Comparison of the synergistic anticancer activity of $AlPcS_4$ photodynamic therapy in combination with different low-dose chemotherapeutic agents on gastric cancer cells

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Abstract. Limited cellular delivery and internalization efficiency of Al(III) phthalocyanine chloride tetrasulfonic acid ($AlPcS_4$) induce poor penetration ability in cells and a slight photodynamic therapy (PDT) effect on gastric cancer. The combination treatment of $AlPcS_4$/PDT with low-dose chemotherapeutic agents may provide a promising treatment strategy to increase the weak delivery efficiency of $AlPcS_4$, reducing the dose of chemical agents without reducing efficacy, and improving apoptosis-inducing abilities, thereby increasing the antitumor effects and decreasing the noxious side effects on gastric cancer. We investigated and compared the synergistic antitumor growth effect on gastric cancer cells by combining $AlPcS_4$/PDT treatment with different low-dose chemotherapeutic agents, namely, 5-fluorouracil (5-FU), doxorubicin (DOX), cisplatin (CDDP), mitomycin C (MMC), and vincristine (VCR). The inhibitory effect was increased in treatments that combined $AlPcS_4$/PDT with all the aforementioned low-dose chemotherapeutic agents, to a different extent. An evident synergistic effect was obtained in the combination treatment of $AlPcS_4$/PDT with low-dose 5-FU, DOX, and MMC by increasing $AlPcS_4$ intracellular uptake ability, improving apoptosis-inducing abilities, and prolonging apoptosis-inducing time. The low-dose chemotherapeutic agents prolonged the apoptosis-inducing period of $AlPcS_4$/PDT, and $AlPcS_4$/PDT quickly improved apoptosis-inducing abilities of chemotherapy even at low doses. Generally, the combination treatment of $AlPcS_4$/PDT with low-dose chemotherapeutic agents had significant antitumor growth effects in addition to a low dark-cytotoxicity effect on gastric cancer, thereby representing an effective and feasible therapy method for gastric cancer.

Introduction

Gastric cancer is a common malignant disease that seriously threatens human life and health (1). In addition to surgery, chemotherapy, and radiotherapy, PDT is another promising curative and palliative treatment approach to eliminate tumor tissue (2). Given its high efficiency, safety, synergy compatibility, repeatability, and relatively low cost, PDT has become attractive to researcher (3). In PDT, the photosensitizer, a light-sensitive drug is administered via systemic injection and is subsequently illuminated by suitable light at the target tissue, leading to generation of reactive oxygen species (ROS), notably singlet oxygen (SOG), thereby causing oxidative stress to damage cellular organelles and membranes and ultimately lead to apoptosis or necrosis of neoplastic tissue (4,5). PDT can be used as monotherapy and in combination with surgery, chemotherapy, or standard radiation therapy (6-9). Monotherapy usually suffers from incomplete tumor killing to ultimately induce poor prognoses and unsuccessful therapy. The combination treatment strategies revealed a more effective antitumor effect preclinically and clinically (10). In the combination of PDT with chemotherapy, chemotherapy can enhance the antitumor effect of PDT by targeting surviving cancer cells and inhibiting regrowth of damaged tumor blood vessels. Furthermore, PDT-mediated vascular permeabilization can enhance the accumulation of chemotherapeuticdrugs in tumors, thereby improving chemotherapy efficacy. Therefore,

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Abbreviations: $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; PDT, photodynamic therapy; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine; ROS, reactive oxygen species; SOG, singlet oxygen; ER, endoplasmic reticulum

Key words: photodynamic therapy, chemical therapy, combination therapy, $AlPcS_4$, low-dose, gastric cancer

the method of combination of PDT with chemotherapy may be an effective treatment strategy for gastric cancermanagement.

$AlPcS_4$, derived from photofrin, is a second-generation photosensitizer. It exhibits near-infrared absorption (position of absorption maxima at 675 nm), thereby providing relatively low tissue absorption and deeper tissue penetration. Furthermore, higher quantum yields, good photostability, and little photobleaching result in the wide use of the photosensitizer in the treatment of coelom cancer in PDT (11,12). It has been reported that $AlPcS_4$ has a superior PDT effect in various cancer cell lines, including breast, pancreatic, ovarian, and colon cancer (13-19). However, to date, whether it can inhibit the growth of gastric cancer cells has not been demonstrated. The antitumor effect of $AlPcS_4$/PDT in combination with chemotherapeuticdrugs on gastric cancer cells is also less known.

Although many researchers have reported that PDT-combined chemotherapy is an effective and feasible therapy for cancer, the combined antitumor effect depends on the selected drug and the dose of the drug. Choosing different chemical drugs can induce different antitumor effects. Thus, researching different chemical drugs for combination with $AlPcS_4$/PDT for an optimal antitumor effect on gastric cancer is important. 5-FU, DOX, CDDP, MMC and VCR are common gold-standard chemotherapeutic agents that are clinically recommended for gastric cancer. In particular, 5-FU, as a thymidylate synthase inhibitor, can rapidly divide cells to cease replication and die by preventing the production of dTMP (20). DOX, an anthracycline chemotherapeutic agent, intercalates into DNA and halts the process of DNA replication by inhibiting the progression of topoisomeraseII (21). CDDP is a metallic coordination compound and can interfere with the DNA repair mechanism, thereby causing DNA damage and inducing apoptosis, which causescell death (22). MMC is an antibiotic that was isolated from Gram-negative bacteria *Streptomyces caespitosus* and can cross-link double-stranded DNA at adenosine and guanine during the G1 or S phase. This antibiotic prevents DNA stranding from separating during the DNA replication process and then halting mitosis. The antibiotic can also bind to the promoter sites of inducible genes, thereby suppressing the synthesis of cellular RNA and protein to control diseases (23). VCR as a vinca alkaloid can interact with β-tubulin in a region adjacent to the GTP-binding site to prevent the formation of spindle microtubules, thereby disabling the function of the cell for aligning and moving the chromosomes to further induce high frequency of micronuclei, chromosome aberration, sister chromatid exchange, DNA damage, and interference with DNA, RNA, and protein synthesis. All of these processes cause cancer cell death (24). Overall, all of these chemotherapeutic agents have an anti-growth effect on cancer cells via DNA or RNA dysfunction. Using them in combination with $AlPcS_4$/PDT for synergistic therapy is expected to achieve a significant antitumor effect on gastric cancer.

Chemotherapy is effective in antitumor treatment. However, chemotherapy requires multiple drug doses that can easily result in severe toxic side effects and multi-drug resistance (25). The chemotherapy agents aforementioned are no exception. Hence, using low-dose chemotherapeutic drugs in combination with $AlPcS_4$/PDT therapy may effectively reduce toxic side effects and multi-drug resistance problems. The low-dose chemical therapy also leads to significant inhibition of the growth activities of gastric cancer cells with the aid of PDT-mediated vascular permeabilization (26-28).

Therefore, in this present study, we attempted to investigate the inhibition of the growth effect by combination treatment between low-dose chemotherapeutic agents (5-FU, DOX, CDDP, MMC and VCR) and $AlPcS_4$/PDT on SGC-7901 gastric cancer cells and compare the antitumor effect between them in order to find promising combination treatment schemes with high anticancer efficiency and low toxic side effects. Given that $AlPcS_4$ was dominant in our design scheme, we evaluated the influence of $AlPcS_4$ intracellular uptake ability and ROS and SOG generation abilities in the presence of low-dose chemotherapeutic agents. The apoptosis-inducing and necrosis-inducing ability was further demonstrated. Low-dose 5-FU, DOX and MMC combination treatment had significant antitumor effects with low dark-cytotoxicity. This combination increased $AlPcS_4$ intracellular uptake ability and ROS and SOG generation abilities, thereby inducing significant apoptosis and necrosis. Low-dose CDDP and VCR combination treatment had a relatively inferior increasing inhibition effect in terms of increasing apoptosis activities. However, low-dose CDDP and VCR indicated a slight adverse effect on $AlPcS_4$ intracellular uptake ability and SOG generation ability.

Materials and methods

Reagents. 5-FU, DOX, CDDP, MMC and VCR were purchased from Sigma-Aldrich; Merck (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck) or sterile PBS (HyClone; GE Healthcare Life Sciences, Logan, UT, USA). The materials were stored at 4˚C and then diluted as needed in RPMI-1640 medium (HyClone; GE Healthcare Life Sciences) on the day of use. $AlPcS_4$ was purchased from Frontier Scientific (Logan, UT, USA) and dissolved in sterile PBS with a concentration of 2 mg/ml and stored at 4˚C in the dark. Then, $AlPcS_4$ was diluted to a range of 1-32 μg/ml following a gradient dilution method in RPMI-1640 medium on the day of use.

Cells. SGC-7901 cells, which are part of a human moderately-differentiated gastric carcinoma cell line, were donated by the State Key Laboratory of Cancer Biology, Digestion Department, Xijing Hospital, affiliated with the Fourth Military Medical University, Xi'an, China. The cells were cultured in RPMI-1640 medium that was supplemented with 10% fetal bovine serum (Sijiqing Co., Ltd., Hangzhou, China) and 1% penicillin/streptomycin in a humidified incubator (Heracell™ 150i CO_2 with copper chambers; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C with 5% CO_2.

Measurement of the absorption spectra and fluorescence spectra of a mixture of $AlPcS_4$ and chemotherapeutic agents. The absorption spectra of free-$AlPcS_4$ (8 μg/ml), $AlPcS_4$ (8 μg/ml) in the presence of 5-FU (20 μM), DOX (0.4 μg/ml), CDDP (5 μM), MMC (0.5 μg/ml) and VCR (0.1 μg/ml) were assessed at 1-h intervals for 6 h by an ultraviolet-visible spectrophotometer (V-550 UV/VIS; Jasco International, Co., Ltd., Tokyo, Japan). Fluorescence spectra of free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU, DOX, CDDP, MMC, and VCR were

recorded using a fluorescence spectrophotometer (F-4500; Hitachi, Ltd., Tokyo, Japan).

