Sinoporphyrin sodium triggered sono-photodynamic effects on breast cancer both in vitro and in vivo

Yichen Liu, Pan Wang, Quanhong Liu, Xiaobing Wang

Key Laboratory of Medicinal Resources and Natural Pharmaceutical Chemistry, Ministry of Education, National Engineering Laboratory for Resource Developing of Endangered Chinese Crude Drugs in Northwest of China, College of Life Sciences, Shaanxi Normal University, Xi’an 710062, Shaanxi, China

Abstract

Sono-photodynamic therapy (SPDT) is a promising anti-cancer strategy. Briefly, SPDT combines ultrasound and light to activate sensitizers that produce mechanical, sonochemical and photochemical activities. Sinoporphyrin sodium (DVDMDS) is a newly identified sensitizer that shows great potential in both sono-photodynamic therapy (SDT) and photodynamic therapy (PDT). In this study, we primarily evaluated the combined effects of SDT and PDT by using DVDMDS on breast cancer both in vitro and in vivo. In vitro, DVDMDS-SPDT elicits much serious cytotoxicity compared with either SDT or PDT alone by MTT and colony formation assays. Terephthalic acid (TA) method and FD500-uptake assay reflected that the combined therapy produced more anti-cancer effect than any monotherapy, and further metastasis may be a potential strategy against highly metastatic breast cancer.

1. Introduction

Breast cancer is the leading malignancy among women in the worldwide [1]. Traditional therapies such as surgery, radiotherapy, chemotherapy, have made great achievements in clinic controlling, but accompanied by severe side effects. Therefore, efficient and novel therapies that reduce the high mortality rate and improve patient quality of life are urgent.

Photodynamic therapy (PDT), proposed by Dougherty et al., is a clinically approved modality for cancer treatment, and first approved by the FDA for certain kinds of cancer in 1998 [2,3]. Since the 1990s, PDT has been gradually applied for treatment of various cancers because it is a non-invasive, highly localized, and relatively safe form of therapy [3–6]. However, PDT application is limited to superficial lesions as the limited penetration of laser light [7]. Inspired by PDT, Yumita used ultrasound to activate sensitizers, which was named sonodynamic therapy (SDT) [8,9]. The major difference between SDT and PDT is the energy source used to activate the sensitizer. Compared with laser light used in PDT, SDT uses ultrasound that can easily penetrate deep tissue layers where some malignancies reside, thereby makes up the major limitation of PDT [9–13].

Sono-photodynamic therapy (SPDT) is an emerging approach in the anti-cancer field using the combination of SDT and PDT. The basis of this therapy is to administer a small amount of sensitizer, which can be activated by both ultrasound and light simultaneously to produce mechanical, sonochemical and photochemical activities [14]. Previous studies have indicated that SPDT can potentiate notable antineoplastic activity against a variety of malignancies at pre-clinical and clinical levels [15–18]. Some researchers reported that the combined therapy produced more obvious anti-cancer effect than any monotherapy, and further decreased the dosage of sensitizer and the energy of ultrasound...
or light [17,18]. Wang et al. report three cases of advanced refractory breast cancer by SDT treatment, the three patients had proven metastatic breast carcinoma and failed to respond to conventional therapy but had significant partial or complete responses after treated with SDT [19]. Kenyon et al. show that the combined therapy can significantly extend predicted median survival time and improve quality of life for patients [20]. Collectively, the above theoretical and clinical researches demonstrate SDT is of worthy further investigation as an alternative strategy for conventional therapy.

Although the PDT mechanism involving type I and type II reactions has been clearly illustrated [21], the mechanisms of SDT are somewhat difficult to explain. In view of the physical properties of ultrasound, various factors such as ROS formation, thermal effect, mechanical effect and cavitational effect are possibly involved in the enhancement of therapeutic effect [22]. While, the combination of SDT and PDT, SPDT refers to several key factors, such as the parameters of ultrasound and light, the sensitizers, the response of tumors, the sequence of ultrasound and light exposure, etc.

Nevertheless, sensitizers are of great importance in SPDT preclinical and clinical trials [14]. Until now, a sensitizer Sonnelux-I-SPDT has treated hundreds of cases with different types of tumors, in which breast cancers showed higher response to SPDT [19,20]. Our recent studies show the sensitizer Sinoporphyrin sodium (also referred to DVDMS) could be highly activated by both light and ultrasound [23–29]. This study aims to explore the preclinical possibilities of DVDMS-SPDT, additionally, the underlying mechanisms such as radical generation, mechanical effect triggered alteration of membrane permeability, and different sequences of light and ultrasound exposure are also carefully analyzed.

