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Chlorin-based photodynamic therapy enhances the effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in bladder cancer cells

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Photodynamic therapy (PDT) is an attractive, emerging therapeutic procedure suitable for the treatment of non-muscle-invasive bladder cancer. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a death ligand that belongs to the TNF superfamily of cytokines. The ability of TRAIL to selectively induce apoptosis in cancer cells but not in normal cells promotes the development of TRAIL-based cancer therapy. However, many tumor cells are resistant to TRAIL-induced apoptosis. The purpose of the study was to overcome TRAIL-resistance in bladder cancer cells by photodynamic therapy (PDT).

Material/Methods: Three human bladder transitional cancer cell lines – T24, 647V and SW780 – were treated with TRAIL and/or chlorin-based PDT. The cytotoxicity was measured by MTT and LDH assays and apoptosis was detected using annexin V by flow cytometry.

Results: Our test confirmed that T24 and 647V bladder cancer cells are resistant to TRAIL, whereas SW780 cells are sensitive to TRAIL. Then we examined the cytotoxic and apoptotic effects of TRAIL in combination with chlorin e6-polyvinylpyrrolidone (Ce6-PVP)-mediated PDT on bladder cancer cells. We showed for the first time that pretreatment with a low dose of PDT significantly sensitizes bladder cancer cells to TRAIL-induced apoptosis. Chlorin-based PDT augments the effect of TRAIL on bladder cancer cells.

Conclusions: PDT with Ce6-PVP photosensitizer enhances the cytotoxic and apoptotic effects of TRAIL on bladder cancer cells. The obtained results suggest that combined treatment by TRAIL and PDT may provide the basis for a new therapeutic approach to induce cell death in bladder cancer.

key words: **PDT • TRAIL • apoptosis • TCC • bladder cancer**

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Background

Transitional cell carcinoma (TCC) of the bladder is the second most common malignancy of the genitourinary tract and the second most common cause of death of all genitourinary tumors [1–3]. Approximately 75–80% of patients with primary bladder cancer present low grade tumors confined to the superficial mucosa. Treatment of non-muscle-invasive bladder tumors is based mainly on transurethral resection combined with intravesical chemo- or immunotherapy [4,5]. Although most cases of bladder cancer are non-muscle-invasive, up to 70% of patients have recurrences and 20% progress to invasive disease [2,3]. Photodynamic therapy (PDT) has been investigated as a new, minimally invasive, alternative modality for non-muscle-invasive bladder tumors. TCC of the bladder is an ideal tumor for PDT because endoscopic access is convenient with intravesically photosensitizer application and good light penetration in cancer cells during irradiation [6,7].

Photodynamic therapy consists of an interaction between a photosensitizer and absorbed light. The extent of photocytotoxicity after PDT is dependent on the photosensitizing molecule, its administered dose, the type of tumor, the light fluence rate and the total light exposure dose [8-10]. The formulation of chlorin e6-polyvinylpyrrolidone (Ce6-PVP), known as Photolon or Fotolon, is a new generation of photosensitizers investigated for their application in PDT [11]. The *in vitro* and *in vivo* studies on bladder cancer models have shown the efficiency of chlorin-based PDT [11–13]. Ce6-PVP demonstrated high sensitivity and specificity for cancers and ability to induce cell death in tumors following PDT without animal toxicity [13]. Lee et al. reported for the first time the clinical use of Ce6-PVP in patients with high risk non-muscle-invasive bladder cancer [14]. Three separate processes contribute to PDT-induced tumor destruction: direct cancer cell death, destruction of tumor vasculature causing tumor ischemia, and activation of an immune response [15–19]. The mechanism of tumor cell killing by PDT depends on the photosensitizer concentration and the light fluence. Low-dose PDT triggers apoptotic cell death, whereas high-dose PDT predominantly causes necrotic cell death [15,19,20].

