Title: Photodynamic therapy agent with a built-in apoptosis sensor for evaluating its own therapeutic outcome *in situ*

Authors: Klara Stefflova, Juan Chen, Diane Marotta, Hui Li & Gang Zheng

Table of contents:

a) Supporting information, Figure 1-purity, UV-Vis, and MALDI-ToF of PP(**3**)

b) Supporting information, Figure 2-purity, UV-Vis, and MALDI-ToF of PPB(**4**)

c) Supporting information, Figure 3-HPLC of scrambled sequence Pyro-GPLGLARK-(BHQ-3) (sPPB) incubated with caspase-3 for 180 minutes

d) Supporting information, Figure 4-accumulation of PPB (**4**) inside KB cells monitored by fluorescence microscopy

e) Supporting information, Figure 5- $HepG_2$ cells incubated with scrambled sequence Pyro-GHSSK(BHQ-3)LQL and Pyro-GDEVDGSGK-(BHQ-3) (**4**) and activated by light monitored by confocal microscopy

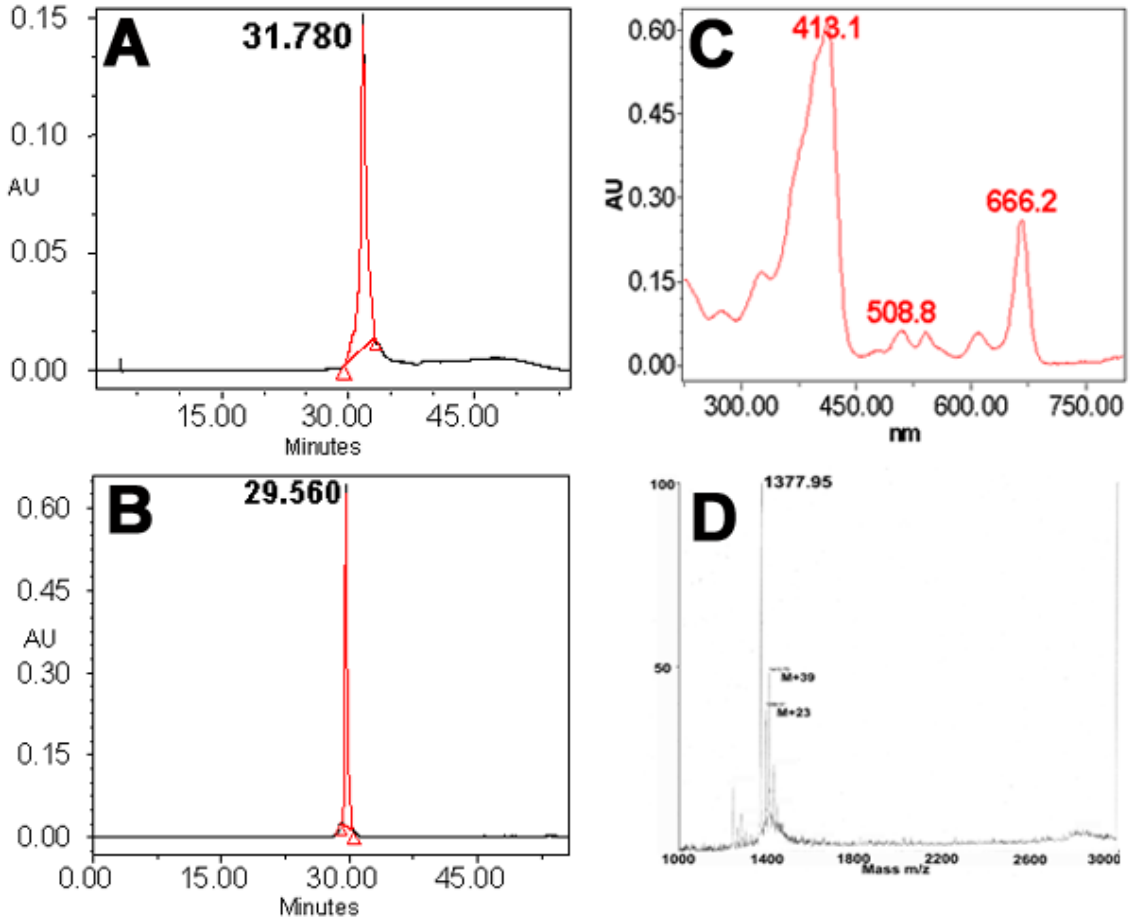
f) ANOVA test for MTT study

g) Supporting information, Figure 6-preliminary in vivo experiment

h) Synthesis of Fmoc-GD(Boc)E(Boc)VD(Boc)GS(Boc)GK(Mtt)-Sieber (1)

