Full Paper

Pyrazolo[3,4-*d*]pyrimidine Derivatives as COX-2 Selective Inhibitors: Synthesis and Molecular Modelling Studies

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The pyrazolo[3,4-*d*]pyrimidine system shows a multitude of interesting pharmacological properties. Owing to the potential anti-inflammatory activity of 5-benzamido-pyrazolo[3,4-*d*]pyrimidin-4-one derivatives and considering the easy synthesis of this class of compounds, a set of new 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-ones has been prepared in 42-80% yields by reacting 5-aminopyrazole-4(*N*-benzoyl)carbohydrazide derivatives and the opportune triethylorthoesters. Compounds **8a**, **b**, **10a – d**, and **11a**, **b** revealed a superior inhibitory profile against COX-2, when compared to that of reference standards NS398 and indomethacin. Molecular modelling studies confirmed the obtained biological results.

Keywords: COX-2 inhibitors / Docking / Pyrazolo[3,4-*d*]pyrimidine / 4(3*H*)-Quinazolinone

Received: August 1, 2008; accepted: September 29, 2008

DOI 10.1002/ardp.200800140

Introduction

Molecules based on the pyrazolo[3,4-*d*]pyrimidine ring system exhibit a multitude of interesting pharmacological properties including antimicrobial [1, 2], antiviral [3, 4], anti-allergic [5], antihypertensive [6], and anti-inflammatory activities [7].

The last activity is very intriguing taking into account that the pyrazolo[3,4-*d*]pyrimidine system **1** is structurally related to the 4(3*H*)-quinazolinone nucleus **2** (Fig. 1). Our research group has long been interested in the *N*-heterocycle-substituted quinazolinones which are characterized by good analgesic and anti-inflammatory activities and, at the same time, very low ulcerogenicity [8 – 14].

In the course of our medicinal chemistry researches, we reacted the pyrazole[3,4-*d*]-1,3-oxazinones **3a – h** with hydrazine hydrate to obtain compounds **4a – h** in 70 – 90% yields together with, in the case of **4a**, **c**, **f**, small amounts of the isomer compounds **5a**, **c**, **f** (Scheme 1) [15].

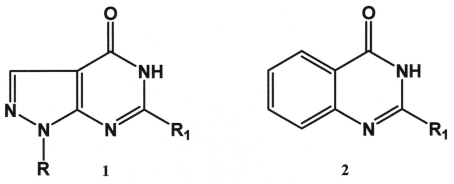

Figure 1. Structurally related pyrazolo[3,4-*d*]pyrimidine **1** and 4(3*H*)-quinazolinone **2** nuclei.

Moreover, by reacting compound **5a** with ethyl orthoformate, the 1-phenyl-5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **8a** was afforded. Compound **8a** was also obtained by benzoylation of the 5-amino-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-one **6** (Scheme 1).

At this point, owing to the easy synthesis of pyrazolo[3,4-*d*]pyrimidine derivative **8a** and considering the potential anti-inflammatory activity of this class of compounds, we thought it would be of interest to extend this synthesis to other analogues of **8a**.

Moreover, using a docking-simulation approach, we have tried to address the structural basis of COX-inhibitor interactions in order to determine differences in the binding mode of compounds in the COX-1 and COX-2 isoforms. Experimental COX-1 and COX-2 activities for com-

