Supporting Information

Potential sonodynamic anticancer activities of artemether and the liposome–encapsulated artemether

Hai-Jun Chen,[†] Xiu-Rong Huang,[†] Xiao-Bin Zhou, Bi-Yuan Zheng, Jian-Dong Huang*

College of Chemistry, State Key Laboratory of Photocatalysis on Energy and Environment, Fuzhou University, Fuzhou, China

[†] These authors contributed equally to this work.

[*] Corresponding author. Tel: +86 591 22866235; Fax: +86 591 22866227; E-mail: jdhuang@fzu.edu.cn

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Experimental Procedures

Reagents: Artemether (**1**) was purchased from Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan). Stock solution of artemether (100 mM) was prepared in dimethyl sulfoxide (DMSO) and stored in the dark at -20 ^{o}C until used. Zinc phthalocyanines (1 mM) were prepared in *N*,*N*-Dimethylformamide (DMF) and stored at 4 °C in the dark. The solution was then diluted to appropriate concentration with PBS (0.01 M, pH 7.4) containing 1 % Cremophor EL (1g, in 100 ml) before use. All other solvents and reagents were of reagent grade and used as received.

Singlet Oxygen Yields: The singlet oxygen yields were determined by a steady-state method using 1,3-diphenylisobenzofuran (DPBF) as the scavenger.[1-3] Generally, the 2-methoxyethanol solution of artemether (5 μM) containing DPBF (50 μM) was prepared and irradiated with ultrasound (1.0 MHz, 2 W/cm^2) using an ultrasound transducer (Therapy Ultrasound 4150, Sonomed V Digital Ultrasound 1.0 MHz & 3.0 MHz, CARCI Company), then DPBF degradation at 413 nm was monitored along with irradiated time. The experimental set-up for ultrasound exposure is showed in **Figure S1**. The transducer with a diameter of 45 mm was submerged in the stainless steel container filled with cold degassed water. The distance between the bottom of the polystyrene tube and the transducer was 10 mm. The quenching rate constant of DPBF (k_Q^{sample}) in different sample is calculated by the equation: $(A_0^{sample} - A_t^{sample})/A_0^{sample} = k_Q^{sample}t + B$, A_0 and A_t are the absorbance at 413 nm under ultrasound treatment for 0 min and t min, respectively.

Liposome Preparation: Artemether-loaded liposomes were prepared by traditional

thin-film hydration method. Briefly, artemether, soybean lecithin, cholesterol and vitamin E at mass ratio of 10:80:20:0.5, were dissolved in 5 mL chloroform. The organic solvent was concentrated to form lipid film, and then the film was hydrated with 5 mL PBS (phosphate buffered saline solution, pH = 7.4) in 30 °C water bath for 2 h. The resultant suspension was sonicated for 10 min (100 W) with a probe (JY92-2D, Ningbo, China) to form liposome, and then the liposome was filtrated through 0.22 μm microporous membrane. The final sample was sealed in vial (the headspace of the vial was filled with nitrogen) and kept in the refrigerator (about 4 °C in the dark).

Physicochemical Characteristics of Artemether-Loaded Liposome: The particle size distribution of artemether-loaded liposomes was measured by dynamic laser-light scattering (DLS) by Zetasizer (Nano-ZS90, Malvern Instruments Ltd., UK). Liposomes were dropped on silica slices for scanning electron microscopy (SEM) (Hitachi S4800, Japan). The amount of artemether incorporated in liposomes was determined by HPLC with following conditions: Akzo Nobel Kromasil C18 column (150 mm × 4.6 mm i.d., pore size 5 μm); the mobile phase, CH_3CN:H_2O (62:38, v/v); flow rate, 1.0 mL/min; the measured wavelength, 216 nm. The encapsulation efficiency (EE) was measured immediately after preparation using centrifugation and ultracentrifugation method and calculated as below:

Encapsulation efficiency (%) = (encapsulated drug in liposome/amount of total drug) × 100%.

Cell Culture: Human hepatocellular carcinoma HepG2 cells (or breast cancer cell lines MCF-7) were obtained from the cell bank of the Chinese Academy of Science,

Shanghai, China. The cells were maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37 ^{o}C in a humidified 5% CO_2 atmosphere.

