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Two arms hydrophilic photosensitizer conjugates with vitamin B for cancer-selective photodynamic therapy

Kyoung Sub Kim[a], Cheol Jun Song[b], Jae Yun Jaung[b] and Kun Na[a]*

Cancer-selective internalization has a great potential for reducing the side effect of photodynamic therapy. Recently, various cancer-targeted delivery carriers have provided enhanced cancer targeting efficiency. Despite significant advancements in cancer-targeted carriers, side effects are still present because of non-selective cellular uptake that occurs in the heterogeneous cancer environment. In this paper, we designed two types of cancer-selectable two arm hydrophilic photosensitizer (CTAHP2K and CTAHP4K) with silicon-tetrapyrazinoporphyrazines (ST), polyethylene glycol and the cancer-specific ligand for cancer-selective theranostics. The synthesized CTAHP4K exhibits a folate receptor-mediated cancer-selective cellular uptake and induces cancer-selective death. The folate receptor-mediated cancer-selective internalization of CTAHP4K was confirmed by competitive interaction with vitamin B in MDA-MB-231 human breast carcinoma. The cancer-selective cytotoxicity of CTAHP4K was confirmed using a 670-nm laser to irradiate Chang Liver cells and MDA-MB-231 cells. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: cancer selective; photodynamic therapy; hydrophilic photosensitizer; vitamin B; theranostic

INTRODUCTION

Accurate detection and treatment of cancer are essential for improving the life rate of cancer patients.[1] To achieve efficient cancer-targeted delivery of anti-cancer drugs and diagnostic agents, various cancer-targeted delivery carriers have been developed, such as polymeric nanoparticles, micelles, and liposomes.[2–5] In spite of the remarkable improvement in cancer-targeted carriers, most of cancer-targeted carriers has a non-selective interaction capacity by their hydrophobic nature and the ionic strength that exists in the heterogeneous tumor environment and side effects are still present.[6–8] Therefore, to minimize the side effects of cancer-targeted carriers, the cancer-selective interaction capacity must also be considered.

Photodynamic therapy (PDT) is approved for the clinical treatment of diverse types of cancers.[9–11] The PDT has a great attraction because of its light-responsive and non-invasive therapeutic effect in disease regions. Photosensitizers are appropriate wavelength light susceptible molecules that are essential for PDT.[12,13] The PSs absorb photon energy at an appropriate wavelength and transfer it to adjacent oxygen molecules to generate singlet oxygen and reactive oxygen species (ROS).[14,15] Silicon tetrapyrazinoporphyrazines (ST) have appeared as excellent candidates for use as PDT agents.[16,17] PEG-substituted silicon–tetrapyrazinoporphyrazines conjugates are especially appealing candidates because of their hydrophilic property leads to increased cancer-selective interactions with cancer targeting moiety and to rapid blood clearance. However, the ST does not have cancer selective targeting ability, which it accumulated in extensively various tissues accompany with side effect of phototoxicity. Therefore, the active cancer targeting moiety in PSs is essential for an effective treatment of cancer. Vitamin B has been established to be an effective cancer targeting moiety.[3,18] The vitamin B receptor is overexpressed in a various human tumors.[19–21] Our group has previously reported that folate is an effective marker for use with cancer targeted delivery carriers.[22–25]

Herein, we designed and synthesized two types of cancer-selectable two arm hydrophilic photosensitizer (CTAHPs) with vitamin B, polyethylene glycol (Mw; 2 kDa, 4 kDa), and ST for combined use in cancer-selective therapy and as diagnostic agents without other chemical drugs or imaging dyes. The synthesized CTAHPs were composed of different molecular weight PEGs, with PEG molecular weights of 2 kDa (CTAHP2K) or 4 kDa (CTAHP4K). The synthesized CTAHPs were confirmed using ^{1}H NMR spectroscopy. The cancer-selective cellular uptake behavior and mechanism of CTAHPs were observed using confocal laser scattering microscopy and flow cytometry. The cancer-selective therapeutic effect of CTAHPs was confirmed in Chang Liver cells and MDA-MB-231 cells. Thus, the synthesized CTAHPs could be effectively applied in cancer-selective theranostics.

