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Inhalable bioresponsive chitosan microspheres of doxorubicin and soluble curcumin augmented drug delivery in lung cancer cells

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ABSTRACT

In present investigation, doxorubicin (Dox) and soluble curcumin (Cur-2-HP-β-CD-complex) combination was simultaneously loaded in inhalable bioresponsive chitosan microspheres (Dox/Cur-2-HP-β-CD-complex-elastin-CMs) bearing a substrate-stimuli, elastin. The mean particle size and mean aerodynamic diameter of inhalable bioresponsive microspheres displayed noteworthy differences after incorporation of elastin. Moreover, combination of Dox and soluble curcumin was molecularly dispersed in microspheres matrix as substantiated by a range of spectral techniques. Inhalable bioresponsive microspheres released astonishingly higher amount of Dox in presence of elastase enzyme at pH ~ 5.5 in comparison to pH ~ 7.4. However, the release of soluble curcumin from tailored bioresponsive microspheres in presence of elastase enzyme was independent of pH. Consistently, inhalable bioresponsive microspheres exhibited outstandingly lower IC_{50} of 3.4-μM in comparison to 6.5-μM of inhalable drug loaded microspheres (Dox/Cur-2-HP-β-CD-complex-CMs) bearing no elastin, against A549, non-small cell lung cancer cells. The superior therapeutic profile of inhalable bioresponsive microspheres may be attributed to enhanced drug release and consequently augmented drug exposure to A549 cells expressing elastase enzyme. In this way, stimuli triggered drug release from tailored inhalable bioresponsive microspheres boosted the phenomena of apoptosis in A549 cells. In conclusion, Dox/Cur-2-HP-β-CD-complex-elastin-CMs warrant further *in-vivo* tumor regression study to prove its therapeutic efficacy.

1. Introduction

Non-small-cell lung cancer (NSCLC) accounts for 75–80% of all lung cancers patients. More than 50% of the patients with advanced NSCLC receive chemotherapy which is often unsuccessful [1–3]. US-FDA has recommended cisplatin/docetaxel for the first-line treatment of advanced NSCLC [4]. Moreover, injectable doxorubicin (Dox) has also been approved by US-FDA for the treatment of NSCLC [5]. However, cardiotoxicity, erratic biodistribution, and drug resistance in addition to short initial plasma half-life (5 min) of Dox hinder the clinical efficacy in NSCLC [6–8]. The cardiotoxicity of Dox may be acclaimed to oxidative stress, like increment in reactive oxygen species (ROS) and lipid peroxidation. In addition, reduced level of protective antioxidants and sulfhydryl groups, inhibition of DNA and protein synthesis, release of vasoactive amines, impaired adrenergic function and decreased expression of cardiac-specific genes also boost the cardiotoxic effect of Dox [9]. This ultimately enhances the frequency of drug administration for achieving the desired clinical goals.

Recently, Dox in combination with curcumin (Cur) exhibited the superior therapeutic efficacy in NSCLC as compared to Dox alone [10]. More interestingly, Cur potentially enhanced the antitumor activity of Dox under both *in vitro* and *in vivo* settings through its ability to suppress NF-kβ and NF-kβ regulated antiapoptotic gene products (Bcl-2 and Bcl-xL) [11,12]. Though, Cur possesses tremendous therapeutic and safety profile; however poor aqueous solubility (0.6 mg/ml) and low oral bioavailability (>1%) deliver a suboptimal therapeutic concentration at the target site [13,14].

Pulmonary drug delivery through inhalation route of administration tenders several merits in comparison to parenteral and

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oral delivery. Stimuli triggered drug release, zero-order release pattern, low dose-dosage regimen, induction of negligible side-effects, quick onset of action, avoidance of hepatic first pass metabolism and high therapeutic bioavailability at the site of action are the lucrative advantages of inhalable drug delivery systems [15,16]. Furthermore, large alveolar surface area (100 m^2), thin absorption membrane (0.1–0.2 μm), and elevated blood flow (5 l/min) make the pulmonary route ideal for administration of chemotherapeutic drugs [17]. In this way, inhalable nanomedicines intended for the management of NSCLC has already gained clinical attention [18–20].

Therefore, to address the physicochemical and biopharmaceutical concerns of Dox and Cur combination for effortless administration through inhalation route of administration, we have designed and optimized Dox and Cur-2-HP-β-CD-complex combination loaded inhalable, bioresponsive chitosan microspheres (Dox-Cur-2-HP-β-CD-complex-elastin-CMs) bearing substrate, elastin. Cur was made soluble in aqueous phase by complexing with 2-HP-β-CD (Cur-2-HP-β-CD-complex), a supramolecular moiety extensively investigated for enhancing the solubility of poorly soluble drugs [21]. On the other hand, chitosan is a biocompatible, bioadhesive and water-soluble polymer [22–25] as well as majorly investigated for delivery of chemotherapeutic drugs through oral [26], parenteral [27] and inhalation route of administration [28]. Next, the function of human neutrophil elastase enzyme is recently identified in NSCLC progression [29]. Moreover, A549, non-small cell lung cancer cells over-express the protein, elastase [30]. Hence, elastin, a substrate for elastase enzyme [31] can be used as a stimulus for triggering the drug release from inhalable bioresponsive particulate carriers.

