Optimization and Validation of a Docking-Scoring Protocol; Application to Virtual Screening for COX-2 Inhibitors

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To exploit available structural information about the cyclooxygenase enzyme for the virtual screening of large chemical libraries, a docking-scoring protocol was tuned and validated. The screening accuracy was assessed using a series of known inhibitors and a set of diverse a priori inactive compounds that was seeded with known active ligands. The major parameters of the DOCK algorithm were investigated. A large improvement of the results was obtained on tweaking some of them. The generated complexes were rescored using six scoring functions. In this way, the striking importance of this step was demonstrated, as well as the good performances of DOCK energy and SCORE for this target. The results were further improved via a consensus approach. As a first application, a subset of a large compound library was screened using this protocol. Among the compounds that were selected for biological testing, a third of them turned out to have a significant enzyme inhibition.

Introduction

The number of therapeutic target three-dimensional structures determined by X-ray diffraction, NMR spectroscopy, or homology modeling is gradually increasing.[1,2] They have been important tools for medicinal chemists to understand essential aspects determining the ligand binding affinity. Many computational methods have been developed to take advantage of this structural information in order to identify small molecules that bind or inhibit the activity of the biological target.[3] The first step in this process is usually referred to as docking and consists of finding the low-energy binding modes of a ligand within the active site of a macromolecule. During a second step, named scoring, the binding energy is estimated with a score or determined more precisely through time-consuming methods. Such structure-based techniques can be involved at all stages of the drug design process.[3a,4,5] They are used to generate new ideas about ways of improving an existing ligand and for the development of new alternative bonding skeletons. Due to computer power and algorithm performance improvements, it is also possible to screen large chemical libraries in a timeline that is useful to the pharmaceutical industry.[5] The biological data for both active and inactive molecules are required to make use of ligand-based techniques, whereas structure-based methods require only the 3D coordinates of the target structure, preferably in complex with ligands. Therefore, they can be used at the very beginning of the drug-design process for preliminary screening. However, limitations remain in this approach, which are the subject of ongoing research efforts. The major problems lie in the accuracy of scoring functions and the way to include the protein flexibility as well as water penetration of the binding site.[6] In this paper, a structure-based approach is applied using the available structural information about COX-2 in order to screen libraries to find new potent inhibitors.

Cyclooxygenase catalyzes the first step of the bioconversion of arachidonic acid to prostaglandins and thromboxanes. The widely used pharmacological class of nonsteroidal antiinflammatory drugs (NSAIDs) acts via inhibition of this enzyme.[7] Two isoforms of this membrane protein have been discovered.[8] The first isoform, COX-1, is constitutively expressed particularly in the gastrointestinal tract and the kidneys and is responsible for the physiological production of prostaglandins. The other isoform, COX-2, is induced during the inflammation process. The COX-2 enzyme is a major therapeutic target for inflammatory diseases, since selective inhibitors have been shown to significantly reduce gastrointestinal and renal side effects compared to classical NSAIDs. Indeed, the nonselective COX-2 NSAIDs are inhibiting both the constitutive COX-1 isoform, which is involved in the gastrointestinal and renal homeostatic functions, and the inducible COX-2 isoform, the expression of which is increased by inflammatory stimuli.[9] Research efforts to design selective COX-2 inhibitor have resulted in a variety of antiinflammatory drugs such as celecoxib (Celebrex),[10] rofecoxib (Vioxx),[11] and valdecoxib (Bextra)[12] (Figure 1). Other therapeutic applications of selective COX-2 inhibitors are also under investigation, including cancer[13] and Alzheimer disease prevention.[14]

Several 3D and 2D QSAR studies of COX-2 inhibitors have been reported.[15] As three-dimensional structures of COX-2 complexed with various ligands are available, structure-based methods can also be used for exploiting this valuable structural information. Prediction of the binding mode and explanation of the selectivity of small

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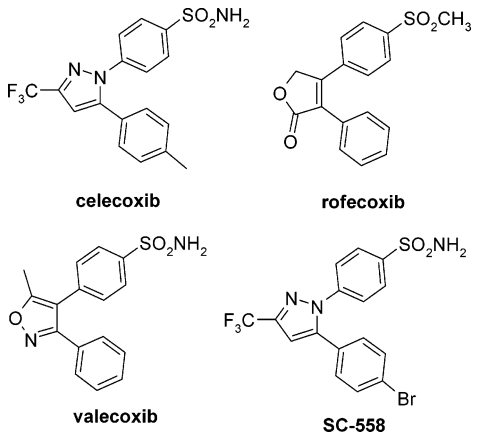


Figure 1. COX-2 specific inhibitors on the market: celecoxib, rofecoxib, valdecoxib, and SC-558.

sets of ligands were attempted with accurate but time-consuming techniques.[16] Otherwise, automated docking methods have been used to estimate the COX-2 affinity for large molecular databases. A few virtual screening studies have been reported in the literature.[17] This target was found to be somewhat demanding for the docking methods, especially with regard to the scoring stage.

This study aimed to find out a reliable and computationally efficient docking-scoring strategy for virtual screening of large libraries for this demanding target. The main docking parameters of the docking software were investigated. They were tuned and validated using two sets of compounds: a series of congeneric ligands, the inhibitory potencies of which have been measured, and another test set dedicated to assess the ability of the protocol to distinguish between active and inactive compounds. The complexes generated with DOCK were rescored using six scoring functions, including some which have never been tested for this target before. The best scoring functions for this target were identified and combined via a consensus approach. As a first application, a subset of a large library made up of commercial compounds was screened using this protocol, a dozen compounds were selected, and their biological evaluation is reported.

Results and Discussion

Protein Structure. As the X-ray crystal protein structure is the experimental basis of docking simulations, its choice and its preparation is of particular importance. First, the two X-ray crystal structures of SC-558 bound to COX-2 (PDB codes 6cox and 1cx2) were compared. The orientations of the sulfonamide substituent differ between the two structures, as can be expected because oxygen and nitrogen atoms cannot be distinguished in the electron density maps at the atomic resolution of these structures. There are also slight residue conformational changes. Concurrently, we considered the histidine 90 ionization state, which can only be assigned by analyzing its hydrogen-bonding pattern with the sulfonyl group of the ligand. The ϵ- and δ-tautomers were tried.

Whatever the protein structure and the histidine ionization state considered, most of the 10 best scored binding modes found for SC-558 are in agreement with the crystal structure. These poses only differ in the conformation of the sulfonamide group. Nevertheless, an oxygen atom of the sulfonyl group is found to interact usually with the histidine. In that case, the His90 should be protonated on the Hϵ to form a favorable hydrogen bond with the ligand. The energy score of the ligand bound to the protein with the ϵ-tautomer of His90 is also a little higher than the score for the alternative ionization state. A majority of the congeneric ligands (about 85%) have a pose very close to the crystallographically determined binding mode of SC-558. This ratio remains roughly constant, whatever the crystal structure and histidine ionization state considered. As the ligands of the congeneric set have the same kind of scaffolds and substituents, they are expected to have similar binding mode. Therefore, these ligands are considered to be correctly positioned in the enzyme pocket by this docking procedure. Among the remaining 15% of the congeneric ligands for which the binding mode predicted differs from the experimentally determined structure, there is a larger ratio of moderately active compounds. The induced fit of the protein that is not taken into account during the docking process may also stand for these wrongly positioned ligands, particularly for spiroheptene series. As for the screening validation set, the enrichment factor obtained with the crystal structure 1cx2, the His90 of which was protonated on Hϵ, is overall higher than using the other form (Table 1). These results suggest that the His90 is eventually more likely in its ϵ-tautomer state. Anyway, the inhibitors that bear a methyl sulfone substituent are known usually to have higher affinity than the corresponding sulfonamide derivatives, and they can only make a hydrogen bond with the ϵ-tautomer of the His90. As a result, this ionization form has been used in the following. No significant variation of the performances was observed using either of the crystal structures. Anyway, it is not really surprising owing to the small conformational changes noticed between the 1cx2 and 6cox structure. In the following, we used the 1cx2 crystal structure.