Apoptosis has been reported as the main mode of low-dose PDT-mediated cell death [15,20]. PDT as well as the members of the tumor necrosis factor (TNF) superfamily mediate apoptosis and may share common intracellular signaling pathways leading to programmed cell death. The tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a death ligand that belongs to the TNF superfamily of cytokines. TRAIL was identified as a powerful activator of apoptosis in tumor cells with no toxicity against normal tissues. TRAIL triggers apoptosis in cancer cells by the activation of death receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5) [21,22]. The recombinant form of TRAIL (rhsTRAIL) or monoclonal antibodies against the TRAIL-R1 and/or TRAIL-R2 (AMG655, CS1008, Mapatumumab, Lexatumumab, Conatumumab) are recently discovered targeted therapeutics in clinical trials. The phase I and phase II studies showed limited toxicity and significant tumor responses after the application of TRAIL [23–25]. However, some tumor cells are resistant to TRAIL-induced apoptosis. The decreased expression of death receptors or proapoptotic proteins and increased expression of anti-apoptotic proteins in cancer cells were involved in TRAIL-resistance [26–28].

TRAIL activity can be augmented through the use of other anticancer agents. *In vitro* and *in vivo* studies have shown that chemotherapeutic drugs or ionizing radiation sensitize cancer cells to TRAIL-induced apoptosis [29–32]. The aim of this study was to enhance apoptotic activity of TRAIL against bladder cancer cells by PDT. We have investigated the cytotoxic and apoptotic effects of TRAIL in combination with low dose of Ce6-PVP mediated PDT in bladder cancer cells. This evidence supports for the first time the therapeutic potential of TRAIL application with PDT against cancer cells derived from solid tumors.

Material and Methods

Bladder cancer cell cultures

The tests were performed on 3 human bladder transitional cancer cell (TCC) lines derived from bladder tumor obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH - German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and ATCC (American Type Culture Collection, Manassas, VA, USA): SW780 cell line – well differentiated transitional cells (G1), 647 cell line – moderately differentiated transitional cells (G2), and T24 cell line – poorly differentiated transitional cells (G3). The bladder cancer cells were grown in monolayer cultures: SW780 cells in Leibovitz's, 647V and T24 cells in DMEM. All media were supplemented with 10% of heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were incubated at 37°C, SW780 cells in 100% air atmosphere, 647V and T24 cells in 95% air atmosphere and 5% CO_2 [28,32,33]. All reagents for bladder cancer cell culture were purchased from PAA The Cell Culture Company (Pasching, Austria).

PDT

Photosensitizer

Chlorin e6-polyvinylpyrrolidone (Ce6-PVP), known as Photolon, was a kind gift from Professor Wieslaw Strek. Stock solutions of Ce6-PVP were prepared in deionized water. Before incubation with bladder cancer cells, further dilutions were made with medium to obtain final concentrations as indicated.

In vitro PDT

Bladder cancer cells were seeded in 96-well plates for 24 hours. Under low light conditions the cells were incubated with Ce6-PVP at the final concentrations of 2–4 µM for 2 hours, then the medium was removed and the cells were washed twice with PBS (phosphate-buffered saline solution). The cells were then irradiated with VIS (400–750 nm, 10 J/cm^2) delivered from the incoherent light source PDT TP-1 (Cosmedico Medizintechnik GmbH, Schwenningen, Germany) [34].

TRAIL

Soluble recombinant human TRAIL (rhsTRAIL) was purchased from PeproTech Inc. (Rocky Hill, NJ, USA).

Cytotoxicity assay

Cytotoxicity was measured by the 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay as previously described [35-38]. The MTT assay is based on the cleavage of the tetrazolium salt MTT to the blue formazan dye by viable cells. The bladder cancer cells (10^6/ml) were seeded for 24 hours before the experiments in a 96-well plate. The TCC cells were pretreated with Ce6-PVP mediated PDT. After 4 hours, the culture medium was removed and 50–100 ng/ml TRAIL was added to the cells for 16 hours. Next, the 20 µl of MTT solution (5 mg/ml) (Sigma Chemical Company, MO, USA) was added to each well for 4 hours. The resulting blue formazan crystals were dissolved in DMSO. Controls included native cells and medium alone. The spectrophotometric absorbance at 550 nm was measured using a microplate reader (ELx 800, Bio-Tek Instruments Inc., Winooski, VT, USA). The percent cytotoxicity was calculated by the formula: percent cytotoxicity (cell death) = (1– [absorbance of experimental wells/absorbance of control wells]) × 100%.

