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•Research article•

Identification of a new azoreductase driven prodrug from bardoxolone methyl and 5-aminosalicylate for the treatment of colitis in mice

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[ABSTRACT] For local treatment of ulcerative colitis, a new azoreductase driven prodrug CDDO-AZO from bardoxolone methyl (CDDO-Me) and 5-aminosalicylate (5-ASA) was designed, synthesized and biologically evaluated. It is proposed that orally administrated CDDO-AZO is stable before reaching the colon, while it can also be triggered by the presence of azoreductase in the colon to fragment into CDDO-Me and 5-ASA, generating potent anti-colitis effects. Superior to olsalazine (OLS, a clinically used drug for ulcerative colitis) and CDDO-Me plus 5-ASA, CDDO-AZO significantly attenuated inflammatory colitis symptoms in DSS-induced chronic colitis mice, which suggested that CDDO-AZO may be a promising anti-ulcerative colitis agent.

[KEY WORDS] Ulcerative colitis; Azoreductase; Prodrug; Bardoxolone Methyl; 5-Aminosalicylate

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Introduction

Ulcerative colitis (UC) together with Crohn's disease (CD), also known as inflammatory bowel disease (IBD), are chronic disorders of the digestive tract characterized by prolonged intestinal inflammation and bowel tissue injury caused by immunodeficiency [1]. There are several types of medicines for the treatment of IBD, including 5-aminosalicylate (5-ASA) prodrugs, immunomodulators, corticosteroids and antibiotics [2]. However, there is currently no cure for IBD, and patient's quality of life may be severely reduced within two or three decades.

In recent years, increasing attention has been drawn on oral colon drug delivery system (OCDDS) for local treatment of colonic diseases. Some strategies have been successfully explored for OCDDS including pH, time, pressure, or osmotic-dependent, and microbially and/or enzymatically driven systems [3-5]. There are various microfloras coexisting in the colon, such as *bifidobacterium*, *bacteroides*, and *eubacterium*, which secrete a wide range of enzymes such as azoreductase, urea hydroxylase, and nitroreductase. Notably, azoreductase driven prodrugs of 5-ASA have been widely used in clinical setting (Scheme 1A). 5-ASA can intervene the metabolism of arachidonic acid to prostaglandins and leukotrienes, scavenge reactive oxygen species, and generate anti-inflammatory effects within the colonic mucosa [6]. However, orally administrated 5-ASA can be completely absorbed into the blood from the upper gut before it reaches the colon. To this end, the prodrugs sulphasalazine (SASP), ipsalazine, balsalazine, and olsalazine (OSZ) are tolerant to the chemical and enzymatic environment in the stomach and small intestine, but can be metabolized by azoreductase in the colon to split into 5-ASA and other active substances, generating additional or synergistic anti-colitis effects [7-9].

Synthetic oleanane triterpenoids 2-cyano-3, 12-dioxooleana-1, 9(11)-dien-28-oic acid (CDDO) and its methyl ester (CDDO-Me, also known as bardoxolone methyl) and