Table 1. Enrichment Factor Obtained with the Two His90 Protonation States

% top scorers	3	6	12
EF for ϵ-tautomer	2.6	1.8	2.6
EF for δ-tautomer	1.1	1.6	2.3

Three sets of spheres were tested to assess which one is the more suitable to define the enzyme binding pocket. First, we checked that the cocrystallized ligand SC-558 is docked correctly using any of the sphere sets. Figure 3 demonstrates that this choice causes a dramatic variation in terms of discrimination between active and inactive compounds for the screening validation set. The calculation speed was also significantly affected. The initial sphere set yielded far better results than the reduced sphere set: 27 active ligands were found among the top scored versus 19, and 9 inactive compounds were found versus 23. However, the calculation is 5 times quicker with the reduced set of spheres (ca. 0.5 min per ligand on average vs 2.6 min). The biased set of spheres derived from the cocrystallized ligand structure exhibits results similar to the initial sphere set, and the calculation duration is about the same (2.3 min per ligand). These results suggest that the initial set made up of 57 spheres represents a

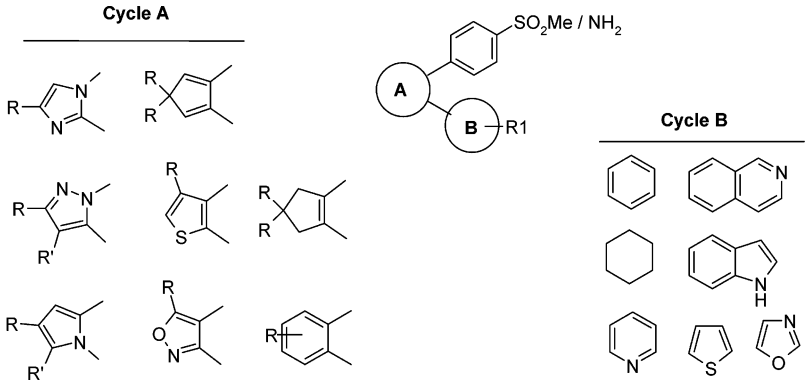


Figure 2. Common scaffolds of the 355 congeneric set ligands.

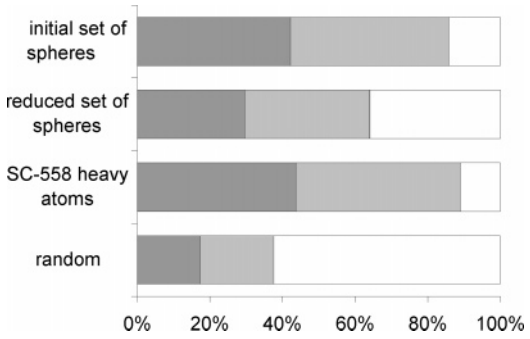


Figure 3. Comparison of the sets of spheres in terms of the percent of active ligands among the 64 top-ranked molecules of the screening validation set and calculation durations.

suitable definition for the binding pocket and it is used in the following.

Conformational Sampling. In an attempt to improve the modest results obtained in preliminary tries using standard default parameters, we investigated the main docking parameters of DOCK. The docking process even failed to generate a correct binding mode for some structural series of the congeneric ligands with these parameters. Therefore, we first considered the ligand conformational sampling. Actually, it turned out not to be sufficient to treat properly such structures and a related issue was noticed in fragment reattachment using the anchor-first search method. Therefore, the flexibility parameters were modified according to the specific structural features of COX-2 inhibitors of the congeneric inhibitors. However, such parameters heavily affect the calculation speed as well, which makes it necessary to find a compromise that is suitable for the screening purpose.

First, a sampling was introduced for the torsion angle between the sulfonyl group and a carbon within an aromatic cycle, while default parameters do not allow rotation of such bonds. The experimental binding mode of SC-558 cannot even be reproduced using these latter parameters. The same preferred torsion positions as for sulfonyl−sp^2-hybridized carbon bonds (±30°, 90°, and 150°) were assigned to sulfonyl−aromatic carbon links, and it was specified that this torsion may be energy-minimized. Using these modified parameters, the binding mode of SC-558 can be reproduced fairly well, as mentioned in the previous section. Using default parameters, most of the congeneric set ligands, the scaffold of which is composed of three phenyl cycles (Figure 2), have poor ranks, although some of them are highly active compounds (three-fourths have a $pIC_{50} > 7.5$). Actually this failure stems from the rigid treatment of this scaffold. Four torsion positions (0°, ±90°, and 180°) were allowed as well as the minimization of the bonds between two aromatic carbons. In this way, better scores are obtained for this series of ligands, so that their ranking is improved.

The default flexibility allowed between the aromatic cycles (only 0° or 180°) appeared not to be sufficient. This causes steric hindrance into the inhibitor scaffold during the fragment-linking step of the anchor-first search method. Indeed, the three five- or six-membered rings cannot be coplanar in such structures (Figure 2). For most of the congeneric inhibitors, these steric repulsions prevent the algorithm from reattaching the fragments and properly building the ligands, during the incremental construction of the anchor-first search method. In an attempt to address this issue, we increased the amount of steric clashes that were allowed during conformational sampling by lowering the clash overlap parameter from 0.5 to 0.3. Despite this energy minimization step, some of the generated conformations were still highly constrained. Therefore, we enlarged the number of torsion positions allowed between the cycles instead. Values of ±90° were allowed as well as the minimization of this torsion angle. In this way, compound conformations are correctly generated and reliable scoring value can be calculated for all the ligands of the congeneric set. Using these modified parameters, the performances on the screening validation set are significantly improved. It increases the enrichment factor from 1.2 to 3. Above all, ranking of the ligands with the triphenyl scaffold is remarkably improved. These parameters are still widely applicable, since they allow us to treat correctly the highly diverse molecules belonging to the screening validation set. Finally, a loss of speed in the calculation by 2 times is the price to pay for the enrichment improvement using these modified conformational sampling parameters.

Conformational Search and Matching. We compared the simultaneous and anchor-first search methods. Using either of the two methods the best scored pose of the cocrystallized ligand is similar to the crystal structure. In addition, roughly the same fraction of congeneric ligands has a binding mode that does not deviate from that of the cocrystallized SC-558. Similar results are also obtained for the congeneric set, but virtually no discrimination between active and moderately active ligands is obtained. In contrast, a better discrimination between the active ligands and the

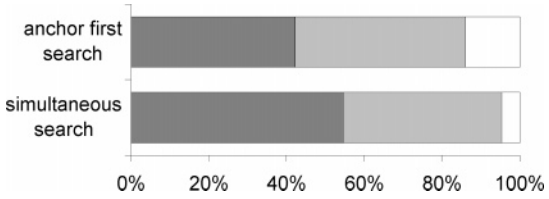


Figure 4. Comparison of the two conformational search methods available in DOCK in terms of the ratio of active ligands among the 64 top-ranked molecules of the screening validation set.

