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# Engineering of multifunctional temperature-sensitive liposomes for synergistic photothermal, photodynamic, and chemotherapeutic effects

Tuan Hiep Tran[a,b,c,1], Hanh Thuy Nguyen[c,1], Nam Van Le[d], Thi Thu Phuong Tran[e], Jong Seong Lee[c], Sae Kwang Ku[f], Han-Gon Choi[g], Chul Soon Yong[c,*], Jong Oh Kim[c,*]

[a] Department for Management of Science and Technology Development, Ton Duc Thang University, Ho Chi Minh City, Viet Nam
[b] Faculty of Pharmacy, Ton Duc Thang University, Ho Chi Minh City, Viet Nam
[c] College of Pharmacy, Yeungnam University, 214-1, Dae-Dong, Gyeongsan 712-749, Republic of Korea
[d] School of Chemistry and Biochemistry, Yeungnam University, 214-1, Dae-Dong, Gyeongsan 712-749, Republic of Korea
[e] The Institute of Molecular Genetics of Montpellier, CNRS, Montpellier, France
[f] College of Korean Medicine, Daegu Haany University, Gyeongsan 712-715, Republic of Korea
[g] College of Pharmacy, Hanyang University, 55, Hanyangdaehak-ro, Sangnok-gu, Ansan 426-791, Republic of Korea

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ABSTRACT

Heterogeneity of cancer cells and drug resistance require multiple therapeutic approaches for comprehensive treatment. In this study, temperature-sensitive liposomes containing anti-cancer agent tanespimycin (17-AAG) and photosensitizer IR 820 were developed for combination of phototherapy and chemotherapy. The temperature-sensitive liposomes composed of DPPC, cholesterol, DSPE-PEG, 17-AAG, and IR 820 (LP-AI) at weight ratio of 35/15/3/2/2 were formulated as a thin film using extrusion and evaluated for particle size, morphology and drug release profile. Furthermore, the anticancer effect of combined therapy was examined *in vitro* and *in vivo* in SCC-7 and MCF-7 cell lines. As a result, LP-AI was prepared at particle size of $166.7 \pm 1.3$ nm, PDI of $0.153 \pm 0.012$, and ζ-potential of $-32.6 \pm 0.8$ mV. After NIR irradiation (660 and 808 nm laser), LP-AI could generate heat and ROS and enhance drug release from nanoparticles which were useful to kill the cancer cells. These were confirmed by *in vitro* cytotoxicity as well as *in vivo* effective ablation of tumors. In conclusion, fast drug release and enhanced treatment efficacy of LP-AI indicate the potential of integrating photo- and chemotherapy for synergistic anti-cancer effects.



## 1. Introduction

Combination therapy is a promising solution for overcoming heterogeneity of cancer cells, drug resistance, and toxicity induced by high and/or repeated drug doses (Choi et al., 2016b; Ramasamy et al., 2014; Tran et al., 2016). Successful combination chemotherapy strategies include dual-drugs (Choi et al., 2016a; Ramasamy et al., 2017; Ruttala and Ko, 2015), gene-chemotherapy (Creixell and Peppas, 2012), photothermal-chemotherapy (Hauck et al., 2008; Sagar and Nair, 2017), photodynamic-chemotherapy (Conte et al., 2013), or gene-photothermal therapy (Kim et al., 2016). These strategies inspired us to develop a multifunctional integrated system for photothermal, photodynamic, and chemotherapy.

Near-infrared (NIR)-absorbing carriers for photothermal cancer therapy offer non-invasive, localized, and controllable treatment (Miao et al., 2015; Nguyen et al., 2017b). After NIR exposure, nanocarriers transform absorbed light to heat, destroying neighboring cancer cells (Wang et al., 2015). In photodynamic therapy, NIR excitation of photosensitizers generates reactive oxygen species (ROS), inducing apoptosis and necrosis in cancer cells (Bechet et al., 2008). Combined photothermal and photodynamic therapy has shown improved anti-cancer effects (Yan et al., 2015; Yang et al., 2015). Indocyanin green (ICG), a near infrared (NIR) dye and other its derivatives, which have been approved by FDA for several applications, have attracted many studies for photodynamic and photothermal therapy (Li et al., 2016; Yan et al., 2016).

