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Original article

NSAIDs revisited: Putative molecular basis of their interactions with peroxisome proliferator-activated gamma receptor (PPARγ)

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Abstract

This paper describes molecular docking studies of a series of classical NSAIDs with PPARγ receptor, which has been pointed as a new target for the design of anti-cancer and anti-inflammatory drugs, and has been found to be responsible for some of the already established pharmacological effects observed for marketed drugs. The results show the molecular basis of PPARγ activation by non-selective COX inhibitors.

Keywords: Molecular docking; PPARγ; NSAIDs; Anti-cancer drugs; Anti-inflammatory drugs

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of prostaglandin biosynthesis through the blockage of cyclooxygenases (COX) [1]. In addition to their general use as anti-inflammatory, analgesic and antipyretic, NSAIDs have an emerging utility in the preventive chemotherapy of human cancer, as observed during the therapy of colon cancer by sulindac (**1**) [2–5]. Epidemiological studies indicated that NSAIDs are able to decrease the risk for developing lung cancer, consistently with an emerging role for eicosanoid biosynthetic pathways in human cancer development [6].

A large number of studies have now demonstrated that NSAIDs may exert some of their cellular actions through COX-independent mechanisms, which are corroborated by the fact that the therapeutic benefits of NSAIDs are typically observed at doses much greater than those required to inhibit the cyclooxygenases, suggesting that they are recognized by other targets [7–13]. This dual therapeutic profile could be very desirable and was recently illustrated by characterization of its molecular basis for COX-2 and p38 MAPK inhibition [14].

Among other potential targets of NSAIDs, the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors has been frequently cited [15–27]. Three isoforms of PPAR have been described, *i.e.* PPARα, δ, and γ, all of which bind to specific DNA sequences as heterodimers with the retinoic acid X-receptors [28]. PPARγ has been shown to be activated by the synthetic antidiabetic thiazolidinediones (TZDs), such as rosiglitazone (**2**) and troglitazone (**3**) (TGZ), as well as by prostaglandin D and J derivatives, which may function as endogenous activators (Fig. 1) [29,30].

TZDs inhibit the expression of various inflammatory proteins like iNOS, TNFα and MMP9 in macrophages and are beneficial in disorders such as inflammatory bowel disease [31,32]. Several anti-inflammatory mechanisms have been suggested, including inhibition of NF-κB, AP1, and STAT transcription factors by PPARγ [33]. Additional studies with classical NSAIDs have shown a potential therapeutic

