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Molecular modelling of the differential interaction between several non-steroidal anti-inflammatory drugs and human prostaglandin endoperoxide H synthase-2 (h-PGHS-2)

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Abstract

The prostaglandin endoperoxide H synthase-1 (PGHS-1) and prostaglandin endoperoxide H synthase-2 (PGHS-2) are the targets of non-steroidal anti-inflammatory drugs (NSAIDs). The high degree of selectivity for inhibition of PGHS-2 shown by certain compounds appears to stem from two mechanisms (time-dependent, time-independent inhibition) by which they interact with each isoform. Molecular models of the complexes between indomethacin, fenamates, 2-phenylpropionic acids and the selective cyclooxygenase-2 (COX-2) inhibitors, with the cyclooxygenase active site of human PGHS-2 have been built by combining homology modelling, conformational searching and automated docking techniques. The stability of the resulting complexes has been assessed by molecular dynamics simulations combined with extended linear response calculations. The results allow us to identify regions of biological significance consistent with both X-ray crystallographic and kinetic results. The selective PGHS-2 inhibitors exploit the extra space of a side-pocket in the active site of PGHS-2 that is not found in PGHS-1. The results obtained point out a marked relationship between the experimental affinity and the electrostatic interaction energy alone for a series of NSAIDs. Analysis of the structural and the energetic data provides evidence supporting that network of hydrogen bonds between Tyr^{355}, Glu^{524}, Arg^{120} and Arg^{513} might be involved in mediating the binding of the time-dependent inhibitors of PGHS-2.

Keywords: Cyclooxygenase inhibitors; h-PGHS-2; COX-2; Anti-inflammatory agents; NSAIDs; Docking; Molecular modelling; Molecular dynamics; QSAR modelling

1. Introduction

Prostaglandins act as potent mediators of pain, fever and inflammation. The prostaglandin H synthase (PGHS) catalyses the conversion of arachidonic acid to the prostaglandin endoperoxide PGG_2 and then to PGH_2, which serves as the precursor for the formation of prostaglandin and thromboxanes [1–4]. Two isoforms of PGHS have been identified [5]. The first isoform, PGHS-1, is constitutively expressed in most tissues and is believed to generate prostaglandins for normal physiological functions. The second isoform, PGHS-2, is characterised by a rapid induction by various stimuli, including mitogens, hormones, cytokines and growth factors. Non-steroidal anti-inflammatory drugs (NSAIDs) [6,7] exert their anti-inflammatory, anti-pyretic and analgesic effects by blocking the production of prostanoids from arachidonic acid through inhibition of PGHS activity. Recent human epidemiological studies suggest an inverse relationship between intake of NSAIDs and the risk of colorectal cancer [8,9], and the severity or incidence of Alzheimer's disease [5].

Kinetic analysis demonstrated that isoform selectivity was attained with the competitive inhibition on PGHS-1, but time-dependent inhibition on PGHS-2 [23]. Owing to variations in assay design and sources of COX activity, it is difficult to compare the kinetic behaviour and inhibitory potency of the various NSAIDs with respect to different PGHS isoforms [10]. There are at least four categories of inhibition of PGHS-2: (a) competitive (ibuprofen); (b) tight binding, time-dependent (indomethacin, flurbiprofen, ketoprofen, meclofenamate, diclofenac, NS-398 and SC-58125); (c) weak binding, mixed (mefenamate and naproxen); and (d) covalent binding (aspirin). The mixed inhibition is characterised by an initial time-dependent loss in enzyme activity, which asymptotically approaches a non-zero limit. This is consistent with a slow, reversible, weakly binding inhibitor.

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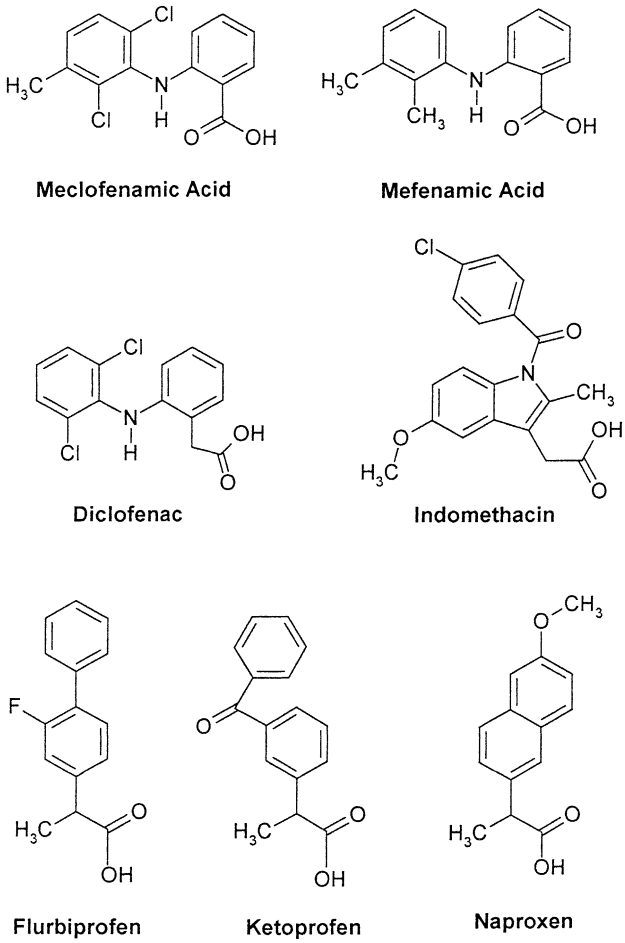


Fig. 1. Structures of several non-selective human PGHS-2 inhibitors.

Not all time-dependent PGHS-2 inhibitors are reversible slow binding inhibitors. NS-398 is selective, irreversible inhibitor of PGHS-2. This compound appears to form an enzyme–inhibitor complex, which promotes inactivation of the enzyme in the absence of substrate.

Essentially all NSAIDs in therapeutic use exhibit non-selective inhibition of both isoforms. However, compounds such as SC-58125, nimesulide, DuP697 and NS-398 are potent, selective inhibitors of PGHS-2 [10].

Fenamic [11,12] and 2-phenylpropionic acids [13] can be classified as competitive inhibitors of substrate binding that cause time-dependent loss of cyclooxygenase activity in the two PGHS isoforms. Meclofenamate, diclofenac, flurbiprofen, ketoprofen and indomethacin show a rapid time-dependent inhibitory effect for the two isoforms. Mefenamate and naproxen are partially time-independent inhibitors for both isoforms (binding mixed in the h-PGHS-2). Inspection of the chemical structures of these compounds (Fig. 1) suggests that properties other than strictly geometrical similarity are necessary to explain their time-dependent/time-independent inhibitory action on PGHS.

Smith et al. [52] using the technique of EPR spectroscopy, observed that binding of NSAIDs induces conformational

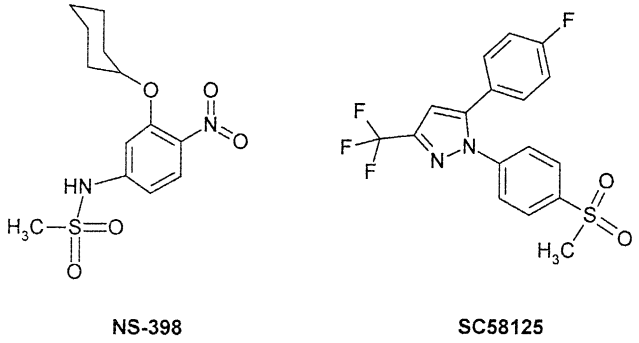


Fig. 2. Structures of two selective human PGHS-2 inhibitors.

changes in the h-PGHS-2. However, interhelical distances determined by EPR for PGHS-2 complexed with flurbiprofen or SC-58125 were in close agreement with those obtained by X-ray analysis. Furthermore, binding of ligand induces a conformational change in the PGHS-2 holoenzyme converting it into a structure similar to those obtained by X-ray analysis [56].

The X-ray crystal structures of ovine PGHS-1 and murine PGHS-2 reveal that carboxylate-containing inhibitors (*S*-flurbiprofen, indomethacin) in the cyclooxygenase active site channel place the carboxylate group close to the guanidinium group of Arg^{120} in PGHS-1 [14] and PGHS-2 [15,16] (residues numbered according to the human PGHS-1 enzyme). Other charged residues in the active site are Glu^{524} in PGHS-1 and Glu^{524} and Arg^{513} in PGHS-2. Moreover, the phenolic side chains of Tyr^{355} in PGHS-1 and PGHS-2 are near the mouth of the channel, opposite to Arg^{120}. This latter residue appears to play a key role in the interaction of PGHS-1 with arachidonic acid and NSAIDs containing carboxylic acid groups [17,18]. However, the fact that all selective PGHS-2 inhibitors reported to date do not contain a carboxylic acid moiety raised the question about the role of the analogous Arg^{120} in PGHS-2. Mancini et al. [19] reported that both diclofenac and fenamates series of NSAIDs bind PGHS-2 so that the carboxylate group of the inhibitor is not close to Arg^{120}. On the other hand, the sulfonyl group of highly selective PGHS-2 inhibitors interacts with Arg^{120} or Arg^{513} in a way similar to the interaction of the carboxylic acid group of flurbiprofen with Arg^{120} in PGHS-1 (Fig. 2) [20].

