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Rationally synthesized coumarin based pyrazolines ameliorate carrageenan induced inflammation through COX-2/pro-inflammatory cytokine inhibition†

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In the present work, coumarin based pyrazolines (**7a–g**) have been synthesized and investigated for their *in vitro* and *in vivo* anti-inflammatory potential. Amongst the synthesized compounds, compounds **7a**, **7d** and **7f** exhibited significant *in vitro* anti-inflammatory activity as compared to the standard etoricoxib. Keeping this in mind, *in vivo* investigations were carried out *via* carrageenan induced inflammation and acetic acid induced writhing models in male Wistar rats and compound **7a** was found to possess appreciable anti-inflammatory and analgesic potential. The mode of action of compound **7a** was also investigated by using substance P as the biomarker, which shows promising results. Further, the selectivity of the most active compound **7a** against the cyclooxygenase enzyme was supported by molecular docking studies which reveal that compound **7a** has greater binding affinity towards COX-2 over COX-1 and 5-LOX enzymes. *In silico* ADME analysis of compound **7a** confirms the drug-like characteristics and the *in vivo* acute toxicity study showed the safety of the compound even up to a 2000 mg kg⁻¹ dose. Thus, compound **7a** was identified as an effective anti-inflammatory agent, and can be explored for further analgesic/anti-inflammatory drug design and development.

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1. Introduction

Inflammation is a body's defense response against injury, infectious agents and autoimmune reactions.¹ However, the outcome of chronic inflammation involves tissue destruction and fibrosis, which may lead to rheumatoid arthritis,² cancer,^{3,4} neurodegenerative disorder⁵ and cardiovascular diseases.^{6,7} Several markers play an important role in inflammation including cytokine receptors, nuclear factor kappa-B (NF-κB), nitric oxide synthase (NOS), tumor necrosis factor alpha (TNF-α), interferons, chemokines and pro-inflammatory enzymes COX-2 and LOX (lipoxygenase).^{8–10} Among them, cyclooxygenase and lipoxygenase are the real culprits, and thus, are the primary targets of anti-inflammatory agents.¹¹ Cyclooxygenases causes inflammation *via* arachidonic acid metabo-

lism by catalyzing the formation of prostaglandin H₂, a precursor for the biosynthesis of prostacyclins, prostaglandins, and thromboxane that affects diverse biological processes such as regulation of immune function, and maintenance of renal blood flow, reproductive biology, and gastrointestinal integrity.¹² Studies have demonstrated that COX exists in multiple isoforms, each with its own physiological expression and function.¹³ Two main isoforms are COX-1, designated as the 'housekeeping' enzyme present in almost all cells and tissues, which regulates homeostasis and blood clotting, and COX-2 which is an inducible enzyme expressed in cells that mediate inflammation such as synoviocytes, macrophages, and monocytes leading to the synthesis of the prostanoids involved in acute and chronic inflammatory conditions. In this context, non-steroidal anti-inflammatory drugs (NSAIDs) are well recognized drugs for the treatment of inflammatory diseases which exert their therapeutic effects by preventing the metabolism of arachidonic acid *via* inhibition of COX enzymes.¹⁴ However, conventional NSAIDs, including non-selective COX-1/2 inhibitors such as aspirin, ibuprofen, indomethacin and diclofenac, are associated with gastric side effects, whereas selective COX-2 inhibitors (COXIBs) such as celecoxib **1** prevent these side effects of non-selective NSAIDs.^{15–18} The severe side effects of clinically used NSAIDs include gastrointestinal

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Fig. 1 Variedly substituted molecules as potential anti-inflammatory/analgesic agents.

lesions,¹⁹ cardiovascular diseases^{20,21} and renal injury,²² which necessitated the development of new chemical entities with higher efficacy and low/no side effects.

Coumarins have attracted intense interest due to their wide range of applications in pharmacological chemistry such as anticancer,²³ anti HIV,²⁴ antimicrobial,²⁵ anticoagulant, antioxidant,^{25,26} antiulcer,²⁷ dyslipidemic,²⁸ antitumor,²⁹ and anti-inflammatory applications.³⁰ Literature reports revealed variedly substituted coumarins, pyrazoles, isoxazoles/isoxazolidines, oxadiazoles *etc.* as potential anti-inflammatory and analgesic agents *via* inhibition of cyclooxygenase/pro-inflammatory cytokines (Fig. 1).^{31–38}

