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Synthesis, Characterization and Biological Potential of Novel Neomycin Prodrug

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Ulcerative colitis; Neomycin; Melphalan; 5-amino salicylic acid.

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, ^{1}H-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.

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)

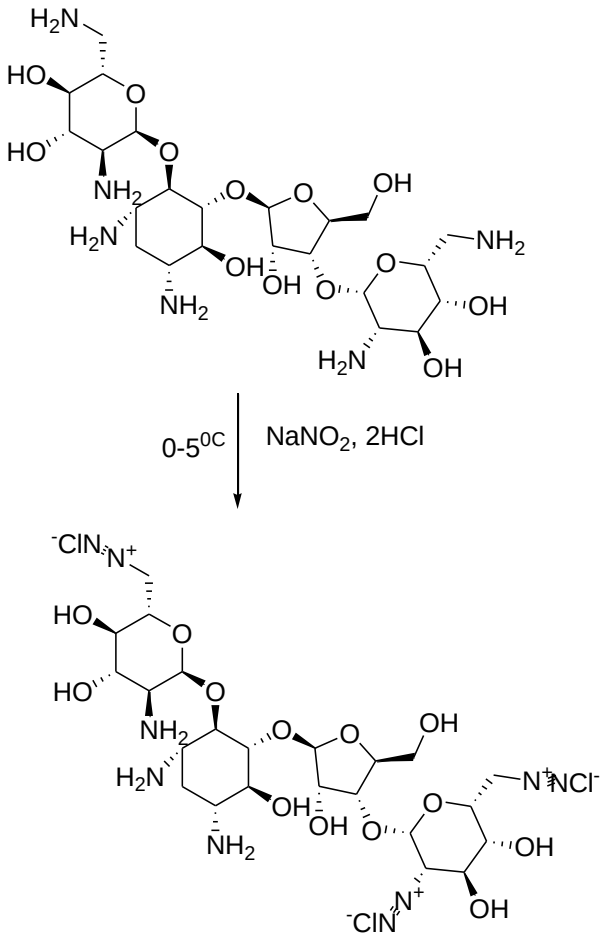
Neomycin (0.01 mol; 6.14 g) was dissolved in a suitable volume of water containing 2.5-3 equivalents of hydrochloride acid (0.02mol; 1.7 ml of 35% HCl), by the application of heat if necessary and then solution was cooled in ice. The temperature was maintained at 0-5^{0}C on a cryostatic bath and an 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^{0}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^{0}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^{0}C. Purified product was dried under vacuum. M.pt- 215^{o}C, Rf- 0.6 (chloroform:methanol, 2:2), percentage yield- 79%, Aq. Solubility- 0.63g/ml, log P- 0.76, λmax 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

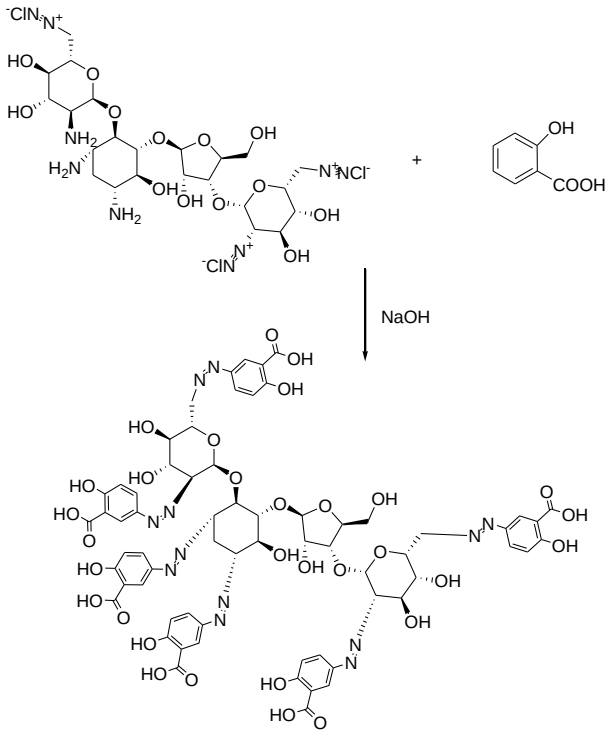
1. Synthesis of diazonium salt of Neomycin



