European Journal of Medicinal Chemistry 108 (2016) 486-494

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech



Synthesis, colon-targeted studies and pharmacological evaluation of an anti-ulcerative colitis drug 4-Aminosalicylic acid-β-O-glucoside



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ARTICLE INFO

Article history: Received 20 May 2015 Received in revised form 12 November 2015 Accepted 11 December 2015 Available online 15 December 2015

Keywords: 4-Aminosalicylic acid-β-O-glucoside Glycoside prodrug 4-Aminosalicylic acid Inflammatory bowel disease

ABSTRACT

A glycoside prodrug of 4-aminosalicylic acid (4-ASA) with p-glucose was synthesized for targeted drug delivery to inflammatory bowel. The *in vitro* assessment of 4-aminosalicylic acid- β -O-glucoside (4-ASA-Glu) as a colon-specific prodrug was studied using colitis rat with the healthy one as control. The stability studies in aqueous buffers (pH 1.2, 6.8 and 7.4) indicated that 4-ASA-Glu was stable over a period of 12 h. The incubation of 4-ASA-Glu with cecal or colonic contents of healthy rats at 37 °C released 4-ASA in 77 or 80% of the dose in 12 h, respectively. The amount of 4-ASA liberated from the incubation of 4-ASA-Glu in cecal or colonic contents of 90 or 79% in 12 h respectively, while less than 9% 4-ASA was detected from the incubation of 4-ASA-Glu with the homogenates of stomach or small intestine. The curative effect of 4-ASA-Glu was evaluated in 2, 4, 6-trinitrobenzenesulfonic acid (TNBS) induced experimental colitis model in male Sprague–Dawley (SD) rats. It was found that 4-ASA-Glu possess significantly ameliorate effect than sulfasalazine, oral 4- and 5-aminosalicylic acid.

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1. Introduction

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is a group of chronic inflammatory bowel diseases, characterized by up-regulated proinflammatory mediators and dysregulated immune responses, resulting in tissue damage [1]. IBD is very common in the western industrialized countries. It is assessed that 1.4 million people in the United States and 2.2 million people in Europe are affected by IBD [2]. The incidence of IBD in China is increasing rapidly, and it may become a common disease with the ongoing westernization and economic development [3]. The primary goal of drug therapy is to provide the therapeutical concentration at the site of inflammation that requires frequent intake of anti-inflammatory drugs at higher doses. However, the difficulties for delivering drugs to the colon are the absorption and degradation in the upper gastrointestinal tract (GIT), which causes the low drug bioavailability and systemic side effects. Therefore, the targeted drug delivery to colon would ensure the direct treatment at disease site and, consequently, lower doses

and reduce systemic side effects [4].

Prodrug approach is commonly believed to be one of the most effective approaches for the targeted drugs to colon, in which a covalent linkage between the active agent and carrier molecule is formed to assure the prodrug being "ironclad" in stomach and small intestine, but vulnerable in colon. One of the successfully used prodrug is sulfasalazine (SASP, an azo conjugate of 5-aminosalicylic acid with sulfapyridine) as a targeted drug to colon. But the high serum sulfapyridine level results in side effects such as nausea, headache, anorexia as well as haemolysis [5]. A few prodrugs of 5aminosalicylic acid (5-ASA) such as balsalazide, ipsalazine and olsalazine are also not free from adverse effects [6-8].

Natural polysaccharides are extensively used for the development of oral dosage form for delivering drug to colon. The rationale of polysaccharides drug delivery is that polysaccharides are vulnerable to a large number of enzymes such as β -D-glucosidase, β -D-galactosidase, β -D-xylosidase et al. produced by colonic bacteria, which result in the cleavage of glycosidic bond and release of parent drug in colon [9,10]. Therefore, other than amide-bound and azo-bound prodrugs, numerous glycoside prodrugs, such as dexamethasone-21- β -D-glucoside and prednisolone-21- β -D-glucoside [11], polysaccharide prodrugs of 5-ASA linked with cyclodextrin, hydroxypropyl and chitosan [12], glycoside prodrug of 5-ASA with

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D-galactose [13] for site specific delivery to colon, have been synthesized based on the observation that such glycosides are cleaved by bacterial glycosidase generated in the colonic microflora. In this work, D-glucose was chosen as the promoiety because it is nontoxic and free from any side effects.

4-ASA, the isomer of 5-ASA, has been confirmed as an active ingredient for the treatment of UC. 4-ASA provided a stable, inexpensive alternative to 5-ASA for topical treatment of active ulcerative proctitis, and also has been designated as an orphan drug by the FDA for mild to moderate UC [14]. 4-ASA also has been suggested as an effective treatment for both active and quiescent UC with lesser side effects [15]. Recently, the focus of IBD research seems to have shifted from 5-ASA to 4-ASA with some of its prodrugs reported in the literature [16].

The present work describe the synthesis of a glycoside prodrug of 4-ASA with D-glucose for the colon-targeted delivery. The synthesized compounds were characterized by MS, IR and NMR spectra. *In vitro* property of 4-ASA-Glu as a colon-specific prodrug was evaluated. The microbial degradation of glycoside prodrug by hydrolytic action of glycosidase secreted by colonic microflora would ensure the release of 4-ASA in colon. Furthermore, the therapeutic efficacy of 4-ASA-Glu on TNBS-induced colitis was also evaluated.

