Development of a Protocol for Virtual Screening of PPARγ Weak Partial Agonists and Their Metabolites: Case Study on Naturally-derived Oleanane Triterpenoids

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***Abstract**: Triterpenoids are well known metabolic syndrome (MetS) modulators. One of the suggested molecular mechanisms of action involves peroxisome proliferator-activated receptor gamma (PPARγ) activation. In this study we aimed to: (i) develop a virtual screening (VS) protocol for PPARγ weak partial agonists, (ii) predict potential metabolic transformations of naturally-derived triterpenoids, and (iii) perform VS of the triterpenoids and their metabolites. The NIH PubMed system was searched for publications about naturally-derived oleanane triterpenoids which are agonists or up-regulators of PPARγ. Structure- and ligand-based methods were combined in the development of the VS protocol. Metabolites were predicted using Meteor Nexus expert system (Lhasa Limited). Two in-house virtual libraries of PPARγ weak partial agonists and naturally-derived triterpenoids with their predicted metabolites were compiled. The pharmacophore-based docking protocol was applied for VS of the collected triterpenoids. Most of the docking poses reproduced the binding mode of caulophyllogenin (a weak partial agonist) in a complex with PPARγ (PDB ID 5F9B). Our results contribute to the mechanistic explanation of the effects of triterpenoids suggesting possible weak partial agonistic activity toward PPARγ. This research can direct further studies on triterpenoids' role in MetS modulation. The developed protocol can be applied for VS of any PPARγ weak partial agonists.*

***Keywords:** PPARγ, Weak partial agonists, Virtual screening, Triterpenoids, Metabolites, Metabolic syndrome.*

Introduction

The metabolic syndrome (MetS) is a complex condition associated with hypertension, type 2 diabetes and obesity and is among the main risk factors for developing cardiovascular diseases [4, 14]. Triterpenoids are shown to ameliorate the MetS, and the modulation of the nuclear receptor PPARγ activity has been suggested as one of the possible mechanisms of this amelioration [6, 11]. The interest in PPARγ-targeting as a treatment strategy for combating metabolic disorders is due to its crucial role in the regulation of lipid and glucose homeostasis.

The PPARγ ligands are generally classified based on their relative efficacy and receptor binding mode into full and partial agonists (Fig. 1).

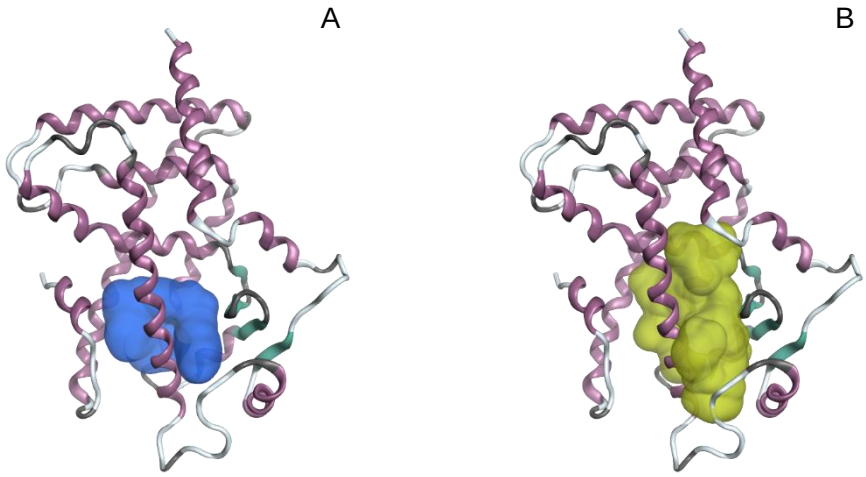


Fig. 1 Prevailing binding regions of the full (A) and weak partial agonists (B) of PPARγ identified by analysis of PDB complexes of the receptor ligand-binding domain [2]

Treatment with PPARγ full agonists has been associated with adverse effects [9], therefore a paradigm has currently shifted toward development of partial agonists [5]. In our previous studies we discriminated weak from strong PPARγ partial agonists reported in the Protein Data Bank (PDB) by analysis of their protein-ligand interaction fingerprints, receptor binding modes and relative efficacies and developed pharmacophores for the two classes of agonists [2]. The pharmacophore of the weak partial agonists has been applied to screen triterpenoids derived from medicinal plants of the genus *Astragalus* [2, 3]. Prediction of possibly coexisting reactive metabolites of an orally administrated compound, which could be produced by Phase I biotransformations, has been shown to allow for a detailed and physiologically relevant *in silico* study of its potential therapeutic modes of action by elucidation of diverse receptor binding patterns [3]. In this study we describe the development of a multi-step virtual screening (VS) protocol for prediction of potential metabolic transformations and PPARγ weak partial agonistic activity of compounds and apply this VS protocol for a mode of action prediction of naturally-derived triterpenoids with experimentally supported relevance to MetS.

