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Site of metabolism prediction on cytochrome P450 2C9: a knowledge-based docking approach

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Abstract A novel structure-based approach for site of metabolism prediction has been developed. This knowledge-based method consists of three steps: (1) generation of possible metabolites, (2) docking the predicted metabolites to the CYP binding site and (3) selection of the most probable metabolites based on their complementarity to the binding site. As a proof of concept we evaluated our method by using MetabolExpert for metabolite generation and Glide for docking into the binding site of the CYP2C9 crystal structure. Our method could identify the correct metabolite among the three best-ranked compounds in 69% of the cases. The predictive power of our knowledge-based method was compared to that achieved by substrate docking and two alternative literature approaches.

Keywords Site of metabolism · Prediction · CYP 2C9 · Docking · Expert system

Introduction

Since in the 1990s most of the attrition from discovery pipelines were due to poor pharmacokinetics [1], ADMET properties are now extensively monitored in the early phase of the drug discovery process. Prediction and optimization of ADMET properties can significantly increase the success rate of the preclinical research, development, and subsequent clinical trials. Accordingly, the prediction of these parameters by in vitro tests became essential in hit-to-lead and lead optimization screening cascades [2]. Metabolic stability has a large impact on crucial ADMET properties, such as the maximal achievable drug concentration and drug toxicity [3, 4]. Hepatic microsomal clearance assay is a feasible in vitro test providing information about the metabolic stability and elimination-rate constant. On the other hand, resolving the structures of the metabolites is still a challenging and labour-intensive task. Therefore, fast and reliable *in silico* prediction of metabolites is in high demand.

Cytochrome P450 (CYP) enzymes are ubiquitous haem-containing monooxygenases [5]. Eukaryotic CYP enzymes are anchored to the microsomal membrane, and they catalyse the oxidative biotransformation of various endogenous and exogenous compounds such as hormones, drugs, environmental compounds and pollutants [6]. Liver is the primary organ for human drug clearance, [7] where CYP enzymes participate in phase I metabolism of 70–80% of currently used drugs. The most important isoforms are CYP1A2 (~5% of current drugs), CYP2C9 and CYP2C19 (~25%), CYP2D6 (~15%) and CYP3A4 (~45%) [8]. These CYP isoforms typically exhibit characteristic, but occasionally overlapping substrate specificities and inhibitor profiles [9]. CYP2C9 is the predominant member of the 2C family with a major contribution to human drug metabolism [9, 10]. CYP2C9 exhibits selectivity for the oxidation of relatively small, lipophilic anions [11]. In particular, anti-inflammatory agents (diclofenac, flurbiprofen, ibuprofen, naproxen, piroxicam), anticoagulant

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compounds (S-warfarin), hypoglycaemic agents (tolbutamide), anticonvulsants (phenytoin) and loop diuretics (torsemide) as well as progesterone are CYP2C9 substrates [10, 12]. There are three crystal structures of CYP2C9 available in PDB including the substrate-free state (PDB ID: 1OG2), [6] and complexes with S-warfarin (1OG5) [6] and flurbiprofen (1R9O) [11].

CYP2C9 is a two-domain protein with an overall fold characteristic of the CYP family consisting of 12 α-helices [6]. The B–C and F-G helices contribute to substrate binding, while the inner haem-binding core is formed by helices E, I, J, K and L [6, 11, 12]. In the 1OG5 crystal structure S-warfarin is in a predominantly hydrophobic pocket lined by residues Arg 97, Gly 98, Ile 99, Phe 100, Leu 102, Ala 103, Val 113, Phe 114, Asn 217, Thr 364, Ser 365, Leu 366, Pro 367 and Phe 476 [6]. The potential site of hydroxylation of S-warfarin is in about 10 Å from the haem iron, therefore the enzyme in the corresponding crystal structure is suggested to be in the inactive conformation [6, 13]. In the 1R9O crystal structure, however, the 4′-oxydation site of flurbiprofen is in a favourable distance (<5 Å) to the haem iron for metabolism [10, 11]. Various studies highlighted the key role of Arg 108 and Phe 114 in the orientation of the substrate [10–12]. Moreover, the co-crystallized CYP2C9 structures suggest that Phe 100 and Phe 114 possess different side chain conformations depending on the structure of the bound substrate. We focused our investigations to holo structures since apo structure (such as 1OG2) is typically less suitable for docking studies. On the other hand, the distant orientation of the ligand in 1OG5 makes the flurbiprofen complex more reliable in respect to site of metabolism studies.

