Evaluation of clinical effectiveness of paclitaxel and ursolic acid co-loaded liposomes as enhanced treatment for head and neck squamous cell carcinoma

Bi-Hua Lv1, Wei Tan2, Xuejun Shang3, Ling Zhang4*

1Department of Pharmacy, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071, 2Department of Radiology, Hospital of Wuhan University of Science and Technology, Wuhan, 430081, 3Department of Andrology, Jinling Hospital Affiliated to Nanjing University School of Medicine, Nanjing 210002, 4Department of Environmental Hygiene and Occupational Medicine, School of Public Health, Hubei Province Key Laboratory of Occupational Hazard Identification and Control, Wuhan University of Science and Technology, Wuhan 430081, China

*For correspondence: Email: zhangling@wust.edu.cn, shangxj98@163.com

Sent for review: 20 July 2018 Revised accepted: 30 October 2018

Abstract

Purpose: To enhance the clinical effectiveness of paclitaxel (PTX) by co-delivery with ursolic acid (UA) for the treatment of head and neck cancer

Methods: Co-loaded liposomes of PTX and UA (UA-PTX-LiP) were prepared by thin-film hydration method. Their size and loading efficiency were determined using dynamic light scattering (DLS) technique and high performance liquid chromatography (HPLC), respectively. The effectiveness of UA-PTX-LiP against HSC-3 human head and neck cancer cell-lines was compared with that of PTX liposome (PTX-LiP) using systemic cell-based in vitro evaluation with MTT assay. Fluorescent microscopy was used for cell uptake studies.

Results: The size of the prepared UA-PTX-LiP was 126.5 ± 3.22 nm. The ratiometric system for PTX and UA as liposomes revealed significantly enhanced cytotoxicity, with comparatively lower IC50, when compared to individual PTX-Lip. Fluorescent microscopy revealed the internalization ability of UA-PTX-LiP by targeted delivery of PTX in HSC-3 human head and neck cancer cell-line.

Conclusion: These results show that UA-PTX-LiP successfully enhances the therapeutic potential and clinical outcomes of PTX in head-and-neck cancer, and also demonstrate the useful effect of combination of UA and PTX in chemotherapy.

Keywords: Paclitaxel, Ursolic acid, Combination chemotherapy, Head-and-neck squamous cancer

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/read), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.


INTRODUCTION

Head-and-neck cancer is ranked amongst the most fatal cancers owing to its poor prognosis. Squamous cell carcinoma of the head and neck (HNSCC) proliferates at the brink of oral cavity, paranasal sinuses and nasal cavity along with larynx and pharynx, and contributes to nearly 900,000 new cases and 350,000 mortalities annually worldwide [1]. Paclitaxel (PTX) is one of the most valued anticancer drugs for the treatment of breast, colon, prostate, melanoma
and various types of solid cancers [2]. However, it has some drawbacks such as poor aqueous solubility, gastrointestinal toxicity and non-specific systemic delivery [3]. Ursolic acid (UA) isolated from the roots of Catharanthus trichophyllus possesses anti-tumor and anti-inflammatory properties [4].

The anticancer properties of PTX and UA can be employed for synergistic combination therapy of HNSCC. The inception of novel drug delivery systems has enhanced the anticancer potential of drugs [5,6]. Therefore, in this study, lipid bilayer liposomes (LIP) that can efficiently co-deliver both hydrophobic and hydrophilic drugs by co-loading them in their hydrophilic core and hydrophobic lipid vesicles were chosen as for the co-delivery of PTX and UA [5,7,8]. In a recent investigation, it was shown that hybrid nanoconstruct of albumin–PTX hybrid nanoparticle (LAPN) encapsulated in liposome significantly enhanced anticancer effectiveness by increasing the accumulation of PTX in tumor, bio-distribution and pharmacokinetic parameters [9].