Inspection of the crystal structure of the ovine PGHS-1–*S*-flurbiprofen complex also shows that Arg^{120} forms a salt bridge with Glu^{524}, and that Tyr^{355}, which is in close proximity to Arg^{120}, could determine the stereochemical specificity of PGHS-1 toward 2-phenylpropionic acid inhibitors [21]. However, the structure of PGHS-2 shows at least two possible hydrogen bonding arrangements near the entrance to the cyclooxygenase-binding pocket: one consists of Arg^{120}, Glu^{524} and Tyr^{355}, and the other of Arg^{513}, Glu^{524} and Tyr^{355}. The disruption of the equilibrium between these two hydrogen-bonding patterns might contribute to the slow binding for the time-dependent inhibition [22]. Indeed, the

high degree of selectivity for inhibition of PGHS-2 shown by certain compounds might be related to the two different mechanisms (time-dependent, time-independent inhibition) that mediate the interaction with each isoform [23].

The recent determination of the three-dimensional cocrystal structures of the complexes of murine PGHS-2 with SC-558 (PDB code 1cx2.pdb), *S*-flurbiprofen (3pgh.pdb) and indomethacin (4cox.pdb) has led to the development of a model for the topography of the NSAIDs binding site in human PGHS-2 [14–16]. Nevertheless, the key features that determine the PGHS-2 (or COX-2) selectivity of certain NSAIDs are not completely known. Particularly, comparison of the crystal structures of PGHS-1 and PGHS-2 provides no definitive explanation for the different behaviour of Arg^{120} between the two isozymes.

The crystal structures of PGHS-1 [58] in complex with reversible competitive inhibitors (ibuprofen and methyl flurbiprofen) and time-dependent inhibitors (flurbiprofen and alclofenac) allow direct comparison of enzyme complexes. The four inhibitors bind to the same site and adopt similar conformations. This suggests that the different apparent modes of NSAIDs binding to the PGHS-2 may result from differences in the efficiency with which inhibitors can perturb the hydrogen bonding networks of residues at the active site.

Understanding the interaction of NSAIDs with PGHS-2 would facilitate the development of NSAIDs with enhanced therapeutic potential and lower toxicity.

In the present paper we address the mode of interaction of fenamic, indomethacin and 2-phenylpropionic acids and of the two relatively selective COX-2 inhibitors SC-58125 and NS-398 with the cyclooxygenase active site of human PGHS-2 making use of automated docking methods and molecular dynamics simulations. The results allow us to discuss the role of Arg^{120}, Tyr^{355} and Arg^{513} in the interaction of PGHS-2 with PGHS-2-specific inhibitors. We interpret our results in the context of a model for inhibitor binding to the human PGHS-2.

2. Methodology

2.1. Human PGHS-2 model

The high sequence identity and functional similarity among PGHS-1 and PGHS-2 enzymes from different sources suggests that they must share very similar overall tertiary and quaternary structures [24]. Human (h)-PGHS-2 was built by using Swiss-Model (Automated Protein Modelling Server) based upon sequence alignment of residues 33-586 of h-PGHS-2 and the crystal structures of mouse PGHS-2 complexed with SC-558 (1cx2.pdb), PGHS-1 dimmer (1prh.pdb), and sheep PGHS-1 complexed with iodosuprofen (1pge.pdb). All ionizable residues were considered in the standard ionisation state at neutral pH. An all atom model was initially built up with the ProModII program [25–28] and then energy-minimised using AMBER5 [34]. No relevant structural change was observed between the active sites of the h-PGHS-2 minimised structure and the three-dimensional structure of unliganded mouse PGHS-2 (5cox.pdb). Our three-dimensional structure of h-PGHS-2 is also in close agreement with the h-PGHS-2 model reported by García-Nieto et al. [54]. The resulting structure was used as the starting co-ordinate file in docking studies.

2.2. Molecular modelling of inhibitors

Molecular modelling of inhibitors was carried out using the Biosym/MSI software [29]. The geometry of indomethacin, diclofenac, meclofenamate, mefenamate, flurbiprofen, ketoprofen, and naproxen acids were obtained from the X-ray data in the Cambridge Structural Database and previous conformational analysis [30–33]. For SC-58125 and NS-398, combining quenched molecular dynamics and energy minimisation techniques using AMBER5 performed conformational analysis. The inhibitors were considered in the standard ionisation state at normal physiological conditions. In all cases the geometry was fully optimised at the ab initio HF/6-31G(d) level using Gaussian 94 [35]. The resulting structures of the inhibitors were used as the starting co-ordinate file in docking studies.

2.3. Docking method

GRID [36,37] was used to explore the most relevant regions of the target in the active site of PGHS-2 for selective interactions with the functional groups present in the ligand. The interaction energies were calculated as Lennard–Jones, electrostatic, and hydrogen bond interactions between a small chemical group (probe) and the enzyme. A distance-dependent dielectric function was used to modulate the electrostatic interaction. Energy calculations were performed using a 0.5 Å grid spacing in a rectangular box measuring 16 Å × 16 Å × 17 Å. The probes selected for this study include the methyl group (C3), aromatic carbon (C1=), hydroxyl group bonded to an aromatic system (OH), nitro oxygen (ON), sp^2 carboxyl oxygen (O::), chlorine or fluorine atom (Cl, F), sp^2 NH group with lone pair (NH=), sp^2 N with lone pair (N:=), ether oxygen (OC2), sulfo oxygen (OS), water and hydrophobic probes.

The AutoDock 3.0 program [38,39] was also used to perform an automatic docking exploration for different conformations of the ligand in the h-PGHS-2 protein model. AutoDock 3.0 uses three search methods (a genetic algorithm, a local search method and an adaptive global–local search method based on Lamarckian genetics (LGA)) in conjunction with an empirical force field that allows the prediction of binding free energies for docked ligand. Two sets of experiments were performed. The first set was designed to test AutoDock's ability to reproduce the binding mode observed experimentally for selected NSAIDs–PGHS-2

complexes. The murine PGHS-2–flurbiprofen complex structure refined at 3.0 Å to an *R*-value of 0.236 (3-pgh.pdb) and murine PGHS-2–indomethacin complex structure refined at 3.0 Å to an *R*-value of 0.219 (4cox.pdb) were used as test systems.

The second set of docking experiments explored indomethacin, diclofenac, meclofenamate, mefenamate, flurbiprofen, ketoprofen, naproxen, SC-58125 and NS-398, in the active site of h-PGHS-2. The exploration was carried out within a 30 Å cube using a 0.25 Å grid spacing. Six affinity grids were calculated for the enzyme. The simulated annealing protocol consisted of 100 runs of 50 cycles. A distance-dependent dielectric constant ($\varepsilon = 4r$) was used. The annealing temperature was set to 310 K during the first cycle and then linearly reduced at the end of each cycle. AutoDock was used to generate conformers within the binding site by randomly changing torsion angles and overall orientation of each molecule. From the 100 simulations performed for each compound, the binding modes with the lowest docked energies from the most populated clusters were selected, and subsequently they were refined using AMBER5.

2.4. Molecular dynamics simulations

The complexes were first energy minimised using AMBER5. Restricted electrostatic potential fitted charges were determined at the HF/6-31G(d) level [57], and the van der Waals parameters were taken from those defined for related atom types in the AMBER95 force field. SHAKE was used to maintain all the bonds at their equilibrium distances. A distance-dependent dielectric constant ($\varepsilon = 4r$) and a cut-off of 11.0 Å were used. In each case, 200 steps of steepest descent were followed by conjugate gradient until the root mean square value of the potential energy gradient was below 0.01 kcal/mol. The complexes were then used for the molecular dynamics simulations. They were heated for 30 ps, equilibrated for 110 ps at 298 K and then a 320 ps molecular dynamics simulation was performed. An integration time-step of 2 fs was used. The non-bonded pair-list was updated every 20 cycles and co-ordinates were saved every 10 ps from the last 200 ps and energy-minimised for further analysis of complexes. In the case of NS-398 and SC-58125 compounds, the two alternate orientations suggested by the automated docking program were considered in molecular dynamics simulations.

