# Effect of Photodynamic Therapy with BPD-MA on the Proliferation and Apoptosis of Human Bladder Cancer Cells

**Chuanshan Xu**[1,4]
**Shiming Wu**[2]
**Zhigang Wang**[1]
**Lehua Yu**[3]
**Qing Yang**[4]

[1] Institute of Ultrasound Imaging, Chong qing University of Medical Sciences, Chongqing 400010, China.
[2] Xinqiao Hospital, The Third Military Medical University, Chongqing 400037, China.
[3] Department of Laser Medical Research, Second Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400010, China.
[4] Southwest Hospital, The Third Military Medical University, Chongqing 400038, China.

Correspondence to: Chuanshan Xu
Tel: 86-23-6060-6025
Fax: 86-23-6381-1304
E-mail: xcshan@163.com

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**OBJECTIVE** To explore the effect of photodynamic therapy with benzoporphyrin derivative monoacid ring A (BPD-MA) on the proliferation and apoptosis of human bladder cancer cells.

**METHODS** Photosensitization of BPD-MA was activated with a red light laser (632.8 nm) delivered at 10 mw/cm$^2$ to give a total dose of 2.4 J/cm$^2$. Cellular proliferative activity was measured using the 3 -(4,5 - dimethylethiazil-2-yl)-2,5-Diph3-eyl tetrazolium bromide (MTT) assay and $^3$H-thymidine incorporation. Cell apoptosis was determined with flow cytometry analysis and the terminal deoxyuridine nicked -labeling (TUNEL) assay.

**RESULTS** At 24 h post photodynamic treatment, photodynamic therapy significantly decreased cellular proliferative activity. The rate of apoptosis in BIU-87 cells 8 h after photodynamic treatment significantly increased up to 26.11± 2.59% as analyzed with flow cytometry. In situ labeling of DNA cleavage products with the terminal deoxyuridine nicked-labeling (TUNEL) assay reinforced these observations, BPD-MA-mediated photosensitization increased the number of TUNEL-positive cells compared to the controls. However, laser irradiation alone, BPD-MA alone and sham radiation did not affect cellular proliferative activity or apoptosis of the human bladder cancer BIU-87 cells.

**CONCLUSION** Photodynamic therapy with BPD -MA significantly decreases cellular proliferative activity and enhances apoptosis. Therapy using this method might be a promising approach to treat patients with bladder cancer.

**KEYWORDS: photodynamic therapy; BPD-MA; human bladder cancer cells; proliferation; apoptosis.**

The treatment of superficial urothelial carcinoma of the bladder with red light after systemic or intravesical administration of photosensitizers, known as photodynamic therapy (PDT), has recently been accepted in many countries. However, several shortcomings of "first-generation" photosensitizers limited the clinical applications of PDT.[1,2] With the development of photochemical technology, "second-generation" photosensitizers with known composition and increased photochemical activities are currently being developed. Benzoporphyrin derivative monoacid ring A (BPD-MA), a new "second-generation" photosensitizer, and has been put forward in view of its advantageous photochemical properties. BPD-MA-based PDT has become a promising approach to treat patients with skin and intraocular tumors and with subfoveal choroidal neovascularisation in age-related macular degeneration.[3,4] The objective of this study was to examine the effect of PDT with BPD-MA on the proliferation and apop-

tosis of human bladder cancer cells.

## MATERIALS AND METHODS

### Reagents and cell culture

Liposomally formulated BPD-MA (QLT PhotoTherapeutics Co., Vancouver. BC. Canada), provided by Dr. Aiqin Liu from the General Hospital of Beijng Military Region PLA, was kept in the dark at -20℃. BPD-MA was diluted in the culture medium without fetal calf serum just before use and added to exponentially growing cells. The 3- (4,5-dimethylethiazil-2-yl)-2, 5-Diph3-eyl tetrazolium bromide (MTT) was obtained from the Sino-America Biotechnology Company. $^3$H-TdR was a generous gift from Prof. Xinze Yan of the Department of Preventative Medicine, Third Military Medical University, Chongqing, China. Propidium iodide was purchased from Sigma (Sigma Chemical Co. St. Louis, MO. USA). Bladder cancer BIU-87 cell lines were obtained from the Research Center of Urologic Surgery, Beijing University, China. The cells were maintained in RPMI 1640 medium (Gibco-BRL. UK) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 U /ml).

