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Molecular docking, synthesis and biological screening of mefenamic acid derivatives as anti-inflammatory agents

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ABSTRACT

Drug induced gastrointestinal ulceration, renal side effects and hepatotoxicity are the main causes of numerous Non-Steroidal Anti-inflammatory Drugs (NSAIDs). Cyclooxygenase-2 (COX-2) inhibitors discovered to decrease the gastrointestinal issues, but unfortunately, most of them are associated with major cardiovascular adverse effects. Along these lines, various new strategies and frameworks were developed wherein basic alterations of the present medications were accounted for. The aim of the study was to prepare derivatives of mefenamic acid to evaluate anti-inflammatory activity with fewer adverse reactions. In this study, molecular docking investigations of outlined derivatives were done utilizing Protein Data Bank (PDB ID-4PH9). Synthesis of heterocyclic compounds was carried out utilizing Dicyclohexylcarbodiimide/4-Dimethylaminopyridine (DCC/DMAP) coupling. Acute toxicity prediction was performed using free online GUSAR (General Unrestricted Structure-Activity Relationships) software. The study indicated most of the compounds under safe category. *In-vitro* pharmacological assessment of heterocyclic compounds was done for COX-1 and COX-2 enzymes for the determination of selectivity. *In vivo* pharmacological screening for anti-inflammatory activity and ED_{50} value were determined utilizing carrageenan induced rat paw edema. Gastro intestinal safety study was carried out on selected compounds and found to be devoid of any gastric ulcer toxicity. Most of the compounds indicated high scores as compared to standard during molecular modelling, analysis and displayed interactions with active amino acids of a COX-2 enzyme. The pharmacological screening uncovered that compound substituted with p-bromophenyl indicated maximum potency.

1. Introduction

Anti-inflammatory drugs are used to treat pain and inflammation associated with musculoskeletal muscle and joints. Inflammation is a result of an increase in the level of prostanoids which are also responsible for the protection of the gastric mucosal membrane (Kalgutkar et al., 2002). Cyclooxygenase (COX) enzyme is involved in the rate-limiting step for the synthesis of different prostaglandins and thromboxanes from arachidonic acid (Marnett et al., 1999). The COX-1 enzyme is responsible for the cytoprotection and the COX-2 enzyme is inducible and responsible for the biosynthesis of Prostaglandins in inflammatory tissues (Dogné et al., 2006; Kalgutkar et al., 2002; Marnett et al., 1999). Unfortunately, anti-inflammatory agents discovered till now suffer from major side-effects. Classical NSAIDs are associated with adverse effects like gastric ulceration, hepatotoxicity, anemia etc. In order to overcome these side effects various selective COX-2 inhibitors were discovered. As a result, celecoxib (Celebrex®) (Penning et al., 1997), rofecoxib (Vioxx®), (Prasit et al., 1999) valdecoxib (Kalgutkar et al., 2000) and etoricoxib (Riendeau et al., 2001) were marketed as selective COX-2 inhibitors for the treatment of inflammation (Bansal et al., 2014). However, in the year 2004, Merck & Co., voluntarily withdrawn rofecoxib from the market due to severe cardiovascular adverse events. The adverse cardiovascular toxicity related to selective COX-2 inhibitors is due to the increased level of TXA_2 in platelet (Patrono and Baigent, 2014). Kalgutkar et al. synthesized various derivatives of classical NSAIDs (Aljadhey et al., 2010). They have synthesized various amide and ester derivatives of meclofenamic acid and indomethacin, which showed selectivity for the COX-2 enzyme. There are several reports which revealed that derivative preparation of well-known NSAIDs showed better results (Kalgutkar et al., 2000; Talley et al., 2000; Woods et al., 2001). Moreover, the gastric irritation associated with NSAIDs and COX-2 inhibitors is free of the route of the administration totally, mitigating any rationalization that removing the carboxylic acid moiety would bring about less gastro-

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intestinal irritation due to some sort of local effect. The points of interest offered by the utilization of the particular COX-2 inhibitors urged to evaluate the pharmacological movement of novel heterocyclic compounds that could be potential anti-inflammatory agents. Hence, preparation of amide derivatives of NSAIDs can be utilized as the classical approach for the development of more efficacious anti-inflammatory agents, which is our real objective.

2. Materials and methods

2.1. Molecular docking studies

Docking was used to predict both ligand orientation and binding affinity with the COX-2 enzyme. The response of designed molecules at the specific active site of the crystallographic structure of the protein was determined. SYBYL-X 1.2 software was used to build all the compounds and energy minimization was carried out using a conjugate gradient algorithm with a gradient convergence value of 0.01 kcal/Mol Å. Partial atomic charges were calculated using the Gasteiger Huckel method. To examine the binding affinities of the designed compounds with the COX-2 enzyme, docking of these compounds using GOLD 5.2 was performed. PDB ID- 4PH9 with a resolution of the 1.8 Å cocrystal structure was downloaded from RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank for docking study. The result of docking analysis was obtained in terms of GOLD score and was compared with the standard drug (Ganga Reddy et al., 2016).

