule ligands within the binding pockets allows better prediction of the suitability of animal models for pharmacological investigations.

In our case the target protein is the human estrogen receptor beta (hERβ). Potential distinct biological roles of the two ERs are suggested by the different mRNA and protein expression patterns,[1–3] and by the phenotypes of the knockout mice.[4–7] The ERαKO mice are infertile (male & female), have decreased bone density, and perturbed breast development. In contrast, ERβKO mice develop normally and do not display a severely hampered reproductive function. When studying knockout animals, however, developmental alterations in several organs can confound the interpretation. Further insight into the actual ligand-dependent functions of both ERs has been provided by applying selective ligands to animals and studying their pharmacological effects. Compounds described for these purposes are the ERα selective agonists propyl-pyra-

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zole-triol (PPT)[8] and 16α-LE$_2$,[9] and the ERβ selective agonists diarylpropionitrile (DPN),[10] ERB-041,[11–13] and 8β-VE$_2$[9] (Figure 1). The ERα agonists induce uterine growth and cause bone-protective effects,[14] reduce luteinizing hormone and follicle-stimulating hormone plasma levels, and increase angiotensin I.[9,15]

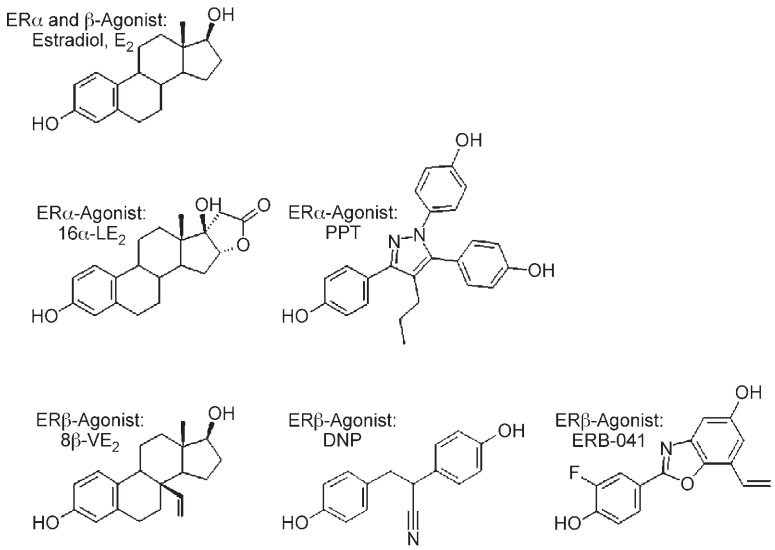

Figure 1. Structures of ERα and ERβ agonistic ligands. The chemical and functional features of the different ligands are presented in the text.

In contrast, ERβ agonists are non-uterotrophic, non-mammotrophic, and do not show bone-sparing effects.[9,11,15,16] However, the compounds display beneficial effects in two rat models for inflammatory diseases, suggesting a potential role of ERβ in immune responses.[11]

In contrast to nonsteroidal subtype-selective ER ligands, 16α-LE$_2$ and 8β-VE$_2$ are close derivatives of the natural steroid hormone estradiol (E$_2$). The selectivity of the compounds was designed by molecular modeling experiments taking into account the plasticity of the hERα and hERβ ligand binding pockets. An analysis of the two receptors' ligand binding pockets revealed two amino acid differences[8] hERα-L384→hERβ-M336 and hERα-M421→hERβ-I373, with hERβ-M336 being positioned above the B- and C-ring (near position 8ß of the steroidal agonist) and I373 below the D-ring (near position 16α and 17α of steroidal agonists) of E$_2$. It was shown that the increased flexibility of the unbranched methionine side chain allows larger substituents to be accommodated. Thus, substitution of E$_2$ with a vinyl group in the 8β position led to a highly selective ERβ agonist (8β-VE$_2$). This compound fits well into the hERβ ligand binding pocket, but colliding with L384 in the case of hERα. Similarly, a substitution with a lactone ring in position 16α/17α led to an ERα agonist (16α-LE$_2$). Again, a larger substituent replaces the flexible methionine side chain (ERα-M421) while interacting unfavorably with the rigidly branched isoleucine (ERβ-I373) below the steroidal D-ring.[9]

For in vitro and ex vivo pharmacological experiments with 16α-LE$_2$, 8β-VE$_2$, and derivatives thereof we intended to use bovine tissues available from slaughterhouses in large quantities.[17] To check the suitability of the bovine receptor for pharmacological investigations with 8β-VE$_2$, we compared the structures of bERβ and hERβ ligand binding domains. We found that the bERβ binding pocket differs from the hERβ in a way that would predict a significant loss of activity of 8β-VE$_2$.

Herein, we present the experimental verification of the predictions made on the basis of the homology models and a subsequent systematic study in which hERα was gradually mutated to embody the hERβ binding pocket and vice versa.

Results

A sequence alignment of ERα and ERβ ligand binding domains is shown in Figure 2. Species included in this analysis are human, mouse, rat, cattle, pig, sheep, chicken, rhesus monkey, common marmoset, golden hamster, and Atlantic croaker. Amino acids that are directly contacting E$_2$ in the hERα/E$_2$ complex[18] are highlighted in black in the alignment. The position corresponding to hERα- L384/hERβ-M336 is marked with a cross and hERα-M421/hERβ-I373 with an asterisk. In Table 1 the sequence identity matrix is given for the full-length ERs (lower left part of the matrix) and the LBD (upper right part of the matrix).

A homology model of bERβ was built based on the X-ray structure of hERα in complex with E$_2$[18] and this sequence alignment. This work was initiated before the structure of the hERβ was published. The homology model was later rebuilt, based on a higher resolution hERα-LBD structure in complex with E$_2$ and a coactivator peptide (PDB-ID: 1GWR[19]) and also the hERβ X-ray structure.[20] bERβ was compared with hERα and hERβ with respect to the binding pocket. Of the 22 amino acids that contact steroid ligands, only one amino acid is different between hERβ and bERβ (hERβ-M336→bERβ-L333) (Figure 2 and 3). hERα and bERβ show only one difference in their binding sites (hERα-M421→bERβ-I370) (Figure 2 and 3), whereas hERβ and hERα differ in two positions (see above). Thus, the bERβ could be described as an intermediate of hERα and hERβ. Interestingly, the amino acid (hERα-L384) that prevents 8β-VE$_2$ from binding to hERα is not exchanged and therefore identical between hERα and bERβ. As in the case of hERα,[9] the 8-vinyl substituent of 8β-VE$_2$ would collide with bERβ-L333, leading to a significant attenuation in binding and transactivation at this receptor. Thus, we predicted that our hERβ-selective compound 8β-VE$_2$ is not active at bERβ and bovine tissue is not suited for pharmacological experiments with certain hERβ-selective compounds. To check the predictions from molecular modeling we tested E$_2$, 16α-LE$_2$, and 8β-VE$_2$ in bERβ driven transactivation assays and compared their activity to human ERβ and ERα, respectively, in the same assays. The results are shown in Figure 4a–c . E$_2$ as an endogenous ligand of bERβ exhibits high potency in the bERβ transactivation assay. 8β-VE$_2$ is at least two orders of magnitude less potent than E$_2$ (EC$_{50}$ = 4.5 × 10^{-9} M, Figure 4a). On hERα, 8β-VE$_2$ shows a similar potency (EC$_{50}$ = 2.3 × 10^{-9} M,

