Investigation of Therapeutic Switching Approach for the Management of Ulcerative Colitis using Codrug Strategy

Nivedita Chatterjee[1*], Suneela Dhaneshwar[2]

[1]Research Scholar, Department of Pharmaceutics, Amity University, Lucknow, Uttar Pradesh, India-226010

[2]Associate Professor, Department of Pharmaceutics, Amity University, Lucknow, Uttar Pradesh, India-226010

Corresponding Email- niveditachatterjee896@gmail.com

Abstract

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract. Ulcerative colitis (UC) is the form of IBD that cause swelling, ulceration and loss of function of the colon and rectum. IL-1 along with TNF-α plays an important role in the pathogenesis of IBD due to its proinflammatory activity. In the present work we have selected two drugs which were mechanistically different. Diacerein is an anti-arthritic drug which has anti-inflammatory, analgesic effect through IL-1 inhibition and 5-ASA is a COX inhibitor used as a first line treatment of UC. A mutual amide prodrug was designed and synthesized by linking diacerein and 5-ASA through amide linkage and evaluated for colon targeted drug delivery. The obtained prodrug was characterized by FTIR, ^{1}H-NMR, ^{13}C-NMR, melting point and R_f value. *Invitro* drug release kinetics was conducted at pH 7.4 and in rat fecal matter. 3.7% release of diacerein was achieved in rat fecal matter over a period of 7 h, following zero order kinetics. The rate constant and half-life were found to be 0.000025 mg $ml^{-1}min^{-1}$ and 1800 min respectively. The results of the study indicate that activation of prodrug was very slow. The synthesized prodrug was also tested to study site-specificity through TNBS-induced colitis model for chronic inflammatory conditions and ulcerogenic tendency on the basis of disease activity score rate, colon/body weight ratio, diameter of colon, colon length, gut pH and histopathology of colon pancreas, liver and stomach.

Keywords: Diacerein, 5-ASA, amide mutual prodrug.

1. Introduction:

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disorder, caused by a dysregulated immune response to host intestinal microflora. The two principal types of inflammatory bowel disease are ulcerative colitis (UC), which is primarily restricted to the colon and rectum, and Crohn's disease (CD), which can affect any segment of the gastrointestinal tract (GIT) from the mouth to the anus[1].

Inflammatory bowel disease (IBD) is a chronic intestinal disease that often has its onset during young adulthood and has a chronic relapsing-remitting course[2].

CD and UC are inflammatory bowel diseases that cause chronic inflammation and damage in the GIT. The GI tract is responsible for digestion of food, absorption of nutrients, and elimination of waste. Inflammation impairs the ability of affected GI organs to function properly, leading to symptoms such as persistent diarrhea, abdominal pain, rectal bleeding, weight loss and fatigue[3].

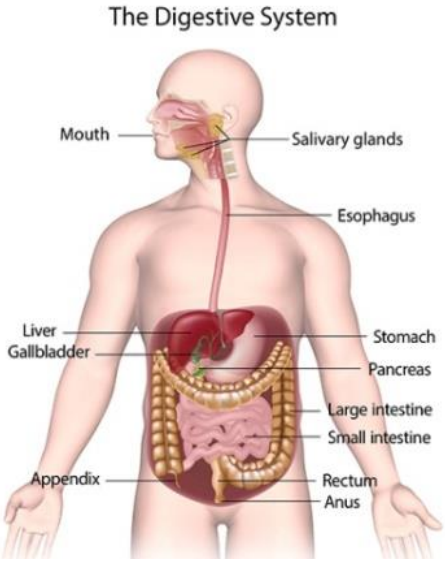


Fig1. Diagram of the digestive system [3]

UC affects the innermost lining of the large intestine (colon) and rectum. Symptoms usually develop over time, rather than suddenly.UC can be debilitating and can sometimes lead to life-threatening complications. While it has no known cure, treatment can greatly reduce the signs and symptoms of the disease and even bring about long-term remission [4].

1.2Therapeutic switching

Therapeutic switching means the application of known drugs and compounds to treat different diseases. Detailed information on an existing drug such as their pharmacology, dose, formulation, and potential toxicity is available as they have already been tested in humans.The drug repurposing approach focuses on the fact that conjoint molecular pathways contribute to the pathophysiology of many different diseases[5].Itis re-investigating existing drugs that failed approval for new therapeutic indications [3].The term “drug repositioning” has been used interchangeably with “drug repurposing” or “drug reprofiling"[6]. Drug repurposing refers to the development of existing drugs for new indications. These drugs may have (i) failed to show efficacy in late-stage clinical trials, without safety issues; (ii) stalled in development for commercial reasons; (iii) passed the point of patent expiry, and (iv) they are being explored in new geographical markets [7].Drug repositioning has many advantages over traditional *de novo* drug discovery approaches in that it can significantly reduce the cost and development time and as many compounds have demonstrated safety in humans it often negates the need for phase I clinical trials. Drug repositioning or drug repurposing is an approach to accelerate the drug discovery process through the identification of a novel clinical use for an existing drug approved for a different indication[7].

1.3 Codrug

Codrugs are used to treat the side effect of other drugs another term used for codrug is ‘mutual prodrug’ which consist of two synergistic drug which chemically linked together, to improve the drug delivery properties of one or both the drugs. Prodrugs are chemically derivatives of active drug which is covalently bonded to an inactive pro-moiety in order to overcome pharmaceutical and pharmacokinetic barriers. The main drawback is to approach the pro-moiety to avoid unwanted ballast when the drug released, they lead to adverse effects. Combining of two different pharmacophores with similar or different pharmacological activities bring out synergistic action or help to target the parent drug to specific site/organ/cells respectively [8].

2. Experiment:

2.1 Materials

Diacerein and 5- Amino salicylic acid (5- ASA)was received as a gift sample from Zim Laboratories**.** Dimethylformamide (DMF), Dimethylformamide (DMF), Dimethylsulphoxide (DMSO), Ethyl acetate and Methanol were purchased from Thermo-Fisher Scientific India Pvt.Ltd. 2,4,6-Trinitrobenzene sulfonic acid (TNBS) was purchased from Sigma - Aldrich Corporation.

2.3 Synthesis of mutual amide prodrug of diacerein with 5-ASA

Synthesis of mutual amide prodrug of diacerein with 5-ASA was carried out by CDI coupling. Diacerein (0.0015M; 0.500 g) was dissolved in 10 ml of DMF in a flat-bottomed flask. CDI (0.0015M; 0.2432g) was added to the above solution and stirred at 20-25˚ Cfor 4.5h. A color change from florescent yellow to light yellow during the course of reaction was observed. A solution of 5-ASA (0.0015M; 0.2297g) in 10 ml DMF was prepared and added to the above reaction mix in small portions and stirred for 1 h at 20-25℃. The reaction mixture was kept for 15.5 h and again stirred for 2 h at the same temperature, a precipitate of yellowish orange colour separated out which was filtered and dried under vacuum. Monitoring of the reaction and purification by preparative TLC was carried out using the mobile phase ethyl acetate: methanol: DMSO: GAA; (2:2:2drop: 1drop; v/v/v/v). The TLC was visualized by iodine vapors and UV light.Scheme of synthesis of is depictedin **Fig 13.**

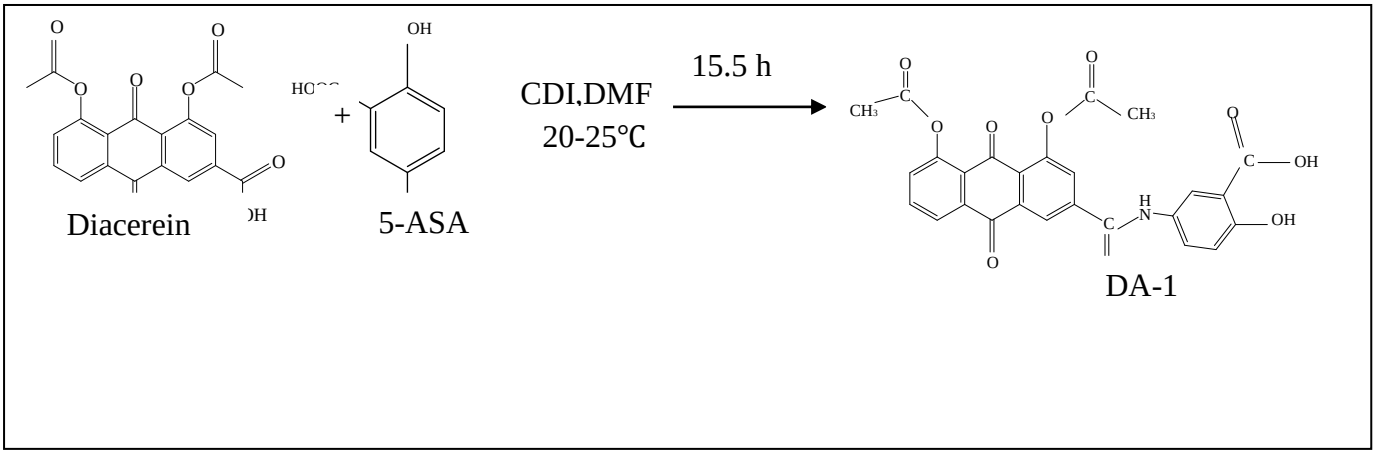


Fig 13. Scheme of synthesis of diacerein-5-ASA mutual amide prodrug

2.4 Physico- chemical characterization

2.4.1 Organoleptic properties

DA-1 was found to be odorless, crystalline and orange in colour.

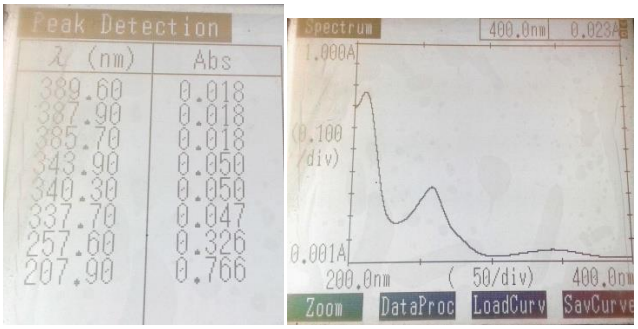
2.4.2 Melting point

Melting point was determined by open capillary method using Ambassador melting point apparatus. The melting point was found to 271.5 ℃ and is uncorrected. DA-1 melted with decomposition [9].

