

## Supporting Information

### **Molecular Imaging of Cancer using X-ray Computed Tomography with Protease Targeted Iodinated Activity-Based Probes**

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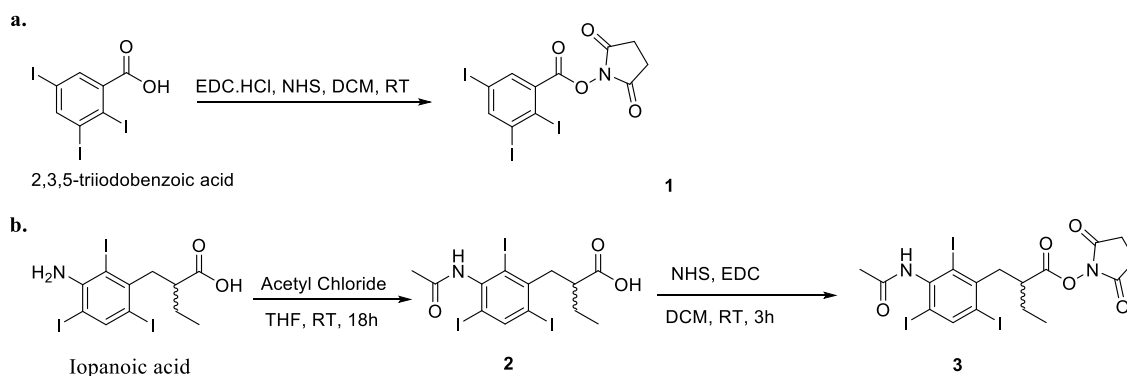
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## 1. Chemistry

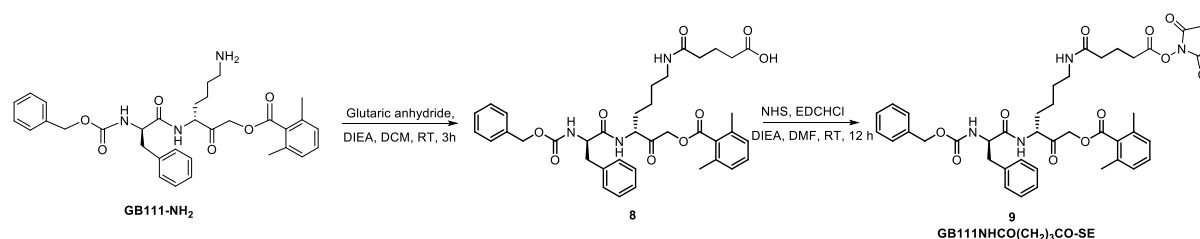
GB111-NH<sub>2</sub> (**3**). The synthesis of the acyloxymethyl ketone (AOMK) started from commercially available Fmoc and Boc-protected Lysine. Standard solid-phase peptide chemistry was used to build the dipeptide portion and related compounds based on<sup>37</sup>. In short, the carboxyl terminus of Fmoc-Lys(Boc)-OH was converted to a bromomethyl ketone by reacting the Fmoc-Lys(Boc)-OH with *N*-methylmorpholine (NMM) and isobutyl chloroformate (IBCF) in the anhydrous conditions. This mixture was then react with diazomethane that was generated in situ after which hydrogen bromide was added. Acyloxymethyl ketone (AOMK) was synthesized by condensing dimethyl benzoic acid and Fmoc-Lys(Boc)-BMK in basic conditions with KF. A di-peptide was synthesized using standard SPPS (solid phase peptide synthesis) on a chlorotrityl resin. The amine protecting group, Boc (tert-Butyloxycabony) was removed with 25% trifluoroacetic acid (TFA). The ε amine was reacted with chlorotrityl chloride resin in basic conditions, then α amine protecting group, Fmoc (Fluorenylmethyloxy carbonyl) was removed with 5% diethyl amine (DEA). Activated carboxybenzyl protected phenyl alanine was reacted with resin bound lysin AOMK by using PyBOP (benzotriazol-1-yl-oxy tripyrrolidinophosphonium hexafluorophosphate) and HOBT (1-Hydroxybenzotriazole). Finally, the elongated peptide was removed from the resin with 10% TFA to obtain compound **GB111-NH<sub>2</sub>**.<sup>37-39,43</sup>

Iodide tags were synthesized starting from commercially available 2,3,5-triiodobenzoic acid or Iopanoic acid. The NHS ester of 2,3,5-triiodobenzoic acid and acetylated Iopanoic acid was generated by using NHS (*N*-hydroxysuccinamide) and EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) in basic conditions (Scheme S1a,b). GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE ester was synthesized by reacting **GB111-NH<sub>2</sub>** with glutaric anhydride and then reaction with NHS to give compound **9**, (Scheme S2).

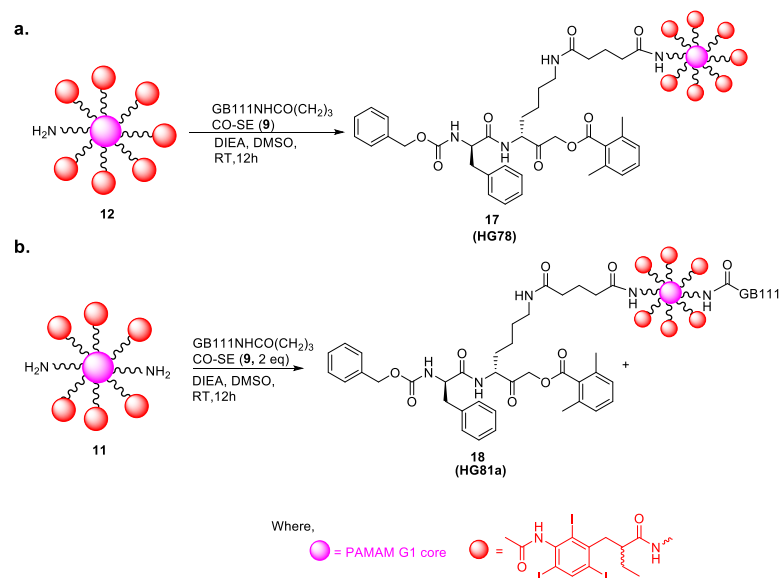
## 2. Schemes



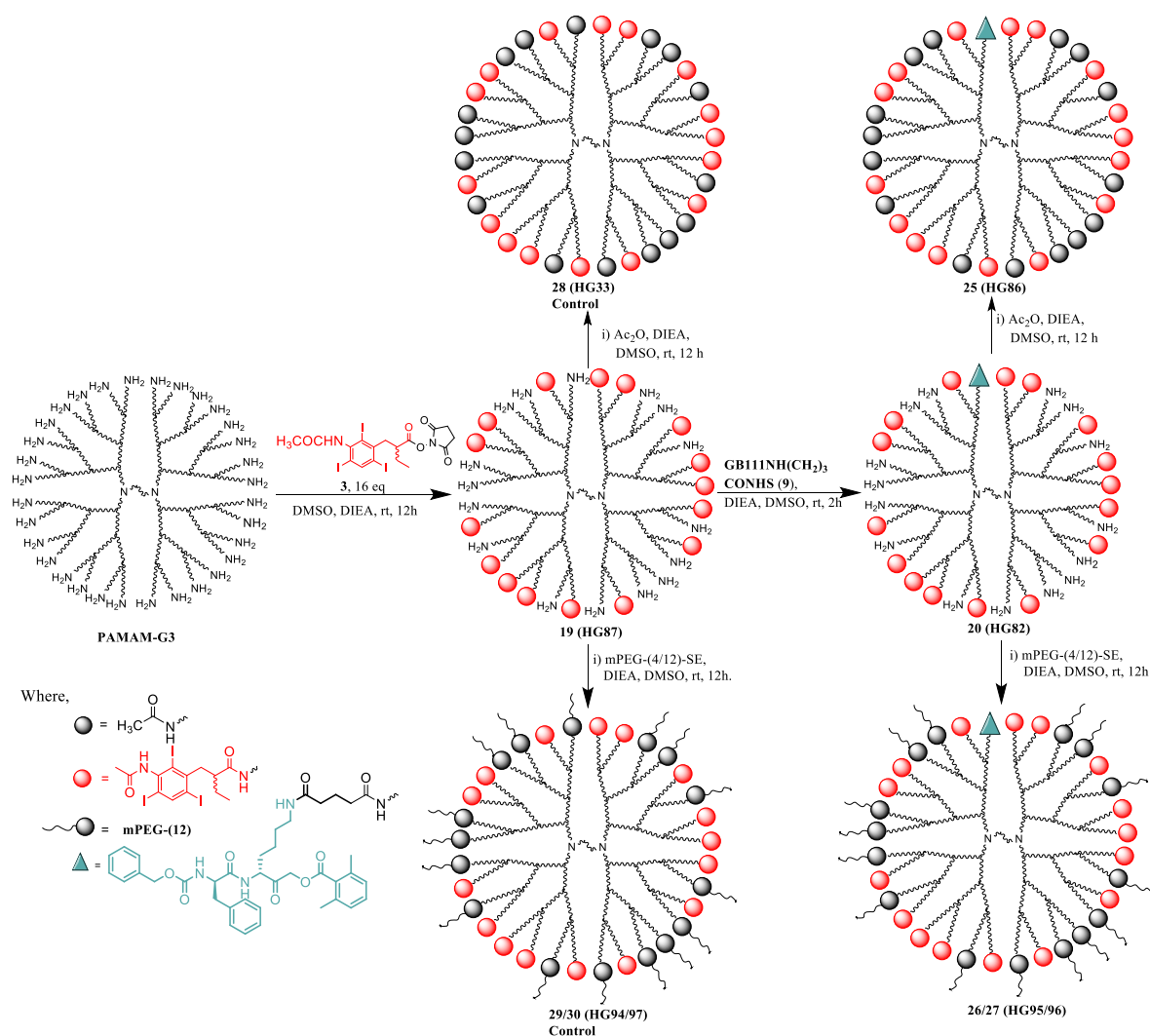
**Scheme S1.** a) Synthesis of 2,3,5-triiodobenzoic acid (TBA) succinamide ester. The TBA iodine tag was generated by reacting TBA with NHS. b) Synthesis of Iopanoic acid succinamide ester.