2.2. LD_{50} prediction using GUSAR software

In silico acute toxicity prediction was performed using GUSAR (General Unrestricted Structure-Activity Relationships) software. GUSAR is a free online software, which include information about the acute toxicity of around 10,000 chemical structures. The software predicts activity of compounds based on QSAR (Quantitative Structure Activity Relationship) models. The data represented as the LD_{50} values (log10 (mmol/kg) of the compounds for different routes of administration like oral, subcutaneous, intravenous and Intraperitoneal. The toxicity class of the given compound was reported, according to the OECD (Organization for Economic Co-operation and Development) classification project of chemical substance (Korobko, 2016).

2.3. Synthesis of amide derivatives of mefenamic acid ***(3a-h)***

Synthesis of amide derivatives was carried out using borosilicate glass wares. REMI rota mantle and magnetic stirrers were used for heating, refluxing as well as stirring of the reaction mixtures. Solvent recovery was done using Rotary Vacuum evaporator (Buchi type). Precoated Silica Gel TLC plates (MERCK) were used for monitoring the progress of the reaction. UV chamber was used for the determination of progress of a reaction. Melting point was measured using the paraffin bath or by melting point apparatus (VEEGO corporation). IR spectra were recorded on JASCO FTIR by KBr dispersion method. Mass Spectra were recorded on BRUKER using ESI as an ion source. ^{1}H NMR and ^{13}C NMR spectra were recorded on BRUKER 400 MHz instrument using TMS as an internal standard.

2.3.1. Synthesis of 2-(2,3-dimethylphenylamino)-N-phenylbenzamide ***3a***

To a mixture of 0.002 mol of 2-(2,3-dimethylphenylamino)benzoic acid and 0.0022 mol of aniline in DCM (dichloromethane) and DMAP (dimethyl amino pyridine) was added with continuous stirring. After 30 min of stirring at 0 °C, the cooled solution of DCC (N,N`-dicyclohexylcarbodiimide) (1.1 equiv) was added in to the above reaction mixture and allowed to stir at room temperature under the N_2 environment for 15 h. The reaction was monitored using TLC using ethyl acetate: hexane (0.5: 4.5) as the solvent system. After completion of the reaction the pale yellow product was extracted using DCM after repeated washing with brine and sodium bicarbonate.

2.3.2. Synthesis of 2-(2,3-dimethylphenylamino)-N-p-tolylbenzamide ***3b***

Same as compound **3a**; in place of aniline, p-toluidine was used (0.002 mol) and the reaction was carried out for 15 h afforded a yellow colour product of **3b**.

2.3.3. Synthesis of 2-(2,3-dimethylphenylamino)-N-(4-fluorophenyl) benzamide ***3c***

Same as compound **3a**; in place of aniline, *p*-fluoroaniline was used (0.002 mol) and the reaction was carried out for 12 h afforded a yellow colour product of **3c**.

2.3.4. Synthesis of 2-(2,3-dimethylphenylamino)-N-(2-chlorophenyl) benzamide ***3d***

Same as compound **3a**; in place of aniline, *o*-chloroaniline was used (0.002 mol) and the reaction was carried out for 15 h afforded a dark yellow colour product of **3d**.

2.3.5. Synthesis of 2-(2,3-dimethylphenylamino)-N-(4-bromophenyl) benzamide ***3e***

Same as compound **3a**; in place of aniline, *p*-bromoaniline was used (0.002 mol) and the reaction was carried out for 15 h afforded a yellow colour product of **3e**.

2.3.6. Synthesis of 2-(2,3-dimethylphenylamino)-N-(2-(piperazin-1-yl)ethyl)benzamide ***3f***

Same as compound **3a**; in place of aniline, 2-aminoethyl piperazine was used (0.002 mol) and the reaction was carried out for 15 h afforded a yellow colour product of **3f**.

2.4. Synthesis of benzimidazole and benzthiazole derivatives ***(4a-4b)***

2.4.1. Synthesis of 2-(1H-benzo[d]imidazole-2-yl)-N-(2,3-dimethylphenyl)benzenamine ***4a***

To 0.1 mol of 3g in a 100 ml round bottom flask, 0.1 mol of glacial acetic acid and Con. HCl was added. The mixture was refluxed at 80 °C for 2.5 h. After the completion of the reaction, crushed ice was added to the reaction mixture with continuous stirring. The reaction mixture was neutralized with $NaHCO_3$ until precipitates were observed. The precipitates were filtered under the vacuum and washed with cold water. The product of **4a,** was purified using column chromatography (5% ethyl acetate: hexane).

2.4.2. Synthesis of 2-(1H-benzo[d]thiazol-2-yl)-N-(2,3-dimethylphenyl)benzenamine ***4b***

Same as compound **4a**; in place of **3g**, **3h** (0.002 mol) and the reaction was carried out for 17 h afforded a pale yellow colour product of **4b**.

2.5. Pharmacological Screening

2.5.1. In vitro COX-1 and COX-2 enzymatic assay

In vitro COX-I and COX-2 inhibition assay was performed for standard and synthesized compounds. Evaluation of COX inhibitory activity was carried out using a COX colorimetric inhibitor screening assay kit (Cayman Chemicals, USA) (Jang, 1997).

The assay kit includes both ovine COX-1 and human recombinant COX-2 enzymes in order to screen isozyme-specific inhibitors. The assay measures the peroxidase activity by monitoring the appearance of oxidized TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) at 590 nm.