Studies have shown that UA attenuates colon cancer by increasing cancer cell apoptosis, and also by arresting angiogenesis through inhibition of the expressions of COX-2 and caspases [10-12]. In the present study, liposomes incorporating both PTX and UA (UA-PTX-LIP) were designed for efficient co-delivery of the two drugs. The resultant UA-PTX-LIP was inspected thoroughly to ensure that it is an effective co-delivery system for combination chemotherapy [13].

The objective of this study was to develop UA-PTX-LIP formulation for the co-delivery of UA and PTX for effective chemotherapy, based on a hypothesis that the combination of UA and PTX could enhance the therapeutic potency of PTX for effective treatment of cancer.

**EXPERIMENTAL**

**Materials**

Paclitaxel (PTX) was purchased from Hefei Bo Mei Biotechnology Co., Ltd. China, while UA was procured from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). HSPC and DSPE-PEG2000 were acquired from Avanti Polar Lipids, Inc; 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was obtained from Sigma Aldrich (St. Louis, MO). All other chemicals used for experiments were of analytical grade. Milli-Q water (Milli-Q plus 185 purification system, Bedford, MA) was used for all aqueous preparations throughout the study.

**Preparation of PTX and UA-PTX liposomes**

The liposomes were prepared using the thin-film dispersion hydration method to formulate liposomes co-loaded with PTX or UA/PTX [14]. The lipid compositions were HSPC/DSPE-PEG2000/Chol at a molar ratio of 65:9:26. The PTX- or UA-PTX–to-lipid ratio was 1:20 (w/w), and the UA:PTX ratio was kept at 1:1 (w/w). The PTX or UA-PTX and lipids were dissolved in chloroform, and the lipid dispersion was dried in a rotary evaporator under reduced pressure at 45 °C to constitute a thin lipid layer. The thin layer of lipid was then hydrated using 4 ml of PBS at pH 6.8, and incubated for 30 min at 50 °C. The resultant lipid vesicles were extruded through polycarbonate membrane (200 nm pore size) 5 times to form homogeneous unilamellar vesicles with aid of a Lipex Extruder driven by pressurized nitrogen at 60 °C. The liposomes were washed and lyophilized to obtain PTX-Lip and UA-PTX-Lip.

**Physicochemical characterization of PTX-LIP and UA-PTX-LIP**

**Particle characterization**

The PTX-Lip and UA-PTX-Lip were evaluated with respect to mean particle size, size distribution and zeta potential by means of Malvern Zetasizer Nano ZS (Malvern 3000HS, France). All measurements were repeated thrice [15].

**Transmission electron microscopy (TEM)**

Freshly-prepared UA-PTX-Lip was spread on a carbon-coated copper grid to form a thin liquid film. The film was stained with 1 % (w/v) phosphotungstic. Excess staining solution was cleared with filter paper and then the film was allowed to air-dry. The stained film was photographed using a transmission electron microscope.

**High performance liquid chromatography (HPLC)**

Following liposome lysis, methanol was used to extract PTX and UA which were quantified using HPLC analysis at maximum absorption wavelengths of 227 nm and 210 nm, respectively [16]. The filtered sample (20 µL) was introduced from the injector port into a C18 column (250 mm, 4.6 mm, 5 µm; Merck). The mobile phase used for simultaneous estimation was 55:45 (v: v) mixture of methanol and acetonitrile at a flow rate of 1.0 ml/min.
Entrapment efficiency (EE) and drug loading (DL)

Entrapment efficiency (EE, %) was estimated by analysing PTX and UA levels in PTX-LiP and UA-PTX-LiP using HPLC method as described above. The liposomes were subjected to low-speed centrifugation at 3000 g for 10 min at room temperature to separate the un-entrapped UA and PTX dispersed in the supernatant liquid. The supernatant was filtered and used for evaluation of free UA and PTX [17].

