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Food flavonoid ligand structure/estrogen receptor-α affinity relationships – toxicity or food functionality?

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ABSTRACT

In silico molecular modelling is used to study interactions between flavonoid phytoestrogens and estrogen receptor (ER)α. Twenty flavonoids from foods were studied; e.g., genistein from soy, naringenin from grapefruit, phloretin from pears, chrysin from oyster mushrooms. These potential ligands' molecular attributes and their spatial arrangements that favour binding to the ligand binding cleft (LBC) of ERα are identified, and Docking Scores calculated. The Docking Score order is the same as the estrogenicity order for 8 of the flavonoids studied in detail. The number and position of flavonoid ring hydroxyls influence the Docking Scores which might relate to ERα's bio-activity. Hydrophobic interactions between ligands and ERα are also important; the number of rotatable C–C bonds in ligands likely affects the magnitude of hydrophobic interactions and ligand fit. Our findings suggest that flavonoids with diverse structural features could have different binding energies and binding affinities with ERα, which might confer different functionalities and toxicities.

1. Introduction

A better understanding of the molecular mechanisms underlying food functionality is crucial to our knowledge of potential health risks and benefits. However, such studies are often time consuming and expensive. Therefore, the application of *in silico* modelling studies to investigate receptor-mediated mechanisms might be useful in exploring food component functionality.

In this paper, we will focus on estrogen receptor (ER)-mediated mechanisms of food functionality and the use of *in silico* molecular modelling (Schrödinger platform) to give insights into the potential benefits and risks of estrogen-mimicking (xenoestrogen) food components.

ERs occur as two isoforms (ERα and ERβ) and are present in many cell types (Heldring et al., 2007); e.g. ERα predominates in mammary cells and ERβ in colon cells (Kuiper et al., 1997). ERs are important in growth and development (Mosselman et al., 1996) with functions far exceeding the conventional concepts of sex hormone receptors (Ye et al., 2018). The most potent natural ligand of ERs is 17β-estradiol (E2, Fig. 1) and when bound to the receptor it initiates a sequence of events that leads to regulation of genes involved in growth and development and expression of sexual characteristics (Reid et al., 2002). In order to activate ERs the ligand ideally has two hydroxyl groups, which hydrogen bond to amino acid residues in the ligand binding cleft (LBC), separated by 9.6 Å of hydrophobicity (Brzozowski et al., 1997). Molecules with similar molecular attributes (i.e. estrogen mimics) activate ERs, but to a lesser extent than E2. For example, the estrogen mimic genistein (a phytoestrogen; Fig. 1) from soy has a hydroxyl separation of 11.6 Å: genistein has a relative estrogenicity of 3.9×10^{-5} (where E2 = 1) (Berckmans et al., 2007) because its ER binding characteristics are not ideal.

Flavonoids are important food components; they include subclasses based on their chemical structures; for example, flavone, flavonol, and isoflavone (Table 1). Flavonoids are present in many commonly consumed fruits, vegetables and herbs (e.g., apigenin in parsley, quercetin in red onion, genistein in soy, bergamottin in grapefruit) (Bailey et al., 2003), and can mimic E2 (Fig. 1) and thus activate ERs (Harris et al., 2005). The binding affinities of both E2 and estrogen mimics to ER isoforms are different this means that they will have differential tissue-based biological activity which is dependent on the distribution of ER isoforms in the tissue (Kuiper et al., 1997). Interestingly, ERβ (predominates in colon cells) (Kuiper et al., 1997) provides a more suitable binding environment for flavonoids and so might play an important role in flavonoid-containing colon-mediated food functionality.

The intimate interactions between flavonoids and ERα have been more extensively studied than their ERβ interactions because of the dominant role of ERα in some important diseases (Ye et al., 2018) including breast cancer (more than 80% of human breast cancers are ERα

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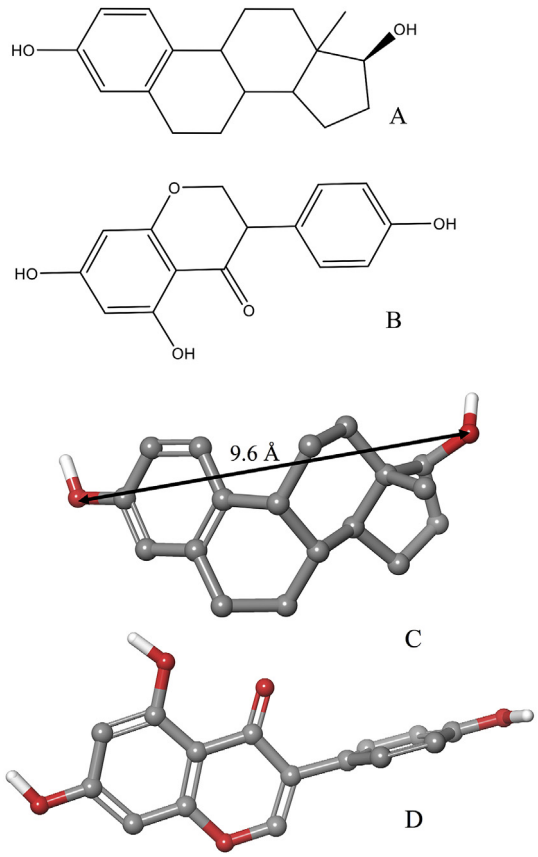


Fig. 1. Top: Two-dimensional structures of E2 (A) and genistein (B) showing that E2's two hydroxyl have approximately the same hydrophobic separations as 2 of genistein's hydroxyls. Bottom: three-dimensional structures of E2 (C) and genistein (D) to show the spatial alignment of their hydroxyl groups. These commonalities of special arrangement of hydroxyls explain why genistein is an estrogen mimic.

positive) (Turner et al., 2015), atherosclerosis (Evangelopoulos et al., 2003), ovarian cancer (Lu et al., 2006), and ERα's potential role in diabetes because of its role in regulation of glucose homeostasis (Bryzgalova et al., 2006).

Since there is more structural information (e.g., X-ray crystallography) (Brzozowski et al., 1997); for ERα than ERβ, we are able to construct a good ERα *in silico* model – for this reason we have focused our food *in silico* functionality molecular mechanism studies on ERα/food flavonoid interactions.

In the context of receptor (e.g., ERα)-mediated biological activity, the structure-affinity relationship is the interdependency between ligand structures and receptor binding affinity (i.e. ligand/receptor fit) (Ye et al., 2018). Structure-affinity relationships are good predictors of biological activity (including toxicity) and are useful screening methods to select potential new receptor-mediated compound for pharmaceutical development (Hall et al., 2003). This has led to *in silico* studies becoming a key tool in drug discovery and development (Ekins et al., 2007) - our studies extend this thinking to food functionality.

