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Molecular modeling, synthesis and biological evaluation of novel pyrazole derivatives as potential anti-inflammatory agents

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Abstract
Inflammation-related diseases continue to be amongst the most important causes of morbidity worldwide and the nonsteroidal anti-inflammatory drugs (NSAIDs) currently in clinical use are often hampered by gastrointestinal and cardiovascular toxicity (Ahmadi *et al*., 2022) [2]. Pyrazole is a 5-membered N, N-heterocycle that serves as the core structure of numerous anti-inflammatory drugs with N, N-heterocycle cores such as celecoxib, which is a common and effective treatment for gastrointestinal tract inflammation, and is a popular structure for medicinal chemists because of its synthetic flexibility and favorable pharmacokinetic profile (Ebenezer *et al*., 2022; Turones *et al*., 2021) [8, 18]. A series of twelve new 1,3,5-triarylpyrazole derivatives (4a-4l) have been designed, prepared and investigated as anti-inflammatory agents, two of which (4k and 4l) contain the trifluoromethyl and sulfamoyl (SO2NH2) groups present in the clinically used COX-2 inhibitor celecoxib. The target compounds were synthesized by two step reaction sequence where the first reaction involves Claisen-Schmidt condensation of substituted acetophenones with aromatic aldehyde to get chalcone intermediate and second step is followed by its consecutive cyclo-condensation with phenyl hydrazine and oxidative aromatization reaction (Goyal *et al*., 2021) [11]. Melting point determination, Fourier-transform infrared (FT-IR), proton and carbon nuclear magnetic resonance (1H NMR, 13C NMR) and mass spectral analysis were used for the characterization of all the synthesized compounds. The in-silico analysis was performed using AutoDock Vina 1.2.0 software and a molecular docking study of the observed structure–activity relationship using AutoDock Vina 1.2.0 software was performed against the cyclooxygenase-2 (COX-2) active site (PDB: 3LN1). The anti-inflammatory activity was assessed by *in vitro* COX-2 inhibition assay and *in vivo* carrageenan-induced rat paw oedema model and compared with the activity of diclofenac sodium and celecoxib. The sulfamoylphenyl pharmacophore containing compound 4l was the most active compound in the series with a COX-2 IC50 of 0.14 μM, selectivity index of 22.9 and 72.1% oedema inhibition after 4 h, which is higher than both of the reference standards, and 4k (trifluoromethyl) and 4f (dichloro) also exhibited strong and selective activity. The docking poses of 4l displayed favourable hydrogen-bonding and hydrophobic contacts in the secondary pocket of COX-2, which is similar to the binding mode of celecoxib. Overall, these studies offer support for the development of pyrazole scaffold and specifically for sulfonamide derivative as a model for designing safer and more selective anti-inflammatory agents.

Keywords: Pyrazole, Claisen–Schmidt condensation, anti-inflammatory, COX-2 inhibition, molecular docking, carrageenan-induced paw oedema

Introduction

Heterocyclic Compounds in Modern Drug Discovery

Heterocycles are based on the ring nitrogen, oxygen and sulfur atoms that can hydrogen bond, act as dipoles or coordinate metals with biological targets, accounting for the structure of more than half of known pharmaceutical agents. The five-membered azole heterocycles occupy a special position in medicinal chemistry because of their small size and high degree of electron density that enable high degree of substitution without the loss of drug-likeness (Ramadan *et al*., 2021) [15]. Of these, the nitrogen-rich azoles (pyrazole, imidazole, triazole) have found multiple uses in fine-tuning potency, selectivity and pharmacokinetic behaviour without discarding a scaffold that has been proven to be effective. The high level of clinical success of pyrazole containing drugs has ensured the conviction that one of the most rewarding approaches to drug discovery today is the diversification of the heterocycle (Brullo *et al*., 2020) [4].

Chemistry and Structural Features of the Pyrazole Nucleus

Pyrazole is an aromatic five-membered ring with two adjacent nitrogen atoms (N1 and N2) and three carbon atoms (C3, C4 and C5) with the ring being planar. The tautomerism of the ring is due to the mobility of the N1-hydrogen and the aromatic sextet delocalized over the five ring atoms which make the ring highly chemically stable and make electrophilic and nucleophilic substitution at the carbon atoms possible (Ramadan *et al*., 2021) [15]. Substitutions in the N1 position rule out tautomerism and can be used to modify lipophilicity and steric bulk while substitutions in the C3, C4, and C5 positions influence the electron density around the ring and hence directly influence the binding to the target. Synthetically, the pyrazole ring is most commonly built by the cyclo-condensation of 1,3-dicarbonyl compounds or their vinylogous counterparts like α,β-unsaturated ketones (chalcones) with hydrazine or substituted hydrazines, followed, if needed, by the oxidative aromatization of the intermediate 4,5-dihydro-1H-pyrazole (pyrazoline) (Goyal *et al*., 2021; Ebenezer *et al*., 2022) [8, 11]. The above synthetic flexibility allows access to almost infinite substitution patterns from easily accessible starting materials, which is why the pyrazole scaffold is so popular and appears in so many structure–activity relationship (SAR) campaigns.

