Short Communication

Synthesis and Selective Inhibitory Activity Against Human COX-1 of Novel 1-(4-Substituted-thiazol-2-yl)-3,5-di(hetero)aryl-pyrazoline Derivatives

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Novel 1-(4-ethyl carboxylate-thiazol-2-yl)-3,5-di(hetero)aryl-2-pyrazoline derivatives were obtained by reacting 3,5-di(hetero)aryl-1-thiocarbamoyl-2-pyrazolines with the ethyl ester of α-bromo-pyruvic acid. The synthesized compounds were confirmed by spectroscopic data and assayed to evaluate their *in vitro* ability to inhibit both isoforms of human cyclooxygenase (hCOX). Some derivatives (compounds **5**, **6**, **13**, **16**, and **17**) displayed promising selectivity against hCOX-1 in the micromolar range and were shown to have a selectivity index similar or better than the reference drugs (indometacin, diclofenac). The introduction of a phenyl or a 4-F-phenyl ring on the C5 associated with a 4-substituted phenyl or a heteroaryl group on the C3 of (4-substituted-thiazol-2-yl)pyrazoline derivatives improved the activity against hCOX-1. Thanks to these preliminary results it could be possible to extend our knowledge of the pharmacophoric requirements for the discovery of new pyrazoline-based hCOX-1 inhibitors.

Keywords: Cyclooxygenase / Microwave-assisted synthesis / Pyrazoline / Selective hCOX-1 inhibitors / Thiazole

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Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most used drugs in the treatment of pain, fever, inflammation, and rheumatic disorders. As many symptoms of inflammation are caused by prostaglandins (PG), the efficacy of NSAIDs has been in part attributed to their ability to inhibit PG synthesis and release. This inhibition occurs through the blockage of the arachidonic acid cascade, which is catalyzed by two isoforms of the cyclooxygenase (prostaglandin G/H synthase, EC 1.14.99.1): COX-1 and COX-2 [1, 2]. The major differences between the two isoenzymes involve their distribution and expression. COX-1 is constitutively distributed in human tissues and is mainly involved in gastroprotection, renal blood flow, and platelet aggregation. Conversely, COX-2 is almost undetectable under normal physiological conditions, while its expression is increased by inflammatory mediators. Traditional NSAIDs are non-selective COX-inhibitors and their use is often associated with the occurrence of a range of side effects. Hence the search for novel analgesic/antiinflammatory agents devoid of severe toxicity continues to be an active area of research in medicinal chemistry [1, 2].

Human COX-1 has been receiving increasing attention in the last years as a reconsidered therapeutic target, because it is involved in a large number of pathologies such as pain, neuro-inflammation, atherosclerosis, endothelium dysfunction, pre-term labor, some types of cancer, and gastrointestinal toxicity (which seems to be induced not by specific hCOX-1 inhibition but by the contemporaneous blockage of both isoenzymes). Moreover, a careful consideration must be given to the cardiovascular side-effects associated with the therapy of inflammation with selective hCOX-2 inhibitors: contrary to hCOX-1 inhibitors, these drugs do not suppress the production of thromboxane A_2, which is a vasoconstrictor and a prothrombotic agent, stimulating platelet activation and

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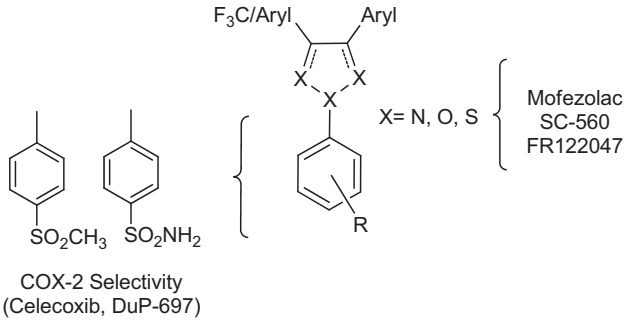


Figure 1. Structural requirements to obtain (diaryl)heterocycles endowed with inhibitory activity oriented towards hCOX-1 or hCOX-2.

aggregation. According to that, the selective inhibition of hCOX-1 could be useful for patients suffering from cardiovascular diseases [3]. Actually, few selective hCOX-1 inhibitors are known and they can be grouped into five chemical classes. Some key structural elements can be recognized to provide a correlation between chemical structure and selective hCOX-1 inhibition (Fig. 1): (i) a five-membered heteroaromatic central ring (pyrazole, thiazole [4], isoxazole, thiophene, and furan); (ii) two (hetero)aryls attached to this core and generally substituted with electron-donating moieties; (iii) the absence of a methylsulfonyl or a sulfamoyl group which shifts the activity and selectivity toward hCOX-2 (due to the reduced side pocket volume of COX-1 with the substitution of Val523 with Ile) [3].

Among these molecules, 1,3,5-trisubstituted-pyrazolines were reported to exhibit anti-inflammatory and analgesic activities by inhibiting both human COX isoenzymes. Some of them were endowed with COX-2 selectivity [5–8], others with contemporaneous inhibition of both isoenzymes or with preferential COX-1 inhibitory activity [9–11].