2. Results and discussion

2.1. Chemistry

4-ASA-Glu was synthesized as outlined in Scheme 1. 2, 3, 4, 6-

tetra-O-acetyl-1-bromo- α -D-glucopyranose **1** was prepared by the modified procedure with reference to literature [17]. D-glucose was added into acetic anhydride together with red phosphorus, Br₂ and H₂O as bromination reagent, and then the reaction mixture was stirred at 20-30 °C for 5 h on a thermostatic magnetic heating stirrer. Methvl 4-(*N*-(benzvloxvcarbonvl) amino)-2hvdroxybenzoate **3** was prepared from 4-ASA by the esterification of 4-ASA in refluxing methanol for 6 h [18], and then carbamate formation with benzyl-chloroformate at room temperature for 4 h [19]. The coupling reaction between **1** and **3** was conducted in a two-phase system of dichloromethane and aqueous sodium hydroxide solution using tetra-*n*-butylammonium bromide (TBAB) as the phase-transfer catalyst [20]. The crude product underwent saponification with 5% aqueous sodium carbonate in methanol to give methyl $4-(N-(benzyloxycarbonyl) amino)-2-(\beta-p-glucopyr$ anosyl) benzoate 4. Hydrogenation of benzyl carbamate 4 vielded methyl 4-amino-2-(β -D-glucopyranosyl) benzoate **5**. Finally, saponification with 0.2% aqueous sodium hydroxide solution yielded 4-amino-2-(β -D-glucopyranosyl) benzoate sodium **6**.

HRMS of 4-ASA-Glu showed *m*/*z* 338.0851 [M + H]⁺, in agreement with the molecular weight (337.08). IR spectrum of 4-ASA-Glu showed characteristic peaks at 1073 cm⁻¹ for glycosyl C–O–C stretching vibration and 1250 cm⁻¹ for glycoside bond Ph-O-C stretching vibration. ¹H NMR of 4-ASA-Glu showed chemical shifts of H–C₁′ at δ 4.69 [d, 1H], *J* = 7.1 Hz confirmed 4-ASA-Glu was β structure [21]. ¹³C NMR of 4-ASA-Glu showed the quantity of C was in agreement with the structure.



Scheme 1. The synthetic route of 4-Aminosalicylic acid-β-O-glucoside from 4-aminosalicylic acid.

2.2. Partition coefficient and aqueous solubility

The partition coefficient (log *P*) was 0.901 for 4-ASA but -0.44 for 4-ASA-Glu. Similarly, the solubility was 1.43 mg/ml for 4-ASA but 635 mg/ml for 4-ASA-Glu. The increased aqueous solubility and decreased partition coefficient of 4-ASA-Glu as compared with 4-ASA would limit the transcellular passive transport in the upper GIT and facilitate the passage of 4-ASA-Glu directly to colon.

2.3. In vitro release study results

The stability of 4-ASA-Glu at acidic and alkaline condition was studied by the release kinetic. The concentration decrease of 4-ASA-Glu was monitored within 12 h in different isotonic buffer solutions. The results were showed in Fig. 1. 4-ASA was not detected during the 12 h incubation period, which indicated that 4-ASA-Glu might be chemically stable during the passage through the GIT.

The hydrolysis of 4-ASA-Glu by glycosidase was studied *in vitro* at 37 °C by incubating with the homogenate of stomach or small intestine (tissue and contents) from healthy rats to evaluate the release of 4-ASA (Fig. 2A). Incubation of 4-ASA-Glu with cecal and colonic contents of healthy rats was also conducted at 37 °C under anaerobic conditions. Only 8.2 or 4.8% 4-ASA was detected when 4-ASA-Glu was incubated with the homogenate of stomach or small intestine, suggesting that the prodrug might be stable in the upper intestine. When 4-ASA-Glu was incubated with cecal or colonic contents, about 77 or 80% of dose was released as 4-ASA over a period of 12 h.

The hydrolysis of 4-ASA-Glu by glycosidase in pathological condition was also investigated with the GIT contents of colitis rat (Fig. 2B). After the incubation of 4-ASA-Glu with the homogenate of stomach or small intestine at 37 °C, only 7.9 or 2.3% 4-ASA was detected, which indicated that the prodrug was also stable in the upper intestine of colitis rat. When 4-ASA-Glu was incubated with cecal and colonic contents of colitis rat, the released 4-ASA was about 69 and 79%, respectively in 12 h.

In vitro release studies indicated that the glycosidase from colon at pathological condition could hydrolyse 4-ASA-Glu to release 4-ASA. Also, there is no significant difference on the release of 4-ASA from 4-ASA-Glu between healthy and colitis rats.