Flavonoids are important food components that impart significant biological activity including estrogenicity, which is a key factor in food functionality (e.g., isoflavone-rich bread is used as partial 'hormone' replacements in peri- and postmenopausal women (Simons et al., 2000)). Further to this, Zhang et al. (2018) studied both ERα interactions *in silico* and biological effects in a cultured cell system of coumarins and meroterpenes from *Cullen corylifolium* (a plant used in Chinese herbal medicine). They showed that the 6 compounds studied (psoralen, angelicin, trioxsalen, psorilidin and bakuchiol) interacted with the ERα LBC in *silico* studies and were estrogenic in luciferase-based ERα cell assays. They concluded that a better fit to the ERα LBC and a greater estrogenicity was associated with longer molecules with hydroxyl groups that could interact with the LBC by forming hydrogen bonds.

In this paper, we report the structure-affinity relationship between ERα and flavonoid ligands *in silico* (using the Schrödinger platform). We use these findings to better understand flavonoid estrogenicity and, importantly, the estrogenicity changes that might result following flavonoid metabolism (e.g., by the gut microbiome); we discuss our findings in the context of food functionality and toxicity.

2. Experimental

2.1. In silico modelling studies

The molecular docking studies were carried out using Schrödinger Small-Molecule Drug Discovery Suit 2017–1 (Schrödinger LLC, New York, USA) to determine the likelihood that the studied molecule (ligand) would bind to the LBC of ERα. Maestro (Schrödinger Release, 2017–1: Maestro, Schrödinger LLC, New York, NY, 2017) was used as the interface for preparing ERα and ligands for study.

2.2. Protein preparation

The X−ray crystallographic coordinate of ERα were taken from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (http://www.rcsb.org), the X-ray crystal structures of ERα complexed with ligands (e.g., genistein in PDB entry 1 × 7R) were used. Protein fragment crystals are derived from different polypeptides of the ERα protein according to the particular preparative methodologies. This means that they might have different polypeptide chains and each chain has a docked ligand (e.g. GEN). One of these chains was arbitrarily used as the ligand–receptor subunit for these docking studies, others were deleted. Missing amino acid residues (Ser 305, Tyr 331, Asp 332, Pro 333, Thr 334, Arg 335, Pro 336, Phe 337, Arg 548, Leu 549) based on ERα's primary sequence were added to complete the protein structure using the Schrödinger 'Prime' command. Besides the ligand, a single water molecule hydrogen bonded to Arg 394 and Glu 353 was kept because it is important in ligand binding) − other water molecules (e.g., solvent water) were removed (Manas et al., 2004). Restrained Minimization was run to provide controls for optimizing the corrected structure, relieving any strain and fine − tuning the placement of specific groups (e.g., hydroxyls). Hydrogen atoms were always optimized fully, which allows relaxation of the H-bond network and alleviates potential steric clashes by user–selected root–mean–square deviations (RMSD) with a tolerance of 0.3 Å (Ignatowicz and Baer-Dubowska, 2001).

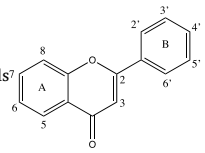
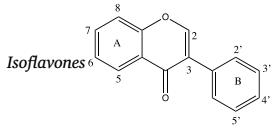
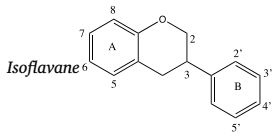
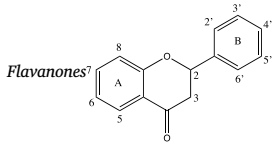
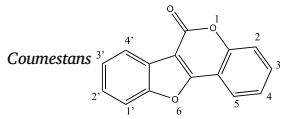
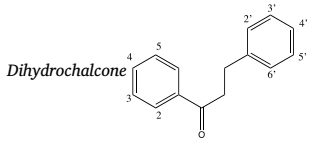
2.3. Receptor Grid Generation

The receptor grid for docking studies was set up and generated from the Receptor Grid Generation panel of Glide (Schrödinger Release, 2017–1: Glide, Schrödinger LLC, New York, NY, 2017). The natural LBC was used for these docking studies, but the original bound ligand (e.g., GEN) was excluded; this determines the position and size of the active site (i.e. the LBC) for ligands docking.

2.4. Preparation of ligands

In this study, 20 flavonoids from different subclasses were selected as potential ligands for ERα. They are primuletin, primetin, chrysin, apigenin, norwogonin, acacetin, scutellarein, diosmetin, artocarpetin, 6-hydroxyluteolin, kaempferol, genistein, glycitein, daidzein, formononetin, biochanin A, naringenin, coumestrol, phloretin. These ligands were built and prepared for docking studies using LigPrep (Schrödinger Release, 2017–1: LigPrep, Schrödinger LLC, New York,

Table 1
The 20 flavonoids used in the Schrödinger platform docking studies.

Subclass	Compound	Substitution position	DockingScore (kcal/mol)	H-bond value* (kcal/mol)	HER# (kcal/mol)
Flavones & Flavonols 	Primuletin	5 (−OH)	−8.13	−1.1	−2.93
	Primetin	5, 8 (−OH)	−8.47	−1.44	−2.82
	Chrysin	5, 7 (−OH)	−8.5	−1.50	−2.7
	Apigenin	5, 7, 4′ (−OH)	−8.64	−1.6	−2.7
	Norwogonin	5, 7, 8 (−OH)	−8.37	−1.44	−2.62
	Acacetin	5, 7, (−OH); 4′ ($-OCH_3$)	−8.58	−1.1	−2.5
	Scutellarein	5, 6, 7, 4′ (−OH)	−9.04	−2.06	−2.2
	Diosmetin	5, 7, 3′ (−OH); 4′ ($-OCH_3$)	−8.47	−1.5	−2.4
	Artocarpetin	5, 3′, 4′ (−OH); 7 ($-OCH_3$)	−7.56	−2.01	−2.3
	6-Hydroxyluteolin	5, 6, 7, 3′, 4′(−OH)	−9.37	−3.36	−2.2
	Kaempferol	5, 2, 7, 4′ (−OH)	−8.81	−2.38	−2.2
Isoflavones 	Genistein	5, 7, 4′ (−OH)	−9.3	−1.69	−2.7
	Glycitein	7,4′(−OH); 6 ($-OCH_3$)	−9.09	−1.6	−2.7
	Daidzein	7, 4′(−OH)	−9.0	−1.3	−2.8
	Formononetin	7(−OH); 4′($-OCH_3$)	−8.7	−0.78	−2.8
	Biochanin A	5, 7, (−OH); 4′ ($-OCH_3$)	−8.9	−1.1	−2.7
Isoflavane 	*S*-equol	7, 4′ (−OH)	−10.44	−1.3	−2.8
Flavanones 	Naringenin	5, 7, 4′ (−OH)	−10.2	−1.44	−2.3
Coumestans 	Coumestrol	3, 2′ (−OH)	−10.4	−1.04	−2.7
Dihydrochalcone 	Phloretin	2, 4, 6′, 4′ (−OH)	−8.3	−2.66	−0.7

NY, 2017); at most 32 ligand poses were generated.