CCK-8 assay. The dark cytotoxicity and anti-growth effect of free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU, DOX, CDDP, MMC and VCR at different doses on SGC-7901 cells was assessed by CCK-8 assay. Briefly, $1x10^4$ SGC-7901 cells/well were seeded in sterile 96-well flat-bottomed plates and incubated overnight at 37˚C. Then, diluted free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU (20 μM), DOX (0.4 μg/ml), CDDP (5 μM), MMC (0.5 μg/ml), and VCR (0.1 μg/ml) were added to each well with final concentrations in the range of 1-32 μg/ml. Wells with cells were divided into different groups. Wells without drug and irradiation treatment comprised the control group, those with drug and no irradiation comprised the dark cytotoxicity group, those with drug and irradiation comprised the synergistic therapeutic group, and wells that contained only complete culture media comprised the blank control group. In the synergistic therapeutic group, the cells were incubated with free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU, DOX, CDDP, MMC and VCR and then illuminated by a 635-nm laser light with a power density of 100 mW/cm^2. All the cells treated with drugs were incubated for 6 h and then washed with PBS twice. Subsequently, they were incubated with fresh RPMI-1640 complete medium for 24 h. Following incubation, the solution containing 100 μl RPMI-1640 medium and 10 μl CCK-8 reagent (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well. The cells were then incubated for 1 h at 37˚C again. Finally, the absorbance levels of the cells were assessed at 450 nm using a microplate reader (Infinite® M200 PRO; Tecan Group, Ltd., Mannedorf, Switzerland). The dark cytotoxicity and anti-growth effect of free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU, DOX, CDDP, MMC and VCR at the doses used was calculated as: [(OD of the drug treated-OD of the blank control)/(OD of the control-OD of the blank control)] x100%.

Detection of intracellular uptake ability of $AlPcS_4$. Intracellular uptake ability of $AlPcS_4$ was evaluated by a fluorescence spectrophotometer (F-4500; Hitachi, Ltd.). In the incubator, $2.5x10^5$ SGC-7901 cells were seeded in six-well plates and allowed to attach overnight. Then, the cells were treated with RPMI-1640 complete media that contained-free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU, DOX, CDDP, MMC and VCR at the same concentrations as aforementioned. Then the cells were incubated for 6 h at 37˚C with 5% CO_2. Following incubation, the cells were washed with PBS twice, digested with trypsin enzyme (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and collected by centrifugation. Then, the emission spectra at 635 nm excitation wavelength were measured.

Detection of cell apoptosis and necrosis by Hoechst 33342/PI assay. Induction of apoptosis and necrosis was detected using Hoechst 33324/PI nuclear staining kit following the manufacturer's instructions. In the incubator, $2.5x10^5$ cells/ml SGC-7901 cells were seeded on sterile coverslips in 6-well plates and allowed to attach overnight. Then, the cells were treated with free-AlPcS4, $AlPcS_4$ in the presence of 5-FU (20 μM), DOX (0.4 μg/ml), CDDP (5 μM), MMC (0.5 μg/ml), and VCR (0.1 μg/ml) at 37˚C with 5% CO_2 for 6 h. Following incubation, the cells were washed with PBS two times, and the medium was replaced with fresh RPMI-1640 culture medium. The cells were irradiated with 635-nm laser light for 5 min and incubated at 37˚C with 5% CO_2 for 24 h. Following treatment, the cells were stained with 5 μl Hoechst 33324 and 5 μl PI reagents for 10 min. After removing the coverslips, the cells were washed with PBS again, mounted on slides with glycerol, and imaged with a fluorescence microscope (Nikon Corp., Tokyo, Japan). To quantify the percentage of apoptosis and necrosis, we counted the number of cells with apoptotic and necrotic characteristics among 200 cells at a high-power field in accordance with the results of stained cell nucleus. All statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). The statistical difference between the means was analyzed by Student's t-distribution test. Significance was set at the 5% level. Data are presented as the mean ± SD of three replicate experiments.

Generation of ROS and SOG assay. Generation of ROS and SOG were assessed by a DCFH-DA fluorescence probe (Beyotime Institute of Biotechnology, Shanghai, China), and singlet oxygen sensor green reagent (SOSGR; Thermo Fisher Scientific, Inc.) via a fluorescence spectrophotometer (F-4500; Hitachi, Ltd.). SGC-7901 cells were seeded in six-well plates at a density of $2.5x10^5$ cells/well and incubated to adhere securely. Then, the cells were treated with free-$AlPcS_4$, $AlPcS_4$ in the presence of 5-FU (20 μM), DOX (0.4 μg/ml), CDDP (5 μM), MMC (0.5 μg/ml), and VCR (0.1 μg/ml) at 1-32 μg/ml for 6 h. The cells were washed twice with PBS and irradiated with laser systems for 5 min. For the detection of ROS, the cells were harvested, incubated with 10 μmol/l DCFH-DA for 20 min at 37˚C in complete darkness again, washed with PBS twice, and assessment was conducted using a fluorescence spectrophotometer under an excitation of 488-nm of light. For the detection of SOSGR, the cells were harvested, permeabilized with 0.5% Triton X-100 in PBS for 10 min, centrifuged at 70 x g for 5 min, washed with PBS, mixed with SOSGR, irradiated with a 635-nm laser system for 5 min, and then detected using a fluorescence spectrophotometer under an excitation of 504-nm of light.

Results

Characterization of $AlPcS_4$ and the chemotherapeutic agent-$AlPcS_4$ mixture. To ensure the influence of $AlPcS_4$ itself by chemotherapeutic agent, the UV-vis absorption spectra and fluorescence intensity of $AlPcS_4$ and the chemotherapeutic agent-$AlPcS_4$ mixture and free-$AlPcS_4$ were assessed. Fig. 1A revealed that the absorption levels of $AlPcS_4$ were derived from the $AlPcS_4$ mixture. The Figure also revealed that the chemotherapeutic agent-$AlPcS_4$ mixture had weaker reduction compared to free-$AlPcS_4$ in deionized water as time changed. Compared to deionized water, the main absorption levels of $AlPcS_4$ were obtained from free-$AlPcS_4$ and the chemotherapeutic agent-$AlPcS_4$ mixture was much more stable in a culture medium that contained FBS (Fig. 1B). However, the main absorption levels of $AlPcS_4$ in the chemotherapeutic agent-$AlPcS_4$ mixture or free-$AlPcS_4$ in a culture medium that contained FBS were lower than those in deionized

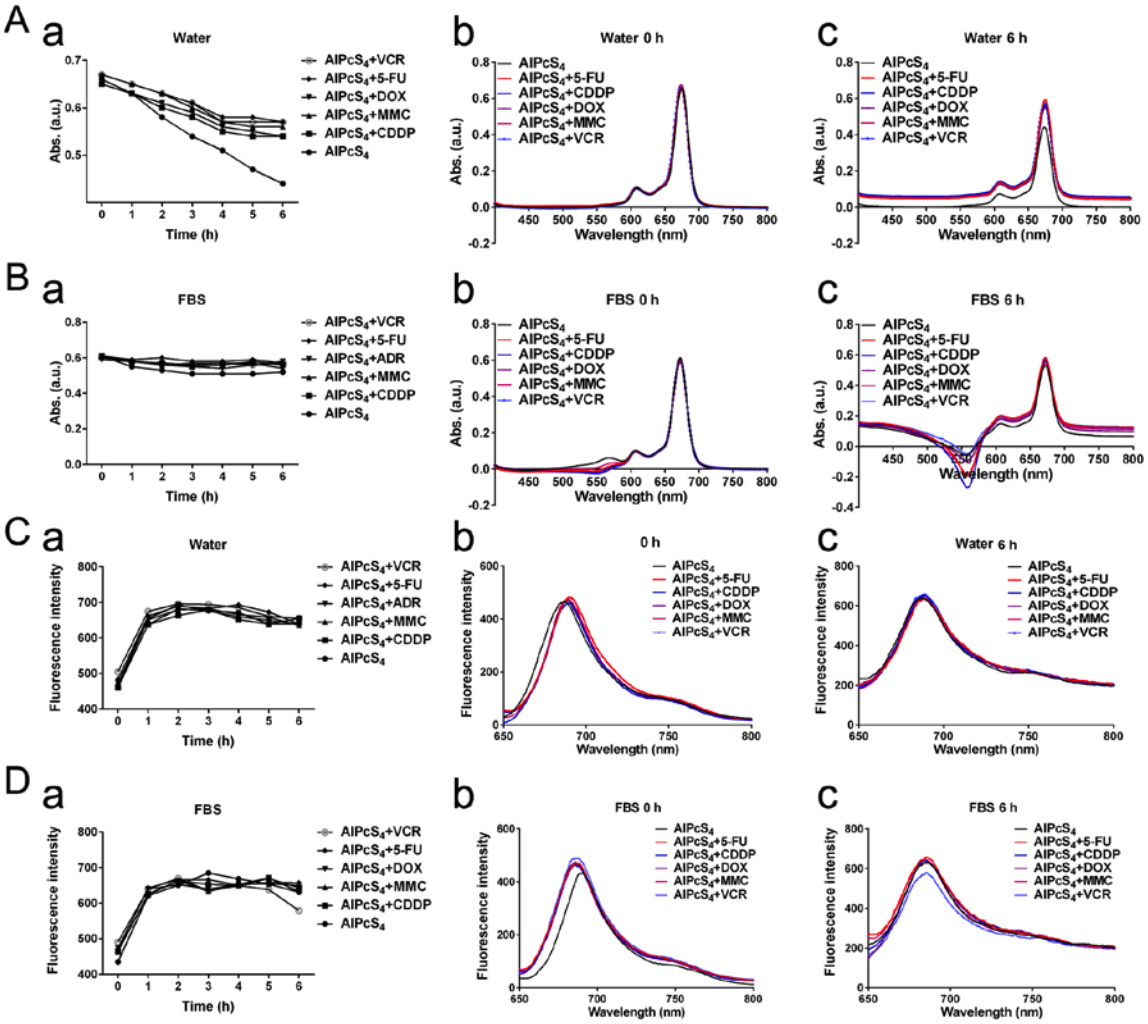

Figure 1. UV-vis absorption spectra and fluorescence intensity of $AlPcS_4$ mixture with 5-FU (20 μm), CDDP (5 μm), DOX (0.4 μm/ml), MMC (0.5 μm/ml), and VCR (0.1 μm/ml) or free-$AlPcS_4$ at 8 μm/ml. (A-a and B-a) Maximum OD values near 675 nm absorption spectra of corresponding agents in deionized water and RPMI-1640 culture medium that contained FBS at 1-6 h. (A-b and B-b) UV-vis absorption spectra of corresponding agents in deionized water and RPMI-1640 culture medium that contained FBS at 0 h. (A-c and B-c) UV-Vis absorption spectra of corresponding agents in deionized water and RPMI-1640 culture medium that contained FBS at 6 h. (C-a and D-a) Maximum fluorescence intensity near 687 nm fluorescence spectra of corresponding agents in deionized water and RPMI-1640 culture medium that contained FBS at 1-6 h. (C-b and D-b) Fluorescence spectra of corresponding agents in deionized water and RPMI-1640 culture medium that contained FBS at 0 h. (C-c and D-c) Fluorescence spectra of corresponding agentsin deionized water and RPMI-1640 culture medium that contained FBS at 6 h. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine; ROS.

