Synthesis and pharmacological evaluation of pyrazolopyrimidopyrimidine derivatives: anti-inflammatory agents with gastroprotective effect in rats

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Received: 17 April 2013 / Accepted: 17 August 2013 / Published online: 4 September 2013
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Abstract We report the synthesis of new anti-inflammatory 1,7-dihydropyrazolo[3',4':4,5]pyrimido[1,6-a]pyrimidine 5 from aminocyanopyrazole. All compounds were characterized by physical, chemical and spectral studies. Preliminary pharmacological evaluation of the resulting products showed that compounds 5a, b, f (50–100 mg/kg, i.p) are active anti-inflammatory agents in carrageenan-induced rat paw oedema assay, and their effects are comparable to that of acetylsalicylic–lysine (300 mg/kg, i.p.), used as a reference drug. The nature of substituent (Y, R3) had a pronounced effect on the anti-inflammatory activity. Studies of structure–activity relationships have led to selection of compound ethyl-3,5-dimethyl-7-imino-N1-phenyl-1,7-dihydropyrazolo[3',4':4,5]pyrimido[1,6-a]pyrimidine-6-carboxylate, 5f which exhibited the most potent anti-inflammatory activity. In addition, the compounds 5a, b, f showed a significant gastroprotective effect against HCl/EtOH-induced gastric ulcer.

Keywords Aminocyanopyrazole · Anti-inflammatory · Gastroprotective · Pyrazolo[3,4-d]pyrimidine · Dihydropyrazolo[3',4':4,5]pyrimido[1,6-a]pyrimidine

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are most widely used to treat variety of acute and chronic inflammatory diseases. Such drugs are being increasingly used for the treatment of postoperative pain (Moote, 1992) with or without supplemental opioid agents. The pharmacological action of these agents was assigned to inhibit two enzymes, known as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Vane et al., 1998). The constitutive isofrom COX-1 is present in most tissues and is involved in the synthesis of prostaglandins vital to normal cell function. In contrast, the inducible isofrom COX-2 appears to be produced primarily in response to growth factors or inflammatory mediators, such as cytokines (Vane and Botting, 1996). Many of the currently available NSAIDs, including indomethacin and piroxicam, are more potent inhibitors of COX-1 than that of COX-2 (Vane and Botting, 1995). This preferential inhibition of COX-1 may be responsible for many of the adverse effects associated with NSAIDs. It has been postulated that NSAIDs which preferentially inhibit COX-2, such as meloxicam (Lipscomb et al., 1998), celecoxib (Simon et al., 1998) and several experimental drugs including NS 398, L-745,337 and DFP, should produce the same or better anti-inflammatory effects with less gastrointestinal, haematological and renal toxicities than classical NSAIDs (Winter et al., 1962). Pyrazolopyrimidines are a class of sedative and anxiolytic drugs such as Zaleplon known by its hypnotic effect (Weitzel et al., 2000). However, pyrazolopyrimidine derivatives become a new chemical resource for searching of novel bioactive compounds in drug development.

On this basis, we directed our attention to the synthesis of novel 1,7-dihydropyrazolo[3',4':4,5]pyrimido[1,6-a]pyrimidines 5a–i related to aminocyanopyrazole with the
aim of improving their anti-inflammatory activity and reducing their ulcerogenic properties as it appeared to be plausible that variation of the active compound structures could exert a pronounced influence on activity, as the case with 5b, f.

Materials and methods

Chemistry

Phenyl hydrazine, malononitrile, triethylorthoester and ammoniac were purchased from Sigma Chemical (Berlin, Germany). Analytical grade solvents (ethanol, HCl, ethyl acetate, chloroform) were obtained from Merck. Melting points (mp) were determined on a Buchi capillary apparatus and were uncorrected. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 300 spectrometer (1H at 300 MHz and 13C at 75 MHz) with deuterio-dimethylsulphoxide (d-DMSO) as solvent and tetramethylsilane as internal standard reference. Infra-red (IR) spectra were recorded on a Bio-rad FTS-6000 spectrometer. Solvents used in reactions were dried and distilled before use. The purity of all synthesized compounds was controlled by thin layer chromatography (TLC; Merck silica gel plates 60F-254). High resolution masses were recorded before use. The purity of all synthesized compounds was controlled by thin layer chromatography (TLC; Merck silica gel plates 60F-254). High resolution masses were recorded on a spectrometer JEOL JMS-Gmante II is composed of a GC/MS system from compounds dissolved in dichloromethane.

