Evaluation of the Cytotoxicity and Immune and Subacute Toxicity of Camptothecin-loaded Nanoparticles

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ABSTRACT

Camptothecin (CPT) is a natural product derived from the oriental tree Camptotheca acuminata, and this product has been reported to have an antitumor activity from a number of experiments [1]. CPT or its derivatives are effective chemotherapeutic agents for the treatment of patients with cancers, such as cancers in liver [2] breast [3,4], lung [5,6], pancreas [7], ovaries [8] and stomach [9] as well as leukemia and lymphoma [10]. CPT interferes with tumor growth by inhibiting DNA topoisomerase I [11]. CPT represents a new class of chemotherapeutic agents with broad antitumor activity since it is a human topoisomerase I inhibitor, which blocks DNA replication in human cancer cells. However, CPT has limited clinical application against cancer because of its water insolubility and toxic side effects [12,13].

The fact that CPT is difficult to apply is mainly due to its insolubility in water as well as severe side effects. To increase the water solubility of CPT and reduce toxicity, researchers developed various CPT delivery systems, including organic non-CPT compounds. Chemicals such as Indolocarbazoles, indenoisoquinolines, and dibenzonaphthyridines have been used in clinical settings [14]. Other delivery systems including pulmonary targeting microparticulate CPT delivery system [15] and nanoparticles (NPs). Among drug delivery systems, polymeric NPs have been extensively investigated because of their unique characteristics. However, the majority of polymer drug carriers have passive targeting characteristics. As such, their clinical application in cancer treatment is limited.

CPT-loaded NPs with polyethylene glycol shells can remain in circulation for a long duration [16,17], thereby prolonging the exposure of tumor cells to antitumor drugs. These drugs subsequently reach their target site during passive targeting through enhanced permeability and retention. However, CPT in passive targeting delivery still has numerous challenges associated with low target-tissue concentrations and unpredictable toxicity. For example, topotecan and irinotecan CPT analogues have been used clinically, but the hematological toxicity of these drugs is still common, including leukopenia, thrombocytopenia and haemoglobin reduction.

In our preliminary study, CPT-loaded biotinylated Pluronic F127/
polycrystalline (biodr-F127-PLA) NPs were prepared via dialysis for non-targeted CPT NPs. The targeted CPT NPs were prepared by conjugating the biodr-F127-PLA NPs with anti-CA125 antibodies. In vitro release and cytotoxicity tests were also conducted. The tissue distribution, antitumor effect in vivo, carbon particle clearance rate, and expression of survivin gene were investigated after the CPT-loaded NPs were intravenously (i.v.) administered. The in vitro release of the targeted CPT NPs exhibited a 40% initial burst within 12 h, followed by a slow release. The cytotoxicity test performed on H22 cells showed that the in vitro antitumor effects of the CPT-loaded targeted NPs were stronger than those of the CPT-loaded non-targeted NPs and the free CPT. The targeted NPs could reduce the side effects of CPT during immunosuppression. The highest CPT concentration was found in the liver of H22 cell-bearing mice that received i.v. injection of the CPT-loaded NPs. The in vivo antitumor effects of the targeted CPT NPs were superior to those of the free CPT and the non-targeted CPT NPs, which might be associated with the reduced mRNA expression of survivin during the treatment period.

Antitumor drugs often affect normal cells while eradicating tumor cells. Pluronic is a commercial family of macromolecular surfactants with the chemical name poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). It is biocompatible to some Pluronic products, such as Pluronic F127, a substance high in poly(ethylene oxide) (PEO-PPO-PEO). 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2.3.2 Each bottle was added with 3 ml of the cell suspension, inverted, and incubated in a CO₂ incubator for 24 h.

Test fluid exchange: After 24 h, the media in all bottles was discarded in a cell culture flask. The negative control group was replaced with a fresh cell culture medium. The test liquid group was also replaced by a fresh culture medium containing 50% of the test liquid. The culturing was resumed at 37°C.

Cell morphology observation and counting: The cells were observed and counted under an inverted microscope on the 2nd, 4th, and 7th days after the media was changed. To calculate the cell concentration, the cells were digested with trypsin to prepare a cell suspension. The number was then counted under a microscope with a hemocytometer.

In vivo effects of CPT NPs on the immune system of normal mice

The ICR mice were injected with free CPT, non-targeted CPT NPs, and targeted CPT NPs consecutively for 14 days, weighed, and injected with Indian ink in the tail vein (0.1 ml/10 g bw). At 2 min (t₁) and 10 min (t₂) post-injection, approximately 50 μl of blood was collected from the eyes. About 20 μl of blood was then immediately added into 2 ml of 0.1% Na₂CO₃ solution. The optical densities (OD) of the dilutions at 2 min (OD₁) and 10 min (OD₂) were measured at 680 nm using a spectrophotometer. After the mice were sacrificed through cervical dislocation, the weights of the liver and spleen were measured. The granule removal (K) and the phagocyte index (α) were calculated as follows:

\[ K = \frac{\log OD_1 - \log OD_2}{t_2 - t_1} \]

\[ \alpha = \frac{\text{Body weight}}{\text{Liver weight + Spleen weight}} \times \sqrt[3]{K} \]

Subacute toxicity of CPT NPs in normal mice

Twenty-four male ICR mice were divided into four groups (n=6 each group): 1 control group, 1 free CPT group, and 2 CPT NP groups. Each mouse was administered with 0.4 ml/20 g bw nanoparticles via i.p. injection for 28 consecutive days.

