Supplemental Material: peptide synthesis

Synthesis of MMAE containing ACPPs (Scheme 1)

The peptides were synthesized by using regular solid phase Fmoc peptide synthesis. Peptides that were used for fluorescence imaging were generated by following similar synthetic schemes that were recently reported by us (Savariar et al *Cancer Res* **2013**, 73, 855). Monomethyl auristatin E (MMAE) containing peptides were synthesized by following Scheme 1. All the reactions involving Cy5 or Cy5 containing peptides were shielded from light. HPLC characterizations and purifications were performed on Agilent 1100 or 1200 with reverse-phase C_{18} column (Phenomenex) using water and acetonitrile solvent system with 0.05% trifluoroacetic acid (TFA) as additive. Electrospray ionization (ESI) mass spectrometry was performed on all the peptides (see Supplementary Table 1) using Agilent HPLC connected to an Agilent LCMS trap XCT

H_2N-e_9-(*Substrate*)-c(SS-*t*Bu)-r_9-c-$CONH_2$ (**1, 8**)

↓ Cy5-Mal

H_2N-e_9-(*Substrate*)-c(SS-*t*Bu)-r_9-c(SuccinimidylCy5)-$CONH_2$ (**2, 9**)

↓ Et_3P

H_2N-e_9-(*Substrate*)-c(SH)-r_9-c(SuccinimidylCy5)-$CONH_2$ (**3, 10**)

↓ Maleimidopropionyl-Val-Cit-PAB-MMAE, NMM, DMSO

H_2N-e_9-(*Substrate*)-c-(Succinimidopropionyl-Val-Cit-PAB-MMAE)-r_9-c(ScuccinimidylCy5)-$CONH_2$ (**4, 11**)

↓ MAL-PEG12-NHS, NMM, DMSO

Mal-PEG-e_9-(*Substrate*)-c(Succinimidopropionyl-Val-Cit-PAB-MMAE)-r_9-c(SuccinimidylCy5)-$CONH_2$

↓ (**5, 12**) Ligand, NMM, DMSO

Ligand-Succinimidyl-PEG12-NH-e_9-(*Substrate*)-c(Succinimidopropionyl-Val-Cit-PAB-MMAE)-r_9-c(Cy5)-$CONH_2$ (**6, 7, 13, 14**)

1-**7** *Substrate* = o-PLGC(Me)AG-o

8-**14** *Substrate* = peg6 $(-NH-CH_2-CH_2-O)_6-CH_2-CH_2-CO-)$

6, **13** *Ligand* = Cyclic(RGDfC)

7, **14** *Ligand* = Cyclic(RADfC)

Cyclic-RGD-PLGC(Me)AG-MMAE (**6**): Ligand = Cyclic(RGDfC)
Substrate= o-PLGC(Me)AG-o
Cyclic-RAD-PLGC(Me)AG-MMAE (**7**): Ligand = Cyclic(RADfC)
Substrate= o-PLGC(Me)AG-o
Cyclic-RGD-PEG6-MMAE (**13**): Ligand = Cyclic(RGDfC)
Substrate= peg6-o
Cyclic-RAD-PEG6-MMAE (**14**): Ligand = Cyclic(RADfC)
Substrate= peg6-o

Scheme1. Synthesis of MMAE containing ACPPs

According to *Scheme 1*, H_2N-e_9–o-PLGCmeAG-o-c(SS-*t*Bu)r_9-c-$CONH_2$ (**1**) was made using common solid phase Fmoc peptide synthesis, where lower case letters refers to D-aminoacids, "o" represents 5-amino-3-oxopentanoyl, and C(Me) is short for S-methylcysteine. The peptides were cleaved from the resin using trifluoroacetic acid (TFA) containing 2% thioanisole, 2% water and 4% triisopropylsilane (TIPS) for 6 h under N_2 and then filtered under N_2 to remove the resin. This filtrate was concentrated and precipitated by adding ice cold 50% hexanes in ethyl acetate, dried under vacuum and purified using HPLC.

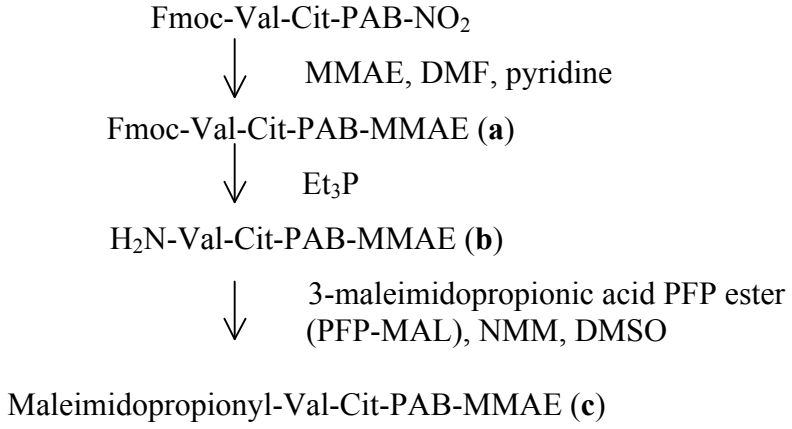
This purified compound **1** (10 mg) was dissolved in 1 ml DMSO (anhydrous) and Cy5 maleimide (~2mg, Cy5-Mal, from GE life sciences) and *N*-methylmorpholine (NMM), (1μL) were added under nitrogen. The completion of the reaction was verified by LCMS (typically in less than 3 h). To this mixture (contains H_2N-e_9–o-PLGC(Me)AG-o-c(SS-*t*Bu)r_9-c(SuccinimidylCy5)-$CONH_2$, purification at this step is not necessary) 30 μL of triethylphosphine (TEP) was added and the reaction was kept at room temperature for another 6 h. The product was precipitated by adding 50 % hexanes in ethyl acetate, which had previously been purged with N_2. The precipitated compound was purified by using HPLC to get **3**.

Compound **3** (H_2N-e_9–o-PLGC(Me)AG-o-c(SH)-r_9-c(SuccinimidylCy5)-$CONH_2$) (5 mg) was dissolved in 0.4 mL DMSO (anhydrous), and maleimidopropionyl-Val-Cit-PAB-MMAE (1.6 mg) was added along with 0.5μL NMM under N_2 and kept at room temperature

for 6 h. After completion of the reaction (note- this reaction mixture contains compound **4,** purification at this stage is not mandatory) MAL-dPEG$_{12}$-NHS ester (~2.0 mg, product number 10284, from Quanta Biodesign Ltd) and NMM (1μL) were added and reacted at room temperature for 24 h (excess of unreacted MAL-dPEG$_{12}$-NHS was removed by washing it with ethyl acetate). Then the product **5** was purified using HPLC and dried using lyophilization. The purified product **5** was dissolved in DMSO (anhydrous) and reacted with ~2 eq (1.4 mg) of Cyclic(RGDfC) (Peptides International) in the presence of NMM (0.5μL) for 1 h and purified by HPLC to get the final product **6**. Compounds **7-14** were synthesized by following same synthetic protocol.