Due to the available structural information and its characteristic role in drug metabolism CYP2C9 quickly became one of the most widely analyzed targets of metabolite prediction studies [10, 12–14]. Between 2000 and 2003 several ligand-based and combined models were published [2, 15–19]. Afzelius et al. applied homology modelling using the rabbit CYP2C5 crystal structure as a template (sharing 77% sequence identity with human CYP2C9) to generate binding conformations of CYP2C9 inhibitors utilized in 3D-QSAR model [16]. Moreover, Sheridan et al. published empirical, structure-based QSAR models for human CYPs including 2C9 for site of metabolism prediction [20]. Several structure-based modelling studies were also published using the available CYP2C9 crystal structures [2, 10, 12, 14, 21].

Up to now the prediction of CYP2C9 catalyzed biotransformation involved two main approaches. Zamora et al. described a site of metabolism tool for predicting CYP2C9 metabolites. This method is based on a comparison between alignment-independent descriptors derived from GRID molecular interaction fields for the active site and a distance-based representation of the substrate [18, 19]. The test set used by Zamora et al. included 43 different substrates (25, 3, 4 and 11 substrates with one, two, three or four sites of metabolism, respectively), [18] representing 87 biotransformations in total. These authors reported that in more than 90% of the investigated biotransformations the hydrogen atom ranked first, second or third by this method was found to be the known site of oxidation [18]. This approach was reconstructed and introduced as the MetaSite software [22]. Sykes et al. described a 3D structure-based method using the molecular alignment program ROCS [23] with the query molecule flurbiprofen [10]. Their database consisted of 70 molecules. Overall, 51 (73%) ligands were aligned successfully, and the site of metabolism was found within 3 Å of the 4′-hydroxylation site of flurbiprofen in 60% of the molecules that can be considered as the overall efficacy of the approach on this particular dataset. These authors also applied a docking-based approach using FRED [24] with the PLP [25] scoring function for the prediction of CYP2C9 metabolites: 31 out of 70 molecules (44%) were correctly positioned [10].

P450cam is the best characterized prokaryotic CYP enzyme, which catalyzes the regio- and stereospecific hydroxylation of camphor [5]. The catalytic cycle of this enzyme has been thoroughly investigated (Fig. 1) and considered to be universal among the CYP enzymes [26, 27].

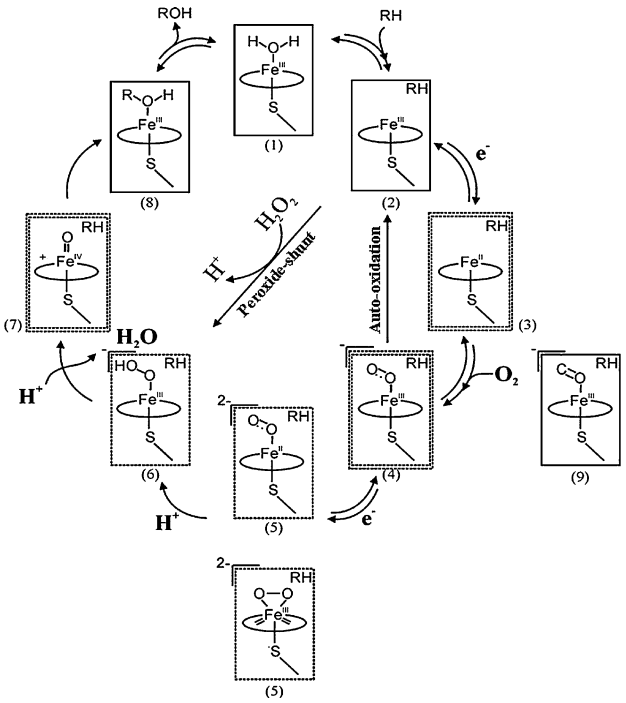

Fig. 1 Catalytic cycle for oxygen activation and transfer by cytochrome P450

After the crystal structure of the substrate-free (1) [28], enzyme-substrate (2) [29], -product (8) [30] and –carbon-monoxy [31] complexes (Fig. 1) became available, the catalytic pathway of P450cam was investigated at cryogenic temperatures by rapid-collection techniques [5]. Direct structural characterisation of normally unstable intermediates (3, 4 and 7) in enzyme-catalysed reactions made it possible to analyze the reaction mechanism in more detail [5]. This work shed light on the fact that the position of the substrate and the intermediates overlap with that of the product during the whole reaction (Fig. 2).

