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Synthesis, biological evaluation of certain pyrazolo [3,4-*d*]pyrimidines as novel anti-inflammatory and analgesic agents

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Abstract In the present study, a series of pyrazolo[3,4-*d*]pyrimidin-4(5*H*)-ones linked at 5-position to thiazoline or thiazolidinone ring systems through imino linkage (**5**–**8**) was designed and synthesized. The compounds were assessed for their anti-inflammatory activity and analgesic in vivo. Also, their ability to inhibit ovine COX-1/COX-2 isozymes was evaluated using in vitro cyclooxygenase (COX) inhibition assay. The newly synthesized compounds **7**, **8d**, and **8e** showed potent anti-inflammatory and analgesic activity. Moreover, compound **7** displayed preferential COX-2 inhibitory potency (IC_{50} = 0.53 μM and COX-2 selectivity index = 10.07) which is more potent than the standard drug meloxicam. Interestingly, the tested compounds showed excellent gastrointestinal safety profile and were well tolerated by experimental animals with high safety margins than the reference drug meloxicam.

Keywords Pyrazolo[3,4-*d*]pyrimidine · Thiazolidinone · Anti-inflammatory · Analgesic · COX-2 inhibition · Ulcerogenic effect

Introduction

Inflammation represents a fundamental host response to a wide range of stimuli, such as trauma, tissue injury, infection, burns, surgery, sepsis, toxic entities or autoimmune injury. Non-steroidal anti-inflammatory drugs (NSAIDs) alleviate pain by counteracting the cyclooxygenase enzymes (COX), a protein essential for prostaglandins (PGs) biosynthesis from arachidonic acid. They compete with arachidonic acid for binding to the COX active site (Smith and Marnett, 1991). These drugs can be subdivided into two classes: (a) classical, ''isozyme non-specific'', NSAIDs and (b) selective, COX-2 inhibitors. Classical NSAIDs not only reduce the production of pro-inflammatory PGs at sites of injury (via COX-2 inhibition) but also the formation of physiological PGs in the stomach and the kidney (via COX-1 inhibition) leading to gastrointestinal (GI) lesions and renal toxicity and at high doses leading to erosions, ulcerations, bleeding, and even death (Blower *et al*., 1997). The experimental and clinical studies available indicate that selective COX-2 inhibitors retain the anti-inflammatory effects characteristic of NSAIDs with a marked increase in GI tolerability as compared to classical non-selective ones. Thus, it is reasonable to speculate that increasing the specificity for COX-2 over COX-1 is one of the strategies that may be employed to improve safety profile and therapeutic potency of NSAIDs.

Pyrazolopyrimidine derivatives were found to possess wide applications in drug development. They are biologically interesting isomeric purine analogs and have important properties as anti-metabolites in purine biochemical reactions (Tollefson *et al*., 2010; El-Enany *et al*., 2010). In the course of research studies devoted for the development of new classes ory drugs, several pyrazolopyrimidine derivatives have been synthesized and have shown potential anti-inflammatory activity associated with remarkable systemic and gastric tolerance (Russo *et al*., 1993; Devesa *et al*., 2004; El-Kerdawy *et al*., 1997). Moreover, thiazole derivatives are known to possess anti-inflammatory, analgesic, and antipyretic activities (Holla *et al*., 2003; Geronikaki *et al*., 2008; Apostolidis *et al*., 2013). For example, meloxicam is a member of the enolic acid group of NSAIDs featuring thiazolyl group. It is a preferential COX-2 inhibitor with less gastrointestinal toxicity than non-selective

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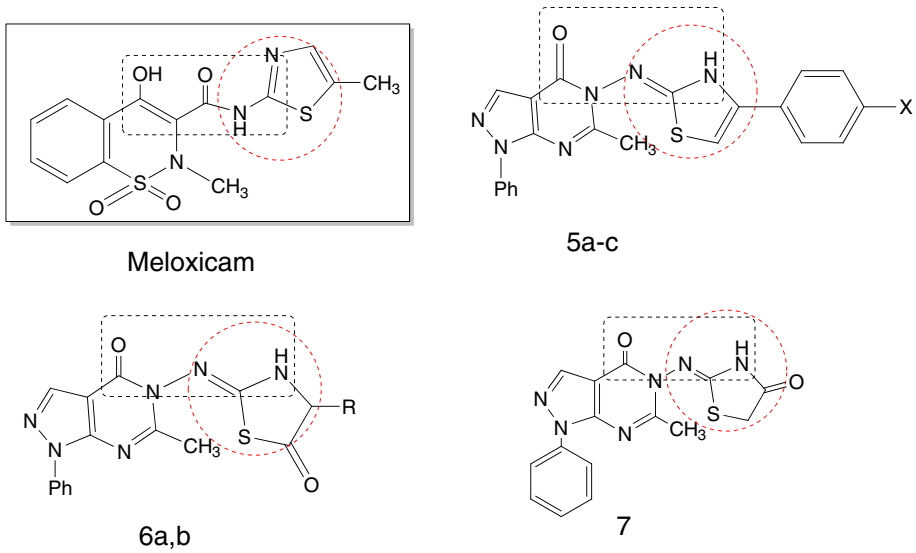


Fig. 1 Structure of Meloxicam and the synthesized compounds

NSAIDs (Beretta *et al*., 2005; Beubler, 2003), as it may combine anti-inflammatory efficacy with improved tolerability (Pairet *et al*., 1998; Engelhardt *et al*., 1995). Thiazolidinones are other important pharmacodynamic heterocyclic nuclei which, when incorporated in different heterocycles templates, have been reported to possess potent anti-inflammatory activity (Goel *et al*., 1999; Srivastava *et al*., 2004; El-Tombary, 2013). The combination of two pharmacophores on the same scaffold is a well-established approach to the synthesis of more potent drugs. Due to the high potency of the compounds containing pyrazolo[3,4-*d*]pyrimidine, thiazole or thiazolidinone moieties as anti-inflammatory, analgesic, and antipyretic agents, it is intended in the present work to synthesize a new pyrazolo[3,4-*d*]pyrimidine derivatives incorporating a known bioactive heterocyclic nuclei thiazole or thiazolidinone, which is attached through imino linkage at the 5-position of the pyrazolopyrimidine nucleus, hoping that these new hybrids could enhance and modulate their anti-inflammatory and/or analgesic activities. Their selectivity as COX-2 inhibitors compared to meloxicam as a structurally related preferential COX-2 inhibitor drug was also measured (Fig. 1). In addition, the ulcerogenic profiles of the newly synthesized compounds were examined.

Materials and methods

Chemistry

General remarks

Melting points are uncorrected and determined in one-end open capillary tubes using Gallen Kamp melting point apparatus MFB-595-010M (Gallen Kamp, London, England). Microanalysis was carried out at Micro-analytical Unit, Faculty of Science, Cairo University. Analyses indicated were within ± 0.4 % of the theoretical values. Infrared Spectra were recorded on Schimadzu FT-IR 8400S spectrophotometer (Shimadzu, Kyoto, Japan), and expressed in wave number (cm^{-1}), using potassium bromide disks. The NMR spectra were recorded on a Varian Gemini 200 MHz and Varian Mercury VX-300 NMR spectrometer. ^1H NMR spectra were run at 300 and 500 MHz and ^{13}C spectra were run at 75 and 125 MHz in dimethylsulfoxide (DMSO-d_6) and $CDCl_3$. Chemical shifts are quoted in δ and related to that of the solvents. Mass spectra were recorded using Hewlett Packard Varian (Varian, Polo, USA) and Shimadzu Gas Chromatograph Mass spectrometer-QP 1000 EX (Shimadzu, Kyoto, Japan). TLC was carried out using Art.DC-Plastikfolien, Kieselgel 60 F254 sheets (Merck, Darmstadt, Germany), the developing solvents were benzene: chloroform: ethanol [7:2:1], and the spots were visualized at 366 and 254 nm by UV Vilber Lourmat 77202 (Vilber, Marne La Vallee, France). Compound **1** (Peet *et al*., 1992), **2** (Schmidt and Druey, 1958), and **3** (Cheng and Robins, 1958) were prepared according to the reported procedure.

