Bone-targeted PAMAM micelle to treat bone metastases of lung cancer

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Abstract

Background: Bone is a frequent site of metastasis in lung cancer patients. So far, the treatment in bone metastasis of lung cancer still has not achieved any satisfactory effects in clinic. In this paper, alendronate (ALN) was selected to be connected with PAMAM via pH sensitive cis-aconitine anhydride (CA) to prepare bone-targeted micelle (DTX@ALN-PAMAM) to treat bone metastasis of lung cancer.

Results: It was discovered that DTX@ALN-PAMAM released docetaxel (DTX) and ALN in pH-dependent manner. Besides, DTX@ALN-PAMAM showed high bind affinity with bone matrix, and quickly desorbed from bone matrix in weak acidic medium due to the rupture of cis-aconitamide bond between ALN and PAMAM. The in vitro results showed that DTX@ALN-PAMAM significantly enhanced the antitumor activity of DTX and decreased bone resorption through inhibiting the formation of osteoclasts in in-vitro 3D bone metastases model of lung cancer. In addition, DTX@ALN-PAMAM accumulated at bone metastases tissues for a relatively long time in tumor-bearing nude mice, which significantly reduced the bone resorption, relieved the pain response of tumor-bearing nude mice, and delayed the growth of bone metastases. Eventually, the therapeutic effect of DTX was improved on bone metastases of lung cancer.

Conclusion: ALN modified PAMAM is a new and an effective platform for the treatment of bone metastasis of lung cancer.

Background

At present, lung cancer is the most commonly diagnosed cancer and the leading cause of cancer death in the world. Nearly 30% to 40% of patients with lung cancer
have developed into bone metastases in the disease course[1, 2]. Bone metastases can cause skeletal-related events (SREs) including pathological fractures, spinal cord compression and hypocalcemia[3], which lead to the shortened life span and the impaired quality of life[4]. In clinic, surgery therapy and chemotherapy are often used to treat bone metastasis of lung cancer. Yet, the therapeutic effect is still unsatisfactory. When lung tumor cells proliferate in bone tissue, they secrete kinds of cytokines, which stimulate osteoblast to release the receptor activator for nuclear factor-κB ligand (RANKL). Consequently, osteoclast is activated by RANKL and bone resorption is enhanced in the bone metastases sites. At the same time, bone resorption results in the release of various growth factors from bone matrix, which accelerates the proliferation of tumor cells in bone metastases site [5–7]. Thus, in regard of the treatment of bone metastases of lung cancer, it is necessary to inhibit tumor growth and reduce bone resorption as well.

Polyamidoamine (PAMAM) dendrimers can form single molecule micelle without critical micelle concentration. It shows greater ability to entrap anti-tumor drugs inside its hydrophobic pockets. Besides, PAMAM exhibits positive charge and facilitates the cellular uptake because there are a large number of amino groups on its surface. However, unmodified PAMAM exhibits obvious hemolytic toxicity and non-specific cytotoxicity on normal tissues, which limits the application in vivo[8]. Fortunately, as PAMAM possesses a large number of amino groups, the modification of diverse functional groups is allowed to modulate its physicochemical properties for different purposes[9, 10].

Alendronate (ALN) is one of the most commonly used bisphosphonates for inhibiting bone resorption. Besides, recent researches find out that ALN has strong binding affinity for bone matrix (hydroxyapatite) because the bite distance of deprotonated
oxygen in ALN molecule is suitable for the chelation of Ca$^{2+}$ in hydroxyapatite[11]. More importantly, the binding affinity of ALN with tumor invasion bone is 10–20 times higher than that with normal bone[12, 13]. However, as a bone-targeted ligand, ALN is usually conjugated with drug carrier via stable chemical bonds in most bone-targeted drug delivery systems. Thus, ALN can hardly be detached from the drug carrier[12, 14, 15]. As a result, the diffusion of drug delivery systems to bone metastases is almost completely inhibited, which results in the significant reduction of the therapeutic effect on bone metastases. Therefore, in order to exert anti-bone resorption and anti-tumor activity, it is essential to detach ALN from drug carrier when drug delivery system accumulates at bone metastases.

