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Soluble telmisartan bearing poly (ethylene glycol) conjugated chitosan nanoparticles augmented drug delivery, cytotoxicity, apoptosis and cellular uptake in human cervical cancer cells

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ABSTRACT

Soluble telmisartan and telmisartan were loaded in to poly (ethylene-glycol) grafted chitosan nanoparticles (S-TEL-PEG-CNPs and TEL-PEG-CNPs) for targeting cervical cancer through non-invasive, intravaginal route. The mean particle size of S-TEL-PEG-CNPs was measured to be 23.4 ± 5.9-nm significantly ($P < 0.05$) higher than 16.2 ± 3.2-nm of TEL-PEG-CNPs. In contrast, the zeta-potential (−21.5 ± 4.6-mV) of S-TEL-PEG-CNPs was insignificantly ($P > 0.05$) different from −23.8 ± 3.7-mV of TEL-PEG-CNPs. In addition, S-TEL-PEG-CNPs exhibited higher percent mucoadhesiveness (40.2%) in comparison ($P < 0.05$) to 31.4% of TEL-PEG-CNPs, although it was lower than CNPs (100%). S-TEL-PEG-CNPs displayed significantly ($P < 0.01$) higher dissolution of drug, 92.5% in comparison to 31.6% from TEL-PEG-CNPs up to 24 h. Furthermore, S-TEL-PEG-CNPs exhibited superior cytotoxicity, apoptosis and cellular uptake, analyzed in human cervical cancer, HeLa cells. The IC_{50} of S-TEL-PEG-CNPs was measured to be 22.3-μM significantly ($P < 0.05$) lower than 40.1-μM of TEL-PEG-CNPs. S-TEL-PEG-CNPs induced higher extent of apoptosis ($P < 0.05$) in HeLa cells as compared to TEL-PEG-CNPs, owing to higher diffusion of drug across biological membrane. Finally, quantitative and qualitative cellular uptake assay confirmed the greater endocytosis of S-TEL-PEG-CNPs in HeLa cells due to diffusion, amorphization, hydrophilicity, and submicron size particularly, below 100 nm. In conclusion, S-TEL-PEG-CNPs warrant further *in vivo* tumour regression study to scale up the technology for clinical translation.

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1. Introduction

Cervical cancer occurs due to the abnormal growth of cells lining in the cervix (the lower part of uterus that ends in vagina). Most of the cervical cancers are caused by a long lasting infection with a virus named as Human Papilloma Virus (HPV). Every year cervical cancer affects nearly 500,000 women globally and responsible for 280,000 deaths annually [1].

In August 2014, the US Food and Drug Administration (FDA) approved bevacizumab (Avastin) for the management of constant, intermittent or late-stage (metastatic) carcinoma of the cervix [2]. Bevacizumab was approved in combination with paclitaxel and cisplatin or with paclitaxel and topotecan for the treatment of cervical cancer. Recently several non-chemotherapeutic drugs have also been explored for their therapeutic efficacy against cancer [3]. Among them, angiotensin II receptor blockers (ARBs) have the potency to inhibit the progression of several types of cancer including prostate and lung cancer [4,5]. Telmisartan (TEL) is an ARB that has high affinity for angiotensin II receptor type 1 (AT_1). Moreover, TEL has 3000-folds greater specificity for AT_1 than AT_2 [6]. TEL also displays peroxisome proliferator-activated receptor (PPAR)-gamma activation activity and therefore alleviated the growth arrest of cancerous cells *via* apoptosis at the dose of 10 mg/kg through oral route of administration [7]. However, poor aqueous solubility (0.078 mg/mL), low oral bioavailability (>50%), and erratic biodistribution limit the scope of TEL in drug delivery to tumour tissues [8–10].

Several chemotherapeutic agents have been targeted to cervical cancer through vaginal route of administration [11]. Intravaginal administration is a beneficent alternative to oral and parenteral route in terms of avoidance of first pass metabolism and gastrointestinal interferences with maximal absorption of drugs. Moreover, intravaginal administration offers large permeation area, high residence time, affluent vascularization and low enzymatic activity [12].

