Polydopamine-based surface modification of paclitaxel nanoparticles for osteosarcoma targeted therapy

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Abstract

In order to achieve the purpose of targeting treatment of osteosarcoma, we developed novel paclitaxel (PTX) nanoparticles (Nps) coated with polydopamine (PDA) and grafted by alendronate (ALN) as ligand. Dopamine can be easily polymerized on various surfaces to form a thin PDA film in alkaline environment, which provided a versatile platform to perform secondary reactions for compounds without functional groups. The targeting Nps had a mean particle size of 290.6 ± 2.2 nm and a zeta potential of −13.4 ± 2.7. It was stable in phosphate buffer saline (PBS, pH 7.4), 5% glucose, plasma and displayed sustained drug release behavior. In vitro assay demonstrated the targeting Nps had stronger cytotoxicity against K7M2 wt osteosarcoma cells than the non-targeting Nps. Furthermore, in vivo distribution study indicated they could accumulate much more in tumor than non-targeting Nps. This is consistent with the in vivo antitumor study, targeting Nps achieved a better therapeutic effect than Taxol (8 mg kg⁻¹, i.v.) (71.85% versus 66.53%) and prominently decreased the side effects of PTX. In general, the PTX-PDA-ALN-Nps may offer a feasible and effective strategy for osteosarcoma targeted therapy. 

Supplementary material for this article is available online

Keywords: polydopamine, nanoparticles, osteosarcoma, alendronate, targeted therapy

(Some figures may appear in colour only in the online journal)

1. Introduction

Osteosarcoma is the most ordinary bone neoplasms, occurring predominantly in teenagers and children with practically 400 new cases every year in the United States [1–5]. It is characterized by high proclivity for early systemic metastases (such as lung metastasis) and local invasion [6, 7]. According to the National Cancer Institute database of the USA, the five year survival rates of patients diagnosed with metastasis...
cancer is only 5%–20% [8, 9]. Although chemotherapy has extremely enhanced the survival in patients with osteosarcoma, some severe problems still remain, including relapse, metastatic disease and serious side effects. In addition, some patients do not respond to chemotherapy and others showed multidrug resistance [10–12]. Thus, improving targeted chemotherapeutic drugs is an important approach for the treatment of osteosarcoma. [13–15].

Paclitaxel (PTX) could congest cancer cells at the G2/M phase and stabilized microtubules to stimulate apoptosis. Furthermore, PTX exhibits significant antitumor activity against osteosarcoma by enhancing nuclear factor-B (NF-B)’s expression, a transcription factor, which adjusts various physiological processes, such as differentiation, apoptosis and inflammation [16, 17]. Hence, we chose PTX as a model drug for the treatment of osteosarcoma. However, the low bioavailability, high toxicity and poor water solubility of PTX extremely hamper its therapeutic effect and limit its clinical application [18]. Among various nanoscale drug delivery systems, Nanoparticles (Nps) formulations have been studied for the transport of insoluble drugs, including chemotherapeutic drugs [19]. Nps always have high drug loading and can be straightforwardly injected intravenously in the form of solid particles, which can be prepared by bottom-up or top-down method [8, 20, 21]. P188 (or Pluronic F68) is a poloxamer which can be used to as a stabilizer and improve the solubility, absorption and bioavailability of Nps [22]. In order to increase the therapeutic efficacy, we prepared PTX into Nps to improve its poor solubility. In this manuscript, Doxorubicin Chloride was used as surfactant, which could decrease surface tension and be used as stabilizer in nanoparticle system [23, 24].

