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Hit Identification and Biological Evaluation of Anticancer Pyrazolopyrimidines Endowed with Anti-inflammatory Activity

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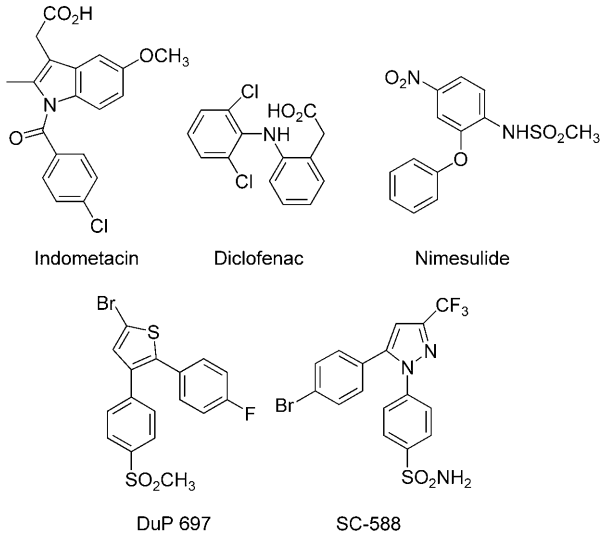
This paper is dedicated to the memory of Prof. Francisco Orallo, who died unexpectedly during the preparation of this manuscript.

In recent years, several studies have proposed the cyclo-oxygenases (COXs) as therapeutic targets in the prevention of cancer.[1] Some epidemiological investigations highlighted that nonsteroidal anti-inflammatory drugs (NSAIDs) reduced the development of several malignant diseases, including colorectal cancer.[2,3,4] NSAIDs inhibit COXs and consequently decrease the levels of prostaglandins (PG) that are significantly involved in carcinogenesis.[5,6]

In fact, COX (also known as prostaglandin G/H synthase) is a heme-containing enzyme that catalyzes the conversion of arachidonic acid (AA) into the hydroperoxide prostaglandin G_2 (PGG_2) and its subsequent reduction to prostaglandin H_2 (PGH_2). PGH_2 is a precursor of several classes of eicosanoids, which modulate central physiological functions, such as the inflammatory response, platelet aggregation, and the protection of gastric mucosa.

Two main COX isoforms are known: the constitutive isoform COX-1 and the inducible isoform COX-2. They show similar catalytic activity and share 65% amino acid sequence homology. COX-1 is involved in regulating vascular tone, and it is mainly present in the gastrointestinal tract and platelets. Inhibition of COX-1 by NSAIDs is believed to cause the adverse side effects associated with these drugs. Conversely, the COX-2 isoform is normally absent in healthy tissues, but it is rapidly induced in response to inflammatory mediators, such as bacterial endotoxin, interleukin 1 and various growth factors.

The COX active site is a narrow cleft, approximately 8 Å wide and 25 Å long. NSAIDs, with the exception of the acetylsalicilic acid, show reversible and competitive COX inhibition. Several scaffolds are able to exert selective COX inhibition, as exemplified by indometacin, diclofenac, nimesulide, DuP 697, SC-588 (shown).



COX-2 can be selectively inhibited despite the very similar structures of the active sites of both proteins. A single mutation of Ile in COX-1 with Val in the COX-2 binding site creates an additional pocket that is hydrophobic in nature.[7]

In tumor cells, high levels of PGE_2 have been highlighted in a number of experimental and clinical studies.[8] COX-2 has been shown to be overexpressed in various cancer types, such as head, neck, breast, skin, lung, bladder, tongue, colorectal, stomach, and prostate tumors.[9] Evidence that COX-2 inhibition can prevent these types of cancer has been reported in the literature.[10–13]

In a recent study, a dimethylamino pyrazolopyrimidine derivative (DPP) was identified as a potent inhibitor of PGE_2 production.[14] A further study confirmed the effect of DPP derivatives in vivo using a 24 h zymosan-injected mouse air pouch model.[15] Moreover, DPP was found to exert acute anti-inflammatory, analgesic and antiangiogenic effects that may be associated with COX-2 inhibition. DPP derivatives showed inhibitory activity comparable to that of the reference compound, NS398, a potent selective COX-2 inhibitor.[15]

