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Synergism of co-delivered nanosized antioxidants displayed enhanced anticancer efficacy in human colon cancer cell lines

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ABSTRACT

Combination of chemopreventive and/or therapeutic agents is the imminent smart approach to cope up with cancer because it may act on multiple targets through different pathways. In the present study, we have synthesized multiple chemopreventive and/or therapeutic agents (Curcumin, Quercetin and Aspirin) loaded nanoparticles by simple cation-anion interaction among the amine groups of chitosan (CS) and phosphate groups of sodium hexametaphosphate (SHMP). These nanosized bioactive materials (CS-SHMP-CQA-NPs) were well characterized and found most effective in colon cancer cell line (HCT-116) compared to other cancer cell lines. Triplex chemopreventive and/or therapeutic agents-loaded NPs were synergistically inducing apoptosis in HCT-116 cells compared to two-chemopreventive agents-loaded NPs as evident by an increase in sub-G_1 cells (percent), and chromatin condensation along with the decrease in mitochondrial membrane potential (MMP). Interestingly, Chou–Talalay analysis revealed that CS-SHMP-CQA-NPs showed strong synergistic effect in its all doses. Thus, our study demonstrates that nanoparticles based bioactive materials significantly inhibit the growth of HCT-116 cells and thus could be a promising approach for colon cancer chemoprevention.

1. Introduction

Cancer is the most challenging and complicated disease nowadays [1–4]. Among all cancers, colorectal cancer is one of the main cause of death worldwide and second foremost cause of death in United States that affects both men and women [5–7]. Although the recent improvement of diagnostic equipments and therapeutic technologies has been able to reduce the mortality [8,9], the treatment of colorectal carcinoma remains complicated [10] due to undesired side effects, such as nephrotoxicity, ototoxicity [11] etc. from traditional chemotherapeutic agents. Therefore, there is an urgent need to search for chemopreventive agents [12–14] that would impede cancer initiation and progression [15]. However, given the complex etiology [16] of cancer, an individual chemopreventive agent may not be sufficient to avert cancer [17]. Therefore, combination of chemopreventive agents targeting multiple pathways is required to achieve enhanced therapeutic effect. In addition, the selection of chemopreventive agents should be based on their high therapeutic index, less side effects and ease of administration.

Curcumin (Cur) and quercetin (Quer) are well known chemopreventive agents and exhibit anticancer activity by [18,19] modulating numerous intracellular signaling pathways associated with inflammation, cellular growth, invasion, mutagenesis, oncogene expression, cell cycle alteration, and apoptosis [20–26]. Cur also prevents chemically induced carcinogenesis in colon [27,28], forestomach [27] and skin [29,30] without any noticeable side effects [31,32]. Interesting to note that Cur is in clinical trial (Phase IIa) for the prevention of colorectal adenomas [33]. Quer also exhibits cancer cell specific anti-proliferative and apoptosis inducing effect leaving normal cells unaffected [34]. Mouat et al. [35] reported that Quer exerts chemoprotective action towards colon cancer cells (SW480). Advantageously, combination of Cur and Quer induces programmed cell death, such as apoptosis, when DNA damage cannot be successfully repaired [36]. On the other hand, aspirin (Acetylsalicylic acid), a member under the classification of

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nonsteroidal anti-inflammatory drugs (NSAIDS), is known to reduce cancer cell progression [37–39] by generating several anti-inflammatory cytokines [40–42]. Recently, found the fact that NSAID drugs, mostly the cyclooxygenase (COX)-2 inhibitors, are the promising candidate for cancer prevention [40,43,44]. In case of growth and invasion of colon cancers, the concentration of prostaglandins, particularly the E_2 series (PGE_2), cyclooxygenase-2 (Cox-2), HGF receptor (c-Met-R), beta-catenin and epidermal growth factor receptor (EGFR) are increased [45]. However, so far, mixed reports are available regarding the use of aspirin (Asp) in colon cancer as a chemopreventive agent [46,47]. Thus, it is important to study the chemopreventive activities of Asp in colon cancer cells in detail.

Therefore, keeping all these in mind that Cur, Quer and Asp have anticancer activities with least side effects and different targets, we questioned whether the combination of Cur, Quer and Asp, could demonstrate synergistic chemopreventive and/or therapeutic effects in colon cancer cell lines. To the best our knowledge, till date, no group has reported the combinatorial anticancer effect of Cur, Quer and Asp in colon cancer cells. However, the therapeutic efficacy of Cur as chemopreventive agent, either single or in combination, is jeopardized by its poor bioavailability which may be overcome by making it nanotized [48,49]. In addition, nanotization of triplex chemotherapeutic agents would improve circulation time through the enhanced permeability and retention (EPR) effect [50,51].

Chitosan (CS), a highly biocompatible and biodegradable matrix [52] was chosen as a nanocarrier to deliver the nanoparticles of triplex chemopreventive and/or therapeutic agents. CS has large number of free amine groups which were cross linked with sodium hexametaphosphate (SHMP) [53] to built CS-SHMP nanoconjugate through electrostatic interactions. Overall, all the three chemopreventive and/or therapeutic agents were entrapped inside the CS-SHMP nanoconjugate which formed self-assembled nanoparticles (CS-SHMP-CQA-NPs).

