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European Journal of Medicinal Chemistry 42 (2007) 885–890
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
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Short communication

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

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Received 13 September 2006; accepted 30 November 2006
Available online 15 December 2006

Abstract

Mutual azo prodrug of 5-aminosalicylic acid with L-tyrosine was synthesized by coupling L-tyrosine with salicylic acid, for targeted drug delivery to the inflamed gut tissue in inflammatory bowel disease. The structure was confirmed by elemental analysis, IR and NMR spectroscopy. In vitro kinetic studies in rat fecal matter showed 87.18% release of 5-aminosalicylic acid with a half-life of 140.28 min, following first order kinetics. Therapeutic efficacy of the carrier system and the mitigating effect of the azo conjugate were evaluated in trinitrobenzenesulfonic acid-induced experimental colitis model. Myeloperoxidase activity was determined by the method of Krawisz et al. The synthesized prodrug was found to produce comparable mitigating effect as that of sulfasalazine on colitis in rats.

Keywords: Mutual azo prodrug; 5-Aminosalicylic acid; Inflammatory bowel disease; L-Tyrosine

1. Introduction

Inflammatory bowel disease (IBD) is characterized by chronic inflammation in the mucosal membrane of the small and/or large intestine. 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 anti-inflammatory 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. 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 abruptly released into proximal colon, which is considered as the optimum site for colon-targeted delivery of the drug [1].

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 [2]. But majority of side effects of sulfasalazine like hepatotoxicity, hypospermia and severe blood disorders

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doi:10.1016/j.ejmech.2006.11.017

are due to sulfapyridine. Even though few prodrugs of 5-ASA like balsalazide, ipsalazine and olsalazine [3–5] have been reported, none of them have reached beyond the stage of clinical trials. 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 the synthesis of azo conjugate of 5-ASA with L-tyrosine (TS) 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 to evaluate the therapeutic efficacy of this drug-carrier system in experimental colitis rat model. L-Tyrosine was chosen as a promoiety due to its marked anti-inflammatory activity [6]. 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.

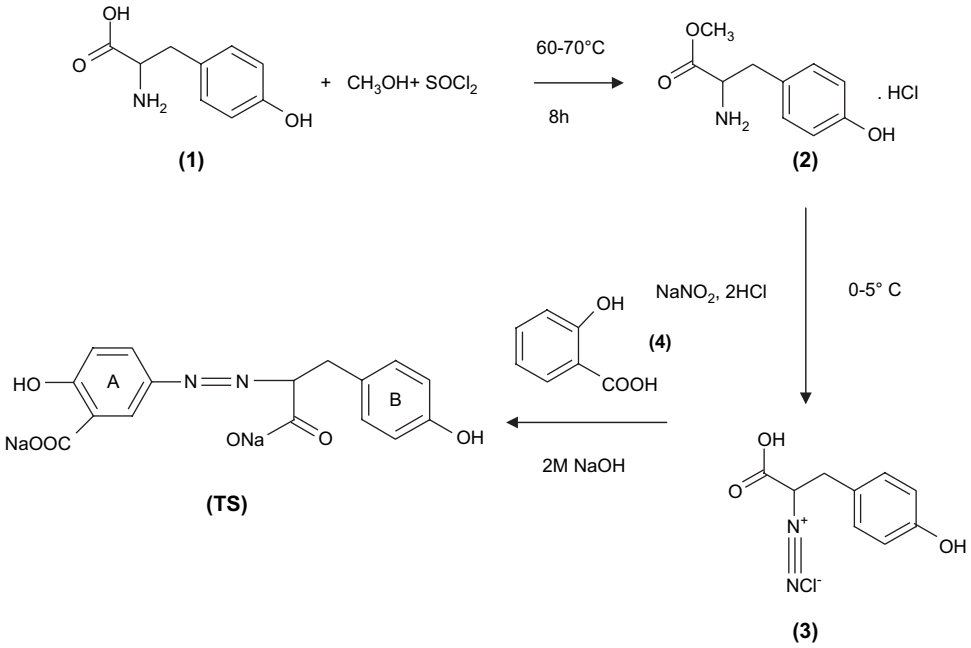
2. Chemistry

The synthesis of derivative is outlined in Scheme 1. Synthesis of methyl ester hydrochloride of L-tyrosine [13] was carried out by adding thionyl chloride to methanol followed by refluxing with L-tyrosine **1** at 60–70 °C for 7 h. L-Tyrosine methyl ester hydrochloride **2** was diazotised [14] at 0–5 °C in cryostatic bath. The coupling [14,15] of diazonium salt of L-tyrosine **3** with salicylic acid **4** was carried out at 0–5 °C in a cryostatic bath (Scheme 1). It was recrystallized by methanol followed by cooling at 0 °C. Purified product (TS) was dried under vacuum.

