Original Article

Functional Role of Novel Anthranilic Acid Derivatives as Anti-inflammatory Agents

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ABSTRACT

Background: The discovery of new cyclooxygenase (COX) inhibitors with high efficacy and safety would greatly aid in developing anti-inflammatory drugs. This study evaluated the anti-inflammatory activity and the expected side effect of two synthesized anthranilic acid derivatives (JS-3 and JS-4). **Materials and Methods:** The COX selectivity of compounds was assessed in a whole blood assay. The results were confirmed by molecular docking studies. *In vivo* anti-inflammatory activity was tested in complete Freund's adjuvant (CFA)-induced rheumatoid arthritis (RA) in rats. The safety profile was determined by administering a dose three times the therapeutic dose. **Results:** *In vitro* COX-2 selectivity of JS-4 was higher than JS-3 (13.70 vs. 5.56). Docking studies supported the higher selectivity of both derivatives against COX-2 than mefenamic acid. Treatment of CFA-arthritic rats with both compounds showed significant ($P < 0.01$) decreases in paw volume, paw thickness, arthritic index, and inflammatory parameters such as rheumatoid factor, interleukin (IL)-1β, IL-6, prostaglandin (PG) E_2, PGI_2, and TXB_2 compared to untreated RA animals. JS-3 and JS-4 improved knee joint histopathology and protected against RA-induced pathological changes: splenomegaly, thymomegaly, and cardiomyopathy. Both derivatives produced minimal effects on liver and renal functions, without any ulcerogenic effects. **Conclusion:** These results suggest that JS-4 is a potential and safe candidate for managing RA without renal, liver, gastrointestinal, or cardiovascular toxicity.

Keywords: Cyclooxygenase inhibitors, Anthranilic acid derivatives, Mefenamic acid, Rheumatoid arthritis, Anti-inflammatory, Safety evaluation, Molecular docking.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder that can be initiated by both environmental and genetic factors.[1-2] During RA, deregulation of the immune system leads to persistent synovial inflammation, cellular infiltration, proinflammatory cytokine production, and ultimately the destruction of cartilage and bone in joints.[3] The prevalence of RA is 0.1-2% of the total population worldwide, depending on the country, with an average global incidence of 0.45%.[4] The pathophysiology of RA involves the interaction of activated T and B cells along with heightened pro-inflammatory cytokines signalling.[1-3]

Cyclooxygenases (COX), the key enzymes responsible for synthesizing the prostaglandins from arachidonic acid, exist mainly in two isoforms: COX-1 and COX-2.[5] Metabolites of arachidonic acid are critical for many biologic processes, including inflammation, ovulation, implantation, angiogenesis, platelet aggregation, and immunologic function.[6-7] Most tissues, including the kidneys, lung, stomach, duodenum, jejunum, ileum,

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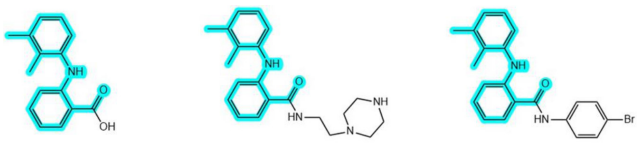
colon, and cecum, constitutively express COX-1.[8] The current general understanding of COX is that COX-1 produces mainly regulatory prostaglandins (PGs), such as prostacyclin (PGI_2) and PGE_2, which act as cytoprotective agents, and maintain the integrity of gastric mucosa, among other functions.[9] COX-2, however, is believed to be an inducible isoform of the enzyme that is upregulated in inflammatory cells following tissue injury or infection. COX-2 produces mainly the prostanoid mediators of inflammation.[9]

PGI_2 is a key player in the complicated COX–prostanoids cascade in cardiovascular physiology and diseases because of its anti-atherogenic and cardioprotective roles.[9-10] PGI_2 counteracts the cardiovascular effects of the platelet-derived thromboxane A_2 (TXA_2) that activates platelet aggregation, vasoconstriction, and vascular proliferation.[6-7] COX-1 produces PGI_2 in many tissues, such as the gastric mucosa and the kidney, while COX-2 is a significant source in the heart.[11] Importantly, selective inhibition of COX-2 reduces PGI_2 synthesis, which can magnify the unopposed TXA_2-mediated adverse effects.[12]

Despite the rising reservoir of freshly synthesized non-steroidal anti-inflammatory drugs (NSAIDs), new compound discovery is necessary due to the negative effects of existing authorized NSAIDs. For example, even with short-term use, a high ibuprofen dosage causes jejunal perforations,[13] and naproxen affects bowel and jejunal integrity.[14] In addition, diclofenac has been shown to increase the risk of heart attacks and strokes by 50% within days after administration.[15] Thus, improving the selectivity of the currently used NSAIDs will lead to the rapid delivery of effective and safer compounds. Although selective inhibition of COX-2 was linked clinically to a higher cardiovascular risk,[16-17] other reports showed better cardiovascular safety of the selective COX-2 inhibitor celecoxib over classic NSAIDs or rofecoxib, a selective COX-2 inhibitor that is no longer in clinical practice.[18-19] Nonetheless, both selective and nonselective NSAIDs impose higher cardiovascular, among other, risks than the placebo or no treatment; hence, the use of the smallest possible effective dosing regimens, especially in high-risk groups, is recommended.[20]

Mefenamic acid and other anthranilic acid derivatives, also known as fenamates, constitute one of the oldest and most used NSAIDs.[21-22] This class of biologically active molecules has attracted the attention of medicinal chemists because of their wide range of pharmacological properties.[23-24] We have recently synthesized a series of amide derivatives of mefenamic acid, which showed promising anti-inflammatory activity both *in vitro* and *in vivo*.[25] Based on our previous results,[25] we selected the two most active compounds with different substitutions (JS-3 and JS-4; Figure 1) to further evaluate their efficacy and safety compared to mefenamic acid. Thus, the current study aimed to evaluate the anti-inflammatory potential of these anthranilic acid derivatives in an animal model of experimental RA and to establish their safety profile. Docking studies were used to explain the observed selectivity of JS-3 and JS-4 on a molecular basis. In addition, the binding modes and interaction profiles with COX-2 were analyzed to show the determinants ligands conformation and selective recognition of COX-2.