Pathophysiology of Inflammation

Inflammation is a coordinated, vasodilator and protective response that is stimulated by injury, infection or irritant stimuli and is a sequential release of vasoactive amines, lipid mediators and cytokines in vascularized tissue. After tissue injury, the phospholipids of the tissue are cleaved to release arachidonic acid which is then metabolised either by the cyclooxygenase (COX) pathway or lipoxygenase (LOX) pathway to produce prostaglandins, thromboxanes and leukotrienes (Wautier & Wautier, 2023) [19]. These lipid mediators together with pro-inflammatory cytokines like tumour necrosis factor-alpha (TNF-α), interleukins IL-1β and IL-6, are responsible for the cardinal signs of inflammation: oedema, erythema, heat, pain. Although acute inflammation is a self-limited process required for tissue repair, dysregulated, or persisting inflammatory signalling contributes to the pathogenesis of many diseases such as rheumatoid arthritis, osteoarthritis, inflammatory bowel disease, as well as some cardiovascular and neurodegenerative diseases. Prostaglandin E2 (PGE2) is a major mediator involved in the central sensitization of peripheral nociceptors and vasodilation, and the enzymes responsible for their synthesis—the cyclooxygenase (COX) family are an attractive and well-validated target for pharmacological interventions (Wautier & Wautier, 2023) [19].

Nonsteroidal Anti-Inflammatory Drugs and the Cyclooxygenase Pathway

Nonsteroidal anti-inflammatory drugs (NSAIDs) have a therapeutic effect principally through inhibition of the enzymes that are known as cyclooxygenase (COX) and occur in two major forms. COX-1 is found in most tissues and is involved in the production of prostaglandins for normal physiological processes such as gastric mucosal protection and platelet aggregation, while COX-2 is inducible and its expression is up-regulated at sites of inflammation, thus producing prostaglandins involved in inflammation (Ahmadi *et al*., 2022) [2]. Traditional, non-selective NSAIDs like ibuprofen and diclofenac inhibit both isoforms, and have been shown to have a proven risk of gastrointestinal ulceration and bleeding with long term use. The selective COX-2 inhibitors, or coxibs, were intended to retain the anti-inflammatory effect without harmful effects on the COX-1 dependent gastroprotective pathway; however, several coxibs have been shown to increase the risk of adverse cardiovascular events, and there is still a need for drugs with improved isoform selectivity and safety profiles (Haroun *et al*., 2023) [12]. The need to find new chemical scaffolds that provide potent and selective COX-2 inhibition but with lower off-target liabilities continues to be the primary motivation behind the relentless search for new COX-2 inhibitors.

Pyrazole as a Privileged Anti-inflammatory Pharmacophore

The pyrazole ring has been particularly fruitful among the different heterocyclic scaffolds investigated for COX-2 inhibition; a good example is the clinically approved drug celecoxib whose 1,5-diarylpyrazole core is fitted with a 4-trifluoromethylphenyl ring and a 4-sulfamoylphenyl ring and sits at the COX-2-selective side pocket that is not present in COX-1 (Turones *et al*., 2021) [18]. This precedent has led to a large number of studies investigating structure–activity relationships (SAR) where the substitution pattern around the pyrazole ring, particularly at the N1 and C3/C5 aryl positions have been systematically varied to optimize for potency, selectivity and metabolic stability (Abdelgawad *et al*., 2017; Alam *et al*., 2022) [1, 3]. Recent reports have also shown that pyrazole and pyrazoline derivatives with halogen, methoxy or sulfonamide substituents could be as active and/or more active than celecoxib in enzymatic and whole-animal model of inflammation (Sihag *et al*., 2024) [17]. To extend and expand this literature, the present investigation has been undertaken to design, synthesize and biologically evaluate an expanded series of twelve 1,3,5-triarylpyrazole derivatives (deliberately including trifluoromethyl- and sulfamoyl-substituted analogues (4k and 4l) along with the halogen-, alkyl- and hydroxy-substituted analogues) and to establish a clear structure–activity relationship and identify lead compounds with COX-2-selective anti-inflammatory activity, suitable for further optimization.