3. Biological investigations

The ulcerogenic activity was determined by Rainsford's cold stress method [8], which is an acute study model and is used to determine ulcerogenic potency of a drug at 10 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 (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 ± S.D.

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 [16]. By this model in vivo characterization of the azo carrier system under the influence of chronic inflammatory symptoms was possible. Wistar rats (average weight 200–230 g; 12–15 weeks; $n = 6$ per group) were used. They were distributed into six different groups i.e. healthy control, colitis control, two standard groups and



Scheme 1.

two test groups. They were housed in a room with controlled temperature (22 °C). The animals were 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 0.25 ml of TNBS (Himedia Laboratories Pvt. Ltd., Mumbai) in ethanol was injected into colon via rubber cannula (dose was 100 mg/kg of body weight of TNBS in 50% v/v ethanol). Animals were then maintained in a vertical position for 30 s and returned to their cages. For three 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-tyrosine and TS, 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 days study. Colitis activity was quantified with a clinical activity score assessing these parameters (Fig. 1) by clinical activity scoring rate. The clinical activity score [17] was determined by calculating the average of the above three parameters for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis). They were sacrificed 24 h after the last drug administration 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. 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 Kolte Pathology Laboratory, Pune. Colored microscopical images of the colon sections were taken on Zeiss optical microscope, Stemi 2000-C, with resolution 10×45 X, attached with trinocular camera.

Further myeloperoxidase (MPO) activity was determined by the method of Krawisz et al. [18]. MPO activity is inversely proportional to the ameliorating effect on disrupted colonic architecture. The intestinal tissue samples (approximately 50–100 mg) were homogenised on ice using a polytron (13,500 rpm, 1 min) in a solution of 0.5% hexadecyltrimethyl ammonium bromide (HTAB, Loba Chemie, Mumbai) in 50 mM potassium phosphate buffer (pH 6.0, 1 ml per 50 mg tissue). The resulting homogenate was subjected to three rapid freezing (70 °C) and thawing (immersion in warm water, 37 °C) cycles. The samples were then centrifuged (4000 rpm, 15 min, 4 °C) to remove insoluble material. The MPO containing supernatant (0.1 ml) was assayed spectrophotometrically after addition of 2.88 ml phosphate buffer (50 mM, pH 6.0) containing 0.167 mg/ml *o*-dianisidine hydrochloride (Himedia Laboratories Pvt. Ltd., Mumbai) and 0.0005% hydrogen peroxide. The kinetics of absorbance changes at 470 nm was measured. Sample enzyme activity was calculated with a standard curve of known MPO unit activity. One unit of MPO activity, defined as the quantity of enzyme able to convert 1 μmol of hydrogen peroxide to water in 1 min at room temperature, was expressed in mU/100 mg of tissue.

For determination of antiarthritic activity, Freund's adjuvant-induced arthritis model was used [19]. Wistar rats of either sex weighing 150–200 g were divided into three groups viz. arthritic control, standard and test containing six animals each. On day one, 0.1 ml of complete Freund's adjuvant (F-5881, Sigma–Aldrich Corporation, USA) was injected into the subplanar region of hind paw of rats. The animals were housed in cages to allow the development of full arthritis up to 13 days. The paw volumes were measured on 5th and 13th day using UGO BASILE Plethysmometer 7140, Italy. On 13th day the drug administration was started and continued up to 21st day. Animals of the standard groups received sulfasalazine and 5-ASA, respectively, while test group received TS. All the doses were calculated on equimolar basis of 5-ASA present in sulfasalazine. The arthritic control group received 1% carboxymethylcellulose only. Finally, paw volume was again measured on 21st day.