water. A possible reason for this finding is that $AlPcS_4$ binds nonspecifically to serum albumin based on electrostatic interaction. The inverted absorption peaks (near 550 nm) appeared in the absorption spectra of the chemotherapeutic agent-$AlPcS_4$ mixture and free-$AlPcS_4$ in culture medium as time increased (Fig. 1B). These peaks may be caused by coagulation at different degrees. The fluorescence intensity of $AlPcS_4$ was derived from $AlPcS_4$, and the chemotherapeutic agent-$AlPcS_4$ mixture also indicated no significant change even after 6 h compared with free-$AlPcS_4$ (Fig. 1C and D). Only the fluorescence intensity of $AlPcS_4$+VCR was slightly reduced after 5 h (Fig. 1D). Furthermore, whether in deionized water or culture medium that contained FBS, the fluorescence intensities markedly increased after dilution of the chemotherapeutic agent-$AlPcS_4$ mixture for 1 h. These may be induced by $AlPcS_4$ at high concentrations, and it had a concentration-dependent fluorescent quenching effect. Overall, the selected chemotherapeutic agents in the present study did not influence $AlPcS_4$ itself. In culture medium, $AlPcS_4$ and the chemotherapeutic agent-$AlPcS_4$ mixture scarcely influenced the absorption levels and fluorescence intensity of $AlPcS_4$. However, the main absorption levels and fluorescence intensity of $AlPcS_4$ (near 675 nm) were not influenced by a chemotherapeutic agent.

Cytotoxicity and anti-growth effect of single or combination treatment. To determine whether the selected chemotherapeutic agents and $AlPcS_4$ under laser light exposure had synergistic antitumor effects on SGC-7901 cells, dark cytotoxicity, and photo-cytotoxicity were determined by CCK-8 assay. As shown in Fig. 2, without light irradiation, the cell viability of SGC-7901 cells incubated with various chemotherapeutic agents, different concentrations of free-$AlPcS_4$, and the mixture of chemotherapeutic agents with different concentrations of $AlPcS_4$ was mostly higher than 80, 90 and 80% respectively. The dark cytotoxicity of $AlPcS_4$ with or

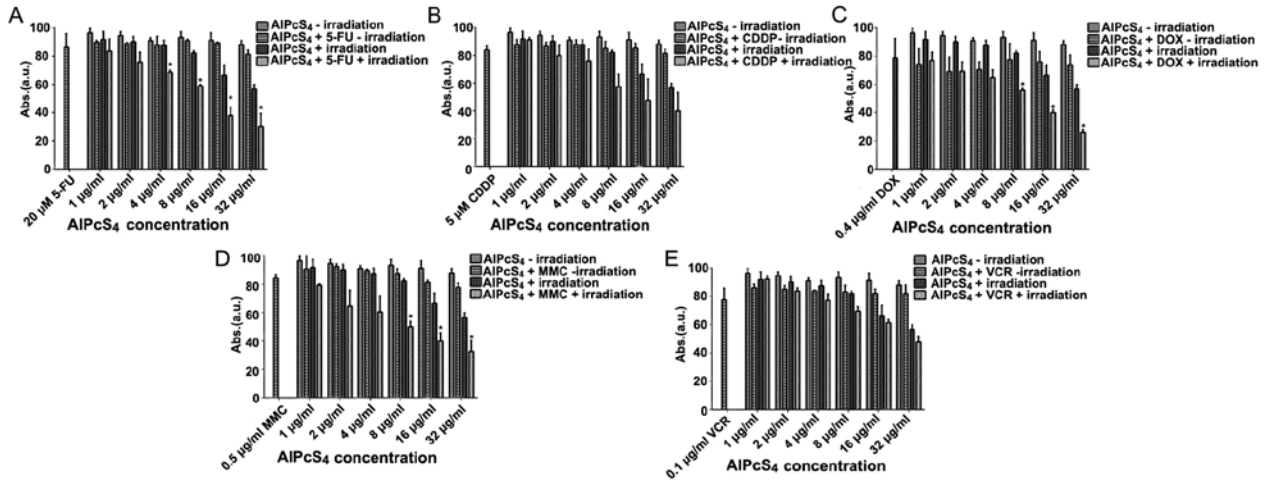

Figure 2. Antitumor growth effect on SGC-7901 cells in single and combination treatment therapy as determined by CCK-8 assay. Dark cytotoxicity and antitumor growth effect on SGC-7901 cells by (A) $AlPcS_4$+5-FU, (B) $AlPcS_4$+CDDP, (C) $AlPcS_4$+DOX, (D) $AlPcS_4$+MMC or (E) $AlPcS_4$+VCR. The cells were treated with 1-32 μm/ml free-$AlPcS_4$ or $AlPcS_4$+5-FU (20 μm), $AlPcS_4$+CDDP (5 μm), $AlPcS_4$+DOX (0.4 μm/ml), $AlPcS_4$+MMC (0.5 μm/ml) or $AlPcS_4$+VCR (0.1 μm/ml) for 6 h. The cells were then incubated again for 24 h with or without 635-nm laser irradiation at 100 mW/cm^2 illumination dosage for 5 min. $^*P<0.05$, represents a statistical difference in antitumor effect between the combination of $AlPcS_4$ with a chemical agent and free-$AlPcS_4$. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

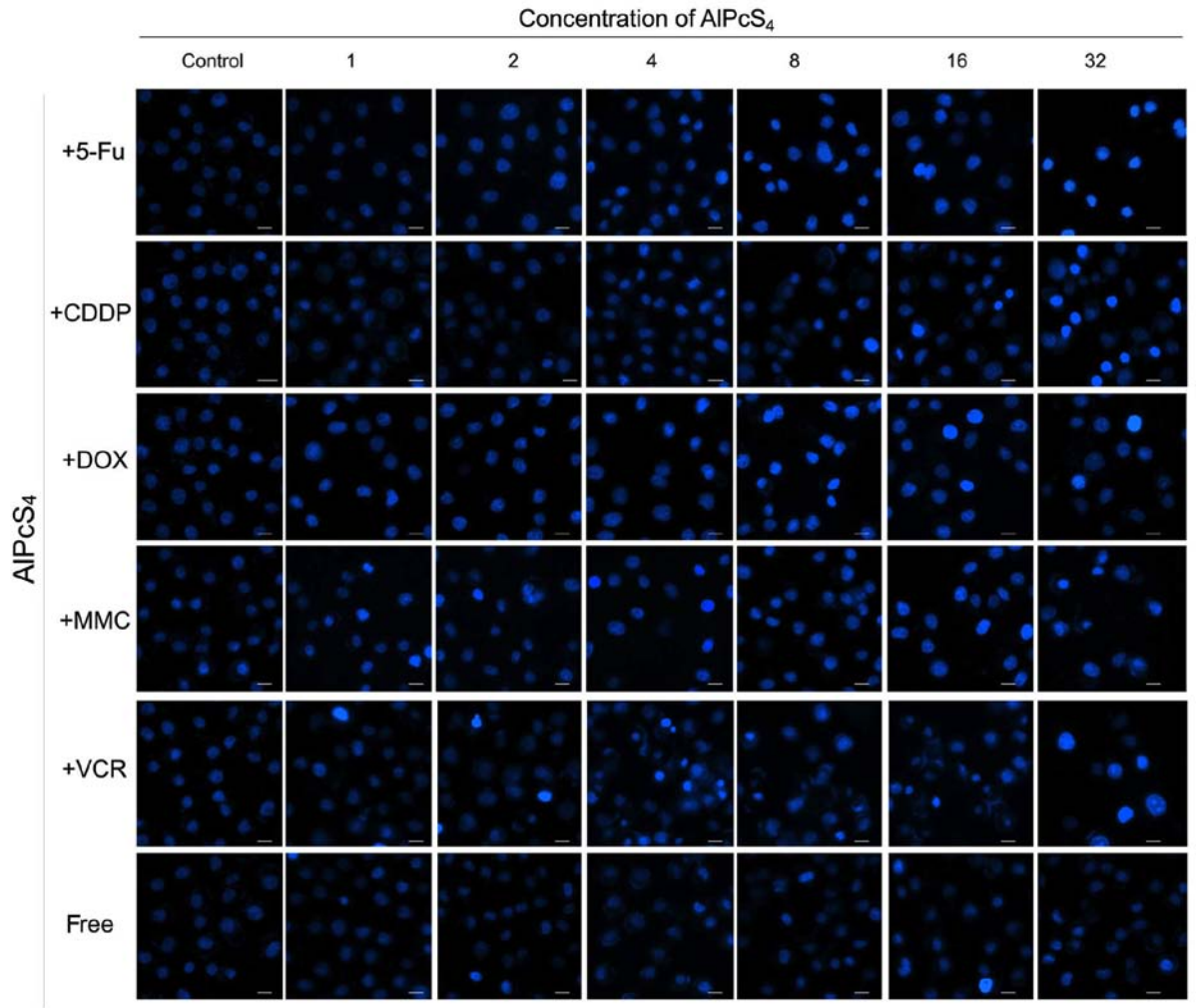

Figure 3. Apoptosis induced by $AlPcS_4$+5-FU, $AlPcS_4$+CDDP, $AlPcS_4$+DOX, $AlPcS_4$+MMC, $AlPcS_4$+VCR and free-$AlPcS_4$ in SGC-7901 cells after being irradiated for 6 h. The cells were then treated with 1-32 μm/ml free-$AlPcS_4$ or $AlPcS_4$+5-FU (20 μm), $AlPcS_4$+CDDP (5 μm), $AlPcS_4$+DOX (0.4 μm/ml), $AlPcS_4$+MMC (0.5 μm/ml), or $AlPcS_4$+VCR (0.1 μm/ml) for 6 h. These samples were then irradiated with 635-nm laser irradiation at 100 mW/cm^2 illumination dosage for 5 min, incubated for 6 h, stained with Hoechst 33342 probe, and then imaged using afluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 μm. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

