Prediction of Metabolism by Cytochrome P450 2C9: Alignment and Docking Studies of a Validated Database of Substrates

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A validated database of 70 molecules known to undergo biotransformation by CYP2C9 was collated. The molecular alignment program ROCS was used with the query molecule flurbiprofen as a basis for predicting the correct active site orientation of the CYP2C9 database molecules. The quality of the results obtained was excellent, with 39 of the first 44 molecules (89%) sorted by ROCS combination score having alignments that accounted for the experimentally observed site of oxidation. Transposition of the first 39 correctly aligned molecules into the CYP2C9 active site yielded an average site of metabolism to iron heme distance of 5.21 Å, in good agreement with previous experimental observations. Molecular docking studies were also undertaken, but the results were less successful than the ROCS-based alignment method, indicating that ligand-based approaches with chemical typing are important in the prediction of metabolism by CYP2C9.

Introduction

Cytochrome P450 (CYP[a]) is a superfamily of enzymes largely involved in the oxidative metabolism of a wide range of drugs, environmental chemicals, and endogenous compounds.[1] The P450 enzymes share common structural characteristics such as a common overall fold despite less than 20% sequence identity across the P450 gene superfamily.[1] In recent years, the elucidation of mammalian and, more recently, human crystal structures has provided further insight into the important structural aspects of these critical biological enzymes.

The major P450 enzymes present in human liver, the principal organ involved in the metabolic clearance of drugs and other chemicals, are classified in the P450 3A, 2C, and 1A subfamilies, which account for approximately 40, 25, and 18% of immunoquantifiable P450s, respectively.[2,3] CYP2C9, which is the predominant member of the 2C family, is an enzyme of major importance in human drug metabolism.[4] CYP2C9 has additionally been shown to be the second most prevalent P450 enzyme in the human small intestine.[2] This enzyme is known to be involved in the metabolism of numerous drugs, such as NSAIDs (e.g., ibuprofen,[5] naproxen,[6] and flurbiprofen[7]), warfarin,[7] oral hypoglycaemic agents, including tolbutamide,[8] the anticonvulsant phenytoin,[9] and the loop diuretic torsemide.[10] Most of these compounds are weak acids. However, they represent just a few of the structurally diverse range of compounds that are oxidized by CYP2C9 and, more recently, the diversity of CYP2C9 substrates has widened with phosphorus-containing thioether pesticides shown to have significant CYP2C9 activity.[11] The importance of CYP2C9 in drug metabolism has led to the enzyme being one of the "standard" enzymes screened during the in vitro investigation of the hepatic metabolism of xenobiotics, particularly newly discovered drugs. Consequently, a large number of substrates have been elucidated and the metabolic profiles have been characterized.

Various attempts have been made to utilize in silico methods for the investigation of the important features of the CYP2C9 active site[12–14] and to use this information to predict positions of oxidation and determine important structural features that confer selectivity. Initially, these studies focused on utilizing homology models of CYP2C9 in the absence of a crystal structure, notably those based on the rabbit CYP2C5 structure, which shares 77% sequence identity with human CYP2C9.[12] Rao et al. reported a ligand-based model for CYP2C9, utilizing the CoMFA model for the prediction of K_i values.[15] The model was proposed to be useful as a pharmacophore-based method to screen for possible drug interactions and to design molecules that will not bind to this enzyme with high affinity. A combined protein and pharmacophore model for CYP2C9 was described by De Groot et al.[16] The approach utilized a combination of pharmacophore modeling, protein homology modeling, and molecular orbital calculations for intermediate and product identification. The major features encapsulated in the model were steric, electronic, and chemical stability properties. The model was subsequently validated with substrates not used to create the initial model. Zamora et al. described a site of metabolism prediction tool for CYP2C9 that was based on comparison between alignment-independent descriptors derived from GRID molecular interaction fields for the active site and a distance-based metric of the substrate.[17] This combination approach ranked all substrate hydrogen atoms in order of likelihood of being located at the site of metabolism. In more than 90% of the 87 CYP2C9-catalyzed reactions investigated, the hydrogen atom ranked first, second, or third as the known site of oxidation.[17]

It is important to note that many of the CYP2C9 pharmacophore/protein models, including those referred to above, made use of homology models based on mammalian P450s, other than CYP2C9. However, two protein structures for CYP2C9 are now available. The proteins were crystallized both with and without substrate present. The presence of substrate yielded the opportunity to investigate the important active-site interactions between protein and substrate. The first structure released was the CYP2C9 protein with bound warfarin (protein databank code: 1OG5),[18] which is known to undergo CYP2C9-catalyzed

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[a] Abbreviations: CYP, cytochrome P450; NSAID, nonsteroidal anti-inflammatory drug; SPZ, sulfaphenazole.

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6- and 7-hydroxylation. It was observed that the warfarin was bound to the 1OG5 structure in the distal end of the active site cavity. Most surprising, however, was the 6- and 7-hydroxylation sites located approximately 10 Å from the iron in the heme moiety. This location is considered unproductive and may reflect the presence of an unidentified molecule bound closer to the heme moiety.

A second crystal structure, 1R9O,[19] was published shortly after, this time with the CYP2C9 substrate flurbiprofen bound, which is known to undergo oxidation, primarily by 4′-hydroxylation. This structure yielded the 4′-oxidation site at an appropriate distance for oxidative attack by the reactive hypervalent iron-oxo intermediate derived from the reduction of molecular oxygen by CYP2C9. This distance was determined to be slightly under 5 Å, in marked contrast to the 10 Å observed in the 1OG5 structure.[18,19] NMR studies based on certain Fe spin-state parameters have determined substrate-heme distances as 6.2, 3.3–4.4, and 4.7–4.9 Å for P450s.[20] The 1R9O structure highlighted several amino acid residues in the binding site that were likely to be important for flurbiprofen binding. In particular, the Arg-108, Asn-289, and Asp-293 residues were flagged as potential substrate recognition moieties, in agreement with previous mutagenesis studies, providing solid evidence for the preference of relatively small lipophilic anionic substrates for CYP2C9. Some of these residues have been previously noted as important in the various in silico approaches referred to earlier,[16] highlighting the utility of the pharmacophore-based approaches.