2.5. Ligand docking, calculations and variability

Rigid-receptor-flexible-ligand docking calculations were performed using Glide in extra precision (XP) mode. Each ligand was conformationally sampled in the LBC, and each pose was scored in terms of its Emodel, which is used for selecting the "best" pose (with lowest-Emodel) of a particular ligand. Glide uses a scoring function (DockingScore) to predict the binding energy then evaluates and semi-quantitatively ranks the binding affinities of potential ligands with their target receptors in a specified conformation (e.g., 1 × 7R of ERα) (Friesner et al., 2004, 2006). DockingScore is an empirical scoring function; it is used to predict the ligand binding free energy and rank different candidate ligands in order of their binding affinities (Friesner et al., 2004). DockingScore is built up from many components, including hydrogen bond (H-bond) value and hydrophobic enclosure reward (HER); these are contributions and factors rewarding or penalizing interactions known to influence ligand binding. In the Glide scoring function used for our docking studies, the optimum distance between two hydrophobic molecules that facilitate hydrophobic interaction with the LBC is 0.5 Å, but if the distance between a ligand's atom (s) and the target atom of an amino acid residues is over 3 Å their hydrophobic interaction (if any) will not be accounted for (Friesner et al., 2006). The DockingScores of ligands were used to predict the order of binding affinities of docked ligands with the receptor. H-bonds and π−π interaction are shown using a Ligand Interaction Diagram.

2.6. DockingScore variability

To check DockingScore variability, 1X7R was prepared, including adding missing amino acid residues as described previously and assigning the rotatable amino acid residue Thr 347). The ligands genistein, phloretin and coumestrol were prepared, docked into the prepared 1X 7R, and DockingScores determined. These ligands were chosen because they have key structural docking differences: genistein is an isoflavone with a single rotatable bond, coumestrol is a coumestan with a more rigid molecular skeleton than genistein, and phloretin is highly flexible. The docking process was run 3 times including separate ligand preparations for each run and DockingScores determined for each run.

3. Results and discussion

3.1. Properties of the ERα binding pocket (1X 7R) – key structural features of genistein that facilitate binding to the LBC

Fig. 2 (a) shows the overall architecture of ERα's LBC - it is formed by parts of helix (H)3 (Met 342 to Leu 354), H6 (Trp 383 to Arg 394),

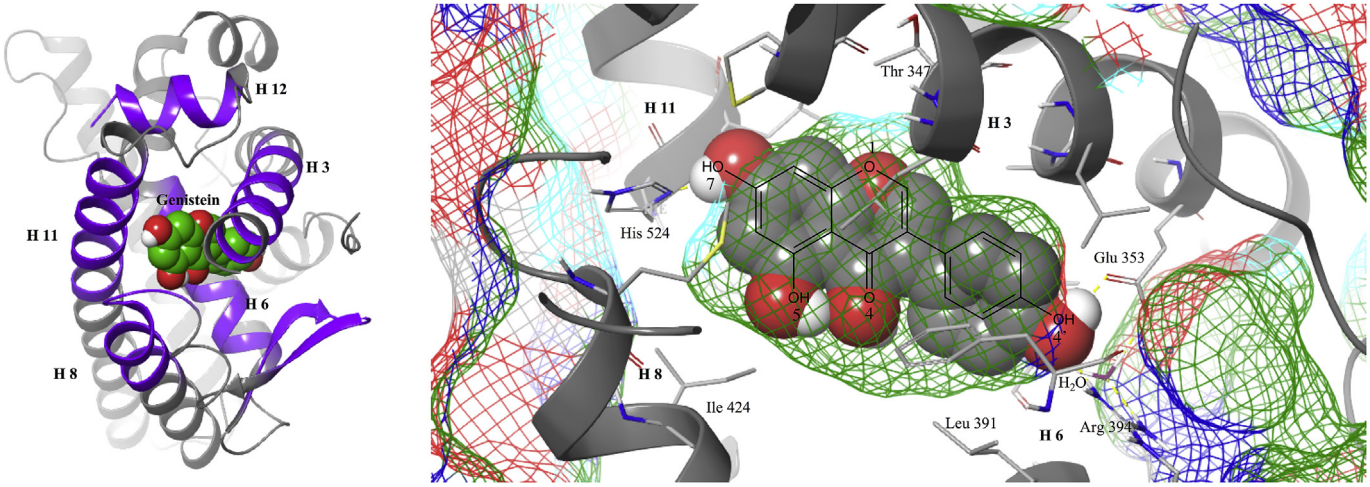


Fig. 2. Structure of estrogen receptor α derived from X-ray crystallographic data (PBA entry 1X7R). (a) Helices represented as ribbons; the important amino acid residues in the ligand binding cleft (LBC) are highlighted in purple; the ligand (genistein) shown in Corey-Pauling-Koltun (CPK) representation. (b) Detail of the LBC of 1X7R. Helices represented as ribbons; amino acid residues shown as sticks; ligand (genistein) shown as CPK representation with a 2D representation superimposed. The topographies of the amino acid residues are shown as coloured meshes to highlight their properties: blue, positive charge; red, negative charge; cyan, polar; green, nonpolar. Yellow dashed lines represent hydrogen bonds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

H8 and its preceding loop (Val 418 to Leu 428), and H11 (Met 517 to Met 528). Fig. 2 (b) shows a more detailed view of the ligand receptor interactions, including hydrogen bonds (yellow dashed lines) between the 7-hydroxy group of genistein and His 524 and the 4′-hydroxy group of genistein and Arg 394, Glu 353 and water. These high bond energy interactions likely lock the genistein ligand in place. The hydrophobic region of the LBC comprises hydrophobic amino acid residues (e.g. Ile 424, Leu 391) (shown as meshed green in Fig. 2). Once locked in place the hydrophobic areas of the ligands probably interact with the hydrophobic region of the LBC via hydrophobic (e.g., van der Waal's) interactions. The energy of both the hydrogen bonds and the hydrophobic interactions initiate the receptor conformational changes which lead to its biological activity (Ye et al., 2018) Therefore, it is likely that the magnitude (in an energy context) of interactions between a ligand and the LBC influence the degree of estrogenicity because the receptor conformational change leads to biological effects. Genistein's 5-hydroxy and 4-keto groups do not favour interaction with the adjacent LBC hydrophobic binding environment (Fig. 2 (a)) because of their δ-negativity due to their electronegative oxygen atoms; this might reduce genistein's possible interactions with the hydrophobic amino acids present in the hydrophobic region of the LBC. This means that genistein is not a perfect fit for ERα′s LBC which might explain why genistein is less estrogenic than the 'natural' ligand E2. Interestingly, the polar Thr 347 residue (Fig. 2(b)) within the LBC's hydrophobic region is available to form a hydrogen bond with an appropriately structured ligand which might increase its binding affinity. In addition, Fig. 2(b) shows that the molecular dimensions of genistein fit well into the receptor – i.e. it is a 'good' ligand. However, there is still available space between genistein and H3 and H6; therefore, a potential ligand with a bigger hydrophobic skeleton could perhaps better utilize this space, thus maximizing interactions with hydrophobic amino acid residues in the LBC which, in turn, might increase its binding energy. This is consistent with Zhang et al. (2018)'s findings which show that the binding affinity of ligands to ERα is related to their molecular length.