Synthesis and spectral data of compounds 2–5

5-Amino-4-cyano-N1-phenyl pyrazoles (2) 5-Amino-4-cyano-1-N1-phenyl pyrazoles prepared via a standard addition of hydrazine derivatives to ketene ethoxymethylene compounds following the reported procedure. Recrystallization from ethanol afforded pure 2 in good yields.

4-Cyano-N1-phenyl pyrazolo[5,1-c][1,2,4]triazole-5-imidates (3) The required 5-amino-4-cyano-N1-phenyl pyrazole (1.0 mmol) was treated with triethylorthoester 6.0 mmol) and a catalytic amount of acetic acid and the mixture was refluxed for 24 h. After cooling, the reaction mixture was evaporated. The product was filtered, washed with diethyl ether then purified by recrystallisation (ethanol) (Gupta et al., 2008; Allouche et al., 2013).

4-Amino-N1-phenyl pyrazolo[3,4-d]pyrimidine (4) A solution of imidate 3 (1.0 mmol) in dry ethanol (5 ml) was treated with ammoniac (2.0 mmol) and a catalytic amount of acetic acid. The reaction mixture was refluxed for 6 h, and the formed solid was collected by filtration, dried and recrystallized from ethanol to give compound 4.

7-Imino-N1-phenyl-1,7-dihydropyrazolo[3′,4′:4,5]pyrimido [1,6-alpyrimidine 5a–e A mixture of compound 4 (1.0 mmol), ketene ethoxymethylene compounds 1 or ethyl-2-cyano-3-ethoxyalkyl-2-enoate (1.0 mmol) and a catalytic amount of acetic acid was refluxed for 2 h in 10 ml ethanol. The formed precipitate was filtered, washed with diethyl ether, dried and recrystallized from ethanol to give compound 5 in good yield.

7-Imino-N1-phenyl-1,7-dihydropyrazolo[3′,4′:4,5]pyrimido [1,6-alpyrimidine 5a–e A mixture of compound 4 (1.0 mmol), ketene ethoxymethylene compounds 1 or ethyl-2-cyano-3-ethoxyalkyl-2-enoate (1.0 mmol) and a catalytic amount of acetic acid was refluxed for 2 h in 10 ml ethanol. The formed precipitate was filtered, washed with diethyl ether, dried and recrystallized from ethanol to give compound 5 in good yield.
8.38 (1H, t, J = 7.3 Hz, ArH2); 7.51 (2H, t, J = 7.3 Hz, ArH4); 9.57 (1H, s, H9); 11.96 (1H, s, NH).
HRMS Calcd. for C15H15N3O2: 287.0976, found: 287.0919.

e) 6-Cyano-7-imino-5-ethyl-N'-phenyl-1, 7-dihydropyrazolo[3',4':5]pyrimido[1,6-alpyrimidine 5e Yield 70 %; mp 168 °C; IR (cm⁻¹); νN-H 3319, νC=O 1747, 1740; RMN 1H (δ ppm, DMSO) 1.26 (3H, t, J = 7.1 Hz, CH3); 2.63 (3H, s, CH3); 4.03 (2H, q, J = 7.1 Hz, CH2); 2.63 (3H, s, CH3); 4.03 (2H, q, J = 7.1 Hz, CH2); 7.49 (1H, t, J = 7.1 Hz, ArH3); 7.59 (1H, s, ArH4); 8.31 (1H, s, ArH5); 8.37 (1H, s, NH); RMN 13C (δ ppm, DMSO): 14.32 (CH3); 89.64 (C-6) 103.64 (C-3a); 111.83 (CN); C arom 120.38 (C-2' and C-6'), 126.65 (C-4'), 138.42 (C-3' and C-5'), 140.12 (C-1'), 143.42 (C-10a), 141.69 (C-3'), 148.47 (C-5'), 162.00 (C-7), C16H13N3O2: 301.105; HRMS Calcd. for C16H13N3O2: 310.1076. found: 301.1084.