2.5. Binding free energy calculations

The differences in binding free energies were calculated as a sum of electrostatic and non-electrostatic contributions: $\Delta G_x = \Delta G_x(\text{ligand–PGHS-2}) - [\Delta G_x(\text{h-PGHS-2}) + \Delta G_x(\text{ligand})]$. The electrostatic component (ΔG_{ele}), which includes the solvent-screened interaction between ligand and enzyme plus the electrostatic contribution due to changes in hydration, was determined from a finite difference solution of the Poisson–Boltzmann equation (PB). Calculations were carried out with the commonly used values of 80 and 4 for the dielectric permittivities of the aqueous and protein environments. Cubic grids with a resolution of 0.8 Å were used. PB calculations were performed using the Delphi module implemented in Insight-II [29]. The non-electrostatic component ($\Delta G_{\text{n-ele}}$) was approximated by adding a Lennard–Jones interaction energy (ΔG_{vdW}) between drug and enzyme to a term related to the change in solvent accessible surface (ΔG_{SAS}; following the linear relationship between SAS and hydrocarbon-transfer free energy observed in solubility studies of small alkanes, a single coefficient of 5 cal/K Å^2 was assigned to the microscopic surface tension) [40,41].

3. Results

3.1. X-ray crystal structure/comparison with the docking model

Mouse and human PGHS-2 have 604 amino acid residues each and they share almost 87% sequence identity and strict sequence conservation in the cyclooxygenase active site. Therefore, their three-dimensional structures are expected to be very similar. Comparison of the active site residues in the two isoforms shows that His^{513} and Ile^{523} of PGHS-1 are not conserved (Arg^{513} and Val^{523} of PGHS-2). While Ile^{523} appears to severely restrict access to the active site, the volume accessible to both substrates and inhibitors in the active site is larger in PGHS-2 relative to PGHS-1 [24].

Our homology modelling of h-PGSH-2 was tested with docking calculations using the AutoDock 3.0 program. Test calculations were performed using *S*-flurbiprofen and *cis*-indomethacin owing to the availability of the three-dimensional structures of murine PGHS-2 complexes with these inhibitors, whose carboxylate group interact Arg^{120}. Binding of indomethacin was examined for the *cis* species (defined according to the benzoil ring relative to the indole) on the basis of previous studies of indomethacin–PGHS-2 in the crystal structure [16], which showed that the *cis* model is favoured over the *trans*. Docking calculations indicated that the cluster of similar conformations with the lowest energy docked structure reproduced very closely the crystallographic binding mode.

These structures obtained from the docking studies were refined using AMBER5. In the optimised model for *S*-flurbiprofen–PGHS-2 complex (Fig. 3 and Table 1), *S*-flurbiprofen is bound to the long hydrophobic channel in a similar way to that observed in the PGHS-1 isoform. The carboxylate group forms a salt bridge with the guanidinium group of Arg^{120} and a hydrogen bond to Tyr^{355}. The distal phenyl ring forms van der Waals contacts with Val^{523}, and the fluorophenyl ring interacts with Val^{349} and Ala^{527} and stacks against Tyr^{385}.

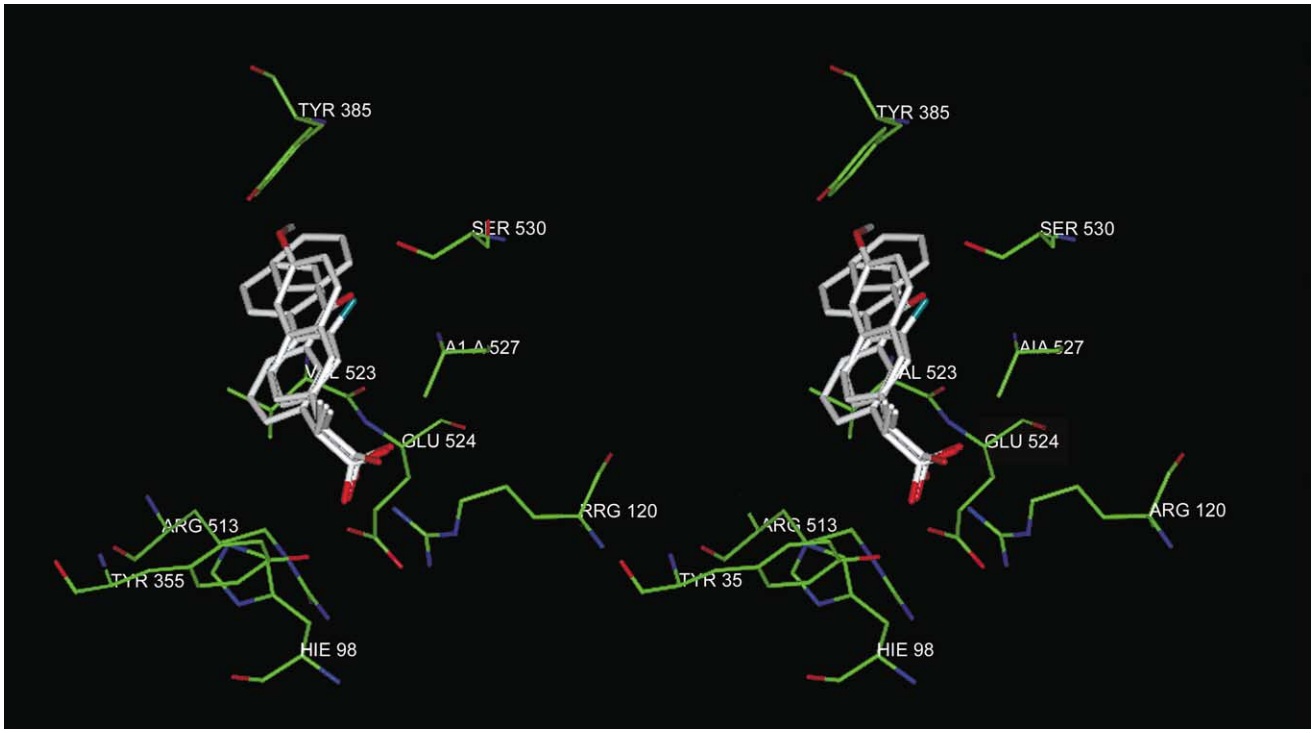


Fig. 3. *S*-flurbiprofen, *S*-naproxen and *S*-ketoprofen bound in the cyclooxygenase binding site channel of human PGHS-2 (in stereoview). Only residues relevant to the discussion are displayed.

In the model for *cis*-indomethacin binding (Fig. 4 and Table 1), the indomethacin fills the entire hydrophobic channel from Tyr^{385} downward a distance of roughly 13 Å and forms a salt bridge with Arg^{120}. The chlorophenyl group lies in a hydrophobic pocket at the top of the channel, the carbonyl group is situated at around 3.5 Å of Ser^{530}, and the indole ring is in contact with several aliphatic side chains, including Val^{349}, Ala^{527}, Leu^{352}, and Val^{523}. The *o*-methoxy group interacts closely with the main-chain atoms of Tyr^{355} and Arg^{513}. Additional contacts are made with the residues His^{89}, Met^{113}, Val^{116}, and Phe^{518}.

Overall, the set of interactions identified from calculations are in agreement with the X-ray three-dimensional structures of murine PGHS-2 complexed with flurbiprofen and indomethacin [22,24,42], as well as with experimental evidences about their implications in modulating the binding affinity [30]. These interactions also agree with the GRID analysis of the active site of the h-PGHS-2 model (data not shown). For instance, the energy contour for OH and NH= probes gives rise to large areas surrounding Arg^{120}, Glu^{524}, Tyr^{355} and Arg^{513}, as less markedly Tyr^{385}, Ser^{530} and Ser^{353}. Likewise, the methyl group produced a completely filled contour at −2.0 kcal/mol on the prime site, indicating that the centre of this region had reduced van der Waals contact. In summary, all the preceding results give support to the computational protocol mentioned above (see Section 2.3), which was subsequently used to examine the binding mode of the NSAIDs inhibitors to the h-PGSH-2 model.

3.2. Docking studies

While interaction with a receptor will certainly perturb the conformational energy of a flexible ligand, high affinity would suggest that the ligand be not highly distorted upon binding. The results of the ligand docking performed with AutoDock 3.0 are summarised in Table 2. With exception of meclofenamate, the differences in the conformational energy between bound state and free solution are about 2 kcal/mol.

Inspection of the docked inhibitors in h-PGHS-2 enzyme reveals that the carboxylate group of NSAIDs is located in a favourable position to interact with the guanidinium group of Arg^{120} or Arg^{513}. The orientation favoured by AutoDock placed the carboxylate of flurbiprofen, ketoprofen, and naproxen (Fig. 3) forming a salt bridge with Arg^{120}, and hydrogen bonded with the phenolic hydroxyl of Tyr^{355}. The three inhibitors bind to the same site and adopt similar conformations to those found in the crystal structures of ovine PGHS-1 complexed with ibuprofen, flurbiprofen and alclofenac [58]. This ionic interaction is expected to be essential for the formation of a tight binding inhibitor complex leading to the time-dependent inhibition of PGHS by flurbiprofen and ketoprofen [43]. The side chain of Ser^{530} lies close to the distal phenyl ring of flurbiprofen and ketoprofen, as has been described for the PGHS-2 complex [24].

