Rapid Imaging of Tumor Cell Death *in vivo* using the C2A domain of Synaptotagmin-I

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Supplemental Methods

Probe expression and chemical labeling

iC2Am (D108N) was produced using a QuickChange Site-Directed Mutagenesis kit (Stratagene). C2Am was labeled with hydrazinonicotinic acid (HYNIC) using methods similar to those used for fluorophore labeling. Briefly, an aliquot of the protein (1 mg/mL) was reduced in HEPES-buffered saline (HBS buffer: 20 mM HEPES, 150 mM NaCl, pH 7.4) containing 10 mM dithiothreitol (DTT) at room temperature for 30 min. The reduced protein was then washed with ice cold HBS in a 5-kDa Vivaspin™ (Sartorius) and concentrated to 1 mg/mL, prior to the addition of maleimide-HYNIC (Solulink) at a 5-fold molar excess in DMSO. Reaction was conducted at room temperature for 1 h with stirring (200 r.p.m) and quenched by addition of glycine (0.6M, pH 9.2) to 0.05 M. A similar method was used to conjugate C2Am to maleimide-DOTA (Macrocyclics B-272).

Active (C2Am) and inactive (iC2Am) forms of C2Am-AF750 were prepared using Alexa-Fluor®750 C5-maleimide (AF750, Invitrogen™, λexc=750 nm, λem=775 nm), following the method described in (*1*). Briefly, the protein was reduced in HBS buffer containing 10 mM DTT, for ½ h at room temperature. The protein was then washed in a 5-kDa Vivaspin concentrator in HNE buffer (20 mM, HEPES, 100 mM NaCl, 5 mM EDTA, pH 7.4). The protein was used at a concentration in the range 50-100 μM, and an approximately 10-fold molar excess of the fluorescent maleimide dye was added to the reaction mixture. The reaction was allowed to proceed at 4 °C for 16 h. The modified protein was separated from unreacted dye by gel filtration using a Hi-Load Superdex 75 26/60 preparation grade column (GE Healthcare, Amersham, UK).

Probe radiolabeling with ^{99m}Tc

C2Am-HYNIC was labeled with ^{99m}-Tc pertechnetate (^{99m}Tc-C2Am), eluted from an Ultra-Technekow™ DTE (Covidien) and supplied at 1.5-2.0 GBq/mL by the Department of Nuclear Medicine (Cambridge University Hospitals), as described previously (*2*). To 50 μL of C2Am-HYNIC (715 nmol in 115 mM tricine buffer, pH 6±1), 200 μL of 0.9% degassed saline were added, followed by 20 μL of pertechnetate (ca. 40 MBq) and 20 μL of degassed stannous tricine solution (0.17 mg/mL $SnCl_2$, 6.7 mg/mL tricine and 0.9% NaCl) and the reaction allowed to proceed at room temperature for 10 min.

Probe radiolabeling with ^{111}In

C2Am-DOTA (60 μg; 20 μL) was labeled by adding 20 μL of acetate buffer 0.2 M pH 5.5, 8 μL 1 M pH 5.5 and 40-50 μL (30–40 MBq) of high purity $^{111}InCl_3$ (supplied at 0.8-1 GBq/mL by the Department of Nuclear Medicine, Cambridge University Hospitals) at 37 °C for 30 min, with orbital shaking (600 r.p.m.). Free ^{111}In was removed using a 5-kDa cut-off spin Amicon-ultra filter (Millipore).

All C2Am conjugates were washed and concentrated using Vivapsin concentrators and analyzed by Electrospray Ionization Mass Spectrometry (ESI-MS) (*1*) (Supplemental Figure 1).

Cell binding assays using ^{99m}Tc-C2Am

Etoposide-treated (15 μM, 24 h) or untreated EL4 cells were washed in ice-cold HBS^+ buffer (HBS with 2 mM $CaCl_2$), pelleted (600g, 4°C, 4min), and re-suspended in the same buffer at $1x10^7$ cells/mL, incubated with ^{99m}Tc-C2Am (1-100 nM), at 37 °C for 30 min in an orbital shaker (250 r.p.m.). The cells were then washed in cold HBS^+ buffer 3 times, prior to measurement of radioactivity in the cell pellets (ISOMED 2100 well counter, MED Nuklear-Medisintechnik GmbH).