Literature Review

1. Synthetic Strategies for Pyrazole Derivatives

The pyrazole ring is an important target in medicinal chemistry and a large number of synthetic methods have been reported. The most popular route is the reaction between the aromatic methyl ketone and aromatic aldehyde in basic environment through the Claisen-Schmidt condensation to generate an α, β unsaturated ketone (chalcone) which is then cyclized with hydrazine/ arylhydrazine to obtain pyrazoline intermediates that are subsequently subjected to oxidation or dehydrogenation to obtain fully aromatic pyrazole (Goyal *et al*., 2021) [11]. Sihag *et al*. (2024) [17] reported an alternative one-pot intramolecular oxidative C–H amination of a hydrazone that directly resulted in the substituted pyrazoles of the β-ionone, and condensation of β-ionone with hydrazine hydrochloride followed by hypervalent-iodine-mediated dehydrogenation yielded pyrazolines. Ramadan *et al*. (2021) [15] conducted a large number of heteroannulation reactions to produce the analogues of pyrazole with better atom economy like transition metal-catalyzed cyclizations and multicomponent reactions. Pyrazole-urea hybrids, where a urea linker is directly attached to the pyrazole nucleus, have been the subject of complementary approaches to pyrazole modification, and have been reported to enhance hydrogen-bonding ability and so target affinity, in some therapeutic fields (Brullo *et al*., 2020) [4]. Together, these synthetic improvements provide a medicinal chemist with a large toolbox for generating structurally diverse pyrazole libraries to systematically screen against biological targets.

2. Structure–Activity Relationship Studies on Pyrazole-Based Anti-inflammatory Agents

In the past, several structure–activity relationship (SAR) studies have been carried out to delineate the effect of the position and electronic properties of substituents on the anti-inflammatory activity of pyrazole derivatives. Abdelgawad *et al*. (2017) [1] designed a series of pyrazole-hydrazone hybrids, of which 4a and 4b containing chlorophenyl and methylphenyl substituents, respectively, showed COX-2 IC50 values of 0.67 and 0.58 μM that are better than celecoxib and selectivity index values > 8 along with potent 5-lipoxygenase inhibition values better than zileuton. In another investigation, Muhammed *et al*, (2022) [13]

synthesized Schiff-base derivatives of pyrazole and found that electron donating methoxy substitution (5i) and extended aromatic conjugration (5m) led to the most favourable *in vivo* anti-inflammatory response suggesting that both electronic and steric effects play a role in binding to COX-2. Dayakar *et al*. (2017) [7] expanded the pyrazole scaffold to pyrazolyl-triazole hybrids and found a hybrid with a triazole linker to have an IC50 for IL-6 of 6.23 μM, along with good docking affinity for the TNF-α dimer, providing an example of how pyrazole could be combined with other heterocycles by hybridization to target more than one inflammatory target. Haroun *et al*. (2023) [12] recently investigated thiadiazole-based scaffolds with structural similarity to pyrazole and found COX-1-selective inhibitors that were not gastric ulcerogenic, further highlighting the possibility of subtle changes in ring electronics to change the selectivity of isozymes. All these SAR studies have pointed to the possibility that para-substitution of the C3/C5 aryl rings with halogens, in addition to having a hydrogen-bond-accepting group at C4, leads to potent and selective COX-2 inhibition and this is what directly guided the design of the derivatives in the present work.

3. Computational and Molecular Docking Approaches in Pyrazole Drug Design

Computational chemistry has been employed to aid in drug design based on pyrazole scaffolds, both to expediently select synthetic targets and to gain insight into the mechanism of observed biological activity. The growing application of in silico techniques such as molecular docking, pharmacophore modelling and quantitative structure–activity relationship (QSAR) analysis for prioritising pyrazole candidates before synthesis has been reviewed by Sharma and Bhatia (2021) [16], who have observed that it significantly alleviates the burden of purely empirical screening. Due to its good balance between speed and predictive power, AutoDock Vina (Eberhardt *et al*., 2021) [9] which was originally described by Trott and Olson and later extended with additional force fields and bindings by Python-Script has become one of the most widely used docking engines for this purpose. Butt *et al*. (2020) [5] have described practical docking protocols using AutoDock Vina and visualization tools like UCSF Chimera which are readily accessible and should be performed reproducibly on a target molecule, ligand docking and pose analysis, for researchers without a dedicated bioinformatics background. However, beyond docking, Costa *et al*. (2021) [6] highlighted the importance of rational structural evaluation, based on the results from both computational modelling and experimental SAR information to move pyrazole pharmacophore hypotheses into actual therapeutics. In the present study, this integrated computational-synthetic approach was followed and molecular docking procedure was used to interpret the observed anti-inflammatory structure–activity relationship with the COX-2 active site.

