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Research paper

Carboxymethylcellulose–tetrahydrocurcumin conjugates for colon-specific delivery of a novel anti-cancer agent, 4-amino tetrahydrocurcumin

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ABSTRACT

Several curcumin derivatives are now becoming increasingly of interest because of their bioactive attributes, especially their action as antioxidants and anti-carcinogenic activities. Tetrahydrocurcumin (THC), an active metabolite of curcumin, was selected to be a proper starting material for the work presented here as it is stable in physiological pH and has the typical pharmacological properties of curcumin. We have now reported that novel synthesized water-soluble polymeric macromolecule prodrugs can specifically deliver the drug to the colon. To study the drug loading and drug release, THC was conjugated with a hydrophilic polymer, carboxymethylcellulose (CMC) with the degree of substitution (DS) values of 0.7 and 1.2. THC was also attached to two different spacers including *p*-aminobenzoic acid (PABA) and *p*-aminohippuric acid (PAH) via an azo bond that was cleaved by the azoreductase activities of colonic bacteria. The novel active molecule, 4-amino-THC, was readily released from the conjugates in the colon (>62% within 24 h) with only very small amounts released in the upper GI tract (<12% over 12 h). The polymer conjugates showed chemical stability at various pH values along the gastrointestinal tract and increased water solubility of up to 5 mg/mL. 4-Amino-THC demonstrated cytotoxic ability against the human colon adenocarcinoma cell lines (HT-29) with an IC_{50} of 28.67 ± 1.01 μg/mL, and even greater selectivity (~4 folds) to inhibit HT-29 cells than to normal human colon epithelial cell lines while curcumin was a non-selective agent against both cell lines. Our study has demonstrated that the use of THC–CMC conjugates may be a promising colon-specific drug delivery system with its sustained release in the colon to be an effective treatment for colonic cancer.

1. Introduction

Colon cancer is a leading cause of morbidity and mortality, and it has been ranked at the third cause of cancer death in the United State [1]. The primary treatment is complete surgical resection of the diseased tissue. Further therapy may include adjunctive chemotherapy and/or radiotherapy depending on the course of the disease. The USA Food and Drug Administration (FDA) has approved several drugs as chemopreventive agents for colon cancer such as folinic acid, 5-fluorouracil, bevacizumab (Avastin®) as well as COX-2 inhibitors such as Celecoxib (Celebrex®). Because of the ineffectiveness, lack of safety and high cost of these agents, many plant-based products have also been developed for the use as alternatives. Curcumin from *Curcuma longa* has also been reported to be an antitumor agent [2] and could be a promising anticancer drug for the use in the near future.

Abbreviations: THC, tetrahydrocurcumin; Na-CMC, sodium carboxymethylcellulose; DS, degree of substitution; PABA, *p*-aminobenzoic acid; PAH, *p*-aminohippuric acid; IBD, inflammatory bowel disease; NMP, 1-methyl-2-pyrrolidinone; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; TFA, trifluoroacetic acid; MWCO, molecular weight cut-off; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; %RSD, percentage relative standard deviation; DMEM, Delbecco's Modified Eagle Medium; EMEM, Eagle's Minimum Essential Medium; CRL-1790, normal human colon epithelial cells CCD 841 CoN; HT-29, human colon adenocarcinoma cell lines; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; mTOR, mammalian target of rapamycin.

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Curcumin derivatives are yellow spices used as food ingredients and additives in certain Asian cuisines. They are naturally concentrated in the rhizomes of Turmeric, and possess several biological activities, and as a result of which they have been used as antioxidants, anti-inflammatory agents, for wound healing, and as antibacterial, anti-Alzheimer and anticancer agents [3]. In a preliminary clinical study in animals, curcumin showed anti-proliferative activity against tumor cells as well as restricting tumor promotion in skin, the oral cavity, lung, intestine, pancreas, prostate and colorectal carcinomas [4]. Preliminary studies have reported that curcumin may produce clinical benefits for patients with colonic inflammatory disease, adenocarcinoma or colorectal cancer with no dose-limiting toxicity at doses of up to 8 g/day for three months [2,5–7]. Curcumin is stable at an acidic pH; nevertheless, it rapidly decomposes at pH values above neutral in the presence of ferulic acid and feruloylmethane to degrade to vanillin and acetone [8–10]. Curcumin was biotransformed to dihydrocurcumin and tetrahydrocurcumin by an endogenous reductase system [11].

Tetrahydrocurcumin, THC, is one of the active metabolites of curcumin. Structurally, THC and curcumin have a β-diketone moiety and phenolic groups, but they differ in that THC lacks the double bond on the hydrocarbon chain. THC is stable at a physiological pH [11], and also possesses a higher antioxidant activity than curcumin in particular [12–14]. THC also expresses more potent tumor angiogenesis than curcumin [15]. Based on these observations in this study THC was chosen to prepare polymer conjugates that could find the use for treating colon cancer.

Due to its low water solubility, THC has only low pharmacological activities. To overcome this drawback, THC was conjugated with carboxymethylcellulose (CMC), a hydrophilic cellulose polymer. CMC, commercially available polymer, is approved as a common excipient and GRAS (generally recognized as safe) by FDA. CMC is an inexpensive pure compound with good compatibility to the skin and mucous membranes; moreover, CMC has suitable functional groups including hydroxyl and carboxyl groups that can allow for chemical reactions.

Colon-specific drug delivery systems (CDDS) are highly desirable for local treatment of a variety of bowel disease, such as inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis as well as colonic cancer. Targeted drug delivery to the colon should not release drugs in the stomach and small intestine, but the systems should allow for drug release and absorption only in the colon to reduce the total amount of drug administered, decrease possible side effects and improve the quality of life for patients suffering from colon-specific diseases [16,17].