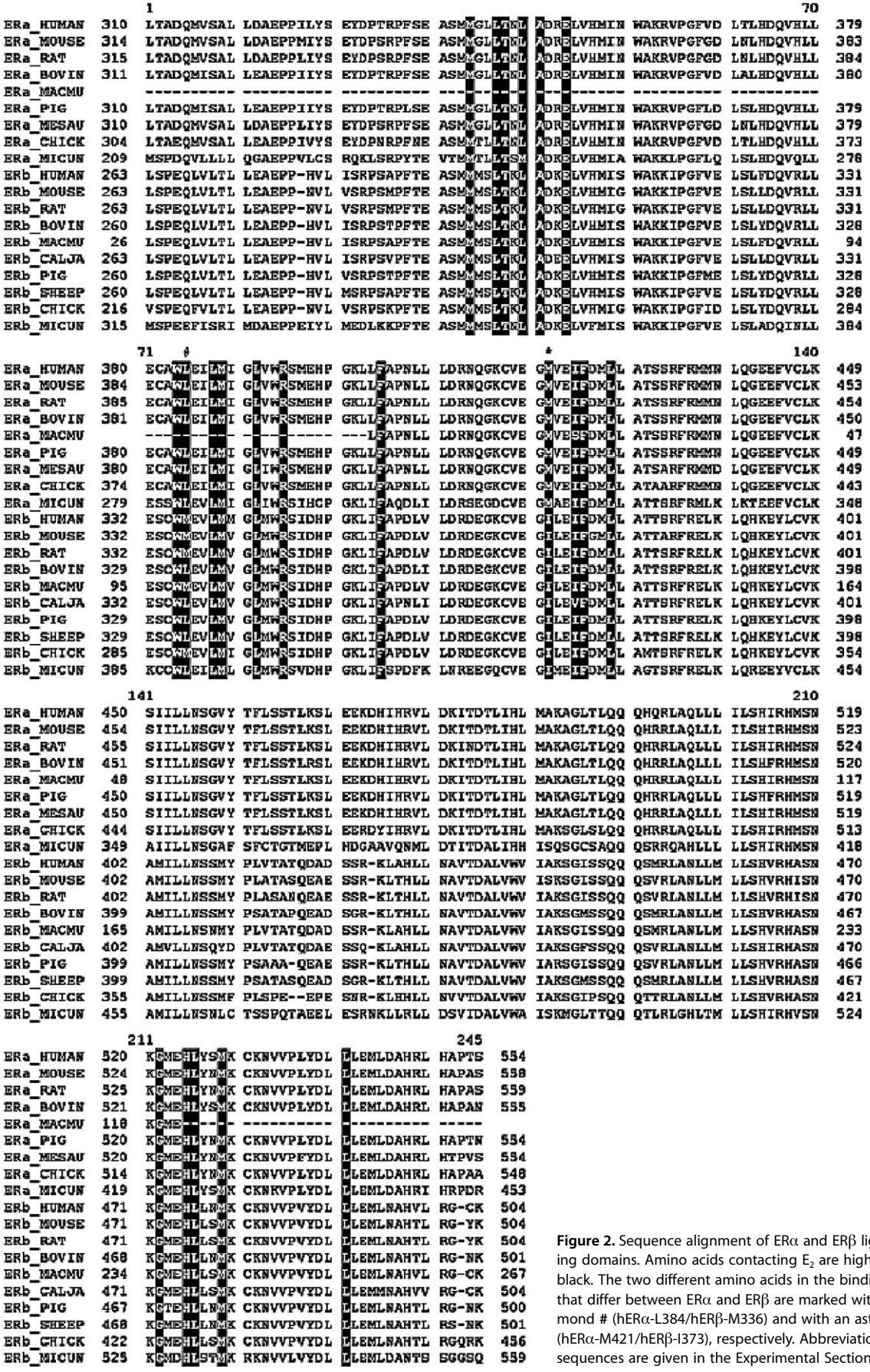

Figure 2. Sequence alignment of ERα and ERβ ligand-binding domains. Amino acids contacting E_2 are highlighted in black. The two different amino acids in the binding pocket that differ between ERα and ERβ are marked with a diamond # (hERα-L384/hERβ-M336) and with an asterisk * (hERα-M421/hERβ-I373), respectively. Abbreviations for the sequences are given in the Experimental Section.

Table 1. Sequence identity matrix of ER proteins.[a]

	ERa_ HUMAN	ERa_ MOUSE	ERa_ RAT	ERa_ BOVIN	ERa_ PIG	ERa_ MESAU	ERa_ CHICK	ERa_ MICUN	ERb_ HUMAN	ERb_ MOUSE	ERb_ RAT	ERb_ BOVIN	ERb_ MACMU	ERb_ CALJA	ERb_ PIG	ERb_ SHEEP	ERb_ CHICK	ERb_ MICUN
ERa_ HUMAN	/	96.7	96.3	96.3	95.9	94.6	93.4	63.2	57.9	56.7	58.3	57.9	58.3	57.5	56.7	57.9	57.5	56.3
ERa_ MOUSE	88.6	/	99.1	95.9	95.9	97.1	93.4	63.2	58.3	56.3	57.9	58.3	57.9	57.1	57.5	58.3	57.5	56.3
ERa_ RAT	88.0	97.6	/	95.5	95.5	97.1	93.0	62.8	57.9	55.9	57.5	57.9	57.5	56.7	57.1	57.9	57.1	56.3
ERa_ BOVIN	91.7	88.5	88.1	/	97.5	93.4	93.0	62.8	57.9	56.7	58.3	57.9	58.3	57.1	56.7	57.9	57.5	56.3
ERa_PIG	91.9	89.1	88.6	94.9	/	93.8	92.6	63.2	57.9	55.9	57.5	57.9	57.5	56.3	57.1	57.9	57.1	55.9
ERa_ MESAU	88.0	92.8	92.6	87.2	88.0	/	91.8	62.8	57.9	56.7	57.5	57.9	57.5	56.7	57.1	57.9	57.1	55.9
ERa_ CHICK	77.7	77.1	76.5	77.2	77.0	78.2	/	63.2	60.0	58.7	59.5	60.0	59.5	58.3	58.7	60.0	58.3	55.9
ERa_ MICUN	40.0	40.0	40.0	40.0	40.5	40.7	40.7	/	60.4	60.4	60.4	61.2	60.8	60.0	60.0	60.8	60.4	55.5
ERb_ HUMAN	40.0	39.5	38.9	39.9	40.1	40.0	40.7	38.6	/	92.1	92.9	94.2	98.7	91.7	92.5	94.2	87.6	64.8
ERb_ MOUSE	39.5	38.8	38.4	39.4	39.3	39.6	40.7	39.0	88.4	/	97.9	92.1	92.5	88.4	92.9	92.5	88.8	64.4
ERb_ RAT	40.1	39.6	39.2	40.2	39.8	40.1	41.0	38.6	88.3	94.7	/	92.9	93.3	89.2	94.2	92.9	90.1	64.8
ERb_ BOVIN	40.1	39.8	39.4	40.2	40.1	40.3	41.1	38.6	87.9	86.6	86.7	/	93.8	89.6	95.4	97.9	87.2	65.7
ERb_ MACMU		–	–	–	–	–	–	–	–	–	–	–	/	92.5	92.1	93.8	87.6	65.3
ERb_ CALJA	39.8	39.1	38.6	39.4	39.1	39.6	39.6	38.0	92.2	85.6	85.0	85.4	48.4	/	88.4	88.4	83.1	63.2
ERb_PIG	39.6	39.5	39.1	39.7	39.8	40.0	40.6	38.3	86.2	86.4	86.9	92.0	45.9	84.1	/	95.0	88.4	64.8
ERb_ SHEEP	40.3	39.8	39.4	40.4	40.3	40.5	41.2	38.8	87.5	86.6	86.6	97.1	46.6	84.5	91.4	/	87.2	65.7
ERb_ CHICK	39.5	38.9	38.7	39.1	39.2	39.4	39.4	41.0	69.7	70.4	70.6	70.6	47.1	67.2	71.3	70.6	/	65.7
ERb_ MICUN	34.8	34.9	34.7	34.6	34.3	34.7	34.7	34.1	43.1	42.9	43.1	43.8	24.8	42.0	42.7	44.1	40.8	/