However, transportation of drugs to the cervix through intravaginal route is a challenging target. Physiologically, cervical tumour resides beneath a sticky mucus layer that protects the cervix against infections. Unfortunately, these mucosal linings also trap and clear the drug-loaded nanoparticles [13,14]. Recent findings indicated that submicron vesicular systems rapidly penetrate cervicovaginal mucus linings and effectively deliver their payload to underlying tumour tissues in a uniform and sustained manner as compared to nanocarriers that do not penetrate efficiently [15,16].

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In this context, mucoadhesive drug delivery systems have been extensively investigated for delivery of drugs through vaginal route of administration [17]. Among them, colloidal (liposomes and niosomes) and particulate carriers (nanoparticles and microparticles) have been widely accepted through intravaginal route owing to biodegradability, low immunogenicity and non-toxic nature [18,19]. However, polymeric particulate carriers of submicron range (10–1000 nm) have demonstrated high stability and reproducibility in comparison to colloidal vesicular systems [20]. Chitosan is a deacetylated derivative of chitin, found largely in the exoskeleton of shell fish and insects [21]. The amino and carboxyl functional groups of chitosan furthermore display affinity for glycoprotein of mucus which eventually promotes the formation of hydrogen bond and induces an adhesive effect [22]. Consequently, chitosan and mucus interaction enhances the residence time as well as augments drug bioavailability at the site of action. In contrast, cervicovaginal mucus in vagina immobilizes the cationic or mucoadhesive drug delivery system and therefore; restricts the penetration of nanovesicles. Alternatively, vaginal drug delivery systems can be anchored with poly (ethylene glycol) (PEG) for neutralizing the cationic charge and thereby improving the penetration of nanoparticles across cervicovaginal mucus [23].

Previously, our group reported the synthesis of soluble nanocomplex of TEL with 2-HP-β-CD (TEL-2-HP-β-CD) and termed it as "soluble telmisartan (S-TEL)" for augmenting the dissolution and transport across biological membrane [24]. Therefore, in present investigation, we have constructed soluble telmisartan and telmisartan bearing poly (ethylene glycol) grafted chitosan nanoparticles (S-TEL-PEG-CNPs and TEL-PEG-CNPs) by ionic gelation method [25]. The tailored nanoformulations were characterized *in vitro* for particles size, zeta-potential, encapsulation efficiency, drug loading capacity, mucoadhesive property, incompatibility, drug release, cytotoxicity, apoptosis and cellular uptake in human cervical cancer, HeLa cells.

2. Materials and methods

2.1. Materials

Telmisartan (TEL, molecular weight ~ 514 Da, purity ~ 98%) was obtained as a gift sample from Glenmark Pharmaceuticals, India. Chitosan (M.W ~ 75,000–85,000 Da) and fluoresceine isothiocynate (Isomer II) were purchased from Himedia Limited, Mumbai, India. Methoxypoly (ethylene) glycol acetic acid (mPEG-CH_2COOH, ≥80%) was purchased from Sigma-Aldrich, USA. Tripolyphosphate (TPP) was procured from Loba Chemie, New Delhi, India. All other chemicals used were of highest analytical grade and used without further purification.

2.2. Cell culture

Human cervical cancer cell line (HeLa) and normal skin cells (HaCaT) were separately grown in 5% CO_2 and 95% air at 37 °C using Dulbecco's modified Eagle's medium (DMEM) (Biologicals, Israel) supplemented with 5% fetal calf serum. All experiments were performed with asynchronous populations in exponential growth phase (24 h after plating) [26].

2.3. Synthesis of poly (ethylene glycol) grafted chitosan

Acetic acid functionalized poly (ethylene glycol) was grafted on to the chitosan by covalent coupling technique [27]. In brief, 50 mg of mPEG-CH_2COOH was dissolved in 5 mL of phosphate buffer (pH ~ 4.9). Subsequently, 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDAC, 0.75% w/v), and *N*-hydroxy succinimide (NHS, 0.75% w/v) were added to the solution for activating the carboxylic acid terminal of mPEG-CH_2COOH. The stirring was continued for 4 h at room temperature. Finally, 5 mL phosphate buffer solution (pH ~ 4.9) of chitosan (50 mg) was added to the reaction mixture. The stirring was continued for next 24 h to complete the reaction. The product, mPEG-CH_2-CONH-Chitosan was subjected to dialysis to completely remove the impurities. The synthesis of mPEG-CH_2-CONH-Chitosan was confirmed by FT-IR spectroscopy. The spectrum of mPEG-CH_2-CONH-Chitosan, chitosan and mPEG-CH_2COOH was captured using spectrum BX (Perkin Elmer, Massachusetts, USA) infrared spectrophotometer. Each sample was prepared in KBr pellet, using a hydrostatic press at a force of 40 psi for 4 min. All samples were scanned thrice between 500 and 4400 cm^{-1} at a resolution of 4 cm^{-1}.

