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Synthesis, anti-inflammatory, antiplatelet and in silico evaluations of (*E*)-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-yl)acrylamides

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A series of (*E*)-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-yl)acrylamides (**7a**–**k**) were synthesized and evaluated for their in vitro inhibitory activities on COX-1 and COX-2 isoforms using a human whole blood assay as well as their antiplatelet profile against human platelet aggregation using arachidonic acid as agonists. Among the synthesized derivatives **7a**–**k**, especially compound **7g** exhibited dual anti-inflammatory and antiplatelet activity with selective COX-2 inhibition.

Key Words: Anti-inflammatory activity, antiplatelet activity, pyrazoles, COX, TXA_2 synthetase

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently prescribed drugs for treatment of pain, fever, and inflammatory and rheumatic diseases. Studies in this field, aimed at discovering better

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tolerated and potent NSAIDs with fewer side effects characteristic of current NSAIDs have been of interest for many years. The non-selective inhibition of the 2-isoforms of COX is considered to have been responsible for the unfavorable side effects associated with the chronic use of NSAIDs. It was assumed that more selective COX-2 inhibitors would have reduced side effects due to the COX-1 inhibition.[1,2] Therefore, it would be useful to develop COX-2 selective inhibitors, which are demonstrated to possess a significantly enhanced gastric safety compared to non-selective NSAIDs. Celecoxib and rofecoxib are 2 well known selective COX-2 inhibitors belonging to the COXIB class. However, rofecoxib and other COX-2 inhibitors have been withdrawn from the market due to their adverse cardiovascular side effects. Recent studies have shown that COX-2 inhibitors are associated with increased thromboembolic risk in cardiovascular disease patients.[3–5] A selective COX-2 inhibitor causes reduction of prostacyclin (PGI_2) amount. Meanwhile, thromboxane A_2 (TxA_2) production is still continued in platelets by COX-1 isoform. It is considered that cardiovascular toxicity of coxibs was generated because of imbalance between PGI_2 and TxA_2 levels.[6] In a patient who requires effective anti-inflammatory treatment and has a high risk for both gastrointestinal bleeding and cardiovascular thrombosis, the use of selective COX-2 inhibitors with antiplatelet agent is recommended.[7] Moreover, there is currently no clear evidence that COX-2 inhibitors represent an independent risk factor in patients at low risk of cardiovascular diseases. Therefore, the clinical rationale for developing compounds with selective COX-2 inhibition still remains to be established.[5,8]

There are several reviews that mainly focus on the molecular and functional bases of the inhibition of COX enzymes by non-selective and COX-2 selective inhibitors.[9,10] The pharmacophores for most of the selective COX-2 inhibitors consist of a central 1,2-diarylsubstituted 5-membered heterocyclic ring.[2,11–13] Some 1,3-diarylpyrazoles derivatives have also been reported as selective COX-2 inhibitors.[14,15] Some studies showed that preparation of the amide derivatives of currently used NSAIDs might be a useful approach to achieve novel COX-2 inhibitors. Kalgutgar et al. showed that amide derivatives of indomethacin[16] and meclofenamic acid[17] selectively inhibited COX-2. Recently, this approach has been used to prepare ester and amide derivatives of ibuprofen, ketoprofen, and mefenamic acid, and the researchers found that especially ester derivative of ketoprofen showed more potent analgesic and anti-inflammatory activity than the parent drug.[18]

The 2-oxo-3*H*-benzoxazole ring has become an important building block in medicinal chemistry and has led to the discovery of a number of derivatives endowed with antispasmodic, antitubercular, antibacterial, antimicrobial, antifungal, and normolipemic effects.[19,20] Several 2-oxo-3*H*-benzoxazole derivatives have been previously reported as analgesic and anti-inflammatory agents.[21,22] Our research group has been interested for some time in studying the effect of substituting selected aromatic rings in current NSAIDs with alternative heteroaromatic moieties such as 2-oxo-3*H*-benzoxazole, 2-oxo-3*H*-benzothiazole and 3(2*H*)-pyridazinone.[23–25] The (3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1*H*-pyrazole derivatives were found to be potent COX-2 inhibitors; especially compound **A** (Figure) demonstrated strong and selective COX-2 inhibitory activity (COX-1 IC_{50} = 1 μM, COX-2 IC_{50} = 0.011 μM).[26]

N-Phenylpyrazole arylhydrazone derivative (**B** in Figure) was found to be active as an analgesic, anti-inflammatory and antiplatelet agent. These types of compounds were very effective in antiplatelet activity on arachidonic acid induced platelet aggregation in rabbit citrated platelet rich plasma possibly acting at the arachidonic acid cascade level.[27,28]

Ozagrel is a cinnamic acid derivative having an imidazol-1-yl substituent (Figure) that acts as a selective

inhibitor of TxA_2 synthetase with an IC_{50} of 11 nM[29] Essential structural features of TxA_2 synthetase inhibitors are as follows: a basic nitrogen atom of a substituted pyridine or imidazole ring, and a carboxylic acid group separated by a unsaturated trans-alkyl chain.[6] 6-Phenyl-3(2*H*)-pyridazinones were reported as antiplatelet agents and the lead compound (IC_{50} = 25 μM) contained a 3-oxoprop-1-en-1-yl fragment as a key structural element in the heterocyclic core.[30] Optimization studies have shown that removal of the phenyl group at C-6 and addition of a benzyl group at N-2 increases the antiplatelet activity (Figure, compound **C** IC_{50} = 4.2 μM).

