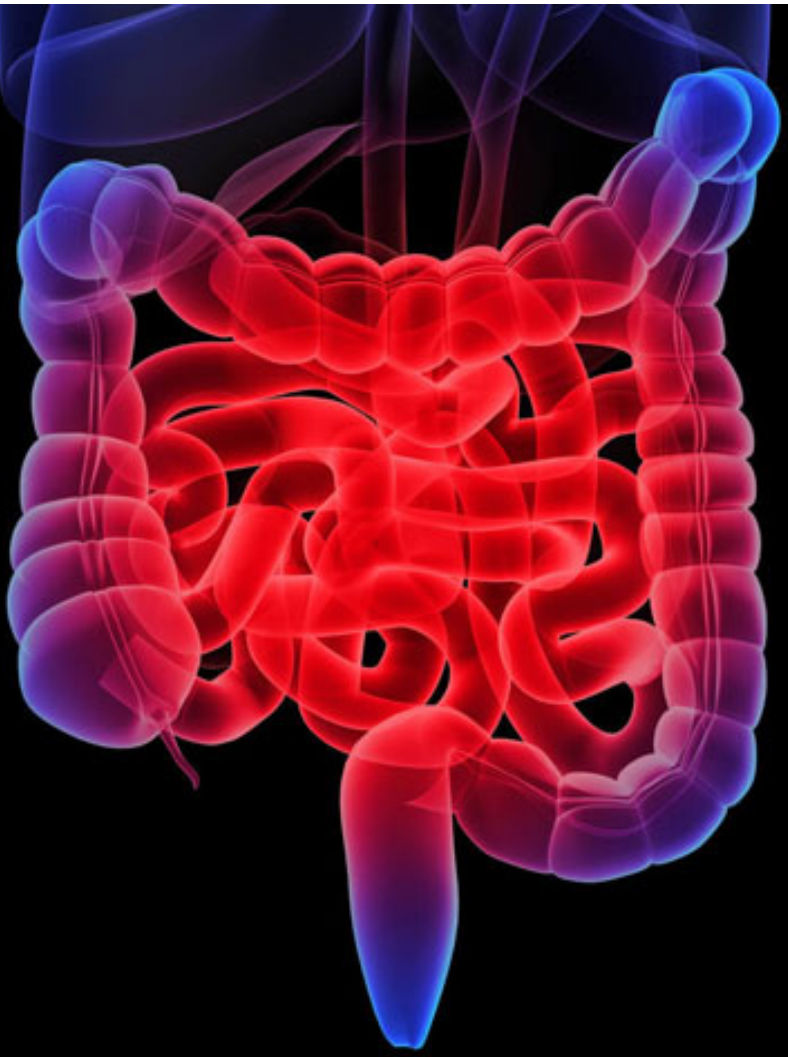

INFLAMMATORY BOWEL DISEASE

MEDDOCS
International

Design, Synthesis, Characterization and Pharmacological Evaluation of Novel Macromolecular Prodrug for the Treatment of Inflammatory Bowel Disease

Shakuntala Santosh Chopade; Suneela Dhaneshwar*

Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, India

Corresponding Author: Suneela Dhaneshwar, M Pharm, PhD

Professor, Department of Pharmaceutical Chemistry, Poona College of Pharmacy, Bharati Vidyapeeth University, Pune-411038, Maharashtra, India.

Director Amity Institute of Pharmacy, Deputy Dean Research Amity University Uttar Pradesh, Lucknow Campus, Lucknow-226028, UP, India.

Tel: +91-20-25437237, +91-2025436898,

Email: suneeladhaneshwar@rediffmail.com & sdhaneshwar1@lko.amity.edu

Published Online: May 15, 2018

eBook: Cervical Cancer

Publisher: MedDocs Publishers LLC

Online edition: http://meddocsonline.org/

Keywords: Prodrug; Inflammatory bowel disease; Mycophenolic acid; Mycophenolate mofetil; Macromolecular carrier

Abstract

Objective: The aim of present work was design, synthesis, characterization and pharmacological evaluation of colon-targeted macromolecular prodrug of mycophenolic acid for the effective management of IBD.

Methods: Macromolecular prodrug (MBS) was synthesized by tethering Mycophenolic Acid (MPA) with β-Cyclodextrin (β-CyD) using EDCI as coupling reagent. Structures were confirmed by IR, NMR, mass and elemental analysis. Release profile of MBS was extensively studied in aqueous buffers, upper GI homogenates, faecal matter and cecal homogenates (in vitro) and rat blood (in vivo). Pharmacological screening was performed using TNBS-induced colitis model in Wistar rats. Statistical evaluation was carried out by applying one-way and two-way ANOVA when compared with disease control.

