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Note

Bone targeting potential of bisphosphonate-targeted liposomes Preparation, characterization and hydroxyapatite binding in vitro

V. Hengst [a,b,c], C. Oussoren [a], T. Kissel [c], G. Storm [a,*]

[a] *Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), University of Utrecht, P.O. Box 80082, The Netherlands*
[b] *MCS Micro Carrier Systems GmbH, Stresemannallee 6, 41460 Neuss, Germany*
[c] *Department of Pharmaceutics and Biopharmacy, Philipps-Universität Marburg, Ketzerbach 63, 35032 Marburg, Germany*

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Abstract

The main constituent of bone is hydroxyapatite (HAP). Since HAP is only present in 'hard' tissues like bone and teeth, it represents a promising target for the selective drug delivery to bone. Due to the exceptional affinity of bisphosphonates (BP) for HAP, cholesteryl-trisoxyethylene-bisphosphonic acid (CHOL-TOE-BP), a new tailor-made BP derivative, was used as bone targeting moiety for liposomes. CHOL-TOE-BP-targeted liposomes were designed for the treatment of bone-related diseases to achieve prolonged local exposure to high concentrations of the bioactive compounds, thereby enhancing therapeutic efficacy and minimizing systemic side effects. The CHOL-TOE-BP-targeted liposomes were characterized regarding particle size and zeta potential. To study the bone targeting potential of these conjugates, an in vitro HAP binding assay was established. The obtained binding data indicate that CHOL-TOE-BP is useful as targeting device for liposomal drug delivery to bone.

Keywords: Bisphosphonates; Hydroxyapatite; Liposomes; Bone targeting

1. Introduction

Bone differs from the rest of the body by the presence of hydroxyapatite (HAP), the mineral phase of bone which is not present in other tissues, except in case of pathological calcifications. Because of this unique feature it is attractive to use HAP as a target for the selective bone delivery of therapeutics to treat bone-related pathologies. Based on their structural similarity to pyrophosphate, bisphosphonates exhibit a strong affinity to HAP and could, therefore, be useful as targeting moieties (Fleisch, 1987; Golomb et al., 1992; Grossmann, 2000). Several approaches for the design of bone-selective delivery systems have been proposed. Gonzalez et al. (2002) developed a bone tissue-targeted bisphosphonate-fullerene. To confer osteotropicity, bisphosphonates have been coupled to non-specific bone therapeutic agents like estradiol, prostaglandin E_2, cisplatin or radiopharmaceuticals (Klenner et al., 1990; Bauss et al., 1996; Gil et al., 1999; Lisic et al., 2001). Here we describe the preparation and the in vitro characterization of a new bone-targeted delivery system composed of liposomes as drug reservoir and cholesteryl-trisoxyethylene-bisphosphonic acid (CHOL-TOE-BP) (Greb et al., 2005) serving as bone targeting molecule (Fig. 1). The cholesteryl-moiety acts as bilayer anchor molecule, the trisoxyethylene-chain as spacer molecule and the bisphosphonic acid residue as targeting device sticking out of the liposomal bilayer. The proposed nano-scale targeting system can potentially be applied to a large variety of drugs for the treatment of bone diseases. It is anticipated that the bone-selective delivery of the liposomally encapsulated drug can lead to locally prolonged drug concentrations potentially enhancing their therapeutic effect. In addition, decreased systemic toxicity is a potential major benefit that can be achieved with this targeting approach (Allen, 1997; Crommelin et al., 2002). The CHOL-TOE-BP-targeted liposomes were characterized with regard to physicochemical characteristics. A HAP binding assay was established under in vitro conditions and the affinity of the targeted liposomes for HAP is reported here.

2. Liposome preparation and characterization

CHOL-TOE-BP-targeted liposomes composed of EPC, cholesterol, DSPE-PEG2000 in a molar ratio of 1.85/1.0/0.15

* Corresponding author. Tel.: +31 30 253 7388; fax: +31 30 251 7839.
E-mail address: g.storm@pharm.uu.nl (G. Storm).