Starting from these assumptions, the aim of our work is to propose the synthesis and the biological evaluation of a new series of 1-(4-substituted-thiazol-2-yl)-3,5-di(hetero)aryl-pyrazoline derivatives as cyclooxygenase inhibitors, improving the structure–activity studies to find out the structural requirements for new selective COX-1 inhibitors. We synthesized novel molecules (**1**–**19**) with different (hetero)aryl groups at positions C3 and C5 and a thiazole ring [4], never explored so far, at position N1 (Table 1). The design of these derivatives explores the change in the electronic density and steric hindrance of the pharmacophoric group. For this reason, we introduced

Table 1. Structures, IC_{50} values, and hCOX-1 selectivity ratios for the inhibitory effects of new compounds and reference inhibitors on the enzymatic activity of hCOX-1 from human platelet microsomes and recombinant human COX-2 expressed in Sf 21 cells.

Compound	Ar/Het	Ar^1/Het^1	hCOX-1 (IC_{50})	hCOX-2 (IC_{50})	Ratio
1	Ph	Ph	**	**	
2	Ph	4-CH_3-Ph	**	**	
3	Ph	Fur-2-yl	**	**	
4	2-CH_3-Ph	4-Cl-Ph	**	**	
5	4-CH_3-Ph	Ph	74.93 ± 3.69 μM	**	>1.3[c)]
6	4-CH_3-Ph	4-F-Ph	70.59 ± 3.21 μM	**	>1.4[c)]
7	4-CH_3-Ph	2,4-Cl-Ph	**	**	
8	4-CH_3-Ph	3,4-Cl-Ph	**	**	
9	4-CH_3-Ph	Thien-2-yl	**	**	
10	4-F-Ph	4-CH_3-Ph	**	**	
11	4-F-Ph	Thien-2-yl	**	**	
12	4-Cl-Ph	4-CH_3-Ph	**	**	
13	4-Cl-Ph	4-F-Ph	68.68 ± 3.56 μM	**	>1.5[c)]
14	4-Cl-Ph	4-Cl-Ph	*	**	
15	Fur-2-yl	4-CH_3-Ph	**	**	
16	Fur-2-yl	4-F-Ph	71.90 ± 3.65 μM	**	>1.4[c)]
17	Pyrrol-2-yl	Ph	29.60 ± 1.58 μM	**	>3.4[c)]
18	Pyrrol-2-yl	4-Cl-Ph	**	**	
19	Pyrrol-2-yl	Thien-2-yl	**	**	
Indometacin			12.16 ± 1.16 μM[b)]	35.20 ± 1.41 μM	2.9
Diclofenac			18.23 ± 1.73 μM	23.62 ± 1.97 μM	1.3
FR122047			93.80 ± 6.55 nM	**	>1066[c)]
DuP 697			22.61 ± 1.56 μM[a)]	126.32 ± 7.41 nM	0.0056

All IC_{50} values shown in this table are the mean ± SEM from five experiments. Level of statistical significance: [a)] $p < 0.01$ or [b)] $p < 0.05$ versus the corresponding IC_{50} values obtained against hCOX-2, as determined by ANOVA/Dunnett's test. *100 μM inhibits the corresponding hCOX activity by approximately 40–50%. At higher concentration the compounds precipitate. **Inactive at 100 μM (highest concentration tested). [c)]Values obtained under the assumption that the corresponding IC_{50} against hCOX-1 or hCOX-2 is the highest concentration tested.

different substituents, electron-withdrawing and -donor groups (CH_3, F, Cl), at the *ortho*, *meta*, and *para* positions of the aryl rings. In addition, we changed the aromatic group at positions C3 and C5 of the pyrazoline scaffold with some heteroaromatic rings such as pyrrole, furan, and thiophene.

Chemistry

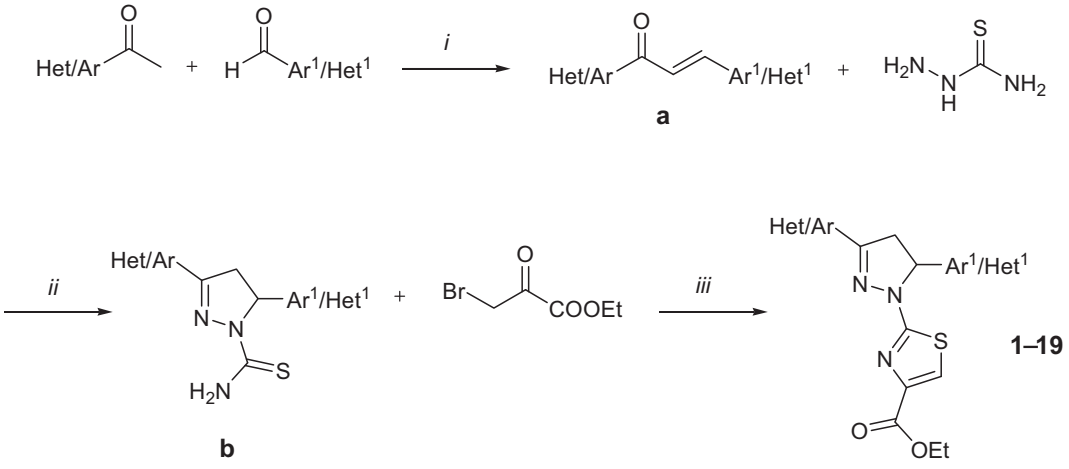
As outlined in Scheme 1, the starting 1,3-di(hetero)aryl-2-propen-1-ones (chalcones) (**a**) have been synthesized by Claisen–Schmidt condensation under microwave irradiation between (hetero)aryl ketones and (hetero)aryl aldehydes in the presence of I_2 and neutral Al_2O_3 [12]. Then, these intermediates were treated with thiosemicarbazide (molar ratio 1:2) in KOH/EtOH at reflux to afford the desired pyrazolines (**b**) [13]. Then, we subjected to cyclization the N1-thiocarbamoyl pendant on these heterocycles with the ethyl ester of α-bromo-pyruvic acid to 4-ethyl carboxylate-thiazole (Hantzsch reaction). The structures of the compounds were confirmed by elemental analysis (see Supporting Information), IR and ^{1}H NMR spectroscopy. In particular, the two methylene protons and the methyne proton, at positions C4 and C5, respectively, of the dihydro-(1*H*)-pyrazole ring, give rise to a well defined system of three double doublets with different *J* values. The IR spectra generally presented C=N stretching bands in the region around 1600 cm^{-1} because of the ring closure.

