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Original Research Article

In vitro and *in vivo* antitumor properties of 7-epidocetaxel, a major impurity of docetaxel

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Abstract

Purpose: *To investigate the antitumor properties and toxicity of 7-epi docetaxel (7-epi DTX) as an active pharmaceutical ingredient, and in formulations.*
Methods: *Docetaxel-loaded albumin nanoparticles (DTX NPs) were prepared by freeze-drying, while 7-epi DTX was detected and isolated by high performance liquid chromatography (HPLC). Their antitumor properties were evaluated in vitro in CT26 cells and in vivo in BALB/c sk-ov-3 xenograft nude mice model. The tissues were histological examined.*
Results: *The in vivo antitumor effects of DTX NPs at different doses of 7-epi DTX were similar. Moreover, the in vitro anti-cancer effect of 7-epi DTX was comparable to that of DTX. However, the in vivo antitumor effectiveness of 7-epi DTX was inferior to that of DTX. In toxicity studies, 7-epi DTX did not elicit any acute toxic effects both as active pharmaceutical ingredients, and as a component of formulations.*
Conclusion: *The results indicate that 7-epi DTX does not elicit acute toxic effects both as an active pharmaceutical ingredient and in bulk formulations. The antitumor property of 7-epi DTX is less than that of DTX.*

Keywords: *7-Epidocetaxel, Impurity, Antitumor properties, Toxicity*

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INTRODUCTION

Impurity profile refers to the identified and unidentified impurities present in a drug substance [1-3]. It is the most important index in pharmaceutical research. Impurities are closely related to the qualities of drugs. These unwanted chemicals can affect the effectiveness and safety of pharmaceutical products, even in a small amounts [4,5]. Therefore, various pharmacopoeias explicitly stipulate the need to identify, determine and quantity the levels of impurities in drugs so as to ensure high drug quality. The amount of impurities depends on the route used in the synthesis of the active pharmaceutical ingredient (API), apart from some other influencing factors [6,7]. In principle, impurity can introduce side effects when drugs are used. These potential dangers can be eliminated by setting rigorous limits for impurities.

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Docetaxel (DTX), an anticancer agent, has been approved by FDA for treating metastatic breast cancer, non-small cell lung cancer (NSCLC), gastric cancer and ovarian cancer [8-10]. Studies have shown that docetaxel-associated impurities include 10-deacetyl baccatin III, 7-epi-10-oxo-docetaxel, and 7-epi docetaxel [11]. One of the impurities in docetaxel is 7-epi docetaxel (7-epi DTX), an isomer of docetaxel with a hydroxyl group epimerization at C7 position [12]. The limit of 7-epi DTX in API and docetaxel formulations has been stipulated [13-15]. Previous studies have reported the isolation and characterization of 7-epi DTX. However, the toxicity and anti-tumor effects of 7-epi DTX have not been reported [16]. The aim of the present study was to investigate the *in vitro* and *in vivo* toxicities and anti-tumor activities of 7-epi DTX.

EXPERIMENTAL

Materials

DTX was purchased from Taihao Pharmaceutical Co. Ltd. (Chongqing, China), while 7-Epi DTX chemical reference substances were obtained from Toronto Research Chemicals (TRC, Canada). Human serum albumin (HSA) was produced by Baxter AG (Vienna, Austria). DTX injection was manufactured by Hengrui Medicine Co., Ltd (Lianyungang, China). Other reagents were purchased from SinoPharm Chemical Reagent Co., Ltd (Beijing, China). Cell counting kit-8 (CCK-8) was supplied by Dojindo Laboratories (Japan). Cell line CT26 were cultured by State Key Laboratory of Pharmaceutical Biotechnology (Nanjing, China), while Sk-ov-3 and NCI-H1650 cells were cultured by Medicilon Pharmaceutical Co. Ltd. (Shanghai, China).

BALB/c nude mice (20 - 25 g) used were purchased from Shanghai Lab Animal Research Center (Shanghai, China). All relative experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Nanjing University.

Preparation of DTX formulation (DTX-loaded albumin nanoparticles (DTXNPs)

Human serum albumin (HSA) and DL-dithiothreitol (DTT) were dissolved in distilled water with stirring. Then, DTX dissolved in ethanol was slowly added to form the DTXNPs. The sample was dialyzed in distilled water to remove DTT, and filtered through 0.22 μm sieve. Subsequently, it was freeze-dried in RD53 lyophilizer (Millirock Inc, USA).