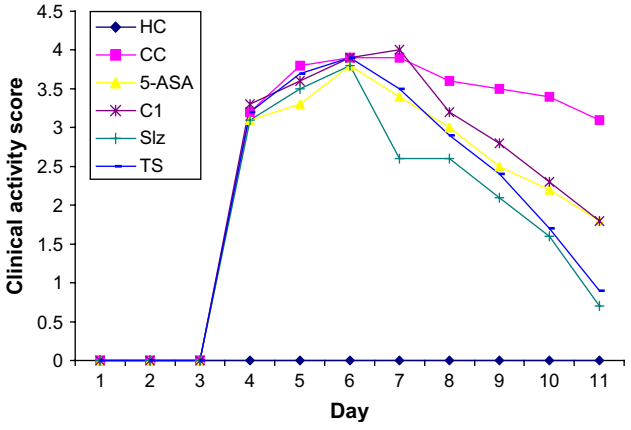


Fig. 1. Clinical activity score rate. HC: healthy control, CC: colitis control, 5-ASA: 5-aminosalicylic acid, C1: carrier (L-tyrosine), Slz: sulfasalazine, TS: prodrug of 5-ASA with L-tyrosine.

4. Results and discussion

The melting point of TS was found to be 283–285 °C (uncorrected). All the results of elemental analysis were in an acceptable error range.

The IR spectra of TS showed characteristic peak at 1494 cm^{-1} of N=N stretching (unsymmetric *p*-substituted azobenzene) which confirms the formation of azo bond. A broad peak of unbonded phenolic OH stretching at 3558–3219 cm^{-1} was also found. It also showed carboxylate anion stretching at 1593 and 1390 cm^{-1} and C–N stretching at 1095 cm^{-1}.

^{1}H NMR spectra of TS showed chemical shifts for protons of Ring A, aromatic OH at δ 5.80 [s, 1H] and Ring B, aromatic OH at 5.83 [s, 1H]; Ring A, CH-benzene at δ 7.74 [s, 1H], δ 7.55 [d, 1H] and δ 7.53 [d, 1H] and Ring B, CH-benzene at δ 6.48 [d, 1H] and δ 7.01 [d, 1H]. The signals of CH (methine) at δ 2.66 [t, 1H] and CH_2 (methylene) at δ 2.93 [d, 2H] were also found.

The aqueous solubility was found to be 0.26 g/ml and partition coefficient in *n*-octanol/phosphate buffer (pH 7.4) was found to be 0.35, 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 264 nm and phosphate buffer (pH 7.4) at 272 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) only 15.17% 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 [7] to confirm the colonic reduction of azo prodrug. $t_{½}$ (average of four trails) of TS was found to be 140.28 min whereas rate constant (K) was found to be $4.94 \times 10^{-3} \pm 0.0001$. Over a period of 7 h, TS gave 87.18% cumulated release of 5-ASA following first order kinetics (Fig. 2). Thus in vitro kinetic studies confirmed that the synthesized conjugate did not release 5-ASA at all in HCl buffer (pH 1.2) but released it in a very negligible extent in phosphate buffer (pH 7.4), whereas the release in rat fecal matter was almost complete.

The synthesized compound was evaluated for ulcerogenic activity by Rainsford's method [8] and the ulcer index was determined that has been shown in Table 1 [9]. The conjugate showed remarkable reduction in the ulcer index (10.76 ± 0.55) as compared to its parent drug (60.03 ± 1.15). This reduction in the ulcer index brought about by the conjugate was comparable to that produced by sulfasalazine (5.83 ± 0.47). Statistical differences between the groups were calculated by One-Way ANOVA followed by Dunnett's post hoc test. All data are expressed as mean ± 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 [10–12]. After inducing the experimental colitis, the clinical activity score increased rapidly and consistently for the next three 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 seven. A significant lowering of clinical activity was shown by TS (0.9 ± 0.13), which was comparable to sulfasalazine (0.7 ± 0.22) but distinctly more than 5-ASA (1.8 ± 0.07). The positive contribution of L-tyrosine towards lowering effect on clinical activity score (1.8 ± 0.06) is obvious from the gross difference in lowering effect of plain 5-ASA and TS. 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).

Table 1
Results of ulcerogenic activity

Compound	Dose (mg/kg)[a]	Ulcer index ± S.D.[b]
HC	–	1.78 ± 0.60
5-ASA	2290	60.03 ± 1.15
Slz	3000	5.83 ± 0.47
TS	3050	10.76 ± 0.55

HC: healthy control, 5-ASA: 5-aminosalicylic acid, Slz: sulfasalazine, TS: prodrug of 5-ASA with L-tyrosine.
[a] Ten times the normal dose.
[b] Average of six readings.