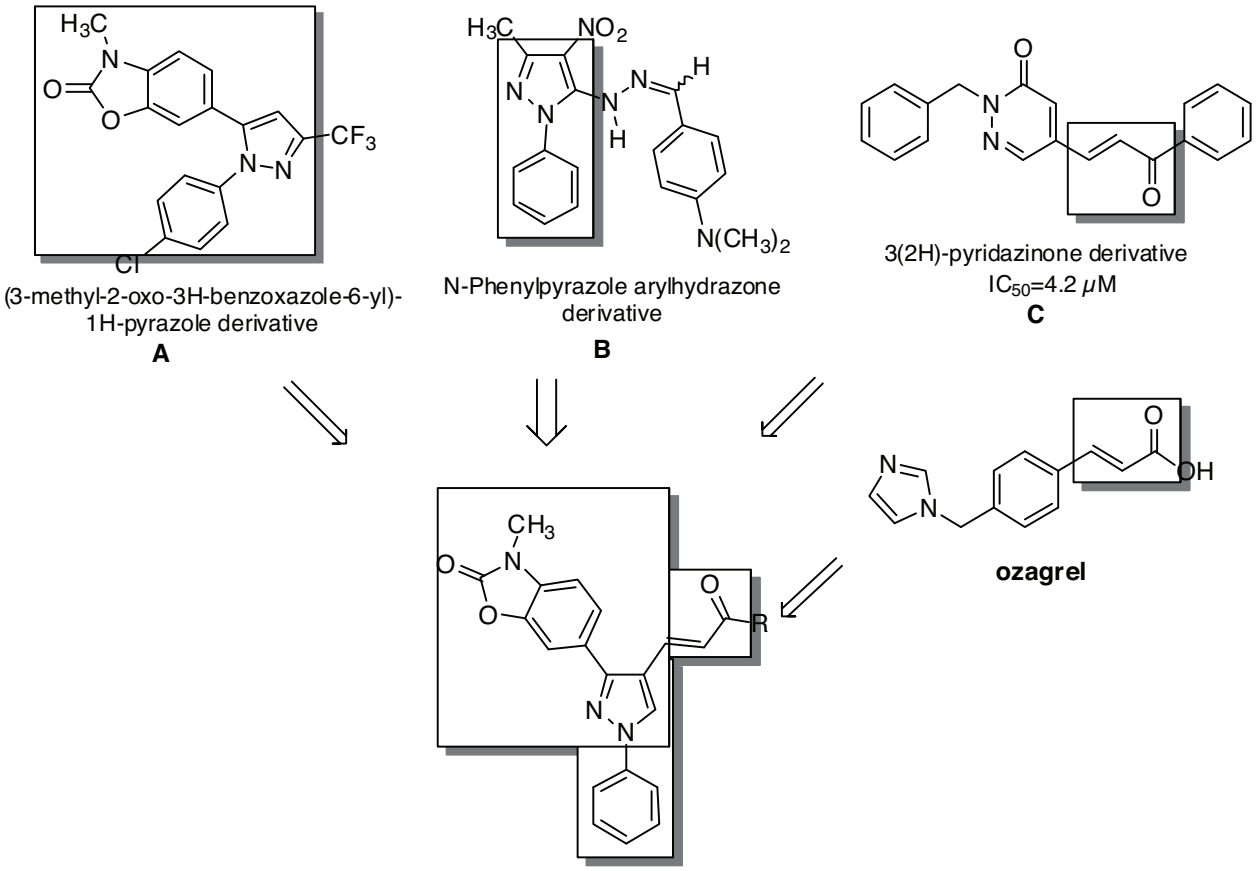


Figure Design of the title compounds.

In the search for new anti-inflammatory and antiplatelet agents, we describe herein the design, synthesis and anti-inflammatory and antiplatelet evaluation of new (*E*)-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)1-phenyl-1*H*-pyrazole-4-yl) acrylamides, which bear a central pyrazole ring whose 1,3,4-positions are substituted with phenyl, 3-methyl-2(3*H*)-benzoxazole moieties and 3-oxoprop-1-en-1-yl fragment, respectively (Figure). Finally, these derivatives were submitted to in silico oral biodisponibility screening to analyze their overall potential to qualify for use as a drug.

Experimental

Chemistry

The chemicals were purchased from commercial vendors and used without purification. Thin-layer chromatography (TLC) was performed on Merck 60F254 plates. The reactions were monitored by TLC on silica gel, with detection by UV light (254 nm) or charring Dragendorff reagent.[31] Melting points were determined with an SMP-II Digital Melting Point Apparatus and are uncorrected (Schorpp Geaetetechnik, Germany). IR spectra were recorded on a Bruker Vector 22 spectrometer as KBr disks. ^{1}H-NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Varian Mercury 400 MHz High Performance Digital FT-NMR spectrometer using tetramethylsilane as the internal standard at the NMR facility of the Faculty of Pharmacy, Ankara University. All chemical shifts were recorded as δ (ppm). High resolution mass spectra data (HRMS) were collected in-house using a Waters LCT Premier XE Mass Spectrometer (high sensitivity orthogonal acceleration time-of-flight instrument) operating in either ESI (+) or ESI (-) methods, also coupled to an AQUITY Ultra Performance Liquid Chromatography system (Waters Corporation, Milford, MA, USA).Flash chromatography was performed with a Combiflash® Rf automated flash chromatography system with RediSep columns (Teledyne-Isco, Lincoln, NE, USA) using dichloromethane-methanol solvent gradients. Elemental analyses were performed with a LECO-932 (C, H, N, S-Elemental Analyzer) at the Faculty of Pharmacy, Ankara University. Microwave-assisted reactions were carried out with a Milestone MicroSYNTH Microwave Synthesis System.

2-Oxo-3*H*-benzoxazole (1)

A dry flask charged with o-aminophenol (10.91 g, 0.1 mol) and urea (12.01 g, 0.2 mol) was placed in the MicroSYNTH Microwave Synthesis System and irradiated at 400 W for 15 min while the temperature was set to 140 °C. After the reaction was completed, the flask was cooled to room temperature and the solid was solved in 5% solution of sodium hydroxide. After acidification with concentrated HCl, the desired product was obtained. Yield 11.61 g (86%). mp 136 °C (Ref.[32]; 137-138 °C).

