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Phytoestrogens and mycoestrogens interacting with breast cancer proteins

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ABSTRACT

Breast cancer is a highly heterogeneous disease influenced by the hormonal microenvironment and the most common malignancy in women worldwide. Some phytoestrogens and mycoestrogens have been epidemiologically linked as risk factors or protectors, however their mechanisms of action are complex and not fully understood. The aim of this study was to predict the potential of 36 natural xenoestrogens to interact with 189 breast cancer proteins using AutoDock Vina. In order to validate our protocol, an *in silico* docking pose and binding site determination was compared with the crystallographic structure and the power of prediction to distinguish between ligand and decoys was evaluated through a receiver operating characteristic curve (ROC) of the resultant docking affinities and *in vitro* data. The best affinity score was obtained for glyceollin III interacting with the sex hormone binding globulin (-11.9 Kcal/mol), a plasma steroid transport protein that regulates sex steroids bioavailability. Other natural xenoestrogens such as beta-carotene, chrysophanol 8-O-beta-D-glucopyranoside and glyceollin I, also presented good affinity for proteins related to this disease and the validation was successful. This study may help to prioritize compounds for toxicity tests or drug development from natural scaffolds, and to elucidate their mechanisms of action.

1. Introduction

Natural xenoestrogens can act as endocrine modulators through mimicking endogenous hormones [1–3]. However, contrary to synthetic endocrine disruptors, which have been extensively associated with reproductive disorders and other diseases, natural xenoestrogens have been reported to exert mostly beneficial effects against breast cancer [4,5].

Among them, phytoestrogens and their derivatives have received special attention due to their high concentrations in food, especially in vegetables and soy products; protective and antiproliferative effects [6–8]. Some of the leading phytoestrogens for cancer treatment or prevention are genistein, coumestrol and glyceollin [9,10]. Interestingly, there are epidemiological studies indicating that countries with high consumption of soy products have a lower incidence of breast cancer in women, compared to the second generation of their emigrants to the United States and countries with different dietary habits. This suggests that phytoestrogen intake plays a substantial role in the susceptibility to this cancer type [11], which is the most common cancer in women worldwide [12].

Phytoestrogens could target multiple systems within the cells, comprising epigenetic regulation, signaling pathways, apoptosis and steroid synthesis [13]. Many of these compounds are able to bind mammalian ER and are weak inhibitors of aromatase, which may alter the risk of breast cancer by lowering the amount of circulating estrogen [14]. The inhibition of cancer cell growth and angiogenesis as well as stimulation of apoptosis, are also under discussion [5,14–16]. Some of the known protein targets for phytoestrogens are cyclin-dependent kinases (p21/p27), the tumor suppressor gene p53, pro-apoptotic and anti-apoptotic genes including Bax and Blc-2 [17]. Besides, their protective effects through changes in the epigenetics patterns of genes with a pivotal role in this disease, such as the genes: breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) [18].

A wide variety of phytoestrogens, such as flavonoids, are good antioxidants with demonstrated both reactive oxygen species (ROS) regulation and anti-cancer effects [19]. However, the evaluation of the potential benefits and risks of phytoestrogens is a complex task [20] and there is not a consensus in this regard [21–24].

Additionally, mycoestrogens are xenoestrogens produced by fungi that can be also present in food. One of the most common is

Abbreviations: AUC, area under the curve; BRCA1, breast cancer 1; BRCA2, breast cancer 2, CAT, catalase; DHT, dihydrotestosterone; EDCs, endocrine disrupting chemicals; ERBB2, human epidermal growth factor receptor 2; ERBBs, epidermal growth factor receptor family; ER-beta, estrogen receptor beta; ESR1, estrogen receptor alpha; IC50, half-maximal inhibitory concentration; NB, nonbonding substances; PDB, Protein Data Bank; PGR, progesterone receptor; ROC, receiver operating characteristic analysis; RORC, retinoic acid-related orphan receptor C; ROS, reactive oxygen species; SHBG, sex hormone-binding globulin; vHTS, virtual high-throughput screening

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zearalenone, which has been reported to be a potential promoter of breast cancer tumorigenesis [25]. This xenoestrogen stimulates the growth of estrogen receptor-positive human breast cells [25–27], and its effects on cell cycle progression of MCF-7 cells may be mediated by the mitogen-activated protein kinase signaling cascade [25], or the inhibition of apoptosis through regulation of Bax/Bcl-2 expression [27]. Moreover, both phytoestrogens and mycoestrogens have been also reported to protect mammary tissue from malignant transformation induced by carcinogens when they are consumed in low doses during prepuberty [28].