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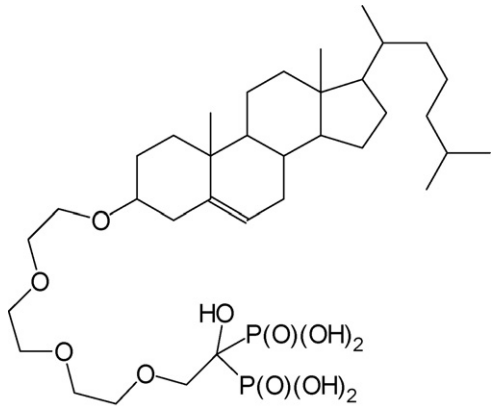

Fig. 1. Chemical structure of cholesteryl-trisoxyethylene-bisphosphonic acid (CHOL-TOE-BP).

and CHOL-TOE-BP concentrations ranging from 3 to 25 mol% were prepared by the lipid film extrusion method. The liposomes were fluorescently labelled with 0.1 mol% 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine 4-chlorobenzenesulfonate sulfonate salt (DiD). Non-targeted liposomes (control liposomes) were composed of EPC, cholesterol, DSPE-PEG2000 in a molar ratio of 1.85/1.0/0.15 and 0.1 mol% DiD.

Mean particle size and size distribution were determined by dynamic light scattering with a Malvern ALV CGS-3 System (Malvern Ltd., Malvern, UK). The average size of the liposomes in all preparations was between 100 and 135 nm with a polydispersity index <0.1 indicating that liposomes with a relative homogenous distribution were obtained (Table 1). It was anticipated that the incorporation of a negatively charged ligand will lead to a negatively charged liposomal surface. The surface charge is an important parameter characterizing the interaction between the CHOL-TOE-BP derivative and HAP. Neves et al. (2002) demonstrated that factors like zeta potential, interfacial tension and the nature of the site groups of bisphosphonates affect binding to HAP, resulting in steric, electrostatic and chelate effects that are involved in the binding mechanism. A negative zeta potential also has impact on the in vivo fate of the liposomes. Although PEG is present in the liposome preparation, a targeting ligand presented at the terminal ends of the PEG chains sticking out of the liposome surface may increase the rate of liposome uptake by liver and spleen (Klibanov, 1998). The zeta potential of CHOL-TOE-BP-targeted liposomes (measured at low ionic strength by dilution 1:100 with 5 mM Hepes buffer, pH 7.4) was measured at 25 °C by laser Doppler velocimetry (Zetasizer 2000, Malvern Instruments Ltd., UK). The data obtained for the various liposome formulations indicate that the zeta potential of liposomes depends on the amount of the CHOL-TOE-BP incorporated in the liposomes: the higher the CHOL-TOE-BP content, the more negative the zeta potential (Table 1).

3. In vitro HAP binding assay

It is well-known that bisphosphonates bind to HAP (Fleisch, 2002). To study the binding of CHOL-TOE-BP-targeted liposomes to HAP, an in vitro HAP binding assay was set up using in vitro HAP binding methods described by Shinoda et al. (1983) and Fujisawa et al. (1996). Briefly, 100 mg pre-equilibrated samples of synthetic HAP (specific surface area: 72.8 m^2/g; determined by BET nitrogen adsorption) were incubated with 50 μl fluorescently (DiD) labelled CHOL-TOE-BP-targeted liposomes (25 mol% CHOL-TOE-BP) overnight at 25 °C. The amount of liposomes not bound to HAP was quantified by fluorimetry (DiD: excitation: 620 nm, emission: 650 nm) in the supernatant after centrifugation of incubated HAP samples at 6000 rpm for 60 min. To determine the initial amount of DiD fluorescence, DiD labelled liposomes were treated the same way as described above but were not incubated with HAP.

