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• BASIC RESEARCH •

Synthesis of an enzyme-dependent prodrug and evaluation of its potential for colon targeting

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Abstract

AIM: To synthesize dexamethasone-succinate-dextran (DSD) conjugate and to evaluate the potentiality of DSD for the treatment of inflammatory bowel diseases.

METHODS: Dexamethasone was attached to dextran (average molecular weight=70 400 Dalton) using succinate anhydride in an anhydrous environment catalyzed by 4-dimethylaminopyridine and 1, 1'-carbonyldiimidazole. The chemical structure of DSD was identified by UV, IR and NMR, and the *in vivo* drug release behavior of this prodrug was investigated after oral administration of DSD suspension.

RESULTS: The DSD conjugate was obtained in two steps and the content of dexamethasone in DSD was 11.28 %. The dextran prodrug was stable in rat stomach and small intestine and negligibly absorbed from these tracts. Four to nine hours after the oral administration, most of the prodrug (>95 %) had moved to the cecum and colon, and was easily hydrolyzed by an endodextranase. Recover of dexamethasone from colon and cecum after administration of DSD conjugate was 6-12 folds higher than the recovery after administration of unmodified dexamethasone(t=2.74, P<0.05). The preferential release of free dexamethasone in cecum and colon over that in the small intestine was statistically significant (t=2.27, P<0.05).

CONCLUSION: The results of this study indicate that dextran conjugates may be useful in selectively delivering glucocorticoids to the colon.

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INTRODUCTION

Inflammatory bowel diseases, which include ulcerative colitis and Crohn's disease are currently treated with glucocoticoids and other anti-inflammatory agents[1,2]. For a steroidal anti-inflammatory drug, e.g. dexamethasone or prednisolone, a long-term administration would produce systemic side effects, including adrenosuppression, Cushinoid symptoms, immunosuppression, and diabetes. In this case, it is desirable to localize the release of dexamethasone insofar as possible to the afflicted sites in the colon. Release of drug in the proximal GI tract should be avoided to circumvent absorption from the small intestine, and consequent drug wastage and systemic side effects[2]. Because of the unique physiological characteristics of the large intestine, drug delivery to the colon can be achieved in different ways, including pH dependent approaches utilizing the changes in pH along the GI tract[3-12], coated dosage forms[13-17], time-controlled or pulsatile release systems[18-24], pressure-controlled colon delivery systems[25-30], coating drugs with bacterially degradable polymers[31-40], and delivery of drugs as prodrugs[41-47].

The bacterial count in the colon is higher than that in the preceding sections of the GI tract by many orders of magnitude in humans and other animals. Enzymes of the colonic bacteria can specifically degrade some kind of polysaccharides and azo-polymers or break the chemical bonds between the parent drug and the carrier, and then the pharmacological active component can be released from natural and synthetic prodrugs. The most important issue for this approach is a selection of the functional groups that can survive the passage through stomach and small intestine, but are degraded by enzymes of the colonic microflora thus specifically releasing the drug into the colon[2].

This project used dexamethasone as the model drug to synthesize a prodrug via a succinate tetracarbon-bridge that links the parent drug to the dextran carrier. Compared with unmodified dexamethasone, dexamethasone-succinate-dextran conjugate is more hydrophilic and has a larger molecular weight, which may decrease its possibility of being absorbed into the systemic circulation through the small intestinal epithelial cells. When it arrives to the colon, the dextran structure is hydrolyzed quickly by endogenous dextranase and then the esterase breaks the ester bond to release the dexamethasone. Distributions of dexamethasone in plasma and luminal contents were investigated after gastric intubation of DSD suspension or equivalent dose of dexamethasone to male SD rats.

MATERIALS AND METHODS

Material and apparatus

Dexamethasone was purchased from Tianjin Pharmacy Ltd., China. 4-dimethylaminopyridine (DMPA), 1,1'-carbonyldiimidazole and dextran (weight-average molecular weight=70 400 Dalton) were obtained from Sigma Chemical Company, St. Louis, MO. Succinate anhydride was purchased from Beijing Medicine Corporation, China. Molecular sieve (5Å) was obtained from Shitian Chemical Ind. China.

Methods

Synthesis of dexamethasone-dextran conjugate

Dexamethasone 3.98 g, succinate anhydride 1.27 g and 4-dimethylaminopyridine 1.55 g were dissolved in 400 ml anhydrous acetone over 5Åmolecular sieves. The reaction solution was stirred at 25 ℃ for 30 minutes, and the resulting solution was evaporated in a rotary evaporator to produce light

yellow solid. After the solid was dissolved in anhydrous ethanol, distilled water was added to achieve a solution of ethanol and water (29:71v/v). The solution was kept at -4 ℃ for 48 h to crystallize and filtered under reduced pressure. The resulting crystals were dried in a P_2O_5 drying pistol with refluxing of 95 % ethanol under vacuum (10 mmHg) for 24 h to produce dexamethasone succinate hemiester (DS). The yield is 85.28±4.57 %.

