Synthesis, Characterization and Biological Potential of Novel Neomycin Prodrug

Deepika Nagpal 1*, Nidhi Aggarwal 2, Arun Nanda 3, D. P. Pathak 4

1. NIET, Pharmacy Institute, Greater Noida, Uttar Pradesh, India
2. IIIMT College of Medical Sciences, Meerut, India
3. Maharshi Dayanand University, Rohtak, Haryana, India
4. Delhi Institute of Pharmaceutical Sciences and Research, New Delhi, India

ABSTRACT: In this study novel prodrug of neomycin with 5-amino salicylic acid (5-ASA) were synthesized and evaluated their potential against In vivo/In vitro model of ulcerative colitis. The presence of azo linkage makes it site specific due to specific cleaves in colon. The synthesized prodrugs are characterized by UV, FTIR, 1H-NMR and MASS spectral analysis. In vitro analysis of synthesized product shown the stability in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4), which substantiate the avoidance of dissolution of prodrugs throughout upper GIT. The results revealed that prodrugs shows reduction in the extent and severity in stomach ulcerations compared to control. © 2015 iGlobal Research and Publishing Foundation. All rights reserved.

INTRODUCTION

Ulcerative colitis (UC) is an inflammatory syndrome of gastrointestinal tract. Indeed, numerous anti-inflammatory therapeutic strategies are available in market but the clinical prevalence of this chronic disorder is still high. Several existing therapeutic strategies are based on the targeting of colon which is the largest area of gastrointestinal tract and hence may be potential delivery systems for targeted specific drug [1-2]. Some study reveals that polymeric azo compounds could be employed for colon targeting, since reduction and splitting of azo bond only occurs in large intestine, which increase the probability of site-specificity for drug. Inflammatory bowel disease (IBD) is a chronic relapsing inflammation due to aberrant mucosal immune system and inappropriate over activation of mucosal response in gastrointestinal tract (GIT) wall. IBD compromises ulcerative colitis (UC) and crohn’s disease (CD) which sometimes leads to life-threatening complications [3-4].

Prodrug is a superlative approach of targeted drug delivery. It has been observed that azo compounds could be used for restricted colon targeting since reduction and subsequent splitting of the azo bond is possible only in the large intestine and therefore they shows highly site-specific nature [5].

Neomycin is antibiotic, it has two activity in ulcerative colitis, the reduction of MPO activity and prevention of the depletion of GSH suggesting that the observed effects may
be mediated through GSH-sensitive processes (antioxidant
effects) and the reduction of changes in vascular
permeability and decreased cellular infiltration in the
mucosa (anti-inflammatory effects) [6].

In presented study, we have synthesized the azo prodrug of
5-ASA and neomycin and evaluate for in vitro and in vivo
analysis. Salicylic acid prodrug (SAP) further evaluated
against ulcerogenic activity and histopathological analysis.

MATERIALS & METHODS
All other chemicals used in the synthesis were of A.R. grade
and those of synthetic grade were purified prior to use.
Sulfasalazine was purchased from Ipca laboratories Ltd.,
Mumbai, salicylic acid were purchased from GlaxoSmithKline
Pharmaceuticals Ltd, Mumbai and Neomycin was obtained
from Mr. Deepak Kannujiya, Production Incharge, Quixotic
Healthcare, Baddi, as a gift sample.

Chemistry
Synthesis of azo prodrug of Neomycin with salicylic acid
(MXP)
Neomycin (0.01 mol; 6.14 g) was dissolved in a suitable
volume of water containing 2.5-3 equivalents of hydrochloride
acid (0.02mol; 1.7 ml of 35% HCl), by the application of heat
if necessary and then solution was cooled in ice. The
temperature was maintained at 0-5°C on a cryostatic bath and
an aqueous solution of sodium nitrite (2 mol, 1.4 g in 10 ml)
was added portion wise, through syringe making sure that the
tip of the syringe was always dipped completely in the
solution. The addition of sodium nitrite solution was continued
till the solution gave an immediate positive test for excess of
nitrous acid with an external indicator i.e. moist potassium
iodide-starch paper. The precipitated neomycin, if any, got
dissolved during the diazotization to give a clear solution of
the highly soluble diazonium salt. To stabilize the diazonium
salt and to minimize secondary reactions, proper condition of
acidity was maintained throughout, by adding excess of acid
(0.5-1 equivalents). The reaction mixture was kept in
cryostatic bath at 0-5°C during the course of reaction (which is
exothermic in nature), in order to avoid the hydrolysis of
diazonium salt to corresponding phenol.