In studies previously reported by us, novel 4-amino-substituted pyrazolopyrimidines were shown to reduce epidermal growth factor (EGF)-stimulated Src activation, causing a decrease in proliferation of A431 epidermoid tumor cells and 8701-BC breast cancer cells.[16–18] These derivatives, by reducing

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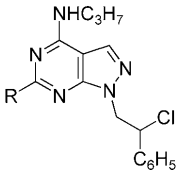
the production of the vascular endothelial growth factor (VEGF) released by tumor cells, also showed a highly selective antiangiogenic effect.[19] Moreover, some of them showed a significant antiproliferative effect on osteosarcoma cells and are active in a xenograft mouse model of this tumor.[20]

On the basis of these reports, and considering the great interest in antitumor agents acting with a dual mechanism (i.e., tyrosine kinase inhibition/anti-inflammatory action), we decided to investigate the potential anti-inflammatory activity of our pyrazolopyrimidines. With this aim, a three-dimensional chemical library containing 423 pyrazolo- and four pyrrolopyrimidines, variously substituted, has been designed and screened in silico against structural models of both COX-1 and COX-2. The compounds in our library possess a wide range of molecular weights (277.32–529.49), and 402 derivatives contain at least one asymmetric carbon atom. Enantiomers and diastereoisomers have been taken into account considering all possible configurations of their asymmetric centers. In total, 427 compounds were modeled.

All of the compounds were evaluated using Instant JChem (v. 1.0)[21] to predict their Log *P*; values ranged from 1.04 to 7.54. As reported previously,[22] the virtual screening tool Glide[23] was used to predict the binding of our compounds to COX-1 and COX-2 obtained from Protein Data Bank (PDB).[24] The crystal structures used for COX-1 and COX-2 were PBD ID: 1Q4G[25] and 1PXX,[26] respectively. The computed ligand–enzyme interaction energies were considered for ranking the theoretical affinity of our molecules with respect to both targets. The highest ranked COX-2 compounds, reporting no interaction with the COX-1 enzymatic cleft, were selected for experimental evaluation (Table 1).

Table 1. Structures of compounds **1**–**3** predicted to be the most selective COX-2 inhibitors together with their enzymatic (K_i) and cell data (IC_{50}).



Compd	R	K_i [μM]		IC_{50}[a] [μM]	
		Src	Abl	A431	8701-BC
1	$N(CH_3)_2$	4.10 ± 0.5	0.32 ± 0.04	20.0 ± 0.9	12.4 ± 0.6
2	S-C_2H_5	0.70 ± 0.1	0.41 ± 0.03	79.1 ± 1.8	29.5 ± 0.9
3	S-*i*C_3H_7	0.52 ± 0.1	0.65 ± 0.2	13.2 ± 0.7	18.1 ± 0.9

[a] A431 epidermoid tumor cells and 8701-BC breast cancer cells. K_i values are the means ± SD of three independent experiments. IC_{50} values are the means ± SEM of four independent experiments.

The binding of the selected compounds to the COX-2 active site was compared by superimposition using the co-crystal structure of the known, selective inhibitor, SC-588 bound to COX-2 (PDB ID: 6COX).[27] The docking experiments suggested to consider especially **1**, since both its enantiomers were able to fit into the COX-2 binding clefts with comparable theoretical affinities. In both cases, the pyrazolopyrimidine scaffold and the 2-chloro-2-phenylethyl substituent of the enantiomers of **1** docked in the same regions as the pyrazole and *p*-bromophenyl moieties of SC-588, respectively. Moreover, in both cases the propylamino group occupied the same trifluoromethyl recognition region of SC-588 (Figure 1). Inversion of the asymmetric carbon only seemed to affect the relative position of the pyrazolopyrimidine scaffold within the binding pocket, maintaining the two extreme phenyl and propyl moieties in the same locations.

