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Complete List of Authors:	Montes-Grajales, Diana; University of Cartagena, Bernardes, Gonçalo; University of Cambridge, Department of Chemistry Olivero-Verbel, Jesus; University of Cartagena,

Urban Endocrine Disruptors Targeting Breast Cancer Proteins

Diana Montes-Grajales [†, ‡], *Gonçalo J. L. Bernardes** [†, §] *and Jesus Olivero-Verbel** [‡].

[†]Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, United Kingdom, [‡]Computational Chemistry Group, Faculty of Pharmaceutical Sciences, University of Cartagena, Campus of Zaragocilla, Cartagena, 130015, Colombia, and [§]Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal.

KEYWORDS: Endocrine disrupting chemical, BPA, breast cancer, circular dichroism, high-throughput virtual screening.

ABSTRACT: Humans are exposed to a huge amount of environmental pollutants called endocrine disrupting chemicals (EDCs). These molecules interfere with the homeostasis of the body, usually through mimicking natural hormones leading to activation or blocking their receptors. Many of these compounds have been associated to a broad range of diseases including the development or increased susceptibility to breast cancer, the most prevalent cancer in woman worldwide, according to the World Health Organization. Thus, this article presents a virtual

high-throughput screening (vHTS) to evaluate the affinity of proteins related to breast cancer, such as ESR1, ERBB2, PGR, BCRA1 and SHBG, among others, with EDCs from urban sources. A blind docking strategy was employed to screen each protein-ligand pair by triplicate in AutoDock Vina 2.0, using the computed binding affinities as ranking criteria. The three-dimensional structures were previously obtained from EDCs DataBank and Protein Data Bank, prepared and optimized by SYBYL X-2.0. Some of the chemicals that exhibited the best affinity scores for breast cancer proteins in each category were 1,3,7,8-tetrachlorodibenzo-p-dioxin, bisphenol A derivatives, perfluorooctane sulfonic acid and benzo(a)pyrene, for catalase, several proteins, sex hormone-binding globulin and cytochrome P450 1A2, respectively. An experimental validation of this approach was performed with a complex that gave a moderate binding affinity *in silico*, the sex hormone binding globulin (SHBG) and bisphenol-A (BPA) complex. The protein was obtained using DNA recombinant technology and the physical interaction with BPA assessed through spectroscopic techniques. BPA binds on the recombinant SHBG, and this results in an increase of its alpha helix content. In short, this work shows the potential of several EDCs to bind breast cancer associated proteins, as a tool to prioritize compounds to perform *in vitro* analysis to benefit the regulation or exposure prevention by the general population.

1. INTRODUCTION

Endocrine disrupting chemicals (EDCs) are a broad range of molecules with potential to affect the endocrine system through different ways, metabolic, epigenetic and DNA damage[1b-e]. These chemicals were classified for first time in the 90's and were defined by the Environmental Protection Agency (EPA) as substances with the ability to interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for

the maintenance of homeostasis, reproduction, development, and/or behavior[2]. Currently, EPA estimates there exist approximately 10,000 EDCs among the common daily exposures that could impose any risk of disease[3]. Some critical points in the debate around these compounds include the potential to affect the health of humans and wildlife, as well as their progeny[4]; the massive volume of production of some of them and the chronic exposure to the general population through the environment and everyday products[5]. These points have enhanced the scientific concern regarding the safety of these chemicals in recent years.

The exposure to EDCs induces adverse effects, specially related to reproduction, development and different cancer types[6], including breast cancer[7] , which according to the World Health Organization (WHO) is the most common cancer in women, both in the developed and less developed world, estimating that worldwide over 508,000 women died in 2011 due to this disease (http://www.who.int/cancer/detection/breastcancer/en/index1.html). The classic hallmark mechanism in breast cancer is the activation of estrogen receptor, for that reason the responses due to EDCs exposures have been mainly explored and attributed to this signaling pathway[1a]. In general, the understanding on the underlying mechanisms of breast cancer[8] are limited because of its complexity and heterogeneity; nevertheless, there are some proteins recognized as important in its initiation and progression, specially hormone receptors such as estrogen receptor (ESR1), progesterone receptor (PGR), and other proteins, in particular, the human epidermal growth factor receptor 2 (HER-2), breast cancer type 1 susceptibility protein (BRCA1) and breast cancer type 2 susceptibility protein (BRCA2)[9].

Currently, thousands of environmental chemicals are subject to regulatory review for their potential as EDCs, and *in vitro* high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for whole animal tests[10]. However, computational toxicology approaches are needed to help to prioritize chemicals for screening[11], focusing on their interaction with critical disease pathways[12]. In this work we evaluated *in silico* the potential of EDCs to target breast cancer proteins, using a virtual high-throughput screening (vHTS) and an *in vitro* validation through spectroscopic methods for one of the predicted protein/ligand complexes.

2. RESULTS

2.1.Text mining

Based on the data mining performed on FABLE, 294 genes/proteins were found to be related to breast cancer (≥100 citations in PubMed) (Suppl. Table 1), and a total of 133 were suitable for docking studies and employed for further analysis. This selection was complemented adding proteins recently discovered as important in breast cancer, as well as with nuclear receptors and cell cycle regulation proteins. Finally, a total of 189 proteins were selected for the vHTS with EDCs.

2.2.High-throughput virtual screening

A total of 305 EDCs were docked with the proteins related to breast cancer selected in the previous step. The EDCs were classified according to the potential sources of exposures in four groups: dioxins and related molecules, plastics and other types of polymers, everyday products and miscellaneous. Results were ranked according to the affinity scores obtained in AutoDock

Vina[13]. Clustered heat maps of the affinity scores of EDCs against breast cancer proteins are presented for each category as supplementary material (Suppl. Figures 1-4).

The virtual screening allowed the identification of protein-ligand pairs with high affinity, candidates to be tested *in vitro* and *in vivo* for breast cancer in further studies. In the following sections the results in each group of EDCs, according to the exposure source, are presented.

2.3.Dioxins and related molecules

The main cluster in the heat map of the virtual screening of breast cancer proteins with dioxins and related molecules (Suppl. Figure 1 and Suppl. Table 2) indicates that these interact mostly with a group of proteins, including catalase (CAT); retinoic acid receptor beta (RARB); SHBG; PGR; phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform (PIK3CA); nuclear receptor ROR-gamma (RORC); nuclear receptor ROR-alpha (RORA); adiponectin (ADIPOQ); cytochrome P450 3A4 (CYP3A4); phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform (PIK3CG) and apolipoprotein D (APOD). Therefore, the underlying mechanisms by which these EDCs elicit their effects could be associated to signaling pathways involved in cell proliferation and oxidative stress, among others.

The complexes that presented the highest affinity score in this group are CAT/1,3,7,8-tetrachlorodibenzo-p-dioxin (Figure 1), and 2,3,4,7-tetrachlorodibenzofuran with the protein cytochrome P450 1A2 (CYP1A2, Figure 2). The top ranking of the complexes formed by EDCs in this group with breast cancer proteins is showed in Table 1.

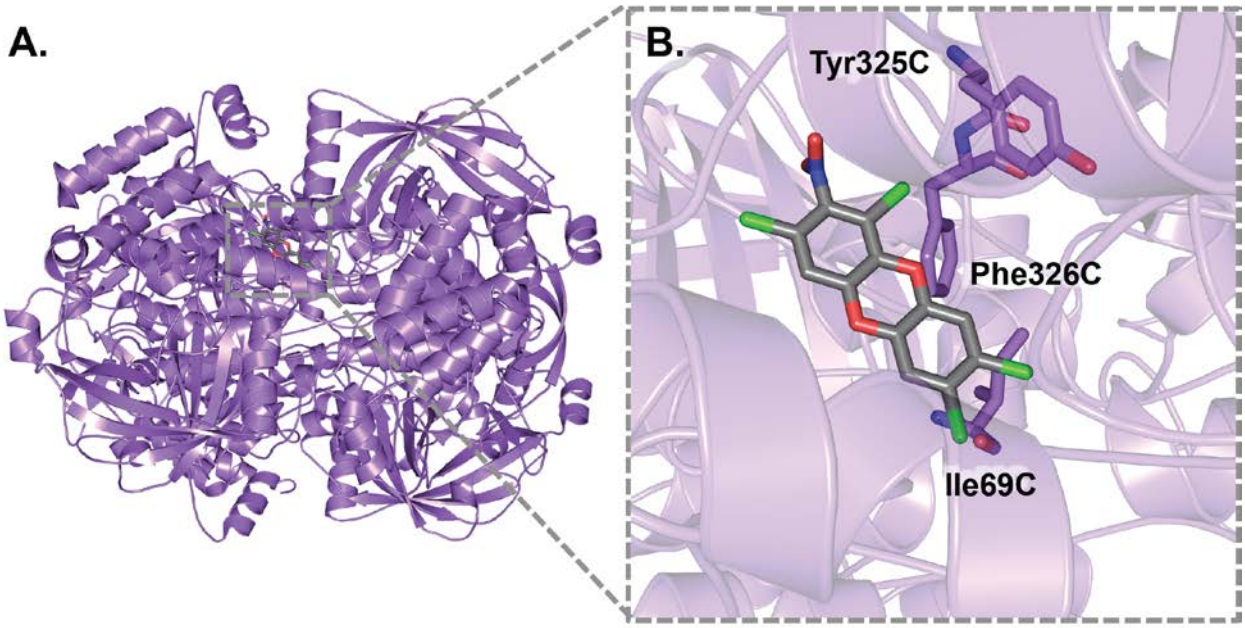

Figure 1. Three-dimensional view of the (A) CAT/1,3,7,8-tetrachlorodibenzo-p-dioxin, (B) showing the binding site and interactions predicted by LigandScout 3.1.