The overall structure of the protein matrix in the 5-exo-hydroxycamphor-bound P450cam (8) was found to be identical to that of the camphor-bound enzyme (2). Furthermore, the RMS shift of the common atoms of the product and the substrate is 0.13 Å only [30]. The 5-hydroxyl group of the product forms a weak interaction with the haem iron atom as evidenced by the continuous electron density between the product OH group and the iron atom [30]. These observations and the principal features of the consensus mechanism suggest that not only the substrates but also the products must fit the active centre of the CYP enzymes. Based on this conclusion, we propose here a novel structure-based approach for site of metabolism prediction consisting of three steps: (1) generation of possible metabolites with a rule-based expert system, (2) docking these metabolites to the active site of the CYP enzyme and (3) selecting the best docked compounds as potential metabolites ranked by their complementarity to the binding site. Since the X-ray diffraction analysis of the enzyme-product complex (1NOO) represented as structure 8 in the catalytic cycle (Fig. 1) revealed that the product must fit to the active site and furthermore it forms direct interaction to haem iron we hypothesize that the real metabolite will possess the best docking pose when docking potential metabolites. Due to its steric, electrostatic and hydrophobic complementary the docking score of the real metabolite is expected to be more favourable than that of the false products. In particular, false metabolites can be distinguished by their higher binding scores or by the complete lack of possible binding poses. This method in general can be used with a variety of expert systems and docking tools. Here we present our results obtained by using MetabolExpert [32] for the generation of potential metabolites and Glide [33] for docking them into the binding site of the CYP2C9 enzyme. Since current structure-based predictions primarily analyse substrate binding modes our results were compared to those obtained by substrate docking. Furthermore, our knowledge-based docking method was benchmarked against the state-of-the-art methodologies published by Zamora et al. [18] and Sykes et al. [10].

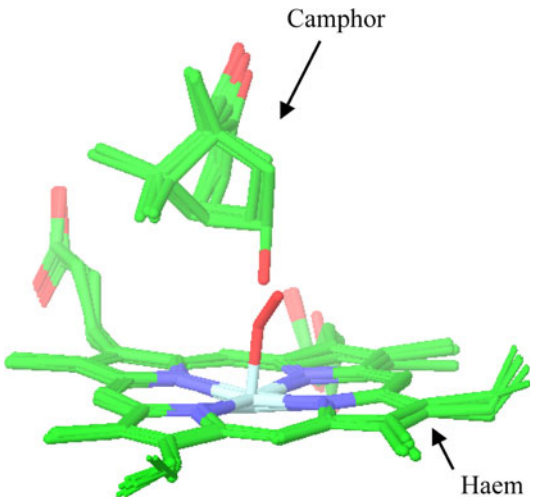

Fig. 2 Superposition of 1DZ4 (ferric enzyme-substrate complex) [5], 1DZ6 (ferrous enzyme-substrate complex) [5], 1DZ8 (iron-bound dioxygen substrate complex) [5], 1DZ9 (enzyme-substrate complex) [5] and 1NOO (enzyme-product) [30] crystal structures of P450cam. Carbon, oxygen and nitrogen atoms are coloured by *green*, *red* and *dark blue*, respectively. The iron atom is coloured by *cyan*

Materials and methods

Substrate database and metabolic reactions

To avoid any bias toward a specific set of substrates and metabolites, and analyze our method using independent datasets, we applied the CYP2C9 substrate libraries originally used by Zamora et al. (43 different molecules with 87 reactions) and Sykes et al. (70 molecules with single site of metabolism) [10, 18]. 21 molecules (58C80, aceclofenac, acenocoumarol, celecoxib, delta-9-tetrahydrocannabinol, diclofenac, flurbiprofen, fluvastatin, indometachin, linoleic acid, lornoxicam, losartan, mefenamic acid, mestranol, montelukast, phenprocoumon, phenytoin, piroxicam, S-MTPPA, warfarin and zafirlukast) were used in both studies. In the case of the overlapping substrates, we used the wider set of biotransformation reactions reported by Zamora et al. In summary, our database contained 92 substrates with 136 metabolic reactions. Compounds were downloaded from the PubChem database [34] or were drawn by MarvinSketch [35]. The substrate database is available from the authors upon request.

MetabolExpert module of the Pallas 3.5.1.4 [32] software was used to generate a pool of possible metabolites of the substrates. MetabolExpert is a rule-based expert system that predicts potential metabolites by the rules constructed from known metabolic transformations covering more than 400 article references [36]. Since the latest article was included in 2004 we applied CYP2C9 specific reaction types and discarded phase II reactions (conjugations) and

non-oxidative transformations updating the implemented set of rules. One general chroman-ring oxidation rule that was already described in the literature [37] was added to the default transformations of MetabolExpert (see Supplementary 1). When the experimentally observed metabolites were missing from the pool generated by MetabolExpert, we added them manually. These data were only used for the independent analysis of the efficacy of metabolite generation and docking.

The reaction data file (rdf) generated by MetabolExpert was converted to structure data file (sdf). All the possible metabolites and substrates were prepared for docking by LigPrep [38]. 3D conformations were optimized by PRCG method and the most probable protomers (pH = 7, Epik module) and tautomers were used for further investigations. The stereochemical information of the substrates was preserved during the metabolite generation protocol.

