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## EXPLORING NOVEL COLON-TARGETING ANTIHISTAMINIC PRODRUG FOR COLITIS

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Present work was inspired by an interesting finding of Raithel *et al.* (11) about remission of steroid-dependent, chronically active ulcerative colitis (UC) in a patient, after treatment with a combination of fexofenadine, disodium cromoglycate and an amino acid-based formula. Literature reports involvement of mast cells activation and increased histamine secretion in the pathogenesis of colitis. The purpose of present work was to evaluate the potential of a novel prodrug of fexofenadine in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in rats. A colon-specific mutual amide prodrug of fexofenadine with D-glucosamine was synthesized. Release was studied in tissue homogenates and rat fecal matter by HPLC. It was further screened in TNBS-induced colitis in rats and also for adverse effects on rat liver, stomach and pancreas. The spectral analysis confirmed the structure of the prodrug. Highly hydrophilic prodrug enabled efficient delivery of fexofenadine to colon. Prodrug furnished negligible release of fexofenadine in upper gastrointestinal tract (GIT) homogenates. About 82% release of fexofenadine was observed in rat fecal matter at the end of 12 hours. The prodrug was twice as effective in lowering the quantifying parameters of colonic inflammation in TNBS- induced colitis than fexofenadine, D-glucosamine, their physical mixture and interestingly oral 5-amino salicylic acid while 2.7 times less effective than sulfasalazine. The prodrug restored disrupted colonic architecture to normal without adversely affecting stomach, liver and pancreas. In conclusion, the results support histamine involvement in the pathogenesis of UC. This novel, dual acting colon-specific prodrug of fexofenadine is promising as combination maintenance therapy with sulfasalazine for UC.

**Key words:** *ulcerative colitis, antihistaminics, colon-targeting prodrug, D-glucosamine, fexofenadine*

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### INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic, recurring and auto-inflammatory intestinal diseases which is divided into two major distinctive entities as ulcerative colitis (UC) and Crohn's disease (CD). IBD is characterized by massive cellular infiltrates due to immunological abnormalities showing increasing numbers of CD4<sup>+</sup> T lymphocytes, mast cells, neutrophils and eosinophils (1). Several immunological, environmental, and genetic factors are believed to be involved in the etiology of IBD (2). Mast cells are innate immune cells that can potentially contribute to IBD through their pro-inflammatory activity and/or effects on immunoregulation (3). Upon activation, mast cells can immediately release large amounts of pro-inflammatory cytokines and can continue to synthesize and release a wide range of pro-inflammatory mediators *de novo* (4). Mast cell-derived mediators can contribute to colitis severity by enhancing neutrophil influx and thus prolonging the ongoing inflammation (5). As mast cells are located adjacent to the intestinal epithelium, their activation may affect the function of the mucosal barrier also (4). A variety of mediators like histamine, prostaglandin D<sub>2</sub>, leukotriene C<sub>4</sub>, platelet activating factor, heparin and neutral proteases are released during mast cell activation and degranulation. These

cells have been implicated in the etiology of inflammatory diseases (6-8), including IBD and its main clinical manifestations such as UC (9) and CD (10).

An interesting report by Raithel *et al.* (11), about induction of remission in a patient with steroid-dependent, chronically active ulcerative colitis, after treatment with a combination of fexofenadine, disodium cromoglycate and an amino acid-based formula, inspired us to design a colon- specific prodrug of fexofenadine. The findings of study by Raithel *et al.* suggest that therapy of UC which does not respond to routine treatment, should focus on an antihistaminic strategy, as pathogenesis of UC involves activated mast cells and elevated levels of histamine because of which a continuous inflammatory activity is observed in the mucosa in spite of steroid therapy. This study also indicated a rapid decrease in serum levels of leukocytes, C-reactive protein and orosomucoid along with lowered clinical disease activity and stool frequency on treatment with above combination formula. Fexofenadine has also been suggested as a good treatment option for ischemic colitis where inadequate blood supply causes inflammation and injury to the large intestine. Unlike other antihistaminics like terfenadine, fexofenadine being more hydrophilic does not cross blood brain barrier causing comparatively less drowsiness (12).

A mutual or chimeric prodrug design was adopted for synthesizing a colon-targeting prodrug of fexofenadine and D-glucosamine was selected as the biologically active moiety. Fexofenadine was chemically linked with D-glucosamine through an amide linkage. Upper gastro-intestinal tract has various peptidases like pepsin, trypsin, chymotrypsin, endopeptidases and carboxypeptidases but N-acyl amidases are those amidases which are found only in colon as they are secreted by colonic microflora, which catalyze hydrolysis of N-acyl linkages formed with amino acids/aminosugars (13, 14). So we hypothesized that this prodrug would be hydrolyzed in colon releasing fexofenadine and D-glucosamine for their local action on the inflamed colon.

We have reported the utility of D-glucosamine; an anti-inflammatory nutraceutical amino sugar; as a promising carrier for targeted delivery of aminosalicylates to colon (15). N-acetyl glucosamine has been implicated to play a significant role in biosynthesis of glucosaminoglycans (GAGs) and intestinal mucus, required for integrity and protection of the gut wall (16, 17). We envisaged that glucosamine supplementation in combination with fexofenadine might synergistically protect deteriorated mucosal linings in UC. Moreover polyhydroxy nature of D-glucosamine would increase hydrophilicity of fexofenadine to such an extent that absorption of the prodrug as a whole in upper GIT would be minimized assuring delivery of intact prodrug to the site of action.

The present work was aimed at rational design and pharmacological screening of colon-targeting, dual acting prodrug (FG1) of fexofenadine with D-glucosamine in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in Wistar rats. An attempt was made to compare the efficacy of this novel antihistaminic approach with the classical aminosalicylate approach in the management of UC.

## MATERIALS AND METHODS

All the experimental procedures and protocols used for pharmacological screening were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of Poona College of Pharmacy, Pune and were in accordance with the guidelines of the Committee for the purpose of Control and Supervision of Experiment on Animals (CPCSEA), Government of India.

### Animals

Male Wistar rats (average weight 200–230 g; 12–15 weeks; n=6/group) were used. They were distributed into 9 different groups *i.e.* healthy control, colitis control, six standard groups and one test group. The animals used for the study were housed under standard environmental conditions of temperature  $23 \pm 1^\circ\text{C}$  and relative humidity of  $50 \pm 5\%$ . A 12 h light/dark cycle was followed. All animals had free access to water and standard pelleted laboratory animal diet. The animals were food fasted 48 hours before experimentation and allowed food and water *ad libitum* after the administration of TNBS.