Biological activity

To study the possible effects of the test drugs (new compounds or reference inhibitors) on human COX isoform enzymatic activity, we evaluated the rate of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine formation, i.e., the increase in absorbance at 600 nm per unit of time ($\triangle A_{600}$ U/min). In these experiments, the inhibitory activity of the tested drugs (new compounds and reference inhibitors) is expressed as IC_{50}, i.e., the concentration of these compounds required for a 50% reduction of the control hCOX isoform enzymatic activity, estimated by least-squares linear regression, using the program Origin 5.0 (Microcal Software, Inc., Northampton, MA, USA), with X = log of tested compound molar concentration and Y = the corresponding percentage of inhibition of control *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine production obtained with each concentration. This regression was performed using data obtained with 4–6 different concentrations of each tested compound which inhibited the control hCOX isoform enzymatic activity by between 20 and 80%. In addition, we calculated the corresponding hCOX-1 selectivity index (SI): IC_{50} (hCOX-2)/IC_{50} (hCOX-1; Table 1). Results shown in the text and tables are expressed as mean ± standard error of the mean (SEM) from five experiments. Significant differences between two means ($p < 0.05$ or $p < 0.01$) were determined by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test.

Results and discussion

Starting from (hetero)aryl ketones and aldehydes, we synthesized chalcones by Claisen–Schmidt condensation under microwave irradiation. Then, the intermediates reacted with thiosemicarbazide in KOH/EtOH at reflux to give a new series of pyrazolines which finally were treated with the ethyl ester of α-bromo-pyruvic acid (Hantzsch reaction) to give the desired derivatives in high yields. All the compounds were tested in order to evaluate their activity against human COX enzymes and some of them were able to inhibit hCOX-1 at micromolar concentrations (compound **17**, IC_{50} = 29.60 ± 1.58 μM) with hCOX-1 selectivity index



Scheme 1. General synthesis of derivatives **1**–**19**. Reagents and conditions: (*i*) I_2-neutral Al_2O_3, MW; (*ii*) KOH/EtOH, reflux; (*iii*) MeOH.

values similar or better than the reference drugs (indometacin, diclofenac), ranging from >1.3 (**5**) to >3.4 (**17**). None of them proved to inhibit the human COX-2 isoform, hence, offering a complete selectivity toward hCOX-1. With regard to the ring on the C5 of the pyrazoline, it is outstanding that an unsubstituted phenyl (**5** and **17**) or a 4-fluoro-phenyl (**6**, **13**, and **16**) endowed compounds with a greater inhibitory activity toward hCOX-1. However, this substitution was well tolerated only with 4-methyl-phenyl (**5** and **6**), 4-chloro-phenyl (**13**), or heteroaryl groups such as furan (**16**) and pyrrole (**17**) at the C3 position. Furthermore, the presence at N1 of a 4-ethyl carboxylate-thiazole positively influenced the COX-1 selectivity. All this information could represent an important tool for the clarification of the pharmacophoric nucleus and for the development of new pyrazoline-based hCOX-1 inhibitors.

Experimental

The compounds, synthesized with the microwave method, were obtained with a Biotage Initiator™ 2.0. The chemicals, solvents for synthesis and spectral grade solvents were purchased from Aldrich (Italy) and were used without further purification. Melting points (uncorrected) were determined automatically on an FP62 apparatus (Mettler-Toledo). ^{1}H NMR spectra were recorded at 400 MHz on a Bruker spectrometer using DMSO-d_6 or $CDCl_3$ as solvent. Chemical shifts are expressed as δ units (parts per million) relative to the solvent peak. Coupling constants J are valued in Hertz (Hz). IR spectra were registered on a Perkin Elmer FT-IR spectrometer Spectrum 1000 in KBr. Elemental analyses for C, H, and N were recorded on a Perkin-Elmer 240 B microanalyzer and the analytical results were within ±0.4% of the theoretical values for all compounds. All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F_{254} Merck).

