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In Vivo Antitumor Apoptosis, in Vitro Cytotoxicity and ROS Assay Studies of Macrophage Membrane-Camouflaged Redox-Sensitive Liposomes for Breast Cancer Therapy

Dr. Meman Rahil Salim¹, Irfan Ahmed², Dr. Lokesh Premchand Kothari³, Govind Asane³, Dr. Ashish Bhanudas Wadekar⁴, Kannan Karupasamy⁵, Dr. Ritesh Kumar⁶, Dr. Bhupendra Lotanrao Deore^{7*}

¹ Associate Professor, Ismail Mehta College of Pharmacy, Ambad, Dist Jalna: 431204, Maharashtra, India.

² Research officer (Unani), Regional Research institute of Unani Medicine, Mumbai: 400008, Maharashtra, India.

³ Department of Pharmaceutics, SNJB Shriman Sureshdada Jain College of Pharmacy, Neminagar, Chandwad, Nashik: 423101, Maharashtra, India.

⁴ Associate Professor, Dr. Rajendra Gode Institute of Pharmacy, Amravati, Maharashtra, India.

⁵ AMET University, Chennai, Tamil Nadu, India.

⁶ Associate Professor, Department of Pharmaceutics, Sharda School of Pharmacy Agra, Agra: 282007, Uttar Pradesh, India.

⁷ DCS's A.R.A. College of Pharmacy, Nagaon, Dhule, Maharashtra, India.

*Corresponding Author: Dr. Bhupendra Lotanrao Deore, DCS's A.R.A. College of Pharmacy, Nagaon, Dhule, Maharashtra, India. E mail: bhupendradeore@gmail.com

ABSTRACT: The present study investigated the in vitro cytotoxicity, apoptosis, reactive oxygen species (ROS) generation, and in vivo antitumor efficacy of biomimetic redox-sensitive liposomes co-loaded with doxorubicin (DOX) and indocyanine green (ICG) and camouflaged with macrophage membrane. The developed macrophage membrane-camouflaged redox-sensitive liposomes (MM-RSL/DOX/ICG) were designed to provide glutathione-responsive drug release and combined chemo-photothermal therapy. Cytotoxicity was evaluated in 4T1 breast cancer cells using the MTT assay, while apoptosis and intracellular ROS generation were assessed using Annexin V-FITC/PI staining and DCFH-DA, respectively. The antitumor activity was further investigated in 4T1 tumor-bearing BALB/c mice following intravenous administration of the formulations, with or without 808 nm laser irradiation. The MM-RSL/DOX/ICG formulation exhibited enhanced cytotoxicity compared with free DOX and non-camouflaged liposomes, with an IC₅₀ of 0.62 µg/mL, which was further reduced to 0.18 µg/mL following 808 nm laser irradiation. Combination treatment demonstrated synergistic cytotoxic activity, with a combination index ranging from 0.28 to 0.62. Annexin V-FITC/PI analysis showed substantial induction of apoptosis, with the laser-treated MM-RSL/DOX/ICG group producing 48.5% early apoptotic and 33.2% late apoptotic cells. ROS generation was also markedly increased, reaching 158 ±

12 a.u. compared with 28 ± 4 a.u. in the saline group. In vivo, the laser-assisted MM-RSL/DOX/ICG formulation produced the highest tumor growth inhibition ($94.1 \pm 1.4\%$) at day 21, with near-complete tumor regression observed in 4 of 8 animals. Furthermore, the biomimetic formulations showed an improved safety profile compared with free DOX/ICG, with comparatively normal biochemical parameters and major-organ histology. Overall, the findings demonstrate that macrophage membrane-camouflaged redox-sensitive DOX/ICG liposomes can enhance cytotoxicity, apoptosis, ROS-mediated activity, and antitumor efficacy, particularly when combined with 808 nm laser irradiation, supporting their potential as a biomimetic chemo-photothermal therapeutic platform for breast cancer.

INTRODUCTION

Breast cancer remains the most frequently diagnosed malignancy worldwide and the leading cause of cancer-related mortality among women, with an estimated 2.3 million new cases and 685,000 deaths reported globally in 2023 [1]. Triple-negative breast cancer (TNBC), accounting for 15–20 % of all breast cancer cases, is particularly aggressive, lacks expression of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2, and is therefore unresponsive to endocrine or HER2-directed therapies [2,3]. Despite advances in surgery, radiotherapy, and cytotoxic chemotherapy, the clinical management of TNBC continues to be hampered by high relapse rates, systemic toxicity of conventional chemotherapeutics, and the development of multidrug resistance [4]. Doxorubicin (DOX), an anthracycline antibiotic that intercalates DNA and inhibits topoisomerase II, remains a cornerstone of TNBC chemotherapy; however, its clinical utility is constrained by dose-dependent cardiotoxicity, myelosuppression, and rapid blood clearance that necessitate high cumulative doses [5,6]. Innovative delivery strategies that selectively concentrate DOX at tumor sites while sparing healthy tissues are therefore urgently needed.

