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Original article

Colon-specific mutual amide prodrugs of 4-aminosalicylic acid for their mitigating effect on experimental colitis in rats

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Abstract

Mutual amide prodrugs of 4-aminosalicylic acid with D-phenylalanine and L-tryptophan were synthesized for targeted drug delivery to the inflamed gut tissue in inflammatory bowel disease. Stability studies in aqueous buffers (pH 1.2 and 7.4) showed that the synthesized prodrugs were stable in both the buffers over a period of 10 h. In rat fecal matter the release of 4-aminosalicylic acid from the prodrugs was in the range of 86–91% over a period of 20 h, with half-lives ranging between 343 and 412 min following first order kinetics. Targeting potential of the carrier system and the ameliorating effect of the amide conjugates were evaluated in trinitrobenzenesulfonic acid-induced experimental colitis model in rats. The prodrugs were assessed for their probable damaging effects on pancreas and liver with the help of histopathological analysis and for their ulcerogenic potential by Rainsford's cold stress method. They were found to have improved safety profile than sulfasalazine, oral 4- and 5-aminosalicylic acid with similar pharmacological spectrum and advantages of sulfasalazine.

Keywords: Mutual amide prodrug; 4-Aminosalicylic acid; Inflammatory bowel disease; D-Phenylalanine; L-Tryptophan; TNBS-induced colitis

1. Introduction

Ulcerative colitis and Crohn's disease collectively referred to as inflammatory bowel disease (IBD) are chronic relapsing conditions characterized by up-regulated proinflammatory mediators and dysregulated immune responses resulting in tissue damage [1] with a high morbidity and remain largely incurable. Routine treatment has not changed significantly over the last 40 years and still relies heavily on steroids and aminosalicylates [2] which 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 anti-inflammatory drugs at higher doses. 5-Aminosalicylic acid (5-ASA) is an active ingredient of agents used for the long term maintenance therapy to prevent relapses of IBD. But its oral administration results in extensive and rapid absorption from the upper gastrointestinal tract (GIT) before it reaches the colon leading to low drug bioavailability and efficiency with significant systemic side effects [3,4]. This formidable barrier of GIT can be circumvented by delivering the drug site-specifically to colon utilizing prodrug approach.

The earliest reported colon-specific prodrug of 5-ASA is sulfasalazine (Slz) (Fig. 1), an azo conjugate of 5-ASA with sulfapyridine (SP) [5]. But its sulfapyridine part used as a carrier is completely absorbed through colon and produces adverse effects like hepatitis and immunoallergic reactions [6]. A total of 2400 suspected adverse drug reactions were reported such as interstitial nephritis, skin reaction, pancreatitis and

Abbreviations: IBD, inflammatory bowel disease; 5-ASA, 5-aminosalicylic acid; GIT, gastrointestinal tract; Slz, sulfasalazine; 4-ASA, 4-aminosalicylic acid; BOC, di-*tert*-butyl dicarbonate; TNBS, 2,4,6-trinitrobenzenesulfonic acid; 4AP, amide conjugate of 4-ASA with D-phenylalanine; 4AT, amide conjugate of 4-ASA with L-tryptophan; MPO, myeloperoxidase; DMSO, dimethyl sulfoxide.

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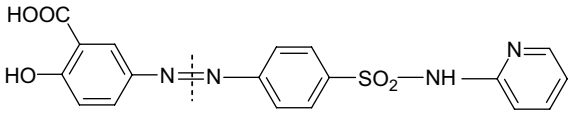


Fig. 1. Sulfasalazine.

blood dyscrasias for sulfasalazine to the Committee on Safety of Medicines (CSM) of the UK between 1st Jan 1991 and 31st Dec 1998 compared with 1100 for mesalazine [7]. Due to adverse effects caused by SP, the efforts were focused on replacing it with 4-aminobenzoyl-β-alanine in balsalazide (Fig. 2) [8] and by another 5-ASA molecule in olsalazine (Fig. 3) [9]. We have reported azo conjugates of 5-ASA with D-phenylalanine [10] and L-tryptophan [11] where mutual prodrug concept has been utilized and these prodrugs have been found to possess comparable effect to that of sulfasalazine on TNBS-induced colitis in rats. Polymeric prodrugs of 5-ASA linked with dextran [12] and polyanhydride [13] have been developed for site specific delivery to colon. Colon-specific amide conjugates of 5-ASA with glutamic acid [14], glycine [15] and taurine [16] are also reported in literature.

4-Aminosalicylic acid (4-ASA) differs from its 5-ASA counterpart by the position of the NH_2 group and is considered as a second line anti-tuberculosis agent in the treatment of drug-resistant tuberculosis caused by *Mycobacterium tuberculosis* [17]. 4-ASA has been used in the treatment of mild to moderate ulcerative colitis in patients who are intolerant of sulfasalazine and in the treatment of Crohn's disease; the drug is designated as an orphan drug by the FDA for use in mild to moderate ulcerative colitis [18,19]. 4-ASA has been claimed to be beneficial in the topical treatment of ulcerative colitis and in contrast to 5-ASA, has no effect on arachidonic acid metabolism in human neutrophils or on the free radical 1,1-diphenyl-2-picrylhydrazyl [20]. 4-ASA has been suggested as an effective treatment for both active and quiescent ulcerative colitis with lesser side effects [21]. 5-ASA-induced pancreatitis is well described on the World Health Organization's adverse reaction database [7,22], which might be of immunoallergic origin [19]. On the other hand, IBD patients on subsequent treatment with 4-ASA enemas show no recurrence of pancreatitis. 4-ASA enema is a safer and well-tolerated therapeutic alternative whenever 5-ASA-induced pancreatitis occurs [19]. Very recently the focus of IBD research seems to have shifted from 5-ASA to 4-ASA with very few of its prodrugs reported in the literature like azo derivatives of 4-ASA with salicylic acid, hydroxybenzene, *N*-salicyloyl glycine acid [23], amide conjugates of 4-ASA with glycine [24], ursodeoxycholic acid [25] and arginine salt of 4-ASA [26]. Research studies suggest that 4-ASA may provide a stable, inexpensive alternative to 5-ASA for the topical treatment of

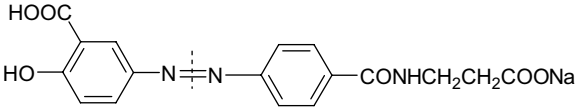


Fig. 2. Balsalazide.

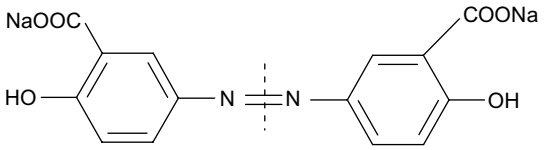


Fig. 3. Olsalazine.

ulcerative colitis or for linking to carrier molecules for release in the colon [27]. Though 4-ASA has so many advantages over 5-ASA, its potential has not yet been fully explored.

