CYP2C9 Structure−Metabolism Relationships: Substrates, Inhibitors, and Metabolites

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The cytochrome P450 (CYP) family is composed of monooxygenases, which mediate the metabolism of xenobiotics and endogenous compounds. The characterization of the interactions between these enzymes and candidate drugs is an important part of the drug discovery process. CYP2C9, one isoform of the CYPs, mediates the oxidation of several important drugs. The aim of this work is to investigate the possibility to study inhibition and substrate interactions with CYP2C9, using docking and the site of metabolism predictions. The model compounds used for the study were the COX-2 inhibitor celecoxib and a series of 13 analogues known to be metabolized by CYP2C9. The results obtained using the two methods gave valuable information about important interactions of inhibitors and substrates with CYP2C9. The two methods could be used to predict the site of metabolism and to determine the productive docking pose for each compound. These predictions were verified by metabolite identification using LC/MS/MS after incubation with recombinant CYP2C9.

Introduction

During recent years, the importance of favorable pharmacokinetics in the development of a successful drug has been widely recognized, which means that absorption, distribution, metabolism, and excretion (ADME)[a] evaluations have become integrated earlier in the drug discovery process. Computational tools are becoming more and more important for screening compounds with regard to the relevant ADME properties.[1] The metabolic behavior of drugs depends not only on the physicochemical properties of the compounds but also on the characteristics of the metabolizing systems involved. The cytochrome P450 (CYP) enzymes are the principal catalysts involved in the oxidative metabolism of xenobiotics.[2] There are currently 57 known CYP isoforms,[3] and 15 of these account for more than 80% of the metabolism of all pharmaceuticals in current clinical use.

CYP2C9, an isoform of the CYPs, is one of the major enzymes responsible for phase I metabolism of drugs.[4] Inhibition of this enzyme may result in undesirable drug−drug interactions or drug toxicity.[5] The ability to predict the substrate and/or inhibitor properties of a compound toward CYP2C9, using computational methods, would be of great value in the drug discovery process. These two properties, metabolic stability and inhibition potential, are interrelated, and the best approach is to study them in parallel. The different approaches used for predicting interactions with CYP2C9 have been extensively reviewed elsewhere.[6−11] The newly solved crystal structures of mammalian CYP2C9[12,13] provide an opportunity to improve our understanding of the interactions between the enzyme and the substrate/inhibitor. Three structures of the CYP2C9 enzyme were used in this study: CYP2C9 cocrystallized with either flurbiprofen (PDB: 1R9O)[13] or warfarin (PDB: 1OG5)[12] and a homology model[14] based on the CYP2C5 (PDB: 1DT6)[15] structure. The site of metabolism of flurbiprofen was positioned at a reasonable distance (4.9 Å) from the iron to facilitate hydroxylation, and the Arg108 residue points into the active site and interacts with the negatively charged substrate. Warfarin on the other hand is located 10 Å from the iron, and Arg108 is oriented away from the active site cavity, which results in a more open structure (Figure 1). These two crystal structures, the half-opened structure with warfarin and the more closed structure with flurbiprofen, demonstrate the conformational flexibility of the enzyme (Figure 1).[16]

MetaSite,[17] a site of metabolism program, has proven to be able to predict the site of metabolism from structurally diverse compounds; that is, in 80% of the cases, MetaSite predicted the correct site of metabolism within the top three predicted sites.[18] This program applies a mixed approach, which means that information related to both the structure of the protein and the ligand are combined to provide a better prediction. The site of metabolism is described by a probability function correlated to the free energy of the CYP substrate recognition and reactivity process. From the docking studies, information is obtained about which amino acids are important for the interaction between the enzyme and the substrate/inhibitor. The prediction of the most likely metabolically labile site(s) in a structure of a drug candidate can be relevant for several reasons. For example, once the liable sites have been identified, the lead compound can be modified to optimize the rate of metabolism. The formation of potential toxic metabolites is also an unwanted property, which can be decreased by structural modifications. Another metabolic aspect that needs to be taken into account is the probability that the compound could inhibit the metabolizing enzyme. Increasing the metabolic stability of a compound can be achieved by replacement of the metabolic "hot spot" with a metabolically stable substituent, such as a trifluoromethyl group, which will resist CYP-catalyzed oxidation.[19] This chemical modification may solve the metabolic stability problem, but

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[a] Abbreviations: ADME, absorption, distribution, metabolism and excretion; COX, cyclooxygenase; CYP, cytochrome P450; PCA, principal component analysis.

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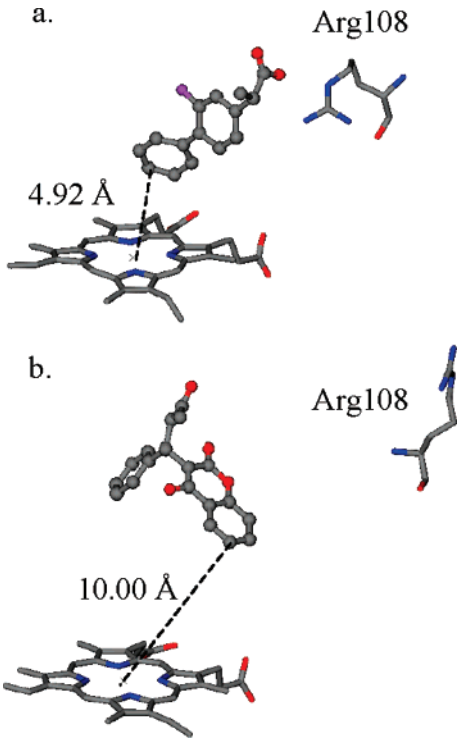


Figure 1. CYP2C9 cocrystallized with (a) flurbiprofen (PDB: 1R9O) and with (b) warfarin (PDB: 1OG5).

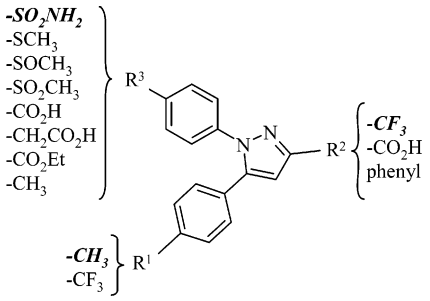


Figure 2. Analogues to celecoxib (the celecoxib substituents are highlighted in bold).

depending on the affinity of the compound to the metabolizing enzyme, there is a risk that the new compound may inhibit the enzyme.

