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Chemical Antagonism between Photodynamic Agent and Chemo-therapeutics: Mechanism and Avoidance

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Content

Materials and methods

Materials

Chlorin e6 trimethyl ester was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DPPC was purchased from Avanti Polar Lipids, Inc. (Alabama, USA). Curcumin (>98%), coumarin 6 (>99%), docetaxel (>98%), vinorelbine bitartrate (>98%) and rapamycin (>98%) were purchased from J&K Chemical Co., Ltd (Shanghai, China). Mertansine (>95%) was purchased from BrightGeneBioMedical Technology Co., Ltd (Suzhou, China). Vinorelbine was precipitated from vinorelbine bitartrate solution via adjusting its pH to 8.0 with ammonia (5 M). Mertansine-(pyridine-2-yldisulfanyl) was prepared by reacting mertansine with an excess amount of 2,2-dipyridyl disulfide in methanol and purified by silicon chromatography with >98% purity. Cell Counting Kit (CCK-8) was obtained from Dojindo China Co., Ltd. (Shanghai, China). Singlet Oxygen Sensor Green (SOSG) was obtained from ThermoFisher Scientific (Waltham, USA). Other organic solvents were of analytical grade and were used as received.

Cell lines Human breast adenocarcinoma cell line MCF-7 and human lung adenocarcinoma epithelial cell line A549 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human colorectal adenocarcinoma cell line HT29, mouse melanoma cell line B16-F10 and multidrug resistant cell line MCF-7/ADR and A549T were purchased from Cell Bank of Shanghai, Chinese Academy of Sciences (CAS, Shanghai, China). A549 and MCF-7 cells and their drug resistant strains were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics (Invitrogen). HT29 and B16-F10 cells were cultured in McCoy's 5a and DMEM medium supplemented with 10% fetal bovine serum and 1% antibiotics, respectively. These cells were maintained in a cell incubator under a humidified atmosphere containing 5% CO_2 at 37 ℃.

Methods

Preparation of liposomes All liposomes were prepared by film hydration and membrane extrusion method. Generally, DPPC and other ingredients were dissolved in a mixture of methanol and dichloromethane (10:1, v/v) in a 250 mL flask. A thin film was formed by removing the solvent using rotavapor and then hydrated with Milli-Q water at 45 ℃. The resulting suspension was extruded sequentially through polycarbonate membranes of 200, 100, and 50 nm in pore size with an extruder (Liposo Fast LF1, Avestin, Ottawa, Canada) according to the reported method,[1] and then purified by ProElut PLS column (Dikma Technologies Inc, Beijing, China). A typical formulation for dual-drug

loaded liposomes (DL-X) contains DPPC, Ce6tM and anti-cancer drug (102:1:0.5, mol/mol). Either Ce6tM or anti-cancer drug would be excluded from the formulation during the preparation of corresponding mono-drug loaded liposomes (ML-X). The amount of anti-cancer drugs would be lowered to one-third of the typical formulation in some cases. ML-X and ML-Ce6tM with DPPC-to-Ce6tM/drug ratio at 68:1 were prepared to obtain a physical mixture of liposomes with either Ce6tM or drugs. The hydrodynamic sizes and ζ-potentials of the prepared liposomes in PBS (pH 7.4) were determined with Zetasizer (Nano ZS90, Malvern, UK). The drug loading and drug encapsulation efficiency of all liposomes were determined using HPLC.

Laser irradiation A 671 nm laser with adjustable power was used to irradiate the liposomes. The power and duration of irradiation, the concentration of sodium azide, and the concentration of solution were varied to investigate their effect on drug decomposition. All the samples were placed in cylindrical glass vials incubated in a water bath of 20 ℃. Whenever necessary, the irradiated samples were lyophilized before HPLC analysis.

Cryo-TEM imaging Cryo-TEM imaging was performed on FEI TF20 Transmission Electron Microscope. All the samples were imaged at 10 mg/mL in DPPC. Three-microliter of the sample solution was deposited on holey carbon grid (BQR2/1 200 mesh, Quantifoil, Germany) pretreated with plasma air. A thin film of the sample solution was produced using Vitrobot (FEI, USA) equipped with a controlled humidity chamber via blotting away excess sample with preset parameters and then plunged instantly into a liquid ethane. The vitrified samples were then transferred to a cryo-holder and cryo-transfer stage that was cooled by liquid nitrogen. To prevent sublimation of vitreous water, the cryo-holder temperature was maintained below -170 °C during the imaging process.

Confocal microscopy Fluoview FV1000 (Olympus, Japan) Confocal Laser Scanning Microscope was used to image the deposition of fluorescent molecules in liposomes. The liposomes for confocal microscopy imaging were not extruded and thus were of suitable size for imaging. ECFP (Ex/Em = 440/476 nm), FITC (Ex/Em = 488/519 nm), and Qdot655 (Ex/Em = 405/655 nm) filters were chosen to visualize curcumin, coumarin 6, and Ce6tM, respectively. Coumarin 6 was chosen as a tracer of the anti-cancer drugs because of its hydrophobicity (log P = 4.53) and high quantum yield.[2]

High-performance liquid chromatography The contents of curcumin, docetaxel, mertansine, vinorelbine, rapamycin, and Ce6tM in liposomes were measured using ultra-performance liquid chromatography system (Acquity H-Class, Waters, USA) equipped with Acquity UPLC BEH C18 column (2.1 × 150 mm,

1.7 μm, Waters) under 30 ℃. The flow rate of elution solvent was 0.3 mL/min. All the samples were diluted with methanol to disrupt the liposomes. Curcumin was monitored at 420 nm, and eluted with a mixture of water and acetonitrile with the following gradient: 60% acetonitrile (0-1 min), 60%-99% acetonitrile (1-4 min), 99% acetonitrile (4-7 min), 99%-60% acetonitrile (7-7.1 min), and 60% acetonitrile (7.1-10 min). Docetaxel was monitored at 227 nm, and eluted with mixture of water and acetonitrile with following gradient: 45% acetonitrile (0-0.5 min), 45%-99% acetonitrile (0.5-4.5 min), 99% acetonitrile (4.5-8.5 min), 99%-45% acetonitrile (8.5-9 min), and 45% acetonitrile (9-11 min). Mertansine was monitored at 232 nm, and eluted with a mixture of water and acetonitrile with the following gradient: 55% acetonitrile (0-1 min), 55%-99% acetonitrile (1-4 min), 99% acetonitrile (4-9.2 min), 99%-55% acetonitrile (9.2-9.3 min), 55% acetonitrile (9.3-12 min). Vinorelbine was monitored at 268 nm, and eluted with mixture of 20 mM pH 4.0 aqueous phosphate buffer and acetonitrile with following gradient: 41% acetonitrile (0-4 min), 41%-99% acetonitrile (4-5 min), 99% acetonitrile (5-11 min), 99%-41% acetonitrile (11-11.1 min), and 41% acetonitrile (11.1-13 min). Rapamycin was monitored at 277 nm, and eluted with a mixture of water and acetonitrile with the following gradient: 70% acetonitrile (0-1 min), 70%-99% acetonitrile (1-5 min), 99% acetonitrile (5-8 min), 99%-70% acetonitrile (8-8.1 nm), and 70% acetonitrile (8.1-11 min). Ce6tM was monitored at 403 nm, and eluted with a mixture of water and acetonitrile with the following gradient: 55% acetonitrile (0-1 min), 55%-99% acetonitrile (1-4 min), 99% acetonitrile (4-9.2 min), 99%-55% acetonitrile (9.2-9.3 min), and 55% acetonitrile (9.3-12 min).

Liquid chromatography-mass spectrometry DL-X with or without irradiation were analyzed with LC-MS system (Agilent 1260 HPLC coupled to Agilent 6460 Triple Quad instrument, Agilent Technologies, USA) to detect the oxidized derivatives of anti-cancer drugs. Samples were disrupted with methanol before analysis. The chemicals were firstly separated on Acquity UPLC BEH C18 (2.1 × 150 mm, 1.7 μm, Waters) using mixture of water and acetonitrile with the following gradient (0.3 mL/min, 30 ℃): 30% acetonitrile (0-1 min), 30%-99% acetonitrile (1-10 min), 99% acetonitrile (10-33 min), 99%-30% acetonitrile (33-33.1 min), and 30% acetonitrile (33.1-40 min). The chemicals with m/z between 200 and 1800 were collected and analyzed using electrospray ionization mode with capillary voltage setting to 4 kV. The gas temperature was 300 ℃ with a flow rate of 5 L/min, and the sheath gas temperature was 350 ℃ with a flow rate of 11 L/min. Species of specific m/z were extracted in MassHunter Quantitative Analysis (version B.04.00, Agilent Technologies) to give chromatography. In this experiment, mertansine-Pyr was used instead of mertansine, because the latter is prone to form dimer which would

complicate data analysis and the formation of dimer would not lead to activity loss of the drug. To further correlate changes between the mass of molecules with their formulas, DL-X with irradiation were analyzed with LC/Q-TOF-MS/MS system (Agilent 1260 HPLC coupled to Agilent 6530 accurate-mass quadrupole-time-of-flight instrument, Agilent Technologies, USA). The chemicals were firstly separated on Agilent Poroshell C18 column (2.1 × 100 mm, 2.7 μm, Agilent) using a mixture of water and acetonitrile with the following gradient (0.35 mL/min, 30 ℃): 30% acetonitrile (0-1 min), 30%-100% acetonitrile (1-7 min), 100% acetonitrile (7-35 min), 100%-30% acetonitrile (35-36 min), and 30% acetonitrile (36-40 min). The parent ions with m/z between 200 and 1700 were collected and analyzed using electrospray ionization mode with capillary voltage setting to 4 kV. The gas temperature was 300 ℃ with a flow rate of 8 L/min, and the sheath gas temperature was 350 ℃. Sheath gas flow was 11 L/min. Fragmentor voltage was set to 175 V. The collision energy was set to 45 V. Data were processed using Agilent Q-TOF LC/MS MassHunter Acquisition Software (version B.06.00, Agilent Technologies).

