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Colon-specific, mutual azo prodrug of 5-aminosalicylic acid with L-tryptophan: Synthesis, kinetic studies and evaluation of its mitigating effect in trinitrobenzenesulfonic acid-induced colitis in rats

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Abstract—Mutual azo prodrug of 5-aminosalicylic acid with L-tryptophan was synthesized by coupling L-tryptophan with salicylic acid, for targeted drug delivery to the inflamed gut tissue in inflammatory bowel disease. The structure of synthesized prodrug was confirmed by elemental analysis, IR and NMR spectroscopy. In vitro kinetic studies in HCl buffer (pH 1.2) showed negligible release of 5-aminosalicylic acid, whereas in phosphate buffer (pH 7.4) 18% release was observed over a period of 7 h. In rat fecal matter, 87.9% of 5-aminosalicylic acid was released with a half-life of 143.6 min, following first order kinetics. The azo conjugate was evaluated for its ulcerogenic potential by Rainsford's cold stress method. The ameliorating effect of the azo conjugate and therapeutic efficacy of the carrier system was evaluated in trinitrobenzenesulfonic acid-induced experimental colitis model. The synthesized prodrug was found to be equally effective in mitigating the colitis in rats as that of sulfasalazine without the ulcerogenicity of 5-aminosalicylic acid.

1. Introduction

Inflammatory bowel diseases (IBD) are chronic relapsing conditions with a high morbidity and remain largely incurable. Crohn's disease and ulcerative colitis are collectively termed as inflammatory bowel diseases, which are characterized by chronic inflammation in the mucosal membrane of small and/or large intestine with periods of remission and relapse over many years. Routine treatment for the same has not changed significantly over last 40 years and still relies heavily on aminosalicylates and steroids, which aim to induce and then maintain 80% of the patients in remission and ameliorate the disease's secondary effects rather than modifying or reversing the underlying pathogenic mechanism.[1,2]

The primary goal of drug therapy is to reduce inflammation in the colon that requires frequent intake of antiinflammatory drugs at higher doses. 5-aminosalicylic acid (5-ASA) is very effective in IBD but it is absorbed so quickly in the upper gastrointestinal tract (GIT) that it usually fails to reach the colon leading to significant adverse effects.[3,4] Therefore, out of the need to overcome this formidable barrier of GIT, colonic drug delivery has evolved as an ideal delivery system for the topical treatment of diseases of colon like Crohn's disease, ulcerative colitis, colorectal cancer and amoebiasis. To achieve successful colonic delivery, a drug needs to be protected from absorption and/or the environment of upper GIT and then be abruptly released into proximal colon, which is considered as the optimum site for colon-targeted delivery of drug.[5]

Prodrug approach is one of the promising approaches for targeting drugs to colon. Colon-specific drug delivery through colon-specific prodrug activation may be accomplished by the utilization of high activity of

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certain enzymes at the target site relative to non-target tissues for prodrug to drug conversion.

Prodrug approach has been successfully utilized in sulfasalazine (an azo prodrug 5-ASA and sulfapyridine) for targeting drugs to colon.[6] But majority of side effects of sulfasalazine like hepatotoxicity, hypospermia and severe blood disorders are due to sulfapyridine. Few prodrugs of 5-ASA like balsalazide, ipsalazine and olsalazine have been reported but most of them suffer from adverse effects due to the carriers used with them.[7–9] The need for a totally safe, colon specific prodrug of 5-ASA with nontoxic carrier still remains. In the present work, concept of mutual prodrug has been adopted for synthesis of azo conjugate of 5-ASA with L-tryptophan (ST) for its colon-targeted delivery, with improved safety profile and comparable activity to sulfasalazine. The aim of this project was to evaluate in vivo, the targeting potential of azo conjugate to inflamed tissue of colon and therapeutic efficacy of this drug-carrier system in experimental colitis rat model. L-Tryptophan was chosen as a promoiety due to its marked antiinflammatory activity.[10] Being a natural component of our body, it would be nontoxic and free from any side-effects. Introduction of azo linkage in the prodrug (similar to sulfasalazine) would ensure release of 5-ASA in colon by the reductive action of azo reductases secreted by the colonic microflora.