Diazonium salt of Neomycin

Scheme-1

2. Coupling of diazotised salt of Neomycin with salicylic acid.



SNP
(5-Aminosalicyclic acid-Neomycin Prodrug)
Scheme-2

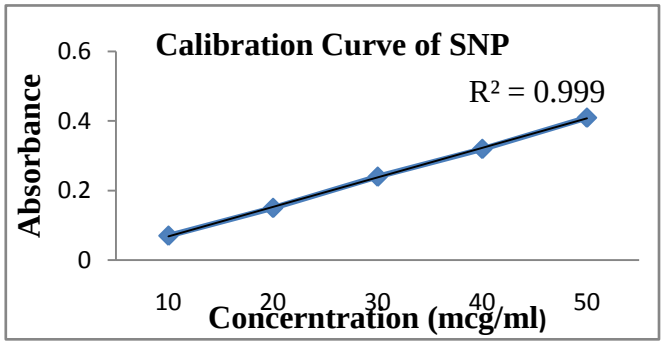
Spectral Data
(SNP): IR (KBr, cm^{-1}): 3626 (OH), 3100 (C-H Ar), 1685 (C=O), 1619 (C=C Ar), 1581 (N=N); 1H NMR (DMSO-*d*6, 400 MHz): 11.99(S, 6H, COOH), 9.06-7.54 (m, 18H, ArH), 4.75(s, 6H, OH), 3.55-3.27(m, 4H, CH tetrahydropyran), 2.76-2.74(d, 6H, CH_2); MS ES+ (ToF): *m/z* 1511.

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 for the amount of SNP remaining. During estimation, it was observed at even after 3 h SNP showed negligible release of the free drug.

Release studies in 0.05 M phosphate buffer (pH 7.4)[7]
Same procedure as described in 5.2.1 was followed; except that the HCl buffer was replaced by phosphate buffer. The kinetics was monitored by the decrease in prodrug concentration with time. During estimation, it was observed at even after 6 h SNP showed only negligible release of the free drug is shown in Fig-1

Fig-1 Calibration curve of SNP



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 concerntration 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 concerntrations of SNP were directly estimated on double beam UV- spectrophotometer (shimadzu uv 1700) 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 1: Release data of SNP in rat fecal matter

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

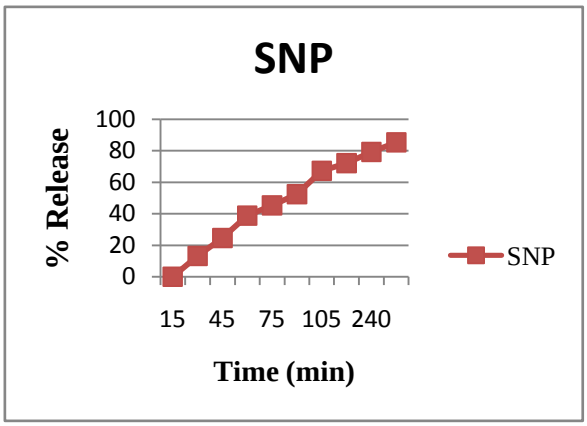


Fig-2 Release profile of 5-ASA from its azo prodrug in rat fecal matter

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 ^{0}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.

Table -2 Scoring of Gastric Ulcers

Sr. No.	Ulcerogenic Response	Score
1	Ulcers less than 1 mm	1
2	Ulcers less than 1-2 mm	2
3	Ulcers less than 2-3 mm	3
4	Ulcers less than 3-4 mm	4
5	Ulcers less than 4-5 mm	5
6	Ulcers greater than 5 mm	10
7	Perforated lesions	25

Table-3 Results of ulcerogenic activity

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

***** 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^{0}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 8cm long was excised and colon/ body weight ratio was determined to quantify the inflammation (Table 5 and Fig 4). Tissue segments 1cm 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 Dunelt's post hoc test. Differences were considered at a *p* value of < 0.05.

