Brief Communication

Synthesis and biological evaluation of a mutual prodrug of norfloxacin and fenbufen

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Abstract

Objectives: The study aimed to synthesize a mutual prodrug of norfloxacin and fenbufen with an objective of obtaining an effective and safer anti-inflammatory drug with useful antimicrobial actions.

Methods: An amide-based mutual prodrug (NF-FN) was prepared following a single-step synthesis by condensing norfloxacin with fenbufen under appropriate laboratory conditions. Its structure was established on the basis of IR, NMR, Mass spectral data and elemental analysis. The prodrug (NF-FN) was evaluated for in-vitro antibacterial activity against two gram positive (Staphylococcus aureus & Bacillus subtilis) and two gram negative bacterial strains (Escherichia coli & Klebsiella pneumonia). The in-vivo anti-inflammatory activity and ulcerogenicity of the synthesized prodrug were investigated in Wistar albino rats at the doses of 10 and 30 mg/kg body weight, respectively.

Results: The synthesized prodrug (NF-FN) showed very good activity against S. aureus & E. coli with MIC-6.25 μg/mL, and good activity against B. subtilis & K. pneumonia with MIC-12.5 μg/mL. Its anti-inflammatory activity was found to be better than that of the parent drug fenbufen. It was also observed to less severe on gastric mucosa in comparison to reference drug, fenbufen.

Conclusion: The prodrug showed promising results as an anti-inflammatory agent however, its antibacterial action was found to be slightly weaker than the other parent drug norfloxacin.

Keywords: Antibacterial; Anti-inflammatory; Cinopal; Fluoroquinolone

Introduction

Fenbufen (4-oxo-4-[biphenyl-4-yl] butanoic acid) is an arylpropionic acid derivative having potent anti-inflammatory actions. It is used clinically to treat and manage various inflammatory conditions. It is available in the market under the name of Cinopal.1 Fenbufen like other members of arylpropionic acid class is a good anti-inflammatory agent but causes gastrointestinal side effects.1,2 One of the synthetic approaches commonly used to improve the NSAIDs safety profile involves chemical modification of the free carboxylic group.3–5 Previously conducted research studies have clearly indicated that chemical derivatization of the carboxylate function of some NSAIDs could result in an increased anti-inflammatory activity with reduced ulcerogenic effect.6,7

Norfloxacin belongs to fluoroquinolone class of antimicrobial agents. A number of derivatives of norfloxacin have been synthesized with an attempt to improve its antimicrobial activities.8,9 Several prodrugs derived from norfloxacin

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have also shown improved pharmacological and pharmacokinetic profile.\textsuperscript{10,11}

Prodrug designing is a concept of retro-metabolic drug design that considers targeting, metabolism, duration of action, biological action, side effect, physico-chemical properties etc. into the drug design process.\textsuperscript{12,13} Prodrug designing so far has been proved to be an exciting and fruitful area of medicinal chemistry research. Generally, in a prodrug, an inert or non toxic carrier group or promoiety is used, whose selection primarily depends on the objectives to be achieved in prodrug designing. In case of mutual prodrug, the promoiety may be another drug.\textsuperscript{14} Prodrugs and mutual prodrugs may exhibit improved biological, pharmacokinetic, pharmacodynamic properties with or without minimum side effects.\textsuperscript{15}

In view of these facts and in continuation of our work on design, synthesis and biologically evaluation of prodrugs,\textsuperscript{10,11} it was considered worthwhile to synthesize a mutual prodrug comprising of norfloxacin and fenbufen i.e., two drugs in one with an aim of obtaining an effective and safer anti-inflammatory drug with useful broad spectrum antimicrobial activity against gram positive and gram-negative bacteria drug with useful antimicrobial actions against gram-positive and gram-negative bacteria (broad spectrum). Such drugs might be useful in the inflammatory conditions associated with infection. The other advantages of using the prodrug could be its sustained release, administration of one drug instead of two and low doses that might be required to produce the desired pharmacological effect.