Forced degradation of DTX and DTXNPs

DTX was dissolved in a mixed solvent (ethanol: water = 70:30, v:v) and then subjected to thermal degradation at 90 ℃ for about 24 h. At this period, mixed solvent was added to prevent evaporation. The formed DTX-NPs were stored at room temperature and 45 ^{o}C to obtain the DTXNPs. Nanoparticles with different doses of 7-epi DTX were obtained in this way.

Determination and isolation of 7-epi DTX (analytical conditions)

High performance liquid chromatography (HPLC) was used in the detection and isolation of 7-epi DTX. Chromatographic separation was performed on LC-2010A HT (Shimadzu, Japan). The analytical condition used were: C18 column (Waters Symetryshield TM), column oven temperature of 45 ℃, and a flow rate of 1 mL/min. The gradient elution made use of water (solvent A) and acetonitrile (solvent B). Detection was performed at a wavelength of 232 nm. The impurities were eluted according using an elution gradient by changing the percentage of solvent B at different times. The mobile phase organic solvent containing 7-epi DTX was collected in about 31 minutes during the elution process. The organic solvent was removed by evaporation, and the 7-epi DTX was freeze-dried to obtain a white powder.

In vivo antitumor effect of DTXNPs

BALB/c sk-ov-3 xenograft nude mice model (tumor size of about 130 mm^3) were used for evaluating the *in vivo* antitumor effect of DTX NPs containing different doses of 7-epi DTX. The mice were assigned to 5 groups (n = 8). The treatment groups were intravenously administered DTX NPs (low dose 7-epi DTX, 7.5 mg/kg), DTX NPs (high dose 7-epi DTX, 7.5 mg/kg), DTX NPs (high dose 7-epi DTX, 15 mg/kg), and DTX injection (7.5 mg/kg; positive control). The injected dose was calculated with DTX. Each treatment was given three times at 7-day intervals. The control group was injected saline only. Tumor size was measured for 21 days after the treatment using a Vernier caliper. Tumor volume (T) was calculated as in Eq 1.

$T = (W^2 . L)/2$ ……………… (1)

where W and L are width and length, respectively.

Tumor size and body weight were monitored twice a week.

In vitro cytotoxicity of DTX and 7-epi DTX

DTX and isolated 7-epi DTX were dissolved in the commercial solvent for DTX injection, to obtain the DTX and 7-epi DTX injections. To study their cytotoxic effects, CT26 cells were maintained and seeded into a 96-well plate at a density of 5×10^3 cells per well. After incubation for 24 h, the cells were treated with DTX and 7-epi DTX injections at different concentrations. After co-incubation for 1 h, the drugs were removed and fresh culture medium was added. Cell viability was tested using CCK-8 kits after 24 h incubation. Cells treated with PBS without irradiation were used as control.

In vivo antitumor effect of DTX and 7-epi DTX

When the tumor size reached approximately 150 mm^3, DTX and 7-epi DTX injections were administered intravenously into the tumor-bearing H1650 nude mice. The mice were randomly divided into five groups (n = 8) treated as follows: saline group, DTX injection (5 mg/kg) group, 7-epi DTX injection (5 mg/kg) group, DTX injection (10 mg/kg) group, and 7-epi DTX injection (10 mg/kg) group. The treatments were given three times at 7-day intervals. During the treatment process, tumor size and body weight were monitored two times every week.

Histological examination

At the end of the treatments, the tumor and organs of each mouse were excised and fixed in 10 % formalin, processed routinely into paraffin, and cut into 8-μm slices. Hematoxylin and eosin (H & E) were used to stain the tumor slices, after which they were examined under the microscope.

Serum biochemical analysis

Whole blood was collected from each mouse and centrifuged to obtain serum. Using a biochemical Autoanalyzer (7170, Hitachi), serum biochemical analysis was carried out to determine the serum levels of alanine transaminase (ALT) and aspartate aminotransferase (AST) so as to evaluate liver function. The functionality of the kidney was assessed by determination of serum levels of urea nitrogen (UREA) and creatinine (CREA).

Statistical analysis

Data were analyzed by one-way analysis of variance for multiple groups, and two-sided Student's *t*-test for two groups. Statistical significance value was set at $p < 0.05$. All the data were analyzed by SPSS software (version 19.0, IBM, USA)

RESULTS

DTX NPs with different levels of 7-epi DTX after thermal degradation

Previous studies reported that DTX can be degraded to 7-epi DTX by heat (Figure 1). Thus, the prepared DTX NPs were subjected to harsh thermal degradation process to obtain a high dose of 7-epi DTX NPs. The quantity of 7-epi DTX in heated DTX NPs reached almost 10 %, as determined using HPLC (data not shown), while the level of 7-epi DTX in DTX NPs kept at room temperature (low dose 7-epi DTX NPs) was only 1 %.

