Liposome-Encapsulated Melphalan Exhibits Potent Antimyeloma Activity and Reduced Toxicity

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ABSTRACT: Multiple myeloma (MM), a plasma cell cancer in bone marrow, remains an incurable disease. Melphalan, an alkylating agent, is a conventional anticancer drug that is still widely used for MM treatment in clinics. However, melphalan-induced organ toxicity and side effects are common. In this study, we loaded melphalan into a liposomal capsule and constituted liposomal melphalan (liposomal MEL). Liposomal MEL particles were approximately 120 nm in size and stable *in vitro*. The liposomal particles could be effectively taken up by MM cells. *In vitro* cytotoxicity assays using MM cell lines and primary MM cells showed that liposomal MEL exhibited similar anti-MM activity compared to an equivalent amount of free melphalan (free MEL) compound. In animal models, liposomal particles had bone marrow

enrichment and prolonged half-life *in vivo*. Liposomal MEL exposure resulted in less liver and colon organ toxicity than exposure to an equivalent amount of free MEL-treated mice. Importantly, liposomal MEL had potent anti-MM activity *in vivo* in a human MM xenograft mouse model. Overall, our findings suggested that liposome-encapsulated melphalan was an effective drug modification of the melphalan compound and showed promise in MM treatment.
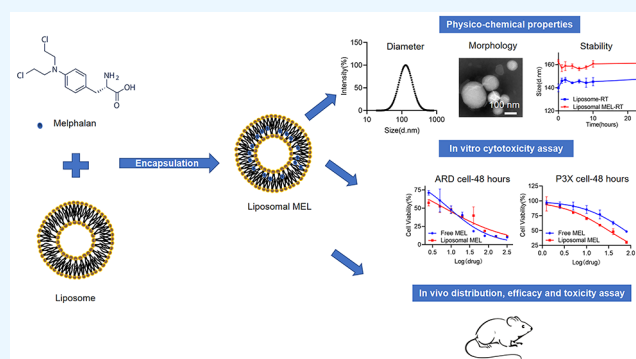
1. INTRODUCTION

Multiple myeloma (MM), characterized by the accumulation of malignant plasma cells in bone marrow, is the second most common hematological cancer in Western countries.[1] In recent decades, with the clinical use of novel agents, MM treatment has experienced revolutionary improvement. According to data from the Surveillance, Epidemiology, and End Results Program (SEER) in the United States, the median survival of MM patients was 6.7 years in the new drug era, compared with 2−3 years 2 decades ago.[2] However, MM is still considered a fatal disease, and patients are looking for curable approaches.

Melphalan is a conventional cytotoxic agent and has been used in MM treatment for more than 50 years. Currently, high-dose melphalan (HDM) induction followed by autologous stem cell transplantation (ASCT) (HDM-ASCT) remains a standard therapy for MM patients who are eligible for stem cell transplantation.[3] Different studies suggest that MM patients receiving a combination therapy of HDM-ASCT and novel agents have superior treatment outcomes.[4] In addition, melphalan is also recommended for elderly patients who are not suitable for other anti-MM treatments.[5] Melphalan is a nitrogen mustard family alkylating agent that causes DNA cross-linking in cells, resulting in DNA damage and cell death in rapidly proliferating cells.[6] However, melphalan is a

cytotoxic agent without targeting features. In theory, any dividing cells, even healthy cells, are sensitive to melphalan-induced cytotoxicity. The side effects of melphalan include nausea, vomiting, diarrhea, oral and intestinal mucositis, and bone marrow suppression.[7] These side effects lead to an increased risk of serious infections and adverse clinical outcomes in MM patients with immunodeficiency who receive melphalan. In addition, melphalan is poorly soluble and unstable at physiological pH and has a short half-life in pharmacological properties.[8] Therefore, a melphalan-loaded nanodrug delivery system that can both maintain therapeutic activity and reduce melphalan-introduced side effects is needed.

Liposome is the first invented drug delivery system to be developed[9] and has been successfully used in clinical practice. The liposomal formulation of doxorubicin Doxil is the most successful one and is widely used in the treatment of
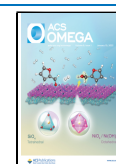
hematological malignancies and some solid tumors.[10] The performance of liposome formulations depends on the enhanced permeability and retention (EPR) effect, similar to other nanomedicines, especially in different solid tumors[11,12] and infectious diseases. However, the mechanism of liposomes in hematologic malignancies has not been well studied. In this study, we constituted liposome-encapsulated melphalan (liposomal MEL). The bilayer matrix of liposomal MEL was formed by egg phosphatidylcholine (EPC), distearylphosphatidylethanolamine-mPEG-2000(mPEG-2000-DSPE), and cholesterol (CHO), as mPEG-2000-DSPE and CHO have been verified to prolong the half-lives of nanosized liposomes in circulation and improve stability in serum.[13] Liposomes consist of phospholipids with both hydrophobic and hydrophilic phases. Hydrophilic molecules can be loaded into the inner core, while hydrophobic molecules can be loaded into the phospholipid bilayer. Liposomes are nontoxic and biodegradable. Previous studies suggest that liposomes facilitate drug delivery for cancer treatment. Moreover, liposomes may also enhance the therapeutic effect of the encapsulated drugs and reduce the side effects. In this study, our aim was to reduce melphalan cytotoxicity to nontumor cells while maintaining the potent anti-MM activity of the agent by liposomal encapsulation. The anti-MM activity and organ cytotoxicity of liposomal MEL were examined and compared with those of the free melphalan compound.