Mefenamic acid JS-3 JS-4

Figure 1: Structures of anthranilic acid derivatives.

The chemical structures of the anthranilic acid derivatives used in the current study including the reference drug 2-(2,3-dimethylphenylamino)benzoic acid (Mefenamic acid), 2-(2,3-dimethylphenylamino)-N-(2-(piperazin-1-yl)ethyl)benzamide (JS-3), and 2-(2,3-dimethylphenylamino)-N-(4-bromophenyl)benzamide (JS-4) are shown. The light blue-highlighted structure represents the mefenamate moiety common to the three chemical structures.

MATERIALS AND METHODS

Chemicals and Kits

Complete Freund's adjuvant (CFA), the calcium ionophore A23187, and lipopolysaccharides (from *Salmonella enterica* serotype enteritidis) were purchased from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany). A serum rheumatoid factor determination kit was purchased from Lab Care Diagnostics (Sarigam, Gujarat, India). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin (IL)-1β, IL-6, PGE_2, PGI_2, and TXB_2 were obtained from Cayman Chemical (Ann Arbor, MI, USA). JS-3 [2-(2,3-dimethylphenylamino)-N-(2-(piperazin-1-yl)ethyl)benzamide] and JS-4 [2-(2,3-dimethylphenylamino)-N-(4-bromophenyl)benzamide] were synthesized, crystallized, and characterized as previously described.[25] Other chemicals were of analytical grade and were obtained from commercial sources.

In vitro Whole Blood Assay

Selective inhibition of the COX activity of the two isoforms (COX-1 and COX-2) was determined using a whole blood assay as previously described.[26-27] In this assay, the production of TXB_2 and PGE_2 is used as surrogate measures for COX-1 and COX-2 activities, respectively. Heparinized blood samples were

incubated with the tested compounds for 1 hr at 37°C before stimulation of either COX-1 by the calcium ionophore A23187 (50 µM, 1 hr, 37°C) or COX-2 by lipopolysaccharides (10 µg/ml, overnight, 37°C). The activity of COX-1 in the first set of aliquots was assessed by measuring the A23187-induced platelet-mediated TXA_2 production as TXB_2, a stable inactive metabolite of TXA_2, using an ELISA assay kit (Cayman Chemical). The COX-2 activity in the second set of aliquots was measured as the concentration of lipopolysaccharides-induced PGE_2 production using an ELISA assay kit (Cayman Chemical).

In vivo CFA-induced RA Model

Experimental Animals

All experiments and protocols were approved by the Institutional Animal Ethics Committee (IAEC) of the Institute of Pharmacy, Nirma University (IP/PCOL/MPH/17/006). Male Wistar rats (250-300 g) were divided into five groups (6 animals each) and maintained in the animal house of Nirma University under controlled conditions of temperature 24 ± 2°C, relative humidity 55 ± 5%, and dark/light schedule (12 hr light and 12 hr dark). Animals had free access to food and purified water and acclimatized for one week before starting the experiment.

Experimental Protocol

The study comprised five groups of Wistar rats: normal control, disease control (RA), RA treated with JS-3 (12.85 mg/kg), RA treated with JS-4 (12.85 mg/kg), and RA treated with mefenamic acid (12.85 mg/kg). The doses were selected based on our previous work.[25,28] Arthritis was induced by a subplantar injection of 0.1 ml CFA in the right hind paw, as previously described.[29] Daily treatment of the animals with the test compounds or vehicle was carried out starting on the 10th day of CFA injection for 10 days.

Determination of Arthritic Index and Paw Inflammation

At the end of the study, the arthritic index was determined as the sum of nodules observed on ears, nose, tail, fore paws, and hind paws, which reflected the severity of arthritis in each group. The paw thickness was measured using a micrometer, while its volume was measured using a plethysmometer on the 19th day.

Determination of Serum Inflammatory Markers

Blood was collected from the retro-orbital plexus of overnight-fasted rats to measure serum rheumatoid factor, IL-1β, IL-6, PGE_2, PGI_2, and TXB_2 as indicators for the progression of inflammation in each group.

Determination of Blood Pressure and Organ Toxicity

Blood pressure and heart rate were determined by a tail-cuff method.[30] At the end of the treatment period, animals were weighed and euthanized. The ratios of spleen and thymus weights to body weight were determined.

Histopathology of the Knee Joint and the Heart

The knee joint and heart tissues were harvested and processed for histopathological studies. Briefly, the tissues were removed and fixed in 10% neutral-buffered formalin. The joint tissue samples were decalcified in 5% formic acid. All tissues were processed for paraffin embedding, sectioned at 5 µm thickness, and subsequently stained with hematoxylin and eosin for examination under a light microscope. The joint sections were examined for the presence of hyperplasia of the synovium, pannus formation, and destruction of the joint space, while heart sections were assessed for signs of cardiomyopathy.

Evaluation of the Safety of Anthranilic Acid Derivatives

Evaluation of Hepatic and Renal Adverse Effects of Anthranilic Acid Derivatives

We evaluated the possible hepatic and renal adverse effects of each test compound after administering a single dose three times higher than the therapeutic anti-inflammatory dose. To assess hepatic toxicity, serum levels of alanine transaminase (ALT) and aspartate transaminase (AST) (Lab Care Diagnostics) were measured, while serum urea, creatinine, and potassium levels were used as markers of renal toxicity. All parameters were determined 4 hr after the acute single high dose exposure.

