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Short communication

Synthesis and biological evaluation of N-substituted-3,5-diphenyl-2-pyrazoline derivatives as cyclooxygenase (COX-2) inhibitors

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ABSTRACT

Eighteen new 1-N-substituted-3,5-diphenyl-2-pyrazoline derivatives have been synthesized and cyclooxygenase (COX-1 and COX-2) inhibitory activities have been evaluated. The results of these biological assays showed that all of new derivatives are not endowed with improved anti-inflammatory activity against COX-1, but some of them showed a good activity against COX-2. To evaluate the binding mode of the most significative compounds (**2d**, **2f**, **2g** and **2k**) docking studies were carried out. These studies confirmed biological data, in fact these compounds were able to fit into the active site of COX-2.

1. Introduction

Cyclooxygenase (COX) or prostaglandin endoperoxide synthase (PGHS) catalyzes the first step in the biosynthesis of the prostaglandins (PGs) from the substrate arachidonic acid (AA) [1]. COX enzyme possesses two distinct catalytic activities: (1) cyclooxygenase activity that catalyzes the oxidation of AA to produce hydroperoxy endoperoxide (PGG2) and (2) peroxidase activity that reduces the hydroperoxide PGG2 to the hydroxy endoperoxide (PGH2). The PGH2 is transformed by a range of enzymic and nonenzymic mechanisms into the primary prostanoids. In addition, arachidonic acid is a substrate for a variety of additional oxidative enzymes such as lipoxygenase, which generates biologically active lipids: hydroperoxyeicosatetraenoic acid (HPETE), hydroxyeicosatetraenoic acid (HETE), and leukotrienes (LTA4, LTB4, LTC4, and LTE4). The cyclooxygenase (COX) enzymes, were identified as the molecular targets of all nonsteroidal anti-inflammatory drugs (NSAIDs) [2–4]. COX-1 is the constitutive isoform and is mainly responsible for the synthesis of cytoprotective PGs in gastrointestinal (GI) tract whereas COX-2 is inducible and plays a major role in PG biosynthesis in inflammatory cells [5–7]. It is believed that the inhibition of COX-1 causes unfavorable GI side effects [8]. Therefore, development of novel compounds having anti-inflammatory activity with an improved safety profile is still a necessity. Literature survey revealed that many pyrazole derivatives [9] have found their clinical application as NSAIDs. Among the highly marketed COX-2 inhibitors that comprise the pyrazole nucleus, celecoxib is the one which is treated as a safe anti-inflammatory and analgesic agent. It is considered as a typical model of the diaryl heterocyclic template that is known to selectively inhibit the COX-2 enzyme. Some other examples of pyrazole derivatives as NSAIDs are mefobutazone, ramifenazone, famprofazone [10–13]. (Fig. 1).

But due to serious adverse effects (such as bone marrow depression, water and salt retention and carcinogenesis), the use of pyrazole derivatives is limited. This limitation has led to the investigation of new pyrazole derivatives with that more potent activity and less toxicity.

Motivated by the aforesaid findings, and pursuing our studies on pyrazole moiety [14–17], we were designed to synthesize a new series of 1-N-substituted-3,5-diphenyl-2-pyrazoline derivatives and tested them as COXs inhibitors.

Compared with Celecoxib, new pyrazoline derivatives were synthesized by replacing pyrazole moiety, with a dihydropyrazole nucleus, linked in C3 and C5 with phenyl group. In particular we have introduced 4-methansulfonyl phenyl, in C3 position, which

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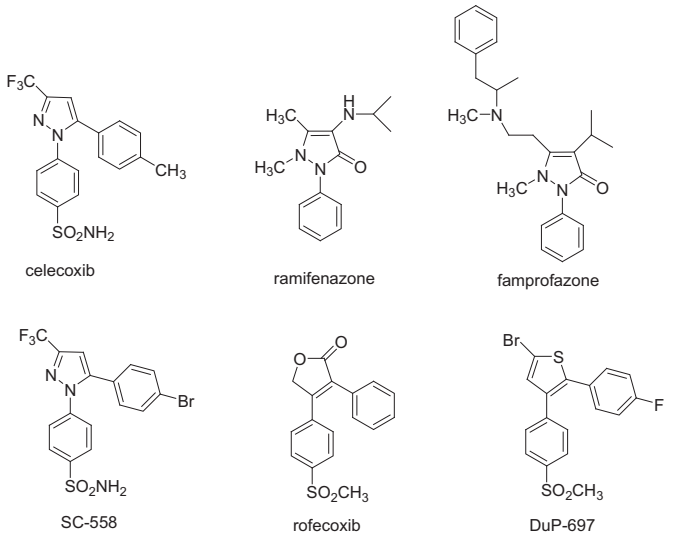


Fig. 1. Chemical structures of some known coxib inhibitors.

seems important in interaction with the secondary pocket of COX-2 active site. (Fig. 2).