Protein preparation

We used the flurbiprofen co-crystallized (1R9O) CYP2C9 crystal structure [11], the camphor (1DZ4) [5] and the 5-exo-hydroxycamphor (1NOO) [30] co-crystallized P450cam for docking studies. Water molecules and co-crystallized glycerine were deleted. Protein Preparation Wizard [33] was used with default settings to prepare the crystal structures. The active site was defined according to the position of the haem-group or the ligand.

Docking

We docked the potential metabolites to the binding site by Glide 5.0 [33] that was previously shown to provide accurate binding modes of small molecules [39–41]. We carried out Standard Precision (SP) calculations with default settings except the number of the poses included in the post-docking minimization which was set to 20.

Since the newly formed groups in the hypothetic product complex should be oriented towards the haem iron as demonstrated by the corresponding P450cam structural data, we tested the effect of filtering the poses based on the distance between the newly formed group and the haem iron. The distance was calculated for the top twenty docking conformations, all the poses possessing value over the distance limit were discarded and the best binding mode was selected according to the default settings available in Glide.

Data analysis

In the case of metabolite docking, we ranked the possible metabolites by GlideEnergy and searched for the rank of the experimentally observed product(s). We calculated the real metabolite ratio of the whole database in two ways. In the first case only the first experimentally verified metabolite was considered in the rank list, while the second approach considered all correct metabolites (if there were more than one). If two metabolites were one after the other in the rank list, we used the same rank as suggested by Zamora et al. [18].

In the case of substrate docking, we used the three lowest energy poses and calculated the percent of the cases where the site of metabolism was the closest heavy atom to the haem iron.

Python scripts were used for data collection and data mining. Graphs and calculations were prepared by MS Excel™, and Origin 7G™.

Results and discussion

According to our hypothesis (1) the correct binding pose of the real metabolite is preferred over the decoy products and (2) docking of metabolites is more efficient approach for prediction of metabolic site than substrate docking. We suggest that true metabolite(s) produced in the catalytic cycle can be more favourably accommodated to the active site than the false ones. In other words, true metabolites possess reasonable binding modes that should reflect in their binding interactions. False metabolites, however, fit less perfectly to the binding site, consequently their interactions should be significantly weaker, or no reasonable binding mode should be generated for them. As a proof of concept we docked the camphor and its possible metabolites into the active site of the cytochrome P450cam (PDB code: 1NOO). During this investigation all the possible hydrogen atoms were substituted into hydroxyl groups one-by-one to test the ranking capability of the method. Ranking of the seven docked molecules by their by GlideEnergy resulted the correct metabolite in the first place. The orientation of the docked product was within 0.56 Å RMSD compared to the experimentally observed binding mode of 5-exo-hydroxycamphor (Fig. 3).

Testing the efficacy of camphor self-docking (protein PDB code:1DZ4) gave converse results, since the site of metabolism is located far away from the haem centre. In this case the carbonyl oxygen interacts with the haem iron instead of forming H-bond with Tyr 96, resulting 2.3 Å RMSD compared to the orientation observed experimentally (Fig. 4).

In the next step we examined the case of flurbiprofen co-crystallized with CYP2C9. For testing purposes each hydrogen atom was substituted to hydroxyl groups and the generated metabolites were docked into the active site of CYP2C9 (PDB code:1R9O) to rank them according to their complementarity. The observed metabolite was the second

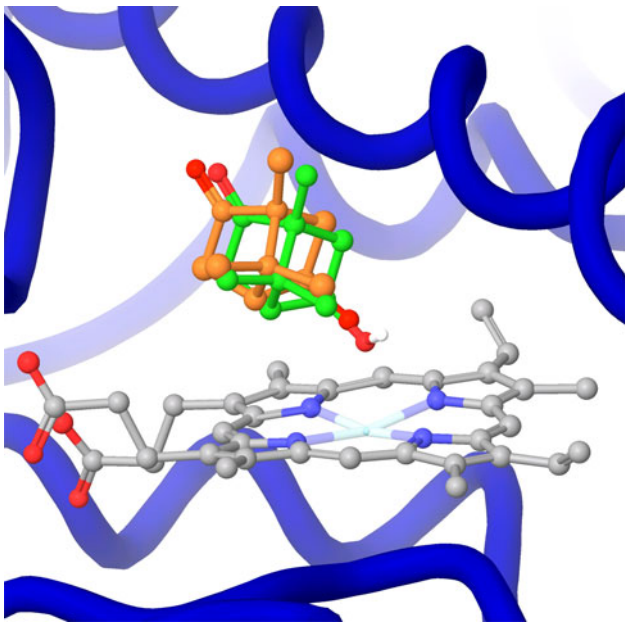

Fig. 3 Binding mode of the 5-exo-hydroxycamphor in the active site of the P450cam (1NOO). Haem carbon, docked ligand carbon, co-crystallized ligand carbon, oxygen, nitrogen and iron atoms are coloured *grey*, *green*, *orange*, *red*, *blue* and *cyan*, respectively

