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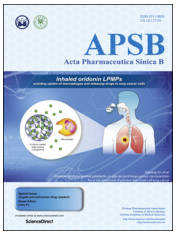

ORIGINAL ARTICLE

Lx2-32c, a novel semi-synthetic taxane, exerts antitumor activity against prostate cancer cells *in vitro* and *in vivo*

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KEY WORDS

Lx2-32c;
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Microtubule;
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Abstract Tubulin has been shown to be an effective target for the development of cytotoxic agents against prostate cancer. Previously, we reported that Lx2-32c is an anti-tubulin agent with high binding affinity to tubulin. In this study, we investigated the potential of Lx2-32c to act as an effective cytotoxic agent in the treatment of prostate cancer. MTT assays showed that Lx2-32c was cytotoxic to all tested prostate cancer cell lines. The Lx2-32c-treated cells typically exhibited a rounded morphology associated with the onset of apoptosis, as evidenced by immunocytochemical staining. Human prostate cancer cell lines treated with Lx2-32c arrest in the G2/M phase of the cell cycle and the treatment is associated with an increased ratio of cells in the sub-G0/G1 phase as determined by flow cytometry. Furthermore, expression of the cleaved form of poly (ADP-ribose) polymerase in prostate cancer cell lines treated with Lx2-32c was shown by Western blotting assay. Xenograft implants of LNCaP and PC3-derived tumors in nude mice showed that Lx2-32c treatment significant inhibited tumor growth with effects equivalent to those of docetaxel. These findings demonstrate the potential of Lx2-32c as a candidate antitumor agent for the treatment of prostate cancer.

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1. Introduction

Prostate cancer is the most common newly diagnosed cancer and the second leading cause of cancer death in men in the United States[1]. Androgen ablation remains the standard, first-line treatment for advanced, androgen-sensitive prostate cancer patients[2]. Even though this treatment can result in the regression of prostate tumors, unfortunately, most cases will subsequently progress to castration-resistant prostate cancer (CRPC)[2]. Once patients develop CRPC, cytotoxic-based chemotherapy offers one of the best strategies in terms of a significant survival benefit[3].

The use of anti-tubulin agents in the clinic was a milestone in the treatment of CRPC with chemotherapy[4]. In the cell, tubulin polymerizes to form long microtubule chains that make up the cytoskeleton. As such, it is responsible for cell shape and mitosis, and balanced microtubule dynamics are critical for cell survival. Docetaxel was the first anti-tubulin, cytotoxic chemotherapy drug that showed a proven survival benefit in the treatment of CRPC[3]. It was consequently approved in 2004 by the FDA for the treatment of CRPC. It has been well documented that docetaxel binds tubulin to shift the microtubule equilibrium toward the polymerized form, thus stabilizing microtubules, arresting cells in the G2/M phase and inducing cell apoptosis[5]. In addition to docetaxel, several other anti-microtubule agents, including cabazitaxel[6], and epothilones[7], have been employed as clinical chemotherapy drugs in the treatment of CRPC. Because of the clear therapeutic benefit of anti-tubulin chemotherapy reagents in treating CRPC, microtubules have been validated as a key target for the research and development of novel anti-prostate cancer drugs.

Lx2-32c (Fig. 1) is a novel taxane which was semi-synthesized from cephalomannine[8]. We have previously shown that Lx2-32c displays robust anti-cancer activity against several cancer types *in vitro* and *in vivo*, including several paclitaxel-resistant cancers[8,9]. Based on its high binding affinity to tubulin, which is stronger than that of docetaxel[10], we here explored the effect of Lx2-32c on prostate cancer cell growth and its mechanism of action, using validated *in vivo* and *in vitro* models.

