Bioorganic & Medicinal Chemistry Letters 19 (2009) 4148–4150

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Synthesis of calcium phosphate-binding liposome for drug delivery

Takahisa Anada [a,*], Yoichi Takeda [b], Yoshitomo Honda [a], Kazuo Sakurai [b], Osamu Suzuki [a,*]

[a] Craniofacial Function Engineering, Tohoku University Graduate School of Dentistry 4-1, Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[b] Department of Chemical Process and Environments, The University of Kitakyushu, 1-1, Hibikino, Wakamatsu-ku, Kitakyushu, Fukuoka 808-0135, Japan

ARTICLE INFO

Article history:
Received 18 March 2009
Revised 13 May 2009
Accepted 29 May 2009
Available online 10 June 2009

Keywords:
Drug delivery system
Liposome
Bone
Hydroxyapatite

ABSTRACT

Metastatic bone disease is often associated with bone pain, pathologic fractures, and nerve compression syndromes. Effective therapies to inhibit the progression of bone metastases would have important clinical benefits. Therefore, we developed a novel calcium phosphate-binding liposome for a bone-targeting drug delivery system. We synthesized a novel amphipathic molecule bearing a bisphosphonate (BP) head group to recognize and bind to hydroxyapatite (HA). We demonstrated that the liposomes having BP moieties show high affinity for HA. Doxorubicin-loaded liposomes adsorbed on the surface of HA significantly reduce the number of viable human osteosarcoma MG63 cells. This shows that the liposomes can be excellent carriers for anticancer drugs because they specifically target bone tissue. This calcium phosphate-binding liposome system could be used with many drugs for bone-related diseases such as osteoporosis, rheumatoid arthritis, and multiple myeloma.

Bone is a common site of breast and prostate cancer metastasis. Metastatic bone disease is often associated with bone pain, pathologic fractures, and nerve compression syndromes. Such complications result in decreased quality of life.[1] Unfortunately, there is no cure for patients with bone metastasis. Therefore, effective therapies to inhibit the progression of bone metastases would have important clinical benefits.

The ideal drug delivery system is thought to be one that restricts its pharmacological activity solely to target sites. If the drug or delivery vehicle has a high affinity for only bone tissue, its therapeutic effect on bone-related disease is maximized and its distribution to other sites is minimized. Hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) is the major inorganic mineral phase found in bone and teeth. HA is not present in other tissues under normal circumstances. Therefore, targeted drug delivery to HA is expected to result in sole delivery to bone tissue. Bisphosphonates (BPs), which are a class of drugs considered to be stable mimics of pyrophosphate, are used as inhibitors of osteoclastic bone resorption. BPs have a marked affinity for HA.[2,3] Thus, a drug or carrier conjugated to BPs has been shown to target bone.[4–7] We report a new approach to deliver doxorubicin (DOX, or adriamycin) to HA using a novel calcium phosphate-binding liposome. DOX is widely used as an anticancer drug, although it can lead to serious systemic side effects such as cardiotoxicity.[8] The main purpose of this Letter is to present preliminary results to demonstrate the superiority of the liposome as a drug carrier for selective drug delivery, assuming that it reaches bone tissue and reduces the systemic side effects of DOX.

We synthesized a novel amphipathic molecule bearing a bisphosphonate head group, 4-*N*-(3,5-ditetradecyloxybenzoyl)-aminobutane-1-hydroxy-1,1-bisphosphonic acid disodium salt (BPA, Fig. 1), using a procedure similar to that used by Xu et al.[9] (see Supplementary data). BPA liposomes were prepared as follows: distearoylphosphatidylcholine (DSPC) and cholesterol (Chol) (DSPC:Chol = 2:1) along with varying amounts of BPA (0, 1, 2, 5, and 10% molar percentage of BPA in the DSPC/Chol solution) were dissolved in chloroform/ethanol/water (131:20:1) and dried under reduced pressure. The thin lipid film was hydrated with water, and the liposomal solution was extruded through a polycarbonate membrane filter with 100-nm pores. The sizes and zeta potentials of the liposomes were obtained by laser scattering. Table 1 summarizes the physicochemical properties of the BPA liposomes. The sizes of the liposomes in all but the 0% BPA preparation were in the range of 120–137 nm, as determined by a dynamic light scattering technique. Analyses of the zeta potentials of the liposomes revealed that the potentials became increasingly negative with increasing amounts of BPA.