3-Methyl-2-oxo-3*H*-benzoxazole (2)

The described method was used,[33] and the reaction product was obtained in a yield of about 90%; mp 83 °C (Ref.[34]; 83-84 °C).

6-Acetyl-3-methyl-2-oxo-3*H*-benzoxazole (3)

A dry flask charged with 3-methyl-2-oxo-3*H*-benzoxazole (7.45 g, 0.05 mol), acetic acid (3.15 mL, 0.055 mol), and PPA (100 g) was placed in the MicroSYNTH Microwave Synthesis System and irradiated at 300 W for 22 min while the temperature was set to 90 °C. The reaction mixture was poured into ice-cold water. The precipitated mixture was filtered off, dried, and purified by recrystallization from ethanol–water mixture. Yield 8.5 g (89%). mp 167-168 °C (Ref.[35]; 166-168 °C).

6-Acetyl-3-methyl-2-oxo-3*H*-benzoxazolephenyl hydrazone (4)

A solution of 6-acetyl-3-methyl-2-oxo-3*H*-benzoxazole (10 g, 0.052 mol), phenyl hydrazine (6.27 g, 0.058 mol) and acetic acid (2 mL, 0.035 mol) in ethanol was stirred for 2 h at reflux and then evaporated. The precipitate was filtered off and dried. Yield 11.64 g (70%). mp 229-230 °C. FT-IR (KBr) cm^{-1} 1754 (C=O).

3-(2,3-Dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-carboxy aldehyde 5

Method A

In a dry flask, $POCl_3$ (2.8 mL, 0.03 mol) was added dropwise to an ice-cold stirred solution of phenyl hydrazone of 3-methyl-6-acetyl-2(3*H*)-benzoxazolone (2.8 g, 0.01 mol) in 10 mL of DMF. The reaction mixture was allowed to attain room temperature, and was then heated at 50 °C for about 4 h. The resulting mixture was poured onto crushed ice, neutralized with dilute NaOH and left overnight. The yellow precipitate obtained was purified by crystallization in toluene. Yield 2.6 g (85%).

Method B

In a dry flask, $POCl_3$ (2.8 mL, 0.03 mol) was added dropwise to an ice-cold stirred solution of phenyl hydrazone of 3-methyl-6-acetyl-2(3*H*)-benzoxazolone (2.8 g, 0.01 mol) in 10 mL of DMF. The reaction mixture was allowed to attain room temperature, and then flask was placed in the MicroSYNTH Microwave Synthesis System and irradiated at 210 W for 5 min while the temperature was set to 50 °C. The resulting mixture was poured onto crushed ice, neutralized with dilute sodium hydroxide and left standing overnight. The yellow precipitate obtained was purified by crystallization in toluene. Yield 3 g (94.3%). mp 255-257 °C. FT-IR (KBr) cm^{-1} 1759 (C=O), 1688 (C=O). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 10.05 (s, 1H, CHO), 8.54 (s, 1H, pyrazole-H^5), 7.78 (m, 4H, ArH), 7.53 (t, 2H, J = 8.0, ArH), 7.41 (d, 1H, J = 7.6, ArH), 7.08 (d, 1H, J = 8.0, ArH), 3.47 (s, 3H, CH_3). Anal. ($C_{18}H_{13}N_3O_3$): C, H, N *calc.* 67.71, 4.10, 13.16 *found* 67.67, 4.11, 13.21.

(*E*)-3-(3-(2,3-Dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-yl)acrylic acid 6

Method A

To a solution of 3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-carboxy aldehyde **5** (3.19 g, 0.01 mol) in pyridine (30 mL) were added malonic acid (4.16 g, 0.04 mol) and piperidine (1.5 mL, 0.015 mol), and the reaction mixture was refluxed for 5 h. On cooling, the reaction mixture was poured onto a solution (100 mL) of crushed ice and concentrated HCl (50% by volume) mixture. The resulting precipitated was filtered off, washed with acidified water and dried. Yield 2.3 g (63.7%).

Method B

A dry flask charged with 3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-carboxy aldehyde **5** (3.19 g, 0.01 mol), malonic acid (4.16 g, 0.04 mol), piperidine (1.5 mL, 0.015 mol) and 30 mL of pyridine was placed in the MicroSYNTH Microwave Synthesis System and irradiated at 600 W for 15 min while the temperature was set to 115 °C. The resulting mixture was poured onto a solution (100 mL) of crushed ice and concentrated HCl (50% by volume) mixture. The resulting precipitated was filtered off, washed with acidified water and dried. Yield 2.6 g (72%). mp 288-290 °C; FT-IR (KBr) cm^{-1} 1779 (C=O), 1689

(C=O); ^{1}H-NMR (400 MHz, DMSO-d_6) δ 12.35 (s, 1H, COOH), 9.22 (s, 1H, pyrazole-H^5), 7.93 (d, 2H, J = 7.6, ArH), 7.59-7.37 (m, 7H, ArH), 6.44 (d, 1H, J = 16, O=C-C<u>H</u>=CH), 3.40 (s, 3H, CH_3). ESI-MS 362.11 $[M+H]^+$

General procedure for the preparation of (*E*)-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)1-phenyl-1*H*-pyrazole-4-yl)acrylamides 7

To a solution of appropriate carboxylic acid derivative (1 mmol) and amine derivative (1 mmol) in 10 mL of DCM-THF mixture (5:1) were added DMAP (0.2 mmol) and EDCI (1.1 mmol) and the resulting solution was stirred overnight at room temperature. The reaction mixture was quenched with 1 N HCl and extracted with DCM. The organic phase was washed with a 1% $NaHCO_3$ solution and brine, dried over Na_2SO_4, and evaporated under vacuum. The residue was purified by flash column chromatography (Combiflash®Rf) using DCM-MeOH as eluents.

