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Synthesis, kinetic studies and pharmacological evaluation of mutual azo prodrug of 5-aminosalicylic acid with D-phenylalanine for colon specific drug delivery in inflammatory bowel disease

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Abstract—Mutual azo prodrug of 5-aminosalicylic acid with D-phenylalanine was synthesized by coupling D-phenylalanine 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) only 15% release was observed over a period of 7 h. In rat fecal matter the release of 5-aminosalicylic acid was almost complete (85%), with a half-life of 160.1 min, following first order kinetics. The azo conjugate was evaluated for its ulcerogenic potential by Ramsford's cold stress method. Therapeutic efficacy of the carrier system and the mitigating effect of the azo conjugate were 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.

Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the mucosal membrane of the small and/or large intestine.[1] Although many treatments have been recommended for IBD, they do not treat the cause but are effective only in reducing the inflammation and accompanying symptoms in up to 80% of patients. 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.[2,3] Therefore, out of the need to overcome this formidable barrier of GIT, colonic drug delivery has evolved as an ideal drug delivery system for the topical treatment of diseases of colon like Crohn's disease, ulcerative colitis, colorectal cancer and amaebiasis. 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.[4]

Prodrug approach is one of the important 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 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.[5] But majority of side effects of sulfasalazine like hepatotoxicity, hypospermia and severe blood disorders are due to sulfapyridine. Few prodrugs of 5-ASA like basalazine, ipsalazine and olsalazine have been reported, but most of them suffer from adverse effects due to the carriers used with them.[6–8] The

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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 5-ASA with D-phenylalanine (SP) for its colon-targeted delivery, which would be safer with comparable activity to sulfasalazine. The aim of this project was to test in vivo the targeting potential of azo conjugate to inflamed tissue of colon and evaluate the therapeutic efficacy of this drug-carrier system in experimental colitis rat model. D-Phenylalanine was chosen as a promoiety due to its marked antiinflammatory activity.[9] 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.

The melting point of SP was found to be 230–235 °C (uncorrected). All the results of elemental analysis were in an acceptable error range.

The IR spectra of 5-ASA conjugate showed characteristic peak at 1485 cm^{-1} of N=N-stretching (unsymmetric *p*-substituted azobenzene) which confirms the formation of azo bond. A broad peak of unbonded phenolic O–H stretching at 3640–3526 cm^{-1} was also found. It also showed carboxylate anion stretching at 1597 cm^{-1} and 1375 cm^{-1} and C–N stretching at 1030 cm^{-1}.

^1H NMR spectra of SP showed chemical shifts for protons of aromatic OH at δ 6.40 [d, 1H], CH benzene 6.34 [d, 1H], δ 6.98 [d, 1H] and δ 7.47 [s, 1H]. The signals of CH-methine at δ 2.8 [s, 1H], CH_2 ethylene at δ 2.9 [d, 2H] were also found. The aqueous solubility found to be 0.25 g/ml and partition coefficient in *n*-octanol/phosphate buffer (pH7.4) was found to be 0.30, 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 236 nm and phosphate buffer (pH 7.4) at 294 nm. Kinetic studies confirmed that the prodrug did not release the parent drug in 0.1 M hydrochloric acid buffer (pH 1.2), whereas in phosphate buffer (pH 7.4) only 15% 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 to confirm the colonic reduction of azo prodrug. $t_{\frac{1}{2}}$ (average of four trials) of SP was found to be 160.1 min, whereas rate constant (*K*) was found to be $4.32 \times 10^{-}$ 0.0001. Over a period of 7 h, SP gave 85% cumulated release of 5-ASA following first order kinetics (Fig. 1). Thus in vitro kinetic studies confirmed that the synthesized conjugate did not release 5-ASA at all in HCl buffer (pH 1.2) but in phosphate buffer (pH 7.4), 15% release was observed. The release in rat fecal matter was almost complete.