*Ethyl 5-amino-1-phenyl-1H-pyrazole-4-carboxylate (**1**)* A mixture of ethyl ethoxymethylenecyanoacetate (93.0 g, 0.55 mol), phenyl hydrazine (54.0 g, 0.5 mol), and ethanol (600 mL) was refluxed for 15 h. The product was filtered and the filtrate was concentrated to half its volume and then cooled. The resulting crystalline solid was filtered and dried to provide the desired compound. It was obtained as white-shining needles (EtOH), Yield 58 %, mp. 99–100 °C; IR ν_{max}/cm^{-1}: 3371.5, 3264.8 (NH_2), 2948.4, 2895,2 (CH aliphatic), 1680.3 (C=O), ^1H NMR ($CDCl_3$, 300 MHz) δ *ppm*: 1.23 (3H, t, $J = 7$ Hz, CH_3), 4.24 (2H,

q, $J = 7$ Hz, CH_2), 6.23 (2H, s, NH_2, exch.D_2O), 7.35–7.92 (5H, m, Ar–H), 8.21 (1H, s, Pyrazole H-3); ^{13}CNMR ($CDCl_3$, 75 MHz) δ *ppm*; 165.8 (C=O), 148.1(C-5), 141.6 (C-3), 137.2 (C-1, Ar–C), 129.4 (C-3, C-5, Ar–C), 126.1 (C-4, Ar–C), 122.6 (C-2, C-6, Ar–C), 108.9 (C-4), 56.6 (CH_2), 18.6 (CH_3); Anal. Calcd for $C_{12}H_{13}N_3O_2$ (231.25): C, 62.33; H, 5.67; N, 18.17. Found: C, 61.90; H, 5.89; N, 17.89.

*5-Amino-1-phenyl-1H-pyrazole-4-carboxylic acid (**2**)* A mixture of Ethyl 5-amino-1-phenyl-1H-pyrazole-4-carboxylate (**1**) (2.31 g, 0.01 mol) and sodium hydroxide (0.8 g, 0.02 mol) in ethanol 95 % (15 mL) was refluxed for 5 h. The solid obtained was filtered and the residue was dissolved in water then acidified with 6 N HCl (15 mL). The resulting crystalline solid was filtered and dried to provide the desired compound. It was obtained as silver scale crystals, Yield 85 %, mp. 176–178 °C, IR ν_{max}/cm^{-1}: 3401.5, 3364.8 (NH_2), 2948.4, (CH), 1682.3 (C=O), ^{1}H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 6.52 (2H, s, NH_2, exch.D_2O), 7.55–7.92 (5H, m, Ar–H), 8.11 (1H, s, Pyrazole H-3), 10.34(1H, s, OH exch.D_2O), ^{13}CNMR(DMSO, 75 MHz) δ *ppm*; 170.2 (C=O), 149.2(C-5), 142.8 (C-3), 139.2 (C-1, Ar–C), 129.4 (C-3, C-5, Ar–C), 126.5 (C-4, Ar–C), 120.6 (C-2, C-6, Ar–C), 109.9 (C-4); Anal. Calcd for $C_{10}H_9N_3O_2$ (203.20): C, 59.11; H, 4.46; N, 20.68. Found: C, 59.10; H, 4.29; N, 20.68.

*6-Methyl-1-phenylpyrazolo[3,4-d][1,3]oxazin-4(1H)-one (**3**)* A mixture of 5-Amino-1-phenyl-1H-pyrazole-4-carboxylic acid (**2**) (2 g, 0.01 mol) and acetic anhydride (5 mL) was refluxed for 5 h then cooled. The crystalline product obtained was filtered, washed with ethanol, and dried to provide the desired compound. It was obtained as white-shining needles (EtOH), Yield 45 %, mp. 180–181 °C, IR ν_{max}/cm^{-1}: 3066.6(CH aromatic) 1772.5(C=O), 2926 (CH aliphatic),1597 (C=C, C=N); ^{1}H NMR ($CDCl_3$, 300 MHz) δ *ppm*: 2.32(3H, s, CH_3), 7.42–8.01 (5H, m, Ar–H), 8.21(1H, s, Pyrazole H-3); ^{13}CNMR($CDCl_3$, 75 MHz) δ *ppm*; 160.2 (C=O), 158.3(C-6),141.9(C-3), 141.9 (C-7′), 139.7 (C-1, Ar–C), 129.1 (C-3, C-5, Ar–C), 126.1 (C-4, Ar–C), 121.1 (C-2, C-6, Ar–C), 109.9 (C-3′), 20.1 (CH_3); Anal. Calcd for $C_{12}H_9N_3O_2$ (227.22): C, 63.43; H, 3.99; N, 18.49. Found: C, 63.44; H, 3.98; N, 18.41.

*1-(6-Methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-yl)thiourea (**4**)* To a solution of 6-methyl-1-phenylpyrazolo [3,4-*d*][1,3] oxazin-4(*1H*)-one (3) (2.27 g, 0.01 mol) in n-butanol (5 mL), thiosemicarbazide (0.91 g, 0.01 mol) was added. The reaction mixture was refluxed for 8 h and after cooling a crystalline product was obtained. It was filtered, dried, and crystallized. It was obtained as white needles (EtOH), Yield 40 %, mp. 165–166 °C; IR ν_{max}/cm^{-1}: 3371.5 (NH), 3263.5, 3178.6 (NH_2), 2970.3 (CH_3), 1643.3 (C=O), 1620.2 (C=N, C=C), 1529.5(NH + C=S + NCS), 1284.5 (C=S + NCS), 1001 (NCN + C=S + C=N); ^{1}H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 2.43 (3H, s, CH_3), 4.49 (2H, s, NH_2, exch.D_2O), 7.19–8.11 (6H, m, Ar–H and Pyrazole H-3), 8.65 (1H, s, NH, exch.D_2O); ^{13}CNMR(DMSO, 75 MHz) δ *ppm*; 180.8 (C=S), 174.1(C=O), 162 (C-6),145.0(C-3), 141.9 (C-7′), 136.2 (C-1, Ar–C), 129.1 (C-3, C-5, Ar–C), 127.1 (C-4, Ar–C), 121.6 (C-2, C-6, Ar–C), 107.9 (C-3′), 21.6 (CH_3); EIMS *m/z* (%): 300 [M^+] (7.83), 91(100). Anal. Calcd for $C_{13}H_{12}N_6OS$ (300.34): C, 51.99; H, 4.03; N, 27.98. Found: C, 51.64; H, 3.99; N, 27.91.

*General procedure for preparation of 6-methyl-1-phenyl-5-[(4-(4-substitutedphenyl) thiazol-2(3H)-ylidene)amino]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-ones **(5a–c)***

A mixture of the **4** (3.0 g, 0.01 mol) and 4-substitutedphenacyl bromide (0.01 mol) in ethanol/chloroform (7:3) mixture (20 mL) was refluxed for 3 h. The mixture was concentrated to half its volume under reduced pressure. After cooling the precipitate was filtered, washed several times with ether, and crystallized.

*6-Methyl-1-phenyl-5-[(4-phenylthiazol-2(3H)-ylidene)amino]-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one **(5a)*** It was obtained as yellow crystals (DMF/H_2O), Yield 51 %, mp. 120–121 °C; IR ν_{max}/cm^{-1}: 3369.6 (NH), 3035.9 (C–H aromatic), 2976.1 (C–H aliphatic), 1670.3 (C=O), 1620.2 (C=N):^{1}H NMR (DMSO-d_6, 300 MHz) δ *ppm*: 2.47 (3H, s, CH_3), 7.34(1H, s, CH of thiazoline), 7.36–8.17 (10H, m, Ar–H), 8.47 (1H, s, pyrazole-H3), 10.23 (1H, s, NH, exch. D_2O); ^{13}C NMR (DMSO, 75 MHz) δ *ppm*; 164.3(C=O), 159.2 (C-6), 156.5(C-4 thiazole), 144.3 (C-2 thiazole), 141.9 (C-3), 138.2 (C-7′), 136.3(N–C-1 Ar–C), 129.2 (C–C-1, Ar–C), 128.5(C-3, C-5, Ar–C), 128.4 (C-3′, C-5′ Ar–C), 127.3(C-6′, Ar–C), 126.8 (C-2′, C-6′-Ar–C), 125.8(C-6-Ar–C), 121.9 (C-2′, C-6′-Ar–C), 107.1(C-5 thiazole), 106.6 (C-3′), 22.0 (CH_3); EIMS *m/z* (%): 400 [M^+](52.78), 239 (100); Anal. Calcd for $C_{21}H_{16}N_6OS$ (400.11): C, 62.98: H, 4.03; N, 20.99. Found: C, 62.63; H, 4.32; N, 21.32.

*6-Methyl-1-phenyl-5-[(4-(4-chlorophenyl)thiazol-2(3H)-ylidene)amino]-1H-pyrazolo[3,4-d] pyrimidin-4(5H)-one **(5b)*** It was obtained as yellowish white crystals (DMF/H_2O), Yield 54 %, mp. 189–190 °C; IR ν_{max}/cm^{-1}: 3286.7 (NH), 3074.5 (C–H aromatic), 2927.9 (C–H aliphatic), 1666.5 (C=O), 1604.7 (C=N):^{1}H NMR (DMSO-d_6, 300 MHz) δ *ppm*: 2.57 (3H, s, CH_3), 7.47 (1H, s, CH of thiazoline), 7.49–7.70 (5H, m, Ar–H) 7.82 (2H, d, $J = 7.5$ Hz, C3-H and C5-H of 4-ClC_6H_4), 8.16.

