Supplemental Materials and Methods

General

Carboxyfluorescein-NHS ((2,5-dioxopyrrolidin-1-yl) 3',6'-dihydroxy-1-oxospiro[2-benzofuran-3,9'-xanthene]-5-carboxylate), DAPI (4',6-diamidino-2-phenylindole), and WGA-Alexa555 (Wheat Germ Agglutinin, Alexa Fluor®555 Conjugate) were purchased from Thermo Scientific (Rockford, IL). DOTA-NHS (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono-N-hydroxysuccinimide ester) was purchased from Macrocyclics Inc. (Dallas, TX). Alendronate and all other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. $^{64}CuCl_2$ prepared by the $^{64}Ni(p,n)$ ^{64}Cu nuclear reaction was obtained from Washington University (St. Louis, MO). All HPLC analyses were performed on a Phenomenex Gemini C18 column (Torrance, CA); flow rate was 1mL/min with mobile phases (A) 0.1% TFA to (B) 5-90% MeCN over 25 minutes. All mass spectroscopy was performed on an LTQ FT mass spectrometer (Thermo, Waltham, MA). Light microscopy was performed using a Zeiss Axio Lab A1 microscope. Confocal microscopy was performed using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena Germany) with Fluar 10x/0.5NA M27, EC Plan-Neofluar 20x/0.5NA M27, LD LCI 25x/0.8NA Water Imm Corr M27 and Plan-Apo 40x/0.95NA Corr M27 objectives. A Zeiss Sigma VP FE SEM equipped with a Gatan 3View2 was used for Serial Block-face scanning electron microscopy (SBEM) and energy dispersive x-ray (EDX) elemental analysis.

Compound Synthesis

Synthesis of DOTA-alendronate: DOTA-NHS (43.7mg, 0.057mmol) and alendronate (31mg, 0.114mmol) were combined in 1.5mL H_2O, basified with 1M NaOH

(to pH 7.3), and rotated under argon at RT for 2.5 hours. The reaction was adjusted to pH 9.0 with 1M NaOH then applied to a 10cm Dowex AG1-X4 (HCO_2^- Form) ion exchange column. The column was washed with 50mL H_2O then eluted with 15mL each of 0.1M, 0.2M, 0.5M, 1.0M, and 2.0M HCO_2H. Fractions 24-26 (1.0M HCO_2H) were pure product as analyzed by LTQ FT mass spectrometry (m/z $\{M+H\}^+$ found: 636.20; calcd: 636.20).

Synthesis of FAM-alendronate: Alendronate (44.5mg, 0.164mmol) was added to 1mL H_2O and vortexed while triethylamine (TEA) was slowly added dropwise over 5 minutes until the solid dissolved. Carboxyfluorescein-NHS (66.1mg, 0.14mmol) was dissolved in DMF (200µl) and added dropwise to alendronate solution over 5 minutes while stirring. The mixture was rotated overnight under argon at RT then purified by HPLC. The product peak at 18.8 minutes was characterized by LTQ FT mass spectrometry (m/z $\{M+H\}^+$ found: 608.07, calcd: 608.07).

Serum Stability

Stability of ^{64}Cu-DOTA-alendronate in serum was measured under simulated biologic conditions at 0, 1, 4, 24, and 48 hours by ITLC and size exclusion HPLC and was found to be greater than 95% at all time points. Aliquots were analyzed for radioactivity and UV absorbance at 280 nm using high-performance liquid chromatography (HPLC) size-exclusion chromatography (SEC) on a Superdex 200 10/300 column (GE Healthcare, Piscataway, NJ, USA) as previously described (*1*).

Briefly, 2µg of DOTA-alendronate (radiolabeled at 37MBq/µg in 0.1M ammonium acetate, pH 7.0 for 30 minutes) or 74MBq of free ^{64}Cu were added to 1mL of fresh rat serum, 1% HSA, or saline, with and without DTPA then incubated for 48 hours. More than 95% of ^{64}Cu remained in DOTA-alendronate (46 minutes) over 48 hours when

incubated in rat serum, HSA, or saline with approximately 5% bound to albumin (39 minutes) and no detectable trace of free ^{64}Cu or ^{64}Cu-DTPA (47 minutes).

Free ^{64}Cu incubated in serum or HSA showed greater than 95% binding to albumin and 5% eluted at an earlier time (20 mins) not associated with albumin. To confirm if the peak at 46 min was ^{64}Cu-DOTA-alendronate and not ^{64}Cu-DTPA, ITLC was performed on the same incubated samples with saline as the running solvent. Less than 1% of the total radioactivity moved with the solvent front indicating little to no ^{64}Cu-DTPA in the samples.

