Contrast Agents

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Synthesis of Conjugatable Bisphosphonates for Molecular Imaging of Large Animals**

*Kumar R. Bhushan, Eiichi Tanaka, and John V. Frangioni**

Mammography is currently the gold standard for the early detection of breast cancer.[1,2] However, mammography suffers from relatively low sensitivity and specificity,[3] and mammographic screening is limited in certain patient populations[4] and breast densities.[5] These limitations have spurred interest in alternate modalities to detect breast cancer. An important diagnostic feature of mammography is the presence of microcalcifications,[1] which result from deposition of calcium salts in breast tissue. Usually these salts are in the form of hydroxyapatite (HA),[6] which is also the first calcium salt deposited by osteoblasts during normal bone growth.

Herein we describe a simplified, reproducible synthesis of 3-amino tetramethyl 1-hydroxypropylidenebisphosphonate (methylester-protected pamidronate) and its use in preparing novel near-infrared (NIR) fluorescent optical contrast agents specific for HA. Such contrast agents show remarkable specificity for HA over other calcium salts. Our synthetic strategy permits preparation in high yield, and thus enables pre-clinical studies of contrast-agent performance in large-animal model systems approaching the size of humans. We also demonstrate that the agent Pam800 provides real-time, intraoperative NIR fluorescence imaging of HA in soft tissue and bone.

Bisphosphonates are widely used for the treatment of bone metastases, and evidence suggests that these compounds provide benefit to breast cancer patients with metastases to bone.[7] Bisphosphonates are analogues of endogenous pyrophosphates in which the hydrolyzable oxygen atom that separates the two phosphate groups is replaced with a more stable carbon atom. The P–C–P unit is responsible for giving bisphosphonates their high affinity for bone, which can be further enhanced by addition of a hydroxy group at the central carbon atom.[8] In vivo, bisphosphonates bind strongly to HA on the bone surface and are preferentially delivered to sites of increased bone formation or resorption. They are potent inhibitors of osteoclast-mediated bone resorption[9] and are effective in lowering serum calcium concentrations in patients with hypercalcemia of malignancy.[10,11] Treatment with bisphosphonates has also been shown to reduce significantly skeletal morbidity, and to improve quality of life in breast-cancer patients with bone metastases.[11]

We have previously reported NIR fluorescence imaging of HA in small-animal model systems using our first generation molecule Pam78.[12–14] These studies were severely limited by the low yield of the contrast-agent synthesis, which precluded development of more relevant large-animal models. The aim of this study was to develop a simplified synthetic method for 1-hydroxy-1,1-bisphosphonate derivatives conjugated to NIR fluorophores, and to validate contrast-agent performance in vivo.

As a consequence of the extreme insolubility of pamidronate in organic solvents, and the base lability of heptamethine indocyanines, previous syntheses were carried out in aqueous media. As such, yields were low (ca. 18–21%) and purification was difficult.[13] These problems prompted us to develop methods for the synthesis of protected pamidronate, and its subsequent conjugation to heptamethine indocyanine fluorophores, under non-aqueous conditions.

Phosphate-protected bisphosphonates, which are a precursor for NIR fluorophore conjugation, can be synthesized by Michaelis–Arbuzov reaction of acyl halide with trialkyl phosphite and dialkyl phosphite.[15] However, published methods utilize strongly basic conditions and elevated temperature, and the reaction is complicated by rearrangement. This rearrangement generates isomeric compounds containing two chemically different phosphorus–carbon bonds, including a tetraalkyl phosphono phosphate.[16,17]

To obtain Pam800 (**7**; Scheme 1), we first devised a synthesis of methylester-protected pamidronate (**4a**), based on the one-pot synthesis method of Tromelin et al.[18] In our method, β-alanine was converted into an acyl halide, followed by one-pot treatment with trimethylphosphite and dimethylphosphite at 0°C to room temperature (RT) over 30 min. Methylester-protected pamidronate (**4a**) was obtained without the use of any base or heating within an hour, in two chemical steps, with an overall yield of 91% (Scheme 1). When performing reactions at elevated temperature or in the presence of strong base, isomerization was a major obstacle, which could be avoided by performing a one-pot reaction of acyl halide and eliminating the isolation steps for the unstable intermediate α-ketophosphonate (**3**). Unwanted isomerized

[*] Dr. K. R. Bhushan, Dr. E. Tanaka, Dr. J. V. Frangioni
Division of Hematology/Oncology,
Department of Medicine and Department of Radiology, Beth Israel Deaconess Medical Center/Harvard Medical School,
330 Brookline Avenue, SLB-05, Boston, MA, 02215 (USA)
Fax: (+1) 617-667-0981
E-mail: jfrangio@bidmc.harvard.edu
Homepage: http://www.frangionilab.org

