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Oral colon targeted delivery systems for treatment of inflammatory bowel diseases: Synthesis, in vitro and in vivo assessment

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ABSTRACT

The aim of this study was to investigate the potential of prodrugs of some non-steroidal anti-inflammatory drugs (NSAIDs) as colon targeted delivery systems for treatment of inflammatory bowel diseases. Naproxen, sulindac and flurbiprofen (Fbp) were used. The carboxylic group of those drugs was conjugated onto the amino group of L-aspartic acid or the hydroxyl group of α- or β-cyclodextrin (CyD). Prodrugs hydrolysis in buffers of pH range 1.2–7.2 and in rat gastrointestinal tract homogenates and the effect of oral pretreatment of rats with clindamycin on the hydrolysis of the prodrugs was examined. Additionally, the effect of oral administration of Fbp-β-CyD prodrug on the experimentally induced colitis in rats was evaluated. The in vivo inflammatory response was assessed macroscopically, histologically and by measurement of reduced glutathione (GSH) levels in colon tissues. No significant hydrolysis of the proposed seven prodrugs in buffers having pH range of 1.2–7.2 was observed over 72 h. Negligible % of drug released from Fbp-α-CyD or Fbp-β-CyD prodrugs was detected in rat stomach contents, intestinal tissues and intestinal contents homogenates. On the other hand, Fbp-α-CyD and Fbp-β-CyD prodrugs released about 60% Fbp within 4 h in rat colon homogenate. Oral pretreatment of rats with clindamycin significantly reduced % Fbp released from Fbp-α-CyD or Fbp-β-CyD prodrugs. Oral administration of Fbp-β-CyD to rats after induction of colitis significantly attenuated the severity of the colonic injury and reduced the score of the macroscopic and microscopic damage. Additionally, there was a significant increase in the level of GSH. The present study provided an evidence that Fbp-β-CyD prodrug may be beneficial in treatment of inflammatory bowel disease.

1. Introduction

Oral colon-specific drug-delivery systems have recently gained importance for delivering a variety of therapeutic agents. The major obstacles to delivering drugs to the colon are the absorption and degradation pathways in the upper GIT. Research interest in the area of colonic delivery has been fuelled by the need to better treat pathologies of the colon. These pathologies range in seriousness from constipation and diarrhea to the debilitating inflammatory bowel diseases (ulcerative colitis and Crohn's disease) through to colon carcinoma, the third most prevalent form of cancer in both men and women (Ibekwe et al., 2004). Targeted drug delivery to the colon would therefore, ensure direct treatment at the disease site and, consequently, lower dosing and reducing systemic side effects. A variety of approaches have been used and systems have been developed for the purpose of achieving colonic targeting. One of the most common approaches is the prodrugs. For colonic delivery of drugs, prodrugs are designed to undergo minimal absorption and hydrolysis in the upper GIT and undergo enzymatic hydrolysis in the colon, releasing the active drug moiety from the carrier (Sinha and Kumria, 2003). The most commonly used naturally occurring colon-targeting carriers are polysaccharides such as cyclodextrins (CyDs), (Anekant et al., 2007; Chourasia and Jain, 2004) and amino acids such as aspartic acid (Jung et al., 2001). CyDs are cyclic oligosaccharides consisting of 6–8 glucose units attached through α-1,4 glucosidic bonds. CyDs are neither hydrolysed nor absorbed from the stomach and small intestine. However, in the colon the vast microflora present breaks these into small saccharides and thus, are absorbed in the large intestine (Sinha and Kumria, 2001). The biodegradable properties of CyDs and L-aspartic acid are useful as a colon-targeting carrier. Thus, prodrugs in which a drug is covalently bound to these enti-

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ties may serve as a source of site-specific delivery of drugs to colon.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of chronic inflammatory states. In addition, they showed a promising activity for prevention and treatment of colitis and colon cancers (Myers et al., 2001; Yamazaki et al., 2002; Jung et al., 2005). However, when they are administered orally, a large amount of the drug is absorbed from the upper GIT, and causes systemic side effects. Therefore, it is preferable to deliver the drug site-specifically to the colon.

In the present investigation, the potential of naproxen, sulindac and flurbiprofen (Fbp) covalent prodrugs (either as aspartate or CyD) as colon delivery system for treatment of inflammatory bowel diseases was explored. Those prodrugs were synthesized by reacting the carboxylic group of NSAIDs and either the amino group of aspartic acid or the hydroxyl group of α- or β-CyD. The effect of pH and rat GIT homogenates on the in vitro hydrolysis of the proposed prodrugs was examined. The effect of oral pretreatment of rats with antibiotic on the release Fbp from its CyD-prodrugs was evaluated. Additionally, the effect of oral administration of the prodrug, Fbp-β-CyD on the experimentally induced acetic acid colitis in rats was assessed using macroscopic, microscopic and biochemical studies.

2. Materials and methods

2.1. Materials

Naproxen (1), sulindac (2), Fbp (3), L-aspartic, α-CyD (α-CyD), β-CyD (β-CyD), reduced glutathione (GSH) and 5,5-dithio-bis(2-nitrobenzoic acid) were purchased from Sigma (St. Louis, MO). DIAION HP-20 was obtained from Mitsubishi Chemical Co. (Tokyo, Japan). All other chemicals were of analytical grade, used without further purification and were obtained from Aldrich Chemical Co. (Milwaukee, WI).