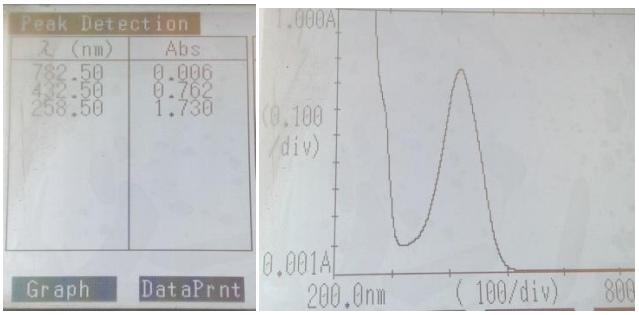
2.4.3 Determination of UV absorption maxima (λmax)

UV absorption is one of the best extensively used methods in drug identification. The compound having chromatophores show distinct absorption peak in the UV- visible range. The λmax of DA-1 was determined in distilled water and aqueous buffers by scanning it on UV- visible spectrophotometer in the range of 200-400nm (UV 1800, Shimadzu). A solution of diacerein/DA-1 was prepared (0.01mg/ml) in various solvents like distilled water, 0.2M HCl buffer (pH 1.2) USP [53] and 0.2 M phosphate buffer (pH 7.4) USP [10]. The solution was scanned on UV-spectrophotometer in the range 200-400 nm to estimate the λmax. The UV scans are depicted in **Fig 14** and **Fig 15**.

(A)



(B)



(C)

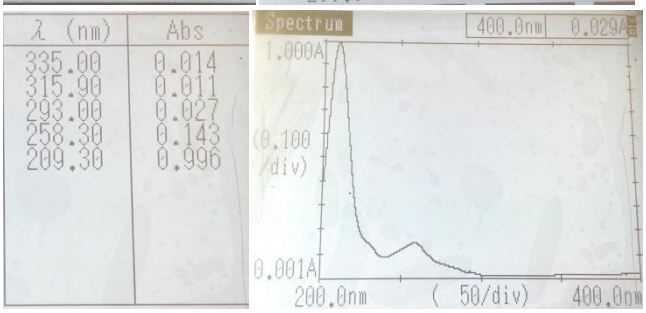
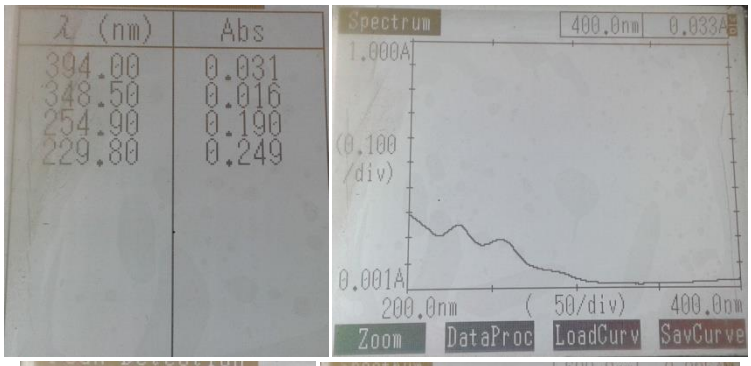
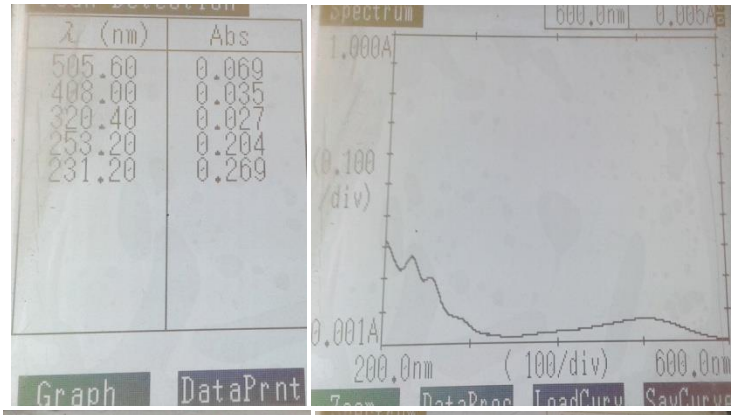


Fig 14. UV spectrum of diacerein in (A) distilled water, (B) HCl buffer pH 1.2, (C) phosphate buffer pH 7.4

(A)



(B)



(C)

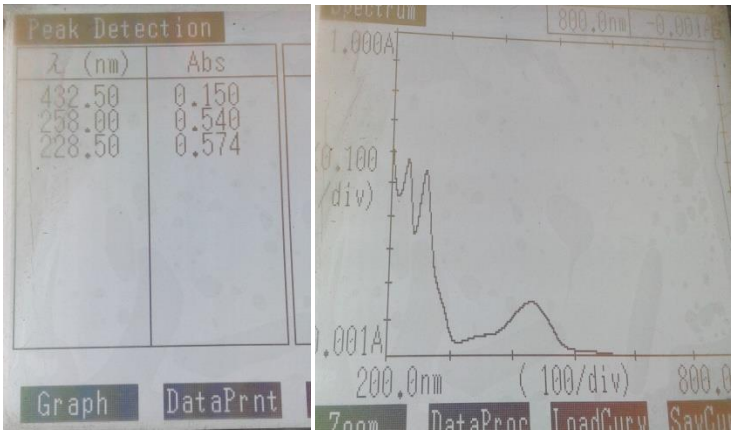


Fig 15. UV spectrum of DA-1 in (A) distilled water, (B) HCl buffer pH 1.2, (C) phosphate buffer pH 7.4

2.4.4 Construction of calibration curves of diacerein and DA-1

The stock solution of diacerein/ DA-1 was prepared (100µg/ml) in respective solvents like distilled water, 0.2M HCl buffer (pH 1.2) USP and 0.2 M phosphate buffer (pH7.4) USP. Serial dilutions were prepared in the range of 2 to 20µg/ml and absorbance was estimated at pre- determined λmax. The constructed calibration curves are shown in **Fig 16** and **Fig 17.**

2.4.4.1 Calibration curves of diacerein

(A)

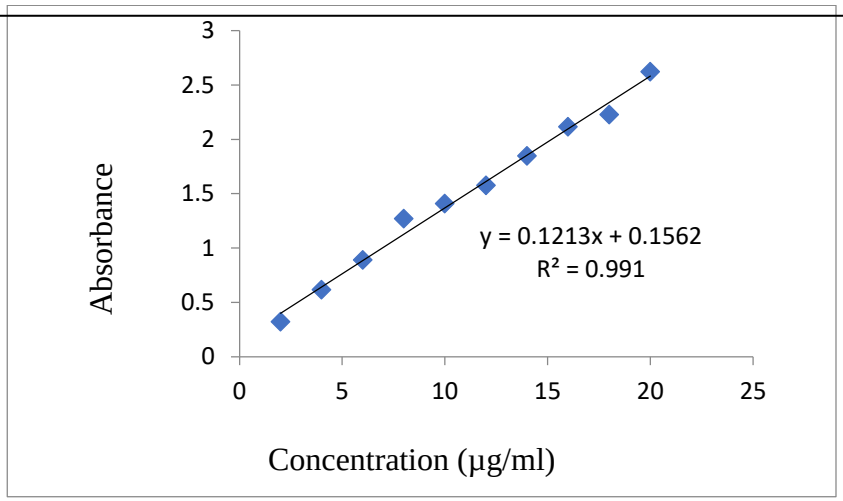


Fig 16. (A) Calibration curve of diacerein in distilled water λ_{max} (207.90 nm)

(B)

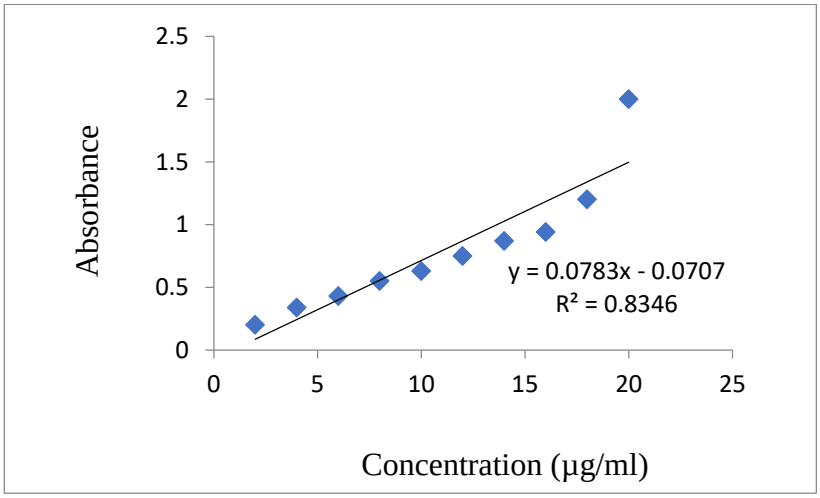


Fig 16. (B) Calibration curve of diacerein in HCl buffer pH 1.2 λ_{max} (258.5 nm)

(C)

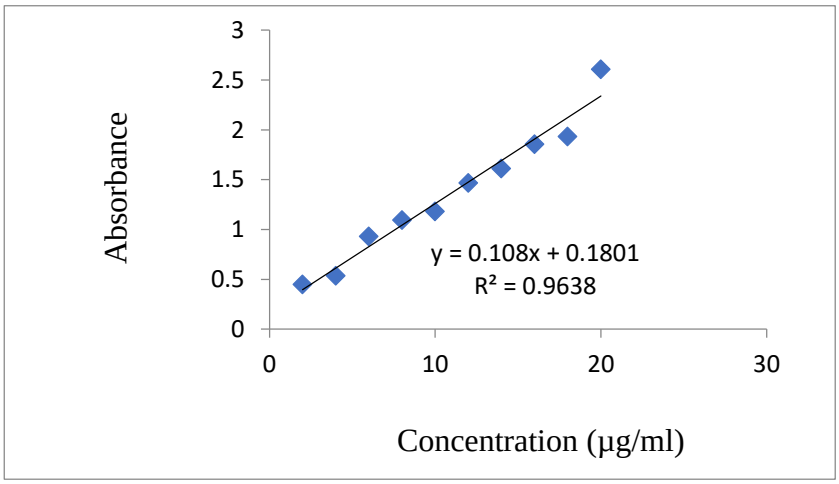


Fig 16. (C)Calibration curve of diacerein in phosphate buffer pH 7.4 λ_{max} (209.3 nm)