To investigate how these substrate and inhibition properties are interrelated, 14 structural analogues were studied. The model compounds were celecoxib,[20,21] which are known to be metabolized by CYP2C9, and 13 synthesized structural analogues of celecoxib.[19] Celecoxib has a fairly rigid core structure with three apparent interaction points R^1, R^2, and R^3. The celecoxib analogues have different substituents in these positions of the molecule (Figure 2).

With this subset of compounds, known to be metabolized by CYP2C9, the inhibition and substrate interactions with CYP2C9 were studied. The approach was to use docking and the site of metabolism predictions. Experimental data were generated to be able to verify the predictions.

Results and Discussion

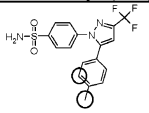
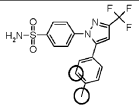
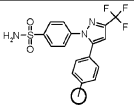
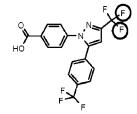
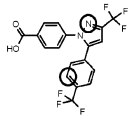
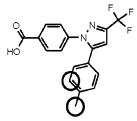
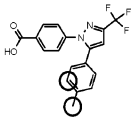
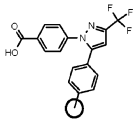
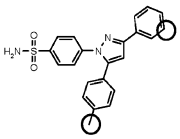
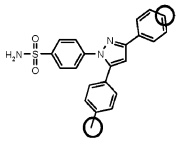
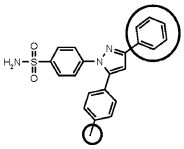
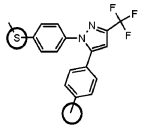
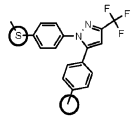
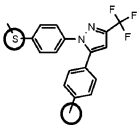
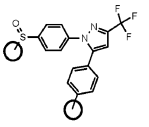
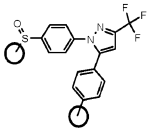
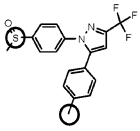
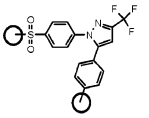
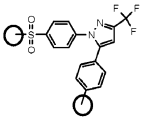
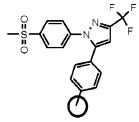
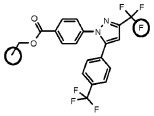
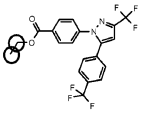
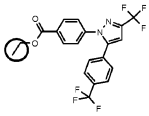
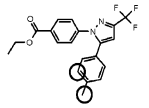
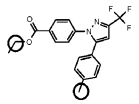
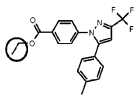
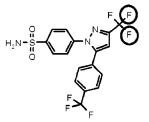
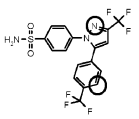
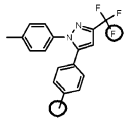
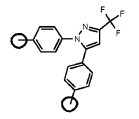
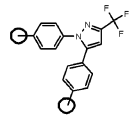
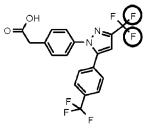
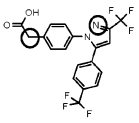
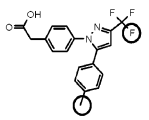
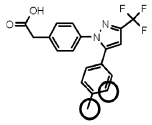
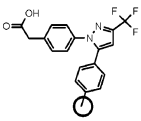
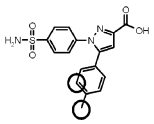
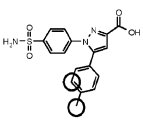
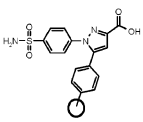
Site of Metabolism Predictions in CYP2C9. MetaSite[17] predicted that the methyl group in celecoxib was the most likely position to be metabolized (Table 1), which could also be verified since celecoxib is known to be metabolized to the corresponding hydroxymethyl derivative.[21] When the methyl group was changed to a trifluoromethyl group (**9**), the 2-nitrogen atom in the pyrazole ring was predicted to be metabolized instead. However, the acidic nature of pyrazole rings makes these heterocycles less susceptible to oxidative metabolism as compared to other five-membered aromatic heterocyclic rings.[22] In addition to this feature, the trifluoromethyl group will sterically hinder the nitrogen atom for metabolism. Therefore, the likelihood that *N*-oxides will be formed in vitro is low. MetaSite made the same predictions for compounds **1** and **11**, which also have a trifluoromethyl group in the R^1 position. In contrast, compound **7**, which also has a trifluoromethyl group in the R^1 position, was not predicted to be metabolized at the 2-nitrogen atom. This compound contains an ester functionality and was instead predicted to be metabolized at the α-carbon atom in the ester moiety.

Analogues to celecoxib containing a methyl substituent in the R^1 position are all predicted to be metabolized in that position (**2**−**6**, **8**, **10**, **12**, and **13c**). In **2**, **5**, **12**, and **13c**, the methyl group is the only position that is predicted to be metabolized to a higher extent than any other atom in the molecule. Compound **10** has an additional methyl group in the R^3 position, which is also predicted to be metabolized but to a lower extent than the other methyl. The sulfide (**4**) was predicted to be equally metabolized at the sulfur atom as the methyl group, while the sulfoxide (**5**) was predicted to be metabolized at the two methyl groups. This prediction was somewhat unexpected since oxidation of a sulfoxide to a sulfone is a reaction known to be mediated by CYP.[23] One explanation to this can be that MetaSite analyzes the sulfoxide group as such, not each individual atom. This means that it is the most sterically favored atom in the group that is predicted to be metabolized, not the most reactive. Consequently, this fact must be considered when looking at the MetaSite results; otherwise, the conclusions drawn from the predictions can be misleading. When adding one more oxygen atom to the compound (**6**), MetaSite predicted the methyl group bound to the sulfur atom to be metabolized to the same extent as the methyl group in the R^1 position.

When interpreting the results of the site of metabolism predictions, it must be considered that it is a ranking of the groups within the compound in relation to each other. This limitation has been solved to some extent by the option "reactivity on" (Experimental Section). When activating this option, the program takes the reactivity of the atom/group into account, not only the distance to the heme and the interaction with the surrounding amino acids. Compounds **8** and **10** were correctly predicted only when the "reactivity on" mode was activated. The site of metabolism predictions of **1**, **7**, **9**, and **11** were not relevant due to the fact that they were not metabolized by CYP2C9. The metabolic "hot spots" predicted for the eight remaining compounds, which were metabolized by CYP2C9, were in agreement with the in vitro metabolite identification for CYP2C9, independently of which reactivity mode that was activated ("reactivity on" or "reactivity off").