2. MATERIALS AND METHODS

2.1. Reagents. Lipids, including egg phosphatidylcholine (EPC) and distearylphosphatidylethanolamine-mPEG-2000 (mPEG-2000-DSPE), were purchased from Avanti Polar Lipids. Cholesterol (CHO) was obtained from Sigma-Aldrich. Melphalan was purchased from Dalian Meilun Biotechnology Co., Ltd. Cell culture media and penicillin−streptomycin were obtained from Corning Corporation (Corning). The hydrophobic fluorescent dyes 3,3′-dioctadecyloxacarbo-cyanine perchlorate (DiO) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) were purchased from Thermo Fisher Corporation.

2.2. Cell Culture. The human MM cell line ARD and murine MM cell line P3x63Ag8U.1 (P3X) were kindly provided by Prof. Yiguo Hu (State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, Sichuan University). The cell lines were negative for mycoplasma contamination and authenticated by short tandem repeat (STR) profiling (GENEWIZ, Inc. Suzhou) before the experiment. ARD cells were cultured in RPMI-1640 medium, and P3x63Ag8U.1 (P3X) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 g/mL streptomycin at 37 °C and 5% CO_2.

2.3. PBMCs and Primary MM Cells Were Isolated by the Density Gradient Technique. PBMCs from healthy volunteers and primary MM cells from newly diagnosed multiple myeloma patients were isolated using standard Ficoll−Paque gradient centrifugation according to the instructions of the manufacturer (Tianjin Haoyang, China). Primary MM cells were isolated from MM patient bone marrow aspiration by CD138+ cell sorting (MoFlo XDP, Beckman Co.). After cell sorting, the primary MM cells were cultured overnight under the same conditions as the MM cell lines. This study was approved by the Ethical Committee of West China Hospital of Sichuan University.

2.4. Construct Liposomal Melphalan. For the preparation of the blank liposome, EPC/CHO/mPEG-DSPE (2:5:2) was dissolved in chloroform and added to a round-bottom flask for evaporation with a rotary vacuum evaporator to a lipid film. Then, the lipid film was rehydrated completely in phosphate-buffered saline (PBS, pH 7.4), and the suspension was sonicated with a probe sonicator for 30 mins. Finally, the suspension was extruded three times through polycarbonate membranes with pore sizes of 450 and 220 nm.

For liposomal melphalan, the melphalan solution dissolved in methanol was mixed with the above EPC/CHO/mPEG-DSPE composition solution. The other methods were the same as above. The liposomal melphalan was stored at 4 °C before use.

2.5. Methods. *2.5.1. Characterization of Liposomal Melphalan.* The mean size and the ζ potential of liposomal melphalan were determined by dynamic light scattering (DLS, NanoBrook Omni particle sizer and ζ potential analyzer) three times at 25 °C. Samples were properly diluted with distilled water (1:100) to avoid multiple scattering effects. Approximately 2 mL of diluted sample was added to a measuring tube, and the measurement was carried out three times. The UV absorbance of liposomal MEL was determined by a UV−vis spectrophotometer (UV-2600, SHIMADZU). Approximately 1.5 mL of sample was added to a measuring tube, and the measurement was carried out three times.

The morphology of liposomal melphalan was examined by transmission electron microscopy (TEM, H6009IV, Hitachi, Japan). Liposomal melphalan was diluted in distilled water and placed on a copper grid covered with nitrocellulose. The samples were negatively stained with phosphotungstic acid and dried at room temperature before testing.

Drug loading (DL) and encapsulation efficiency (EE) were examined and analyzed with high-performance liquid chromatography (HPLC). The DL and EE of liposomal MEL were calculated according to the following equations

$$DL = \frac{drug}{copolymer + drug} \times 100\%$$

$$EF = \frac{experimental\ drug\ loading}{theoretical\ drug\ loading} \times 100\%$$

The release behavior of melphalan was evaluated by a dialysis method. In brief, the liposomal MEL and free MEL were placed in a dialysis bag (MWCO = 3500 Da), which was immersed in phosphate buffer solution (PBS, pH 7.4) containing Tween 80 (0.5 wt %). Then, they were incubated under gentle shaking at 100 rpm at 37 °C. At predetermined time points, the release medium was collected and replaced with prewarmed fresh medium. The concentration of melphalan in the collected medium was quantified by HPLC at 261 nm.