Materials and methods

Data extraction and organization

The NIH PubMed system was queried for publications about naturally-derived triterpenoids [15]. The structural information was retrieved from the NIH PubChem system or from literature sources [16]. The PDB was used as a source of X-ray complexes of human PPARγ and its ligands with weak partial agonistic activity ($E_{max} \leq 35\%$ [2]) [17]. The corresponding ligands were extracted from the complexes, while the available structural and biological data for homologue series of their chemical analogues were retrieved from the literature [15]. The DUD-E database was used as a source of PPARγ decoys [18].

In silico methods

Prediction of Phase I metabolism

Metabolite prediction was performed in the Meteor Nexus system (v. 3.1.0; Knowledge Base: Meteor KB 2018 1.0.0) using the default settings of the Site of Metabolism Scoring (SOM, with

Molecular Mass Variance) method. The likelihood of biotransformations in its knowledge base are determined using experimental data for compounds that match the same biotransformation, have similar molecular weights and are chemically similar around the site of metabolism to the query compound [7, 8]. Only Phase I biotransformations were selected for prediction.

Molecular modelling and analysis of the protein-ligand interactions

The MOE software (v. 2019.0102) was used for generation of a virtual library of compounds, structure preparation, assignment of ionization states at pH 7.4 ("Molecule Wash" tool) pharmacophore search, molecular docking and protein-ligand interaction fingerprint analysis ("PLIF" tool) [10]. At the steps involving molecular docking a London dG scoring function was applied, based on our previous study on optimization of the docking simulation [1]. Venn diagrams of the frequencies of simultaneous interactions with PPARγ amino acids were generated using "Venn Diagram Maker Online" [19].

Statistics

The parameters used for estimation of the performance of the VS protocol are as follows:

Sensitivity (*Sn*)

$$Sn = \frac{\mathrm{TP}}{\mathrm{TP} + \mathrm{FN}} \tag{1}$$

Specificity (*Sp*)

$$Sp = \frac{\mathrm{TN}}{\mathrm{TN} + \mathrm{FP}} \tag{2}$$

Accuracy (*Acc*)

$$Acc = \frac{\mathrm{TP} + \mathrm{TN}}{\mathrm{TP} + \mathrm{TN} + \mathrm{FP} + \mathrm{FN}} \tag{3}$$

where TP is true positive, TN – true negative, FP – false positive and FN – false negative.

Results and discussion

Development of virtual libraries of compounds

Virtual library of PPARγ weak partial agonists

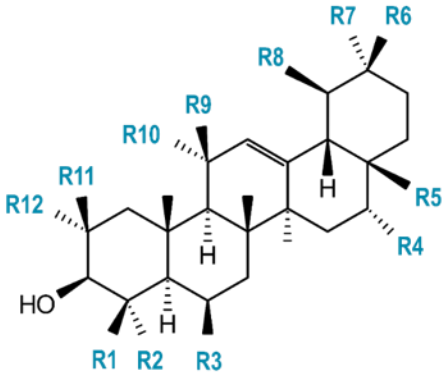
In total, 125 structures were collected and the corresponding biological data for transactivation activity (EC_{50}, μM) and relative maximal activation (relative efficacy, E_{max}, %) were harvested (Supplementary Table 1). A previously established maximal relative efficacy threshold value of $E_{max} \leq 35\%$ [2] was used as an inclusion criterion for the preparation of a virtual library with known PPARγ weak partial agonists. The virtual library contains weak partial agonists either extracted from PPARγ-ligand complexes in PDB (26 compounds) or retrieved from reported in the literature homologue series of these PDB ligands (99 compounds).

Virtual library of naturally-derived triterpenoid modulators of PPARγ

Structural data for 24 naturally-derived oleanane-type triterpenoid agonists and/or up-regulators of PPARγ were retrieved from the literature. Only the aglycons of the triterpenoids were studied (Supplementary Table 2). Among them caulophyllogenin is the only oleanane-type triterpenoid deposited in PDB in a complex with PPARγ (PDB ID 5F9B). Caulophyllogenin is an

experimentally proven weak partial agonist of the receptor with a relative maximum activation of 9.4%. The 24 structures share 10 common aglycons and the substituents that differ from those in the referent triterpenoid caulophyllogenin are shaded in grey (Table 1). The triterpenoids were selected based on experimental evidence for relevance to MetS, positive modulation of PPARγ activity and/or PPARγ expression and mono-treatment with a pure compound. The PPARγ modulation is accomplished by enhancing its expression (for esculentoside A and glycyrrhizic acid), by agonistic effect combined with increased expression (for asiaticoside) or by agonistic effect alone (for the triterpenoids sharing the other 7 aglycons).