(2H, d, $J = 7.5$ Hz, C2-H and C6-H of 4-ClC_6H_4), 8.46(1H, s, pyrazole –H3), 10.89 (1H, s, NH, exch. D_2O); ^{13}C NMR (DMSO, 75 MHz) δ *ppm*; 167.7(C=O), 161.1 (C-6), 155.3(C-4 thiazole), 149.5 (C-2 thiazole), 148.7 (C-3), 137.9 (C-7′), 136.3(N–C-1 Ar–C), 132.7 (C–C-1, Ar–C), 132.1 (C–Cl), 129.2 (C-3, C-5, Ar–C), 128.5 (C-3′, C-5′Ar–C), 127.3(C-2′, C-6′, Ar–C), 127.2(C-6, Ar–C), 121.8 (C-2, C-6, Ar–C), 106.3(C-5 thiazole), 105.8 (C-3′), 21.9 (CH_3); EIMS *m/z* (%): 436.75 [M + 2] (6.82), 434.7[M^+](27.31), 209.9 (100); Anal. Calcd for $C_{21}H_{15}ClN_6OS$ (434.9): C, 58.00; H, 3.48; N, 19.32. Found: C, 58.12; H, 3.56; N, 19.43.

*6-Methyl-1-phenyl-5-[(4-(4-bromophenyl)thiazol-2(3H)-ylidene)amino]-1H-pyrazolo[3,4-d] pyrimidin-4(5H)one (**5c**)* It was obtained as brown crystals (DMF/H_2O), Yield 57 %, mp. 150–151 °C; IR ν_{max}/cm^{-1}: 3292.4 (NH), 3072.6 (C–H aromatic), 2931.8 (C–H aliphatic), 1666.5 (C=O), 1602.8 (C=N); ^1H NMR (DMSO-d_6, 500 MHz) δ *ppm*: 2.46 (3H, s, CH_3), 7.57 (1H, s, CH of thiazoline), 7.59–7.84 (6H, m, Ar–H and pyrazole –H3), 7.61 (2H, d, $J = 8.4$ Hz, C3-H and C5-H of 4-BrC_6H_4), 7.84 (2H, d, $J = 8.4$ Hz, C2-H and C6-H of 4-BrC_6H_4), 9.89 (1H, s, NH, exch.D_2O); ^{13}C NMR (DMSO, 125 MHz) δ *ppm*; 173.4(C=O), 168.5 (C-6), 161.5(C-4 thiazole), 150.3 (C-2 thiazole), 138.5 (C-3), 137.2 (C-7′), 129.8(N–C-1 Ar–C), 129.1 (C–C-1, Ar–C), 128.5 (C-3′, C-5′Ar–C), 127.8(C-3, C-5, Ar–C), 127.3(C-2′, C-6′, Ar–C), 127.2(C-6, Ar–C), 122.4 (C–Br), 121.8 (C-2, C-6, Ar–C), 107.0(C-5 thiazole), 105.8 (C-3′), 22.5 (CH_3); EIMS *m/z* (%): 481.80 [M + 2](0.10), 479.80 [M^+] (0.14), 477.8 [M-2](0.14), 172.95 (100); Anal. Calcd for $C_{21}H_{15}BrN_6OS$ (479.35): C, 52.62; H, 3.15; N, 17.53. Found: C, 52.68; H, 3.42; N, 17.46.

*General procedure for preparation of 4-alkyl-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d] pyrimidin-5(4H)-ylimino)thiazolidin-5-ones (**6a, b**)*

A mixture of **4** (3.0 g, 0.01 mol), anhydrous potassium carbonate (2.76 g, 0.01 mol) and either chloroacetyl chloride or 2-chloropropionyl chloride (0.015 mol) in dry benzene (15 mL) was heated under reflux for 10 h. The reaction mixture was filtered while hot. The formed precipitate was washed with water, dried, and crystallized.

*2-(6-Methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-5-one (**6a**)* It was obtained as grayish white crystals (MeOH), Yield 41 %, mp. 270–271 °C; IR ν_{max}/cm^{-1}: 3323.3 (NH), 3072.6 (C–H aromatic), 2983.8 (C–H aliphatic), 1718.5 (C=O), 1701.2 (C=O), 1616.3 (C=N); ^1H NMR (DMSO-d_6, 300 MHz) δ *ppm*: 2.47 (3H, s, CH_3), 5.20 (2H, s, CH_2 of thiazolidinone), 7.47–8.13 (5H, m, Ar–H), 8.16 (1H, s, pyrazole H-3), 11.85 (1H, s, NH, exch.D_2O); ^{13}C NMR (DMSO, 75 MHz) δ *ppm*; 173.3(C=O), 168.6 (C=O), 160.0 (C-6), 158.6 (C-2 thiazole), 156.9 (C-3), 139.1(N–C-1 Ar–C), 137.2 (C-7′), 129.1 (C-6, Ar–C), 126.8(C-3, C-5, Ar–C), 121.6 (C-2, C-6, Ar–C), 107.0 (C-3′), 61.6 (CH_2) 22.3 (CH_3); EIMS m/z (%): 340 [M^+] (17.42), 77(100); Anal. Calcd for $C_{15}H_{12}N_6O_2S$ (340.36): C, 52.93; H, 3.55; N, 24.69. Found: C, 52.61; H, 3.76; N, 24.65.

*4-Methyl-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d] pyrimidin-5(4H)-ylimino)thiazolidin-5- one (**6b**)* It was obtained as white crystals (MeOH) 0, Yield 35 %, mp. 262–263 °C; IR ν_{max}/cm^{-1}: 3417.8 (NH), 2976.1, 2954.9 (C–H aliphatic), 1718.5 (C=O), 1701.2(C=O), 1608.6 (C=N);^1H NMR (DMSO-d_6, 300 MHz) δ *ppm*: 1.55(3H, d, $J = 6.6$ Hz, CH_3 of thiazolidinone), 2.44 (3H, s, CH_3), 4.16 (1H, q, $J = 6.6$ Hz, CH of thiazolidinone), 7.27–8.35 (5H, m, Ar–H), 8.38 (1H, s, pyrazole H-3), 12.45 (1H, s, NH, exch.D_2O); ^{13}C NMR (DMSO, 75 MHz) δ *ppm*; 190.2(C=O), 162.1 (C=O), 158.6(C-2 thiazole), 157.6 (C-6), 142.9 (C-3), 141.2 (C-7′), 136.3(N–C-1 Ar–C), 129.1 (C-6, Ar–C), 127.8(C-3, C-5, Ar–C), 121.6 (C-2, C-6, Ar–C), 107.0 (C-3′), 44.1 (C-5 thiazole) 21.9 (CH_3), 18.4(CH_3); EIMS *m/z* (%): 354 [M^+] (17.42), 77 (100); Anal. Calcd for $C_{16}H_{14}N_6O_2S$ (354.39): C, 54.23; H, 3.89; N, 23.71. Found: C, 54.51; H, 3.57; N, 23.78.

*2-(6-Methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-4-one (**7**)* To a suspension of the appropriate thiosemicarbazide **4** (3.0 g, 0.01 mol) in glacial acetic acid (10 mL), anhydrous sodium acetate (1.7 g, 0.02 mol) and monochloroacetic acid (1.98 g, 0.02 mol) were added. The reaction was refluxed for 12 h. After cooling, the mixture was poured onto ice water (10 mL) and allowed to stand overnight in the fridge. The product was filtered, dried, and crystallized.

It was obtained as white crystals (DMF/H_2O), Yield 66 %, mp. 265–266 °C; IR ν_{max}/cm^{-1}: 3327.2 (NH), 3078.3 (C–H aromatic), 2987.7, 2924.0 (C–H aliphatic), 1724.3 (C=O), 1707.0(C=O), 1616.3 (C=N); ^1H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 2.42 (3H, s, CH_3), 4.06 (2H, s, CH_2 of thiazolidinone), 7.39–8.09 (5H, m, Ar–H), 8.34 (1H, s, pyrazole H-3), 11.75, 12.55 (1H, 2 s, tautomer NH, OH, exch. D_2O); ^{13}C NMR (DMSO,75 MHz) δ *ppm*: 173.6(C=O), 168.6 (C=O), 160.0 (C-6), 158.6 (C-2 thiazole), 152.8 (C-3), 149.5 (C-7′),138.3(N–C-1 Ar–C),129.1 (C-6, Ar–C), 127.8(C-3, C-5, Ar–C), 121.6 (C–2, C-6, Ar–C), 105.7 (C-3′), 33.0 (CH_2), 21.8 (CH_3); EIMS *m/z* (%): 340.1 [M^+] (39.95), 293.1(100); Anal. Calcd for $C_{15}H_{12}N_6O_2S$ (340.36): C, 52.93; H, 3.55; N, 24.69. Found: C, 52.94; H, 3.57; N, 24.65.

