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Clathrin-mediated internalization of micelles based on chondroitin

sulfate-*graft*-poly(ε-caprolactone) causing distinct CPT-induced deaths in lung cancer

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Abstract

Our previous research has synthesized a chondroitin sulfate-*graft*-poly(ε-caprolactone) copolymer with a high content of poly(ε-caprolactone) (18.7 mol%), named H-CP, which self-assembled in water into a rod-like micelle to encapsulate the hydrophobic camptothecin in the core (micelle/CPT) for tumor-targeting delivery. Owing to recognition of the micelle to CD44, the micelle/CPT entered CRL-5802 cells efficiently and released CPT efficaciously, thus showing higher tumor suppression than a commercial product, CPT-11. In this study, H1299 cells were found to have higher CD44 expression than CRL-5802 cells. However, the lower CD44-expressing CRL-5802 cells had higher cell death and higher cellular uptake of the micelle/CPT compared with the higher CD44-expressing H1299 cells. Examining the internalization pathway of the micelle/CPT in the presence of different endocytic chemical inhibitors showed that CRL-5802 cells involved clathrin-mediated endocytosis which was not found in H1299 cells. Analysis on the cell cycle of the two cell lines exposed to the micelle/CPT revealed that CRL-5802 cells arrested mainly in the S phase and H1299 cells, mainly in the G2-M phase. A consistent result was also found in the evaluation of γ-H2AX expression, showing around three-fold higher in CRL-5802 cells than in H1299 cells. A near-IR dye, IR780 was encapsulated into the micelle to observe the *in vivo* biodistribution of micelle/IR780 in tumor-bearing mice. The CRL-5802 tumor indeed showed higher fluorescence intensity than the H1299 tumor at any tracing time points following 1 hour. Thus, we tentatively conclude CRL-5802 utilized the clathrin-mediated

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internalization pathway and arrested in the S phase upon exposure to the micelle/CPT; all are possible

reasons for better therapeutic outcome in CRL-5802 cells than in H1299 cells.

Introduction

Chondroitin sulfate (CS) is a natural polysaccharide, having many merits such as biocompatibility,

biodegradability,[1] serving as an anti-inflammatory agent,[2] and a natural ligand for the cluster

determinant (CD44) receptor.[3] The CD44 receptor is one of the membrane receptors overexpressing on

many solid tumor surfaces.[4, 5] Thus, CS can be used as a specific molecular ligand for targeting cancer

cells through CD44-mediated endocytosis.[3]

A copolymer based on CS and polycaprolactone (PCL), named CP, was synthesized as an

anticancer drug carrier.[6] Both CS and PCL are FDA-approved nontoxic materials. Owing to

amphiphilicity, the CP copolymer could self-assemble into micelles in water, which could be utilized to

encapsulate a hydrophobic anticancer drug, camptothecin (CPT), in the core for tumor-targeting

delivery. CPT is an antineoplastic agent and has a low aqueous solubility and short-term stability of the

lactone ring structure, thus limiting its development in clinical settings.[7, 8] The advantages of using the

CP self-assembled micelles in drug delivery include not only recognition to CD44 but also saving the

lactone ring of CPT in blood circulation. Moreover, a polysaccharide-modified nanoparticle prevents

recognition by the reticuloendothelial system (RES),[9, 10] similar to the role of poly(ethylene glycol).
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The physicochemical properties of the micelle and CPT-loaded micelle (micelle/CPT) were thoroughly characterized and the therapeutic efficacy of the micelle/CPT was demonstrated in non-small cell lung cancer (NSCLC) CRL-5802 cells, expressing the CD44 receptor on cell surface.[6] The CD44-expressing intensity on the surface of several NSCLC cell lines have been examined, and H1299 cells were found to have the highest expression of CD44. When exposed to the micelle/CPT, the higher CD44-expressing H1299 cells showed lower cytotoxicity and apoptosis than the lower CD44-expressing CRL-5802 cells. To account for the higher therapeutic efficacy of CRL-5802 cells, experiments were conducted using endocytosis chemical inhibitors to understand if internalization of the micelle/CPT into the two cell lines involved different pathways and how this affected cell cycle arrest, apoptosis, and γ-H2AX formation. The *in vivo* biodistribution of the micelle in tumor-bearing mice was examined using a near-infrared optical imaging to determine the amount of micelles accumulated in the two tumors.

Materials and methods

Materials

Copper(I) bromide (CuBr), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), sodium borate, Tween 20, dimethyl sulfoxide-*d* (DMSO-d_6), chloroform-*d* (CDCl$_3$) and deuterium oxide (D$_2$O) were purchased from Aldrich (St. Louis, MO). 2,2'-Bipyridine

(Bpy), 2-bromo-2-methylpropionyl bromide, Amberlite® IR120, Dowex 50W×8 (H) were purchased

from Acros (Morris Plains, NJ). Chondroitin sulfate-*g*-poly(ε-caprolactone) copolymer containing a

high content of poly(ε-caprolactone) (H-CP) and Rhodamine 123-conjugated H-CP (Rh123-H-CP)

were prepared as previously described.[6] Camptothecin (CPT) was kindly acquired from Industrial

Technology Research Institute of Taiwan. Bovine serum albumin (BSA) was purchased from

MDbio Inc. (Taipei, Taiwan). Fetal bovine serum (FBS) was purchased from Biological Industries

(Beit Haemek, Israel). Dulbecco's modified Eagle's medium (DMEM) and Tris-glycine gels were

purchased from Invitrogen (Carlsbad, CA). An annexin V-FITC apoptosis detection kit was purchased

from Strong Biotech (Taipei, Taiwan). For western blot assay, the primary antibodies against XIAP,

γ-H2AX and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and primary

antibodies against p27[Kip-1], CDK2 and cyclin A were purchased from GenTex Inc. (Irvine, CA).

Preparing H-CP micelle

A H-CP copolymer was synthesized according to our previous publication.[6] The H-CP micelle was

prepared by a simple dialysis method. Briefly, the H-CP copolymer (10 mg) was dispersed in 5 mL of

DMSO containing 4 μL of trifluoroacetic acid at 60 ºC. The solution was placed in a dialysis bag (Mw

cut-off 1000 membrane, Spectrum Labs, Rancho Dominguez, CA) and dialyzed against DD water for 1

day, followed by freeze-drying to produce the micellar product.

CPT or IR780-encapsulated micelles (micelle/CPT or micelle/IR780)

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The method for preparing CPT or IR780-loaded micelles was similar to that for preparing the H-CP

micelle. In brief, CPT or IR780 powder was dissolved in DMSO at 1 mg/mL to yield a stock solution.

The H-CP (10 mg) powder was dissolved in 5 mL of DMSO containing 4 µL of trifluoroacetic acid at

60 ºC, followed by adding 1 mL of the CPT or IR780 stock solution. The solution was dialyzed against

DD water using Mw cut-off 6000 membrane (Spectrum Labs, Rancho Dominguez, CA) for 1 day and

then filtered to remove the unencapsulated CPT or IR780. Finally, the micelle/CPT or micelle/IR780

was obtained by freeze-drying.

Cell lines

CRL-5802 and H1299 non-small cell lung carcinoma cell lines were obtained from Dr. Cheng at the

Biomedical Science and Environmental Biology Department of Kaohsiung Medical University in

Taiwan and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C

under humidified 5% CO_2.

Expression of CD44 receptor

An immuno-phenotype analysis was used to evaluate the expression of CD44 receptor in several cell

lines.[11] The cells of 5×10^5 cells/tube were suspended with 90 µL PBS in 1.5 mL Eppendorf tubes and

10 µL of CD44-FITC (50 µg/mL) (eBioscience, San Diego, CA) was added to co-incubate at 4°C for

30 minutes in darkness. The cells were washed three times with 0.1M PBS and resuspended in 1 mL

PBS. The fluorescence intensity of FITC was measured using flow cytometry (Becton-Dickinson,

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Mansfield, MA). Data were averaged from three experiments.

Cytotoxicity

The cytotoxicities of CPT and micelle/CPT were measured using the MTT assay. The CPT powder was

dissolved in DMSO to yield a stock solution (2 mg/mL), followed by dilution to a concentration of

0.0025 – 5.0 µg/mL in DMEM containing 10% FBS for the cytotoxic test of CPT. The equivalent CPT

concentration of the micelle/CPT was controlled within 0.0025 – 5.0 µg/mL as well. Cells were seeded

in 96-well culture plates at a density of 5×10^3 cells per well in DMEM containing 10% FBS for 24

hours. The culture medium was replaced with 100 µL medium containing various concentrations of

CPT. Following 24 hours, the cells were washed three times with 0.1M PBS and replenish fresh

medium to post-incubate for another 24 or 48 hours. The number of viable cells was measured by

estimating their mitochondrial reductase activity using the tetrazolium-based colorimetric method.