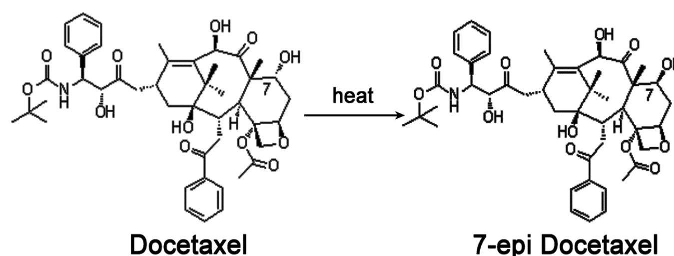

Figure 1: Degradation of docetaxel by heat

In vivo antitumor effect of DTX NPs

Figure 2 (A) shows the tumor growth curves obtained in *in vivo* assessment of the antitumor effect of TX NPs. In the saline group, the tumor volume increased rapidly from the initial average volume of about 150 mm^3 to 832.08 mm^3. Tumor growth was significantly inhibited in the groups given DTX formulations (both DTX injection and DTX NPs) on day 21. Tumor volume increases in the DTX injection and DTX NPs groups were lower than that in the control group (tumor volumes were 366.01, 444.76 and 378.64 mm^3, respectively at 7.5 mg/kg). Tumor inhibition was similar in the different DTX formulation groups administered the same dose of 7.5mg/kg. In addition, better tumor inhibition was exhibited at the higher DTX dose of 15 mg/kg, which resulted in an average tumor volume of 108.09 mm^3.

The body weights of mice treated with DTX injection and DTX NPs were both slightly decreased, relative to the weight of mice in the saline groups, with an average weight loss from 23g to 21g (Figure 2B).

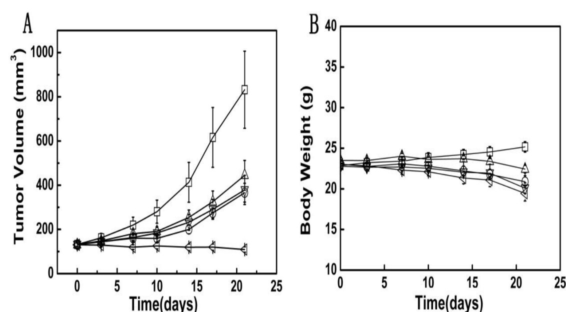

Figure 2: (A) Tumor growth curves of nude mice bearing sk-ov-3 tumor after various treatments as indicated. (B) Body weight data of mice after different treatments. □Saline; ∆DTX NPs, 7.5mg/kg, low dose 7-epi DTX; ∇DTX NPs, 7.5mg/kg, high dose 7-epi DTX; ○DTX Injection,7.5mg/kg; ◁DTX NPs, 15 mg/kg, high dose 7-epi DTX. Data are shown as mean ± SEM (n = 8)

Toxicological properties of DTX NPs

At the end of the treatment, the mice were sacrificed. Liver and kidney tissues, as well as tumor tissues were excised, processed for light microscopy and stained with hematoxylin and eosin (HE) so as to study their microstructures and apoptosis. The tumor site showed different levels of lesions, except in the saline group. Figure 3 A does not show noticeable tissue lesions in the main organs. The results demonstrate that the amount of 7-epi DTX in DTX NPs did not induce obvious tissue toxicity in the mice model. Mice serum samples were subjected to assay of liver and kidney function indices such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum urea, and serum creatinine (CREA). The results (Figure 3 B) showed that serum ALT in the group injected high dose 7-epi DTX NPs (67.14 U/L) was a little higher than ALT levels in the other groups. None of other biochemical parameters showed any statistical difference between the control and the experimental mice.

Isolated 7-epi DTX

As shown in Figures 4A & 4B, the retention time of separated 7-epi DTX was 31.18 min, consistent with the 7-epi DTX chemical reference substances (31.19 min). The purity of separated 7-epi DTX was measured and calculated by area normalization method.

In vitro antitumor effects of DTX and 7-epi DTX

As shown in Figure 5, dose-dependent cytotoxicity was observed both in DTX and 7-epi DTX groups. The cytotoxicity of DTX and 7-epi DTX increased with increase in drug contentration ranging from 0.8 to 8000 ng/mL. In addition, 7-epi DTX displayed the same level of cytotoxicity as DTX, indicating similarities in their *in vitro* anti-cancer effects.

