EXERCISE III.1

STRUCTURAL DATA: THE BASIS FOR MOLECULAR MODELING

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Theoretical Introduction

Proteins can properly and correctly work only when they fold into their native structures. The possibility of observing the three-dimensional structure of a protein, through X-ray diffraction or NMR techniques, is essential to understand how a protein works and to rationally design potential ligands, capable of inhibiting or activating a biological target. We can, in fact, assume that structural data represent the milestone for reliable molecular modeling and computer-aided drug design approaches. Nevertheless, determining the exact structure into which a protein naturally folds is a very complex and time-consuming procedure. Thus, the molecule has to be purified, crystallized, exposed to X-radiation, and the diffraction diagram must be transformed into an intelligible electron density map. Unfortunately, some proteins are not easy to crystallize and others are not able to generate clear diffraction patterns after X-ray exposure, so, different computational approaches and simulations must be applied, in order to find new potential lead compounds. Actually, there are two different methods used to design new drugs: the ab initio approach and the in silico virtual screening. The first is based on the knowledge of the three-dimensional structure of the target binding pocket and aims to design drugs sterically and physicochemically complementary to the active site properties and shape. The second one is able to screen thousand of compounds belonging to a large database of known molecules, and to computationally evaluate their binding affinity toward the target protein (Lengauer 2003).

If we are very lucky and we know both the three-dimensional structure of our biological target and of the potential lead compound, or at least of the potential chemical scaffold, we can simply apply structure-based drug design procedures, trying to improve the inhibition potency of the potential new drugs. If the protein structure is known, but we do not have any clue about the structure of a possible inhibitor, or activator, we can use combinatorial chemistry techniques to generate millions of molecules, to be subsequently virtually tested, with docking and scoring simulations. Knowing the structure of the receptor and the shape and chemical nature of the binding pocket, it is also possible to model a lead compound, in order to achieve an almost perfect geometrical and chemical complementarity, thus combining random virtual screening and rational drug design processes, or to choose a complete de novo design approach. On the contrary, ligand-based design can be used when only the ligand configuration is known, but the target structure is not available. Quantitative structure–activity relationship (QSAR) investigation could represent a useful tool to improve the inhibition properties of a lead compound, computationally modifying its chemical properties. Moreover, QSAR techniques could elucidate also the chemical peculiarities of the binding pocket, driving to the prediction of a pharmacophore model. At last, when no structural information is available on either the biological target or the ligands, the unique possibility is to generate thousands of different chemical compounds through combinatorial chemistry and to experimentally screen them. Virtual screening is the computational in silico variant of high-throughput screening, and even if the accuracy of virtual predictions is usually lower than the accuracy of experimental measurements, virtual screening presents some benefits. The calculations, in fact, are cheaper and faster, and it is possible to test and model compounds not yet purchased or synthesized.

Three-dimensional modeling, even though it is still far from complete exactness, represents a useful tool for the prediction of unknown pharmaceutical target structures. Amino acid residues are characterized by specific electrostatic fields, and used to assume particular shapes. Through several techniques, such as force field and molecular mechanics calculations, homology modeling, and fold recognition, it is sometimes possible to model and predict the folding pathway that a specific protein might follow and also the behavior and dynamics of the folded protein. Proteins sharing similar sequences or functions often adopt the same overall fold. Nevertheless, it is interesting to observe that proteins with no sequence similarity and even with different functions, may also exhibit similar foldings. Thus, it has been suggested that there is a limited number of 1000–7000 different families, to which almost all proteins may belong, that have been adapted by duplication, mutation, or natural selection processes, to perform all the existent biological functions (Lengauer 2003). Members belonging to the same family and functionally analogous sites assume the same folding patterns. This makes sense, since proteins performing the same functions should have the same general structure. Usually, the tertiary structures of evolutionary related proteins are better conserved than their primary sequences. However, differences between three-dimensional structures are known to increase with decreasing sequence identity, thus leading to corresponding model accuracy fall-off.

