Synthesis and in vitro kinetic study of novel mutual azo prodrug for inflammatory bowel disease

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ABSTRACT

Background: Inflammatory bowel disease (IBD) refers to idiopathic inflammatory diseases of the intestine, principally ulcerative colitis and Crohn's disease. IBD is characterized by chronic inflammation in the mucosal membrane of large intestine. 5-ASA is the gold standard for the treatment of IBD and when searched for a better 5-ASA prodrug, a novel mutual azo prodrug was designed and synthesized.

Methods: A mutual prodrug was synthesized by coupling *p*-phenetidine with salicylic acid. The stability of this prodrug in HCl buffer, in phosphate buffer and in rat fecal matter were monitored.

Results: The chemical structure of mutual prodrug was characterized by physical and spectroscopic techniques using FTIR, UV/Visible, ^{1}H-NMR and ^{13}C-NMR spectra. In vitro kinetic studies in HCl buffer (pH 1.2) showed negligible release of 5-ASA and *p*-phenetidine, whereas in phosphate buffer (pH 7.4) only (22.04 %) release was observed over a period of (6 hr.). In rat fecal matter, the hydrolysis of mutual prodrug was almost complete (77.96 %), with a half-life of 182.67 min, following zero order kinetics.

Conclusion: The mutual prodrug was split in colon by the action of bacterial azoreductase into 5-ASA and *p*-phenetidine that constitute two anti-inflammatory compounds with different mechanisms of action. Therefore, this mutual prodrug is a promising colon specific prodrug for IBD and worthy of further study.

Keywords: IBD, 5-ASA, *p*-phenetidine, azo coupling, mutual prodrug.

الخلاصة

الخلفية: مرض الأمعاء التحريضي يشير إلى مجموعة من الأمراض التحريضية المجهولة الأسباب والتي تصيب الأمعاء خاصا التهاب القولون المتقرح ومرض كرونز، ويتميز مرض الأمعاء التحريضي بوجود التهاب مزمن في الغشاء المخاطي للأمعاء الغليظة. يعتبر ٥- أمينو حامض الساليسيليك المعيار الذهبي لمعالجة هذا المرض، وعند البحث عن أفضل بادئ دواء لعقار٥- أمينو حامض الساليسيليك، تم تصميم وتصنيع بادئ دواء تبادلي جديد من نوع الآزو.

طرق العمل: تم تصنيع بادئ الدواء التبادلي باقتران البارا- فينتيدين مع حامض الساليسيليك. إن استقرارية بادئ الدواء التبادلي في محلول حامض الهايدروكلوريك البفري، محلول الفوسفيت البفري وفي مادة براز الجرذان قد تمت مراقبتها.

النتائج: تم تشخيص الشكل الكيميائي لبادئ الدواء الجديد باستخدام الوسائل الفيزيائية والطيفية كطيفي الأشعة تحت الحمراء وفوق البنفسجية والرنين النووي المغناطيسي للبروتون والكاربون، كما أظهرت الدراسات الحركية خارج جسم الكائن الحي استقرارية بادئ الدواء الجديد في محلول حامض الهايدروكلوريك البفري (درجة حموضة ٢,١) وتحرير فقط (٠٤,٢٢%) في محلول الفوسفيت البفري (درجة حموضة ٤,٧) خلال ٦ساعات. أن تحلل بادئ الدواء الجديد في مادة براز الجرذان كان شبه كامل (٧٧,٩٦%) وبعمر نصف (١٨٢,٦٧ دقيقة) متبعا حركيات رتبة الصفر.

الاستنتاج: في القولون وتحت تأثير إنزيم الازوريدكتيز البكتيري، سينشطر بادئ الدواء التبادلي ليحرر٥- أمينو حامض الساليسيليك والبارا- فينتيدين موفرا بذلك مركبين من مضادات التحريض باليات عمل مختلفة. لذلك يمكن أن يعتبر بادئ الدواء التبادلي هذا دواء واعد لعلاج مرض الأمعاء التحريضي كما انه جدير بدراسة إضافية.

