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ORIGINAL PAPER

Roles of key residues specific to cyclooxygenase II: an ONIOM study

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Abstract Binding energy calculations of flurbiprofen to the binding pocket of the cyclooxygenase (COX) enzyme were performed based on quantum chemical calculations. The interaction energies between flurbiprofen and two types of COX binding sites were studied. Quantum chemical calculations were used, based on the B3LYP hybrid functional and the MP2 method, with 6-31G(d) and 6-31G(d,p) basis sets. The results show that the main interaction between flurbiprofen and two COX isozymes (COX-1 and COX-2) is due to Arg120. In addition, selective SC558 COX-2 inhibitor was also compared. It was found that repulsive interaction plays a significant role in its inhibition of COX-2. ONIOM2(B3LYP/6-31G(d):PM3) calculations indicate that flurbiprofen interacts via moderate hydrogen bonding with Arg120 in the COX-2 binding site, while no hydrogen bond was detected with either Tyr355 or Val523. The ONIOM2 method can be used to describe the specific interaction of the inhibitor and is helpful in designing a specific COX inhibitor.

Keywords Cyclooxygenase · Flurbiprofen · Binding energy · Quantum chemical calculations · ONIOM

Introduction

Cyclooxygenase (COX) is a bifunctional enzyme catalyzing conversion of arachidonic acid into prostaglandins. This enzyme has been classified into two different isozymes, COX-1 and COX-2. COX-1 is a constituent of most tissues and creates prostaglandins used for basic housekeeping throughout the body, while COX-2 is more selective, and is induced by inflammation and pain [1–3]. Various side effects—such as ulcers, antiplatelet activity, gastrointestinal irritation, and suppression of renal function—can arise from the inhibition of both isozymes by non-steroidal anti-inflammatory drugs (NSAIDs) [4–6]. Traditional NSAIDs, such as aspirin, ibuprofen, indomethacin, and flurbiprofen [7–9], can inhibit prostaglandin synthesis and display anti-inflammatory action. However, these compounds show no selectivity and cause side effects by the inhibition of COX-1, whereas their anti-inflammatory activity results from the inhibition of the inducible COX-2. Therefore, specific COX-2 inhibitors—such as SC558, celecoxib (Celebrex), rofecoxib (Vioxx), valdecoxib, etoricoxib, and lumiracoxib [10–13]—provide a new class of anti-inflammatory drugs with significantly reduced side effects, although they pose an increased risk of cardiovascular events [14, 15].

COX-1 and COX-2 are bifunctional homodimer enzymes, in which each subunit consists of both cyclooxygenase and peroxidase active sites. Firstly, cyclooxygenase catalyzes conversion of arachidonic acid into prostaglandin G2. Then, the peroxidase enzyme converts prostaglandin G2 into prostaglandin H2. The entrance of the COX active site is a long hydrophobic channel consisting of three main residues: Arg120, Tyr355, and Glu524. The active sites of both COX enzymes are very similar. Significantly different residues are found only at position 513 (His in COX-1 vs. Arg

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in COX-2) and position 523 (Ile in COX-1 vs. Val in COX-2). Deletion of a methylene group at position 523 in COX-2 allows access to an additional pocket by the side chains of selective COX-2 inhibitors. Therefore, the structural class of selective COX-2 inhibitors mostly consists of diaryl heterocyclic compounds, with methylsulfonyl (SO_2Me) or sulfonamide (SO_2NH_2) moieties substituted at the para-position of the benzene ring to fit with Arg at position 513, thus retaining COX-2 inhibitory selectivity.

Flurbiprofen (Fig. 1a), or (+/−)-2-(2-fluoro-4-biphenyl)propionic acid, is a chiral 2-arylpropionic acid. *(S)*-Flurbiprofen exhibits non-selective competitive COX inhibition. This compound produces side effects due to its inhibition of both COX isozymes. It inhibits human recombinant COX-1 and COX-2 with IC_{50} values of 0.04 and 0.51 nM [9]. The structure of flurbiprofen is both hydrophilic and hydrophobic at the carboxylate group and biphenyl moiety. Another class of inhibitor is SC558 (Fig. 1b), or 4-[5-(4-bromophenyl)-3-trifluoromethyl-1*H*-1-pyrazolylbenzenesulfonamide. The structure of this inhibitor is composed of three important parts: a central five-membered ring, two aryl heterocyclic rings containing substituted bromine, and a substituted sulfonamide in the benzene ring. The sulfonamide side chain of SC558 is the most important moiety, specific to the COX-2 binding site.