Singlet oxygen detection SOSG was used to monitor $^1O_2$ generated by Ce6tM upon irradiation, based on its capability to emit green fluorescence after reacting with $^1O_2$. The fluorescence from activated SOSG was imaged qualitatively using ChemiDoc imaging system (ChemiDoc XRS+, BioRad, USA), and was determined quantitatively using microplate reader (Enspire, PerkinElmer, Singapore) at Ex/Em 504/525 nm. The DL-SOSG was prepared from a mixture of DPPC, Ce6tM and SOSG at 102:0.03:2 in molar. To determine whether anti-cancer drug could quench $^1O_2$ or not, liposomes loaded with Ce6tM, anti-cancer drug and SOSG was prepared at a molar ratio of 475:1:6:0.75. In some cases, 30 mM sodium azide was added to quench the generated $^1O_2$.

Drug release The release of encapsulated drugs and Ce6tM from the liposomes was carried out by incubating ML-X or DL-X in RPMI 1640 containing 10% FBS. Samples were collected at 2 and 8 h after incubation and eluted through ProElut PLS column to remove salts, biomolecules, and intact liposomes. The released drugs and Ce6tM were then flushed out with methanol, and their contents were determined using HPLC.

Cell viability assay A549, B16-F10, HT29 and MCF-7 cells were seeded into 96-well plate at 4×10^3 cells/well and allowed to attach for 24 h. The medium was then replaced with fresh medium containing anti-cancer drug in different formulations (with or without 2 min irradiation with 671 nm laser at 2 W/cm^2) at predetermined concentrations (Vinorelbine: 0.5 ng/mL − 30 μg/mL; Mertansine: 0.3 ng/mL − 20 μg/mL; Rapamycin: 1 ng/mL − 40 μg/mL; Curcumin: 1 ng/mL − 50 μg/mL) and incubated for another

48 h. Cell viabilities were determined by CCK-8 kit according to the manufacturer's protocol using a microplate reader (Enspire, PerkinElmer, Singapore). IC_{50} was defined as the concentration at which only 50% of cells survived when compared with control group. To investigate the synergistic effect between vinorelbine and Ce63tM, A549, MCF-7 and their drug resistant strains A549T and MCF-7/ADR were used. The sensitivity of the cells to vinorelbine was first confirmed. A549T and MCF-7/ADR cells were seeded into 96-well plate at 4×10^3 cells/well and allowed to attach for 24 h. The medium was then replaced with fresh medium containing anti-cancer drug in different formulations (with or without 2 min irradiation with 671 nm laser at 2 W/cm^2) at predetermined concentrations (Vinorelbine: 0.5 ng/mL − 30 μg/mL) and incubated for another 48 h. Cell viabilities were determined by CCK-8 kit according to the manufacturer's protocol using a microplate reader (Enspire, PerkinElmer, Singapore). IC_{50} was defined as the concentration at which only 50% of cells survived when compared with control group. To investigate the synergistic effect , cells were seeded into 96-well plate at 4×10^3 cells/well and allowed to attach for 24 h. The medium was then replaced with fresh medium containing the vinorelbine in different formulations at predetermined concentrations (0.01 ng/mL – 0.1 μg/mL). After 4 h incubation, the cells were irradiated with 671 nm laser (0.625 W/cm^2, 20 s). Then the cells were further incubated for another 48 h, and their viabilities were determined by CCK-8 kit. IC_{50} was defined as the concentration at which only 50% of cells survived when compared with control group.

Estimation of inter-liposome distance The inter-liposome distance (l) in a solution of liposome could be roughly estimated based on the assumption that each liposome occupies a cubic whose side length is l. The amount of DPPC (m) and the volume of the solution (V) could be defined as equations **1** and **2**, based on which the concentration of solution could be expressed as equation **3**. The inter-liposome distance−which equals to the side length l−could be calculated using equation **4**. In equation 4, ρ is the density of DPPC (1.05g/cm^3); r is the radius of the liposome (35 nm); d is the thickness of lipid bilayer (5 nm); and c is the concentration of DPPC in the solution. The inter-liposome distances l are 488 and 157 nm when the concentration of DPPC in solution (c) are 0.6 and 18 mg/mL.

$$m = \frac{4}{3}n\pi\rho\left[r^3 - (r-d)^3\right] \quad \textbf{1}$$

$$V = nl^3 \quad \textbf{2}$$

$$c = \frac{m}{V} = \frac{4\pi\rho\left[r^3 - (r-d)^3\right]}{3l^3} \quad \textbf{3}$$

$$l = \sqrt[3]{\frac{4\pi\rho\left[r^3 - (r-d)^3\right]}{3c}} \quad \textbf{4}$$

Estimation of the mean first collision time The mean time required for two liposomes under a Brownian motion to have the first collision (mean first collision time) was estimated by simulating and monitoring the positions of 15625 liposomes. Generally, increasing the number of simulated liposomes will decrease the value of mean first collision time. If we increase the number of the liposome to infinity (it is computational impossible though), the mean first collision time will converge to a limiting value corresponding to the value in the real world experiment. The current number 15625 is chosen such that further doubling the number will not decrease the mean first collision time by the current standard deviation. The initial distance between two closely positioned liposomes was set to 488 nm and 157 nm. The integration time steps were Δt = 10 μs and 1 μs respectively. The random displacement Δs in Δt in each of x, y and z direction of a liposome undergoing Brownian motion has a zero mean and standard deviation given by equation **5**. D in equation **5** is the Diffusion coefficient defined in equation **6**. All the liposomes moved Δs towards a random direction from their original positions in Δt. The distance between any two liposomes (d_{lipo}) was then calculated and compared with a cutoff distance (d_{cutoff} = 2r + 20 nm). If the d_{lipo} are larger than d_{cutoff}, the liposomes are allowed to move for another Δt until d_{lipo} is equal or smaller than d_{cutoff}. The number of steps that took for the system to reach this status is then recorded. In equation **6**, κ is the Boltzmann constant ($1.38 \times 10^{-23} m^2 \cdot kg \cdot s^{-2} \cdot K^{-1}$); T is the thermodynamic temperature of solution (293 K); η is the dynamic viscosity of the solvent ($1 \times 10^{-3} Pa \cdot s$); and r is the radius of the liposome (35 nm).

$$std(\Delta s) = \sqrt[2]{2D\Delta t} \quad \textbf{5}$$

$$D = \frac{\kappa T}{6\pi\eta r} \quad \textbf{6}$$

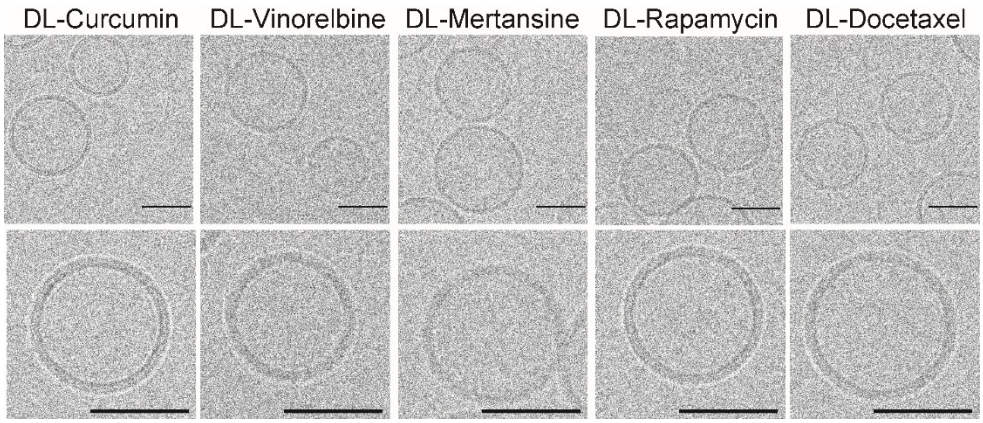

Figure S1. Representative cryo-TEM images of DL-X. The circles with higher electron density (darker than background) are lipid bilayer formed by DPPC with a thickness of about 5 nm. The scale bars represented a distance of 50 nm.

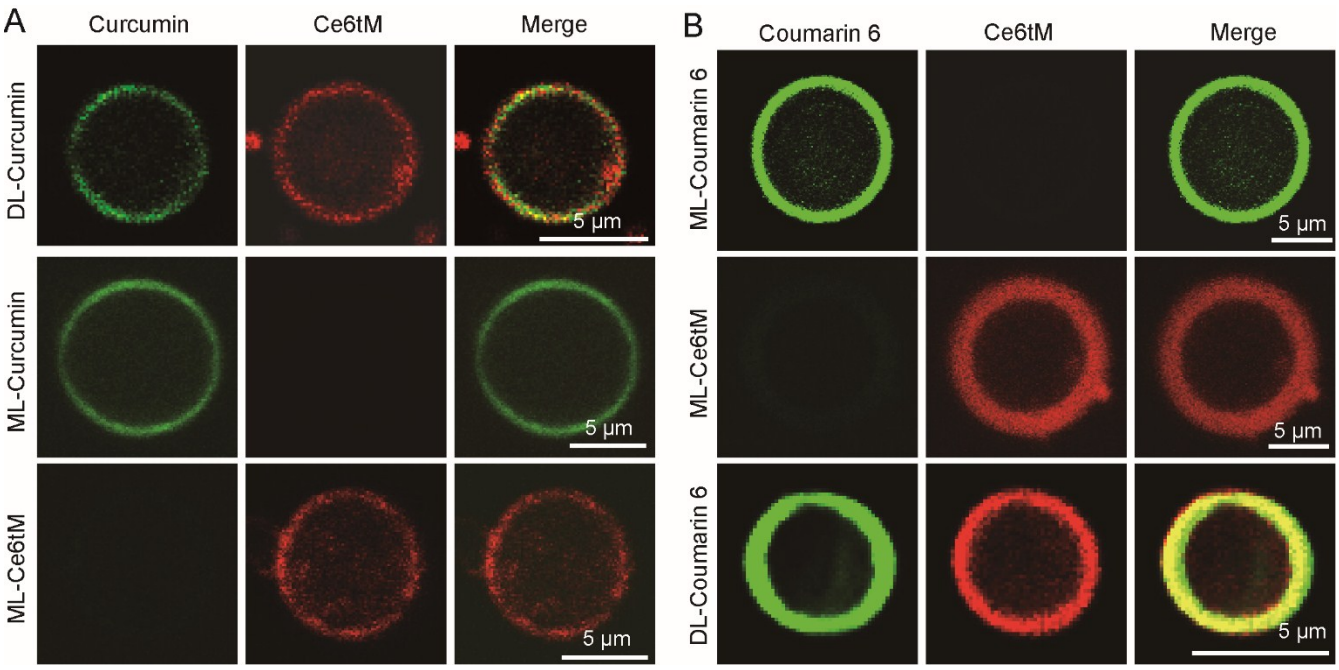

Figure S2. Confocal microscopic images of liposomes. (A)) Confocal microscopic images of the prepared DL-Curcumin, ML-Curcumin, and ML-Ce6tM. (B) Confocal microscopic images of the prepared ML-Coumarin 6, ML-Ce6tM and DL-Coumarin 6. Coumarin 6 here was used as a location indicator of non-fluorescent drugs including docetaxel, maytansine, vinorelbine, and rapamycin. To be captured with a confocal microscope, the liposomes were not extruded through a polycarbonate membrane (pore size = 50 nm) and thus are of a size in micrometer scale.

