CT Imaging of Enzymatic Activity in Cancer using Covalent Probes Reveal a Size-Dependent Pattern.

Darya Tsvirkun,[†] Yael Ben-Nun,[†] Emmanuelle Merquiol,[†] Ivan Zlotver,[†] Karen Meir,[±] Tommy Weiss-Sadan[†], Ilan Matok[†], Rachela Popovtzer[‡] and Galia Blum*[,†]

[†]The Institute for Drug Research, The School of Pharmacy, The Faculty of Medicine, Campus Ein Karem, The Hebrew University, Jerusalem 9112001, Israel. [±] Department of Pathology, Hadassah Medical Center, Jerusalem 9112001, Israel.

[‡]Faculty of Engineering & The Institute of Nanotechnology and Advanced Materials, Bar-Ilan University, Ramat Gan 52900, Israel.

S1

Chemical synthesis of GB111-NH_2:

Synthesis was followed the method described in Blum G. et al[18], *(Nature Chemical Biology* volume 3, 2007): Fmoc-Lys(Boc)-dimethyl benzoyl acyloxymethyl ketone (Fmoc-Lys(Boc) AOMK) (365.2 mg, 0.594 mmol), purchased from Fountainbridge Ltd, was de-protected by 25% trifluoroacetic acid (TFA)/dichloromethane (DCM) (v/v) during 30 minutes to receive the free amine, solvent was removed by co-evaporation with toluene in vacuo. 2-Chlorotrityl chloride resin (371.1 mg) was loaded by shaking of resin with the Fmoc-Lysine(NH_2)-AOMK (1 eq.) dissolved in anhydrous DCM and diisopropylethylamine (DIEA) (3.5 eq, 360 µl) for 1.5 hours. Methanol (1 ml/gr resin) was added to cap unreacted resin and the resin was shaken for 30 minutes followed by washing with DCM and DMF. The Fmoc protecting group was removed by two quick washes with 5% diethylamine (DEA)/DMF (v/v) followed by a 5 minute incubation with 5%DEA and then washed with DCM and DMF. Deprotection was verified by Kiser test. The peptide was elongated by addition of a solution of N -benzyloxycarbonyl-phenyalanine (Cbz-Phe), (3 eq., 226 mg), Hydroxybenzotriazole (HOBT; 3 eq., 101.3 mg) and benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate (PyBOP; 3 eq., 116 mg) in DMF for 2 hours. The resin was then washed with DCM and DMF and the final peptide product was cleaved from the resin by addition of 7% TFA/DCM (v/v) for 7 to 15 minutes. The cleaved solution was collected and solvent was removed by co-evaporation with toluene. Crude GB111-NH_2 was further dried in vacuo and purified by HPLC, compound was eluted with 44% Acetonitrile (ACN) to yield a white powder, 78.94 mg, 0.138 mmol, M/z+1 574, 56% yield relative to resin loading.