The present work describes concept-based mutual prodrug design and synthesis of amide conjugates of 4-ASA with amino acids for its colon-targeted delivery, with improved safety profile than sulfasalazine, 5-ASA and 4-ASA. The aim of this project was to test, *in vivo* the targeting potential of amide conjugates to inflamed tissue of colon and comparatively evaluate the therapeutic efficacy of this drug-carrier system over azo prodrugs of 5-ASA (with same carriers as used in the present study) reported by us, in experimental rat colitis model. The amino acid carriers have been found to increase the bioavailability and efficacy of the drugs and at the same time reduces their toxicity. D-Phenylalanine and L-tryptophan were chosen as promoieties due to their marked anti-inflammatory activity [10,11,28]. When linked with 4-ASA, they would improve its efficiency in mitigating the colonic inflammation. Being the natural components of our body, they would be nontoxic and free from any side effects. *N*-Aromatic acyl-amino acid conjugates are reported in the literature, they are stable in upper intestine and hydrolyzed when incubated with mammalian cecal content [29]. Introduction of amide linkage in the prodrug would increase aqueous solubility thus transcellular absorption by lipid membrane permeation might be limited in upper intestine. This would facilitate delivery of intact prodrug to colon. Microbial degradation of amide prodrugs by hydrolytic action of amidases secreted by the colonic microflora would further ensure the release of 4-ASA only in colon.

2. Materials and methods

2.1. Synthesis

2.1.1. Materials and general methods

^{1}H NMR spectra of the synthesized compounds were recorded in DMSO using ^{1}H NMR Varian Mercury 300 Hz with super conducting magnet using TMS as internal standard. Chemical shift values are reported in ppm downfield on δ scale. The IR spectra of the synthesized compounds were recorded on JASCO, V-530 FTIR in potassium bromide (anhy. IR grade). 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 vapors as detecting agent.

All chemicals used in the synthesis were of AR grade. 4-ASA and D-phenylalanine were purchased from Himedia Laboratories Pvt. Ltd., Mumbai while L-tryptophan was purchased from Loba Chemie, Mumbai.

2.1.2. Synthesis of methyl ester hydrochloride of phenylalanine (PME·HCl) and tryptophan (TME·HCl)

Freshly distilled thionyl chloride (0.05 mol + 30% extra) was slowly added to methanol (100 ml) with cooling and amino acid (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 methyl ester hydrochlorides of tryptophan and phenylalanine, respectively. The crude products were triturated with 20 ml portions of cold ether at 0 °C, until excess dimethyl sulphite was removed. The resulting solid products were collected and dried under high vacuum. The products were then 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 PME·HCl and TME·HCl.

PME·HCl: m.p.: 150–156 °C (uncorrected), $R_f = 0.81$ in chloroform:methanol (2:1), % yield: 70, IR (KBr): 3390 cm^{-1} and 3270 cm^{-1} N–H stretching primary aromatic amine, 3070 cm^{-1} aromatic C–H stretching, 1740 cm^{-1} C=O saturated ester stretching, 1470 cm^{-1} C–H bending CH_2, 1250 cm^{-1} C–O saturated ester stretching, ^{1}H NMR (DMSO-d_6): δ 7.26 [m, 5H] aromatic–CH, δ 3.84 [m, 1H] methine, δ 3.67 [s, 3H] methyl, δ 3.16 [d, 2H] methylene, δ 2.0 [s, 2H] amine.

TME·HCl: m.p.: 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.

2.1.3. Protection of amino group of 4-ASA by BOC

A solution of 4-ASA (10 mmol) in a mixture of dioxane (20 ml), water (10 ml) and 1 M sodium hydroxide (10 ml) was stirred and cooled in an ice-water bath. Di-*tert*-butyl dicarbonate (11 mmol) was added dropwise over a period of 3 h at room temperature and further stirring was continued at room temperature for 30 min. The solution was concentrated under high vacuum to about 10–15 ml, cooled in ice-water bath, covered with a layer of ethyl acetate (30 ml) and acidified with dilute aqueous potassium hydrogen sulphate solution to pH 2–3 (Congo red). The aqueous phase was extracted with ethyl acetate (2 × 15 ml). The ethyl acetate extracts were pooled, washed with water (3 × 20 ml), dried over anhydrous sodium sulphate and dried under vacuum to give BOC protected 4-ASA (*N-tert*-butylcarbonyl-4-ASA). It was recrystallized by dissolving in mixture of ethyl acetate and hexane (4:1 v/v) and cooling at 0 °C.

4-ASA-BOC: m.p.: 125 °C, $R_f = 0.66$ in benzene:chloroform:ethyl acetate (2:1:1 v/v/v), % yield: 76, IR (KBr): 3620 cm^{-1} phenolic O–H stretching, 3070 cm^{-1} aromatic C–H stretching, 1685 cm^{-1} C=O stretching (amide I), 1590 cm^{-1} C=O stretching (amide II), 1470 cm^{-1} C–H bending CH_2, 1430 cm^{-1} and 1370 cm^{-1} C–H bending CH_3, 1360 cm^{-1} *tert*-butyl stretching, ^{1}H NMR (DMSO-d_6): δ 11.0 [s, 1H] OH-carboxylic acid, δ 8.0 [s, 1H] NH-sec. amide, δ 7.94 [m, 4H] CH-methine, δ 5.0 [s, 1H] C–OH aromatic, δ 1.40 [t, 3H] CH_3-methyl.

2.1.4. Protection of hydroxyl group of BOC-4-ASA by acetylation

Well dried BOC protected 4-ASA (0.040 mol) was placed carefully in freshly prepared admixture of 10 ml each of acetic anhydride and glacial acetic acid in a clean and dry 100 ml round bottom flask and stirring was continued at room temperature for the duration of 12 h. The resulting mixture was directly poured into 100 ml cold water, contained in a 500 ml beaker in one lot; the contents were stirred vigorously with clean glass rod till the shining tiny crystals separated out. The product was filtered off on a Buchner funnel fitted with an air suction device and the residue was washed with sufficient cold water, was drained well and finally the excess of water removed by pressing it between the folds of filter paper and spreading it in the air to allow it to dry completely. It was recrystallized by dissolving in methanol and cooling at 0 °C.

M.p.: 105 °C, $R_f = 0.45$ in benzene:chloroform:ethyl acetate (2:1:1 v/v/v), % yield: 85, IR (KBr): 3060 cm^{-1} aromatic C–H stretching, 1688 cm^{-1} C=O stretching (amide I), 1595 cm^{-1} C=O stretching (amide II), 1472 cm^{-1} C–H bending CH_2, 1434 cm^{-1} and 1370 cm^{-1} C–H bending CH_3, 1368 cm^{-1} *tert*-butyl stretching, ^{1}H NMR (DMSO-d_6): δ 8.0 [s, 1H] NH-sec. amide, δ 7.94 [m, 4H] CH-methine, δ 1.40 [t, 3H] CH_3-methyl.

2.1.5. Synthesis of acid chloride of protected 4-ASA

Redistilled thionyl chloride (3.3 mmol) in methylene chloride (2 ml) was added dropwise to a suspension (3.32 mmol) of protected 4-ASA in 15 ml of anhydrous methylene chloride cooled at 0 °C containing pyridine (0.4 mmol). The mixture was allowed to attain room temperature and stirring was continued for 24 h. The product was dried under vacuum and immediately used in the next step.

M.p.: 200 °C (melts with decomposition), $R_f = 0.62$, chloroform:methanol:benzene (4:1:1 v/v/v), % yield: 82%, IR (KBr): 3060 cm^{-1} aromatic C–H stretching, 1810 cm^{-1} C=O stretching (acid halide), 1688 cm^{-1} C=O stretching (amide I), 1595 cm^{-1} C=O stretching (amide II), 1472 cm^{-1} C–H bending CH_2, 1434 cm^{-1} and 1370 cm^{-1} C–H bending CH_3, 1368 cm^{-1} *tert*-butyl stretching, ^{1}H NMR (DMSO-d_6): δ 8.0 [s, 1H] NH-sec. amide, δ 8.56 [m, 4H] CH-methine, δ 1.40 [t, 3H] CH_3-methyl.