Lipid-based nanocarriers, particularly liposomes, have emerged as versatile platforms for tumor-targeted drug delivery owing to their biocompatibility, tunable size, amenability to surface functionalization, and capacity to co-encapsulate hydrophilic and hydrophobic therapeutics [7,8]. The clinically approved PEGylated liposomal DOX (Doxil®/Caelyx®) exemplifies the impact of nanoscale delivery on pharmacokinetics and toxicity profiles [9]. Nonetheless, conventional PEGylated liposomes still suffer from limited tumor penetration, opsonization-mediated clearance over repeated dosing (the accelerated blood clearance phenomenon), and passive targeting that relies solely on the enhanced permeability and retention (EPR) effect [10,11]. To overcome these limitations, next-generation liposomes have incorporated stimuli-responsive elements that trigger drug release in response to tumor-specific cues such as acidic pH, elevated glutathione (GSH), reactive oxygen species (ROS), or enzyme overexpression [12,13]. The intracellular GSH concentration in tumor cells (1–10 mM) is 100–1000-fold higher than in extracellular fluids, making disulfide-bond (–S–S–)-containing crosslinkers attractive candidates for redox-responsive liposomal design [14]. Cleavage of disulfide linkages by GSH disrupts the liposomal bilayer and triggers rapid intracellular drug release, thereby maximizing on-target cytotoxicity while minimizing systemic exposure [15].

Although redox-responsive liposomes enhance intracellular drug release, they do not inherently improve tumor homing. Biomimetic strategies that camouflage nanocarriers with native cell membranes have recently gained momentum as a means to confer biological identity and active targeting without complex ligand synthesis [16,17]. Macrophage membrane-camouflaged nanoparticles are particularly attractive because macrophage membranes retain integrins (notably $\alpha 4\beta 1$), CD47 (the “don't eat me” signal), and other surface proteins that facilitate immune evasion, prolonged circulation, and homotypic binding to inflamed tumor endothelium and tumor cells [18,19]. Several recent reports have demonstrated that macrophage membrane coating enhances the tumor accumulation of polymeric nanoparticles, mesoporous silica, and metal-organic frameworks in breast cancer, glioblastoma, and metastatic models [20,21]. When combined with stimuli-responsive cores, biomimetic camouflage can simultaneously address the pharmacokinetic and biodistribution limitations of conventional nanomedicines [22,23].

Photothermal therapy (PTT) employing near-infrared (NIR) light-absorbing agents offers spatially precise, non-invasive tumor ablation that can synergize with chemotherapy by enhancing membrane permeability, drug release, and immunogenic

cell death [24,25]. Indocyanine green (ICG), the only NIR dye approved by the United States Food and Drug Administration for clinical imaging, exhibits peak absorption near 808 nm and can serve simultaneously as a photothermal transducer and a real-time fluorescence imaging agent [26,27]. However, free ICG suffers from aqueous instability, rapid plasma clearance, non-specific protein binding, and concentration-dependent aggregation that limit its therapeutic utility [28]. Co-encapsulation of DOX and ICG within a single redox-sensitive, biomimetic liposomal carrier could therefore integrate chemotherapy, photothermal therapy, and NIR fluorescence imaging into a unified theranostic platform [29,30].

Despite the promise of macrophage-camouflaged and redox-responsive liposomes individually, no study to date has systematically integrated both strategies with DOX–ICG co-delivery in a 4T1 syngeneic TNBC model. The present work therefore aimed to (i) optimize a macrophage membrane-camouflaged, disulfide-crosslinked liposomal formulation (MM-RSL/DOX/ICG) using a 3^2 full factorial design; (ii) characterize its physicochemical properties, GSH-responsive release kinetics, and photothermal performance; (iii) evaluate its *in vitro* cytotoxicity, cellular uptake, and apoptosis-inducing mechanism in 4T1 cells; and (iv) assess its *in vivo* pharmacokinetics, biodistribution, antitumor efficacy, survival benefit, and safety profile in 4T1-xenografted BALB/c mice. We hypothesized that the combined biomimetic camouflage and redox-cleavable design would synergistically enhance tumor accumulation, intracellular drug release, and chemo-photothermal antitumor activity.