### Materials

2,4,6-trinitrobenzene sulfonic acid (TNBS) was purchased from Sigma-Aldrich Corporation, USA; fexofenadine hydrochloride was obtained as a gift sample from Dr. Reddy's Laboratories, Hyderabad, India while D-glucosamine hydrochloride was purchased from Himedia Pvt. Ltd. Mumbai, India. Sulfasalazine (SLZ) was gifted by Wallace Pharmaceutical Pvt. Ltd., Goa. 5-aminosalicylic acid (5-ASA) was purchased from Himedia Chemicals Ltd., Mumbai, India. All other

chemicals used in the synthesis were of A.R. grade. The melting point of the prodrug was determined by open capillary method and is uncorrected. Pre-coated silica gel plates - 60 F<sub>264</sub> (Merck) were used for monitoring the reactions and checking the purity of the synthesized compound by thin layer chromatography. Ultraviolet light and iodine vapors were used as detecting agents. The  $\lambda_{\text{max}}$  of synthesized prodrug was determined on JASCO V530, UV-Visible double-beam spectrophotometer in various solvents like, methanol, distilled water, hydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4).

The IR spectrum of synthesized compound was recorded on JASCO, V-530 FTIR in potassium bromide (anhydrous I.R. grade) pellets. The <sup>1</sup>H-NMR spectrum was recorded in DMSO-*d*<sub>6</sub> while <sup>13</sup>C-NMR was recorded in CDCl<sub>3</sub> using <sup>1</sup>H-NMR Varian Mercury 300 MHz with super conducting magnet at the Department of Chemistry, University of Pune, Pune.

For *in vitro* kinetic studies, a new HPLC method was developed for simultaneous estimation of FG1 in presence of its metabolites fexofenadine and glucosamine that might be released after its possible activation. The HPLC system used for this purpose consisted of a pump (Jasco LC Net II/ ADC, Serial no: B224461095), with sampler programmed at 20  $\mu\text{l}$  capacity per injection and a UV/VIS detector (Jasco UV 2075). Data was integrated using Jasco Borwin version 1.5. The column used was HiQ Sil C<sub>18</sub>HS (4.6 mm I.D.  $\times$  250 mm; Batch: #080253; Column Number: 0HS00422) in the reversed phase partition chromatographic condition. The system was used in an air-conditioned HPLC laboratory atmosphere ( $20 \pm 1^\circ\text{C}$ ). Before analysis, the mobile phase was degassed using sonicator and filtered through a 0.45  $\mu\text{m}$  membrane filter. Sample solutions were also filtered through the same. The system was equilibrated before making an injection. The column was monitored for UV absorbance at a detection wavelength 220 nm for estimation of FG1. All the kinetic studies were carried out in triplicate. The K values from the plots were calculated separately and average K and S.D. value was determined.

### Synthesis of amide prodrug of fexofenadine with D-glucosamine (Fig. 1)

To a solution of fexofenadine [1] (1 g; 0.0019 M) in DMF (50 ml), DCC (0.431 g; 0.00209 M) was added with stirring at  $0^\circ\text{C}$  for 3 hours. Then, D-glucosamine [2] (0.374 g, 0.00209 M) was added to the reaction mixture and stirred mechanically at  $0^\circ\text{C}$  for 12 hours and then at room temperature for 48 hours. After filtration, the filtrate was evaporated under reduced pressure to remove the solvent. The residue thus obtained was purified by preparative TLC using ethyl acetate: methanol: glacial acetic acid (3:1.5:3 drops) to obtain the final prodrug FG1 [3].

FG1 (prodrug of fexofenadine with D-glucosamine): (2-(4-{1-hydroxy-4-[4-(hydroxy-diphenyl- methyl)-piperidin-1-yl]-butyl}-phenyl) N(2,4,5-trihydroxy-6-hydroxymethyl- tetrahydropyran-3-yl)-isobutyramide) m.p.  $120^\circ\text{C}$  (d) (uncorrected), yield: 40%, *R*<sub>f</sub>: 0.90 ethyl acetate: methanol: glacial acetic acid (3:1.5:3 drops), Log P: 0.046 (n-octanol: phosphate buffer pH 7.4),  $\lambda_{\text{max}}$ : 220 nm (distilled water), 221 nm (methanol), 220 nm (HCl buffer pH 1.2), 223 nm (phosphate buffer pH 7.4), IR (KBr;  $\text{cm}^{-1}$ ): 3472; primary and secondary OH stretching, 3300; NH stretching secondary amide, 3200; C-H stretching aromatic, 3057; CH<sub>3</sub> stretching aliphatic, 2930, 2054; C-H stretching aliphatic, 1644; C=O stretching secondary amide, 1590, 1506, 1448; C=C stretching aromatic. <sup>1</sup>H-NMR (DMSO-*d*<sub>6</sub>; ppm):  $\delta$  9.37 (bs, 1H) NH-amide,  $\delta$  7.13-7.50 (m, 14H) aromatic C-H,  $\delta$  5.65 (s, 1H) benzylic OH,  $\delta$  5.32 (d, 1H) C<sub>1</sub>-H of tetrahydropyran,  $\delta$  4.52 (t, 1H) C-H-methine,  $\delta$  3.34-3.60 (m, 7H) C-H tetrahydropyran, CH<sub>2</sub> methylene, sec. alcoholic OH,  $\delta$  3.03-3.2 (m, 4H) CH<sub>2</sub>-piperidine,  $\delta$  2.75 (s, 1H) CH-piperidine,  $\delta$  2.5 (t, 2H) CH<sub>2</sub> methylene,  $\delta$  1.6-