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 hemorrhagic ulcerations and histopathological features included transmural necrosis, edema, absence of epithelium, a massive mucosal/submucosal infiltration of inflammatory cells. In vivo treatment with TS 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 TS (Fig. 4f). These results were found to be comparable with those obtained for sulfasalazine treated group. 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.

Myeloperoxidase (MPO) activity, which is an important quantitative index for colonic inflammation was determined

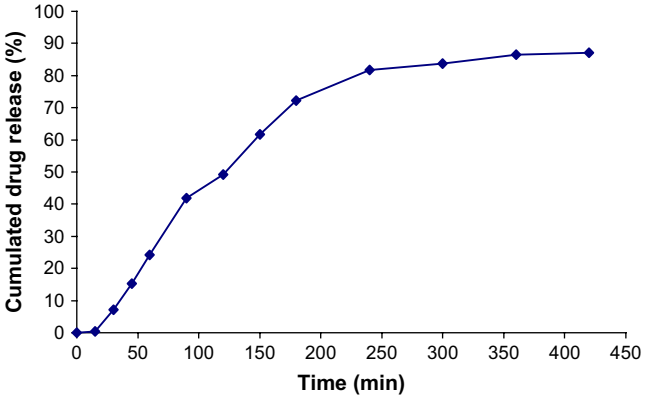


Fig. 2. Release profile of 5-ASA from TS in rat fecal matter.

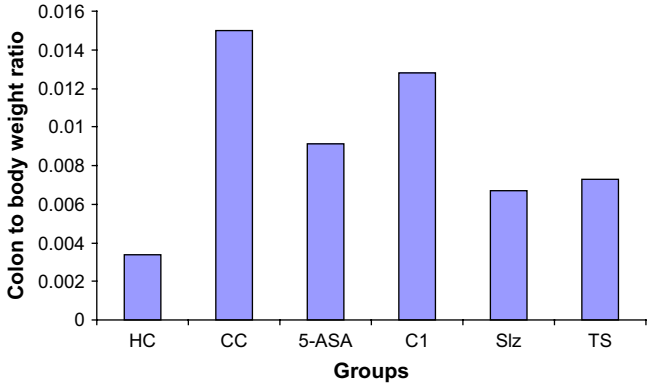


Fig. 3. Colon to body weight ratio. HC: healthy control, CC: colitis control, 5-ASA: 5-aminosalicylic acid, C1: carrier (L-tyrosine), Slz: sulfasalazine, TS: prodrug of 5-ASA with L-tyrosine.