SPECT studies

A NanoSPECT system (Bioscan Inc.) with 4 detector heads (230×215 mm; sodium iodide crystals) with multiple pinholes (36×2.0mm) was used. A minimum of 50,000 counts/projection were collected and the data reconstructed to give an isotropic resolution of 300 μm. CT images were acquired by helical acquisition using a microtomography system (NanoPET/CT, Mediso) and reconstructed using a modified cone beam filtered back projection method using a Butterworth filter to give an isotropic resolution of 212 μm. SPECT and CT images were fused and radioactivity quantified using VivoQuant ^{TM} 1.22 (InviCRO), and rendered in 3D for detailed visualization.

Immunohistochemistry and *ex vivo* imaging of tissue fluorescence

Excised tissues were weighed, radioactivity measured as described above and approximately half of each specimen was placed in 10% neutral buffered formalin (NBF, 10%, Acquascience). Unstained tissue sections were de-waxed and rehydrated and slides mounted using Prolong Gold™ antifade reagent (Life Technologies), cured for 24 h at room temperature, prior to

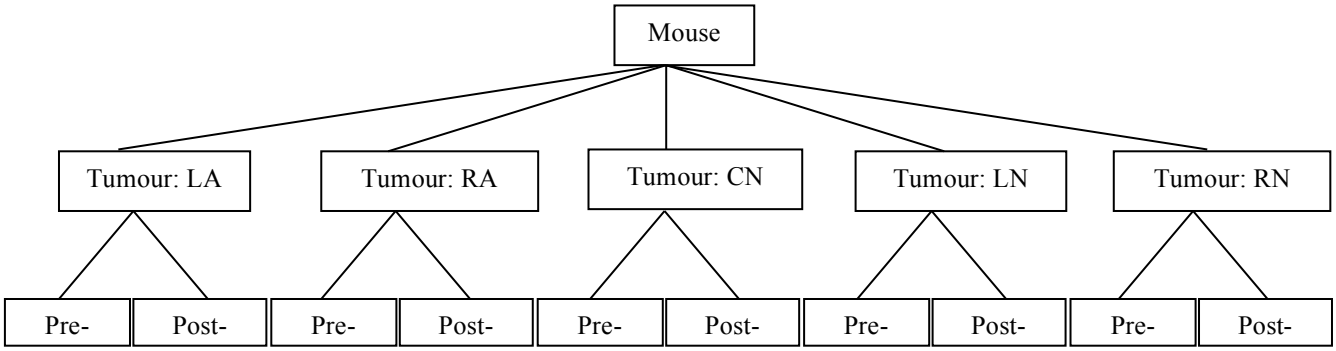
microscopic NIRF imaging at a resolution of 21 μm, using a Odyssey™ (Li-Cor) flat-bed scanner. Tissues fixed for 24 h in NBF were transferred into 70% ethanol and sections (3-μm) cut using a rotary microtome. Hematoxylin and eosin (H&E) staining was performed on a Leica ST5020/CV5030 workstation (Leica Biosystems). For cleaved-caspase-3 (CC3) staining, a rabbit monoclonal anti-CC3 antibody (Cell Signaling Technology™ Inc., used at a 1:100 dilution) and a donkey anti-rabbit secondary biotinylated antibody (Jackson ImmunoResearch Laboratories Inc., diluted 1:250 in Bond™ diluent) were used with a Leica Microsystems Bond™-Max (Leica Biosystems) system using a LM Bond™ Intense-R Detection Kit, which is based on streptavidin-horse-radish-peroxidase for detection of the secondary antibody. An avidin-biotin blocking kit was used (Vector Laboratories Inc). A hematoxylin nuclei counterstain was used. A Zeiss Mirax™ Scan 150 (Carl Zeiss) with a 20× objective was used to digitize tissue images (at 0.369 μm/pixel), which were stored using a Spectrum™ digital pathology information database (Aperio™). Aperio's ImageScope™ software was used for image analysis.