In the present work, various theoretical methods were used to investigate COX/inhibitor interaction. Quantum chemical calculations and combined quantum mechanics, a method known as ONIOM (our own N-layered integrated molecular orbital and molecular mechanics) [16–18], were applied to flurbiprofen in order to examine the particular interaction of the inhibitor with the COX-2 binding site. Use of the ONIOM method has recently been applied to large chemical and biological systems. This method is mostly applied to large biomolecules, organometallic complexes, and reaction mechanisms [19–25].

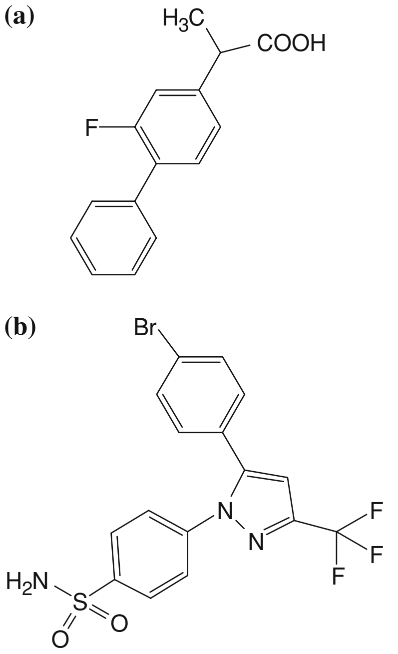

Fig. 1 Molecular structures of flurbiprofen (**a**) and SC558 (**b**)

Calculations were based on the particular interaction of the flurbiprofen complex with COX-1 and COX-2. In addition, neutral and ionic forms of flurbiprofen, and some residues at the binding site, were also considered. Moreover, the interaction of SC558 with the COX-2 binding site was studied for comparison with flurbiprofen. The results obtained give greater insight into the specific and non-specific interactions in the binding pocket of cyclooxygenase. This structural information can be a guide for the design and development of a specific COX-2 inhibitor.

Results and discussion

Quantum chemical calculations of inhibitor and enzyme interaction–particular interaction of flurbiprofen with the COX-2 binding site

The interaction energies between flurbiprofen and each residue in the binding pockets of COX-1 and COX-2 were calculated by B3LYP/6-31G(d), B3LYP/6-31G(d,p), and MP2/6-31G(d) methods, as shown in Table 1. The results show that B3LYP/6-31G(d) and B3LYP/6-31G(d,p) calculations do not give significantly different interaction energies (less than 0.50 kJ/mol). This indicates that polarization of the hydrogen atom does not affect the system. The results obtained from the B3LYP and MP2 methods with a 6-31G(d) basis set clearly demonstrate that the key residues in the COX-2 binding site are Arg120 and Tyr355, which show important attractive (−16.62 kJ/mol) and repulsive (14.44 kJ/mol) interactions. According to the results, the MP2 method can exhibit attractive interaction for several residues, in contrast to the B3LYP method [23]. Therefore, in order to achieve a deeper understanding of COX/inhibitor interaction, the MP2 method was used in the next investigation.

The results obtained from MP2/6-31G(d) calculations indicate that attractive interaction plays a significant role in flurbiprofen binding. It is important to note that the main contribution is due to the interaction with Arg120, as indicated by an interaction energy of −16.62 kJ/mol. However, there are some repulsive interactions, especially with Tyr355, Val116, and Leu359 (14.44, 10.47, and 9.50 kJ/mol). Graphical representation of the attractive and repulsive interactions of flurbiprofen with the COX-2 binding site is shown in Fig. 2.

Table 1 Interaction energies (*INT*) of flurbiprofen with individual amino acids (X_i) of the neutral system of binding site of COX isozymes (in kJ/mol), calculated at the B3LYP/6-31G(d), B3LYP/6-31G(d,p), and MP2/6-31G(d) levels of theory

	Interaction energies (kJ/mol)			
	COX-2			COX-1
	B3YLP		MP2	MP2
	6-31G(d)	6-31G(d,p)	6-31G(d)	6-31G(d)
Val116	13.10	12.77	10.47	7.74
Arg120	−11.30	−10.80	−16.62	−50.40
Val349	15.70	15.57	−0.38	−3.60
Leu352	2.22	2.22	−5.57	−5.15
Ser353	1.09	1.13	−4.02	−3.52
Tyr355	22.19	22.19	14.44	7.95
Leu359	12.85	12.35	9.50	−0.71
Tyr385	14.99	14.57	7.41	−8.12
Met522	−1.47	−1.34	−5.19	−6.24
Val/Ile523*	2.80	2.97	−6.03	−6.57
Gly526	0.75	0.75	−4.56	−7.16
Ala527	10.93	10.80	−8.20	−8.29
Ser530	1.34	1.42	−6.78	−0.04
Leu531	5.02	4.81	−5.78	−5.65