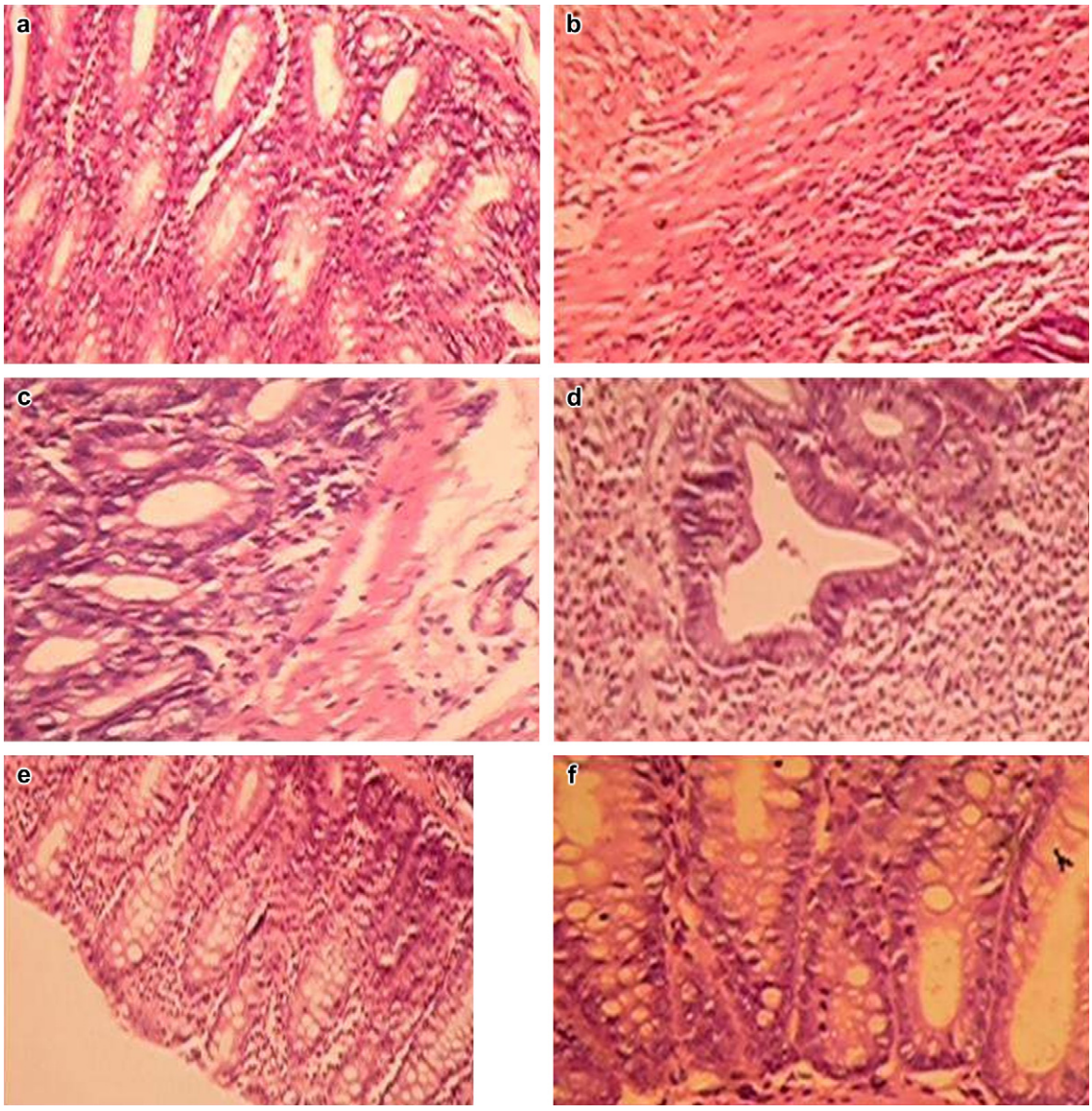


Fig. 4. Histology of colon of rats subjected to TNBS: (a) healthy control; (b) colitis control showing mucosal injury characterized by absence of epithelium and a massive mucosal/submucosal infiltration of inflammatory cells; (c) 5-ASA; (d) L-tyrosine, both showing slight mucosal abscess and inflammatory infiltrate. (e) Sulfasalazine; (f) TS showing corrected morphology of colon with comparable results to that of sulfasalazine.

in terms of mU/100 mg tissue. MPO activity for TS was found to be 53.51 mU/100 mg tissue, which is comparable to sulfasalazine (46.63 mU/100 mg tissue), but much less than plain 5-ASA (60.85 mU/100 mg tissue). The results are depicted in Fig. 5. Finally, antiarthritic activity was determined in Freund's adjuvant-induced arthritis, where sulfasalazine was taken as the standard disease modifying anti-rheumatoid drug. Percent inhibition of arthritis using TS was found to be 62%, which is slightly less than sulfasalazine (73%), while plain 5-ASA showed only 47% inhibition of arthritis (Table 2).

5. Conclusion

The data generated as an outcome of this work demonstrates that this new prodrug has a remarkable ameliorating effect on the disruption of colonic architecture and suppresses

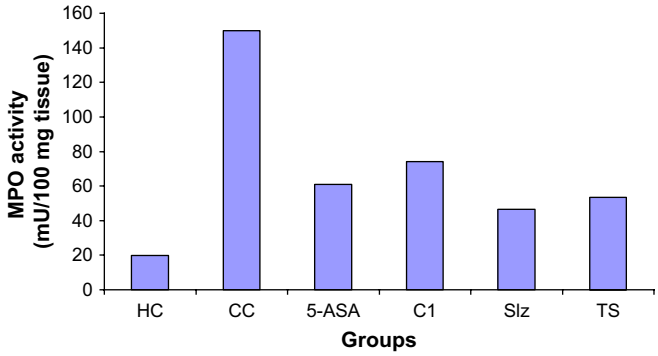


Fig. 5. Myeloperoxidase activity. HC: healthy control, CC: colitis control, 5-ASA: 5-aminosalicylic acid, C1: carrier (L-tyrosine), Slz: sulfasalazine, TS: prodrug of 5-ASA with L-tyrosine.

