Comparison of the synergistic anticancer activity of AlPcS4 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 (AlPcS4) induce poor penetration ability in cells and a slight photodynamic therapy (PDT) effect on gastric cancer. The combination treatment of AlPcS4/PDT with low-dose chemotherapeutic agents may provide a promising treatment strategy to increase the weak delivery efficiency of AlPcS4, 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 AlPcS4/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 AlPcS4/PDT with all the aforementioned low-dose chemotherapeutic agents, to a different extent. An evident synergistic effect was obtained in the combination treatment of AlPcS4/PDT with low-dose 5-FU, DOX, and MMC by increasing AlPcS4 intracellular uptake ability, improving apoptosis-inducing abilities, and prolonging apoptosis-inducing time. The low-dose chemotherapeutic agents prolonged the apoptosis-inducing period of AlPcS4/PDT, and AlPcS4/PDT quickly improved apoptosis-inducing abilities of chemotherapy even at low doses. Generally, the combination treatment of AlPcS4/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 chemotherapeutic drugs in tumors, thereby improving chemotherapy efficacy. Therefore,
the method of combination of PDT with chemotherapy may be an effective treatment strategy for gastric cancer management. AlPcS₄, 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 photosensitivity, 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 AlPcS₄ 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 AlPcS₄/PDT in combination with chemotherapeutic drugs 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 AlPcS₄/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 causes cell death (22). MMC is an antibiotic that was isolated from Gram-negative bacteria Streptomyces caesipitosus 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 AlPcS₄/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 AlPcS₄/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 AlPcS₄/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 AlPcS₄ was dominant in our design scheme, we evaluated the influence of AlPcS₄ 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 AlPcS₄ 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 AlPcS₄ 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. AlPcS₄ 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, AlPcS₄ 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™ 150 i CO₂ with copper chambers; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C with 5% CO₂.

Measurement of the absorption spectra and fluorescence spectra of a mixture of AlPcS₄ and chemotherapeutic agents. The absorption spectra of free-AlPcS₄ (8 µg/ml), AlPcS₄ (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-AlPcS₄, AlPcS₄ in the presence of 5-FU, DOX, CDDP, MMC, and VCR were
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, AlPcS₄ 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% CO₂ for 24 h. Following treatment, the cells were stained with 5 µl Hoechst 33324 and PI reagents for 10 min at 37˚C in complete darkness again, washed with PBS twice, and assessment was conducted using 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 2.5x10⁵ cells/well and incubated to adhere overnight. Then, the cells were treated with free-AlPcS₄, AlPcS₄ 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 for 6 h. Following incubation, 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 AlPcS₄ and the chemotherapeutic agent-AlPcS₄ mixture.** To ensure the influence of AlPcS₄ itself by chemotherapeutic agent, the UV-vis absorption spectra and fluorescence intensity of AlPcS₄ and the chemotherapeutic agent-AlPcS₄ mixture and free-AlPcS₄ were assessed. Fig. 1A revealed that the absorption levels of AlPcS₄ were derived from the AlPcS₄ mixture. The Figure also revealed that the chemotherapeutic agent-AlPcS₄ mixture had weaker reduction compared to free-AlPcS₄ in deionized water as time changed. Compared to deionized water, the main absorption levels of AlPcS₄ were obtained from free-AlPcS₄ and the chemotherapeutic agent-AlPcS₄ mixture was much more stable in a culture medium that contained FBS (Fig. 1B). However, the main absorption levels of AlPcS₄ in the chemotherapeutic agent-AlPcS₄ mixture or free-AlPcS₄ in a culture medium that contained FBS were lower than those in deionized
water. A possible reason for this finding is that AlPcS₄ 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-AlPcS₄ mixture and free-AlPcS₄ in culture medium as time increased (Fig. 1B). These peaks may be caused by coagulation at different degrees. The fluorescence intensity of AlPcS₄ was derived from AlPcS₄, and the chemotherapeutic agent-AlPcS₄ mixture also indicated no significant change even after 6 h compared with free-AlPcS₄ (Fig. 1C and D). Furthermore, whether in deionized water or culture medium that contained FBS, the fluorescence intensities markedly increased after dilution of the chemotherapeutic agent-AlPcS₄ mixture for 1 h. These may be induced by AlPcS₄ at high concentrations, and it had a concentration-dependent fluorescent quenching effect. Overall, the selected chemotherapeutic agents in the present study did not influence AlPcS₄ itself. In culture medium, AlPcS₄ and the chemotherapeutic agent-AlPcS₄ mixture scarcely influenced the absorption levels and fluorescence intensity of AlPcS₄. However, the main absorption levels and fluorescence intensity of AlPcS₄ (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 AlPcS₄ 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-AlPcS₄, and the mixture of chemotherapeutic agents with different concentrations of AlPcS₄ was mostly higher than 80%, 90% and 80% respectively. The dark cytotoxicity of AlPcS₄ 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) AlPcS4 + 5-FU, (B) AlPcS4 + CDDP, (C) AlPcS4 + DOX, (D) AlPcS4 + MMC or (E) AlPcS4 + VCR. The cells were treated with 1-32 µm/ml free-AlPcS4 or AlPcS4 + 5-FU (20 µm), AlPcS4 + CDDP (5 µm), AlPcS4 + DOX (0.4 µm/ml), AlPcS4 + MMC (0.5 µm/ml) or AlPcS4 + 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/cm² illumination dosage for 5 min. *P<0.05, represents a statistical difference in antitumor effect between the combination of AlPcS4 with a chemical agent and free-AlPcS4. AlPcS4, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.

