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The UV-filter benzophenone-1 inhibits 17β-hydroxysteroid dehydrogenase type 3: Virtual screening as a strategy to identify potential endocrine disrupting chemicals

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ABSTRACT

The prevalence of male reproductive disorders and testicular cancer is steadily increasing. Because the exposure to chemicals disrupting natural hormone action has been associated with these diseases, it is important to identify endocrine disrupting chemicals (EDCs) and their targets of action. Here, a 3D-structural database that can be applied for virtual screening approaches to facilitate the identification of EDCs was constructed. The database was screened using pharmacophores of 17β-hydroxysteroid dehydrogenase type 3 (17β-HSD3), which catalyzes the last step of testosterone synthesis in testicular Leydig cells and plays an essential role during male sexual development. Among other chemicals, benzophenone (BP) UV-filters were predicted as potential 17β-HSD3 inhibitors. Biological analyses revealed (2,4-dihydroxyphenyl)-phenylmethanone (also known as benzophenone-1, BP-1) as an inhibitor of human 17β-HSD3 (IC_{50} 1.05 μM). BP-1 also efficiently blocked conversion of androstenedione to testosterone by mouse and rat 17β-HSD3 in whole-organ enzyme assays. Moreover, BP-1 antagonized the testosterone-dependent activation of androgen receptors (IC_{50} 5.7 μM), suggesting synergistic anti-androgenic effects of BP-1 by preventing testosterone formation and blocking receptor activation. In addition, analyses of several commonly used UV-filters on estrogen- and androgen-metabolizing 17β-HSD enzymes revealed 3-benzylidene camphor (3-BC) and 4-methylbenzylidene camphor (4-MBC) as low micromolar 17β-HSD2 inhibitors. In conclusion, screening of virtual chemical structure libraries can facilitate the identification of compounds interfering with hormone action. The potential disruption of 17β-HSD enzyme function by the UV-filters BP-1, 3-BC and 4-MBC requires further investigation and should be considered for safety assessment of these chemicals.

1. Introduction

In the industrialized world, there is a rising incidence of male reproductive disorders, designated as testicular dysgenesis syndrome and comprising cryptochidism, hypospadias, decreased testosterone concentrations in adulthood, infertility, and testicular cancer [1–3]. It has been proposed that testicular dysgenesis syndrome may originate from disturbed hormone homeostasis during fetal life [2,4]. Testosterone, together with anti-Müllerian hormone and insulin-like factor 3, is one of three hormones secreted by fetal testes and responsible for male sexual differentiation. Androgen-dependent processes include testicular development, masculinization of the brain, and adult sexual behavior [4]. Therefore, disruption of hormone action by endocrine disrupting chemicals (EDCs) during development could be potentially harmful later in adult life.

It has been shown that fetal exposure of male rats with the plasticizers di(2-ethylhexyl)phthalate or di(n-butyl)phthalate causes sexual maldevelopment due to abnormal aggregation of Leydig cells and decreased testosterone levels [5,6]. Moreover, a study in adult men demonstrated an association of low sperm

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counts and changes in testicular volume with the exposure to pesticides [7]. However, the mechanisms of action of many EDCs remain unclear, as many of these chemicals, including phthalates, do not interfere with androgen receptor (AR) function [8].

So far, the major focus of studies on EDCs was on the disruption of nuclear hormone receptor function, especially on sex-steroid receptors [9,10]. However, environmental chemicals can interfere with multiple steps of steroid hormone regulation, including biosynthesis, plasma binding, transport into/out of the target cell, intracellular binding and metabolism, receptor activation, signaling, and degradation. Thus, novel, efficient methods are required to analyze multiple potential targets and to test large numbers of chemicals for interference with these targets. Due to time and cost limitations, the biological *in vitro* testing of all potentially harmful chemicals is unrealistic. Therefore, computational tools that are also used in drug discovery, might be applied in a first step and used as filters to predict potential EDCs and to decrease the number of chemicals to be tested *in vitro* [11–13].

In the present project, a 3D-structure database of potential EDCs that is suitable for *in silico* screening using pharmacophore models of any potential target protein was generated. This library was then tested in a virtual screening experiment choosing 17β-HSD3, which is almost exclusively expressed in testicular Leydig cells and is one of the major enzymes responsible for the synthesis of testosterone from 4-androstene-3,17-dione (AD) during male development [14]. The importance of 17β-HSD3 is best demonstrated in male patients with loss-of-function mutations, suffering from pseudohermaphroditism, with malformations of the male reproductive organs and elevated ratios of AD to testosterone [14,15]. Because of the development of female external genitalia they are often raised as females until puberty [15,16]. Thus, impaired testosterone synthesis due to inhibition of 17β-HSD3 by EDCs could potentially be harmful for sexual development. Despite the importance of 17β-HSD3 in early development and prepuberty, only few studies investigated the interaction of this enzyme with environmental chemicals [17,18].

2. Materials and methods

2.1. Hardware and software specifications

Calculations were performed using PipelinePilot version 6.1.5 (Accelrys Inc., San Diego, CA, USA) on a personal computer running Fedora 8 Linux and with the CatDB module of Catalyst 4.11 (Accelrys Inc., San Diego, CA, USA) on a personal computer running Fedora 6 Linux.

2.2. Construction of a 3D-structure database of potential EDCs

A database of 3D-structures of potential EDCs was constructed. As data source, the publicly available version 2 of the endocrine disruptors priority setting database of the U.S. Environmental Protection Agency was used [19]. This database collects mainly information from *in vitro* and *in vivo* investigations of effects of chemicals on sex-steroid receptors. Briefly, two lists of EDCs containing chemical names and CAS numbers were retrieved from the database (Fig. 1A). To convert these data into a format suitable for virtual screening, all entries in the unified database were queried against the PubChem database with a cheminformatic toolkit for mining biomedical knowledge and using the chemical names or CAS numbers [20,21]. The structures that returned as hits from the PubChem database were merged, the combined entries filtered for errors, and the final screening database was constructed using Catalyst catDB module.

The PubChem Substance database was downloaded as a collection of compressed SD files by FTP [22], containing over 17.8 million entries from different databases. "PubChem Substances" was chosen over "PubChem Compounds", because it contains only one record for each structure. This facilitates the removal of erroneous structures based on the assumption that a structure reported in multiple original databases has a higher probability to be correct.