* Val523 in COX-2 and Ile523 in COX-1

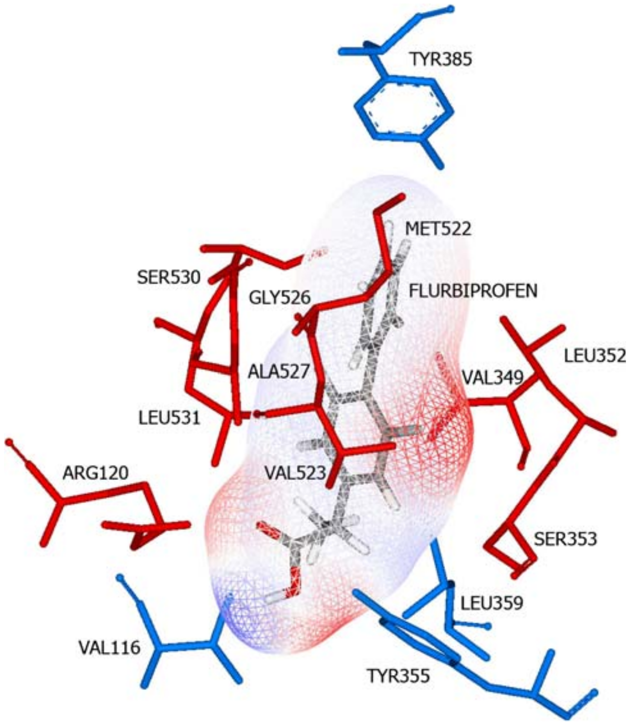

Fig. 2 Attractive (*red*) and repulsive (*blue*) interactions of flurbiprofen with individual residues in the binding site, calculated at the MP2/6-31G(d) level of theory

Comparison of interactions between flurbiprofen and COX-1 and COX-2

It is interesting to note that flurbiprofen can exhibit different interactions with the other isoform, COX-1. Therefore, similar calculations were performed; the results obtained from the MP2/6-31G(d) method are shown in Table 1 in which all residues were preformed in a neutral form. There is a large difference in interaction energies between flurbiprofen and the COX-1 and COX-2 binding sites: about 34 kJ/mol. The results confirm that Arg120 is the key residue in both COX active sites. Flurbiprofen exhibits stronger interaction with Arg120 in COX-1 than in COX-2 because of two hydrogen bonds between the carboxylic moiety of flurbiprofen and the guanidine side chain of Arg120 (1.80 and 1.86 Å). In the case of Tyr385, there are opposite results in COX-1 and COX-2 binding. In COX-1, flurbiprofen demonstrates energetically favorable attractive interaction with this residue (−8.12 kJ/mol), whereas in COX-2, the inhibitor shows repulsive interaction (7.41 kJ/mol). Considering Tyr355 residue, the interaction with flurbiprofen in the COX-2 enzyme provides a higher repulsion (about 7 kJ/mol) than in COX-1. Another residue in both isozymes that shows different interaction energies is Ser530. Interaction between flurbiprofen and Ser530 in the COX-2 binding site gives higher attractive energy (about 6.7 kJ/mol) than that of the complex in COX-1. Van der Waals surfaces of these three residues in both enzymes binding with flurbiprofen are shown in Fig. 3. These results indicate that there is a different orientation of the hydroxyl group in the tyrosyl moiety of the inhibitor, which causes the different binding energies.

When considering the main amino acids at position 523 in the binding sites of COX-1 and COX-2, which are Ile and Val, similar interactions were found (−6.57 and −6.03 kJ/mol). This means that upon binding, flurbiprofen is specific to COX-1, and the main contribution comes from the interaction between the inhibitor and Arg120.

Analysis of different interactions of flurbiprofen and SC558 in binding with COX-2

The structures of flurbiprofen and SC558 are roughly equivalent in relation to the COX-2 binding site, based on the fact that the fluorophenyl ring of flurbiprofen overlaps with the bromophenyl ring of SC558. The sulfonamide moiety of SC558 attached to the pyrazole ring causes its selective inhibition of COX-2. For a comparison of the interactions at the same binding site, 23 amino acids in the binding site are shown in Table 2 in which basic and acidic chains were ionized at pH 7.0 based on their pK_a values. The calculated interaction energies between each inhibitor