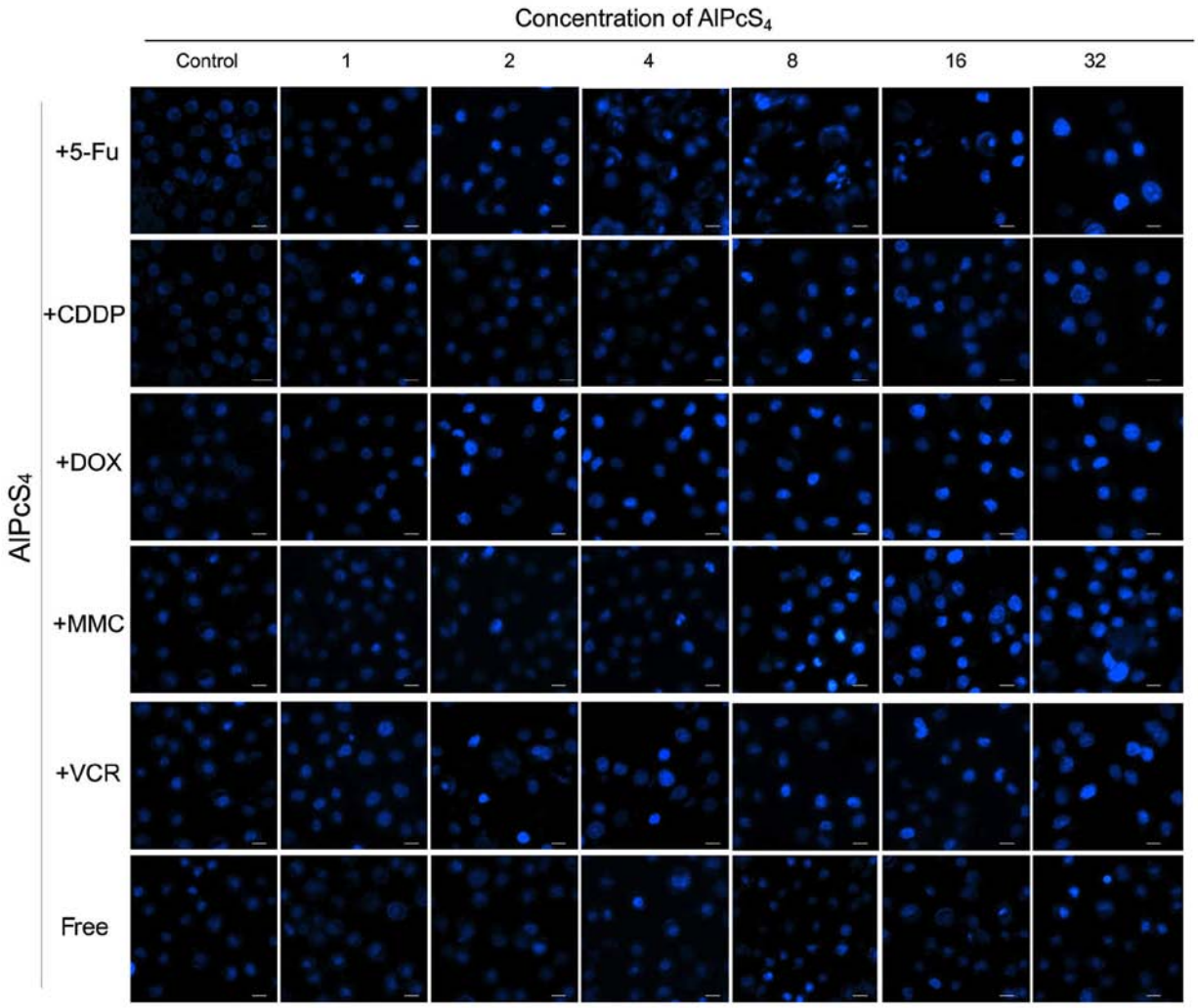

Figure 4. Apoptosis induced by AlPcS$_4$+5-FU, AlPcS$_4$+CDDP, AlPcS$_4$+DOX, AlPcS$_4$+MMC, AlPcS$_4$+VCR, and free-AlPcS$_4$ in SGC-7901 cells after being irradiated for 12 h. The cells were treated with 1-32 μm/ml free-AlPcS$_4$ or AlPcS$_4$+5-FU (20 μm), AlPcS$_4$+CDDP (5 μm), AlPcS$_4$+DOX (0.4 μm/ml), AlPcS$_4$+MMC (0.5 μm/ml) or AlPcS$_4$+VCR (0.1 μm/ml) for 6 h. The cells were then irradiated with 635-nm laser irradiation at 100 mw/cm^2 illumination dosage for 5 min, incubated for 12 h, stained with Hoechst 33342 probe, and then imaged using afluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 μm. AlPcS$_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

without low-dose chemotherapeutic agents was markedly low. The dark cytotoxicity induced by the combination treatment of AlPcS$_4$ with a low-dose chemotherapeutic agent was lower than the summation of the dark cytotoxicity induced by free-AlPcS$_4$ as well as the low-dose chemotherapeutic agent alone. The dark cytotoxicity was even lower than that from the low-dose chemotherapeutic agent alone. Hence, without laser irradiation, the antagonistic effects in the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents were obtained. The antagonistic effects of AlPcS$_4$+VCR and AlPcS$_4$+CDDP were higher than those of AlPcS$_4$+5-FU, AlPcS$_4$+DOX, and AlPcS$_4$+MMC.

After 635 nm laser irradiation of 100 mW/cm^2 illumination dosage for 5 min, the cell viability of free-AlPcS$_4$ slightly decreased as the concentration increased. Even at 32 μg/ml, the cell viabilities of cells remained at ~57%. However, the viability of cells treated with AlPcS$_4$+5-FU, AlPcS$_4$+DOX, AlPcS$_4$+CDDP, AlPcS$_4$+MMC and AlPcS$_4$+VCR after irradiation by laser light had a significant decrease, especially at high concentrations. At 32 μg/ml, the cell viabilities of cells were decreased to roughly 30, 25, 39, 32 and 47%, respectively. Notably, the anti-growth effect by PDT combined with chemical therapy of AlPcS$_4$+5-FU, AlPcS$_4$+DOX, and AlPcS$_4$+MMC was higher than the efficiency summation of free-AlPcS$_4$ and 5-FU, DOX, and MMC. When we discarded the antitumor growth effect induced by free-AlPcS$_4$ at the highest concentration and 5-FU, DOX, or MMC, the inhibitory effects of the combination treatment increased at average values of 12.49, 14.67 and 10.3%, respectively. The results of the statistical analysis revealed that there were significant differences between free-AlPcS$_4$ and AlPcS$_4$+5-FU, AlPcS$_4$+DOX, and AlPcS$_4$+MMC concerning the antitumor effect, compared with AlPcS$_4$+CDDP andAlPcS$_4$+VCR.

Apoptosis/necrosis-inducing abilities of single or combination treatment. Apoptosis is the main cause of death in PDT. Hence, to ascertain whether the selected chemotherapeutic agents and AlPcS$_4$ under laser light exposure induced apoptosis in the cells, a Hoechst 33324 and PI staining assay was conducted. As shown in Figs. 3-5, cell shrinkage and nuclear

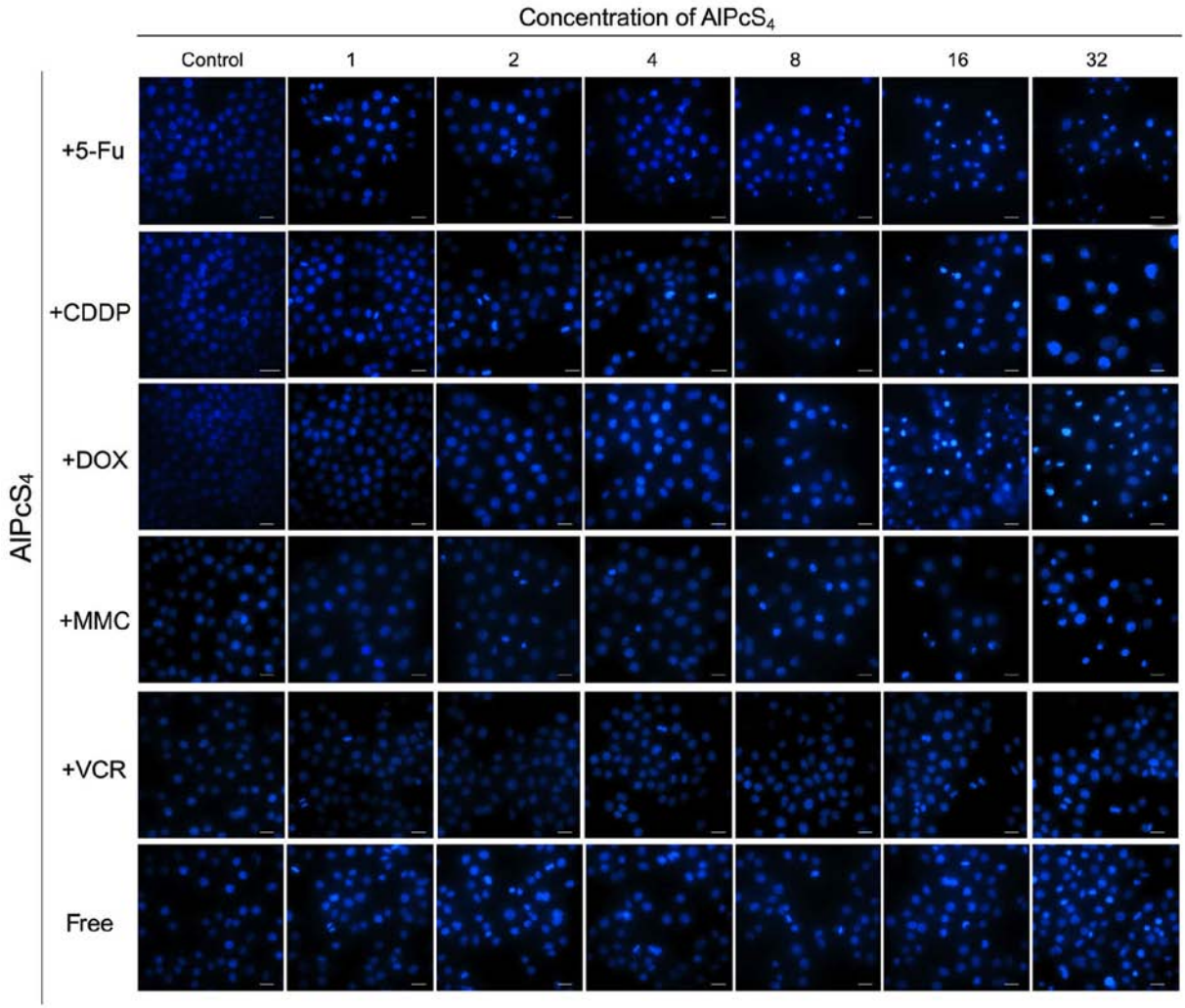

Figure 5. Apoptosis induced by $AlPcS_4$+5-FU, $AlPcS_4$+CDDP, $AlPcS_4$+DOX, $AlPcS_4$+MMC, $AlPcS_4$+VCR and free-$AlPcS_4$ in SGC-7901 cells after being irradiated for 24 h. The cells were then treated with 1-32 μm/ml free-$AlPcS_4$ or $AlPcS_4$+5-FU (20 μm), $AlPcS_4$+CDDP (5 μm), $AlPcS_4$+DOX (0.4 μm/ml), $AlPcS_4$+MMC (0.5 μm/ml) or $AlPcS_4$+VCR (0.1 μm/ml) for 6 h. The cells were then irradiated with 635-nm laser irradiation at 100 mW/cm^2 illumination dosage for 5 min, incubated for 24 h, stained with Hoechst 33342 probe, and then imaged using afluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 μm. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