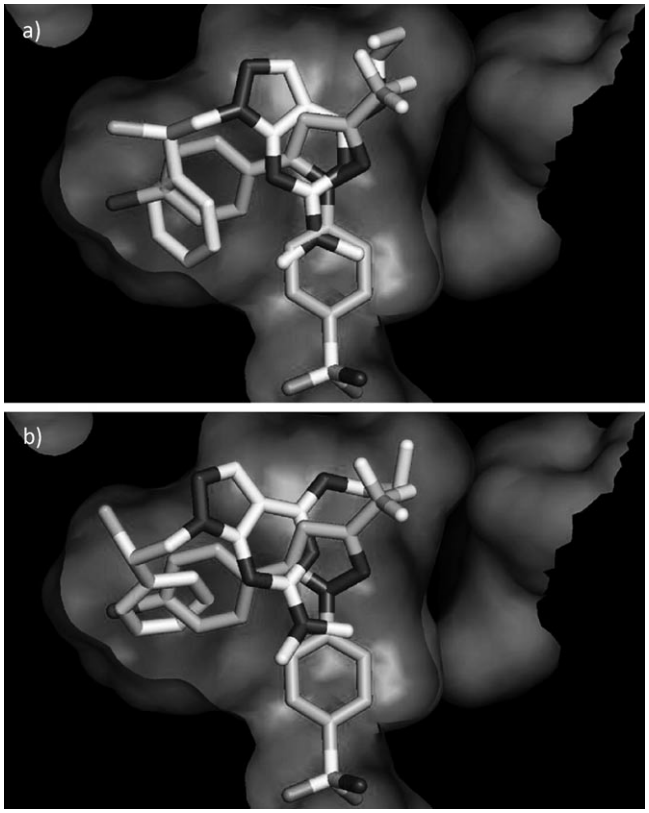


Figure 1. The best predicted docking poses of a) (*R*)-**1** and b) (*S*)-**1** (white carbon sticks) superimposed on that of the reference COX inhibitor SC588 (gray carbon sticks), modeled in the COX-2 binding site (PDB ID: 1PXX). The binding site is shown as a surface.

Compound **2**, the previously reported anticancer agent,[16–20] and the novel parent compound **3**, were also well-ranked, with larger differences in the predicted binding affinities of the enantiomers. In particular, compound **3** appeared as interesting as **1** due to their similar binding modes. In fact, both enantiomers of compound **1** and the (*S*)-enantiomer of **3** were predicted to bind with the propylamino substituent oriented similarly to the trifluoromethyl moiety of SC-588, and the R group in the same region as the phenylsulfonamide group of SC-588 (data not shown). Since we were mainly interested in the hit identification of dual action compounds, we selected **1**–**3** for biological evaluation.

Moreover, among our ligands, only the enantiomers of derivative **1** were predicted to establish a hydrogen bond with the enzyme through the Ser 530 residue of COX-2. This may ex-