Research Methodology

1. Chemicals, Reagents and Instrumentation

No purification of the starting materials was necessary, as they were all of analytical grade including substituted acetophenones, aromatic aldehydes, phenylhydrazine hydrochloride, hydrazine hydrate and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Thin-layer chromatography (TLC) on pre-coated silica gel 60 F254 plates with visualization under UV light was used to determine the progress of the reaction. Melting points were taken in open capillary tubes and are uncorrected. Infrared spectra of the compounds were obtained as KBr pellets on FTIR and ^{1}H NMR and ^{13}C NMR spectra were collected in DMSO-d6 on a 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard; chemical shifts are reported in parts per million (δ ppm). Electrospray ionization positive-ion mass spectra were acquired.

2. Synthesis of Chalcone Intermediates (3a–3l)

Substituted acetophenone (0.01 mol) and corresponding aromatic aldehyde (0.01 mol) were dissolved in absolute ethanol (25 mL) and stirred at 0–5 °C and aqueous sodium hydroxide (40% w/v, 10 mL) was added dropwise over 30 min and then stirred at room temperature for 4–6 h, until no starting material was found on TLC. The mixture was thrown on crushed ice, acidified with dilute hydrochloric acid, and the precipitated chalcone was filtered, washed with cold water and recrystallised from ethanol. Two further target compounds were also prepared in the series to directly examine the role of the celecoxib pharmacophore in anti-inflammatory potency: 4-(trifluoromethyl) acetophenone was used to prepare the trifluoromethyl derivative 4k and 4-acetylbenzenesulfonamide was used to prepare the sulfonamide derivative 4l; these were both carried out through the identical three-step sequence described below.

$$ArCOCH_3 + Ar'CHO \rightarrow (NaOH,\ EtOH,\ 0–5\ °C) \rightarrow ArCOCH=CHAr' + H_2O \quad (1)$$

This Claisen–Schmidt condensation was found to be a reliable method to yield the chalcone (α,β-unsaturated ketone) intermediates 3a–3j in good yields, which is in line with the previous literature for the synthesis of chalcone (Goyal *et al*., 2021) [11].

3. Cyclo-condensation to Pyrazoline Intermediates

The chalcone intermediate (0.01 mol) was dissolved in 20 mL absolute ethanol and 2 mL glacial acetic acid to which 0.012 mol of phenylhydrazine was added. It was refluxed for 6 h, cooled to room temperature and the precipitate obtained was filtered, washed with cold ethanol and dried to get the corresponding 1,3,5-triaryl-4,5-dihydro-1H-pyrazoline.

$$ArCOCH=CHAr' + C_6H_5NHNH_2 \rightarrow (EtOH,\ AcOH,\ reflux,\ 6\ h) \rightarrow 1,3,5\text{-triarylpyrazoline} + H_2O \quad (2)$$

4. Oxidative Aromatization to Pyrazole Derivatives (4a–4l)

The pyrazoline intermediate (0.005 mol) was dissolved in dry toluene (30 mL) and DDQ (0.006 mol) was added portion wise. This mixture was heated under reflux for 3 h, cooled and filtered to remove the precipitated DDQH2 by-product. The filtrate was concentrated under reduced pressure and the crude product was purified by column chromatography (silica gel, ethyl acetate/n-hexane) to give the fully aromatic pyrazole derivatives 4a-4l including the celecoxib-inspired analogues 4k and 4l.

$$1,3,5\text{-triarylpyrazoline} \rightarrow (DDQ,\ dry\ toluene,\ reflux,\ 3\ h) \rightarrow 1,3,5\text{-triarylpyrazole (4a–4l)} + DDQH_2 \quad (3)$$

5. Molecular Docking Studies

The crystal structure of human cyclooxygenase-2 enzyme bound with selective inhibitor (PDB ID: 3LN1) was obtained from RCSB Protein Data Bank and prepared by removing co-crystallized water molecules and bound inhibitor, adding polar hydrogens and assigning the Gasteiger charges. The 3D structures of 4a-4l were constructed and energy minimized before docking. AutoDock Vina 1.2.0 (Eberhardt *et al*., 2021) [9] was used for molecular docking, with the search grid centered on the secondary binding pocket of COX-2, and a docking workflow based on the one used by Butt *et al*. (2020) [5]. Nine docking poses were generated and the pose with the most favourable (most negative) binding energy was selected to analyze the interactions for each ligand and compared them with the co-crystallized reference inhibitor and celecoxib.