PET Imaging

The energy window of the emission scans was set to 350-605keV with a time resolution of 6ns. Dynamic PET scans were 1 hour duration and separated into six 10 minute scans for practical analysis. For static PET Scans, rats were anesthetized and injected with a single intravenous dose (1-2μg) of ^{64}Cu-DOTA-alendronate in 1% HSA buffered saline using a 30g tuberculin syringe then allowed to recover before and between imaging points. Static scans were 10 minutes in duration. Data were sorted into two-dimensional sinograms using the Fourier rebinning method and corrected for intrascan radiodecay, detector non-uniformity, and random coincidence noise. Images were reconstructed by the iterative three-dimensional ordered subsets expectation maximization (OSEM) method (4 iterations).

Confocal and Electron Microscopy

Confocal microscopy: Fresh samples were collected, cut to less than 1cm^3, and immediately placed in 4% PFA for 4 hours. Tissues were dehydrated then paraffin embedded, sectioned (5 μm thickness), fixed on a slide, dissolved with xylene, then

stained with FAM-alendronate, DAPI, and WGA-Alexa555 (as well as a control slide stained with hematoxylin and eosin) then washed with water.

Serial block face scanning electron microscopy: tissue samples were prepared following the procedure outlined in SBEM Protocol v7_01_10 (https://www.ncmir.ucsd.edu/sbem-protocol/). During image collection (18500X mag, 1.1kV), 1007 sections were sliced from the sample block at a cut depth of 70 nm. Images were viewed using Gatan Digital Micrograph, aligned using Cygwin and IMOD, and segmented for 3D reconstruction using Amira 7.0 software. After SBEM, the elemental composition was determined using EDX on the exposed block face.

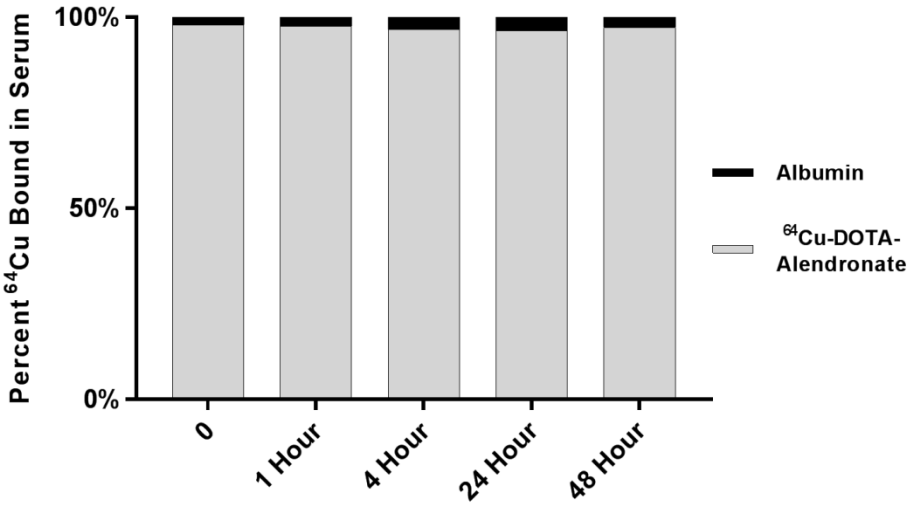
Biodistribution and Dosimetry

Following euthanasia by CO_2, cardiocentesis was performed prior to tissue excision beginning with the urinary bladder, then all major organs (heart, lung, liver, spleen, pancreas, stomach, large intestine, small intestine, kidneys, muscle, femur, stifle joint, and lumbar vertebrae) and mammary tissue.

