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RESEARCH ARTICLE

Synthesis, cyclooxygenase inhibition, anti-inflammatory evaluation and ulcerogenic liability of new 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives

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Abstract

A new group of 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives **14a**–**d**–**21** were synthesized from 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (**12**). All the synthesized compounds were evaluated for their cyclooxygenase (COX) inhibition, anti-inflammatory activity and ulcerogenic liability. All the target compounds were more potential in inhibiting COX-2 than COX-1. Compounds having pyrazolyl moiety in a hybrid structure with pyrazolo[3,4-*d*]pyrimidine scaffold (**14a**–**d**, **16** and **17**) showed higher edema inhibition percentage activities (34–68%) and the 5-aminopyrazole derivative (**14c**, $ED_{50} = 87.9\,\mu mol/kg$) was the most potent one > celecoxib ($ED_{50} = 91.9\,\mu mol/kg$). While, the *in vivo* potent compounds (**14a**–**d**, **16**, **17** and **21**) caused variable ulceration effect (ulcer index = 0.33–4.0) comparable to that of celecoxib (ulcer index = 0.33), the pyrazol-3-one derivative (**16**) and the acetohydrazide (**21**) were the least ulcerogenic derivatives showing the same ulcerogenic potential of celecoxib.

Keywords

Anti-inflammatory, cyclooxygenase inhibition, pyrazolo [3,4-d] pyrimidine

History

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Introduction

Inflammation is an essential physiological response to a wide variety of stimuli (infection, trauma, burns, surgery and injury), and aimed at limiting damage and promoting tissue healing[1]. Non-steroidal anti-inflammatory drugs (NSAIDs) treat pain and inflammation via inhibition of cyclooxygenase enzyme (COX), a protein responsible for prostaglandins (PGs) biosynthesis from arachidonic acid[2,3]. COX exists in at least two distinct isoforms: a constitutive form (COX-1) and an inducible form (COX-2)[4]. While, the constitutive isoform (COX-1) is essential for the synthesis of cytoprotective PGs, biosynthesis of pro-aggregatory thromboxaneA2 (TXA2) and maintenance of renal function, the inducible one (COX-2) is induced in response to pro-inflammatory stimuli and is responsible for the progression of inflammation[5–7]. Traditional NSAIDs such as aspirin (**1**), indomethacin (**2**) and ibuprofen (**3**) inhibit both the isoforms leading to side effects ranging from ulcers to perforation and bleeding[8]. In an attempt to circumvent these side effects, selective COX-2 inhibitor drugs, such as celecoxib (**4**), rofecoxib (**5**) and valdecoxib (**6**), have been developed where they exhibited equivalent anti-inflammatory/analgesic activities to nonselective COX inhibitors, but with less GI toxicity[9] (Figure 1). Unfortunately, some cardiovascular side effects such as myocardial infarction and increased incidences of high blood pressure caused by the highly selective COX-2 inhibitors led to the withdrawal of rofecoxib and valdecoxib from the market[10,11].

Pyrazolo[3,4-*d*]pyrimidine represents one of the most frequently found scaffold in a wide variety of anti-inflammatory agents[12,13]. The 5-benzamido-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-one derivative (**7**) showed superior inhibitory profile against COX-2 when compared to that of reference standards *N*-[2-(cyclohexyloxy)4-nitrophenyl]methane sulfonamide (NS398) and indomethacin[14]. Also, the sulfamoylphenylpyrazolopyrimidine derivative (**8**) was reported to exhibit comparable anti-inflammatory activity with celecoxib (**4**) at a dose of 25 mg/kg[15]. Furthermore, the 4-substituted-1-phenylpyrazolo[3,4-*d*]pyrimidine derivative (**9**) showed considerable anti-inflammatory activity[16]. Guided by the previously mentioned studies and as a continuation of our previous work[17–21] for the development of safe anti-inflammatory derivatives,here wedescribe the synthesis, *in vitro* evaluation as COX-1/COX-2 inhibitors, *in vivo* anti-inflammatory (AI) activity and ulcerogenic liability for some new 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a**–**d**–**21**) with the hope of realizing compounds with improved anti-inflammatory activity and diminished side effects.

Experimental

Chemistry

Melting points were determined using a Griffin apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Shimadzu 435 spectrometer (Palo Alto, CA) using KBr discs. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 400 MHz spectrometer (Faculty of Pharmacy, Beni-Suef University, Beni-