Lactate dehydrogenase release assay

Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon membrane damage in necrotic cells. LDH activity was measured using a commercial cytotoxicity assay kit (Roche Diagnostics GmbH, Mannheim, Germany), in which the LDH released in culture supernatants is measured with a coupled enzymatic assay, resulting in the conversion of a tetrazolium salt into a red formazan product. The TCC cells were pretreated with Ce6-PVP mediated PDT and then incubated with 50–100 ng/ml TRAIL for the indicated period of time. The sample solution (supernatant) was removed, and the LDH released from the cells into the culture medium was detected. The spectrophotometric absorbance at 490 nm wavelength was measured using a microplate reader. The maximal release was obtained after treating control cells with 1% Triton X-100 (Sigma Chemical Company, St. Louis, MO, USA) for 10 minutes at room temperature [39–43]. The necrotic percentage was expressed using the formula: (sample value/maximal release) × 100%.

Detection of apoptosis by flow cytometry

Apoptosis was measured using flow cytometry to quantify the levels of phosphatidylserine (PS) on the outer membrane of apoptotic cells. Externalized PS on the outer surface of the cytoplasmic membrane becomes labelled by annexin V-FITC, which has a high affinity for PS-containing phospholipids bilayers. The annexin V assay was performed using the Apoptest-FITC Kit (Dako, Glostrup, Denmark). The TCC cells (10^6/ml) were seeded in 24-well plates for 24 hours and then exposed to PDT and/or 50–100 ng/ml TRAIL for 16 hours. After this time, the cancer cells were washed twice with PBS and resuspended in 1 ml of binding buffer. The cell suspension (500 µl) was then incubated with 5 µl of annexin V-FITC and 10 µl of propidium iodide (PI) for 10 minutes at room temperature in the dark. The population of annexin V-positive cells was evaluated by flow cytometry (BD LSRII, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) [44–46].

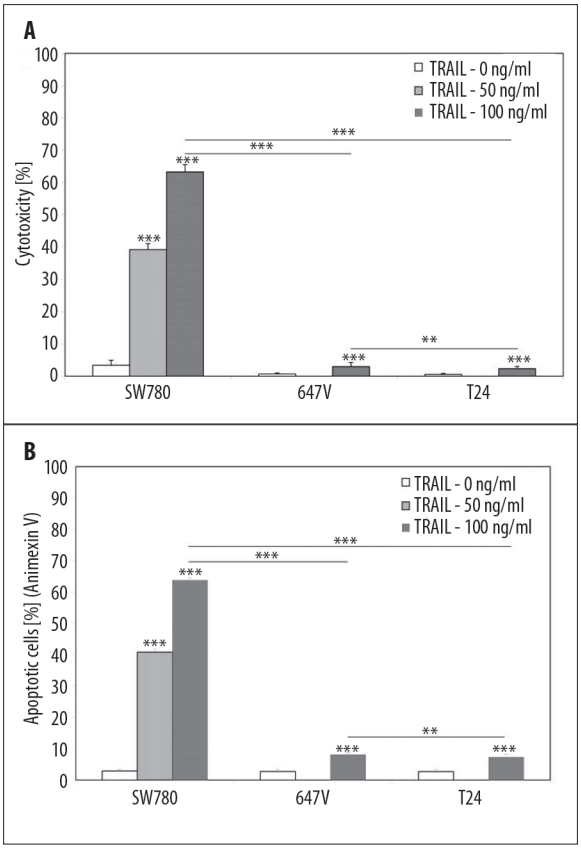

Figure 1. Cytotoxic and apoptotic effects of TRAIL on bladder cancer cells. Cells were incubated for 16 hours with TRAIL at concentrations of 50–100 ng/ml. (**A**) Cytotoxic activity of TRAIL in bladder cancer cells. The percentage of dead cells was measured by the MTT cytotoxicity assay. (**B**) TRAIL induced apoptosis in bladder cancer cells. Apoptotic cell death was detected by annexin V-FITC staining using flow cytometry. The values represent mean±SD of three independent experiments performed in quadruplicate for cytotoxicity n=12 or duplicate for apoptosis n=6 (*** $P<0.001$ compared with the corresponding control and ** $P<0.01$ or *** $P<0.001$ compared between cell types).