2.2. Methods

2.2.1. Synthesis

Melting points were determined on a Mettler FP80 melting point apparatus (Mettler Toledo, USA) and are uncorrected. Conjugates were purified by ion-exchange chromatography using DIAION HP-20. Solvents used for extraction were dried over $MgSO_4$, filtered and distilled. Elemental analyses was performed on a PerkinElmer apparatus (PerkinElmer, USA). ^{1}H NMR spectra were obtained in DMSO-d_6 at 500 MHz, Brucker instrument (Brucker company, USA), with TMS as an internal standard. Compounds **10** and **11** (Scheme 2) were synthesized according to literature citation (Uekama et al., 1997).

General procedure for the synthesis of 2-[2-(6-methoxy-naphthalen-2-yl)-propionylamino]-succinic acid (**5**), 2-{2-[6-fluoro-3-(4-methanesulfinyl-benzylidene)-2-methyl-3H-inden-1-yl]-acetyl-amino}-succinic acid (**6**), and 2-[2-(2-fluoro-biphenyl-4-yl)-propionylamino]-succinic acid (**7**).

To a stirred solution of L-aspartic acid (**4**, 2.66 mg, 0.02 mol), and the appropriate NSAID (**1**–**3**, 0.01 mol) in dry CH_2Cl_2 (25 ml), dicyclohexylcarbodiimide (DCC) (3.24 mg, 0.015 mol) was added at room temperature. The mixture was stirred for 24 h then filtered and evaporated to dryness under vacuum. The obtained residue was extracted with ethyl acetate (50 ml ×3), dried over anhydrous sodium sulphate, filtered, evaporated and then purified by the use of column chromatography using 10% ethyl acetate/*n*-hexane as eluting system to give **5**–**7** (Table 1). ^{1}H NMR (DMSO-d_6), **5**: δ 7.77–7.73 (t, 2H), 7.66 (s, 1H), 7.41–7.39 (d, 1H), 7.17–7.15 (d, 1H), 7.13 (s, 1H), 4.13–4.09 (d, 2H), 3.93 (s, 3H,), 3.63–3.62 (t, 1H), 1.96–1.94 (d, 1H), 1.54–1.53 (d, 3H). **6**: δ 7.74–7.72 (d, 2H), 7.67–7.66 (d, 2H), 7.16–7.14 (t, 2H), 6.88–6.86 (t, 1H), 6.58–6.54 (m, 1H), 4.03 (s, 1H), 2.82 (s, 3H), 2.20 (s, 3H), 1.95 (s, 2H), 1.83–1.82 (d, 2H). **7**:δ 7.52–7.51 (d, 2H), 7.45–7.40 (m, 3H), 7.38–7.35 (t, 1H), 7.22–7.18 (t, 2H), 4.09–4.03 (m, 2H), 3.65–3.60 (m, 1H), 2.01–1.95 (m, 1H), 1.51–1.50 (d, 3H), *m*/*z* (359, 19%).

Table 1
Physicochemical properties of the synthesized prodrugs **5**–**7** and **12**–**15**

Prodrug	Solvent	Yield (%)	MP (°C)	Molecular formulae
5	Ether/CH_2Cl_2	83	184–185	$C_{18}H_{19}NO_6$
6	Ether	72	180–181	$C_{24}H_{22}FNO_6S$
7	EtOAc/hexane	79	231–232	$C_{19}H_{18}FNO_5$
12	Acetone/H_2O	70	158–195	$C_{40}H_{72}O_{32}$
13	MeOH/H_2O	68	146–147	$C_{56}H_{82}O_{37}$
14	Acetone/H_2O	30	197–198	$C_{51}H_{71}FO_{31}$
15	Acetone/H_2O	30	138–139	$C_{57}H_{81}FO_{36}$

General procedure for the synthesis of 6^A-*O*-[2-(6-methoxy-naphthalen-2-yl)-propionyl]-α-CyD (**12**), 6^A-*O*-[2-(6-methoxy-naphthalen-2-yl)-propionyl]-β-CyD (**13**), 6^A-*O*-[2-(2-fluoro-biphenyl-4-yl)-propionyl]-α-CyD (**14**), and 6^A-*O*-[2-(2-fluoro-biphenyl-4-yl)-propionyl]-β-CyD (**15**).