The orientation of diclofenac, meclofenamate and mefenamate (Fig. 5) suggests that the ionic interaction between their carboxylate group with Arg^{120} of h-PGHS-2 contribute little to the binding. Rather, diclofenac has the carboxylic

Table 1
Residue-based energy decomposition of the interaction energy (kcal/mol) between inhibitors and the cyclooxygenase active site of h-PGHS-2 in the orientations bound studied

Protein	*S*-flurbiprofen		*S*-ketoprofen		*S*-naproxen		Meclofenama		Mefenama		Diclofenac		Indomethac		NS-398		SC-58125 residue	
	vdW	ele	vdW	ele	vdW	ele	vdW	ele	vdW	ele	vdW	ele	vdW	ele	vdW	ele	vdW	ele
His^{98}	−0.4	−0.3	−1.9	−0.2	−1.8	−1.0	−1.3	−1.5	−0.2	−0.1	−0.0	0.1	−3.0	−0.3	−2.6	−0.2	−3.6	−0.6
Arg^{120}	−2.0	−4.9	−4.2	−4.9	−2.1	−9.0	−2.5	−2.0	−1.9	−4.1	−2.8	−3.2	−2.0	−7.6	−5.5	−1.2	−4.9	−0.1
Val^{349}	−2.0	0.0	−0.5	0.0	−2.1	0.0	−3.1	0.0	−2.4	0.2	−1.3	0.1	−1.9	−0.1	−4.4	0.1	−2.9	0.0
Leu^{352}	−3.5	−0.1	−0.1	0.0	−1.0	−0.1	−0.1	−0.2	−3.1	−0.2	−2.2	0.2	−4.1	−0.3	−4.5	0.4	−4.4	0.1
Ser^{353}	−1.1	−0.1	−1.5	−0.2	−1.0	−0.2	−1.7	−2.7	−1.0	0.4	−4.5	−1.3	−4.0	−0.5	−4.0	0.2	−4.8	0.0
Tyr^{355}	−2.2	−2.2	−2.7	−0.6	−1.9	0.0	−2.2	−0.6	0.9	0.0	−2.6	−1.3	−2.3	−2.8	−3.1	−0.4	−2.5	−0.5
Arg^{513}	−0.1	−0.8	−0.1	−0.9	−0.1	−1.0	−0.4	−4.8	−1.3	−6.0	0.0	−1.1	−2.9	−3.5	−1.7	−0.8	−3.7	−2.2
Phe^{518}	−1.1	0.0	−2.2	0.0	−2.3	−0.1	−1.9	−0.4	−2.2	−0.2	−0.2	0.0	−4.2	−0.0	−0.4	0.1	−2.2	0.1
Val^{523}	−4.9	0.2	−2.7	0.0	−2.6	0.1	−3.7	−0.2	−3.5	−0.3	−1.5	0.1	−4.8	−0.2	−3.5	−0.1	−4.7	−0.1
Ala^{527}	−3.3	−0.2	−4.2	0.0	−4.5	−0.4	−3.3	−0.25	−3.6	0.0	−3.7	−0.2	−3.0	0.0	−1.2	0.0	−2.2	0.0
Ser^{530}	−1.2	−0.7	−1.8	−0.3	−2.1	0.0	−1.7	0.0	−2.5	−0.1	−1.9	0.2	−1.3	−0.2	−1.1	0.0	−0.5	0.0

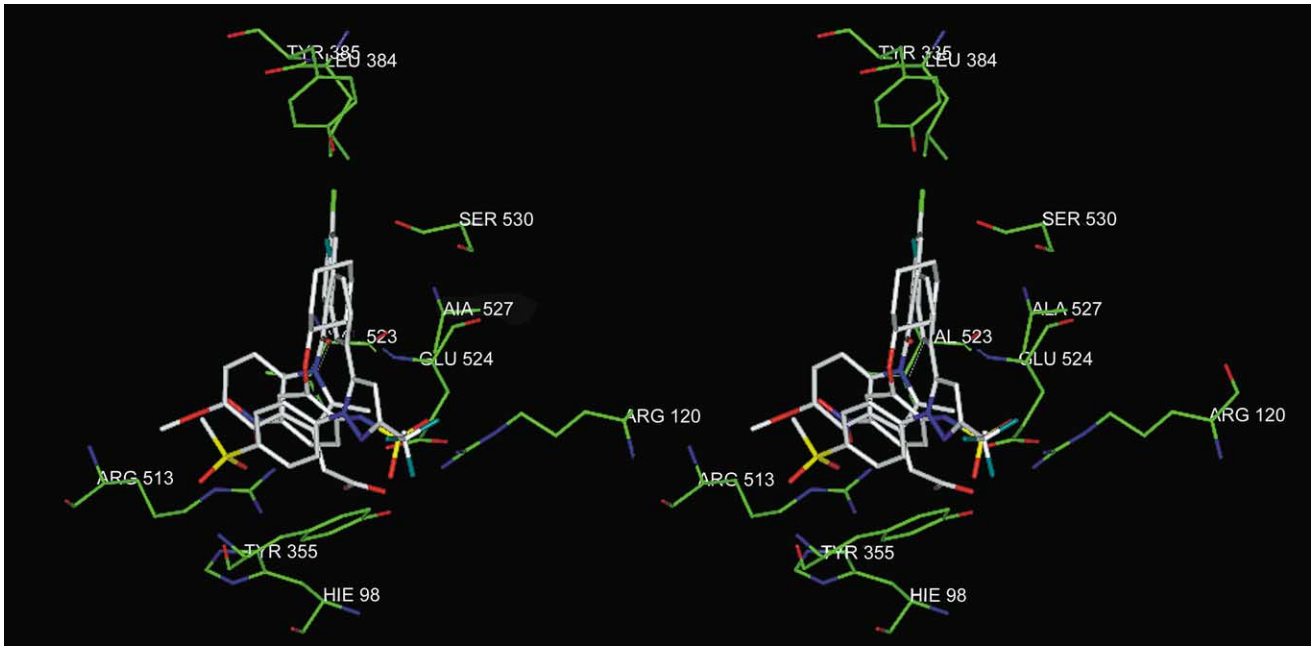


Fig. 4. *Cis*-indomethacin, SC-58125 and NS-398 bound in the cyclooxygenase active site channel of human PGHS-2 (in stereoview). Only residues relevant to the discussion are displayed.

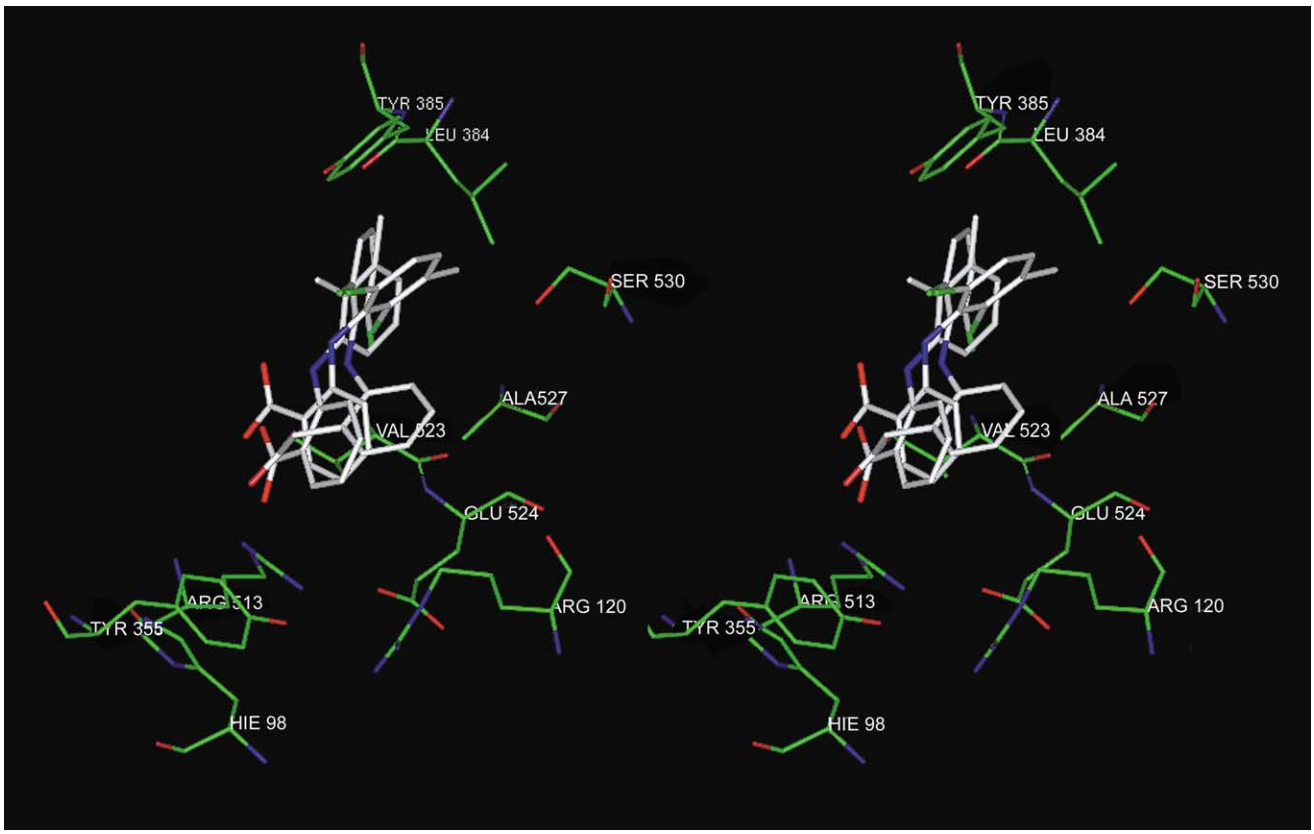


Fig. 5. Diclofenac, meclofenamate and mfenamate bound in the cyclooxygenase active site channel of human PGHS-2 (in stereoview). Only residues relevant to the discussion are displayed.