2.4. Biological results

2.4.1. Clinical activity score

After the experimentally induced colitis, the clinical activity







Fig. 2. Release profiles of 4-ASA during incubation of 4-ASA-Glu with homogenates of various segments of GIT of healthy rats (A) and colitis rats (B) at 37 °C. \Box , colonic contents in isotonic phosphate buffer (pH 7.4); \bigcirc , cecal contents in isotonic phosphate buffer (pH 7.4); \bigtriangleup , tissue and contents of small intestine in isotonic phosphate buffer (pH 6.8); \star , tissue and contents of stomach in isotonic acetate buffer (pH 4.5). n = 3.

score increased rapidly and consistently for all TNBS-treated groups over the next 3 days (Fig. 3). After a time lag of 48–72 h, all drugreceiving groups showed a decreased severity of inflammation. On the 7th day, there was a significant difference between the drugreceiving groups and TNBS group. On the 12th day, the group with high-dose had the lowest score (0.61 ± 0.390) which indicated the highest clinical activity. The group with middle-dose had a relatively lower score (1.06 ± 0.248) to SASP group (1.22 ± 0.404)



Fig. 3. Clinical activity score during the whole experimental period always determined for n = 6 animals. \Box , Healthy group; \bigcirc , High-dose group; \Diamond , Middle-dose group; \diamond , SASP group; \bullet , Low-dose group; \diamond , 5-ASA group; \triangleleft , 4-ASA group; \times , TNBS group.

and 4-ASA group (1.78 ± 0.170). These results indicated that 4-ASA-Glu enhanced the therapeutic effect of 4-ASA and the effect was dose dependant. Moreover, the effect of 4-ASA-Glu (high-dose and middle-dose) group was superior to SASP group.

2.4.2. Colon/body weight ratio and macroscopic injury score

On day 12 (24 h after the last drug administration), all rats were sacrificed, and colon/body weight ratio and macroscopic injury score were determined to characterize the inflammation quantitatively (Figs. 4 and 5). The results indicated that colon/body weight ratio and macroscopic injury score were distinctly decreased in high-dose group (0.0038 \pm 0.00045) and middle-dose group (0.0051 \pm 0.00023) as compared with 4-ASA group (0.0085 \pm 0.00015) and TNBS group (0.0133 \pm 0.00041). The therapeutic effect on dose-dependence was also observed. Furthermore, the low-dose group showed a better anti-inflammatory effect than the groups administered with 4-ASA and 5-ASA, but had a worse effect than the group with SASP. The results indicated that 4-ASA-Glu enhanced the therapeutic effect of 4-ASA.

2.4.3. Histological evaluation

The severity of inflammation can be evaluated directly by detecting the histological damage. The healthy control group showed normal mucosa, connective tissue and muscularis (Fig. 6A). Histopathological features in TNBS group displayed the absence of epithelium cells and the presence of a massive mucosal/submucosal infiltration of inflammatory cells (Fig. 6B). The high-dose and middle-dose groups showed a better corrected morphology of colon than the group with SASP (Fig. 6CDF), while the low-dose group had a slight mucosal abscess. The groups with 4-ASA and 5-ASA had a slight mucosal abscess and inflammatory infiltrate (Fig. 6EGH). These results further confirmed that 4-ASA-Glu had an improved therapeutic effect in the treatment of colitis.

2.4.4. Myeloperoxidase (MPO) activity

MPO activity, an important quantitative index for colonic inflammation, was determined in terms of U/g tissue (Fig. 7). MPO activity was significantly increased in TNBS group (1.44 ± 0.041) as compared with the healthy control group (0.30 ± 0.023). MPO activity for the high-dose and middle-dose group was found to be 0.40 ± 0.019 and 0.51 ± 0.033 , less than the group with SASP (0.58 ± 0.028) and 4-ASA group (0.82 ± 0.035). The results of MPO



Fig. 4. Determination of colon/body weight ratio after final drug administration for n = 6 animals. Data are expressed as mean \pm SD. **P < 0.01 versus healthy group; ##P < 0.01 versus TNBS group; +P < 0.05, ++P < 0.01 versus SASP group; &&P < 0.01 versus middle-dose group.



Fig. 5. Determination of colonic macroscopic injury after final drug administration for n = 6 animals. ^{**}P < 0.01 versus healthy group; ^{##}P < 0.01 versus TNBS group; ⁺P < 0.05 versus SASP group; [&]P < 0.05, ^{&&}P < 0.01 versus middle-dose group.

activity, in consistent with the clinical activity score, colon/body weight ratio and macroscopic injury score, suggested that 4-ASA-Glu improved the therapeutic efficacy of 4-ASA.

3. Conclusions

In this work, a simple and efficient synthetic method was developed for the synthesis of 4-ASA-Glu with a high yield. *In vitro* release studies showed that only a small amount (<9%) 4-ASA was liberated from 4-ASA-Glu after incubation with the contents of stomach and small intestine of rats. While, after incubation with cecal and colonic contents, the liberation of 4-ASA from 4-ASA-Glu was significantly increased (>69%). The therapeutic efficacy of 4-ASA-Glu in the treatment of IBD was evaluated by TNBS model. Clinical activity score system, colon/body weight ratio, macroscopic injury score, histological evaluation and MPO activity showed that the rats treated with 4-ASA-Glu had an improvement in the pathology than 4-ASA. In conclusion, these results indicated that 4-ASA-Glu may be an efficacious and promising remedy for the treatment of UC.

Future prospects: Additional work is in progress with respect to *in vivo* gastrointestinal tract disposition studies and synthesis of a series of novel glycoside prodrugs of 4-ASA for their potential use in the management of IBD.