Sodium salt of the appropriate NSAID (1, 3, 0.01 mol) was added to the tosylated α-CyD (9.9 g, 0.01 mol) or the tosylated β-CyD (12.0 g, 0.01 mol), in dimethylformamide (DMF, 100 ml), and the mixture was stirred at 100 °C for 48 h. The reaction solution was concentrated under reduced pressure, a large amount of acetone (1000 ml) was added, and the precipitate was collected under vacuum. The solid was dissolved in DMF (10 ml) and the solution was added to about 100 ml of acetone. This procedure was repeated several times. The conjugate was then purified by ion-exchange column chromatography (DIAION HP-20) eluted with methanol/water mixture with gradual increase of methanol content. The elutes were monitored by TLC, and the prodrugs appeared in the 20–40% methanol in water fraction. Methanol was removed under reduced pressure, and the aqueous solution was lyophilized to yield the purified conjugate **12**–**15** (Table 1). ^{1}H NMR (DMSO-d_6), **12**: δ 7.74–7.79 (m, 2H), 7.69 (s, 1H), 7.37–7.39 (d, 1H, J = 3.5 Hz), 7.26 (s, 1H), 7.13–7.15 (dd, 1H, J = 2.5, 6.5 Hz), 5.43–5.50 (m, 12H, CyD, 2,3-OH), 4.78 (s, 6H, CyD, 1H), 4.47 (s, 5H, CyD, 6-OH), 3.85 (s, 3H, OCH3), 3.74–3.80 (m, 13H, CyD; 3,5-H, Nap CH), 3.56–3.64 (m, 12H, CyD; 6H), 3.27–3.40 (m, 12H, CyD; 2,4, H overlap with solvent peak), 1.42–1.44 (d, 3H, CH3; J = 6.0 Hz). **13**: δ 7.86–7.87 (d, 1H, J = 8.0 Hz), 7.72–7.77 (m, 1H), 7.69 (s, 1H), 7.43–7.45 (d, 1H, J = 8.5 Hz), 7.26 (s, 1H), 7.12–7.14 (dd, 1H, J = 2.0, 8.0 Hz), 3.85 (s, 3H, OCH3), 3.70–3.71 (q, 1H, J = 6.0 Hz), 3.48 (s, 14H, CyD, 2,3-OH), 1.51–1.76 (m, 25H, CyD), 1.37–1.38 (d, 3H, CH_3; J = 6.5 Hz), 1.08–1.25 (m, 30H, CyD). **14**: δ 7.53–7.54 (d, 2H, J = 6.0 Hz), 7.46–7.49 (t, 3H, J = 7.5 Hz), 7.40–7.41 (d, 1H, J = 7.5 Hz), 7.22–7.24 (t, 2H, J = 6.5 Hz), 5.50 (s, 12H, CyD, 2,3-OH), 4.80 (s, 6H, CyD, 1H), 4.50 (s, 5H, CyD, 6-OH), 3.74-3.80 (m, 13H, CyD; 3,5-H, CH), 3.58-3.68 (m, 12H, CyD; 6-H), 3.27–3.40 (m, 12H, CyD; 2,4,-H overlap with solvent peak), 1.40–1.41 (d, 3H, CH3; J = 6.0 Hz). **15**: δ 7.43-7.44 (d, 2H, J = 7.0 Hz), 7.44–7.47 (t, 3H, J = 7.0 Hz), 7.39–7.41 (t, 1H, J = 7.5 Hz), 7.21–7.23 (t, 2H, J = 6.0 Hz), 5.70 (s, 14H, CyD, 2,3-OH), 4.83 (s, 7H, CyD, 1-H), 3.75–3.79 (m, 15H, CyD; 3,5-H, CH), 3.57–3.67 (m, 14H, CyD), 3.31–3.39 (m, 20H, CyD), 1.40–1.41 (d, 3H, CH_3; J = 6.0 Hz).

2.2.2. High-performance liquid chromatography (HPLC) assay of NSAIDs

The free sulindac (Swanson and Boppana, 1981), naproxen (Rozou and Antoniadou-Vyza, 2004) and Fbp (Sajeev et al., 2002) were assayed using HPLC assay. Calibration curves were constructed from peak-area of standard concentrations of pure drug. The chromatographic mobile phase consisted

of 63:37 (v/v) methanol:0.4 N sodium acetate pH 4; 40:60 (v/v) methanol:phosphate buffer pH 7 and 40:20:40 v/v methanol:acetonitrile:phosphate buffer pH 5.6 for sulindac, naproxen and Fbp, respectively. The flow rates and detection wavelengths were 1.2, 1 and 0.75 ml/min and 254, 230 and 248 nm for sulindac, naproxen and Fbp, respectively. The operating temperature was ambient and the injected volume was 20 μl. HPLC system (Shimadzu, Japan) consisted of Shimadzu pumping system LC-20AT, Rheodyne injector with 20 μl loop and UV–vis spectrophotometric detector SPD-20A, data recording unit, Shimadzu LC solution software, Version 1.1. Copyright® 1998–2003. μ Bondapak™ C_{18} Column (3.9 × 150 mm with particle size of 4 μm) (Waters, USA) was used.

2.2.3. In vitro evaluation of the proposed prodrugs

2.2.3.1. Hydrolysis of prodrugs in aqueous buffer solution. The study was carried out by incubating 200 μl of prodrug solutions (10 mg/ml methanol) with 2 ml of: artificial gastric juice without pepsin (2 g sodium chloride and 80 ml HCl 1 M in 1 L of water); acetate buffer pH 4.5; simulated duodenal fluid (pH 6.8 without pancreatin), and phosphate buffer pH 7.2. The solutions were incubated at 37 °C and shaken at 50 rpm and sampled at specified time intervals up to 72 h. The samples were analyzed by HPLC assay for free drug contents. Each experiment was performed in triplicate.

2.2.3.2. Hydrolysis of prodrugs after incubation with biological homogenates. Male rats about 250 ± 25 g without starvation were anesthetized by diethyl ether and midline incision was made. The rat stomach and small intestine segments were extracted. The small intestine contents were collected in a vial and the small intestine tissues were cut into small pieces, and both were homogenized separately in 50 mM isotonic phosphate buffer (pH 6.8) using a tissue homogenizer (Ultra Turrax® T18 basic, IKA Works Inc., Wilmington, NC) at 4 °C for 2 min to give homogenate of final concentration of 20%. The stomach contents were also homogenized and diluted with cold isotonic acetate buffer (pH 4.5). The homogenates were filtered through gauze to remove large particulates. The solution of prodrug (200 μl) of 10 mg/ml in propylene glycol was added to the filtrates (1 ml) and incubated at 37 °C. Colon tissues were collected separately in vials containing cold isotonic phosphate buffer pH 7.2 previously flushed with nitrogen. The mixture was homogenized under stream of nitrogen at 4 °C to form 20% homogenate. Portions of 1 ml of colon homogenate and 200 μl of the prodrug solution (10 mg/ml in propylene glycol) were added to each vial and the mixture was incubated at 37 ± 0.5 °C under anaerobic conditions. The anaerobic conditions were simulated by using airtight vessels containing gas-generating kits for anaerobic system (Oxoid Ltd., Wade road, Basingstoke, Hants, UK). At appropriate intervals samples were withdrawn and centrifuged at 5000 rpm for 5 min. To 1 ml portion of supernatant 1 ml of methanol was added, vortex-mixed for 1 min and filtered through a Millipore membrane filter (0.45 um). The concentration of free drug was determined by HPLC under the aforementioned conditions. Each experiment was performed in triplicate.