S. No	Weight Loss	Stool Consistency	Rectal Bleeding	Score Rate
1	No loss	Well formed pellets	No blood	0
2	1-5%	----	----	1
3	5-10%	Pasty and semi formed stools, not sticking to anus	Positive finding	2
4	10-20%	----	----	3
5	> 20%	Liquid stools, sticking to anus	Gross bleeding	4

Table 4 Scoring Rate of Clinical Activity

Sr.No.	Compound	Colon to body weight ratio (w/w) ± S.D.
1	Healthy control	0.006 ± 0.0004
2	Colitis control	0.04 ± 0.0005
3	5-Aminosalicylic acid	0.014 ± 0.002
4	Neomycin	0.02 ± 0.0006
5	Sulfasalazine	0.013 ± .0006
6	SNP	0.008 ± 0.0006

Table 5 Colon to Body Weight Ratio*

* Average of five readings $p < 0.05$.

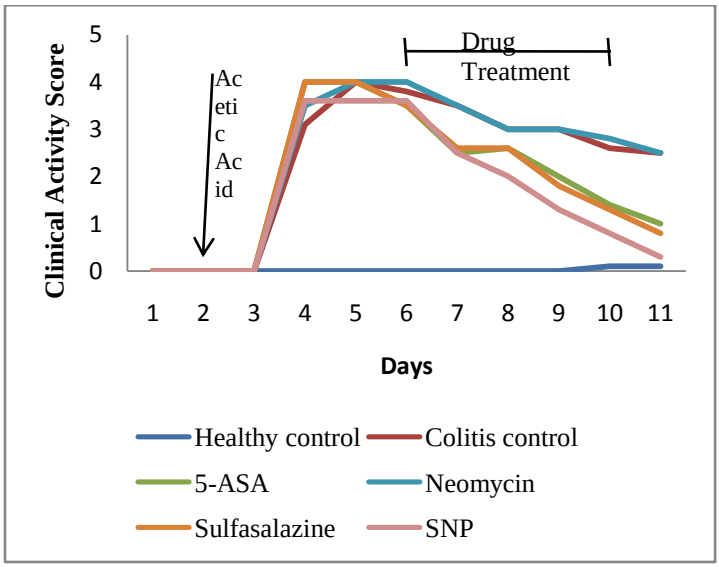


Fig 3 Graph of Clinical Activity Score Rate

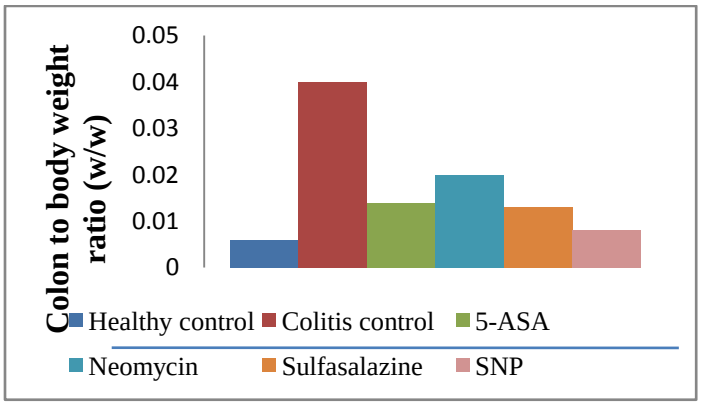


Fig 4 Graph of Colon to Body Weight Ratio

Table 6: Histopathology Report.

Gross Examination	
Compounds	**Dilation, Congestion, Wall thickness**
SAP (A)	Thin walled, not dilated
Neomycin (C)	Partly dilated & congested
Colitis contol (D)	Partly dilated, congested & wall thickened
Healthy (E)	Thin walled, not dilated
5-ASA (F)	Congestion
Sulfasalazine (G)	Thin walled

RESULTS & 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) negligble 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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CONFLICT OF INTEREST

No conflict of interest.

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