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Supplementary Materials for

A mouse-human phase 1 co-clinical trial of a protease-activated fluorescent probe for imaging cancer

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Supplementary Materials

Materials and Methods

Human study safety evaluations

A safety evaluation consisting of history and physical exam, vital signs, electrocardiogram (ECG), blood and urine laboratory studies, and pulmonary function tests (PFTs) were conducted prior to peripheral intravenous (i.v.) administration of LUM015. Safety evaluations during the 24-hour period after i.v. injection consisted of vital signs, ECGs, blood and urine laboratory studies and documentation of any adverse pharmacological activity (APA). APA was defined as any functional effect on the major physiological systems that is at least probably attributable to LUM015. Laboratory studies and APA evaluation were performed again at an optional 48-hour time point after LUM015 injection and subjects returned to the study site once per week for 2 weeks after surgery for evaluations consisting of vital signs, laboratory studies, and documentation of any APA. PFTs were repeated at the 2 week visit. An end of study assessment occurred 30-35 days post LUM015 administration for APA.

Murine cancer models

Primary STS were generated using the previously described alleles, *LSL-Kras*^{G12D}*(32)*, *Braf*^{CA}*(33)*, *p53*^{flox} (*34*), and *LSL-YFP (35)*. *LSL-YFP* mice were obtained from Jackson Laboratory. STS were initiated by intramuscular injection of an adenovirus expressing Cre-recombinase as described previously (*36*) into the hind limb of mice with the genotype *Braf*$^{CA/+}$*;p53*$^{flox/flox}$ (BP) or *LSL-YFP;LSL-Kras*$^{G12D/+}$*; p53*$^{flox/flox}$ (KPY). BP mice were used for all experiments unless noted.

Orthotopic breast cancers were generated using MMTV-PyVT, FVB and BALB/c mice originally obtained from Jackson Laboratories and bred and maintained in the Cox-7 gnotobiotic animal facility of the Edwin L. Steele Laboratory at MGH. Spontaneous tumors were excised when they reached ~8 mm in diameter and a ~1 mm^3 piece of viable tumor tissue was transplanted orthotopically into syngeneic FVB female mice (*39*). Alternatively, single breast tumors were established by implanting 10^5 4T1 cells into the third mammary fat pad (*40*). 4T1 cells (ATCC) were cultured in high-glucose DMEM (ATCC) with 10% FBS and 1% MEM NEAA (Invitrogen). Cell lines were authenticated in 2013 by IDEXX laboratories (IDEXX RADIL Case #14116-2013) and found to be identical to the profile established for the 4T1 cell line (ATCC #CRL-2539).

Fluorescence imaging and image analysis

Excitation illumination from a 300-W xenon lamp (Sunoptic Technologies) was collimated and reflected toward a 627.5-672.5 nm band-pass excitation filter and then reflected toward the specimen. The fluorescence emission was filtered by a 685-735 nm band pass element and relayed onto a charge coupled device (CCD) (PixelFly QE) which was connected to a computer for image acquisition and display. A dark field background image was subtracted from both the image under study and a flat field correction image in order to eliminate background signal. The image under study was then divided by the background-corrected flat-field image to correct for signal variations across the field of view. A 6-mm diameter cropping mask centered on the two dimensional CCD array was applied to the image under study prior to the generation of image statistics. Brightness and contrast settings were determined based on the tumor image histogram and then applied to all images from the same patient or mouse.

Immunohistochemistry and immunofluorescence

FFPE tissue samples from mice and humans injected with LUM015 were obtained. Five-µm–thick unstained sections were deparaffinized with xylene and rehydrated with a graded series of ethanol and water washes before immunostaining. For PEG immunohistochemistry and immunofluorescence studies, the primary antibody was rat anti-PEG used at concentrations of 1:175 and 1:200, respectively (Abcam). For immunohistochemistry, the secondary antibody was biotinylated rabbit anti-rat IgG (1:200, Vector) and eight 40X fields were scored for intensity of PEG staining according to the following scale: 0- no staining, 1- minimal staining, 2- moderate staining, 3- intense staining. Scoring was done by three independent researchers who were blinded to the identity of the sample and the average score was used, rounded to the nearest whole number. For immunofluorescence, the secondary antibody was Alexa Fluor 488-conjugated goat anti-rat immunoglobulin M (1:200, Life Technologies). Nuclear staining was performed using Hoechst 33342 dye (1:1000, Life Technologies). Pictures were acquired with a Leica SP5 inverted confocal microscope (Leica Microsystems) using Leica Suite software (Leica microsystems).