* *Correspondence to: Kun Na, Center for Photomedicine, Department of Biotechnology, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon-si, Gyeonggi do 420-743, Republic of Korea.*
E-mail: kna6997@catholic.ac.kr

a *K. S. Kim, K. Na*
Center for Photomedicine, Department of Biotechnology, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon-si, Gyeonggi do, 420-743, Republic of Korea

b *C. J. Song, J. Y. Jaung*
Department of Organic and Nano Engineering, Hanyang University, 17, Haengdang-dong, Seongdong-gu, Seoul 133791, Republic of Korea

EXPERIMENTAL

Materials

Polyethylene glycol (PEG, Mw 2, and 4 kDa), vitamin B (folic acid, FA), N-hydroxysuccinimide (NHS), 1, 3-dicyclohexyl carbodiimide (DCC), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), methanol (MeOH), dimethyl sulfoxide (DMSO), 9, 10-dimethylanthracene (DMA), 4′,6-diamidino-2-phenylindole (DAPI, blue fluorescence, DAKO), and Sephadex® LH-20 were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Flash chromatography was performed using a Merck-EM type 60 (230–400 mesh) silica gel (flash). The dialysis membrane (molecular weight cut-off 1 kDa) was purchased from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). RPMI 1640 medium without FA, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), and Dulbecco's phosphate buffered saline (DPBS) were purchased from Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). ^{1}H and ^{13}C NMR spectra were recorded on a 500-MHz NMR Spectrometer (AVANCE III 500 MHz, Bruker, Germany). Fluorescence spectra were measured using a Shimadzu RF-5301PP spectrophotometer.

Synthesis and characterization of TAHPs

Silicon-tetrapyrazinoporphytazine (ST) was synthesized according to previous report.[26] CTAHP2K and CTAHP4K were conjugated as described below. FA (33 mg, 75 μmol), DCC (20 mg, 97 μmol), and NHS (11 mg, 97 μmol) were dissolved in DMSO. PEG2K-ST (24 mg and 5 μmol) or PEG4K-ST (44 mg and 5 μmol) was dissolved in DMSO.[26] The FA and PEG-ST solutions were mixed slowly, and the reaction mixture was stirring for 24 hr at room temperature (RT). Sequentially, the reaction mixture was dialyzed using dialysis membrane of cut-off 1 kDa for 3 days in distilled water (DW). The final solution was lyophilized. The ^{1}H NMR spectrum was recorded in deuterated dimethyl sulfoxide (DMSO-d6) at RT using a 500-MHz NMR spectrometer.

Measurement of singlet oxygen generation

Singlet oxygen ($^{1}O_2$) was measured chemically by the detection of DMA (singlet oxygen quencher) utilizing fluorescence spectroscopy. DMA (20 μM) was mixed with CTAHP2K and CTAHP4K in DW. The solution was irradiated with a 670-nm laser source at 3 J/cm^2. The DMA fluorescence intensity (Ex 360 nm and Em 380–550 nm) was recorded using an RF-spectrofluorometer (RF-5301PP, Shimadzu, Japan) for 45 min at RT. The ST in DW was used as control group.

Cell culture

MDA-MB-231 (human breast carcinoma) cells and Chang Liver cells were obtained from the Korean Cell Line Bank (KCLB No. 30026) and ATCC (CCL-13) and were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin without FA supplementation. The cells were cultured at 37°C in 100% humidity and at 5% CO_2. Each group was dissolved in serum-free (SF) medium. The untreated cells were irradiated or kept in the dark and used as a reference standard.

Cancer-selective cellular uptake of CTAHPs

To confirm the cancer-selective cellular uptake of CTAHPs, Chang Liver cells and MDA-MB-231 cells (1×10^6 cells/well in a six-well plate) were treated with CTAHP2K and CTAHP4K at concentrations of 5 μg/ml for 3 hr. The cells were then washed twice with DPBS, fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI and visualized using a confocal laser scanning microscope (CLSM, LSM 710 Meta; Zeiss, Germany).The fluorescence images were analyzed using LSM Image Browser software (Zeiss).