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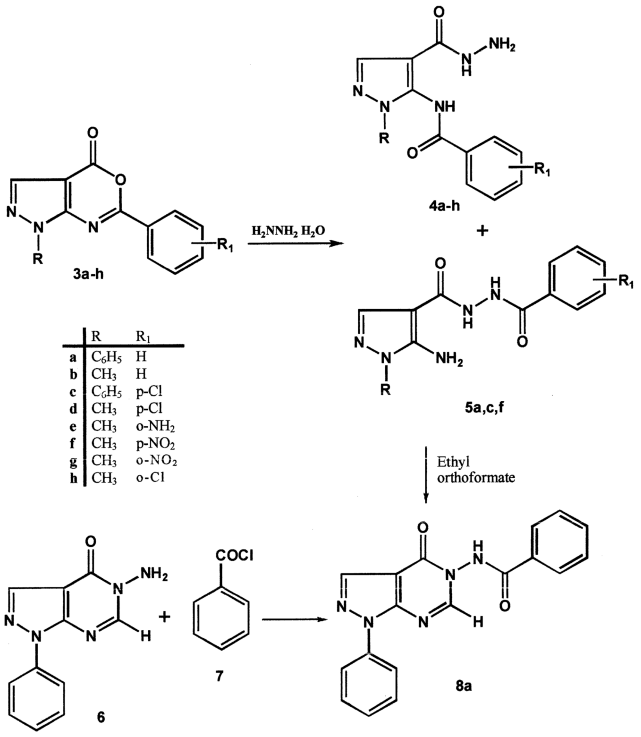
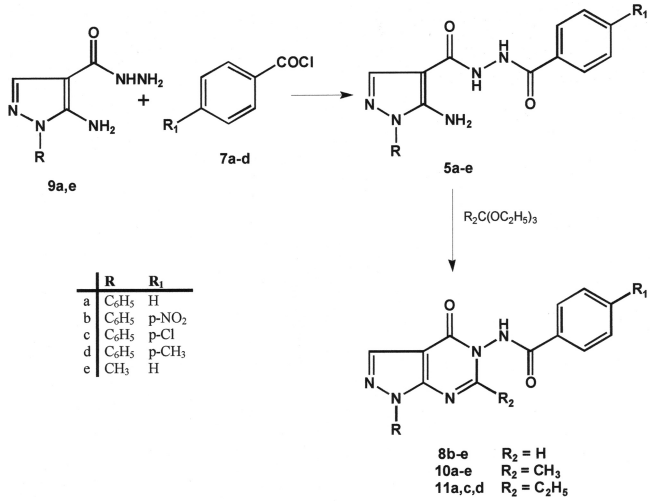


Scheme 1. Synthetic pathway for the formation of compound **8a**.



Scheme 2. Synthetic pathway for the formation of compounds **8**, **10**, and **11**.

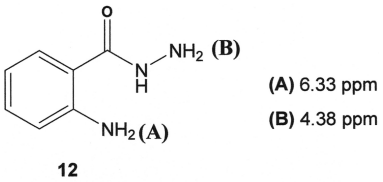


Figure 2. The amino NMR signal of anthranoylhydrazide **12**.

pound-**8a** analogues were finally performed with the aim to confirm the *in silico* results.

Results and discussion

The 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-one derivatives **8b–e**, **10a–e**, and **11a**, **c**, **d** were synthesized according to the procedure described in Scheme 2.

In particular, condensation of 5-amino-1*H*-pyrazole-4-carbohydrazide **9a**, **d** with the opportune benzoyl chloride **7a–d** in acetonitrile afforded the 5-aminopyrazole-4-(*N*-benzoyl)carbohydrazides **5a** [15], **b**, **c** [15], **d**, **e**. We excluded the possibility to obtain derivatives of type **4** (Scheme 1) on the basis of spectroscopic as well as bibliographic data comparison. In particular, the ^{1}H-NMR spectra of the compounds **5** showed the presence of the pyrazole amino group signal at about 6 ppm as reported for the compounds **5a**, **c** [15] and for the anthranoylhydrazide **12** [16] and, the absence of the signal at about 4.38 ppm for the more shielded hydrazide amino group (Fig. 2) [15, 16].

Finally, the reaction of the 5-aminopyrazole-4-(*N*-benzoyl)carbohydrazides **5** with the opportune triethylorthoesters afforded the corresponding pyrazolo[3,4-*d*]pyrimidine **8b–e**, **10a–e**, and **11a**, **c**, **d** (Scheme 2). The structures of the above mentioned compounds was assigned on the basis of satisfactory elemental analyses as well as spectroscopic data. In the case of derivatives **11**, the ^{1}H-NMR spectra indicated hindered internal rotation around the bond that links the pyrazolo[3,4-*d*]pyrimidine nucleus to the benzamido moiety. In fact, the methylene protons of the ethyl group, bonded at 6-position of the pyrazolo[3,4-*d*]pyrimidine ring are diastereotopic and showed signals that appeared as multiplets in the 2.60 to 2.97 ppm region, due to geminal and vicinal coupling.