*General procedure for preparation of 2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)-5-substituted benzylidenethiazolidin-4-ones **(8a–f)***

An equimolar mixture of **7** (3.4 g, 0.01 mol) and the appropriate aldehyde (0.01 mol) in glacial acetic acid (10 mL) in the presence of anhydrous sodium acetate(0.8 g, 0.01 mol) was refluxed for 8 h. The reaction mixture was concentrated, cooled, and poured onto crushed ice (10 mL). The formed precipitate was filtered, dried, and crystallized from DMF/water.

*5-Benzylidene-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino) thiazolidin-4-one **(8a)*** It was obtained as white crystals, Yield 59 %, mp. 212–213 °C; IR ν_{max}/cm^{-1}: 3387.0 (NH), 3078.3 (C–H aromatic), 2958.8 (C–H aliphatic), 1712.7 (C=O), 1701.2(C=O), 1612.4 (C=N); ^{1}H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 2.49 (3H, s, CH_3), 7.47–8.19 (10H, m, Ar–H), 8.38 (1H, s, pyrazole H-3), 8.42 (1H, s, HC =), 11.74, 12.60 (1H, 2 s, tautomer NH, OH, exch. D_2O); ^{13}C NMR (DMSO,75 MHz) δ *ppm*: 173.6(C=O), 171.8 (C=O), 158.6 (C-6), 157.1 (C-2 thiazole), 152.8 (C-3), 149.5 (C-7′),138.3 (C=C), 138.1(N–C-1 Ar–C),136.0 (C–C-1, Ar–C),129.7 (C-3, C-5, Ar–H), 129.1 (C-6′, Ar–C), 128.1(C-3′,C-5′, Ar–C), 126.9 (C-2′, C-6′, Ar–H),126.7(C-6, Ar–H), 121.6 (C-2, C-6, Ar–C), 121.3(C-5Thiazolidine), 106.5 (C-3′), 21.8 (CH_3); EIMS *m/z* (%): 428 [M^+] (11.55), 429 [M + 1] (6.58), 77 (100); Anal. Calcd for $C_{22}H_{16}N_6O_2S$ (428.47): C, 61.67; H, 3.76; N, 19.61. Found: C, 61.78; H, 3.89; N, 19.65.

*5-(4-Methoxybenzylidene)-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-4-one **(8b)*** It was obtained as a yellow crystals, Yield: 64 %, mp. 248–249 °C; IR ν_{max}/cm^{-1}: 3394.7 (NH), 3070.4 (C–H aromatic), 2978.0, 2933.7 (C–H aliphatic), 1718.5 (C=O), 1693.5 (C=O), 1614.4 (C=N); ^{1}H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 2.49 (3H, s, CH_3), 3.83(3H, s, OCH_3), 7.03–8.39 (6H, m, Ar–H and pyrazole H-3), 7.10 (2H, d, $J = 7.8$, C3-H and C5-H of 4-$OCH_3C_6H_4$), 7.79 (2H, d, $J = 7.8$ Hz, C2-H and C6-H of 4-$OCH_3C_6H_4$), 9.03 (1H, s, HC =), 11.74, 11.88 (1H, 2 s, tautomer NH, OH, exch. D_2O); ^{13}C NMR (DMSO,75 MHz) δ *ppm*: 173.6(C=O), 171.8 (C=O), 164.4 (C-6), 158.6 (C-OCH_3), 157.1(C-2 thiazole), 152.8(C=C), 149.5 (C-7′),138.3 (C-3), 138.1(N–C-1 Ar–C),136.1 (C–C-1, Ar–C),129.1 (C-3, C-5, Ar–H), 127.1(C-2′, C-6′, Ar–C), 124.9 (C-2, C-6, Ar–H), 121.6(C-6, Ar–H), 121.3 (C-3′, C-5′, Ar–C), 111.5(C-5Thiazolidine), 108.5 (C-3′), 55.6(OCH_3), 22.0 (CH_3); EIMS *m/z* (%): 458 [M^+] (10.16), 77 (100); Anal. Calcd for $C_{23}H_{18}N_6O_3S$ (458.49): C, 60.25; H, 3.96; N, 18.33. Found: C, 60.45; H, 3.98; N, 18.34.

*5-(3,4-Dimethoxybenzylidene)-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-4-one **(8c)*** It was obtained as yellow crystals, Yield: 66 %, mp. 206–207 °C; IR ν_{max}/cm^{-1}: 3385.0 (NH), 3068.7 (C–H aromatic), 2983.8, 2929.8 (C–H aliphatic), 1718.5 (C=O), 1689.6 (C=O), 1610.5 (C=N); ^{1}H NMR (DMSO-*d*6, 300 MHz) δ *ppm*: 2.50(3H, s, CH_3) 3.90–3.92 (6H, br S, 2OCH_3), 7.17–8.15 (8H, m, Ar–H), 8.42 (1H, s, pyrazole H-3), 9.06 (1H, s, HC=), 11.83, 12.62 (1H, 2 s, tautomer NH, OH, exch.D_2O); ^{13}C NMR (DMSO) δ *ppm*: 173.6(C=O), 173.2 (C=O), 164.4 (C-6), 158.6 (C-OCH_3), 157.1(OCH_3), 152.8(C-2 thiazole), 149.5 (C=C),144.2 (C-7′),138.3 (C-3), 138.1(N–C-1 Ar–C),136.1 (C–C-1, Ar–C),129.1 (C-3, C-5, Ar–H), 127.1(C-2, C-6, Ar–C), 124.9 (C-6′, Ar–H), 121.6(C-5 Thiazolidine), 121.3 (C-5′, Ar–C), 111.5(C-2′, Ar–C), 108.5 (C-3′), 55.6(2OCH_3), 22.0 (CH_3); EIMS *m/z* (%): 488 [M^+] (30.73), 77.05 (100); Anal. Calcd for $C_{24}H_{20}N_6O_4S$ (488.52): C, 59.01; H, 4.13; N, 17.20. Found: C, 59.32; H, 4.29; N,17.45.

*5-(4-Fluorobenzylidene)-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-4-one **(8d)*** It was obtained as yellow crystals, Yield: 51 %, mp. °C; IR ν_{max}/cm^{-1}: 3414.0 (NH), 3053.3 (C–H aromatic), 2926.0 (C–H aliphatic), 1710.5 (C=O), 1703.1 (C=O), 1618.2 (C=N); ^{1}H NMR (DMSO-*d*6,300 MHz) δ *ppm*: 2.48 (3H, s, CH3), 7.29–7.95 (5H, m, Ar–H), 7.39 (2H, d, $J = 8.4$ Hz, C2-H and C6-H of 4-FC_6H_4), 8.09 (2H, d, $J = 8.7$ Hz, C3-H and C5-H of 4-FC_6H_4), 8.38 (1H, s, pyrazole H-3), 10.16 (1H, s, HC =), 11.76, 12.57 (1H, 2 s, tautomer NH, OH, exch.D_2O); ^{13}C NMR (DMSO,75 MHz) δ *ppm*: 181.7 (C–F), 173.5(C=O), 171.7 (C=O), 160.3 (C-6), 152.8(C-2 thiazole), 149.6 (C=C),138.1 (C-7′),138.0 (C-3), 136.4(N–C-1 Ar–C),136.1 (C–C-1, Ar–C),129.2 (C-3, C-5, Ar–H), 127.1(C-2′, C-6′, Ar–C), 122.0 (C-2, C-6, Ar–H), 121.8(C-3, C-5Ar-H)121.3 (C-5 Thiazolidine), 106.3 (C-3′), 21.8 (CH_3); EIMS *m/z* (%): 446 [M^+] (21.99), 89 (100); Anal. Calcd for $C_{22}H_{15}FN_6O_2S$ (446.46): C, 59.18; H, 3.39; N, 18.82. Found: C,59.56; H, 3.42; N, 18.67.