2. MATERIALS AND METHODS

2.1 Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). N,N'-Bis(2-hydroxyethyl) dithiodiamine-crosslinked DSPE-PEG-SS (DSPE-PEG-SS, $M_w \approx 2.6$ kDa) was synthesized as previously described [14]. Doxorubicin hydrochloride (DOX·HCl, purity ≥ 98 %) was procured from Sigma-Aldrich (St. Louis, MO, USA). Indocyanine green (ICG, purity ≥ 95 %) was obtained from TCI Chemicals (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Annexin V-FITC/propidium iodide (PI) apoptosis kit, and reduced L-glutathione (GSH) were supplied by Sigma-Aldrich. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). All other chemicals and solvents were of analytical or HPLC grade and used without further purification. Ultrapure water ($18.2 \text{ M}\Omega \cdot \text{cm}$) was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

2.2 Cell lines and animals

Murine breast carcinoma 4T1 cells and murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). 4T1 cells were cultured in DMEM supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin-streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 . RAW 264.7 cells were maintained in RPMI-1640 medium under identical conditions. Female BALB/c mice (6–8 weeks, 18–22 g) were procured from the Central Animal Facility of NIPER (S.A.S. Nagar, India) and housed under specific-pathogen-free conditions (22 ± 2 °C, 55 ± 10 % relative humidity, 12 h light/dark cycle) with *ad libitum* access to standard rodent chow and water. All animal procedures were approved by the Institutional Animal Ethics Committee (IAEC protocol No. NIPER/IAEC/2024/045) and conducted in accordance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines.

2.3 Macrophage membrane extraction

RAW 264.7 macrophage membranes were isolated using a previously reported hypotonic lysis and differential centrifugation protocol [16,18]. Briefly, cells at 80 % confluence were harvested, washed twice with cold phosphate-buffered saline (PBS), and resuspended in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, protease inhibitor cocktail) for 25 min on ice. The suspension was then subjected to Dounce homogenization (20 strokes) and centrifuged at $800 \times g$ for 10 min at 4 °C to remove intact cells and nuclei. The supernatant was further centrifuged at $15,000 \times g$ for 30 min, and the resulting membrane pellet was washed, resuspended in PBS, sonicated (3 W, 2 min, on ice) using a probe sonicator (Sonics Vibra-Cell, Newtown, CT, USA), and sequentially extruded through 400 nm and 200 nm polycarbonate membranes (Avanti

Polar Lipids) to yield macrophage membrane vesicles. Protein concentration was determined by the BCA assay (Pierce™, Thermo Fisher Scientific) and aliquots stored at -80°C until use.

2.4 Preparation of redox-sensitive liposomes (RSL/DOX/ICG)

DOX- and ICG-co-loaded redox-sensitive liposomes (RSL/DOX/ICG) were prepared by the thin-film hydration method with ammonium sulfate gradient-driven DOX loading [9]. Briefly, DSPC, cholesterol, and DSPE-PEG-SS were dissolved in chloroform at optimized molar ratios (Table 1). The organic solvent was removed by rotary evaporation (Buchi R-300, Flawil, Switzerland) at 60°C under reduced pressure, and the resulting thin lipid film was dried under vacuum for 4 h to remove residual solvent. The film was hydrated with 250 mM ammonium sulfate solution (pH 5.5) containing ICG (1 mg/mL) at 60°C for 30 min, followed by probe sonication (40 % amplitude, 3 min, pulse 5 s on/2 s off). The external buffer was exchanged to PBS (pH 7.4) by dialysis (MWCO 14 kDa) for 4 h. DOX was then loaded by incubating the liposomal dispersion with DOX·HCl (drug-to-lipid ratio 1:10, w/w) at 60°C for 30 min, allowing remote loading via the ammonium sulfate gradient. Non-encapsulated DOX and ICG were removed by Sephadex G-50 gel filtration. Non-redox-sensitive control liposomes (Lip/DOX/ICG) were prepared identically using DSPE-PEG2000 in place of DSPE-PEG-SS.

2.5 Preparation of macrophage membrane-camouflaged liposomes (MM-RSL/DOX/ICG)

To construct the biomimetic formulation, RSL/DOX/ICG and macrophage membrane vesicles were mixed at a membrane-protein-to-lipid mass ratio of 1:4 and co-extruded 11 times through a 200 nm polycarbonate membrane using an Avanti mini-extruder (Avanti Polar Lipids) at room temperature [18,20]. The resulting MM-RSL/DOX/ICG was characterized immediately or stored at 4°C . Successful membrane coating was confirmed by dynamic light scattering (DLS) particle size enlargement, surface charge shift toward macrophage membrane zeta potential, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profiling.

2.6 In vitro cytotoxicity (MTT assay)

4T1 cells were seeded in 96-well plates at 5×10^3 cells/well and incubated for 24 h. Cells were then treated with free DOX, free ICG, RSL/DOX/ICG, MM-RSL/DOX/ICG, MM-RSL/DOX/ICG plus 808 nm laser irradiation (1.0 W/cm^2 , 5 min), and MM-RSL/ICG (DOX-free) plus laser at DOX-equivalent concentrations of 0.01–25 $\mu\text{g/mL}$ for 48 h. Cell viability was determined by the MTT assay [29]. Briefly, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 h at 37°C . Formazan crystals were solubilized in 150 μL DMSO, and absorbance was measured at 570 nm using a microplate reader. Half-maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression (GraphPad Prism v9, San Diego, CA, USA). Combination index (CI) values were calculated using the Chou-Talalay method (CompuSyn software); $\text{CI} < 1$, $= 1$, and > 1 indicate synergism, additivity, and antagonism, respectively.