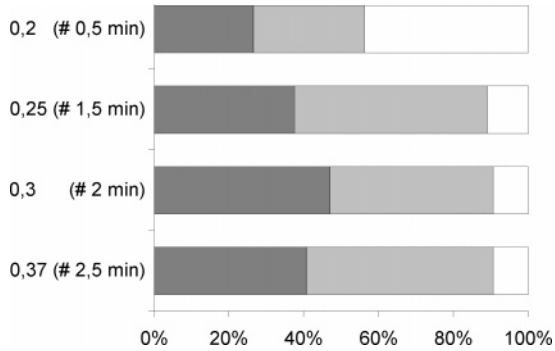


Figure 5. Variation of the active ligand ratio among the top-ranked molecules of the screening validation set according to the distance tolerance values.

diverse inactive compounds of the screening validation set is achieved with a simultaneous search method (Figure 4). 55% of active compounds of the validation set are found among the top-ranked ligands, whereas only 43% of them are retrieved using the anchor-first search method. The enrichment factor corresponding to active compounds reaches 3.1 (vs 2.4 with the anchor-first search) and only three inactive compounds are found among the top-scored ligands. The other molecules are moderately active compounds, as expected for virtual screening. Furthermore, the calculations are only slightly quicker using the anchor-first search method when a set of diverse compound is docked. The poorer results obtained with anchor-first method may be attributable to the difficulty in finding an appropriate anchor for COX-2 inhibitors, since protein−ligand interactions are mainly hydrophobic. Even the multiple anchor parameter did not preclude that problem. Intramolecular constraints in the tricyclic moiety, which is common to the inhibitors of the congeneric set, may also prevent the algorithm from performing properly.

The performances and calculation speed depend critically on the value of another parameter: the distance tolerance. Consequently, special attention was brought to find a suitable value for this parameter for both conformational search methods. Using the anchor-first search method, the best discrimination ability on the screening validation set is obtained with 0.3 (Figure 5). In addition, the speed of the calculations decreases, when the distance tolerance is enlarged, so that the value of 0.3 is a good compromise between the screening performances and the computational speed. With the simultaneous search method, the trend is more dramatic. Only the default value of 0.25 leads to calculation sufficiently quick to allow application to virtual screening. Larger or smaller values decrease dramatically the calculation speed (more than 10 min per ligand).

Other parameters were also inspected to assess their influence on the protocol performances. The most suitable value for the node minimum parameter turned out to be 4. Calculations are slower with the default value of 3 and it does not yield better results. Besides, the discrimination power on the screening validation set is lower using a value of 5. For the conformation cutoff factor, which controls the sampling of the simultaneous search method, values of 4 and 5 gave similar performances. There is a slight decrease in calculation speed with 4. However, owing to the slender difference, the default value of 5 was chosen in this study so as to keep parameters as widely applicable as possible, in agreement with the purpose of the virtual screening.

The score convergence with up to 10 runs using different random seed numbers was investigated. An average difference between one score and the minimum value among five scores of 1.6 kcal is observed for the 355 ligands of the congeneric set. These score variations are rather large, as most of the scores range from −30 to −50. In contrast, the rank variations are less significant, since the correlation coefficient between the rankings corresponding to above-mentioned case is higher than 0.9. For the screening validation set, no significant improvement is detected using the minimum value of three or five estimates for each compound. The results are just steadier, but the calculation durations rise proportionally. For these reasons, a single seed value was used for the screening application.

The above-described tuning of some parameters that may affect the docking step accuracy led to a significant improvement of the results compared with use of the default parameters. In particular, a good discrimination between active COX-2 inhibitors and inactive compounds was obtained via the simultaneous search method combined with an appropriate distance tolerance value. Suitable modifications of the ligands flexibility parameters were also required to treat properly series of known inhibitors. Despite these alterations related to the studied target, the docking parameters are still widely applicable, as demonstrated by the correct treatment of the inactive compounds belonging to the screening validation set. Besides, the calculation speed has been kept at a reasonable level (ca. 2 min per ligand) for the purpose of virtual screening. These docking parameters combined with the DOCK energy scores perform fairly well in discriminating between active and inactive compounds of the screening validation set. This represents the usual virtual screening conditions. In contrast, this protocol did not allow us to distinguish as well the known active from the moderately active ligands of the congeneric set. Although such discrimination is not really required for virtual screening application, we tried to improve this latter result as well as the discrimination ability on the screening validation set by investigating the scoring step.

Rescoring. In an attempt to improve the results obtained beforehand with DOCK energy, a rescoring approach has been carried out using a variety of other scoring functions. Figures 6 and 7 display the results of the two validation sets for DOCK contact, SCORE, FlexX, GOLD, ChemScore, and PMF. The performances of the initial scoring function DOCK energy are also reported for the purpose of comparison, as well as the

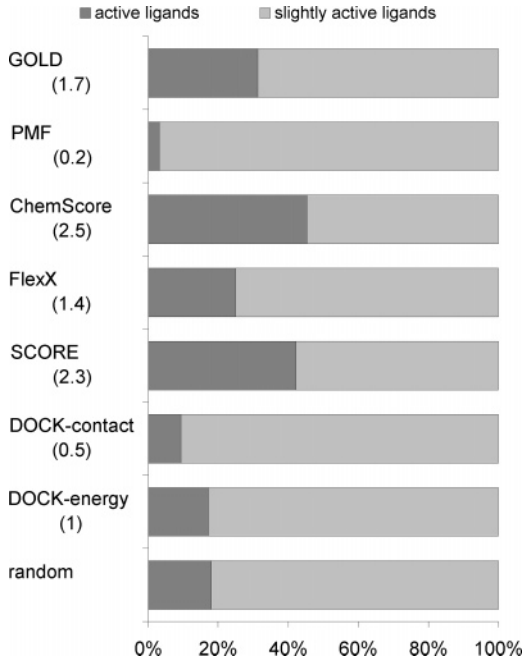


Figure 6. Comparison of the rescoring results on the congeneric ligand set. The enrichment factors are reported in brackets.

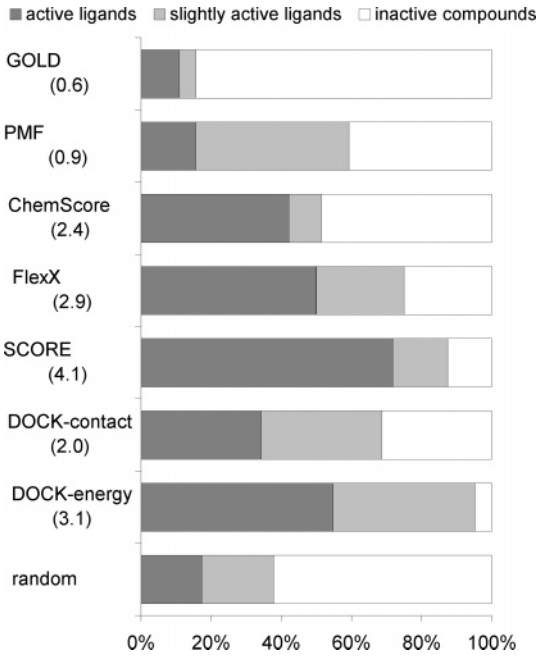


Figure 7. Comparison of the rescoring results on the screening validation set.

constitution of the whole data set. This latter case illustrates the results that would have been obtained if the same number of molecules were randomly picked from the whole database.