Coupling of diazonium salt of Neomycin with salicylic acid
Salicylic acid (0.01 mol; 1.38 g) was completely dissolved in
sodium hydroxide solution (2 mol; 0.08g/ml). The solution
was cooled below 5°C. Then slowly diazotised salt of
neomycin was added with continuous stirring, through
syringe. Alkaline condition was constantly maintained. After
completing the reaction, water was evaporated and crude
product was recovered. It was recrystallized by dissolving in
methanol and cooling at 0°C. Purified product was dried under
vacuum. M.pt- 215°C, Rf- 0.6 (chloroform:methanol, 2:2),
percentage yield- 79%. Aq. Solubility- 0.63g/ml, log P- 0.76,
λ.max in HCl buffer (pH 1.2): 302 nm and in phosphate buffer
(pH 7.4): 308 nm.

Scheme: Synthesis of azo conjugates of salicylic acid with
Neomycin

1. Synthesis of diazonium salt of Neomycin
2. Coupling of diazotised salt of Neomycin with salicylic acid.

\[ \text{SNP} \]
\[ (5\text{-Aminosalicylic acid-Neomycin Prodrug}) \]

Spectral Data (SNP): IR (KBr, cm\(^{-1}\)) 3626 (OH), 3100 (C-H Ar), 1685 (C=O), 1619 (C=C Ar), 1581 (N=N); 1H NMR (DMSO-d6, 400 MHz): 11.99 (S, 6H, COOH), 9.06-7.54 (m, 18H, ArH), 4.75 (s, 6H, OH), 3.55-3.27 (m, 4H, CH tetrahydropyran), 2.76-2.74 (d, 6H, CH\(_2\)); MS ES+ (ToF): m/z 1511.

In-vitro kinetic studies of synthesized compounds

Release studies in 0.05 M hydrochloric acid buffer (pH 1.2) [7]
SNP (10 mg) were introduced in 900 ml of HCl buffer taken in two different baskets and were kept in a constant temperature bath at 37 ± 1°C. The solutions were occasionally stirred and 5 ml aliquot portions were withdrawn at various time intervals. The aliquots were directly estimated on UV spectrophotometer at 302 nm. The concentration of prodrug remaining was determined from calibration curve of SNP. The percent release data of free drug from its prodrug is quoted in tables 1 and Fig 2.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Amount of 5-ASA release</th>
<th>% Release</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>0.132</td>
<td>13.2</td>
</tr>
<tr>
<td>45</td>
<td>0.245</td>
<td>24.5</td>
</tr>
<tr>
<td>60</td>
<td>0.387</td>
<td>38.7</td>
</tr>
<tr>
<td>75</td>
<td>0.452</td>
<td>45.2</td>
</tr>
<tr>
<td>90</td>
<td>0.523</td>
<td>52.3</td>
</tr>
<tr>
<td>105</td>
<td>0.672</td>
<td>67.2</td>
</tr>
<tr>
<td>120</td>
<td>0.721</td>
<td>72.1</td>
</tr>
<tr>
<td>240</td>
<td>0.792</td>
<td>79.2</td>
</tr>
<tr>
<td>360</td>
<td>0.852</td>
<td>85.2</td>
</tr>
</tbody>
</table>

Release studies in rat fecal matter (pH 7.4)[8-9]
All the prodrug were dissolved in phosphate buffer (pH 7.4) so that final concentration of solution was 250 μg/ml. Fresh fecal material of rats was weighed (about 1g) and placed in different sets of test tubes. To each test tube, 1 ml of the prodrug solution was added and diluted to 5 ml with phosphate buffer (50 μg/ml). The test tubes were incubated at 37°C for different intervals of time. For analysis, the concentrations of SNP were directly estimated on double beam UV- spectrophotometer (shimadzu uv 1700) at 302 nm. The percent release data of free drug from its prodrug is quoted in tables 1 and Fig 2.