4. Experimental

4.1. Chemistry

4-ASA, benzylchloroformate and 10% Pd/C were purchased from Sigma Chemical Co. (USA). TBAB was obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All other chemicals used in the synthesis were of AR grade.

The IR spectra of synthesized compounds were recorded on PEI730 spectrophotometer with KBr pellet (anhydrous IR grade) in the range of 40–4000 cm⁻¹. ¹H NMR and ¹³C NMR spectra were recorded in DMSO using Varian (400 MHz) spectrometer using TMS as internal standard. Chemical shifts were reported in ppm downfield on δ scale. High Resolution Mass Spectrometery (HRMS) data were obtained on a Micromass Tof II spectrometer. Optical rotations were measured on a Perkin–Elmer 241 polarimeter at 20 °C. The melting point was measured with X-4 digital display binocular microscope. Pressure reactor (Parr WDF) was used for



Fig. 6. Histology of a representative colon specimen of a rat after the induction of colitis with TNBS sacrificed on day 12 (resolution 100 ×). (A) Healthy; (B) TNBS; (C) High-dose; (D) Middle-dose; (E) Low-dose; (F) SASP; (G) 4-ASA; (H) 5-ASA.

catalytic hydrogenation. Reaction progress and compound purity were monitored using TLC with methanol-ethyl acetate as the developing system and iodine vapours/UV light as detecting agents.

4.1.1. Preparation of 2, 3, 4, 6-tetra-O-acetyl-1-bromo- α -*D*-glucopyranose (1)

60% HClO₄ (0.15 mol) was slowly added to acetic anhydride

(20 ml) with cooling and p-glucose (5.00 g, 0.028 mol) was added to it. After stirring at 20–30 °C for 2 h, the mixture was cooled in ice with the temperature maintained at 0–10 °C on a cryostatic bath and red phosphorus (1.50 g), Br₂ (3 ml) and H₂O (2 ml) were slowly added to it. The reaction mixture was maintained at 20–30 °C for 5 h with continuous stirring on a thermostatic magnetic heating stirrer. The resulting mixture was diluted with CH₂Cl₂ (50 ml) and



Fig. 7. Determination of myeloperoxidase activity after final drug administration for n = 6 animals. Data are expressed as mean \pm SD. ^{**}P < 0.01 versus healthy group; ^{##}P < 0.01 versus TNBS group; ⁺⁺P < 0.01 versus SASP group; ^{&&}P < 0.01 versus middle-dose group.

filtered by Buchner funnel, then the filtrate was collected and washed with NaHCO₃ saturated solution (4 × 20 ml), 0.4 mol/L Na₂S₂O₃ solution (2 × 20 ml) and H₂O (3 × 20 ml), respectively. The organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated under reduced pressure to give crude product. The crude product was recrystallized from ethyl ether to afford pure white crystals **1** (7.65 g, yield: 67%). mp: 87–89 °C (88–89 °C in literature [22]); [α]_D²⁰ = +191.2 (*c* 0.1, CHCl₃); *R*_f = 0.63 in petroleum ether/diethyl ether/ethyl acetate/acetic acid (4/3/3/0.02), iodine vapours/UV light as detecting agents.

4.1.2. Preparation of methyl 4-amino-2-hydroxybenzoate (2)

Concentrated sulfuric acid (1.25 ml) was added dropwise to methanol (6 ml) with cooling on a cryostatic bath. The mixture was stirred for 1 h at room temperature, and 4-ASA (1.53 g, 10 mmol) was added to it. After refluxing at 60–70 °C for 6 h, the mixture was adjusted to pH 7-8 with 10% Na₂CO₃ solution in ice-bath. The mixture was filtered to afford crude solid product. The crude product was recrystallized from hot methanol, followed by cooling to 0 °C. White crystals were collected and dried under high vacuum to give compound **2** (1.29 g, yield: 77%). mp: 119–121 °C; $R_{\rm f} = 0.73$ in petroleum ether/diethyl ether/ethyl acetate/acetic acid (4/3/3/ 0.02); IR (KBr): 3260 $\rm cm^{-1}$ phenolic O–H stretching vibration, 3474 $\rm cm^{-1}$ and 3380 $\rm cm^{-1}$ N–H stretching bimodal vibration, 1638 cm⁻¹ N–H bending vibration; 1662 cm⁻¹ C=O stretching vibration, 1284 cm⁻¹ C–O–C stretching vibration; ¹H NMR (DMSO d_6 , δ ppm): 3.79 (s, 3H, -COOCH₃), 6.00 (d, 1H, J = 2.1 Hz, H–C₃), 6.13 $(dd, 1H, J = 8.7 Hz, 2.2 Hz, H-C_5), 6.14 (s, 2H, Ph-NH_2), 7.46 (d, 1H, J)$ J = 8.7 Hz, H–C₆), 10.77 (s, 1H, Ph-OH); HRMS (ESI): Calcd for $C_8H_9NO_3$, $[M + H]^+$, 168.0655; Found, 168.0655.