Targeted Nps are a feasible method to reduce the side effects and improve the locally effective drug concentration at tumor [25–27]. By the way of grafting a variety of active targeting agents (peptides, antibodies and nucleic acids), drugs can be delivered specifically by the formulation of Nps to the tumor cells and tissues. Tetracycline, small peptide aspartic acid, alizarin red and bisphosphonate are recognized to the tumor cells and tissues. Tetracycline, small peptide targeting agents [13], etidronate, which are commonly adopted as bone-targeting ligands [33], can be changed by the added ligands, the pre-functionalized materials can not be modiﬁed diametrically through functional ligands [33]. In addition, the polymers’ chemical properties could be changed by the added ligands, the pre-functionalized polymers may lose its capacity to encapsulate and keep the drug. To resolve this problem, dopamine polymerization was used as a method to functionalize the surfaces of Nps [34]. Dopamine can self-polymerized and deposition on various materials’ surface to emerge a thin polymerized dopamine (PDA) film. More importantly, PDA can easily immobilize functional ligands (nucleophiles containing amine and thiol groups) on its surface by Schiff-base reaction and Michael addition reaction [35]. All this method required was just a short incubation of Nps in weak alkaline solution with dopamine, then added ligands in the solution for secondary reaction [17, 36, 37]. Using this method, we have fabricated a PDA-coated PTX-Nps with ALN (PTX-PDA-ALN-Nps) through surface modification for osteosarcoma targeted therapy.

2. Materials and methods

2.1. Materials

PTX with 99% purity was obtained from Coupling Technology Co. Ltd (Beijing, China). ALN was supplied by Wuhan Jingchu Chen Pharmaceutical Chemical Co. Ltd (Wuhan, China). Poloxamer (P188) and Doxacurium Chloride were supplied by Beijing Benove Biological Technology Co. Ltd (Beijing, China). Dopamine-Hcl and Tris-Hcl (PH 8.8) were obtained from the Milky Way Beijing Tianhong Chemical Co. Ltd (Beijing, China). PTX injections were purchased from the Beijing union pharmaceutical factory (Beijing, China). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-triazolium bromide (MTT) was provided by Sigma-Aldrich Co. Ltd (St Louis, MO, USA). DiR was supplied by AAT Bioquest Inc. (Sunnyvale, CA, USA). Phosphate buffer saline (PBS) was purchased from Broadcom industrial biotechnology Co., Ltd (Beijing, China). DMEM fetal bovine serum was purchased from Gibco, Invitrogen Corp. (Carlsbad, CA, USA). Deionized water was used in the experiments. All the other chemicals were commercially available reagents of at least analytical grade.

2.2. Cell line and animals

The K-M2 wt osteosarcoma cell lines were obtained from the Cell Culture Center, Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences (Shanghai, People’s Republic of China). Six-to eight-week-old BALB/c mice (female, 20 ± 2 g) were supplied by the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). The Guidelines for Ethical and Regulatory for Animal Experiments as defined by Institute of Medicinal Plant Development (IMPLAD), China were performed in all animal experiments.

2.3. Preparation of PTX-Nps and PTX-PDA-ALN-Nps

Briefly, PTX (50 mg) was put in ethanol (1 ml) then transferred into 10 ml aqueous with poloxamer 188 (50 mg) and doxacurium chloride (20 mg) under magnetic stirring (1200 rpm) for 20 min at room temperature. Then the suspension was stirred for 20 min at the same speed and ultrasound at 100 W for 5 min. The PTX-Nps were obtained by centrifugation (13 000 rpm, 15 min) and washed three times by deionized water (preparation procedure one).

The resultant PTX Nps were immersed by 1 ml Tris buffer (10 mM, pH 8.8) containing 0.5 mg of dopamine and stirred constantly for 2 h at room temperature (preparation procedure two). Subsequently, the PDA coated PTX Nps were collected by centrifugation (13 000 r min⁻¹, 10 min).
ALN was adopted for particle surface functionalization by conjugating with PDA coatings. Simply, 10 mg of ALN was dissolved in 10 mM Tris buffer with pH 8.8 and PDA coated PTX Nps were added in. After constant stirring for 3 h, the resultant Nps were centrifuged (13 000 rpm, 10 min) and washed three times with deionized water to obtain PTX-PDA-ALN-Nps (preparation procedure three).

To obtained Dir labeled Nps, ‘PTX and Dir (w/w = 40:1)’ was put together in ethanol instead of ‘PTX’, then ‘preparation procedure one, two and three’ were repeated. To obtained drug-free Nps, ‘1 ml ethanol (without PTX)’ was transferred into 10 ml aqueous with poloxamer 188 (50 mg) and doxorururide chloride (20 mg), then ‘preparation procedure one, two and three’ were repeated.