Statistical analysis of results

The results are expressed as mean ±S.D. obtained from 3 independent experiments performed in quadruplicate (n=12) or duplicate (n=6). Statistical significance was evaluated using the Mann-Whitney U test and Student's T test, followed by analysis of variance (ANOVA). A *P*-value <0.05 was considered significant.

Results

Cytotoxic and apoptotic effects of TRAIL on bladder cancer cells

The cytotoxic and apoptotic effects of TRAIL on bladder cancer cells are demonstrated in Figure 1. The cytotoxicity of TRAIL at the concentrations of 50–100 ng/ml was 39.2±1.8–63.2±2.3% for SW780. The cytotoxicity of TRAIL at the concentration of 100 ng/ml was 2.9±1.3% for 647V cells

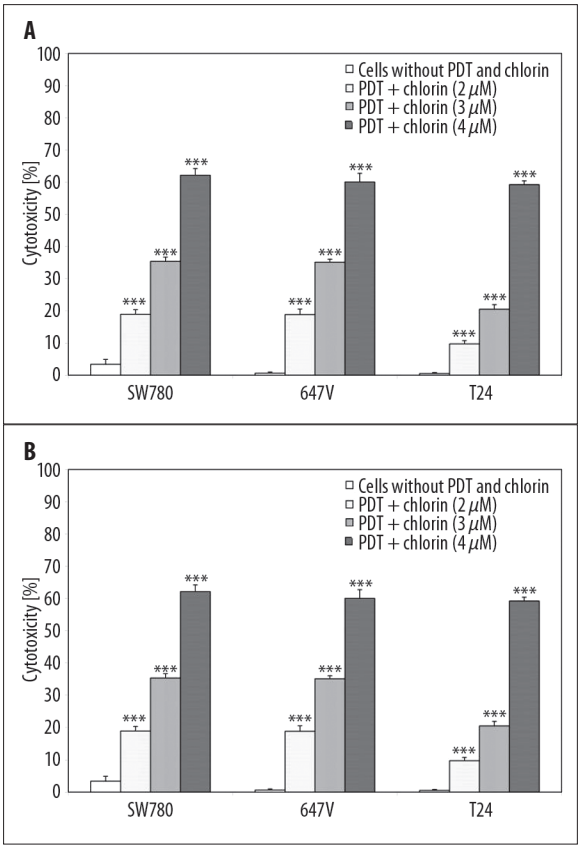

Figure 2. Cytotoxic and apoptotic effects of chlorin-based PDT on bladder cancer cells. Cells were incubated with 2–4 μM Ce6-PVP for 2 hours and subsequent light irradiation of 10 J/cm^2). (**A**) Cytotoxic activity of chlorin-based PDT in bladder cancer cells. The percentage of dead cells was measured by the MTT cytotoxicity assay. (**B**) Chlorin-based PDT induced apoptosis in bladder cancer cells cells. Apoptotic cell death was detected by annexin V-FITC staining using flow cytometry. The values represent mean ±SD of three independent experiments performed in quadruplicate for cytotoxicity n=12 or duplicate for apoptosis n=6 (*** $P<0.001$ compared with the corresponding control).

and 2.3±0.8% for T24 cells. TRAIL-induced apoptosis in bladder cancer cells was determined by annexin V staining. The exposure of SW780 cells to 50–100 ng/ml TRAIL increased the percentage of apoptotic cells to 40.8±0.9–63.8±0.9%. SW780 cells were sensitive to TRAIL. In contrast, the 2 other bladder cancer cell lines, 647V and T24, were resistant to TRAIL-mediated apoptosis. The treatment with 100 ng/ml TRAIL resulted in 8.1±0.7% apoptotic cells for 647V cells and 7.3±0.9% for T24 cells. The necrotic cell death percentage of TCC cells examined by Apoptest-FITC, and the LDH assay was near zero. TRAIL concentrations higher than 100 ng/ml resulted in no significant increase in cytotoxic and apoptotic activities against cancer cells.