The synthesized compound was evaluated for ulcerogenic activity by Rainsford's method[11] and the ulcer index was determined[12] (Table 1). The conjugate showed remarkable reduction in the ulcer index (11.3 ± 1.1) as compared to its parent drug (59.6 ± 4.7). This reduction

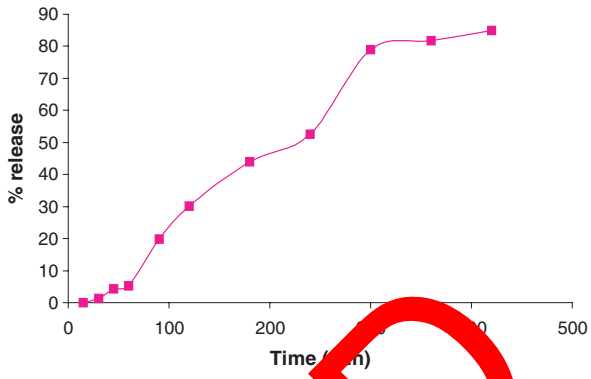


Figure 1. Release profile of 5-ASA from SP in rat fecal matter.

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
SP	3050	1.33 ± 1.1

HC, healthy control; 5-ASA, 5-amino salicylic acid; Slz, sulfasalazine; SP, prodrug of 5-ASA with D-phenylalanine.
[a] Average of six readings.

in the ulcer index brought about by the conjugate was compared 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.[13–15] 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 SP (1.39 ± 0.39), which was comparable to sulfasalazine (0.83 ± 0.42) but distinctly more than 5-ASA (2.09 ± 0.27). The positive contribution of D-phenylalanine towards lowering effect on clinical activity score (1.92 ± 0.08) is obvious from the gross difference in lowering effect of plain 5-ASA and SP. To ensure the synergistic effect of D-phenylalanine further, two test groups of animals were subjected to rectal administration of plain D-phenylalanine and 5-ASA+ D-phenylalanine, respectively. The lowering of clinical activity score by rectally administered D-phenylalanine was (1.06 ± 0.35) less than that of sulfasalazine (0.83 ± 0.42) but better than D-phenylalanine administered orally (1.92 ± 0.08). Co-administration of 5-ASA+ D-phenylalanine showed comparable lowering of clinical activity score as that of sulfasalazine (0.83 ± 0.42) but better than D-phenylalanine (1.92 ± 0.08) or 5-ASA (2.09 ± 0.27) administered orally. This

particular finding supports positive contribution of D-phenylalanine 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 D-phenylalanine as well as 5-ASA+ D-phenylalanine was comparable to sulfasalazine. 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, a massive mucosal/submucosal infiltration of inflammatory cells. In vivo treatment with SP 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 SP. These results were found to be comparable with those obtained for sulfasalazine treated group. Histopathological features of rectally administered D-phenylalanine and 5-ASA + D-phenylalanine 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. Differences were considered at a P value of <0.01 in relation to control.

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 criterion for selection of D-phenylalanine as carrier has also proven correct, as it has effectively delivered 5-ASA to colon. Moreover, its synergistic ameliorating effect on disrupted colonic architecture strengthens the hypothesis of mutual prodrug design.

^{1}H NMR spectra of the synthesized compound were recorded in DMSO using ^{1}H NMR Varian Mercury 300 Hz with superconducting magnet using TMS as internal standard. Chemical shift values are reported in ppm downfield from δscale. The IR spectra of the synthesized compound were recorded on JASCO, V-530 FTIR in potassium bromide (anh. IR grade). The absorbance maxima (λ_{max}) of synthesized compound was 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).

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 values were 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. SP (10 mg) was introduced in 900 ml 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 SP remaining.