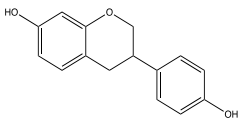
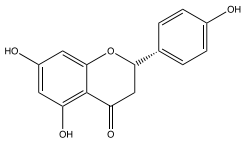
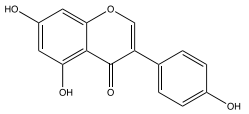
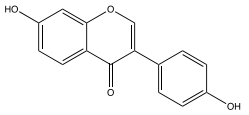
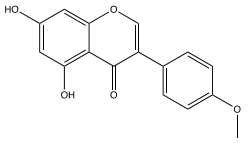
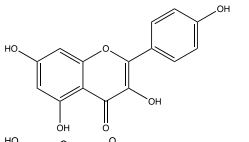
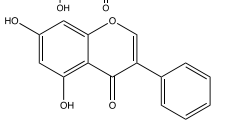
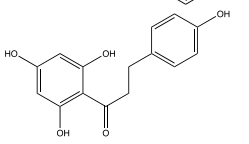
The reliability of Shrödinger predictions of binding energies is a very important consideration when attempting to extrapolate from *in silico* studies to biological effect scenarios. Friesner et al. (2004) showed a good relationship between Shrödinger scoring functions (mathematical functions used to calculate binding energy) and ERα *in vitro* binding affinity which augurs well for the accuracy of Shrödinger predictions.

3.2. Estrogenic activity of food flavonoids and their DockingScores

Flavonoids are present in many foods (e.g., genistein in soy) and dietary supplements (e.g., food phytoestrogen supplements); many are estrogen mimics - they comprise part of the complex dietary cocktail that humans are exposed to daily, and are possibly responsible for the functionality of some foods (Waring and Harris, 2005). Flavonoid phytoestrogen are thought to have significant biological effects at the population level (e.g., reduced sperm quality (Lim and Shaw, 2016)). For this study, 19 flavonoids from different subclasses with structural diversity were selected for molecular modelling studies (Table 1). The selected flavonoids fall into five subclasses which have different key structural components, including their molecular skeletons which form the hydrophobic cores of the individual ligands and determines the separation of important LBC binding groups (e.g., −OH). Within each subclass of the flavonoids the individual compounds have varying numbers and distributions of hydroxyl and methoxy groups. These structural differences contribute to the different H-bond values, HER, and thus different DockingScores. The DockingScore predicts the binding energy of ligands, which relates to ligand binding affinity with the receptor; this, in turn, might relate to the ligand's biological activity (e.g., estrogenicity).

Over 5000 naturally occurring flavonoids have been characterized from plants (Ullah and Khan, 2008). However, surprisingly few have been studied using functional assays (e.g., MCF-7 proliferation) to determine, for example, estrogenicity (Ververidis et al., 2007). Table 2 shows structures, DockingScores and published estrogenicity values from a single published study (Breinholt and Larsen, 1998) for the 8 flavonoids we studied. Having the estrogenicity values from a single study reduces data variability which might occur when studies are carried out in different laboratories. Their molecular structures show the two key structural features: namely, hydroxyl groups and a hydrophobic backbone which have similar structural and spatial arrangements to E2's key binding moieties. These two crucial structural features facilitate interactions with the LBC by hydrogen bond formation and hydrophobic interactions. Interestingly, the order of estrogenicity of the compounds studied is the same as the order of their DockingScores (Table 2). This indicates a potential association between the ligand/receptor docking energy (i.e. DockingScore) and estrogenicity (Breinholt and Larsen, 1998; Matsumoto et al., 2004).

Table 2
Estrogenicity and Docking Scores for 8 selected flavonoids[a].

Compounds	Examples of food sources	Structure	Relative estrogenicity[b]	DockingScore (kcal/mol)
***S*-equol**	Gut microbiome-mediation		2.3×10^{-2}	−10.44
Naringenin	Grapefruit, oranges		7.8×10^{-3}	−10.2
Genistein	Fava beans, soybeans		4.5×10^{-3}	−9.3
Daidzein	Soybeans		2.8×10^{-4}	−9
Biochanin A	Peanuts, chickpea, soy		2.5×10^{-4}	−8.9
Kaempferol	Apple, potato, blackberry		1.1×10^{-4}	−8.81
Chrysin	Oyster mushroom		2.5×10^{-4}	−8.5
Phloretin	Apple and pear skin		9.4×10^{-5}	−8.3

[a] Data from Breinholt and Larsen (1998).
[b] Percentage EC_{50} for E2 relative to EC_{50} for test compounds.

3.3. DockingScore variability

DockingScore values determined following 3 separate ligand docking runs for genistein, coumestrol and phloretin were identical for each ligand. This means that there is no variability between these parameters following rigid docking studies.

3.4. H-bond values determine a ligand's binding to ERα

H-bond (i.e., the total hydrogen-bond energy between a ligand and the corresponding receptor in the Shrödinger system) is the sum of the individual hydrogen bond energies. H-bond values are affected by the geometry of individual hydrogen bonds, based on both the angles and distances between the donor and acceptor atoms in the bond (Eldridge et al., 1997).

There are two interesting trends in the estrogenicity and DockingScore data in Table 2. Firstly, the ligands which have more phenolic hydroxyls have larger H-bond values. This is likely due to the increased number of hydrogen bonds formed between the ligand's hydroxyl groups and ERα's LBC amino acid residues. This is exemplified by the following flavones which have different numbers of hydroxyls and differential H-bond values: primuletin (monohydroxy, −1.1 kcal/mol), primetin (dihydroxy, −1.4 kcal/mol), apigenin (trihydroxy, −1.6 kcal/mol) and two isoflavones, daidzein (dihydroxy, −1.3 kcal/mol) and genistein (trihydroxy, −1.69 kcal/mol). Furthermore, genistein has higher estrogenic activity than daidzein (Table 2) which suggests that H-bond values could be related to estrogenicity. This is logical since more hydroxyl group-mediated hydrogen bonds between the ligand and the receptor would likely lead to more ligand/receptor interactions, a greater receptor conformational change and thus perhaps a greater receptor-mediated biological response (in this case, estrogenicity). In addition, previous research indicated that H-bond value might be associated with other receptor-mediated biological activities (e.g., cathepsin D and its inhibitor pepstatin) (Baldwin et al., 1993). An understanding of the importance of hydrogen bond interactions in the ligand-receptor binary system might also explain why triphenol pyrazole ligands show better ERα binding affinity than monohydroxy- and dihydroxyphenols (Stauffer et al., 2000).