In this paper, ALN was connected with PAMAM via pH sensitive cis-aconitine anhydride (CA) to prepare docetaxel (DTX) loaded micelle. ALN was used as bone-targeting ligand and also as bone resorption inhibitor. DTX was loaded in the core of PAMAM for antitumor effects. After intravenous injection, micelle could assemble in bone metastases tissue due to the targeting effect of ALN. ALN detached from PAMAM in acid bone metastases tissue due to the rupture of cis-aconitamide bond between ALN and PAMAM. After that, ALN was uptaken by osteoclast and then inhibited bone resorption, whereas PAMAM was uptaken by tumor cell and then released DTX in tumor cell to play its role as anti-tumor by inhibiting microtubule protein[16].

Results

Characterization of ALN-PAMAM

The molecular structure of ALN-PAMAM was characterized by $^1$HNMR and FTIR. As
shown in Figure S1A, ALN exhibited a peak at 2.97 ppm [-NH-CH$_2$-CH$_2$-CH$_2$-C(OH)P$_2$-]. CA exhibited a peak at 1.8 ppm [-COCH=C(COOH)-]. PAMAM exhibited multiple peaks at 2.2 ppm [-HNCH$_2$CH$_2$CO-], 2.7 ppm [-CONHCH$_2$CH$_2$NH-], 3.2 ppm [-CONHCH$_2$CH$_2$NH$_2$] and 3.3 ppm [-NHCH$_2$CH$_2$NHCO-]. Figure S1B showed the FTIR spectrum of ALN-PAMAM. The absorption peak at 1643 cm$^{-1}$ confirmed the amide bond of ALN-PAMAM. The absorption peak at 1375 cm$^{-1}$ and 610 cm$^{-1}$ indicated the presence of ALN. These results confirmed the successful synthesis of ALN-PAMAM.

**Characterization of DTX@ALN-PAMAM**

The drug loading, particle size and zeta potential of DTX@ALN-PAMAM were 5.2±0.4%, 99±13 nm and 4.35±0.28 mV (Figure 1A), respectively. TEM images showed that DTX@ALN-PAMAM displayed a spherical appearance (Figure 1B). DTX@ALN-PAMAM was stable within 7 days (Figure S2) in PBS (pH 7.4). As shown in Figure 1C, after being incubated with different PBS, the zeta potential of DTX@ALN-PAMAM increased with the decrease of medium pH. The *in vitro* drug release profiles of DTX@ALN-PAMAM showed pH dependent manner (Figure 1D). Less than 40% of DTX was released from DTX@ALN-PAMAM in pH7.4 medium in 60 h, and more than 70% of DTX was released from DTX@ALN-PAMAM in pH5.0 medium in 60 h. The faster rate of drug release in pH 5.0 medium was due to the partial protonation of the PAMAM [17]. The sustained release property of DTX from DTX@ALN-PAMAM in neutral medium suggested that DTX was well embedded in the cavities of PAMAM [18]. Moreover, the hemolysis rate of DTX@ALN-PAMAM showed a dose-dependent profile. The hemolytic rate of DTX@ALN-PAMAM was 0.60% and 1.56% when its concentration was 1 mg/mL and 3 mg/mL, respectively.

**Adsorption and desorption of DTX@ALN-PAMAM with hydroxyapatite**
Figure 2A showed that DTX@ALN-PAMAM could quickly bind with hydroxyapatite (HAp). The binding ratios of DTX@ALN-PAMAM with HAp reached a plateau after 30 min, which indicated a strong adsorption of DTX@ALN-PAMAM with HAp. Figure 2B shows the desorption of DTX@ALN-PAMAM from HAp in different pH media. More than 70% of DTX@ALN-PAMAM could desorb from HAp in the medium of pH 6.5 within 2 h. These results suggested that DTX@ALN-PAMAM exhibited high binding affinity with bone matrix, and PAMAM micelle could quickly desorp from bone matrix in weak acidic medium. Besides, ALN released from the nanoparticle was further determined by HPLC, and the result is shown in Figure S3. In an acidic medium, the release of ALN reached the plateau stage in 1 h. The above data indicated that ALN could be released from DTX@ALN-PAMAM through the cleavage of acid-sensitive chemical bonds in an acidic environment.