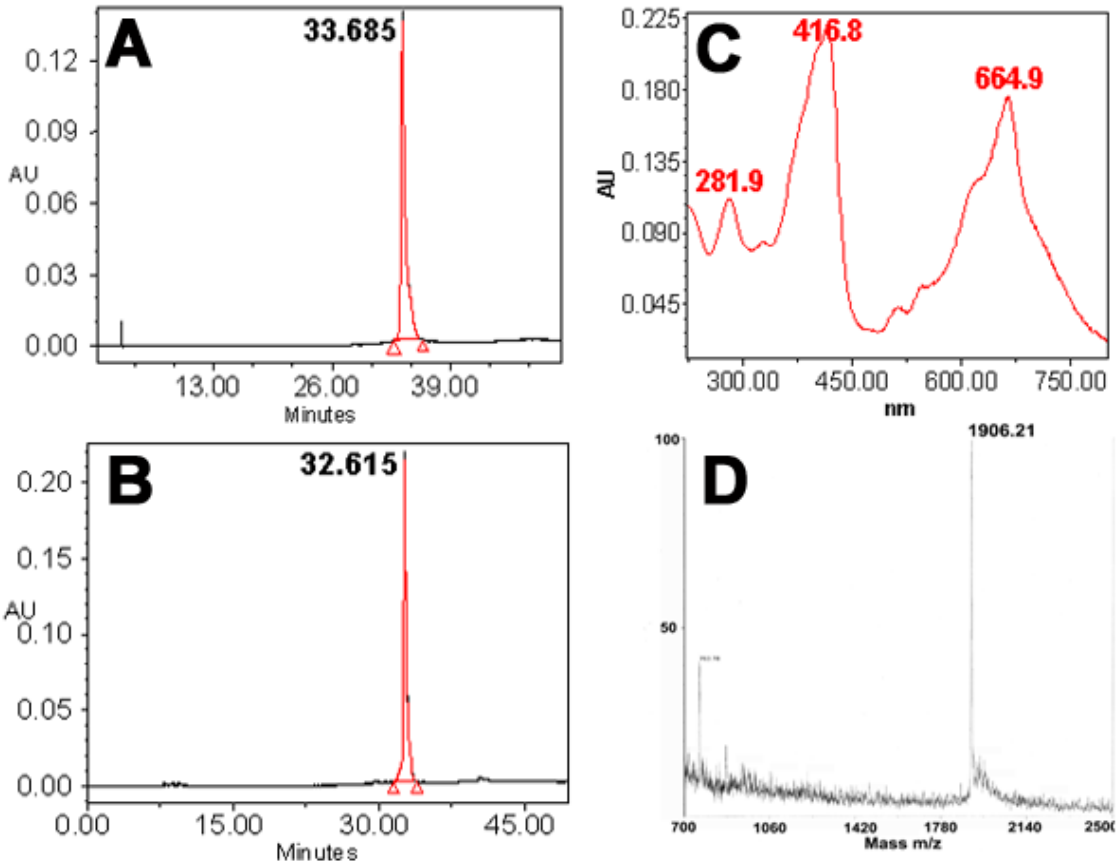
Supporting information:

a)



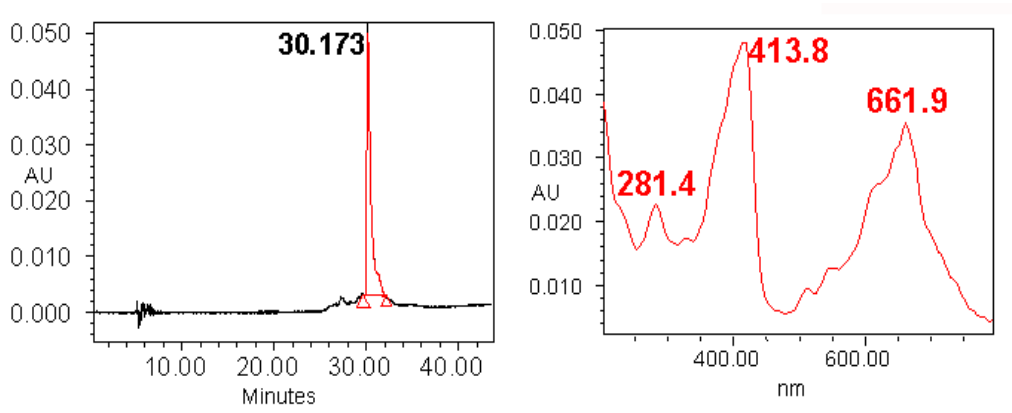
Supporting information, Figure 1. (**A+B**) HPLC of purified Pyro-GDEVDGSGK (**PP, 3**) at 410 nm, using methods: **A**) 100% of 0.1%TFA to 100% of acetonitrile (ACN) in 50 min, flow: 1mL/min, RT=31.780min, and **B**) 90% of TEAA and 10% of ACN to 100% of ACN in 45 min, flow: 1.5mL/min, RT=29.560min. **C**) UV-Vis of corresponding peak, **D**) MALDI-ToF of Pyro-GDEVDGSGK (mass calculated: 1377.64, found: 1377.95).