6-(4-((*E*)-3-Morpholino-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7a)

Elution with DCM-MeOH (0%-4%) afforded **7a** as a white solid (yield 82%); mp 113-115 °C; FT-IR (KBr) cm^{-1} 1757 (C=O), 1643 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.19 (s, 1H, pyrazole-H^5), 7.90 (d, 2H, J= 8.4, ArH), 7.57 (t, 3H, J=8, ArH), 7.58-7.39 (m, 4H, ArH), 7.19 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 3.68-3.56 (m, 8H, morpholine), 3.40 (s, 3H, CH_3); HRMS $C_{24}H_{22}N_4O_4$ $[M+H]^+$ *calc.* 431.1719, *found* m/z 431.1732.

(*E*)-N-Benzyl-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazol-6-yl)-1-phenyl-1*H*-pyrazole-4-yl) acrylamide (7b)

Elution with DCM-MeOH (0%-5%) afforded **7b** as a white solid (yield 83%); mp 245-246 °C; FT-IR (KBr) cm^{-1} 1759 (C=O), 1648 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 8.99 (s, 1H, pyrazole-H^5), 8.63 (t, 1H, J= 6, ArH), 7.95 (d, 2H, J= 9.2, ArH), 7.58-7.24 (m, 11H, ArH), 6.54 (d, 1H, J= 15.6, O=C-C<u>H</u>=CH), 4.38 (d, 2H, J= 6.4, OCNH-C<u>H</u>$_2$), 3.40 (s, 3H, CH_3); HRMS $C_{24}H_{22}N_4O_3$ $[M+H]^+$ *calc.* 451.1719, *found* m/z 451.1725.

6-(4-((*E*)-3-(4-(4-Chlorophenyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7c)

Elution with DCM-MeOH (0%-5%) afforded **7c** as a white solid (yield 71%); mp 243-245 °C; FT-IR (KBr) cm^{-1} 1773 (C=O), 1632 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.20 (s, 1H, pyrazole-H^5), 7.91 (d, 2H, J= 7.6, ArH), 7.57-7.37 (m, 9H, ArH), 7.24 (d, 1H, J = 9.2, ArH), 7.22 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 6.98 (d, 1H, J= 8.8, ArH), 3.74 (m, 4H, piperazin-H^{2,6}), 3.37 (s, 3H, CH_3), 3.16 (m, 4H, piperazin-H^{3,5}); HRMS $C_{30}H_{26}ClN_5O_3$ $[M+H]^+$ *calc.* 540.0121, *found* m/z 540.1802.

6-(4-((*E*)-3-(4-(4-Fluorophenyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7d)

Elution with DCM-MeOH (0%-4%) afforded **7d** as a white solid (yield 68%); mp 250-252 °C; FT-IR (KBr) cm^{-1} 1776 (C=O), 1632 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.20 (s, 1H, pyrazole-H^5), 7.89 (d, 2H, J= 7.6, ArH), 7.57-7.36 (m, 8H, ArH), 7.22 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 7.08-6.97 (m, 3H, ArH), 3.74 (m, 4H, piperazin-H^{2,6}), 3.37 (s, 3H, CH_3), 3.09 (m, 4H, piperazin-H^{3,5}); HRMS $C_{30}H_{26}FN_5O_3$ $[M+H]^+$ *calc.* 524.2098, *found* m/z 524.2098.

6-(4-((*E*)-3-(4-(4-Fluorobenzyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7e)

Elution with DCM-MeOH (0%-5%) afforded **7e** as a white solid (yield 65%); mp 126-128 °C; FT-IR (KBr) cm^{-1} 1758 (C=O), 1643 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.20 (s, 1H, pyrazole-H^5), 7.90 (d, 2H, J= 8, ArH), 7.56 (m, 3H, ArH), 7.46-7.34 (m, 6H, ArH), 7.19-7.14 (m, 3H, ArH), 3.55 (m, 4H, piperazin-H^{2,6}), 3.50 (s, 2H, CH_2), 3.39 (s, 3H, CH_3), 2.42-2.34 (m, 4H, piperazin-H^{3,5}); HRMS $C_{31}H_{28}FN_5O_3$ $[M+H]^+$ *calc.* 538.2254, *found* m/z 538.2277.

6-(4-((*E*)-3-(4-(4-Chlorobenzyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7f)

Elution with DCM-MeOH (0%-5%) afforded **7f** as a white solid (yield 72%); mp 197-198 °C; FT-IR (KBr) cm^{-1} 1780 (C=O), 1640 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.19 (s, 1H, pyrazole-H^5), 7.90 (d, 2H, J= 7.6), 7.56 (m, 3H, ArH), 7.46-7.34 (m, 8H, ArH), 7.17 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 3.68-3.56 (m, 4H, piperazin-H^{2,6}), 3.51 (s, 2H, CH_2), 3.39 (s, 3H, CH_3), 2.43-2.35 (m, 4H, piperazin-H^{3,5}); HRMS $C_{31}H_{28}ClN_5O_3$ $[M+H]^+$ *calc.* 554.1959, *found* m/z 554.1985.

6-(4-((*E*)-3-(4-(Pyridin-4-yl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7g)

Elution with DCM-MeOH (0%-4%) afforded **7g** as a white solid (yield 57%); mp 263-265 °C; FT-IR (KBr) cm^{-1} 1776 (C=O), 1648 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.22 (s, 1H, pyrazole-H^5), 8.19 (d, 2H, J= 6.4, pyridine-H^{2,6}), 7.91 (d, 2H, J = 8.8, ArH), 7.60-7.39 (m, 7H, ArH), 7.23 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 6.87 (d, 2H, J= 6.4, pyridine-H^{3,5}), 3.82-3.69 (m, 4H, piperazin-H^{2,6}), 3.45-3.31 (m, 7H, CH_3, piperazin-H^{3,5}); HRMS $C_{29}H_{26}N_6O_3$ $[M+H]^+$ *calc.* 507.2145, *found* m/z 507.2141.