DOCK contact performs worse than DOCK energy for both validation sets. Only six active compounds of the congeneric set are found among the top-scored ligands versus 11 with DOCK energy scores. Even a random choice would be better, but the performance stems from variations due to the rather small number of compounds considered. Concerning the screening validation set, 22 active ligands are found among the 64 first ranked compounds against 35 with DOCK energy. And, moreover, there are 20 inactive compounds in the top-ranked

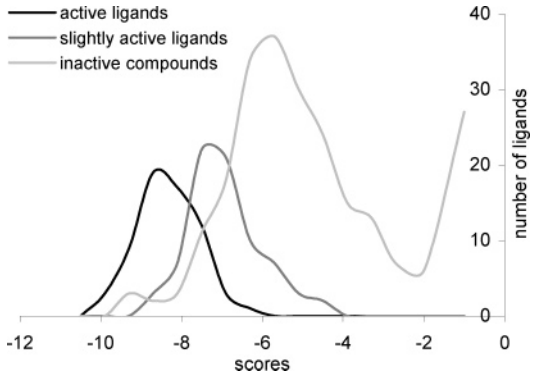


Figure 8. The SCORE scores distribution of the active, moderately active ligands, and inactive compounds in the screening validation set.

ligand subset, whereas only three were recovered with DOCK energy. It is worth noting that this latter scoring function is quite attractive for virtual screening on this target, as illustrated by the results obtained for the screening validation set. Fifty-five percent of the active ligands are found among the 64 top-scored molecules, while only a little more than 1% of the 230 inactive molecules have a good score. Unfortunately, this scoring function does not deal as well with the congeneric set.

The rescoring with SCORE results in a large improvement for the set of congeneric ligands (Figure 6). Forty-two percent of the top-ranked compounds are active. Despite the overlap of the score profiles (Figure 8), the enrichment factor reaches 2.3. Even with these very attractive results, a rather poor correlation ($r = 0.44$) between the scores and the pIC_{50} values is observed. Nevertheless, this correlation is slightly improved when the pyrazole-type inhibitors are discarded ($r = 0.55$). In addition, this scoring function does a more satisfactory job than DOCK energy scores for the screening validation set as well. Seventy-two percent of active ligands are among the top-scored compounds. Accordingly, the enrichment factor attains 4.1, which corresponds to around three-fourths of the maximum possible enrichment factor for this set of compounds. However, the ratio of inactive compounds among the top scorers (false positives) is larger in comparison with DOCK energy results (12% vs 5%). The remaining compounds are moderately active ligands, but they might be regarded as hits in a virtual screening process.

Owing to these promising results obtained with the rescoring approach, the scoring functions available in CScore were also considered for further improvement. Regarding the congeneric set, ChemScore performed the best, leading to slightly better performances than SCORE. Forty-five percent of the active ligands are among the top-scored molecules, and the associated enrichment factor is 2.5 (vs 2.3 with SCORE). The correlation between the scores and the pIC_{50} values does not appear to be higher using ChemScore than with SCORE. An r coefficient of 0.47 is obtained when all the congeneric ligands are considered, and it reaches 0.58 when the imidazole-type inhibitors (nearly one-third of the set) are excluded. On the contrary, there is no discrimination between active and moderately active ligands using the FlexX scoring function. ChemScore and FlexX result in slightly poorer estimates than DOCK energy scores on the screening validation set.

Thus, 42% and 50% of the active ligands are respectively found among the top-ranked molecules, and moreover, the fractions of inactive compounds are clearly higher compared to DOCK energy score (7% and 14% versus less than 2%). The corresponding enrichment factors are respectively 2.4 and 2.9 (against 3.1 with DOCK energy). The scoring function of GOLD and PMF performed poorly for rescoring the molecules docked into the COX-2 enzyme with DOCK. No enrichment is observed on the screening validation set. Regarding the congeneric set, GOLD yields a slight enrichment (1.7), whereas PMF performs poorly. We also performed the rescoring of the 10 first poses generated with DOCK and reranked them using each of the scoring functions. However, no improvement of the enrichment has been obtained by so doing.

Some explanations could account for these results and may give evidence for further improvement of the docking-scoring protocol. Concerning the characteristics of the COX enzyme cavity, the inhibition is mainly mediated via lipophilic interactions within the very confined binding pocket, whereas hydrophilic interactions are supposedly not a predominant feature.[18,17d] Estimation of the lipophilic interactions is one of the main limitations of most of the current scoring functions. Nevertheless, the SCORE, ChemScore, and FlexX scoring functions include a specific term accounting for this kind of interactions. That may explain their somewhat better results for this target compared to the others. Due to the predominant lipophilic characteristics of the binding pocket, the desolvation and entropic phenomena are supposed to be of particular importance. Only a few of the current scoring functions include a term that takes them into account. This could explain the good results of SCORE and ChemScore. As for FlexX, the entropic term is reported to mainly reduce the molecule size bias of this scoring function.[17d] PMF is supposed to treat implicitly such phenomena through the knowledge-based principle, but it has not turned out to be successful in this case.

An electrostatic term was demonstrated to be also required to evaluate properly the COX-2 ligand affinity, since DOCK contact performs less well than DOCK energy. On the other hand, this kind of interaction may be overestimated in FlexX and Gold scoring functions, so that compounds predicted to make mainly hydrophilic interactions with the rim of the pocket turn out to be falsely positive. These scoring functions also appeared to perform worse than others like SCORE, which is more tolerant of deviations of the hydrogen-bond interactions from the ideal orientation.

Around 80% of the congeneric ligands bear at least one fluorine atom, the interactions of which are known to be hardly evaluated properly. However, no significant correlation was found between the presence of this atom and the ranking errors. On the other hand, PMF is reported to have a poor potential for the halogen atoms, due to an insufficient number of occurrences in the PDB.[19] This could explain why this scoring function failed to treat correctly COX-2 ligands.

The COX-2 binding pocket is known to be globally rigi,d except for a small region.[20] Therefore, the induced-fit phenomenon is not supposed to be of particular importance. However, small alterations of the ligand−protein contact surface can have a significant influence on the hydrophobic interaction prediction. Thus, fine flexibility treatment of the protein may be required to obtain more accurate ligand placements and scores. Anyway, slight differences in the congeneric ligand structures yield large activity variations. This makes this target very demanding for the scoring functions that have to be all at once error-tolerant for the inaccuracy and ligand induced fit of the structural data and very sensitive to minor differences in the protein−ligands interactions. More time-consuming techniques are known to provide more accurate interaction energy estimates,[21] but it is not practically feasible to make use of them for the purpose of high-throughput screening.