Materials and Methods

Synthesis

Melting points were recorded in open capillary tube and are uncorrected. The IR spectrum of the mutual prodrug was recorded in KBr pellet using a Win IR FTS135 spectrophotometer. \textsuperscript{1}H NMR spectrum was recorded on Bruker spectrophin DPX-300 MHz (Rheinstetten, Germany) with tetramethylsilane (TMS) as an internal standard in solvent CDCl\textsubscript{3}. Mass spectrum was recorded on a Jeol JMS-D 300 (Tokyo, Japan) instrument fitted with a JMS 2000 data system at 70 eV. Microanalysis of the compound was done on Perkin-Elmer model 240 analyzer and the values were found within ±0.4\% of the theoretical values. The progress of the reaction was monitored on silica gel G coated plates by TLC. Iodine chamber and UV-lamp were used for visualization of TLC spots. Dry solvents were used throughout the study. The reaction involved in the synthesis of the mutual prodrug is given in Scheme 1. The starting material 1, fenbufen i.e., 4-oxo-4-(biphenyl-4-yl)butanoic acid required for the study was prepared as per the reported method.\textsuperscript{3}

Scheme 1: Protocol for synthesis of mutual prodrug (NF-FN).

The reaction proceeded. The contents were then further stirred for 3 h. After completion of reaction, the reaction mixture was decomposed by adding on to the ice cold water (100 mL). A solid mass separated out, which was filtered, washed with water, dried and crystallized from methanol:chloroform mixture (1:1) to furnish TLC pure light reddish brown crystals of NF-FN.

Yield: 46\%, m.p.: 228–229 °C. R\textsubscript{f}: 0.38 (Toluene:Ethyl acetate:Formic acid, 5:4:1), IR (KBr/V\textsubscript{max}) cm\textsuperscript{-1}: 3281 (–COOH), 3018 (aryl C–H), 2986 & 2834 (C–H), 1731 (C=O), 1658 (CONH), 1621 (C=O, pyridine), 1468 (C–N) and 1237 (C–F). \textsuperscript{1}H NMR (CDCl\textsubscript{3}): (δ ppm) 1.60 (t, 3H, –CH\textsubscript{2}CH\textsubscript{3}), 4.37 (q, 2H, –CH\textsubscript{2}–), 2.87 & 3.42 (t, each, 2x-CH\textsubscript{2}–), 3.29 & 3.87 (m, each, 4x-CH\textsubscript{2}–), pipеразине moeity), 6.87 (s, 1H, proton ortho to fluorine), 7.42–7.63 (m, 5H, Ar–H of pphenyl ring), 7.69 & 8.08 (d, each, AzBz, 4H, p-benzoyl ring), 8.05 (s, 1H, proton meta to fluorine), 8.70 (s, 1H, pyridine ring), MS: m/z 555 (M\textsuperscript{+}), C\textsubscript{32}H\textsubscript{30}FN\textsubscript{3}O\textsubscript{5}; Calculated C, 69.18; H, 5.44; N, 7.56; Found C, 68.93; H, 5.32; N, 7.48.

Antibacterial activity

The in-vitro antibacterial activity of the newly synthesized mutual prodrug of (NF-FN) was determined against 4 bacterial strains; 2 gram positive bacteria – Staphylococcus aureus (MTCC 96) & Bacillus subtilis (MTCC 121), and two gram negative bacteria – Escherichia coli (MTCC 1652) & Klebsiella pneumonia (ATCC 13883). The minimum inhibitory concentration (MIC) of the test compound and standard drug norfloxacin was determined according to the turbidity method.\textsuperscript{16}

Anti-inflammatory activity

The synthesized mutual prodrug was also investigated for its anti-inflammatory activity by using carrageenan-induced paw edema method of Winter et al.\textsuperscript{17} The animal studies were approved by the Institutional Animal Ethics Committee [IAEC] of Jamia Hamdard University, New Delhi and utmost care was taken to ensure that the animals were treated in the most humane and acceptable manner. The experiment was performed on Wistar Albino rats of
either sex, weighing 180–200 g. The animals were randomly divided into three groups comprising of six animals in each group. Group I was kept as control, and received only 0.5% carboxymethyl cellulose (CMC) solution. Groups II and III were labeled as test group and standard group, and received NF-FN and fenbufen, respectively, in the dose of 10 mg/kg p.o. 0.1 mL of Carrageenan solution (0.1% in sterile 0.9% NaCl solution) was injected subcutaneously into the sub-plantar region of the right hind paw of each rat, 30 min after the administration of the test compound (FN-NF) and standard drug (fenbufen). The paw volume was measured using a digital Plethysmometer (Panlab LE 7500) at 4 h after carrageenan injection. The percentage inhibition of edema was calculated using the formula:

\[
\text{Anti - inflammatory activity(\% inhibition)} = 1 - \left( \frac{a - x}{b - y} \right) \times 100
\]

where, \(a\) = paw edema at 4 h after carrageenan in treated groups; \(b\) = paw edema at 4 h after carrageenan in control group; \(x\) = normal paw volume of treated group; \(y\) = normal paw volume of control group.