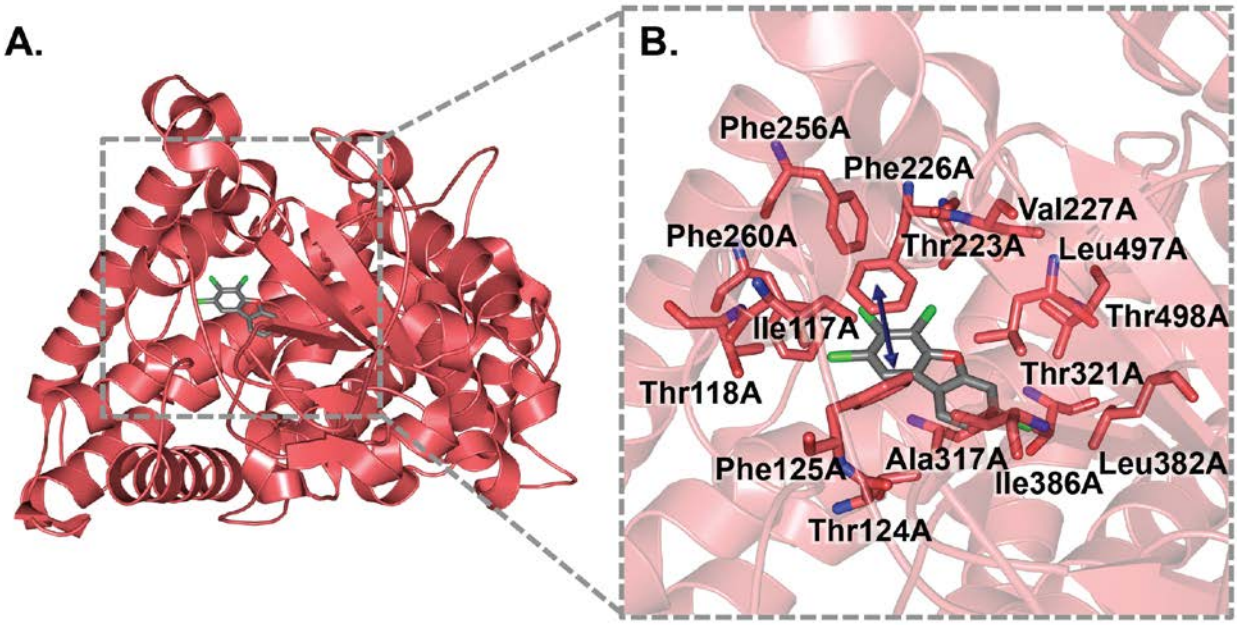

Figure 2. Three-dimensional view of the (A) CYP1A2/2,3,4,7-tetrachlorodibenzofuran, (B) showing the binding site and interactions predicted by LigandScout 3.1. The blue arrows represent aromatic ring interactions.

Table 1. Dioxins and related molecules with best affinity scores (<-10.0 kcal/mol) after docking with breast cancer associated proteins.

Short name	PDB	EDCs	CID	Affinity (kcal/mol)
CAT	1DGF	1,3,7,8-tetrachlorodibenzo-p-dioxin	149104	-10.3
CYP1A2	2HI4	2,3,4,7-tetrachlorodibenzofuran	55111	-10.2

2.4.Plastics and other types of polymers

The docking simulation showed that several EDCs in this category have the potential to bind breast cancer proteins (Suppl. Table 3 and Suppl. Figure 2). However, the derivatives of the well-known plasticizer BPA presented the best affinities, being located the top of the ranking table (Table 2).

Table 2. EDCs in plastics and other types of polymers with best affinity scores (<-10.0 kcal/mol) after docking with breast cancer associated proteins.

Short name	PDB	EDCs	CID	Affinity (kcal/mol)
SRC	2H8H	bisphenol M	3292100	-10.8
ESRRG	2E2R	bisphenol AF	73864	-10.8
RXRB	1H9U	bisphenol M	3292100	-10.7
VDR	1IE9	bisphenol A dimethacrylate	76739	-10.3
VDR	1IE9	bisphenol M	3292100	-10.3
SHBG	1F5F	bisphenol M	3292100	-10.3
RARB	4DM6	bisphenol M	3292100	-10.2

ESRRG	2E2R	bisphenol B	66166	-10.2
CYP1A2	2HI4	dihydroxymethoxychlor olefin	84677	-10.1
HBA1	1A01	2,4,6-triphenyl-1-hexene	45356241	-10.1
CYP1A2	2HI4	diphenyl-p-phenylenediamine	6319	-10.1

The chemical structures of BPA and its analogs are presented in Figure 3, as well as their potential targets in breast cancer according to the vHTS, and a functional protein association network generated by STRING[14].

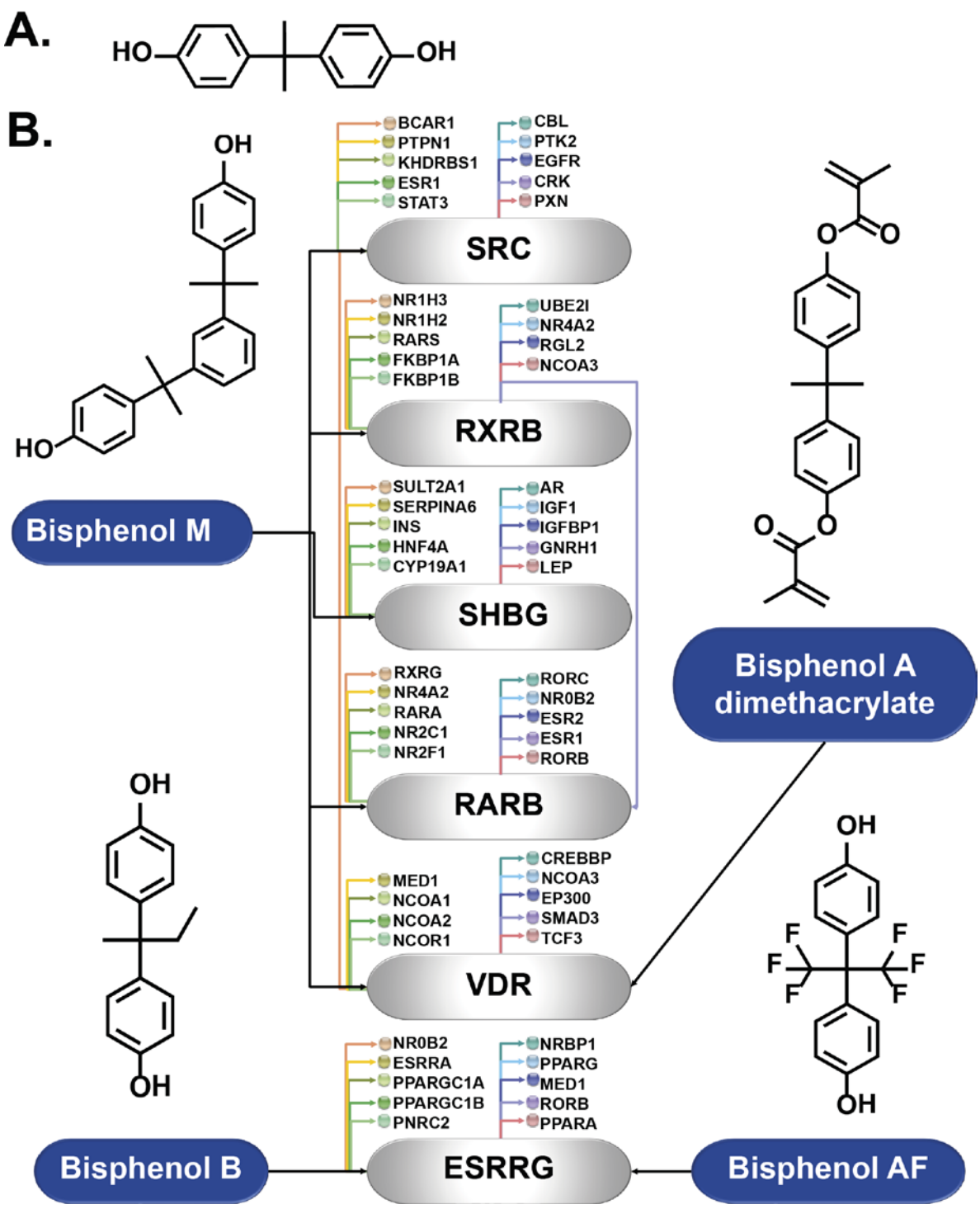

Figure 3. A) Chemical structure of BPA and B) its analogs showing the interactions with breast cancer proteins according to docking studies, and the analysis of the protein-protein interactions by STRING v.10[14].

The two complexes with the best affinity score were proto-oncogene tyrosine-protein kinase Src (SRC)/bisphenol M and estrogen-related receptor gamma (ESRRG)/ bisphenol AF (-10.8 kcal/mol). This is an interesting finding, as bisphenol AF (BPAF), is considered a new bisphenol analogue used as raw material in plastic industry; however, little is known about its occurrence in the environment and the potential associated risk[15]. The three-dimensional view of these complexes and their interactions are presented in Figures 4-5. Most of the interactions were hydrophobic in nature; however there are some hydrogen bond donor features that suggest the presence of hydrogen bonds between the ligand and the surrounding residues.

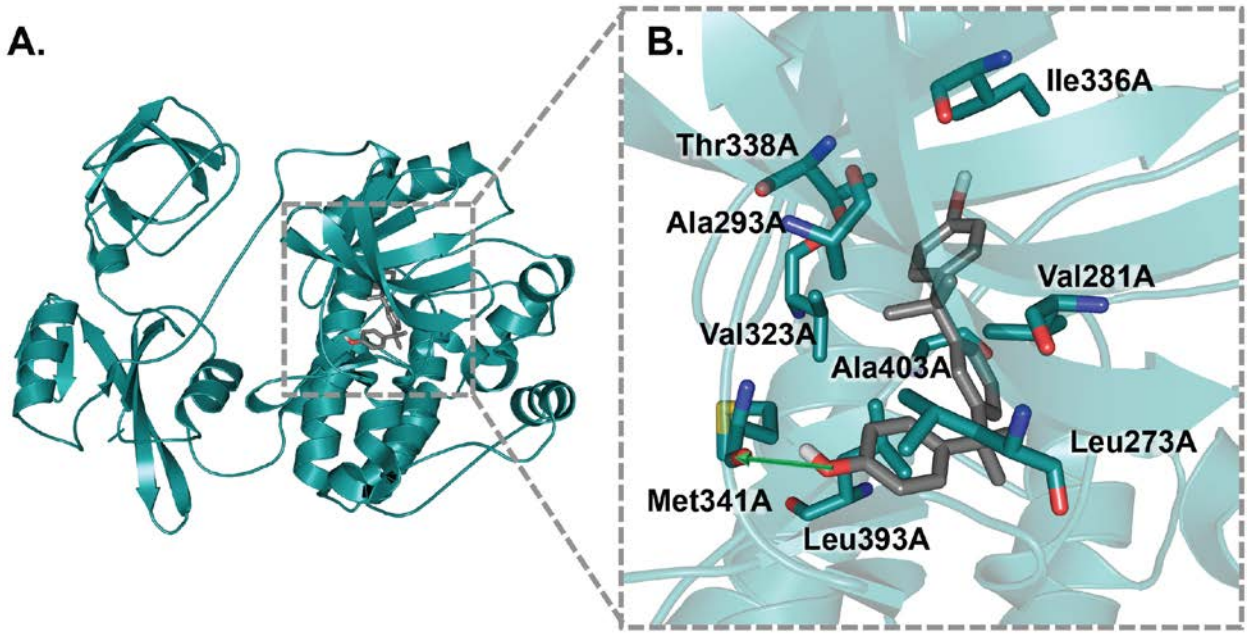

Figure 4. Three-dimensional view of the (A) SRC/bisphenol M complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features.