\[
EE (\%) = \frac{W_1}{W_2} \times 100 \quad \cdots \cdots \cdots \text{(1) for PTX}
\]
\[
EE (\%) = \frac{W_3}{W_4} \times 100 \quad \cdots \cdots \cdots \text{(2) for UA}
\]
\[
DL (\%) = \frac{W_2}{W_3} \times 100 \quad \cdots \cdots \cdots \text{(3) for PTX}
\]
\[
DL (\%) = \frac{W_4}{W_5} \times 100 \quad \cdots \cdots \cdots \text{(4) for UA}
\]

where \(W_1\) is the weight of PTX in PTX-LiP; \(W_2\) is the weight of PTX initially taken; \(W_3\) is the weight of UA in UA-PTX-LiP; \(W_4\) is the weight of UA initially taken; \(W_5\) is the weight of PTX-LiP, and \(W_6\) is the weight of UA-PTX-LiP.

Cellular uptake studies

The cellular uptakes of PTX-LiP and UA-PTX-LiP formulations were determined in HSC-3 cells so as to appraise drug accumulation in the cancer cells. The internalization of PTX-LiP and UA-PTX-LiP in HSC-3 human head and neck cancer cell-lines was evaluated using FACS. The HSC-3 human head-and-neck cancer cells were cultured in McCoy’s 5A media (Sigma Aldrich) supplemented with 10 % fetal bovine serum (Gibco BRL), antibiotics and L-glutamine at 37 °C. Fluorescein isothiocyanate (FITC)-loaded PTX-LiP (FITC-LiP) and FITC-loaded UA-PTX-LiP (UA-FITC-LiP), were prepared by adding 1 % (w/v) FITC in PBS used for hydration of thin lipid layer during the preparation of PTX-LiP and UA-PTX-LiP.

All the procedures were performed in the dark. The HSC-3 cells were seeded in 6-well plates using fresh culture media, and incubated for 24 h. Thereafter, FITC-LiP and UA-FITC-LiP were added to the culture plates and incubated for 4 h, after which the cells were washed thrice with PBS and processed for flow cytometric analysis at an excitation wavelength of 480 nm and an emission wavelength of 550 nm.

Apoptosis assay

Annexin V/PI double stain assay was used for determination of apoptosis of HSC-3 cells in 12-well plates. The cells were incubated up to 24 h so that they attained adherence at the bottom. Then, the medium was changed for a fresh one, after which the cells were treated separately with control, PTX-LiP and UA-PTX-LiP, and again incubated for 24 h. Following the incubation, the HSC-3 cells were washed thrice with PBS, followed by trypsinization and cell harvesting. The collected cells were subjected to centrifugation for 5 min at 1500 rpm and suspended in 500 µl of PBS. Finally, the cells were suspended in binding buffer cocktail, double-stained with FITC-Annexin V and PI, and incubated in the dark for 15 min. The cells were immediately analysed using BD FACS Calibre flow cytometer.

Determination of intracellular reactive oxygen species (ROS)

The intracellular production of ROS was determined using 2′, 7′-dichlorofluorescein diacetate (DCFDA), based on the principle that on oxidation, DCFDA is de-esterified inside the cell matrix into a highly fluorescent 2′,7′-dichlorofluorescein (DCF). The HSC-3 cells were cultured in a 6-well plate and incubated for 24 h. Then, the cells were treated with UA-LiP, UA-PTX-LiP and blank liposomes for the indicated durations at 37 °C in a 5 % CO\(_2\) incubator, after which they were flushed with PBS and incubated with 5 µM of DCFDA in the dark for 30 min. Then, the cells were washed again with PBS and reconstituted in 500 µl of PBS. Thereafter, the levels of cellular ROS were determined using fluorescence-activated cell sorter (FACS, Calibur, BD Biosciences, USA). The DCF data were recorded at an excitation wavelength of 488 nm and emission wavelengths of 515–540 nm.