In vitro cancer-selective cellular uptake and competitive inhibition

To evaluate the FA receptor-mediated cancer-selective internalization, the FA (50 μg/ml) was pre-treated in MDA-MB-231 cells (1×10^6 cells/well in a six-well plate) and then CTAHP2K and CTAHP4K (ST concentration; 5 μg/ml) were treated in MDA-MB-231 cells (3-hr incubation at 37°C). After 3 hr, the cells were then washed twice with DPBS, fixed with 4% paraformaldehyde, and the nuclei were stained with DAPI and visualized using a CLSM. Cellular uptake was quantitatively analyzed using flow cytometry (Beckman, SanJose, CA, USA). For each group, 1×10^4 cells (gated events) were counted, and the ST fluorescence was detected using logarithmic settings (FL4; Em = 670 nm). Each experiment was measured statistically using the CXP analysis program (Beckman). Fluorescence intensity was analyzed at RT using a plate reader (Tecan Genios, NC, USA) with an Ex 635 nm and Em 675 nm.

Cancer-selective cytotoxicity of CTAHPs

Chang Liver cells and MDA-MB-231 cells (1×10^4 cells/well in a 96-well plates) were treated with various concentrations (0–1 mg/ml) of CTAHP2K and CTAHP4K. After 3-hr incubation, the cells were rinsed twice with DPBS, and then fresh culture medium was added to each well. The 670-nm laser source was irradiated in culture plate with maximum 5 J/cm^2. After additional 24-hr incubation, the cell viability was analyzed using the MTT assay. The absorbance was analyzed at 570 nm using a microplate reader (Bio-Tek, VT, USA).

Efficacy of cancer-selective PDT with CTAHPs

To assess the cytotoxicity of CTAHP2K and CTAHP4K, MDA-MB-231and Chang Liver cells (1×10^4 cells/well) were added to a 96-well plate in 100 μl of complement medium and incubated overnight. CTAHP2K and CTAHP4K were added to each well, and the plates were returned to the incubator for 4 hr. After incubation, the wells were rinsed three times with DPBS. Fresh medium was then added to each well, and the plates were irradiated using the 670-nm laser source (0–5 J/cm^2). The cells were then incubated for an additional 24 hr. After incubation, the cell viability was assessed using the MTT assay. The resulting formazan crystals were dissolved in DMSO and transferred to a new plate. The absorbance intensity was measured at 570 nm using a microplate reader (Bio-Tek, VT, USA).

RESULTS AND DISCUSSION

Synthesis of CTAHPs

To prove our hypothesis, we synthesized CTAHPs as shown in Fig. 1 according to our previous report.[27] Diaminomaleonitrile (DAMN) is a well-established starting material for the synthesis of 2,3-dicyanopyrazines and related compounds.[28] 5,9,9-