In vitro **SDT Treatment Protocols:** HepG2 cells (or MCF-7 cells, 2×10^5 cells per well) in the exponential phase were collected and divided randomly into four groups: (1) control, (2) drug alone, (3) ultrasound alone, and (4) ultrasound plus drug. For drug alone and ultrasound plus drug groups, the cells were incubated with 100 μM of artemether or 20 μM of liposome–encapsulated artemether (LM) for 45 min, allowing sufficient time for cellular uptake of the sensitizer to reach a maximum level. Instead of drug, an equivalent quantity of medium was used for the control group and ultrasound alone group. Polystyrene tube containing 0.5 mL of cell suspension (2×10^5 cells mL^{-1} in RPMI 1640 medium) was fixed vertically on the focal area of the transducer. The distance between the bottom of the polystyrene tube and the transducer was 10 mm. The cells in ultrasound and ultrasound plus drug groups were exposed to ultrasound at a frequency of 1.0 MHz and an intensity of 2 W/cm^2 for 3 min. The ultrasound system (Therapy Ultrasound 4150) was manufactured by the CARCI Company. For all experiments, the cold degassed water was used as the ultrasonic coupling medium, thereby reducing thermal effect caused by ultrasound irradiation. The experimental set-up for ultrasound exposure is showed in **Figure S1**. After the treatment procedure, cells were cultured for an additional time as specified in the text and then subjected to different analysis.

Cytotoxicity: The 3-(4,5-dimthylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide

(MTT) assay was used to determine the cell viability. After SDT treatment, the treated cells or control cells (100 μL) were seeded on a 96-well plate and incubated overnight at 37 °C under 5% CO_2. The medium was then removed from each well. After that, a 10 μL of MTT solution (5 mg mL^{-1} in PBS) and 90 μL of the medium were added into each well, followed by incubation for 4 h at 37 °C under 5% CO_2. Subsequently, the MTT-containing medium was removed and 150 μL DMSO was added into each well. After shaking for 10 min, the optical density (OD) at 490 nm was measured using microplate reader (Tecan M200Pro). The killing rate was calculated using the following equation:

Cytotoxicity (%) = (OD sham group-OD treatment group)/OD sham group × 100%.

Photocytotoxicity Assay. To assess the photocytotoxic effect of artemether, about 1.0 × 10^4 HepG2 cells (or MCF-7 cells) per well in the culture medium were seeded in 96-multiwell plates and incubated at 37 °C for 24 h in a humidified 5% CO_2 atmosphere. Artemether were first dissolved in DMSO to give 10 mM solutions, which were diluted to 1 mM with the culture medium. These served as the stock solutions for the following in vitro studies. For cytotoxicity studies, the solutions were further diluted with the culture medium. The cells, after being rinsed with PBS, were incubated with 100 μL of the artemether solutions (100 μM) for 2 h at 37 °C under 5% CO_2. The cells were then rinsed again with PBS and refed with 100 μL of the culture medium before being illuminated at ambient temperature. For dark cytotoxicity, the above cells, rinsed with PBS and refed with 100 μL of the culture medium, were incubated for 24 h without light exposure. The cell survival was assessed using the MTT assay. For light

cytotoxicity, after incubation with artemether for about 2 h, the cells were exposed to light (for control and artemether, $\lambda > 200$ nm, for ZnPc, $\lambda > 610$ nm) for 30 min at a dose of 27 $J \cdot cm^{-2}$ and then incubated again for 24 h, and finally the MTT cell viability assay was performed and each experiment was performed in triplicate.

Determination of Intracellular ROS: Intracellular reactive oxygen species (ROS) production was detected using flow cytometry with 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. DCFH-DA, a non-fluorescent cell-permeant compound, is deacetylated by intracellular esterase and converted to the reduced probe dichlorodihydrofluorescein (DCFH), which is oxidized rapidly to the highly fluorescent product dichlorofluorescein (DCF) in the presence of ROS. DCFH-DA (10 μM, dilute in serum-free medium) was added for 45 min after cells were exposed to ultrasound and incubated at 37 °C for 30 min with gentle shaking. After incubation with the fluorochrome, cells were washed twice with PBS and analyzed immediately by flow cytometer through FL-2 filter with an excitation wavelength of 488 nm.

Statistical Analysis: Data were described as mean values ± standard deviation. The statistical analysis of the data was processed using a statistical package SPSS 13.0. Differences between groups were analyzed by the one-way analysis of variance (One-Way ANOVA). A P-value of less than 0.05 was considered as a statistically significant difference.