### Photodynamic therapy and treatment of cells

For photodynamic studies, cells were incubated for a total of 90 min at 37℃ with BPD-MA (625 ng/ml ) in RPMI1640 without fetal calf serum. After incubation with BPD-MA, cells were exposed to red light (632.8 nm) delivered at 10 mw/cm$^2$ to give a total dose of 2.4 J/cm$^2$. After photodynamic treatment, the medium containing BPD-MA was removed, and the cells washed twice with 2 ml of D-Hanks buffered saline without phenol red. Then the cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum.

### MTT assay

The cells ($2.5\times10^4$) were loaded into 3 replicate wells (0.2 ml per well) of 96-well microtitre plates. After 18 h, 20 μl of a MTT solution (5 mg/ml) was added to each well. The cells were incubated for a further 6 h at 37℃. The reaction was stopped by the addition of 150 μl dimethl sulfoxide (DMSO). The degree of color development was analyzed with an automated densitometer microtitre plate reader (Huadong Electronic Factory, Nanjing, Jiangsu, China) using a 490 nm filter.

### $^3$H-Thymidine incorporation

Eight hours before collecting the cells, 0.5 μCi of $^3$H-thymidine was added to each well. The labeled cell monolayer was washed twice with phosphate-buffer saline (PBS), and the cells were detached with 0.25% trypsin (Sigma Chemical Co. St. Louis, MO. USA) and collected by a cell harvester. The cell-associated radioactivities were determined using a liquid scintillation counter (262 Industry Factory, Xi'an,China).

### PCNA immunohistochemical staining

The BIU-87-treated cells were rinsed with PBS and fixed with acetone at 4℃ for 10 min. The positive percentage of PCNA expression was detected by using the SABC method. Non-specific binding sites were blocked with 10% normal horse serum for 30 min. When the serum was removed, the cells were incubated with monoclonal mouse anti-human PCNA antibody (4℃, 24h) and goat antimouse IgG (25℃, 30 min). The cells were then treated with avidin DH- biotinylated horseradish peroxidase H complex (ABC) and counterstained with haematoxyin.

### Analysis of DNA status

The propidium iodide fluorescence analysis procedure was used to detect changes in the status of cellular DNA. Cells ($1\times10^6$) were scraped out of the plates, washed twice with ice-cold PBS, and permeabilized and fixed in 70% ethanol at 4℃ for 12 h. The cells were washed twice in ice-cold PBS and treated with RNase A (5 μ/ml, DNase-free) and stained with propidium iodide (50 μg/ml) in PBS. Samples were analyzed by flow cytometry and the percentage of cells containing hypodiploid levels of DNA calculated from single-parameter flow cytometry for propidium iodide fluorescence using a flow cytometer (Coulter Electronics Enc, Hialeah, FL).

### TUNEL assay

Terminal deoxyuridine nicked-labeling (TUNEL) assays were performed by using the TUNEL label mix and tunnel enzyme according to the procedure described by the manufacturer (Wuhan Boster Biotechnology Co., Ltd. Wuhan, China). Briefly, after photodynamic treatment, cells were cultured in RMPI 1640 medium supplemented with 10% FCS for 8 h prior to fixation. The cells were then washed twice in PBS, fixed in paraformaldehyde (4%) and permeabilized in 70% ethanol. The cells were digested with proteinase K (2 mg/L) for 3 min. After 2 washes in PBS, the cells were kept in labeling buffer at room temperature and then labeled with Dig-dUTP in the presence of terminal deoxynucleotidyl transferase by incubating for 2 h

at 37℃ in a humidified atmosphere in the dark. After 3 washes in PBS, the cells were treated with Stop/Wash buffer for 30 min at 37℃, and then incubated for 30 min at 37℃ in anti-Dig-biotin antibody and SABC. The cells were analyzed by the DAB-produced color reaction.

### Statistical analysis

All data were expressed as the mean ± standard deviation ($\bar{x}$±SD) and were analyzed by one-way analysis of variance. Groups with adducts were compared with the control. A *P*-value of less than 0.05 was considered significant.

## RESULTS

### The optical density at the wavelength of 490 nm

The optical density of the BIU-87 cells post PDT with BPD-MA significantly decreased to 0.1683 ± 0.0337 and was lower than the controls. However, there was no significant change in the optical density of the cells in the groups treated with BPD-MA, or laser irradiation alone or in the sham radiation group (Table 1).