2.1.6. Coupling of amino acid methyl ester hydrochloride with protected 4-ASA acid chloride

Methyl ester hydrochloride of amino acid (0.11 mol) was accurately weighed and dissolved in 4 ml water containing sodium hydroxide (0.11 mol) and the resultant solution was cooled in ice bath. Acid chloride of protected 4-ASA (0.12 mol) and solution of sodium hydroxide (0.11 mol) in

2 ml of water were added in small lots over a period of 2 h, into the previously chilled amino acid ester solution with constant stirring. The stirring was continued for a further duration of 36 h at 5–10 °C so as to complete the reaction. The resultant reaction mixture was acidified to Congo red by careful dropwise addition of diluted HCl in chilled condition. The resultant solid was filtered off on Buchner funnel fitted with an air suction device and the residue was washed with sufficient cold water, drained and finally the excess of water removed by pressing it between the folds of filter paper and was dried under vacuum. The crude product was purified by preparative chromatography using the solvent system dichloromethane:-methanol:benzene (4:3:1 v/v/v) and amino acid conjugate of protected 4-ASA was obtained.

Protected 4AP: m.p.: 150 °C (melts with decomposition), $R_f = 0.68$, dichloromethane:methanol:benzene (4:3:1 v/v/v), % yield: 72, IR (KBr): 3065 cm^{-1} aromatic C–H stretching, 1682 cm^{-1} C=O stretching (amide I), 1590 cm^{-1} C=O stretching (amide II), 1474 cm^{-1} C–H bending CH_2, 1430 cm^{-1} and 1368 cm^{-1} C–H bending CH_3, 1360 cm^{-1} *tert*-butyl stretching, ^{1}H NMR (DMSO-d_6): δ 8.0 [s, 1H] NH-sec. amide, δ 8.56 [m, 4H] CH-methine, δ 3.40 [d, 2H] CH_2-methylene, δ 2.0 [s, 1H] NH-amine, δ 1.40 [t, 3H] CH_3-methyl.

Protected 4AT: m.p.: 158 °C $R_f = 0.67$, chloroform:methanol:benzene (4:1:1 v/v/v), % yield: 78, IR (KBr): 3592 cm^{-1} indole N–H stretching, 3069 cm^{-1} aromatic C–H stretching, 1680 cm^{-1} C=O stretching (amide I), 1595 cm^{-1} C=O stretching (amide II), 1478 cm^{-1} C–H bending CH_2, 1432 cm^{-1} and 1367 cm^{-1} C–H bending CH_3, 1365 cm^{-1} *tert*-butyl stretching, ^{1}H NMR (DMSO-d_6): δ 10.1 [s, 1H] NH-indole, δ 8.56 [m, 4H] CH-methine, δ 8.0 [s, 1H] NH-sec. amide, δ 7.55–6.45 [s, 1H] CH-indole, δ 3.40 [d, 2H] CH_2-methylene, δ 2.0 [s, 1H] NH-amine, δ 1.40 [t, 3H] CH_3-methyl.

2.1.7. Deprotection of acetyl and BOC groups to form final prodrug

Protected 4-ASA amino acid conjugate (0.0031 mol) was treated with 30 ml of 1 M solution of hydrochloric acid in acetic acid and was stirred for 12 h at room temperature. Then it was neutralized with 25% sodium hydroxide solution to give the crude product which was purified by preparative chromatography using the solvent system chloroform:methanol (4:1) to give pure final product, which was stored in well-closed amber coloured bottle in refrigerator.

4AP: m.p.: 160 °C (melts with decomposition), $R_f = 0.62$, chloroform:methanol:benzene (5:1:1 v/v/v), % yield: 78, IR (KBr): 3616 cm^{-1} unbonded phenolic OH stretching, 3425 cm^{-1} amide NH stretching, 3018 cm^{-1} aromatic C–H stretching, 1633 cm^{-1} amide C=O stretching, 1574 cm^{-1} carboxylate anion stretching, 1474 cm^{-1} C–H bending CH_2, 1430 cm^{-1} and 1368 cm^{-1} C–H bending CH_3, ^{1}H NMR (DMSO-d_6): δ 5.21 [s, 1H] aromatic OH, δ 6.29 [s, 1H], δ 6.38 [s, 1H], δ 6.98 [t, 3H], δ 7.11 [t, 3H], δ 7.22 [d, 3H], δ 7.34 [d, 3H], and δ 7.66 [d, 3H] CH-benzene, δ 3.26 [t, 2H] CH-methine, δ 3.02 [d, 1H] CH_2-methylene, δ 4.31 [s, 1H] NH_2-primary aromatic amine, δ 9.88 [s, 1H] NH stretching in benzamide.

4AT: m.p.: 165 °C (melts with decomposition), $R_f = 0.66$, chloroform:methanol:benzene (5:1:1 v/v/v), % yield: 73, IR (KBr): 3616 cm^{-1} unbonded phenolic OH stretching, 3414 cm^{-1} NH stretching (sec. amide), 3018 cm^{-1} aromatic C–H stretching, 1631 cm^{-1} amide C=O stretching, 1521 cm^{-1} carboxylate anion stretching, 1473 cm^{-1} C–H bending CH_2, 1432 cm^{-1} and 1364 cm^{-1} C–H bending CH_3, ^{1}H NMR (DMSO-d_6): δ 5.31 [s, 1H] aromatic OH, δ 6.29 [s, 1H], δ 6.28 [s, 1H], δ 6.99 [t, 3H], δ 7.12 [t, 3H], δ 7.31 [d, 3H], δ 7.34 [d, 3H] and δ 7.63 [d, 3H] CH-benzene, δ 3.30 [t, 2H] CH-methine, δ 3.02 [d, 1H] CH_2-methylene, δ 3.91 [s, 1H] NH_2-primary aromatic amine stretching, δ 9.98 [s, 1H] NH-stretching in benzamide.

2.2. Partition coefficient and aqueous solubility

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).

2.3. In vitro stability studies

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. All chemicals used in the preparation of buffer were of AR grade.

In vitro stability studies were carried out in hydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4) [30,31]. 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 hydrolysis of amide linkage by amidases 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. 4AP and 4AT (10 mg each) were introduced in 900 ml of HCl buffer taken in two different baskets and 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 directly estimated on UV spectrophotometer at 251 nm and 285 nm, respectively, for the amount of 4AP and 4AT remaining. 4-ASA, which was supposed to be released by the synthesized prodrugs, did not interfere with absorption of 4AP and 4AT because its λ_{max} was found to be 274 nm, which was substantially different from 4AP and 4AT. Therefore simultaneous estimation of prodrugs in presence of released 4-ASA was carried out over a period of 10 h. 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 prodrugs concentration with time over a period of

10 h. The aliquots were directly estimated on UV spectrophotometer at 255 nm and 290 nm, respectively, for the amount of 4AP and 4AT remaining. To study the release of 4-ASA from 4AP and 4AT in rat fecal matter [32], prodrugs were 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 (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 under anaerobic conditions in CO_2 incubator for different intervals of time. For analysis, the aliquots of 4AP and 4AT were removed from the test tubes at different time intervals and estimated directly on double-beam UV spectrophotometer (JASCO, V-530 model, Japan) at 255 nm and 290 nm, respectively. The concentration of prodrugs remaining was determined from the calibration curve of 4AP and 4AT in phosphate buffer.

2.4. Biological investigations

2.4.1. General methods

Pharmacological screening of the synthesized compounds 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.