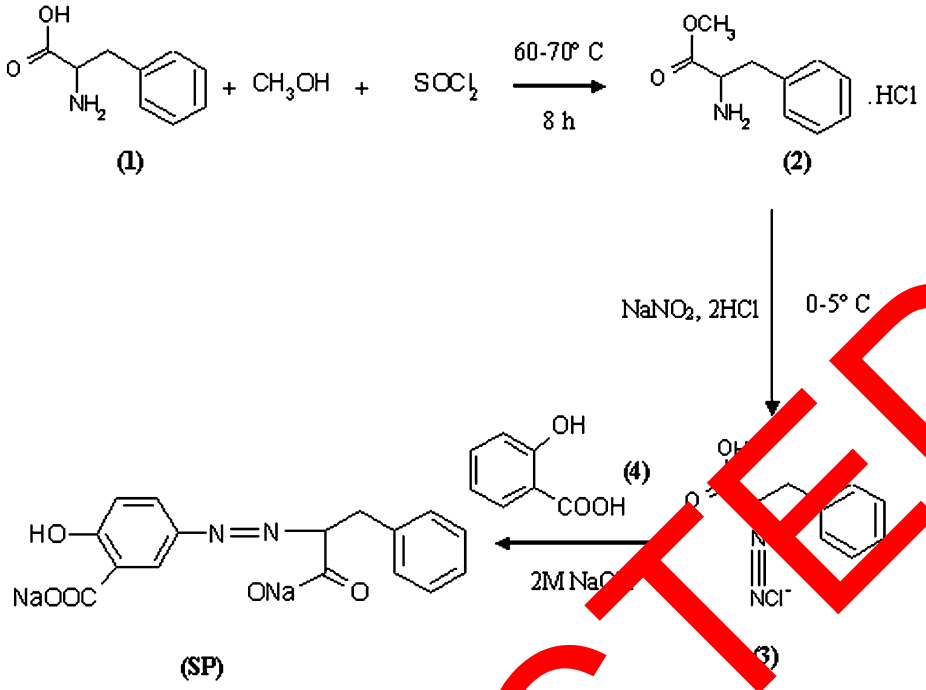
Same procedure as described earlier was followed; except that the HCl buffer was replaced by phosphate buffer. The kinetics was monitored by the decrease in prodrug concentration with time.

To study the release of 5-ASA from SP in rat fecal matter,[18] SP was dissolved in sufficient volume of phosphate buffer (pH 7.4) so that final concentration of solution was 2 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 SP 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.

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.

All chemicals used in the synthesis were of AR grade. Sulfasalazine was obtained as gift sample from Wallace Pharmaceutical Pvt. Ltd. Goa; salicylic acid and D-phenylalanine were purchased from Loba Chemie, Mumbai. 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.

Synthesis of methyl ester hydrochloride of D-phenylalanine[19] was carried out by adding thionyl chloride to methanol followed by refluxing with D-phenylalanine (1) at 60–70° C for 7 h. D-Phenylalanine methyl



Scheme 1.

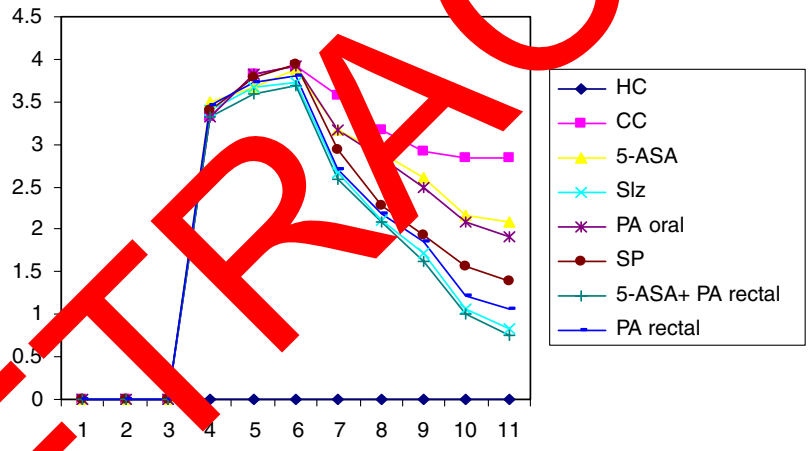


Figure 2. Clinical activity score rate. HC, healthy control; CC, colitis control; 5-ASA, 5-amino salicylic acid; Slz, sulfasalazine; PA oral, D-phenylalanine oral; SP, prodrug of 5-ASA with D-phenylalanine; 5-ASA with PA rectal, co-administration of 5-ASA with PA by rectal route; PA rectal, rectal administration of D-phenylalanine.

ester hydrochloride (2) was diazotised[20] at 0–5 °C in cryostatic bath. The coupling[20,21] of diazonium salt of D-phenylalanine (3) with salicylic acid (4) was carried out at 0–5 °C in a cryostatic bath (Scheme 1). It was recrystallized from methanol followed by cooling at 0 °C. Purified product (SP) was dried under vacuum.