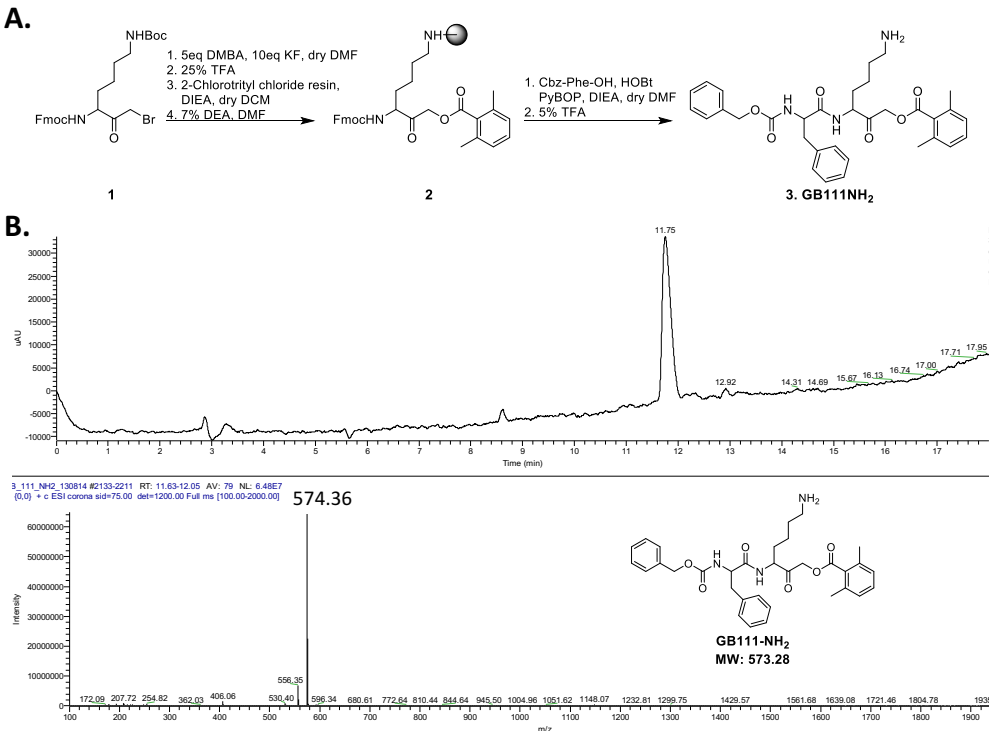


Figure S1. (A) Chemical synthesis and (B) characterization of GB111-NH_2 by LCMS- top chromatogram is UV trace at 254 nm of purified compound and bottom chromatogram shows the mass of the peak eluted at 11.75 minutes.

S2

Chemical synthesis of Acetylated-Aspartic acid-Tryptophan-Lysine-amide (Ac-DWK-amide):

Ac-DWK-amide was prepared using a Rink resin (Advanced Chemtech) using standard solid phase peptide synthesis methods. The resin was loaded by shaking with Fmoc-Lys(Boc)-OH (3 eq, 567 mg, 1.21 mmol), Hydroxybenzotriazole (HOBT; 3 eq; 163 mg) and benzotriazol-1-yl-oxytripyrrolidino phosphonium hexafluorophosphate (PyBOP; 3 eq; 629 mg) dissolved in anhydrous DMF and diisopropylethylamine (DIEA; 4 eq; 285 µl) for two hours. The resin was then washed with DCM and DMF. The Fmoc protecting group was removed by incubation with 20% piperidine/DMF (v/v) for 20 min followed by DCM and DMF washes. The peptide was elongated by addition of a solution of Fmoc-Tryptophan (Boc)-OH (3 eq; 620 mg), HOBT (3eq; 159 mg) and PyBOP (3 eq; 613 mg) in anhydrous DMF and DIEA (4 eq; 278 µl) for two hours. The resin was washed with DCM and DMF. After additional Fmoc de-protection by incubation with 20% piperidine/DMF (v/v) the last amino acid was conjugated. A solution of Fmoc-Aspartic acid (OtBu)-OH (3 eq; 402 mg), HOBT (3eq; 132 mg) and PyBOP (3 eq; 508 mg) in anhydrous DMF and DIEA (4 eq; 168 µl) was added for five hours and then washed with DCM and DMF. The Fmoc was removed with 20% piperidine/DMF (v/v) and free amine was acetylated by adding a solution of acetyl chloride (AcCl; 6eq; 131 µl), DIEA (3 eq; 163 µl) in anhydrous DCM for 1 hour. The final product Ac-DWK-amide was cleaved from resin by addition of 95% TFA, 4% water and 1% triisopropylsilane (TIS) for 2 hours. The crude peptide was further dried in vacuo and purified by HPLC. Ac-DWK-amide eluted at 45% ACN to yield a white powder, 143 mg, 0.294 mmol, M/z+1 489, 73% yield relative to resin loading.