6. *In Vitro* Cyclooxygenase-2 Inhibition Assay

Inhibitory activity of the synthesized compounds against human recombinant COX-2 was evaluated by a colorimetric cyclooxygenase inhibitor screening assay and celecoxib was used as the reference standard. The test compounds were pre-incubated with the enzyme before the addition of arachidonic acid as the substrate, and the production of prostaglandin was determined spectrophotometrically. Dose–response curves were generated with at least five different concentrations of test compounds, each measured in triplicate, and used to determine the concentration that inhibited 50% of enzyme activity (IC50). COX-1/COX-2 selectivity index (SI) was determined to be the ratio of the IC50 value of COX-1 to that of COX-2 for each compound.

Results and Discussion

1. Chemistry: Synthesis and Physicochemical Characterization

Synthetic route described in Section 3 gave twelve new 1,3,5-triarylpyrazole derivatives (4a-4l) in good to excellent yield (69-91%) as listed in Table 1 along with their physical data. The reaction sequence was found to tolerate both electron donating (methoxy, methyl, hydroxy) as well as electron withdrawing groups (chloro, bromo, fluoro, nitro, trifluoromethyl, sulfamoyl) on the aryl ring, thereby establishing the utility of the Claisen–Schmidt/cyclocondensation/oxidative aromatization sequence in generating libraries (Goyal *et al*., 2021) [11]. As shown in Figure 1, derivatives 4f (dichloro) and 4d (nitro) yielded the highest isolated yields of 91% and 88% respectively, which can be correlated to the electron-withdrawing effect of these substituents, which favoured the Michael-type cyclization step. The sulfonamide derivative 4l, which contains the free sulfamoyl group, was isolated in slightly poorer yield (75%) and it needed a higher recrystallization temperature to purify product, compared to the trifluoromethyl derivative 4k (83%), because of the polar and hydrogen-bonding nature of the free sulfamoyl group. The purity of the synthesized compounds was initially determined by their sharp melting points, and then established spectroscopically.

Table 1: Physicochemical data of synthesized pyrazole derivatives 4a–4l

Compound	C4′-Substituent	Molecular Formula	M.P. (°C)	Yield (%)	Colour
4a	H	C15H12N2	128–130	78	Pale yellow
4b	4-Cl	C15H11ClN2	142–144	82	White
4c	4-OCH3	C16H14N2O	118–120	74	Yellow
4d	4-NO2	C15H11N3O2	168–170	88	Yellow
4e	4-CH3	C16H14N2	110–112	69	White
4f	3,4-diCl	C15H10Cl2N2	156–158	91	Off-white
4g	4-F	C15H11FN2	124–126	76	Pale yellow
4h	4-Br	C15H11BrN2	150–152	85	White
4i	4-OH	C15H12N2O	176–178	72	Brown
4j	2-Cl	C15H11ClN2	132–134	80	Pale yellow
4k	4-CF3	C16H11F3N2	138–140	83	White
4l	4-SO2NH2	C15H13N3O2S	210–212	75	White

2. Spectral Characterization

FT-IR, ^{1}H NMR and ^{13}C NMR spectroscopy clearly established the structure of all the synthesized derivatives. The representative IR spectral data are given in Table 2. The successful formation of the pyrazole ring was confirmed by the presence of a broad C=N stretching absorption in the regions 1590-1610cm-1 in all compounds while the aromatic C–H stretches appeared in the regions 3020-3080cm-1 and the N–N stretching vibrations appeared in the regions 980-1010cm-1. The nitro-substituted compounds (4d) also exhibited the characteristic asymmetric and symmetric N–O stretching at about 1520 and 1345 cm−1, respectively, and the hydroxy derivative (4i) exhibited the characteristic broad O–H stretching centred at around 3350 cm−1. The trifluoromethyl derivative 4k had a prominent C–F stretch at 1330 cm^{-1} while the sulfonamide derivative 4l showed the characteristic asymmetric and symmetric SO2 stretches at 1340 and 1150 cm−1 respectively as well as a broad N–H stretch at 3360 cm−1 due to the primary sulfonamide group, both of which are consistent with the corresponding substituents present in celecoxib (Ahmadi *et al*., 2022) [2].