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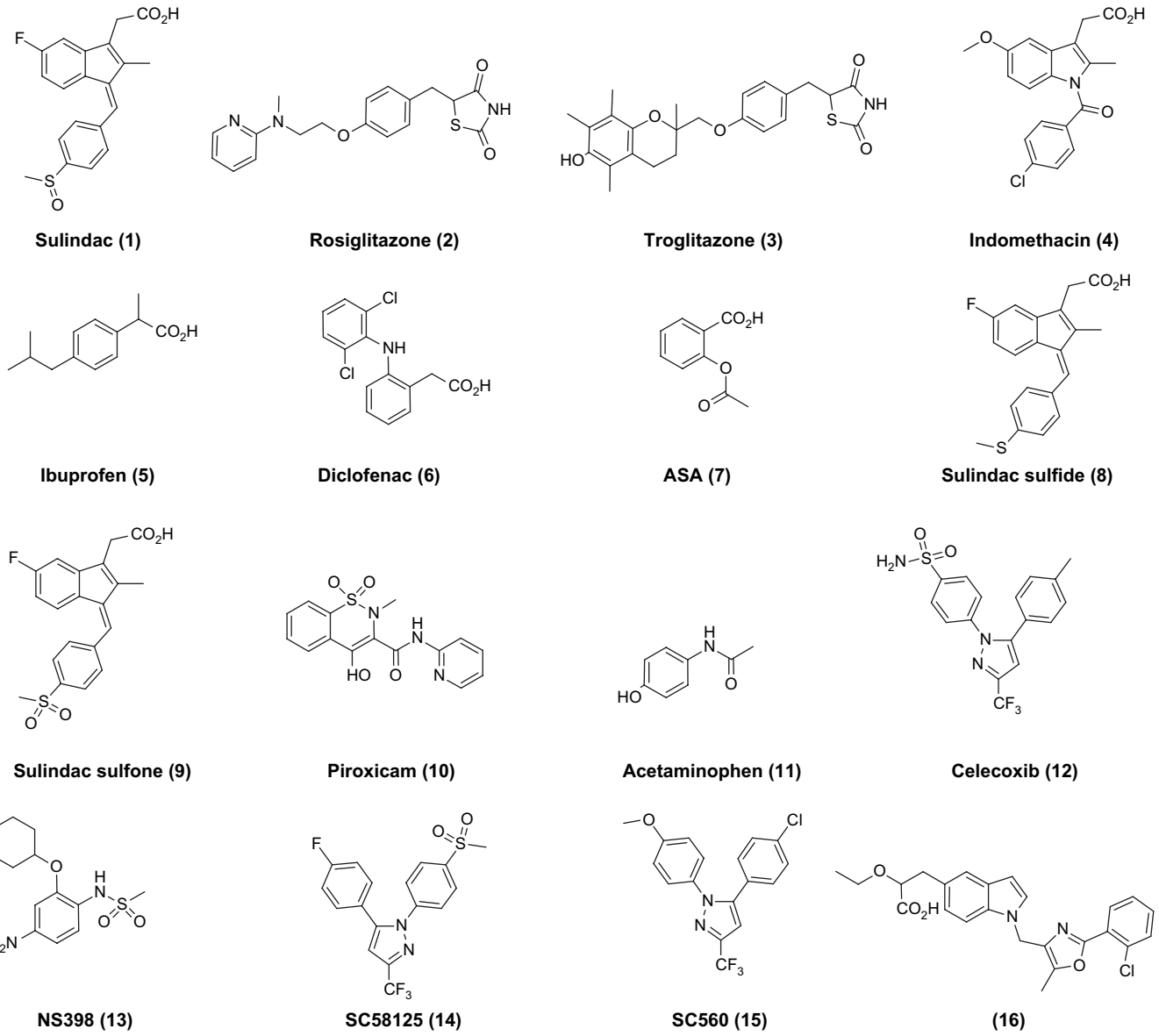

Fig. 1. PPARγ agonists and some NSAIDs evaluated in the docking studies with PPARγ.

role of these drugs in Alzheimer's disease, acting as anti-amyloidogenic substances through PPARγ activation, among other inter-connected pathways of the disease [24]. PPARγ has been implicated both as a tumor suppressor and tumor promoter and is expressed in many cancers, including colon, breast and prostate and PPARγ ligands are generally antiproliferative in these settings [34–37].

In addition, a variety of reports have shown the activation of PPARγ by NSAIDs and have correlated them to their anti-tumorigenic action [16–20]. Most importantly, it has been observed that this action seems to be dependent on the degree of selectivity they have for cyclooxygenases 1 and 2. For instance, the ability of various compounds to bind/activate PPARγ was examined in a study performed by Nixon and colleagues to assess the potential influences of this biochemical pathway on their reported anti-tumorigenic activities [16]. Many potential PPARγ ligands, including various eicosanoids and NSAIDs, were examined for PPARγ luciferase reporter activation in colorectal carcinoma (Caco-2) cells. This study revealed that, in general, products derived from lipoxygenases have a greater affinity for PPARγ than products derived from cyclooxygenases. Conventional NSAIDs that inhibit both COX-1 and COX-2 bind to PPARγ, in contrast, COX-1/2 selective inhibitors do not [16].

In general, conventional NSAIDs served as PPARγ ligands and induced luciferase activity from about 2–6.5-fold. Indomethacin (**4**) [38] (6.43-fold, at 50 μM) and ibuprofen (**5**) [39] (5.81-fold at 1000 μM) induced luciferase activity as effectively as TGZ (5.22-fold, at 10 μM) (**3**) (Fig. 1) [16]. Diclofenac (**6**) [40], ASA (**7**) [41], and sulindac sulfide (**8**) [42] also displayed relatively strong binding activity, 4.06-, 4.02- and 3.81-fold at 200, 10,000 and 50 μM, respectively (Fig. 1). Interestingly, the prodrug, sulindac (**1**), and its metabolite sulindac sulfone (**9**), weak COX inhibitors [42], were not as

effective in binding to PPARγ, showing binding activities of 2.51- and 3.33-fold, at 100 and 400 μM, respectively. On the other hand, sulindac sulfide (**8**), that inhibits COX non-selectively (IC_{50} ratio COX-1/COX-2 = 0.258) [42], showed the strongest binding activity (3.81-fold) (Fig. 1) [16].