2. Methods

2.1. Materials

Lx2-32c is prepared by the State Key Laboratory of Bioactive Substances and Functions of Natural Medicines at Institute of Materia Medica (Chinese Academy of Medical Sciences) following the previous protocol with a purity of 98% checked by HPLC[8]. Docetaxel was purchased from Beijing Shongshuo Pharmaceutical Technology Development Co., Ltd. (Beijing, China) with a purity of 99.5%. Lecithin was provided by Avanti Polar Lipids Inc., (Alabaster, AL, USA). Cholesterol was purchased from Hubei KangBaoTai Fine Chemicals Co., Ltd. (Wuhan, China). In experiments *in vitro*, Lx2-32c and docetaxel were both dissolved in DMSO and stored at −20 °C for less than 1 month before use. The vehicle (DMSO) was used as a control in all experiments at a maximum concentration of 0.1%. In the animal model, Lx2-32c liposomes were prepared according to our previously published protocol[11] and the docetaxel injection solution was obtained from Qilu Pharmaceutical Co., Ltd. (Jinan, China).

2.2. Cell lines and cell culture

The human prostate cancer cell lines LNCaP, PC3 and Du145 and the murine prostate cancer cell line RM-1 were purchased from the Cell Culture Center at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). All cell lines were cultured in RPMI-1640 media supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) (Gaithersburg, MD, USA), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. All cells were harvested during the exponential growth phase.

2.3. Animals

The BALB/c nude mice were purchased from Beijing HFK Bioscience Co., Ltd. (China). The animals were housed in a light- and temperature-controlled room (21–22 °C; relative humidity 60–65%) and maintained on a standard diet and water. All animal studies comply with the ARRIVE guidelines, and all of the experimental protocols were approved by the Committee of the Ethics of Animal Experiments of Yantai University (Yantai, China).

2.4. Cell viability assay

Cell viability was measured using an MTT assay according to our previously published protocol[12]. Briefly, the cells were seeded in a 96-well plate and incubated for 24 h, then treated with vehicle control or Lx2-32c for 72 h. MTT solution (5 mg/mL) was added into each well and incubated for another 2 h. DMSO was added and the optical density was measured at 570 nm using a Molecular Devices SpectraMax M5 (Sunnyvale, CA, USA). The 50% inhibitory concentration (IC_{50}) values were calculated.

2.5. Immunofluorescence assays

The effect of Lx2-32c on microtubule morphology was visualized by immunofluorescence microscopy[8]. LNCaP and PC3 cells were

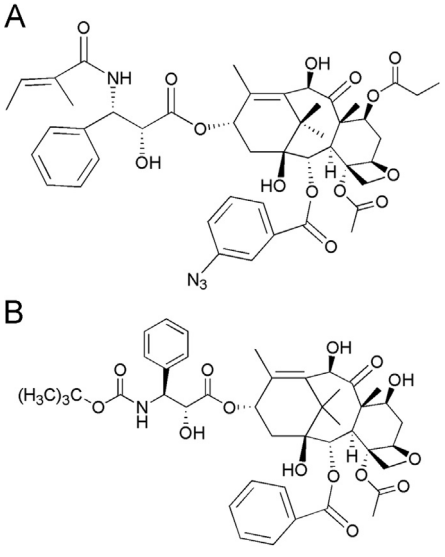

Figure 1 Chemical structures of Lx2-32c (A) and docetaxel (B).

plated at 5×10^3 cells/well in a 24-well cell culture plate and cultured overnight. After treatment for 24 h, the cells were fixed with cold 3.7% formaldehyde in phosphate-buffered saline (PBS) for 10 min, and then incubated in 0.1% Triton X for 10 min. The cells were incubated with anti–α-tubulin antibody (St. Louis, MO, USA) at 37 °C for 1 h, and re-incubated with a FITC-conjugated secondary antibody and then co-stained with Hoechst 33258 (10 μg/mL). The cells were observed under a fluorescence microscope (Tokyo, Japan), and representative images were taken of cellular microtubules.