Cytotoxicity studies

In vitro cytotoxicities of PTX, PTX-LiP and UA-PTX-LiP were determined in HSC-3 cells using MTT assay [18]. The HSC-3 cells were cultured and maintained as described in previous sections. Briefly, the cells were seeded at a density of 5000 cells/well in 96-well plates for 24 h. Then, the cells were treated with PTX, PTX-LiP and UA-PTX-LiP at comparable concentrations of PTX, i.e. 0.001, 0.01, 0.1, 1 and 10 µg/ml, followed by incubation for 24 h. Thereafter, MTT solution was added, and the cells were further incubated for 3 h. After discarding culture medium from each well, the resultant formazan crystals were dissolved in 150 µl DMSO.
μl of DMSO, and the absorbance of each solution was measured in a Bio-Rad 680 micro plate reader at 570 nm. The experiments were performed in triplicate, and cell viability (V) was calculated as in Eq 5.

\[ V = \frac{(At/Ac)}{100} \]  

where At and Ac are the absorbance of treated and control groups, respectively.

**Lactate dehydrogenase (LDH) release assay**

The HSC-3 cells were seeded in 96-well plate for 24 h at a density of 1 x 10⁴ cells per well. Then, the cells were treated with PTX, PTX-LiP and UA-PTX-LiP at equivalent concentration of 1µg/mL. After 24 h, LDH assay was performed as specified in the manufacturer’s protocol [19]. In this assay, 1mL of 3 % Triton X 100 and 1mL PBS (pH 7.4) were used as positive and negative controls, respectively. The level of LDH released was used as a marker of toxicity [20].

**Statistical analysis**

Graph Pad Instat software (Graph Pad Software Inc., CA, USA) was used for one-way analysis of variance (ANOVA), followed by Turkey–Kramer test for comparing multiple tests. The significance level was fixed at \( p < 0.05 \). Values of \( IC_{50} \) were calculated using Prism 5 software.

**RESULTS**

**Characteristics of PTX-LiP and UA-PTX-LiP**

The mean sizes of PTX-LiP and UA-PTX-LiP were 109.7 ± 2.11 and 126.5 ± 3.22 nm, respectively. The zeta potentials of PTX-LiP and UA-PTX-LiP were -24.9 ± 1.22 and -26.4 ± 2.11 mV, respectively. Their zeta potential values were negative, which might be due to their phospholipid contents [21]. The encapsulation efficiency (EE) and drug loading of PTX were 97 and 5.7 % in UA-PTX-LiP, and 89 and 6.2 % in PTX-LiP, respectively. These results are presented in Table 1. Figure 1 shows size distribution and TEM images of UA-PTX-LiP which reveal its size (about 150 nm). This was in line with the results obtained from DLS analysis. The discrepancies in sizes were due to that fact that TEM image of UA-PTX-LiP was acquired in the dry state under vacuum, whereas the DLS profile was obtained in an aqueous dispersion.

**Cytotoxicity**

Free PTX, taxol, PTX-LiP and UA-PTX-LiP dose-dependently induced cytotoxicity in HSC-3 cancer cells (Figure 2). In particular, UA-PTX-LiP showed higher cytotoxic effect than pure PTX or PTX-LiP at all tested concentrations. At a ratio of 1:1, PTX and UA were significantly more cytotoxic (\( IC_{50} = 0.2 \) µg/mL) than pure PTX at a lower dose. These results (including those from cellular uptake studies) establish that UA-PTX-LiP is effective in causing more accumulation of intracellular drugs through endocytosis, resulting in greater ability to activate caspase-3 and induce more cell apoptosis [22]. They also indicate the enhancement of anticancer activity through ratiometric loading of PTX and UA, relative to single drugs.
Apoptosis

The results obtained from double staining assay using Annexin V/PI with respect to the effects of UA-PTX-LiP, PTX-LiP and control on apoptosis in HSC-3 cell lines are shown in Figure 3. The apoptotic cells were more in cells treated with UA-PTX-LiP than in cells treated with PTX-LiP or control. Thus, UA-PTX-LiP induced apoptosis specifically in HSC-3 cell lines.

