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Synthesis and evaluation of azo pro-drugs of flurbiprofen for colon targeting

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Abstract

This investigation for revealing the new synthsized azo prodrug of flurbiprofen which increase the potency of approach and decrease the associated adverse effects. Different class of amino acids which have synergestic effect with flurbiprofen for the treatment of UC and safely used in IBD, have been selected for synthesis of azo prodrug with fenoprofe. These azo prodrugof different classes has been further evaluated against physicochemical property, *in vitro* stability test and *in vivo* ulcer models in rats. The associated consequence reveals the successful synthesis of azo prodrug and also explored the significant therapeutic potential against TNBS induced UC model. Thin layer chromatography of all the intermediates and final compounds were performed on silica gel plates, using UV and iodine chamber for visualization of spots andsingle spots were obtained for the same,. Open capillary method was used to determine the melting points of the final products, which was found to be uncorrected. The objective of synthesizing such azo prodrugs of flurbiprofen which have improved aqueous solubility and lower log P values than that of flurbiprofen has been achieved. Such high values for aqueous solubility of these novel prodrugs confirm that they will reach intact into colon and will not get absorbed in the upper GIT. All physical and microscopical alteration has also been evaluated in standard (sulfasalazine) and all other azo prodrug groups, and significantly compared with colitis control. This evaluation confirmed that azo prodrugs significantly protect against TNBS induced colitis and show the similar results with sulfasalazine group. This finding proves the therapeutic potential of azo prodrug against ulcerative colitis as similar or better to sulfasalazine.

Keywords: Azo prodrug; Ulcerative colitis; Sulfasalazine; Flurbiprofen

1. Introduction

The largest portion of the large intestine is colon. Indeed, colon refers to the entire large intestine. The section of colon expands from cecum (a joint between large and small intestine) to the right side of the abdomen in upper direction (ascending colon) then parallel to diaphragm, across to the left side (transverse colon), and downward to left side or parallel to asending colon (descending colon) and then loops (at sigmoid flexure, or sigmoid colon) which further join the rectum as shown in Fig 1. Colon is the second large site for absorption of fluid and salts; simultaneously it lubricates the waste products and store waste products until they passed away from the body. Ascending and transverse sections significantly participate in the absorptions of various liquid material received from the small intestine and dehydrated to fecal mass. [1].

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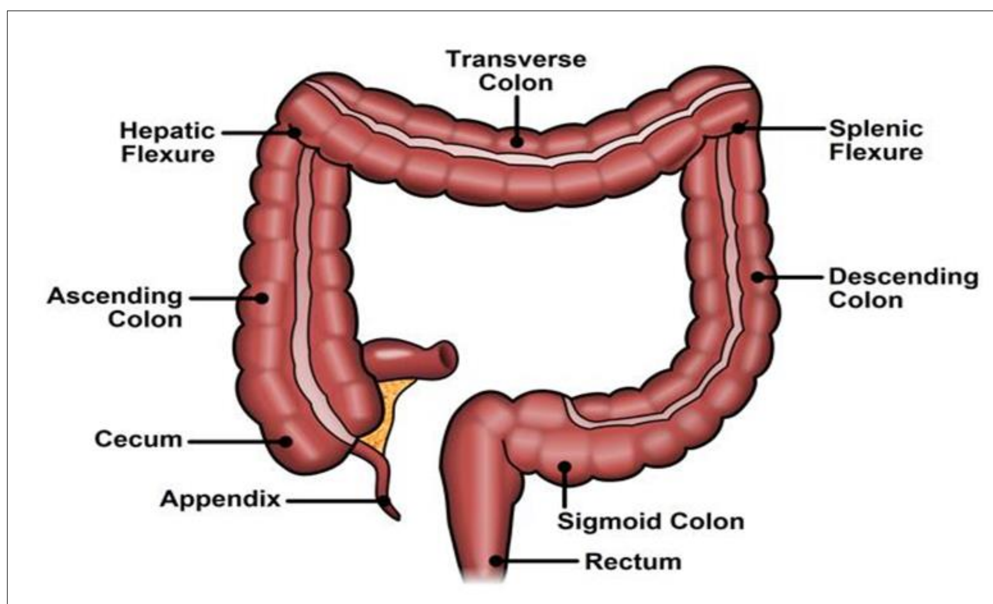


Figure 1 Human Intestine

Inflammatory bowel disease (IBD) is a chronic state which aasociated with severe inflammatory condition in mucosal membrane of small and/ or large intestine.Ulcerative colitis (UC) and Crohn's disease (CD) are communally accredited as IBD. Although the mentioned complications are seems to be relevant but havedistinctive features. UC is categorized as persistent mucosal inflammation in colon site of large intestine and rectum. Whereas, CD is the advanced stage of transmural inflammation in entire or partial of gastrointestinal tract. This terrible inflammatory stage significantly directs to expansion and maturity of ulcerations which might leads to diarrhea, abdominal pain and blood loss in fecal material. Instead of several insidious diseases, the appearance of symptoms results in more dangerous complications including hemorrhage, obstruction, perforation or cancer.