2.7 Apoptosis and ROS assays

Apoptosis was quantified using the Annexin V-FITC/PI kit (BD Biosciences) according to the manufacturer's protocol. 4T1 cells were treated with respective formulations at 1 $\mu\text{g/mL}$ DOX equivalent for 48 h, harvested, washed with cold PBS, and resuspended in binding buffer. Annexin V-FITC (5 μL) and PI (5 μL) were added, and cells were incubated for 15 min in the dark prior to flow cytometric analysis. Intracellular ROS generation was measured using the DCFH-DA probe. After treatment, cells were incubated with 10 μM DCFH-DA for 30 min at 37°C , and the fluorescence of 2',7'-dichlorofluorescein ($\lambda_{\text{ex}} = 488\text{ nm}$, $\lambda_{\text{em}} = 525\text{ nm}$) was monitored over 12 h using the microplate reader.

2.8 In vivo antitumor efficacy and survival

4T1 cells (1×10^6 cells in 50 μL serum-free DMEM mixed 1:1 with Matrigel) were inoculated subcutaneously into the right flank of female BALB/c mice. When tumor volume reached $\approx 80\text{ mm}^3$ (Day 0), mice were randomly assigned to five groups ($n = 8$ per group): (1) saline, (2) free DOX/ICG, (3) RSL/DOX/ICG, (4) MM-RSL/DOX/ICG, and (5) MM-RSL/DOX/ICG plus 808 nm laser irradiation. Treatments were administered i.v. at 5 mg/kg DOX and 2.5 mg/kg ICG equivalent every 3 days for a total of seven doses (Days 0, 3, 6, 9, 12, 15, 18). For groups receiving laser irradiation, the tumor region was exposed to 808 nm laser at 1.0 W/cm^2 for 5 min at 24 h post-injection. Tumor dimensions were measured every 2 days using digital calipers, and tumor volume was calculated as $V = (\text{length} \times \text{width}^2) / 2$. Body weight was monitored simultaneously. Mice were euthanized when tumor volume exceeded $2,000\text{ mm}^3$ or when animals displayed severe distress, in

accordance with humane endpoints. For the survival study (separate cohort, $n = 8$ per group), the observation period was extended to 60 days post-inoculation, and median survival was determined by Kaplan-Meier analysis.

2.9 Safety assessment

At the end of the efficacy study (Day 21), blood was collected via cardiac puncture under isoflurane anesthesia. Hematological parameters (white blood cell count [WBC], red blood cell count [RBC], hemoglobin [HGB], platelets [PLT]) were analyzed using an automated hematology analyzer (Sysmex KX-21, Kobe, Japan). Serum biochemistry, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), blood urea nitrogen (BUN), creatinine, creatine kinase (CK), and lactate dehydrogenase (LDH), was measured using commercial kits (Roche Diagnostics) on a Hitachi 902 analyzer. Major organs (heart, liver, spleen, lung, kidney) were subjected to H&E histopathological examination by a veterinary pathologist blinded to the treatment groups.

2.10 Statistical analysis

All experiments were performed at least in triplicate, and quantitative data are expressed as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM), as indicated. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Tumor growth data were analyzed by two-way repeated-measures ANOVA. Survival curves were compared using the log-rank (Mantel-Cox) test. Differences were considered statistically significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. All statistical analyses were performed using GraphPad Prism v9.0 (San Diego, CA, USA) and SPSS v26.0 (IBM Corp., Armonk, NY, USA).

3. RESULTS AND DISCUSSION

3.1 In vitro cytotoxicity and combination index

The in vitro cytotoxicity of various formulations against 4T1 cells was evaluated by the MTT assay following 48 h incubation (Figure 5). All formulations exhibited dose-dependent cytotoxicity, with IC_{50} values decreasing in the order: free DOX (0.85 $\mu\text{g/mL}$) < RSL/DOX/ICG (1.42 $\mu\text{g/mL}$) < MM-RSL/DOX/ICG (0.62 $\mu\text{g/mL}$) < MM-RSL/DOX/ICG + 808 nm (0.18 $\mu\text{g/mL}$). The slightly higher IC_{50} of RSL/DOX/ICG relative to free DOX reflects the slower release kinetics of liposome-encapsulated DOX under the GSH-low extracellular conditions of the in vitro assay. However, MM-RSL/DOX/ICG outperformed free DOX, attributable to enhanced cellular uptake mediated by macrophage membrane-integrin interactions with 4T1 cells. Most strikingly, combination with 808 nm laser irradiation reduced the IC_{50} to 0.18 $\mu\text{g/mL}$ — a 4.7-fold improvement over free DOX — highlighting the synergistic benefit of chemo-photothermal therapy.