2.4.2. Trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis

Trinitrobenzenesulfonic acid (TNBS)-induced experimental colitis model was selected to study the ameliorating effect of amide prodrugs of 4-ASA and *in vivo* characterization of the amide carrier system on the inflamed tissue of colon in IBD. It is the most relevant model for chronic inflammation of colon [33]. Wistar rats (average weight 200–250 g; 12–15 weeks; $n = 6$/group) were used. They were distributed into 14 different groups, i.e. healthy control, colitis control, three standard groups and nine 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 (Sigma–Aldrich Chemicals Pvt. Ltd., Schweiz) in ethanol was injected into colon via rubber canula (dose was 100 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 4-ASA, 5-ASA, sulfasalazine, D-phenylalanine, L-tryptophan, 4AP and 4AT orally while 4-ASA, D-phenylalanine, L-tryptophan, 4-ASA + D-phenylalanine and 4-ASA + L-tryptophan rectally, 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 prodrugs. 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 (Fig. 4) by clinical activity scoring rate. 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) [34]. They were sacrificed 24 h after the last drug administration by isoflurane anesthesia and a segment of colon, 8 cm long was excised and colon/body weight ratio was determined to quantify the inflammation (Fig. 5). Tissue segments, 1 cm in length were then fixed in 10% buffered formalin for histopathological studies. Histopathological studies of the colon (Fig. 6A–N), pancreas (Fig. 7A–C), and liver (Fig. 8A–C) were carried out using haematoxylin and eosin stains, at Kolte Pathology Laboratory, Pune. Colored microscopical images of the colon sections were taken on Nikon Electronic microscope ECLIPSE E200 with resolution (10×), attached with Nikon COOL-PIX 5400 (Type: E5400 Digital Camera) of magnification, 0.26–0.92×.

2.4.3. Myeloperoxidase (MPO) activity

Myeloperoxidase (MPO) activity was determined by the method reported by Krawisz et al. [35], the results of which are depicted in Fig. 9.

2.4.4. Ulcerogenic activity

The ulcerogenic activity was determined by Rainsford's cold stress method [36] at 10 times higher dose as described in our earlier work [37] with 4-ASA, 5-ASA and sulfasalazine as standards. Wistar rats (average weight 120–150 g; $n = 6$/group) were used for the same.

3. Results and discussion

3.1. Chemistry

Synthesis of methyl ester hydrochlorides of amino acids [38] was carried out by adding thionyl chloride to methanol followed by refluxing with amino acid (**1**) at 60–70 °C for 7 h. Amino group of 4-ASA was protected by using di-*tert*-butyl dicarbonate (BOC). The reaction was carried out at ambient temperature for 30 h [39]. The hydroxyl group of BOC-4-ASA (**2**) was protected by using acetic anhydride in presence of glacial acetic acid [40]. The reaction was carried out at ambient temperature for 12 h. The carboxylic group of protected 4-ASA (**3**) was activated by treating it with thionyl chloride [41]. The reaction was carried out at room temperature for 24 h. The coupling between acid chloride of protected 4-ASA (**4**) and methyl ester hydrochloride of amino acid was carried out by Schotten–Bauman procedure using 10% sodium hydroxide [40]. Reaction was carried out at 5–10 °C for 36 h. Finally, the amino and hydroxyl groups were

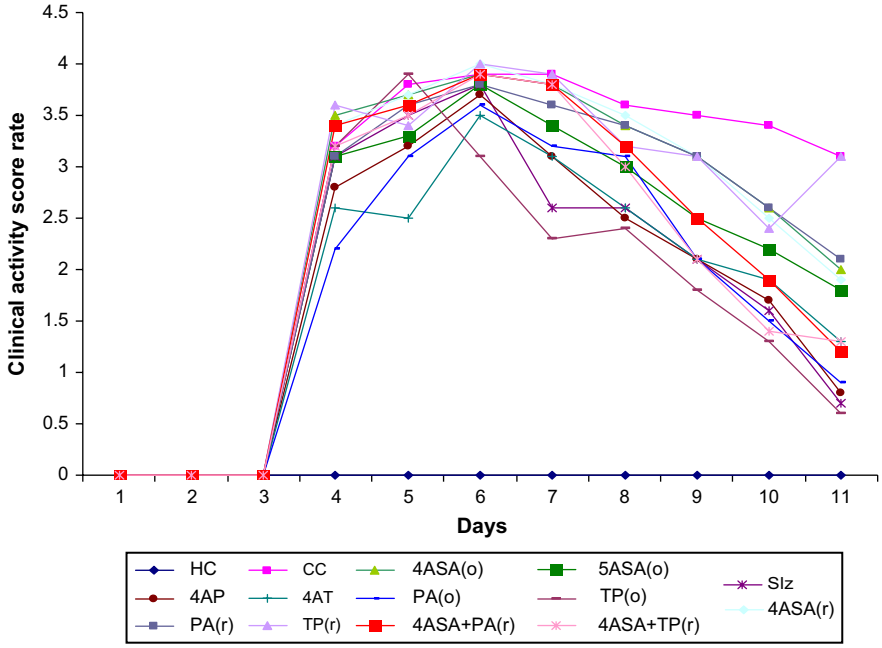


Fig. 4. Clinical activity score rate. Average of six readings; $P < 0.05$. HC: healthy control; CC: colitis control; 4-ASA (o): administration of 4-aminosalicylic acid by oral route; 5-ASA (o): administration of 5-aminosalicylic acid by oral route; Slz (o): administration of sulfasalazine by oral route; 4AP (o): administration of amide conjugate of 4-ASA with D-phenylalanine by oral route; 4AT (o): administration of amide conjugate of 4-ASA with L-tryptophan by oral route; PA (o): administration of D-phenylalanine by oral route; TP (o): administration of L-tryptophan by oral route; PA (r): administration of D-phenylalanine by rectal route; TP (r): administration of L-tryptophan by rectal route; 4-ASA (r): administration of 4-aminosalicylic acid by rectal route; 4-ASA + PA (r): co-administration of 4-aminosalicylic acid + D-phenylalanine by rectal route; 4-ASA + TP (r): co-administration of 4-aminosalicylic acid + L-tryptophan by rectal route.