Table 1: Release data of SNP in rat fecal matter

Concentrations of SNP were determined from calibration curve of SNP. The concentration of prodrug remaining was determined from calibration curve of SNP. The percent release data of free drug from its prodrug is quoted in tables 1 and Fig 2.

Fig-1 Calibration curve of SNP
Pharmacological Evaluation

Biological evaluation of the synthesized compounds was carried out in the Department of Pharmaceutical Technology, NIET and its animal facility is approved by CPCSEA (1121/ac/CPCSEA/07). The experimental protocols for the same have been approved by the Institutional Animal Ethical Committee.

Wistar albino rats of either sex weight 120-150 g were divided in control and experimental groups having N=5 for each groups. The ulcerogenic activity was evaluated by the Rainsford’s cold stress method.[8] Salicylic acid and sulfasalazine were considered as standard drugs. Test and standard compounds were administered orally (suspended in CMC). Doses of all the drugs were first calculated on equimolar basis of 5-ASA present in sulfasalazine and then were converted into ten times higher doses. With oral administration of 5 ml of the aqueous drug suspensions (at 10 times the normal dose), the animals were stressed by exposure to cold (-15 °C for 1 h). The animals were placed in separate polypropylene cages to ensure equal cold exposure. After 2 h of drug administration, the animals were sacrificed. The stomach and duodenal part were opened along the greater curvature and the number of lesions was examined by means of a magnifying lens. All ulcers larger than 0.5 mm were counted. The ulcer index was determined by scoring ulcers as described by Cioli et al (1979),[10] (Table 2).

The results of ulcerogenic activity are summarized in Table 3. Average of six reading was calculated and all data was expressed as mean ± S.D. Statistical differences between the groups were calculated by Student’s t test. Differences were considered at a p value of < 0.05.

![SNP Release profile](image)

**Fig-2 Release profile of 5-ASA from its azo prodrug in rat fecal matter**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ulcerogenic Response</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ulcers less than 1 mm</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ulcers less than 1-2 mm</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>Ulcers less than 2-3 mm</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Ulcers less than 3-4 mm</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>Ulcers less than 4-5 mm</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Ulcers greater than 5 mm</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Perforated lesions</td>
<td>25</td>
</tr>
</tbody>
</table>

**Table 2-2 Scoring of Gastric Ulcers**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Ulcer index ± S.D.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>----</td>
<td>0.6 ± 0.97</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>3000</td>
<td>1.4 ± 0.97</td>
</tr>
<tr>
<td>Salicylic acid</td>
<td>1140</td>
<td>5.6 ± 1.8</td>
</tr>
<tr>
<td>SNP</td>
<td>5710</td>
<td>1.6 ± 1.2</td>
</tr>
<tr>
<td>Neomycin</td>
<td>4570</td>
<td>5.8 ± 1.8</td>
</tr>
</tbody>
</table>

* Average of five readings; p< 0.05.

**Tri Nitro Benzene Sulfonic Acid (TNBS) induced experimental colitis model**

**Animals**

Male Wister rats (average weight 200–230 g; 12-15 weeks; n=5/group) were used. They were distributed into 8 different groups’ i.e. Healthy control, colitis control, two standard groups and four test groups. They were housed in a room with controlled temperature (22°C). The animals were food fasted 48 h before experimentation and allowed food and water *ad libitum* after the administration of acetic acid.

**Induction of Colitis**[11]

The ulcerogenic activity was determined by the Rainsford’s cold stress method, which is an acute study model and is used to determine ulcerogenic potency of a given drug at ten times higher dose. Salicylic acid and sulfasalazine were taken as standards. It was found that the presence of suspending agents like carboxy methylcellulose decreases the incidence of gastric ulcers. Hence, the test compounds and standards were administered orally, as fine particles suspended in CMC by continuous stirring. The volume of vehicle or suspensions was kept constant. Wistar rats of either sex weighing between 120-150 g were randomly distributed in control and experimental group of five animals each. Doses of all the drugs were first calculated on equimolar basis of 5-ASA present in sulfasalazine and then were converted into ten times higher doses. Following oral administration of 5 ml of the aqueous drug suspensions (at 10 times the normal dose), the animals were stressed by exposure to cold (-15 °C for 1 h). The
animals were placed in separate polypropylene cages to ensure equal cold exposure. After 2 h of drug administration, the animals were sacrificed. The stomach and duodenal part were opened along the greater curvature and the number of lesions was examined by means of a magnifying lens. All ulcers larger than 0.5 mm were counted. The ulcers were scored according to the method reported by Cioli et. al. (1979).