Statistics

Data are expressed as mean ± SD, unless stated otherwise. Two-tailed Student T-test, with Tukey's post-hoc non-parametric correction, was used for pairwise comparisons. Two-way ANOVA with Bonferroni post-test correction was used for multi-parametric analysis (GraphPad Prism version 5, Sigma Software). $P<0.05$ was considered statistically significant. Statistical analysis of SPECT imaging data was performed by the Bioinformatics Core of the CRUK Cambridge Institute (see Supplemental Materials and Methods).

Statistical Analysis of SPECT data (Figure 6).

Some animals could only be imaged either before or after treatment. For this reason, a mixed statistical model was fitted to the data in Figures 6, the structure of which is shown below. There were 6 mice in total, one of which had only pre-dose data and one of which had only post-dose data.



LA, RA: left, right axillary tumors, respectively; CN, LN, RN: central, left, right cervical nodal tumors, respectively.

Fixed effects for dose, protein and the dose*protein interaction were considered. Random effects were fitted for dose nested within tumor nested within mouse. During the model building process, random effects were compared using restricted maximum likelihood (REML) and fixed effects were compared using maximum likelihood (ML). The final model was fitted using REML. The analysis was conducted in R software vs. 2.14.1, using the nlme package (version 3.1.102). The R output for the final model is given below.

In a mixed effect model, usually only the parameter estimates for the fixed effects are interpreted. The results of the fixed effects part of the model are given in Table A.

Table A *Fixed effects*

Parameter	**Reference level**	**Estimate**	**Standard error**	**95% confidence interval**	**P-value**
Intercept	-	0.218	0.065	0.09 to 0.35	0.0028
Protein	C2Am	0.127	0.098	-0.06 to 0.32	0.2628
Dose	Post-dose	0.034	0.043	-0.05 to 0.12	0.4398
Protein*Dose	C2Am, Post-dose	0.284	0.079	0.13 to 0.44	0.0022

The results in Table A can be interpreted as follows:

- The difference between the pre- and post-treatment stages is 0.318 (0.034+0.284) SUV units for C2Am.

Residual plots were used to check for normality of the model residuals and these were satisfactory.

Post-hoc tests were performed to compare pre- and post-treatment measurements for each protein separately. This part of the analysis was conducted in R 2.14.1, using the multcomp package (version 1.2.17). The results are given in Table B. The P-values were adjusted for multiple testing using a Bonferroni correction.

