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) E2, PGI2, and TXB2 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.
colon, and cecum, constitutively express COX-1. 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. COX-2, however, is believed to be an inducible isozyme of the enzyme that is upregulated in inflammatory cells following tissue injury or infection. COX-2 produces mainly the prostanoid mediators of inflammation.

\(\text{PGI}_2\) is a key player in the complicated COX–prostanoids cascade in cardiovascular physiology and diseases because of its anti-atherogenic and cardioprotective roles. \(\text{PGI}_2\) counteracts the cardiovascular effects of the platelet-derived thromboxane \(\text{A}_2\) (TXA\(_2\)) that activates platelet aggregation, vasoconstriction, and vascular proliferation. COX-1 produces \(\text{PGI}_1\) in many tissues, such as the gastric mucosa and the kidney, while COX-2 is a significant source in the heart. Importantly, selective inhibition of COX-2 reduces \(\text{PGI}_2\) synthesis, which can magnify the unopposed TXA\(_2\)-mediated adverse effects.

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, and naproxen affects bowel and jejunal integrity. In addition, diclofenac has been shown to increase the risk of heart attacks and strokes by 50% within days after administration. 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, 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. 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 regimen, especially in high-risk groups, is recommended.

Mefenamic acid and other anthranilic acid derivatives, also known as fenamates, constitute one of the oldest and most used NSAIDs. This class of biologically active molecules has attracted the attention of medicinal chemists because of their wide range of pharmacological properties. We have recently synthesized a series of amide derivatives of mefenamic acid, which showed promising anti-inflammatory activity both in vitro and in vivo. Based on our previous results, 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.

**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. 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 isomers (COX-1 and COX-2) was determined using a whole blood assay as previously described. 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

![Figure 1: Structures of anthranilic acid derivatives.](image)
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 TXA₂ production as TXB₂, a stable inactive metabolite of TXA₂, 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 PGE₂ 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. Arthritis was induced by a subplantar injection of 0.1 ml CFA in the right hind paw, as previously described.

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, PGE₂, PGI₂, and TXB₂, 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. 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 with slight modifications. The Schrödinger Maestro suite
Morsy, et al.: Anthranilic Acid Derivatives as Anti-inflammatory agents

* COX-2 selectivity was calculated for a compound by dividing its COX-1 IC₅₀ (concentration inhibited TXB₂ production by 50%) by the COX-2 IC₅₀ (concentration to determine its median inhibitory concentration (IC₅₀) against either enzyme. 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 TXB₂ and PGE₂ 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 PGE₂ production (IC₅₀ = 4.3 µM) while inhibiting TXB₂ production at an IC₅₀ 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.

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

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

#: 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.
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, PGE₂, PGI₂, and TXB₂

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 PGE₂, PGI₂, and TXB₂ compared to the normal controls. Mefenamic acid-, JS-3-, and JS-4-treated RA rats, on the other hand, demonstrated significantly decreased levels of PGE₂, PGI₂, and TXB₂ relative to the untreated RA rats. Neither mefenamic acid-, JS-3-, nor JS-4-treated groups showed significant decreases in the levels of PGE₂, PGI₂, and TXB₂ 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. Microscopic 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).