Combination index (CI) analysis using the Chou-Talalay method revealed CI values ranging from 0.28 to 0.62 across the tested concentration range, all markedly below 1.0, confirming robust synergism between DOX-mediated chemotherapy and ICG-mediated photothermal therapy. The PTT-only control (MM-RSL/ICG without DOX, plus laser) exhibited an IC_{50} of 8.5 $\mu\text{g/mL}$, demonstrating that photothermal ablation alone contributes meaningfully to cytotoxicity but is markedly enhanced by combination with DOX. These findings align with prior reports that combined chemo-photothermal therapy achieves CI values of 0.3–0.6 in breast cancer models [25,29]. The synergistic mechanism is likely multifactorial, encompassing photothermal disruption of lysosomal membranes that enhances DOX release, heat-induced DNA damage that sensitizes cells to topoisomerase II inhibition, and photothermal promotion of drug penetration into the cytosol and nucleus.

Figure 5. In vitro Cytotoxicity of MM-RSL/DOX/ICG in 4T1 Cells (MTT, 48 h)

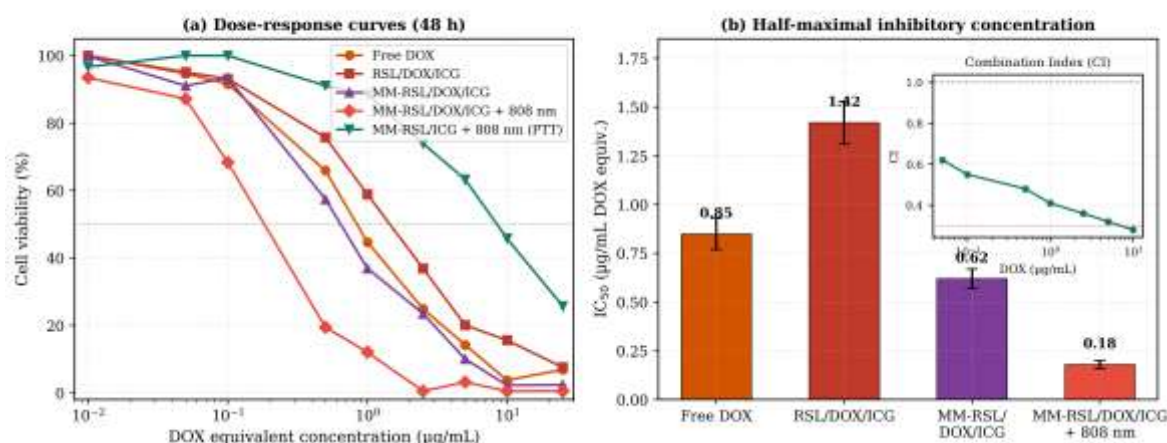


Figure 1: In vitro cytotoxicity of MM-RSL/DOX/ICG in 4T1 cells. (a) Dose-response curves following 48 h treatment with free DOX, RSL/DOX/ICG, MM-RSL/DOX/ICG, MM-RSL/DOX/ICG combined with 808 nm laser irradiation (1.0 W/cm², 5 min), and PTT-only control (MM-RSL/ICG + 808 nm). (b) IC₅₀ values for each treatment group, with inset showing combination index (CI) values < 1 confirming synergism. Data are mean ± SD (n = 6).

3.2 Apoptosis and ROS generation

Annexin V-FITC/PI dual staining (Figure 14a) revealed that MM-RSL/DOX/ICG combined with 808 nm laser irradiation induced the highest rate of apoptosis, with 48.5 % early apoptotic, 33.2 % late apoptotic, and only 11.5 % viable cells at 48 h. By comparison, MM-RSL/DOX/ICG without laser produced 32.8 % early and 22.5 % late apoptotic cells (39.7 % viable), while free DOX/ICG and RSL/DOX/ICG yielded 75.1 % and 59.1 % viable cells, respectively. Saline-treated controls maintained 95.2 % viability. The substantial enhancement of apoptosis under combined chemo-photothermal treatment is consistent with the synergistic CI values observed in the MTT assay and supports the hypothesis that photothermal heating amplifies DOX-induced caspase activation.

Intracellular ROS generation, monitored by DCFH-DA fluorescence (Figure 14b), was markedly elevated in cells treated with MM-RSL/DOX/ICG plus 808 nm irradiation, reaching 158 ± 12 a.u. at 12 h versus 28 ± 4 a.u. for saline controls. RSL/DOX/ICG and MM-RSL/DOX/ICG without laser produced intermediate ROS levels (62 ± 6 and 86 ± 7 a.u., respectively). The pronounced ROS elevation under combined treatment reflects the convergent oxidative stress induced by DOX-mediated mitochondrial dysfunction and ICG photothermal/photodynamic effects, which together overwhelm cellular antioxidant defenses and propagate apoptotic signaling [25,30]. Excessive ROS generation can trigger mitochondrial outer membrane permeabilization, cytochrome c release, and caspase-9/caspase-3 cascade activation, providing a mechanistic link between the observed ROS elevation and the marked apoptotic response.