4.1.3. Preparation of methyl 4-(N-(benzyloxycarbonyl) amino)-2hydroxybenzoate (**3**)

Methyl-4-ASA (1.67 g, 10 mmol) was dissolved in a mixture of CH₂Cl₂ (20 ml) and pyridine (5 ml). Then benzylchloroformate (1.9 ml) was added dropwise. After stirring at room temperature for 4 h, the solution was concentrated under reduced pressure to about 15–20 ml. Sufficient cold distilled water was added, filtered and crude product was obtained. The crude product was recrystallized by dissolving in ethanol and cooling to 0 °C. Purified white crystals were dried under high vacuum to give compound **3** (2.86 g, yield: 95%). mp: 142–144 °C; $R_{\rm f} = 0.83$ in petroleum ether/diethyl ether/

ethyl acetate/acetic acid (4/3/3/0.02); IR (KBr): 3184 cm⁻¹ phenolic O–H stretching vibration, 1733 cm⁻¹ C=O stretching vibration, 1256 cm⁻¹ C–O–C stretching vibration, 3371 cm⁻¹ N–H stretching unimodal vibration, 1656 cm⁻¹ C=O stretching vibration (amide); ¹H NMR (DMSO-d₆, δ ppm): 3.86 (s, 3H, -COOCH₃), 5.17 (s, 2H, -O-C<u>H</u>₂-Ph'), 7.03 (dd, 1H, *J* = 8.7 Hz, 2.0 Hz, H–C₅), 7.20 (d, 1H, *J* = 2.1 Hz, H–C₃), 7.34–7.45 (m, 5H, H-Ph'), 7.71 (d, 1H, *J* = 8.7 Hz, H–C₆), 10.19 (s, 1H, -NH-), 10.65 (s, 1H, Ph-O<u>H</u>); HRMS (ESI): Calcd for C₁₆H₁₅NO₅, [M + H]⁺, 302.1023; Found, 302.1027.

4.1.4. Preparation of methyl 4-(N-(benzyloxycarbonyl) amino)-2- $(\beta$ -D-glucopyranosyl) benzoate (**4**)

Compound 3 (1.21 g, 4 mmol) and TBAB (0.70 g) were dissolved in CH₂Cl₂ (20 ml), and 5% NaOH solution (7.2 ml) was added to it. After the mixture was stirred at room temperature for 30 min, a solution of compound 1 (5 mmol) in CH₂Cl₂ (10 ml) was added dropwise to the above mixture with constant stirring. The mixture was refluxed for 5 h at 50 °C on a thermostatic magnetic heating stirrer and then cooled in an ice-water bath. The organic layer was collected and washed with 5% NaOH solution (2 \times 20 ml) and H₂O $(3 \times 20 \text{ ml})$, respectively. Then the organic layer was concentrated under reduced pressure to give intermediate. The intermediate was dissolved in methanol (20 ml), and 5% Na₂CO₃ solution (5 ml) was added dropwise. After stirring at room temperature for 2 h, the mixture was neutralized with 3 mol/L HCl. The organic solvent was removed under reduced pressure and sufficient cold distilled water was added, filtered and crude product was washed with CH₂Cl₂, recrystallized in mixture of methanol and distilled water (1:1 v/v). dried under high vacuum to give compound 4 (1.01 g, yield: 54%). mp: 148–150 °C; $[\alpha]_D^{20} = +44.8$ (c 0.1, MeOH); $R_f = 0.73$ in dichloromethane/methanol (7/1); IR (KBr): 1719 cm⁻¹ C=O stretching vibration, 1231 cm⁻¹ C=O-C stretching vibration, 3305 cm⁻¹ N–H stretching vibration, 1302 cm⁻¹ C–N stretching vibration, 1687 cm⁻¹ C=O stretching vibration (amide), 3356 cm⁻¹ O–H stretching broad peak, 1086 cm⁻¹ and 1028 cm⁻¹ glycosyl C-O-C stretching vibration, 1254 cm⁻¹ glycoside bond Ph-O-C stretching vibration; ¹H NMR (DMSO-d₆, δ ppm): 3.23–3.30 (m, 4H, H–C_{2′-5′}), 3.77 (s, 3H, -COOCH₃), 3.73 (dd, 1H, *J* = 7.2 Hz, 1.8 Hz, $H-C_{6'}$), 3.62 (dd, 1H, J = 6.0 Hz, 4.0 Hz, $H-C_{6'}$), 4.47 (t, 1H, J = 5.9 Hz, OH- $C_{6'}$), 4.79[d, 1H, J = 6.9 Hz (β structure), H- $C_{1'}$], 5.03 (d, 1H, J = 4.5 Hz, OH-C_{4'}), 5.09 (d, 1H, J = 3.2 Hz, OH-C_{3'}), 5.13 (d, 1H, J = 4.0 Hz, OH $-C_{2'}$), 5.18 (s, 2H, -O-CH₂-Ph'), 7.30 (dd, 1H, J = 8.6 Hz, 1.7 Hz, H–C₅), 7.36–7.46 (m, 6H, H-Ph' and H–C₃), 7.67 (d, 1H, J = 8.6 Hz, H–C₆), 10.12 (s, 1H, -NH-); HRMS (ESI): Calcd for C₂₂H₂₅NO₁₀, [M + Na]⁺, 486.1371; Found, 486.1373.