2.4. Characterization of PTX-PDA-ALN-Nps

2.4.1. Particle size, zeta potential and morphology. The particle size, zeta potentials and polydispersity index (PDI) of PTX-Nps, PTX-PDA-ALN-Nps were measured by dynamic light scattering (Zetasizernano 2S, Malvern Instruments, UK) at room temperature. Every specimen was measured in three times and the average ± standard deviation were used. The Transmission Electronic Microscopy (TEM, JEOL Ltd, Tokyo, Japan) was employed to ascertained the morphology of Nps. The Nps were suspended in 1% double color pomelo and dropped on copper grids.

2.4.2. Drug loading capacity (LC) and encapsulation efficiency (EE). The amount of PTX was detected by high-performance liquid chromatography (HPLC; Ultimate 3000, DIONEX, USA) for measuring the LC and EE of nanoparticles. The drug-loaded nanoparticles were suspended in methanol and vortexed for 3 min. After that, the supernatant was obtained by centrifugation (13 000 r min⁻¹, 5 min) and transferred into mobile phase, which consist with water and acetonitrile (50:50, v/v). A C18 column (5 μm, 250 × 4.6 mm, Waters Symmetry) was adopted at 35 °C under a UV–vis absorption at 266 nm, with a flow rate of 1.0 ml min⁻¹, and an injection volume of 20 μl. The following equations (1), (2) were used to calculated the LC and EE of nanoparticles, respectively

\[
LC(\%) = \frac{\text{The weight of PTX in liposomes}}{\text{Weight of liposomes}} \times 100\% \tag{1}
\]

\[
EE(\%) = \frac{\text{Total PTX} - \text{uncapsulated PTX concentration}}{\text{Total PTX concentration}} \times 100\% \tag{2}
\]

2.4.3. Fourier transform infrared (FT-IR) spectroscopy analysis. The FT-IR spectroscopy analysis of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps was measured by FT-IR spectrophotometer (Thermo Nicolet, Madison, WI, USA) using KBr.

2.4.4. X-ray photoelectron spectroscopy (XPS). PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps was recorded by XPS (Thermo escalab 250Xi, USA) with monochromatic Al Kα (hv = 1486.6 eV). Survey and high-resolution scans were obtained for C 1s (284.80 eV) and N 1s (160 and 20 eV).

2.4.5. Stability of PTX-PDA-ALN-Nps in various physiological solutions. To evaluate the suitability of PTX-PDA-ALN-Nps for intravenous injection, the size change in various physiological media including physiological saline, isotonic glucose (5% glucose), phosphate buffer saline (PBS, pH 7.4) and plasma were performed. PTX-PDA-ALN-Nps were mixed (1:1, v/v) with 2 × PBS (pH 7.4), 10% glucose, 1.8% NaCl and mice plasma (1:4, v/v), then incubated at 37 °C. Particle size and distribution at particular times were measured and each sample was performed for three times.

2.5. Hemolytic assay

The fresh anticoagulated blood from the health mice eye canthus was centrifuged (5000 rpm, 15 min) to eliminate the fibrous proteins, then washed by normal saline twice and diluted with the ratio of 4% (v/v). Different concentrations of PTX-PDA-ALN-Nps (0.06–1 mg ml⁻¹) adjusted to isotonic by NaCl were confused with 4% red blood cell suspensions (1:1, v/v) and the suspensions were incubated for 4 h at 37 °C after that centrifuged for 5 min (5000 rpm). The supernatants were collected to measure the absorbance at 540 nm by ELISA plate reader (Biotek, Winooski, VT). The negative control and positive control were 0.9% NaCl and deionized water, respectively. The hemolysis percentage (%) was counted by the following equation (3):

\[
\text{Hemolysis percentage(\%) = } \frac{A_{\text{sample}} - A_{\text{positive}}}{A_{\text{positive}} - A_{\text{negative}}} \times 100\% \tag{3}
\]

where, \(A_{\text{sample}}\) is the absorbance of the PTX-PDA-ALN-Nps, \(A_{\text{negative}}\) is the negative control’s absorbance, and \(A_{\text{positive}}\) is the positive control’s absorbance. Each samples were analyzed in three times.

