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The augmented anticancer potential of AP9-cd loaded solid lipid nanoparticles in human leukemia Molt-4 cells and experimental tumor

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ABSTRACT

AP9-cd, a novel lignan composition from *Cedrus deodara* has significant anticancer potential, and to further enhance its activity, it was lucratively encumbered into solid lipid nanoparticles (SLNs). These nanoparticles were formulated by micro-emulsion technique with 70% drug trap competence. AP9-cd-SLNs were regular, solid, globular particles in the range of 100–200 nm, which were confirmed by electron microscopic studies. Moreover, AP9-cd-SLNs were found to be stable for up to six months in terms of color, particle size, zeta potential, drug content and entrapment. AP9-cd-SLNs have 30–50% higher cytotoxic and apoptotic potential than the AP9-cd alone. The augmented anticancer potential of AP9-cd-SLNs was observed in cytotoxic IC_{50} value, apoptosis signaling cascade and in Ehrlich ascites tumor (EAT) model. AP9-cd-SLNs induce apoptosis in Molt-4 cells via both intrinsic and extrinsic pathway. Moreover, the dummy nanoparticles (SLNs without AP9-cd) did not have any cytotoxic effect in cancer as well as in normal cells. Consequently, SLNs of AP9-cd significantly augment the apoptotic and antitumor potential of AP9-cd. The present study provides a podium for ornamental the remedial latent via novel delivery systems like solid lipid nanoparticles.

1. Introduction

During the last few years drug development focus has been shifted to the natural chemotherapeutic agents from plants, which can be further modified to enhance their potential with less side effects. Plants have been utilized as medicines for thousands of years and around 70% of anticancer drugs are either natural products or their derivatives [1,2]. Plant derived anticancer agents can be clinically categorized into four main classes: Vinca alkaloids, epipodophyllotoxins, taxanes, and camptothecin [3]. Podophyllotoxin derivatives and congeners have strong anticancer potential but their toxicities, resistance and poor bioavailability limit their clinical use [4]. Therefore, the discovery and development of novel podophyllotoxin analogues are needed to be explored. In that concern, a novel lignan, AP9-cd was isolated from dry waste wood powder of *Cedrus deodara* [5]. AP9-cd is a standardized lignan composition containing wikstromal, matairesinol and dibenzylbutyrolactol. It has significant cytotoxic, apoptotic and antitumor potential in different human cancer cell lines and tumor models [6,7]. AP9-cd seems to be a potent anticancer leads and further study will require on how to improve its efficacy.

Discovery of a new drug alone is not sufficient in its clinical development because most of the new drugs follow poor *in vivo* results. Several anticancer drugs have such pattern due to poor tumor site distribution, toxicity, drug-resistant, efflux, permeation barriers and instability [8–10]. Therefore, most of the active anticancer drugs cannot achieve intra tumors therapeutic concentration [9]. All these hallmarks of cancer cells lead to compromised clinical outcomes in spite of significant *in vitro* efficacy. There were several ways to culminate all these cancer therapeutics hurdles and

Abbreviations: SLNs, solid lipid nanoparticles; SEM, Scanning Electron Microscopy; TEM, transmission electron microscopy; MTT, 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide; PI, propidium iodide; EAT, Ehrlich ascites tumor; PARP, poly-ADP-Ribose polymerase.

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formulation development by means of solid lipid nanoparticles (SLNs) was among one of them [11]. SLNs are particle of size from 50 nm to 1000 nm having a lipid core, which remains solid at room and body temperature. These are relatively new class of drug delivery system with excellent future potential in anticancer drug development. It is anticipated that, in the near future, anticancer compounds may be delivered in a more efficient, specific and non-toxic forms of SLNs.

AP9-cd have poor aqueous solubility and partition coefficient (log P), which limits its *in vivo* anticancer potential. Therefore, there is unmet need to design its appropriate formulation to overcome these limitations and to enhance in *vivo* therapeutic potential. In that concern, it was formulated into a lipophilic system, Solid lipid nanoparticles (SLNs). AP9-cd-SLNs have significant higher cytotoxic, apoptotic and antitumor potential than AP9-cd alone in human leukemia Molt-4 cells and experimental Ehrlich ascites tumor (EAT) model. We are first time reporting the *in vitro* and *in vivo* anticancer potential of AP9-cd-SLNs, which were formulated through micro-emulsion method [12,13]. The present formulation of AP9-cd was successfully clear all the desired points, which were thought before designing, like high therapeutic potential and low side effects.

