Supporting information

NO-donor COX-2 inhibitors. New nitrooxy-substituted 1,5-diarylimidazoles endowed with COX-2 inhibitory and vasodilator properties

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Elemental analyses

Compound	Formula	Calculated			Found		
		%C	**%H**	**%N**	**%C**	**%H**	**%N**
6	$C_{15}H_{11}ClFN_3O_3S \cdot 0.25\ H_2O$	48.39	3.11	11.29	48.45	2.93	11.21
8	$C_{17}H_{14}ClFN_4O_6S$	44.70	3.09	12.26	44.85	3.26	12.16
8a	$C_{17}H_{15}ClFN_3O_4S \cdot H_2O$	47.50	3.99	9.78	47.60	3.59	9.53
9	$C_{18}H_{16}ClFN_4O_6S$	45.92	3.42	11.90	45.97	3.58	11.74
9a	$C_{18}H_{17}ClFN_3O_4S$	50.77	4.02	9.87	50.79	4.07	9.79
10	$C_{16}H_{12}ClFN_2O_3S$	52.39	3.30	7.64	52.33	3.31	7.51
11	$C_{18}H_{15}ClFN_3O_6S$	47.43	3.32	9.22	47.14	3.27	9.18
11a	$C_{18}H_{16}ClFN_2O_4S$	52.62	3.92	6.82	53.23	4.04	6.83
14	$C_{19}H_{17}ClFN_3O_6S$	48.57	3.65	8.94	48.32	3.64	8.58
14a	$C_{19}H_{18}ClFN_2O_4S \cdot 0.75\ H_2O$	52.06	4.48	6.39	51.96	4.40	6.24
15	$C_{19}H_{16}ClFN_2O_3S$	56.09	3.96	6.88	56.04	4.22	6.69
16	$C_{19}H_{16}ClFN_4O_9S$	42.99	3.04	10.55	42.84	3.10	10.29

Detailed computational procedures

Computational setup. All molecules used in the simulations were constructed using standard bond lengths and angles with the MOE modelling suite. The ground state geometries were fully optimized without geometry constraints using a RHF/6-31G(d) *ab initio* method until the largest component of the gradient was less than $1 \cdot 10^{-4}$ Hartree/Bohr. Vibrational frequency analysis allowed characterising the stationary points as minima (no imaginary frequencies). The crystal structures of murine COX-2 in complex with SC-558 (Kurumbail, R. G.; Stevens, A. M.; Gierse, J. K.; McDonald, J. J.; Stegeman, R. A.; Pak, J. Y.; Gildehaus, D.; Miyashiro, J. M.; Penning, T. D.; Seibert, K.; Isakson, P. C.; Stallings, W. C. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature* **1996**, *384*, 644-648) and ovine COX-1 in complex with α-methyl-4-biphenylacetic acid (Gupta, K.; Selinsky, B. S.; Kaub, C. J.; Katz, A. K.; Loll, P. J. The 2.0 Å resolution crystal structure of prostaglandin H$_2$ synthase-1: structural insights into an unusual peroxidase *J. Mol. Biol.* **2004**, *335*, 503-518) were downloaded from the RCSB

Protein Data Bank (PDB codes *1cx2* and *1q4g*). Missing hydrogens and standard PARM99 AMBER force field parameters were assigned to the protein using the LEaP module of the AMBER 8 software package. Histidine tautomeric states were assumed to be ε for all but heme-coordinated His388 in the case of COX-2, while for COX-1 δ-tautomers were assumed for His90, His388 and His513. These assignments were made on the basis of previous work (Mozziconacci, J.-C. et al., cit. ref.) and visual inspection, following the criterion of favouring hydrogen bonding between the imidazole N-H and the sulfonyl oxygen present in all inhibitors. Atom types and parameters for heme were taken from literature (Giammona, D. A. Ph.D. thesis, University of California, Davis (1984), modified by Bayly, C.; http://pharmacy.man.ac.uk/amber/cof/heme_all_inf.html). The proteins underwent full conjugate-gradient minimization (dielectric constant = $4r$, non-bonded cutoff = 16 Å) until the gradient rms (drms) was smaller than 0.01 kcal/mol Å, keeping non-hydrogen atoms fixed to their crystallographic positions with a harmonic potential (force constant = 1000 kcal/mol Å^2); all minimizations and molecular dynamics (MD) were performed with the SANDER module. Atom types and GAFF parameters for ligands were assigned with the ANTECHAMBER module; missing parameters were empirically derived from equilibrium geometries found with *ab initio* methods. Atomic charges were fitted to the HF/6-31G(d) electrostatic potential according to the RESP procedure (Cornell, W. D.; Cieplak, P.; Bayly, C. I.; Gould, I. R.; Merz, K. M.; Ferguson, D. M.; Spellmeyer, D. C.; Fox, T.; Caldwell, J. W.; Kollman, P. A. A second generation force field for the simulation of proteins, nucleic acids, and organic molecules *J. Am. Chem. Soc.* **1995**, *117*, 5179-5197. The RESP fit was made by a in-house BASH script, based on the CSH script by De Winter, H.; http://amber.scripps.edu/Questions/resp2.txt). In order to check the consistency of the new force field parameters, all ligands were minimized with a dielectric constant = 1 until the drms was lower than $1 \cdot 10^{-4}$ kcal/mol Å; the resulting geometry was found to be in good agreement with the one found by *ab initio* calculations (RMSD = 0.2-0.5 Å).