In this study, the anti-proliferation and metastatic inhibition elicited by DVDMS-mediated SPDT was investigated on breast cancer cells both in vivo and in vitro. The findings may provide important implications for DVDMS application in the treatment of cancer.

2. Materials and methods

2.1. Sensitizers

Sinoporphyrin sodium (DVDMS) was kindly provided by Professor Qicheng Fang from the Chinese Academy of Medical Sciences (Beijing, China). It has a purity of 98.5%. DVDMS was dissolved in a physiological saline solution to a final storage concentration of 1 mM and was stored in the dark at −20 °C. The chemical structure of DVDMS is shown in Fig. 1.

2.2. Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide tetrazolium (MTT), paraformaldehyde, Triton X-100, bovine serum albumin (BSA), crystal violet, terephthalic acid (TA), fluorescein isothiocyanate-dextran (FD500) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2′,7′-Dichlorodihydrofluorescein-diaacetate (DCSF–DA) was from Molecular Probes Inc. (Eugene, OR, USA). Dihydroethidium (DHE) was purchased from Invitrogen (Thermo Scientific Inc., US). Primary antibody against proliferating cell nuclear antigen (PCNA) was purchased from Abcam (Cambridge, UK). Secondary antibodies were obtained from Zhong Shan Golden Bridge Biotechnology (Beijing, China).

2.3. Tumor cell lines

Mouse mammary cancer 4T1 cell line was obtained from the department of basic medicine, Union Medical College, Beijing, China. Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the cell bank of the Chinese Academy of Science, Shanghai, China. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Life Technologies, Inc., USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 1 mM L-glutamine, in an incubator with 5% CO2 and 100% humidity at 37 °C. Cells in the exponential phase of growth were used in each experiment.

2.4. Animals

The BALB/c mice (female, 18–20 g body weight) were supplied by the Experimental Animal Center of Fourth Military Medical University (FMMU) (Xi’an, China) and housed at room temperature with a 12 h light/dark cycle and allowed free access to food and water. After 1 week’s acclimation, BALB/c mice were subcutaneously injected at the right flanks with 0.1 ml 4T1 cells (1 × 10⁷ cells/ml). When the tumor reached a size of 60–70 mm³, the tumor-bearing mice were randomly assigned to different groups and ready for experiment. All animal experiments were carried out with the approval of the university’s institutional animal care and use committee.

2.5. In vitro laser light and ultrasound treatment

Cells in the exponential phase were collected and re-suspended in a complete culture medium at cell densities of 2 × 10⁶ cells/ml in 35 mm cell culture dish (Corning Inc. Tewksbury MA, USA) for 12 h. Then all samples were randomly divided into different groups: control group (Control), ultrasound group (US), 0.5 μM DVDMS group (DVDMS), DVDMS plus ultrasound group (SDT), DVDMS plus laser light group (PDT), DVDMS plus ultrasound plus laser light group (SPDT) and DVDMS plus laser light plus ultrasound group (PSDT). For all groups except the control and ultrasound, cells were incubated with 0.5 μM DVDMS for 3 h, allowing sufficient time for cells to uptake the sensitizer rapidly to achieve a maximum level. It is worth mentioning that, because the breast cancer 4T1, MDA-MB-231 and MCF-7 cells are adherent-dependent growth, in order to investigate the natural biological response to ultrasound treatment, we exposed cells to ultrasound and light irradiation when they were normal adherent growth without any trypsinization.

For laser light, a semiconductor laser (excitation wavelength: 635 nm; manufacturer: Institute of Photonics & Photon Technology, Department of Physics, Northwest University, Shaanxi, China) was used as previous report [25]. Laser irradiance was measured...
using a radiometer system (Institute of Photonics & Photon Technology, Department of Physics, Northwest University). As for \textit{in vitro} experiments, the laser was used with a power intensity of 23 mW/cm\(^2\) and an irradiation time of 60 s such that the final dose of light is 1.4 J/cm\(^2\).

For the \textit{in vitro} ultrasound set-up [30]. The planar ultrasound apparatus (Fig. 2), manufactured by Sheng Xiang High Technology Co., LTD (China), was applied in this study. Ultrasonic frequency of this apparatus was 0.84 MHz. The diameter of its planar transducer was 35 mm. The ultrasound intensity was calibrated before it was displayed in the LED screen of the apparatus. An intensity of 0.25 W/cm\(^2\) and duration of 60 s was used for ultrasound treatment. For irradiation, the interval between transducer and cell culture plate was filled with ultrasound couplant to facilitate ultrasound transmission. Temperature increase inside the culture plates was measured before and after ultrasound treatment with a digital thermometer, and no significant variation of temperature was detected (±1 °C). Thus, any bio-effects observed in this study were considered to be non-thermal.