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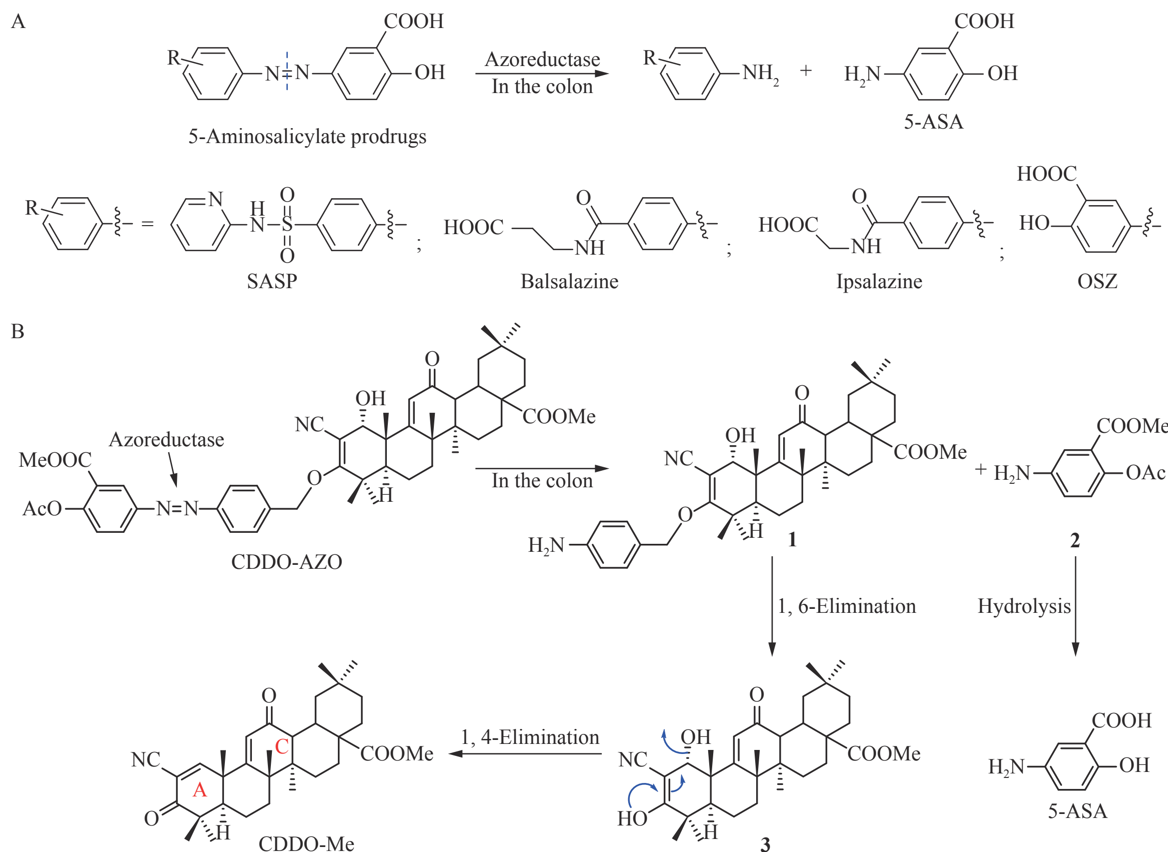
amide derivative CDDO-Im, bearing *α*-cyano-substituted *α*, *β*-unsaturated ketone (CUK) in ring A, display potent anti-inflammatory and anti-oxidant activities by activating Keap1/Nrf2/ARE and inhibiting nuclear factor kappa-B kinase (IKK)/NF-*κ*B signaling though the reactions of the CUK moiety with Cys-151 in Keap1 and Cys-179 in IKK, respectively, which suggesed that CDDOs may be effective against IBD due to oxidative stress and inflammation [10]. Further studies revealed that CDDO-Im significantly reduced colonic STAT3 activation, inhibited IL-17 secretion from splenocytes and colonic strips, and improved histological and biochemical parameters in DSS-induced colitis in mice [11]. Additionally, it was reported that CDDO-Me not only suppressed colitis-associated colon cancer in mice through elevating the expression of 15-hydroxyprostaglandin dehydrogenase (15-PGDH) [12], but also protected human colon epithelial cells against radiation-induced carcinogenesis [13]. Therefore, CDDO-Me and its analogues exhibit promising therapeutic effects against IBD.

Nevertheless, the CUK moiety in ring A of CDDO-Me and its analogues is highly reactive, which can interact with 577 cellular proteins, as indicated by proteomic analysis [14]. To avoid the reactions of the CUK moiety with undesirable and off-target proteins to generate side effects, our research group developed a prodrug strategy to mask the CUK moiety by conversion of CUK to the enol followed by etherification or esterification to generate stable prodrugs [15-16]. The obtained CUK-modified CDDO-Me prodrugs can be specifically cleaved into CDDO-Me in the target tissues and display potent efficacy for the intervention of diabetic nephropathy (DN) and pulmonary arterial hypertension (PAH) with improved safety. With respect to the anti-colitis efficacy of 5-ASA and CDDO-Me, we hypothesized that azoreductase-triggered prodrug CDDO-AZO (Scheme 1B), which was obtained through CUK pharmacophore masking and azo linker, may be relatively stable in the upper digestive tract before it reaches the colon. Once triggered by azoreductase in the colon, CDDO-AZO will split into CDDO-Me derivative (**1**) and methyl 2-acetoxy-5-aminobenzoate (**2**). The former produces enol (**3**) through 1, 6-elimination, and subsequently produces CDDO-Me through 1, 4-elimination, while the latter produces 5-ASA through hydrolysis, generating additional or synergistic anticolitis effects. To verify this hypothesis, we synthesized CDDO-AZO and investigated its therapeutic effects on DSS-induced colitis mice.