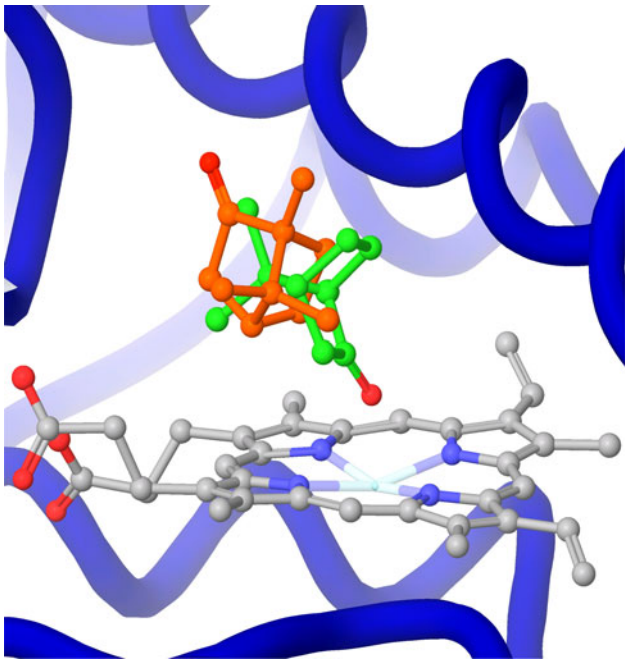

Fig. 4 Binding mode of camphor in the active site of the P450cam (1DZ4). Haem carbon, docked ligand carbon, co-crystallized ligand carbon, oxygen, nitrogen and iron atoms are coloured *grey, green, orange, red, blue* and *cyan*, respectively

ranked compound using GlideEnergy. In the case of the best ranked decoy, the corresponding hydroxyl group formed upon the haem mediated biotransformation is located in 12.5 Å distance from the haem iron, therefore

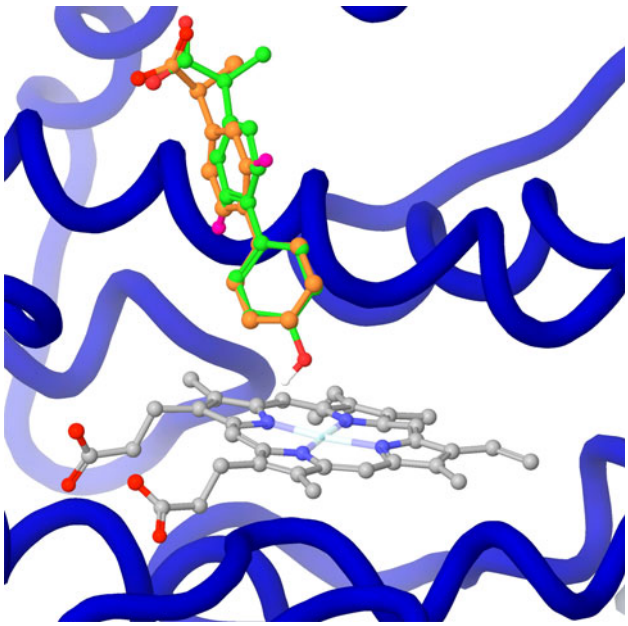

Fig. 5 Binding mode of the 4′-hydroxy-flurbiprofen and the co-crystallized ligand in the active site of the CYP2C9 (1R9O). Haem carbon, docked ligand carbon, co-crystallized ligand carbon, oxygen, nitrogen, fluorine and iron atoms are coloured *grey, green, orange, red, blue, pink* and *cyan*, respectively

this metabolite might be discarded. Binding mode of the real metabolite overlaps with that of the native ligand considering the carbon atoms (Fig. 5), moreover the oxygen atom is located in an appropriate distance (3.95 Å) from haem iron.

The binding mode of 4′-hydroxy-flurbiprofen matches the interaction field of the substrate and forms an additional interaction with the haem iron. Therefore, we assume that modelling the oxygenated product complex (Fig. 1, structure 8) is a feasible way of distinguishing between decoy and real metabolites.