2. Chemistry

The synthesis of the target compounds is shown in Scheme 1. The intermediate chalcones **1a**–**i**, were obtained by direct Claisen–Schmidt condensation between different aromatic aldehyde and 4-methyl sulfonyl acetophenone in a basic medium in ethanol 96%. A solution of appropriate 4′-methyl sulfonyl chalcone **1a**–**i** in acetic acid and hydrazine hydrate or thiosemicarbazide in 50 mL of ethanol and sodium hydroxide was refluxed for 8 h, to obtain the new derivatives **2a**–**r** (see Supplementary Data).

3. Biochemistry

The potential effects of the test drugs on total hCOX activity (*bis*dioxygenase and peroxidase reactions) were investigated by measuring their effects on the oxidation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) to *N,N,N′,N′*-tetramethyl-*p*-phenylenediimine, using AA as common substrate for both hCOX-1 and hCOX-2, microsomal COX-2 prepared from insect cells (Sf 21 cells) infected with recombinant baculovirus containing cDNA inserts for hCOX-2 (Sigma–Aldrich Química S.A., Alcobendas, Spain) and COX-1 from human platelet microsomes (obtained as described in the above paragraph since, unlike hCOX-2, hCOX-1 is not available commercially).

4. Results and discussion

The *in vitro* activity of derivatives **2a**–**r**, is reported in Table 1; we depicted only COX-2 activity, because all derivatives were inactive

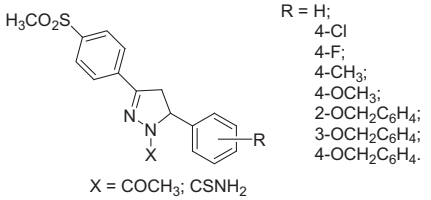
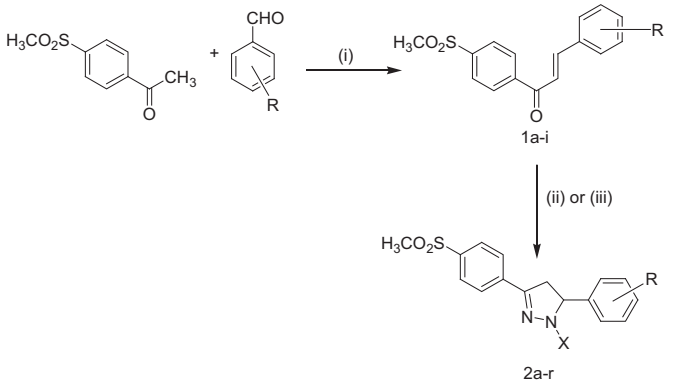


Fig. 2. 1-N-substituted-3,5-diphenyl-2-pyrazoline derivatives.



Scheme 1. Reagent and conditions: (i) $Ba(OH)_2 \cdot 8H_2O$, EtOH 96%, 25–30 °C, 8 h; (ii) NH_2NH_2, CH_3COOH, EtOH dry, 78 °C, 24 h; (iii) $NH_2NHCSNH_2$, NaOH, EtOH dry, 78 °C, 24 h (for assignment of **a**–**r** see Table 1).

as COX-1 inhibitors. N-acetyl derivatives (**2a**–**i**), were found to be more potent, than corresponding N-thiocarbamoyl derivatives (**2j**–**r**).

Derivatives **2d**, **2f** and **2g** are the most potent compounds of N-acetyl series, whereas, among N-thiocarbamoyl derivatives the most active compound is **2k**. For the most active compounds we have carried out HPLC enantioseparations (Supplementary Data) to investigate which enantiomer was the best inhibitor. The biological results showed a slight difference of activity between the two isomers with respect to each other and with respect to the racemic mixture. In order to deeply investigate the recognition of our most potent and COX-2 selective pyrazoline derivatives, docking simulations were carried out onto both enantiomers of compounds **2d**, **2f**, **2g** and **2k**. The Protein Data Bank (PDB) [18] was searched for COX-1 and COX-2 experimental models. Human enzyme isoforms were not found and, consequently, the crystallographic structures 1Q4G, [19] from *Ovis aries*, and 6COX, [20] from *Mus musculus*, were selected for mimicking the cyclooxygenase 1 and 2 respectively. The

Table 1
IC_{50} values for the inhibitory effects of test drugs (new compounds and reference inhibitors) on the enzymatic activity of recombinant human COX-2 expressed in Sf 21 cells.

