Doxorubicin-Conjugated Bisphosphonate Nanoparticles for the Therapy of Osteosarcoma

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Abstract
Osteosarcoma is amongst the most common primary malignant tumors of bone and occurs in adolescents and young adults. Current treatment is a combination of both surgery and chemotherapeutics. However, the use of anticancer drugs is still associated with serious side effects. In this article we describe a doxorubicin drug delivery system based on bisphosphonate nanoparticles with a narrow size distribution. We have shown that the doxorubicin-conjugated bisphosphonate nanoparticles preferentially target the bone tumor, thereby increasing the anti-cancer drug bioavailability to the tumor. Furthermore, we have been able to demonstrate that the doxorubicin-conjugated bisphosphonate nanoparticles have significantly higher activity than the free drug both in vitro and in vivo. Testing the doxorubicin-conjugated bisphosphonate nanoparticles on an osteosarcoma xenograph in a chicken embryo model demonstrated that these nanoparticles specifically targeted the tumor and thus decreased the tumor size.

ABBREVIATIONS

INTRODUCTION
Osteosarcoma (OS) is amongst the most common primary malignant tumors of bone occurring in both adolescents and young adults [1,2]. Current treatment is a combination of both surgery and chemotherapeutics. Combinations of high doses of doxorubicin [3] methotrexate [4,5], cisplatin and ifosfamide [6] have led to a significant improvement in survival rate. However, the use of anticancer drugs is still associated with serious side effects, due to nonspecific uptake and in the case of OS a poor bone blood supply [7] necessitating the use of toxic high dosages. In addition, drug-resistant phenotypes and “secondary malignancies” [8] occur. Therefore, the development of bone-targeted anti-tumor agents with minimal or no side effects for the prevention and treatment of cancer-associated bone diseases remains a priority [9].

Bisphosphonates (BP) are the primary drug used for the treatment of bone diseases such as osteoporosis, Paget’s disease, benign and malignant bone diseases, etc. [10-14]. BP are a chemical analog to endogenous pyrophosphate, have a High Affinity to Hydroxyapatite (HAP) and exhibit potent bone resorption inhibitory activity [15,16], due to their similarity to pyrophosphate. Whereas in pyrophosphate the oxygen atom binds to two phosphorus atoms (P–O–P) in BP the oxygen is substituted by a carbon atom (P–C–P), thus increasing BP resistance to chemical and enzymatic degradation [16,17]. Due to BP compounds high affinity to Ca²⁺ ions they can used for bone targeting in areas with high resorption activity [18,19].

Submicron-sized polymeric particles (3-200 nm) [20] have aroused considerable interest in the area of drug delivery [21,22]. Many scientific groups have focused their efforts to improve Nanoparticles (NPs) targeting to recipient cells and tissues [23-27]. In addition, due to NPs submicron size they are...
able to bypass the body’s drug resistance mechanism and thus increase the intracellular drug concentration in cancer cells while avoiding toxicity of normal cells [28-30].

A novel BP NPs has been recently synthesized in our laboratory [31-34]. The BP NPs are constructed of three monomers: Methacrylate PEG Bisphosphonate (MA-PEG-BP) monomer, a monomer containing a primary amine group (N-(3-aminopropyl) methacrylamide hydrochloride, APMA) and a crosslinker monomer to form a crosslinked particle. The novel BP NPs possess high concentration of PEG in order to increase the half-life time of the NPs in the blood [35] as well as a dual functionality: chelation to the bone mineral - HAP, through the BP group, and covalent attachment to carboxylate (or carboxylate derivatives) compounds via the primary amine groups. These BP NPs have shown insignificant toxicity and high affinity to bone, and therefore suggest that they can be a good candidate for drug delivery to bone tumors [31,34].

In this article we describe the drug delivery and therapeutic ability of doxorubicin- conjugated BP NPs. Doxorubicin was conjugated covalently to the primary amine group on the BP NPs through a PEG spacer. The doxorubicin-conjugated BP NPs have shown high affinity to osteosarcoma cells, as well as high affinity to osteosarcoma tumors in a chicken embryo model.