**Table 1. The optical density in BIU-87 cells post PDT with BPD-MA.**

| Groups | Optical density |
|---|---|
| PDT with BPD-MA | 0.1683 ± 0.0337# |
| Laser irradiation alone | 0.7775 ± 0.0932* |
| BPD-MA alone | 0.7223 ± 0.0355* |
| Sham radiation | 0.7210 ± 0.0369* |

# The group of PDT with BPD-MA vs. laser irradiation or BPD-MA alone or sham radiation $P<0.01$. *Difference among laser irradiation alone and BPD-MA alone and sham radiation $P>0.05$.

### DNA synthesis of BIU-87 cells

The proliferative activity in BIU-87 cells under sham radiation was maintained at a rate of $^3$H-thymidine incorporation of 7,701±300 cpm. Treatment of cells with PDT markedly decreased cellular proliferative activity measured with $^3$H-thymidine incorporation. But the rate of $^3$H-thymidine incorporation in cells among the groups treated with BPD-MA or laser irradiation alone or with sham radiation was similar (Table 2).

### The rate of apoptosis in BIU-87 cells after photodynamic treatment

The results from flow cytometry showed that photodynamic treatment with BPD-MA significantly enhanced apoptosis in the BIU-87 cells (26.11±2.59)%. Treatment with laser irradiation alone (5.03±1.68)%, BPD-MA alone (4.71±1.84)% or sham radiation (5.28 ±1.49)% did not significantly affect the apoptosis of the BIU-87 cells (Fig.1). In situ labeling of DNA cleavage products with the TUNEL assay reinforced these observations, as BPD-MA-mediated photosensitization increased the number of TUNEL-positive cells compared to the controls (data not shown).

**Table 2. DNA synthesis of BIU-87 cells post PDT with BPD-MA.**

| Groups | DNA synthesis (cpm) |
|---|---|
| PDT with BPD-MA | 1264 ± 207# |
| Laser irradiation alone | 8,009 ± 64* |
| BPD-MA alone | 7,953 ± 794* |
| Sham radiation | 7,701 ± 300* |

# The group of photodynamic therapy with BPD-MA vs. laser irradiation or BPD-MA alone or sham radiation $P>0.05$. * Difference among laser irradiation alone and BPD-MA alone and sham radiation $P>0.05$.

## DISCUSSION

The management of bladder cancer remains a challenge despite significant improvements in preventing disease progression and improving survival.[5,6] Recently, PDT with hematoporphyrin derivatives (HpD), a "first-generation" photosensitizer, was introduced as a new treatment for superficial transitional cell carcinoma. [7,8] However, skin photosensitivity and reduced bladder capacity remain the limitations for PDT with HpD on bladder cancer [1,2]. BPD-MA is a new second-generation photosensitizer with properties of an ideal photosensitizer such as chemical purity, minimal dark toxicity, significant absorption at larger wavelength (630~690 nm), light quatum yield, preferential tumor localization and rapid clearance from normal tissues. [3,4,9,10] BPD-MA-based PDT will probably become a new promising approach to treat patients with bladder cancers.

Several early studies [11,12] showed that BPD-MA mainly localizes on the mitochondrial membranes, and the treatment using PDT with BPD-MA can cause widespread mitochondrial damage when it was directly applied to the cells. Therefore BPD-MA-mediated PDT can halt cellular metabolism and affect cellular proliferative activity. In our study, we found that PDT with BPD-MA could effectively inhibit cellular proliferative activity of the human bladder cancer cell line