In this study, we have collated a database of 70 known CYP2C9 substrates, either with CYP2C9 as the primary P450 metabolizing enzyme or with CYP2C9 having a more minor role. However, we have only included compounds as CYP2C9 substrates if they meet at least one of the following two conditions in the primary literature: (1) Kinetic data (K_m and V_{max}) exists for metabolism by recombinant CYP2C9. (2) There is a minimum of 20% inhibition of the human liver microsomal CYP2C9-catalyzed reaction by sulfaphenazole, a highly selective inhibitor of this enzyme[10,21]

For each molecule, we noted the primary site of oxidation, unless there is no clear evidence for a dominant CYP2C9 metabolite. The present study has several main goals: (1) To investigate whether the flurbiprofen structure (as taken from the 1R9O crystal structure) is a suitable template molecule for the alignment of CYP2C9 substrates. (2) Quantification of the overlays by comparing the distance between the known 4′-flurbiprofen hydroxylation site and the site of metabolism in each overlaid molecule from the CYP2C9 database. Correctly aligned database molecules would be expected to have a small distance between their principal site of metabolism and the 4′-hydroxylation site in flurbiprofen. (3) Score the flurbiprofen-aligned CYP2C9 substrates with a docking program and determine the substrate oxidation site-heme distance. The alignment scores could potentially be used as a quality control strategy. (4) Identify important amino acid interactions from the docking analysis.

Many of the substrates in our CYP2C9 database were obtained from literature searches, and from a paper that used support vector machines to classify compounds as substrates or nonsubstrates of CYP2C9.[22] Additional substrates were sourced from previous modeling studies.[16,17]

Database and Conformers

The CYP2C9 database comprised 70 molecules obtained from literature searches and from previously published studies.[16,17,22] The molecules used in this study are shown in Figure 1, with the principal site of CYP2C9 metabolism indicated in each case. The primary literature was consulted in all cases (Table 1).

A conformational database was created for the 70 molecule data set using the Omega conformer generation software.[23] Omega has been shown previously to accurately reproduce the structures of protein-bound ligands and has been compared with the Catalyst conformer generator in a recent paper.[24,25] Omega creates initial models of structures by utilizing a fragment-based approach. The fragments are usually assembled by means of predefined fragment libraries built with the "makefraglib" module. The model-building phase of conformer generation is followed by a torsion-driving stage that generates conformers up to the limits predefined by the user in terms of energy and ensemble size, among other parameters.

The molecules were stored initially as SMILES strings, without specific enumeration of stereochemistry or *cis*/*trans*-isomerism. A fragment library was created using the "makefraglib" module of Omega and was utilized in the final conformation creation process. Subsequently, the "flipper" module in Omega was used to enumerate the stereochemical features of the molecules, which were employed by the main module of Omega for conformer generation. Default parameters were used, apart from the energy window command (ewindow), which was set to 30 kcal mol^{-1} instead of the default value of 25 kcal mol^{-1}. This was found to yield better results in the overlay procedure discussed below.

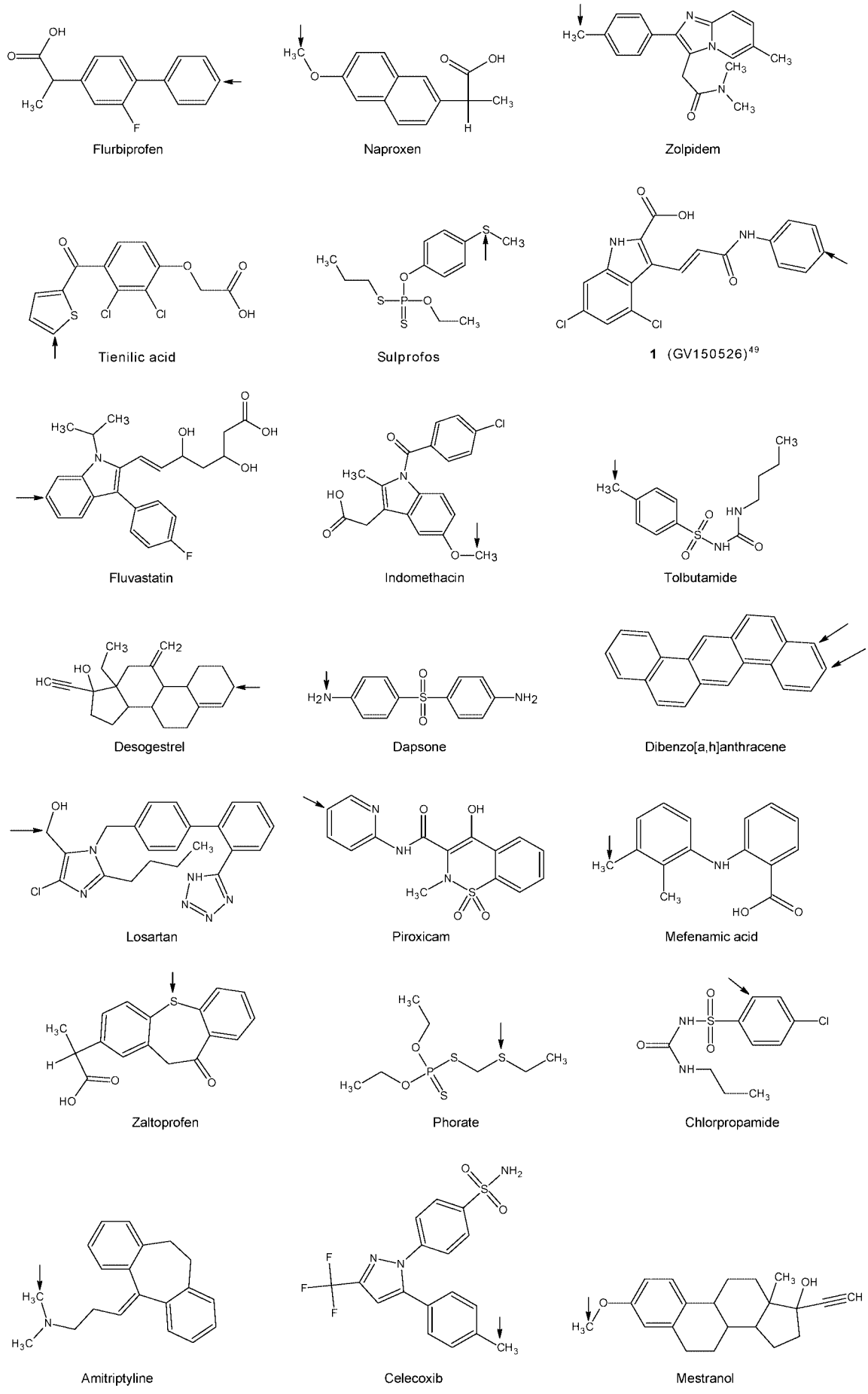
Methods

We have examined the use of the ROCS overlay method[26] and molecular docking with the program FRED[27] to investigate the structural similarity of CYP2C9 substrates and the location of their oxidation sites with respect to the heme iron, as well as confirming the important amino acids in the CYP2C9 binding modes.