2. Chemistry

Synthesis of methyl ester hydrochloride of L-tryptophan[11] (**1**) was carried out by adding thionyl chloride to methanol followed by refluxing the amino acid at 60–70 °C for 7 h. L-Tryptophan methyl ester (**2**) was diazotised[12] at 0–5 °C in cryostatic bath. The coupling[12,13] of diazonium salt of L-tryptophan (**3**) with salicylic acid (**4**) was carried out at 0–5 °C in a cryostatic bath (Scheme 1). It was recrystallized with methanol followed by cooling at 0 °C. Purified product (ST) was dried under vacuum.

3. Results and discussion

The melting point of ST was found to be 243 °C (uncorrected). All the results of elemental analysis were in an acceptable error range. The aqueous solubility was found to be 0.28 g/ml and partition coefficient in *n*-octanol/ phosphate buffer (pH 7.4) was found to be 0.26 which was decreased as compared to 5-ASA (0.64). The kinetics was monitored by the decrease in prodrug concentration with time in HCl buffer (pH 1.2) at 288 nm and phosphate buffer (pH 7.4) at 290 nm. Kinetic studies confirmed that the prodrug did not release the parent drug in 0.05 M hydrochloric acid buffer (pH 1.2), whereas in phosphate buffer (pH 7.4) 18% release was observed after 7 h. Thus, the objective of bypassing the upper GIT without any free drug release was achieved. The release kinetics was further studied in rat fecal matter[14] to confirm the colonic reduction of azo prodrug. $t_{1/2}$ (average of four trails) of ST was found to be 143.6 min, whereas rate constant (K) was found to be $4.82 \times 10^{-3} \pm 0.0001\ s^{-1}$. Over a period of 7 h, ST gave 87.9% cumulated release of 5-ASA following first order kinetics (Fig. 1). Thus in vitro kinetic studies confirmed that the synthesized conjugate was stable in HCl

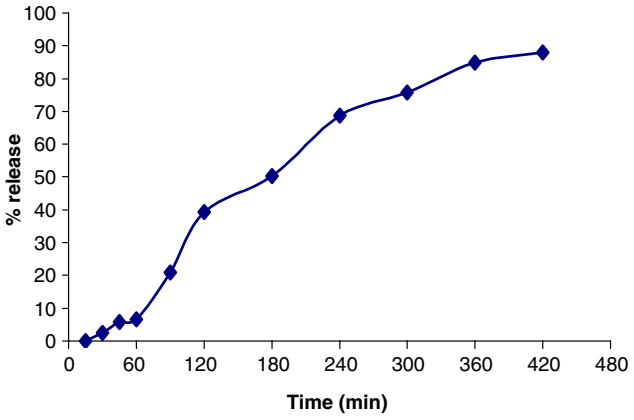
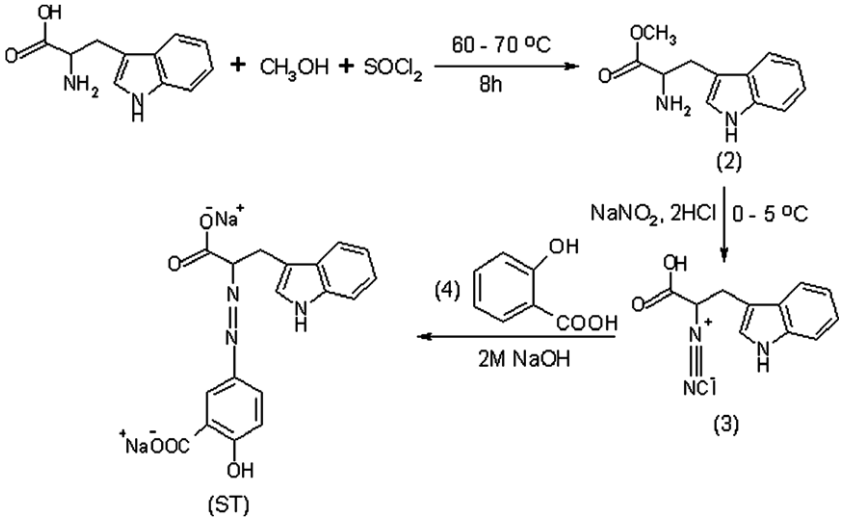


Figure 1. Release profile of 5-ASA from its azo prodrug in rat fecal matter.



Scheme 1.