b) 6-Cyano-7-Imino-3,5-Dimethyl-N'-phenyl-1, 7-dihydropyrazolo[3',4':5]pyrimido[1,6-alpyrimidine 5b Yield 54 %; mp 182 °C; IR (cm⁻¹); νN-H 3324, νC=O 1747; RMN 1H (δ ppm, DMSO): 2.50 (3H, s, CH3); 2.64 (3H, s, CH3); 7.26 (1H, t, J = 7.3 Hz, ArH2); 7.51 (2H, t, J = 7.3 Hz, ArH3 and ArH4); 7.54 (2H, d, J = 7.3 Hz, ArH2 and ArH3); 8.27 (1H, s, NH); RMN 13C (δ ppm, DMSO): 13.01 (CH3); 24.45 (CH3); 66.03 (CH3); 105.28 (C-3); 115.10 (C-3a); 121.07 (C-2' and C-6'), 125.50 (C-4'), 129.12 (C-3' and C-5'), 138.88 (C-1'), 142.79 (C-10a), 146.88 (C-3), 148.30 (C-5), 154.14 (C-9), 156.21 (C-4a), 156.48 (C-7), 164.27 (C-7); HRMS Calcd. for C16H13N3O2: 362.149, found: 362.1478.

c) 6-Cyano-7-imino-9-Methyl-N'-phenyl-1,7-dihydropyrazolo[3',4':5]pyrimido[1,6-alpyrimidine 5c Yield 71 %; mp 166 °C; IR (cm⁻¹); νN-H 3321, νC=O 1747; RMN 1H (δ ppm, DMSO): 2.62 (3H, s, CH3); 7.40 (1H, t, J = 7.3 Hz, ArH4); 7.49 (2H, t, J = 7.3 Hz, ArH3 and ArH4); 7.68 (2H, d, J = 7.3 Hz, ArH2 and ArH3); 8.19 (1H, s, ArH4); 8.41 (1H, s, ArH3); 8.73 (1H, s, NH); RMN 13C (δ ppm, DMSO): 14.32 (CH3); 89.64 (C-6) 103.64 (C-3a); 111.83 (CN); C arom 120.38 (C-2' and C-6'), 126.65 (C-4'), 138.42 (C-3' and C-5'), 140.12 (C-1'), 143.42 (C-10a), 141.69 (C-3'), 148.47 (C-5'), 162.00 (C-7), C16H13N3O2: 301.105; HRMS Calcd. for C16H13N3O2: 310.1076. found: 301.1084.
and C-5'), 134.35 (C-1'), 138.10 (C-10a), 148.14 (C-3), 151.37 (C-5), 153.53 (C-9), 154.00 (C-4a), 155.18 (C-7), 163.36 (CO). 120.62-126.73-129.20-134.35, C_17H_14N_6O_2: 334.1171; HRMS Calcd. for: C_17H_14N_6O_2: 334.1178; found: 334.1171.

The anti-inflammatory activity of compounds (5b, 5f, 5g) on carrageenan-induced rat paw oedema was determined according to Winter et al. (1962). The animals were divided into three groups of six rats each. The control group received intraperitoneally 2.5 ml/kg of vehicle solution (Twee 80/Absolute ethanol/Saline solution (0.9 %): 1/1/18). The reference group received acetylsalicylic–lysine (300 mg/kg i.p.), and the test groups received compounds 5a, b, f, g (50 and 100 mg/kg, i.p.). After 30 min, 0.05 ml of 1 % carrageenan suspension was injected into the left hind paw. The paw volume up to the tibiotarsal articulation was measured using a plethysmometer (model 7150, UgoBasile, Italy) at 0 h (V_0) (before carrageenan injection) and 1, 3 and 5 h later (V_T) (after carrageenan injection). Paw swelling was determined for each rat and the difference between V_T and V_0 was taken as the oedema value. The percent inhibition was calculated according to the following formula:

\[
\% \text{Inhibition} = \left(\frac{V_T - V_0}{V_T - V_0}\right) \times 100
\]