ROCS is a shape comparison program that has an additional built-in chemical forcefield that can be used to compare the chemical features of molecules, as well as their shape.[26] The shape comparison in ROCS is performed by use of the simple, but effective, concept of volume overlap. Generally a query molecule (or guide structure) is used to compare with the shapes of molecules in a database. Shape similarity is computed by way of a Tanimoto score, with a score of 1 representing a perfect shape match. In other words, the closer the shape score is to 1, the better the shape match with the chosen query molecule.

However, shape is only one facet of molecular recognition. Additionally, chemical features have an important role to play in determining a molecule's likely interactions with a protein. In ROCS, the chemical similarity between a query molecule and a database molecule is computed by way of a color forcefield, which is defined by the use of SMARTS patterns. The forcefield considers chemical features such as hydrophobic fragments, rings, anionic and cationic moieties, hydrogen bond donors and acceptors, and how these centers interact.[26] The ROCS chemical forcefield has default settings, but these can be adjusted and weighted differently as required. An ImplicitMillsDean color forcefield is used in this work, which includes a pK_a model based on pH = 7.

Previously we have used the scaled color forcefield score to discriminate between strong and weak hepatic microsomal binders.[28] The scaled color score on its own was found to give the greatest discrimination between strong and weak microsomal binders and, additionally, yield the important chemical character-



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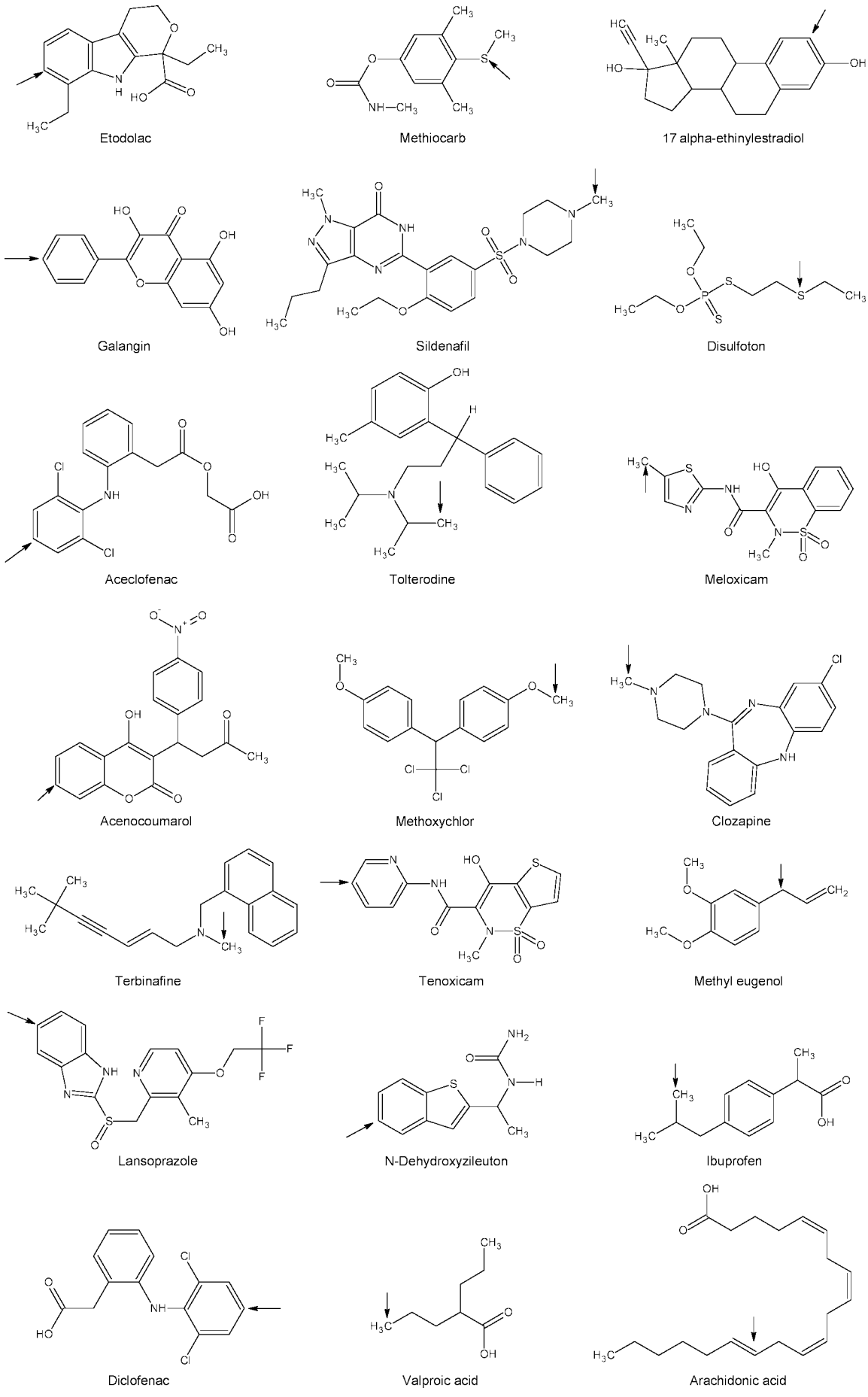

