Selective Photodetection and Photodynamic Therapy for Prostate Cancer through Targeting of Proteolytic Activity

Maria-Fernanda Zuluaga¹, Nawal Sekkat¹, Doris Gabriel¹, Hubert van den Bergh², and Norbert Lange¹

Abstract

Frequent side effects of radical treatment modalities and the availability of novel diagnostics have raised the interest in focal therapies for localized prostate cancer. To improve the selectivity and therapeutic efficacy of such therapies, we developed a minimally invasive procedure based on a novel polymeric photosensitizer prodrug sensitive to urokinase-type plasminogen activator (uPA). The compound is inactive in its prodrug form and accumulates passively at the tumor site by the enhanced permeability and retention effect. There, the prodrug is selectively converted to its photoactive form by uPA, which is overexpressed by prostate cancer cells. Irradiation of the activated photosensitizer exerts a tumor-selective phototoxic effect. The prodrug alone (8 μmol/L) showed no toxic effect on PC-3 cells, but upon irradiation the cell viability was reduced by 90%. In vivo, after systemic administration of the prodrug, PC-3 xenografts became selectively fluorescent. This is indicative of the prodrug accumulation in the tumor and selective local enzymatic activation. Qualitative analysis of the activated compound confirmed that the enzymatic cleavage occurred selectively in the tumor, with only trace amounts in the neighboring skin or muscle. Subsequent photodynamic therapy studies showed complete tumor eradication of animals treated with light (150 J/cm² at 665 nm) 16 hours after the injection of the prodrug (7.5 mg/kg). These promising results evidence the excellent selectivity of our prodrug with the potential to be used for both imaging and therapy for localized prostate cancer. Mol Cancer Ther; 12(3); 1–8. ©2012 AACR.

Introduction

Prostate cancer is the most prevalent cancer in the male population (1). The gold standard for the treatment of localized disease is radical prostatectomy or radiotherapy. A minority of low-risk patients can be kept under active surveillance, but this often only delays the final treatment (2). The excellent results obtained with the radical treatment come at the cost of frequent side effects (mainly sexual or urinary dysfunction) and their long-lasting impact on the quality of life. Mapping biopsies and imaging with endorectal coil MRI have laid the foundation for local therapies, which might cause fewer side effects.

Current options for localized therapy include brachytherapy, cryotherapy, high intensity focused ultrasound, laser ablation, and photodynamic therapy (PDT; ref. 3). The latter requires 3 main elements: a photosensitizer, light, and oxygen. After administration, the photosensitizer accumulates to some extent in the target tissue and subsequently can be selectively activated by light to produce reactive oxygen species. With recent progress in light delivery and dosimetry, the use of PDT is no longer restricted to the skin. Fairly superficial lesions in hollow organs can be treated (4, 5) and prostate cancer is also open to PDT if one inserts optical devices into the lesions.

HpD, a hematoporphyrin derivative, which is a complex mixture of porphyrins was among the first photosensitizers assessed clinically. This was followed by the use of a somewhat purified mixture called Photofrin that was used to treat prostate cancer (6). Subsequently, small prospective clinical trials using Foscan (7) and 5-aminolevulinic acid (8), have been reported. Despite promising PDT responses, one observed prolonged skin sensitization in the case of Foscan and occasional extraprostatic tissue injury. This encouraged further research efforts, which aimed mainly at improving PDT selectivity and reducing side effects. In this context, LuTex and Tookad specifically targeting the vasculature...
combined with local light delivery to the prostate were evaluated (9). Trials for the treatment of primary and recurrent prostate cancer using these agents showed good tolerability. However, some patients did not respond to the treatment or presented urinary and rectal damage (10–13). Even with the improved formulation of Tookad, insufficient therapeutic responses and collateral damage have been reported recently (Emberton, IPA Congress, Innsbruck, 2011).