**Scheme S2.** GB111-NH succinamide ester was generated by reacting GB111-NH<sub>2</sub> with glutaric anhydride followed by a reaction with NHS to give compound **9**. A detailed procedure is described in the supplementary experimental section.



**Scheme S3.** a) A seven iodide tagged IN-ABP, **17**, was synthesized starting from compounds **12**, which was reacted with GB111-NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**) under basic condition. b) a six iodide tagged IN-ABP **18** attached to two GB111 targeting moieties, was synthesized starting from compounds (**11**), which was reacted with 2 equivalents of **9**, under basic condition. Detailed procedures are described in the experimental.



**Scheme S4.** Synthesis of PAMAM dendrimer core multiple tagged IN-ABPs (Acetylated and PEGylated): PAMAM G3 was linked to 16 iodine tags by reacting with IPA SE **3**, one GB111-NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**) was coupled and the remaining free amines were then capped with acyl, PEG (4/12) or left free, generating **25 (HG86)**, **26** and **27 (HG95/96)** and **20 (HG82)** respectively. Control compounds lacking the GB111-NH moiety were generated by acetylation or PEGylation (by PEG 4 or PEG 12) or left as free amines making **28 (HG33)**, **29/30 (HG94/97)** and **19 (HG87)**, respectively. Detailed procedures are described in the experimental.

### 3. EXPERIMENTAL SECTION

**Synthesis of 2,5-dioxopyrrolidin-1-yl 2,3,5-triiodobenzoate (1).** A solution of 2,3,5-triiodobenzoic acid (0.2 mmol, 1 eq.) in 5 mL anhydrous dichloromethane (DCM) under argon was mixed with *N*-hydroxysuccinimide (NHS; 0.4 mmol, 2 eq.), *N,N*-Diisopropylethylamine (DIEA; 0.6 mmole, 3 eq.) and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl; 0.2 mmol, 2 eq.). The reaction mixture was stirred for 3h at ambient temperature. DCM was added to the crude residue and the resulting organic phase was washed with water, brine and saturated NaHCO<sub>3</sub>. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. Crude product was purified by HPLC and eluted with 70% acetonitrile (ACN), to obtain (1) as a white solid (0.16 mmol, 82 % yield). ESI-MS (*m/z*): 613.66 [M+H]<sup>+</sup>, 635.58 [M+Na]<sup>+</sup>.

**Synthesis of 2-(3-acetamido-2,4,6-triiodobenzyl)butanoic acid (2).** A solution of Iopanoic acid (IPA; 0.88 mmol, 1 eq.) in tetrahydrofuran (THF; 10 mL) and acetyl chloride (1.76 mmol, 2 eq.) was stirred at room temperature for 18h. The reaction mixture was diluted with DCM, washed with water and brine. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. The crude product (0.81 mmol, 92%) obtained was used for next step without purification. ESI-MS (*m/z*): 613.66 [M+H]<sup>+</sup>, 635.58 [M+Na]<sup>+</sup>.

**Synthesis of 2,5-dioxopyrrolidin-1-yl 2-(3-acetamido-2,4,6-triiodobenzyl)butanoate (3).** A solution of 2 (0.81 mmol, 1 eq.) in anhydrous DCM (10 mL) under argon, was mixed with *N*-hydroxysuccinimide (NHS) (1.62 mmol, 2 eq.), DIEA (2.43 mmol, 3 eq.) and EDC·HCl (1.62 mmol, 2 eq.). The reaction mixture was stirred for 3h at ambient temperature. DCM was added to the crude residue and the resulting organic phase was washed with water, brine and saturated NaHCO<sub>3</sub>. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. The crude product (3) was purified by HPLC and eluted with 60% ACN, to obtain (3) as a white solid (0.58 mmol, 72 % yield), ESI-MS (*m/z*): 711.51 [M+H]<sup>+</sup>, 733.50 [M+Na]<sup>+</sup>. <sup>1</sup>H-NMR (300 MHz, DMSO-*d*<sup>6</sup>): δ 9.96 (d, *J* = 9.8 Hz, 1H, amide NH), 8.40 (s, 1H, Ar-H), 3.45 (m, 2H, CH<sub>2</sub>), 3.10 (m, 1H, CH), 4.32 (m, 2H, ), 2.81 (s, 4H, 2XCH<sub>2</sub>), 2.03 (s, 3H, CH<sub>3</sub>), 1.81 (s, 1H, CH), 1.47 (m, 1H, CH), 0.93 (t, 3H, CH<sub>3</sub>).

**Synthesis of Iodide tagged ABP (4a-b).** A solution of GB111-NH<sub>2</sub> (0.009 mmol, 1 eq.), iodine tag Succinamide ester (SE) (1 or 3; 0.14 mmol, eq.1.5) and DIEA (0.027 mmol, 3 eq.) in anhydrous DMSO (10 mL) was stirred at room temperature for 12h. The reaction mixture was diluted with DCM (5 mL), washed with water, brine and saturated NaHCO<sub>3</sub>. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. The crude product was

purified by HPLC and eluted with 60% to 65% ACN, to obtain (**4a-b**) as a white solid. **4a**: ESI-MS (m/z): 1057.16 [M+2]<sup>+</sup>, 1079.49 [M+H+Na]<sup>+</sup>. **4b**: ESI-MS (m/z): 1170.63 [M+2]<sup>+</sup>.

**Synthesis of 5-benzyl-8-(2-((2,6-dimethylbenzoyl)oxy)acetyl)-3,6,14-trioxo-1-phenyl-2-oxa-4,7,13-triazaheptadecan-17-oic acid (5)**. A solution of GB111-NH<sub>2</sub> (0.087 mmol, 1 eq.) in DCM (5 mL) and succinic anhydride (0.096 mmol, 1.1 eq.) was stirred at room temperature for 3h. The reaction mixture was diluted with DCM, washed with water and brine. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. The crude product was purified by HPLC and the product eluted with 57% ACN, to obtain (**5**) as a white solid (0.074 mmol, 85% yield). ESI-MS (m/z): 674.93 [M+H]<sup>+</sup>.

**Synthesis of GB111NH-PAMAM-Go (6)**. PAMAM-Go (0.3 mmol, 1.5 eq.) and a mixture of **5** (0.2 mmol, 1 eq.), benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 0.4 mmol, 2 eq.), Hydroxybenzotriazole (HOBt; 0.4 mmol, 2 eq.) and DIEA (0.6 mmol, 3 eq.) were dissolved in anhydrous DMF at 0 °C. Then the reaction mixture was stirred for 12h at ambient temperature. DCM was added to the crude residue and the resulting organic phase was washed with water, brine and saturated NaHCO<sub>3</sub>. The organic phase was dried over MgSO<sub>4</sub> and solvent was removed in *vacuo*. The crude product was purified by C4 reverse phase HPLC and the product eluted with 25% ACN, to obtain (**6**) as a white solid (0.134 mmol, 67% yield). ESI-MS (m/z): 1173.27 [M+H]<sup>+</sup>.