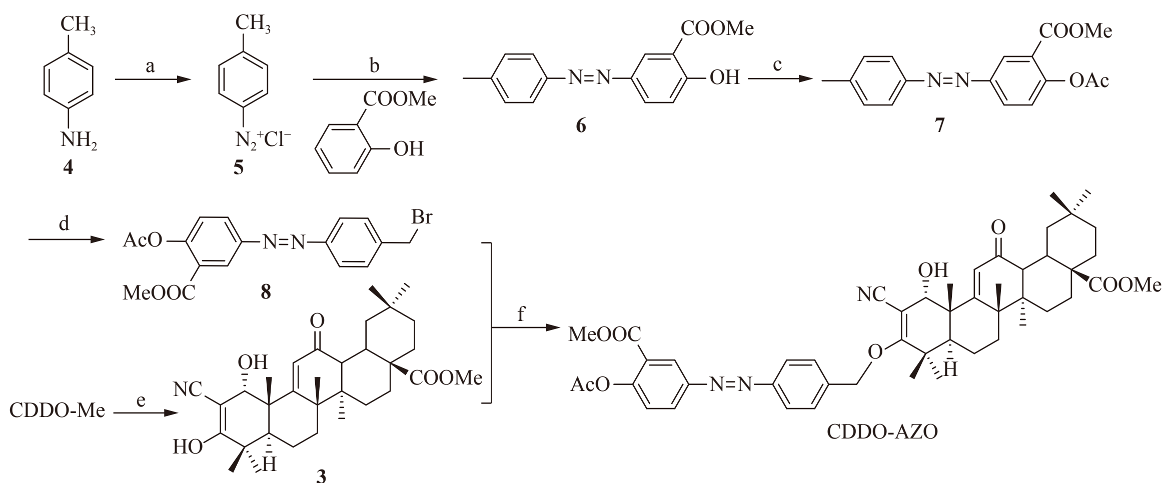
Results

Synthesis of CDDO-AZO

The synthetic route of CDDO-AZO is depicted in Scheme 2. The diazotization of 4-aminotoluene **4** produced



Scheme 1 A) Structures of azoreductase driven prodrugs of 5-ASA; B) Rational for the design of an azoreductase-triggered and CUK-modified prodrug CDDO-AZO



Scheme 2 The synthetic route of CDDO-AZO. Reagents and conditions: (a) $NaNO_2$, 6% HCl, 0 °C for 1 h; (b) NaOH, H_2O, 0 °C for 0.5 h, 65% for (a) and (b); (c) H_2SO_4, Ac_2O, 40 °C for 1 h, 82%; (d) NBS, AIBN, CH_3CN, reflux, 85%; (e, f) K_2CO_3, DMF, rt, 12 h, 37%

diazonium salt **5**, followed by condensation reaction with methyl salicylate producing azo compound **6**. Subsequent acetylation of **6** offered **7** which was brominated to furnish intermediate **8**. Meanwhile, starting from oleanolic acid, CDDO-Me was obtained using a previously reported method containing 9-step reactions [17]. Subsequently, CDDO-Me was treated with K_2CO_3 in the presence of H_2O in DMF to offer enol **3** [15], which was not purified and directly reacted with bromide compound **8** to give the target compound CDDO-AZO.