Figure 3. Apoptosis induced by AlPcS4 + 5-FU, AlPcS4 + CDDP, AlPcS4 + DOX, AlPcS4 + MMC, AlPcS4 + VCR and free-AlPcS4 in SGC-7901 cells after being irradiated for 6 h. The cells were then treated with 1-32 µm/ml free-AlPcS4 or AlPcS4 + 5-FU (20 µm), AlPcS4 + CDDP (5 µm), AlPcS4 + DOX (0.4 µm/ml), AlPcS4 + MMC (0.5 µm/ml), or AlPcS4 + VCR (0.1 µm/ml) for 6 h. These samples were then irradiated with 635-nm laser irradiation at 100 mW/cm² illumination dosage for 5 min, incubated for 6 h, stained with Hoechst 33342 probe, and then imaged using a fluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 µm. AlPcS4, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.
The dark cytotoxicity induced by the combination treatment of AlPcS4 with a low-dose chemotherapeutic agent was lower than the summation of the dark cytotoxicity induced by free-AlPcS4 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 AlPcS4/PDT with low-dose chemotherapeutic agents were obtained. The antagonistic effects of AlPcS4+VCR and AlPcS4+CDDP were higher than those of AlPcS4+5-FU, AlPcS4+DOX, and AlPcS4+MMC.

After 635 nm laser irradiation of 100 mW/cm² illumination dosage for 5 min, the cell viability of free-AlPcS4 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 AlPcS4+5-FU, AlPcS4+DOX, AlPcS4+CDDP, AlPcS4+MMC and AlPcS4+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 AlPcS4+5-FU, AlPcS4+DOX, and AlPcS4+MMC was higher than the efficiency summation of free-AlPcS4 and 5-FU, DOX, and MMC. When we discarded the antitumor growth effect induced by free-AlPcS4 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-AlPcS4 and AlPcS4+5-FU, AlPcS4+DOX, and AlPcS4+MMC concerning the antitumor effect, compared with AlPcS4+CDDP and AlPcS4+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 AlPcS4 under laser light exposure induced apoptosis in the cells, a Hoechst 33342 and PI staining assay was conducted. As shown in Figs. 3-5, cell shrinkage and nuclear

![Figure 4. Apoptosis induced by AlPcS4+5-FU, AlPcS4+CDDP, AlPcS4+DOX, AlPcS4+MMC, AlPcS4+VCR, and free-AlPcS4 in SGC-7901 cells after being irradiated for 12 h. The cells were treated with 1-32 µm/ml free-AlPcS4 or AlPcS4+5-FU (20 µm), AlPcS4+CDDP (5 µm), AlPcS4+DOX (0.4 µm/ml), AlPcS4+MMC (0.5 µm/ml) or AlPcS4+VCR (0.1 µm/ml) for 6 h. The cells were then irradiated with 635-nm laser irradiation at 100 mw/cm² illumination dosage for 5 min, incubated for 12 h, stained with Hoechst 33342 probe, and then imaged using a fluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 µm. AlPcS4, Al(III) phthalocyanine chloride tetrasulfonic acid; 5-FU, 5-fluorouracil; DOX, doxorubicin; CDDP, cisplatin; MMC, mitomycin C; VCR, vincristine.](image-url)
fragmentation appeared in SGC-7901 cells induced by both free-AlPcS4 and the chemotherapeutic agent-AlPcS4 mixture after irradiation at 6, 12 and 24 h, only the number of cell induced by free-AlPcS4 was low. In other words, apoptosis is active in AlPcS4/PDT. The apoptosis-inducing abilities were increased in SGC-7901 cells in AlPcS4/PDT synergistic treatment with low-dose chemical drug agents. Apoptosis induced by single AlPcS4/PDT was mainly active at 6-12h. 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 AlPcS4/PDT with low-dose CDDP and VCR increased.