The ability of compound **8a–c**, **10a-d**, and **11a–c** to inhibit the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) was determined using a COX-1 / COX-2 inhibitor screening assay kit (kit n#560101;Cayman Chemical, Ann Arbor, MI, USA) using *N*-[2-(cyclohexiloxy)4-nitrophenyl]methane sulphonamide (**NS398)** and indomethacin (**INDO**) as reference compounds. Results are shown in Table 1.

Crystal structures (6COX [17] and 1Q4G [18]) from the Brookhaven Protein Data Bank provided Cartesian coordinates for COX-2 in complex with SC558 **13** and ovine COX-1 in complex with α-methyl-4-biphenylacetic acid **14**, respectively.

Insigth II program 2005 (Accelrys Software Inc., San Diego, CA, USA) was used to check the 6COX and 1Q4G

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Table 1. Percent-inhibition of COX-1 and COX-2 at 10 μM concentration.

Compound	COX-1	COX-2
8a	19.8	78.9
8b	ns	75.9
8c	24.9	ns
10a	ns	10.2
10b	27.6	75.4
10c	ns	62.8
10d	29.8	66.0
11a	47.2	77.9
11b	ns	29.2
11c	36.4	ns
NS398	22.8	54.0
INDO	36.4	29.2

ns = not significant (%-inhibition <10%).

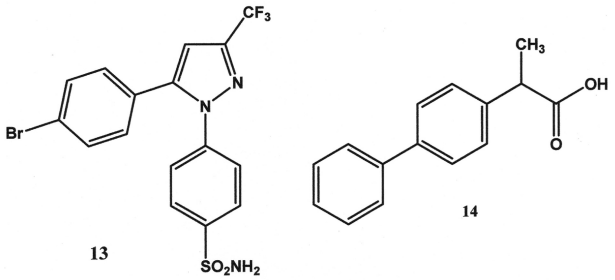

Figure 3. Structures of ligands: SC558 **13** and α-methyl-4-biphenylacetic acid **14**.

structures for missing atoms, bound and contacts. Cerius[2] 4.10 program (Accelrys Software Inc.,) was used to add hydrogen to the enzyme structures, to manually delete ligands and water molecules.

The structures of SC558 **13** and α-methyl-4-biphenylacetic acid **14** (Fig. 3) were obtained with Cerius[2] 4.10 by selecting and cutting the ligands from the protein-ligand complex.

Structures of the pyrazolo[3,4-*d*]pyrimidine **8a – c**, **10a – d**, and **11a – c** were generated by molecular modelling with Cerius[2] 4.10 software by optimizing their geometry using the Dreiding 2.21 force field with Gasteiger charges.

The active sites of 6COX and 1Q4G were defined by the "docked-ligand" procedure in the program Cerius[2] 4.10 according to the shape of the ligands SC558 **13** and α-methyl-4-biphenylacetic acid **14**, respectively, as grid points around the ones which are unoccupied by protein atoms. A rigid docking experiment was performed for SC558 **13** and α-methyl-4-biphenylacetic acid **14**, the ligand fit predicted conformations with their x-ray crystallographic structures show a very high overlap with a RMS of 0.37 Å for **13** and 0.18 Å for **14**.