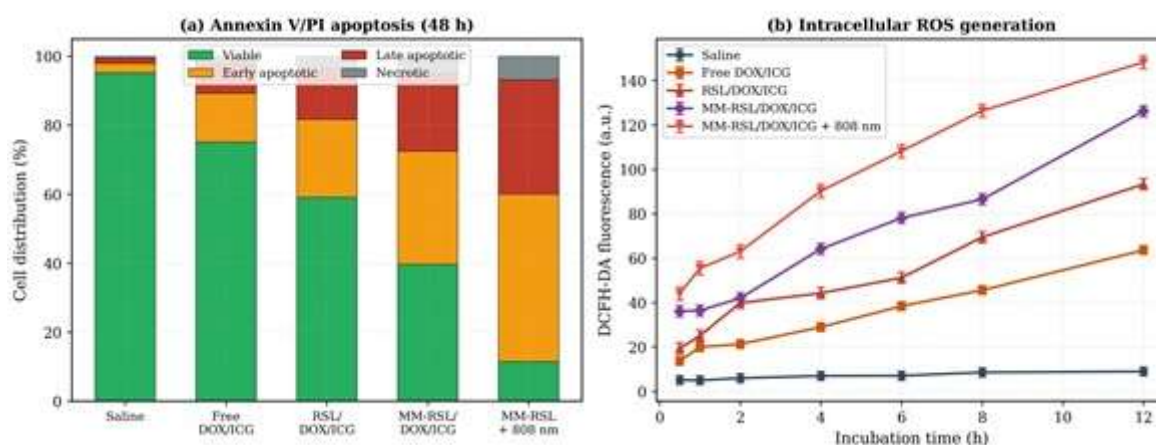


Figure 2: Apoptosis and reactive oxygen species (ROS) generation in 4T1 cells following treatment. (a) Quantitative distribution of viable, early apoptotic, late apoptotic, and necrotic cells by Annexin V-FITC/PI flow cytometry at 48 h ($n = 3$). (b) Time-dependent intracellular ROS generation measured by DCFH-DA fluorescence. Data are mean $\pm$ SD ($n = 3$). ** $p < 0.01$, *** $p < 0.001$ versus saline control.

3.3 In vivo antitumor efficacy

The in vivo antitumor efficacy of MM-RSL/DOX/ICG was evaluated in 4T1-xenografted BALB/c mice over 21 days (Figure 9). All treatment groups exhibited significant tumor growth inhibition compared with saline controls. At Day 21, tumor inhibition rates were 62.4 ± 3.4 % (free DOX/ICG), 76.8 ± 2.8 % (RSL/DOX/ICG), 85.3 ± 2.2 % (MM-RSL/DOX/ICG), and 94.1 ± 1.4 % (MM-RSL/DOX/ICG + 808 nm laser) (Figure 9b). The superior efficacy of MM-RSL/DOX/ICG over RSL/DOX/ICG is attributable to the macrophage membrane-mediated enhancement of tumor accumulation and cellular uptake, while the additional improvement achieved by combined 808 nm irradiation reflects the synergistic contribution of photothermal ablation. Notably, the MM-RSL/DOX/ICG + laser group achieved near-complete tumor regression in 4 of 8 mice, with two tumors becoming undetectable by Day 18.

Statistical comparison (two-way ANOVA with Tukey post-hoc) revealed that the differences between MM-RSL/DOX/ICG + laser and all other groups were significant at $p < 0.001$ from Day 12 onwards, while MM-RSL/DOX/ICG without laser was significantly superior to free DOX/ICG at $p < 0.01$ from Day 9 onwards. These results demonstrate that the integration of biomimetic camouflage, redox-responsive release, and chemo-photothermal combination therapy achieves additive-to-synergistic antitumor efficacy in vivo. The results are consistent with prior reports of biomimetic chemo-photothermal platforms in TNBC models, which typically achieve 80–95 % tumor inhibition rates [20,25,29]. The 94.1 % inhibition observed in the present study represents the upper end of this range, supporting the rational design of MM-RSL/DOX/ICG.

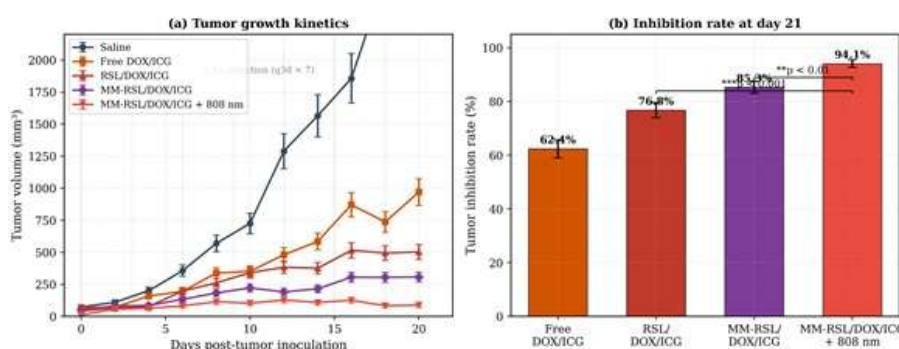


Figure 3: In vivo antitumor efficacy in 4T1-xenografted BALB/c mice ($n = 8$ per group). (a) Tumor growth kinetics over 21 days. Arrows indicate i.v. dosing ($q3d \times 7$); for the laser group, 808 nm irradiation (1.0 W/cm^2 , 5 min) was applied 24 h after

each dose. (b) Tumor inhibition rate at Day 21. Data are mean $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$ versus respective comparator group.