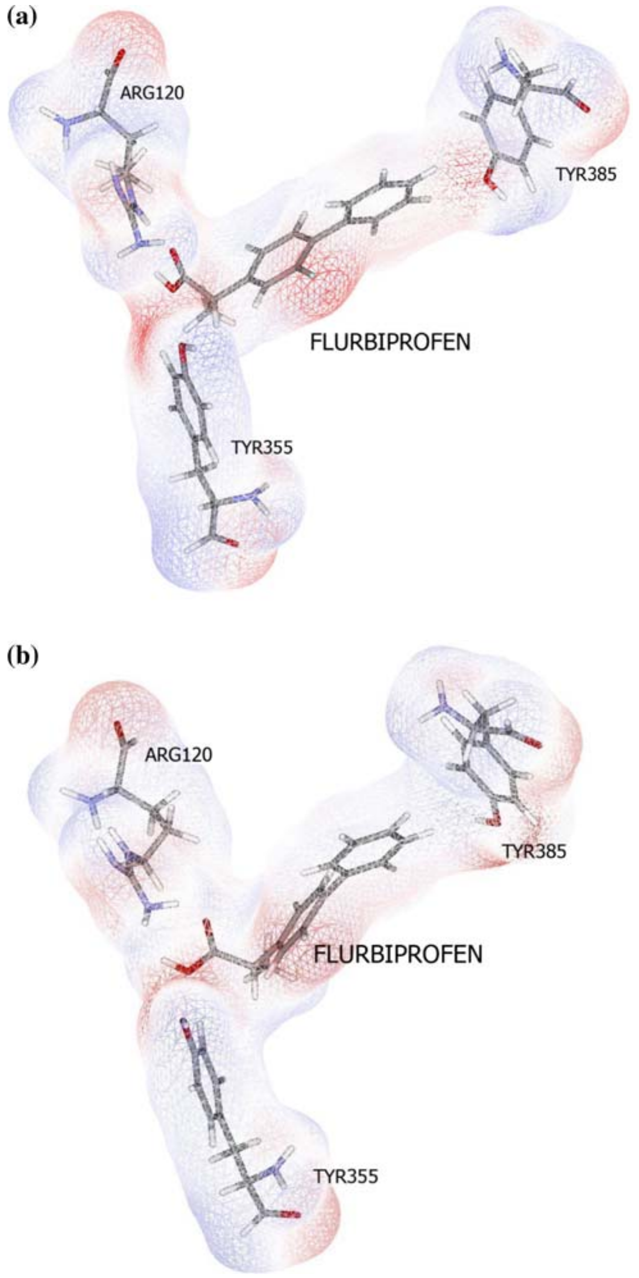

Fig. 3 Van der Waals surface of flurbiprofen with Arg120, Tyr355, and Tyr385 in COX-1 (**a**) and in COX-2 (**b**)

Table 2 Interaction energies (*INT*) of flurbiprofen and SC558 with individual amino acids (X_i) (in kJ/mol) of the ionic system of the COX-2 binding pocket, calculated at the MP2/6-31G(d) level of theory

	Interaction energies (kJ/mol)	
	Flurbiprofen	SC558
His90	0.17	13.81
Val116	0.88	−2.26
Arg120	−39.18	−16.20
Gln192	−0.21	8.29
Val349	−0.59	−3.47
Leu352	−5.57	6.45
Ser353	−4.02	−9.08
Gly354	−0.42	−2.39
Tyr355	12.89	−15.20
Leu359	13.56	−2.13
Tyr385	7.12	11.13
Trp387	−3.18	−0.38
Arg513	−2.43	89.79
Ala516	−0.13	−5.90
Ile517	−0.29	−7.53
Phe518	−2.34	18.71
Gly519	0.13	−3.39
Met522	−5.11	−7.49
Val523	−5.90	6.57
Gly526	−3.47	−3.43
Ala527	−8.25	−2.80
Ser530	−6.57	−3.60
Leu531	−5.90	−2.01
Total	−58.81	67.48

and individual residues are shown in this table. It is interesting to note that the general interaction within the binding site of flurbiprofen demonstrates attractive interaction with COX-2, whereas SC558 exhibits repulsive interaction. The results indicate that Arg120, Tyr355, Leu359, Arg513, Phe518, and Val523 residues show widely different interactions with flurbiprofen and SC558. There is a greater attractive interaction between flurbiprofen and Arg120 (−39.18 kJ/mol), while Tyr355 interacts more favorably with SC558 (−15.20 kJ/mol) than flurbiprofen (12.89 kJ/mol). The Val523 residue, which is the key residue for discrimination of both isozymes, shows selectivity with the sulfonyl phenyl ring of SC558. These results clearly indicate that SC558 interacts with COX-2 better than flurbiprofen does, based on repulsive interaction—particularly in the case of Arg513.

ONIOM calculations

The main focus of our study is to investigate the specific interaction of flurbiprofen with key amino acids in the binding sites: Arg120, Tyr355, and Val 523. The binding energies obtained from ONIOM2 calculations are given in Table 3. The results show that the binding energies of flurbiprofen/Arg120 are higher than for flurbiprofen/Val523. This suggests that the hydrogen bonding between the carboxylic group of flurbiprofen and the guanidine group of Arg120 is stronger. Hydrogen bond distances are in the range of 1.99–2.28 Å (obtained by models A–D), whereas no hydrogen bonding is observed between Tyr355 and flurbiprofen.