Oral pretreatment of six rats with clindamycin was performed as follows: six rats each received 25 mg clindamycin/kg body weight suspended in 0.5 ml of 1% sodium carboxymethyl cellulose (Na CMC). Clindamycin was given twice daily for 3 days before sacrificing the animal and separation of colon tissues. Blanks for all of the incubations were obtained by mixing corresponding amounts of homogenate without addition of prodrug.

2.2.4. In vivo evaluation of Fbp β-CyD prodrug

2.2.4.1. Induction of experimental colitis in rats. The induction of colitis was performed using acetic acid method reported by Millar et al., 1996 with little modification. The study was conducted in accordance with the standards of care and use of laboratory animals established by King Saud University. Rats were fasted for 24 h, with access to water ad libitum before the induction of colitis. Each rat was sedated by inhalation of ether. Two milliliters of acetic acid (3% (v/v) in 0.9% saline) was infused for 30 s using a polyethylene tube (2 mm in diameter). The tube was inserted through the rectum into the colon, to a distance of 8 cm. The acetic acid was retained in the colon for 30 s, after which the fluid was withdrawn.

2.2.4.2. Treatment of experimentally induced colitis. Thirty-two adult male albino rats (180 ± 20 g) were used throughout the study. The animals were randomly divided into four groups, each consists of eight animals: normal control group, acetic acid group, vehicle treated group (1% Na CMC), and prodrug treated group. The treated group received prodrug **15**, β-CyD-Fbp, in a dose equivalent to 20 mg Fbp /kg body weight. The prodrug was suspended in 0.5 ml of 1% Na CMC and administered orally. The doses were given once daily for 5 consecutive days. Food and water were allowed through out the period of treatment. After 5 days the rats were sacrificed and the colon segments were removed, and the contents were removed gently. The damage of the colon specimen was assessed by macroscopic scoring, histological examination, and determination of reduced GSH. The weights of rats were recorded though out the period of experiment.

2.2.4.3. Assessment of colitis.

2.2.4.3.1. Macroscopic scoring. Colon (6 cm), extending proximally for 2 cm above the anal orifice, was removed. The tissue was split longitudinally. The macroscopic appearance of the colonic mucosa was scored according to arbitrary scale ranging from 0 to 4 as follows: 0 = no macroscopic change, 1 = mucosal erythema, 2 = mild mucosal edema, slight bleeding or small erosion, 3 = moderate edema, bleeding ulcers or erosions, 4 = severe ulceration, erosion, edema and tissue necrosis (Millar et al., 1996).

2.2.4.3.2. Histopathological study. Full thickness biopsy specimens were fixed in 10% formalin phosphate buffer solution (pH 7.4) prior to embedding, sectioning and staining with haemotoxylin. Histological evaluation of colonic damage was assessed by light microscope equipped with digital camera (Motic China Group Co. Ltd., China).

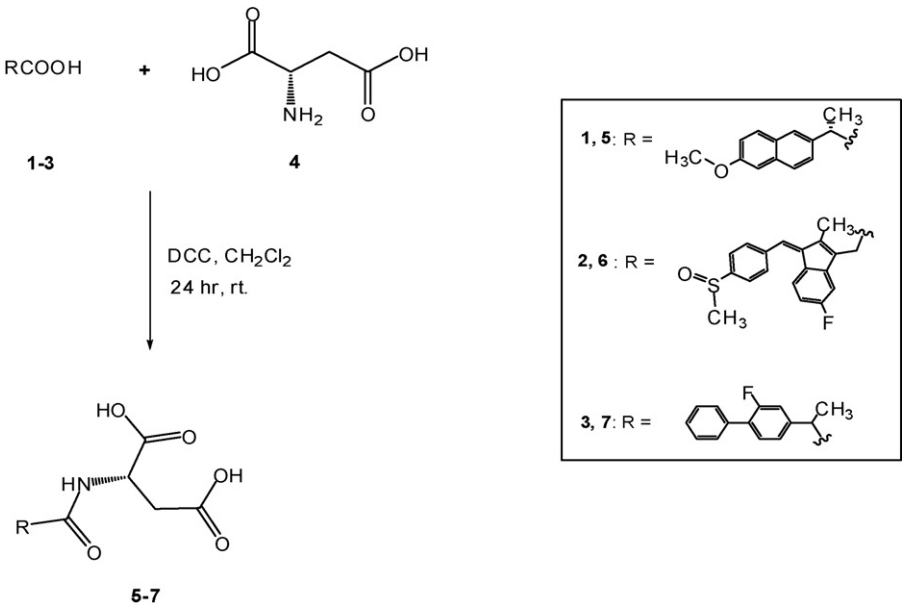
2.2.4.3.3. Determination of reduced GSH. Colonic tissue samples were homogenized in ice cold 10 mmol/L Tris–HCL buffer of pH 7.2, and centrifuge at 2500 rpm for 10 min at 4 °C. The supernatant was used for the measurement of GSH. Reduced GSH was determined using the method described by Owens and Belcher, 1965 which is based on the reaction of 5,5-dithiobis-(2-nitrobenzoic acid) with GSH present in the tissue. The absorbance was measured spectrophotometrically at 412 nm. The amount of GSH present in the sample was calculated using a standard of GSH containing 1 mg of GSH/ml of 3% metaphosphoric acid. The increase in the extinction at 412 nm is proportional to the amount of GSH present.

2.2.5. Statistical analysis

Student *t*-test was used to test the differences between the calculated parameters using SPSS Statistical Package (Version 10, SPSS Inc., 1999, USA). Statistical differences yielding $p \leq 0.05$ were considered to be significant.