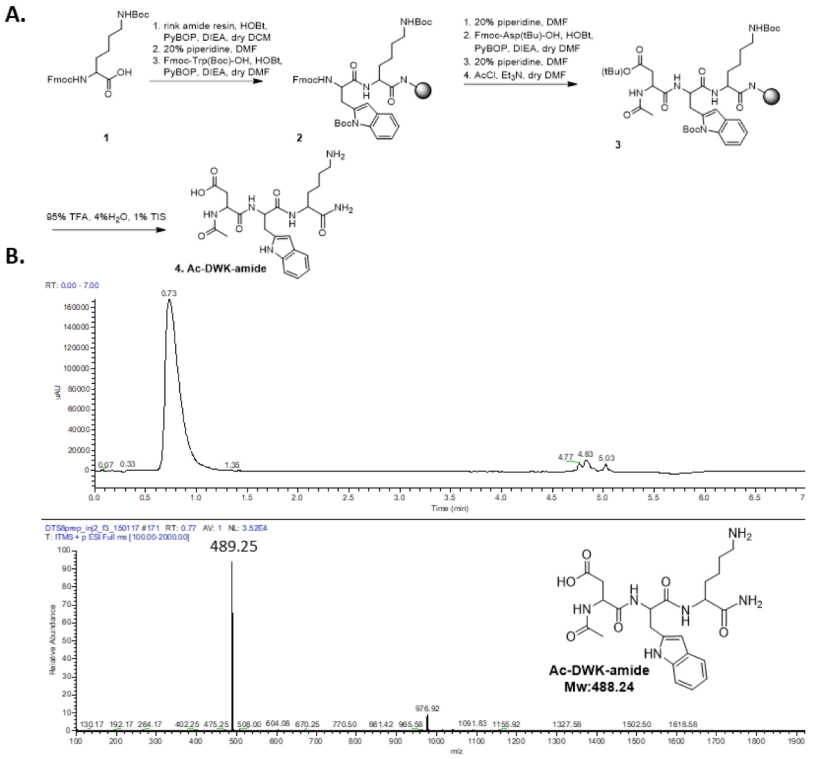


Figure S2. (A) Chemical synthesis and (B) characterization of Ac-DWK-amid by LCMS- top chromatogram is UV trace at 254 nm of purified compound, bottom mass chromatogram the peak (at 0.73 minutes).

S3

A.

Tag	Concentration [mg/ml]	Attenuation [HU]	Beam E [keV]
GNP	0.05	13 ± 3	35keV
GNP	0.1	16 ± 8	85keV
Gadolinium	0.05	17 ± 3	35keV
Gadolinium	0.1	38 ± 8	85keV
Iodine	0.05	10 ± 3	35keV
Iodine	0.5	15 ± 8	85keV

B.

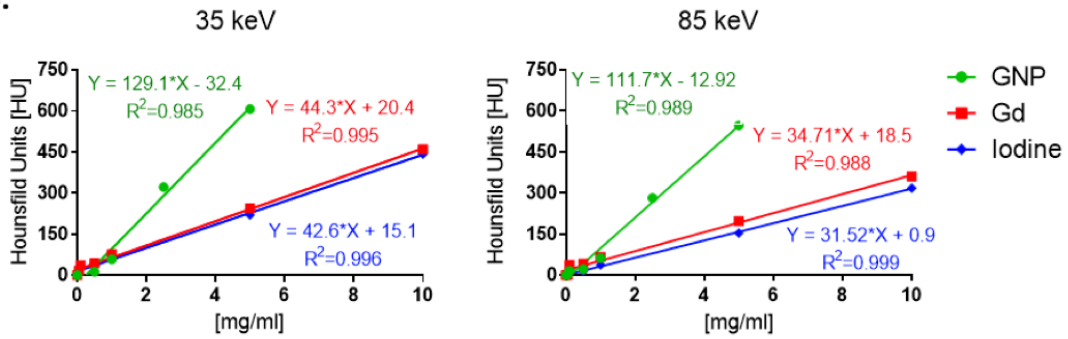
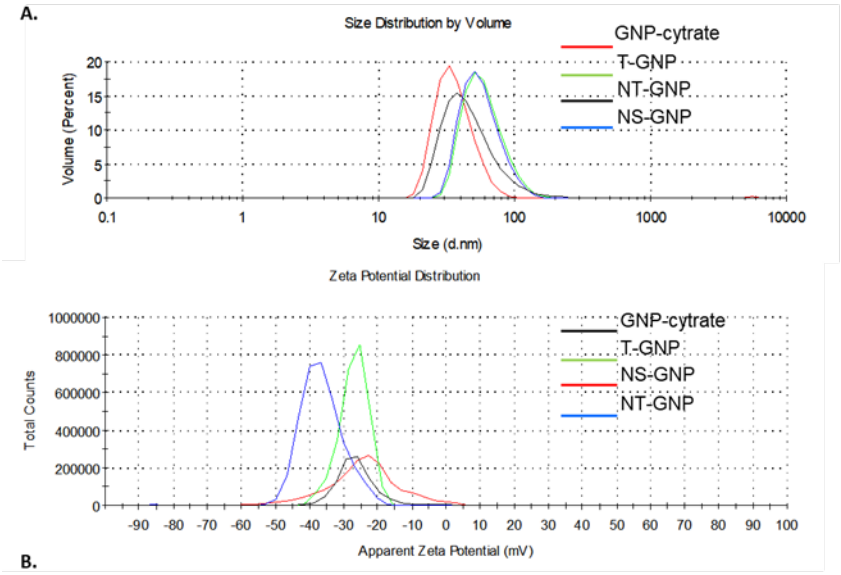