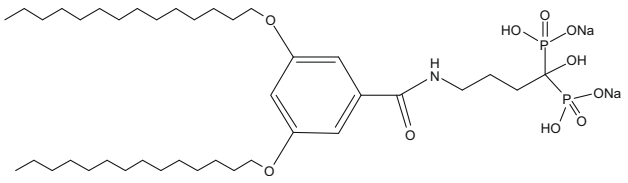

Figure 1. Chemical structure of a novel amphipathic molecule bearing a bisphosphonate head group 4-*N*-(3,5-ditetradecyloxy-benzoyl)-aminobutane-1-hydroxy-1,1-bisphosphonic acid disodium (BPA; see supplementary data).

* Corresponding authors. Tel.: +81 22 717 7636; fax: +81 22 717 7637 (T.A.).
E-mail addresses: anada@mail.tains.tohoku.ac.jp (T. Anada), suzuki-o@mail.tains.tohoku.ac.jp (O. Suzuki).

0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.05.117

Table 1
Characterization of BPA-liposomes

Liposomes	Mean particle size[a] (nm)	Zeta potential[a] (mV)	DOX encapsulation efficiency[a] (%)
0% mol BPA	295.4 ± 94.0	−1.1 ± 1.1	94 ± 5
1% mol BPA	137.4 ± 65.8	−6.8 ± 0.4	91 ± 6
2% mol BPA	129.5 ± 63.2	−9.0 ± 2.1	89 ± 5
5% mol BPA	123.4 ± 37.9	−11.8 ± 1.0	62 ± 7
10% mol BPA	120.4 ± 38.4	−21.8 ± 0.4	44 ± 8

[a] The data represent the mean ± SD (n = 3).

These data suggest that the efficient incorporation of BPA into liposomes is responsible for increasing the negative charge on the liposomal surface. The mean size of the particles in the 0% BPA liposome was larger than that in the other BPA liposomes. One possible explanation for this may be the aggregation of particles corresponding to the zeta potential of the 0% BPA liposome being near zero.

We evaluated the binding capacity of the BPA liposomes to HA. The preparation of HA has been described previously.[10] A binding assay was performed as follows: synthesized HA was suspended in 50 mM Tris–HCl (pH 7.4) at 1, 2, 3, 5, and 10 mg/mL. BPA liposomes were added to the HA suspensions (final concentration of total lipids = 100 μM), and the mixture was then gently shaken for 6 h at room temperature. After centrifugation at 5000 rpm, the light-scattering intensities of the supernatants were measured. The decrease in the intensity expresses the difference to the initial concentration, which corresponds to the amount bounds to HA. Figure 2 shows the affinity of the BPA liposomes to HA. The binding between BPA liposomes and HA depended on the amount of BPA present. When the volume of HA was over 5 mg/mL, in both the 5% and 10% mol BPA lipid, almost all the liposomes bound to HA. In contrast, the liposome with no BPA (0% mol BPA lipid) did not bind to HA. These data indicate that BP groups on the liposomal surfaces play an important role in determining the capacities of the liposomes for HA.

DOX-loaded liposomes were prepared by a modification of the remote-loading method developed by Mayer et al.[11] In this process, the lipid film was hydrated with 0.3 M sodium citrate (pH 4.0), the liposomal solution was extruded through a filter with a pore size of 100 nm, the solution was adjusted to pH 7.4, DOX (molar ratio of DOX:total lipid = 1:5) was added to the solution, and the solution was maintained at 60 °C for 10 min. Unentrapped DOX was removed by gel filtration, and the loading efficiency was estimated by measuring the absorbance at 480 nm. The DOX encapsulation efficiency is listed in Table 1. The liposomes of all compositions exhibited DOX encapsulation. The encapsulation yields in liposomes with 0%, 1%, and 2% mol BPA lipid were almost all the same. However, the loading yields in liposomes with 5% and 10% mol lipid were lower, and the yield in liposomes with 10% mol BPA lipid solutions was lower than that with 5% mol lipid. These results suggest that DOX can interact with a negatively charged bisphosphonate group through electrostatic interaction owing to the positive charge of the aminosugar moiety, and that the interaction may inhibit the encapsulation of DOX in the liposomes in the 5% and 10% solutions.[12]