2.5.2. Study of effect of heterocyclic derivatives on the animal model of carrageenan-induced paw edema

All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee (IAEC) of the Institute of Pharmacy, Nirma University, Ahmedabad as per guidelines of the committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), the Ministry of Social Justice and Empowerment, the Government of India. Protocol numbers are IP/PCOL/MPH/17/006 and IP/PCEM/FAC/20/028. Healthy Wistar rats (250–300 g) were procured from the Torrent Research Centre, Gandhinagar, Ahmedabad. Animals were housed in groups of 6 animals in the animal house of Nirma University, Ahmedabad under controlled conditions of temperature 23 ± 2 °C, relative humidity, 55 ± 5%, and photo-schedule (12 h light, and 12 h dark). Animals had free access to food and purified water ad *libitum*. Animals were acclimatized for one week before starting the experiment and randomized into different groups.

The animals were divided in to five groups- normal control group, disease control group, animals treated with mefenamic acid (12.8 mg/kg), animal treated with test compounds **3e** (12.85 mg/kg) and **3f** (12.85 mg/kg). 0.1 ml of 1% w/ v carrageenan solution was administered into the plantar side of the right hind paw of the rats (Chigayo et al., 2014). In the plethysmometer, mercury displacement method was used for the determination of the volume of the injected paw. The paw volume was measured 5hr after the injection of carrageenan. The difference between the left paw volume and the right paw volume gave the actual edema volumes. Paw volumes are physical indicators of inflammation in early as well as the chronic phase of the disease. Using the following formula the % inhibition of paw volume was calculated.

$$\textit{Increase in Paw Volume} = VD - VO$$

Where, VD=Paw Volume at 5 h, VO=Paw Volume at 0 min

$$\%\ \textit{Inhibition in Paw Volume} = VD - VO/VO \times 100$$

2.5.3. Determination of ED_{50} value of the selected compound on an animal model of carrageenan-induced paw edema

ED_{50} value for the selected compound was calculated to determine the dose required to cause a therapeutic effect in 50% of a population. In another set of experiment animals were randomized into eight groups- normal control group, disease control group, mefenamic acid (6.4 mg/kg, 12.8 mg/kg, 25.6 mg/kg), test compounds treated with **3e** (6.4 mg/kg, 12.8 mg/kg, 25.6 mg/kg). Using the same method paw edema was induced and calculated. ED_{50} values were estimated by back-transforming a linear regression model for percent inhibition as a function of log (dose).

2.5.4. The effect of heterocyclic derivative on the peptic ulcer

GI side-effects were evaluated after single-dose administration of drugs. After one hour of administration of selected compounds, various parameters were evaluated for gastrointestinal toxicity. All animals were killed and stomachs were removed, opened along the greater curvature and rinsed with saline to remove gastric contents and blood clots. Each stomach was examined grossly and the degree of ulceration was graded as 0, 0.5, 1, 2, 3, 4, 5 and 6 for no lesions or normal stomach, hyperemia (red coloration), hemorrhagic spots. Grade 1–5 describes small ulcers, many small ulcers, many small and large ulcers and stomach full of ulcers with perforations respectively.

3. Results

3.1. Docking studies

To inspect the binding affinities of compounds with the COX-2 enzyme, docking of these compounds utilizing both SYBYL X1.2 and GOLD 5.2 (PDB ID-4PH9 for mefenamic acid) was performed. Among the mefenamic acid derivatives, phenyl, 2-chlorophenyl, 4-tolyl and 4-bromophenyl analogs demonstrated higher scores than standard except **3c** as shown in the Table 1.

Table 1
Docking results of mefenamic acid analogs.

Compounds	GOLD score	Interaction
Mefenamic acid	47.17	TYR 386
3a	60.51	TYR386, ASN383, THR213
3b	57.92	HIS389
3c	45.77	TYR386
3d	58.51	TYR386, HIS208
3e	57.7	TYR386, ASN383
3f	54.35	TYR386
4a	58.66	HIS208, THR207, TYR 386
4b	57.43	TYR386, HIS208

3.2. Computer aided acute toxicity prediction

The free online GUSAR software has been employed for the prediction of acute toxicity of compounds under investigation. The values obtained for different routes of the administration of mefenamic acid derivatives as shown in Table 2. The LD_{50} values by Intravenous and oral administration were found to be between 55 to 130 and 866–2735 respectively.

3.3. Physical characterization of mefenamic acid derivatives

The synthetic protocol for the target molecules involves a single step. Mefenamic acid forms amide bond via DCC/DMAP coupling using different primary amines **(**Scheme 1**)**. Structure elucidation of synthesized compounds was done by using IR, Mass, NMR (both ^{1}H NMR and ^{13}C NMR) spectroscopy (Bali et al., 2012; Vasantha et al., 2015).