These observations constitute the basis of the two most successful, and widely applied, protocols for protein modeling: homology modeling and protein threading.

Homology modeling combines two different computational tools: sequence analysis and molecular modeling. This approach is based on the assumption that, usually, homologous proteins share very similar structures. Therefore, given the amino acid primary sequence of an unknown structure and the experimentally solved structure of a homologous protein, homology modeling uses the known protein as a template to model the new structure, mutating each different amino acid in the solved structure. Whereas, when no homologous structures are available, protein threading endeavors to find a protein fold compatible with the model sequence.

The basic protocol followed by homology modeling approaches is made up of four steps: (i) a template or parent structure, related to the unknown one, is identified and the two sequences are aligned; (ii) the backbone coordinates of the well-conserved regions are borrowed from the template structure; (iii) non-conserved sequences, like loop or regions in which several amino acids have been inserted or deleted, are virtually modeled, using methods based on the knowledge of protein structure determinants; (iv) side chains are added, in according with the backbone modulation and the generation of a low-energy model.

Even when the three-dimensional structure of the pharmaceutical target has been determined through experimental techniques, such as X-ray diffraction, the computational modelers must face different structural problems: (i) hydrogen atoms position and (ii) water molecules orientation. In fact, being hydrogens too small to be clearly identified, these atoms must be computationally added and minimized, to avoid steric clashes and negative repulsive interactions. Similarly, only the water oxygen position can be unambiguously determined, so important water molecules must be optimized, in order to clearly investigate their potential role of protein-ligand bridges (Ladbury 1996; Cozzini et al. 2004). Another big problem is represented by the resolution of the crystallographic structure, which can significantly affect the quality of models and simulations. At high resolution values, lower than 1.5 Å, the model is probably more than 95 % a consequence of the observed data, while at lower resolution values, bigger than 2.5 Å, the model is much more subjective than widely appreciated (Davis et al. 2003; Kleiwegt et al. 1999). As a consequence, unless the resolution is high, the presence or absence of water molecules cannot be determined with certainty, and, sometimes, the addition of waters is used to artificially reduce the differences between observed and calculated structure-factors amplitude (Davies et al. 2003).

Usually, quick methods treat the receptor as a rigid object, thus neglecting any kind of protein flexibility. This represents one of the most common errors occurring in computational studies (Carlson 2002a). The binding of a ligand within an enclosed binding site, in fact, requires that part

of the receptor is flexible, in order to allow the ligand access. For this reason, several binding sites are characterized by large flexible regions, able to open and close, in according with molecule entrance or release (Carlson 2002b). The capability of properly modeling protein flexibility is also extremely important to understand the real inhibition activity of a drug. Exploiting the possible conformations induced by the ligand entrance in the binding site could prevent several prediction errors and the elimination of potentially active compounds (Teague 2003).

A case study: The estrogen receptor
(green boxes outline practical sessions)

To execute the following exercise, you are required to download a graphic molecular modeling program (e.g., Pymol, RasMol, RasTop, Swiss-PDB Viewer, or Chime), which are freely available on the web at the following addresses:

Pymol: http://pymol.sourceforge.net/
RasMol: http://www.bernstein-plus-sons.com/software/rasmol/
RasTop: http://www.geneinfinity.org/rastop/
Swiss-PDB Viewer: http://www.expasy.ch/spdbv/
Chime http://www.mdli.com/

Using one of the listed programs, you will be able to directly observe the reported protein structures, identify the critical amino acids, and observe the different shapes assumed by the flexible binding pocket of the studied protein. All structures are freely available on the Protein Data Bank web site (www.pdb.org).