Although nanoparticle technology has already been applied to improve the water solubility of curcuminoids [18–20], polymer conjugates of these natural products have not been reported as being used for colon-specific drug delivery. In this study we have designed polymer conjugates with microflora-activated strategy which was preferable triggering component in colon specificity by the increased bacteria population and enzyme activities in the colon [21,22]. THC prodrugs were expected to release a new compound, 4-amino-THC (Fig. 1) by the azoreductase activities of colonic bacteria. In this article we report on the synthesis and characterization, *in vitro* drug release in the rat gastrointestinal contents as well as the investigations on the toxicity of these THC–CMC conjugates. The cytotoxic activity of the novel active compound, 4-amino-THC, was compared to curcumin and THC with normal and colon cancer cell lines.

2. Materials and methods

2.1. Materials

Sabiwhite (tetrahydrocurcumin ultra pure) was from Sabinsa Corporation (Piscataway, NJ, USA). *p*-Aminohippuric acid (PAH) and *N*-Boc-1,6-hexanediamine were from Sigma (Buchs, Switzerland). Carboxymethylcellulose sodium salt molecular weight 250,000 with degree of substitution (DS) 0.7 and 1.2 as well as phosphate buffered saline pH 7.4 was from Sigma (Missouri, USA). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) hydrochloride was from Fluka (Buchs, Switzerland). Ethyl cyanoglyoxylate-2-oxime (Oxyma Pure®) was from Merck (Hohenburnn, Germany). Dialysis tubing cellulose membrane was from CelluSep (Texas, USA). *p*-Aminobenzoic acid (PABA) was from Merck (Xiamen, P.R. China). Methanol (HPLCgrade) was from RCI Labscan (Bangkok, Thailand). 3,(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) was from Invitrogen (Oregon, USA). 0.5% Trypsin–EDTA 10X was from Gibco, Invitrogen (Ontario, Canada). Dulbecco's Modified Eagle Medium (DMEM) powder and Fetal Bovine Serum (FBS) were from Gibco, Invitrogen (New York, USA). Human colon epithelial cell lines CCD 841 CoN (CRL-1790) and Eagle's Minimum Essential Medium (EMEM) were from ATCC (Virginia, USA). Dimethyl sulfoxide (DMSO) was from Amresco (Ohio, USA). All other reagents and solvents were from commercial products and of analytical or reagent grade.

2.2. Synthesis of THC–CMC (**IV**)

The synthesis of the THC–CMC conjugate (**IV**) consisted of 3 steps as follows (Scheme 1).

2.2.1. THC–PABA/PAH (**ia/b**)

A solution of PABA/PAH (1.37/1.94 g, 10 mmol) in water (5 mL) and a solution of $NaNO_2$ (0.69 g, 10 mmol) in water (5 mL) were mixed. The mixture was stirred in an ice bath (3–5 °C) for 15 min and acidified with 3 M HCl and then stirred again for a further 5 min. Complete diazotization was observed from the presence of excess nitrous acid tested by potassium iodide/starch indicator paper. THC (3.72 g, 10 mmol) was dissolved in 0.5 M NaOH and stirred at 3–5 °C. The diazonium salt solution was added to this solution, and the pH was then adjusted to 10 with 1 M NaOH. The reddish brown solution obtained was further stirred for 30 min. The mixture was acidified with 3 M HCl, and the reddish-brown solid was filtered off and washed with water [23]. Finally, the product (**IA/B**) was purified by silica gel column chromatography with dichloromethane:methanol (9:1) as a mobile phase. The dried product was characterized by IR, ^{1}H NMR and ^{13}C NMR.

THC–PABA (**IA**): Yellow powder, yield 85.10%: IR (KBr, ν cm^{-1}): 1035 (C—O), 1430, 1514 (N=N), 1650–1678 (C=O), 2900–2940 (C—H), 3413 (O—H). ^{1}H NMR (DMSO-d_6, 500 MHz): 2.73–2.80 (4H, sex, CH_2 THC), 3.10–3.16 (4H, qn, CH_2 THC), 3.70 (3H, s, CH_3 THC), 3.72 (3H, s, CH_3 THC), 6.57–6.66 (4H, m, ArH THC), 6.73–6.75 (4H, m, ArH THC), 7.58–7.61 (2H, sex, ArH PABA), 7.93–7.96 (2H, sex, ArH, PABA), 8.63–8.65 (2H, d, OH THC), 13.52 (1H, s, enol THC). ^{13}C NMR (DMSO-d_6, 300 MHz): 29.0, 29.9, 44.5, 55.6, 55.7, 112.7, 112.7, 115.4, 115.5, 115.8, 120.4, 120.5, 126.7, 131.1, 131.8, 132.1, 134.8, 144.8, 144.8, 145.7, 147.5, 166.9, 198.2, 199.4.

THC–PAH (**IB**): Dark yellow powder, yield 78.23%: IR (KBr, ν cm^{-1}): 1029 (C—O), 1427, 1530 (N=N), 1608–1739 (C=O), 2850–2924 (C—H), 3403 (O—H). ^{1}H NMR (DMSO-d_6, 500 MHz): 2.72–2.81 (4H, m, CH_2 THC), 3.12–3.17 (4H, sex, CH_2 THC), 3.70 (3H, s, CH_3 THC), 3.72 (3H, s, CH_3 THC), 3.91–3.92 (2H, d, CH_2 PAH), 6.57–6.66 (4H, m, ArH THC), 6.75–6.80 (2H, dd, ArH THC),