*In vitro* cytotoxicity of DTX@ALN-PAMAM

The cytotoxicity of DTX@ALN-PAMAM on A549 cells and MDA-MB-231 cells is shown in Figure 2C-D. ALN-PAMAM showed no significant cytotoxicity on A549 cells and MDA-MB-231 cells. Moreover, the cell viability in ALN-PAMAM treated group was significantly higher than that in PAMAM treated group, indicating that modification of PAMAM with ALN significantly decreased the cytotoxicity of PAMAM. Furthermore, both free DTX and DTX@ALN-PAMAM showed obvious cytotoxicity in dose-dependent manner on A549 cells and MDA-MB-231 cells, and DTX@ALN-PAMAM exhibited higher cytotoxicity on both A549 cells and MDA-MB-231 cells as compared with free DTX (Figure 2E-F). This result implied that DTX@ALN-PAMAM significantly increased the anti-tumor efficacy of free DTX.

Cellular uptake of DTX@ALN-PAMAM in A549 cells and RAW264.7 cells

Confocal laser scanning microscopy (CLSM) and HPLC were used to study cellular
uptake profiles of DTX@ALN-PAMAM in A549 cells. As shown in Figure 3, the cellular uptake of DTX@ALN-PAMAM in A549 cells exhibited time-dependent characteristic. Moreover, the cellular uptake of DTX@ALN-PAMAM in A549 cells was significantly increased in acidic culture medium as compared with that in neutral culture medium (Figure 3A-B). HPLC result was consistent with the CLSM result (Figure 3C). The increased cellular uptake of DTX@ALN-PAMAM in acidic medium was due to the exposure of positive charge PAMAM, which emerged after the cleavage of pH-sensitive cis-aconityl linkage between ALN and PAMAM in acidic medium.

Non-specific phagocytosis of macrophage affects the assembly of nanoparticles at the target tissue[19], thus the cellular uptake of DTX@PAMAM and DTX@ALN-PAMAM by RAW264.7 cells was investigated by CLSM. The uptake of DTX@PAMAM and DTX@ALN-PAMAM by RAW264.7 cells exhibited time-dependent profile, and the modification of PAMAM with ALN could significantly decrease the uptake of DTX@ALN-PAMAM by RAW264.7 cells (Figure 3D-E). This indicated that the non-specific phagocytosis of DTX@ALN-PAMAM by macrophages could be reduced in blood circulation. Consequently, the accumulation of DTX@ALN-PAMAM at bone metastases would be enhanced. Furthermore, as shown in Figure S4, ATP depletion and incubation in 4 °C could decrease the uptake of DTX@ALN-PAMAM by A549 cell, which indicated the cellular uptake of DTX@ALN-PAMAM was an energy-dependent endocytosis by A549 cells. In addition, sucrose also inhibited the uptake of DTX@ALN-PAMAM by A549 cells, and this indicated the endocytosis of DTX@ALN-PHAMA in A549 cells was also mediated through clathrin pathway.

**Anti-bone resorption and anti-tumor activity of DTX@ALN-PAMAM on 3D model of bone metastases of lung cancer**

Figure 4A shows SEM image of tumor cells in *in-vitro* 3D bone metastases model of
l lung cancer, and the results showed that treatment with DTX@ALN-PAMAM decreased the number of tumor cells as compared with control group and free DTX group (Figure 4B). This indicated that the growth of tumor cells in 3D bone metastases model of lung cancer was inhibited by DTX@ALN-PAMAM. In general, bone lacunae are formed after osteoclasts absorb organic components and minerals from the bone matrix. Figure 4C shows the SEM images of bone lacunae in *in-vitro* 3D bone metastases model of lung cancer, and the results showed that treatment with DTX@ALN-PAMAM reduced the number of bone lacunae as compared with control group and free DTX group (Figure 4D). This implied that DTX@ALN-PAMAM inhibited the formation of bone lacunae in 3D bone metastases model of lung cancer. Besides, osteoclast number is an indicator of bone resorption. Trap and methyl green were used to stain the osteoclast in *in-vitro* 3D bone metastases model of lung cancer. Figure 4E-F showed that treatment with DTX@ALN-PAMAM markedly reduced the number of osteoclasts as compared with control group and free DTX group. The above data demonstrated that DTX@ALN-PAMAM attenuated bone resorption by inhibiting the maturation of osteoclasts in *in-vitro* 3D bone metastases model of lung cancer.