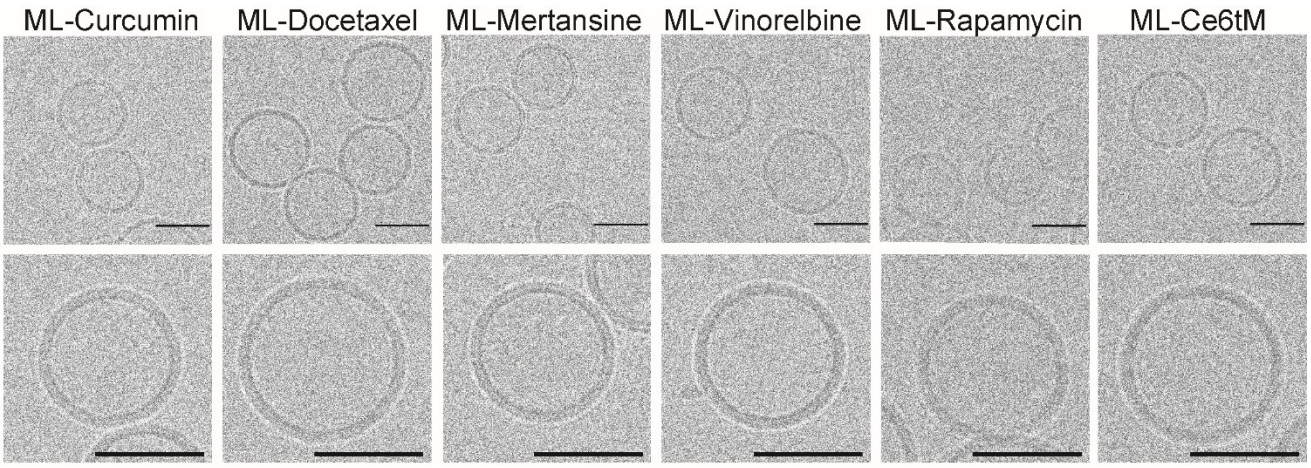

Figure S3. Representative cryo-TEM images of ML-X. The circles with higher electron density (darker than background) are lipid bilayer formed by DPPC with a thickness of about 5 nm. The scale bars represented a distance of 50 nm.

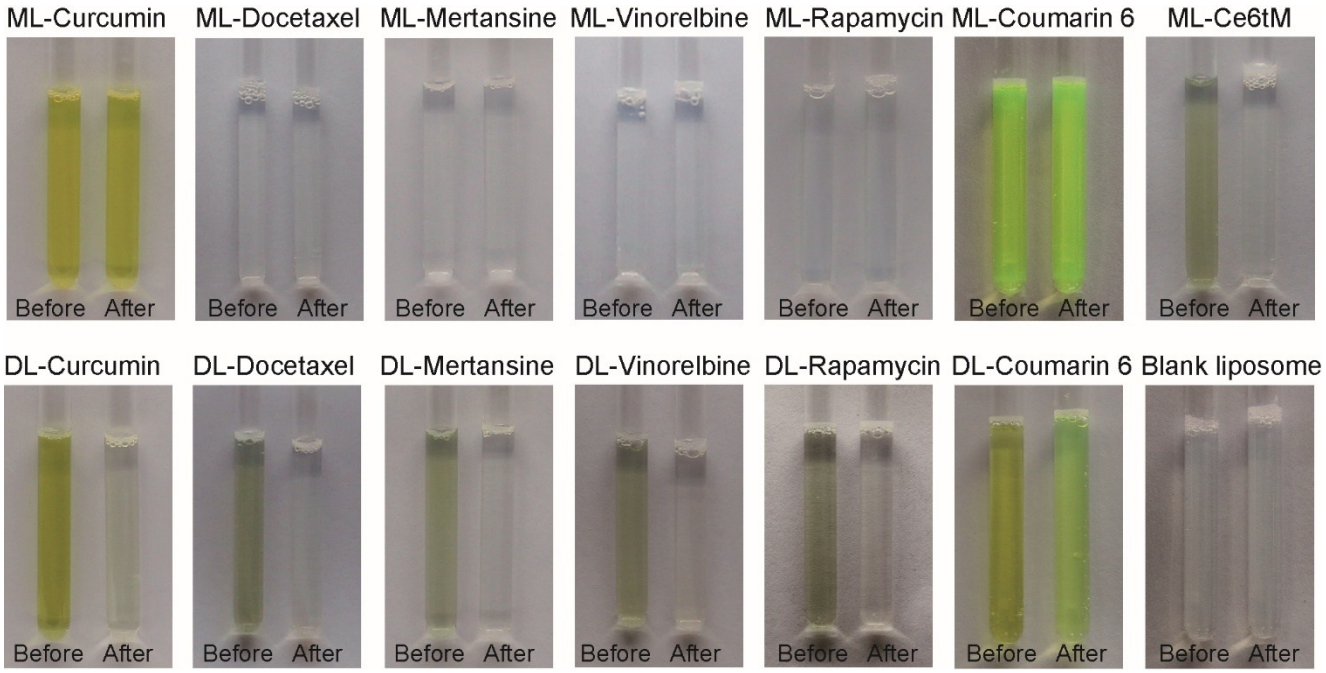

Figure S4. Photographic images of liposomes before and after irradiation. The solutions of different liposomes were irradiated with 671 nm laser (2 W/cm^2, 10 min), and the color of the solution before and after irradiation was monitored.

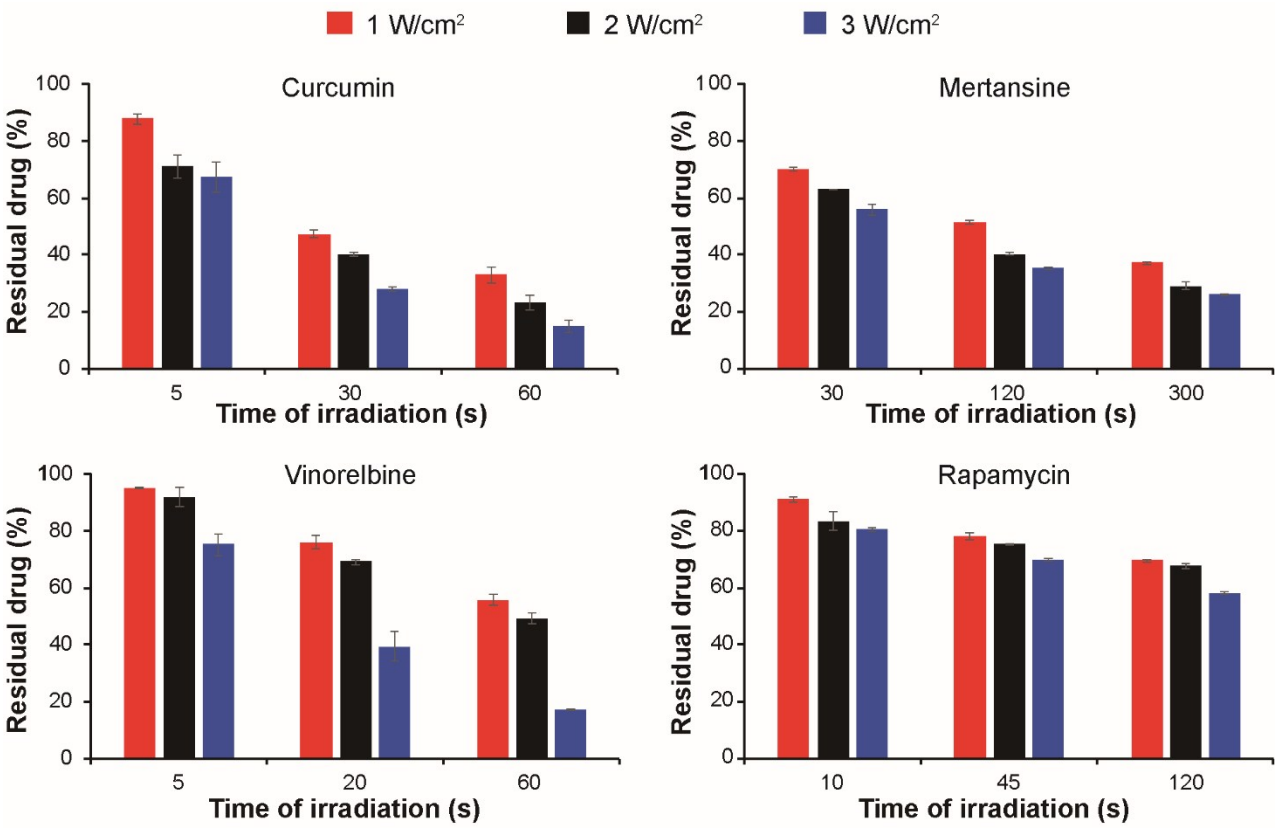

Figure S5. The effect of laser power on the degradation of anti-cancer drugs. DL-X were irradiated with 671 nm laser for a pre-determined time, and the amount of intact drug was measured by HPLC. Higher irradiation power resulted in faster drug degradation. The data were presented as mean ± s.d. (n=3).