2.6. In vitro drug release kinetics

The in vitro release characteristic of PTX from PTX-Nps, PTX-PDA-Nps, PTX-PDA-ALN-Nps were investigated by using dialysis. Briefly, Nps were suspended in regenerated cellulose dialysis bag (MWCO, 8000–14,000, Sigma, USA) with 40 ml phosphate buffer saline (PBS, pH 7.4) and 0.2% Tween 80. The tube was moved into a water bath with the temperature of 37 °C and shaken at 120 rpm. Then 1 ml sampled was removed at the indicated time, and centrifuged at 10 000 rpm for 10 min. The sampled supernatant was used for HPLC analysis and replaced with fresh release medium. Each batch of experiments was performed in three times.

2.7. In vitro cytotoxicity assay

The cytotoxicity of the PTX-PDA-ALN-Nps was calculated by the MTT assay. Typically, 150 μl K2Mg2 wt osteosarcoma cells were plated on 96-well plates (8000 cells/well) and grown overnight in a humidified atmosphere of 5% CO₂ at
After that, the K7M2 wt cells were incubated with different PTX concentrations (1, 5, 10, 50, and 100 μg ml\(^{-1}\)) of PTX-Nps, PTX-PDA-Nps, PTX-PDA-ALN-Nps, PTX injection and blank Nps for 24, 48, and 72 h. The medium was replaced with 20 μl of DMEM medium containing 5 mg ml\(^{-1}\) MTT and incubated for 4 h. Finally, the medium was replaced by 150 μl DMSO, then the absorbance was investigated by an ELISA plate reader (Biotek, USA) at 570 nm. The cell inhibitory rate was analyzed as follows:

\[
\text{Cell inhibitory rate} \% = 1 - \frac{OD_{\text{experiment}}}{OD_{\text{control}}} \times 100.
\]  

(4)

The GraphPad Prism, Version 5 (Inc., La Jolla, CA) was used to calculate the half maximal inhibitory concentration (IC\(_{50}\)).

### 2.8. In vivo distribution study

The real-time fluorescent imaging was adopted to evaluate the distribution of PTX in the Balb/c mice with K7M2 wt tumors (volume about 100 mm\(^3\)). The mice were intravenously injected with 2 mg kg\(^{-1}\) PTX/DiR-Nps, PTX/DiR-PDA-Nps, PTX/DiR-PDA-ALN-Nps (PTX: DiR = 40:1), respectively. Living Image Software 4.4 (PerkinElmer USA) was used to detect fluorescent images at 1, 4, 8, 12, 24 h. All the mice were used in quantitative analysis. The relative intensity was calculated according to the following equation (5):

\[
\text{Relative intensity} = \frac{\text{fluorescence}_{\text{tumor}}}{\text{fluorescence}_{\text{liver}}}. 
\]  

(5)

### 2.9. In vivo antitumor activity

*In vivo* antitumor effect of PTX-PDA-ALN-Nps was performed on K7M2 wt tumor-bearing Balb/c mice. The mice were inoculated subcutaneously with 0.2 ml of K7M2 wt tumor cells (5.0 × 10\(^6\) cells ml\(^{-1}\)) on the right armpit. The K7M2 wt tumor-bearing mice were stochastically grouped into five (eight mice per group), while the tumor volume reached almost 100 mm\(^3\). The mice were treated with

\[
V = \frac{\text{length} \times \text{width}^2}{2}.
\]  

(6)

The tumor inhibition rate (TIR) was calculated using the following equation (7):

\[
\text{TIR(\%)} = 1 - \frac{\text{Tumor weight}_{\text{experimental group}}}{\text{Tumor weight}_{\text{negative group}}} \times 100\%.
\]  

(7)

Liver (Spleen) index

\[
= \frac{\text{Liver (Spleen) weight}}{\text{body weight}} \times 100\%.
\]  

(8)

### 2.10. Statistical analysis

The statistical analysis was carried out by the software of IBM SPSS Statistics, version 19 (IBM Co., Armonk, NY). Comparison of liver and spleen index effect between Taxol and normal saline solution by the independent samples T test. Other comparisons (cell inhibitory rate, antitumor rate) were based on a one-way analysis of variance and post hoc analysis (F-test). \(p < 0.05\) was considered statistically significant.
3. Results and discussion