Table 1. Results of ulcerogenic activity

Compound	Dose (mg/kg)	Ulcer index ± SD[a]
HC	—	2 ± 1
5-ASA	2290	59.6 ± 4.7
Slz	3000	9 ± 2
ST	2829	13 ± 1.1

HC, healthy control; 5-ASA, 5-aminosalicylic acid; Slz, sulfasalazine; ST, prodrug of 5-ASA with L-tryptophan.
[a] Average of six readings; $p < 0.01$.

buffer (pH 1.2) but in phosphate buffer (pH 7.4), 18% release was observed. The release in rat fecal matter was almost complete.

The synthesized compound was evaluated for ulcerogenic activity by the method of Rainsford.[15] The ulcers were scored according to the method reported by Rainsford[16] and the ulcer index was determined (Table 1). The conjugate showed remarkable reduction in the ulcer index (13 ± 1.1) as compared to its parent drug (59.6 ± 4.7). This reduction in the ulcer index brought about by the conjugate was comparable to that produced by sulfasalazine (9 ± 2). Statistical differences between the groups were calculated by Kruskal–Wallis test followed by Dunn's post hoc test. All data are expressed as means ± SD. Differences were considered at a p value of <0.01 in relation to control.

In order to study the feasibility of azo prodrug of 5-ASA for targeted oral drug delivery to the inflamed tissue of colon in IBD, TNBS-induced experimental colitis model was selected.[17–19] After inducing the experimental colitis, the clinical activity score increased rapidly and consistently for the next 3 days for all groups. All drug-receiving groups showed a decrease of inflammation severity after a lag time of 24–48 h. The difference between the drug treated group and colitis control group became significant on day 7. A significant lowering of clinical activity was shown by ST (1.06 ± 0.51), which was comparable to sulfasalazine (0.83 ± 0.42) but distinctly more than 5-ASA (2.09 ± 0.27). The positive contribution of L-tryptophan towards lowering effect on clinical activity score (1.83 ± 0.33) is obvious from the gross difference in lowering effect of plain 5-ASA and ST. To ensure the synergistic effect of L-tryptophan further, two test groups of animals were subjected to rectal administration of plain L-tryptophan and 5-ASA + L-tryptophan, respectively. The lowering of clinical activity score by rectally administered L-tryptophan was (1.05 ± 0.35) less than that of sulfasalazine (0.83 ± 0.42) but better than L-tryptophan administered orally (1.83 ± 0.08). Rectal co-administration of 5-ASA + L-tryptophan showed comparable lowering of clinical activity score (0.72 ± 0.38) as that of sulfasalazine (0.83 ± 0.42) but better than L-tryptophan (1.83 ± 0.08) or 5-ASA (2.09 ± 0.27) administered orally. This particular finding supports positive contribution of L-tryptophan and hence its synergistic effect. On day 11 (24 h after the drug administration), the animals were sacrificed and colon/body weight ratio was determined to quantify inflammation. The prodrug treated group showed a distinct decrease in the colon/body weight ratio compared to colitis control group (Fig. 3). Decrease in colon/body weight ratio produced by rectally administered L-tryptophan (0.0073 ± 0.00053) as well as 5-ASA + L-tryptophan (0.0089 ± 0.00053) was comparable to that of sulfasalazine (0.0080 ± 0.00036). During the evaluation of macroscopic damage of colon segments in colitis control, the colons appeared flaccid and filled with liquid stool. The cecum, colon and rectum all had evidence of mucosal congestion, erosion and haemorrhagic ulcerations and histopathological features included transmural necrosis, oedema, absence of epithelium, and a massive mucosal/submucosal infiltration of inflammatory cells. In vivo treatment with ST resulted in the significant decrease in the extent and severity of colonic damage. Its histopathological features clearly indicated that the morphological disturbances associated with TNBS administration were corrected by treatment with ST. These results were found to be comparable with those obtained for sulfasalazine treated group. Histopathological features of rectally administered L-tryptophan and 5-ASA + L-tryptophan groups also indicated correction of disrupted morphology of the colon. Statistical differences between the groups were calculated by Kruskal–Wallis test followed by Dunn's post hoc test. All data are expressed as means ± SD. Differences were considered at a p value of <0.01 in relation to control.