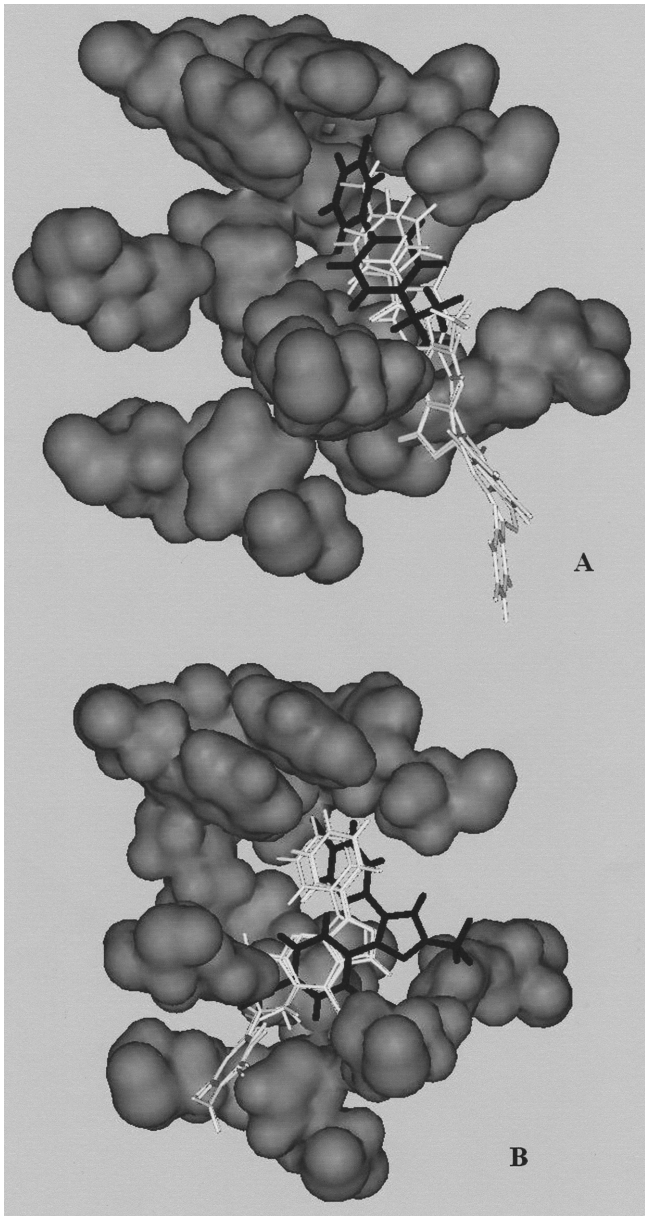

Figure 4. Overlay of the docked orientations for: (A) α-methyl-4-biphenylacetic acid **14** (black) and 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-one derivatives **8a**, **b**, **10b** (white) in the active site of COX-1; (B) SC558 **13** (black) and 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-one derivatives **8a**, **b**, **10b** (white) in the active site of COX-2.

All inhibitors were docked into the active sites of 6COX and 1Q4G to generate the docked conformations using the flexible docking procedure of "ligand fit" in the program Cerius[2] 4.10.

The cyclooxygenase-binding site in both isoenzymes is a long, narrow, hydrophobic channel extending from the membrane-binding region of the protein [19]. A se-

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Table 2. Analytical and spectroscopical data of compounds **5b**, **d**, **e**.

Comp.	Composition	Yield (%)	M.p. (°C)	IR (cm^{-1})	^{1}H-NMR (DMSO-d_6) (ppm)
5b	$C_{17}H_{14}N_6O_4$ calc.: C, 55.74; H, 3.85; N, 22.94; found: C, 55.93; H, 3.78; N, 22.64.	75	255	3450-3100 (NH_2 and NH-NH), 1673 (CO).	6.43 (s, 2H, NH_2); 7-45-8.40 (a set of signals, 10H, C_6H_4, C_6H_5 and pyrazole H-3), 10.60 (s, 1H, NH); 10.70 (s, 1H, NH).
5d	$C_{18}H_{17}N_5O_2$: calc.: C, 64.47; H, 5.11; N, 20.88; found: C, 64.43; H, 5.30; N, 20.62.	90	230	3400-3100 (NH_2 and NH-NH), 1675 (br, CO).	2.38 (s, 3H, CH_3); 6.42 (s, 2H, NH_2); 7.31-8.04 (a set of signals, 10H, C_6H_4, C_6H_5 and pyrazole H-3); 9.88 (s, 1H, NH); 10.25 (s, 1H, NH, with D_2O).
5e	$C_{12}H_{13}N_5O_2$ calc.: C, 55.59; H, 5.05; N, 27.01; found: C, 55.53; H, 5.28; N, 26.98.	85	273–275	3400-3100 (NH_2 and NH-NH), 1685-1650 br, CO).	3.57 (s, 3H, CH_3); 6.04 (s, 2H, NH_2); 7.58-7.93 (a set of signals, 6H, C_6H_5 and pyrazole H-3); 9.85 (s, 1H, NH); 10.83 (s, 1H, NH).

Crystallization solvent: ethanol.

Table 3. Analytical and spectroscopical data of compounds **8b–e**.