**Synthesis of Iodide tagged ABP (7a-b)**. A solution of **6** (0.009 mmol, 1 eq.), iodine tag SE (**1** or **3**; 0.027 mmol, 3.0 eq.), and DIEA (0.027 mmol, 3 eq.) in anhydrous DMSO (2 mL) was stirred at room temperature for 12h. The reaction mixture was diluted with DCM and worked up as described above. The crude product was purified by HPLC and the product eluted with 58% to 65% ACN, to obtain (**7a-b**) as a white solid. **7a**: ESI-MS (m/z): 1310.40 [M/2+2]<sup>+</sup>. **7b**: ESI-MS (m/z): 1479.89 [M/2+H]<sup>+</sup>.

**Synthesis of 5-benzyl-8-(2-((2,6-dimethylbenzoyl)oxy)acetyl)-3,6,14-trioxo-1-phenyl-2-oxa-4,7,13-triazaheptadecan-17-oic acid (8)**. A solution of GB111-NH<sub>2</sub> (0.087 mmol, 1 eq.) in DCM (5 mL) and glutaric anhydride (0.096 mmol, 1.1 eq.) was stirred at room temperature for 3h. The reaction mixture was diluted with DCM, worked up and purified by HPLC as described above. The product eluted with 57% acetonitrile, to obtain (**8**) as a white solid (0.074 mmol, 95%). ESI-MS (m/z): 688.35 [M+H]<sup>+</sup>, 710.38 [M+Na]<sup>+</sup>.

**Synthesis of GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (9)**. A solution of **8** (0.81 mmol, 1 eq.) in anhydrous DMF (5 mL) under argon was mixed with NHS (1.62 mmol, 2 eq.), DIEA (2.43 mmol, 3 eq.) and EDC·HCl (1.62 mmol, 2 eq.). The reaction mixture was stirred for 12 at

ambient temperature. Ethyl acetate was added to the crude residue that was worked up and purified by HPLC as described above. The product eluted with 45% acetonitrile, to obtain (**9**) as a white solid (0.58 mmol, 82 % yield). ESI-MS (m/z): 785.08 [M+H]<sup>+</sup>, 817.00 [M+Na]<sup>+</sup>. <sup>1</sup>H-NMR (300 MHz, DMSO-d<sup>6</sup>): δ 8.50 (d, *J* = 7.2 Hz, 1H, amide NH), 7.79 (s, 1H, amide NH), 7.64 (d, *J* = 7.9 Hz, 1H, amide NH), 7.27 (m, 10H, Ar-H), 7.20 (m, 1H, Ar-H), 7.10 (d, *J* = 15.4 Hz, 2H, Ar-H), 5.09 (d, *J* = 10.1 Hz, 1H, CH Aomk), 4.95 (s, 2H, CH<sub>2</sub>), 4.84 (m, 2H, 2XCH), 4.32 (m, 2H, ), 3.00 (s, 2H, CH<sub>2</sub>), 2.79 (s, 4H, 2XCH<sub>2</sub>), 2.64 (s, 2H, CH<sub>2</sub>), 2.29 (s, 6H, CH<sub>3</sub>), 2.15 (s, 2H, CH<sub>2</sub>), 1.80 (s, 2H, CH<sub>2</sub>), 1.54 (m, 2H, CH<sub>2</sub>), 1.35 (m, 2H, CH<sub>2</sub>), 1.28 (m, 2H, CH<sub>2</sub>).

**Synthesis of six, seven and eight iodine tagged compounds (11, 12, 13).** A solution of PAMAM G1 (0.069 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with **3** (0.28 mmol, 4.0 eq) and DIEA (0.55, 8 eq.). The solution was stirred at room temperature for 12h. The reaction mixture was purified by HPLC and the products were eluted with 40% to 46% ACN, to obtain six, seven and eight iodine tagged products (**11**, **12**, **13** respectively) as a white solid (12, 9, 22 % yields respectively). **11**: ESI-MS (m/z): 1250.91 [M/4+H]<sup>+</sup>, 1000.89 [M/5+H]<sup>+</sup>. **12**: ESI-MS (m/z): 1399.56 [M/4+H]<sup>+</sup>. **13**: ESI-MS (m/z): 1548.08 [M/4+H]<sup>+</sup>.

**Synthesis of six iodine tagged probe HG81 (14).** A solution of **11** (0.009 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**, 0.009 mmol, 1.0 eq) and DIEA (0.027, 3 eq.). The solution was stirred at room temperature for 12h. The reaction mixture was purified by HPLC and the product eluted with 41% ACN, to obtain **HG81 (14)** as a white solid (0.0074 mmol, 82 % yield). LCMS: ESI-MS (m/z): 1885.76 [M/3+H]<sup>+</sup>, 1414.71 [M/4+H]<sup>+</sup>.

**Synthesis of Cy5 labeled IN-ABP, HG92 (15).** A solution of **HG81 (14)**, (0.009 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with Cy5-SE (0.009 mmol, 1.0 eq) and DIEA (0.027, 3 eq.), the mixture was stirred in the dark at room temperature for 12h. The reaction mixture was purified by HPLC and the product eluted with 47% ACN, to obtain **HG92 (15)** as a blue solid (0.007 mmol, 78 % yield). LCMS: ESI-MS (m/z): 1577.83 [M/4+H]<sup>+</sup>.

**Synthesis of Cy5 labeled control compound, HG31 (16).** A solution of **12** (0.009 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with Cy5-SE (0.009 mmol, 1.0 eq) and DIEA (0.027, 3 eq.), the mixture was stirred in the dark at room temperature for 12h. The reaction mixture was purified by HPLC and the product eluted with 51% ACN, to obtain (**16**) as a blue solid (0.0077 mmol, 86 % yield). ESI-MS (m/z): 1558.83 [M/4+H]<sup>+</sup>, 1247.25 [M/5+H]<sup>+</sup>.

**Synthesis of seven iodine tagged probe, HG78 (17).** A solution of **12** (0.009 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**, 0.009 mmol, 1.0 eq) and DIEA (0.027, 3 eq.). The solution was stirred at room temperature for 12h then purified by HPLC and the product eluted with 47% ACN, to obtain **HG78 (17)** as a white solid (0.0074 mmol, 81 % yield). ESI-MS (m/z): 1563.72 [M/3+H]<sup>+</sup>.

**Synthesis of six iodine tagged probe HG81a (18).** A solution of **11** (0.009 mmol, 1 eq.) in anhydrous DMSO (2 mL) was mixed with GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**, 0.018 mmol, 2.0 eq) and DIEA (0.027, 3 eq.). The mixture was stirred at room temperature for 12h then purified by HPLC and the product eluted with 45% ACN, to obtain **HG81a (18)** as a white solid (0.0078 mmol, 87 % yield). ESI-MS (m/z): 1578.45 [M/4+H]<sup>+</sup>.

**Synthesis of PAMAM-G3 conjugated (IN-ABPs).** A solution of G3 PAMAM dendrimers ((NH<sub>2</sub>)<sub>32</sub>-G3, 100 mg; 0.014 mmol) in anhydrous DMSO (3 mL), was mixed with DIEA (0.46 mmol, 32 eq.) and iodine tag SE (**3**, 0.23 mmol, 16.0 eq). The mixture was stirred at room temperature for 12 h then dialyzed by an 8 kD strip against methanol and deionized water for 3 runs each followed by lyophilization to yield (IPA)<sub>16</sub>(NH<sub>2</sub>)<sub>16</sub>-G3 (**19**) as a sticky white solid (211 mg; 88 %). To the resulting conjugate, (IPA)<sub>16</sub>(NH<sub>2</sub>)<sub>16</sub>-G3 (**19**, 200 mg, 0.0122 mmol, 1.0 eq)) in anhydrous DMSO (2 mL), were added GB111NHCO(CH<sub>2</sub>)<sub>3</sub>CO-SE (**9**, 0.0122 mmol, 1.0 eq) and DIEA (0.39 mmol, 32 eq.). The mixture was stirred at room temperature for 2 h, dialyzed as above and then lyophilized to yield (IPA)<sub>16</sub>(NH<sub>2</sub>)<sub>15</sub>(GB)<sub>1</sub>-G3 (**20**) as a sticky white solid (190 mg; 91 %).

