Identification of a Second Binding Site in the Estrogen Receptor

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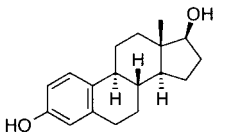
Fluorescence spectrometry data by Tyulmenkov and Klinge (*Arch. Biochem. Biophys.* **2000**, *381*, 135−142) suggest the presence of a second binding site in both subtypes ERα and ER*β* of the estrogen receptor (ER). A cavity previously described as a solvent channel was located in close proximity to the steroid binding site of both ER subtypes. Derivatives of a tetrahydrochrysene (THC) compound, speculated in the literature to bind to a second binding site, were docked successfully in the second sites identified. However, computation of accurate interaction scores indicates preferred binding to the steroid binding site over the second binding site of both ERα and ER*β* for all THC derivatives. Therefore, binding to this second site is probably not the reason the THC derivatives are agonists on ERα and antagonists on ER*β*. Most likely, the smaller steroid binding site of ER*β* compared to ERα and/or the apparent larger flexibility of helix 12 of ER*β* make ER*β* more readily adopt an antagonist conformation.

Introduction

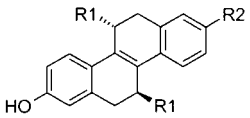
The estrogen receptor (ER) is a member of the nuclear receptor (NR) gene family binding the steroid hormone estradiol **1**.[2−4] Two subtypes are known of ER, designated ERα and ER*β*.[5] Both ER subtypes are important targets in pharmaceutical industry.[6] Estradiol and derivatives are constituents of the oral contraceptive pill. Selective estrogen receptor modulators (SERMs), which show tissue-dependent agonistic or antagonistic behavior, are used as first line treatment for estrogen-responsive breast cancer and for anti-osteoporotic therapy.[7,8] Finally, pure estrogen antagonists (or anti-estrogens) are currently in clinical development for anti-breast-cancer treatment.[9]

Like other NR family members, the full-length ER receptor consists of a ligand independent transactivation domain AF1 at the amino terminus, a central DNA binding domain and the ligand binding domain at the carboxy terminus. The ER ligand binding domain changes conformation upon binding of an agonist, which subsequently allows recruitment of one or more coactivators. The complex thus formed activates DNA transcription by binding to the estrogen response element.[10−13] The agonist is completely enclosed by ER and forms part of the hydrophobic core of the protein. The orientation of helix 12, located at the carboxy-terminus of the ligand binding domain, is pivotal in distinguishing between agonists and antagonists. In the latter case, it blocks access to a groove located between helices 3, 4, and 5, the binding site for coactivators during transcription (AF2 site).

In a recent paper, Tyulmenkov and Klinge[1] used fluorescense spectrometry of tetrahydrochrysene ketone (THCK) to measure the kinetics of estradiol binding to ERα and ER*β*. THCK was found to bind to both ER subtypes in the presence of estradiol **1** and certain known ER antagonists, leading to the conclusion that there is a second binding site in ER. The measured dissociation constants suggest that THCK binds to the same second site in ERα and ER*β*, but the fluorescense spectra indicate that the microenvironments of the second sites are not identical. Tyulmenkov and Klinge claimed that crystallographic studies of the ligand binding domain of ER have not revealed a possible second binding site, hence the second binding site must be located in another domain of ER.[1] Jensen et al.[14,15] had previously proposed a second binding site in the ER based on their immunoassay results. In this model, ligand binding to the steroid binding site would exclusively mediate an agonist response, while binding to the second site would be responsible for antagonistic behavior. Note that this model is at odds with crystal structures showing that antagonists bind to the steroid binding site.[10,16−18]



1, estradiol



	R1	R2
2	Me	OH
3	Et	OH
4	Pr	OH
5	Et	$(CO)CH_3$

The group of Katzenellenbogen has synthesized multiple THC derivatives and determined their (ant)-agonistic behavior on both ERα and ER*β*.[19−21] All compounds **2**−**4** were agonists on ERα. Increasing the size of R1 increases antagonism on ER*β*, **2** still being an agonist, **3** a partial agonist, and **4** an antagonist. The structure of THCK was not given in the paper by Tyulmenkov and Klinge,[1] but reference to work by Katzenellenbogen et al.[21] established THCK is compound **5**. The relative binding affinity of **5** for ERα is reported as 40%-68% of estradiol **1**,[19,21] and it is an ERα agonist.[22]

