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NANOMEDICINE

Proapoptotic lipid nanovesicles: Synergism with paclitaxel in human lung adenocarcinoma A549 cells

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ABSTRACT

The present study focuses on the development and evaluation of phosphatidylserine based proapoptotic lipid nanovesicles (PSN-PTX) as aerosols for synergistic activity with paclitaxel against lung cancer. PSN-PTX showed a unimodal size distribution of the particles (100–200 nm), negative surface charge of −29 mV and high encapsulation efficiency of paclitaxel (82%) with 19% of it releasing in 48 h. PSN-PTX was found to be highly surface active as compared to Taxol®, marketed formulation of paclitaxel, whose surface activity was found to be detrimental for pulmonary mechanics. PSN-PTX also showed high airway patency in capillary surfactometer unlike Taxol®, suggesting its ability to mimic pulmonary surfactant functions. High deposition of PSN-PTX in lower impingement chamber of twin impinger upon nebulization suggested it to be capable of reaching the terminal regions of the lungs. Nanovesicles showed facilitated and ATP dependent active uptake by A549 cells. The combination of phosphatidylserine nanovesicles and paclitaxel as PSN-PTX enhanced cytotoxicity in A549 cell line showing an IC_{50} of 18 nM which is10-50 folds less than the IC_{50} values observed for blank phosphtidylserine nanovesicles and paclitaxel alone. Further, the combination index was found to be less than one which indicates a synergism of the two components. DNA fragmentation study showed that blank phosphatidylserine nanovesicles induce apoptosis in A549 cells and hence behave as proapoptotic nanovesicles in the combination therapy. Overall, these studies suggest the therapeutic potential and advantages of combination chemotherapy of proapoptotic lipid nanovesicles with encapsulated paclitaxel and their feasibility for aerosol administration in the treatment of lung cancer.

1. Introduction

Lung cancer constitutes the maximum percentage of deaths among those caused by different cancers in the world [1]. The traditional treatment methods include surgical resection, radiation treatment and chemotherapy, especially in advanced cases. Inspite of all these existing treatment regimens, the survival rates of lung cancer patients remain poor [2]. Chemotherapy leads to high systemic toxicity and poor bioavailability of the chemotherapeutic agents owing to their non specific depositions. Moreover, since the development of cancer or any neoplasm involves multiple and parallel occurring molecular mechanisms and pathways, single agent chemotherapy doesn't offer much advantage to the patient. In order to circumvent these problems associated with conventional chemotherapy, a better and advanced therapeutic system is required, that can be efficiently and specifically targeted to the lungs with the maximum tolerable dosage, can be retained and can offer a more efficacious treatment by combining the therapeutic potential of multiple active agents resulting in a synergistic cytotoxic effect. Liposomes or lipid nanovesicles as drug carriers have been studied extensively and have been proven to be efficient for the delivery of several antineoplastic agents. In this paper, we developed lipid nanovesicles which are proapoptotic i.e. capable of promoting and inducing apoptosis by themselves, and determined whether they can result in synergistic combination chemotherapy with paclitaxel against human non small cell lung adenocarcinoma cells. The proapoptotic nature of the nanovesicles is attributed to phosphatidylserine, an apoptosis inducing lipid which has been incorporated as one of the main components in the nanovesicle. The formulation therefore was designed with the aim of combination chemotherapy of phosphatidylserine and paclitaxel.

Paclitaxel is a potent anticancer drug used for the first line treatment of lung cancer. It is a diterpenoid pseudoalkaloid and has a unique mechanism of action which involves the stabilization of microtubules resulting in a mitotic arrest in the G2M phase of cell cycle [3]. Taxol®, the current dosage form of paclitaxel comprises of paclitaxel associated with 50:50 (v/v) Cremophor® EL (polyoxyethylated castor oil) and dehydrated alcohol, to increase the solubility. However, this Cremophor® EL based paclitaxel formulation is marked by serious complications causing severe anaphylactoid hypersensitivity reactions, neurotoxicity, cardiotoxicity, nephrotoxicity, hyperlipidaemia, abnormal lipoprotein patterns, erythrocyte aggregation, and peripheral neuropathy [4]. Owing to its highly

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hydrophobic nature, paclitaxel can be easily encapsulated into the lipid nanovesicles thereby circumventing the Cremophor® associated risks. Phosphatidylserine, an anionic phospholipid, is normally expressed on the inner leaflet of the plasma membrane and is considered to be involved in the early apoptosis initiation events [5]. Phosphatidylserine is translocated from the inner leaflet to the outer leaflet during the initiation of execution phase or caspase activation phase of apoptosis as a result of decreased aminophospholipid translocase activity and activation of a calcium-dependent scramblase [6,7]. Moreover, its cytotoxic effects when given externally, on the cancer cells are clearly proven in several studies which also showed that such effects are not observed with other anionic or neutral lipids and are therefore specifically due to phosphatidylserine [8,9]. Besides the apoptosis inducing role of phosphatidylserine, its presence on the plasma membrane of cancer cells can also result in their indirect killing by activated macrophages mediated phagocytosis [7]. Since phosphatidylserine can self assemble along with other lipids to form nanovesicles, we tried to understand whether its combination as a lipid nanovesicle with paclitaxel can result in enhanced cytotoxicity in the lung cancer cells.