**In vivo biodistribution of DTX@ALN-PAMAM**

Figure 5 shows the biodistribution results of DTX@ALN-PAMAM-cy7.5 in tumor-bearing mice. The result revealed that most nanoparticles accumulated in the liver and tumor tissue in 12 h after it was intravenously injected, and less fluorescence was observed in brain, kidneys, spleen, lungs, and heart. Even 24 h after intravenous injection, there were still more DTX@ALN-PAMAM-cy7.5 accumulated in tumor tissue. This implied that DTX@ALN-PAMAM could assemble at bone metastases tissues for a relatively long time.
**In vivo anti-tumor activity of DTX@ALN-PAMAM**

During the treatment for bone metastases, the change of tumor volume is shown in Figure 7A. DTX@ALN-PAMAM (20 mg/kg) significantly delayed the growth of tumor in dose-dependent profile. Compared with free DTX and ALN, DTX@ALN-PAMAM exhibited the strongest anti-tumor activity. Besides, bone pain of tumor-bearing nude mice was investigated through counting the number of paw lifting. DTX@ALN-PAMAM (20mg/kg) could significantly decrease the number of paw lifting as compared with saline-treated group, ALN-treated group or DTX-treated group (Figure 7B).

As shown in Figure 7C, there was no significant body weight reduction in DTX@ALN-PAMAM group. This implied that DTX@ALN-PAMAM did not lead to obvious systemic toxicity. In addition, H&E staining showed that there were no significant histomorphology changes in the main organ of tumor-bearing nude mice (Figure 7D). Compared with normal saline-treated group and ALN-treated group, DTX and DTX@ALN-PAMAM significantly decreased the number of atypical cells and abnormal nucleus in tumor tissue. The above results implied that DTX@ALN-PAMAM exerted great toxicity on tumor tissue with less toxicity on normal tissue. Furthermore, the bone mineral density (BMD) is an important index for bone resorption. The BMD of the right hind limb of tumor-bearing nude mice was 2154 mg/cc, which is significantly higher than that of normal saline-treated, ALN-treated and DTX-treated tumor-bearing nude mice[20]. This indicated that DTX@ALN-PAMAM could inhibit the bone resorption at bone metastases sites in tumor-bearing mice.

**Discussion**

PAMAM is a synthetic polymer with five unique characteristics[18, 21–23]. The first
characteristic is the hydrophobic cavity of PAMAM can effectively entrap hydrophobic drug such as DTX and paclitaxel. Another characteristic is the particle size of PAMAM is about 1.9–11.4 nm, which ensures great cellular uptake of drug delivery systems[24]. Besides, the positive charge of PAMAM can further facilitate its cellular uptake. After that, there are many reactive amino groups in PAMAM that can be used to link with bioactive substances such as peptides or antibodies. The last characteristic is, when PAMAM is in acidic microenvironment, the cavity rapidly opens and releases the entrapped drug[25].

Currently, in the designation of bone targeted drug delivery system for the treatment of bone metastases, the researchers mainly focus on how to improve the accumulation of drug delivery system at the site of bone metastases[26]. They usually neglect the desorption of drug delivery system from bone matrix and efficient entry into tumor cell of bone metastases. Actually, there are two ways to modify ALN with drug carrier. One way is to adsorb ALN directly to the surface of the drug carrier, and the other way is to connect ALN with drug carrier by stable chemical bonds. The former modification method is simple, but ALN is easy to fall off from the drug carrier in blood circulation, which results in the loss of bone targeting effect [27]. The latter modification method can keep bone targeting for a long time in vivo, but the drug carrier can be easily adsorbed in bone matrix [28]. The result is it will decrease the cellular uptake of entrapped drug by tumor cell of bone metastases and reduce the anti-tumor effect of drug delivery system [12].

In general, the bone resorption is extremely active at bone metastases sites, and the local pH of the bone resorption cavity decreases to 4–4.5[29]. In this paper, ALN was conjugated with PAMAM by using acid sensitive cis-aconitine anhydride through beta-carboxylamide. The experimental results indicated that hemolytic effect of
PAMAM was significantly reduced by modification of ALN with PAMAM. In addition, ALN-PAMAM was stable in blood circulation and reduced the non-specific uptake of ALN-PAMAM by macrophages, and ALN could be detached from ALN-PAMAM in acidic medium. This implied that in the acidic environment of bone metastases, ALN could be detached from ALN-PAMAM and was uptaken by osteoclasts, subsequently inducing the apoptosis of osteoclasts and inhibited bone resorption. Meanwhile, after the shedding of ALN, PAMAM micelle also got rid of the strong adsorption of bone matrix and could be uptaken by bone metastatic tumor cells to inhibit the proliferation of tumor cells.