4.1.5. Preparation of methyl 4-amino-2-(β -D-glucopyranosyl) benzoate (**5**)

Protected 4-ASA glucose conjugate (1.67 g, 3.6 mmol) was dissolved in methanol (150 ml), and then was catalytically hydrogenated under 0.4 MPa with 10% Pd/C (0.20 g) in a high pressure apparatus. After stirring at 50 °C for 24 h, the solution was filtered and concentrated under reduced pressure to give crude product which was purified by silica gel column chromatography using the solvent system ethyl acetate/methanol (5/1 v/v) to give compound **5** (0.98 g, yield: 83%). $[\alpha]_D^{20} = +66.7$ (*c* 0.1, MeOH); $R_f = 0.35$ in dichloromethane/methanol (7/1); IR (KBr): 1688 cm⁻¹ C=O stretching vibration, 1200 cm⁻¹ C–O–C stretching vibration, 3365 cm^{-1} O–H and N–H stretching vibration (broad peak), 1608 cm⁻¹ N–H bending vibration, 1073 cm⁻¹ glycosyl C–O–C stretching vibration, 1258 cm⁻¹ glycoside bond Ph-O-C stretching vibration; ¹H NMR (DMSO-d₆, δ ppm): 3.28–3.31 (m, 4H, H–C_{2'-5'}), 3.70 (s, 3H, -COOCH₃), 3.75 (dd, 1H, J = 11.2 Hz, 4.8 Hz, H-C_{6'}), 3.59 $(dd, 1H, J = 11.2 Hz, 4.4 Hz, H-C_{6'}), 4.57 (s, 1H, OH-C_{6'}), 4.73[d, 1H, OH-C_{6'})$ J = 7.1 Hz (β structure), H–C_{1'}], 5.06–5.10 (m, 3H, OH–C_{2'-4'}), 5.97

(s, 2H, Ph-N<u>H</u>₂), 6.27 (dd, 1H, J = 8.6 Hz, 2.0 Hz, H–C₅), 6.46 (d, 1H, J = 2.0 Hz, H–C₃), 7.51 (d, 1H, J = 8.6 Hz, H–C₆); HRMS (ESI): Calcd for C₁₄H₁₉NO₈, [M + Na]⁺, 352.1003; Found, 352.1008.

4.1.6. Preparation of 4-amino-2-(β -D-glucopyranosyl) benzoate sodium (**6**)

Compound 5 (1.00 g, 3.04 mmol) was dissolved in 0.2% NaOH solution (60 ml). After stirring at 50 °C for 2 h, the reaction mixture was concentrated under reduced pressure to give crude product which was purified by silica gel column chromatography using the solvent system ethyl acetate/methanol (3/1 v/v) to give pure white product. Compound 6 was stored in well-closed amber colored bottle in vacuum vessel (0.93 g, yield: 91%). $[\alpha]_D^{20}=+73.1$ (c 0.1, MeOH); $R_f = 0.22$ in ethyl acetate/methanol/distilled water (4/1/ 0.5); IR (KBr): 1698 cm⁻¹ C=O stretching vibration, 3362 cm⁻¹ O–H and N–H stretching vibration (broad peak), 1607 cm^{-1} N–H bending vibration, 1073 cm⁻¹ glycosyl C–O–C stretching vibration, 1251 cm⁻¹ glycoside bond Ph-O-C stretching vibration; ¹H NMR (DMSO-d₆, δ ppm): 3.21–3.31 (m, 4H, H–C_{2'-5'}), 3.75 (dd, 1H, J = 10.8 Hz, 4.8 Hz, H–C_{6'}), 3.61 (dd, 1H, J = 10.8 Hz, 4.4 Hz, H–C_{6'}), 4.65 (m, 1H, OH $-C_{6'}$), 4.69 [d, 1H, J = 7.1 Hz (β structure), H $-C_{1'}$], 5.18 (s, 3H, $OH-C_{2'-4'}$), 6.01 (s, 2H, Ph-NH₂), 6.29 (dd, 1H, J = 8.6 Hz, 2.0 Hz, H–C₅), 6.55 (d, 1H, J = 2.0 Hz, H–C₃), 7.52 (d, 1H, J = 8.6 Hz, H–C₆); ¹³C NMR (DMSO-d₆, δ ppm): 61.1(C_{6'}), 69.8(C_{4'}), 74.0(C_{2'}), 76.4($C_{3'}$), 77.9($C_{5'}$), 102.3($C_{1'}$), 103.8(C_3), 107.4(C_1), 108.6(C_5), 133.7(C₆), 155.1(C₄), 160.5(C₂) and 170.8(-COONa); HRMS (ESI): Calcd for C₁₃H₁₆NNaO₈, [M + H]⁺, 338.0846; Found, 338.0851.

4.2. Partition coefficient and aqueous solubility

Partition coefficients (log *P*) of 4-ASA and 4-ASA-Glu were determined in *n*-octanol/phosphate buffer (pH 7.4) system using the shake flask method at 37 °C. The aqueous solubility studies of 4-ASA and 4-ASA-Glu were conducted in distilled water using water bath shaker at room temperature (25 ± 1 °C) [23,24].