Effect of Anthranilic Acid Derivatives on Gastrointestinal Toxicity

Four hours after acute dosing, animals were killed, and their stomachs were removed. Each stomach was opened longitudinally along the greater curvature, and the gastric content along with blood clots were removed by rinsing with cold saline. The gastric luminal surface was examined to determine the degree of ulceration and graded as follows: 0, no lesions (normal stomach); 0.5, hyperemia (red coloration); 1, hemorrhagic spots; 2, small ulcers (< 5); 3, many (> 5) small ulcers; 4, many small and large ulcers; 5, stomach full of ulcers; 6, stomach full of ulcers with perforations.

Docking Studies

The protein, ligands preparation, and docking procedures were performed as previously described[31-32] with slight modifications. The Schrödinger Maestro suite

(Schrödinger, New York, NY, USA) was used in all docking steps. The compounds were prepared and 3D-optimized by LigPrep. The protein structure file was obtained from the Protein Data Bank (PDB, 5IKR). The structure comprises human COX-2 bound with mefenamic acid at 2.34 Å resolution.[33]

The structure was optimized for docking using the protein preparation module. The solution's crystallographic compounds and water molecules were eliminated. The protein was protonated by adding polar hydrogens, the structures were optimized, and energy was decreased by employing the OPLS2005 force field. For docking grid generation, mefenamic acid was used as a center for a 20-Å grid box. The standard precision SP glide docking protocol was used and the output results were ranked by the docking scores. The accuracy of the docking run was assessed by redocking of mefenamic acid, and the docking pose showed perfect complementarity and low route mean square deviations (RMSD), compared with the bound ligand.

Statistical Analysis

The data are represented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., CA, USA). Statistical differences between the means of various groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's test for comparison of different groups. The gastrointestinal toxicity ulcer scores were analyzed using the Kruskal-Wallis nonparametric test followed by Dunn's multiple comparison test. The difference between two groups was considered statistically significant at $P < 0.05$.

RESULTS

Effect of Anthranilic Acid Derivatives on COX Activity

The present study compared the relative potencies of mefenamic acid and the synthesized derivatives (JS-3 and JS-4) to determine their selectivity against the COX enzymes. Inhibition of TXB_2 and PGE_2 production was determined in samples of whole blood pre-treated with the test compounds. The results in Table 1 show that JS-4 is a highly selective inhibitor of PGE_2 production (IC_{50} = 4.3 μM) while inhibiting TXB_2 production at an IC_{50} of 59 μM, which indicates higher COX-2 selectivity. On the other hand, mefenamic acid showed little preference for COX-2 enzyme selectivity compared to JS-3 or JS-4.

Table 1: Effect of anthranilic acid derivatives on cyclooxygenase (COX) activity.

Group	IC_{50} (μM)		COX-2 selectivity*
	COX-1	COX-2	
Mefenamic acid	21.2	7.3	2.9
JS-3	45	8.1	5.56
JS-4	59	4.3	13.7

Whole blood samples were incubated with varying concentrations of the test compound before activation of either COX-1 or COX-2 (see Methods for details) to determine its median inhibitory concentration (IC_{50}) against either enzyme. * COX-2 selectivity was calculated for a compound by dividing its COX-1 IC_{50} (concentration inhibited TXB_2 production by 50%) by the COX-2 IC_{50} (concentration inhibited PGE_2 production by 50%).

Effect of Anthranilic Acid Derivatives on the Progression of CFA-Induced RA

Effect of Anthranilic Acid Derivatives on Arthritic Index

Injection of CFA for induction of RA in rats significantly increased the arthritic index of untreated RA animals. Compared to the RA group, mefenamic acid-treated rats showed a modest but significant decrease in the arthritic index. Treatment of RA rats with either of the anthranilic acid derivatives showed significant reductions in the arthritic index scores, which were comparable to those shown in the mefenamic acid-treated rats (Table 2).

Effect of Anthranilic Acid Derivatives on Paw Volume and Paw Thickness

Data in Table 2 show that induction of RA by CFA significantly ($P < 0.01$) increased the paw volume and thickness of untreated rats compared to the normal controls. Conversely, administration of mefenamic acid or the novel anthranilic acid derivatives significantly attenuated the severity of paw inflammation, as shown by the decrease in paw volume and thickness.

Effect of Anthranilic Acid Derivatives on Rheumatoid Factor

Untreated RA rats showed significantly increased rheumatoid factor agglutination values (Table 3), which were significantly mitigated by treating the animals with

Table 2: Effect of anthranilic acid derivatives on arthritic index, paw volume, and paw thickness.

Group	Arthritic index	Paw volume (ml)	Paw thickness (cm)
Nornal control	0.0 ± 0.0	0.37 ± 0.021	0.35 ± 0.021
Disease control	3.5 ± 0.22#	0.75 ± 0.056#	0.75 ± 0.047#
Mefenamic acid	2.7 ± 0.21*#	0.38 ± 0.028***	0.56 ± 0.018***#
JS-3	2.5 ± 0.22**#	0.30 ± 0.013***	0.47 ± 0.017***#
JS-4	2.3 ± 0.21**#	0.37 ± 0.017***	0.57 ± 0.033**#

#: Significantly different from normal control group ($P < 0.05$); *,**,***: Significantly different from disease control group at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Each group comprised 6 animals. Values are expressed as mean ± SEM.

Table 3: Effect of anthranilic acid derivatives on biochemical parameters.