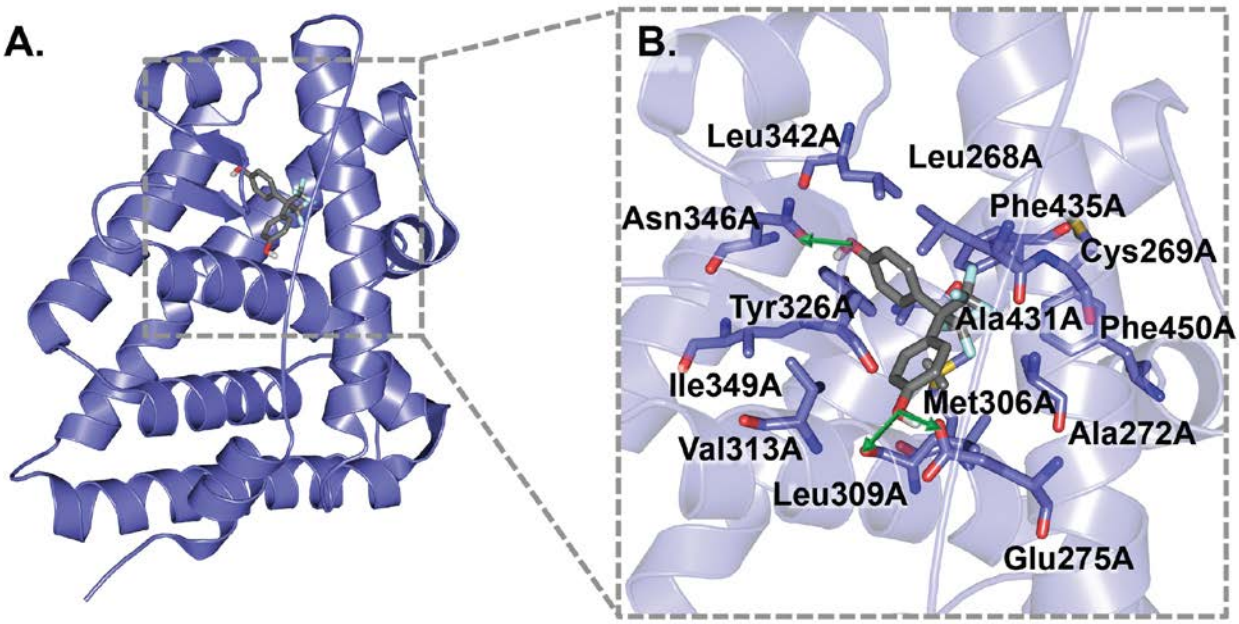

Figure 5. Three-dimensional view of the (A) ESRRG/bisphenol AF complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features.

Everyday products

A broad range of EDCs presents in cosmetics, household products, drugs and personal care products, among others, exhibited good affinity for proteins involved in breast cancer (Suppl. Table 4. and Suppl. Figure 3). The complexes with the best affinity scores in this group were the perfluorooctane sulfonic acid with the hormone transporter SHBG (Figure 6), and the RARB/AHTN complex (Figure 7).

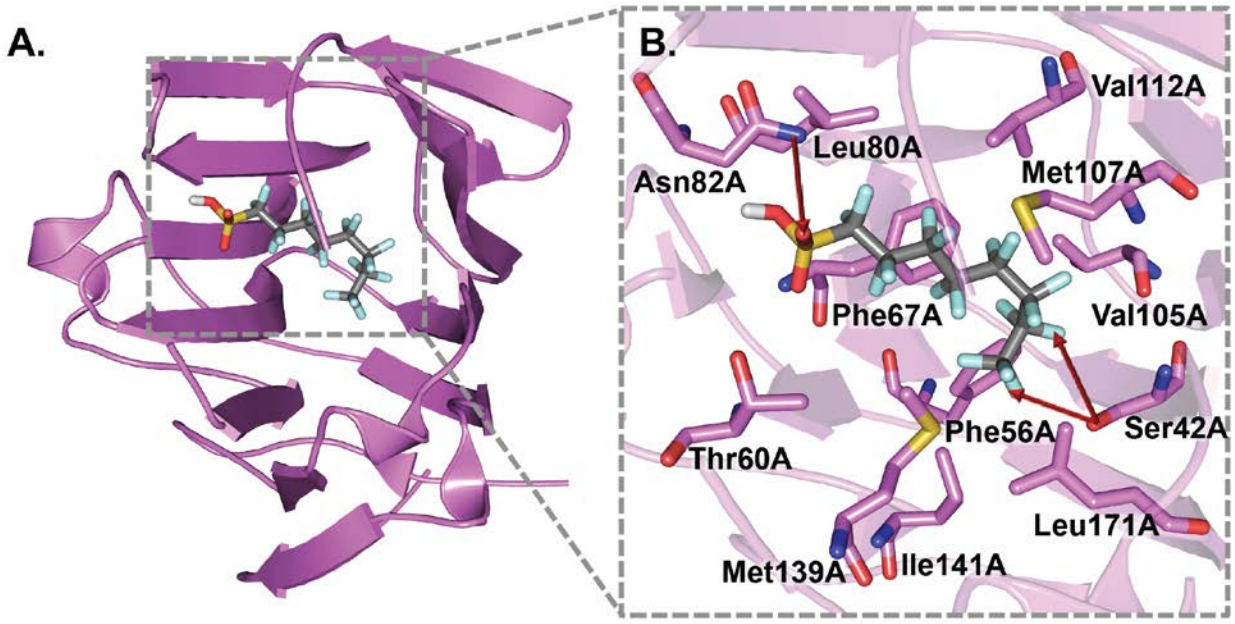

Figure 6. Three-dimensional view of the (A) SHBG/perfluorooctane sulfonic acid complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The red arrows represent hydrogen-bond acceptor features.

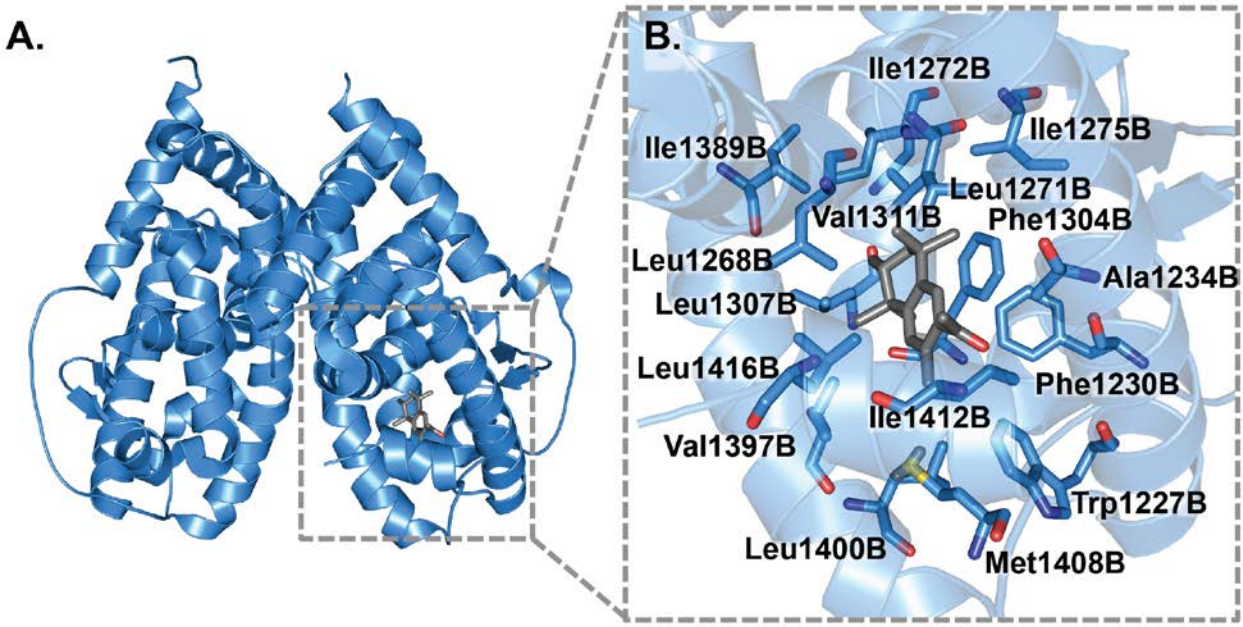

Figure 7. Three-dimensional view of the (A) RARB/AHTN complex, (B) showing the binding site and interactions predicted by LigandScout 3.1.

Other complexes that also exhibited good affinity scores are presented in Table 3.

Table 3. EDCs in everyday products with best affinity scores (<-10.0 kcal/mol) after docking with breast cancer associated proteins.

