The effect of photochemical internalization of bleomycin in the treatment of urothelial carcinoma of the bladder: An in vitro study

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Abstract

Objectives: In this in vitro study, we determined whether meso-tetraphenyl chlorin disulphonate (TPCS2a)-based photochemical delivery of bleomycin was able to potentiate the cytotoxicity of bleomycin on bladder cancer cells.

Materials and methods: The human RT4, RT112, 253J, T24, and rat AY-27 urothelial carcinoma cell lines were used. Cells were seeded in 96-well plates. TPCS2a was added to the growth medium and the plates were incubated overnight. Cells were then resuspended in TPCS2a-free culture medium and incubated for 3 hours. Subsequently, cells were treated for 60 minutes with increasing doses of epirubicin, gemcitabine, mitomycin C, or bleomycin followed by illumination for different periods. Cell viability was measured with a colorimetric assay after 72 hours.

Results: For the single treatments, in all 5 cell lines a dose-dependent inhibition of cell proliferation was observed. This was seen both after treatment with TPCS2a-based photodynamic therapy (PDT), as well as after treatment with either bleomycin or one of the control chemotherapeutic agents.

After treatment with PDT (240-s illumination), bleomycin 9.0 μM, and the combination of these treatments, relative survival percentages were 89.2 ± 11.0, 70.2 ± 8.9, and 30.5 ± 6.1, respectively, in the T24 cell line. After treatment with PDT (120-s illumination), bleomycin 27 μM and the combination of these treatments, relative survival percentages were 93.6 ± 15.7, 74.7 ± 9.6, and 30.0 ± 11.1, respectively, in the AY-27 cell line. In both cell lines, PDT combined with bleomycin showed significantly (P < 0.001) higher cell kill than the sum of the single treatments, suggesting a photochemical internalization effect.

Conclusions: TPCS2a-based photochemical internalization of bleomycin showed a significant, at least, additive antiproliferative activity against human and rat urothelial carcinoma cells in vitro. Thus, photochemical internalization may have therapeutic potential as an intravesical strategy against bladder cancer. As the effect is heterogeneous, biomarker studies are warranted to be able to predict the effects of a photochemical internalization-based treatment.

Keywords: Urinary bladder neoplasms; Photochemical internalization; Photodynamic therapy; Urothelial carcinoma; Cytotoxicity

1. Introduction

The initial treatment of non–muscle invasive bladder cancer (NMIBC) is transurethral resection, followed by intravesical therapy (i.e., chemotherapy or immunotherapy) [1]. However, intravesical therapy is not without toxicity, and a substantial percentage of treated patients still experience tumor recurrences or progression to muscle-invasive bladder cancer. Therefore, improved treatment modalities are urgently needed.

Photodynamic therapy (PDT) is a potential treatment modality for NMIBC. PDT involves the administration of a photosensitizer and its subsequent activation by light of an appropriate wavelength. The result is the destruction of cells containing the photosensitizer. Clinical trials with PDT have shown promising results in the treatment of bladder cancer [2,3]. Photochemical internalization (PCI) is a new technology that can be regarded as an enhanced PDT modality. PDT and PCI share many fundamental
photodynamic properties, but PCI acts as a light-directed drug-delivery system by triggered release of endocytosed macromolecules into the cytosol [4,5]. Thus, PCI can help therapeutic molecules reach their intracellular target of action, realizing their therapeutic potential, instead of being degraded by lysosomal hydrolases. The PCI effect is achieved by photosensitizing compounds specifically localizing in the membranes of endocytic vesicles, destroying these membranes by an oxidative process after illumination.

Bleomycin is used in multiple, standard cancer chemotherapy regimens, and has also been studied as intravesical treatment for NMIBC with only limited success [6,7]. The hydrophilic and relatively large chemical structure limits the ability of bleomycin to penetrate membranous structures, but in many cell types bleomycin can be taken up by endocytosis. In this case, bleomycin accumulates in endocytic vesicles, where it may be degraded; or it can enter slowly into the cytosol, where it can be degraded by bleomycin hydrolase before reaching its therapeutic target in the nucleus. However, bleomycin cytotoxicity is highly increased when the cytosol is exposed to similar amounts of bleomycin following electroporation [8]. Thus, bleomycin may become a very efficient and specific chemotherapeutic agent when it is combined with a treatment modality that activates its therapeutic potential only in the target environment. PCI of bleomycin inhibits tumor growth in different animal tumor models in a synergistic fashion [9,10].

Firstly, as bladder cancer is potentially well suited for effective treatment by PCI because it is easily accessible for both intravesical instillation and illumination, we studied the relative cell-kill effect of meso-tetraphenyl chlorin (TPCS2a)-based PDT [11] in 4 human bladder cancer cell lines and a rat bladder cancer cell line (anticipating future animal studies). Secondly, we studied whether TPCS2a-based photochemical delivery of bleomycin potentiated the cytotoxicity of bleomycin on these bladder cancer cell lines. Three commonly used antibladder cancer intravesical chemotherapeutic agents (i.e., epirubicin, gemcitabine, and mitomycin C) were used as controls.