3. Results and discussion

The strategy of synthesis of the target prodrugs is depicted in Schemes 1 and 2. The acid drugs (naproxen, sulindac and Fbp (**1**–**3**)) were allowed to react with aspartic acid (**4**) in



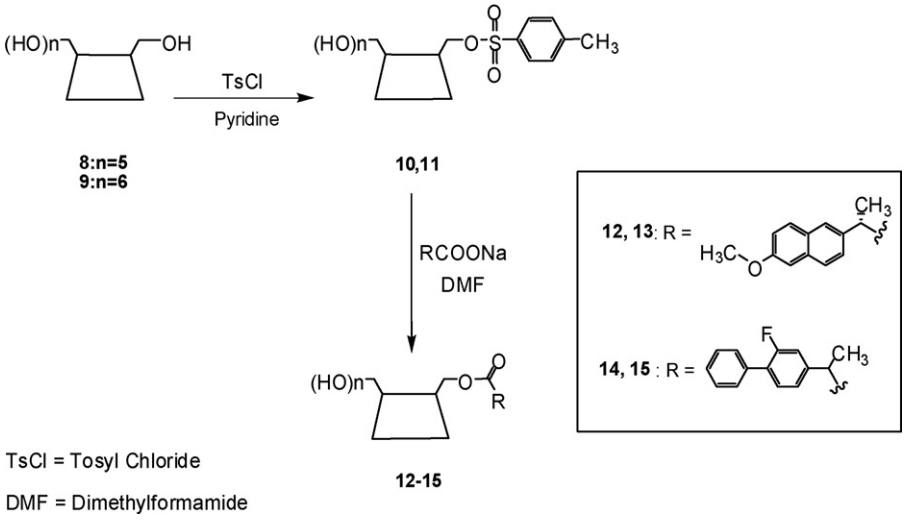
Scheme 1. .

presence of DCC as catalyst to obtain 2-[2-(6-methoxy-naphthalen-2-yl)-propionylamino]-succinic acid (**5**), 2-{2-[6-fluoro-3-(4-meth-anesulfinyl-benzylidene)-2-methyl-3*H*-inden-1-yl]-acetyl-amino}-succinic acid (**6**), and 2-[2-(2-fluoro-biphenyl-4-yl)-propionylamino]-succinic acid (**7**), respectively. On the other hand, the two NSAIDs (**1**, **3**) were also allowed to react with the tosylated α- or β-CyD (**10**, and **11**) in DMF to afford 6^A-*O*-[2-(6-methoxy-naphthalen-2-yl)-propionyl]- α- or β-CyD (**12** and **13**), and 6^A-*O*-[2-(2-Fluoro-biphenyl-4-yl)-propionyl]-α- or β-CyD (**14**, **15**). Compounds **10** and **11** were previously synthesized according to literature citation (Uekama et al., 1997). All of the obtained new prodrugs (**5**–**7**, and **12**–**15**) were identified on the basis of elemental analyses and NMR data.

3.1. In vitro hydrolysis of the proposed prodrugs

The colon targeting prodrug must pass through the stomach and the small intestine, to reach the colon as an intact form, and to be degraded by enzymes of colonic flora. These enzymes act as triggers for releasing drug molecules from prodrug. Long transit time encountered in the colon also gives sufficient time for action of these enzymes on the prodrug substrates (Friend, 2005). Therefore, the drug release behavior from the prodrugs **5**–**7** and **12**–**15** in buffers having the pH range of GIT and in rat biological homogenates was evaluated. It is worth mentioning that, the applied HPLC assays were specific for each free drug and the corresponding prodrugs were not detected under the adapted conditions.

The results of in vitro hydrolysis studies revealed that none of the prodrugs succeeded to release the carried drugs for over 72 h at 37 ± 0.5 °C in aqueous buffers (pH 1.2, 4.5, 6.8 and 7.2) under the current experimental conditions. This indicated that the prepared prodrugs were chemically stable in the pH environment of the GIT. The same result was obtained when the proposed prodrugs were incubated with homogenate of rat stomach contents. On other hand, there were negligible percentage of Fbp (**3**) released in small intestine and small intestine contents homogenates from Fbp-α-CyD (**14**) and Fbp-β-CyD (**15**) as



Scheme 2. .

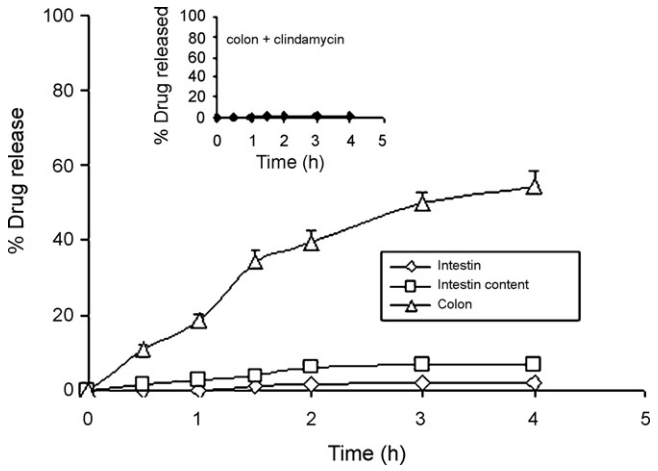


Fig. 1. Time course of flurbiprofen appearing in the medium during incubation of the prodrug **14** (Fbp-α-CyD) with rat gastrointestinal homogenates. The inserted figure shows the effect of oral pretreatment of rats with 25 mg/kg clindamycin twice daily on % Fbp released from prodrug **14**. $n = 3$.

shown in Figs. 1 and 2. However, in the rat colon homogenate, only prodrugs **14** and **15** released Fbp (**3**) gradually, being about 60 % released within 4 h, the other prodrugs **5**–**7**, **12** and **13** did not show any sign of hydrolysis to release the carried drug at this stage. These results suggested that Fbp-CyD prodrugs hydrolysis takes place in the colon and the resulting small saccharide ester prodrugs are hydrolyzed to free Fbp (**3**) by the action of enzymes produced by microflora in rat colon as was reported previously by Hirayama and Uekama, 1999. It is worth mentioning that, there was no significant difference ($p > 0.05$) detected in percentage of Fbp (**3**) released form α or β-CyD-Fbp prodrugs as shown in Figs. 1 and 2.