Liquid chromatography/mass spectrometry

To prepare plasma samples, 20 µl of plasma was incubated at 45º C for 4 hours in a 2-ml polypropylene screw-cap vial with 8 µl of 1 µg/ml Fragment 1–$^{13}C_{10}{}^{15}N_6$ (internal standard) and 20 µl of 2.5 mg/ml trypsin in PBS. After cooling to room temperature, 100 µl of 2% formic acid in acetonitrile was added to precipitate proteins followed by centrifugation for 5 minutes at

16,000 *g* and removal of 20 µl of the supernatant for liquid chromatography/mass spectrometry (LC/MS/MS). For tissue measurements, 50 µl of sample containing one part tissue homogenate and two parts H_2O were incubated with 10 µl of 1 µg/ml Fragment 1–$^{13}C_{10}{}^{15}N_6$ (internal standard) and 25 mg/ml trypsin in PBS. Following protein precipitation and centrifugation, 10 µl of the supernatant was used for LC/MS/MS analysis. Calibration samples were prepared by adding known amounts of pure Fragment 1 or LUM015 to control plasma or tissue homogenate and processed as above.

A Shimadzu 20A series liquid chromatography system with binary pumps, an autosampler, and column oven was used. We used a 4.6 × 50 mm C18 Phenomenex Kinetex column with a 4 × 3 mm Kinetex guard column at 45ºC. Samples were kept at 4ºC. Mobile phase A: 0.1% formic acid in 80:20 water:acetonitrile. Mobile phase B: acetonitrile. A 1 ml/min flow rate was used with the following elution gradient: 20-95% B for 0-1 min, 95% B for 1-2.5 min, 95-20% B for 2.5-2.6 min, and 20% B for 2.6-4 min for equilibration. Total run time was 5 min.

For electrospray ionization mass spectrometry (ESI-MS/MS) an AB/SCIEX API 4000 QTrap tandem-mass spectrometer was used. Optimization of parameters for positive mode electrospray (declustering potential, temperature, gas flow) and mass spectrometer (parent/daughter ions, quadrupole potential) was performed by direction infusion of the pure analyte Fragment 1 into the ESI-LC/MS/MS system. The following multiple reaction monitoring (MRM) transitions were monitored for quantification (*m*/*z* units): Fragment 1 parent ion $(M+H)^{2+}$/daughter ion fragment as 533.2/371 and Fragment 1-$^{13}C_{10}{}^{15}N_6$ parent ion $(M+H)^{2+}$/daughter ion fragment as 541.1/371. Analysis of calibration samples in the range of 7.0-870 nM for plasma and 3.5-870 nM for tissue showed a linear response (r=0.999 criterion)

and the low limits of quantification (LLOQ) were confirmed as 7 nM for plasma and 3.5 nM for tissue at a ± 80% accuracy criterion.

HPLC with absorbance and fluorescence detection

To prepare samples, 30 µl of plasma or tissue homogenate (1 part tissue + 2 parts H_2O) were shaken vigorously for 20 s at speed 4 in a FastPrep apparatus (Thermo-Savant) with 60 µl methanol and 10 glass beads (1 mm, Bio Spec Inc.) in a 200 µl polypropylene conical vial. The supernatant was added to 30 µl of 0.1% formic acid in water and 50 µl was injected into the high pressure liquid chromatography (HPLC) system.

For HPLC, a Waters 2695 pump/autosampler/column heater module was used with a 3 × 250 mm Phenomenex Hydro-RP column and 4 × 3 mm Kinetex guard column at 50º C. Samples were kept at 10º C. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was methanol. The flow rate was 0.7 ml/min and the elution gradient was as follows: 10-30% B for 0-2 min, 30-90% B for 2-12 min, 90% B for 12-16 min, 90-10% B for 16-17 min, and 10% B for 17-27 min as equilibration. The total run time was 27 minutes.

We used a Waters 2475 fluorescence detector (FD) with excitation at 649 nm and emission at 670 nm and a gain of 10 along with a Waters 2487 UV/Vis absorbance detector at 649 nm. The solvent flow from the column was split and adjusted to be equal (0.35 ml/min to each detector) to provide for equal peak retention time on both fluorescence and absorption chromatograms, allowing for Fragment 2 identification. Separate sets of calibration samples in the 0.0375-300 µM range were prepared for LUM015, Fragment 2, and Fragment 3 by adding pure standards to control tumor homogenates. Linear calibration responses were obtained for all

three analytes in the concentration range measured (r=0.999 criterion) and the LLOQs were confirmed to be 37.5 nM at ± 80% accuracy criterion.