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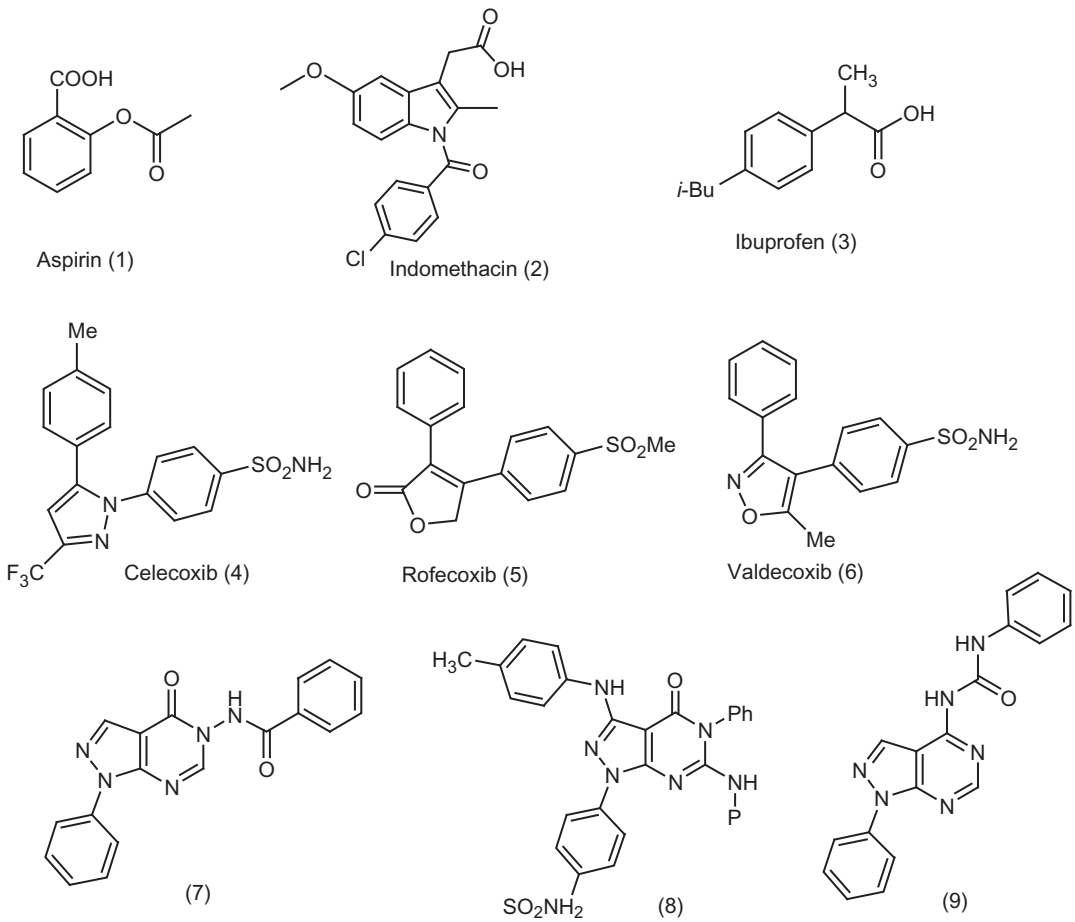


Figure 1. Chemical structures of traditional NSAIDs; aspirin (1), indomethacin (2) and ibuprofen (3), selective COX-2 inhibitors; celecoxib (4), rofecoxib (5), and valdecoxib (6) and reported pyrazolo[3,4-*d*]pyrimidine derivatives (7–9) with anti-inflammatory activity.

Suef, Egypt) in D_2O, DMSO-d_6 with TMS as the internal standard, where *J* (coupling constant) values were estimated in Hertz (Hz). Mass spectra were run on Hewlett Packard 5988 spectrometer. Microanalysis was performed for C, H, N at the Micro Analytical Center, Cairo University, Egypt, and was within ± 0.4% of theoretical values. All other reagents, purchased from the Acros Chemical Company (Milwaukee, WI), were used without further purification. 6-Methyl-1-phenyl-1,5-dihydropyrazolo[3,4-*d*]pyrimidin-4-one (**10**)[23], 2-ethoxymethylenemalononitrile (**13a**)[24], 2–(1-ethoxy-ethylidene)malononitrile (**13b**)[25], 2-cyano-3-ethoxy-acrylic acid ethyl ester (**13c**)[25], 2-cyano-3-ethoxybut-2-enoic acid ethyl ester (**13d**)[25] and 4–(2-chloroacetylamino)-benzoic acid ethyl ester (**18**)[26] were prepared according to reported procedures.

Ethyl 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetate (11)

A mixture of 6-methyl-1-phenyl-1,5-dihydropyrazolo[3,4-*d*]pyrimidin-4-one **(10,** 10 mmol, 2.26 g), ethyl chloroacetate (1.22 g, 10 mmol) and anhydrous potassium carbonate (1.38 g, 10 mmol) in dry acetone (20 mL) was heated under reflux for 6 h. After cooling, the reaction mixture was poured into ice-cold water. The separated solid was filtered, and crystallized from methanol to give pure compound **11**. Physical and spectral data are listed below.

Yellow solid; Yield 71%; m.p. 115–116 °C; IR (KBr) 3062 (CH aromatic), 2924 (CH aliphatic), 1731 (C = O), 1549 (C = N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.24 (t, 3H, $J = 7.2$ Hz, $C\underline{H}_3CH_2$), 2.59 (s, 3H, pyrimidine CH_3), 4.20 (q, 2H, $J = 7.2$ Hz, $CH_2C\underline{H}_3$), 4.95 (s, 2H, CH_2O),7.40–7.45 (m, 1H, phenyl H-4), 7.56–7.59 (m, 2H, phenyl H-3, H-5), 8.05 (d, $J = 7.5$ Hz, 2H, phenyl H-2, H-6), 8.35 (s, 1H, pyrazole H-3); ^{13}C NMR ($CDCl_3$) δ 14.42, 23.92, 45.57, 61.89, 105.05, 122.21, 127.65, 129.73, 136.56, 138.53, 150.81, 157.49, 159.77, 168.37; EIMS (*m/z*) 312 ($M^{+\cdot}$, 11.32%), 59 (100%). Anal.Calcd for $C_{16}H_{16}N_4O_3$: C, 61.53; H, 5.16; N, 17.94. Found: C, 61.55; H, 5.03; N, 18.13.

2-(6-Methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (12)

A mixture of compound **(11,** 3.12 g, 10 mmol), hydrazine hydrate (99.9%) (1 mL, 20 mmol) in ethanol (20 mL) was heated under reflux for 5 h. After cooling, the separated solid was filtered, dried and crystallized from acetone to afford the target compound**12**. Physical and spectral data are listed below.