4. Conclusions

The data generated as an outcome of this work demonstrate that this new prodrug has a remarkable ameliorating effect on the disruption of colonic architecture and suppresses the course of TNBS induced colitis effectively. The criteria for selection of L-tryptophan as carrier have also proved to be correct, as this particular drug carrier system has delivered 5-ASA specifically to colon, bypassing the release of free drug in upper GIT thus lowering the ulcerogenic potential of 5-ASA effectively. Moreover, its synergistic ameliorating effect on disrupted colonic architecture strengthens the hypothesis of mutual prodrug design.

5. Experimental

5.1. In vitro hydrolysis kinetics

In vitro stability studies were carried out in hydrochloric acid buffer (pH 1.2), phosphate buffer (pH 7.4)[16,17]. The total buffer concentration was 0.05 M and a constant ionic strength (μ) of 0.5 was maintained for each buffer by adding a calculated amount of potassium chloride. The feasibility of reduction of azo linkage by azo reductase secreted by intestinal microflora was tested with the help of release study in rat fecal matter at 37 ± 1 °C. All the kinetic studies were carried out in triplicate. The K values from the plots were calculated separately and average K and SD value was determined. The half lives were calculated using software 'PCP Disso' developed by Department of Pharmaceutics, Poona College of Pharmacy, Pune. The process was validated as per U.S.P. XXIV

edition using different parameters like accuracy, selectivity, sensitivity and reproducibility. ST (10 mg) was introduced in 900 ml of HCl buffer taken in a basket and was kept in a constant temperature bath at 37 ± 1 °C. The solution was occasionally stirred and 5 ml aliquot portions were withdrawn at various time intervals. The aliquots were shaken with equal amount of chloroform in order to remove the interference by 5-ASA which was supposed to be released by the synthesized prodrug and the aliquots were estimated on UV spectrophotometer at 294 nm for the amount of ST remaining.

Same procedure as described earlier was followed; except that the HCl buffer was replaced by phosphate buffer. The kinetics was monitored by the decrease in prodrug concentration with time.

To study the release of 5-ASA from ST in rat fecal matter,[19] ST was dissolved in sufficient volume of phosphate buffer (pH 7.4) so that final concentration of solution was 250 μg/ml. Fresh fecal material of rats was weighed (about 1 g) and placed in different sets of test tubes. To each test tube containing weighed amount of rat fecal matter, 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 aliquots of ST were removed from the test tubes at different time intervals and shaken with chloroform so as to extract free drug from the aliquots. The concentration of 5-ASA was directly estimated from the chloroform layer on double beam UV-spectrophotometer (JASCO, V-530 model, Japan) at 322 nm.

5.2. Ulcerogenic activity

The ulcerogenic activity was determined by cold stress method (Rainsford),[15] which is an acute study model and is used to determine ulcerogenic potency of a drug at ten times higher dose. 5-ASA and sulfasalazine were taken as standards. The test compounds and standards were administered orally, as fine particles suspended in carboxymethylcellulose by continuous stirring. The volume of vehicle or suspensions was kept constant. Wistar rats of either sex weighing between 120 and 150 g were randomly distributed in control and experimental groups of six animals each. 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. Average of six readings was calculated and was expressed as means ± SD.

5.3. Trinitrobenzenesulfonic acid (TNBS) induced experimental colitis model

In order to study the ameliorating effect of azo prodrug of 5-ASA on the inflamed tissue of colon in IBD, 2,4,6-trinitrobenzenesulfonic acid induced experimental colitis model was selected which is simple and reproducible. Moreover it is the most relevant model as it involves the use of immunological haptens and develops a chronic inflammation rather than an acute mucosal injury.[20] By this model in vivo characterization of the azo carrier system under the influence of chronic inflammatory symptoms was possible. Sprague–Dawely rats (average weight 200–230 g; 12–15 w; n = 6/group) were used. They were distributed into six different groups i.e., healthy control, colitis control, two standard groups and two 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 TNBS. To induce an inflammation, all the groups except healthy control group were treated by a procedure discussed below. After light narcotizing with ether, the rats were catheterized 8 cm intrarectal and 500 μl of TNBS (Himedia Laboratories Pvt. Ltd., Mumbai) in ethanol was injected into colon via rubber canulla (dose was 150 mg/kg of body weight of TNBS in ethanol, 50% solution). Animals were then maintained in a vertical position for 30 s and returned to their cages. For 3 days the rats were housed without treatment to maintain the development of a full inflammatory bowel disease model. The animals of standard and test groups received orally 5-ASA, sulfasalazine, L-tryptophan and ST, respectively, once daily for five continuous days at doses equimolar to 5-ASA present in sulfasalazine. The healthy control and colitis control groups received only 1% carboxymethylcellulose instead of free drug or prodrug. The animals of all groups were examined for weight loss, stool consistency and rectal bleeding throughout the 11-day study. Colitis activity was quantified with a clinical activity score assessing these parameters (Fig. 2) by clinical activity scoring rate.[21] 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

