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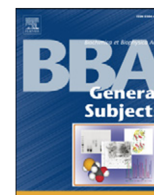

Combinatorial anticancer effects of curcumin and 5-fluorouracil loaded thiolated chitosan nanoparticles towards colon cancer treatment

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ABSTRACT

Background: Evaluation of the combinatorial anticancer effects of curcumin/5-fluorouracil loaded thiolated chitosan nanoparticles (CRC-TCS-NPs/5-FU-TCS-NPs) on colon cancer cells and the analysis of pharmacokinetics and biodistribution of CRC-TCS-NPs/5-FU-TCS-NPs in a mouse model.
Methods: CRC-TCS-NPs/5-FU-TCS-NPs were developed by ionic cross-linking. The in vitro combinatorial anticancer effect of the nanomedicine was proven by different assays. Further the pharmacokinetics and biodistribution analyses were performed in Swiss Albino mouse using HPLC.
Results: The 5-FU-TCS-NPs (size: 150 ± 40 nm, zeta potential: +48.2 ± 5 mV) and CRC-TCS-NPs (size: 150 ± 20 nm, zeta potential: +35.7 ± 3 mV) were proven to be compatible with blood. The in vitro drug release studies at pH 4.5 and 7.4 showed a sustained release profile over a period of 4 days, where both the systems exhibited a higher release in acidic pH. The in vitro combinatorial anticancer effects in colon cancer (HT29) cells using MTT, live/dead, mitochondrial membrane potential and cell cycle analysis measurements confirmed the enhanced anticancer effects (2.5 to 3 fold). The pharmacokinetic studies confirmed the improved plasma concentrations of 5-FU and CRC up to 72 h, unlike bare CRC and 5-FU.
Conclusions: To conclude, the combination of 5-FU-TCS-NPs and CRC-TCS-NPs showed enhanced anticancer effects on colon cancer cells in vitro and improved the bioavailability of the drugs in vivo.
General significance: The enhanced anticancer effects of combinatorial nanomedicine are advantageous in terms of reduction in the dosage of 5-FU, thereby improving the chemotherapeutic efficacy and patient compliance of colorectal cancer cases.

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1. Introduction

Colorectal cancer constitutes about 40% of cancers diagnosed yearly and is the third most leading cause of mortality among cancer patients (lung, female breast, colorectal and stomach cancers) [1,2]. 5-FU, a chemo drug used against colon cancer acts by inhibiting the S phase of the cell cycle, thereby blocking the synthesis of DNA, and triggering cell death [3–9]. Chemotherapy using 5-FU has a major limitation in relation to non-specificity [10,11] and moreover 5-FU is resistant to colon cancer [12]. Literatures suggest that the effectiveness of 5-FU as a chemo drug in colon cancer treatment could be improved by combinatorial approach, wherein either conventional chemo drugs [2] or other nontoxic plant derived materials such as genistein [13,14], geraniol [13,14], and curcumin, CRC, [15,16] were used. The combinatorial approach with CRC makes it more important because of its well proven anticancer potential towards many cancers [16–19] and its safety in preclinical/clinical trials [20]. In colon cancer, CRC induces anticancer effects through multiple mechanisms [21].

Even though CRC has wide potential as a chemotherapeutic agent, the usage is limited due to its low bioavailability [22] which is a result of rapid metabolism [22] and the inactive metabolites [22]. Bioavailability of CRC could be enhanced either by absorption enhancers such as piperine [23] or encapsulating the drug CRC in nano, as well as microparticle system made from different polymeric carrier systems [23–27]. The combinatorial anticancer effects of 5-FU with CRC has been proven, the challenges being toxicity and low bioavailability