Group	RF (IU/ml)	IL-1β (µg/ml)	IL-6 (µg/ml)	PGE_2 (µg/ml)	PGI_2 (µg/ml)	TXB_2 (µg/ml)
NC	5.31 ± 0.54	16.1 ± 1.53	124.0 ± 4.43	367.5 ± 13.1	337.8 ± 15.81	253.3 ± 15.76
DC	26.6 ± 2.76#	38.4 ± 2.24#	216.9 ± 4.39#	967.7 ± 6.8#	1063 ± 31.49#	421.3 ± 22.54#
Mef	10.2 ± 0.40*	26.3 ± 1.38	146.0 ± 21.54**	824.9 ± 21.2#	681.1 ± 48.25***#	261.6 ± 26.29**
JS-3	8.46 ± 0.62*	19.4 ± 2.15**	117.1 ± 11.99***	504.1 ± 78.9***	734.5 ± 22.78***#	263.2 ± 15.9**
JS-4	5.29 ± 0.45*	19.2 ± 1.54**	124.8 ± 15.91***	610.5 ± 40.6***#	876.3 ± 24.30**#	273.03 ± 41.5**

Levels of rheumatoid factor (RF), interleukin (IL)-1β, IL-6, and prostanoids [prostaglandin (PG) E_2, PGI_2, and thromboxane (TX) B_2] were measured in serum samples isolated from the retro-orbital plexus blood of overnight-fasted rats. #: Significantly different from normal control (NC) group ($P < 0.05$); **,***: Significantly different from disease control (DC) group ($P < 0.01$, $P < 0.001$, respectively). Each group comprised 6 animals. Values are expressed as mean ± SEM. Mef: Mefenamic acid.

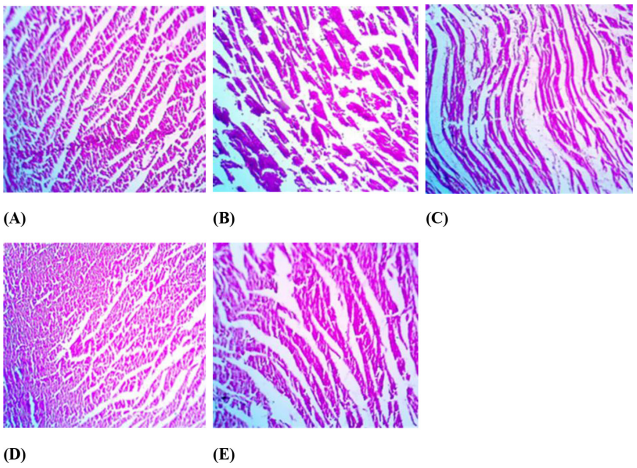


Figure 2: Effect of anthranilic acid derivatives on histology of heart tissue.

(A) Normal control group, (B) Disease control group, (C) Disease treated with mefenamic acid (12.85 mg/kg), (D) Disease treated with JS-3 (12.85 mg/kg), and (E) Disease treated with JS-4 (12.85 mg/kg).

Table 4: Effect of anthranilic acid derivatives on cardiovascular and immune system.

Group	Blood pressure (mm Hg)	Heart rate	Spleen to body weight ratio	Thymus to body weight ratio
NC	126.00 ± 2.3	480 ± 40	0.51 ± 0.011	0.150 ± 0.016
DC	152.66± 4.3#	632 ± 21#	0.81 ± 0.015##	0.092 ± 0.006#
Mef	128.00 ± 1.9**	515 ± 14	0.64 ± 0.008**	0.135 ± 0.002*
JS-3	125.00 ± 2.6**	490 ± 35*	0.65 ± 0.028**	0.130 ± 0.007
JS-4	122.67 ± 3.2**	465 ± 32*	0.58 ± 0.018**	0.150 ± 0.014**

#,##: Significantly different from normal control (NC) group at $p < 0.05$ and $P < 0.01$, respectively; *,**: Significantly different from disease control (DC) group ($P < 0.05$, $P < 0.01$, respectively). Each group comprised 6 animals. Values are expressed as mean ± SEM. Mef: Mefenamic acid.

mefenamic acid or its novel analogs; JS-3 and JS-4. Interestingly, treatment with JS-4 showed completely normalized rheumatoid factor values (5.29 ± 0.45 vs. 5.31 ± 0.54 in normal control rats).

Effect of Anthranilic Acid Derivatives on serum levels of IL-1β, IL-6, PGE_2, PGI_2, and TXB_2

The untreated RA rats showed significantly elevated levels of IL-1β and IL-6 compared to the normal controls. Treatment of the RA animals with mefenamic acid, JS-3, or JS-4 significantly decreased serum levels of IL-1β and IL-6 compared to the untreated RA group. Similarly, the CFA-induced arthritic animals showed significantly higher levels of PGE_2, PGI_2, and TXB_2 compared to the normal controls. Mefenamic acid-, JS-3-, and JS-4-treated RA rats, on the other hand, demonstrated significantly decreased levels of PGE_2, PGI_2, and TXB_2 relative to the untreated RA rats. Neither mefenamic acid-, JS-3-, nor JS-4-treated groups showed significant decreases in the levels of PGE_2, PGI_2, and TXB_2 compared to the normal control rats (Table 3).

Effect of Anthranilic Acid Derivatives on Cardiovascular Parameters

Rats in the untreated RA group in the current study showed significantly increased blood pressure ($P < 0.01$) and heart rate ($P < 0.01$) values compared to the normal controls. Treatment of RA rats with the standard NSAID mefenamic acid or its derivatives has not significantly changed these parameters compared to the normal control group (Table 4). The histopathology of the heart showed normal structure and architecture of cardiac fibers in the normal control group of animals. The RA disease control group showed mild to moderate cardiomyopathic changes. Microscopical examination of hearts from the NSAIDs (mefenamic acid, JS-3, and JS-4) treatment groups revealed a normal architecture of cardiac muscle fibers in these groups (Figure 2).

Effect of Anthranilic Acid Derivatives on the Immune System

A significant increase in the spleen to body weight ratio was observed in the untreated disease control group compared to the normal control group. Standard- and anthranilic acid derivatives-treated groups showed significant reductions in the spleen to body weight ratio compared to untreated RA animals. However, the mefenamic acid and JS-3 treatment groups still showed significantly higher than normal spleen to body weight ratios (Table 4), while JS-4 treatment normalized this parameter. Administration of CFA also showed a significant reduction of the thymus to body weight ratio in the disease RA group. A statistically significant improvement was observed in the mefenamic acid ($P < 0.05$) and the JS-4-treated rats ($P < 0.01$), while the JS-3-treated rats showed an improvement, albeit statistically insignificant (adjusted $P = 0.0883$).