Inflammatory bowel disease (IBD) encompasses several chronic inflammatory conditions, most significantly ulcerative colitis and Crohn's disease[1]. IBD is characterized by chronic inflammation in the mucosal membrane of the small and/or large intestine[2]. The etiology of IBD remains unknown; however, two primary theories have been proffered focusing on either a specific persistent infectious agent[3,4] or an abnormal host immune response to ubiquitous antigens in the luminal constituents. Evidence support the observation that patients with IBD are genetically susceptible to this disease and the defect targets are unable to effectively down-regulate the inflammatory response to specific antigens or luminal bacteria[5]. Although many treatments have been recommended for IBD, they do not treat the cause but are effective only in reducing the inflammation and accompanying symptoms in up to 80% of patients[6].

Oral delivery is the most common and preferred route of drug administration, this is the ideal route to deliver compounds to colonic sites to treat IBD; however; the digestive tract exhibits several obstacles to drug delivery including gut motility,[7] stomach intraluminal pH profiles[8] and degradative enzymes[9].

In order to achieve an effective colonic delivery, a drug needs to be protected from absorption and/or the environment of upper GI tract and then rapidly released into the proximal colon, which is the optimal site for colon-targeted delivery of the drug. Colonic drug delivery through colon-specific prodrug activation may be accomplished by the utilization of the high activity of certain enzymes at the target site relative to non-target tissues, enabling for prodrug conversion to active drug[10].

The intestinal microflora consists of a coexisting mixture of aerobic, facultative anaerobic and strict anaerobic bacteria in a complex ecosystem. These bacteria produce a wide range of enzymes such as β-glucuronidase, β-xylosidase, α-arabinosidase, β-galactosidase, nitroreductase, azoreductase, deaminase, urea hydroxylase, etc[11,12].

5-Aminosalicylic acid (5-ASA) is an effective compound to attenuate the inflammatory response in IBD while its mechanism of action is not fully understood. Because 5-ASA usually fails to reach the colon leading to significant adverse effects,[13] a prodrug approach for colonic delivery of 5-ASA has become a rational system of drug delivery for the topical treatment of IBD[14].

5-ASA triggers the peroxisome proliferator-activated receptor (PPAR-γ) family of nuclear receptors, which regulate inflammation, cell proliferation, apoptosis, and metabolic function. PPAR-γ receptors are highly expressed in colonic epithelia and their expression is up-regulated by the gut bacteria[15].

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 IBD[16, 17]. When they are administered orally, a large amount of the NSAIDs are absorbed from the upper GIT and causes systemic side effects. Therefore, it is preferable to deliver the drug site-specifically to the colon.[18]

As amide or azo prodrugs, selective delivery of NSAIDs to colon can be useful in terms of reducing the administered dose and undesirable side-effects[19, 20] but the most important disadvantages of these prodrugs are the low bioavailability and the

irritation cause by their carboxylic acid groups[21, 22].

P-phenetidine is a minor metabolite of phenacetin and is a more potent inhibitor, even at a nanomolar level, of the prostaglandins synthesis than indomethacin, with greater selectivity to COX-2 inhibition[23, 24].

It was believed that *p*-phenetidine may cause renal toxicity and methemo-globinemia, but recently, researches confirm that the N-hydroxy metabolite which results from hepatic oxidation of *p*-phenetidine is responsible for these toxicities[25, 26] .

Experimental

Materials *P*-phenetidine was synthesized in laboratory according to Williamson synthesis of ether[27, 28] via condensation of sodium *P*-aminophenoxide and ethyl chloride. All other chemicals were of analytical reagent grade and those of synthetic grade were purified prior to use.

Instruments

Thin layer chromatography (TLC) of the synthesized compound was performed on precoated plates of silica gel 60 F 5 (Merck) using iodine vapor and UV light for visualization. The solvent mixture employed for TLC was composed from chloroform: acetone (4:1).

Melting point of the product was determined by open capillary method on electrothermal CIA 9300 and is uncorrected.

Chemical structures were drawn by Chemdraw Office 2001 software.

Ultraviolet spectrum of the synthesized compound was determined on Carrywinn U.V. Varian UV/Visible double-beam spectrophotometer in hydrochloric acid buffer (pH 1.2), phosphate buffer (pH 7.4), chloroform and distilled water.