Abbreviations: 5-FU, 5-fluorouracil; CRC, curcumin; TCS, thiolated chitosan; FT-IR, Fourier transform infrared spectroscopy; DDA, degree of deacetylation; DLS, dynamic light scattering; PBS, phosphate buffered saline; SEM, scanning electron microscope; FBS, fetal bovine serum; RPM I, Roswell Park Memorial Institute medium; HPLC, high pressure liquid chromatography; EPR, enhanced permeability and retention effect; NPs, nanoparticles; BSA, bovine serum albumin; 5-FU-TCS-NPs, 5-fluorouracil loaded thiolated chitosan nanoparticles; CRC-TCS-NPs, curcumin loaded thiolated chitosan nanoparticles; MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]; Rh 123, rhodamine 123; PI, propidium iodide; RNase, ribonuclease; Hb, hemoglobin; PT, prothrombin time; aPTT, activated partial thromboplastin time; PPP, platelet poor plasma; Mw, molecular weight; TPP, pentasodium tripolyphosphate; CO_2, carbon dioxide; EDTA, ethylene diamine tetra acetic acid; OD, optical density; JC-1, 5,5′,6,6′-tetraethylbenzidazolylcarbocyanine iodide; AUC, area under the curve; ACD, acid citrate dextrose; H and E, Harris's Hematoxylin and Eosin; FdUMP, fluorodeoxyuridine monophosphate; FdUTP, fluorodeoxyuridine triphosphate; FUTP, fluorouridine triphosphate; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; COX-2, cyclooxygenase-2; pH, potenz hydrogen; HT29, human colon adenocarcinoma; IEC 6, mouse intestinal epithelial cells

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respectively. Nanoencapsulation technique is widely used for entrapping diverse drugs, and in a way advantageous as it increases the bioavailability of CRC and 5-FU [5,25,28]. In addition, the nanosized particles help in more tumor accumulation because of their enhanced permeability and retention effect (EPR) [25]. There have been several reports suggesting the potential of CRC as a suitable replacement for genistein and geraniol and it was shown to promote the efficacy of 5-FU in various cancers including colon cancer [14,28]. The augmented combinatorial antitumorigenic potential of CRC with 5-FU towards colon cancer has already been reported [13,14,28]. The mechanism of combinatorial effect involves the inhibition of cyclooxygenase-2 (COX-2) in the mRNA and protein levels, which is overexpressed in many kinds of colon cancers [13,14,28].

The current thesis work focuses on an effective strategy to improve the efficacy of 5-FU assisted chemotherapy against colon cancer. This has been addressed by combinatorial approach in which CRC was used in combination with 5-FU. The potential of both drugs was improved by nanoencapsulation, wherein a non-toxic polymeric carrier system; 'thiolated chitosan' (TCS) [29,30] was used. Thus the developed 5-FU-loaded-thiolated chitosan nanoparticles (5-FU-TCS-NPs) and CRC-loaded-thiolated chitosan nanoparticles (CRC-TCS-NPs) were characterized, and evaluated for their in vitro combinatorial anticancer effects and in vivo plasma concentration–time profile.

2. Materials and methods

2.1. Materials

Chitosan (Mw: 100–150 kDa and DDA—80%) was purchased from Koyo Chemical Co Ltd., Japan, TCS (degree of thiol substitution: 60 ± 6%) was prepared based on the reported literatures [29,30]. 5-FU, CRC, BSA, pentasodium tripolyphosphate (TPP), dialysis tubings (Mw cut-off 12 kDa), Triton X-100, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], rhodamine 123 (Rh 123), propidium iodide (PI) and RNase were purchased from Sigma Aldrich. Colon cancer (HT29) and mouse intestinal epithelial cells (IEC 6) were purchased from NCCS Pune, India. A mitochondrial membrane potential assay kit was purchased from Invitrogen and all other chemicals used are of analytical grade and used without further purification.

2.2. Synthesis of 5-FU-TCS-NPs

5-FU was dissolved in methanol (1 mg/ml) and it was incubated with TCS solution, 45 ml (22.5 mg of TCS and 5 mg of 5-FU) overnight. Further the drug incubated TCS samples were cross-linked with TPP (1%) solution for a volume ratio of (TCS:TPP, 45:1), followed by incubating with 0.1 ml of 1% BSA solution for 30 min. The drug loaded-nanoparticles were separated from the suspension by centrifugation at 15,000 rpm for 30 min. The supernatant was discarded and the pellet was redispersed in saline and it was used for further characterization and studies.

2.3. Synthesis of CRC-TCS-NPs

CRC incorporated TCS solutions [CRC in ethanol, 1 mg/ml for a ratio of 45 ml (22.5 mg of TCS, 5 mg of CRC)] was cross-linked with TPP (1%) followed by incubating with 0.1 ml of 1% BSA solution for 30 min. The drug loaded-nanoparticles were separated by centrifugation (15,000 rpm for 30 min). The resulting supernatant and pellet were collected and were used for further characterization and studies.