CDDO-AZO attenuated DSS-induced chronic colitis in mice

To investigate the anti-colitis activity of CDDO-AZO, a mouse model of DSS-induced chronic colitis was established through four administration cycles of 2% dextran sulfate sodium (DSS, 36−50 kDa, MP Biomedicals, Solon, OH, USA) [18]. The experimental protocol is shown in Fig. 1A. The disease activity index (DAI) has been widely used as an indicator of colitis severity and is associated with erosion and inflammation. The mice challenged with DSS displayed noticeable body weight loss, loose feces, and rectal bleeding, resulting in significant elevation of DAI compared with those in the control group (Fig. 1B). It was found that CDDO-AZO significantly attenuated the increase of DAI in a dose-dependent manner (Fig. 1C). Notably, CDDO-AZO 16.5 $mg \cdot kg^{-1}$ displayed lower DAIs than the same mole dose of CDDO-Me (10 $mg \cdot kg^{-1}$) and CDDO-Me (10 $mg \cdot kg^{-1}$) plus 5-ASA (3 $mg \cdot kg^{-1}$), whereas CDDO-AZO 49.5 $mg \cdot kg^{-1}$ exhibited more potent therapeutic effects than the same weight dose of olsalazine (OLS, Fig. 1C). Furthermore, according to morphological examination, the model group presented significantly shorter colon length and more diffuse colonic inflammation than the control group (Fig. 1D). Oral administration of CDDO-AZO obviously relieved the ulcers, which is superior to CDDO-Me, OLS and CDDO-Me plus 5-ASA. Hematoxylin and eosin staining (H&E, Fig. 1E) also showed significant nuclear hyperchromasia and enlargement, a high nucleus-to-cytoplasm ratio, dysplasia, goblet cell loss, and inflammatory cell infiltration in the lamina propria, as well as squamous epithelium metaplasia of the intestinal mucosa near the anus in the colon tissues of the model group. CDDO-AZO treatment attenuated inflammation severity. Histologically, CDDO-AZO produced similar effects as other positive drugs (Fig. 1E). Taken together, these results showed CDDO-AZO exerts therapeutic effects on DSS-induced chronic colitis in mice. It may be because the azoreductase-triggered and CUK-modified prodrug CDDO-AZO can protect the pharmacophores of 5-ASA and CUK in the upper digestive tract before it reaches the colon, and regenerate two pharmacophores in the colon to produce additional or synergistic anti-colitis effects.

Conclusion

Colon targeting strategy, which can avoid drug metabolism in the upper intestine and deliver drugs into the colon to increase distribution in the colon, is preferred for the treatment of ulcerative colitis. Based on the therapeutic efficacy of 5-ASA and CDDO-Me, we designed and synthesized an azoreductase-triggered prodrug CDDO-AZO through CUK-based modification and an azo linker. It is proposed that CDDO-AZO is stable before reaching the colon, while it can also be triggered by the presence of azoreductase in the colon to fragment into CDDO-Me and 5-ASA, generating potent anti-colitis effects. In DSS-induced chronic colitis mice, CDDO-AZO exhibits more potent therapeutic effects than OLS, CDDO-Me, and CDDO-Me plus 5-ASA, which suggests that CDDO-AZO may be a promising anti-ulcerative colitis agent and further investigations are necessary to explain the pharmacokinetics and mechanism of actions in detail.

Experimental

General Chemical Methods

Nuclear magnetic resonance (NMR) spectra were obtained from a Bruker Avance 300 (^1H 300 MHz; ^{13}C 75 MHz)