Assessment of colonic damage by clinical activity score [12]
The animals of all groups were examined for weight loss, stool consistency and rectal bleeding throughout the 11 days study. Colitis activity was quantified with a clinical activity score assessing these parameters as previously applied by [Hartmann et al] (Table 4 and Fig-3). The clinical activity score was determined by calculating the average of the above three parameters for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis). They were sacrificed 24 h after the last drug administration and a segment of colon 8 cm long was excised and colon/ body weight ratio was determined to quantify the inflammation (Table 5 and Fig 4). Tissue segments 1 cm in length was then fixed in 10% formalin for histopathological studies. Histological evaluation has revealed in Table 6.

Statistical Analysis
All data are expressed as mean ± S.E.M.; n refers to number of animals in each group. Statistical differences between groups were calculated by one and two-way ANOVA followed by the Dunetl’s post hoc test. Differences were considered at a p value of < 0.05.

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Compound</th>
<th>Colon to body weight ratio (w/w) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Healthy control</td>
<td>0.006 ± 0.0004</td>
</tr>
<tr>
<td>2</td>
<td>Colitis control</td>
<td>0.04 ± 0.0005</td>
</tr>
<tr>
<td>3</td>
<td>5-Aminosalicylic acid</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>4</td>
<td>Neomycin</td>
<td>0.02 ± 0.0006</td>
</tr>
<tr>
<td>5</td>
<td>Sulfasalazine</td>
<td>0.013 ± 0.0006</td>
</tr>
<tr>
<td>6</td>
<td>SNP</td>
<td>0.008 ± 0.0006</td>
</tr>
</tbody>
</table>

Table 5 Colon to Body Weight Ratio*
* Average of five readings p< 0.05.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Weight Loss</th>
<th>Stool Consistency</th>
<th>Rectal Bleeding</th>
<th>Score Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No loss</td>
<td>Well formed pellets</td>
<td>No blood</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>1-5%</td>
<td>----</td>
<td>----</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>5-10%</td>
<td>Pasty and semi formed stools, not sticking to anus</td>
<td>Positive finding</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>10-20%</td>
<td>----</td>
<td>----</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>&gt; 20%</td>
<td>Liquid stools, sticking to anus</td>
<td>Gross bleeding</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4 Scoring Rate of Clinical Activity

![Fig 3 Graph of Clinical Activity Score Rate](image)

![Fig 4 Graph of Colon to Body Weight Ratio](image)
RESULT & DISCUSSION

Kinetic studies of SNP for the release of 5-ASA confirmed that these prodrugs did not release the parent drug in 0.05 M hydrochloric acid buffer (pH 1.2), whereas in phosphate buffer (pH 7.4) negligible release was observed after 6 hrs. Thus, the objective of bypassing the upper GIT without any free drug release was achieved. Synthesized prodrug was confirmed by their spectral studies. The results of ulcerogenic activity reveal that salicylic acid when directly administered orally, shows maximum ulcer index (5.6 ± 1.8), whereas sulfasalazine- a prodrug of 5-ASA shows a lower ulcer index (1.4 ± 0.97) as it delivers 5-ASA directly to colon with minimum release of 5-ASA in upper GI tract. The synthesized prodrugs SNP show comparable lowering of ulcer index as that of sulfasalazine, which proves that like sulfasalazine, SNP also deliver 5-ASA specifically to colon with very negligible release in upper GI tract. These results are consistent with the results obtained for in vitro release studies in HCl buffer (pH 1.2) and phosphate buffer (pH 7.4). The mitigating effect of SNP as well as standards was determined by clinical score system, colon/ body weight ratio and histopathological studies of colon.

Their histopathological features clearly indicated that the morphological disturbances associated with Tri Nitro Benzene Sulfonic Acid (TNBS) administration were corrected by treatment with SAP. These results were found to be comparable with those obtained for free 5-ASA and sulfasalazine treated groups.

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