3.2. Applications in Toxicology

3.2.1. Anti-Target Screening

Although the actual virtual screening process is analogous to lead identification, anti-target screening pursues a different aim. Lead identification focuses on the discovery of ligands for therapeutically relevant targets, whereas anti-target screening aims at predicting the interaction of molecules with macromolecules mediating potentially harmful effects (so-called anti-targets). These investigations support the identification of (serious) adverse events already at an early stage in drug development. This strategy is powerful, as recently shown by Kratz *et al.* [148], who successfully applied pharmacophore models to identify inhibitors of the human ether-a-go-go-related gene (hERG) potassium channel, thereby predicting the cardiotoxic potential of the investigated molecules [148].

3.2.2. Parallel Screening

Parallel screening represents an extension to lead identification and anti-target screening protocols. It investigates not a single target but a whole collection of macromolecules with the aim of obtaining activity profiles of compounds of interest in order to prioritize further investigation. Thus, the focus of this technique shifts from the target of interest to the compound of interest, which is screened against a collection of pharmacophores, representing a plethora of different targets. Parallel screening has the potential to identify macromolecular interaction partners of the investigated molecule, thereby providing novel insight into its biological activities. These activities may include beneficial (*i.e.*, therapeutic) and harmful (*i.e.*, toxic) effects. Therefore, the results support the evaluation of a compound both with regard to the occurrence of adverse events and potential novel application fields (whenever this aspect represents the main aim of the parallel screening, this technique is also referred to as drug repurposing or drug repositioning). In the attempt to explore the biological activity of leoligin, a lignan isolated from the alpine plant Edelweiss (*Leontopodium alpinum*), the compound was screened against the Inte:Ligand pharmacophore collection in the course of a parallel screening [149]. Among the proposed targets, wascholesteryl ester transfer protein (CETP), a target involved in lipoprotein metabolism, was shown to be activated by leoligin in subsequent experimental testing. On the other side, leoligin was also predicted to inhibit the cytochrome P450 (CYP) isoforms 1A2, 2C9, and 3A4 [150], which are involved in the metabolic clearance of exogenous compounds. While it was not active on CYP1A2, it was a weak inhibitor on CYP2C9, and a sub-micromolar IC_{50} was determined for CYP3A4 [150]. Inhibition of CYP enzymes can cause severe drug-drug interactions that may lead to serious adverse effects and eventually require the termination of a drug development project. Accordingly, both potentially beneficial (CETP activation) and potentially harmful (CYP inhibition) effects can be detected during a parallel screening.

3.2.3. Examples

There is a great demand for improved methods for the safety assessment of man-made chemicals released into the environment [68,151]. Endocrine-disrupting chemicals (EDCs) are exogenous substances interfering with hormone synthesis, metabolism and/or hormonal regulation, thereby adversely affecting human health by contributing to developmental and reproductive disorders, cardio-metabolic diseases, cancer, and immune-related diseases and psychiatric disorders [152]. EDCs include substances used in agriculture, industrial production, dyes, food preservatives, or body care products and cosmetics. Several SDRs are essentially involved in the control of the local availability of active glucocorticoids, androgens and estrogens, and these enzymes should therefore be considered in the assessment of potential EDCs. *In silico* tools are well established in the drug discovery process; however, they can also display a valuable part in the identification of new EDCs or the mechanism of action of known EDCs [68].

Nashev and Vuorinen *et al.* [70] reported a pharmacophore-based virtual screening using a ligand-based 11β-HSD pharmacophore model preferentially focusing on 11β-HSD2 [106]. The 11β-HSD2

protects the mineralocorticoid receptor (MR) from activation by cortisol and renders specificity for the much less abundant aldosterone to activate this receptor. Genetic defects of this enzyme cause the syndrome of apparent mineralocorticoid excess (AME), characterized by hypokalemia, hypernatremia, and severe hypertension [153,154]. In addition, placental 11β-HSD2 protects the fetus from enhanced maternal cortisol exposure [155,156]. Therefore, disrupting corticosteroid action by EDCs can be expected to cause substantial adverse health effects. VS of an EDC database predicted 29 compounds fitting into the model of which five hits were selected for biological evaluation. Two compounds were found to inhibit 11βHSD2, the silane coupling agent AB110873 and the antibiotic lasalocid, with IC_{50} values of 6.1 μM and 14 μM, respectively. The silane AB110873 is widely used as a rubber additive for the production of tires, mechanical goods, or shoe soles and lasalocid is used as a feed additive for the prevention of infections in the breeding of chicken and turkeys. Docking studies were implemented to understand the binding mode of AB110873 in 11β-HSD2 (Figure 17) and MR.