Synthesis of Maleimidopropionyl-Val-Cit-MMAE (Scheme 2)

To a dry reaction vessel, MMAE (2mg, Concortis, CA, USA), Fmoc-Val-Cit-PAB-NO_2 (6.5mg, 3 equivalents, Concortis, CA, USA), HOBT (0.5 mg), DMF (100 μl) and pyridine (30 μl) were added and stirred for two days while monitoring the formation of the product **a** using LCMS. The product (**a)** was purified by HPLC (this procedure was adapted from Fransico et al *Blood* **2003**, 102, 1458). The compound **a** was treated with 20% piperidine in DMF for 20 min to get (**b)**, which was vacuum dried and redissolved in DMSO. To this solution 3-maleimidopropionic acid PFP ester (PFP-MAL, 2eq), (Molecular Biosciences, CO, USA.), and NMM (2 eq) were added and reacted for 6 h to get maleimidopropionyl-Val-Cit-MMAE (**c**). This final compound (**c**) was purified using preparative HPLC and characterized using LCMS.



Scheme 2. Synthesis of Maleimidopropionyl-Val-Cit-PAB-MMAE

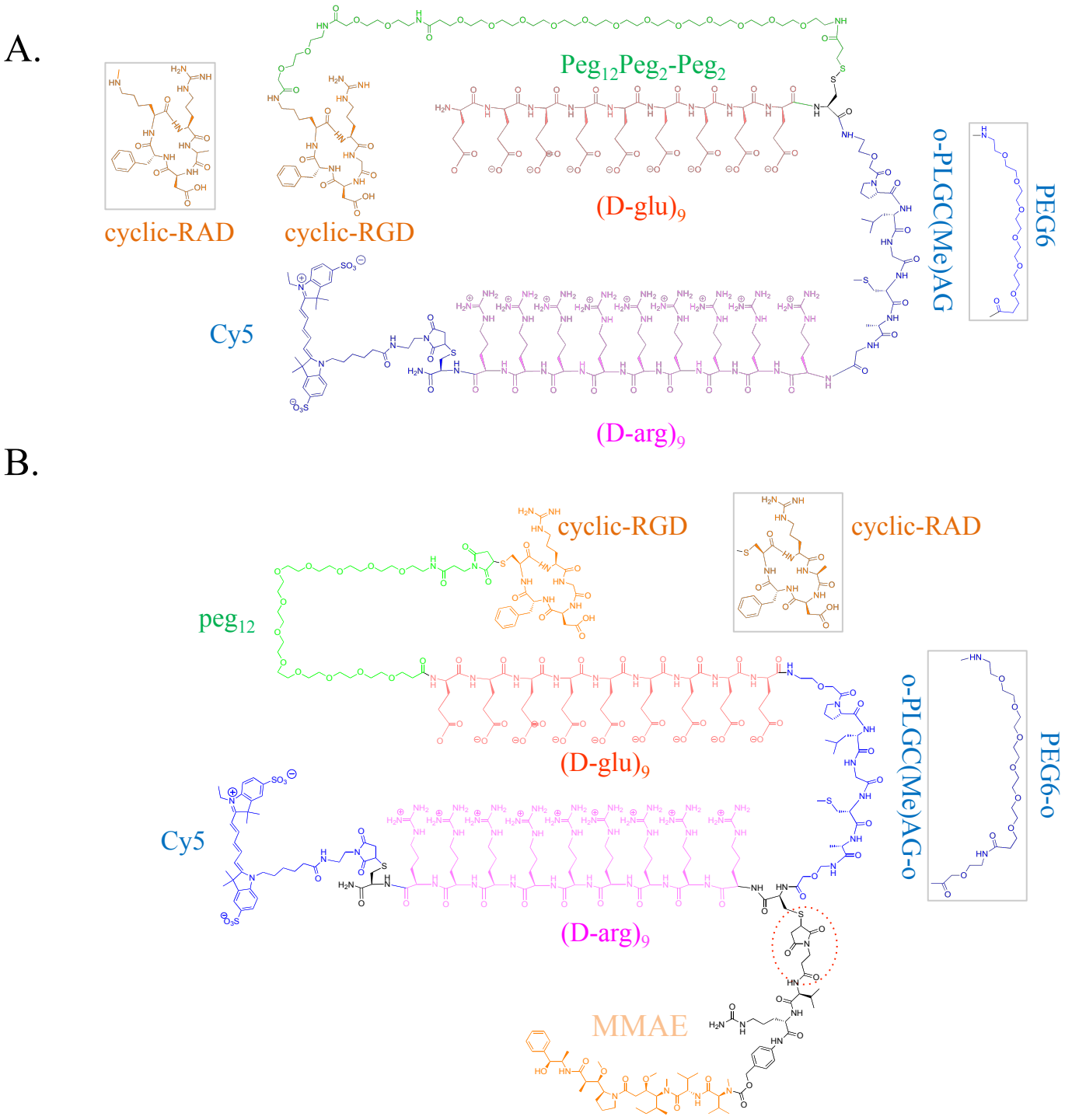
*Synthesis of cyclic-RGD-PLGC(Me)AG-MMAE-ACPP (**17**)*

To a dry reaction vessel compound **3** (H_2N-e_9–o-PLGC(Me)AG-o-c(SH)-r_9-c(SuccinimidylCy5)-$CONH_2$) (5 mg), anhydrous DMSO (0.4 mL), maleimidocaproyl-Val-Cit-PAB-MMAE (2 mg), NMM (0.5μL) were added in sequence and kept at room temperature for 6 h under N_2. After completion of the reaction (note- this reaction mixture contains compound **15,** purification at this stage is not mandatory) MAL-dPEG$_{12}$-NHS ester (~2.0 mg, from Quanta Biodesign) and NMM (1μL) were added and the reaction was kept at room temperature for 24 h (excess of unreacted MAL-dPEG12-NHS was removed by washing it with ethyl acetate). Then the product **16** was purified using HPLC and lyophilized. The product **16** was dissolved in DMSO (anhydrous) and reacted with 2 mg of Cyclic(RGDfC) in the presence of NMM (0.5μL) for 1 h and purified by HPLC to get the final product **17**.