b)



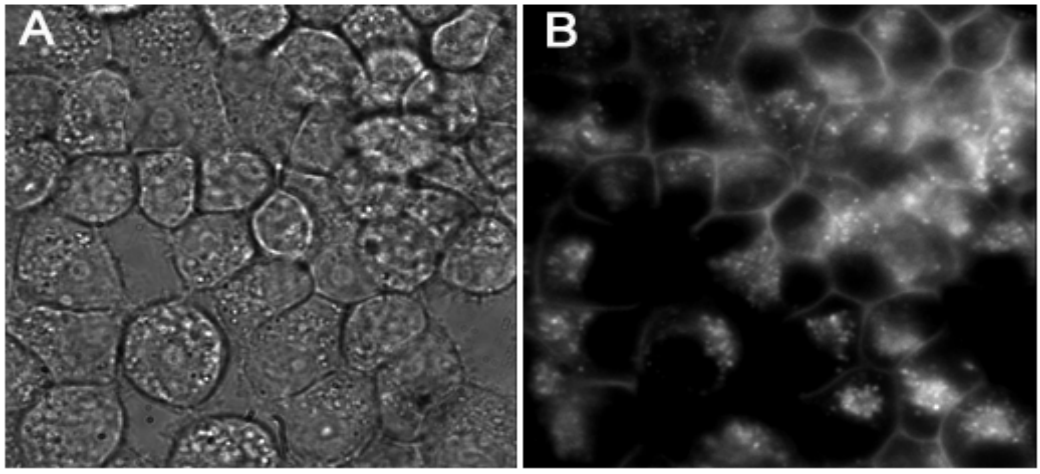
Supporting information, Figure 2. (**A+B**) HPLC of purified Pyro-GDEVDGSGK-(BHQ-3) (**PPB, 4**) at 410 nm, using methods: **A**) 100% of 0.1%TFA to 100% of ACN in 50 min, flow: 1mL/min, RT=33.685min, and **B**) 90% of TEAA and 10% of ACN to 100% of ACN in 45 min, flow: 1.5mL/min, RT=32.615min. **C**) UV-Vis of corresponding peak, **D**) MALDI-ToF of Pyro-GDEVDGSGK-(BHQ-3) (mass calculated: 1906.91, found: 1906.21).

c)



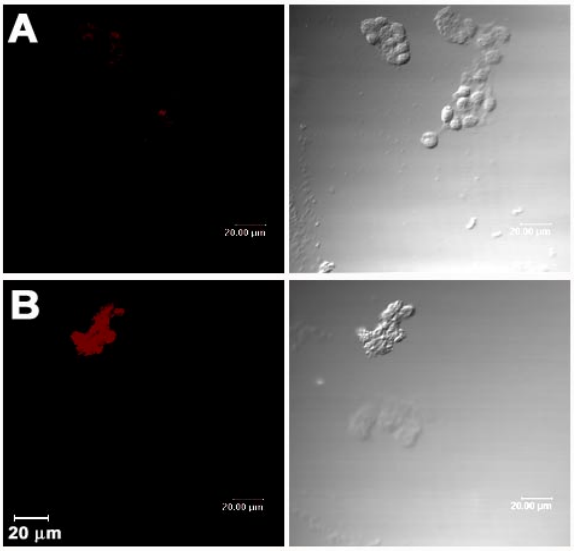
Supporting information, Figure 3. HPLC (left, method 90% of 0.1%TFA and 10% of ACN to 100% of ACN in 45 min, flow: 1 mL/min, 410nm) and UV-Vis spectrum (right, spectrum of the 30.173min peak) of Pyro-GPLGLARK-(BHQ-3) incubated with caspase-3 (ratio 200 : 1) for 180 minutes. There is only one peak RT=30.173min corresponding to the uncleaved molecule.

d)



Supporting information, Figure 4. Accumulation of **PPB** (15μM, 2hrs) in living KB cells (human epidermoid carcinoma cells) monitored using an Olympus IX71 inverted fluorescence microscope (TRITC filter). **A**) DIC image and **B**) corresponding fluorescent image of **PPB** accumulation in cytoplasm.