Comp	R	X	COX-2 IC_{50}
2a	H	$COCH_3$	**
2b	4Cl	$COCH_3$	21.70 ± 1.86 μM
2c	4F	$COCH_3$	19.63 ± 1.62 μM
2d	$4CH_3$	$COCH_3$	6.77 ± 0.48 μM
2e	$4CF_3$	$COCH_3$	19.83 ± 1.76 μM
2f	$4OCH_3$	$COCH_3$	7.61 ± 0.58 μM
2g	$2OCH_2Ph$	$COCH_3$	3.20 ± 0.24 μM
2h	$3OCH_2Ph$	$COCH_3$	**
2i	$4OCH_2Ph$	$COCH_3$	**
2j	H	$CSNH_2$	50.31 ± 4.06 μM
2k	4Cl	$CSNH_2$	9.35 ± 0.76 μM
2l	4F	$CSNH_2$	**
2m	$4CH_3$	$CSNH_2$	31.85 ± 2.57 μM
2n	$4CF_3$	$CSNH_2$	*
2o	$4\ OCH_3$	$CSNH_2$	*
2p	$2OCH_2Ph$	$CSNH_2$	*
2q	$3OCH_2Ph$	$CSNH_2$	*
2r	$4OCH_2Ph$	$CSNH_2$	*
Indometacin			35.20 ± 1.41 μM
Diclofenac			23.62 ± 1.97 μM
Nimesulide			231.40 ± 19.84 μM
DuP-697			126.32 ± 7.41 nM

All IC_{50} values shown in this table are the mean ± S.E.M. from five experiments. IC_{50} values obtained against COX-2, as determined by ANOVA/Dunnett's. * Inactive at 25 μM (highest concentration tested). ** Inactive at 100 μM (highest concentration tested).

first PDB model reported the complex between the COX-1 and the α-methyl-4-biphenylacetic acid and was characterized by the highest resolution (2.0 Å) available for cyclooxygenase structures into the PDB at the search time. The 6COX was selected taking into account its co-crystallized ligand, the SC-588, which represents a COX-2 selective inhibitor with some structural similarities with respect to our compounds. After a preliminary pre-treatment (Supplementary Data) the above indicated PDB models were considered the targets of our docking simulations carried out using the AutoDock–Vina software [21]. Theoretical results were submitted to both visual and energy analysis. Interestingly within the COX-1 all our compounds were not able to fit into the known active site preferring the recognition of other areas, i.e. that related to the iron protoporphyrin IX (HEME) cofactor (Supplementary Data). Such an observation was not a surprise, in fact, the original PDB model 1Q4G reported a glycerol molecules interacting to the cofactor iron ion, and, recently [22], and we have described the HEME recognition of anti-inflammatory oleuropein semi-synthetic derivatives. The cofactor could be considered as an accessory binding site modulating the COX activity but we are not confident about its relevance into the anti-inflammatory properties of our compounds. We can suggest that molecules with a large chemical structure could be not able to fit into the know COX-1 binding site and, consequently, their target recognition, in computational simulation, moves toward more accessible sites. Actually, in order to verify the steric hindrance role into the COX-1 recognition, we have performed, first of all, the docking of the co-crystallized ligand and, then, of known non-selective COX inhibitors, such as flurbiprofen, nimesulide and diclofenac. In all these cases the resulting configurations have highlighted the recognition of both classic binding sites and HEME moiety, in particular the complex with the α-methyl-4-biphenylacetic acid have reproduced the PDB model 1Q4G geometries with the most stable ligand orientation and, at higher interaction energies directly in contacts with the cofactor. With the aim to stronger validate our computational procedure, we have docked to COX-1 two known COX-2 selective inhibitors such as the SC-588 and the DuP-697. The results of these last compounds were quite similar to that indicated for our derivatives, actually both have interacted to the HEME and were not able to fit into the classic binding site. On the base of the above reported evidences, and taking into account the experimental COX-1 IC_{50} values of our pyrazoline derivatives, we focused our computational study onto their COX-2 interaction only. The COX-2 docking experiments were validated including in our simulations the selective inhibitors SC-588 and DuP-697. Such molecules allowed us to verify both the theoretical complexes geometries and the adopted scoring functions. Into the COX-2 the best scored configurations of all compounds have fitted into the known classic binding site, i.e. the cleft occupied by SC-588 into the PDB original model (Supplementary Data). In order to evaluate the binding modes of all docked compounds we have considered both the default AutoDock–Vina scoring function and the MolInE method [23,24] highlighting a good qualitative agreement with respect to the experimental inhibition IC_{50} (Table 2).