Cytotoxic and apoptotic effects of chlorin-based PDT on bladder cancer cells

Treatment of bladder cancer cells with 2–4 μM Ce6-PVP and subsequent irradiation with VIS (400–750 nm, 10 J/cm^2) showed a dose-dependent increase of cytotoxicity, whereas photosensitizer or VIS alone had no effect. The photoactivated Ce6-PVP showed a similar cytotoxicity in all TCC cells lines. The photocytotoxic effects of PDT with 2 μM, 3 μM, 4 μM Ce6-PVP were: 18.9±1.4%, 35.4±1.3% and 62.1±2.1% for SW780 cells; 18.8±1.7%, 35.1±0.9% and 60.1±2.7% for 647V cells; 9.7±1.0%, 20.5±1.4% and 59.2±1.2% for T24 cells (Figure 2A). The necrotic cell death percentage of bladder cancer cells examined after PDT with Ce6-PVP at the concentration of 2 μM and 3 μM by Apoptest-FITC and the LDH assay was near zero. PDT with 4 μM Ce6-PVP induced apoptosis and partially necrosis in bladder cancer cells. During further tests, PDT with Ce6-PVP at the concentration of 2 μM and 3 μM (low dose PDT) was used. Chlorin-based PDT increased apoptotic cells to 18.8±1.0–29.6±1.1% for SW780 cells, to 17.2±0.8–25.9±0.9% for 647V and to 10.3±0.9–19.6±1.0% for T24 cells (Figure 2B).

Cytotoxic and apoptotic effects of combined treatment of TRAIL with chlorin-mediated PDT on bladder cancer cells

The bladder cancer cells were pretreated with a low dose of PDT (Ce6-PVP at concentrations of 2–4 μM and VIS: 400–750 nm, 10 J/cm^2) and 4 hours after irradiation a subsequent incubation of TCC cells with TRAIL at the concentration of 50 ng/ml (TRAIL-sensitive SW780 cells) or 100 ng/ml (TRAIL-resistant 647V and T24 cells) was done for 16 hours. The cytotoxic and apoptotic effects of TRAIL with chlorin-based PDT against bladder cancer cell lines are shown in Figures 3 and 4. The combined treatment of TRAIL and PDT with Ce6-PVP at concentrations of 2–4 μM induced cytotoxic activity against bladder cancer cells as follows: 54.8±3.5–85.1±1.3% for SW780, 26.5±1.1–87.7±1.1% for 647V, 20.5±1.4–87.6±1.2% for T24 cells. The cytotoxicity of TRAIL with low dose PDT in TCC cells was causing through apoptosis. Therefore, in further tests Ce6-PVP at the concentration of 2 μM and 3 μM (low dose PDT) was applied in combination with TRAIL. We found that chlorin-mediated PDT strongly cooperated with TRAIL to induce apoptosis in cancer cells. The percentage of the apoptotic cells stained with annexin V-FITC detected by flow cytometry after pretreatment with PDT with Ce6-PVP at concentrations of 2–3 μM and subsequent TRAIL exposure was elevated to 57.1±0.9–84.2±0.8% in SW780 cells, to 33.4±0.9–74.6±1.1% in 647V cells and to 26.5±1.0–73.4±1.0% in T24 cells. The necrotic cell death percentage of bladder cancer cells examined by Apoptest-FITC and the LDH assay was near zero. Chlorin-based PDT significantly augmented TRAIL-induced cell death in all bladder cancer lines. PDT with Ce6-PVP enhanced the apoptosis-inducing potential of TRAIL and sensitized TRAIL-resistant 647V and T24 bladder cancer cells.