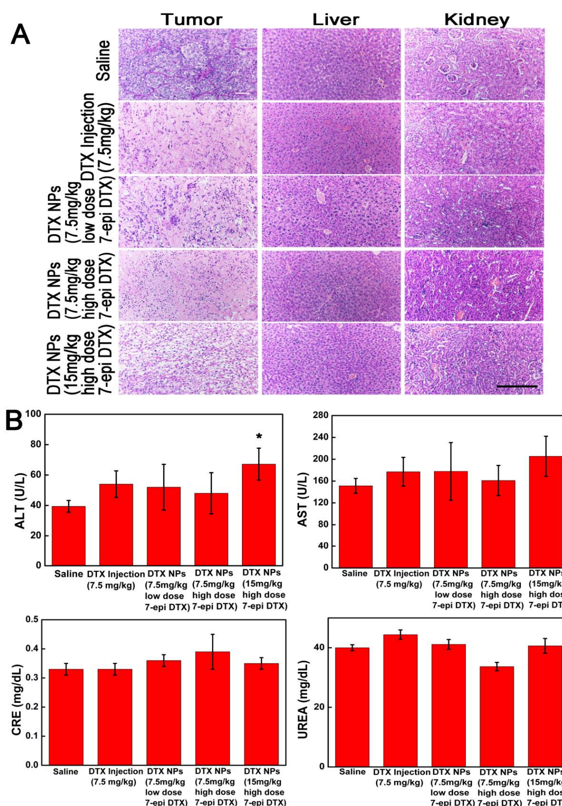

Figure 3: Toxicity of docetaxel formulations. (A) hematoxylin and eosin (H & E) staining of tumor, liver and kidney, scale bar= 50μm (B) Serum biochemistry data of mice after the various treatments. Results are mean ± SEM (n = 8)

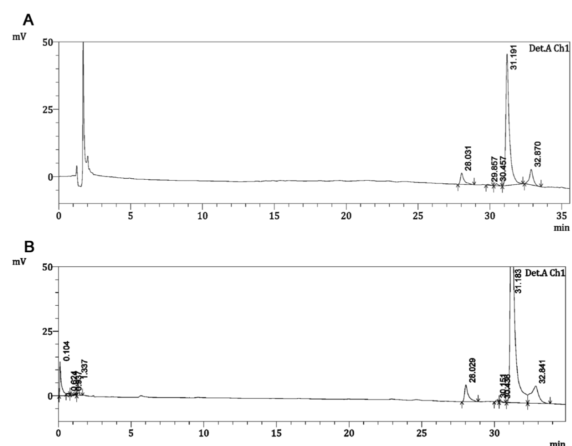

Figure 4: HPLC chromatogram of: (A) 7-epi docetaxel chemical reference substances; (B) separated 7-epi docetaxel

In vivo antitumor effects of DTX and 7-epi DTX

As shown in Figure 6 A, the tumor size of the

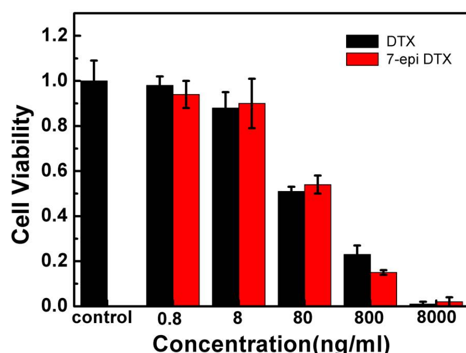

Figure 5: *In vitro* cytotoxicity of DTX and 7-epi DTX. CT26 cell viability was determined after 24 h co-incubation with 7-epi DTX and DTX injections. Each data point is mean ± SD (n = 6)

saline group increased rapidly from the initial volume of 186.84 to 1713.55 mm^3. The tumor size of the 7-epi DTX-injected group was similar to that of the control group (volume increase from 187.05 to 1813.22 mm^3). At the same dose (5 mg/kg), DTX exhibited a better tumor inhibition performance than 7-epi DTX. Moreover, a significant tumor inhibition was observed in the high-dose DTX groups. At DTX dose of 10 mg/kg, tumor inhibition was higher than that in any other group. The body weight of mice did not change significantly after the treatments (Figure 6 B).