[a] The identity score is expressed as a percentage. The LBD identity is depicted in the upper right part of the matrix. The identity for the full-length ERs is given in the lower left part of the matrix. The identity is calculated with BioEdit.[28] For Rhesus macaque ERβ (ERb_MACMU) only the sequence of the LBD was available.

Figure 4c and Table 2) but is two orders of magnitude more potent on hERβ ($EC_{50} = 5 \times 10^{-11}$ M, Figure 4b and Table 2). Consistent with our prediction, 16α-LE_2 displays no activity on bERβ ($EC_{50} \gg 10^{-6}$ M, Figure 4a). On hERβ, 16α-LE_2 is also a poor agonist ($EC_{50} = 2.3 \times 10^{-8}$ M, Figure 4b and Table 2), whereas on hERα it displays a potency close to that of E_2 ($EC_{50} = 1.4 \times 10^{-11}$ M, Figure 4c and Table 2).

To further investigate the effect of the two variable amino acids in the ER binding pockets we generated variants of hERα and hERβ through in vitro site-directed mutagenesis containing, respectively:

- hERα-L384M, hERα-M421I, and the double mutant hERα-L384M/M421I,
- hERβ-M336L, hERβ-I373M, and the double mutant hERβ-M336L/I373M,

and performed transactivation assays in U2-OS human osteosarcoma cells in the presence of the two subtype-selective ligands and E_2 as reference compound.

Table 2. Transcriptional potency of ER ligands on hERα and hERβ variants.[a]

	hERα-wt		ERα-L384M		ERα-M421I		ERα-L384M/M421I	
	EC_{50} [M]	RTP	EC_{50} [M]	RTP	EC_{50} [M]	RTP	EC_{50} [M]	RTP
E_2	6.1e-12	100	3.2e-11	100	2.3e-10	100	1.4e-10	100
8β-VE_2	2.3e-9	0.26	5.2e-11	61.5	> 1e-7	nd[b]	4.8e-10	29.2
16α-LE_2	1.4e-11	43.6	7.2e-11	44.4	2.6e-7	0.09	3.2e-7	0.04
	hERβ-wt		ERβ-M336L		ERβ-I373M		ERβ-M336L/I373M	
	EC_{50} [M]	RTP	EC_{50} [M]	RTP	EC_{50} [M]	RTP	EC_{50} [M]	RTP
E_2	2.3e-11	100	3e-11	100	4.4e-11	100	> 1e-6	nd
8β-VE_2	5e-11	46	1.3e-8	0.23	5e-11	88	> 1e-6	nd
16α-LE_2	2.3e-8	0.1	3e-8	0.1	1.3e-10	34	> 1e-6	nd

[a] EC_{50} is determined from the respective transactivation assays (Figure 5 hERα, Figure 6 hERβ). RTP represents the relative transcriptional potency and is calculated as described in the Experimental Section. [b] nd: Not determined.

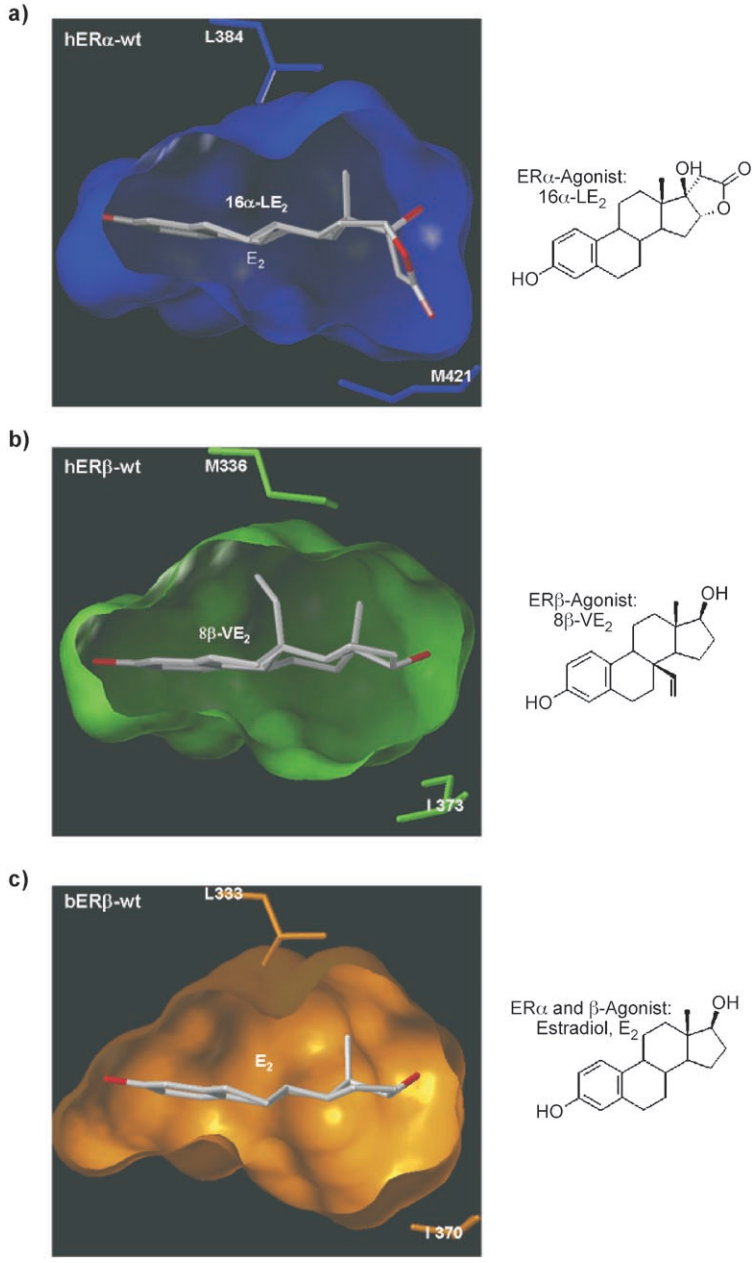

Figure 3. ER ligands in the ER binding pockets. Schematic representation of the predicted binding mode of the ER subtype-selective ligands in the binding pockets of a) hERα, b) hERβ, and c) bERβ.