To generate (**21**), (IPA)<sub>16</sub>(NH<sub>2</sub>)<sub>15</sub>(GB)<sub>1</sub>-G3 (**20**, 20 mg, 0.0012 mmol, 1.0 eq) was dissolved in anhydrous DMSO (2 mL) then Cy5-SE (0.0017 mmol, 1.0 eq) and DIEA (0.075 mmol, 64 eq.) were added and stirred at room temperature in the dark for 2 h, then acetic anhydride (0.056 mmol, 32 eq) was added and stirred at room temperature for 12 h. The mixture was then dialyzed and lyophilized to yield (IPA)<sub>16</sub>(GB)<sub>1</sub>(Cy5)<sub>1</sub>(Ac)<sub>14</sub>-G3 (**21**) as a blue solid (25 mg; 78.1%). Similarly compound (**22**) generated by using PEG-12-SE (0.037 mmol, 32 eq) instead of acetic anhydride to yield (IPA)<sub>16</sub>(GB)<sub>1</sub>(Cy5)<sub>1</sub>(PEG-12)<sub>14</sub>-G3 (**22**) as a blue solid (27 mg; 86.8 %).

The negative controls, G3 dendrimers without GB111 were synthesis as follow: The free amine conjugate, (IPA)<sub>16</sub>(NH<sub>2</sub>)<sub>16</sub>-G3 (**19**, 20 mg, 0.0012 mmol, 1.0 eq), was mixed with Cy5-SE (0.0017 mmol, 1.0 eq) and DIEA (0.075 mmol, 64 eq.) in anhydrous DMSO (2 mL). The mixture was stirred at room temperature for 2 h in the dark, then acetic anhydride (0.056 mmol, 32 eq) was added and stirred at room temperature for 12 h. The mixture was then



dialyzed and lyophilized to yield (IPA)<sub>16</sub>(Cy5)<sub>1</sub>(Ac)<sub>15</sub>-G3 (**23**) as a blue solid 19 mg; 89.3 %). Similarly compound (**24**) generated by using PEG-12-SE (0.037 mmol, 32 eq) instead of acetic anhydride to yield (IPA)<sub>16</sub>(Cy5)<sub>1</sub>(PEG-12)<sub>15</sub>-G3 (**24**) as a blue solid (26 mg; 83.3 %).

Similar compounds lacking the Cy5 tag were generated as described in above method, without usage of Cy5 fluorophore. (IPA)<sub>16</sub>(GB)<sub>1</sub>(Ac)<sub>15</sub>-G3 (**25**) as a brown solid (19 mg; 91 %), (IPA)<sub>16</sub>(GB)<sub>1</sub>(PEG-4)<sub>15</sub>-G3 (**26**) as a white solid (20 mg; 83.9 %), (IPA)<sub>16</sub>(GB)<sub>1</sub>(PEG-12)<sub>15</sub>-G3 (**27**) as a white solid (28 mg; 89.9 %), (IPA)<sub>16</sub>(Ac)<sub>16</sub>-G3 (**28**) as a brown solid (18 mg; 86.3 %), (IPA)<sub>16</sub>(PEG-4)<sub>16</sub>-G3 (**29**) as a white solid (21 mg; 86.6 %) and (IPA)<sub>16</sub>(PEG-12)<sub>16</sub>-G3 (**30**) as a white solid (29 mg; 89.5 %).

The number of conjugated IPA molecules per dendrimer was determined to be 16, on average using <sup>1</sup>H-NMR spectroscopy. The characteristic peaks at δ (ppm) 8.3-8.4 belong to the phenyl proton (16 H's) of the aromatic ring of IPA. The number of conjugated GB111 molecules per dendrimer was determined to be one, on average, using <sup>1</sup>H-NMR spectroscopy. The characteristic peaks at δ (ppm) 2.3 belong to the dimethyl proton (6 H's) of the aromatic ring of GB111. The number of conjugated Acetyl, PEG-4 and PEG-12 per dendrimer was determined to be on average, using <sup>1</sup>H-NMR spectroscopy.

**Recombinant cathepsin labeling, competition assay.** Recombinant human Cathepsin L (0.5 μg) or Cathepsin B (0.5 μg), a kind gift from Prof. Boris Turk, J. Stefan Institute, Ljubljana, Slovenia, were incubated with indicated concentrations of targeted or control IN-ABP in reaction buffer (50 mM acetate, 2 mM DTT and 5 mM MgCl<sub>2</sub>, pH 5.5) for two hours at 37°C. After probe incubation, residual cathepsin activity was labeled with GB123<sup>43</sup> for 30 minutes. The reaction was stopped by addition of sample buffer x4 (40% glycerol, 0.2 M Tris/HCl, pH 6.8, 20% beta-mercaptoethanol, 12% SDS and 0.4 mg/ml bromophenol blue). Samples were then boiled, separated on a 12.5% SDS gel and scanned for fluorescence by a Typhoon scanner FLA 9500 at excitation/emission wavelengths of 635/670 nm.

**Recombinant cathepsin direct labeling.** Recombinant human Cathepsin L (0.5 μg) or Cathepsin B (0.5 μg) were incubated with indicated concentrations of targeted or control Cy5 labelled IN-ABP in reaction buffer for 2 hours as described above. The reaction was stopped by addition of sample buffer x4 and analyzed by in gel fluorescence as described above.

**Cell cultures.** NIH-3T3 mouse fibroblast cells and 4T1 mouse mammary gland epithelial cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin and 1% streptomycin and cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C.

**Evaluation of probes permeability to intact cells, competition assay.** NIH-3T3 cells ( $1 \times 10^5$  cells/well) were seeded in a twelve-well plate one day before treatment. Cells were treated with indicated concentrations of compounds that were pre-dissolved in 0.1% DMSO and 3% Ethanol in the culture medium. After 24 hours of probe incubation, residual cathepsin activity was labelled with GB123 ( $1 \mu\text{M}$ ) for two hours (final DMSO and ethanol concentration were maintained at 0.2% and 3%, respectively). Cells were washed with PBS and lysed by addition of RIPA buffer (PBS, 1% NP40, 0.5% DOC and 0.1% SDS). Lysates were boiled for 5 minutes, centrifuged, and equal protein amounts were separated by 12.5% SDS-PAGE. Residual labelled proteases in cells were visualized by scanning the gel for fluorescence as described above.

**Evaluation of probes permeability and selectivity in intact cells, direct labelling assay.** NIH-3T3 ( $1 \times 10^5$  cells/well) were seeded in a twelve-well plate one day before treatment. Cells were treated with indicated concentrations of Cy5 labelled compounds that were pre-dissolved in 0.1% DMSO in culture medium for 24 hours. Cells were then washed with PBS and lysed by addition of RIPA buffer. Labelled proteases in cells were analysed by in gel fluorescence as described above.

**In vitro Cytotoxicity Assays.** NIH-3T3 cells ( $5 \times 10^3$  cells/well) were seeded in a 96-well plate one day before treatment. Cells were treated with indicated concentrations of compounds that were pre-dissolved in 0.1% DMSO in the culture medium. After 24h or 48h incubation cells were fixed with 2.5% glutaraldehyde for 10 min. Cells were washed with water and borate buffer (0.1M, pH = 8.5), and stained by 1% Methylene Blue in borate buffer (100  $\mu\text{l}$ /well), for at least 1 hour at room temperature. The stain was aspirated, the plates were rinsed by immersion in deionized water, air-dried and then the stain was released from cells by 0.1 N HCl (200  $\mu\text{l}$ /well). After 1 hour incubation at  $37^\circ\text{C}$ , the absorbance of each well at 620 nm was determined on a Cytation 3 microplate reader.

**Fluorescent *in vivo* imaging.** 4T1 cells (that express high cathepsin activity)<sup>41,42</sup> were grown to subconfluency, followed by detachment with trypsin, spin down and resuspension in 0.5% BSA in sterile PBS and 25% matrigel. Cells ( $1 \times 10^6$  per spot in a total volume of 20  $\mu\text{l}$ ) were injected subcutaneously on the back into 3-4 week-old male BALB/c mice under isoflurane anaesthesia (n=3). Tumors were typically established ( $\sim 100 \text{ ml}^3$ ), 9-11 days after cells injections. Then fur was removed from mice and a 100  $\mu\text{l}$  solution of a 0.5 mg iodine/mouse of targeted or control Cy5 labelled iodinated compounds (in PBS with 60% DMSO, 10% Tween80) were injected intravenously via the tail vein. Mice anesthetized with

isoflurane were then imaged at indicated time points after compound injection using an IVIS Kinetic (Perkin Elmer, MA, USA) equipped with a 630/690 nm excitation/emission filter.