Intracellular reactive oxygen species (ROS)

The results obtained showed that UA enhanced ROS levels in HSC-3 cells, but UA-PTX-LiP produced highly ROS in HSC-3 cells than UA. Intracellular ROS accumulation by LiP could be one of the key factors involved in induction of apoptosis in cancer cells. Oxidative stress is caused by direct ROS accumulation in cancer cells or inhibition of endogenous antioxidants. Thus, UA manipulates the redox state of cancer cells by initiating intracellular ROS, thereby leading to cellular apoptosis [23].

Effect of treatments on LDH levels

The pattern of LDH release after cell treatment using PTX, UA-PTX-LiP and PTX-LiP is presented in Figure 4. The release of LDH due to UA-PTX-LiP treatment was minimal.

DISCUSSION

Even after being continuous progress in the diagnosis, chemotherapy and adjuvant therapies for HNSCC, toxicity remained an urgent issue for patients with HNSCC. PTX is widely used as an anticancer drug for various cancer including HNSCC, but has been associated with severe toxicity [26]. The combination of different chemotherapeutics is of great advantage; especially the herbal combination can provide reduced toxicity and enhance anticancer activity [27]. Here we report the liposomal co-delivery of PTX and UA with the aim to reduce the toxicity control. Cells in general can take up liposomes non-specifically via the general biochemical reactions with the receptors or via endocytosis [24]. High cellular internalization of UA-PTX-LiP may be due to the presence of UA [25].
and increase the potency of PTX. The functionality of the nanotechnology has been exploited to load UA and PTX. The particle size of UA-PTX-LiP measured using DLS was slightly higher than that of measured by TEM which might be attributed to the aqueous dispersion of the liposomes measured by DLS. Further, we evaluated the in-vitro efficacy of UA-PTX-LiP in HSC-3 cell lines for their potency in killing the cells and also the mechanism of its enhanced activity was also established. The results suggest that liposomal drug delivery system (UA-PTX-LiP) show significant advantages over free UA and PTX in assessing in vitro cellular uptake through improved drug diffusion in the cells [28].

PTX and UA combination was significantly more cytotoxic than PTX alone at lower doses. In vitro antitumor evaluation, i.e., FACS, immunofluorescence assay and apoptosis indicated that UA-PTX-LiP inhibited the growth of head and neck cancer cells and increased cellular uptake which induced cellular apoptosis more powerfully than PTX-LiP and free PTX. UA-PTX-LiP was capable of inducing apoptosis greater than PTX-LiP, which might be attributed to the presence of UA which have inhibited COX-2 and induced apoptosis [21]. It was also observed that UA-PTX-LiP showed higher potency than PTX-LiP and free PTX as UA exerted increased oxidative effect on HSC-3 cells which facilitates its cytotoxic effect, our results were consistent with previous research [29]. Furthermore, the results show that UA a natural triterpenoid have a significant effect in chemosensitizing HSC-3 cells. UA-PTX-LiP could provide a more potent combination than PTX alone as indicated in in-vitro experiments. It will be of great value if further studies including the animal model could be done to establish the actual efficacy of the combination therapy.

CONCLUSION

PTX and UA co-loaded liposomes has been successfully developed and evaluated for their anticancer potency. UA-PTX-LiP is more cytotoxic than PTX alone even at low concentrations. Therefore, UA-PTX-LiP is a potential combination system for cancer therapy, especially head and neck cancer tumors.

DECLARATIONS

Acknowledgement

This project was supported by Funding of Health and Family Planning Commission of Hubei Province (nos. WJ2017M174 and WJ2017M240).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Bi-Hua Lv and Wei Tan contributed equally to this work. The project work was designed by Bi-Hua Lv, Ling Zhang. The experiments were carried out by Bi-Hua Lv, Wei Tan, Xuejun Shang. The manuscript writing and editing was carried out by Bi-Hua Lv, Wei Tan, Xuejun Shang, Ling Zhang.

REFERENCES


