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Oral delivery of zoledronic acid by non-covalent conjugation with lysine-deoxycholic acid: In vitro characterization and in vivo anti-osteoporotic efficacy in ovariectomized rats

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ABSTRACT

We assessed the possibility of changing the route of administration of zoledronic acid to an oral dosage form and its therapeutic efficacy in an estrogen-deficient osteoporosis rat model. To enhance oral bioavailability, we formed an ionic complex by electrostatic conjugation of zoledronic acid with lysine-linked deoxycholic acid (Lys-DOCA, an oral absorption enhancer). After forming the complex, the characteristic crystalline features of pure zoledronic acid disappeared completely in the powder X-ray diffractogram and differential scanning calorimetry thermogram, indicating that zoledronic acid existed in an amorphous form in the complex. In vitro permeabilities of zoledronic acid/Lys-DOCA (1:1) (ZD1) and zoledronic acid/Lys-DOCA (1:2) (ZD2) complex across Caco-2 cell monolayers were 2.47- and 4.74-fold higher than that of zoledronic acid, respectively. Upon intrajejunal administration to rats, the intestinal absorption of zoledronic acid was increased significantly and the resulting oral bioavailability of the ZD2 complex was determined to be 6.76 ± 2.59% (0.548 ± 0.161% for zoledronic acid). Ovariectomized (OVX) rats showed 122% increased bone mineral density versus the OVX control at 12 weeks after treatment with once weekly oral administration of ZD2 complex (16 μg/kg of zoledronic acid). Furthermore, rats treated with ZD2 complex orally showed significant improvement in the parameters of trabecular microarchitecture and bone strength: 149% higher bone volume fraction (BV/TV), 115% higher trabecular number (Tb.N), and 56% higher mean maximum load (F_{max}) than in the OVX group. The trabecular microstructure and bone mechanical properties in the oral zoledronic acid group were not significantly changed compared with the OVX control. Thus, the oral ZD2 complex inhibited osteoporosis progression effectively by promoting osteogenesis and trabecular connectivity. The oral ZD2 complex would be expected to improve patient compliance by replacing the conventional injectable form and expand the indications, to include prophylaxis for osteoporosis and bone metastases.

1. Introduction

Zoledronic acid is a potent nitrogen-containing parenteral bisphosphonate, used clinically as an anti-resorptive agent in treating osteoporosis, Paget's disease, hypercalcemia, multiple myeloma, and bone metastases arising from solid tumors, such as breast, prostate, and lung cancers (Black et al., 2007; Boissier et al., 2000; Novartis Pharmaceutical Corp., 2003). The primary mode of action of zoledronic acid is inhibition of bone resorption via induction of osteoclast apoptosis (Green et al., 1994; Hornby et al., 2003). As is true of other bisphosphonates, zoledronic acid exhibits a high affinity for hydroxyapatite and binds directly to mineralized zones at sites of drug release. The drug then becomes internalized by osteoclasts that are engaging in bone resorption, compromising osteoclast formation, function, and survival. It also induces apoptosis of various types of cancer cells (Corey et al., 2003; Dunford et al., 2001; Khajuria et al., 2015).

Zoledronic acid is currently only available in a parental dosage form for infusion over at least 15 min (Novartis Pharmaceutical Corp., 2003). However, too rapid an injection of bisphosphonate can cause the formation of complexes with calcium in the blood, leading to renal failure due to the complex being retained in the kidney (Blanchette and Pritchard,

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2015). Moreover, local delivery of bisphosphonates, such as subcutaneous or intramuscular administration increases the risk of tissue irritation, inflammation, pain, and necrosis at the injection site (Ezra and Golomb, 2000; Lin, 1996). Given this background, there is a demand for the development of other dosage forms of zoledronic acid, particularly an oral dosage form, because orally administered drugs are becoming more widespread in many therapeutic areas, including the treatment of cancer.

However, all bisphosphonates, including zoledronic acid, exhibit low oral bioavailability (<1%), attributable to the drugs being highly polar and hydrophilic and the fact that they form insoluble metal complexes in the upper intestine, most commonly with calcium, resulting in low gastrointestinal (GI) permeation (Lin, 1996). For these reasons, oral delivery of zoledronic acid has been challenging. There have been various attempts to generate novel oral formulations by crystallization and the formation of metal salts, including Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, mono- and trihydrates, and amorphous forms of zoledronic acid, to improve its aqueous solubility, permeability, and subsequent oral bioavailability (Aronhime et al., 2005; Mohakhud et al., 2006; Novartis Ag et al., 2008). Additionally, Merrion Pharmaceuticals prepared an oral dosage form of zoledronic acid wherein a medium chain fatty acid or a salt of a medium chain fatty acid having a carbon chain length of 6–20 carbon atoms was used as an oral enhancer (Merrion Research Ill Ltd., 2010) and Thar Pharmaceuticals used modified amino acid carriers by complex formation with amino acids to enhance the oral absorption of zoledronic acid (Thar Pharmaceuticals, Inc., 2011).