6-(4-((*E*)-3-(4-(4-Tolyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7h)

Elution with DCM-MeOH (0%-4%) afforded **7h** as a white solid (yield 78%); mp 217-219 °C; FT-IR (KBr) cm^{-1} 1776 (C=O), 1648 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.23 (s, 1H, pyrazole-H^5), 7.91 (d, 2H, J= 8, ArH), 7.59-7.39 (m, 7H, ArH), 7.26 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 7.05 (d, 2H, J= 8.4, ArH), 6.89 (d,

2H, J = 8.4, ArH), 3.82-3.71 (m, 4H, piperazin-$H^{2,6}$), 3.40 (s, 3H, N-CH_3), 3.13-3.09 (m, 4H, piperazin-$H^{3,5}$), 2.21 (s, 3H, Ar-CH_3); HRMS $C_{31}H_{29}N_5O_3$ $[M+H]^+$ *calc.* 520.2349, *found* m/z 520.2359.

6-(4-((*E*)-3-(4-(4-(Trifluoromethyl)phenyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7i)

Elution with DCM-MeOH (0%-4%) afforded **7i** as a white solid (yield 63%); mp 235-237 °C; FT-IR (KBr) cm^{-1} 1776 (C=O), 1632 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.23 (s, 1H, pyrazole-H^5), 7.93 (d, 2H, J = 8.8, ArH), 7.60-7.39 (m, 9H, ArH), 7.23 (d, 1H, J= 15.2, O=C-C<u>H</u>=CH), 7.13 (d, 2H, J= 8.8, ArH), 3.85-3.73 (m, 4H, piperazin-$H^{2,6}$), 3.40-3.31 (m, 7H, CH_3, piperazin-$H^{3,5}$); HRMS $C_{31}H_{26}F_3N_5O_3$ $[M+H]^+$ *calc.* 574.2066, *found* m/z 574.2075.

6-(4-((*E*)-3-(4-(4-(Methylbenzyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7j)

Elution with DCM-MeOH (0%-4%) afforded **7j** as a white solid (yield 77%); mp 207-208 °C; FT-IR (KBr) cm^{-1} 1778 (C=O), 1640 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.18 (s, 1H, pyrazole-H^5), 7.90 (d, 2H, J= 8, ArH), 7.56 (m, 3H, ArH), 7.46-7.34 (m, 4H, ArH), 7.20-7.13 (m, 5H, ArH), 3.67-3.55 (m, 4H, piperazin-$H^{2,6}$), 3.46 (s, 2H, CH_2), 3.39 (s, 3H, CH_3), 2.42-2.33 (m, 4H, piperazin-$H^{3,5}$), 2.28 (s, 3H, CH_3); HRMS $C_{32}H_{31}N_5O_3$ $[M+H]^+$ *calc.* 534.2505, *found* m/z 534.2526.

6-(4-((*E*)-3-(4-(4-(Trifluoromethyl)benzyl)piperazin-1-yl)-3-oxoprop-1-enyl)-1-phenyl-1*H*-pyrazole-3-yl)-3-methyl-2-oxo-3*H*-benzoxazolone (7k)

Elution with DCM-MeOH (0%-4%) afforded **7k** as a white solid (yield 68%); mp 118-120 °C; FT-IR (KBr) cm^{-1} 1757 (C=O), 1643 (C=O); ^{1}H-NMR (400 MHz, DMSO-d_6)δ 9.19 (s, 1H, pyrazole-H^5), 7.90 (d, 2H, J = 7.6, ArH), 7.71 (d, 2H, J = 8.4, ArH), 7.58-7.55 (m, 5H, ArH), 7.47-7.38 (m, 4H, ArH), 7.17 (d, 1H, J = 15.2, O=C-C<u>H</u>=CH), 3.70-3.56 (m, 6H, CH_2, piperazin-$H^{2,6}$), 3.39 (s, 3H, CH_3), 2.43-2.35 (m, 4H, piperazin-$H^{3,5}$); HRMS $C_{32}H_{28}F_3N_5O_3$ $[M+H]^+$ *calc.* 588.2222, *found* m/z 588.2230.

Biological assays

Cyclooxygenase inhibition

Human whole blood COX-1 and COX-2 assay

The human whole blood assay, originally developed by Patrignani et al.[36] is considered the more biologically relevant way to assess the inhibition of the cyclooxygenase isoenzymes, COX-1 and COX-2, by a test compound[37] In this assay, platelets stimulated upon coagulation are thought to be the main source of COX-1, whereas monocytes stimulated with LPS are thought to be the source of COX-2. COX-1 activity is determined by the production of thromboxane B_2 (TxB_2), while COX-2 activity is determined by the production of prostaglandin E_2 (PGE_2).

For testing the COX-1 activity, fresh blood samples from healthy volunteers who had not taken any NSAIDs for at least 7 days prior to blood extraction were collected in vacutainer tubes without any anticoagulant. Aliquots of 500 μL of blood were incubated either with 1 μL of vehicle (DMSO) or 1 μL of test compound (synthesized compound or indomethacin as reference) solution (10 μM) for 1 h at 37 °C. Plasma was separated by centrifugation (5 min at 13,000 rpm, 4 °C) and TXB_2 levels were measured using the Correlate-EIA^{TM} TXB_2 Enzyme Immunoassay Kit from Assay Design Inc. (Ann Arbor, MI, USA). Indomethacin was used as reference.