Short name	PDB	EDCs	CID	Keywords	Affinity (kcal/mol)
SHBG	1F5F	perfluorooctane sulfonic acid	74483	Household products	-10.4
RARB	4DM6	AHTN	89440	Cosmetics	-10.4
GSTP 1	3N9J	Emodin	3220	Drugs	-10.2
VDR	1IE9	3-(4-methylbenzylidene)camphor	6434217	Cosmetics	-10.2
SHBG	1F5F	3-(4-methylbenzylidene)camphor	6434217	Cosmetics	-10.1
SERPINB5	1WZ9	Triclocarban	7547	Antimicrobial, personal care products, household products	-10.1
CAT	1DGF	Emodin	3220	Drugs	-10.1

2.5.Miscellaneous

The molecules that exhibited the best affinities in this category were mostly related to combustion, some of them are 3-hydroxy-benzo(a)pyrene, (benzo(a)pyrene and 6-hydroxychrysene (Suppl. Table 5 and Suppl. Figure 4.). The best affinity score, was predicted for the complex CYP1A2/benzo(a)pyrene (Figure 8).

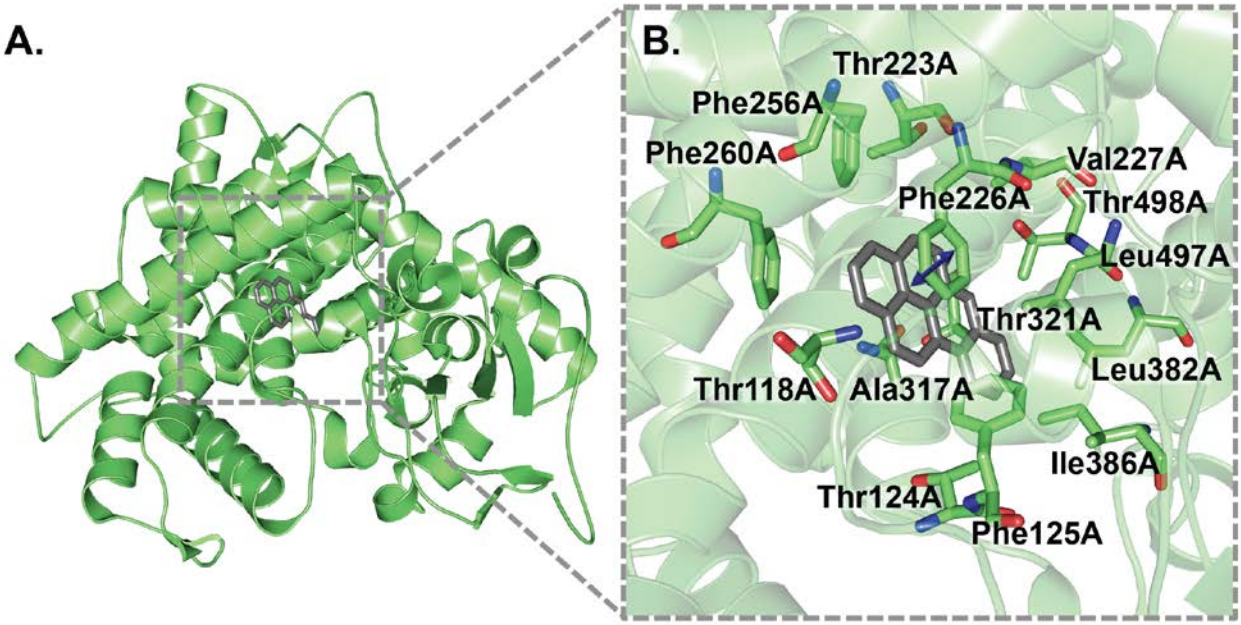

Figure 8. Three-dimensional view of the (A) CYP1A2/benzo(a)pyrene complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The blue arrows represent aromatic ring interactions.

Other complexes with EDCs from different sources also exhibited good affinity scores (Table 4). However, most of them were polycyclic aromatic hydrocarbons (PAHs).

Table 4. Miscellaneous EDCs with best affinity scores (<-10.0 kcal/mol) after docking with breast cancer associated proteins.

Short name	PDB	EDCs	CID	Keywords	Affinity (kcal/mol)
CYP1A2	2HI4	benzo(a)pyrene	2336	combustion, wood, cigarette	-13.2
CYP1A2	2HI4	benzanthrone	6697	industrial pollutant, combustion	-13.0
CYP1A2	2HI4	6-hydroxychrysene	37766	metabolite, combustion	-12.1
CYP1A2	2HI4	1-hydroxypyrene	21387	metabolite, combustion	-12.0
MYLK4	2X4F	3-hydroxy-benzo(a)pyrene	25890	combustion	-12.0
RARB	4DM6	benzo(a)pyrene	2336	combustion, wood, cigarette	-12.0
MYLK4	2X4F	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.9
SHBG	1F5F	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.8
CYP1A2	2HI4	1,9-dimethylphenanthrene	34454	environmental pollutant, cigarette	-11.8
CAT	1DGF	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.8
RARB	4DM6	benzanthrone	6697	industrial pollutant, combustion	-11.7
CAT	1DGF	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.6
CAT	1DGF	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-11.6
CHEK2	2W0J	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.5
CHEK2	2W0J	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-11.5
MYLK4	2X4F	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-11.5
SHBG	1F5F	2-hydroxybenzo(a)	42027	metabolite, combustion	-11.4

		pyrene			
CYP3A4	1TQN	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.4
CHEK2	2W0J	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.4
CYP3A4	1TQN	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.3
CYP3A4	1TQN	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-11.3
CYP2B6	3IBD	6-hydroxychrysene	37766	metabolite, combustion	-11.3
SHBG	1F5F	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.3
PIK3CA	3HHM	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.2
ADIPOQ	4DOU	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.2
ADIPOQ	4DOU	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-11.2
PIK3CA	3HHM	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.2
ESR2	1QKM	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.1
CHEK2	2W0J	6-hydroxychrysene	37766	metabolite, combustion	-11.1
ADIPOQ	4DOU	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.1
CYP1A2	2HI4	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.1
CDK2	1AQ1	3-hydroxy-benzo(a)pyrene	25890	combustion	-11.0
SHBG	1F5F	6-hydroxychrysene	37766	metabolite, combustion	-11.0
HPGDS	2VCQ	2-hydroxybenzo(a)	42027	metabolite, combustion	-11.0

		pyrene			
ADIPOQ	4DOU	6-hydroxychrysene	37766	metabolite, combustion	-11.0
CDK2	1AQ1	benzo(a)pyrene	2336	combustion, wood, cigarette	-11.0
CDK2	1AQ1	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.9
SRC	2H8H	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.9
RARG	2LBD	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.9
MYLK4	2X4F	6-hydroxychrysene	37766	metabolite, combustion	-10.9
MYLK4	2X4F	benzanthrone	6697	industrial pollutant, combustion	-10.9
SHBG	1F5F	benzanthrone	6697	industrial pollutant, combustion	-10.8
NME1	1UCN	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.8
RXRG	2GL8	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.8
SRC	2H8H	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.8
SRC	2H8H	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.8
PIK3CA	3HHM	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.8
CYP2B6	3IBD	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.8
RARB	4DM6	1-hydroxypyrene	21387	metabolite, combustion	-10.8
RXRB	1H9U	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.7
RXRB	1H9U	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.7
AR	2AM9	2-hydroxybenzo(a)	42027	metabolite, combustion	-10.7

		pyrene			
PPARD	2AWH	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.7
RXRA	2P1T	HHCB	91497	fragrance	-10.7
MYLK4	2X4F	1-hydroxypyrene	21387	metabolite, combustion	-10.7
STRADA/ MO25	3GNI	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.7
RARB	4DM6	1,9-dimethylphenanthrene	34454	environmental pollutant, cigarette	-10.7
HPGDS	2VCQ	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.6
HPGDS	2VCQ	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.6
AR	2AM9	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.6
APOD	2HZQ	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.6
APOD	2HZQ	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.6
RXRA	2P1T	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.6
CAMK2B	3BHH	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.6
RARB	4DM6	6-hydroxychrysene	37766	metabolite, combustion	-10.6
GSTP1	3N9J	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.6
CAT	1DGF	6-hydroxychrysene	37766	metabolite, combustion	-10.6
AR	2AM9	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.6
CAT	1DGF	benzanthrone	6697	industrial pollutant, combustion	-10.5
RARB	4DM6	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.5

NME1	1UCN	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.5
PPARD	2AWH	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.5
APOD	2HZQ	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.5
STRADA/MO25	3GNI	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.5
MAPK1	3I60	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.5
RPS6KB1	4L3J	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.5
RPS6KB1	4L3J	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.5
ESR2	1QKM	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.5
RPS6KB1	4L3J	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.5
CYP1A2	2HI4	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.4
NME1	1UCN	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.4
PPARD	2AWH	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.4
RXRG	2GL8	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4
RPS6KA1	2WNT	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4
PIK3CG	3MJW	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4
GSTP1	3N9J	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4
MET	1R0P	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4

RELA	2O61	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.4
CAMK2B	3BHH	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.4
CAMK2B	3BHH	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.4
NQO1	1D4A	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.3
RORA	1N83	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.3
PGR	1SQN	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.3
RXRA	2P1T	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.3
RORC	3L0L	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.3
PIK3CG	3MJW	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.3
CXCR4	3ODU	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.3
SHBG	1F5F	1-hydroxypyrene	21387	metabolite, combustion	-10.2
MET	1R0P	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
MET	1R0P	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.2
PGR	1SQN	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.2
PGR	1SQN	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
NR3C2	2AA2	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
AR	2AM9	6-hydroxychrysene	37766	metabolite, combustion	-10.2