3.08 g of DS and 1.78 g of 1,1' -carbonyldiimidazole were dissolved in 15 ml of anhydrous dimethyl sulfoxide (DMSO). The reaction was run at 25 ℃ with stirring for 30 min. Then a solution of dextran in anhydrous DMSO(200 ml) and triethylamine(17.5 ml) was added, and the mixture was stirred at 25 ℃ for 21 h. The dextran conjugate was precipitated by adding 300 ml of ethanol/ether (50:50v/v) to the DMSO solution with stirring. The resulting polymer was dispersed in ethanol again and filtered under a stream of dry nitrogen. The precipitate was collected by filtration under reduced pressure, then washed with anhydrous ether three times to produce DSD white powder (yield: 81.27±5.09 %): UV λmax: 242 nm (ε14 500); IR(KBr): 3420(OH), 2930(CH_2),1740(C=O), 1660 (C=C), 1020(C-O-C) 898 cm^{-1}; ^{1}HNMR (DEXO-d_6): δ7.310, 7.285(d,1H,C-1), 6.233, 6.207(d,1H,C-2), 6.002 (s,1H, C-4), 3.488, 3.508, 3.623, 3.742,4.668 (s,1H,C-5' ,C-4' ,C-3' ,C-2' , C-1'), 3.202(s,2H,C-6'), 2.054(s,2H, C-21), 1.464 (s,3H,C-19), 0.860(s,3H, C-18), 0.758, 0.774 (d,3H,C-16).

The content of dexamethasone in DSD was measured by HPLC after alkaline hydrolysis.

Preparation of DSD granules and DSC test To evaluate the potential colon specificity of DSD *in vivo* test, granules of DSD or dexamethasone were prepared with the following ingredients: DSD or dexamethasone, cornstarch and lactose (5:50:45). A wet granulation method was applied. The granules were partially dissolved and suspended in water before dosing. Before granulation, all the ingredients were subjected to Differential Scanning Calorimetry (DSC). Measurements were performed on a calorimeter DSC7 connected to a Thermal Analysis Data Station 3 700 (Perkin-Elmer, Germany). Five mg of bulk materials were accurately weighed into standard aluminum pans. Thermograms were recorded from 303 to 573 K at a heating rate of 10 K • min^{-1}.

***In vivo* test** Male SD rats (weighing about 150 g) were fed a standard diet (R-2, Chengdu) and were fasted for 18 h prior to drug administration with free access to drinking water. The rats were divided into the test group and control group randomly. Each group was subdivided further into seven subgroups. The test groups were administered with suspension of DSD (equivalent to 3 mg of dexamethasone per Kg of rat body weight) by gastric intubation, and the control groups were administered with suspension of dexamethasone. After the drug administration, blood samples of the test subgroups and the control subgroups were collected at each predetermined time (1,3,4,5,6,7,9 h). Then the rats were sacrificed by decapitation and the stomach, proximal small intestine (PSI), distal small intestine (DSI), cecum and colon were removed. The contents of the GI tract were removed by gently squeezing the GI segments. The separated contents and tissues were quickly frozen to -20 ℃ and stored until analysis. Rat blood samples were collected and centrifuged at 700 g for 10 min. The plasma was frozen to -20 ℃ and stored until analysis was performed.