White solid; Yield 62%; m.p. 245–246 °C; IR (KBr) 3439–3205 (br, NH and NH_2), 3044 (CH aromatic), 2947 (CH aliphatic), 1652 (C = O), 1569 (C = N) cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.48 (s, 3H, CH_3), 4.32 (s, 2H, NH_2, D_2O exchangeable), 4.76 (s, 2H, CH_2O), 7.40–7.43 (m, 1H, phenyl H-4), 7.56–7.60 (m, 2H, phenyl H-3, H-5), 8.07 (d, $J = 7.5$ Hz, 2H, phenyl H-2, H-6), 8.32 (s, 1H, pyrazole H-3), 9.44 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 23.96, 44.82, 105.25, 122.17, 127.62, 129.76, 136.52, 138.58, 150.91, 157.82, 160.43, 166.62; EIMS (*m/z*) 298 ($M^{+\cdot}$, 1.34%), 210 (100%). Anal.Calcd for $C_{14}H_{14}N_6O_2$: C, 56.37; H, 4.73; N, 28.17. Found: C, 56.62; H, 4.30; N, 28.00.

General procedure for preparation of 5-(aminopyrazol-1-yl) pyrazolo[3,4-*d*]pyrimidine derivatives (14a–d)

To a solution of 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (**12**, 2.98 g, 10 mmol) in ethanol

(20 mL), the appropriate ethoxymethylenemalononitrile or ethyl ethoxymethylenecyanoacetate derivative (**13a–d**, 10 mmol) was added and the reaction mixture was heated under reflux for 10 h. The reaction mixture was concentrated under reduced pressure and the product was left overnight in refrigerator. The separated solid was filtered and crystallized from acetic acid to afford the corresponding pyrazole derivatives **14a–d** for which physical and spectral data are listed below.

5-Amino-1-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-1*H*-pyrazole-4-carbonitrile (14a)

66% yield; white crystals; m.p. 260–261 °C; IR (KBr disk) 3419, 3339 (forked, NH_2), 2952 (C–H aliphatic), 2214 (C≡N), 1697 (C = O), 1602 (C = N); ^{1}H NMR (DMSO-d_6) 2.58 (s, 3H, pyrimidine CH_3), 4.90 (s, 2H, CH_2O), 7.37 (s, 1H, pyrazole H-3′), 7.40–7.44 (m, 3H, phenyl H-4 & NH_2, D_2O exchangeable), 7.56–7.60 (m, 2H, phenyl H-3, H-5), 8.06 (d, $J = 7.6$ Hz, 2H, phenyl H-2, H-6), 8.34 (s, 1H, pyrazole H-3);^{13}C NMR (DMSO-d_6) δ 23.87, 44.63, 105.23, 121.54, 122.11, 124.28, 127.55, 128.79, 129.75, 136.64, 138.65 150.87, 154.80, 157.54, 160.19, 166.44; MS (*m/z*): 374 ($M^{+\cdot}$, 6.92%), 210 (100%); Anal. Calcd for $C_{18}H_{14}N_8O_2$: C, 57.75; H, 3.77; N, 29.93; Found: C, 57.60; H, 4.00; N, 29.68.

5-Amino-3-methyl-1-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-1*H*-pyrazole-4-carbonitrile (14b)

61% yield; white crystals; m.p. 250–252 °C; IR (KBr disk) 3425, 3350 (forked, NH_2), 2935 (C–H aliphatic), 2218 (C≡N), 1689 (C = O), 1601 (C = N); ^{1}H NMR (DMSO-d_6) 2.24 (s, 3H, pyrazole CH_3), 2.62 (s, 3H, pyrimidine CH_3), 5.52 (s, 2H, CH_2O), 7.41–7.45 (m, 1H, phenyl H-4), 7.57–7.61 (m, 2H, phenyl H-3, H-5), 8.02–8.07 (m,4H, phenyl H-2, H-6 and NH_2, D_2O exchangeable), 8.36 (s, 1H, pyrazole H-3); ^{13}C NMR (DMSO-d_6) δ 16.22, 24.15, 43.22, 105.12, 122.82, 124.15, 127.62, 128.82, 129.60, 136.65, 138.49, 150.81, 154.70, 157.55, 160.22, 166.45; MS (*m/z*): 388 ($M^{+\cdot}$, 93%), 77 (100%); Anal. Calcd for $C_{19}H_{16}N_8O_2$: C, 58.76; H, 4.15; N, 28.85 Found: C, 58.50; H, 4.12; N, 28.50.

Ethyl 5-amino-1-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-1*H*-pyrazole-4-carboxylate (14c)

82% yield; white crystals; m.p. 260–262 °C; IR (KBr disk) 3465, 3337 (forked, NH_2), 3108 (C–H aromatic), 2931 (C–H aliphatic), 1710 (ester C = O), 1685 (C = O), 1564 (C = N); ^{1}H NMR (DMSO-d_6) δ 1.29 (t, $J = 7.2$ Hz, 3H, $C\underline{H}_3CH_2$), 2.65 (s, 3H, pyrimidine CH_3), 4.24 (q, 2H, $J = 7.2$ Hz, $CH_2C\underline{H}_3$), 5.59 (s, 2H, CH_2O), 7.41–7.45 (3, 3H, phenyl H-4 and NH_2, D_2O exchangeable), 7.57–7.61 (m, 2H, phenyl H-3, H-5), 7.94 (s, 1H, pyrazole H-3′), 8.07 (d, $J = 8$ Hz, 2H, phenyl H-2, H-6), 8.37 (s, 1H, pyrazole H-3); ^{13}C NMR (DMSO-d_6) δ 14.85, 24.03, 47.49, 59.93, 94.15, 105.05, 122.26, 127.68, 129.77, 136.59, 138.58, 144.58, 150.92, 153.34, 157.55, 160.17, 163.23, 169.87; MS (*m/z*): 421 ($M^{+\cdot}$, 17%) 80 (100%); Anal. Calcd for $C_{20}H_{19}N_7O_4$: C, 57.00; H, 4.54; N, 23.27 Found: C, 56.62; H, 4.34; N, 23.54.