2.4. Labeling of Rh 123 to CRC-TCS-NPs and 5-FU-TCS-NPs

Rh 123 was labeled to the CRC-TCS-NPs and 5-FU-TCS-NPs using ionic interaction and physical adsorption. The redispersed nanoparticle pellet of 5-FU-TCS-NPs and CRC-TCS-NPs was separately incubated with Rh 123 (5 mg/ml, 0.5 ml) overnight and stirred in the dark. The resulting Rh 123 labeled nanoparticles were centrifuged (15,000 rpm for 15 min), and washed (3 times with water). The washed pellet was redispersed in saline and was used for cellular uptake studies.

2.5. Entrapment efficiency (EE) and loading efficiency (LE) of 5-FU-TCS NPs and CRC-TCS-NPs

The EE and LE of the 5-FU-TCS-NPs/CRC-TCS-NPs were quantified using spectrophotometry [31,32]. For this, the drug loaded-nanoparticle pellets of 5-FU-TCS-NPs and CRC-TCS-NPs were separated from the nanoparticle suspension by centrifugation (15,000 rpm for 30 min), lyophilized, and weighed. The lyophilized drug loaded-nanoparticles of 5-FU-TCS-NPs and CRC-TCS-NPs were used for extracting the entrapped 5-FU and CRC. The extraction procedure is explained below. For the CRC-TCS-NPs, the lyophilized pellets were treated with ethanol to completely redissolve the entrapped CRC (vortexing, and probe sonication) and centrifuged. The yellowish supernatant was collected, and the absorbance value was measured using a spectrophotometer at 429 nm. The corresponding drug concentration was calculated from the calibration graph of CRC. Similarly for the 5-FU-TCS-NPs, the lyophilized 5-FU-TCS-NP pellet was treated with methanol to completely redissolve the entrapped 5-FU (vortexing, and probe sonication), centrifuged, and the supernatant was collected. The absorbance value was measured using a UV–vis spectrophotometer at 262 nm. The corresponding drug concentration was calculated from the calibration graphs of 5-FU. The EE and LE of the 5-FU-TCS-NPs/CRC-TCS-NPs can be calculated using the following equations:

$$\mathrm{EE}(\%) = \frac{\text{Amount of entrapped drug(CRC or FU)present in the pellet}}{\text{Initial amount of 5-FU or CRC used for the drug encapsulation}} \times 100$$

$$\mathrm{LE}(\%) = \frac{\text{Amount of entrapped drug(CRC or FU)present in the pellet}}{\text{Weight of the lyophilized CRC-TCS-NPs/5-FU-TCS-NPs(yield)}} \times 100.$$

2.6. Characterizations of 5-FU-TCS-NPs and CRC-TCS-NPs using DLS, SEM, zeta potential and FT-IR

The particle size of the developed nanoparticle was measured using DLS (DLS-ZP/Particle Sizer Nicomp™ 380 ZLS) and SEM (JEOLJSM-6490LA). Stability was analyzed using zeta potential measurements (Zeta sizer, DLS-ZP/Particle Sizer Nicomp™ 380 ZLS). Fourier transform infrared spectroscopy (FT-IR) was used to analyze the potential chemical interaction between the TCS and the drugs (Perkin Elmer Spectrum RXI Fourier Transform Infrared spectrophotometer).

2.7. Drug release studies

The in vitro release profile of 5-FU from 5-FU-TCS-NPs and CRC from CRC-TCS-NPs was carried out based on the reported protocols [31,32] using dialysis, for 5-FU [32] and the Eppendorf method for CRC [31] and the studies were carried out at pH 4.5 and 7.4 at 37 °C under sink conditions [31]. The protocol for 5-FU release is explained as follows. The drug loaded-nanoparticle pellet was redispersed in 3 ml water, mixed homogeneously and transferred to dialysis tubes. The dialysis tubing containing nanoparticle sample was kept in a beaker containing phosphate buffered saline (PBS) at pH 7.4 and 4.5 separately. The resulting system was incubated in a water bath shaker at 37 °C under shaking. At predetermined time intervals, 0.75 ml of release media was taken from the beaker and replenished with fresh PBS. The released drug in PBS was measured using a spectrophotometer at 262 nm. This was continued for 4 days and the released drug at each time point was quantified using calibration graphs.

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