Discussion

TRAIL was discovered independently by 2 groups, Wiley et al., 1995 and Pitti et al., 1996, and since this time it has been intensively investigated as a potent antitumor agent in preclinical and clinical studies [22,25]. However, a variety of cancer cells have different sensitivity or resistance to TRAIL-induced cytotoxicity. During the TRAIL application, only the TRAIL-sensitive cancer cells are deleted and cancer cells with acquired TRAIL resistance develop. The expression of death receptors and proapoptotic or antiapoptotic

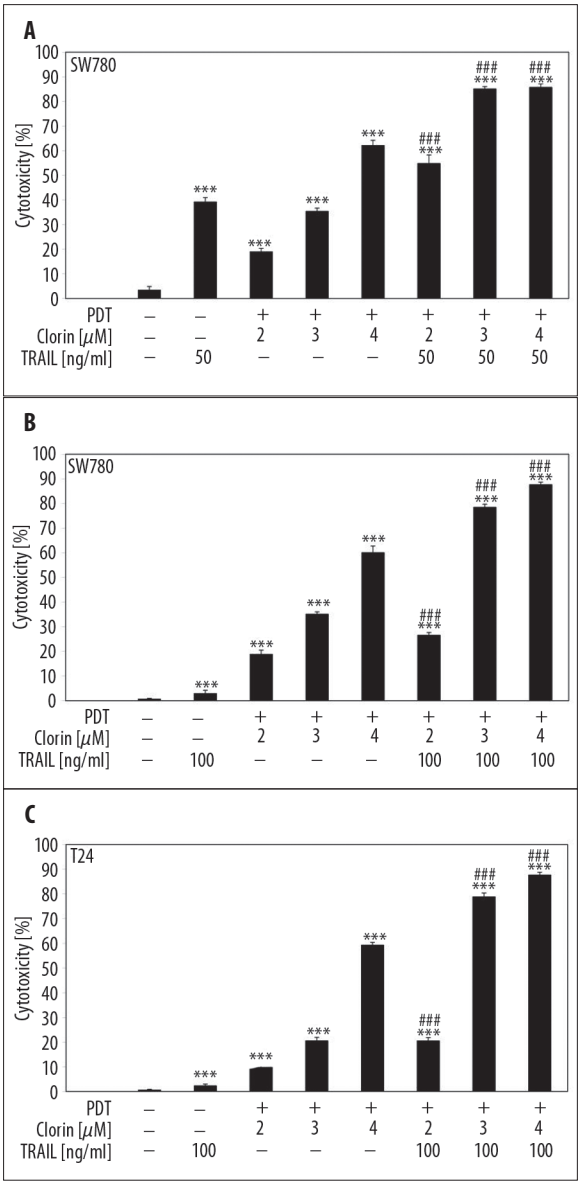

Figure 3. Cytotoxic effect of TRAIL in combination with chlorin-based PDT on bladder cancer cells. Cells were pretreated with chlorin-based PDT (2–4 μM Ce6-PVP; light irradiation of 10 J/cm^2) and then incubated for 16 hours with TRAIL at concentrations of 50 or 100 ng/ml. Cytotoxic activity of TRAIL in combination with chlorin-based PDT in (**A**) SW780, (**B**) 647V and (**C**) T24 bladder cancer cells. The percentage of dead cells was measured by the MTT cytotoxicity assay. The values represent mean ±SD of three independent experiments performed in quadruplicate n=12 (*** $P<0.001$ compared to control, ### $P<0.001$ compared to TRAIL).