Docking Studies in Two CYP2C9 Crystal Structures. When ligands are docked into the active site of an enzyme, the goal is usually to optimize the interactions between the ligand and the binding site. The affinity should be increased so the compound of interest binds tightly to the target, resulting in the desired pharmacological effect. However, working with docking studies on CYPs, another approach is needed. Because binding of ligands to CYPs can cause drug−drug interactions, tight binding is instead of interest to avoid. Thus, the structural optimization should lead to a decreased or diminished affinity. To investigate if docking studies of celecoxib and its synthesized

Table 1. Experimentally Determined and Predicted Site of Metabolism in CYP2C9, for Celecoxib and Some Synthesized Analogues[a]

Compound	CYP2C9 Site of Metabolism Prediction Reactivity (off)	CYP2C9 Site of Metabolism Prediction Reactivity (on)	Experimentally Determined Site of Metabolism in CYP2C9
Celecoxib			
1			No metabolite
2			
3			
4			
5			
6			
7			
8			
9			No metabolite
10			
11			No metabolite
12			
13c			

[a] The site of metabolism predictions with or without the reactivity factor activated, performed by MetaSite, are shown and the two highest ranked atoms from the prediction are highlighted with circles.

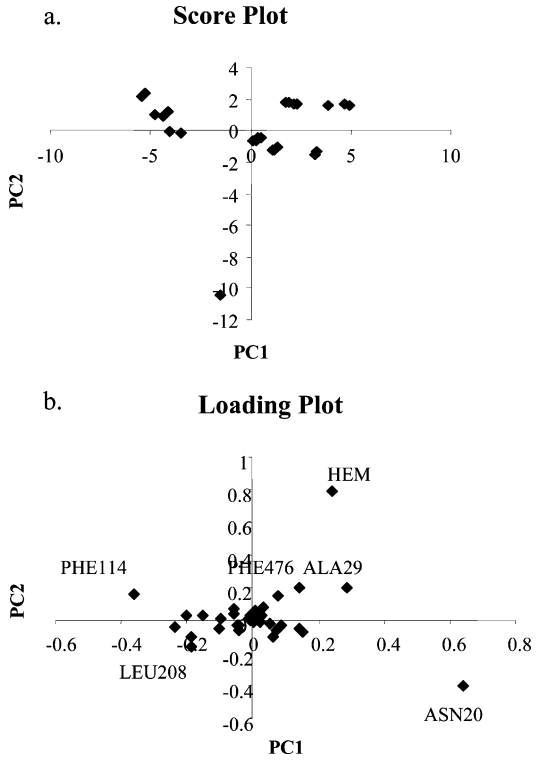


Figure 3. PCA for the interaction matrix obtained from the docked poses for celecoxib into the open form of CYP2C9. (a) In the score plot, each cluster contains docking solutions with similar docking binding modes. (b) The loading plot shows the contribution of the different amino acids in the binding site for the different binding modes.

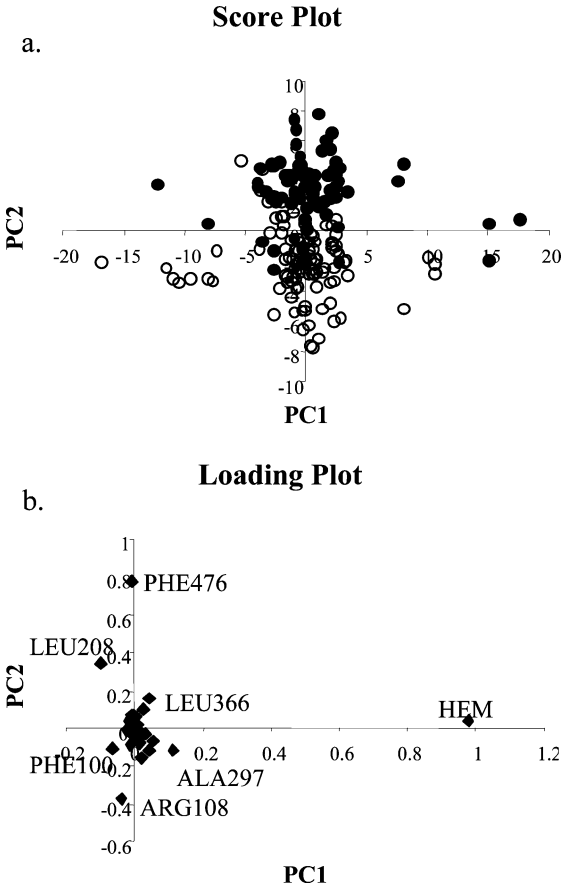


Figure 4. PCA for the interaction matrix obtained for both the open (open circles) and the closed (filled circles) form of CYP2C9 used in the docking study. The first two components explain 44% of the variance. (a) The score plot shows that the major difference between all docking poses is due to which protein structure is used. (b) The loading plot shows the amino acids that are discriminative for the two CYP2C9 crystal structures.

analogues could be used to predict their interaction with CYP2C9, 10 conformers of each compound were generated in MacroModel (v. 7.0.110) and docked into the active site of CYP2C9 using the docking program GLUE.[24] The two crystal structures of CYP2C9 used for the docking study were the closed CYP2C9 structure cocrystallized with flurbiprofen (PDB: 1R9O) and the open CYP2C9 structure cocrystallized with warfarin (PDB: 1OG5).

Docking experiments result in numerous docking poses that need to be analyzed manually. To efficiently analyze the docking results, a new approach that involves energy calculations of the different docking poses was applied.[25] The analysis could be simplified by just selecting one conformation/compound from each cluster, based on the fact that poses with similar positions in the score plot also have a similar interaction pattern. GOLPE was used for the principal component analysis (PCA) of the interaction data obtained from the dockings. One compound was analyzed at a time, and only the docking poses, which had any atom within a radius of 5 Å or less from the heme, were selected for the PCA. The amino acids in the binding site represented the variables. Five principal components were extracted, but only the first two components were used for the analysis since the higher components did not give any additional interpretable information. Each cluster in the PCA score plot represents one docking binding mode (Figure 3a). Moreover, the loading plots showed the individual contribution of the amino acids in the different binding modes (Figure 3b). In addition, the differences between the two crystal structures and the identification of which amino acids that were discriminative were investigated (Figure 4). It was shown that Phe476 was discriminative for the open form of CYP2C9, whereas Arg108 was discriminative for the closed form of the enzyme. These two amino acids adopt different positions in the two crystal structures 1R9O and 1OG5