2.6. Cell viability assays

Cell viability was evaluated using conventional MTT assay and colony formation assay. For MTT assay, cytotoxicity was detected at 4 h after different treatments as set forth [26]. Cell viability was calculated as follows equation: Cell survival (%) = OD\(_{\text{treatment group}}\)/OD\(_{\text{control group}}\) × 100%.

Moreover, the colony-formation assay was adopted for examination of long-term proliferative potential in this study. After different treatment, cells were seeded onto 35 mm culture dishes at a density of 1000 cells/well, and subsequent protocols were performed as previous described [25]. Then the total number of colonies was counted and proliferation potential was calculated using the following equation: relative colony formation rate (%) = number of colonies with at least 50 cells in the control group/number of colonies with at least 50 cells in the treatment group = number of colonies with at least 50 cells in the control group × 100%. The experiment was conducted in triplicate. Representative views were also photographed.

2.7. Cell motility

Cell motility was evaluated using transwell assays as previously reported [25]. 100 μl 2.5 × 10\(^5\) cells/ml were seeded into the top chamber of a Corning chamber (Corning Pharringen, San Diego, CA) in serum-free medium containing 0.3% BSA. Medium containing 10% serum was placed in the lower chamber. After 20 h, cells that migrated to the underside of the membrane were detected using a 0.1% crystal violet solution. The assay was repeated three times with three replicates each. Cells that migrated to the underside of the membrane were quantified using light microscopy (Nikon Eclipse TE2000-S, Japan). Crystal violet was dissolved using a 0.1% crystal violet solution.

2.8. Determination of intracellular ROS and superoxide anion

Intracellular ROS level was assessed by measuring the fluorescence intensity of dichlorofluorescein (DCF) as described in our previous article [31]. Briefly, cells were incubated with 10 μM DCFH-DA at 37 °C for 30 min prior to laser light and/or ultrasound treatment. At 2 h after treatment, the cells were washed in phosphate-buffered saline and then imaged using an E-600 fluorescence microscope (Nikon Corporation, Tokyo, Japan). Multilabel Reader was used to further validate the intensity of DCF. The treatment was the same with above. At 2 h after treatment, cells were harvested and lysed in 100 μl phosphate-buffered saline. Fluorescence of the supernatant was measured on a Multilabel Reader (PE EnSpire, USA) at an excitation wavelength 488 nm with emission wavelength at 500–550 nm.

Dihydoroethidium (DHE), a selective probe for superoxide anion \((O_2^-)\), was used to further identify intracellular ROS [32]. Briefly, cells were incubated with 5 μM DHE at 37 °C for 30 min prior to laser light and/or ultrasound treatment. At 2 h after treatment, the samples were washed in phosphate-buffered saline and harvested by trypsinization, then detected immediately by flow cytometry (Guava easyCyte 8HT, Millipore, Billerica, MA).

2.9. Evaluation of ultrasound cavitation

Acoustic cavitation of ultrasound was evaluated using TA (terephthalic acid) method. When the acoustic power is above a threshold in a liquid system, some active species such as hydroxyl radicals (OH\(^-\)) and hydrogen peroxide (H\(_2\)O\(_2\)) are formed on thermalysis of H\(_2\)O during ultrasound exposure [33]. TA solution (1 mM) as dosimeter solution reacts with a hydroxyl radical formed during ultrasound irradiation (0.5-, 1.0- and 1.6 W/cm\(^2\) for 60 s), forms 2-hydroxyterephthalic acid (HTA) which are detected using fluorescence photometer (LS-55, PE, USA) at 426 nm.

2.10. Detection of cell membrane permeability

In order to measure the changes of membrane permeability induced by ultrasound used in SPDT/PSDT, FD500-uptake assay was performed. FD500 is the conjugate of fluorescein FITC and dextran with a molecular weight of 500,000 that cannot freely penetrate the cell membrane. Once membrane permeability is enhanced due to some stress, such as sonication, FD500 may enter the cell membrane. Then the samples were washed in phosphate-buffered saline and harvested by trypsinization, then detected immediately by flow cytometry.