Among the conventional NSAIDs tested as PPARγ ligands, piroxicam (**10**) [43] displayed the weakest binding activity (1.92-fold, at 1000 μM), while acetaminophen (**11**) [44] does not inhibit COX and also did not bind to PPARγ. Conversely, the COX-2-selective inhibitors were poor ligands for PPARγ. Celecoxib (**12**) [45], NS398 (**13**) [46] and SC58125 (**14**) [47] displayed no PPARγ binding activity. Likewise, the COX-1-selective inhibitor, SC560 (**15**) [48], did not bind to PPARγ. In general, the conventional NSAIDs that inhibit both COX-1 and COX-2 non-selectively could serve as PPARγ ligands and it was concluded that they may either exert their anti-tumorigenic actions by activating PPARγ or competing with the endogenous ligand(s) for PPARγ. However, any effects of COX-1 or COX-2-selective inhibitors on tumorigenesis have been concluded to be mediated by other pathways [16].

Based on these reports, the goal of the present study was to investigate the molecular basis of the interaction of NSAIDs and PPARγ with flexible docking studies using FlexE software [49]. This strategy provided models of ligand–receptor complexes that have been compared to PPARγ activation data reported by Nixon and colleagues [16]. The results provided a rational basis for future design of PPARγ ligands using as template the core of classical non-steroidal anti-inflammatory drugs.

2. Methods

2.1. General procedures

All calculations have been performed on a PC running under Linux Red Hat Enterprise version 3.0 platform. Structural manipulations were performed using Sybyl 7.3 [50].

2.2. Construction of the ligands

The construction of the ligands for FlexE was performed as for FlexX [51], using Sybyl version 7.3 [50]. The molecular structure of rosiglitazone (**2**, Fig. 1) has been withdrawn from the crystal structure of this drug bound to PPARγ (PDB code: 1FM6) [52]. Troglitazone (**3**, Fig. 1) has been drawn upon modifications from the crystal structure of rosiglitazone. The four possible diastereoisomers of troglitazone have been modeled in their neutral and ionized states. The other ligands' coordinates have been generated using the program Sketcher, available in Sybyl version 7.3. Next, the correct atom types (including hybridization states) and correct bond types were defined, hydrogen atoms were added and charges were assigned to each atom. Carboxylate groups present in the structure of classical NSAIDs were modeled in their ionized states at physiological pH. Rosiglitazone and troglitazone have been modeled in their neutral and ionized states, for comparison. Finally, the structures were energy-minimized, using the semiempirical AM1 method [53] available in the PC Spartan '04 software [54]. After this procedure, AM1 and PM3 point charges were assigned to the ligands [53].

2.3. Selection of protein crystal structures

Ligand-bound crystallographic structures of PPARγ are available in the Protein Data Bank [55]. In this study, 1FM6, 2GTK, 2F4B, 1KNU, 1ZEO and 2ATH were evaluated and selected for docking [52,56–60]. The active recognition site of the ensemble has been defined as the collection of residues within 10.0 Å of the bound inhibitor and comprised the union of all ligands of the ensemble. All atoms located less than 10.0 Å from any ligand atom were considered. 2GTK was used as a reference structure for the united protein preparation [56].

Proteins were prepared for the docking studies, using the Biopolymer module of Sybyl 7.3. Amber7 FF99 charges were attributed to the protein atoms [50]. The first step in the generation of suitable protein structures for the ensemble superimposition is the deletion of extra chains. In this work, chain A was kept while the others were deleted, since the software is able to deal only with monomers. Next, biopolymer protein analysis tool was used, in a stepwise process of analysis and correction of geometry parameters. For each structure, the description of an ensemble contains the definition of the protein atoms (via chain identifiers and hetero groups), the resolution of ambiguities in the PDB file (alternate location indicators, etc.), the location of hydrogen atoms at heteroatoms, and the definition of the active site atoms. The assignment of hydrogen positions has been made on the basis of default rules except for the definition of the hydrogen positions inside the histidine side-chain. The side-chains of lysine, arginine and the carboxylate groups of aspartic and glutamic acids have been modeled in their ionized states. Water molecules contained in the PDB file have been removed. All carboxylic acid and amino groups were modeled in their ionized forms.