**Gastroprotective activity**

The gastroprotective activity of pyrazolopyrimidopyrimidines 5a, b, f, g was studied in 150 mM HCl/EtOH-induced gastric ulcer (Hara and Okabe, 1985). Rats were fasted for 24 h prior receiving any treatment and were divided into six groups of six animals each. Group I was kept as control group and received the vehicle (Twee 80/Absolute ethanol/Saline solution (0.9 %): 1/1/18). Group II and III received compound 5a (50, 100 mg/kg, i.p.), respectively, and Group IV and V received compound 5b (50, 100 mg/kg, i.p.), respectively. Group VI and VII received compound 5f (50, 100 mg/kg, i.p.), respectively, and group VIII and IX received compound 5g (50, 100 mg/kg, i.p.), respectively. Group X received cimetidine (100 mg/kg, i.p.) as reference drug. After 30 min, all groups were orally treated with 1 ml/100 g of 150 mM HCl/EtOH (40:60, v/v) solution for gastric ulcer induction. Animals were sacrificed 1 h after the administration of ulcerogenic agent; their stomachs were excised and opened along the great curvature, washed and stretched on cork plates. The surface was examined for the presence of lesions and the extent of the lesions was measured. The summative length of the lesions along the stomach was recorded (mm) as lesion index.

**Statistics**

Results are expressed as the mean ± SEM of six animals per group. The data were analysed using Student’s t test, \(*p < 0.05, **p < 0.01 and ***p < 0.001\) was considered significant.

**Results and discussion**

**Chemistry**

The synthetic routes to target compounds 5a-i are outlined in Scheme 1. The 5-amino-4-cyano-N^1-phenylpyrazole 2,
used as a starting material, was prepared in two steps fol-
lowing a similar method reported by Petrie et al. (1985),
Anderson et al., (1990), Aggarwal et al., (2011). The first
step involves acid-catalysed condensation of orthoester with
malonate to form ethoxymethylene malononitrile 1. Thi
later reacts then with substituted hydrazine to give the am-
inocyanopyrazole 2. Treatment of 2 with orthoester in the
presence of catalytic amount of acid furnished the corre-
sponding cyano-pyrazoloimidates 3 which subsequently
were transformed to the corresponding amino pyrazolopyr-
imidines 4 (Booth et al., 1999; Gupta et al., 2008; Oliveira-
Campos et al., 2007; Bakavoli et al., 2010) upon treatment
with ammoniac. Reaction of compound 4 with ketene eth-
oxymethylene compounds 1 in ethanol in presence of cata-
lytic amount of acid furnished the desired 6-cyano-1,7-
dihydropyrazolo[3′,4′:4,5]pyrimido[1,6-a]pyrimidine 5a–e
in 70 % yield as a yellow solid. The same procedure gave a
crystalline ethyl-1,7-dihydro pyrazolo [3′,4′:4,5]pyrimido
[1,6-a]pyrimidine-6-carboxylate 5f–i from ethyl-2-cyano-3-
ethoxyalkyl-2-enoate in 80 % yield. Scheme 1 shows the
synthetic strategy to obtain the target compounds by the
four-steps method, yielding the compounds with structure
5a–i listed in Table 1.

It is interesting to note that time reaction and yield of
products are directly related to the nature of substituent
(R3 and Y). The yields of compounds 5h and 5d are 89
and 77 %, respectively. Hydrogen substituent R3 gave
superior yields in short time. In all cases, reaction leads
to pyrazolo pyrimido pyrimidine only when R1 or R2 is a
hydrogen atom. However, steric effect decreased yields
of the reaction, as in the case of 5g, and may even
prevent the progress of the reaction when R2 and R3 are
methyl groups. Analysis of the NMR and IR spectra
indicated that compounds 5f–i has ester functional group
in their structures so ethoxymethylene cyanoacetate reacts
with pyrazolopyrimidine and in both cases Y is CN or
CO2Et, nitrogen attacked on the nitrile function as the
first attack.