PPARD	2AWH	6-hydroxychrysene	37766	metabolite, combustion	-10.2
MSH2	2O8B	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.2
RPS6KA1	2WNT	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.2
FGFR2	3B2T	2-hydroxybenzo(a) pyrene	42027	metabolite, combustion	-10.2
ARF-BP1	3H1D	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.2
MAPK1	3I60	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.2
MAPK1	3I60	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
GSTP1	3N9J	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
CHKA	2CKO	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
RARG	2LBD	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
HPGDS	2VCQ	aurin	5100	analytical chemistry	-10.2
RPS6KA1	2WNT	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
PIK3CA	3HHM	6-hydroxychrysene	37766	metabolite, combustion	-10.2
MTOR	4JSN	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.2
PIK3CA	3HHM	benzanthrone	6697	industrial pollutant, combustion	-10.1
CDK2	1AQ1	6-hydroxychrysene	37766	metabolite, combustion	-10.1
CAT	1DGF	1-hydroxypyrene	21387	metabolite, combustion	-10.1
SHBG	1F5F	1,9-dimethylphenant	34454	environmental pollutant, cigarette	-10.1

		hrene			
RORA	1N83	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.1
NR3C2	2AA2	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.1
NR3C2	2AA2	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.1
ESRRG	2E2R	3-monobromobisphenol A	656688	metabolite, flame retardants	-10.1
MYLK4	2X4F	1,9-dimethylphenanthrene	34454	environmental pollutant, cigarette	-10.1
ESR1	3ERT	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.1
RORC	3L0L	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.1
PPARG	3LMP	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.1
GSTP1	3N9J	benzanthrone	6697	industrial pollutant, combustion	-10.1
ADIPOQ	4DOU	benzanthrone	6697	industrial pollutant, combustion	-10.1
MTOR	4JSN	benzo(a)pyrene	2336	combustion, wood, cigarette	-10.1
ESR2	1QKM	3-hydroxy-benzo(a)pyrene	25890	combustion	-10.1
ADIPOQ	4DOU	bis(4-hydroxyphenyl)[(2-phenoxysulfonyl)phenyl]methane	130780	analytical chemistry	-10.1
CXCR4	3ODU	2-hydroxybenzo(a)pyrene	42027	metabolite, combustion	-10.1

2.6.BPA/SHBG interaction

The interaction of BPA or its derivatives and breast cancer associated proteins is of special concern, as humans are exposed to these chemicals through contact with different products that contain them, especially through foods that have been in contact with packaging materials that may release these pollutants[16]. Several authors have proposed these chemicals affect the function of multiple organs and increase the risk of breast cancer in mice[17]. In breast cancer, many proteins play a pivotal role in the pathogenesis of this disease. However, SHBG is a small and stable protein with high affinity for estrogens and androgens that has been found in breast tissue and cell lines through immunostaining[18], being the major and specific binding protein for testosterone and estradiol. Besides, decreased circulating levels of this protein have been observed in breast cancer patients possibly indicating higher bioavailable estrogens[19]. These were the main reasons we use to choose it to obtain it and to validate our docking predictions. Therefore, we selected the hormone transporter SHBG[20], as this exhibited a good affinity score in AutoDock Vina (-8.2 kcal/mol), to computationally assess their interactions and conformational changes by CD. Moreover, the data reported in the scientific literature suggest human SHBG may transport some xenostrogens into the plasma and modulate their bioavailability to cell tissues[21], which could disrupt the natural hormones balance.

The conformational analysis performed in LigandScout 3.1[22] suggests that BPA interacts in the binding pocket of SHBG (Figure 9). Most of the interactions of BPA were hydrophobic (Met139A, Leu80A, Val112A), although one residue in the binding site exhibited hydrogen bond

donor and acceptor features (Thr60A), and an aromatic ring interaction was predicted for BPA and one of the amino acids of the contact residues (Phe67A).

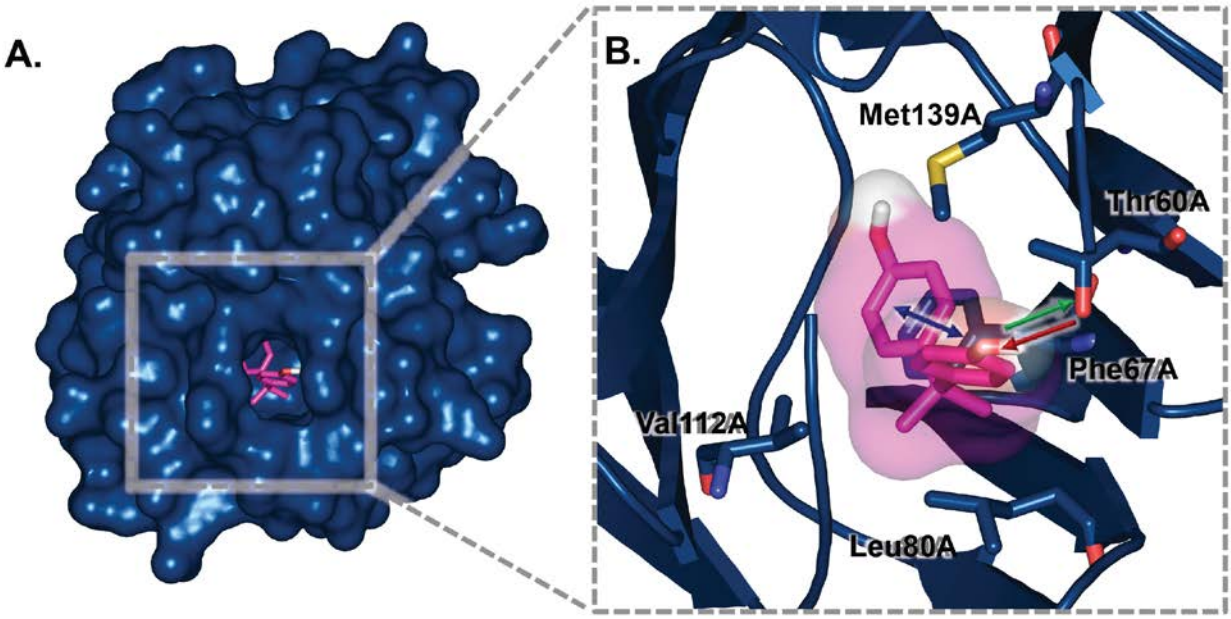

Figure 9. Three-dimensional view of the (A) SHBG/ bisphenol A complex, (B) showing the binding site and interactions predicted by LigandScout 3.1. The green arrows represent hydrogen-bond donor features, the red arrows show the hydrogen-bond acceptor features, and the blue arrows indicate aromatic ring interactions.

The three-dimensional view of the complex also indicates the position of BPA deep in the binding site, which can contribute to an elevated affinity. It should be pointed out that conformational changes should have occurred to allow this docking pose.

2.7.Protein expression and purification

Recombinant SHBG was expressed and purified from *E. coli* C41(DE3) cells using Dynabeads His-Tag Isolation and Pulldown. SDS- PAGE electrophoresis gel of the purified protein is presented in Suppl. Figure 5.

2.8.Microscale thermophoresis

After FPLC purification a clear separation between the protein and the dye, and a small portion of the total protein was labeled in the collected fractions (75-77) was observed in the spectra. Therefore, this study was used with qualitative purposes, which was enough to evaluate the functionality of the recombinant protein to bind the ligand.

The curve of the MST analysis and the fluorescence data is presented in Suppl. Figure 6. This shows that SHBG has the tendency to bind BPA at around 1µM of BPA, although a saturation point was not reached. This could be the result of a low protein labelling with a high non-labeled protein ratio, which makes more difficult to reach the saturation point as the total protein concentration was high in the sample.

2.9.Circular dichroism

The correct folding of the recombinant protein was assessed by CD. According to PDB[23], the secondary structure of SHBG (PDB ID: 1F5F) consist of 4% alpha helix and 40% beta sheets. This correlates with the values obtained by circular dichroism of the SHBG dissolved in PBS at pH 7.4: helix=6.3% and beta sheets=40.6% (strand 1=26.3% and strand 2=14.3%). The results of the secondary structure analysis, according to the DichroWeb[24] software are presented in Figure 10.

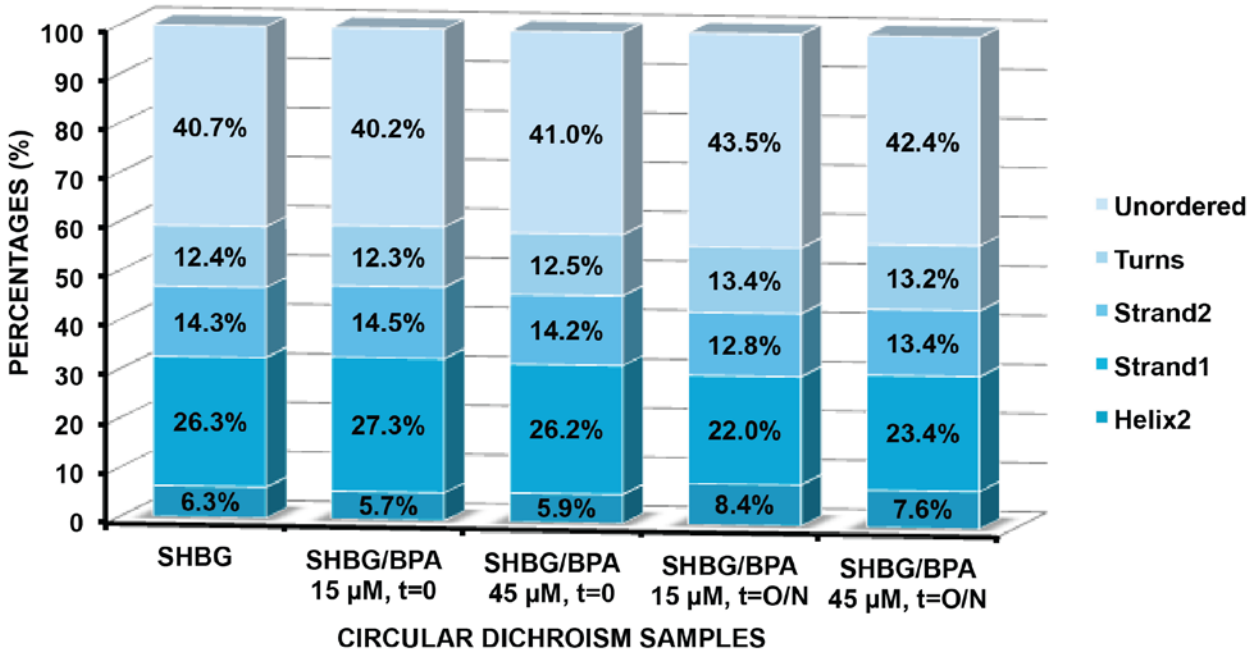

Figure 10. Changes in the secondary structure of SHBG after incubation with BPA.