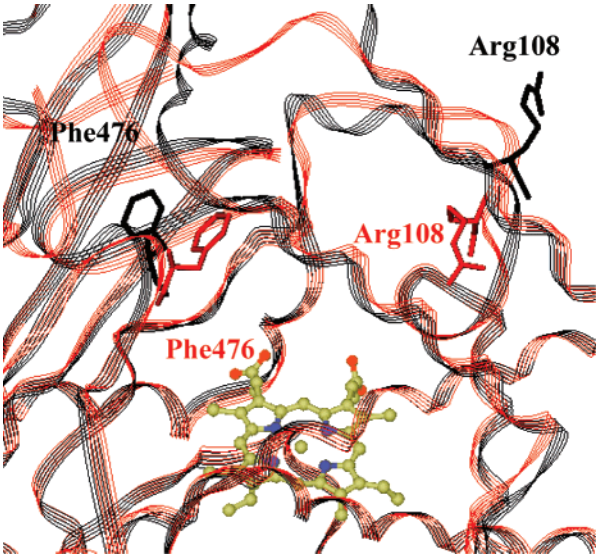


Figure 5. Two crystal structures, 1R9O (closed, red) and 1OG5 (open, black), were aligned using the heme as a reference. Arg108 (10 Å) and Phe476 (5 Å) are shifted away from the active site in the 1OG5 crystal structure.

(Figure 5). The analysis tables for the PCA in Figures 3 and 4 can be found in the Supporting Information.

One major difference between the docking result from the closed (PDB: 1R9O) and the open (PDB: 1OG5) forms of

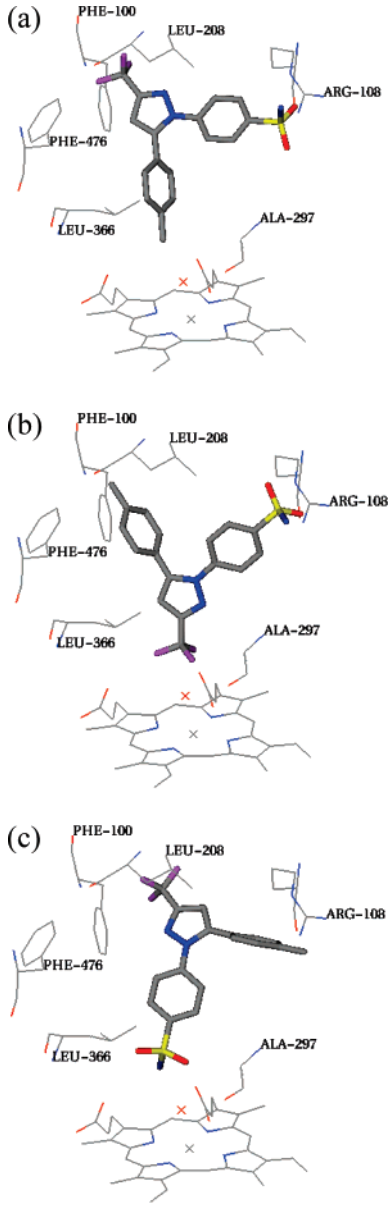


Figure 6. Example of the possible docking positions of celecoxib {4-[5-(4-methylphenyl)-3-trifluoromethyl-1*H*-pyrazol-1-yl]benzenesulfonamide} in the active site of CYP2C9 (PDB: 1R9O). When the methyl group or the trifluormethylgroup is oriented toward the heme, the oxygen atom in the sulfonamide moiety is within a preferable distance from the Arg108 residue. Because celecoxib is known to be metabolized at the methyl group, it was possible to determine that it was binding mode (a) that represented the productive binding mode.

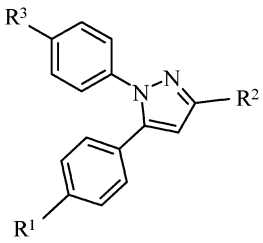
CYP2C9 was that the compounds, which were docked into the closed form of CYP2C9, gave fewer or no poses in agreement with the experimentally identified site of metabolism (see In Vitro Studies of CYP2C9 Metabolism). This was probably due to the fact that the closed form of the protein is cocrystallized with flurbiprofen, which is a smaller ligand than celecoxib. Warfarin is also smaller than celecoxib but is positioned further away from the heme iron, which causes the active site to enlarge. When docking into the closed form of CYP2C9, seven out of the 14 docking poses were in agreement with the prediction of the site of metabolism, whereas for the open form productive binding modes were found for all 14 compounds.

From the visual inspection of the docking poses, it was concluded that celecoxib and its analogues could be docked in three main positions. In Figure 6, celecoxib is shown as an example where the methyl, trifluoromethyl, or sulfonamide group was docked close to the oxyferryl. When the methyl group is oriented toward the heme, the oxygen atom in the sulfonamide moiety is within a preferable distance from the Arg108 residue. This pattern with three different docking poses was applicable to all celecoxib analogues. Because celecoxib is known to be metabolized at the methyl group,[21] it was possible to determine which of these three docking poses represented the productive binding mode. The metabolism of the celecoxib analogues was not known; hence, it was not possible to identify which of the docking poses that was correct. From the docking study, it was possible to obtain information about probable metabolites but not to determine the productive binding mode. It is known that the major drawback of docking programs is that the scoring functions are not successful in correctly identifying the binding mode. The ability of different scoring algorithms to predict the protein-bound conformation of a cocrystallized ligand has been evaluated.[26] The study demonstrated that when the size of the binding site or complexity of the compounds increased, fewer protocols were able to generate poses that were close to that formed in the crystal structure.

The docking results obtained in the present study show that the orientation of the compound into the active site with respect to the heme would depend greatly on the protein structure used. The score and loading plots in Figure 4 show that apart from direct binding to the heme it was the protein structure that was discriminative for all of the docking poses. The docking tool provided the correct site of metabolism orientation among its docking solutions, but the poor performance of the scoring function made it difficult to distinguish it from the nonproductive binding modes. This decreased the usefulness of docking studies to design a compound with diminished affinity for CYP2C9. The energy calculations used for the analysis of the different poses helped in the interpretation and clarification of the information obtained from the dockings and decreased the analysis time of the docking poses. To be able to draw conclusions from docking studies, it is preferable to have access to a few compounds for which the metabolite pattern is known for a specific enzyme isoform. If this information is available, docking could be a valuable tool when the aim is to design analogues with improved metabolic stability within a specific compound class.