Ethyl 5-amino-3-methyl-1-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)-acetyl]-1*H*-pyrazole-4-carboxylate (14d)

White solid; Yield 62%; m.p. 264–265 °C; IR (KBr) 3443, 3333 (forked, NH_2), 3064 (CH aromatic), 2927 (CH aliphatic), 1721 (ester C = O), 1694 (C = O), 1553 (C = N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.30 (t, $J = 6.4$ Hz, 3H, $C\underline{H}_3CH_2$), 2.34 (s, 3H, pyrazole CH_3), 2.64 (s, 3H, pyrimidine CH_3), 4.25 (q, 2H, $J = 6.4$ Hz, $CH_2C\underline{H}_3$), 5.56 (s, 2H, CH_2O), 7.37 (s, 2H, NH_2, D_2O exchangeable), 7.41–7.45 (m, 1H, phenyl H-4), 7.58–7.61 (m, 2H, phenyl H-3, H-5), 8.07 (d, $J = 7.2$ Hz, 2H, phenyl H-2, H-6), 8.35 (s, 1H, pyrazole H-3); ^{13}C NMR (DMSO-d_6) δ 14.74, 15.03, 23.97, 47.43, 59.89, 92.90, 105.03, 122.31, 127.74, 129.78, 136.55, 138.50, 150.91, 153.97, 154.22, 157.62, 160.17, 163.88, 169.32; EIMS (*m/z*) 435 ($M^{+\cdot}$, 6.71%), 40 (100%). Anal.Calcd for $C_{21}H_{21}N_7O_4$: C, 57.93; H, 4.86; N, 22.52. Found: C, 57.82; H, 4.90; N, 22.48.

1-[2-(6-Methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-[1,2]diazetidin-3-one (15)

A mixture of 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (**12,** 2.98 g, 10 mmol), chloroacetyl chloride (0.8 mL, 10 mmol) and anhydrous potassium carbonate (1.38 gm, 10 mmol) in dry dimethylformamide was stirred at room temperature for 24 h. The reaction mixture was poured into ice-cold water and the separated product was filtered, dried and crystallized from benzene to give compound **15.** Physical and spectral data are listed below.

White solid; Yield 55%; m.p. 272–273 °C; IR (KBr) 3432 (NH), 3053 (CH aromatic), 2942 (CH aliphatic), 1692 (2C = O), 11599 (C = N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.60 (s, 3H, CH_3), 4.15 (s, 2H, diazetidinone CH_2), 4.91 (s, 2H, CH_2O), 7.42–7.45 (m, 1H, phenyl H-4), 7.57–7.61 (m, 2H, phenyl H-3, H-5), 8.06 (d, $J = 7.5$ Hz, 2H, phenyl H-2, H-6), 8.34 (s, 1H, pyrazole H-3), 10.51 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 23.85, 41.21, 44.64, 105.19, 122.16, 127.16, 129.76, 136.60, 138.58, 150.86, 157.60, 160.20, 165.51, 166.24; EIMS (*m/z*) 338 ($M^{+\cdot}$, 5.97%), 77 (100%). Anal.Calcd for $C_{16}H_{14}N_6O_3$: C, 58.80; H, 4.17; N, 24.84. Found: C, 58.92; H, 4.00; N, 24.50.

5-Methyl-2-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-2,4-dihydropyrazol-3-one (16)

A mixture of hydrazide (**12**, 2.98 g, 10 mmol), ethyl acetoacetate (1.30 g, 10 mmol) in ethanol (20 mL) was heated under reflux for 12 h. After cooling, the reaction mixture was poured onto ice-water, the separated solid was filtered, dried and crystallized from dioxane to afford compound **16**. Physical and spectral data are listed below.

White solid; Yield 68%; m.p. 260–261 °C; IR (KBr) 3069 (CH aromatic), 2942 (CH aliphatic), 1685, 1668 (2C = O), 1560 (C = N) cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 2.65 (s, 3H, pyrimidine CH_3), 2.71 (s, 3H, pyrazolone CH_3), 5.08 (s, 2H, pyrazolone $COCH_2$), 5.40 (s, 2H, CH_2O), 7.42–7.45 (m, 1H, phenyl H-4), 7.57–7.61 (m, 2H, phenyl H-3, H-5), 8.06 (d, $J = 7.5$ Hz, 2H, phenyl H-2, H-6), 8.36 (s, 1H, pyrazole H-3); ^{13}C NMR (DMSO-d_6) δ 23.92, 24.25, 37.16, 45.70, 105.18, 122.21, 127.66, 129.77, 136.64, 138.58, 149.85, 150.90, 157.42, 157.67, 160.15, 167.51; EIMS (*m/z*) 364 ($M^{+\cdot}$, 12%), 77 (100%). Anal.Calcd for $C_{18}H_{16}N_6O_3$: C, 59.34; H, 4.43; N, 23.06. Found: C, 59.50; H, 4.40; N, 23.35.

1-(3,5-Dimethylpyrazol-1-yl)-2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)-ethanone (17)

A mixture of hydrazide (**12,** 2.98 g, 10 mmol), and acetylacetone (10 mmol, 1 g) in acetic acid (10 mL) was heated under reflux for 6 h. After cooling, the reaction mixture was poured onto ice-water. The colorless powder obtained was crystallized from ethanol to give compound **17** for which physical and spectral data are listed below.

White crystals; Yield 55%; m.p. 189–190 °C; IR (KBr disk) 3079 (C–H aromatic), 2898 (C–H aliphatic), 1692 (C = O), 1609

(C = N); ^{1}H NMR (DMSO-d_6) δ 2.58 (s, 3H, pyrimidine CH_3), 2.73 (s, 3H, pyrazole CH_3), 2.78 (s, 3H, pyrazole CH_3), 4.88 (s, 2H, CH_2O), 6.65 (s, 1H, pyrazole H-4′), 7.40–7.43 (m, 1H, phenyl H-4), 7.56–7.60 (m, 2H, phenyl H-3, H-5), 8.06 (d, J = 7.5 Hz, 2H, phenyl H-2, H-6), 8.34 (s, 1H, pyrazole H-3);^{13}C NMR (DMSO-d_6) δ 14.12, 15.17, 23.90, 45.51, 105.10, 112.5, 114.4, 121.78, 122.24, 127.67, 129.76, 136.55, 138.52, 150.83, 157.58, 159.88, 169.82; MS (*m/z*): 362 ($M^{+\cdot}$, 8.5%) 173 (100%); Anal. Calcd for $C_{19}H_{18}N_6O_2$: C, 62.97; H, 5.01; N, 23.19; Found: C, 62.86; H, 5.00; N, 23.10.