Results: The in vitro studies confirmed that the prodrug of MPA with β-CyD (MBS) was stable in HCl and phosphate buffer over a period of 3 h and 7 h respectively, indicating negligible hydrolysis in the upper GIT. MBS prodrug showed 89% release of MPA in colon while 55% release in faecal matter. In vivo study revealed 75% release of MBS prodrug which clearly indicates that activation of prodrug was mediated by hydrolysis which in turn is catalysed by weak alkaline pH of blood (7.4) in combination with N-acyl esterases present in colon. The findings of TNBS-induced colitis model proved that this gastro-sparing prodrug showed comparable pharmacological profile to Mycophenolate Mofetil (MMF) with absence of MMF related side effects which was evidenced by significant lowering of clinical activity score. Moreover colon-to-body weight ratio was markedly reduced by prodrug-treated group (1.5times when compared to MPA) indicating mitigating effect on colonic inflammation.

Conclusion: This novel, macromolecular prodrug holds a potential to be used as promising therapeutic agent and thus can be used successfully for the treatment of UC.

Introduction

Inflammatory Bowel Disease (IBD) is collective name for Crohn's Disease (CD) and Ulcerative Colitis (UC), characterized by uncontrolled immune activation and inflammation of the alimentary tract in genetically predisposed individuals [1]. Smoking is probably the strongest environmental factor for the development of CD. Epidemiology of IBD reveals that around one in 700 people are affected with IBD in the UK, which commonly occurs between the age of 15 and 40, although any age can be affected [2].

Mycophenolic Acid (MPA) was first licensed for transplantation in 1995 and rapidly grew popularity, becoming the second most widely used immunosuppressant in United States in 2004. It was initially marketed as mycophenolate Mofetil (MMF); a prodrug of MPA to improve oral bioavailability. More recently, the salt mycophenolate sodium has also been introduced. It

acts as a non-competitive, selective and reversible inhibitor of Inosine-5'- Monophosphate Dehydrogenase (IMPDH) [3,4]. Use of MMF in patients with refractory IBD has been documented, particularly in steroid-dependent individuals, or patients who are intolerant to one or more conventional therapies. However two most recurrently observed adverse events with both MPA and MMF are leukopenia and GI disorders, especially diarrhea has raised cautionary flag for their use in IBD [5]. Markus Ahlheim (2006) synthesized a salt or prodrug of mycophenolic acid used for parenteral administration suitable for acute situations such as prior or immediately after surgery [6]. Many patents have been filed for improving bioavailability of MPA such as amino ester derivatives MPA [7], hydroxamic derivatives of MPA [8], 4 and 6-substituted derivatives of MPA [9] and 5- hexanoic acid side chain derivatives of MPA [10] in the last two decades. The current international scenario indicates that there is more focus on preparing derivatives of MPA and on clinical trials of various biologicals in the treatment of IBD rather than attempts to design colon-specific prodrugs which we propose in the present work [11].

Cyclodextrins are a family of cyclic oligosachharides with hydrophilic outer surface and lipophilic central cavity. The lipophilic environment of central cavity of CyDs offers ability to form inclusion complexes by taking whole molecule or rather some nonpolar part in its hydrophobic cavity [12]. Taking this in advantage β-CyD enhances the bioavailability of drugs by increasing drug solubility, dissolution and/or drug permeability. In addition, it also improves other desirable properties of drugs such as stability, gastrointestinal tolerability and can be used to mask undesirable taste of drugs [13]. β-CyD unlike γ-CyD cannot be hydrolyzed by human salivary and pancreatic α –amylases, however vast microbiota present in colon, especially bacteroids, break these into small saccharides that are absorbed in large intestine. Moreover, fermentation of cyclodextrin leads to production of short chain fatty acid that can contribute to the maintenance of health and integrity of colonic epithelium. It has been proved through study in healthy human volunteers that β-CyD is poorly digested in small intestine but is completely degraded by the microflora of the colon.

There are no reports of macromolecular prodrugs of MPA with β-CyD so far. On the basis of above facts, the study has been focused on synthesis, characterization and pharmacological evaluation of macromolecular prodrug of MPA in TNBS-induced colitis rat model.

Materials

All chemicals used in the synthesis were of AR grade and purchased from Merk Speciality Pvt. Ltd., Mumbai. Mycophenolate sodium was obtained from Emcure Pharmaceuticals Pvt. Ltd., Pune (India). β-CyD was purchased from Sigma-Aldrich, St. Louis, USA. The IR spectra were recorded on JASCO, V-530 FTIR. NMR spectra were recorded on NMR Varian Mercury 400 MHz at SAIF, Panjab University, Chandigarh. For determination of aqueous solubility and partition coefficient JASCO V530, UV-Visible double-beam spectrophotometer was used. In vitro and in vivo studies were carried out by using HPLC comprised of a pump (Jasco PU-1580), a UV/VIS detector (Jasco UV 1575) and Waters Xterra RP 18 column.

Methods

Synthesis of MBS prodrug

MPA [1mol] in DMF was activated using EDCI at 0°C. After 1h, β-CyD [1.2 mol] in DMSO was mixed with 2 drops of triethylamine which was added to MPA-EDCI activated complex. The reaction was continued at room temperature and completion was confirmed by TLC using chloroform: methanol: glacial acetic acid; (74:25:1; v/v/v). Then reaction mixture was poured in 15ml of cold water and extracted with ethyl acetate. The organic layer was separated and dehydrated with sodium sulphate, filtered and concentrated and purified using preparative TLC. Structure of synthesized prodrug was confirmed by spectroscopic methods [3] (Figure 1).

