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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and evaluation of anti-inflammatory, analgesic, ulcerogenicity and nitric oxide-releasing studies of novel ibuprofen analogs as nonulcerogenic derivatives

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Abstract Since the last 41 years, Ibuprofen has been one of the most widely used Non-Steroidal Anti-Inflammatory Drug (NSAID) due to its anti-inflammatory actions. As all the NSAIDs are suffering from the deadlier GI toxicities, Ibuprofen also is no exception to these toxicities. The free –COOH group is thought to be responsible for the Gastrointestinal (GI) tract toxicity associated with all the traditional NSAIDs. Therefore, the main aim of this study was to develop new chemical entities as potential anti-inflammatory agents with less GI toxicities. In this article, synthesis of a series of Hybrid molecules containing important pharmacophore of Ibuprofen and substituted diaryl rings on 5-membered heterocycle similar to coxibs and Nitric oxide-releasing moiety are described. All the synthesized compounds were tested in vivo for their anti-inflammatory, analgesic, ulcerogenic properties, and histopathological studies and in vitro for their nitric oxide-releasing properties. Out of the six synthesized compounds, four compounds showed significant anti-inflammatory and analgesic activity which was compared with standard. All the synthesized compounds exhibited significant nitric oxide-releasing and reduced GI ulcerogenic activity.

Keywords Ibuprofen · Nitric oxide · Anti-inflammatory · Analgesic · Ulcerogenicity · Vasodilator

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Introduction

Non-steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most commonly prescribed drugs to reduce pain, inflammation, and fever. These drugs reduce pain and edema by suppressing the formation of prostaglandins, by inhibiting the activity of the enzyme Cyclooxygenase (COX-1 and COX-2). Selective COX-2 inhibitors elicit less or no GI damage and bleeding compared with conventional NSAIDs, although the magnitude of this reduction continues to be debated in the literature (Szabó *et al*., 2008; Biava *et al*., 2010). As widely reported in the laypress, the selective COX-2 inhibitors also cause significant adverse effects in the renal and cardiovascular systems, possibly more serious than those caused by conventional NSAIDs. However, their use is limited by their significant side effects upon the stomach and the kidney. Their side effects as well as therapeutic actions are related to their ability to inhibit cyclooxygenase enzymes involved in the first step of the arachidonic acid cascade (Insel, 1996; Wolfe *et al*., 1999).

Recent strategies adopted to minimize the side effects of NSAIDs include the use of the dual LOX/COX inhibitors, the use of selective COX-2 inhibitors, and the use of hybrid molecules made up of non-selective or selective COX inhibitors together with a nitric oxide-releasing functional group (Bias *et al*., 2004; Doggrell, 2005; Velazquez *et al*., 2005).

Recent data revealed serious cardiovascular side effects associated with selective COX-2 inhibitors (Bias *et al*., 2004; Dogne *et al*., 2005). In addition, such drugs only minimize the development of new gastric ulcers but do not cure the existing ones (Hunt *et al*., 2002). The strategy involving the use of hybrid molecules made up of non-selective COX inhibitors together with a nitric oxide-releasing moiety,

constitutes one of the most promising approaches, because nitric oxide supports several endogenous GI defense mechanisms, including increase in mucus, bicarbonate secretions, increase in mucosal blood flow and inhibition of the activation of proinflammatory cells (Wey *et al*., 2007; Muscará and Wallace, 2006; Chegaev *et al*., 2007). Moreover, because of the beneficial cardiovascular effects (vasodilation) of Nitric Oxide, such drugs are expected to be devoid of the cardiovascular adverse effects associated with the use of selective COX-2 inhibitors (Doggrell, 2005; Muscará and Wallace, 2006).

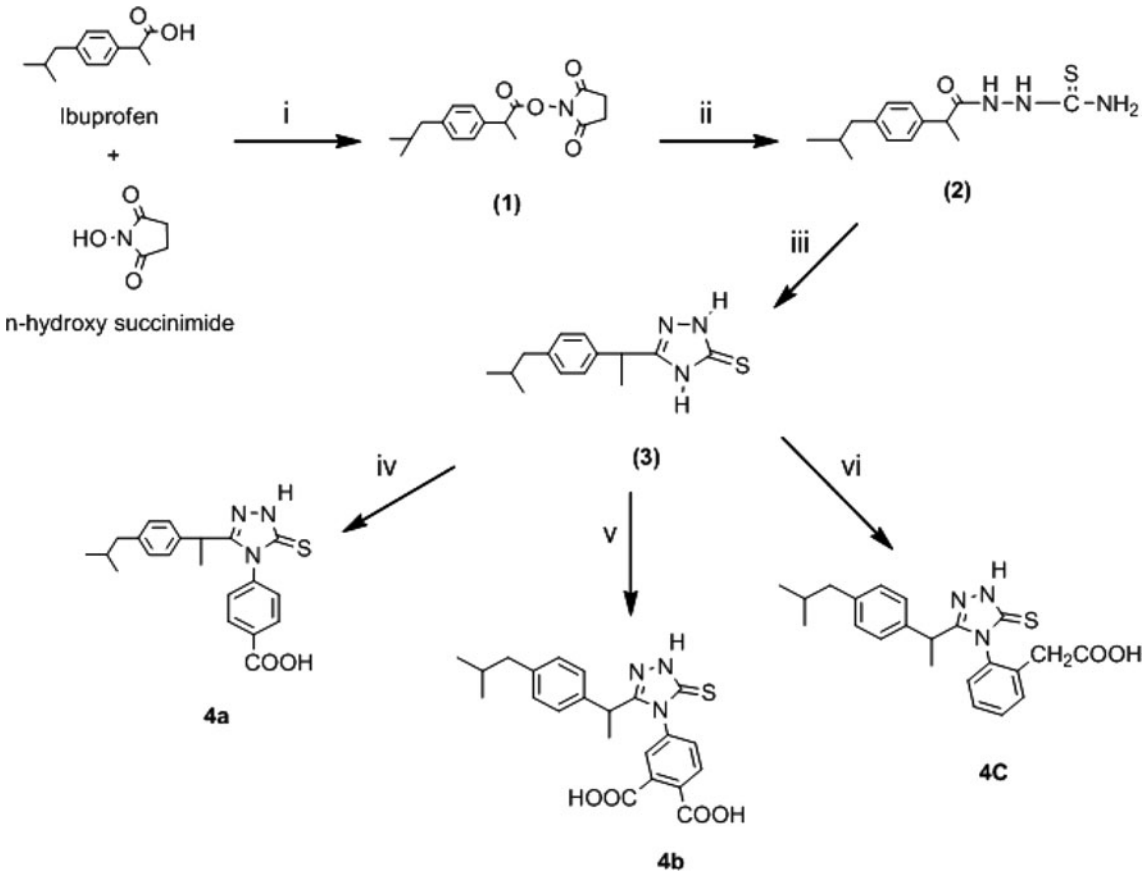
Among those nitric oxide-NSAIDs that came into clinical trials are Nitroaspirin, Nitronaproxene, Nitroketoprofen, Nitroibuprofen, etc. Among the nitric oxide donors adopted to prove the validity of this principle are furoxans, oximes, hydrazides, and organic nitrates. (Mann, 2003; Keeble and Moore, 2002; Rehse and Brehme, 2000).

Synthetic approaches based on chemical modification of NSAIDs have been taken with the aim of improving safety profile and in turn therapeutic window of the resultant NSAIDs. Our previous studies had described the synthesis of hybrid molecules with nitric oxide-releasing group that resulted in an increased anti-inflammatory activity with reduced GI-ulcerogenicity (Bhandari *et al*., 2010; Bhandari *et al*., 2009). In our attempt to continue to discover new, safer, and potent agents for the treatment of inflammatory diseases, we have synthesized hybrid compounds containing pharmacophore of Ibuprofen, 1,2-diaryl triazole ring, the pharmacophore similar to coxibs and nitric oxide-releasing group to accentuate potency and reduce GI toxicities associated with the traditional NSAIDs. The compounds designed so were found to possess much significant analgesic, anti-inflammatory, vasodilatory profile with significant reduction in ulcerogenic toxicities.