EXPERIMENTAL PART

Materials
The following analytical-grade chemicals were purchased from commercial sources and used without further purification: polyethylene glycol methacrylate (MA-PEG, Mn 360), Tetraethylene Glycol Dimethacrylate (TETEGDA), polyethylene glycol methacrylate ether (MA-PEG-OCH3, Mn 300), Potassium Persulfate (PPS), O-[(N-Succinimidyl) succinylaminoethyl-O'-methylpolyethylene glycol (PEG-NHS, Mw 750), polyvinylpyrrolidone (PVP, Mw 360K), sodium hydroxide (NaOH, 1 N), hydrochloric acid (HCl, 1 N), anhydrous dichloromethane, anhydrous N,N-Dimethylformamide (DMF), chromium oxide, isopropanol, magnesium sulfate (97%), triethylamine (99%), methanesulfonyl chloride, sodium chloride, sodium azide (99.5%), triphenylphosphine, glycine and O,O′- Bis[2-(N-Succinimidyl-succinylamino)ethyl]polyethylene glycol (NHS-PEG-NHS MW 3,000) from Sigma (Rehovot, Israel); N-(3-aminopropyl)methacrylamide hydrochloride, (APMA) from Polysciences, Inc. (Warrington, PA) Dialysis membrane (1000 K-16MM), bicarbonate buffer (BB, 0.1 M, pH 8.4), sodium carbonate and sodium bicarbonate from Bio-Lab Ltd. (Jerusalem, Israel); Cy 3-NHS ester and Cy 7-NHS ester from Lumiprobe Corporation (Florida, USA); Doxorubicin hydrochloride from wonda science (Massachusetts, USA). Dulbecco’s phosphate-buffered saline (PBS), Dulbecco’s Minimum Essential Medium (DMEM), fetal bovine serum, glutamine, penicillin and streptomycin from Biological Industries (Bet Haemek, Israel); Human cell lines Saos-2 and U-2OS from the American Type Culture Collection (Manassus, VA); Marigel from Sigma (Germany); Water was purified by passing deionized water through an Elgastat Spectrum reverse osmosis system (Elga Ltd., High Wycombe, UK).

Synthesis of the BP NPs
BP NPs were prepared similarly to that described in the literature [31,32]. Briefly, 45 mg MA-PEG-BP, 5 mg APMA and 50 mg TETEGDA (total monomers concentration was 5 % w/v) were added to a vial containing 8 mg of PPS (8 % w/v) as initiator and 20 mg PVP 360K (1 % w/v) as stabilizer dissolved in 2 mL of BB. For the polymerization, the vial containing the mixture was purged with N2 to exclude air and then shaken at 83°C for 8h. The obtained BP nanoparticles were washed of excess reagents by extensive dialysis cycles (cut-off of 1000 k) with purified water.

Synthesis of the NIR fluorescent BP NPs
Near IR (NIR) fluorescent BP NPs were synthesized similarly to describe in the literature [32]. In brief, NIR fluorescent BP NPs were prepared by reaction of the primary amino groups on the BP NPs with Cy7-NHS ester. Cy7-NHS ester (2 mg) was dissolved in 0.5 mL of anhydrous DMSO. 250 µL of the Cy7-NHS ester solution was then added to 5 mL of the BP NPs dispersion in BB (2 mg/mL), and the reaction was stirred overnight at rt. Blocking of residual amine groups was then accomplished by adding 0.5 mg of PEG-NHS to the obtained NIR fluorescent BP nanoparticles aqueous dispersion. The reaction was then stirred 30 min at rt. The obtained NIR fluorescent-conjugated BP nanoparticles were then washed from excess reagents by extensive dialysis in water. Cy3- NHS ester was conjugated to the BP NPs in a similar manner.

NIR fluorescent control nanoparticles possessing OCH3 groups instead of the BP groups, for the chicken embryo body distribution experiments, were prepared similarly substituting the monomer MA-PEG-BP for MA-PEG-OCH3.