Seeking for binding mode of flurbiprofen substrate gave similar results to the case of camphor substrate docking. In the case of the best binding mode of the substrate the experimentally observed site of metabolism (the 4′-oxdiation site) of flurbiprofen was positioned 11.8 Å away from the haem iron (Fig. 6). Binding mode of flurbiprofen was significantly different form that found experimentally, the carboxyl group was oriented towards the haem iron instead of interacting with Arg 108 and Asn 204. Interestingly, skipping post-docking minimization gives the correct pose (RMSD is within 0.5 Å) as the top one, but in case of extended post-docking minimization the false pose outperforms the correct one in terms of the GlideEnergy.

Based on these observations we investigated a larger set of available substrates and published metabolites to validate the methodology.

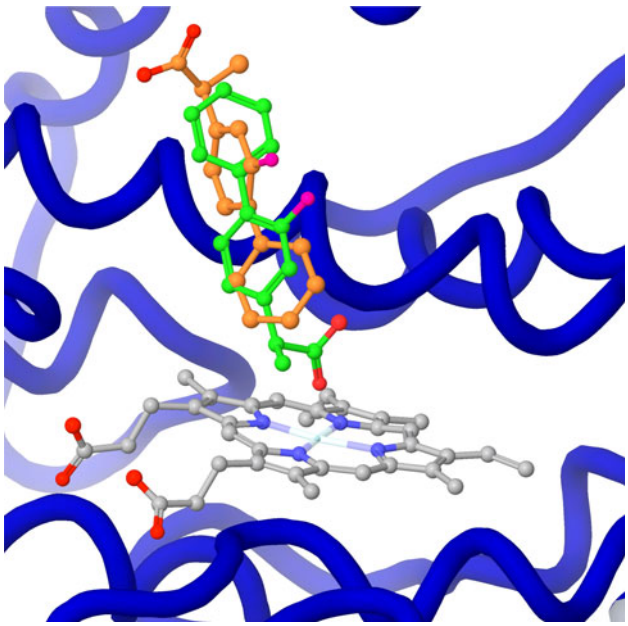

Fig. 6 Binding mode of flurbiprofen in the active site of the CYP2C9 (1R9O). Haem carbon, docked ligand carbon, co-crystallized ligand carbon, oxygen, nitrogen, fluorine and iron atoms are coloured *grey, green, orange, red, blue, pink* and *cyan*, respectively

Metabolite generation

Considering the 92 substrates and 136 biotransformation reactions MetabolExpert generated 74% of all true metabolites. This rate is significant, however one quarter of the correct metabolites was still lost. MetabolExpert provides the possibility of editing and expanding its database. New rules can be based on in-house metabolism studies or on the growing literature of the CYP enzymes. In our case, expanding the rules of MetabolExpert by one recently described hydroxylation-reaction [37] at the chroman ring increased the efficacy up to 82%.

Metabolite docking

We applied docking to select the correct metabolites from the potential ones generated by MetabolExpert. GlideEnergy values were used to rank order all possible metabolites based on their complementarity to the active site. We investigated the impact of active site definition (haem centred or substrate centred) and the use of post-docking filter monitoring the distance between the atom added to the substrate upon the biotransformation and the haem iron. Results are summarized in Table 1.

Modelling the correct binding mode of oxygenated product complex is a crucial point of our methodology, accordingly we analysed the impact of the distance filter defined between the metabolic site and haem iron. Twenty poses were generated and filtered by a certain distance cut-off to select the best binding mode for each possible metabolite. Setting an appropriate filter gives the opportunity to discard poses not fulfilling the requirements of our hypothesis, since it eliminates the poses which are not in agreement with the approximated product complex. Analysing the percentage of the cases ranked first, second or third with the best setting (flurbiprofen centred active site) using filtering (shown in Fig. 7) revealed 6 Å as an optimal distance filter which is in agreement with the experimental observations on P450cam [42].

Considering first ranked poses docking efficacy is decreasing after 6 Å with increasing distances and is continuously turning into the non-filtered situation. On the other hand, the efficacy calculated for second and third ranked cases increases and reaches maximum at higher distance. Based on these observations we conclude that the distance filter of 6 Å applied for the first, second and third ranked poses could significantly improve the overall efficacy of our method.

Table 1 Docking results shown as the percent of the cases where the correct metabolite could be found among the first, first and second or at the three best-ranked candidates

Active site	Rank	Post-docking filter	
		(−)	(+)
Haem centered	I.	47.8	51.1
	I–II.	72.8	71.7
	I–III.	83.7	79.3
Substrate centered	I.	45.6	57.6
	I–II.	67.4	75.0
	I–III.	79.3	83.7

6 Å cut-off between the haem iron and the atom added in the metabolic reaction was applied for post-docking filtering