*3.3.1. Characterization of 2-(2,3-dimethylphenylamino)-N-phenylbenzamide **3a***

75% yield, m.p. 205–210 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 8.98 (s, 1 H) 7.78 (s, 1 H) 7.48 (t, *J*=7.56 Hz, 3 H) 7.29 (t, *J*=8 Hz, 2 H) 7.16 (t, *J*=7.96 Hz, 1 H) 7.06 (t, *J*=8.28 Hz, 2 H) 6.98 (t, *J*=7.76 Hz, 1 H) 6.88 (d, *J*=7.32 Hz, 1 H) 6.82 (d, *J*=8.4 Hz, 1 H) 2.24 (s, 3 H) 2.12 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 166.92, 146.63, 138.17, 137.08, 136.69, 131.72, 130.28, 128.09 (2 C), 126.30, 124.97, 124.79, 123.61, 120.43, 119.60 (2 C), 115.82 (2 C), 114.09, 19.63, 12.94. ESI-MS: *m/z*=316 $[M]^{+}$.

*3.3.2. Characterization of 2-(2,3-dimethylphenylamino)-N-p-tolylbenzamide **3b***

81% yield, m.p. 200–205 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 9.04 (s, 1 H) 7.78 (s, 1 H) 7.54 (d, *J*=7.88 Hz, 1 H) 7.45 (d, *J*=8.36 Hz, 2 H) 7.22 (d, *J*=7.12 Hz, 1 H) 7.14 (m, *J*=7.88 Hz, 3 H) 7.05 (t, *J*=7.76 Hz, 1 H) 6.95 (d, *J*=7.36 Hz, 1 H) 6.89 (d, *J*=7.84 Hz, 1 H) 6.75(t, *J*=7 Hz, 1 H) 2.34 (s, 3 H) 2.31 (s, 3 H) 2.19 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 167.91, 147.60, 139.28, 138.11, 135.12, 134.40, 132.65, 131.29, 129.64 (2 C), 127.31, 125.95, 125.82, 121.40, 120.79 (2 C), 117.02, 116.84, 115.10, 20.95, 20.68, 13.99. ESI-MS: *m/z*=330 $[M]^{+}$.

*3.3.3. Characterization of 2-(2,3-dimethylphenylamino)-N-(4-fluorophenyl)benzamide **3c***

48% yield, m.p. 190–195 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 9.18 (s, 1 H) 7.87 (d, *J*=7.88 Hz, 2 H) 7.15 (d, *J*=9.68 Hz, 2 H) 7.01 (t, *J*=7.68 Hz, 2 H) 6.93 (d, *J*=7.12 Hz, 2 H) 6.67 (d, *J*=8.48 Hz, 2 H) 6.56 (t, *J*=7.44 Hz, 1 H) 5.22 (s, 1 H) 2.255 (s, 3 H) 2.107 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 169.17, 149.44, 138.73, 138.24, 134.19,

Table 2
Acute toxicity prediction using GUSAR online software.

Compound code	R^1/X	LD_{50} (mg/kg)		
		Intraperitoneal route	Intravenous route	Oral route
3a	C_6H_5	2171 out of AD	130.3 (Class IV)	2032 (Class V)
3b	4-MeC_6H_4	2895 out of AD	119.5 (Class IV)	2735 (Class V)
3c	4-F-C_6H_4	2206 (Non-toxic)	115.6 (Class IV)	2028 (Class V)
3d	2-Cl-C_6H_4	1852 (Non-toxic)	77.550 (Class IV)	1395 (Class IV)
3e	4-Br-C_6H_4	2289 out of AD	157.6 (Class IV)	1931(Class IV)
3f	*N*-(2-piperazin−1-yl)ethyl	369.6 (Class IV)	55.330 (Class IV)	866.9 (Class IV)
4a	X=NH	277.9 out of AD	72.480 (Class IV)	1451 (Class IV)
4b	X=SH	757.1 out of AD	127.2 (Class IV)	1724 (Class IV)

Out of AD: Compound is out of applicability domain of models

132.46, 131.49 (2 C), 126.76 (2 C), 125.94 (2 C), 123.06 (2 C), 116.07 (2 C), 113.69 (2 C), 110.77, 20.68, 14.03. ESI-MS: *m/z*=334.8 $[M]^+$.

3.3.4. Characterization of 2-(2,3-dimethylphenylamino)-N-(2-chlorophenyl)benzamide ***3d***

80% yield, m.p. 200–202 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 9.18 (s, 1 H) 7.87 (dd, *J*=6.52 Hz, 2 H) 7.17 (m, 3 H) 7.15 (m, *J*=7.08 Hz, 2 H) 7.08 (d, *J*=7.6 Hz, 1 H) 7.01 (d, *J*=7.8 Hz, 2 H) 6.93 (d, *J*=7.28 Hz, 1 H) 5.22 (s, 1 H) 2.257 (s, 3 H) 2.108 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 168.11, 148.38, 137.67, 137.18, 133.13(2 C), 131.41, 130.43 (2 C), 125.70 (2 C), 124.88 (2 C), 122.00 (2 C), 115.01 (2 C), 112.63 (2 C), 109.71, 19.62, 12.97. ESI-MS: *m/z*=350.1 $[M]^+$.