In Vitro Studies of CYP2C9 Inhibition. Recombinant CYP2C9 was used in the fluorescence inhibition assay to measure the inhibition properties of celecoxib and its analogues (Table 2). The trifluoromethyl analogue **9** (IC_{50} = 17 μM) proved to be metabolically stable,[19] but the inhibition potential was in the same range as that of celecoxib (IC_{50} = 10 μM). These two compounds were docked in the exact same position; hence, **9** is positioned with the trifluoromethyl group directed toward the heme. Changes in the R^2 position to prevent the interaction with CYP2C9 as in **3** and **13c**, which contain a phenyl and a carboxylic acid group, respectively, were studied. Both compounds have shown decreased metabolism.[19] From the docking studies of celecoxib, the conclusion was drawn that the addition of a bulky group in the R^2 position (**3**) would cause a clash with the protein and that the compound will no longer fit close to the heme. The decreased metabolism indicated that the interaction with the active site was altered, but the compound showed inhibitory potential (IC_{50} = 16 μM), which means that the compound still can fit into the enzyme. The carboxylic acid group added in the R^2 position had a larger impact on the interaction with the active site. In addition to a decreased metabolism, the compound also showed a decreased inhibition

Table 2. Summary of the CYP2C9 Inhibition Data for Celecoxib and Its Analogues

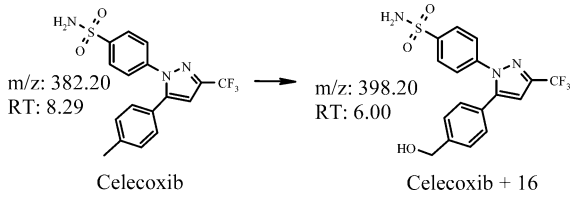


compound	R^1	R^2	R^3	IC_{50} (μM)
celecoxib	$-CH_3$	$-CF_3$	$-SO_2NH_2$	10 ± 0.8
1	$-CF_3$	$-CF_3$	$-CO_2H$	>40
2	$-CH_3$	$-CF_3$	$-CO_2H$	>40
3	$-CH_3$	phenyl	$-SO_2NH_2$	16 ± 2.8
4	$-CH_3$	$-CF_3$	$-SCH_3$	>40
5	$-CH_3$	$-CF_3$	$-SOCH_3$	26 ± 3.1
6	$-CH_3$	$-CF_3$	$-SO_2CH_3$	16 ± 1.4
7	$-CF_3$	$-CF_3$	$-CO_2Et$	>40
8	$-CH_3$	$-CF_3$	$-CO_2Et$	>40
9	$-CF_3$	$-CF_3$	$-SO_2NH_2$	17 ± 0.9
10	$-CH_3$	$-CF_3$	$-CH_3$	>40
11	$-CF_3$	$-CF_3$	$-CH_2CO_2H$	25 ± 3.9
12	$-CH_3$	$-CF_3$	$-CH_2CO_2H$	>40
13c	$-CH_3$	$-CO_2H$	$-SO_2NH_2$	>40

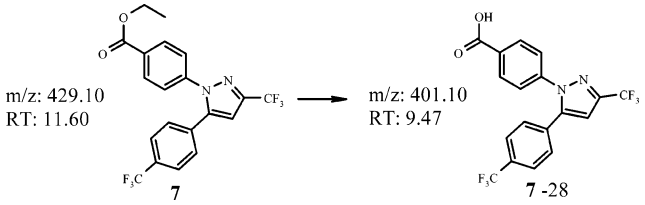
potential (IC_{50} > 40 μM). In the case of compound **10** (IC_{50} > 40 μM), a methyl group replaces the sulfonamide group of celecoxib. The metabolism was decreased with 70%,[19] and no inhibition was detected; hence, the interactions with CYP2C9 were considerably decreased. This is probably due to the disruption of the interaction between the sulfonamide group and the Arg108. The inhibition data from the three compounds representing different oxidation states on the sulfur atom showed that for each oxygen atom that was added the inhibitory capacity increased (IC_{50} values for **4**, **6**, and **5** were >40, 26, and 16 μM, respectively). These results are compatible with the hypothesis that the oxygen atom could interact with Arg108, which was suggested from the docking analysis.

The compounds with a carboxylic acid group in the R^3 position (**1** and **2**) did not show any inhibitory potential, and the same was observed for the corresponding esters **7** and **8** (IC_{50} > 40 μM). One structural characteristic for a compound to be selectively recognized by CYP2C9 is the presence of an anionic site.[27] Therefore, this result was surprising since an interaction between the Arg108 and the negatively charged group in **1** and **2** was anticipated. The orientation of the carboxylic acid moiety was considered to be important; therefore, the elongated derivatives **11** and **12** were synthesized and analyzed. Compound **12** (IC_{50} > 40 μM) did not show any inhibition, but **11** with a trifluoromethyl group in the R^1 position showed some inhibition (IC_{50} = 25 μM). Compounds **1** and **11**

Scheme 1



Scheme 2



give indications of the influence of the distance between the site of metabolism and the anionic site in the binding pocket.

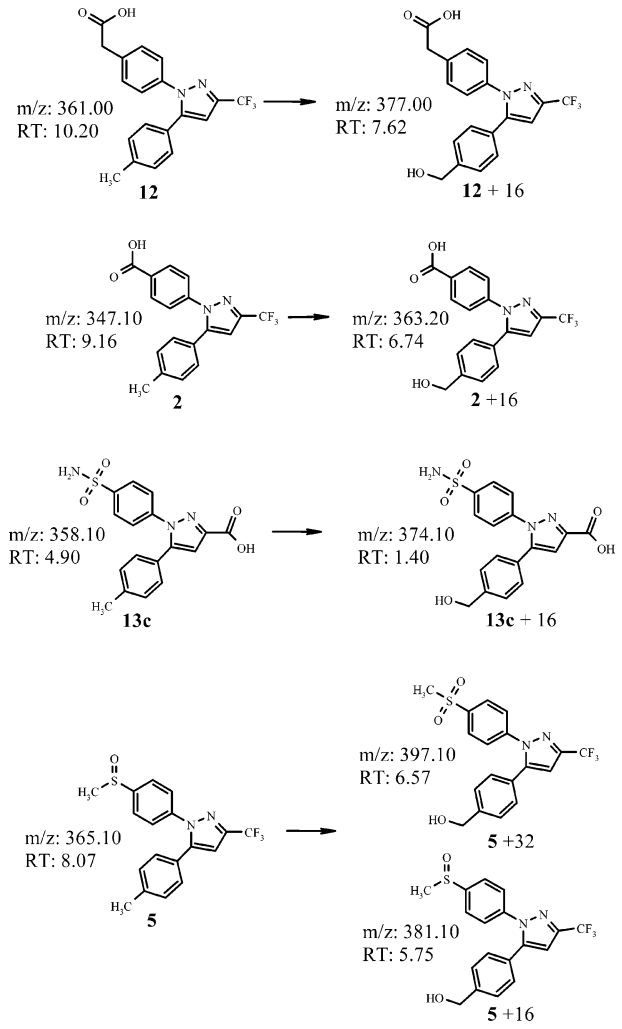
In Vitro Studies of CYP2C9 Metabolism. Celecoxib and its analogues were examined for their metabolic properties using assays based on recombinant CYP2C9. The different analogues with chemical modifications in the R^1, R^2, and R^3 positions (Table 1) allowed an analysis of the relationship between the structure and the metabolic properties. The metabolite identification was based on mass addition or mass loss from the parent compound; no fragmentation analysis of the metabolites was performed. Celecoxib (*m*/*z* 382.2) is known to be metabolized to the hydroxymethyl derivative by CYP2C9,[21] and for this compound, only one metabolite was detected with a *m*/*z* 398.2. Thus, the assumption was made that the *m*/*z* +16 metabolite was the hydroxymethyl derivative (Scheme 1).