### Table 1: Synthesis of 7-imino-N1-phenyl-1,7-dihydro
pyrazolo[3′,4′:4,5]pyrimido [1,6-a]pyrimidine 5a–i

| Compounds | R1 | R2 | R3 | Y    | Yields (%) | Reaction time (h) |
|-----------|----|----|----|------|-----------|------------------|
| 5a        | CH3| H  | H  | CN   | 68        | 24               |
| 5b        | CH3| H  | CH3| CN   | 54        | 71               |
| 5c        | H  | CH3| H  | CN   | 71        | 24               |
| 5d        | H  | H  | H  | CN   | 77        | 5                |
| 5e        | H  | H  | H  | CH3  | 70        | 48               |
| 5f        | CH3| H  | CH3| CO2Et| 71        | 75               |
| 5g        | CH3| H  | CH3| CO2Et| 69        | 84               |
| 5h        | H  | H  | H  | CO2Et| 89        | 7                |
| 5i        | H  | H  | CH3| CO2Et| 78        | 24               |
Biological activity

Anti-inflammatory and gastroprotective activities of compounds 5a, b, f, g

The pyrazolopyrimidine derivatives are a well-known class of NSAIDs with several products in market (Russo et al., 1992; El-Kateb et al., 2012) (Figs. 1, 2).

The structure–activity relationships (SAR) for these compounds have been extensively explored for optimization of anti-inflammatory activity last three decades, since this class was introduced (Lombardino and Wiseman, 1972; Farré et al., 1986; Berq et al., 1999; Lee et al., 1999).

In continual efforts to find potentially safer and more efficacious parent agents through further exploration of SAR of this class, we decided to study the pharmacological profiles of compounds 5a, b, f, g belonging to pyrazolopyrimidopyrimidine family. We examined the effect of modification of the electronic nature of substituents on various portions of type NSAIDs. For this objective the hydrogen atom (position 5) is replaced by methyl or ethyl group, even and for more important anti-inflammatory activity, the cyano function is replaced by ester function.

Table 2 reveals the results of the intraperitoneal administration of the compounds 5a, b, f, g in carrageenan-induced rat paw oedema. The compounds 5a, b, f, g tested at 50 and 100 mg/kg, i.p. produced a significant reduction of the oedema throughout the entire period of observation in a dose-dependent manner. The highest reduction of the oedema was at 3 h after carrageen injection with a percent inhibition ranged, from 40.64 to 56.81 % for compound 5a, from 58.98 to 71.36 % for compound 5b, from 60.02 to 82.83 % for compound 5f and from 28.75 to 42.87 % for compound 5g, whereas the reference drug (acetylsalicylic–lysine, 300 mg/kg, i.p.) produced 48.03 % reduction in paw volume. The influence of the substituent R2 on activity is remarkable. Compound 5a is less potent than the 5-methyl derivatives 5b, so a methyl group linked to the pyrimidine cycle increases the activity compared to the case of a hydrogen atom. At the same dose (100 mg/kg), compound 5b produced 71.36 % inhibition of oedema against 56.81 % for 5a. In addition, the compound 5f is
more potent than the ethyl derivatives 5g, so an ethyl group linked to the pyrimidine cycle decreases the activity compared to the methyl group.

On the other hand, mucosal erosion and ulceration are produced by most NSAIDs with varying degrees. Inhibition of synthesis of gastroprotective prostaglandins (PGE2) is clearly involved (Nezamis et al., 1967) and due to the inhibition of the constitutive isoform COX-1 (Main and Whittle, 1973; Cryer and Feldman, 1992). Thus, deficiency of PGs reduces the mucosal secretions along with hydrogen carbonate that ultimately aggravates the lethal effects of acid on the stomach lining leading to mucosal damage (Fig. 3).