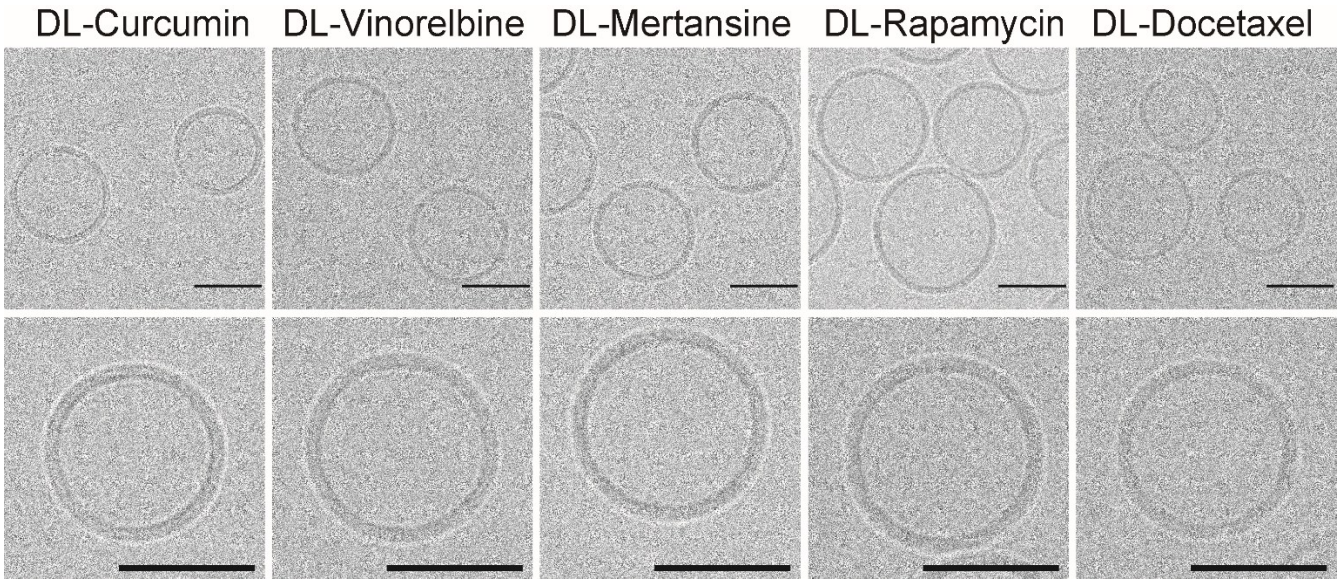

Figure S6. Representative cryo-TEM images of DL-X after irradiation. DL-X were irradiated with 671 nm laser (2 W/cm^2, 10 min), and were imaged using cryo-TEM for a possible change in morphologies before and after laser irradiation. The scale bars represented a distance of 50 nm.

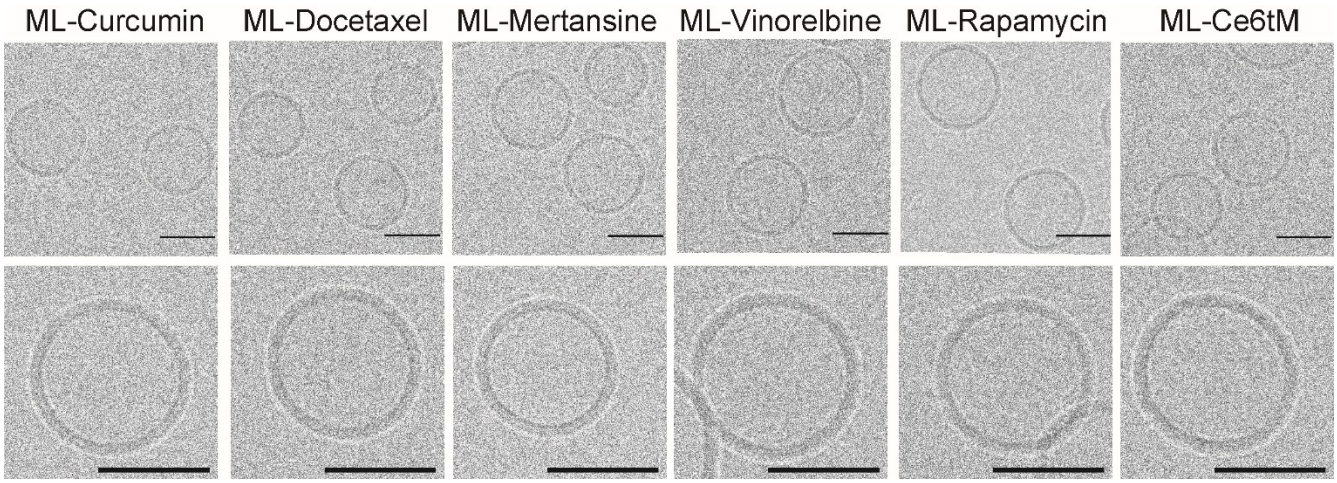

Figure S7. Representative cryo-TEM images of ML-X after irradiation. ML-X were irradiated with 671 nm laser (2 W/cm^2, 10 min), and were imaged using cryo-TEM for a possible change in morphologies before and after laser irradiation. The scale bars represented a distance of 50 nm.

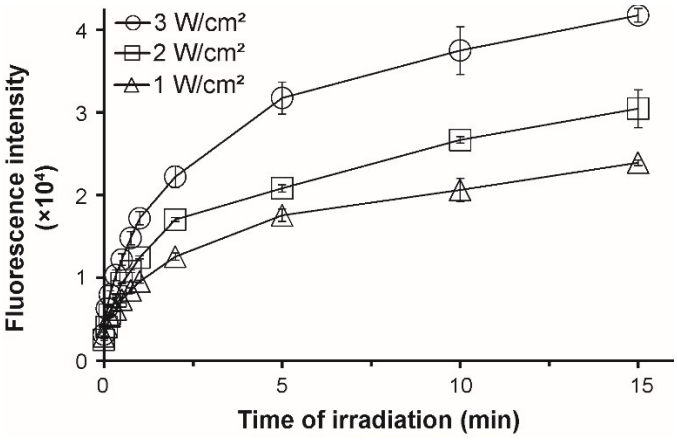

Figure S8. The effect of laser power on the generation of $^1O_2$ by Ce6tM. Hydrophobic $^1O_2$ indicator SOSG was co-encapsulated with Ce6tM into liposomes (0.37 mg/mL) to monitor the generation of $^1O_2$ under the irradiation with 671 nm laser of different power. The fluorescence intensity from $^1O_2$-activated SOSG was laser power- and time-dependent. The data were presented as mean ± s.d. (n=3).

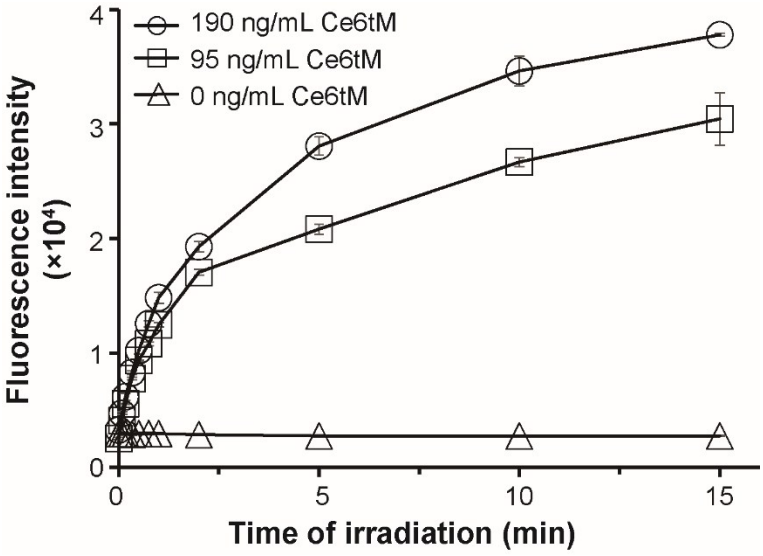

Figure S9. The effect of Ce6tM concentration on the generation of $^1O_2$. SOSG was co-encapsulated with Ce6tM of different amounts into liposomes (0.37 mg/mL) to monitor the generation of $^1O_2$ under laser irradiation (671 nm, 2 W/cm^2). The fluorescence from $^1O_2$-activated SOSG was measured using a fluorospectrometer. The fluorescence intensity highly relied on the concentration of Ce6tM. The data were presented as mean ± s.d. (n=3).

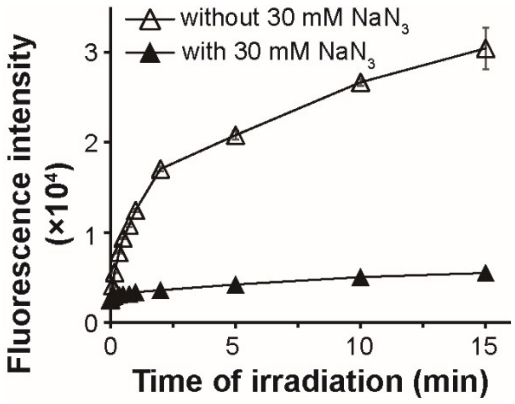

Figure S10. The effect of sodium azide on $^1O_2$ release. The amount of released $^1O_2$ in the presence and absence of sodium azide (30 mM), a $^1O_2$ quencher, was measured using a SOSG-based method. The initial concentration of liposomes was 0.37 mg/mL in DPPC. The data were presented as mean ± s.d. (n=3).