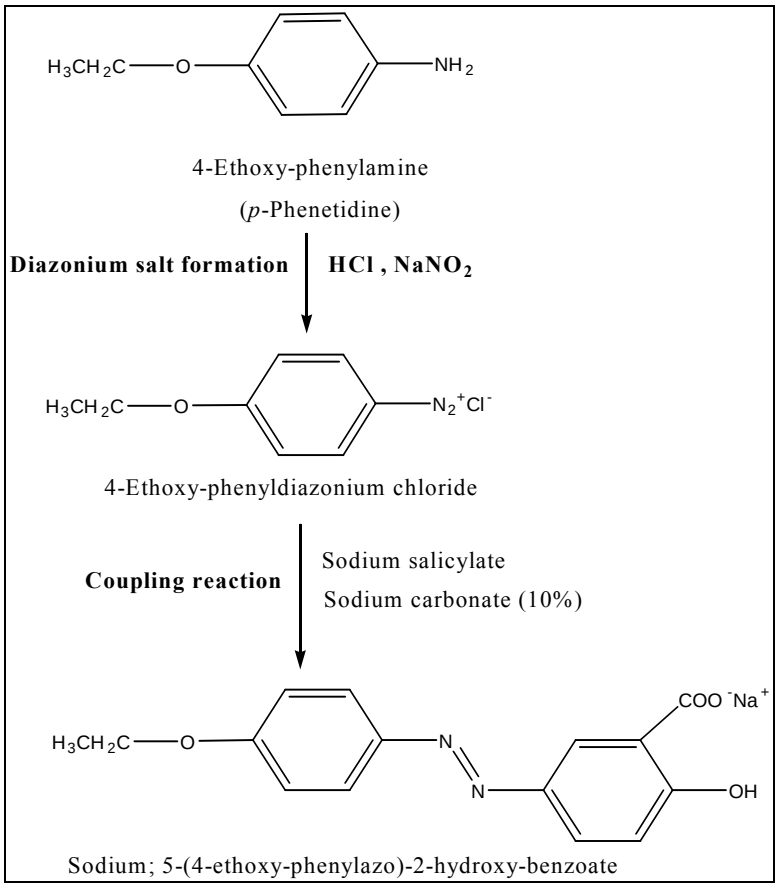
FTIR spectrum of the synthesized compound was recorded by Buck 500 scientific I.R. spectrophotometer in anhydrous potassium bromide (IR grade) pellet.

The ^{1}H-NMR and ^{13}C-NMR spectra of the synthesized compound were recorded by Varian Mercury 400 MHz. (France)

Synthesis of azo prodrug [29]

A concentrated hydrochloric acid (16 ml) was added to a well stirred suspension of *p*-phenetidine citrate (3.425 g, 0.025 mol) in water (12 ml) and the mixture was heated up to 70°C and maintained at that temperature till a clear solution obtained. After cooling the solution to 3°C in a cryostatic bath, a solution of sodium nitrite (2 g, 0.028 mol) in water (10 ml) was added dropwise over a period of 10 minutes with stirring. The reaction mixture was stirred at a temperature below 5°C for one hour. The excess of nitrous acid (tested by using moist starch iodide paper) was removed by adding required amount of sulphonic acid solution (10%). The clear diazonium salt solution was obtained and used immediately in the coupling reaction.

Salicylic acid (3.45 g, 0.025 mol) was dissolved in sodium hydroxide solution (25 ml, 10% w/v). The solution was cooled to 3°C in cryostatic bath. To this well stirred solution, the above diazonium solution was added dropwise and the temperature kept below 5°C. The reaction mass was further stirred for two hours at 5°C maintaining the pH 8.0 by adding required amount of 10% w/v of sodium carbonate solution. The reaction mass was diluted with hot water (80 ml) until the washings were neutral. Then diluted solution of HCl was added dropwise. The product was filtered off, dried and recrystallized from ethanol (scheme 1).



Scheme 1. Synthesis of azo prodrug

The purity of compound was established by TLC and its result showed that only a single spot was observed. The melting point was 217-219°C, the percentage of yield was 78% and the R_f value was 0.43.

