Abstract. The aim of the present study was mainly to assess the advantage of docetaxel-loaded PEG-albumin nanoparticles (PEG-DANPs) against non-small cell lung cancer (NSCLC) compared with the commercial product of docetaxel (Aisu®) and docetaxel-albumin nanoparticles (DANPs). We made systematic assessments on these three drugs against NSCLC both in vitro and in vivo. Based on our experiments, PEG-DANPs showed a dose- and time-dependent efficacy in the in vitro cytotoxicity studies; the tumors growth and the metastases in the livers of NSCLC-bearing nude mice in vivo were reduced markedly by PEG-DANPs, and the PEG-DANP-treated mice had a minimum of weight loss; furthermore, the mice which were treated with PEG-DANPs can survive longer than the other groups. In conclusion, the PEG-DANPs have the lowest side-effects, and the highest antitumor and metastases activity of the three drugs, and it may provide an alternative to patients with NSCLC.

Introduction

Lung cancer is the first ranked malignant tumor with high incidence and mortality worldwide, and more than one million patients are diagnosed with lung cancer each year. Among the various types of lung cancer, NSCLC accounts for 75-85%, and elderly patients aged more than 65 years account for more than 50% (1). Generally when patients are diagnosed at a late stage (stage III or IV) the 5-year survival rate is only 12-15% (2,3), as the patients lost the opportunity for surgical treatment (4,5).

For elderly advanced NSCLC patients, chemotherapy is still the first choice of treatment (6,7), it is relatively effective treatment for elderly NSCLC patients who cannot tolerate surgical operation. It can effectively reduce the progress of lung cancer and the recurrence, enhance the effect of clinical treatment, prolong patient survival rate and improve their quality of life (8).

There are various chemotherapy regimens and drugs for elderly NSCLC patients, such as cisplatin, VP-16, gemcitabine, paclitaxel and docetaxel (9). However, there is still lack of a mature and effective way for NSCLC due to the side-effects of the drugs. Thus, how to choose a drug with benefits and little side effects is a hotspot in tumor research (10,11). Docetaxel is a type of anti-microtubule taxane drug, and it is the only one approved for first- and second-line chemotherapy for NSCLC treatment by the US Food and Drug Administration (FDA), and the EU. According to the existing clinical data of current studies, DTX has been proven very effective for NSCLC (12,13).

Biodegradable albumin nanoparticles are thought more and more important during the past few decades (14). Previous studies have been proven that drug-loaded polymeric nanoparticles accumulated in certain tumors more efficiently than other carriers by enhancing permeability and retention (EPR) effect (15,16). Furthermore, another advantage is long circulating half-life and lower systemic toxicity which is superior to conventional drug formulations (17-20).

Polyethylene glycol (PEG) is one of medicinal synthetic polymer injections which can be used for the body as approved by FDA. There are numerous advantages of PEG drugs such as: i) extending biologic half-life of drugs, enhancing long-acting and sustained-release effect; ii) improving the solubility and stability of the drug; iii) reducing immunogenicity and antigenicity; iv) reducing enzyme degradation; v) enhancing the targeting function of drugs; and vi) reducing the toxicity of various drugs. Based on the above advantages, we chose PEG to formulate polymeric nanoparticles due to its excellent biocompatibility and biodegradability.

Aiming to develop a good nanoparticle carrier for DTX, we synthesized PEG-DANPs via the emulsion-evaporation cross-link method, and we carried out a series of experiments both in vitro and in vivo, the results demonstrated that PEG-DANPs were quite a promising modality for NSCLC.
Materials and methods

Materials. All reagents and solvents were used as received, without further purification. Monomethoxy PEG with a molecular weight of 20,000 kDa (mPEG 20,000), D,L-lactide and stannous octoate were purchased from Sigma-Aldrich Chemical Corp. (Shanghai, China); DTX was purchased from Beijing Norzer Pharmaceutical Co., Ltd. (Beijing, China); free-DMTX (Aisu®) is manufactured by Hengrui Pharmaceutical Co., Ltd. (Jiangsu, China); and 3-(4,5)-dimethylthiazol-(2-y1)-3,5-di-phenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA); Annexin V-FITC apoptosis detection kit was purchased from 4A Biotech Co., Ltd. (Beijing, China); VivoGlo® luciferin was purchased from Promega Corporation (Madison, WI, USA). Trypsin, fetal bovine serum (FBS) and RPMI-1640 medium were purchased from HyClone Laboratories (Beijing, China). All reagents and solvents were used as received, without further purification.