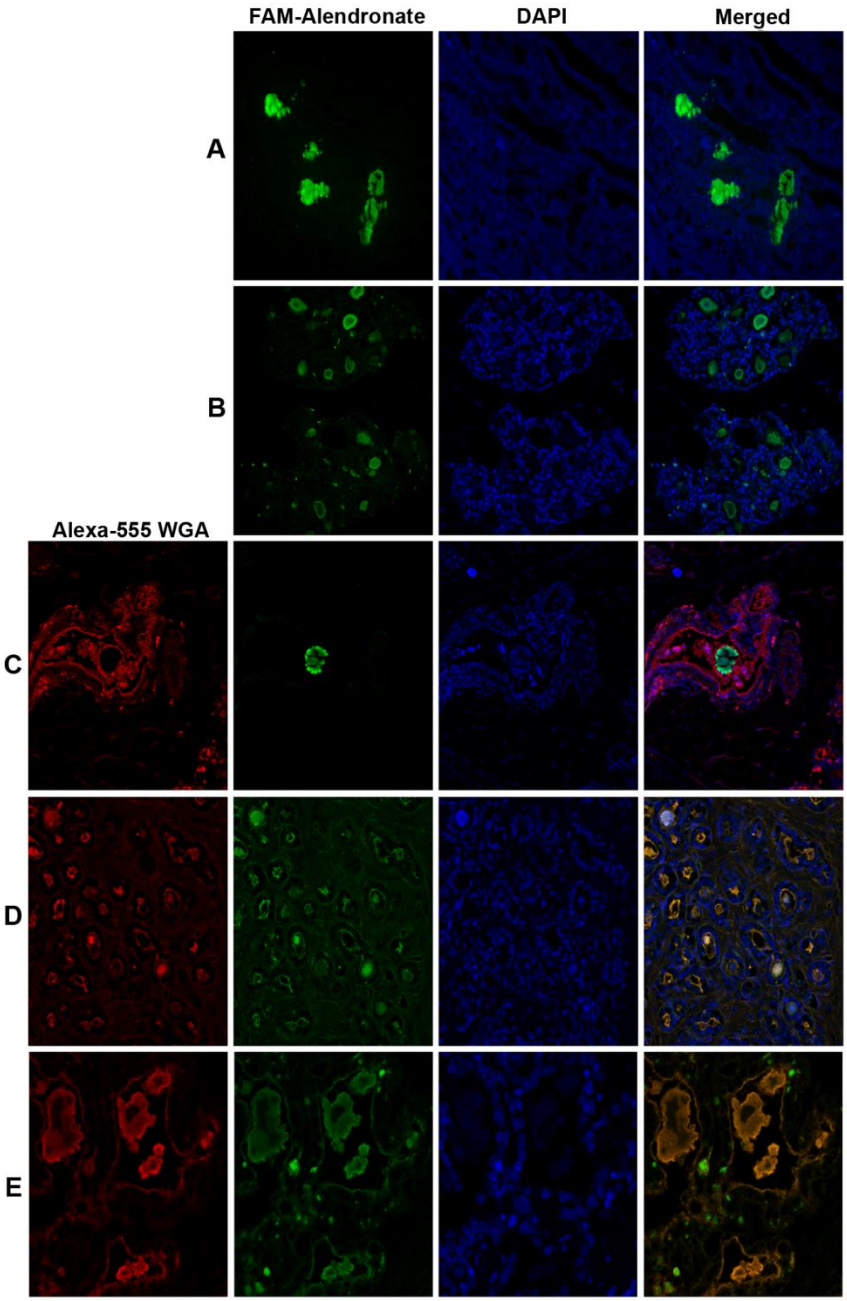
Radioactivity counts (cpm) were corrected for background and radioactive decay, and the percent of the injected dose (%ID) and %ID/g of each tissue/organ were calculated. The %ID in blood, muscle, and bone were estimated assuming a whole-blood volume of 64mL/kg, a total muscle mass of 40%, and a total bone mass of 10% of the total body weight, respectively (*2*). The radioactivity in the stomach and intestines included the radioactivity in their contents.

For each time-point, the activity concentration (%ID/g) was measured in each of the 14 delineated organs and multiplied by the organ volume to obtain the injected dose per organ. Additionally, region of interest (ROI) tracing of PET images was performed every 5 minutes for the first hour on two rats followed by 20 different time points for the