3.4.4.2Calibration curves of DA-1

(A)

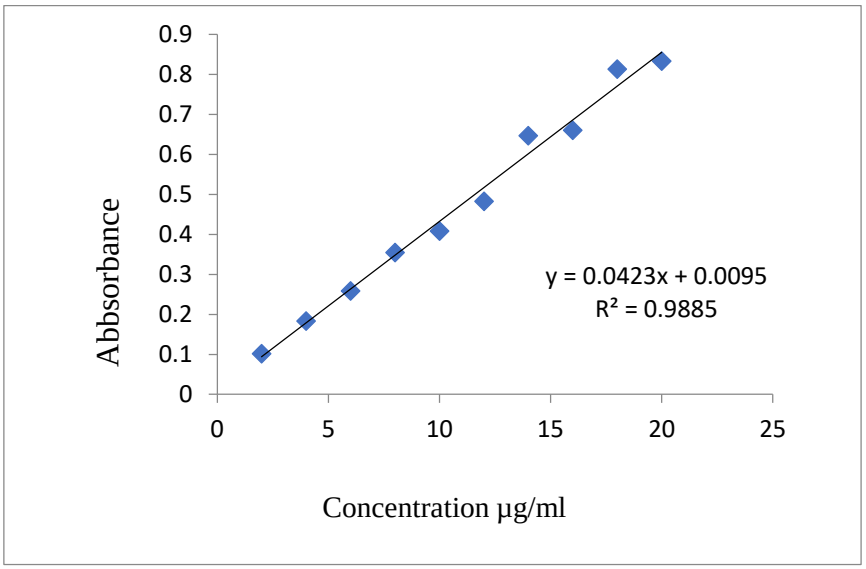


Fig 17. (A) Calibration curve of DA-1 in distilled water λ_{max} (229.80 nm)

(B

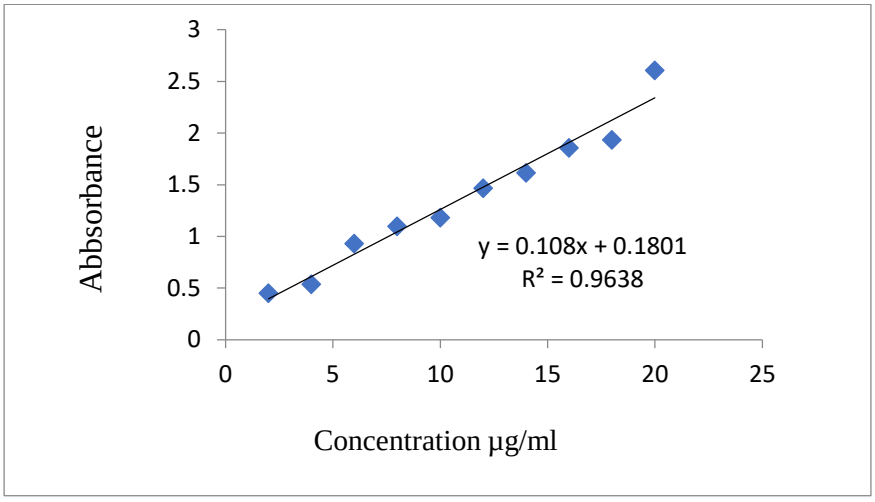


Fig 17. (B) Calibration curve of DA-1 in HCl buffer pH 1.2 λ_{max} (231.20 nm)

(C

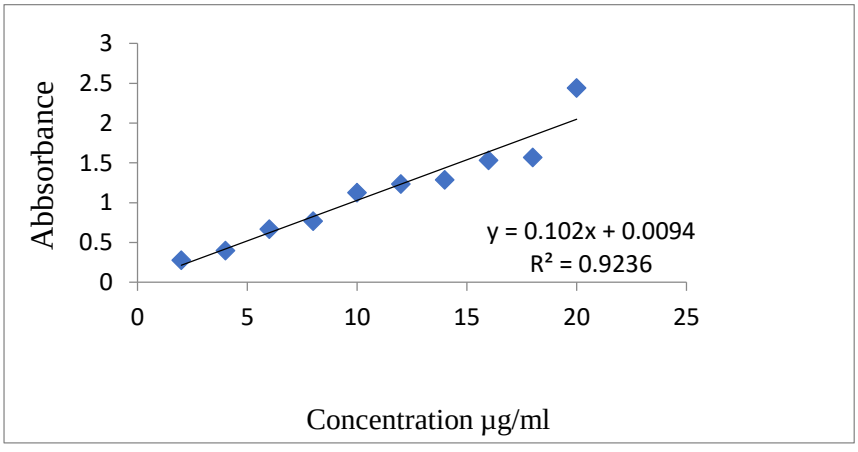


Fig 17. (C)Calibration curve of DA-1 in phosphate buffer pH 7.4 λ_{max}(228.50 nm)

2.5 Aqueous solubility determination

The aqueous solubility of diacerein/ DA-1 was determined at room temperature (33 ± 1℃). Excess amount of the diacerein /DA-1 was added to distilled water in stoppered conical flask so as to make it saturated and was mechanically stirred for 24 h. It was ensured that saturation equilibrium was established. Upon filtration, an aliquot of the filtrate was diluted with an appropriate amount of distilled water and unknown concentration was determined by UV-spectrophotometer [11,12].

2.6 Partition coefficient determination

The partition coefficient of the diacerein/ DA-1 was determined in n-octanol/ distilled water at room temperature (33 ± 1℃). Sample (10 mg) was dissolved in n-octanol (10 ml) and 10 ml distilled water was slowly added to it and the n-octanol-distilled water mixture was shaken for 24 h on wrist shaker to reach distribution equilibrium. The n-octanol and distilled water were mutually saturated prior to use. Aqueous layer was separated using separating funnel and estimated on UV-spectrophotometer at pre-determined wavelength. The partition coefficient P was calculated from the following equation.

$$P = \frac{C_i - C_w}{C_w} - \frac{V_w}{V_o}$$

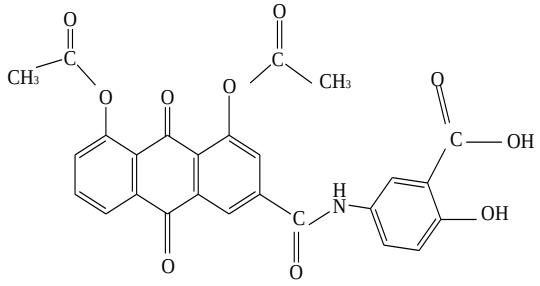
Where C_i and C_w are the solute concentrations in the aqueous phase before and after distribution respectively. $V_w$is the volume of the aqueous phase and V_o is the volume of n-octanol. The results are reported as logarithm of P value (Log P_{oct}) [13].

2.7 Spectral analysis

FTIR spectroscopy of diacerein and synthesized prodrugs was performed on Agilent Cary 630 at CSIR-CDRI, Lucknow.^{1}H-NMR of diacerein and synthesized prodrug was performed on Bruker DRX-300 at 400 MHz at CSIR-CDRI, Lucknow. ^{13}C-NMR was performed on Bruker DRX-300 at 400 MHz at CSIR-CDRI, Lucknow.

2.8 Amide mutual prodrug of diacerein with 5-ASA (DA-1)

Structural formula:



IU PAC name: 5-(1,8-Diacetoxy-9,10-dioxo-9,10-dihydroanthracene-6-carboxamido)-2-hydroxybenzoic acid

Molecular formula: $C_{26}H_{17}NO_{10}$

Melting point: 271.5℃ d (uncorrected; melts with decomposition)

R_f: 0.67 (ethyl acetate: methanol: GAA: DMSO; 2:2:2drops:1drops; v/v/v/v)

Solubility:

- Freely soluble in dimethyl formamide and dimethyl sulfoxide.
- Insoluble in dichloromethane.
- Sparingly soluble in ethanol and methanol.

Aqueous solubility: 0.008 mg/ml

Log P_{oct}: 2.85(n-octanol: distilled water)

λ_{max}:

- **Distilled water:** 229.80 nm
- **0.2M HCl buffer (pH 1.2) USP:** 231.20 nm
- **0.2M Phosphatebuffer (pH 7.4) USP:** 228.50 nm