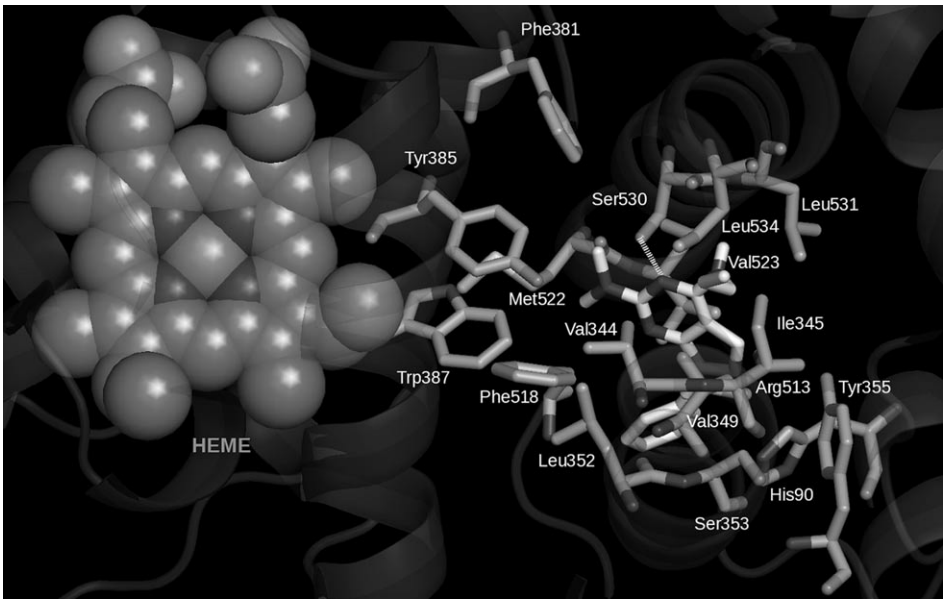
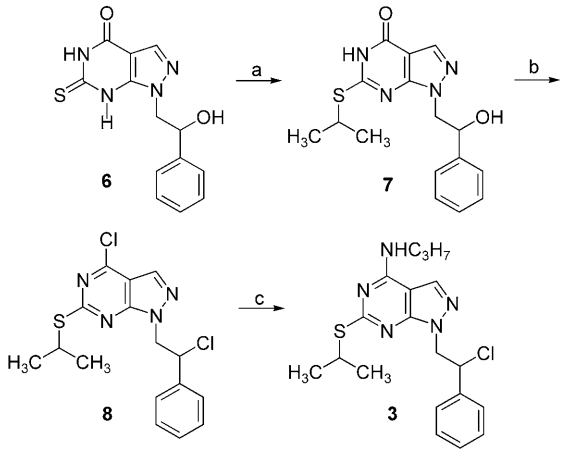


Figure 2. Predicted recognition of the COX-2 active site by (*S*)-**1**. The ligand (white carbon sticks) is shown interacting with key residues in the active site (gray carbon sticks); the heme cofactor is shown in slate spacefill model, and the rest of the enzyme is in transparent gray cartoon. The dotted line indicates a hydrogen bond.

plain the better affinity of compound **1** for COX-2 compared with the other studied compounds. Figure 2 shows the predicted, most stable binding mode of the (*S*)-**1**–COX-2 complex.

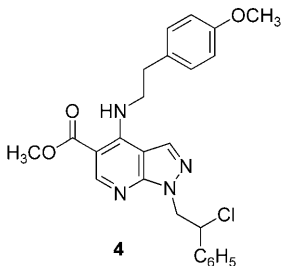
The synthesis of compounds **1** and **2** was previously reported.[28,29] Compound **3** was prepared as shown in Scheme 1, starting from intermediate **6**.[30] Synthetic details regarding the preparation of compound **3** are reported in the Supporting Information.



Scheme 1. Synthesis of compound **3**. *Reagents and conditions*: a) $(CH_3)_2CHBr$, K_2CO_3, DMF, RT, 8 h, 57% yield; b) $POCl_3$/DMF, $CHCl_3$, reflux, 8 h, 74% yield; c) *n*-propylamine, anhyd toluene, RT, 48 h, 97% yield.

A direct method for the enantiomeric separation was used to obtain sizeable amounts of the individual enantiomers of compounds **1**–**3**. Separation was performed on a semipreparative Chiralpak AS column, using *n*-hexane and 2-propanol as the eluant. The enantiomeric excess was then determined by analytical Chiracel OD column using an UV detector. The absolute stereochemistry of the chiral center on the side chain of compounds **1**, **2** and **3** was established via CD spectra comparison with those of compound **4** of known absolute configuration, obtained through X-ray crystal structure,[31] and confirmed with that of a very similar compound **5** reported by Da Settimo and co-workers.[32]

The structure of compound **5** and CD spectra analyses are reported in the Supporting Information. Stereogenic assignment demonstrated that the (−) and (+) isomers of compounds **1**–**3** correspond to the *R* and *S* absolute configurations, respectively.