On hERα wild-type (Figure 5a) E_2 and 16α-LE_2 display a similar potency (EC_{50} 6.1×10^{-12} M E_2 and 1.4×10^{-11} M 16α-LE_2, Table 2), while the hERβ ligand (8β-VE_2) is about two orders of magnitude less potent[9] (EC_{50} 2.3×10^{-9} M, Table 2). On the hERα-L384M variant (Figure 5b) all three compounds are equipotent. 8β-VE_2 is clearly more potent than on hERα wild-type (EC_{50} 5.2×10^{-11} M, Table 2), confirming the ERβ-binding character of this hERα mutant. Apparently, in the hERα-M421I variant (Figure 5c) the two subtype-selective estrogens do not induce transactivation. Even estradiol is about 100-fold less potent than on hERα wild-type (EC_{50} 2.3×10^{-10} M, Table 2). In the hERα-L384M/M421I double mutant (Figure 5d), selectivities are reversed. 8β-VE_2 is active, while the hERα compound 16α-LE_2 displays low potency in the transactivation assay. Interestingly, E_2 shows an activation profile on this hERα double mutant similar to that on hERβ wild-type (EC_{50} 1.4×10^{-10} M, Table 2).

Subsequently, we analyzed the same compounds on the respective hERβ variants (Figure 6a–d). As clearly shown on hERβ wt, the 8β-VE_2 compound displays a ~500-fold higher potency than the hERα ligand (Figure 6a, EC_{50} 5×10^{-11} M 8β-VE_2 and 2.3×10^{-8} M 16α-LE_2, Table 2). On hERβ-M336L both compounds, 16α-LE_2 and 8β-VE_2, are about three orders of magnitude less active than E_2 (Figure 6b, EC_{50} 1.3×10^{-8} M 8β-VE_2 and 3×10^{-8} M 16α-LE_2, Table 2). The ligand binding site in this mutant protein most closely resembles that of the bERβ wild-type protein (Figure 4a). On hERβ-I373M all three ligands are nearly equally potent and the subtype-selective character of 16α-LE_2 and 8β-VE_2 is lost (Figure 6c). Competition binding experiments confirm these results (Figure 7). Whereas E_2, 16α-LE2, and 8β-VE2 are equally potent in competing with the binding of radio-labeled E_2 to the hERβ-I373M mutant (Figure 7, lower panel), both 16α-LE2 and 8β-VE2 are weaker competitors in comparison with E_2 in binding to the hERβ-M336L mutant receptor (Figure 7, upper panel). The hERβ-M336L/I373M double mutant cannot be activated by any of the three steroidal ligands (Figure 6d). Both western-blot analysis and saturation-binding experiments using radio-labeled E_2 show lack of detectable ERβ protein expression or E_2 binding in U2-OS cells transfected with a hERβ-M336L/I373M expression construct (data not shown). This indicates reduced stability of the double mutant protein.

Discussion

Of the 22 amino acids within the ER-LBDs that are in the direct vicinity of the ligand E_2, two amino acids differ between hERα and hERβ (Figure 3). Interestingly, the conservation at these two positions appears to have undergone different selection during evolution. In fact, the leucine in ERα (equivalent to hERα-L384) is conserved throughout all species, whereas the corresponding methionine in ERβ (equivalent to hERβ-M336) is maintained in humans, mice, rats, rhesus monkeys, and chickens, but is changed to leucine in other mammals such as cow, sheep, pig, marmoset, and fish (Figure 2). On the other hand, the methionine in ERα (hERα-M421) and the corresponding isoleucine in ERβ (hERβ-I373) demonstrate a strict conservation throughout all aligned receptors. All other amino acids involved in steroid ligand contacts are identical in the two receptors. These observations allow the conclusion that the two variable positions represent the dominant structural clues of the ligand selectivity in the ERs.

Based on a comparison of the bovine ERβ with human ERα and ERβ LBDs, we predicted that the bovine receptor is not