3.3.5. Characterization of 2-(2,3-dimethylphenylamino)-N-(4-bromophenyl)benzamide ***3e***

38% yield, m.p. 210–215 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 8.948 (s, 1 H) 6.626 (d, *J*=7.2 Hz, 1 H) 6.808 (d, *J*=8.36 Hz, 1 H) 7.22 (d, *J*=7.12 Hz, 1 H) 7.14 (m, *J*=7.88 Hz, 3 H) 7.05 (t, *J*=7.76 Hz, 1 H) 6.95 (d, *J*=7.36 Hz, 2 H) 6.89 (d, *J*=7.84 Hz, 1 H) 6.75(t, *J*=7 Hz, 1 H) 5.18 (s, 1 H)2.34 (s, 3 H) 2.31 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 167.91, 147.60, 139.28, 138.11, 135.12, 134.40, 132.65, 131.29, 129.64 (2 C), 127.31, 125.95, 125.82, 121.40, 120.79 (2 C), 117.02, 116.84, 20.68, 13.99. ESI-MS: *m/z*=395.3 $[M]^+$.

3.3.6. Characterization of 2-(2,3-dimethylphenylamino)-N-(2-(piperazin-1-yl)ethyl)benzamide ***3f***

73.7% yield, m.p. 150–152 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 9.08 (s, 1 H) 7.71 (d, *J*=7.2 Hz 1 H) 7.30 (d, *J*=7.62 Hz, 1 H) 6.89 (m, *J*=7.9 Hz, 2 H) 6.41 (d, *J*=7.12 Hz, 1 H) 6.30 (d, *J*=7.24 Hz, 2 H) 5.05 (s, 1 H) 4.12(m, *J*=12.3 Hz, 2 H) 3.89 (m, *J*=12.8 Hz, 2 H) 3.5 (t, *J*=11.8 Hz, 4 H) 3.40 (t, *J*=12.0 Hz, 4 H) 2.89 (s, 1 H) 2.3 (s, 3 H) 2.22 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 169.15, 143.12, 142.56, 141.17, 140.67, 138.51, 132.23, 129.85, 126.55, 124.34, 121.04, 120.42, 119.53, 43.76, 47.12, 49.83, 51.66, 34.35, 37.78, 20.78, 14.13. ESI-MS: *m/z*=352.47 $[M]^+$.

In the second step ring closure was carried out using Con. HCl and glacial acetic acid as shown in ring closure mechanism is involved as shown in the Scheme 2.

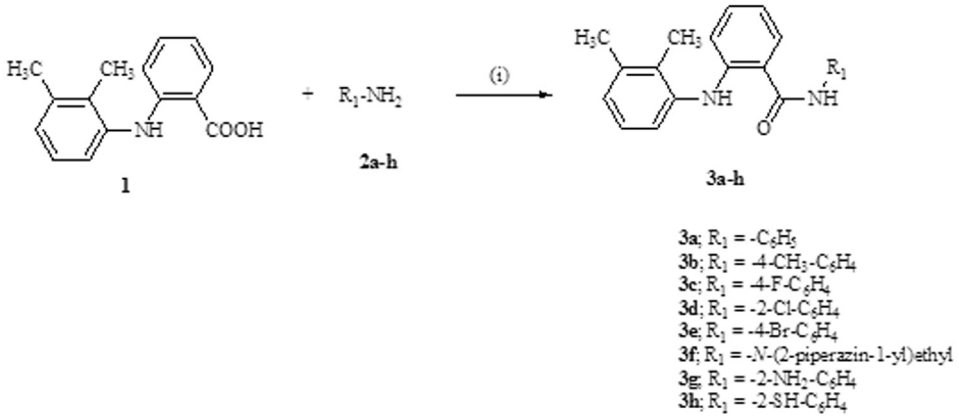
3.4. Physical Characterization of benzimidazole and benzthiazole derivatives ***(4a-4b)***

*3.4.1. Characterization of 2-(1H-benzo[*d*]imidazole-2-yl)-N-(2,3-dimethylphenyl)benzenamine* ***4a***

