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Improved efficacy of cisplatin in combination with a nano-formulation of pentacyclic triterpenediol

Noor Alam [b], Arem Qayum [a,c], Ashok Kumar [a,c], Vaibhav Khare [b], Parduman Raj Sharma [c], Samar Singh Andotra [d], Shashank K. Singh [a,c], Surinder Koul [d,*], Prem N. Gupta [a,b,**]

[a] Academy of Scientific and Innovative Research (AcSIR), Anusandhan Bhawan, 2 Rafi Marg, New Delhi 110001, India
[b] Formulation & Drug Delivery Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
[c] Cancer Pharmacology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India
[d] Natural Product Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu 180001, India

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ABSTRACT

Cisplatin is widely used for the treatment of various cancers including cervical, ovarian, lung and head and neck, however, its clinical success is limited owing to the dose-dependent adverse effects, mainly nephrotoxicity and neurotoxicity. In order to address this limitation, the present study was undertaken to investigate growth inhibitory effect of cisplatin in combination with a triterpenediol (3a, 24-dihydroxyurs-12-ene and 3a, 24-dihydroxyolean-12-ene, TPD) on human ovarian cancer cell line. Poly(dl-lactic-*co*-glycolic) acid nanoparticles loaded with TPD (TPD-PLGA-NPs) were successfully developed by emulsion solvent evaporation method. The TPD-PLGA-NPs were characterized for size distribution and zeta potential which was in order of 152.56 ± 3.01 nm and −17.36 ± 0.37 mV respectively. The morphological evaluation was carried out by transmission electron microscopy and the formulation was also characterized using Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). The drug loading of the optimized formulation was 51.03 ± 1.52 μg/mg and the formulation exhibited sustained drug release profile. The *in vitro* cellular uptake study of coumarin-6 loaded PLGA nanoparticles in OVCAR-5 cells demonstrated a time dependent increase in uptake efficiency. Further, growth inhibitory effect of cisplatin was investigated in combination with TPD-PLGA-NPs. The combination index (CI) was <1, indicating a synergistic interaction. Further, at 75% of cell growth inhibition (ED_{75}) the dose of cisplatin was reduced to 3.8 folds using this combination. The results indicated the potential of cisplatin and TPD-PLGA-NPs combination in order to reduce to dose limiting toxicities of the former.

1. Introduction

Conventional single drug chemotherapy (mono-therapy) offers limited accessibility of drug to tumor tissues that requires a higher dose, leading to adverse effect [1]. Also, repeated treatment with single drug and administration of higher dose can result in resistance to the chemotherapeutics. For a decade, combination chemotherapy has been envisaged as a potential strategy to limit the draw-backs associated with single chemotherapeutic agent. Unlike mono-therapy, combination chemotherapy offers benefits such as countering different biological targets in cancer cells, thereby maximizing therapeutic efficacy and reduces the adverse effect [2].

Cisplatin is one of the potent chemotherapeutic agents for the treatment of various solid tumors including cervical, ovarian, head and neck, and lung cancer [3]. The biochemical mechanisms of cisplatin cytotoxicity involve the binding of the drug to guanines base of DNA and formation of inter-strand and intra-strand DNA cross links. These complexes may interfere with normal DNA replication and/or transcription mechanisms [4]. Besides the binding with DNA, cisplatin may covalently bind to proteins and other cellular targets, and modulate the activity of enzymes, receptors, and other proteins that ends up in cytotoxic phenomena [5]. The severe side-effects, including nephrotoxicity, neurotoxicity, myelotoxicity, bone-marrow depression and acquired chemo-resistance have limited the usage of cisplatin [6–8]. In order to overcome the dose-limiting toxicities in clinical use of cisplatin, combined chemotherapy with other anticancer agents is a potential option [9]. A number of drug combination with platinum compounds have been investigated which improved clinical outcome and exhibited a positive impact on survival of patient [10–12].