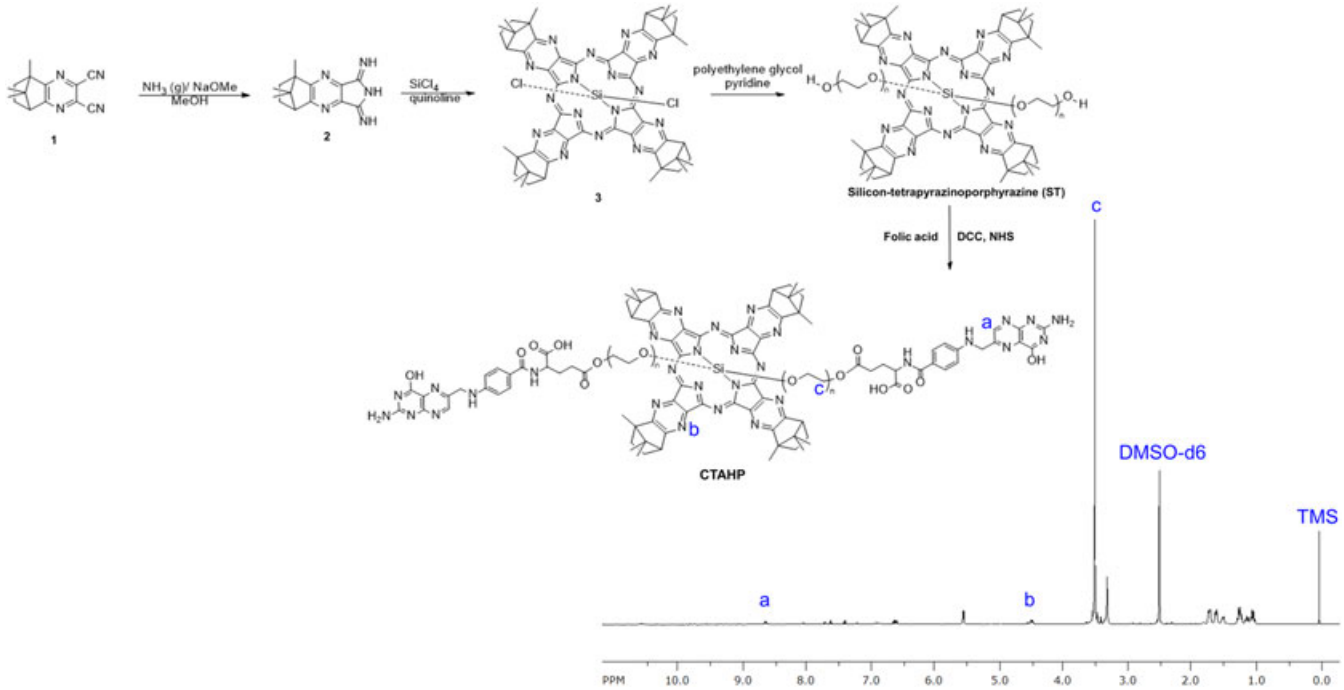

Figure 1. Synthesis and characterization of CTAHPs. ^{1}H NMR analysis of CTAHPs. The synthesized CTAHPs had a —CH peak from ST at 4.50 ppm, —CH peaks from FA at 7.64 and 8.64 ppm, and a —CH_2 peak from PEG at 3.60 ppm. [Colour figure can be viewed at wileyonlinelibrary.com]

Trimethyl-5,6,7,8-tetrahydro-5,8-ethanoquinoxaline-2,3-dicarbonitrile (compound 1) was synthesized by a condensation reaction using camphorquinone and DAMN. 5,10,10-Trimethyl-5,6,7,8-tetrahydro-1H-5,8-methanopyrrolo[3,4-b]quinoxaline-1,3 (2H)-diimine (compound 2) was synthesized by bubbling dry ammonia through a MeOH solution containing 5,9,9-trimethyl-5,6,7,8-tetrahydro-5,8-methanoquinoxaline-2,3-dicarbonitrile in the presence of pyridine.[29] Then, reaction of compound 2 with $SiCl_4$ in quinoline under reflux produced silicon pyrazinoporphyrazine dichloride (compound 3).[30] The ST were then prepared by refluxing silicon pyrazinoporphyrazine dichloride with PEG2K or PEG4K in the presence of pyridine in toluene for 18 hr.[31] The CTAHPs were synthesized via a DCC and NHS coupling reaction as shown in Fig. ure 1. The synthesized CTAHPs were confirmed using ^{1}H NMR spectroscopy (Fig. 1).

Characterization of CTAHPs

PEGylation of the photosensitizer is a process that introduces a hydrophilic property and also improves the water solubility, as shown in Fig. 2a, in which the UV–Vis absorption spectra of ST, CTAHP2K and CTAHP4K demonstrate that PEGylation improves the absorbance of the photosensitizer (Fig. 2b).[32] The suitability of CTAHPs for use as a PDT agent was evaluated by singlet oxygen measurements. The singlet oxygen generation efficacy of ST, CTAHP2K, and CTAHP4K was determined by MDA (singlet oxygen quencher). DMA absorbs nearby singlet oxygen, which it induces a decrease of DMA fluorescence intensity at 460 nm. Figure 2c showed that the DMA fluorescence intensity of ST was not decreased by 670-nm laser irradiation. This result indicates that it does

