Rational Design of a Receptor-Targeted Photodynamic Molecular Beacon for the Multi-level Control of Singlet Oxygen Production and PDT Activity in Cancer Cells

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ABSTRACT

Photodynamic therapy (PDT) involves the combined action of light, oxygen and a photosensitizer (PS). It offers unique control in the PS's action because the key cytotoxic agent, singlet oxygen ($^1O_2$), is only produced *in situ* upon irradiation. The $^1O_2$ production can be controlled in three levels. The first level involves the judicious use of fiber optics to selectively deliver light to disease tissues. The second level is to exert control over the PS's localization by selectively delivering PS to cancer cells. The third level is to exert control of the PS's ability to generate $^1O_2$ in responding to specific cancer biomarkers. Here, we present two PDT agents based on the latter two levels of $^1O_2$ control. The first PDT agent "PPF" contains a PS (Pyro) and a tumor homing molecule (folate) and a peptide linker. PPF was found to be selectively accumulated in cancer cells via folate receptor (FR) pathway. The second PDT agent "$PP_{MMP7}B$" is a matrix metalloproteinase-7 (MMP7)-triggered photodynamic molecular beacon (PMB) containing a PS (Pyro), a $^1O_2$ quencher (BHQ3) and a MMP7-cleavable peptide linker. Thus, the $^1O_2$ production of $PP_{MMP7}B$ is highly sequence-specific and its photodynamic cytotoxicity is MMP7-dependent. Since these agents are designed to share functional modules (PS and peptide linker) and common cancer cell model (KB cells overexpress both FR and MMP7), it forms the basis for rational design of receptor-targeted PMB for achieving a multi-level control of $^1O_2$ production in cancer cells, which in term, could provide a much higher level of PDT selectivity.

Keywords: Photodynamic therapy, molecular beacon, photodynamic molecular beacon (PMB), photosensitizer (PS), singlet oxygen ($^1O_2$), control of $^1O_2$ production, MMP7, folate receptor, targeted delivery, KB cells.

1. INTRODUCTION

Photodynamic therapy (PDT) is an emerging cancer treatment modality involving the combination of light, a photosensitizer (PS) and molecular oxygen. Each factor is ineffective by itself, but when the PS is irradiated with light of an appropriate wavelength in the presence of oxygen, cytotoxic reactive oxygen species, mainly singlet oxygen ($^1O_2$), are produced [1]. $^1O_2$ is a powerful, fairly indiscriminate oxidant that reacts with a variety of biological molecules and destroys various cellular assemblies, particularly membranes. Its short lifetime limits its diffusion range (~ 20nm) in cellular media [2], so that the site of the primary generation of $^1O_2$ determines which subcellular structures are damaged [3]. Furthermore, it has been demonstrated that $^1O_2$ near-infrared (NIR) luminescence correlates well with PDT-induced cell kill, regardless of the treatment fluence, fluence rate, triplet oxygen concentration, initial PS concentration and rate of PS photobleaching [4]. This suggests that, if control and selectivity can be applied in the photosensitized production of $^1O_2$ in cancer cells, then PDT-induced cell death should be highly tumor specific. In general, PDT selectivity could be achieved by controlling the $^1O_2$ production at three different levels: 1) controlling the selectivity of light irradiation to the disease tissue, 2) enhancing the selectivity of the delivery of PS to tumor tissue, and 3) exerting control of the PS's ability to produce $^1O_2$ in responding to specific cancer-associated biomarkers.

The first approach controls $^1O_2$ production by controlling how the light is delivered to targeted tissues. This approach is the easiest to implement as light can be readily manipulated and positioned, particularly through the judicious use of advanced fiber optics (e.g., prostate interstitial fibers) [5]. Thus, a certain level of PDT selectivity can be achieved by irradiating only the diseases tissues. However, this approach can not achieve a high level of selectivity due to the limited