SUPPLEMENTARY FIGURES

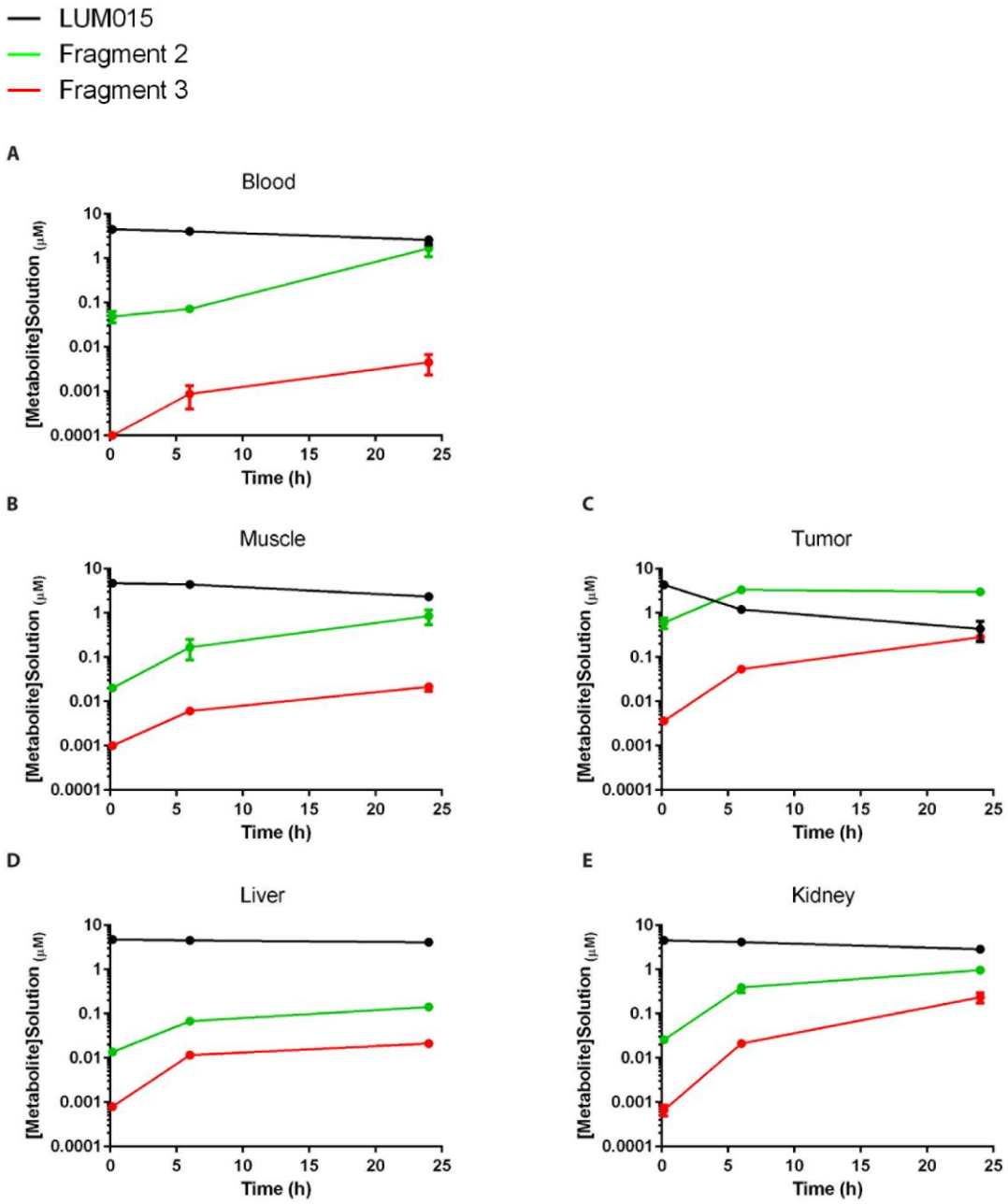


Figure S1. In vitro incubation of LUM015 with mouse tissues. (**A** to **E**) Whole blood, muscle, tumor, liver, and kidney tissues were isolated from three mice with primary STS and homogenized. One part tissue was mixed with two parts H_2O and LUM015 was added to the solution to a concentration of 4.65 µM followed by incubation at 37ºC. The samples were analyzed by HPLC-FLD to show the concentration of LUM015 and fluorescent metabolites by tissue type at 10 minutes, 6 hours, and 24 hours.