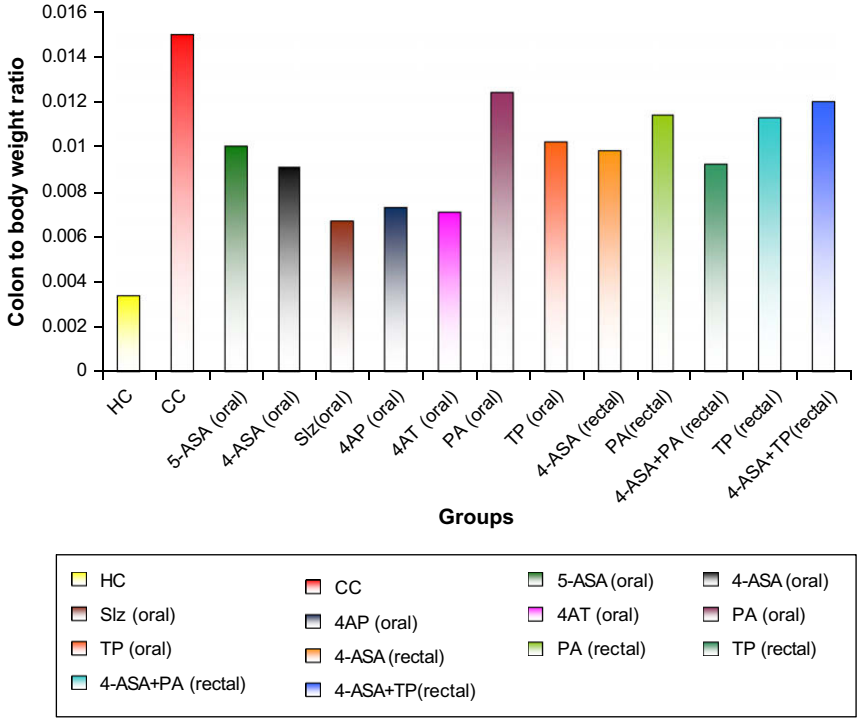


Fig. 5. Colon to body weight ratio. Average of six readings; $P < 0.05$. HC: healthy control; CC: colitis control; 4-ASA (o): administration of 4-aminosalicylic acid by oral route; 5-ASA (o): administration of 5-aminosalicylic acid by oral route; Slz (o): administration of sulfasalazine by oral route; 4AP (o): administration of amide conjugate of 4-ASA with D-phenylalanine by oral route; 4AT (o): administration of amide conjugate of 4-ASA with L-tryptophan by oral route; PA (o): administration of D-phenylalanine by oral route; TP (o): administration of L-tryptophan by oral route; PA (r): administration of D-phenylalanine by rectal route; TP (r): administration of L-tryptophan by rectal route; 4-ASA (r): administration of 4-aminosalicylic acid by rectal route; 4-ASA + PA (r): co-administration of 4-aminosalicylic acid + D-phenylalanine by rectal route; 4-ASA + TP (r): co-administration of 4-aminosalicylic acid + L-tryptophan by rectal route.

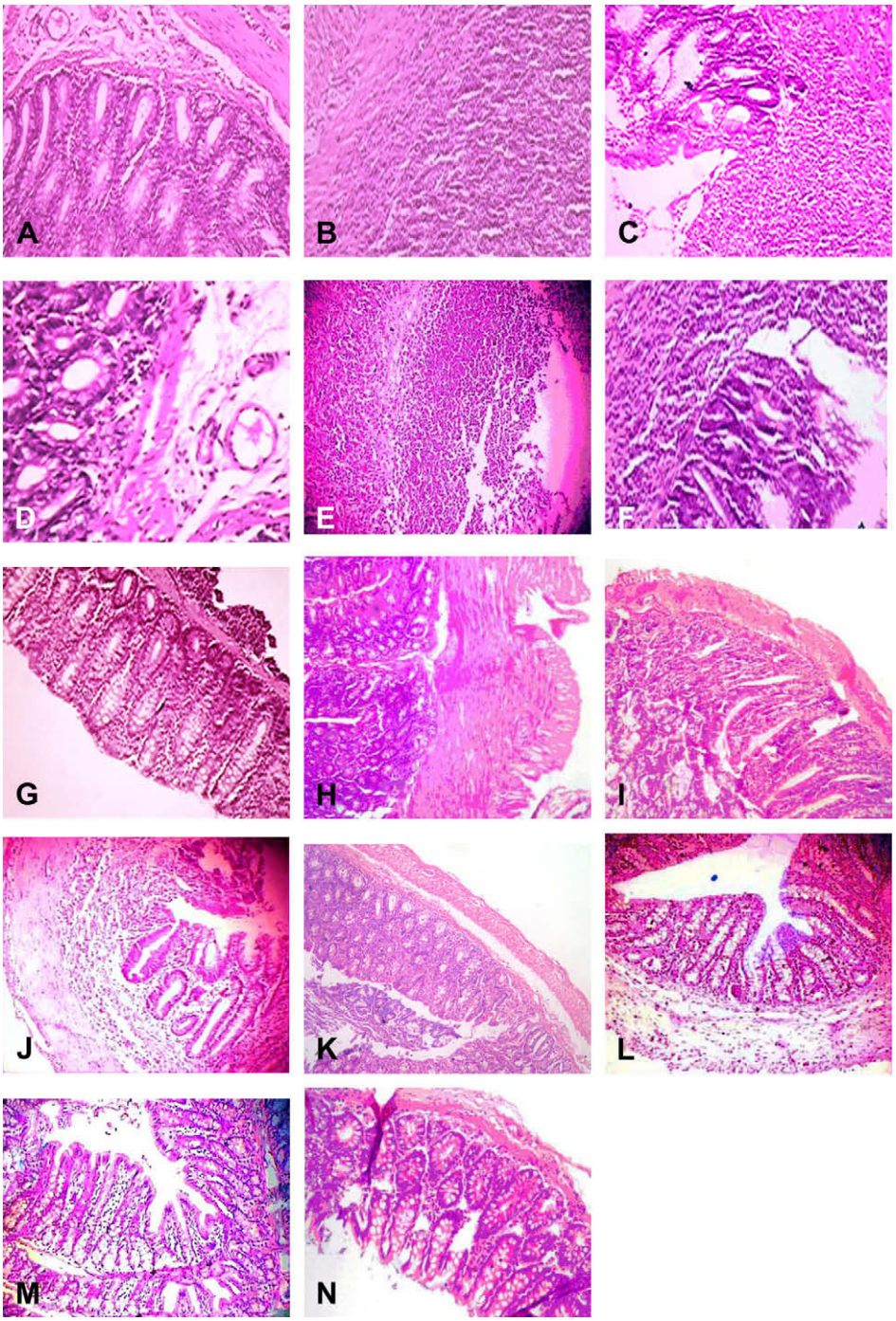


Fig. 6. Histology of colon of rats subjected to TNBS. (A) Healthy control and (B) colitis control showing mucosal injury characterized by absence of epithelium and a massive mucosal/submucosal infiltration of inflammatory cells. (C) 5-ASA, (D) 4-ASA, (E) D-phenylalanine and (F) L-tryptophan, all showing slight mucosal abscess and inflammatory infiltrate on oral administration. (G) Sulfasalazine, (H) 4AP and (I) 4AT showing corrected morphology of colon with comparable results to that of sulfasalazine. (J) d-Phenylalanine, (K) 4-ASA, (L) L-tryptophan, (M) 4-ASA + D-phenylalanine and (N) 4-ASA + L-tryptophan show comparable results as that of colon-specific prodrugs thus indicating a positive contribution of carriers towards anti-inflammatory activity on rectal administration.

deprotected using acetic acid [42]. Reaction was carried out at room temperature for 12 h (Scheme 1).

The melting points of 4AP and 4AT were found to be 160 °C and 180 °C, respectively, which melted with decomposition (uncorrected). All the results of elemental analysis were in an acceptable error range. The IR spectra of 4AP and 4AT showed characteristic peaks at 1633 cm^{-1} and 1631 cm^{-1} for amide C=O stretching, 3425 cm^{-1} and 3414 cm^{-1} for amide NH-stretching, 3616 cm^{-1} and 3501 cm^{-1} for unbonded phenolic O–H stretching, 1574 cm^{-1} and 1521 cm^{-1} for carboxylate anion stretching, 3468 cm^{-1} and 3305 cm^{-1} for NH stretching in primary aromatic amine, respectively.