The fundamental role played by estrogens in reproductive endocrinology has been known from the middle of the 20th century. Through the interaction with the two different receptors ERα and ERβ, belonging to the big nuclear receptor superfamily, estrogens participate in the regulation of the female reproductive system, and in particular, of the uterus, ovaries, and breast (Jordan 2003; Amari et al. 2004). Estrogens and progestins have been used as oral contraceptives, and are also applied in estrogen replacement therapy, to alleviate symptoms and urogenital atrophy, during menopause. Jensen and Jacobson hypothesized, in the late 1950s, that an estrogen receptor (ER), able to start all the biochemical processes associated with estrogen actions, should be present in the target tissues (Jensen et al. 1960; 1962). The ER was then isolated and characterized, and subsequently found also in breast tumors, providing the base for the development of antiestrogenic therapy as an alternative to ablative surgery. The first molecules developed to contrast the estrogen-simulated growth of breast cancer were tamoxifen (Wakeling et al. 1983) and raloxifen. These two molecules are currently on the market for the treatment of hormone-dependent breast cancer and for the prevention and cure of osteoporosis (Dutertre et al. 2000). The actions of estrogen and antiestrogen compounds are mediated by the interaction with ERα and ERβ, respectively identified in 1966 and 1999 (Toft et al. 1966; Gustafsson 1999). The two receptors share a similar size and also a similar structure, formed by a variable amino terminal region involved in transactivation processes, a central DNA binding domain (DNB), a ligand binding domain (LBD) characterized by a 53 % identity in ERα and ERβ, and a carboxy terminal region (Jordan 2003). When natural estrogens bind to the ERs, inducing the dissociation from heat shock protein, the dimerization, the binding to a specific DNA region, and the transcription of responsive genes.

Tamoxifen and raloxifen belong to the SERMs drug category (selective ER modulators), which comprises ligands able to act both as estrogen and as antiestrogen compounds. SERMs may, in fact, show agonistic or antagonistic activity, according to the cellular and promoter contest and also to the receptor isoform (Pike et al. 2001). It is generally accepted that different ligands induce

different conformational changes in the ERs, able to interfere with its ability to interact with other proteins, such as coactivators and corepressors.

The first attempt to model the structure of ERα was made by Höltje and Dall in 1993 (Höltje et al. 1993). The binding site was identified through homology modeling approach, comparing the ER sequence with the sequences of other steroid receptors and steroid binding proteins. The sequence between Leu379 and Met388 was identified as a region of particular interest. Analogous sequences were localized into several related proteins, like the progesterone receptor, the androgen receptor, the glucocorticoid receptor, the mineralcorticoid receptor, the steroid dehydrogenase, and the Na^+/K^+-ATPase. A conserved triptophan (Trp383) was identified in all the compared macromolecules. This residue could favorably interact with the hydrophobic scaffold of steroid skeleton, while Glu380, sometimes substituted by Gln380, could easily act as a hydrogen bond donor, capable of interacting with the estradiol hydroxyl group. These chemical and structural properties are usually shared by common SERMs. This sequence was identified as a part of the binding site arranged in an α-helix secondary structure. A second conserved region was located in the α-helix formed by the peptide comprised between Cys447 and Ser456. The two helixes were joined together, and the estradiol was docked into them. In the proposed model, Glu380, Trp383, Leu387 on the first helix and Lys449, Ile452, and Leu453 on the second, seemed to be really important for the complex formation.

- **Download from the Protein Data Bank the structure of ERα complexed with estradiol (1ere): observe the complex and the secondary structure of the receptor.**
- **Identify the region delimited by Leu379 and Met388.**
- **Identify the region delimited by Cys447 and Ser456.**
- **Localize the following residues: Glu 380, Trp383, Leu387, Lys449, Ile452, and Leu453.**