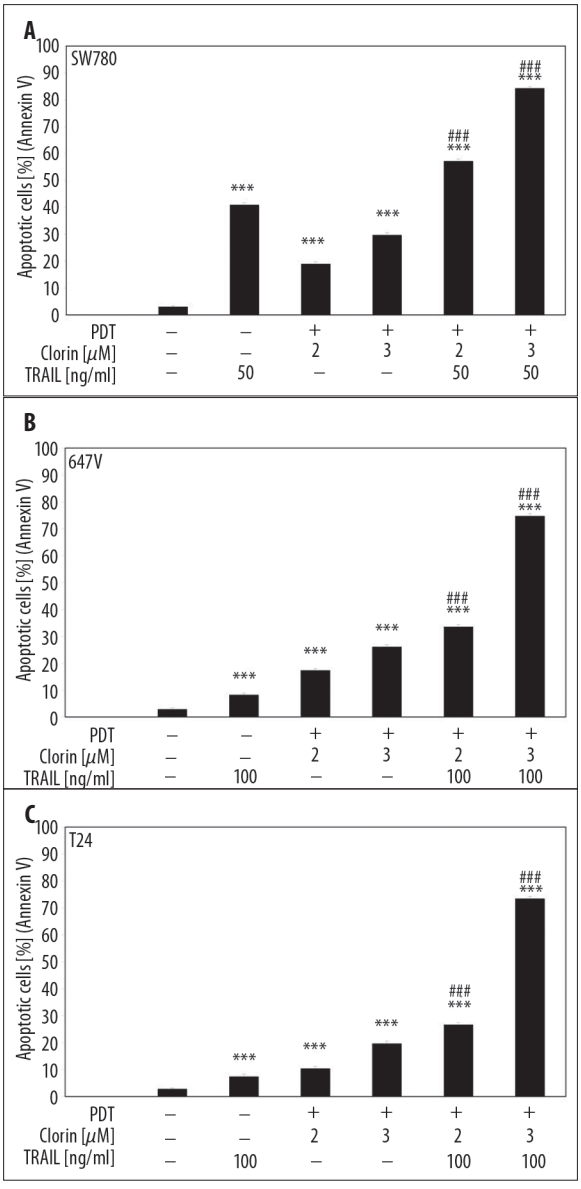

Figure 4. Apoptotic effect of TRAIL in combination with chlorin-based PDT on bladder cancer cells. Cells were pretreated with chlorin-based PDT (2–3 μM Ce6-PVP; light irradiation of 10 J/cm^2) and then incubated for 16 hours with TRAIL at concentrations of 50 or 100 ng/ml. TRAIL-induced apoptosis in combination with chlorin-based PDT in (**A**) SW780, (**B**) 647V and (**C**) T24 bladder cancer cells. Apoptotic cell death was detected by annexin V-FITC staining using flow cytometry. The values represent mean ±SD of three independent experiments performed in duplicate n=6 (*** $P<0.001$ compared to control, ### $P<0.001$ compared to TRAIL).

proteins in cancer cells is involved in TRAIL resistance [26–28]. New treatment modalities have been designed to destroy TRAIL-resistant tumors. TRAIL resistance in bladder cancer cells can be overcome by a combination of TRAIL with chemotherapeutic drugs or ionizing radiation [29–32]. Our research focused on the combined effect of TRAIL with PDT on bladder cancer cells. The principle of PDT is based on the fact that photosensitizer is selectively absorbed and retained by malignant tissues. Recently, there has been abundant evidence that PDT can trigger apoptosis both *in vitro* and *in vivo* [8,9,15,19,20]. TRAIL is a cytokine able to induce apoptosis in cancer cells with no toxicity to normal cells [21–24]. The ideal anticancer therapy should selectively kill cancer cells without harming normal cells. Therefore, PDT and TRAIL showed the same valuable properties, tumor-specific affinity and apoptotic activity against

cancer cells. PDT may influence the sensitivity of cancer cells to TRAIL-mediated apoptosis.

The management of urologic malignancy was one of the earliest clinical applications of PDT. Numerous findings confirmed that PDT could be effectively and safely used in treatment of non-muscle-invasive bladder cancer. The photosensitizer is deposited directly in bladder cancer lesion, and the urinary bladder allows good conditions for irradiation. Therefore, PDT of bladder tumors has a good therapeutic prospect [4–7]. From the clinical point of view, the bladder cancer cell lines are a proper cellular model suitable to explore PDT. There is great interest in the synthesis of new generation photosensitizers with improved PDT characteristics. Chlorins are particularly strong under investigations in preclinical and clinical fluorescence diagnostics and photodynamic therapy of bladder tumors. Formulation of chlorin e6 in combination with the hydrophilic polyvinylpyrrolidone exhibited *in vitro* and *in vivo* high fluorescence intensity and selective phototoxicity against bladder cancer [11,13,47]. Ce6-PVP showed efficacy and safety for use in patients with bladder cancer. Lee et al. presented the first clinical experience with PDT using intravesically applied Ce6-PVP for transitional bladder cancer. The results of a pilot study suggest that Ce6-PVP-based PDT is a promising technique in treating high risk and refractory non-muscle-invasive transitional bladder cancer [14].

