Molecular Modeling Used to Evaluate CYP2C9-Dependent Metabolism: Homology Modeling, Molecular Dynamics and Docking Simulations

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Abstract: Cytochrome P450 (CYP) 2C9 is the principal isoform of the CYP2C subfamily in the human liver and is involved in the oxidation of several endogenous and xenobiotic compounds, including many therapeutic drugs. The metabolism of drugs by CYP2C9 can yield either safe or toxic products, which may be related to the recognition and binding modes of the substrates to this isoform. These interactions can be studied using *in silico* methods such as quantum chemistry, molecular dynamics and docking simulations, which can also be useful for predicting the structure of metabolites. In these types of studies, the ligand and the protein must be tridimensional models; thus, the protein can be built by homology modeling or retrieved from the Protein Data Bank. Therefore, the current review emphasizes the importance of using *in silico* methods to predict the metabolism of CYP2C9 because these computational tools have allowed the description of the principal characteristics of the active site of this isoform at the molecular level and the chemical properties of its ligands.

Keywords: Cytochrome P-450 (CYP), CYP2C9, docking simulations, drug metabolism, homology modeling, molecular dynamics simulations.

1. INTRODUCTION

In the last few years, computational methods have been used to obtain more information about the absorption, distribution, metabolism, excretion and toxicity (ADMET) of diverse compounds [1-3]. One of the major pharmacokinetic parameters that can be studied using in silico methods is metabolism because these data can be correlated with the experimental results. Thus, several software packages, such as MetaSite [4], Meta-Expert [5], and SMARTCyp [6], have been developed to accurately predict the reaction site of a molecule and consequently identify whether the produced metabolite is toxic, non-toxic, active or inactive.

The cytochrome P450 (CYP) is a family of enzymes used to analyze the metabolism by *in silico* methods. CYPs catalyze the phase I metabolism of xenobiotics in humans and other mammals through a variety of reactions, including aliphatic and aromatic hydroxylation, epoxidation, N- and O-dealkylation, and N- and S-oxidation. In humans, approximately 15 enzymes that belong to the CYP1-3 families are responsible for the metabolic clearance of the most lipophilic chemicals. In particular, CYP2C9 is a human CYP that contributes significantly to the hepatic metabolism of therapeutic drugs [7].

CYP2C9 constitutes 18 to 30% of the total hepatic CYP [8, 9] and is distinguished from other human CYP2C isoforms by its preference for negatively charged substrates at physiological pH; however, neutral or positively charged substrates may also be metabolized. Some drugs that are CYP2C9 substrates and that have a narrow therapeutic index are the anticoagulant (*S*)-warfarin and the antiepileptic phenytoin. Other important substrates of this enzyme are non-steroidal anti-inflammatory drugs, such as flurbiprofen, ibuprofen, naproxen, and diclofenac; the hypolipidemic agent gemfibrozil; [10]. These substrates interact with different amino-acid residues in the catalytic site of CYP2C9, which contains principally basic residues that stabilize the binding of anionic substrates [11-14].

In this sense, several *in silico* methods have been used to describe the interactions exhibited between CYP2C9 and its substrates to explain the principal mechanism by which this isoform metabolizes each substrate. In these studies CYP2C9 crystallized structures have been used because with them is possible to have tridimensional models and know at molecular level the recognition between the enzyme and their ligands. Two substrates that have been crystallized bound to CYP2C9 are warfarin and flurbiprofen [15, 16].

Furthermore, the use of *in silico* tools has helped predict the formation of safe or toxic products. Therefore, due to the important participation of CYP2C9 in the metabolism of many compounds and the quantity of information that can be obtained by *in silico* methods, the aim of this review is to explain how the homology modeling, molecular dynamics (MD) and docking simulations have been used to study the interactions between CYP2C9 and its substrates. In addition, *in silico* methods are used in this work to analyze the interactions of four of the principal molecules that produce toxicity when they interact with CYP2C9: dapsone, sulfaphenazole, and valproic and tienilic acids.

2. CYP2C9 CRYSTALLIZED STRUCTURES

The 3D structures of CYP2C9 that have been solved using X-ray methods allow the more precise determination of the interactions between the amino acids and the ligand. The first two crystallized structures of CYP2C9 were reported by Williams *et al.*; one is shown without a substrate (PDB code 1OG2) and the other is shown forming a complex with warfarin (PDB code 1OG5) [15]. However, these proteins have important modifications in their sequences because they were obtained from a mutated form that contains seven amino-acid substitutions: K206E, I215V, C216Y, S220P, P221A, I223L and I224L (Fig. 1). The residue P221 in CYP2C9 is located at position P220 in the CYP2C8 and CYP2C5 isoforms, where it forms a tight turn between helices F′ and G′. In the 1GO2 crystallized structure, the N-terminal transmembrane domain was removed (approximately 22 amino acids residues) and a MAKKT sequence was added to facilitate the determination of

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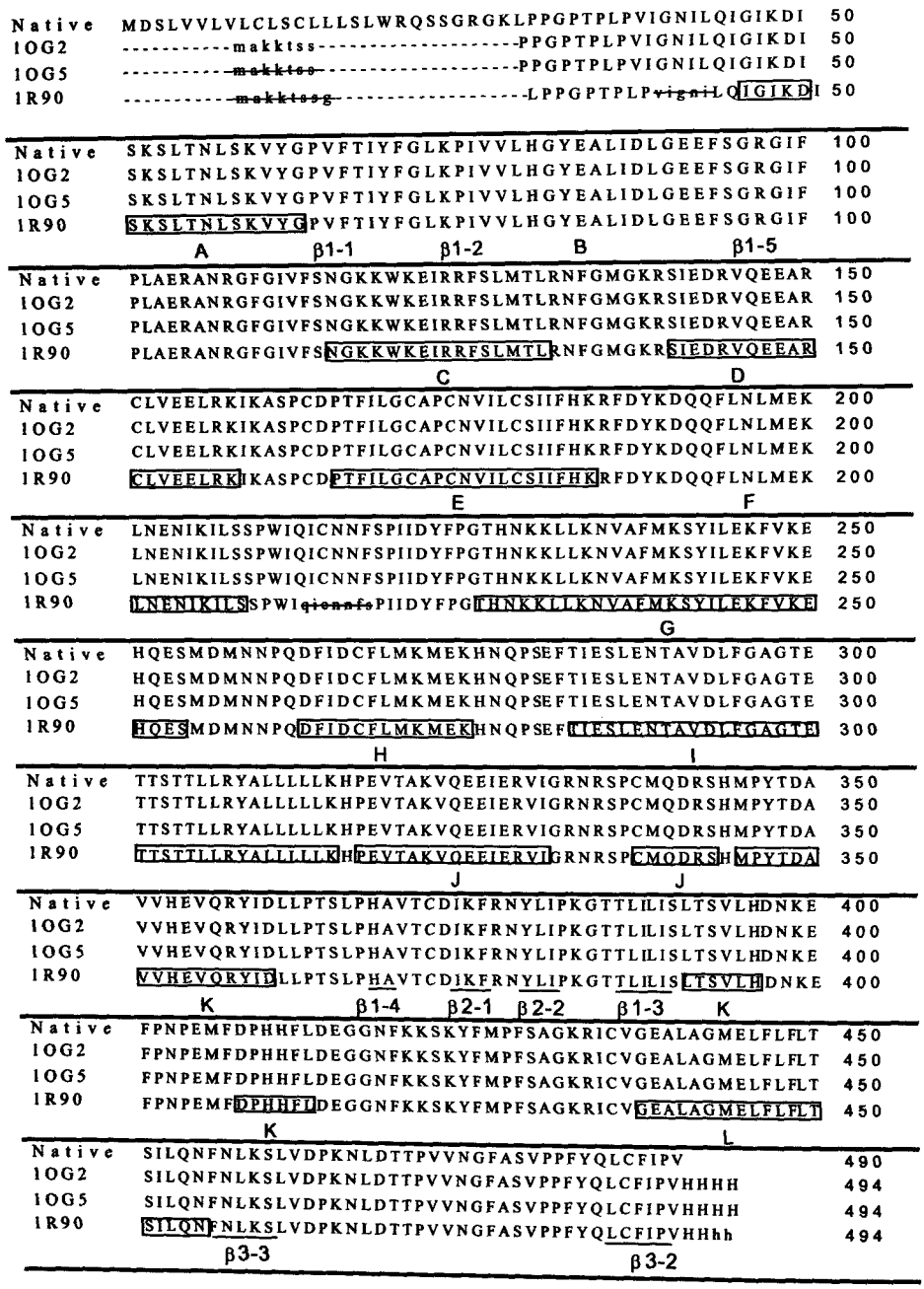