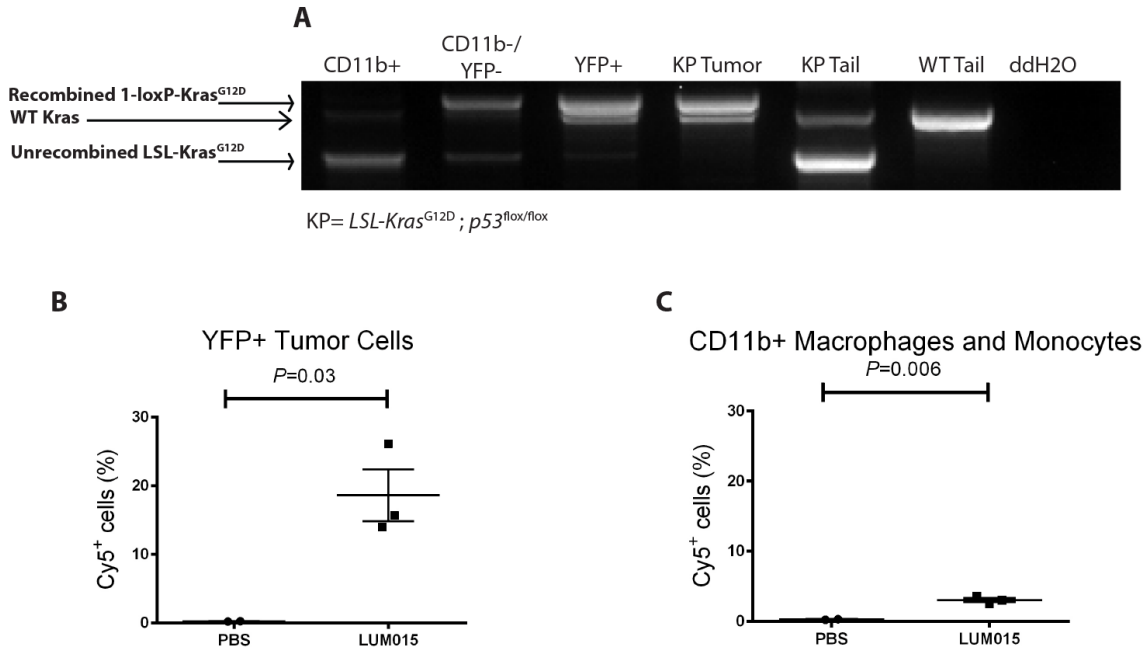


Figure S2. **The percentage of tumor cells that are Cy5$^+$ after administration of LUM015.** (**A**) PCR genotyping of genomic DNA from the three different Cy5$^+$ populations. Stromal cells should amplify the WT *Kras* and unrecombined *LSL-Kras*^{G12D} bands. YFP$^+$ tumor cells should amplify the WT *Kras* and recombined *1-loxP-Kras*^{G12D} bands. The KP Tumor lane is a KP lung tumor that served as a positive control for the recombined allele. The KP Tail lane served as a positive control for the unrecombined allele. The WT tail lane served as a positive control for the WT allele. (**B**) The mean percentage (± SEM) of YFP$^+$ tumor cells that were Cy5$^+$ after i.v. LUM015 (n_{PBS}=2 mice, n_{LUM015}= 3 mice). (**C**) The mean percentage (± SEM) of CD11b$^+$ tumor-associated macrophages and monocytes that were Cy5$^+$ after i.v. LUM015 (n_{PBS}=2 mice, n_{LUM015}= 3 mice). *P*-values in (B and C) determined by unpaired t-tests.