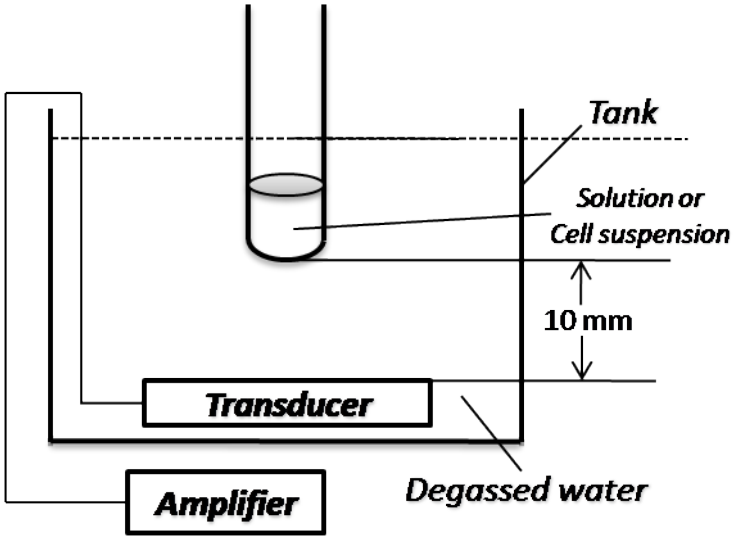

Figure S1. Diagram of the ultrasonic exposure apparatus.

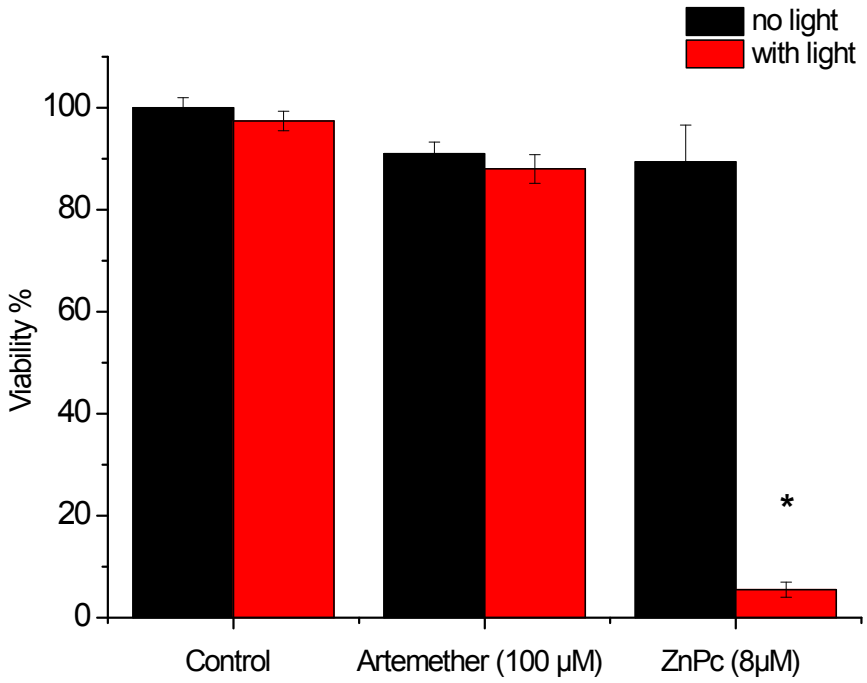

Figure S2. Cytotoxic effects of artemether (100 μM) and ZnPc (8μM) against HepG2 cells in the absence and presence of light (for control and artemether, $\lambda > 200$ nm, for ZnPc, $\lambda > 610$ nm). ZnPc was used as a positive control (*, $p < 0.01$).