Table 1. Ten common aglycons for 24 oleanane triterpenoids



No	Name	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12
1	Glycyrrhizic acid	CH_3	CH_3	H	H	CH_3	COOH	CH_3	H	= O		H	H
2	Compounds 9[13]; 1, 2, 3-6[12]; Oleanolic acid	CH_3	CH_3	H	H	COOH	CH_3	CH_3	H	H	H	H	H
3	Compound 2[13]	CH_3	CH_3	OH	OH	COOH	CH_3	CH_3	H	H	H	H	H
4	Esculentoside A	CH_3	CH_2OH	H	H	COOH	CH_3	$COOCH_3$	H	H	H	OH	H
5	Asiaticoside	CH_3	CH_2OH	H	H	COOH	H	CH_3	CH_3	H	H	H	OH
6	Compounds 3, 5-8, 10, 11[13]; Hederagenin	CH_3	CH_2OH	H	H	COOH	CH_3	CH_3	H	H	H	H	H
7	Compound 1[13]	CH_3	CH_2OH	OH	OH	COOH	CH_3	CH_3	H	H	H	H	H
8	Platycodin D	CH_2OH	CH_2OH	H	OH	COOH	CH_3	CH_3	H	H	H	OH	H
9	Platyconic acid	COOH	CH_2OH	H	OH	COOH	CH_3	CH_3	H	H	H	OH	H
10	Caulophyllogenin	CH_3	CH_2OH	H	OH	COOH	CH_3	CH_3	H	H	H	H	H

Development of a virtual screening protocol for PPARγ partial agonists

Description of the protocol

A four-step protocol was developed (Fig. 2):

1. Prediction of Phase I biotransformations of a compound intended for oral administration.
2. Docking of the input structure and its predicted metabolites in the receptor binding site.
3. Filtering the best docking poses with a 5-point pharmacophore.
4. Re-docking of the best poses in the same binding site using a 6-point pharmacophore at the placement step of the docking algorithm.

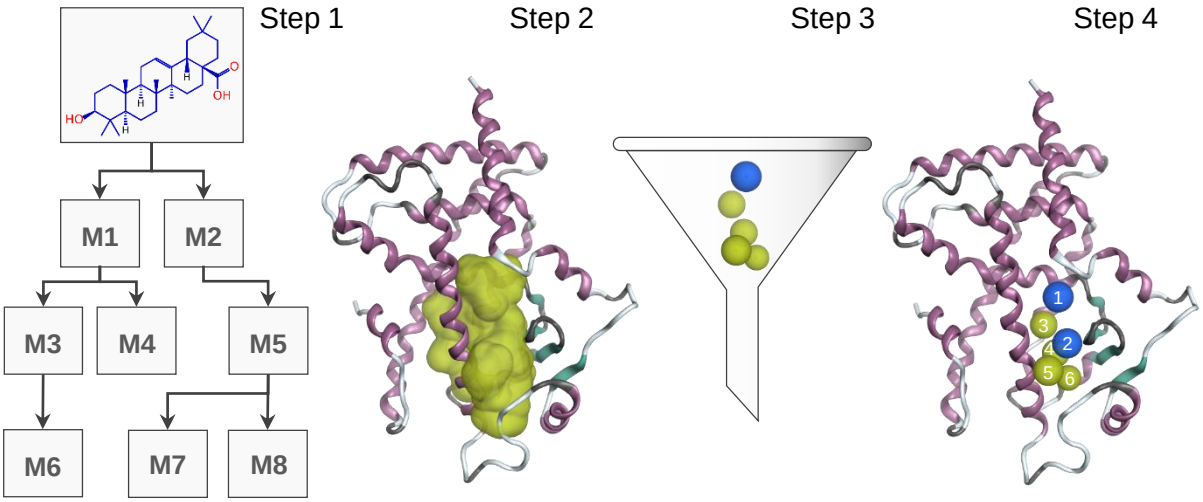


Fig. 2 Virtual screening protocol. Step 1: Metabolism prediction; Step 2: Docking in PPARγ; Step 3: Pharmacophore filtering; Step 4: Pharmacophore-based re-docking; pharmacophore features F1-F6 are numbered accordingly.

The 1st step of the protocol comprises metabolite prediction in the Meteor Nexus system. By default, a maximum of three generations of metabolites is considered (Fig. 2, Step 1). At the 1st step the input structures are considered neutral. The application of the next steps of the protocol requires additional structure processing including assignment of: (i) the physiologically relevant ionization at pH 7.4, and (ii) the possible stereochemistry at the sites of metabolism.

At the 2nd step, a number of conformations of the input structures and their metabolites are generated by molecular docking in the binding pocket of PPARγ as defined by the available crystallographic poses of known weak partial agonists (a training set of 17 structures, shown in bold in Supplementary Table 1). This yields in reasonable three-dimensional representations of the ligand structures in the context of the receptor binding site (Fig. 2, Step 2).

The 3rd step allows filtering of possible weak partial agonists through a 5-point pharmacophore with 4 hydrophobic points (F3-F6) and one hydrogen bond (HB) acceptor point (F1) (Fig. 2, Step 3).

At the final 4th step a pharmacophore-based re-docking is performed to predict the binding mode of the filtered weak partial agonists in the receptor pocket. This pharmacophore includes an additional point (F2), which comprises a HB acceptor feature (Fig. 2, Step 4). At this step the docking algorithm is set to allow flexibility not only of the ligands but also of the side chains of the amino acid residues (the "Induced Fit" option in MOE).