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Genetically Engineered and Optical Probes for Biomedical Applications IV, Samuel Achilefu, Darryl J. Bornhop, Ramesh Raghavachari, Alexander P. Savitsky, Rebekka M. Wachter, Eds., Proc. of SPIE Vol. 6449, 644906, (2007) · 1605-7422/07/$18 · doi: 10.1117/12.699754

tumor localization of existing PDT agents, which in term, causes the treatment-related toxicity to surround normal tissues as well as the sunlight-induced skin toxicity. Here we focus on the latter two approaches by developing two PDT agents designed to have common functional modules (both containing a similar length peptide sequence attached to the same PS) with the ultimate goal of integrating them into PDT agent with a multi-level control of $^1O_2$ production. The first PDT agent, PPF, comprises a pyropheophorbide (Pyro) as the PS and a folate moiety as homing folate receptors (FR) [6], linked together through a peptide linker (GDEVDGSGK). The purpose of this design is to enable the PS to accumulate selectively in cancer cells and to mimic the pharmacological-behavior of the second PDT agent, $PP_{MMP7}B$, which is a matrix metalloproteinase-7 (MMP7)-triggered photodynamic molecular beacon (PMB). PMB is a novel extension of the well known concept of molecular beacon [7] that uses fluorescence resonance energy transfer (FRET) to offer control of fluorescence emission in response to specific cancer targets. By combining the FRET principle with PDT, we seek to enable the cancer biomarker-controlled $^1O_2$ production to achieve a higher level of PDT selectivity [8]. $PP_{MMP7}B$ features of a PS (Pyro) and a $^1O_2$ quencher (BHQ3) linked together through a MMP7-cleavable peptide sequence (GPLGLARK). It is designed to be photodynamically inactive until triggered by the cancer-associated MMP7. Taking advantage of the fact that KB cancer cells overexpress both FR [6] and MMP7 [9], we designed a FR-targeted and MMP7-triggered PMB (TaPMB) by integrating functional modules of PPF and $PP_{MMP7}B$ in order to achieve a multi-level control of $^1O_2$ production in cancer cells, which in term, could provide a much higher level of PDT selectivity. The concept of TaPMB is depicted in Figure 1. Thus, TaPMB comprises a folate moiety as the tumor homing molecule, a MMP7-cleavable peptide as the linker, a Pyro as the PS and a BHQ3 as the $^1O_2$ quencher (Q). Proximity of PS and Q can either quench $^1O_2$ generation through PS excited-state energy transfer and/or can scavenge the $^1O_2$ that has been generated, so that the PS's photoactivity is silenced until this TaPMB selectively accumulates in KB cancer cells via FR pathway and the peptide linker interacts with MMP7. Therefore, the higher degree of PDT selectivity could be achieved through a triple-level control of $^1O_2$ production: 1) selective delivery light to tumors, 2) selective localization of PS in tumors, and 3) selective $^1O_2$ production in responding to cancer-associated biomarkers.

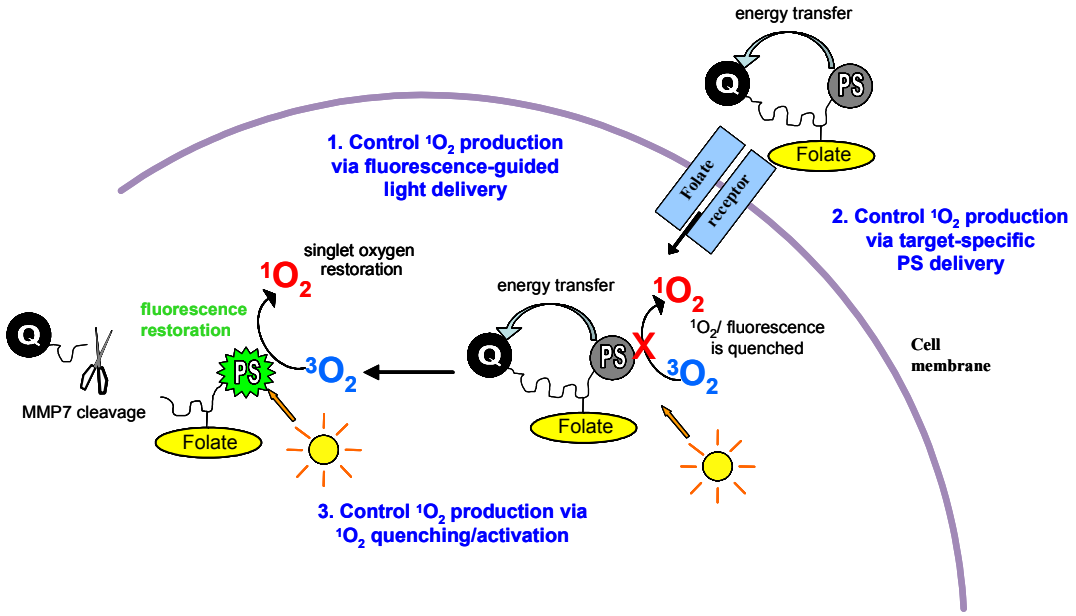

Figure 1. The concept of multi-level control of $^1O_2$ production using a receptor-targeted photodynamic molecular beacon (TaPMB).