Therefore, improvements in the tumor selective delivery of photosensitizer are needed to avoid collateral damage of the urethra, rectum, and urinary sphincter (14). With this goal in mind, we have developed polymeric protease-sensitive photosensitizer prodrugs (PPP) following a triple targeting strategy: (i) selective delivery of the PPP into tumor tissue is promoted by the polymeric carrier through the enhanced permeability and retention effect (15). In its prodrug form, photoactivity is impeded through efficient intramolecular quenching between closely positioned photosensitizer molecules on the polymeric carrier. (ii) Proteolytic activation occurs via cleavage of the peptide linkers by urokinase-type plasminogen activator (uPA), which is overexpressed by prostate cancer cells (16). Release from the polymeric backbone thus reestablishes the photosensitizer’s photoactivity selectively in the target tissue. (iii) Local irradiation further increases selectivity and induces toxic radicals.

In a previous study, we have reported on a prodrug candidate (uPA-PPP-4) capable of accumulating in prostate cancer tumors and being activated by upregulated uPA (17). The present report investigates the therapeutic potential of this prodrug by evaluating its phototoxic effect in vitro in PC-3 and luciferase-transfected PC-3M-luc-C6 cancer cells as well as in vivo in a prostate cancer xenograft model.

Materials and Methods

Compounds
uPA-PPP-4 (Fig. 1) consisted of multiple copies of the photosensitizer pheophorbide a (Pba) attached to a poly-L-lysine backbone via a GSGRSAG peptide sequence. It was synthesized and characterized as described previously (17, 18) as well as in more detail in the Supplementary Materials and Methods. The purity of the prodrug was confirmed by reversed phase high-performance liquid chromatography, with monitoring at 280, 330, and 450 nm. A prodrug mass of approximately 108 kDa was confirmed by SEC-MALLS-RI-UV using a column Waters Ultrahydrogel linear (column temperature: 35°C ± 0.2°C; mobile phase: 0.15 mol/L acetic acid, 0.1 mol/L sodium acetate, 0.05% NaN3 at a pH of 4.0; flux: 0.4 mL/min). This system contains a pump: Waters Alliance HPLC System, and 3 detectors: a Schambeck RI detector (Bad Honnef), a light-scattering detector Wyatt MiniDawn, and a UV–VIS detector Waters Lambda-Max.

Cell culture
PC-3 cells (American Type Culture Collection) from human prostate cancer origin were cultured in F-12 growth medium supplemented with 10% FBS. Luciferase-transfected PC-3M-luc-C6 cells, a kind gift of Caliper LifeSciences, were maintained in Minimum Essential Medium with Earle’s Balanced Salts with 10% FBS, nonessential amino acids, l-glutamine, sodium pyruvate, and Minimum Essential Medium Vitamin Solution. Both cell lines were grown as monolayers at 37°C in a humidified incubator containing 5% CO2. The cells were harvested using TrypLE Express, and
passaged every 4 to 5 days. Cell lines used in this study were not authenticated.

**In vitro PDT**

Phototoxicity was tested on PC-3 and luciferase-transfected PC-3M-luc-C6 cells. All lots of 1.2 × 10^4 and 1.0 × 10^4 cells, respectively, in 100 µL complete medium were seeded in 96-well plates and cultured for 12 hours to 70% confluence. Cells were given fresh complete medium containing uPA-PPP-4 at final concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0 µmol/L Pb equivalents for 6 hours. Cells were washed twice with sterile Hank’s Balanced Salt Solution (HBSS) and fresh medium was added. Plates were either placed on a light table equipped with OSRAM L 18W/67 Blue light tubes (PCI Biotech) or kept in the dark. The radiation intensity was 7.5 mW/cm². Cells were irradiated at light doses of 2.5, 5.0, and 10 J/cm². Cell viability was measured using a mitochondrial MTT solution (HBSS) and fresh medium was added. Plates were washed twice with sterile Hank’s Balanced Salt Solution (HBSS) and fresh medium was added. Plates were either washed with 200 µL HBSS and 50 µL MTT (1 mg/mL) in complete medium was added into each well. After 3 hours, dimethyl sulfoxide (DMSO; 200 µL) was added to dissolve formed violet formazan crystals. After brief agitation on a microplate shaker, the absorption at 525 nm was measured with a plate reader (Saphire). Positive and negative controls were treated with complete medium or 0.1% Triton in NaOH 5 mol/L, respectively. Percentage cell survival was calculated with respect to control samples, as follows: [A (test-conc.) — A (100% dead)]/[A (100% viable) — A (100% dead)] × 100. All conditions were tested in sextuplicates.