The two pharmacophores used in the VS protocol are variations of a previously reported pharmacophore, developed by a comprehensive analysis of weak partial agonists' structural features and protein-ligand interactions fingerprints [2]. It should be noted that the pharmacophores applied at steps 3 and 4 of the protocol can be set to different number of pharmacophore points depending on the required level of tolerance. In the particular case at the 3rd step a requirement for the best match to the centres of all 5 pharmacophore points was set, i.e. the best docking poses corresponded to the lowest root mean square deviation (RMSD) value. At the 4th step, however, the pharmacophore was used as a means of guiding the

generation of docking poses (using a "Pharmacophore" placement in MOE). In this case matching at least three essential points (F2, F3 and F4) of the 6-point pharmacophore was enough for a docking pose to be successful (Fig. 2, Step 4).

Validation of steps 2, 3 and 4 of the protocol

The internal validation of the protocol resulted in nearly 50% of the training set successfully passing the filtering step 2 (Table 2). This is due to the fact that the majority of the known weak partial agonists are usually small in size and hardly match at once all points of the restrictive 5-point pharmacophore. At this step the X-ray poses were reproduced with RMSD values between 0.9 and 3 Å. In particular, the caulophyllogenin's X-ray pose (PDB ID 5F9B) was reproduced with an RMSD of 1.8 Å (Fig. 3).

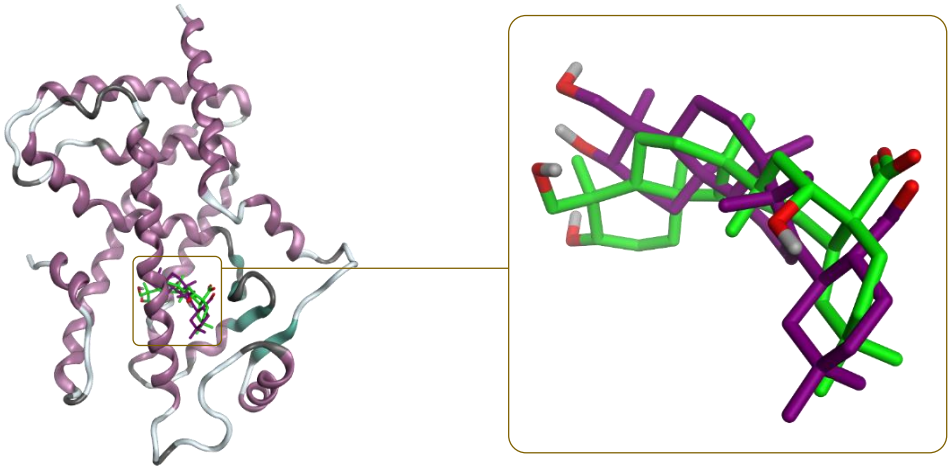


Fig. 3 The best docking pose of caulophyllogenin (green), reproducing its crystallographic pose (purple) with an RMSD value of 1.8

The external validation of the protocol was performed using the test set of 108 known weak partial agonists and 2527 decoy structures. The statistical performance of the VS protocol in this validation underlined how the high restrictiveness for simultaneous matching to all 5 pharmacophoric points, although reducing the sensitivity (*Sn*) to 40%, allowed for a higher specificity (*Sp*) of 70%. The overall accuracy (*Acc*) of the developed protocol was estimated to be 68%.

Table 2. Results from the validation of the VS protocol using 5-point pharmacophore filtering of weak partial agonists of PPARγ

Number of compounds in / **Step**	**Training set**	**Test set**	**Decoys**
2. Docking in PPARγ	17	108	2527
3. Pharmacophore filtering	8	43	771
4. Pharmacophore-based re-docking	8	43	771

Virtual screening of triterpenoid aglycons

Six types of biotransformations were predicted for the parent structures of the 10 aglycons, including: allylic hydroxylation; hydroxylation of methyl carbon adjacent to an aliphatic ring; oxidation of primary alcohols; oxidation of secondary (alicyclic) alcohols; hydrolysis of sterically hindered acyclic carboxylic esters; and reduction of α,β-unsaturated compounds. After assignment of stereochemistry to the predicted metabolites, a library of 122 structures (112 metabolites and 10 parent aglycons) was generated. Nine of the metabolites appear twice in the library by duplicating 7 metabolites and 2 parent aglycons in other metabolic trees (shown in bold in Supplementary Table 2). The number of the predicted metabolites per aglycon varied between 6 and 14 (Fig. 4).

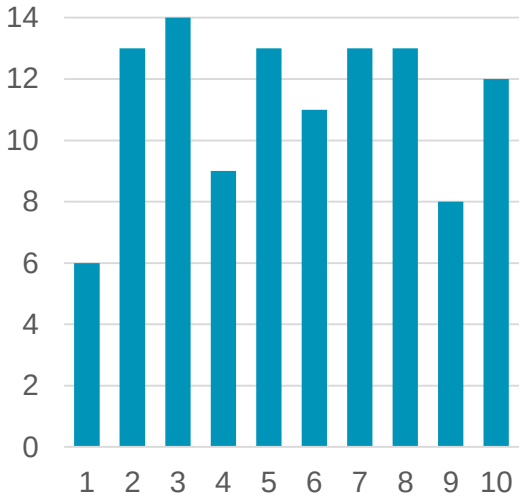


Fig. 4 Number of structures per aglycon in the virtual library.
The 10 aglycons are assigned according to their sequential numbering in Table 1.