When this metabolic "hot spot" was changed into a trifluoromethyl group as in **9**, no metabolite was detected; hence, the metabolic stability was improved. This was also the result for two of the other compounds having a trifluoromethyl group in the R^1 position (**1** and **11**). Compound **7** also has the same group in the R^1 position but contains an ethyl ester functionality in the R^3 position. MetaSite predicted the α-carbon atom in the ester moiety to be hydroxylated, resulting in the formation of the corresponding carboxylic acid derivative. This was verified by metabolite identification, using a synthesized compound (**1**) as standard and comparing the retention times and *m*/*z* values (Scheme 2).

Analogues to celecoxib containing a methyl group in the R^1 position were all predicted to be metabolized in that position (**2**−**6**, **8**, **10**, **12**, and **13c**). For compounds **2**, **5**, **12**, and **13c**, that was the only position that was predicted to be metabolized to a much higher extent than any other position in the molecule (Scheme 3). These predictions were verified for compounds **2**, **12**, and **13c**. In addition to the predicted metabolite of compound **5**, the sulfoxide was further oxidized to the corresponding sulfone.

Compound **10** has an additional aromatic methyl group in the R^3 position, which was predicted to be metabolized but to a lower extent than the methyl group in the R^1 position (Scheme 4). From the experimental data, it was possible to detect two metabolites with *m*/*z* +16. The amount of the metabolite with the longer retention time (11.82 min) was only one-third of that for the metabolite with the shorter retention time (11.12 min). Thus, the prediction that one of the methyl groups is favored for metabolism was validated, but it was not possible to assign which of the methyl groups was preferred. The metabolite *m*/*z*

Scheme 3



+32 was also detected, which indicates that the compound has two binding modes in the active site of CYP2C9.

The sulfide (**4**) was predicted to be metabolized equally well at the sulfur atom and the methyl group. Three metabolites were formed (Scheme 5), one *m*/*z* +32 metabolite and two *m*/*z* +16 metabolites, which indicates metabolism in two different parts of the compound. When the sulfide was oxidized to give compound **5**, the sulfoxide was not predicted to be further oxidized. That prediction was inaccurate as two metabolites were detected, one *m*/*z* +32 and one *m*/*z* +16 (Scheme 3). For **6**, MetaSite predicted the methyl group bound to the sulfone to be metabolized to the same extent as the methyl in the R^1 position, but only the hydroxymethyl was detected (Scheme 5).

Compound **3** was predicted to be mainly metabolized in the para-position of the phenyl ring connected to the R^2 position in the pyrazole ring (Scheme 6). Further detailed metabolite identification needs to be carried out to be able to verify this prediction. The experimental data show that only one *m*/*z* +16 metabolite is formed; whether the hydroxylation occurs in the phenyl ring could however not be concluded. The circles in the structure of the metabolite of **3** (Scheme 6) show possible oxidation positions.

Compound **8** was predicted to be metabolized both at the methyl group (R^1) and at the ester functionality (R^3). The experimental data confirmed these predictions; the carboxylic acid metabolite was detected but was minor (1/20) as compared to the hydroxymethyl metabolite (Scheme 7). The hydrolyzed metabolite (*m*/*z* −28) was verified using synthesized **2** (for the structure, see Scheme 3) to compare the retention time.

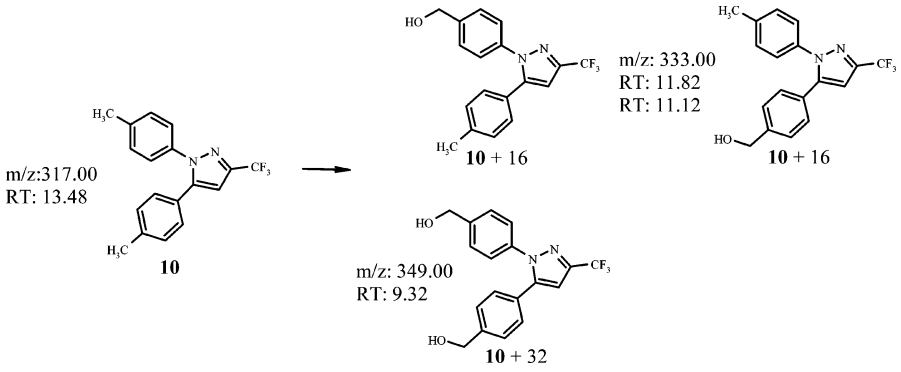
Conclusions

Drug metabolism in the context of drug discovery is a complex process that includes issues relating to metabolic stability, enzyme identification, metabolite identification, reactive metabolites, and enzyme inhibition properties. All of these parameters are interrelated and need to be considered in parallel in the development of new therapeutic agents. To solve one of these issues by making a structural modification in the compound could lead to a problem with another. The aim of this study was to investigate the utility of information obtained from the docking program GLUE and the site of metabolism prediction tool MetaSite as an aid for understanding and predicting the interactions between CYP2C9 and potential inhibitors/substrates. The model compounds used in the study were celecoxib[20,21] and 13 of its analogues known to be inhibitors of COX-2 and to be metabolized by CYP2C9.[19] Changing the methyl group in celecoxib to a trifluoromethyl group decreased the rate of metabolism,[19] but the affinity to the active site of CYP2C9 remained, which lead to retained inhibition of the enzyme. Hence, even though the substitution may solve the potential metabolic stability issue, the CYP2C9 inhibition problem remains. The information needed to guide toward improved metabolic properties can be achieved by combining the docking results with the MetaSite predictions. From the results obtained in this study, a three-step procedure is suggested as follows: (i) prediction of the metabolic "hot spot" in a structure using MetaSite, (ii) modification of the metabolic "hot spot" in silico, and (iii) docking of the new compound into the active site. If the new moiety is positioned toward the heme, there is a high probability that the compound will have similar interactions as the parent compound i.e. the inhibitory potential is retained. Because of the fact that the active site of CYP2C9 is large and thereby unspecific and flexible, several docking solutions of each compound were obtained. The step in this procedure that needs improvement is how to distinguish between these different docking solutions, as the available scoring programs are not reliable enough for correct ranging of docking poses. When combining the information gained from MetaSite predictions with a docking program with optimized scoring function, a highly valuable prediction method for optimization of metabolic stability and inhibition properties will be available.