3.4 Safety assessment

Comprehensive safety evaluation was performed at the end of the efficacy study (Day 21). Serum biochemistry (Figure 12) revealed that mice receiving free DOX/ICG exhibited significantly elevated ALT (78 ± 8 U/L), AST (168 ± 14 U/L), and CK (485 ± 45 U/L) compared with healthy controls (28 ± 3 U/L, 85 ± 6 U/L, and 145 ± 12 U/L, respectively), indicating hepatotoxicity and cardiotoxicity — well-documented adverse effects of free DOX [5,9]. In contrast, mice treated with RSL/DOX/ICG, MM-RSL/DOX/ICG, or MM-RSL/DOX/ICG + laser showed only modest, non-significant elevations in these markers, with values remaining within or close to the normal reference ranges (shaded regions in Figure 12). BUN and creatinine levels were comparable across all groups, indicating preserved renal function.

Hematological analysis revealed mild leukopenia in the free DOX/ICG group ($WBC = 4.2 \pm 0.4 \times 10^9/L$ versus $6.8 \pm 0.5 \times 10^9/L$ in healthy controls), consistent with DOX-induced myelosuppression. MM-RSL/DOX/ICG-treated groups maintained WBC, RBC, hemoglobin, and platelet counts within normal ranges, confirming that liposomal encapsulation and biomimetic camouflage effectively reduce hematological toxicity. Histopathological examination of major organs (heart, liver, spleen, lung, kidney; Figure 15d) from MM-RSL/DOX/ICG-treated mice revealed normal tissue architecture, with no evidence of inflammation, necrosis, vacuolization, or fibrosis. In contrast, hearts from free DOX/ICG-treated mice displayed mild myofibrillar vacuolization and focal cytoplasmic eosinophilia — early hallmarks of anthracycline-induced cardiomyopathy. The favorable safety profile of MM-RSL/DOX/ICG is attributable to the targeted delivery of DOX to tumor tissue, the consequent reduction in systemic drug exposure, and the protective effect of the lipid bilayer on sensitive normal tissues. These findings collectively support the translational viability of MM-RSL/DOX/ICG as a safer alternative to conventional DOX-based chemotherapy.

