J|A|C|S COMMUNICATIONS

Published on Web 08/28/2004

# Protease-Triggered Photosensitizing Beacon Based on Singlet Oxygen Quenching and Activation

Juan Chen,† Klara Stefflova,‡ Mark J. Niedre,§ Brian C. Wilson,§ Britton Chance,‖ Jerry D. Glickson,† and Gang Zheng*,†

*Departments of Radiology, Chemistry, Biochemistry, and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Medical Physics, Ontario Cancer Institute/University of Toronto, Toronto, Ontario MSG 2M9, Canada*

Received May 4, 2004; E-mail: Gang.Zheng@uphs.upenn.edu

In type-II photosensitization the singlet oxygen, $^1O_2$, is generated by energy transfer from the triplet state of a photosensitizer molecule (PS), following intersystem crossing of the PS from its photoexcited singlet to triplet state.[1] $^1O_2$ is widely believed to be the major cytotoxic agent involved in photodynamic therapy.[1]

In 1990, Matayoshi et al.[2] first used fluorescence resonance energy transfer (FRET) to design activated probes for imaging retroviral proteases. FRET-based imaging probes ("molecular beacons") have since been used in many applications,[3,4] including protease-activated near-infrared (NIR) fluorescent probes developed by Weissleder and colleagues for cancer imaging.[5] These yield high tumor-to-background ratios, since they are nonfluorescent in the native state.

Here we combine these two concepts, i.e., type-II photosensitization and molecular beacons, to design a photosensitizing beacon (PS-beacon) comprising a disease-specific linker, a PS, and a $^1O_2$ quencher/scavenger, such that there is no photosensitization until the linker interacts with a specific target molecule, such as a tumor-specific enzyme.

As proof-of-principle of this approach, we designed a protease-triggered PS-beacon, depicted in Figure 1A. This molecule consists of a short peptide sequence specific to a protease that is over-expressed in cancer cells. A PS and a $^1O_2$ quencher/scavenger (Q) are conjugated to the opposite ends of this sequence. Proximity of PS and Q can quench $^1O_2$ generation through PS triplet-state energy transfer and/or can scavenge the $^1O_2$ that has been generated. We hypothesize that, in the presence of tumor-specific enzyme, the substrate sequence will be cleaved and PS and Q will separate so that the PS can be photoactivated.

To build the model construct, a cleavable caspase-3 substrate, GDEVDGSGK (recognition site underlined), was chosen as the peptide sequence, for which there is a well-established assay for the caspase-3-specific fluorogenic substrate.[6] Pyropheophorbide *a* (Pyro) was used as the PS. This is a chlorophyll analogue that has long-wavelength absorption at 667 nm and is an efficient $^1O_2$ producer with a quantum yield >50%.[7] A carotenoid (CAR) was selected as the Q moiety, since CARs are well known both to quench triplet excited states and also to scavenge singlet oxygen.[8] Hence, when the CAR moiety is held in close proximity to the PS by the short peptide sequence, it should efficiently decrease $^1O_2$ generation and lifetime.

To synthesize the Pyro-peptide-CAR (PPC) PS-beacon (Figure 1B), a GDEVDGSGK peptide was synthesized. Pyro was coupled to the N-terminal glycine of peptide on solid support. This conjugate was then cleaved from the support and deprotected. CAR conjugation was then carried out in solution, since it is acid-labile.

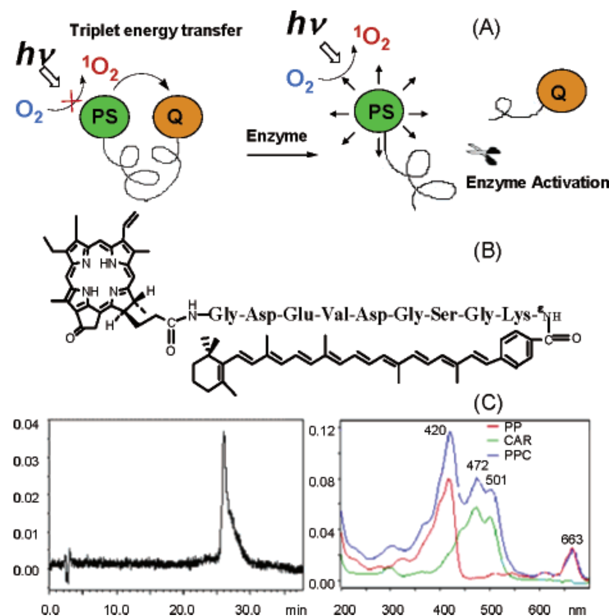


***Figure 1.*** (A) Concept of $^1O_2$ quenching/scavenging and activation. (B) Structure of caspase-3 activatable PPC beacon. (C) HPLC retention time[9] of PPC and the corresponding absorption spectra of PPC compared to Pyro-peptide (PP) and CAR.