fragmentation appeared in SGC-7901 cells induced by both free-$AlPcS_4$ and the chemotherapeutic agent-$AlPcS_4$ mixture after irradiation at 6, 12 and 24 h, only the number of cell induced by free-$AlPcS_4$ was low. In other words, apoptosis is active in $AlPcS_4$/PDT. The apoptosis-inducing abilities were increased in SGC-7901 cells in $AlPcS_4$/PDT synergistic treatment with low-dose chemical drug agents. Apoptosis induced by single $AlPcS_4$/PDT was mainly active at 6-12 h. Furthermore, given the presence of chemotherapeutic agents, especially 5-FU, DOX and MMC, the duration of apoptosis-inducing time increased. Even at 24 h, higher apoptosis-inducing abilities were obtained (Fig. 7). In addition, the increased apoptosis-inducing abilities were obtained in the combination therapy even without the synergistic effects. However, apoptosis was induced quickly at 6 h (Fig. 3). The increasing trend may be due to the low-dose chemotherapeutic agent. Hence, although the apoptosis-inducing abilities decreased at 12 and 24 h (Figs. 4 and 5), the final inhibitory effects of cell viabilities in combination treatment of $AlPcS_4$/PDT with low-dose CDDP and VCR increased.

In the synergistic therapy, higher apoptosis-inducing abilities induced by $AlPcS_4$/PDT+DOX were obtained, especially at high concentrations of $AlPcS_4$ at 24 h, compared to $AlPcS_4$/PDT+MMC (Fig. 5). In the treatment of $AlPcS_4$+DOX or$AlPcS_4$+MMC and irradiation with laser light after 6 and 24 h, the percentage of apoptotic bodies increased at average values of 3.8-, 1.9-, 2.8- and 1.7-fold, at 6 and 24 h, respectively (Fig. 7). Additional late apoptotic and necrotic cells were obtained even at 6 and 12 h after treatment by $AlPcS_4$/PDT+DOX (Fig. 6). In contrast to $AlPcS_4$/PDT+DOX and $AlPcS_4$/PDT+MMC, apoptosis was induced quickly by $AlPcS_4$/PDT+5-FU at 6 h. This increasing trend was similar to that of $AlPcS_4$/PDT+VCR. In addition, the apoptosis-inducing abilities increased (Figs. 3 and 7). At 32 μg/ml, the percentage of apoptotic bodies of $AlPcS_4$+5-FU at 6, 12 and 24 h reached ~38, 80 and 88%, respectively (Fig. 7). The apoptosis-inducing abilities at12 and 24 h were higher than those in $AlPcS_4$/PDT+DOX and $AlPcS_4$/PDT+MMC. In addition, more necrotic cells were observed at 12 h after treatment with $AlPcS_4$/PDT+5-FU (Fig. 6).

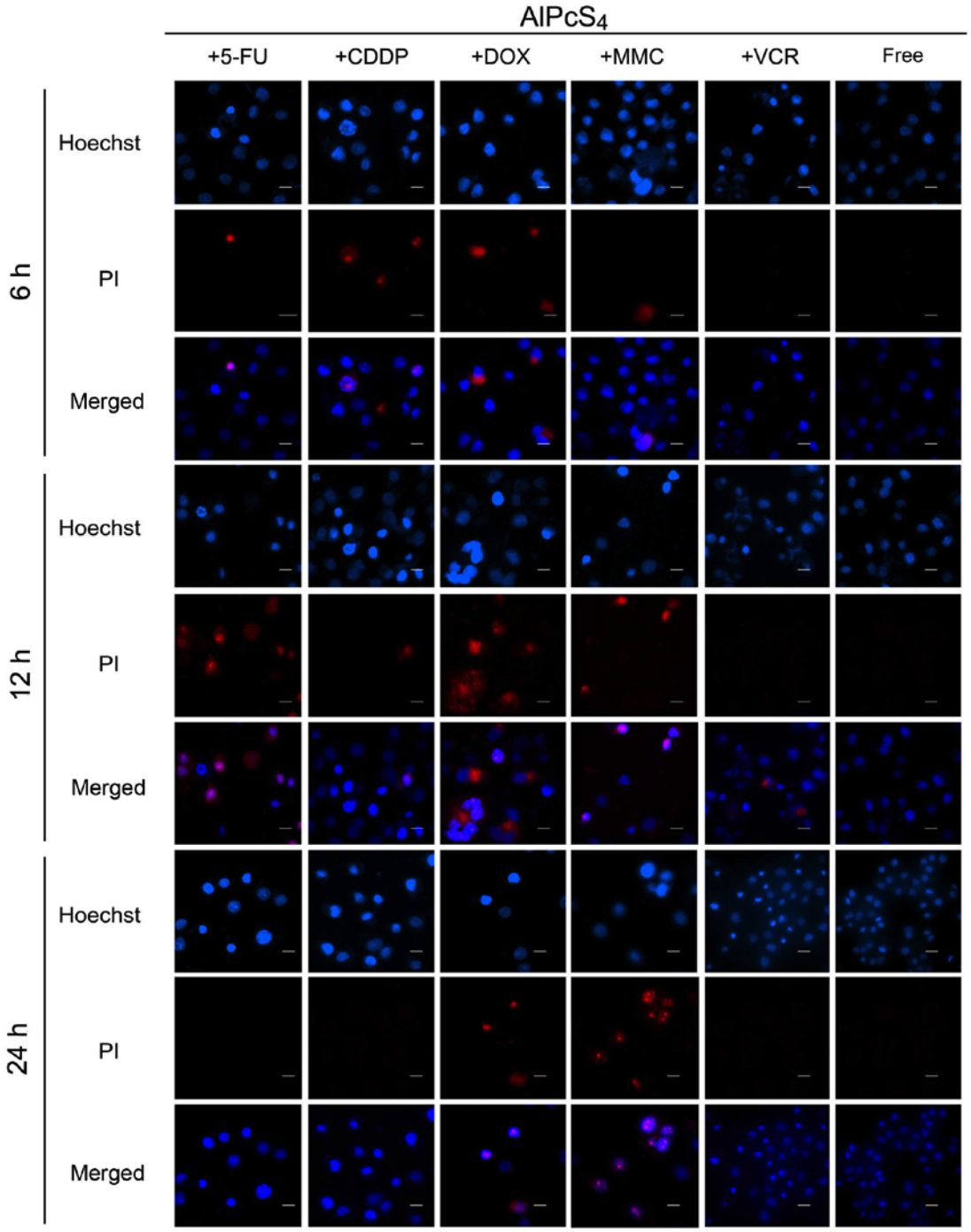

Figure 6. Apoptosis and necrosis induced by AlPcS$_4$+5-FU, AlPcS$_4$+CDDP, AlPcS$_4$+DOX, AlPcS$_4$+MMC, AlPcS$_4$+VCR and free-AlPcS$_4$ in SGC-7901 cells after being irradiated for 6, 12 and 24 h. The cells were treated with 1-32 μm/ml free-AlPcS$_4$ or AlPcS$_4$+5-FU (20 μm), AlPcS$_4$+CDDP (5 μm), AlPcS$_4$+DOX (0.4 μm/ml), AlPcS$_4$+MMC (0.5 μm/ml) or AlPcS$_4$+VCR (0.1 μm/ml) for 6 h. The cells were then irradiated with 635-nm laser irradiation at 100 mW/cm^2 illumination dosage for 5 min. The cells were incubated for 6, 12 and 24 h, stained with Hoechst 33342 and PI probes, and imaged usingfluorescence microscopy. Below 32 μm/ml, no necrosis was obtained. Therefore, the Hoechst staining and PI images are shown at 32 μm/ml. All the Hoechst staining and PI images were acquired at an x400 magnification. The scale bar represented 20 μm. AlPcS$_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

In addition, the apoptosis assay results revealed that AlPcS$_4$/PDT+5-FU evidently improved apoptosis-inducing abilities even at low concentrations of AlPcS$_4$. AlPcS$_4$/PDT+DOX slightly increased apoptosis-inducing abilities at low concentrations of AlPcS$_4$ at 24 h. AlPcS$_4$/PDT+MMC indicated fewer increased apoptosis-inducing abilities at a low concentration of AlPcS$_4$. At 4 μg/ml AlPcS$_4$, the percentage of apoptotic bodies of AlPcS$_4$/PDT+MMC reached~16 and 29%, at 6 and 24 h, respectively. Generally, 5-FU and DOX have optimal apoptosis-inducing abilities of AlPcS$_4$ even at low concentrations. MMC markedly improved the apoptosis-inducing abilities of AlPcS$_4$ at a high concentration. CDDP and VCR slightly improved apoptosis-inducing abilities even when significantly inhibited.