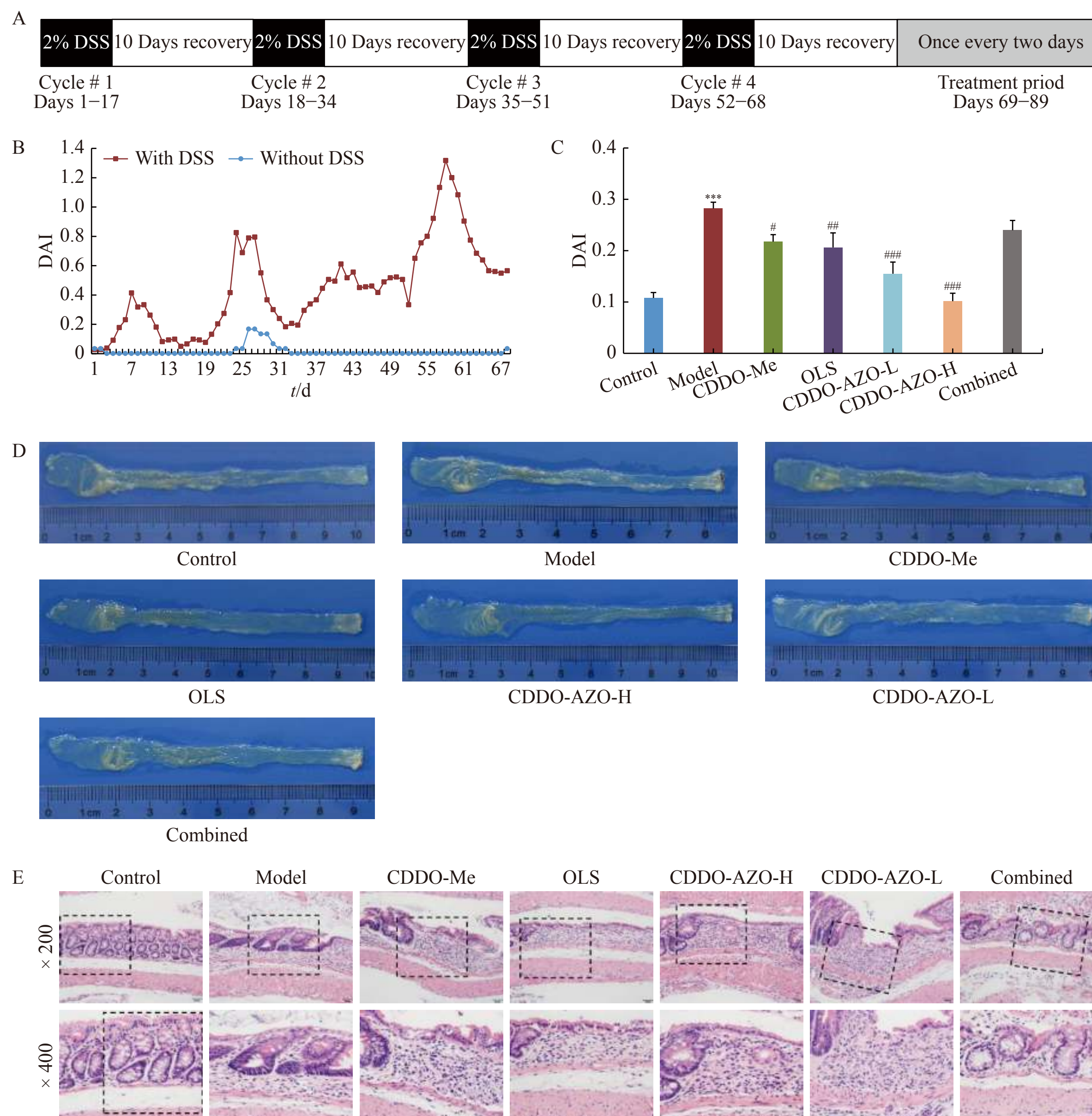


Fig. 1 (A) The experimental protocol for evaluating the therapeutic effects of CDDO-AZO in DSS induced inflammatory colitis. (B) Disease activity index (DAI) of the DSS group increased, compared with that of the control group. (C) DAI was improved after treatment (values are expressed as the mean of DAI throughout the whole treatment period). CDDO-AZO-H indicates the high-dose (49.5 mg·kg^{-1}) CDDO-AZO group, CDDO-AZO-L indicates the low-dose (16.5 mg·kg^{-1}) CDDO-AZO group, and the combined indicates the group of CDDO-Me plus 5-ASA. (D) Representative image of colon tissues from each group. (E) Representative colon tissues were sectioned and subjected to H&E staining. The rectangle in each upper micrograph (scale bar = 50 μm) is magnified on the bottom (scale bar = 20 μm). Data represent the means ± SEM of independent experiments (n = 10). $^{***}P$ < 0.001 *vs* the control group, $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001 *vs* the model group

spectrometer at 300 K using TMS as an internal standard. Mass spectrometry (MS) spectra were recorded on a Mariner mass spectrometer. TLC was performed on silica gel GF/UV 254, and column chromatography was conducted by silica gel (200−300 mesh). The purities of target compounds were characterized by HPLC (Shimadzu DGU-20A3R) and HRMS (Agilent Technologies LC/MSD TOF).

*Methyl 2-hydroxy-5-(p-tolyldiazenyl)benzoate (**6**)*

To a mixture of *p*-toluidine (1 g, 9 mmol) with dilute HCl (6%, 25 mL) under ice bath was added dropwise 5 mL of $NaNO_2$ (0.65 g, 9 mmol) aqueous solution for 30 min. The reaction mixture was stirred under ice bath for additional 30 min, and the diazo-reaction was monitored by the starch-potassium iodide test papers. The resultant diazonium salt solution was slowly added into the solution of methyl salicylate (1.36 g, 9 mmol) in aqueous sodium hydroxide solution under ice bath, and a reddish brown solid precipitated. One hour later, the reaction mixture was filtrated and the filter