2.11. In vivo sonodynamic and photodynamic therapy

For \textit{in vivo} experiments, the laser light treatment equipment was same as the \textit{in vitro} one. The laser was utilized with a power...
intensity of 417 mW/cm² and an irradiation time of 2 min such
that the final dose of light is 50 J/cm². For the in vivo ultrasound
treatment, because previously we found the tumor tissues
responded well to the focused ultrasound with a frequency of
1.90 MHz [27]. Here, we would like to use this focused ultrasound
to treat tumors. As described in previous paper, the focused ultra-
sound transducer with a frequency of 1.90 MHz, manufactured by
the Institution of Applied Acoustics, Shaanxi Normal University
(Xi'an, China), was submerged in degassed water in the tank facing
directly upward. A continuous sine-wave signal was generated and
amplified by a multifunctional generator (AG1020; T&C Power
Conversion Inc., Rochester, New York, USA) before feeding the
transducer. An average intensity of 1.6 W/cm² (Iswana) was used
for ultrasound treatment for 3 min. For the experiments, cold
degassed water (4 °C) was used as the ultrasound coupling med-
ium, thus reducing hyperthermia during treatment. The tempera-
ture close to the exposed region showed no significant variation
(<3 °C).

The tumor-bearing mice were randomly divided into five
groups: Control, PDT, SDT, SPDT and PSDT. The control mice did
not receive any treatment. The other four groups of mice were
injected with DVDMS (2 mg/kg) via the caudal vein, and after
24 h post injection, they were exposed to laser light (PDT), ultra-
sound (SDT), or ultrasound immediately followed by laser light
(SDPT) or laser light immediately followed by ultrasound (SDST).
Prior to laser light and ultrasound applications, hair from the
tumor region was removed using depilatory cream. The mice were
anesthetized with 1% pentobarbital sodium and placed on a plate
for PDT treatment or on a plexiglass plate with degassed water
for SDT treatment. For SDT, the naked tumor region was immersed
into the degassed water and the center of the tumor was exposed
to the focused ultrasound spot for 3 min. The overview protocol is
shown in Fig 3.

The therapeutic results of each group were evaluated by mea-
suring the tumor volumes for 12 days, and the body weight was
also measured. The long (a) and short (b) diameters of the tumors
were measured using slide calipers every day after treatment. The
mean tumor volume was calculated using the formula: ab²/2, and
the volume inhibition ratio was calculated as follows: (1-average
tumor volume of treated group/average tumor volume of the con-
trol group) × 100%. 12 days after treatment, the mice were
sacrificed, and the tumors were removed and weighed. In addition,
the lungs were removed and fixed in Bouin’s solution for 24 h. The
pulmonary nodules were photographed and counted.

2.12. Hematoxylin and eosin (H&E) straining

Tumors and major organs was fixed using 10% formalin for at
least 24 h. Samples were then paraffin-embedded, sectioned, and
stained with H&E. Histopathological changes were observed using
light microscopy (Nikon E600, Japan).

2.13. Immunohistochemistry

Paraffin-embedded tumor tissue sections (7 μm thick) were
dewaxed, rehydrated, and then treated with heat-mediated anti-
gen retrieval using 10 mM citrate buffer (pH 6.0) for 15 min.
Sections were permeabilized with 0.2% Triton X-100 for 15 min.
Sections were immersed in 3% hydrogen peroxide solution for
10 min to quench endogenous peroxidase activity. Non-specific
binding was prevented by incubation with 5% normal goat serum
for 15 min. The sections were then incubated with anti-PCNA anti-
body overnight at 4 °C. Antibody binding was detected using
horseradish peroxidase-conjugated secondary antibody for
20 min at 37 °C. Sections were visualized using diaminobenzidine
(DAB) solution counterstained with hematoxylin, and observed
using light microscopy.

2.14. Statistical analysis

Quantitative data in all statistical analyses was set as
mean ± standard deviation of three samples obtained from three

Fig. 3. Diagram of in vivo DVDMS-SPDT treatment protocol. Tumor-bearing mice were randomly divided into five groups: Control, PDT, SDT, SPDT and PSDT. The control mice
did not receive any treatment. The other four groups of mice were injected with DVDMS (2 mg/kg) via the caudal vein, and after 24 h post injection, they were exposed to
laser light (PDT), ultrasound (SDT), or ultrasound immediately followed by laser light (SPDT) or laser light immediately followed by ultrasound (PSDST). The laser was utilized
with a power intensity of 417 mW/cm² and an irradiation time of 2 min such that the final dose of light is 50 J/cm². The in vivo frequency of the ultrasound was 1.90 MHz.
Mice were exposed to ultrasound for 1.6 W/cm², 3 min in this study.
3. Results