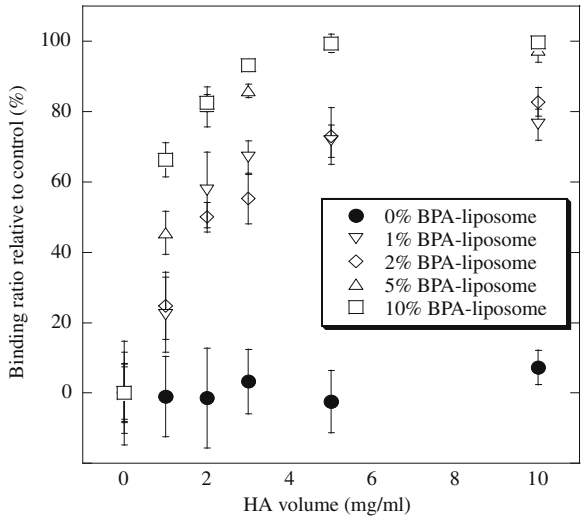

Figure 2. Affinity of the BPA-liposomes to HA. The percentage of the liposomes bound to HA was determined by the measuring light-scattering intensities of the supernatant. The results are shown as the mean ± SD (n = 3). The binding ratio (%) = 100 − [(light-scattering intensity of the supernatant of each sample)/(light-scattering intensity of the supernatant of each control (without HA)) × 100].

Finally, the in vitro cytotoxicities of the DOX-loaded liposomes in the 0%, 5%, and 10% mol BPA lipid, which were adsorbed onto HA, were estimated using a human osteosarcoma cell line (MG63). HA was coated onto a 48-well tissue culture plate (6 mg/well). We evaluated the carrier performance from the viability of MG63 after 3 days of culture. The liposomes in the 5% and 10% mol BPA lipid were used in the present experiment because of their high affinity for HA. Since their DOX encapsulation efficiencies were different, as shown in Table 1, the DOX concentrations, which were determined from the encapsulation efficiency, were adjusted so that they would be the same for each. Before seeding the cells on the HA-coated plates, the plates were allowed to adsorb the liposomes on the surfaces of the HA coatings for 6 h, and then each well was washed with water to remove the nonspecifically bound liposomes. Figure 3A shows the cytotoxicities of the DOX-loaded liposomes that were adsorbed onto HA as functions of the initial DOX concentrations in a 250 μL culture medium. There was no noticeable change in the cytotoxicity of the liposomes in the 0% mol BPA lipid throughout the DOX concentration range of 0–10 μM. However, the cytotoxicities of the liposomes in the 5% and 10% mol BPA lipid were significantly enhanced as the concentration of DOX increased. The viabilities of the MG63 cells cultured on the plates containing adsorbed liposomes from the 10% mol BPA lipid were lower than those of the cells cultured on the plates containing adsorbed liposomes from the 5% mol BPA lipid. Moreover, Figure 3B shows the cytotoxicities of the liposomes as functions of the liposome concentrations of the MG63 cells. The proliferation capacity was unchanged even when the concentrations of the BPA liposomes were increased in the 0%, 5%, and 10% mol BPA lipid; this observation was similar to that made for the 0% mol BPA lipid. These data indicate that the ability of the DOX-loaded BPA liposomes adsorbed on the HA to effectively suppress tumor cell growth depended on the amount of BPA in the liposome. These results may be explained by the release of DOX in the vicinity of the cells and/or the uptake of the liposomes by the cells. However, the latter case, electrostatic interaction may not be the main mechanism for cellular uptake of the liposomes, because both the cellular membrane and the BPA liposomes are negatively charged. Therefore, the reason that the liposomes in the 10% mol BPA lipid performed the best may be attributable to their high affinity for HA and their drug release properties (see Fig. S1).

To summarize the present findings, liposomes having BP moieties show high affinity for HA. DOX-loaded liposomes adsorbed on the surface of HA significantly reduce the number of tumor cells. This shows that BPA liposomes can be excellent carriers for anticancer drugs because they specifically target bone tissue. This calcium phosphate-binding liposome system could be used with many drugs for bone-related diseases such as osteoporosis, rheumatoid arthritis, and multiple myeloma. The BPA-liposomes may be selectively distributed to the skeleton and favor sites of high turnover. Moreover, enhanced permeability and retention (EPR) ef-