Table 2
Results of simulated-annealing docking by AutoDock. ΔE_c (kcal/mol) is the conformational energy difference between bound and unbound states ($\Delta E_c = E_{c,bound} - E_{c,unbound}$)[a]

Compound	ΔE_c	ΔG_{bind}
S-flurbiprofen	2.10	−12.04
S-ketoprofen	2.18	−11.77
S-naproxen	1.64	−11.04
Diclofenac	1.92	−11.09
Indomethacin	1.20	−10.19
Mefenamate	2.93	−10.76
Meclofenamate	0.10	−11.45
NS-398	1.58	−10.49
SC-58125	1.40	−13.70

[a] ΔG_{bind} (kcal/mol) is the interaction free energy between enzyme and inhibitor.

group located in a favourable position for interaction with the hydroxyl of Tyr^{355}, and appears to form a hydrogen bond with that residue. In PGHS-2 the residue 503 is a leucine whose small size allows the Leu^{384} side chain to orient itself towards the protein away from the active site [15]. The PGHS-2 active site has more space in this region in the form of a small lipophilic pocket. The halogen substitution in the second ring of the fenamic inhibitors (meclofenamate and diclofenac) allows better binding to PGHS-2 than to PGHS-1.

The weakly acidic inhibitors possessing an aryl methyl sulfonyl group, NS-398 and SC-58125 (Fig. 4), interact with Arg^{120} and Arg^{513} by two different binding orientations. Both binding modes are chemically reasonable, yielding similar interaction energies with the enzyme. The orientation of NS-398 favoured by AutoDock places the nitrogen and oxygen atoms of the sulphonamide group interacting with Arg^{120}, the nitro group in the pocket close to Arg^{513}, and the phenoxy ring occupies a position perpendicular to the aromatic ring of the Tyr^{385}. This binding mode could reproduce the experimental evidence available for this drug [46–48], and (Kurumbail, personal communication). For SC-58125 the phenylsulfonamide group is accommodated in the pocket vicinal to Val^{523} with the oxygen atoms of the sulphonamide group hydrogen-bonded to Arg^{513}. In this case, the docking calculations showed that SC-58125 occupies a position comparable to that of SC-558 in its complex with mouse COX-2 [24,48].

In summary, as observed with ovine PGHS-1 [17,58], the Arg^{120} group of h-PGHS-2 is necessary for the efficient binding of non-PGHS-2-selective inhibitors. Furthermore, the guanidinium group of Arg^{120} actually interferes with the binding of the PGHS-2 selective inhibitor [47], which interact closely with main-chain atoms of Leu^{352}, Ser^{353} and Val^{523} in the zones surrounding the first aromatic ring. Thus, the single Val^{523} to Ile mutation of PGHS-2 results in a loss of sensitivity to inhibition by NS-398 and SC-58125, suggesting that these atoms enhance the time-dependent affinity for PGHS-2 [42]. On the other hand, no significant relationship was found between the experimental inhibitory potencies (Table 3) and the binding free energy (ΔG_{bind}) determined by AutoDock (Table 2) ($r_p = 0.45$).

3.3. Molecular dynamics simulations

The behaviour of the docked receptor–ligand complexes was studied in a dynamic context to account for protein flexibility and conformational changes. The analysis showed that in all cases the inhibitor–enzyme complex remains stable along all the simulation without suffering remarkable structural changes.

In order to examine the relative contributions of the residues to the ligand binding, the collected structures were energy-minimised, and the interaction energy between inhibitor and the binding site was decomposed on a residue basis using the ANAL module of AMBER for the most favourable binding obtained for each inhibitor (Table 1).

The differences in the interaction energy between ligand and the cyclooxygenase active site of h-PGHS-2 arise mainly from electrostatic and van der Waals contributions emanating from the carboxylate and sulphonamide group. It can be seen that the major residue contributors to the binding energy are Arg^{120}, Tyr^{355}, Arg^{513}, Val^{523} and Ala^{527}. The Arg^{120},

Table 3
Electrostatic and non-electrostatic contributions (kcal/mol) to the binding free energy, and their inhibitory value pIC_{50} for human PGHS-2 values relative to those compound SC-58125[a]

Compound	ΔG_{ele}	ΔG_{vdW}	ΔG_{SAS}	ΔG_{bind}	pIC_{50} [19]	IC_{50} (COX-1)/IC_{50} (COX-2)	Predicted values pIC_{50} (Eq. (1))
S-flurbiprofen	−11.8	12.4	0.8	1.4	8.3	0.15	8.4
S-ketoprofen	−6.8	12.8	0.3	6.3	7.8		7.6
S-naproxen	−4.2	14.0	0.5	10.3	6.5 [51]	0.24	7.1
Meclofenamate	−4.3	15.2	0.5	11.5	7.3	0.8	7.2
Mefenamate	16.4	12.9	0.6	29.9	3.1 [51]	0.03	3.7
Diclofenac	−4.1	14.0	0.6	10.5	7.2	2	7.15
Indomethacin	−0.3	−5.4	−0.3	−6.0	5.5	0.16	6.5
NS-398	5.3	3.2	0.0	8.5	7.1	2000	5.6
SC-58125	0.0	0.0	0.0	0.0	7.2 [51]	2500	6.5

[a] The absolute values (kcal/mol) for the interaction of SC-58125 are −40.0, −52.5 and −3.85 for the electrostatic, Lennard–Jones, and solvent-accessible surface contributions. IC_{50} values for the h-PGHS-2 inhibition "in vitro".

Tyr^{355} and Arg^{513} residues form salt bridge or H-bond contacts with the carboxylate/sulphonamide groups. The interactions of the drug with Leu^{384}, Val^{523} and Ala^{527} residues are very important in the structural transition that underlies time-dependent inhibition by the PGHS-2-selective agents [49].

In the AMBER-refined complexes for NS-398, the overall intermolecular interaction energy is very similar in the two possible arrangements. The orientation that places the sulphonamide group close to Arg^{120} (Fig. 4) gives rise to a slightly more favourable van der Waals interaction. Only SC-58125 (Table 1) places the sulphonamide group close to Arg^{513} and His^{98} (one of the oxygen atom forms a hydrogen bond to Arg^{513}, the other oxygen is hydrogen-bonded to His^{98}) and it gives rise to a more favourable electrostatic interaction in this orientation. The cyclooxygenase active site forks at the SC-58125 binding site. Hydrophobic residues Leu^{352}, Phe^{518} and Val^{523} surround the phenyl ring. The fluorophenyl ring of SC-58125 is bound in a hydrophobic cavity formed by Phe^{381}, Leu^{384}, Tyr^{385}, Trp^{387} and Ser^{530}, with contributions from Gly^{526} and Ala^{527}. The trifluoromethyl group is bound in an adjacent pocket formed by Met^{113}, Val^{116}, Val^{349}, Tyr^{355}, Leu^{359} and Leu^{531}. These two binding regions have similar features to the binding of arylacetics and indomethacin. The distal ring of arylacetics overlaps with the fluorophenyl ring of SC-58125, the first ring superimposes on the pyrazole of SC-58125, and the carboxylate of arylacetics and the trifluoromethyl group of SC-58125 fill the same cavity. The most striking result is that both meclofenamate and diclofenac bind h-PGHS-2 so that their carboxylate group is not in close proximity to Arg^{120} but forms two hydrogen bonds near the entrance to the cyclooxygenase binding pocket involving Arg^{120}, Arg^{513} and Tyr^{355} residues. The Leu^{384} sidechain into the upper part of the active site has a small lipophilic region and allows that both meclofenamate and diclofenac bind better to PGHS-2.

3.4. Energy analysis

In vitro determination of IC_{50} values is subject to some uncertainty owing to a variety of factors like the source of enzyme activity, species variability, use of recombinant enzymes and of cells versus purified enzymes. Most assays rely on measurement of the final product, PGE_2, either by RIA, ELISA or HPLC, each method requiring pre-incubation of enzyme with inhibitor ranging from 2 to 15 min. As a result, comparisons of potency and selectivity become difficult. For instance, published selectivity ratios for one of the earliest PGHS-2 selective inhibitors, NS-398, have ranged from 11 to >1000-fold [43–45].