**Prostate cancer model**

Female Swiss Nu/Nu mice (5 to 6 weeks, 17–22 g) were supplied by Charles River Laboratories. The mice were maintained with ad libitum access to sterile food and acidified water in a light cycled room acclimatized at 22°C ± 2°C under pathogen-free conditions. All experimental procedures on animals were carried out in compliance with the Swiss Federal Law on the Protection of the Animals, according to a protocol approved by the local veterinary authorities. To induce xenografts, 1.5 × 10⁶ cells were injected subcutaneously into the dorsal region of mice. Tumors of approximately 200 mm³ in size were formed within 3 weeks after inoculation.

**In vivo PDT**

PC-3M-luc-C6 xenograft bearing mice (n = 7) were injected retro-orbitally with uPA-PPP-4 (7.5 mg Pb equivalents/kg) when tumors had an estimated volume of 200 mm³ (3–4 weeks after inoculation). Tumors were irradiated with a light dose of 150 J/cm² at 665 ± 5 nm (Ceralas I 670, Biolitec) 16 hours after conjugate administration. The radiation intensity was 70 mW/cm². Animals were maintained under 1% to 2% isoflurane inhalation during irradiation. Two other groups of animals received drug alone (n = 4) and light alone (n = 4). PDT effects were followed up to 90 days by bioluminescence imaging of animals using an IVIS 200 small-animal imaging system (Caliper Life Sciences Inc.). Ten to 15 minutes before in vivo bioluminescence imaging, animals received an intraperitoneal injection of α-luciferin (150 mg/kg in Dulbecco’s Phosphate-Buffered Saline). Mice were sacrificed when tumors reached volumes bigger than 1,000 mm³ or at the end of the study (90 days after treatment). Data were analyzed with Living Image 3.0 software (Caliper Life Sciences Inc.).

**Qualitative analysis of prodrug cleavage products**

Cleavage products were qualitatively analyzed in tumor, skin, and muscle homogenates of the corresponding tissues 16 hours after systemic administration of uPA-PPP-4 (7.5 mg Pb equivalents/kg). Briefly, frozen tissues were weighed and homogenized with a solution containing a protease-inhibitor cocktail (5 µL per 100 mg tissue) and acetonitrile:water (1:1; 1 mL per 100 mg tissue) by means of a tissue homogenizer (Eurostar digital IKA; Werke). The suspensions were sonicated (15 minutes at 14 kHz) and centrifuged (15 minutes at 1,450 rpm). The supernatant was collected and extraction was repeated twice as described. Collected supernatants were lyophilized and subsequently reconstituted in acetonitrile:water (1:1; 1 mL/100 mg tissue). Samples were sonicated (5 minutes, 14 kHz), filtered and subjected to analytic HPLC (LaChroma, Merck) with a fluorescence detector (λex = 405 nm; λem = 670 nm). Separation was conducted on a C18 column (Nucleodur gravity 3 µC 125/4; Macherey-Nagel) using a 0.01% TFA/water/acetonitrile gradient.

**Statistical analysis**

Mean ± SD values were used for expression of data. Statistical analyses of data were done using Student t test. Differences of P < 0.05 were considered statistically significant.

**Results**

The phototoxic effect induced by uPA-PPP-4 was investigated in the uPA-overexpressing prostate cancer cells PC-3 (19, 20) and its luciferase-expressing mutant PC-3M-luc-C6 cells. The latter was chosen for the subsequent quantitative assessment of PDT studies in vivo. The effect of PDT on cells treated with prodrug (0.5, 1.0, 2.0, 4.0, and 8.0 µmol/L Pb equivalents), either irradiated with a light dose of 2.5, 5.0, and 10 J/cm² or kept in the dark is summarized in Fig. 2.