*5-(4-Chlorobenzylidene)-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino) thiazolidin-4-one **(8e)*** It was obtained as yellow crystals, Yield: 56 %, mp.260–261 °C; IR ν_{max}/cm^{-1}: 3369.6 (NH), 3072.6 (C–H aromatic), 2981.9 (C–H aliphatic), 1710.5 (C=O), 1701.2 (C=O), 1618.2 (C=N); 1H NMR (DMSO-*d*6,300 MHz) δ *ppm*: 2.49 (3H, s, CH3), 7.53–7.87 (5H, m, Ar–H), 7.55 (2H, d, $J = 8.1$ Hz, C3-H and C5-H of 4-ClC_6H_4), 7.94 (2H, d, $J = 8.1$ Hz, C2-H and C6-H of 4-FC_6H_4), 8.49 (1H, s, pyrazole H-3), 9.30 (1H, s, HC =), 10.60, 11.74 (1H, 2 s, tautomeric OH, NH, exch.D_2O); ^{13}C NMR (DMSO) δ *ppm*: 173.2(C=O), 166.9 (C=O), 152.9 (C-6), 149.5 (C-2 thiazole), 138.1 (C=C),136.3 (C-7′), 133.5

(C–Cl), 134.8 (C-3), 131.6 (N–C-1 Ar–C),131.4 (C–C-1, Ar–C),130.0 (C-3, C-5, Ar–H), 129.3(C-3′, C-5′, Ar–C), 129.2 (C-2′, C-6′, Ar–H), 127.1(C-6, Ar–H), 121.6(C-2, C-6, Ar–H)121.4 (C-5 Thiazolidine), 105.7 (C-3′), 22.1 (CH_3); EIMS *m/z* (%): 464 [M + 2] (36.32), 462 [M^+] (26. 01), 74 (100), 76 (31.39); Anal. Calcd for $C_{22}H_{15}ClN_6O_2S$ (462.91): C, 57.08; H, 3.27; N, 18.15. Found: C, 57.45; H, 3.29; N, 18.34.

*5-(4-Bromobenzylidene)-2-(6-methyl-4-oxo-1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-5(4H)-ylimino)thiazolidin-4-one (**8f**)* It was obtained as yellow crystals, Yield: 47 %, mp. 238–240 °C; IR ν_{max}/cm^{-1}: 3387.0 (NH), 3074.5 (C–H aromatic), 2985.8 (C–H aliphatic), 1712.7 (C=O), 1701.2 (C=O), 1600.9 (C=N); 1H NMR (DMSO-*d*6,300 MHz) δ *ppm*: 2.49 (3H, s, CH3), 7.71–7.80 (5H, m, Ar–H), 7.28 (2H, d, $J = 8.4$ Hz, C2-H and C6-H of 4-BrC_6H_4), 7.77 (2H, d, $J = 8.7$ Hz, C3-H and C5-H of 4-BrC_6H_4), 7.99 (1H, s, pyrazole H-3), 9.30 (1H, s, HC =), 11.85 (1H, s, NH, exch.D_2O); ^{13}C NMR (DMSO,75 MHz) δ *ppm*: 173. 2(C=O), 167.4 (C=O), 161.1 (C-6), 149.5 (C-2 thiazole), 138.1 (C=C),136.1 (C-7′), 132.1 (C-3), 131.8 (N–C-1 Ar–C),131.5 (C–C-1, Ar–C),129.8 (C-3′, C-5′, Ar–H), 129. 1(C-3, C-5, Ar–C), 127.2 (C-2′, C-6′, Ar–H),122.3(C–Br), 121.8(C-6, Ar–H), 121.6(C-2, C-6, Ar–H)121.3 (C-5 Thiazolidine), 105.7 (C-3′), 22.0 (CH_3); EIMS *m/z* (%): 508 [M + 2] (54.92), 506 [M^+] (61.90), 105 (100); Anal. Calcd for $C_{22}H_{15}BrN_6O_2S$ (507.36): C, 52.08; H, 2.98; N, 16.56. Found: C, 52.41; H, 3.12; N, 16.66.

Pharmacological screening

Materials and methods Animals-Adult Albino rats of both sexes weighing 150–200 g were used in the experiments. Animals were housed under standardized conditions for light and temperature and received standard rat chow and tap water add libitum. Animals were randomly assigned to different experimental groups, each kept in a separate cage. All animal procedures were performed after approval from the Ethics committee of the National Research Center and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985).

Drugs and chemicals Carrageenan Iambda Sigma-Aldrich chemical company (USA), meloxicam Khahira Pharmaceutical and Chemical Company (Cairo, Egypt).

Anti-inflammatory evaluation (in vivo screening) All the target compounds were evaluated for their anti-inflammatory activity using carrageenan-induced rat paw edema method of Winter *et al.* (1962). The employed technique is based on the ability of the tested compounds to inhibit the edema produced in the hind paw of the rat after injection of carrageenan. The animals were randomly divided into 14 groups of five rats each. The initial hind paw volume of rats was determined volumetrically by means of plethysmometer 7150 (UGO Basile, Italy). Meloxicam (reference standard) and the tested compounds suspended in 2 % Tween 80 were administered intraperitoneally at a dose of 1.5 mg/kg body weight, while the control group received only 2 % Tween 80, 1 h before induction of inflammation. The paw edema was induced by sub-plantar injection of 1 % carrageenan solution in saline (0.9 %) (0.1 mL). Carrageenan caused visible redness and pronounced swelling that was well developed by 4 h and persisted for more than 48 h. Right hind paw was measured with a plethysmometer before and at 1, 2, 3, and 4 h after carrageenan injection. The difference of average values between treated and control group is calculated for each time interval and evaluated statistically. Quantitative variables from normal distribution were expressed as mean ± standard error (SEM). The anti-inflammatory activity was expressed as percentage inhibition of edema volume in treated animals in comparison with the control group according to the following equation:

$$\% \text{ Inhibition } = (\text{Vc} - \text{Vt})\,100/\text{Vc},$$

where Vc is the mean of edema volume of rat paw after administration of carrageenan in the control group, Vt is the mean of edema volume of rat paw after administration of the tested compounds or the reference drugs.

Analgesic activity The hot-plate test was performed on rats by using an electronically controlled hot-plate (ugo Basile, Italy) heated to 50 °C (±0.1 °C) for possible centrally mediated analgesic effect of the drugs. For three consecutive days preceding the experiment, rats were adapted on the hot plate by placing them on a plate maintained at room temperature for 15 min each day. Fourteen groups of rats, each were given saline and/or the different compounds and the last group received meloxicam (1.5 mg/kg) 60 min prior to testing. Each animal was then placed gently onto a 50 °C hot plate to perform the test. Latency to exhibit nociceptive responses, such as licking paws or jumping off the hot plate was determined 60 and 90 min post treatment (Laviola and Alleva, 1990).

Ulcerogenic effects Groups of five male albino rats weighing 150–175 g were used. They were starved 48 h prior to drug administration. The test compounds were administered orally in 1.5 mg/kg as aqueous suspension. The animals were sacrificed after 7 h. Stomachs were removed and placed on saline-soaked filter paper until inspection. A longitudinal incision along the greater curvature was made with fine scissor. The stomach was

inverted over the index finger and the presence or absence of gastric irritation is determined. The presence of a single or multiple lesions (erosion, ulcer or perforation) is considered to be positive. The results are expressed as the severity of lesions/rat (Mozsik *et al.*, 1982). Statistical analysis was carried out using Kruskal–Wallis non-parametric one way ANOVA.

In vitro cyclooxygenase (COX) inhibition assays The COX-1 and COX-2 activities of the compounds were measured using ovine COX-1 and human recombinant COX-2 enzymes included in the 'Colorimetric COX (ovine) inhibitor screening assay kit' (Catalog No. 760111, Cayman Chemicals Inc., Ann Arbor, MI, USA). Kit provided by Cayman (Cayman Chemical Co., Ann Arbor, MI) which measures the peroxidase component of COXs by the method of Kulmacz and Lands (Kulmacz and Lands, 1983). The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) at 590 nm. The assay was performed as follows: prepare first the background wells by adding 160 µL of assay buffer (0.1 M Tris–HCl, PH8), 10 µL of heme to three wells then we prepare the 100 % initial activity wells by adding 150 µL of assay buffer, 10 µL of heme and 10 µl of enzyme (either COX-1 or COX-2) to three wells. Finally, inhibitor wells were prepared by adding 150 µL of assay buffer, 10 µL of heme and 10 µL of enzyme (either COX-1 or COX-2) to three wells. Add 10 µL of tested compounds dissolved in DMF to the inhibitor wells and 10 µL of DMF to the 100 % initial activity wells and background wells. Carefully shake the plate for a few seconds and incubate for 5 min at 25 °C. Add 20 µL of colorimetric substrate solution to all of the wells used. Add 20 µL of arachidonic acid to all of the wells used. Carefully shake the plate for a few seconds and incubate for 5 min at 25 °C. Read the absorbance at 590 nm using a plate reader. Determine the average absorbance of all the samples. Subtract the absorbance of the background wells from absorbance of 100 % initial activity and

Inhibitor wells. Calculate the % inhibition as follows:

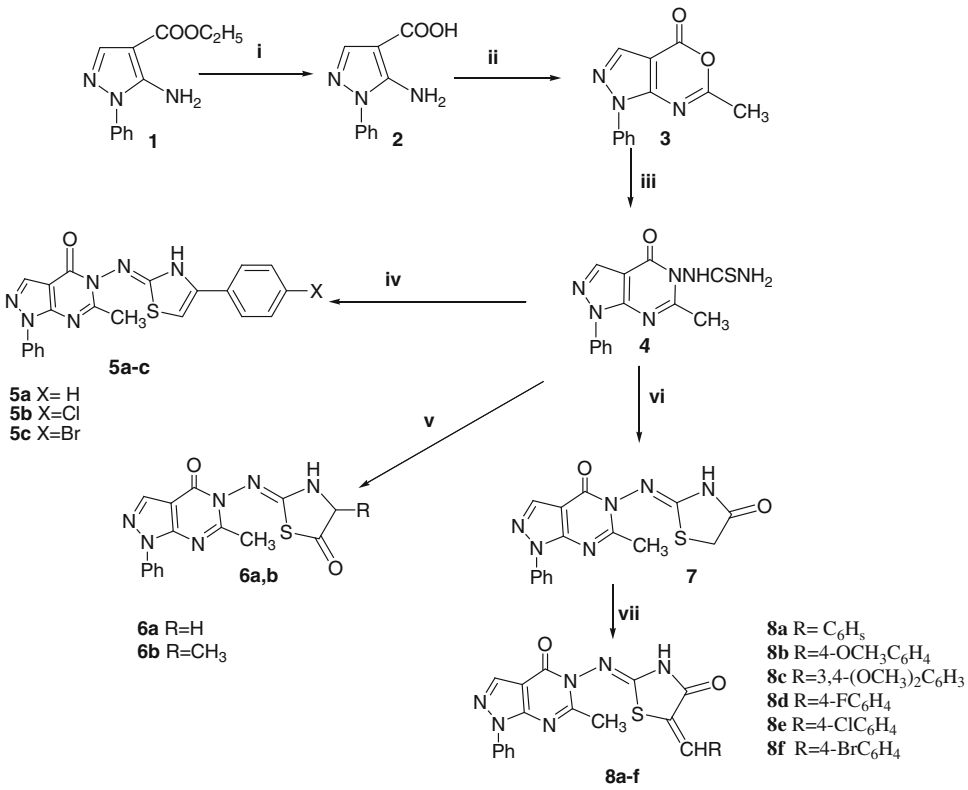
$$\%\ \text{inhibition} = \frac{\text{Absorbance of } 100\ \%\ \text{initial activity} - \text{Absorbance of inhibitor sample}}{\text{Absorbance of } 100\ \%\ \text{initial activity}} \times 100$$

Either graph the % inhibition or % initial activity by the inhibitor concentration to determine the IC_{50} values here (concentration at which there was 50 % inhibition). The IC_{50} values were calculated for all of the compounds. Meloxicam was used as positive control.

Results and discussion

Chemistry

The target compounds **5–8** were synthesized as shown in Scheme 1. 1-(6-Methyl-4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidin-5(4*H*)-yl) thiourea (**4**) was selected as the primary starting material for this series of reactions, and was prepared via three step procedure which involved the hydrolysis of ethyl ester of 5-amino-1-phenylpyrazole carboxylic acid (**1**) (Peet *et al.*, 1992) with alcoholic sodium hydroxide followed by neutralization to afford the corresponding carboxylic acid **2** (Schmidt and Druey, 1958) which was then refluxed with acetic anhydride to give 6-methyl-1-phenylpyrazolo [3,4-*d*][1,3] oxazin-4(1*H*)-one (**3**) (Cheng and Robins, 1958). The oxazinone derivative **3** was converted to pyrazolopyrimidinylthiourea **4** by its nucleophilic substitution reaction with thiosemicarbazide. Insertion of nitrogen in the ring was characterized by the disappearance of the band at 1,180 cm^{-1} of C–O and the shift of carbonyl band from 1,770 to 1,643 which is a very low value due to H-bond between C=O and NH of thiosemicarbazide at position-5. Also the IR spectrum was characterized by the appearance of absorption bands at 1,529, 1,284, and 1001 cm^{-1} attributed to N–C=S function (Omar and AboulWafa, 1984). Moreover, appearance of new peaks at 3,371, 3,263, and 3,178 cm^{-1} due to NH and NH_2 stretching which appeared also as two D_2O exchangeable signals at 4.49 and 8.64 *ppm* in ^1H NMR spectra. Nucleophilic reaction of thiosemicarbazide **4** with 4-substituted phenacyl bromide yield 2-imino thiazolines derivatives **5a–c**. The structure of compounds **5a–c** was elucidated from their spectral data. The IR spectra lacked the mixed vibrational bands due to N–C=S function present in their precursors **4** and also showed shifting of carbonyl absorption to 1,670 cm^{-1}, indicating disappearance of H-bond together with disappearance of the absorption bands for the NH_2 group. The ^1H NMR spectra were consistent with the proposed structures. Treatment of thiosemicarbazone **4** with chloroacyl chloride in refluxing dry benzene, following the reaction conditions reported for the preparation of related compounds (Taher *et al.*, 2012), in the presence of anhydrous potassium carbonate gave thiazolidinone derivatives **6a**, **b**. The IR spectra of compounds **6a**, **b** proved as useful in tracing the appearance of another C=O of thiazolidinone ring at 1,718 cm^{-1} and



Scheme 1 Illustrate the synthetic pathway of compounds. Reagent and condition: *i* ethanolic NaOH, reflux 6 h *ii* Acetic anhydride, reflux 5 h. *iii* Thiosemicarbazide, n-butanol, reflux 8h. *iv* p-substituted phenacyl bromide, ethanol/chloroform, reflux 3 h. *v* chloroacyl chloride, potassium carbonate, benzene, reflux 10 h. *vi* chloroacetic acid, sodium acetate, glacial acetic acid, reflux 12 h. *vii* substituted aldehyde, sodium acetate, glacial acetic acid, reflux 8 h.

disappearance of the mixed vibrational bands due to N–C=S function present in their precursors **4**. ^{1}H NMR spectrum of **6a** showed that CH_2 of thiazolidinone appeared as a singlet peak at δ5.1 *ppm* integrated for two protons, in addition to a D_2O exchangeable singlet at δ 11.75 *ppm* integrated for one proton assigned for NH. Moreover, the ^{1}H NMR spectra of **6b** showed doublet signal at δ 1.54 ppm integrated for three protons due to the methyl group at thiazolidinone C-4 and quartet signal at δ 4.27 ppm integrated for one proton due to thiazolidinone CH-4. The ^{13}C NMR spectrum of **6a** revealed a shielded signal due to the methylene carbon C-4 at δ 61.6 *ppm*. While ^{13}CNMR spectrum of compound **6b** disclosed three signals at δ 18.4, 21.9, and 44.1 *ppm* corresponding to the CH_3 of thiazolidinone, CH_3 of pyrimidine and stereogenic carbon of the thiazolidinone nucleus, sequentially. Cyclo-condensation of the thiourea derivative **4** with chloroacetic acid in refluxing glacial acetic acid, in the presence of anhydrous sodium acetate afforded the corresponding 2-(6-methyl-4-oxo-1-phenyl-1*H*-pyrazolo [3,4-*d*] pyrimidin-5(4*H*)-ylimino) thiazolidin-4-one.(**7**). The ^{1}H NMR spectra showed the singlet at δ 4.06 *ppm* integrated for two protons due to the thiazolidinone CH-5. Condensation of **7** with commercially available aldehydes in the presence of anhydrous sodium acetate in refluxing glacial acetic acid afforded the corresponding 2-(6-methyl-4-oxo-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-5(4*H*)-ylimino)-5-substituted benzylidene thiazolidin-4-one **8a–f**. The ^{1}HNMR spectra showed significant absorption bands at δ 8.4–10.1 *ppm* corresponding to =CH proton of compounds **8a–f**. Both the analytical and spectral data (IR, ^{1}H-NMR, ^{13}C-NMR and MS) of all the newly synthesized compounds were in full agreement with the proposed structures.