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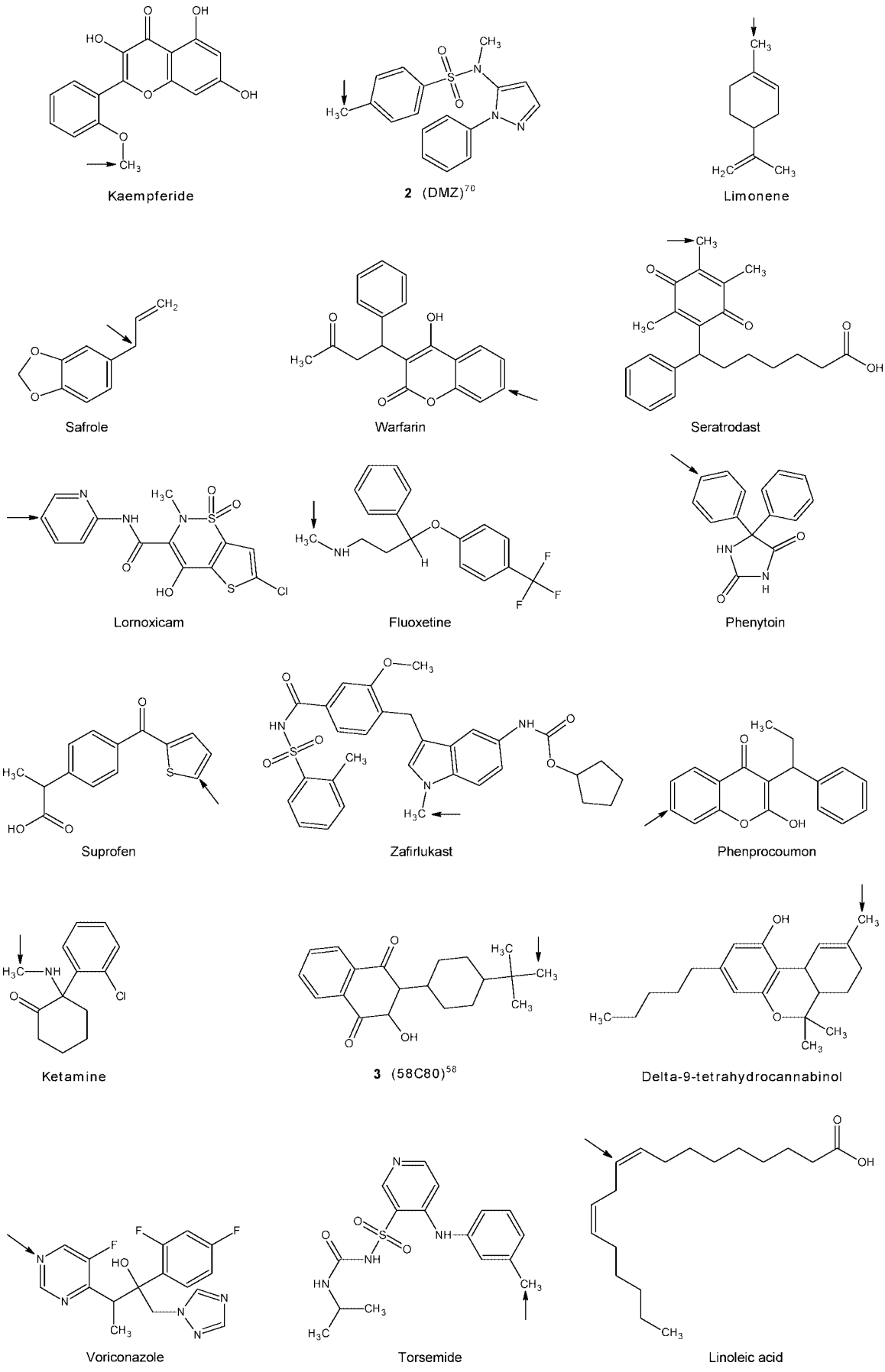

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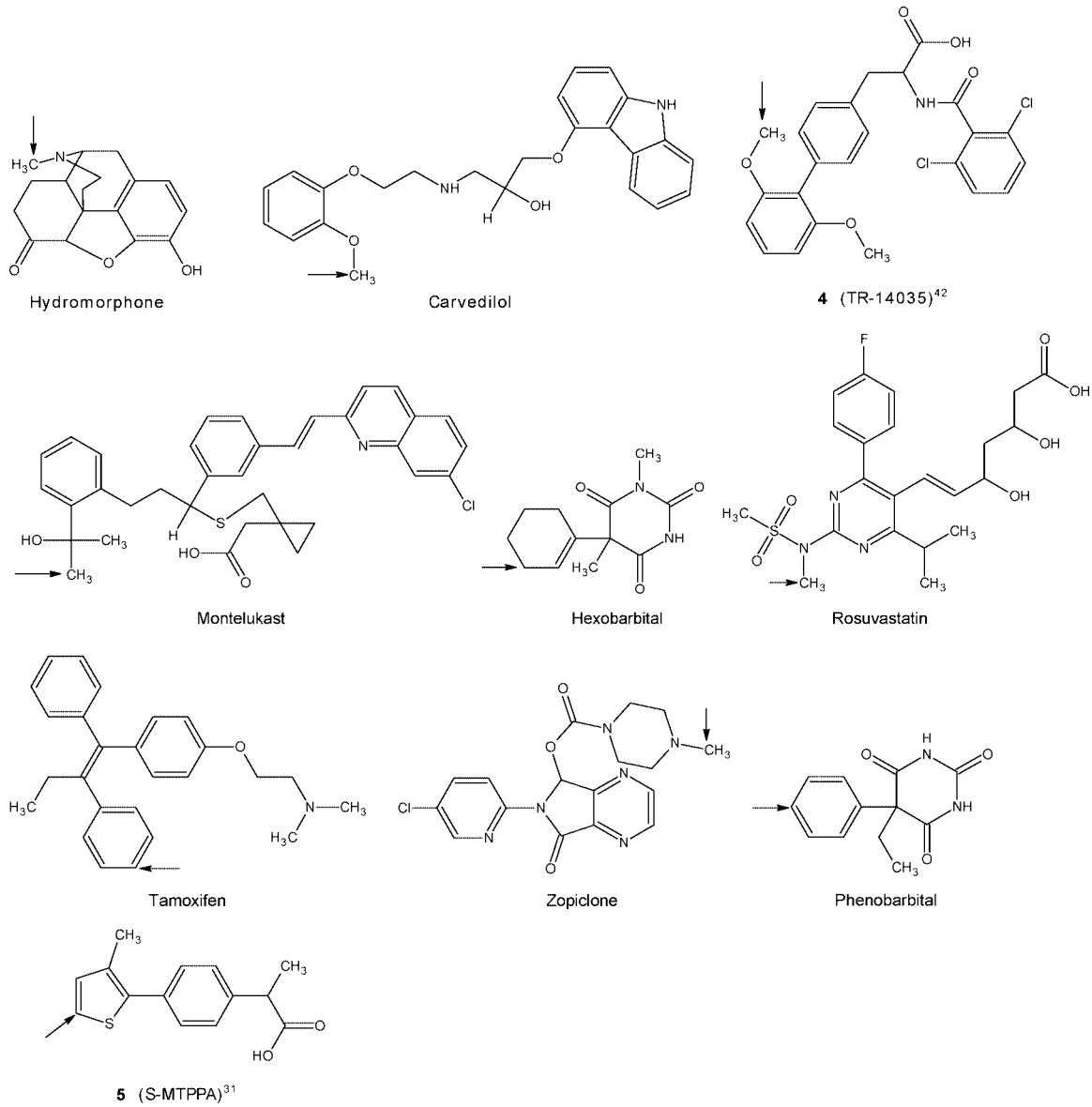

Figure 1. Structures of molecules in the CYP2C9 database with the principal site of CYP2C9 metabolism indicated by the arrow. It should be noted in the case of dibenzo[*a*,*h*]anthracene, a diol metabolite is formed.

istics relevant to nonspecific binding. As is the case for shape, color has a perfect match score of 1, via the scaled color score. Therefore, a linear combination of color and shape leads to a perfect match score of 2. This is called the combination score (referred to as the combo score). The combo score was used in this study as the overlay score of choice, because both shape and chemical features are likely to be important for binding in the active site. To include both color and shape in the optimization, the setting "optchem" must be included on the ROCS command line.