The biological tests were carried out using a chromogenic assay based on the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) during the reduction of PGG_2 to PGH_2. The enzyme activity was measured by estimation of the initial reaction rate as followed by the increase in absorbency at 600 nm. Control experiments using H_2O_2 (0.4 mM) as the substrate showed that inhibitors **1**–**3** did not affect the peroxidase activity of COX under the assay conditions. The reference COX inhibitors were preincubated at 37 °C for 5 min before initiation of the enzymatic reaction by the addition of 1 μM arachidonic acid. Our compounds were submitted to the same analysis using pure enantiomers after stereogenic assignment (Supporting Information). The results are reported in Table 2.

Among the known COX inhibitors, DuP 697 resulted the most potent and selective against COX-2 with inhibitory activity in the nanomolar range. As reported in Table 2, our compounds exhibited significant COX-2 selectivity, with inhibitory activity in the micromolar range. In particular, both enantiomers of **1** were more potent than the reference inhibitors indomethacin and diclofenac. It is worth noting that the inhibitory activities for these two reference compounds were recalculated by us since no convergence to similar IC_{50} values were found in the literature. Compound **2** showed inhibitory activity com-

Table 2. IC_{50} values and COX-1 selectivity ratio obtained using the pure enantiomers of compounds **1**–**3** with respect to reference compounds.

Compd	IC_{50} values[a] [μM]		Selectivity ratio[b]
	COX-1[d]	COX-2	
Indometacin[c]	12.16 ± 1.16	35.20 ± 1.41	2.9
Diclofenac	18.23 ± 1.73	23.62 ± 1.97	1.3
Nimesulide	***	231.40 ± 19.84	< 0.46[e]
DuP 697[c]	22.61 ± 1.56	0.12632 ± 0.00741	0.0056
(R)-(−)-**1**	**	21.60 ± 1.18	< 0.22[d]
(S)-(+)-**1**	**	20.35 ± 1.87	< 0.20[e]
(R)-(−)-**2**	**	36.73 ± 3.16	< 0.37[e]
(S)-(+)-**2**	**	30.85 ± 2.79	< 0.31[e]
(R)-(−)-**3**	**	65.26 ± 2.41	< 0.65[e]
(S)-(+)-**3**	**	66.48 ± 5.30	< 0.66[e]

[a] All IC_{50} values are the mean ± SEM ($n = 5$). [b] COX-1 ratio = [IC_{50} (COX-2)]/[IC_{50} (COX-1)]. [c] Level of statistical significance: $P < 0.01$ (for indometacin) and $P < 0.05$ (for DuP 697) versus the corresponding IC_{50} values obtained against COX-2, as determined by ANOVA/Dunnett's test. [d] **, inactive at 100 μM (highest concentration tested); ***, inactive at 500 μM (highest concentration tested). [e] Values obtained under the assumption that the corresponding IC_{50} against COX-1 or COX-2 is the highest concentration tested.

parable to indometacin, but with improved COX-2 selectivity. Despite the binding modes predicted by the docking experiments, both enantiomers of compound **3** showed moderate COX-2 inhibition and selectivity.

In conclusion, we have demonstrated that antiproliferative pyrazolopyrimidines can exert a dual activity, with anti-inflammatory effects comparable to known COX inhibitors. Even through the anti-inflammatory potency of these compounds was not as high as the known COX-2-selective inhibitor DuP 697, compound **1** revealed interesting COX-2 activity and selectivity compared with the other three reference drugs. This aspect, together with the sub-micromolar activity of all three compounds against cytoplasmic tyrosine kinases Src and Abl, highlights the potential of this class of molecules as dual anti-inflammatory/tyrosine kinase inhibitors that could represent a therapeutic opportunity for the prevention and treatment of cancer.

Experimental Section

The Supporting Information contains full experimental details for the docking simulations, synthesis, enantiomeric separation and biological evaluation.