e)



Supporting information, Figure 5. $HepG_2$ cells incubated with 50µM of: **A**) scrambled sequence Pyro-GHSSK(BHQ-3)LQL, or **B**) Pyro-GDEVDGSGK-(BHQ-3) (**4**) for 24 hours and then treated with 5 J/cm^2. There is a minimal fluorescence increase in the case of the scrambled sequence Pyro-GHSSK(BHQ-3)LQL, suggesting that it is not cleaved as a consequence of light-triggered events and that PDT-BIAS is indeed apoptosis specific.

f) ANOVA test for MTT study

Based on an ANOVA test, we found a significant difference ($P < 0.05$) between these pairs:

a) 1µM PPB – 0 J/cm^2 and 7.5 J/cm^2

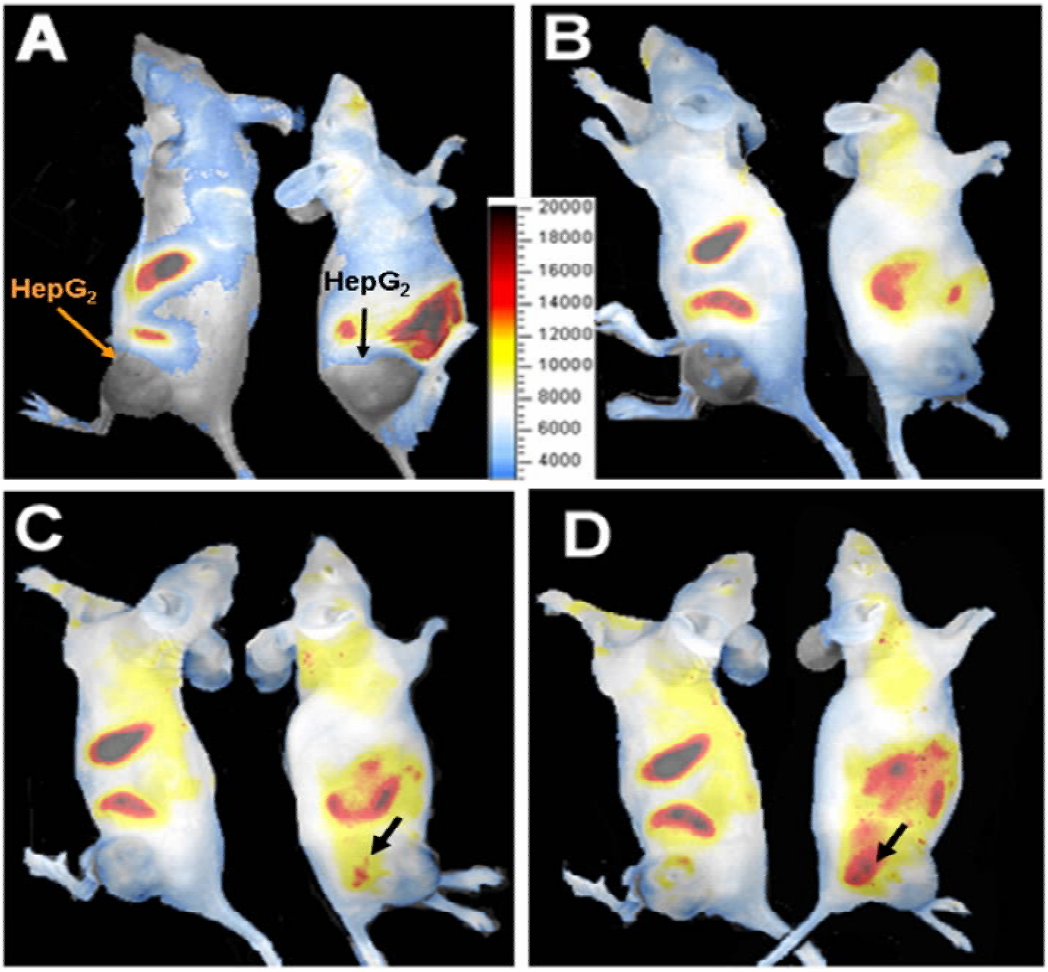
b) 5µM PPB – 0 J/cm^2 and 5 J/cm^2, 0 J/cm^2 and 7.5 J/cm^2

c) 15µM PPB – 0 J/cm^2 and 1 J/cm^2, 0 J/cm^2 and 5 J/cm^2, 0 J/cm^2 and 7.5 J/cm^2

d) 30µM PPB – 0 J/cm^2 and 1 J/cm^2, 0 J/cm^2 and 5 J/cm^2, 0 J/cm^2 and 7.5 J/cm^2

suggesting that with increasing drug dose we need a lower light dose to achieve significantly lower viability of cells treated with light compared to that of cells incubated with the same drug dose but kept in the dark.