In vitro release study in aqueous buffers

The in vitro study was carried out in various incubation media such as 0.05M HCl buffer pH 1.2 (corresponding to pH of stomach) and 0.05M phosphate buffer pH 7.4 (simulating pH of small intestine and blood). The procedure employed by Dhaneshwar, et al. (2017) was followed to carry out in vitro study [14]. The amount of prodrug hydrolysed was estimated on HPLC at 225nm wavelength using phosphate buffer (pH4.5): acetonitrile (40:60; v/v) as mobile phase. The Rt values for MPA and MBS were 5.6 and 15.2 min respectively with the flow rate of 0.6 ml/min. All the kinetic studies were carried out in triplicate and their standard deviation was calculated.

In vitro release study in different incubation media

In vitro release of MPA from the prodrug was further studied in stomach, colon, intestinal homogenates and faecal matter following the method reported by Dhaneshwar, et al. (2017) [14]. Samples were withdrawn after predefined intervals for the next 3h, 7h, 10h and 13h for stomach homogenates, small intestinal homogenates, colon homogenates and faecal matter respectively and analysed by HPLC.

In vivo study

In vivo study was carried out in male Wistar rats (average weight 200–230 g; 12–15 weeks; n=6/group). MBS prodrug was given orally (83 mg/kg) and blood samples were collected in EDTA tubes at predefined time intervals up to 13th and finally at 24th h. In vivo study was carried out by following the procedure reported by Dhaneshwar et al. (2017) [14].

Pharmacological evaluation

Pharmacological screening of synthesized conjugate was carried out in Poona College of Pharmacy, Pune. All the experimental procedures and protocols used for animal study were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) (CPCSEA/PCH/15/2014-15).

TNBS-induced colitis model

The prodrug was screened for anti-colitic activity by TNBS-induced colitis model [14,15]

Doses: Healthy control: saline, Disease control: 2, 4, 6-trinitrobenzoic acid (TNBS) 100 mg/kg of body weight in 50% v/v ethanol solution, MPA:18.5mg/kg , MMF:25mg/kg , MBS:83mg/kg , β-CyD : 65mg/kg and physical mixture of β-CyD and MPA: 18.45+65.27 mg/kg, all doses were calculated on an equimolar basis to the dose of MPA.

As previously reported by Hartmann, et al. (2000), the clinical activity score was determined by calculating the average of the three parameters viz; weight loss, stool consistency and rectal bleeding for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis) [16]. On 11th day

animals were sacrificed by isoflurane anaesthesia and their colon/body weight ratio was calculated on the basis of dissected sections of colon. Rat stomach, colon, liver and pancreas were removed and sent for histopathological evaluation. Gastric ulcers were scanned and ulcer index was calculated by scoring the ulcers as per method reported by Cioli,et al. (1979) [17].

Results and discussion

Physico-chemical characterisation and spectral data of MBS prodrug

The IR spectrum of MBS showed characteristic C=O stretching at 1762 cm^{-1} for ester whereas broad peak at 3424 cm^{-1} corresponding to β-CyD confirmed the structure of anticipated macromolecular ester. The results of mass spectroscopy revealed that calculated molecular weight of prodrug was in accordance with its predicted molecular weight. In this work the obtained mole compositions from elemental analysis, 1H NMR and 13C NMR were relatively in good agreement.

Ester prodrug of β-CyD and MPA: Aqueous solubility

0.098mg/ml; Log Poct: 0.62. FTIR (Anhydrous KBr; cm^{-1}): 3424(OH stretch), 2923 (C=C stretch aromatic ring), 1762 (C=O stretch ester), 1529 (C=C bend aromatic ring), 1318 (phenol C-O stretch aromatic ring), 1132 (C-O stretch ester). 1HNMR(DMSO; 400MHz) MPA backbone: δ2.30[s,3H] = C-CH_3, 2.13 [s, 3H] Ar-CH_3, 2.50-2.57 [m, 4H] O=C-CH_2-CH_2, 3.7 [t,3H] Ar–OCH_3, 3.0-3.15 [m, 3H] Ar-CH_2-CH=, 5.10 [s, 2H] lactone ring, 5.3 [s, H] –OH; β-CyD backbone: δ 1.54-2.07 [d, 20H] –OH cyclohexyl ring, 2.82-3.01 [d, 7H] –CH_2–OH, 3.43-3.49 [m, 14H]-CH and –O-CH cyclohexyl ring, 4.09-4.29 [t, 7H]-CH- cyclohexyl ring. 13CNMR (400 MHz, DMSO): δ15.9,16.06,16.15,22.41,22.82,25.01,31.85,33.63,33.93,34.16,34. 77,54. 95,60.54,60.81,61.04,68.55,79. 15,106. 90,115. 12,122. 38, 122. 76,123. 20,133. 53,133.67,134. 51,144. 75,145. 76,147. 10,153. 07,162. 02,162.51,167.89,170.14, 170.55. Mass: m/z M+2: 1440. 4, (Molecular weight: 1438.5). Elemental analysis: Calculated for $C_{59}H_{90}O_{40}$; C, 49.23; H, 6.3. Found: C, 49.25; H, 6.2. DSC study: 195.5°C (crystalline nature)

