SYNTHESIS AND BIOLOGICAL EVALUATION OF MUTUAL PRODRUGS OF 2-{{[3-(TRIFLUOROMETHYL)PHENYL]AMINO}BENZOIC ACID

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ABSTRACT

For reducing the gastrointestinal (GI) toxicity associated with flufenamic acid (FA), its carboxylic group was masked by synthesizing its mutual prodrugs with propyphenazone by direct coupling and by using spacer technique. The structures of the synthesized mutual prodrugs (FE and FG) were confirmed by IR, $^1$H NMR, $^{13}$C NMR, mass spectroscopy and their purity were established by elemental analysis. In vitro hydrolysis study of both prodrugs (FE & FG) in enzyme-free simulated intestinal fluid (SIF, pH 7.4) furnished 43.97% and 53.46% release of parent drug FA with a half life of 9.13 and 7.54 hours respectively, following first order kinetics. While in simulated gastric fluid (SGF, pH 1.2) they were found to be stable. Both FA prodrugs were retaining anti-inflammatory activity intact and exhibited better analgesic activity along with much reduced ulcerogenicity as compared to parent drug. However prodrug FE showed better percentage anti-inflammatory activity (61.91%) than FG (45.22%) at the end of 6th h and it also exhibited better percentage analgesic activity (76.85%) than FG (71.12%) at the end of 2nd h. Hence FE could be considered as a better candidate for prodrug among the two.

KEYWORDS: Flufenamic acid, NSAIDs, Mutual prodrug and Ulcerogenicity.

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INTRODUCTION

Flufenamic acid (FA), one of the non-steroidal anti-inflammatory drugs, is an anthranilic acid derivative with analgesic, anti-inflammatory and antipyretic properties. It could not be used as up to its potential, because of its irritancy towards the gastric mucosa offered due to presence of free carboxylic acid group. GI mucosal injury produced by NSAIDs is generally believed to be caused by two different mechanisms\(^1\). The first mechanism involves a local effect composed of a direct contact while the other has an indirect effect on the GI mucosa \(^2\). The local contact effect can be attributed to the local inhibition of prostaglandin (PG) synthesis in the GI tract. The indirect effect can be attributed to a combination of ion-trapping mechanism of NSAIDs in mucosal cells and back diffusion of H\(^+\) ions from the lumen into the mucosa. Topical irritation by the free carboxylic group of the NSAIDs is considered an important factor in establishing superficial stomach erosion, particularly in the corpus region of the stomach. The second mechanism is based on the generalized systemic action occurring after absorption and can be manifested even after intravenous dosing\(^5\). The systemic effects are manifested due to inhibition of synthesis of gastric PGs like PGE\(_2\) and PGI\(_2\). In the past, there has accordingly been a lot interest in the area as a drug development opportunity because the design of a successful prodrug represents a significant and interesting scientific conundrum\(^6\). Reported flufenamic acid derivatives include dextran-flufenamic acid ester\(^7\), cholesteryl ester prodrugs\(^8\), butyl flufenamate\(^9\), aminocarbonyloxymethyl esters prodrugs\(^10\), hydrophobic monomeric and dimeric nanoprodugs\(^11\), (N,N,N-trialkylammonium)alkyl ester and thioester derivatives\(^12\) etc. Propyphenazone is a non-acidic pyrazole drug and has a good analgesic and antipyretic activity with no anti-inflammatory activity. The metabolism of propyphenazone has been reported to proceed via the formation of 3-hydroxymethyl-propyphenazone (HMP), which is pharmacologically active as the parent drug\(^13\)\(^-\)\(^14\). Naproxen-propyphenazone esters\(^15\) and mafenamic acid-propyphenazone ester\(^16\) as mutual prodrugs have already been synthesized with the aim of improving the therapeutic index through prevention of gastrointestinal irritation and bleeding. Taking into considerations of these findings, both NSAIDs (flufenamic acid and propyphenazone) were coupled as mutual prodrugs via direct esterification and by using spacer to achieve many advantages related to synergistic effect with reduced GI irritation and a study on their various physicochemical characters, hydrolysis kinetics, anti-inflammatory, analgesic activity and ulcerogenicity as mutual prodrugs.