The mechanisms of action of phytoestrogens and mycotoxins on breast cancer are intricate and poorly understood [29]. Consequently, and given the presence of these chemicals in foods, the aim of this work was to evaluate *in silico* the potential of endocrine disrupting chemicals (EDCs) from natural sources to bind breast cancer-related proteins, in order to guide further studies that may help to elicit the role of these molecules in protective or toxicity mechanisms of breast carcinoma through protein–ligand interactions.

2. Methods

An *in silico* approach was employed to identify phytoestrogens with potential to target breast cancer proteins. Moreover, a validation with available experimental data was performed to assess the prediction power of our protocol.

2.1. High-throughput virtual screening

A virtual high-throughput screening (vHTS) was carried out among 36 natural xenoestrogens and 189 proteins involved in breast cancer. These lasts were selected, prepared and optimized as reported in our previous work [30]. On the other hand, the natural compounds were downloaded from EDCs DataBank [31], using as selection criteria that they appear reported in the scientific literature as phytoestrogens, mycoestrogens or their metabolites. The docking parameters were set by AutoDock Tools [32], using a blind docking strategy; the grid coordinates were centered in the macromolecule adjusted to cover the whole surface of the protein with a spacing of 0.357 Å. Kollman charges and polar hydrogen atoms were added to the protein structures and exported as .pdbqt format files for docking calculations. The virtual screening was carried out in AutoDock Vina [33] running on Linux Red Hat 6. Each ligand/protein pair was docked by triplicate, and the averages of the best affinity scores (Kcal/mol) of each protein–ligand pair were used to rank the complexes. In order to easily visualize the results and establish comparisons, a hierarchical cluster was built in R version 3.1.2 [34], using the "heatmap.2" function of the gplot library [35]. The color key was set employing the predicted docking affinity scores by the RColorBrewer package of the same software, good affinity scores (< -7.5 Kcal/mol) were colored in red, weak affinity (-7.4 to -6 Kcal/mol) in white, and the predicted non-binders (> -5.9 Kcal/mol) in blue.

2.2. Protein-ligand interaction prediction

The contact residues participating in the protein–ligand interactions of the complexes with the best affinity scores were predicted *in silico* by LigandScout 3.1.4 [36], using default settings.

2.3. Validation

A two-step validation was carried out to evaluate the power of prediction of AutoDock Vina [33] to identify the binding site and to distinguish between ligands and decoys according to the docking affinity cut-off (-7.5 Kcal/mol) and experimental data [37]. The validation of the docking pose prediction was carried out through the superposition of one of the predicted complexes by AutoDock Vina [33] and its crystallographic structure available on Protein Data Bank (PDB) -estrogen receptor beta (ER-beta)/genistein; PDB: 1X7J-.In addition, a receiver operating characteristic analysis (ROC) was performed using the estrogen receptor alpha (ESR1; PDB: 1L2I) and estrogenic compounds, including some phytochemicals, in order to assess the selectivity and sensitivity of our methodology. A dataset of 70 EDCs with strong, moderate and non-binder ligands, including natural compounds [37], was used to evaluate the efficiency of our protocol to predict correctly the ligand–protein complexes according to the docking affinity cut-off (-7.5 Kcal/mol) compared to experimental data (half-maximal inhibitory concentration, IC_{50}). The type of binder was kept as in the original articles [37,38], however, weak binders were removed from ROC study. Inactive substances were selected as those reported as nonbonding substances (NB) with log $IC_{50} > -4.0$, and active were the strong and moderate binders with log $IC_{50} < -6.0$. The area under the ROC curve was calculated using "ROCR" package [39] in R [34]. The specificity and sensitivity in the affinity cut-off (< -7.5 Kcal/mol) were also calculated by the same software.