The failure of prodrugs **5**–**7**, **12** and **13** to release their own carried drugs could be explained by comparing the structures of the three used drugs **1**–**3**, which contain naphthalen, inden, and biphenyl groups, respectively. The comparison revealed that both naphthalen, and inden are two bulky moieties surrounding the amide functions (prodrugs **5** and **6**), and naphthalene surrounding

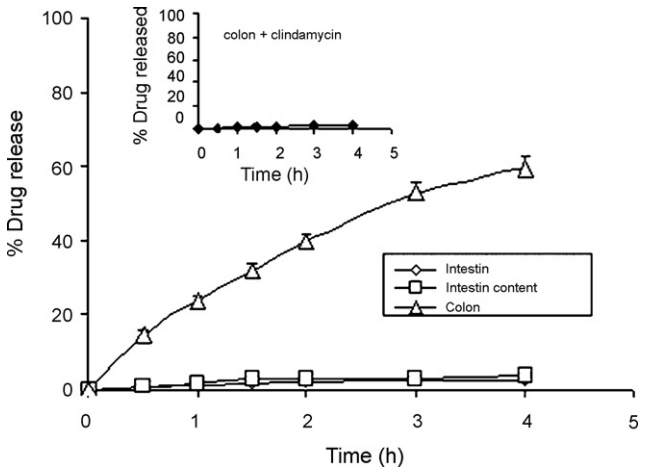


Fig. 2. Time course of flurbiprofen appearing in the medium during incubation of the prodrug **15** (Fbp-β-CyD) with rat gastrointestinal homogenates. The inserted figure shows the effect of oral pretreatment of rats with 25 mg/kg clindamycin twice daily on % Fbp released from prodrug **15**, $n = 3$.

the ester groups (prodrugs **12** and **13**), needed to be hydrolyzed, causing stric hindrance preventing the enzymatic attack. Biphenyl group is less bulky and the ester and amide functions are more easily accessible to the enzymes. Only the esters in **14** and **15** prodrugs containing the biphenyl moieties get hydrolyzed easily, while the biphenyl amide in prodrug **7** did not cleave because of the steric effect of the two carboxylic groups of the succinic acid in the vicinity, in addition to the fact that esters are easier to hydrolyze than amides. This explanation was in agreement with Minami et al., 1998, who reported that the amide linkage may be resistant to hydrolysis by bacterial enzymes. What is left to discuss is the bulkiness of the CyD itself. This bulkiness was overcame by the ester group acting as spacer, giving a gap to facilitate the enzymatic action as appeared in case of prodrugs **14** and **15**.

As was indicated by the results, the hydrolysis of Fbp-α-CyD (**14**) or Fbp-β-CyD (**15**) prodrugs was preferentially occurred in colon due to the bacterial count in the colon that has been estimated to be 10^{11} per gram and consists of around 400 species being predominantly anaerobic in nature compared with 10^4 per gram in the proximal small intestine (Ibekwe et al., 2004). In addition, clinical studies have shown clear evidence that β-CyD (**9**) is poorly digested in the small intestine but is almost completely degraded by the colonic microflora (Flourie et al., 1993). Colonic bacteria are capable of degrading CyDs for carbon source by stimulating CyDase activity (Flourie et al., 1993; Gerloczy et al., 1985). The release of Fbp (**3**) from prodrugs **14** and **15** was in agreement with Zou et al., 2005, who reported significantly higher release of 5-ASA from CyDs-5 ASA in cecal and colonic contents than that in stomach and small intestine contents. These results were also in accordance with Uekama et al. (1997) and Minami et al. (1998), who reported the same behavior for α-, β- and γ-CyD with biphenyl acetic acid ester. Similarly, Kamada et al., 2002, reported that CyD conjugate of ketoprofen released ketoprofen only in rat cecum and colonic content.

Based on the obtained results Fbp-CyDs prodrugs were chosen to examine the effect of oral pretreatment of rats with clidamycin on the hydrolysis of **14** or **15** prodrugs. Clindamycin was known to inhibit the growth of aerobic and anaerobic bacteria (Guay, 2007). The oral pretreatment of rats with clindamycin resulted in a significant reduction of % Fbp (**3**) released from **14** or **15** prodrugs as shown in the inserted figures in Figs. 1 and 2. This result supported the hypothesis that normal flora in the colon tissue have a significant contribution to the hydrolysis of Fbp-α (**14**) or β-CyD (**15**) prodrugs. This result was in agreement with Nakamura et al., 1992, who reported that following oral pretreatment with kanamycin sulfate and trnidazole there was a significant inhibition of salicylic acid formation from salicyl-tyrosin and salicyl-methionine.

Based on the results of in vitro assessment studies prodrug **15** (Fbp-β-CyD) was chosen to be evaluated in vivo on rats after induction of colitis by acetic acid.

3.2. *In vivo evaluation of Fbp β-CyD prodrug*

Acetic acid-induced colitis was chosen as a method for ulcer induction because the formed ulcer resembles human ulcerative colitis in histology, eicosanoid production and excessive oxygen-derived free radicals release by inflamed mucosa (Millar et al., 1996).