2.6. Cell cycle distribution analysis

A flow cytometry assay was used to analyze the cell cycle distribution as previously reported[12]. Briefly, the cells were plated into 6-well plates and incubated overnight. After treatment with the test substances for 24 or 48 h, the cells were harvested and fixed in cold 70% ethanol overnight at −20 °C, and then washed with PBS and stained with PI solution (20 mg/mL PI and 20 mg/mL RNaseA in PBS) for 30 min. The cell fluorescence was measured using FACS Cytometry (San Jose, CA, USA) and the cell cycle distribution was analyzed.

2.7. Western blotting assay

Cells were treated with the test compounds for 48 h and then lysed in RIPA cell lysis buffer. After centrifugation at 12,000 rpm using the Eppendorf centrifuge (Model 5424, Germany) for 10 min at 4 °C, the cell lysates were subjected to SDS-PAGE and proteins were transferred to PVDF membranes. The membranes were blocked with 5% fat-free dried milk in TBST buffer for 2 h and probed overnight at 4 °C with rabbit monoclonal antibodies against poly ADP-ribose polymerase (PARP, Danvers, MA, USA). After incubation with the corresponding secondary antibodies, the proteins were visualized using an enhanced chemiluminescence system (Pittsburgh, PA, USA) according to the manufacturer's instructions.

2.8. In vivo anti-cancer efficacy studies

Male nude mice (4–6 weeks old, BALB/c) were introduced to established xenograft tumor models of LNCaP and PC 3 according to our previous protocols[8]. Briefly, the tumors were isolated from donor mice and implanted in the dorsa of recipient mice. When the tumors reached an average volume of 100–300 mm^3, the animals were randomized into four groups (five or six per group): (a) vehicle, (b) 10 mg/kg docetaxel injection, (c) 10 mg/kg Lx2-32c liposomes, and (d) 20 mg/kg Lx2-32c liposomes. Treatments were administered once every 3 days *via* intraperitoneal injection. Tumor diameters and body weight were measured every 3 days during treatment and the relative tumor volume was calculated. At the end of the treatment, the mice were sacrificed and tumors were removed. The rate of tumor inhibition in each treatment was subsequently calculated based on the tumor weight.

2.9. Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons were made by a one-way analysis of variance (ANOVA) test and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Lx2-32c inhibits the proliferation of prostate cancer cells

The effect of Lx2-32c on the proliferation of prostate cancer cell lines was detected by MTT assay, and the data showed that Lx2-32c displayed robust cytotoxic activity against all the tested prostate cancer cells, including the androgen-sensitive LNCaP cell line and the androgen-resistant PC3, Du145 and RM-1 cell lines (Table 1). The *in vitro* antitumor activity of Lx2-32c was stronger than, or at least comparable to, that of docetaxel when assayed under comparable conditions.

Table 1 Anti-proliferative profiles of Lx2-32c in prostate cancer cell lines.

Cell line	Sensitivity to androgen	IC_{50} (nmol/L)	
		Docetaxel	Lx2-32c
LNCaP	Sensitive	4.41 ± 1.64	2.57 ± 0.71
PC3	Resistant	9.37 ± 2.30	2.94 ± 0.57
DU145	Resistant	11.89 ± 1.56	7.17 ± 1.28
RM-1 (Murine)	Resistant	12.45 ± 2.89	4.22 ± 0.39

3.2. Lx2-32c enhances microtubule polymerization in LNCaP and PC3 cells

The effect of Lx2-32c treatment on cell microtubules was examined by fluorescence microscopy following immunofluorescent staining of tubulin[8]. As shown in Fig. 2, the untreated LNCaP and PC3 cells exhibited extensive microtubule formations in the cytoplasm. However, following the treatment with Lx2-32c, most cells exhibited an enlarged and rounded morphology with multiple nuclei. After treatment, the cells were observed to include the presence of DNA fragments, an indication of mitotic catastrophic induction[13]. Normal metaphase plates with characteristic spindle poles were rarely observed in Lx2-32c-treated cells, though they were present within control-treated cultures.