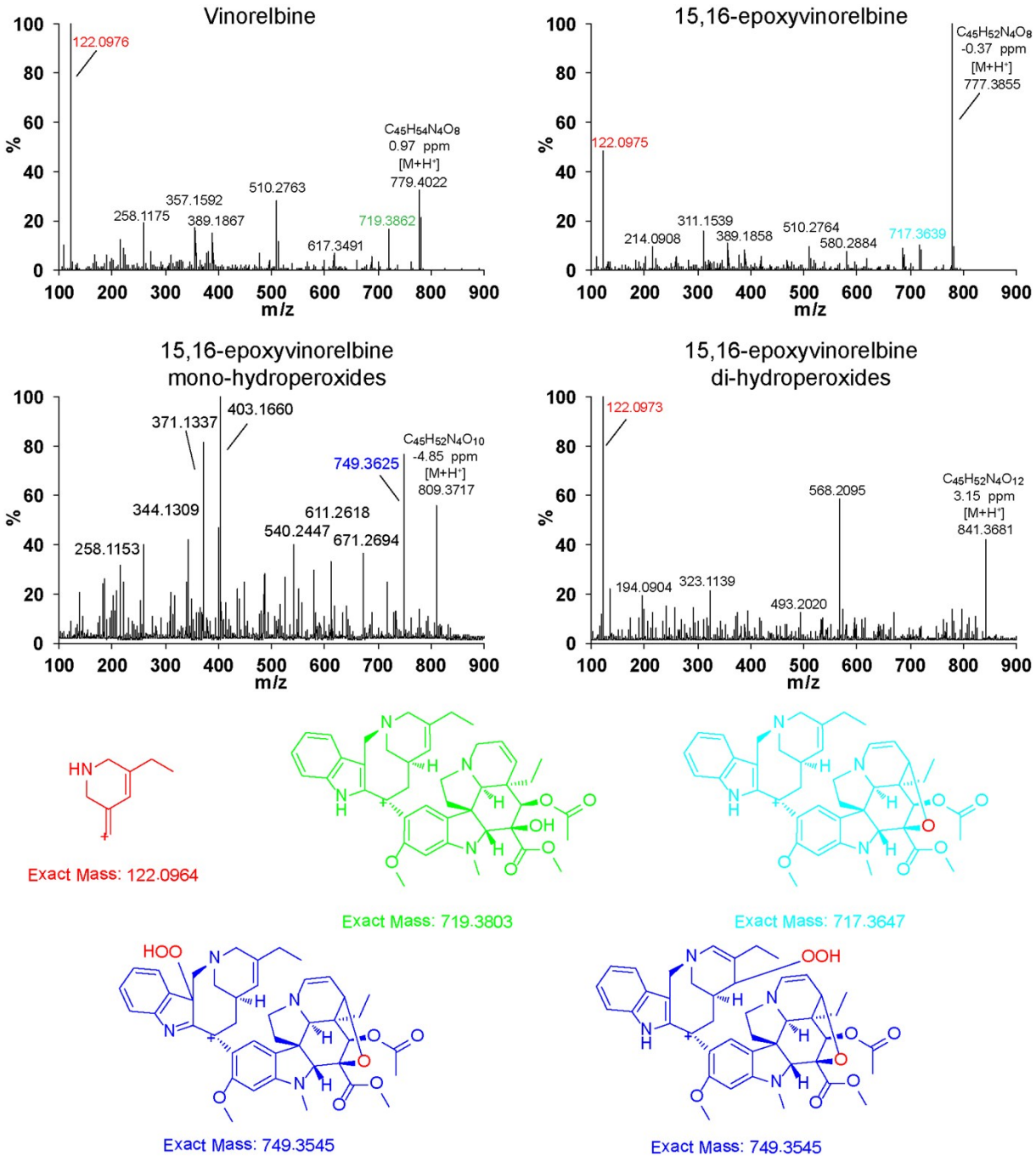

Figure S11. Mass spectra of DL-Vinorelbin before and after irradiation. The possible structures of the detected fragments were listed.

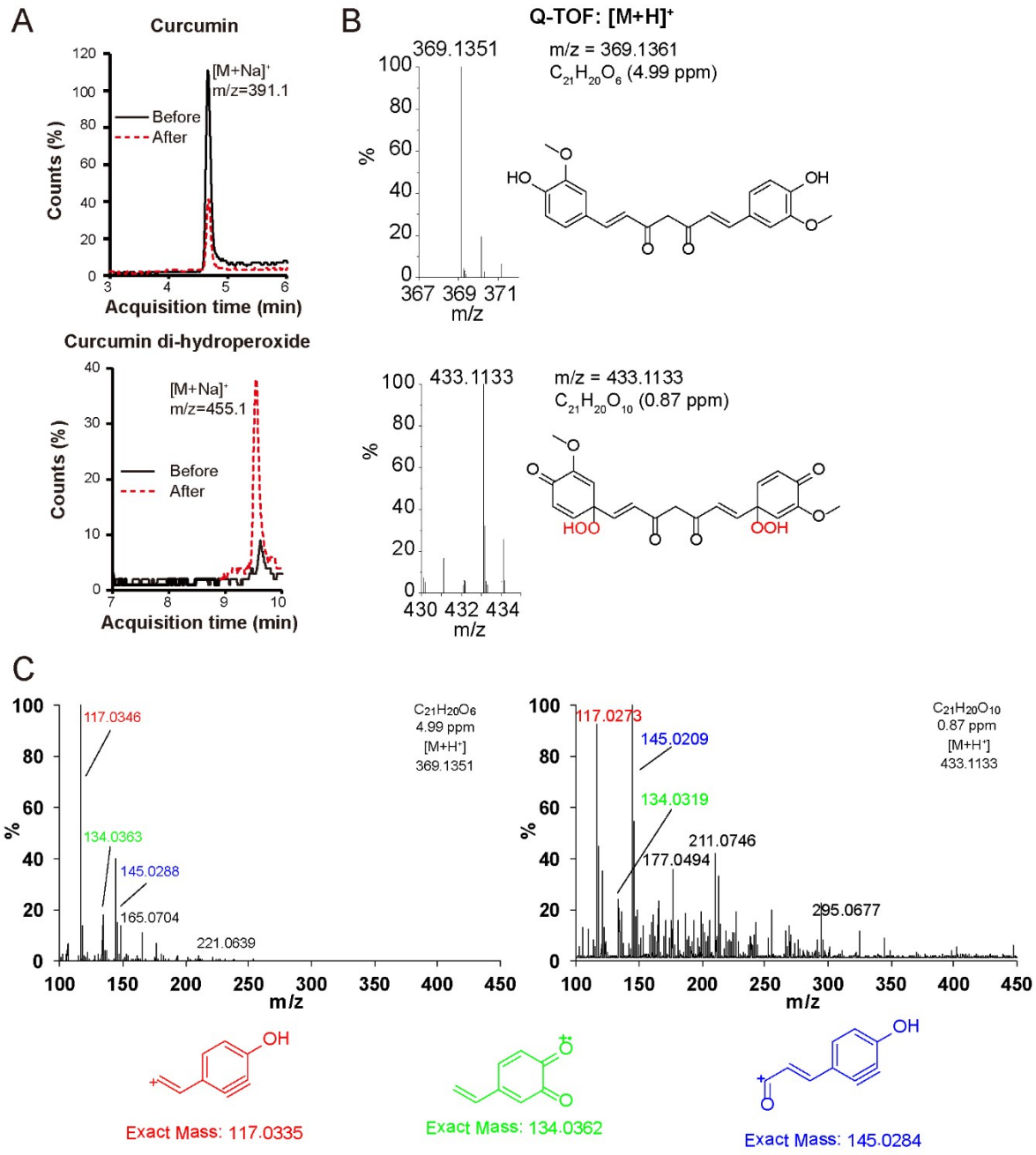

Figure S12. Mass spectra of DL-Curcumin before and after irradiation. (A) LC-MS spectra of DL-Curcumin (18 mg/mL) before and after irradiation (671 nm laser, 2 W/cm2). (B) Q-TOF high-resolution mass spectra of curcumin and its oxidized species. (C) Characteristic fragments of curcumin detected and their possible structures.