Figure S3. Detection level of various contrast agents by micro-CT. Dilutions of elements were scanned by a dual beam micro CT at 35 keV and 85 keV. (**A**) The minimal detectable concentration of GNP, gadolinium and iodine at both energies is presented. (**B**) Curves of concentration vs HU are plotted showing a linear correlation obtained between X-ray attenuation and tag concentration, slopes reveal three-time higher attenuation efficiency of GNP relative to other contrast agents in both energies.

S4



Size [d,nm]										
DTS-vi	Day 0		Day 4		Day 15		Day 32		Day 69	
	± SD	PdI	± SD	PdI	± SD	PdI	± SD	PdI	± SD	PdI
10 nm	33.4± 0.4	0.16	30.4 ± 0.8	0.36	35.2 ± 0.9	0.23	32.7 ± 0.1	0.38	34.8 ± 0.2	0.41
30 nm	57.7 ± 0.2	0.16	58.8 ± 1.4	0.18	63.1 ± 1.6	0.23	66.6 ± 0.7	0.17	64.7 ± 1.4	0.25
100 nm	137.4 ± 0.1	0.07	135.6 ± 1.6	0.20	137.5 ± 1.5	0.06	134.3 ± 0.8	0.13	136.2 ± 1.0	0.11
Zeta [mV± SD]										
	Day 0		Day 4		Day 15		Day 32		Day 69	
10 nm	-24.8 ± 1.2		-23.1 ± 1.8		-21.6 ± 0.4		-26.3 ± 1.6		-24.7 ± 2.3	
30 nm	-26.2 ± 0.9		-28.8 ± 1.2		-30.1 ± 0.4		-27.1 ± 1.4		-25.8 ± 2.5	
100 nm	-24.3 ± 0.6		-22.6 ± 0.3		-21.9 ± 0.6		-26.5 ± 0.4		-24.4 ± 1.0	

Figure S4. Characterization of GNP-ABPs. (A) Representative measurement of 30 nm GNP derivatives, top: the hydrodynamic diameter (HD) measured by dynamic light scattering (DLS) revealing increase in the HD after PEGylation and targeting/control moiety coupling. Bottom: Zeta-potential measurements of the same derivatives indicating minimal change of the nanoparticle surface. **(B)** T-GNPs (DTS-iv) were kept at 4 ºC in 10% DMF:DDW solution through ten weeks. Measurements of HD (top) and Zeta potential (bottom) over time are presented. Particles were found stable.