As a bisphosphonate, ALN has a natural affinity for the bone, especially for tumor invasion bone. Therefore, DTX@ALN-PAMAM was accumulated at metastatic bones. This resulted in the improvement of bone mineral density and the reduction of bone resorption at the site of bone metastases. In addition, DTX@ALN-PAMAM effectively attenuated bone pain, which was resulted from the reduction of bone resorption at the site of bone metastases[30]. Furthermore, H&E staining showed that the tumor tissue in DTX@ALN-PAMAM treated group exhibited a large amount of necrosis areas. However, other major organs such as heart, liver, spleen, lung and kidney did not appear histomorphological changes after administration of DTX@ALN-PAMAM. These results indicated that DTX@ALN-PAMAM showed high toxicity on tumor tissue and less toxicity on normal organ tissue.

Methods

All materials are particularly described in supplementary information (SI).

Synthesis of ALN-PAMAM

Figure S5 showed the synthetic route of ALN-PAMAM. To synthesize PAMAM-CA,
PAMAM (20 mg) was dissolved in deionized water, and CA (30 mg) was dissolved in 1, 4-dioxane. Then, CA solution was dropwise added into PAMAM solution in ice bath under the condition of stirring. The pH was adjusted to 9.0 by using NaOH. After being stirred for 3 h, the solution was transferred to a dialysis bag (3500 Da) and dialyzed in deionized water for 6 h, and water was changed every 2 h. Finally, the liquid in the dialysis bag was freeze-dried and solid powder of PAMAM-CA was obtained. Then, PAMAM-CA (30 mg) was dissolved in deionized water, EDCI (76.2 mg) and NHS (45.7 mg) were added into PAMAM-CA solution and stirred for 6 h. Then ALN (130 mg) was added into the solution and stirred for 24 h at room temperature. The solution was transferred to a dialysis bag (molecular cutoff was 3500 Da) and dialyzed in deionized water for 24 h, and deionized water was replaced with fresh one every 6 h. Finally, the liquid in the dialysis bag was freeze-dried and ALN-PAMAM solid powder was obtained. The structure of ALN-PAMAM was confirmed by $^1$HNMR on INOVA-400 MHz spectrometer (Varian, USA, D$_2$O) and Fourier-transform infrared (FTIR) spectra (Perkin Elemer, USA).

**Preparation and characterization of DTX@ALN-PAMAM**

The solvent evaporation method was used to prepare DTX-loaded nanoparticles (DTX@ALN-PAMAM). In short, 5 mL mixture of methanol and acetone (v/v=1:1) containing DTX (2 mg) was added dropwise to a solution of ALN-PAMAM (10 mg ALN-PAMAM was dissolved in 10 mL distilled water) and stirred for 12 h. Then rotary evaporator was used to remove the methanol and acetone. At last, 0.8 μm polypropylene filter membrane was used to filtrate the mixture solution, and the filtrate was freeze-dried to obtain the solid of DTX@ALN-PAMAM micelle.

The size distribution, poly dispersity index (PDI), and zeta potentials of DTX@ALN-
PAMAM were measured by dynamic light scattering (DLS, Beckman Coulter Particle Analyzer). The transmission electron microscope (JEOL-100CXII) was used to observe the shape of nanoparticles. The drug loading (DL%) was determined by HPLC (Waters 2695/2996, Massachusetts, USA) through a Diamonsil C$_{18}$ column (250 mm×4.6 mm, 5 µm). The mobile phase consisted of acetonitrile and water (55:45, v/v), and the flow rate was 1.0 mL/min. The detection wavelength was 230 nm, and the injection volume was 20 µL. The equation of linear regression was $y=375.71x-143.62$, $r^2=0.9999$. The drug loading was calculated by the following equation: drug loading=$(\text{the weight of DTX in DTX@ALN-PAMAM})/(\text{the weight of DTX@ALN-PAMAM}) \times 100\%$.

**In vitro drug release**

DTX@ALN-PAMAM (10 mg) was dissolved into 2 mL PBS containing 0.5% tween 80 (w/v) with different pH medium. The mixture was sealed in a dialysis bag (molecular cutoff was 10000) and then immersed into the release medium (20 mL PBS, pH 7.4, 6.5 or 5.5). At pre-designed time point, the samples were taken out from release medium out of dialysis bag and replaced with an equal volume of fresh PBS. DTX concentrations were determined by the HPLC.