Effect of Anthranilic Acid Derivatives on Histopathology of the Knee Joint

Histopathological examination of the knee joint sections revealed normal joint structure, healthy cartilage, and absence of signs of inflammation in the normal control group of animals. However, the joints of the RA-diseased untreated rats showed moderate to severe hyperplasia of the synovium, focal cartilage destruction, pannus formation, and the destruction of the joint space. Treatment with mefenamic acid or its derivatives showed significant improvements in hyperplasia of the synovium and inflammatory cell infiltration and mild to moderate cartilage destruction (Figure 3).

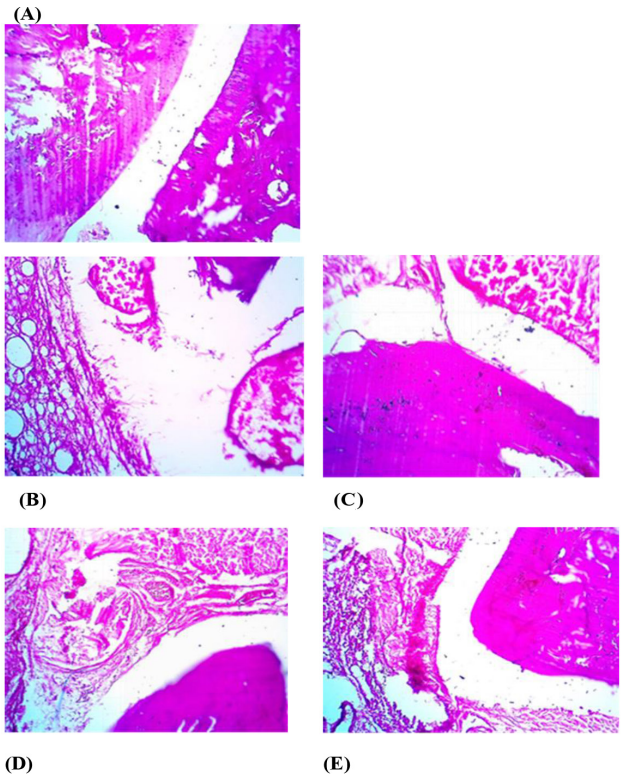


Figure 3: Effect of anthranilic acid derivatives on the knee joint.

(A) Normal control group, (B) Disease control group, (C) Disease treated with mefenamic acid (12.85 mg/kg), (D) Disease treated with JS-3 (12.85 mg/kg), and (E) Disease treated with JS-4 (12.85 mg/kg).

Evaluation of Adverse Effects of Anthranilic Acid Derivatives

Evaluation of Hepatic and Renal Side Effects

To assess the safety of the studied compounds, we evaluated the adverse effects of the selected compounds after administering a single high dose, three times higher than the therapeutic dose, in experimental animals. The levels of ALT and AST, common surrogates of hepatic function, were not significantly altered by treatment with the standard or anthranilic acid derivatives after acute high dose administration (Figure 4A, B). Mefenamic acid treatment slightly, but not significantly, increased urea levels in the serum. The other anthranilic acid derivatives (JS-3 and JS-4) did not affect serum urea levels. Similarly, neither mefenamic acid nor its derivatives significantly changed serum creatinine levels. Although JS-3 produced slightly increased serum creatinine levels, this change was not statistically significant (Figure 4C, D). Administration of mefenamic acid or its derivatives induced slight, but statistically significant, elevations in potassium levels after a single administration of the supratherapeutic doses (Figure 4E).

Effect of Anthranilic Acid Derivatives on Gastric Ulceration

Acute gastric toxicity of the compounds under study was evaluated by visually examining the gastric mucosae and scoring peptic ulcers. No significantly elevated ulcer scores were found in any of the treated groups after acute administration of triple the therapeutic doses of mefenamic acid or its derivatives (Figure 4F). The least scores were observed in the JS-3, and JS-4 treated rats.

Molecular Docking of Anthranilic Acid Derivatives with Human COX-2

The docking study showed a common binding site for mefenamic acid, JS-3, and JS-4 composed of a hydrophobic pocket comprising VAL116, TYR348, VAL349, LEU352, TYR355, MET522, VAL523, ALA527, LEU531, and LEU539 (Figure 5). The docking scores were comparable among the compounds, with a higher score for mefenamic acid followed by JS-4 and JS-3 (Table 5). The binding of mefenamic acid is supported by two hydrogen bonds with TYR385 and SER530 (Figure 5A). JS-3 formed one hydrogen bond

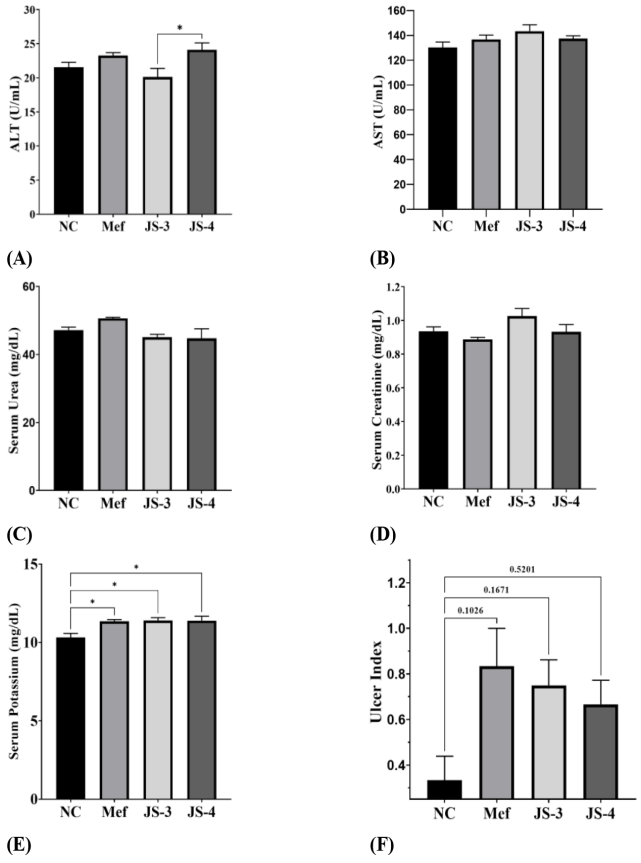


Figure 4: Effect of anthranilic acid derivatives on hepatic and renal functions.