In the synergistic therapy, higher apoptosis-inducing abilities induced by AlPcS4/PDT+DOX were obtained, especially at high concentrations of AlPcS4 at 24 h, compared to AlPcS4/PDT+MMC (Fig. 5). In the treatment of AlPcS4+DOX or AlPcS4+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 AlPcS4/PDT+DOX (Fig. 6). In contrast to AlPcS4/PDT+DOX and AlPcS4/PDT+MMC, apoptosis was induced quickly by AlPcS4/PDT+5-FU at 6 h. This increasing trend was similar to that of AlPcS4/PDT+VCR. In addition, the apoptosis-inducing abilities increased (Figs. 3 and 7). At 32 µg/ml, the percentage of apoptotic bodies of AlPcS4+5-FU at 6, 12 and 24 h reached ~38, 80 and 88%, respectively (Fig. 7). The apoptosis-inducing abilities at 12 and 24 h were higher than those in AlPcS4/PDT+DOX and AlPcS4/PDT+MMC. In addition, more necrotic cells were observed at 12 h after treatment with AlPcS4/PDT+5-FU (Fig. 6).

Figure 5. Apoptosis induced by AlPcS4+5-FU, AlPcS4+CDDP, AlPcS4+DOX, AlPcS4+MMC, AlPcS4+VCR and free-AlPcS4 in SGC-7901 cells after being irradiated for 24 h. The cells were then treated with 1-32 µm/ml free-AlPcS4 or AlPcS4+5-FU (20 µm), AlPcS4+CDDP (5 µm), AlPcS4+DOX (0.4 µm/ml), AlPcS4+MMC (0.5 µm/ml) or AlPcS4+VCR (0.1 µm/ml) for 6 h. The cells were then irradiated with 635-nm laser irradiation at 100 mW/cm2 illumination dosage for 5 min, incubated for 24 h, stained with Hoechst 33342 probe, and then imaged using a fluorescence microscope. All the Hoechst staining images were acquired at an x400 magnification. The scale bar represented 20 µm. AlPcS4, 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 AlPcS4/PDT + 5-FU evidently improved apoptosis-inducing abilities even at low concentrations of AlPcS4. AlPcS4/PDT + DOX slightly increased apoptosis-inducing abilities at low concentrations of AlPcS4 at 24 h. AlPcS4/PDT + MMC indicated fewer increased apoptosis-inducing abilities at a low concentration of AlPcS4. At 4 µg/ml AlPcS4, the percentage of apoptotic bodies of AlPcS4/PDT + MMC reached ~16 and 29%, at 6 and 24 h, respectively. Generally, 5-FU and DOX have optimal apoptosis-inducing abilities of AlPcS4 even at low concentrations. MMC markedly improved the apoptosis-inducing abilities of AlPcS4 at a high concentration. CDDP and VCR slightly improved apoptosis-inducing abilities even when significantly inhibited.
Influence of AlPcS₄ fluorescence intensity on combination treatment. To estimate the influence of AlPcS₄ delivery efficiency on combination treatment, a fluorescence intensity assay of AlPcS₄ was evaluated after treatment with different chemotherapeutic agents and AlPcS₄. As revealed in Fig. 8, the fluorescence intensity of AlPcS₄ significantly increased with the help of low-dose 5-FU and DOX. Notably, AlPcS₄+5-FU exhibited a significant difference at all the used concentrations compared to free-AlPcS₄, but AlPcS₄+DOX exhibited a statistical difference only at a high concentration. At a high concentration of AlPcS₄, the increasing fluorescence intensity trend in the presence of DOX was greater than 5-FU. At the highest concentration of AlPcS₄, DOX and 5-FU fluorescence 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 AlPcS₄, did the fluorescence intensity exhibit a statistical difference between free-AlPcS₄, but AlPcS₄+DOX exhibited a statistical difference only at a high concentration.