General procedure for the synthesis of derivatives 1–19

Different substituted aryl or heteroaryl ketones (5 mmol), substituted aryl or heteroaryl aldehydes (5 mmol), and I_2-neutral Al_2O_3 (carbonyl compound/catalyst 1:2 w/w) were homogenized in a mortar. Then the mixture was irradiated in a 10 mL sealed vessel suitable for an automatic single-mode microwave reactor (2.45 GHz high-frequency microwaves, power range 0–300 W). The mixture was pre-stirred for 30 s and then heated by microwave irradiation for 10 min at 60–80°C (irradiation power reaches its maximum at the beginning of the reaction, then it decreases to lower and quite constant values). The internal vial temperature was controlled by an IR sensor probe. After cooling with pressurized air, the reaction mixture was poured onto ice and chloroform (50 mL) was added to the reaction mixture. The solid catalyst was removed by filtration under vacuum. After washing the filtrate with $Na_2S_2O_3$ solution (20 mL), the separated organic layer was concentrated under reduced pressure and the product was recrystallized from ethanol. The obtained chalcone (**a**, 5 mmol) was dissolved in 100 mL of a mixture of KOH in ethanol and vigorously stirred with thiosemicarbazide (10 mmol) The desired product was filtered off under vacuum and crystallized from suitable solvent (ethanol or 2-propanol) and dried. The obtained *N*-thiocarbamoylpyrazolines (**b**, 50 mmol) were subsequently converted to (4-substituted-thiazol-2-yl)pyrazolines by reaction with the ethyl ester of α-bromo-pyruvic acid (50 mmol) in methanol (50 mL). The crude products were purified by chromatography (SiO_2, ethyl acetate/*n*-hexane 2:1).

Chemical–physical data for new compounds

*Ethyl 2-(3,5-diphenyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**1**)*

Mp 163–165°C, 82% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 7.0 Hz, 3H, CH_3), 3.28–3.34 (dd, J_{gem} = 16.8 Hz, J_{cis} = 5.4 Hz, 1H, C_4H-pyrazoline), 3.88–3.96 (dd, J_{gem} = 15.8 Hz, J_{trans} = 11.7 Hz, 1H, C_4H-pyrazoline), 4.29–4.33 (q, J = 7.0 Hz, 2H, CH_2), 5.73–5.78 (dd, J_{cis} = 5.6 Hz, J_{trans} = 12.0 Hz, 1H, C_5H-pyrazoline), 7.33–7.32 (m, 5H, Ar), 7.44–7.45 (m, 3H, Ar), 7.52 (s, 1H, C_4H-thiazole), 7.75–7.78 (m, 2H, Ar).

*Ethyl 2-(3-phenyl-5-p-tolyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**2**)*

Mp 93–94°C, 90% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.23–1.26 (t, J = 7.1 Hz, 3H, CH_3), 3.30–3.32 (dd, J_{gem} = 17.5 Hz, J_{cis} = 5.6 Hz, 1H, C_4H-pyrazoline), 4.00–4.05 (dd, J_{gem} = 17.5 Hz, J_{trans} = 12.1 Hz, 1H, C_4H-pyrazoline), 4.18–4.20 (q, J = 7.1 Hz, 2H, CH_2), 5.65–5.69 (dd, J_{cis} = 5.6 Hz, J_{trans} = 12.1 Hz, 1H, C_5H-pyrazoline), 7.14–7.20 (m, 4H, Ar), 7.47–7.49 (m, 3H, Ar and C_4H-thiazole), 7.76–7.78 (m, 3H, Ar).

*Ethyl 2-(5-(furan-2-yl)-3-phenyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**3**)*

Mp 146–147°C, 90% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.37–1.41 (t, J = 7.1 Hz, 3H, CH_3), 3.64–3.68 (dd, J_{gem} = 18.1 Hz, J_{cis} = 6.2 Hz, 1H, C_4H-pyrazoline), 3.75–3.79 (dd, J_{gem} = 18.1 Hz, J_{trans} = 11.3 Hz, 1H, C_4H-pyrazoline), 4.33–4.38 (q, J = 7.1 Hz, 2H, CH_2), 5.86–5.90 (dd, J_{cis} = 6.2 Hz, J_{trans} = 11.3 Hz, 1H, C_5H-pyrazoline), 6.33–6.34 (m, 1H, C_4H-furan), 6.56–6.57 (m, 1H, C_3H-furan), 7.33 (s, 1H, C_5H-furan), 7.44–7.45 (m, 3H, Ar), 7.55 (s, 1H, C_4H-thiazole), 7.78–7.80 (m, 2H, Ar).

*Ethyl 2-(5-(4-chlorophenyl)-3-o-tolyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**4**)*

Mp 169–170°C, 91% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34–1.37 (t, J = 7.1 Hz, 3H, CH_3), 2.73 (s, 3H, CH_3–Ar), 3.36–3.42 (dd, J_{gem} = 17.3 Hz, J_{cis} = 5.6 Hz, 1H, C_4H-pyrazoline), 3.95–4.03 (dd, J_{gem} = 17.3 Hz, J_{trans} = 11.8 Hz, 1H, C_4H-pyrazoline), 4.29–4.33 (q, J = 7.1 Hz, 2H, CH_2), 5.68–5.72 (dd, J_{cis} = 5.6 Hz, J_{trans} = 11.8 Hz, 1H, C_5H-pyrazoline), 7.28–7.34 (m, 8H, Ar), 7.54 (s, 1H, C_4H-thiazole).