Table 3 reports the experimental inhibitory potency ($p\mathrm{IC}_{50} = \log(1/\mathrm{IC}_{50})$) together with electrostatic and non-electrostatic contributions to the binding free energy (see Section 2.3). The relative differences in electrostatic and van der Waals energies between bound and unbound states varies up to 28 and 20 kcal/mol for the subset of compounds. Attachment of fluorine at position 3 in the flurbiprofen makes the electrostatic interaction more favourable compared to the unsubstitued compounds ketoprofen and naproxen. Likewise, replacement of methyl by chlorine in the second ring of the fenamic acids largely favours the electrostatic interaction between inhibitor and enzyme. Finally, all of the inhibitors are largely buried in the enzyme, and the changes in the SAS between bound and unbound states of the inhibitor (ΔG_{SAS}) mostly reflect the differences in the SAS of the inhibitor in solution. On the other hand, no significant relationship ($r_{\mathrm{p}} = 0.33$) was found between the experimental inhibitory potencies (Table 3) and the $\log p$ determined by $C\log p$ 4.0 [59].

Inspection of the separate contributions to the binding free energy revealed a marked dependence of the inhibitory activity with the electrostatic term (Table 3); the partial least squares method [50] was employed to validate the models. Thus, a good correlation between the experimental $p\mathrm{IC}_{50}$ and the electrostatic term is found (Pearson's correlation coefficient of 0.87). On the other hand, no significant correlation was observed between the experimental inhibitory potencies and either the van der Waals interaction energy or the change in the solvent-accessible surface, which otherwise are highly correlated ($r_{\mathrm{p}} = 0.91$). The relationship between experimental data and the electrostatic term is given in Eq. (1), which predicts reasonably well the $p\mathrm{IC}_{50}$ of the complete series (Table 3), the largest deviation being found for indomethacin:

$$\log\left(\frac{1}{\mathrm{IC}_{50}}\right) = 6.48 - 0.165\Delta G_{\mathrm{ele}} \tag{1}$$

$$Q^2 = 0.958, \qquad F = 21.0, \qquad \mathrm{SDEP} = 0.088,$$
$$R^2 = 0.75, \qquad \mathrm{SDEE} = 0.827$$

Reparametrization of Eq. (1) to include other contributions led to Eq. (2), which was found to be statistically less significant:

$$\log\left(\frac{1}{\mathrm{IC}_{50}}\right) = 6.19 - 0.147\Delta G_{\mathrm{ele}} + 0.022\Delta G_{\mathrm{vdW}} - 0.34\Delta G_{\mathrm{SAS}} \tag{2}$$

$$Q^2 = 0.624, \qquad F = 15.5, \qquad \mathrm{SDEP} = 2.277,$$
$$R^2 = 0.69, \qquad \mathrm{SDEE} = 0.923$$

Looking at the adjustable parameters, the coefficients of the electrostatic and SAS terms are negative. However, the coefficient of the van der Waals component is positive, which means that a better van der Waals interaction with the enzyme decreases the inhibitory potency. This finding seemed unreasonable, though it might imply some kind of steric collapse with the protein. Keeping in mind the large correlation between the van der Waals interaction energy and the change in the solvent-accessible surface of the inhibitor ($r_{\mathrm{p}} = 0.91$), we rather believe that their role in Eq. (2) simply reflects a spurious cancellation of their contributions.

4. Discussion

Amino acid sequences of three PGHS-1 and four PGHS-2 enzymes are known. Comparison of any PGHS-1 with any PGHS-2 enzyme shows a sequence identity of 60%, which indicates that their overall structures are highly conserved [24]. The COX active sites of PGHS-1 and PGHS-2 are very similar, the only residue that differs is the Ile → Val^{523} [16]. With 87% identity and strict sequence conservation in the COX active site, the structure of h-PGHS-2 is expected to be very similar to the murine enzyme, and lends credence to the present model. The PGHS-2 structure revealed a second internal pocket extending off the NSAIDs binding site. In PGHS-2 the volume of the primary inhibitor binding site and the secondary pocket is calculated to be 394 $Å^3$, whereas the volume of the NSAID binding site in PGHS-1 is 316 $Å^3$ [45].

The structural studies on murine PGHS-2 have shown that the conformations of the free and complexed enzymes are very similar in the crystal. However, the structure of the solubilized h-PGHS-2 holoenzyme measured in solution differs from the crystal structure of h-PGHS-2 holoenzyme obtained by X-ray analysis, but the binding of NSAIDs would shift the equilibrium between different conformations of the solubilized holo-PGHS-2 toward the most favoured conformation for crystallisation [52].

The role of Val^{523} as a determinant in the differential interaction of PGHS-2 with selective and non-selective inhibitors is clear from experiments with the Ile^{523} → Val PGHS-1 mutant, which show increased potency of NS-398 to PGHS-1. The van der Waals interactions (Table 1) between ligand and Val^{523} were larger for indomethacin (4.8 kcal/mol), *S*-flurbiprofen (4.9 kcal/mol), SC-58125 (4.7 kcal/mol), meclofenamate (3.7 kcal/mol) and NS-398 (3.5 kcal/mol) compared to diclofenac (1.5 kcal/mol), while van der Waals interactions between ligand and Ala^{527} in the region surrounding the first aromatic ring were larger for diclofenac (3.7 kcal/mol).

The Val^{523} mutations in PGHS-2 produced diverse responses to the inhibitor [49]. The Val^{523} → Ala mutant was susceptible to time-dependent inhibition by SC-58125 and NS-398, whereas the single mutation Val^{523} → Ile, Glu, or Lys of PGHS-2 increased the IC_{50} to >100 μM, which looks like PGHS-1 for the selective PGHS-2 inhibitors. The differences between the behaviour of wild-type PGHS-2 and the Val^{523} mutant demonstrate that even subtle changes at position 523 influenced the susceptibility to time-dependent inhibition, the increases in size or charge (Val → Ile, Glu or Lys) decreased the tendency of time-dependent inhibition, whereas a decrease in size (Val → Ala) had little effect. Only naproxen had no inhibitory effect with the mutant compared to PGHS-1 and PGHS-2 [51]. The other NSAIDs, indomethacin, diclofenac, mefenamate and flurbiprofen, showed no change in selectivity with the mutant.

The cyclooxygenase NSAID binding site consists of a long, narrow, hydrophobic channel and the presence of Arg^{120} at the mouth of the channel provides a suitable anchoring for ligands bearing a carboxylate group. Other charged residues in the mouth of the cyclooxygenase active site include Glu^{524} and Arg^{513}.

In our model (Fig. 3) the carboxylate moiety of all (2*S*)-phenylpropionics inhibitors forms a salt bridge with Arg^{120} in an identical fashion as bind to *o*-PGHS-1 in crystal structures [58]. The two-carboxylate oxygen atoms of the inhibitor are between 2.40 and 2.60 Å from one of guanidine nitrogenous of the arginine, and the electrostatic contributions showed a significant influence on the overall inhibitory effect. In addition, one carboxylic acid oxygen of indomethacin (Fig. 4) lies at <2.5 Å from the phenolic hydroxyl of Tyr^{355}. Side chains lining the channel below the inhibitor surround it and render it inaccessible to solvent. The efficiency of PGHS-2 inhibition by (2*S*)-phenylpropionics and indomethacin increased when the interaction with Arg^{120} was enhanced. In our model for NS-398 the sulphonamide group interacts with Arg^{120} and Tyr^{355} in a manner similar to the carboxylic group of (2*S*)-phenylpropionics, while the nitro group interacts with Arg^{513}.

Flurbiprofen is representative of the 2-phenylpropionic acid class of NSAIDs that are relatively non-specific for PGHS isozymes. Flurbiprofen causes a time-dependent inhibition of both PGHS, but it failed to cause a time-dependent inhibition of Arg^{120} → Glu^{120} mutant h-PGHS-2 [47,48]. In contrast, no significant inhibition was observed with indomethacin. A 1000-fold decrease in potency occurs for NS-398 [23], the change in inhibitory potency of NS-398 on mutation of h-PGHS-2 (Arg^{106} → Glu) being related to a difference in the kinetics of inhibition (time-dependent → time-independent). Diclofenac and meclofenamate retained some inhibitory effects (potency was decreased five- to six-fold) against the mutant (Arg^{120} → Glu) h-PGSH-2 enzyme. The time-dependent inhibition by diclofenac and meclofenamic is similar for the mutant h-PGHS-2 albeit at a higher concentration of inhibitor. This suggests that the contribution to inhibitor binding of an ionic interaction with Arg^{120} of h-PGHS-2 is minimal [48].

SC-58125 binds in the COX active site with the sulphonamide group, which interacts with His^{89}, Gln^{192} and Arg^{513}. One oxygen atom forms a hydrogen bond to His^{89}, the other oxygen being hydrogen bonded to Arg^{513}. The amide nitrogen forms a hydrogen bond to the carbonyl oxygen of Phe^{518}.