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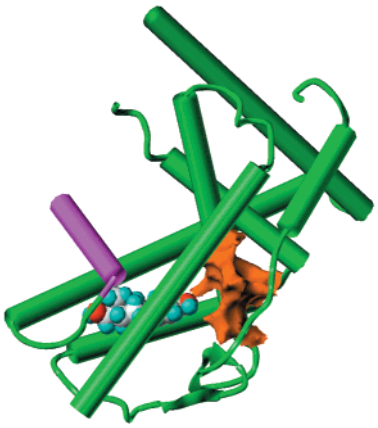

Figure 1. Cartoon representation of ERα with the second binding site identified (orange). The natural ligand estradiol **1** is shown in space-filling representation. Helix 12 is colored magenta.

The steroid binding sites of ERα and ER*β* are nearly identical, only two residues lining the binding site being different:[17] Leu384/Met421 in ERα are equivalent to Met336/Ile373 in ER*β*. A second binding site would offer novel opportunities to create subtype-selective compounds, especially since the fluorescense data indicate this binding site to be more differentiated between the ER subtypes than the steroid binding sites. Furthermore, if the ER subtype selective (ant)agonistic behavior of THC derivatives is connected to binding to the second binding site, more selective ER modulators might be designed. This modeling study shows that the ligand binding domain of ER does contain a second binding site and that the experimental observations by Tyulmenkov and Klinge[1] can be explained by its presence.

Results and Discussion

The crystal structures of the ligand binding domains of both ERα and ER*β* were submitted to a program developed in-house to search for cavities and grooves in protein structures. The steroid binding site was readily identified, as was the AF2 site, which is the groove in which co-activators bind to ER. Only one other sizable cavity was found, located directly next to the steroid binding site (Figure 1). Inspecting other ER structures in the PDB revealed this cavity present in all of them, as well as in the homologous progesterone receptor. This site was qualified as a solvent channel by Brzozowski et al. when they solved the first crystal structures of ERα.[10] The size of the second site was independent whether ER was binding an agonist or antagonist. Helix 12, which changes conformation dependent on whether an agonist or antagonist is bound, is located away from the second pocket. The second site of ER*β* is slightly larger than the second site of ERα, while the opposite applies to the steroid binding sites (Figure 2). The second binding site is less hydrophobic in both ERα and ER*β* as the steroid binding site. Four residues lining the second binding site differ between the ER subtypes: Ile326, Leu327, Ile386, and Phe445 in ERα are equivalent to His279, Val280, Val338, and Tyr397 in ER*β*, respectively.

The program Gold was used to dock compounds **2**−**5** in both sites of both ERα and ER*β*. As a control, estradiol **1** was docked as well. The X-ray crystal structure conformation of **1** in the steroid binding site of ERα was reproduced exactly, as was expected. However, **1** was exclusively found to bind 'upside down' in ER*β* compared to ERα (Figure 3). In subsequent discussion, the binding mode predicted by docking in ER*β* is referred to as the upside down mode, and the binding mode in ERα as X-ray mode. The existence of the upside down binding mode has been proposed to explain the similar antiestrogenic behavior of 7α- and 11*β*-substituted estradiols by Jordan,[23] Abul-Hajj,[24] and Tedesco[25] and has recently been observed in the crystal structure of ER*β* with ICI-164,384.[18] For these compounds, the large 7α substituents were thought to drive the upside down mode. The upside down mode of **1** can be rationalized by looking at both the ER binding sites and estradiol itself. The steroid binding site of both ER subtypes is structurally identical apart from two residue pairs. Focusing on the pair Leu384(ERα)/Met336(ER*β*), it can clearly be seen that the Met residue in ER*β* clashes with the methyl group of estradiol in the X-ray binding mode. Both residues of the Met421(ERα)/Ile373(ER*β*) pair can accommodate a methyl substituent of estradiol in the down position, so this pair does not seem to be important in determining the estradiol binding orientation. Apart from the methyl substituent, the estradiol molecule itself is approximately flat and can be considered semisymmetric with respect to rotation around the hydroxy−hydroxy axis.[23−25] This enables estradiol to make almost the same hydrophobic contacts in both binding modes despite the different orientation. The existence of two binding modes is further facilitated by the, apart from the phenolic ring, not very tight binding of estradiol by ER in either mode. The protein model used for the docking study in ERα was that determined in the crystal structure with estradiol. For ER*β*, the protein structure was taken from the crystal structure with the partial agonist genistein. Since the protein structure is a rigid template during docking, the ERα structure was therefore the ideal template for docking estradiol. Similarly, the ER*β* structure was the ideal template for docking genistein, but not necessarily estradiol, especially when taking into account that genistein lacks the methyl group causing the estradiol-Met336 clash in ER*β*. To investigate this further, estradiol in X-ray mode was placed in the binding site of ER*β* by overlapping both ER crystal structures using the Cα atoms. Residue Met336 was energy minimized while the rest of the protein plus estradiol were kept rigid. This yielded a structure in which the conformation of the Met336 side chain is slightly different from that observed in the crystal structure. The conformational energy was raised by 6.2 kcal/mol, and the RMS displacement excluding hydrogen was 0.31 Å (Met336 only). Estradiol was subsequently docked in the steroid binding site of this ER*β* structure. The most favorable docked orientation was still the upside down binding mode. However, the X-ray binding mode was now found as the second ranked dock. Moreover, the Gold fitness score differed only by 0.7 units (29.3 for the upside down mode, 28.6 for the X-ray mode, respectively). The X-ray binding orientation seems therefore feasible in ER*β* as well. To compare both binding modes further, estradiol was energy minimized in both binding orientations in ERα, ER*β* and ER*β* with Met336 modified as described