Supplemental Table 1: List of peptides and synthetic intermediates with corresponding molecular weights

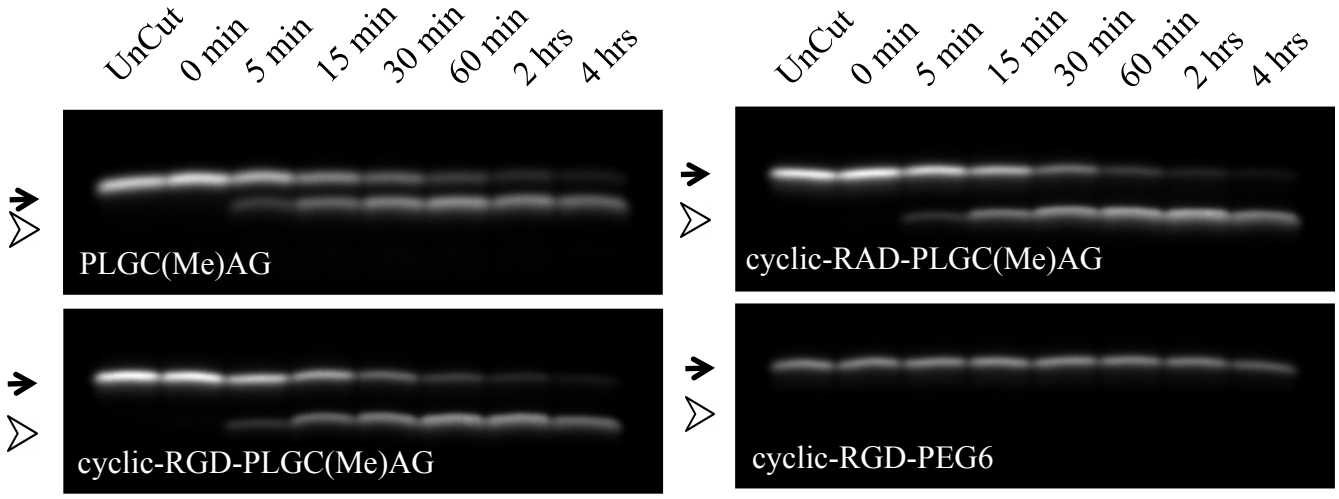
Peptide name	#	Peptide sequence	Mass obtained (Da)	Average Mass calculated (Da)
cyclic-RGD		cyclic(RGDfC(SuccinimidylCy5))	1358.4	1358.6
PLGC(Me)AG		H_2N-e_9-o-PLGC(Me)AG-r_9-c(SuccinimidylCy5)	4080.9	4080.5
cyclic-RGD-PLGC(Me)AG		H_2N-e_9-C[peg_{12}-cyclic(RGDfK)]-o-PLGC(Me)AG-r_9-c (SuccinimidylCy5)	5763.6	5763.5
cyclic-RAD-PLGC(Me)AG		H_2N-e_9-C[peg_{12}-cyclic(RADfK)]-o-PLGC(Me)AG-r_9-c (SuccinimidylCy5)	5777.4	5777.5
cyclic-RGD-PEG6		H_2N-e_9-c[peg_{12}-cyclic(RGDfK)]-peg_6-r_9-c(SuccinimidylCy5)	5485.2	5485.2
cyclic-RAD-PEG6		NH_2-e_9-c[peg_{12}-cyclic(RADfK)]-peg_6-r_9-c(SuccinimidylCy5)	5499.0	5499.2
	1	H_2N-e_9-o-PLGC(Me)AG-o-c(SS*t*Bu)r_9-c-$CONH_2$	3593.6	3594.0
	2	H_2N-e_9-o-PLGC(Me)AG-o-c(SS*t*Bu)r_9-c(SuccinimidylCy5)-$CONH_2$	4372.8	4372.9
	3	H_2N-e_9-o-PLGC(Me)AG-o-c(SH)r_9-c(SuccinimidylCy5)-$CONH_2$	4284.8	4284.8
	4	H_2N-e_9-o-PLGC(Me)AG-o-c(Succinimidopropionyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	5558.7	5559.3
	5	MAL-peg12-NH-e_9-o-PLGC(Me)AG-o-c(Val-Cit-PAB-MMAE)r_9-c (SuccinimidylCy5)-$CONH_2$	6310.0	6310.2
cyclic-RGD-PLGC(Me) AG-MMAE	**6**	Cyclic(RGDfC)-Succinimidyl-PEG12-NH-e_9-o-PLGC(Me)AG-o-c (Succinimidopropionyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6889.0	6888.8
cyclic-RAD-PLGC(Me) AG-MMAE	**7**	Cyclic(RADfC)- Succinimidyl-PEG12-NH-e_9-oPLGC(Me)AG-o-c (Succinimidopropionyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6903.0	6902.8
	8	H_2N-e_9-peg6-o-c(SS*t*Bu)r_9-c-$CONH_2$	3315.2	3315.7
	9	H_2N-e_9-peg6-o-c(SS*t*Bu)r_9-c(SuccinimidylCy5)-$CONH_2$	4094.4	4094.6
	10	H_2N-e_9-peg6-o-c(SH)r_9-c(SuccinimidylCy5)-$CONH_2$	4006.0	4006.4
	11	H_2N-e_9- peg6-o-c(Succinimidyl-Val-Cit-PAB-MMAE)r_9-c(Cy5)-$CONH_2$	5281.0	5281.0
	12	MAL-peg12-NH-e_9- peg6-o-c(Succinimidopropionyl Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6031.6	6031.8
cyclic-RGD-PEG6-MMAE	**13**	Cyclic(RGDfC)- Succinimidyl-PEG12-NH-e_9-peg6-o-c (Succinimidopropionyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6610.8	6610.5
cyclic-RAD-PEG6-MMAE	**14**	Cyclic(RADfC) Succinimidyl-PEG12-NH-e_9-peg6-o-c (Succinimidopropionyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6624.0	6624.5
	15	H_2N-e_9- o-PLGC(Me)AG-o-c(Succinimidocaproyl- Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	5601.5	5601.4
	16	MAL-peg12-NH-e_9- o-PLGC(Me)AG-o -c(Succinimidocaproyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6352.8	6352.2
	17	Cyclic(RGDfC)-Succinimidyl-PEG12-NH-e_9- o-PLGC(Me)AG-o-c (Succinimidocaproyl-Val-Cit-PAB-MMAE)r_9-c(SuccinimidylCy5)-$CONH_2$	6931.2	6930.9
	a	Fmoc-Val-Cit-PAB-MMAE	1345.6	1345.7
	b	H_2N-Val-Cit-PAB-MMAE	1123.2	1123.4
	c	Maleimidopropionyl-Val-Cit-PAB-MMAE	1274.4	1274.5