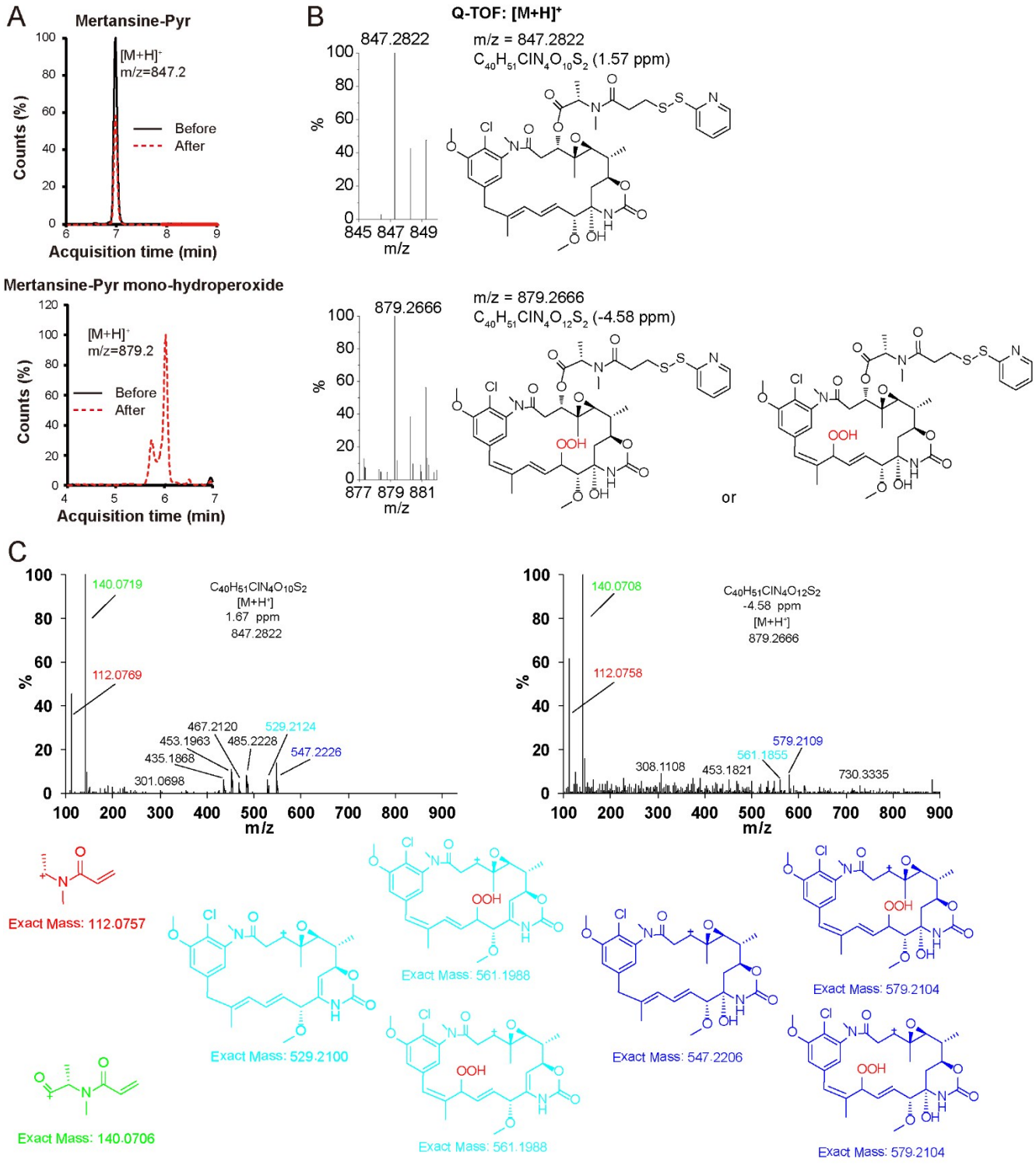

Figure S13. Mass spectra of DL-Mertansine-Pyr before and after irradiation. (A) LC-MS spectra of DL-Mertansine-Pyr (18 mg/mL) before and after irradiation (671 nm laser, 2 W/cm^2). (B) Q-TOF high-resolution mass spectra of mertansine-Pyr and its oxidized species. (C) Characteristic fragments of mertansine-Pyr detected and their possible structures.

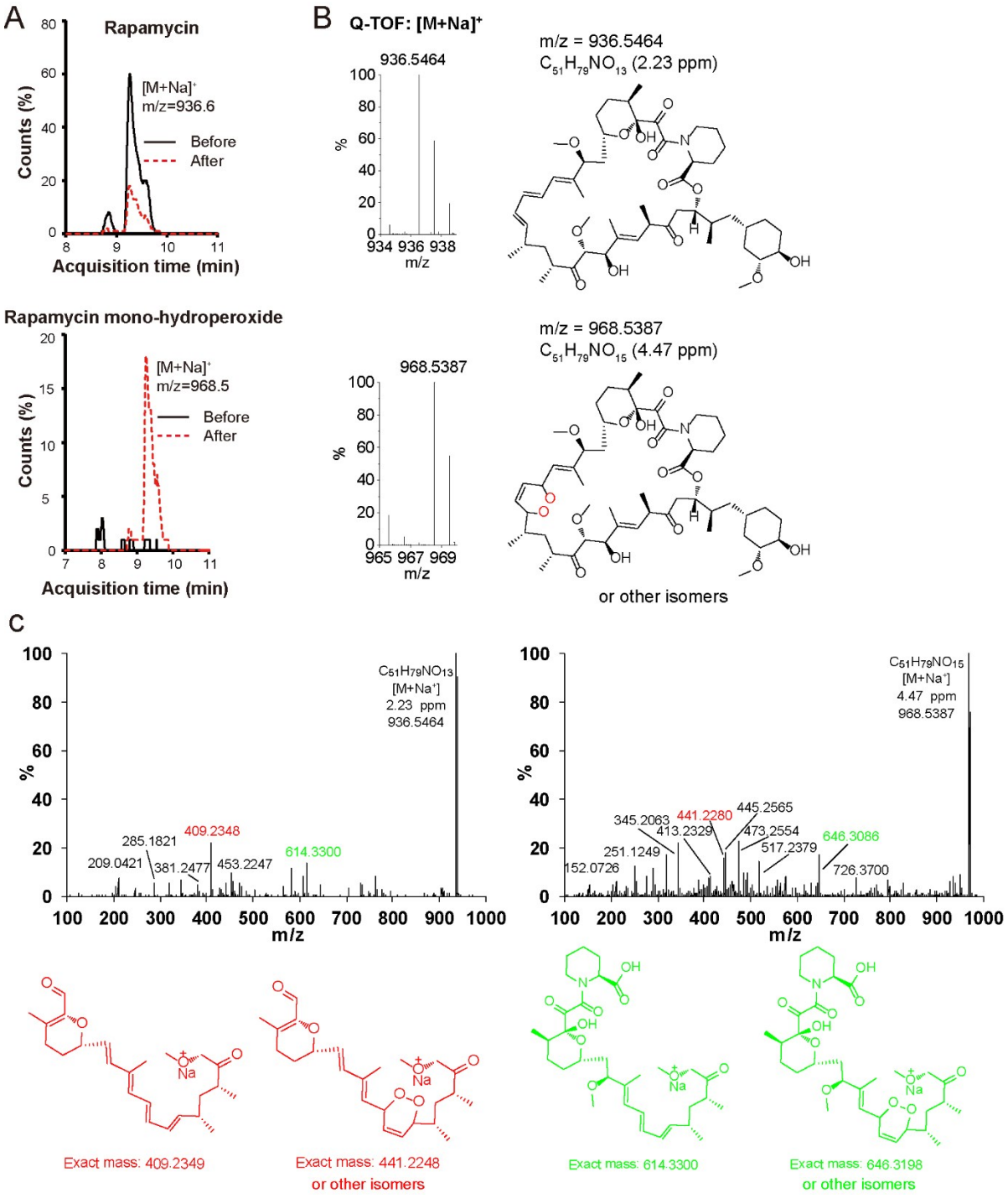

Figure S14. Mass spectra of DL-Rapamycin before and after irradiation. (A) LC-MS spectra of DL-Rapamycin (18 mg/mL) before and after irradiation (671 nm laser, 2 W/cm^2). (B) Q-TOF high-resolution mass spectra of rapamycin and its oxidized species. (C) Characteristic fragments of rapamycin detected and their possible structures.

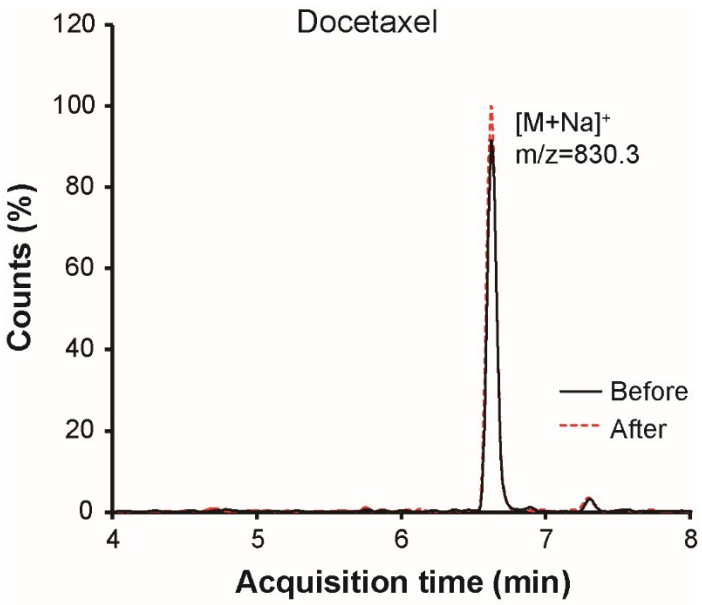

Figure S15. LC-MS spectra of docetaxel in DL-Docetaxel before and after irradiation. DL-Docetaxel (18 mg/mL in DPPC) was irradiated with 671 nm laser (2 W/cm^2) for 15 min before analysis. No significant change in the retention time, exact mass, and amount of docetaxel was observed.

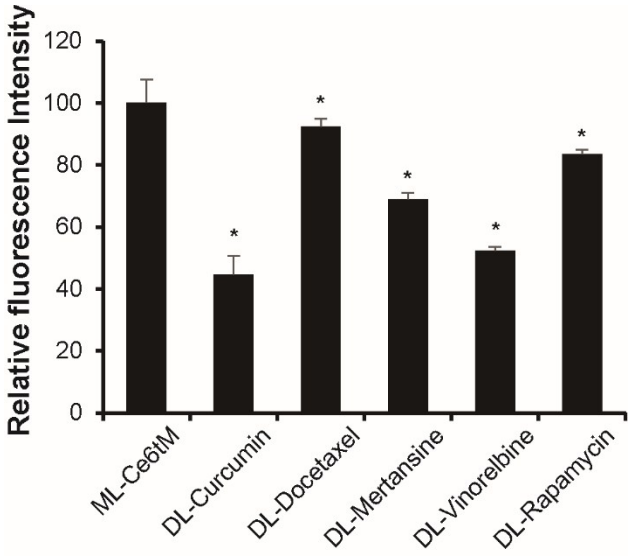

Figure S16. Depletion of $^1O_2$ by anti-cancer drugs. DL-X (0.7 mg/mL in DPPC) were irradiated with 671 nm laser (2 W/cm^2) for 10 s. The amounts of $^1O_2$ released by DL-X were compared with that released by ML-Ce6tM using SOSG-based fluorospectrometry. A 55%, 8%, 31%, 48%, and 18% decrease in the $^1O_2$ release was observed from DL-Curcumin, DL-Docetaxel, DL-Mertansine, DL-Vinorelbine, and DL-Rapamycin, respectively, when compared with ML-Ce6tM. Data were presented as mean ± s.d. (n = 3). *p<0.0005 compared with ML-Ce6tM.