Fig. (1). Alignment of crystallized CYP2C9 (1OG2, 1OG5 and 1RO9) and the native sequence.
In bold are shown the amino acids mutated during the crystallization of 1OG2 and 1OG5, and in cross out those missing in the 1RO9 structure.

the catalytic domains of CYP2C9 [17]. In addition, the C-terminus of the enzyme was modified, including a tag of four histidine residues to facilitate the purification of the truncated enzymes (Fig. 1). In the 1GO5 crystallized structure, certain regions that are considered important were identified during the recognition of the substrate. Some of these regions are the B-C loop involving the 101-106 residues and the F-G loop (212-222 residues), which had not been previously observed in any other CYP structure. Furthermore, Williams *et al.* reported that the residues R97 and F114 are also important in the 1GO2 and 1GO5 crystallized structures, referencing some mutagenesis studies [14, 18, 19]. In Table **1**, the most important residues identified in both crystallized structures and their respective functions are shown. Although the CYP2C9 crystallized structures obtained by Williams *et al.* were of great help for identifying important regions of this isoform, the ligand in the 1OG5 structure (warfarin) was positioned 10 Å away from the heme (located between helices I and L), which is considered to be too far for the oxidation of that ligand. This observation led to the proposal that the CYP2C9 structure captured by Williams *et al.* only reflects one conformation that the enzyme can adopt during the catalysis and that warfarin was bound in a transition state of the protein. For this reason, 1GO5 was suggested to be the open structure of CYP2C9, similar to that which occurs for CYP2B4 [20].

One year after Williams *et al.* reported the first crystallized structures of CYP2C9, Wester *et al.* published a crystallized structure of this isoform bound to flurbiprofen (PDB code 1RO9) [16].

Table 1. Principal Amino Acids Identified as Important in the 1GO5 Structure

Amino Acids	Importance
Thr 301	Participates in the proton transfer path due to its involvement in a hydrogen bond between the iron and water molecule. (Haines et al 2001)
Trp 120, Arg 124, His 368, Arg 433, Arg 97	Participate in the stabilization of the heme group by hydrogen bonds with the propionates groups.
Phe 114	Interacts with the substrate
Asp 293, Glu 300	Located in the active sites and show different degrees of flexibility more Glu 300 than Asp 293.
Gln 214, Asn 217	Interact with the substrate through a hydrogen bond
Phe 476	Interacts with the substrate through a pi-pi stacking interaction.
Arg 108, Arg 204	Interact with the substrate through a salt bridge or hydrogen bonds
Asp 293	Interacts with the substrate through a hydrogen bond.

CYP2C9 was named by these authors as CYP2C9dH because N-terminal is truncated (partially deleted) and at the C-terminal there are four histidine added. The obtained structure is similar to that of 1OG5 in many regions but with a root mean square deviation (RMSD) of 0.74 Å. However, the conformation of the crystallized 1RO9 structure is significantly different from that of 1GO5. The former shows an extra turn near residue 53 at the N-terminal of helix A, a second between residues 97 and 121 of helices B and C, a third in the F helix between residues 197 and 232 and the last between residues 467 and 478 in the β antiparallel sheets of the C-terminal. Furthermore, the observed movement of the B helix to the region of the C helix in the 1RO9 structure differs significantly in the 1OG5 structure, which has a conformation more typical of CYP2C5 (PDB codes: 1NR6 and 1N6B), CYP2C8 (PDB code: 1PQ2), and CYP2B4 (PDB code: 1SUO). This region exhibits significant flexibility in the structures of the isoforms CYP2C5 [21] and CYP2B4 [20], which can adopt alternative conformations without greatly altering their secondary structure. In addition, it has been reported that the opening and closing of these structures to allow substrate binding is possible in the final portion of the B-C loop [21]. The flurbiprofen is located 4.9 Å from the iron of the heme group of CYP2C9 (PDB code: 1RO9), which is consistent with the distances observed in other CYP-substrate complexes. Some of the amino acids of the CYP2C9 catalytic site that interacts with the flurbiprofen were identified previously recognized warfarin in the 1GO5 crystallized structure [15, 16]. However, as observed in Table **2**, there are some amino acids that differ between the structures. For example, the R108 residue was identified as important for ligand recognition in the 1RO9 crystallized structure, which was demonstrated by the fact that flurbiprofen has a high affinity for CYP2C9 due to its interaction with this residue. This observation is consistent with other reports in which the substitution of R108 with other amino acids alters the flurbiprofen binding [22] and greatly diminishes the oxidation of warfarin [22] and diclofenac [18, 22]. Interestingly, the hydroxylation of pyrene, a neutral substrate that is not capable of forming a hydrogen bond with R108, was unaffected by the R108F mutation [22], showing that the recognition between the enzyme and the substrate is dependent on the chemical structure of the latter. Thus, the 1R9O crystallized structure contributes significantly to the interpretation of the mutagenesis studies that have been previously reported; these studies indicated that the residues R108 [18, 22-24], N289 [23-25], and D293 [26] are important participants in substrate recognition by the CYP2C9 enzyme. Flanagan *et al.* have reported the importance of the D293 residue in the CYP2C9 structure based on site-directed mutagenesis studies in which they observed that bacteria cannot correctly incorporate the heme group when the mutations D293Q and D293N are present [27].

Table 2. Principal Amino Acids that Interact with the Substrate Identified in 1GO5 and 1R09

1GO5 Structure	1R09 Structure
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.	Val-113, Phe-114, Ile-205, Leu-208, Val-237, Met-240, Val-292, Gly-296, Ala-297, Leu-366 Arg-108, Asn-289 and Asp-293

Therefore, the results of these studies lead to the conclusion that, despite the difference in the sequences used for the crystallization of CYP2C9, the 1R9O and 1OG5 structures reflect the flexibility of the protein conformation. However, it is important to emphasize that the conformational changes observed in 1RO9 have not been identified in other crystallized structures of the CYPs of different mammals.

3. COMPUTATIONAL METHODS

Several CYP enzymes have been the subject of many computational studies due to interest in knowing their metabolic properties and the structures of their products. In this sense, the use of *in silico* methods in these types of studies has experienced a recent resurgence, as attested by the increase in the number of recent publications [28-31]. This interest is based on the idea that combining chemical knowledge and empirical data obtained from *in vitro* systems complements existing information and allows for comparisons. In this sense, many theoretical studies have used computational methods, such as homology modeling, molecular dynamics (MD) and docking simulations, which have been useful for knowing the structure of a protein, its recognition sites, and its interaction type with its ligands. With these and other computational methods, it is also possible to predict the oxidation sites of the CYPs, the chemical features that cause their metabolic instability and the inhibitory potency of a substrate [32]. These data in turn allow chemical modifications of the substrate to avoid the production of toxic metabolites by these enzymes. In this sense, due to the important participation of CYP2C9 during the metabolism of diverse drugs, the results are relevant at the molecular level for determining the interactions between this enzyme and its substrates and inhibitors. Furthermore, models based on computational methods may enable the discovery of new substrates or inhibitors of CYP2C9,

thus ensuring that only those with desirable properties are synthesized and evaluated *in vitro*.

3.1. Homology Modeling

Before CYP2C9 was crystallized and placed in the Protein Data Bank (PDB), a variety of theoretical studies were performed to determine its structure. Because other CYPs had been crystallized, many of these studies were based on homology models for which the coordinates of the bacterial CYP crystallized structure were taken (CYP101, -102, -107, and -108) [33-34]. However, the construction of these models was complicated because of the dissimilarity between the bacterial and mammalian amino acid sequences (20-30%) [18]. In addition, this issue meant that the molecular basis of drug binding to human CYPs was difficult to determine even though many models of different bacterial CYP-substrates complexes have been extrapolated and correlated with the former [35-38]. For instance, Payne *et al.* reported some homology models of CYP2C9 using bacterial and fungal templates; in this case, the sequence identities were 17.4%, 19.0%, 19.1%, and 17.7% for CYP101, CYP102, CYP107A, and CYP108, respectively. They considered that a number of structural motifs were conserved among the four templates, allowing the definition of structurally conserved regions (SCRs) despite the low sequence homology [39]. However, the structure of the first mammalian CYP solved from the CYP2C5 rabbit isoform was deposited in the Brookhaven Protein Data Bank (PDB 1dt6) on January 2000 [40]. This experimental evidence allows researchers to develop numerous homology models using this crystallized structure as template and has been used to evaluate the specificity, interactions and metabolic and pharmacophore sites of the substrates [41-44].