At time zero (t=0), no main differences were observed for the contents of helix or strands of the samples of SHBG with BPA 1.5 x 10^{-5} and 4.5 x 10^{-5} M. However, after overnight incubation (t=O/N), an increase in the helix content of the protein was observed, with a percentage increase of more than 20% in both cases. The CD spectra for 1.5 x 10^{-5} and 4.5 x 10^{-5} M of BPA at time zero and overnight are presented in Figure 11. The Dunn's test did not show statistical significant differences between the medians of the SHBG sample and the SHBG/BPA samples in time zero. However, a statistical difference was revealed for the comparison of each sample after overnight incubation with the protein sample, and the SHBG/BPA samples in time zero (Table 5).

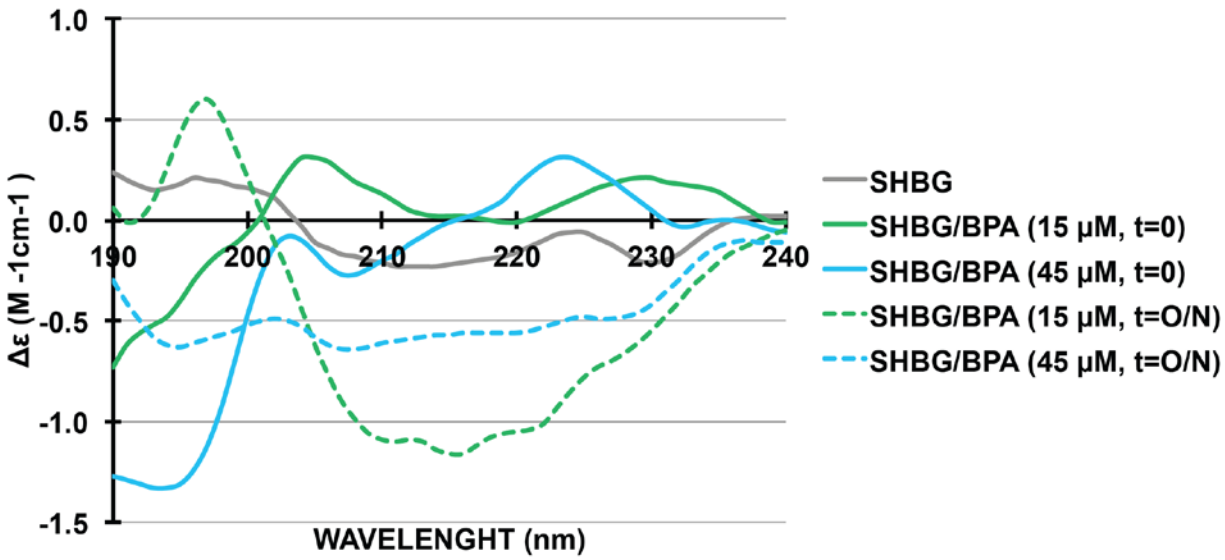

Figure 11. Processed circular dichroism spectra of SHBG and SHBG incubated with 1.5 x 10^{-5} and 4.5 x 10^{-5} M of BPA at time zero (t=0) and overnight (t=O/N) by DichroWeb[24].

Table 5. Results of the statistical analysis.

Dunn's multiple comparisons test	Mean rank diff,	Significant
SHBG vs. SHBG/BPA (15µM, t=0)	-26,71	No
SHBG vs. SHBG/BPA (45µM, t=0)	7,735	No
SHBG vs. SHBG/BPA (15µM, t=O/N)	101,5	Yes
SHBG vs. SHBG/BPA (45µM, t=O/N)	78,19	Yes
SHBG/BPA (15µM, t=0) vs. SHBG/BPA (45µM, t=0)	34,44	No
SHBG/BPA (15µM, t=0) vs. SHBG/BPA (15µM, t=O/N)	128,2	Yes
SHBG/BPA (15µM, t=0) vs. SHBG/BPA (45µM, t=O/N)	104,9	Yes
SHBG/BPA (45µM, t=0) vs. SHBG/BPA (15µM, t=O/N)	93,78	Yes
SHBG/BPA (45µM, t=0) vs. SHBG/BPA (45µM, t=O/N)	70,45	Yes
SHBG/BPA (15µM, t=O/N) vs. SHBG/BPA (45µM, t=O/N)	-23,33	No

3. DISCUSSION

In this work we studied the potential of EDCs from urban sources to target breast cancer proteins using an *in silico* approach. The macromolecules CAT, RARB, SHBG, PGR and PIK3CA presented the highest number of theoretical complexes with xenoestrogens (affinity ≤ -8.0 kcal/mol). These are pivotal in breast cancer and could be modulating the response to EDCs exposure through metabolic ways. CAT is an antioxidant enzyme that helps to control oxidative stress and DNA damage associated to EDCs exposure and breast cancer development[25]; RARB modulates the proliferation of breast cancer cells by limiting the growth and promoting the apoptosis[26]; SHBG transport steroid hormones through the bloodstream, limiting their free fraction, its binding with xenoestrogens could increase the bioavailable estradiol augmenting the risk of breast cancer[27]; PGR is a well-known marker that together with the ER status is used in the immunohistochemically prognosis of this disease, except for the triple negative subtype, that comprises around 15% of the cases[28]; and PIK3CA has an important role in neoplasia, and aberrations in its pathway or in the gene that codifies it results in an increased risk of cancer[29].

Several of the tested small molecules exhibited high docking affinity in computer simulations and should be prioritized for *in vitro* and *in vivo* assays, in addition some of them showed to be frequent hitters as they act as promiscuous compounds binding different targets[30]. The visual inspection of the docking affinity heat maps (see Supplementary material) indicates that the highest density of protein-ligand pairs with potential to interact between them is presented for dioxins and related molecules. Therefore, this may be the principal source of EDCs interacting

with the metabolic pathways involved in breast cancer. The number of potential targets was followed for the categories: miscellaneous, everyday products, and plastics and other types of polymers, respectively.

The compounds with the highest number of theoretical targets among the breast cancer proteins were the PAHs: 2-hydroxybenzo(a)pyrene, benzo(a)pyrene and 3-hydroxy-benzo(a)pyrene. In the group of plastics and other type of polymers, the most frequent hitter was bisphenol M, followed by the synthetic compound used in the production of thermally stable polyesters and polycarbonates, 4,4'-(octahydro-4,7-methano-5H-inden-5-ylidene) bisphenol, and the styrene trimer, 2,4,6-triphenyl-1-hexene, generated in the decomposition of plastics and released from containers to food[31]. For everyday products, the most promiscuous compound found was the perfluorooctane sulfonic acid. This is interesting, as EDCs can be interacting with many different pathways through protein-ligand interactions making their mechanisms and effects more difficult to understand and control.

The two complexes with the highest affinity scores in the group of dioxins and related molecules were: CAT/1,3,7,8-tetrachlorodibenzo-p-dioxin and CYP1A2/2,3,4,7-tetrachlorodibenzofuran. However, other chemicals in this group also presented very good affinity (≤-9.5 kcal/mol) for CYP1A2 (See suppl. Material Rank_Dioxins), which is in agreement with the scientific literature that indicates this kind of compounds have been found to bind and induce CYP1A2[32]. The eventual binding of 1,3,7,8-tetrachlorodibenzo-p-dioxin to CAT may have an effect in the hydrogen peroxide binding an then in the oxidative stress response.

EDCs from plastics and other types of polymers constitute a special category due to the increased use of these materials in human life, and the annual tendency to augment progressively their already high volumes of production[33]. One of the plasticizers that have received more attention in the last decade is the BPA, a monomer used in polycarbonate plastics and epoxy resins frequently found in food containers, which as a result of restrictive regulations has started to be replaced by other analogues[34]. Surprisingly, some of these derivatives (bisphenol M, bisphenol B, BPAF, dihydroxymethoxychlor olefin and bisphenol A dimethacrylate) are on the top of the complexes with highest docking affinity for breast cancer proteins *in silico* (Table 2), with greater affinity than BPA; therefore we suggest them as priority compounds to be tested against this disease. The two best affinity scores in this group were obtained for bisphenol M in complex with SRC, a protein that may promote the growth of tumor cells and is overexpressed in breast cancer[35]; and for BPAF with ESRRG, an orphan nuclear receptor widely implicated in the transcriptional regulation of energy homeostasis[36] that acts as tumor suppressor in several types of cancers[37]. Interestingly BPAF, has been found to promote breast cancer cell proliferation *in vitro*[38], and bisphenol M exhibited high predicted affinity (<-10.0 kcal/mol) for several proteins related to breast cancer.

A small number of compounds released from household products, cosmetics, drugs and personal care products were found to bind strongly breast cancer proteins *in silico* (<-10.0 kcal/mol, Table 3). Among these, perfluorooctane sulfonic acid, used in the manufacture of plastics, textiles, electronics, and many other industrial products[39]; AHTN found in cosmetics[40]; emodin a medicine that has been found to suppress tumor growth[41]; 3-(4-methylbenzylidene)camphor employed as UV filter in cosmetics[42]; and triclocarban, frequently

utilized in detergents and soaps as antimicrobial agent[43]. Nevertheless, there were many molecules in this group that presented moderate and weak binding that eventually could have a role in the homeostasis disruption in relation to the development or progression of this disease. On the other hand, several of the targeted proteins for these compounds are shared with the top list for plastics and related molecules, as well as with the proteins most frequently found in theoretical EDCs-breast cancer protein complexes, some of them are SHBG, RARB, CAT and vitamin D3 receptor (VDR).

In contrast, a large number of PAHs, derived from combustion, occupied the top ranking of complexes formed for miscellaneous EDCs. Some of these are the well-known carcinogen benzo(a)pyrene, benzanthrone, 6-hydroxychrysene and 1-hydroxypyrene. This type of molecules has been classically associated to breast cancer through genetic damage by DNA adduct formation[44], however they could also be acting throw protein-ligand interactions. In addition, other molecules from diverse sources also exhibited high affinity for breast cancer proteins such as: HHCB, used in fragrances[40]; the chemical indicator aurin[45]; and the flame retardant 3-monobromobisphenol A[46]; among others (Table 4).