3.3. Lx2-32c arrests the cell in the G2/M phase and induces cell apoptosis

To explore the effect of Lx2-32c on the cell cycle and cell apoptosis, PI-stained cells were analyzed by flow cytometry. The data show that 20 nmol/L Lx2-32c treatment for 24 h was sufficient to arrest prostate cancer cells in the G2/M phase (Fig. 3). Additionally, the ratio of cells in the sub-G0/G1 phase was observed to increase dramatically after treatment with Lx2-32c for 24 and 48 h (Fig. 4A).

3.4. Lx2-32c induces the cleavage of PARP

To re-confirm the effect of Lx2-32c on cell apoptosis, the expression of cleaved (CL)-PARP, a biomarker of apoptosis[14], was detected by Western blot. As shown in Fig. 4B, treatment with

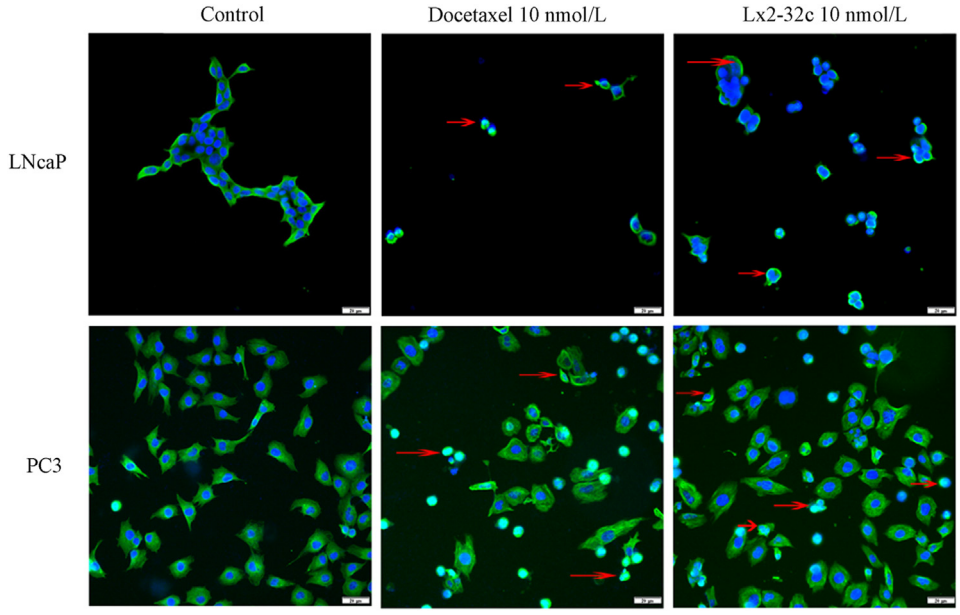

Figure 2 The effect of Lx2-32c on microtubules in LNcaP and PC3 cells. Cells were treated for 24 h with 0.1% DMSO, 10 nmol/L Lx2-32c or 10 nmol/L docetaxel. After fixation, cells were incubated with an anti-α-tubulin antibody followed by a FITC-conjugated secondary antibody and stained with Hoechst 33258 to visualize DNA. Images were overlaid electronically after cells were examined by fluorescent microscopy and the representative pictures were taken. Arrowheads indicate the altered microtubule morphology (scale bar: 20 μm).