3.1. Cytotoxicity assessment

Cytotoxicity of DVDMS-mediated SDT and/or PDT in vitro was evaluated on 4T1, MDA-MB-231 and MCF-7 cell lines by MTT assay 24 h after different treatments. As shown in Fig. 4A–C, DVDMS alone (0.5 μM) and ultrasound alone groups did not show obvious cytotoxicity on all breast cancer cell lines. Compared with control, two single treatment increased the cytotoxicity of 4T1, MDA-MB-231 and MCF-7 cells to 27.36%, 34.88% and 24.44% for SDT, and 36.69%, 40.16% and 38.58% for PDT respectively (p < 0.01 vs. control). When treated by the combination of SDT and PDT, markedly loss in cell viability was observed among all breast cancer cell lines. Therein, the cell viability loss of 4T1, MDA-MB-231 and MCF-7 cells after treated with SPDT was 80.49%, 81.69% and 70.69%, respectively. After PSDT treatment, the viability loss of the above cell lines was 85.01%, 86.13% and 77.48% respectively. These data indicate a synergistic enhancement of cytotoxicity when compared with SDT alone (p < 0.001) or PDT alone (p < 0.001).

A colony formation assay on 4T1 cells was further confirmed the reduced proliferative potential induced by the combination of SDT and PDT. As shown in Fig. 5, numerous colony formed in control and DVDMS alone groups had no effect on colony formation ability. In contrast, colony formation ability of 4T1 cells in SPDT and PSDT groups decreased significantly stronger than that in SDT and PDT treatment alone. The colony formation rate in DVDMS alone, SDT, PDT, SPDT, PSDT groups were 99.02%, 68.78% (p < 0.01 vs. control), 59.51% (p < 0.01 vs. control), 12.68% (p < 0.001 vs. control), 8.29%, respectively (p < 0.001 vs. control, p < 0.001 vs. SDT/PDT). This result is consistent well with the MTT assay above.

3.2. DVDMS mediated SPDT/PSDT inhibits cell migration in 4T1 cells

Since metastasis is the primary cause of mortality in breast cancer patient, we evaluated the effect of cell migratory ability treated by DVDMS-SPDT on cell migration using a transwell assay [34]. Numerous cells in the control group migrated to the underside of the well at 20 h post treatment, while a significant decrease of cell migration was observed in PDT alone. When compared with SDT and PDT alone, the combined therapies further decreased the rate of cell migration to a lower level (Fig. 6). Analysis of the OD ratio of crystal violet indicated similar results. The findings showed 44.29% (p < 0.01), 59.94% (p < 0.01) decreases in cell migration rate in SDT and PDT groups with 0.5 μM DVDMS exposure, respectively. While a markedly decrease with 87.57% (p < 0.001 vs. control), and 91.59% (p < 0.001 vs. control) was seen in the SPDT and PSDT groups at the same drug dose.

3.3. DVDMS-mediated combination treatment (SPDT/PSDT) drastically enhance the ROS production

Cell damage induced by PDT or SDT is closely related to the generation of intracellular ROS [25,31]. We examined whether combination treatment can enhance the ROS production in 4T1 cells. Therefore, intracellular ROS level was measured using DCFH-DA staining and DHE staining after SDT and/or PDT treatment. As indicated in Fig. 7, compared with control, PDT increased ROS generation for about 50 times (p < 0.01), whereas SDT showed little DCF fluorescence intensity enhancement for 15 times per milligram protein. Importantly, SPDT and PSDT treatments dramatically enhanced intracellular ROS levels, which were about 180–240 times higher compared with control (p < 0.001) and were about 4–5 times higher compared with SDT and PDT (p < 0.01). DHE staining (Fig. 8) showed that, compared with the control, SDT and PDT caused 19.2% (p < 0.05) and 27.5% (p < 0.05) high DHE fluorescence. Under the same conditions, much high DHE fluorescence were observed when cells were treated with SPDT and PSDT, in which the high DHE fluorescence increased to 55.3% (p < 0.01) and 62.15% (p < 0.01), respectively.

3.4. Ultrasound-induced cavitational effect

Acoustic cavitation is a complicated process that leads to both mechanical shear stress and free radicals formation arising from the collapse of oscillating bubbles [35]. In our study, TA (terephthalic acid) method was used to detect the degree of cavitation caused by ultrasound treatment. Fig 9 shows that at 1.9 MHz focused ultrasound treatment, HTA fluorescence intensity increased slightly after 0.5 W/cm² (p < 0.05) and increased dramatically after 1- and 1.6 W/cm² (p < 0.01) ultrasound treatment, which indirectly implies the utilized parameters would cause cavitational effect.