***In vivo* imaging of Iodinated compounds by CT.** Tumor bearing mice (n = 4-7) as described above were injected intravenously via the tail vein with a solution of targeted IN-ABP or control nanomaterials dissolved in PBS with 60% DMSO, 10% Tween80 in a total volume of 100  $\mu$ l, the iodine concentration was kept constant at 0.5 mg/mouse. Mice anesthetized with isoflurane were imaged before, and 5 and 24 hours post injection. An X-ray CT scanner (Skyscan High Resolution Model 1176) equipped with 64 detectors with a nominal resolution of 35  $\mu$ m with a 0.5 mm aluminum filter, at 60 kVp and 350 mA was used. Reconstruction was performed using SkyScanNRecon software, ring artifact reduction, Gaussian smoothing (3%), and beam hardening correction (25%) were applied in all experiments. ROI were manually drawn around the tumor, ROI's were linearly correlated between slices with tumor volume for each animal at each time point. Volume rendered three-dimensional (3D) images were generated using SkyScan CT-Volume ("CTVol") software.

***In vivo* labeling of cathepsins within mice tumor and other main organs.** Mice were sacrificed, tumor and major organs from IN-ABP, HG92, and control, HG31, injected mice were collected 24 hours post injection. Tissues were lysed and equal protein amount were separated by 12.5 % SDS PAGE that was scanned for fluorescence as described above.

**Fluorescent microscopy of IN-ABP in tumors.** Tumor from IN-ABP and control injected mice were collected 24 hours post injection. Tumor tissues were incubated for 4 hours with 4% paraformaldehyde, followed by an overnight incubation with 30% sucrose at 4°C. Samples were then embedded in OCT and frozen at -80°C. Tissues were sectioned into 10  $\mu$ m thick slices using a CM 1900 cryotome (Leica Microsystems, Wetzlar, Germany). The samples were mounted with DAPI Fluoromount-G (Southern Biotech, AL, USA). Fluorescent images were taken with an Olympus FV10i confocal microscope (Olympus, Tokyo, Japan) equipped with Cy5 and DAPI filters.

**Chemical Characterizations.** Chemical reactions were analysed by a Liquid Chromatography Mass Spectrometer (LC-MS) (Thermo Scientific MSQ-Plus), attached to an Accela UPLC system. Proton NMR spectra were recorded on Varian Mercury 500 MHz spectrometer in deuterated solvent. The nanoparticle size, size distribution, and zeta potential of compounds were measured using a Malvern Zeta Sizer Nano ZS (Malvern Instruments, Worcestershire, UK). Transmission electron microscopy (TEM) images were taken using a JEOL JEM-1400Plus electron microscope operating at different kV.

#### 4. <sup>1</sup>H-NMR data of PAMAM-G3 conjugated (IN-ABPs):

**HG87 (19):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.89 (bs, 16H, amide NH), 8.31 (s, 16H, Ar-H), δ 7.94- 7.70 (brs, 60H, amide protons), 3.23-2.91 (bs, ~120H), 2.71-2.53 (brs, ~184H), 2.42-2.29 (brs, ~58H), 2.23-2.07 (bs, ~120H), 2.03 (s, 48H, CH<sub>3</sub>), 1.61 (m, 16H, CH), 1.30 (m, 16H, CH), 0.71 (t, 48H, CH<sub>3</sub>).

**HG82 (20):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.93 (bs, 16H, amide NH), 8.35 (s, 16H, Ar-H), δ 7.96- 7.76 (brs, 60H, amide protons), 7.34-7.23 (m, 10H, Ar-H), 7.23-7.16 (m, 3H, Ar-H), 7.13-7.07 (m, 3H, Ar-H), 5.78 (m, 2H, CH<sub>2</sub>), 3.53-2.96 (bs, ~120H), 2.71-2.53 (brs, ~184H), 2.46-2.33 (m, ~58H), 2.31 (s, 8H, CH<sub>3</sub>), 2.26-2.07 (bs, ~120H), 2.02 (s, 48H, CH<sub>3</sub>), 1.87 (s, 2H, CH<sub>2</sub>), 1.68 (m, 16H, CH), 1.54 (m, 2H, CH<sub>2</sub>), 1.35 (m, 16H, CH), 1.21 (m, 2H, CH<sub>2</sub>), 0.71 (t, 48H, CH<sub>3</sub>).

**HG90 (21):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.93 (bs, 16H, amide NH), 9.86 (m, 6H, cy5H), 9.43 (m, 9H, cy5H), 9.33 (m, 3H, cy5H), 8.35 (s, 16H, Ar-H), 8.24 (m, 4H, cy5H), δ 7.95- 7.76 (brs, 60H, amide protons), 7.65 (m, 8H, cy5H), 7.34-7.24 (m, 14H, Ar-H), 7.22-7.18 (m, 3H, Ar-H), 7.11-7.08 (m, 3H, Ar-H), 6.97 (m, 5H, cy5H), 6.79 (m, 9H, cy5H), 6.31 (m, 8H, cy5H), 4.97 (brs, 2H, CH<sub>2</sub>), 4.33 (m, 12H, cy5H), 3.26-3.15 (bs, ~60H), 3.24 (brs, ~60H), 3.14-3.09 (brs, ~120H), 2.74-2.56 (brs, ~120H), 2.46-2.35 (brs, ~60H), 2.30 (s, 20H), 2.26-2.13 (brs, ~120H), 2.02 (s, 48H, CH<sub>3</sub>), 1.97 (s, 4H), 1.80 (s, 45H, CH<sub>3</sub>), 1.69 (m, 16H, CH), 1.54 (m, 2H, CH<sub>2</sub>), 1.34 (m, 16H, CH), 1.24 (m, 2H, CH<sub>2</sub>), 1.14 (s, ~20H), 0.75 (t, 48H, CH<sub>3</sub>).

**HG93 (22):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.93 (bs, 16H, amide NH), 9.85 (m, 6H, cy5H), 9.46 (m, 9H, cy5H), 9.34 (m, 3H, cy5H), 8.35 (s, 16H, Ar-H), δ 7.95- 7.76 (brs, 60H, amide protons), 7.34-7.24 (m, 14H, Ar-H), 7.22-7.18 (m, 3H, Ar-H), 7.11-7.08 (m, 3H, Ar-H), 6.95 (m, 5H, cy5H), 6.80 (m, 9H, cy5H), 6.33 (m, 8H, cy5H), 4.97 (brs, 2H, CH<sub>2</sub>), 3.57-3.46 (bs, ~400H), 3.24 (brs, ~60H), 3.20-3.16 (brs, 120H), 3.14-3.01 (brs, ~120H), 2.64 (brs, ~50H), 2.33-2.27 (brs, ~58H), 2.34-2.19 (brs, ~120H), 2.02 (s, 48H, CH<sub>3</sub>), 1.68 (m, 16H, CH), 1.54 (m, 2H, CH<sub>2</sub>), 1.34 (m, 16H, CH), 1.24 (m, 2H, CH<sub>2</sub>), 1.14 (s, ~20H), 0.75 (t, 48H, CH<sub>3</sub>).

**HG99 (23):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.91 (bs, 16H, amide NH), 9.86 (m, 6H, cy5H), 9.41 (m, 9H, cy5H), 8.34 (s, 16H, Ar-H), 8.21 (m, 4H, cy5H), δ 7.93- 7.75 (brs, 60H, amide protons), 6.96 (m, 5H, cy5H), 6.77 (m, 9H, cy5H), 6.30 (m, 8H, cy5H), 4.31 (m, 12H, cy5H), 3.29-3.13 (bs, ~60H), 3.21 (brs, ~60H), 3.13-3.07 (brs, ~120H), 2.73-2.55 (brs,

~120H), 2.47-2.36 (brs, ~60H), 2.27-2.11 (brs, ~120H), 2.03 (s, 48H, CH<sub>3</sub>), 1.79 (s, 45H, CH<sub>3</sub>), 1.67 (m, 16H, CH), 1.35 (m, 16H, CH), 1.13 (s, ~20H), 0.73 (t, 48H, CH<sub>3</sub>).

**HG32 (24):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.94 (bs, 16H, amide NH), 9.86 (m, 6H, cy5H), 9.43 (m, 9H, cy5H), 9.35 (m, 3H, cy5H), 8.33 (s, 16H, Ar-H), δ 7.96- 7.75 (brs, 60H, amide protons), 6.93 (m, 5H, cy5H), 6.83 (m, 9H, cy5H), 6.31 (m, 8H, cy5H), 3.56-3.41 (bs, ~400H), 3.21 (brs, ~60H), 3.19-3.16 (brs, 120H), 3.13-3.02 (brs, ~120H), 2.63 (brs, ~50H), 2.32-2.26 (brs, ~58H), 2.33-2.19 (brs, ~120H), 2.01 (s, 48H, CH<sub>3</sub>), 1.67 (m, 16H, CH), 1.35 (m, 16H, CH), 1.13 (s, ~20H), 0.73 (t, 48H, CH<sub>3</sub>).