Results and discussion

Chemistry

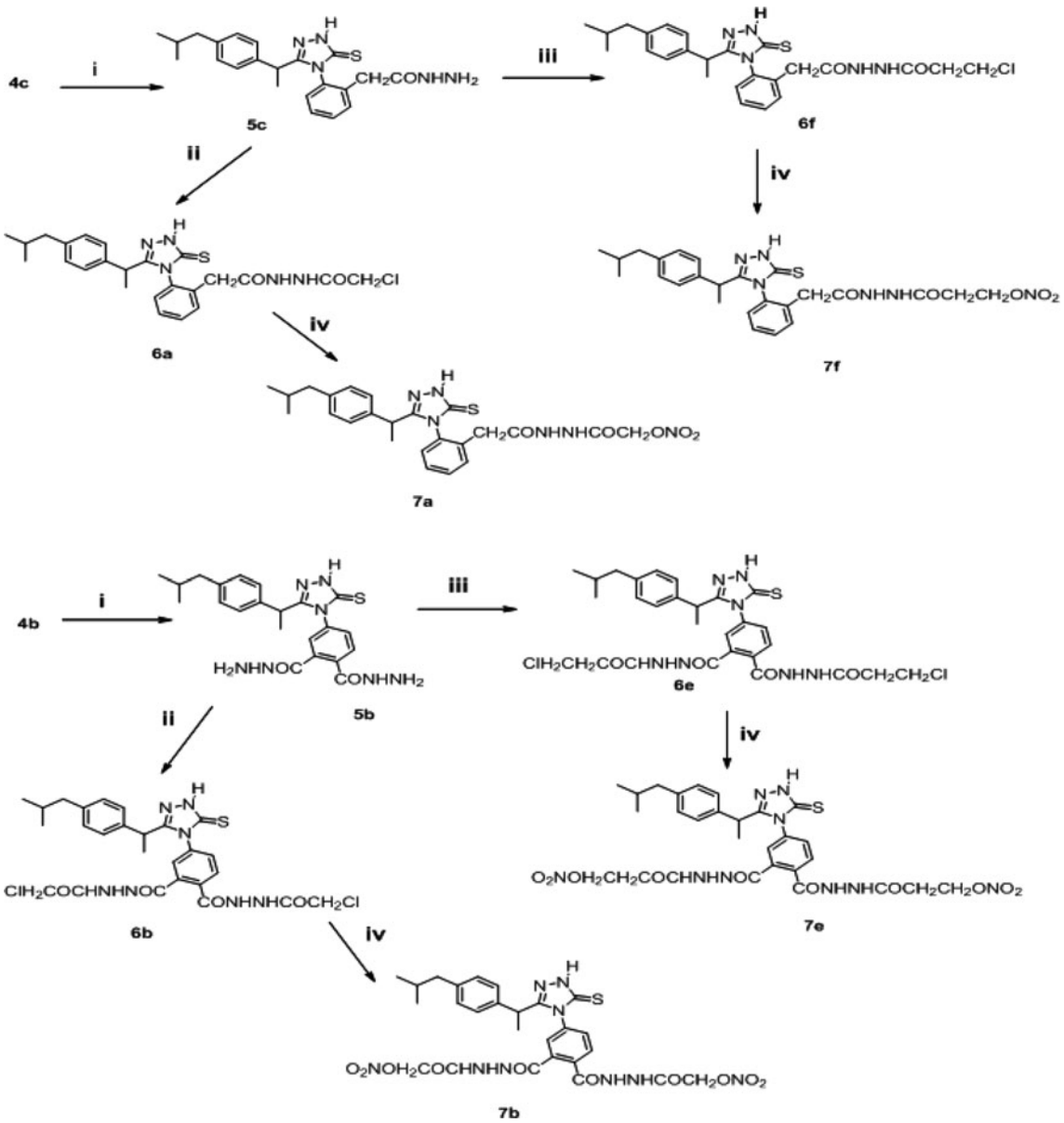
The synthetic route, utilized to synthesize title compounds, is outlined in Schemes 1, 2 and 3. The compound, 2,5-dioxopyrrolidin-1-yl-2-(4-isobutylphenyl)propanoate (**1**), was prepared according to the method reported in the literature



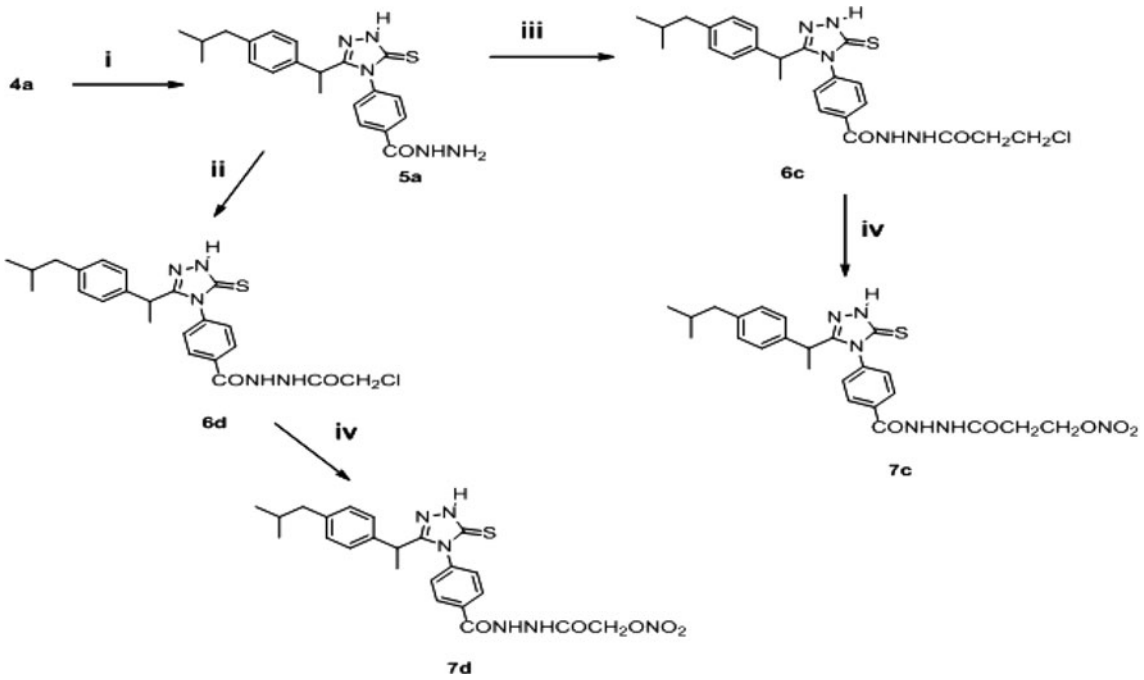
Scheme 1 Synthesis of Compounds **4a**–**4c**[a]. [a] Reagents and Conditions: (i) DCC, THF, overnight at 5°C; (ii) THF, thiosemicarbazide, reflux 12 h; (iii) 10% aq. KOH, dil. HCl, reflux; (iv) 4-chlorobenzoic acid, ethanol, reflux 4 h; (v) 4-chlorophthalic acid, ethanol, reflux 4 h and (vi) 2-(2-chlorophenyl)acetic acid, ethanol, reflux 4 h

(Birsen *et al*., 2000) using Ibuprofen. The 2-(2-(4-isobutylphenyl)propanoyl)hydrazincarbothioamide (**2**) was prepared by reacting (**1**) with thiosemicarbazide in tetrahydrofuran. Ring formation yielding 5-(1-(4-isobutylphenyl) ethyl)-2H-1,2,4-triazole-3(4H)-thione (**3**) was carried out by reacting (**2**) with 10% KOH. The different acid derivatives (**4a**–**4c**) were prepared by reacting (**3**) with different acids. The reaction of (**4a**–**4c**) with thionyl chloride followed by hydrazine hydrate yielded corresponding hydrazides (**5a**–**5c**). The reaction of these hydrazides with the substituted chloro acetyl chloride gave the corresponding haloalkyl derivatives (**6a**–**6f**). The terminal chloro function is then replaced with nitric oxide by reacting (**6a**–**6f**) with silver nitrate to get nitrate derivatives (**7a**–**7f**) using acetonitrile as solvent. The structures of various synthesized compounds were assigned on the basis of results of different chromatographic and spectral studies. The physical data, FTIR, ^{1}H-NMR, Mass spectral data, and elemental analysis data for all the synthesized compounds are given in experimental protocols.