Outliers and Consensus. The rescoring results demonstrated that a few of the scoring functions tested yielded good discrimination results. Therefore, a consensus approach, which consists of combining estimates from a variety of scoring functions into a single consensus score,[17d,22a,23b,c,f] may be profitable. The consensus approach performs all the better because the scoring functions have complementary ability; i.e., they do not fail to identify the same active ligands or inactive compounds. Therefore, we considered the compounds that were poorly ranked by each scoring function. The false-positive compounds represent the most awkward case, because they would be wrongly chosen for biological testing and increase the cost of this experimental validation. In this study, compounds referred to as inactive have not actually been assayed, but they are probably so (see description of the screening validation set composition). However, these false-positive compounds could ideally correspond to the tiny number of compounds that might be active. Seventeen molecules are found to be among the 30 top-scored inactive compounds for at least two of the best scoring functions (SCORE, DOCK energy, ChemScore, and FlexX). By the way, some of these molecules have a structure rather similar to known COX-2 inhibitors, so they could turn out to be promising compounds that are worth being assayed. Conversely, the false-negative compounds (i.e. for which a poor score is predicted while they are active) would be missed during the virtual screening process. There are three compounds for which the pIC_{50} is about 8 among the 30 poorest ranked active ligands with both DOCK and FlexX. Some triphenyl-type ligands still have rather poor ranking with DOCK energy, despite the improvement obtained thanks to the conformational sampling alteration. Conversely, none of the 30 poorest scored active compounds of SCORE and ChemScore have a pIC_{50} higher than 7.6, which is consistent with the good results of these scoring functions in ranking of the congeneric set ligands. No common structural features were identified among these false-positive compounds.

A consensus combination was carried out so as to take advantage of the above-mentioned complementarity of the scoring functions for this target. Only the scoring functions that were found to perform fairly well for the validation sets (DOCK energy, SCORE, FlexX, and ChemScore) were considered. The results are summarized in Table 2. With the consensus C1, all the molecules that are found among the 64 top-ranked compounds by at least one scoring function are consid-

Table 2. Consensus Results of the Four Best Scoring Functions

	consensus				single scoring functions			
	C1	C2	C3	C4	DOCK	SCORE	FlexX	ChemScore
% active ligands retrieved[a]	97	73	38	13	55	73	50	42
% active ligands[b]	39	75	83	100	55	73	50	42
enrichment factor	2.3	4.3	4.8	5.8	3.1	4.2	2.9	2.4

[a] The ratio of active ligands retrieved is the number of active ligands among the top-scored compounds divided by the total number of active ligands in the initial database. [b] The ratio of active ligands is the number of active ligands among the top-scored compounds divided by the number of top-scorers considered.

Table 3. Consensus Results after Active-inactive Threshold Tweaking

	DOCK, SCORE, FlexX, ChemScore				DOCK, SCORE
	C1	C2	C3	C4	C2
% active ligands retrieved	98	95	78	36	65
% active ligands	27	43	81	96	83
enrichment factor	1.6	2.5	4.6	5.5	4.7

ered as active. The consensus C2 corresponds to the molecules that are predicted as active according to at least two scoring functions, and so on. Finally, the molecules are active according to the consensus C4 if they are predicted as active by the four scoring functions. Here the consensus C2 performs as well as the best scoring function (enrichment factor of 4.3 vs 4.2 with SCORE). It combines the four independent estimates; as a result, we can assume the prediction is more reliable. Additionally, the consensus C3 and C4 enable us to select reduced subsets of compounds that are even more likely to contain active compounds.

To further improve the performance of this consensus approach, the score thresholds between the compounds considered as active and inactive are tweaked according to score profiles (such as in Table 3). In that way, the number of molecules regarded as active is altered. This fraction is slightly reduced for the scoring functions that perform the best on the congeneric set in order to take advantage of their discrimination ability, especially regarding to the congeneric ligands. The score thresholds were shifted from −8.1 to −8.2 for SCORE and from −39.3 to −39 for ChemScore. On the other hand, the fraction of compounds considered as active was increased for the scoring functions which showed a modest discrimination ability by changing the score limits from −41.2 to −35 for DOCK and from −16.9 to −12 for FlexX. So it enables us to make the most of the discrimination ability of the first scoring functions on the congeneric set of ligands as well as to take advantage of the second ones, which are widely applicable and thus perform well on the screening validation set. In that way, the consensus C3 performs even better than the consensus C2 previously with an enrichment factor of 4.6 compared to 4.3. (Table 3) The same trend is observed with consensus C4 compared to consensus C3 using the previous limits. Finally, this approach allows us to improve both the accuracy and the robustness of the predictions.

Application of the Protocol to Virtual Screening. Since the above-presented results demonstrate that the optimized docking-scoring protocol can serve as a practical virtual screening tool to find COX-2 inhibitors, it was applied to the virtual screening of a 1.2 million compound library subset. As a first application, the 13 711 compounds of this library containing at least one

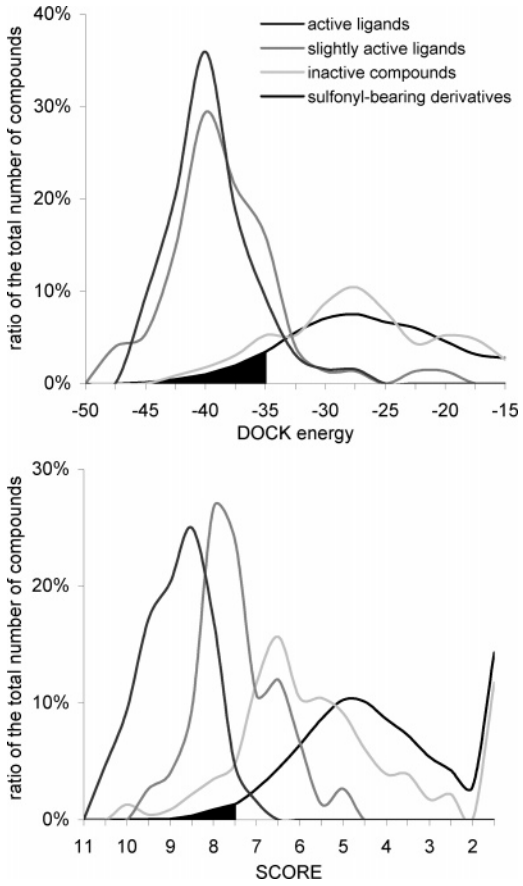


Figure 9. The DOCK energy (top) and SCORE (bottom) scores distribution of the compounds in the screening validation set and in the set of compounds that bear a sulfonyl group.

methyl sulfone or unsubstituted sulfonamide group were docked into the COX-2 enzyme structure.

First, we focus on the top-scored compounds. According to the validation step results, we used the SCORE and DOCK energy scoring functions. However, it has been clearly demonstrated these scores can only be used as a qualitative filter to select an active ligand enriched subset. According to the comparison of the score profile of the whole screened database with those of the active and of the slightly active inhibitors and along with the results of the consensus study, we chose score cutoffs that would enable us to recover most of the active ligands. By the way, it is worth noting that the best score estimates for the screened molecules are roughly the same as for the known active ligands for both of the scoring functions, as we might anticipate. Using a limit of −35 for DOCK scores, 7% of the screened compounds and most of the known active inhibitors (95%) are retrieved (Figure 9). This fraction of molecules is clearly too large to imagine that they are all active, so a more restricted selection is needed. However, the process is

expected to discard most of the inactive compounds and the molecules that DOCK failed to dock correctly. Using a threshold of 7.5 for the SCORE estimates, only a little more than 1% of the screened compounds are retrieved (Figure 9), which is much more in agreement with the common HTS hit rates.[24] Nevertheless, this limit would have enabled us to identify 98% of the active ligands of the congeneric set and about 67% of the slightly active ligands. The score limits that have been chosen are rather generous compared to the validation step because a virtual screening protocol is mainly expected to identify original structures, even if they are not highly active. Anyway, the hits found with virtual screening need to be chemically optimized later. At this stage, a set of 149 compounds was selected with the two score thresholds.