The ER case represents a typical example of computational failure. Four years later, in fact, in 1997, Brzozowski and co-workers identified through crystallography the real structure and the real binding pocket of the LBD, solving the structures of the receptor complexed with estradiol and raloxifen (Brzozowski et al. 1997). The LBD is formed by 2 β-sheets and 12 α-helixes, organized in 2 layers (H1-H4 and H7, H8, H11) surrounding a central core (H5, H6, H9, and H10). H12 flanks the three-layer motif and is characterized by high mobility. Estradiol occupies diagonally the binding site, forming two H-bonds with its two hydroxyl groups. The phenolic hydroxyl interacts with Glu353, Arg394, and a water molecule, the 17-β hydroxyl contacts His524 (Figs. 2a and 3a), while the lipophilic estradiol portion occupies the LBD hydrophobic core, formed by segments of helixes H3, H6, H8, H11, and H12. The recognition of the receptor and the stabilization of the complex are obtained through a strong chemical and structural complementarity between the ligand and the binding pocket.

- **Identify residues Glu353, Arg 394, and His524 in 1ere.pdb.**
- **Highlight the H-bonds formed by the estradiol molecules with these three residues.**
- **Identify the hydrophobic amino acids surrounding the hydrophobic core of the ligand: Ala350, Trp383, Leu346, Leu387, Leu391, Ile 424, Leu428, Leu525, Leu540…**
- **Compare the experimentally determined location of the binding site with the model proposed by Höltje and Dall in 1993.**

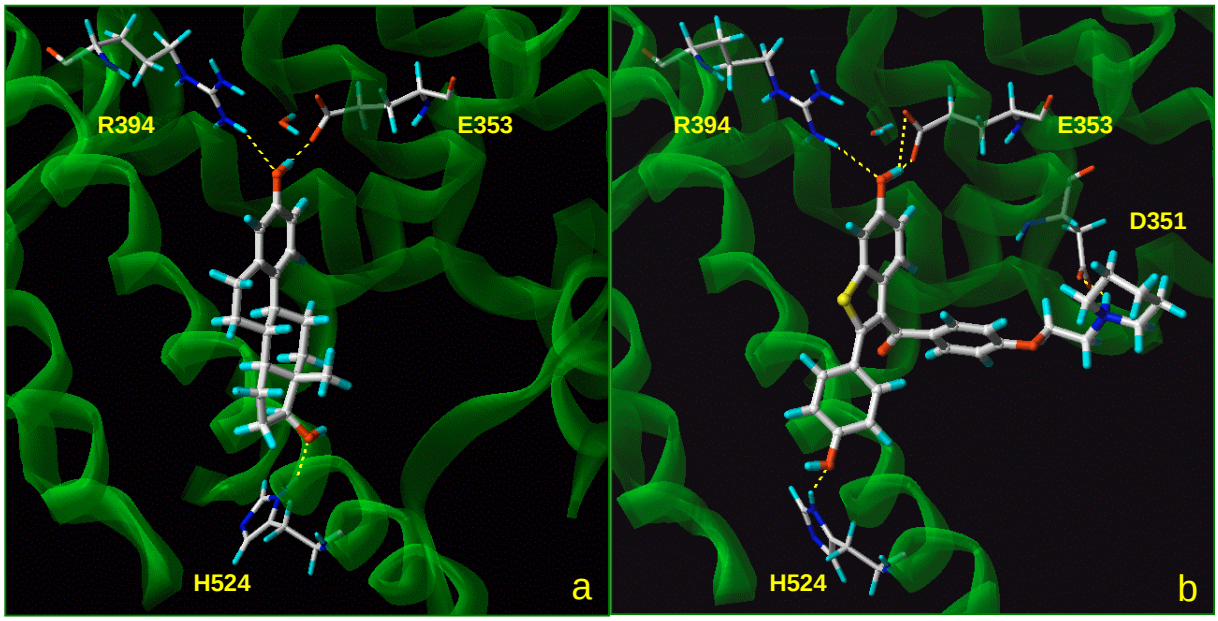


Fig. 1 (a) Estradiol in ERα binding pocket (1ere). (b) Raloxifen in ERα binding pocket (1err). The ligands are displayed in ball and stick, while the protein residues in capped stick. H-bonds are identified by yellow dotted lines.