3.1. Preparation of nanoparticles

The preparation technique of PTX-PDA-ALN-Nps is schematized in figure 1. The PTX-PDA-ALN-Nps were prepared in three steps. Firstly, PTX Nps were synthesized according to the procedure one (prepared at 2.3). Secondly, PTX-Nps were immersed in 1 ml Tris buffer (10 mM, pH 8.8) with 0.5 mg of dopamine to coat polydopamine on the Nps. Finally, ALN was added in the weak alkaline solution to conjugate on the surface of PTX-PDA-ALN. When dopamine hydrochloride was added, the suspensions began to turn dark, which indicated the dopamine had smoothly polymerized [34]. Then, the amine group of ALN covalently conjugated to PDA via Schiff base or Michael addition reaction. Based on the above three parts, we successfully conjugated ligand (ALN) to the PDA-coated PTX-Nps.

The principle of PDA was first discovered in 2007 and published in Science, which could be used on various surface of inorganic and organic materials (noble metals, oxides, polymers, semiconductors, and ceramics) [35]. The catechol of dopamine

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**Figure 2.** DLS size distribution (A)–(C) and TEM images (D)–(F) of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps ($n = 3$).
was oxidized to quinone, and then reacts with other catechols and/or quinones to form polymerized dopamine (pD) [38]. While the PDA method has not been used in the modification of polymeric nano-carriers, until 2014. Joonyoung Park et al. functionalized the surface of PLGA NPs with folate, cRGD, and a stealth polymer, and observed the influence of pH, initial DA concentration and incubation time on the film formation [33]. As the concentration of dopamine increases, the increase in particle size can be interpreted as an indication of micron pD aggregates. Increased size of PTX-PDA-ALN-Nps and PTX-PDA-Nps can be used as an indicator of quantitative density. The size of PDA coated nanoparticles were stable during 14 d at room temperature (figure S1 is available online at stacks.iop.org/NANO/30/255101/mmedia), which means the PDA film was stable. However, there is some evidence that the structures of PDA were stable at physiological pH, but unstable in acidic conditions [39, 40], which is why some PDA-coated nanoparticles are pH sensitive.

3.2. Verified the conjugation of ALN on PTX-PDA-Nps

Size and surface properties of Nps induces significant differences in drugs cellular uptake, drug release, bio-distribution and in vivo pharmacokinetics. Small size Nps with relatively high surface area can pass through the leaky tumor micro vessels into tumor site, which is known as enhanced permeability and retention effect (EPR effect) [41, 42]. As shown in table 1, The particle sizes were compared between different groups with the result that PTX-Nps (251.8 nm) < PTX-PDA-Nps (286.4 nm) < PTX-PDA-Nps-Aln (290.6 nm). Perhaps due to the presence of PDA film, the size increased approximately 40 nm, which also proves the successful preparation of PTX-PDA-ALN-Nps [43]. The size distribution of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps was illustrated in figures 2(A)–(C). The PTX-PDA-ALN-Nps have high LC (34.14%) and EE (80.32%).

Zeta potential is crucial for the stability of the Nps. We can reach a conclusion that the value of zeta potential of Nps were slightly increased after coating with dopamine. This result may be caused by PDA coating, which is a kind of negative polyelectrolytes.

The morphology of Nps can be investigated through the transmission electron microscope (TEM), and showed in figures 2(D)–(F). However, there was no noticeable difference nor visible surface feature attributable to PDA aggregates between these three groups of Nps under TEM. This may be due to the low magnification of transmission electron microscopy and the concentration of dopamine [33]. To verify the surface chemical group composition of Nps and the successful modification of PDA film, FT-IR spectroscopy was adopted in this study. There are some new absorption signals emerged after surface modification (figure 3). All of the three experimental groups exhibited a strong peak at 3477 cm\(^{-1}\), indicating the presence of hydroxyl groups stretching vibration, including surface adsorbed water. At the same time, due to the N–H and O–H stretching modes of ALN and PDA, we can notice that PTX-PDA-Nps and PTX-PDA-ALN-Nps were increased than PTX-Nps at 3477 cm\(^{-1}\). The 1736 cm\(^{-1}\) band significantly decreased in PTX-PDA-Nps that proving the PDA-coating was conjugated on Nps [44]. The absorption peaks at 1654 and 1579 cm\(^{-1}\) were caused by the N–H bending vibrations and C=C resonance vibrations in the aromatic ring [41]. The increased or decreased of these characteristic absorption bands was all from PDA or ALN.