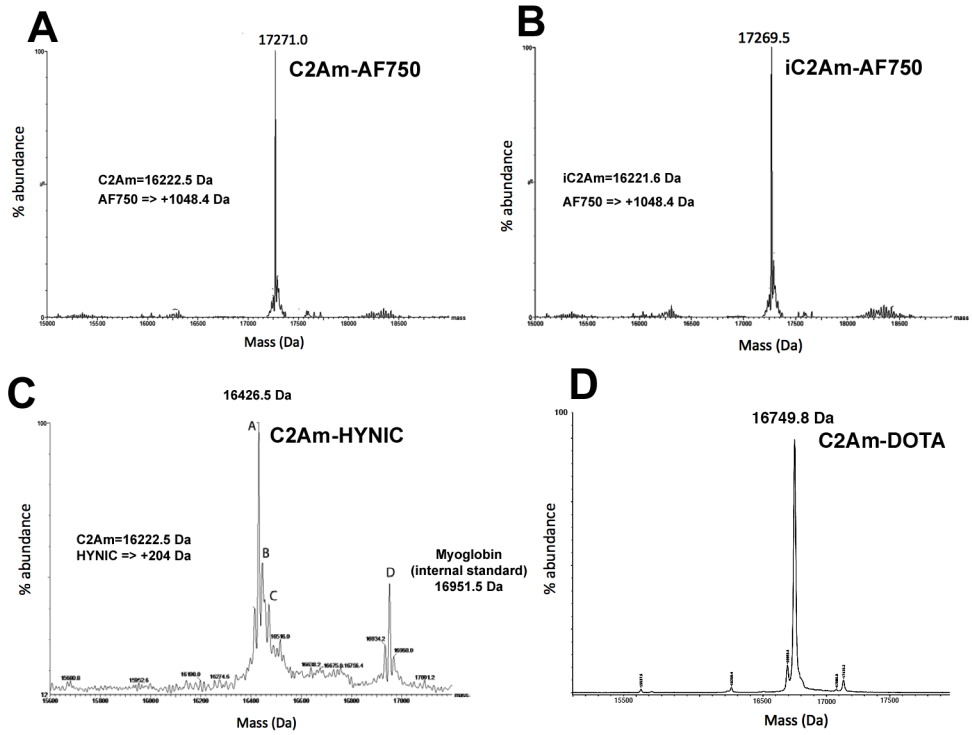
Table B *Post-hoc comparisons*

Comparison	Estimated difference	Standard error	95% confidence interval	Adjusted P-value
Pre- v post-treatment for C2Am	0.318	0.067	0.19 to 0.45	<0.0001

The difference between the pre- and post-treatment measurements was significant for C2Am.

R output for final model:

```
> #Final model
>
> m.final <- lme(Response ~ Protein + relevel(Dose,ref="Pre") + Protein*relevel(Dose,ref="Pre"),
random=~1|ID/Tumour/Dose, data=mydata, method="REML", na.action=na.omit)
> summary(m.final)
Linear mixed-effects model fit by REML
 Data: mydata
       AIC      BIC   logLik
  12.60281 27.23194 1.698597

Random effects:
 Formula: ~1 | ID
        (Intercept)
StdDev: 2.22047e-05

 Formula: ~1 | Tumour %in% ID
        (Intercept)
StdDev:   0.2247239

 Formula: ~1 | Dose %in% Tumour %in% ID
        (Intercept)    Residual
StdDev:   0.1165061 0.006787696

Fixed effects: Response ~ Protein + relevel(Dose, ref = "Pre") + Protein * relevel(Dose,      ref
= "Pre")
                                          Value  Std.Error DF  t-value p-value
(Intercept)                            0.21783215 0.06538123 24 3.331723  0.0028
ProteinC2A                             0.12696367 0.09751878  4 1.301941  0.2628
relevel(Dose, ref = "Pre")Post         0.03366731 0.04261415 18 0.790050  0.4398
ProteinC2A:relevel(Dose, ref = "Pre")Post 0.28407845 0.07943183 18 3.576380  0.0022
 Correlation:
                                       (Intr) PrtC2A r(Dr="
ProteinC2A                             -0.670
relevel(Dose, ref = "Pre")Post         -0.326  0.218
ProteinC2A:relevel(Dose, ref = "Pre")Post  0.175 -0.407 -0.536

Standardized Within-Group Residuals:
         Min           Q1          Med           Q3          Max
-0.116832335 -0.011816817 -0.002297878  0.009583274  0.196308461

Number of Observations: 50
Number of Groups:
                     ID           Tumour %in% ID Dose %in% Tumour %in% ID
                      6                       30                      50
> anova(m.final)
                                  numDF denDF  F-value p-value
(Intercept)                           1    24 66.98628  <.0001
Protein                               1     4  9.12222  0.0392
relevel(Dose, ref = "Pre")            1    18 10.30247  0.0049
Protein:relevel(Dose, ref = "Pre")    1    18 12.79050  0.0022
```



Supplemental Figure 1.