Supplemental Figure 1: Chemical Structure of dual targeted peptides



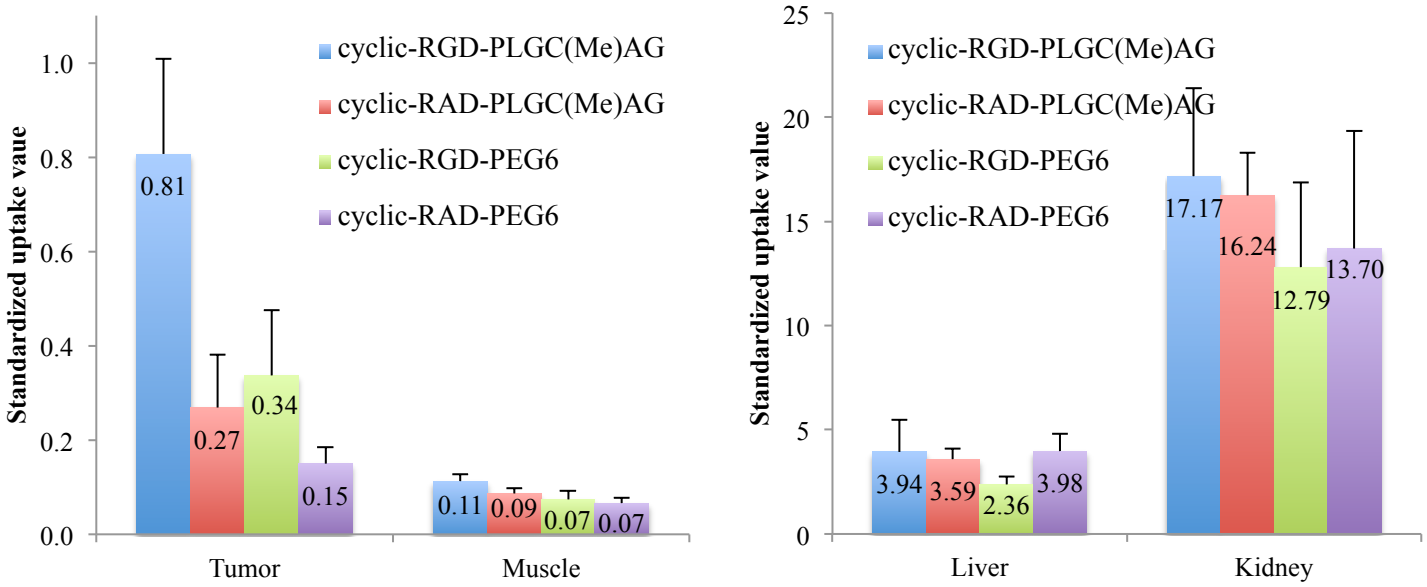
Supplemental Figure 1: A) Chemical structures of imaging and B) therapeutic dual targeted ACPPs. Control moieties are shown in the boxed regions. C) Succinimidopropionyl- and Succinimidocaproyl- linkers (location indicated by the dotted red circle in B) used for therapeutic peptide synthesis.

Supplemental Figure 2: Gel images for MMP-2 cleavage assay



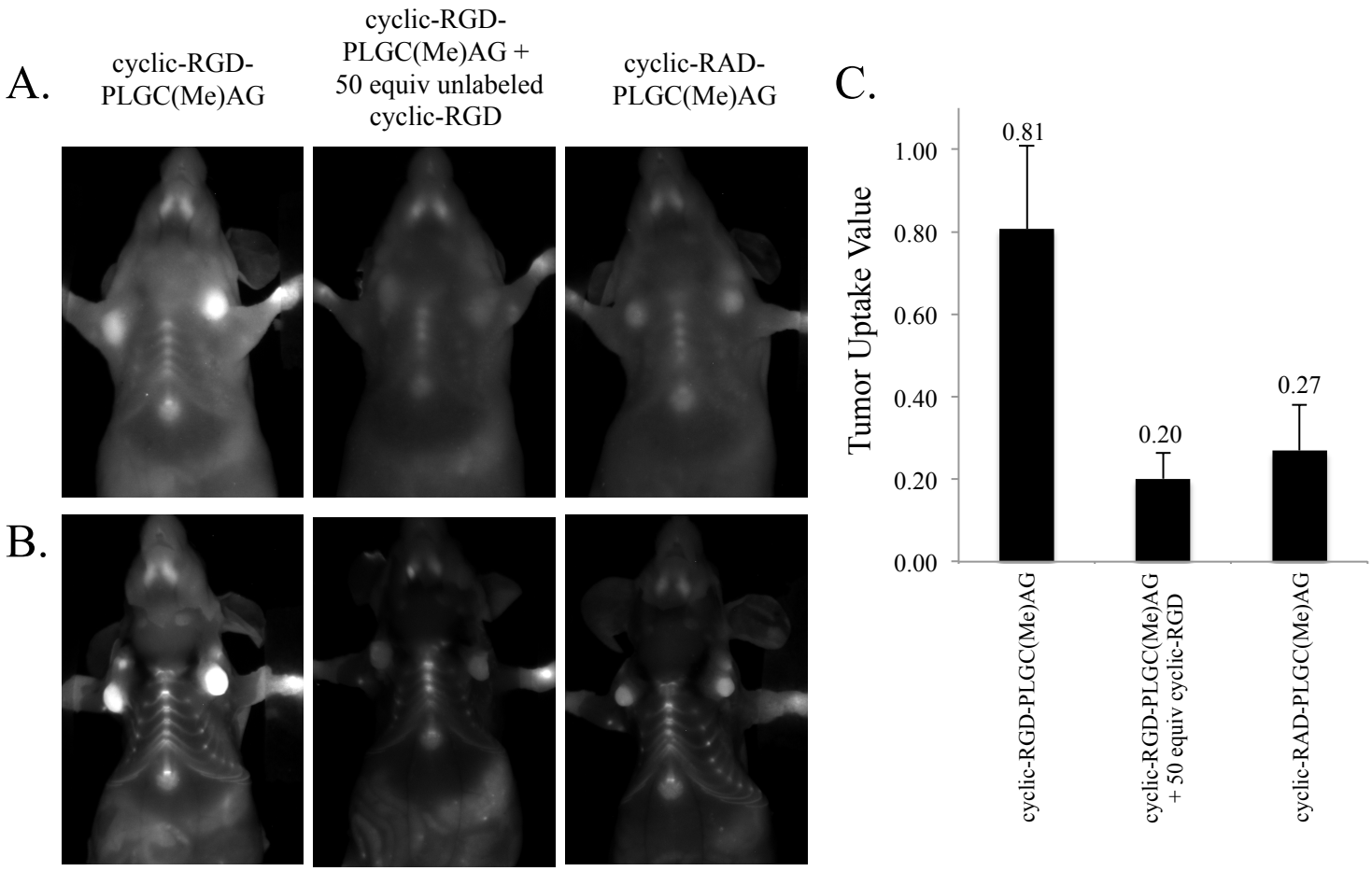
Supplemental Figure 2: For reaction conditions and concentrations see materials and methods. Samples from the reactions were subjected to electrophoresis on 10-20% tricine gradient gels for 90 minutes at 110V. Gels where removed from their casings and imaged on a Maestro Imager using an excitation filter of 620/22nm and an emission filter of 670/20nm. The black arrows indicate in-tact peptide and the white arrow heads denote the Cy-5 labeled portion of the cleavage products.