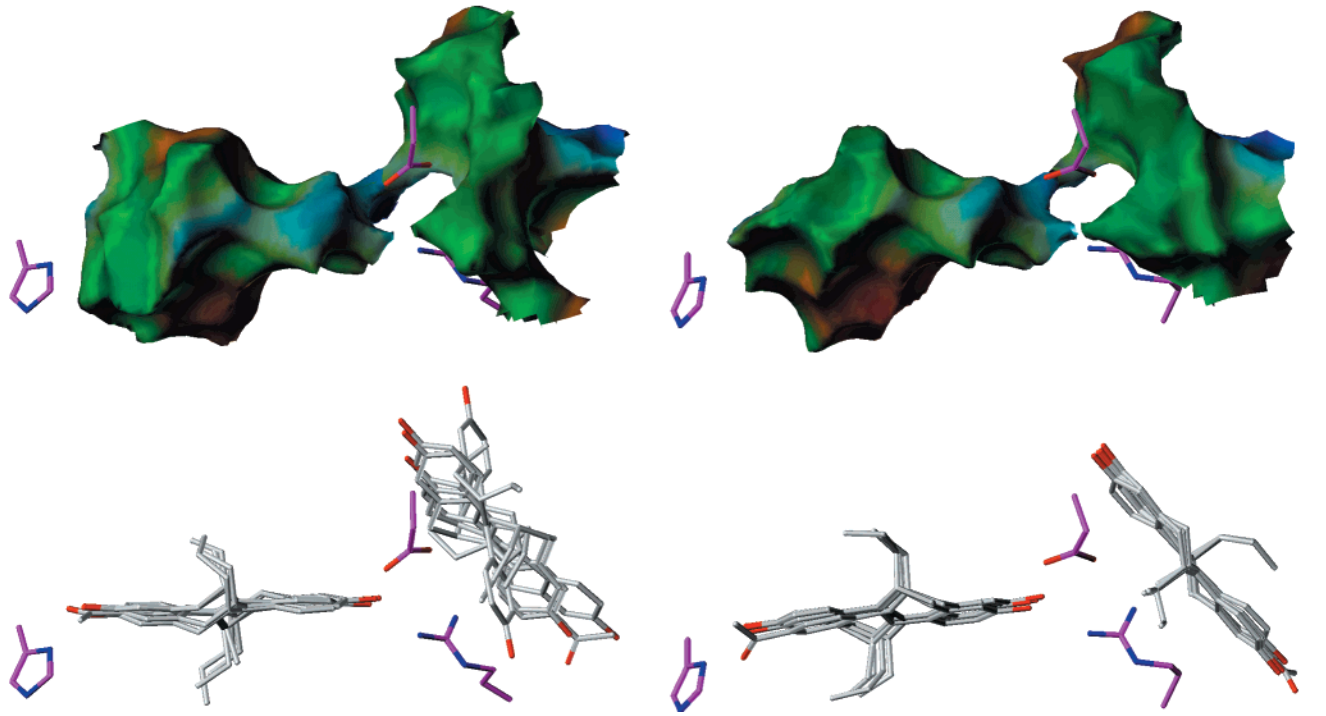

Figure 2. Binding modes of THC derivatives **2**−**5** in ERα (left) and ERβ (right). The scale and orientation of the molecules in the lower half of the figure matches exactly the surfaces of the binding sites in the upper half. The surfaces are colored by lipophilicity, ranging from brown (most lipophilic) to blue (least lipophilic). Three key side chains are shown: Glu353, Arg394, and His524 in ERα, which are equivalent to Glu305, Arg346, and His475 in ERβ. The carbon atoms of these residues have been colored magenta for clarity. Hydrogen atoms of the THC compounds have been omitted for clarity.