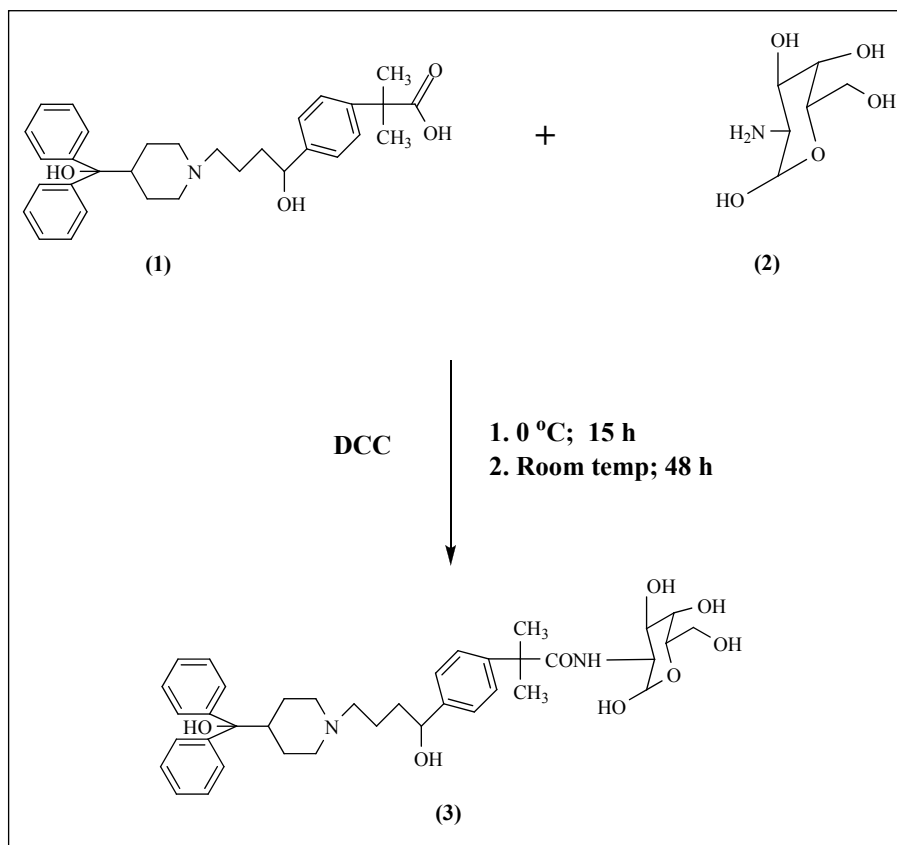


Fig. 1. Scheme of synthesis of amide prodrug of fexofenadine with D-glucosamine

1.8 (m, 2H) CH<sub>2</sub> methylene,  $\delta$  1.518 (s, 4H) OH-tetrahydropyran,  $\delta$  1.05-1.25 (m, 6H) 2  $\times$  CH<sub>2</sub> piperidine, CH<sub>2</sub> methylene. D<sub>2</sub>O exchange NMR: diminished signals for exchangeable protons of tetrahydropyran ( $\delta$  1.518/1.54). <sup>13</sup>C-NMR(CDCl<sub>3</sub>; ppm): fexofenadine backbone: 175.45 NHC=O, 147.59, 144.68, 144.02, 131.61, 128.21, 128.15, 126.14. Ring A,B,C 18  $\times$  CH aromatic, 78.84 -C-OH, 72.21 -CH-OH, 58.14 -N-CH<sub>2</sub>, 55.86, 53.73, 43.60, 37.86, 5  $\times$  CH<sub>2</sub> piperidine, CH<sub>2</sub> butyl, 48.06 CH<sub>3</sub>-C-CH<sub>3</sub>(OH), 39.92, 2  $\times$  CH<sub>3</sub>, 21.5 CH<sub>2</sub> butyl, glucosamine backbone: 125.31, 72.21, 71.5, 68.12, 58.14, 53.61, 5  $\times$  CH-tetrahydropyran (18, 19).

#### *In vitro release kinetics of FG1 in stomach homogenates of rat*

Four Wistar rats were anesthetized by ether and sacrificed and midline incisions were made. Sections of stomach were collected separately, washed to remove their contents, homogenized using Remi overhead homogenizer and diluted to half concentration with isotonic hydrochloric acid buffer (pH 1.2). FG1 (12.5 mg) was dissolved in HCl buffer (pH 1.2) and volume was made up to 10 ml (1250  $\mu$ g/ml). 5 ml of this solution was added to 10 ml volumetric flask and volume was made up to 10 ml with HCl buffer (pH 1.2) (625  $\mu$ g/ml). This was considered as the stock solution. To each eppendorf tube (1 ml capacity), 0.8 ml of the stock solution of prodrug and 0.2 ml of stomach homogenate was added and kept in incubator at 37 $\pm$ 1°C. The first eppendorf tube (0 min) was taken out, centrifuged at 5,000 rpm at 4°C for 10 min. This drug-homogenate solution (0.1 ml) was taken in fresh eppendorf tube and 0.9 ml of methanol was added to it with help of micropipette and centrifuged again at 5,000 rpm and 4°C for 10 min. The sample (20  $\mu$ L) was injected in the C<sub>18</sub> column and eluted with the mobile phase methanol: ammonium acetate buffer (pH 4 adjusted with glacial acetic acid) (25:75 v/v) at a flow rate of 1 ml/min and elute was monitored at wavelength

of 220 nm and chromatograms of all the components were taken by measuring the absorption with a sensitivity of AUFS 0.01. Similarly appropriate eppendorf tubes were taken out of incubator and subjected to same treatment as mentioned above at particular time intervals till 3 hours (20).

#### *In vitro release kinetics of FG1 in intestinal homogenates of rat*

Four Wistar rats were anesthetized by ether and sacrificed and midline incision was made. Sections of small intestine were collected separately, washed to remove their contents, homogenized using Remi overhead homogenizer and diluted to half concentration with isotonic phosphate buffer (pH 7.4). Same procedure was applied for sample preparation and subsequent treatment as mentioned above. The release was studied over a period of 6 hours (20).

#### *Release studies in fecal matter*

Fresh rat fecal matter was collected from animals kept in metabolic cages. FG1 (12.5 mg) was dissolved in phosphate buffer (pH 7.4) and volume was made up to 10 ml (1250  $\mu$ g/ml). 5 ml of this solution was added to 10 ml volumetric flask and volume was made up to 10 ml with isotonic phosphate buffer (pH 7.4) (625  $\mu$ g/ml). This was considered as the stock solution. To each eppendorf tube, 0.9 ml of the stock solution of prodrug and 0.1 ml of fecal matter was added and kept in incubator at 37 $\pm$ 1°C in anaerobic conditions (5% CO<sub>2</sub>). The first eppendorf tube (0 min) was taken out, centrifuged at 10,000 rpm at 4°C for 20 min. This sample (20  $\mu$ L) was injected in the column and eluted with the mobile phase methanol: ammonium acetate buffer (pH 4 adjusted with glacial acetic acid) (25:75 v/v) at a flow rate of 1 ml/min and elute was monitored at wavelength of 220 nm. Similarly appropriate eppendorf tubes of that particular

time interval were taken out of incubator and subjected to same treatment as mentioned above. The release was observed over a period of 12 hours (20).

### Pharmacological evaluation

#### 1. Induction of colitis

To induce an inflammation, all the groups except healthy control group were treated by the following procedure: after light narcotizing with ether, the rats were catheterized 8 cm intra-rectal and 0.25 ml of TNBS in ethanol was injected into colon *via* rubber cannula (dose of TNBS was 100 mg/kg of body weight in 50% v/v ethanol 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 received orally fexofenadine, D-glucosamine, physical mixture of fexofenadine and D-glucosamine, sulphasalazine and 5-aminosalicylic acid (5-ASA) oral, 5-ASA rectal and FG1 respectively, once daily for five continuous days. The healthy control and colitis control groups were given only saline instead of free drug or prodrug (21).

#### Doses

Healthy control: saline, colitis control: 2,4,6-trinitrobenzene sulfonic acid (TNBS) 100 mg/kg, fexofenadine: 25 mg/kg, D-glucosamine: 0.9 mg/kg, sulfasalazine (SLZ): 66.5 mg/kg, physical mixture of fexofenadine and D-glucosamine: 25+0.9 mg/kg, prodrug FG1: 33 mg/kg (equimolar basis to dose of fexofenadine), 5-aminosalicylic acid (5-ASA) (oral): 25.5 mg/kg, 5-aminosalicylic acid (5-ASA) (rectal): 25.5 mg/kg.

#### TNBS-induced experimental colitis in rats

Protective effect of FG1 on inflamed rat colon was evaluated in TNBS-induced experimental colitis as per protocols described by Yamada *et al.* (21). Dose of FG1 was calculated on equimolar basis to fexofenadine. Standard parameters like disease activity score, colon to bodyweight ratio, myeloperoxidase activity were assessed in the 11 day study model. Histopathology of inflamed rat colons was also performed. Effect of FG1 on rat liver, pancreas and stomach was studied for its safety evaluation and compared with plain fexofenadine.