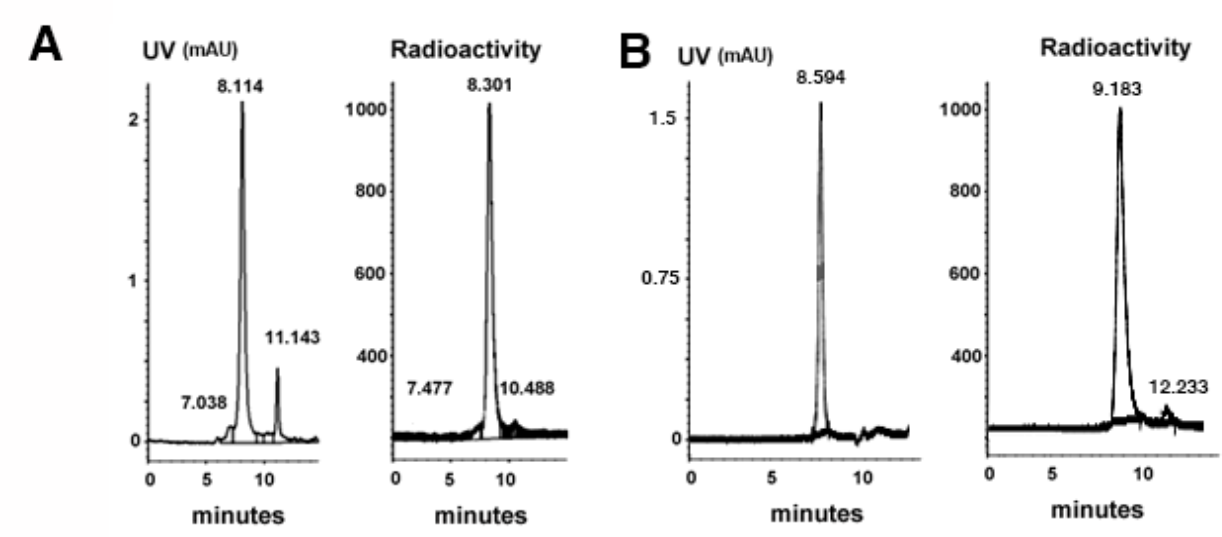
Analysis of imaging probes using electron spray ionization mass spectrometry (ESI-MS).

(A) ESI spectrum of active C2Am-AF750 showed a single peak at 17271.0 Da, corresponding to the addition of one molecule of Alexa Fluor™ 750 (+1048.4 Da) to C2Am (16222.5 Da).

(B) ESI spectrum of inactive-C2Am-AF750 (iC2Am-AF750) showed a single peak at 17269.5 Da, corresponding to the addition of one molecule of Alexa Fluor™ 750 to iC2Am (16221.6 Da).

(C) Complete modification of C2Am with maleimido-HYNIC ligand to form C2Am-HYNIC (A). A heavier peak (B) is seen at 15 Da above (A) but is much smaller. Peak (D) represents myoglobin (16951.5 Da) added for calibration purposes. Note also the small heavier peak (C) (40 Da heavier than A), likely representing the K^+ bound form of C2Am-HYNIC.

(D) Complete modification of C2Am with maleimido-DOTA to form C2Am-DOTA (16749.8 Da).

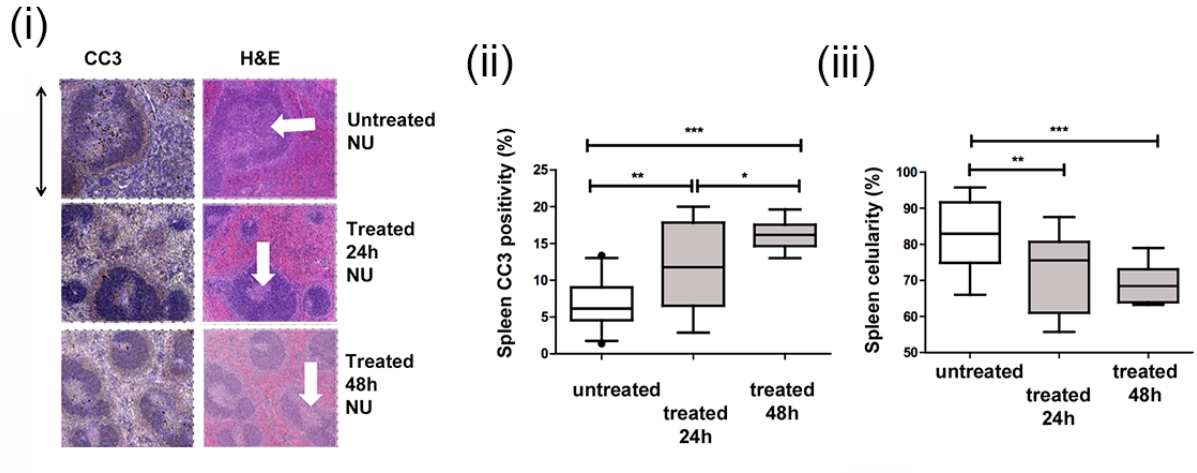


Supplemental Figure 2.