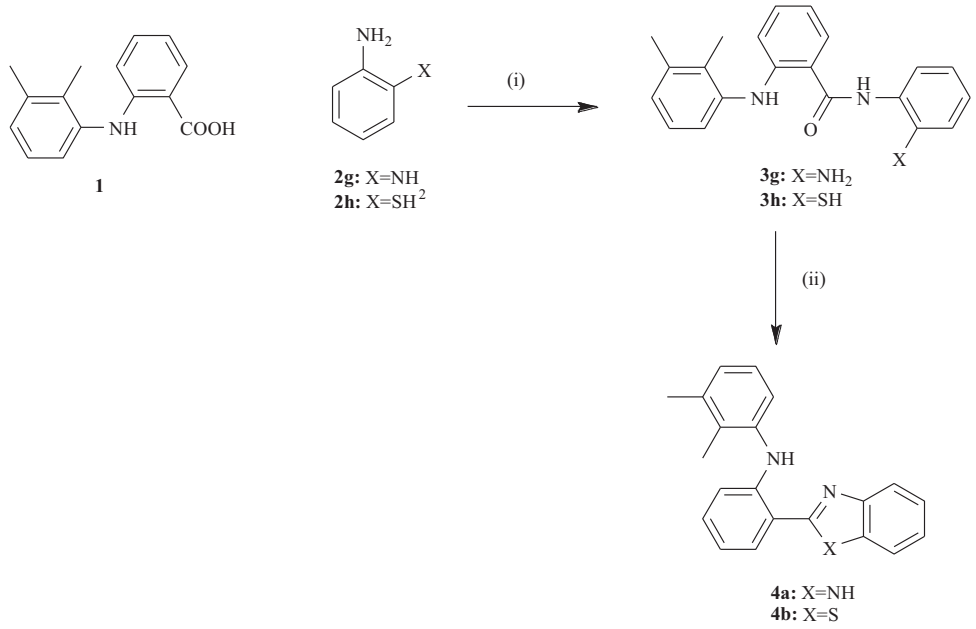
68.3% yield, m.p. 190–195 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 10.59 (s, 1 H) 7.827 (m, *J*=8.28 Hz, 2 H) 7.706 (dd, *J*=7.92 Hz, 1 H) 7.34 (t, *J*=7.0 Hz, 1 H) 7.25 (t, *J*=7.04 Hz, 1 H) 7.20 (d, *J*=7.84 Hz, 1 H) 7.15 (t, *J*=7.2 Hz, 1 H) 7.05 (t, *J*=7.48 Hz, 1 H) 6.93 (m, *J*=7.12 Hz, 2 H) 6.76 (t, *J*=8.28 Hz, 1 H) 5.2 (s 1 H) 2.24 (s, 3 H) 2.09 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 169.24, 153.42, 14p5.42, 139.45, 138.23, 134.19, 131.61(2 C), 130.66, 127.70, 126.78 (2 C), 124.68 (2 C), 123.68, 119.82, 118.69, 116.61 (2 C), 115.78, 20.76, 14.24. ESI-MS: *m/z*=313 $[M]^+$.

*3.4.2. Characterization of 2-(1H-benzo[*d*]thiazol-2-yl)-N-(2,3-dimethylphenyl)benzenamine* ***4b***

42.1% yield, m.p. 150–155 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ PPM: 10.59 (s, 1 H) 7.84 (m, *J*=7.5 Hz, 2 H) 7.71 (dd, *J*=7.8 Hz, 1 H) 7.2 (t, *J*=7.3 Hz, 1 H) 7.17 (t, *J*=7.7 Hz, 1 H) 7.08 (d, *J*=7.3 Hz, 1 H) 6.97 (t, *J*=7.6 Hz, 1 H) 6.83 (t, *J*=7.2 Hz, 1 H) 6.80 (m, *J*=7.4 Hz, 2 H) 6.54 (t, *J*=7.6 Hz, 1 H) 2.25 (s, 3 H) 2.09 (s, 3 H) ^{13}C NMR (400 MHz, $CDCl_3$) δ PPM: 169.31, 153.40, 146.54, 138.40, 137.25, 134.19, 131.68(2 C), 130.81, 127.45, 126.27 (2 C), 125.47 (2 C), 123.68, 119.81, 118.63, 115.84 (2 C), 115.95, 20.75, 14.15. ESI-MS: *m/z*=330 $[M]^+$.



Scheme 1. Reagents and conditions: (i) DCC, DMAP, DCM, 15 h stirring.



Scheme 2. Reagents and conditions: (i) DCC, DMAP, DCM, 15 h stirring; (ii) Conc. HCl, Glacial acetic acid, reflux at 80 °C for 2.5 h with stirring.

3.5. Biological screening

3.5.1. In-vitro pharmacological evaluation of heterocyclic derivatives using colorimetric assay kit

The *in-vitro* screening results showed that celecoxib exhibited very weak inhibitory activity against COX- 1 enzyme (4.76%) when compared with mefenamic acid (42.86%). The synthesized compounds **3e** and **3 f** exhibited COX-1 inhibitory activity 42.86% and 33.33% respectively. On the other hand, celecoxib exhibited the superior inhibitory profile against the COX-2 enzyme (100%) when compared with mefenamic acid (75.71%) and other investigated compounds **3e** (80.05%) and **3 f** (68.78%). However, **3 f** has more selectivity towards COX-2 enzyme and was more than that of mefenamic acid. The COX-2 to COX-1 selectivity ratio of compound **3 f**, was found to be 2.063, while mefenamic acid produced an approximate selectivity ratio of 1.767 **(**Table 3**).**

3.5.2. In-vivo pharmacological evaluation

From the *in-vitro* evaluation, compounds which showing better results were subjected for *in-vivo* pharmacological evaluation. During the late phase of edema formation, the volume of the carrageenan injected paw increased rapidly and progressively to reach a maximum of 58% after 5 h as compared to the normal control group. All the selected compounds produced a higher inhibition of edema formation in the carrageenan treated rats as shown in Fig. 1. Their anti-inflammatory activities were comparable to or higher than that of mefenamic acid. Compound **3e** showed significant anti-inflammatory activity, was subjected to ED_{50} determination for further comparison between different standard drugs. Compound **3e** dose-dependently reduced carrageenan-induced paw edema compared with vehicle-treated rats, showing an ED_{50} value of 17.09 mg kg. Mefenamic acid also reduced paw edema (An ED_{50} value of 20.17 mg kg) **(**Table 4**).**