Influence of AlPcS₄ 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 AlPcS₄ and evaluate the influence of AlPcS₄ 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-AlPcS₄. However, compared with free-AlPcS₄, the fluorescence signal significantly increased after treatment with AlPcS₄+5-FU and AlPcS₄+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-AlPcS₄) or combination (AlPcS₄+chemical agent) treatment was assessed. 5-FU and DOX improved SOG generation abilities of AlPcS₄ (Fig. 9). Compared with free-AlPcS₄, 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 AlPcS₄ 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 AlPcS₄. These results were consistent with the results obtained in the fluorescent intensity assay of AlPcS₄+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 7. Statistical analysis of apoptosis and necrosis induced by AlPcS₄+5-FU, AlPcS₄+CDDP, AlPcS₄+DOX, AlPcS₄+MMC, AlPcS₄+VCR and free-AlPcS₄ 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 AlPcS₄ with a chemical agent and free-AlPcS₄. AlPcS₄, 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 AlPcS4 combination treatment with chemical agents is necessary. As shown in Fig. 10, after pre-treatment with AlPcS4 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 AlPcS4, treatments with AlPcS4+5-FU, AlPcS4+DOX, AlPcS4+MMC, AlPcS4+CDDP, and AlPcS4+VCR resulted in increases by 10.5-, 8.7-, 3.1-, 2.9- and 2-fold on average, respectively, compared with free-AlPcS4. At all concentrations of AlPcS4, treatments with AlPcS4+5-FU, AlPcS4+DOX, AlPcS4+MMC, AlPcS4+CDDP, and AlPcS4+VCR resulted in 6.3-, 5.6-, 2.9-, 2- and 1.6-fold increases, respectively, compared with free-AlPcS4/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 AlPcS4+5-FU and AlPcS4+DOX exhibited significant differences compared with free-AlPcS4.

Discussion

AlPcS4 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 AlPcS4/PDT and etoposide, an antitumor drug, on K562 human leukemic cells to improve the antitumor effect of AlPcS4/PDT (31). This proposal which points to the combination treatment of AlPcS4/PDT with chemical agents, may be a
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 AlPcS4/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 AlPcS4/PDT combination treatment to improve the effect of AlPcS4/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 AlPcS4/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-AlPcS4 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 AlPcS4/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 AlPcS4/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 much higher than that of 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 AlPcS4/PDT with low-dose chemotherapeutic agents used in our study with 635-nm laser irradiation were assessed. The combination treatment of AlPcS4/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-AlPcS4 and 5-FU, DOX, or MMC, respectively. In other words, the synergistic anticancer activity via the combination of AlPcS4/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 AlPcS4/PDT with low-dose CDDP or VCR was lower than the efficiency summation of free-AlPcS4 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-AlPcS4. 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 AlPcS4 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 AlPcS4. The antitumor effect of the combination of AlPcS4/PDT with low-dose DOX was optimal. Thus, the mass of increased antitumor effect in AlPcS4/PDT+DOX may be induced by the increased AlPcS4/PDT effect. The results of apoptosis-inducing and necrosis-inducing abilities revealed that AlPcS4 with low-dose chemotherapeutic agents increased apoptosis and necrosis abilities compared with free-AlPcS4. 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 AlPcS4/PDT+DOX was higher than that of AlPcS4/PDT+5-FU and much higher than that of AlPcS4/PDT+MMC. The results of ROS generation related to apoptosis revealed that the increased ability of ROS generation induced by AlPcS4/PDT+5-FU was higher than that by AlPcS4/PDT+DOX and much higher than that by AlPcS4/PDT+MMC. These results revealed that the combination treatment of AlPcS4/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 AlPcS4/PDT. In addition, the results of the synergistic effect revealed that the combination antitumor growth effect of AlPcS4/PDT+DOX was higher than that of AlPcS4/PDT+DOX and much higher than that of AlPcS4/PDT+MMC. Hence, the increased degree of the apoptosis-inducing activities of AlPcS4/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 AlPcS4/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). AlPcS₄ can be accumulated in the mitochondria, and local damage induced by AlPcS₄ 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 AlPcS₄/PDT with low-dose chemotherapeutic agents may provide promising treatment strategies to increase the weak delivery efficiency of AlPcS₄ 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 AlPcS₄/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 AlPcS₄/PDT with a low-dose chemotherapeutic agent. An evident synergistic effect was obtained in the combination treatment of AlPcS₄/PDT with low-dose 5-FU, DOX, and MMC. These combination treatments increased AlPcS₄ 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 AlPcS₄/PDT. In general, the combination treatments of AlPcS₄/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 AlPcS₄ intracellular uptake ability, improving the apoptosis-inducing abilities induced by chemotherapeutic agents in a short time and prolonging the apoptosis-inducing period of AlPcS₄/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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