The results of gastroprotective activity of compounds 5a, b, f, g on gastric ulcer induced by HCl/ethanol solution are shown in Table 3. Oral administration of the ulcerogenic agent to the control group clearly showed a mucosal damage characterized by multiple haemorrhage red bands of different sizes along the long axis of the glandular stomach as described in other studies (Shay et al., 1945; Yassir et al., 1999). When we compared the gastroprotective activity of compounds 5a, b, f, g we observed that

### Table 2

| Sample                              | Dose (mg/kg) | Oedema (10⁻² ml) (mean ± SEM) | Oedema inhibition (%) |
|-------------------------------------|--------------|------------------------------|-----------------------|
|                                     |              | 1 h      | 3 h        | 5 h        | 1 h      | 3 h        | 5 h        |
| Vehicle (2.5 ml/kg)                 | –            | 35.87 ± 4.48 | 50.66 ± 3.68 | 56.04 ± 2.91 | –        | –          | –          |
| Acetylsalicylic–lysine (reference drug) | 300          | 13.23 ± 2.69** | 26.32 ± 2.44** | 29.15 ± 2.87** | 63.10      | 48.03      | 47.98      |
| 5a                                  | 50           | 20.59 ± 2.51* | 30.07 ± 3.51* | 33.73 ± 4.16* | 42.59      | 40.64      | 39.8       |
|                                     | 100          | 7.01 ± 3.41** | 21.88 ± 1.89** | 23.45 ± 2.5**  | 80.44      | 56.81      | 58.15      |
| 5b                                  | 50           | 14.62 ± 3.21* | 20.78 ± 2*    | 23.56 ± 2*    | 59.25      | 58.98      | 57.95      |
|                                     | 100          | 2.81 ± 2.06*** | 14.51 ± 2.98*** | 20.86 ± 2.21*** | 92.17      | 71.36      | 62.76      |
| 5f                                  | 50           | 13.51 ± 3.4** | 20.25 ± 2.8** | 22.74 ± 3.2** | 62.31      | 60.02      | 59.42      |
|                                     | 100          | 2.07 ± 2.8*** | 8.69 ± 2.3*** | 17.45 ± 2.5*** | 94.22      | 82.83      | 68.85      |
| 5g                                  | 50           | 24.37 ± 2.7* | 36.09 ± 2.9* | 41.95 ± 2.8 | 32.04      | 28.75      | 25.13      |
|                                     | 100          | 12.31 ± 3.2** | 28.94 ± 2.4* | 33.52 ± 2.3 | 65.66      | 42.87      | 40.18      |

The values represent the mean difference of volume of paw ± SEM (n = 6)

* p < 0.05, ** p < 0.01, *** p < 0.001 significantly different from control group

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### Table 3

| Treatment                  | Dose (mg/kg) | Ulcer index (mm) | Inhibition (%) |
|----------------------------|--------------|------------------|----------------|
| Vehicle (2.5 ml/kg) (control) | –            | 85 ± 2.82        | –              |
| Compounds                  |              |                  |                |
| 5a                         | 50           | 43.66 ± 2.58     | 48.63          |
|                            | 100          | 30 ± 3.03*       | 64.7           |
| 5b                         | 50           | 26.83 ± 3.43***  | 68.43          |
|                            | 100          | 11.83 ± 0.75***  | 86.08          |
| 5f                         | 50           | 23.34 ± 2.9**    | 72.53          |
|                            | 100          | 7.29 ± 0.3***    | 91.42          |
| 5g                         | 50           | 50.81 ± 3.2      | 40.22          |
|                            | 100          | 40.65 ± 2.8      | 52.17          |
| Cimétidine (reference drug) | 100          | 22.07 ± 2.12**   | 74.03          |

Data expressed as mean ± SEM (n = 6)

* p < 0.05, ** p < 0.01, *** p < 0.001 significantly different from control group
pyrazolopyrimidopyrimidine 5b (100 mg/kg) demonstrated the higher significant inhibition of gastric lesion (91, 42 %).

In conclusion, we have synthesized a new series of 1,7-dihydropyrazolo [3′,4′:4,5]pyrimido [1,6-α]pyrimidine 5a-i derivatives. The yield of the reaction seems to be significantly influenced by the nature of substituent. The highest yield is obtained for more hydrogen atom substituent. However, test (or experimental) compounds 5a, b, f showed that the methyl group increases the anti-inflammatory activity, contrary to ethyl group which decreases this activity. The same interpretation is found with gastroprotective effect. Indeed, our results on the gastroprotective effects of compounds 5a, b, f compared with cimetidine indicate that replacement of hydrogen by methyl reduces the gastrointestinal adverse effects.

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