Synthesis of the doxorubicin-conjugated BP NPs
Doxorubicin-conjugated BP NPs were prepared by an initial reaction of the primary amine group on the BP NPs with NHS-PEG-NHS followed by the addition of doxorubicin. Briefly, NHS-PEG-NHS (10 mg) was dissolved in Double Distilled Water (DDW) (1 mL). 500 µL of the NHS-PEG-NHS solution was then added to 5 mL of the BP NPs dispersion in BB (2 mg/mL), and the reaction was stirred at rt. After 10 min, 1 mg doxorubicin, which initially dissolved in DDW, was added to the dispersion and was stirred for an additional 1 h. Blocking of residual amine groups was then accomplished by adding 50 mg of glycine to the doxorubicin BP NPs aqueous dispersion. The reaction was then stirred for a further 30 min at rt. The obtained doxorubicin-conjugated BP NPs were then washed from excess reagents by extensive dialysis in water. The concentration of the conjugated doxorubicin was determined using fluorescence intensity (3ex 470 nm; 3em 590 nm).

Extent of doxorubicin leakage
Leakage of doxorubicin conjugated to the BP NPs into the continuous phase (PBS containing 4% HSA) was evaluated according to the following procedure: doxorubicin-conjugated BP NPs dispersed in PBS (1 mg/mL) containing 4% HSA were shaken at 37°C for 12 h and then filtered via a 300 kDa filtration tube (VS0241 Viva Spin) at 4000 rpm (Centrifuge CN-2200 MRC). The fluorescence intensity of the supernatant was then measured at 3ex=470 nm; 3em=590 nm.

Characterization of the BP nanoparticles
Dried particle size and size distribution were measured with
Central group contained 7 embryos. The fluorescence intensity of the for different time periods of 4, 24, 48 and 72 h. Each experiment cellotape, and the chicken embryos were returned to incubation After injection, the window in the egg's shell was sealed with were Injected Intravenously (IV) into a large CAM blood vessel. BP groups were substituted by OCH3 groups) dispersed in PBS dispersed in PBS and of the control fluorescent NPs (wherein the 100 µL of 0.1 mg/mL of the Cy7-conjugated cross linked BP NPs implanted on a plastic ring placed on the CAM. On E13, a total of day (E) E8 of incubation, a window was opened in the shell, and the chorioallantoic membrane (CAM) was exposed. A total experiments. The cell lines were maintained in DMEM supplemented with heat-inactivated fetal bovine serum 10%, penicillin 100 IU/mL, streptomycin 100 µg/mL, and L-glutamine 2 mM. Cell lines were screened using a mycoplasma detection kit to ensure that they remained mycoplasma-free [36].

Cell viability test (XTT)

In vitro toxicity of the doxorubicin-conjugated BP NPs was tested using two types of cancer cell lines, human osteosarcoma Saos-2 and U-2OS [37-39]. XTT assay was used in order to assess cell viability. The doxorubicin- conjugated BP NPs were freshly dispersed in PBS and then added to the 95% confluent cell culture in culture medium so that the final concentration of conjugated doxorubicin BP NPs was 8 and 40 µg/mL giving a final concentration of doxorubicin of 50 and 250 ng/mL respectively. The cell cultures were further incubated at 37°C in a humidified 5% CO2 incubator and then checked for cellular toxicity after 48 and 72 h. The percentage of cell cytotoxicity was calculated as shown in the manufacturer’s protocol of the XTT toxicity detection kit. All samples were tested in six folds.