Table 2
Comparison among experimental IC_{50} (μM) and theoretical binding energies (kcal/mol). Best and average values were computed by AutoDock–Vina software, ΔΔG was calculated applying the MolInE method to the AutoDock–Vina interaction energies. Compounds **2d**, **2f**, **2g** and **2k** results were obtained averaging the values of the corresponding enantiomers.

Compound	Binding energies (kcal/mol)			IC_{50} (μM)
	Best	Average	ΔΔG	
DuP-697	−7.70	−6.65	−7.07	0.13
2d	-2.35^{S}	−2.20	−2.22	6.77
2f	-1.65^{S}	−1.13	−1.38	7.61
2g	-3.55^{S}	−2.37	−2.56	3.20
2k	-0.45^{R}	−0.03	−0.15	9.35

$^{R, S}$ energy value related to (*R*) - and (*S*)- enantiomers respectively.

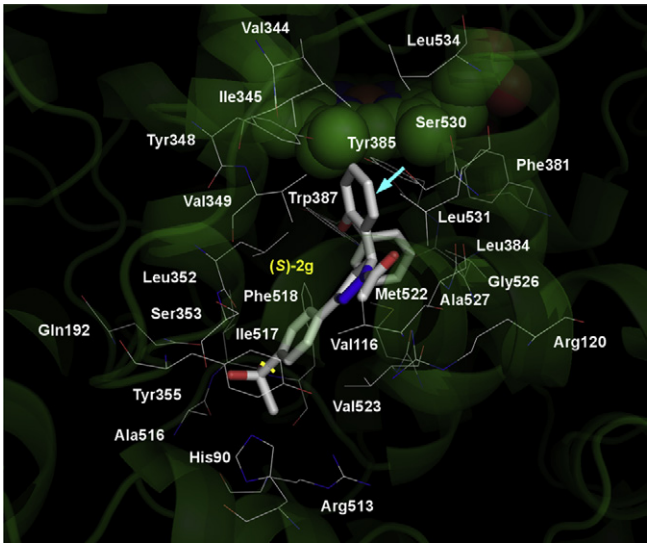


Fig. 3. Best pose of **2g** into the COX-2 binding site. The ligand is depicted in polytube model, interacting residues are labelled and displayed in wireframe, HEME cofactor is in green carbon coloured spacefill. Yellow dotted line and cyan arrow indicate intermolecular hydrogen bond and hydrogen-pi interaction respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The graphical analysis of the most stable COX-2 complexes revealed similar configuration for all docked pyrazoline derivatives. In all cases our compounds showed their 2-aryl-R substituent directed to the inner cyclooxygenase side while the 4-methylsulfonylphenyl moiety was located into the same pocket occupied by the SC-588 sulfonamide group into the 6COX original PDB model (Fig. 3 and Supplementary Data).

Such an additional binding cleft is known to be exclusive for the COX-2 and its recognition is on the basis of the coxibs selectivity. The number of recognized residues was quite similar among our derivatives ranging from 19 amminoacids to 24. Excluding (*S*)-**2d** and both enantiomers of **2k**, our pyrazoline derivatives demonstrated hydrogen-pi interaction to Ser530. A deeper analysis highlighted that only the (*R*) - and the (*S*) - enantiomers, of the most potent inhibitor **2g**, were able to produce hydrogen bonds to Arg513 and Phe518 respectively. Moreover the phenoxy-group of **2g** was well accommodated into an aromatic cage delimited by Phe518, Trp387, Tyr385 and Phe384. We addressed to those evidences the rational of the higher COX-2 inhibition of **2g** with respect to the other analogues.

In particular derivatives **2d**, **2f**, **2g** and **2k**, were the most interesting compounds, with IC_{50} values in range micromolar. Because these compounds have a carbon chiral we separated them in two enantiomers and then re-tested; then we completed the experimental part, using molecular modeling studies. No significant differences between both enantiomers and racemic inhibition activities were confirmed by the molecular modeling studies too.

5. Conclusion

Molecular modeling studies confirm biological data, in fact all our compounds were not able to fit into the known active of COX-1, while, into the COX-2 the best scored configurations of all compounds have fitted into the known classic binding site. Our studies have underlined the importance of 4-methylsulfonylphenyl in C3 position on pyrazoline, but, very interesting results obtained

with binding studies about the phenyl on C5; in fact respect to the coxib series we have inserted a methylene group among two aryl groups, to realize if the activity maintained. This shift of phenyl group produced derivatives several selective COX-2 inhibitors, but less active than references. Also we have investigated the importance of substituents on the C5 aryl and molecular modeling study underline that some of these e.g. phenoxy-group seem to help the interaction with active site of enzyme. These preliminary results encouraged us to persevere the search in this field, and these novel compounds could be an interesting template for the design of new derivatives.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version.at doi:10.1016/j.ejmech.2010.10.005.

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