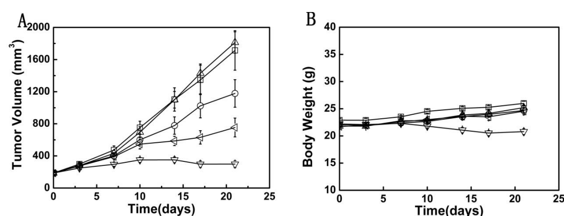

Figure 6: (A) Tumor growth curves of nude mice bearing H1650 tumor after various treatments as indicated (B) Body weight data of mice after different treatments. □Saline; △7-epi DTX Injection, 5mg/kg; ▽ DTX Injection, 10mg/kg; ○DTX Injection, 5mg/kg; ◁ 7-epi DTX Injection, 10mg/kg. Data are shown as mean ± SEM (n = 8)

Toxicological properties of DTX NPs

In Figure 7 A, the tumor site showed different levels of lesion except in the saline group. However, there were no significant injuries in the major organs (liver and kidney). To determine if DTX and 7-epi DTX produce liver of renal toxicities, liver and kidney function indices (ALT , AST, urea and creatinine) were measured in mice. There were no significant differences in biochemical indices between the different groups (Figure 7 B), indicating that DTX and 7-epi DTX did not induce toxicities in the liver and kidney.

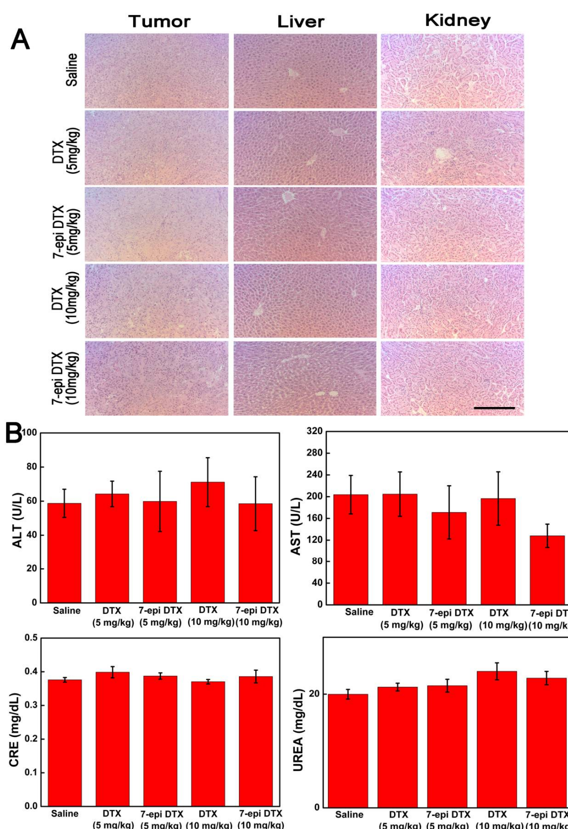

Figure 7: Toxicity of 7-epi docetaxel. (A) hematoxylin and eosin (H & E) staining of tumor, liver and kidney,scale bar= 50μm; (B) serum biochemistry data of mice after the different treatments. Values are presented as mean ± SEM (n = 8)

DISCUSSION

Many drugs are sensitive to heat and tropical temperature which result in thermal degradation. Different reactions such as hydrolysis, isomerization, rearrangement and polymerization take place during thermal degradation [17,18]. In particular, DTX can change into 7-epi DTX after a thermal degradation process. The *in vivo* antitumor efficacy of DTX NPs were not significantly affected by the dose of 7-epi DTX. The better tumor inhibition displayed by DTX NPs may be due to the high therapy dose of DTX (15 mg/kg).

Serum levels of transaminases (AST and ALT) are commonly used as sensitive markers of tissue damage, particularly liver damage. The high ALT levels indicate that DTX NPs exerted toxic effects in the liver. In addition, the organ toxicity of DTX NPs was not influenced by 7-epi DTX content. A slight decrease in body weight of mice occurred during the treatment. This may be due to the immune-toxicity of HSA [19,20].

The HPLC chromatogram demonstrated successful isolation of 7-epi DTX from DTX bulk drugs. The results of assays of *in vitro* antitumor

effect of DTX and 7-epi DTX showed that they exerted dose- dependent cytotoxic effects. Thus, it may be speculated that the pharmacological effect of 7-epi DTX is similar to that of DTX in vitro. The in vivo antitumor efficacy of DTX and 7-epi DTX(API) indicating that DTX had a better antitumor effect than 7-epi DTX at the same doses (5 mg/kg and 10 mg/kg). Toxicological studies indicated that DTX and 7-epi DTX did not elicit any acute toxic effects. The limitations of 7-epi DTX should be addressed in subsequent studies.

CONCLUSION

The results obtained in this study have demonstrated the antitumor properties and toxicity of 7-epi DTX, both as an active pharmaceutical ingredient and in bulk formulations. The findings may serve as a guide for further studies on DTX.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work

Contribution of authors

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Yiqiao Hu, Jinhui Wu; Qing Li, Ahu Yuan collected and analysed the data; Ke Jiang, Haoran Wang wrote the text and all authors have read and approved the text prior to publication.

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