In vitro study

The in vitro kinetics confirmed that the prodrug was stable in HCl and phosphate buffer over a period of 3 h and 7 h respectively, indicating negligible hydrolysis in the upper GIT. In addition to this, MBS showed 88.9% release of MPA in colon homogenates (K± S.D.; 0.0019±0.0014) while 54.8% release in faecal matter. This may be because of faster activation in colon homogenates compared to faecal content which can be accredited to higher microbial population in colon than faeces. Release of MPA from prodrug in rat colon homogenates and faecal matter confirmed its colon-specific activation. (Figure 2). Half-life of MBS was found to be 348 and 797 min in rat colon homogenates and faecal matter respectively following first order kinetics.

In vivo study

In vivo behaviour of orally administered plain MPA and MBS prodrug was compared in order to have a comparative account for justifying the advantage of a colon-targeted delivery system over plain MPA.

Orally administered MPA (18.5 mg/kg) appeared in blood after 1h, reaching a maximum at 7h (68%) indicating ready absorption from stomach and small intestine, which then gradually started declining reaching negligible concentration at 24 h.

In contrast, when MBS was administered orally (83 mg/kg), till 5h neither MBS nor MPA was observed in blood indicating that the prodrug bypassed absorption in stomach. First appearance of MBS was observed in blood 6 h indicating partial absorption from small intestine. Concentration of MBS consistently increased thereafter reaching 75% at 10h, indicating major absorption of intact MBS from large intestine. MPA was observed in blood from 10th h onwards indicating hydrolysis of MBS into MPA in large intestine by cyclodextranases (esterases) which due to high lipophilicity must have penetrated colonic mucosa, entering systemic circulation. MPA concentration reached 60% at 13h. The amount of MPA and MBS started decreasing after 13h reaching negligible level at 24h (Figure 3).

24h pooled samples of urine and faeces also showed presence of MBS and MPA revealing their excretion through kidneys and large intestine.

Pharmacological evaluation

The alleviating effect of synthesized prodrug as well as standard was evaluated for clinical activity score rate and colon/body weight ratio in TNBS-induced experimental colitis model in Wistar rats [14,15]. Till 5th day the clinical activity score increased rapidly and consistently for all TNBS-treated groups (Table 1). Full-puffed colonic inflammation was evidenced by the high clinical activity score (3.30±2.0) in colitis control group. The anti-colitic activity of prodrug was compared with MMF and MPA. Outcomes of animal study showed that prodrug-treated group showed comparable lowering of clinical activity score rate to MMF but 3.5 times more lowering effect than MPA. Results also indicated that prodrug-treated group showed 1.5 times decrease in the colon/body weight ratio compared to MPA (Figure 4), which was in agreement with its superior lowering effect on clinical activity score rate.

Outcomes of histopathological studies revealed that the colitis control was characterised by severe erosion with absence of mucosal layer, goblet cells depletion, distorted crypts architecture, lymphocytic infiltration, thickening of muscularis mucosa leading to complete destruction of colon mucosal architecture. Histopathological features of prodrug-treated group showed marked decrease in the extent and severity of colonic damage (Figure 5), soft faeces and cecal enlargement which is one of the side effect associated with β-CyD but no evidence of inflammatory responses, cell degeneration or cell death was observed.

MBS prodrug showed a significant reduction in ulcer index compared to MPA which was comparable with MMF. Relatively low ulcer index and partition coefficient of MBS suggest that its absorption from upper GI tract would be negligible and it would be stable at stomach pH, thus might successfully by-pass the formidable barrier of upper GI tract (Figure 6). These results were not only consistent with their release pattern in various incubation media but also found to be statistically significant when compared with healthy control.

Conclusion

In the existing study, macromolecular prodrug design was adopted for synthesizing a colon-targeting prodrug of MPA with β-CyD for the effective management of IBD. In MBS prodrug the morpholinoethyl group of MMF was replaced by β-CyD which not only spared from MMF-related side effects but also assisted in increasing MPA solubility, dissolution and bioavailability. Fur-

thermore it significantly helped in maintaining health and integrity of colonic epithelium. TNBS-induced colitis was ameliorated by oral administration of MBS alone without any concurrent treatment of any aminosalicylate or sulfasalazine with absence of untowardly effect on GIT. However soft stools and cecal enlargement were observed in prodrug-treated group. Thus it can be concluded that the developed colon-targeted strategy for MPA holds a lot of promise and potential as compared to oral administration of plain MPA in TNBS-induced colitis in Wistar rats. It presents a promising opportunity which can be explored further for its probable utility in management of IBD.