Therefore, in present investigation, the processing conditions for the preparation of Dox/Cur-2-HP-β-CD-complex-elastin-CMs were optimized by Central Composite Design, CCD [32] and Response Surface Methodology, RSM [33]. The optimized conditions were further used in emulsion polymerization method [34] for the preparation of chitosan microspheres. Microspheres were tested *in vitro* for particle size, zeta-potential, aerodynamic size, surface morphology, encapsulation efficiency, drug loading capacity and dissolution testing in simulated lungs fluid (SLF, pH ~ 7.4 and ~5.5) in presence of elastase enzyme. The therapeutic potential of tailored microspheres was investigated against A549, non-small cell lung cancer cells by employing standard cell proliferation assay [35].

2. Material and methods

2.1. Materials

Doxorubicin (Dox) was obtained as a gift sample from Fresenius Kabi Oncology Limited, Himachal Pradesh, India. Elastin substrate and elastase enzyme (Porcine pancreas) were purchased from Sigma-Aldrich, USA. Chitosan, a linear polysaccharide of deacetylated β-1,4-D-glucosamine (M_W ~ 1,50,000 Da, 75–85% Deacetylated) was acquired from Himedia Limited, Mumbai, India. Light liquid paraffin, heavy liquid paraffin, glutaraldehyde (25% v/v aqueous solution) and span 80 were obtained from Loba Chemie, Mumbai, India. Uppermost analytical grade chemicals were used without further purification.

2.2. Cell line and mediums

Human non-small cell lung cancer, A549 cell line was cultured in 95% air and 5% CO_2 at 37 °C using DMEM (Dulbecco's Modified Eagle's Medium) comprising 10% fetal bovine serum. All experiments were performed with asynchronous cell populations in exponential growth phase (24 h after plating) [36].

2.3. Optimization of processing conditions using quality by design (QbD)

The processing conditions in preparation of inhalable chitosan microspheres were optimized by CCD [32] to get RSM [33] that selected an object, noticed the contributing factors and investigated the relationship between responses and factors. CCD enabled several independent variables to be investigated at the same time using a relatively small number of experiments, while RSM optimization analyzed the interaction between variables. Therefore, CCD-RSM defined the interaction between factors, avoided unwanted experiments and optimized the results. Our preliminary investigations indicated that variables, such as chitosan concentration (% w/v), acetic acid volume (ml), amount of glutaraldehyde (ml) and mixing speed (rpm) were the main contributing factors that influenced the particle size of inhalable microspheres. A CCD model was used to statistically optimize the factors that affected the particle size of microspheres. For each factor, experimental range was selected on the basis of previous investigations and probability of preparing the microspheres at extreme values. The values of different variables were considered to be in following range: chitosan concentration (X_1): 2–4 w/v%, acetic acid (X_2): 0.5–1.5 ml, glutaraldehyde concentration (X_3): 10–14 ml and mixing speed (X_4): 2000–4000 rpm. The design consisted of 30 runs (16 factorial points, 8 axial points, and 6 centre points) that yielded total 30 experiments (Suppl. Table 1). The purpose of replication was to estimate the experimental errors and increase the accuracy. Each experimental run was repeated thrice (n = 3). Star points represented the extreme values (low and high) for each factor in the design and allowed estimation of second-order effects. Furthermore, star points were at some distance, alpha, from the centre, based on the properties desired for design and number of factors in the design. Alpha in coded units was the axial distance from the centre point and made the design rotatable. A rotatable design provided equally good predictions at points equally distant from the centre, a very desirable property for RSM [37]. A design matrix comprising 30 experimental runs (Suppl. Table 1) was constructed and responses were modeled by the following surface linear equation:

$$Y = 3.25917 + 0.52083 * \text{chitosan} - 0.31667 * \text{aceticacid} - 0.093750 * \text{glutaraldehyde} - 2.79167E\text{-}004 * \text{speed}$$

Where Y is the measured response associated with each factor level combinations. The statistical analysis was performed by using the software Design Expert (Version 9.0), where analysis of relationship between the response variable "Y" and the entire set of "X" variables at 95% level (ANOVA) was significant, when $P < 0.05$.

2.4. Preparation of inhalable bioresponsive microspheres

Inhalable bioresponsive microspheres, Dox/Cur-2-HP-β-CD-complex-elastin-CMs were prepared by emulsion polymerization method [34]. In brief, 3% w/v of chitosan dispersion comprising 60 mg of Dox, Cur-2-HP-β-CD-complex (~100 mg Cur) and 1 mg of elastin substrate was prepared in 10 ml of distilled water containing 1.25 ml of acetic acid. The oil phase was prepared by adding 75 ml of light liquid paraffin to 75 ml of heavy liquid paraffin that was admixed with 1.5 g of span 80. The oil phase was maintained at 70 °C. The emulsion was prepared by pouring the aqueous phase in oil phase in drop-wise fashion followed by stirring at 3000 rpm for 1 h. After stirring, a mixture of 12 ml glutaraldehyde saturated with 30 ml of toluene was added to the emulsion to cross-link the microspheres. The temperature was lowered down to 50 °C and stirred again for next 1 h (Scheme 1). In last, microspheres were washed with n-hexane and dried in a desiccator. Correspondingly,