Fig. 3 shows the macroscopic characteristics of colon 5 days after induction of colitis and 5 days after treatment with the prodrug **15** in comparison with normal colon. The acetic acid treatment induced severe macroscopic hemorrhage and inflammation in the colon after rectal administration (Fig. 3b) as assessed by the colonic damage score (2.5) as shown in Fig. 4. The acetic acid treated rats,

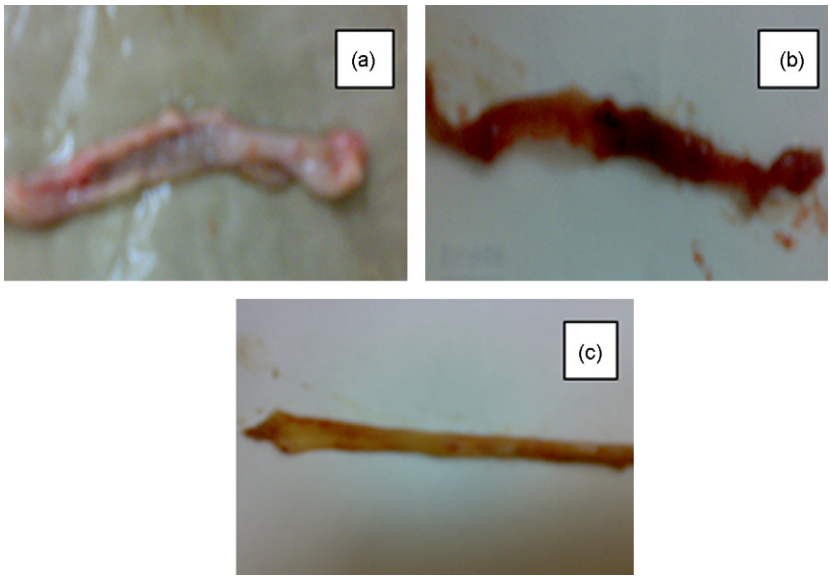


Fig. 3. Macroscopic appearance of (a) normal colon, (b) acetic acid treated colon and (c) acetic acid treated colon after oral administration of prodrug **15** (Fbp-β-CyD) once daily for 5 days. The dose of Fbp was equivalent to 20 mg/kg. $n = 8$.

which had received the vehicle, suffered from general weakness and less activity and a significant loss in body weight (Fig. 5). Treatment for 5 days with the prodrug **15** significantly reduced the severity of the hemorrhage (Fig. 3c) and gross lesion score (0.45) as shown in Fig. 4. In addition, no illness signs appeared on the rats and the loss in body weight was significantly prevented (Fig. 5).

The histopathological features of acetic acid treated rats included necrotic destruction of mucosa and submucosa, areas of hemorrhage and diffuse inflammatory cell infiltration in the mucosa (Fig. 6b). Five days after induction of colitis the group administered 1% Na CMC showed evidence of mucosal congestion, and hemorrhagic ulceration in mucosa and submucosa (Fig. 6c). In other word, it has the same histological features like the acetic acid treated group. Treatment of rats with the prodrug **15** significantly attenuated the extent and severity of the histological signs of cell damage (Fig. 6d). It has normal appearance and shows no signs of ulceration or bleeding. Histopathological studies confirmed the intestinal anti-inflammatory effect exerted by the prodrug **15** on inflamed colonic tissues.

Fig. 7 illustrates that the GSH concentration in the colon tissues was significantly decreased after induction of colitis as compared to normal control group ($p \leq 0.001$). This was in agreement with Koch et al., 2000, who reported that GSH levels were significantly depleted in ulcerative colitis compared to control colon. The depletion of GSH in ulcerative colitis may be related to inflammation (Koch et al., 2000; Ruan et al., 1997).

After treatment with the prodrug **15** for 5 consecutive days there was a significant increase in the GSH concentration in colonic tissues compared with acetic acid treated tissues ($p \leq 0.001$). It has been suggested that the elevated level of GSH may be attributed to the radical scavenging capacity of the released anti-inflammatory drug (El-Medany et al., 2005). Fbp has been reported to act as anti-inflammatory by direct inhibition of eicosanoid formation or by indirect inhibition of eicosanoid formation, by inhibiting expression of enzymes involved in eicosanoid synthesis (Myers et al., 2001). It is worth mentioning that, eicosanoids, such as prostaglandins, are molecules made by oxygenation of 20-carbon essential fatty acids. They exert complex control over many body reactions, mainly in inflammation.

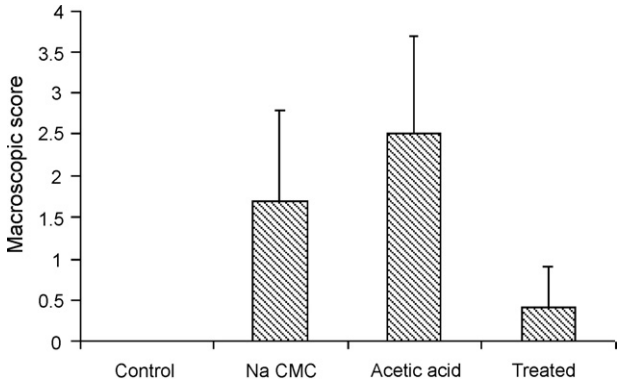


Fig. 4. Macroscopic scoring of rat colon biopsy of normal colon, acetic acid treated colon and acetic acid treated colon after oral administration of prodrug **15** (Fbp-β-CyD) once daily for 5 days. The dose of Fbp was equivalent to 20 mg/kg. $n = 8$.