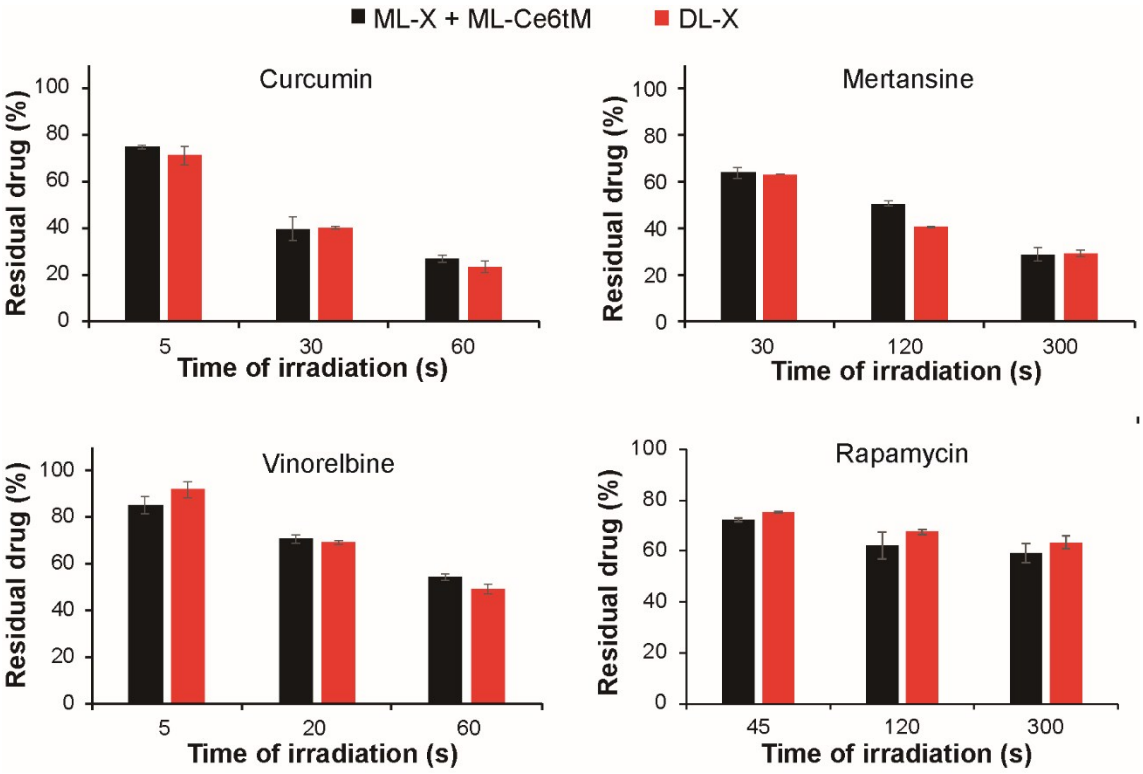

Figure S17. The effect of formulation on drug decomposition. The degradation of anti-cancer drugs in DL-X and physical mixture of ML-X and ML-Ce6TM (18 mg/mL in DPPC) upon irradiation (671 nm laser at 2 W/cm^2) was monitored by HPLC. No significant difference was observed between the two formulations in their rates of anti-cancer decomposition. The data were presented as mean ± s.d. (n=3).

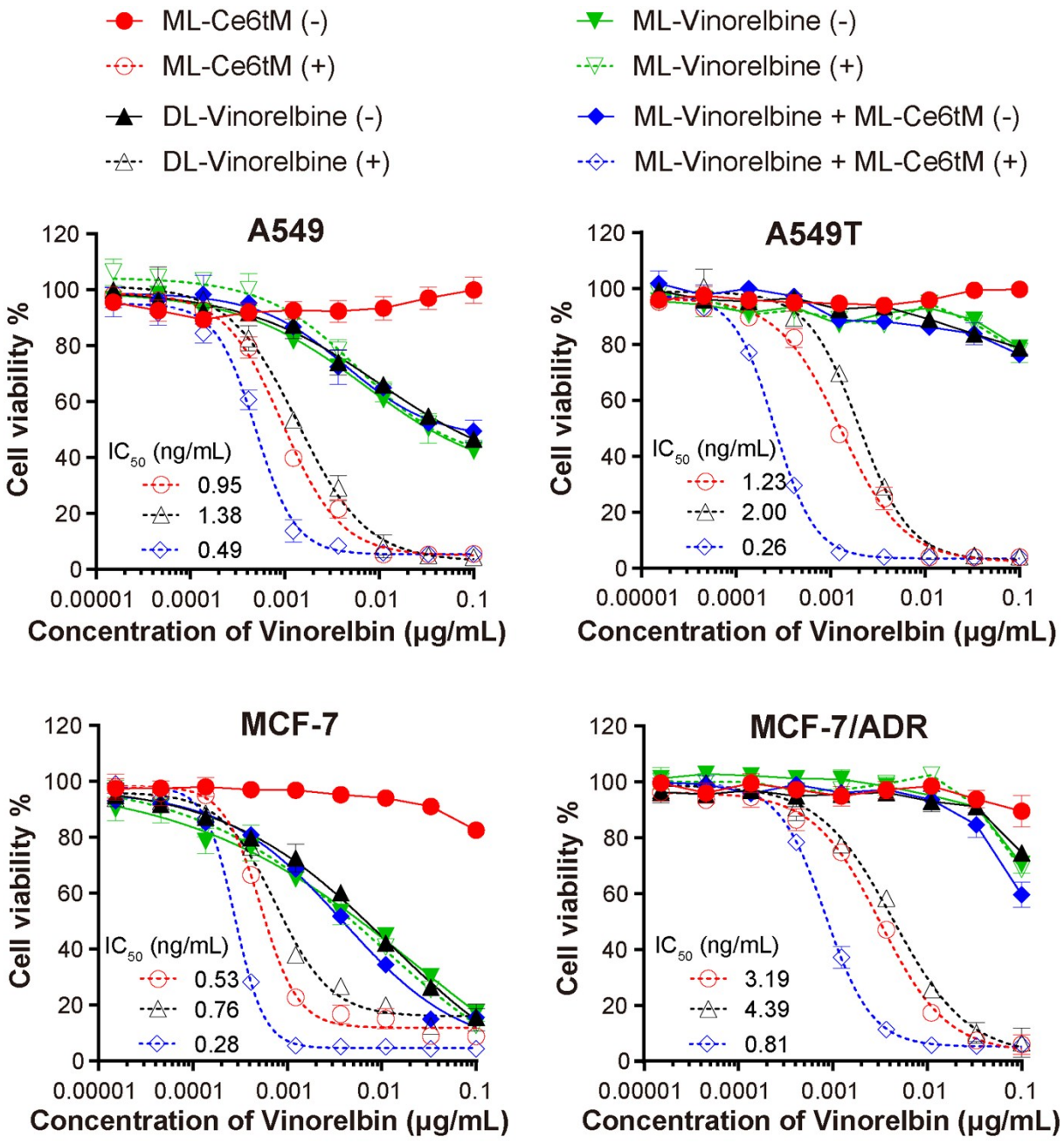

Figure S18. Cytotoxicity of different formulations on A549, A549T, MCF-7, and MCF-7/ADR. The formulations were incubated with cells for 4 h, followed by 671 nm laser irradiation (0.625 W/cm^2, 20 s). The cell viabilities were determined with CCK-8 kit 48 h after treatment. The data were presented as mean ± s.d. (n=3), and fitted with GraphPad Prism 7 to obtain IC_{50} for ML-Ce6tM, DL-Vinorelbine, ML-Ce6tM + ML-Vinorelbine.

Table S1. The size, size distribution and ζ-potential of the prepared liposomes before and after irradiation characterized with DLS.

	LogP[a]	Ce6tM	Before irradiation			After irradiation[b]		
			Size (nm)	PDI	ζ-potential (mV)	Size (nm)	PDI	ζ-potential (mV)
Curcumin	2.39	−	80.1 ± 2.3	0.109	0.13 ± 0.54	80.3 ± 1.0	0.117	0.08 ± 0.26
		+	79.6 ± 1.8	0.118	-0.04 ± 0.67	80.3 ± 1.2	0.102	-0.35 ± 0.57
Docetaxel	2.89	−	80.0 ± 0.6	0.093	0.20 ± 0.54	81.7 ± 1.8	0.093	-0.54 ± 0.26
		+	81.0 ± 1.3	0.112	0.10 ± 0.25	85.1 ± 3.1	0.099	-0.67 ± 0.11
Mertansine	3.47	−	82.0 ± 2.8	0.127	-0.05 ± 0.16	82.0 ± 2.3	0.129	0.12 ± 0.97
		+	81.0 ± 2.2	0.157	0.25 ± 0.63	82.9 ± 1.6	0.147	-0.53 ± 0.12
Coumarin 6	4.53	−	82.7 ± 1.0	0.131	0.19 ± 0.41	83.6 ± 0.4	0.140	-0.20 ± 0.54
		+	81.1 ± 0.4	0.130	-0.11 ± 0.85	82.0 ± 1.0	0.137	0.15 ± 0.26
Vinorelbine	4.92	−	81.0 ± 6.1	0.102	0.24 ± 0.30	82.3 ± 11.3	0.096	0.19 ± 0.88
		+	80.8 ± 1.2	0.117	-0.16 ± 0.22	83.4 ± 2.0	0.138	-0.21 ± 0.54
Rapamycin	5.13	−	83.1 ± 0.9	0.131	0.19 ± 0.61	83.2 ± 1.6	0.152	-0.05 ± 1.05
		+	82.1 ± 1.6	0.139	0.31 ± 0.87	82.1 ± 1.3	0.145	-0.17 ± 0.56
Ce6tM	6.24	−	79.8 ± 2.7	0.077	0.22 ± 0.50	79.8 ± 2.0	0.092	0.16 ± 0.68
		+	81.3 ± 3.9	0.114	0.00 ± 0.74	83.6 ± 3.3	0.149	-0.64 ± 0.38

[a] The Log P values of all molecules were calculated with Advanced Chemistry Development (ACD/Labs) Software version 11.02 (© 1994-2016 ACD/Labs).

[b] The liposomes were each irradiated for 10 min with a 671 nm laser at 2 W/cm^2.

Table S2. The drug loading of prepared liposomes. The data were presented as mean ± s.d. (n = 3).

