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Ligand-based NMR spectra demonstrate an additional phytoestrogen binding site for 17β-hydroxysteroid dehydrogenase type 1

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ABSTRACT

The enzyme 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) has become an important drug target for breast cancer because it catalyzes the interconversion of estrone to the biologically more potent estradiol which also plays a crucial role in the etiology of breast cancer. Patients with an increased expression of the 17β-HSD1 gene have a significantly worse outcome than patients without. Inhibitors for 17β-HSD1 are therefore included in therapy development. Here we have studied binding of 17β-HSD1 to substrates and a number of inhibitors using NMR spectroscopy. Ligand observed NMR spectra show a strong pH dependence for the phytoestrogens luteolin and apigenin but not for the natural ligands estradiol and estrone. Moreover, NMR competition experiments show that the phytoestrogens do not replace the estrogens despite their similar inhibition levels in the *in vitro* assay. These results strongly support an additional 17β-HSD1 binding site for phytoestrogens which is neither the substrate nor the co-factor binding site. Docking experiments suggest the dimer interface as a possible location. An additional binding site for the phytoestrogens may open new opportunities for the design of inhibitors, not only for 17β-HSD1, but also for other family members of the short chain dehydrogenases.

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1. Introduction

Elevated levels of estradiol, the most potent human estrogen, stimulate the growth of breast cancer cells [1]. The enzyme responsible for the high levels of estradiol in malignant breast cells, 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) [2–4], is therefore an important target for drug discovery in breast cancer treatment. Several attempts have been undertaken to develop targeted and specific drugs for 17β-HSD1 [5–22], however, the lead compounds developed need further improvements. Naturally occurring phytoestrogens have been shown to inhibit several 17β-hydroxysteroid dehydrogenase types [23,24] and anticipated to become potential candidates in steroid-related cancer therapies. Understanding of the protein binding site and ligand binding mode is an important step in the design and optimization of new inhibitors. Previous work has been focused on two major classes of 17β-HSD1 inhibitors (Fig. 1), the steroidal compounds and non-steroidal compounds. The first class is based on the steroidal scaffold of estrone and estradiol. Compounds that belong to the second class are the phytoestrogens which have flavonoid and isoflavonoid scaffolds [23,24]. Compounds based on the steroidal scaffold were designed to fit into the cavity of the active site of 17β-HSD which is well characterized from crystal structures of 17β-HSD1 with estradiol [25,26], equilin [27] and a compound termed EM-1745 based on the estradiol scaffold and a substituent in the 16β position which points into the co-factor binding site [18,20]. Binding of different classes of inhibitors at the active site is also supported by docking studies [10,28]. Amongst them are the well-known phytoestrogen 17β-HSD1 inhibitors which were previously believed to bind to the estrogen binding site. However, to our knowledge there is to date no structure of a 17β-HSD1/phytoestrogen complex which would provide evidence in support of this assumption. Since crystallization of complexes has been difficult and NMR experiments observing protein signals were impossible considering the limited solubility of 17β-HSD1 and its tendency to aggregate, we have used waterLOGSY NMR spectra to study ligand binding. Ligand-based NMR experiments to characterize protein ligand interactions such as waterLOGSY [29,30], SALMON [31] and Saturation Transfer Difference (STD) [32] are commonly used for screening