Several investigations reveal that the intitiation and development of these diseases is only concern with genetic susceptibility that enables an agent such as a virus or bacteria to trigger an abnormal immune response [2-5].

Immune system is the natural defecnce mechanism of our body which provide capability to fight against various infection and diseases. Commonly the lymphocytes and leukocytes are the major unitof white blood cells which act against primary infection. Furthermore, lymphocytes include two subtypes namly *T-cell*s and *B-cells.* These both subtypes are able to distinguish foreign invaders (antigens) and also to offensive attack on the antigens sothat defensive mechanism become intitiate. Particularly, B-cells generate antibodies, which are separate agents that can either ride along with a B-cell or travel on their own to attack the antigen. In-addition, T-cells are specific for receptor interaction which can recognize the specific antigen. Furthermore, T-cells are further characterized as killer T-cells or helper T-cells (TH cells). Killer T-cells may cause direct attack on antigens in all cells having nucleus. Subsequently, helper T-cells can also identify antigens, but their role is multi facilated. They may stimulate B-cells andalso can direct the several mediators which can produce auto antibodies (which are directed against the body's own cells) [6].

2. Material and methods

The amino acids were obtained from M/s Hi-Media Ltd., Mumbai, India and drugs flurbiprofen was obtained as gift sample from FDC, Ltd, Mumbai India. Other reagents and solvents were of analytical grade. Sulfasalazine was purchased from Ipca laboratories Ltd., Mumbai. The melting points were recorded by melting point determination apparatus (Sigma Instrument, Mumbai, India) and are uncorrected. The infrared spectra were recorded on IR spectrophotometer (Shimadzu 8201 PC) in KBr phase, . 1H NMR was recorded in NMR spectrophotometer (Bruker DRX 300, USA). The samples were made in D2O and DMSO-D6 using tetra methyl silane (TMS) as internal standard The hydrolysis data and drug content determination were performed by UV spectrophotometer (Shimadzu, Japan).

2.1. Scheme for the synthesis of azo compounds

- Synthesis of azo conjugates of flurbiprofen
- Esterification of amino acids

- Diazotization of amino acids
- Coupling of diazotised salt amino acids with flurbiprofen

2.1.1. Procedure

Step 1: Synthesis of methyl ester hydrochlorides of amino acids

Freshly distilled (0.05 M, 6 ml) of thionyl chloride was slowly added to methanol (100 ml) with cooling and amino acid (0.1 M) was added to it. The mixture was refluxed for 6-8 h at 60-70ºC with continuous stirring on magnetic stirrer to obtain crude amino acid methyl ester hydrochloride. The resulting solid product was collected and dried under vacuum. It was recrystallized from hot methanol followed by cooling at 0ºC. The crystals were collected.

Step 2: Diazotization of amino acid

Amino acids (0.01 mol) was dissolved in a suitable volume of water containing 2.5-3 equivalents of hydrochloric acid (0.02 mol; 1.7 mL of 35% HCl), by the application of heat (if necessary) and then solution was cooled in ice.The temperature was maintained at 0-5^{0}C on a cryostatic bath and an aqueous solution of sodium nitrite (2 mol, 1.4 g in 10 mL) was added (portion wise), To stabilize the diazonium saltand to minimize secondary reactions (proper condition of acidity was maintained throughout) by adding excess of acid (0.5-1 equivalents). The reaction mixture was kept in cryostatic bath at 0-5^{0}C during the course of reaction (which is exothermic in nature), in order to avoid the hydrolysis of diazonium salt tocorresponding phenol [7-9].

2.1.2. Coupling of diazotised salt of amino acids with flurbiprofen

Flurbiprofen (0.01 mol) was completely dissolved in sodium hydroxide solution (2 mol; 0.08g/mL). The solution was then cooled at below 5^{0}C. Then slowly diazotised salt of amino acid as added with continuous stirring, through syringe.

Crude product was recovered. It was recrystallized by dissolving in methanol. Purified product was dried under vacuum. The reaction was monitored by TLC using chloroform:methanol (4:1.5), as a solvent system.

2.2. Physicochemical characterization of prodrugs

The thin layer chromatography of the synthesized compounds was performed on plates of silica gel plates using iodine vapours and UV light as detecting agents.T he solvent system is methanol:water (3:1 v/v)The melting points of intermediates and the final products were determined by open capillary method.

The absorbance maxima (λ_{max}) of synthesized compounds were determined on Shimadzu UV 1700, UV-Visible double-beam spectrophotometer in solvents, likehydrochloric acid buffer (pH 1.2) and phosphate buffer (pH 7.4).