2. METHODOLOGY

Materials and General Methods.

UV-vis and fluorescence spectra were recorded on a Perkin-Elmer (Boston, MA, USA) Lambda 20 spectrophotometer and LS-50B spectrofluorometer, respectively. MALDI-ToF mass spectroscopy was performed on an Applied Biosystems

(Foster City, CA, USA) Voyager DE system. Reverse phase (RP) analytical HPLC was performed on a Zorbax 300SB-C8 column using a Waters 600 Controller with a 2996 photodiode array detector (HPLC method: Solvent A= 0.1% TFA/Water; Solvent B= Acetonitrile; Gradient: from 80% of A and 20% of B to 100% of B over 40min; flow rate:1.0mL/min). Confocal microscopy images were acquired using a Zeiss LSM510 META laser scanning confocal microscope (Heidelberg, Germany), and *in vivo* fluorescence images were acquired on a Xenogen IVIS imager (Hopkinton, MA, USA).

$^1O_2$ measurement

PDT-generated $^1O_2$ was quantified in solution by directly measuring its NIR luminescence at 1270 nm using an instrument and a protocol that have been described before [4]. Briefly, 10ns pulsed 532 nm laser excited the solution and the luminescence spectrum was sampled, after rejection 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.

Cell lines

KB cells (human nasopharyngeal epidermoid carcinoma cells with high MMP7 and folate receptor expression, $MMP7^+$, FA^+), BT20 cells (human breast cancer cells, lack of MMP7 expression, $MMP7^-$) and HT1080 cells (human fibrosarcoma cells, lack of FA expression, FA^-) were purchased from ATCC (Manassas, VA, USA). All cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 2mM L-glutamine, 17.9mM sodium bicarbonate, 0.1mM non-essential amino acids, 1.0mM sodium pyruvate, and 10% fetal bovine serum (FBS) and were grown at 37°C in a humidified atmosphere containing 5% CO_2.

Confocal microscopy

Cells were grown for 2d in 4-well Lab-Tek chamber slides. The MEM medium containing indicated amount of PDT probe was added and the cells were incubated at 37°C. The cells were washed 5 times with ice-cold Phosphate Buffered Saline (PBS) and then fixed for 20min with 1% formaldehyde in PBS at room temperature. The chamber slides were then imaged by confocal microscopy with 633 nm excitation and >650 nm detection.

MTT assay

Cells were grown for 2d in 96-well plate. Experiments were started, after one quick wash with ice-cold PBS buffer, by addition of 100μL of cell growth medium containing indicated amounts of PDT probes. After incubation at 37°C with 5% CO_2, the cells were washed twice with ice-cold PBS buffer and then 100μL of cell growth medium was added. PDT treatment was performed using a 670nm laser with one of 3 different light fluences (1, 5 or 7.5J/cm^2). The cells were allowed to continue growth for 24 h, at which time the MTT tracer, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (ATCC, Manassas, VA, USA) was added to the medium at 0.5mg/mL. Two hours later, the medium was removed and replaced with 150μL of 1: 1 DMSO: 70% isopropanol in 0.1M HCl. The absorbance at 570 nm was measured on a Bio-tek ELx model 800 (MTX Lab System Inc., Vienna, VA, USA).

***In vivo* fluorescence imaging**

Nude mice (N=3) were inoculated with each 10^7 of HT1080 and KB cells on the left and right flanks respectively and the tumors were grown for 7d with the mice maintained on a low-fluorescence diet. These double tumor bearing mice were intravenously injected with 30nmol of PPF and scanned by Xenogen imager using a Cy 5.5 filter over time.