The degree of HAP binding was assessed as mean of three independent experiments according to the following formula:

$$\text{HAP binding}\,(\%) = \frac{(X - Y)100}{X}$$

where X is the initial amount of DiD fluorescence corrected for the blank value and the degree of dilution and Y is the amount of DiD fluorescence in the supernatant corrected for the blank value and the degree of dilution.

The data presented in Fig. 2 clearly demonstrate the capability of CHOL-TOE-BP-targeted liposomes to bind to HAP. Non-targeted liposomes hardly show any binding demonstrating that the ligand plays an important role in the binding process (Fig. 2). However, it is not clear yet whether specific ligand–HAP interactions are involved, as these initial binding experiments do not distinguish between HAP affinity due to increased negative zeta potential and HAP affinity due to CHOL-TOE-BP specificity.

Fig. 3 shows a positive correlation between the degree of binding and the HAP amount present during incubation. A higher HAP amount resulted in higher degree of binding (Fig. 3).

Table 1
Physicochemical liposome characteristics

Liposome type	Incorporated amount of ligand (mol%)	Mean diameter (nm)[a]	Polydispersity index[a,b]	Zeta potential (mV)[a]
EPC/CHOL/DSPE-PEG2000	–	99 ± 1	0.02 ± 0.01	−18 ± 2
EPC/CHOL/DSPE-PEG2000/CHOL-TOE-BP	3	134 ± 1	0.04 ± 0.01	−28 ± 2
EPC/CHOL/DSPE-PEG2000/CHOL-TOE-BP	14	117 ± 1	0.08 ± 0.03	−34 ± 1
EPC/CHOL/DSPE-PEG2000/CHOL-TOE-BP	25	128 ± 1	0.09 ± 0.01	−40 ± 2

[a] Values are mean ± S.D. ($n = 3$).
[b] A polydispersity index of 0 indicates that a complete monodisperse system is obtained and a value of 1 indicates that an absolutely heterodisperse system is present.

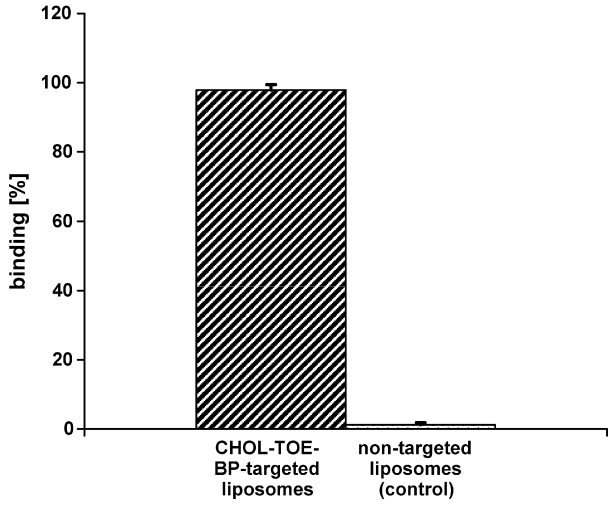

Fig. 2. Degree of HAP binding of CHOL-TOE-BP-targeted liposomes (25 mol% CHOL-TOE-BP) vs. non-targeted liposomes. Data represent the mean and standard deviation of three independent experiments.

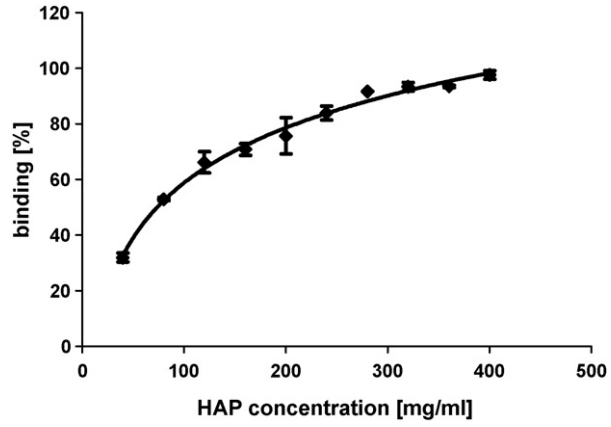

Fig. 3. Effect of HAP concentration on the degree of HAP binding of CHOL-TOE-BP-targeted liposomes (25 mol% CHOL-TOE-BP). Data represent the mean and standard deviation of three independent experiments.