MATERIALS AND METHODS

Propyphenazone was obtained as a gift sample from the Vani Pharma Labs Limited, Hyderabad, AP, India and drug FA was purchased from sigma Aldrich. The other reagents and solvents used were of analytical grade. The melting points of synthesized compounds were determined in open capillary using elbica melting point apparatus and recorded in °C without correction. The TLC of the compounds was performed on silica gel G coated glass plates with Ethyl acetate: n-Hexane (2:1) as solvent. Iodine vapors were used as detecting agent. The infrared spectra were recorded by BRUKER FT-IR. Proton nuclear magnetic resonance spectra (\(^1\)H NMR) and \(^13\)C NMR were recorded on Bruker Avance II 400 NMR Spectrophotometer using tetramethyl silane as an internal standard. NMR spectra were recorded with DMSO as a solvent and the chemical shift data were expressed as values relative to TMS (Chemical shift δ in ppm). Mass spectra of the compounds were obtained using LC-MS (SHIMADZU-2010 AT, Software class VP). Elemental analyses were performed at the Analysis centre, Chemistry department, Faculty of Science, Delhi University. The hydrolysis study was carried out using a Veego Paddle Type dissolution apparatus (VDA-8DR USP Standard). The hydrolysis data and drug content determination were performed by a UV-Visible Spectrophotometer Pharma Spec-1700 (SHIMADZU). Number of animals and the experimental protocols were approved by Institutional Animal Ethics Committee, Hindu college of Pharmacy, Sonepat, Haryana (registration no.585/02/c/CPCSEA, dated 27/11/2012).
1. **Chemistry**

Mutual prodrugs of FA were prepared by two methods

(a) Direct esterification (FE)

(b) Spacer technique (FG)

**Synthesis of Flufenamic–propyphenazone mutual prodrug by direct esterification (FE):**

**Step-1: Preparation of 3-bromomethyl propyphenazone (BMP)**

3-bromomethyl propyphenazone, C_{14}H_{17}BrN_{2}O, was synthesized from propyphenazone and bromine, according to Meister, Lucius, Bruning in Hochst, 1907, with some modifications to increase yield and purity as follows; Propyphenazone (10 g) was dissolved in 25 ml dichloromethane (DCM) in 100 ml round bottom flask fitted with dropping funnel; it was kept over an ice bath and bromine solution (6.95 g; 2.23 ml bromine dissolved in 5 ml DCM) was added drop-wise and very slowly with the aid of stirring for 1 hr. Temperature was maintained 10-15°C throughout the reaction. The reaction progress was monitored by TLC using ethyl acetate: Hexane (2:1) ratio. An aliquot of 30 ml 10% cold aqueous sodium carbonate solution was added with vigorous shaking. The brown color of the bromine faded rapidly upon its addition. The mixture was kept for 30 min and a solution of HMP (0.5g, 2 mmol) in dry DMF (10 ml) was added dropwise. The reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with water and extracted with DCM. Organic layer was separated and washed with cold diethyl ether to get more pure crystals of BMP.

**Step-2: Preparation of 3- hydroxymethyl propyphenazone (HMP)**

BMP (0.309 g, 1 mmol) was refluxed with 5.0 ml of water. The reaction progress was monitored by TLC, using EA: Hexane (2:1). Once the reaction get completed, reaction mixture was cooled, extracted with DCM and the DCM layer was collected. It was washed with water and dried over anhydrous sodium sulphate. Concentration of DCM layer resulted in white hydroxyl derivative. Recrystallization with hot water resulted in 0.21g (85.36%) pure white crystals of HMP.