Intracellular uptake and endocytosis inhibition

The synthesis of rhodamine 123-conjugated H-CP (Rh123-H-CP) was referred to our previous

publication.[6] The cellular uptake of Rh123-H-CP was studied using a flow cytometer. CRL-5802 or

H1299 cells were seeded at a density of 2×10^5 cells per well in 6-well plates in DMEM supplemented

with 10% FBS and incubated for 24 hours. The culture medium was removed and replaced with 2 mL

DMEM containing Rh123-H-CP (100 µg/mL). The cells containing Rh123-H-CP were incubated at 37

ºC for another 30 minutes or 2 hours. The cells were washed three times with 0.1M PBS, collected and

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analyzed using the flow cytometer.

Three chemical inhibitors were utilized to study endocytosis pathways using following

concentrations: wortmannin (50 nM), chlorpromazine (0.5, 1.0, 5.0 and 10 μg/mL), and genistein (50,

100 and 200 μM).[12] CRL-5802 or H1299 cells were seeded at a density of 1×10^5 cells per well in

6-well plates in DMEM supplemented with 10% FBS and incubated for 24 hours. The cells were

pretreated with various concentrations of inhibitors at 37 ºC for 30 minutes. Next, the cells were

washed and replaced with 2 mL DMEM containing Rh123-H-CP (100 μg/mL). The cells were further

incubated at 37 ºC for 2 hours, washed three times with 0.1M PBS, collected, and analyzed using flow

cytometry.

Cell cycle analysis

The effect of CPT and micelle/CPT internalization on cycle progression of cancer cells were studied by

cell cycle analysis. Cells (CRL-5802 or H1299) were seeded at a density of 2×10^5 cells per well in

6-well plates and incubated in DMEM supplemented with 10% FBS for 24 hours. Subsequently, CPT

and micelle/CPT were respectively added to the cells at an equivalent CPT concentration of 0.1 μg/mL

in the same culture medium. The cells containing the drug were then incubated at 37 ºC for 24 hours.

The culture mediums were removed and the cells were washed and detached using 0.1% 1X trypsin (1

mL/well). The cells were collected by centrifugation at 1000 rpm for 5 minutes and fixed using 70%

ethanol overnight. Following fixation, the cells were washed and incubated with 20 μg/mL PI and 20

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μg/mL RNase in 0.1M PBS at room temperature for 15 minutes. The pellets of cells were obtained by

centrifugation at 1000 rpm for 5 minutes and resuspended in 0.1M PBS to analyze the distribution of

cell cycle using flow cytometry.

Cell apoptosis

To estimate the apoptosis-inducing efficiency of CPT and micelle/CPT, the annexin-V/PI dual staining

assay was utilized. The cells were plated at a density of 2×10^5 cells per well into 6-well culture plates

and incubated in DMEM containing 10% FBS for 24 hours. Next, the cells were respectively treated

with CPT and micelle/CPT at a CPT concentration of 0.1 μg/mL in DMEM without 10% FBS at 37 ºC

for 24 hours. The H-CP micelle and cultured cells were used as blank and control groups, respectively.

The cells were washed three times with 0.1M PBS, collected and labeled with annexin V-FITC (10

μg/mL) and PI (20 μg/mL) at 37 ºC for 30 minutes. After labeling, the cells were analyzed using flow

cytometry. The experiment was repeated three times.

Evaluation of γ-H2AX activation

The degree of DNA damage was assessed using γ-H2AX as a marker.[13] Cells were seeded at a density

of 2×10^5 cells/well in 6-well tissue culture plates and incubated in DMEM medium containing 10%

FBS for 24 hours. CPT and micelle/CPT was respectively added at a CPT concentration of 0.1 μg/mL

in the culture medium and then incubated at 37 ºC for 24 hours. The cells were washed and harvested

using 0.1% 1X trypsin (1 mL/well), collected into Eppendorf tubes and centrifuged at 3000 rpm for 10

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minutes. Next, 70% ethanol was added dropwise into the tubes to fix the cells at -20 ºC overnight. After

fixation, the cells were washed in BSA-T-PBS solution (1% bovine serum albumin and 0.2% Triton

X-100 in PBS; Sigma, St. Louis, MO) and incubated with 100 mL of the BSA-T-PBS solution

containing 0.2 mg p-histone H2AX (Ser 139) monoclonal antibody (#sc-101696, Santa Cruz

Biotechnology, Santa Cruz, CA) on a shaker at 4 ºC overnight. Following centrifugation, the

supernatant was discarded from the tubes and the residue was washed twice in the BSA-T-PBS solution.

Alexa Fluor 488-tagged secondary antibody (Jackson Laboratory, Bar Harbor, ME) was diluted to 1:

200 with the BSA-T-PBS solution and mixed with the cells at 37 ºC for 1 hour. Following two washes

with the BSA-T-PBS solution, the cells were incubated with 20 µg/mL of PI at room temperature for

30 minutes. The fluorescent intensity of the cells was recorded using flow cytometry.

Western blot analysis

CRL-5802 or H1299 cells were plated at a density of 2×10^5 cells per well into 6-well culture plates

and incubated in DMEM (1mL) containing 10% FBS for 24 hours. The cells were respectively treated

with CPT and micelle/CPT at a CPT concentration of 0.1 µg/mL and at 37 ºC for 1 hour. The cells

were scraped and collected to 1.5 mL Eppendorf tubes, and proteins were extracted from the cells by

mixing with lysis buffer (0.05 mol/L Tris-HCl at pH 6.8, 2% SDS, and 6% β-mercaptoethanol) in ice

for 30 minutes. Following lysis, the supernatant solution was separated from the cell pellets by

centrifugation at 13,000 rpm for 30 minutes and the protein concentration was measured using a

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BCA^{TM} protein assay kit (Pierce, Rockford, IL). Next, each protein (30 μg) was mixed with a 5X

sample dye followed by boiling it at 95 ^{o}C for 5 minutes. An equal amount of protein (30 μg) was

separated using 10% SDS-PAGE gel and transferred to a PVDF membrane. The PVDF (polyvinylidene

difluoride; Merck Millipore Life Science, Darmstadt, Germany) membrane was blocked in blocking

buffer (5% nonfat milk powder dissolved in Tris-buffered saline buffer containing 0.1% Tween 20

[TBST]) at room temperature. Following 1 h of incubation, the blocked PVDF membrane was probed

with a 1:1000 dilution of phosphorylated histone H2AX, γ-H2AX (Ser^{139}) primary antibody in 1%

nonfat milk-TBST buffer at 4 °C overnight. The next day, the PVDF membrane was washed three

times with TBST and incubated with 1:4000 dilution of a horseradish peroxidase (HRP)-conjugated

secondary antibody (Thermo Fisher Scientific, San Jose, CA) in TBST at room temperature for 1 h.

The PVDF membrane was washed three times with TBST and developed using an enhanced

chemiluminescence (ECL) detection kit (Amersham, Piscataway, NJ).

Confocal laser scanning microscopy (CLSM)

CRL-5802 or H1299 cells were seeded at a density of 1×10^5 cells per well in 12-well plates

containing one glass coverslip per well, in DMEM supplemented with 10% FBS for 24 hours. The

culture medium was replaced with 1 mL medium without 10% FBS, which contains either CPT or

micelle/CPT at a CPT concentration of 0.1 μg/mL. The H-CP micelle (blank) and cultured cells were

used as control groups. The cells with or without CPT were incubated at 37 ºC for 24 hours. The

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coverslips containing the cells were removed, washed gently twice with PBS, and then treated with 4%

paraformaldehyde to fix cells for 30 minutes. After fixing, the cells on the coverslips were washed

twice with PBS and then incubated with blocking buffer (1% BSA in PBS) at room temperature for 15

minutes. The supernatant was removed and then the cells were mixed with p-histone H2AX (Ser^{139})

monoclonal primary antibody (dilution 1: 300 with blocking buffer) at room temperature. Following 1

hour of standing, the cells on coverslips were washed 3 times with blocking buffer and stained with

Alexa Fluor 488-tagged secondary antibody, which was diluted to 1: 200 with the blocking buffer and

incubated at 37 ºC for 1 hours. Following two washes with the blocking buffer, the coverslips

containing the cells were mounted with Fluoromount-G^{TM} (eBioscience) for CLSM observation

(Olympus Fv 1000, Tokyo, Japan).