2.5.2. Examination of the Cellular Uptake of the Liposomal Particles. To examine the uptake of the liposomal particles by MM cells, we constructed a reporter liposomal particle encapsulated with a hydrophobic fluorescent dye, 3,3′-dioctadecyloxacarbo-cyanine perchlorate(DiO) (liposomal DiO). Liposomal DiO was added to MM cell culture medium at a final concentration of 100 ng/mL and incubated for 4 h. After incubation, the cells were washed three times with ice-cold PBS. Then, the cells were fixed with 4% paraformaldehyde. The cells were stained with Hoechst (10 μg/mL) to
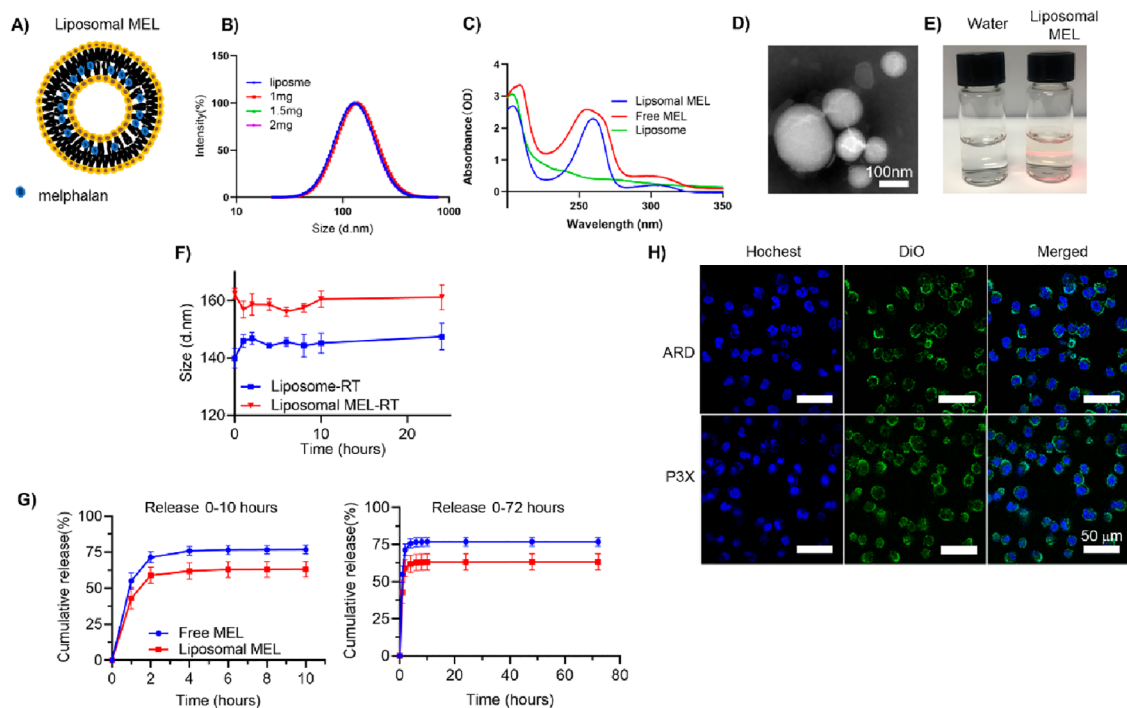
Figure 1. Liposomal melphalan morphology, stability, and cellular uptake. (A) Schematic illustration of liposomal MEL, which was melphalan encapsulated into an mPEG-egg phosphatidylcholine (EPC)-cholesterol (CHO) lipid bilayer; (B) size distribution of liposomal MEL particles; (C) UV absorbance for free MEL, liposomal MEL, and blank liposomes; (D) transmission electron microscopy image of liposomal MEL; (E) macroscopic morphology of liposomal MEL. Laser beam through the water (left), liposomal MEL (right). (F) Stability of liposome or liposomal MEL at room temperature; (G) in vitro drug release profiles showing the release of melphalan from free melphalan (MEL) in DMSO or from liposomal MEL in water; (H) the cellular uptake of DiO by ARD and P3X cells. Representative confocal fluorescence image of ARD and P3X cells. Nuclei were stained with Hoechst 33258. Data are presented as the mean ± SD (n = 3). All results were obtained from three independent experiments.

visualize nuclei. After staining, the cells were examined by confocal microscopy (Nikon, Japan).

2.5.3. Cell Viability Assay and Apoptosis Assay. Cell viability was determined by Cell Counting Kit 8 (CCK8) (#BX-6005, BioX Technology) according to the manufacturer's protocol. Cells were seeded and cultured at a density of 1.5×10^4 cells/well in 100 μL of medium in 96-well microplates. Then, the cells were treated with various concentrations of liposomal MEL, free MEL, and blank liposomes (0, 1.25, 2. 5, 5, 10, 20, 40, 80 μM). After treatment for 72 h, 10 μL of CCK8 reagent was added to each well and then incubated for 2 h. The absorbance was analyzed at 450 nm using a microplate reader.

The cells were seeded into 12-well culture plates at a density of 1×10^5/well in 1 mL of medium and treated with liposomes, free MEL, or liposomal MEL at a concentration of 15 μM for 48 h. Cell apoptosis was examined by flow cytometry using an Annexin V-Alexa Fluor 647/PI (#FXP023, 4A Biotech, China) apoptosis kit according to the manufacturer.