3. Results

3.1. Virtual High-throughput screening

A total of 36 natural products were docked against 189 proteins related to breast cancer selected by text mining. The theoretical affinity scores obtained for each protein–ligand pair calculated by AutoDock Vina [33] are presented in Suppl. Table 1. This data is also available as a heat map (Suppl. Fig. 1) to facilitate the identification of clusters of proteins and natural xenoestrogens with similar docking affinity patterns. The complex that exhibited the best affinity score *in silico* was the phytoalexin glyceollin III coupled to the sex hormone binding globulin (SHBG; -11.9 Kcal/mol). The hydrophobic interactions in the binding site of this complex were predicted to be mediated by the contact residues Trp84A, Phe67A, Val105A, Thr60A, Met139A, and Leu171A; similarly, according to LigandScout, some hydrogen bonding interactions were predicted for Ser128A and Trp66A, (Fig. 1). Other phytoestrogens that also presented strong affinity for breast cancer proteins (< -11.0 Kcal/mol), are glyceollin II/SHBG, beta-carotene/catalase (CAT), glyceollin I/CAT, chrysophanol 8-O-beta-D-glucopyranoside/CAT, glyceollin I/SHBG, glyceollin II/ CAT and beta-carotene/retinoid acid-related orphan receptor (RORC).

In order to analyze the theoretical binding mode of natural xenoestrogens with the best affinity score for proteins highly correlated with breast cancer such as: ESR1, human epidermal growth factor receptor 2 (ERBB2), progesterone receptor (PGR) and BRCA1, the contact residues and protein–ligand interactions of the complexes with the best affinity score for each protein are presented in this section. The natural xenoestrogen that exhibited the best binding score for ESR1 was beta-carotene with a docking affinity of -9.2 Kcal/mol, which present mostly hydrophobic interactions with the residues of the binding site. In addition, the ligand is extended outside of the ligand binding pocket, which is an unusual prediction (Fig. 2). The phytoestrogens: coumestrol, daidzein, genistein, chrysin, equol, oxyfluorfen, glyceollin I, chrysophanol, apigenin and glabridin, also presented good affinity scores ($\leq$-8.0 Kcal/mol) for this protein.

The molecule with the best docking affinity score for ERBB2 was chrysophanol 8-O-beta-D-glucopyranoside, which exhibited a moderate binding affinity (-8.8 Kcal/mol), by interacting with different chains of the protein, the contact residues predicted by LigandScout [36] are Tyr387A, His415C, Asn416C and Leu355C with hydrophobic and hydrogen bonding interactions (Fig. 3). Other compounds with good affinity ($\leq$-8.0 Kcal/mol) for this protein were alizarin, glyceollin I, glyceollin II, 8-prenylnaringenin, 6-prenylnaringenin, beta-carotene, glyceollin IV, hesperetin, glyceollin III, luteolin, and alpha-zearalenol.

The strongest binding affinity score for PGR was obtained for chrysophanol 8-O-beta-D-glucopyranoside (-10.1 Kcal/mol). However,

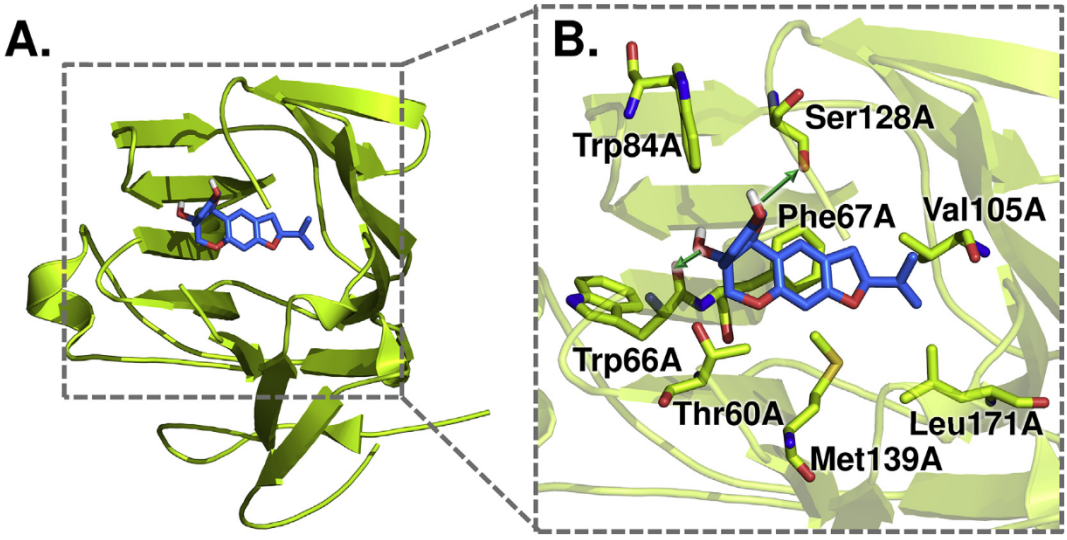

Fig. 1. Three-dimensional view of the (A) SHBG/glyceollin III complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features.