Supplemental Figure 3: Peptide SUVs (MDA-MB-231 tumors)



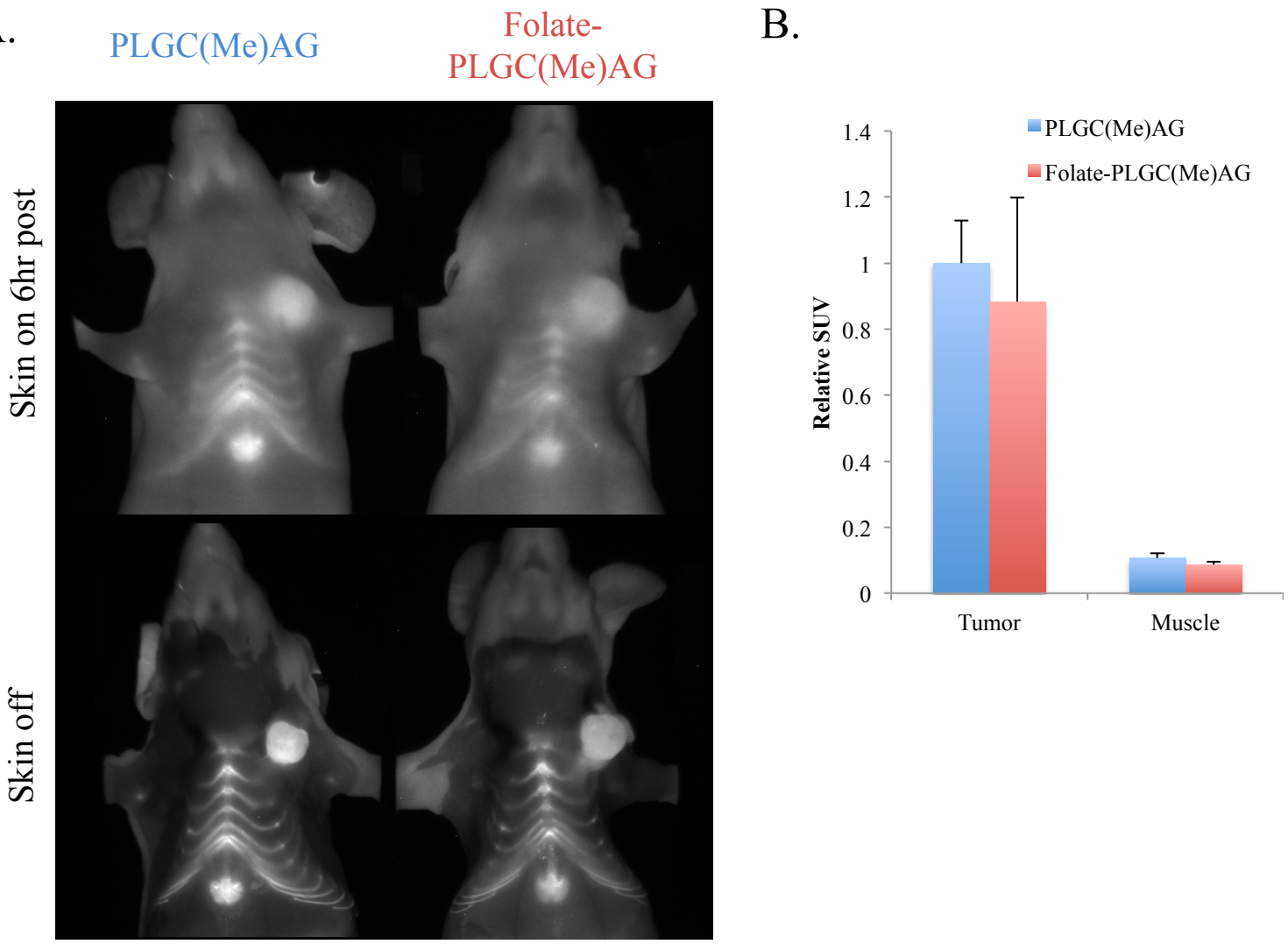
Supplemental Figure 3: Standardized uptake values (SUV) for the cyclic-RGD-PLGC(Me)AG-ACPP along the various control peptides (n=5) are presented for the tumor, muscle, liver and kidney. SUV can be defined by the following equation: (moles of peptide in the given tissue/weight of given tissue)/(moles of peptide injected/animal body weight).

Supplemental Figure 4: Injection of cyclic-RGD-PLGC(Me)AG-ACPP with excess of unlabeled cyclic-RGD



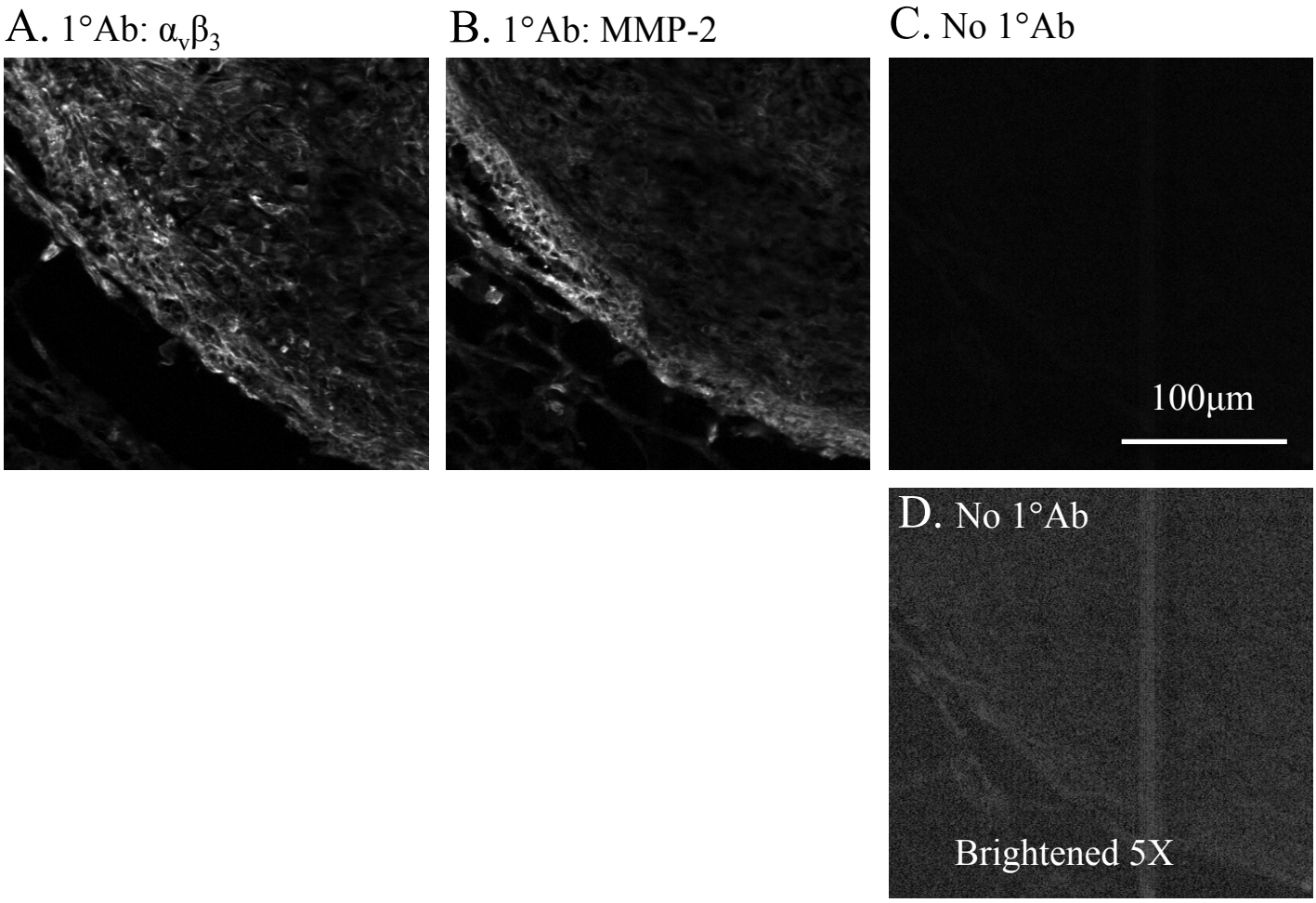
Supplemental Figure 4: A) Mice with MDA-MB-231 tumors were injected with 10 nanomoles of either cyclic-RGD-PLGC(Me)AG-ACPP, cyclic-RGD-PLGC(Me)AG-ACPP with 50 molar equivalents (500 nanomoles) of unlabeled cyclic(RGDfK), or cyclic-RAD-PLGC(Me)AG-ACPP. Six hours after injection, mice were imaged to evaluate tumor targeting. B) Skin was resected to expose the tumor and surrounding tissue. C) Standardized uptake value for the tumor of the various peptides tested, n=5 for each group.