The IR spectra of the synthesized compounds were recorded on Agilent Resolution Pro, FTIR. The ^{1}H-NMR spectra of the synthesized compounds were recorded in DMSO using ^{1}HNMR Varian Mercury 400 MHz with super conducting magnet.

The partition coefficients of the synthesized compounds were performed in n-octanol/ phosphate buffer (pH 7.4) at room temperature (25 ± 1^{0}C). The compounds were separately dissolved in 10 mL n- octanol and 10 mL phosphate buffer was slowly added to it and the octanol- phosphate buffer mixture was shaken for 48 h on a wrist shaker to reach distribution equilibrium. The volumes of each phase were chosen so that the solute concentrations could readily be determined by UV spectrophotometer to calculate partition coefficient.

The aqueous solubility of all the synthesized compounds was determined at room temperature (25 ± 1^{0}C). Excess amount of the compound was added to distilled water in stoppered conical flask and was mechanically stirred for 24 hr. It was ensured that saturation equilibrium was established. Then solute was determined on UV-spectrophotometer [10-12].

2.3. *In vitro* kinetic evaluation

As the present work aims at delivery of flurbiprofen to colon through itsazo prodrugs, it is necessary to assess their suitability with respect to stability in stomach and small intestine. Therefore, after the azo conjugates were synthesized and, there *in vitro* stability studies were carried out in hydrochloric acid buffer (pH 1.2) (simulated to stomach pH), phosphate buffer (pH 7.4)(simulated to intestinal pH). 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 potassiumchloride. As these

conjugates have been developed with an aim to deliver flurbiprofen to colon for localized inflammatory bowel disease (IBD), the feasibility of reduction of azo linkage by azo reductase secreted by intestinal microflora was tested with help of release study in rat fecal matter at 37 ± 1˚C.All the kinetic studies were carried out in triplicate [13-15].

2.4. *In vivo* (Biological) Evaluation

2.4.1. Ulcerogenic Activity

The ulcerogenic activity was determined by the Rainsford'scold stressmethod, which is an acute study model and is used to determine ulcerogenic potency of a given drug at ten time's higher dose. flurbiprofen and sulfasalazine were taken as standards. It was found that the presence of suspending agents like carboxy methylcellulose decreases the incidence of gastric ulcers. Hence, the test compounds and standards were administered orally, as fine particles suspended in CMC by continuous stirring. The volume of vehicle or suspensions was kept constant. Wistar rats of either sex weighing between 200–230 g were randomly distributed in control and experimental group of four animals each. Doses of prodrugs were calculated on equimolar basis of flurbiprofen. They were then converted into ten times higher doses. Following oral administration of 5 mL of the aqueous drug suspensions (at 10 times the normal dose), the animals were stressed by exposure to cold (-15 °C for 1 h). The animals were placed in separate polypropylene cages to ensure equal cold exposure. After 2 h of drug administration, the animals were sacrificed. The stomach and duodenal part were opened along the greater curvature and thenumber of lesions was examined by means of a magnifying lens. All ulcers larger than 0.5 mm were counted. The ulcers were scored according to the method reported by Cioli *et. al.* (1979) and the ulcer index was determined. The mean ulcer index (UI) was calculated by severity of gastric mucosal lesions which are graded as grade 1: less than1 mm erosions, grade 2: 1-2 mm erosions and grade 3: more than 2 mm erosions.

The UI was calculated as

UI = [1× (number of lesions of grade 1) + 2× (number of lesions of grade 2) + 3× (number of lesions of grade 3)]/10

Average of five reading was calculated and all data was expressed as mean ± S.D.

Table 1 Scoring of Gastric Ulcers

Sr. No.	Ulcerogenic Response	Score
1	Ulcers less than 1 mm	1
2	Ulcers less than 1-2 mm	2
3	Ulcers less than 2-3 mm	3
4	Ulcers less than 3-4 mm	4
5	Ulcers less than 4-5 mm	5
6	Ulcers greater than 5 mm	10
7	Perforated lesions	25

2.5. TNBS induced experimental colitis model

In order to study the use of azo prodrug of flurbiprofen for targeted oral drug delivery to the inflamed tissue of colon in IBD, TNBS induced experimental colitis model was selected, because site specificity can only be studied by treating the inflammation that occurs in colon. This model is simple andreproducible.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. Induction of colitis by TNBS in rats is one of standardized methods to produce an experimental model of inflammatory bowel disease.