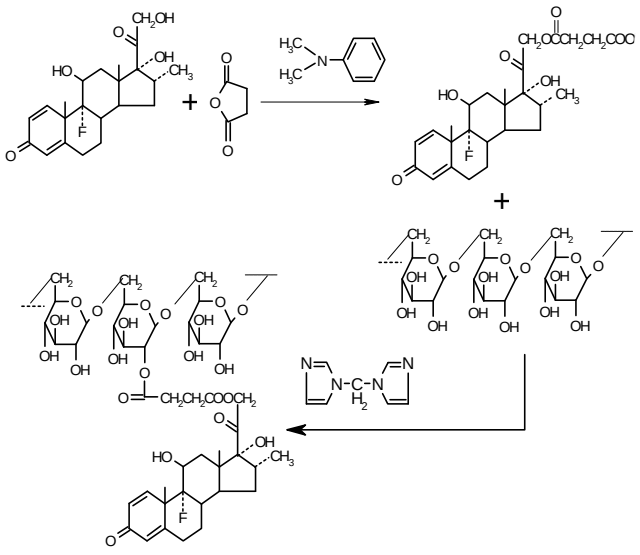
Analysis The frozen intestinal contents were thawed, weighed, and diluted to 50 %(w/v) with phosphate buffer (pH6.8). The suspended samples were homogenized by vortexing, and then 0.5 g of the diluted contents was placed in a 5-ml centrifuge tube. 200 μl of isotonic phosphate buffer (pH2), 100 μl of internal standard solution (0.1336 mg • ml^{-1}) and dexamethasone solution in different concentrations were added to the 5-ml centrifuge tube. The samples were extracted with acetic ester (3 ml) by vortexing for 2 min. After centrifuging for 10 min at 1000 g, 2 ml of the organic phase was removed and evaporate at 45 ℃ under vacuum. The residue was redissolved in mobile phase solution and centrifuged for 10 min at 1000 g. 20 μl of the supernatant fluid was subjected to HPLC analysis under the following conditions: Shimadzu CTO-10A system controller, LC-10AT pumps, SPD-10A variable wavelength detector, a Shimpack CLC C18 column(5 μm,4.6×150 mm). The mobile phase consisted of 35 % acetonitrile and 65 % buffer (50 mM trisodium citrate adjusted to pH4.6 with phosphoric acid). A flow rate of 1 ml.min^{-1} and a detection wavelength of 241nm were used. Prednisolone acetate was used as the internal standard. The plasma samples were treated by the same method described above.

RESULTS

Chemistry

DSD was prepared in two steps with a modified Mcleod reaction[2]. It is essential to keep the reaction continuing under the anhydrous condition to ensure high yield. Succinate anhydride was coupled to the dexamethasone hydroxyl group in anhydrous acetone in the presence of 4-dimethylaminopyridine to produce hemiester. Then the hemiester was coupled to dextran in DMSO using 1,1' -carbonyldiimidazole as catalyzer (Scheme 1).

The chemical structure was identified by ^{1}HNMR and IR, confirming the procedure of scheme 1. The content of dexamethasone in DSD was 11.28 equals to about 20 dexamethasone (molecular weight=392 Dalton) molecules were coupled to one dextran molecule (average molecular weight=70 400 Dalton).



Scheme 1 Preparation of DSD

Differential scanning calorimetry (DSC)

Study on interaction between the supplementary ingredients of suspension and DSD was performed by DSC. The thermogram displayed two transition peaks at 343K and 561K corresponding to DSD, and another two peaks at 420K and 486K corresponding to mixed ingredients. No new transition peak was observed when the physical mixture of DSD and ingredients were subjected to DSC, indicating that there was no interaction between DSD and the supplementary ingredients.

In vivo testing

The recovery of free dexamethasone from rat blood and GI tract at various times following oral administration of DSD suspensions is shown in Table 1. During the whole observation period (0-9 h), no dexamethasone was detected in blood. This observation indicated that DSD conjugate was so stable that it could not be degraded in upper GI tract and could not be absorbed into blood. Three hours after dosing, only very small amount (<3 % of total recovery) of dexamethasone was detected in small intestine in spite of the high level of esterase in small intestine. After 6h, the recovery of dexamethasone in small intestine further decreased. At the same time, a large portion (>95 % of total recovery) of the prodrug reached the cecum and colon intact.

Control experiments in which unmodified dexamethasone was administered showed that dexamethasone was absorbed primarily from the small intestine and the blood concentration of dexamethasone was much higher than test groups. Meanwhile, very small amount of dexamethasone was observed either in the cecum or in the colon (Table 2).

Table 1 Recovery of free dexamethasone from the rat blood and GI tract at various times after administration of DSD suspensions [a, b] (equivalent to 15mg of dexmethasone per Kg body weight)

t	T	Recovery of dexamethasone(%)					
h	μg	B	S	PSI	DSI	Ce	Co
1	5.2	nd	74	26	nd	nd	nd
3	7.3	nd	54	nd	46	nd	nd
4	14	nd	8.8	6.6	10.2	54	20.5
5	27.6	nd	9.2	5.0	13.4	54.4	18.0
6	133	nd	4.0	0.27	2.2	83	10.2
7	128	nd	4.5	nd	0.557	80	15.2
9	105	nd	12.4	3.49	3.72	62	18.2

Table 2 Recovery of free dexamethasone from the rat blood and GI tract at various times after administration of dexamethasone suspensions[a,b] (equivalent to 15 mg of dexmethasone per Kg body weight)

t	T	Recovery of dexamethasone (%)					
h	μg	B	S	PSI	DSI	Ce	Co
1	1076	8.9	54	15.8	20.8	0.05	nd
3	857	4.58	94.6	0.05	1.08	nd	nd
4	390	26	58	10.2	5.0	0.40	nd
5	230	36	22	1.19	4.2	16	2.56
6	203	33	30	6.7	3.63	24.7	2.25
7	139	39	49	2.02	5.6	3.74	0.79
9	134	40	42	1.52	7.8	6.0	3.1

[a]Values represent the average of three animals. [b]t, Time; T, Total recovery; B, Blood; S, Stomach; PSI, Proximal small intestine; DSI, Distal small intestine; Ce, Cecum; Co, Colon. nd: Not detected

The dexamethasone recoveries from cecum and colon after the oral administration of DSD suspensions or dexamethasone suspensions were also shown graphically (Figure 1 and Figure 2). It was obvious that the recovery of test groups from cecum and colon after administration were higher than that of control groups by 6-12 folds (t=2.74, P<0.05).