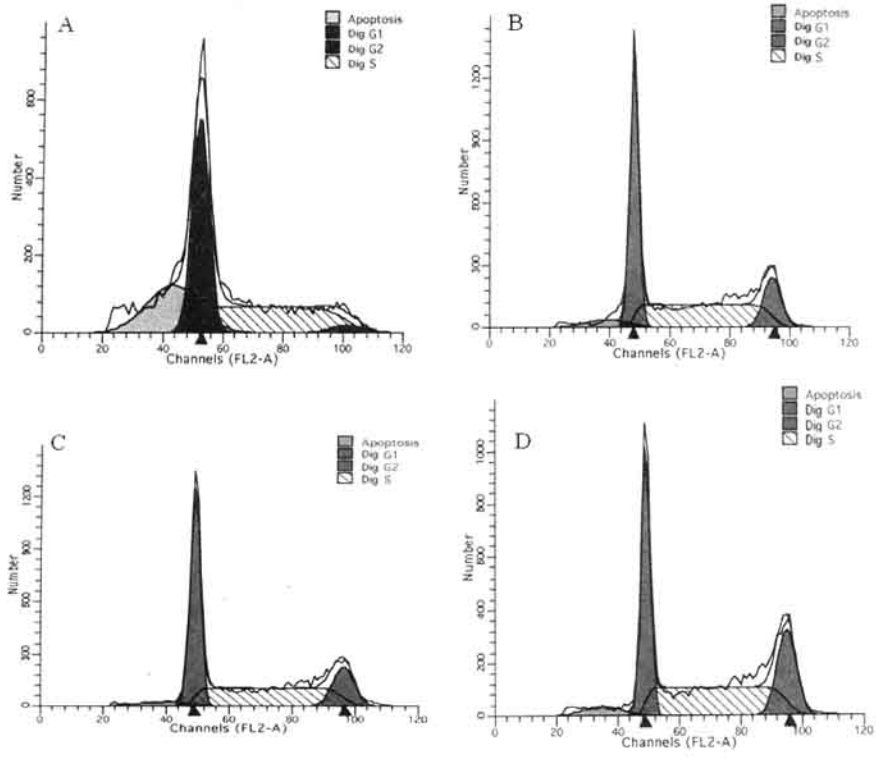


**Fig.1.** The rate of apoptosis in BIU-87 cells after BPD-MA-mediated photodynamic treatment was measured by flow cytometry with PI staining (BPD-MA 625 ng/ml, light dose 2.4 J/cm$^2$). A: photodynamic treatment with BPD-MA. B: Laser irradiation alone. C: BPD-MA treatment alone. D: sham radiation. ☐ Sub-diploid cells stand for apoptosis; ■ Dig G1 stands for cells in G1 phase of the cell cycle; ■ Dig G2 stands for cells in the G2 phase of the cell cycle; ▨ Dig S stands for the cells in S phase of the cell cycle.

BIU-87. After the treatment with BPD-MA-mediated PDT, cellular proliferative activity of BIU-87 cells measured by the MTT assay and $^3$H-thymidine incorporation significantly decreased, indicating that PDT with BPD-MA might inhibit cellular metabolism and hinder the DNA synthesis of the BIU-87 cells.

Proliferating cell nuclear antigen (PCNA), an auxiliary polymerase protein, plays an important role in DNA synthesis, and its expression represents the proliferative activity of tumor cells. Moreover, PCNA has become an important marker reflecting the proliferative ability of carcinomas.[13,14] Our immunohistochemical staining showed that the rate of positive expression of PCNA in BIU-87 cells increased up to 91.8±2.05%, however, the positive expression of PCNA in BIU-87 cells post PDT with BPD-MA markedly declined(data not shown), which suggests that BPD-MA-based PDT might significantly decrease the expression of PCNA, hinder DNA synthesis and inhibit the proliferative activity of BIU-87 cells.

Recent studies have shown that PDT can induce cell death through necrosis or apoptosis both in vitro and in vivo. An apoptotic response has been observed in a variety of tumor cell lines after photosensitization with different porphyrin and phthalocyanine derivatives.[15-17] Our studies showed that BPD-MA-mediated PDT also could induce apoptosis in BIU-87 cells. After PDT with BPD-MA the rate of apoptosis of BIU-87 cells significantly increased to 26.11± 2.59%, and positive nuclei cells after TUNEL staining were obviously enhanced. At the present time, it has become clear that multiple pathways are involved in PDT-mediated apoptosis. Mitochondria play a central role in apoptotic control by releasing the apoptosis-inducing factor and cytochrome c into the cytosol, which in turn activate the downstream apoptotic-signaling events. [18-22] PDT, with BPD-MA localizing preferentially in mitochondria, triggered BIU-87 cells into apoptosis, probably because the rapid release of cytochrome c initiates the caspase signaling cascade by the photochemical damage of mitochondria.

Recent studies have shown that cancer is a disease

with unusual proliferation, differentiation and abnormal apoptosis. It has been proved to be an useful treatment for cancer patients to inhibit the proliferation of tumor cells and enhannce apoptosis.[23,24] In our studies, BPD-MA-mediated PDT significantly inhibited the proliferation of bladder cancer cells and induced cellular apoptosis. Our data demonstrate that photodynamic therapy with BPD-MA might be a promising approach to treat patients with bladder cancer. Therefore, its in vivo study and clinical application awaits further study.

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