<table>
<thead>
<tr>
<th>Group</th>
<th>RF (IU/ml)</th>
<th>IL-1β (µg/ml)</th>
<th>IL-6 (µg/ml)</th>
<th>PGE₂ (µg/ml)</th>
<th>PGI₂ (µg/ml)</th>
<th>TXB₂ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>5.31 ± 0.54</td>
<td>16.1 ± 1.53</td>
<td>124.0 ± 4.43</td>
<td>367.5 ± 13.1</td>
<td>337.8 ± 15.81</td>
<td>253.3 ± 15.76</td>
</tr>
<tr>
<td>DC</td>
<td>26.6 ± 2.76#</td>
<td>38.4 ± 2.24#</td>
<td>216.9 ± 4.39#</td>
<td>967.7 ± 6.8#</td>
<td>1063 ± 31.49#</td>
<td>421.3 ± 22.54#</td>
</tr>
<tr>
<td>Mef</td>
<td>10.2 ± 0.40*</td>
<td>26.3 ± 1.38</td>
<td>146.0 ± 21.54**</td>
<td>824.9 ± 21.2#</td>
<td>681.1 ± 48.25**#</td>
<td>261.6 ± 26.29**</td>
</tr>
<tr>
<td>JS-3</td>
<td>8.46 ± 0.62*</td>
<td>19.4 ± 2.15**</td>
<td>117.1 ± 11.9***</td>
<td>504.1 ± 78.9***</td>
<td>734.5 ± 22.78***#</td>
<td>263.2 ± 15.9***</td>
</tr>
<tr>
<td>JS-4</td>
<td>5.29 ± 0.45*</td>
<td>19.2 ± 1.54**</td>
<td>124.8 ± 15.91***</td>
<td>610.5 ± 40.6***#</td>
<td>876.3 ± 24.30***#</td>
<td>273.03 ± 41.5**</td>
</tr>
</tbody>
</table>

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

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

$#,$##: 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.
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).

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.
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. 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 and whether they are safer than classical NSAIDs. In addition, the physiological expression of COX-2 in the heart, kidney, and other tissues 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 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 IC₅₀ 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. In this assay, inhibiting the synthesis of the COX-1-dependent TXA₂ (assayed as its inactive metabolite TXB₂), and the COX-2-dependent PGE₂ by a chemical compound reflect its COX-1 and COX-2 inhibitory function, respectively. Our results showed that JS-4 has the highest

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Docking score</th>
<th>Glide ligand efficiency</th>
<th>Glide hbond</th>
<th>Glide vdw</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mefenamic acid</td>
<td>-9.587</td>
<td>-0.532</td>
<td>-0.7</td>
<td>-32.9849</td>
</tr>
<tr>
<td>JS-3</td>
<td>-7.976</td>
<td>-0.306</td>
<td>-0.3</td>
<td>-35.1206</td>
</tr>
<tr>
<td>JS-4</td>
<td>-9.00</td>
<td>-0.358</td>
<td>-0.7</td>
<td>-19.935</td>
</tr>
</tbody>
</table>

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**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).

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**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-crystallized ligand (blue sticks).
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.41-42,45 RA animals with both derivatives (JS-3 and JS-4), like mefenamic acid, significantly reduced the levels of these autoantibodies, notably rheumatoid factor, IL-1β, and IL-6,49 which indicates a dysregulated immune function in response to autoimmunity.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, mafenamic 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.45

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.40,49,50,52 On the other hand, treating the RA animals with both derivatives (JS-3 and JS-4), like the mafenamic acid-treated rats, displayed normalized levels of TXB2, a surrogate of TXA2 production.27 Although all treated groups decreased the levels of PGE2 and PGI2 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.66 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. 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. The two anthranilic acid derivatives tested here showed good COX-2 selectivity in the current study and in our previous report, 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. 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, 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.

Some clinical reports linked selective COX-2 inhibition with a higher risk of cardiovascular events. 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. 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 isosforms in normal healthy human tissues, including neuronal, renal, and colonic samples, which questions the accepted concept of 'constitutive' vs. 'inducible' isosforms. In line with these findings, Yu, Ricciotti 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. 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. 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. 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. 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.

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. 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, 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. 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, these adverse effects are expected to be less with the selective COX-2 inhibitors, manifested as a significant increase in urea and creatinine levels. 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. 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\textsubscript{2} and PGI\textsubscript{2}), critical for upper gastrointestinal mucosal cytoprotection and modulation of gastric acid secretion. 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\textsubscript{50}: Half maximal inhibitory concentration; IL: Interleukin; NSAIDs: Non-steroidal anti-inflammatory drugs; PG: Prostaglandin; PGI\textsubscript{2}: Prostacyclin; RA: Rheumatoid arthritis; RMSD: Route mean square deviations; TXA\textsubscript{2}: Thromboxane A\textsubscript{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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