2.5.4. Animal Experiments. BALB/c mice (female, 6−8 weeks) were purchased from Dossy Co., Ltd., Sichuan, China. B-NDG mice (NOD-Prkdcscid IL2rgtm1/Bcgen) were purchased from Biocytogen Co., Ltd., Jiangsu, China (female, 5 weeks old). All animal experiments were approved by the West China Hospital Animal Ethics Committee.

To examine liposomal particle distribution *in vivo*, we constructed a reporter liposomal particle encapsulated with a hydrophobic fluorescent dye, 1,1′-dioctadecyl-3,3,3′,3′-tetra-methylindotricarbocyanine iodide (DiR) (liposomal DiR).

BALB/c mice (n = 6) were randomly divided into two groups (three mice per group) and treated with free DiR (0.4 μg/mice) and liposomal DiR (0.4 μg/mice) through intravenous injection. Then, at the determined time points, the distribution of the liposomes was monitored through the IVIS spectrum *in vivo* fluorescence imaging system. At the last time point, the major organs, including the heart, liver, spleen, lung, kidney, tibia, and femur, were isolated to detect the fluorescence distribution after the mice were sacrificed.

To examine the toxicity of liposomal MEL *in vivo*, Balb/c mice (n = 10) were randomly divided into two groups (five mice per group) and treated with saline, blank liposomes, free MEL (5 mg/kg), or liposomal MEL (5 mg/kg) every 3 days three times. After the treatment, peripheral blood was collected for liver and renal function and blood cell counts. Then, the major organs, including the heart, liver, spleen, lung, kidney, and colon, were isolated for pathological examination by hematoxylin and eosin (H&E) after the mice were sacrificed.

To examine the treatment efficacy, we established a human MM xenograft mouse model. The B-NDG mice (n = 20) were intravenously inoculated with ARD cells (2×10^6 cells/mouse) with consistent luciferase gene expression. Approximately 10 days after tumor cell inoculation, the tumor-bearing mice (n = 20) were randomly divided into four groups (five mice per group) and treated with saline, blank liposomes, free MEL, or liposomal MEL at a dose of 5 mg/kg every three days three times. Then, bioluminescence imaging (BLI) was monitored to investigate the treatment efficacy every 4 days by the IVIS Spectrum Imaging System. The mice were taken for the BLI signal after the mice were injected intraperitoneally with D-

luciferin potassium salt at a dose of 150 mg/kg for 15 min. The animal weight was measured every 2 days. The serum of the mice was harvested every 4 days to examine the secretion of M protein.

2.5.5. Hematoxylin and Eosin Staining. The major organs, including the heart, liver, spleen, lung, kidney, and colon, were isolated after the mice were sacrificed. The organs were fixed in 4% paraformaldehyde immediately for 48 h, exposed to 70% ethanol overnight, and embedded in paraffin. Then, the embedded tissues were sliced into 5 μm sections and stained with hematoxylin and eosin (H&E). The stained sections were imaged under an inverted phase contrast microscope.

2.6. Statistical Analysis. Statistical analysis was performed with SPSS 19.0 software. The results are expressed as the mean ± SD. Statistical significance for comparisons among two or three groups was analyzed using Student's t test or one-way ANOVA, respectively. P values of less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Characterization of Liposomal Melphalan. Liposomal MEL was prepared as described in the Materials and Methods section. In liposomal MEL particles, multiple melphalan molecules were packed in liposome bilayers. The hydrophilic mPEG segment formed the shell, which provided good stability and solubility in water (Figure 1A). The average particle sizes of blank liposomes and liposomal MEL were approximately 120 and 130 nm, respectively (Figure 1B). After MEL was loaded into the liposome, the size may be variable for different drug loadings (Table 1). The ζ potentials of blank

Table 1. Characteristics of Liposomal MEL at Various Weight Ratios

MEL: EPC/CHO/mPEG-DSPE feeding radio (wt)	drug loading (%)	encapsulation efficiency (%)	mean size (nm)
2:2:5:2	14.74	77.8	137.76 ± 1.26
3:2:5:2	17.76	64.8	152.53 ± 1.32
4:2:5:2	27.26	84.3	131.95 ± 2.62

liposomes and liposomal MEL were 22.87 ± 1.37 and 31.34 ± 3.11 mV, respectively. The UV absorbance (Figure 1C) showed peaks at 261 nm for both free MEL and liposomal MEL but not for blank liposomes. The TEM image (Figure 1D) revealed that liposomal MEL was distributed uniformly with a spherical shape, which was consistent with the particle size determination. Both blank liposomes and liposomal MEL displayed stability for 24 h (Figure 1F).

The dialysis method was introduced to study drug release behavior. The release profiles demonstrated that the release of liposomal MEL was slower than that of free MEL (Figure 1G). In the first 10 h, the cumulative release was 76.8% of free MEL and 63.2% of liposomal MEL. This property indicated that EPC/CHO/mPEG-DSPE liposomes might be a drug delivery system to reduce the exposure of normal tissues while reducing organ damage and drug side effects. At the same time, it might prolong the drug metabolism time *in vivo* and increase the exposure time of the tumor. Then, the MM cell lines were incubated with liposomal DiO for 4 h, which demonstrated that the liposomes could be taken up well by both ARD and P3X cells (Figure 1H).