2. Materials and methods

2.1. Chemicals and antibodies

RPMI-1640 medium, propidium iodide (PI), DNase-free RNase, proteinaseK, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenol:chloroform: isoamylalcohol, penicillin, streptomycin, L-glutamine, pyruvic acid, ethidium bromide, EDTA, bromophenol blue, agarose, phosphate buffer saline (PBS), Hoechst 33258, triton-X, camptothecin, Soy lecithin and tween-80 were purchased from Sigma chemical Co. Fetal bovine serum was obtained from the GIBCO Invitrogen Corporation (#16000-044, lot No. 1237517) USA. AnnexinV-FITC apoptosis detection kit, were from B.D. Clontech, USA. Compritol 888 ATO® (medium chain triglycerides) gift sample from Colorcon Asia Pacific Pvt. Ltd., Singapore. Mouse anti-human antibodies to Bax (#SC20067), Bcl-2 (#SC7382), PARP-1 (#SC8007), TNF-R1 (#SC8436), β-Actin (#SC-47778) and goat anti-mouse IgG-HRP (#SC2031) were from Santa Cruz Biotechnology, USA. Electrophoresis reagents, Protein estimation kit, PVDF membrane and protein markers were from Bio-Rad Laboratories, USA. Hyper film and ECL Plus western blotting detection kit were from Amersham Biosciences, UK. All other chemicals used were of analytical grade and available locally.

2.2. Source of AP9-cd

Samples of AP9-cd were received from Dr. A.K. Saxena, Indian Institute of Integrative Medicine, Jammu, India. The composition of the lignan mixture (AP9-cd) was assayed by HPLC as described earlier [5].

2.3. Grounding of SLNs

Solid lipid nanoparticles (SLNs) were prepared by micro-emulsification technique as described earlier [14]. A mixture of soy lecithin, tween-80 in water (1:3:8) was heated up to ~72 °C, which was mixed with preheated (82–85 °C) mixture of AP9-cd and Compritol 888 ATO (1:5). The prepared hot micro-emulsion was dispersed in cold distilled water (~2 °C) under continuous stirring at 5000 rpm for 1.5 h.

2.4. Measurement of total drug content and entrapment

Total drug content is calculated to determine any significant losses, which might have occurred during the production of solid lipid nanoparticles. The value of total drug content will used as a standard to estimate the amount of drug entrapped in prepared nanoparticles. The prepared SLNs dispersion was ultracentrifuge at 90,000 rpm and drug content was calculated in both the supernatant and the pellet by getting a clear solution with chloroform: methanol (1:1). Blank SLNs were treated in a similar manner and act as an internal control to compensate any drug-ingredients interaction. Amount of drug in the pellet gave a direct measure of the extent of drug entrapped [14].

2.5. Particle size analysis and zeta potential

The mean particle size and zeta potential of the AP9-cd loaded SLNs was determined as describe earlier by using Zetasizer (Malvern Instruments, England) [14]. The prepared SLNs were diluted with water (1:10) and made for particle size determination and zeta potential (surface charge) measurement through photon correlation spectroscopy and electrophoretic mobility, respectively.

2.6. Transmission electron microscopy (TEM) analysis

The morphology of SLNs was examined by transmission electron microscopy (TEM). The samples were diluted 1:25 with ultra pure water. The specimens were made by placing a small drop of 2% (w/v) phosphotungstic acid (PTA) on a carbon coated, 400-mesh specimen grid and adding an approximately equal quantity of diluted material. The material was sucked up once into a drawn out capillary tube and a small quantity were returned to the grid. Most of this liquid was then removed after a few seconds by touching momentarily the edge of the grid with a filter paper. The grids were then examined with a JEOL 100CXII electron microscope at 60 kV [15].

2.7. Scanning Electron Microscopy (SEM) analysis

The surface morphology of prepared SLNs was examined by SEM [16]. The diluted sample in water was spread over a cover slip and dry at room temperature. The cover slips were mounted over the stubs. The samples were coated with gold using a Sputter coater (Polaron) and examined under an electron microscope.

2.8. In vitro drug release

The *in vitro* drug release of prepared AP9-cd-SLNs was carried out by dialysis membrane method [17]. Phosphate buffer, pH 7.4, pre-equilibrated at 37 °C was used as receptor media for the studies. Aqueous SLN dispersion was placed in the sealed dialysis tubing, dipped into the receptor media and stirred continuously at 200–300 rpm. Samples were collected from the receptor medium at indicated time intervals and the amount of free or released AP9-cd was calculated.

2.9. Stability studies

The prepared AP9-cd-SLNs (20 ml) was filled into 20 ml glass vials and stored under varying conditions. Storage of vials was carried out at refrigerated (4 °C) and ambient temperature (25 °C). After 6 months dispersions were evaluated for the change in total drug content, drug entrapment, color, particle size, and zeta potential [18].