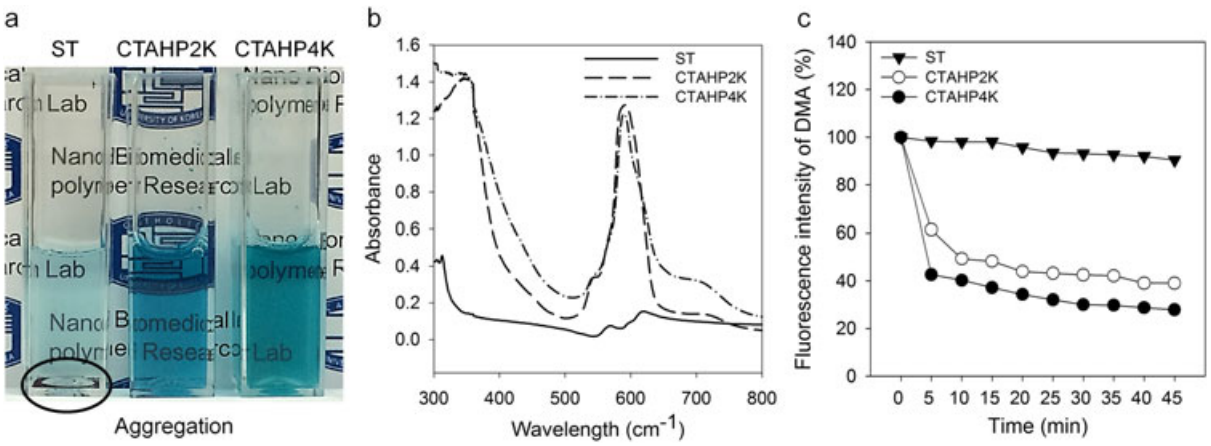

Figure 2. Characterization of ST conjugates. a) Enhanced water solubility of CTAHPs. b) UV–Vis spectral analysis of ST and CTAHPs. c) Singlet oxygen generation ($^{1}O_2$) from CTAHPs measured with DMA in DW. [Colour figure can be viewed at wileyonlinelibrary.com]

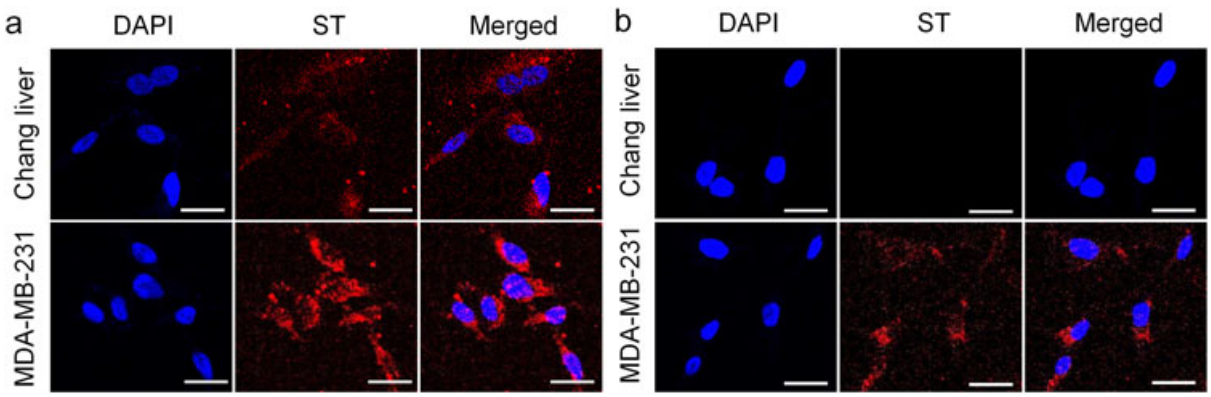

Figure 3. Cancer-selective cellular uptake of CTAHPs in Chang Liver cells and MDA-MB-231 cells. CLSM images after treatment with a) CTAHP2K and b) CTAHP4K. The white scale bar represents 20 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