**Step-3: Preparation of Flufenamic - 3-hydroxypropyphenazone ester prodrug (FE)**

A solution of carbonyldimidazole (CDI) {0.443g, 2.7 mmol} in anhydrous dimethylformamide (2.5 ml) was added to a cold solution of NSAID (2 mmol) dissolved in anhydrous DMF (3.5 ml). The mixture was kept for 30 min and a solution of HMP (0.5g, 2 mmol) in dry DMF (10 ml) was added dropwise. The reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with water and extracted with DCM. Organic layer was collected, washed with water and dried over anhydrous sodium sulphate. It was concentrated to yield (69%) pure ester prodrug. IR (cm^{-1}): 2963.22 (aliphatic C-H str.), 1733.25 (C-O str. of ester), 1161.84 (C-O-C str.), 1064.96 (O-C-C, bond of ester from 10 instantly alcohol), 1520.68(C=C, phenyl nucleus), 3325.05 (N-H, 2° amine), 1580.35 (N-H, 2° amine), 693.11, 660.70 (O-C-N def, amide), 1330.14 (C-F str.), 795.07, 693.11, 864.23 (1,3- disubstituted Ar ring); ^{1}H NMR(δ (ppm)): 1.232 (d, 6H, CH3 of –CH(CH3)₂, J=7.2 Hz), 3.077 (s, 3H, CH3 of CH₃N), 2.804-2.891 (m, 1H, CH of –CH(CH₃)₂, 7.257-7.336 (m, 4H, Ar-H), 7.352-7.376 (m, 4H, Ar-H), 7.426-7.545 (m, 5H, Ar-H), 4.688 (s, 2H, CH₂ of -COOCH₂), 5.518 (s, 1H, Ar-NH-Ar); ^{13}C NMR: 21.15 (CH₃ of –CH(CH₃)₂), 23.44 (CH₃ of –CH(CH₃)₂), 30.68 (CH of –CH(CH₃)₂), 164.32 (CO of pyrazole), 162.25 (COO), 114.34-145 (Ar-carbons), 39.38 (N-CH₃), 154.52 (C-CH₂ of pyrazole), 125.69 (-CF₃); Mass spectra: Molecular ion peak at m/z 509. Elemental analysis: Calculated: C, 66.00%; H, 5.14%; N, 8.25%. Found: C, 66.12%; H, 5.27%; N, 8.10%.

**Synthesis of FA–propyphenazone mutual prodrug by using spacer (FG)**

**Step-1:** Preparation of glycyl-3-hydroxymethyl propyphenazone (Gly-HMP)

To an ice- cold solution of Boc-glycine (1.75 g, 10 mmol) in 30 ml DCM were added to HMP.
Step-2: Preparation of Flufenamic -Glycine-3-hydroxypropyphenazone (FG)

A solution of carbonyldiimidazole (1 g, 6.16 mmol) in anhydrous DMF (5 ml) was added drop-wise, at 4°C to a solution of FA (1 g) dissolved in anhydrous DMF (10 ml). To the cold mixture, an equimolar solution of Gly-HMP in dry DMF (20 ml) was added drop-wise. The reaction mixture was stirred for 10 min at 4°C, for 3 h at room temperature and then evaporated under vacuum. The residue was purified by column chromatography (hexane / ethyl acetate; 2: 1) to yield (75.2%) of pure compound FG. IR (cm⁻¹): 2937.70 (C-H str. of methyl group), 1741.15 (C=O, ester), 1064.58, 1201.30 (C-O-C str.), 1579.65 (N-H inplane bending 2\(^{\text{nd}}\) amide), 3043.81 (=C-H str, Ar), 695.22, 660.14 (Mono substituted Ar ring), 747.96 (1,2 Disubstituted Ar ring), 793.91 (1,3 Disubstituted Ar ring), 1095.87(C-N str), 1330.77(C-F str). \(^{1}H\) NMR(δ (ppm)): 3.478 (s, 1H, aromatic NH), 6.833-6.972 (m, 4H, Ar-H), 7.199-7.295 (m, 4H, Ar-H), 7.361-7.531(m, 5H, Ar-H), 4.475 (s, 2H, CH\(_2\) of –COOCH\(_2\)-), 1.194 (d, 6H, CH\(_3\) of –CH(CH\(_3\))\(_2\), J=7.4 Hz), 3.029 (s, 3H, CH\(_3\) of –CH\(_3\)N), 2.508-2.564 (m, 1H, CH of –CH(CH\(_3\))\(_2\)), 8.432 (s, 1H, NH of –CONH\(_2\)); \(^{13}C\) NMR: 21.380 (CH of –CH(CH\(_3\))\(_2\)), 21.603 (CH\(_3\) of –CH(CH\(_3\))\(_2\)), 31.144 (CH of –CH(CH\(_3\))\(_2\)), 164.843 (CO of pyrazole), 171.138(COO), 114.682-145.478 (Ar-carbons), 39.467 (N-CH\(_3\)), 40.580 (NH-CH\(_2\)-COO), 154.855 (C-CH\(_2\) of pyrazole), 162.836 (-NHC(O)-), 129.348 (-CF\(_3\)); Mass spectra: Molecular ion peak at m/z 566; Elemental analysis: Calculated: C, 63.60%; H, 5.16%; N, 9.89% Found: C, 63.46%; H, 5.02%; N, 9.99%.