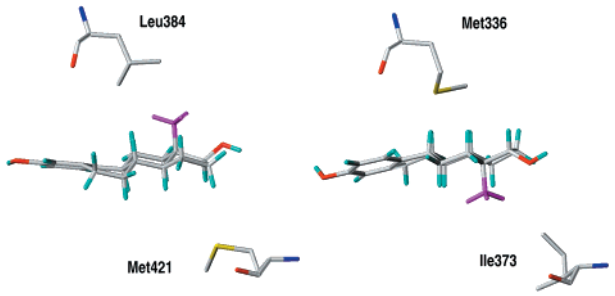

Figure 3. Binding mode of estradiol in ERα (left, X-ray) and in ERβ (right, docked structure). The 13-methyl is shown in magenta. The two residues that are different between the ERα and ERβ binding sites are indicated.

previously. After minimization, the structural difference between ERβ and ERβ with modified Met336 has almost completely disappeared for both binding modes.

The fitness scoring routine of Gold relies heavily on hydrogen donor−acceptor pairs, while the binding of both the THC derivatives **2**−**5** and estradiol **1** is mainly driven by hydrophobic interactions. The Gold scores may not be too reliable in this case, and therefore the docked structures have been ranked with more sophisticated scoring algorithms: The CScore (consensus score) algorithm of Sybyl, a combination of the FlexX, Dock, and Gold* scoring schemes, and the in-house developed Potential of Mean Force (PMF) score[26,27] (Table 2). Note that the Gold* score implemented in Sybyl is not the same as the fitness scoring used by the program Gold. For ERα, all three scoring components of CScore rank **1** more favorable in the second binding site than in the steroid binding site (X-ray mode). This is clearly a contradiction of experimental results, which prompted us not to rely on (any component of) the consensus score.

Table 1. Gold Fitness Scores of Best Ranking Docks[a]

	ERα binding site		ERβ binding site	
compd	steroid	second	steroid	second
1	35.42	15.0	37.15[b]	24.04
2	50.72	18.5	58.96	46.97
3	58.56	5.42	56.95	55.01
4	53.66	3.24	39.71	42.16
5	35.31	16.65	34.20	52.53

[a] Higher scores indicate more favorable binding. [b] Upside down binding mode.

In general, the ranking of the PMF scores closely matches the ranking of the Gold fitness score in Table 1. The difference is the ranking of **4** and **5** between the two binding sites in ERβ: The Gold score favors the second binding site, whereas the PMF score favors the steroid site. The PMF score, as well as the Gold fitness score, do rank **1** in ERα correctly as more favorable in the steroid binding site. The relative binding affinities of **2**−**5** compared to **1** for ERα binding are 8%, 126%, 48%, and 40%, respectively.[21] Later, the same affinities for **2**−**4** were measued by the same group as 222%, 221%, and 34% for ERα, and 254%, 432%, and 92% for ERβ.[19,20] The difference may be attributed to the use of full-length ER versus the ligand binding domain only.[19,20] Assuming the binding takes place in the steroid binding site, the PMF score matches the latter ranking better as does the Gold fitness score. Therefore, in subsequent discussion, the PMF score will be used exclusively.