3. Results and discussion

All the selected molecules for the molecular docking studies are depicted in Fig. 1. Care was taken to include in the studies the PPARγ agonists rosiglitazone (**2**) and troglitazone (**3**), and an indomethacin derivative belonging to the indole acetic acid class (**16**), which has shown to be a potent PPARγ agonist [56]; non-selective cyclooxygenase inhibitors such as ASA (**7**) [41], sulindac (**1**), sulindac sulfide (**8**) and sulfone (**9**) [42]; a selective COX-1 inhibitor, SC560 (**15**) [48] and a selective COX-2 inhibitor, celecoxib (**12**) (Fig. 1) [45].

The proposed interaction modes of the ligands in the active site of PPARγ were determined as the highest scored conformations (best-fit ligands) among 30 conformations and binding modes generated according to FlexX scoring function [50,51], which correspond to the structures with the most favorable free energy of binding, *i.e.* ΔG_{bind} (kJ/mol) (Fig. 2). It is worth of note that ΔG values shown in this study have been based on PM3 charges [53] (Fig. 2) due to a better

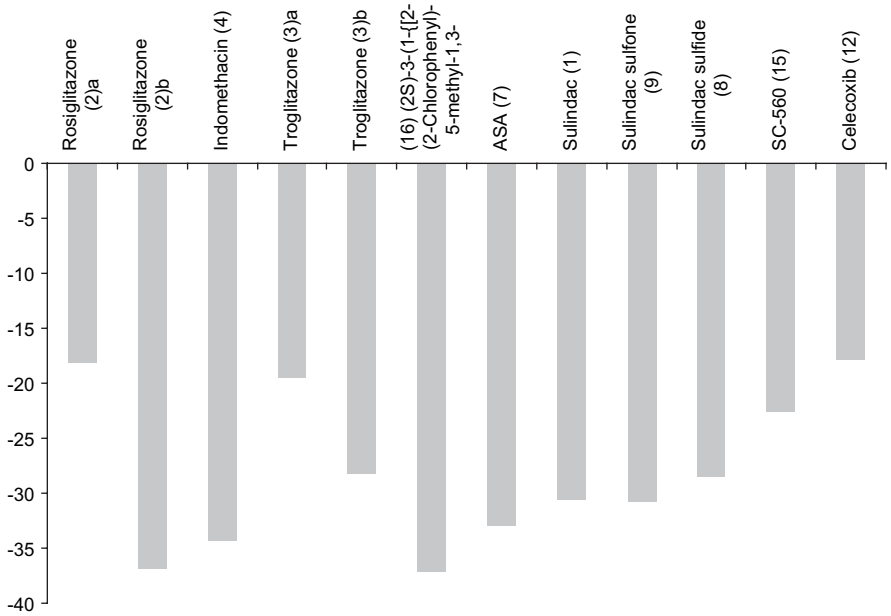

Fig. 2. *In silico* ΔG_{bind} values (kJ/mol) obtained for docking of rosiglitazone (**2**), troglitazone (**3**) and the selected NSAIDs to PPARγ by using FlexE. (a) and (b) Refer to the neutral and ionized states of rosiglitazone and troglitazone, respectively.

qualitative correlation of the binding energy (ΔG_{bind}) to PPARγ activation, since non-selective cyclooxygenase inhibitors, such as sulindac (**1**), sulindac sulfide (**8**), sulindac sulfone (**9**) and ASA (**7**), showed the most favorable binding energies, while highly potent and selective COX-1 (SC560) or COX-2 inhibitors (celecoxib) showed the worst energy values, corroborating PPARγ activation data reported in the literature [16] (Fig. 2).

The docking analysis was firstly performed with the co-crystallized ligands rosiglitazone (**2**) and (2*S*)-3-(1-{[2-(2-chlorophenyl)-5-methyl-1,3-oxazol-4-yl]methyl}-1*H*-indol-5-yl) 2-ethoxypropanoic acid (**16**) (PDB codes: 1FM6 and 2GTK) [52,56], to assess the efficiency of the FlexE program for the docking known ligands.