When the amount of HAP present during the incubation was kept constant, an increased amount of CHOL-TOE-BP-liposomes resulted in a lower binding likely due to saturation of the HAP binding sites (Fig. 4). Altogether, these results demonstrate that

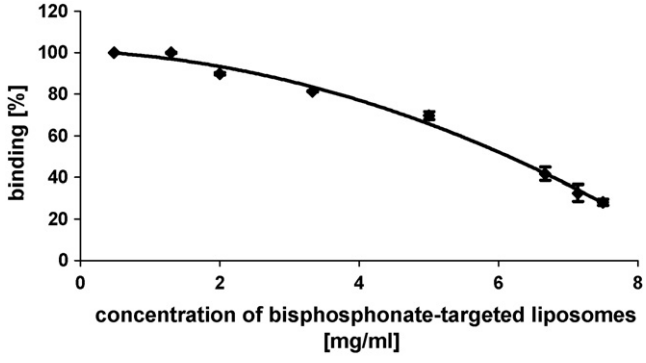

Fig. 4. Effect of liposome concentration on the degree of HAP binding of CHOL-TOE-BP-targeted liposomes (25 mol% of CHOL-TOE-BP). Data represent the mean and standard deviation of three independent experiments.

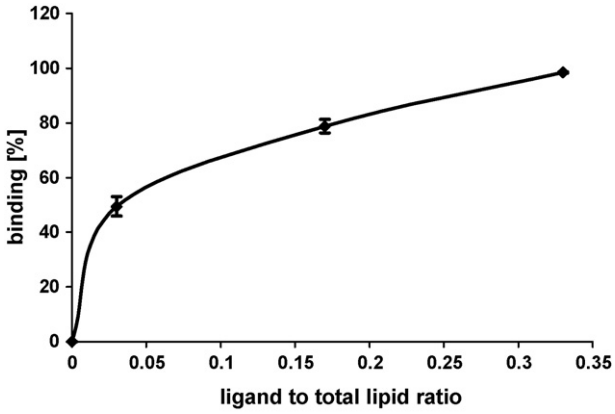

Fig. 5. Effect of CHOL-TOE-BP density on the degree of HAP binding of CHOL-TOE-BP-targeted liposomes. Data represent the mean and standard deviation of three independent experiments.

maximal binding can be reached at an optimal ratio of CHOL-TOE-BP-targeted liposomes to HAP. In our experimental setup, this ratio was about 0.005 (25 mol% CHOL-TOE-BP). In addition, the density of the CHOL-TOE-BP-ligand on the surface of the liposomes proved to be an important factor influencing the binding capacity. A higher CHOL-TOE-BP-ligand density resulted in a higher degree of HAP binding in a biphasic pattern (Fig. 5). The steepness of the slope at the beginning of the curve may reveal a good accessibility of HAP binding site to bisphosphonate groups present on the liposomal surface. At a ligand-to-total lipid ratio higher than 0.04, the curve levels off demonstrating that a higher CHOL-TOE-BP-ligand density is needed to reach maximum binding as the most accessible binding sites have been occupied already. These data suggest that the effect of the density of the CHOL-TOE-BP concentration with regard to in vivo studies should be carefully studied to obtain the maximum affinity of CHOL-TOE-BP-targeted liposomes to bone.

In order to develop an optimal bone-targeted drug delivery system, the next experimental steps to be taken will involve the study of pharmacokinetics and tissue distribution of CHOL-TOE-BP-targeted liposomes, with special emphasis on the degree and mechanism of bone localization. Based on the in vitro data presented here, we suggest that CHOL-TOE-BP-targeted liposomes have potential for drug delivery to bone. Therefore, further investigation of this bone targeting system is warranted.

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