Preparation of DANPs and PEG-DANPs. DTX was dissolved in chloroform and ethanol to form solution A, albumin was dissolved in sterile water to form solution B. The solution A and B was mixed and stirred by homogenate machine under a light microscope. Lung tumor and other sections were routinely stained with hematoxylin and eosin (H&E) and evaluated under a light microscope.

Cell line and animals. Human non-small lung cancer A549 cell line was provided by the Department of Pathology in Institute of Medicinal Biotechnology in Peking Union Medical College. A549 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Female BALB/c mice (6-8-weeks old) were used for antitumor efficacy studies and were purchased from Beijing Vital River Laboratories (Beijing, China).

Animals were acclimatized in the holding facility prior to beginning of the present study. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences. All surgeries were performed under sodium pentobarbital anesthesia (5 mg/ml solution), and all efforts were made to minimize suffering. Lung tumor and other sections were routinely stained with hematoxylin and eosin (H&E) and evaluated under a light microscope.

Cell viability. The in vitro cytotoxic activity of DANPs and PEG-DANPs was evaluated by the MTT assay. Briefly, the A549 cells (8x10^4 cells/ml) were seeded into 96-well plates and incubated for 24 h to allow cell attachment. The cells were then treated with a series of phosphate-buffered saline (PBS) (control), Aisu®, DANPs and PEG-DANPs at 37°C (0.001, 0.01, 0.1, 1, 10 and 100 µg/ml). At the incubation-time points of 48 h, 20 µl of MTT (5 mg/ml) was added and incubated for 4 h, MTT was aspirated off and 180 µl/well of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and the plate was gently shaken for 10 min. The optical density (OD) was measured at 490 nm by Synergy H1m monochromator-based multi-mode microplate reader (BioTek, Winooski, VT, USA). The cell inhibition was calculated according to the below formula: Cell inhibition (%) = [1 - (ODsample - ODblank)/(ODsample - ODblank)] x 100%. The results were expressed as means ± SD of 3 measurements. No precipitation of DTX was found during incubation procedure (23).

Cell apoptosis assay. Apoptotic cells were determined by dual staining with an Annexin V and propidium iodide (PI) kit (4A Biotech Co., Ltd.) according to the manufacturer’s instructions (24). After 48 h of incubation in the exponential stage, A549 cells seeded in 12-well plates were treated for a further 48 h with 10 nmol/ml Aisu®, DANPs and PEG-DANPs, respectively. After treatment, cells were washed twice with warm PBS, detached by trypsin without EDTA, then through the following steps: collection, centrifugation, washing with warm PBS, further staining with PI and Annexin V-FITC for 15 min at room temperature in the dark. Apoptosis was then analyzed using a FACScan cytometer. Quadrant analysis was performed and cells that stained positive for both Annexin V-FITC and PI were designated as apoptotic, while unstained cells were designated as viable.

In vivo antitumor and metastasis inhibition. All experimental procedures were performed in conformity with institutional guidelines and protocols for the care and use of laboratory animals. We chose 40 BALB/c mice to divide into two parts, one part was to establish the transplantation tumor model (25,26) to observe the inhibition of drugs for transplantation tumor and weight changes of nude mice; the other part was to establish the in situ carcinoma model to observe the inhibition of drugs for in situ carcinoma and metastasis. Each group contained 20 mice and they were equally divided into four groups, respectively, negative control (glucose injection group), positive control (Aisu® group), DANPs and PEG-DANPs groups with 5 animals each. The lung cancer A549 cells were suspended in BD Matrigel, and the mice in each group were implanted with 3x10^6 cells in alar or in the lung. When the tumor volume in transplantation tumor group reached ~120 mm³, the mice were treated 4 times at 7-day intervals with 5% glucose injection (negative control), with Aisu®, DANPs or PEG-DANPs at the same time in both parts, respectively. All formulations were injected intravenously via the tail vein at a DTX dose of 20 mg/kg. The body weight and tumor volume were measured simultaneously. Tumor volume was calculated using the equation of V = w² x l/2. Here, w and l are the width and length of the tumor. Forty-eight hours after the last treatment, the mice were sacrificed. The tumors with lung and other major organs (including heart, liver, spleen and kidney) were removed, fixed in 10% formalin solution, and subjected to paraffin embedding for H&E staining.
Survival rate of the nude mice after treatment. Additional 32 nude mice were used to establish the transplantation tumor model and divided into negative control group (group glucose injection), positive control group (Aisu®), DANPs and PEG-DANPs group, the mice were treated until natural death, observe and the survival rate was drawn.