BP NPs targeting and therapeutic activity in a chicken embryo model seeded with Saos-2 cells

Experiments were performed according to the protocols of the Israeli National Council for Animal Experiments. Fertile chicken eggs obtained from a commercial supplier were incubated at 37°C at 60–70% humidity in a forced-draft incubator, as described in the literature [36,40]. On embryonic day (E) E8 of incubation, a window was opened in the shell, and the chorioallantoic membrane (CAM) was exposed. A total of 6x10⁵ Saos2 cells were mixed in 30 µL of Matrigel and then implanted on a plastic ring placed on the CAM. On E13, a total of 100 µL of 0.1 mg/mL of the Cy7-conjugated cross linked BP NPs dispersed in PBS and of the control fluorescent NPs (wherein the BP groups were substituted by OCH3 groups) dispersed in PBS were injected Intravenously (IV) into a large CAM blood vessel. After injection, the window in the egg’s shell was sealed with cellophane, and the chicken embryos were returned to incubation for different time periods of 4, 24, 48 and 72 h. Each experiment group contained 7 embryos. The fluorescence intensity of the NIR fluorescent BP and control NPs was studied by the Maestro II in vivo imaging system, 2D planar fluorescence imaging of small animals (Cambridge Research & Instrumentation, Inc., Woburn, MA). A NIR excitation/emission filter set was used for our experiments (3ex: 710–760 nm, 3em > 750 nm). The Liquid Crystal Tunable Filter (LCTF) was programmed to acquire image cubes from 790 nm to 860 nm with an increment of 10 nm per image. Fluorescence intensity measurements were performed using ImageJ software [41]. The tumor samples were transferred onto black paper and then imaged.

In a similar manner, doxorubicin-conjugated BP NPs (0.1 mg/ml containing 0.5 µg conjugated doxorubicin) and free doxorubicin (10 µg/ml) were injected on E12. The tumor was extracted after 96 h and weighed. All the experiment with chicken embryos were repeated twice with similar results.

To decrease animal suffer, the experiment with chicken embryos were terminated after E16.

RESULTS AND DISCUSSION

Synthesis and characterization of the doxorubicin-conjugated BP NPs

Doxorubicin-conjugated BP NPs for osteosarcoma therapy were prepared according to Figure 1, as described in the experimental section. The measured dry diameter of the BP NPs was 52.3 ± 21 nm. The doxorubicin was then covalently conjugated to the BP NPs, via a PEG spacer, through the free amine groups on the surface of the BP NPs. Due to the conjugation the dry diameter of these NPs increased slightly from 52.3 ± 21 nm to 62.7 ± 21 nm (Figure 2A). An inverse behavior was observed for the hydrodynamic diameters, as shown in Figure 2B, e.g., the hydrodynamic diameter decreased from 160 ± 17 nm for the BP NPs to 155 ± 20 nm for the doxorubicin-conjugated BP NPs. The slight decrease in the hydrodynamic diameter could be attributed to the increased surface hydrophobicity as a result of the doxorubicin binding. The leakage of doxorubicin from the BP NPs to PBS containing 4% HSA was investigated according to the description in the experimental section. After 12 h incubation at 37°C no detected leakage was observed.

The concentration of the doxorubicin conjugated to the BP NPs was determined by measuring the fluorescence of the bound drug ((3ex 470; (3em 590) as described in the experimental section. Investigation of the binding of doxorubicin to the BP NPs (10 mg) using different concentrations of doxorubicin (0.2, 1 and 2 mg) and of NHS-PEG-NHS (0.2, 1, and 5 mg) was evaluated. It was found that in all combinations 5 µg of doxorubicin was conjugated to 1 mg of the BP NPs. Hence we can conclude that the concentrations which we used for the binding were in excess. The doxorubicin binding yields for the different concentrations of doxorubicin (0.2, 1 and 2 mg), with a constant concentration of NHS-PEH-NHS (5 mg) were calculated to be 3, 5 and 30 %, respectively. Increasing the concentration of NHS-PEG-NHS (0.2, 1, and 5 mg) while using a constant concentration of doxorubicin (1 mg), did not affect the concentration of the bound doxorubicin. It should be noted that in the continuation of the present study we used 5 µg conjugated doxorubicin for each mg of the BP NPs as described above.

The change in the charge of the doxorubicin-conjugated BP NPs as a function of pH was evaluated using zeta (ξ) potential
Figure 1 Scheme of the synthesis of the doxorubicin-conjugated BP NPs.

Figure 2 TEM image (A), size histogram (B) and ζ-potential (C) of the doxorubicin-conjugated BP NPs (Error bars represent standard deviation).