Percentage of drug in liposome (in molar)	**ML-X**	**DL-X**			
		2:1 (Ce6tM:drug in mole)		**6:1 (Ce6tM:drug in mole)**	
		X	**Ce6tM**	**X**	**Ce6tM**
Curcumin	0.486 ± 0.013	0.475 ± 0.009	0.958 ± 0.008	0.163 ± 0.002	0.965 ± 0.011
Docetaxel	0.488 ± 0.025	0.480 ± 0.004	0.944 ± 0.021	0.161 ± 0.001	0.953 ± 0.015
Mertansine	0.494 ± 0.011	0.487 ± 0.003	0.962 ± 0.004	0.161 ± 0.001	0.978 ± 0.010
Vinorelbine	0.493 ± 0.012	0.486 ± 0.002	0.962 ± 0.006	0.161 ± 0.001	0.950 ± 0.037
Rapamycin	0.486 ± 0.005	0.482 ± 0.002	0.979 ± 0.012	0.165 ± 0.005	0.999 ± 0.030
Ce6tM	0.975 ± 0.003	-	-	-	-

Table S3. The drug encapsulation efficiency of the prepared liposomes. The data were presented as mean ± s.d. (n = 3).

Encapsulation Efficiency (%)	ML-X	DL-X			
		2:1 (Ce6tM:drug in mole)		6:1 (Ce6tM:drug in mole)	
		X	Ce6tM	X	Ce6tM
Curcumin	98.33 ± 0.85	98.71 ± 1.54	98.43 ± 0.99	98.81 ± 0.88	97.87 ± 2.47
Docetaxel	98.08 ± 0.55	99.15 ± 1.16	99.11 ± 0.40	99.93 ± 0.37	98.74 ± 0.35
Mertansine	99.36 ± 2.46	98.19 ± 1.60	98.61 ± 0.97	97.44 ± 1.96	99.42 ± 2.26
Vinorelbine	98.17 ± 0.27	98.47 ± 1.61	98.52 ± 0.68	97.64 ± 2.59	98.91 ± 1.19
Rapamycin	98.69 ± 0.58	99.12 ± 1.06	99.31 ± 1.25	98.47 ± 0.37	98.93 ± 2.56
Ce6tM	99.12 ± 1.62	-	-	-	-

Table S4. The half-lives of anti-cancer drugs co-encapsulated with Ce6tM at different molar ratio after irradiation with 671 nm laser at 2 W/cm^2. The concentration of liposomes was 18 mg/mL in DPPC. The data were presented as mean ± s.d. (n = 3).

	Molar ratio (Ce6tM : drug)			p
	0[a]	2:1 (min)	6:1 (min)	
Curcumin	−	0.29 ± 0.01	0.09 ± 0.01	0.00002
Docetaxel	−	−	−	−
Maytansine	−	1.27 ± 0.01	0.72 ± 0.08	0.00013
Vinorelbine	−	0.95 ± 0.16	0.31 ± 0.01	0.00103
Rapamycin	−	13.46 ± 0.83	0.81 ± 0.05	0.00001

[a] Less than 50% of encapsulated anticancer drugs were decomposed for ML-X and DL-Docetaxel after a 15-minute irradiation.

Table S5. Drug release from ML-X and DL-X after incubation in RPMI 1640 containing 10% FBS. The data were presented as mean ± s.d. (n = 3).

Drug release (%)	ML-X		DL-X 6:1 (Ce6tM:drug in mole)			
	2 h	8 h	2 h		8 h	
			X	Ce6tM	X	Ce6tM
Curcumin	1.79 ± 0.28	6.21 ± 0.75	1.08 ± 0.76	1.48 ± 0.41	7.28 ± 1.03	7.24 ± 1.49
Docetaxel	1.40 ± 0.40	6.85 ± 1.64	1.95 ± 0.38	0.90 ± 0.14	5.92 ± 1.99	5.74 ± 1.78
Mertansine	1.24 ± 0.41	6.61 ± 1.46	1.53 ± 0.32	1.41 ± 0.29	7.51 ± 0.93	7.34 ± 3.16
Vinorelbine	1.55 ± 0.11	7.10 ± 0.38	1.28 ± 0.22	1.45 ± 0.35	7.12 ± 0.81	5.77 ± 1.92
Rapamycin	1.53 ± 0.34	6.80 ± 0.14	1.57 ± 0.43	1.26 ± 0.40	7.39 ± 2.82	5.78 ± 2.15
Ce6tM	1.32 ± 0.19	5.91 ± 2.26	-	-	-	-

Table S6. Efficacy of vinorelbine-containing formulations against A549, B16-F10, HT29, and MCF-7 cells[a].

IC_{50} (ng/mL)	Laser[b]	A549	B16-F10	HT29	MCF-7
DL-Vinorelbine	−	11.15 (7.87−15.81)	116.40 (71.60−189.30)	2.54 (1.23−5.28)	94.10 (66.49−133.20)
	+	389.40 (320.30−473.30)	3518.00 (2428.00−5096.00)	185.50 (156.30−220.10)	1261.00 (939.80−1693.00)
ML-Vinorelbine + ML-Ce6tM	−	10.29 (7.35−14.40)	105.40 (67.63−164.40)	2.37 (1.09−5.12)	94.92 (71.07−126.80)
	+	40.88 (30.12−55.47)	753.40 (582.40−974.70)	7.64 (4.87−11.97)	255.80 (209.70−312.10)
ML-Vinorelbine	−	9.63 (6.87−13.50)	106.10 (63.94−176.00)	2.46 (1.13−5.36)	108.70 (78.87−149.80)
	+	9.27 (6.77−12.70)	103.60 (61.92−173.30)	2.31 (1.17−4.58)	105.80 (78.96−141.90)

[a]The results were presented as IC_{50} values and their 95% confidence interval.

[b] A 671 nm laser was applied to the liposomes at a power of 2 W/cm^2 for 2 min.

Table S7. Efficacy of mertansine-containing formulations against A549, B16-F10, HT29, and MCF-7 cells[a].

IC_{50} (ng/mL)	Laser[b]	A549	B16-F10	HT29	MCF-7
DL-Maytansine	−	6.07 (4.71−7.82)	39.33 (23.13−66.89)	3.91 (3.14−4.88)	18.34 (12.43−27.06)
	+	50.57 (43.71−58.51)	250.80 (150.10−418.80)	13.42 (9.93−18.14)	271.20 (200.00−367.90)
ML-Maytansine + ML-Ce6tM	−	6.06 (4.71−7.80)	40.52 (24.73−66.37)	3.93 (3.14−4.92)	18.97 (12.78−28.16)
	+	11.92 (9.43−15.07)	88.92 (55.85−172.30)	6.16 (5.19−7.31)	41.08 (28.50−59.20)
ML-Maytansine	−	6.11 (4.77−7.84)	42.01 (26.03−67.81)	3.53 (2.87−4.34)	18.46 (12.17−28.02)
	+	5.83 (4.61−7.35)	43.99 (26.10−74.13)	3.67 (2.97−4.54)	19.97 (13.42−29.72)

[a]The results were presented as IC_{50} values and their 95% confidence interval.

[b] A 671 nm laser was applied to the liposomes at a power of 2 W/cm^2 for 2 min.

Table S8. Efficacy of rapamycin-containing formulations against A549, B16-F10, HT29, and MCF-7 cells[a].

IC_{50} (μg/mL)	Laser[b]	A549	B16-F10	HT29	MCF-7
DL-Rapamycin	−	0.07 (0.04−0.11)	1.14 (0.77−1.68)	0.32 (0.22−0.47)	0.80 (0.57−1.12)
	+	1.33 (1.06–1.67)	6.09 (4.40−8.43)	1.67 (1.40−2.00)	3.71 (2.95−4.66)
ML-Rapamycin + ML-Ce6tM	−	0.077 (0.05−1.19)	1.13 (0.80−1.60)	0.31 (0.21−0.46)	0.68 (0.51−0.92)
	+	0.16 (0.10−0.24)	2.06 (1.35−3.16)	0.58 (0.39−0.86)	1.34 (0.91−1.96)
ML-Rapamycin	−	0.07 (0.05−0.11)	1.05 (0.74−1.50)	0.32 (0.22−0.47)	0.70 (0.52−0.95)
	+	0.07 (0.05−0.10)	1.12 (0.76−1.65)	0.32 (0.21−0.47)	0.73 (0.53−1.01)

[a]The results were presented as IC_{50} values and their 95% confidence interval.

[b] A 671 nm laser was applied to the liposomes at a power of 2 W/cm^2 for 2 min.

Table S9. Efficacy of curcumin-containing formulations against A549, B16-F10, HT29, and MCF-7 cells[a].

IC_{50} (μg/mL)	Laser[b]	A549	B16-F10	HT29	MCF-7
DL-Curcurmin	−	13.13 (11.86−14.54)	35.59 (33.58−37.73)	1.23 (1.07−1.43)	8.45 (7.14−10.00)
	+	45.49 (40.10−51.60)	N/A	5.29 (6.04−8.24)	99.27 (71.81−137.20)
ML-Curcurmin + ML-Ce6tM	−	11.88 (10.68−13.21)	32.10 (29.84−34.53)	1.43 (1.23−1.67)	8.43 (7.43−9.57)
	+	43.86 (35.79−53.75)	N/A	3.01 (2.78−3.51)	69.01 (45.64−104.30)
ML-Curcurmin	−	13.95 (12.84−15.16)	31.98 (29.79−34.34)	1.27 (1.06−1.52)	10.71 (9.32−12.31)
	+	13.30 (11.87−14.90)	30.86 (28.64−33.27)	1.29 (1.14−1.47)	9.70 (8.33−11.30)

[a]The results were presented as IC_{50} values and their 95% confidence interval.

[b] A 671 nm laser was applied to the liposomes at a power of 2 W/cm^2 for 2 min.

Table S10. Efficacy of vinorelbine-containing formulations against A549T and MCF-7/ADR cells[a].

IC_{50} (ng/mL)	Laser[b]	A549T	MCF-7/ADR
DL-Vinorelbine	−	209.60 (136.20−322.40)	701.7 (275.00−1791.00)
	+	–[c]	–[c]
ML-Vinorelbine	−	181.10 (111.90−293.20)	891.00 (468.50−1694.00)
	+	157.40 (105.60−234.60)	939.90 (504.00−1753.00)

[a]The results were presented as IC_{50} values and their 95% confidence interval.

[b] A 671 nm laser was applied to the liposomes at a power of 2 W/cm^2 for 2 min.

[c] 50% cell killing was not achieved within the tested range of concentrations (0.5–30 μg/mL)

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