Statistical analysis. Results are presented as means ± SD. Statistical comparisons were carried out by t-test or ANOVA analysis. The level of significance was set at p<0.05.

Results

Determination of DANPs and PEG-DANPs via PAGE gel electrophoresis. The PAGE gel electrophoresis displayed PEG-DANPs existing in two bands, PEG and protein, respectively; whereas there was only one band in DANPs, which represents the protein (Fig. 1).

Cell viability. Fig. 2 shows the result of the cytotoxicity of Aisu®, DANPs and PEG-DANPs against A549 lung cancer cells. A549 cells were exposed to a series of equivalent concentrations of Aisu®, DANPs and PEG-DANPs for 48 h, and the inhibition rates were determined via the MTT method. The cell survival rate had a dose-dependent inverse relationship with the drug concentrations. PEG-DANPs accelerated cellular uptake of the drug and induced higher cytotoxicity in cancer cells than Aisu® and DANPs particularly at lower DTX concentrations (0.001-0.1 µg/ml). However, A549 cells were more sensitive to Aisu® than DANPs and PEG-DANPs at higher concentrations (1-100 µg/ml). Nanoparticles are internalized into cancer cells via endocytic mechanisms (27), while the free-drug diffuses into cells according to the concentration gradient between the intracellular and extracellular environments. It is why Aisu® is more cytotoxic at higher concentrations.

PEG-DANPs increase DTX-induced apoptosis in A549 cells. DTX was described as an antimitotic agent which could bind to β-tubulin, resulting in blocking the cell cycle at the G2/M phase and apoptosis of cells (28,29). According to a previous study, encapsulation of DTX in nanoparticles could increase apoptosis of prostate cancer cells (30). Given that PEG-DTX-HANPs demonstrated stronger in vitro cytotoxicity than DANPs and Aisu®, we performed apoptosis assays using Annexin V-FITC and PI staining to compare apoptosis induction. As predicted, PEG-DANPs (55.65%) increased late apoptosis in A549 cells compared with DANPs and Aisu® (25.85 and 43.00%) (Fig. 3).

Anticancer and metastasis inhibition of PEG-DANPs in vivo. Tumor-bearing nude mice were injected with Aisu®, DANPs and PEG-DANPs, and their therapeutic effects were examined by measuring the suppression of body weight and tumor growth. The body weight of the mice in the negative control...
groups was basically unchanged or slightly increased, while the mice in the drug groups all lost weight in the process of the treatment. However, the reduction range in the Aisu group was more significant than the other two groups (p<0.01) (Fig. 4), suggesting severe systemic toxicity in addition to tumor toxicity. It was found that all the tumor volumes treated with Aisu, DANPs and PEG-DANPs were much smaller than those of negative control groups treated with the same dose (p<0.05), and the tumors of PEG-DANPs groups were obviously smaller than those of Aisu groups (Fig. 5A and B), implying that these new drug delivery systems showed improved antitumor efficiency against NSCLC.

Figure 4. Changes in the body weight of A549 tumor-bearing nude mice of the Aisu, DANPs and PEG-DANPs (male, n=10). The drugs were intravenously administered 4 times at seven-day intervals at a dose of 20 mg/kg. The body weights of the Aisu group were significantly lighter than those of the PEG-DANPs group (*p<0.01).

Figure 5. Changes in the tumor volume of A549 tumor-bearing nude mice of the Aisu, DANPs and PEG-DANPs (male, n=10). The drugs were intravenously administered 4 times at 7-day intervals at a dose of 20 mg/kg. The tumor volume of the DANPs and PEG-DANPs groups were much smaller than those of the negative control group, and PEG-DANPs group is more significant (*p<0.05, **p<0.01).