In a previous study, we have shown that the intestinal permeability of bisphosphonates such as ibandronate could be significantly improved by generation of molecular complexes with lysine-linked deoxycholic acid (Lys-DOCA) (Park et al., 2013). The aim of the current study was to expand our technology to develop an oral formulation of zoledronic acid, resulting in a new dosage form for long-term administration by conjugating the Lys-DOCA as an absorption enhancer to increase the intestinal permeability and prove its therapeutic efficacy in vivo. After characterization of the zoledronic acid/Lys-DOCA (ZD) complexes in terms of crystallinity and water solubility, we confirmed the in vitro drug permeability through an artificial intestinal membrane and a Caco-2 cell monolayer, followed by assessment of the oral bioavailability of the ZD complex in vivo. Finally, the ZD complex was administered orally to ovariectomized (OVX) rats once per week, after which we evaluated its anti-osteoporotic efficacy compared with oral or intraperitoneally injected zoledronic acid by analyzing bone properties, such as bone mineral density (BMD), trabecular bone microarchitecture in tibiae, and biomechanical strength.

2. Materials and methods

2.1. Materials

Zoledronic acid was obtained as the hydrated disodium salt from Ultratech India Ltd. (Mumbai, India). Deoxycholic acid (DOCA), ethyl chloroformate, N-methylmorpholine, N_ε-Boc-L-lysine methyl ester hydrochloride (H-Lys(Boc)-OMe·HCl), lithium aluminum hydride ($LiAlH_4$), ibandronate, trimethylsilyl diazomethane (TMSD; 2 M solution in hexane), ammonium acetate, and formic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Tetrahydrofuran (THF), chloroform, methanol, acetyl chloride, and *n*-hexane (analytical grade) were obtained from Merck Co. (Darmstadt, Germany). Solvents for high-performance liquid chromatography (HPLC) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) analyses were from Merck KGaA (Darmstadt, Germany) and Fisher Scientific (Pittsburgh, PA, USA).

2.2. Animals

Sprague Dawley rats (females, 200–250 g) were purchased from Orient Co., Ltd. (Gyunggi-do, Republic of Korea). The animals were acclimated for 1 week in an animal facility under controlled conditions of temperature (23 ± 2 °C), relative humidity (55 ± 10%), and light (12/12-h light/dark, with no ultraviolet exposure). The animals had free access to a laboratory diet (Purina Co., St. Louis, MO, USA) and ion-sterilized tap water.

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Seoul, Republic of Korea; approval date, 12/10/2014; ref. no., SNU-13081-3-1). All experiments were performed in accordance with the NIH guidelines for the Care and Use of Laboratory Animals and the guidelines of the IACUC.

2.3. Preparation of zoledronic acid/absorption enhancer complex

The oral absorption enhancer, N^α-deoxycholyl-L-lysyl-methylester (Lys-DOCA), was synthesized by conjugating DOCA with L-lysine, as described previously (Park et al., 2013). Briefly, ethyl chloroformate (6.4 mL) and N-methylmorpholine (7.4 mL) were dropped into 3.25% (w/v) DOCA solution in THF in an ice bath, and stirred for 30 min. Next, H-Lys(Boc)-OMe·HCl (20 g) and N-methylmorpholine (7.4 mL) were added to the mixture at room temperature, and the reaction mixture was refluxed for 2 h. After cooling to room temperature, the mixture was stirred overnight. The precipitates were filtered and the solvent was evaporated. Lys(Boc)DOCA was obtained by purification of the residue using column chromatography (chloroform/methanol). Lys(Boc)DOCA was dissolved in a solvent mixture of acetyl chloride (23.4 mL) and methanol (100 mL) in an ice bath and stirred for 30 min. After solvent evaporation, the residue was dissolved in water and washed with chloroform three times. Finally, the aqueous layer was lyophilized and Lys-DOCA was obtained as a white powder.

The ZD complex was prepared by the ionic interaction of zoledronic acid and Lys-DOCA under aqueous conditions. Briefly, 5 mg/mL solutions of zoledronic acid and Lys-DOCA were prepared separately in distilled water, and the ZD complex was then formed by the addition of Lys-DOCA solution into a zoledronic acid aqueous solution with continuous stirring. The complexation molar ratios of zoledronic acid to Lys-DOCA were 1:1 (ZD1) and 1:2 (ZD2). The solutions were then centrifuged and lyophilized at −80 °C to obtain white powders.

2.4. Characterization of zoledronic acid/absorption enhancer complex

Complex formation between zoledronic acid and Lys-DOCA was confirmed by comparing the characteristic crystalline features of pure zoledronic acid, Lys-DOCA, physical mixtures of zoledronic acid and Lys-DOCA, and the ZD complexes using powder X-ray diffraction (PXRD) and differential scanning calorimetry (DSC). PXRD patterns were collected with a Rigaku-D/MAX-IIIV diffractometer (D5005, Bruker, Germany) at 40 mA and 40 kV using Ni-filtered Cu-Kα radiation. The powdered samples were deposited on an adhesive support, 0.5 mm thick, and placed in the diffractometer. PXRD patterns were recorded in step-scan mode in the range $3° \le 2\theta \le 40°$ with a scanning rate of $0.02°s^{-1}$.

Thermal analysis of the samples was carried out using a DSC 204A/G Phoenix Instrument (Netzsch, Germany). Approximately 5 mg of each sample were weighed into a non-hermetically sealed aluminum pan and scanned at a heating rate of 5 °C/min over a temperature range of 25–250 °C. All DSC measurements were made under a nitrogen atmosphere at a flow rate of 100 mL/min.

2.5. Solubility and partition coefficient of the zoledronic acid/absorption enhancer complex

The solubilities of the ZD1 and ZD2 complexes in phosphate-buffered saline (PBS, pH 6.8) were determined. Briefly, excess ZD1 or ZD2 complex was added to 5 mL of PBS at pH 6.8 in a sealed glass container. The samples were agitated at 100 rpm for 24 h in a shaking water