other ligands with great affinities (< -9.0 Kcal/mol) were glyceollin I, glyceollin II, glabridin, alizarin, glyceollin III, 6-prenylnaringenin, chrysin, glyceollin IV and coumestrol. The interacting residues of the complex PGR/chrysophanol 8-O-beta-D-glucopyranoside are Gln815, Lys822B, Glu695B, Leu758B, Gln725B, Ile699B, and Val698B, which participate in hydrophobic and hydrogen bonding interactions (Fig. 4).

Beta-carotene and glyceollin I were the molecules that presented the highest affinity score for BRCA1. These were predicted to share the binding site and some contact residues such as Leu1679A, Leu1701A, Val1654A, and Phe1662A. Besides, other molecules exhibited moderate affinity for this protein, such as beta-carotene and glyceollin I, both with a docking affinity score of -8.0 Kcal/mol (Fig. 5).

3.2. Validation

Validation of the docking pose prediction compared to the crystallographic structure of the complex ER-beta/genistein (PDB: 1X7J; Fig. 6A.) showed a good predictability of the binding site by AutoDock Vina [33]. In order to develop a ROC graph, 70 molecules (17 ligands and 53 decoys) were docked against the estrogen receptor alpha ESR1 (PDB:1L2I); the results of the docking affinities as well as the reported IC_{50} and log IC_{50} are presented in Suppl. Table 2. The area under the curve AUC) is 0.914 (P value < 0.0001, Fig. 6B). For the cut-off (< -7.5 Kcal/mol) the sensitivity and selectivity were 88.24% and 79.25%, respectively.

4. Discussion

4.1. Virtual high-throughput screening

The virtual high-throughput screening results suggest that several natural xenoestrogens have the potential to target multiple proteins involved in breast cancer (Suppl. Fig. 1), with a predominance of hydrophobic and hydrogen bonding interactions in the binding pocket of the complexes analyzed by LigandScout. The affinity scores ranged from very strong (-11.9 Kcal/mol) to moderate and low interactions (Suppl. Table 1).

The heat map and cluster constructed with the docking scores (Suppl. Fig. 1) show a very promiscuous theoretical behavior of most of phytoestrogens and mycoestrogens. The natural xenoestrogens with the poorest affinity scores for most of the breast cancer proteins were: caffeine, nicotine, m-coumaric acid, catechol, and resorcinol. In