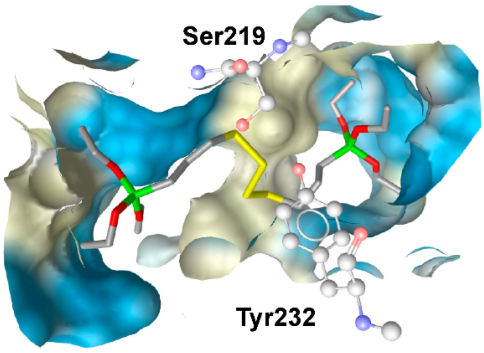

Figure 17. Docking of silane into the homology model of 11β-HSD2 [78] suggests hydrogen bond interactions with Ser219 and Tyr232 [70].

Genetic defects resulting in 17β-HSD3 deficiency cause 46,XY disorder of sex development [142,157,158]. Inhibition of 17β-HSD3 activity by EDCs might reduce plasma testosterone levels, thereby interfering with male sexual development and contributing to male reproductive disorders. To identify potential EDCs inhibiting 17β-HSD3, Nashev and Schuster *et al.* generated a ligand-based pharmacophore model [74]. VS of an EDC database predicted several organic UV filters containing a benzophenone as a bioactive chemical scaffold. UV filters are a structurally diverse class of chemicals widely used in sunscreens and cosmetics as well as plastic additives. *In vitro* testing of selected virtual hits and similar environmentally relevant derivatives led to the identification of benzophenone-1 (BP-1) as the most potent 17β-HSD3 inhibitor with an IC_{50} of 1.05 μM in intact cells. BP-2,3-benzylidene camphor (3-BC) and 4-methylbenzylidene camphor (4-MBC) moderately inhibited 17β-HSD3 with IC_{50} values between 10.7 μM and 33.3 μM, but showed substantial inhibitory activity on 17β-HSD2 with IC_{50} between 5.9 μM and 10.3 μM. Importantly, the most active compound, BP-1, as well as 3-BC and 4-MBC were not included in the initial virtual hit list but added to the biological testing due to their use as UV filters. Hence, VS displays an initial filter for the identification of potential EDC compound classes and aims at prioritizing the compounds to be included for biological investigations. In analogy to the drug discovery process, it is important to test structurally related enzymes in order to know whether they are affected by a given EDC. Importantly, major metabolites should also be included in the analysis. For example, BP-3 showed no activity against 17β-HSD3, but it is demethylated *in vivo* to the potent inhibitor BP-1 [159]. To explain the differential inhibitory activities of the tested UV filters, Schuster and Nashev conducted pharmacophore-based SAR studies, suggesting that the ether group on BP-3 and BP-8 instead of a hydroxyl group on BP-1 and BP-2 was the reason for the loss of activity of BP-3 and BP-8 (Figure 18). To further study the toxicological relevance of 17β-HSD3 inhibition by BP-1, concentrations reached *in vivo*, especially in the testes, need to be determined.

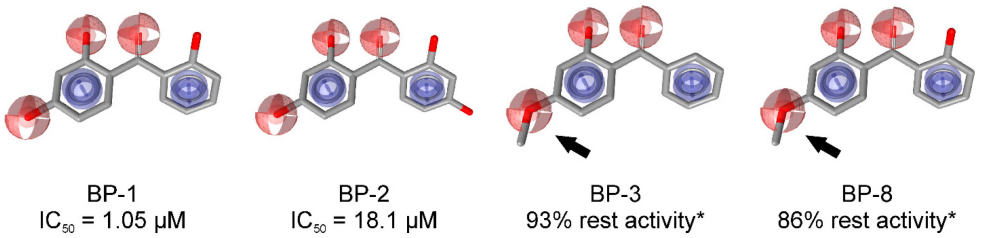

Figure 18. SAR analysis revealed that the etherification of the hydroxyl group (as indicated by the arrows) was responsible for the loss of activity observed for BP-3 and BP-8 [74]. *Remaining enzyme activity at a compound concentration of 20 µM compared to vehicle control.