High-revolution XPS spectra were used for further prove the successful modification of PDA and conjugation of ALN (Sirova et al. 2016, Kim et al. 2017). XPS (N1s and C1s) of the Nps were displayed in figure 4. PTX-PDA-Nps and PTX-PDA-ALN-Nps had two nitrogen peaks (N1s) at \(~398.6\) eV (non-protonated amine groups) and \(~402.4\) eV (protonated amine groups), but PTX-Nps only owned one peak at \(~398.6\) eV. Moreover, the nitrogen peaks of PTX-PDA-ALN-Nps were more intense than that of PTX-PDA-Nps and PTX-Nps. This provides some evidence that the conjugation of ALN on the surface of PTX-PDA-Nps [45]. The narrow scan for C1s peaks was used to verify the coating of polydopamine and the functionalization of Nps as well (figures 4(D)–(F)). The carbon peaks (C1s) increase intensity of PTX-PDA-Nps and PTX-PDA-ALN-Nps at \(~286.3\) and \(~284.7\) eV were resulted from the C–O groups and C–C groups of PDA films. Furthermore, the intensity of peaks at \(~286.3\) and \(~284.7\) eV for PTX-PDA-ALN-Nps were both larger than the other two groups, demonstrating the ALN were conjugated on PTX-PDA-Nps successfully. Both FT-IR and XPS experiments verified the successful coated of PDA film and conjugation of ALN on the surface of Nps [46].

3.3. PTX-PDA-ALN-Nps were stable in physiological media and showed no hemolysis

PTX-PDA-ALN-Nps were stable in isotonic glucose (5% Glucose), phosphate buffer saline (PBS, pH 7.4) and plasma after incubation at 37 °C for 8 h. There were no visible particle size increase or aggregation among the three physiological media (\(P ^{+} 0.05\)). Many enzymes or serum albumin in plasma
Figure 4. XPS spectra analysis of PTX Nps, PTX-PDA- Nps and PTX-PDA-ALN- Nps. (A)–(C) Narrow scan for N1s peaks; (D)–(F) narrow scan for C1s peaks.
can be adsorbed on the surface of Nps, which in some cases leads to aggregation or clogging of blood [47]. Besides, PTX-PDA-ALN-Nps retained their size in plasma during 8 h, suggesting its excellent plasma stability (figure S2). Furthermore, PTX-PDA-ALN-Nps revealed no hemolysis under 1 mg ml$^{-1}$, which proved that PTX-PDA-ALN-Nps were suitable for intravenous administration (figure S3).

3.4. PTX-Nps possessed sustained release property

In vitro drug accumulation release of the PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps during the 120 h were shown in figure 5. The drug release of PTX-PDA-ALN-Nps was from 1% to 69% within 120 h. The PTX-Nps showed steady, continuous release patterns, just like the drug release profiles of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps, which indicating that PDA coating had no effect on the drug release. Perhaps PTX was excellently wrapped in Nps, which keeping a sustained release effect [44]. The release kinetic models (zero-order release) and t$^{1/2}$ (half the time of the release of the drug) of each Nps were calculated and shown below