2. Materials and methods

2.1. Cell lines and culture conditions

The human urothelial carcinoma (UC) cell lines RT4, RT112, 253J, T24 [17], and rat UC cell line AY-27 (kindly provided by Dr. Ronald Moore, University of Alberta and Cross Cancer Institute, Edmonton, Alberta, Canada) were grown as a monolayer culture in RPMI-1640 medium with l-glutamine (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), penicillin G (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen) at 37°C in a humidified 95% air/5% carbon dioxide atmosphere. Medium was changed twice weekly and cells were passaged using trypsin ethylenediaminetetra-acetic acid (Invitrogen) when confluent.

2.2. Chemotherapeutic- and TPCS2a-based PDT experiments

For TPCS2a-based photodynamic treatments, cells were harvested, washed, resuspended and plated at 1.0 × 10^4 cells per well in 96-well microtiter plates (Corning Inc., Corning, New York; black plate, clear bottom). TPCS2a (supplied by PCI Biotech AS, Oslo, Norway) was added at a final concentration of 0.2 μg/ml, and the plates were incubated overnight. Cells were then washed 3 times with TPCS2a-free culture medium, resuspended in TPCS2a-free culture medium and incubated for 4 hours. Cells were then illuminated for different periods (0–10 min), using a LumiSource illumination device (PCI Biotech AS, Oslo, Norway). LumiSource was delivered with a bank of 4 light tubes (4 × 18 W Osram L 18/67, Blue) emitting mainly blue light with a peak wavelength of approximately 420 nm, with an average irradiance of 13.5 mW/cm².

For combination therapy experiments, cells were prepared as described previously and after incubation in TPCS2a-free culture medium for 3 hours, cells were subsequently exposed to increasing doses of epirubicin (Pfizer bv, Capelle a/d IJssel, The Netherlands) (0–48.6 μM), gemcitabine (Sun Pharmaceutical Industries Europe B.V., Hoofddorp, The Netherlands) (0–65.6 μM), mitomycin C (Kyowa Hakko Kirin Co Ltd., Tokyo, Japan) (0–65.6 μM), or bleomycin (Euro Nippon Kayaku GmbH, Frankfurt, Germany) (0–81.0 μM). All solutions were prepared on the day of use. After incubation for 1 hour more, the cells were illuminated for different periods (0–5 min) as described earlier. After illumination, cells were washed 3 times with culture medium. Cell proliferation was measured after 72 hours. Treatment doses used in these studies were selected to evaluate whether additive effects occurred and were not optimized for treatment outcome.

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay

The effect of chemotherapy and PCI was determined by MTT assay. After 72 hours of treatment, 30 μl of 5 mg/ml MTT solution (Sigma-Aldrich) prepared in phosphate-buffered saline was added to the medium. Blue dye taken up by the cells after 4 hours of incubation was dissolved in dimethyl sulfoxide (100 μl per well) and optical density at 595 nm was read on an automated microplate reader (BioRad 3550, BioRad Laboratories, Hercules, CA). The bleomycin experiments were performed in quadruplicate and repeated one time by another laboratory worker; the control experiments with epirubicin, gemcitabine, and mitomycin C were performed at least once in quadruplicate.

2.4. Statistical analysis

In each combination experiment (with epirubicin, gemcitabine, mitomycin C, and bleomycin), the Wald test was
used to test for statistical significance of differences in relative cell survival between the treatment groups (control, PDT alone, chemotherapy alone, and combination). \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Chemotherapeutic- and TPCS2a-based PDT single treatment effects

PDT alone showed a clear phototoxic illumination time-dependent inhibition of cell proliferation in all 5 cell lines (Fig. 1). The viability of all cell lines was reduced to \(< 40\%\) of control with 360-second illumination. A further increase of the light dose resulted in approximately 99\% cell death at 600 seconds (data not shown).

TPCS2a pretreatment followed by chemotherapy treatment alone (without illumination) demonstrated a clear dose-dependent inhibition of cell proliferation in all 5 cell lines tested (Fig. 2).

3.2. Chemotherapeutic- and TPCS2a-based PDT combination effects

To evaluate the PCI therapy effect, chemotherapy was combined with TPCS2a and light treatment (Fig. 2). Combination of PDT and bleomycin was clearly superior to the single treatment modalities in the human T24 cell line and the rat AY-27 cell line, indicating at least an additive effect, suggesting a PCI effect. In contrast, additive treatment effects were observed for the other treatment regimens and in the other cell lines, as indicated by the parallel dose-response curves.

The treatment effects of PDT combined with bleomycin and PDT combined with epirubicin, gemcitabine, or mitomycin C in the T24 and AY-27 cell lines are shown in Fig. 3. To be able to compare the 4 individual chemotherapeutic agents, we selected the chemotherapeutic doses with a relative cell survival of \(> 70\%\): epirubicin 5.4 \(\mu\)M, gemcitabine 0.8 \(\mu\)M, mitomycin C 65.6 \(\mu\)M, and bleomycin 9.0 \(\mu\)M for the T24 cell line and epirubicin 0.2 \(\mu\)M, gemcitabine 0.3 \(\mu\)M, mitomycin C 0.8 \(\mu\)M, and bleomycin 27 \(\mu\)M for the AY-27 cell line. As shown in Fig. 3, PDT combined with any chemotherapeutic drug showed a significantly higher cell kill compared with PDT alone in both cell lines (\(P < 0.05\)). Similarly this combination was superior to chemotherapy alone (\(P < 0.05\)), except for epirubicin and gemcitabine treatment in the AY-27 cell line. PDT combined with bleomycin showed significantly (\(P < 0.001\)) higher cell kill than the sum of the single treatments in both cell lines, suggesting a PCI effect.