4. Limitations

As with every method, pharmacophore modeling and pharmacophore-based virtual screening also have their limits. A recent study compared the performances of two pharmacophore modeling programs, LigandScout and Discovery Studio, on the identification of novel cyclooxygenase inhibitors [160]. Intriguingly, although both programs succeeded in the identification of novel bioactive molecules, the virtual hit lists retrieved with the two tools were highly complementary. It is of note that not a single overlap in the hit lists was observed, even when the identical crystal structure of a ligand-target complex was employed for model generation. This illustrates that neither of the two programs was capable of comprehensively covering the active space and that models from different programs need to be combined whenever a more complete retrieval of active molecules is required. The authors suggested that the reasons for this finding may be found in the different screening algorithms and feature definitions deployed by the programs.

Feature definitions can be improved in general, as highlighted by the treatment of halogens. Some pharmacophore modeling programs consider halogens solely as hydrophobic moieties in the default settings [7,8,161]. LigandScout, in addition, matches fluorine to HBA features [6]. In 2013, a study by Sirimulla *et al.* [162] revealed that in many ligand-target complexes, halogens participate in strong halogen bond formation, e.g., with aromatic rings, and thereby considerably contribute to the interaction between the ligand and the target. These types of interactions, although often employed by medicinal chemists to improve the binding affinity of compounds [162], are not yet implemented in common pharmacophore-based virtual screening tools.

A major limitation, not only for pharmacophore modeling but for virtual screening tools in general, is the fact that the quality of a pharmacophore model critically depends on the data employed for model generation, refinement, and theoretical validation. Many public data repositories are available that can be explored to build a model. However, caution is required as, apparently, parts of the data are erroneous. Fourches *et al.* investigated six different datasets, and after curation, up to 10% of the original structures were removed [163]. Besides several other preventive measures, the authors suggest to include a final manual inspection step to check the structures of the input compounds. We fully support this recommendation and would even go one step further: Not only the structures of the compounds included in the modeling dataset need to be critically evaluated, but also the annotated biological data. This starts with inclusion/exclusion criteria for appropriate/inappropriate testing systems applied to determine the biological activity of a compound (for example, data obtained from intact cell assays or from animal tissue preparations are of limited use for human enzyme models), the application of suitable activity cut-offs (distinguishing between specific and unspecific effects, depending on the investigated target), and ends at a critical comparison with the original literature as errors can also happen during the transfer of data to depositories. These procedures may be quite elaborate; nevertheless, they are crucial for the generation of high quality models and every modeler is well advised to carefully review the data on which the models are based.

Another limitation is a lack of compounds confirmed as inactive for a specific target. Results on proven inactives for model validation are often not accessible because, unfortunately, negative

results are rarely published. The information from confirmed inactive compounds is important for the balancing between selectivity and sensitivity of a model during the validation step. In the drug development process, restrictive models are required because, finally, only one or a few lead compounds are selected for further optimization steps, whereas in a toxicology screening, it is important to correctly find preferably all of the potentially harmful substances. Albeit considerably more successful than random screening, the success rate of VS may still be a limiting factor for toxicological projects. However, it has the ability to identify structural compound classes that then can be further evaluated. Obviously, the database used for VS might be self-limiting as not all potential active compounds are included.