Table

Table 1: Clinical Activity Score#

Days → Groups ↓	1	2	3	4	5	6	7	8	9	10	11
HC	0	0	0	0	0	0	0	0	0	0	0
DC	0	0.67±1.15	0.87±1.5	2.53±0.5	2.63±0.65	2.63±0.65	2.97±0.85	3.12±1.8	3.22±1.7	3.23±2.0	3.30±2.0
MMF	0	0.33±0.58	0.67±1.15	1.07±1.29	1.53±0.5	2.53±0.5	2.10±0.85	1.07±0.51	0.73±0.81	0.23±0.68	0***
MPA	0	0.43±0.75	0.67±1.15	2.50±0.17	2.63±0.35	2.63±0.35	2.33±0.58	1.97±0.65	1.63±0.91	0.97±0.85	0.77±0.68 ***
MBS	0	0.43±0.58	0.67±1.15	2.3±0.85	2.3±0.89	2.63±0.50	2.1±0.85	1.06±0.75	0.53±0.17	0.40±0.40	0.22±0.23 ***
β -CyD+MPA	0	0.33±0.58	0.33±0.58	1.83±0.68	2.17±0.75	2.17±0.75	1.87±0.51	1.63±0.7	1.30±0.58	1.29±0.50	1.27±0.35**
β –CyD	0	0.33±0.58	0.43±0.75	1.53±0.81	2.10±0.17	2.33±0.58	2.17±1.03	1.93±0.58	1.87±0.81	1.63±0.35	1.73±0.1*

#Average of six readings; Two-way ANOVA followed by Bonferroni's Test, statistical significance considered at P < 0.05*; P<0.01**; P<0.001*** when compared to disease control.
HC: Healthy control; DC: Disease control; MMF: Mycophenolate mofetil; MPA: Mycophenolic acid; β -CyD +MPA: Physical mixture of β -CyD and MPA.

Figures

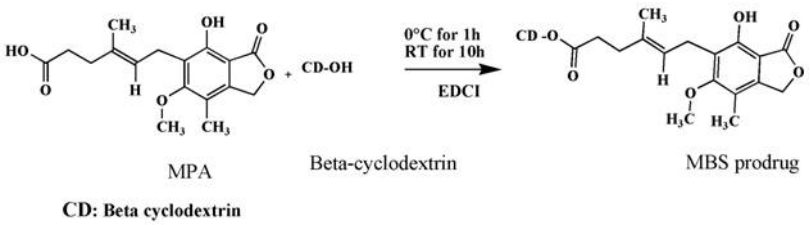

Figure 1: Synthesis of MBS prodrug

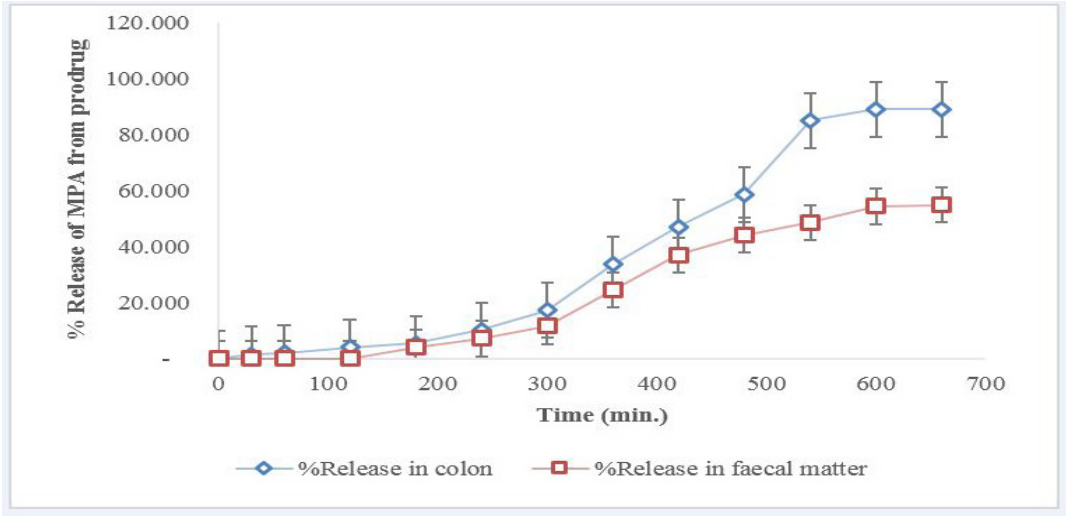

Figure 2: In vitro release of MPA from MBS prodrug in colon homogenates and faecal matter