S5

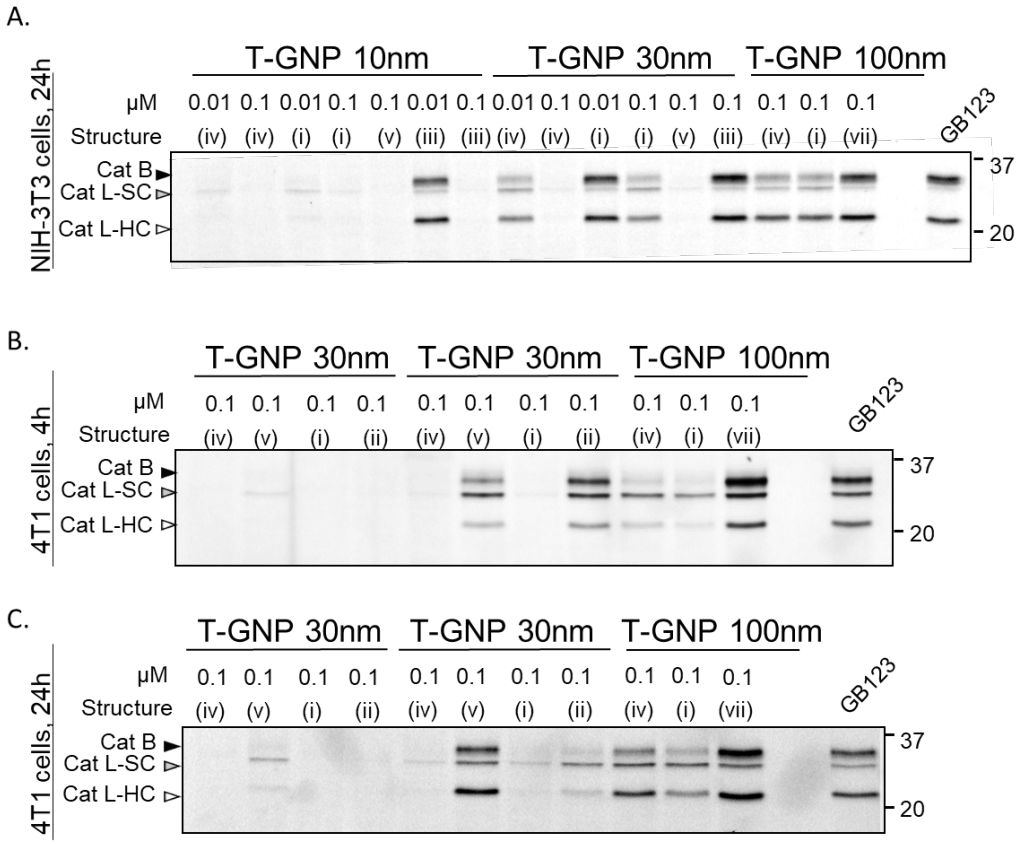
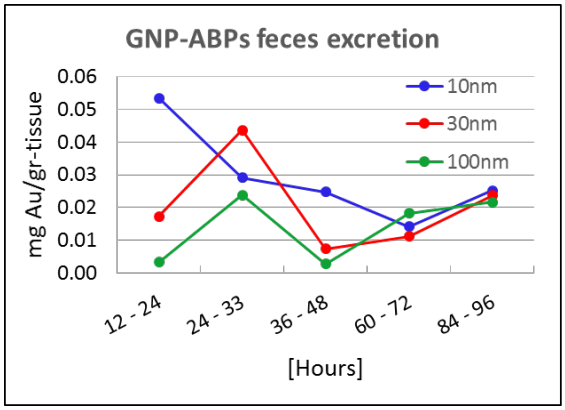


Figure S5. Biochemical evaluation of GNP-ABPs. Inhibition of endogenous cathepsin activity in (**A**) NIH-3T3 mouse fibroblast cells, treated with indicated concentrations of inhibitor moiety (GB111) attached to GNPs for 24 h, samples were analyzed as in Fig 2C. (**B, C**) 4T1 murine mammary carcinoma cells treated with indicated concentrations of inhibitor moiety (GB111) attached to GNPs for 4 h and 24 h respectively. Cells were treated with indicated probes for 4 or 24 hours in growth medium followed by labeling of residual enzyme activity by GB123 for 2 hours. After protein determination, residual labeled proteases in cells were visualized by scanning the gel with a Typhoon scanner FLA 9500, excitation/emission wavelengths of 635/670 nm.

S6



T-GNP Total Excretion

	ID% / gr-tissue
Probe	**exp2**
10 nm	3.3 %
30 nm	2.3 %
100 nm	1.5 %

Figure S6. Targeted GNPs excretion is size depended *in-vivo.* Preliminary data, feces and urine of animals injected with indicated GNPs were collected for indicated hours post injection. Gold secreted in feces was measured by FAAS showing a size depended pattern. No T-GNP were detected in the urine.