In vitro stability studies

The stability of azo prodrug in 0.05 M hydrochloric acid buffer (pH 1.2) and in 0.05 M phosphate buffer (pH 7.4) was monitored according to the following procedure [30]:

A sample (10 mg, 0.035 mmol) of azo prodrug was introduced into a conical flask containing 900 ml of HCl buffer; the resulting solution was kept at a constant temperature (37 ± 1°C) using a water bath with gentle stirring. When the UV spectra of 5-ASA, *p*-phenetidine and prodrug were overlaid, it was observed that the UV spectrum of prodrug did not interfere with the absorption ranges of 5-ASA and *p*-phenetidine, as is obvious from the difference in the λ_{max} values of 5-ASA (303 nm), *p*-phenetidine (247 nm) and the prodrug (385 nm). Therefore the aliquots were directly estimated on UV/Visible spectrophotometer at 385 nm every 30 minutes for three hours to monitor the amount of prodrug remaining.

In order to examine the stability of azo prodrug in phosphate buffer, the same procedure as described above was followed, except that the phosphate buffer replaced the HCl buffer and the UV/Visible data were taken at 476 nm every 30 minutes for six hours.

Table 1. Kinetic data obtained from the stability studies

Type of buffer	A	λ_{max} (nm)	a (mmole)	ε
HCl buffer (pH 1.2)	0.0413	385	0.035	589.75
Phosphate buffer (pH 7.4)	0.0537	476	0.035	766.81

A = absorbance, a = conc. of mutual prodrug at zero time and ε = absorbance coefficient.

Release study in rat fecal matter [31]

The azo prodrug was dissolved in phosphate buffer (pH 7.4), so that the final concentration of the solution was 250 mg/ml. Fresh fecal material of rats was weighed (1 g) and placed in set of test tubes. To each test tube, (1 ml) of the prodrug solution was added and diluted to (5 ml) with phosphate buffer to achieve a final concentration of 50 mg/ml. The test tubes were incubated at 37°C.

Every (30 minutes) for six hours, one test tube was removed from a water bath and the concentration of azo prodrug was directly estimated on a double beam UV/Visible spectrophotometer at 476 nm. All the kinetic studies were carried out in triplicate and monitored by the decrease in prodrug concentration with time.

Results and Discussion

Colonic drug delivery has gained a great importance not just for the delivery of drugs for the treatment of local diseases associated with colon like Crohn's disease and ulcerative colitis but also for the potential it holds for the systemic delivery of proteins and therapeutic peptides[32]. The large intestine, though difficult to reach by peroral delivery, is still deemed to be the ideal site for the delivery of agents to cure the local diseases of colon.[33] The most critical challenge in such drug delivery approach is to preserve the formulation during its passage through the stomach and about first six meters of the small intestine [34, 35].

Targeted drug delivery to the colon would therefore, ensure direct treatment at the disease site and, consequently, lower the administered dose and systemic side effects.[36] A variety of approaches have been developed for the purpose of achieving colonic targeting, one of the most common approaches is azo prodrugs[37]. This type of prodrugs is designed to undergo minimal absorption and hydrolysis in the upper GIT and undergo enzymatic hydrolysis via azoreductase to release the active drug moiety in colon[38].

In treatment of IBD, 5-ASA usually coupled with a carrier for colon targeting,[39] the most commonly used naturally occurring colon-targeting carriers are polysaccharides such as cyclodextrins [40] and amino acids such as aspartic acid, glutamic acid, glycine, lysine and tyrosine, these carriers are not toxic but have no pharmacological activity[41-43].

The potential of some NSAIDs as colon targeted delivery systems for treatment of IBD was studied, but these systems have several disadvantages such as low bioavailability and irritation caused by their carboxylic acid group[44].

In this study, a novel mutual azo prodrug of 5-ASA with *p*-phenetidine was synthesized. The azo linkage of this mutual prodrug was proposed to be broken in colon by the action of azoreductase produced by colonic microflora to release two compounds with different anti-inflammatory mechanisms of action. This study proposed that this novel prodrug may be beneficial in treatment of IBD.