Ulcrogenic activity

**In-vivo** acute ulcerogenic activity was done as per the method of Cioli et al.\(^1\) Wistar albino rats (180–200 g) which were used for anti-inflammatory activity were again used after washing period for determining acute ulcerogenic activity of NF-FN. The rats were divided into three groups consisting of six animals in each group. Ulcrogenic activity was evaluated after oral administration of test compound (NF-FN) or fenbufen at the dose of 30 mg/kg. Control rats received oral administration of vehicle (suspension of 1% carboxy methyl cellulose). Food but not water was removed 24 h before administration of the test compound. After the drug treatment, the rats were fed normal diet for 17 h and then sacrificed. The stomach was removed and opened along the greater curvature, washed with distilled water and cleaned gently by dipping in saline. The gastric mucosa of the rats was examined by means of a magnifying glass. For each stomach, the severity of mucosal damage was assessed according to the following scoring system: 0.5 – redness; 1.0 – spot ulcers; 1.5 – hemorrhagic streaks; 2.0 – ulcers >3 but \(\leq 5\); 3.0 – ulcers >5. The mean score of each treated group minus the mean score of the control group was considered as severity index of gastric mucosal damage.

Statistical analysis

Data were analyzed with the help of a computer program SPSS ver 19 and the results are presented as the mean ± standard error (SE). One way analysis of variance (ANOVA) test was used to analyze the statistical significance in tested groups for ulcerogenic and anti-inflammatory activity. A value of \(P < 0.01\) was considered statistically significant.

Results and discussion

Synthesis

The title prodrug (NF-FN) was synthesized in a single step as outlined in Scheme 1. Norfloxacin (NF) was condensed with fenbufen (FN) in dry pyridine in presence of POCl\(_3\) at a temperature 0–5 °C. Usually work up of the reaction mixture followed by crystallization from methanol:chloroform mixture gave the desired prodrug (NF-FN) as red colored crystalline compound.

Structure elucidation of NF-FN

The structure of NF-FN was established on the basis of IR, \(^1\)H NMR, mass spectral data and elemental analysis results. The IR spectrum of the mutual prodrug showed a peak at 3281 cm\(^{-1}\) due to stretching of carboxylic –OH group. The stretching bands due to C=H of aromatic ring, methyl and methylene groups of ethyl side chain and piperazine ring were obtained at 3018, 2986 & 2834 cm\(^{-1}\), respectively. The three characteristic bands at 1731, 1655 and 1625 were due to C==O stretching in ester group, tertiary amide group and pyridine group, respectively. Bands were also present at 1468 (C= N) and 1237 (C=F) cm\(^{-1}\). The \(^1\)H NMR spectrum of the prodrug NF-FN showed a triplet and a quartet located at \(\delta\) 1.60 and \(\delta\) 4.37 arising from the methyl and methylene group of ethyl moiety in norfloxacin. There were two triplets located at \(\delta\) 2.87 and \(\delta\) 3.42 integrating for the protons of two methylene groups. There appeared two multiplets located at \(\delta\) 3.29 and \(\delta\) 3.87 arising from the protons of four methylene groups of piperazines moiety. There was located a singlet at \(\delta\) 6.87 arising from the lone proton ortho to fluorine atom. Another singlet located at \(\delta\) 8.15 could be accounted for another lone proton meta to fluorine. There was a multiplet centered at \(\delta\) 7.42 which integrated for three protons of the phenyl ring, the other two protons of this ring appeared as another multiplet centered at \(\delta\) 7.63. There appeared two doublets arising from the p-substituted phenyl ring. The lone proton of pyridine ring appeared as a singlet located at \(\delta\) 8.70. These data are satisfactory for the structure assigned to the compound. The mass spectrum of NF-FN showed a molecular ion peak at m/z 555, and M\(^+\) + 1 peak at m/z 556. Other peaks observed were of good intensity and were consistent with the structure of prepared compound. The elemental analysis values were found within ±0.4% of the theoretical values.