**HG86 (25):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.91 (bs, 16H, amide NH), 8.34 (s, 16H, Ar-H), 8.01- 7.71 (m, 60H, amide protons), 7.33-7.24 (m, 13H, Ar-H), 7.21-7.17 (m, 3H, Ar-H), 7.11-7.07 (m, 3H, Ar-H), 4.96 (m, 2H, CH<sub>2</sub>), 3.25-3.15 (m, ~120H), 3.14-2.99 (brs, ~154H), 2.76-2.53 (m, ~120H), 2.46-2.33 (m, ~58H), 2.30 (s, 9H, CH<sub>3</sub>), 2.27-2.10 (brs, ~120H), 2.01 (s, 48H, COCH<sub>3</sub>), 1.79 (s, 42H, COCH<sub>3</sub>), 1.90 (s, 4H, CH<sub>2</sub>), 1.66 (m, 16H, CH), 1.52 (m, 4H, CH<sub>2</sub>), 1.34 (m, 16H, CH), 1.24 (m, 2H, CH<sub>2</sub>), 0.74 (t, 48H, CH<sub>3</sub>).

**HG95 (26):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.92 (bs, 16H, amide NH), 8.35 (s, 16H, Ar-H), δ 7.95- 7.76 (brs, 60H, amide protons), 7.33-7.23 (m, 15H, Ar-H), 7.22-7.18 (m, 4H, Ar-H), 7.12-7.08 (m, 4H, Ar-H), 4.97 (brs, 2H, CH<sub>2</sub>), 3.52-3.44 (brs, 20H), 3.25-3.15 (brs, 50H), 3.14-2.99 (bs, 120H), 2.72-2.59 (brs, ~108H), 2.45-2.34 (brs, ~58H), 2.33-2.27 (brs, ~40H), 2.26-2.11 (brs, ~120H), 2.01 (s, 48H, CH<sub>3</sub>), 1.66 (m, 16H, CH), 1.53 (m, 2H, CH<sub>2</sub>), 1.33 (m, 16H, CH), 1.23 (m, 2H, CH<sub>2</sub>), 0.75 (t, 48H, CH<sub>3</sub>).

**HG96 (27):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.93 (bs, 16H, amide NH), 8.35 (s, 16H, Ar-H), δ 7.95- 7.81 (brs, 60H, amide protons), 7.34-7.24 (m, 16H, Ar-H), 7.22-7.18 (m, 4H, Ar-H), 7.11-7.08 (m, 4H, Ar-H), 4.97 (bs, 2H, CH<sub>2</sub>), 3.57-3.46 (bs, ~500H), 3.27-3.43 (m, ~400H), 3.25-3.15 (brs, 120H), 3.14-3.01 (brs, ~120H), 2.42-2.35 (brs, ~58H), 2.33-2.27 (brs, ~40H), 2.34-2.19 (brs, ~120H), 2.02 (s, 48H, CH<sub>3</sub>), 1.66 (m, 16H, CH), 1.54 (m, 2H, CH<sub>2</sub>), 1.34 (m, 16H, CH), 1.23 (m, 2H, CH<sub>2</sub>), 0.75 (t, 48H, CH<sub>3</sub>).

**HG33 (28):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.89 (bs, 16H, amide NH), 8.31 (s, 16H, Ar-H), 8.03- 7.72 (m, 60H, amide protons), 7.10-7.06 (m, 3H, Ar-H), 3.23-3.16 (m, ~120H), 3.13-2.98 (brs, ~154H), 2.77-2.52 (m, ~120H), 2.47-2.32 (m, ~58H), 2.26-2.11 (brs, ~120H), 2.01 (s, 48H, COCH<sub>3</sub>), 1.78 (s, 42H, COCH<sub>3</sub>), 1.64 (m, 16H, CH), 1.32 (m, 16H, CH), 0.75 (t, 48H, CH<sub>3</sub>).

**HG94 (29):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.95 (brs, 16H, amide NH), 8.36 (s, 16H, Ar-H), δ 7.94- 7.70 (brs, 60H, amide protons), 3.52-3.46 (brs, ~160H), 3.23-2.91 (brs, ~120H), 2.71-2.53 (brs, ~184H), 2.42-2.29 (brs, ~58H), 2.23-2.07 (brs, ~120H), 2.02 (s, 48H, CH<sub>3</sub>), 1.68 (m, 16H, CH), 1.35 (m, 16H, CH), 0.75 (t, 48H, CH<sub>3</sub>).

**HG97 (30):** <sup>1</sup>H-NMR (500 MHz, DMSO-d<sub>6</sub>): δ 9.91 (bs, 16H, amide NH), 8.34 (s, 16H, Ar-H), δ 7.96- 7.80 (brs, 60H, amide protons), 3.59-3.45 (bs, ~500H), 3.42-3.25 (m, ~400H), 3.23-3.14 (brs, 120H), 3.12-2.98 (brs, ~120H), 2.41-2.33 (brs, ~58H), 2.31-2.18 (brs, ~120H), 2.01 (s, 48H, CH<sub>3</sub>), 1.67 (m, 16H, CH), 1.33 (m, 16H, CH), 0.73 (t, 48H, CH<sub>3</sub>).

## 5. Table

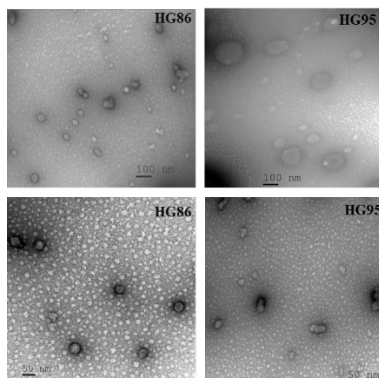
**Table S1.**

**Zeta Potential and size of multiple tagged IN-ABPs**

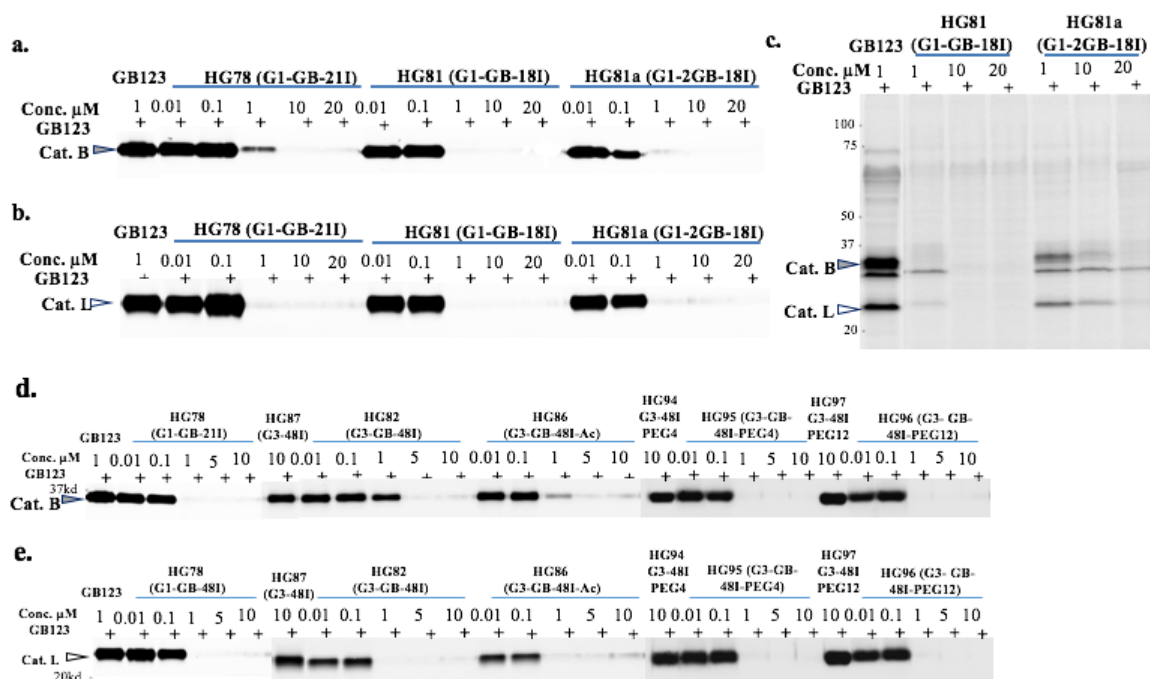
| Probes       | Zeta (mv) | Size by vol. (nm) | PDI   |
|--------------|-----------|-------------------|-------|
| PAMAM-G3     | 9±1       | 4±1               | 0.327 |
| <b>HG33</b>  | 24±1      | 6±2               | 0.325 |
| <b>HG32*</b> | 25±0.2    | -                 | -     |
| <b>HG82</b>  | 34±3      | 6±2               | 0.38  |
| <b>HG86</b>  | 25±4      | 4±1               | 0.468 |
| <b>HG87</b>  | 34±1      | 4±1               | 0.474 |
| <b>HG90*</b> | 8±1       | -                 | -     |
| <b>HG93*</b> | 34±4      | -                 | -     |
| <b>HG94</b>  | 10±0.5    | 5±1               | 0.456 |
| <b>HG95</b>  | 18±0.3    | 5±2               |       |
| <b>HG96</b>  | 21±2      | 5±2               | 0.81  |
| <b>HG97</b>  | 9±0.5     | 7±2               | 0.477 |
| <b>HG99*</b> | 17±2      | -                 | -     |