Table 3 ONIOM2 binding energies with BSSE-CP correction of flurbiprofen bound into the COX-2 binding pocket, and key residues were set in the inner layer

ONIOM2 method	Binding energy (kJ/mol)			Hydrogen bonding distance (Å)	
	Arg120	Tyr355	Val523	N_{Arg120}–H-O_{FLP}	O_{Tyr355}–H-O_{FLP}
Model A	−39.73	−45.00	−39.06	2.24	4.17
Model B	−41.15	−36.04	−39.22	2.28	4.18
Model C	−54.29	−48.06	−43.45	1.97	4.10
Model D	−52.45	−57.06	−43.12	1.99	4.09

Hydrogen bonding distances are also indicated

Concerning the interaction between flurbiprofen and Tyr355, the bond distance between O_{TYR355}-H-O_{FLP} from model D is about 1.4 Å longer than that observed in the X-ray crystallographic structure (3.09 Å) (3PGH.pdb). The calculated results indicate no hydrogen bond formation between the thyroxyl side chain of Tyr355 and flurbiprofen (see Fig. 4). It is also known that a significant difference between COX-1 and COX-2 is due to the difference at position 523: Ile in COX-1 and Val in COX-2. Deletion of a methylene group at 523 in COX-2 allows access to an additional pocket. Van der Waals interaction between Val523 and flurbiprofen implies that the interaction might not be significant in binding with COX-2. Therefore, the selective COX-2 inhibitor exploits the additional pocket for enhanced binding through the sulfonamide or methylsulfone moiety; this clearly discriminates selective from non-selective COX inhibitors.

Interaction of key residues with flurbiprofen

In order to better understand the particular interaction of the key residues in the binding site with flurbiprofen, a comparison of their interactions was performed. In this case, two different states, neutral and ionic charge environments, were also considered. The ONIOM2(B3LYP/6-31G(d):PM3) method was used, and calculated results are shown in Table 4.

Effects of neutral and ionic charges on binding energy

Flurbiprofen and each key residue were included at a high level of the ONIOM2 model, while the other residues were set at a low level, for both neutral and ionic systems. It was clearly seen that in the neutral system, the binding energies of the three models are not significantly different. However, in ionic systems, binding energies between flurbiprofen and each key residue are in the following order: flurbiprofen—Arg120 $\gg$ Val523 > Tyr355. This means that ionic charges in the system play an important role in inhibitor-enzyme interaction, particularly the interaction between flurbiprofen and Arg120. However, too high-binding energies of the