cake was purified by silica gel column chromatography (petroleum ether-ether acetate, 40 : 1) to get the title compound **6** as a reddish brown solid (1.6 g, 65%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.09 (s, 1H), 8.43 (d, J = 2.37 Hz, 1H), 8.07 (d, J = 8.91 Hz, 1H), 7.78 (d, J = 8.22, 2H), 7.28 (t, J = 8.16 Hz, 2H), 7.08 (d, J = 8.94 Hz, 1H), 4.00 (s, 3H), 2.42 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 170.5, 163.6, 150.6, 145.4, 141.3, 129.6, 128.8, 126.4, 122.7, 118.4, 112.4, 52.6, 21.5; ESI-MS *m/z* 269 [M + H]$^+$.

*Methyl 2-acetoxy-5-(p-tolyldiazenyl)benzoate (**7**)*

A mixture of **6** (510 mg, 1.9 mmol) in acetic anhydride (289 mg, 2.8 mmol) and concentrated sulfuric acid (30 mg) was stirred at 40 °C for 1 h. After the addition of 50 mL of ice water, the mixture was stirred for 10 min. Then the mixture filtrated and the filter cake was purified by silica gel column chromatography (petroleum ether-ethyl acetate, 40 : 1) to obtain the title compound **7** as a reddish brown solid (495 mg, 82%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.56 (d, J = 2.37 Hz, 1H), 8.09 (dd, J = 2.43, 8.58 Hz, 1H), 7.84 (d, J = 8.25 Hz, 2H), 7.32 (d, J = 8.13 Hz, 2H), 7.25 (d, J = 8.34 Hz, 1H), 3.88 (s, 3H), 2.44 (s, 3H), 2.18 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.5, 164.5, 152.1, 150.2, 142.2, 129.8, 127.3, 126.6, 124.7, 123.8, 123.1, 77.5, 77.0, 76.6, 52.4, 21.6, 21.0; ESI-MS *m/z* 335 [M + Na]$^+$.

*Methyl 2-acetoxy-5-(((4-bromomethyl)phenyl)diazenyl)benzoate (**8**)*

A mixture of **7** (100 mg, 0.37 mmol), NBS (69 mg, 0.38 mmol) and AIBN (5 mg, 0.03 mol) in 10 mL acetonitrile was heated under reflux for 30 min. After the solvent was removed under vacuum condition, 50 mL ethyl acetate was added. The organic solvent was washed three times with saturated brine, and dried over anhydrous sodium sulfate. After the organic layer was filtrated, the filtrate was concentrated to get the residue which was purified by silica gel column chromatography (petroleum ether−ethyl acetate, 20 : 1) to obtain the title compound **8** as a reddish brown solid (467 mg, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.48 (d, J = 2.37 Hz, 1H), 8.01 (dd, J = 2.4, 8.58 Hz, 1H), 7.78 (d, J = 8.34 Hz, 2H), 7.44 (d, J = 8.34 Hz, 2H), 7.18 (d, J = 8.58 Hz, 1H), 4.45 (s, 2H), 4.04 (s, 3H), 2.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 169.8, 164.4, 152.5, 152.0, 149.9, 141.1, 130.0, 127.6, 127.4, 124.8, 123.9, 123.5, 123.3, 77.5, 77.1, 76.7, 52.5, 32.7, 21.0; ESI-MS *m/z* 413, 415 [M + Na]$^+$.

Methyl 1-hydroxyl-2-cyano-3-(4-((4-acetoxy-3-(methoxycarbonyl)phenyl)diazenyl)benzyloxy)-12-oxo-oleana-2(3), 9(11)-dien-28-oate (CDDO-AZO)