3. RESULT AND DISCUSSION

Control $^1O_2$ production through the targeted delivery of PS

To control the localization of PS in cancer cells, a FR-targeted PDT agent, Pyro-GDEVDGSGK-Folate (PPF), was synthesized. The PPF contains three functional modules. The first module is a fluorescent photosensitizer pyropheophorbide (Pyro) for NIR imaging (with a long-wavelength absorption at 665 nm and emission at 675 nm and 720 nm) and PDT (over 50% singlet oxygen yield) [10, 11]. This semisynthetic photosensitizer, obtained by three steps from *Spirulina* algae, has minimal dark toxicity and its derivative Photochlor® is in phase I/II clinical trial [12]. The second module is a folate moiety, serving as a tumor homing molecule that guides the photosensitizer into folate receptor

(FR)-overexpressing cancer cells [13, 14] via receptor-mediated endocytosis [15]. FRs are mainly overexpressed on ovary, breast, colon, lung, nose, prostate, and brain cancer cells and activated macrophages [16] but have limited expression on normal cells (e.g. kidney, intestine, lung) with restricted accessibility for blood-circulating drugs [17]. The third module is a short peptide sequence GDEVDGSGK inserted between the photosensitizer and folate. It serves multiple purposes: A) it is a stable and hydrophilic linker that prevents the separation of folate and Pyro and enhances water solubility, B) it separates the Pyro from the Folate to avoid the hindrance of FR-targeting [17], C) it serves as a pharmacomodulator for better delivery efficiency and decreased normal tissue toxicity, and D) it is possible to exchange it with other peptide sequences (e.g., MMP7-specific peptide) thus allowing easier integration with the peptide-based PMB. The detailed synthesis of PPF and the evaluation of the critical roles of peptide and folate for its selective uptake in FR^+ cells will be reported elsewhere. This study will focus on the results that are relevant to the rationales of the TaPMB design.

1) PPF selectively accumulation in cells.

A confocal microscopy study was preformed on KB (FR positive, FR^+) and HT1080 (FR negative, FR^-) cells incubated with 50μM or 100μM of PPF. As shown in Figure 2, strong accumulations of PPF were observed through the whole KB cell (FR+) at both concentrations, while minimal uptakes of PPF were found in HT1080 cells (FR-). This clearly indicated that the uptake of PPF is mediated by the FR pathway.

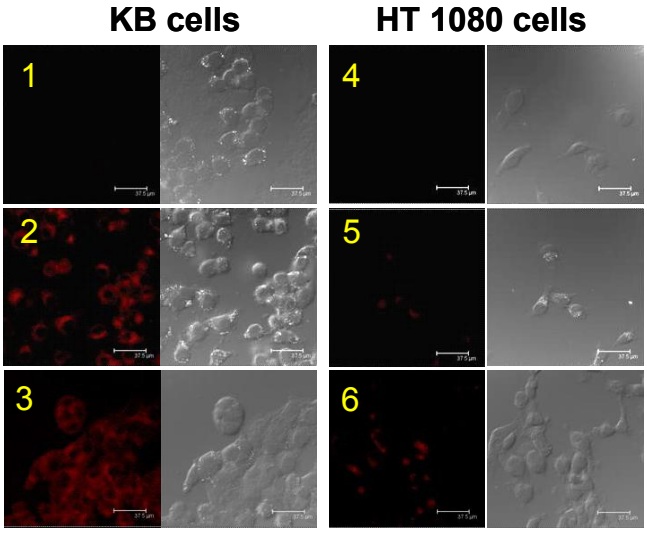

Figure 2. Confocal images of 1) KB cell alone, 2) KB+50μM PPF, 3) KB+100μM PPF, 4) HT1080 cell alone, 5) HT1080+50μM PPF, and 6) HT1080+100μM PPF.

2) Selective PDT-induced killing by PPF

The logical result of PPF's preferential accumulation in FR^+ cells should be a selective killing of these cells as the result of controlling the localization of $^1O_2$ production. To compare the post-PDT viability reduction for cell lines with different levels of FR expression, KB (FR^+), HT 1080 (FR^-) cells were incubated with 5 μM PPF for 24h and then subjected to PDT treatment with light doses ranging from 0, 1, 5, to 10 J/cm^2 using a 670nm tunable laser with a fluence rate of 20mW/cm^2. The MTT study (Figure 3) shows that the viability of KB cells drops bellow 10% upon PDT treatment with a 5 J/cm^2 light dose. However, using the highest light dose (10 J/cm^2) does not effectively kill the HT 1080 cells. This result clearly suggests that a high degree control of PDT activity is achieved by controlling of the localization of $^1O_2$ production in FR^+ cells.