3.2. Antimyeloma Activity of Liposomal Melphalan In Vitro. To examine the cytotoxicity of liposomal MEL and free melphalan in vitro, human MM cells ARD and murine MM cells P3X were treated with titration doses of the drugs for 72 h. The drugs inhibited cell growth in a dose-dependent manner in both cell lines (Figure 2A,B). Liposomal MEL and free MEL induced similar MM cell growth inhibition. After 72 h of treatment with ARD cell lines, the IC50 values for liposomal MEL and free MEL were 3.356 and 2.519 μM, respectively. For P3X cell lines, the IC50 values for liposomal MEL and free MEL were 2.283 and 8.002 μM, respectively. Liposome vehicle had no cytotoxicity to MM cells (Figure 2C). Liposomal MEL had minimum cytotoxicity to nontumoric peripheral blood mononuclear cells (PBMCs) (Figure 2D). In addition, both liposomal MEL and free MEL induced apoptosis in MM cell lines (Figure 2E,F) and primary MM cells isolated from the patients' bone marrow aspirations (Figure 2G,H). Overall, our data suggested that liposomal MEL and melphalan exhibited similar anti-MM activity in vitro. Under the working concentration, liposomal MEL had limited cytotoxicity to nontumoric cells.

3.3. Real-Time Distribution of Liposomal Particles In Vivo. To examine the liposomal particle distribution *in vivo*, we intravenously injected liposomal DiR, a liposomal particle encapsulated with florescent dye, into experimental mice. Free DiR-injected mice were used as controls. As shown in Figure 3A, after injection, liposomal particles were enriched in the bones of limbs. Furthermore, liposomal particles were stable *in vivo* during the test time. Forty-eight hours after injection, the mice were sacrificed, and the fluorescence intensity in each organ was examined (Figure 3B). Of note, leg bones from liposomal DiR-injected mice had significantly higher fluorescent intensity than those from free DiR-injected mice (Figure 3C). Overall, our findings showed that liposomal particles had bone marrow enrichment. Since MM is a bone cancer characterized by malignant cell accumulation in bone marrow, the feature of bone marrow enrichment of liposomal particles might facilitate drug delivery to the tumor site.

3.4. Organ Toxicity of Liposomal Melphalan In Vivo. To examine the organ toxicity of the drugs *in vivo*, we injected either liposomal MEL or free MEL into Balb/c mice. After treatment, peripheral blood was collected for liver and renal function and blood cell counts. Then, the mice were sacrificed, and organ biopsies were examined by H&E staining. As shown in Figure 4A, free MEL induced hepatocyte swelling and blurred cell boundaries and sheet necrosis in liver and colon mucosal cell necrosis, while in the liposomal MEL group, we only observed mild edema of hepatocytes without obvious hepatocyte necrosis and colonic mucosal necrosis. Furthermore, liver function tests showed that the levels of alanine aminotransferase (ALT), total bilirubin (TBIL), and lactate dehydrogenase (LDH) in the free MEL group mice were significantly higher than those in the liposomal MEL group mice ($p < 0.05$), all of which suggested that liposomal MEL reduced hepatotoxicity *in vivo* (Figure 4B–D). Overall, our data suggested that liposomal MEL had reduced organ toxicity *in vivo* in experimental mice compared with free MEL.