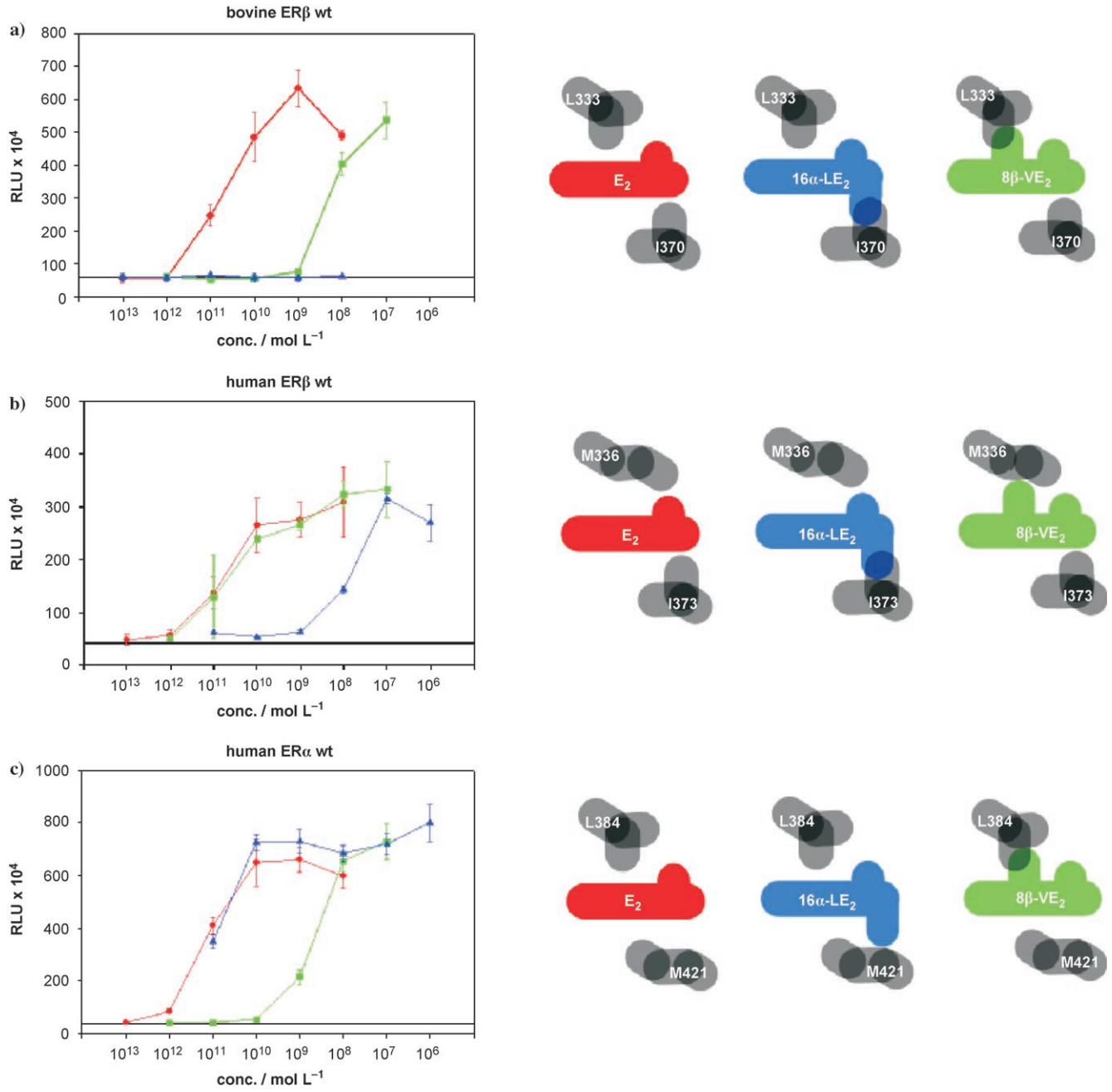

Figure 4. Activity of ER ligands on bovine ERβ. The transactivation assay in U2-OS cells is as described in Experimental Section. a) bovine ERβ wild-type; b) human ERβ wild-type; c) human ERα wild-type. The structural explanation of the ligand activities is provided in the cartoon on the right side. The steroidal ligands are depicted in side-view (along the plane of the aromatic A-ring; rings A-B-C-D from left to right). Dark grey spheres indicate the variable amino acids (branched amino acids: Leu and Ile, linear amino acid: Met). Overlapping spheres indicate repulsive interactions between amino acids and steroidal substituents leading to weaker (right-shift) transactivation. The different ER ligands are identified by colors (graph and cartoon): E_2, red; 8β-VE_2, green; 16α-LE_2, blue; vehicle, black baseline.

suited for the pharmacological characterization of ER subtype-selective compounds, 16α-LE_2 and 8β-VE_2. The bERβ is characterized by a single amino acid difference within the binding pocket with respect to hERβ. The flexible hERβ-M336 is exchanged to the rigid bERβ-L333 (Figure 3c). Consistent with this docking, the transactivation experiments on bERβ (Figure 4) show that 8β-VE_2 is a very weak activator because of the clash of the 8-vinyl group with the rigid L333. Even more severely impaired, 16α-LE_2 does not fit at all into the binding pocket because of the repulsion of the lactone moiety with I370. Thus, only E_2 is a potent agonist on bERβ. The experimental evidence shows that bERβ is not suited for pharmacological studies with the type of selective estrogens described here.[9] As the situation is presumably identical for pig, sheep, and the common marmoset, it is predicted that these animals are unsuitable for pharmacological studies with 8β-VE_2. On the other hand, rats, mice, and rhesus macaques show a completely conserved ligand binding pocket in comparison with hERβ. Rats

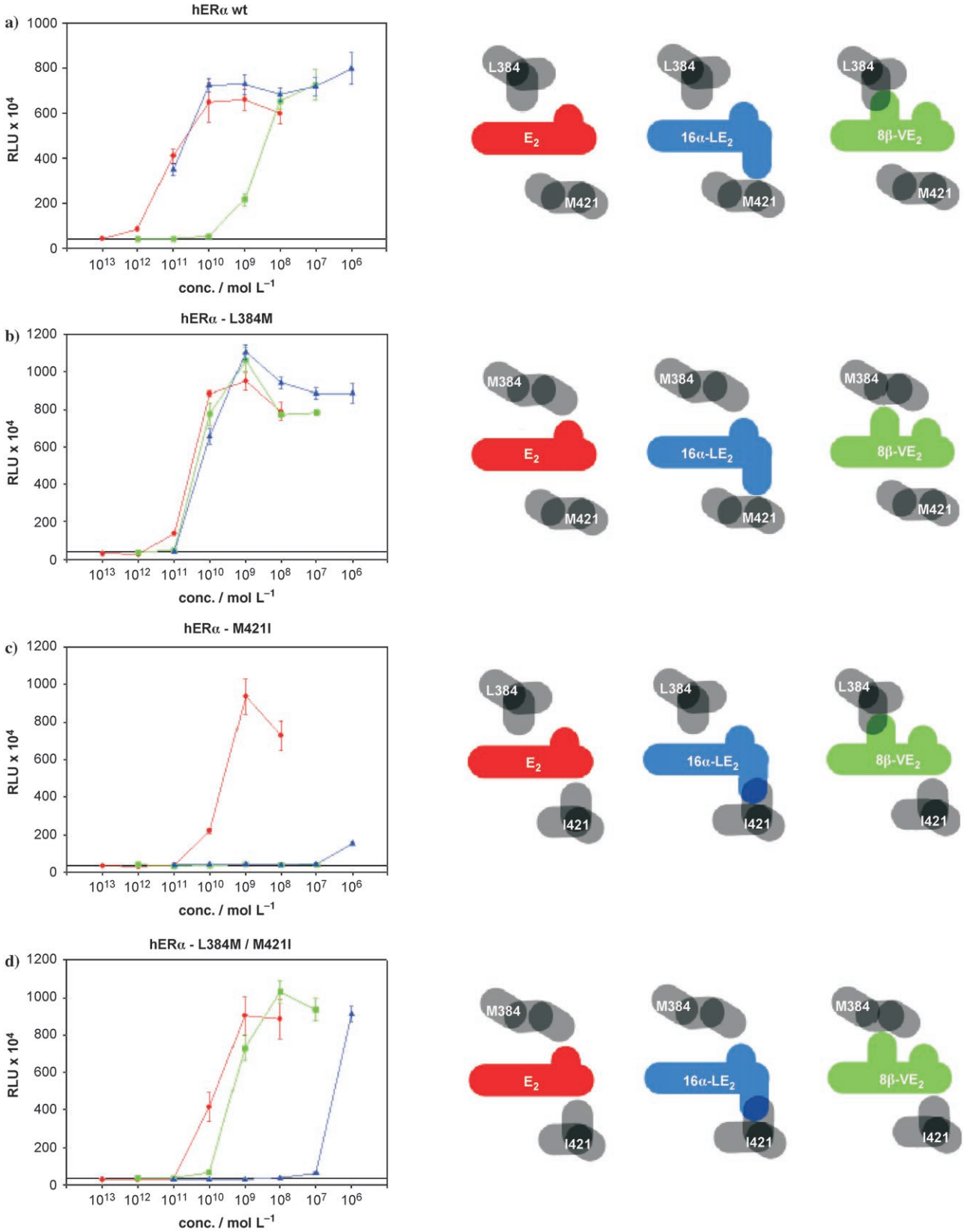

Figure 5. Activity profile of ER ligands on human ERα variants. The transactivation assay in U2-OS cells is as described in Experimental Section. a) hERα wild-type; b) hERα-L384M; c) hERα-M421I; d) hERα-L384M/M421I. The structural explanation of the ligand activities is provided in the cartoons on the right side (see also Figure 4 legend).