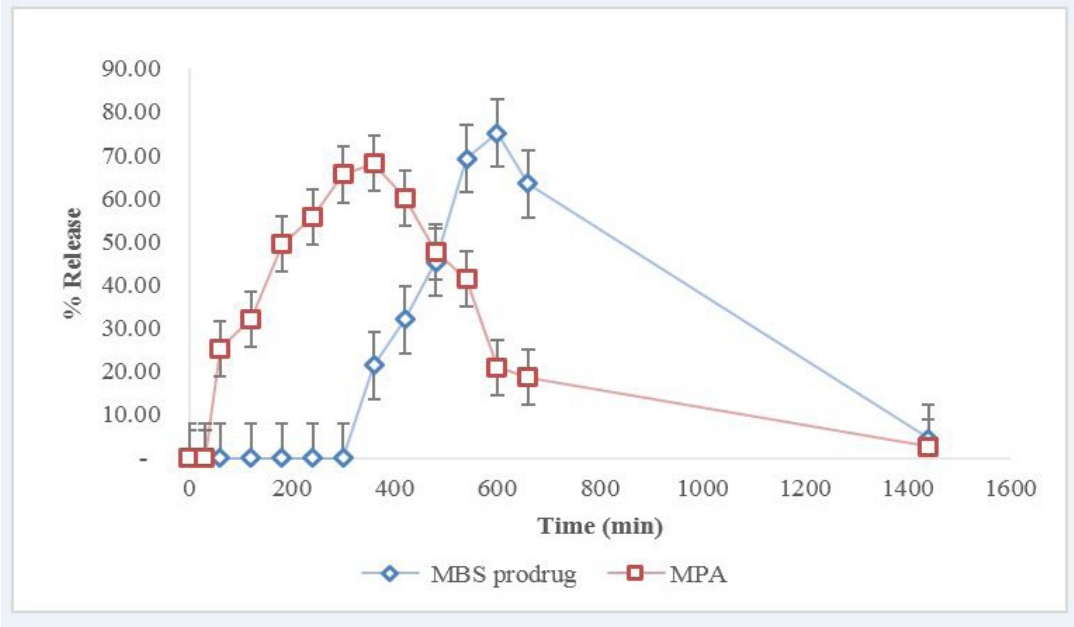

Figure 3: In vivo release study

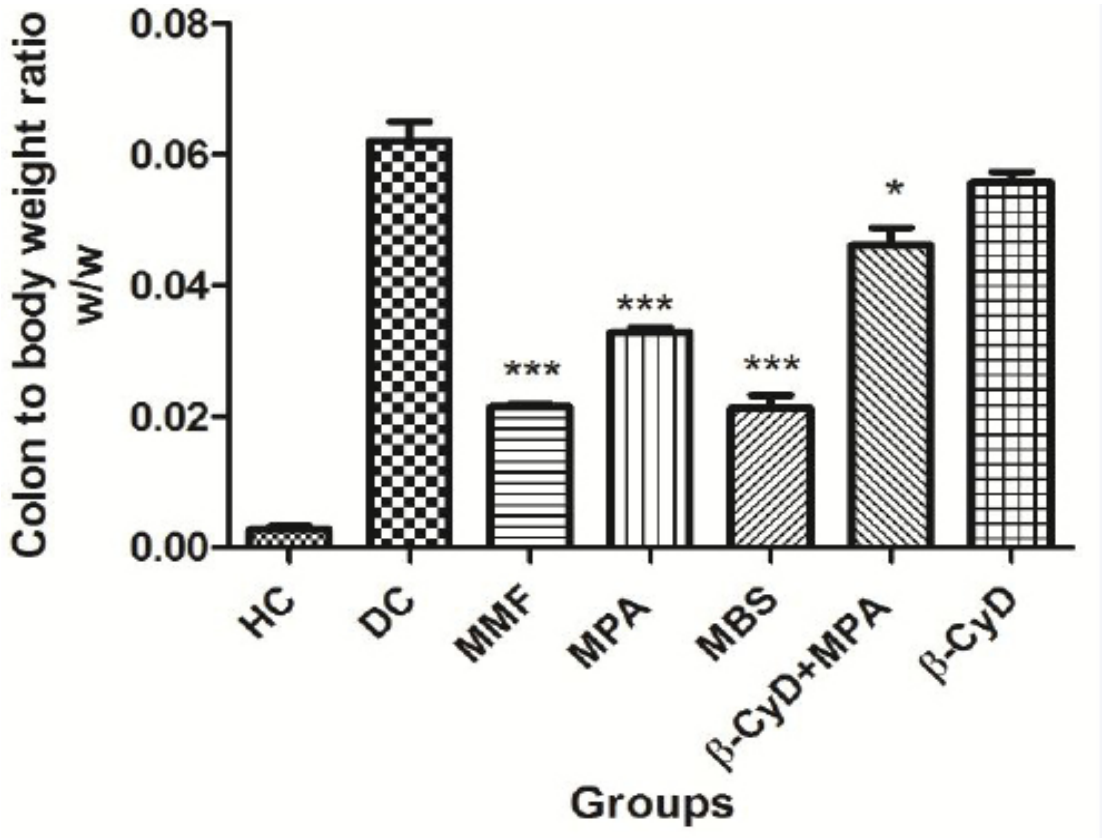

Figure 4: Colon to body weight ratio#

#Average of six readings; One-way ANOVA followed by Dunnett's Multiple Comparison Test, statistical significance considered at P < 0.05*; P<0.01**; P<0.001*** when compared to disease control.