The FTIR spectra of the title compounds (**7a**–**7f**) exhibited very similar features and showed the expected bands for the characteristic groups which are present in the compounds such as C=N stretching vibrations, amide C=O stretching, and another specific band for $-NO_2$ vibrations.



Scheme 2 Synthesis of Compounds **7a**, **7b**, **7e**, and **7f**[a]. [a] Reagents and conditions: (i) Thionyl chloride, hydrazine hydrate, ethanol, reflux 6 h; (ii) chloro-acetyl chloride, dry benzene, triethylamine, reflux 10 h; (iii) chloro-propionyl chloride, dry benzene, triethylamine, reflux 10 h; and (iv) $AgNO_3$, acetonitrile, stirring, RT, 3 h



Scheme 3 Synthesis of Compounds **7c** and **7d**[a]. [a] Reagents and conditions: (i) Thionyl chloride, hydrazine hydrate, ethanol, reflux 6 h; (ii) chloro-acetyl chloride, dry benzene, triethylamine, reflux 10 h; (iii) chloro-propionyl chloride, dry benzene, triethylamine, reflux 10 h; and (iv) $AgNO_3$, acetonitrile, stirring, RT, 3 h

In the ^{1}H-NMR spectral data, all the protons were seen according to the expected chemical shift and integration values. The aromatic protons appeared as multiplet peaks within the range of 7.07–7.6 δ ppm, and the synthesized derivatives had shown the peak at 6.9 δ for –NH of triazole ring that confirms the formation of triazole ring.

Pharmacology

The synthesized new chemical entities (NCEs) were subjected to the evaluation of anti-inflammatory activity, analgesic activity, and acute ulcerogenicity studies as well as investigated for their nitric oxide-releasing properties. Ibuprofen and Diclofenac were used as reference standards. The experiments were performed using albino rats of Wistar strain of either sex, weighing in the range of 140–160 g. The animals were maintained at 25 ± 2°C, 50 ± 5% relative humidity, and 12-h light/dark cycle. All the animals were fasted for 24 h before the experiments, and water was provided ad libitum. The test compounds were suspended in 1% aqueous carboxy methyl cellulose (CMC) solution and administered orally to experimental animals for all the studies.

Anti-inflammatory activity

Anti-inflammatory activity of the synthesized compounds was evaluated by carrageenan-induced rat paw edema model, and equimolar doses are equivalent to Ibuprofen. Sub-planter injection of 0.1 ml of 1% Carrageenan in rat paw increased the paw volume (edema) in all the animals of various groups. The onset of action was evident during 1 h in various test groups. The significant ($P < 0.01$) reduction of rat paw edema was observed for most of the test compounds, at 3 h compared to control group and Ibuprofen (Table 1). Compounds with significant anti-inflammatory profile were subjected to GI-ulcerogenicity potential studies at 12 times the therapeutic doses with additional physical (cold) stress. A thorough examination of the results of histopathological studies indicated the absence of the disruption of morphology of the gastric epithelial cells and the absence of ulcers/erosion in test (test compd.-treated) group animals which were compared to reference standard, Diclofenac acid, and control group animals. The results of the ulcerogenicity studies are presented in Table 3, and the results of Histopathological studies are depicted in Fig. 1a–f. Out of the synthesized six NCEs, compounds **7a**, **7c**, **7d**, and **7f** (61.25–62.82%) exhibited very significant anti-inflammatory activity compared to standard drug Ibuprofen (62.82% at 3 h). Thus, the compounds with nitrate-containing moiety at ortho or para position of phenyl ring show higher anti-inflammatory activity whereas the bi-substitution of nitrate-containing moiety at both ortho and para positions of phenyl in compounds (**7b** and **7e**) shows decreased anti-inflammatory activity. Results indicated that the length of the chain by

Table 1 Results of anti-inflammatory activity of synthesized compounds against carrageenan-induced rat paw edema model in rats

Compound code/ dose (mg/kg, p.o)	Change in paw volume in (ml) after drug treatment (±SEM)			Anti-inflammatory activity (% inhibition)		
	1 h	2 h	3 h	1 h	2 h	3 h
Control	1.65 ± 0.017	1.77 ± 0.02	1.91 ± 0.03	–	–	–
Ibuprofen (20)	0.76 ± 0.072**	0.74 ± 0.009**	0.71 ± 0.02**	53.93	58.19	62.82
7a (49.70)	0.75 ± 0.074**	0.73 ± 0.009**	0.71 ± 0.05**	54.54	58.75	62.82
7b (63.98)	1.04 ± 0.042**	1.06 ± 0.03**	1.11 ± 0.07**	38.78	40.11	41.88
7c (49.70)	0.79 ± 0.009**	0.76 ± 0.01**	0.73 ± 0.01**	52.12	57.06	61.78
7d (48.34)	0.78 ± 0.02**	0.77 ± 0.01**	0.74 ± 0.01**	52.72	56.49	61.25
7e (66.69)	1.19 ± 0.06**	1.24 ± 0.09**	1.30 ± 0.09**	27.87	29.94	31.93
7f (51.06)	0.80 ± 0.03**	0.77 ± 0.02**	0.73 ± 0.01**	51.51	56.49	61.78

Data analyzed by one way ANOVA followed by Dunnett's '*t*' test, ($n = 6$), * $P < 0.05$, ** $P < 0.01$ significant from control