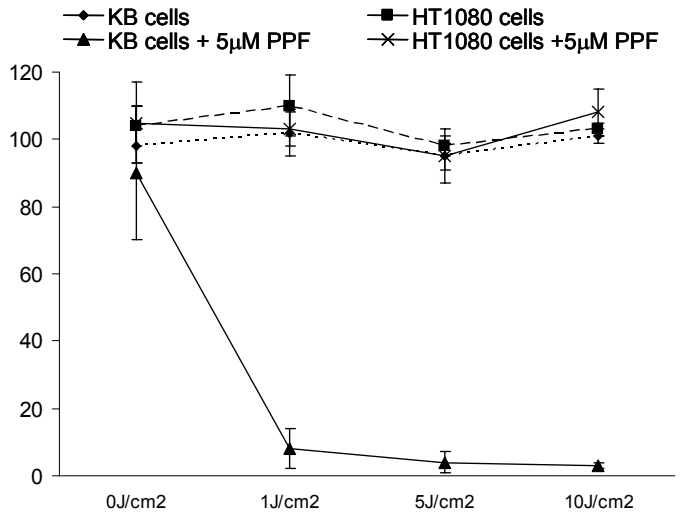

Figure 3. The viability of KB (FR^+) and HT1080 (FR^-) cells after incubated with 5μM PPF for 24h and treated with different PDT light doses (0, 1, 5, 10J/cm^2) was determined by the MTT assay. Data shown here was based on 3 different experiments and the results were expressed as mean ± standard error.

3) Selective in vivo accumulation of PPF

To validate the FR-targeting specificity of PPF *in vivo*, nude mice bearing an HT 1080 tumor (FR-) on the left flank and a KB tumor (FR+) on the right flank were used for *in vivo* imaging studies. Mice were intravenously injected with 30nmol of PPF and scanned by Xenogen imager using a Cy 5.5 filter at different time points (prescan, 5 min, 6h and 24h). As shown in Figure 4, five minutes after injection, PPF is distributed rapidly throughout the animal including to the tumor xenografts. By 6h post-injection, the diffuse systemic fluorescence pattern begins to recede (washout effect) and regions of particle retention are clearly defined. PPF can be seen within the upper abdomen and PPF accumulates significantly in KB tumor more than HT 1080. Finally at 24h a strong fluorescence is only emitted from the KB tumor and minimal fluorescence was observed else where in the animal including HT1080 tumor, liver, etc., suggesting minimal nonspecific uptake of PPF. This study suggests that in the highly likelihood, the localization of $^1O_2$ production can be confined selectively in FR^+ cancer cells *in vivo*.

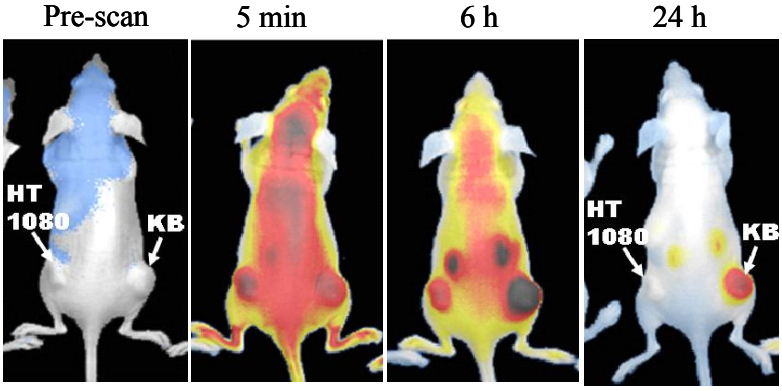

Figure 4. Real time *in vivo* fluorescence imaging of the dual tumor bearing mouse intravenously injected with 30nmol of PPF.