Infrared spectrum of azo prodrug

The infrared spectrum (KBr) of azo prodrug showed a weak band at 1494

cm^{-1} for unsymmetrical *p*-substituted azo group. The band at 3356 cm^{-1} indicating the presence of phenolic-OH (H-bonded). The bands at 1612 cm^{-1}, 1085 cm^{-1} can be attributed to aromatic ether. The characteristic bands at 1754 cm^{-1} and at 2935 cm^{-1} are due to the stretching of carbonyl and hydroxyl groups of carboxylic acid while the bands appear at 3095 cm^{-1}, 2885 cm^{-1} are corresponding to the stretching of methylene and methyl groups respectively. The strong bands at 824 cm^{-1}, 805 cm^{-1} refer to the bending of C-H of disubstituted and trisubstituted aromatic rings respectively.

UV/Visible spectrum of azo prodrug

The ultraviolet spectrum of azo prodrug gave different λ_{max} values in different solvents such as: λ_{max} in an aqueous acidic solution (pH 1.2) = 385 nm, λ_{max} in an aqueous phosphate buffer solution (pH 7.4) = 476 nm, λ_{max} in chloroform = 412 nm and the λ_{max} in distilled water =454 nm. The λ_{max} values of azo prodrug in different solvents showed an increasing in magnitude compared with 5-ASA, this red shift or bathochromic shift is due to the increase in conjugation indicating the formation of azo bond.[45, 46]

^{1}H-NMR spectrum of azo prodrug

^{1}H-NMR (DMSO-d_6) spectrum of azo prodrug showed the chemical shifts for the protons of methyl and methylene groups at δ 1.91-2.07 ppm (t, 3H) and at δ 3.06-3.29 ppm (q, 4H) respectively. This spectrum clearly indicated the proton of phenolic-OH at δ 6.23 ppm (s, 1H). The protons of aromatic rings resonated at δ 6.90-6.96 ppm (m, 3H) and at δ 7.13-7.34 ppm (dd, 4H) while the proton of carboxylic acid group resonated at δ 11.14 ppm (s, 1H).

^{13}C-NMR spectrum of azo prodrug

^{13}C-NMR spectrum of azo prodrug reported that the carbons of methyl and methylene groups resonated at δ 14.72 ppm and at δ 67.94 ppm respectively. The carbon of aromatic ring attached to COOH resonated at δ 129.48 ppm while the carbon atoms of aromatic rings attached to azo bond resonated at δ 132.94 ppm and at δ 134.61 ppm which confirmed the formation of azo bond.

The carbon atom of aromatic ring attached to OH resonated at δ 153.45 ppm while the carbon atom of aromatic ring attached to ether group resonated at δ 164.12 ppm. This spectrum clearly indicated that the carbon atom of (C=O) group resonated at δ 170.71 ppm.

In vitro kinetic studies

The azo prodrug in (0.05 M) hydrochloric acid buffer (pH 1.2) showed negligible release of 5-ASA and *p*-phenetidine. Whereas in phosphate buffer (pH 7.4), only (22.04 %) release was observed over a period of six hours. The objective of bypassing the upper gastrointestinal tract with minimum prodrug release was achieved. Further study in rat fecal matter was carried out to confirm the colonic reduction of azo prodrug over a period of six hours; azo prodrug gave (77.96 %) cumulative release of 5-ASA and *p*-phenetidine.

Table 2 shows the kinetic data obtained from the release study of azo prodrug in rat fecal matter at 37°C and λ_{max} (476 nm).

Table 2. Kinetic data of the release study in rat fecal matter

Absorbance	Time (min.)	(a-x) (mol×10 ^{6})	x (mol×10 ^{6})	Cumulated drug release (%)
0.0537	0	35	0	0
0.0492	30	32.05	2.95	8.43
0.0458	60	29.88	5.12	14.63
0.0397	90	25.91	9.09	25.97
0.0355	120	23.18	11.82	33.77
0.0307	150	20.05	14.95	42.71
0.0267	180	17.43	17.57	50.20
0.0224	210	14.59	20.41	58.31
0.0180	240	11.73	23.27	66.49
0.0132	270	8.58	26.42	75.49
0.0093	300	6.09	28.91	82.60
0.0036	330	2.38	32.62	93.20
0	360	0	35	100

(a)= conc. of azo prodrug at time zero and equal to (35×10^{-6} mole), (a-x) = conc. of azo prodrug remaining for any time.

The release study of azo produg in rat fecal matter followed zero order kinetics (Figures 1, 2), the $t_{1/2}$ (average of three trials) of azo prodrug was found to be (182.67 min), whereas the rate constant (k) was found to be ($0.0958 \times10^{-6} \pm 0.0001$).