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Keywords: COX · dual activity · enantiomeric separation · pyrazolopyrimidines · virtual screening

[1] M. J. Thun, M. M. Namboodiri, C. W. Heath Jr. , *N. Engl. J. Med.* **1991**, *325*, 1593 – 1596.
[2] D. M. Schreinemachers, R. B. Everson, *Epidemiology* **1994**, *5*, 138 – 146.
[3] D. C. Farrow, T. L. Vaughan, P. D. Hansten, J. L. Stanford, H. A. Risch, M. D. Gammon, W. H. Chow, R. Dubrow, H. Ahsan, S. T. Mayne, J. B. Schoenberg, A. B. West, H. Rotterdam, J. F. Fraumeni Jr. , W. J. Blot, *Cancer Epidemiol., Biomarkers Prev.* **1998**, *7*, 97 – 102.
[4] M. J. Langman, K. K. Cheng, E. A. Gilman, R. J. Lancashire, *BMJ* **2000**, *320*, 1642 – 1646.
[5] P. Ben-Av, L. J. Crofford, R. L. Wilder, T. Hla, *FEBS Lett.* **1995**, *372*, 83 – 87.
[6] J. L. Masferrer, K. M. Leahy, A. T. Koki, B. S. Zweifel, S. L. Settle, B. M. Woerner, D. A. Edwards, A. G. Flickinger, R. J. Moore, K. Seibert, *Cancer Res.* **2000**, *60*, 1306 – 1311.
[7] R. M. Garavito, M. G. Malkowski, D. L. DeWitt, *Prostaglandins Other Lipid Mediators* **2002**, *68–69*, 129 – 152.
[8] A. Raz, *Biochem Pharm.* **2002**, *63*, 343 – 347.
[9] A. J. Dannenberg, N. K. Altorki, J. O. Boyle, C. Dang, L. R. Howe, B. B. Weksler, K. Subbaramaiah, *Lancet Oncol.* **2001**, *2*, 544 – 551.
[10] R. E. Harris, J. Beebe-Donk, G. A. Alshafie, *BMC Cancer* **2008**, *8*, 237.
[11] Y. Dai, W. H. Wang, *World J. Gastroenterol.* **2006**, *12*, 2884 – 2889.
[12] L. Gallicchio, K. Visvanathan, A. Burke, S. C. Hoffman, K. J. Helzlsouer, *Int. J. Cancer* **2007**, *121*, 211 – 215.
[13] S. Jain, G. Chakraborty, R. Raja, S. Kale, G. C. Kundu, *Cancer Res.* **2008**, *68*, 7750 – 7759.
[14] J. M. Quintela, C. Peinador, L. Gonzàlez, I. Devesa, M. L. Ferràndiz, M. J. Alcaraz, R. Riguera, *Bioorg. Med. Chem.* **2003**, *11*, 863 – 868.
[15] I. Devesa, M. J. Alcaraz, R. Riguera, M. L. Ferràndiz, *Eur. J. Pharmacol.* **2004**, *488*, 225 – 230.
[16] F. Carraro, A. Pucci, A. Naldini, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, C. Brullo, P. Fossa, G. Menozzi, L. Mosti, F. Manetti, M. Botta, *J. Med. Chem.* **2004**, *47*, 1595 – 1598.
[17] S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, C. Brullo, P. Fossa, L. Mosti, G. Menozzi, F. Carraro, A. Naldini, C. Bernini, F. Manetti, M. Botta, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2511 – 2517.
[18] S. Schenone, O. Bruno, F. Bondavalli, A. Ranise, L. Mosti, G. Menozzi, P. Fossa, S. Donnini, A. Santoro, M. Ziche, F. Manetti, M. Botta, *Eur. J. Med. Chem.* **2004**, *39*, 939 – 946.
[19] S. Donnini, M. Monti, C. Castagnini, R. Solito, M. Botta, S. Schenone, A. Giachetti, M. Ziche, *Int. J. Cancer* **2007**, *120*, 995 – 1004.
[20] F. Manetti, A. Santucci, G. A. Locatelli, G. Maga, A. Sreafico, T. Serchi, M. Orlandini, G. Bernardini, N. P. Caradonna, A. Spallarossa, C. Brullo, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, O. Hoffmann, M. Bologna, A. Angelucci, M. Botta, *J. Med. Chem.* **2007**, *50*, 5579 – 5588.
[21] a) Instant JChem, version 1.0 (ChemAxon Ltd); Full product details and a user's guide can be found here: http://www.chemaxon.com/products/instant-jchem/; b) V. N. Viswanadhan, A. K. Ghose, G. R. Revankar, R. K. Robins, *J. Chem. Inf. Comput. Sci.* **1989**, *29*, 163 – 172; c) G. Klopman, J.-Y. Li, S. Wang, M. Dimayuga, *J. Chem. Inf. Comput. Sci.* **1994**, *34*, 752 – 781; d) F. Csizmadia, A. Tsantili-Kakoulidou, I. Panderi, F. Darvas, *J. Pharm. Sci.* **1997**, *86*, 865 – 871; e) G. Bouchard, P. A. Carrupt, B. Testa, V. Gobry, H. H. Girault, *Pharm. Res.* **2001**, *18*, 702 – 708.
[22] A. Procopio, S. Alcaro, M. Nardi, M. Oliverio, F. Ortuso, P. Sacchetta, D. Pieragostino, G. Sindona, *J. Agric. Food Chem.* **2009**, *57*, 11161 – 11167.
[23] a) Glide: A complete solution for ligand–receptor binding (Schrödinger, LLC); Full product details and a list of publications can be found here: http://www.schrodinger.com/products/14/5/ (Last accessed: June 02, 2010); b) M. D. Eldridge, C. W. Murray, T. R. Auton, G. V. Paolini, R. P. Mee, *J. Comput.-Aided Mol. Des.* **1997**, *11*, 425 – 445.
[24] H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucl. Acids Res.* **2000**, *28*, 235 – 242.
[25] K. Gupta, B. S. Selinsky, C. J. Kaub, A. K. Katz, P. J. Loll, *J. Mol. Biol.* **2004**, *335*, 503 – 518.
[26] S. W. Rowlinson, J. R. Kiefer, J. J. Prusakiewicz, J. L. Pawlitz, K. R. Kozak, A. S. Kalgutkar, W. C. Stallings, R. G. Kurumbail, L. J. Marnett, *J. Biol. Chem.* **2003**, *278*, 45763 – 45769.