Then the selection was filtered out using geometric criteria that derived from the putative binding modes generated by docking. First, the compounds whose sulfonyl group is not predicted to interact with the same residues as in the crystal structures were discarded. This is related to the initial selection of molecules for docking the sulfonyl group, which is supposed to confer COX-2 selectivity to them. However, the sulfonyl group needs to be correctly located so that the choice of this kind of ligands would be worthwhile. Second, the compounds that clearly protruded from the enzyme binding pocket were discarded by checking on the 3D coordinates. Such binding modes do not make sense, since the COX enzyme is opened toward the cell membrane, which is not present in the 3D structure used in this study.

The putative binding mode of the remaining 69 molecules was eventually inspected to check visually the ligand−protein interactions. Eighteen of these molecules have a diaryl heterocycle moiety. We expected to find such compounds that are similar to known inhibitors with a binding mode comparable to the experimentally determined structure. This represents an extra validation of the docking-scoring protocol. However, such a virtual screening study aims to discover alternate chemotypes. For that reason, we focused on the other selected molecules. Thirty-eight of them have a linear structure; i.e., they are not branched, and as a consequence they cannot fill the whole binding pocket of the enzyme, unlike known selective inhibitors. Strong interactions could provide these ligands with a significant affinity, but they are less likely to be selective of the COX (and COX-2 primarily) enzyme because of the defective fit with the binding site. The 13 remaining compounds are all the more appealing in such an approach because they fill quite well the binding site and they are less similar to known COX-2 inhibitors than the first mentioned molecules. Among all these compounds, 20 were chosen for in vitro biological testing and 12 ended up being available from suppliers (Table 4).

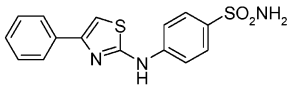
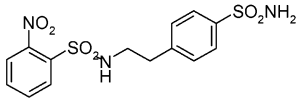
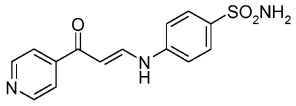
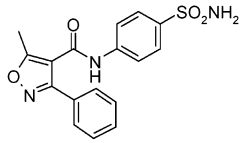
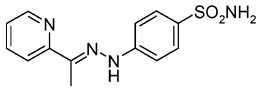
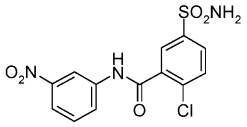
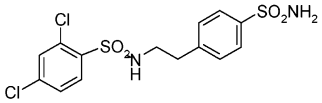
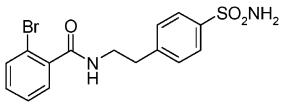
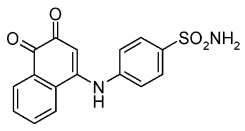
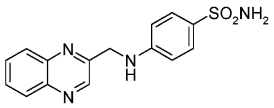
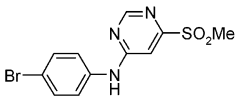
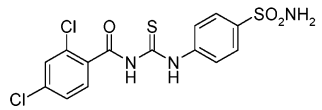
These compounds were assayed for their ability to inhibit COX-2 using a human enzyme immunoassay according to the manufacturer's instructions.[25] Four of the 12 tested compounds were found to have an enzyme inhibition greater than about 50% at a concentration of 1 μM (Table 4). Moreover, two molecules have an inhibition greater than 60% and the inhibition is lower than 25% for only three compounds. This ratio of active compounds has validated our virtual screening protocol. Moreover, the choice of molecules that all bear a sulfonyl group as a first subset to be screened does not introduce a significant bias in this study, because this structural feature is related to the inhibitor selectivity for COX-2 rather than their affinity. Furthermore, compounds containing a sulfonamide group are extensively used as therapeutics (e.g. carbonic anhydrase inhibitors,[26] antidiabetic, antibacterial, antimalarial drugs). Therefore, this chemical group is far from specific to COX-2 inhibitors. As the selectivity is of special importance for the pharmacological use of the COX-2 inhibitors, the compounds were also assayed for their ability to inhibit COX-1 isoenzyme, and these results are reported in Table 4. Compounds **10** and **11** end up having both a good inhibitory activity and some selectivity. However, these experimental data are not related to any of the docking results. By the way, the techniques employed in this study are definitely not accurate enough to investigate such a subtle phenomenon that has been hardly treated by more precise methods. Even the best measured enzyme inhibition values do not necessarily ensure an especially high COX-2 affinity. Anyway, these hits would have to be optimized and they turn out to be attractive enough for this virtual screening approach owing to their originality. In fact, the introduction of an heteroatom or a carbonyl link between the heterocyclic and the phenyl ring lacking the methyl sulfone is reported to result in active and selective compounds in the diaryl heterocycle-type series.[27] On the contrary, in compound **4**, an amide linkage is introduced between the heterocyclic ring and the phenyl ring which bears the sulfonamide group. Compound **6** is somewhat structurally related to the nimesulide series, but it differs in many points. Concerning the compounds **10** and **11**, they are loosely linked to sulfonamide analogues of the fenamic acid NSAIDs. No significant correlation was noticed between the activity values and the DOCK energy or SCORE scores. However, the three ligands that have the highest SCORE estimates turned out to be among the four most active molecules.

Conclusion

This study aimed to exploit the structural information available about the COX-2 enzyme to adapt a virtual screening protocol that can be used for identifying new original inhibitors. First, the main docking parameters of DOCK were optimized so as to deal correctly with a series of congeneric diaryl heterocycle inhibitors. The choice of the sphere set that defines the active site, some conformational sampling, and the matching parameters appear to be of special importance. Compared to the default settings, these tweaked parameters led to a significant improvement of the discrimination ability between active and inactive compounds, while the calculations were kept sufficiently quick to allow application to virtual screening. For the same purpose, we also made sure that this protocol could still be applied to diverse molecules.

Then the rescoring of the protein−ligand complexes generated with DOCK via six scoring functions suggested that this step is crucial. DOCK energy turned out to perform better than most of the other commonly

Table 4. Structures, Inhibitory Potencies, and Calculated Scores of the 12 Molecules Chosen for Biological Testing

compound	structure	DOCK energy	SCORE	enzyme inhibition (%)[d]	
				COX-2	COX-1
1[a]		-39.6	7.3	29	61
2[a]		-43.1	7.1	22	61
3[a]		-35.1	7.1	42	11
4[a]		-40.2	7.8	48	23
5[a]		-35.1	7.6	39	38
6[b]		-37.6	8.0	57	23
7[b]		-41.8	7.6	30	23
8[b]		-36.2	7.1	10	46
9[c]		-37.6	7.2	27	14
10[c]		-37.9	7.2	54	11
11[c]		-35.5	7.9	60	14
12[c]		-38.6	7.0	22	8

[a] Ordered from ChemDiv[56] [b] Ordered from ChemBridge[57] [c] Provided by the NCI[58] [d] Measured at a concentration of 1 μM using a human enzyme immunoassay[25]

used scoring functions. However, SCORE dramatically improved the results for the congeneric set of ligands while this scoring function has demonstrated a good discrimination ability on the screening validation set as well. Assumptions were proposed to explain the scoring function performances on this target. This comparison study also provides a deeper understanding of their advantages and limitations and may serve as a starting point for an equivalent work on targets that have similar properties. In addition, a consensus approach turned out to further improve the results as it combines the estimates of the four scoring functions that were found to perform the best and appeared to complete each other well.