(A) Alanine transaminase (ALT), (B) Aspartate transaminase (AST), (C) Urea, (D) Creatinine, (E) Potassium, and (F) Ulcer index. *: Significantly different from normal control (NC) group ($p < 0.05$). Each group comprised 6 animals. Values are expressed as mean ± SEM. NC–normal control, Mef–disease treated with mefenamic acid (40 mg/kg), JS-3–disease treated with JS-3 (40 mg/kg), JS-4–disease treated with JS-4 (40 mg/kg).

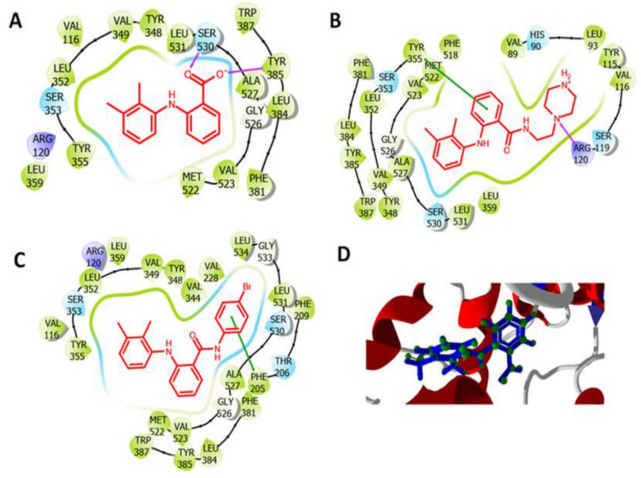


Figure 5: The docking site and ligand interactions of mefenamic acid, JS-3, and JS-4 with human cyclooxygenase-2.

(A) The ligand interaction of mefenamic acid. (B) The ligand interaction of JS-3. (C) The ligand interaction of JS-4. Positively charged residues are in blue, and hydrophobic residues are in green. Stacking interactions are represented by green lines and hydrogen bonds are in violet lines. (D) The redocked pose of mefenamic acid (green balls and stick) compared with the co-crystalized ligand (blue sticks).

Table 5: The docking score and binding parameters for mefenamic acid, JS-3, and JS-4 with human cyclooxygenase-2.

Group	Docking score	Glide ligand efficiency	Glide hbond	Glide vdw
Mefenamic acid	-9.587	-0.532	-0.7	-32.9849
JS-3	-7.976	-0.306	-0.3	-35.1206
JS-4	-9.00	-0.358	-0.7	-19.935

with ARG120 and a stacking interaction with TYR355. JS-4 formed a stacking interaction with PHE205. The docking findings revealed that hbonds and vdw forces contributed positively to the binding of all compounds (Table 5). According to the docking data, mefenamic acid had the greatest ligand effacing score, followed by JS-4 and JS-3, respectively. The quality checks of the docking run showed low RMSD and perfect alignment of docked and co-crystallized mefenamic acid (Figure 5D).

DISCUSSION

Extensive research efforts have focused on the discovery of selective inhibitors of COX-2 with the aim of reducing their gastrointestinal toxicity compared with classical NSAIDs.[9,34] However, the reports associating cardiovascular adverse effects such as the increased risk of myocardial infarction, and other thrombotic events, with the use of selective COX-2 inhibitors raised important questions on the safety of these drugs[35] and whether they are safer than classical NSAIDs. In addition, the physiological expression of COX-2 in the heart,[36] kidney,[37-38] and other tissues[39] contradicts our previous understanding that this isoform is only inducible in inflammatory conditions.

In the present study, we aimed to assess the anti-inflammatory activity and the expected adverse effects of two new anthranilic acid derivatives,[25] compared to the parent compound mefenamic acid. We selected the two compounds that showed the best results in preliminary anti-inflammatory screening assays and the highest *in vitro* activity. To further characterize the compounds pharmacologically, we used the whole blood assay to determine the corresponding IC_{50} for each compound against COX-1 and COX-2. The whole blood assay is a standard approach that determines the COX selectivity of a compound.[26-27,40] In this assay, inhibiting the synthesis of the COX-1-dependent TXA_2 (assayed as its inactive metabolite TXB_2), and the COX-2-dependent PGE_2 by a chemical compound reflect its COX-1 and COX-2 inhibitory function, respectively. Our results showed that JS-4 has the highest

COX-2 selectivity, followed by JS-3, compared to the very low selectivity of mefenamic acid. Therefore, it can be concluded that treatment of inflammatory conditions with JS-4 might induce minimal side effects compared to mefenamic acid. Thus, we tested this hypothesis in a model of RA.[41-42]

Induction of RA by CFA is a well-established working model of chronic inflammation that is widely used in the assessment of new anti-inflammatory agents.[41-43] CFA initially induces edema and soft tissue thickening at the injection site, followed by a late phase flare, with activation of the immune system, and the development of overt RA and other systemic inflammatory events.[42,44] The development of nodules in different organs such as the ears, nose, and tail is a characteristic clinical manifestation of RA associated with disease severity,[41-42] which was evident in the current study. In addition, a reduction in the arthritic score is a direct function of a compound's anti-inflammatory activity.[45] All NSAID-treated groups in the current study showed reduced arthritic scores indicative of anti-inflammatory and anti-arthritic effects. On the other hand, the determination of paw edema in the CFA model is a simple, sensitive, and fast method for evaluating the therapeutic effects of anti-rheumatic agents. CFA increases the injected paw volume and thickness due to T-cell proliferation.[46] In line with their anti-inflammatory effects, mefenamic acid and the studied derivatives reduced paw volume and thickness compared with the disease control group.