2.8.1 FTIR spectrum of DA-1 (Anhydrous KBr)

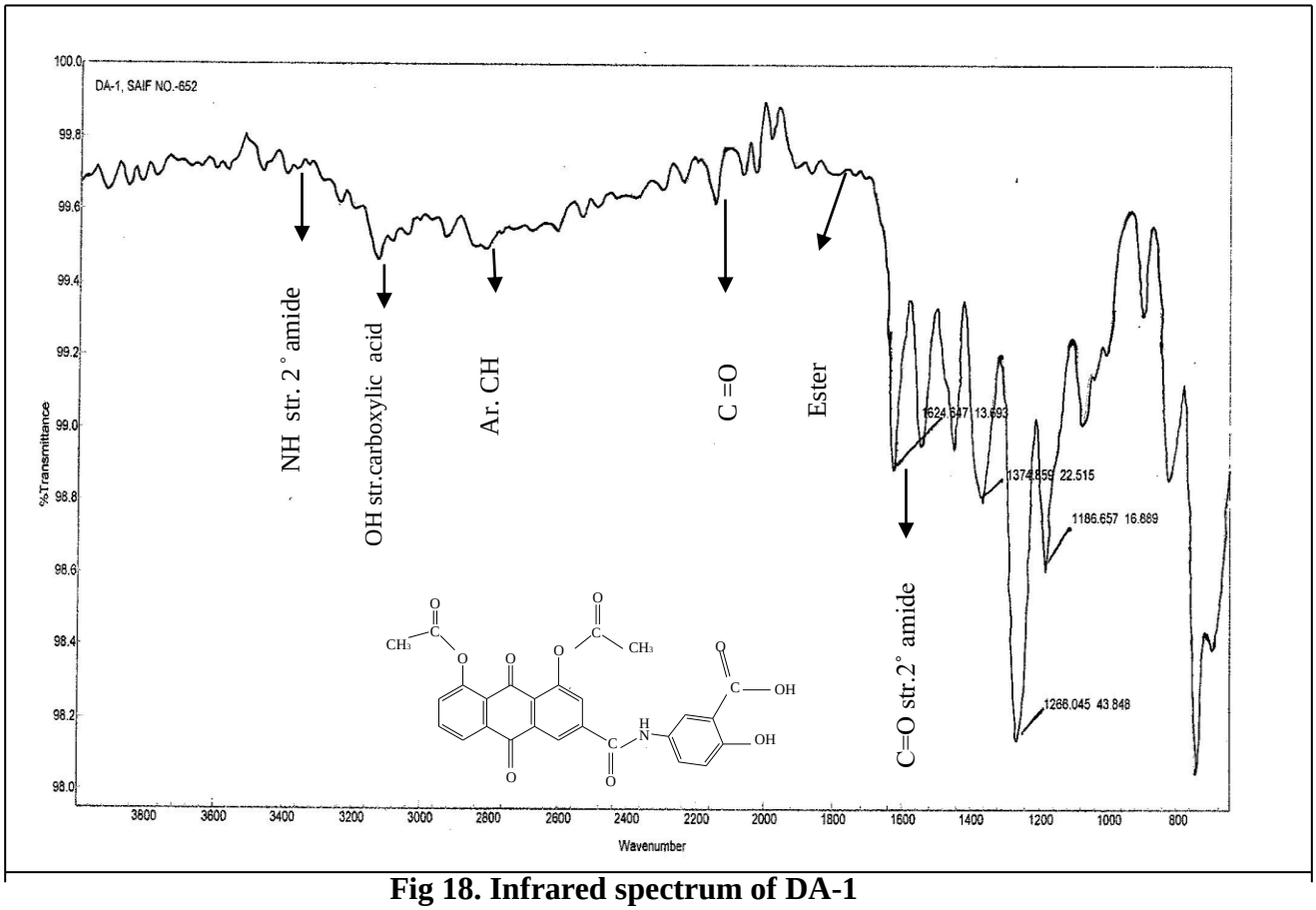


Fig 18. Infrared spectrum of DA-1

Table 5:Interpretation of IR spectrum of DA-1 (Anhydrous KBr)

S.N.	Type of group	IR ranges (cm^{-1})	
		Expected	**Observed**
1	C=O stretch 2˚amide	1640-1690	1624
2	C=O stretch ester	1760-1700	1760
3	Aromatic sp^2 CH	4000-3070	3080
4	OH stretch carboxylic acid	3500-3000	3120
5	NH stretch 2˚ amide	3500-3000	3400

2.8.2 [1]H-NMR spectrum of DA-1 (DMSO-d_6)

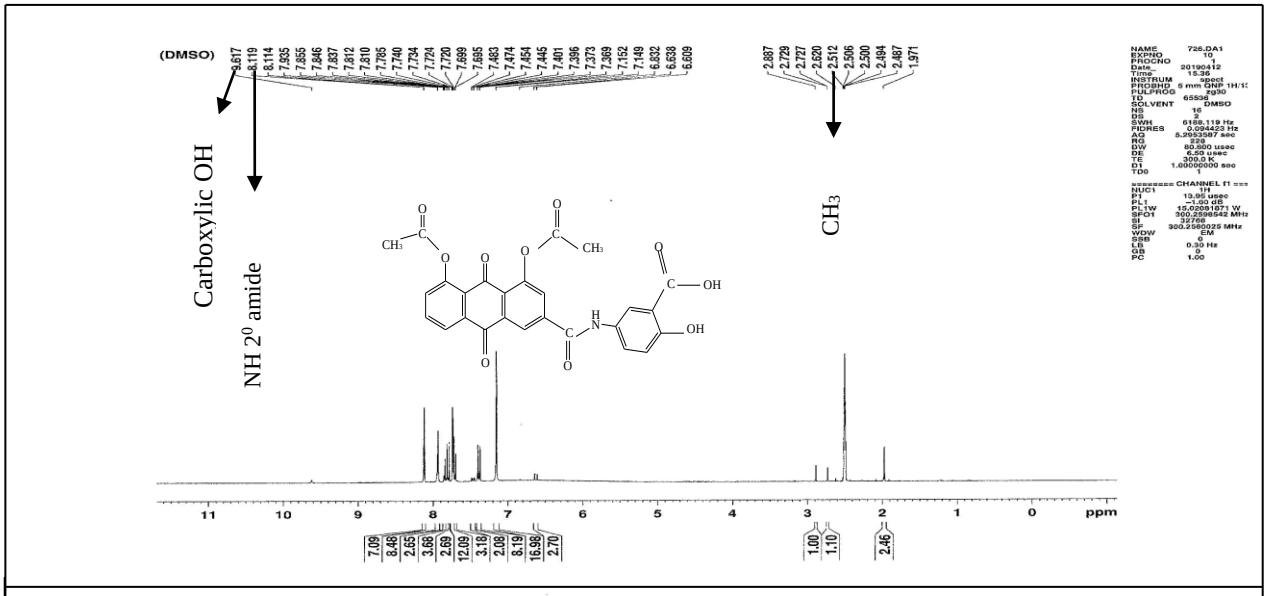


Fig 19. (A) [1]HNMR spectrum of DA-1 (DMSO-d_6)

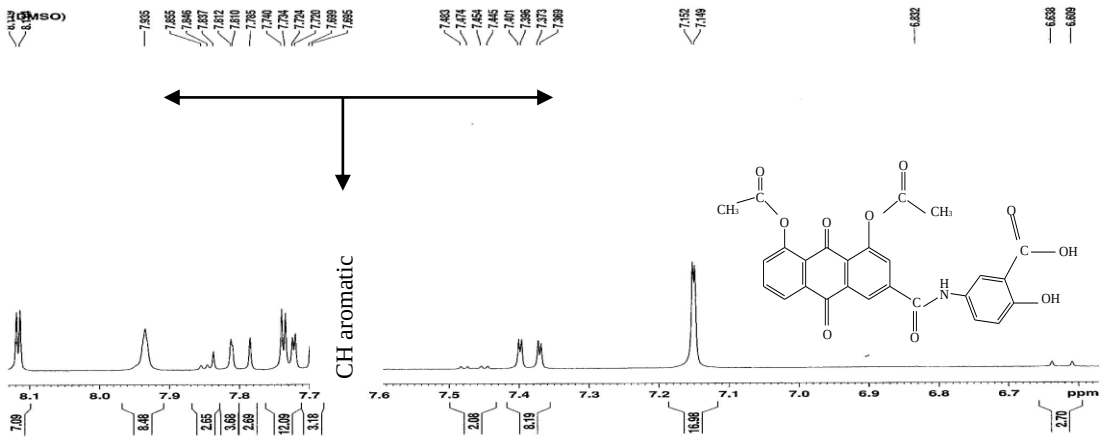


Fig19. (B) Expanded spectrum of DA-1 (DMSO-d_6)

Table 6: Interpretation of [1]H-NMR spectrum of DA-1 in DMSO-d_6

S.N.	Assignment	Chemical shifts (ppm)
1.	Carboxylic OH	δ 9.67 [s, 1H]
2.	- NH 2^0 amide	δ 8.119 [s, 1H]
3.	-CH aromatic	δ 7.36-7.93 [m, 8H]
4.	-CH_3	δ 2.512 [s, 6H]

2.8.3 ^{13}C-NMR spectrum of DA-1 (DMSO-d_6)

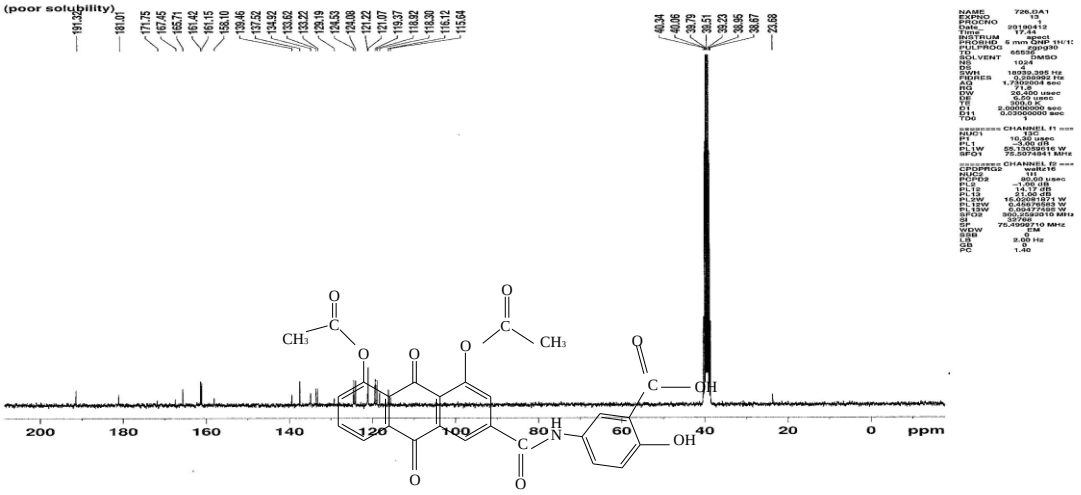


Fig 20. ^{13}C-NMR spectrum of DA-1 (DMSO-d_6)

2.9 Discussion

All the chemicals used in the synthesis were of analytical reagent grade and those of laboratory grade were purified prior to use. Thin layer chromatography of the synthesized prodrug was performed on pre-coated silica gel plates – 60 F_{254} using iodine chamber and UV light for visualization of spots and single spot was obtained for the same, using ethyl acetate: methanol: n-hexane:2 drops of GAA:1 drops of DMSO (2:2:2drops:1drops;v/v/v/v) solvent system indicating purity of the compound. Melting point of the final product was determined by open capillary method and is uncorrected. The prodrug melted with decomposition.

The main objective of the project was to target diacerein to colon by linking covalently with 5-ASA through enhancing aqueous solubility of diacerein so that absorption of prodrug would be restricted to upper GIT. Conjugation of diacerein with 5-ASA brought about moderate improvement in aqueous solubility of diacerein from being practically insoluble to 0.08 mg/ml. Log P_{oct} of diacerein was found to be 3.65 which was lowered to 2.85 for prodrug DA-1. The lowering of partition coefficient was in accordance with the enhanced aqueous solubility.

The IR spectrum (**Fig 18**) of the synthesized prodrug showed characteristic absorption bands for NH stretching of secondary amide at 3400 cm^{-1} and C=O stretching of secondary amide at 1624 cm^{-1} confirming the formation of amide prodrug. The prominent peak of C=O stretching of ester linkage of diacerein backbone was evident at 1760 cm^{-1}. The characteristic peak of OH stretch carboxylic acid of the 5-ASA backbone was observed at 3120 cm^{-1} conforming that group did not react during the course of reaction and was free in the prodrug structure.