The computer simulations of the docking poses and protein-ligand interaction analysis in the top complexes of each category showed that the most frequent binding forces were due to hydrophobic, hydrogen bond donor, hydrogen bond acceptor and aromatic ring features of the ligands and contact residues in the binding site. The conformational study of the protein-ligand pair selected for *in vitro* assays, SHBG/BPA, showed that the predicted interacting residues (Met139A, Thr60A, Phe67A, Leu80A and Val112A), are the same found experimentally on the

binding site of endogenous hormones and xenoestrogens, that binds this protein with high affinity[47]. Therefore, BPA may occupy the pocket for natural estrogens in the body, reducing the fraction of bound estrogen and increasing its bioavailability[48], which is considered a risk factor to develop this disease[18].

The recombinant human SHBG used in our spectroscopic analysis was obtained without any mutation, and according to the MST analysis it was functional, and their folding was the expected, as assessed by the circular dichroism spectra of the native protein without the ligand. The differences in the circular dichroism spectra of SHBG and SHBG with BPA may be the result of both BPA binding and the dimerization of the protein, as has been reported with steroid ligands that bind this protein with high affinity[49]. The main conformational change occurred after BPA binding was an increment of the alpha helix content after overnight incubation, this result has also been observed for the binding of this molecule to other proteins, such as human serum albumin[50]. The Dunn's test showed statistical differences in the spectra for overnight incubation with BPA, but not for the immediate CD recorded after BPA addition (time zero), suggesting that the reaction between BPA and SHBG, may be time-dependent. This is not surprising, as related studies regarding binding assays, used the same physiologic conditions of pH and temperature as in our protocol, as well as an incubation time of at least one hour[48a].

The results discussed in this section showed the plausibility of some EDCs to interact with proteins involved in signaling pathways regarding breast cancer. Therefore further analysis in this field is needed to support regulatory actions, being of special concern the case of the new BPA analogs.

4. CONCLUSIONS

This study presents a virtual screening that helps to understand how endocrine disruptors could be plausible ligands for the breast cancer proteome. This approach provided, important candidates to be evaluated *in vitro* against models of this disease, predicting protein patterns of proteins that are more commonly affected by EDCs. Many of the predicted hits have no scientific reports regarding its experimental evaluation; therefore further studies are required in this field. Computational studies are an important tool that can improve the velocity and efficiency of the evaluations of EDCs overcoming the known limitations regarding the high number of this compounds and diseases associated to them. Several endocrine disruptors have the theoretical capability to bind proteins related to breast cancer, some of them are more promiscuous being able to bind different targets, therefore this are proposed as priority for experimental test. BPA binds SHBG, showing a conformational change of the protein.

5. EXPERIMENTAL SECTION

A four-step approach has been used to identify EDCs with potential to target breast cancer proteins. This includes the selection of the proteins by data mining tools, vHTS, evaluation of *in silico* interactions and experimental validation by spectroscopic methods.

5.1.Text mining

Proteomics studies of breast cancer have provided relevant information regarding the identification of proteins of interest in the diagnosis and treatment of this disease[51], even though there is not a consensus list or repository with this information. Therefore, a meta-analysis was performed to identify proteins and genes associated with breast cancer in PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), using the text mining tool FABLE (Fast Automated

Biomedical Literature Extraction; http://fable.chop.edu/), employing the key words "breast cancer". The results were ranked according to their number of hits, and a cutoff of ≥100 references related to breast cancer in PubMed was used. This methodology was carried out in order to include the proteins more frequently cited with a role in breast cancer. Some important nuclear receptors and cell cycle targets were also included, as they have been reported to be deregulated in human breast cancer[1a, 52].

5.2.High-throughput virtual screening

Molecular docking simulations were carried out to find EDCs with the potential to target breast cancer proteins *in silico*. Calculations were run on Linux RHEL6 for IBM Power, utilizing a System X rack with Power 730 processors. AutoDock Vina[13], an open-source program for molecular docking and virtual screening, was used to assess the binding affinity of each EDC/protein complex, as this software has been largely employed and formally validated for target identification of this kind of molecules, having a high prediction performance when compared to experimental data[53], not only regarding the binding affinity but also the crystallographic binding modes[54]. A total of 305 EDCs from urban sources were downloaded from EDCs DataBank[55] and docked with the selected breast cancer proteins, using the same protocol reported in our previous work for inverse virtual screening[1a]. The crystallographic coordinates of the proteins, with resolution suitable for docking studies (in average, 2.0 Å), were downloaded from Protein Data Bank (PDB)[23] in pdb text format file which were then employed for preprocessing in SYBYL-X 2.0 program package (Tripos, St. Louis, MO). All ions, water molecules and other substructures were removed from the coordinated files and the biopolymer structure preparation tool was used for analyzing and fixing the 3D-structures with default

settings. Optimization of the proteins was also carried out by the same software package employing the Powell method, Kollman united and Kollman all atoms force fields, AMBER charges, dielectric constant 1.0, NB cutoff 8.0, maximum interactions 100 and termination gradient 0.001 kcal/mol. The resultant pdb file was then submitted to AutoDock Tools[56] for preparing the grid parameters and the required pdbqt files for the docking studies. Kollman charges and polar hydrogen atoms were added to the three-dimensional structures of the proteins, the grid was centered in the macromolecule and the size adjusted to include the whole protein surface with a spacing of 0.357 Å. The structures of the EDCs were directly downloaded from EDCs DataBank[55], and used for virtual screening in Autodock Vina[13]. Each ligand/protein pair was docked by triplicate and the best Affinity scores of the single runs were then used to calculate the averages and the results ranked according to these values.

The EDCs were separated according to their source of exposure in four groups: dioxins and related molecules, plastics and other types of polymers, everyday products and miscellaneous. The results ranked and the best protein/ligand complexes of each category were then selected to determine their non-covalent interactions by computer aided simulations. In addition, a hierarchical clustering, which is a powerful tool to blindly explore proteomic data[57], was performed in R (http://www.r-project.org/) for each group, using the “heatmap.2” function of the gplot library[58]. The color key of the heat map, was selected using the RColorBrewer package of R, to present in red the protein-ligand pairs with strong and moderate docking affinity (-15.0 to -8.0 kcal/mol), in white those with weak affinity (-8.0 to -7.0 kcal/mol) and in blue the proteins-ligand pairs that are not likely to interact according to the simulation (-7.0 to 5.0 kcal/mol). As a result, patterns in the behavior of EDCs against breast cancer proteins and vice versa were

identified. The employed affinity cutoff (< -8.0 kcal/mol) was the same used in the protocol of our previous studies with AutoDock Vina[13], which allowed a good correlation between *in silico* binding affinity and experimental data[54, 59].

5.3.Evaluation of protein-ligand interactions

An *in silico* approach was employed to evaluate the contact residues participating in the protein-ligand interaction in selected complexes formed between breast cancer related proteins and EDCs. This was achieved using LigandScout 3.1[22]. The best docking pose of the EDC resulting from the docking with the target protein by AutoDock Vina[13] was isolated in AutoDock Tools[56] and merged with the optimized file of the protein structure in pdb format by SYBYL X-2.0 (Tripos, St. Louis, MO). This file was then used as input in LigandScout, the parameters utilized for the pharmacophore features and interacting residues were those established by default in the program.

In order to validate our protocol, a protein-ligand pair, was selected for studying its interactions *in silico* and *in vitro*. The criteria used to pick the complex that exhibited an Affinity score near the cutoff (around -8.0 kcal/mol), a common EDC with generalized exposure in the population and a protein with certain impact in breast cancer.

5.4.Experimental validation of the existence of protein-ligand interactions for a predicted EDC/protein complex

The protein ligand interaction between bisphenol A (BPA), one of the most common EDCs used in plastics[60], and the steroid transporter protein sex hormone-binding globulin (SHBG) was

analyzed by LigandScout software[22], using the same protocol described above; and the validation of the binding and conformational changes of this complex was achieved by spectroscopic methods[61], employing microscale thermophoresis (MST) and circular dichroism (CD). The protein was obtained by recombinant DNA technology and BPA (≥99%; 239658-50G) was purchased from Sigma Aldrich.

5.5.Protein expression and purification

The blank plasmid pET15-MHL was transfected into the gene for human SHBG (PDB: 1F5F) (obtained from Eurofins, Ebersberg), and then harbored in *Escherichia coli* DH5α cells. The plasmid was purified and transformed into chemically competent *E. coli* C41(DE3). Transformed cells were plated onto LB agar containing 100 μg/mL of ampicillin, and incubated overnight at 37 °C. A single colony was used to inoculate 100 mL of LB/ampicillin for overnight growth. 20 mL of the starting solution was used to inoculate 400 mL of LB/ampicillin medium. The culture was incubated at 37 °C (180 rpm) until the A_{600} was 0.6–0.8 at which point protein expression was induced by the addition of isopropyl 1-thio-β-d-galactopyranoside (IPTG) to a final concentration of 0.5 mM. After overnight incubation at 20 °C[62], the induced cells were harvested by centrifugation, the pellet was resuspended in cold PBS buffer and washed twice, resuspended again in 15 mL of cold lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 50 mM Tris, pH 8.0), maintained in agitation 30 min (4°C), and centrifuged (20 min, 12,000 rpm). This pellet was discarded and the supernatant was placed on ice, and stored at −20 °C until protein purification (http://www.abcam.com/index.html?pageconfig=resource&rid=11379). Magnetic beads (Dynabeads His-Tag Isolation and Pulldown (Life technologies) were used for protein purification according to the protocol provides by the supplier

(http://tools.lifetechnologies.com/content/sfs/manuals/DynabeadsHisTagIsolationPulldown_man.pdf). A dialysis procedure was also employed to remove small molecules using a slide-A-Lyzer Dialysis Cassette, 20K MWCO (Thermo scientific) according to the protocol provided by the supplier, and a SDS electrophoresis gel was run to check the presence and quality of the protein.

5.6.Protein storage

In order to avoid frequent freezing and thawing, the protein sample was divided into aliquots, stored frozen at -20°C, and kept at physiological pH (PBS buffer pH 7.4).