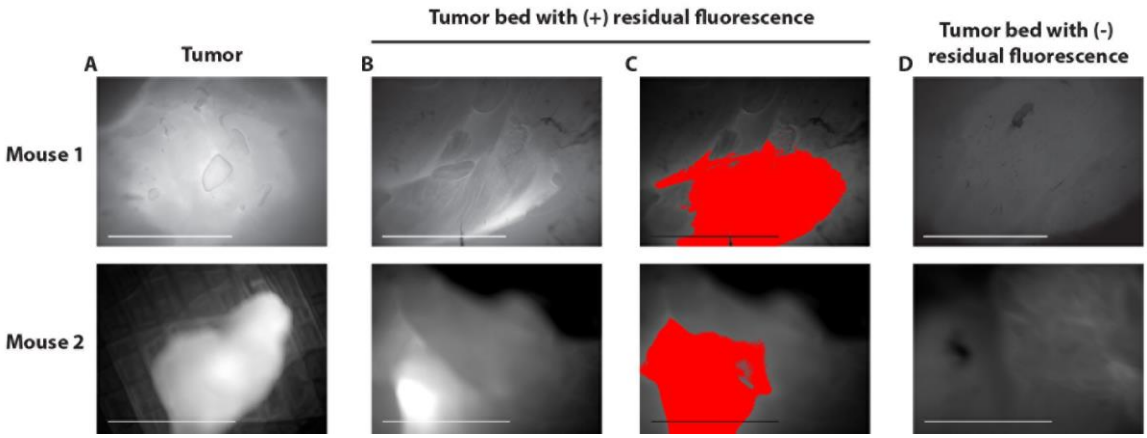


Figure S3. In vivo detection of residual fluorescence within the mouse tumor bed. (**A**) After tumor resection, the tumor was bisected and an ex vivo image was obtained using the LUM imaging device. Two representative tumor images are shown. A threshold for residual fluorescence was set at 80% of the minimum pixel value in the tumor image. Then, the tumor bed was imaged in vivo and assessed for the presence of pixels that exceeded the threshold value followed by closure of the surgical wound. The mice were then followed for local recurrence. (**B** and **C**) Two representative in vivo images of tumor beds with positive residual fluorescence (B), with the pixels exceeding the threshold colored red (C). (**D**) Two representative in vivo images of tumor beds with negative residual fluorescence. Scale bars, 5 mm.

A

	Mouse	Human		
PK Parameter	1.5 mg/kg	0.5 mg/kg	1.0 mg/kg	1.5 mg/kg
T_{max} (h)	0.17 +/ 0.00	0.11 +/- 0.17	0.06 +/- 0.13	0.00 +/- 0.00
C_{max} (µg/mL)	31.70 +/- 10.91	15.46 +/- 2.26	28.41 +/- 6.55	45.76 +/- 3.78
AUC_{last} (µg/mL * h)	137.20 +/- 20.80	78.36 +/- 12.91	159.65 +/- 45.94	252.08 +/- 57.98
$T_{1/2}$ (h)	11.32 +/- 5.00	5.21 +/- 1.33	5.08 +/- 0.86	5.42 +/- 0.67