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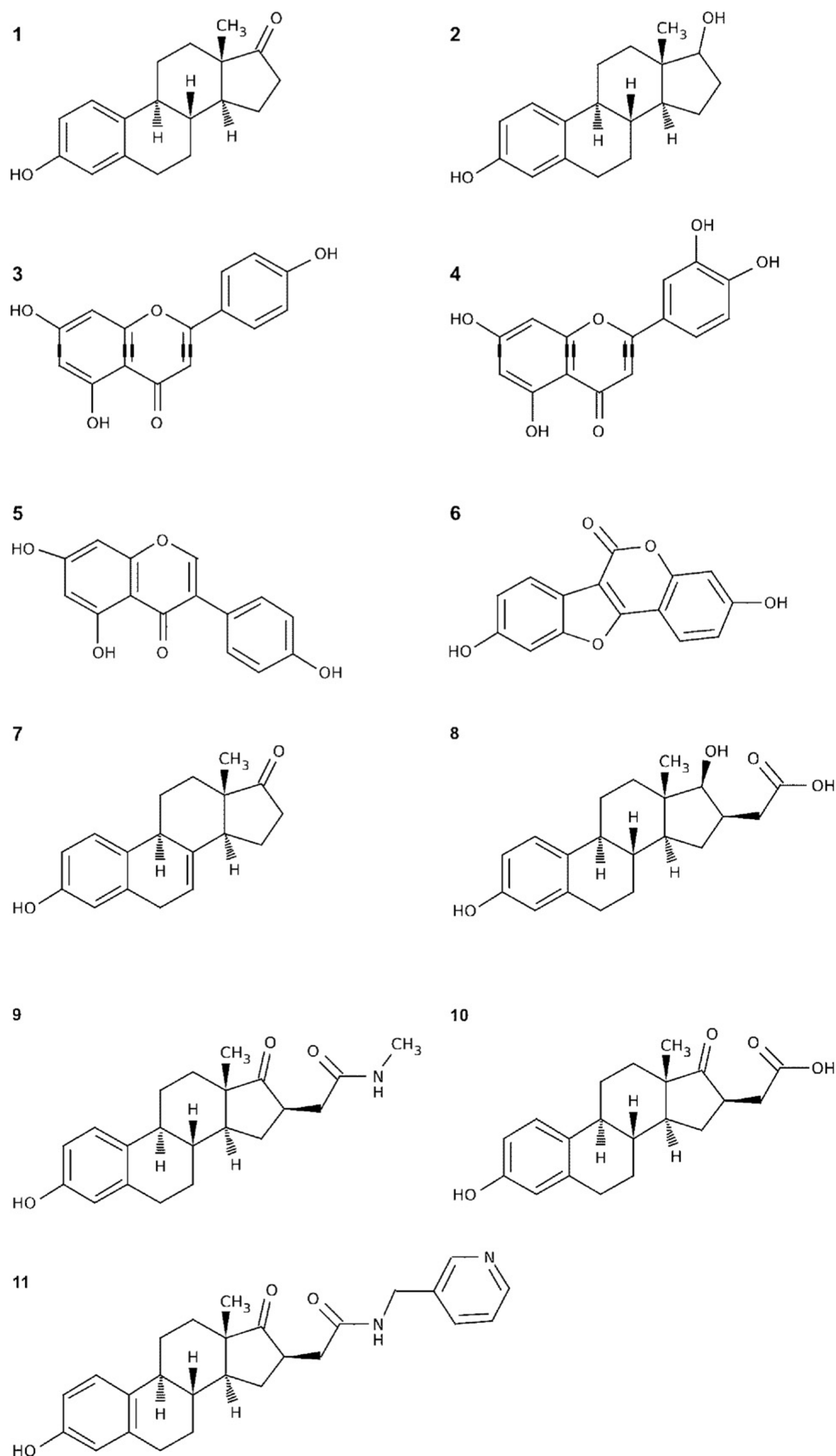

Fig. 1. Chemical structures of the natural ligands estrone (1) and estradiol (2), the phytoestrogens, apigenin (3), luteolin (4) and genistein (5), coumestrol (6), and the estrone derivative equilin (7), and of the steroidal inhibitors (8–11) (5, 21) designed to occupy the substrate and co-factor binding pockets.

and for the determination of ligand binding epitopes. These methods can directly monitor the protein ligand interaction without any interference from fluorophores or other types of labels on the ligand or the protein and are not limited by the size of the protein. Although these experiments do not provide direct information on the structure of the ligand binding site, they can be used to derive indirect information about the binding site from competition experiments between different ligands. Since these methods select resonances arising from protein bound inhibitors it is possible to distinguish uncompetitive vs competitive binding as the latter shows replacement of signals whereas uncompetitive binding shows spectra of both compounds in parallel.

In this study the binding characteristics of the natural ligands estrone and estradiol (1 and 2 in Fig. 1), the phytoestrogens apigenin, luteolin, genistein and coumestrol as non-steroidal inhibitors (3–6 in Fig. 1), and four estrone and estradiol derivatives as steroidal inhibitors (8–11 in Fig. 1) [5,21] were characterized. By recording waterLOGSY experiments for competing inhibitors and by studying the pH dependences of binding we probed for binding modes of both inhibitor types.