In this sense, Lewis *et al.* and Afzelius *et al.* have reported the homology modeling of CYP2C9 based on the CYP2C5 crystallized structure due to the high similarity between both sequences. The obtained results show that CYP2C9 shares 78% sequence identity with CYP2C5 [45, 46]. Also, Oda created a CYP2C9 model based on the CYP2C5 structure, finding an 87% sequence identity between them [47]. After CYP2C9 was crystallized, Afzelius *et al.* carried out a study that compared the CYP2C9 and CYP2C5 structure conformations obtained by crystallization and homology modeling, respectively. In addition, they used MD simulations to determine whether they overlap in chemical space and how their correlation depends on the modeling technique used. They found that homology models are useful theoretical tools to obtain 3D models of CYP enzymes because they show good quality from a stereochemical point of view, and this was demonstrated by the fact that the most favored regions in these models have a higher percentage in a Ramachandran plot than in the templates. In this study, it was observed that great changes occur in the merging region of the B-C loop, F-G loop, and N-terminal, that the C-terminal loop (V473, N474, G475, and F476) and the N-terminal loop (VI47 and K48) are closer to each other, and that the distance between the C-terminal and F-G loops is larger than in the CYP2C5 structure [48].

Although several reports have used the CYP2C5 crystallized structure as a template for the construction of CYP2C9 homology models because of its high similarity, there are now several crystallized structures of this isoform that serve as a template for improving the homology models of diverse CYP2C enzymes, such as CYP2C19 [49]. These data show that the homology-modeling method is still used because crystallized structures often contain several mutations and missing residues [50].

3.2. Molecular-Dynamics (MD) Simulations

Several studies have been performed using homology modeling to determine the CYP2C9 3D structure. However, because these 3D models do not show protein movements, MD is used. In this sense, the 1OG2 and 1OG5 crystallized structures of CYP2C9 and the substrate warfarin have been used with the AMBER program to describe the molecular basis of substrate binding and regioselectivity. During the MD simulations, some fluctuations were observed in the F-G and B-C loops, which interact with the endoplasmic-reticulum membrane to form a channel in which the hydrophobic substrate can access to the enzyme, preventing contact with the water solvent. These fluctuations were observed in both free-form CYP2C9 and the CYP2C9–warfarin complex. However, this narrow channel was stabilized and widened in the presence of the substrate. In addition, the presence of second and third channels was reported by MD simulations. The second, referred to as the solvent channel, is located in the region between the helices F and I and turns in the C-terminal antiparallel β-sheet, allowing contact between the solvent and the heme group. The third channel is located between the B' helix/B-C loop and the G and I helices. Furthermore, one of the transition states during the MD simulations allows the identification of the close proximity between the CYP oxyferryl radical and the warfarin hydrogen atoms of positions 6 and 7, from which it is possible to conclude that, as result of this reaction, warfarin is hydroxylated at those positions by CYP2C9 [51].

It is well known that CYPs are promiscuous enzymes because each can catalyze different types of substrates or produce different metabolites from one substrate. One of the CYPs functions is to catalyze the hydroxylation of several substrates, as is demonstrated by CYP2C9, which can hydroxylate fluvastatin and tamoxifen despite their different chemical structures. It has been suggested that the promiscuity of CYP enzymes is attributed to their flexibility and malleability. These structural properties were found by studying MD simulations of the movements of CYP2A6, CYP2C9, and CYP3A4, which are considered pharmacologically important enzymes in human hepatic metabolism. This work shows that CYP2C9 is intermediate in terms of both its substrate specificity and malleability. Its fluctuations are spread over the B'/C, C/D, G'/G, G/H, and H/I loops, the meander region and the $\beta3_1/\beta3_2$ loop (residues 463-466). These flexible regions are similar to those identified by X-ray analysis. The same MD-simulation study but at high temperatures shows some conformational changes, principally in the flexible regions, which affect the egress path. In the initial structure, the solvent channel is preferred and the pw2b channel is closed, but the shifts of the F helix C-terminal and F/F' loop close the solvent channel. The pw2b opens due to a shift (~5 Å) of the loop between residues 68-72 during the MD simulation (Fig. 2) [52].

Taking into account all of the different movements in the CYP2C9 structure and other CYP enzymes, a model of gating in CYP enzymes was recently proposed in which the pw2b channel participates in the opening and closing of the CYP2C9 structure (Fig. 2) [53]. In addition, Afzelius *et al.* used MD simulations to explore the openings and closings of the 3D structures that correspond to access channels. They measured the distances between the backbone carbons of the secondary structure elements, exploring each snapshot obtained from two separate MD-simulation runs. During the first run, one major conformational freedom was observed in the opening between the B-C loop and the ß1-1 sheet. It has been suggested that the substrate recognition and access occur in structures in which K72 participates in the gates [14]. In the second run, this region shows only minor flexibility, which emphasizes the difficulty in making structural assumptions on the basis of MD simulations for the highly flexible CYP2C enzymes. However, the overall RMSD and the movement range were similar for both MD simulations [48]. Furthermore, MD simulations and Raman-resonance (RR) and ultraviolet-visible (UV/VIS) spectroscopy at normal pressure and high pressure of up to 300 MPa (HP) have been performed to obtain information about the CYP2C9 active-site cavity and its compressibility. All MD simulations and RR and UV/VIS spectroscopic data indicate that CYP2C9, CYP2D6 and CYP3A4 exhibit considerably greater flexibility or malleability than CYP2A6 and CYP1A2, given that the overall topology of

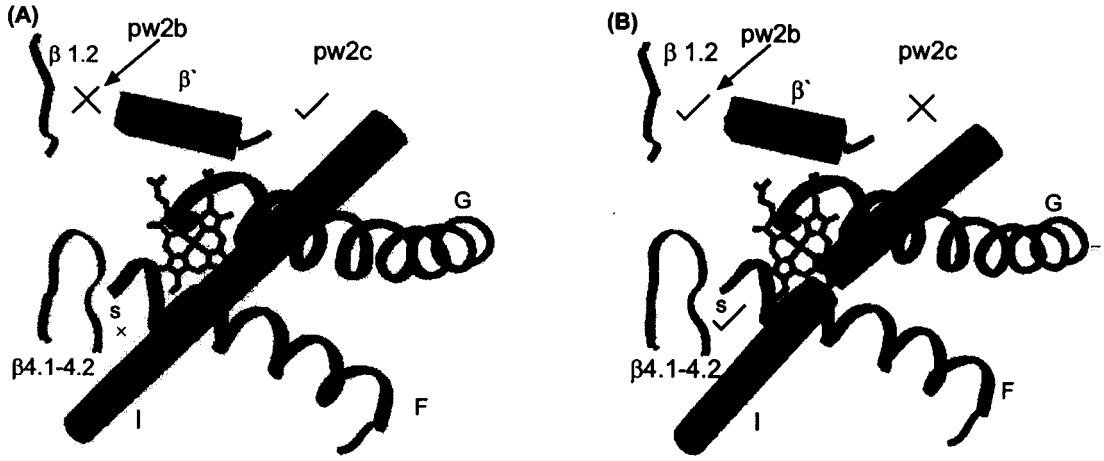


Fig (2). Principal secondary structures that participate in the open and closed conformations of CYP2C9.
(**A**) In the closed state the β4.1-4.2 sheet and the F and G helices are blocking the way of the drug(s) channels; the β' helix together with the β1.2 sheet are closing the channel pw2b, while the displacement of the β' helix open the channel pw2c. (**B**) In the open state the β' sheet near to the F, G and I helices close the pw2c channel opening the pw2b, while the displacement of the β1.2 and β4.1-4.2 sheets away from the binding site allow the opening of the channels.

CYP2C9 was similar to the X-ray structure during 10 ns of MD. The regions with more flexibility at NP and HP were those exposed to the water solvent and the substrate-binding site [28].

Oda *et al.* has used MD simulations to analyze the changes in the CYP2C9-(S)-mephenytoin complex using several mutants [47]. In addition, Bikádi and Hazai performed MD simulations of the CYP2C9-methoxychlor complex to explain the chiral preference between CYP2C9 and others CYPs, such as CYP2C19, CYP2C5 and CYP2C3. They found that the amino acids in the CYP2C9 active site that interact with methoxychlor are more rigid than those of CYP2C3, producing a stereoselective metabolism of this substrate by the former that was not observed with CYP2C3. Therefore, they conclude that the rigidity and stability analyses of the CYP2C9-methoxychlor complex during MD are important in differentiating between possible chiral preferences in methoxychlor metabolism by CYP2C enzymes, including CYP2C9 [50].