Analysis of the structural and the binding free energy data (Tables 1, 3 and 4) allows us to suggest that Tyr^{355} participates in the time-dependent inhibition of PGHS-2 through its interactions with the inhibitor, and/or the hydrogen bonding network. This hypothesis is consistent with the proposal by So et al. [22], and Luong et al. [46].

So et al. [22] proposed a role for Tyr^{355} in the time-dependent inhibition and allosteric activation through its interactions with the substrate, the inhibitor, and/or the hydrogen-bonding network. The structures of PGHS-2 show at least two possible hydrogen bonding conformations near

Table 4
Residue-based energy decomposition of the interaction energy (kcal/mol) between residues $Arg^{513}/Glu^{524}/Tyr^{355}$ and $Arg^{120}/Glu^{524}/Tyr^{355}$ in the inhibitor–h-PGHS-2 complexes and h-PGHS-2

Inhibitor–h-PGHS-2 complex	Glu^{524}/Arg^{120}		Glu^{524}/Arg^{513}		Tyr^{355}/Arg^{120}	
	vdW	ele	vdW	ele	vdW	ele
S-flurbiprofen	−2.3	−8.8	−2.5	−5.1	−0.6	0.2
S-ketoprofen	−2.8	−8.1	−1.6	−5.4	−0.1	0.1
S-naproxen	1.0	−8.5	−0.1	−0.8	−0.1	0.1
Meclofenamate	−2.9	−8.1	−1.5	−6.0	−0.9	0.5
Mefenamate	−2.7	−8.3	−0.2	−1.2	−0.1	0.0
Diclofenac	−3.1	−4.1	−1.2	−7.2	−4.0	−1.3
Indomethacin	−2.1	−9.5	−0.1	−1.0	−0.3	0.1
NS-398	−2.1	−6.9	−1.3	−5.9	−0.8	0.1
SC-58125	−2.9	−6.3	−1.6	−6.4	−0.8	0.1
h-PGHS-2	−1.5	−7.7	−1.2	−8.0	−2.5	−1.2

the entrance to the COX binding pocket, one consisting of Arg^{120}, Glu^{524}, and Tyr^{355} and another consisting of Arg^{513}, Glu^{524}, and Tyr^{355}.

Smith et al. [52] observed that the arachidonate/enzyme conformation is likely stabilised by formation of the same network of hydrogen bonds between amino acid chains at the mouth of the cyclooxygenase active site that stabilises binding of time-dependent inhibitors and that the disruption of this equilibrium contributes to the slow binding inhibition.

In our model the network of hydrogen bonds between Tyr^{355}, Glu^{524}, Arg^{120} and Arg^{513} participate in stabilising the binding of the time-dependent inhibitors of PGHS-2 with fenamics, (2*S*)-phenylpropionics and PGHS-2-selective inhibitors. To look into such a possibility, we monitored interatomic distances between NH1, NH2 of Arg^{120} and Arg^{513}; O1, O2 of Glu^{524} and OH of Tyr^{355}. Typical examples of the distance fluctuations between NH1 and NH2 of Arg^{120} and Arg^{513} with O1 and O2 of Glu^{524} in *S*-flurbiprofen–PGHS-2, *S*-naproxen–PGHS-2, NS-398–PGHS-2 and SC-58125–PGHS-2 complexes are shown in Fig. 6. We also monitored interaction energies of Arg^{120}, Arg^{513}, and Tyr^{355} with Glu^{524} residues in the cyclooxygenase cavity for all the inhibitor–PGHS-2 complexes. We observed those electrostatic interactions of Arg^{120}/Arg^{513} with *S*-naproxen, indomethacin and mefenamate are responsible for the fact that the disruption of the hydrogen bond between Arg^{513} and Glu^{524} contributes to the slow mixed binding inhibition (Table 1). However, electrostatic interactions of Arg^{120}/Tyr^{355} with *S*-flurbiprofen and *S*-ketoprofen contributing to the H-bond network may predominate in the tightened form. Likewise, the interactions of $Arg^{120}/Arg^{513}/Tyr^{355}$ with meclofenamate, diclofenac, SC-58125 and NS-398 are responsible for the fact that the tightened form of the H-bond network contributes to the slow binding inhibition (Table 1).

The equilibrium between these two hydrogen-bonding arrangements forms a constriction and is an important determinant of NSAID binding (Table 4 and Fig. 6). The $Arg^{513}/Glu^{524}/Tyr^{355}$ H-bond network may predominate in the relaxed form, leaving Arg^{120} free to interact with substrate, whereas in the tightened conformation the Arg^{120}/Glu^{524} hydrogen-bonding network locks the substrate into a catalytically competent conformation. Since time-dependent NSAIDs cannot undergo oxygenation, the unproductive hydrogen bond-stabilised complex they form dissociates only slowly.

Moreover, meclofenamate was incapable of inhibiting the mutation of h-PGHS-2 ($Tyr^{355} \rightarrow$ Phe) [22], thus suggesting a role for Tyr^{355} in the time-dependent inhibition by diclofenac and meclofenamate. However, if these interactions are reinforced, as it is assumed, through the simultaneous presence of other interactions, they become much stronger and are able to last much longer.

A hydrogen bond also appears to be formed between the carbonyl bridge oxygen of the indomethacin and the side chain hydroxyl of Ser^{530} (Fig. 4). Acetylation of Ser^{530} or mutation of this residue to methionine does not greatly affect the binding and inhibitory properties of *S*-flurbiprofen, *S*-ketoprofen, indomethacin and NS-398. In contrast, diclofenac and meclofenamic acid have lost all inhibitory potency for the serine-modified forms of PGHS-2 [19]. This is consistent with the hypothesis that the increased bulk by a methionine residue or acetyled serine results in a steric hindrance, which prevents their interaction with the Tyr^{355} residue in the NSAIDs binding site of cyclooxygenase.

This study shows that the two different types of inhibitors utilise the same binding and adopt similar conformations. To discern the feasibility of the different binding modes, the computed binding free energies were compared in light of the inhibitory data. Inspection of the separate contributions to the binding free energy revealed a marked dependence of the inhibitory activity with the electrostatic term, which indicate that *S*-flurbiprofen is more effective in inhibiting the enzyme than the other compounds. These results suggest that competitive inhibitors and slow tight-binding inhibitors bind and inhibit PGHS-2 by the same mechanism, and differ principally in the efficiency with which can open the polar active site entrance of the cyclooxygenase channel and that disruption of the two hydrogen bonding equilibrium ($Arg^{120}/Glu^{524}/Tyr^{355}$ and $Arg^{513}/Glu^{524}/Tyr^{355}$)

contributes to the slow binding inhibition. They suggested that the ability of the channel to move between open and closed conformations modulate the capabilities of both substrates and inhibitors to reach the internal binding site.

Our structural data (Table 2) are in accord to the hypothesis that time-dependent inhibitors adopt one particular conformation to enter the active site, and then, after their arrival, isomerize to a different conformation that is not capable of exiting [5]. The CoMFA studies by Marot et al. [53] suggested a good correlation between the two molecular fields (steric and electrostatic) and the activities registered for the selective COX-2 inhibitors.

The central channel of the NSAID-binding pocket of PGHS-2 has been estimated to be about 17% larger than that of PGHS-1. This increased size may simply allow inhibitors to bind more tightly in the PGHS-2 active site,

