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Research paper

In vitro combinatorial anticancer effects of 5-fluorouracil and curcumin loaded *N*,*O*-carboxymethyl chitosan nanoparticles toward colon cancer and *in vivo* pharmacokinetic studies

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ABSTRACT

Colon cancer is the third most leading causes of death due to cancer worldwide and the chemo drug 5-fluorouracil's (5-FU) applicability is limited due to its non-specificity, low bioavailability and overdose. The efficacy of 5-FU in colon cancer chemo treatment could be improved by nanoencapsulation and combinatorial approach. In the present study curcumin (CUR), a known anticancer phytochemical, was used in combination with 5-FU and the work focuses on the development of a combinatorial nanomedicine based on 5-FU and CUR in *N*,*O*-carboxymethyl chitosan nanoparticles (*N*,*O*-CMC NPs). The developed 5-FU-*N*,*O*-CMC NPs and CUR-*N*,*O*-CMC NPs were found to be blood compatible. The *in vitro* drug release profile in pH 4.5 and 7.4 showed a sustained release profile over a period of 4 days. The combined exposure of the nanoformulations in colon cancer cells (HT 29) proved the enhanced anticancer effects. In addition, the *in vivo* pharmacokinetic data in mouse model revealed the improved plasma concentrations of 5-FU and CUR which prolonged up to 72 h unlike the bare drugs. In conclusion, the 5-FU and CUR released from the *N*,*O*-CMC NPs produced enhanced anticancer effects *in vitro* and improved plasma concentrations under *in vivo* conditions.

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1. Introduction

The third most leading causes of death due to cancer in the world arise from colorectal cancer and this accounts for 40% of all cancer cases diagnosed yearly [1,2] The treatment modalities for colon cancer include surgery, radiofrequency ablation, cryosurgery, chemotherapy, radiation therapy and targeted therapy [3]. Among which chemotherapy utilizes different drugs or drug combination to reduce the cancer cell growth. The FDA approved chemo drugs or drug combinations used in colon cancer include adrucil (5-fluorouracil, 5-FU), avastin (bevacizumab), camptosar (irinotecan hydrochloride), capecitabine, cetuximab, eloxatin (oxaliplatin), erbitux (cetuximab) or drug combinations such as capox, folfiri, folfiri–bevacizumab, folfiri–cetuximab, folfox, and zelox [4]. In colon cancer, chemotherapy is given adjuvant after surgery and the first line chemo drug used is 5-FU [4,5]. Being a thymidylate synthase inhibitor, it inhibits the cancer cell growth by the arrest of cells in the S phase [6] and its major drawback is systemic toxicity arising from its non-specificity, low plasma half life, leading to the use of high doses [7] and its inefficacy in chemo treatment results from cyclooxygenase 2 (COX-2) over expression in colon cancers [8–12].

Abbreviations: 5-FU, 5-fluorouracil; CUR, curcumin; *N*,*O*-CMC, *N*,*O*-carboxymethyl chitosan; FT-IR, Fourier transforms Infrared spectroscopy; DDA, degree of deacetylation; kDa, kilo Dalton; Mw, molecular weight; DLS, dynamic light scattering; PBS, phosphate buffered saline; SEM, scanning electron microscope; FBS, fetal bovine serum; RPMI, Roswell park memorial institute medium; HPLC, high pressure liquid chromatography; EPR, enhanced permeability and retention effect; NPs, nanoparticles; BSA, bovine serum albumin; 5-FU-*N*,*O*-CMC NPs, 5-fluorouracil loaded *N*,*O*-carboxymethyl chitosan nanoparticles; CUR-*N*,*O*-CMC NPs, curcumin loaded *N*,*O*-carboxymethyl chitosan nanoparticles; EE, entrapment efficiency; LE, loading efficiency; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; Rhod 123, rhodamine 123; PI, propidium iodide; RNase, ribonuclease; Hb, hemoglobin; PT, prothrombin time; aPTT, activated partial thromboplastin time; PPP, platelet poor plasma; TPP, pentasodium tripolyphosphate; CO_2, carbon dioxide; EDTA, ethylene diamine tetra acetic acid; JC-1, 5,5′,6,6′-tetraethylbenzidazolylcarbocyanine iodide; AUC_{0-t}, area under the curve; C_{max}, concentration maximum; T_{max}, time of maximum concentration; ACD, acid citrate dextrose; H and E, Harris's Hematoxylin and Eosin; FdUMP, fluorodeoxyuridine monophosphate; FdUTP, fluoro-deoxyuridine triphosphate; FUTP, fluorouridine triphosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; COX-2, cyclooxygenase-2; pH, potenz hydrogen; HT 29, human colon adenocarcinoma; IEC 6, mouse intestinal epithelial cells.