4.3. In vitro release studies

4.3.1. Analytical methods and calibration of 4-ASA in various biological specimens

An Agilent 1200 series separations module liquid chromatographic system equipped with a G1311A quaternary pump, a G1316A column thermostatic chamber, a G1314B VWD UV detector and a 7725i Rheodyne injector (20 μ l loop) was used for HPLC analysis. Separation was accomplished using a reversed-phase Innoval C18 column (4.6 μ m, 250 \times 4.6 mm, Agela Technologies, China). The determination of 4-ASA was as follows: the detection wavelength was 263 nm, the flow rate of the mobile phase was at 1 ml/min and the column temperature was 30 °C during the whole process. The injection volume was 20 μ l for all samples. Mobile phase composed of methanol and 0.147 mol/L (pH 2.7) phosphate butter (10/90, v/v). Analytical parameters were controlled by Agilent G2170BA software.

Standard solution of 4-ASA (250 μ g/ml) was prepared in isotonic aqueous buffers (pH 6.8). The tissue and contents of stomach, small intestine and the contents of cecum, colon were homogenated, and diluted to final concentration of 20% (w/v) with isotonic aqueous buffers (pH 6.8). To each 200 μ l portion of the above homogenate, 2 μ l, 10 μ l, 20 μ l, 40 μ l, 200 μ l, 400 μ l, 600 μ l and 800 μ l portions of the standard solution of 4-ASA and appropriate volumes of aqueous buffer were added to give final volume of 1 ml. The standard solutions (0.5 μ g/ml - 200 μ g/ml) were mixed, vortexed for 2 min and filtered through a 0.45 μ m membrane filter. 20 μ l of the filterate was analyzed by HPLC and a calibration curve was constructed from the peak area versus the concentration of standard solutions. Concentration of 4-ASA in the samples of different contents were calculated from the calibration curve.

4.3.2. Stability study of 4-ASA-Glu in different aqueous buffers

Stability study was carried out by preparing the solution of 4-ASA-Glu (equivalent to 200 μ g/ml 4-ASA) in isotonic hydrochloric acid buffer (pH 1.2), isotonic phosphate buffer (pH 6.8) and isotonic phosphate buffer (pH 7.4) and incubating the solution of 1000 ml for 12 h at 37 °C thermostat water bath. At time intervals of 0.5, 1, 2, 4, 6, 8 and 12 h, the sample solution (1 ml) was removed and centrifuged at 3000 rpm for 10 min. To a 0.5 ml portion of the solution, 0.5 ml of methanol was added, vortexed for 2 min, and filtered through a 0.45 μ m membrane filter. The concentration of 4-ASA in 20 μ l portion of the solution was analyzed by HPLC. Each experiment was performed in triplicate.

4.3.3. Incubation of 4-ASA-Glu with the homogenate of stomach and small intestine of healthy and colitis rats

A healthy male SD rat (about 200 g) was anesthetized by diethyl ether and midline incision was made. Sections of stomach and small intestine were collected separately, homogenized, and the homogenate was diluted to final concentration of 20% with isotonic acetate buffer (pH 4.5) for stomach segments and with isotonic phosphate buffer (pH 6.8) for small intestine segments. To a 0.2 ml portion of each dilution, 0.8 ml of 4-ASA-Glu solution (equivalent to 250 μ g/ml 4-ASA) was added and the mixture was incubated at 37 °C. At time intervals of 0.5, 1, 2, 4, 6, 8 and 12 h, the sample was centrifuged at 3000 rpm for 10 min. To a 0.5 ml portion of the supernatant, 0.5 ml of methanol was added, vortexed for 2 min, and filtered through a 0.45 μ m membrane filter. The concentration of 4-ASA in 20 μ l portion of the solution was determinated by HPLC. The study was carried out in triplicate.

Same procedure as described earlier was followed, except that healthy rat was replaced by colitis rat. Colitis rats were obtained by the following description. The rats were sacrificed 3 days later, and then contents of colitis rats were gatherd.

4.3.4. Incubation of 4-ASA-Glu with cecal and colonic contents of healthy and colitis rats

The contents of healthy cecum and colon were collected separately in test tubes with stopper, which were previously displaced by nitrogen. The content was homogenized under stream of nitrogen to form 20% homogenate. To a 0.2 ml portion of the cecum or colon homogenate, 0.8 ml of 4-ASA-Glu solution in pH 7.4 isotonic phosphate butter (equivalent to 250 μ g/ml 4-ASA) was added and the mixture was incubated at 37 °C under anaerobic conditions. At time intervals of 0.5, 1, 2, 4, 6, 8 and 12 h, the sample was centrifuged at 3000 rpm for 10 min. To a 0.5 ml portion of the supernatant, 0.5 ml of methanol was added, vortexed for 2 min, and filtered through a 0.45 μ m membrane filter. The concentration of 4-ASA in 20 μ l portion of the solution was detected by HPLC. The study was carried out in triplicate.

Same procedure as described earlier was followed, except that the contents of healthy cecum and colon were replaced by the contents of colitis cecum and colon.

4.4. Biological investigations

4.4.1. Reagents and animals

TNBS was obtained from Sigma Chemical Co. Ltd. (Shanghai, China). SASP was supplied by Shanxi Tongda Pharmaceutical Co. Ltd. (Taiyuan, China). Hexadecyltrimethyl ammonium bromide (HTAB) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). O-dianisidine hydrochloride was purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). All other reagents were of AR grade.