Table 2
Antiarthritic activity

Compounds	Paw volume				Difference in paw volume[a]		% Inhibition	
	1 day	5 day	13 day	21 day	13 day	21 day	13 day	21 day
Arthritic control	2.27 ± 0.03	2.53 ± 0.11	3.12 ± 0.05	3.58 ± 0.06	0.85 ± 0.03	1.31 ± 0.02	–	–
5-ASA	2.27 ± 0.02	2.94 ± 0.05	3.02 ± 0.04	2.96 ± 0.03	0.75 ± 0.04	0.69 ± 0.02	12	47
Slz	2.17 ± 0.03	2.64 ± 0.03	2.87 ± 0.06	2.53 ± 0.02	0.70 ± 0.04	0.36 ± 0.05	18	73
TS	2.23 ± 0.02	2.79 ± 0.04	2.97 ± 0.03	2.72 ± 0.04	0.74 ± 0.02	0.49 ± 0.06	13	62

[a] Average of six readings.

the course of TNBS-induced colitis effectively. The criteria for the selection of L-tyrosine as carrier has also proven correct, as it has effectively delivered 5-ASA to colon.

6. Experimental protocols

^{1}H NMR spectra of the synthesized compound were recorded in DMSO using ^{1}H NMR Varian Mercury 300 Hz with super conducting magnet using TMS as internal standard at Dept. of Chemistry, University of Pune, Pune. Chemical shift values are reported in parts per million downfield on δ scale. The IR spectra of the synthesized compound were recorded on JASCO, V-530 FTIR in potassium bromide (anhydrous IR grade). The absorbance maxima (λ_{max}) of synthesized compounds were determined on JASCO V-530, UV–vis 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 was 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 L-tyrosine 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.

Acknowledgement

Authors thank the AICTE for providing financial assistance and to Wallace Pharmaceutical Pvt. Ltd., Goa, for providing the gift sample of sulfasalazine.

References

[1] M.K. Chourasia, S.K. Jain, J. Pharm. Sci. 6 (2003) 33.
[2] B.E. Sands, Gastroenterology 118 (2000) S68.
[3] L.R. Sutherland, D.E. Rothy, P.L. Beck, Inflamm. Bowel Dis. 3 (1997) 65.
[4] N. Mahmud, M.A. Kamm, J.L. Dupas, Gut 49 (2001) 552.
[5] S.S.C. Rao, N.W. Read, C.D. Holdsworth, Gut 28 (1987) 1474.
[6] K. Takagi, S. Okabe, US Patent 3,988 (1976) 466.
[7] A. Rubinstein, D. Nakar, A. Sintov, Pharm. Res. 9 (1992) 276.
[8] K.D. Rainsford, Proc. Br. Pharm. Soc. (1980) 226P.
[9] K.D. Rainsford, Agents Actions 5 (1975) 553.
[10] G.P. Morris, P.L. Beck, M.S. Herridge, W.T. Drew, Gastroenterology 96 (1989) 795.
[11] B. Zingarelli, F. Squadrito, P. Graziani, R. Camerini, A.P. Caputi, Agents Actions 39 (1993) 150.
[12] T. Li, J. World, Gasteroenterology 9 (2003) 1028.
[13] Dhaneshwar Suneela, S.C. Chaturvedi, Indian Drugs 31 (1994) 374.
[14] B.S. Furniss, A.J. Hannaford, P.W.G. Smith, A.R. Tatchell, Vogel's Textbook of Practical Organic Chemistry, in: A.I. Vogel (Ed.), fourth ed. The English Language Bookman, London, 1978, pp. 688–690.
[15] J. March, in: Advanced Organic Chemistry – Reactions, Mechanisms and Structure, third ed. Wiley Eastern Ltd., 1986, pp. 355–356.
[16] T. Yamada, S. Marshall, R.D. Specian, M.B. Grisham, Gastroenterology 102 (1992) 1524.
[17] G. Hartmann, C. Bidlingamaier, B. Seigmund, S. Albrich, J. Schulze, K. Tschoep, A. Eigler, H.A. Lehr, S. Endres, J. Pharmacol. Exp. Ther. 292 (2000) 22.
[18] J.E. Krawisz, P. Sharon, W.F. Stenson, Gastroenterology 87 (1984) 1344.
[19] A.S. Hunjra, K. Dharmendra, N. Gairola, M. Bhojani, S.S. Dhaneshwar, Indian J. Pharm. Edu. Res. 40 (2006) 40.