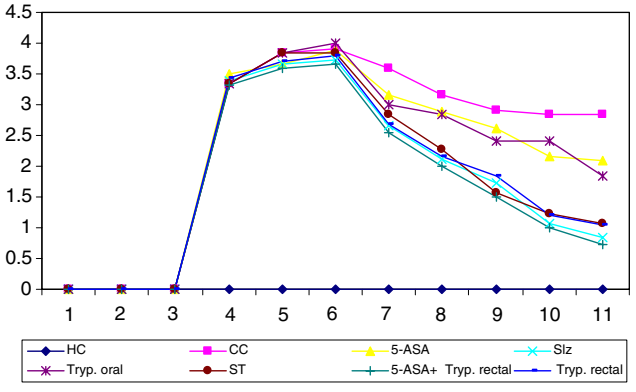


Figure 2. Clinical activity score rate. Average of six readings; $p < 0.01$. HC, healthy control; CC, colitis control; 5-ASA, 5-aminosalicylic acid; Slz, sulfasalazine; Tryp. Oral, L-tryptophan oral; ST, prodrug of 5-ASA with L-tryptophan, 5-ASA + Tryp. rectal: co-administration of 5-ASA with L-tryptophan by rectal route; Tryp. rectal, rectal administration of L-tryptophan.

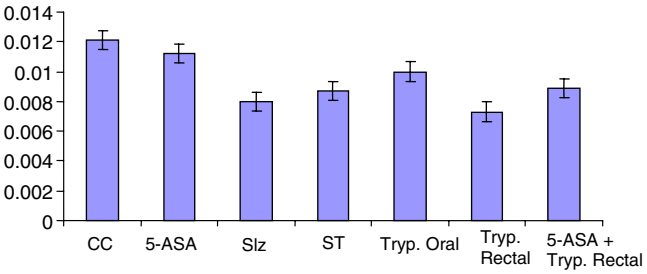


Figure 3. Colon to body weight ratio. Average of six readings; $p < 0.01$. HC, healthy control; CC, colitis control; 5-ASA, 5-aminosalicylic acid; Slz, sulfasalazine; Tryp. Oral, L-tryptophan oral; ST, prodrug of 5-ASA with L-tryptophan; 5-ASA + Tryp. rectal, co-administration of 5-ASA with L-tryptophan by rectal route; Tryp. rectal: rectal administration of L-tryptophan.

24 h after the last drug administration by isoflurane anaesthesia and a segment of colon, 8 cm long, was excised and colon/ body weight ratio was determined to quantify the inflammation (Fig. 3). Tissue segments 1 cm in length were then fixed in 10% buffered formalin for histopathological studies. Histopathological studies (Fig. 4a–e) of the colon were carried out using haematoxylin and eosin stains, at Nucleus Pathology Laboratory, Pune. Coloured microscopical images of the colon sections were taken on Zeiss optical microscope, Stemi 2000-C, with resolution 5×20X, attached with trinocular camera at Kolte Pathology Laboratory, Pune.

5.3.1. General procedure. All chemicals used in the synthesis were of AR grade. Sulfasalazine was obtained as gift sample from Wallace Pharmaceutical Pvt. Ltd. Goa, India. Salicylic acid and L-tryptophan were purchased from Loba Chemie, Mumbai, India. The reactions were monitored on TLC, which was performed on precoated silica gel plates-60 F_{264} (Merck) using solvent system of chloroform/methanol (4:1.5) and iodine vapours/UV light as detecting agents.