#### Assessment of colonic damage by disease activity score and colon to body weight ratio

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 disease activity score assessing these parameters as previously applied by Hartmann *et al.* (22) (Table 1). The disease activity score was determined by calculating the average of the above three parameters for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis). They were

sacrificed 24 hours after the last drug administration by isoflurane anesthesia and a segment of distal colon 8 cm long was excised and colon/ body weight ratio was determined to quantify the inflammation. The dissected colon was used for myeloperoxidase assay and tissue segments (1 cm) were then fixed in 10% buffered formalin for histopathological studies.

#### Quantitative assessment of colonic damage by determination of myeloperoxidase activity

The histological feature of colitis is marked by the presence of inflammatory cells; neutrophils, lymphocytes and histiocytes. The more acute the illness, the prominent the neutrophil component of the inflammatory infiltrate. The determination of myeloperoxidase activity in the intestine is a simple biochemical assay that can be used to quantitate inflammation. The activity of intestinal myeloperoxidase (MPO), was measured using the method of Krawisz *et al.* <http://gut.bmjournals.com/cgi/content/full/43/6/783> - B20 with minor modifications (23, 24).

Briefly, intestinal tissue samples (approximately 50–100 mg) were homogenized on ice using a polytron (13,500 rpm, one minute) in a solution of 0.5% HTAB in 50 mM potassium phosphate buffer (HTAB, 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 (4,000 rpm, 15 minutes, 4°C) to remove insoluble material. The MPO containing supernatant (0.1 ml) was assayed spectrophotometrically after addition of 2.9 ml phosphate buffer (50 mM, pH 6.0) containing 0.17 mg/ml *o*-dianisidine hydrochloride and 10 µl of 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 one minute at room temperature, was expressed in mU/100 mg of tissue.

#### Histopathological analysis

Histopathological studies of the stomach, colon, liver and pancreas were carried out at Satyam Pathology Laboratory, Pune. The pathologist was unaware of the experimental protocols. The histopathological sections were stained with haematoxylin and eosin. Colored microscopical images of the sections were taken on the Nikon optical microscope, Eclipse E-200, with resolution 10×40X, attached with trinocular camera at Poona College of Pharmacy, Pune.

#### Statistical analysis

All data are expressed as mean ± S.E.M.; n refers to number of animals in each group. Statistical differences between groups were calculated by One-Way ANOVA followed by the Dunnett's post hoc test. Differences were considered at a P value of <0.001–0.05.

Table 1. Scoring rate of disease activity (Hartmann *et al.*, 2000).

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

Table 2. *In vitro* release kinetics data of FG1.

S. N.	Medium	Order of kinetics	$t_{1/2}$ (min <sup>-1</sup> )	K(sec <sup>-1</sup> )*	% Release of fexofenadine
1.	Stomach homogenate	--	–	–	Negligible
2	Intestinal homogenate	1 <sup>st</sup>	726	0.00026 ± 0.00001	19.5% at the end of 6 h
3	Fecal matter	1 <sup>st</sup>	260	0.00051 ± 0.000026	82 % at the end of 12 h

\*Average of three readings ±S.D.

Table 3. Histopathology of rat colon.

Group	Gross examination	Microscopic examination
HC	Normal	Within normal limit
CC	Congested, dilated & ulcerated mucosa	Shows ulcerations, lymphocytic infiltrate
F	Normal	Within normal limit
G	Subnormal	Large lymphoid collection
SLZ	Normal	Within normal limit
F+G	Appears congested	Shows ulcerated mucosa
FG1	Normal	Shows mild lymphocytic infiltrate
5- ASA (oral)	Congested	Shows mucosal distortion, inflammation
5- ASA (rectal)	Normal	Shows no mucosal distortion, very less inflammation

## RESULTS

### TNBS-induced colitis

#### Partition coefficient

The success of a well designed colon-specific prodrug depends on how much hydrophilicity has been imparted by the covalent linkage of the carrier to the parent drug so as to restrict the trans-membrane passage of the prodrug through upper GIT. This ensures that maximum amount of orally administered prodrug reaches colon, bypassing its absorption in upper GIT. Therefore partition coefficient of the prodrug was experimentally determined in terms of log P and was found to be 0.046, which was 109 folds lower than fexofenadine (log P: 5).

#### Spectral analysis

The IR spectrum of the synthesized compound showed absorption bands at 1644 and 3300 cm<sup>-1</sup>, for carbonyl stretching and NH stretching of secondary amide respectively. The <sup>1</sup>H-NMR spectrum of FG1 showed chemical shifts for protons of amide group and tetrahydropyran. Moreover D<sub>2</sub>O exchange NMR showed diminished signals for exchangeable protons of tetrahydropyran ( $\delta$  1.518/1.54). Results of <sup>13</sup>C-NMR also supported formation of FG1.

#### Kinetic studies

Stability and release profile of FG1 (Table 2) in stomach homogenates exhibited no release of fexofenadine at the end of 3 h but 19.5 % release was observed on incubation with small intestinal homogenates at the end of 6 h. *In vitro* kinetic studies in rat fecal matter indicated 82% release of fexofenadine at the end of 12 h with a half life of 260 min following first order kinetics.

Protective effect of FG1 was evaluated in eleven day model of pre-existing TNBS-induced experimental colitis in rats against chronic inflammatory conditions and compared with fexofenadine, D-glucosamine, their mixture and sulfasalazine because site specificity can only be studied by treating the inflammation that occurs in colon. TNBS model is efficiently able to mimic both acute and chronic colitis resembling the human UC (25). Results of disease activity score, colon/body weight ratio, myeloperoxidase activity and histopathological parameters are depicted in Figs. 2, 3, 4 and Table 3 respectively while the photomicrographs of the rat colon, liver, pancreas and stomach are shown in Figs. 5, 6, 7 and 8, respectively.

Full blown colonic inflammation was evidenced by the high disease activity score (3.1±0.08) in colitis control group. All the drug- treated groups started showing decreased inflammation severity from 9<sup>th</sup> day onwards as evident from lowered disease activity scores, reaching the minimum score on 11<sup>th</sup> day except for physical mixture of F and G. Plain F and G- treated groups lowered disease activity score comparably by 42%, 5-ASA (rectal), SLZ and FG1- treated groups by 81%, 78% and 75%, respectively, while groups treated with 5-ASA (oral) and 5-ASA (rectal) brought about 61% and 81% lowering respectively. Physical mixture of F and G was not effective in lowering the disease activity score.

After sacrificing the animals on 11<sup>th</sup> day, colon/body weight ratio was determined. The healthy control showed lowest (0.0034±0.00014) while colitis control group had highest (0.±0.00061) colon/body weight ratio. Maximum lowering of the ratio was seen with groups treated with 5-ASA (rectal) (68%), SLZ (55%) and FG1 (53%). Results of plain fexofenadine and 5-ASA (oral)-treated groups were comparable (39% and 40% respectively) while glucosamine- treated group exhibited 30% lowering of the ratio. Physical mixture was ineffective in this respect.

