Urological Science xxx (2016) 1–6

Contents lists available at ScienceDirect

Urological Science

journal homepage: www.urol-sci.com

Original article

Comparison of therapeutic efficacy of lipo-doxorubicin and doxorubicin in treating bladder cancer

Dah-Shyong Yu [a, *, †], Hui-Yu Yan [b], Chia-Lun Wu [c], Shun-Hsing Hung [d, †]

[a] Uro-Oncology Laboratory, Division of Urology, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan
[b] Graduate Institute of Biomedical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan
[c] Graduate Institute of Life Science, National Defense Medical Center, Taipei, Taiwan
[d] Division of Urology, Department of Surgery, Chi-Mei Medical Center, Tainan, Taiwan

ARTICLE INFO

Article history:
Received 8 April 2016
Received in revised form
16 August 2016
Accepted 29 August 2016
Available online xxx

Keywords:
bladder cancer
doxorubicin
endocytosis
lipo-doxorubicin

ABSTRACT

Objectives: Doxorubicin is commonly used in the treatment of superficial bladder cancer, but more side effects and shorter intracellular retention time hamper its clinical application. Since lipo-doxorubicin (Lipodox) has the advantages of longer half-life and lower clearance rate than doxorubicin, it should improve the efficacy of tumor therapy and reduce the normal tissue toxicity of doxorubicin.

Materials and Methods: In this study, we compared the cytotoxicity of Lipodox and doxorubicin in different treatment durations on bladder cancer cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Drug distribution was tracked under fluorescence microscopy. The metabolic rate after treatment was measured by serial flow cytometry. Finally, an *in vivo* orthotopic MBT-2 bladder tumor model was established for comparing the differences of therapeutic efficacy, including tumor weight and survival rate.

Results: The 50% inhibitory concentration (IC_{50}) of doxorubicin and Lipodox for MBT-2 cells was 0.62 μg/mL and 130 μg/mL, respectively, after 48 hours treatment. Lipo-dox presented higher cytotoxicity than doxorubicin at 6 hours (93% vs 73%) and 12 hours (93% vs 80%) treatment. After drug treatment, Lipodox fluorescence distribution was observed mostly in the cell membrane, lysosomes, and nuclei of tumor cells, while doxorubicin was concentrated in the nuclei. Initial fluorescence intensity of doxorubicin was 27.3 times that of Lipodox ($p < 0.001$) at time of treatment. The fluorescence intensity of doxorubicin decreased to 12% after 24 hours culture but that of Lipodox remained at 81%. In an orthotopic model, the average tumor weight and survival were: control group: 1.0 ± 0.3 g, 25%; doxorubicin treatment group: 0.7 ± 0.05 g, 43%; and Lipodox treatment group: 0.2 ± 0.1 g, 57%.

Conclusion: Our study demonstrated that Lipodox can enhance doxorubicin cytotoxicity in bladder cancer cells and inhibit tumor growth in orthotopic bladder cancer with improved survival rate. Therefore, we suggest Lipodox may act as an alternative to doxorubicin in the treatment of local bladder cancer.

1. Introduction

Doxorubicin is a cytotoxic anthracycline antibiotic isolated from *Streptomyces peucetius* var. *caesius*, and exerts its antitumor effect via inhibition of topoisomerase II. Using liposomes as a vehicle for delivering cytostatic agents was first described in the 1960s. Liposomes were conceived as drug delivery systems to modify drug pharmacokinetics and distribution, with the goal of reducing the toxicity of chemotherapy. These liposomes improve the pharmacological properties of some cytostatic agents, allowing for an increased proportion of the drug to be delivered to the tumor tissue while substantially reducing the exposure to normal tissues.

Liposomal doxorubicin — doxorubicin hydrochloride encapsulated in liposomes coated with methoxy polyethylene glycol (PEG) — has shown diminished uptake by the reticuloendothelial immune system, a longer half-life, and theoretically increases liposomal deposition in tumor tissue. In comparison to conventional or liposomal doxorubicin formulas, the PEGylated liposomal doxorubicin (PLD) has extended blood circulation time, improved treatment localization, and better tolerance than conventional

* Corresponding author. Uro-Oncology Laboratory, Division of Urology, Department of Surgery, Tri-Service General Hospital, National Defense Medical Center, Number 325, Section 2, Chenggong Road, Neihu Dist, Taipei 114, Taiwan.
E-mail address: yuds@ms21.hinet.net (D.-S. Yu).
† These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.urols.2016.08.001

formulations.[1] Since its approval by the US Food and Drug Administration (FDA), PLD has become a major component in the routine management of epithelial ovarian cancer and AIDS-related Kaposi's sarcoma.[2,3] Doxorubicin has been used as one of the common intravesical chemotherapeutics for superficial bladder cancer. Its effectiveness is limited by short duration of tumor contact and affordable drug concentration. Hence, we tried to assess the potential of lipo-doxorubicin (Lipodox) in treating bladder cancer as an alternative to doxorubicin.

2. Materials and methods

2.1. Culture of cell lines

Low-grade human TCC TSGH-8301 cells established at our laboratory were cultured in RPMI-1640 medium (Thermo Scientific HyClone, Logan, UT, USA), supplemented with 10% fetal bovine serum and antibiotics. One mouse TCC cell line, MBT-2, was purchased from American Type Culture Collection and also cultured in RPMI-1640 medium. The cultured cells were incubated in 5% CO_2 at 37°C.