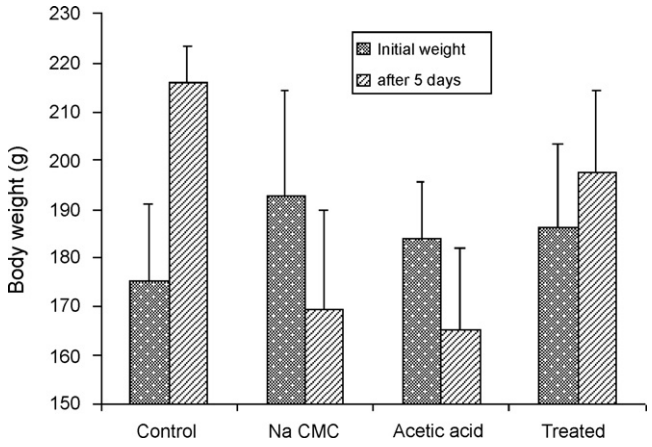


Fig. 5. The effect of acetic acid-induced-colitis on the body weight of rats before and after oral administration of prodrug **15** (Fbp-β-CyD) once daily for 5 days. The dose of Fbp was equivalent to 20 mg/kg. $n = 8$.

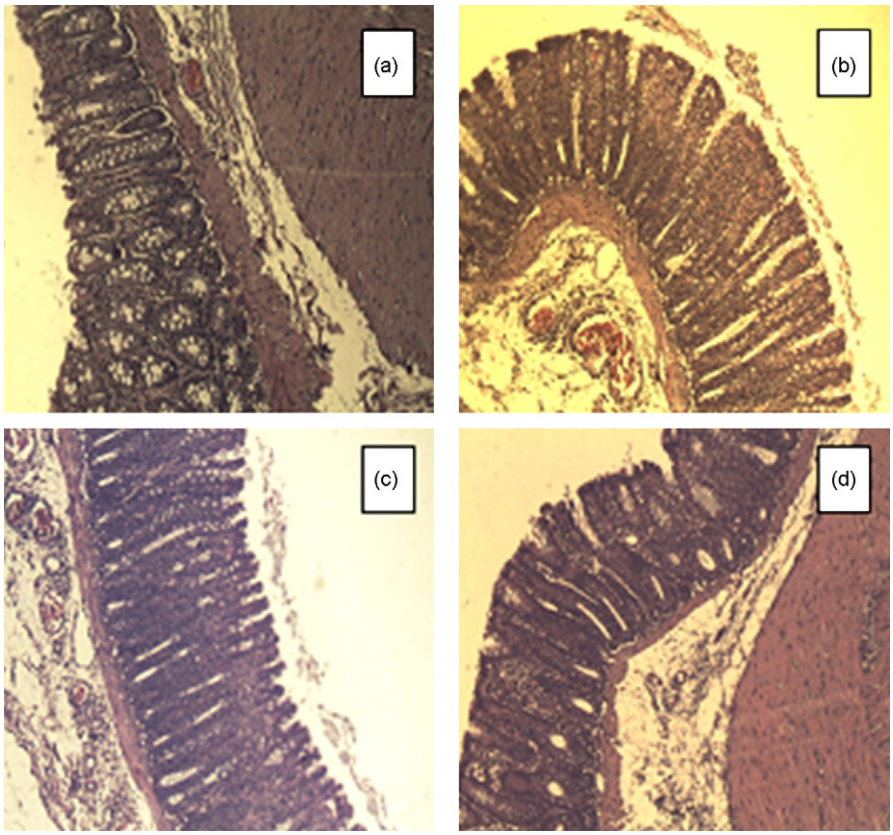


Fig. 6. Histological appearance of colonic tissues (a) normal, (b) acetic acid treated, (c) acetic acid treated after administration of 1% Na CMC for 5 days, and (d) acetic acid treated after oral administration of prodrug **15** (Fbp-β-CyD) once daily for 5 days. The dose of Fbp was equivalent to 20 mg/kg. $n = 8$.

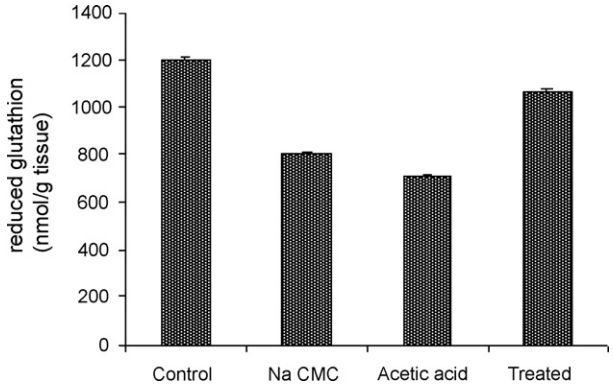


Fig. 7. Levels of reduced glutathione (GSH) in the colonic tissue of rats after acetic acid treatment; after administration of 1% Na CMC to acetic acid treated rats and after oral administration of prodrug **15** (Fbp-β-CyD) once daily to acetic acid treated rats for 5 days. The dose of Fbp was equivalent to 20 mg/kg. $n = 8$.

4. Conclusion

In conclusion, successful colonic delivery of prodrugs of NSAIDs requires careful consideration of number of factors, including the properties of the drug and the properties of colon targeting carrier in addition to the type of bond involved in prodrug synthesis. The present data suggested that CyDs, which are natural polysaccharides, can function as colon targeting carrier by covalent conjugation with Fbp. Fbp-CyDs prodrugs are chemically stable in the pH environment of the GIT and hydrolyzed to free Fbp by the action of enzymes produced by microflora in rat colon. Furthermore, Fbp-β-CyD prodrug showed a great potential in treatment of colitis induced by acetic acid since it significantly decreased the extent and severity of colonic damage induced by acetic acid.

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