4. Discussion

In this in vitro study, we evaluated the therapeutic potential of TPCS2a-based photochemical delivery of bleomycin in 4 human bladder cancer cell lines and a rat bladder cancer cell line. In the T24 and AY-27 cell lines, a significant and at least additive antiproliferative effect of the combined PDT and bleomycin treatment was observed, a clear indication of the PCI effect. For all the single treatment regimens, a clear dose-dependent inhibition of cell proliferation was observed for all 5 cell lines.

Chemotherapeutics require efficient penetration into the cytosol of the tumor cells to reach their intracellular targets and exert their therapeutic activity. The prerequisite to penetrate the plasma membrane limits the exploitable chemical structure of chemotherapeutic agents to mostly small and lipophilic compounds penetrating the plasma membrane by passive diffusion. In addition, drugs (also more hydrophilic drugs) can be taken up by means of various transporters, e.g., gemcitabine [12].

The PDT mechanism relies on the in situ generation of cytotoxic agents by the activation of a light-sensitive drug, resulting in cell death [13]. The PCI technology is based on the same principles as PDT, i.e., the activation of a photosensitizer by light followed by formation of reactive oxygen species. Unlike PDT, the concept of PCI is based on the use of designed photosensitizers that localize preferentially in the membranes of endocytic vesicles of the targeted cells. The most efficient PCI photosensitizers have an amphiphilic structure (e.g., TPCS2a [11]) with a hydrophilic part inhibiting penetration through cellular membranes. The site-specific PCI-induced drug delivery adds to the well-described cytotoxic [13], vascular, and immunostimulatory effects of PDT. PCI of bleomycin has already shown to induce a synergistic inhibition of tumor growth in vivo [9,10]. Adigbli et al. [14] demonstrated an increased cytotoxic (i.e., PCI) effect of the chemotherapeutic agent, mitoxantrone, combined with the photosensitizer hypericin and illumination against multidrug-resistant bladder cancer
Fig. 2. Relative survival of human 253J, RT4, RT112, and T24 and rat AY-27 urothelial carcinoma cells after TPCS2a-based photodynamic treatment in combination with epirubicin, gemcitabine, mitomycin C, or bleomycin. The cells were incubated with TPCS2a at 0.2 µg/ml for 24 hours, washed and incubated for 3 more hours in TPCS2a-free culture medium and subsequently treated with an increasing dose of epirubicin, gemcitabine, mitomycin C, or bleomycin. After incubation for 1 more hour, the cells were illuminated with increasing illumination times (0–300 s). Cells were then washed and incubated again. Relative cell survival was assessed 72 hours later. The percentage (mean ± SD) of viable cells from the corresponding control is shown. The curves with 0 seconds of illumination represent the dose-response curves after treatment with chemotherapy alone. SD = standard deviation.
The effect of bleomycin varies widely between different tumors and cell lines. It has been well established that the cytotoxic effect would be greatest in areas that are illuminated, decreasing systemic toxicity [15], in line with the cytotoxic effect seen for the other chemotherapeutics are most likely the consequence of their lower molecular weight and higher hydrophilicity, or their cellular uptake is dependent on the activity of transport proteins, promoting easier cytoplasmic accumulation.

Only in the T24 and AY-27 cell lines, the treatment effects of PDT combined with bleomycin were significantly higher than the sum of the single treatments. The heterogeneous response of the bladder cancer cell lines is probably a reflection of the heterogeneity of the disease. It is possible that the endocytosis and transport mechanisms of bleomycin or TPCS2a or both differ between the various cell lines. It has been well established that the cytotoxic effect of bleomycin varies widely between different tumors as well as between different organs [16]. This might be due to differences in the cells’ DNA-repair capacity, bleomycin hydrolase activity, cellular uptake mechanisms, and possibly the rate of bleomycin efflux. In our experiments, TPCS2a represents an additional element. It is possible that different cell types use different endocytosis pathways leading to intracellular TPCS2a accumulation resulting in cell death without endosomal release. Another possible factor for the heterogeneous antiproliferative effects are the cellular resistance mechanisms to bleomycin. A better understanding of tumor biology and pathways critical for tumor genesis may provide personalized treatment opportunities for patients with urothelial cancer.

In conclusion, TPCS2a-based PCI of bleomycin showed a significant, at least, additive antiproliferative activity against human and rat UC cells in vitro. Therefore, PCI may have therapeutic potential as an intravesical strategy against NMIBC, provided patient stratification can be achieved based on a predictive biomarker (panel). Further studies are needed to explore whether such predictive biomarkers can be defined.

References