The good enrichment factor obtained on the screening validation set suggests that this protocol is appropriate for searching potential COX-2 inhibitors through virtual screening of chemical libraries. The first application of this protocol to screen a set of compounds bearing a sulfonyl group was presented. Attractive results were obtained. Some of the best scored and correctly located compounds are fairly similar to known potent inhibitors. In addition, other more original molecules were assayed and one-third of the dozen tested compounds ended up inhibiting the COX-2 enzyme. This represents an experimental validation of the docking-scoring protocol setup in this study.

Now, we plan to apply this promising protocol to the screening of larger and more diverse chemical libraries gathered in the laboratory in order to propose more original molecules for biological testing. We have been working on the prefiltering of these libraries so as to avoid wasting computational power with molecules which are not 'druglike' by today's standards.[28a] In addition, some terms of the best scoring functions identified in this work could be altered to adapt them to this target and further improve the results. The hydrophobic terms would be probably the most critical to refine. We have also compared and investigated the combination of this docking-scoring method and 2D-QSAR techniques that are used in the laboratory.[28b,29] The ligand alignment derived from docking could also be used as a basis for 3D-QSAR studies that may yield more precise affinity predictions.

Materials and Methods

Docking. The widely distributed molecular docking software DOCK 4.01[30] was used to perform flexible docking of the molecules into the protein structure, which was kept rigid. It utilizes a sphere-matching algorithm to fit ligand atoms into a set of spheres that fills the binding pocket. The ligand is divided into rigid fragments, and a conformational search prior to docking or incremental construction is used to treat ligand flexibility. After on-the-fly energy minimization of the generated complexes, ligands are ranked according to a gridded energy score. A large number of DOCK parameters can be altered to control each stage of the algorithm. Starting from the default values[31] and previous studies,[32] we tweaked these parameters in accordance with the specificity of the target considered. Moreover, due to the screening application purpose, it was necessary to make a compromise between the speed and the precision of the calculations. A brief description of the algorithm and the associated parameters investigated in this work is given below.

The first docking stage is a conformational search. A collection of rigid segments separated by rotatable bonds is identified in the ligand structure. The ring flexibility is ignored in the DOCK algorithm. Using the torsion drive method, low-energy dihedral values are tried for each torsion previously defined. These low-energy angles are stored in flat files that can be edited. Alterations of these parameters have been tested. During this process some intramolecular overlaps are allowed using the default value of 0.5 for this parameter, except when it is mentioned explicitly. Afterward, two methods are proposed in DOCK: simultaneous search and anchor-first search. Using the simultaneous search method, the entire set of molecule conformations is generated in one step. All torsions are searched prior to the orientation search, so that each conformation is docked independently. The comprehensiveness of the conformational sampling is mainly controlled via the value of the conformational cutoff factor to which the default value was assigned. In contrast, in the anchor-first search method, the molecule conformation is constructed and energy minimized one segment at a time, starting from an anchor segment via an incremental construction approach.[33] As the choice of this one was not obvious primarily in our case, the multiple fragment option was used (with anchor size = 5) to consider several possible anchor fragments. Minimize anchor, torsion minimize, reminimize layer, and ligand parameters were also employed during this stage and they were assigned their standard default value.

The geometric matching algorithm of DOCK superimposes the ligand atoms onto the spheres that represent the negative image of the binding site. As recommended for library screening, the manual matching mode was used so that this step could be finely controlled through parameters such as node minimum and maximum, distance minimum, and, above all, distance tolerance. Neither critical point nor chemical label was specified. Fifty configurations per ligand building cycle with the anchor-first search method and 5000 maximum orientations were generated.

The score evaluates the interactions between the ligand and the protein target in each complex generated. Three scoring functions are available in the DOCK4 program:[31] energy, contact, and chemical score. For pose generation, the energy score was used, as it is reported in the literature to be the most robust method.[22] The gridded calculation mode was used; otherwise, the score evaluation would be far too slow for screening purposes.[34] Both the intra- and intermolecular terms were calculated. The bump filter is a parameter that controls the preliminary checking of the ligand−protein overlap. It was set to 6 in order to discard the widely overlapping orientations, but not those that could fit pretty well in the binding site after optimization. The complexes generated were energy-minimized using default parameters, except for the maximum iteration number and the cycle number, which were enlarged to respectively 100 and 10. As recommended,[35] some of the calculations were repeated using several values of seed, so that the score variability was assessed.

Rescoring. The scoring step is crucial and is usually reported as a limitation of the structure-based approach in the literature, especially with respect to the COX-2 enzyme. In an attempt to address this issue, we used a rescoring approach which has proved to be successful in several cases.[17d,22a,23] The docking algorithm and the energy scoring function of DOCK[31b,36] were still used to fit compounds to the enzyme binding pocket. Then, the best scored protein−ligand complex for each molecule was re-evaluated with other scoring functions. DOCK contact,[31] the different scoring functions available in Cscore,[23f,37,38] i.e., GOLD,[39] FlexX,[40,41] ChemScore,[42] PMF,[19] and the one of SCORE[43] were tested. All these scoring functions are based on force fields or empirical principles, except for PMF, which derives from a knowledge-based approach. Rescoring in this way is technically feasible for large library screening because tens to thousands of protein−ligand complexes can be scored per minute with the scoring functions used herein. These scoring functions are briefly detailed in the Supporting Information for the purpose of comparison.

Protein Structure. The starting data were crystal structures of murine COX-2 bound with SC-558 (Figure 1), a related *p*-bromo derivative of celecoxib (PDB code 1cx2 and 6cox).[18]

They were chosen since the cocrystallized ligand has a structure similar to the inhibitors used in this study. However, they have been determined with a rather poor resolution of 3.0 Å. A single protein chain was extracted from the tetrameric crystal structure. The ligand was removed and the ionization state of the binding site residues was checked. However, histidine 90 can adopt two virtually indistinguishable orientations that can only be assigned on the basis of the hydrogen-bonding pattern with the cocrystallized ligand. But the conformation of the ligand sulfonamide group cannot be derived unambiguously from the X-ray diffraction data. Thus, the two reasonable ionization states (histidine protonated on Hδ or Hϵ) were tried. Kollman united-atom charges were assigned for the protein. The Connolly surface of the binding site was calculated with MOLCAD[44] using a 1.4 Å radius spherical probe. This surface was used by the program SPHGEN[31a] to fill the active site with spheres of varying sizes (between 1.4 and 4 Å radii). The set of spheres was reduced manually to a final cluster made up of 57 spheres. In an attempt to assess the importance of this feature, a more reduce set of 29 spheres was also tested as well as a set consisting of 26 spheres, the centers of which are located where the heavy atoms of SC-558 are as it is bound to the enzyme in the crystal structure. A 23 × 21 × 21 Å box which encloses the sets of spheres was gridded with a spacing of 0.3 Å. In this way, the enzyme atoms contributions to the score are stored at each grid point within a 15 Å cutoff. Energy (steric and electrostatic terms) and contact scoring grids were used in this study.