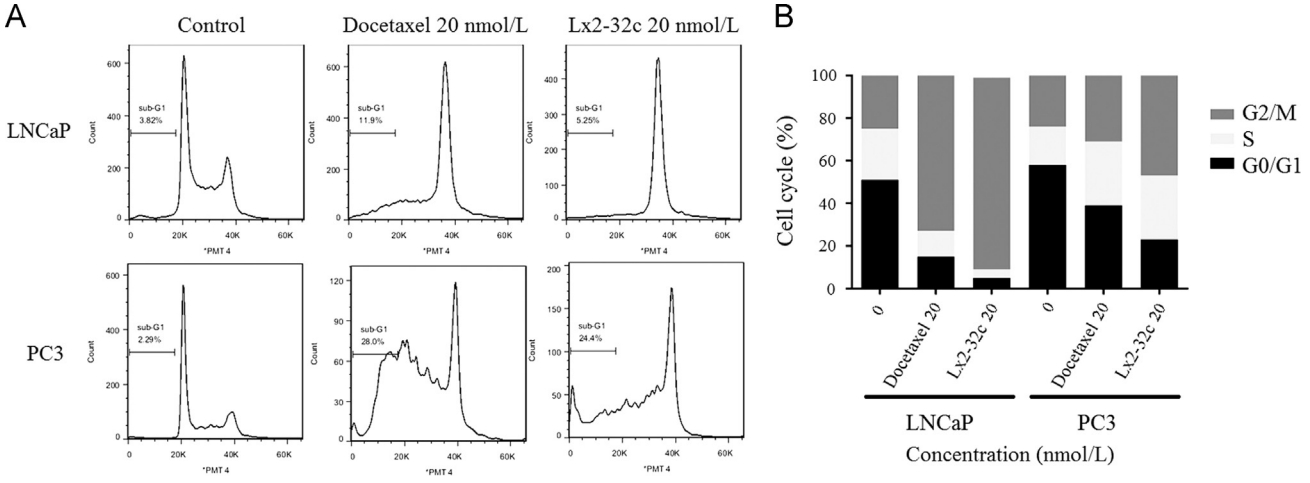

Figure 3 The effect of Lx2-32c on the cell cycle in LNcaP and PC3 cells. Cells were seeded into 6-well plates and treated with Lx2-32c (20 nmol/L) or docetaxel (20 nmol/L) for 24 h. The cells were then subjected to flow cytometry assay to determine the cell cycle (A) and analyzed (B).

Lx2-32c for 48 h resulted in the cleavage of PARP in both LNcap and PC3 cells, which is consistent with the findings of the flow cytometry assay.

3.5. *Lx2-32c possesses robust antitumor activity in vivo*

To evaluate the antitumor activity of Lx2-32c *in vivo*, xenograft models of LNCaP and PC3 were established. As shown in Fig. 5, Lx2-32c treatment was able to significantly suppress the growth of tumors derived from LNCaP and PC3 in a dose-dependent manner. The observed antitumor potency of Lx2-32c was equivalent to that for the docetaxel. As noted in Tables 2 and 3, the growth of LNCaP and PC3 tumors was inhibited by more than 87% following the administration of Lx2-32c at 10 or 20 mg/kg. An equivalent significant decrease in body weight of the treated animals was observed in both the Lx2-32c-treated and docetaxel-treated mice.

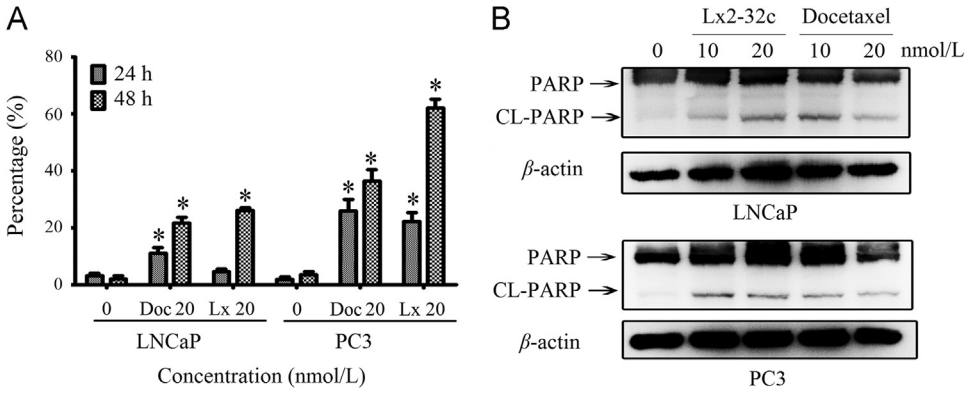

Figure 4 The effect of Lx2-32c on the apoptosis in LNcaP and PC3 cells. Cells were seeded into 6-well plate and treated with Lx2-32c (20 nmol/L) or docetaxel (20 nmol/L) for 24 h. The cells were then subjected to flow cytometry assay to determine the cell apoptosis (A) or the cells were lysated to detect the CL-PARP by Western blot (B). $^{*}P<0.05$ compared with control group. Doc, docetaxel; Lx, Lx2-32c.