Ethyl 4-(2-*N*′-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]hydrazineacetylamino)benzoate (19)

A mixture of 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (**12,** 2.98 g, 10 mmol), and ethyl 4-(2-chloroacetamido)benzoate (**18**, 2.41 g, 10 mmol) in absolute ethanol (30 mL) was heated under reflux for 8 h. After cooling, the formed precipitate was filtered, washed with ethanol and crystallized from ethanol to give compound **19**. Physical and spectral data are listed below.

White crystals; Yield 76%; m.p. 189–190 °C; IR (KBr disk) 3441–3352 (3NH), 3064 (C–H aromatic), 2980 (C–H aliphatic), 1720–1689 (3C = O), 1562 (CN); ^{1}H NMR (DMSO-d_6) δ 1.32 (t, J = 6.4 Hz, 3H, $C\underline{H}_3CH_2$), 2.58 (s, 3H, pyrimidine CH_3), 4.30 (q, 2H, J = 6.4 Hz, $CH_2C\underline{H}_3$), 4.76 (s, 2H, CH_2O), 5.44 (s, 2H, CH_2CO), 7.36–7.43 (m, 1H, phenyl H-4), 7.58–7.62 (m, 2H, phenyl H-3, H-5), 7.79 (s, 1H, NH, D_2O exchangeable), 7.90 (d, J = 6.8 Hz, 2H, ethyl benzoate H-3, H-5), 7.97 (d, J = 6.8 Hz, 2H, ethyl benzoate H-2, H-6), 8.08 (d, J = 7.5 Hz, 2H, phenyl H-2, H-6), 8.32 (s, 1H, pyrazole H-3), 10.60 (s, 1H, NH, D_2O exchangeable), 12.30 (s, 1H, NH, D_2O exchangeable);^{13}C NMR (DMSO-d_6) δ 14.65, 24.12, 45.72, 45.91, 61.05, 105.27, 122.11, 122.22, 127.64, 129.74, 130.64, 136.54, 138.45, 142.99, 150.92, 157.75, 160.27, 161.57, 165.80, 168.80, 169.74; MS (*m/z*): 503 ($M^{+\cdot}$, 1.32%), 192 (100%); Anal. Calcd for $C_{25}H_{25}N_7O_5$: C, 59.64; H, 5.00; N, 19.47; Found: C, 59.70; H, 5.20; N, 19.50.

Benzoic acid *N*′-[2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetyl]-hydrazide (20)

A mixture of hydrazide (**12,** 2.98 g, 10 mmol) and benzoyl chloride (1.40 g, 10 mmol) in absolute ethanol (30 mL) was heated under reflux for 6 h. After cooling, the formed precipitate was filtered and crystallized from butanol to give compound **20**. Physical and spectral data are listed below.

White crystals; Yield 86%; m.p. 238–239 °C; IR (KBr disk) 3444–3256 (2NH), 3055 (C–H aromatic), 1693 (2C = O), 1599 (C = N); ^{1}H NMR (DMSO-d_6) δ 2.66 (s, 3H, pyrimidine CH_3), 4.97 (s, 2H, CH_2O), 7.40–7.44 (m, 1H, phenyl H-4), 7.49–7.52 (m, 1H, benzoyl H-4), 7.57–7.61 (m, 4H, phenyl H-3, H-5 and benzoyl H-3, H-5), 7.87 (d, J = 6.8 Hz, 2H, benzoyl H-2, H-6), 8.07 (d, J = 7.5 Hz, 2H, phenyl H-2, H-6), 8.34 (s, 1H, pyrazole H-3), 10.45 (s, 1H, NH, D_2O exchangeable), 10.51 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 23.91, 44.69, 105.27, 122.12, 127.56, 127.90, 128.08, 128.98, 129.76, 132.42, 136.67, 138.68, 150.90, 157.58, 160.29, 166.00, 166.81; MS (*m/z*): 402 ($M^{+\cdot}$, 5.86%),77 (100%); Anal. Calcd for $C_{21}H_{18}N_6O_3$: C, 62.68; H, 4.51; N, 20.88; Found: C, 62.50; H, 4.66; N, 20.50.

N′-4-chlorophenylaminocarbonyl-2-(6-Methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (21)

A mixture of hydrazide (**12,** 2.98 g, 10 mmol) and 4-chlorophenyl isocyanate (1.53 g, 10 mmol) in dioxane (20 mL) was heated under reflux for 4 h. The obtained solid was filtered, washed with dioxane, dried and crystallized from acetic acid to give compound **21**.

White crystals; Yield 81%; m.p. 160–161 °C; IR (KBr disk) 3435–3276 (3NH), 1728, 1691 (2C = O)1593 (C = N); ^{1}H NMR (DMSO-d_6) δ 2.67 (s, 3H, pyrimidine CH_3), 5.20 (s, 2H, CH_2O), 7.32–7.42 (m, 4H, phenyl H-4 and chlorophenyl H-2, H-6 and NH, D_2O exchangeable), 7.49–7.60 (m, 4H, phenyl H-3, H-5 and chlorophenyl H-3, H-5), 8.06 (d, J = 7.5 Hz, 2H, phenyl H-2, H-6), 8.38 (s, 1H, pyrazole H-3), 10.40 (s, 1H, NH, D_2O exchangeable), 11.07 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ 24.11, 45.06, 105.14, 122.17, 123.08, 127.62, 128.52 129.26, 129.76, 136.70, 136.84, 138.61, 150.88, 152.58, 157.78, 160.33, 169.01; MS (*m/z*): 451 ($M^{+\cdot}$, 1.13%),154 (100%); Anal. Calcd for $C_{21}H_{18}ClN_7O_3$: C, 55.82; H, 4.02; N, 21.70; Found: C, 55.50; H, 4.00; N, 21.50.