In vivo biodistribution

Nude mice (Balb/cAnN.Cg-*Foxnl*^{nu}/CrlNarl, male, ~ 6 weeks old) were purchased from National

Laboratory Animal Center (NLAC) of Taiwan. The animal experiment was approved by Institutional

Animal Care and Use, Committee of Kaohsiung Medical University. The biodistribution of

micelle/IR780 was monitored by non-invasive fluorescence in tumor-bearing mice with two tumors

grafted on the right (H1299) and left (CRL-5802) hind legs. After tumors grew to approximately 150

mm^3 in volume, the micelle/IR780 (1.75 mg/kg of IR780) was intravenously injected via the lateral tail

vein into tumor-bearing mice.[14] Following the injection, the micelle/IR780 was imaged at several time

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points (1, 4, 8, 12, 24, 36 and 48 hours) using an IVIS Spectrum System 3D (Caliper Life Sciences,

Hopkinton, MA). The exposure time was set at 2 seconds and the wavelength of excitation and

emission was selected at 745nm and 840nm with an ICG filter. Following 48 hours, relevant organs,

tissues, and tumors were harvested from the mice and imaged immediately to quantify intensities using

the region of interest (ROI) function of the Living Image® software and then the heart, liver and kidney

were sectioned into slices for histopathological examination with Hematoxylin & Eosin (H&E)

staining.

Statistical analysis

Means and standard deviations (SD) of data were calculated. Comparison between groups was tested

using Student's *t*-test and *P*< 0.05 was considered to be significant.

Results and discussion

CD44 expression and cytotoxicities

A chondroitin sulfate-*g*-poly(ε-caprolactone) copolymer with a high content of PCL (18.7 mol%),

named H-CP, had been synthesized and utilized to encapsulate the hydrophobic CPT (micelle/CPT) in

the core for tumor-targeting delivery.[6] With recognition of the H-CP micelle to CD44, the micelle/CPT

entered CRL-5802 cells and released CPT efficaciously, thus showing higher tumor suppression

compared with the commercial CPT-11. Examination of CD44 expression on the surface of many

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NSCLCs revealed that H1299 cells show higher CD44 expression than CRL-5802 cells (Figure 1).

Thus, it was hypothesized that the therapeutic outcome of CPT released from the micelle/CPT against

H1299 cells should be better than that against CRL-5802 cells.

To test this hypothesis, the cell viabilities of the two cell lines exposed to free CPT and the

micelle/CPT were promptly screened after 24 and 48 hours of incubation (Figure 2). The cytotoxicities

of the micelle/CPT were found to be higher than that of free CPT in both cell lines and after both

incubation durations. The anticancer drug-encapsulated NPs usually have advantages over their parent

drugs including: (1) an increase in water solubility of low soluble or insoluble anticancer drugs and an

enhancement in biodistribution and therapeutic efficacy; (2) an accumulation of anticancer drugs in

tumor tissues by the enhanced permeation and retention (EPR) effect and a reduction in systemic side

effects; (3) protection of anticancer drugs against deactivation during blood circulation, transport to

targeted organs or tissues and intracellular trafficking. In CRL-5802 cells, the micelle/CPT showed

higher potency in cell-killing activity (IC_{50}= 0.01 µg/mL for 24 hours, IC_{50}= 0.004 µg/mL for 48 hours)

than in H1299 cells (IC_{50}= 1.36 µg/mL for 24 hours, IC_{50}= 0.15 µg/mL for 48 hours). This result

completely contradicted the hypothesis that higher CD44 expression on H1299 cell membrane should

enhance the cellular uptake of the micelle/CPT, leading to better therapeutic outcome of CPT.

Intracellular uptake and endocytosis inhibition

To further confirm the hypothesis, flow cytometry was utilized as an alternative measurement to

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examine the internalization efficacy of the H-CP micelle into two cell lines. A fluorescent dye,

rhodamine 123 (Rh123), was conjugated to the micelle. As seen in Figure 3 (A&B), the cellular uptake

of the Rh123-H-CP micelle into CRL-5802 cells was profoundly higher than that into H1299 for 30

minutes and for 2 hours of incubation. This result agreed with the finding in cytotoxicity (Figure 2).

The higher CD44-expressing H1299 cells were less able to internalize the micelle, which may be

attributed to other pathways, besides the CD44-mediated endocytosis, involved in uptake of the micelle

in the two cell lines.

Different endocytosis pathways of nanomedicine involved in cellular uptake have been widely

reported.[15] Thus, the cells were pretreated with three chemical inhibitors before being treated them with

the Rh123-H-CP micelle. Wortmannin was selected for macropinocytosis; chlorpromazine, for

clathrin-mediated endocytosis (CME); and genistein, for caveolae-mediated endocytosis.[6] The

inhibition effect of these three inhibitors on cellular uptake of the micelle was preliminarily tested at

the following concentrations: 50 nM wortmannin, 10 μg/mL chlorpromazine, and 200 μM genistein.

Flow cytometric diagrams showed a left shift when CRL-5802 and H1299 cells were respectively

pretreated with chlorpromazine and genistein, indicating the inhibition effect, while the diagrams

remained intact when the cells were treated with wortmannin (Supporting Figure 1S). Thus, the

dose-dependent inhibition of chlorpromazine and genistein was further tested. Flow cytometric

diagrams showed an apparent shift to the left when CRL-5802 cells were pretreated with

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chlorpromazine at 5 and 10 μg/mL, and genistein at 100 and 200 μM, as compared with the control

group (Supporting Figure 1S). Subsequently, the cellular accumulation of fluorescence intensity was

quantified according to the control group. The fluorescence intensity decreased significantly for the

chlorpromazine- and genistein-pretreated cells at the aforementioned concentrations (Figure 3C). The

same test was done on H1299 cells. No inhibition effect was observed in H1299 cells pretreated with

different doses of chlorpromazine. In contrast, there is an apparent shift to the left when the cells were

pretreated with genistein at a low dose of 10 μM (Supporting Figure 1S and Figure 3D). The flow

cytometric results suggest that the cellular uptake of the micelle was indeed cell line-dependent. The

cellular uptake of the micelle into CRL-5802 cells utilized CD44-, clathrin-, and caveolae-mediated

endocytosis; however, in H1299 cells, only CD44- and caveolae-mediated endocytosis pathways were

involved.

Cellular internalization and subsequent intracellular processing of nanoparticles are key barriers to

successful *in vitro* and *in vivo* delivery of drugs. Clathrin- and caveolin-dependent endocytosis have

been extensively studied in the past decade.[15] Clathrin-dependent endocytosis yields acidified vesicles

(pH 5 - 6) that fuse with lysosomes (pH ~4.5). Caveolin-dependent uptake is less well characterized but

is associated with the formation of caveosomes that are less acidified and are believed to avoid

trafficking to lysosomes. The differences in endocytic vesicles and subsequent processing may

significantly impact the intracellular destination of drug-encapsulated nanoparticles. There are several

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examples in which CME appears to be defined as the most prominent mechanism for cellular entry.[16-20]

For example, the nanoparticles made of D,L-polylactide (PLA) and poly(ethylene glycol-*co*-lactide)

(PEG-*co*-PLA) have been explored for drug delivery. Owing to the presence of the partially hydrolyzed

PLA, the surface of the nanoparticles was negatively charged and usually altered with cationic

surfactants such as stearylamine.[16, 21] In the polarized MDCK epithelial cells, the nanoparticles used

CME independent of their charge. In contrast, in non-polarized HeLa cervical cancer cells, the anionic

particles used multiple pathways (CME and caveolae-mediated), while cationic particles appeared to be

restricted to CME and macropinocytosis. Hyaluronic acid (HA) conjugated with hydrophobic

poly(L-histidine) (PHis) was utilized to prepare a pH-responsive and tumor-targeted copolymer,

hyaluronic acid-g-poly(L-histidine) (HA-PHis), for use as a carrier for anti-cancer drugs.[22] The results

demonstrated the pH-responsive HA-PHis micelles were taken up via CD44 receptor-mediated

endocytosis as well as via CME, and delivered to lysosomes, which contribute to trigger the release of

doxorubicin (DOX) into the cytoplasm. A review of these examples showed that using the CME

pathway seems to enhance cellular uptake of biodegradable micelles and results in better therapeutic

outcome.