The top docking of pose obtained for rosiglitazone (**2**, Fig. 3a) shows good correspondence to the crystallographic structure [52], with a RMS value of 0.68 Å [52], and reproduces the binding mode of this compound, showing hydrogen bonding interactions of the thiazolidinedione ring with Ser289, Tyr473, His223, His449 [52]. Interestingly, the ionized form of this compound gave a better ΔG_{bind} value (−36.84 kJ/mol) in comparison to its neutral form (−18.17 kJ/mol) (Fig. 2). These results are in opposition to what has been observed in rigid receptor docking of this compound and PPARγ [61].

FlexE docked (**16**) at the same pocket as in the crystal structure, with a RMS value of 0.11 Å (Fig. 3b). According to the structural studies reported recently, hydrogen bonding interactions of the carboxylic acid groups of known PPARγ agonists with Tyr473 and His449 in the AF-2 helix of the receptor represent the structural basis for the PPARγ agonist activity due to stabilization of this secondary structure [56]. Also, interaction with His323 gives extra stability to the ligand–PPARγ complexes. Correspondingly, Fig. 3b shows a network of hydrogen bonding interactions of the carboxylate group of the indomethacin derivative (**16**) with Ser289, His323, His449 and Tyr473. This region is known as arm I, and is composed mostly of polar residues [62]. Furthermore, additional van der Waals interactions of the 2-Cl–C_6H_4 moiety of **16** with Val339, Ile341 and Leu353 (Fig. 3b) in the vicinity of a hydrophobic region named arm II of the receptor [62] explain the affinity of this compound to PPARγ. This result shows a reasonable qualitative assessment of binding mode prediction with FlexE method [49].

Since it has been reported that troglitazone (**3**) is an equal mixture of four stereoisomers involving two asymmetric centers, with each of them having similar pharmacologic effects, we have modeled the four stereoisomers and found that the *R*,*R*-isomer presented the best ΔG_{bind} value (Fig. 2) [63]. Surprisingly, it presented a higher (unfavourable) ΔG_{bind} value (−28.193 kJ/mol) when compared to indomethacin (**4**) (−34.37 kJ/mol) (Fig. 2). We hypothesized that this result is related to some limitations in the calculation of the charge distribution in the thiazolidinedione ring in its ionized form, although the ΔG_{bind} observed for this compound in the neutral form was even worse (−19.50 kJ/mol) as observed for rosiglitazone (**2**, Fig. 2). However, since the latter also bears the thiazolidinedione moiety, steric effects generated by the trimethyl-chromanol ring in the evaluation of ΔG_{bind} by FlexE are probably being overestimated.

The next step in this work was the docking evaluation of COX inhibitors indomethacin (**4**), acetyl salicylic acid (ASA) (**7**), sulindac (**1**), sulindac sulfide (**8**), sulindac sulfone (**9**), SC560 (**15**) and celecoxib (**12**). The top ligand–receptor poses obtained for these compounds with FlexE are depicted in Figs. 3–5.

The best docking pose obtained for indomethacin (**4**) shows that its carboxylate group hydrogen bonds to His323, His449,