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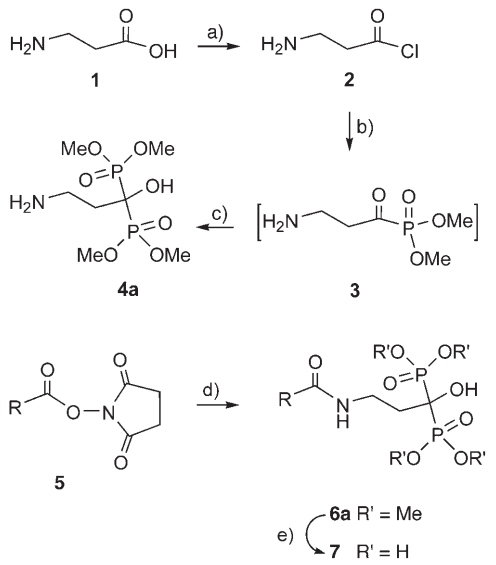

Scheme 1. Preparation of **4a** and **7**. Reagents and conditions: a) $SOCl_2$, CH_2Cl_2, reflux, 1 h; b) $(MeO)_3P$, 0 °C–RT, 30 min; c) $(MeO)_2P(O)H$, 0 °C–RT, 30 min; d) **4**, DMSO, *N*-methylmorpholine, RT, 4 h; e) Me_3SiBr, DMF, RT, 18 h, and $MeOH/H_2O$ (4:1), RT, 30 min. R = IRDye 800CW.

product **4b** was then less than 5% of total during analytical scale synthesis (Figure S1, Supporting Information).

The conjugation of NIR fluorophore IRDye 800CW *N*-hydroxysuccinimide ester (**5**) to methylester-protected pamidronate (**4a**) was performed in DMSO solution in the presence of *N*-methyl morpholine. The reaction was performed in the dark at room temperature, and formation of Pam800 methyl ester (**6a**) was monitored by HPLC. Nucleophilic attack by the primary amine of methylester-protected pamidronate (**4a**) resulted in displacement of the *N*-hydroxysuccinimide group and formation of a stable amide linkage between IRDye 800CW and protected pamidronate (Scheme 1). Note that approximately 8% of the undesired isomeric compound **6b** was observed (Figure S2, Supporting Information).

Dealkylation of bisphosphonic ester functions was carried out by using bromotrimethylsilane in DMF followed by methanolysis. The final compound, Pam800 (**7**), was purified by preparative HPLC and analyzed by liquid chromatography mass spectrometry (LCMS) and NMR spectroscopy. The typical overall yield of **7** was 71%.

Prior to in vivo experiments, Pam800 (**7**) was fully characterized. The maximum absorption (778 nm) and emission (799 nm) of Pam800 (Figure 1) are located within the "NIR window,"[19] an area of the electromagnetic spectrum that maximizes photon penetration and recovery in living tissue. In phosphate-buffered saline, pH 7.4 (PBS), the extinction coefficient for **7** at 778 nm was $174\,000\,M^{-1}cm^{-1}$ and its quantum yield was 6.4%. In 100% fetal bovine serum, its quantum yield was 6.0%. The specificity of Pam800 for HA over other calcium salts was determined (Figure 2 A). We incubated HA and the phosphate (CP), oxalate (CO), carbonate (CC), and pyrophosphate (CPP) salts of calcium with 100 nM Pam800 for 30 min at room temperature with

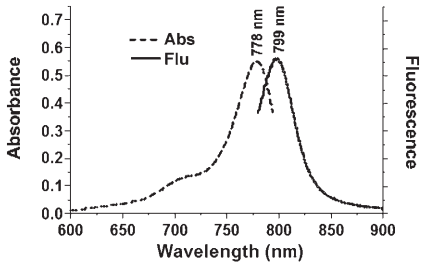

Figure 1. Absorption and fluorescence spectra of Pam800 (**7**) at a concentration of 2 μM in PBS buffer. Maximum absorption and fluorescence emission wavelengths are indicated.