^{1}H NMR spectrum of the synthesized compound was recorded in DMSO using ^{1}H NMR Varian Mercury 300 Hz with superconducting magnet using TMS as internal standard. Chemical shift values are reported

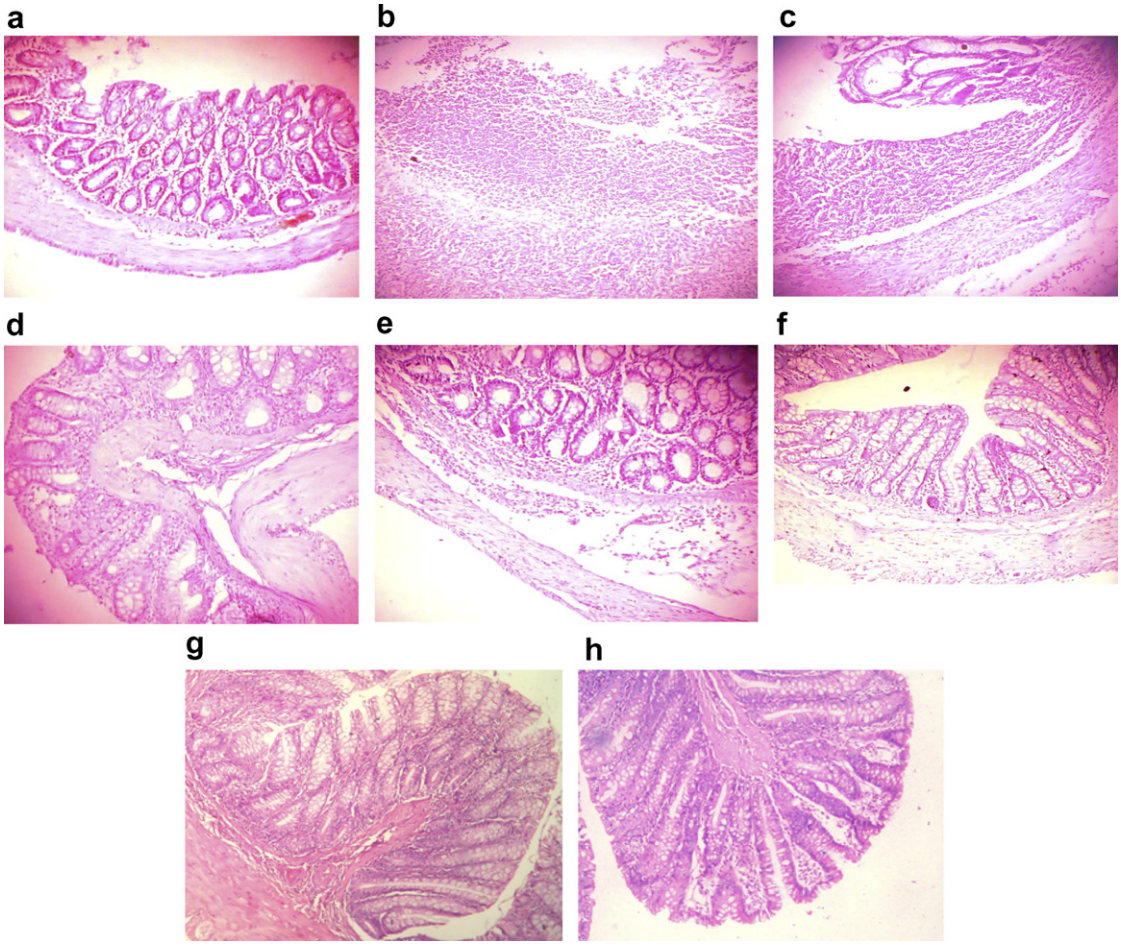


Figure 4. Histology of colon of rats subjected to TNBS. (a) Healthy control. (b) Colitis control showing mucosal injury characterized by absence of epithelium and a massive mucosal/submucosal infiltration of inflammatory cells. (c) 5-ASA, showing slight mucosal abscess and inflammatory infiltrate on oral administration. (d) Sulfasalazine. (e) ST showing corrected morphology of colon with comparable results to those of sulfasalazine. (f) L-Tryptophan showing no mucosal injury with slight inflammatory infiltrate on oral administration. (g) L-Tryptophan showing corrected morphology of colon with comparable results to those of sulfasalazine on rectal administration. (h) Co-administration of 5-ASA and L-tryptophan by rectal route, showing comparable ameliorating effect as that of sulfasalazine.