The ^{1}H-NMR spectrum **(Fig 19 A, B)** of DA-1 exhibited characteristic chemical shift of protons of NH secondary amide atδ 8.11 ppm conforming the formation of amide prodrug. The chemical shifts of all other protons observed in ^{1}H-NMR of DA-1 were in accordance with its anticipated structure.[13] C- NMR of DA-1**(Fig 20)** exhibited chemical shifts for carbons at relevant δ ppm values.

The number of carbon atoms observedin^{13} C- NMR were matching with the anticipated structure of DA-1. The sample of synthesize prodrug has been sent to CSIR-CDRI for D_2Oexchange NMR and the results are awaited.

2.10 *in vitro* release kinetics:

The design of a prodrug is predicted on the ability of the host tissue or organism to regenerate the bioactive parent molecule from the inactive bio-reversible drug derivative because, intact/stable/non-bio-reversible prodrug implies that the drug is not getting activated *in vivo* and therefore is not available at the site which was targeted by that specific delivery system. Therefore, it is required to test the prodrug ability to deliver the drug to the site of action in intact form and evaluate the exact mechanism of activation of prodrug once it reaches the target site. Biotransformation of prodrug to active drug can take place either chemically or enzymatically. Most common mechanism is hydrolytic cleavage mediated by enzymatic catalysis although oxidative and reductive reactions have also been used. GIT microflora has several enzymes that cleave bonds like glycosidic, azo, carboxylic esters, sulphate and nitrate esters, amides and sulfamates. The amino acid derivatives are hydrolyzed by endopeptidases [58]. *In vitro* release kinetics is an important tool to understand the behavior of prodrug with respect to its stability and ability to release the drug *in vitro* conditions that simulate the ones in the body with respect to pH and enzymes. *In vitro* dissolution testing has been an integral component for characterization of a colon-specific drug delivery system. Physico-chemically and hydrodynamically defined conditions that simulate with the environment that is encountered in GI tract should be provided ideally for carrying out *invitro* release studies from colon-targeting delivery systems. Basket method, paddle method, Bio-Dis method and flow-through cell method are the four dissolution apparatus recommended in the USP. However, sometimes it is necessary to modify USP dissolution methods due to certain constraints observed during dissolution evaluation of controlled release drug delivery systems meant for oral administration [59].Several activation mechanisms are utilized in different, novel designs of colon-specific delivery systems. For evaluation of such systems, the ideal environment in which the release is to be studied should principally simulate the pH, type of bacterial population, type and specific enzymatic activity, fluid volume and mixing intensity of the *in vivo* environment. Conventional basket method is simple and convenient, but essentially provides insight on qualitative profile and specifications or functionality of a delivery system rather than validity of its design. This method has been regularly employed using aqueous buffers of varied pH 1.2, pH 4.5, pH 6.8, pH 7.4, with or without enzymes, simulating the transit time, changing pH and enzymatic set up throughout the GIT [60, 61]. The studies are carried out over different time periods depending on the stability of the prodrug and the rate at which it releases the activated drug. The technique of incubation with tissue homogenates of stomach and small intestine of rats to check stability of colon-specific prodrugs in upper GIT may also used many times [62]. USP Dissolution Apparatus III is used to assess the drug release in different media taking advantage of the unique technique of moving dissolution tubes along successive rows of vessels which could be pre-programmed. Most of the colon-targeting delivery systems are activated by colonic microflora and therefore, conventional basket method could not predict the *in vivo* performance of such systems.

Alternatively, fecal and caecal contents of rats, pigs and rabbits have been utilized as dissolution medium with which the prodrug are incubated in CO_2 incubator or in nitrogen atmosphere to maintain anaerobic conditions at 37±1ºC. Samples are withdrawn at different intervals for analysis. The predominant presence of *Bifidobacterium, Bacteroides* and *Lactobacillus* in rat caecal contents is similar to human colonic microflora and is therefore it is most preferred in the dissolution studies. Colon-specific targeting has also been studied by incubating prodrug with colonic segments to get a proof of concept. As the present work was aimed to design the colon-targeted mutual amide prodrug of diacerein with 5-ASA,it is necessary to assess its suitability with respect to stability in small intestine.The feasibility of hydrolysis of amide linkage by N-acyl amidases secreted by colonic microflora was tested with the help of release study in fresh rat fecal matter at 37 ± 1℃.All the kinetic studies were carried out in triplicate. The K values were calculated from the plot, and then K was determined separately along with $t_{1/2}$ and percentage of diacerein released over the course.

Following formulae were used to calculate the half-life and order of reaction:

1. **Zero order reaction:**

Rate constant (K) in mg $ml^{-1}min^{-1}$:

$$\mathbf{x = Kt}$$

Half life ($t_{1/2}$) in min:

$$\mathbf{t_{1/2} = \frac{a}{2K}}$$

2. First order reaction:

Rate constant (K) in min^{-1}:

$$\mathbf{\log \frac{a}{(a-x)} = \frac{K}{2.303} \times t}$$

Half life ($t_{1/2}$) inmin:

$$\mathbf{t_{1/2} = \frac{0.693}{K}}$$

Where,

a = Initial concentration of prodrug in µg/ml

x = Amount of drug released in µg/ml

(a – x) = Amount of prodrug remaining in µg/ml

t = time in min

2.11Release studies in rat fecal matter

Fresh rat fecal matter was collected from animal cages. A solution of DA-1 of (90µg/ml) was prepared in 0.2 M phosphate buffer (pH 7.4) USP and fecal matter was homogenized in distilled water and it was diluted with the help of 0.2 M phosphate buffer (pH 7.4) USP. Test tubes were numbered and arranged in the holder. In every test tubes 0.9 ml prodrug solution and 0.1 ml of fecal matter solution was poured and all the test tubes were closed properly with the help of cotton plug and incubated at 37± 1℃ in an incubator. At every hour that is from 0 to 7 h test tubes were taken out and the content was filtered with the help of membrane filter and absorbance was noted at λ_{max} 209.30nm for calculating unknown concentration of diacerein released by the prodrug by Shimadzu UV 1800 spectrophotometer.

The results of release study of diacerein from DA-1 are compiled in**Table 7.**

Table 7: Release profile DA-1 on incubation with rat fecal matter

Time (t) (min)	x (mg/ml)	K ($mg\ ml^{-1}\ min^{-1}$)	$t_{1/2}$ (min)	Cumulative % release
60	0.0045	0.000075	1800	3.6
120	0.0046	0.000038		3.7
180	0.0031	0.000017		2.52
240	0.0041	0.000017		3.3
300	0.0045	0.000015		3.6
360	0.0029	0.000008		2.3
420	0.0046	0.00001		3.7
		Mean=0.000025		

*Initial concentration of prodrug was 90µg/ml, x= concentration of diacerein released by prodrug, K= Rate constant, $t_{1/2}$ = Half life

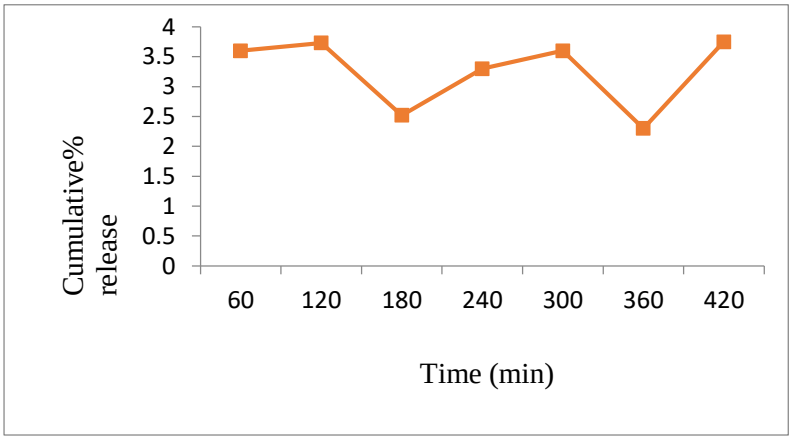


Fig 21. Release profile of diacerein from DA-1 on incubation with rat fecal matter

Table8: Release data of diacerein from DA-1

Incubation medium	Order of reaction	K ($mg\ ml^{-1}\ min^{-1}$)	$t_{1/2}$ (min)	Cumulative % release
Rat fecal matter	Zero order	0.000025	1800	3.7%

2.12 Discussion

In vitro release kinetics of DA-1 was studied in fresh rat fecal matter in order to assess the ability of colonic enzymes such as N-acyl amidase to hydrolyze amide linkage between diacerein and the carrier 5-ASA. The release kinetics was studied over a period of 7h at 37±1˚C in an incubator. DA-1furnished 3.7% release of diacerein in fecal matter over a

period of 7h following zero order kinetics. The rate constant and half life were found to be 0.000025mg ml^{-1} min^{-1} and 1800min respectively. The results of the study indicated that activation of prodrug was very slow which could be ascribed to the steric hindrance of the anthracene nucleus present adjacent to the amide linkage which must have resisted the approach of enzyme towards the linkage for its hydrolysis.