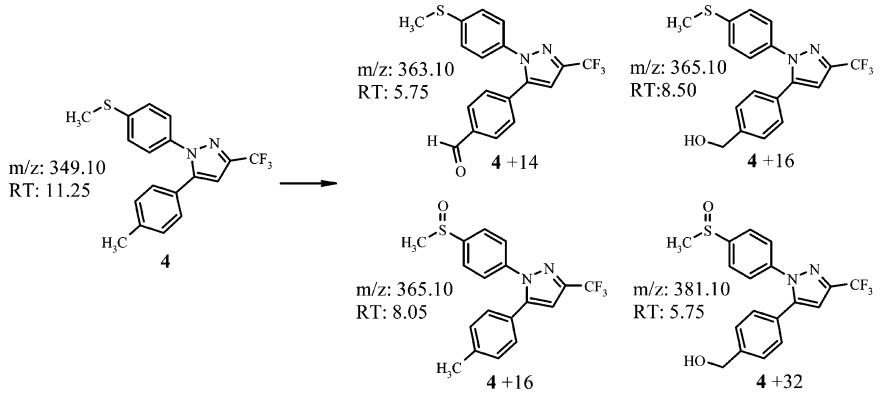
Experimental Section

Materials. The chemicals used in the assays were 7-methoxy-4-trifluoromethylcoumarin (MFC), sulfaphenazole, and NADPH purchased from Sigma Chemical Co. (St. Louis, MO). Celecoxib was purchased from AApin Chemicals Limited (Abingdon, United Kingdom). Tris-hydroxymethyl-aminomethane was purchased from ICN Biomedicals Inc. (Irvine CA). K_2HPO_4 and KH_2PO_4 were purchased from Kebo Lab (Stockholm, Sweden). Acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). Recombinant human CYP2C9, heterologously expressed in *Saccharomyces cerevisiae*, was obtained from AstraZeneca Biotech Laboratory (Södertälje, Sweden).[28] Human CYP2C9HR Bactosomes expressing human CYP2C9 were purchased from Cypex Ltd. (Dundee, Scotland, United Kingdom). All calculations were performed in a Linux environment on a 32 MB personal computer. The software utilized in the computational analysis was GRID v. 22 and MetaSite v. 2.7.5 (Molecular Discovery Ltd., PG, ITALY), GOLPE v. 4.6.0 (MIA, http://www.miasrl.com), SYBYL 7.3.2 (Tripos Associates Inc., St. Louis, MO), and Maestro v. 7.5.116 (Schrödinger, NY).

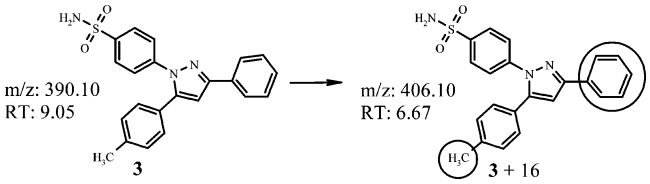
Scheme 4



Scheme 5



Scheme 6



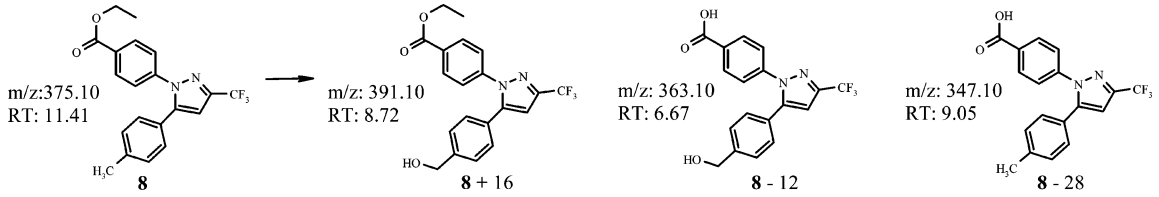
Site of Metabolism Predictions in CYP2C9. Celecoxib and synthesized analogues were submitted to the program MetaSite, which is a fully automated procedure.[17] The molecular interaction field for CYP2C9 obtained from the GRID package is precomputed and stored inside the software. Once the structures of the compounds are provided, conformation generation, the semiempirical calculations, pharmacophoric recognition, descriptor handling, similarity computation, and the reactivity computation are all carried out automatically. The chemical reactivity represents the activation energy required to produce a reactive intermediate in oxidative biotransformation reactions. These hydrogen abstraction processes have been simulated by ab initio calculations of small fragments from druglike substrates for human CYPs and stored in MetaSite. When a fragment in the target molecule is recognized as one in the MetaSite database, all atoms in that fragment are assigned the corresponding reactivity value. This reactivity contribution can be switched on and off. When the reactivity is taken into account, the final ranking for potential metabolic sites is the product of the similarity analysis and the chemical reactivity. In the off mode, MetaSite predicts how the protein orients the compounds in the enzyme active site without considering the reactivity of the atoms.

Docking Studies in Two CYP2C9 Crystal Structures. Prior to the docking studies, the structures of celecoxib and its analogues were inserted in Maestro, where MacroModel was used for fast conformational analysis. In each compound, three torsions allowed to rotate were defined. Otherwise, default settings were used. Ten low-energy conformers of each compound were saved and docked into two different X-ray structures, a closed form of CYP2C9 (PDB: 1R9O)[13] and an open form of CYP2C9 (PDB: 1OG5),[12] using the GRID[29,30] based docking program GLUE.[24] The docking program maps the active site using hydrophobic, hydrogen-bond donor/acceptor, and electrostatic probes. All tetrahedrals obtained from four minimal energy points from GRID are computed. These four-point pharmacophores derived from the interaction for the active sites are then used as templates to compare with the ligand. GLUE identifies the polar and hydrophobic heavy atoms of the ligand and calculates all tetrahedrals between these atoms. When a pharmacophore is recognized, the ligand is aligned in the enzyme cavity followed by an energy computation. If there are any conflict contacts between the ligand and the protein, an induced fit process is started to accommodate the substrate in the protein cavity. The same process is repeated for all possible four-point pharmacophore templates.