As indicated previously, flurbiprofen was the substrate cocrystallized in the 1R9O CYP2C9 structure. Flurbiprofen is considered to be a prototypic anionic, hydrophobic substrate for CYP2C9, similar to a number of other nonsteroidal antinflammatory drugs such as naproxen and ibuprofen. Flurbiprofen has been found in various studies to have low K_m values, both with human liver microsomes and CYP2C9 as the enzyme source.[7,29] On the basis of this body of evidence, we have chosen flurbiprofen as the query compound for the ROCS analysis of the CYP2C9 database. The coordinates of flurbiprofen, as given in the 1R9O crystal structure, were used to ensure correct alignment in the CYP2C9 active site for ligand−protein interaction studies.

Results and Discussion

ROCS overlays with flurbiprofen as the query molecule were performed on the multiconformer database described earlier. Additional command line settings other than those already described were "randomstarts" set to 50 and "besthits" set to 1500. The "randomstarts" parameter is used to alleviate problems that might occur due to selection of the initial overlay orientation, which is chosen based on inertial properties. Problems can occur if the query and database molecules are of vastly different size.[26] "Besthits" simply describes the number of overlays that are written to the output sdf file and was set to 1500 such that at least one conformer of each of the 70 molecule CYP2C9 database was present in the results sdf file. The results were sorted by combo score from highest to lowest.

The sorted list was then examined, and the first instance (i.e., the conformer with the highest ROCS combo score) of each of the 70 database molecules was retained for further analysis.

Table 1. Results of the ROCS Overlays with the 1R9O Flurbiprofen Structure[a]

molecule	SPZ	K_m	combo	distance (Å)	aligned	ref.
flurbiprofen	✓	✓	1.811	0.24	yes	*7*, *29*
cmpd **5**	✓	-	1.658	0.79	yes	*31*
suprofen	-	✓	1.643	0.32	yes	*32*
naproxen	✓	✓	1.616	0.71	yes	*6*
tienilic acid	-	✓	1.485	1.30	yes	*33*
ibuprofen	✓	-	1.408	3.50	yes	*5*
indomethacin	✓	✓	1.387	9.59	no	*34*
zaltoprofen	✓	-	1.349	4.10	yes	*35*
seratrodast	✓	✓	1.337	1.51	yes	*36*
desogestrel	✓	✓	1.335	0.52	yes	*37*
17α-ethinylestradiol	✓	✓	1.323	1.48	yes	*38*
losartan	✓	✓	1.315	3.15	yes	*39*
etodolac	✓	-	1.314	1.43	yes	*40*
mestranol	✓	-	1.303	2.16	yes	*41*
cmpd **4**	✓	✓	1.244	4.24	yes	*42*
fluvastatin	✓	✓	1.226	0.57	yes	*43*
aceclofenac	✓	-	1.186	0.96	yes	*44*
Δ-9-tetrahydrocannabinol	✓	✓	1.167	2.73	yes	*45*
rosuvastatin	✓	-	1.161	2.18	yes	*46*
tenoxicam	-	✓	1.148	0.24	yes	*47*
lornoxicam	-	✓	1.127	0.24	yes	*48*
cmpd **1**	✓	-	1.120	0.52	yes	*49*
piroxicam	-	✓	1.116	0.24	yes	*47*
galangin	✓	✓	1.101	0.47	yes	*50*
arachidonic acid	✓	-	1.101	2.73	yes	*51*
chlorpropamide	✓	✓	1.087	2.32	yes	*52*
meloxicam	✓	-	1.080	1.35	yes	*53*
kaempferide	✓	✓	1.076	2.18	yes	*50*
phenprocoumon	-	✓	1.059	9.65	no	*54*
diclofenac	✓	✓	1.058	1.83	yes	*47*, *55*
acenocoumarol	✓	✓	1.050	2.69	yes	*56*
N-dehydroxyzileuton	✓	✓	1.046	1.83	yes	*57*
cmpd **3**	✓	-	1.041	9.27	no	*58*
methiocarb	-	✓	1.030	3.10	yes	*11*
lansoprazole	✓	✓	1.028	3.24	yes	*59*
zolpidem	✓	✓	1.024	1.24	yes	*60*
linoleic acid	✓	-	1.011	0.62	yes	*61*
dibenzo[*a*,*h*]anthracene	-	✓	1.010	0.08	yes	*62*
celecoxib	✓	✓	1.009	7.83	no	*63*
sulprofos	-	✓	1.003	2.02	yes	*11*
torsemide	✓	✓	1.001	2.64	yes	*10*
warfarin	✓	✓	0.998	0.26	yes	*7*, *47*
voriconazole	✓	✓	0.993	9.72	no	*64*
tolbutamide	✓	✓	0.990	1.57	yes	*8*, *47*
ketamine	✓	✓	0.987	5.02	no	*65*
mefenamic acid	-	✓	0.949	8.67	no	*47*
sildenafil	✓	✓	0.948	9.96	no	*66*
terbinafine	✓	✓	0.943	7.60	no	*67*
montelukast	✓	-	0.934	3.28	yes	*68*
valproic acid	✓	-	0.933	5.23	yes	*69*
cmpd **2**	-	✓	0.923	11.36	no	*70*
fluoxetine	✓	✓	0.919	5.46	no	*71*
hexobarbital	✓	-	0.918	1.16	yes	*72*
dapsone	✓	✓	0.917	1.82	yes	*73*
clozapine	-	✓	0.915	1.44	yes	*74*
carvedilol	✓	-	0.911	10.82	no	*75*
tolterodine	✓	-	0.906	8.25	no	*76*
methyl eugenol	✓	✓	0.905	8.36	no	*77*
methoxychlor	✓	-	0.901	2.61	yes	*78*
safrole	✓	✓	0.889	8.35	no	*79*
hydromorphone	✓	-	0.887	2.70	yes	*80*
phenobarbital	✓	-	0.874	0.65	yes	*81*
zopiclone	✓	-	0.870	10.99	no	*82*
amitriptyline	✓	-	0.855	9.11	no	*83*
phenytoin	✓	✓	0.843	0.75	yes	*9*, *47*
tamoxifen	✓	-	0.843	0.54	yes	*84*
zafirlukast	✓	-	0.831	2.49	yes	*85*
phorate	-	✓	0.812	7.61	no	*11*
limonene	✓	✓	0.809	2.97	yes	*86*
disulfoton	✓	✓	0.789	8.81	no	*11*

[a] The distance shown is from the flurbiprofen 4′-hydroxylation site to the reported site of metabolism for the database molecule, as shown in Figure 1.