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The effectiveness of 5-FU as a chemo drug can be improved by two approaches, nanoencapsulation and combinatorial treatment, where the former involves the entrapment of 5-FU in a polymer based nanoparticle system, which reduces the non-selective exposure and improves the plasma half life [13–18], whereas the latter involves the use of nontoxic COX-2 inhibitors such as CUR (the well known anticancer phytochemical) in combination with 5-FU. The improvement in chemo efficacy in combinatorial treatment results from the CUR induced suppression of COX-2 expression, leading to improved effectiveness of 5-FU in conventional chemotherapy.

In addition, the nanoparticles are advantageous in terms of the ability of enhanced permeability and retention effect (EPR) which helps in the accumulation of drug loaded NPs in the tumor tissues in comparison with normal tissues [19,20]. There has been a reported study for the potential enhancement of 5-FU anticancer efficacy with CUR/hexahydrocurcumin under *in vitro* [21–23] and *in vivo* [24] conditions by the inhibition of COX-2 gene/proteins. In another work, the synergistic growth inhibitory effects of CUR with 5-FU were proven in gastric carcinoma cells [25]. Even though the *in vitro* and *in vivo* anticancer effects of hexahydrocurcumin with 5-FU have been proven, the major challenge is the low bioavailability of CUR/hexahydrocurcumin [26,27]. The technique of nanoencapsulation has been widely explored to improve the bioavailability of CUR [28–31] as well.

Hence 5-FU and CUR were individually entrapped in a biodegradable, and biocompatible chemically modified chitosan based nanoparticles (NPs); *N*,*O*-carboxymethyl chitosan (*N*,*O*-CMC) [32–34]. The developed systems, 5-FU loaded *N*,*O*-CMC NPs (5-FU-*N*,*O*-CMC NPs) and CUR-loaded *N*,*O*-CMC NPs (CUR-*N*,*O*-CMC NPs) were characterized for its *in vitro* hemocompatibility, drug release profile, cellular internalization, and *in vitro* combinatorial anticancer effects (in HT 29 cells by MTT, live dead, mitochondrial membrane potential and propidium iodide staining; cell cycle analysis). In addition the plasma concentration time profile of the developed NPs was evaluated by pharmacokinetics in Swiss Albino mouse model up to 72 h. The resulting animals were euthanized and analyzed for biodistribution and histopathological assessment.

2. Materials and methods

2.1. Materials

Chitosan (Mw: 100–150 kDa and DDA – 80%) was obtained from Koyo Chemical Co. Ltd., Japan, *N*,*O*-CMC (degree of carboxymethyl substitution – 57 ± 8%) was prepared based on the reported literatures [32], acetic acid and isopropanol from Merck, chloroacetic acid from Nice Chemicals, 5-FU, CUR, BSA, pentasodium tripolyphospahte (TPP), dialysis tubings (Mw cut-off 12 kDa), triton X-100, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], xylene, paraffin wax, hematoxylene, eosin, rhodamine 123, propidium iodide, and RNase were purchased from Sigma Aldrich. Human colon adenocarcinoma (HT 29) and mouse intestinal epithelial cells (IEC 6) were purchased from NCCS Pune, India. Live dead and mitochondrial membrane potential assay kits (JC-1) were purchased from Invitrogen. All other chemicals used are of analytical grade and used without further purification.