Nevertheless, several cavities still remain unoccupied after the estradiol binding. It is, in fact, well known, that LBD, being extremely flexible, can accept a number of very different hydrophobic groups, adapting its shape to the ligand conformation (Anstead et al. 1997). The raloxifen lateral chain is, in fact, really too long to be contained in the estradiol binding pocket, thus, H12 is displaced from its natural position and forced to protrude from the pocket formed by H3 and H11. All steroidal and non-steroidal antiestrogen compounds, characterized by voluminous side chains, displace H12, inducing the same different binding pocket conformation (Fig. 2b).

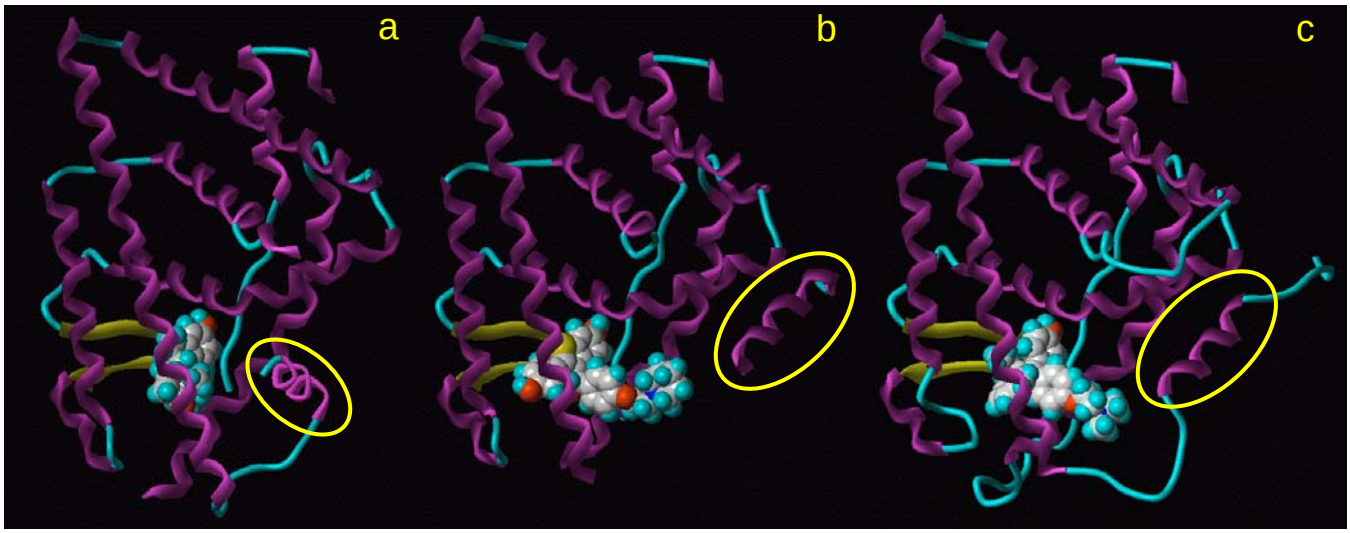


Fig. 2 (a) ERα complexed with estradiol (1ere). (b) ERα complexed with raloxifen (1err). (c) ERα complexed with 4-OH tamoxifen (3ert). The receptor is represented in ribbon tube, while the ligands are shown in spacefill cartoons. The helix12 is highlighted by yellow circles.