Biological evaluation

COX-1/COX-2 inhibition colorimetric assay

The *in vitro* inhibition of ovine COX-1/COX-2 was measured using an enzyme immuno assay (EIA) kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions and as reported before[27].

In vivo anti-inflammatory activity

Animals

Male Wister albino rats with average body weight of 120–150 g were used in the experiments. The animals were kept under controlled environment, humidity 60 ± 10%, light period of 12 h/day and temperature 27 ± 2° C with access to food and water. The experimental procedures were carried out in compliance with the Institutional Animal Ethics Committee regulations. All experiments were performed in the morning according to the guidelines for the care of laboratory animals.

Carrageenan-induced rat paw edema

The anti-inflammatory activity of the synthesized compounds was determined *in vivo* by carrageenan-induced paw edema method in rats[28]. Rats were divided into 12 groups of 3 animals each. The first group was administered with vehicle; the second one was administrated with celecoxib (50 mg/kg), while the remaining groups were administrated with test compounds (**14a–d**–**21**, 50 mg/kg, and one group per one compound). One hour after administration of vehicle, test compounds or celecoxib, paw edema was induced by subcutaneous injection of 1% carrageenan in saline (0.05 mL/rat) into the left hind paw of each rat. Paw thickness of each rat was measured after 3 h of carrageenan injection, and then the change in thickness and the % inhibition of paw edema were calculated.

Additionally, the ED_{50} values for the most potent derivatives (**14a–d**, **16**, **17** and **21**) were calculated using at least three different doses (three rats per group for each dose) and paw thickness of each rat was measured after 3 h of carrageenan injection according to the reported procedure[27].

Ulcerogenic liability study

Ulcerogenic liability for the most biologically active synthesized compounds (**14a–d**, **16**, **17**, **21**) and celecoxib was evaluated using the previously reported procedures[29]. Thirty rats were used in this study, divided into 10 groups and fasted for 18 h before drug administration. The control group received the vehicle (2.5% Tween 80). Other groups were received test compounds or celecoxib as a reference drug at a dose of 50 mg/kg. After 2 h, animals were fed. Rats were given the required dose orally for

three successive days. After 2 h of the last dose, rats were sacrificed; the stomach of each rat was removed and then, opened along the greater curvature and rinsed with saline. In order to examine the stomach, it was stretched by pins on a corkboard. The gastric mucosa was carefully inspected for the occurrence of ulcers with the aid of an illuminated magnifying lens (l0x), and ulcer index was calculated according to the method described by Cho and Ogle[28]. Lesions were counted and measured along the greater diameter using transparent ruler. Every five hemorrhagic spots were considered equivalent to 1 mm of ulcer. The ulcer index (mm) was calculated from the sum of the total length of ulcers and hemorrhagic spots in each stomach.

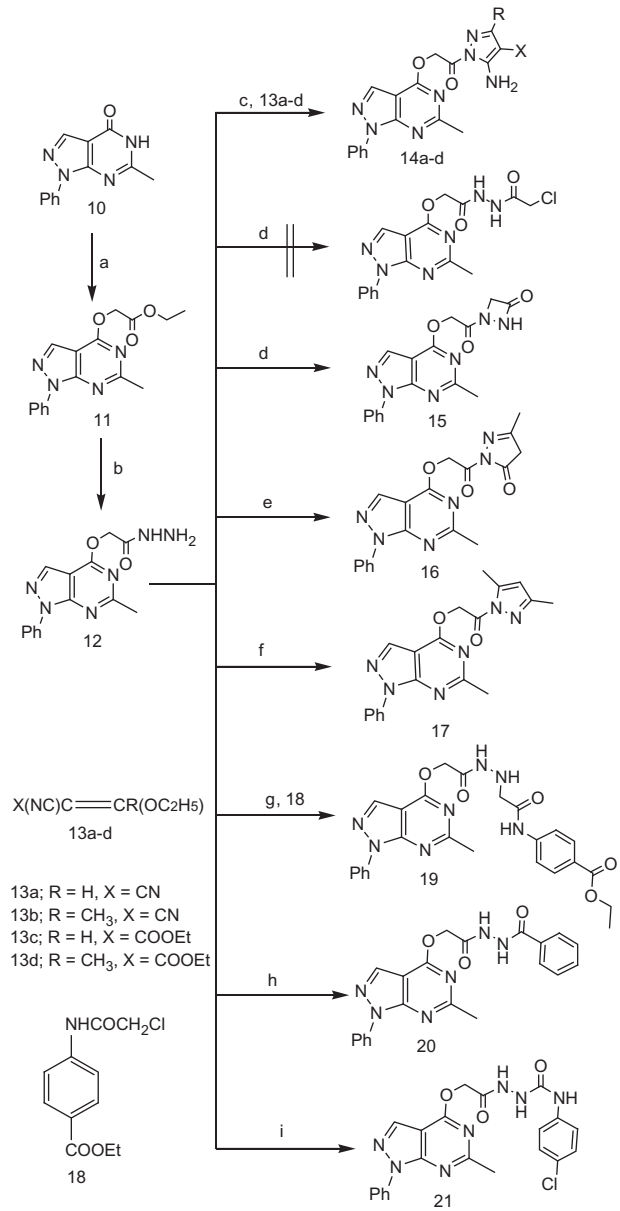
Statistical analysis

Significant difference among groups was assessed using one-way ANOVA followed by Dunnett's test. The results were expressed as mean ± standard error (SEM). Differences were considered significant at $*p > 0.05$, $**p > 0.01$ and $***p > 0.001$.