2. In-vitro hydrolysis studies of FA prodrugs

The hydrolysis study was conducted at 37±0.5°C using apparatus. Two hydrolysis media were used: 900 ml of non-enzymatic simulated gastric fluid (SGF, pH 1.2) and simulated intestinal fluid (SIF, pH 7.4). An accurate amount of 100 mg of the prodrug was used for the study. From the matrix, aliquots of 1 ml were withdrawn at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 h and were immediately replaced with 1.0 ml of fresh hydrolysis media equilibrated at 37±0.5°C. Free FA released after the hydrolysis of prodrugs was extracted with 5 ml methanol. The methanol layer was estimated on UV spectrophotometer for the amount of free FA released after hydrolysis of prodrugs in SGF and SIF. The kinetics of hydrolysis was monitored by increase of free drug concentration with time and order of reaction and half life (t\(_{1/2}\)) were also calculated. The rate of hydrolysis was calculated using equation, k= (2.303/t) log (a/a-x) where k represents hydrolysis constant, t is the time in min, ‘a’ is the initial concentration of prodrug, x is the amount of prodrug hydrolysed and (a-x) is the amount of prodrug remaining.

3. In-vivo Evaluation

The prodrugs prepared were screened for analgesic and anti-inflammatory activity as compared with that exerted by FA and HMP, separately. Wistar rats of Albino strain (150-200 g) were used. All the animals were obtained from Animal House of Hindu college of Pharmacy, Sonipat. The selected animals were housed in polypropylene cages at standard environmental conditions at 22 ± 2°C, relative humidity of 45–55 %, in a well ventilated room maintained at 12: 12 h light: dark cycle, fed with standard pellet diet and water ad libitum. All the animals were acclimatized for a week before experiment. All animal experiments were carried out according to the guidelines of the Committee for the Purpose of Control of Experiments on Animals and approval of the Institutional Animal Ethics Committee was obtained. The animals were fasted with free access to water for 12 h prior to the tests. The tested compounds were prepared for oral administration in aqueous
0.5% Carboxymethylcellulose (CMC) solution. The experimental protocol was approved by Institutional Animal Ethics Committee (registration no.585/02/c/CPSEIA, dated 27/11/2012). All the results were expressed as Mean ± Standard error (SEM). Data was analyzed using one-way ANOVA followed by Tukey-Kramer multiple comparisons test. p-values < 0.05 were considered as statistically significant.

(a) Analgesic activity

The analgesic activity was assessed by tail flick method\textsuperscript{18}. Rats were divided into five groups (n=6 in each group). Group I served as control (received 0.5% w/v CMC (10 ml/kg) only). Group II and III received standard drugs i.e FA (20 mg/Kg body weight, 71.1 µmol/kg) and HMP (23 mg/Kg body weight, 13.97 µmol/kg), Group IV and V received prodrug FE (36.24mg /Kg body weight, 71.1 µmol/kg) and FG (40.30mg/Kg body weight, 71.1 µmol/kg), where the dose was molecular equivalent to the free drug. The control, standard and test drugs were given by oral route. Rats were treated with drug vehicle or standard or test drugs. The reaction time was recorded with a stopwatch. Each animal served as its own control and two readings were obtained for the control at 0 and 10 min interval. The average of the two values was the initial reaction time (T\textsubscript{a}). The reaction time (T\textsubscript{b}) for the test groups was taken at intervals 0.5, 1, 2, 3 and 4 h. The percentage analgesic activity was calculated using the formula: Percentage analgesic activity = \[1-(T\textsubscript{a}/T\textsubscript{b})\] \times 100