One caveat of pharmacophore modeling is that a modeler needs to be aware of concerns about detailed interaction patterns of the active compounds in the dataset. Although high quality experimental data confirming their binding and activity may be available for these ligands, the exact binding site is still not clearly defined for most of them. Many molecules may occupy a similar yet slightly different part of the binding pocket, e.g., compared to the co-crystallized ligand in an X-ray crystallographic complex employed for model generation. Accordingly, the interaction patterns may differ. This factor is even more pronounced in ligands affecting the function of a protein by binding to allosteric sites, disrupting conformational changes, or interfering with post-translational modifications. Similar concerns also apply for the experimental validation of the *in silico* predictions, as it is, in the end, often not known whether the newly identified compounds indeed exert the predicted binding mode [164]. An X-ray crystal structure of the ligand-target complex would provide the ultimate confirmation of the exact interaction patterns; however, 3D structure resolution of transmembrane proteins by crystallization remains difficult. Several SDRs belong to this class of proteins such as, for instance, 11βHSD2, 17βHSD2, and 17βHSD3. For these proteins, structure-based pharmacophore modeling is currently not possible, and homology modeling remains challenging due to the low sequence similarity of SDRs. However, for these cases, ligand-based pharmacophore modeling displays an elegant solution.

Pharmacophore-based VS proved to be a powerful tool to support drug discovery and development, especially concerning the enrichment of active molecules among test compounds. Nevertheless, expectations concerning the results of VS need to remain realistic. Although sometimes potent compounds are discovered via VS, the majority of virtual hits usually display only weak activity. For this concern, the initial virtual hits from VS should be considered similar to initial experimental hits discovered in a HTS campaign, which also require further chemical optimization steps to develop to potential drug candidates [164].

5. Conclusions

The current work summarizes prospective pharmacophore-based studies conducted in the field of steroid biology, with special focus on SDRs, and highlights success stories reported in this area. Pharmacophore models are suitable to address a wide range of issues relevant for both drug discovery and toxicology. This is of special relevance for SDRs, because members of this target class are both associated with therapeutic value (e.g., 17β-HSD1 inhibition for the treatment of hormone-sensitive cancers) and toxicological liabilities (disruption of 11β-HSD2 actions). Although the method itself still has room for improvement as pointed out in the "Limits" section, the caveats associated with pharmacophore modeling largely also apply for other virtual screening techniques. In addition, in case of a lack of available structural data on macromolecular targets, ligand-based modeling strategies offer a useful alternative. The identification of structurally diverse molecules may, to a certain extent, be restricted to the data employed for model generation and refinement. However, the extraction of crucial interactions and their representation via abstract chemical features proved to be a powerful approach to step beyond the initial chemical space. As highlighted in this review, pharmacophore-based VS is a valuable scaffold-hopping tool. Importantly, this allows for the application of pharmacophore-based virtual screening also for compound classes that do not fall into the category of "small drug-like

compounds" or whose properties differ from that of synthetic compounds: For example, natural products provide a vast resource for bioactive compounds that can be exploited for therapeutic purposes. On the other hand, the *in silico*-driven investigation of environmental chemicals, which often chemically differ from drug-like molecules, facilitates the rapid identification of potentially harmful compounds that need to be prioritized for experimental evaluation. Given the many application fields of pharmacophore-based virtual screening and the successful examples summarized in this review, an increasing number of studies, also in the field of SDR research, can be expected in the future.

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Abbreviations

The following abbreviations are used in this manuscript:

3-BC	3-benzylidene camphor
4-MBC	4-methylbenzylidene camphor
AKR	aldo-keto reductase
AME	apparent mineralocorticoid excess
AR	aromatic features
BP	benzophenone
CETP	cholesterylester transfer protein
CYP	cytochrome P450
DHEA	dehydroepiandrosterone
DHT	5α-dihydrotestosterone
DUD-E	Directory of Useful Decoys, Enhanced
EDCs	endocrine disrupting chemicals
ER	estrogen receptor
GA	glycyrrhetinic acid
H	hydrophobic feature
HBA	hydrogen bond acceptor
HBD	hydrogen bond donor
hERG	human ether-a-go-go related gene
HSD	hydroxysteroid dehydrogenase
HTS	high-throughput screening
IUPAC	International Union of Pure and Applied Chemistry
MD	molecular dynamics
MOE	Molecular Operating Environment
MR	mineralocorticoid receptor
NADP	nicotinamide adenine dinucleotide phosphate
PAINS	Pan-Assay Interference Compounds
PDB	Protein Data Bank
PPAR γ	peroxisome proliferator-activated receptor γ
ROC-AUC	area under the receiver operating characteristics curve
SAR	structure-activity relationship
SDR	short-chain dehydrogenase/reductase

VS	virtual screening
XVols	exclusion volumes

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