Synthesis and Preclinical Pharmacology of 2-(2-Aminopyrimidino) Ethyldene-1,1-Bisphosphonic Acid Betaine (ISA-13-1)—A Novel Bisphosphonate

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Purpose. To validate our hypothesis that a bisphophonate (BP) having a nitrogen-containing heterocyclic ring on the side chain, and with no hydroxyl on the geminal carbon would possess increased activity, and better oral bioavailability due to enhanced solubility of its calcium complexes/salts and weaker Ca chelating properties.

Methods. A novel BP, 2-(2-aminopyrimidino)ethyldene-1,1-bisphosphonic acid betaine (ISA-13-1) was synthesized. The physicochemical properties and permeability were studied in vitro. The effects on macrophages, bone resorption (young growing rat model), and tumor-induced osteolysis (Walker carcinosarcoma) were studied in comparison to clinically used BPs.

Results. The solubility of the Ca salt of ISA-13-1 was higher, and the log \( \beta_{Ca, BP} \) stability constant and the affinity to hydroxyapatite were lower than those of alendronate and pamidronate. ISA-13-1 exhibited effects similar to those of alendronate on bone volume, on bone osteolysis, and on macrophages, following delivery by liposomes. ISA-13-1 was shown to have 1.5–1.7 times better oral absorption than the other BPs with no deleterious effects on the tight junctions of intestinal tissue.

Conclusions. The similar potency to clinically used BPs, the increased oral absorption as well as the effect of drug administration on activity of ISA-13-1 warrant its further consideration as a potential drug for bone diseases.

KEY WORDS: bisphosphonates (diphosphonates); calcium-related disorders; bone-related disorders; drug administration; drug absorption tight junctions; manniotil.

INTRODUCTION

Several bisphosphonates (Fig. 1) are in use for the treatment of various calcium-related disorders such as Paget’s disease, hypercalcemia of malignancy, tumor osteolysis, and most recently, in osteoporosis (1). The macrophage suppressive effect of BPs has attracted interest recently as a possible approach to pharmacotherapy in rheumatoid arthritis (2). The biological effects of the BPs stem from their incorporation in bone, enabling direct interaction with osteoclasts and/or osteoblasts through a variety of biochemical pathways (3). A significant drawback of the BPs is their very poor oral absorption (less than 1%) which is further diminished by concomitant food intake (4). The oral administration of BPs is associated with GI disturbances (5), and it is believed that they are absorbed via the paracellular route with accompanied disruption of the tight junctions (6,7). Therefore, the development of potent BPs possessing increased oral absorption with no toxic effect is of importance.

It appears that the incorporation of nitrogen-containing heterocyclic rings increases their pharmacological activity (8,9). The capacity of BPs to accumulate in bone is attributed to their high affinity to HAP due to a tridentate interaction of the phosphonic and hydroxyl functions with calcium. In the absence of a hydroxyl group on the geminal carbon only bidentate complexes can be formed resulting in lower affinities to calcium and a better solubility of the calcium/salts complexes (10). The latter characteristics could contribute to better compatibility with food and increased oral absorption. We hypothesized that a BP with a nitrogen-containing heterocyclic ring in the side chain, and with no hydroxyl on the geminal carbon could show increased activity as well as better oral bioavailability.

MATERIALS AND METHODS

Syntheses

Alendronate, \([^{14}C]\)-Alendronate (Sp.Act.1 \( \mu \)Ci/mg), Pamidronate, and \([^{13}C]\)-Pamidronate (Sp.Act.1 \( \mu \)Ci/mg) were synthesized as described by Alferiev et al. (11) with some modifications. Both the "cold" and radioactive 2-(2-Aminopyrimidino) ethyldene-1, 1-bisphosphonic acid betaine (ISA-13-1) were synthesized by the nucleophilic addition of 2-aminopyrimidine to the activated double bond of vinylidene-1, 1-bisphosphonic acid, using the methodology developed by Alferiev et al. (12) as shown in Fig. 1. Elemental analysis and spectroscopic methods showing the expected results identified the products.

Physicochemical Properties

Dissociation and Stability Constants

The dissociation constants (pKa) of alendronate and ISA-13-1, and the calcium-complex stability constants (\( \log \beta \)) were determined by high precision, potentiometrically controlled titration (SCHOTT Geräte, Hofheim, Germany) and subsequent iteration of titration data with the program ITERAX 2.01. For pKa determinations, 50 mL of a solution consisting of 0.25 mmol BP, 1.5 mmol NaOH and 3.5 mmol NaCl was titrated vs. 0.1 M HCl in equidistant steps of 0.1 mL at 25 \( \pm 0.1 \)°C. The pH was monitored using a glass electrode calibrated by

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the method of blank titration (8 and 2 titrations were performed for pamidronate and alendronate, and for ISA 13-1, respectively).