not generate singlet oxygen because of the high hydrophobicity of ST aggregates in DW. In contrast, the DMA fluorescence intensity of CTAHP2K and CTAHP4K was remarkably decreased after laser irradiation. This result indicates that the generation of singlet oxygen by CTAHP2K and CTAHP4K on laser irradiation is because of the increased hydrophilicity that was added to the ST by PEG, and consequently the CTAHPs completely dissolved in DW. These results suggest that CTAHPs can be used for PDT.

Efficacy of cancer-selective detection

To demonstrate the cancer-selective detection of CTAHPs for cancer cell diagnosis, cellular uptake was analyzed using CLSM. The CLSM images of CTAHP2K were observed in MDA-MB-231 as well as Chang Liver cells (Fig. 3a) because of the hydrophobicity of ST and the insufficient hydrophilicity of PEG2K. In contrast, the CLSM images of CTAHP4K were observed only in MDA-MB-231 cells because of cancer-selective uptake (Fig. 3b). These

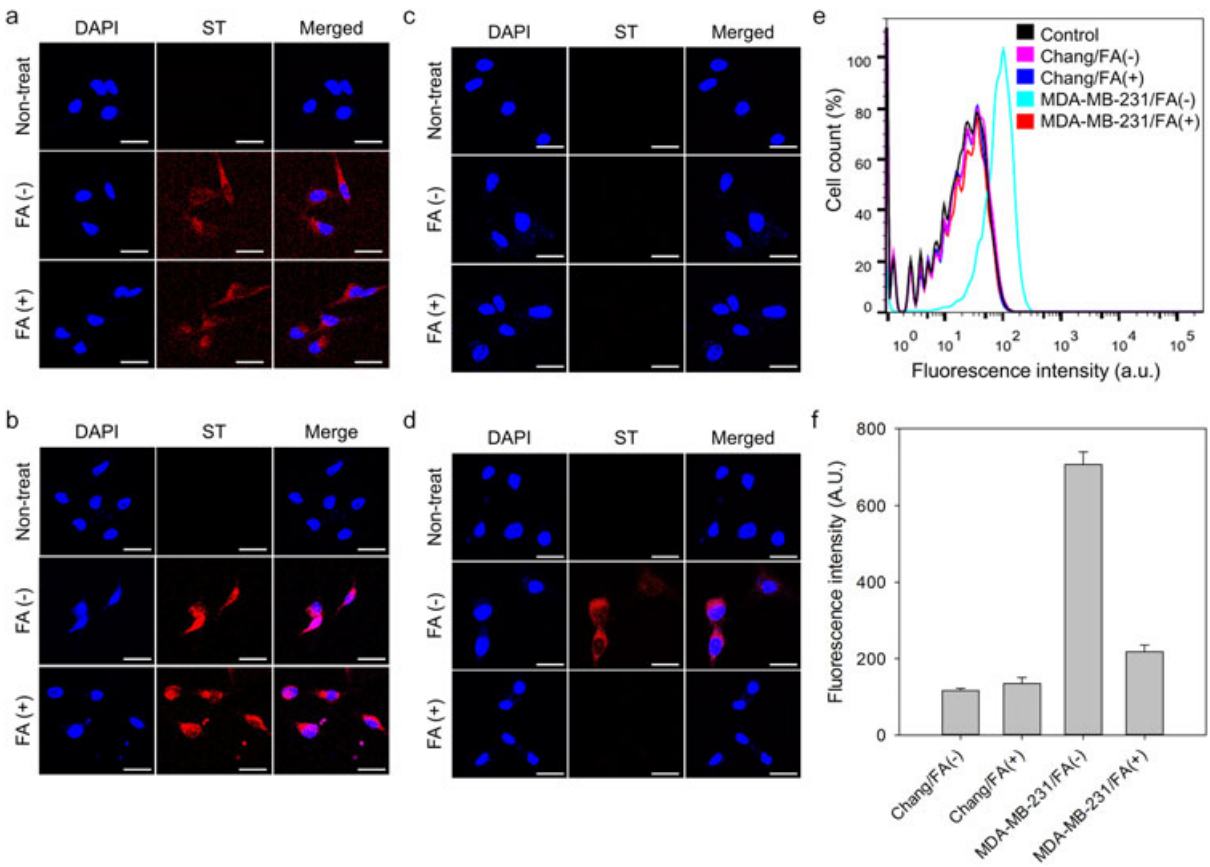

Figure 4. Cancer-selective cellular uptake of CTAHP2K and CTAHP4K. CLSM images after treatment with CTAHP2K (ST concentration, 5 μg/ml) and free FA in a) Chang Liver cells and b) MDA-MB-231 cells. CLSM images after treatment with CTAHP4K (ST concentration, 5 μg/ml) and free FA in c) Chang Liver cells and d) MDA-MB-231 cells. (FA(−), free FA non-treatment; FA(+), free FA pre-treatment). The white scale bar represents 20 μm. e) Flow cytometry analysis of CTAHP4K. f) Fluorescence intensity of each group after CTAHP4K treatment. [Colour figure can be viewed at wileyonlinelibrary.com]

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results indicate that not only adding a targeting moiety but also incorporating appropriate hydrophilic and hydrophobic properties are important factors for promoting selective uptake by target cells.[7]

Cancer-selective cellular uptake and competitive inhibition

To ensure the FA receptor-mediated cancer-selective cellular uptake of CTAHPs, we followed the cellular uptake using CLSM and the intracellular fluorescence intensity in Chang Liver cells and MDA-MB-231 cells for competitive interactions after pre-treatment with FA (FA+). The CLSM images of CTAHP2K exhibited folate receptor independent red