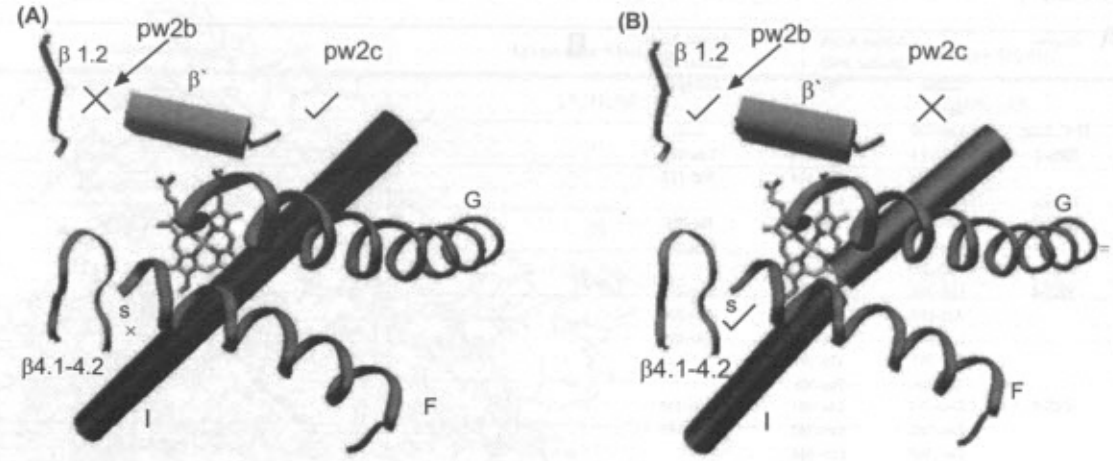
3.3. Docking Simulations

One of the most important in silico techniques used to analyze the binding of different ligands on a protein at molecular level is docking. The use of this technique with mutagenesis studies has been helpful in determining the amino acids that are important during the interaction between CYP2C9 and their ligands [54, 55]. The results show that the hydrophobic residues F114, V113, and F476 are important in its binding site [56]. The sites where substrates and inhibitors have at least two non-coplanar aromatic rings and anionic and/or hydrogen-bond acceptor sites are located at 6-10 Å from the hydroxylation site [57]. Taking into account that CYP2C9 accepts weakly acidic substrates, several reports have suggested that the active site of CYP2C9 has positive residues that may interact electrostatically with negatively charged groups [12, 17, 58, 59]. For instance, several groups that have employed a variety of computer-modeling techniques have proposed that R105 and R108 are charged residues in CYP2C9 that are important in drug metabolism [14, 39, 60]. All of these contributions lead to the hypothesis that CYP2C9 has an anionic binding site. However, this enzyme also has hydrophobic and hydrogen-bond donor or acceptor residues in its active site [12, 14, 58].

CYP2C9 metabolize a great variety of substrates whose metabolism has been studied by *in vitro* analysis, as shown in Table **3**. For instance, Chesné *et al.* studied the involvement of human hepatic CYP in meloxicam metabolism using several *in vitro* models, including human liver microsomes, hepatocyte cultures and heterologously expressed CYP isoforms. The results show that meloxicam is hydroxylated by CYP2C9 more than by CYP3A4. However, subsequent studies demonstrated that the 5-hydroxymethyl metabolite of meloxicam is converted to a carboxyl product by further oxidation of the methyl group only in primary human hepatocyte cultures and not in microsomes. These results suggest that CYPs are not involved in the oxidation of the alcohol group of meloxicam and support the use of cultured hepatocytes for analyzing drug metabolic profiles [61]. However, few of these ligands (substrates and inhibitors) have been studied by *in silico* methods (Table **3**). Before CYP2C9 was crystallized, docking calculations were performed using the models obtained by homology modeling. Lewis described the binding of diclofenac, which forms π-π stacking interactions with the side chains of F114 and F473, and the side chain of Q214 to form a hydrogen bond with the carboxylate moiety of diclofenac, allowing the 4-hydroxylation of this compound [45]. Lewis *et al.* evaluated the binding of (S)-naproxen, phenytoin, and progesterone after creating their homology models. They found that there are important amino-acid residues for substrate recognition in the binding site of CYP2C9, including R105 and R97 as key cationic residues and N202, D293, P101, L102, G296, and F476 [41].

Afzelius *et al.* achieved docking calculations using 40 CYP2C9 inhibitors (including in Table **3**) to select the best conformer for a 3D-QSAR analysis [46]. From these studies, Lewis and Afzelius identified important amino-acid residues in the CYP2C9 active site from different substrate recognition sites (SRS) (Fig. **3A**). Afzelius reported 17 amino-acid residues in the active site, of which two were polar (T301 and T304), two acidic (D293 and E300) and the rest hydrophobic (Fig. **3B**).

Zamora *et al.* reported a theoretical method to predict the site where the molecules can be metabolized using a CYP2C9 homology model [62]. They employed 43 compounds with 87 metabolic putative reactions. Their method is based on a comparison between alignment-independent descriptors derived from GRID molecular-interaction fields for the CYP2C9 active site. The results are given as a ranking list of all of the hydrogen atoms of each substrate molecule that can be metabolized; they reported that the oxidation site of the substrates ranked as the first in 50%, at least second in 75% and at least third in more than 90% of the cases. Actually, this methodology is commercialized as MetaSite software and has been



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One of the most important in silico techniques used to analyze the binding of different ligands on a protein at molecular level is docking. The use of this technique with mutagenesis studies has been helpful in determining the amino acids that are important during the interaction between CYP2C9 and their ligands [54, 55]. The results show that the hydrophobic residues F114, V113, and F476 are important in its binding site [56]. The sites where substrates and inhibitors have at least two non-coplanar aromatic rings and anionic and/or hydrogen-bond acceptor sites are located at 6-10 Å from the hydroxylation site [57]. Taking into account that CYP2C9 accepts weakly acidic substrates, several reports have suggested that the active site of CYP2C9 has positive residues that may interact electrostatically with negatively charged groups [12, 17, 58, 59]. For instance, several groups that have employed a variety of computer-modeling techniques have proposed that R105 and R108 are charged residues in CYP2C9 that are important in drug metabolism [14, 39, 60]. All of these contributions lead to the hypothesis that CYP2C9 has an anionic binding site. However, this enzyme also has hydrophobic and hydrogen-bond donor or acceptor residues in its active site [12, 14, 58].

CYP2C9 metabolize a great variety of substrates whose metabolism has been studied by *in vitro* analysis, as shown in Table **3**. For instance, Chesné *et al.* studied the involvement of human hepatic CYP in meloxicam metabolism using several *in vitro* models, including human liver microsomes, hepatocyte cultures and heterologously expressed CYP isoforms. The results show that meloxicam is hydroxylated by CYP2C9 more than by CYP3A4. However, subsequent studies demonstrated that the 5-hydroxymethyl metabolite of meloxicam is converted to a carboxyl product by further oxidation of the methyl group only in primary human hepatocyte cultures and not in microsomes. These results suggest that CYPs are not involved in the oxidation of the alcohol group of meloxicam and support the use of cultured hepatocytes for analyzing drug metabolic profiles [61]. However, few of these ligands (substrates and inhibitors) have been studied by *in silico* methods (Table **3**). Before CYP2C9 was crystallized, docking calculations were performed using the models obtained by homology modeling. Lewis described the binding of diclofenac, which forms π-π stacking interactions with the side chains of F114 and F473, and the side chain of Q214 to form a hydrogen bond with the carboxylate moiety of diclofenac, allowing the 4-hydroxylation of this compound [45]. Lewis *et al.* evaluated the binding of (S)-naproxen, phenytoin, and progesterone after creating their homology models. They found that there are important amino-acid residues for substrate recognition in the binding site of CYP2C9, including R105 and R97 as key cationic residues and N202, D293, P101, L102, G296, and F476 [41].

Afzelius *et al.* achieved docking calculations using 40 CYP2C9 inhibitors (including in Table **3**) to select the best conformer for a 3D-QSAR analysis [46]. From these studies, Lewis and Afzelius identified important amino-acid residues in the CYP2C9 active site from different substrate recognition sites (SRS) (Fig. **3A**). Afzelius reported 17 amino-acid residues in the active site, of which two were polar (T301 and T304), two acidic (D293 and E300) and the rest hydrophobic (Fig. **3B**).