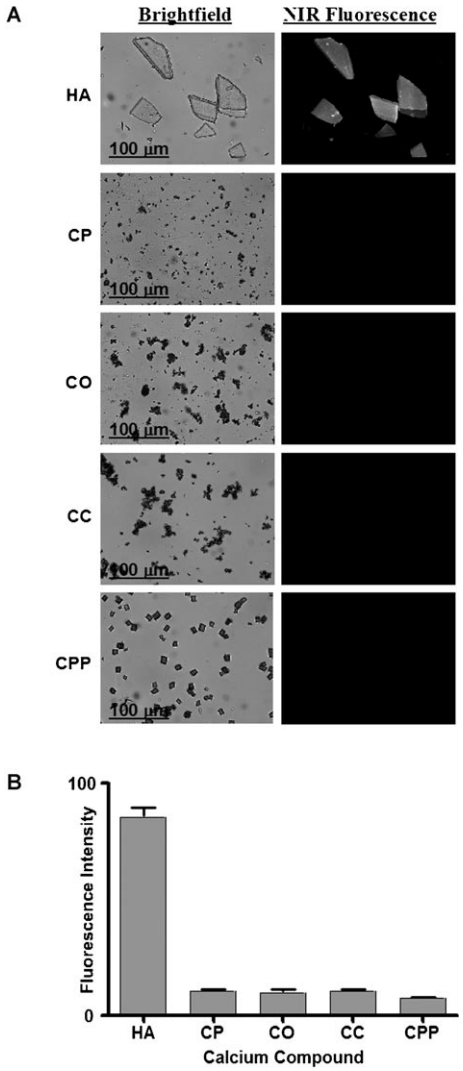

Figure 2. The specificity of Pam800 (**7**) for HA and other calcium salts. A: Crystals of HA, calcium phosphate (CP), calcium oxalate (CO), calcium carbonate (CC), and calcium pyrophosphate (CPP) were stained with Pam800, washed, and viewed by brightfield microscopy (left) and NIR fluorescent microscopy (right). NIR fluorescence images have identical exposure times and normalizations. The results are representative of three independent experiments. B: Quantification (mean ± SD) of crystals from (A) using NIR fluorescence imaging. All measurements (3 independent experiments) were from identically sized and shaped regions of interest.

constant motion, then washed four times with a 100-fold excess of PBS. As shown in Figure 2B, Pam800 has a greater than eightfold specificity for HA over other calcium salts found in the body, and permits NIR fluorescence detection of HA with high sensitivity.

We characterized Pam800 for its in vivo performance during image-guided surgery. Surgery was performed on 30-kg Yorkshire pigs whose organs are roughly the same size as human organs. Pam800 was administered intravenously to anesthetized pigs at a dose of 0.06 μmol kg^{-1} (1.8 mg total). After 2–4 h of clearance, image-guided surgery was performed using a custom intraoperative NIR fluorescence imaging system.[20] As shown in Figure 3A, Pam800 provided high-sensitivity detection of normal bones, and could guide surgery both prior to, and after, the skin incision, since NIR penetrates relatively deeply into living tissue. To replicate breast cancer microcalcification as would be encountered during breast surgery, 5 mg each of HA and calcium oxalate crystals were injected subcutaneously. Importantly, Pam800 could also detect soft tissue-embedded HA crystals with high sensitivity and specificity (Figure 3B). In particular, Pam800 correctly identified HA, which is common in malignant breast disease, but not calcium oxalate, which is typically deposited in benign lesions and is rarely seen in malignancies.[21,22]

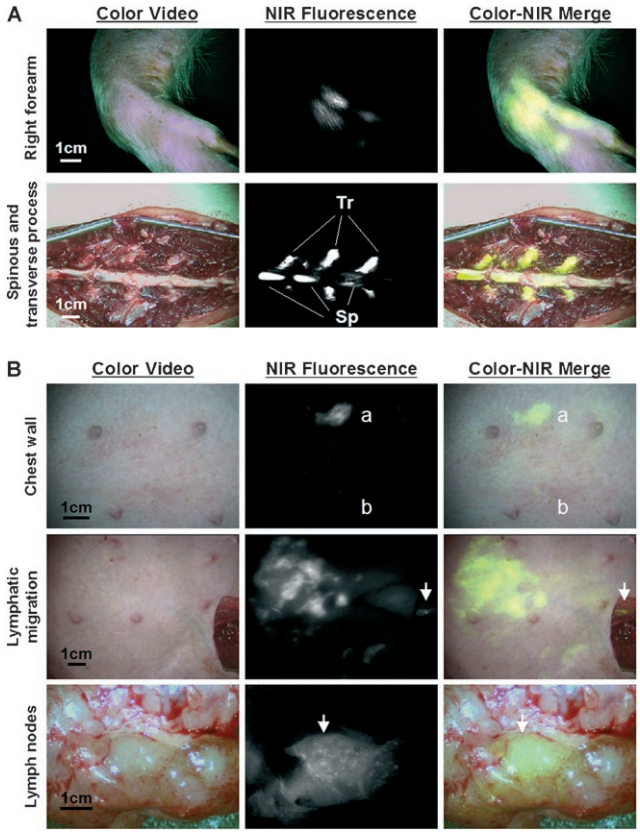

Figure 3. Real-time intraoperative NIR fluorescence imaging in 30 kg Yorkshire pigs. A: Bones of the forearm (top), and traverse (Tr) and spinous processes (Sp) of vertebrae (bottom) are shown. B: Subcutaneously administered HA crystals (a) and calcium oxalate crystals (b). Pam800 identified HA deposits through skin (top), and permitted real-time tracking of HA crystals as they migrated (middle) from the injection site to regional lymph nodes (bottom). Arrows mark location of lymph nodes. Figure data are representative of 4 independent experiments in 4 different animals.

In conclusion, we have described the simple and reliable production of a methylester-protected pamidronate derivative that can be used in organic solvent to produce novel optical contrast agents, such as Pam800 (**7**). Pam800 can be produced in preparative quantities, at high yield, and provides real-time image guidance to surgeons who require visualization of bone and/or tissue calcification. We have developed a straightforward, reproducible large-animal model system that could be used to validate optical contrast agents specific for breast-cancer microcalcifications.

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