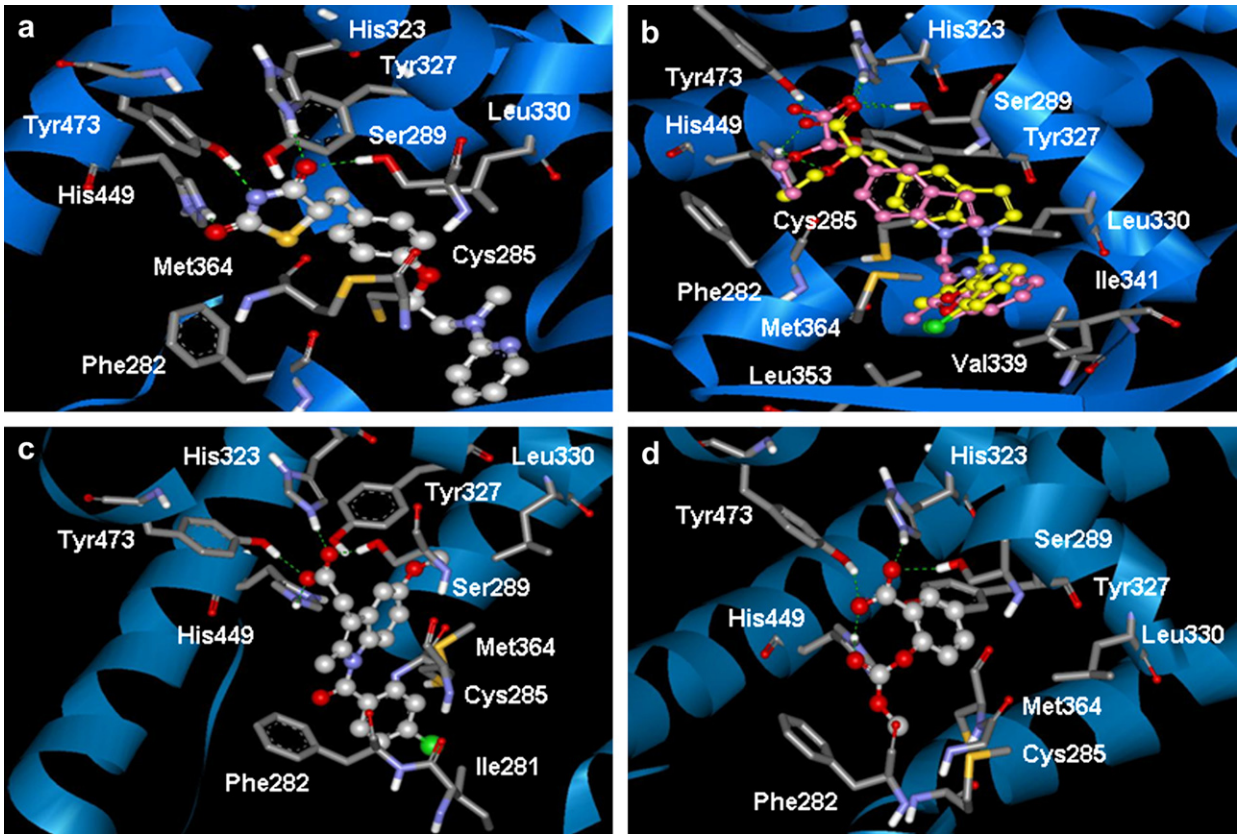

Fig. 3. (a) Top pose obtained for rosiglitazone (**2**); (b) superimposition of co-crystallized indole acetic acid ligand (**16**) (yellow) and top pose obtained with FlexE (magenta); (c) top pose obtained for indomethacin (**4**) and (d) top pose obtained for ASA (**7**). Hydrogen bonds are shown as green dashed lines (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Tyr473 and Ser289 (Fig. 3c). Additionally, its *p*-Cl group is in close van der Waals contact with Ile281, which is part of arm II of PPARγ. This result supports the excellent ΔG_{bind} found for this drug and further corroborates PPARγ activation by this compound (Fig. 3c). Following the analysis, we inspected the best docking pose obtained for ASA, which showed, as expected, that this NSAID is able to perform the same hydrogen bonding interactions observed for indomethacin (Fig. 3d). Also, the oxygen atom of the ester carbonyl group hydrogen bonds to His449 (Fig. 3d). However, no van der Waals contacts have been observed.

Docking of sulindac (**1**), which bears a sulfoxide group, has revealed new interacting possibilities at the ligand binding site. Even though its carboxylate group hydrogen bonds to His449 and the fluorine atom hydrogen bonds to His323, we have also observed a strong interaction at the vicinity of the ligand binding site, between the oxygen atom of the sulfoxide group and Arg288 (Fig. 4a).

The visual inspection of the top pose obtained for sulindac sulfide (**8**) (Fig. 4b) also reveals key hydrogen bonding interactions with Tyr327, His323 and Ser289. An extra feature presented by this NSAID is the proximity of the methylthio moiety to the hydrophobic amino acid residue Leu356, within van der Waals contact (Fig. 4b). The hydrophobic interaction performed by this compound may be related to a pharmacophoric demand to enhance PPARγ activation. This assumption is supported by the fact that the ligands' binding site, south to the AF-2 helix [56], is mostly lined with hydrophobic amino acid residues.

Finally, analysis of the top pose of sulindac sulfone with PPARγ (Fig. 4c) shows hydrogen bonding interactions of the carboxylate group of sulindac sulfone (**9**) with Tyr473, His323 and Ser289. An additional hydrogen bond is performed by the sulfone group with the −NH moiety in the backbone of Phe363.