Herein, we demonstrate that co-delivery of Cur, Quer and Asp loaded chitosan-SHMP nanoconjugate exhibited synergistic chemopreventive and/or therapeutic effects through apoptosis in colon cancer cell lines (HCT-116). Although, Cur and Quer combination have been studied as a chemopreventive agent in gastric cancer (MGC-803) cells [36] but chemopreventive efficacy of the combination of Cur, Quer and Asp in encapsulated form is not reported hitherto.

2. Materials and methods

2.1. Chemicals

All the specified chemicals and reagents viz., chitosan (75% deacetylated), sodium hexametaphosphate (SHMP), curcumin (Cur), quercetin (Quer) and aspirin (Asp) were purchased from Sigma (Sigma St Louis, MO, USA) unless otherwise stated. Culture media, fetal bovine serum (FBS), antibiotics-antimycotic solution and trypsin-EDTA were purchased from Gibco BRL, USA. Plastic wares and culture wares used in the study were procured commercially from Nunc, Denmark. Milli Q water (deionized water, double distilled) was used in all the experiments.

2.2. Cell culture

Cell lines used in the study viz., human epidermoid carcinoma (A-431), human breast carcinoma (MCF-7), human colon carcinoma (HCT-116) and immortalized human keratinocyte (HaCaT) cell lines were initially procured from National Centre for Cell Sciences, Pune, India and since then have been maintained at, CSIR-Indian Institute of Toxicology Research, Lucknow, India, following the standard protocols.

2.3. Preparation of curcumin, quercetin and aspirin loaded chitosan nanoparticles (CS-SHMP-CQA-NPs)(*2*)

Initially, CS was dissolved in acetic acid to give a final concentration of 1 mg/mL. The CS solution was adjusted to pH 5 with 2 M NaOH. Drug (Cur:Quer:Asp) ratios (w/w) were kept at 1:1:3.5. The drug ratios have been chosen by following Zhang et al. [36] (Cur and Quer ratio) and Zhou et al. [54] (Cur and Asp ratio) reports to obtain maximum therapeutic index and synergism. Cur and Quer were dissolved in ethanol (50 mL), each separately and Asp was dissolved in water (20 mL). Solution of Cur and Quer in ethanol was added drop wise into the chitosan solution, followed by Asp in water solution. After that, SHMP solution (0.133% w/v in purified water) [53] was added drop-wise into the chitosan solutions under constant magnetic stirring as the nanoparticles formed spontaneously. The above solution was stirred for 15 h at 25 ± 2 °C. Subsequently, the resulting solution was dialyzed against double distilled water with stirring for 24 h with the water being changed at least 6 times to remove unwanted materials. It has been found that 24 h was sufficient to remove unwanted materials in a controlled experiment. The dialyzed solution was lyophilized for 24 h to obtain yellow solid [CS-SHMP-CQA (**2**)]. Nanoparticles were stored at 4 °C under anhydrous conditions in dark till use.

Similarly, two-drug-loaded nanoparticles [Cur and Quer entrapped NP, CS-SHMP-CQ (**1a**); Quer and Asp entrapped NP, CS-SHMP-QA (**1b**); Cur and Asp entrapped NP, CS-SHMP-CA (**1c**)] were also prepared without altering drug ratios.

2.4. Characterization of the NPs

2.4.1. Percent yield

After achieving the constant weight, yield (%) of NPs was calculated by using the following formula:

$$\text{Yield } (\%) = \frac{\text{Weight of nanoparticle}}{\text{Weight of (drug + polymer)}} \times 100$$

2.4.2. Particle size measurement

The mean particle size and the polydispersity index (PDI) of **1a**, **1b**, **1c** and **2** were determined by dynamic light scattering (DLS) technique employing a nominal 5 mW He-Ne laser operating at 633 nm wavelength. The freeze dried NP was dispersed in aqueous buffer and the size was measured. The measurements were carried out at 25 °C with the following settings: 10 measurements per sample; refractive indices of CS, 1.523; viscosity of water, 0.89 cP. The particle size was measured in triplicate.

2.5. Drug loading and entrapment efficiency

The drug loading and encapsulation efficiency were determined by analyzing the NPs spectrophotometrically using Lambda Bio 20 UV/VIS Spectrophotometer (Perkin Elmer, USA). The amount of Cur, Quer and Asp present in the nanoparticles was estimated as follows: a known amount of NPs (1 mg/mL) was dispersed in the mixture of double distilled water: ethanol (1:1) solution by stirring the sample vigorously and the absorbance of the solution was measured at 265, 373 and 426 nm for Asp, Quer and Cur, respectively, and the amount of drug present was calculated from a previously drawn calibration curve of concentration *vs.* absorbance with different known concentrations of the drugs. The percent drug