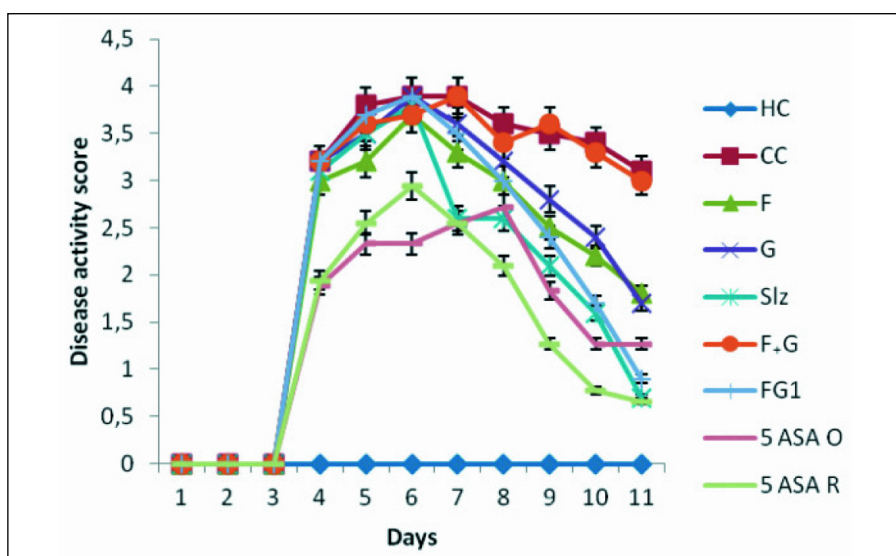


Fig. 2. Disease activity score rate\*.

\* Average of three parameters, *i.e.* weight loss, stool consistency and rectal bleeding + S.D;  $P < 0.05$ .

HC: Healthy control, CC: colitis control, F: fexofenadine, G: D-glucosamine, SLZ: sulfasalazine, F+G: physical mixture of fexofenadine and D-glucosamine, FG1: prodrug of fexofenadine with D-glucosamine 5-ASA: 5-aminosalicylic acid.

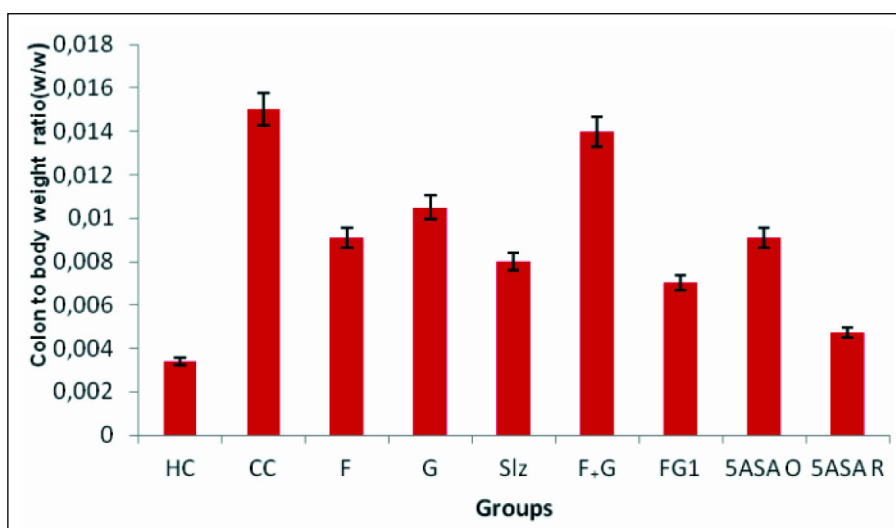


Fig. 3. Colon to body weight ratio\*.

\*Average of six readings  $\pm$  S.E.M.;  $P < 0.05$ .

HC: Healthy control, CC: colitis control, F: fexofenadine, G: D-glucosamine, SLZ: sulfasalazine, F+G: physical mixture of fexofenadine and D-glucosamine, FG1: prodrug of fexofenadine with D-glucosamine 5-ASA: 5-aminosalicylic acid.

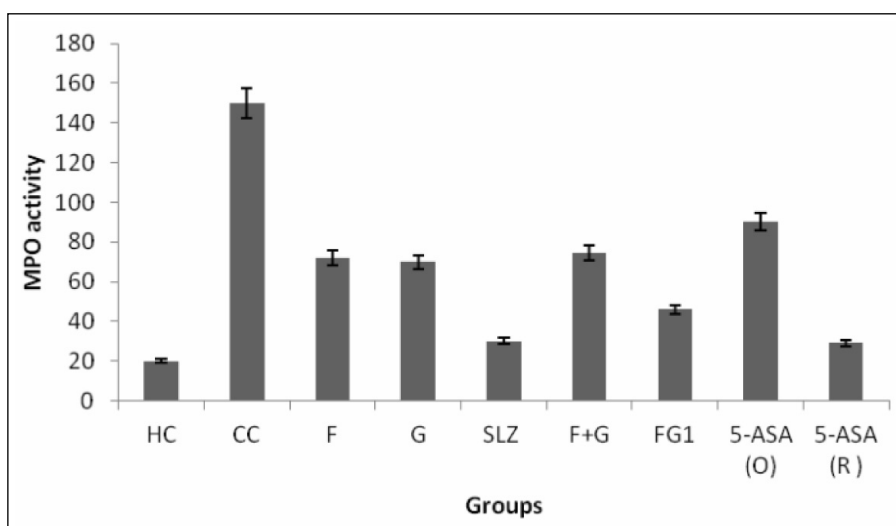


Fig. 4. Myeloperoxidase activity.

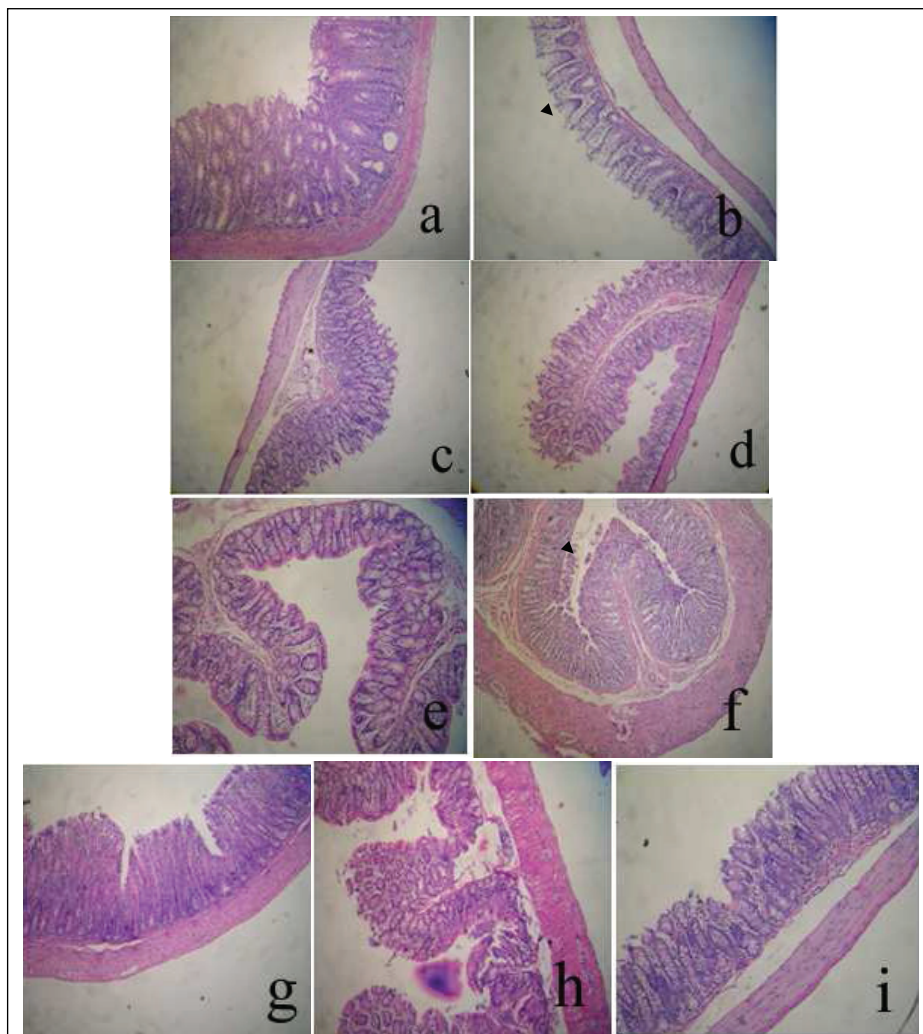
\*Average of six readings  $\pm$  S.E.M.;  $P < 0.01$