2.6. Animal requirements

Male Wister rats (average weight 200–230 g; 12-15 weeks) were used. They weredistributed into eight different groups, each containing five animals. Animals were divided in different groups. They were housed in a room with controlled temperature (22^{0}C). The animals were food fasted 48 h before experimentation and allowed food and water *ad libitum*. Animal experiment was approved by the Institution animal ethical committee the approval no. are as Ocp/IAEC/2023/04

2.7. Induction of Colitis

Rats were fasted for 24 h before experimentation. Rats were lightly anesthetized with ketamine and xylazine (20 mg/kg and 5 mg/kg, i.m.). A polyethylene catheter with 2 mm diameter was inserted through the rectum intothe colon to a distance of 8 cm. For ulcerative colitis induction, TNBS dose was150 mg/kg of body weight of TNBS in ethanol, 50% solution) was infused into the colon of all rats (except the normal control group) through the catheter, held in place for 30 sec. The catheter was left in place for few seconds then gentlyremoved. For 3 days the rats were housed without treatment to maintain the development of a full inflammatory bowel disease modelwith full access of food and water *ad libitum*. The animals of standard and test groups received orally flurbiprofen and prodrugs respectively, once daily for five continuous days.. The normal control and colitis control groups received only 1% carboxy methylcellulose instead of free drug or prodrug.

2.8. Assessment of colonic damage by clinical activity score

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 as previously applied by Hartmann *et al*. 2000 . The clinical activity score was determined by calculating the average of the above three parameters for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis).They were sacrificed 24 h after the last drug administration and a segment ofcolon 8cm long was excised and colon/ body weight ratio was determined to quantify the inflammation. Tissue segments 1cm in length was then fixed in 10% formalin for histopathological studies.

2.9. Histopathological analysis

Histopathological studies of the colon were carried out and colored microscopical images of the colon sections were taken on Radical Instruments Microscope, RXLR-3T, with resolution 10 x 40 X, attached with trinocular camera.

Table 2 Scoring Rate of Clinical Activity

S. No	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

3. Results and discussion

3.1. Physico- chemical and spectral characterization of prodrug (FLI)

The physico- chemical and spectral charecterization of azo prodrug of flurbiprofen and isoleucine (FLI) are discussed as follows: Melting point. 190˚C, , percentage yield- 76%, aq. Solubility- 0.21g/mL, log P- 1.79, □max in HCl buffer (pH 1.2):282nm and in phosphate buffer (pH 7.4): 299nm. Rf value is 0.61,

(FLI): IR (KBr, cm^{-1}): 2870 (OH), 3030 (C-H Ar), 2920 , 2872 (CH , Ali.),1710 (C=O),1638,1580, 1460,1370 (C=C Ar),1493(N=N) 1276(-OCH3) 1070 (C-F str).

^{1}H NMR (DMSO-*d6*, 400 MHz, δ) 7.76-7.20 (m, 7H, ArH), 4.57 (s, 1H, OH) 2.12 (q, 1H, CH methine) 3.85 (s, 3H, COOCH3) , 11.9I(s 1H, COOH)

The physico- chemical and spectral charecterization of azo prodrug of flurbiprofen and serine (FLS) are discussed as follows: Melting point. 210˚C, , percentage yield- 79%, Aq. Solubility- 0.23g/mL ,log P- 1.45, □max in HCl buffer (pH 1.2):281nm and in phosphate buffer (pH 7.4): 296 nm. Rf value is 0.65,

(FLS):IR (KBr, cm^{-1}): 2860 (OH), 3010 (C-H Ar), 2930 , 2772 (CH , Ali.),1690 (C=O), 1618,1560, 1470,1459 (C=C Ar),1491(N=N) 1271(-OCH_3) 1076 (C-F str).

^{1}H NMR (DMSO-*d6*, 400 MHz, δ) 7.75-7.10 (m, 7H, ArH), 4.59(s, 1H, OH) 2.52 (q, 1H,CH methine) 3.84 (s, 3H, $COOCH_3$) , 10.91(s 1H, COOH)

3.2. Physico- chemical and spectral characterization of prodrug (FLL)

The physico- chemical and spectral charecterization of azo prodrug of flurbiprofen and lysine (FLL) are discussed as follows: Melting point. 215˚C, , percentage yield- 89%, aq. Solubility- 0.26 g/mL, log P- 1.15, max in HCl buffer (pH 1.2):289nm and in phosphate buffer (pH 7.4): 305 nm. Rf value is 0.66, **(FLL):**IR (KBr,cm^{-1}): 2770 (OH), 3002 (C-H Ar), 2942 , 2762 (CH , Ali.),1695 (C=O),1595,1560, 1460,1396 (C=C Ar),1490(N=N) 1251(-OCH_3) 1042 (C-F str).;^{1}H NMR (DMSO-*d6*, 400 MHz, δ), 7.35-7.10 (m, 7H, ArH), 3.59(s, 1H, OH) (q, 1H, CH methine) 3.79 (s, 3H, $COOCH_3$) , 11.62(s 1H, COOH) 5.6 (t,2H, NH_2)