The pretreatment of the protein before the docking procedure included deletion of the water molecules and removal of the ligand from the binding site. To decrease the importance of the electrostatic interactions and hinder the docked compounds to interact directly with the iron in the heme, a dummy atom was added 2 Å above the iron. The dummy atom used was OES, which represents an explicit tetrahedral ester oxygen atom that does not accept any hydrogen bonds. The PDB file had to be modified before being imported into GLUE, which is performed by GREATER that converts the PDB format to the format of the input files (kout files) required to run the docking procedure. The box size used was 20 Å × 20 Å × 20 Å with the crystallographic ligand positioned in the center. The probes selected for this study were the probes representing the different atom types that could be found in the docked compounds. These probes were selected manually when performing the binding site precalculations in GLUE. Default values were used when performing the docking, and the result was analyzed by performing a PCA analysis on the docking poses.

To analyze the docked poses, a new procedure involving energy calculations of the different docking was applied.[25] Each atom in the docked compounds was assigned an appropriate GRID probe, that is, functional groups representing different physicochemical properties.[29,30] At these *x*-, *y*-, and *z*-coordinates, the energy of

Scheme 7



interaction was calculated by GRID in the protein cavity, using the POSI directive. Subsequently, the energy values for all atoms of the same amino acid residue were summarized. The output was a table with the amino acid residues as columns and the docking poses as rows, containing the accumulative energies. The accumulated energy provided a representation of interactions between the ligand and the protein.

PCA. GOLPE was used for the PCA of the interaction data obtained from the dockings. From the PCA, the impact of the different amino acids in the discrimination between the closed and the open form of CYP2C9 could be detected and the docking results could be analyzed without inspection of each docking pose. The PCA analysis was performed for both of the interaction matrices obtained for the closed and open form of CYP2C9. Docking poses 5 Å or less from the heme were selected for the PCA analysis. For each atom, a GRID probe was assigned, and Tripos force field atom types were used to do this straightforward classification of the atoms. The description of each probe can be found in the grub.dat file. The atomic positions for all of the selected docking poses classified by their interaction type were submitted to GRID using the appropriate probe to compute point interactions (POSI option in GRID) instead of a GRID field. The interaction energy between an atom in the docking pose and the atoms in the enzyme active site was calculated. The atom in the active site giving rise to the strongest interaction is tracked back to identify the amino acid to which it belongs. This calculation was performed for each atom in all of the docking poses. Only negative energies were taken into account, and these energies were accumulated for each pose, giving rise to a matrix containing all of the docking poses and all of the amino acids that interact with the atoms within these docking poses.

In Vitro Studies of CYP2C9 Inhibition. The fluorometric assays[31] were done in black Costar 96-well plates (Corning Inc., Corning, NY). The addition of reagents to the 96-well plates was done by a multipipet. The total reaction volume was 200 μL, and each reaction mixture contained 3 pmol of CYP2C9 (*S. cerevisiae*), 1 mM NADPH, 50 μM MFC, 0.025 mM KPO_4 buffer, pH 7.4, and inhibitor in eight different concentrations. Test compounds and positive control inhibitors were dissolved in 50% acetonitrile in water. The final acetonitrile concentration in the assay was 2%. All compounds were tested for fluorescence at the excitation (405 nm) and emission (535 nm) wavelengths to verify that the compounds did not interfere with the assay, that is, fluorescence at the same wavelength as the MFC metabolite. The inhibitors were serially diluted to give final concentrations, ranging from 0.018 to 40 μM. Sulfaphenazole was used as a positive control and was serially diluted to give final concentrations, ranging from 0.005 to 12 μM. The reactions were started by the addition of NADPH after a preincubation of 10 min at 37 °C. To stop the reaction, 75 μL of 20% 0.5 M Tris and 80% acetonitrile were added. The fluorescence was measured using a SpectraMax Gemini XS (Molecular Devices Corp., Sunnyvale, CA). The compounds were assumed to be competitive inhibitors, and the IC_{50} values could be determined by nonlinear least-squares regression analysis using GraFit version 4.0.13 (Erithacus Software Limited, Middlesex, United Kingdom).

In Vitro Studies of CYP2C9 Metabolism. The total reaction volume was 200 μL, and the experiments were performed in duplicates. Each reaction mixture contained 10 pmol of CYP2C9 (Bactosomes), 1 mM NADPH, 0.1 mM KPO_4 buffer, pH 7.4, and 10 μM test compound. Test compounds were dissolved in 50% acetonitrile in water giving a final assay concentration of solvent of 2.5%. Incubations were done at 37 °C for 60 min with and without NADPH. Samples were collected at times 0 and 60 min. The reactions were started by the addition of NADPH after a preincubation of 10 min at 37 °C. The reactions were stopped with 70 μL of ice-cold acetonitrile. After centrifugation at 4000*g* for 20 min at 4 °C, 10 μL of supernatant was injected into the LC/MS/MS system.

The HPLC system used included a HP 1100 serial LC pump and column oven (Agilent Technologies Deutschland, Waldbronn, Germany) and a CTC HTS autosampler (CTC Analytics, Zwingen, Switzerland). Chromatography was performed on a HyPURITY C18 analytical column (50 mm × 2.1 mm i.d., 5 μm, ThermoQuest, Runcorn, United Kingdom) with a HyPURITY C18 guard column (2.1 mm × 10 mm, 5 μm). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The linear gradient started at 10% B and increased to 90% B over 15 min, stayed there for 2.0 min, and then returned to 10% for 3.0 min to equilibrate the column at a flow rate of 0.75 mL/min. The mass spectrometrical analyses were performed using an API4000 instrument (Applied Biosystems/MDS Sciex, CA). The mass spectrometer was operated in both positive and negative ion modes. The tuning parameters were optimized for the compounds by infusing an 50% acetonitrile/H_2O solution containing 1 μM analyte at a flow rate of 10 μL/min into the mobile phase (0.75 mL/min) using a T connection. The scan parameters for multiple reaction monitoring (MRM) of the 14 compounds investigated can be found in the Supporting Information. Instrument control, data acquisition, and data evaluation were performed using Analyst 1.4 software (Applied Biosystems/MDS sciex, CA).

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Supporting Information Available: Scan parameters for MRM of the 14 compounds investigated and analysis tables for the PCA in Figures 3 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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