Comp.	Composition	Yields (%)	M.p. (°C)	IR (cm^{-1})	^{1}H-NMR (DMSO-d_6) (ppm)
8b	$C_{18}H_{12}N_6O_4$ calc.: C, 57.45; H, 3.21; N, 22.33; found: C, 57.15; H, 3.38; N, 22.64.	62	304–305	3172 (br, NH), 1709 (CO), 1686 (CO).	7.44-8.68 (a set of signals, 11H, C_6H_5, C_6H_4, quinazolinone H-2 and pyrazole H-3); 12.26 (s, 1H, NH).
8c	$C_{18}H_{12}ClN_5O_2$ calc.: C, 59.11; H, 3.31; N, 19.15; found: C, 59.12; H, 3.50; N, 19.21.	69	279–280	3180 (br, NH), 1707 (CO), 1690 (CO).	7.41-8.59 (a set of signals, 11H, C_6H_5, C_6H_4, quinazolinone H-2 and pyrazole H-3); 12.06 (s, 1H, NH).
8d	$C_{19}H_{15}N_5O_2$ calc.: C, 66.08; H, 4.38; N, 20.28; found: C, 65.85; H, 4.06; N, 20.61.	62	262–263	3180 (br, NH), 1693 (CO).	2.41 (s, 3H, CH_3); 7.40-8.64 (a set of signals, 11H, C_6H_5, C_6H_4, quinazolinone H-2 and pyrazole H-3); 11.83 (s, 1H, NH).
8e	$C_{13}H_{11}N_5O_2$ calc.: C, 57.99; H, 4.12; N, 26.01; found: C, 57.92; H, 4.27; N, 26.38.	75	167–168	3260-3150 (NH), 1660–1630 (2 × CO).	3.78 (s, 3H, CH_3); 7.55-8.08 (a set of signals, 6H, C_6H_5 and pyrazole H-3); 10.70 (br s, 1H, NH).
10a	$C_{19}H_{15}N_5O_2$ calc.: C, 66.08; H, 4.38; N, 20.28; found: C, 66.34; H, 4.30; N, 20.11.	48	240–241	3215 (br, NH), 1726 (CO), 1677 (CO).	2.54 (s, 3H, CH_3); 7.42-8.43 (a set of signals, 11H, 2 x C_6H_5, and pyrazole H-3); 11.67 (s, 1H, NH).
10b	$C_{19}H_{14}N_6O_4$ calc.: C, 58.46; H, 3.61; N, 21.53; found: C, 58.39; H, 3.52; N, 21.32.	46	176–177	3269 (br, NH), 1718 (CO), 1693 (CO).	2.54 (s, 3H, CH_3); 7.42-8.47 (a set of signals, 10H, C_6H_5, C_6H_4 and pyrazole H-3); 12.00 (s, 1H, NH).
10c	$C_{19}H_{14}Cl\ N_5O_2$ calc.: C, 60.09; H, 3.72; N, 18.44; found: C, 59.85; H, 3.39; N, 18.57.	53	284–285	3180 (br, NH), 1710–1690 (2 × CO).	2.54 (s, 3H, CH_3); 7.45-8.43 (a set of signals, 10H, C_6H_5, C_6H_4 and pyrazole H-3); 11.79 (s, 1H, NH).
10d	$C_{20}H_{17}N_5O_2$ calc.: C, 66.84; H, 4.77; N, 19.49; found: C, 67.15; H, 4.71; N, 19.53.	48	261–262	3260 (NH), 1700–1691 (2 × CO).	2.43 (s, 3H, CH_3); 2.52 (s, 3H, CH_3); 7.40-8.42 (a set of signals, 10H, C_6H_5, C_6H_4 and pyrazole H-3); 11.58 (s, 1H, NH).
10e	$C_{14}H_{13}N_5O_2$ calc.: C, 59.36; H, 4.63; N, 24.72; found: C, 59.65; H, 4.44; N, 24.68.	80	149–150	3250 (br, NH), 1680–1635 (2 × CO).	2.46 (s, 3H, CH_3); 3.77 (s, 3H, CH_3); 7.55-8.04 (a set of signals, 6H, C_6H_5 and pyrazole H-3); 10.63 (s, 1H, NH).
11a	$C_{20}H_{17}N_5O_2$ calc.: C, 66.84; H, 4.77; N, 19.49; found: C, 66.89; H, 4.58; N, 19.51.	42	172–173	3233 (br, NH), 1726 (CO), 1667 (CO).	1.27 (t, 3H, CH_3); 2.69-2.97 (m, 2H, CH_2) 7.41-8.43 (a set of signals,11H, 2 x C_6H_5 and pyrazole H-3); 11.58 (s, 1H, NH).
11c	$C_{20}H_{16}ClN_5O_2$ calc.: C, 60.99; H, 4.09; N, 17.78; found: C, 60.86; H, 3.99; N, 17.62.	46	184–185	3393 (br, NH), 1715 (CO), 1691 (CO).	1.26 (t, 3H, CH_3); 2.66-2.95 (m, 2H, CH_2) 7.44-8.43 (a set of signals, 10H, C_6H_5, C_6H_4, and pyrazole H-3); 11.72 (s, 1H, NH).
11d	$C_{21}H_{19}N_5O_2$ calc.: C, 67.55; H, 5.13; N, 18.76; found: C, 67.71; H, 5.27; N, 18.59.	45	229–230	3250 (NH), 1722–1699 (2 × CO).	1.26 (t, 3H, CH_3); 2.42 (s, 3H, CH_3); 2.67-2.95 (2 m, H, CH_2) 7.40-8.43 (a set of signals, 10H, C_6H_5, C_6H_4, and pyrazole H-3); 11.46 (s, 1H, NH).