2. Materials and methods

2.1. Cloning, expression and purification

The sequence for 17β-HSD1 was generated using two primers: 5′-GCGGGATCCCTGGAG ACGTTGCAGCTGGA-3′ and 5′-GCCAAGCTTTTACTGCGGGG-CGCCCGGAGGATC-3′. The PCR product was cloned into a His6-tagged protein G (B1 domain) (GB1) [33] containing pET30a (+) vector between the BamHI and HindIII restriction sites. A fusion with an N-terminal GB1 domain was used to increase the solubility of the protein. GB1–17β-HSD1 was expressed in BL21-DE3 cells by induction with 0.5 mM IPTG for 16 h at 20 °C. Cells were resuspended in 50 mM sodium phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, pH 7.5 including protease inhibitors (c@mplete, Roche) and consecutively lysed using a french press at 30 kPsi. The lysate was spun in a Beckmann centrifuge, JA 25.50 rotor, for 1 h at 24 krpm. The supernatant was purified using a 20 ml anion-exchange Q sepharose HP column (Amersham). After extensive washing with 100 ml of resuspension buffer the protein was slowly eluted with 240 ml 50 mM sodium phosphate, 1 mM DTT, 1 mM EDTA, 20% glycerol, pH 7.5 with a gradient of NaCl ranging from 0 to 600 mM. GB1–17β-HSD1 eluted at approximately 300 mM NaCl. Further purification was performed by size exclusion using a Superdex 200 26/60 pg (Amersham). The running buffer was 50 mM sodium phosphate, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 20% glycerol, pH 7.5 and GB1–17β-HSD1 eluted at an early stage suggesting a molecular size larger than expected for the dimer.

2.2. Compounds

The steroidal inhibitors 8–11 were synthesized by M.S. (Phosphoenix, France) as previously described [5,21]. All other inhibitors were obtained from Sigma–Aldrich. Inhibitors were all dissolved in 100 mM stock solutions in DMSO.

2.3. In vitro inhibition assay

In 450 μl sodium phosphate buffer at pH 6.5, GB1–17β-HSD1 solution and ^{3}H-labeled estrone (PerkinElmer) were added to a concentration of 1 and 25 nM, respectively. The enzymatic reaction at 37 °C was started by adding 50 μl of the co-factor NADPH (5 mg/ml). The final concentration of the compounds estrone, estradiol, apigenin, coumestrol, luteolin, genistein, equilin, and steroidal inhibitors 8–11 [5,21] (Fig. 1) in the reaction mixture was 2 μM. The enzymatic reaction was stopped with 100 μl 0.21 M ascorbic acid in methanol:acetic acid 99:1 (v:v). The substrate and product were trapped on a pre-equilibrated Strata C18-E column (Phenomenex). The SPE column was washed with water before the substrate and product were eluted with methanol. The steroids were separated by HPLC using a Luna 5 μ C18(2), 125 mm × 4.00 mm column from Phenomenex, with a acetonitrile–water mixture (43:57) using a flow rate of 1 ml/min. Quantification of the substrate and product was achieved by online-scintillation counting of the radioactivity (ReadyFlowIII, Beckman, and Berthold LB506D). Percentage of inhibition for the individual compounds results from the difference between the amount of radioactive estradiol synthesized with and without inhibitor present in the reaction mixture [34].

2.4. NMR experiments

A modified waterLOGSY [31] pulse sequence which produced spectra void of difference artifacts was used for NMR data acquisition. WaterLOGSY spectra [29,30] of 10 μM GB1–17β-HSD1, 50 mM sodium phosphate, 10 mM NaCl, 0.05 mM DTT, 0.05 mM EDTA, 1% glycerol, 40% DMSO-d6 (Sigma–Aldrich), pH 6.8 or pH 8.9 with 500 μM compound were acquired on Varian INOVA 800 and 900 MHz spectrometers equipped with a cryogenically cooled probe. A mixing time of 750 ms was optimized for optimal signal intensity without contributions of the free ligand. For each spectrum 4096 data points at a spectral width of 15.6 ppm was recorded using 128 transients and a retention time of 4 s. Excitation sculpting with polychromatic pulses was used for multiple solvent suppression [35].