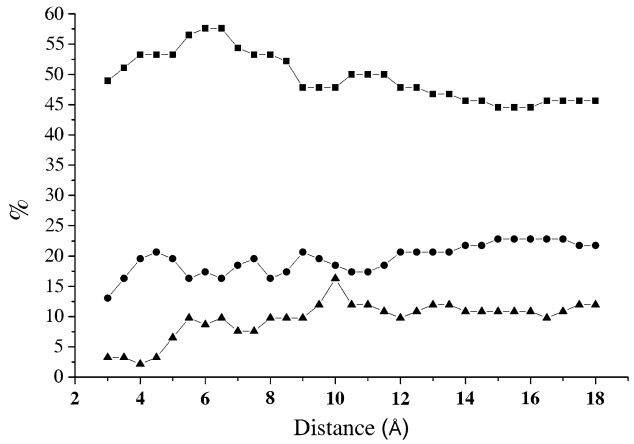

Fig. 7 Docking efficacy plotted by the distance cut-off of filtering. *Square, circle* and *triangle* indicates the percentage of the cases ranked first, second and third, respectively. Docking was performed using the 1R9O crystal structure with flurbiprofen centred active site

Our optimization protocol also assessed the influence of the active site definition. Accordingly, we defined the active site based on the flurbiprofen substrate or haem. This parameter, which sets the number of possible initial locations (site-points) of the molecule in the enzyme, had only a slight effect on the docking efficacy (Table 1). Our results suggest that using the flurbiprofen centred active site in combination with the post-docking filter gave the highest fraction of first ranked cases.

It should be noted that the only role of the energy function is ranking possible metabolites. Here we use GlideEnergy, a modified Coulomb—van der Waals interaction energy that is not scaled to reproduce binding affinity. Although it is a proper function to sample the main interaction pattern and complementarity of the metabolite with the protein it cannot estimate its binding affinity. The higher GlideEnergy of false metabolites indicates that these potential products could not fit the active site or if reasonable binding mode exists they can not form interactions similar to that of the real metabolite. Therefore, preferred GlideEnergy of the real metabolite over the other potential products do not indicate product based inhibition.

The best docking performance was achieved by using the 1R9O crystal structure, flurbiprofen active site definition and filtering. Analysing the cases where our method failed to predict the true metabolite among the top three ranked product, 8 out of 15 had more than 6 rotatable bonds (for example carvediolol (10) and linoleic acid (14)), 3 had molecular weight (Mw) over 500 (montelukast, zafirlukast and the hydroxylated derivative of zafirlukast [18]) and there were 3 steroid derivatives (desogestrel, 17-alpha-ethinylestradiole and mestranol). This result might be explained by (1) the absence of receptor flexibility during Glide SP docking, since recognition of high Mw metabolites or huge rigid molecules like steroids are probably accompanied by a considerable conformational change in the protein, and (2) docking ligands with numerous rotatable bonds expands the conformational space resulting more challenging task for docking.

Substrate docking

Currently used site of metabolism approaches are primarily based on the structures of substrates. We therefore docked the substrates to the binding site as well and compared the efficacies with those obtained by metabolite docking.

Substrate docking calculations were carried out in SP mode using the 1R9O crystal structure and flurbiprofen centred active site. 43 out of 92 substrates (47%) were docked in optimal orientation considering the three best ranked poses. The method outlined in this paper consists of two subprocesses, consequently the total performance can be calculated from the metabolite generation (82%) and docking (84%) steps, which gives 69% as an overall efficacy. We therefore concluded that our knowledge-based metabolite docking approach is significantly more accurate than substrate docking on the enzyme investigated (CYP2C9) and metabolite dataset used. It is important to note that our method combines the advantages of the expert system and the structural information of the CYP enzyme. The possible metabolites contain more hydrophilic interacting points than the substrates. This might result in more efficient docking of metabolites, since hydrophilic interactions are considered in the initial steps of the Glide docking process ("subset test"). Another advantage of our metabolite docking based prediction is that substrate docking needs manual evaluation while the evaluation in our method can be automated.

Comparison to other methods

Our substrate database included 43 compounds reported by Zamora et al. and 70 molecules reported by Sykes et al. providing us a benchmarking challenge. Zamora et al. utilized 87 metabolic reactions. They reported the correct hydrogen atom as the site of oxidation ranked as first 50%, second 75% or third in more than 90% of the cases, using their dataset (43 substrates with 87 true metabolites), we obtained 59, 65 and 71% overall efficacy, respectively (see Table 2).

Sykes et al. reported a site of metabolism study based on ROCS alignments. In this study the site of metabolism was found within 3 Å of the 4′-hydroxylation site of flurbiprofen in 60% of the cases. Considering the three best-ranked metabolites, our method demonstrated 66% maximal efficiency on the same dataset (see Table 2).

It is important to mention that we applied the substrate sets of Zamora and Sykes et al., which ensures that our substrate selection is not biased against our method. It is also important to emphasize that our method provide the exact metabolite structures, while substrate-based methods suggest the site of metabolism only.