3.5. Antimyeloma Activity of Liposomal Melphalan In Vivo in a Myeloma Mouse Model. Finally, we examined the anti-MM activity of liposomal MEL *in vivo*. The animal study using a human MM xenograft mouse model was designed, as shown in Figure 5A. MM tumor growth *in vivo* was visualized by IVIS imaging (Figure 5B), and the luciferase intensity was
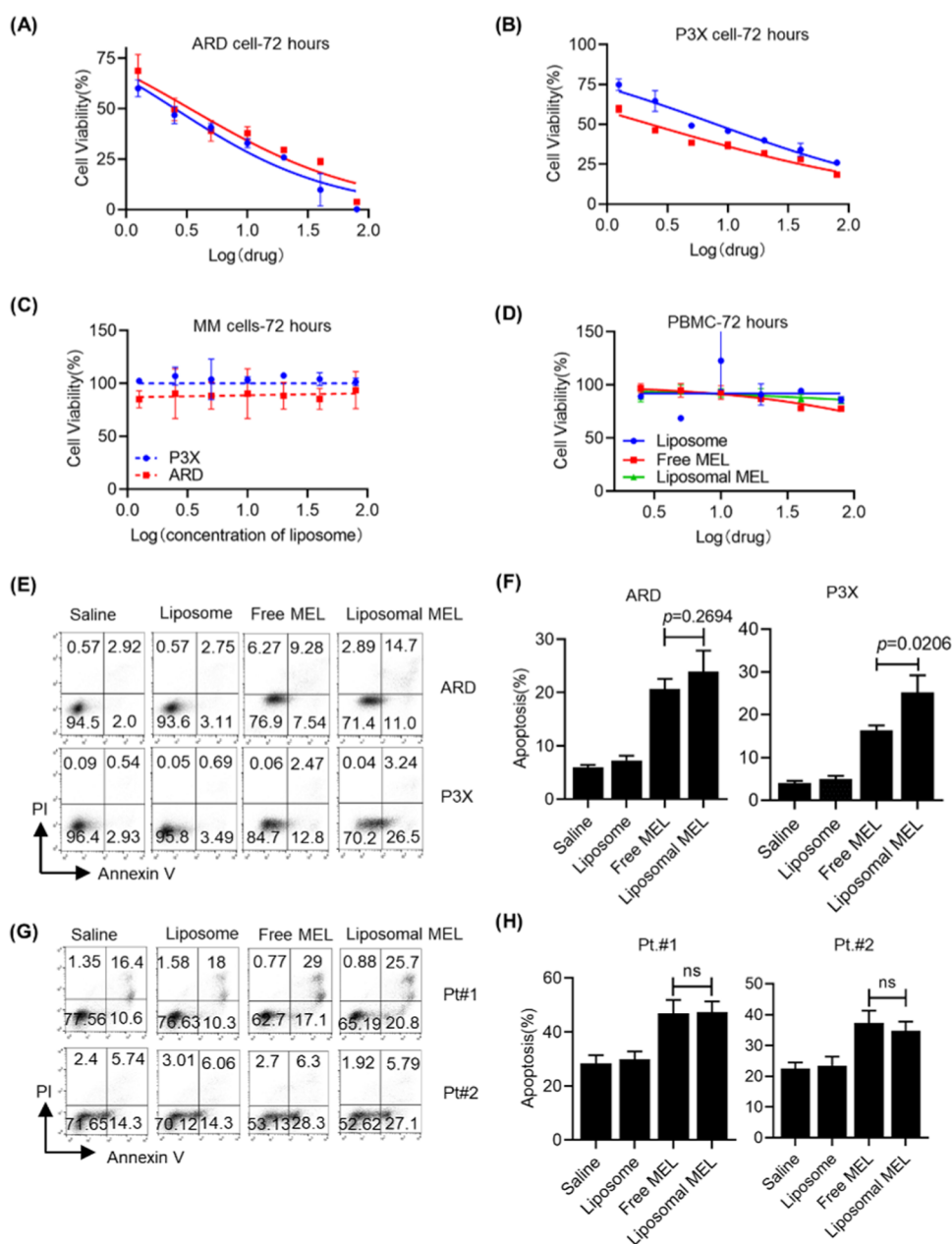
Figure 2. Cytotoxicity of liposomal melphalan in myeloma cell lines in vitro. (A) Cell viability of ARD cells after the treatment of titrations of liposomal MEL and free MEL for 72 h; (B) same experiment in P3X cells; (C) cell viability of ARD or P3X cells after treatment of titrations of liposome for 72 h; (D) cell viability of PBMC cells after treatment of titrations of liposome, free MEL, or liposomal MEL for 72 h; (E) cell apoptosis of ARD or P3X cells after treatment of liposomal MEL (15 μM), MEL (15 μM), or liposome (15 μM) for 48 h; (F) result quantification; (G) same apoptosis assay using primary MM cells isolated from newly diagnosed MM patients' bone marrow aspirations; and (H) result quantification. Data are presented as the mean $\pm$ SD ($n = 3$). All results were obtained from three independent experiments.

quantified (Figure 5C). The data suggested that both liposomal MEL and free MEL exhibited strong anti-MM effects *in vivo*. The changes in tumor burden after treatment were also examined by circulating M protein in mice (Figure 5D). Tumor-bearing mice treated with either liposomal MEL or free MEL had prolonged survival (Figure 5E). The body weight of mice during treatment suggested that liposomal MEL or free MEL was well tolerated by the animals (Figure 5F). In summary, liposomal MEL exhibited potent anti-MM activity *in vivo*, no worse than the well-established anti-MM agent melphalan.