ns not significant

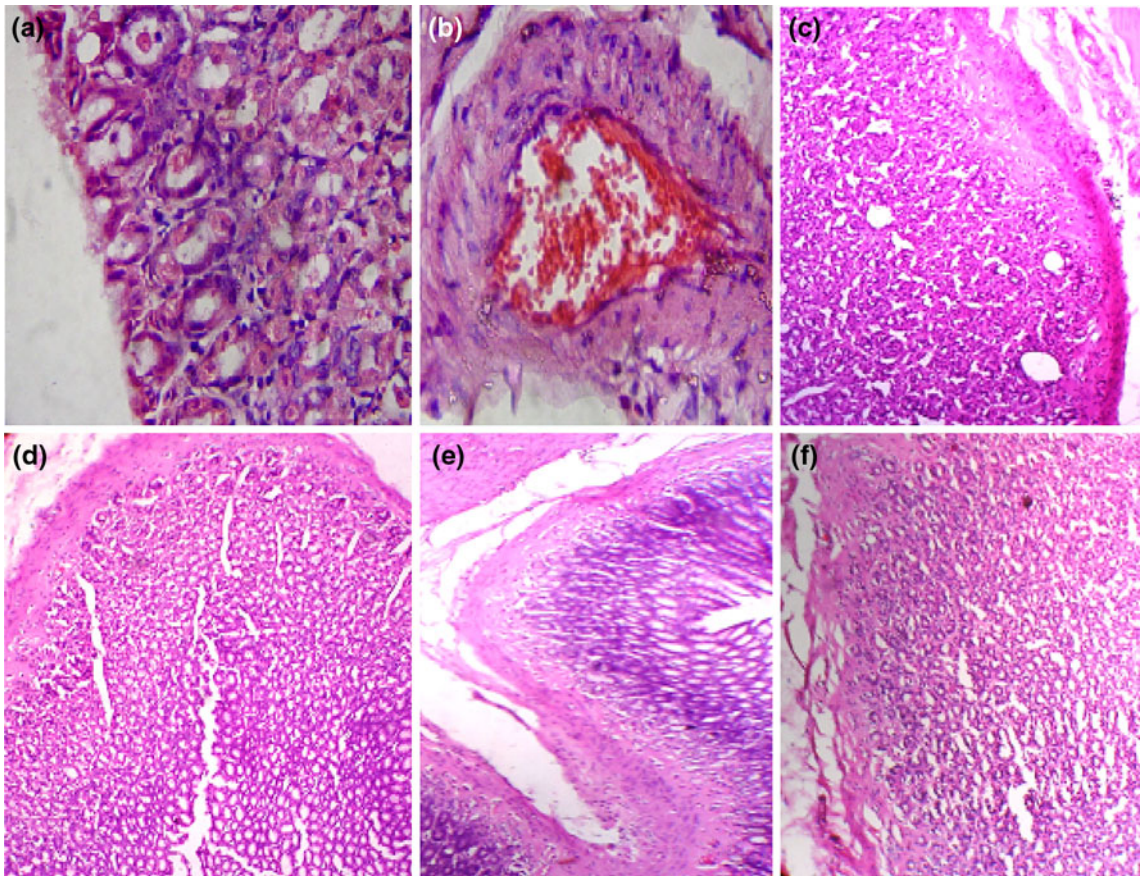


Fig. 1 Haematoxylin and Eosin Immunohistochemical staining of gastric ulcers after ulcer induction in rats. As illustrated in Fig 1, specimen **a** shows intact mucous membrane in treated control rat showing granular tissues composed of macrophages, fibroblasts, and endothelial cells forming microvessels. Congestion of mucosal blood vessels was observed in Diclofenac-treated group, specimen (**b**). No damage was seen to mucosa of rat treated with test compound, **7a**, specimen (**c**), **7c**, specimen (**d**), **7d**, specimen (**e**), and **7f**, specimen (**f**) these specimens **c**–**f** were identical to that of the control, specimen (a). Original magnification 200×

which nitrate-containing moiety attached to phenyl ring did not affect the anti-inflammatory activity of the compounds. Based on the findings of these preclinical results, further studies need to be carried out to investigate the other specifications, such as in vitro assays, chronic ulcerogenecity studies, toxicological studies, and mechanism by which these drugs exhibit potential analgesic, anti-inflammatory activities.

Analgesic activity

The analgesic activity of the compounds was studied by using acetic acid-induced writhing test in mice. The analgesic activity was evaluated at equimolar doses equivalent to 15 mg/kg (Ibuprofen) body weight. The important analgesic profile of the compounds was measured by the classical acetic acid-induced writhing model. From the results, it was noticed that all the compounds possess significant analgesic activity (Table 2). The analgesic effects of compounds **7c** (64.75%) and **7d** (63.57%) were found to be better than that of Ibuprofen (62.43%). Similar to anti-inflammatory activity, the presence of nitrate-containing moiety at ortho or para position of phenyl ring in compounds shows higher analgesic activity as compared to the bi-substitution of this moiety at both the ortho and para positions of phenyl in compounds **7b** and **7e**. It was exhibited that the presence of nitrate-containing moiety at para position of phenyl ring (Compounds **7c** and **7d**) show higher analgesic activity as compared to substitution at ortho position of phenyl ring (Compounds **7a** and **7f**).

Acute ulcerogenecity studies

The compounds, which exhibited significant anti-inflammatory and analgesic activity comparable to that of Ibuprofen, were subjected to acute ulcerogenecity studies. Therefore ulcerogenic effect of the compounds **7a**, **7c**, **7d**, and **7f** with best overall profile in animal efficacy model was evaluated for gastric ulcerogenic potential in rat stress model at 12 times the therapeutic doses of Diclofenac acid (Table 3). When compared with Diclofenac acid, all the four compounds did not cause any gastric ulceration and disruption of gastric epithelial cells at the above mentioned oral doses. The results are shown in Table 3. Thus, it was indicated that presence of nitrate-containing moiety will decrease the adverse effect of compounds (NSAIDS.).

Table 2 Results of analgesic activity of synthesized compounds against acetic acid-induced writhing test in mice

Compound no.	Dose (mg/kg, p.o)	No. of Writhes in 5–15 min after treatment (Mean ± SE)	% Inhibition
Control	Acetic acid (1% v/v)	28.83 ± 0.65**	–
Ibuprofen	15	10.83 ± 0.60**	62.43
7a	37.28	11.83 ± 0.94**	58.96
7b	47.98	13.66 ± 0.33**	52.61
7c	37.28	10.16 ± 0.47**	64.75
7d	35.85	10.5 ± 0.42**	63.57
7e	49.46	14.83 ± 0.60**	48.56
7f	37.87	12.66 ± 0.33**	56.08

Data analyzed by one way ANOVA followed by Dunnett's '*t*' test, ($n = 6$), ** $P < 0.01$ significant from control

Table 3 Ulcerogenic effects of synthesized compounds in comparison with diclofenac acid

Compound code	Dose (mg/kg, p.o)	Ratio of ulcerated animals	Ulcer index (mean ± SE)
Diclofenac	24	6/6	2.3 ± 0.3
7a	59.64	Nil	–
7c	59.64	Nil	–
7d	57.96	Nil	–
7f	61.2	Nil	–

Histopathological studies

The stomach specimen of Diclofenac acid-treated rats was characterized by complete disruption of protective mucosal layer (Fig. 1b). Histopathological analysis also showed characteristic features of ulceration in diclofenac acid-treated group of animals. The tissue of Diclofenac-acid treated rats showed that some epithelial cells in the ulcer margin were proliferated and migrated over and into the ulcer crater. This indicated the complete disruption of gastric epithelial layer. Scanning of stomach specimens using electron microscope revealed that in the rats no injury was observed in stomach mucosa when treated with synthesized derivatives (**7a**, **7c**, **7d**, and **7f**). As illustrated in Fig. 1, specimen c, d, e, and f are seen identical to that of the control (Fig. 1 Specimen a).

Nitric oxide-release study

In isolated Wistar rat aorta rings, compounds **7a**–**7f** competitively inhibited norepinephrine-induced contraction effects, causing a shift to the right of the norepinephrine concentration response curves. EC_{50} (μg/ml) values were calculated from the cumulative concentration response curves. In order to prove the involvement of nitric oxide in the relaxation process, nitric oxide-releasing properties of synthesized compounds were assessed in phosphate buffer, pH 7.4, in the presence of L-cysteine, relative to nitric oxide released from standard sodium nitrite solution (Table 4).

Conclusions

Six hybrid derivatives were synthesized and screened for analgesic, anti-inflammatory, ulcerogenic potential, and nitric oxide-releasing studies. Most of the compounds (4 out

Table 4 EC_{50} values and nitric oxide-releasing properties of the compounds (**7a–7f**)

Compound code	EC_{50}	% NO release[a]
7a	55.26	0.35
7b	38.68	0.42
7c	30.52	0.39
7d	42.78	0.48
7e	34.88	0.57
7f	58.56	0.32

[a] Percentage of NO released ($n = 2$) relative to a theoretical maximum release of 1 mol NO/mol of test compound; determined by Griess reagent in the presence of 5 mM L-cysteine, at pH 7.4

of 6) exhibited significant analgesic and anti-inflammatory activity. Compounds (**7c**) and (**7d**) showed strong analgesic activity in the acetic acid-induced writhing tests. Among all the synthesized compounds, compound (**7a**) exhibited most prominent and consistent anti-inflammatory activity. From the detailed analysis of the results of histopathological studies, we conclude that the synthesized compounds have not only retained but showed enhanced anti-inflammatory profile, which are devoid of the deadlier gastrointestinal toxicities, but also all the synthesized derivatives exhibited significant vasorelaxant activity that is devoid of CVS toxicities associated with coxib families of COX-2 inhibitors. Therefore, it can be concluded that the rational, based on which these NCEs were designed, has been proven to be superior compared to the currently used NSAIDs. Outcome of this research study is very promising. The only concern to be addressed now is the pharmacokinetic profile of the designed NCEs, since molecular weight of all the designed compounds is beyond 500.