*Ethyl 2-(5-phenyl-3-p-tolyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**5**)*

Mp 109–110°C, 85% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34–1.35 (t, J = 7.0 Hz, 3H, CH_3), 2.33 (s, 3H, CH_3–Ar), 3.32–3.38 (dd, J_{gem} = 17.6 Hz, J_{cis} = 5.2 Hz, 1H, C_4H-pyrazoline), 3.88–3.95 (dd, J_{gem} = 17.6 Hz, J_{trans} = 11.8 Hz, 1H, C_4H-pyrazoline), 4.29–4.32 (q, J = 7.0 Hz, 2H, CH_2), 5.80–5.84 (dd, J_{cis} = 5.2 Hz, J_{trans} = 11.8 Hz, 1H, C_5H-pyrazoline), 7.13–7.15 (d, J_o = 7.8 Hz, 2H, Ar), 7.27–7.28 (d, J_o = 7.8 Hz, 2H, Ar), 7.44–7.45 (m, 3H, Ar), 7.50 (s, 1H, C_4H-thiazole), 7.76–7.78 (m, 2H, Ar).

*Ethyl 2-(5-(4-fluorophenyl)-3-p-tolyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**6**)*

Mp 169–170°C, 89% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 7.1 Hz, 3H, CH_3), 2.42 (s, 3H, CH_3–Ar), 3.28–3.33 (dd, J_{gem} = 17.4 Hz, J_{cis} = 5.5 Hz, 1H, C_4H-pyrazoline), 3.86–3.93 (dd, J_{gem} = 17.4 Hz, J_{trans} = 11.9 Hz, 1H, C_4H-pyrazoline), 4.25–4.35 (q, J = 7.1 Hz, 2H, CH_2), 5.72–5.76 (dd, J_{cis} = 5.5 Hz, J_{trans} = 11.9 Hz, 1H, C_5H-pyrazoline), 6.99–7.04 (m, 2H, Ar), 7.24–7.26 (d, J_o = 7.9 Hz, 2H, Ar), 7.33–7.37 (m, 2H, Ar), 7.51 (s, 1H, C_4H-thiazole), 7.75–7.77 (d, J_o = 7.9 Hz, 2H, Ar).

*Ethyl 2-(5-(2,4-dichlorophenyl)-3-p-tolyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**7**)*

Mp 234–235°C, 80% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35–1.36 (t, J = 7.1 Hz, 3H, CH_3), 2.41 (s, 3H, CH_3–Ar), 3.16–3.22 (dd, J_{gem} = 17.6 Hz, J_{cis} = 5.9 Hz, 1H, C_4H-pyrazoline), 3.99–4.04 (dd, J_{gem} = 17.6 Hz, J_{trans} = 11.9 Hz, 1H, C_4H-pyrazoline), 4.27–4.33 (q, J = 7.1 Hz, 2H, CH_2), 5.99–6.03 (dd, J_{cis} = 5.9 Hz, J_{trans} = 11.9 Hz, 1H, C_5H-pyrazoline), 7.15–7.16 (m, 2H, Ar), 7.23–7.25 (d, J_o = 7.8 Hz, 2H, Ar), 7.45 (m, 1H, Ar), 7.56 (s, 1H, C_4H-thiazole), 7.71–7.73 (d, J_o = 7.8 Hz, 2H, Ar).

*Ethyl 2-(5-(3,4-dichlorophenyl)-3-p-tolyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**8**)*

Mp 166–167°C, 90% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34–1.38 (t, J = 7.0 Hz, 3H, CH_3), 2.42 (s, 3H, CH_3–Ar), 3.26–3.31 (dd, J_{gem} = 17.4 Hz, J_{cis} = 5.7 Hz, 1H, C_4H-pyrazoline), 3.87–3.95 (dd, J_{gem} = 17.4 Hz, J_{trans} = 11.8 Hz, 1H, C_4H-pyrazoline), 4.28–4.34 (q, J = 7.0 Hz, 2H, CH_2), 5.67–5.71 (dd, J_{cis} = 5.7 Hz, J_{trans} = 11.8 Hz, 1H, C_5H-pyrazoline), 7.22–7.25 (m, 3H, Ar), 7.40–7.41 (d, J_o = 7.9 Hz, 1H, Ar), 7.42 (s, 1H, Ar), 7.48 (s, 1H, C_4H-thiazole), 7.58–7.59 (d, J_o = 7.9 Hz, 2H, Ar).

*Ethyl 2-(5-(thiophen-2-yl)-3-p-tolyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**9**)*

Mp 126–127°C, 89% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34–1.38 (t, J = 7.0 Hz, 3H, CH_3), 2.42 (s, 3H, CH_3–Ar), 3.26–3.31 (dd, J_{gem} = 17.4 Hz, J_{cis} = 5.7 Hz, 1H, C_4H-pyrazoline), 3.87–3.95 (dd, J_{gem} = 17.4 Hz, J_{trans} = 11.8 Hz, 1H, C_4H-pyrazoline), 4.28–4.34 (q, J = 7.0 Hz, 2H, CH_2), 5.67–5.71 (dd, J_{cis} = 5.7 Hz, J_{trans} = 11.8 Hz, 1H, C_5H-pyrazoline), 7.22–7.25 (m, 3H, Ar), 7.40–7.41 (d, J_o = 7.9 Hz, 1H, Ar), 7.42 (s, 1H, Ar), 7.48 (s, 1H, C_4H-thiazole), 7.58–7.59 (d, J_o = 7.9 Hz, 2H, Ar).