The specificity of dexamethasone release was further evaluated by comparing the amount of free dexamethasone recovered in the small intestine with that in the colon in the test group. A paired t-test indicated that the preferential release of free dexamethasone in cecum and colon over that in the small intestine was statistically significant (t=2.27, P<0.05). Meanwhile, a similar analysis in the control group showed that the difference between the dexamethasone concentration in the colon and that in the small intestine was not statistically significant.

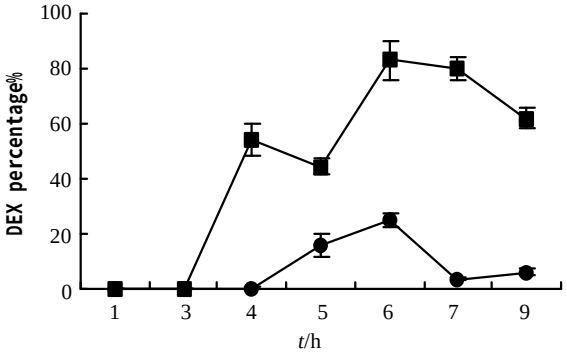


Figure 1 The dexamethasone contents-time curves in cecum after the oral administration dexamethasone suspensions (●-●) and DSD suspensions (■-■)

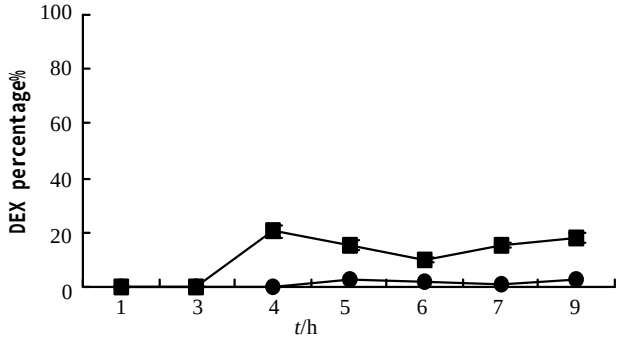


Figure 2 The dexamethasone contents-time curves in colon after the oral administration dexamethasone suspensions (●-●) and DSD suspensions (■-■)

DISCUSSION

The bacterial count in the colon is much higher than that in upper GI tract[2]. The colonic micro flora produce a variety of enzymes, including azoreductase, various glycosidases and amidases, which are not present in the stomach or the small intestine. Therefore, enzyme dependent drug release, which relies on the existence of enzyme-producing microorganisms in the colon, could be used to deliver drug to the colon after enzymatic cleavage of degradable carrier bonds and premature drug release does not occur in this case.

Besides treating inflammatory bowel diseases, colon-specific drug delivery system might be useful in other situations. The delivery of certain antineoplastic agents to the

colon might be beneficial in controlling colon cancer[48]. Enzyme prodrug gene therapy for colon cancer is also investigated by several researchers[49,50]. Antibiotics might be delivered specifically to the colon via cyclodextrin carriers[51-53]. In each of these cases, colon-specific delivery would allow the use of higher doses of potent agents with fewer systemic side effects.

The present results showed that the ester type prodrugs of dexamethasone/dextran release dexamethasone preferentially on cecal and colonic contents after the hydrolysis of dextran to small oligosaccharides, suggesting that dextran could serve as a new class of colon-specific drug carrier. The dextran conjugate survives the passage through upper GI tract although the high level of esterase in small intestine, indicating that dextran protects ester bond from hydrolysis by esterase. This result, together with the observation mentioned above, suggests that bacterial enzymes in the colon are responsible for hydrolysis of dextran conjugates. When DSD reached the colon, dextran was completely hydrolyzed into smaller oligosaccharides and exposed the ester bonds to esterase, which led to the rapid release of dexamethasone.

In summary, a colon-specific drug-delivery system has been developed based on drug-dextran conjugation and the unique glycosidase activity of the colonic microflora. Colonic drug delivery can be achieved with carriers by making prodrugs that survive the passage through stomach and small intestine, but the active moiety is released by enzymes specifically produced in colon.

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