**Adsorption and desorption of DTX@ALN-PAMAM with hydroxyapatite**

A hydroxyapatite (HAp) binding assay was performed as previously reported method[31]. DTX or DTX@ALN-PAMAM (equivalent DTX dose was 0.25 mg) were suspended in 5 mL of PBS (pH7.4) containing tween 80 (0.5%, w/v). Then, HAp (50 mg) was added into DTX@ALN-PAMAM suspension or free DTX suspension. The samples were gently shaken at room temperature. At pre-designed time intervals, the above mixture was centrifugated at 5000×g for 5 min. 50 µL of the supernatant
was collected and mixed with 50 μL PBS (pH7.4) containing tween 80 (0.5%, w/v), and the DTX concentration in supernatant was measured by using HPLC. The binding rate of DTX@ALN-PAMAM with HAp was calculated by the following equation: binding rate (\%) = [(C_{without HAp} - C_{with HAp})/C_{without HAp}] \times 100\%

For desorption assay, after DTX or DTX@ALN-PAMAM suspension (equivalent DTX dose was 0.25 mg) was incubated with HAp for 2 h, the mixtures were centrifuged for 5 min (5000 \times g). The supernatant and precipitation was separated, and the initial amount of DTX in supernatant \((m_0, \mu g)\) was detected by HPLC. 5 mL of PBS (pH6.5) containing tween 80 (0.5%, w/v) was added into precipitation, and the precipitation was resuspended. Then the suspension was incubated in a water bath at 37 °C. At 5, 15, 30, 60, and 120 min, the suspension was centrifuged at 5000 \times g for 5 min. 50 μL of supernatant was taken out and 50 μL of PBS (pH 6.5) containing tween 80 (0.5%, w/v) was added into mixture, and the precipitation was resuspended and was incubated at 37 °C in a water bath. The amount of DTX in supernatant \((m_x, \mu g)\) was measured by HPLC. Under the same conditions, PBS (pH7.4) containing tween 80 (0.5%, w/v) was used as control. Finally, the following equation was used to calculate the desorption rate of DTX@ALN-PAMAM from HAp. Desorption rate (\%) = [m_x/(250-m_0)] \times 100\%.

**Hemolytic experiment**

The hemolytic effect of ALN-PAMAM was evaluated according to the procedure described in the literature previously [32]. Briefly, 5 mL fresh blood from a SD rat was collected and then centrifuged at 1500 \times g for 5 min. Then the sediment was washed with physiological saline until the supernatant became free of red color. The red blood cells (RBCs) were diluted to 2% (v/v) suspension by using physiological
saline. Different concentrations of PAMAM and ALN-PAMAM were incubated with RBCs suspension for 40 min at 37 °C. The samples were centrifuged at 5000×g for 5 min, and the absorbance of supernatant was measured at 398 nm by using an ultraviolet spectrophotometer. In addition, distilled water and physiological saline were used as positive and negative controls, respectively. The hemolysis rate (HR) was calculated. HR (%)=(A_{sample}-A_{negative control})/(A_{positive control}-A_{negative control})×100%.

**ALN release from ALN-PAMAM**

ALN-PAMAM (10 mg) were dissolved in 5 mL PBS (pH 5.0) and then moved to a dialysis bag (retained molecular weight 3500 Da). The dialysis bag was immersed in 20 mL PBS (pH 5.0) and incubated at 37 °C. At the pre-designed time point, 1 mL of release medium out of dialysis bag was taken and 1 mL of the same medium was added into the release medium. ALN in the release medium was determined through HPLC with Diamonsil C_{18} column (250 mm×4.6 mm, 5 µm). The mobile phase consisted of acetonitrile and water (0.4% EDTA, pH7.2) at a ratio of 35/65, and the flow rate was 0.5 mL/min. The detection wavelength was 264 nm, and the injection volume was 20 µL. The equation of linear regression was y=2347.2x-10292, \(r^2=0.9996\).

**In vitro cytotoxicity of DTX@ALN-PAMAM**

A549 cells and MDA-MB-231 cells were seeded at a density of 5×10^4 cells/well in 96-well transparent plate, and they were incubated for 24 hours at 37 °C. Then DTX and DTX@ALN-PAMAM with different concentrations (1-50 µg/mL) were added to the cells. After cells were incubated for 48 h, the cell culture medium was removed and the cells were incubated with fresh culture medium containing MTT (0.5 mg/mL) for
4 h at 37 °C. The culture medium was discarded and 150 µL DMSO was added, and the absorbance of each well was determined by the microplate reader at wavelength 490 nm (Bio-Rad Laboratories, Inc., California, USA). Through dividing the OD values of samples by the OD values of blank, the cell viability was calculated.