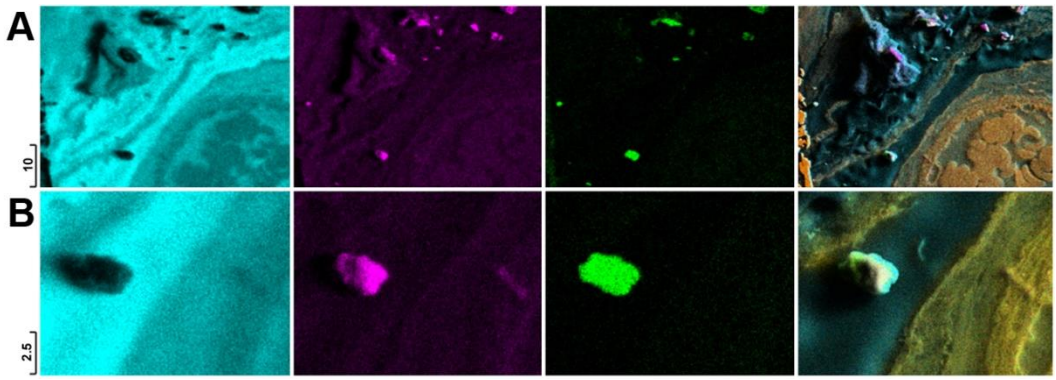
next 47 hours on the other six rats. ROI values were normalized for each rat by calculating a conversion coefficient for each organ in each rat using the gamma counted tissue values as a standard for the traced value from the PET image at the same time point. Disintegrations accumulated per organ were generated with OLINDA for all tissues except the bladder. After modeling the total excreted activity using the third-term exponential modeling feature in OLINDA, the activity within the bladder was assumed to be the difference between estimated values at urination points of 1, 2, and 4 hours then every 4 hours following. To calculate bladder dosimetry, the area between the time activity curves for excretion and bladder wall was calculated by modified trapezoidal rule assuming complete voiding of the bladder and linear accumulation between urination points.



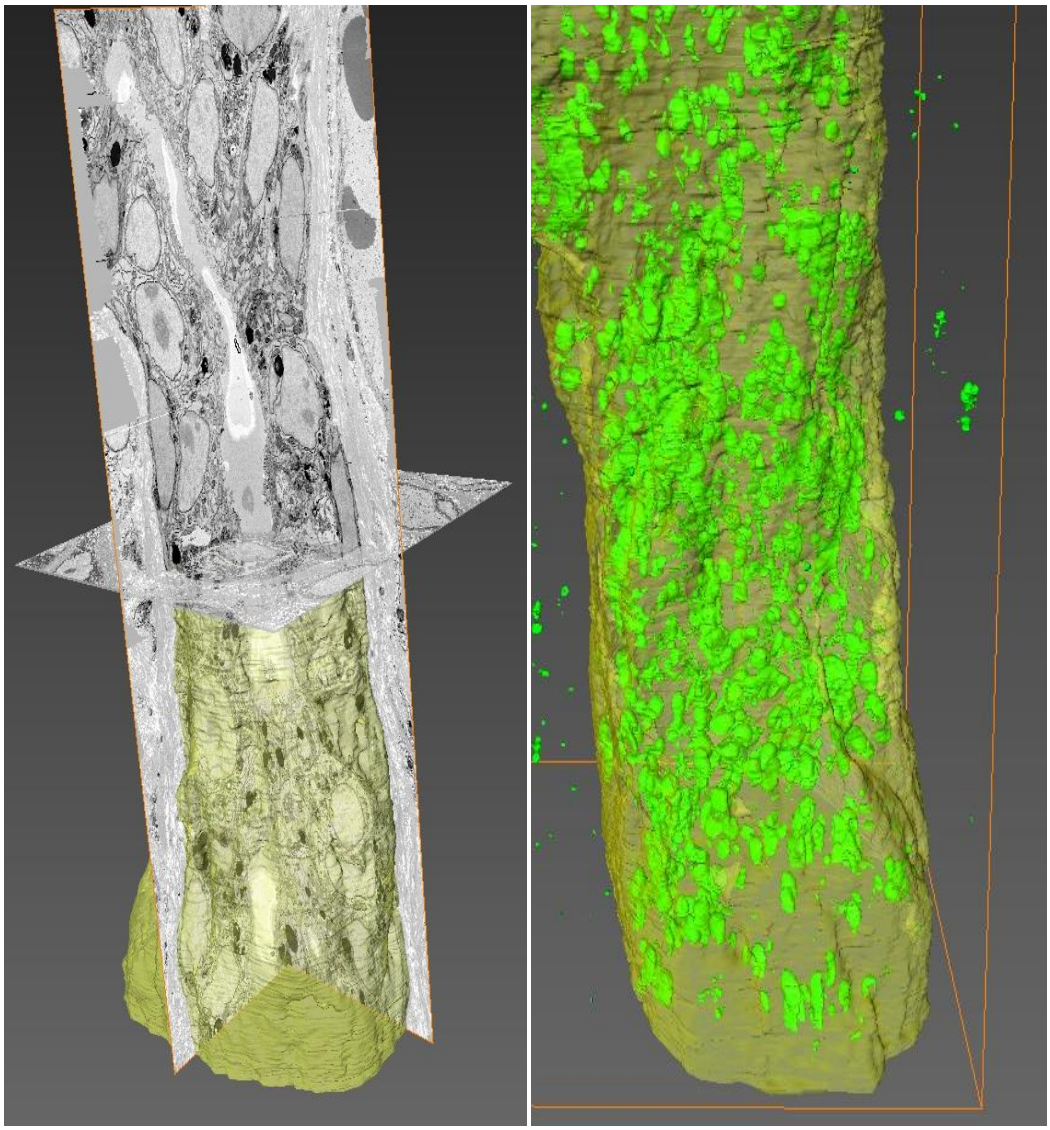
SUPPLEMENTAL FIGURE 1. Stability of ^{64}Cu-DOTA-alendronate in rat serum. Two μg of DOTA-alendronate radiolabeled with ^{64}Cu (37MBq/μg) in 0.1M ammonium acetate, pH 7.0 for 30 minutes was incubated with 1 mL of rat serum at 37^{o}C for 48hr with aliquots removed at indicated time points for analysis by SEC for ^{64}Cu-albumin and ^{64}Cu-DOTA-alendronate.