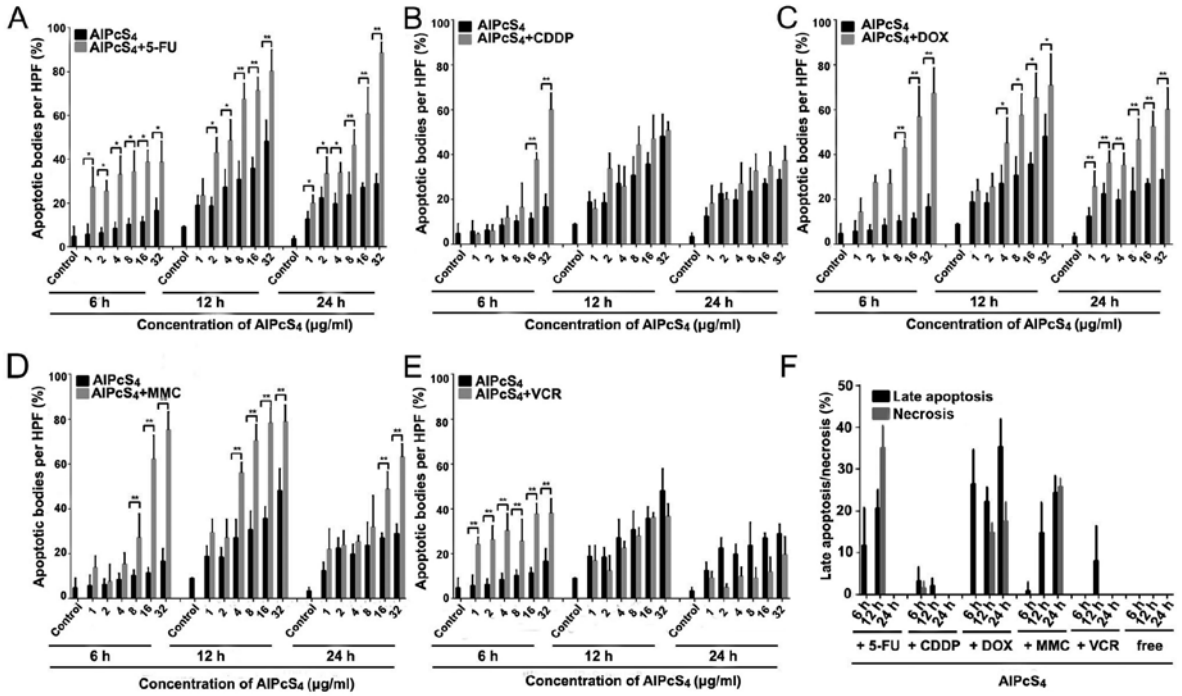

Figure 7. Statistical analysis of apoptosis and necrosis induced by $AlPcS_4$ + 5-FU, $AlPcS_4$ + CDDP, $AlPcS_4$ + DOX, $AlPcS_4$ + MMC, $AlPcS_4$ + VCR and free-$AlPcS_4$ in SGC-7901 cells after being irradiated for 6, 12 and 24 h. (A-E) The histograms represent the percentage of cells with apoptotic and necrotic characteristics among 200 cells at a high-power field. The data represent the average of three experiments. The bar is the SD. $^{*}P<0.05$ and $^{**}P<0.01$ represented a statistically significant difference in the number of apoptotic bodies between the combination of $AlPcS_4$ with a chemical agent and free-$AlPcS_4$. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

Influence of $AlPcS_4$ fluorescence intensity on combination treatment. To estimate the influence of $AlPcS_4$ delivery efficiency on combination treatment, a fluorescence intensity assay of $AlPcS_4$ was evaluated after treatment with different chemotherapeutic agents and $AlPcS_4$. As revealed in Fig. 8, the fluorescence intensity of $AlPcS_4$ significantly increased with the help of low-dose 5-FU and DOX. Notably, $AlPcS_4$+5-FU exhibited a significant difference at all the used concentrations compared to free-$AlPcS_4$, but $AlPcS_4$+DOX exhibited a statistical difference only at a high concentration. At a high concentration of $AlPcS_4$, the increasing fluorescence intensity trend in the presence of DOX was greater than 5-FU. At the highest concentration of $AlPcS_4$, DOX and 5-FUfluorescence intensity was increased by 300 and 270%, respectively. Compared with DOX and 5-FU, MMC resulted in inferior increases by 150% on average. Furthermore, only at 32 μg/ml $AlPcS_4$, did the fluorescence intensity exhibit a statistical difference between $AlPcS_4$+MMC and free-$AlPcS_4$. Conversely, CDDP and VCR did not markedly improve the fluorescent intensity of $AlPcS_4$. VCR even reduced the fluorescence intensity of $AlPcS_4$. The fluorescent intensity of $AlPcS_4$ could be used to reveal the efficiency of cellular internalization of $AlPcS_4$. Thus, DOX and 5-FU could prominently increase the efficiency of cellular internalization of $AlPcS_4$. MMC could slightly increase the efficiency of cellular internalization of $AlPcS_4$. CDDP and VCR could not increase the efficiency of cellular internalization of $AlPcS_4$, even when reduced.

Influence of $AlPcS_4$ intracellular location on combination treatment. The intracellular location of the photosensitizer is a significant factor that influences the PDT effect. To observe the intracellular location of $AlPcS_4$ and evaluate the influence of $AlPcS_4$ intracellular location in the presence of low-dose used chemical agents, fluorescence imaging was carried out. As shown in Fig. 8F, intracellular staining remained mainly in the cytoplasm and did not change compared with free-$AlPcS_4$. However, compared with free-$AlPcS_4$, the fluorescence signal significantly increased after treatment with $AlPcS_4$+5-FU and $AlPcS_4$+DOX. These results were consistent with the results of fluorescent intensity assay.

SOG generation of single or combination treatment. Underlight activation, the photosensitizer generated SOG to induce cancer cell death. Thus, the concentration of SOG in SGC-7901 cells induced by single (free-$AlPcS_4$) or combination ($AlPcS_4$+chemical agent) treatment was assessed. 5-FU and DOX improved SOG generation abilities of $AlPcS_4$ (Fig. 9). Compared withfree-$AlPcS_4$, 5-FU and DOX resulted in significant increases at average values of 1.7- and 1.9-fold and exhibited statistical differences. MMC resulted in inferior increases, it could improve $AlPcS_4$ SOG generation abilities by 120% on average but it did not exhibit statistical differences (Fig. 9). Compared with 5-FU, DOX and MMC, CDDP and VCR were not able to increase the concentration of SOG (Fig. 9). At high concentrations, VCR reduced SOG generation abilities of $AlPcS_4$. These results were consistent with the results obtained in the fluorescent intensity assay of $AlPcS_4$+VCR.

ROS generation of single or combination treatment. Given the help of SOG generated by the photosensitizer, the ROS concentration associated with reticulum stress may be easily