Control $^1O_2$ production using MMP7-triggered PMB

To control $^1O_2$ production using our novel concept based on protease-triggered $^1O_2$ quenching and activation, a

MMP7-activatable PMB, Pyro-GPLGLARK(BHQ3) ($PP_{MMP7}B$), was synthesized. Matrix metalloproteinases (MMPs) are known to be important in normal tissue remodeling, but also play critical roles in many diseases including cancer [18-20]. MMP7 has been a particularly important target because of its epithelial origin and its high expression in pancreatic, colon, breast [21, 22], and non-small cell lung cancer [19, 23, 24]. In addition, there is a common cancer cell model (KB) overexpressing both FR and MMP7, thus results obtained from PPF and $PP_{MMP7}B$ can provide rationales for the design of the fully integrated TaPMB. The detailed synthesis protocol of $PP_{MMP7}B$ will be reported elsewhere. This study will focus on the results that are relevant to the rationales of the TaPMB design.

Validation of MMP7-triggered $PP_{MMP7}B$ activation and its corresponding $^1O_2$ activation in solution

In order to evaluate the efficiency of $^1O_2$ and fluorescence quenching in intact $PP_{MMP7}B$ as well as the specificity of its activation by MMP7, fluorescence and $^1O_2$ near-infrared (1270nm) luminescence were measured respectively in solutions of PP_{MMP7}, PP_{MMP7}+BHQ3 (1:1 molar ratio), $PP_{MMP7}B$, $PP_{MMP7}B$+MMP7 and $PP_{MMP7}B$+MMP7+MMP7 inhibitor. As shown in Figure 5, comparing with $PP_{MMP7,}$ 13-fold less of fluorescence and 18-fold less of $^1O_2$ production were observed in $PP_{MMP7}B$, while physical mixture of BHQ3 and PP_{MMP7} did show noticeable fluorescence and $^1O_2$ quenching, confirming that the close proximity of Pyro and BHQ3 by the MMP7 associated peptide (GPLGLARK) effectively inhibits both $^1O_2$ and fluorescence production of Pyro. Moreover, adding MMP7 to the $PP_{MMP7}B$ (molar ratio: 1: 60; 3h incubation) restored the quenched $^1O_2$ and fluorescence by 19-fold and 12-fold respectively. These restoration were completely blocked by co-incubation with the MMP7 inhibitor (molar ratio = 1:60:1200 for MMP7:$PP_{MMP7}B$:inhibitor), confirming that MMP7-induced separation of BHQ3 and Pyro allows the photoactivation of the latter.

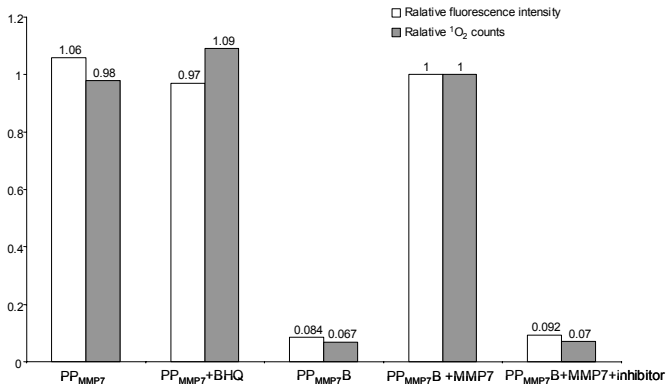

Figure 5. The relative fluorescence intensity and relative $^1O_2$ counts of PP_{MMP7}, PP_{MMP7}+BHQ, $PP_{MMP7}B$, $PP_{MMP7}B$+MMP7 and $PP_{MMP7}B$+MMP7+inhibitor.

Validation of MMP7-triggered $PP_{MMP7}B$ activation in cancer cells

To prove that $PP_{MMP7}B$ can be specifically activated by MMP7 *in vitro*, confocal fluorescence microscopy studies were performed on KB ($MMP7^+$) and BT20 ($MMP7^-$) cells after 4 hours incubation with 60 μM of $PP_{MMP7}B$. As shown in Figure 6, strong fluorescence signal was observed in KB cells incubated with $PP_{MMP7}B$, whereas BT20 cells incubated with $PP_{MMP7}B$ showed minimal fluorescence. In addition, there is no overlap between the Pyro and DAPI fluorescence showing that the $PP_{MMP7}B$ is localized throughout the KB cells except for the nucleus. These results validated that $PP_{MMP7}B$ is triggered by MMP7 selectively.