Following the docking studies, we have analyzed the putative interactions of SC560 (**15**), a selective COX-1 inhibitor, and PPARγ (Fig. 5a). Only one hydrogen bond, between one fluorine atom belonging to the CF_3 group and −NH in the backbone of Phe282, was observed. However, the *p*-Cl−C_6H_4 ring of SC560 is in van der Waals contact with the side-chain of Leu330 and Met364. Additional hydrophobic contacts have also been observed between the CF_3 group of SC560 (**15**) and the side-chain of Ile281 (Fig. 5a).

The top pose obtained for celecoxib (**12**) complexed with PPARγ (Fig. 5b) showed hydrogen bonding interactions between the sulfone group of this selective COX-2 inhibitor with Tyr473, His323, His449 and Ser289. Also, the *p*-CH_3−C_6H_4 ring is in van der Waals contact with Met364 and Leu330. Finally, the CF_3 group of celecoxib is in van der Waals contact with Ile281 (Fig. 5b).

Comparing the interactions performed by these two selective COX inhibitors and the other structures, it becomes very obvious that, even when they are able to stabilize the AF-2

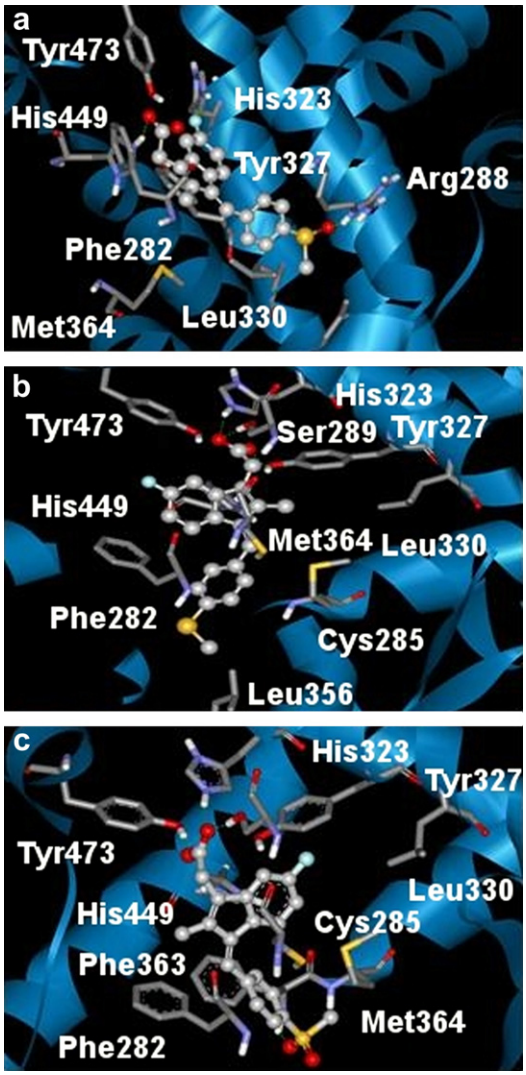

Fig. 4. Top poses obtained by molecular docking of (a) sulindac (**1**), (b) sulindac sulfide (**8**) and (c) sulindac sulfone (**9**), with PPARγ. Hydrogen bonds are shown as green dashed lines (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

helix by means of hydrogen bonds, they may not be strong enough because they do not involve ionic groups.

Finally, in order to better understand the molecular basis of the differential activation of PPARγ performed by selective and non-selective COX inhibitors, we investigated the molecular surfaces of the amino acid residues pertaining to the regions named arm I and arm II of the ligand binding domain (LBD) [62].

The visual analysis of the best ligand–receptor docking poses of rosiglitazone (**2**), sulindac sulfide (**8**), celecoxib (**12**) and SC560 (**15**) (Fig. 6a–d) clearly demonstrates that rosiglitazone (Fig. 6a) possesses a polar head and also a hydrophobic tail that is able to reach part of arm II in PPARγ, as observed experimentally (Fig. 6a) [52].