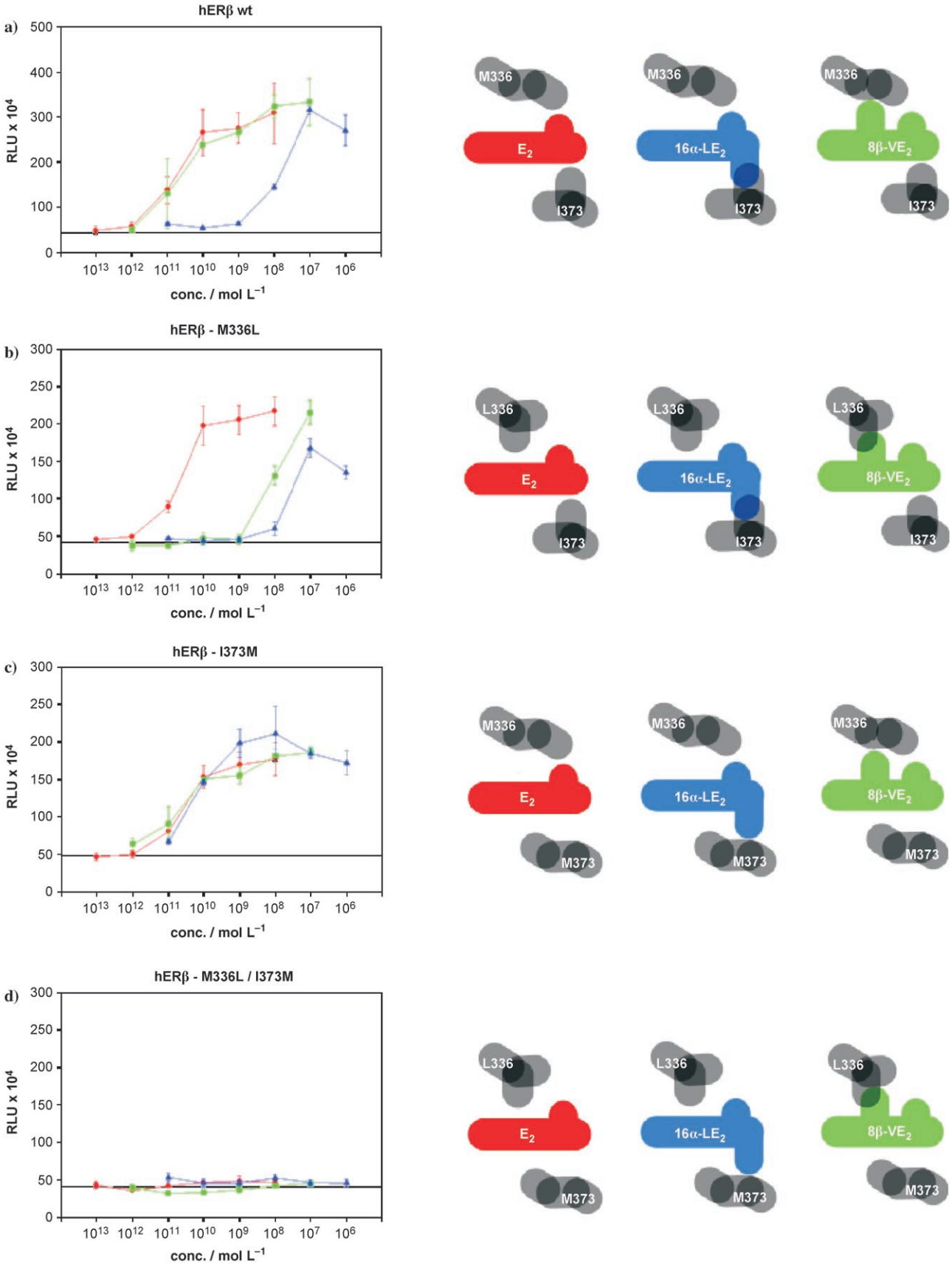

Figure 6. Activity profile of ER ligands on human ERβ variants. The transactivation assay in U2-OS cells is as described in Experimental Section. a) hERβ wild-type; b) hERβ-M336L; c) hERβ-I373M; d) hERβ-M336L/I373M. The structural explanation of the ligand activities is provided in the cartoons on the right side (see also Figure 4 legend).

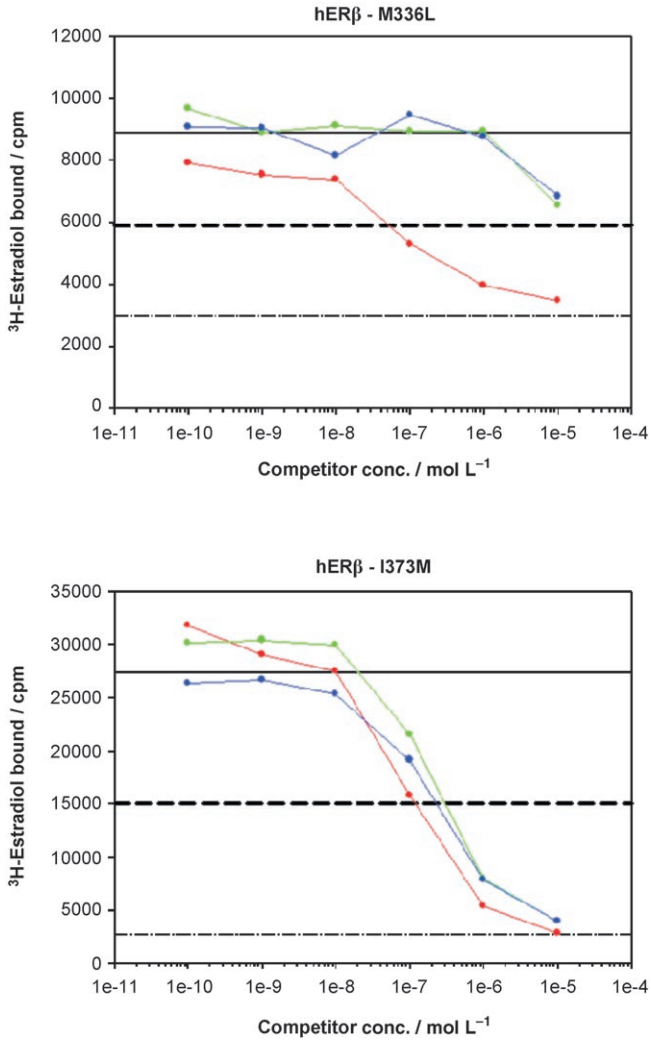

Figure 7. Ligand-binding assay on hERβ variants. Ligand-binding competition assays in U2-OS cells are as described in Experimental Section. Upper panel: hERβ-M336L, lower panel: hERβ-I373M. ER ligands are identified by colors: E_2, red; 8β-VE_2, green; 16α-LE_2, blue.