4. DISCUSSION

Melphalan is a conventional anti-MM therapeutic that has been used in MM treatment for more than 50 years. After long-term use of the drug, both the strength (e.g., potent anti-MM activity, quick effectiveness) and weakness (e.g., organ toxicity and side effects) of melphalan are well recognized. Many efforts have been made to modify melphalan to improve its anti-MM activity and reduce its side effects. The most successful melphalan-based drug development might be melphalan flufenamide, also known as melflufen. Melflufen is a peptide-conjugated melphalan, chemically ethyl ester of a dipeptide consisting of melphalan and para-fluoro-L-phenyl-
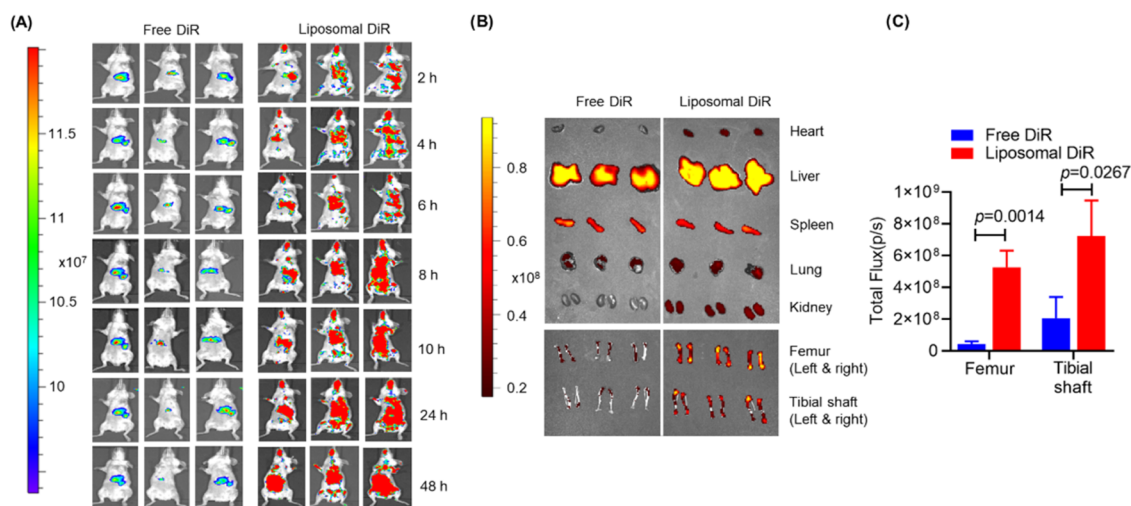
Figure 3. Liposomal particles have bone marrow enrichment in vivo. (A) Liposomal DiR or free DiR was injected into the tail vein of Balb/c mice. The distribution of *liposomes in vivo* was examined by IVIS. (B) Forty-eight hours after dye inoculation, the mice were sacrificed. Different organs were isolated for fluorescence intensity examination by IVIS. (C) Quantification of liposomal particle distribution in leg bones. $p < 0.05$ (Student's t test). Data are presented as the mean $\pm$ SD ($n = 3$).
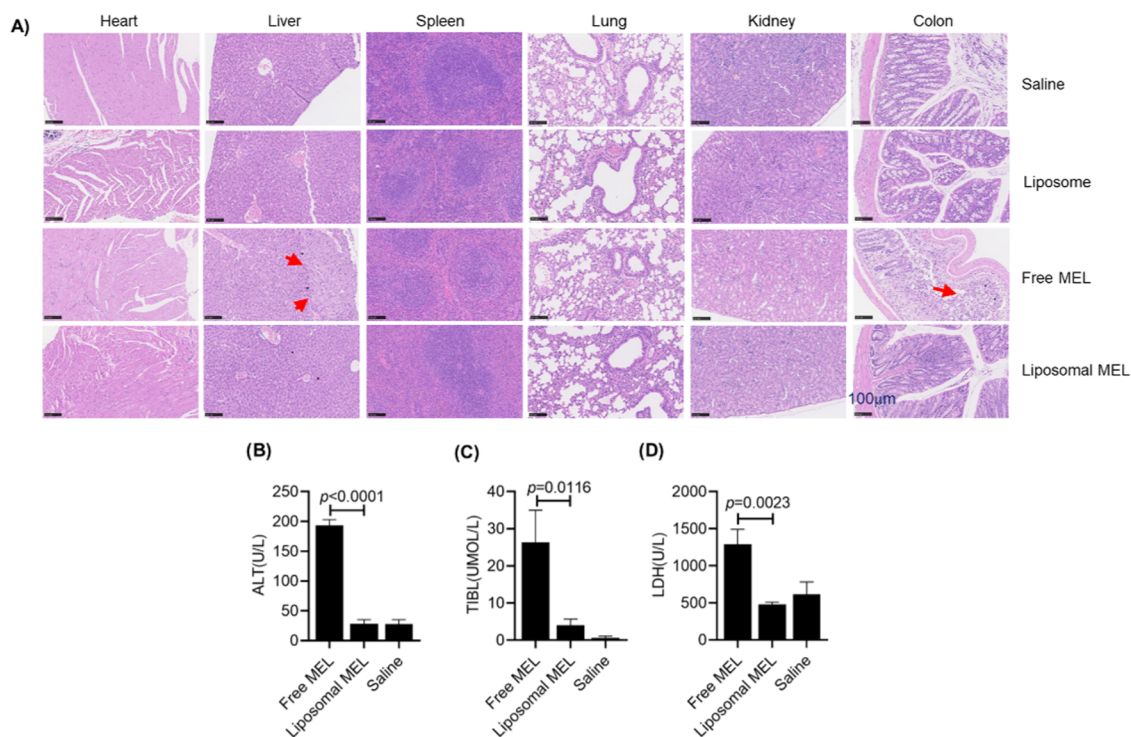

Figure 4. Liposomal melphalan exhibits reduced toxicity in vivo. (A) H&E staining images of the major organs of mice after drug administration for 7 days (saline, liposomes, free MEL (5 mg/kg), or liposomal MEL (5 mg/kg)). Hepatocyte swelling, blurred cell boundaries, and sheet necrosis in the liver and colon mucosal cell necrosis were obviously observed in the free MEL group (the red arrows shown). (B)−(D) Examination of hepatic function (ALT, TBIL, and LDH) ($n = 3$). Data are presented as the mean $\pm$ SD ($n = 3$).