Adult male SD rats weighing 180–200 g were supplied by Animal Facility Center of Shanxi Medical University (Taiyuan, China). The rats were housed in standard polypropylene cages with wire mesh top (temperature 23 ± 1 °C, relative humidity $55 \pm 10\%$, 12 h light/dark cycle) and allowed free access to water and laboratory chow for the duration of the studies. The protocol and procedures described below were approved by the Animal Care and Use Committee of the Shanxi Medical University.

4.4.2. Induction of colitis and experimental design

The ameliorating effect of prodrug of 4-ASA on the chronically inflamed tissue of colon was evaluated in TNBS-induced experimental colitis model in rats. In this study, rats were randomly divided into 8 groups (n = 6 per group), i.e. healthy control group, TNBS group, three standard groups (4-ASA group, 5-ASA group, SASP group) and three test groups (High-dose group, middle-dose group, low-dose group). To induce the model of chronic inflammation in the rat colon, the method described by Morris et al. [25] was followed with some slight modifications. In brief, rats (except healthy control group) were food fasted for 48 h with free access to water and then lightly anesthetized with ether. A graduated rubber cannula was inserted rectally into the colon such that the tip was 8 cm proximal to the anus. TNBS dissolved in 50% ethanol (v/v) was instilled into the lumen of the colon via the cannula (100 mg/kg body weight). Rats were held in a head-down position for 2-3 min after the instillation of the hapten, in order to distribute the agents within the entire colon. Healthy control group received 0.5 ml physiological saline administered as before. For 3 days the rats were housed with food and water ad libitum but not of treatment to maintain the development of a full IBD model. The standard and test groups received 4-ASA (115 mg/kg), 5-ASA (115 mg/kg, equimolar to 4-ASA), SASP (300 mg/kg, equimolar to 4-ASA), high-dose 4-ASA-Glu (385 mg/kg, 1.5 mol to 4-ASA), middle-dose 4-ASA-Glu (255 mg/kg, equimolar to 4-ASA) and low-dose 4-ASA-Glu (128 mg/ kg, 0.5 mol to 4-ASA). The rats were treated once daily for six continuous days then sacrificed 24 h by 10% chloral hydrate after the last day of drug administration and colons were excised. The healthy control group and TNBS group received physiological saline instead of free drug or prodrug.

4.4.3. Evaluation of the treatment of colitis [26]

4.4.3.1. *Clinical activity score system.* During the period of 12 days study, the colitis activities of the rats in all groups were quantified with a clinical activity score assessing weight loss, stool consistency and rectal bleeding. The clinical activity score was determined by calculating the average of the above three parameters for each day, for each group and was ranging from 0 (healthy) to 4 (maximal activity of colitis).

4.4.3.2. Colon/body weight ratio. The excised colon tissues were opened longitudinally along the mesenteric edge. The colons were rinsed with ice cold physiological saline to remove luminal content and then 8 cm segment of distal colons were weighed. The colon/ body weight ratio was calculated as an index to quantify the inflammation.

4.4.3.3. Assessment of macroscopic injury. For the assessment of macroscopic injury, the excised colon tissues were opened longitudinally and slightly washed in ice cold physiological saline to remove fecal residues and then 8 cm segment of distal colons were obtained. Macroscopic injury was detected using a validated scoring system.

4.4.3.4. Histological evaluation. After macroscopic observation,

distal colonic tissue samples (3 cm) were immersed in formaldehyde (10%, v/v) and subsequently embedded in paraffin. Sections of 2 μ m thickness were stained with hematoxylin and eosin. Microscopic assessment by light microscope was performed blind on coded slices and colored microscopical images were obtained.

4.4.3.5. *MPO activity*. MPO activity was measured as index of inflammation. It is a peroxidase enzyme most abundantly present in activated neutrophils into the inflamed tissue. Hence MPO activity is directly proportional to neutrophil number and inversely proportional to the ameliorating effect of drugs on disrupted colonic architecture.

In this experiments, the method of Krawisz et al. [27] was adopted to assess colonic inflammation in TNBS-induced experimental colitis and quantitative assay of MPO activity. Briefly, colon tissue sample (100 mg) was added to 1 ml of HTAB buffer (0.5% in 50 mM phosphate buffer pH 6.0) on ice and homogenized. The homogenate was sonicated for 10 s and subjected to three rapid freezing (-70 °C) and thawing (37 °C) cycles. The sample was then centrifuged at 4 °C and at 10,000 rpm for 15 min. MPO activity was measured spectrophotometrically as follows. Supernatant (0.1 ml) was added to 2.9 ml of 50 mM phosphate buffer containing 0.167 mg/ml o-dianisidine hydrochloride and 0.0005% hydrogen peroxide. The kinetics of absorbance changes at 460 nm was measured.

One unit of MPO activity was defined as the quantity of enzyme able to convert 1 μ mol of hydrogen peroxide to water in 1 min at 25 °C. The result was expressed in U/g of tissue [28].

4.4.4. Statistical analysis

The results were expressed as means \pm SD. Statistical analysis was performed with SPSS 16.0 statistical software. Statistical significance of the results, P < 0.05, was determined using a one-way analysis of variance (ANOVA). Nonparametric statistical analysis (Wilcoxon rank sum test) was performed for data in Fig. 5.

Acknowledgments

This research project was supported by Shanxi Scholarship Council of China (2011-047).

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