g)



Supporting information, Figure 6. Monitoring the increase of fluorescence after intratumor injection of Pyro-GDEVDGSGK-(BHQ-3) (**PPB**, 80nmol) (both mice) and PDT (150 J/cm^2, the right mouse). Xenogen images (composite of photograph and fluorescence image) of two mice each bearing a $HepG_2$ tumor: A) before the **PPB** injection (the $HepG_2$ tumors are very dark and have no fluorescence, therefore they appear gray due to the photograph portion of the image and not the fluorescence overlay), B) 2 hours after PPB injection and 1.5 hours after PDT (only the mouse on the right), C) 3 hours after injection and 2.5 hours after PDT (the right mouse), and D) 4 hours after injection and 3.5 hours after PDT (the right mouse). There is a visible increase of fluorescence in the tumor of the PDT and PPB treated mouse (left side of the tumor marked by an arrow) compared to the tumor that was injected by PPB but not treated by

light (the left mouse). At 4 hours after injection there is a slightly increased signal in the tumor of the mouse treated by PPB only (probably attributed to the PPB *in vivo* decomposition), but it is comparably lower than in the PDT-treated tumor.

Preliminary *in vivo* data. Nude mice were subcutaneously inoculated on the upper side of the leg with 10^7 HepG_2 cells resuspended in 100μL of HBSS and the tumor was grown for about 20 days. Mice were anesthetized and scanned using a Xenogen IVIS imager with Cy 5.5 filter (λ_{exc}=615-665 nm, λ_{em}=695-770 nm) before the injection (A). 80nmol of **PPB** in 100μl was injected into the tumor of each mouse. The right mouse received PDT treatment (150 J/cm^2) for the whole tumor area immediately following the injection. Both mice were scanned by Xenogen 2 hours after the injection (B, 1.5 hours after PDT), 3 hours after injection (C, 2.5 hours after PDT) and 4 hours after injection (D, 3.5 hours after PDT). Xenogen images were acquired at the Bioluminescence Molecular Imaging Core facility at UPENN on an IVIS Xenogen Imager.

While these data support the function of PDT-BIAS *in vivo*, concerns regarding the unsuitability of dark HepG_2 tumor for fluorescence imaging and the lack of specific tumor targeting should be considered for future *in vivo* studies. Nevertheless, this preliminary study is encouraging, suggesting that there is an increase in Pyro fluorescence in the area of PDT treatment as a consequence of *in vivo* cleavage of our caspase-3-specific peptide linker.

h) Fmoc-GD(Boc)E(Boc)VD(Boc)GS(Boc)GK(Mtt)-Sieber resin (1). The peptide was synthesized by manual SPPS using commercially available Fmoc amine protected amino acids as building blocks and Sieber resin (cleavable by 1 % TFA) as a solid phase,

all purchased from Novabiochem. The peptide synthesis cycle consists of three main steps separated by washing cycles (NMP/DCM 1:1):

1. Fmoc cleavage by 20 % piperidine/*N*,*N*-Dimethylformamide (DMF) 3 x 5 mL x 5 min. followed by wash with 10% *N*,*N*-Diisopropylethylamine (DIPEA) in DCM/NMP (1:1)

2. Amino acid coupling in the presence of activators HOBt and HBTU. This cycle has two steps: 1) the amino acid/HOBt/HBTU ratio to the resin is 3/3/3:1 and the coupling time is 15 min in dry NMP, 2) the ratio is decreased to 3/3/3:2 and the coupling time decreases to 10 min.

3. Capping by 5 mL of 0.3M Acetylimidazole/NMP once for 10 min.

These steps are periodically repeated with the exception of the first amino acid (Lys) coupling that uses a 1:2/2/2 ratio of Lys/HOBt/HBTU to resin Fmoc-loading (only 50% of the resin's capacity is used to ensure the coupling efficiency will be constant even at the end of the peptide synthesis). The capping step (#3) after Lys coupling is also more extensive (3 x 5 mL x 15 min). After every step, a small sample was transferred out, cleaved by 50% TFA, and HPLC and ESI-MS were run for purity control. This sequence, containing Asp followed by Gly should not be kept in very basic conditions even for short periods of time (Fmoc cleavage for 15 minutes using 1,5-Diazabicyclo[5.4.0]-5-undecene (DBU)) or in very acidic conditions (90% TFA) for a longer time period (>5 hours), otherwise the caspase-3 cleavage site is inactivated.