It is well established that a common structural feature of selective COX-2 inhibitors is the presence of two vicinal aryl rings or 1,3-aryl groups attached with a central five or six-membered heterocyclic or carbocyclic motif. On the other hand, various marketed COX-2 inhibitors comprise a sulfone moiety which was found to be responsible for their pro-oxidant activity. This pro-oxidant effect could be related to the adverse effects observed with rofecoxib and etoricoxib due to which these drugs have not been approved by the FDA for the U.S. market.^{39a} On the basis of these considerations, the present work describes the rational design and synthesis of coumarin-pyrazoline in which the coumarin nucleus was envisaged as a probable replacement for one of the aryl rings in a selective COX-2 inhibitor (celecoxib 1), and 3-acetyl pyrazoline as a central five membered carbocyclic ring with the underlying anticipation that the designed compounds would have a selective COX-2 inhibitory effect with low/no side-effects. Further, the *ortho*- two aryl groups present in marketed selective COX-2 inhibitors were replaced with *meta*-

two aryl moieties in the newly synthesized compounds (7a–g) resulting in more bulky compounds, which could maximize the interaction with the hydrophobic residues within the COX-2 active site and enhance the COX-2 selectivity (Fig. 2).

Earlier coumarin-pyrazolines have been reported for their anti-tumor activity;^{39b} however, to the best of our knowledge, their anti-inflammatory activity has not been reported yet, therefore, keeping in mind their pharmacological potential and their structural resemblance to selective COX-2 inhibitors such as celecoxib, herein, we have re-synthesized coumarin-pyrazoline derivatives 7a and 7e along with some novel derivatives 7b, 7c, 7d, 7f and 7g and evaluated them for detailed anti-inflammatory and analgesic activities.

2. Results and discussion

2.1. Chemistry

The synthetic methodology employed for the synthesis of target compounds is illustrated in Scheme 1. The reaction of salicylaldehyde 2 with ethyl acetoacetate 3 in the presence of piperidine at RT yielded 3-acetyl coumarin 4.⁴⁰ The literature reports both acid- and base-catalyzed methods for the preparation of coumarin-chalcone derivatives, for instance, in the presence of piperidine (at 80 °C),^{41,42} 10% NaOH⁴³ and 40% NaOH.⁴⁴ Herein, we have reported the synthesis of coumarin-chalcones by the reaction of 3-acetyl coumarin 4 with substituted benzaldehydes 5 using a few drops of conc. H₂SO₄ in glacial acetic acid in excellent yields. Subsequently, chalcone 6 was cyclized in the presence of hydrazine hydrate in glacial acetic acid to furnish the desired coumarin-pyrazolines 7a–g in high yields (Table 1).⁴⁵

Fig. 2 Marketed COX-2 selective inhibitors and design considerations for targeted coumarin-pyrazolines (7a-g).

Scheme 1 Synthesis of coumarin-pyrazolines 7a-g.

The synthesized compounds were purified by column chromatography over silica gel and characterized spectroscopically (IR, ^1H NMR, ^{13}C NMR and HRMS). For instance, in the ^1H -NMR spectrum of compound 7b, the appearance of a double doublet (dd) of one proton at δ 5.50 ppm was ascribed to the chiral C-5 proton of pyrazoline, whereas the

methylene hydrogens of the pyrazoline ring appeared as two double doublets at 3.88 (1H) and 3.42 (1H) ppm. The two singlets at δ 3.81 ppm and δ 3.77 ppm were ascribed to OCH_3 protons, whereas the singlet at δ 2.43 ppm corresponds to CH_3 protons. The ^{13}C NMR resonance of acetyl carbonyl appeared at 169.15 and others corroborated well with the structure of compound 7b. The peak corresponding to the mass of 7b with m/z 423.1569 (calcd m/z 423.1556 $[\text{M} + \text{H}]^+$) was observed in the high resolution mass spectra (HRMS), which is in agreement with the molecular formula of the assigned structure.

2.2. Pharmacological evaluation

2.2.1. *In vitro* anti-inflammatory activity. All the prepared compounds (7a-g) were evaluated³² for their potential to

Table 1 Reaction time and (%) yields for compounds 7a-g

Compound	R	Time (h)	Yield (%)
7a	4-OCH ₃	8	82.1
7b	3,4,5-Tri-OCH ₃	8	85.5
7c	4-Cl	8	78.1
7d	4-NO ₂	8	73.5
7e	H	8	66.1
7f	4-OH	8	65.0
7g	2-OCH ₃	8	75.9

inhibit COX-1 and COX-2 using an ovine COX-1/COX-2 assay kit (Catalog No. 560101, Cayman Chemicals Inc., Ann Arbor, MI, USA) and the results obtained are presented in Table 2. The results of the inhibition assay reveal that all the prepared compounds exhibit COX-2 selective inhibition as compared to COX-1. In particular, compound 7a selectively inhibits COX-2 ($IC_{50} = 0.41 \mu M$) over COX-1 which is much comparable to etoricoxib ($IC_{50} 0.23 \mu M$). In addition, compounds 7d and 7f also showed potent inhibitory activity against COX-2 with $IC_{50} = 1.79$ and $2.39 \mu M$, respectively.