*Ethyl 2-(3-(4-fluorophenyl)-5-p-tolyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**10**)*

Mp 162–163°C, 85% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 7.2 Hz, 3H, CH_3), 2.33 (s, 3H, CH_3–Ar), 3.29–3.34 (dd, J_{gem} = 16.2 Hz, J_{cis} = 4.5 Hz, 1H, C_4H-pyrazoline), 3.84–3.89 (dd, J_{gem} = 16.2 Hz, J_{trans} = 10.3 Hz, 1H, C_4H-pyrazoline), 4.27–4.35 (q, J = 7.2 Hz, 2H, CH_2), 5.67–5.69 (dd, J_{cis} = 4.5 Hz, J_{trans} = 10.3 Hz, 1H, C_5H-pyrazoline), 7.11–7.16 (m, 4H, Ar), 7.25 (s, 2H, Ar), 7.50 (s, 1H, C_4H-thiazole), 7.74–7.78 (m, 2H, Ar).

*Ethyl 2-(3-(4-fluorophenyl)-5-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**11**)*

Mp 162–163°C, 83% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.36–1.40 (t, J = 7.1 Hz, 3H, CH_3), 3.47–3.53 (dd, J_{gem} = 17.4 Hz, J_{cis} = 5.0 Hz, 1H, C_4H-pyrazoline), 3.83–3.90 (dd, J_{gem} = 17.4 Hz, J_{trans} = 11.4 Hz, 1H, C_4H-pyrazoline), 4.32–4.37 (q, J = 7.1 Hz, 2H, CH_2), 6.08–6.12 (dd, J_{cis} = 5.0 Hz, J_{trans} = 11.4 Hz, 1H, C_5H-pyrazoline), 6.94–6.96 (m, 1H, C_4H-thiophene), 7.13–7.22 (m, 4H, C_3H-thiophene, C_5H-thiophene, and Ar), 7.58 (s, 1H, C_4H-thiazole), 7.81–7.83 (m, 2H, Ar).

*Ethyl 2-(3-(4-chlorophenyl)-5-p-tolyl-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**12**)*

Mp 116–117°C, 86% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 7.0 Hz, 3H, CH_3), 2.32 (s, 3H, CH_3–Ar), 3.27–3.32 (dd, J_{gem} = 16.1 Hz, J_{cis} = 4.6 Hz, 1H, C_4H-pyrazoline), 3.83–3.90 (dd, J_{gem} = 16.1 Hz, J_{trans} = 12.2 Hz, 1H, C_4H-pyrazoline), 4.28–4.32 (q, J = 7.0 Hz, 2H, CH_2), 5.75–5.77 (dd, J_{cis} = 4.6 Hz, J_{trans} = 12.2 Hz, 1H, C_5H-pyrazoline), 7.13–7.15 (d, J_o = 7.7 Hz, 2H, Ar), 7.24–7.26 (d, J_o = 7.7 Hz, 2H, Ar), 7.40–7.42 (d, J_o = 8.3 Hz, 2H, Ar), 7.51 (s, 1H, C_4H-thiazole), 7.68–7.70 (d, J_o = 8.3 Hz, 2H, Ar).

*Ethyl 2-(3-(4-chlorophenyl)-5-(4-fluorophenyl)-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**13**)*

Mp 231–233°C, 81% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 6.9 Hz, 3H, CH_3), 3.32–3.33 (dd, J_{gem} = 17.1 Hz, J_{cis} = 4.2 Hz, 1H, C_4H-pyrazoline), 3.88–3.89 (dd, J_{gem} = 17.1 Hz, J_{trans} = 10.7 Hz, 1H, C_4H-pyrazoline), 4.29–4.32 (q, J = 6.9 Hz, 2H, CH_2), 5.81–5.82 (dd, J_{cis} = 4.2 Hz, J_{trans} = 10.7 Hz, 1H, C_5H-pyrazoline), 7.03–7.04 (m, 2H, Ar), 7.34–7.35 (m, 2H, Ar), 7.41–7.43 (m, 2H, Ar), 7.53 (s, 1H, C_4H-thiazole), 7.69–7.71 (m, 2H, Ar).

*Ethyl 2-(3,5-bis(4-chlorophenyl)-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**14**)*

Mp 159–160°C, 80% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.37 (t, J = 7.0 Hz, 3H, CH_3), 3.25–3.30 (dd, J_{gem} = 17.7 Hz, J_{cis} = 5.6 Hz, 1H, C_4H-pyrazoline), 3.86–3.93 (dd, J_{gem} = 17.7 Hz, J_{trans} = 12.0 Hz, 1H, C_4H-pyrazoline), 4.26–4.35 (q, J = 7.0 Hz, 2H, CH_2), 5.74–5.78 (dd, J_{cis} = 5.6 Hz, J_{trans} = 12.0 Hz, 1H, C_5H-pyrazoline), 7.30–7.32 (m, 4H, Ar), 7.41–7.43 (d, J_o = 8.4 Hz, 2H, Ar), 7.53 (s, 1H, C_4H-thiazole), 7.68–7.70 (d, J_o = 8.7 Hz, 2H, Ar).