Supplemental Figure 5: Dual targeting of PLGC(Me)AG-ACPP with folate does not improve tumor uptake in SK-OV-3 tumors



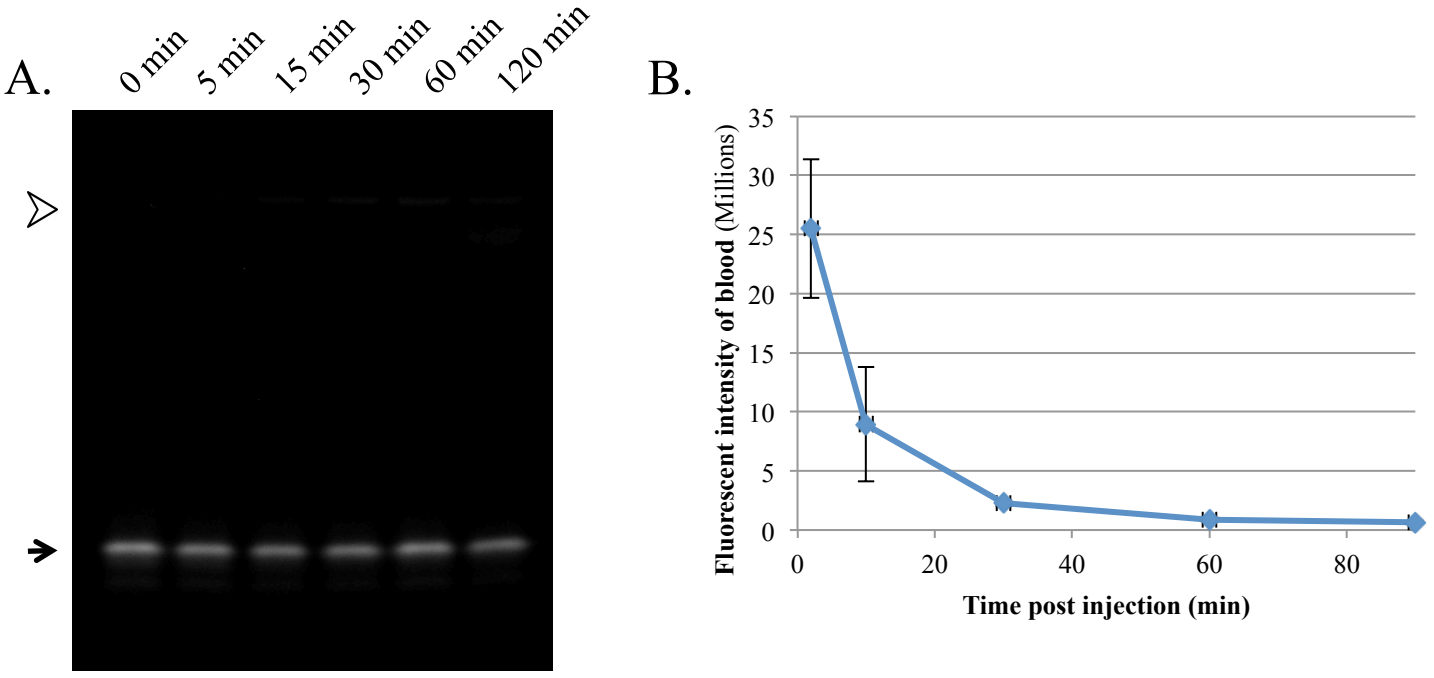
Supplemental Figure 5: A) Athymic nu/nu mice were fed on a low folate diet for three weeks prior to injection of SKOV3 cells that had also been cultured in low folate media for one month prior to tumor cell injection. Mice remained on the low folate diet for the duration of the experiment. Tumors were allowed to grow to approximately 5 mm in diameter prior to animal injection with 10 nanomoles of either PLGC(Me)AG-ACPP or folate-PLGC(Me)AG-ACPP (folate-peg$_4$-e$_9$-o-PLGC(Me)AG-r$_9$-c(Cy5)). Tumor contrast was present in anesthetized mice six hours post peptide injection (top panel). Post mortem, skin was removed to reveal tumors and surrounding tissue (bottom panel). B) Tissues were harvested from the mice and homogenized to calculate the tissue SUVs. Values are normalized the the tumor SUV of the PLGC(ME)AG-ACPP