Results and discussion

Chemistry

A group of 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a–d**–**21**) were synthesized using the reaction sequence illustrated in Scheme 1. Accordingly, the reaction of 6-methyl-1-phenyl-1,5-dihydropyrazolo[3,4-*d*]pyrimidin-4-one (**10**) with ethyl chloroacetate in presence of anhydrous potassium carbonate provided the corresponding ethyl acetate ester **11,** which upon reaction with hydrazine hydrate yielded the key intermediate 2-(6-methyl-1-phenyl-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-yloxy)acetohydrazide (**12**) in 62% yield. Cyclization of the hydrazide **12** with the appropriate ethoxymethylenemalononitrile or ethyl ethoxymethylenecyanoacetate derivative (**13a–d**) gave the respective 5-aminopyrazole derivatives **14a–d** in high yields (61–82%). Reaction of hydrazide **12** with chloroacetyl chloride and anhydrous potassium carbonate in dimethylformamide yielded the cyclic diazetidin-3-one derivative **15** rather than the open chloroacetyl derivative. Formation of **15** was confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analyses. Also, mass spectrum confirmed the formation of **15** through i) presence of peak at $m/z = 338$, which corresponds to molecular ion peak of **15**, ii) absence of any peaks at $m/z = 374$ or 376, which corresponds to $M^{+\cdot}$ or M^{+2} of the open form, respectively, and iii) absence of any isotopic peaks attributed to chlorine atom of the open form. Moreover, while reacting the hydrazide **12** with ethyl acetoacetate gave the pyrazol-3-one derivative **16**, reaction of **12** with acetylacetone yielded the 4,5-dimethylpyrazole derivative **17**. Also, compound **12** was coupled with ethyl 4-(2-chloroacetamido)benzoate (**18**) to yield the corresponding ethyl benzoate ester **19**. Finally, the key intermediate **12** was reacted with benzoyl chloride and 4-chlorophenyl isocyanate to give the target 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives **20** (86% yield) and **21** (81% yield), respectively.



Scheme 1. Reagents and conditions: (a) ethyl chloroacetate, acetone, K_2CO_3, reflux, 6 h; (b) hydrazine hydrate, ethanol, reflux, 5 h; (c) $CR(OC_2H_5)=C(CN)X$, ethanol, 10 h; (d) chloroacetyl chloride, DMF, K_2CO_3, RT, overnight; (e) ethyl acetoacetate, ethanol, reflux, 12 h; (f) acetylacetone, CH_3COOH, reflux, 6 h; (g) 4-$COOC_2H_5$-C_6H_4-$NHCOCH_2Cl$, ethanol, reflux, 8 h; (h) benzoyl chloride, ethanol, reflux, 6 h; (i) 4-chlorophenylisocyanate, dioxane, reflux, 4 h.

Biological evaluation

In vitro COX inhibition assay

The potency of the synthesized target compounds **14a–d**–**21** as COX inhibitors was determined as the concentration causing 50% inhibition (IC_{50}) for ovine COX enzyme using an enzyme immunoassay (EIA) kit. Also, the COX-2 selectivity indexes (SI values) that is defined as IC_{50} (COX-1)/IC_{50} (COX-2) were calculated and compared with that of celecoxib (**4**) as a COX-2-selective reference drug. The results showed that the target compounds (**14a–d**–**21**) exhibited a broad range (moderately potent to weakly potent) of COX-1 ($IC_{50} = 3.97$–10.11 μM), and (moderately potent to highly potent) COX-2 ($IC_{50} = 0.56$–5.89 μM range, see data in Table 1), inhibitory activities. All the tested compounds had more potential in inhibiting COX-2 isozyme than COX-1 isozyme. Compounds having pyrazolyl moiety (5-aminopyrazole derivatives **14a–d**, pyrazol-3-one derivative **16** and 4,5-dimethylpyrazole derivative **17**) in a hybrid structure with the pyrazolo[3,4-*d*]pyrimidine scaffold were generally less potent inhibitors of COX-1, more potent inhibitors of COX-2 and in turn more COX-2 selective (COX-2 S.I. = 3.11–11.99) than compounds having the other moieties (**15**, **19**, **20** and **21**) (COX-2 S.I. = 1.38–5.60). The 4,5-dimethylpyrazole derivative **17** was the most potent COX-2 inhibitor ($IC_{50} = 0.56$ μM), while the 5-aminopyrazole derivative **14b** was the most COX-2 selective (S.I. = 11.99) in comparison with the reference COX-2 selective drug celecoxib (COX-2 $IC_{50} = 1.11$ μM, S.I. = 6.61).

Table 1. *In vitro* COX-1 and COX-2 inhibition of 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a–d–21**) and celecoxib.

	IC_{50} (μM)[a]		
Compound No.	COX-1	COX-2	COX-2 S.I.[b]
14a	5.22	1.23	4.24
14b	9.11	0.76	11.99
14c	4.11	1.32	3.11
14d	4.51	0.86	5.24
15	8.14	5.89	1.38
16	7.50	0.74	10.12
17	3.97	0.56	7.09
19	7.62	5.22	1.46
20	10.11	4.50	2.25
21	9.74	1.74	5.60
Celecoxib	7.34	1.11	6.61

[a]IC_{50} value represents the compound concentration that is required. to produce 50% inhibition of COX-1 or COX-2 which is the mean. value of two determinations where the deviation from the mean is. < 10% of the mean value.
[b]Selectivty index (COX-1 IC_{50}/COX-2 IC_{50}).