2.5. Docking experiments

Autodock 3.0 [36] was used to dock apigenin into the structure of a 17β-HSD1 homodimer [37] which comprises two estradiol ligands and two co-factor molecules. For the docking experiments one of the estradiol molecules was removed from the three-dimensional structure. The docking grid size was 30 Å × 30 Å × 30 Å and each grid point was separated by 0.375 Å and centered around the free binding pocket. A total of 10 docking attempts were performed and ranked by their calculated inhibition constants.

3. Results and discussion

3.1. Preparation of 17β-HSD1 for NMR experiments

The characterization of the protein binding site and binding modes is an important step in the design of new inhibitors. For 17β-HSD1 this has been difficult because the protein tends to aggregate and precipitate which limits crystallization and NMR spectroscopy. Nevertheless, a GB1 fusion construct was soluble and active in the *in vitro* assays and in an NMR assay even at higher DMSO concentrations (*vide infra*). HSQC spectra of ^{15}N-labeled protein were, however, not feasible because the protein aggregates to large soluble clusters of larger than 700 kD as determined by ultracentrifugation causing broad lines in NMR spectra. Nevertheless, the purified unlabeled protein could be used for ligand-based NMR experiments. The activity of the protein is largely preserved at DMSO concentrations of 10–20% thus facilitating ligand observed NMR experiments with inhibitors of low solubility in H_2O.

3.2. Probing interactions using an in vitro binding assay and by ligand-based NMR experiments

Known inhibitors of 17β-HSD1 bind either in the hormone binding pocket or in the co-factor binding site. Estrone, estradiol, equilin, phytoestrogens [38] and a series of previously published inhibitors with an estrone and estradiol scaffold (Figs. 1 and 2)

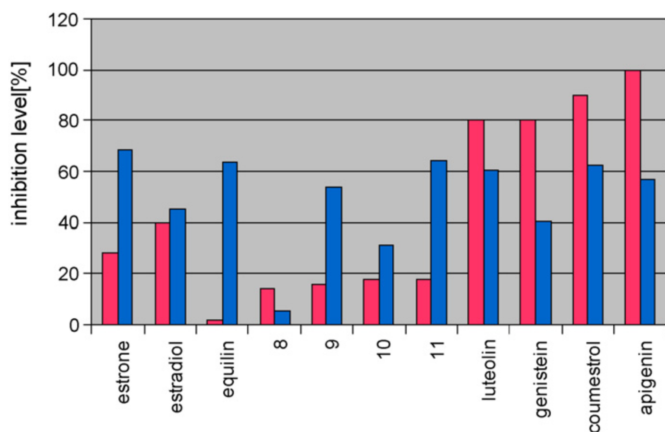

Fig. 2. Inhibition level (in % reduction of estradiol production) from the *in vitro* assay (blue bars) compared to signal intensity in waterLOGSY spectra (red bars). WaterLOGSY intensities were normalized to 100% for the strongest signal of apigenin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

[5,8,11,19–21,39] were used in the *in vitro* inhibition assays and waterLOGSY NMR experiments (Fig. 3). In the waterLOGSY experiment signals originate from magnetization transfer starting from water molecules. For ligands bound to proteins the sign of signals is opposite compared to those that do not interact with any protein. The intensity of signals in waterLOGSY experiments depends primarily on the life-time of the protein ligand interaction which is inversely correlated to the off-rate and the binding affinity ($k_{off} = K_d \times k_{on}$). Since the on-rate k_{on} is generally limited by diffusion the signal intensity in waterLOGSY spectra should predominantly be determined by the off-rate. For increasing affinities (decreasing off-rates) the signal intensity reaches a maximum as a consequence of more prolonged magnetization transfer before it decreases for higher affinities where the concentration of the free ligand becomes too low to observe an NMR signal. In waterLOGSY spectra recorded for several 17β-HSD1 ligand complexes the largest intensities were observed for the phytoestrogen apigenin (Fig. 3). Where spectra showed SALMON [31] effects arising from water accessibility the largest signal of the spectrum was used.