PTX-Nps,

$$y = 0.6353x + 2.1958 \ (R^2 = 0.9826), \ t^{1/2} = 75.40 \ h.$$ 

PTX-PDA-Nps,

$$y = 0.6257x + 1.2689 \ (R^2 = 0.9825), \ t^{1/2} = 77.88 \ h.$$ 

PTX-PDA-ALN-Nps,

$$y = 0.5694x + 1.4354 \ (R^2 = 0.9882), \ t^{1/2} = 85.29 \ h.$$ 

3.5. PTX-PDA-ALN-Nps exhibited greater cytotoxicity on K$_{aM_2}$ wt cells

MTT assay was carried out in k$_{aM_2}$ wt cells to assess the cytotoxicity of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps.
Taxol and drug-free Nps was selected as control group. K7M2 wt cells were treated with four groups Nps and Taxol at 1, 5, 10, 50 and 100 μg ml$^{-1}$ for 24, 48 and 72 h respectively. Figures 6(A)–(C) indicated that the cell viability declined with the enhance of incubation time for all experimental groups, showing time and dose-dependent effect, especially for PTX-PDA-ALN-Nps\[29\]. Furthermore, these data also clearly demonstrated the PTX-PDA-ALN-Nps had better cytotoxicity than clinical used Taxol and other non-targeted Nps at 48 and 72 h. This may be related to the slow release of PDA-modified of Nps and the targeting effect of ALN, as we know, ALN could be combined with protein tyrosine phosphatase that is over expressed in osteosarcoma cells (Morelli et al 2011). Drug-free Nps did not exhibited significant cytotoxicity on K7M2 wt cells, demonstrating the copolymer, PDA coating and ALN have no toxicity and good biocompatibility to cells.

The half maximal inhibitory concentration (IC$_{50}$) means that causes 50% cells decease in a designated period to assess the therapeutic effects. The IC$_{50}$ values of K7M2 wt cells after 24, 48 and 72 h incubation were showed in Table 2. PTX-PDA-ALN-Nps (48.12 ± 2.16, 7.89 ± 0.22 and 5.81 ± 0.16 mg ml$^{-1}$ for 24, 48 and 72 h, respectively) were significantly lower than other experience groups (>50 mg ml$^{-1}$).

### Table 2. Targeting PTX-PDA-ALN-Nps exhibited higher accumulation at the tumor site.

| Incubation time (h) | IC$_{50}$ (mg ml$^{-1}$) |
|---------------------|--------------------------|
|                     | PTX Nps | PTX-PDA- Nps | PTX-PDA-ALN- Nps | Taxol |
| 24                  | >50     | >50          | 48.12 ± 2.16     | 17.24 ± 1.26 |
| 48                  | >50     | >50          | 7.89 ± 0.22      | 9.08 ± 0.14  |
| 72                  | 18.20 ± 2.23 | 14.90 ± 0.23 | 5.81 ± 0.16      | 7.12 ± 0.18  |

Figure 7. The distribution of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps in K7M2 wt tumor-bearing mice (A) The distribution of different PTX formulations in K7M2 wt tumor mice at various time point. (B) Average fluorescence intensity of tumors/average fluorescence intensity of liver at various time point. All data represent the mean ± SD ($n=5$ $^*$$P<0.05$, $^{**}P<0.01$).

Figure 8. In vivo anti-tumor activity of PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps towards K7M2 wt tumor-bearing mice (A) Tumor growth curves, (B) changes of body weight. (mean ± SD, $n=8$). $^*$$p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. 

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for 24 h or 48 h and >14 mg ml\(^{-1}\) for 72 h). PTX-Nps and PTX-PDA-Nps also showed similar cytototoxicity under different concentrations, which further evidenced that PDA coating had no effect on cell viability, toxicity and biocompatibility [31].

3.6. Targeting PTX-Nps exhibited higher accumulation at the tumor site

Non-invasive near-infrared optical imaging technology was adopted to evaluate the bio-distribution and tumor-targeting efficiency of the Nps. The Dir was entrapped in PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps before injecting to K\(_2\)M\(_2\) wt cell-bearing Balb/C mice. Figure 7 illustrated the \textit{in vivo} fluorescence intensity of mice at 1, 4, 8, 12 and 24 h after tail intravenous injection. The bio-distribution study demonstrated that the PTX-PDA-ALN-Nps accumulated more than PTX-Nps and PTX-PDA-Nps in the tumor site. With the increase of time, fluorescence in tumor commence to gather and got to the maximum. In addition, the relative fluorescence intensity of the PTX-PDA-ALN-Nps in tumor was higher than other groups at 8 h (PTX-PDA-ALN-Nps (0.24) > PTX-PDA-Nps (0.18) > PTX-Nps (0.164)), which clearly indicated the \textit{in vivo} targeting ability of PTX-PDA-ALN-Nps. It may be owing to protein tyrosine phosphatases, a bis-phosphonate binding receptor, over expressed in osteosarcoma [10]. Furthermore, the absorption of macrophages of the reticuloendothelial system especially the Kupffer cells in the liver could also lead to the liver’s high fluorescence density for all PTX formulations.