The most potent derivatives are in the process for chronic toxicity and pharmacokinetic studies; based on those results, further action plan will be finalized.

Experimental

Synthetic studies

All the compounds were synthesized using the reported literature procedures, and synthetic procedures were set and optimized as and where required. All the chemicals were procured from Merck, Sigma-Aldrich, Mumbai. Melting points were determined by open capillary tubes and were uncorrected. FTIR spectra of the powdered compounds were recorded using KBr on a Jasco FTIR V 430 + spectrometer with Diffuse Reflectance Attachment and are reported in cm^{-1} and ^1H NMR spectra were recorded on a Varian Mercury YH300 (300 MHz FT NMR) spectrophotometer using TMS as an internal reference (Chemical shift represented in δ ppm). Mass spectra were recorded on GC-MS QP5050ASystem (benchtop quadrupole mass spectrophotometer). Elemental analysis was recorded on FLASHEA112 series at Shimadzu Analytical Centre and NMR Facility, the Department of Chemistry, University of Pune. Purity of the compounds was checked on TLC plates using silica gel G as stationary phase and iodine vapors as visualizing agent. The 2-(4-isobutylphenyl)propanoic acid (Ibuprofen) was procured from Inland Pharmaceuticals Ltd., Pune, India.

Synthesis of 2,5-dioxopyrrolidin-1-yl2-(4-isobutylphenyl)propanoate (**1**)

Compound (**1**) was synthesized as per the previously reported procedure (Birsen *et al*., 2000). Yield: 80%; and M.P: 82–84°C. FTIR spectra of the compound showed bands at 3053 (Ar C–H); 2933 (Aliphatic C–H); and 1676 (C=O).

Synthesis of 2-(2-(4-isobutylphenyl)propanoyl) hydrazinecarbothioamide (**2**)

The product (**2**) was obtained as per the previously reported procedure (Birsen *et al*., 2000). Yield: 70%; and M.P: 92–94°C. FTIR spectra of the compound showed bands at 3465 (N–H); 3043 (Ar C–H); 2941 (Aliphatic C–H); and 1680 (C=O).

Synthesis of 5-(1-(4-isobutylphenyl) ethyl)-2H-1,2,4-triazole-3(4H)-thione (**3**)

The product (**3**) was obtained as per the previously reported procedure (Birsen *et al*., 2000). Yield: 74%; and M.P: 128–130°C. FTIR spectra of the compound showed bands at 3455 (N–H); 3061 (Ar C–H); 2941 (Aliphatic C–H); and 1546 (C=N).

*Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) benzoic acid (**4a**) (McCall et al., 1986)*

Equimolar amounts of intermediate (**3**) (1 g, 0.003 M) and 4-chlorobenzoic acid (0.46 g 0.003 M) were refluxed in ethanol (10 ml) for 4 h. After completion of 3 h from the starting of reflux, $NaHCO_3$ (0.252 g, 0.003 M) was added in reaction mixture to increase the rate of forward reaction. After 4-h duration, the reaction mixture was cooled, ethanol was evaporated to yield product (**4a**). Yield: 76%; and M.P: 152–154°C. FTIR spectra of the compound showed bands at 3541 (O–H); 3463 (N–H); 3043 (Ar C–H); 2943 (Aliphatic C–H); 1685 (C=O); and 1546 (C=N). ^1H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.89 (d, 6H, $-(CH_3)_2$);

1.82–1.84 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.0 (q, 1H, –CH–); 1.2 (d, 3H, $–CH_3$); 6.90 (s, 1H, triazole NH); 6.20–7.81 (m, 4H, 2,3,5,6 ArH); and 12.79 (s, 1H, –OH). Mass spectra of the compound exhibited molecular ion peak at *m/z* 381(M^+); other important fragments were observed at 382 ($M^+ + 1$), 338, and 248.

*Synthesis of 4-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phthalic acid (**4b**)*

The product **4b** was obtained as per the procedure reported in "Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) benzoic acid (**4a**) (McCall *et al*., 1986)" section. Yield: 70%; and M.P: 142–144°C. FTIR spectra of the compound showed bands at 3532 (O–H); 3433 (N–H); 3053 (Ar C–H); 2921 (Aliphatic C–H); 1685 (C=O); and 1549 (C=N). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.92 (d, 6H, $–(CH_3)_2$); 1.78–1.80 (m, 1H, –CH–); 2.45 (d, 2H, $–CH_2–$); 7.13– 7.17 (m, 4H, 2,3,5,6 ArH); 3.2 (q, 1H, –CH–); 1.2 (d, 3H, $–CH_3$); 6.88 (s, 1H, triazole NH); 6.54–8.09 (m, 3H, 2,5,6 ArH); and 12.34 (s, 2H, $–(OH)_2$). Mass spectra of compound exhibited molecular ion peak at *m/z* 425 (M^+); other important fragments were observed at 426 ($M^+ + 1$), 427 ($M^+ + 2$), 382, 292.

*Synthesis of 2-(2-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl) acetic acid (**4c**)*

The product **4c** was obtained as per procedure reported in "Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) benzoic acid (**4a**) (McCall *et al*., 1986)" section. Yield: 73%, and M.P: 148–150°C. FTIR spectra of the compound showed bands at 3549 (O–H); 3445 (N–H); 3043 (Ar C–H); 2932 (Aliphatic C–H); 1678 (C=O); and 1539 (C=N). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.88 (d, 6H, $–(CH_3)_2$); 1.80–1.82 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 2.9 (q, 1H, –CH–); 1.1 (d, 3H, $–CH_3$); 6.89 (s, 1H, triazole NH); 6.34–6.82 (m, 4H, 2,3,4,5 ArH); 3.49 (s, 2H, $–CH_2–$); and 12.28 (s, 1H, –OH). Mass spectra of compound exhibited molecular ion peak at *m/z* 395 (M^+); other important fragments were observed at 396 ($M^+ + 1$), 397 ($M^+ + 2$), 352, 262, and 380.

*Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzohydrazide (**5a**)*

The product (**5a**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005). A mixture of (1 g, 3 mmol) of **4a** and excess of thionyl chloride (0.36 ml, 5 mmol) was refluxed for 4 h, excess thionyl chloride was neutralized by using formic acid, and the residue was refluxed with excess hydrazine hydrate (0.11 ml, 5 mmol) in ethanol (6 ml) for 6 h. The mixture was left to cool and then evaporated under reduced pressure. The residue obtained was filtered, washed with water, dried, and crystallized from absolute alcohol. Yield: 68%; and M.P. 174–176°C. FTIR spectra of the compound showed bands at 3431 (N–H); 3042 (Ar C–H); 2938 (Aliphatic C–H); 1680 (C=O); and 1555 (C=N). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.91 (d, 6H, $–(CH_3)_2$); 1.84–1.86 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 2.9 (q, 1H, –CH–); 1.2 (d, 3H, $–CH_3$); 6.84 (s, 1H, triazole NH); 6.17–7.53 (m, 4H, 2,3,4,5 ArH); 9.48 (s, 1H, –NH–); and 4.35 (s, 2H, $–NH_2$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 395 (M^+); other important fragments were observed at 396 ($M^+ + 1$), 397 ($M^+ + 2$), 352, 262, and 380.