All the steroidal inhibitors 8–11 [5,21] (Fig. 1) designed to bind in the estrone binding pocket showed signal intensities in waterLOGSY spectra similar to those observed for the natural ligands estrone and estradiol. These interactions are also well established by docking studies [5,13]. Furthermore, the level of inhibition in the *in vitro* binding assay was similar to those measured for autoinhibition by unlabeled estrone and estradiol. This shows that the steroid analogue inhibitors 8–11 have similar affinity for 17β-HSD1 as the natural steroidal ligands. The hypothesis that steroidal inhibitors act as competitive inhibitors is also supported by a crystal structure of the complex of 17β-HSD1 with equilin [27], an estrogen analog from horses, which shows binding in the estradiol binding pocket.

Interestingly, compared to estradiol, estrone and the steroidal inhibitors 8–11, the phytoestrogens apigenin, luteolin, coumestrol and genistein showed approximately twice the waterLOGSY signal intensities. Considering the similar level of inhibition in the *in vitro* assay this apparent discrepancy suggests a variation of kinetic on- and off-rates compared to estradiol leading to similar dissociation constants (Fig. 3).

3.3. pH dependence of ligand binding

Besides the reductase activity at physiological pH, 17β-HSD1 also shows oxidase activity at higher pH where it converts estradiol back into estrone. We therefore studied ligand binding at the two pH values, pH 6.8 and pH 8.9. The signal intensities in the waterLOGSY spectrum for both estrone and estradiol were similar at pH 6.8 and pH 8.9 (Fig. 3) suggesting a similar affinity. However, waterLOGSY signal intensities for luteolin and apigenin show a significant difference in their pH dependence. For both phytoestrogens, luteolin and apigenin, we observe strong signals in waterLOGSY spectra at pH 6.8 but weak signals at pH 8.9 as exemplified for luteolin in Fig. 3. This suggests that these compounds bind to another more pH dependent binding site because a pH dependence of the NMR signals of the phytoestrogens is not likely.

3.4. Probing for a second binding site

To probe the possibility of a second binding site competition experiments were carried out between apigenin, luteolin, and estradiol. The choice of estradiol reflects the availability of structures [25,26] and the better solubility in aqueous solution of estradiol compared to estrone. Considering that all three compounds have similar affinities for 17β-HSD1, we expected displacement of resonances in waterLOGSY spectra for competing ligands. Fig. 4A shows that luteolin does indeed compete with apigenin as the signal of apigenin at 8.1 ppm almost disappears at equimolar concentra-

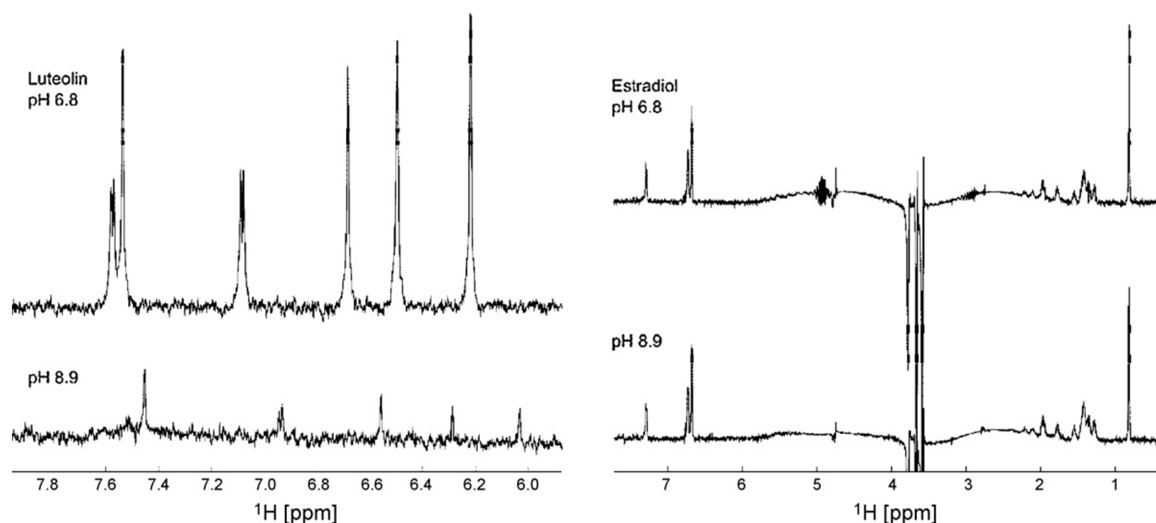

Fig. 3. WaterLOGSY spectra of luteolin and estradiol in the presence of 17β-HSD1 at different pH values showing pH dependence in intensities for luteolin but not for estradiol.