For COX-2, fresh blood samples from healthy volunteers who had not taken any NSAIDs for at least 7 days prior to blood extraction were collected in EDTA-containing tubes. Aliquots of 500 μL of blood were incubated either with 1 μL of vehicle (DMSO) or 1 μL of test compound (synthesized compound or indomethacin as reference) solution (10 μM) in the presence of LPS (10 μg/mL) for 24 h at 37 °C to induce COX-2 expression. Plasma was separated by centrifugation (5 min at 13000 rpm, 4 °C) and PGE_2 levels were measured using the Correlate-EIA^{TM} PGE_2 Enzyme Immunoassay Kit from Assay Design Inc. (Ann Arbor, MI, USA). Indomethacin was used as reference.

Effect of compounds on platelet aggregation

The platelet aggregation study was carried out using the turbidometric method described by Born et al.[38] Freshly drawn venous human citrated blood (sodium citrate 3.8%, 1:9 v/v) from healthy subjects who had not taken drugs with antiplatelet activity for 10 days were centrifuged with 800 rpm for platelet-rich plasma (PRP) and 1500 rpm for platelet-poor plasma (PPP). Platelet count in PRP was adjusted to 3.8×10^8 platelets/mL using PPP.

Aggregation was measured at 37 °C with constant stirring at 1100 rpm by utilizing a Lumi-Aggregometer (Chrono-Log). The test compound (or the standard inhibitor, aspirin) was dissolved in DMSO. The final concentration of DMSO in the test cuvette was fixed at 1% (v/v). Final concentrations of synthesized compounds for antiplatelet activity assay were selected according to their solubility in DMSO. A 400 μL sample of PRP was placed in the cuvette of the aggregometer and incubated for 5 min with 5 μL of test compound. Then 5 μL of inducer (arachidonic acid [AA], 600 μM, final concentration) was added and the change in the light transmission was recorded for 5 min. Aspirin was used as reference drug for antiplatelet activity

In silico oral biodisponibility

The theoretical study of oral bioavailability was performed using the molinspiration on-line program (http://www.molinspiration.com). Lipinski's rule-of-five describes molecular properties important for a drug pharmacokinetics in the human body. Poor absorption and permeation are more likely to occur when there are more than 5 hydrogen-bond donors (HBD), more than 10 hydrogen-bond acceptors (HDA), the molecular mass (MM) is greater than 500, or the log P value (clogP) is greater than 5. The active compound must be consistent with at least 3 of the 4 rules.[39]

Results and discussion

Chemistry

We focused our synthetic efforts on diaryl heterocyclic ring systems as illustrated in the Scheme. The starting compound, 3-methyl-2-oxo-3*H*-benzoxazole (**2**), was prepared by methylation with dimethyl sulfate of 2-oxo-3*H*-benzoxazole (**1**) which was readily synthesized via the reaction of o-aminophenol and urea. Compound **2** was then converted to 6-acetyl-3-methyl-2-oxo-3*H*-benzoxazole (**3**) via Friedel–Crafts acylation under microwave conditions (Scheme). Subsequently, the hydrazone derivative **4** was generated by carrying out condensation in the presence of phenylhydrazine and acetic acid in refluxing ethanol. This hydrazone derivative was then reacted with $POCl_3$ and DMF using 2 different methods (conventional synthesis and reaction under microwave conditions) resulting in 1,3-diaryl pyrazole **5** with an aldehyde group at the 4 position. 3-(2,3-Dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)-1-phenyl-1*H*-pyrazole-4-carboxy aldehyde **5** was then treated with malonic acid in pyridine to prepare the corresponding α,β-unsaturated carboxylic acid via Knoevenagel condensation reaction using both conventional and microwave irradiation methods. By treatment of **6** with appropriate amines in the presence of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI), dimethylaminopyridine (DMAP), which was used as the carboxylate activator, the resulting amide derivatives **7** were prepared in good yield (57%-83%).

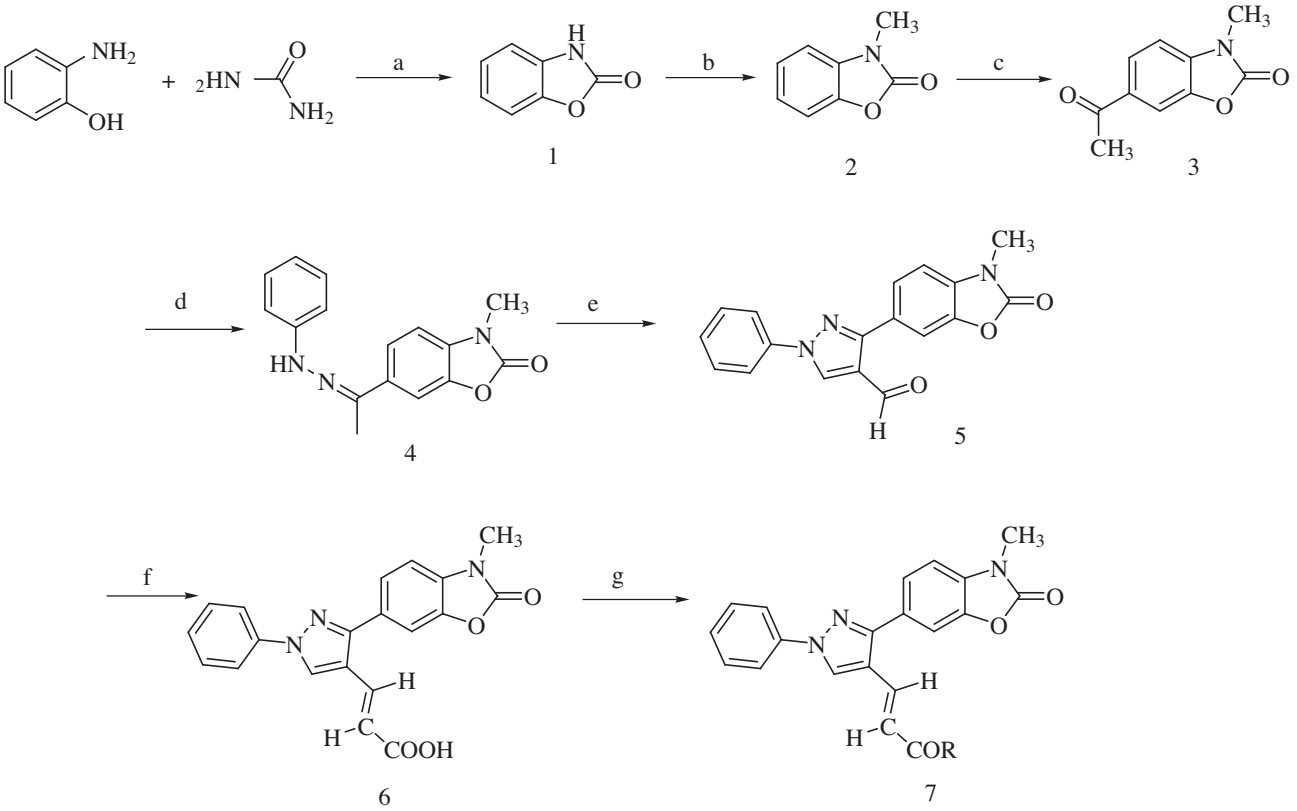
The chemical structures of these compounds were elucidated by their elemental analysis, and IR, HRMS and ^{1}H-NMR spectral data.