Cell cycle

CPT is a potent anticancer drug which inhibits DNA topoisomerase I and causes apoptosis of cancer

cells.[23] To understand further the mechanism of drug action, the effect of CPT on the cell cycle of two

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cell lines was evaluated. Cell cycle arrest was analyzed using propidium iodide (PI)-labeled RNase in

CRL-5802 and H1299 cells after 24 hours of post-incubation. Flow cytometric analysis showed 21.3%,

40.4%, and 18.3% of arrest for CPT-treated CRL-5802 cells and 26.0%, 37.4%, and 13.0% for

micelle/CPT-treated CRL-5802 cells in G_1, S and G_2-M phases, respectively (Figure 4A). Similarly,

flow cytometric analysis was done on H1299 cells. There were 19.4%, 27.8%, and 45.3% of arrest for

CPT-treated cells and 7.89%, 23.8%, and 58.8% for micelle/CPT-treated cells found in G_1, S, and

G_2-M phases, respectively (Figure 4B). Cell cycle analysis revealed that CPT or micelle/CPT arrested

mainly CRL-5802 cells in the S phase and H1299 cells in the G_2-M phase. Although CPT has been

shown to induce an accumulation of cells at the intra-S-phase checkpoint,[24] other studies also showed

that both low-dose CPT and CPT-resistant cancer cells could induce accumulations at G_1/S transition or

G_2/M boundary instead of the intra-S arrest alone.[25] This fact will attenuate the therapeutic outcome of

CPT.

The S-phase cells of the myelogenous leukemias HL-60 or KG-1 are susceptible to undergo

apoptosis in the presence of CPT. However, glioma cells U-251 MG and D-54 MG which express

ectopic p16 lead to G_1 phase arrest and are resistant to CPT derivative topotecan.[26] On the contrary,

human lymphocytic leukemia cells MOLT-4 or mouse L1210 cells are resistant to CPT and no

significant CPT-induced accumulation in S phase was detected.[27, 28] In addition, Goldwasser's work

showed that human colon cancer cell lines SW620 and KM12 trigger accumulations in two distinct

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phases following CPT treatment.[25] The CPT-resistant KM12 was arrested in G_2, whereas the

CPT-sensitive SW620 was arrested in S phase irreversibly. The effect of CPT on the cell cycle arrest

was indeed cell line-dependent.

The S-phase selectivity reflects that the formation of double-strand DNA breaks when the

advancing replication forks collide with CPT-stabilized topo I-DNA complexes.[29, 30] Nevertheless,

additional studies revealed that further exposure of exponentially growing cells to high CPT

concentrations produces a subsequent G_2 arrest in synchronized HeLa cells as a consequence of

impaired activation of cdc2-cyclin B complexes.[31, 32] Their results suggested that the alteration in

checkpoints of DNA damage may be a critical factor for defining the chemosensitivity of cancer cells

to CPT.

P27 [Kip-1] is a member of Cip/Kip family and modulates the progression of cell cycle by binding

cyclin/Cdk complexes. Furthermore, p27 acts as a regulator of cell cycle progression at both G_1/S and S

to G_2/M transition phases.[33] In normal cells, p27 is a tumor suppressor and has been reported to inhibit

the cell-cycle progression. However, the expression of p27 has been shown to be a negative indicator

that correlated with the prognosis of patients with oral squamous cancer,[34] chronic B-cell lymphocytic

leukemia,[35] and non-small cell lung cancer.[36] Here, the CPT/micelle causes a dramatically decreased

level of p27 protein in CRL-5802 cells, but not in H1299 cells (Figure 4C). The down-regulation of

p27 is presumed to enhance the cytotoxicity of CRL-5802 cells treated with the CPT/micelle.

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Cell apoptosis

To examine the induction of cell apoptosis in the CPT-treated or micelle/CPT-treated cells at a CPT

concentration of 0.1 μg/mL, an annexin-V/PI dual staining assay was conducted using flow cytometry

(Figure 5A). The four quadrants display necrotic cells stained with PI in the first quadrant (Q1), late

apoptotic cells stained with PI and annexin-V in the second quadrant (Q2), early apoptotic cells stained

with annexin-V in the third quadrant (Q3), and healthy cells not stained with PI and annexin-V in the

fourth quadrant (Q4). The quantification of apoptotic cells was plotted in Figure 5(B). The percentages

of late/early apoptotic cells (Q2+Q3) of micelle/CPT-treated CRL-5802 cells (~ 34%) were

significantly higher than that of CPT-treated cells (~ 25%); however, no significant difference was

found in H1299 cells exposed to either micelle/CPT or CPT. The relative apoptosis ratios between cells

treated with the micelle/CPT and empty H-CP in CRL-5802 and H1299 cells were calculated and

plotted in Figure 5(C). The relative apoptosis ratio was about 3.5 for CRL-5802 cells and about 1.5 for

H1299 cells. These results demonstrated the micelle/CPT has significantly higher cell-killing efficiency

in CRL-5802 cells than in H1299 cells and using the micelle/CPT is more potent than using CPT

against CRL-5802 cells.

Evaluation of γ-H2AX

The nucleus contains genomic materials (DNA and RNA), regulates the gene expression and controls

the replication of DNA during the cell cycle. Hence, damaging the DNA in the nucleus of cancer cells

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would lead to inhibition of rapid cellular division. The most common assay of DNA fragmentation

relies on labeling DNA double-strand breaks (DSBs) with fluorochrome-tagged deoxynucleotides. The

induction of DSBs by genotoxic agents provides a signal for histone H2AX phosphorylation on Ser[139];

the phosphorylated H2AX is named γ-H2AX. Hence, γ-H2AX has evolved as one of the important

biomarkers for observing DNA damage.[37]

To understand the effect of CPT or micelle/CPT on the nucleus of CRL-5802 and H1299 cells, the

cells were treated with the equivalent CPT concentration of 0.1 μg/mL for 2 hours. The cells were

stained with green fluorescent Alexa fluor 488 antibody specific for γ-H2AX and the expression of

γ-H2AX was determined using flow cytometry, western blot analysis, and CLSM of cells exposed to

H-CP, CPT, or micelle/CPT. As seen in the flow cytometric diagrams, the expression of γ-H2AX was

profoundly high in both cells exposed to CPT and micelle/CPT as compared with the control group and

H-CP-treated group (Figure 6A&B). In CRL-5802 cells, it was much clearer that micelle/CPT-treated

cells induced a significantly higher level of γ-H2AX expression than CPT-treated cells. When the

expression of γ-H2AX in micelle/CPT-treated cells was quantified, around 3-fold increase in γ-H2AX

expression could be found in CRL-5802 as compared with that in H1299 cells (Figure 6B). Similar

result was obtained from the western blot image (Figure 6C), illustrating a higher γ-H2AX expression

in CRL-5802 cells than in H1299 cells. Table 1 summarizes the *in vitro* anticancer superiority of

CRL-5802 cells over that of H1299 cells when the cells were exposed to micelle/CPT.

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Figure 7 shows the expression of γ-H2AX evaluated by CLSM. As can be seen, invisible γ-H2AX

expression was found in the nuclei of untreated CRL-5802 and H1299 cells, slightly visible expression

in cells treated with empty H-CP, and highly visible expression in cells treated with CPT and

micelle/CPT. Both CPT and micelle/CPT had higher ability in causing DNA damage in CRL-5802

cells than in H1299 cells because the higher intensity of green fluorescence indicates higher expression

of γ-H2AX in the nucleus.