*Synthesis of 4-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phthalohydrazide (**5b**)*

The product (**5b**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and reported in "Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzohydrazide (**5a**)" section. Yield: 88%; and M.P: 188–190°C. FTIR spectra of the compound showed bands at 3445 (N–H); 3032 (Ar C–H); 2933 (Aliphatic C–H); 1682 (C=O); and 1559 (C=N). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.87 (d, 6H, $–(CH_3)_2$); 1.82–1.84 (m, 1H, –CH–); 2.47 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.0 (q, 1H, –CH–); 1.3 (d, 3H, $–CH_3$); 6.89 (s, 1H, triazole NH); 6.42–7.88 (m, 3H, 3, 5,6 ArH); 9.56 (s, 2H, $–(NH)_2–$); and 4.33 (s, 4H, $(–NH_2)_2$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 453 (M^+); other important fragments were observed at 454 ($M^+ + 1$), 455 ($M^+ + 2$), 410, and 438.

*Synthesis of 2-(2-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl)acetohydrazide (**5c**)*

The product (**5c**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzohydrazide (**5a**)" Yield: 79%; and M.P: 178–180°C. FTIR spectra of the compound showed bands at 3455 (N–H); 3043 (Ar C–H); 2939 (Aliphatic C–H); 1678 (C=O); and 1549 (C=N). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.88 (d, 6H, $–(CH_3)_2$); 1.78–1.80 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.3 (q, 1H, –CH–); 1.2 (d, 3H, $–CH_3$); 6.92 (s, 1H, triazole NH); 6.34–6.82 (m, 4H, 3,4,5,6 ArH); 3.44 (s, 2H, $–CH_2–$); 9.08 (s, 1H, –NH–); and 4.35 (s, 2 H, $–NH_2$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 409 (M^+); other

important fragments were observed at 410 ($M^+ + 1$), 411 ($M^+ + 2$), 366, and 394.

*Synthesis of 2-chloro-N′-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)acetohydrazide (**6a**)*

The product (**6a**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005). A mixture of (1 g, 0.0024 M) of intermediate **5c** was refluxed with 0.19 ml of chloro-acetyl chloride in dry benzene (15 ml) and in the presence of triethylamine (0.1 ml) for 10 h. The solution was evaporated to dryness, the residue was washed with acetone, the precipitate was filtered, washed with water, and crystallized from absolute ethanol. Yield: 72%; and M.P: 208–210°C. FTIR spectra of the compound showed bands at 3455 (N–H); 3044 (Ar C–H); 2936 (Aliphatic C–H); 1684 (C=O); 1541 (C=N); and 685 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.89 (d, 6H, $–(CH_3)_2$); 1.82–1.84 (m, 1H, –CH–); 2.44 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.0 (q, 1H, –CH–); 1.4 (d, 3H, $–CH_3$); 6.83 (s, 1H, triazole NH); 6.34–6.82 (m, 4H, 3,4,5,6 ArH); 3.46 (s, 2H, $–CH_2–$); 10.08 (s, 2H, $–(NH)_2–$); and 4.27 (s, 2 H, $–CH_2–$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 485 (M^+); other important fragments were observed at 486 ($M^+ + 1$), 487 ($M^+ + 2$), 442, and 470.

*Synthesis of N′1, N′2-bis(2-chloroacetyl)-4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phthalohydrazide (**6b**)*

The product (**6b**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-chloro-*N′*-(2-(2-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl) acetohydrazide (**6a**)" section. Yield: 80%; and M.P: 222–224°C. FTIR spectra of the compound showed bands at 3465 (N–H); 3034 (Ar C–H); 2939 (Aliphatic C–H); 1687 (C=O); 1551 (C=N); and 689 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.84 (d, 6H, $–(CH_3)_2$); 1.85–1.87 (m, 1H, –CH–); 2.39 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 2.9 (q, 1H, –CH–); 1.2 (d, 3H, $–CH_3$); 6.87 (s, 1H, triazole NH); 6.42–7.88 (m, 3H, 2,5,6 ArH); 10.74 (s, 2H, $–(NH)_2–$); 10.05 (s, 2H, $–(NH)_2–$); and 4.25 (s, 4 H, $–(CH_2)_2–$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 605 (M^+); other important fragments were observed at 606 ($M^+ + 1$), 607 ($M^+ + 2$), 562, and 590.

*N′-(3-chloropropanoyl)-4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzohydrazide (**6c**)*

The product (**6c**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-chloro-*N′*-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl)acetyl)acetohydrazide (**6a**)" section. Yield: 75%; and M.P: 210–212°C. FTIR spectra of the compound showed bands at 3459 (N–H); 3042 (Ar C–H); 2934 (Aliphatic C–H); 1680 (C=O); 1547 (C=N); and 681 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.87 (d, 6H, $–(CH_3)_2$); 1.82–1.84 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.0 (q, 1H, –CH–); 1.3 (d, 3H, $–CH_3$); 6.89 (s, 1H, triazole NH); 6.17–7.53 (m, 4H, 2,3,5,6 ArH); 10.75 (s, 1H, –NH–); 10.08 (s, 1H, –NH–); 2.46 (t, 2 H, $–CH_2–$); and 3.66 (t, 2 H, $–CH_2–$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 485 (M^+); other important fragments were observed at 486 ($M^+ + 1$), 487 ($M^+ + 2$), 442, and 470.

*Synthesis of N′-(2-chloroacetyl)-4-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzohydrazide (**6d**)*

The product (**6d**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-chloro-*N′*-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl)acetyl)acetohydrazide (**6a**)" section. Yield: 82%; and M.P: 228–230°C. FTIR spectra of the compound showed bands at 3452 (N–H); 3043 (Ar C–H); 2926 (Aliphatic C–H); 1675 (C=O); 1554 (C=N); and 683 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.84 (d, 6H, $–(CH_3)_2$); 1.83–1.85 (m, 1H, –CH–); 2.41 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.2 (q, 1H, –CH–); 1.1 (d, 3H, $–CH_3$); 6.82 (s, 1H, triazole NH); 6.17–7.53 (m, 4H, 2,3,5,6 ArH); 10.71 (s, 1H, –NH–); 10.05 (s, 1H, –NH–); and 4.27 (S, 2 H, $–CH_2–$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 471 (M^+); other important fragments were observed at 472 ($M^+ + 1$), 473 ($M^+ + 2$), 428, and 456.

*Synthesis of N′1, N′2-bis(3-chloropropanoyl)-4-(3-(1-(4-butylphenyl) ethyl)-5-thioxo1H-1,2,4-triazol-4(5H)-yl)phthalohydrazide (**6e**)*

The product (**6e**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-chloro-*N′*-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl)acetyl)acetohydrazide (**6a**)" section. Yield: 78%; and M.P: 214–216°C. FTIR spectra of the compound showed bands at 3441 (N–H); 3046 (Ar C–H); 2939 (Aliphatic C–H); 1681 (C=O); 1552 (C=N); and 687 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.87 (d, 6H, $–(CH_3)_2$); 1.82–1.84 (m, 1H, –CH–); 2.43 (d, 2H, $–CH_2–$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.0 (q, 1H, –CH–); 1.2 (d,

3H, $-CH_3$); 6.86 (s, 1H, triazole NH); 6.42–7.88 (m, 3H, 2, 5, 6 ArH); 10.75 (s, 2H, $-(NH)_2-$); 10.08 (s, 2H, $-(NH)_2-$); 2.46 (t, 4 H, $-(CH)_2-$); and 3.66 (t, 4 H, $-(CH)_2-$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 633 (M^+); other important fragments were observed at 634 ($M^+ + 1$), 635 ($M^+ + 2$), 590, and 618.