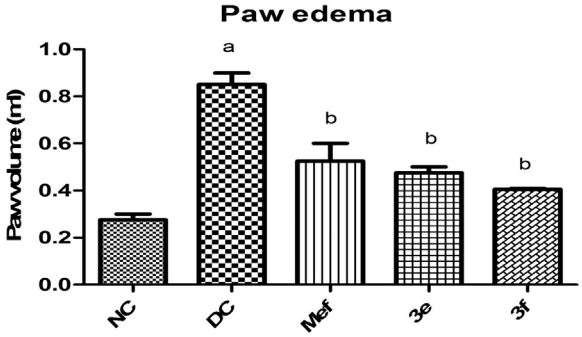


Fig. 1. Anti-inflammatory activity of heterocyclic compounds. [a]P < 0.05 represents the significance level when compared with normal control group, [b]P < 0.01 compared with disease control group, Each group consists of 6 animals. Values are expressed as Mean ± S.E.M, normal control, disease control, disease treated with mefenamic acid(12.85 mg/kg), **3e**-disease treated with **3e** (12.85 mg/kg), **3f**-disease treated with **3f** (12.85 mg/kg).

Table 3
% inhibition and IC_{50} values of heterocyclic derivatives.

Compound Code	% inhibition at 10 μM		Selectivity	COX-II IC_{50}
	COX-I	COX-II		
3a	38.095	13.53	0.355	NP
3b	33.333	20.294	0.609	NP
3c	23.810	29.314	1.231	NP
3d	19.048	23.677	1.243	NP
3e	42.857	80.05	1.868	2.131
3f	33.333	68.775	2.063	NP
4a	38.095	24.804	0.651	NP
4b	52.381	47.353	0.904	NP
Celecoxib	4.762	100.34	21.071	NP
Indomethacin	61.905	78.571	1.269	NP
Mefenamic acid	42.857	75.714	1.767	5.342
Aspirin	28.571	29.313	1.026	NP

Table 4
Determination of ED_{50} Value.

S.No	Groups	Dose in mg	Difference in volume	% inhibition
1	Disease Control	–	1.3	100
2	Mefenamic acid	6.4	0.3	15.5
3	Mefenamic acid	12.8	0.55	28.1
4	Mefenamic acid	25.6	0.4	65.2
5	Treated with **3e**	6.4	1.075	17.31
6	Treated with **3e**	12.8	0.875	32.7
7	Treated with **3e**	25.6	0.275	78.85

3.5.3. Effect of heterocyclic derivative on peptic ulcer

No lesions were found in the rat treated with heterocyclic derivatives at 12.8 mg/kg dose. Similarly, an oral administration of mefenamic acid at 12.8 mg/kg dose did not showed incidence and size of ulcers in treated rats.

4. Discussion

Cyclooxygenase is a key enzyme responsible for metabolism of arachidonic acid into several prostaglandins. Prostaglandins formed via COX-1 activity control renal perfusion, promote platelet aggregation and provide gastro protection by regulating mucous secretion. Prostaglandins formed via COX-2 activity mediate pain, inflammation, fever and inhibit platelet aggregation. COX-2 exclusively expressed in an inducible way in the sites of inflammation while COX-1 is the source of the same prostaglandins in the gastric epithelium, where they act as cytoprotective. Research efforts have focused, on the discovery of selective COX-2 inhibitors. It was initially believed that this class of drugs would have reduced gastro-intestinal toxicity compared to classical NSAIDs (FitzGerald, 2003). Although inhibition of both COX-1 and COX-2 is generally well tolerated, it is associated with a wide spectrum of potential clinical toxicities. Many adverse events are attributed to inhibition of the constitutively expressed COX-1 enzyme, and some of these appear to be significantly reduced through the use of COX-2-specific inhibitors (Roth, 1988). However, confusion still surrounds the role of COX-2 selective inhibitors because of an increased risk of myocardial infarction and other thrombotic events (Dogné et al., 2004). The points of interest offered by the utilization of particular COX-2 inhibitors urged to evaluate the pharmacological action of novel heterocyclic subordinates that could be potential COX-2 inhibitors and to principally examine their expected side effects. The introduction of new COX-2 inhibitors comparable in efficacy to celecoxib, the model medication, with lower adverse reactions would be a milestone and a point of reference to the improvement of mitigating treatment, which is our main objective.

Due to the adverse effects connected with COX-2 inhibitors, researchers have been endeavoring to change the structure of commonly used anti-inflammatory agents, especially concentrating on the acidic group. Empowered by the above perceptions numerous derivatives of mefenamic acid were designed. Diverse amines and in addition, acids were utilized for the synthesis. Compounds were in anticipation that these would be more active, potent and of having less toxic effects than existing drugs.

Molecular docking investigation uncovered that the vast majority of the compounds displayed the same hydrogen bonding interactions as exhibited with standard with amino acids like TYR 386, ASN 383, THR 213 etc. An obtained docking score revealed that the designed compounds showed more selectivity for the COX-2 enzyme.

In silico acute toxicity prediction was performed using GUSAR software. Most of the compounds were found to be out of the applicability domain of the models during the acute toxicity study when considered intraperitoneal route except compounds **3c**, **3d** and **3 f**. An *In silico* investigation of GUSAR software revealed that compounds **3d–f** and **4a-b** are slightly toxic (class 4), whereas compounds **3a–c** are practically nontoxic (class 5).