3.5. Ultrasound-induced membrane permeability

Cell membrane integrity induced by ultrasound used in our study was evaluated by FD500-uptake assay to demonstrated mechanical effects of ultrasound. FD500 does not stick on the cell membrane, and rarely enters the dead cells [18]. Thus, the uptake of FD500 can be a credible sign to reflect the membrane permeability [18]. As shown in Fig. 10, proportions of 4T1 cells exhibiting high fluorescence intensities of FD500 can be a credible sign to reflect the membrane permeability. Therefore, intracellular ROS level was measured using DCFH-DA staining and DHE staining after SDT and/or PDT treatment
Fig. 5. *In vitro* cytotoxicity assessment of DVDMS-SPDT on 4T1 cells determined by colony formation assay. Error bars represent the SD from three independent experiments. *p < 0.01 versus control, **p < 0.001 versus control, ***p < 0.001 SPDT/PSDT group versus SDT group, &&& p < 0.001 SPDT/PSDT group versus PDT group.

Fig. 6. Analyses of change in cell migration on 4T1 cells using a transwell assay. Cells in each group move to the lower surface of the filter were stained with crystal violet and photographed under a light microscope (a–e). a: Control, b: SDT, c: PDT, d: SPDT, e: PSDT. The OD ratio of crystal violet were measured (bottom right corner). Error bars represent the SD from three independent experiments. *p < 0.01 versus control, **p < 0.001 versus control, ***p < 0.001 SPDT/PSDT versus SDT, &&& p < 0.001 SPDT/PSDT versus PDT.

Fig. 7. Intracellular ROS generation in 4T1 cells at 2 h post treatment. The cells were labeled with DCFH-DA and observed by fluorescence microscope (Left side). a: Control, b: DVDMS alone, c: SDT, d: PDT, e: SPDT, f: PSDT. The mean fluorescence intensity of the oxidized product DCF in cells was detected by Multifunctional microplate reader (Right side).
34.50% and 45.62% after 0.25-, 0.5-, 0.75- and 1 W/cm² planar ultrasound treatment, respectively. These results confirm that ultrasound process mode used in this study could cause injury of cell membrane with an ultrasonic intensity dependent manner indicating the mechanical effects involved in SPDT/PSDT as well as SDT in our study.

3.6. DVDMS mediated SPDT/PSDT enhance inhibition effect on tumor growth

To investigate the in vivo anti-cancer efficacy of combination treatment, a 4T1 mouse mammary cancer model was utilized. Tumor-bearing mice were divided into five experimental groups, and result was shown in Fig. 11. Representative mice were also photographed at 12 days after the corresponding treatments (Fig. 11A). DVDMS mediated SDT/PDT markedly inhibited tumor growth (volume and weight) (Fig. 11B–D), and the effect was noticeably enhanced with the combination of these two method. The tumor weight inhibiting ratios in the SDT and PDT groups were 46.66% and 55.54% (p < 0.01 vs. control). Importantly, for SPDT and PSDT, the tumor weight inhibition ratio was 81.45% and 84.83% (p < 0.001 vs. control, p < 0.01 vs. SDT/PDT), indicating that DVDMS mediated SDT and PDT repressed tumor growth and that the effect was strengthened when the two therapy combined.

3.7. DVDMS mediated SPDT/PSDT inhibits lung metastasis in vivo

Mice were sacrificed and assessed for the extent of metastasis to the lungs by examining the gross appearance of pulmonary nodules after fixation with Bouin’s solution for 24 h. Lungs of mice in the control group displayed multiple metastasized tumors of various sizes on their surface (Fig. 12). In contrast, the surfaces of the lungs from both SPDT and PSDT treated mice, which were very similar to the normal lung. While either single treatment of SDT and PDT reduced lung metastases partly. The pulmonary nodules were manually counted and the average numbers of Control, SDT, PDT, SPDT, PSDT groups were 22 ± 2.38, 12 ± 2.22 (p < 0.001 vs. control), 10.2 ± 2.18 (p < 0.001 vs. control), 2 ± 2.18 (p < 0.001 vs. control, p < 0.01 vs. SDT/PDT), 1.6 ± 1.26 (p < 0.001 vs. control, p < 0.01 vs. SDT/PDT), respectively.

3.8. Histological and immunohistochemical analysis of in vivo anti-tumor effects

For histopathological analysis using H&E staining (Fig. 13), we observed that the tumor tissue from the control group displayed compact tumor cells with an intact structure. In the SDT or PDT groups, the tumor tissues were no longer structurally integrated: there were sections devoid of cells and there were numerous nuclear fragments. The tissue damage was more severely extensive...
Fig. 10. Detection of cell membrane permeability by FD500-uptake assay. 4T1 cells were sonicated in the presence of 1 mg/ml of FD500 conjugated with fluorescein FITC and then the FD500-positive cells were quantified by flow cytometry.