The developed VS protocol was applied to all 113 unique structures (10 aglycons and 103 metabolites) by keeping the top-scored 10 poses per structure at step 2 and the top-scored pose at step 3. At the final re-docking step 4 the top 5 poses, which performed protein-ligand interactions with the highest number of amino acids in the pocket, were kept (Fig. 2). For all 560 poses of aglycons and their metabolites, the estimated docking scores ranged between -19 (the best scoring) and -12 kcal/mol. For comparison, the scores of the caulophyllogenin's poses ranged between -15 and -13 kcal/mol. The direct contacts with 19 different amino acids in the binding pocket were predicted, and each pose was shown to interact simultaneously with 2 to 6 amino acids. Most often, the contacts included HB donor, HB acceptor and ionic interactions and rarely arene-arene attraction. A detailed analysis of the predicted poses outlined five amino acids that were the most frequently involved in direct protein-ligand interactions. The most frequently referred amino acid, located in the β-sheet, was Ser342 (491 occurrences in the PLIF), followed by amino acids from α-helix 3 (H3), like Arg288 (362), Cys285 (95), and Ser289 (93), and from α-helix 7 (H7), like Met364 (95). Further, an analysis of the frequencies of co-occurrence of interactions involving these amino acids was performed for the predicted ligands' poses. This analysis is illustrated by four Venn diagrams in Fig. 5 A-D. Almost all occurrences of Arg288 (H3) in the protein-ligand fingerprints of the analysed poses occurred simultaneously with interactions involving Ser342 (β-sheet). More than a half of the interactions with Ser289 (H3) were predicted for poses that simultaneously interacted also with Arg288 and Ser342. The protein-ligand interactions involving amino acid residues Cys285 (H3) and Met364 (H7), which had identical frequencies

of occurrence in the PLIFs, were also predicted to co-exist in most of the cases and in 50% of them these interactions were also accompanied by interactions with Ser342 and Arg288.

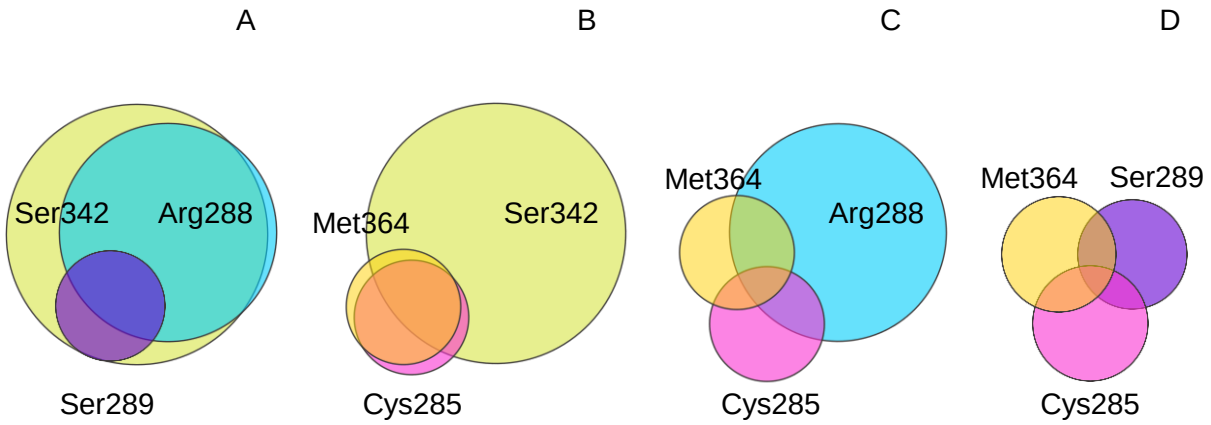
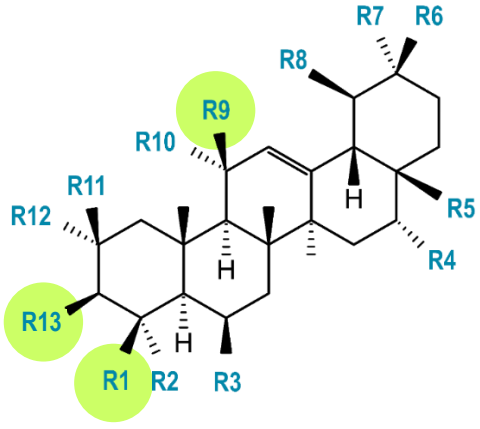


Fig. 5 Venn diagrams representing the frequencies of simultaneous interaction of the ligands with amino acids in the receptor pocket. The sphere size correlates with the number of occurrences of given residue in the PLIFs.

The co-occurrence rates of the amino acid pairs Cys285-Ser289, Met364-Cys285 and Met364-Ser289 in the PLIFs were comparable. We clustered the predicted poses of the triterpenoids and their corresponding metabolites according to the belonging of the structures to the metabolic trees of the ten originally outlined aglycons. The groups of asiaticoside and platycodin D included poses interacting with the maximal number of amino acids. For these poses the changes in the ligand structures at the sites of metabolism compared to the original aglycons are presented in Table 3.