The stability constants were determined in titrated solutions containing a metal: ligand ratio of 1:1 (Ca:BP) of 0.002 M Ca and BP, and 0.01 M NaOH, ionic strength of 0.1 M (NaCl) with 0.1 M HCl, using combined glass-electrode for pH determinations.

Solubility of the Bisphosphonates

Solubility of the Ca salts of the BPs was determined by analyzing the amount of BP that remained in the supernatant of Tris buffer solutions (pH 7.4) containing 1 mM BP, and 1.2 mM CaCl₂ (ionic Ca physiological concentration). The solutions were shaken (100 rpm) and incubated at 37°C. After 1 and 24 h, the solutions were centrifuged (1900 g, 10 min.), and the BPs concentrations in the supernatants were determined at 305 nm for ISA-13-1, and by HPLC for alendronate (13).

Binding Affinity to HAP

The binding affinities of the BPs to HAP were calculated from the BPs adsorption isotherms as described elsewhere (14). The concentration of ISA-13-1 was determined at λ = 305 nm.

Inhibition of HAP Formation and Dissolution

The inhibition of HAP formation and dissolution in the presence of the tested compound were studied as described previously (14). Following preliminary studies, the drug concentrations were chosen to be 0.1 mM and 0.048 M for the inhibition of HAP formation and dissolution, respectively.

Intestinal Permeability

The permeability and oral bioavailability of ISA-13-1 were studied in comparison to pamidronate. Pamidronate’s oral absorption in animals and humans as well as its physicochemical characteristics are similar to those of alendronate and other BPs (1,8,10). All experimental animal studies adhered to the “Principles of Laboratory Animal Care” (NIH publication #85-23), and the guidelines of the Hebrew University of Jerusalem.

The permeability of ISA-13-1 and pamidronate across a segment of jejunal tissue were conducted in diffusion cells (1.88 cm², 8 ml of bicarbonate-Ringer buffer, 37°C, and gas lift of 95% O₂-5% CO₂). ISA-13-1 and [¹⁴C]-pamidronate ([¹⁴C] 1 μCi/mg) at concentrations of 1 mM were added to the mucosal bathing solution. Samples (1 ml) were taken from the serosal bathing solutions at 15-min intervals, and were replaced with buffer. Since mannitol is slowly transported via the paracellular route and is used as a marker of the tight junction integrity (15), the permeability of the jejunal tissue to D-mannitol (10 mM and a tracer amount of its ³H isotope, 30 Ci/mmol) was examined in the presence of both drugs.

In Vivo Absorption and Disposition

Absorption and disposition of [¹⁴C]-pamidronate and of [¹⁴C]-ISA-13-1 (10 mg/kg) were examined in rats following peroral administration of the drugs’ solution (via a stomach tube). Male Sabra rats (250–300 g) were acclimated in metabolic cages one week prior to the investigation, with free access to water and food except for 16 h prior to drug administration when only water was made available. The rats were sacrificed 24 h after drug administration, and drug amount in the tibia, femur, kidney, liver, intestine, spleen, muscle, brain, urine and feces were determined by radioactive measurements following digestion of the specimens to CO₂ by means of a SampleOxidizer (Packard, USA).

Effects on Bone Development

In preliminary experiments ISA-13-1 was found more effective than pamidronate. Therefore, the activity of ISA-13-1 was compared to that of alendronate, the most potent BP in clinical use (1). Three-week-old male rats (Sabra) were treated by daily intramuscular injections of ISA-13-1 and alendronate for 14 days at a dosage of 0.01 mg/kg/day. The control group received normal saline. The bones were analyzed as described previously (14).

Walker Carcinosarcoma (WCS) Model

This model was used to evaluate ISA-13-1 effect on bone resorption in a tumor osteolysis model (14). On day 2 of the experiment, the rats were divided into 3 groups: Control (saline), alendronate, and ISA-13-1 that received 50 μmol/kg, 0.5 ml s.c. injections on days 2, 3, and 4. On days 6 and 9 the rats were weighed. The amount of calcium in the urine were measured on days 2, 3, 4, 6, 8 and 9, as well as the levels of pyridinium crosslinks excreted in urine. Total urinary pyridinoline (PYD) and deoxypyridinoline (DPD) were determined by HPLC as described previously (16).

Effects on Macrophages

The drug was encapsulated in negatively charged DSPG-cholesterol (67:33) liposomes as described in detail elsewhere (17). The growth inhibitory properties of free and liposome-encapsulated ISA-13-1 were studied with murine macrophage cell line, RAW 264 (17). At the end of growth period, cell growth was evaluated using an MTT assay except that serum-containing medium was replaced with medium without serum just prior to the addition of MTT (18). The effect