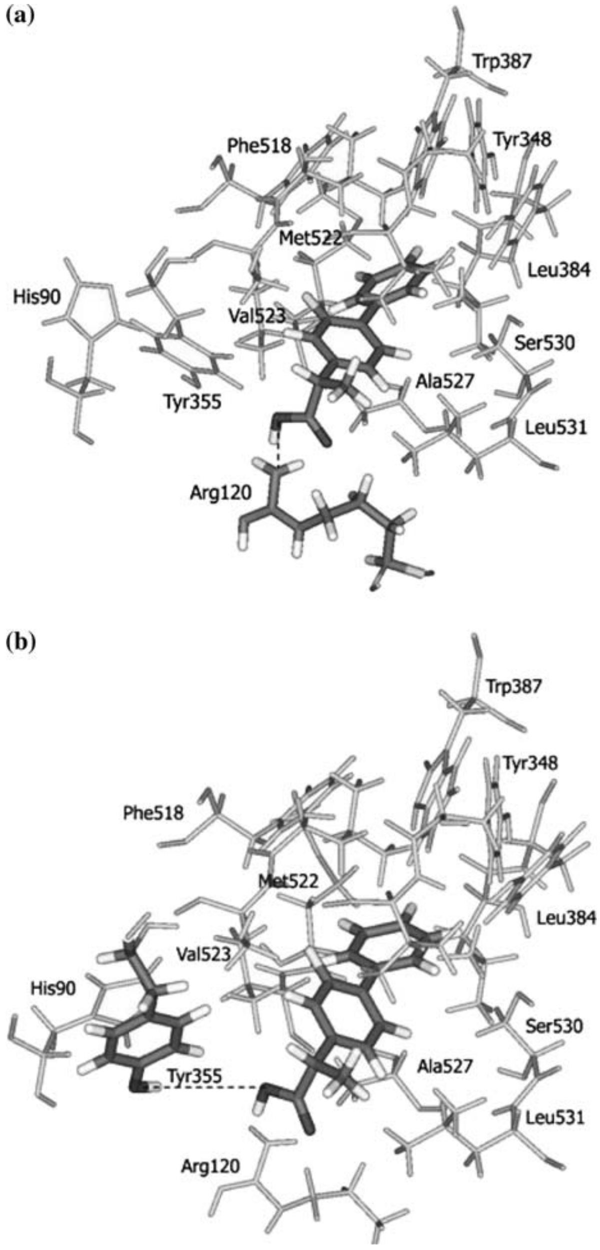

Fig. 4 Hydrogen bonding between flurbiprofen/Arg120 (1.99 Å) (**a**) and flurbiprofen/Tyr355 (4.10 Å) (**b**), obtained from ONIOM2 (model D) calculations

Table 4 Binding energy of flurbiprofen bound into the COX-2 binding pocket, calculated by ONIOM2 (B3LYP/6-31G(d):PM3) method

Inner layer Outer layer	Binding energies (kJ/mol) Flurbiprofen/ Arg120 Rest residues	 Flurbiprofen/ Tyr355 Rest residues	 Flurbiprofen/ Val523 Rest residues
Neutral	−54.29	−48.06	−43.45
Ionic	−429.40	−145.34	−176.44

Flurbiprofen and each key residue (Arg120, Tyr355, and Val523) are set in different states (neutral and ionic) (in kJ/mol)

ionic system calculated from ONIOM calculations might come from electrostatic interaction between the carboxylate group of flurbiprofen and ionized residues such as Arg120. This point will be further investigated.

Effects of neutral and ionic charges on hydrogen bonding

Considering the hydrogen bonding interaction in the flurbiprofen-Arg120 ONIOM model, ionic charges produce a stronger hydrogen bond, with a distance of 1.68 (2.65) Å as compared to 1.97 (2.90) Å in the neutral system (values in parentheses are the intermolecular distance between the atom of the hydrogen bond donor and the atom of the hydrogen bond acceptor). X-ray crystallographic data provide a hydrogen bond distance of about 2.96 Å. Superimposition of the neutral and ionic charge structures, as well as the X-ray structure, are shown in Fig. 5.

In the flurbiprofen-Tyr355 model, the ionic system shows hydrogen bonding between carboxylate of the inhibitor and the hydroxyl group of the tyrosine residue of about 1.77 (2.76) Å, while the neutral system does not show hydrogen bonding. In contrast, in the flurbiprofen-Val523 model no hydrogen bonding is detected in either system. This confirms that most of the interaction energy of flurbiprofen/COX-2 binding comes from the main interaction with Arg120 (a result found in both inner and outer layers of model calculations).

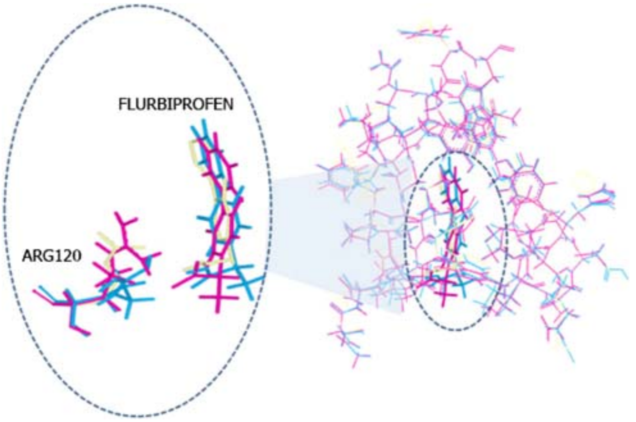

Fig. 5 Superimposed structures of neutral (*blue*) and ionic (*red*) systems obtained from ONIOM2 calculations, compared with X-ray structure (*yellow*). Arg120 and flurbiprofen are in inner layer, and the other residues are in outer layer

Conclusions

The individual interaction energy of flurbiprofen with the COX-2 binding pocket was investigated. The results show that MP2/6-31G(d) is an appropriate method for studying enzyme-inhibitor interaction. A comparison of the interactions of flurbiprofen and SC558 in the COX-2 binding site clearly indicates that Arg120 strongly interacts with both inhibitors. However, in the case of SC558, the repulsive interaction with Arg513 in the COX-2 binding site plays a significant role in specific inhibition. ONIOM2 calculation was a useful method to investigate the interactions between flurbiprofen and key amino acids in the binding pocket. The results obtained indicate that flurbiprofen can produce moderate hydrogen bonding with Arg120, while no hydrogen bond is detected with Tyr355 or Val523.