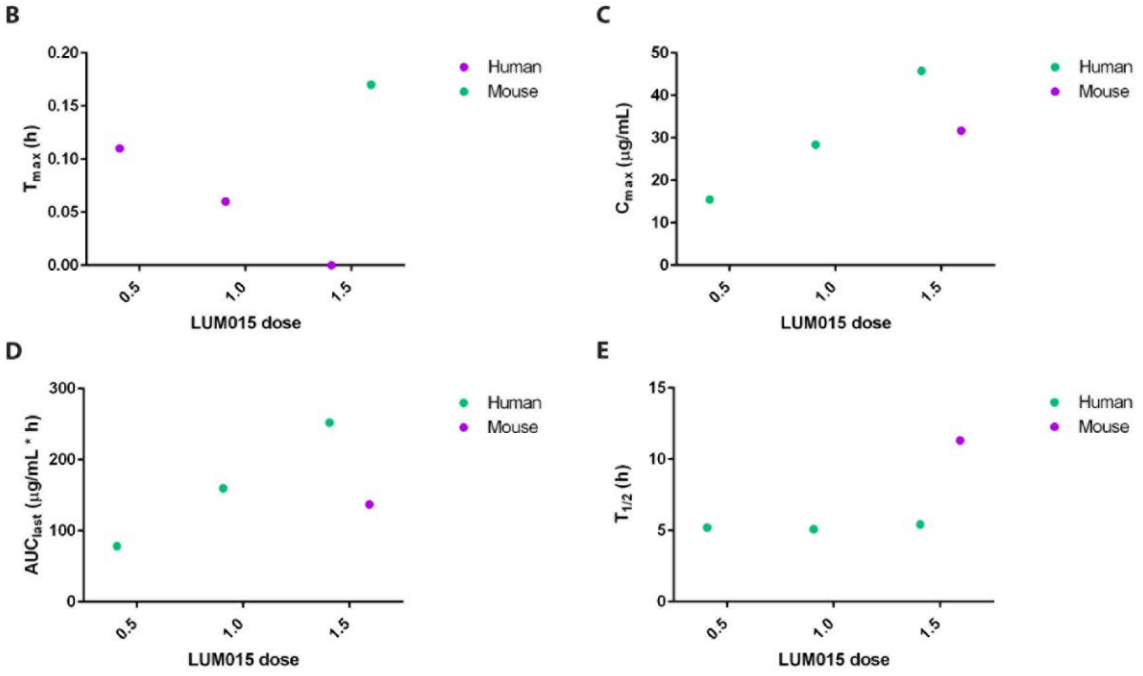


Figure S4. Summary pharmacokinetic data from mice and humans. (**A**) Mean PK parameters (± SD) are shown for mice administered 1.5 mg/kg LUM015 as well as for the three dose cohorts of the Phase I clinical trial of LUM015 (B-E (n_{mouse}= 4; $n_{human\text{-}0.5\ mg/kg}$= 6; $n_{human\text{-}1.0\ mg/kg}$= 6; $n_{human\text{-}1.5\ mg/kg}$= 3). T_{max} represents the time in hours after administration of LUM015 at which the maximum plasma concentration (C_{max}) was reached. The total area under the curve from administration to 22 hours is reported as AUC_{last} and the total drug exposure over that time. The terminal half-life, $T_{1/2}$, is based on pharmacokinetic data up to 22 hours after injection of LUM015 and represents the main elimination process of the probe. (**B** to **E**) Individual subject data used to compile the table in (A).

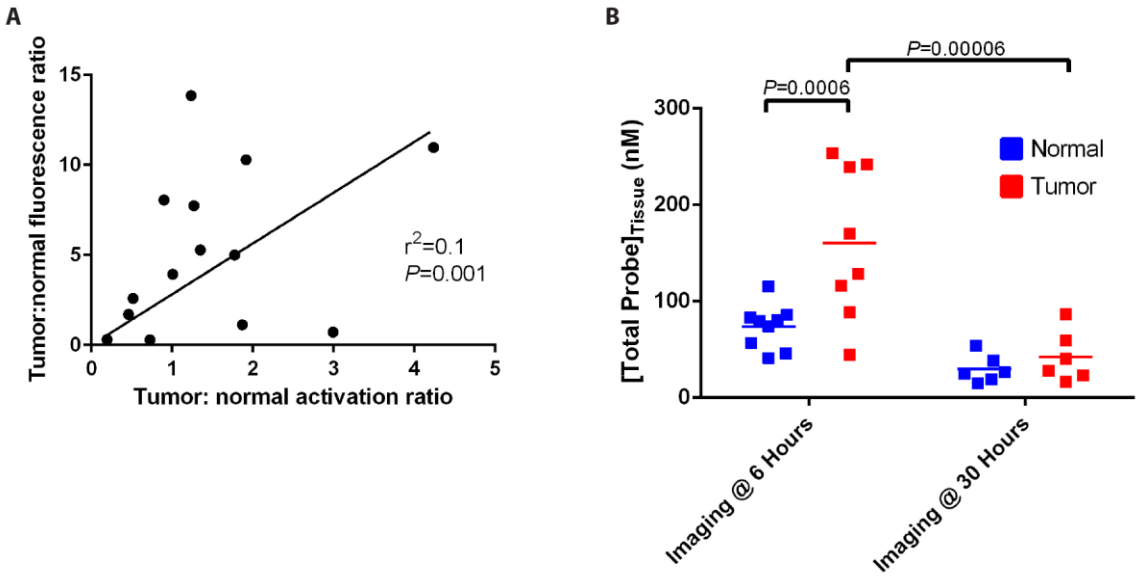


Figure S5. Contribution of protease-activation and LUM015 distribution for tumor-selective fluorescence. (**A**) Correlation between the tumor:normal fluorescence ratio and the fraction activated probe (FAP) ratio in all humans (n = 14). P value determined by F-test on the linear regression model. (**B**) The total concentration of probe was determined for 15 normal and 14 tumor tissue samples from human subjects, using the equation: $[\text{Total Probe (nM)}]_{\text{tissue}} = [\text{LUM015 (nM)}]_{\text{tissue}} + [\text{Fragment 2 (nM)}]_{\text{tissue}} + [\text{Fragment 3 (nM)}]_{\text{tissue}}$. P values determined by multiple comparison t-test with Bonferroni correction.