When we compare the same surfaces with the top docking pose of sulindac sulfide (Fig. 6b), it becomes clear that it possesses not only the same polar head as rosiglitazone but also the hydrophobic tail that is represented by the methylthio-

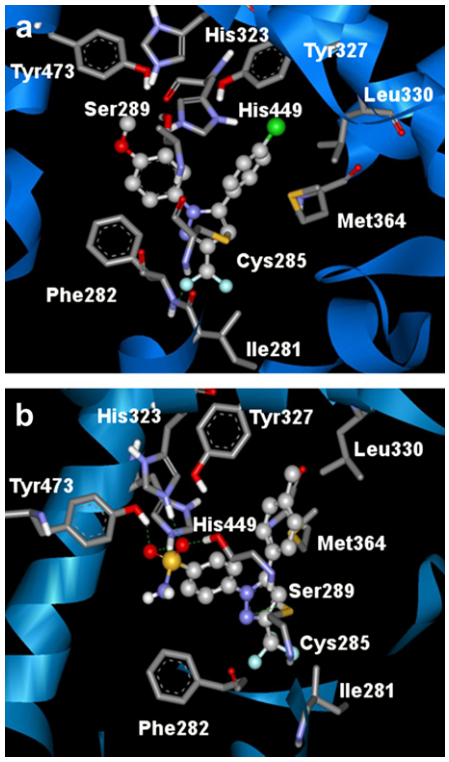

Fig. 5. Top poses obtained by molecular docking of (a) SC560 (**15**) and (b) celecoxib (**12**), with PPARγ. Hydrogen bonds are shown as green dashed lines (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

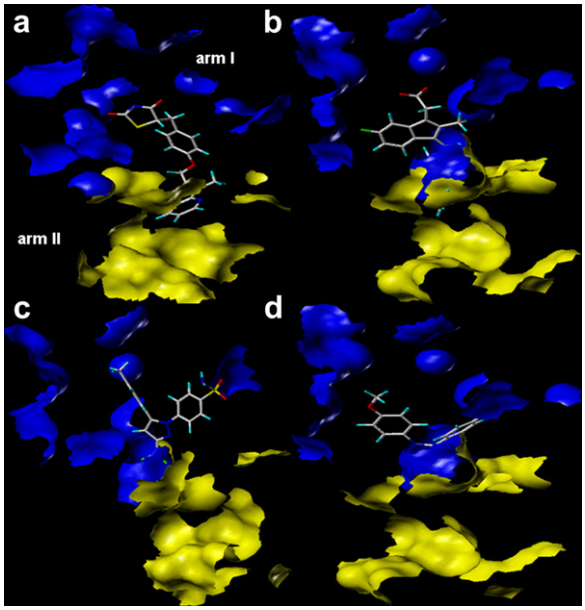

Fig. 6. Molecular surfaces of the amino acid residues pertaining to the ligand binding site of PPARγ and the top poses obtained by molecular docking of (a) rosiglitazone (**2**), (b) sulindac sulfide (**8**), (c) celecoxib (**12**) and (d) SC560 (**15**), with PPARγ. Blue and yellow surfaces indicate amino acids from arm I (mostly polar residues) and arm II (mostly hydrophobic residues), respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

benzilidene group, which is able to partially reach arm II. However, neither celecoxib (Fig. 6c) nor SC560 (Fig. 6d) are able to reach arm II and also do not bind tightly to arm I as already observed above (Fig. 5a–b). Thus, we may hypothesize that both factors keep selective COX inhibitors from stabilizing the AF-2 helix that is important for keeping the receptor in its active conformation.

4. Conclusions

In this paper we used flexible molecular docking tools to investigate putative binding modes of NSAIDs with PPARγ that may help in the design of new ligands of this receptor, using as template the core of classical non-steroidal anti-inflammatory drugs, as it has already been reported for indomethacin derivatives [57]. The binding scores (ΔG_{bind}) of the ligands from FlexE were found in accordance with their relative PPARγ activation.

Additionally, the investigation of the predicted binding mode of sulindac sulfide has shown that it possesses the pharmacophoric requisites to bind PPARγ, that is, a polar head and a hydrophobic tail, which is partially buried in the region encompassed by arm II, a hydrophobic region of the receptor, what may be accounting for its better activity. On the other hand, celecoxib and SC560, selective COX inhibitors, do not bear the same structural requisites, being poor PPARγ ligands evidentiated by weak binding in arms I and II. In short, based on these data, the design of new molecules bearing either the core of classical non-steroidal anti-inflammatory drugs or modified skeletons of selective COX inhibitors that fulfill these pharmacophoric requisites may be useful in the search for new candidates to the therapy of a variety of diseases.

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