Six of the 19 flavonoids studied with different numbers of hydroxyl groups were selected for more detailed investigation of the hydrogen bond interaction between the ligand and LBC amino acid residues. Fig. 3 shows that methylation of vicinal dihydroxy flavonoids (e.g., biochanin A; Table 2) changes the ligand's interactions with ERα because methylation sterically hinders interactions, forcing the unmethylated hydroxyl groups to interact with different amino acid residues (Fig. 3 (e) and (f)). Interestingly, a methylbenzene group (e.g., in

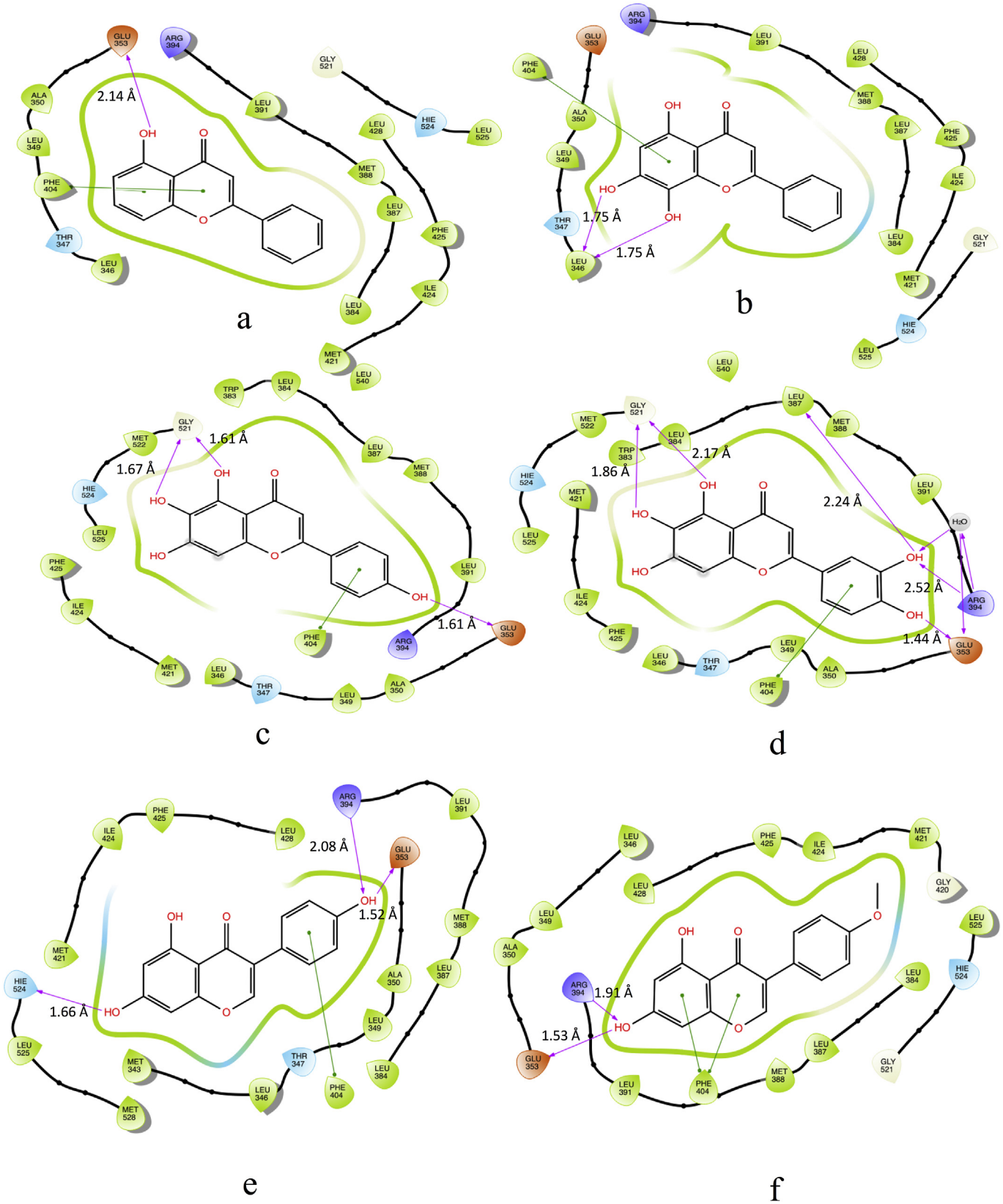


Fig. 3. Ligand interactions with amino acid residues in ERα visualised in Shrödinger. Purple arrows represent hydrogen bonds; direction of arrow denotes electron donor to electron acceptor in the hydrogen bond. Different coloured residues represent amino acid properties: blue, positive charge; red, negative charge; cyan, polar; green, nonpolar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

biochanin A; Table 2) might not form a hydrogen bond with amino acid residues in the LBC predicted by the docking study (Palusiak and Grabowski, 2002). This finding might explain why genistein has stronger estrogenicity than biochanin A (Le Bail et al., 2000). Furthermore, this extra polar substituent might interfere with ligand ERα hydrophobic interactions as discussed above; this is the case for 2, 3-diarylindans (Anstead et al., 1989). It is likely that this extra polar substituent could cause an unfavourable interaction with the LBC of ERα; for example, kaempferol has one more hydroxyl than genistein and is a weaker estrogen mimic (Table 2). On the other hand, Zhang et al. (2018) suggested that keto groups could form hydrogen bonds (keto groups can be hydrogen bond donors) with key amino acid residues in the LBC of ERα so having a favourable interaction effect.

In summary, the potential defining effects of polar groups on ligand binding and estrogenicity depend on both their positions on the skeletal ring structure and their spatial orientations.

3.5. Ligand HER determines theoretical binding energy with ERα

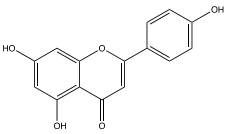
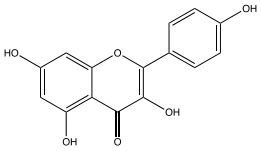
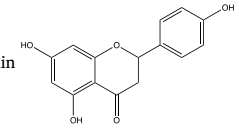
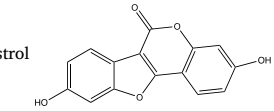
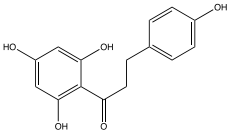
HER is the term used to differentiate the relationship between the hydrophobicity of ligands and the geometric receptor environment. It describes the spatial arrangement of hydrophobic amino acid residues (e.g. Leu) in the ligand binding cleft of, for example, a receptor (in this case ERα) which supports hydrophobic interactions between the ligand and its receptor. For the perfect interaction, a hydrophobic ligand would be enclosed in the hydrophobic microenvironment of the LBC to give a maximum potential for hydrophobic interactions - ideally, this would involve the ligand sandwiched between two hydrophobic regions. The distance between ligand atoms that might interact with target atoms of the LBC's hydrophobic region is important; there is an optimum distance to facilitate a high energy hydrophobic interaction (i.e. a strong ligand/LBC hydrophobic interaction). Thermodynamically, a hydrophobic ligand 'prefers' to sit amongst hydrophobic residues in a hydrophobic binding cleft; conversely, increased hydrophilic character likely reduces the ideal thermodynamic fit to a hydrophobic cleft. It is the balance between the hydrophobic and hydrophilic character of the ligand that influences its binding to the LBC.

In this study, 5 compounds from different flavonoid subclasses were selected to study the importance of the ligands' molecular skeletons for ERα binding. As can be seen from Table 3 and Fig. 4, when coumestan binds to the LBC it sits parallel to the cleft's hydrophobic region, this means it has the highest HER. On the other hand, phloretin has highly rotatable bonds and so shows the lowest HER value. This indicates that if a potential ligand in the same binding environment sits parallel to the hydrophobic binding region, it will bind more strongly than a similar ligand with a non-parallel molecular arrangement (i.e. with groups outside the parallel plane; e.g. kaempferol (Fig. 3)).