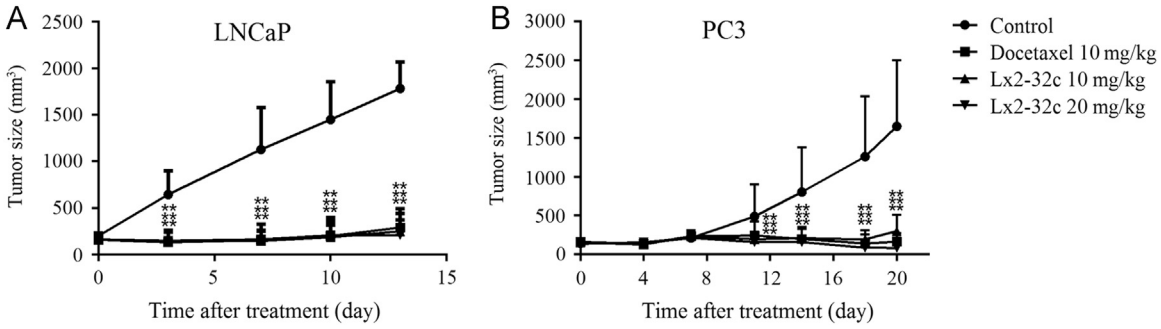

Figure 5 Effect of Lx2-32c on the growth of LNCap tumor (A) and PC3 tumor (B) in nude mice. Tumor diameter was serially measured with a vernier caliper, and the relative tumor volume was calculated and the growth curve was drawn. Vehicle (●); 10 mg/kg docetaxel (■); 10 mg/kg Lx2-32c (▲); 20 mg/kg Lx2-32c (▼). $^{**}P<0.01$ compared with control group.

Table 2 Inhibitory effects of Lx2-32c on the tumor growth of LNCaP in nude mice.

Group	Dosage (mg/kg)	Number	Body weight (g)[a]		Tumor weight (g)[a]	Inhibitor rate (%)
		Initial/end	Initial	End		
Control	0	5/5	25.9±1.2	28.4±1.1	1.34±0.67	0
Docetaxel	10	5/5	24.4±1.5	24.9±1.0*	0.08±0.07*	94.4
Lx2-32c	10	5/5	24.6±1.7	25.2±1.5*	0.19±0.10*	88.8
	20	5/5	24.8±1.7	22.4±3.2*	0.03±0.02*	97.9

[a]Data are expressed as mean±SD ($n=5$).
*$P<0.01$ compared with control.

Table 3 Inhibitory effects of Lx2-32c on the tumor growth of PC3 in nude mice.

Group	Dosage (mg/kg)	Number	Body weight (g)[a]		Tumor weight (g)[a]	Inhibitor rate (%)
		Initial/end	Initial	End		
Control	0	5/5	25.6±2.6	28.1±1.5	1.35±0.29	0
Docetaxel	10	6/5	25.1±2.7	24.8±2.9	0.13±0.11*	90.1
Lx2-32c	10	6/6	26.5±2.4	26.3±2.5	0.16±0.19*	87.8
	20	6/6	24.4±1.5	21.4±1.3*	0.14±0.15*	89.5

[a]Data are expressed as mean±SD ($n=5$ or 6).
*$P<0.01$ compared with control.