In vivo biodistribution

To evaluate the *in vivo* accumulation of micelles in CRL-5802 and H1299 xenografted tumors, a

near-IR dye, IR780, was encapsulated into micelles. The biodistribution of micelle/IR780 was

monitored by non-invasive fluorescence in tumor-bearing mice with the tumor grafted on the right

(H1299) and left (CRL-5802) hind legs. After tumors grew to approximately 150 mm^3 in volume, the

micelle/IR780 was intravenously injected via the lateral tail vein into tumor-bearing mice.[14] Following

the injection, the micelle/IR780 was imaged at different time points as shown in Figure 8(A). The

fluorescence intensity of IR780 at both tumor sites increased with increasing circulation time. It was

clear the fluorescence intensity is always higher on the CRL-5802 tumor than on the H1299 tumor in

mice if compared at the same time points. To confirm whether the higher accumulation of the

micelle/IR780 on the CRL-5802 tumor was due to the different tumor sizes, tumors were isolated after

48 hours. The image of Figure 8(B) illustrates clearly tumors of similar sizes. The relevant organs,

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tissues, and tumors were also dissected from mice at 48 hours after instillation and optically imaged

immediately to determine the remaining fluorescence intensity. In Figure 8(C), high IR780

fluorescence intensities were observed at both tumor sites because of enhanced permeation and

retention (EPR) effect.[38] The optical imaging intensity of the CRL-5802 tumor was significantly higher

than that of the H1299 tumor (Figure 8D), indicating that the CLR-5802 tumor indeed had higher

ability in internalization of the micelle. To evaluate the safety of the H-CP micelle *in vivo*, the heart,

liver, and kidney were harvested for histopathological examination with Hematoxylin & Eosin (H&E)

staining as well. Neither noticeable organ damage nor inflammation was observed in the micelle/IR780

group compared with the saline group (Figure 8E).

Conclusions

This study demonstrated why lower CD44-expressing CRL-5802 cells caused higher DNA damage and

higher cellular uptake as compared with higher CD44-expressing H1299 cells when exposed to CPT or

micelle/CPT. Endocytic chemical inhibitors were utilized to examine the internalization pathways of

the micelle/CPT into the cells. CRL-5802 cells involved CD44-, clathrin-, and caveolae-mediated

pathways, while H1299 cells involved only CD44- and caveolae-mediated pathways. Using CME

seems to be superior because micelles encapsulated with endosomes could reach the acidic lysosomes,

where the abundance of enzymes helps degrade the H-CP micelle and accelerate the release of CPT.

From the cell cycle analysis of CPT-treated cells, CRL-5802 cells arrested mainly in the S phase and

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H1299 cells, mainly in the G_2-M phase. The qualitative and/or quantitative evaluation of γ-H2AX

expression, a biomarker for DNA damage, was obtained from flow cytometry, CLSM, and western blot

studies. All results demonstrated that CRL-5802 cells had higher γ-H2AX expression than H1299 cells.

The *in vivo* biodistribution of micelle/IR780 in tumor-bearing mice showed that the CRL-5802 tumor

had higher fluorescence intensity than the H1299 tumor. It can be concluded that the therapeutic

outcome of a drug does not correlate only with the amount of CD44 on the surface of cancer cells. The

cells used multiple internalization pathways and arrested in the S phase as demonstrated in CRL5802

cells, showing a higher ability in causing cell death as compared with H1299 cells.

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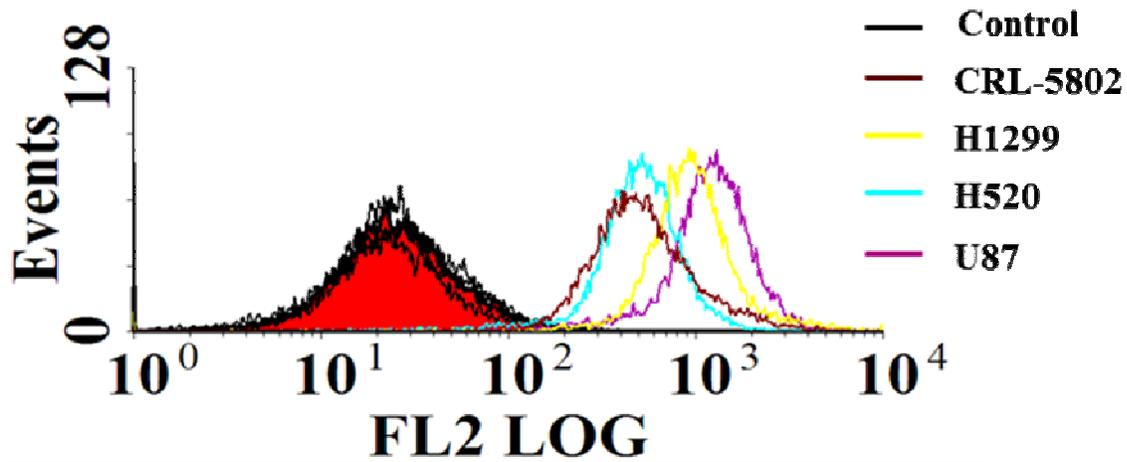
Caption Legends