For the EDC database, lists containing names and CAS numbers were obtained and checked for duplicates. Searches were performed on the local copy of "PubChem Substances" by comparing the names with the fields PUBCHEM_EXT_DATASOURCE_REGID and PUBCHEM_SUBSTANCE_SYNONYM, and the CAS numbers with the field PUBCHEM_GENERIC_REGISTRY_NAME. Initial hits were processed as follows: small counter ions and solvent molecules were removed and the molecules neutralized, compounds labeled by radioactive isotopes were removed and the remaining hits first checked for chemical consistency (no R-groups; presence of chemical structure) and then for structural consistency. To perform these connectivity matches, the IUPAC International Chemical Identifier (InChI) was used [23,24], which can be computed from within

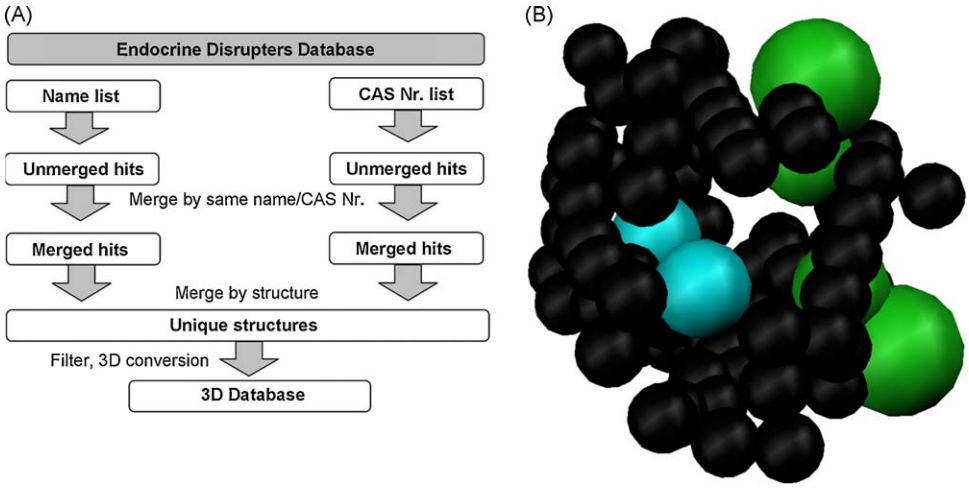


Fig. 1. (A) Scheme of the construction of the virtual 3D-endocrine disruptor library. A 'hit" refers to a compound of the initial lists, for which a corresponding 3D-structure could be found in PubChem. Before 3D conversion, inorganic compounds and substances that the used virtual screening program could not handle (e.g. Pb-containing compounds) were removed from the compound collection (filtered). (B) Pharmacophore model of 17β-HSD3. Chemical features are color-coded: hydrogen bond acceptors – green, hydrophobic features – cyan, exclusion volume spheres – grey.

PipelinePilot. InChI describes chemical structure in the form of a string, consisting of layers and sub-layers, including information about chemical formula, connectivity, charges, protonation states, stereochemistry, isotopes, and tautomerism. While these strings are usually not humanly readable, InChI has some advantages when comparing two structures: the use of different layers allows choosing from different levels of similarity or identity for comparison. Most tautomeric forms can be compared, although keto–enol and ring-chain tautomerisms are currently not supported. Since stereo-information is not always included for the retrieved search results originating from different databases, all stereo-information from the structures was removed and compared via their stereo-stripped InChI strings. Identical compounds were merged, and previously available stereo-information was restored, choosing those structures which had the more completely defined stereo-information (and thus the longer SMILES string). Initial 3D-structures were generated in PipelinePilot, and then exported as structure data (SD) files. Catalyst databases were generated using the "fast" algorithm and a maximum number of 255 conformations per molecule.

2.3. Construction of an inhibitor-based pharmacophore model of 17β-HSD3

3D-structures for the compounds used for model development were generated within Catalyst 4.11. For each compound, a set of low-energy conformers was computed using the "best" mode with a maximum of 250 conformers per molecule and an energy maximum of 20 kcal/mol above the calculated minimum. For pharmacophore model generation, the HipHop Refine algorithm of Catalyst 4.11 was employed. This algorithm aligns highly active compounds from the training set in a three-dimensional way so that common chemical functionalities lie at similar locations. Based on the alignment, pharmacophore features are placed where common chemical functionalities among the active compounds are observed. Chemical features considered in the hypothesis generation process were hydrogen bond acceptors and hydrophobic groups (Fig. 1B). Next, "forbidden" areas for compounds (exclusion volume spheres) are strategically placed where steric interactions contributing to biological (in-)activity can be approximated. This information is taken from the inactive compounds included in the training set (Table 1). For example, if compound ABC is active and ABCD is not – even though ABCD contains the same pharmacophore as ABC – differences in the steric bulk are estimated to carry responsibility for the absence of ABCDs biological activity. Accordingly, an exclusion volume sphere is placed at the 3D-location of D.

2.4. Virtual screening of the EDC database against the 17β-HSD3 inhibitor pharmacophore model

The EDC database was virtually screened using the "best flexible search" algorithm of Catalyst 4.11. Hits returned from this search were ranked by computing the geometric fit of the compound into the pharmacophore model.

2.5. Chemicals

2-Ethylhexyl-α-cyano-β-phenylcinnamate, 4-tert-butyl-4′-methoxy-dibenzoylmethane, benzylsalicylate, octylsalicylate, ethyl-2-cyano-3,3-diphenylacrylate, and ρ-aminobenzoic acid were purchased from Sigma–Aldrich Chemie GmbH, Buchs, Switzerland. Benzophenone derivatives, 3-BC ((3Z)-3-benzylidene-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one) and 4-MBC ((3E)-1,7,7-trimethyl-3-[(4-methylphenyl)methylidene]bicyclo[2.2.1]heptan-2-one) were obtained from Merck AG, Glattbrugg, Switzerland, and were of the highest purity available (>98%).