**Cellular uptake of DTX@ALN-PAMAM on A549 cells and RAW264.7 cells**

A549 cells and RAW264.7 cells in the logarithmic growth phase were seeded in the in 24-well plate containing coverslips at $2 \times 10^4$ cells/well and cultured at 37 °C for 24 h. Then serum-free culture medium containing DTX@ALN-PAMAM-Cy7.5 was added to the cells and incubated for 30 min and 3 h, respectively. After that, cells were washed with PBS for three times. Then 4% paraformaldehyde was used to fix cells. Finally, DAPI (600 ng/mL, 50 µL) was used to stain the cell. The fluorescence in A549 cells and RAW264.7 cells was observed by confocal laser scanning microscope (CLSM, Nikon Corporation, Tokyo, Japan).

HPLC was also used to quantitively analyze the cellular uptake of DTX@ALN-PAMAM on A549 cells. Briefly, A549 cells were added in 6-well plates at a density of $2 \times 10^5$ cells per well. After the cells were cultured for 24 h, the culture medium was replaced with fresh serum-free medium at different pH (pH6.5 or pH7.4) containing DTX or DTX@ALN-PAMAM (the equivalent DTX dose was 20 µg/mL), and the cells were incubated for 30 min, 1 h and 3 h. 50 µL of culture medium was taken out and the content of DTX was measured by HPLC. The amount of cellular uptake was calculated by subtracting DTX in culture medium from the total amount of DTX added in the culture medium at the beginning.

**The endocytic mechanism of DTX@ALN-PAMAM on A549 cells**

After the cells were cultured for 24 h, the culture medium was replaced with fresh
serum-free medium containing different endocytic inhibitors including sucrose (150 mg/mL, clathrin pathway inhibitor), colchicine (800 μg/mL, macropinocytotic pathway inhibitor), methyl-β-cyclodextrin (5 μg/mL, caveolin pathway inhibitor) and 2-deoxyglucose (0.95 mg/mL, ATP inhibitor). The cell in control group was treated with normal culture medium. The cell in low temperature group was incubated at 4 °C. The cells were incubated for 1 h, and then DTX@ALN-PAMAM (the equivalent DTX dose was 20 μg/mL) was added and incubated for another 4 h. Finally, 50 μL of culture medium was taken out and the content of DTX was measured by HPLC. The amount of cellular uptake was calculated by subtracting DTX in culture medium from the total amount of DTX added in the culture medium at the beginning.

**Anti-bone resorption and anti-tumor activity of DTX@ALN-PAMAM in *in-vitro* 3D bone metastases model of lung cancer**

The craniums of 1 day old neonatal CD-1 mice were isolated under sterile conditions and placed into the 48-well plate after being washed with normal saline[33]. A549 cells were seeded at a density of 5×10⁵ cells per well in 48-well transparent plates containing cranium in culture medium. The cranium without seeding A549 cells was set as negative control. After being incubated for 2 days, the craniums were exposed to fresh medium containing DTX (10 μg/mL) or DTX@ALN-PAMAM (the equivalent DTX dose was 1, 10 and 100 μg/mL). The craniums were incubated for another 48 h and then fixed with glutaraldehyde and dehydrated in a series of ethanol solutions. After that, gold palladium was sprayed on the cranium to observe tumor cells on cranium tissue by field emission scanning electron microscope (SEM, Hitachi High-Technologies Corporation, Japan). For observing bone lacunae, the tumor cells on the surface of cranium were removed by trypsin. Then, the craniums were fixed with glutaraldehyde and dehydrated in a series of ethanol solutions.
After that, gold palladium was sprayed on the cranium to observe bone lacunae on cranium tissue by SEM. For observing osteoclasts, the cranium was stained with Trap and methyl green, then the osteoclasts on cranium tissue was observed by using microscope[34, 35].