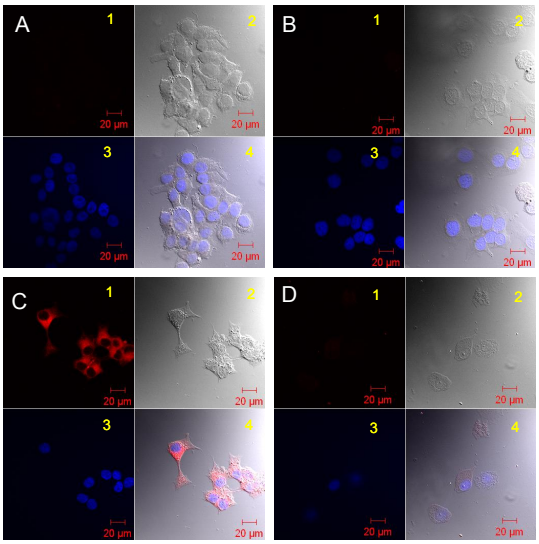

Figure 6. Confocal microscopy images of $PP_{MMP7}B$ in KB ($MMP7^+$) and BT20 ($MMP7^-$) cells. The numbers refer to each set of images: 1) fluorescence images, 2) bright field images, 3)DAPI images and 4) overlaid images. **A)** KB cell alone, **B)** BT20 cell alone, **C)** KB cells+60 µM $PP_{MMP7}B$, **D)** BT20 cells+60 µM $PP_{MMP7}B$.

Validation of the MMP7-dependent PDT selectivity of $PP_{MMP7}B$ in cancer cells

KB ($MMP7^+$) and BT20 (FR^-) cells were incubated with 2.5 µM $PP_{MMP7}B$ for 24h and then subjected to PDT treatment with light doses ranging from 0, 1, 5, to 7.5 J/cm^2 using a 670nm tunable laser with a fluence rate of 20mW/cm^2. As shown in Figure 7, the viability of KB cells drops bellow 10% upon PDT treatment with a 1J/cm^2 light dose. Whereas at the same dose, no noticeable reduction of BT20 cell viability was observed. Taking together, $PP_{MMP7}B$ is specifically photoactivated by MMP7 and its photodynamic cytotoxicity is MMP7 sequence specific. This study clearly demonstrated that control of $^1O_2$ production can be achieved by controlling the PS's ability to generate $^1O_2$ in responding to a MMP7 trigger.

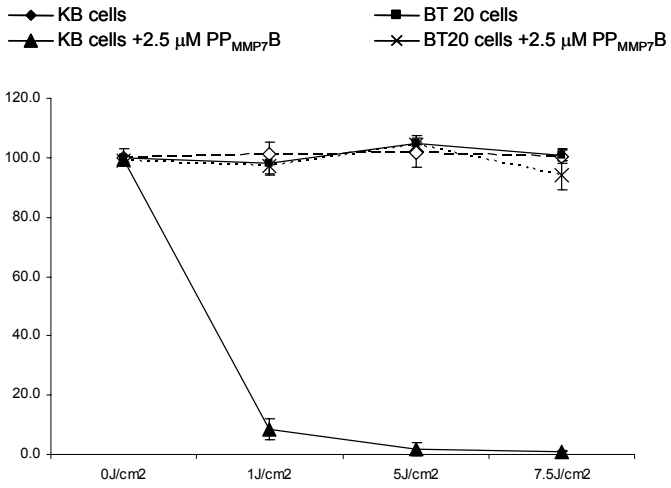

Figure 7. The viability of KB ($MMP7^+$) and BT20 ($MMP7^-$) cells after incubated with 2.5µM $PP_{MMP7}B$ for 16h and treated with different PDT light doses (1, 5, 7.5J/cm^2) was determined by the MTT assay. Data shown here was based on 3 different experiments and the results were expressed as mean ± standard error.