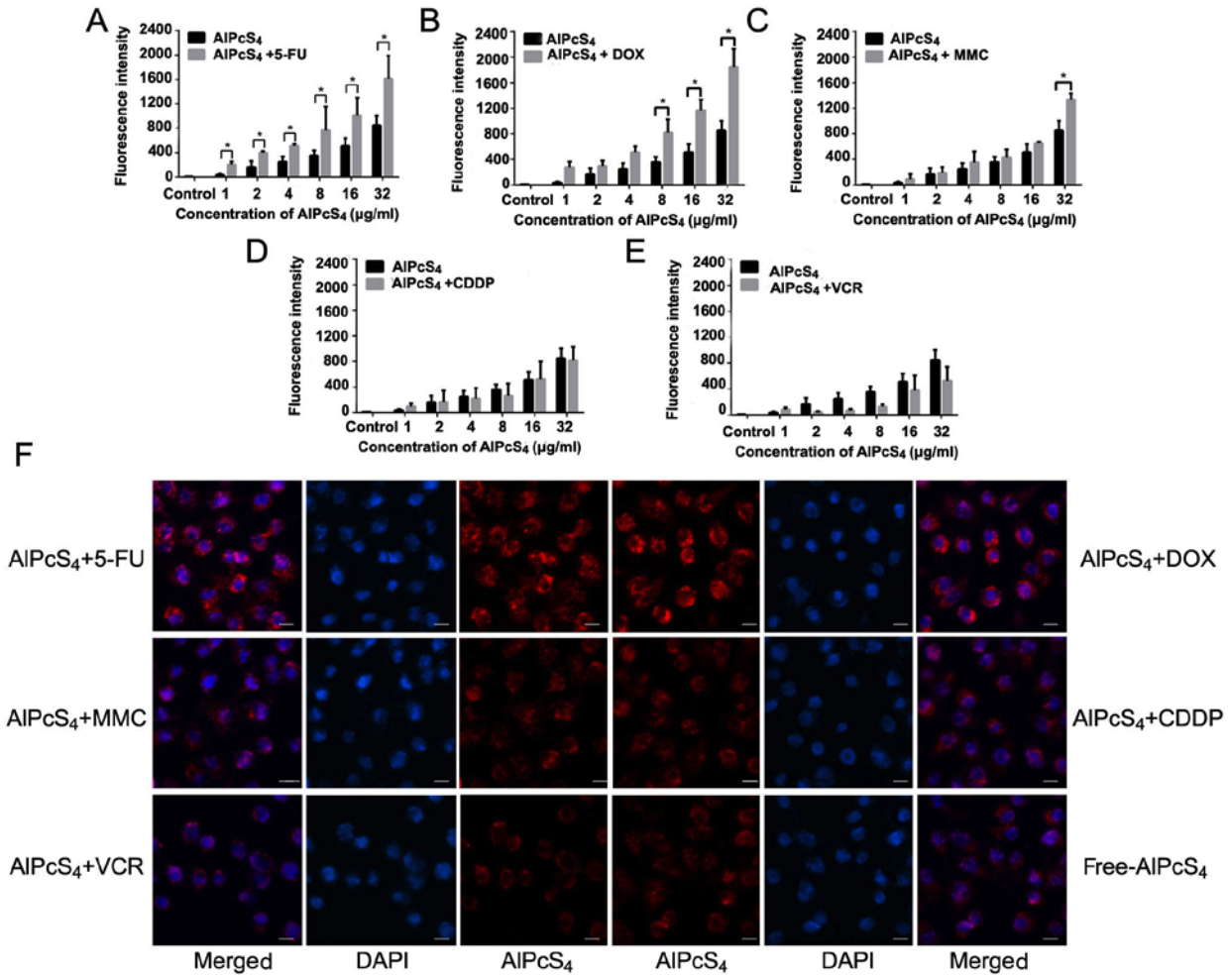

Figure 8. Fluorescence intensity analysis and fluorescence imaging of $AlPcS_4$ in SGC-7901 cells after treatment with $AlPcS_4$+5-FU, $AlPcS_4$+DOX, $AlPcS_4$+MMC, $AlPcS_4$+CDDP, $AlPcS_4$+VCR and free-$AlPcS_4$. (A-E) Fluorescent intensity analysis of $AlPcS_4$ in SGC-7901 cells after treatment with 1-32 μm/ml free-$AlPcS_4$, $AlPcS_4$+5-FU (20 μm), $AlPcS_4$+DOX (0.4 μm), $AlPcS_4$+MMC (0.5 μm/ml), $AlPcS_4$+CDDP (5 μm) or $AlPcS_4$+VCR (0.1 μm/ml) for 6 h and measured by using a fluorescence spectrophotometer. The data represents the average of three experiments and the bar is the SD. *P<0.05 and **P<0.01 represented a statistically significant difference in the fluorescence intensity of $AlPcS_4$ in cells between the combination therapy of $AlPcS_4$ with a chemical agent and single therapy of free-$AlPcS_4$. (F) The fluorescent images of $AlPcS_4$ in SGC-7901 cells after treatment with 32 μm/ml free-$AlPcS_4$, $AlPcS_4$+5-FU (20 μm), $AlPcS_4$+DOX (0.4 μm/ml), $AlPcS_4$+MMC (0.5 μm/ml), $AlPcS_4$+CDDP (5 μm) or $AlPcS_4$+VCR (0.1 μm/ml) for 6 h and measured using a fluorescence microscope. All the images were acquired at an x400 magnification. The scale bar represented 20 μm. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

increased by chemical agents and the photosensitizer, thereby triggering apoptosis pathway activation and leading to cell death (29,30). Hence, the evaluation of the ROS generation induced by $AlPcS_4$ combination treatment with chemical agentswas necessary. As shown in Fig. 10, after pre-treatment with $AlPcS_4$ and chemical agents and irradiation with laser light, DCFH-DA fluorescence intensity of SGC-7901 cells increased at different levels. At the highest concentration of $AlPcS_4$, treatments with $AlPcS_4$/PDT +5-FU, $AlPcS_4$/PDT +DOX, $AlPcS_4$/PDT +MMC, $AlPcS_4$/PDT +CDDP, and $AlPcS_4$/PDT+VCR resulted in increases by 10.5-, 8.7-, 3.1-, 2.9- and 2-fold on average, respectively, compared with free-$AlPcS_4$/PDT. At all concentrations of $AlPcS_4$, treatments with $AlPcS_4$/PDT +5-FU, $AlPcS_4$/PDT +DOX, $AlPcS_4$/PDT +MMC, $AlPcS_4$/PDT +CDDP, and $AlPcS_4$/PDT+VCR resulted in 6.3-, 5.6-, 2.9-, 2- and 1.6-fold, increases, respectively, compared with free-$AlPcS_4$/PDT. In contrast to MMC, CDDP, and VCR, 5-FU and DOX increased ROS concentration in a dose-dependent manner, and the increasing fluorescence intensity trend of 5-FU was higher than that of DOX. Statistical analysis also revealed that only $AlPcS_4$+5-FU and $AlPcS_4$+DOX exhibited significant differences compared with free-$AlPcS_4$.

Discussion

$AlPcS_4$ is a second-generation photosensitizer that may be a promising antitumor agent in PDT for gastric cancer therapy due to its emission spectra in the NIR region, deep penetration in tissue, high quantum yields, good photostability, and little photobleaching. However, its low delivery efficiency induces slight penetration ability in cancer cells, thereby leading to a limited PDT effect on gastric cancer cells. These issues warrant resolution. Gantchev *et al* proposed a combination treatment strategy via $AlPcS_4$/PDT and etoposide, an antitumor drug, on K562 human leukemic cells to improve the antitumor effect of $AlPcS_4$/PDT (31). This proposal which points to the combination treatment of $AlPcS_4$/PDT with chemical agents, may be a

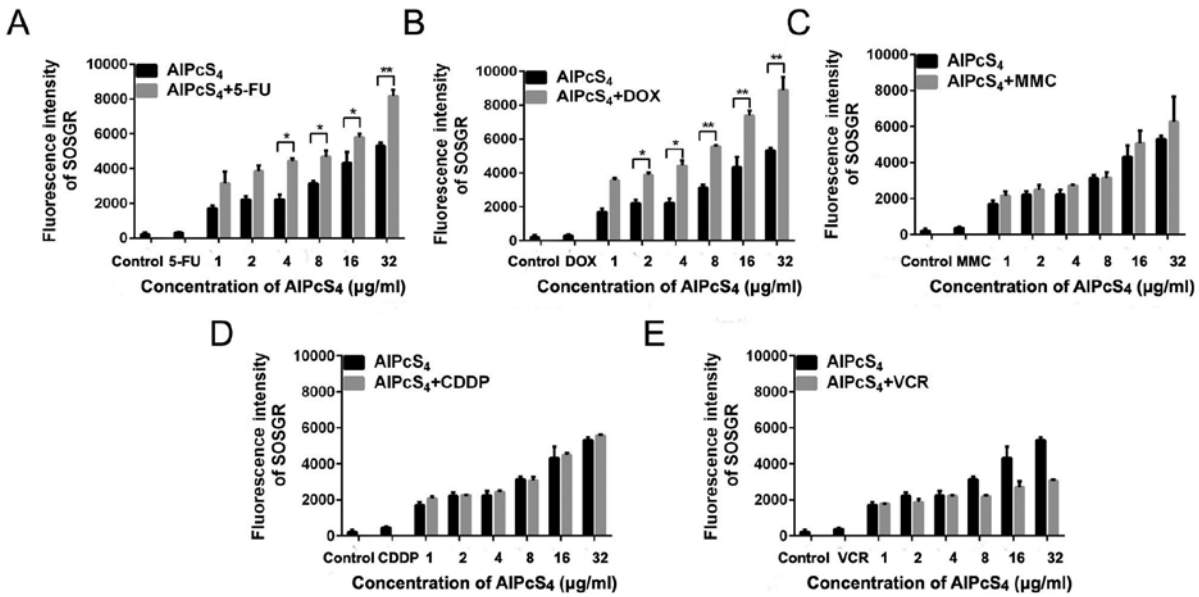

Figure 9. SOG production in SGC-7901 cells treated with $AlPcS_4$+5-FU, $AlPcS_4$+CDDP, $AlPcS_4$+DOX, $AlPcS_4$+MMC, $AlPcS_4$+VCR and free-$AlPcS_4$. (A-E) Fluorescence intensities of SOSGR probes were measured to analyze SOG in SGC-7901 cells after treatment with 1-32 μm free-$AlPcS_4$. (A) $AlPcS_4$+5-FU (20 μm), (B) $AlPcS_4$+DOX (0.4 μm/ml), (C) $AlPcS_4$+MMC (0.5 μm/ml), (D) $AlPcS_4$+CDDP (5 μm) or (E) $AlPcS_4$+VCR (0.1 μm/ml) and irradiation with 635-nm laser light for 5 min. Data represent the average of three experiments and the bar is the SD. *P<0.05 and **P<0.01 represent a statistically significant difference in the fluorescence intensity of SOSGR in cells between the combination therapy of $AlPcS_4$ with a chemical agent and single therapy of free-$AlPcS_4$. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine; SOG, singlet oxygen.

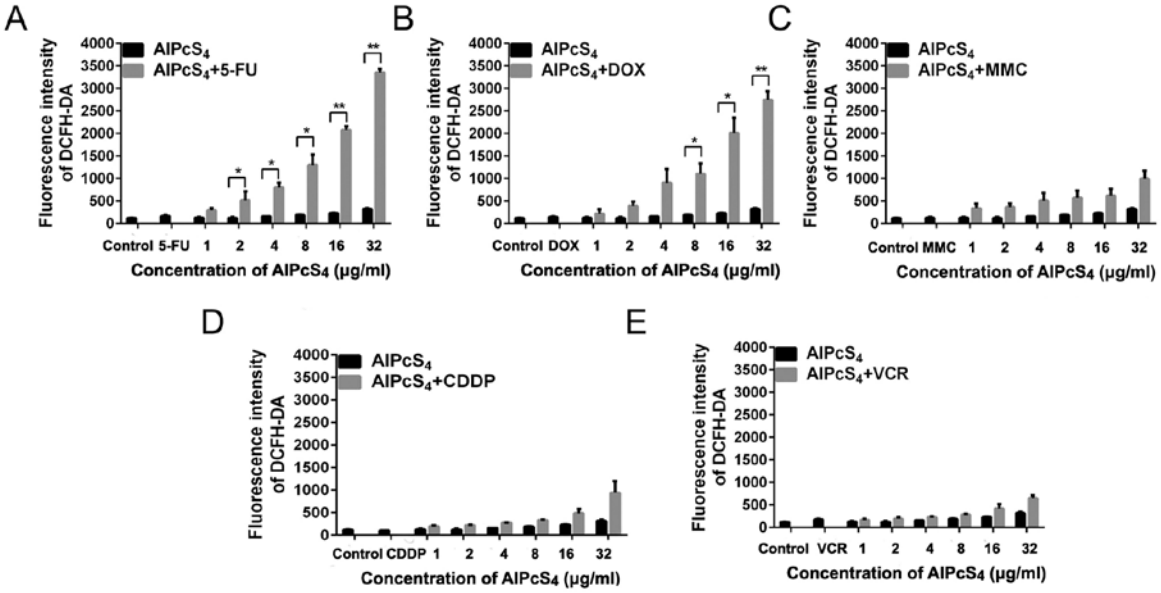

Figure 10. ROS production in SGC-7901 cells treated with $AlPcS_4$+5-FU, $AlPcS_4$+CDDP, $AlPcS_4$+DOX, $AlPcS_4$+MMC, $AlPcS_4$+VCR and free-$AlPcS_4$. (A-E) Fluorescence intensities of DCFH-DA probes were measured to analyze ROS in SGC-7901 cells after treatment with 1-32 μm/ml free-$AlPcS_4$. (A) $AlPcS_4$+5-FU (20 μm/ml), (B) $AlPcS_4$+DOX (0.4 μm/ml), (C) $AlPcS_4$+MMC (0.5 μm/ml), (D) $AlPcS_4$+CDDP (5 μm) or (E) $AlPcS_4$+VCR (0.1 μm/ml) and irradiation with 635-nm laser light for 5 min. Data represent the average of three experiments and the bar is the SD. *P<0.05 and **P<0.01 represent a statistically significant difference in the fluorescence intensity of DCFH-DA in cells between the combination therapy of $AlPcS_4$ with a chemical agent and single therapy of free-$AlPcS_4$. $AlPcS_4$, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine; ROS, reactive oxygen species.

promising method for gastric cancer therapy. Little research has focused on the combination of PDT with conventional chemotherapeutics for gastric cancer therapy. Thus, using different chemical drugs for combination with $AlPcS_4$/PDT to optimize treatment effects on gastric cancer should be investigated.