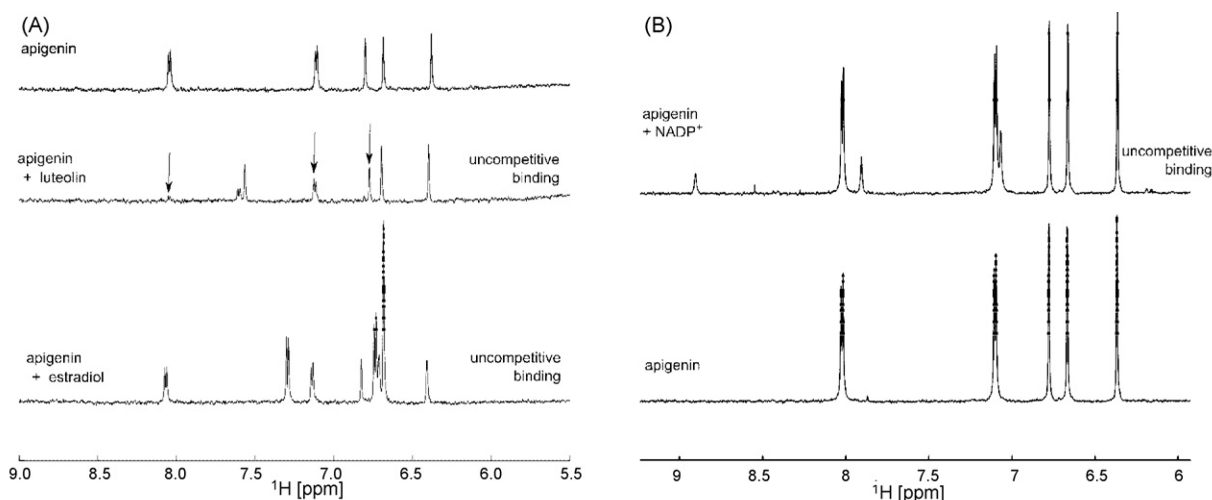

Fig. 4. (A) WaterLOGSY spectra of apigenin, apigenin + luteolin and apigenin + estradiol in the presence of 17β-HSD1. The upper spectrum was recorded with 500 μM apigenin in the presence of 10 μM 17β-HSD1. The middle spectrum shows the competition spectrum of 500 μM luteolin and apigenin where most of the apigenin signals show reduced intensity (arrows). (Bottom) Addition of equimolar amounts of estradiol to 500 μM apigenin does not show replacement owing to competition, all apigenin signals are still there. (B) WaterLOGSY spectra recorded for competition of apigenin and $NADP^+$. The lower spectrum shows 500 μM apigenin in the presence of 10 μM 17β-HSD1. The top spectrum shows the spectrum after adding an equal amount of 1 mM $NADP^+$. The signals corresponding to apigenin are identical in both spectra, some lower intensity signals arising from $NADP^+$ are observed in the presence of the co-factor.

tions of luteolin. However, when estradiol was added to apigenin in the presence of 17β-HSD1, the signal intensity of apigenin remained unchanged. At the same time the signals of estradiol were observed. These results strongly suggest that apigenin and estradiol bind simultaneously at different sites of the protein.

These results raise the question where another binding site might be located. One possibility could be the co-factor binding site, in particular if the protein has lost the co-factor during purification. In this case inhibition would be achieved by competing with the co-factor NADPH which is required for the reduction of estrone. To probe for this type of inhibition we recorded ^{1}H NMR spectra during the conversion of estrone to estradiol catalyzed by 17β-HSD1 in the presence of NADPH. This experiment showed conversion of estrone to estradiol for the methyl signal which has a different chemical shift for the two steroids and proves the activity of the protein under the conditions used for NMR experiments and that the co-factor can bind to its dedicated binding site.