The VIDA program[30] was used to analyze and visualize the ROCS results and to calculate the distance from the 4′-hydroxylation site of flurbiprofen to the site of CYP2C9 metabolism for each database molecule, as shown in Figure 1. The smaller the distance, the more closely the sites of metabolism match between flurbiprofen and the database molecule. It should be noted that the site of metabolism of some molecules in Figure 1 has topologically equivalent atoms; in these instances, the topologically equivalent atom closest to the 4′-hydroxylation site in flurbiprofen was chosen. Examples of four representative overlays are given in Figure 2 for the substrates naproxen, warfarin, tolbutamide, and phenytoin. In each case shown in Figure 2, the site of CYP2C9 metabolism in the overlaid molecule is within 1.6 Å of the 4′-hydroxylation site in flurbiprofen, indicating that the molecules are well-aligned.

A summary of the results obtained from the ROCS analysis is shown in Table 1, sorted by combo score. In each case, the combo score for each molecule is given, along with the distance from the flurbiprofen 4′-hydroxylation position to the site of metabolism in the molecule of interest. The success or failure of the alignment is additionally shown. The initial criteria for selection as a CYP2C9 substrate are additionally given in Table 1; SPZ indicates that inhibition studies of the human liver microsomal reaction by sulfaphenazole were performed and yielded a minimum of 20% inhibition of activity. K_m indicates that kinetic data were obtained with recombinant CYP2C9. At least one of these criteria is satisifed for every molecule in the database, indeed almost half of the molecules satisfy both criteria. The primary references consulted for each molecule are given.

A summary of the overlay parameters is presented in Figure 3. Overall, 51 of the 70 database molecules (i.e., 73%) have successful alignments based on their sites of metabolism, with 60% of all molecules having their site of metabolism aligned to within 3 Å of the 4′-hydroxylation site in flurbiprofen. Upon inspection of Table 1, it is evident that the number of incorrect alignments increases as the ROCS combo score decreases. This is to be expected, as a lower combo score indicates a lower match of shape/chemistry with the query molecule flurbiprofen. However, this is a favorable aspect of this method because a ROCS threshold score could be proposed as a cutoff to decrease the number of false positives. This dependence of prediction quality on the combo score is well illustrated when it is noted that, of the top 28 molecules in Table 1 ranked by combo score, only one molecule, indomethacin, is incorrectly aligned. A threshold ROCS combo score of 0.99 is proposed as a "reliability" cutoff. Application of this cutoff combo score yields 39 correct alignments from 44 molecules, with a resulting accuracy of 89%.

As the ROCS combo score decreases, the molecules have a lower degree of similarity with the original query molecule, flurbiprofen. Flurbiprofen may be considered to be a respresentative molecule for a large number of CYP2C9 substrates, but clearly there are additional substrates that have less similarity with the shape/chemistry of flurbiprofen. Safrole, limonene, and methyl eugenol, which have ROCS combo scores of 0.91 or less, are neutral, relatively small molecules that would not be expected to interact with the amino acid residues in CYP2C9 implicated in polar interactions (e.g., Arg-108, Asn-289, and Asp-293). Another class of molecules that tend to have reasonable CYP2C9 activity but have little similarity to flurbiprofen are the relatively large tertiary amines, such as amitriptyline, clozapine, tolterodine, zopiclone, and hydromor-

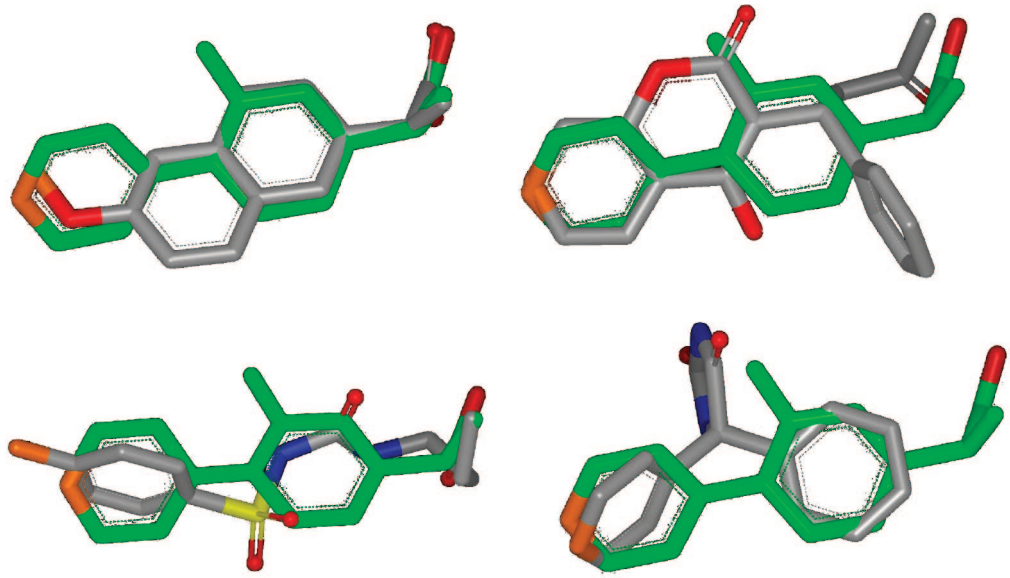

Figure 2. Representative examples of ROCS combo score overlays with flurbiprofen. The sites of metabolism are shown in orange. Clockwise, from top left: naproxen, combo = 1.616, distance = 0.71 Å; warfarin, combo = 0.998, distance = 0.26 Å; tolbutamide, combo = 0.990, distance = 1.57 Å; phenytoin, combo = 0.843, distance = 0.75 Å.