Clinical observation and mortality during the study period: All of the mice were observed daily for clinical signs of toxicity, morbidity, and mortality. Detailed clinical observations were recorded. The mice from each group were weighed once every 4 days.

Clinical pathology: At the end of the study, the mice were anesthetized with carbon dioxide, followed by an overnight fast. The mice’s blood samples were obtained through retro-orbital bleeding. Hematology measurements included white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), lymphocyte (LYM), granulocyte RDW, and platelet (PLT). Clinical chemistry measurements covered total bilirubin (BIL-T), alanine aminotransferase (ALT), aspartate aminotransferase (ALB), globulin (GLB), alkaline phosphatase (ALP), urea nitrogen (BUN), creatinine (CREA), potassium (K), sodium (Na), and chlorine (Cl).

Urinalysis: At the end of the study, each mouse was subjected to urinalysis, and results were read using a Uritest-180 urine analyzer. The measured parameters were appearance (color and clarity), glucose (uGLU), bilirubin (uBIL), ketone (KET), occult blood (RCB), acidity (pH), protein (PRO), urobilinogen (uBG), nitrite (NIT), and leukocytes (LEU).

Necropsy: All animals in the study were subjected to a detailed gross necropsy that included careful examination of the external surface of the body, all the orifices, cranial, thoracic, and abdominal cavities, as well as their contents. The control and high-dose groups were subjected to gross necropsy after an observation period of 14 days. The liver, kidneys, adrenals, testes, epididymis, uterus, ovaries, thymus, spleen, brain, and heart of all the mice were trimmed of any adherent tissue as appropriate, and their wet weight was taken as soon as possible. Organ coefficients were calculated according to organ weights and body weights. The following tissues were preserved in the most appropriate fixation medium for histopathologic examination: all gross lesions, brain (representative regions, including the cerebrum, cerebellum, and medulla/pons), spinal cord, stomach, thyroid, thymus, small and large intestines, liver, kidneys, adrenals, pancreas, spleen, heart, trachea, lungs (preserved by inflating with a fixative and then immersed), gonads, uterus, accessory sex organs, prostate, urinary bladder, lymph nodes, peripheral nerve (sciatic or tibial) preferably in proximity to the muscle, and a section of the bone marrow. Clinical and other findings might suggest the need to examine additional tissues. The relative organ weights were calculated according to the following formula: relative organ weight (%) = weight of organ (g)/body weight (g) × 100.

Histopathologic examination: Tissue and organ (all gross lesions, brain, spinal cord, stomach, thyroid, thymus, small and large intestines, liver, kidneys, adrenals, spleen, heart, trachea and lungs, gonads, uterus, accessory sex organs, prostate, urinary bladder, lymph nodes, peripheral nerve, and bone marrow) samples from the control and treated mice were cut into thin slices and then fixed with 4% formaldehyde for more than 1 week. The tissues and organs were then processed through a graded series of ethanol and xylene and embedded in paraffin. Organ and tissue sections were stained with hematoxylin and eosin (H&E) for histopathological examination under a microscope, and abnormal conditions were recorded.

A complete histopathological examination was carried out on the preserved organs and tissues of all mice in the control and high-dose groups. This examination was extended to the mice in other dosage groups if treatment-related changes were observed in the high-dose group.

Statistical analysis

Data were presented as means ± SD and examined for normal distribution by performing homogeneity of variance. One-way ANOVA was conducted to analyze the data with homogeneity of variance. Each group was compared with the control group via Dunnnett’s t test. If the data were abnormally distributed or had heterogeneity of variance, Wilcoxon–Wilcox rank sum test was used instead. Fisher’s exact probability test was used to enumerate data. The results of urinalysis from the reagent strips were analyzed using Kruskal-Wallis test, followed by multiple comparisons with Dunnnett’s t test. Clinical signs necropsy findings, and histopathological findings were represented as frequencies and subjected to Fisher’s exact probability test as appropriate. The significant probability values p<0.05(*) or p<0.01(**) were represented as asterisks.
RESULTS AND DISCUSSION

Cytotoxicity of CPT NPs

On the 2nd and 4th days, the L-929 cells of the targeted and non-targeted CPT NP groups were adherent and had triangular or irregular shapes. In spite of this, the growth experienced no suppression or interruption. On the 7th day, the cells grew well and showed normal morphology. They were adherent and had a columnar or irregular triangular shape. The suspension cells of these two groups in the test solution were slightly higher than those of the negative control group. The CPT group exhibited significantly lower growth than the targeted and non-targeted CPT NPs groups did on the 2nd, 4th, and 7th days. The CPT group had significantly higher cell death than the two CPT NP groups. CPT NPs had grade 1 cytotoxicity, whereas free CPT had grade 2 (2 and 4 days). After 7 days, the cytotoxicity of CPT NPs remained at grade 1, whereas the cytotoxicity of the free CPT reached grade 3 (Table 1).