Fig. 11. DVDMS-SPDT inhibits tumor growth. (A) Representative photos of 4T1 tumor-bearing mice from different groups at day 12 after treatment. (B) Tumor weight of different groups of tumor-bearing mice after treatment. (C) Image of tumors excised from each group of mice 12 days after treatment. (D) Tumor growth curves of different groups of tumor-bearing mice after treatment. Error bars represent the SD from three independent experiments. **p < 0.01 versus control, ***p < 0.001 versus control, ##p < 0.01 SPDT/PSDT group versus SDT group, &&p < 0.01 SPDT/PSDT group versus PDT group.
in the SPDT and PSDT group compared with either single treated group.

To determine the effect of combination on cell proliferation in vivo, immunohistochemistry analysis was performed to evaluate the expression level of PCNA, a representative marker of proliferation [36]. PCNA was highly expressed in the control group and had a reduced expression level in the SDT and PDT groups; the level was further greatly decreased in the SPDT and PSDT groups than either single treatment group (Fig. 14).

3.9. Evaluation of side effects using DVDMS mediated SPDT/PSDT

The potential in vivo toxicity of DVDMS mediated combination treatment of SDT and PDT was also preliminary examined. We did not detect any overt signs of toxic side effects or changes in body weight with drug dose at 2 mg/kg (Fig. 15A). Furthermore, we harvested the major organs including the heart, liver, spleen, and kidney. We were unable to detect any organ damage using H&E staining (Fig. 15B).

4. Discussion

Sono-photodynamic therapy (SPDT) combines ultrasonic irradiation and laser light with drugs known as sensitizers that amplify its ability to inflict preferential damage on malignant cells [16]. Both preliminarily pre-clinical and clinical studies have indicated that the combined therapy can overcome major limitations of PDT and enhance the deep-preferentially ability with selectively targeting of the tumor tissue [15,16,19,20]. Ultimately, the combined therapy performed stronger anti-cancer effect than any monotherapy and may decrease the sensitizers dosage and ultrasound or light energy, which can further reduce the side effects caused by the sensitizer [20–22].

Sensitizer is key component in both PDT and SDT. DVDMS is a kind of porphyrins photosensitizer isolated from Photofrin II. Compare with Photofrin II, DVDMS has advantages of 98.5% chemical purity, high water solubility, good targeting and brief skin sensitivity [24]. Previous work have shown that DVDMS preformed great activities in both SDT and PDT treatment, which indicated that DVDMS may be a great candidate sensitizer in SPDT treatment [25,27–29]. Therefore, we agog to assess the anti-cancer effect of DVDMS-SPDT, and investigate whether DVDMS-SPDT can perform a markedly combination effect.

Herein, we used the BALB/c-derived mouse mammary carcinoma cell line 4T1, which share numerous characteristics with human mammmary carcinoma, including immunogenicity, growth characteristics, and metastatic properties, to investigate the effects of sono-photodynamic therapy on cell metastasis and proliferation both in vivo and in vitro.

Firstly, we investigate the tumor inhibition effect of DVDMS-SPDT both in vitro and in vivo. For in vitro studies, we preliminarily assess the cytotoxicity of DVDMS-mediated SPDT in 4T1, MDA-MB-231 and MCF-7 cell lines. A significant cell viability loss (Fig. 4A–C) was found in combined treatment groups with presence of 0.5 μM DVDMS 24 h after treatment in each cell line. Colony formation assay on 4T1 cells support the conclusion from the point of long-term proliferative ability (Fig. 5). The two assays confirm that combination treatment evoked by DVDMS is more efficiency than SDT/ PDT alone at the same DVDMS dose. Moreover, in vivo study, tumor volume and weight in SPDT and PSDT groups were significantly inhibited in comparison with those in each single treatment group.

Fig. 12. DVDMS-SPDT inhibits lung metastasis in vivo. (A) Photos of lungs after soaking in Bouin’s solution showing spontaneous pulmonary breast cancer metastases (black arrows and dotted circle). (B) The pulmonary nodules were manually counted and the average numbers were calculated in different groups. *p < 0.01 versus control, **p < 0.001 versus control, ***p < 0.001 SPDT/PSDT group versus SDT group, ###p < 0.001 SPDT/PSDT group versus PDT group.

Fig. 13. Tumor sections were stained with hematoxylin and eosin (HE) after different treatments. Histopathological changes were observed under a light microscope.
group (Fig. 11A–D). H&E staining showed obvious tissue destruction with large spaces post SPDT and PSDT treatment (Fig. 13). Furthermore, the expression level of PCNA were significantly downregulated in SPDT and PSDT groups compared with control, SDT and PDT groups (Fig. 14). Besides, comparing with our previous work on DVDMS-PDT, despite promising results used 150 J/cm² on the treatment of same xenograft mode, SPDT using the light dose of 50 J/cm² with a more efficiency treatment effect are even safer than conventional PDT [25]. One probable explanation is that the increased treatment efficiency may owe to subsequent SDT could compensate for the unavoidable attenuation PDT effect on the deeper tissues.