Methods

Systems studied

Molecular structures of flurbiprofen bound with COX-1 and COX-2 were obtained from the 3.10 and 2.50 Å resolved crystal structures 1CQE.pdb and 3PGH.pdb [26, 27]. Based on these structures, only chain A was selected. The systems studied were defined for both COX enzymes consisting of 23 residues surrounding the flurbiprofen inhibitor in COXs' binding pockets with at least one atom interacting with any atom of flurbiprofen within the interatomic distance of 6 Å. In order to compare different enzyme/inhibitor interactions, SC558 was selected; the bound structure was obtained from the 3.0 Å resolved crystal structure (1CX2.pdb). Residues surrounding the SC558 within 6 Å were selected using the same criteria as in the before-mentioned system. Therefore, the residues consisting in 6 Å of both COXs/flurbiprofen and COX-2/SC558 are His90, Val116, Arg120, Val349, Leu352, Ser353, Gly354, Tyr355, Leu359, Tyr385, His/Arg513, Ser/Ala516, Ile517, Phe518, Gly519, Met522, Ile/Val523, Glu524, Gly526, Ala527, Ser530, and Leu531. Terminated cutting residues, the N- and C-terminal ends of cut residues were capped with an acetyl group ($CH_3CO–$) and a methyl amino group ($–NHCH_3$) from the adjacent residues in all systems. Hydrogen atoms were added to the

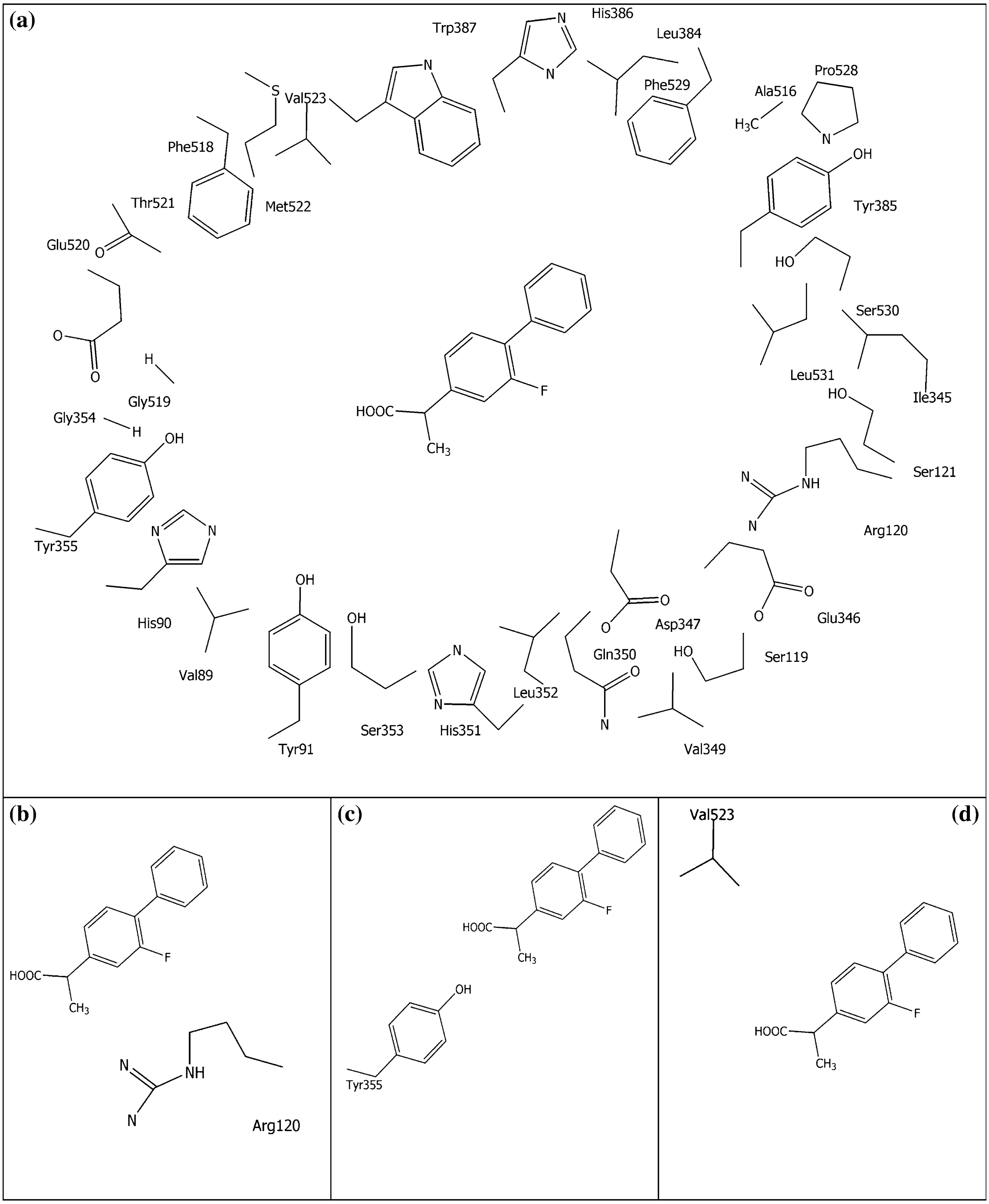

Fig. 6 Schematic representation of the flurbiprofen bound to the COX-2 binding site, consisting of 32 residues (**a**). For ONIOM2 calculations, each key residue (Arg120, Tyr355, and Val523) was added in the inner layer (**b**–**d**)