Docking. All inhibitors were docked into the active site of both cyclooxygenase isozymes using AutoDock 3.0.5. For each protein a PDBQS file was generated adding AMBER PARM99 all-atom charges and AutoDock solvation parameters to the PDB file. Two different atom types were defined for polar and non-polar hydrogens for both proteins and ligands, in order to perform docking in an all-atom fashion, modelling hydrogen bonds explicitly with a directional 12-10 Lennard-Jones potential. The starting coordinates for the various inhibitors were determined by superposition with the co-crystallized ligand, respectively SC-558 for COX-2 and α-methyl-4-biphenylacetic acid for COX-1; RESP-fitted charges were employed. Rotatable bonds were defined with the AutoTors module, then the energy grid maps were computed with AutoGrid. The Lamarckian Genetic Algorithm (LGA) as implemented in AutoDock was used to find the most favourable enzyme-inhibitor (EI) complexes. Default parameters were used except for the number of generations, energy evaluations and docking runs, which were set respectively to $4 \cdot 10^5$, $1.5 \cdot 10^5$ and 100. The inhibitor conformations thus obtained were clustered according to a rather loose similarity criterion (rms tolerance = 1.5 Å). While for COX-1 the docking scores indicated very poor affinity for all ligands, thus confirming the COX-2 selectivity found experimentally (see Results and discussion), in the case of COX-2 the scoring function implemented in AutoDock was not able to discriminate between the different affinities of the ligands. For this reason, a refinement of the EI complexes, followed by more extensive conformational sampling through MD, were carried out.

EI complexes refinement and MD simulations. Among the 100 poses found for each ligand, the lowest energy pose from the most populated cluster was chosen. Its complex with *1cx2* was minimized in two steps (dielectric constant = 4r, non-bonded cutoff = 16 Å, drms = 0.01 kcal/mol Å), restraining protein heavy atoms with a harmonic force constant of 1000 kcal/mol Å^2 first, and then reduced to 5 kcal/mol Å^2. The minimized complex coordinates were used as the starting point for an implicit solvent MD simulation using the Generalized Born (GB) model as implemented in the SANDER module (*igb* = 2; Onufriev, A.; Bashford, D.; Case, D. A. Exploring protein native states and large-scale conformational changes with a modified Generalized Born

model. *Proteins* **2004**, *55*, 383-394). Van der Waals radii for Fe, F, Cl were taken from literature ((a) Bondi, A. Van der Waals volumes and radii. *J. Phys. Chem.* **1964**, *68*, 441-451; (b) Hawkins, G. D.; Cramer, C. J.; Truhlar, D. G. Parametrized models of aqueous free energies of solvation based on pairwise descreening of solute atomic charges from a dielectric medium. *J. Phys. Chem.* **1996**, *100*, 19824-19839). During the simulation protein heavy atoms were lightly restrained with a force constant of 5 kcal/mol Å^2, in order to avoid large distortions of the overall enzyme structure; bonds involving hydrogen atoms were constrained with the SHAKE algorithm (Ryckaert, J. P.; Ciccotti, G.; Berendsen, H. J. C. Numerical integration of cartesian equations of motion of a system with constraints – molecular dynamics of *N*-alkanes. *J. Comput. Phys.* **1977**, *23*, 327-341). Standard dielectric constants of 1 for the interior and 80 for the exterior were chosen, and a 16 Å cutoff value was adopted. The temperature of the system was raised from 0 to 300 K in a 5-ps heating run, then a production run of 40 ps (40000 steps, time step = 1 fs) was carried out, keeping the temperature constant by coupling to a Berendsen thermostat (Berendsen, H. J. C.; Postma, J. P. M.; van Gunsteren, W.; DiNola, A.; Haak, J. R. Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* **1984**, *81*, 3684-3690), time constant = 1 ps. 800 snapshots were collected, one every 50 MD steps, and the last 400 snapshots were used for the following step as both energy and RMSD values appeared well converged along the trajectory.

Energy evaluation. For each of the 400 snapshots the coordinates of unbound enzyme and inhibitor were extracted from the EI complex, and three separate energy evaluations allowed calculating G_{water} for complex, protein and ligand respectively, and thus $\Delta G_{MM\text{-}GBSA}$, according to the following equations:

$G_{water} = E_{MM} + G_{solvation}$

$E_{MM} = E_{bond} + E_{angle} + E_{torsion} + E_{electrostatic} + E_{vdW}$

$G_{solvation} = G_{polar} + G_{nonpolar}$

$\Delta G_{MM\text{-}GBSA} = G_{water}(\text{complex}) - [G_{water}(\text{enzyme}) - G_{water}(\text{ligand})]$

Both E_{MM} and $G_{solvation}$ were determined with the SANDER module; no cutoff was used for non-bonded interactions. G_{polar} was calculated with the GB model (*igb* = 2, *gbsa* = 1), while $G_{nonpolar}$ was derived from the solvent-accessible surface area (SASA) of the solute through the equation $G_{nonpolar} = 0.00542 \cdot SASA$. With respect to the original MM-GBSA formulation (Massova, I.; Kollman, P. A. Computational alanine scanning to probe protein-protein interactions: a novel approach to evaluate binding free energies. *J. Am. Chem. Soc.* **1999**, *121*, 8133-8143) the entropic term was omitted in the calculation of G_{water}. As explained in the Results section, the purpose of this molecular modelling study was not to assess true binding energies, but rather to score a series of inhibitors, correlating their inhibitory potency with their binding behaviour. Due to the great computational demands requested by normal mode analysis or quasi-harmonic analysis, and to the intrinsic difficulties connected with a reliable evaluation of the entropic contributions by these methods over a short trajectory (only 40 ps), it seemed preferable to forego the most rigorous theoretical approach in favour of affordable CPU times. The price to pay is that $\Delta G_{MM\text{-}GBSA}$ values calculated without considering the entropic cost of restraining translational and rotational degrees of freedom are significantly more negative than the experimental ones, as already outlined in previous work ((a) Froloff, N. et al., cit. ref.; (b) Windemuth, A. et al., cit. ref.).