3.3. Physico- chemical and spectral characterization of prodrug (FLM)

The physico- chemical and spectral charecterization of azo prodrug of flurbiprofen and methionine (FLM) are discussed as follows: Melting point. 217˚C, percentage yield- 82%, aq. Solubility- 0.28 g/mL log P- 0.97, ƛmax in HCl buffer (pH 1.2):294nm and in phosphate buffer (pH 7.4): 312 nm. Rf value is 0.62, **(FLM):** IR (KBr, cm^{-1}): 2870 (OH), 3012 (C-H Ar), 2842 , 2761 (CH , Ali.),1705 (C=O), 1585,1540, 1450,1396 (C=C Ar),1496(N=N) 1249(-OCH_3) 1079(C-F str). ^{1}H NMR (DMSO-*d6*, 400 MHz, δ) 10.03 (s, H, COOH) 6.95- 7.92 (m, 4H, ArH) 4.65(s, 1H, OH) 3.37-3.88 (m, 2H, CH_2) 2.49(q 1H, CH)

3.4. *In vitro* kinetic study of prodrugs

3.4.1. Release studies in 0.05 M hydrochloric acid buffer (pH 1.2)

FLI, FLS, FLL and FLM(10 mg) are introduced in 900 mL of HCl buffer taken in dissolution apparatus and were kept in a constant temperature bath at 37 ± 1˚C. The solutions were occasionally stirred and 5 mL aliquot portions were withdrawn at various time intervals. The aliquots were directly estimated on UV spectrophotometer. During estimation, it was observed that at even after 3 h FLI, FLS ,FLL and FLM showed negligible release of the free drug.

3.4.2. Release studies in 0.05 M phosphate buffer (pH 7.4)

FLI, FLS ,FLL and FLM (10 mg) were introduced in 900 mL of phosphate buffer taken in dissolution apparatus and were kept in a constanttemperature bath at 37 ± 1˚C. The solutions were occasionally stirred and 5 mL aliquot portions were withdrawn at various time intervals. The aliquots were directly estimated on UV spectrophotometer During estimation, it was observed that at even after 3 h prodrugs showed negligible release of the free drug. During estimation, it was observed that after 6 h FLI, FLS ,FLL and FLM showed only 15.7% release of the free drug.

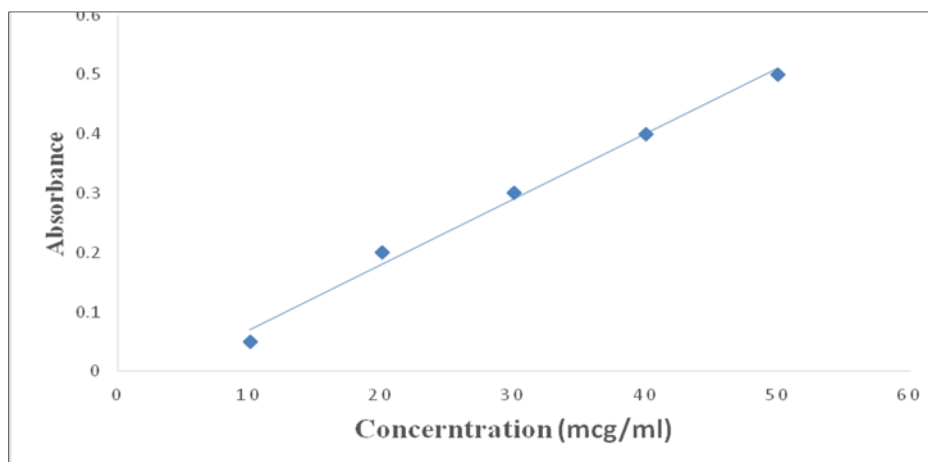


Figure 2 Standard curve of flurbiprofen

3.5. Release studies in rat fecal matter (pH 7.4)

All the prodrug were dissolved in phosphate buffer (pH 7.4) so that final concentration of solution was 250 μg/mL. Fresh fecal material of rats was weighed (about 1g) and placed in different sets of test tubes. To each test tube, 1 mL of the prodrug solution was added and diluted to 5 mL with phosphate buffer (50 μg/mL). The test tubes were incubated at 37˚C for different intervals of time.. The percent release data of free drug from its prodrug is determined. During estimation, it was observed that FLI, FLS ,FLL and FLM showedsignificant release .

Table 3 Percentage of flurbiprofen released in rat fecal matter

Time(min)	% flurbiprofen released			
	FLI	FLS	FLL	FLM
15	0	0	0	0
30	12	13.3	12.3	12.3
45	23.3	24.4	23.7	26.6
60	34.3	38.7	36.4	39.2
75	37.6	45.2	44.3	47.8
90	43.3	52.7	51.1	57.2
105	52.3	67.3	65.7	68.2
120	62.7	72.3	71	74.4
240	75.5	79.7	80.4	81.5
360	81.2	85.3	88.7	91.2

3.6. Biological Evaluation

3.6.1. Ulcerogenic Tendency

GI side effects constitute the most frequent of all the adverse reactionsassociated with NSAIDs. Most workers generally accept the fact that GIT lesions produced by NSAIDs are the result of two different mechanisms:

- A direct contact effect.
- A generalized systemic effect, which may be manifested after intravenous dosing.