HC: Healthy control, CC: colitis control, F: fexofenadine, G: D-glucosamine, SLZ: sulfasalazine, F+G: physical mixture of fexofenadine and D-glucosamine, FG1: prodrug of fexofenadine with D-glucosamine 5-ASA: 5-aminosalicylic acid.

Myeloperoxidase assay was performed on the dissected colon segments after sacrificing the rats. Healthy control had lowest level of MPO ( $19.9 \pm 1.45$ ) while colitis control showed highest level ( $150 \pm 4.04$ ). SLZ, rectally administered 5-ASA and FG1 produced maximum lowering of MPO level (80%, 81.35 and 70%

respectively), plain F, G and their physical mixture lowered MPO concentration comparably by approximately 52% while orally administered 5-ASA could reduce the level only by 40%.

After sacrificing the animals, an 8 cm long segment of distal colon was excised for damage evaluation macroscopically



**Fig. 5.** Histopathology of rat colon. **a.** Healthy control: intact colonic cyto-architecture; **b.** Colitis control: on receiving TNBS inflammatory infiltrate and ulceration in the mucosal layer is evident (arrowhead); **c.** Fexofenadine (oral): moderate protection against TNBS; **d.** Glucosamine (oral): intact colonic morphology; **e.** Sulfasalazine (oral): conservation of disrupted colonic cyto-architecture; **f.** Physical mixture of fexofenadine and glucosamine (oral): partial deformation in the villi region (arrowhead); **g.** Prodrug (oral): conservation of disrupted colonic cyto-architecture; **h.** 5-Amino salicylic acid (oral): mild protection against TNBS; **i.** 5-Amino salicylic acid (rectal): intact colonic morphology.

(Table 3). The colons of colitis control group were characterized by congested, dilated and ulcerated mucosa. Microscopic examination of the dissected sections of colons of colitis control group indicated total disruption of natural architecture, showing massive ulcerations and lymphocytic infiltrate. Fexofenadine-treated colons appeared normal, D-glucosamine group exhibited large lymphoid collection, animals treated with physical mixture showed ulcerated mucosa while FG1 restored the disrupted colonic architecture to normal with mild lymphocytic infiltrate. Prodrug had no adverse effects on stomach, liver and pancreas.

## DISCUSSION

### Partition coefficient

Covalent linkage of glucosamine with fexofenadine significantly enhanced the hydrophilicity of the latter owing to polyhydroxy nature of glucosamine. The higher hydrophilicity of synthesized prodrug would minimize its absorption in the upper GIT directing the intact prodrug to the colon more efficiently. This would assure effective delivery of fexofenadine and glucosamine at the site of action *i.e.* colon.

### Spectral analysis

The structure of the synthesized prodrug was confirmed by spectral analysis. IR spectrum exhibited peaks which were

characteristic of the anticipated structure.  $^1\text{H}$ -NMR spectrum showed characteristic chemical shifts for protons of amide group and tetrahydropyran, which were in accordance with its predicted structure. Diminished signals for exchangeable protons of tetrahydropyran proved that its OH groups were intact and did not react during the course of reaction. Results of  $^{13}\text{C}$ -NMR also matched with the predicted number of total carbons in the structure of FG1. All the above results confirmed the structure of FG1.

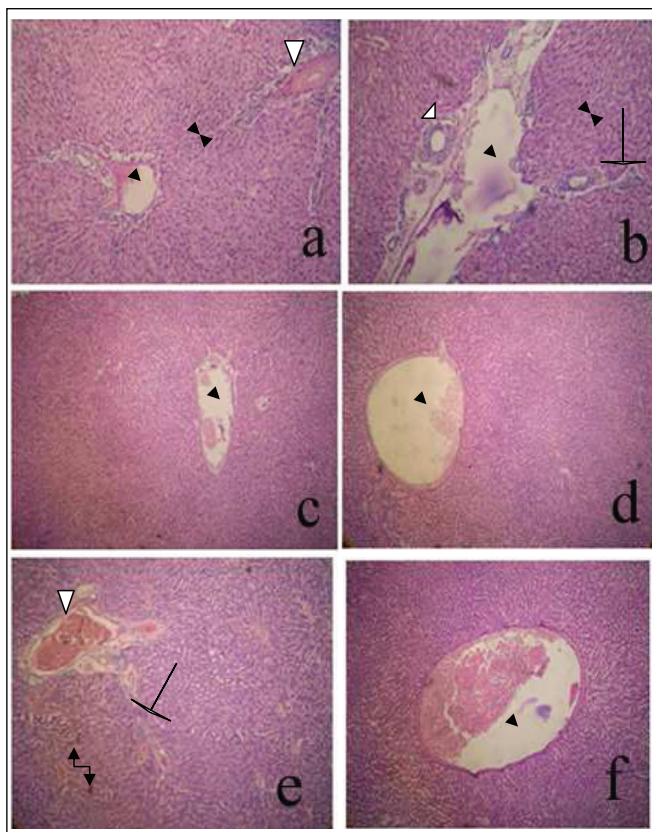
### Kinetic studies

Stability and *in vitro* release of fexofenadine from FG1 was studied by incubating the prodrug with upper GIT homogenates at  $37^\circ\text{C}$ . Kinetic studies of FG1 for the release of fexofenadine (Table 2) confirmed that FG1 was stable in stomach homogenates till 3 h, while furnishing minimal release in small intestinal homogenates at the end of 6 h. The kinetics of release pattern was further studied in rat fecal matter to confirm the colonic activation of amide prodrug which indicated 82% release of fexofenadine at the end of 12 h with a half life of 260 min.