Purification of this product was achieved by using two connected Diol and C18 Sep-pak columns and confirmed by HPLC (Figure 1C). The structure of PPC was confirmed by MALDI-TOF (calculated 1895.24, found 1896.03). The presence of all three structural components in PPC was further confirmed by UV−vis (Figure 1C), which shows characteristic peaks of the Pyro (420, 663 nm) and CAR (472, 501 nm) moieties.

The PPC was then tested for caspase-3 cleavage using HPLC. As shown in Figure 2A, addition of caspase-3 clearly induced cleavage, as demonstrated by the diminishing PPC peak at 26.7 min and the rise of Pyro and CAR residues at 12.4 and 20.7 min, respectively. The cleavage was proved to be caspase-3-specific by using a caspase-3-specific inhibitor (Ac-DEVD-CHO) that completely blocked the enzyme activity.[6] The stability of both Pyro and CAR upon caspase-3 cleavage was demonstrated by the fact that their absorption spectra remain unchanged (Figure 2B).

To test this concept, $^1O_2$ was measured directly in solutions of PPC alone, PPC incubated with caspase-3, and PPC incubated with caspase-3 plus a caspase-3 inhibitor. Pyro-peptide (PP) alone, without the CAR moiety, was used as a positive control. $^1O_2$ generation was quantified by measuring its NIR luminescence at 1270 nm, using an instrument that has been described previously.[10] Briefly, a 10 ns pulsed 523 nm laser (75 mW/cm$^2$) excites the solution and the luminescence spectrum is sampled, after rejection

† University of Pennsylvania Radiology.
‡ University of Pennsylvania Chemistry.
§ Ontario Cancer Institute/University of Toronto.
‖ University of Pennsylvania Biochemistry/Biophysics.



10.1021/ja047392k CCC: $27.50 © 2004 American Chemical Society

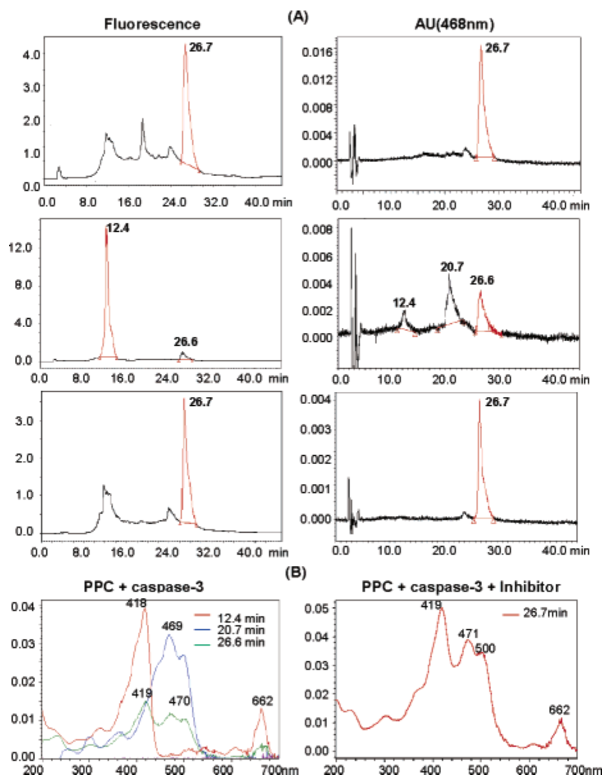


***Figure 2.*** (A) HPLC chromatograms monitoring caspase-3 cleavage by Pyro fluorescence (left) and CAR absorption (right): PPC alone (first row), PPC + caspase-3 (second row), and PPC + caspase-3 + inhibitor (third row). (B) Absorption spectra corresponding to the dominant HPLC peaks for PPC + caspase-3 (left) and PPC + caspase-3 + inhibitor (right).

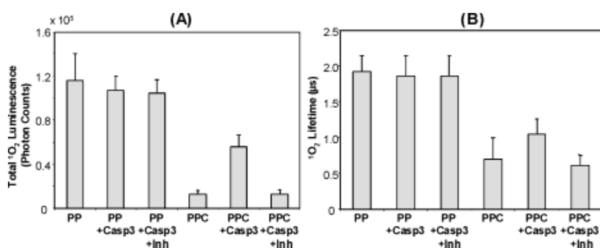


***Figure 3.*** (A) Total $^1O_2$ luminescence counts for PP, PP + caspase-3, PP + caspase-3 + inhibitor, PPC, PPC + caspase-3, and PPC + caspase-3 + inhibitor. (B) Corresponding $^1O_2$ lifetime.

of PS fluorescence, using a set of interference filters and a high-sensitivity NIR photomultiplier tube operating in the time-resolved single-photon counting mode.