Fig. 1. Fluorescent intensities of CD44 expression in non-small cell lung cancer cell lines and U87 cells (a human glioblastoma cell line).
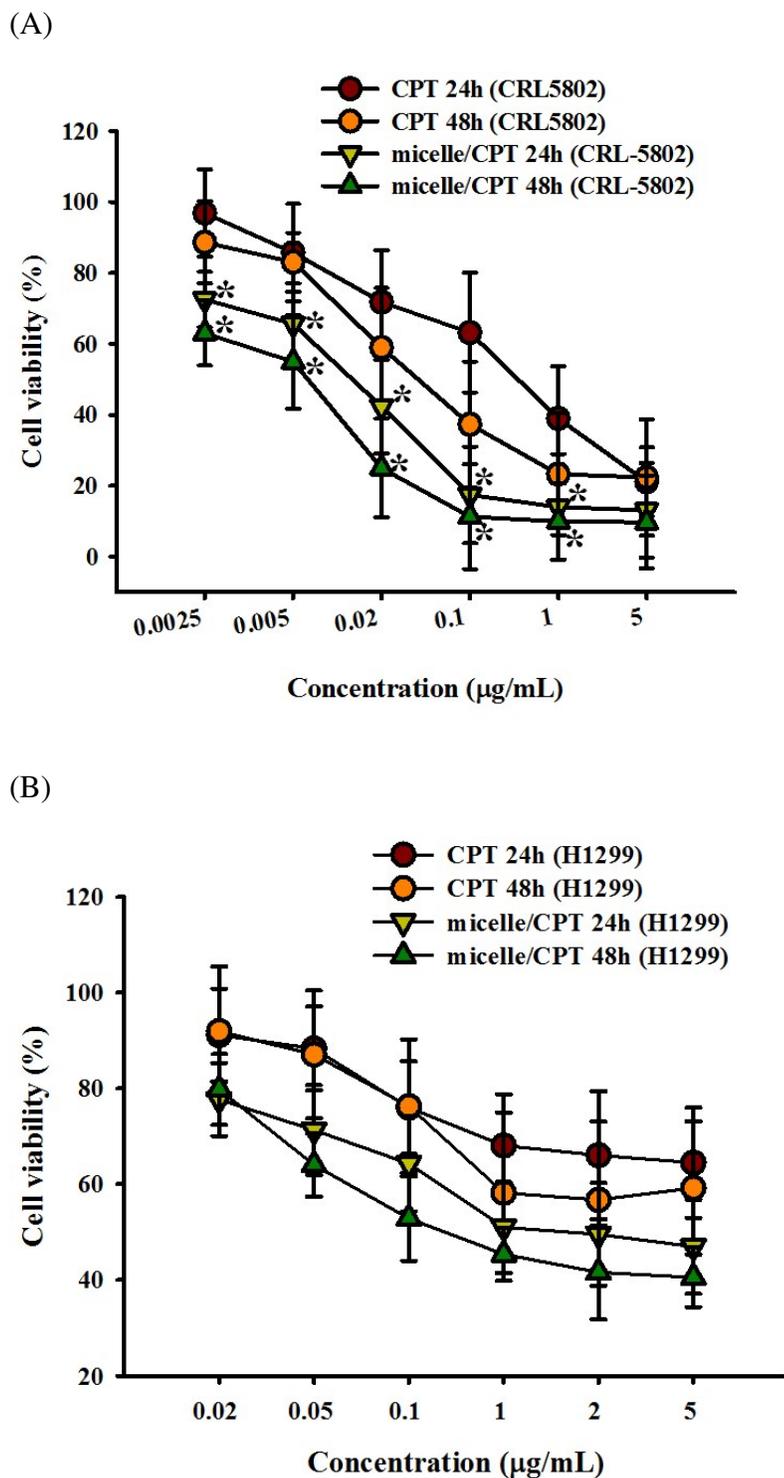
Fig. 2. Cell viability tests of CPT and CPT-loaded micelle (micelle/CPT) against (A) CRL-5802 cells and (B) H1299 cells for 24 and 48 hours of incubation using an MTT assay.
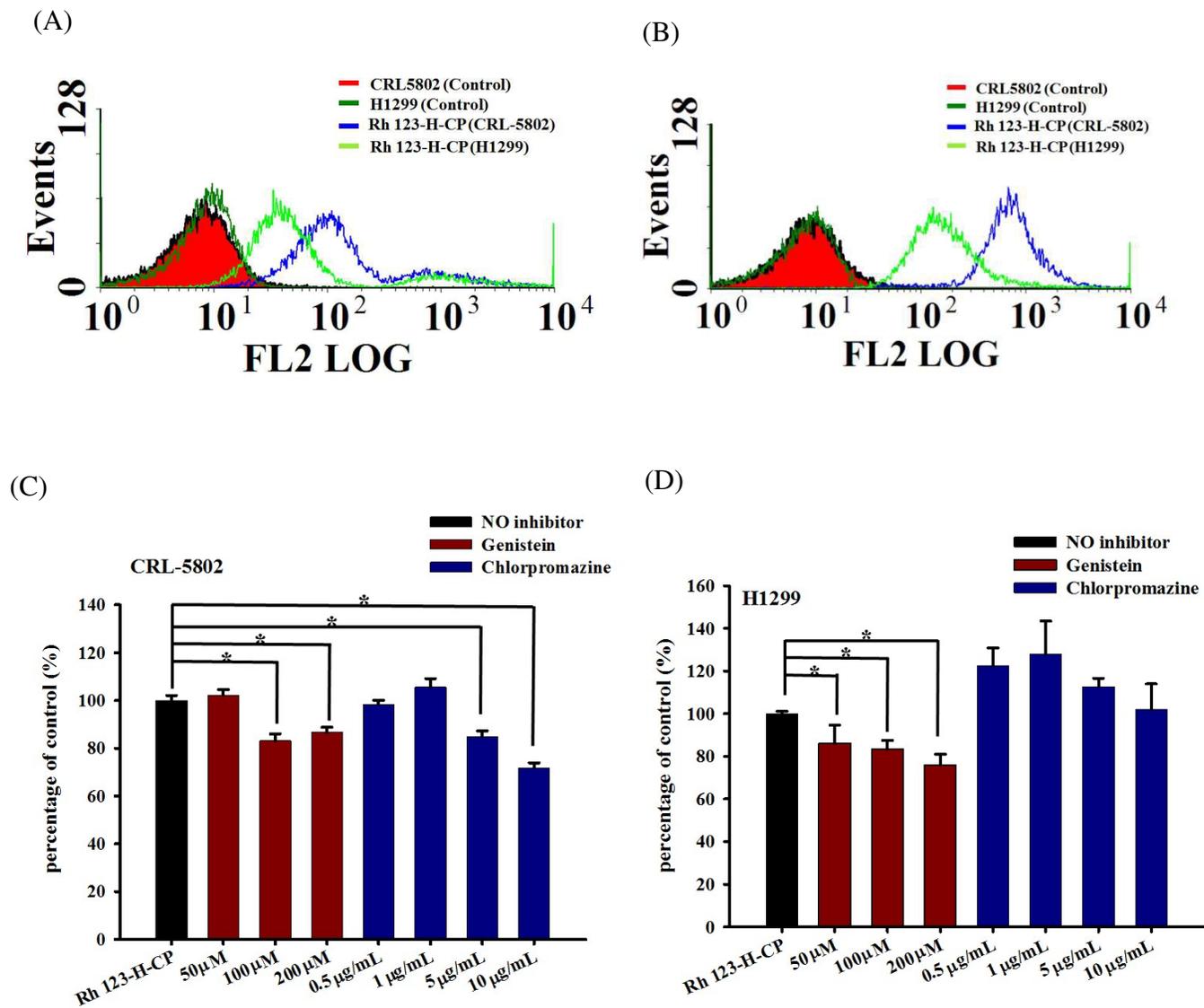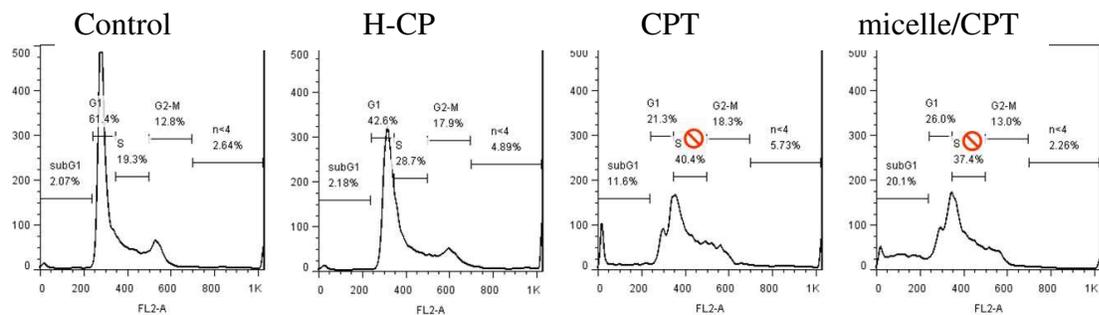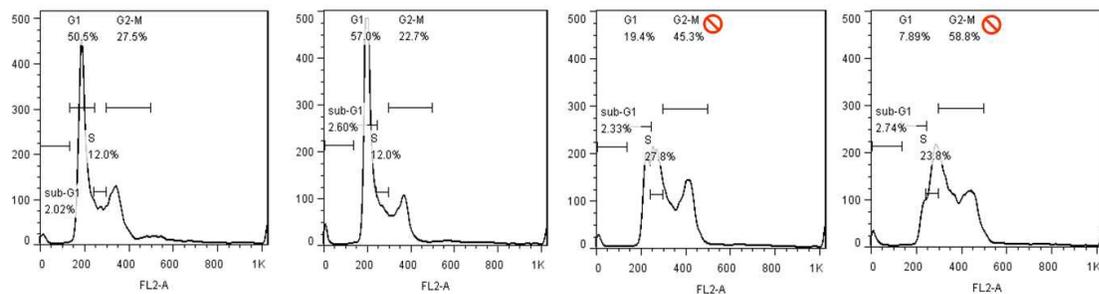
Fig. 3. Flow cytometric histograms of CRL-5802 and H1299 cells exposed to Rh123-linked micelle for (A) 30 minutes and (B) 2 hours of incubation. The inhibition of intracellular uptake in (C) CRL-5802 cells and (D) H1299 cells pretreated with dose-dependent chlorpromazine and genistein inhibitors for 30 minutes before the cells were exposed to the micelle for 2 hours of incubation.

Fig. 4. Cell cycle analysis of H-CP, CPT, and micelle/CPT against (A) CRL-5802 cells and (B) H1299 cells for 24 hours of incubation using the equivalent CPT concentration of 0.1 µg/mL. (C) The protein-expression amounts of cell cycle modulators p27, CDK2 and cyclin A, and pro-survival protein XIAP. The results of western blot analysis showed that the micelle/CPT causes a dramatically decreased level of p27 protein in CRL-5802 cells as compared with H1299 cells. β-Actin was used as an internal control for equal loading.

Fig. 5. Induction of apoptosis in CRL-5802 and H1299 cells exposed to H-CP micelle, CPT, and micelle/CPT for 24 hours of incubation at 37 ^{o}C using the equivalent CPT concentration of 0.1µg/mL. (A) the annexin-V/PI dual staining assay, (B) the percentage of apoptosis cells calculated from (A). (C) The relative apoptosis ratios between cells treated with the micelle/CPT and empty H-CP (n=3, *P< 0.05).

Fig. 6. Formation of γ-H2AX in (a) CRL-5802 cells and (b) H1299 cells exposed to micelle, free CPT, and micelle/CPT for 2 hours of incubation using the equivalent CPT concentration of 0.1µg/mL. (A) The expression of γ-H2AX was analyzed by flow cytometry, (B) plotted in percentage of γ-H2AX-positive cells, and (C) done by western blotting analysis (n=3, *P< 0.05). β-Actin was used as an internal control for equal loading.

Fig. 7. CLSM images of γ-H2AX in CRL-5802 and H1299 exposed to micelle, free CPT, and micelle/CPT for 2 hours of incubation using the equivalent CPT concentration of 0.1µg/mL. The cells nuclei were stained with DAPI in blue and γ-H2AX was stained with p-histone H2AX (Ser 139) monoclonal primary antibody and Alexa Fluor 488-tagged secondary antibody. The formation of γ-H2AX foci was indicated in green fluorescence.