2.6. Measurement of 17β-HSD3 and 17β-HSD5 activities

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4.5 g/l glucose, 50 U/ml penicillin/streptomycin, 2 mM glutamine, and 1 mM HEPES, pH 7.4, and transfected by the calcium phosphate precipitation method with expression plasmids for human 17β-HSD3 and 17β-HSD5. After 24 h, 15,000 cells/well were distributed in 96-well plates pre-coated with poly-L lysine. Cells were allowed to adhere for 24 h, followed by washing with serum- and steroid-free (doubly charcoal-treated) medium and incubation for 30–60 min to measure 17β-HSD3 activity. To measure 17β-HSD5 activity, cells were incubated for 3–5 h. The incubation time was chosen such that enzyme activity was in the linear range and total substrate conversion was below 30%. In both assays 200 nM [1,2,6,7-^{3}H]-AD was used as substrate, in the presence of the corresponding UV-filter. UV-filters (stock solutions in DMSO) were freshly diluted in serum- and steroid-free medium and used immediately. Final solvent concentrations were kept below 0.1%. Reactions were stopped by adding 2 mM unlabeled AD and testosterone dissolved in methanol, followed by separation of steroids by TLC using chloroform:ethyl acetate at a ratio of 3:1 as solvent system. Product formation was detected by scintillation counting.

To assure that the observed inhibition was not caused by cell death, cytotoxicity of UV-filters at 40 μM was assessed in parallel to the activity assay under identical conditions. Cells were incubated with the corresponding UV-filter for 5 h at 37 °C, washed with PBS and incubated in fresh medium containing 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After conversion of MTT, the medium was removed and 200 μl of DMSO were added to the insoluble fraction. Conversion of MTT was kept below OD 0.9$^{(A570-A690)}$.

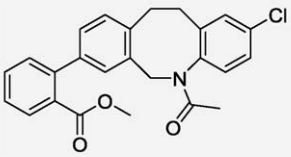
2.7. Activity assays for 17β-HSD1 and 17β-HSD2

HEK-293 cells were cultured and transfected as described above with plasmids for human 17β-HSD1 and 17β-HSD2. 17β-HSD2 activity was measured in intact cells seeding initially 6000 cells/well and treating as described above. Cells were incubated for 40 min at 37 °C with 200 nM radiolabeled estradiol. For 17β-HSD1 activity measurements, transfected cells were centrifuged, and the pellet was resuspended in a buffer containing 50 mM potassium phosphate, 20% glycerol and 1 mM EDTA, followed by sonication and immediate determination of the conversion of 200 nM radiolabeled estrone to estradiol in the presence of 500 μM cofactor NADPH for 15 min at 37 °C. Reactions were stopped by adding 2 mM of unlabeled estrone and estradiol, followed by separation of steroids by TLC and scintillation analysis.

2.8. Ex vivo mouse and rat testis assays to determine testosterone formation

Testes were isolated from mice and rats, decapsulated, and 40 mg fresh tissue was incubated with 1 μM [1,2,6,7-^{3}H]-AD in 200 μl DMEM containing 1% FCS. Reactions were carried out at 37 °C for 15 min in the presence of vehicle (0.1% DMSO) or the corresponding concentration of benzophenone-1 (BP-1). Steroids were extracted from the medium with ethyl acetate, the solvent was evaporated under nitrogen flow and steroids were dissolved in 2 mM unlabeled AD and testosterone. Results were normalized per milligram of tissue.

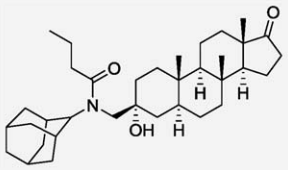
Table 1
Training compounds for the 17β-HSD3 inhibitor pharmacophore model.



1

IC_{50} = 0.02 nM [55]

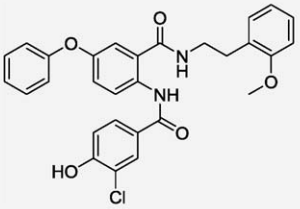
2-(5-acetyl-2-chloro-5,6,11,12-tetrahydrodibenz[b,f]azocin-8-yl)-benzoic acid methyl ester



2

IC_{50} = 35 nM [56]

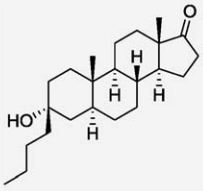
N-[[(3?,5?)-3-hydroxy-17-oxoandrostan-3-yl]methyl]-N-(tricyclo[3.3.1.13,7]dec-2-ylmethyl)-butanamide



3

IC_{50} = 60 nM [57]

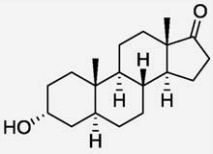
2-[(3-chloro-4-hydroxybenzoyl)amino]-N-[2-(2-methoxyphenyl)ethyl]-5-phenoxy-benzamide



4

IC_{50} = 116 nM [58]

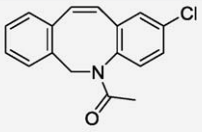
3α-butyl-3-hydroxy-5α-androstan-17-one



5

IC_{50} = 182 nM [59]

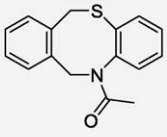
androsterone



6

IC_{50} = 240 nM [55]

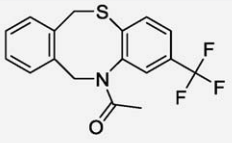
5-acetyl-2-chloro-5,6-dihydro-dibenz[b,f]azocine



7

IC_{50} = 400 nM [55]

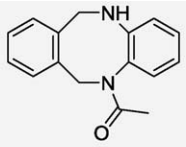
12-acetyl-11,12-dihydro-6H-dibenzo[b,f][1,4]thiazocine



8

IC_{50} = 2810 nM [55]

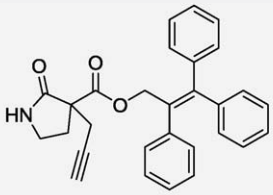
12-acetyl-11,12-dihydro-2-(trifluoromethyl)-6H-dibenzo[b,f][1,4]thiazocine



9

IC_{50} = 4740 nM [55]

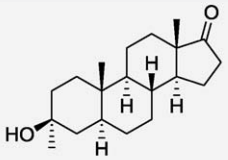
5-acetyl-5,6,11,12-tetrahydro-dibenzo[b,f][1,4]diazocine



10

IC_{50} = 9150 nM [60]

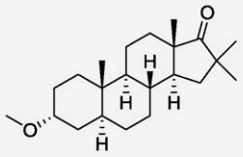
2,5-dioxo-3-(2-propyn-1-yl)- 3-pyrrolidinecarboxylic acid 2,3,3-triphenyl-2-propen-1-yl ester



11

inactive [58]

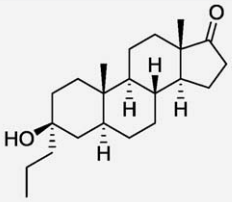
3β-hydroxy-3-methyl-5α-androstan-17-one



12

inactive [61]