(HC) Healthy Control; (DC) Disease Control; (MMF) Mycophenolate Mofetil; (MPA) Mycophenolic Acid; (β -CyD +MPA) Physical mixture of β -CyD and MPA.

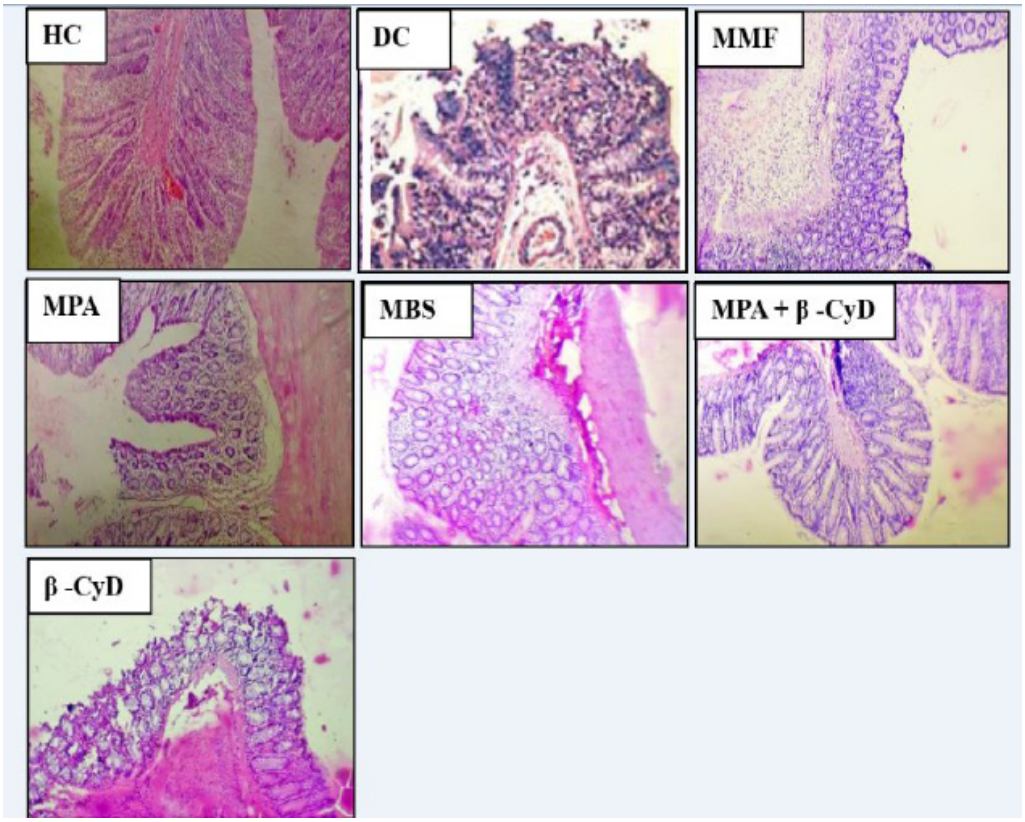

Figure 5: Photomicrographs of colon

(HC) Healthy Control showing normal architecture of colon mucosa; (DC) Disease Control showing severe haemorrhages in submucosa of colon with infiltration of inflammatory cells; (MMF) Mycophenolate Mofetil showing normal colon architecture; (MPA) Mycophenolic acid showing mild infiltration of inflammatory cells and mild degeneration of lining of epithelium; (MBS) Prodrug of MPA and β-CyD showing normal colon architecture with minimal infiltration of inflammatory cells; (MPA+ β-CyD) physical mixture of MPA and β-CyD showing mild infiltration of inflammatory cells; (β-CyD) - showing mild degeneration of lining of mucosa.