Both cell lines display a light and drug dose-dependent cell survival. uPA-PPP-4 alone presented little to no toxic effects as shown by cell survival percentages around 100% for all prodrug concentrations. Phototoxic effects were particularly evident at photosensitizer dose of 4.0 µmol/L or higher. In PC-3 cells at 8 µmol/L of Pb equivalents approximately 50% of cells survived irradiation with 2.5 or 5 J/cm² of light, whereas at a dose of 10 J/cm² only 5% of cells remained viable. In PC-3M-luc-C6 cells similar dose-response curves were observed. Cell survival after
treatment with 4.0 and 8.0 μmol/L of Pb equivalents at all light doses were not statistically different between PC-3 and PC3-3M-luc-C6 cells (P values > 0.05) except for the condition 8 μmol/L -5 J/cm² (P = 2.11E-05).

We have used PC-3M-luc-C6 as basis for our experimental animal model for prostate cancer, as they allow noninvasive monitoring of tumor growth through bioluminescence in a quantitative manner (21). In this study, bioluminescence was used to assess the photodynamic efficacy of uPA-PPP-4 on tumors. Using whole body fluorescence imaging, we found that tumors became selectively fluorescent 16 hours after prodrug administration (17). Figure 3 illustrates the typical colocalization of fluorescence (rainbow-color scale) and bioluminescence (yellow-hot-color scale) signals at this time point. Therefore, we selected this condition as drug-light interval in further PDT studies.

HPLC analysis of tissue extracts confirmed the presence of the expected photoactive Pb-GSGR fragment inside tumors (see Fig. 4). Concentration of this compound in tumor was 27 times higher than in the skin. Some smaller fragments with longer retention times presumably due to further proteolytic processing were also found in the tumor and also to a much smaller extend in the skin. In contrast, no photoactive fragments were found in muscle.

For PDT, 7.5 mg Pb equivalents/kg of prodrug was given to the mice via retro-orbital injection and 16 hours later, tumors were irradiated with 150 J/cm² at 665 ± 5 nm. The radiation intensity was 70 mW/cm². Animals receiving the drug alone or irradiated with light alone were used as controls. Figure 5A shows a sequence of images before and after PDT taken on one mouse, which ended up with complete remission. Bioluminescent images taken 15 minutes after administration of α-luciferin were used to quantify prostate cancer cells. On the average, a tumor volume of 200 mm³ corresponds to 2.5 × 10⁷ photon s⁻¹. Macroscopically, 1 day after treatment a local inflammatory response was visible. Inflammation developed into necrosis that appeared as a dark crust on the skin by day 3 and this was succeeded by healing and complete elimination of the tumor as confirmed by bioluminescence imaging. The absence of a bioluminescent signal, which persisted over 90 days, indicated complete destruction of the tumor-associated cells. Figure 5B summarizes the regions of interest analysis of sequences of images obtained for the 3 treatment regimes (PDT, drug alone, and light alone) until day 15 after treatment. Mice receiving both light and drug showed a 3 log reduction of tumor bioluminescence already the day after treatment. In this group, the mean bioluminescent signal remained below the initial value for at least 30 days (Supplementary Fig. S1). In contrast to PDT, light alone showed a slight reduction on tumor bioluminescence (P = 0.002). No reduction in bioluminescence was observed for animals...
representative traces of mouse tissues 16 hours after retro-orbital cleavage products were detected at all. Chromatograms show compounds were detected in skin in trace amounts. In muscle, no to the cleaved Pb extracts. In tumor, a major peak was found at 7.5 minutes, corresponding minutes are other minor cleavage Pb peptidyl fragments. The same compounds were detected in skin in trace amounts. In muscle, no cleavage products were detected at all. Chromatograms show representative traces of mouse tissues 16 hours after retro-orbital injection of uPA-PPP-4 (7.5 mg Pba equivalents/kg).

receiving prodrug alone ($P = 0.001$). In both control groups, we observed a 4-fold increase in tumor bioluminescence until day 15 after treatment, day at which the animals were euthanized. No significant difference between control groups could be established ($P = 0.6$).