Figure 6. H&E staining of A549 tumor bearing nude mice (magnification, x200). (A) Tumor cells in transplant model. (B) Tumor cells in situ model. As seen in the control groups, the tumor cells were of different sizes with abundant deep blue stained nuclei and there was loss of polarity, tightly packed cells with ill-defined nuclei, we can even see the tumor emboli in lungs of in situ model. In the PEG-DANPs groups, the number of cancer cells was significantly reduced, the color of nuclei was stained red without any nuclear division.
indicating that PEG-DANPs is the most effective to inhibit tumor growth among the three.

A typical tumor cellular morphology was seen under the microscope in the negative control groups both in transplant model and \textit{in situ}. Regardless of the tissue, tumor or lung, we could see that the tumor cells were of different sizes with abundant deep blue stained nuclei, and the nuclear division was significant. There was also loss of polarity, tightly packed cells with ill-defined nuclei and visible focal degeneration or necrosis of cancer cells. This showed that the model had been established successfully (Fig. 6A and B). In the PEG-DANPs groups, the number of cancer cells was significantly reduced, the color of nuclei was stained red rather than light blue in DANPs and Aisu\textsuperscript{a} group, and large necrotic areas could be seen without any nuclear division.

Extensive metastases were found in the livers of control group mice, and there were large tumor nests, we could also see liver metastases both in Aisu\textsuperscript{a} and DANPs-treated mice which were significant smaller than the control groups. In contrast, liver tissue from PEG-DANP-treated mice showed barely measurable levels of tumor cells but only few neutrophils infiltrated by tumor cells (Fig. 7), these results suggest that PEG-DANPs could suppress metastases in other organs such as liver more effectively than DANPs and Aisu\textsuperscript{a}.

\textit{Survival rate of the nude mice after treatment.} Nude mice in control group were all dead on the 36th day, and the mice in PEG-DANPs group were dead on the 56th day. Fig. 8 shows that the mice which were treated with PEG-DANPs survived longer than the other groups, and the differences are significant.

\textbf{Discussion}

According to the results of our previous experiments (31), we demonstrated that PEG-DANPs presented a more sustained manner of release \textit{in vitro}, this is since DTX is encapsulated in the core portion, it has to go through the process of diffusion before release which leads to the delayed effect. Furthermore, PEG-DANPs are superior to DANPs \textit{in vitro} drug release due to the PEG on the surface of the nanoparticles. Moreover, PEG-DANPs could be also used as a platform for the incorporation of active targeting moiety (28). PEG-DANPs could not only minimize the exposure of normal tissues but also increase the accumulation of the therapeutic drug in the tumor site compared to Aisu\textsuperscript{a} (32), thus showing its potential applicability as a drug delivery system.

The PEG chain of PEG-DANPs is hydrophilic, it is currently thought to act as a protector to achieve long circulation time of drugs in the blood. It accumulated in the liver, spleen and lung, and finally was released from these organs to blood circulation according to the drug concentration gradient (29), which resulted in a sustained blood level compared to Aisu\textsuperscript{a} and DANPs. From the above results, we can make a conclusion that PEG-DANPs can not only increase the concentration and uptake of antitumor drugs in the tumors, but also prolong the time that drugs are sustained in the blood (33,34), which are the main approaches to increase antitumor activity and inhibit tumor growth in chemotherapy.

PEG-DANPs with an appropriate particle size can significantly accumulate in the tumor via the EPR effect, this is called size-dependent passive targeting. The size of PEG-DANPs is \textasciitilde{169} nm (31), it can preferentially accumulate and stay in tumor tissues compared to normal tissues. Moreover, the higher DTX concentration in blood of the PEG-DANPs group could lead to delay tumor development significantly better than the other two drugs both in transplant model and \textit{in situ}. In Fig. 4, the body weight changes in the tested mouse groups are shown. The body weight loss of the mice in Aisu\textsuperscript{a} groups was more significant than the DANPs and PEG-DANPs groups, particularly the PEG-DANPs group. These results show that
PEG-DANPs has less toxicity to normal organs and less systemic toxicity so that it can provide longer survival time. Collectively, our experiments were reported on the PEG-DANPs against NSCLC in vitro and in vivo. The in vitro cytotoxicity study proved the dose- and time-dependent manner against A549 lung cancer cells; the in vivo PEG-DANPs had superior antitumor and metastasis effects compared to DANPs and Aisu\textsuperscript{a}, and also relatively lower side-effects providing longer survival time. PEG-DANPs exerted promising therapeutic effects on NSCLC, and it is a good drug-delivery platform for the treatment of NSCLC.

References