(b) Anti-inflammatory activity

The carrageenan induced rat hind paw edema method described by Winter et al.\textsuperscript{19} was used to evaluate the acute anti-inflammatory activity of the prodrugs. Rats were divided into control, standard and test groups of six animals each. Pretreatment initial paw volumes of all animals were measured using a mercury plethysmometer. The control group was given only an appropriate volume of 0.5% CMC. Standard group received FA and HMP (equivalent dose, 13.97 µmol/kg) respectively. To the test group, prodrugs (FE and FG) were administered orally using the similar doses as employed in the analgesic activity. One hour after treatment, edema in the left hind paw of the rat was induced by injection of 0.1 ml of 1% (w/v) carrageenan solution in normal saline solution (0.9% w/v). The marking of the paw was done with ink at the level of lateral malleolous and then immersed in mercury up to this mark. The relative change in paw volume was taken as a measure of edema which was determined by measuring the paw volume immediately after injection and at 1, 2, 3, 4, 6 and 8 hour intervals following the carrageenan administration. The percent inhibition of edema, as an indication of anti-inflammatory activity was compared with the controls. The percentage inhibition of swelling was calculated using the following formula:

\[\text{Inhibition} (%) = \frac{(V\textsubscript{o} - V\textsubscript{t}) \text{control} - (V\textsubscript{o} - V\textsubscript{t}) \text{treated} \times 100}{(V\textsubscript{o} - V\textsubscript{t}) \text{control}}\]

V\textsubscript{o} and V\textsubscript{t} relates the average volume in the hind paw of rats (n=6) before any treatment and after anti-inflammatory agent treatment, respectively.

(c) Ulcerogenic Activity

Gastrointestinal toxicity was determined using the method as described by Kunchandy et al.\textsuperscript{20}. Five groups of six rats each were fasted for 12 hour prior to the administration of drug. The first group served as control and received vehicle (0.5% CMC, 10 ml/kg) only. Group II and III received FA and HMP (in equivalent doses, 71.1 µmol/kg) respectively. Group IV and V received equivalent doses of prodrugs FE (36.24 mg/kg, 71.1 µmol/kg) and FG (36.24 mg/kg, 71.1 µmol/kg) separately. All the test drugs or standard of vehicle were administered orally to rats over a period of seven successive days. All the rats were fasted for 24 hour on the 8\textsuperscript{th} day. The animal was sacrificed with excessive anesthesia. The stomach was removed, opened along the greater curvature and washed gently in running tap water. The gastric mucosa of the rat was examined by means of magnifying lens. The score of ulcers were assessed according to the following scoring system: 0.0 - normal colored stomach; 0.5 - pink to red coloration of stomach; 1.0-spot ulcer; 1.5 - haemorrhagic streak; 2.0 - number of ulcers < 5; 3.0 - number of ulcers > 5; 4.0 - ulcers with bleeding.
RESULTS AND DISCUSSION

1. Chemistry
Bromopropyphenazone (BMP) was prepared by bromination of propyphenazone according to Meister, Lucius, Bruning in Hochst, 1907 with some modifications to increase yield and purity. Bromopropyphenazone (BMP) was used for preparation of hydroxymethyl-propyphenazone (HMP) by reflux with water. Flufenamic-propyphenazone ester (FE) was synthesized by coupling of HMP and FA as shown in scheme 1.

General steps for synthesis:
- Scheme 1 General steps for the synthesis of FE prodrug.
  - a) Synthesis of 3-bromomethyl propyphenazone (BMP),
  - b) synthesis of HMP,
  - c) synthesis of FE ester prodrug

The CDI method gave better yield of the corresponding ester with minimal reaction by-products. In order to avoid or minimize the steric hindrance effect on the hydrolysis of flufenamic-HMP ester prodrug (FE), a spacer was introduced. The spacer chosen was glycine. N-Protected glycine
coupled with HMP by DCC followed by N-deprotection and coupling with FA using CDI yielded flufenamic amide prodrug, FG as illustrated in scheme II.