Figure 3 A photomicrograph (A) and histogram (B) showing the kinetics of the tumor marking by the NIR fluorescent BP and the control NPs in a chicken embryo model. On E8 of incubation, a total of 6x10^5 Saos-2 cells were implanted in a plastic ring placed on the CAM. On E13, a total of 100 µL of 0.1 mg/mL of the Cy7-conjugated BP NPs or the Cy7-conjugated control NPs dispersed in PBS were IV injected into a large CAM blood vessel. The chicken embryos were then returned to incubation for different time periods (4, 24 48 and 72 h).

(Doxorubicin-conjugated BP NPs, exhibit a decrease in the ζ-potential as the pH increases up to about 5.0, then up to about pH 10.0 the ζ-potential did not significantly affected by the increase in the pH. Around physiological pH (pH 7.4) the ζ-potential is found to be -40 mV, as seen in Figure 2C. The pI of doxorubicin-conjugated BP NPs as shown in Figure 2C was measured to be 2.9 mV. These finding support that the doxorubicin-conjugated BP NPs maintain their stability in physiological pH.

Tumor targeting and therapeutic activity of the BP NPs

Previous in vivo testing on a chicken embryo model, by our group, demonstrated a high affinity of the NIR fluorescent BP NPs to bones as opposed to other organs [31]. In the present study
we examined the BP NPs ability to target osteosarcoma tumor in a CAM model, by initially implanting Saos-2 cells on the CAM of a chicken embryo, as described in the experimental section [32]. Saos-2 cell were chosen due to their ability to form solid tumors, as opposed to U-2OS cells that did not form a prominent solid tumor in the CAM model. The NIR fluorescent BP NPs and the NIR fluorescent control NPs (similar particles except the BP groups were replaced with methoxy groups, as described in the experimental section) were IV injected to the chicken embryo and the kinetics of the fluorescence in the blood and tumor were followed for 4, 24, 48 and 72 h. 48 h after the IV injection both the Cy7-conjugated BP NPs and the Cy7-conjugated control NPs could not be detected in the blood. In contrast, the fluorescence intensity of the Saos-2 tumor (Figure 3) containing the NIR fluorescent BP NPs increases with time while the fluorescent intensity of the NIR fluorescent control NPs were significantly lower and of the same intensity level at all time periods. This behavior indicates the high specificity of the BP NPs to the osteosarcoma tumor, due to the increase in Ca$^{2+}$ concentration in the Saos-2 tumor environment [42,43], causing an increased uptake of the BP NPs.

After establishing the tumor targeting ability of the BP NPs, the therapeutic ability of the free doxorubicin doxorubicin-conjugated BP NPs were examined. Before testing on the CAM model, cell toxicity was evaluated on osteosarcoma cells. For this purpose, we compared the activity of 50 and 250 ng/ml free doxorubicin and the same concentration of doxorubicin conjugated to the BP NPs on Saos-2 and U-2OS cell lines. Figure 4 summarizes the results of these trials. This figure first of all exhibits that the non-conjugated BP NPs do not possess any toxicity to both cell lines. In addition, this figure illustrates that the % cell viability of the Saos-2 cell line treated with free doxorubicin is significantly higher than that of the doxorubicin-conjugated BP NPs. The treatment with 50 ng/ml non-conjugated or conjugated doxorubicin over 48 h exhibits 77 and 46 % cell viability, respectively, and a further decrease in the cell viability was shown after 72 h, 53 and 35 %, respectively. When treated with a concentration 250 ng/ml doxorubicin a more dramatic difference between the free doxorubicin to the doxorubicin-conjugated BP NPs was illustrated. Treatment for 48 h showed 58 and 10 % viability, respectively, and for 72h 44 and 11 %, respectively. A similar effect was noticeable when U-2OS cells were treated with free doxorubicin and doxorubicin-conjugated BP NPs. For treatment of 50 ng/ml over 48 h the cell viability was 89% compared to 26%, respectively and for 72 h 74 and 21 % viability, respectively. A slight smaller difference was noticeable when treated with 250 ng/ml doxorubicin, over 48h was 40 and 23 % viability, and over 72 h was 30 and 22 % viability, respectively.