Supplemental Figure 6: No primary antibody control for IHC



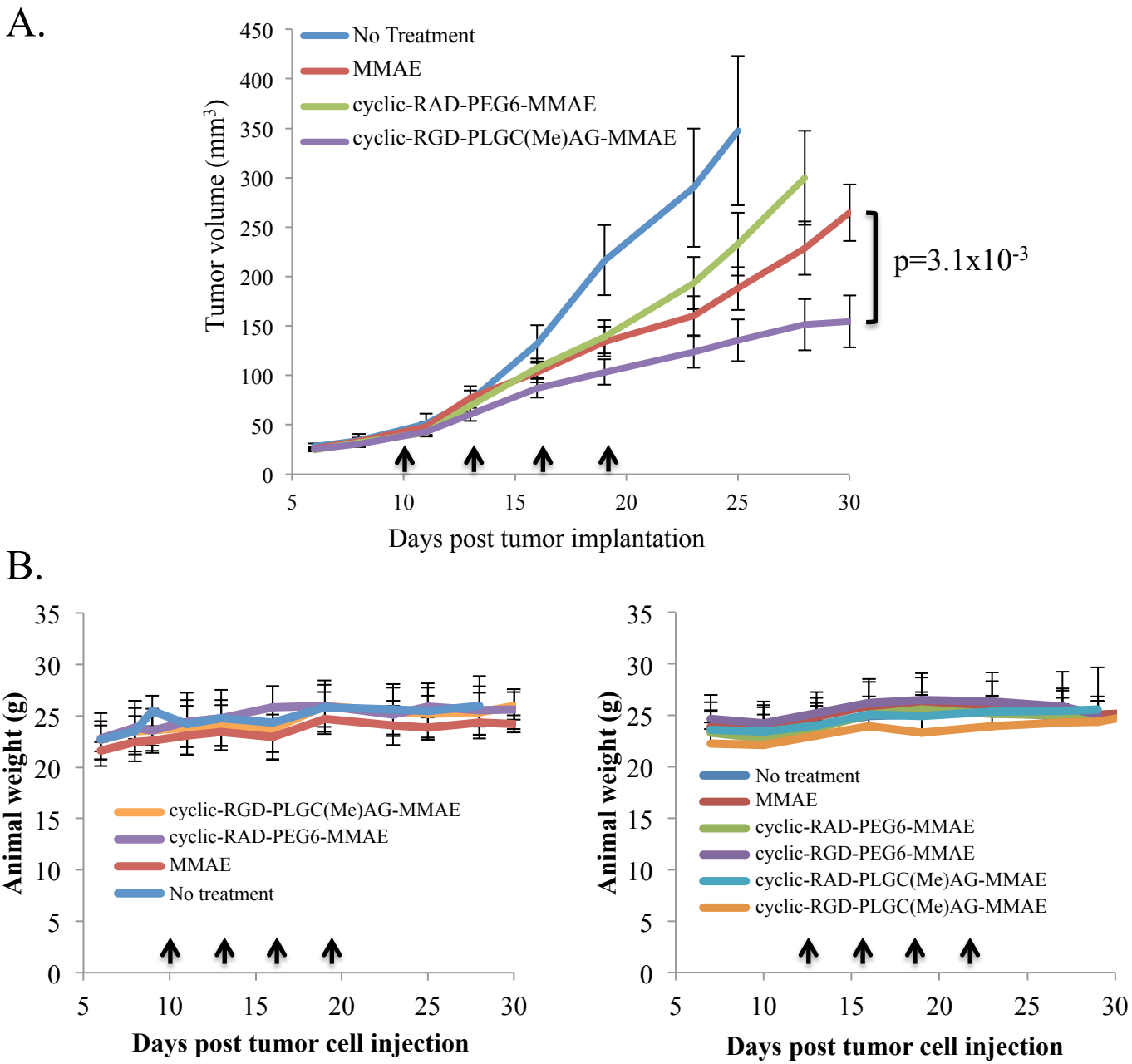
Supplemental Figure 6: A-C) Immunohistochemistry was performed on serial sections as described in materials and methods. Briefly, slides were incubated with either primary antibodies against $\alpha_v\beta_3$ (A) MMP-2 (B) or no antibody (C) prior to being treated with a FAM-labeled secondary and visualized with confocal microscopy. D) Image in C brightened 5X to show tissue margins.

Supplemental Figure 7: Plasma stability and blood clearance of cyclic-RGD-PLGC(Me)AG-ACPP



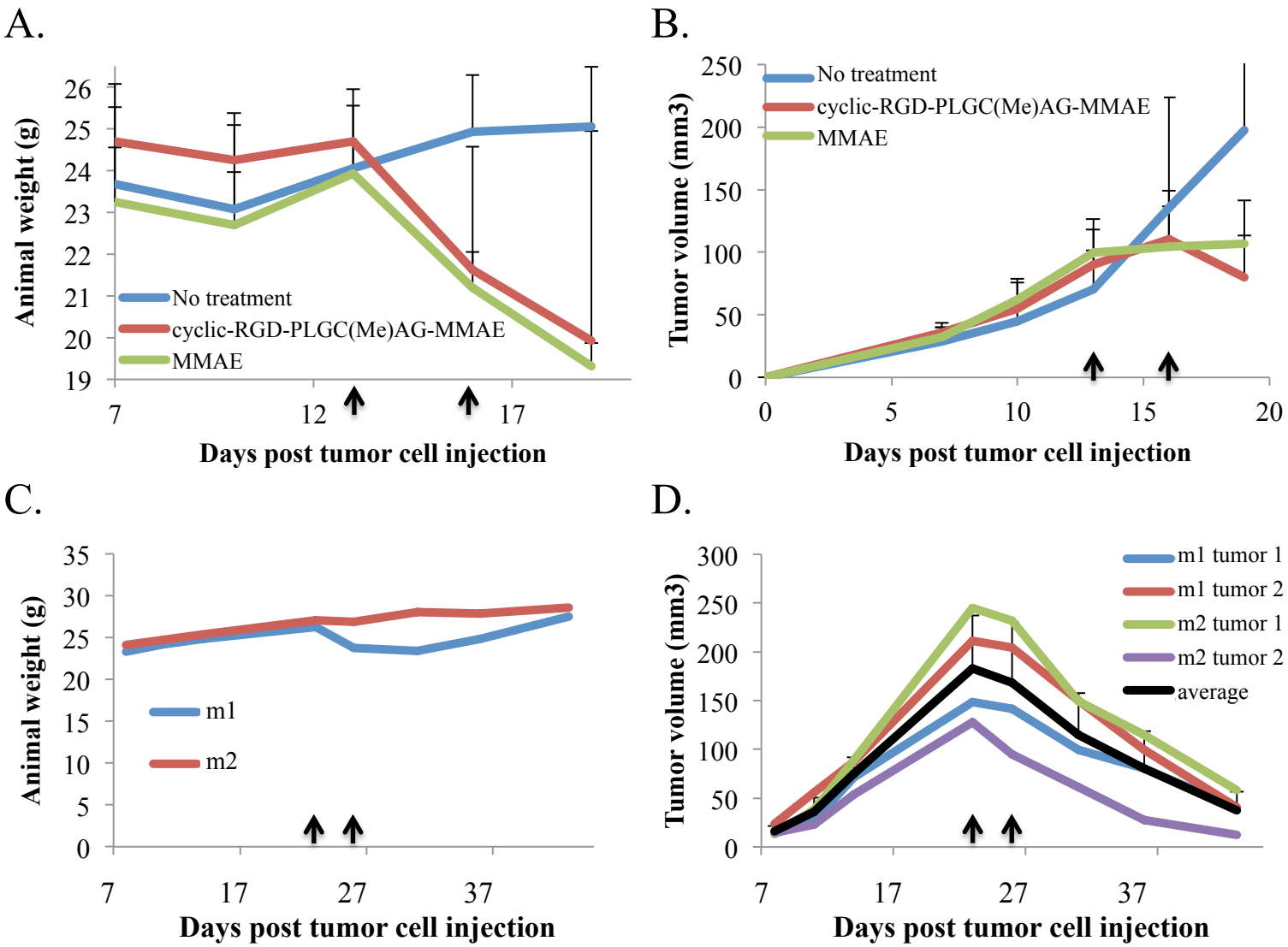
Supplemental Figure 7: A) cyclic-RGD-PLGC(Me)AG ACPP (5μM) was incubated in fresh mouse plasma at 37°C and aliquots were taken at the time points indicated. Samples were run on 10-20% tricine gels and then imaged on the Maestro imager for Cy5 fluorescence. The black arrow denotes the location of the intact peptide. B) Mice (n=3) were injected with 10nmole of cyclic-RGD-PLGC(Me)AG-ACPP and at various timepoints after injection the tail was pricked and 5-10μL of blood was collected in heparinized capillary tubes. These tubes were imaged on the Maestro imager using an excitation filter of 620/22nm and an emission filter of 670/20nm. Image J was used to measure the integrated fluorescent intensity of the blood, and those values were plotted as a function of time post injection.