S7

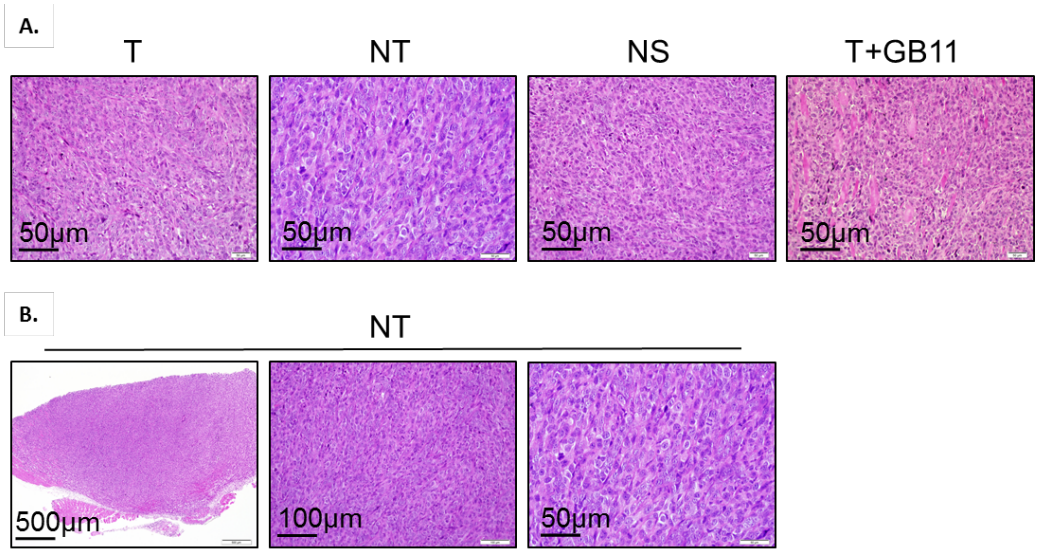


Figure S7. Pathological sections of tumor tissue. Tumors from animals described in Figure 6 were resected at the end of the experiment, part of the tissue was used for Flame atomic absorption spectroscopy and part was taken for pathological analysis as described in the methods. (**A**) Representative H/E staining of tumor sections taken from tumor bearing mice 72 h post IV injection of 30 nm Targeted (T), Non-Targeted (NT), Non-Specific (NS) and T mice that were pretreated with GB111-NH_2 inhibitor (T+GB111)**.** (**B**) Representative enlarged images of tumor taken from a mouse treated with NT-GNPs.
All tumors were found to demonstrate similar histological features without necrotic tissue except for one tumor (from the non-targeted group).

S8

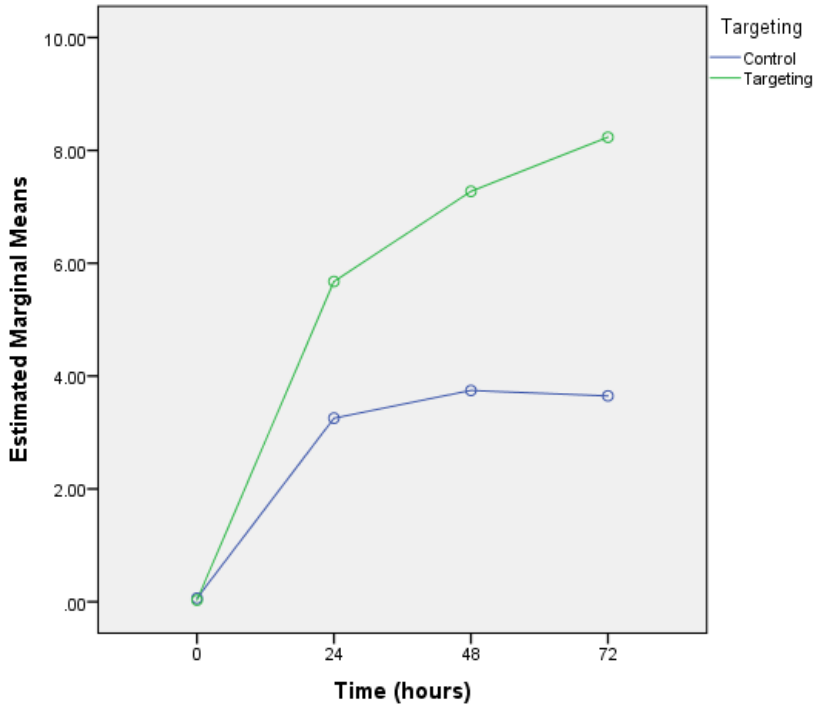


Figure S8. Estimated Marginal Means of Measures. Repeated measures ANOVA was performed on data presented in Figure 6D by comparing the targeted group and a combined group that consist of all other groups (NS, NT and T + Inhibitor). The estimated Marginal Means of the CT attenuation is shown, targeted particles shown in green and the combined controls in blue.

For Time: In within subjects, since P value in Mauchly's Test of Sphericity was 0.043, we used the results from Greenhouse-Geisser test. For time, the Greenhouse-Geisser $P < 0.001$, and for the interaction between Time and Targeting the $P=0.002$. The between subject effects ($P = 0.004$), Post-hoc analysis (alfa = 0.0083). For the time analysis, between 0-24 h, 0-48 h, 0-72 h there was a significant difference with P value < 0.001. Between 24-48 h there was a trend with $p=0.076$ and between 24-72h there was a trend as well with a $P = 0.033$. No significance was found between 48-72 h ($P = 1$).