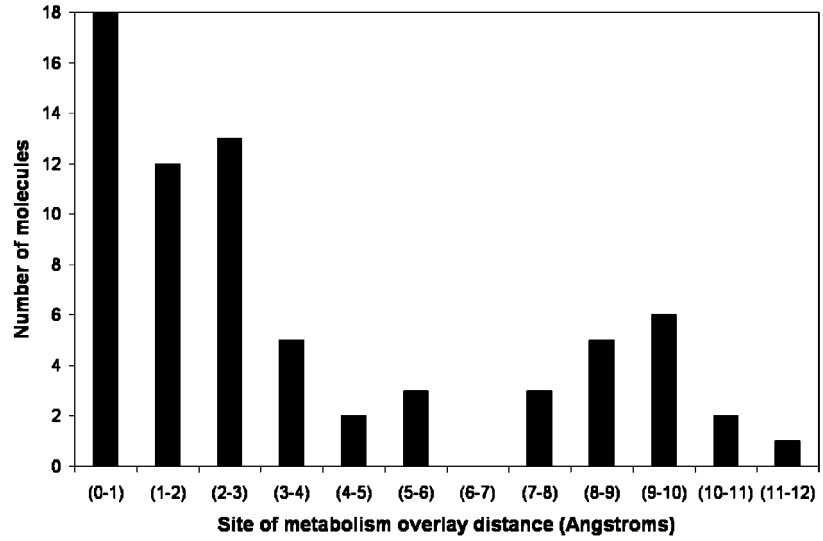

Figure 3. ROCS overlay results for the CYP2C9 substrates. The molecules are grouped together according to the distance from the flurbiprofen 4′-hydroxylation position to the site of metabolism in the database molecule of interest. These are the distances shown in Table 1.

phone. CYP3A4 is generally considered to be the major contributor to the metabolism of these compounds, with CYP2C9 having a lesser role.[74,76,80,82,83] Indeed, the majority of the molecules toward the bottom of Table 1 would generally be considered to have forms other than CYP2C9 as the major biotransformation enzyme. It would seem likely that these CYP2C9 substrates require a different molecular scaffold to facilitate their alignments based on the site of metabolism in CYP2C9.

Thus, we investigated a number of other substrates as query compounds, including diclofenac, tolbutamide, warfarin, fluoxetine, and amitriptyline. Overall, none performed as well as flurbiprofen. Diclofenac was marginally less successful as the query compound, but the other molecules were generally poor in facilitating correct alignments. However, while fluoxetine failed to align the prototypic acidic substrates of CYP2C9, this compound was successful at aligning the "atypical" amine substrates, such as clozapine, zopiclone, tolterodine, and amitriptyline. Table 2 shows the first eight ROCS hits sorted by combo score when fluoxetine is used as the query compound. The site of metabolism for fluoxetine is *N*-demethylation.

Table 2. Results of the ROCS Overlays with Fluoxetine[a]

molecule	combo	distance (Å)	aligned
fluoxetine	2.000	0.00	yes
clozapine	1.329	1.30	yes
carvedilol	1.251	5.38	no
zopiclone	1.251	2.67	yes
lansoprazole	1.218	8.47	no
tolterodine	1.199	2.30	yes
voriconazole	1.186	1.44	yes
amitriptyline	1.161	1.39	yes

[a] The distance shown is from the site of fluoxetine *N*-demethylation to the reported site of metabolism for the database molecule, as shown in Figure 1.

It should also be noted that some "minor" CYP2C9 substrates, such as pitavastatin and nateglinide, provided successful alignments with the flurbiprofen/ROCS alignment-based procedure but were excluded on the basis that they did not meet the

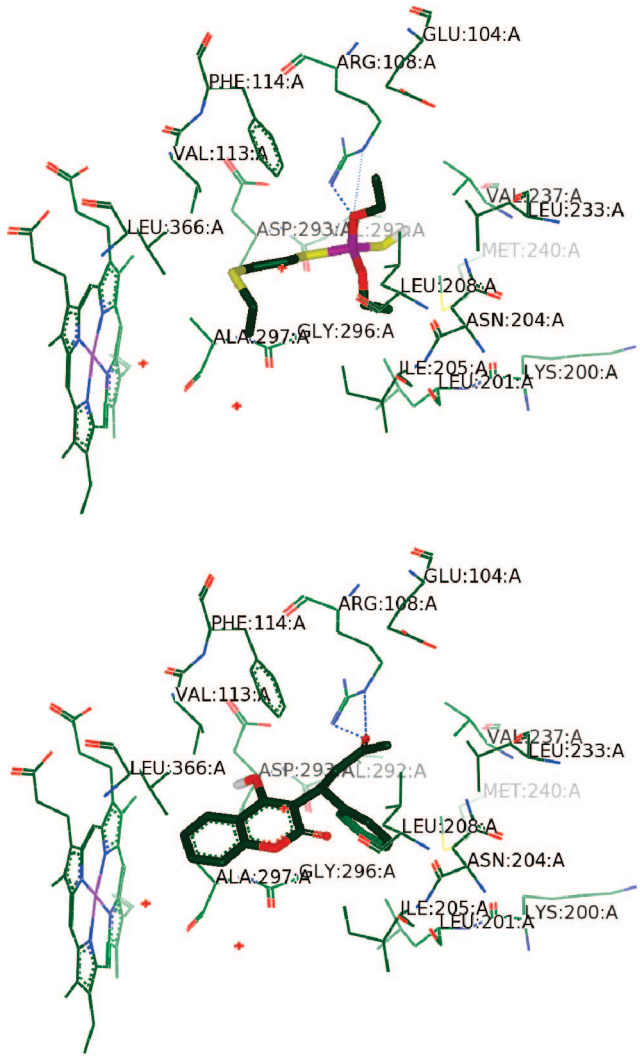

Figure 4. Docked structures of disulfoton (top) and warfarin (bottom) in the active site of 1R9O, with hydrogen bonding interactions shown with dotted blue lines. The disulfoton molecule is setup for sulfoxidation, with its site of metabolism 7.38 Å from the heme iron, whereas the warfarin molecule is aligned in position for hydroxylation, 5.15 Å from the heme iron. The PLP scoring function was used in both cases.

stringent criteria for being included as CYP2C9 substrates (i.e., the existence of kinetic data or sulfaphenazole inhibition data in the primary literature).

Until now we have looked at a purely ligand-based view of the alignment process; how do these ROCS alignments translate into the protein environment? We investigated the proximity of the sites of metabolism to the heme iron in the 39 successfully aligned CYP2C9 substrates with a ROCS combo score greater than our cutoff of 0.99. Over these 39 molecules, the average distance from the site of metabolism to the iron heme was determined to be 5.21 ± 0.95 Å, with 32 molecules in the 4–6 Å region, 4 between 6 and 7 Å, 2 < 4 Å, and 1 > 7 Å. These statistics agree well with previously observed substrate−heme distances of 6.2 Å, 3.3–4.4 Å, and 4.7–4.9 Å for P450-mediated reactions.[20]

Docking Studies. Docking studies were undertaken using the program FRED from Openeye Software.[27] The active site was defined using the "box" command line parameter incorporating the location of flurbiprofen according to the 1R9O crystal structure. This box size was not sufficiently large enough to encompass the variety of CYP2C9 structures in this study, and thus was augmented by 2 Å using the "addbox" parameter. Additional larger boxes were investigated, however, 2 Å was found to give the best docking results overall. Scoring functions included in FRED are chemscore, chemgauss2, PLP, screenscore, shapegauss, and a consensus score.[27]