Fig. 8. (A) The optical images of CLR-5802 and H1299 tumors xenografted in male Balb/c mice (~ 6 weeks old, n=3 labeled as #1, 2, 3) using a near-infrared non-invasive optical imaging technique. (B) The tumors isolated to validate similar sizes. (C) The fluorescent images and (D) relative fluorescence intensities of isolated tissues after mice were injected with the IR-780-loaded micelle (micelle/IR 780)

for 48 hours. (E) HE analysis of the effect of the micelle on heart, liver, and kidney. (The equivalent concentration of IR780 is 1.75 mg/kg using EX 745 nm and EM 840 nm).

Table 1. The summary of the *in vitro* data obtained from the study in two cell lines

		CRL-5802	H1299
CD 44 expression		Low	High
IC_{50}	24 hr	0.01 µg/mL	1.36 µg/mL
(micelle/CPT)	48 hr	0.004 µg/mL	0.15 µg/mL
Endocytosis pathways		clathrin- and caveolae-	caveolae-
Cell cycle		S phase	G_2-M phase
Cell apoptosis (micelle/CPT)		33.06 ± 2.49 %	14.14 ± 2.68 %
γ-H2AX (micelle/CPT)		High	Low

Fig. 1. Fluorescent intensities of CD44 expression in non-small cell lung cancer cell lines and U87 cells (a human glioblastoma cell line).

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(A)

(B)

Fig. 2. Cell viability tests of CPT and CPT-loaded micelle (micelle/CPT) against (A) CRL-5802 cells and (B) H1299 cells for 24 and 48 hours of incubation using an MTT assay.

(A)

(B)

(C)

(D)

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(A)   **CRL-5802**

(B)   **H1299**

(C)

Fig. 4. Cell cycle analysis of H-CP, CPT, and micelle/CPT against (A) CRL-5802 cells and (B) H1299 cells for 24 hours of incubation using the equivalent CPT concentration of 0.1 μg/mL. (C) The protein-expression amounts of cell cycle modulators p27, CDK2 and cyclin A, and pro-survival protein XIAP. The results of western blot analysis showed that the micelle/CPT causes a dramatically decreased level of p27 protein in CRL-5802 cells as compared with H1299 cells. β-Actin was used as an internal control for equal loading.

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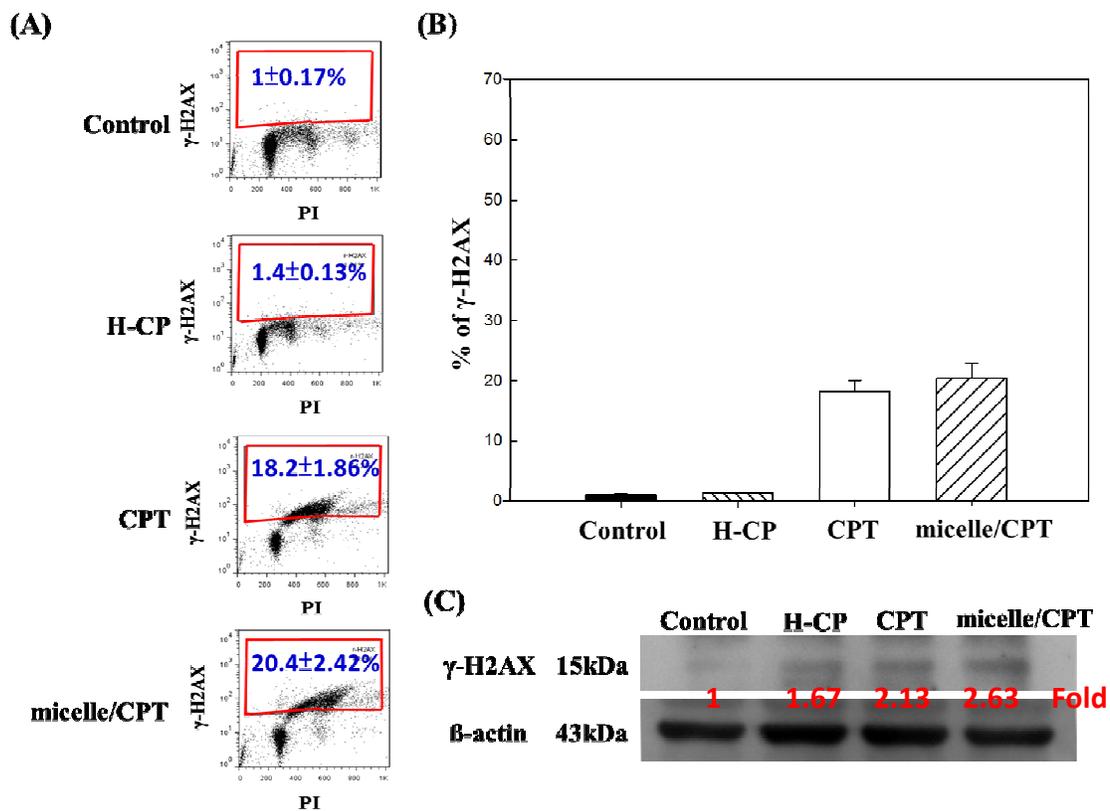
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(a)   **CRL-5802**