Crystallization solvent: dioxane for **8b–e**, **10a**, **b**, **d**, and **11a**, **b**; ethyl acetate for **10e**; ethanol for **10c** and **11d**.

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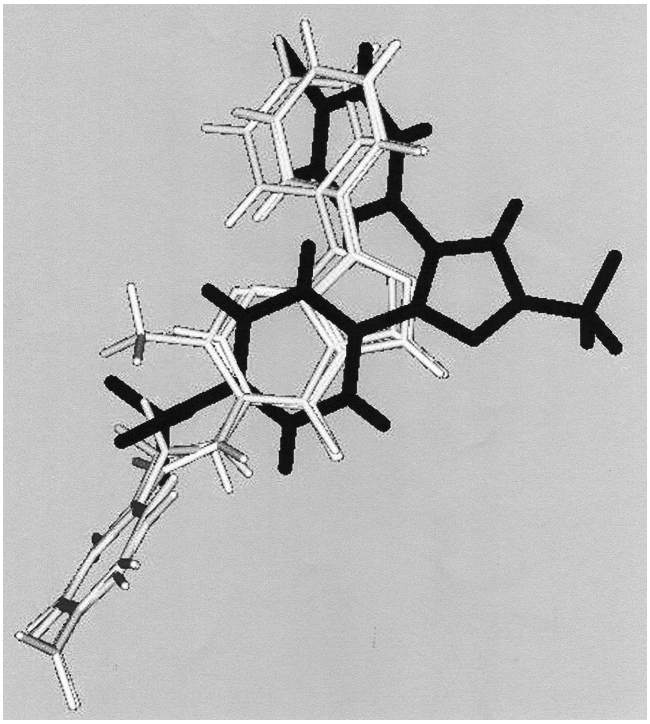

Figure 5. Overlay of the docked orientation for compounds **8a**, **b**, **10b** (white) and SC558 **13** (black) docked in the COX-2.

cond cavity branched off from the main channel that lead to the cyclooxygenase active site is observed in COX-2. A similar pocket exists in COX-1, but it is smaller and inaccessible because of the bulkier isoleucine at position 523 present in place of the valine in COX-2 [17]. So, the bulky 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidine-4-one structures are allowed to bind in the active site of COX-2 in a better way than the active site of COX-1 (Fig. 4).

In particular, analysis of the docked structure of the synthesized pyrazolo[3,4-*d*]pyrimidines **8a**, **b**, **10b** in the active sites of 6COX and 1Q4G reveals that these compounds are not capable to take up the COX-1 binding site and their structures are partially outside.

On the contrary, the bigger COX-2 binding site accommodates well the compounds **8a**, **b**, and **10b**. Comparison of the different docking results of the most active compounds **8a**, **b**, **10b** and SC588 **13** shows that, in principle, they adopt the same binding mode in the COX-2 binding site (Fig. 5). The ligands are oriented so that their pyrazole *N*-phenyl ring overlapped with the 5-bromophenyl ring of SC558 **13**. Furthermore, concerning the pyrazolo[3,4-d]pyrimidine nucleus, the pyrimidinone ring overlappes with the phenylsulphonamide moiety of SC558 **13** and both take up the second cavity branched off from the main channel which is also capable of accommodating the 5-benzamido group of the 1*H*-pyrazolo[3,4-*d*]pyrimidine-4-ones **8a**, **b, 10b** (Figs. 4 and 5).