Table 3. Results of the ROCS Overlays with the 1R9O Flurbiprofen Structure for 30 Compounds Known to have P450 Enzymes other than CYP2C9 as their Primary Metabolizing P450 Enzyme[a]

compound	ROCS combo score	principal P450 enzyme
proguanil	1.041	CYP2C19
7-ethoxyresorufin	1.035	CYP1A2
midazolam	0.988	CYP3A4
nicotine	0.961	CYP2A6
2-aminofluorene	0.944	CYP1A2
verapamil	0.943	CYP3A4
chlorpheniramine	0.925	CYP2D6
clobazam	0.923	CYP2C19
mephenytoin	0.921	CYP2C19
dextromethorphan	0.914	CYP2D6
diazepam	0.913	CYP2C19
quinidine	0.900	CYP3A4
codeine	0.868	CYP2D6
nortriptyline	0.856	CYP2D6
tacrine	0.855	CYP1A2
debrisoquine	0.855	CYP2D6
nifedipine	0.838	CYP3A4
perhexiline	0.829	CYP2D6
terfenadine	0.821	CYP3A4
chlorzoxazone	0.819	CYP2E1
felodipine	0.813	CYP3A4
fluphenazine	0.808	CYP2D6
coumarin	0.791	CYP2A6
caffeine	0.771	CYP1A2
saquinavir	0.763	CYP3A4
theophylline	0.759	CYP1A2
toluene	0.626	CYP2E1
sevoflurane	0.561	CYP2E1
halothane	0.472	CYP2E1
ethanol	0.219	CYP2E1

[a] A total of 28 of the 30 molecules have combo scores lower than the threshold value of 0.99 chosen in the first part of the paper.

Docking was performed on the unaligned database of CYP2C9 molecules. Overall, the results were relatively poor in comparison with using the ROCS site of metabolism overlays with flurbiprofen. For direct comparison with the ROCS-based method to which a cutoff combo score of 0.99 was applied (yielding 44 CYP2C9 substrates), we have examined the lowest energy docking pose for each of the first 44 different database molecules obtained with each docking scoring function. As noted previously, 39 out of the first 44 molecules selected with the ROCS method were correctly aligned. The best docking results were obtained with the PLP scoring function, with 31 of the first 44 molecules selected correctly aligned in the CYP2C9 active site. The various other scoring functions gave less successful results, ranging from 22 of 44 for shapegauss to 30 of 44 for chemscore.

While the docking performance was less successful, insight was gained into some CYP2C9 substrates that were previously found to not fit well with the predictive model substrate for CYP2C9.[11] The substrates concerned are thioether pesticide compounds, namely, phorate, disulfoton, sulprofos, and methiocarb (Figure 1). All four compounds were docked in orientations that facilitated oxidation using the PLP scoring function. Two of these compounds, sulprofos and methiocarb, were also aligned successfully with the ROCS-based method. Aromatic stacking interactions with phenylalanine 114 are likely to be important for both of these compounds, with edge-to-face interactions dominating. However, phorate and disulfoton do not contain aromatic moieties and yielded very low ROCS

combo scores with the flurbiprofen alignment method. Inspection of the docked structures for these two compounds with PLP suggests that the ethoxy group attached to the central phosphorus atom may be able to form hydrogen bonds with arginine 108, which is well recognized as an important residue in the CYP2C9 active site. This leaves the sulfur group between 7 and 8 Å from the iron heme. This may reflect the fact that the thioether sulfur atom is an energetically favorable site of metabolism, and hence, both alignment and energetics may be important considerations. The docked structure of disulfoton is shown in Figure 4. For comparison, the docked structure of warfarin, which has its site of metabolism 5.15 Å from the iron heme, is also shown.

Applications of the Alignment Method. The ROCS method is a rapid procedure for evaluating the shape and chemical similarity of a query compound with a large database. In this regard, the proposed ROCS alignment procedure with flurbiprofen as a query compound in conjunction with the cutoff combo score of 0.99 would be a useful database searching tool for investigating potential CYP2C9 substrates.

The ROCS combo score cutoff of 0.99 was chosen to minimize the number of incorrectly aligned compounds. To further investigate the applicability of this cutoff score, we selected 30 drug molecules known to be metabolized by P450 enzymes other than CYP2C9. The 30 compounds included seven CYP2D6 and CYP3A4, five CYP1A2 and CYP2E1, four CYP2C19, and two CYP2A6 substrates. Table 3 shows the results of the overlays with flurbiprofen for the 30 molecule non-CYP2C9 data set. It should be noted that the non-CYP2C9 database was prepared in an identical fashion to the CYP2C9 database described earlier in the manuscript. A total of 28 of the 30 molecules data set have ROCS combo scores less than the threshold score of 0.99. Thus, it is our expectation that, in the majority of cases, molecules with a ROCS score of less than 0.99 would be expected to have a P450 enzyme other than CYP2C9 as the major metabolizing enzyme, provided flurbiprofen is used as the query molecule. In the case of a flurbiprofen query and a molecule with a combo score of less than 0.99, the method presented here would need to be considered in parallel with procedures relevant to other P450 enzymes. The obvious exception to this in the database of 70 substrates is phenytoin, which is principally a CYP2C9 substrate.[9]

Conclusion

This study has presented a novel application of the ROCS alignment tool for predicting the orientation of substrate drug molecules in the active site of the CYP2C9. The proposed method is based on using the shape and chemistry of the known CYP2C9 substrate flurbiprofen as the query molecule. Application of a ROCS cutoff score gave a high degree of accuracy, resulting in 39 of 44 molecules having alignments that could account for the experimentally observed site of metabolism by CYP2C9. Transposition of the correctly aligned ROCS structures into the protein environment resulted in an average site of metabolism to iron heme distance of 5.21 Å, which was in agreement with previous experimental observations. Docking studies with several scoring functions using the program FRED yielded results, which were of lower predictive quality than the alignment-based method. Docking studies yielded some insight into the CYP2C9 substrate features of thioether pesticides. A particularly interesting aspect of this work is that the ROCS ligand-based approach yielded superior results to the combined protein-docking approach. Interestingly, a recent evaluation of virtual screening benchmarking studies showed that, averaged over multiple targets, 3D ligand-based methods outperformed docking algorithms when chemical typing was included.[87] This may indicate that it is prudent to consider ligand approaches alongside protein-based approaches. Indeed, we note that a recently released version of FRED has an added ligand-based scoring function, the chemical Gaussian overlay (cgo). The application of this scoring function to the site of metabolism problem is currently under investigation.

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