Integration of PPF and $PP_{MMP7}B$ into TaPMB design

In general, there are three levels of $^1O_2$ production control in cancer cells: 1) controlling the selectivity of light irradiation to the disease tissue, 2) enhancing the selectivity of the delivery of PS to tumor tissue, and 3) exerting control of the PS's ability to produce $^1O_2$ in responding to specific cancer-associated biomarkers. Each mechanism offers a certain degree of control of PDT activity but also each has major limitations. Site specific illumination by the use of optic fibers coupled to appropriate laser sources can reach tumors not only by endoscopy in hollow organs (e.g., esophagus, bronchial tree and bladder), but also by the direct intraoperative surface (e.g., brain) or by the interstitial illumination (e.g., prostate) [5]. However, control of light delivery has serious limitation as $^1O_2$ production and its corresponding PDT activity is indiscriminant towards tumor and normal tissues due to the nonspecific nature of current PS. Therefore, if a PS can be localized in tumor cells via targeted delivery, the PDT selectivity can be improved since the nature of $^1O_2$ (in situ production with extremely short lifetime) [4] determines that it can only cause PDT-induced cell damage in its immediate vicinity, such as at cellular binding sites specific to the particular PS. However, this approach of controlling the localization of the $^1O_2$ production depends on the degree of receptors overexpression in cancer cells versus normal healthy cells. Therefore, it is highly desirable to develop methods by which the efficiency of $^1O_2$ production can be selectively controlled. The PMB concept we recently introduced has great potential in addressing this issue as it seeks to protect normal cells from photodamage by masking its $^1O_2$ production and its associated photodynamic toxicity in these cells. Therefore, in theory, it will shift the burden of PDT selectivity from how selectively the PS can be delivered to cancer cells to how selective a biomarker is to cancer cells and how selective the interaction of PMB is to this biomarker. However, in a practical sense, it still heavily depends on the delivery efficiency of the PMB to the targeted tissues. Therefore, integration these levels of $^1O_2$ production into a single PDT agent could make PDT a more safe and selective clinical technique. Here, we present our rationales for designing the PDT agent with triple level control of $^1O_2$ production in cancer cells. First, we developed a tumor targeted PDT agent, PPF, which have enhanced PS's selective accumulation in folate receptor-overexpressing KB cancer cells thus enabled the $^1O_2$ to be generated preferentially in these cells. Next, we developed a MMP7-triggered PMB, $PP_{MMP7}B$, which offers a very high level control of $^1O_2$ production and PDT activity in MMP7-overexpressing KB cancer cells. In addition, PPF and $PP_{MMP7}B$ have many common features by design: 1) share the same PS (Pyro), 2) contain similar lengths of peptide sequence, and 3) similar synthesis protocols. Therefore, it is highly probable that PPF and $PP_{MMP7}B$ can be integrated into a single TaPMB that resembles many features of PPF and $PP_{MMP7}B$. More importantly, both PPF and $PP_{MMP7}B$ were evaluated using the common cancer cell model as KB cells overexpressing both FR and MMP7. Therefore, we hypothesized that this new construct should enter KB cells preferentially via folate receptor mediated endocytosis and subsequently activated by MMP7 overexpressed by the same cancer cells to restore its fluorescence and its ability to generate $^1O_2$. Upon irradiating with light guided by MMP7-triggered fluorescence emission, the TaPMB will destroy cancer cells while leaving normal cells unharmed. This new paradigm has the potential to generate extremely high tumor specificity for the PS as its tumor specificity will depend on the unprecedented triple level control of $^1O_2$ production. Thus, one can envisage using this high-specificity PDT to 'see' and photoablate the targeted tumor tissues, while sparing adjacent non-cancer tissues to minimize complications.

4. CONCLUSION

We have developed two PDT agents with different mechanisms of $^1O_2$ production control. The first PDT agent PPF was found to be selectively accumulated in cancer cells via folate receptor (FR) pathway thus enabled the $^1O_2$ to be generated preferentially in these cells. The second PDT agent $PP_{MMP7}B$ was found to have a MMP7-dependent $^1O_2$ production and photodynamic cytotoxicity. Based on these findings, we have designed a receptor-targeted photodynamic molecular beacon (TaPMB) for achieving a multi-level control of $^1O_2$ production in cancer cells, which in term, could provide a higher degree of PDT selectivity.

ACKNOWLEDGMENT

This work was supported by DOD DAMD17-03-1-0373, NIH R21-CA95330 and Oncologic Foundation of Buffalo (to GZ), and the National Cancer Institute of Canada (to BW).

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