The antagonist properties of raloxifen and other antiestrogenic compounds are specifically based on the induced movement of H12, which disrupts the surface topography of AF-2, the LBD's transcriptional activation function, usually able to interact with putative transcriptional coactivatots, in a ligand-dependent manner (Beato et al. 1996; Cavailles et al. 1994). The coactivator recruitment

surface of LBD is, indeed, centered on helix12, thus, its movement and displacement after raloxifen binding, prevents the formation of a competent AF-2, capable of properly interacting with coactivators. In particular, the tissue selectivity of different inhibitors is based on their capability to occlude specific coactivator recruitment sites. Consequently, the binding mode of raloxifen is quite different from the position assumed by estradiol into the pocket, even though several contacts and interactions are maintained (Fig. 1b). One of the antagonist hydroxyl groups still interacts through H-bond with Gln353, Arg394, and a water molecule. Analogously, the second hydroxyl group contacts the side chain of His524, which is, however, displaced 5.1 Å from the position occupied by the 17-β hydroxyl in the complex between ER and estradiol. Moreover, an additional H-bond formed between Asp351 and the piperazine ring nitrogen is observable. The hydrophobic core of the compound is still involved in nonpolar contacts with several residues forming the binding pocket, as observed in the complex with the natural substrate (Brzozowski et al. 1997).

- **Download from the Protein Data Bank the structure of ERα complexed with raloxifen (1err).**
- **Identify the key residues Glu353, Arg 394, His524, and Asp351 and the bridging water molecule.**
- **Highlight the H-bonds formed by the raloxifen molecules with these four residues.**
- **Identify the hydrophobic amino acids surrounding the hydrophobic core of the ligand.**
- **Compare the structure of ER complexed with estradiol and with raloxifen and observe the movement of helix12.**

The structure of ERα complexed with tamoxifen has been solved in 1998 by Shiau and co-workers (Shiau et al. 1998). In this complex, helix12 is formed by the sequence comprised between residues 536 and 544. As observed in the ER-raloxifen complex, H12 occupies the part of the coactivator-binding groove formed by several residues located on H3, H4, and H5 and is not able to cover the ligand binding pocket as when an agonist is placed in it. The phenolic hydroxyl group still interacts with Glu353, Arg394, and the conserved water molecule previously identified in both ER-estradiol and ER-raloxifen complexes. Being the inhibitor lateral chain really large and bulky, is not completely contained in the binding pocket, but exits between H3 and H11 (Fig. 2c). The dimethylamino group, placed on the side chain, forms a salt bridge with Asp351, but there is no significant contact with His524.

The first antiestrogenic effect mediated by tamoxifen is due to the motion of helix12, precluded to assume the agonist-induced conformation, so a different LBD conformation is promoted by tamoxifen binding. In addition, the B aromatic ring of tamoxifen forces some of the residues forming the binding pocket to assume a conformation extremely different from that adopted in agonism presence. As a consequence, a new LBD conformation is stabilized. This conformation allows H12 to reach the static region of AF-2 surface, mimicking coactivator binding. These two conformational effects both contribute to tamoxifen antiestrogenic activity (Shiau et al. 1998).

The binding pockets of ERα complexed with raloxifen and tamoxifen are quite different in shape and geometry: a 1.58 Å r.m.s. was calculated on the overall structures. This is obviously due to the different inhibitor structures and conformations (Fig. 3) and to the different amino acids involved in the complex formation (Cozzini et al. 2004).

- **Download from the Protein Data Bank the structure of ERα complexed with 4-OH tamoxifen (3ert).**
- **Identify the relevant amino acids forming the binding pocket.**
- **Compare the structure of ER complexed with estradiol, raloxifen, and tamoxifen.**
- **Try to superimpose the complexes structures and observe the differences in receptor and ligand conformations.**