(b)   **H1299**

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Fig. 6. Formation of ɤ-H2AX in (a) CRL-5802 cells and (b) H1299 cells exposed to micelle, free CPT, and micelle/CPT for 2 hours of incubation using the equivalent CPT concentration of 0.1µg/mL. (A) The expression of ɤ-H2AX was analyzed by flow cytometry, (B) plotted in percentage of ɤ-H2AX-positive cells, and (C) done by western blotting analysis (n=3, *P< 0.05). β-Actin was used as an internal control for equal loading.
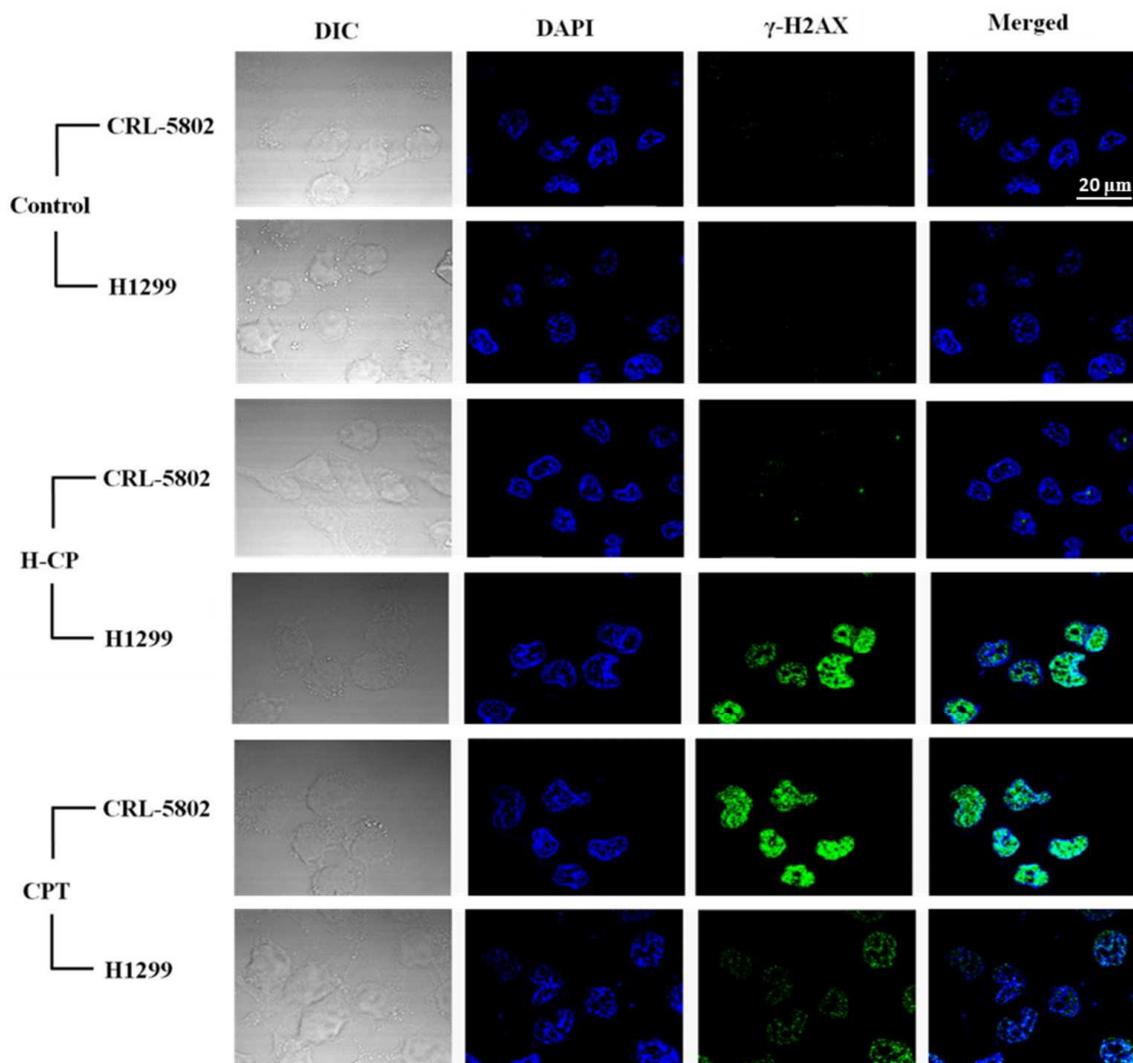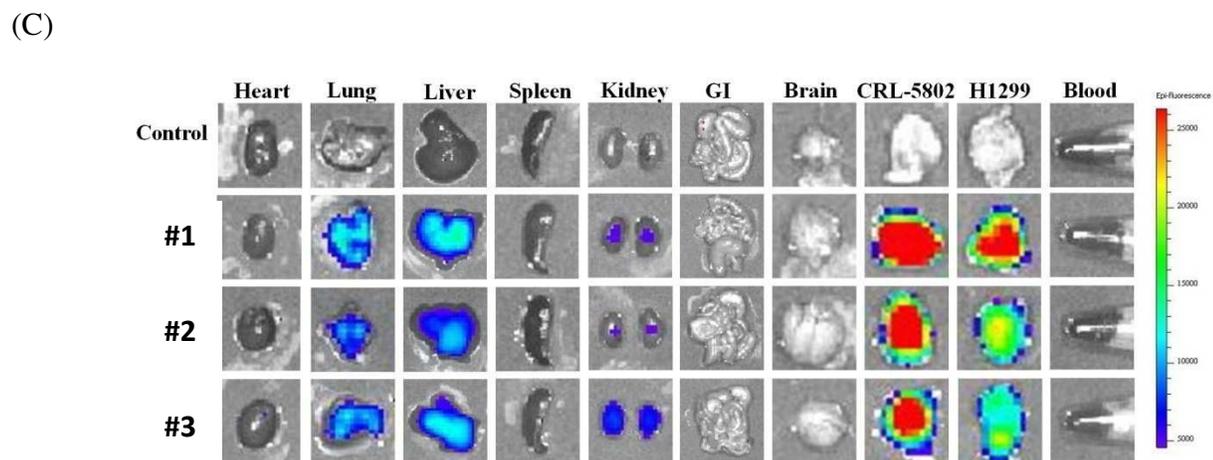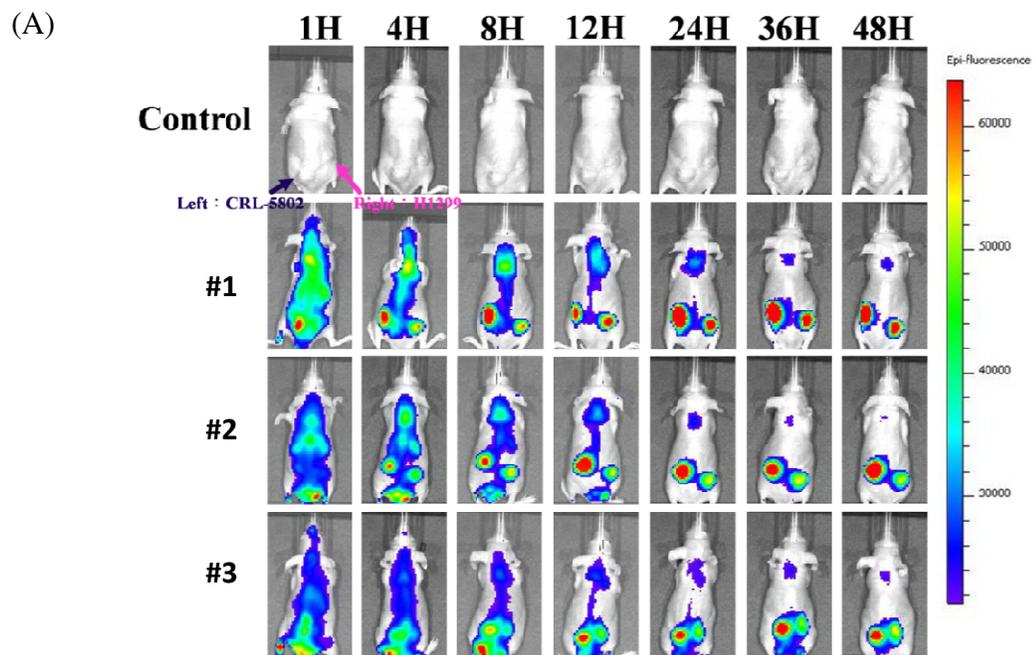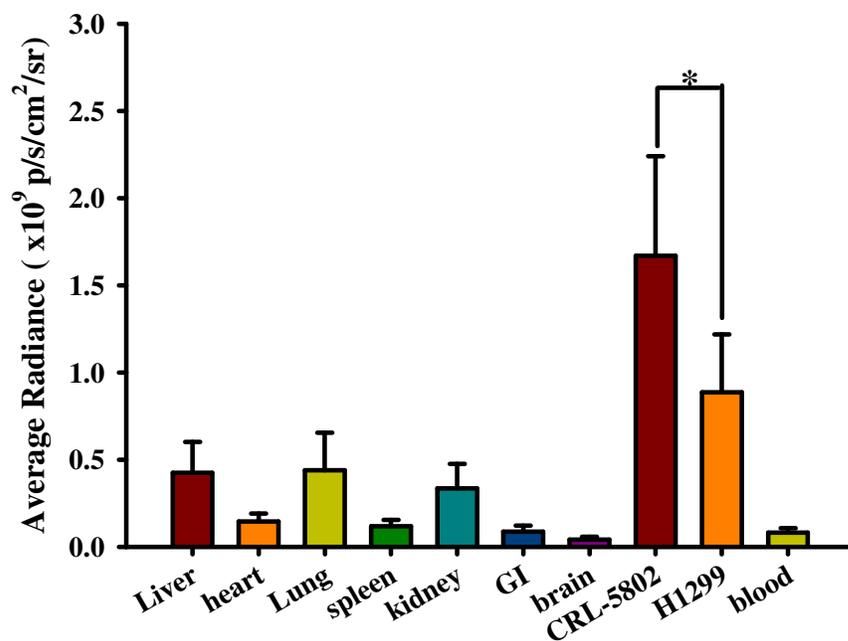
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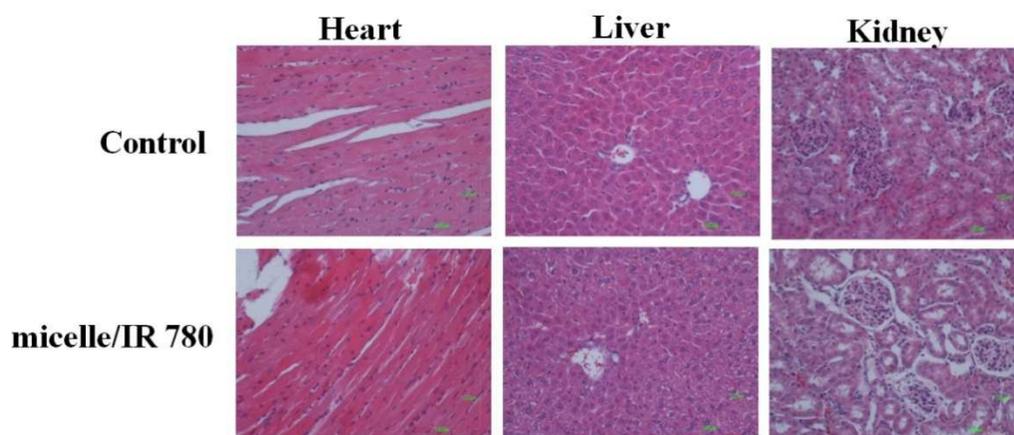
(A)

(B)

(C)

(D)

(E)

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