5.7.Microscale Thermophoresis (MST)

In order to evaluate the likelihood of a possible functionality of BPA binding on SHBG, a qualitative MST analysis was carried out. This method reflects the directed movement of particles in a microscopic temperature gradient, and enables the analysis of molecular interactions in solution at microliter scale[63], with low sample consumption[64].

The SHBG protein was labeled using the blue fluorescent dye NT-495-NHS (NanoTemper Technologies) and incubated for 1 hour at room temperature. In order to separate the free dye from the protein, a superdex 200 10/300GL column and an AKTA PURE FPLC system (GE Healthcare) were utilized with PBS buffer pH 7.4 at 0.5 mL/s flow rate. Fractions of 0.25 mL were collected with a fraction collector F9-R (GE Healthcare). The labeled SHBG was incubated for 5 min at room temperature with different concentrations of BPA in PBS containing 5% ethanol as vehicle, testing 1:2 concentrations from 0.03 to 1000 nM (1 µM). The samples (3–5 µL) were loaded into hydrophilic glass capillaries (Monolith NT Capillaries) and the

thermophoresis analysis was performed (LED 40%, IR laser 20%) using a NanoTemper Monolith NT.115 (NanoTemper Technologies) instrument. The capillary used for the MST analysis with BPA and SHBG was the hydrophilic one, as this generated the expected Gaussian curve.

5.8.Circular Dichroism

A stock solution consisted of a 30 mM BPA solution in absolute ethanol. The spectra of SHBG (1 μM in Tris pH 7.4) was analyzed to characterize the non-bonded folding state of the recombinant protein, by comparison with the information provided in PDB[23]. The changes in the secondary structure of the protein after BPA binding were accessed by recording the circular dichroism (CD) spectra of SHBG with two concentrations of BPA (1.5×10^{-5} and 4.5×10^{-5} M) at time zero and after overnight incubation. The solutions were prepared adding 0.2 and 0.6 μL of the BPA stock solution (30 mM) to 400 μL of SHBG (1 μM in Tris pH 7.4), respectively. The TRIS buffer was used as background and subtracted from all runs. The CD spectra were recorded using the applied photophysics Chriscan, wavelength (nm): 190-350; step: 0.5; time per point (s): 0.5 repeats: 5, temperature: 25°C and auto-subtraction of the background. All the spectra were recorded in both delta A and mdeg units, with 5 repetitions, using a quartz cuvette of 10 mm path length, volume: 0.7 mL, inside: 2 mm; spectral range 190-2500 nm; 4 transparent windows and dimensions 45 mm x 12.5 mm x 12.5 mm, in a nitrogen atmosphere[50]. The data was recorded using the programs Prodata viewer, Chirascan and APLData Converter. The circular dichroism spectra were saved as ASCII files (.kin file format), employing the Pro-data Chriscan software, and utilized as input files in DichroWeb[24] for the secondary structure determination. The parameters used were: file format=free, input units=milidegrees, initial wavelength=190, final

wavelength=350, wavelength step=0.5, lowest datapoint to use in the analysis=190, analysis program=CONTIN, reference set=SMP 180 (Optimized for 190-240 nm), Optional scaling factor=1, Output units=delta epsilon. A statistical analysis of the results of the CD was performed by GraphPad Prism 6.01 (GraphPad Software Inc., San Diego, CA, USA). A non-parametric multiple comparison test Kruskal-Wallis was performed using the raw data of the spectra recorded for SHBG and SHBG/BPA, besides a post-hoc Dunn's multiple comparison test of the row data based on Kruskal-Wallis analysis was also applied to compare the medians of the individual groups. Statistical significance was accepted at $p \leq 0.05$.

ASSOCIATED CONTENT

Supporting Information.

Suppl. Table 1. List of proteins and genes associated to breast cancer in the literature according to FABLE (Accessed: 12 dic 2012).

Suppl. Table 2. Docking affinity scores for dioxins and related molecules with breast cancer associated proteins.

Suppl. Table 3. Docking affinity scores for EDCs from plastics and other polymers with breast cancer associated proteins.

Suppl. Table 4. Docking affinity scores for EDCs from everyday products with breast cancer associated proteins.

Suppl. Table 5. Docking affinity scores for miscellaneous EDCs with breast cancer associated proteins.

Suppl. Figure 1. Cluster heat map of the docking affinity scores obtained for EDCs in the group of dioxins and related molecules (columns) and breast cancer proteins (rows).

Suppl. Figure 2. Cluster heat map of the docking affinity scores obtained for EDCs in the group of plastics and other types of polymers(columns) and breast cancer proteins (rows).

Suppl. Figure 3. Cluster heat map of the docking affinity scores obtained for EDCs in the group of Everyday Products (columns) and breast cancer proteins (rows).

Suppl. Figure 4. Cluster heat map of the docking affinity scores obtained for EDCs in the group of Miscellaneous (columns) and breast cancer proteins (rows).

Suppl. Figure 5. SDS-PAGE of the purified SHBG protein.

Suppl. Figure 6. Microscale thermophoresis (MST) study of the protein-ligand interaction of fluorescently labeled SHBG and BPA.

AUTHOR INFORMATION

Corresponding Author

*J O-V: e-mail: joliverov@unicartagena.edu.co, phone: +57 5 6698179.

*GB: e-mail: gb453@cam.ac.uk, phone: +44 0 1223336305.

Author Contributions

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ABBREVIATIONS

ADIPOQ, adiponectin; APOD, apolipoprotein D; BPA, bisphenol A; BPAF, bisphenol AF; BRCA1, breast cancer type 1 susceptibility protein; BRCA2, breast cancer type 2 susceptibility protein; CAT, catalase; CD, circular dichroism; CDK2, cyclin-dependent kinase 2; CHEK2, serine/threonine-protein kinase Chk2; CYP1A2, cytochrome P450 1A2; CYP2B6, cytochrome P450 2B6; CYP3A4, cytochrome P450 3A4; EDCs, endocrine disrupting chemicals; EPA, Environmental Protection Agency; ESR1, estrogen receptor; ESRRG, estrogen-related receptor gamma; FABLE, fast automated biomedical literature extraction; GSTP1, glutathione S-transferase P; HBA1, hemoglobin subunit alpha; HER-2, human epidermal growth factor receptor 2; HPGDS , hematopoietic prostaglandin D synthase; HTS, high-throughput screening; IPTG, isopropyl 1-thio-β-d-galactopyranoside MET , hepatocyte growth factor receptor; MMP9,

matrix metalloproteinase-9; MST, microscale thermophoresis; MYLK4, myosin light chain kinase family member 4; NME1, nucleoside diphosphate kinase A; NP2, nitrophorin-2; NQO1, NAD(P)H dehydrogenase [quinone] 1; NR3C1, glucocorticoid receptor; OH-PCBs Hydroxylated polychlorinated biphenyls; O/N, overnight; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; PDB, Protein Data Bank; PGR, progesterone receptor; PIK3CA, phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform; RARB, retinoic acid receptor beta; RXRG , retinoic acid receptor RXR-gamma; RORA, nuclear receptor ROR-alpha; RORC, nuclear receptor ROR-gamma; RPS6KB1, ribosomal protein S6 kinase beta-1; SHBG, sex hormone-binding globulin; SRC , proto-oncogene tyrosine-protein kinase Src; VDR, vitamin D3 receptor; vHTS, virtual high-throughput screening; WHO, World Health Organization.

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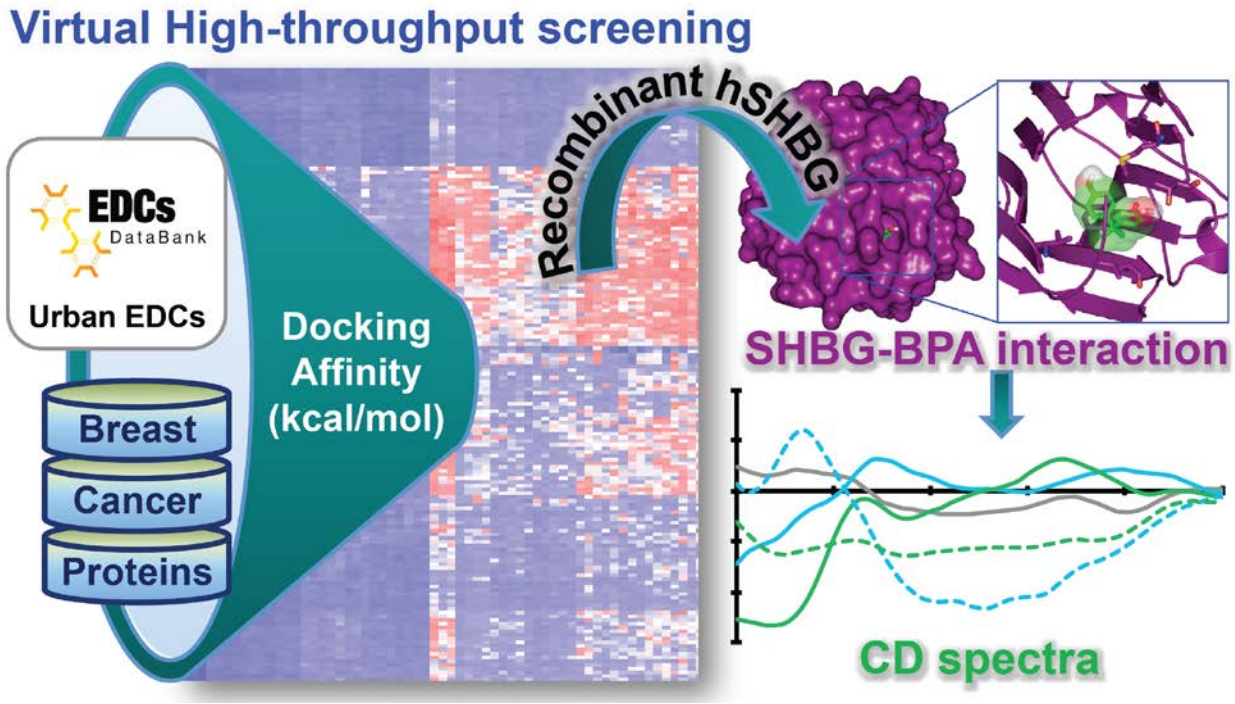
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