The IR spectra of hydrazone **4** showed disappearance of the carbonyl peak at 1675 cm^{-1} belonging to the acetyl group of 6-acetyl-3-methyl-2-oxo-3*H*-benzoxazole (**3**), and a secondary N-H stretching band was observed at 3344 cm^{-1} as a singlet. The IR spectra of compound **5** exhibited a characteristic strong absorption for the aldehyde carbonyl group at 1687 cm^{-1}. In the IR spectra of compound **6,** a strong absorption band at 1689 cm^{-1} and intense O-H stretching absorption in the region of 3300-2500 cm^{-1} for α,β-unsaturated carboxylic acid were observed. Final amide derivatives exhibited a characteristic strong absorption in the area of 1648-1632 cm^{-1} attributable to the C=O of the amide group.

In the ^{1}H-NMR spectra of compound **5**, 2 singlets were displayed due to the aldehyde group at 10.05 ppm and pyrazole at 8.54 ppm, each showing the integration for 1 proton. In the ^{1}H-NMR spectra of compounds **6**, the signal of the carboxylic acid was observed at 12.35 ppm. One of the olefinic protons of the alkyl chain was observed as a doublet at 6.45 ppm with a coupling constant of 16 Hz indicating the (*E*) isomer. In the ^{1}H-NMR spectra of the final amide derivatives **7a-k** that olefinic proton was observed at about 7.26-7.17 ppm as a doublet with a coupling constant of 15.2 Hz. Pyrazole gave a singlet at about 9.23-8.99 ppm.

Biological evaluation

The compounds reported herein were tested for their ability to inhibit COX-2 and/or COX-1 using in the vitro human whole blood assay described by Patrignani et al.[36] which is considered the more biologically relevant way to assess the inhibition of the cyclooxygenase isoenzymes, COX-1 and COX-2, by a test compound[37] Preliminary screenings of both title compounds and indomethacin as reference were performed at a final concentration of 10 μM to determine the percent inhibition of the COX-1 and COX-2 isoforms.



Scheme Reagents and conditions: a) MW irradiation, 400 W, 140 °C, 15 min; b) dimethyl sulfate, NaOH, 30 min; c) acetic acid, PPA, MW irradiation, 90 °C, 20 min; d) phenyl hydrazine, acetic acid, ethanol, 2 h reflux; e) *Method A*; dimethyl formamide, phosphoroxy chloride ($POCl_3$), 50 °C, 4 h; *Method B*; dimethyl formamide, phosphoroxy chloride ($POCl_3$), MW irradiation, 210 W, 50 °C, 5 min; f) *Method A*; malonic acid, pyridine, piperidine, 115 °C, 5 h; *Method B*; malonic acid, pyridine, piperidine, MW irradiation, 600 W, 115 °C, 15 min; g) appropriate amine derivative, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI), dimethylaminopyridine (DMAP), dichloromethane, rt, overnight.

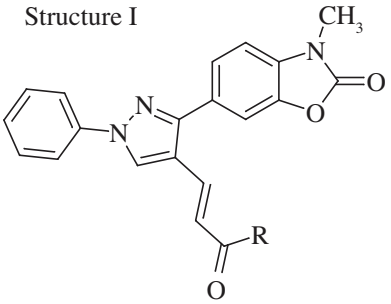
With regard to COX-1 inhibitory activities (Table 1) only compound **7a** (22%), carrying a morpholine ring at the side chain showed moderate inhibition at 10 μM. Although the inhibitory activities were not very pronounced, all derivatives demonstrated COX-2 inhibition (1%-39%), except **7a**. Compound **7e**, having 4-floro benzyl piperazine at the amide portion, inhibited COX-2 but not COX-1, although this compound was a poor inhibitor of COX-2 (20%). Interestingly, 4-chlorobenzyl substitution on the piperazine ring resulted in a derivative (**7f**) that was inactive against COX-1 at the concentration range tested (10 mM) and COX-2 inhibitory activity of this compound was less pronounced (3%).

With respect to COX selectivities, compounds **7g, 7h, 7i,** and **7j** showed COX-2 selective inhibitory properties. Insertion of the 4-pyridyl, 4-methyl and trifluoromethylphenyl group in compounds **7g**, **7h, 7i** improved inhibitory action (∼28%). The 4-methyl benzyl piperazine derivative **7j** showed the best inhibitory activity (38%).