Elevated rheumatoid factor is an important surrogate of RA and, together with serum levels of inflammatory cytokines, can be used to monitor disease progression and treatment outcomes.[47-48] Experimentally, induction of RA by injecting CFA elevates blood levels of rheumatoid factor,[43] IL-1β, and IL-6,[49] which indicates a dysregulated immune function in response to autoimmunity.[50] Rheumatoid factor generation in arthritis involves B cell activation and several genetic predispositions to arthritic diseases.[51] RA animals in this work showed elevated serum rheumatoid factor, IL-1β, and IL-6 compared to control rats. These results corroborate previous reports showing increased RF and inflammatory interleukins in CFA-induced RA.[45,49,50,52] On the other hand, treating the RA animals with both derivatives (JS-3 and JS-4), like mefenamic acid, significantly reduced the levels of these inflammation biomarkers, which indicates protective, and possibly immunomodulatory, effects against RA.

TXA_2, PGE_2, and PGI_2 are vasoactive prostanoids produced by the action of COXs. TXA_2 is a strong platelet activator and inducer of smooth muscle contraction, whereas PGE_2 and PGI_2 are potent aggregation inhibitors and smooth muscle relaxants.[53-54] Indeed, selective inhibition of COX-2 leads to inhibition of PGE_2 and PGI_2 production without affecting TXA_2. This effect, in theory, increases the risk of pathologic platelet activation and thrombus formation.[10,55-56] In the present study, CFA administration produced a significant increase in the levels of all studied prostanoids, notably TXA_2, in the disease control group. Both JS-3- and JS-4-treated groups, like the mefenamic acid-treated rats, displayed normalized levels of TXB_2, a surrogate of TXA_2 production.[27] Although all treated groups decreased the levels of PGE_2 and PGI_2 than the untreated ones, their levels are still higher than the normal controls. This latter effect might contribute to cardiovascular protection or lower risk in these animals.[10,20]

The results of the current study showed increased blood pressure and heart rate in the untreated RA animals compared to the normal rats. Clinically, inflammatory conditions such as RA increase the risk of cardiovascular disease, including hypertension and atherosclerosis.[57] Furthermore, several studies illustrated the connection between systemic inflammation and cardiovascular dysregulations, such as endothelial dysfunction and myocardial toxicity, which might explain the increased blood pressure of untreated CFA-arthritis rats observed in our results.[58-62] Indeed, histological examination of myocardial tissue of these rats showed signs of mild cardiomyopathy. Interestingly, RA rats that received the different anti-inflammatory treatments showed normal blood pressure values. However, one important factor to consider while one tries to explain these results would be the lowered pain threshold expected in the RA untreated rats, which can alter the autonomic control of the heart and/or blood vessels and contribute to increased blood pressure.[63-65]

One important limitation of the current study is that we used the tail-cuff method to measure blood pressure and heart rate. This method involves the inflation of a cuff around the base of the rat tail, which can be a painful stressor during blood pressure measurement. While we did not have the tools to verify whether this was the case, this possibility cannot be excluded. All NSAID-treated rats showed normal blood pressure and heart rate values, besides their normal myocardial histology. While this can be a COX-dependent effect of these compounds by suppressing inflammation and disease progression and halting the cytokine/immune cell/inflammation cycle, the analgesic effect of these drugs can also modulate the level of stress during blood pressure measurement. Nevertheless, in a similar adjuvant arthritis model, the authors reported no change in blood pressure after disease induction.[60] However, that study involved

using an invasive carotid artery cannulation technique under general anesthesia, which is expected to abolish pain and stress-related factors during blood pressure measurement.[60] Interestingly, in their work, treatment with diclofenac, but not the selective COX-2 inhibitor celecoxib, elevated the rat blood pressure levels while surprisingly improving *in vitro* endothelial dysfunction of the aorta isolated from the same animals.[60] The two anthranilic acid derivatives tested here showed good COX-2 selectivity in the current study and in our previous report,[25] but mefenamic acid lacks this relative selectivity. However, the cardioprotection is observed almost equally with the three treatments, which might support the involvement of pain regulation besides the anti-inflammatory effects in these results.

The obtained docking scores and hydrogen bonds profile in the current results suggests a stronger affinity of mefenamic acid over its derivatives toward human COX-2 binding. However, the better selectivity of the compounds JS-3 and JS-4 in biochemical experiments is supported by the configuration of chemical changes of mefenamic acid in the COX-2 binding site. While mefenamic acid is well placed in the COX's inhibitor cavity, it has no interactions with ARG120 and TYR355 at the constriction leading to the active site. The slight marginal improvement in the selectivity of JS-3 is contributed by forming a hydrogen bond with ARG120. The obtained higher selectivity of JS-4 was supported by the hydrophobic binding between its bromophenyl group and the COX-2 hydrophobic pocket composed of LEU531, GLY533, and LEU534. This resulted in increased selectivity of JS-4 over mefenamic acid. In this context, COX-2's adoption of alternate substrate conformations and accommodation of bulky substrates are almost invariably accompanied by a rotation of LEU531 away from the constriction residues to make more space. This movement has not been seen in COX-1 structures.[66] This observed space adoption for COX-2 allowed for the inclusion of a larger derivative of JS-4 that cannot be deployed in the COX-1 structure. Another important aspect of JS-4 selectivity is the projection towards GLY533. Mutation of GLY533 to alanine resulted in a complete loss of COX activity,[67] implying the importance of this residue in the COX's activity. The bulky substituents of mefenamic acid in compounds JS-3 and JS-4 had led to a marked alteration in their binding pockets compared to mefenamic acid and included some of the binding patterns of diaryl heterocycle inhibitors as coxibs. The piperazine ring of JS-3 formed a bond with ARG120 and lightly filled an upper cavity formed by LEU93, TYR115, and VAL116, which lead to partial modification of the mefenamic acid ring position to interact with PHE518, MET522, VAL523, ALA527, and SER530, which shares on hosting the selective coxib drugs.[68]