In vivo anti-inflammatory activity

The anti-inflammatory activity of the prepared 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a–d–21**) were evaluated using carrageenan-induced rat paw edema assay. Each compound was administered orally (50 mg/kg) immediately prior to the induction of inflammation by carrageenan subcutaneous injection. The anti-inflammatory activity was then calculated based on paw-volume changes at 3 h after carrageenan injection as presented in Table 2.

The obtained *in vivo* data was compatible with the *in vitro* results consequently, compounds having pyrazolyl ring (**14a–d**, **16** and **17**) showed higher edema inhibition percentage activities (34–68%), while the other derivatives (**15**, **19**, **20** and **21**) showed lower edema inhibition percentage activities (17–46%) in comparison with celecoxib (72%).

Moreover, the ED_{50} values for the most potent derivatives (**14a–d**, **16**, **17** and **21**) were calculated in comparison with celecoxib. The seven derivatives showed good anti-inflammatory activities (ED_{50} = 87.9–170.1 μmol/kg), especially the 5-amino-pyrazole derivative (**14c**, ED_{50} = 87.9 μmol/kg) was more potent than celecoxib (ED_{50} = 91.9 μmol/kg).

Ulcerogenic liability

The most potent anti-inflammatory compounds (**14a–d**, **16**, **17** and **21**) were subjected to ulcerogenic liability in comparison with celecoxib, low ulcerogenic reference drug, which was reported[18,22] by our group to be about seven folds less ulcerogenic than ibuprofen as traditional NSAID and the results are shown in Table 3. It was clear that the tested compounds caused variable ulceration effect (ulcer index = 0.33–4.0) comparable to that of celecoxib (ulcer index = 0.33). The pyrazol-3-one derivative (**16**) and the acetohydrazide (**21**) were the least ulcerogenic derivatives showing the same ulcerogenic potential of celecoxib. The low ulcerogenic potential of **16** and **21** (ulcer index = 0.33) could be attributed to its low potency against COX-1 isozyme (IC_{50} = 7.50, 9.74 μM, respectively) in addition to its good COX-2 selectivity indices (S.I. = 10.12, 5.60). Similarly, the relatively high ulcerogenic potential of 3,5-dimethylpyrazole derivatives **14c**, **14d** (ulcer index = 3.0, 4.0 respectively) was attributed to their considerable potency against COX-1 isozyme (IC_{50} = 4.11 and 4.51 μM, respectively) in addition to their relatively low COX-2 selectivity indices (S.I. = 3.11, 5.24).

Table 2. Anti-inflammatory activities for 50 mg/kg dose of 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a–d–21**), (ED_{50}, μmol/kg) of most potent derivatives (**14a–d**, **16**, **17** and **21**) and celecoxib.

Compound	Increase in paw volume following carrageenan administration and % of anti-inflammatory activity (AI)[a]	ED_{50} (μmol/kg)[b]
14a	$0.29 \pm 1.40^{***}$(48%)	139
14b	$0.09 \pm 1.45^{***}$(46%)	139.2
14c	$0.12 \pm 0.87^{***}$(68%)	87.9
14d	$0.24 \pm 1.77^{*}$(34%)	170.1
15	$0.28 \pm 2.08^{*}$(22%)	ND[c]
16	$0.18 \pm 1.33^{***}$(50%)	137.4
17	$0.14 \pm 1.38^{***}$(48%)	143.6
19	$0.21 \pm 1.90^{*}$(29%)	ND[c]
20	$0.09 \pm 2.23^{*}$(17%)	ND[c]
21	$0.09 \pm 1.45^{***}$(46%)	119.9
Celecoxib	$0.09 \pm 0.75^{***}$(72%)	91.9

Values represent mean ± SEM ($n = 3$), Significance levels
$^{*}p > 0.05$,
$^{**}p > 0.01$ and
$^{***}p > 0.001$.as compared to the control group.
[a]Inhibitory activity in a carrageenan-induced rat paw edema assay using a dose of 50 mg/kg at 3 h after oral administration of the test compound.
[b]The ED_{50} value (μmol/kg) at 3 h after oral administration of the test compound was calculated using three different doses.
[c]Not determined.

Table 3. Ulcerogenic effect for most potent 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives (**14a–d**, **16**, **17** and **21**) and celecoxib.

Compound	Ulcer index	Relative ulcerogenicity to Celecoxib
14a	2.33	7.06
14b	1.67	5.06
14c	3.00	9.09
14d	4.00	12.12
16	0.33	1
17	1.00	3.03
21	0.33	1
Celecoxib	0.33	1

Conclusion

A new series of 1-phenylpyrazolo[3,4-*d*]pyrimidine derivatives **14a–d–21** were synthesized for its evaluation as COX inhibitors, anti-inflammatory agents and ulcerogenic liability. Structure-activity data acquired and biological studies showed that (i) all compounds were more potential in inhibiting COX-2 than COX-1, (ii) Compounds having pyrazolyl moiety in a hybrid structure with the pyrazolo[3,4-*d*]pyrimidine scaffold **14a–d**, **16**, **17** were generally more potent inhibitors of COX-2 and in turn more COX-2 selective than compounds having the other moieties, (iii) Compounds having pyrazolyl moiety **14a–d**, **16**, **17** in addition to the acetohydrazide derivative **21** showed good anti-inflammatory activity, especially **14c**, which was more potent than celecoxib and compound **21** that had approximately 77% of celecoxib potency, (iv) both the compounds **16** and **21** showed the same ulceration effect of the low ulcerogenic reference drug (celecoxib) and (v) coupling of the pyrazolyl moiety with pyrazolo[3,4-*d*]pyrimidine scaffold in one hybrid structure offers a potential drug design concept for the development of NSAIDs that have good anti-inflammatory activity and low adverse ulcerogenic side effects.

Declaration of interest

The authors have declared no conflict of interest.

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