* Corresponding authors.
*E-mail addresses:* trantuanhiep@tdt.edu.vn (T.H. Tran), csyong@ynu.ac.kr (C.S. Yong), jongohkim@yu.ac.kr (J.O. Kim).
[1] These authors contributed equally.

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Among derivatives of ICG, IR 820 has reported as a modified form with the addition chlorobenzene ring which makes IR 820 more stable and have longer circulation time in the body, compared to ICG (Kumar and Srivastava, 2015). However, owning to low stability and short half-life, a variety of nanopartilces such as micelles (Li et al., 2016) or liposomes (Nguyen et al., 2017b; Yan et al., 2016) have been developed as drug delivery of NIR dye and chemotherapeutic agent for enhancing stability and inducing synergistic effect in treatment of cancers.

HSP90, the 90 kDa heat shock protein, has been indicated to involve in malignant processes that are crucial to the growth and survival of cancer cells (Dimopoulos et al., 2011). HSP90 expression increase has been reported in several cancers, including breast cancer (Yano et al., 1999) and squamous cell carcinoma (Huang et al., 2014) thus, it is suggested to use as an anticancer drug target (Lin et al., 2016). In addition, the heat shock proteins amplification is also indicated to contribute to cellular thermo-tolerance during photothermal therapy, resulting in limited treatment efficiency (Huang et al., 2011; Kim and Lee, 2017; Richardson et al., 2011). HSP90 inhibitors like tanespimycin (17-AAG) bind to HSP90 and reduce cancer cell survival *in vitro* and *in vivo* (Richardson et al., 2011). Furthermore, 17-AAG can suppress pro-survival and angiogenic signaling subunits mediated by phototherapy (Lin et al., 2016; Richardson et al., 2011), which suggest that the combination tanespimycin (17-AAG) and photothermal therapy is proposed to improve anti-tumor effects (Huang et al., 2011; Lin et al., 2016).

In this study, the temperature-sensitive liposomes, incorporating IR 820 for photodynamic (PDT) and photothermal therapy (PTT) and tanespimycin (17-AAG), a HSP90 inhibitor, were designed. In response of NIR irradiation, IR 820 can induce heat and increase temperature above the physiological temperature of liposome. This leads to burst release of 17-AAG and it acts as an effective anti-cancer agent. Combining heat from PTT, ROS production from PDT, and chemotherapeutic effects of 17-AAG, this carrier could ensure therapeutic efficiency and open new trends in anti-cancer treatment.

## 2. Materials and methods

### 2.1. Materials

17-AAG and IR 820 were purchased from Sigma (St. Louis, MO, USA). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,2-Distearoyl-sn-glycero-3-phosphorylethanolamine-polyethyleneglycol 5000 (DSPE-PEG5000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were of reagent grade and used without further purification. SCC-7 and MCF-7 cell lines were originally obtained from the Korean Cell Bank (Seoul, South Korea). Cell lines were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and incubated at 37 °C in a 5% $CO_2$ humid incubator.

### 2.2. Preparation of LP-AI

The thermal-sensitive liposomes were optimized based on our previous study and prepared by lipid film hydration followed by extrusion (Choi et al., 2016a; Kuijten et al., 2015; Nguyen et al., 2017b). Briefly, 17-AAG and DPPC: cholesterol: DSPE-PEG5000 lipid mixture with ratio 2:35:15:5 (w/w) were dissolved in 3:1 (v/v) chloroform/methanol in a round-bottomed flask. Organic solvents were removed by a rotary evaporator at 50 °C for 2 h. The dry lipid film was hydrated by adding 5 mL of IR 820 at concentration of 0.4 mg/mL aqueous solution in a 50 °C water bath for 30 min. Dispersions were homogenized 10 times with a mini-extruder through polycarbonate membrane filters (Whatman, Maidstone, UK) with a pore diameter of 200 nm. LP-AI was lyophilized using mannitol (5%, w/v) as the cryoprotectant. The obtained liposome dispersions were freeze-dried using a lyophilizer (FDA5518, Ilshin, South Korea). The powder was collected for further studies.