Antibacterial activity

**In-vitro** antibacterial activity was carried out against the bacterial strains gram positive (S. aureus & B. subtilis) and gram negative (E. coli & K. pneumonia). Minimum inhibitory concentration was determined and results presented in Table 1 indicate that the prodrug (NF-FN) showed very good activity against S. aureus & E. coli with MIC-6.25 μg/mL, and good activity against B. subtilis & K. pneumonia with MIC-12.5 μg/mL. Norfloxacin showed MIC value ranging from 3.12 to 6.25 μg/mL against the tested microbes. In-

| Table 1: Antimicrobial activity (MIC) of the synthesized mutual prodrug (NF-FN). |
|---------------------------------|---|---|---|---|
| **Compound**                   | **S. aureus** | **B. subtilis** | **E. coli** | **K. pneumonia** |
| NF-FN                           | 6.25         | 12.5           | 6.25        | 12.5             |
| Norfloxacin                     | 3.12         | 3.12           | 3.12        | 6.25             |
| **MIC:** minimum inhibitory concentration (μg/mL). |
Table 2: Anti-inflammatory and ulcerogenic activities of the mutual prodrug (NF-FN).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Anti-inflammatory activity (% Inhibition in rat paw edema)</th>
<th>Ulcerogenic activity (Severity index)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal paw volume (x)</td>
<td>Paw edema 4 h after carrageenan (a)</td>
<td>% inhibition</td>
</tr>
<tr>
<td>Control</td>
<td>0.68 ± 0.02</td>
<td>0.99 ± 0.03</td>
</tr>
<tr>
<td>NF-FN</td>
<td>0.72 ± 0.05</td>
<td>0.84 ± 0.03</td>
</tr>
<tr>
<td>Fenbufen</td>
<td>0.71 ± 0.03</td>
<td>0.85 ± 0.04</td>
</tr>
</tbody>
</table>

**P < 0.01 compared to the parent drug (fenbufen), and data were given in mean ± SEM and analyzed by ANOVA.

In-vivo antibacterial activities are required to further ascertain its usefulness; which are under progress in our laboratory.

Anti-inflammatory activity

The in-vivo anti-inflammatory activity of NF-FN was evaluated by carrageenan-induced rat paw edema method. The compound was tested at 10 mg/kg oral dose and was compared with the parent drug fenbufen at the same oral dose. The obtained pharmacological results revealed that the amide prodrug NF-FN was highly active compound with 61.30% inhibition of inflammation, and its activity was better than that of the parent drug fenbufen (54.83%) at the same dose level (10 mg/kg) (Table 2). This increase in anti-inflammatory activity could be due to synergistic actions of the norfloxacin and fenbufen. Also, the amide linkage between fenbufen and norfloxacin is likely to be hydrolyzed by several amidases present in intestine, thus, free anti-inflammatory molecule will be released in intestine preventing its toxic effects on gastric mucosa.

Ulcerogenic activity

The title compound was screened for ulcerogenic activity in Wistar albino rats according to the method of Cioli. Ulcerogenic activity was evaluated after oral administration of test compound (NF-FN) or fenbufen at the dose of 30 mg/kg. The tested compound showed low ulcerogenic activity, 0.6, whereas the parent drug showed high severity index, 1.5 (Table 2). So the mutual prodrug (NF-FN) exhibited better GI profile as compared to the parent drug fenbufen which could possibly be due to the conversion of the free carboxylic group of fenbufen to the amide linkage thereby reducing the gastric irritation caused by the direct contact of carboxylic acid with the gastric lining. The amidases found in intestine would likely to cleavage the amide linkage of the prodrug and therefore, the NF-FN would pass through the stomach as intact molecule. The synthesized prodrug is not completely free from ulcerogenic effects due to non selective inhibition of cyclooxygenase enzyme.

Conclusion

A mutual prodrug (NF-FN) was successfully synthesized by establishing an amide (–CONH–) linkage between norfloxacin (NF) with fenbufen (FN) in a single step. The prodrug (NF-FN) showed significant in-vitro antibacterial activity against the tested four bacterial strains, and better anti-inflammatory activity together with low ulcerogenic action than that of the parent drug fenbufen. It is expected that after in-vivo hydrolysis (by amidases and/or other enzymes) the mutual prodrug would release norfloxacin and fenbufen into the system which have established antibacterial and anti-inflammatory activity, respectively. Hydrolysis studies are in progress in our laboratory to establish the suggested hypothesis. These results affirm the importance of exploring old drugs as safer templates to built new mutual prodrug candidates.

Recommendations

The mutual prodrug exhibited enhanced anti-inflammatory activity along with the reduced gastric toxicity. More detailed in-vivo studies focusing on pharmacokinetics of drug are needed to ascertain its usefulness in inflammatory conditions associated with bacterial infections.

Conflict of interest

Authors declare no conflict of interest.

Authors’ contributions

AA and SAK carried out the literature survey and performed the experiment; AH supervised the work. SAK and AH analyzed spectral data and wrote the manuscript. All authors read, reviewed and approved the final draft of the manuscript before submission.

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