fluorescence signals in folate receptor negative Chang Liver cells (Fig. 4a) as well as in MDA-MB-231 cells that were pre-treated with FA (Fig. 4b), which was because of the hydrophobicity of CTAHP2K. On the other hand, the CLSM images of CTAHP4K show that FA non-treated (FA-) MDA-MB-231 cells exhibit selective red fluorescence signals, while Chang Liver cells (Fig. 4c) and FA pre-treated MDA-MB-231 cells (Fig. 4d) did not produce red fluorescence signals. These results were confirmed with flow cytometry (Fig. 4e) and intracellular fluorescence intensity (Fig. 4f). These results suggest that CTAHP4K can be a target for folate receptor-mediated cancer-selective internalization, and the CTAHP4K can be used as a cancer target diagnostic agent.

Cancer-selective therapeutic effect of CTAHPs

To evaluate the cancer-selective therapeutic effect of CTAHPs, we optimized the non-toxic concentrations of CTAHPs in Chang Liver cells and MDA-MB-231 cells. CTAHP2K (Fig. 5a) and CTAHP4K (Fig. 5b) were treated with various concentrations (0.01–1.00 mg/ml) in Chang Liver cells and MDA-MB-231 cells. The results demonstrate that CTAHP2K and CTAHP4K exhibit concentration-dependent cytotoxicity at concentrations greater than 0.10 mg/ml. Consequently, a concentration of 0.05 mg/ml was selected for CTAHP2K and CTAHP4K for further experiments. We determined the efficacy of PDT with CTAHP2K and CTAHP4K at various levels of laser power irradiation (0–5 J/cm^2) in Chang Liver cells and MDA-MB-231 cells. The viability of Chang Liver cells and MDA-MB-231 cells was measured via MTT assays. The cytotoxicity of CTAHP2K in MDA-MB-231 as well as in Chang Liver cells was dependent upon the laser power (Fig. 5c). CTAHP2K had a high cellular uptake efficacy in both Chang Liver cells and MDA-MB-231 cells because of its hydrophobicity. In contrast, CTAHP4K exhibited increased cancer-selective cell cytotoxicity. Notably, the cytotoxicity of CTAHP4K was significantly increased to 67.5% at a laser irradiation power level of 5 J/cm^2. The CTAHP4K exhibited folate-mediated cancer-selective cellular uptake and induced cell death in MDA-MB-231 cells. These results demonstrate that CTAHP4K can be used not only as a cancer diagnosis agent but also as a therapeutic agent.