Inhibition of albumin denaturation. Denaturation of proteins is a well-documented cause of inflammation.^{46,47} Thus, the synthesized compounds were investigated for their protective ability against albumin denaturation which could indicate their anti-inflammatory capability. The results shown in Table S1† and Fig. 3A indicate that the synthesized compounds were effective in protecting against heat induced albumin denaturation in a concentration dependent manner. Particularly, compound 7a has shown more significant protection against denaturation. At $100 \mu g ml^{-1}$, compound 7a has shown 70% protection as compared to the standard (72%).

Membrane stabilization assays. Human red blood cell (HRBC) membrane stabilization has been used as an approach to examine the *in vitro* anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the compounds may well stabilize the lysosomal membranes.^{48,49} Stabilization of lysosomes is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils, such as bacterial enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release. Therefore, to check the effect of the synthesized compounds on the lysosomal membrane, membrane stabilization assays (heat and hypotonicity induced hemolysis) have been performed.

Heat induced hemolysis. The synthesized coumarin-pyrazolines (7a–g) inhibited the heat induced hemolysis in a concentration dependent manner as shown in Table S2† and Fig. 3B. Among all the synthesized compounds, compound 7a has shown the most significant protection against the damaging effect of heat compared to the control. At 10, 25, 50 and $100 \mu g ml^{-1}$, compound 7a has exhibited protection

against heat induced hemolysis by 60, 63, 67 and 69%, respectively, as compared to the standard etoricoxib (65, 73, 78 and 76%, respectively).

Hypotonicity induced hemolysis. The results showed that the synthesized coumarin-pyrazolines in the concentration range $10\text{--}100 \mu g ml^{-1}$ protect the erythrocyte membrane against lysis induced by hypotonic solution (Table S3† and Fig. 3C). Among all the synthesized compounds, compound 7a has shown the most significant protection against the damaging effect of the hypotonic solution compared to the control. At 10, 25, 50 and $100 \mu g ml^{-1}$, compound 7a has exhibited protection against hypotonicity induced hemolysis by 62, 63, 67 and 68%, respectively, as compared to the standard etoricoxib (52, 62, 66 and 69%, respectively).

2.2.2. *In vivo* anti-inflammatory activity. The synthesized coumarin-pyrazolines were further tested for *in vivo* anti-inflammatory activity using the carrageenan paw edema method.³⁸ Male Wistar rats (4–6 weeks old), weighing between 150 and 200 g, were selected for the *in vivo* study. The animals were divided into the following groups: group I was treated with the vehicle and served as the control while group II was treated with carrageenan (0.05 ml of 1%). In other groups (group III–X), the compounds were administered at doses of 25 and $50 mg kg^{-1}$. After 30 min of treatment with etoricoxib or the test compounds, acute inflammation was induced by subplantar injection of 0.05 mL of freshly prepared 1% suspension of carrageenan. The volume displacement was measured by using plethysmography and recorded after 3 h, 6 h and finally at 24 h. The displacement volume was significantly increased in the carrageenan treated group of animals as compared to the control group. Among the synthesized compounds, compounds 7a, 7d and 7f showed a decrease in the displacement volume as compared to the carrageenan group in a time dependent manner. There was no significant reduction in the displacement volume when doses were increased from 25 to $50 mg kg^{-1}$. Among all the tested compounds, at 0.5, 3, 6 & 24 h, compound 7a has shown the most significant reduction in displacement volume as compared to the standard drug (Fig. 4). Particularly, compound 7a at 24 h reduced the displacement volume remarkably by 67.39% which is similar to the standard etoricoxib, whereas compounds 7d and 7f reduced the displacement volume by 52.17 and 55.43%, respectively, as compared to the carrageenan.