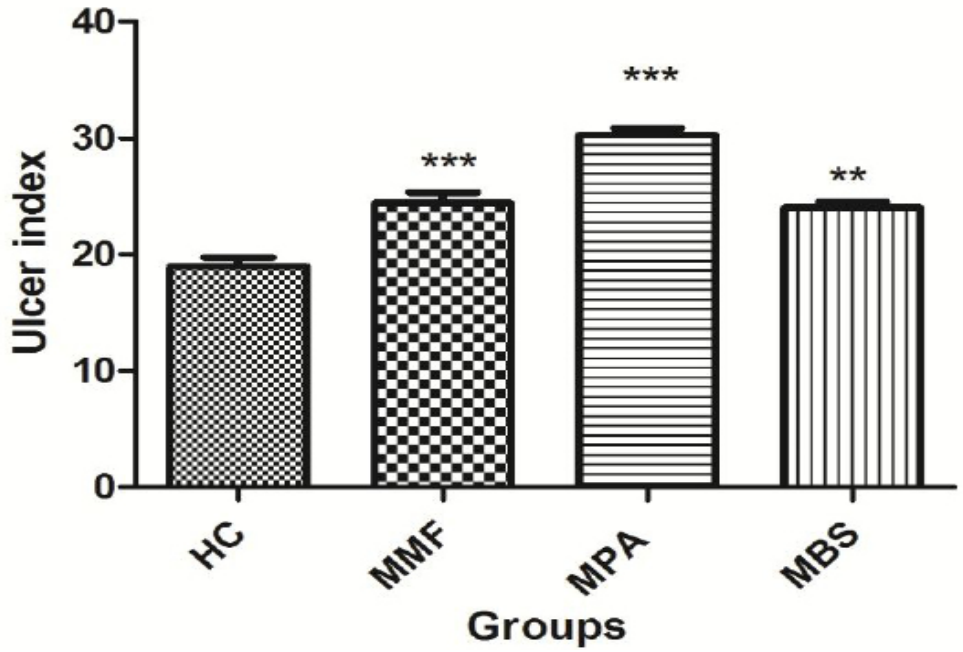

Figure 6: Photomicrographs of colon

#Average of six readings; One-way ANOVA followed by Dunnett's Multiple Comparison Test, statistical significance considered at P < 0.05*; P<0.01**; P<0.001*** when compared to healthy control.

(HC) Healthy Control; (MMF) Mycophenolate Mofetil, (MPA) Mycophenolic Acid; (MBS) Prodrug of MPA and β-CyD.

Acknowledgements

The authors are grateful to Department of Science and Technology, New Delhi (DST- WOS-A) for financial assistance to carry out this project and Emcure Pharmaceuticals Pvt. Ltd. Pune, Maharashtra for providing gift sample of mycophenolate sodium. The authors would like to thank Dr. K.R. Mahadik, Principal, Poona College of Pharmacy, Pune for providing necessary facilities to carry out the work.

References

1. Friend D. Colon-specific drug delivery. Adv. Drug Deliv. Rev. 1991; 7: 149-199.
2. Dhaneshwar S, Vadnerkar G. Rational design & development of colon-specific prodrugs. Curr. Top. Med. Chem. 2011; 11: 2318-2345.
3. Dorota I, Grzegorz C, Agata K, Piotr T, Krystyna D. Synthesis and biological activity of mycophenolic acid-amino acid derivatives Eur. J. Med. Chem. 2013; 69: 863-871.
4. www.drugbank.com
5. http://en.wikipedia.org/wiki/Mycophenolate_mofetil
6. Ahlheim. Parenteral formulation of MPA, a salt or prodrug thereof, US Patent No. 2006/0189683A1.
7. Lee W, Leo G, Miksztal A, Chu N, Leung K, Nelson PH. Bioavailability improvement of mycophenolic acid through amino ester derivatization. Pharm Res. 1990; 7: 161-166.
8. Batovska D, Kim D, Mitsuhashi S, Cho Kwon H, Ubukata M, Kwon HJ. Hydroxamic acid derivatives of mycophenolic acid inhibit histone deacetylase at the cellular level. Biosci Biotechnol Biochem. 2008; 72: 2623-2631.
9. Patterson. US Patent 5, 554,612, 4 and 6-substituted derivatives of MPA. 1996.
10. David Morgans, Jr.David B. Smith, Francisco X. Talam as, Dean R. Artis, Alicia Cervantes, Todd R. Elworthy, et al. US Patent 5,633,279, 5-hexanoic acid side chain derivatives of MPA. 1997.
11. http://www.rocheusa.com/products/cellcept/pi.pdf.
12. Loftson T, Masson M, Brewster ME. Self-association of cyclodextrins and cyclodextrin complexes, J Pharm. Sci. Tech. 2004; 93: 1091-1099.
13. Giardina C, Inan MS. Nonsteroidal anti-inflammatory drugs, short chain fatty acids and reactive oxygen metabolism in human colorectal cancer cell, Biochim Biophys Acta. 1998; 1401: 277-288.
14. Dhaneshwar SS, Sarkate A. Investigation of mitigating effect of colon-specific prodrugs of boswellic acid on 2,4,6-trinitrobenzene sulfonic acid induced colitis in Wistar rats: Design, kinetics and biological evaluation. World J Gastroenterol. 2017; 23: 1147-1162.
15. Yamada T, Marshall S, Specian RD, Geisham MB. A comparative analysis of two models of colitis in rats. Gastroenterology. 1992; 102: 1524-1534.
16. Hartmann G, Bidlingmaier C, Seigmund B, Albrich S, Schulze J, Tschoep K, et al. Specific type IV phosphodiesterase inhibitor rolipram mitigates experimental colitis in mice. J. Pharmacol. Exp. Ther. 2000; 292: 22-30.
17. Cioli V, Putzolu S, Rossi V, Barcellona P, Corradino C. The role of direct tissue contact in the production of gastrointestinal ulcers by anti-inflammatory drugs in rats. Toxicol. Appl. Pharmacol. 1979; 50: 283-289.