Zamora *et al.* reported a theoretical method to predict the site where the molecules can be metabolized using a CYP2C9 homology model [62]. They employed 43 compounds with 87 metabolic putative reactions. Their method is based on a comparison between alignment-independent descriptors derived from GRID molecular-interaction fields for the CYP2C9 active site. The results are given as a ranking list of all of the hydrogen atoms of each substrate molecule that can be metabolized; they reported that the oxidation site of the substrates ranked as the first in 50%, at least second in 75% and at least third in more than 90% of the cases. Actually, this methodology is commercialized as MetaSite software and has been

A

Region	Amino Acids *Afzelius 2001* Active site	Amino Acids *Afzelius 2001* Model	Amino Acids *Lewis 2002* Active site
B-C.loop	Leu-102	Leu-102	——
SRS-1	Val-113	Val-113	Leu-102
	Phe-114	Phe-114	Val-113
F-helix	Leu-201	Leu-201	
SRS-2	Ile-205	Ile-205	Ile-205
	Gln-214		Leu-208
I-helix	Asp-293	Asp-293	
SRS-4	Gly-296	Gly-296	Asp-290
	Ala-297	Ala-297	Ala-294
	Gln-300	Gln-300	Thr-298
	Thr-301	Thr-301	
	Thr-304	Thr-304	
SRS-5	Leu-361	Leu-361	Leu-359
	Leu-362	Leu-362	Leu-363
	Leu-366	Leu-366	
B2	Phe-476	Phe-476	
SRS-6	Ala-477	Ala-477	Phe-473
			Ala-474

B

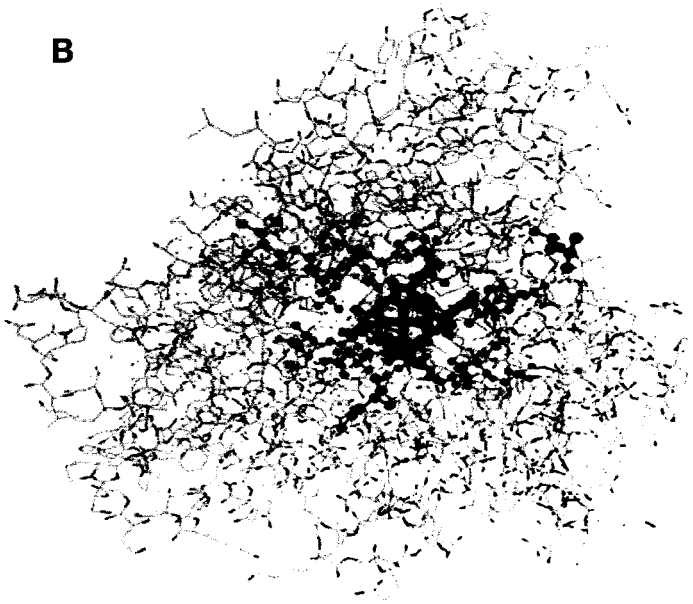


Fig. (3). Amino acids of SRS in CYP2C9.
(**A**) Amino acids residues reported by Afzelius and Lewis. (**B**) Location of the amino acids of SRS in the tertiary structure of CYP2C9, B-C loop (blue), SRS-1 (red), F-helix (gray), SRS-2 (orange), I-helix (tan), SRS-4 (green), SRS-5 (pink), B2 (purple) and SRS-6 (lime).

Table 3. CYP2C9 Ligands Studied by *In Silico* and *In Vitro* Methods

Substrates	Molecular Modeling	*In Vitro* Studies
17-α-ethinylestradiol		
58C80	13, 96	
Aceclofenac		
Acenocoumarol		105
Mefenamic Acid	55	106
Tienilic Acid	96	
Valproic Acid		104
Amitriptyline		
Carvedilol		107
Celecoxib	69	69
Chlorpropamide		
Clozapine		108, 109
Dapsone	77	109
Desogestrel		110
Dibenzo(a,h)anthracene		
Diclofenac	13, 96	17, 19, 22, 53, 109, 111-117
Disulfoton		118
DMZ		
Etodolac		

(Table 3) Contd....

Substrates	Molecular Modeling	*In Vitro* Studies
Phenytoin	13, 38, 96	108, 109
Phenobarbital		
Phenprocoumon		
Fluoxetine	55	
Flurbiprofen	63, 70	70, 109, 111, 113
Fluvastatin		
Phorate		118
Galagin		72, 119
GV150526		
Hexobarbital		
Hydromorphone		
Ibuprofen	13, 55, 96	
Indomethacin		
Kaempferol		72, 119
Ketamine		117, 118
Lansoprazole		
Limonene		122
Lornoxicam		123-127
Losartan		115
Meloxicam		61
Mestranol		
Methyl Eugenol		
Methiocarb		118
Methoxychlor		
Montelukast		
Naproxen	13, 38, 55	
N-Dehidroxyzileuton		
Piroxicam		109
Rosuvastatin		
Safrole		
Seratrodast		128
Sildenafil		129
S-MTTPA		
Sulprofos		118
Suprofen	55	
Tamoxifen	96	
Tenoxicam		100

A

Region	Amino Acids *Afzelius 2001* Active site	Amino Acids *Afzelius 2001* Model	Amino Acids *Lewis 2002* Active site
B-C.loop	Leu-102	Leu-102	------
SRS-1	Val-113	Val-113	Leu-102
	Phe-114	Phe-114	Val-113
F-helix	Leu-201	Leu-201	
SRS-2	Ile-205	Ile-205	Ile-205
	Gln-214		Leu-208
I-helix	Asp-293	Asp-293	
SRS-4	Gly-296	Gly-296	Asp-290
	Ala-297	Ala-297	Ala-294
	Gln-300	Gln-300	Thr-298
	Thr-301	Thr-301	
	Thr-304	Thr-304	
SRS-5	Leu-361	Leu-361	Leu-359
	Leu-362	Leu-362	Leu-363
	Leu-366	Leu-366	
B2	Phe-476	Phe-476	
SRS-6	Ala-477	Ala-477	Phe-473
			Ala-474

B

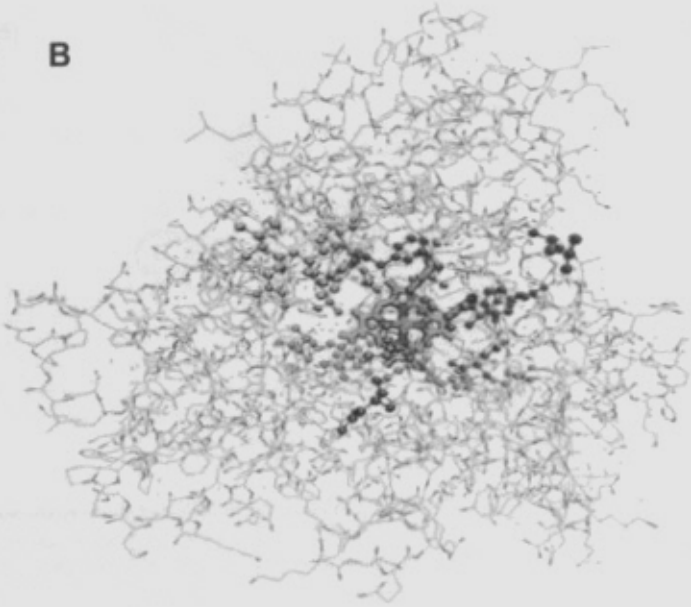


Fig. (3). Amino acids of SRS in CYP2C9.
(**A**) Amino acids residues reported by Afzelius and Lewis. (**B**) Location of the amino acids of SRS in the tertiary structure of CYP2C9, B-C loop (blue), SRS-1 (red), F-helix (gray), SRS-2 (orange), I-helix (tan), SRS-4 (green), SRS-5 (pink), B2 (purple) and SRS-6 (lime).

Table 3. CYP2C9 Ligands Studied by *In Silico* and *In Vitro* Methods

Substrates	Molecular Modeling	*In Vitro* Studies
17-α-ethinylestradiol		
58C80	13, 96	
Aceclofenac		
Acenocoumarol		105
Mefenamic Acid	55	106
Tienilic Acid	96	
Valproic Acid		104
Amitriptyline		
Carvedilol		107
Celecoxib	69	69
Chlorpropamide		
Clozapine		108, 109
Dapsone	77	109
Desogestrel		110
Dibenzo(a,h)anthracene		
Diclofenac	13, 96	17, 19, 22, 53, 109, 111-117
Disulfoton		118
DMZ		
Etodolac		

(Table 3) Contd....