In a study the importance of the direct contact effect in GIT toxicity of flurbiprofen was examined in rats. The results of this study suggest that direct tissue contact of drugs plays an important role in production of GIT lesions. Drug when administered orally seems to produce irritancy and ulcerogenicity in the upper GI tract. The use of azo prodrug does not allow free drug to be liberated in upper GI tract, thus it has been postulated as an approach todecrease the GIT toxicity. Therefore azo conjugates as prodrugs of Drug were synthesized, assuming that they would not release parent drug in the stomach and small intestine, so that gastric side effect of parent drug would be minimized.

The release studies of azo conjugates of Drug have suggested that they are stable at acidic pH and show negligible release of parent drug at intestinal pH. Evaluation of ulcerogenic potential of these conjugates would further ensure us whether the synthesized compounds are less irritating to gastric mucosa than the parent drug or not and whether the azo prodrugs actually deliver drug specifically to colon or not. Hence the synthesized compounds wereinvestigated for their ulcerogenic potential.

3.7. TNBS induced experimental colitis model:

The ulcerogenic activity of drug associated prodrugs, was determined by TNBS induced experimental colitis model in rats. TNBS wasinfused into the colon of all rats (except the normal control group) through the catheter, held in place for 30 sec for induction of experimental colon ulcer. A significant appearance of experimental ulcer in animal colons which received TNBS treatment has been observed. In-addition, loss of body weight, stool consistency, rectal bleeding has observed on the basis of score.

Additionally, the clinical activity score has been determined bycalculating the average of above three parameters for every day against each groups. Moreover, colon to body weight ratio has been determined to quantify the inflammation,. About 1 cm length of colon tissue was stored in 10% formalin and further evaluated for histological examination. The consequences from these analysis reveals the protective potential of FLI, FLS ,FLL and FLM in comparison to standard, flurbiprofen and diseases control group, which have remarkable colon ulcer.

Table 4 Results of ulcerogenic activity

Compound	**Ulcer index ± S.D.***
Normal Control	0.6 ± 0.12
Standard (Sulfasalazine)	5.3 ± 0.15
Flurbiprofen Control	29.62 ± 1.8
FLI	5..1 ± 0.13
FLS	4.7 ± 0.2
FLL	4.8 ± 0.97
FLM	4.5 ± 0.17

Table 5 Clinical activity score rate

Groups	**Days**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Normal control	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0 ± 0.02	0.1 ±0.02	0.11 ± 0.02
Diseases control	0 ± 0.02	0 ± 0.02	0 ± 0.02	3.1 ± 0.02	4.0 ± 0.05	3.8 ± 0.02	3.5 ± 0.05	3.0 ± 0.05	3.0 ± 0.03	2.6 ± 0.02	2.4±0.05
Standard	0 ± 0.02	0 ± 0.02	0 ± 0.02	4.0 ± 0.02	4.0 ± 0.05	3.5 ± 0.02	2.6 ± 0.05	2.6 ± 0.02	1.8 ± 0.02	1.3 ± 0.02	0.82 ± 0.02
flurbiprofen	0 ± 0.02	0 ± 0.02	0 ± 0.02	4.0 ± 0.05	4.0 ± 0.02	3.5 ± 0.03	2.5 ± 0.05	2.6 ± 0.02	2.0 ± 0.05	1.4 ± 0.02	1.0 ± 0.02
FLI	0 ± 0.02	0 ± 0.02	0 ± 0.02	4.0 ± 0.05	3.5 ± 0.02	3.6 ± 0.05	3.0 ± 0.05	2.0 ± 0.05	1.3 ± 0.05	0.5 ± 0.04	0.3 ± 0.05
FLS	0 ± 0.01	0 ± 0.05	0 ± 0.02	3.6 ± 0.02	3.6 ± 0.04	3.6 ± 0.05	2.5 ± 0.04	2.0 ± 0.05	1.3 ± 0.02	0.8 ± 0.05	0.4 ± 0.03
FLL	0 ± 0.02	0 ± 0.02	0 ± 0.02	3.5 ± 0.02	3.5 ± 0.02	3.2 ± 0.05	2.5 ± 0.02	2.0 ± 0.05	1.6 ± 0.02	1.0 ± 0.02	0.3 ± 0.04
FLM	0 ± 0.02	0 ± 0.02	0 ± 0.02	4.0 ± 0.04	3.5 ± 0.02	3.1 ± 0.05	2.6 ± 0.02	2.0 ± 0.05	1.5 ± 0.03	0.8 ± 0.02	0.3 ± 0.02