\* Size not determined by DLS because of Cy5 fluorescence

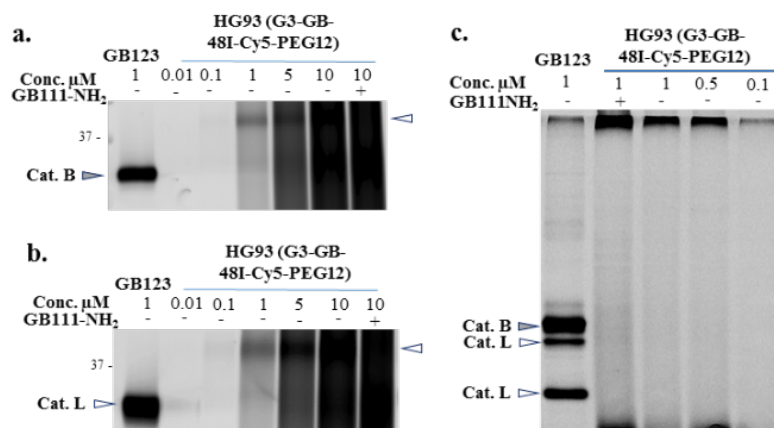
## 6. Figures



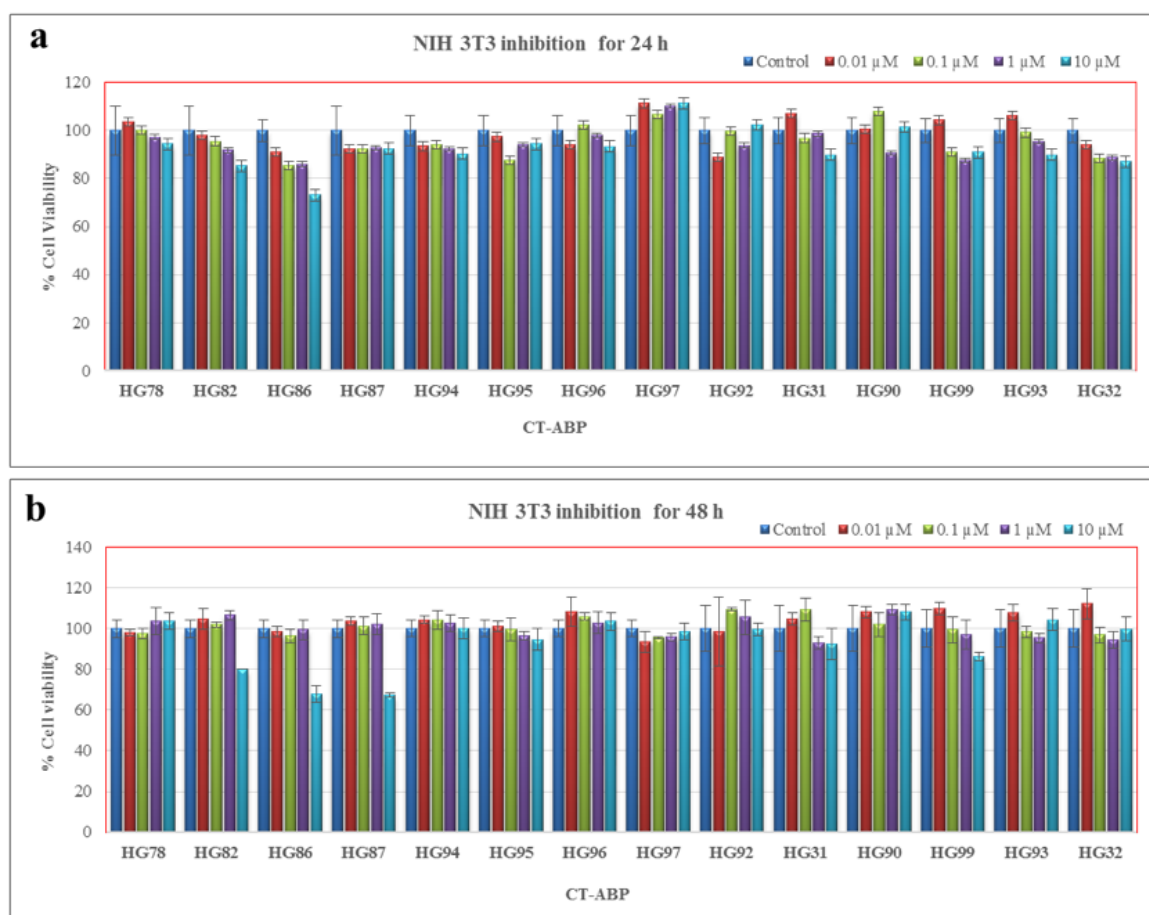
**Figure S1. TEM images of iodinated activity based probe particles.** Acetylated and PEGylated particles are shown in 100 and 50 nm bar scales.



**Figure S2. Inhibition of cathepsins activity.** a, b) Competitive inhibition of recombinant cathepsin B and L by G1 IN-ABPs, as was described in the experimental. c) Inhibition of endogenous cathepsin activity within intact NIH-3T3 cells as was described in the experimental. (d, e) Inhibition of recombinant cathepsin B and L by G1 and G3 IN-ABPs, as described in experimental.

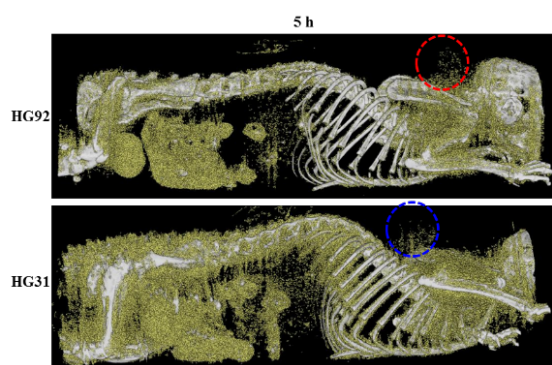


**Figure S3. Biochemical Evaluations.** a, b) Labeling of recombinant cathepsins B and L by PEG IN-ABP **HG93** as described in the experimental. c) Direct labeling of endogenous cathepsin in intact NIH-3T3 as described in the experimental.

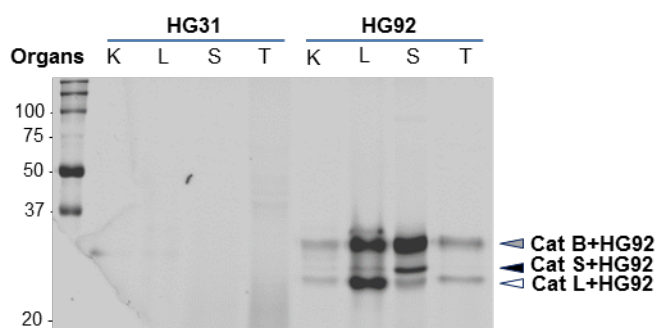


**Figure S4. Viability assay of IN-ABPs.** Viability of NIH-3T3 cells was measured by methylene blue assay 24 hours (a) and 48 hours (b) after incubation with indicated concentrations of compounds.