The physicochemical properties were determined (Table 1). The yields of prodrugs were good. The structures of prodrugs formed were confirmed by UV, $^1$H and $^{13}$C NMR, Mass and FT-IR spectral methods. The purity was determined by TLC. The results of elemental analysis of synthesized prodrugs were in all case within 0.4% of theoretical values and were in confirmation of desired structure.

<table>
<thead>
<tr>
<th>Prodrug Code</th>
<th>Molecular formula</th>
<th>Mol. wt.</th>
<th>Colour</th>
<th>m.p.(°C)</th>
<th>% Yield</th>
<th>R&lt;sub&gt;f&lt;/sub&gt; value</th>
<th>Log P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FE</td>
<td>C&lt;sub&gt;20&lt;/sub&gt;H&lt;sub&gt;26&lt;/sub&gt;F&lt;sub&gt;3&lt;/sub&gt;N&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;</td>
<td>509</td>
<td>Yellow</td>
<td>110-115</td>
<td>69.00</td>
<td>0.57</td>
<td>5.76</td>
</tr>
<tr>
<td>FG</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;32&lt;/sub&gt;F&lt;sub&gt;3&lt;/sub&gt;N&lt;sub&gt;4&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;</td>
<td>566</td>
<td>Yellow</td>
<td>Semisolid</td>
<td>75.20</td>
<td>0.48</td>
<td>4.62</td>
</tr>
</tbody>
</table>

Ethyl Acetate : n-Hexane (2 : 1)

2. **In-vitro hydrolysis of ester**
Since the carboxylic group of FA is essential for the therapeutic action, prodrugs of prolonged action were designed in a form which the biologically active moiety can be released in its original state with time. Therefore the release of FA from its prodrugs was studied *in vitro* in order to evaluate the possible time span in which the drug could be available from different prodrugs. The comparative patterns of hydrolysis of these prodrugs in SGF and SIF were studied (Figure 1 and 2).  

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Comparative pattern of hydrolysis of FE and FG in SGF (pH-1.2)

Figure 1

Amount of FA regenerated on hydrolysis of FE and FG in SGF (pH-1.2) was found as 10.50% and 6.90% respectively over a period of 2 hours.

Comparative pattern of hydrolysis of FE and FG in SIF (pH-7.4)

Figure 2

The amount of FA regenerated on hydrolysis of FE and FG in SIF (pH-7.4) was found as 43.97% and 53.46% respectively over a period of 10 hours.

Both prodrugs showed negligible hydrolysis in acidic medium (pH 1.2) for 2 hours. From these results, this is confirmed that the release of FA should occur predominantly at higher pH of the intestine. This may be due to the fact that ester hydrolysis is a reversible reaction in acidic pH and in alkaline pH it is irreversible and complete. The predominant release of FA from its prodrugs at pH 7.4 indicates potential of the prodrug to reduce the gastric complications caused by direct contact of free carboxyl group of the drug to gastric mucosa.

Kinetic parameters for hydrolysis of mutual prodrugs at 37 °C were calculated (Table 2). The corresponding half lives for FE and FG were found to be 13.02 and 20.69 hours (in SGF, pH 1.2) and 9.12 and 7.54 hours (in Phosphate buffer, pH 7.4) respectively. The half-lives and the rate constants for prodrug hydrolysis Table 2 indicated that an esterification of carboxylic group of FA rendered its prodrugs more stable at pH 1.2, but less stable at pH 7.4.
Table 2

Kinetic Parameters for Hydrolysis of Mutual prodrugs FE and FG at 37 °C

<table>
<thead>
<tr>
<th>Prodrug code</th>
<th>$K_{\text{obs}}$ (hr$^{-1}$)$^\text{a}$</th>
<th>$T_{1/2}$ (hr)$^\text{a}$</th>
<th>$K_{\text{obs}}$ (hr$^{-1}$)$^\text{b}$</th>
<th>$T_{1/2}$ (hr)$^\text{b}$</th>
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</thead>
<tbody>
<tr>
<td>FE</td>
<td>0.053217</td>
<td>13.02204</td>
<td>0.075925</td>
<td>9.127442</td>
</tr>
<tr>
<td>FG</td>
<td>0.033479</td>
<td>20.69929</td>
<td>0.0919</td>
<td>7.540809</td>
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</tbody>
</table>