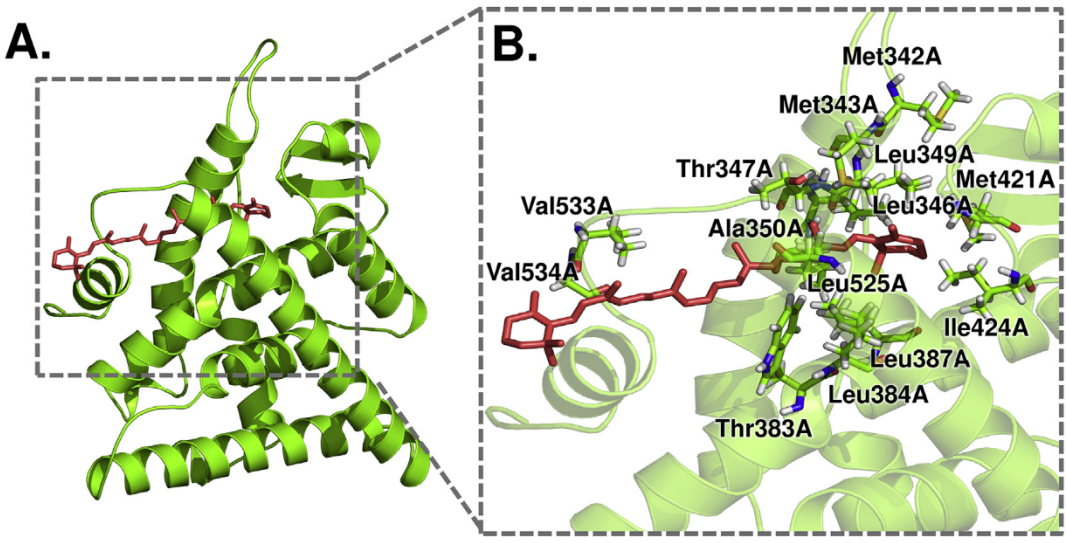

Fig. 2. Three-dimensional view of the (A) ESR1/beta-carotene complex, (B) showing the binding site and interactions predicted by LigandScout 3.1.

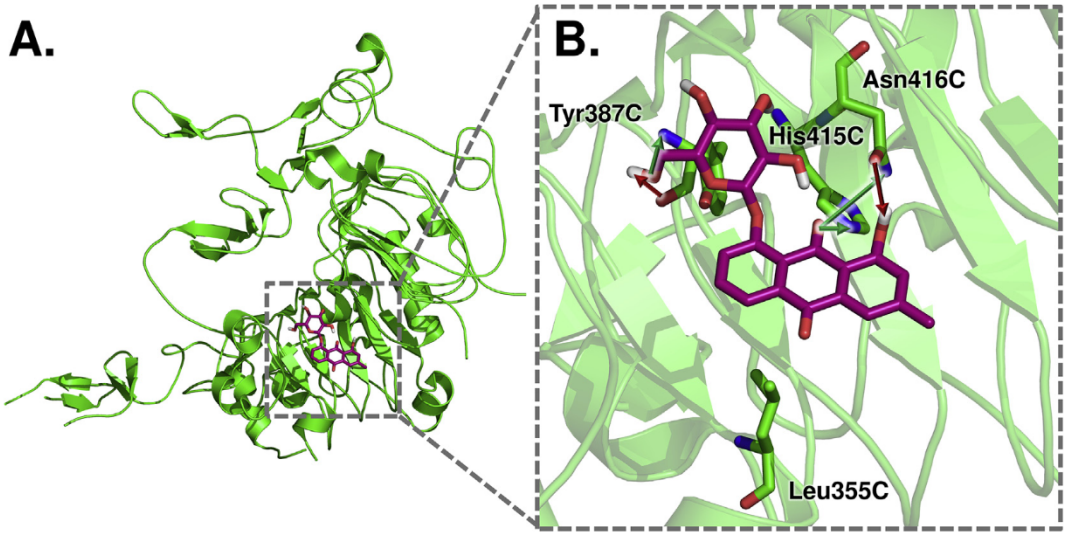

Fig. 3. Three-dimensional view of the (A) ERBB2/chrysophanol 8-O-beta-D-glucopyranoside complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features and the red arrows show the hydrogen-bond acceptor features.