The proposed activation mechanism is depicted in **Fig 22.**

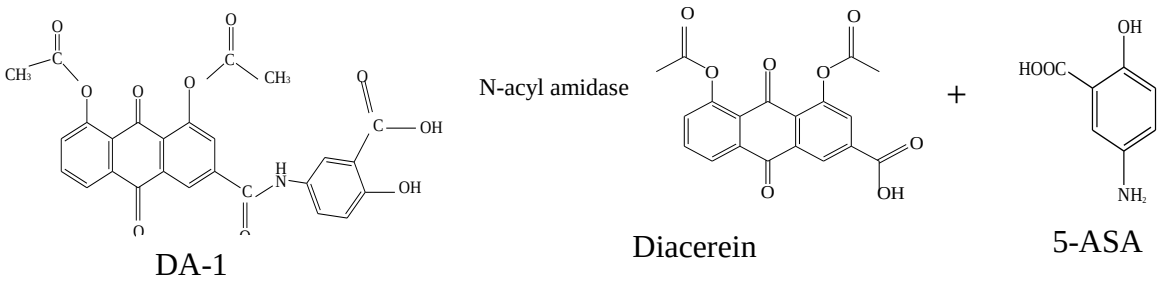
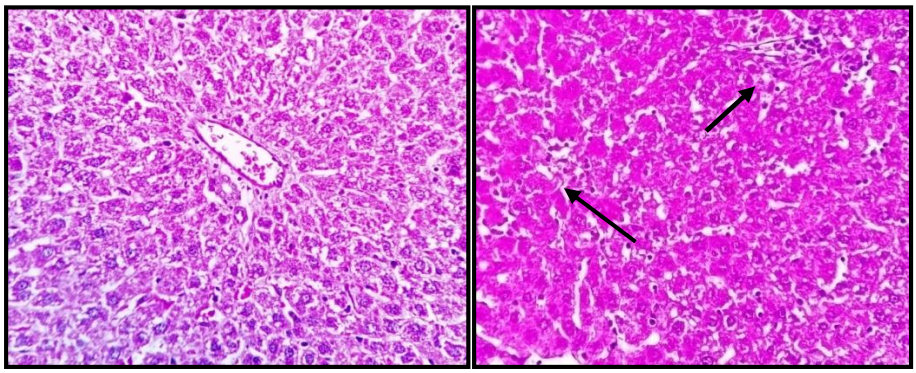


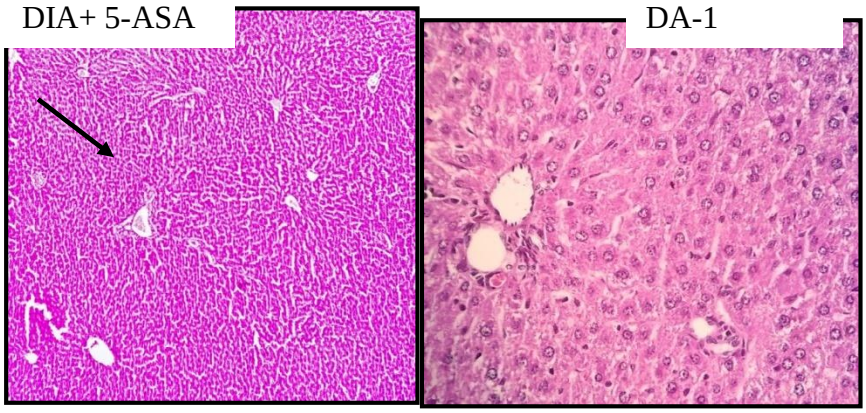
Fig 22. Proposed mechanism of activation

Because of time constraint the release kinetics of prodrug could not be studied in colon homogenate where the population of anaerobic bacteria and concentration of N-acyl amidases would have been more which might have given a better release of diacerein.

Diacerein 5-ASA



DIA+ 5-ASA DA-1



Staining by hematoxyline and eosine, (H.& E.X100)

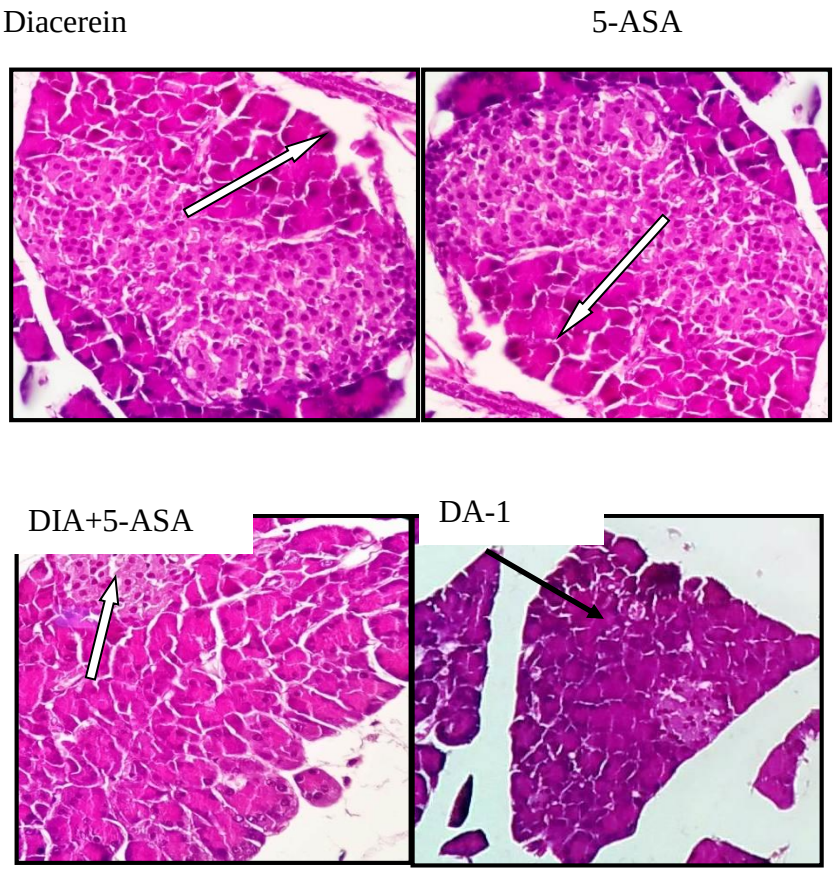
Diacerein: Cloudy swelling in hepatocytes

5-ASA:Cloudyswelling (arrow) in hepatocytes and focal infiltration of lymphocytes in hepatic parenchyma

Diacerein+5-ASA: Normal histology

DA-1: Normal

Fig 31. Histopathology of Wister rat liver



Staining by hematoxyline and eosine, (H.& E.X100)

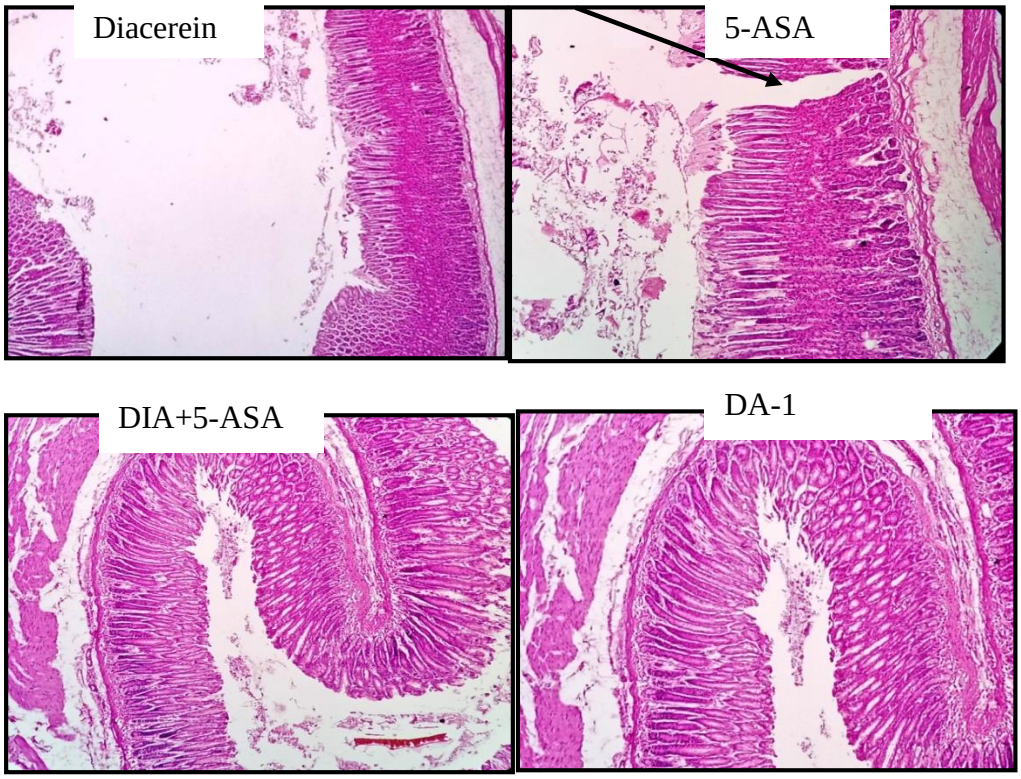
Diacerein: Normal acini (arrow) and islets (arrowhead). Clear spaces are indicative of high adipose tissue

5-ASA: Normal acini (arrow) and islets (arrowhead). Clear spaces are indicative of high adipose tissue

DIA+ 5- ASA:Normal acini (arrow) and islets (arrowhead). Clear spaces are indicative of high adipose tissue

DA-1: Normal islets (arrow) and acini

Fig 32. Histopathology of Wister rat pancreas



Staining by hematoxyline and eosine, (H.& E.X100)

Diacerein: Gastritis characterized by infiltration of mononuclear cells (MNCs) in mucosa

5-ASA: Gastritis characterized by erosion (arrow) in focal area, degeneration and desquamation of epithelial cells along with mild infiltration of mono-nuclear cells

DIA+5-ASA Slight degeneration and desquamation of epithelial cells

DA-1: Stomach: Slight degeneration and desquamation of epithelial cells

Fig 33. Histopathology of Wister rat stomach

2.13 Discussion

Having synthesized the prodrug of diacerein and 5-ASA and subsequent confirmation of its structure and studying its release kinetics, it was essential and important to study how the synthesized prodrug would behave when administered to animals to determine site-specificity through TNBS-induced colitis model for chronic inflammatory conditions and ulcerogenic tendency.5-ASA and diacerein were used as standards and the ameliorating effect of synthesized prodrug was compaired with the standards as well as physical mixture of 5-ASA and diacerein. All standards and test compounds were administered orally. The mitigating effect of all test groups was determined by disease activity score rate, colon/body weight ratio, and histopathological studies of colon. The prodrug was also assessed for its safety profile in comparison to 5-ASA-induced gastric ulcers/pancreatitis and sulfapyridine-induced hepatitis with the help of histopathological analysis of stomach, pancreas and liver.