Nonaka *et al* demonstrated that the combination of Photofrin/PDT with CDDP enhanced cytotoxic and apoptotic effects, thereby inhibiting the cell growth in lymphoma cancer and esophageal carcinoma (32). Casas *et al* evaluated the synergistic effect between 5-ALA/PDT and DOX on

mammary adenocarcinomas, and the anticancer effect was significantly enhanced by the combined treatment. Datta *et al* observed that combined 5-ALA/PDT and MMC treatment was a workable therapeutic approach on superficial bladder cancer (33). Martin *et al* demonstrated that the enhancement of antitumor growth effectiveness could be obtained by combining 5-ALA/PDT and 5-FU in non-melanoma skin cancer (34). Dima *et al* revealed that a clearly positive inhibition effect was obtained by using the combination of Photofrin/PDT with VCR in ovarian cancer (35). CDDP, DOX, MMC, 5-FU and VCR are also common gold-standard chemotherapeutic agents that are clinically recommended for gastric cancer. Hence, we can infer that CDDP, DOX, MMC, 5-FU, and VCR may be used for AlPcS$_4$/PDT combination treatment to improve the effect of AlPcS$_4$/PDT. However, all the aforementioned chemotherapeutic agents have high systemic toxicity, drug resistance, high rates of tumor metastasis, and recurrence during use. Therefore, a combined treatment must be used to enhance therapeutic efficacy, reduce toxic side effects, and elude drug resistance. Ilaria *et al* investigated the antitumor growth effect of the combination of indocyanine green/PDT with low-dose CDDP on breast cancer cells. Wei *et al* evaluated the inhibitory effect of the combination of 5-ALA/PDT with low-dose CDDP on HeLa cells (28,36). The results revealed that indocyanine green or 5-ALA/PDT combination with low-dose CDDP have mutual reinforcement of antitumor efficacy while having few toxic side effects. Therefore, in the present study, we investigated the combined effect of AlPcS4/PDT with low-dose CDDP, DOX, MMC, 5-FU and VCR on SGC-7901 gastric cancer cells, respectively.

The inhibition of cell viability induced by the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents without 635-nm laser irradiation was lower than the summation of the inhibition of cell viability induced by free-AlPcS$_4$ or alone and low-dose chemotherapeutic agents without 635-nm laser irradiation. This value was even lower than the inhibition of cell viability induced by low-dose chemotherapeutic agents used alone. The antagonistic effects in the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents without laser irradiation were obtained. The antagonistic effect of low-dose VCR in combination therapy without 635-nm laser irradiation was more pronounced, whereas the effect of low-dose CDDP was inferior. Compared with VCR and CDDP, the inhibition of cell viability induced by the combination treatment of AlPcS$_4$/PDT with low-dose 5-FU, DOX, and MMC without 635-nm laser irradiation was more evident. The antagonistic effects of low-dose 5-FU, DOX and MMC in combination therapy without 635-nm laser irradiation were weaker. The degree of the antagonistic effect of low-dose DOX was higher than 5-FU and was much higher than MMC. Low-dose chemotherapy usually induced resistance emergence to limit the treatment effect of the antitumor drugs (37). Hence, the antagonistic effect in combination therapy without laser irradiation may be caused by drug resistance.

Furthermore, the antitumor growth effects of the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents used in our study with 635-nm laser irradiation were assessed. The combination treatment of AlPcS$_4$/PDT with low-dose 5-FU, DOX and MMC had significant inhibitory effects on gastric cancer cells. The inhibitory effects of combination treatment were higher than the efficiency summation of free-AlPcS$_4$ and 5-FU, DOX, or MMC, respectively. In other words, the synergistic anticancer activity via the combination of AlPcS$_4$/PDT with low-dose DOX was optimal; at low-dosages, 5-FU or MMC was inferior. Compared with 5-FU, DOX, or MMC, the inhibitory effect of the combination treatment of AlPcS$_4$/PDT with low-dose CDDP or VCR was lower than the efficiency summation of free-AlPcS$_4$ and CDDP or VCR, respectively. However, the antitumor growth effect continued to increase at average values of 16.5 and 8.7% compared with free-AlPcS$_4$. The increased antitumor effect in combination therapy with laser irradiation was contrary to the antagonistic effects in the combination therapy without laser irradiation. Therefore, irradiation of laser light and PDT therapy may improve the drug resistance of low-dose chemotherapeutic agents.

To determine the reason for the enhancement of therapeutic efficacy, the fluorescence intensity of AlPcS$_4$ with low-dose chemotherapeutic agents, including CDDP, DOX, MMC, 5-FU and VCR, in SGC-7901 cells was determined. The increasing trend of DOX was higher than that of 5-FU and much higher than that of MMC, thereby revealing that DOX was superior in effectively improving the efficiency of cellular internalization of AlPcS$_4$. The antitumor effect of the combination of AlPcS$_4$/PDT with low-dose DOX was optimal. Thus, the mass of increased antitumor effect in AlPcS$_4$/PDT+DOX may be induced by the increased AlPcS$_4$/PDT effect. The results of apoptosis-inducing and necrosis-inducing abilities revealed that AlPcS$_4$ with low-dose chemotherapeutic agents increased apoptosis and necrosis abilities compared with free-AlPcS$_4$. In addition, the results of the apoptosis-inducing ability revealed that low-dose chemotherapeutic agents quickly increased the apoptosis-inducing ability. Furthermore, in the presence of low-dose chemotherapeutic agents, the apoptosis activation time was prolonged. High apoptosis-inducing ability was induced.

The results of SOG generation demonstrated that the increased SOG effect of AlPcS$_4$/PDT+DOX was higher than that of AlPcS$_4$/PDT+5-FU and much higher than that of AlPcS$_4$/PDT+MMC. The results of ROS generation related to apoptosis revealed that the increased ability of ROS generation induced by AlPcS$_4$/PDT+5-FU was higher than that by AlPcS$_4$/PDT+DOX and much higher than that by AlPcS$_4$/PDT+MMC. These results revealed that the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents exhibited a higher antitumor growth effect not only by improving the apoptosis-inducing activities of the chemotherapeutic agents but also by increasing the apoptosis-inducing activities of AlPcS$_4$/PDT. In addition, the results of the synergistic effect revealed that the combination antitumor growth effect of AlPcS$_4$/PDT+DOX was higher than that of AlPcS$_4$/PDT+DOX and much higher than that of AlPcS$_4$/PDT+MMC. Hence, the increased degree of the apoptosis-inducing activities of AlPcS$_4$/PDT was much more significant. However, the general trend of the ROS generation and antitumor growth effect revealed that ROS-related triggers that induce apoptosis are important factors that lead to cell death in the combination treatment of AlPcS$_4$/PDT with low-dose chemotherapeutic agents.

Therefore, the possible molecular pathway involved in the combination treatment-induced apoptosis may involve the activation of the mitochondrial apoptosis pathway or via the endoplasmic reticulum (ER) stress-induced apoptosis pathway (38,39). $AlPcS_4$ can be accumulated in the mitochondria, and local damage induced by $AlPcS_4$ may be propagated to the mitochondria by various means; chemical therapy can also activate mitochondrial apoptosis pathways to lead cell apoptosis (40). Mitochondrial ROS can damage DNA and activate an aberrant apoptosis signaling pathway (41). Furthermore, the generation of ROS could trigger ER stress (42). This stress can further trigger several specific signaling pathways, including ER-associated protein degradation and the unfolded protein response (38). The unfolded protein response can trigger apoptosis activities by inducing cytoprotective and destructive functions when ER stress is prolonged or adaptive responses fail. In a future study, the expression levels of a series of proteins involved in the mitochondrial apoptosis pathway and ER-stress apoptosis pathway will be detected by western blot analysis in combination therapy, such as Bcl-2 family proteins, caspase-related proteins, phosphorylated eIF2α, GADD153, ATF6, GRP78 and GRP94. Our aim is to verify the enhanced synergistic antitumor activity induced by activating the mitochondrial apoptosis pathway and the ER stress-induced apoptosis pathway.

Finally, we conclude that the combination treatment of $AlPcS_4$/PDT with low-dose chemotherapeutic agents may provide promising treatment strategies to increase the weak delivery efficiency of $AlPcS_4$in gastric cancer cells and further effectively improve the antitumor effect on gastric cancer. The treatment also decreases the toxic side effects. Hence, we investigated the antitumor growth effect on the SGC-7901 gastric cancer cell line by combination treatment of $AlPcS_4$/PDT with various low-dose chemotherapeutic agents, including 5-FU, DOX, CDDP, MMC and VCR. The antitumor growth effect could be increased through a combination treatment of $AlPcS_4$/PDT with a low-dose chemotherapeutic agent. An evident synergistic effect was obtained in the combination treatment of $AlPcS_4$/PDT with low-dose 5-FU, DOX, and MMC. These combination treatments increased $AlPcS_4$ intracellular uptake ability and ROS and SOG generation abilities, thereby inducing significant apoptosis and necrosis. In addition, low-dose chemotherapeutic agents improved apoptosis-inducing abilities quickly and prolong the apoptosis-inducing period of $AlPcS_4$/PDT. In general, the combination treatments of $AlPcS_4$/PDT with low-dose chemotherapeutic agents had a significant antitumor growth effect and a low dark-cytotoxicity effect on gastric cancer via the increase of $AlPcS_4$ intracellular uptake ability, improving the apoptosis-inducing abilities induced by chemotherapeutic agents in a short time and prolonging the apoptosis-inducing period of $AlPcS_4$/PDT.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JX, ZZ and CY conceived and designed the study. JX, SW, BX, YH, JW and SW performed the experiments. JX wrote the paper. LZ and LS review and edited the manuscript. All authors read and approved the final manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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