Analysis of ^{99m}Tc- and ^{111}In- labeling of C2Am using gel filtration radio-HPLC.

(A) ^{99m}Tc-C2Am-HYNIC, the dominant peak at 8.30 min in the radioactivity channel, was ^{99m}Tc-labeled C2Am, corresponding to 8.114 min in the UV channel. Another minor peak visible at 10.48 min in the radioactivity channel is likely to be reduced pertechnetate. Tricine was visible in the UV channel at 11.14 min. Labeling efficiency of ^{99m}Tc-labeled C2Am was 94%.

(B) ^{111}In-C2Am-DOTA, the main species at 9.183 min in the radioactivity channel, was ^{111}In-labeled C2Am, corresponding to 8.594 min in the UV channel. The minor peak visible at 12.233 min in the radioactivity channel is ^{111}InCl3. Labeling efficiency of ^{111}In -labeled C2Am was 97%.



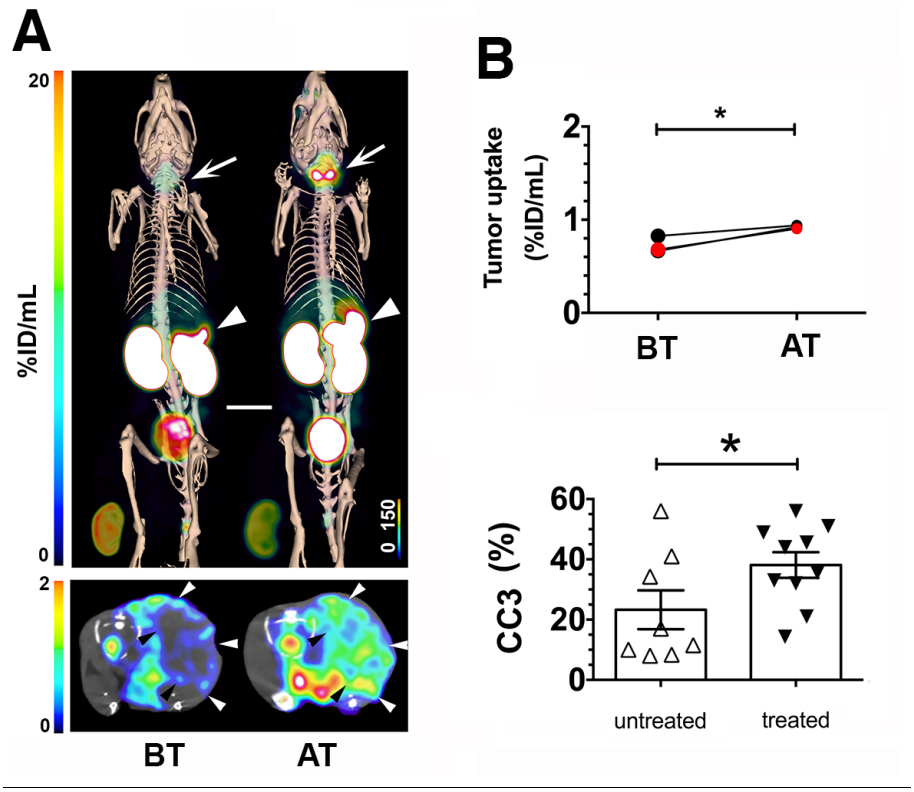
Supplemental Figure 3. Splenic response to etoposide treatment.

(i) H&E stained spleen sections in treated and untreated BalbC/NU animals.

(ii) Analysis of CC3 staining.

(iii) Spleen cellularity decreases with treatment.

Spleen sections (ii,iii) from untreated (n=20) or treated (n=10) mice were analyzed in each group. *P<0.05, **P<0.005, ***P<0.001, differences between groups (i, ii) were significant t-test.



Supplemental Figure 4.

SPECT imaging of cell death *in vivo* in EL4 tumors.