2.2.3. Effect on the cytokine level. Immune cells modulate inflammatory responses by release of pro-inflammatory cytokines and contribute to inflammatory and autoimmune disorders. Thus, the cytokine level was estimated by using rat TNF- α , IL-6, and IL-1 β immunoassay kits (KRISHGEN Biosystem, Ashley Ct, Whittier, CA).³⁸ The tested compounds 7a, 7d and 7f have shown a significant decrease in the level of TNF- α , IL-6 and IL-1 β which were increased by the carrageenan. However, compound 7a has shown more significant ($p < 0.001$) reduction in the level of TNF- α , IL-6, and IL-1 β as compared to compounds 7d and 7f (Fig. 5).

Table 2 *In vitro* COX-1 and COX-2 inhibitory activity of coumarin-pyrazolines 7a–g

Compound code	COX inhibition (IC_{50} , μM)	
	COX-1	COX-2
7a	22.4	0.41
7b	>30	4.35
7c	ND	ND
7d	16.2	1.79
7e	ND	ND
7f	20	2.39
7g	>30	4.37
Etoricoxib	>30	0.23

ND – not determined.

Fig. 3 *In vitro* anti-inflammatory activity. A) Inhibition of albumin denaturation, B) inhibition of heat induced hemolysis and C) inhibition of hypotonicity induced hemolysis. Data are expressed as mean $\pm$ S.D. Significantly different ^a $p < 0.05$ as compared to etoricoxib, ^b $p < 0.05$ as compared to 7a, and ^c $p < 0.05$ as compared to 7b using ANOVA.

2.2.4. *In vivo* analgesic activity. The analgesic activity of the most potent compound 7a was assessed by the acetic acid induced abdominal constriction (writhing) test.³⁸ The animals were divided into three groups of six animals each. Group 1 animals were administered with the vehicle and served as the vehicle control. Group II were administered with compound 7a and group III animals were administered with etoricoxib. One hour after vehicle and drug administration, each animal was injected with 3% (v/v) acetic acid, 300

mg kg⁻¹ i.p. The number of stretches or writhes (arching of the back, elongation of the body and extension of the forelimbs) was counted cumulatively over a period of 20 min. Compound 7a has been observed to significantly reduce the abnormal writhing by 70.5% as compared to the control, whereas etoricoxib produced 57.6% inhibition of writhing (Fig. 6).

2.2.5. Studies supporting the probable mode of action of the compounds. It is well documented that substance P (SP)

Fig. 4 *In vivo* anti-inflammatory activity of the synthesized compounds (7a–g). Data are expressed as mean $\pm$ S.D. ^a p < 0.001 vs. control, ^b p < 0.001 vs. carrageenan.

Fig. 5 Effect of i.p. administration of compounds 7a, 7d and 7f on the serum levels of cytokines. Data are expressed as mean $\pm$ S.D. [#] p < 0.001 vs. control, ^{*} p < 0.05, ^{**} p < 0.01, ^{***} p < 0.001 vs. carrageenan.

is an important neuropeptide involved in neurogenic inflammation and it exerts various pro-inflammatory actions on immune cells, including macrophages. Prostaglandin E₂ (PGE₂)

Fig. 6 Effect of compound 7a on acetic acid induced writhing. All the values are expressed as mean $\pm$ S.E.M. ^a p < 0.05 vs. control, ^b p < 0.05 vs. etoricoxib.

is a potent lipid mediator produced in nearly every cell type where it is synthesized from arachidonic acid *via* the actions of cyclooxygenase enzymes (COX-1 and COX-2).⁵⁰ COX-1 is constitutively expressed and important for normal regulation of vascular activity and gastrointestinal function, whereas COX-2 is an inducible enzyme stimulated by numerous mitogens, cytokines, oxidants, and microbial products.⁵¹ In particular, COX-2 is primarily responsible for the synthesis of PGE₂, and elevates inflammation by increasing vasodilation, vascular permeability, and edema in numerous respiratory diseases, such as acute lung injury, chronic obstructive pulmonary disease, pulmonary fibrosis, and lung cancer. Some studies suggest that COX-2 and PGE₂ might be associated with substance P-related inflammatory responses.^{52–54} Thus, to study the potential target of this compound, substance P (COX and LOX pathway stimulator) was administered to the

Fig. 7 Effect of substance P pretreatment on the analgesic effect of compound 7a. All the values are expressed as mean $\pm$ S.E.M. ^a $p < 0.05$ vs. control, ^b $p < 0.05$ vs. compound 7a.

animals 30 min before the administration of the most potent compound 7a and then acetic acid was injected after 30 min.⁵⁵ Pretreatment of rats with substance P results in the reversal of the analgesic effect of compound 7a as shown in Fig. 7. Further, compound 7a exhibits poor inhibition of 5-LOX (IC_{50} of 12 μ M) as compared to COX-2 (IC_{50} of 0.41 μ M) which clearly indicates that COX-2 may be the probable target of 7a. These results suggest that compound 7a has an

analgesic effect in acetic acid induced algnesia due to inhibition of the cyclooxygenase-2 pathway.