As shown in Figure 3, caspase-3 and its inhibitor have no effect on the $^1O_2$ production of PP, and PPC itself has 8-fold less $^1O_2$ production than PP. Thus, the $^1O_2$ quenching in PPC is due to the presence of CAR. Moreover, addition of caspase-3 to the PPC (molar ratio 1:60; incubation time 1 h) resulted in a 4-fold increase in $^1O_2$ signal, an effect that was completely prevented by co-incubation with the caspase-3 inhibitor (8 × PPC concentration with the same incubation time). The 2-fold difference in $^1O_2$ luminescence as well as $^1O_2$ lifetime between the PP and PPC + caspase-3 is probably due to the presence of free CAR quenchers in solution after cleavage. This effect will likely be less in vivo since there will be less free CARs available. The difference in $^1O_2$ luminescence between PPC alone and PPC + caspase-3 is likely due to both photosensitizer triplet-state quenching and $^1O_2$ scavenging by CAR. These data demonstrate that $^1O_2$ generation is effectively inhibited by the CAR quencher and that caspase-3-induced separation of the quencher and the photosensitizer molecules allows photoactivation of the latter.

In addition, the ratio of the PPC to PPC + caspase-3 in $^1O_2$ lifetime (Figure 3B) is 3:4, whereas this ratio in total $^1O_2$ (Figure 3A) is 1:4. Since $^1O_2$ luminescence is directly proportional to $^1O_2$ lifetime, Pyro triplet state quenching by CAR seems to be more important for the intact PPC molecule than $^1O_2$ scavenging. This is consistent with the fact that the requirement for efficient triplet state quenching is less rigid than the direct $^1O_2$ quenching (five double bonds in CAR required for the former but nine required for the latter).[11]

All of the above experiments were repeated at least in triplicate and were statistically significant ($p < 0.04$).

In conclusion, a new photosensitization concept has been developed, based on $^1O_2$ quenching and activation, and the first PS-beacon designed using this concept has been validated in solution by direct $^1O_2$ luminescence and lifetime measurements. It is planned to test this next in cancer cells in vitro and in vivo with a tumor-specific peptide linker[5] and to extend the concept to other activation mechanisms, such as the use of tumor-specific antisense linker.[12]

**Acknowledgment.** This work was supported by DOD DAMD17-03-1-0373 (G.Z.), NIH R21-CA95330 (G.Z.), the Oncologic Foundation of Buffalo (G.Z.), and the National Cancer Institute of Canada (B.W., M.N.). Partial support from NIH R24-CA83105 (J.D.G.) is also acknowledged.

## References


(1) Dougherty, T. J.; Gomer, C. J.; Henderson, B. W.; Jori, G.; Kessel, D.; Korbelik, M.; Moan, J.; Peng, Q. *J. Natl. Cancer Inst.* **1998**, *90*, 889−905.
(2) Matayoshi, E. D.; Wang, G. T.; Krafft, G. A.; Erickson, J. *Science* **1990**, *247*, 954−958.
(3) Zhang, J.; Campbell, R. E.; Ting, A. Y.; Tsien, R. Y. *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 906−918.
(4) Tyagi, S.; Kramer, F. R. *Nat. Biotech.* **1996**, *14*, 303−308.
(5) Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov, A. *Nat. Biotech.* **1999**, *17*, 375−378.
(6) Nicholson, D. W.; Ali, A.; Thornberry, N. A.; Vaillancourt, J. P.; Ding, C. K.; Gallant, M.; Gareau, Y.; Griffin, P. R.; Labelle, M.; Lazebnik, Y. A.; Munday, N. A.; Raju, S. M.; Smulson, M. E.; Yamin, T.; Yu, V. L.; Miller, D. K. *Nature* **1998**, *17*, 1295−1304.
(7) Zhang, M.; Zhang, Z. H.; Blessington, D.; Li, H.; Busch, T. M.; Madrak, V.; Miles, J.; Chance, B.; Glickson, J. D.; Zheng, G. *Bioconjugate Chem.* **2003**, *14*, 709−714.
(8) Gust, D.; Moore, T. A.; Moore, A. L.; Jori, G.; Reddi, E. *Ann. N.Y. Acad. Sci.* **1993**, *691*, 32−47.
(9) HPLC method: column, RP C8 300 Å; solvents, A = 0.1% TFA in water, B = $CH_3CN$, C = MeOH; gradient, 60% A and 40% B to 10% A and 90% B in the first 20 min, then to 90% B and 10% C for another 10 min, finally to 80% B and 20% C for 10 min.
(10) Niedre, M.; Secord, A. J.; Patterson, M. S.; Wilson, B. C. *Cancer Res.* **2003**, *63*, 7986−7994.
(11) Foote, C. S.; Chang, Y. C.; Denny, R. W. *J. Am. Chem. Soc.* **1970**, *92*, 5216−5218.
(12) Sokol, D. L.; Zhang, X.; Lu, P.; Gewirtz, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11538−11543.


JA047392K