The PMF score was applied on the minimized complexes of **1** in both ERα and ERβ in both binding modes (Table 3). As expected, for ERα the X-ray binding mode is clearly preferred over the upside down mode, but it is the preferred binding mode in ERβ as well. This highlights the sensitivity of protein conformation during

Table 2. Sybyl CScore (FlexX, Gold*, and Dock) and PMF Scores of Best Ranking Docks[a]

	ERα binding site		ERβ binding site	
compd	steroid	second	steroid	second
		FlexX		
1	−37.9	−49.4	−41.8[b]	−29.6
2	−37.7	−31.3	−49.1	−41.1
3	−42.9	−47.9	−55.5	−44.4
4	−48.4	−51.4	−56.2	−50.2
5	−44.9	−50.5	−55.7	−43.9
		Gold*		
1	−172	−291	−247[b]	−195
2	−211	−217	−269	−244
3	−255	−347	−332	−290
4	−298	−357	−384	−332
5	−293	−323	−377	−290
		Dock		
1	−89.2	−93.9	−99.7[b]	−82.3
2	−92.3	−66.4	−99.2	−70.3
3	−93.7	−100	−103	−78.5
4	−94.3	−93.1	−103	−76.9
5	−114	−96	−123	−82.0
		PMF		
1	−431	−176	−407[b]	−159
2	−429	−262	−457	−230
3	−485	−54	−480	−237
4	−426	−71	−491	−199
5	−400	−165	−477	−241

[a] Lower scores indicate more favorable binding. [b] Upside down binding mode.

Table 3. PMF Scores of Different Binding Modes of Estradiol in ERα and ERβ after Energy Minimization

	binding mode	
receptor	X-ray	upside down
ERα	−448	−421
ERβ	−431	−421
ERβ[a]	−426	−422

[a] Met336 modified (see text).

docking as one realizes that this binding mode was not found prior to minor tweaking of the conformation of Met336. In conclusion, the X-ray conformation is preferred in both ER subtypes, although the upside down conformation is only marginally less favorable.

Gold could be forced to fit **1** in the second binding sites, but only by excluding binding to the steroid site, as can be seen from the fitness scores in Table 1. For all THC derivatives, there is a clear preference for the steroid binding site of ERα over the second site. However, for ERβ, the second binding site becomes more favorable than the steroid binding site with increasing size of R or with the introduction of the keto functionality. This is mainly caused by a decrease of the fitness score in the steroid site, rather than an increase of the score in the second site. However, comparing the scores between the two second binding sites, binding to the second site of ERβ is clearly preferred. The binding modes of **5** in the second binding sites are depicted in Figure 4 as an illustration for all THC compounds. In ERβ, all THC derivatives donate a hydrogen bond to carbonyl oxygen of Glu305. In ERα this binding mode is blocked by Ile386, and the hydroxy group pointing in the cavity cannot form a hydrogen bond, which explains the more favorable binding in the second binding site of ERβ compared to ERα. Visual inspection of the docked THC derivatives (Figure 2) reveals that the smaller steroid cavity of ERβ compared to ERα results in more

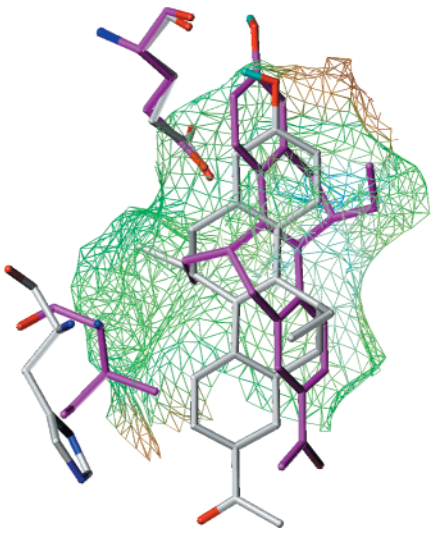

Figure 4. Top view of **5** as docked into the second binding sites of ERα and ERβ. The proteins have been overlaid using equivalent Cα atoms. Carbon atoms of ERα and **5** docked into ERα have been colored magenta. The wire surface is the second binding site of ERβ. The Glu residues are the same as in Figure 2 (Glu353 for ERα, Glu305 for ERβ). In ERα, Ile326 clashes with the aromatic ring of **5** bearing the keto functionality, preventing the more favorable binding mode observed in ERβ. In the latter protein, His279 leaves enough space for **5** to form a hydrogen bond with the backbone carbonyl of Glu305. Note that in both docking modes the keto functionality is fully solvent exposed.

restrained, energetically and entropically unfavorable conformations of **2**−**5**.