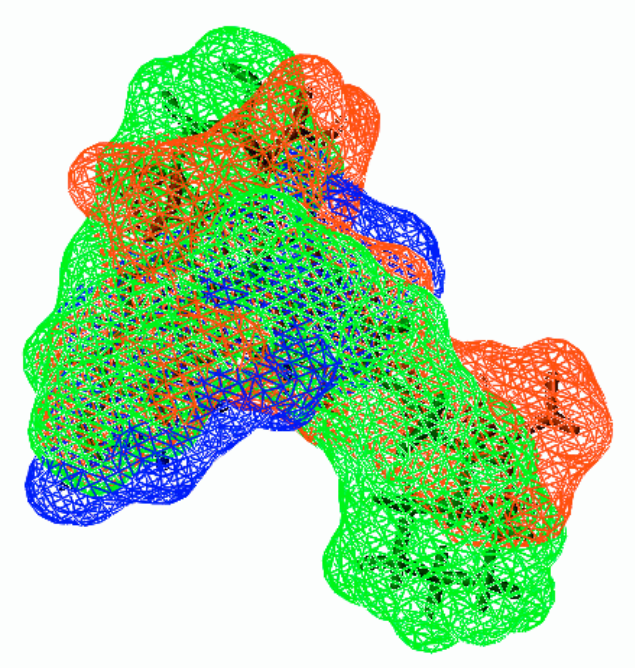
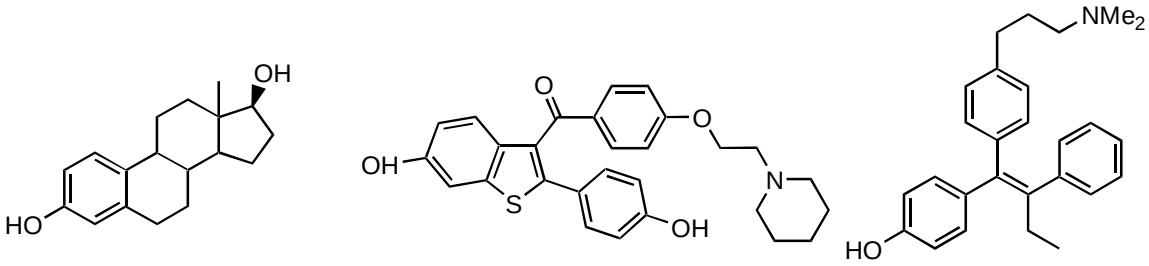


Fig. 3 Superimposition of estradiol (blue), raloxifen (green) and tamoxifen (red) placed into the ERα binding pocket. The contour surfaces represent the Connolly surface calculated for each molecule.

- **Search in the literature the structure of other ER agonists/antagonists and try to predict their activity, observing their structures and comparing them with estradiol, raloxifen, and tamoxifen, reported in Scheme 1.**



estradiol **raloxifen** **4-OH tamoxifen**

Scheme 1 Ligand compounds.

It is generally assumed that the ER flexibility and its capability to assume different conformational states represents the keys leading to transcription and protein production, or to selective transcription inhibition, when the binding pocket is occupied by antiestrogenic compounds (Luck et al. 2000). A three-state model has been proposed by Carlson and co-workers, to explain the ER mechanism of action (Carlson et al. 1997). The three different conformations have been recognized as a collapsed pocket without the ligand, a closed pocket with the ligand, and an open pocket where ligand exchange is very fast.

Recently, a second binding site has been identified in the ERα and ERβ LBD (Tyulmenkov et al. 2000). The residues forming this second binding pocket are Ile326, Leu327, Ile386, Phe445 in

ERα, and His 279, Val280, Val338, Tyr397 in ERβ. The differences in amino acids could be used to design subtype-selective compounds, unfortunately, until now, no obvious function seems to be attributable to this second pocket. Its presence and nature has been explained as an evolutionary remnant, when a larger ancestral binding site was modified to bind estradiol (van Hoorn 2002).