After a washing period of two days, on day 3, all animals received an intra-rectal application of TNBS except the healthy control group. Before this time point, animals showed no clinical problems. After inducing the experimental colitis, the disease activity score increased rapidly and consistently for the next 3 days for all groups.Starting from day 6, rats received orally, standard and test drugs daily for five consecutive days. Only the colitis control group received saline (0.9%). The disease activity score rate was used to evaluate the severity of colonic inflammation and the colitis control group proved to be an excellent model of inflammation as evidenced by the highly increased disease activity score.All drug- receiving groups showed a decrease of inflammation severity after a lag time of 24 h. The difference between the drug-treated groups and colitis control became significant on day 8. There is an inverse correlation between lowering of disease activity score rate and degree of ameliorating effect of a drug on the colonic inflammation. Lower the disease activity score rate, higher is the ameliorating effect.The colitis control group at the end of 11 days, exhibited highest disease activity score rate (2.74 ± 0.68) which was significantly brought down by orally administered diacerein and 5-ASA by 73.73% (0.72 ± 0.152) and 75.92% (0.66± 0.0) respectively. Prodrug-treated group provided 95.25% protection (0.13 ± 0.29) which proved to be 21% more effective in ameliorating colonic inflammation than the standard drugs i.e. diacerein and 5-ASA which could be ascribed to the synergistic anti-inflammatory effect offered by the carrier 5-ASA . Physical mixture of diacerein and 5-ASA furnished 90.52% (0.26 ± 0.36) protection against TNBS-induced colitis which was slightly less than prodrug.

On day 11, the animals were sacrificed and colon/body weight ratio was determined to quantify inflammation, as there is an inverse relationship between lowering of colon/body weight ratio and extent of amelioration of colonic inflammation. The prodrug-treated group and physical mixture showed a distinct decrease in the colon to body weight ratio (0.0098± 0.0002) as compared to colitis control group (0.047±0.001). Diacerein and 5-ASA- treated groups significantly reduced colon/body weight ratio to 0.007 ± 0.0002 that was comparable to the healthy control. These results were in accordance with the observations for disease activity score rate. After sacrificing the animals, colon length, colon diameter and gut pH were also determined. The colon length reduces while the diameter increases due to colitis [84]. All the treatment groups significantly increased the colon length and reduced the diameter back to normal as that of healthy control indicating prominent protective effect on the colonic inflammation.

The pH of colon becomes acidic during IBD [14]. Gut pH of colitis control was 4.6 which was brought to normal (6.6-9) by all the treatment groups indicating significant mitigating effect.

Histopathological characterization of colon sections was performed by considering four independent parameters viz. mucosal distortion, inflammation, fibrosis and cryptitis. In healthy control the colonic architecture was found to be normal with all the four layers intact **(Fig 31 HC)**. In colitis control group (**Fig 31 CC**) the colons appeared flaccid and filled with liquid stool. The caecum, colon and rectum all had evidence of mucosal congestion, erosion and hemorrhagic ulcerations. The histopathological features included transmural necrosis, edema and absence of epithelium and a massive mucosal and a sub-mucosal infiltration of inflammatory cells.

In vivo treatment with the diacerein**(Fig 31 Diacerein)** and DA-1 **(Fig 31 DA-1)** resulted in enteritis characterized by degeneration of glandular epithelial cells (2) and infiltration of MNCs in mucosa (3) indicating mild protection against TNBS- induced colitis. 5-ASA-treated group **(Fig 31 5-ASA)** showed marked protection of disrupted colon against TNBS. Goblet cells and mucosal epithelial cells were preserved. Physical mixture of diacerein and 5-ASA **(Fig 31 DIA+5-ASA)** exhibited enteritis showing degeneration and desquamation of epithelial cells from mucosa, goblet cell hyperplasia along with infiltration of mononuclear cells in mucosa and sub-mucosa of colon.

To evaluate and compare the safety profile of synthesized prodrug with respect to 5-ASA-induced pancreatitis/gastric ulcers and sulfapyridine-induced hepatitis, the prodrug was also assessed for its probable damaging effect on liver, pancreas and stomach with the help of histopathological analysis.

Treatment with DA-1**(Fig 32 DA-1)** and physical mixture of diacerein and 5-ASA **(Fig 32 DIA+5-ASA)** resulted in normal histology of liver indicating absence of any adverse effect on the hepatocytes. Diacerein**(Fig 32 Diacerein)** and 5-ASA-treated groups**(Fig 32 5-ASA)** showed cloudy swelling in hepatocytes and focal infiltration of lymphocytes in hepatic parenchyma.

Histological assessment of pancreas of all treatment groups**(Fig 32 Diacerein, 5-ASA, DIA+5-ASA, DA-1)**showed normal islets and acini and the results were comparable to that of healthy control.

Assessment of histology slides of rat stomach indicated that in DA-1 **(Fig 33 DA-1)** and physical mixture-treated group **(Fig 33 DIA+5-ASA)** slight degeneration and desquamation of epithelial cells was observed while in **d**iacerein-treated group **(Fig 33 Diacerein)** gastritis characterized by infiltration of mononuclear cells (MNCs) in mucosa was evident. 5-ASA – treated group **(Fig 33 5-ASA)** showed gastritis characterized by erosion in focal area, degeneration and desquamation of epithelial cells along with mild infiltration of mono-nuclear cells which is in accordance with the GI ulcerogenicity reported for oral 5-ASA [15].

2.14 Summary and conclusion

Summary

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disorder, caused by a disregulated immune response to host intestinal microflora. The two principal types of inflammatory bowel disease are ulcerative colitis (UC), which is primarily restricted to the colon and rectum, and Crohn's disease (CD), which can affect any segment of the gastrointestinal tract (GIT) from the mouth to the anus. Inflammatory bowel disease (IBD) is a chronic intestinal disease that often has its onset during young adulthood and has a chronic relapsing-remitting course. UC affects the innermost lining of the large intestine (colon) and rectum. Symptoms usually develop over time, rather than suddenly. UC can be debilitating and can sometimes lead to life-threatening complications. While it has no known cure, treatment can greatly reduce the signs and symptoms of the disease and even bring about long-term remission. UC symptoms can vary, depending on the severity of inflammation and where it occurs.The exact cause of IBD is not known. The genetic (antimicrobial peptides, handling of bacteria, chemokines and cytokines) and environmental factors (diet, microbes, infections, stress, NSAIDs, smoking) start damaging the barrier of GIT, then the translocation of the microbial products occur, which results to activation of immune cell.

So, the failure of instruction and activation of immune cells leads to the edifice of pro-inflammatory mediators which cause tenderness. Due to inflammation, fibrosis, stereosis, abscesses and fistula occur. TNF-α is a pro-inflammatory cytokine that mediates the local inflammatory immune response and induces the expression of other pro-inflammatory cytokines and chemokines. Chemokines play a role in maintaining homeostasis of mucosal immunity and enhance the recruitment and activation of lymphocytes that contribute to the pathogenesis of IBD.IL-1 along with TNF-α plays an important role in the pathogenesis of IBD due to its pro-inflammatory activity. IL-1such as IL-1α and IL-1β induce the production of type 2 cyclooxygenase, phospholipase A and inducible nitric oxide syntheses (INOS) leading to inflammation of the intestinal mucosa.

In IBD, exposure to antigens gives rise to antibodies followed by inflammatory response after immune system activation leading to generation of cytokines.Pro-inflammatory cytokines include interleukin (IL), IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α.The first evidence for the involvement of IL-1β in IBD was by Mahida*et al*. (1989), they reported enhanced production of this cytokine in mononuclear cells isolated from the actively inflamed mucosa of IBD patients. IL is also involved in pathogenesis of both acute and chronic inflammatory disorders.Inhibition of the cytokine generation attenuates inflammatory conditions in IBD.Diacerein directly inhibits IL-1 synthesis and release and down modulates IL-induced activities. Diacerein also plays an inhibitory role in superoxide production and also affects macrophage migration and phagocytosis which are involved in the inflammatory response.Therefore IL-1 inhibitors can prove to be an alternate option for the management of IBD.Till date there are no reports in the literature of colon- specific prodrugs of diacerein for UC. 5-ASA is the drug of choice for IBD since ages in spite of its severe side effects on GIT. In the present work, we planned to covalently link diacerein with 5-ASA by applying co-drug concept and investigate the utility of diacerein (anti-arthritic drug) in the management of UC by targeting them to colon.

In the present work we have selected two drugs which are mechanistically different where diacerein is an anti-arthritic drug which has anti-inflammatory, analgesic effect through IL-1 inhibition and 5-ASA is a 5-LOX inhibitor used as a first line treatment of UC.By chemically combining diacerein with 5-ASA through a bioreversible covalent linkage, we synthesized a mutual prodrugs which maximum provide multiple benefits through their multi-targeted pharmacological actions, where colonic inflammation will be treated by 5-ASA and IL-1 will be simultaneously inhibited by diacerein which has been implicated in the pathophysiology of UC.The prodrug was screened in 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) - induced colitis in rats and its mitigating effect was compared with the parent drugs and their physical combination.

Plan of work

Mutual amide prodrug was synthesized by CDI coupling reaction, purification was carried out by preparative TLC followed by physico-chemical characterization. Structural conformation was performed by spectral analysis; *in vitro* release kinetics was undertaken in rat fecal matter and biological evaluation carried out in TNBS-induced experimental colitis in Wister rats.

All the chemicals used in the synthesis were of analytical reagent grade and those of laboratory grade were purified prior to use.

Thin layer chromatography of the synthesized prodrug was performed on pre-coated silica gel plates – 60 F_{254} using iodine chamber and UV light for visualization of spots and single spot was obtained for the same, usingethyl acetate:methanol: n-hexane: ethyl acetate (2:2:2drops:1drops;v/v/v/v) solvent system indicating purity of the compound. Melting point of the final product was determined by open capillary method and is uncorrected. The prodrug melted with decomposition.

The main objective of the project was to target diacerein to colon by linking it covalently with 5-ASA through enhancing aqueous solubility of diacerein so that absorption of prodrug would be restricted to upper GIT. Conjugation of diacerein with 5-ASA brought about moderate improvement in aqueous solubility of diacerein from being practically insoluble 0.08 mg/ml. Log P_{oct} of diacerein was found to be 3.65 which was lowered to 2.85 for prodrug DA-1. The lowering of partition coefficient was in accordance with the enhanced aqueous solubility.

The IR spectrum (**Fig 18**) of the synthesized prodrug showed characteristic absorption bands for NH stretching of secondary amide at 3400 cm^{-1} and C=O stretching of secondary amide at 1624 cm^{-1} confirming the formation of amide prodrug. The prominent peak of C=O stretching of ester linkage of diacerein backbone was evident at 1760 cm^{-1}. The characteristic peak of OH stretch carboxylic acid of the 5-ASA backbone was observed at 3120 cm^{-1} confirming that group did not react during the course of reaction and was free in the prodrug structure.