Interestingly, the flavanone naringenin (found in grapefruit) does not have a C2 = C3, and so it has a more flexible skeletal ring structure than the flavone apigenin (found in parsley and celery) which does have a C2 = C3 (Table 3). In a different setting, Naringenin is a potent inhibitor of the enzyme xanthine oxidase (XO), while apigenin is a much weaker inhibitor (Lin et al., 2015). This further illustrates and supports the importance of bond flexibility in facilitating close ligand/protein interactions in the binding cleft (in this example XO's active site). Here we extrapolate the concept of bond flexibility supporting good ligand fit to protein binding sites (active site in the case of XO) to the LBC of ERα.

These interaction with the LBC likely result in an ERα conformational change which influences interaction with the nuclear estrogen response element (ERE) and so drives the ultimate biological response. Different ligands might lead to different levels of biological response due to differences in their interactions with the LBC (Ye et al., 2018). Che et al. (2016) carried out studies to explore the dynamics of conformational changes in ERα with its LBC occupied by different

Table 3
Examples of flavonoid subclasses and their hydrophobic enclosure reward.

Subclass	Example compound	HER (kcal/mol)
Flavone	Apigenin 	−2.7
Flavonol	Kaempferol 	−2.2
Flavanone	Naringenin 	−2.3
Coumestan	Coumestrol 	−2.7
Dihydrochalcone	Phloretin 	−0.7

phytoestrogens or mycoestrogens. They utilised $^{2}H_{2}O$ exchange followed by ER pepsin fragmentation and column chromatographic separation to show that different ligands resulted in different levels of deuterium exchange. Interestingly, this suggests that different ligands interact with the ERα LBC resulting in different conformational changes. This, of course, might explain the differential biological activity of these ligands. Our work has shown that the interactions of different food flavonoids with the LBC is different and structure (particularly hydroxyl group) dependent. Bringing Chen et al. (2016)'s and our findings together suggests that different interactions at the LBC might lead to different conformational changes; this might, in turn, modulate ERα-driven biological response.

3.6. Gut biotransformation might increase the estrogenicity of flavonoids

Gut microbiome-mediated biotransformation of some flavonoids results in the formation of metabolites with different estrogenicities to their parent compounds. For example, daidzein is metabolized to the more estrogenic *S*-equol (Table 2, Fig. 5) (Breinholt and Larsen, 1998), and dihydrogenistein is less estrogenic than genistein (Pfitscher et al., 2008). The differences in the estrogenicities of these metabolites are likely due to the different hydrophobicities of their molecular skeletons. Increased estrogenicity is likely due to the different hydrophobicities of daidzein and its metabolite, S-equol which leads to S-equol having a more favourable hydrophobic interaction with the LBC than daidzein – S-equol does not have a keto group, and so has a more hydrophobic skeleton (Fig. 5). This might lead to both health benefits and risks. For example, increasing estrogenic load could be beneficial in peri-menopausal women who are responding physiologically to the natural reduction in their estrogen levels as the menopause progresses. Dietary flavonoids, or flavonoid dietary supplements, might ameliorate these symptoms (Huntley, 2009), especially if microbiome-mediated biotransformation of the flavonoids leads to increased estrogenicity. It is also possible that cytochromes P450-mediated cellular metabolism could result in flavonoid hydroxylation, which might have similar

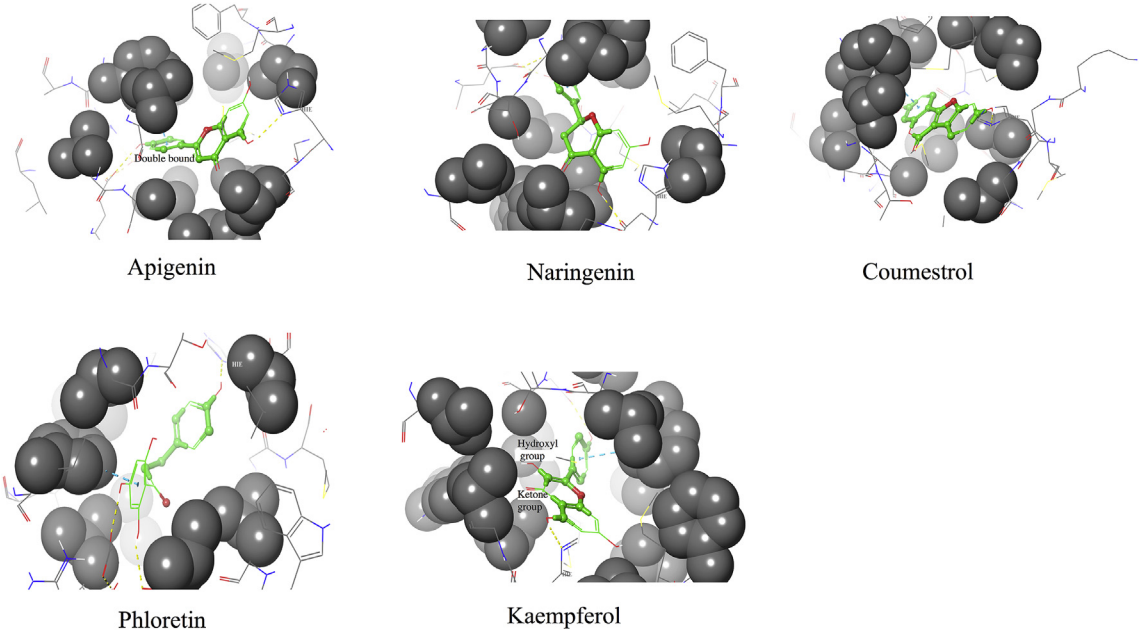


Fig. 4. Selected flavonoids and their hydrophobic enclosures in the ERα ligand binding cleft (LBC). hydrophobic enclosures, CPK representation; hydrophobic atoms in the ligand (green), ball and stick representation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

biological effects; and interestingly, flavonoids are known to affect cytochromes P450 expression (Sergent et al., 2009) which could also influence flavonoid cellular biotransformation. On the risk side, men exposed to dietary flavonoids might develop ERα-mediated adverse effects at a population level, including reduced sperm count (Sharpe et al., 1995) and gynecomastia (Degen and Bolt, 2000). Similarly, prepubertal girls (they have low estrogen levels (Cutler Jr, 1997)) might be triggered to enter puberty by dietary flavonoid exposure because female puberty requires an estrogen stimulus (Wolff et al., 2008). This will likely be exacerbated if microbiome-mediated biotransformation increases dietary flavonoids' estrogenicities. Indeed, it is thought that increased exposure to environmental estrogen mimics is related to an increased incidence of precocious puberty in girls worldwide (Wolff et al., 2008).

The quantitative production of *S*-equol from daidzein differs between individuals, and this possibly reflects their gut microbiome composition because it is likely that not all bacterial species convert daidzein to *S*-equol (Chang and Nair, 1995). This points to the possibility that inter-individual variation in gut microbiome composition might influence the impact of dietary flavonoids on human health in a functional food setting.