### TNBS-induced colitis model

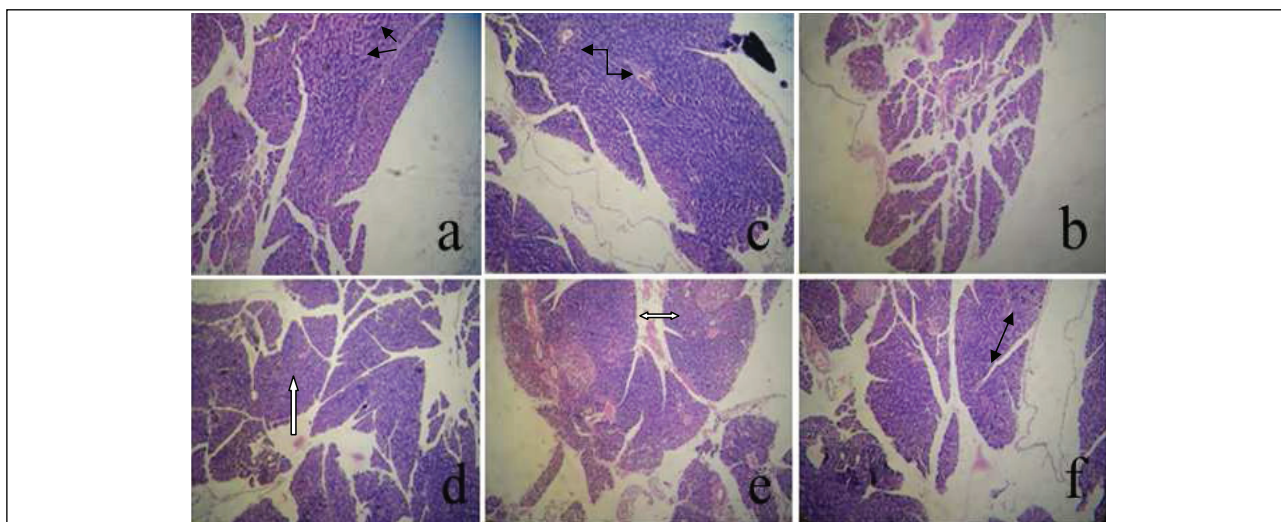
TNBS- induced colitis offers an excellent tool for the pre-clinical testing of anti-tumor necrosis factor therapeutics targeting ulcerative colitis, as tumor necrosis factor (TNF) is an already established therapeutic target for the same and its clinical





*Fig. 6.* Histopathology of rat liver.

**a.** Healthy control: showing normal liver architecture characterized by central vein (black arrowhead), portal triad (white arrowhead) and parenchyma or hepatocytes (double black arrowheads); **b.** Colitis control: showing normal liver architecture; **c.** Fexofenadine (oral): showing central veins (black arrowhead), portal tracts (white arrowhead), hepatocytes (double black arrowheads) and sinusoids (double white arrowheads) appear normal with no significant pathological changes; **d.** Glucosamine (oral): appears normal with no significant pathological changes; **e.** Physical mixture of fexofenadine and glucosamine (oral): showing normal liver morphology with Kupfer's cells (double-headed twisted arrow); **f.** Prodrug (oral): appears normal with no significant pathological changes.



*Fig. 7.* Histopathology of rat pancreas.

**a.** Healthy control: showing normal pancreas architecture with characteristic islets of Langerhans (black arrows); **b.** Colitis control: showing normal pancreas architecture; **c.** Fexofenadine (oral): showing absence of fibrosis and signs of distortion, irregular size or dilatation in ducts (twisted double-headed arrow); **d.** Glucosamine (oral): appears normal with characteristic lobules (white arrow) with any significant pathological changes; **e.** Physical mixture of fexofenadine and glucosamine (oral): showing normal pancreas architecture showing septa (white double-headed arrow); **f.** Prodrug (oral): showing normal pancreas architecture with acini (black double-headed arrow).

application has given impressive results (26). It is the most relevant model as it involves the use of TNBS; an immunological hapten that acts as a contact sensitizing allergen and develops a chronic inflammation rather than an acute mucosal injury in a reproducible manner (21). Extent of mitigating effect offered by FG1 on TNBS-induced colitis was compared with four standard drugs: fexofenadine, D-glucosamine, sulfasalazine and physical

mixture of fexofenadine and D-glucosamine on the basis of quantifying parameters, characteristic of experimental colitis in rats. Histopathological studies of colon, pancreas, liver and stomach were used for evaluating safety of FG1. Severity of colonic inflammation is reflected by elevated scores of three important parameters namely disease activity score (average of stool consistency, rectal bleeding and weight loss), colon/body



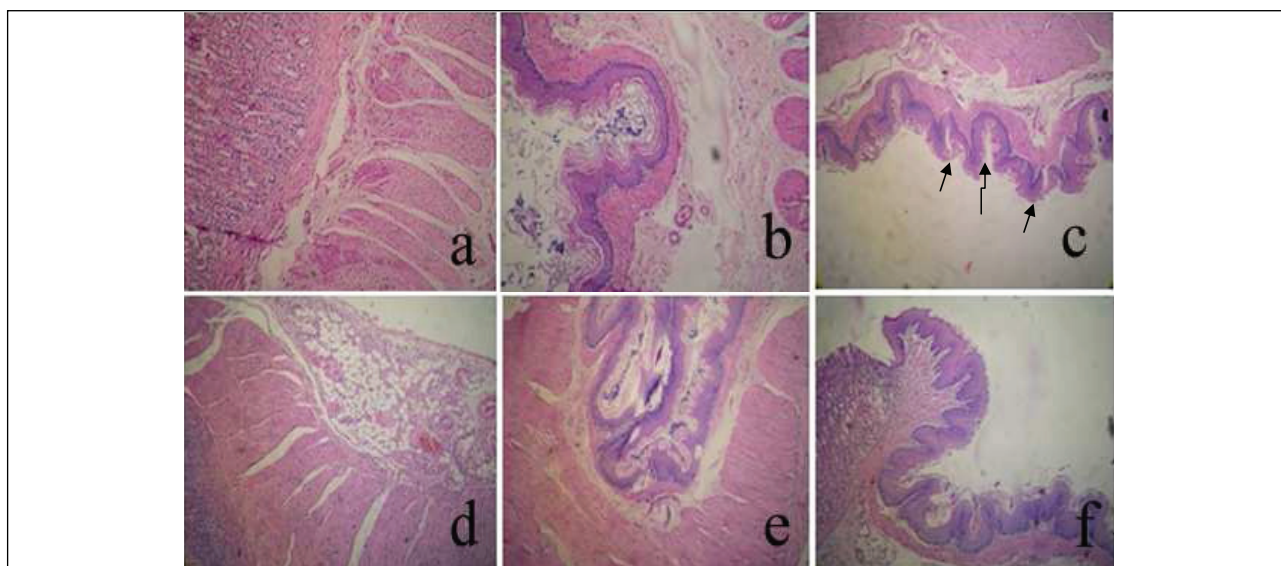


Fig. 8. Histopathology of rat stomach.