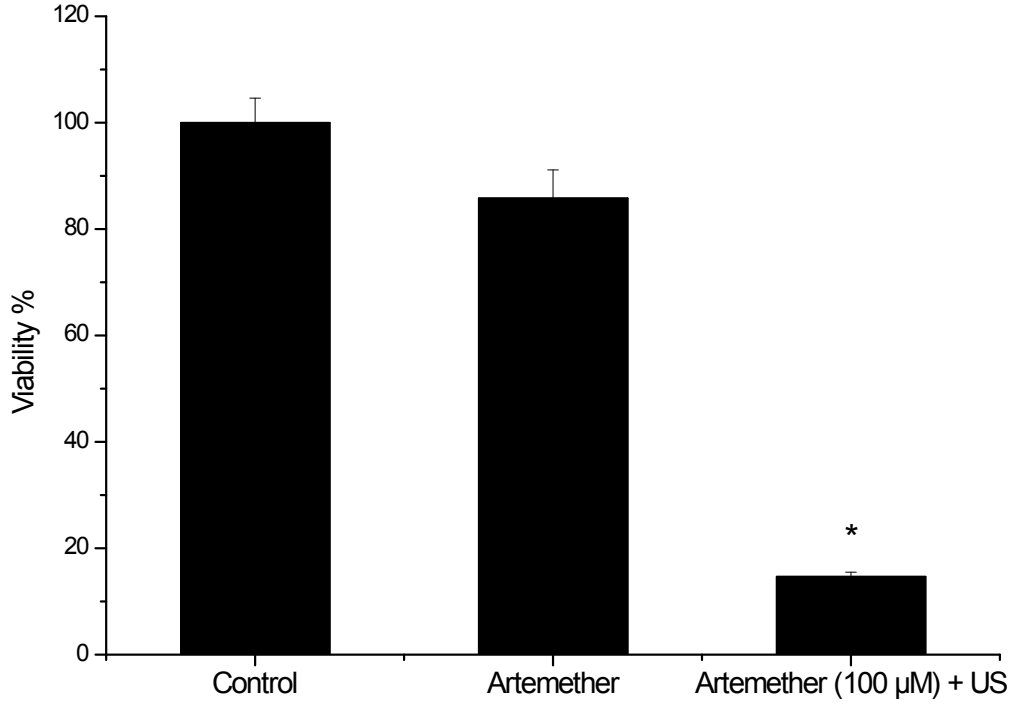

Figure S3. Sonocytotoxicity of artemether (100 μM) against MCF-7 cells (*, $p < 0.01$).

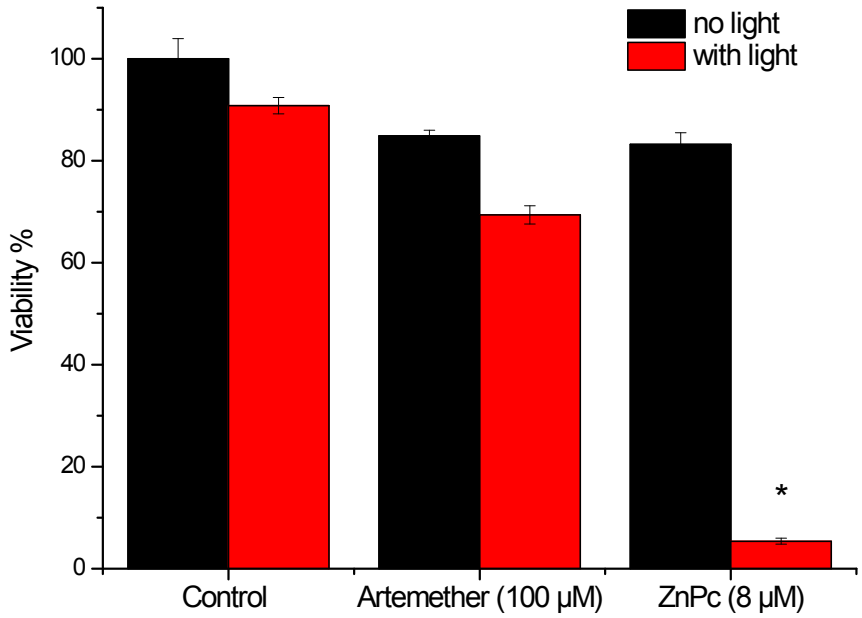

Figure S4. Cytotoxic effects of artemether (100 μM) and ZnPc (8 μM) against MCF-7 cells in the absence and presence of light (for control and artemether, $\lambda > 200$ nm, for ZnPc, $\lambda > 610$ nm). ZnPc was used as a positive control (*, $p < 0.01$).

Table S1. The sonodynamic activity of artemether.

Event	Quenching rate of DPBF, k_Q^{sample} (10^{-3}/min)	k_Q^{sample}-$k_Q^{solvent}$ (10^{-3}/min)
Only ultrasound	3.15	-
Artemether plus ultrasound	13.48	10.33

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