Imaging of ^{99m}Tc-labeled C2Am was performed 2 h after probe administration, and 26 h after drug treatment. (A) SPECT-CT fusion images of representative EL4 tumor-bearing mice before (A, left) and after (A, right) etoposide treatment and 2 h post administration of ^{99m}Tc-C2Am. Tumor location is indicated by the horizontal white line. The insets (top left) represent kidney signal on a larger vertical scale. ^{99m}Tc-C2Am accumulation in the salivary glands and spleen is indicated by the thin arrows and arrowheads. Arrowheads (A, lower) delineate tumor edges defined on CT. The red circles in (B) correspond to the %ID/mL values for the tumors of the animals shown in (A). (B, lower panel) CC3 staining in untreated and drug-treated tumors. *P<0.05, n=3 tumors/group (A), n=8-10 tumors/group (B). Abbreviations: BT, AT, before and after treatment, respectively. 3D rendering of SPECT data are shown in Supplemental video 1.

Supplemental Table 1. Surface Plasmon Resonance (SPR) analysis of imaging probes.

	kd (1/s)	**ka (1/Ms)**	**KD (nM)**	**Rmax**
iC2Am-AF750	N/D	N/D	N/D	N/D
C2Am-AF750	3.89×10^{-2} $\pm2\times10^{-4}$	7.11×10^{5} $\pm4.5\times10^{3}$	54.7 ±0.4	421.7 ±0.54
C2Am-HYNIC	9.46×10^{-2} $\pm1.3\times10^{-3}$	1.392×10^{6} $\pm2\times10^{4}$	68.0 ±1.4	505.9 ±1.2
C2Am-DOTA	1.16×10^{-2} $\pm1.1\times10^{-4}$	1.299×10^{5} $\pm1.4\times10^{3}$	89.3 ±0.2	469.8 ±9.3

Kinetic data analysis using Biacore T100 Evaluation 1.1.1 software. Concentration ranges 0-150 nM. Materials, methods and analysis were described previously (1). KD: thermal equilibrium dissociation constant (nM), ka, kd: kinetic association (1/Ms) and dissociation (1/s) constants, Rmax: maximum analyte binding capacity of the surface in response units (RU); values quoted are means ± standard deviation obtained from the fit to a 1:1 kinetic binding model. N/D-No detectable binding.

Supplemental Table 2 – Biodistribution of NIR-labelled imaging agents, in tumor-bearing EL4 mice, 24 h after etoposide treatment and at the indicated times after probe injection.

Tissue (TFI/mg)	**2 hours**		**24 hours**	
AnxV-AF750	Mean	SD	Mean	SD
Muscle	4.3	0.6	0.95	0.03
Spleen	7.7	0.8	2.68	0.25
Tumor	4.8	0.6	2.53	0.47
Liver	13.4	0.9	4.1	0.7
Kidney	88.9	5.6	60.0	5.0
Tumor-to-muscle	1.12	0.21	2.66	0.50
C2Am-AF750	Mean	SD	Mean	SD
Muscle	4.5	0.9	0.47	0.04
Spleen	3.6	0.4	0.63	0.02
Tumor	10.0	1.0	1.71	0.12
Liver	9.8	0.8	0.63	0.03
Kidney	161	6	38.6	1.1
Tumor-to-muscle	2.2	0.49	3.63	0.40
iC2Am-AF750	Mean	SD	Mean	SD
Muscle	6.1	1.8	0.32	0.02
Spleen	1.6	0.3	0.32	0.03
Tumor	4.9	0.3	0.35	0.02
Liver	4.1	0.2	0.3	0.03
Kidney	183	10	19.1	0.8
Tumor-to-muscle	0.80	0.24	1.1	0.1

TFI- total fluorescence intensity; data reported as TFI per mg of wet tissue mass, collected *post mortem*.

Data availability. The raw data acquired during this study and on which the results presented in this paper are based can be found at http://content.cruk.cam.ac.uk/kblab/jnm2017a.zip

Supplemental References

1. Alam IS, Neves AA, Witney TH, Boren J, Brindle KM. Comparison of the C2A domain of Synaptotagmin-I and Annexin-V as probes for detecting cell death. *Bioconjug Chem*. 2010;21:884-891.
2. Blankenberg FG, Vanderheyden J-L, Strauss HW, Tait JF. Radiolabeling of HYNIC-annexin V with technetium-99m for in vivo imaging of apoptosis. *Nat Protoc*. 2006;1:108-110.