4. Conclusions

This study shows, firstly, that both the number of phenolic hydroxyls on a ligand molecule and their relative positions and special arrangements can affect the hydrogen bond interaction between the ligand and key amino acid residues in the ERα's LBC. In addition, different flavonoid electron donors (e.g., methoxy or hydroxy) likely result in different hydrogen bond energies. This, in turn, might result in different binding energies which would likely influence estrogenicity. For example, genistein has 5-, 7- and 4′-hydroxyl groups, whereas biochanin A has 5- and 7-hydroxyl groups with a methoxy group at the 4′ position (Table 2). This might explain the estrogenicity differences between these two compounds (Table 2).

Secondly, a ligand sitting parallel to the hydrophobic binding region in ERα's LBC likely binds more strongly than a similar molecule with a non-parallel orientation because the former is closely aligned with the hydrophobic amino acid residues with which its hydrophobic region interacts. The number of rotatable bonds in, and the degree of hydrophobicity of, the ligand are also important molecular features that very likely influence the magnitude of hydrophobic interaction with the LBC. The rotatable bonds are particularly important because they facilitate reorientation of the ligand's hydrophobic regions allowing them to interact more efficiently with the LBC's hydrophobic amino acid residues.

A detailed understanding of the interaction of ligands with ERα gives insight into their potential biological activity (i.e., functionality and/or toxicity); this could be in a pharmacological or a functional food setting and so might play an important role in understanding food flavonoid toxicity, designing pharmacologically active ligands (e.g., in the treatment of ER + breast cancer) or understanding the functionality of flavonoid-containing foods and their usefulness in a human health context.

The balance between dietary phytoestrogen and endogenous estrogen levels should be considered; for example, isoflavone supplements are sometimes used to alleviate symptoms of menopause (Potter et al., 1998) because they enhance total plasma estrogenicity, thus ameliorating the biochemical and physiological effects of declining natural estrogens. On the other hand, women with high circulating estrogen levels (e.g., child-bearing age women) would be unlikely to benefit from isoflavone dietary supplementation. A similar argument applies to the implications of dietary phytoestrogen to breast cancer

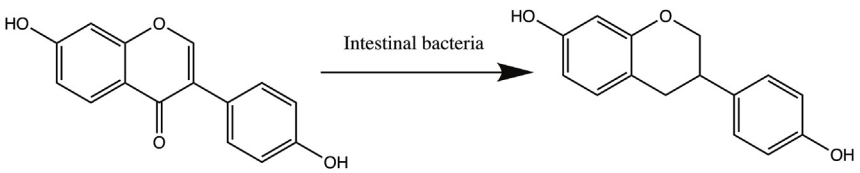


Fig. 5. Gut microbiome-mediated biotransformation of daidzein (left) to equol (right).

risk where high isoflavone (e.g., genistein) doses appear to prevent breast cancer cell proliferation, whereas low doses promote proliferation (He and Chen, 2013). And, importantly, dietary phytoestrogens can interfere with the action of estrogen receptor-based (e.g., tamoxifen) treatment for breast cancer because they might compete with tamoxifen for occupancy of the LBC, even though the phytoestrogens have a very much lower binding affinity for the LBC than tamoxifen (Ju et al., 2002).

In silico modelling studies might aid our understanding of the functionality and potential biological activity of food and, in the long-term they might underpin the development of personalised diets to reduce health risks and help us to better understand pharmaceutical/food interactions.