The ulcerogenic activity was determined by Rainsford's cold stress method,[11] 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 experi-

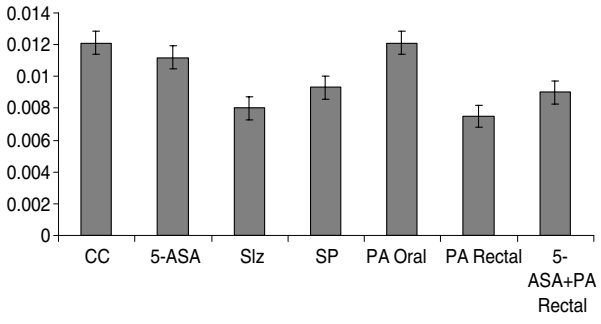


Figure 3. Colon to body weight ratio. HC, healthy control; CC, colitis control, 5-ASA, 5-amino salicylic acid; Slz, sulfasalazine; PA oral, D-phenylalanine oral; SP, prodrug of 5-ASA with D-phenylalanine; 5-ASA with PA rectal, co-administration of 5-ASA with PA by rectal route; PA rectal, rectal administration of D-phenylalanine.

mental 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 mean ± SD.

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) SP showing corrected morphology of colon with comparable results to that of sulfasalazine. (f) D-Phenylalanine showing no mucosal injury with slight inflammatory infiltrate on oral administration. (g) D-Phenylalanine showing corrected morphology of colon with comparable results to that of sulfasalazine on rectal administration. (h) Co-administration of 5-ASA and D-phenylalanine by rectal route, showing comparable ameliorating effect to that of sulfasalazine.

In order to study the ameliorating effect of azo prodrug of 5-ASA on the inflamed tissue of colon in IBD, trinitrobenzenesulfonic acid (TNBS)-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.[22] By this model in vivo characterization of the azo carrier system under the influence of chronic inflammatory symptoms was possible. Sprague–Dawley 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 canula (dose was 150 mg/kg of body weight of TNBS in ethanol, 50% solution). Animals were then maintained in a vertical position for 30 sec 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 rec[illegible] orally 5-ASA, sulfasalazine, D-phenylalanine and [illegible]P, respectively, once daily for five continuous days at do[illegible] equimolar to 5-ASA present in sulfas[illegible]ine. Th[illegible] healthy control and colitis control grou[illegible] rece[illegible]d only 1% carboxymethylcellulose instead of [illegible]ee drug [illegible]or prodrug. The animals of all groups w[illegible] exa[illegible] for weight loss, stool consistency [illegible]and [illegible] bleeding throughout the 11 days study. [illegible]olitis activ[illegible] was quantified with a clinical activity [illegible]assessing th[illegible] parameters (Fig. 2) by clinical ac[illegible]vity sc[illegible]ng rate. The clinical activity score was dete[illegible]ined by cal[illegible]ating the average of the above three [illegible]arameters for ea[illegible] day, for each group, and was r[illegible]ging fr[illegible] 0 (healthy) to 4 (maximal activity of colitis).[23] The[illegible] were sacrificed 24 h after the last drug administ[illegible]n by isof[illegible]rane anaesthesia and a segment [illegible]lon, [illegible]m lon[illegible] was excised and colon/body w[illegible]ght rat[illegible] was de[illegible]ined to quantify the inflammati[illegible] (Fig. 3). [illegible]issue segments 1 cm in length were then fixed [illegible] 10% [illegible] buffe[illegible] formalin for histopathological studies. [illegible]stopathological studies (Fig. 4a–e) of the colon were [illegible]arried 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.

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