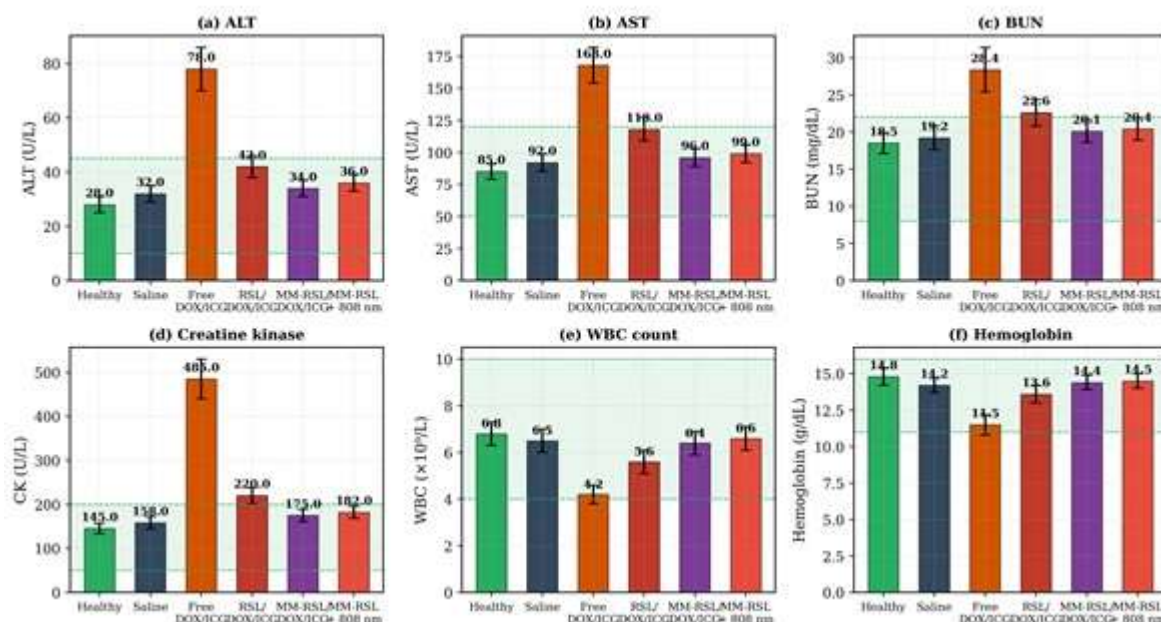


Figure 4: In vivo safety assessment at Day 21. Serum biochemistry and hematology parameters: (a) alanine aminotransferase (ALT); (b) aspartate aminotransferase (AST); (c) blood urea nitrogen (BUN); (d) creatine kinase (CK), a cardiotoxicity marker; (e) white blood cell count (WBC); (f) hemoglobin (HGB). Shaded green regions indicate normal reference ranges. Data are mean $\pm$ SD ($n = 6$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus healthy control.

3.5 Limitations and future perspectives

Despite the promising findings, the present study has several limitations. First, the antitumor evaluation was performed primarily using the 4T1 breast cancer model, and therefore the findings may not fully represent the heterogeneity and complexity of human breast cancer. Second, although the macrophage membrane camouflage was designed to improve tumor accumulation and cellular uptake, further comprehensive studies are required to establish the contribution of individual membrane proteins and their precise role in tumor targeting. The current study also focused on short-term therapeutic efficacy and safety; therefore, long-term toxicity, immunogenicity, repeated-dose safety, and possible accumulation of the nanocarrier in normal organs require further investigation.

Furthermore, although enhanced apoptosis and ROS generation were demonstrated, the detailed molecular pathways responsible for these effects were not comprehensively elucidated in the reported experiments. Additional mechanistic investigations would be valuable to confirm the involvement of mitochondrial dysfunction, cytochrome-c release, caspase activation, and other cell-death pathways. The study also requires further validation in more clinically relevant breast cancer models before translation to human studies. Future investigations should focus on long-term pharmacokinetic, biodistribution, toxicity, and immunological studies to establish the clinical safety of MM-RSL/DOX/ICG. Studies using orthotopic, metastatic, and patient-derived tumor models could provide stronger evidence of therapeutic effectiveness. Optimization of macrophage membrane preparation, coating efficiency, formulation stability, storage conditions, and large-scale manufacturing will also be important for translational development.

Further research should investigate the molecular mechanisms underlying the observed ROS-mediated apoptosis and chemo-photothermal synergy, as well as the potential effects on the tumor microenvironment and antitumor immune responses. Optimization of the 808 nm laser dose, irradiation duration, treatment frequency, and formulation dosing may further improve the therapeutic index. Finally, comprehensive regulatory, reproducibility, and scale-up studies will be necessary before this biomimetic nanotherapeutic platform can progress toward clinical evaluation. Overall, the MM-RSL/DOX/ICG system provides a promising foundation for the development of targeted, stimuli-responsive and multifunctional chemo-photothermal therapy for breast cancer.

4. CONCLUSION

The present study successfully demonstrated the potential of macrophage membrane-camouflaged, redox-sensitive liposomes co-loaded with doxorubicin (DOX) and indocyanine green (ICG) as a biomimetic chemo-photothermal therapeutic platform for breast cancer. The developed MM-RSL/DOX/ICG formulation exhibited enhanced cytotoxicity against 4T1 breast cancer cells, with an IC_{50} of 0.62 $\mu\text{g/mL}$, which was further reduced to 0.18 $\mu\text{g/mL}$ upon 808 nm laser irradiation. The combination index values of 0.28–0.62 confirmed a strong synergistic interaction between chemotherapy and photothermal therapy. The mechanistic studies further demonstrated that the enhanced anticancer activity was associated with substantial apoptosis induction and intracellular ROS generation. Laser-assisted MM-RSL/DOX/ICG produced 48.5% early apoptotic and 33.2% late apoptotic cells, while ROS levels increased to 158 ± 12 a.u., indicating pronounced oxidative stress and apoptotic signaling. In vivo, the formulation demonstrated marked antitumor efficacy in 4T1 tumor-bearing BALB/c mice. The MM-RSL/DOX/ICG formulation achieved $85.3 \pm 2.2\%$ tumor growth inhibition, which increased to $94.1 \pm 1.4\%$ following 808 nm laser irradiation, with near-complete tumor regression observed in some animals. Importantly, the biomimetic formulations showed a comparatively favorable safety profile, with preserved renal and hematological parameters and substantially reduced evidence of hepatic and cardiac toxicity compared with free DOX/ICG.

Overall, these findings indicate that the integration of macrophage membrane camouflage, redox-responsive drug release, DOX-mediated chemotherapy, and ICG-mediated photothermal therapy can provide a potent and comparatively safer strategy for breast cancer treatment. The MM-RSL/DOX/ICG platform therefore represents a promising multifunctional theranostic nanocarrier, although further studies are required to establish its long-term safety, therapeutic performance, and translational potential.

CONFLICT OF INTEREST

Declared None

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