As tumor metastasis is one of the major challenges in cancer treatment, in this study, we also focused on metastatic ability of 4T1 cells after different treatments [34]. Transwell assay demonstrated that SDT and PDT treatment could mildly suppress cell migration, whereas SPDT and PSDT can almost completely inhibit cell migration 20 h after treatment (Fig. 6). Furthermore, as breast cancer cells usually metastasize to lungs [37,38]. The lung metastasis assay was performed to assess the metastatic ability after different treatments. Results in Fig. 12 demonstrated that the number of pulmonary nodules in the PSDT and SPDT groups were significantly less than that in control and single treatment groups, indicating that the combined treatments greatly inhibited the metastasis of tumor cells to the lungs. This result is consistent well with the in vitro findings, suggesting that DVDMS-SPDT can efficiently suppress migration and metastasis in 4T1 cell both in vitro and in vivo.

Above results confirm that DVDMS-SPDT can achieve better therapeutic results on inhibition effect of tumor proliferation and metastasis compared with either single treatment, that was similar to what have been found in previous studies on SPDT [19,20], which indicating that the newly identified sensitizer, DVDMS, can be potentially used in SPDT treatment.

Next, it is necessary to explore the possible underlying mechanisms on enhancement of cell killing by the combination. It is well known that oxidation stress is one of the key factors in the tumor destruction of PDT and might be also involved in mechanism of SDT [39]. Excessive ROS can damage lipids, proteins, and DNA as well as cause mitochondrial dysfunction, deregulate ion balance, and cause loss of membrane integrity [31]. Therefore, the ROS level was assessed in order to explore whether the production of ROS plays a crucial role in anti-cancer effect of DVDMS-SPDT. The result (Figs. 7 and 8) suggested a markedly increased oxidative stress in 4T1 cell responded to DVDMS-SPDT, which might provide strong contribution to the enhanced anti-cancer effect of the combination treatment.

Although oxidative stress has been proved to be involved in mechanisms of SDT and PDT, unlike light, due to the physical properties of ultrasound, various mechanisms other than ROS such as thermal effect, mechanical effect and cavitation effect are possibly responsible for the enhancement of therapeutic effect [9,40]. Acoustic cavitation is a complicated process that leads to both mechanical shear stress and free radicals formation arising from the collapse of oscillating bubbles. The degree of bio-effects depends on the given ultrasound intensity and frequency and the other experimental conditions [40]. In this study, TA method was used to detect the degree of cavitation caused by ultrasound treatment. Result in Fig. 9 confirms that the present parameter can cause acoustic cavitation and may induce a series of chemical reactions and mechanic shearing, ultimately resulting in enhancement of cell damage. Meanwhile, multiple reports have indicated that ultrasound can increase the permeability of the plasma membrane [41,42]. In this paper, we use FD500-uptake assay to measure the
changes of membrane permeability induced by ultrasound. The data suggest that ultrasound used in in-vitro study can cause permeability alteration of cell membrane with an ultrasonic intensity dependent manner [Fig. 10]. The increased permeability of cell membrane may further improve induction of drug uptake, and work to delivery of sensitizer into tumor cell, which possibly cause more efficiency anticancer effect [43].

Moreover, we evaluated the effect of combined therapy with different treatment orders and interestingly found that when PDT was performed before SDT, the combination effect was relatively stronger than that in case of SDT prior to PDT. Wang et al. and Jin et al. (from two independent groups) reported similar results in their study [16,39]. Based on these results, we tentatively interpret this to the combination of PDT with subsequent SDT has a first choice of PDT dependent on oxygen concentration followed by SDT, which is not completely dependent of oxygen. Finally, during the whole treatment period, there were no obvious side effects, such as significant body weight drop and detectable internal organs damage (Fig. 15A and B), which indicating the relative safety of this combined treatment.

5. Conclusions

In this study, DVDMS mediated SPDT produce stronger therapeutic effects on breast cancer over SDT and PDT alone both in vitro and in vivo and DVDMS-SPDT may be a potential strategy against highly metastatic breast cancer. The excess level of ROS may plays a crucial role in cell death, cavitations effect and changes of membrane permeability induced by ultrasound also work on the enhancement of combination therapy. Due to the complicated system of SPDT, the mechanism becomes more elusive and there is no certain theory about SPDT. Thereby, more in depth preclinical studies and clinical trials are certainly needed to verify and prefect this new method.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Nos. 81472846, 81571834) and the Fundamental Research Funds for the Central Universities (Grant No. GK201502009).

References