and mice have already been successfully used for pharmacological characterizations of hERβ-selective compounds.[9,16] Consequently, rhesus monkeys are also strongly suggested as an in vivo pharmacological model for 8β-VE_2. These correct predictions on bERβ have been made possible because of the detailed 3D structure of the receptor–ligand complexes. It is important to note that sequence comparison of the full-length protein and LBD (Table 1) between human ERβ and those of suitable species such as the mouse (88.4%/92.1%), rat (88.3%/92.9%), rhesus monkey (–/98.7%), and unsuitable species such as cattle (87.9%/94.2%), pig (86.2%/92.5%), sheep (87.5%/94.2%), and marmoset (92.2%/91.7%) does not reveal this crucial difference and, in fact, would have led to an inappropriate choice of species.

The residues building the ERα binding pockets are completely conserved throughout the majority of species included in this analysis (Figure 2). These receptors are thus highly similar to the hERα and supposed to be suited for pharmacological studies. This is especially true for bovine, rat, mouse, and chicken ERα. Tissues (calf uterus cytosol) and in vivo models (Allen-Doisy test, chick oviduct method) have been used in studying estrogens for decades.

The fact that single amino acid mutations can completely reverse pharmacological responses has recently been demonstrated with the hERα-A353Q mutation that renders testosterone (in addition to E_2) a potent ligand of this mutant ER.[21] Another well known example is the abolished binding of the progesterone receptor antagonist mifepristone (RU486) to chicken and hamster progesterone receptor (PR).[22] A single point mutation (human PR-Gly722→chicken PR-Cys575) prevents antiprogestins such as RU486 from binding to chicken (and hamster) PR and excludes for example, hamsters from pharmacological studies with antiprogestins.[23,24]

The bERβ transactivation results prompted us to assess our prediction on hERα and hERβ variants in the two amino acids of the respective LBDs. Thus, we gradually mutated hERα to embody the hERβ binding pocket and vice versa. Four single point and two double mutant ERs were assayed for transactivation with E_2 and the two ER subtype-selective compounds. Nearly all results of the transactivation experiments can be explained with simplified schemes highlighting the interactions of the substituents in the steroidal ligands with the mutated amino acids (Figure 5 and 6). In the hERα-wt E_2 and 16α-LE_2 are potent transactivators, whereas 8β-VE_2 shows drastically reduced activity. In the hERα-L384M mutant, the binding pocket now contains two flexible methionines rendering the three compounds almost equally potent agonists. The hERα-M421I mutant is characterized by a rigid binding pocket including two branched amino acids (L384, I421). Only the smallest compound, E_2, activates this variant whereas the binding of 16α-LE_2 is reduced because of repulsive forces between the lactone moiety and I421, and the binding of 8β-VE_2 is completely abolished because of unfavorable contacts with M384. The hERα-L384M/M421I double mutant is characterized by a binding site that is identical in sequence with the hERβ-wt binding pocket. Indeed the transactivation profile of the three steroid ligands resembles those observed for the hERβ-wt (compare Figure 5d and 6a, see Table 2). 8β-VE_2 is a potent activator on this hERα mutant.

Acting through the hERβ-wt, E_2, and 8β-VE_2 are potent agonists, whereas 16α-LE_2 shows drastically reduced activity. In the hERβ-M336L mutant, the binding pocket now contains two rigid branched amino acid (L336, I373). This binding pocket is identical in sequence with that of the hERα-M421I mutant protein. Again, only E_2 is a potent agonist in this case. The hERβ-I373M is characterized by a flexible binding pocket including two methionine residues (M336, M373). All three ligands act as potent agonists. Their subtype selectivity is lost because of the larger and more flexible binding pocket of this mutant protein. These results were confirmed in ligand competition experiments (Figure 7), indicating that different ligand affinities cause the differences in transactivation potency (as opposed to, for example, differential cofactor recruitment). The hERβ-M336L/I373M double mutant should in principle behave like the

hERα-wt. However, none of the ligands lead to detectable transactivation. This is the only mutant which could not be activated with E_2. As neither saturable binding of E_2 or 8β-VE2, nor detectable levels of this protein could be shown in U2-OS cells transiently transfected with the double mutant construct, we conclude that the exchange of the two amino acids in hERβ destabilizes the protein or interferes with its correct folding, and thus precludes pharmacological analysis of this mutant receptor.

From the functional analysis of the ER variants, their differing impact on E_2 activity is evident. Within the hERα mutants E_2 potency decreases two orders of magnitude from hERα wild-type (EC_{50} 6.1×10^{-12} M, Table 2) to hERα-L384M/M421I (EC_{50} 1.4×10^{-10} M). On the other hand, E_2 displays an almost identical potency (EC_{50} $2–4\times10^{-11}$ M) on all the hERβ variants, except for the presumably unstable double mutant. Moreover, on hERβ wild-type, E_2 is still 10-fold more active than on hERα-L384M/M421I, though they possess the same binding pocket. Potential explanations for this could reside in different amino acid sequences and domain structures outside the binding pockets. The impact of these mutations on the folding of more distant protein regions such as the AF1 domain or the cofactor interaction boxes for instance should not be underestimated.

The transactivation results provide conclusive evidence that the lactone moiety of 16α-LE_2 directly interacts with hERα-M421 within the binding pocket. All receptors having a methionine at that position (hERα-wt, hERα-L384M, hERβ-I373M) can be activated by 16α-LE_2. Similarly, the vinyl group of 8β-VE_2 interacts with hERβ-M336. All proteins having a methionine in the position above the steroidal B/C-rings (hERβ-wt, hERβ-I373M, hERα-L384M) are activated by 8β-VE_2. The consistency in this interaction pattern rules out any drastically different binding mode than that previously described.[9]

These findings are in agreement with transactivation experiments and site-directed mutagenesis studies published for diarylpropionitrile (DPN).[25] Docking of the *R* and *S* enantiomer of DPN into hERα and hERβ revealed that, especially for the *S*-DPN, the high hERβ selectivity stems from repulsive interactions of the nitrile group with hERα-L384. On the other hand, the ERβ selectivity of the nonsteroidal ERB-041[13] depends on favorable interactions of its vinyl group with hERβ-I373. Comparing the binding mode of ERB-041 with 8β-VE_2 shows that the two vinyl substituents in these compounds are far apart from each other and undergo completely different interactions. It is predicted here that ERB-041 should not be affected by the exchange of hERβ-M336→bERβ-L333 that, in contrast to 8β-VE_2, should render this compound a selective bERβ agonist.