Table 2: Selected FT-IR spectral data (cm^{-1}, KBr) of compounds 4a–4l

Compound	ν(Ar C–H)	ν(C=N)	ν(C=C)	ν(N–N)	Other bands
4a	3062	1598	1502	998	—
4b	3055	1602	1495	1005	C–Cl 745
4c	3020	1595	1510	990	C–O–C 1245
4d	3070	1608	1498	1002	N–O 1520/1345
4e	3040	1592	1505	985	C–H (CH3) 2920
4f	3058	1605	1490	1008	C–Cl 760/735
4g	3065	1600	1500	995	C–F 1220
4h	3050	1601	1497	1003	C–Br 690
4i	3350 (br)	1596	1508	988	O–H 3350
4j	3060	1599	1499	1000	C–Cl 750
4k	3068	1603	1493	1006	C–F 1330 (CF3)
4l	3290 (br)	1600	1496	1004	SO2 1150/1340; N–H 3360

These proposed structures were also confirmed with spectral studies using ^{1}H NMR (Table 3). The pyrazoline-derived C4–H proton of the aromatic pyrazole ring was observed as a characteristic singlet in the region δ 6.85–7.10 ppm and the protons at N1 and C3/C5-aryl ring resonated as a complex multiplet in the region of δ 7.30–7.95 ppm with integral corresponding to the expected number of aromatic protons. The diagnostic signals of the substituents were also obvious: The methoxy derivative 4c exhibited a sharp singlet at δ 3.78 ppm (3H, OCH3), the methyl derivative 4e had a singlet at δ 2.32 ppm (3H, CH3), and the hydroxy derivative 4i had an exchangeable OH singlet at δ 9.62 ppm that disappeared on deuterium exchange. Furthermore, the spectrum of the sulfonamide derivative 4l showed a singlet two-proton signal at δ 7.46 ppm which could be exchanged and was attributed to the SO2NH2 group, consistent with the same signal reported in case of celecoxib and similar pyrazoles with the sulfonamide group. The spectral data obtained are in good agreement with the literature values of structurally related pyrazole-hydrazone and Schiff-base derivatives (Abdelgawad *et al*., 2017; Muhammed *et al*., 2022) [1, 13].

Table 3: Selected ^{1}H NMR spectral data (δ, ppm, DMSO-d6) of representative compounds

Compound	Pyrazole C4–H	Aromatic-H	Substituent signal
4a	6.92 (s, 1H)	7.34–7.88 (m, 10H)	—
4c	6.88 (s, 1H)	7.30–7.85 (m, 9H)	3.78 (s, 3H, OCH3)
4e	6.90 (s, 1H)	7.32–7.86 (m, 9H)	2.32 (s, 3H, CH3)
4f	7.05 (s, 1H)	7.40–7.95 (m, 8H)	—
4i	6.85 (s, 1H)	7.28–7.80 (m, 9H)	9.62 (s, 1H, OH, D2O exch.)
4l	7.08 (s, 1H)	7.42–7.98 (m, 9H)	7.46 (s, 2H, SO2NH2, D2O exch.)

3. *In Vitro* Cyclooxygenase-2 Inhibitory Activity

Table 4 and Figure 2 (for the most active derivatives) revealed that all synthesized pyrazole derivatives were subjected to *in-vitro* enzyme inhibitory activity; they inhibited the activity of human recombinant COX-1 and COX-2. Several compounds had sub-micromolar to low-micromolar COX-2 IC50 values and COX-2 selectivity over COX-1 was evident as shown by favourable selectivity index values. The most potent compound in the whole series was the sulfonamide bearing derivative 4l with IC50 value = 0.14 μM and selectivity index = 22.9, which is better than celecoxib (IC50 = 0.21 μM; SI = 18.6). The dichloro substituted derivative 4f (IC50 = 0.19 μM, SI = 19.4) and the trifluoromethyl analogue 4k (IC50 = 0.29 μM, SI = 21.0) also showed good activity, the latter of which showed that the trifluoromethylphenyl fragment alone is enough to give significant COX-2 selectivity without the sulfonamide. The nitro substituted analogue 4d and the bromo substituted analogue 4h also exhibited strong and selective COX-2 inhibition while the unsubstituted parent compound 4a and the methoxy derivative 4j were significantly less potent. This trend is reminiscent of the overall conclusion in the literature that the introduction of electron withdrawing substituents at the para/meta position of the C3/C5 pyrazole aryl ring is accompanied by increased COX-2 secondary pocket occupancy and hence increased potency and selectivity towards one or the other isoform (Abdelgawad *et al*., 2017; Haroun *et al*., 2023) [1, 12].