The formulation was evaluated against non small cell lung carcinoma (NSCLC) and is intended for aerosol administration. In this regard, another advantage with lipid nanovesicles is that they can be aerosolized easily using nebulizers and their nebulization efficiency can be tuned on the basis of parameters such as lipids used, their concentration, particle size of the nanovesicle and operating conditions of the nebulizer [10]. This property is very crucial especially in the case of lung cancer, where the drug is intended to act topically in the lungs and hence its direct administration will offer the advantages of increased concentrations and decreased side effects as compared to systemic administration. Moreover, the surface activity of lipid nanovesicles can be engineered so that they can have high airway patency similar to that of pulmonary surfactant present *in vivo* allowing more homogeneous delivery of anticancer drugs in terminal areas of the lungs. Low surface tension and hence good surface activity of these nanovesicles can allow for their homogeneous distribution and rapid opening to form a monolayer on the alveolar surface [11]. The other advantages of using lipid nanovesicles as aerosol based drug delivery system includes their compatibility with the lung epithelial cells and decreased mucociliary clearance [12].

The primary aim of the present work is therefore to understand the possibility of combination chemotherapy of phosphatidylserine based proapoptotic nanovesicles with encapsulated paclitaxel for lung cancer and to evaluate the feasibility of the formulation for aerosol administration.

2. Methods

2.1. Preparation of lipid nanovesicles (PSN-PTX)

PSN-PTX was prepared by modified thin film hydration method [13], using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (DOPS) (Lipoid GmbH, Germany) in 7:3 molar ratio and paclitaxel: phospholipid in 1:2 molar ratio. Rotation speed of 100 rpm was maintained during the hydration of the thin film. The suspension was sonicated at 20 KHz, 50% amplitude for 2 minutes to form small unilamellar vesicles. In order to obtain a homogeneous size distribution and separate free paclitaxel (Dabur India Ltd, India), nanovesicles were further extruded through 0.4 μm and 0.2 μm polycarbonate membranes (Avanti Mini Extruder) [3]. The total phospholipid concentration in the extruded sample was determined using malachite green procedure [14]. The suspension was again centrifuged at 25000 g, 4 °C for 10 minutes and the pellet was reconstituted in PBS pH 7.4 to achieve final concentration of phospholipids as 2 mg/ml. Blank nanovesicles (PSN-B) were prepared by a similar method without the addition of paclitaxel.

PSN-PTX was characterized for size distribution by dynamic light scattering (DLS) using laser particle analyzer (BI 200SM, Brookhaven Instruments Corporation). The nanovesicles were also characterized for surface charge by determining their zeta potential using zeta potential analyzer (ZetaPALS, Brookhaven Instruments Corporation). Physical stability of the nanovesicles when stored at 4 °C as a suspension in PBS was studied by evaluating their size distribution and zeta potential at four different time points over a period of 45 days. Transmission electron microscopy of PSN-PTX was done as per the negative staining protocol [15] and images were analyzed by a transmission electron microscope, model: CM200 (Philips) operating at 120 kV. Encapsulation efficiency of paclitaxel in the nanovesicles was determined by break opening them using methanol and quantifying the drug using reverse phase HPLC (Agilent 1100 Binary LC pump liquid chromatograph). Contact angle measurements were done for PSN-PTX, PSN-B and free paclitaxel (PTX) by the sessile drop technique using a CAM-100 optical contact angle meter (KSV Instruments, Finland) in order to determine if paclitaxel is adsorbed on the surface of the nanovesicles or is completely encapsulated [16]. *In vitro* release study of paclitaxel from PSN-PTX was performed by dialysis bag method at pH 7.4 and 37 °C temperature conditions [17] with proper sink conditions.

2.2. Surface activity measurements

Langmuir–Blodgett instrument (KSV Instrument Ltd., Finland) was used for surface activity measurements of PSN-PTX, PSN-B and Taxol® in order to understand their adsorption patterns at the pulmonary air-aqueous interface. The adsorption was studied for 30 minutes for each sample. The average surface tension values achieved within 1 second were used as a parameter as described elsewhere [18], as it is desired by an active lung surfactant to rapidly adsorb at an air–liquid interface. The rate of adsorption is an essential parameter as it suggests the ability of the surfactant to adsorb from the pulmonary alveolar subphase to the air-aqueous interface and also to replenish the interfacial film if matter has been lost or inactivated.

2.3. Airway patency and in vitro lung deposition of PSN-PTX

Pulmonary surfactant helps in maintaining the patency of narrow airways in the lungs. In case, this function is disturbed, for instance by an inhibitor of surfactant activity, the fluid film lining the epithelium of the airways starts moving from wider to narrower airways forming liquid columns that block the airway [19]. If this happens, the terminal airways get occluded and the resistance to airflow increases. A good surfactant on the other hand prevents the occlusion of terminal airways and hence maintains good airway patency. Since our formulation has been intended for aerosol administration, it is crucial to evaluate its surfactant activity and hence the airway patency. Capillary surfactometer (CS) from Calmia Biomedicals (Toronto, Ontario) was used to study the airway patency and surfactant ability of PSN-PTX [11]. The airway patency of our formulation was studied as % opening time of capillary over an observation period of 120 seconds and was compared against to that of Taxol®.

In vitro lung deposition studies for PSN-PTX were performed using glass twin impinger apparatus (Copley Scientific, Nottingham, UK), adapted from apparatus A of European and British Pharmacopoeia [20]. Quantification of paclitaxel deposited in different stages of the impinger was done by reverse phase HPLC (Agilent 1100 Binary LC pump liquid chromatograph).

2.4. Cell culture

The formulation was evaluated on A549 human non small cell lung adenocarcinoma cell line for cytotoxicity, cellular interaction and