Substrates	Molecular Modeling	*In Vitro* Studies
Terbinafine		130
Tolbutamide	13, 96	22, 109, 112-114, 116, 131, 132
Tolterodine		
Torsemide		
TR-14035		133
Voriconazole		
Warfarin	13, 96	67, 109
Zafirlukast		
Zaltoprofen		
Zolpidem		134
Zopiclone		
Δ-9-tetrahydrocannabinol	13	
Inhibitors	**Molecular Modeling**	***In vitro* studies**
(R)-Pantoprazole		
(R)-Rabeprazole		135
(R)-Warfarin		67
(S)-Miconazole		109, 136
(S)-Pantoprazole		
(S)-Rabeprazole		135
(S)-Warfarin	95	19, 67, 109, 111, 113, 131
Dicoumarol		
Phenylbutazone		
Phenytoin	97	137
Omeprazole Sulfone		109, 114
Pyrimethamine		
Progesterone	41	
Quercetin		111, 121
Quinine		111
SFID		
Sulfafenazol	46, 96	111, 112, 126
Thiabendazole		111

used by several researchers to predict the metabolism of diverse compounds [63, 64]. We added a paragraph in Docking simulations section line 327 "In this sense, MetaSite and other programs to predict the metabolism are based in the use of computational techniques such as quantum mechanics (QM), which was used to predict the rates of oxidation catalyzed by CYP450. Korzekwa *et al.* performed a QM model based on the hydrogen abstraction using *p*-nitrosophenoxy (PNR) and the semiempirical AM1 Hamiltonian [65]. This technique together with MD was used after by Jones and Korzekwa in order to predict the rates and regioselectivity [66]. They followed a methodology to do MD runs, but they employed CYP101 and CYP102 during the analyses because they did not have a crystallized structure of a mammalian CYP450. Their methodology consisted in 8 steps which included MD and docking, however they concluded that in this study their PNR model gives a good quantitative prediction of rates for hydrogen atom abstraction,

but is inappropriate when is applied to reactions that not occur by hydrogen atom abstraction or substrates that have limited motion in the active site, which can be improved by MD models.

After Zamora *et al.* reported their *in silico* methodology to predict metabolism, Sykes reported the use of the ROCS alignment method for predicting the orientation of substrate drug molecules in the CYP2C9 active site. This method was based on using the shape and chemistry properties of the known CYP2C9 substrate flurbiprofen as the query molecule. The results of this method show a high degree of accuracy, superior to that of docking. However, the molecules evaluated by ROCS must have the same shape because this method is based on the use of the simple but effective concept of volume overlap [67].

Years later, a method based on the docking procedure with the aim of predicting CYP2C9 metabolism was used, employing 92 substrates with 136 reactions selected from the substrate library reported by Zamora *et al.* and Sykes *et al.* [62, 67]. This method consists of the construction of the possible metabolites using the MetabolExpert software, which were posteriori docked at the CYP2C9 binding site to select the best. The results obtained by this method are better than those reported by Zamora *et al.* and Sykes *et al.*, suggesting that docking is a good approach to predict metabolism because it is possible to obtain the metabolite structure instead of merely the site in the molecule where the metabolism occurs [68]. In addition, these methods can be applied to studying the metabolism of the named suicide enzymes.

Furthermore, molecular-docking calculations have the advantage of explaining the stereoselective metabolism by CYP2C enzymes. In this sense, Bikádi and Hazai reported a study in which the CYP2C9 enantioselectivity on methoxychlor was analyzed. They found that this property was impossible to determine by the docking energies because these values are similar in all CYP2C enzymes (6 kcal/mol approximately). However, the position of the prochiral methoxychlor at the CYP2C9 binding site allowed the identification of the yield of the *S*-mono-OH-M metabolite, which has been obtained experimentally. Thus, these results led to the conclusion that it is not possible to construct any quantitative model of chiral recognition based only on docking energies [49].

However, in some studies, the free-energy values obtained by docking allow the prediction of the better ligand or metabolism site. Yao *et al.* found that the principal metabolite is methyl-hydroxygliclazide during the metabolism of gliclazide by CYP2C9. They identified and analyzed two complexes. The first is CYP2C9- gliclazide in which the 6β-carbon atom is placed near to the heme group (4.33 Å) and R108, explaining the production of the 6β-hydroxygliclazide metabolite. In relation to the second complex, the distance from the methyl-carbon to the heme is 3.89 Å, yielding methyl-hydroxy-gliclazide. They found that this complex has a lower and more favorable total interaction energy than first complex [50]. These results are comparable to experimental data in which the second complex has a lower K_M value than the first. In addition, they found that F114 and F476 are important amino-acid residues for ligand binding and selectivity, as has been previously reported [55].

Due to computational methods, Wang *et al.* described the CYP2C9 binding pockets for fluoxetine, ibuprofen, naproxen, suprofen, and mefenamic acid. Their results were very useful for mutagenesis studies, providing insights into the metabolic mechanism that may be of relevance to the personalization of each drug [69]. Furthermore, Jones *et al.* used this method widely to show the binding of ibuprofen, naproxen, phenytoin, warfarin, diclofenac, delta 1-tetrahydrocannabinol, 58C80, tolbutamide, and the selective inhibitor sulfaphenazole on CYP2C9 [13].

After CYP2C9 was crystallized, the number of docking studies increased because a different crystal structure of the same protein can show different conformational changes. For instance, Ahlstrom *et al.* studied the interaction between CYP2C9 and celecoxib using two crystallized structures of CYP2C9 (1RO9 and 1OG5). The principal amino acids that interact with celecoxib are F100, L208, F476, L366, A297, and R108, but the oxygen atom located in the sulfonamide moiety interacts with the R108 residue when the methyl and trifluoromethyl groups are oriented toward the heme. Therefore, they concluded that the binding mode of the ligand depends on the structure employed (1RO9 and 1OG5). Furthermore, they also evaluated different analogs of celecoxib by docking; however, due to their unknown *in vitro* metabolism, they were not able to establish the correct binding mode of these compounds. Thus, they concluded that docking studies are useful for obtaining information about probable metabolites but not for determining the ligand-binding mode [70]. In a docking study by Hummel *et al.*, the interaction between flurbiprofen and benzbromarone on CYP2C9 was reported. They found that these substrates interact with the same amino-acid residues reported by Ahlstrom *et al.*, reproducing their experimental data [71].

The docking method is not only useful for studying the substrate binding but also that of inhibitors. Dayong *et al.* reported the CYP2C9 inhibition by 6-hydroxyflavone, finding that the competitive inhibitors luteolin, apigenin, baicalein, quercetin, and morin bind close to the heme and occupy the same binding site as flurbiprofen in the 1R9O crystal structure [16]. The CYP2C9-6-hydroxyflavone complex identified by docking simulation indicated that 6-hydroxyflavone is bound by a π-π stacking interaction with the phenyl group of F100 and by two hydrogen bonding interactions with L102 and F100 [72].

During the metabolism with CYP2C9 and other CYPs, some substrates yield toxic metabolites. Although great efforts have been made to explain why CYP2C9 produces these metabolites, the answer remains unclear. However, *in silico* methods are a good tool to predict the production of toxic metabolites. Recently, Rossat *et al.* described an *in silico* protocol to predict the formation of toxic metabolites by CYP2C9, identifying the ligand-binding mode. The method consisted of using pharmacophore prealignment, automated flexible docking, and multidimensional quantitative structure-activity relationship (mQSAR). In this study, they employed 85 compounds (68 training, 17 tested); they found that the correlation between the experiments and their results was 0.678 and concluded that this method is suitable for predicting the binding affinities [73]. This review is also focused on analyzing whether *in silico* methods have been used to predict the toxicity of four of the principal molecules that produce toxicity when they interact with CYP2C9: dapsone, sulfaphenazole, and valproic and tienilic acids (Table 4).

3.3.1. Dapsone

Also called 4,4'diamino-diphenyl sulphone (DDS), dapsone is a compound used to treat *Pneumocystis carinii* pneumonia in acquired-immunodeficiency-syndrome patients. Dapsone displays two types of toxicity, both of which are thought to be due to the N-oxidation mediated by CYP2C9, CYP3A4 and CYP2E1 [74-76]. The hematoxicity of this drug is attributed to the hydroxylamine metabolite (Table 4), which is capable of being co-oxidized with hemoglobin (Hb) in red blood cells to produce nitroso dapsone and metahemoglobin (met-Hb). Although it has been reported in *in vitro* studies that dapsone can produce this toxic metabolite by CYP2C9, its formation mechanism is unknown; thus, computational methods have been used to understand this recognition. Docking studies using AUTODOCK 3.05 [77] were used to examine the potential fit of dapsone in the structure of the flurbiprofen complex (1RO9). The predicted location of the dapsone molecule is under helix F, between flurbiprofen and the C-terminal β sheet (SRS 6) (Fig. 4A). The docking results suggest that the dapsone binding in this position may limit the motion of flurbiprofen in the active site, allowing it to remain in an appropriate position for oxidation. Furthermore, this binding may displace the solvent molecules and shield the perferryl oxo intermediate of hydrogen-ion transfer from the bulk sol-