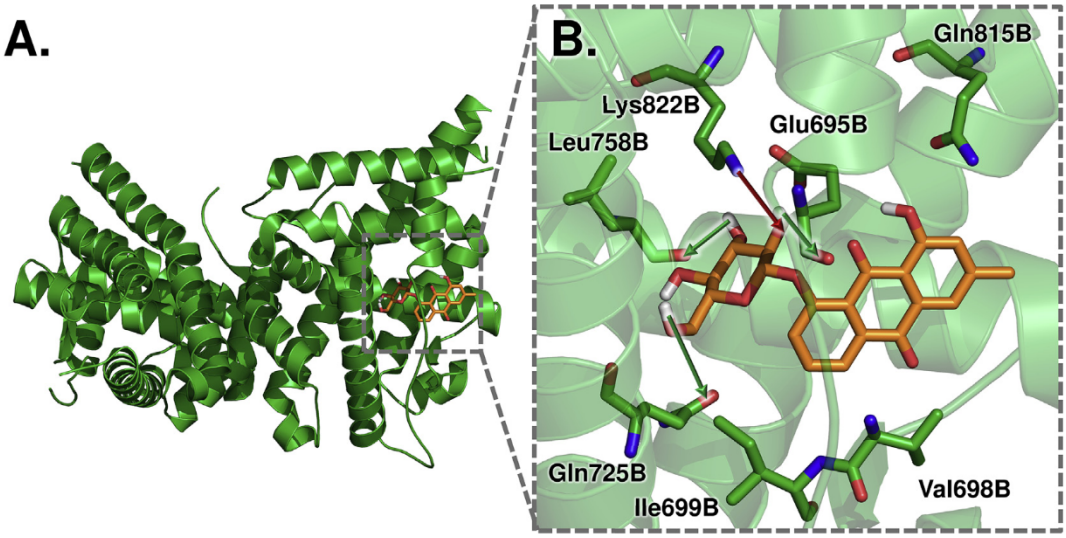

Fig. 4. Three-dimensional view of the (A) PGR/chrysophanol 8-O-beta-D-glucopyranoside, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features and the red arrows show the hydrogen-bond acceptor features.

addition, several proteins involved in antioxidant effect, cell cycle regulation and signaling pathways of breast cancer presented high and moderate affinity scores patterns for numerous natural xenoestrogens. Therefore, they could be mediating the biological response associated with these chemicals.

Mycoestrogens and phytoestrogens showed similar patterns of binding for several proteins, which is consistent to the found experimentally for the rat uterine estrogen receptor binding for of a group of structurally diverse xenoestrogens from plant and fungal origin [40].

The best *in silico* affinity score was obtained for the complex SHBG/glyceollin III (-11.9 Kcal/mol). This phytoestrogen has been reported to be a cancer cell growth inhibitor *in vitro* and *in vivo* [41], and to bind plasma proteins such as SHBG [42]. The SHBG binding pocket for glyceollin III is the same of endogenous hormones such as estradiol and dihydrotestosterone (DHT), which act as agonist and antagonist, respectively [43]. Glyceollin shows a similar orientation than estradiol in the binding site. However, the predicted *in silico* interaction showed hydrogen bonds formation with Ser128A and Trp66A, instead of Asn82 and Asp65; and similar hydrophobic interactions with the residues Phe67A and Met139 [44]. This is interesting, as agonists and antagonists share the same binding site of SHBG but with different orientations [45]. Glyceollin I and II also presented high affinity for SHBG.

According to the *in silico* studies, several phytoestrogens present in food, and used in natural medicine, were found to bind strongly to the antioxidant enzyme CAT, some of them were beta-carotene, glyceollin I and chrysophanol 8-O-beta-D-glucopyranoside with affinity scores of −11.6, −11.5, and −11.3 Kcal/mol, respectively. This is interesting as other phytoestrogens belonging to the group of flavonoids have been previously reported to interact with catalase *in vitro* [46]. Therefore, there may be protein–ligand interaction inducing the antioxidant effect of these compounds.

The best complexes and interaction analysis of natural xenoestrogens with the proteins highly related to breast cancer: ESR1, ERBB2, PGR, and BRCA1, are discussed below.

The estrogen receptor is one of the immunohistochemical markers used for breast cancer diagnosis [47]. ESR1 belongs to important pathways in the development of this cancer type [48], and is widely used as a target for the prevention and treatment of this disease [49]. Beta-carotene, as well as other phytoestrogens such as coumestrol,