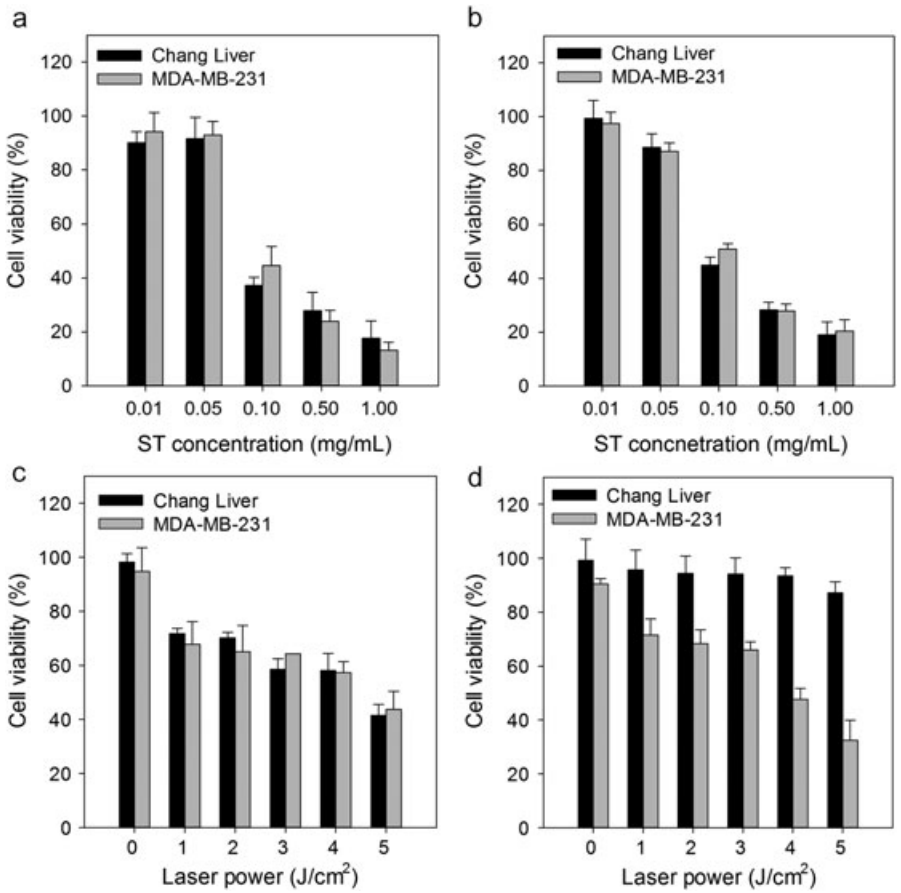

Figure 5. Cancer-selective cytotoxicity by CTAHPs. Cell cytotoxicity was measured after treatment with various concentrations a) CTAHP2K and b) CTAHP4K in Chang Liver cells and MDA-MB-231 cells ($n = 4$). The cytotoxicity test analyzed after treatment with c) CTAHP2K and d) CTAHP4K (ST concentration; 50 μg/ml) at various laser irradiation power levels in Chang Liver cells and MDA-MB-231 cells ($n = 4$).

CONCLUSIONS

In this study, we demonstrated that cancer-selectable two arm hydrophilic photosensitizer (CTAHPs) with PEG and cancer-specific ligands can be used simultaneously for cancer-selective theragnosis agent without the use of other drugs and imaging dyes. The CTAHP4K that we synthesized was effective at diagnosing cancer that overexpresses the FA receptor. Moreover, the CTAHP4K generated singlet oxygen inside cancer cells after laser irradiation and effectively induced cancer-selective death by cell membrane peroxidation. We believe that CTAHP4K is a suitable candidate for the simultaneous diagnosis and treatment of cancer and that it has great potential for application in cancer-targeted delivery.

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