### 2.3. Physicochemical characterization of LP-AI

Size distribution of liposomes was determined using dynamic light scattering (DLS) with a Zetasizer Nano–Z (Malvern Instruments, Worcestershire, UK). Samples were diluted with distilled water prior to determination. LP-AI morphology was characterized by transmission electron microscopy (Hitachi H-7600, Tokyo, Japan). The samples were prepared on a carbon-coated copper grid in the presence of 2% phosphotungstic acid and dried at room conditions before measurement. Atomic force microscopy images were collected in tapping mode using Nanoscope IIIa (Digital Instruments Co., USA) (Tran et al., 2015a).

Differential scanning calorimeter (DSC Q200, TA Instruments, USA) was used to determine the melting phase transition temperature ($T_m$) of the lipid and liposomes. Samples in 2–3 mg were added in aluminum pans. The DSC scans were recorded over the temperature range of 20 °C to 150 °C at 10 °C/min of heating rate, under nitrogen flow of 50 mL/min and empty pan as reference sample (Truong et al., 2016).

Loading capacity of 17-AAG and IR 820 was analyzed indirectly by measuring the free drug in suspension. Briefly, LP-AI was centrifuged using an Amicon® centrifugal tube (Millipore, USA) at a speed of 5000 rpm for 15 min. The 17-AAG concentration in the supernatant was determined using high-performance liquid chromatography (HPLC) with a mobile phase of acetonitrile: 10 mM ammonium acetate with 0.1% (v/v) acetic acid (pH 4.8) (60:40, v/v), at a flow rate of 0.8 mL/min, and detection wavelength of 334 nm (Pradhan et al., 2015). IR 820 concentration was detected by UV–vis spectrophotometer at 790 nm (Nguyen et al., 2017b).

### 2.4. NIR irradiation temperature profile

Temperature profile of the samples after NIR laser irradiation was determined using an infrared thermal camera (Therm-App® TH, Vumii Imaging Inc, Roswell, GA, USA). Samples (150 μL) of various concentrations (1–40 μg/mL) of free IR 820 and LP-AI were placed in microtubes and exposed to 808 nm NIR laser (FC-W-808, Changchun New Industries Optoelectronics Technology, China) at 2 W/cm$^2$ for 5 min.

### 2.5. In vitro *drug release studies*

Drug release profiles were evaluated in medium at 37 °C and 42 °C. Briefly, a dialysis bag (MWCO, 3.5 KDa) with 1 mL of LP-AI dispersion was placed in 35 mL of release medium (phosphate-buffered saline, pH 7.4, with or without 5% fetal bovine serum) inside a shaking water bath (HST – 205 SW, Hanbaek ST Co., Seoul, Korea) and continuously shaken at 100 rpm. At predetermined time intervals, 0.5 mL of the incubated solution was withdrawn and replaced with an equal volume of fresh medium. Released 17-AAG was analyzed using HPLC as described above.

### 2.6. In vitro *cellular uptake*

SCC-7 and MCF-7 cells grown in 12-well plates at a density of $1 \times 10^5$ cells per well for 24 h were treated with coumarin 6-loaded liposomes (LP-C6) in DMEM at 1 and 5 μg/mL for 30 and 60 min. After treatment, the cells were collected, washed three times with PBS, and re-suspended in 1 mL PBS for flow cytometry analysis (FACSVerse, BD Biosciences, San Jose, CA, USA) (Sun et al., 2016;