If apigenin or luteolin were bound in the co-factor binding site they should be replaced by NADPH or by $NADP^+$ which would be reflected by displacement of signals in waterLOGSY spectra. However, the addition of the co-factor $NADP^+$ showed no changes for the signals of apigenin in the presence of 17β-HSD1 (Fig. 4B) but some additional low-intensity signals corresponding to $NAPD^+$, most likely as a consequence of the high affinity and low off-rate of the co-factor.

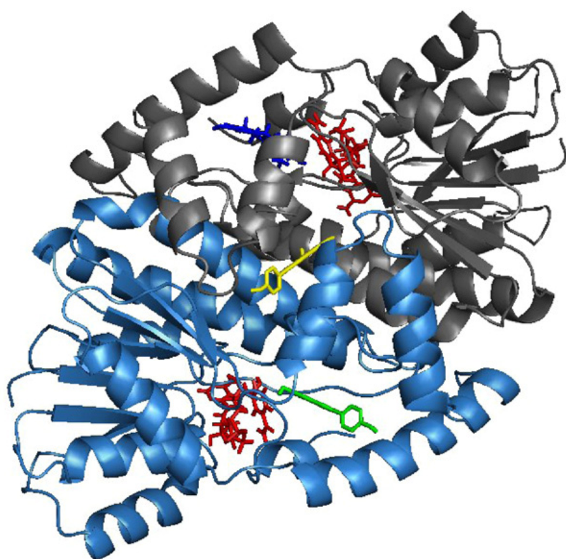

Fig. 5. Three dimensional structure of 17β-HSD1 (1FDU) (25) with estradiol (blue), apigenin (green and yellow) and $NADP^+$ (red). Apigenin can either occupy the estradiol binding (green) site or position at the interface of two 17β-HSD1 monomers (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.5. Computational docking

A computational docking approach was used to interpret these NMR results in structural terms, i.e. to evaluate where a putative phytoestrogen binding site might be located. For this purpose apigenin was docked into the dimer of the crystal structure using the Autodock software after removing estradiol from one of the two binding sites of the homodimer [37]. Out of ten docking attempts, eight showed apigenin bound in the estradiol binding site. However, in two out of the ten cases the apigenin molecule was found at the interface of the two protein subunits near the C-terminal α-helices (Fig. 5) suggesting a possible binding site in this region. The final intermolecular energy of the two complexes where apigenin was bound at the dimer interface has a similar intermolecular energy as the best orientation in the active site of the protein (see

Table 1
Docking results.

Conformation/location	Intermolecular energy [kcal/mol]	Internal energy of ligand [kcal/mol]	Torsional free energy [kcal/mol]
1/active site	−7.93	+2.45	+1.25
2/active site	−7.92	+2.45	+1.25
3/active site	−8.00	+2.87	+1.25
4/active site	−7.39	+2.51	+1.25
5/dimer interface	−8.91	+2.58	+1.25
6/dimer interface	−8.84	+2.57	+1.25
7/active site	−8.86	+2.49	+1.25
8/active site	−8.01	+2.54	+1.25
9/active site	−7.75	+2.45	+1.25
10/active site	−7.95	+2.44	+1.25

Table 1). This putative binding site is further supported by higher B-factors for residues in its vicinity [37] reflecting additional mobility. Increased flexibility at this binding site would also explain the higher intensity of waterLOGSY signals reflecting higher off-rates.

4. Conclusions

Although further investigation is required to confirm the location of an additional binding site in 17β-HSD1, waterLOGSY competition experiments and the pH dependency of signal intensities in waterLOGSY spectra strongly emphasize the existence of different 17β-HSD1 binding sites for phytoestrogens and steroids. Docking calculations provide a plausible rationale for the observed effects in ligand binding suggesting an additional binding site at the dimer interface. These observations are likely to open new avenues for the design of 17β-HSD1 inhibitors as breast cancer therapeutics which simultaneously could target the different binding sites of the protein.

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