Table 3. Variations in the substituents of selected aglycons as compared to their metabolites



Name	R1	R9	R13
Asiaticoside	**CH_3**	**H**	**OH**
Asiaticoside, M1	CH_3	H	=O
Asiaticoside, M45.S	CH_2OH	OH	OH
Platycodin D	**CH_2OH**	**H**	**OH**
Platycodin D, M45.SS	COOH	CH_2OH	OH

Asiaticoside and its metabolites M1 and M45.S reproduced the receptor binding mode of caulophyllogenin (Fig. 6 A), while platycodin D and its metabolite M45.SS occupied the binding site parallel to α-helix H3 and literally anchored to the pocket entrance (Fig. 7 A). The codes of the amino acids, which are most frequently occurring in the PLIFs, are shown in black, while the rest – in grey.

The protein-ligand interaction diagrams of asiaticoside and M1 (Fig. 6 B, C) illustrate how metabolising hydroxyl substituent to carbonyl oxygen at the terminal ring (R13, Table 3) changed the pose in a way that favoured some interactions rather than others. In this case, the HB donor interaction with Arg288 (H3) was interrupted, due to slight shift enhancing the interactions with amino acids from the β-sheet (Ser342 and Ile341). The diagrams outline only the HB donor and acceptor interactions and, in addition to the direct contacts, a contact with Glu343 from the β-sheet, mediated by a bridging water molecule, is shown.

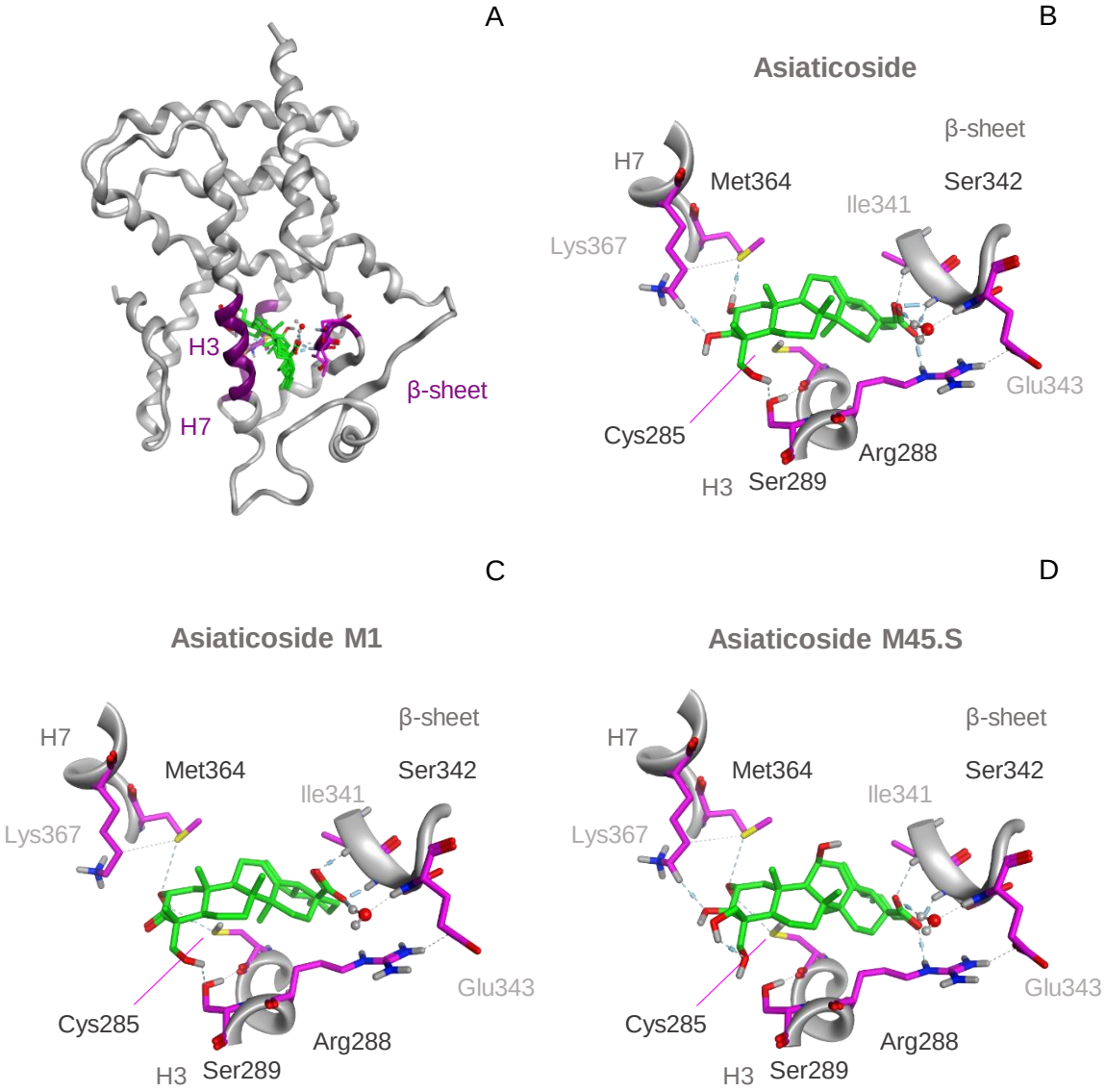


Fig. 6 Binding mode in the receptor (A) and protein-ligand interaction diagrams of asiaticoside (B) and its predicted metabolites M1 (C) and M45.S (D)