The ^{1}H-NMR spectrum **(Fig 19 A, B)** of DA-1 exhibited characteristic chemical shift of protons of NH secondary amide atδ 8.11 ppm conforming the formation of amide prodrug. The chemical shifts of all other protons observed in ^{1}H-NMR of DA-1 were in accordance with its anticipated structure.[13] C- NMR of DA-1**(Fig 20)** exhibited chemical shifts for carbons at relevant δ ppm values. The number of carbon atoms observed in[13] C- NMR were matching with the anticipated structure of DA-1. The sample of synthesize prodrug has been sent to CSIR-CDRI for D_2Oexchange NMR and the results are awaited.

In vitro release kinetics of DA-1was studied in fresh rat fecal matter in order to assess the ability of colonic enzymes such as N-acyl amidase to hydrolyze amide linkage between diacerein and the carrier 5-ASA. The release kinetics was studied over a period of 7h at 37±1℃ in an incubator. DA-1 furnished 3.7% release of diacerein in fecal matter over a period of 7h following zero order kinetics. The rate constant and half life were found to be 0.000025 mg ml^{-1} min^{-1} and 1800 minrespectively. The results of the study indicated that activation of prodrug was very slow which could be ascribed to the steric hindrance of the anthracene nucleus present adjacent to the amide linkage which must have resisted the approach of enzyme towards the linkage for its hydrolysis. The proposed activation mechanism is depicted in **Fig 25.**

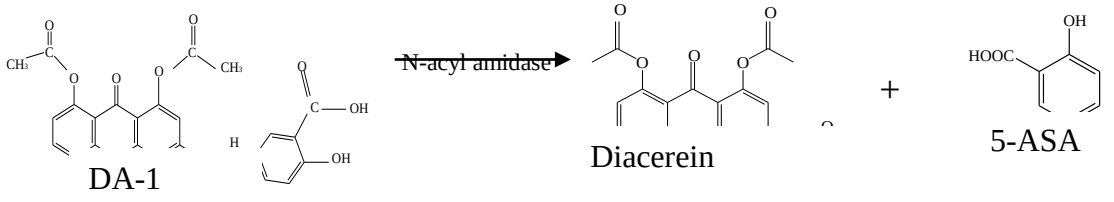


Fig 25. Proposed mechanism of activation of mutual amide prodrug of diacerein with 5-ASA.

Because of time constraint release kinetics of prodrug could not be studied in colon homogenate where the population of anaerobic bacteria and concentration of N-acyl amidases would have been more which might have given better release of diacerein.

Having synthesized the prodrug of diacerein and 5-ASA and subsequent confirmation of its structure and studying its release kinetics, it was essential and important to study how the synthesized prodrug would behave when administered to animals to determine site-specificity through TNBS-induced colitis model for chronic inflammatory conditions and ulcerogenic tendency.5-ASA and diacerein were used as standards and the ameliorating effect of synthesized prodrug was compared with the standards as well as physical mixture of 5-ASA and diacerein. All standards and test compounds were administered orally. The mitigating effect of all test groups was determined by disease activity score rate, colon/body weight ratio, and histopathological studies of colon. The prodrug was also assessed for its safety profile in comparison to 5-ASA-induced gastric ulcers/pancreatitis and sulfapyridine-induced hepatitis with the help of histopathological analysis of stomach, pancreas and liver.

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On day 11, the animals were sacrificed and colon/body weight ratio was determined to quantify inflammation, as there is an inverse relationship between lowering of colon/body weight ratio and extent of amelioration of colonic inflammation. The prodrug-treated group and physical mixture showed a distinct decrease in the colon to body weight ratio (0.0098± 0.0002) as compared to colitis control group (0.047±0.001). Diacerein and 5-ASA-treated groups significantly reduced colon/body weight ratio to 0.007 ± 0.0002 that was comparable to the healthy control. These results were in accordance with the observations for disease activity score rate. After sacrificing the animals, colon length, colon diameter and gut pH were also determined. The colon length reduces while the diameter increases due to colitis.

All the treatment groups significantly increased the colon length and reduced the diameter back to normal as that of healthy control indicating prominent protective effect on the colonic inflammation. The pH of colon becomes acidic during IBD. Gut pH of colitis control was 4.6 which was brought to normal (6.6-9) by all the treatment groups indicating significant mitigating effect. Histopathological characterization of colon sections was performed by considering four independent parameters viz. mucosal distortion, inflammation, fibrosis and cryptitis. In healthy control the colonic architecture was found to be normal with all the four layers intact **(Fig 31 HC)**. In colitis control group (**Fig 31 CC**) the colons appeared flaccid and filled with liquid stool. The caecum, colon and rectum all had evidence of mucosal congestion, erosion and hemorrhagic ulcerations. The histopathological features included transmural necrosis, edema and absence of epithelium and a massive mucosal and a sub-mucosal infiltration of inflammatory cells. *In vivo* treatment with the diacerein**(Fig 31 Diacerein)** and DA-1 **(Fig 31 DA-1)** resulted in enteritis characterized by degeneration of glandular epithelial cells (2) and infiltration of MNCs in mucosa (3) indicating mild protection against TNBS- induced colitis. 5-ASA-treated group **(Fig 31 5-ASA)** showed marked protection of disrupted colon against TNBS. Goblet cells and mucosal epithelial cells were preserved. Physical mixture of diacerein and 5-ASA**(Fig 31 DIA+5-ASA)** exhibited enteritis showing degeneration and desquamation of epithelial cells from mucosa, goblet cell hyperplasia along with infiltration of mononuclear cells in mucosa and sub-mucosa of colon. To evaluate and compare the safety profile of synthesized prodrug with respect to 5-ASA-induced pancreatitis/gastric ulcers and sulfapyridine-induced hepatitis, the prodrug was also assessed for its probable damaging effect on liver, pancreas and stomach with the help of histopathological analysis.

Treatment with DA-1**(Fig 31 DA-1)** and physical mixture of diacerein and 5-ASA **(Fig 31 DIA+5-ASA)** resulted in normal histology of liver indicating absence of any adverse effect on the hepatocytes. Diacerein**(Fig 31 Diacerein)** and 5-ASA-treated groups**(Fig 31 5-ASA)** showed cloudy swelling in hepatocytes and focal infiltration of lymphocytes in hepatic parenchyma.

Histological assessment of pancreas of all treatment groups**(Fig 32 Diacerein, 5-ASA, DIA+5-ASA, DA-1)**showed normal islets and acini and the results were comparable to that of healthy control.Assessment of histology slides of rat stomach indicated that in DA-1 **(Fig 33 DA-1)** and physical mixture-treated group **(Fig 33 DIA+5-ASA)** slight degeneration and desquamation of epithelial cells was observed while in diacerein-treated group **(Fig 33 Diacerein)** gastritis characterized by infiltration of mononuclear cells (MNCs) in mucosa was evident. 5-ASA-treated group **(Fig 33 5-ASA)** showed gastritis characterized by erosion in focal area, degeneration and desquamation of epithelial cells along with mild infiltration of mono-nuclear cells which is in accordance with the GI ulcerogenicity reported for oral 5-ASA.

Conclusion:

A mutual amide prodrug of diacerein and 5-ASA was designed and synthesized successfully with a goal of exploring the possibility of repurposing an interlukein-1 inhibitor diacerein for the management of IBD. *In vitro* release kinetics in rat fecal matter indicated extremely slow activation and release of diacerein (only3.7%) due to steric hindrance of the anthracene, which might have been mediated by N-acyl amidases secreted by colonic microflora. Contrary to this, synthesized prodrug provided 95% protection against TNBS-induced colonic inflammation characteristic of colitis without adversely affecting pancreas and stomach. The outcome of this study suggests that significantly high extent of protective effect produced by DA-1 in TNBS-induced experimental colitis might be due to the intact prodrug and not due to the released diacerein or 5-ASA which was not sufficient to elicit such high level of ameliorating effect. More extensive studies are required to establish stability of prodrug at varied pH conditions and homogenates of GIT to establish it as a new chemical entity (hybrid molecule) capable of managing IBD not as prodrug but as a hybrid molecule.

Conflict of Interest:

The authors have no conflict of interest.

References:

1. Shahrokh: Introduction of inflammatory bowel disease biomarkers pannel using protein protein 6interaction (PPI) network analysis. 2016;1:S8-S13.
2. Ashwin N: Environmental risk factors for inflammatory bowel disease. Gastroenterol Hepatol 2014; 9: 367–74.
3. Lapidus A, Bernell O, Hellers G, Lofberg R: Clinical course of colorectal Crohn's disease: a 35-year follow-up study of 507 patients. Gastroenterology 1998; 114:1151-1160.
4. Shanahan F,Hawkey CJ, Bosch J, Richter JE, Garcia-Tsao G, Chan FKL :*Textbook of:* Ulcerative colitis. *Clinical Gastroenterology and Hepatology* Oxford Wiley-Blackwell 2012; 2:355–371.
5. Cavalla D: Therapeutic switching: a new strategic approach to enhance R& D products 2005; 8(11): 914-8.
6. Sleigh SH: Repurposing Strategies for Therapeutics. Pharmaceutical Medicine 2012; 24 (3): 151–159.
7. Franko ML: Chapter 13 - DNA Methyl transference Inhibitors for Cancer Therapy. Academic press 2015; 265-290.
8. Kumar VM, Meher CM: Approach of prodrug on drug design 2019; 1-3.
9. Rautio: Prodrug design and clinical application2008; (3): 272.
10. Huttunen K., Raunio H., Rautio J: Prodrugs design and clinical applications. Pharmacol. Rev. 2011; 63:750–771.
11. Philip AK, Dabas S, Pathak K.Optimized prodrug approach: a means for achieving enhanced anti-inflammatory potential in experimentally induced colitis. J Drug Target 2009;17(3):235-241.

12. Oluwatoyin AO, John TF. In vitro evaluation of khaya and albizia gums as compression coating for drug targeting to the colon. J Pharm Pharmacol 2005;57:63-168.
13. Akala EO, Elekwachi O, Chase V, Johnson H, Lazarre M, Scott K. Organic redox-initiated polymerization process for the fabrication of hydrogels for colon-specific drug delivery. Drug Dev Ind Pharm 2003;29(4):375-386
14. Chourasia MK, Jain SK. Pharmaceutical approaches to colon targeted drug delivery systems. J Pharm Pharm Sci 2003;6(1):33-66
15. Basit A, Bloor J. Prespectives on colonic drug delivery, Business briefing. Pharmtech 2003; 185-190.