[27] R. G. Kurumbail, A. M. Stevens, J. K. Gierse, J. J. McDonald, R. A. Stegeman, J. Y. Pak, D. Gildehaus, J. M. Miyashiro, T. D. Penning, K. Seibert, P. C. Isakson, W. C. Stallings, *Nature* **1996**, *384*, 644 – 648.
[28] S. Schenone, C. Brullo, O. Bruno, F. Bondavalli, L. Mosti, G. Maga, E. Crespan, F. Carraro, F. Manetti, C. Tintori, M. Botta, *Eur. J. Med. Chem.* **2008**, *43*, 2665 – 2676.
[29] F. Carraro, F. Naldini, A. Pucci, G. A. Locatelli, G. Maga, S. Schenone, O. Bruno, A. Ranise, F. Bondavalli, C. Brullo, P. Fossa, G. Menozzi, L. Mosti, M. Modugno, C. Tintori, F. Manetti, M. Botta, *J. Med. Chem.* **2006**, *49*, 1549 – 1561.
[30] S. Schenone, O. Bruno, F. Bondavalli, A. Ranise, L. Mosti, G. Menozzi, P. Fossa, F. Manetti, L. Morbidelli, L. Trincavelli, C. Martini, A. Lucacchini, *Eur. J. Med. Chem.* **2004**, *39*, 153 – 160.
[31] M. Botta, *unpublished results*.
[32] F. Da Settimo, G. Primofiore, C. La Motta, S. Taliani, F. Simorini, A. M. Marini, L. Mugnaini, A. Lavecchia, E. Novellino, D. Tuscano, C. Martini, *J. Med. Chem.* **2005**, *48*, 5162 – 5174.

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