SUPPLEMENTARY TABLES

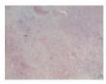
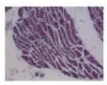
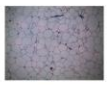
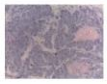
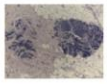
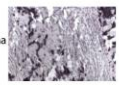
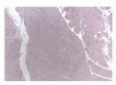
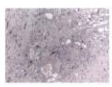
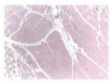
Table S1. **No adverse pharmacological activity of LUM015 in humans.** The terminal half-life, $t_{1/2}$, is given based on pharmacokinetic data up to 22 hours after injection of LUM015.

Patient #	Sex	Age	Weight (kg)	Total dose (mg)	C_{max} (µg/ml)	$t_{1/2}$ (h)	Adverse pharmacological activity	Hypersensitivity reaction
1	M	60	90.7	45.35	17.24	7.60	None	None
2	F	38	89.4	44.70	11.65	4.31	None	None
3	M	67	88.6	44.30	15.40	3.82	None	None
4	F	40	104.9	104.90	31.55	5.74	None	None
5	M	49	98.2	98.20	20.96	4.54	None	None
6	M	56	86.6	86.67	37.48	5.85	None	None
7	M	32	90.6	90.61	21.40	5.41	None	None
8	M	59	115.2	115.15	32.32	5.34	None	None
9	F	51	57	56.97	26.74	3.60	None	None
10	F	70	84.1	126.21	45.83	5.57	None	None
11	F	66	66	98.94	41.96	4.69	None	None
12	F	56	89.3	133.94	49.51	6.01	None	None
13	F	38	97.9	48.94	17.40	5.11	None	None
14	F	33	81.5	40.76	14.11	5.63	None	None
15	F	35	51.5	25.76	16.96	4.79	None	None

Table S2. Liver function tests. Patient aspartate aminotransferase (AST) and alanine transaminase (ALT) levels values before and after administration of LUM015.

	AST (U/l) Normal range: 15-41							**ALT (mg/dl)** Normal male range: 17-63 Normal female range: 14-54						
Patient #	**Pre-study**	**8 h**	**22-24 h**	**2 d**	**7 d**	**14 d**	**30-35 d**	**Pre-study**	**8 h**	**22-24 h**	**2 d**	**7 d**	**14 d**	**30-35 d**
1	28	20	21	28	14	23	.	30	27	30	24	19	20	.
2	19	19	16	17	29	29	.	17	18	16	15	24	31	.
3	19	22	20	18	20	18	.	25	30	29	21	26	23	.
4	18	21	17	17	18	20	.	16	14	16	13	19	19	.
5	23	21	19	109	91	76	25	16	16	13	38	58	139	22
6	34	33	27	27	45	48	.	27	27	30	25	35	47	.
7	33	31	29	28	29	31	.	42	31	29	27	26	33	.
8	20	22	24	29	21	22	26	16	20	18	15	20	17	22
9	19	20	27	18	17	22	.	13	11	10	11	11	11	.
10	21	39	27	25	24	20	18	26	28	27	29	23	21	23
11	25	59	37	25	25	23	.	19	58	44	31	33	21	.
12	25	23	33	26	47	51	26	25	25	23	18	51	81	26
13	28	26	32	20	27	18	.	34	28	30	19	24	17	.
14	22	26	21	27	46	38	18	40	50	36	38	76	92	31
15	15	33	30	22	20	21	.	12	16	14	12	18	21	.

Table S3. **Ex vivo imaging of human tissues.** A gross image of the resection specimen is provided as well as H&E histology of tumor and adjacent normal tissues. The mean fluorescence intensity of tumor and normal tissue for each patient is reported along with the tumor to normal fluorescence ratio.