The survival of mice treated with PDT, prodrug alone, and light alone is presented in Fig. 6. Animals treated with either prodrug alone or light alone had to be sacrificed before or on day 15 after treatment because of high tumor burden. The PDT survival curve was significantly different from these 2 groups ($P = 0.001$). Four animals that presented partial response to PDT were sacrificed on day 30 or 45 after treatment (57% survival). Complete remission to PDT treatment was observed in the 3 remaining mice (43% survival), which were sacrificed at the end of the study (90 days).

Discussion

Today, uPA is recognized as one of the key players in tumor progression in a wide panel of pathologies. Therefore, it has been identified as a target to specifically release cytotoxic agents. The first uPA-sensitive prodrug was reported by Chung and Kratz (22). It consisted of an albumin-bound doxorubicin containing a uPA substrate. This compound was stable in human plasma and the maximum-tolerated dose was 4.5-folds the dose of free doxorubicin as determined in a single nude mouse experiment. Subsequently, other uPA-sensitive prodrugs of TNF (23) and anthrax toxin (24) containing motifs recognized by uPA have been evaluated, providing in vivo evidence of potent antitumor effects. Recently, a doxorubicin analog was used for the development of an uPA-sensitive prodrug platform (25). The evaluation of one of these prodrugs in a variety of cancer cells lines showed a powerful inhibition of cell growth when activated in vitro.

The first polymeric photosensitizer prodrugs were developed by Choi and colleagues (26) for a more selective PDT. In this first-generation PPP, multiple copies of the photosensitizer are tethered to a protease-sensitive polymeric backbone (26, 27). A major drawback of these compounds is their limited selectivity, as all proteases recognizing a Lys-Lys motif are able to activate them. To circumvent this problem, a second-generation PPPs have been developed introducing a small peptide linker between the photosensitizer and the polymeric backbone (18). In this new design the linker-sequence is constructed according to the specific cleavage requirements of proteolytic enzymes of the target site.
Because of the known overexpression of uPA in prostate cancer (16, 28), we began to explore the potential of uPA-sensitive PPPs for a selective PDT of prostate cancer. From the known uPA-sensitive substrates, we have chosen the GSGRSAG peptide sequence for our PPPs (29). We have further shown a selective cleavage of uPA-PPPs by uPA in the test tube and in the prostate cancer cell lines DU145 and PC-3 overexpressing this protease (30). We have shown the selective cleavage of uPA-PPPs by uPA in the test tube. Sixteen hours after administration, tumors were irradiated with a light dose of 150 J/cm² at 665 ± 5 nm. The radiation intensity was 70 mW/cm².*, the study concluded after 90 days.

To further improve the therapeutic outcome, repetitive PDT can be envisaged to address the occasional partial response. This concept of repetitive PDT has been already studied in spheroids models, in vivo and in the clinic mostly for the treatment of brain cancer (35–39). According to these studies, the use of multiple sessions enhanced elimination of deep tumor cells infiltrating the surrounding brain. Combination treatments might also help to improve PDT efficacy. It is now widely accepted that stress induced through photodynamic insult in certain cases initiates signaling pathways, leading to VEGF increase in prostate cancer cells (40), which in turn contributes to tumor survival and regrowth. In this context,
PDT in combination with antiangiogenic agents for prostate cancer might result in an increased anticanic response.

Conclusions

We developed a uPA-sensitive prodrug that is not toxic to prostate cancer cells but efficiently inactivates cells in vitro after enzymatic activation and exposure to light. Activation of the prodrug occurs selectively in the tumors and is correlated with uPA overexpression. In vivo PDT can completely eliminate prostate cancer xenografts as shown by bioluminescence imaging. More research in orthotopic prostate cancer models is envisioned to confirm the potential advantages of our strategy over other current PDT approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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