Biological activity

Anti-inflammatory activity

All the targeted compounds were evaluated for their anti-inflammatory activity via carrageenan-induced rat paw edema according to the reported method (Winter *et al.*, 1962) using meloxicam as reference drug. The results were recorded in Table 1, and illustrated graphically in Fig. 2. According to Table 1, administration of many tested compounds 1 h prior to carrageenan injection at dose of 1.5 mg/kg caused significant inhibition of paw edema response. Compounds **7**, **8c**, **8d**, and **8e** caused significant decrease in paw edema after 1, 2, 3, and 4 h after drug administration, while **8f** gave delayed response after 2 h of administration and continued to the fourth hour. However, compounds **5a** and **5b** gave their response after 3 h and persisted to the fourth hour, but compound **5c** significantly decreased the paw edema 4 h post administration. On the

Table 1 Anti-inflammatory effect and percentage inhibition of meloxicam and synthesized compounds on carrageenan-induced edema of the hind paw in rats ($n = 5$)

Cpd. no.	Edema (mm) ± SEM				% inhibition			
	1 h	2 h	3 h	4 h	1 h	2 h	3 h	4 h
Control	56.07 ± 2.920	80.10 ± 5.764	101.3 ± 1.333	116.7 ± 5.278	–	–	–	–
Meloxicam	29.92 ± 3.567*	42.02 ± 6.462*	49.65 ± 6.510*	56.59 ± 4.699*	46.63	47.56	50.98	51.51
5a	47.63 ± 5.958	59.22 ± 2.648	64.69 ± 7.178*	71.48 ± 7.548*	15	26	36.14	38.74
5b	43.79 ± 2.114	60.33 ± 3.569	73.57 ± 7.061	81.78 ± 7.370*	21.9	24.68	27.37	29.92
5c	44.73 ± 4.158	65.41 ± 4.159	75.80 ± 1.924	81.58 ± 1.643*	20.22	18.33	25.17	30
6a	54.83 ± 7.630	77.77 ± 5.756	89.60 ± 3.767	98.97 ± 6.584	2.2	2.9	11.54	15.2
6b	47.96 ± 2.273	71.05 ± 4.374	84.28 ± 7.949	88.97 ± 8.207	14.46	11.29	16.8	23.76
7	33.08 ± 3.705*	40.60 ± 1.327*	43.65 ± 3.672*	49.01 ± 7.559*	41	49.3	56.91	58.05
8a	47.01 ± 3.164	71.87 ± 7.176	76.07 ± 6.391	84.35 ± 6.394	16.07	10.27	24.9	27.72
8b	49.00 ± 5.715	67.99 ± 8.612	77.31 ± 7.493	86.65 ± 7.906	12.6	15.11	23.68	25.74
8c	37.20 ± 7.634*	52.14 ± 4.211*	60.03 ± 11.41*	64.00 ± 2.29*	33.65	34.9	40.7	45.15
8d	31.27 ± 1.411*	46.56 ± 1.088*	56.80 ± 4.190*	64.18 ± 4.529*	44.23	41.87	43.93	45.15
8e	32.95 ± 6.739*	46.16 ± 2.148*	56.14 ± 5.620*	64.24 ± 3.709*	41.23	42.37	44.58	44.95
8f	49.08 ± 3.311	51.16 ± 5.327	60.03 ± 8.579	86.01 ± 3.574	23.16	36.12	40.74	48.43

Values represent the mean ± SE of five animals for each groups

* $P < 0.05$: Statistically significant from control (one way Anova followed by Tukey test)

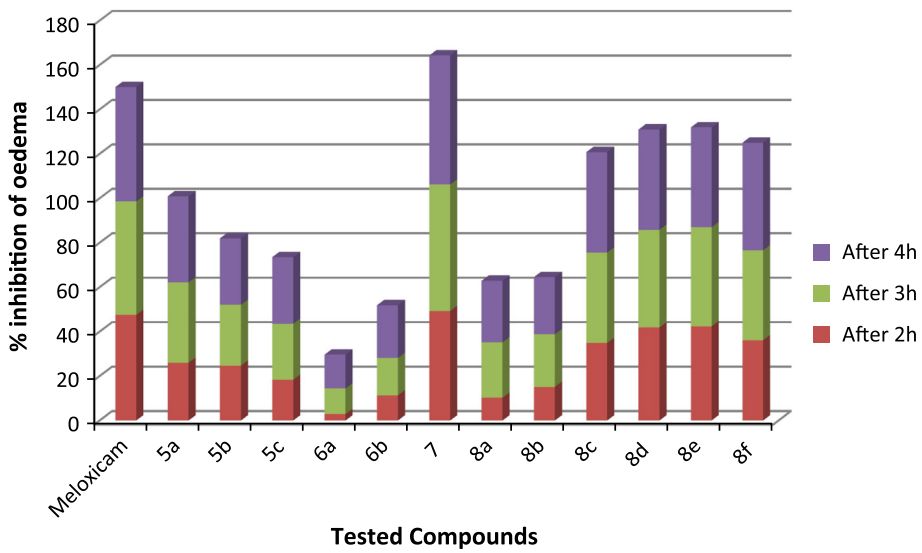


Fig. 2 The anti-inflammatory activity of tested compounds

other hand, compounds **6a**, **6b**, **8a**, and **8b** were less active toward carrageenan-induced edema in comparison to the standard reference meloxicam, which markedly and significantly inhibited the paw edema 1, 2, 3, and 4 h after carrageenan injection. Thus, compounds **5a**, **5b**, **5c**, **7**, **8c**, **8d**, **8e**, and **8f** have good anti-inflammatory activity and compound **7** is the most potent derivative.

Structure–activity relationship

Results listed in Table 1 revealed that the pyrazolopyrimidine derivatives bearing thiazolidinone moiety **7**, **8a**–**f** exhibited higher anti-inflammatory activity than those bearing thiazoline moiety **5a**–**c**. Considering the anti-inflammatory activity of the thiazole derivatives **5a**–**c**, it was noticed that the 4-unsubstituted phenyl derivative **5a** showed higher activity than the 4-substituted phenyl congeners **5b**, **c** which gave almost the same results. Considering the anti-inflammatory activity of the pyrazolopyrimidine derivatives bearing thiazolidinone moiety **6a**, **6b**, and **7**, it was noticed that the thiazolidin-4-one derivative **7** exhibited higher activity than their thiazolidin-5-one congeners **6a**, **b**, as compound **7** showed four folds increase in activity than **6a** (% inhibition of **7** = 58 vs 15.2

of **6a**). While substitution of methylene group in **6a** with methyl group increase its activity (23.7 vs 15.2). However, on the formation of the derivatives **8a–f**, they exhibited moderate potency compared with their starting compound **7**. Moreover, the nature of the aryl substituent attached to the methene group has an observable effect on the activity, as shown in compounds **8d**, **8e**, and 8f bearing 4-electron withdrawing substituent (F, Cl, Br group) exhibiting improved activity than their congeners with 4-electron donating substituent (methoxy group) **8b**, **8c** or with unsubstituted phenyl analogs **8a**. Also, increasing the number of methoxy groups on the arylidene moiety was accompanied by enhancement of anti-inflammatory activity (% inhibition of **8c** = 45.15 vs 25.74 of **8b**).

Table 2 Analgesic effect

Group	Reaction time (s)		
	Basal	60 min	90 min
Control	4.95 ± 0.558	5.36 ± 0.452	4.40 ± 0.148
Meloxicam	4.50 ± 0.369	9.35 ± 0.776*	10.23 ± 1.067*
5a	4.03 ± 0.260	6.68 ± 0.629	10.33 ± 1.408*
5b	4.41 ± 0.151	9.93 ± 0.8241*	8.40 ± 1.016
5c	3.68 ± 0.238	7.13 ± 0.746	9.61 ± 0.819*
6a	5.58 ± 0.397	9.46 ± 0.824*	9.11 ± 1.047
6b	5.30 ± 0.478	7.86 ± 0.906	11.15 ± 1.476*
7	5.80 ± 0.425	9.65 ± 0.641*	11.62 ± 1.089*
8a	4.30 ± 0.319	7.16 ± 0.727	9.16 ± 1.168
8b	4.98 ± 0.417	7.15 ± 1.007	9.63 ± 1.053
8c	4.28 ± 0.474	7.48 ± 0.843	9.36 ± 0.636*
8d	5.55 ± 0.558	10.03 ± 0.609*	10.92 ± 0.716*
8e	5.10 ± 0.501	9.38 ± 0.663*	10.33 ± 1.160*
8f	4.80 ± 0.293	7.36 ± 0.682	9.98 ± 0.340*

Values represent the mean ± SE of five animals for each groups

* $P < 0.05$: Statistically significant from control (one way Anova followed by Tukey test)

Analgesic Activity

The analgesic activity of all the synthesized compounds was also evaluated by applying Hot-plate test (Laviola and Alleva, 1990), using meloxicam as a standard reference. Results were expressed as mean ± SE. Difference between vehicle control and treated groups were tested using one way ANOVA followed by Tukey test. According to Table 2, compounds **7**, **8d**, and **8e** showed significant analgesic activity higher than that obtained by meloxicam 60 and 90 min post administration. While compound **6a** exhibited equipotent analgesic effect or slightly less than that of meloxicam after 60 and 90 min of their administration. After 90 min of administration, compounds **5a**, **5c**, **6b**, exhibited significant analgesic activity more potent than meloxicam, while compounds **8a**, **8b**, **8c**, and **8f** exhibited comparable potency to meloxicam. Compound **5b** exhibited the analgesic effect after 60 min of administration only. Thus, it can be concluded that all compounds have significant analgesic activity and compound **7** with the highest anti-inflammatory activity exhibited the best analgesic activity. Results were illustrated by Fig. 3.