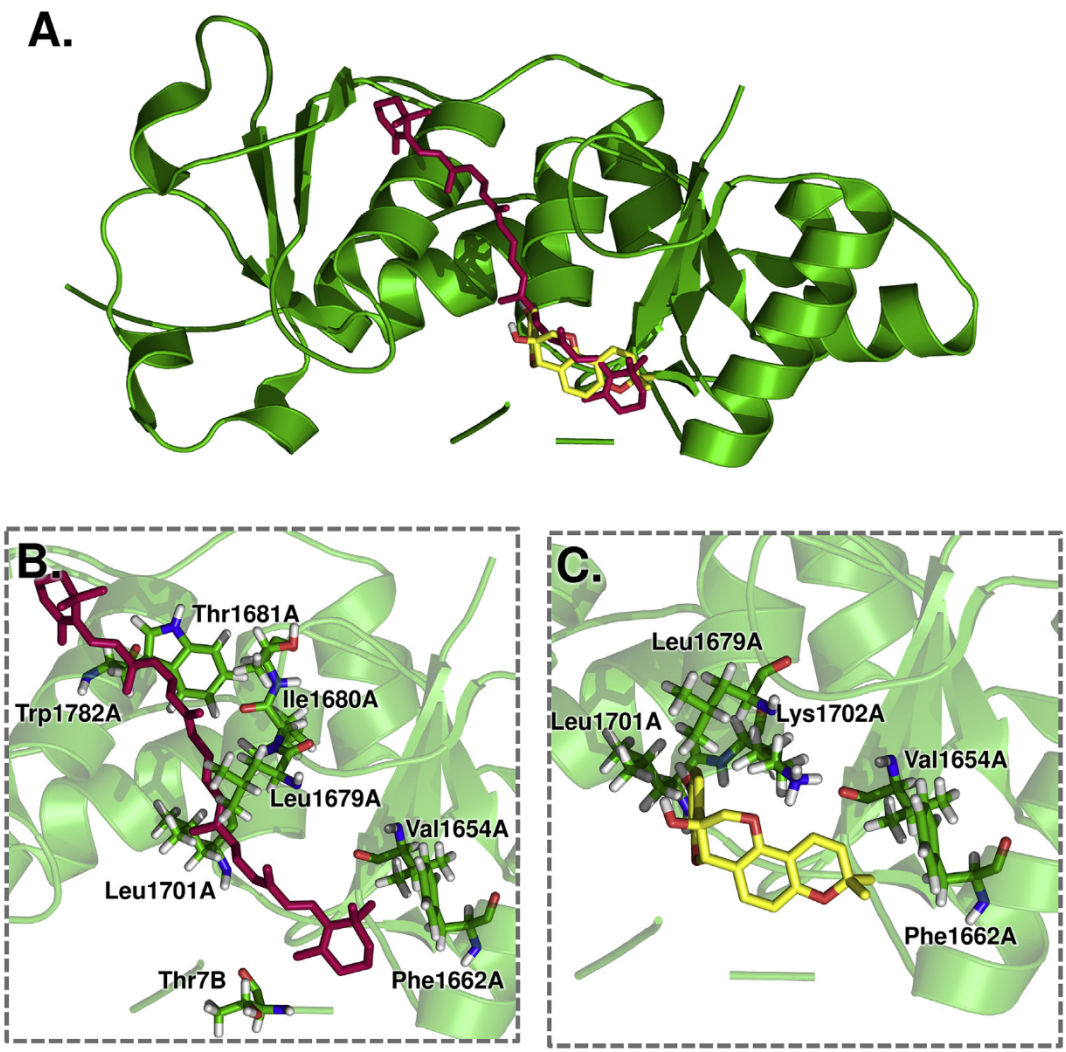

Fig. 5. Three-dimensional view of (A) the docking and (B) interactions of BRCA1/beta-carotene and (C) BRCA1/ glyceollin I predicted by LigandScout 3.1.

daidzein, genistein, chrysin, equol, oxyfluorfen, glyceollin I, chrysophanol, apigenin, and glabridin, showed high affinity *in silico* for this protein. Therefore they could have a potential as chemopreventive agents for breast cancer. Actually, some of them have been already reported to produce a reduction in breast cancer cell proliferation *in vitro*, such as the carotenoids [50].

ERBB2 gene is commonly overexpressed in certain breast cancer types and is associated with resistance to chemotherapeutic agents [51]. In recent years, ERBB2 protein has been proposed as a target for the development of new treatments for this disease acting on signaling pathways involving this and other epidermal growth factor receptors [52], as it is involved in the cell growth control, differentiation and survival [53], and their deregulation has been linked to tumorigenesis [52]. This protein is considered an orphan receptor [54], however, there has been reported its interaction with estradiol *in vitro* [55]. This surprising result, however, has not since been independently replicated