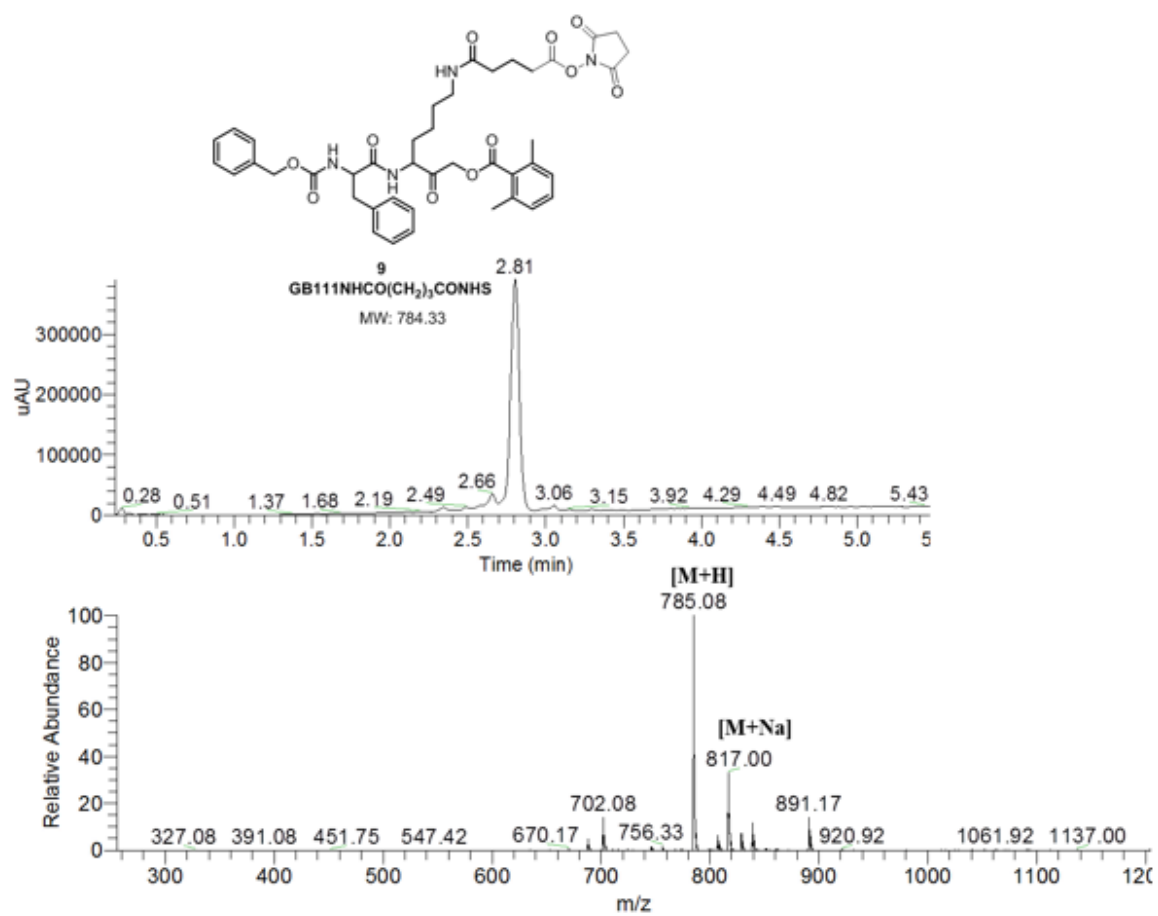


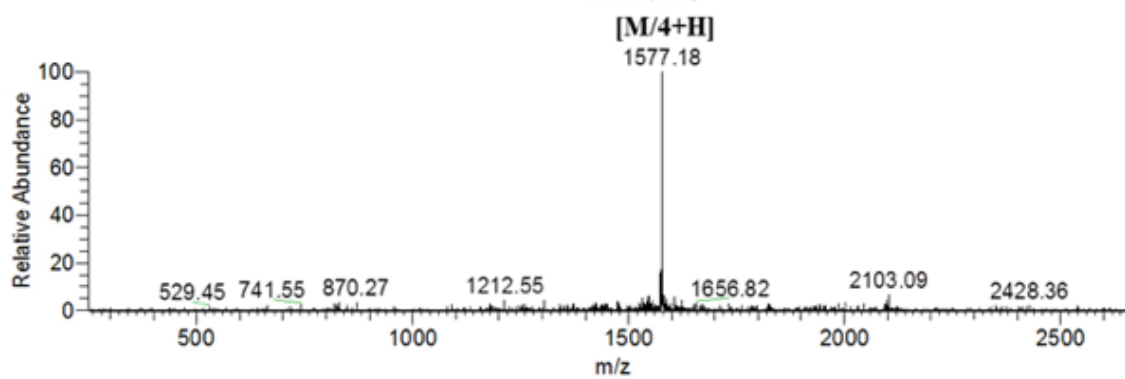
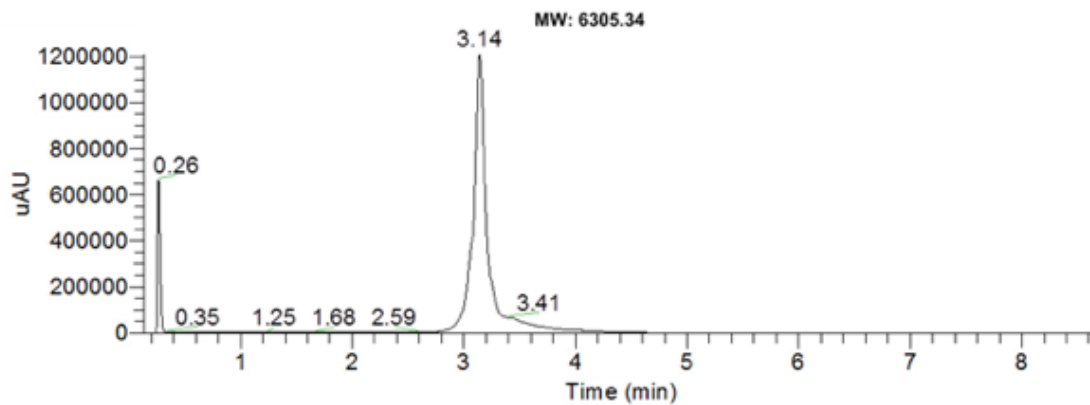
**Figure S5.** *In vivo* X-ray computed tomography, a representative picture of a mouse 5 h post-injection of IN-ABP **HG92** and respective control **HG31**.

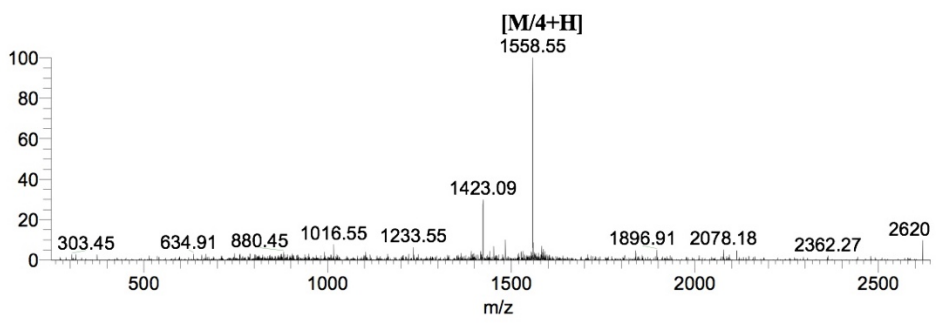
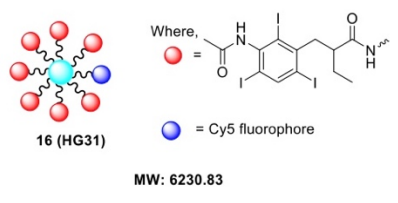
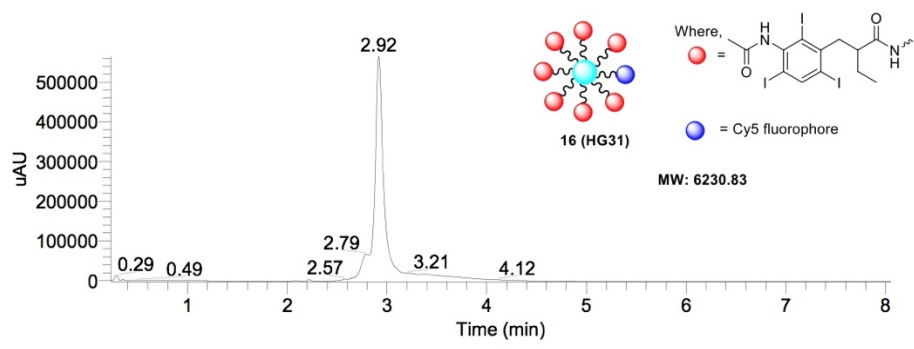


**Figure S6.** Tissues from mice 24 hours post injection of **HG92** or control **HG31** were lysed and separated by SDS PAGE that were scanned for fluorescence. Fluorescent bands reflect covalent binding of the **HG92** to tissue cathepsins. The extremely high *in vivo* specificity of the compound is detected by the indicative bands between 22 and 35 kDa that are lacking in the control samples. K = Kidney, L = Liver, S = Spleen, T = Tumor.

## 7. LCMS







## 8. $^1\text{H-NMR}$ spectroscopy