**a.** Healthy control: showing normal stomach architecture; **b.** Colitis control: appears normal with no significant pathological changes; **c.** Fexofenadine (oral): showing thick glandular mucosa (black arrow), packed with gastric glands (black twisted arrow) appear normal with no significant pathological changes; **d.** Glucosamine (oral): showing intact morphology; **e.** Physical mixture of fexofenadine and glucosamine (oral): appears normal with no significant pathological changes; **f.** Prodrug (oral): appears normal with no significant pathological changes

weight ratio and myeloperoxidase activity. Lower values of these parameters correlate with better ameliorating effect on the inflammation of colon.

Disease activity score is a marker of progression of colitis characterized by three important symptoms: diarrhoea, rectal bleeding and colonic inflammation. The prodrug was comparable to sulfasalazine (SLZ) and rectally administered 5-ASA while 1.2 times more effective than 5-ASA (oral) and 1.7 times more effective than fexofenadine and glucosamine in lowering the disease activity score.

Increased colon to body weight ratio reflects severity of colonic inflammation. For lowering effect on colon to body weight ratio, FG1 was comparable to SLZ while 1.35 times more effective than 5-ASA (oral) and fexofenadine and 1.76 times more effective than glucosamine. However FG1 was 1.3 times less effective in reducing colon to body weight ratio than rectally administered 5-ASA.

The histologic feature of IBD is marked by the presence of inflammatory cells; neutrophils, lymphocytes and histiocytes. The more acute the illness, the prominent the neutrophil component of the inflammatory infiltrate (23). Myeloperoxidase (MPO) is a heme-containing enzyme stored in the azurophilic granules of neutrophilic polymorphonuclear leukocytes (PMNs) and in the lysosomes of monocytes in humans (27). The determination of MPO activity in the intestine is a simple biochemical assay used to quantitate local inflammation. MPO is a marker used to assess neutrophil infiltration to the site of inflammation in both human and experimental models of IBD. Attenuation of MPO level by FG1 was slightly less (1.1 times) than SLZ but 1.3-1.45 times more than fexofenadine, glucosamine and 5-ASA (oral). The plausible explanation is that diminution of MPO level by FG1 is due to its ability to decline neutrophil infiltration to the inflamed tissue. Therefore, it may preclude the release of components that might worsen inflammatory conditions. Overall we can conclude that the prodrug was almost comparable to sulfasalazine and rectally administered 5-ASA in lowering the three quantifying

parameters of colonic inflammation (disease activity score, colon/body weight ratio and myeloperoxidase activity) in TNBS-induced colitis while significantly more effective than fexofenadine, D-glucosamine, their physical mixture and oral 5-ASA (oral).

Histopathological studies of colon of rats treated with fexofenadine, SLZ and 5-ASA (rectal) exhibited restored colonic architecture. Large lymphoid collection was observed in colon of rats treated with glucosamine. It can be explained on the basis of a reported finding of Sadeghi *et al.* that treatment with glucosamine can exert immunostimulatory effects by activating T lymphocytes in healthy individuals (28). Colons treated with chemically conjugated prodrug showed normal colon morphology with mild lymphocytic infiltrate which might be due to immunostimulation by glucosamine while for physical mixture of F+G, colons appeared congested with ulcerated mucosa. FG1 proved to be better than physical mixture because it was able to release F and G locally in colon in effective concentration for their protective effect while F and G administered orally were unable to reach the colon in required concentration to mitigate colonic inflammation. Prodrug as well as fexofenadine and glucosamine had no adverse effects on stomach (as against gastric ulcers produced by orally administered 5-ASA), liver and pancreas (as against adverse effects of 5-ASA and SLZ on liver and pancreas) proving the safety of this prodrug in the management of IBD.

Histamine has been suggested as participating in intestinal inflammation (10) and there are reports of increased histamine secretion during active CD (29). It is the main mast cell mediator that increases vascular permeability, leukocyte infiltration, and smooth muscle contraction. Protective effect of fexofenadine and its prodrug on the TNBS-induced colonic inflammation resulting in colonic mucosal defense can be explained on the basis of its antihistaminic effect.

Although there are lot of contradictory and complimentary viewpoints available in the literature about mechanism of 5-ASA and its colon-specific prodrug sulphasalazine, a number of

possibilities seem likely viz: free radical scavenging leading to reduced leukotriene production, inhibition of chemotactic response to leukotriene B<sub>4</sub>, reduced synthesis of platelet activating factor and inhibition of leucocyte adhesion molecule upregulation. All these mechanisms seem to interplay towards their mitigating effect in IBD (30).

Mucosal glycoprotein and mucus synthesis are involved in maintaining cytoarchitecture of colonic mucosa through their cytoprotective effect. Abnormalities or impaired glycoprotein/mucus biosynthesis are implicated in pathogenesis of IBD. Glucosamine acts as a building block for the biosynthesis of glycoproteins and glucosaminoglycans, the rate determining step being glycosylation catalyzed by glucosamine synthetase (31). Plain glucosamine showed significant lowering effect on all the quantifying parameters of colitis and its ameliorating effect on colonic inflammation was comparable to fexofenadine. The cytoprotective effect of glucosamine released locally in the colon after colon-specific activation (hydrolysis) of FG1 might be responsible for enhanced efficacy of prodrug than the parent drug, in suppressing the course of TNBS-induced colitis.

It is interesting to note that the present study is the first one indicating alleviation of immune-based animal model of IBD *i.e.* TNBS-induced colitis in rats by fexofenadine and its prodrug with glucosamine. The prodrug healed/suppressed colonic macroscopic and histological damages, diminished disease activity score, colon to body weight ratio and tissue MPO which were elevated in colitis control animals due to TNBS-induced colitis.

In the present work, D-glucosamine was explored as a colon-targeting carrier for mutual prodrug strategy that culminated into successful design and synthesis of colon-specific prodrug of fexofenadine. The results of the present work indirectly support the hypothesis of involvement of histamine in the pathogenesis of UC. This conclusion is based on the fact that TNBS-induced colitis was ameliorated by oral administration of FG1 alone without any concurrent treatment of any aminosalicilate or sulfasalazine. However more in depth and extensive studies are required to justify this hypothesis. This novel, dual acting prodrug of fexofenadine with D-glucosamine holds a lot of promise and could be used in combination with sulfasalazine as a maintenance therapy to counteract the relapse of UC.

**Acknowledgements:** The authors are thankful to Dr. Reddy's Laboratories, Hyderabad, India and Wallace Pharmaceutical Pvt. Ltd., Goa, for providing gift sample of fexofenadine and sulfasalazine, respectively. The authors are also thankful to the Department of Chemistry, University of Pune, for spectral analysis of the compound.

**Conflict of interests:** None declared.

**Abbreviations:** HC: healthy control, CC: colitis control, F: fexofenadine, G: D-glucosamine, SLZ: sulfasalazine, F+G: physical mixture of fexofenadine and D-glucosamine, FG1: prodrug of fexofenadine with D-glucosamine 5-ASA: 5-aminosalicylic acid.

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Received: May 8, 2012

Accepted: August 25, 2012

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