^{1}H NMR spectra of 4AP showed chemical shifts for protons of aromatic OH at δ 5.21 [s, 1H], CH-benzene δ 6.29 [s, 1H],

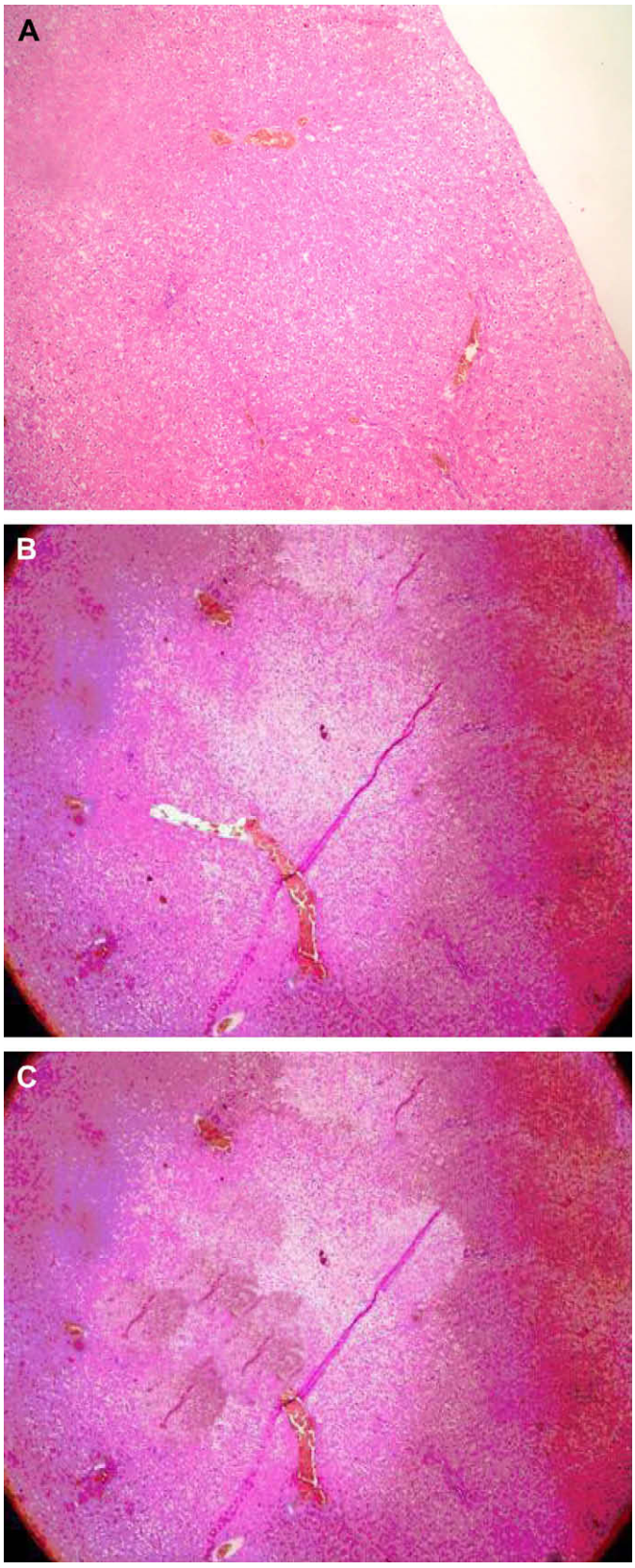


Fig. 7. Histology of pancreas of rats after treatment with prodrugs. (A) Healthy control, (B) 4AP and (C) 4AT showing no evidence of pancreatitis.

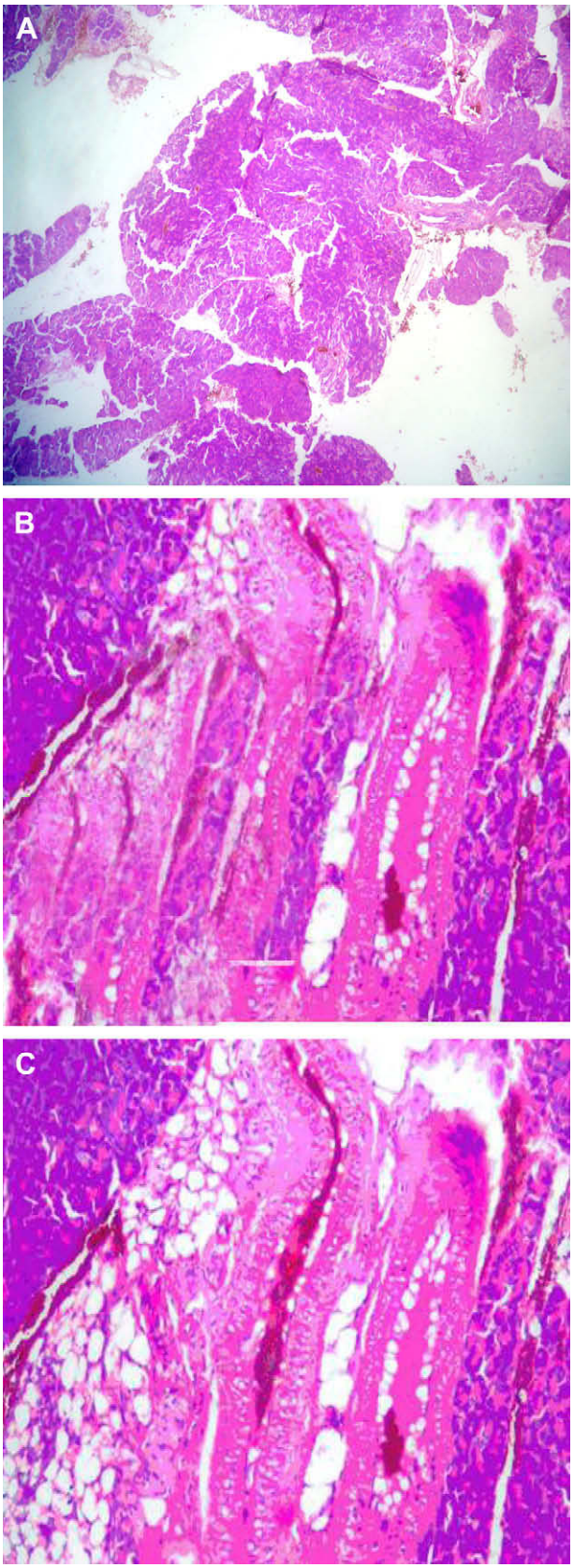


Fig. 8. Histology of rat livers after treatment with prodrugs. (A) Healthy control, (B) 4AP and (C) 4AT showing no adverse effects on liver.

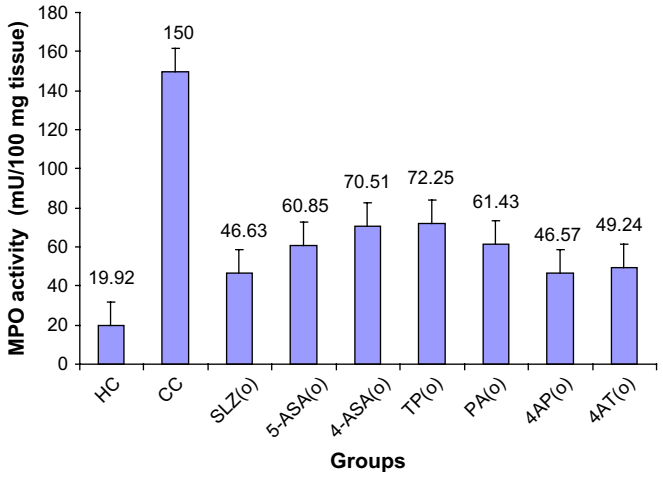


Fig. 9. Myeloperoxidase activity. HC: healthy control; CC: colitis control; 4-ASA (o): administration of 4-aminosalicylic acid by oral route; 5-ASA (o): administration of 5-aminosalicylic acid by oral route; Slz (o): administration of sulfasalazine by oral route; 4AP (o): administration of amide conjugate of 4-ASA with D-phenylalanine by oral route; 4AT (o): administration of amide conjugate of 4-ASA with L-tryptophan by oral route; PA (o): administration of D-phenylalanine by oral route; TP (o): administration of L-tryptophan by oral route.