3.7. Higher tumor inhibition rate and lower toxicity of PTX-PDA-ALN-Nps

Based on the advance \textit{in vitro} cytototoxicity on K\(_2\)M\(_2\) wt tumor cells experiments, the PTX-PDA-ALN-Nps can be employed in tumor therapeutic research as a prospective drug. The antitumor effect of the PTX-loaded Nps was inspected in K\(_2\)M\(_2\) wt tumor cell-bearing mice. The mice were grouped into five (\(n = 8\)), treated with PTX-Nps, PTX-PDA-Nps and PTX-PDA-ALN-Nps. Taxol and physiological saline were used as positive and negative control groups, respectively. The mice were given an injection through a tail vein every 2 d, in the meantime, the mouse’s weights and volume of tumor were measured. Figure 8(A) showed the curve of tumor growth in 14 d treatment. According to the figure 8(A), compared with saline, Taxol and Nps, PTX-PDA-ALN-Nps significantly inhibited the growth of tumor. Moreover, according to the table 3, the tumor inhibition rate of targeted PTX-PDA-ALN-Nps was also superior to Taxol (71.85% versus 66.53%), which was consistent with the previous cytototoxicity results. Body weights, liver index and spleen index changes could reflect the safety characters of PTX Nps. Notably, although there was no significant change in weight between each group, but the liver and spleen index of Taxol reduced extremely compared with the saline group (\(P < 0.05\)), while other Nps nearly had no differences (figure 8(B) and table 3). This means that the Nps could reduce liver and spleen toxicity of Taxol. The antitumor efficacy experiment showed that PTX-PDA-ALN-Nps have a higher tumor inhibition rate and lower toxicity, could release PTX and maintain its bioactivity for osteosarcoma therapy [48].

4. Conclusions

In summary, PTX-Nps with PDA for surface modification, were prepared for targeted therapy osteosarcoma. Through the novel dopamine polymerization method, PTX-PDA-Nps were simply functionalized by alendronate. The size of PTX-PDA-ALN-Nps is about 290 nm with smooth surface. In addition, XPS and FT-IR was used to testify the successful incorporation of PDA and conjugation of ALN on PTX-Nps. PTX-PDA-ALN-Nps were stable in physiological media and showed no hemolysis. \textit{In vitro} cytotoxicity experiment demonstrated the PTX-PDA-ALN-Nps remarkably inhibit cell proliferation with PTX-Nps and PTX-PDA-Nps comparing. \textit{In vivo} distribution essay through living imaging investigations declared that PTX-PDA-ALN-Nps could be eligible for a potential drug delivery system for malignant osteosarcoma targeting treatment. Moreover, the antitumor efficacy experiment showed that PTX-PDA-ALN-Nps have a higher TIR. All experiments and data showed that PTX-PDA-ALN-Nps is a promising drug for targeting treatment of osteosarcoma in the future.

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Table 3. The \textit{in vivo} antitumor effects of different groups of PTX against Balb/C tumors in mice. (mean ± SD, \(n = 8\), ***\(p < 0.01\) versus normal saline).

| Sample            | Tumor weight (g) | Inhibition rate (%) | Liver index | Spleen index |
|-------------------|-------------------|---------------------|-------------|--------------|
| Normal saline     | 2.45 ± 0.13       | NA                  | 0.047 ± 0.009 | 0.029 ± 0.007 |
| Taxol             | 0.82 ± 0.17       | 66.53               | 0.031 ± 0.002** | 0.017 ± 0.005** |
| PTX- Nps          | 1.05 ± 0.33       | 57.08               | 0.041 ± 0.005 | 0.025 ± 0.007 |
| PTX-PDA- Nps      | 0.91 ± 0.23       | 62.74               | 0.043 ± 0.004 | 0.026 ± 0.004 |
| PTX-PDA-ALN-Nps   | 0.69 ± 0.18       | 71.85               | 0.046 ± 0.007 | 0.027 ± 0.002 |
Disclosure statement

No potential conflict of interest was reported by the authors.

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