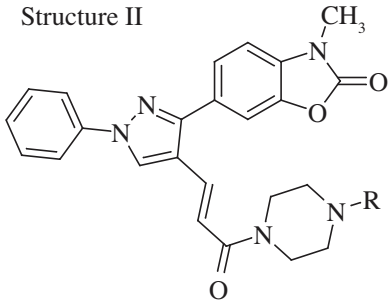
Indomethacin, meclofenamic acid and zomepirac are known to show their activity primarily by inhibition of COX-1 isoform. By preparing amides and esters through the free carboxylate group in these drugs, COX-2 selective compounds were achieved[11,16,17] As expected like these well-established COX-2 inhibitor compounds, the derivatization of the carboxyl function to amide derivatives in our final compounds did produce molecules with potent anti-inflammatory activities and with COX-2 selectivity to some extent.

Table 1. Effects of synthesized compounds on in vitro COX-2 and COX-1 enzyme inhibition in human whole blood assay and in vitro platelet aggregation induced by arachidonic acid.

Structure I



Structure II



Comp.	R	Anti-inflammatory activity		Antiplatelet activity (inducer-600 μM AA)	
		COX-2 inhibition (%)	COX-1 inhibition (%)	Final Concentration (μM)	Inhibition (%)
	Structure I				
6	OH	NI	NI	122	4.9
7a	morpholin-1-yl	1.17	**21.88**	122	1.2
7b	$NCH_2C_6H_4$	16.6	NI	122	11.2
	Structure II				
7c	4-ClC_6H_4	9.11	NI	47.7	4.9
7d	4-FC_6H_4	8.65	NI	61	2.9
7e	CH_2(4-FC_6H_4)	**20.32**	**1.55**	122	3.7
7f	CH_2(4-ClC_6H_4)	3.04	NI	122	NI
7g	**pyridine-4-yl**	**28.50**	NI	**61**	**39.6**
7h	4-$CH_3C_6H_4$	**27.57**	NI	122	4.9
7i	4-$CF_3C_6H_4$	**27.33**	NI	81.3	NI
7j	CH_2(4-$CH_3C_6H_4$)	**38.78**	NI	122	NI
7k	CH_2(4-$CF_3C_6H_4$)	13.32	NI	122	11.8
Indomethacin[a)]		**89.14**	**68.57**	NT	NT
Aspirin[b)]		NT	NT	122	100

[a)] Indometacin was used as nonselective COX reference. Indometacin (10 μM) was also tested for determining % inhibition as reference drug; [b)] Aspirin was used as reference drug for antiplatelet activity; NI: No inhibition; NT: Not tested.

The antiplatelet effects obtained in the Born test[38] with compounds **6** and **7a-7k** are summarized in Table 1. We used arachidonic acid as inducer of the platelet aggregation and aspirin as reference drug. Most compounds exhibited no antiplatelet action. Derivatives **7b** and **7k** which bear benzyl amine and 4-triflоromethylbenzylpiperazine at the amide portion respectively, were found to be poor inhibitors of platelet aggregation. However, 4-pyridylpiperazine derivative **7g** exhibited moderate inhibitory activity on platelet aggregation at 61 μM concentration. The structure-activity relationship of TXA_2 synthetase inhibitors has already been well established. It is found that there should be appropriate length between nitrogen atom of pyridine residue, which is known to selectively inhibit TXA_2 synthetase via making chelate with iron, and carboxylic acid moiety for selective TXA_2 synthetase inhibitors[40,41] Introducing pyridine moiety to the amide portion of title compounds resulted in a potent antiplatelet compound and this result was consistent with the literature.

In silico oral biodisponibility—molecular modeling approach

The synthesized compounds **(6, 7a-k)** were submitted to an in silico evaluation using a molecular modeling approach. Good absorption after oral administration is obligatory for anti-inflammatory and antiplatelet medical

Table 2. Parameters for Lipinski rule-of-five for synthesized compounds predicted using a molecular modeling approach.

Compound	Predicted oral bioavailibility (Lipinski rule-of-five)[a)]			
	HBA	HBD	M.M.	clog*P*
6	7	1	361.357	2.337
7a	8	0	430.464	2.288
7b	7	1	450.498	3.596
7c	8	0	540.023	4.709
7d	8	0	509.541	4.127
7e	9	0	537.595	3.896
7f	8	0	554.050	4.410
7g	8	0	506.566	2.742
7h	8	0	519.605	4.480
7i	8	0	573.575	4.926
7j	8	0	533.632	4.181
7k	8	0	587.602	4.628

[a] To present good theoretical oral bioavailability – number of hydrogen bond acceptors (HBA) ≤ 10 and donors (HBD) ≤ 5, clog$P \leq 5$ and molecular mass ≤ 500.

use purposes. To predict the drug-like properties of the synthesized compounds, we analyzed these derivatives according to the rule-of-five developed by Lipinski et al. (Table 2).[39] This rule theoretically determines if a chemical compound has suitable properties to be an orally active drug in humans. According to the rule-of-five, an orally active drug has fewer than 10 hydrogen bond acceptors and fewer than 5 donors, clogP less than 5 and molecular mass (MM) below 500. Molecules violating more than one of these rules may have problems with bioavailability. The results showed that compounds **6** and **7a,b** fulfilled the Lipinski rule-of-five (Table 2). The rest of the compounds violated only the criterion for molecular mass.

Conclusions

The synthesis of a series of (*E*)-3-(3-(2,3-dihydro-3-methyl-2-oxo-3*H*-benzoxazole-6-yl)1-phenyl-1*H*-pyrazole-4-yl)acrylamide derivatives is described along with their preliminary evaluation as potential anti-inflammatory and antiplatelet agents. The results of the biological evaluation revealed that especially compound **7g** exhibited dual anti-inflammatory and antiplatelet activity with selective COX-2 inhibition. We think that the preliminary in vitro activity results of this class of compounds might lead to further studies to develop better candidates for COX-2 selectivity and with potent anti-inflammatory and antiplatelet activities.

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