δ 6.38 [s, 1H], δ 6.98 [t, 3H], δ 7.11 [t, 3H], δ 7.22 [d, 3H], δ 7.34 [d, 3H], and δ 7.66 [d, 3H]. The signals of CH-methine at δ 3.26 [t, 2H], CH_2-methylene at δ 3.02 [d, 1H], NH_2-primary aromatic amine stretching at δ 4.31 [s, 1H], NH-stretching in benzamide at δ 9.88 [s, 1H] were also found. ^{1}H NMR spectra of 4AT showed chemical shifts for protons of aromatic OH at δ 5.31 [s, 1H], CH-benzene δ 6.29 [s, 1H], δ 6.28[s, 1H], δ 6.99 [t, 3H], δ 7.12 [t, 3H], δ 7.31 [d, 3H], δ 7.34 [d, 3H] and δ 7.63 [d, 3H]. The signals of CH-methine at δ 3.30 [t, 2H], CH_2-methylene at δ 3.02 [d, 1H] NH_2-primary aromatic amine stretching at δ 3.91 [s, 1H], NH-stretching in benzamide at δ 9.98 [s, 1H] were also found.

The aqueous solubilities of 4AP and 4AT were found to be 0.33 g/ml and 0.26 g/ml, respectively. Partition coefficients (log P) of 4AP and 4AT in n-octanol/phosphate buffer (pH 7.4) were found to be −0.21 and −0.19, which were much lower as compared to 4-ASA (1.012).

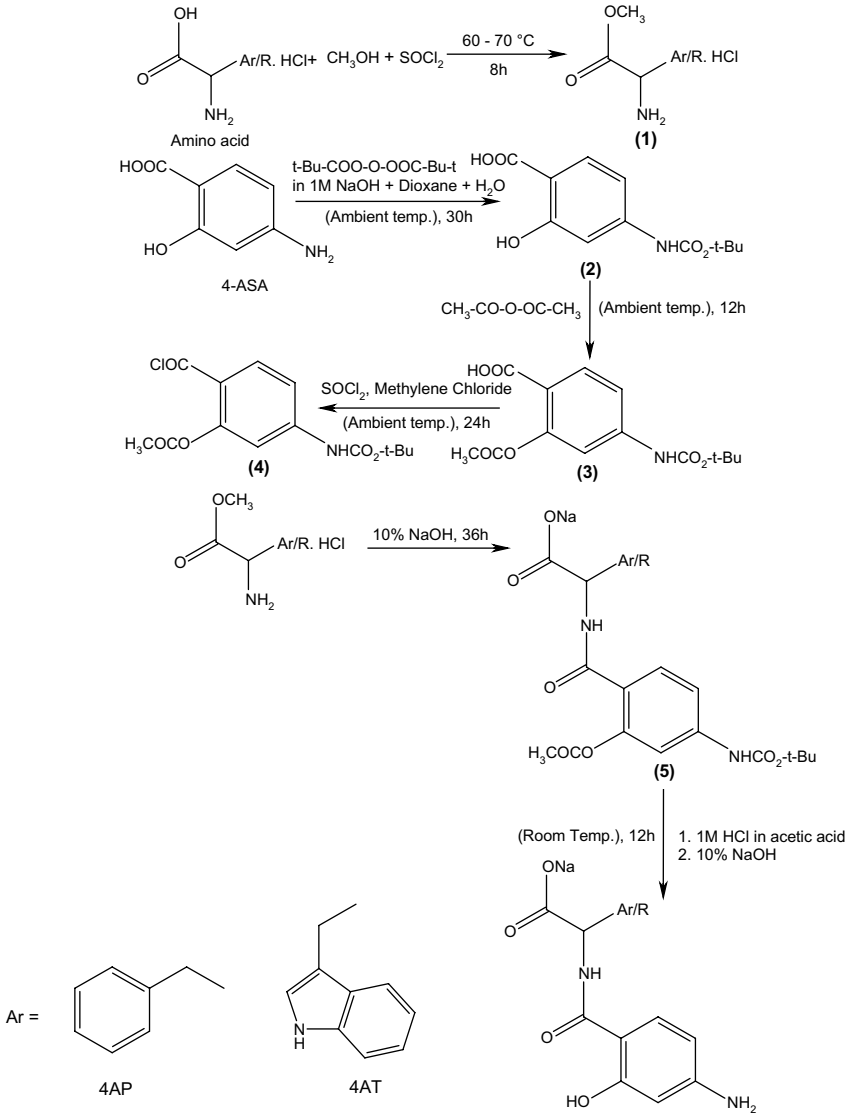
3.2. Kinetic study results

In order to check the stability of these prodrugs at acidic and alkaline pH, their release kinetics was studied by monitoring the decrease in concentration of 4AP and 4AT with time, in HCl buffer (pH 1.2) at 251 nm and 285 nm and in phosphate buffer (pH 7.4) at 255 nm and 290 nm, respectively.

Kinetic studies confirmed that these prodrugs were stable and did not release 4-ASA in aqueous buffers of pH 1.2 and 7.4. Thus, the objective of bypassing the upper GIT without any free drug release was achieved. The hydrolysis kinetics was further studied in rat fecal matter [43] to confirm the colonic hydrolysis of amide prodrugs, over a period of 20 h. Half-lives (average of four trails) of 4AP and 4AT were found to be 412 min and 343 min whereas rate constants (K) were found to be $2.015 \times 10^{-3} \pm 0.0001\ s^{-1}$ and $1.673 \times 10^{-3} \pm 0.0001\ s^{-1}$, respectively. The cumulative percent release of 4-ASA from the prodrugs followed first order kinetics (Fig. 10) with 86% release from 4AP and 91% from 4AT. *In vitro* kinetic studies confirmed that the release of 4-ASA from synthesized conjugates in rat fecal matter was almost complete over a period of 20 h.

3.3. Biological results

In order to evaluate the feasibility of orally administered amide prodrugs of 4-ASA for targeted drug delivery to the inflamed colon in IBD, 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced experimental colitis model was chosen [30,44,45]. Severity of inflammation was quantified by determining clinical activity score, colon to body weight ratio and myeloperoxidase activity. There is direct correlation between the severity of inflammation and these parameters, i.e. more severe the inflammation, higher are the values for these parameters and lower the mitigating effect of the prodrugs. After induction of experimental colitis, the animals were housed without treatment for the next 3 days to maintain the development of a full IBD model. During these 3 days, the clinical activity score for all groups increased consistently. After a lag time of 24–48 h, all drug-receiving groups showed a decrease in severity of inflammation. On 7th day, a significant difference between the drugs treated groups and a colitis control group was observed. 4AP produced a comparable lowering of clinical activity (0.8 ± 0.09) to sulfasalazine (0.9 ± 0.7) while effect of 4AT on lowering of clinical activity was moderate (1.3 ± 0.12), but for both, it was distinctly more than 4-ASA (2.0 ± 0.08). The positive contribution of carriers towards lowering effect on clinical activity score (2.4 ± 0.13 and 2.2 ± 0.08 for D-phenylalanine and L-tryptophan, respectively) is obvious from the gross difference in lowering effect of plain 4-ASA, 4AP and 4AT. To ensure the synergistic effect of these carriers further, four test groups of animals were subjected to rectal administration of plain D-phenylalanine and L-tryptophan, 4-ASA + D-phenylalanine and 4-ASA + L-tryptophan, respectively. The lowering of clinical activity score by rectally administered D-phenylalanine and L-tryptophan was 2.1 ± 0.03 and 1.8 ± 0.15, respectively, which was less than that of sulfasalazine (0.9 ± 0.07) but better than D-phenylalanine (2.4 ± 0.08) and L-tryptophan (2.2 ± 0.08) administered orally. Co-administration of 4-ASA + D-phenylalanine and 4-ASA + L-tryptophan showed comparable lowering of clinical activity score (1.2 ± 0.07 and 1.3 ± 0.04, respectively) as that of sulfasalazine (0.9 ± 0.07) but better than 4-ASA or carriers administered orally. This particular finding supports the positive contribution of carriers, their synergistic effect and hence the mutual prodrug hypothesis. On day 11 (24 h after the drug administration), the animals were sacrificed and colon/body weight ratio was determined to quantify inflammation. 4AP (0.0073 ± 0.00049) and 4AT (0.0071 ± 0.00060) treated groups showed a distinct decrease in the colon/body weight ratio compared to colitis control group (Fig. 5). Significant decrease in colon/body weight ratio