2.2. Experimental

2.2.1. Synthesis of 5-FU loaded N,O-CMC nanoparticles (5-FU-N,O-CMC NPs) and CUR loaded N,O-CMC nanoparticles (CUR-N,O-CMC NPs)

The methodology of 5-FU-*N*,*O*-CMC NPs and CUR-*N*,*O*-CMC NPs synthesis was based on our own reported methods [33,34] in which slight modification was carried out to increase the redispersibility of the NPs. For the 5-FU-*N*,*O*-CMC NPs, methanolic 5-FU solution (1 mg/mL) was incubated with *N*,*O*-CMC solution in water, (22.5 mg of *N*,*O*-CMC and 10 mg of drug) for overnight followed by TPP cross-linking (1%) for a volume ratio of 45: 1(*N*,*O*-CMC: TPP), followed by incubating with 0.1 mL of 1% BSA solution for 30 min. The methodology of CUR-*N*,*O*-CMC NPs synthesis [33] was repeated in the same way as explained above. CUR incorporated *N*,*O*-CMC solution (22.5 mg of *N*,*O*-CMC, and 5 mg of drug) was cross-linked with TPP (1%) followed by BSA incubation (0.1 mL, 1%) for 30 min. The drug loaded nanoparticles of 5-FU-*N*,*O*-CMC NPs and CUR-*N*,*O*-CMC NPs were separated by centrifugation (15,000 rpm for 30 min). The pellets were collected and used for further characterization and studies.

2.3. Entrapment efficiency (EE) and loading efficiency (LE)

The EE and LE of NPs sample was quantified spectrophotometrically. The protocol involves the complete extraction of 5-FU/CUR from the nanoparticle pellet through methanol/ethanol followed by spectrophotometric quantification. The EE and LE were calculated with respect to the amount of entrapped drug and the nanoparticle yield respectively. The EE and LE values were calculated using the following equations (Eqs. (1) and (2)) as reported earlier [33,34].

$$\mathrm{EE}\ (\%) = \frac{\text{Total amount of CUR/5-FU present in the pellet}}{\text{Initial amount of 5-FU/CUR used for drug loading}} \times 100 \quad (1)$$

$$\mathrm{LE}\ (\%) = \frac{\text{Amount of CUR/5-FU within the pellet}}{\text{Yield of nanoparticles}} \times 100 \quad (2)$$

2.4. Characterizations of 5-FU-N,O-CMC NPs and CUR-N,O-CMC NPs by DLS, SEM, zeta potential and FT-IR

Particle size and surface morphology of the 5-FU-*N*,*O*-CMC NPs and CUR-*N*,*O*-CMC NPs was analyzed using DLS (DLS-ZP/Particle Sizer Nicomp™ 380 ZLS) and SEM (JEOLJSM-6490LA). The colloidal stability and surface charge of the developed nanoparticles were analyzed using zeta potential measurements (Zeta Sizer (DLS-ZP/Particle Sizer Nicomp™ 380 ZLS). The potential chemical interaction between the drugs, TPP and carrier *N*,*O*-CMC was analyzed by Fourier transform infrared spectroscopy, FT-IR (Perkin Elmer Spectrum RXI Fourier Transform Infrared spectrophotometer).

2.5. In vitro drug release profile

The *in vitro* release profile of 5-FU/CUR from 5-FU-*N*,*O*-CMC NPs and CUR-*N*,*O*-CMC NPs was studied using the reported protocols [33–35] through dialysis and Eppendorf method respectively for 5-FU [34,35] and CUR [33]. The experiments were carried out in phosphate buffered saline (PBS) of pH 4.5 and 7.4 at 37 °C. Both release studies were performed by maintaining the sink conditions. For the 5-FU release studies, the 5-FU-*N*,*O*-CMC NPs (12 mg of 5-FU-*N*,*O*-CMC NPs containing 4 mg of entrapped 5-FU) were dispersed in 3 mL of water, transferred to dialysis tubes, and kept in a vessel containing PBS (Volume of dialysate: 60 mL) of pH 4.5 and 7.4. The whole system was incubated at 37 °C under shaking. At fixed time intervals, 0.75 mL of release media was taken out and replenished with fresh PBS. The collected PBS at each time point was analyzed for 5-FU quantification by an absorption spectrophotometer at 262 nm.

The drug release experiments with the CUR-*N*,*O*-CMC NPs were carried out using dialysis method in the initial phase of the research work. But the results were inconsistent and no reproducibility was observed. This could be due to the hydrophobicity of

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