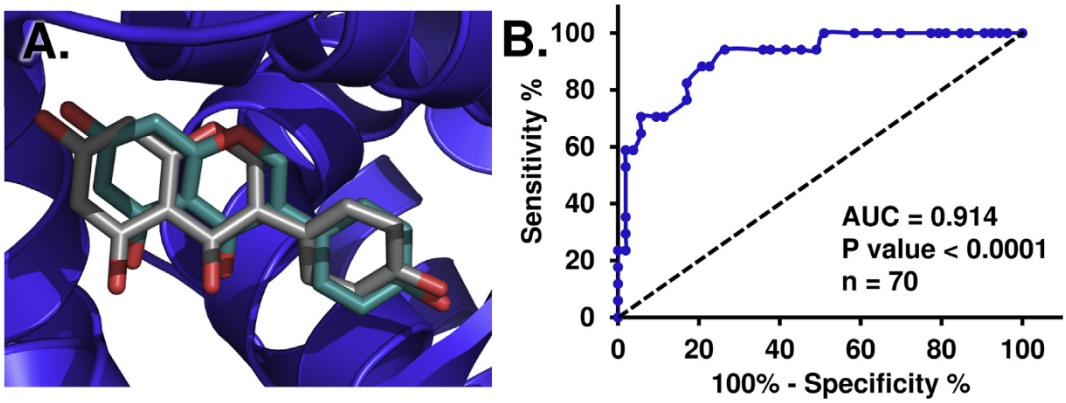

Fig. 6. A. Superposition between the crystallographic structure of the complex ER-beta/genistein (PDB: 1X7J) with the resultant docking pose of genistein (The crystallographic structure is represented in gray). B. ROC and area under the curve (AUC) for ERS1 ligands and decoys.

in other literature reports. According to the *in silico* study, the natural xenoestrogen that exhibited the best affinity score for ERBB2 was the phytoestrogen derived from rhizomes: chrysophanol 8-O-beta-D-glucopyranoside [56], and the contact residues in the extracellular domain are Tyr 387A, His415C, Asn416C, and Leu355C. Interestingly, the dimerization of the extracellular domains activates the phosphorylation of the intracellular domains, important for the signal transduction of the epidermal growth factor receptor family (ERBBs) [57]. Therefore, natural xenoestrogens could be affecting this process through the protein–ligand interaction with ERBB2.

Other important macromolecule for the prognosis and treatment of breast cancer is the progesterone receptor [58], as the hormonal microenvironment is very influential in this disease [59]. Among the molecules that presented high docking affinity (> -9.0 Kcal/mol) for this protein are the phytoestrogens glyceollin I, glyceollin II, glabridin, alizarin, glyceollin III, 6-prenylnaringenin, chrysin, glyceollin IV and coumestrol. The natural xenoestrogen that presented the best affinity score for PGR was 8-O-beta-D-glucopyranoside (-10.1 Kcal/mol), and the predicted contact residues in the binding site are Gln815, Lys822B, Glu695B, Leu758B, Gln725B, Ile699B, and Val698B. In addition, a hydrogen bond formation with Gln725B was predicted. This amino acid have been reported to participate in the interaction with estradiol in the hormone binding site [60], and other surrounding amino acids with the presence of hydrogen bonds and hydrophobic interactions.

On the other hand, the tumor suppressor protein BRCA1 plays a pivotal role in the DNA damage response through protein–protein interactions that regulates the cell cycle progression and homologous recombination for DNA repair [61], and has been proposed as a target for breast cancer treatments [62]. The molecules that obtained the highest docking affinity scores for this protein were glyceollin I, which is a phytoalexin found in soybean [63], and beta-carotene, present in many foods such as carrots, cantaloupe, squash, pawpaw, sweet potatoes and mangoes [64]. Consequently, some phytoestrogens have the potential to target this protein. Besides, there has been reported that diets based on a high consumption of plant-derived estrogenic compounds also modulate the expression of the BRCA1 gene [65].

Natural xenoestrogens from fungi and plant sources showed similar binding patterns and most of them are multi-target compounds interacting with a broad range of breast cancer proteins (Suppl. Fig. 1). Therefore, many phytoestrogens and mycoestrogens have the potential to interact with proteins involved in several pathways of this disease through a protein–ligand mechanism. Furthermore, the validation process demonstrated that AutoDock Vina [33] and our protocol is suitable for this system with a good power of prediction according to the specificity and selectivity obtained by the ROC curve for the selected affinity cut-off (< −7.5 Kcal/mol), and the predictability of the binding site and docking pose of our protocol compared to the crystallographic structure of one of the tested complexes ER-beta/genistein (PDB: 1X7J).

5. Conclusions

Many natural xenoestrogens exhibit good theoretical affinity for numerous proteins involved in breast cancer, with a similar pattern for phytoestrogens and mycoestrogens. Therefore, these compounds may be emulating multi-target mechanisms in the prevention, treatment or development of breast cancer by protein–ligand interactions on different signaling pathways involved in this disease.

6. Conflict of interest

The authors declare that there is no conflict of interest.

7. Funding

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.steroids.2018.03.010.

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