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

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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.

In this communication, we describe a simplified, reproducible synthetic scheme for 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 scheme permits preparative production 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 structure is responsible for giving bisphosphonates their high affinity for bone, which can be further enhanced by addition of a hydroxyl 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

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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 contrast agent synthesis, which precluded development of more relevant large animal models. The aim of this study was to develop a simplified synthetic scheme for 1-hydroxy-1,1-bisphosphonate derivatives conjugated to NIR fluorophores, and to validate contrast agent performance *in vivo*.

Due to the extreme insolubility of pamidronate in organic solvents, and the base lability of heptamethine indocyanines, previously described synthetic schemes were carried out in aqueous media. As such, yields were low ($\approx$ 18-21%) and purification was difficult.[13] This prompted us to develop methods for the synthesis of protected pamidronate, and its subsequent conjugation to heptamethine indocyanine fluorophores, under non-aqueous conditions.

From the literature,[15] we found that by Michaelis-Arbuzov reaction of acyl halide with trialkyl phosphite and dialkyl phosphite, one can synthesize phosphate-protected bisphosphonates, a precursor for NIR fluorophore conjugation. However, previously 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 phosphonophosphate.[16,17]

To obtain Pam800 (**7**), 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 to its acyl halide form, followed by one-pot treatment with trimethylphosphite and dimethyl phosphite 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). While 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 product (**4b**) was then less than 5% of total during analytical scale synthesis (Figure 1, Supporting Information, SI).

The conjugation of NIR fluorophore IRDye®800CW NHS ester (**5**) to methylester-protected pamidronate (**4a**) was performed in DMSO in the presence of N-methyl morpholine. The reaction was performed in the dark at RT, 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 NHS group and formation of a stable amide linkage between IRDye®800CW and protected pamidronate (Scheme 2). Note that approximately 8% of the undesired isomeric compound (**6b**) was observed (Figure 2, SI).

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 LCMS and NMR. The typical overall yield of Pam800 (**7**) during preparative synthesis was 71%.

Prior to *in vivo* experiments, Pam800 (**7**) was fully characterized for its spectral properties (Figure 1) and calcium salt specificity (Figure 2). Peak absorption (778 nm) and emission (799

nm) of Pam800 are located within the “NIR window,”[19] an area of the electromagnetic spectrum that maximizes photon penetration and recovery in living tissue. In PBS, Pam800's extinction coefficient at 778 nm was $174,000 \text{ M}^{-1}\text{cm}^{-1}$ and its quantum yield was 6.4%. In 100% fetal bovine serum, its quantum yield was 6.0%. To determine the specificity of Pam800 for HA over other calcium salts, we incubated HA and the phosphate, oxalate, carbonate, and pyrophosphate salts of calcium with 100 nM Pam800 for 30 min at RT with constant motion, then washed 4 times with a 100-fold excess of PBS. As shown in Figure 2B, Pam800 has a greater than 8-fold specificity for HA over other calcium salts found in the body, and permits NIR fluorescence detection of HA with high sensitivity.

We finally 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 $0.06 \mu\text{mol/kg}$ (1.8 mg total). After 2-4 hours 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]

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. Finally 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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Figure 1. Absorption and fluorescence spectra of Pam800 (7) at a concentration of 2 μ M in PBS buffer. Peak absorption and fluorescence emission wavelengths were 778 nm and 799 nm, respectively.

Figure 2.

The specificity of Pam800 for HA and other calcium salts. A, Crystals of HA, calcium phosphate (CP; $\text{Ca}_3\text{O}_8\text{P}_2$), calcium oxalate (CO; $\text{Ca}(\text{COO})_2$), calcium carbonate (CC; CaCO_3), and calcium pyrophosphate (CPP; $\text{Ca}_2\text{O}_7\text{P}_2$) were stained with 100 nM Pam800, washed, and viewed by NIR fluorescent microscopy and brightfield microscopy. NIR fluorescence images have identical exposure times and normalizations. The results are representative of three independent experiments. B, Quantification (mean $\pm$ SD) of crystals from (A) using NIR fluorescence imaging. All measurements (N=3) were from identically sized and shaped regions of interest.

Figure 3.

Real-Time Intraoperative NIR Fluorescence Imaging in 30 kg Yorkshire pigs. (A) After intravenous injection and 2-4 hours of clearance, Pam800 permits high-sensitivity, real-time image guidance of normal bones during surgery. Shown are bones of the forearm (top), and traverse (Tr) and spinous processes (Sp) of vertebrae (bottom). (B) Subcutaneously administered HA crystals (a) and calcium oxalate crystals (b) create a large animal model of breast cancer microcalcification. Pam800 identified HA deposits through skin, and permitted real-time tracking of HA crystals as they migrated from the injection site to regional lymph nodes (arrows). Figure is representative of N=4 animals.

Scheme 1. Reagents and conditions

(a) SOCl₂, CH₂Cl₂, reflux, 1h; (b) (MeO)₃P, 0°C-RT, 30 min; (c) (MeO)₂P(O)H, 0°C-RT, 30 min.

Scheme 2. Reagents and conditions

(a) Compound **4**, DMSO, NMM. RT, 4 h; (b) $(Me)_3SiBr$, DMF, RT, 18 h & MeOH- H_2O (4:1), RT, 30 min.