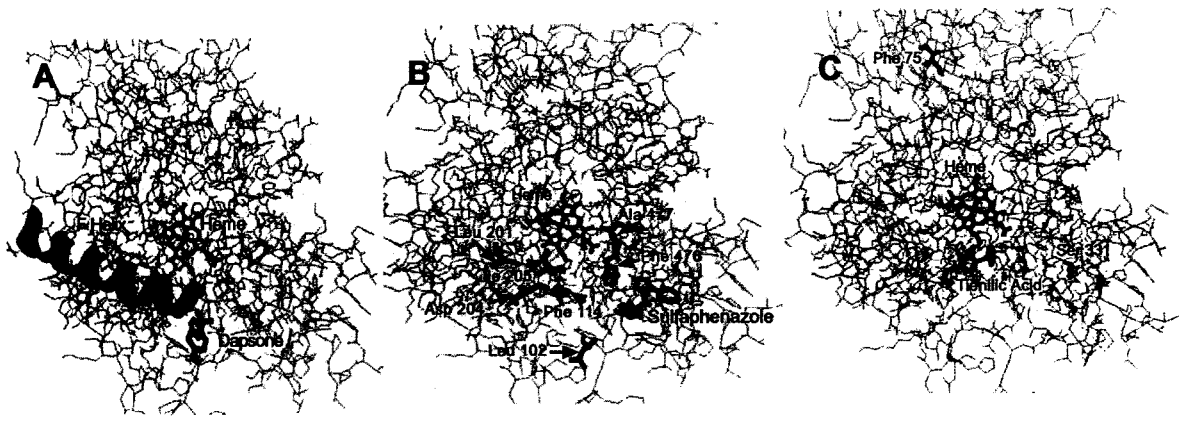
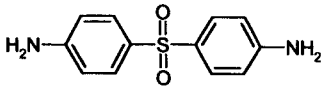
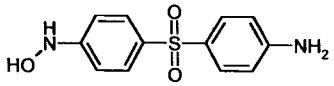
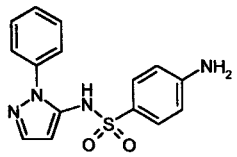
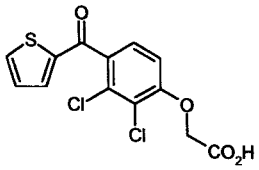
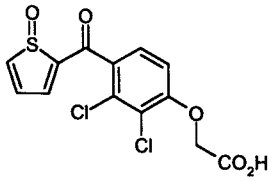
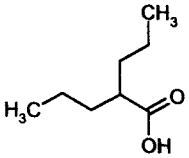
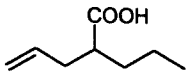


Fig. (4). Docking of dapsone, sulfaphenazole and tienilic acid on CYP2C9 (PDB code: 1RO9).
Interaction of (A) dapsone with the F helix; (B) sulfaphenazole principally with the residues P476 and P114; (C) tienilic acid with the residue S331and the heme group.

Table 4. Principal Molecules that Produce Toxicity when Interact with CYP2C9

Molecule	Reactive Metabolite
Dapsone 	*N*-hydroxylamine, nitroso dapsone 
Sulfaphenazole 	
Tienilic acid 	Tienilic acid isomer S-oxide 
Valproic acid 	4-ene valproic acid 

vent [78]. The relatively large active-site cavity compared with the size of flurbiprofen suggests that there might be sufficient space to accommodate dapsone together with flurbiprofen in the 1R9O structure [79].

Other reports have proposed that dapsone may cause the activation of other compounds by binding in the active site of CYP2C9. This scenario is analogous to that of 7,8-benzoflavone, which activates the phenanthrene CYP3A4-mediated metabolism at the same time that it acts as a CYP3A4 substrate [80]. This effect has been observed during the hydroxylation of flurbiprofen in human liver microsomes and baculovirus-expressed CYP2C9 [81], where the presence of dapsone does not displace this substrate from the CYP2C9 active site [82]. Despite docking studies that have been performed with CYP2C9 and dapsone, there is currently no expla-

nation for why CYP2C9 produces the toxic metabolite in relation to its dapsone binding mode.

3.3.2. Sulfaphenazole (SPZ)

This antibacterial drug belongs to the family of sulfonamides, commonly known as sulfas. The metabolism of this drug has been studied *in vitro* and *in silico*. Some *in vitro* reports have shown the inhibitory effect of sulfaphenazole and some of its derivatives on human liver CYPs. The results demonstrate that this drug fails to inhibit the activities catalyzed by yeast microsomes expressing CYP1A1, 1A2, 3A4 and 2C19. However, sulfaphenazole and its dichlorophenyl derivative act as very strong competitive inhibitors of CYP2C9. The authors have suggested that the effect of both compounds is attributed to the binding of their NH_2 nitrogen atom to CYP2C9 iron, an electrostatic interaction of their SO_2N^- anionic site with a cationic residue of the isoform and an interaction of its *N*-phenyl residue with a hydrophobic part of the protein active site [11].

In addition, the interaction of recombinant CYP2C9 with sulfaphenazole and some substrates (tienilic acid, lauric acid and diclofenac) was evaluated. The studies were carried out using UV-VIS and H^1 nuclear magnetic resonance (NMR) spectroscopy. The paramagnetic relaxation effects of the CYP2C9 heme with the protons of the substrates were determined from the NMR studies of the complex CYP2C9-substrate, with the aim of determining the distances between the links of the substrate and the CYP iron for which the Solomon-Bloembergen equation was used. Subsequently, the relative position of the substrates with the CYP2C9 iron was determined based on the data obtained from the NMR and molecular-modeling methods. Most had similar energies (5 kcal/mol) with the exception of the tienilic acid (8 kcal/mol) [83].

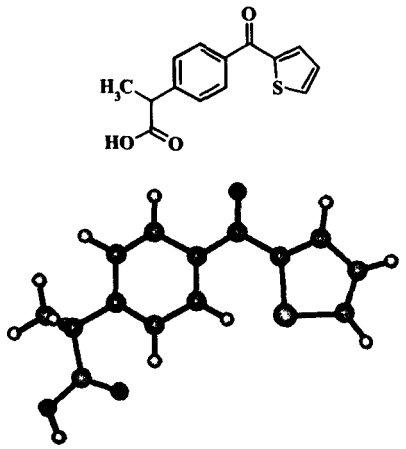
The binding of sulfaphenazole has also been described by Afzelius [46] where F114 (SRS-1) forms π-π stacking interaction with sulfaphenazole and with L366 (SRS-5) and F476 (SRS-6). L201 and I205 (SRS-2) in the F-helix, constitutes another hydrophobic pocket for interaction with sulfaphenazole (Fig. **4B**). The backbone of I205 in close proximity probably forms a hydrogen bond to the aniline nitrogen of sulfaphenazole. The amino acid A297 in region SRS-4 forms a third hydrophobic pocket with V113 (SRS-1) and L366. A direct hydrophobic interaction is suggested from L362 (SRS-5). The A477 in the SRS-6 region (β2) also shows very strong hydrophobic interactions. L102 builds a fourth hydrophobic pocket together with V292 in helix I. The polar grid interacts with D293 in SRS-4 and, for the more potent inhibitors, with D204 in the F helix (SRS-2).

3.3.3. Tienilic Acid

(2,3Dichloro-4-(2-thienylcarbonyl)phenoxyacetic acid) is an antihypertensive drug that is believe to be activated in the active site of CYP2C9 producing a thiophene S-oxide. However, the formation of this metabolite has never been demonstrated but only proposed. What has been demonstrated is the formation and trapping of tienilic acid isomer S-oxide [84]. This metabolite covalently binds to CYP2C9, producing the suicide inactivation of this enzyme [85-87]. In addition, after the binding of the sulfoxide to CYP2C9, an autoimmune hepatitis is induced [88, 89]. This effect of tienilic acid was discovery thanks to several *in vitro* assays to analyze its toxicity were done. For instance, Nishiya *et al.* studied the CYP-mediated metabolism of tienilic acid to determine whether some of its metabolites produce hepatoxicity *in vivo*. A GS-tienilic-acid adduct was identified in the bile of rats given an oral dose of tienilic acid co-treated with the GSH biosynthesis inhibitor L-buthionine-(S,R)-sulfoximine (BSO) and the CYP inhibitor 1-aminobenzotriazole (ABT). The results indicate that the decrease in GSH levels and upregulation of electrophilic/oxidative stress-related heme oxygenase-1 (Ho-1), NADPH dehydrogenase quinine 1 (Nqo1) and glutamate-cysteine ligase modifier subunit (Gclm) genes produced by the treatment with tienilic acid are completely prevented in rat livers co-treated with ABT. Furthermore, the elevation of serum alanine aminotransferase (ALT) and severe hepatocyte necrosis by a combination of tienilic acid and the inhibitor BSO were also prevented by the CYP2C9 inhibitor. Therefore, the authors suggested that the oxidative stress is produced on the hepatocytes of rats treated with tienilic acid and leads to tienilic-induced hepatotoxicity under impaired glutathione (GSH) biosynthesis [90-92].