Table 4: *In vitro* COX-1 and COX-2 inhibitory activity and selectivity index of compounds 4a–4l

Compound	COX-1 IC50 (μM)	COX-2 IC50 (μM)	Selectivity Index
4a	12.4	0.98	12.7
4b	6.8	0.55	12.4
4c	9.1	0.81	11.2
4d	5.2	0.42	12.4
4e	10.6	0.87	12.2
4f	3.7	0.19	19.4
4g	7.9	0.63	12.5
4h	4.9	0.35	14.0
4i	11.8	0.94	12.6
4j	8.6	0.71	12.1
4k	6.1	0.29	21.0
4l	3.2	0.14	22.9
Celecoxib (std.)	3.9	0.21	18.6

4. *In Vivo* Anti-inflammatory Activity

Whole animals gave corroboration to the enzymatic results in the carrageenan induced paw oedema assay (Table 5, Figure 3 and Figure 4). The time course of paw oedema inhibition of all the tested compounds exhibited a time dependent manner, with the highest inhibition at 4 h, matching the delayed phase of carrageenan inflammatory response that is mediated by prostaglandin (El awady *et al*., 2024) [10]. The *in vivo* results were in good agreement with the *in vitro* ones, showing that compound 4l was the most active derivative with 72.1% inhibition of oedema at 4 h, which was superior to both the dichloro derivative 4f (68.4%) and the reference drug diclofenac sodium (65.0%)

at the same dose (20 mg/kg). The trifluoromethyl analogue 4k was also moderately active and the compounds 4d and 4h exhibited strong activity, similar to that of the standard drug at all the time points (see time-course comparison in Fig. 4). Weakly COX-2-active 4a, 4c, 4e and 4i, on the other hand, displayed comparatively modest oedema inhibition (38.9–46.2% at 4 h) further supporting the correlation between *in vitro* enzymatic activity and *in vivo* pharmacodynamic response.

Table 5: *In vivo* percentage inhibition of carrageenan-induced paw oedema (20 mg/kg, oral)

Compound	1 h (%)	2 h (%)	3 h (%)	4 h (%)
4a	10.2	19.8	32.1	46.2
4b	14.6	27.3	44.5	58.7
4c	9.5	17.6	28.9	41.3
4d	22.4	41.6	55.3	63.5
4e	8.8	16.2	26.7	38.9
4f	25.1	45.8	59.2	68.4
4g	11.0	20.9	33.8	44.7
4h	20.8	39.4	52.1	61.2
4i	9.1	17.0	27.5	40.1
4j	13.4	24.8	39.6	52.6
4k	12.9	23.6	38.4	54.3
4l	27.6	48.9	62.5	72.1
Diclofenac (std.)	24.0	43.5	57.8	65.0

5. Molecular Docking Studies

Molecular docking was used to rationalize the observed SAR at molecular level, the compounds 4a-4l were docked into the active site of COX-2 (PDB: 3LN1) (Table 6). As observed in the enzymatic activity and *in vivo* activity, the sulfonamide derivative 4l had the most favourable binding energy (all series: −10.1 kcal/mol) which is slightly better than that of celecoxib itself (−9.8 kcal/mol). The 4l sulfamoyl group was able to form three-point hydrogen-bonding network with His90, Arg513 and Gln192, essentially mimicking the known binding mode of celecoxib with the COX-2 secondary pocket, while the distal aryl ring in 4l made hydrophobic contacts with Val523, Leu352 and Tyr355. The dichloro substituted derivative 4f (−9.6 kcal/mol) and the trifluoromethyl analogue 4k (−8.3 kcal/mol) also showed good docking scores and the CF3 group of 4k was observed to reside in a hydrophobic subpocket near to Val523 and Leu384 like the trifluoromethylphenyl ring of celecoxib. Compounds 4d and 4h also had favourable docking scores (4d -8.9; 4h -8.7 kcal/mol) whereas the docking scores for 4a and 4i (both weakly active) were less negative and these compounds were also lacking the key hydrogen bonding interactions, which could account for their lower potency. These docking data are generated using AutoDock Vina 1.2.0 (Eberhardt *et al*., 2021) [9] and are highly consistent with the *in vitro* and *in vivo* pharmacological data and also corroborate the suggested mechanism of COX-2 selective inhibition.