$^\text{a}$ In SGF (pH-1.2), $^\text{b}$ In SIF Phosphate buffer (pH- 7.4)

3. In vivo Biological evaluation

Analgesic, anti-inflammatory and ulcerogenic activities of the prodrugs were studied in comparison to equivalent doses (71.1µmol/kg) of FA. Results of anti-inflammatory activity by parent drug and its mutual prodrugs in terms of difference in paw volume and percentage inhibition at various time intervals were obtained (Tables 3 and 4) respectively.

Table 3

Change in paw volume of treated groups FA, HMP and its mutual prodrugs at various time intervals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Change in Paw Volume (ml) (Mean± SEM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>0.38± 0.012</td>
</tr>
<tr>
<td>FA</td>
<td>20.00</td>
<td>0.24± 0.01***</td>
</tr>
<tr>
<td>HMP</td>
<td>23.00</td>
<td>0.25± 0.025**</td>
</tr>
<tr>
<td>FE</td>
<td>36.24</td>
<td>0.28± 0.019*</td>
</tr>
<tr>
<td>FG</td>
<td>40.30</td>
<td>0.30± 0.03</td>
</tr>
</tbody>
</table>

Oedema is expressed as mean change in paw volume ± SEM n= 6 animals. *p<0.05, **p<0.001, ***p < 0.0001 as compared to control. $^\Delta$p<0.05, $^{\Delta\Delta}$p <0.01as compared to FA. $^\sigma$p <0.05, $^{\sigma\sigma}$p < 0.01, $^{\sigma\sigma\sigma}$p <0.001as compared to HMP.

Table 4

% Inhibition of acute inflammation by FA and its mutual prodrugs as compared to control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>00.00</td>
</tr>
<tr>
<td>FA</td>
<td>20.00</td>
<td>36.94</td>
</tr>
<tr>
<td>HMP</td>
<td>23.00</td>
<td>32.61</td>
</tr>
<tr>
<td>FE</td>
<td>36.24</td>
<td>26.08</td>
</tr>
<tr>
<td>FG</td>
<td>40.30</td>
<td>19.56</td>
</tr>
</tbody>
</table>

FA exhibited maximum anti-inflammatory effect (42.85%) at 2nd hour where as second parent drug HMP showed it maximum effect (37.49%) at 3rd hour. Both mutual prodrugs showed better maximum inhibition and for longer time as compared to both parent drugs. FE ester prodrug displayed maximum activity (61.91%) at 6th hour where as FG prodrug displayed its maximum activity (55.26%) at 4th hour. FE prodrug showed relatively better % inhibition i.e. 51.61, 52.63, 61.91and 59.36% during 3rd, 4th, 6th and 8th hour respectively.
Change in paw volume of groups treated with FA and its mutual prodrugs at various time intervals

![Graph showing change in paw volume of treated groups at various time intervals](image1.png)

Figure 3
Both prodrugs exhibited highly significant results ($p < .001$) as compared to control and also possessed significantly better activity as compared to FA.

% Inhibition of acute inflammation by FA and its mutual prodrugs as compared to control

![Graph showing % inhibition as compared with control versus time](image2.png)

Figure 4
FE mutual prodrug displayed maximum activity (61.91%) at 6th hour whereas FG prodrug displayed its maximum activity (55.26%) at 4th hour.