in ppm downfield on δ scale. The IR spectrum of the synthesized compound was recorded on JASCO, V-530 FTIR in potassium bromide (anhyd IR grade). The absorbance maxima (λ_{max}) of synthesized compounds were determined on JASCO V530, UV–visible double-beam spectrophotometer in hydrochloric acid buffer (pH 1.2), phosphate buffer (pH 7.4) and distilled water. Partition coefficient was determined in *n*-octanol/phosphate buffer (pH 7.4), whereas the aqueous solubility was determined in distilled water at room temperature (25 ± 1 °C). Pharmacological screening of the synthesized compound was carried out in the Department of Pharmacology, Poona College of Pharmacy and its animal facility is approved by CPCSEA. The experimental protocols for the same were approved by the Institutional Animal Ethical Committee.

5.3.2. Synthesis of tryptophan methyl ester hydrochloride (TME·HCl). Freshly distilled thionyl chloride (0.05 mol + 30% extra) was slowly added to methanol (100 ml) with cooling and L-tryptophan (0.1 mol) was added to it. The mixture was refluxed for 7 h at 60–70 °C with continuous stirring on a magnetic stirrer. Excess of thionyl chloride and solvent was removed under reduced pressure giving crude TME·HCl. The crude product was triturated with 20 ml portions of cold ether at 0 °C, until excess dimethyl sulfite was removed. The resulting solid product was collected and dried under high vacuum. It was recrystallized from hot methanol by slow addition of 15–20 ml of ether, followed by cooling at 0 °C. Crystals were collected on the next day and washed twice with ether/methanol mixture (5:1) followed by pure ether and dried under vacuum to give pure TME·HCl.

Mp 220–223 °C (Uncorrected), R_f 0.78 in chloroform/methanol (2:1), % yield 73. IR (KBr) 3590 cm^{-1} indole N–H stretching, 1735 cm^{-1} C=O saturated ester stretching, 1470 cm^{-1} C–H bending CH_2, 1430 cm^{-1} and 1370 cm^{-1} C–H bending CH_3, 1240 cm^{-1} C–O saturated ester stretching. ^{1}H NMR (DMSO-d_6): δ 10.65 [d, 1H] NH-indole, δ 7.10–7.16 [m, 4H] and δ 6.9 [d, 1H] CH-indole, δ 4.15 [s, 3H] CH_3-methyl, δ 2.5 [t, 1H] CH-methine, δ 2.6 [d, 2H] CH_2-methylene.

5.3.3. Diazotisation of tryptophan methyl ester hydrochloride. Tryptophan methyl ester hydrochloride (0.01 mol) was dissolved in a suitable volume of water containing 2.5–3 equiv of hydrochloric acid (0.02 mol 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 in 10 ml) was added portionwise, 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 until the solution gave an immediate positive test for excess of nitrous acid with an external indicator, that is, moist potassium iodide-starch paper. The precipitated tryptophan methyl ester hydrochloride, if any, got dissolved during the diazotisation 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 equiv). 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.

5.3.4. Coupling of diazotised L-tryptophan methyl ester with salicylic acid. Coupling of salicylic acid with diazotized tryptophan methyl ester was carried out in situ. Salicylic acid (0.01 mol) was completely dissolved in sodium hydroxide solution (2 mol/ml). The solution was cooled below 5 °C. Then slowly diazotised salt of tryptophan methyl ester 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. The reaction was monitored by TLC using chloroform: methanol (4:1.5), as a solvent system. The route of synthesis is described in Figure 1. Mp 243 °C (uncorrected), R_f 0.60, IR (KBr) 1487 cm^{-1} –N=N– stretching (non-symmetric *p*-substituted azobenzene), 3466–3422 cm^{-1} unbonded phenolic O–H stretching, 3590 cm^{-1} indole N–H stretching, 1597 and 1383 cm^{-1} carboxylate anion stretching, 2320 cm^{-1} aromatic C–H stretching, 1030 cm^{-1} C–N stretching. ^{1}H NMR (DMSO-d_6): δ 6.59 [d, 1H] protons of aromatic OH, δ 6.9 [d, 1H] and δ 7.10–7.16 [m, 4H] CH-indole, δ 10.65 [d, 1H] NH-indole, δ 6.64 [d, 1H] and δ 7.2 [d, 1H] CH-benzene, δ 2.5 [t, 1H] CH-methine, δ 2.6 [d, 2H] CH_2-methylene.

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