A mixture of CDDO-Me (297 mg, 0.59 mmol) in 10 mL of DMF with pure water (106 mg, 5.9 mmol) and K_2CO_3 (162.8 mg, 1.18 mmol) was stirred overnight at room temperature. After the addition of compound **8** (231 mg, 0.59 mol), the mixture was stirred for 0.5 h. Then 5 mL of 1 mol·L^{-1} diluted hydrochloric acid was added to terminate the reaction. After diluted with dichloromethane (50 mL), the mixture was washed three times with saturated sodium bicarbonate solution and saturated brine. The organic layer was dried over anhydrous sodium sulfate and filtrated. The filtrate was concentrated and purified by silica gel column chromatography (petroleum ether−ethyl acetate, 6: 1) to get the title compound CDDO-AZO as a yellow solid (178 mg, 37%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 8.51 (d, J = 2.37 Hz, 1H), 8.05 (d, J = 8.58 Hz, 1H), 7.88 (d, J = 8.28 Hz, 2H), 7.47 (d, J = 8.34 Hz, 2H), 5.80 (s, 1H), 5.52 (q, J = 10.23 Hz, 2H), 3.86 (s, 3H), 3.72 (t, J = 12.42 Hz, 4H), 3.70 (s, 4H), 3.55 (t, J = 4.80 Hz, 4H), 3.05 (d, J = 13.56 Hz, 1H), 2.88 (d, J = 4.47Hz, 1H), 2.66 (s,1H), 2.32 (s, 3H), 1.27, 1.23, 1.20, 1.15, 1.13, 1.03, 0.90 (s, each 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 199.6, 178.3, 175.3, 173.4, 169.5, 164.4, 152.5, 152.2, 149.9, 139.1, 128.8, 127.4, 126.8, 124.8, 123.9, 120.1, 85.3, 77.5, 77.1, 76.7, 74.2, 73.3, 72.3, 61.7, 52.5, 52.0, 49.9, 47.3, 45.2, 44.5, 42.5, 40.8, 35.9, 34.5, 33.3, 32.8, 31.5, 30.7, 28.6, 28.2, 23.8, 23.1, 22.6, 21.6; ESI-MS *m/z* 856 [M + Na]$^+$.

Animals and DSS-induced chronic colitis

Seventy male C57BL6/J mice, aged seven weeks, were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China) and acclimatized under a constant environment (22 °C, 55%−65% humidity, and 12 h light/12 h dark cycles) with food and water easily accessible. All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University.

Chronic colitis was induced in sixty mice by four cycles of "7 days of 2% dextran sulfate sodium (DSS, 36−50 kDa, MP Biomedicals, Solon, OH, USA) in drinking water, alternating with 10 days of ultrapure water", for a total of 68 days (Fig. 1A). Then, the mice were randomly divided into the following six groups (n = 10): (i) a model group (i.g. with vehicle 1% CMC-Na); (ii) a CDDO-Me group (i.g. with 10 mg·kg^{-1} CDDO-Me); (iii) an OLS group (i.g. with 49.5 mg·kg^{-1} OLS); (iv) a high-dose CDDO-AZO group (i.g. with 49.5 mg·kg^{-1} CDDO-AZO, designated as CDDO-AZO-H); (v) a low-dose CDDO-AZO group (i.g. with 16.5 mg·kg^{-1} CDDO-AZO, designated as CDDO-AZO-L); and (vi) a CDDO-Me plus 5-ASA group (i.g. with 10 mg·kg^{-1} CDDO-Me and 3 mg·kg^{-1} 5-ASA, designated as combined). Meanwhile, ten mice without DSS treatment were served as a control group (n = 10). The above treatment was performed every two days for three consecutive weeks.

Body weight, food and water consumption were measured daily throughout the experiments. Stool consistency and visible blood in feces were also examined. DSS-induced colitis was scored as DAI based on weight loss, stool consistency, and bloody excreta as follows. Weight loss score = 0: normal, 1: 1%−5%, 2: 5%−10%, 3: 10%−20%, and 4: > 20%; stool consistency score = 0: normal, 1: soft, 2: pasty, 3: soft & pasty, and 4: liquid; blood in excreta score = 0: normal; 1: trace, 2: mild hemoccult, 3: obvious hemoccult, and 4: gross bleeding. At the end of the experiments, the mice were sacrificed through cervical dislocation and the colons were rapidly collected, rinsed with ice-cold PBS, longitudinally opened, laid flat on an ice board, and photographed. Some of the colon specimens were kept frozen at −80 °C,

while the others were fixed in 10% phosphate-buffered formalin over 24 h for H&E staining.

Pathological examination

To study colon tissue morphology, H&E staining was used. The colon tissues were fixed with 10% phosphate-buffered formalin and embedded in paraffin, cut into sections, and stained with H&E before pathologcial analysis.

Statistical analysis

Statistical analyses were performed using one-way ANOVA and Tukey's multiple comparison test by IMB SPSS Statistics 21.0. Data are expressed as mean ± SEM. $P < 0.05$ is considered statistically significant.

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