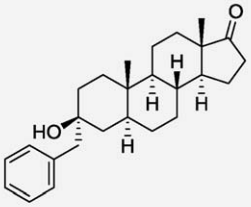
3α-methoxy-16,16-dimethyl-5α-androstan-17-one



13

inactive [58]

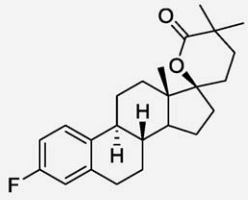
3β-hydroxy-3-propyl-5α-androstan-17-one



14

inactive [58]

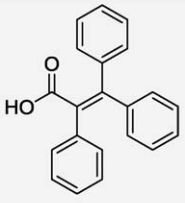
3β-hydroxy-3-phenylmethyl-5α-androstan-17-one



15

inactive [62]

3-fluoro-17-hydroxy-23-methyl-19,21-dinorchola-1,3,5(10)-triene-23-carboxylic acid δ-lactone



16

inactive [60]

triphenylacrylic acid

2.9. Gene transactivation assay with human androgen receptor

HEK-293 cells (150,000 cells/well) were incubated in 24-well plates for 24 h and transfected with plasmid for human AR (0.1 μg/well), MMTV-lacZ β-galactosidase reporter (0.15 μg/well) and pCMV-LUC luciferase transfection control (0.05 μg/well). After 24 h, cells were washed twice with charcoal-treated DMEM and incubated with 0.2 nM testosterone and various concentrations of

UV-filters for 24 h. Cells were washed with PBS and lyzed with 50 μl lysis buffer (Tropix, Applied Biosystems, Foster City, CA, USA) supplemented with 0.5 mM dithiothreitol, and lysates (20 μl) were analyzed for luciferase (D-luciferin, sodium salt, Synchem) and β-galactosidase activities (Galacto-light plus kit, Tropix).

3. Results

3.1. Generation of an EDC database

The Endocrine Disruption Priority Setting Database (EDC-DB) Version 2 consists of 142,975 entries including environmental chemicals such as pesticides, commercial chemicals, cosmetic ingredients, food additives, nutritional supplements, and other substances of priority for endocrine disruptors screening. The database contains no chemical structures, but compound names and CAS numbers. To convert these data into a 3D-compound library suitable for virtual screening, a series of Pipeline Pilot scripts that assigned this information to chemical structures taken from the PubChem database were developed (Fig. 1A).

Name-database: From the initial 142,975 entries in the EDC-DB, 65.1% had a name indexed. Of these compounds, entries lacking a CAS number, such as "roofing paper", "putrescent whole egg solids", "red pepper", and others, were excluded. For proper assignment of chemical structures, names containing "polymer", "derivative", or "analogue" were discarded. Additionally, names shorter than four characters were filtered because they represent truncated names that cannot be identified unambiguously. After this filtering process 62,305 (43.6%) unique compounds remained. The names of these unique compounds (in many cases more than one name per compound) were submitted to search the PubChem Substances database, consisting of 17.8 million entries in the version used. From this search, more than 85,000 (46.6%) substances returned as hits (unmerged hits).

CAS-database: In parallel, all entries with a CAS number (97.0%) were submitted to the PubChem Substances search resulting in approximately 179,000 hits (83.5%, unmerged hits). Thus, the CAS number list covered nearly all unique compounds. Only 3060 entries were exclusively found using their name and not by their CAS number.

Merging by the same name/CAS number: The two resulting databases still included duplicate entries (several compounds with the same names or CAS number), mostly because of entries from different original databases. To find the correct and unique structure for all entries, the structures with the same names/CAS numbers were directly compared using their InChI codes. For an accurate stereochemistry representation, in cases where different amounts of stereochemistry information was present in the PubChem Substance hit list, the structure with the highest amount of stereo-information was kept. Using this workflow, the majority of multiple entries could be assigned to unique structures. However, 4.5% (name-list)/7.9% (CAS number list) returned multiple structures for the same name or CAS number. In these cases, a preferred structure among all solutions from the PubChem was sought. Structure solutions provided from different original database sources were preferred over structures from single sources. That way, over 80% of all problematic entries could be assigned satisfactorily. Finally, salts and mixture substances were merged by removing small counter ions, mixture components, and neutralizing compounds. Structures with wrong valences were excluded.

Merge by structure: In the last step of database preparation, the structures from the names and CAS number lists were merged.

Filtering and 3D conversion: Before the generated and filtered database was suitable for pharmacophore-based screening, compounds including B, Al, Si, Ge, As, Se, Sn, Sb, Te, or Pb were excluded. Additionally, compounds were required to possess at least one carbon atom and a molecular mass between 70 and 1000 g mol^{-1} l^{-1}. The remaining 76,754 compounds (63.9% of the initial search list) constituted the basis for 3D-database generation. Of these, the catDB algorithm of Catalyst 4.11 was able to convert 76,677 compounds (99.9%) into the 3D EDC-DB.

Table 2
UV-filters recognized as hits from screening the EDC database using the 17β-HSD3 pharmacophore model.

Name	Synonym	BestFit
2-Ethylhexyl-α-cyano-β-phenylcinnamate	α-Phenylcinnamate	3.329
2,2′-Dihydroxy-4-methoxybenzophenone	Benzophenone-8	3.162
2-Hydroxy-4-(octyloxy)benzophenone	Benzophenone-12	2.577
5-Chloro-2-hydroxybenzophenone	Benzophenone-7	2.523
2,2′-Dihydroxy-4,4′-dimethoxybenzophenone	Benzophenone-6	2.401
2-Hydroxy-4-methoxybenzophenone	Benzophenone-3	2.392
Octylsalicylate		2.059
Benzylsalicylate		1.936
4-tert-Butyl-4′-methoxy-dibenzoylmethane	Avobenzone	1.928
Ethyl-2-cyano-3,3-diphenylacrylate	Etocrilene	1.539
2,2′,4,4′-Tetrahydroxybenzophenone	Benzophenone-2	0.474

3.2. Construction of a ligand-based pharmacophore model for human 17β-HSD3 inhibitors

A chemical feature-based 3D-pharmacophore model for 17β-HSD3 inhibitors based on 16 compounds with known effect on the enzyme was generated (Table 1). Compounds **1**–**3** were classified as highly active inhibitors for the model generation process, compounds **4**–**7** were included in the medium active inhibitors group, which is used by the model generation algorithm for deciding which of the many computed models are subjected to model refinement by exclusion volume sphere placement, and the remaining compounds **8**–**16** served for exclusion volume spheres calculation. The resulting 3D-pharmacophore model consisted of two hydrogen bond acceptors, two hydrophobic features, and 76 exclusion volume spheres forming a binding pocket shape in which active compounds must fit (Fig. 1B).