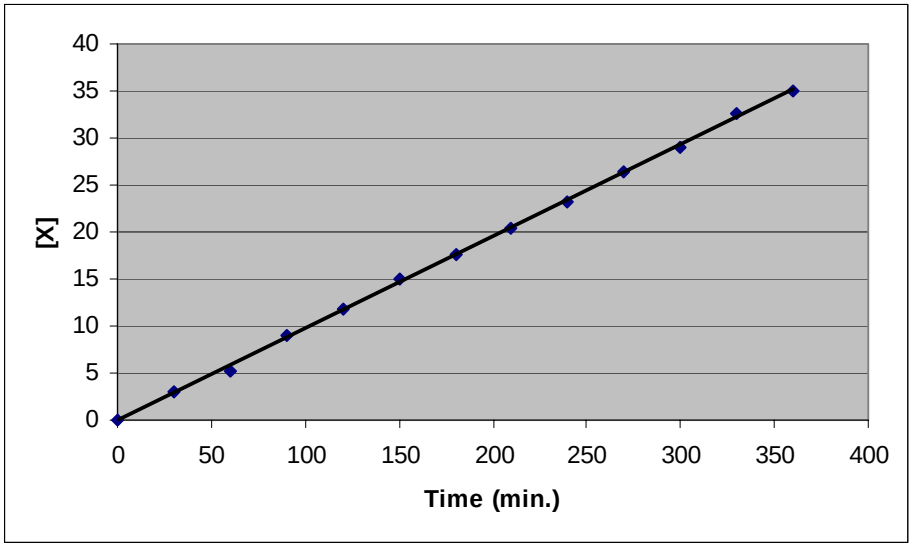


Figure 1. The slope for release study of the azo prodrug in rat fecal matter

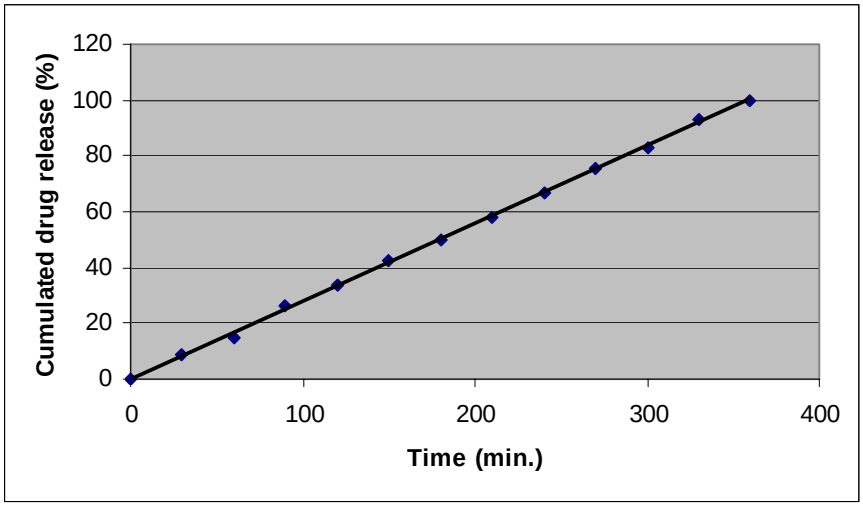


Figure 2. Release profile of 5-ASA and *p*-phenetidine from their azo prodrug in rat fecal matter.

Conclusion

In the present work, a novel mutual azo prodrug was synthesized by coupling *p*-phenetidine with salicylic acid and its chemical structure was characterized by physical and spectroscopic techniques, as FTIR, UV/Visible, ^{1}H-NMR and ^{13}C-NMR spectra. Properties of this prodrug acting as a colon-specific compound was evaluated depending on in vitro kinetic studies in HCl buffer, in phosphate buffer and in rat fecal matter. The results showed that only a small fraction of novel prodrug was hydrolyzed in upper gastrointestinal tract and the most fraction was delivered to the colon and split by bacterial azoreductase to liberate *p*-phenetidine and 5-ASA. Therefore, this prodrug is a promising colon specific prodrug for IBD and worthy of further study.

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