*Ethyl 2-(3-(furan-2-yl)-5-p-tolyl-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**15**)*

Mp 104–105°C, 89% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.21–1.25 (t, J = 7.1 Hz, 3H, CH_3), 2.26 (s, 3H, CH_3–Ar), 3.13–3.19 (dd, J_{gem} = 17.6 Hz, J_{cis} = 5.6 Hz, 1H, C_4H-pyrazoline), 3.91–3.98 (dd, J_{gem} = 17.6 Hz, J_{trans} = 10.1 Hz, 1H, C_4H-pyrazoline), 4.30–4.32 (q, J = 7.1 Hz, 2H, CH_2), 5.63–5.65 (dd, J_{cis} = 5.6 Hz, J_{trans} = 10.1 Hz, 1H, C_5H-pyrazoline), 6.65–6.67 (m, 1H, C_4H-furan), 6.97–6.98 (m, 1H, C_3H-furan), 7.21 (m, 1H, C_5H-furan), 7.74 (s, 1H, C_4H-thiazole), 7.88–7.89 (m, 2H, Ar), 7.90–7.91 (m, 2H, Ar).

*Ethyl 2-(5-(4-fluorophenyl)-3-(furan-2-yl)-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**16**)*

Mp 167–168°C, 84% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.36 (t, J = 7.1 Hz, 3H, CH_3), 3.24–3.29 (dd, J_{gem} = 17.5 Hz, J_{cis} = 5.6 Hz, 1H, C_4H-pyrazoline), 3.83–3.90 (dd, J_{gem} = 17.5 Hz, J_{trans} = 12.0 Hz, 1H, C_4H-pyrazoline), 4.28–4.32 (q, J = 7.1 Hz, 2H, CH_2), 5.72–5.76 (dd, J_{cis} = 5.6 Hz, J_{trans} = 12.0 Hz, 1H, C_5H-pyrazoline), 6.53–6.55 (m, 1H, C_4H-furan), 6.77 (s, 1H, C_3H-furan), 7.00–

7.04 (m, 2H, Ar), 7.33–7.35 (m, 2H, Ar), 7.47 (s, 1H, C_4H-thiazole), 7.58 (s, 1H, C_5H-furan).

*Ethyl 2-(5-phenyl-3-(1H-pyrrol-2-yl)-4,5-dihydropyrazol-1-yl)-thiazole-4-carboxylate (**17**)*

Mp 191–192°C, 85% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.33–1.35 (t, $J = 7.1$ Hz, 3H, CH_3), 3.15–3.20 (dd, $J_{gem} = 17.0$ Hz, $J_{cis} = 3.8$ Hz, 1H, C_4H-pyrazoline), 3.32–3.38 (dd, $J_{gem} = 17.0$ Hz, $J_{trans} = 10.1$ Hz, 1H, C_4H-pyrazoline), 4.30–4.32 (q, $J = 7.1$ Hz, 2H, CH_2), 4.72–4.74 (dd, $J_{cis} = 3.8$ Hz, $J_{trans} = 10.1$ Hz, 1H, C_5H-pyrazoline), 6.22–6.23 (m, 1H, pyrrole), 6.91–6.92 (m, 1H, pyrrole), 7.32–7.38 (m, 6H, Ar and C_4H-thiazole), 7.51–7.52 (m, 1H, pyrrole), 11.30 (br s, 1H, NH, D_2O exch.).

*Ethyl 2-(5-(4-chlorophenyl)-3-(1H-pyrrol-2-yl)-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**18**)*

Mp 86–86°C, 82% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.22–1.25 (t, $J = 7.2$ Hz, 3H, CH_3), 3.16–3.19 (dd, $J_{gem} = 17.2$ Hz, $J_{cis} = 5.7$ Hz, 1H, C_4H-pyrazoline), 4.15–4.20 (q, $J = 7.2$ Hz, 2H, CH_2), 4.45–4.49 (dd, $J_{gem} = 17.2$ Hz, $J_{trans} = 11.5$ Hz, 1H, C_4H-pyrazoline), 6.15–6.16 (dd, $J_{cis} = 5.7$ Hz, $J_{trans} = 11.5$ Hz, 1H, C_5H-pyrazoline), 6.96 (m, 1H, pyrrole), 7.05 (m, 1H, pyrrole), 7.31–7.33 (d, $J_o = 8.0$ Hz, 2H, Ar), 7.41–7.43 (d, $J_o = 8.0$ Hz, 2H, Ar), 7.50 (s, 1H, C_4H-thiazole), 8.98 (s, 1H, pyrrole), 11.74 (s, 1H, NH, D_2O exch.).

*Ethyl 2-(3-(1H-pyrrol-2-yl)-5-(thiophen-2-yl)-4,5-dihydropyrazol-1-yl)thiazole-4-carboxylate (**19**)*

Mp 154–155°C, 89% yield; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.23–1.27 (t, $J = 7.0$ Hz, 3H, CH_3), 3.17–3.28 (dd, $J_{gem} = 17.6$ Hz, $J_{cis} = 5.9$ Hz, 1H, C_4H-pyrazoline), 4.16–4.22 (q, $J = 7.0$ Hz, 2H, CH_2), 4.74–4.76 (dd, $J_{gem} = 17.6$ Hz, $J_{trans} = 10.9$ Hz, 1H, C_4H-pyrazoline), 6.02–6.03 (dd, $J_{cis} = 5.9$ Hz, $J_{trans} = 10.9$ Hz, 1H, C_5H-pyrazoline), 6.17–6.19 (m, 1H, C_4H-thiophene), 6.88–6.90 (m, 1H, C_3H-thiophene), 6.97–6.99 (m, 1H, pyrrole), 7.07 (s, 1H, C_5H-thiophene), 7.35–7.37 (m, 1H, pyrrole), 7.53 (s, 1H, C_4H-thiazole), 9.06 (s, 1H, pyrrole), 11.80 (br s, 1H, NH, D_2O exch.).