The results showed that the mutual prodrug synthesized by direct esterification was found to be more effective than prodrug made by using spacer technique of parent drugs. The anti-inflammatory activity of prodrugs significantly improves over time. This means that the prodrug per se is devoid of anti-inflammatory activity and the observed latent activity results from hydrolysis to the parent drugs. Both prodrugs exhibited highly significant results ($p < .001$) as compared to control and also possessed significantly better activity as compared to FA. The analgesic activity was determined by tail flick method and the latency period at different time interval was observed (Table 5).
Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Latency period in seconds (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>1.10±0.056</td>
</tr>
<tr>
<td>FA</td>
<td>20.00</td>
<td>1.09±0.134</td>
</tr>
<tr>
<td>HMP</td>
<td>23.00</td>
<td>1.10±0.133</td>
</tr>
<tr>
<td>FE</td>
<td>36.24</td>
<td>0.92±0.086</td>
</tr>
<tr>
<td>FG</td>
<td>40.30</td>
<td>1.29±0.189</td>
</tr>
</tbody>
</table>

Reaction time is expressed as mean ± SEM
n=6 animals; p < 0.0001, **p < 0.001, *p < 0.05 as compared to control. ∆p < 0.05, ∆∆p < 0.01, ∆∆∆p < 0.001 as compared to FA. *p < 0.05, **p < 0.001, ***p < 0.0001 as compared to HMP

The percentage analgesic activity of FA and its mutual prodrugs at various time intervals was determined (Fig 5). The results indicated that FE ester (36.24 mg/kg) showed maximum analgesic activity (76.85%, p<0.001) at 2nd hour which is significantly better as compared to parent drug FA (66.02% at 1st hour) where as FG prodrug showed significantly better activity at 3rd hour (72.12%, p<0.0001) as compared to parent drug FA. The results indicated that both prodrugs have better and long lasting analgesic activity when compared to parent drug.

% Analgesic activity of FA and its mutual prodrugs at various time intervals

![Graph showing % Analgesic activity of Fluufenamic prodrugs vs Time (hrs)]

Figure 5

Maximum analgesic activity was shown by FE at 2nd hour (76.85%, p<0.001) and by FG at 3rd hour (72.12%, p<0.0001) respectively which is significantly better than parent drug FA (66.02% at 1st hour)

Ulcerogenic potential of synthesized prodrugs was tested in comparison to the parent drugs following oral administration for 7 days in rats and the results were obtained in the form of average number of ulcer score (Table 6). Screening for ulcerogenic activity showed that synthesized compounds had less tendencies (p < 0.001) to form ulcer when compared to that of the parent drugs (Fig 6). The results indicated that GI toxicity due to direct contact of the carboxylic group has been protected. Photographs illustrated the gastric mucosal injury (Fig 7).
Table 6
Ulcerogenic potential of FA and its prodrugs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Average number of ulcer score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>FA</td>
<td>20.00</td>
<td>3 ± 0.258***</td>
</tr>
<tr>
<td>HMP</td>
<td>23.00</td>
<td>1.33 ± 0.167****</td>
</tr>
<tr>
<td>FE</td>
<td>36.24</td>
<td>1.083 ± 0.154**</td>
</tr>
<tr>
<td>FG</td>
<td>40.30</td>
<td>0.58 ± 0.154*</td>
</tr>
</tbody>
</table>

Ulcerogenic potential is expressed as average number of ulcer score ± SEM. *n* = 6 animals. ****p < 0.0001, **p < 0.01, as compared to control, #p < 0.0001 as compared to FA. σp < 0.05 as compared to HMP.

Ulcerogenic potential of FA, HMP, and its mutual prodrugs

Figure 6
Both prodrugs showed less tendencies (p < 0.001) to form ulcer when compared to that of the parent drugs.

Illustration of the mucosal injury in rats following oral administration of FA, HMP and their mutual prodrugs

Figure 7
(a) Number of ulcers > 5 (3.0)-FA  (b) Spot ulcer (1.0)-HMP  (c) Pink to red coloration(0.5) – FG  (d) Spot ulcer (1.0)-FE
CONCLUSION

The mutual prodrugs of FA were successfully synthesized and structures were confirmed based on spectral analysis. Both prodrugs showed encouraging hydrolysis rate in SIF and excellent pharmacological response. The in vitro and in vivo evaluation of synthesized prodrugs revealed improvement in the therapeutic index of parent drugs. Both FA prodrugs were retaining anti-inflammatory activity intact and exhibited better analgesic activity along with much reduced ulcerogenicity as compared to parent drug. However prodrug FE showed better anti-inflammatory and analgesic activity than FG and hence it could be considered as a better candidate for prodrug among the two.

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REFERENCES