*Synthesis of 3-chloro-N′-(2-(2-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)propanehydrazide (**6f**)*

The product (**6f**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-chloro-*N′*-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl) phenyl)acetyl)acetohydrazide (**6a**)" section. Yield: 74%; and M.P: 204–206°C. FTIR spectra of the compound showed bands at 3459 (N–H); 3051 (Ar C–H); 2946 (Aliphatic C–H); 1676 (C=O); 1551 (C=N); and 688 (C–Cl). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.89 (d, 6H, $-(CH_3)_2$); 1.85–1.87 (m, 1H, –CH–); 2.46 (d, 2H, $-CH_2-$); 7.13–7.17 (m, 4H, 2,3,5,6 ArH); 3.3 (q, 1H, –CH–); 1.1 (d, 3H, $-CH_3$); 6.88 (s, 1H, triazole NH); 6.34–6.82 (m, 4H, 3, 4, 5, 6 ArH); 3.44 (s, 2 H, $-CH_2$); 10.08 (s, 2H, $-(NH)_2-$); 2.46 (t, 2H, $-CH_2-$); and 3.66 (t, 2 H, $-CH_2-$). Mass spectra of compound exhibited molecular ion peak at *m/z* 499 (M^+); other important fragments were observed at 500 ($M^+ + 1$), 501 ($M^+ + 2$), 456, and 484.

*Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-2-oxoethyl nitrate (**7a**)*

The product (**7a**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005). A solution of the intermediate 6a (0.85 g) in dry acetonitrile (2 ml) was treated portionwise with a solution of $AgNO_3$ (0.34 g) in dry acetonitrile (5 ml), and the whole mixture was stirred at room temperature for 3 h. The mixture was then filtered, evaporated to dryness, and the residue was recrystallized from absolute ethanol. Yield: 76%; and M.P: 236–238°C. FTIR spectra of the compound showed bands at 3468 (N–H); 3043 (Ar C–H); 2951 (Aliphatic C–H); 1686 (C=O); 1549 (C=N); and 1518 ($C-NO_2$). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 1.2 (d, 9H, $-(CH_3)_3$); 2.02–2.08 (m, 1H, –CH–); 2.4 (d, 2H, $-CH_2-$); 7.07–7.1 (m, 4H, 2,3,5,6 ArH); 3.02 (q, 1H, –CH–); 6.9 (s, 1H, triazole NH); 7.21–7.4 (m, 4H, 2, 3, 4, 5 ArH); 3.5 (s, 2 H, $-CH_2$); 8.18 (s, 2H, $-(NH)_2-$); and 4.6 (s, 2H, $-CH_2-$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 512 (M^+), other important fragments were observed at 497, 455, 379, 252, 134, and 104. Elemental analysis was observed as C (56.33%), H (5.47%), and N (16.45%).

*Synthesis of 2-(2-(4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)-2-(2-(2-nitroacetyl)hydrazinecarbonyl) benzoyl) hydrazinyl)-bis-2-oxoethyl nitrate (**7b**)*

The product (**7b**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-2-oxoethyl nitrate (**7a**)" section. Yield: 70%; and M.P: 244–246°C. FTIR spectra of the compound showed bands at 3453 (N–H); 3045 (Ar C–H); 2949 (Aliphatic C–H); 1689 (C=O); 1561 (C=N); and 1519 ($C-NO_2$). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 1.2 (d, 9H, $-(CH_3)_3$); 2.02–2.09 (m, 1H, –CH–); 2.4 (d, 2H, $-CH_2-$); 7.18–7.3 (m, 4H, 2,3,5,6 ArH); 3.02 (q, 1H, –CH–); 6.9 (s, 1H, triazole NH); 7.4–7.52 (m, 3H, 2, 5, 6 ArH); 8.23 (s, 4 H, $-(NH)_4-$); and 4.4 (s, 4H, $-(CH_2)_2-$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 659 (M^+); other important fragments were observed at 644, 602, 498, 324, 238, and 152. Elemental analysis was observed as C (47.36%), H (4.49%), and N (19.03%).

*Synthesis of 3-(2-(4-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzoyl)hydrazinyl)-3-oxopropyl nitrate (**7c**)*

The product (**7c**) was obtained as per reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-2-oxoethyl nitrate (**7a**)" section. Yield: 80%; and M.P: 232–234°C. FTIR spectra of the compound showed bands at 3475 (N–H); 3053 (Ar C–H); 2933 (Aliphatic C–H); 1676 (C=O); 1546 (C=N); and 1515 ($C-NO_2$). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 1.2 (d, 9H, $-(CH_3)_3$); 2.23–2.38 (m, 1H, –CH–); 2.58 (d, 2H, $-CH_2-$); 7.3–7.41 (m, 4H, 2,3,5,6 ArH); 3.1 (q, 1H, –CH–); 6.88 (s, 1H, triazole NH); 7.6–7.78 (m, 4H, 2, 3, 5, 6 ArH); 8.22 (s, 2 H, $-(NH)_2-$); 2.58 (t, 2H, $-CH_2-$); and 3.94 (t, 2H, $-CH_2-$). Mass spectra of the compound exhibited molecular ion peak at *m/z* 512 (M^+); other important fragments were observed at 469, 379, 252, 133, and 90. Elemental analysis was observed as C (56.10%), H (5.46%), and N (16.47%).

*Synthesis of 2-(2-(4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)benzoyl)hydrazinyl)-2-oxoethyl nitrate (**7d**)*

The product (**7d**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and iss reported in "Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)

hydrazinyl)-2-oxoethyl nitrate (**7a**)" section. Yield: 83%; and M.P: 254–256°C. FTIR spectra of the compound showed bands at 3466 (N–H); 3042 (Ar C–H); 2943 (Aliphatic C–H); 1680 (C=O); 1551 (C=N); and 1523 (C–NO_2). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 0.84 (d, 9H, –$(CH_3)_3$); 2.01–2.3 (m, 1H, –CH–); 2.49 (d, 2H, –CH_2–); 7.43–7.6 (m, 4H, 2,3,5,6 ArH); 3.22 (q, 1H, –CH–); 6.9 (s, 1H, triazole NH); 6.63–6.77 (m, 4H, 2, 3, 5, and 6 ArH); 8.3 (s, 2 H, –$(NH)_2$–); and 4.3 (s, 2H, –CH_2–). Mass spectra of the compound exhibited molecular ion peak at *m/z* 498 (M^+); other important fragments were observed at 365, 337, 162, 119, and 76. Elemental analysis was observed as C (55.52%), H (5.20%), and N (16.79%).

*Synthesis of 3-(2-(4-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)-2-(2-(2-nitroacetyl) hydrazinecarbonyl) benzoyl) hydrazinyl)-bis-3-oxopropyl nitrate (**7e**)*

The product (**7e**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-2-oxoethyl nitrate (**7a**)" section. Yield: 65%; and M.P: 258–260°C. FTIR spectra of the compound showed bands at 3456 (N–H); 3031 (Ar C–H); 2935 (Aliphatic C–H); 1675 (C=O); 1541 (C=N); and 1519 (C–NO_2). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 1.24 (d, 9H, –$(CH_3)_3$); 2.17–2.22 (m, 1H, –CH–); 2.5 (d, 2H, –CH_2–); 7.4–7.58 (m, 4H, 2,3,5,6 ArH); 3.18 (q, 1H, –CH–); 6.5 (s, 1H, triazole NH); 7.75–7.88 (m, 3H, 2, 5, 6 ArH); 8.18 (s, 4 H, –$(NH)_4$–); 2.72 (t, 4H, –$(CH_2)_2$–); and 3.9 (t, 4H, –$(CH_2)_2$–). Mass spectra of the compound exhibited molecular ion peak at *m/z* 687 (M^+); other important fragments were observed at 644, 526, 342, 266, 180, and 124. Elemental analysis was observed as C (49.04%), H (4.72%), and N (18.40%).