Patient #	Gross Resection	Tumor Type	Tumor Histology	Mean Tumor Fluorescence	Normal Tissue Type	Normal Tissue Histology	Mean Normal Fluorescence	Tumor:Normal Fluorescence Ratio
1		Well-differentiated Liposarcoma		2.3×10^{10}	Fibrous/Adipose		2.4×10^{10}	1.0
2		UPS		5.4×10^{10}	Muscle		6.6×10^{9}	8.2
3		UPS		2.3×10^{10}	Muscle		1.2×10^{10}	2.0
4		Malignant Peripheral Nerve Sheath Tumor		3.4×10^{10}	Nerve Sheath		2.0×10^{10}	1.7
5		Myxoinflammatory fibroblastic sarcoma/ hemosiderotic fibrolipomatous tumor		2.4×10^{9}	Adipose		3.1×10^{9}	0.8
6		UPS		3.4×10^{10}	Adipose		1.6×10^{10}	2.2
7	No Image	MetastaticClear Cell Sarcoma		1.3×10^{11}	Adipose		1.2×10^{11}	1.1
8		UPS		1.5×10^{11}	Muscle		3.8×10^{10}	3.9
9		IDC: ER+, PR-, Her2+		5.4×10^{11}	Breast		1.1×10^{11}	5.0
10		IDC: ER+, PR+, Her2-	Not Obtained		Breast		1.5×10^{10}	N/A
11		Metastatic Spindle Cell Sarcoma		8.0×10^{10}	Muscle		5.8×10^{9}	13.9
12		Myxofibrosarcoma		4.5×10^{11}	Muscle		4.8×10^{10}	9.5
13		Synovial Sarcoma		1.3×10^{11}	Muscle		2.3×10^{10}	5.6
14		Spindle Cell Sarcoma		3.8×10^{10}	Muscle		2.8×10^{10}	1.3
15		IDC: ER+, PR+, Her2-		3.2×10^{10}	Breast		2.6×10^{10}	1.3

Fluorescence given in counts/s/cm^2

Supplementary Table 3

Table S4. Correlating tissue fluorescence with metabolite concentration. One representative tumor and normal tissue sample from each patient was used for metabolite concentration analysis by LC-MS/MS and HPLC. The mean tissue fluorescence value for that tissue is reported and used for correlation analyses.

Patient #	Tissue type	Tissue fluorescence	[LUM015] (nM)	[Fragment 3] (nM)	[Fragment 2] (nM)
1	Liposarcoma	1.4×10^{10}	17.0	5.9	0.0
	Colonic mucosa	4.8×10^{10}	24.6	8.9	5.0
2	Undifferentiated pleomorphic sarcoma (UPS)	8.2×10^{9}	21.7	5.9	0.0
	Muscle	8.5×10^{8}	20.0	3.0	3.3
3	UPS	2.9×10^{10}	34.3	5.9	0.0
	Muscle	1.7×10^{10}	12.6	5.9	0.0
4	Malignant peripheral nerve sheath tumor	3.1×10^{10}	51.9	17.7	16.9
	Muscle	7.9×10^{9}	32.1	21.2	0.0
5	Myxoinflammatory fibroblastic sarcoma/ hemosiderotic fibrolipomatous tumor	1.6×10^{9}	10.4	5.9	0.0
	Muscle	2.2×10^{9}	21.4	3.0	0.0
6	UPS	3.4×10^{10}	47.4	5.9	5.8
	Skin	1.1×10^{11}	0	11.9	3.0
7	Metastatic clear cell sarcoma	1.3×10^{11}	146.2	14.8	8.0
	Adipose	1.2×10^{11}	76.3	5.9	0.0
8	UPS	2.9×10^{11}	92.1	35.5	0.0
	Muscle	3.8×10^{10}	31.6	8.9	0.0
9	Invasive ductal carcinoma (IDC)	5.6×10^{11}	127.7	38.5	89.5
	Breast	1.1×10^{11}	52.8	20.7	0.0
10	Breast	1.2×10^{10}	67.2	14.8	3.3
11	Metastatic spindle cell sarcoma	8.0×10^{10}	190.0	50.3	4.8

	Muscle	5.8×10^{9}	64.1	14.8	0.0
12	Myxofibrosarcoma	4.9×10^{11}	180.5	50.3	10.1
	Muscle	4.8×10^{10}	99.7	8.9	6.1
13	Synovial sarcoma	2.5×10^{11}	60.9	50.3	4.1
	Muscle	2.3×10^{10}	70.6	8.9	0.0
14	Spindle cell sarcoma	7.3×10^{10}	41.2	3.0	0.0
	Muscle	2.8×10^{10}	39.7	5.9	0.0
15	IDC	5.7×10^{10}	73.3	8.9	3.7
	Breast	1.1×10^{10}	50.1	5.9	0.0