Some clinical reports linked selective COX-2 inhibition with a higher risk of cardiovascular events.[16-17] A research group found that despite the low expression of COX-2 in normal cardiomyocytes, both COX-1 and COX-2 were important for tissue recovery after myocardial infarction in humans.[36] The same group studied the expression of both COX-1 and COX-2 in human autopsy and biopsy samples. They reported the expression of both isoforms in normal healthy human tissues, including neuronal, renal, and colonic samples, which questions the accepted concept of 'constitutive' vs. 'inducible' isoforms.[8] In line with these findings, Yu, Ricciotti[69] introduced further evidence on the importance of COX-2 in normal vascular physiology. In particular, this study showed the regulation of endothelial nitric oxide synthase (eNOS)/NO-mediated vascular relaxation by COX-2 activity, hence the importance of COX-2 in controlling blood pressure and thrombus formation.[69] On the other hand, other studies showed that celecoxib, a selective COX-2 inhibitor, was superior to conventional NSAIDs, including indomethacin, or rofecoxib, in cardiovascular safety.[18-19] Furthermore, an analysis of published clinical trials concluded that the incidence of serious adverse effects in patients treated with selective COX-2 inhibitors is not higher than those on classical NSAIDs.[70] Thus, whether selective COX-2 inhibition predisposes patients to higher cardiovascular risk than classical NSAIDs remains an open debate.

Inflammation increases the release of cytokines, which might activate extramedullary haematopoiesis resulting in disruption of spleen histology as splenomegaly.[71] Administration of CFA induced splenomegaly in the untreated disease control groups compared to the normal control group, which was mitigated by treating the rats with mefenamic acid or its derivatives indicating their effectiveness as anti-inflammatory agents. On the other hand, the preservation of thymus weight by these drugs reflects their beneficial effects as immunomodulators.[42,72]

Histopathological evaluation of the knee joints of RA-diseased rats showed considerable damage to articular structures, hyperplasia of the synovium, focal cartilage destruction, and pannus formation, indicating joint damage and inflammation, which is supported by previous reports.[29,41-42] However, treatment with anthranilic acid derivatives showed a protective effect on histopathology of synovium supporting its anti-arthritic effect.

The administration of either the classical "older" NSAIDs or even the more recent COX-2 selective inhibitors can activate mechanisms that enhance blood pressure, induce or worsen liver function, impair kidney function to the point of renal failure, and precipitate gastric ulceration,[17,20,69,73-74] especially with high doses. Thus, the present study evaluated the safety profile of anthranilic acid derivatives at supratherapeutic doses compared to the standard drug mefenamic acid. COX-1 is distributed in the renal blood vessels, collecting tubules, glomeruli, and interstitial cells, while COX-2 is distributed in the loop of Henle and interstitial cells of the kidney.[36-39] Thus, inhibition of both isoforms with traditional NSAIDs or COX-2 by the selective COX-2 inhibitors can have variable effects on renal function and might contribute to nephrotoxicity, especially when combined with other insults.[37-38] However, these adverse effects are expected to be less with the selective COX-2 inhibitors,[73,75] manifested as a significant increase in urea and creatinine levels.[75] In the present study, both the standard drug and derivatives did not produce any significant increase in urea and creatinine levels, indicating a lack of acute nephrotoxic effects. However, the long-term safety of such compounds on the kidney is yet to be established.

Patients with RA have an approximately 11-fold greater risk of developing liver injury.[74,76] Serum levels of ALT and AST are commonly used biomarkers of liver injury. In the present study, acute administration of all the studied NSAIDs did not later the levels of ALT and AST in serum, which suggests a lack of acute adverse effects on the liver. Several studies suggested the causative role of different NSAIDs in the etiology of peptic ulcers, another important gastrointestinal condition. NSAIDs inhibit the synthesis of prostaglandins (PGE_2 and PGI_2), critical for upper gastrointestinal mucosal cytoprotection and modulation of gastric acid secretion.[34,70,77] In the present study, the stomachs dissected from animals exposed to high doses of the anthranilic acid derivatives showed only slight ulcerative potential 4 hr after exposure. These results indicate the lack of acute gastrointestinal toxicity by the tested compounds, which does not preclude the possible toxicity with chronic dosing.

CONCLUSION

The results of the current study provide evidence of the effectiveness and safety of two newly introduced anthranilic acid derivatives as anti-inflammatory agents in a well-documented model of RA. Furthermore, the results illustrate that both JS-3 and JS-4 have comparable anti-inflammatory activities, despite the superior COX-2 selectivity of the latter. In addition, both JS-3 and JS-4 displayed slightly better anti-inflammatory actions, especially at the cytokine expression level, than the standard mefenamic acid as well as an improved safety profile. Therefore, both drugs might be good candidates for further clinical examination and assessment of long-term toxicity.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

ALT: Alanine transaminase; **AST:** Aspartate transaminase; **CFA:** Complete Freund's adjuvant; **COX:** Cyclooxygenase; **ELISA:** Enzyme-linked immunosorbent assay; **eNOS:** Endothelial nitric oxide synthase; **IC_{50}:** Half maximal inhibitory concentration; **IL:** Interleukin; **NSAIDs:** Non-steroidal anti-inflammatory drugs; **PG:** Prostaglandin; **PGI_2:** Prostacyclin; **RA:** Rheumatoid arthritis; **RMSD:** Route mean square deviations; **TXA_2:** Thromboxane A_2.

SUMMARY

- JS-3 and JS-4 (an anthranilic acid derivatives) is a potential and safe candidate for managing rheumatoid arthritis.
- They have no renal, liver, gastrointestinal, or cardiovascular toxicity.
- Both derivatives have higher selectivity against cyclooxygenase-2 than mefenamic acid.

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