- **Observe Fig. 4 and try to identify the second binding site in 1ere.pdb.**
- **Identify residues Ile326, Leu327, Ile386, and Phe445.**

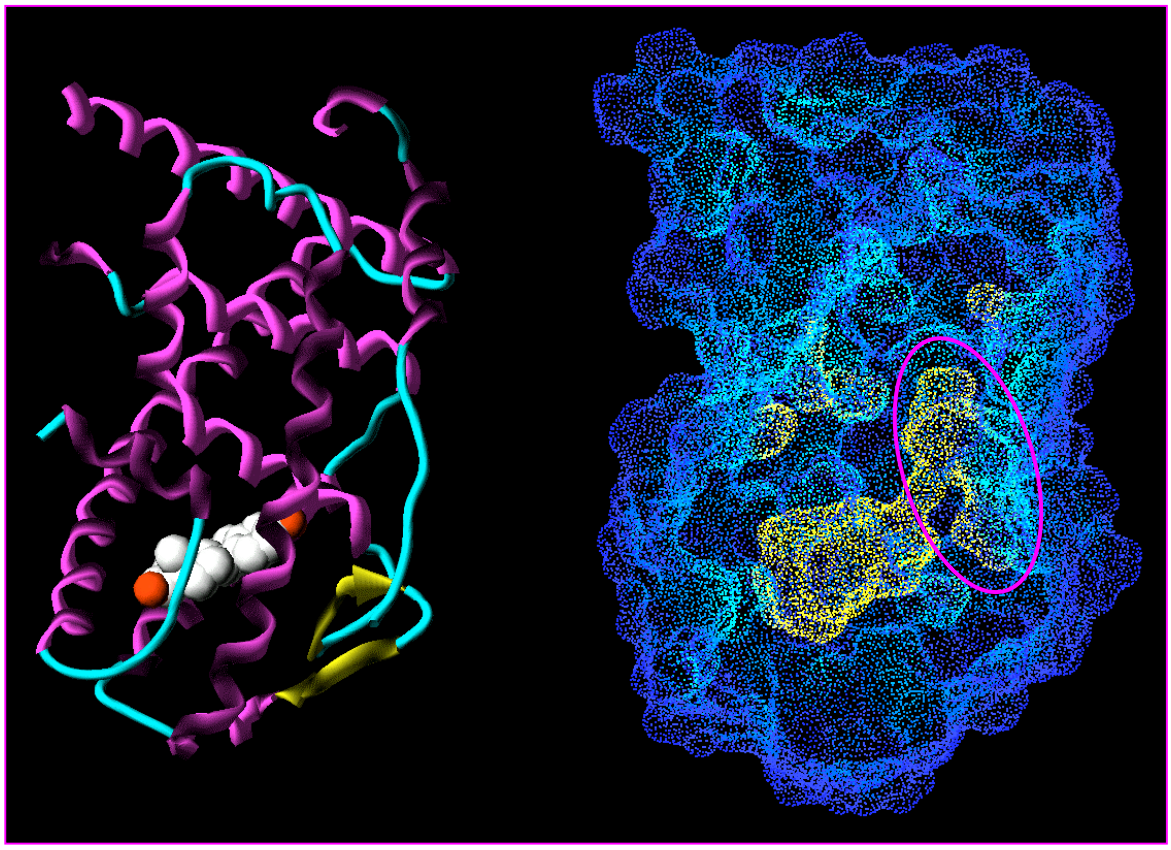


Fig. 4 ERα complexed with estradiol (1ere). **Left side: t**he receptor is displayed in ribbon tube, while the ligand, placed into the first binding pocket, is represented in spacefill cartoons**. Right side:** Connolly surface of the ERα receptor. External protruding regions are colored blue, while cavities and clefts are, respectively, colored green, yellow, and orange. The magenta circle locates the second binding site.

All these findings testify the importance of producing correct models and properly simulating flexibility, in particular when target molecules are characterized by extremely flexible binding sites. Moreover, a correct knowledge or prediction of protein flexibility could lead to an increase ing ligand affinity and could be really useful to understanding drug mechanisms of action, binding site location, and binding orientation, thus facilitating the design of new potential drugs.

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- See the papers by Cozzini et al. (Practice I.12) and Giurato et al.(Practice II.4) for further useful readings on log *P*.

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