2.2.6. Acute toxicity studies. Acute toxicity studies on the most active compound were carried out on either sex of rats as stated in OECD guidelines.⁶¹ Four groups of animals were included in this study with three animals per group. The first group of animals served as the control and the second, third, and fourth groups were treated with the tested compound 7a at varied doses up to 2000 mg kg⁻¹, respectively, after 4 h of fasting. All the animal groups were continuously observed for the first 4 h and periodically for 24 h. After 14 days, histological studies were performed *via* H and E staining by sacrificing one animal each from the control and the highest dose (2000 mg kg⁻¹). The animals treated with under investigation compound 7a at a dose of 50 mg kg⁻¹ displayed no behavioral changes, whereas the animals administered with doses of 300 and 2000 mg kg⁻¹ experienced some irritation, itching, and sneezing occasionally after 1–4 h of treatment. However, it should be mentioned here that these symptoms diminish within the next 6 h and no significant changes were observed in the post-mortem analysis of the myocardium, liver or kidney tissues of the animals administered with the highest dose of compound 7a (2000 mg kg⁻¹) as compared to the control group (Fig. 8).

Fig. 8 Photomicrographs of hematoxylin–eosin stained sections of the control myocardium (A), kidney (B), and liver (C) and compound 7a (2000 mg kg⁻¹)-treated myocardium (D), kidney (E), and liver (F). The pictures were taken with a light microscope at $\times 200$ magnification.

Fig. 9 A) Docking of compound 7a in the active site of COX-2; B) overlay representation of celecoxib with compound 7a.

2.2.7. Molecular docking study. A molecular docking study was performed using Gold score 5.1 software to predict and compare the selectivity of coumarin-pyrazoline derivatives against COX-2 (PDB 3LN1),^{56,57} COX-1 (PDB 3KK6)^{56,58} and 5-LOX (PDB 3V99) enzymes.^{56,59} The affinities of the synthesized compounds were evaluated in terms of Gold score and it was observed that the synthesized compounds 7a–g showed good binding interaction with the active site of the COX-2 enzyme as compared to COX-1 and 5 LOX, which were also taken into consideration to evaluate the selectivity (Table S4[†]). Further, molecular docking results revealed that the most potent compound 7a was fitted well to the active site of COX-2 with Gold score = 88.25, as compared to celecoxib (Gold score = 96.42; Table S4[†]). The carbonyl of coumarin is involved in hydrogen bonding with Tyr341 and the acetyl group of pyrazoline interacts with Ser516. The aromatic ring of coumarin also showed π -H hydrophobic interaction with Val 509 (Fig. 9A). This may explain the selectivity of the synthesized coumarin pyrazolines against the COX-2 enzyme. The complete overlay of the standard celecoxib was observed with compound 7a as shown in Fig. 9B. The docking results suggest that compound 7a may potentially and selectively target the COX-2 enzyme. An *in silico* ADME study on synthesized compounds 7a–g was performed using the Swiss ADME predictor to predict the absorption, distribution, metabolism and elimination (ADME) parameters (Table S5[†]). It was observed that all the coumarin pyrazolines 7a–g showed ADME properties in the acceptable range, with no violation according to Lipinski's rule of five.⁶⁰ Therefore, these compounds can be further utilized to be developed as drug candidates.

3. Conclusion

Coumarin based pyrazolines 7a–g were synthesized and evaluated for *in vitro* and *in vivo* anti-inflammatory activity. From the *in vitro* and *in vivo* experiments, compound 7a was identi-

fied as the highly active anti-inflammatory agent comparable to the standard etoricoxib. Compound 7a significantly suppressed the cytokine level and showed remarkable analgesic and anti-inflammatory activities with minimum toxicity risk. Testing of this compound on Wistar rat showed reversal of algnesia and inflammation with comparable efficacy to the standard drug etoricoxib by targeting specifically COX-2. Further, molecular docking studies supported that COX-2 is the possible selective target for the mode of action of compound 7a, which was also evidenced by *in vivo* mechanistic studies using substance P as the biomarker. The obtained results suggest that compound 7a could be used as a potential lead for the development of efficient anti-inflammatory agents.

Conflicts of interest

The authors declare that they have no conflict of interests.

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