The combinations of cisplatin with the phytochemicals are also receiving recognition in order to limit cytotoxic manifestation of the

* Correspondence to: S. Koul, Natural Product Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu-Tawi 180001, India.
** Correspondence to: P.N. Gupta, Senior Scientist Formulation & Drug Delivery Division CSIR-Indian Institute of Integrative Medicine Canal Road, Jammu-Tawi-180001, India.
E-mail addresses: skoul@iiim.ac.in (S. Koul), pngupta10@gmail.com, pngupta@iiim.ac.in (P.N. Gupta).

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cisplatin [13]. In this study a natural compound derived from the *Boswellia serrata* is investigated in combination with cisplatin. The gum exudate of *Boswellia serrata*, was traditionally explored in the management of various diseases [14,15]. Boswellic acids (BAs), a group of pentacyclic triterpenoids are obtained from gum resin of *Boswellia serrata*. The major constituents of gum resin, β-boswellic acid, 11-keto-β-boswellic acid, 3-*O*-acetyl-β-boswellic acid and 3-*O*-acetyl-11-keto-β boswellic acid have been indicated for anti-neoplastic and anti-inflammatory activities [16,17]. The other pentacyclic triterpenediol obtained from *Boswellia serrata*, occurs in the form of an isomeric mixture of 3α, 24-dihydroxyurs-12-ene and 3α, 24-dihydroxyolean-12-ene (TPD) in nature. One 3α, 24-dihydroxyurs-12-ene isomer was isolated previously from the gum resin of *Boswellia serrata* [18] and when this was synthesized chemically, an isomeric mixture of 3α,24-dihydroxyurs-12-ene and 3α,24-dihydroxyolean-12-ene (TPD) was obtained [19]. It was reported that a pentacyclic ring bone of boswellic acid is decisive characteristics for anti-topoisomerase activity [17]. The TPD induces apoptosis through extrinsic pathway by huge generation of reactive oxygen species and nitric oxide, activation of tumor necrosis factor (TNF) family proteins and activation of caspase-8. Further, the loss of mitochondrial membrane potential leads to release of cytochrome *c* from the mitochondria, activate caspase-9 and finally cleavage of poly ADP ribose polymerase (PARP) that induce apoptosis through intrinsic pathway [20,21].

Combination chemotherapy with drugs having different mode of action is advantageous compared to monotherapy [22]. As the biological targets of cisplatin and TPD are different [4,20] and both the agents are active on the same cancerous cell lines, hence, we investigated TPD as a potential candidate for combination chemotherapy with cisplatin. Further, the low aqueous solubility and high lipophilicity of TPD [21], necessitating the development of appropriate formulation for intravenous administration for its clinical application. Therefore TPD loaded PLGA nanoparticles (TPD-PLGA-NPs) were developed and characterized for various parameters including morphology, particle size distribution, zeta potential, drug loading and drug release profile. The developed formulation was also characterized using Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC). Additionally, the nano-formulation was evaluated for cellular uptake behavior. Subsequently, *in vitro* growth inhibition of combination of cisplatin with TPD or TPD-PLGA-NPs was investigated for possible synergistic effect in order to address dose associated toxicities of the cisplatin.

2. Material and methods

2.1. Materials

PLGA (MW 24–38 kDa) was purchased from Boehringer Ingelheim (Germany). Cisplatin, polyvinyl alcohol (PVA, MW 31–50 kDa), coumarin-6, dichloromethane (DCM), 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI), RPMI-1640 medium, sulforhodamine B (SRB) dye, streptomycin and penicillin were purchased from Sigma-Aldrich (India). Fetal bovine serum (FBS) was obtained from Gibco Life technologies, U.S.A. All other chemicals and excipients used were of analytical grade. Ultrapure water (Millipore, India) was used throughout the experiments.