For metabolite M45.S of asiaticoside a hydroxylation of methyl carbon adjacent to the terminal ring was predicted (R1, Table 3). This transformation became a prerequisite for establishment of an intramolecular interaction at the terminal ring of the structure between the newly formed and the adjacent hydroxyl groups which in turn disrupted the interaction of the ligand with Ser289 (H3). For both metabolites of asiaticoside a rotation of a hydroxyl substituent at the same terminal ring of the structure was predicted to weaken the strength of the interaction with H7 (Met364) while stabilizing the pose by new weak interaction with H3 (Cys285) (Fig. 6,

C and D). Thus, the predicted binding triad of asiaticoside and its metabolites in the receptor pocket is characterized by an obligate interaction with the β-sheet (both direct and water-mediated) accompanied by a dynamic switching between H3- or H7-dependent stabilization of the pose.

In the case of platycodin D and its metabolite M45.SS (Fig. 7) the replacement of a hydroxymethyl by a carboxyl substituent at the terminal ring (R1, Table 3) led to the participation of the latter in the formation of intramolecular bonds with the two adjacent hydroxyl groups. This reorganization resulted in a complex HB-networking between H3 and the H1/H2 turn by: (i) association of one of the adjacent hydroxyl groups to the interaction with H3, and (ii) formation of a new direct contact between the carboxyl group and the H1/H2 turn. The hydroxylation at the central ring, facilitating further contact with the β-sheet, limited even more the flexibility of the ligand in the pocket and underlined the predicted platycodin D binding triad involving H3, H1/H2 turn and the β-sheet.