*Synthesis of 3-(2-(2-(2-(3-(1-(4-isobutylphenyl) ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-3-oxopropyl nitrate (**7f**)*

The product (**7f**) was obtained as per the previously reported procedure (Abadi and Gehan, 2005) and is reported in "Synthesis of 2-(2-(2-(2-(3-(1-(4-isobutylphenyl)ethyl)-5-thioxo-1H-1,2,4-triazol-4(5H)-yl)phenyl)acetyl)hydrazinyl)-2-oxoethyl nitrate (**7a**)" section. Yield: 72%; and M.P; 236–238°C. FTIR spectra of the compound showed bands at 3446 (N–H); 3032 (Ar C–H); 2945 (Aliphatic C–H); 1685 (C=O); 1545 (C=N); 1527 (C–NO_2). ^{1}H NMR chemical shifts at ($CDCl_3$, δ ppm): 1.2 (d, 9H, –$(CH_3)_3$); 1.98–2.17 (m, 1H, –CH–); 2.39 (d, 2H, –CH_2–); 7.21–7.4 (m, 4H, 2,3,5,6 ArH); 3.08 (q, 1H, –CH–); 6.84 (s, 1H, triazole NH); 7.14–7.21 (m, 4H, 3, 4, 5, 6 ArH); 3.5 (s, 2 H, –CH_2–); 8.17 (s, 2H, –$(NH)_2$–); 2.65 (t, 2H, –CH_2–); and 3.82 (t, 2H, –CH_2–). Mass spectra of the compound exhibited molecular ion peak at *m/z* 526 (M^+); other important fragments were observed at 483, 365, 190, 148, and 118. Elemental analysis was observed as C (56.08%), H (5.61%), and N (16.34%).

Pharmacology

Animals

Swiss albino mice of either sex weighing 20–25 g and Wistar rats weighing in the range 140–160 g were obtained from National Centre for Cell Sciences (NCCS), Pune, India. All the animals were housed under standard ambient conditions of temperature (25 ± 2°C) and relative humidity of 50 ± 5%. A 12/12-h light/dark cycle was maintained. All the animals were allowed to have free access to water and standard palletized laboratory animal diet 24 h before pharmacological studies. All the experimental procedures and protocols used in this study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of College, Pune, constituted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/257, CPCSEA/300), Government of India.

Preparation of test compounds

After suspending the test compounds in 1.0% aqueous solution of sodium carboxymethyl cellulose (CMC), the test samples were administered to the test animals orally. The positive and negative control group animals received the same experimental handling as those of the test groups except that the drug-treated control group animals received only appropriate volumes of vehicle and of the reference drug, Ibuprofen.

Anti-inflammatory activity

Anti-inflammatory activity was evaluated using the well-known Carrageenan-induced rat paw edema model of Winter *et al*., 1962, using eight groups of six animals each. A freshly prepared aqueous suspension of Carrageenan (1.0% w/v, 0.1 ml) was injected in the subplanter region of right hind paw of each rat. One group was kept as control, and the animals of the other group were pretreated with the test compounds, 1 h before the Carrageenan treatment. The volume was measured before and after carrageenan treatment at 60-min intervals with the help of digital plethysmometer (Panlab LE 7500, USA).

Analgesic activity

The analgesic activity was evaluated using the acetic acid induced writhing method (Koster *et al*., 1959), using groups of six animals each. A solution of acetic acid (1% v/v) in distilled water was prepared and injected intraperitoneally in a volume of 0.1 ml. One group was kept as control, and the animals of the other group were pretreated with the test compounds, 1 h before the acetic acid (1% v/v) treatment. The writhing episodes were recorded for 15 min; stretching movements consisting of arching of the back, elongation of body, and extension of hind limbs were counted.

Acute ulcerogenicity studies

Acute ulcerogenicity screening was done according to method reported by Cioli *et al*. (1979). The mucosal damage was examined by means of an electron microscope. For each stomach specimen, the mucosal damage was assessed according to the following scoring system.

Score description

0.0 Normal (no injury, bleeding, and latent injury);
0.5 Latent injury or widespread bleeding (>2 mm);
1.0 Slight injury (2–3 dotted lines);
2.0 Severe injury (continuous lined injury or 5–6 dotted injuries);
3.0 Very severe injury (several continuous lined injuries);
4.0 Widespread lined injury or widened injury.

The mean score of each treated group minus the mean score of control group was regarded as severity index of gastric mucosal damage. Data are expressed as mean ulcer score ± SEM, data analyzed by one way ANOVA followed by Dunnett's '*t*' test to determine the significance of the difference between the standard group and the rats treated with the test compounds. The differences in results were considered significant when P was found to be <0.01.

Histopathological studies (Motilva *et al*., 2004; Omar *et al*., 1996; Palaska *et al*., 2002)

For the histopathological study, rats were sacrificed 4 h after the cold stress and their stomach specimens were removed and put into 10% formalin solution. A longitudinal section of stomach along the greater curvature, which included the ulcer base and both sides of the ulcer margin, was taken and fixed in 10% formalin for 24 h at 4°C and embedded in white solid paraffin. Morphological examination was performed with Haematoxylin and Eosin staining to analyze histological changes and examined under electron microscope.

In vitro and in vivo screening of nitric oxide

Vasorelaxing activity (Calderone et al., 1996; Ferrarini et al., 1998)

In order to determine a possible vasodilator mechanism of action, the compounds were tested on isolated aortae of male normotensive Wistar rats (250–350 g). The rats were sacrificed by cervical dislocation under light ether anesthesia and bled. The aortae were immediately excised, freed of extraneous tissues, and prepared as multiple-ring preparations. Then the vessels were suspended, under a preload of 2 g, in 10-ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37°C and continuously bubbled with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (BIOPAC System, Inc., MP 35, USA).

After an equilibration period of 60 min, the endothelial integrity was confirmed by Acetylcholine (ACh) (55 μM)-induced relaxation of Norepineohrine (NE. 20 μg/ml) precontracted tissues. A relaxation ≥70% of the NE-induced contraction was considered representative of an acceptable presence of the endothelial layer, while the organs, showing a relaxation <70%, were not used in the experimental procedures. At 30–40 min after the confirmation of the endothelial integrity, the aortic preparations were contracted by treatment with a single concentration of NE (20 μg/ml) or KCl (30 mM), and when the contraction reached a stable plateau, the test compounds in concentration (0.1 mg/ml) were added cumulatively.

Detection of nitrite (Abadi and Gehan, 2005)

A solution of the appropriate compound (20 μl) in dimethylsulfoxide (DMSO) was added to 2 ml of 1:1 v/v mixture either of 50 mM phosphate buffer (pH 7.4) or of an HCl solution (pH 1) with MeOH, containing 5 × of 10^{-4} M L-cysteine. The final concentration of the test compound was 10^{-4} M. After 1 h at 37°C, 1 ml of the reaction mixture was treated with 250 μl of Griess reagent [sulfanilamide (4 g), *N*-naphthylethylenediamine dihydrochloride (0.2 g), and 85% phosphoric acid (10 ml) in distilled water (final volume: 100 ml)]. After 10 min at room temperature, the absorbance was measured at 540 nm. Sodium nitrite standard solutions (10–80 μmol/ml) were used to construct the calibration curve. The results were expressed as the percentage of NO released ($n = 2$) relative to a theoretical maximum release of 1 mol NO/mol of test compound.

Statistical analysis

Data obtained for each set of anti-inflammatory model were expressed as the mean of change in paw volume ± SD and analyzed by one-way ANOVA followed by Dunnett's test. Data from acetic acid-induced writhing model were expressed as the mean of number of writhes ± SEM and analyzed by one way ANOVA followed by Dunnett's '*t*' test. Level of significance was set to $P < 0.05$. All the statistical calculations were performed using evaluation version of Graph Pad_ Prism 3.0 (USA) statistical software.

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