4. Discussion

Microtubules are dynamic polymeric proteins that play a key role in mitosis. They have been validated as one of the most important and successful drug targets for the treatment of different malignant tumors, including CRPC[4,15]. We report that Lx2-32c, a novel microtubule-stabilizing agent, may act as a potential antineoplastic compound against prostate cancer. By disrupting microtubule dynamics, Lx2-32c was able to arrest human prostate cancer cell lines in the G2/M phase and induce cell apoptosis. Furthermore, Lx2-32c displayed robust antitumor activity in xenograft models of prostate cancer in nude mice, indicating that Lx2-32c might be a potential drug candidate for prostate cancer treatment.

Although androgen deprivation therapy is typically used as a standard interventional treatment for advanced metastatic prostate cancer, almost all patients eventually progress to CRPC[2]. Upon reaching this stage, the microtubule-stabilizing agents, such as docetaxel and cabazitaxel, have been proven to be the most effective chemotherapy regimen[16]. The tubulin and the microtubule, therefore, had been validated as the most potential drug target for the research and development of novel agents against CRCP. Based on our previous data that the binding affinity of Lx2-32c to β-tubulin is stronger than that of docetaxel[10], we hypothesized that Lx2-32c might exert a more powerful or at least comparable effect to docetaxel in the treatment of prostate tumors. Indeed, in the MTT assay, Lx2-32c displayed stronger cytotoxic activity against all the tested prostate cancer cell lines, including androgen-sensitive LNCaP cells and androgen-independent cells PC3, Du145 and RM-1, indicating that Lx2-32c may be effective in targeting prostate cancer.

As a microtubule-stabilizing agent, Lx2-32c promotes microtubule polymerization and inhibits its depolymerization in prostate cancer cells. This was easily visualized using immunofluorescence staining. As shown in Fig. 2, the increase in the density of cellular microtubules, as well as the obvious thick bundles of microtubules, both of which are characteristic features of microtubule stabilizers[17,18], were observed in both LNCaP cells and PC3 cells treated with Lx2-32c. As microtubule dynamics were disrupted by the presence of Lx2-32c in these cells, the mitotic spindles could not be assembled correctly and cell mitosis was blocked. Consequently, the cell cycle was disrupted and mitotic catastrophe was induced, indicated by the presence of DNA fragments within the cells.

Flow cytometry was conducted to further analyze the effect of Lx2-32c on the cell cycle. Consistent with our previous findings using other cancer cell lines[8], Lx2-32c arrested both LNCaP and PC3 cells in the G2/M phase. Arresting the cell cycle in this manner appears to induce apoptosis in these cells, as demonstrated by the increased ratio of cells in the sub-G0/G1 phase following treatment with Lx2-32c. Similar results were observed in the Western blot assay, in which CL-PARP, a well-known apoptosis biomarker, was induced in Lx2-32c-treated cells. Taken together, these findings show clearly that Lx2-32c is able to disrupt microtubule dynamics by binding tubulin to arrest prostate cancer cells in the G2/M phase and subsequently inducing apoptosis.

Finally, the xenograft model of prostate cancer cells was established using nude mice to explore the *in vivo* growth inhibitory activity of Lx2-32c. To avoid any hypersensitivity reactions induced by Cremophor EL castor oil[19], the common solvent for taxanes, Lx2-32c liposomes were prepared and used in all animal studies[11]. The data show that Lx2-32c is able to inhibit the growth of xenograft tumors from both prostate cancer cell lines tested, and that the antitumor activity was comparable to that of docetaxel, the first-line drug currently used to treat prostate cancer in clinics. Both Lx2-32c and docetaxel were observed to exhibit slight to moderate toxic effects to the animals based on the evidence of weight loss in treated animals.

5. Conclusions

In summary, our study demonstrates that Lx2-32c, a novel microtubule-polymerizing agent, can arrest prostate cancer cells in the G2/M phase and induce cell apoptosis by disrupting microtubule dynamics, and that this compound displays robust antitumor efficacy *in vivo*. These findings indicate the potential of Lx2-32c as a candidate antitumor agent for the treatment of prostate cancer.

Acknowledgments

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