3.3. Virtual screening of the EDC database

Screening of the generated 3D-structural database of EDCs with the 17β-HSD3 pharmacophore yielded, among other chemicals, several representatives of the so-called organic UV-filter chemicals (Table 2). UV-filters are structurally diverse chemicals (Fig. 2) capable to absorb UVA and UVB light and thus protect from UV-radiation. Several UV-filters have previously been found to interfere with endocrine functions, and evidence for a disruption of sex-steroid receptor function by some of these chemicals was provided [25–28].

3.4. Inhibition of 17β-HSD3-dependent testosterone synthesis by UV-filters in transfected HEK-293 cells

Because the virtual screening with the 17β-HSD3 pharmacophore yielded several benzophenone-derived UV-filters, the effect of this group of chemicals was investigated in bioassays. HEK-293 cells transiently expressing human 17β-HSD3 were incubated with 20 μM of the corresponding chemical and 200 nM AD for 45 min, followed by determination of testosterone formation. Several benzophenone UV-filters inhibited the conversion of AD to testosterone (Table 3). BP-1 was most potent and concentration-dependently inhibited 17β-HSD3 (IC_{50} 1.05 μM) (Table 4 and Fig. 3A). Weaker effects were observed for BP-2 (IC_{50} 18.1 μM) and BP-6 (39% inhibition of 17β-HSD3 activity at 20 μM). In contrast, BP-3, which is

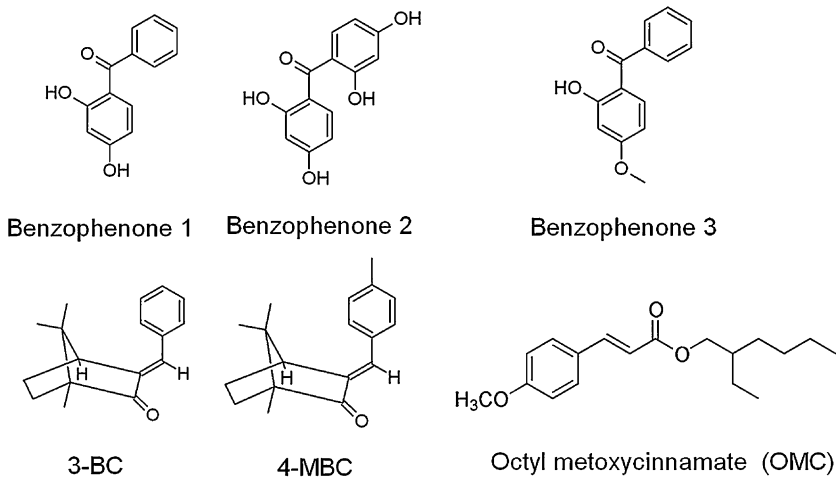


Fig. 2. Chemical structures of selected UV-filters tested in this study.

metabolized *in vivo* to BP-1 [29], did not significantly inhibit 17β-HSD3. BP-4, BP-7, BP-8 and BP-12 showed very weak or no inhibitory effect. In addition, the widely used UV-filters 3-benzylidene camphor (3-BC), 4-methylbenzylidene camphor (4-MBC) and octyl-methoxycinnamate (OMC) were tested. 3-BC and 4-MBC were not recognized by our pharmacophore model, and they were included in the bioassay because they showed anti-androgenic activity in other systems [25,26]. While OMC did not significantly reduce 17β-HSD3-dependent testosterone formation, 3-BC and 4-MBC both concentration-dependently inhibited 17β-HSD3 with estimated IC_{50} of 33.3 and 10.7 μM, respectively (Table 4 and Fig. 3A). Other UV-filters tested include methyl- benzyl- and octylsalicylate, octyl-dimethyl-PABA, homosalate, α-phenylcinnamate, isoamylmethoxycinnamate and ethyl-2-cyano-3,3-diphenylacrylate that all did not inhibit 17β-HSD3 as well as avabenzene that was a very weak inhibitor.

Table 3
Inhibitory effect of different UV-filter chemicals (20 μM) on the activities of 17β-HSD3 and AR. For measurement of 17β-HSD3 activity, 200 nM androstenedione was used. To assess AR antagonist activity of the tested compounds, 0.2 nM testosterone (T) was used as a positive control.

UV-filter	17β-HSD3 activity (% of control)	AR activation (% of activation with 0.2 nM T)
BP-1	1.1 ± 0.1	32 ± 10
BP-2	47 ± 4	30 ± 1
BP-3	93 ± 1	20 ± 7
BP-4	97 ± 5	110 ± 12
BP-6	61 ± 19	44 ± 7
BP-7	84 ± 9	74 ± 18
BP-8	86 ± 3	54 ± 17
BP-12	111 ± 5	Not determined
3-BC	56 ± 1	69 ± 22
4-MBC	33 ± 8	84 ± 21
OMC	89 ± 1	114 ± 2

Table 4
Inhibition of androgen-metabolizing 17β-HSD enzymes by UV-filters. 17β-HSD enzymes were expressed in HEK-293 cells, and the conversion of 200 nM AD to testosterone (17β-HSD3, 17β-HSD5), estrone to estradiol (17β-HSD1), or estradiol to estrone (17β-HSD2), respectively, was measured in the presence of various concentrations of UV-filters in intact cells. 17β-HSD1 activity was measured in cell lysates in the presence of 500 μM NADPH, the activities of the other enzymes were measured in intact cells. Data (IC_{50} [μM] ± SD) were obtained from at least three independent experiments.

UV-filter	17β-HSD3	17β-HSD5	17β-HSD2	17β-HSD1
BP-1	1.05 ± 0.08	No inhib.	>20	65 ± 6.1[a]
BP-2	18.1 ± 3.8	No inhib.	10.3 ± 2.5	42 ± 0.8[a]
3-BC	33.3 ± 7.5	No inhib.	6.3 ± 1.4	72 ± 14[a]
4-MBC	10.7 ± 0.9	No inhib.	5.9 ± 1.3	70 ± 4.7[a]

[a] Data represent percentage of control enzyme activity measured in cell lysates in the presence of 20 μM of the corresponding chemical. No inhib., no inhibition detectable at 40 μM.