2.4. Determination of density of poly (ethylene glycol) on chitosan polymer

The amount of PEG grafted on to the chitosan polymer was determined quantitatively. In brief, 50 mg of chitosan or mPEG-CH_2-CONH-Chitosan was dispersed separately in 5 mL of deionised water. To this solution, 2 mL of the ninhydrin reagent was added and heated on a boiling water bath for 30 min. The tubes were then cooled below 30 °C in a cold water bath, followed by dilution with 3 mL of 50% (v/v) ethanol/water. The solutions were then vigorously stirred on a vortex mixer and filtered through 0.22 μm membrane filter (MDI, Ambala, India). Finally, the absorbance of the filtrate was measured at 570 nm using a UV–Visible spectrophotometer (1800, Shimadzu, Kyoto, Japan) [28].

2.5. Preparation of soluble telmisartan loaded poly (ethylene glycol) grafted chitosan nanoparticles

Soluble telmisartan loaded poly (ethylene glycol) grafted chitosan nanoparticles (S-TEL-PEG-CNPs) were prepared by ionic gelation method [25]. In brief, 60 mg of S-TEL [24] was added to 3 mL of 5% glacial acetic acid solution containing mPEG-CH_2-CONH-Chitosan. The mixture was stirred at 3000 rpm (Remi, Mumbai, India) for few min and 1.2 mL of 0.4% aqueous TPP solution was added to the mixture in drop-wise manner. Stirring was continued at 3000 rpm for 60 min. S-TEL-PEG-CNPs were spontaneously formed upon drop-wise addition of cross-linking agent at room temperature. The nanoparticles were separated by centrifugation (Sorvall Ultracentrifuge, Thermoscientific) at 30,000 rpm and 15 °C for 1 h, lyophilized (Lark Technology, India) and stored under refrigerated conditions. Correspondingly, TEL-PEG-CNPs were prepared by using 15 mg of TEL and 3 mL of 5% glacial acetic acid solution containing mPEG-CH_2-CONH-Chitosan under identical conditions to obtain the lyophilized powder of TEL-PEG-CNPs. Blank nanoparticles (PEG-CNPs and CNPs) were also prepared without incorporation of drug for comparative studies.

2.6. Labelling of fluorescent dye to nanoparticles

S-TEL-PEG-CNPs and TEL-PEG-CNPs were labelled with FITC using coupling chemistry [29]. In brief, a 5 mL of FITC solution (1 mg/mL in borate buffer pH ~ 8.0) was incubated with 100 mg quantity of each nanoparticle sample suspended in 5 mL of borate buffer (pH ~ 8.0). After 5 h of incubation period in dark at room temperature, the FITC-labelled S-TEL-PEG-CNPs and TEL-PEG-CNPs were separated from free FITC using dialysis membrane. To determine the labelling efficiency, FITC-labelled S-TEL-PEG-CNPs and TEL-PEG-CNPs were separately dissolved in 0.1 M acetic acid solution and diluted with PBS (pH ~ 7.4) to achieve a final concentration of 500 μg/mL. Percent labelling efficiency was calculated as the percent weight of FITC to the weight of FITC-labelled S-TEL-PEG-CNPs or TEL-PEG-CNPs. A calibration curve of FITC was prepared between 20 and 80 μg/mL in PBS (pH ~ 7.4).

2.7. Characterization of nanoparticles

2.7.1. Particle size and zeta-potential

Particle size distribution and zeta-potential of nanoparticles were measured by using a zeta-sizer (HAS 3000, Malvern Instruments, Worcestershire, UK). Briefly, 10 mg quantity of each sample was

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