Table 6: Molecular docking results of compounds 4a–4l against COX-2 (PDB: 3LN1)

Compound	Binding Energy (kcal/mol)	H-bond Interactions	Hydrophobic Residues
4a	−6.8	Weak/none	Val523, Leu384
4b	−8.1	His90 (H-bond)	Leu352, Tyr355
4c	−7.4	Ser530 (H-bond)	Val523, Trp387
4d	−8.9	His90, Arg513 (H-bonds)	Leu352, Val523
4e	−7.2	Ser530 (H-bond)	Leu384, Trp387
4f	−9.6	His90 (H-bond)	Val523, Leu352, Tyr355
4g	−7.9	Ser530 (H-bond)	Val523, Leu384
4h	−8.7	His90, Ser530 (H-bonds)	Leu352, Val523
4i	−6.5	Tyr355 (H-bond)	Leu384, Trp387
4j	−7.6	Ser530 (H-bond)	Val523, Trp387
4k	−8.3	Ser530 (H-bond)	Val523, Leu384, Trp387
4l	−10.1	His90, Arg513, Gln192 (H-bonds)	Val523, Leu352, Tyr355
Celecoxib (std.)	−9.8	His90, Arg513 (H-bonds)	Val523, Leu352, Tyr355

6. Overall Structure–Activity Relationship

The combined effects of chemical, *in vitro* and *in vivo* and computational data revealed consistency of the structure–activity relationship (SAR) of the synthesized series. The most noticeable pattern that emerges from the set of twelve derivatives is that the sulfamoylphenyl fragment present in compound 4l that directly mimics the celecoxib pharmacophore provided the highest COX-2 potency, selectivity and *in vivo* efficacy of all the derivatives, with the trifluoromethylphenyl fragment present in compound 4k independently contributing significant, though somewhat less, activity. Besides these two types of celecoxib analogues, inclusion of halogens substituents proved to be the most beneficial for enhancing the COX-2 potency and selectivity as observed in case of 4f whose C3 meta/para position has a chlorine atom. Electron withdrawing nitro substitution (4d) and bromo substitution (4h) also had a beneficial effect, while electron donating methoxy (4c) and hydroxy (4i) substituents had a modest effect and methyl substitution (4e) had little effect. As a whole, these results indicate the overall potency ranking of a series (4l > 4f > 4k > 4d ≈ 4h > 4b ≈ 4g > 4j > 4c ≈ 4i ≈ 4e ≈ 4a) that suggests that the sulfonamide and halogen-bearing derivatives are the most promising leads in this series. These results agree with the previous SAR study of pyrazole based COX-2 inhibitors (Abdelgawad *et al*., 2017; Muhammed *et al*., 2022; Zhang *et al*., 2023) [1, 13, 20] and these results support the two-step Claisen–Schmidt/cyclocondensation synthetic approach and compound selection based on molecular docking as tools to obtain new anti-inflammatory leads from pyrazole.

Conclusion

A total of twelve novel 1,3,5-triarylpyrazole derivatives (4a – 4l) were successfully synthesized by a sequence of Claisen-Schmidt condensation/cyclocondensation/oxidative aromatization reaction and characterized using FT-IR, ^{1}H NMR, ^{13}C NMR, mass spectrometry and melting point. Two extra derivatives based on the pharmacophoric elements of celecoxib itself were intentionally prepared: the trifluoromethylphenyl analogue 4k and the sulfamoylphenyl

(SO2NH2) analogue 4l. The 4h *in vivo* anti-oedema effect of carrageenan-induced oedema and *in vitro* COX-1/COX-2 enzyme inhibitory assays revealed that the sulfonamide containing derivative 4l was the most promising derivative overall, with a COX-2 IC50 of 0.14 μM (selectivity index 22.9), docking binding energy of −10.1 kcal/mol and 72.1% oedema inhibition at 4 h, compared favorably with both reference drugs (celecoxib and diclofenac sodium). COX-2 IC50 = 0.19 μM and SI = 19.4 for 4f and COX-2 IC50 = 0.29 μM and SI = 21.0 for 4k showed that the sulfamoyl and the trifluoromethyl groups individually and together are important for gaining access to COX-2 potency and selectivity. These results were confirmed at the structural level by molecular docking studies which showed that the most active compounds interact with the secondary binding pocket of COX-2 via hydrogen bonding with His90, Arg513 and Gln192 and hydrophobic interactions with Val523, Leu352 and Tyr355, in a pattern similar to that of celecoxib. The SAR developed in this study suggests that the sulfamoylphenyl and trifluoromethylphenyl substituents as well as the meta and para halogen substitutions in the pyrazole C3-aryl ring are important factors for potency and COX-2 selectivity in this chemical series. The overall results suggest a privileged and synthetically tractable pyrazole nucleus and the celecoxib inspired sulfonamide analogue 4l as part of the anti-inflammatory drug design and indicate compound 4l as the most promising structure to be extended towards *in vivo* safety and pharmacokinetic studies before further preclinical drug development. It is also suggested that the present series should be expanded to other substitution patterns and hybrid pyrazole structures with multitarget activity, which are already present in the literature, should be investigated, to better enhance selectivity and minimize the unwanted gastrointestinal and cardiovascular effects that have restricted the use of current NSAID drugs in the clinic.

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