3.5. Inhibition of testosterone synthesis by BP-1 in freshly isolated mouse and rat testis tissue

To assess whether BP-1 inhibits testosterone synthesis in an endogenous system, *ex vivo* assays with mouse and rat testes were performed. Freshly isolated tissue from decapsulated testes was incubated with 1 μM radiolabeled AD for 15 min in the presence or absence of BP-1. Inhibition of testosterone formation was observed in testes isolated from either mice or rats. Conversion of AD to testosterone in mouse testis tissue was inhibited by more than 80% at BP-1 concentrations of 5 μM and higher (Fig. 3B). Similar observations were made in assays with rat testis tissue (Fig. 3C).

3.6. Effect of selected UV-filters on other 17β-HSD enzymes involved in androgen metabolism

Next, it was examined whether the selected UV-filters might inhibit other androgen-metabolizing 17β-HSD enzymes. None of the UV-filters tested inhibited 17β-HSD5 (also known as AKR1C3) (Table 4), which catalyzes the conversion of AD to testosterone in testis and prostate. While the other 17β-HSD enzymes are members of the short-chain dehydrogenase/reductase family, 17β-HSD5 belongs to the aldo-ketoreductase enzymes. It is also expressed in placenta, liver, kidney, adipose tissue and ovaries [30,31] and metabolizes other substrates including prostaglandins. In contrast to 17β-HSD5, the SDRs 17β-HSD1 and 17β-HSD2 were inhibited by several of the selected UV-filters. 17β-HSD2, which is expressed in uterus, liver, kidney, intestine and adipose tissue and converts testosterone to AD and estradiol to estrone, was inhibited by BP-2, 3-BC and 4-MBC with IC_{50} of 10.3, 6.3, and 5.9 μM, respectively (Table 4). BP-3 had no effect and BP-1 showed weak inhibition of 17β-HSD2 at 40 μM. 17β-HSD1, which is expressed in placenta, uterus, breast and adipose tissue and is important for the formation of estradiol from estrone but can also convert AD to testosterone [32], was weakly inhibited by BP-1, BP-2, 3-BC and 4-MBC but not by BP-3. OMC, one of the most widely used UV-filters, and BP-4 did not inhibit any of the 17β-HSD enzymes analyzed.

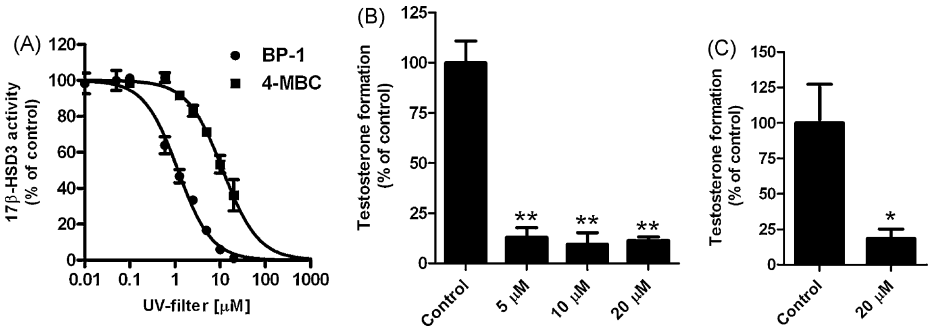


Fig. 3. Effect of selected UV-filters on 17β-HSD3 activity. (A) Concentration-dependent inhibition of 17β-HSD3 by BP-1 and 4-MBC in HEK-293 cells. HEK-293 cells expressing human 17β-HSD3 were incubated for 45 min with 200 nM AD and 0.1–20 μM of BP-1 or 4-MBC, respectively, followed by determination of testosterone (T) formation. Data represent mean ± SD from at least three independent experiments. (B and C) Inhibition of the synthesis of testosterone from AD *ex vivo* in isolated mouse (B) and rat (C) testes. Testes were decapsulated and incubated with 1 μM AD and either vehicle or BP-1 at the concentration indicated for 15 min, followed by determination of testosterone formation. Data represent mean ± SD from three independent experiments. Repeated measurements were analyzed for significance by one-way ANOVA and comparisons between controls and BP-1 treated samples were performed using *t*-test in GraphPad Prism 5. $^{**}<0.01$ and $^{*}<0.05$ compared with control.

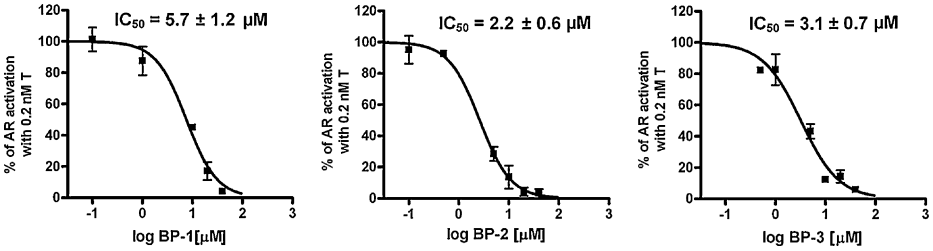


Fig. 4. Inhibition of the transactivation activity of AR by UV-filters. HEK-293 cells were transfected with plasmids for human AR, β-galactosidase reporter (MMTV-LacZ) and luciferase (CMV-luc, transfection control). Cells were incubated with 0.2 nM testosterone (T) and either vehicle (0.1% DMSO) or the corresponding UV-filter for 24 h, followed by determination of the ratio of β-galactosidase and luciferase activities. Concentration–response curves are shown for the three most potent AR antagonists BP-1, BP-2 and BP-3. Data represent mean ± SD from three independent experiments, each measured in triplicates ($n = 3$).

3.7. Antagonist activity of UV-filters on human AR

Previous studies provided evidence for antagonist effects of some UV-filters on AR [25,26]. To evaluate the relevance of the observed anti-androgenic activities of the UV-filters inhibiting 17β-HSD3-dependent testosterone synthesis, their direct effects on AR in reporter-gene transactivation assays were determined. None of the UV-filters tested activated the AR at concentrations up to 20 μM. However, several benzophenones as well as 3-BC and 4-MBC inhibited testosterone-dependent AR activation (Table 3). The most potent AR antagonists were BP-1, BP-2 and BP-3 with IC_{50} values of 5.7 μM, 2.2 μM and 3.1 μM, respectively (Fig. 4). BP-4 neither showed agonist nor antagonist activity on AR. Thus, the IC_{50} value of BP-1 for 17β-HSD3 was about five times lower than that for the AR.