On the basis of the PMF scores, the preference of THC compounds **4**−**5** for the second binding site of ERβ disappears, mainly because the score for the steroid binding site increases. One has to take into account that the PMF score does not contain the deformation energy of the ligand. However, given that the conformational differences for the THC derivatives as caused by the different binding environments are small, the steroid binding site is probably preferred for both ER subtypes. Concluding, the docking study predicts the steroid binding site as the preferred binding site for all THC derivatives **2**−**5**, but relatively less so for ERβ. Estradiol **1** shows a clear preference for the steroid binding site of both receptors. A mixture of **1** and **5** competing for ER binding is predicted to yield **1** binding exclusively to the steroid site of both ERα and ERβ, with the fluorescent probe **5** competing with **1** for binding to this site. The remainder of **5** will bind in the second binding site. This scenario can explain the mixture of uncompetitive and competitive binding observed for **1** and **5** by Tyulmenkov and Klinge[1] but contradicts their claim that that **5** does not bind in the steroid binding site.

What is the function of the second binding site? Since it is not physically close to the receptor sites required for dimerization or for recruitment of activation factors, ER antagonism is not expected by interfering with protein−protein interactions. If the second binding site is part of the entrance or exit channel for **1**, binding to the second site may be antagonistic. However, the steroid binding site is located close to the protein surface near His524 (ERα numbering), i.e., at the opposite end of the steroid binding site as where the second binding site is located (Figure 2). Access to the steroid binding

site seems at least equally likely via this route as via the second binding site. Finally, apo-ER is thought either to exist in a partially unfolded state or to be stabilized by chaperone proteins in an apo-receptor complex.[13] The ER conformation in either state is not known, but given the proximity of the second binding site to the steroid binding site, it is likely that the second binding site does not even exist, at least in the partially unfolded state, prior to binding of a ligand in the steroid binding site.[28] Thus, there seems no obvious function for the second binding site.

From an evolutionary perspective, steroid binding receptors are relatively new additions to the NR family.[29,30] If one assumes that the ancestor of the ER had a larger binding site, the second site may be a left-over when the Glu353/Arg394 (ERα) pair divided the larger ancestral site in a smaller steroid binding site and the second binding site (Figure 2). The evolutionary closest nonsteroid binding cousin of ER is the retinoic X receptor (RXR).[30] The natural ligand of RXR, 9-*cis*-retinoic acid (9-*c*RA), is indeed larger than estradiol. Moreover, in the RXRα/9-*c*RA crystal structure,[31] the carboxylic acid group of 9-*c*RA forms an ion pair with Arg316 identical to the Glu353/Arg394 pair in ERα. The ER may have evolved from the RXR receptor by the protein furnishing the acid functionality originally provided by the ligand. The second binding site as evolutionary remnant is in accordance with the apparent lack of function of this binding site and with the absence of the binding site in other NRs such as RARγ.

The antagonistic behavior of the larger THC derivatives in ERβ can be explained by the binding mode in the steroid site alone. This site is smaller in ERβ than in ERα, and therefore likely to be more sensitive of substituents overlapping with helix 12. In ERβ, one of the R-substituents of **2**−**5** comes close to residue Val487, a residue in the hinge region of helix 12. ERβ itself might inherently be easier to antagonize, since the crystal structure of ERβ with the known (partial) agonist genistein shows helix 12 is an antagonist-like conformation, despite genistein lacking a side chain that pushes helix 12 away.[11]

Conclusions

A second binding site has been identified within the ligand binding domain of both ERα and ERβ. Results of docking experiments with the fluorescent probe tetrahydrochrysene ketone **5** in both the steroid and the second binding sites of both ER subtypes were in full agreement with fluorescent data from literature. The increasing antagonistic behavior of tetrahydrochrysene derivatives **2**−**4** in ERβ is most likely not related to binding in the second site but an inherent property of ERβ itself. The presence and apparent lack of function of the second binding site can be explained as an evolutionary remnant when a larger ancestral binding site was modified to bind estradiol. On the technical side, the PMF scoring algorithm was found to be superior to both the Gold fitness score and the Sybyl CScore methods. The sensitivity of ligand docking to minor conformational changes in the protein was highlighted.

Experimental Methods

Docking studies were performed with crystal structures 1ere (ERα)[10] and 1qkm (ERβ)[17] taken from the PDB.[32] Sybyl version 6.6 was used to add hydrogen atoms, perform energy minimizations, and generate computer graphics.[33] An in-house program based on the SURFNET algorithm by Laskowski[34] was used to search for cavities in the ER structures.[35] Docking was performed with version 1.1 of Gold.[36] The CScore method was used as implemented in Sybyl 6.6.[33] The PMF score has been described in the BLEEP algorithm.[26,27] Protein structures were overlapped using MNYFIT.[37] The default settings of the programs indicated were used in all cases.

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