2.2. Preparation of triterpenediol 3a, 24-dihydroxyurs-12-ene and 3a, 24-dihydroxyolean-12-ene (TPD)

The isomeric mixture of TPD was obtained from boswellic acids by the method as described previously by our group [20]. Briefly, a weighed amount of mixture of (α + β) boswellic acid was taken in dry ether and diazomethane solubilized in diethyl ether was dropped in the reaction mixture, until completion of the reaction. The solvent of the reaction mixture and excess of diazomethane were removed to obtain the mixture comprising the methyl esters. Subsequently, the methyl ester derivative of boswellic acid was dissolved in dry ether and lithium aluminum hydride (LiAlH4) was added to the reaction mixture and stirred for 1 h under nitrogen conditions. Further, the ethyl acetate was added to the solution to neutralize excess LiAlH4 and the organic layer was washed with purified water. The desired product (3α, 24-dihydroxyurs-12-ene and 3α, 24-dihydroxyolean-12-ene, TPD) was obtained after concentrating the solvent and crystallized as white crystals using hexane-ethylacetate solution.

2.3. Preparation and optimization of TPD loaded PLGA nanoparticles (TPD-PLGA-NPs)

TPD loaded nanoparticles were prepared by emulsion solvent evaporation method [23], with minor modification [Fig. 1]. The drug and polymer ratio was optimized by taking different concentration of TPD and a fixed PLGA (35 mg) concentration, which were subsequently, dissolved in dichloromethane and emulsified (Labsonic M Ultrasonic Homogenizer, Sartorious, Germany) into aqueous phase containing PVA (2%, w/v). The emulsion was agitated by magnetic stirrer until complete evaporation of dichloromethane. The nanoparticles were collected by centrifugation (19,000 rpm, 15 min; Sigma 3–30 K, Germany), and reconstituted the pellet with water and centrifuged again. This step was repeated thrice for washing of nanoparticles. Finally, the pellet was dispersed in Milli-Q water and freeze dried (Advantage freeze dryer, VirTis, Gardiner, NY, USA) with mannitol as cryoprotectant. The freeze dried formulations were stored in desiccators till further use.

2.4. Characterization of nanoparticles

2.4.1. Particle size, polydispersity index and zeta potential measurement

Particle size, polydispersity index and zeta potential of the nanoparticles were determined by dynamic light scattering (Zetasizer Nano ZS90, Malvern Instruments, UK) at 25 °C after resuspension of formulation in appropriate volume of phosphate buffer saline (PBS, pH 7.4). Each measurement was performed in triplicate.

2.4.2. Morphological examination

The morphology of TPD loaded PLGA nanoparticles was examined using transmission electron microscope (TEM, Philips Morgagni-268) operating at 70 kV. The nanoparticles suspension was sonicated and a drop of suspension placed on the copper grid. After drying, samples were treated with phosphotungstic acid (2%) and subsequently, observed under the TEM.

2.4.3. Drug loading

Weighed amount of TPD-PLGA nanoparticles was dissolved in acetonitrile and vigorously vortexed and sonicated. Subsequently, this solution was centrifuged at 19,000 rpm for 15 min, supernatant was collected and analyzed by high performance liquid chromatography (HPLC) [20]. The analysis was performed at 30 °C by a RP-18 column (E-Merck, 5 μm, 4.0 × 250 mm). The mobile phase was a mixture of acetonitrile: water (0.5% acetic acid) in 95:05 ratios at a flow rate of 1.5 mL/min and detection was performed at 210 nm.

2.4.4. In vitro release profile

In order to determine the *in vitro* release pattern of TPD from PLGA nanoparticles, weighted amount of nanoparticles were dispersed in phosphate buffer saline (PBS, pH 7.4 and pH 5.5) containing tween-80 (0.5%, v/v) in a microcentrifuge tubes. The tubes were placed in a water bath shaker incubator (New Brunswick Scientific, USA) at 37 °C under gentle agitation. At a definite time intervals, the tubes were centrifuged (19,000 rpm, 10 min) and the known volume of supernatants were collected. After sampling, the equal volume of fresh PBS containing tween-80 (0.5%, v/v) was added to the release media. The collected samples were analyzed as described above using HPLC.