4. Discussion

Regarding the safety assessment of chemicals, there is a great demand for novel strategies to identify compounds that disrupt physiological functions and to uncover the targets responsible for effects of toxic chemicals. Regulatory offices are running vast projects on compound safety focusing on putative endocrine disruptors, *e.g.* the Endocrine Disruptor Screening Program (EDSP) of the U.S. Environmental Protection Agency [19] and the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation of the European Union [33]. The EU-funded "Reproductive Effects of Environmental Chemicals in Females" (REEF) project addresses the question if such chemicals can be passed from mother to fetus, and how this affects fetal development [34].

Commonly, assemblies of relevant compounds, independent from their intended use or function, are communicated as lists. For theoretical calculations on these compounds, *e.g.* the prediction of their solubility or their effects on the human organism, additional information on 2D- or 3D-structure is required. It would be tedious to assemble these data for thousands of compounds manually. The workflow featured in this study provides an effective means of rapidly and accurately transforming compound lists into 3D-structural databases suitable for computational chemistry calculations. This opens a door to the broad application of computational methods to identify putative EDCs and rank them for thorough *in vitro* testing.

The virtual library of 76,677 potential EDCs, constructed in our approach, can be extended by including an unlimited number of new suspected chemicals. The library can be subjected to screening with all available pharmacophores of targets relevant for endocrine regulation, including receptors, metabolizing enzymes, binding proteins, and transporters.

Because of its importance for testosterone synthesis in Leydig cells [14,15] and because this target has been neglected in the assessment of endocrine disruptors so far, 17β-HSD3 was chosen for this proof-of-concept study. The screening hits included several chemicals that are widely used as UV-filters in sunscreens and other cosmetic products and as light stabilizers in many plastic products. UV-filters are a relatively new, structurally diverse group of chemicals (Fig. 2), which gained attention because of recent evidence for their interference with the functions of sex-steroid receptors [25–28]. Their use is steadily increasing, following the demand of sun lotions with higher protection factors.

Pharmacophore-based virtual screening is generally used as a rapid *in silico* filter for identifying potential lead compounds for a

specific pharmacological target. Frequently, this *in silico* filtering process successfully identifies active compound classes; however, it sometimes misses just the most active compound(s) from a given chemical scaffold. Compared to the modest hit rate of random high-throughput screening approaches (*e.g.* 0.02% for protein tyrosine phosphatase 1B inhibitors [35]), pharmacophore-based screenings usually have higher hit rates, in the % range. Therefore, it is common also to submit chemically closely related compounds to biological testing once a bioactive hit is identified (as it is done in large compound optimization programs in drug discovery). In the presented study, virtual screening has successfully led to the identification of a bioactive chemical scaffold (benzophenone), which was consequently further explored by testing similar and environmentally relevant derivatives of this compound class. This led to the identification of BP-1 as the most active compound, which has not been included in the initial hit list from virtual screening. Other UV-filters predicted by the *in silico* approach (α-phenylcinnamate, octyl- and benzylsalicylate and etocrilene) did not inhibit and others (avabenzone) showed weak inhibition at 20 μM (data not shown).

Our results show for the first time that BP-1 inhibits 17β-HSD3-dependent testosterone formation in intact cells (IC_{50} 1.05 μM) as well as *ex vivo* in freshly isolated mouse and rat testes. BP-1 also directly antagonized testosterone-dependent AR activation with an IC_{50} of 5.7 μM, when measured in charcoal-treated medium containing 10% serum (Fig. 4), and an IC_{50} of 3.6 μM in serum- and steroid-free medium (data not shown). Our findings of effects of benzophenone-derived UV-filters on AR are in line with a study by Molina-Molina et al. [36]. Despite some differences between the two studies in the absolute inhibitory concentrations that are probably due to the different cell systems used, BP-2 showed the most potent direct antagonist effect on AR, followed by BP-1 and BP-3. Nevertheless, the IC_{50} value of BP-1 for 17β-HSD3 was 3–6-fold lower than that for AR, emphasizing the relevance of 17β-HSD3 inhibition for over-all anti-androgenic effects of BP-1. BP-1 efficiently inhibited the conversion of AD to testosterone in freshly isolated mouse and rat testes, indicating that the chemical has access to the relevant target, and that its effect is independent of the species. In addition to BP-1, weak inhibitory effects were observed for BP-2, BP-6, 3-BC and 4-MBC. Thus, because most products contain several UV-filters, additive effects of mixtures [27,37] and synergistic effects due to inhibition of 17β-HSD3 by some chemicals and blocking of AR by others need to be considered for safety assessment.

The benzophenone-type 17β-HSD3 inhibitors showed a clear structure–activity relationship (Fig. 5). The two most active compounds (BP-1 and BP-2) share five common chemical groups: two hydroxyls on positions 2 and 4, the linker carbonyl, and two aromatic rings. Three parameters have a profound impact on compound activity: (i) derivatization of the 4-hydroxyl, (ii) substitution on position 5 of the first aromatic ring, and (iii) substitution on the second aromatic ring. Ether formation (BP-3, BP-4, BP-6, BP-8, and BP-12) on position 4 leads to dramatically reduced activity. Also, substitution on position 5 of the first aromatic ring (BP-4 and BP-7) and 2′ substitution on the second aromatic ring (BP-2, BP-6, and BP-8) contributes to inactivity of the compound. In contrast, position 4′ adds to benzophenone activity: adding the 4′-methoxy group to the inactive compound BP-8 leads to the moderately active BP-6, even equaling the negative effect of the 4-methoxy substitution. A hydroxyl on this position is also favorable (BP-2). Accordingly, the free hydroxyls in positions 4 and 4′ may form hydrogen bonds with 17β-HSD3, which are crucial for enzyme inhibition. Thus, they may orient similarly to the endogenous ligands into the binding pocket: the polar groups on the benzophenone scaffold at positions 4 and 4′ have a distance of approximately 10 Å—the same distance of the polar positions 3 and 17 in AD and testosterone. Both benzophenones and endogenous steroids share a mostly hydrophobic character between those polar parts. They could therefore bind in a substrate-competitive position.