Effect of NPs on the carbon particle clearance of mice

Immunoregulation is a complex balance in the body and any imbalance in the immunological mechanism would lead to pathogenesis. Immunity is suppressed in cancer. Many free anticancer drugs used in chemotherapy have strong side effects that deteriorate the immune system. The phagocytic capacity of peritoneal macrophages is one of the parameters that measure the nonspecific immune function of the body, and this capacity is assessed by a carbon clearance test. Table 2 shows that the clearance rate of charcoal particles increased in the free CPT group. However, the carbon clearance rate of the CPT NP group did not significantly differ from that of the control mice. The CPT NPs did not enhance phagocytosis in the normal mice, and the difference comparing to free CPT was significant (p). No significant difference was observed when these findings were compared with those of the control group. This result might be attributed to the high concentration of CPT NPs in the target tissue and their low side effects (Table 2).

Subacute toxicity of CPT NPs

Clinical signs and mortality: There was no treatment-related mortality was observed in the mice treated with CPT NPs for 4 weeks at any of the dosages tested. Also no treatment-related clinical findings were recorded in each dose group. Changes in body weight: There was no difference was observed in the body weight gain between the CPT NP group and the non-target CPT NP group compared with that of the control group. However, the body weight of the free CPT group was significantly lower than data from the two other CPT groups and the control group (p<0.05) (Figure 1).

Hematologic examination: Blood parameter analysis is essential for the evaluation of hematological lesions. The subacute toxicity of CPT NPs was examined 28 days after the drug was administered. The total number of WBC was significantly different between the free CPT and those CPT NP groups (p<.01). There was no

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**Table 1:** Cytotoxicity of CPT NPs of the mice fibroblast-like L-929 cell line.

| Groups          | 2 h RGR (%) | Toxicity level | 48 h RGR (%) | Toxicity level | 72 h RGR (%) | Toxicity level |
|-----------------|-------------|----------------|--------------|----------------|--------------|----------------|
| Control         | 100         | 0              | 100          | 0              | 100          | 0              |
| CPT             | 60.13       | 2              | 54.26        | 2              | 45.33        | 3              |
| Non-targeted CPT NPs | 81.53       | 1              | 89.21        | 1              | 91.62        | 1              |
| Targeted CPT NPs | 83.27       | 1              | 90.69        | 1              | 94.15        | 1              |

**Table 2:** Effects of CPT NPs on the carbon particle clearance in mice.

| Group                   | Carbonaceous granule removal (K) | Phagocytic index (a) |
|-------------------------|----------------------------------|----------------------|
| Control                 | 0.028 ± 0.011                    | 3.49 ± 0.72          |
| Free CPT                | 0.042 ± 0.016*                   | 5.17 ± 0.63*         |
| Non-targeted CPT NPs    | 0.030 ± 0.013*                   | 3.89 ± 0.51*         |
| Targeted CPT NPs        | 0.026 ± 0.009*                   | 3.21 ± 0.32*         |

*p<0.05 compared with the control group; *with free CPT group, p<0.05

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**Figure 1:** The body weight of subacute toxicity of CPT NPs. No difference was observed in the body weight gain between the CPT NPs group and the non-target CPT NPs group compared with that of the control group. However, the body weight of the free CPT group was significantly lower than that of the two other CPT groups and the control group (p<0.05).
significant difference was observed between the targeted CPT NPs group and the control group. The two CPT NP groups had no effects on the number of RBCs. A difference was observed in the average volume and hemoglobin content of RBCs between the CPT NP and free CPT groups, but no difference was found between the CPT NP and control groups (Table 3).

**DISCUSSION AND CONCLUSIONS**

CPT was incorporated into biotin-F127-PLA or F127-PLA polymeric NPs through a dialysis method. The targeted NPs were prepared by conjugating biotin-F127-PLA NPs with anti-CA125 antibodies. Results of this experiment indicated that the new targeted CPT NPs may reduce the toxicity of cellular and organism level. Most human nanoparticle preparations are intravenously injected. In this experiment, intraperitoneal injection is used. The drug is administered through portal vein or inferior vena cava into the systemic circulation, and there is a first-pass elimination effect, which may lead to intraperitoneal administration cannot reach the level of intravenous administration. Therefore, the different effects of the two methods need to be further observed in future experiments.

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