These results are in agreement with the inhibitory activity against the purified human COX-2 and ovine COX-1 reported for compounds **8a – c**, **10a – d**, and **11a – c** (Table 1). In particular, as predicted by the docking experiments, the pyrazolo[3,4-*d*]pyrimidines **8a**, **b**, **10b** showed the best inhibitory activity against COX-2 (75.4 to 78.9% at 10 μM) and a very high COX-2 / COX-1 selectivity.

Analytical and spectroscopical data of compounds **5** and **8b-e** are reported in Tables 2 and 3, respectively.

Financial support from MIUR (fund 60%) is gratefully acknowledged.

The authors have declared no conflict of interest.

Experimental

Chemistry

All benzoyl chloride 7a – d were purchased from Aldrich Chemical Company (Sigma-Aldrich, Italy) and were used as received. TLC was performed on silica gel plates (Merck 60, F_{254}, 0.2 mm; Merck, Germany). Melting points were determined on a Büchi 530 capillary melting point apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected; IR spectra were recorded with a Jasco IR-810 spectrophotometer (Jasco, Tokyo, Japan) as nujol mulls; ^{1}H-NMR spectra were obtained in DMSO-d_6 on a Bruker AC-E 250 MHz spectrometer (TMS as internal standard; Bruker Bioscience, USA).

*General procedure for 5-aminopyrazole-4-(N-benzoyl)carbohydrazides **5b, d, e***

A solution of pyrazole-4-carbohydrazides **9a**, **b** (0.01 mol) in acetonitrile (50 mL) was refluxed with 0.01 mol of the opportune benzoyl chloride **7b**, **d**, **e** for 7 h. After this time, the solid that separated off was collected and recrystallized from ethanol to give pure **5**; yields 85 to 90%.

*General procedure for 5-benzamido-1H-pyrazolo[3,4-d]pyrimidine-4-ones **8, 10, 11***

1-*R*-5-Aminopyrazole-4-(*N*-benzoyl)carbohydrazides **5** (0.03 mol) were refluxed with 10 mL of the opportune triethylorthoester for 4 h. After this time, the solid which separated off was collected and recrystallized from the respective solvent (dioxane for **8b – e**, **10a**, **b**, **d**, and **11a**, **b**; ethyl acetate for **10e**; ethanol for **10c** and **11d**) to give pure **8**, **10**, and **11**; yields 44 to 75%.

Enzyme assay

The reference compounds indomethacin and NS398 were purchased from Cayman Chemical, Ann Arbor, MI, USA (cat. N° 70270 and 70590, respectively).

The ability of the test compound to inhibit the conversion of arachidonic acid to prostaglandin H_2 (PGH_2) was determined

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using a COX1/ COX2 inhibitor screening assay kit (kit n#560101; Cayman Chemical) according to the manufacturer's protocol.

Cyclooxygenase catalyzes the first step in the biosynthesis of arachidonic acid to PGH_2. The COX (ovine) Inhibitor Screening Assay directly measures $PGF_{2\alpha}$ produced by $SnCl_2$ reduction, by enzyme immunoassay (ACE™ competitive EIA). This assay is based on the competition between PG and PG-acetylcholinesterase (AChE) conjugate (PG-tracer) for a limited amount of PG monoclonal antibody.

Since the concentration of PG varies, the amount of PG-tracer that is able to bind to the PG monoclonal antibody will be inversely proportional to the concentration of PG in the well. This antibody-PG complex binds to goat polyclonal anti-mouse IgG that has been attached to the well previously. The plate was washed to remove any unbound reagents and Ellman's Reagent (which contains the substrate to acetylcholinesterase) is added to the well.

The product of this enzymatic reaction absorbs at 405 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of PG trace bound to the well, which is inversely proportional to the amount of free PG present in the well during the incubation: Absorbance $\propto$ [bound PG-tracer] $\propto 1/[PG]$.

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