Preparation of microsomes from human platelets

Human platelets were isolated by centrifugation from buffy coats obtained from the Centro de Transfusión de Galicia (Santiago de Compostela, Spain) and prepared as we have previously described [14, 15]. Briefly, the buffy coat was diluted 1:1 with washing buffer of the following composition at pH 6 (mM): NaCl (120), KCl (5), trisodium citrate (12), glucose (10), sucrose (12.5), and then centrifuged at 400 *g* for 8 min in a centrifuge (Omnifuge 2.0 RS, Heraeus Sepatech, Osterade, Germany) at 25°C to obtain platelet rich plasma. The upper layer obtained in this centrifugation, containing platelet rich plasma, was gently removed and centrifuged at 850 *g* for 20 min at 4°C in a centrifuge (J2-MI, Beckman Instruments, Inc., Palo Alto, California, USA). The platelet pellet was recovered, resuspended with washing buffer, and centrifuged again at 850 *g* for 20 min at 4°C. To prepare human platelet microsomes, the resultant platelet pellet of the above centrifugation was resuspended in 7 mL of sodium phosphate buffer (10 mM, pH 7.4), sonicated at 50 W for 50 s (5 pulses of 10 s), and centrifuged at 850 *g* for 20 min at 4°C in a refrigerated centrifuge. The pellet was discarded and the supernatant was subsequently centrifuged at 10 000 *g* for 10 min at 4°C in the same centrifuge. The pellet obtained in this centrifugation was discarded and the supernatant was finally centrifuged at 100 000 *g* for 1 h at 4°C in a centrifuge. The resultant pellet containing platelet microsomes was resuspended in 1 mL of sodium phosphate buffer (50 mM, pH 7.4) and the protein concentration in the platelet microsome suspension (approximately 2 mg/mL) was measured by the method of Bradford [16], using a protein assay kit from BioRad Laboratories (Alcobendas, Spain). Platelet microsome aliquots were stored at −80°C for several days (without apparent loss of COX activity) until use.

Determination of human cyclooxygenase isoform activity

The biological evaluation of the test drugs on total hCOX activity (bisdioxygenase and peroxidase reactions) was 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 arachidonic acid 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 commercially available). The formation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine (a coloured compound) from *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine catalyzed by COX can be detected spectrophotometrically at 600 nm. In this study, hCOX activity was evaluated using the above spectrophotometric method following the general procedure described previously [17] with several modifications. Briefly, 0.1 mL of Tris–HCl buffer (100 mM, pH 8) containing 1 μM hematin, 100 μM TMPD, various concentrations of the test drugs (new compounds or reference inhibitors), and appropriate amounts of hCOX-1 and hCOX-2 required and adjusted to obtain under our experimental conditions the same control absorbance increase (0.08 A600 U/min) were incubated for short periods of time (3–5 min) to avoid a notable loss of COX activity at 37°C in a flat-bottom 96-well microtest plate (BD Biosciences, Franklin Lakes, NJ, USA) placed in the dark multimode microplate reader chamber. After this incubation period, the reaction was started by adding (final concentration) 100 μM arachidonic acid and the formation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediimine from TMPD, i.e., the increase in absorbance at 600 nm was measured at 37°C in a multi-mode microplate reader (Fluostar Optima, BMG Labtech GmbH, Offenburg, Germany) for 25 s, a period in which the absorbance increased linearly from the beginning. The specific absorbance (used to obtain the final results) was calculated after subtraction of the background absorbance generated in wells containing a blank solution, i.e., all components except the COX isoforms, which were replaced by a Tris–HCl buffer solution. Under our experimental conditions, this background activity was practically negligible. Control experiments were carried out simultaneously by replacing the test drugs (new compounds and reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above test drugs to modify the absorbance of the reaction mixture due to non-enzymatic inhibition (e.g., for directly reacting with TMPD) was determined by adding these drugs to solutions containing only TMPD in a Tris–HCl buffer solution.

Drugs and chemicals

The drugs, vehicle, and chemicals used in the experiments were the new compounds, dimethyl sulfoxide, hematin porcine, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride, arachidonic acid sodium salt, indometacin (purchased from Sigma–Aldrich Química S.A., Alcobendas, Spain), diclofenac sodium salt, FR122047 monohydrochloride hydrate, and DuP 697 (purchased from Cayman Chemical, Ann Arbor, MI, USA). Appropriate dilutions of some of the above drugs were prepared every day immediately before use in deionized water from the following concentrated stock solutions kept at −20°C: the new compounds, diclofenac sodium salt, FR122047 hydrate, and DuP 697 (0.1 M) in DMSO. In addition, stock solutions of the following drugs were prepared daily before use: arachidonic acid (30 mM) and indometacin (10 mM) in ethanol; hematin (0.2 mM) and TMPD (40 mM) in DMSO. Due to the instability of some chemicals (e.g., DuP 697, arachidonic acid), the corresponding solutions of these chemicals were maintained in inert atmosphere until use. In all assays, neither deionized water (Milli-Q, Millipore Ibérica S.A., Madrid, Spain) nor appropriate dilutions of the vehicle used (DMSO) had significant pharmacological effects.

Supporting Information

Elemental analyses and other chemical–physical data for the new compounds are freely available in the full-text version of the article in Wiley InterScience.

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The authors have declared no conflict of interest.

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