A comparison of different benzophenone derivatives and representatives of two other classes of UV-filters revealed compounds inhibiting either 17β-HSD3 or AR, compounds inhibiting both and, importantly, compounds neither inhibiting testosterone synthesis nor interfering with AR activation and which may be

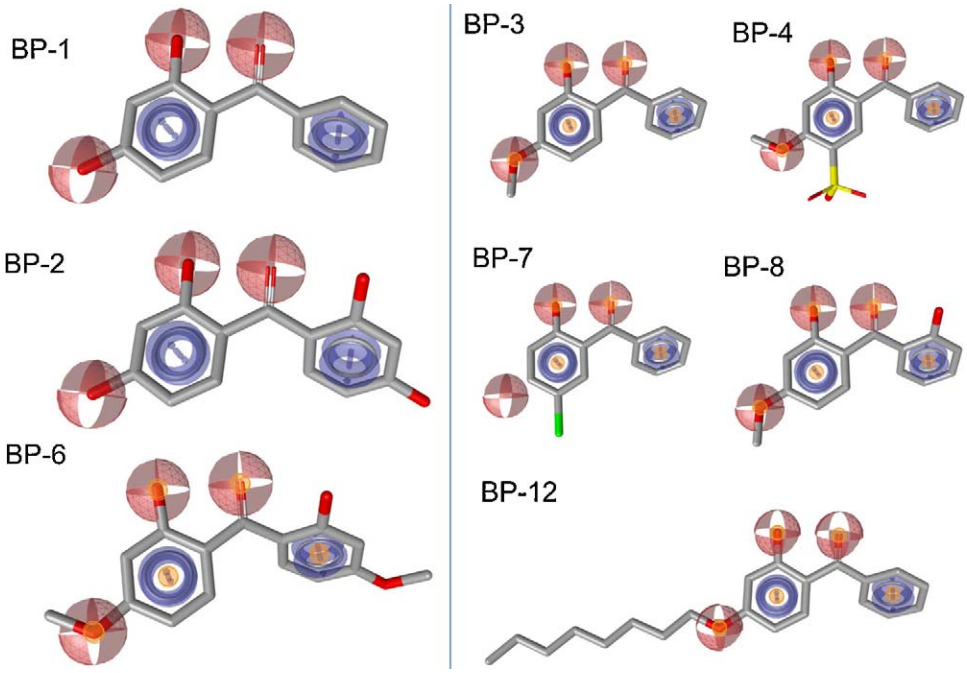


Fig. 5. Structure–activity relationship of the benzophenone-type 17β-HSD3 inhibitors discussed in this study. All compounds are aligned to a LigandScout [53,54] pharmacophore model representing common chemical groups of the active compounds. Chemical groups are color-coded: red – hydroxyl group (hydrogen bond acceptor); blue – aromatic ring.

considered as safe from this point of view. However, *in vivo* metabolism of benozophenone derivatives needs to be carefully investigated. BP-3, for example, which did not inhibit 17β-HSD3 in our assay, is metabolized *in vivo* mainly to the potent inhibitor BP-1 [29]. OMC did not inhibit 17β-HSD3; however, a weak agonist activity on AR at 20 μM was observed, in agreement with a study by Kunz and Fent [38].

Analysis of other 17β-HSD enzymes revealed inhibition of 17β-HSD2 in intact cells by 3-BC and 4-MBC with IC_{50} values of 6.3 and 5.9 μM, respectively. 17β-HSD2 and 17β-HSD3 are both expressed in adipose tissue, which plays an important role for peripheral hormonal regulation. 17β-HSD2 is also expressed in the uterus, gastro-intestinal tract and liver, where it controls the availability of active estrogens and androgens. Disturbed regulation of this enzyme has been associated with endometriosis, colon cancer and prostate cancer [39,40]. 17β-HSD2 is also expressed in skin, particularly in hair follicles, where inhibition by 3-BC and 4-MBC might be relevant because testosterone is a regulator of hair growth [41].

Most UV-filters are relatively stable in the aquatic environment, lipophilic, and tend to accumulate in aquatic organisms [42]. BP-1, which is widely used as UV-absorber in polymers and contained in food packaging and cosmetics, is classified as an irritant, with low acute toxicity and an LD_{50} of 8600 mg/kg body weight in rats (Merck safety data sheet for BP-1). Data on BP-1 concentrations in the environment and in living organisms are missing. However, data on BP-3, 4-MBC and OMC are available [43]. In fat tissue of fish from German lakes and Swiss rivers 4-MBC levels of 3.8 mg/kg (approximately 15 μM) and 0.42 mg/kg (approximately 1.6 μM) have been measured [44,45]. Thus, humans may be exposed to UV-filters via the food chain. For the risk assessment of UV-filters, the concentrations reached in human tissues are relevant. UV-filters can penetrate through the skin, and BP-3 and 4-MBC could be detected in human plasma and urine after topical applications [46,47]. Schlumpf et al. recently could detect UV-filters in breast milk samples from 76% of all tested women [48], indicating exposure of babies during the lactation period. Treatment of rats with 3-BC and 4-MBC during pregnancy led to delayed onset of puberty [49]. The timing of puberty is dependent on optimal testosterone synthesis [50], thus some of the effects reported by Schlumpf et al. may be caused by inhibition of 17β-HSD3. Skin penetration and toxicokinetics of 4-MBC have been studied in humans [51]. Maximal plasma levels in males were 238 ng/ml for BP-3 and 18 ng/ml for 4-MBC (approximately 1 μM and 70 nM). These plasma 4-MBC concentrations are significantly lower than the IC_{50} for 17β-HSD3 obtained in the present study; however, accumulation of UV-filters in adipose tissue has to be taken into account. Interestingly, a recent study demonstrated an association of higher maternal concentrations of BP-3 with decreased birth weight in girls, but increased birth weight in boys [52].

5. Conclusions

A 3D-structural library of chemicals with proven and suspected endocrine disrupting effects that is suitable for virtual screening approaches using chemical feature-based pharmacophores was constructed. The presented approach should facilitate the identification of chemicals acting on a given target of interest as well as the identification of the target(s) responsible for an observed adverse effect. We propose a novel mechanism for the previously reported endocrine effects of UV-filters and demonstrate that some of these chemicals interfere with androgen metabolism by inhibiting 17β-HSD enzymes. BP-1 was identified as a potent inhibitor of 17β-HSD3-dependent testosterone formation. In addition, BP-1 acts as a direct AR antagonist, suggesting that BP-1 causes synergistic effects *in vivo* by inhibiting testosterone synthesis and blocking AR activation. Animal experiments with pre- or neonatal treatment with relevant doses of BP-1 and 4-MBC and examination of testosterone concentrations and testosterone-dependent phenotypes are necessary to further investigate potential health consequences of these chemicals.

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