Supplemental Figure 8: Tumor growth curve corresponding to Figure 5B and animal body weights corresponding to Figure 5 B&C



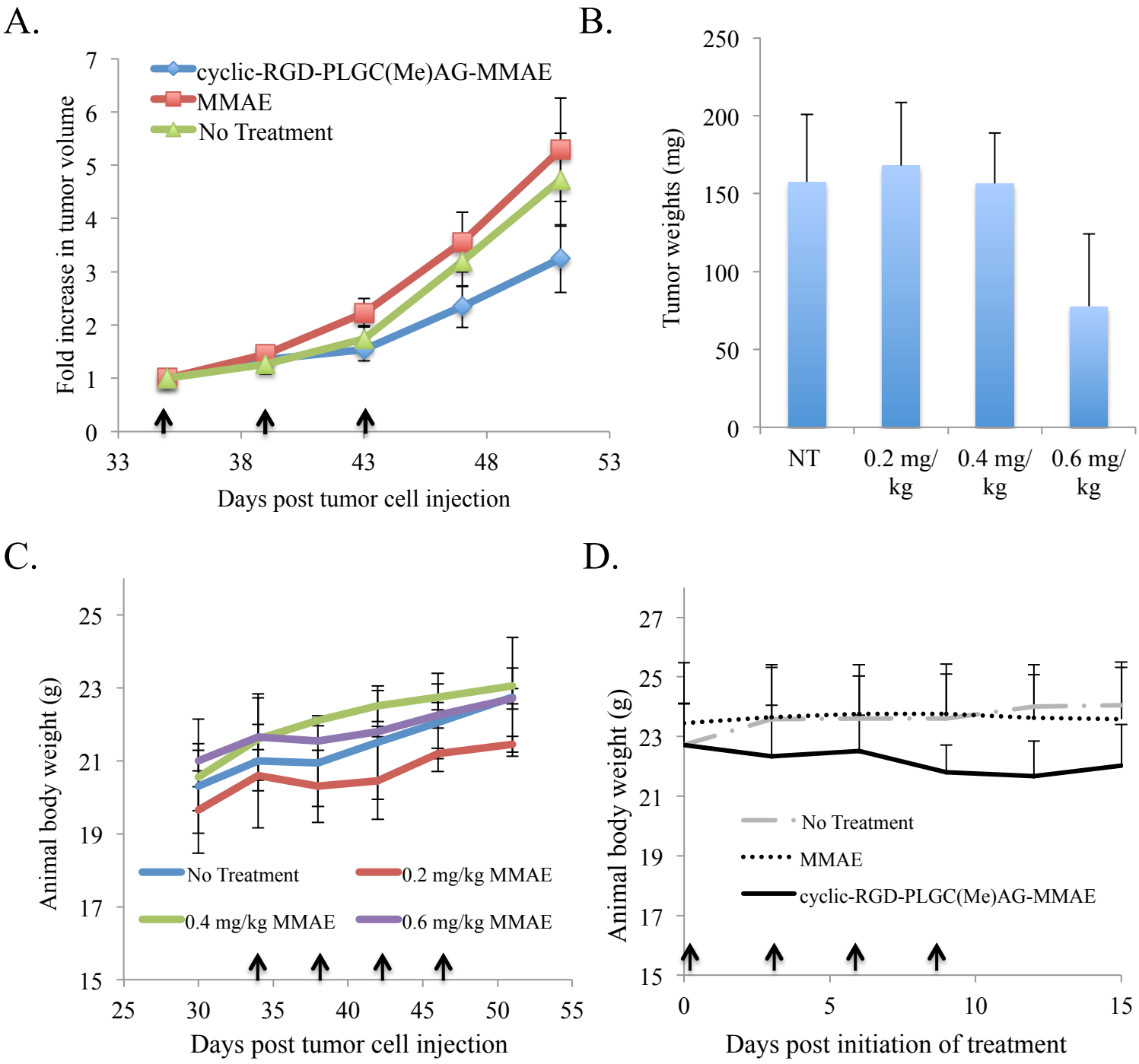
Supplemental Figure 8: A) Tumor growth curves corresponding to the experiment presented in Figure 5 B. Animals with MDA-MB-231 orthotopic mammary tumors in both the left and right thoracic fat pads (~50mm^3) were divided into treatments groups (n=7) and received no treatment, MMAE alone, cyclic-RGD-PLGC(Me)AG-MMAE-ACPP or cyclic-RAD-PEG6-MMAE-ACPP. The MMAE dose for each therapeutic agent is equivalent to 0.2 mg/kg. Treatment was given as indicated by the black arrows and tumor size was determined using caliper measurements. P values were calculated using a students t-test in Excel. B) Animal body weights corresponding to the experiments presented in Figure 5 B and C. Left panel, n=7 per treatment group and for the right panel, n=4 per treatment group. Black arrows indicate when therapy was administered

Supplemental Figure 9: Animal body weight and tumor growth curves for therapy studies at 1.2mg/kg (~40nmol) and 0.6mg/kg (~20nmol)



Supplemental Figure 9: A) Animal body weight and B) tumor growth curves for MDA-MB-231tumor bearing mice that were injected with either MMAE or cyclic-RGD-PLGC(ME)AG-MMAE-ACPP at a dose of 1.2mg/kg MMAE (~40nmol of peptide conjugate). Black arrows indicate injection dates and n=4 mice per treatment group C) Animal body weight and D) tumor growth curves for MDA-MB-231tumor bearing mice (n=2 with bilateral tumors) that were dosed cyclic-RGD-PLGC(ME)AG-MMAE-ACPP at a concentration of 0.6mg/kg MMAE (~20nmol of peptide conjugate). Black arrows indicate injection dates.

Supplemental Figure 10: Targeting of MMAE in Py230 syngeneic tumor model



Supplemental Figure 10: A) Tumor growth curves for Py230 tumor bearing mice that were injected with either MMAE or cyclic-RGD-PLGC(ME)AG-MMAE-ACPP at a dose of 0.2 mg/kg. Black arrows indicate injection dates and n=4 mice per treatment group. B) Final Py230 tumor mass after 4 injections of MMAE (given every 4 days) at the doses indicated (n=2 mice, 2 tumors per mouse). Tumors were harvested 5 days post end of treatment C) Animal body weights corresponding to (B) D) Animal body weights (n=4) for mice given 0.5mg/kg MMAE or the molar equivalent of cyclic-RGD-PLGC(Me)AG-MMAE-ACPP as indicated by the black arrows. This panel corresponds to the experiment in Figure 5C of the main text.