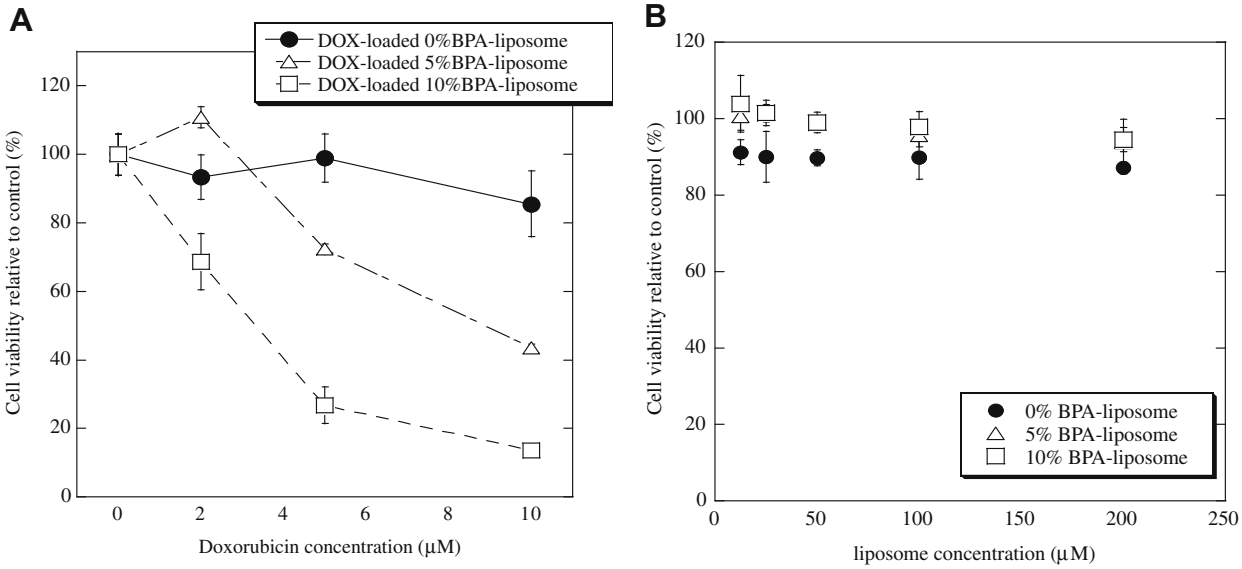

Figure 3. Cytotoxicities of the DOX-loaded liposomes, with and without BPA, as functions of the initial drug concentrations (A), and a comparison of the cytotoxicities of the liposomes without DOX as functions of liposome concentrations (B). The results are shown as the mean ± SD (n = 3).

fect[13] may play a important role for bone-targeting drug delivery system using BPA-liposomes. The in vivo evaluation of BPA liposomes is currently in progress.

Acknowledgment

This study was supported in part by Grants-in-Aid (17076001 and 19750134) from the Ministry of Education, Science, Sports, and Culture of Japan.

Supplementary data

Supplementary data (Experimental details, materials, chemical modification procedures, the assay for HA binding, the assay of drug release, and the cytotoxicity assay for the carries) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.05.117.

References and notes

1. Mundy, G. R. *Nat. Rev. Cancer* **2002**, *2*, 584.
2. Fleisch, H. *Drugs* **1991**, *42*, 919.
3. Papapoulos, S. E. *Bone* **2006**, *38*, 613.
4. Gittens, S. A.; Bansal, G.; Zernicke, R. F.; Uludag, H. *Adv. Drug Delivery Rev.* **2005**, *57*, 1011.
5. Hengst, V.; Oussoren, C.; Kissel, T.; Storm, G. *Int. J. Pharm.* **2007**, *331*, 224.
6. Hirabayashi, H.; Fujisaki, J. *Clin. Pharmacokinet.* **2003**, *42*, 1319.
7. Wang, D.; Miller, S. C.; Kopeckova, P.; Kopecek, J. *Adv. Drug Delivery Rev.* **2005**, *57*, 1049.
8. Singal, P. K.; Natasha, I. *N. Engl. J. Med.* **1998**, *339*, 900.
9. Xu, G.; Yang, C.; Liu, B.; Wu, X.; Xie, Y. *Heteroat. Chem.* **2004**, *15*, 251.
10. Suzuki, O.; Nakamura, M.; Miyasaka, Y.; Kagayama, M.; Sakurai, M. *Tohoku J. Exp. Med.* **1991**, *164*, 37.
11. Mayer, L. D.; Bally, M. B.; Hope, M. J.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *816*, 294.
12. Fonseca, M. J.; van Winden, E. C. A.; Crommelin, D. J. A. *Eur. J. Pharm. Biopharm.* **1997**, *43*, 9.
13. Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, *46*, 6387.