2.2. Chemicals

Doxorubicin was purchased from Sigma (St. Louis, MO, USA). Lipodox was generously provided by Don-Yang Pharmaceutical Co. (Taiwan) without conflict of interest in this study.

2.3. Cell viability measurement

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.[4] MBT-2 cells were seeded in triplicate at 2×10^4 cells/well on a 96-well plate and incubated with various concentration of doxorubicin or Lipodox for 2 hours and then the medium was changed. At 6-hour, 12-hour, and 24-hour intervals, the culture media were removed and MTT (0.5 mg/mL) was added, followed by incubation at 37°C for 3 hours in a CO_2 incubator. After insoluble crystals were completely dissolved in dimethyl sulfoxide, the absorbance at 560 nm was measured using a microplate reader (Thermo Scientific Instruments, Salzburg, Austria).

2.4. Fluoroscopic observation of intracellular distribution of doxorubicin and Lipodox in TCC cells

In and on cellular doxorubicin and Lipodox were studied by fluorescence microscopy distribution. TSGH-8301 TCC cells were left in sterilized slides for hours until near confluence. Doxorubicin (1.74 μg/mL) and Lipodox (20 μg/mL) were added to the medium and coculture for 2 hours. The medium was removed and the slides treated by 1:1 acetone/methanol solution and rinsed using phosphate-buffered saline solution. The slides were covered by microslide in 1:1 glycerol/Q water solution and the cells were observed for drug fluorescence distribution in intracellular structures under a fluorescence microscope (Leica DMI6000B, Wetzlar, Germany).

2.5. Changes of intracellular drug concentration measured by flow cytometry

Flow cytometry was used to detect the intracellular drug fluorescence intensity at different time periods. MBT-2 tumor cells were cultured in 24-well microplates and treated by 20 μg/mL doxorubicin or Lipodox for 2 hours. Then the medium was replaced by drug free RPMI-FCS and the cells were continuously cultured for 48 hours. The cells were harvested at 24-hour and 48-hour intervals and the intracellular drug fluorescence measured by EPICS XL-MCL flow cytometer (Beckman–Coulter, Miami, FL, USA) and analyzed by Expo 32 software. The fluorescence intensity was compared with the initial fluorescence of tumor cells at 2 hours after drug treatment and expressed as a percentage ratio of initial drug fluorescence at 24-hour and 48-hour intervals.

2.6. MBT-2 orthotopic tumor implantation

The animal study received institutional animal care and use committee approval. MBT-2 tumor cells were subjected to passage *in vitro*. Single-cell suspensions were prepared from tissue culture flasks by trypsinization and adjusted to the required concentration of 5×10^6 cells/mL. Mice were anesthetized with a single dose of intraperitoneal injection of 2, 2, 2-tribromoethanol: 2-methyl-2-butanol: Q water = 1 g: 2.5 mL: 80 mL mixture (20 μL/g body weight per mouse) before the study. The bladder was catheterized via the urethra with a 24-gauge plastic intravenous cannula under sterile conditions. The bladder was then traumatized by instilling 0.1 mL of a 0.1N HCl solution for 15 seconds, which was neutralized with 0.1 mL 0.1N KOH and then flushed with sterile saline. The tumor cell suspension (5×10^5 cells in 0.1 mL 50% normal mouse serum) was instilled via the cannula. The urethra was compressed with a serrefine clamp for 30 minutes to prevent premature bladder evacuation. Under these conditions, macroscopic tumors usually developed in mice, which resulted in death within 6 weeks without treatment.

2.7. Intravesical treatment of orthotopic bladder tumors using doxorubicin and Lipodox

For intravesical therapy, the mice were anesthetized, catheterized, and administered 100 μL doxorubicin or Lipodox (160 μg/mL) and retained for 1 hour in both groups of mice ($n = 7$). Treatment began 3 days after MBT-2 tumor implantation, and it was given twice weekly for a total of six doses. In addition, negative control mice ($n = 12$) were only injected with 0.1 mL phosphate-buffered saline. The activity, body weight, and survival of mice were monitored daily. After a mouse died, it was frozen and kept in a deep freeze for further histopathological evaluation until the end of the study. The wet bladder was resected and weighed till the end of study. Cumulative survival rates of treated MBT-2-bearing mice were periodically determined during 60 days of observation and each study was repeated twice.

2.8. Statistical analysis

Data are presented as mean ± standard error of at least three independent experiments. For comparisons between three or more groups, two-way analysis of variance was used followed by the Student method for multiple pair-wise comparisons. Differences were considered statistically significant at a value of $p < 0.05$.

3. Results

3.1. Lipodox has higher cytotoxicity than doxorubicin for TCC cells

Comparison of cytotoxicity between doxorubicin and Lipodox over the period of 12 hours after drug treatment with different serial concentrations revealed that doxorubicin had higher cytotoxicity for MBT-2 tumor cells than Lipodox had (Figure 1). At a cut-off concentration of 15.6 g/mL, doxorubicin and Lipodox had similar cytotoxicity in MBT-2 tumor cells at the 12-hour mark with viability of <10%. The lipodox-treated cells presented with persistent

Please cite this article in press as: Yu D-S, et al., Comparison of therapeutic efficacy of lipo-doxorubicin and doxorubicin in treating bladder cancer, Urological Science (2016), http://dx.doi.org/10.1016/j.urols.2016.08.001