To summarize, these findings show that the doxorubicin-conjugated BP NPs were significantly more potent and toxic than the free doxorubicin in both types of osteosarcoma cells. In contrast, the BP NPs as control, do not exhibit any toxicity to these cells.

Further studies on the uptake of the BP NPs were performed, by initially conjugating the fluorescent dye Cy3 to the free amine of the BP NPs (as described in the experimental part), to enable the use of a fluorescent and confocal microscopy with a filter of ex 512 em 570 nm. For this purpose U-2OS cells were incubated for 1, 4 and 24 h with the Cy3-conjugated BP NPs (0.16 mg/ml). Cell uptake was analyzed using fluorescence microscope (Zeiss, Axio imager Z1). The images (Figure 5) clearly show that after 1 h the Cy3-conjugated BP NPs penetrate the cells, and after 24 h the Cy3-conjugated BP NPs are more concentrated in the cytoplasm, as seen in Figure 5C. The cell uptake to the cytoplasm was confirmed by confocal microscope. The experiment cannot repeat with the doxorubicin-conjugated BP NPs due to cell apoptosis, however we hypothesize that doxorubicin-conjugated BP NPs will also penetrate the cell. The doxorubicin will then be cleaved
The therapeutic activity of the doxorubicin conjugated to the BP NPs was further investigated using the CAM model. Experiments were carried out similar to those described for the targeting ability of the BP NPs (paragraph 2.8). 0.1 mg/ml BP NPs, 0.1 mg/ml doxorubicin-conjugated BP NPs (0.5 µg doxo/ml) and free doxorubicin (10 µg/ml) dispersed/dissolved in PBS were injected IV into the chicken embryos and the tumor was excised and weighed after 96 h. Figure 6 indicates that even though the concentration of the conjugated doxorubicin was 20 times less than that of the free doxorubicin (0.5 and 10 µg, respectively) it in the cytoplasm by enzymes allowing the free doxorubicin to reach the DNA and inhibit the enzyme topoisomerase II thus preventing DNA replication [44].
prevented the tumor growth significantly (p < 0.05). On the other hand, no significant differences were noted between BP NPs, free doxorubicin and untreated tumors. Hence we can conclude that the doxorubicin-conjugated BP NPs have a high affinity to osteosarcoma tumors attributed to the presence of the BP groups in comparison to the free doxorubicin which probably dispersed unselectively all over the chicken embryos body. The greater therapeutic effect of the doxorubicin-conjugated BP NPs is due to the polyvalency effect, which is a cause of a high concentration delivered to the tumor by the NPs. This confirms results shown in earlier studies that suggest that nanoparticle drug delivery improves both the uptake and therapeutic response of anticancer drugs by tumors [45].

SUMMARY AND DISCUSSION

In this study, we engineered a new doxorubicin-conjugated BP NPs for the treatment of osteosarcoma. These doxorubicin-conjugated BP NPs may prove to be very useful for the in vivo treatment of bone tumors, due to their high affinity to Ca++, allowing the delivery of high concentrations of doxorubicin directly to the tumor and thus reducing the side effects. We have demonstrated that conjugation of doxorubicin to BP NPs significantly increases the anti-cancer activity of the drug against osteosarcoma cell lines in comparison to the free drug. This was further investigated in a CAM model were we verified the affinity and the therapeutic activity of the doxorubicin-conjugated BP NPs to osteosarcoma tumors. The results obtained showed that the targeted delivery of doxorubicin significantly increased the efficacy of the anti-cancer drug, thus allowing the efficient use of low concentration of doxorubicin.

In future work, we plan to extend this study to in vivo mouse models, allowing us to study the body distribution and t1/2 of the BP NPs, as well as confirming the targeting and therapeutic activity of the doxorubicin-conjugated BP NPs. Moreover, by conjugating other suitable drugs, these BP NPs may be used for treatment of other bone diseases where there is a high rate of bone resorption.

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