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References

Anstead, G.M., Peterson, C.S., Katzenellenbogen, J.A., 1989. Hydroxylated 2, 3-diarylindenes: synthesis, estrogen receptor binding affinity, and binding orientation considerations. J. Steroid Biochem. 33, 877–887.
Bailey, D.G., Dresser, G.K., Bend, J.R., 2003. Bergamottin, lime juice, and red wine as inhibitors of cytochrome P450 3A4 activity: comparison with grapefruit juice. Clin. Pharmacol. Ther. 73, 529–537.
Baldwin, E.T., Bhat, T.N., Gulnik, S., Hosur, M.V., Sowder, R.C., Cachau, R.E., Collins, J., Silva, A.M., Erickson, J.W., 1993. Crystal structures of native and inhibited forms of human cathepsin D: implications for lysosomal targeting and drug design. Proc. Natl. Acad. Sci. Unit. States Am. 90, 6796–6800.
Berckmans, P., Leppens, H., Vangenechten, C., Witters, H., 2007. Screening of endocrine disrupting chemicals with MELN cells, an ER-transactivation assay combined with cytotoxicity assessment. Toxicol. Vitro 21, 1262–1267.
Breinholt, V., Larsen, J.C., 1998. Detection of weak estrogenic flavonoids using a recombinant yeast strain and a modified MCF7 cell proliferation assay. Chem. Res. Toxicol. 11, 622–629.
Bryzgalova, G., Gao, H., Ahrén, B., Zierath, J., Galuska, D., Steiler, T., Dahlman-Wright, K., Nilsson, S., Gustafsson, J.-Å., Efendic, S., 2006. Evidence that oestrogen receptor-α plays an important role in the regulation of glucose homeostasis in mice: insulin sensitivity in the liver. Diabetologia 49, 588–597.
Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engström, O., Öhman, L., Greene, G.L., Gustafsson, J.-Å., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753.
Chang, Y.-C., Nair, M.G., 1995. Metabolism of daidzein and genistein by intestinal bacteria. J. Nat. Prod. 58, 1892–1896.
Chen, X., Uzuner, U., Li, M., Shi, W., Yuan, J., 2016. Phytoestrogens and mycoestrogens induce signature structure dynamics changes on estrogen receptor. Int. J. Environ. Res. Public Health 13, 869. https://doi.org/10.3390/ijerph13090869.
Cutler Jr., G., 1997. The role of estrogen in bone growth and maturation during childhood and adolescence. J. Steroid Biochem. Mol. Biol. 61, 141–144.
Degen, G., Bolt, H., 2000. Endocrine disruptors: update on xenoestrogens. International Archives of Occupational and Anvironmental Health 73, 433–441.
Ekins, S., Mestres, J., Testa, B., 2007. In silico pharmacology for drug discovery: methods for virtual ligand screening and profiling. Br. J. Pharmacol. 152, 9–20.
Eldridge, M.D., Murray, C.W., Auton, T.R., Paolini, G.V., Mee, R.P., 1997. Empirical scoring functions: I. The development of a fast empirical scoring function to estimate the binding affinity of ligands in receptor complexes. J. Comput. Aided Mol. Des. 11, 425–445.
Evangelopoulos, D., Alevizaki, M., Lekakis, J., Cimponeriu, A., Papamichael, C., Kominakis, A., Kalofoutis, A., Moutsatsou, P., 2003. Molecular analysis of the estrogen receptor alpha gene in men with coronary artery disease: association with disease status. Clin. Chim. Acta 331, 37–44.
Friesner, R.A., Banks, J.L., Murphy, R.B., Halgren, T.A., Klicic, J.J., Mainz, D.T., Repasky, M.P., Knoll, E.H., Shelley, M., Perry, J.K., 2004. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. J. Med. Chem. 47, 1739–1749.
Friesner, R.A., Murphy, R.B., Repasky, M.P., Frye, L.L., Greenwood, J.R., Halgren, T.A., Sanschagrin, P.C., Mainz, D.T., 2006. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein− ligand complexes. J. Med. Chem. 49, 6177–6196.
Hall, L.M., Hall, L.H., Kier, L.B., 2003. Modeling drug albumin binding affinity with e-state topological structure representation. J. Chem. Inf. Comput. Sci. 43, 2120–2128.
Harris, D., Besselink, E., Henning, S., Go, V., Heber, D., 2005. Phytoestrogens induce differential estrogen receptor alpha-or beta-mediated responses in transfected breast cancer cells. Exp. Biol. Med. 230, 558–568.
He, F.-J., Chen, J.-Q., 2013. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: differences between Chinese women and women in Western countries and possible mechanisms. Food Science and Human Wellness 2, 146–161.
Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Ström, A., Treuter, E., Warner, M., 2007. Estrogen receptors: how do they signal and what are their targets. Physiol. Rev. 87, 905–931.
Huntley, A., 2009. The health benefits of berry flavonoids for menopausal women: cardiovascular disease, cancer and cognition. Maturitas 63, 297–301.
Ignatowicz, E., Baer-Dubowska, W., 2001. Resveratrol, a natural chemopreventive agent against degenerative diseases. Pol. J. Pharmacol. 53, 557–569.
Ju, Y.H., Doerge, D.R., Allred, K.F., Allred, C.D., Helferich, W.G., 2002. Dietary genistein negates the inhibitory effect of tamoxifen on growth of estrogen-dependent human breast cancer (MCF-7) cells implanted in athymic mice. Cancer Res. 62, 2474–2477.
Kuiper, G.G., Carlsson, B., Grandien, K., Enmark, E., Häggblad, J., Nilsson, S., Gustafsson, J.-A.k., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 138, 863–870.
Le Bail, J.-C., Champavier, Y., Chulia, A.-J., Habrioux, G., 2000. Effects of phytoestrogens on aromatase, 3β and 17β-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci. 66, 1281–1291.
Lim, D., Shaw, I.C., 2016. Is there a link between dietary phytoestrogens and reproductive health in men? A meta-analysis of data from the USA and China. Int. J. Food Sci. Technol. 51, 23–29.
Lin, S., Zhang, G., Liao, Y., Pan, J., Gong, D., 2015. Dietary flavonoids as xanthine oxidase inhibitors: structure–affinity and structure–activity relationships. J. Agric. Food Chem. 63, 7784–7794.
Lu, L., Katsaros, D., Wiley, A., de la Longrais, I.A.R., Risch, H.A., Puopolo, M., Yu, H., 2006. The relationship of insulin-like growth factor-II, insulin-like growth factor binding protein-3, and estrogen receptor-alpha expression to disease progression in epithelial ovarian cancer. Clin. Cancer Res. 12, 1208–1214.
Manas, E.S., Xu, Z.B., Unwalla, R.J., Somers, W.S., 2004. Understanding the selectivity of genistein for human estrogen receptor-β using X-ray crystallography and computational methods. Structure 12, 2197–2207.
Matsumoto, T., Kobayashi, M., Moriwaki, T., Kawai, S.i., Watabe, S., 2004. Survey of estrogenic activity in fish feed by yeast estrogen-screen assay. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 139, 147–152.
Mosselman, S., Polman, J., Dijkema, R., 1996. ERβ: identification and characterization of a novel human estrogen receptor. FEBS (Fed. Eur. Biochem. Soc.) Lett. 392, 49–53.
Palusiak, M., Grabowski, S.J., 2002. Methoxy group as an acceptor of proton in hydrogen bonds. J. Mol. Struct. 642, 97–104.
Pfitscher, A., Reiter, E., Jungbauer, A., 2008. Receptor binding and transactivation activities of red clover isoflavones and their metabolites. J. Steroid Biochem. Mol. Biol. 112, 87–94.
Potter, S.M., Baum, J.A., Teng, H., Stillman, R.J., Shay, N.F., Erdman Jr., J.W., 1998. Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women. Am. J. Clin. Nutr. 68, 1375S–1379S.
Reid, G., Denger, S., Kos, M., Gannon, F., 2002. Human estrogen receptor-α: regulation by synthesis, modification and degradation. Cell. Mol. Life Sci. 59, 821–831.
Sergent, T., Dupont, I., Van Der Heiden, E., Scippo, M.-L., Pussemier, L., Larondelle, Y., Schneider, Y.-J., 2009. CYP1A1 and CYP3A4 modulation by dietary flavonoids in human intestinal Caco-2 cells. Toxicol. Lett. 191, 216–222.
Sharpe, R.M., Fisher, J.S., Millar, M.M., Jobling, S., Sumpter, J.P., 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ. Health Perspect. 103, 1136.
Simons, L.A., von Konigsmark, M., Simons, J., Celermajer, D.S., 2000. Phytoestrogens do not influence lipoprotein levels or endothelial function in healthy, postmenopausal women. Am. J. Cardiol. 85, 1297–1301.
Stauffer, S.R., Coletta, C.J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B.S., Katzenellenbogen, J.A., 2000. Pyrazole ligands: structure− affinity/activity relationships and estrogen receptor-α-selective agonists. J. Med. Chem. 43, 4934–4947.
Turner, N.C., Ro, J., André, F., Loi, S., Verma, S., Iwata, H., Harbeck, N., Loibl, S., Huang Bartlett, C., Zhang, K., 2015. Palbociclib in hormone-receptor–positive advanced breast cancer. N. Engl. J. Med. 373, 209–219.
Ullah, M.F., Khan, M.W., 2008. Food as medicine: potential therapeutic tendencies of plant derived polyphenolic compounds. Asian Pac. J. Cancer Prev. APJCP 9, 187–196.
Ververidis, F., Trantas, E., Douglas, C., Vollmer, G., Kretzschmar, G., Panopoulos, N., 2007. Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part I: chemical diversity, impacts on plant biology and human health. Biotechnol. J. 2, 1214–1234.
Waring, R., Harris, R., 2005. Endocrine disrupters: a human risk? Mol. Cell. Endocrinol. 244, 2–9.
Wolff, M.S., Britton, J.A., Boguski, L., Hochman, S., Maloney, N., Serra, N., Liu, Z., Berkowitz, G., Larson, S., Forman, J., 2008. Environmental exposures and puberty in inner-city girls. Environ. Res. 107, 393–400.
Zhang, T., Zhong, S., Hou, L., Li, T., Xing, X., Guan, T., Zhang, J., Wang, Y., 2018. Estrogenic properties of coumarins and meroterpene from the fruits of *Cullen corylifolium*: experimental and computational studies. Phytochemistry 152, 148–153.
Ye, H., Dudley, S.Z., Shaw, I.C., 2018. Intimate estrogen receptor-α/ligand relationships signal biological activity. Toxicology 408, 80–87.