In vivo anti-bone resorption and anti-tumor activity of DTX@ALN-PAMAM

A bone metastasis model was produced according to the method described by Arguello et al[36]. Briefly, the nude mice were anesthetized by inhalation of isoflurane, and A549 cells were injected in the tibial bone marrow cavity of right hind limb of mice (100 μL, 1×10^7/mL). One week later, normal saline, DTX, ALN, or DTX@ALN-PAMAM was intravenously administered once a week for 3 weeks. Besides, the pain response of tumor-bearing mice was evaluated according to the method described by Vermeirsch H [37]. Vernier caliper was used to measure the length (L) and width (W) of tumor, and the tumor volume (V) was calculated through

V=L×W^2/2. At the end of this experiment, the tumor-bearing nude mice were executed euthanasia and the main tissues such as brain, heart, liver, spleen, lung, kidney and tumors were all removed and fixed in paraformaldehyde (4%) for 24 h. Then the tissues were cut for H&E staining. The slices were observed under microscope. Additionally, leg bones (femur, tibia and fibula) were dissected and stored in 4% paraformaldehyde solution. Micro-CT (Siemens, Munich, Germany) was used to determine the bone density.

In vivo biodistribution of DTX@ALN-PAMAM

Cy7.5 marked DTX@ALN-PAMAM (DTX@ALN-PAMAM-Cy7.5) was injected via the tail vein to tumor-bearing nude mice to evaluate the biodistribution of DTX@ALN-PAMAM-Cy7.5. At 12 h or 24 h after the injection, the tumor-bearing nude mice were
executed euthanasia and the main tissues such as brain, heart, liver, spleen, lung, kidney and tumor tissues were removed. The distribution of DTX@ALN-PAMAM-Cy7.5 in organs and tumor tissues was observed by using an in vivo imaging (Caliper Life Sciences Corporation, USA).

**Statistical analysis**

All data were expressed as mean ± standard deviation. The comparison of each group was performed by one-way ANOVA with SPSS 18.0 statistical software. A value of P less than 0.05 was considered statistically significant.

**Conclusion**

DTX@ALN-PAMAM delivered DTX and ALN to bone metastasis simultaneously to break the “vicious circle” between tumor growth and bone resorption. The combination of ALN and DTX in DTX@ALN-PAMAM exhibited synergistic effects on the treatment of bone metastases of lung cancer, which resulted in the enhancement of the therapeutic effect in vivo. Therefore, ALN modified PAMAM is a new and useful platform for the treatment of bone metastasis of lung cancer.

**Abbreviations**

ALN: alendronate; DTX: docetaxel; RANKL: receptor activator for nuclear factor-κB ligand; PAMAM: olyamidoamine; CLSM: confocal laser scanning microscopy; BMD: bone mineral density; H&E: hematoxylin and eosin.

**Declarations**

**Authors’ contributions**

SB performed the nanoparticle preparation and characterization; YC conducted in
vitro release experiments; DL conducted in vitro cellular uptake studies; SB and YC conducted cell in vitro activity studies; SB and QJ carried out the distribution experiment. YC and ML carried out the in vivo experiment. YC drafted this paper; SZ initiated the study and provided intellectual input; BZ and QM provided intellectual input. SB and YC contributed equally to this paper and were co-first authors. All authors did a proofreading for the final draft.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

All data generated and analyzed in this research are included in this manuscript.

**Consent for publication**

All authors agree to the publication of this paper.

**Ethics approval and consent to participate**

Animal experiments were approved by the Fourth Military Medical University Animal Care and Use Committee (Approval No: 20171101).

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FIGURES

Figure 1

Characterization of DTX@ALN-PAMAM. Size distribution (A); TEM image (B); zeta potential (C); in vitro DTX release (D). Data were expressed as mean ± SD (n=3).
Figure 2

The bioactivity of DTX@ALN-PAMAM in vitro. The adsorption of DTX@ALN-PAMAM
Figure 3

The cellular uptake of DTX@ ALN-PAMAM -Cy7.5. CLSM images of cellular uptake of DTX@ ALN-PAMAM -Cy7.5.
Anti-bone resorption and anti-tumor activity of DTX@ALN-PAMAM in in-vitro 3D bone metastases model.
In vivo biodistribution of DTX@ALN-PAMAM. Ex vivo imaging of organs, tumor tissue and bone tissue after in vivo imaging of nude mice (A). Statistical result of fluorescence intensity (B). Data were expressed as mean±SD (n=5).

Figure 6

In vivo anti-tumor activity of DTX@ALN-PAMAM. The tumor volume change of tumor-bearing nude mice (A). The number of focies (B). The body weight change (C). Data were expressed as mean±SD (n=5). **P<0.01, vs same dose of free DTX. ##P<0.01, vs dose of 10 mg/kg.
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