SUPPLEMENTAL FIGURE 2. Individual, non-merged confocal images from Figure 4.



SUPPLEMENTAL FIGURE 3. Scanning electron microscopy with energy dispersive X-ray (EDX) for elemental identification and compositional analysis of (A) intercellular microcalcifications and (B) an intraductal microcalcification within a malignant rat mammary tumor. Elements chosen for analysis were carbon, oxygen, and calcium.



SUPPLEMENTAL VIDEO 1. Serial section electron microscopy and three-dimensional reconstruction of a mammary duct within a mammary carcinoma. This video demonstrates the spatial distribution of intercellular and intracellular osmiophilic structures (green) within a mammary duct (yellow).

Supplemental Table 1

Converted volume of interest measurements*

	Time (min)	Kidney (%ID/g)	Femur (%ID/g)	Blood (%ID/g)	Liver (%ID/g)
Rat 1	5	0.34	0.16	0.23	0.07
	10	0.80	0.39	0.23	0.12
	15	1.04	0.60	0.19	0.14
	20	1.29	0.80	0.29	0.17
	30	1.52	1.06	0.20	0.20
	40	1.54	1.36	0.27	0.23
	50	1.33	1.56	0.24	0.25
	60†	1.29	1.69	0.15	0.27
	CF	1.11	2.02	3.33	1.40
Rat 2	240†	1.25	1.87	0.24	0.26
	CF	2.02	1.87	4.03	0.89
Rat 3	780†	0.82	2.35	0.17	0.36
	CF	2.76	2.35	2.26	1.62
Rat 4	840†	0.99	2.27	0.15	0.35
	CF	2.63	2.27	2.16	1.48
Rat 5	120	1.89	2.44	0.18	0.56
	360	1.68	2.22	0.23	0.57
	1440†	0.98	2.06	0.22	0.53
	CF	2.75	2.22	2.85	1.54
Rat 6	180	1.88	2.55	0.14	0.52
	300	1.69	1.72	0.22	0.52
	1320	0.95	1.91	0.27	0.48
	1680†	0.89	1.81	0.25	0.52
	CF	2.83	1.96	2.72	1.60
Rat 7	180	1.94	2.52	0.20	0.64
	1080	1.20	2.52	0.23	0.59
	2400†	0.71	2.25	0.17	0.49
	CF	3.22	2.29	2.78	2.05
Rat 8	5	3.93	1.22	0.65	0.92
	10	2.79	1.88	0.51	0.82
	15	2.34	2.12	0.22	0.81
	20	2.04	2.31	0.25	0.79
	30	1.85	2.51	0.18	0.76
	40	1.79	2.51	0.17	0.74
	50	1.79	2.51	0.15	0.70
	60	1.82	2.51	0.13	0.69
	120	2.08	1.93	0.11	0.60
	240	2.19	2.51	0.17	0.60
	1440	1.23	1.89	0.12	0.59
	2580	0.84	1.93	0.14	0.46
	2880†	0.83	2.12	0.17	0.52
	CF	3.02	1.93	1.62	1.95

*Using gamma counted tissues as timepoint standards, a conversion factor (CF) coefficient was calculated for each tissue in each rat and used to convert volume of intensity image intensity measurements into absolute uptake values (%ID/g).

†Gamma counted tissue value

Supplemental References

1. Yazaki PJ, Wu AM. Expression of recombinant antibodies in mammalian cell lines. *Methods Mol Biol.* 2004;248:255-268.

2. Sharp PE, Villano JS. *The laboratory rat*. 2nd ed. Boca Raton, FL: CRC Press; 2013.