Ulcerogenic activity

The ulcerogenic effect of all synthesized compounds and meloxicam was evaluated by the reported method (Meshali *et al.*, 1983), and the ulcer index was calculated according to the reported method (Mozsik *et al.*, 1982). Gross observation of the isolated rat stomachs showed a normal stomach texture for all tested compounds with no

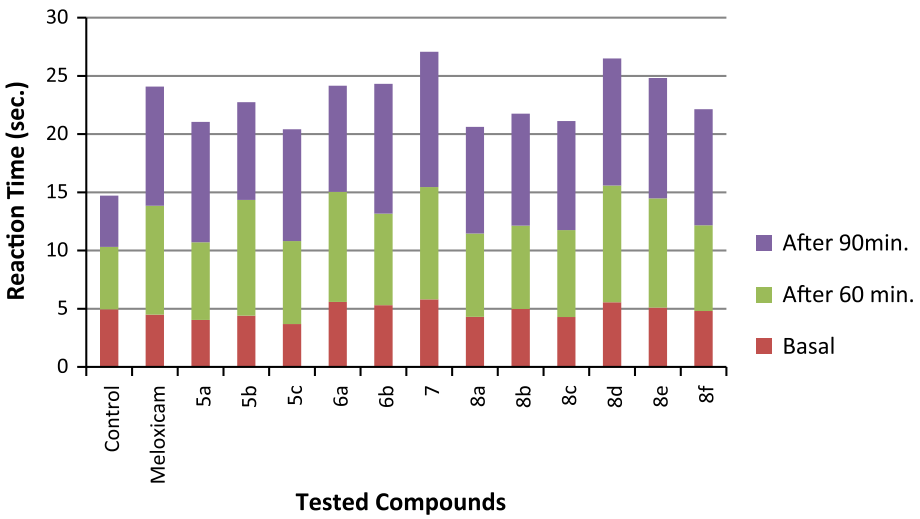


Fig. 3 The analgesic effect of the tested compounds

ulcerogenic effect in all of the experimental animals. This indicates a superior gastrointestinal safety profile (0 % ulceration). It is worth mentioning that meloxicam was found to cause ulcer severity 5.44 ± 1.25 under the same experimental conditions. Therefore, the potential medicinal value of these compounds as anti-inflammatory and analgesic agents is that they have better safety margin than meloxicam, on gastric mucosa.

Table 3 In vitro COX-inhibition data for the synthesized compounds

Compound	COX-1[a] (IC_{50}, μM)	COX-2[a] (IC_{50}, μM)	COX-2 SI[b]
5a	8.54	1.94	4.40
5b	7.32	1.60	4.58
5c	6.61	1.47	4.49
6a	12.48	2.37	5.27
6b	9.02	2.46	3.67
7	5.34	0.53	10.07
8a	16.33	4.94	3.30
8b	14.61	4.65	3.14
8c	13.34	3.77	3.53
8d	10.34	1.39	7.44
8e	7.75	1.19	6.51
8f	6.10	0.84	7.26
Meloxicam	18.64	5.11	3.65

[a] Values are means of two determinations acquired using an ovine COX-1/COX-2 assay kit (catalog no. 760111, Cayman Chemicals, MI, USA) and the deviation from the mean is <10 % of the mean value

[b] In vitro COX-2 selectivity index (COX-1 IC_{50}/COX-2 IC_{50})

In vitro COX-inhibition assay

The synthesized compounds were evaluated for their ability to inhibit COX-1 and COX-2, using an ovine COX-1/COX-2 assay kit. According to the protocol recommended by the supplier (Kulmacz and Lands, 1983), IC_{50} (μM) was determined as the means of two determinations acquired and the deviation from the mean is <10 % of the mean value. The selectivity index (SI values) was defined as IC_{50} (COX-1)/IC_{50} (COX-2). In the assay system, the IC_{50} on COX-1 and COX-2 of the synthesized compounds compared to the reference drug meloxicam were recorded in Table 3 and the results were represented graphically in (Fig. 4).

Results revealed that all the new compounds showed very good activity against both COX-1 and COX-2 more than the reference drug meloxicam. Selectivity index of the compounds also showed that they were more selective toward COX-2 than COX-1. IC_{50} values in μM (Table 3) acquired by determination of the in vitro ability of the tested compounds to inhibit COX-2 showed that compound **7** with thiazolin-4-one derivative was more potent and selective COX-2 inhibitor (IC_{50} = 0.53 μM, SI = 10.07) compared with the reference drug meloxicam (IC_{50} = 5.11 μM, SI = 3.65). Some of the tested compounds (**6b**, **8c**) were found to be preferential COX-2 inhibitor as meloxicam. It was also noticed that formation of Schiff's derivative substituted at 4-position of phenyl ring with fluoro, chloro or bromo derivatives **8d**, **8e**, and **8f** exhibited decline in potency (IC_{50} = 1.39, 1.19, 0.84 μM, SI = 7.44, 6.51, 7.26, respectively), compared with their starting compound **7**. Replacement of halogen substituent with methoxy group or unsubstituted derivative led to diminish in the COX-2 activity. In addition, replacement of the thiazolidinone nucleus with a thiazoline moiety (compounds **5a–c**) showed moderate COX-2 inhibition activity.

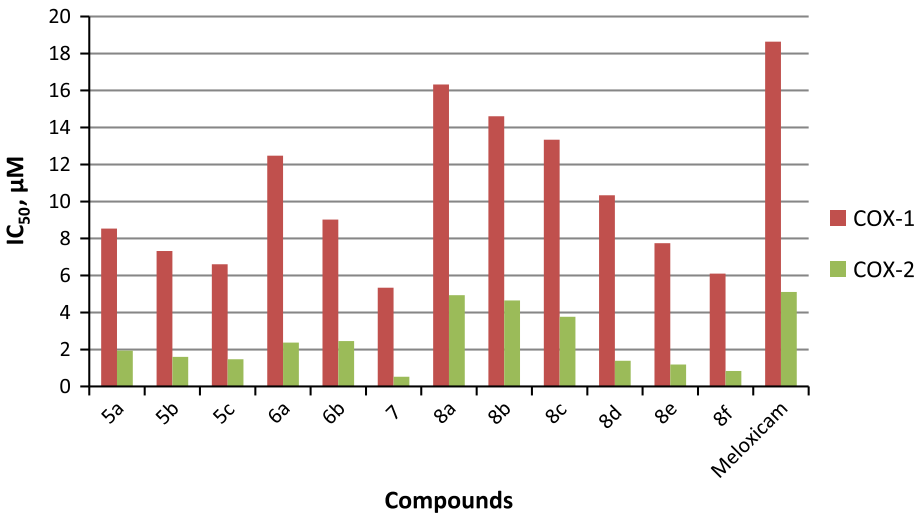


Fig. 4 Graphic comparison of IC_{50} values (COX-1 and COX-2) of the synthesized compounds and meloxicam

Conclusion

The present study reported the synthesis of novel derivatives of pyrazolo[3,4-*d*] pyrimidin-4(1*H*)-ones attached to thiazoline or thiazolidinone ring system. The synthesized compounds were evaluated for their COX-1/COX-2 inhibitory activity in vitro. All Compounds were effective as COX-1 and COX-2 inhibitors. Compound **7** was found to be potent and selective COX-2 inhibitors ($IC_{50} = 0.53$ μM) and selectivity (SI) = 10.07 which is more potent than the standard drug meloxicam. In addition, all compounds were assessed for their anti-inflammatory activity, analgesic and ulcerogenic liability in vivo. All the tested compounds exhibited moderate to significant anti-inflammatory and analgesic activities. Interestingly, compounds **7**, **8d**, and **8e** showed potent anti-inflammatory and analgesic activity. In addition, all of the tested compounds possessed excellent gastrointestinal safety profile and were well tolerated by experimental animals with high safety margins than the reference drug meloxicam. It can be concluded that compounds containing thiazolidinones exhibit better analgesic and anti-inflammatory activity than compounds having thiazoline moiety. In particular, compound **7** demonstrated potential anti-inflammatory and analgesic agents, also it showed high selectivity against COX-2 which makes it a good lead-candidate for further optimization and development of potent and safe anti-inflammatory agents.

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