A completely different binding mode is provided by the ERα-selective, nonsteroidal compound PPT.[26] More extensive conformational rearrangements within the ligand binding pocket must occur to accommodate PPT.[8] This is in contrast to 16α-LE_2 and 8β-VE_2 which are designed to be structurally close to 17β-estradiol and effectively exploit the few differences within the binding pockets with only relatively small substituents. It is thus supposed that 16α-LE_2 and 8β-VE_2 do not induce larger conformational rearrangements of the LBD (with respect to E_2) and that ligand selectivity is predominantly dependent on direct contacts of these ligands within the binding pocket.

We have shown herein that knowledge about the interaction of ligands with the amino acids constituting the ligand-binding pocket can be used to predict suitable animal models. Our example shows that a detailed three-dimensional understanding of ligand–protein interactions can be used to plan pharmacological experiments, an application of structural biology/structural bioinformatics to drug discovery which goes far beyond structure-based drug design. This method is not limited to the estrogen receptor or nuclear receptors per se. It should be applicable in those cases, where solid structural information on protein–ligand complexes is available and respective orthologues show a significant conservation to guarantee the generation of reliable homology models.

The predictions only focus on the conservation of ligand-binding sites in target proteins and do not reflect differences between species that stem from dissimilar signal transduction pathways, different expression levels of the target proteins, or the interaction of the test compounds with other proteins in respective species. Thus, the predictions involve certain aspects of pharmacodynamics and do not reflect differences in pharmacokinetics such as distribution, metabolism, and excretion.

Experimental Section

Homology modeling and structure-based ligand design: The full-length sequences of estrogen receptors α and β from various species were aligned using the program ClustalW, Version 1.8.[27] ERα sequences comprise (name: Swiss-Prot accession number): human (ERa_HUMAN: P03372), mouse (ERa_MOUSE: P19785), rat (ERa_RAT: P06211), bovine (ERa_BOVIN: P49884), pig (ERa_PIG: Q29040), chicken (ERa_CHICK: P06212), rhesus macaque (ERa_MACMU: P49886, fragment 121 aa), golden hamster (ERa_MESAU: Q9QZJ), Atlantic croaker (ERa_MICUN: P57753). ERβ sequences included in the study are: human (ERb_HUMAN: Q92731), mouse (ERb_MOUSE: O08537), rat (ERb_RAT: Q62986), bovine (ERb_BOVIN. Q9XSB5), pig (ERb_PIG: Q9XSW2), sheep (ERb_SHEEP: Q9TU15), chicken (ERb_CHICK: Q9PTU5), rhesus macaque (ERb_MACMU: Q9TTE5, fragment 279 aa), common marmoset (ERb_CALJA: Q95171), Atlantic croaker (ERb_MICUN: P57781). In ClustalW gap opening and extension penalties of 10 and 0.2 respectively, were used together with the Gonnet series matrices for the multiple alignments. The sequence identity matrix for the full-length sequences and the LBD (ligand binding domain) was calculated with BioEdit.[28] The homology model of ERβ was built on the basis of the crystal structure of ERα in complex with E_2.[18] Since helix 12 of the ERα-LBD forms contacts with a neighboring protein molecule in this crystal structure, a conformation of this helix similar to helix 12 in the PR-LBD was modeled. This model of ERα was used as the template for ERβ. The program SYBYL[29] (module COMPOSER) was used to build the model. Energy minimization with the AMBER 4.1 force field implemented in SYBYL led to the final model of ERβ. For a more general description of homology modeling, see reference [30]. The volume of the amino acid side chains methionine, leucine, and isoleucine was calculated using MOLCAD in SYBYL. Compounds 16α-LE_2 and 8β-VE_2 were manually placed into the binding pockets of wild-type and mutant receptors by superimposition with E_2 and subsequently docked with energy minimization

using the Merck molecular force field (MMFFs)[31] implemented in SYBYL.

Site-directed mutagenesis and transactivation assays: The expression plasmids for human ERα and ERβ have been already described elsewhere.[32] The respective mutants were generated with the QuikChange XL Site-directed mutagenesis kit (Stratagene, CA, USA) according to manufacturer's instructions. The estrogenic potency of the ER ligands "in vitro" was determined by transactivation assay as described.[32] Briefly, U2-OS human osteosarcoma cells (ATCC, Manassas, Virginia, USA) were transiently co-transfected with hERα or hERβ and respective mutant forms and an $(ERE)_2$-luciferase reporter gene. Estrogen-induced reporter gene activity was determined 24 h after treatment of the cells. 17β-estradiol (E_2) was used as a reference. The relative transcriptional potency (RTP) is defined as (EC_{50} E_2/EC_{50} test compound) × 100 where E_2 is characterized by an $EC_{50} = 6.2 \pm 1.6 \times 10^{-12}$ M ($n = 8$) in the ERα transactivation assay and an $EC_{50} = 2.3 \pm 1.4 \times 10^{-11}$ M ($n = 8$) in the ERβ transactivation assay.

Ligand-binding assays: U2-OS cells were transiently transfected with expression vectors for wild-type hERβ, hERβ-M336L, hERβ-I373M, and the double mutant hERβ-M336L/I373M. For saturation binding, cells were trypsinized 48 h after transfection, and 10000 cells/data point were incubated with ^{3}H-estradiol at concentrations between 5×10^{-10} M and 10^{-7} M in DMEM. Nonspecific binding was determined in the presence of 10^{-5} M unlabelled E_2. After 1 hr of incubation at room temperature, cells were transferred on a 96well-filter plate. ^{3}H-scintillation of the immobilized cells (corresponding to receptor-bound radiolabeled E_2) was measured on the filter plate by a Packard TopCount NXT device. Specific binding was calculated as difference between total and nonspecific binding for each concentration of ^{3}H-estradiol. For competition binding, cells were trypsinized 48 h after transfection, and 10000 cells/data point were incubated with different concentrations of unlabeled E_2 or test substance and 10^{-7} M of ^{3}H-Estradiol in DMEM. Receptor-bound ^{3}H-E_2 was measured as outlined above, and plotted against the concentration of unlabeled test substance. All data points were in triplicate.

Compounds: All compounds were provided by the Schering Medicinal Chemistry. The synthesis of the 8β-VE_2 (8-vinylestra-1,3,5(10)-triene-3,17β-diol, named 8β-VE_2) and 16α-LE_2 (3,17-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone) is described in patent applications.[33,34]

Acknowledgements

We are grateful to Dr. Norbert Walther (Institute for Hormone and Fertility Research, University of Hamburg, Hamburg, Germany) for the provision of the bovine ERβ expression vector.

Keywords: animal models · estrogen receptors · mutagenesis · protein models · selective agonists · structure–activity relationships

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Received: July 18, 2006
Published online on September 26, 2006