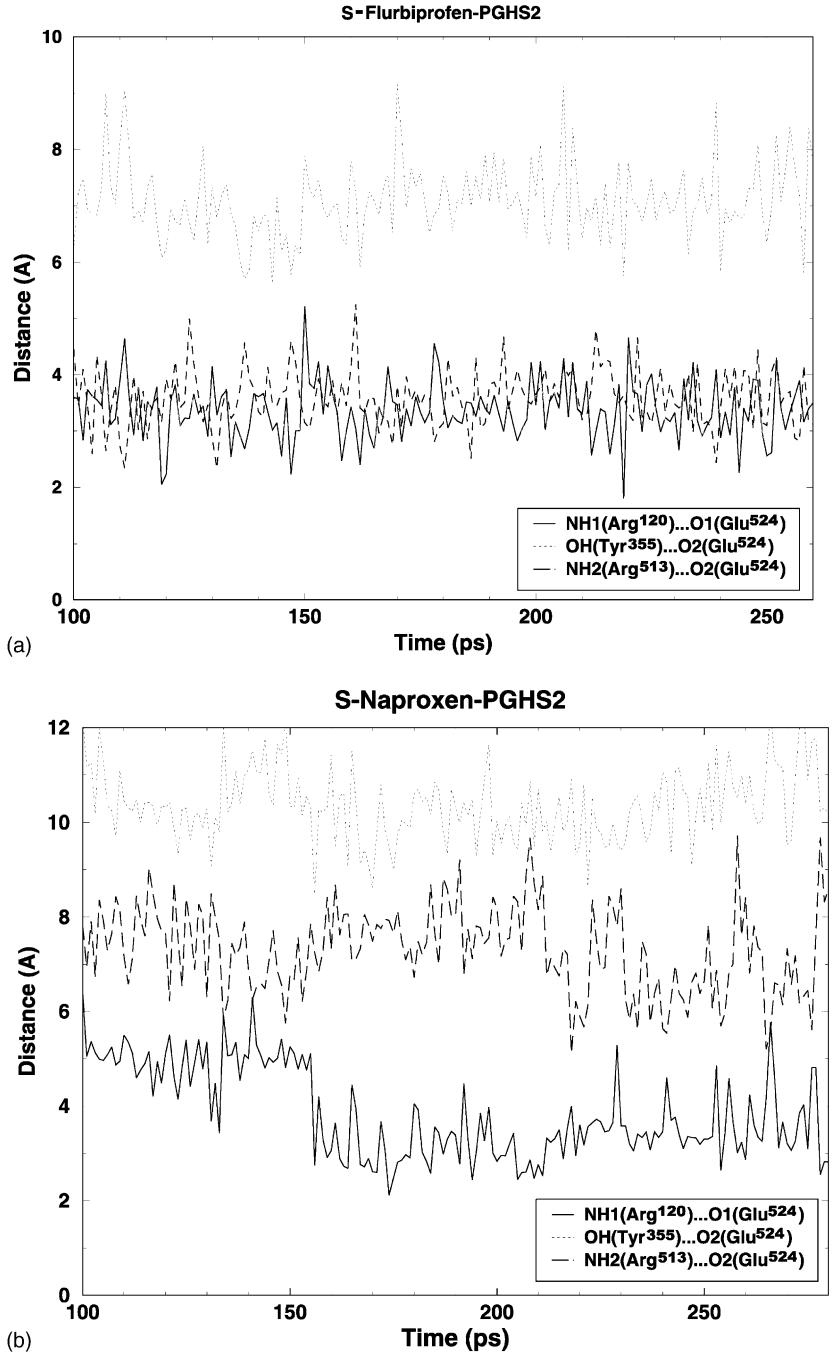
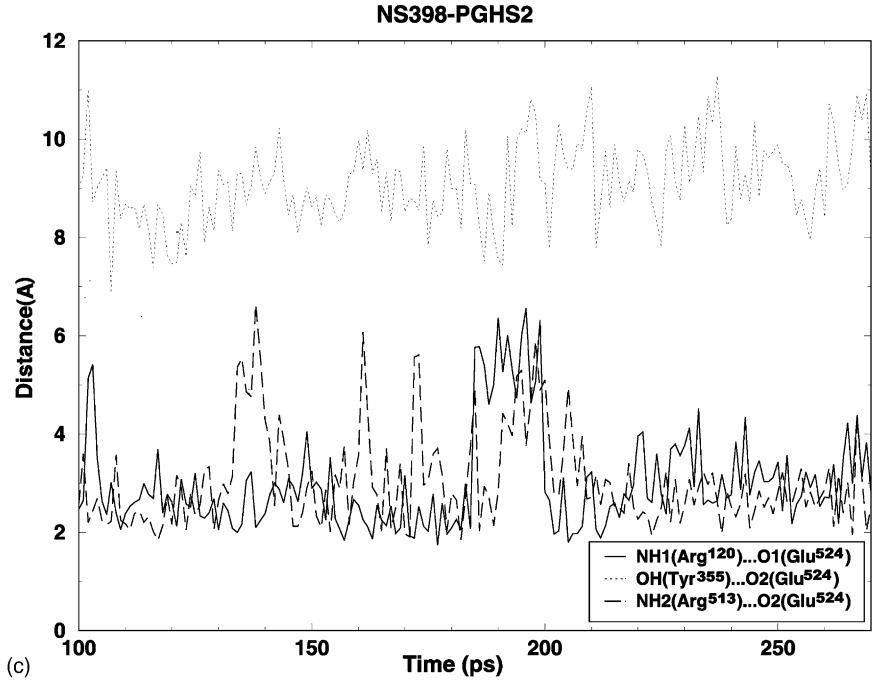


Fig. 6. Variation of distances of NH1 and NH2 of Arg^{120} with respect to O1 and O2 of Glu^{524}, of OH of Tyr^{355} with respect to O1 and O2 of Glu^{524}, and of NH1 and NH2 of Arg^{513} with respect to O1 and O2 of Glu^{524} in: (a) *S*-flurbiprofen–PGHS-2; (b) *S*-naproxen–PGHS-2; (c) NS-398–PGHS-2; (d) SC-58125–PGHS-2.



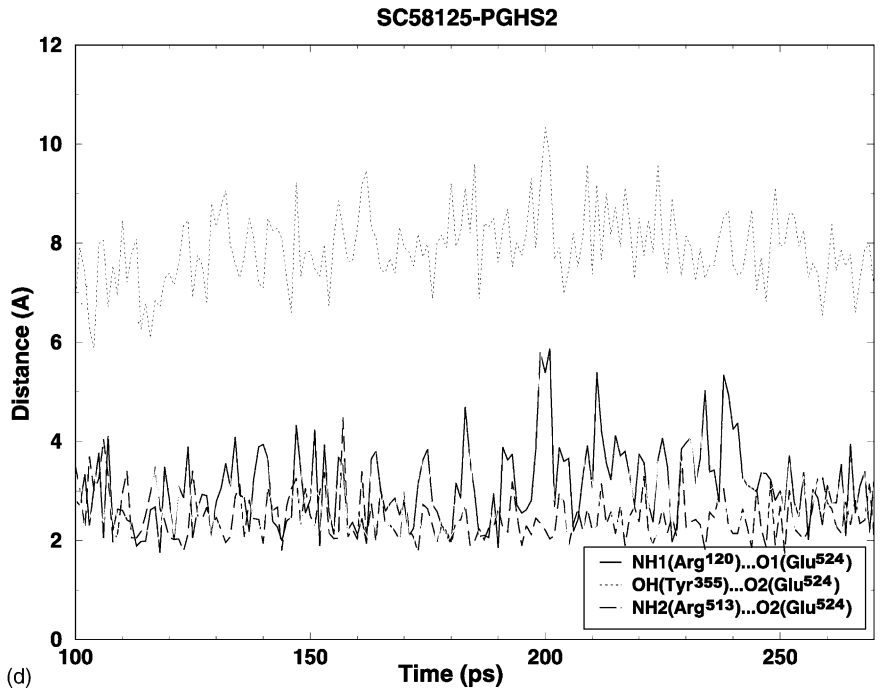


Fig. 6. (*Continued*).

reducing the relative importance of ionic interactions with Arg^{120} or permitting more avid binding of non-acidic inhibitors in PGHS-2. Moreover, the substitution of Val^{523} in PGHS-2 for Ile^{523} in PGHS-1 permits access to a pocket, or nook, near the mouth and adjacent to the central channel of the binding pocket, increasing the volume of the PGHS-2 NSAID binding site about 25% larger in PGHS-2 than in PGHS-1. This extra size is essential [54,55] for selective inhibition of PGHS-2 by NS-398 and SC-58125 (Fig. 4), and binding in this nook is probably also facilitated by Arg^{513}, which is within bonding distance of the sulphonamide group of SC-58125.

5. Conclusions

This paper examines the interactions of time-dependent PGHS-2 inhibitors with the receptor. Even though previous

experimental studies [58] have shown that time-dependent and time-independent inhibitors elicit almost identical enzyme conformations, the potential involvement of diverse conformational states cannot be completely ruled out. Therefore, a structure-based ligand design strategy based on X-ray crystallographic date in conjunction with a variety of molecular modelling techniques has been adopted to examine which interactions make a significant contribution to the biological activity. Our results point out that the combined use of Grid/AutoDock can provide such information.

Inspection of the separate contributions to the binding free energy revealed a marked dependence of the inhibitory activity with the electrostatic term.

Increasing the volume of the PGHS-2 NSAID binding site compared to that in PGHS-1 is essential for selective inhibition of PGHS-2 by NS-398 and SC-58125, and binding in this nook is probably also facilitated by Arg^{513}, which is within bonding distance of the nitro group or sulphonamide group of both compounds.

Likewise, results suggest that Tyr^{355} seems to be involved in the time-dependent inhibition of PGHS-2 through its interactions with the inhibitor, and/or the hydrogen-bonding network.

At the entrance of the cyclooxygenase cavity, we found H-bonding between Arg^{513} and Glu^{524} in all inhibitor–COX-2 complexes, except indomethacin–COX-2, *S*-naproxen–COX-2 and mefenamate–COX-2 complexes. These conformational differences may affect the kinetics of interaction. All the selective PGHS-2 compounds show the lowest van der Waals interaction energies, and the maximum molecular volumes. These features allow them to have specific contacts with His^{98}, Arg^{120}, Val^{349}, Leu^{352}, Ser^{353}, Tyr^{355}, Arg^{513} and Val^{523}.

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