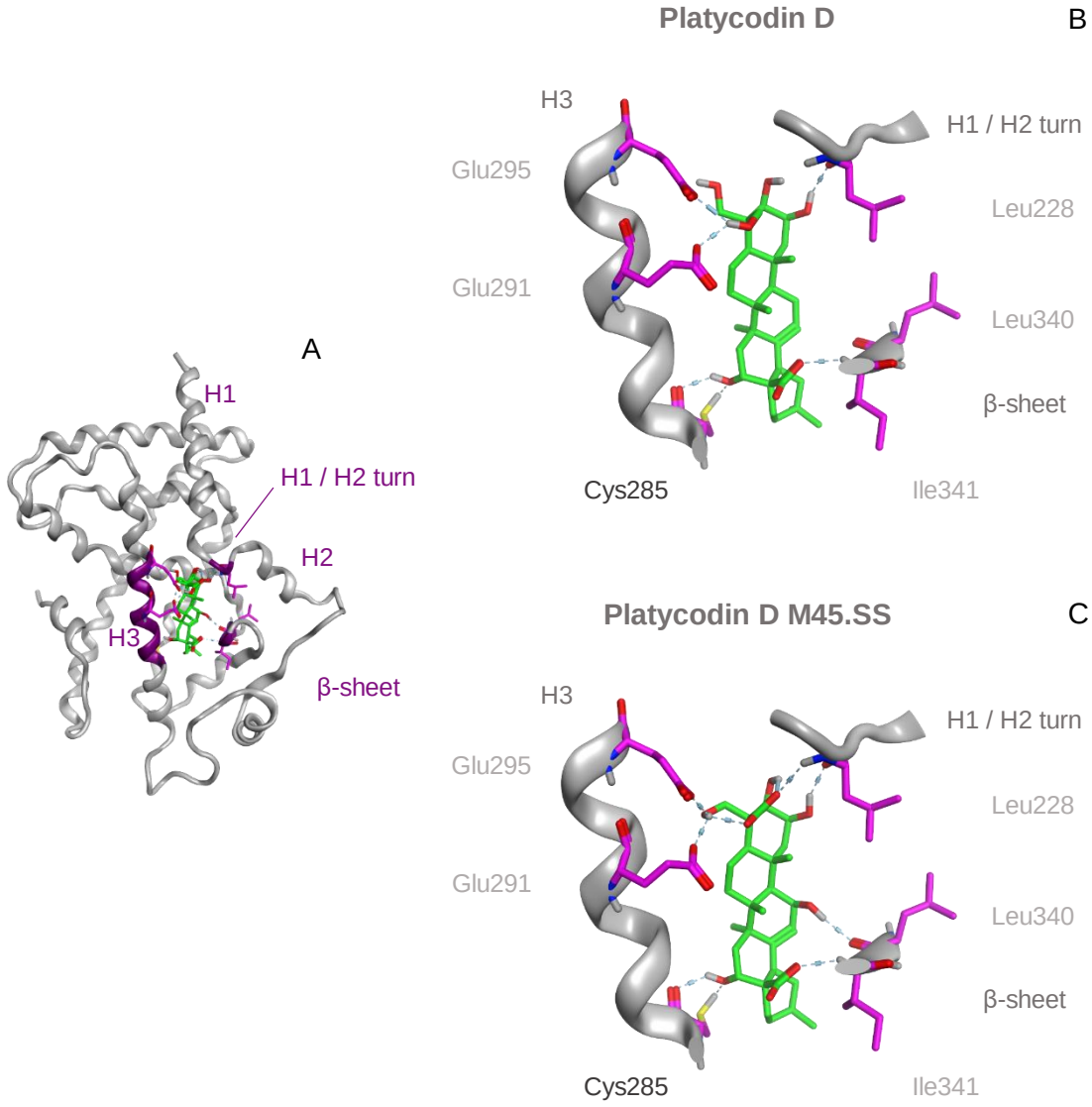


Fig. 7 Binding mode in the receptor (A) and protein-ligand interaction diagrams of platycodin D (B), and its predicted metabolite M45.SS (C)

The examples of asiaticoside and platycodin D show that taking into account the pharmacologically active form of the compound is essential when carrying out *in silico* simulations to predict receptor-mediated molecular action. In the context of the modes of action this implies both: (i) preparation of physiologically relevant ionization forms, and (ii) considering the possibility of metabolic activation or deactivation of the structures.

While the metabolic transformations were not shown to significantly change the molecular shape of asiaticoside and platycodin D and the molecular surface charges of the asiaticoside's metabolites (Fig. 8 A), for the metabolite of platycodin D a significant redistribution of the electron density was recorded (Fig. 8 B). This observation confirms the necessity for consideration of the metabolites when estimating the effects of the interactions of weak partial agonists with the receptor.

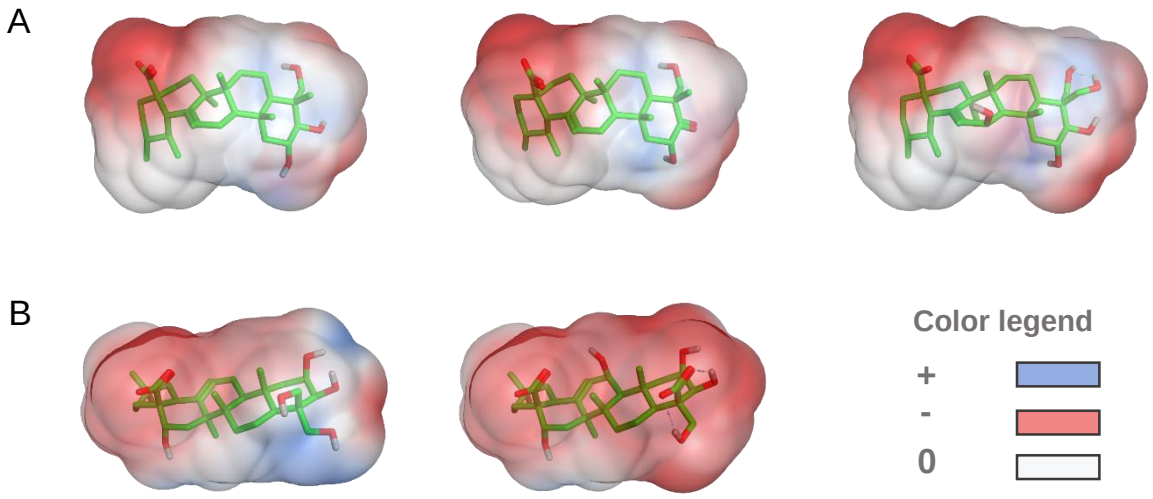


Fig. 8 Electrostatic properties of the poses of asiaticoside and its predicted metabolites (M1 and M45.S, left to right) (A) and platycodin D and its predicted metabolite M45.SS (B) mapped on the van der Waals interaction molecular surfaces by the MOE "Surfaces and Maps" tool within 4.5 Å of the atoms

Conclusion

The developed and validated VS protocol proved its ability to discriminate weak partial agonists from PPARγ decoys. It could be useful for rapid screening and prioritization of a large number of compounds in the development of drugs, nutritional supplements or components of novel functional foods. Our results from the application of the VS protocol to triterpenoids suggest possible PPARγ weak partial agonistic binding mode for all investigated aglycons and their predicted metabolites.

This research contributes to the mechanistic explanation of the effects of triterpenoid saponins/sapogenins by a potential PPARγ-mediated mode of action and can direct further studies of these compounds for their use as MetS modulators. The approach can be applied for VS of other naturally-derived compounds to predict the possible PPARγ weak partial agonists among them.

All data collected were processed, refined and organised in two separate datasets with: (i) structural and experimental data for PPARγ weak partial agonists (Supplementary Table 1), and (ii) library with aglycons of oleanane-type triterpenoids, which are relevant to PPARγ

modulation and MetS, supplemented by predicted metabolites (Supplementary Table 2). These sets are freely accessible and can be used as data sources in other studies of PPARγ and its ligands.

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