The diversity of the benchmarking database is a key aspect to consider when different methods are compared.

Table 2 Site of metabolism prediction efficacy for benchmarking datasets

Substrate set	Zamora et al. [18]		Sykes et al. [10]	
Rank	Docking Efficacy (%)	Overall	Docking	Overall
I.	72	59	47	39
I–II.	79	65	70	57
I–III.	87	71	80	66

Efficacy data are shown as the percent of the cases where the correct metabolite could be found among the top ranked candidates

Table 3 Diversity assessment of the datasets

	Zamora et al. [18]	Sykes et al. [10]	This work
Tanimoto mean	0.317	0.229	0.250
Tanimoto SD	0.177	0.105	0.127
Number of substrates	43	70	92

The preferable method should provide reliable results on diverse databases. We calculated the 2D-UNITY fingerprints [43] and the corresponding Tanimoto matrix of the databases (see Table 3).

Table 3 shows that the molecules used by Zamora et al. are less diverse than the substrates utilized by Sykes et al. This is partially reasoned by the fact that Zamora et al. adopted 13 coumarin-like molecules and 7 enantiomer pairs in their database. Furthermore, the performance of the GRID based approach by Zamora et al. (commercialized as MetaSite) was shown to depend on the applied substrate database. Truzner et al. published a prediction data for 18 compounds and concluded that MetaSite predicted 14 molecules (78%) correctly, considering the best ranked three options [3]. Zhou et al. reported metabolite prediction for CYP3A4 substrates by MetaSite with 78% accuracy [44].

A QSAR based metabolic site prediction approach was recently reported by Sheridan et al. for the prediction of the oxidation set of a diverse set of substrates [20]. This method yielded 73% ($N = 68$) and 67% ($N = 10$) efficacy on CYP2C9 for the calibration and external sets, respectively considering the top two atoms [20]. The authors also predicted the metabolic site for these molecules by MetaSite that resulted in 69 and 67% efficacy values for the calibration and test sets, respectively [20].

Based on the results achieved by our methodology, we suggest that knowledge-based docking is a competitive approach with the generally used substrate-based prediction methods. Moreover, an important advantage of our approach is the direct structural prediction of the metabolite instead of giving the site of metabolism points for the molecules. It is important to mention that higher efficacy might be achieved by occasionally updating the expert system responsible for the generation of potential metabolites. Another possibility is to use more accurate and sophisticated docking programs with further optimized parameters.

Conclusions

We report here a novel methodology for predicting the metabolites of CYP enzymes. The underlying phenomenon behind this approach is the overlap between the substrate and the product structures demonstrated by the crystal structures representing the different stages of the P450cam catalytic cycle. Consequently, we suggest that the correct metabolite can be selected from the set of potential metabolites by docking to the CYP crystal structure. As a proof of concept, we used CYP2C9, which is a major contributor of human drug metabolism. We constructed a database comprising of 92 substrates, generated the corresponding possible metabolites by MetabolExpert and applied Glide as a docking tool.

MetabolExpert generated 74% of the true metabolites with default metabolite rules, while incorporating one recently described rule this efficacy was increased to 82%. Regarding the optimization of the docking phase we conclude that filtering the poses by their distance between the metabolic site and the haem iron can improve the efficacy. We achieved the best result using the 1R9O crystal structure co-crystallized with flurbiprofen with ligand centred active site, applying post-docking distance filter of 6 Å. Using the best options, one correct metabolite was found among the three best-ranked options in 69% of the cases, since metabolite generation and docking accuracy was 82 and 84%, respectively.

Our method gave comparable results with those achieved by literature approaches used for site of metabolism prediction. It was also demonstrated that metabolite docking performed better than substrate docking on our dataset. The main advantages of our method are automatic evaluation of the results and the direct prediction of the metabolite structures instead of the site of metabolism only. Furthermore the application of the knowledge-based docking method does not necessarily require the use of specific commercial metabolism prediction software. The possible metabolite pool can be generated by chemists and pharmacokinetic experts, providing opportunity testing their ideas, discussing and interpreting the results easily.

Our knowledge-based docking approach can be adopted for different CYP enzymes. The efficacy of the knowledge based method depends on both metabolite generation and docking. If the metabolite generation algorithm is not capable to provide the correct metabolite among the decoys, it can not be predicted by the subsequent docking step that underlines the importance of expert systems tailored for the given isoform. On the other hand, docking protocols could also be optimized to achieve the best performance on the CYP enzyme under investigation.

Predicting the metabolically vulnerable points and corresponding metabolites is useful for metabolic stability optimization. We therefore suggest our novel method as a reliable and fast metabolite prediction tool to support lead optimization programs.

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