X-ray structure to generate a complete structure of the model using the SYBYL 7.0 program [28]. The structure was optimized with the semiempirical PM3 method [29], in which all the heavy atoms of the amino acids in the pocket were fixed in the X-ray structure. Therefore, only the geometry and position of the inhibitor molecules, as well as the positions of all the hydrogen atoms, were optimized. These structures were used to generate the starting geometries for all calculations.

Inhibitor-individual residue interaction model setup

Interaction energies between the inhibitor (flurbiprofen or SC558) with the individual residue (defined as X_i) were calculated using the optimized complex structures. For flurbiprofen, there are residues within 6 Å of the binding pocket consisting of 14 amino acids: Val116, Arg120, Val349, Leu352, Ser353, Tyr355, Leu359, Tyr385, Met522, Val523, Gly526, Ala527, Ser530, and Leu531. Seven amino acids within 6 Å of the binding pocket of the SC558 inhibitor were added due to the sulfonamide moiety. These residues are His90, Gln192, Arg513, Ala516, Ile517, Phe518, and Gly519. The interaction energy of each inhibitor-residue pair was calculated by three methods: B3LYP/6-31G(d), B3LYP/6-31G(d,p), and MP2/6-31G(d). The definition of the interaction energy (*INT*) is shown in Eq. 1:

$$INT_{(\text{Inhibitor}-X_i)} = E_{(\text{Inhibitor}-X_i)} - E_{(\text{Inhibitor})} - E_{(X_i)} \tag{1}$$

Calculations by the B3LYP/6-31G(d), B3LYP/6-31G(d,p), and MP2/6-31G(d) methods were used to search for the most appropriate method for this system. Then, interactions between the two COX isozymes and flurbiprofen were analyzed. In addition, interaction energies between the two inhibitors (flurbiprofen and SC558) and individual residues were compared. All calculations were performed using the GAUSSIAN 03 program package [30], implemented on a Linux PC-based multiprocessor (3.4 GHz).

Analysis of COX-2/flurbiprofen interaction using the ONIOM2 method

The ONIOM2 method was used to investigate the interaction between flurbiprofen and the residues in the COX-2 binding site (Fig. 6). In this study, we focused on the interactions between flurbiprofen and three important residues: Arg120, Tyr355, and Val523. Arg120 is a key amino acid of the active site of the substrate, in which the guanidinium group functions to stabilize the carboxylate of the inhibitor; while Tyr355 is a key amino acid for the flurbiprofen binding site. Both residues are located near the hydrophobic channel-binding region of the protein. Moreover, Val523 is considered to be the amino acid that differentiates the COX-1 and COX-2 isozymes. Therefore, these three residues were particularly investigated.

The ONIOM2 systems were set up by separation of the model into two layers. The inner layer, or the "interaction region," was composed of flurbiprofen and the residues Arg120, Tyr355, and Val523, using a high level of calculation, while the outer layer (the "environmental region") consisting of the remaining residues was treated at a lower level of calculation. Therefore, different ONIOM2 methods—ab initio and density functional theory combined with PM3—were applied to calculate the structural information and estimate interaction energies between flurbiprofen and the COX-2 binding site. The calculation setup also included partitioning into a model layer and a real layer. Combinations of different levels of theory—HF/6-31G(d), HF/6-31G(d,p), B3LYP/6-31G(d), and B3LYP/6-31G(d,p)—with PM3 for ONIOM2 setup were performed, resulting in Models A, B, C, and D, as follows:

Model A : ONIOM2(HF/6-31G(d):PM3)
Model B : ONIOM2(HF/6-31G(d,p):PM3)
Model C : ONIOM2(B3LYP/6-31G(d):PM3)
Model D : ONIOM2(B3LYP/6-31G(d,p):PM3)

In addition, the ionic system was also considered and calculated by the ONIOM2(B3LYP/6-31G(d):PM3) method. In this case, flurbiprofen was set as the ionic form, as well as ionic residues in the binding site. All ONIOM2 calculations were corrected by basis set superposition error using counterpoint (BSSE-CP) correction [23].

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