The synthetic protocol for the target molecules involves a single step. Mefenamic Acid forms amide bond via DCC/DMAP coupling using different primary amines. The ring closure reaction involves the same mechanism as in amide coupling. In the second step ring closure was carried out using Con. HCl and glacial acetic acid. It was observed that the yield of the compound with bromo substitution at the 4th position of the phenyl ring found to be lower as compared to chloro substitution. It was also observed that the yield of the compound with methyl substitution at the 4th position of the phenyl ring was found to be higher as compared to other derivatives.

In the present study, the heterocyclic derivatives were subjected to COX-1 and COX-2 evaluation for inhibition activities using a COX colorimetric screening assay method consisting of ovine COX-1 and human recombinant COX-2 enzymes. The IC_{50} values of potent compounds against COX-1 and COX-2 (μM) were calculated from the concentration inhibition response curve. The outcomes demonstrated that all the selected compounds were very weak inhibitors of COX-1 contrasted with mefenamic acid except from **4b**. All the compounds were found to be strong inhibitors of COX-2, however, their selectivity for the COX-2 protein was not as much as that of celecoxib. Compounds **3e** and **3 f** had the highest selectivity ratio among all the investigated compounds. In spite of the fact that the selected compounds were less potent inhibitors of COX-2 contrasted with celecoxib.

There has been considerable exertion in the pharmaceutical industry to recognize a COX-2 selective inhibitor with mitigating properties, yet without the unfavorable effects related with conventional NSAIDs (Bansal et al., 2014). In the present study, the heterocyclic derivatives were subjected to COX-1 and COX-2 evaluation of inhibition activities using a COX Colorimetric Screening assay kit consisting of ovine COX-1 and human recombinant COX-2 enzymes. The IC50 values of potent compounds against COX-1 and COX-2 (μM) were calculated from the concentration inhibition response curve.

Carrageenan-induced rat paw edema is used as a working model of inflammation in search for new anti-inflammatory drug (Vinegar et al., 1969). Both the selected compounds produced a higher inhibition of edema formation in the carrageenan- induced rat paw edema. Their anti-inflammatory activities were comparable to or higher than that of mefenamic acid. The results of carrageenan-induced rat paw edema assay suggest that the vast majority of the recently examined compounds hindered intense periods of inflammation. It can be expected that they may interact with known mediators of inflammation, for example, cytokines (Süleyman and Büyükokuroğlu, 2001).

In addition, carrageenan is known to actuate macrophages and polymorphonuclear cells. These activated cells secrete COX-2 enzyme, the protein which is responsible for the synthesis of cytokines involved in inflammation (Li et al., 2005). In this manner, inhibition of COX-2 can add to the mechanism of action underlying the anti-inflammatory effect of compounds under test. Subsequently authors suggested that investigated compounds have suppressed the acute stage of inflammation by hindering the synthesis of PGs by COX-2 inhibition. Various reviews have proposed that NSAID may assume an essential part in the etiology of perforated peptic ulcers. Non-steroidal calming drugs inhibit the synthesis of prostaglandins which have cytoprotective impacts in the upper gastrointestinal tract, and in addition suppressing gastric acid secretion. In the present study, the stomach was analyzed from the animal and scoring was carried out. Mefenamic acid indicated slight ulcers while none of the newly synthesized compounds demonstrated any ulcerogenic impact on the gastric mucosa of treated rats.

It has been accounted for that, rather than being exclusively because of inhibition of gastric protection managed by COX-1, stomach ulceration brought on by NSAIDs may likewise because of topical toxicity as an outcome of their physical and chemical characteristics (Smale and Bjarnason, 2003). Their acidic attributes disrupt the defensive mucus layer and uncover the underlying cells to the acidic pH of the stomach. Moreover, acidic NSAIDs may accumulate because of ion trapping inside intestinal enterocytes achieving concentration that leads to uncoupling of mitochondrial oxidative phosphorylation (Krause et al., 2003).

5. Conclusion

Due to the importance and the emerging use of COX-2 inhibitors, several researchers have been investigating and trying to solve the issues of toxicity related to the NSAIDs. The amide derivatives of mefenamic acid were synthesized and tested for anti-inflammatory activity. According to docking studies by both Sybyl and GOLD suite,

all compounds were showing high docking scores except **3c** and with the same interaction as that of standard i.e. mefenamic acid. The computer aided acute toxicity prediction of synthesized compounds revealed that all compounds were found to be safe. The synthetic protocol involved two steps wherein the first step was to synthesize amide derivative via acid-amine coupling using DCC/DMAP as reagents. In the second step, the formation of the ring occurs from the amide derivative via Con. HCl and glacial CH_3COOH under reflux condition. The *in-vivo* pharmacology studies on the selected compounds were performed, followed by computer aided drug design and *in vitro* studies. Compound **3a** exhibited very good interactions and also score in molecular docking analysis, yet during *in vitro* screening of COX-2 protein, not demonstrated selectivity. Mefenamic acid derivatives substituted with p-bromophenyl and piperazine heterocyclic ring indicated promising anti-inflammatory activity amongst other compounds. The present study revealed that synthesized derivatives, by all accounts, to be a promising and safe alternative for the management of acute and chronic inflammatory conditions.

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