Our tests were performed on 3 human bladder transitional cancer cell lines: SW780, 647 and T24 cells. The moderately differentiated transitional 647V cells and poorly differentiated transitional T24 cells were resistant to TRAIL, in contrast to well differentiated transitional SW780 cells sensitive to TRAIL-induced cytotoxicity. We examined the effect of TRAIL in combination with chlorin-based PDT on bladder cancer cells. PDT could induce cell death through both apoptosis and necrosis in a PDT dose-dependent manner [19,20]. We have used only low dose PDT (Ce6-PVP at the concentrations of 2 μM and 3 μM) in combination with TRAIL to trigger apoptotic death in bladder cancer cells. Application of PDT with 4 μM Ce6-PVP (high dose PDT) caused also partial necrosis. Necrotic cell death involved inflammatory mediators released in the course of inappropriate pathological response [19]. Our studies confirmed that pretreatment of bladder cancer cells with a low dose of PDT significantly augments TRAIL-induced cell death. PDT with Ce6-PVP sensitizes TRAIL-resistant 647V and T24 bladder cancer cells to TRAIL-induced apoptosis.

The combined effect of TRAIL and low dose PDT was previously described only once by Granville et al. in human Jurkat lymphoma cells, indicating that the TNF family members, TRAIL and Fas ligand augment the apoptotic activity of PDT with verteporfin in Jurkat cells [48]. We investigated for the first time the apoptotic and cytotoxic effects of the combined treatment of TRAIL with PDT on cancer cells derived from solid tumors. Our results demonstrated that PDT enhanced the potential of TRAIL in bladder cancer cells by about 30% compared to PDT or TRAIL alone. The use of TRAIL with PDT in our study aimed to reduce the effective dosage for these agents and to lower their adverse effects in normal cells. The mechanism by which PDT acts on TRAIL-induced cell death of bladder cancer is not clear and a further study are required to examine the signaling pathways by which PDT sensitizes bladder cancer cells to TRAIL-induced apoptosis. The combined therapy of TRAIL with PDT in the future may be a potential method for treating non-muscle-invasive bladder cancer and preventing recurrence or cancer progression.

Conclusions

Chlorin-based photodynamic therapy might enhance the effect of TRAIL in bladder cancer cells. Pretreatment with low dose of chlorin e6-polyvinylpyrrolidone-mediated PDT significantly sensitizes TRAIL-resistant cancer cells to TRAIL-induced apoptosis. The obtained results suggest that the combined treatment of TRAIL and PDT may provide the basis for a new therapeutic approach to induce cytotoxicity in bladder cancer cells.

Acknowledgements

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Abbreviations

ATCC – American Type Culture Collection; **Ce6-PVP –** chlorin e6-polyvinylpyrrolidone; **DMSO –** dimethylsulfoxide; **DMEM –** Dulbecco's Modified Eagle Medium; **DR –** death receptor; **DSMZ –** Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures); **FACScan –** fluorescent activating cell scan; **FBS –** fetal bovine serum; **FITC –** fluorescein isothiocyanate; **LDH –** lactate dehydrogenase; **MTT –** 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide; **PBS –** phosphate-buffered saline solution; **PDT –** photodynamic therapy; **PI –** propidium iodide; **PS –** phosphatidylserine; **SD –** standard deviation; **TCC –** transitional cell carcinoma; **TNF –** tumor necrosis factor; **TRAIL –** tumor necrosis factor-related apoptosis-inducing ligand; **VIS –** visible.

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