Ligands Sets. The 4D energy minimization method implemented in MOE[45] was used for generating an initial 3D structure for each molecule. Then, the ligands were prepared for docking by means of DOCK tools sdf2mol2 and sybdb.[31a] By so doing, the atom types and connectivities were corrected. The hydrogens were added to ligands, with special caution to the protonation state of groups assumed to be ionized at a physiological pH. Partial charges were assigned using the Gasteiger−Marsili method as implemented in Sybyl.[46]

Two sets of ligands were used to adjust the parameters and validate the docking-scoring protocol. First, a set of 355 congeneric COX-2 inhibitors was selected from the literature. Their structures (Figure 2) are close to that of the cocrystallized ligand SC-558 and the marketed selective COX-2 inhibitors. The molecules belong to nine chemical families according to their central heterocyclic scaffold, including spiroheptene[47] (30 ligands), spiroheptadiene[47] (2 ligands), pyrrole[48] (22 ligands), imidazole[49] (128 ligands), cyclopentene[50] (40 ligands), benzene[51] (44 ligands), thiophene[52] (1 ligand), pyrazole[52] (86 ligands), and isoxazole[53] (2 ligands). The structures of these series of diaryl heterocycle-type inhibitors can be found in Table 1 of the Supporting Information. They have been used for QSAR studies before.[15b,d] Available inhibition values were measured with the same biological protocol using human COX-2 recombinant enzyme and the detection of the transformed arachidonic acid into PGE_2 with an immuno-enzymatic test ELISA (*e*nzyme *l*inked *i*mmunosorbent *a*ssay).[54] The pIC_{50} values of these inhibitors range from 4 to 9. The inhibitors with $pIC_{50} > 8$ were referred to as active and the other were considered as moderately active. This limit was chosen with the aim of obtaining a suitable ratio between the two classes of compounds. It resulted in a set of 64 active ligands, leaving about 82% of the molecular database as a moderately active compounds set.

A second set of compounds was built to evaluate how successful the protocol is at identifying active ligands among inactive molecules in as realistic as possible screening conditions. Using the software MOE, 52 2D P−VSA molecular surface descriptors[55] were calculated for 1.2 million compounds that were gathered from 15 commercial libraries devoted to screening.[28] First, we extracted around 183 000 compounds for which the descriptors are in the same range (expanded by 20%) as for the congeneric ligand set. Compounds containing over 15 rotatable bonds or atom types which are not parametrized in DOCK (mainly metals)[31a] were also removed. By so doing the compounds that are the most unlikely to bind to the enzyme were filtered out. A total of 230 compounds was eventually extracted according to a maximum molecular diversity with MOE. Since most of the compounds from the 1.2 million compound library do not probably have inhibitory potency for the COX-2 enzyme, this subset is comprised of a priori inactive molecules as well, even if some of them could be active. Actually, active compound rates are typically less than 1% for HTS performed on pharmaceutical screening libraries.[24] As a result, in a statistical point of view, no more than two or three compounds out of the 230 ought to inhibit COX-2 enzyme. It is very unlikely that this ratio would be increased by the preliminary filters, because they are too crude to provide an active ligand enrichment. On the other hand, this filtering step makes the validation set more realistic and more demanding for the docking protocol, since the 230 compounds selected have similar characteristics to known COX-2 inhibitors. This group of 230 compounds is named inactive ligands in the following. This set was seeded with the 64 most potent inhibitors ($pIC_{50} > 8$) and the 75 least potent inhibitors ($pIC_{50} < 6$) of the abovementioned congeneric set. This set of 369 compounds is referred to as the "screening validation set", since it reproduces real screening conditions where small number of active ligands are searched for in a large, diverse collection.

Even after optimization, the docking protocol is not fast enough to allow us to screen millions of compounds considering the moderate computational power that was available in the laboratory. Accordingly, as a preliminary investigation, a subset was extracted from a 1.2 million commercial compound library that had been collected before.[24] Since most of the selective COX-2 inhibitors discovered so far bear either a methyl sulfone or an unsubstituted sulfonamide group, 3095 sulfone and 10 616 sulfonamide functionalized molecules were collected with a substructural search using MOE from the whole library.

Comparison Criteria. The docking-scoring protocols were evaluated according to several criteria. First, binding modes generated for the cocrystallized ligand as well as for the inhibitors of the congeneric set were compared with the crystal structure of the SC-558 bound to COX-2. As the inhibitory potencies of ligands belonging to the congeneric set have been determined experimentally, the correlation between these values and the calculated scores can be considered. However, due to the approximations of the employed methods, such correlation is likely to be poor. In addition, the results for the screening validation set can only be compared qualitatively. For these reasons, a qualitative way of comparison based on the compound ranking was preferred.

As 64 inhibitors have a $pIC_{50} > 8$ in the congeneric set, after scoring the 64 first-ranked molecules were retrieved and the ratio of active compounds among them was assessed. Ideally, all these 64 best-scored molecules should be active, but in reality only a fraction of them are true positive compounds (i.e., active compounds for which high scores are evaluated), whereas the other are false-positive compounds (i.e. high scores are predicted for them while they have no inhibitory potency). The same kind of analyses were performed on the screening validation set considering that the number of top-ranked compounds and the number of active compounds in the set is the same. However, three molecules classes were discerned: the active, moderately active ligands, and inactive compounds.

To evaluate the ability of the docking-scoring protocols to help discover active compounds, the enrichment factor was calculated by dividing the fraction of the selected molecules that were active by the fraction of active compounds in the source pool:

$$EF = (\text{hits}_{\text{sampled set}}/N_{\text{sampled set}})/(\text{hits}_{\text{total database}}/N_{\text{total database}})$$

$$EF_{\max} = N_{\text{total database}}/\text{hits}_{\text{total database}}$$

Noteworthy, the possible enrichment values depend strongly on the initial database size and composition. Therefore, the maximum possible enrichment is also reported for the purpose

of comparison. It is calculated using the function described above with the assumption that all possible active compounds were selected.

Material. Ligand and protein preparation, complex visualization, and CScore calculations were performed with Sybyl6.8 running on a SGI workstation. The SCORE estimates were also performed on the workstation. The docking calculations were carried out with DOCK on PCs running the Linux operating system, as well as the diversity calculations using MOE. In the following, calculation times are reported for a 2GHz P4 Linux box.

Biological Testing. Samples of the selected compounds were obtained from various suppliers.[56−58]. They have been tested using a COX (human) inhibitor screening assay according to the protocol recommended by the manufacturer.[25]

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Supporting Information Available: A brief description of the scoring function terms, tables of the 355 COX-2 inhibitor structures, respective pIC_{50} and score values calculated with SCORE, a plot of the enrichment factor obtained using one and several seed values, DOCK energy score profiles for active and inactive compounds, and a scatter plot depicting the correlation between affinities and scores calculated with SCORE for the congeneric ligands. This material is available free of charge via the Internet at http://pubs.acs.org.

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