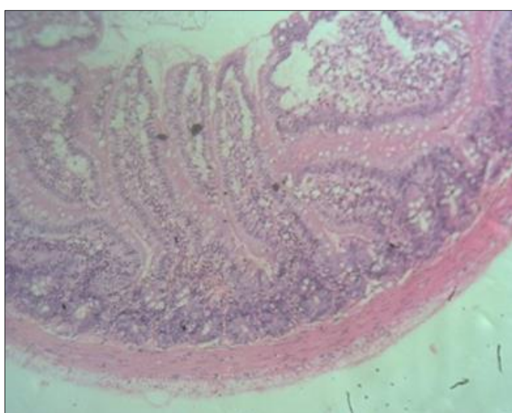
Table 6 Colon to Body Weight Ratio*

Sr. No.	Compound	Colon to body weight ratio ($^{w}/w$) ± S.D.
1	Normal control	0.006 ± 0.0004
2	Diseases control	0.04 ± 0.0005
3	Standard	0.009 ± 0.0006
4	flurbiprofen	0.011 ± 0.0002
5	FLI	0.010 ± 0.0004
6	FLS	0.008 ± 0.0006
7	FLL	0.009 ± 0.0001
8	FLM	0.009 ± 0.0006

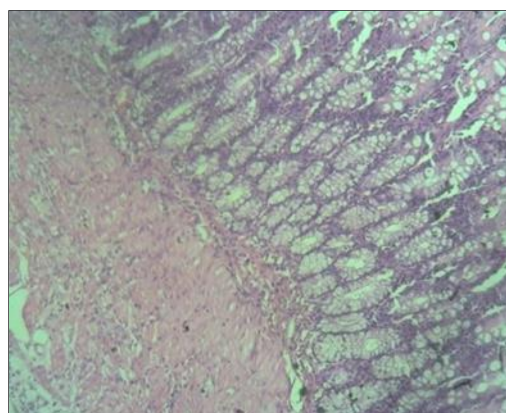
Table 7 Histopathology Report (Microscopic study)

Microscopic Study				
Compounds	**Mucosa**	**Inflammatory infiltrate**	**Mucus**	**Necrosis**
Normal Control	Flattened	----	Absent	-----
Diseases Control	Ulcerated	+++	+++	+++
Standard	Flattened	+	Absent	+
flurbiprofen	Flattened	++	Scanty	++
FLI	Flattened	+	Absent	+
FLS	Flattened	+	Absent	+
FLL	Flattened	+	Absent	+
FLM	Flattened	+	Absent	+