Due to these disadvantages, tienilic acid was withdrawn from the market [93]. In this sense, suprofen, a derivate of tienilic acid, produces similar toxicity because this drug also has a thiophene group (Fig. **5**) where CYP2C9 catalyzes the formation of thiophene-4,5-epoxide [94].

Suprofen



Tienilic Acid

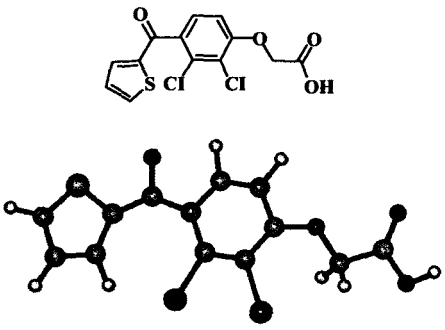


Fig. (5). Chemical structures of suprofen (A) and tienilic acid (B), that are molecules with a thiophene group.

Therefore, taking into account the similarity in the chemical structure, it is possible that all molecules with a thiophene group metabolize in the same way any substrates of CYP2C9 reported in Table **3** that have a thiophene group except tienilic acid and suprofen. Several efforts were made to describe the mechanism of CYP2C9 inactivation by tienilic acid by *in silico* methods [95]. For instance, Mancy *et al.* reported a predictive model using biochemical studies and molecular modeling showing that CYP2C9 substrates interact through their anionic site with a cationic residue of the protein [57]. However, specific details about the interaction between CYP2C9 and tienilic acid were only elucidated by Lewis;

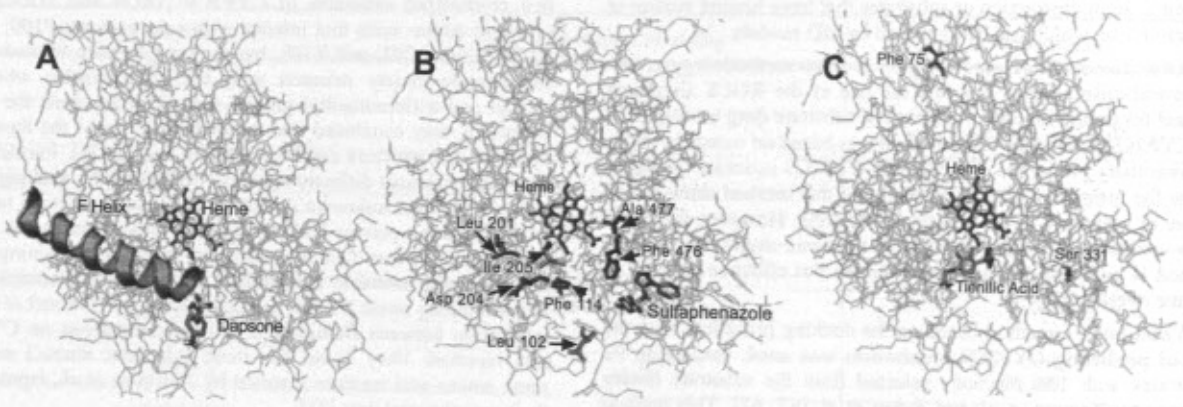
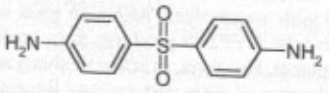
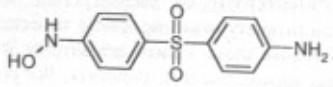
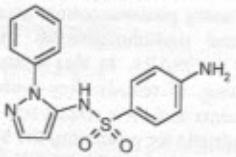
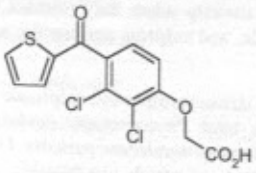
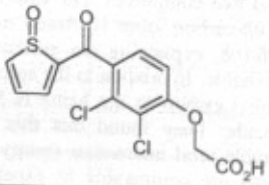
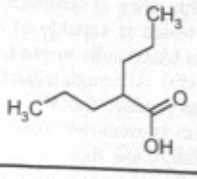
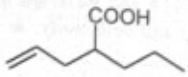


Fig. (4). Docking of dapsone, sulfaphenazole and tienilic acid on CYP2C9 (PDB code: 1RO9).
Interaction of (**A**) dapsone with the F helix; (**B**) sulfaphenazole principally with the residues P476 and P114; (**C**) tienilic acid with the residue S331and the heme group.

Table 4. Principal Molecules that Produce Toxicity when Interact with CYP2C9

Molecule	Reactive Metabolite
Dapsone 	*N*-hydroxylamine, nitroso dapsone 
Sulfaphenazole 	
Tienilic acid 	Tienilic acid isomer S-oxide 
Valproic acid 	4-ene valproic acid 

vent [78]. The relatively large active-site cavity compared with the size of flurbiprofen suggests that there might be sufficient space to accommodate dapsone together with flurbiprofen in the 1R9O structure [79].

Other reports have proposed that dapsone may cause the activation of other compounds by binding in the active site of CYP2C9. This scenario is analogous to that of 7,8-benzoflavone, which activates the phenanthrene CYP3A4-mediated metabolism at the same time that it acts as a CYP3A4 substrate [80]. This effect has been observed during the hydroxylation of flurbiprofen in human liver microsomes and baculovirus-expressed CYP2C9 [81], where the presence of dapsone does not displace this substrate from the CYP2C9 active site [82]. Despite docking studies that have been performed with CYP2C9 and dapsone, there is currently no expla-

however, he employed a CYP2C9 structure obtained by homology modeling as a CYP102 template [96]. In this work, it was identified that the carboxylic group of tienilic acid form a hydrogen bond with S331 and π-stack with F75 (Fig. 4C). In this sense, additional studies using one of the CYP2C9 crystallized structures were not performed; thus, it is not possible to know whether the recognition between CYP2C9 and tienilic acid is different. Therefore, the prediction of new drugs that do not have the same toxicity but do have the same beneficial effects of tienilic acid is not possible.

3.3.4. Valproic Acid (VPA)

2-Propylpentanoic acid, or dipropylacetic acid, is an antiepileptic drug with clinical use as an anticonvulsant, primarily in the treatment of epilepsy and bipolar disorder. Current interest in VPA is based on its inhibition of histone deacetylation [97] and its potential therapeutic application in cancer [98] and human-immunodeficiency-virus infection [99]. Clinical use of VPA is associated with a rare but potentially fatal hepatotoxicity [100]. Based on retrospective studies [101], this idiosyncratic toxicity has been linked to several risk factors, including the concurrent administration of a CYP-inducing drug (e.g., phenobarbital), especially in patients younger than 2 years of age. The mechanism of VPA-induced hepatotoxicity is not known, but it has been postulated that 4-ene-VPA, which is an oxidative metabolite of VPA, may be biotransformed to an electrophilic diene that inhibits mitochondrial β-oxidation enzymes and depletes cellular GSH [102]. In addition, it was concluded in one study that CYP2C9 plays a role in the formation of 4-ene-VPA [103] and catalyzes the VPA 4-hydroxylation and VPA 5-hydroxylation [104]. As shown in Table 4, VPA is quite different in chemical structure from the other CYP2C9 substrates that produce toxic metabolites. However, its binding to CYP2C9 has not been completely explained despite its use in *in silico* studies [67].

In conclusion, all of the contributions described previously demonstrate that *in silico* methods are a good tool for describing the binding of CYP2C9 substrates because the amino-acid residues identified by crystallization during the binding of flurbiprofen and warfarin are the same as those identified by docking studies with different substrates. In addition, MD simulations allow the identification of conformational changes, which is of great importance because they occur during the interaction between the protein and its ligand *in vivo*. Furthermore, it is possible to identify important regions in the protein, such as channels and binding sites. However, the *in silico* methods have not been widely used to describe the toxicity of different substrates and inhibitors, as shown with dapsone, sulfaphenazole, tienilic and valproic acids. Therefore, there are many computational techniques that could be used to describe to determine whether there are similar characteristics during the binding of substrates that yield toxic metabolites.

LIST OF NON-STANDARD ABBREVIATIONS

CYP-cytochrome P-450	=	CYP2A6, 2B4, 2C3, 2C5, 2C8, 2C9, 2C19, 2E1, 3A4-cytochrome P450 2A6, 2B4, 2C3, 2C5, 2C8, 2C9, 2C19, 2E1, 3A4
MD	=	Molecular dynamics
mQSAR	=	Multidimensional quantitative structure-activity relationship
NMR	=	Nuclear magnetic resonance
PDB	=	Protein Data Bank
RR	=	Raman resonance
RMSD	=	Root mean square deviation
SPZ	=	Sulfaphenazole
3D	=	Third-dimensional
UV-VIS	=	Ultraviolet-visible
VMD	=	Visual Molecular Dynamics
VPA	=	Valproic acid

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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