Scheme 1.

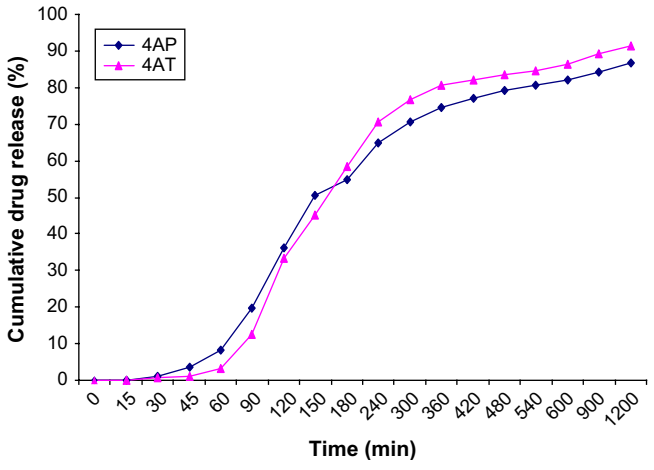


Fig. 10. Release profile of 4-ASA from 4AP and 4AT in rat fecal matter. 4AP: amide conjugate of 4-ASA with D-phenylalanine; 4AT: amide conjugate of 4-ASA with L-tryptophan.

produced by rectally administered carriers as well as co-administration of 4-ASA with carriers was comparable to sulfasalazine and indicated an intestinal anti-inflammatory activity. Myeloperoxidase (MPO) activity was measured according to the technique described by Krawisz et al.; the results were expressed as MPO units per gram of wet tissue and one unit of MPO activity was defined as that degrading 1 mmol min^{-1} of hydrogen peroxide at 25 °C [35]. Colonic MPO activity for 4AP and 4AT in mU/100 mg tissue was found to be 46.57 and 49.24, respectively, which was comparable to sulfasalazine (46.63), but much less than plain 4-ASA (70.5) suggesting a lower neutrophil infiltrate in the inflamed colon. Macroscopic damage of colon segments in colitis control was characterized by severe mucosal necrosis and ulceration with thickening of bowel wall accompanied by hyperemia, edema of submucosa, epithelial disruption, mucosal erosions with goblet cell depletion and a mixed inflammatory infiltrate containing polymorphonuclear leucocytes and lymphocytes. *In*

vivo treatment with 4AP and 4AT 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 4AP and 4AT. These results were found to be comparable with those obtained for sulfasalazine treated group. Histopathological features of rectally administered D-phenylalanine, L-tryptophan, 4-ASA + D-phenylalanine and 4-ASA + L-tryptophan groups also indicated correction of disrupted morphology of the colon. To evaluate and compare the safety profile of synthesized prodrugs with respect to 5-ASA-induced pancreatitis and sulfapyridine-induced hepatitis, the prodrugs were assessed for their probable damaging effects on pancreas and liver with the help of histopathological analysis. The histopathological sections of prodrug-treated rat livers and pancreas showed no adverse effects on liver or any signs of pancreatitis. From these particular findings, it can be concluded that the synthesized prodrugs have improved safety profile than 5-ASA or sulfasalazine. Statistical differences between the groups were calculated by One-way ANOVA followed by Dunnett's post hoc test. Differences were considered at a P value of <0.05 in relation to control.

The synthesized compounds were evaluated for ulcerogenic activity by Rainsford's cold stress method [38] and the ulcer index was determined [46,47] according to the method reported by Cioli et al. (Table 1). 4AP and 4AT showed remarkable reduction in the ulcer index 6.0 ± 2.74 and 8.4 ± 2.608, respectively, as compared to 4-ASA (69.61 ± 14.6). Their ulcer indices were comparable to sulfasalazine (5.83 ± 0.47). One-way ANOVA followed by Dunnett's post hoc test was used for calculation of statistical differences between the groups. All data were expressed as mean ± SD. Differences were considered at a P value of <0.01 in relation to control.

A comparative study was carried out between amide prodrugs of 4-ASA (4AP and 4AT) and azo prodrugs of 5-ASA with D-phenylalanine (SP) and L-tryptophan (ST) which were reported earlier by us [10,11] with respect to their therapeutic efficacy in TNBS-induced colitis. The comparative data revealed that amide prodrugs of 4-ASA with same carriers required more time for releasing 4-ASA by colonic hydrolysis ($t_{1/2}$ 6–7 h) than 5-ASA azo prodrugs to release 5-ASA by colonic reduction ($t_{1/2}$ 2 h) which could be accounted by the fact that amides are reported to be resistant to hydrolysis. Amongst the two amino acids, D-phenylalanine proved to be the better carrier, may it be an amide or an azo prodrug, as it showed better results than L-tryptophan with respect to synergistic mitigating effect on TNBS-induced colitis. Out of the four prodrugs, 4AP and SP (both having the carrier D-phenylalanine) showed comparable results to each other and sulfasalazine. ST and 4AT (both having the carrier L-tryptophan) showed slightly lower activity spectrum.

Table 1
Results of ulcerogenic activity

Compound	Dose (mg/kg)[a]	Ulcer index[b] ± SD
Healthy control	–	1.78 ± 0.60
5-ASA	1154.30	60.03 ± 1.15
4-ASA	1154.30	69.61 ± 1.46
Slz	3000	5.83 ± 0.47
4AP	2263.75	6.0 ± 2.74
4AT	2258.01	8.4 ± 2.608

5-ASA: 5-aminosalicylic acid; 4-ASA: 4-aminosalicylic acid; Slz: sulfasalazine; 4AP: amide prodrug of 4-ASA with D-phenylalanine; 4AT: amide prodrug of 4-ASA with L-tryptophan.

[a] Ten times the equimolar dose.

[b] Average of six readings; $P < 0.01$.

4. Conclusion

The amide prodrugs of 4-ASA demonstrated a significant ameliorating effect on TNBS-induced colitis in rats. The synergistic ameliorating effect of D-phenylalanine and L-tryptophan on disrupted colonic architecture strengthens the hypothesis of concept-based mutual prodrug design. The synthesized prodrugs have noticeably improved safety profiles than sulfasalazine, oral 4- and 5-aminosalicylic acid with similar pharmacological spectrum and advantages of sulfasalazine.

Future prospects. Additional work is in progress with respect to *in vivo* kinetic studies and synthesis of a series of novel mutual prodrugs of 4-ASA for their potential use in the management of IBD.

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