3.8. Histological evaluation



Normal Control



Disease Control

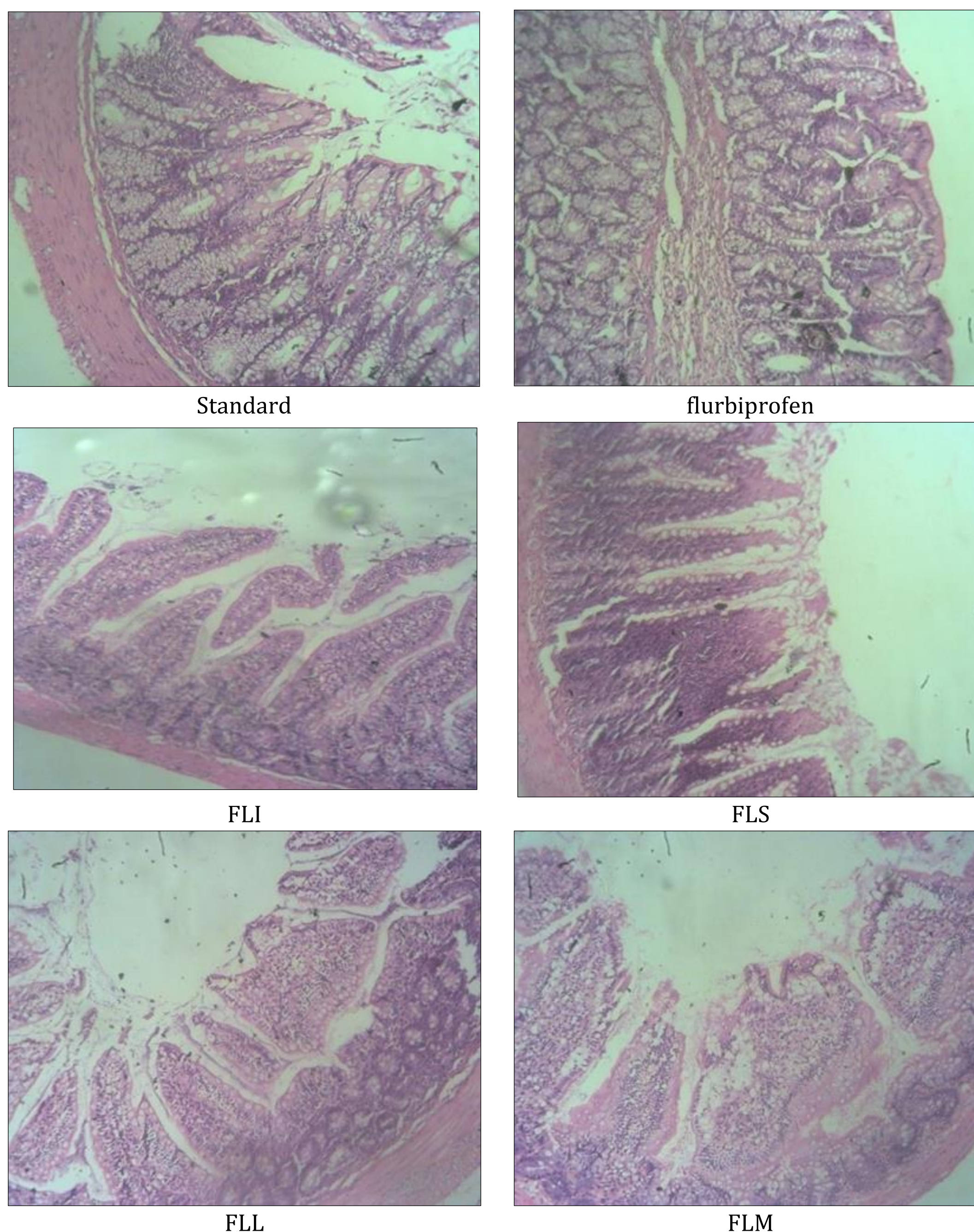


Figure 3 Histological examination of rat colon for all groups

Targeting for the treatment of IBD is comparatively difficult with conventional drugs because of lack of concentration of active drug reaching at this site. Oral treatment of IBD is full of riddle of limitations including first pass metabolism, which reduces the potency of active ingredient, toxic effect on liver and other organ and unnecessary delay of drug avalibility at the site of action. To target the site specific delivery of drug, prodrug is considered as the novel approach. This methodology has been efficiently utilized in sulfasalazine. Sulphasalazine is the most prescribed drug of choice for the treatment of IBD. This drug is formed by the azo conjugation sulfapyridine and 5-ASA. The 5-ASA is an active moiety which is used to cure the disease, sulfapyridine act as transporter and protects 5- ASA from the acidic pH of stomach and prevents its absorption from smallintestine, delivering it to colon. Several adverse reactions have been reported - associated with sulfasalazine due to sulfapyridine. Thus, there was the need of revisit to explore some alternative for the betterment of this chronic pathology. We proposed this investigation for revealing the new synthsized azo prodrug of flurbiprofen which increase the potency of approach and decrease the associated adverse effects. Different class of amino acids which have synergestic effect with flurbiprofen for the treatment of UC and safely used in IBD, have been selected for synthesis of azo prodrug with fenoprofe. These azo prodrugof different classes has been further evaluated against physicochemical property, *in vitro* stability test and *in vivo* ulcer models in rats. The associated consequence

reveals the successful synthesis of azo prodrug and also explored the significant therapeutic potential against TNBS induced UCmodel. Thin layer chromatography of all the intermediates and final compounds were performed on silica gel plates, using UV and iodine chamber for visualization of spots andsingle spots were obtained for the same,. Open capillary method was used to determine the melting points of the final products, which was found to be uncorrected. The objective of synthesizing such azo prodrugs of flurbiprofen which have improved aqueous solubility and lower log P values than that of flurbiprofen has been achieved. Such high values for aqueous solubility of these novel prodrugs confirms that they will reach intact into colon and will not get absorbed in the upper GIT.

The IR spectra of the synthesized compounds show absorption bands in the range of 1500 to 1490 cm^{-1}, for unsymmetrical p-substituted azo bond, which is characteristic of the anticipated structures of synthesized compounds. These results confirm the formation of mutual azo prodrugs and also the accuracy of the anticipated structures drawn for these novel prodrugs.

The prodrug treated groups showed a distinct decrease in the colon to body weight ratio compared to colitis control group. The difference between sulfasalazine & synthesized prodrugs was not significant. In colitis control group, the colons were filled with liquid stooland appeared to be flaccid. The colon, cecum and rectum all parts were having evidence of mucosal congestion, erosion and hemorrhagic ulcerations. Histopathological features of this group, included transmural necrosis, edemaand absence of epithelium. There was massive mucosal and submucosal infiltration of inflammatory cells. These all physical and microscopical alteration has also been evaluated in standard (sulfasalazine) and all other azo prodrug groups, and significantly compared with colitis control.

4. Conclusion

This evaluation confirmed that azo prodrugs significantly protect against TNBS induced colitis and show the similar results with sulfasalazine group. This finding proves the therapeutic potential of azo prodrug against ulcerative colitis as similar or better to sulfasalazine.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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