alanine. Peptide conjugation conferred a hydrophilic nature to melflufen, which entered cells more efficiently than the parental melphalan compound. Within cells, melflufen can be processed by aminopeptidase to release free melphalan for function.[14] By 2021, melflufen had been temporally approved by the FDA for relapsed or refractory MM (RRMM) treatment.[15] The other melphalan modifications were mainly in preclinical stages. Several researchers synthesized melphalan derivatives, chemically modified melphalan, to achieve superior anti-MM activity.[16] Some modifications to melphalan aimed to

alter the biophysical features of melphalan. For example, Ajazuddin et al. synthesized a pegylated melphalan conjugate to improve the drug's aqueous solubility.[17] Polyethylene glycol-conjugated melphalan might have improved drug delivery *in vivo*, thus enhancing drug activity.[18] In addition to the chemical modification of the melphalan backbone, researchers have also attempted to integrate the drug with different delivery systems. Kalimuthu et al. prepared a peptide−melphalan conjugate and loaded it into gold nanoparticles with further surface modification on the particles.
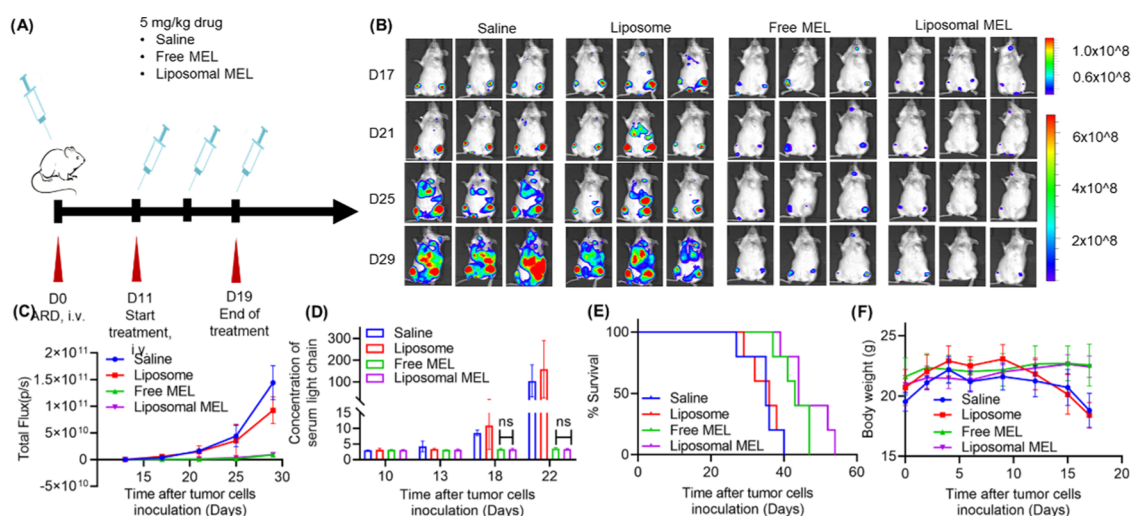
Figure 5. Liposomal melphalan inhibits myeloma tumor growth in vivo. (A) Illustration of the treatment assay in a human MM xenograft mouse model; (B) after tumor establishment, the tumor-bearing mice were randomly divided into four groups with five mice per group (results are presented for three out of five mice). The mice were treated with tail vein injection of saline, liposomes, free MEL (5 mg/kg), or liposomal MEL (5 mg/kg). Tumor growth in vivo was examined by in vivo bioluminescence spectroscopy. (C) Serum monoclonal globulin levels were examined by ELISA. (D) Total luminous flux in each group was examined. (E) Survival of mice in each group. (F) Body weight of mice in each group. Data are presented as the mean ± SD (n = 3).

Such nanoparticles retained the cytotoxicity of melphalan with slow drug release.[19] Alternatively, melphalan or melphalan prodrug might be loaded into liposomes to improve the drug's efficacy.[20,21] Liposomes have a phospholipid bilayer and are widely used as vesicles for drug encapsulation. Liposomal reconstitution of drugs might confer new physicochemical features to the drug and improve the drug's stability, solubility, delivery in vivo, and targeting.[22] For example, CPX-351 (also known as Vyxeos), a liposomal encapsulated daunorubicin and cytarabine, has been used in acute myeloid leukemia treatment.[23] As early as the 1980s, researchers constituted liposomal melphalan particles and investigated their distributions in vivo in experimental animals.[24] Later, the anticancer activity of liposomal melphalan was tested in different tumor-bearing animal models, such as models of plasmacytoma,[25] melanoma,[26] retinoblastoma,[27] and breast cancer.[28] In general, liposomal melphalan exhibited potent anticancer activity with reduced systemic toxicity.

Melphalan is a potent chemotherapeutic agent in MM treatment, and melphalan-based drug development and delivery optimization have been investigated since the 1980s. The studies for liposomal melphalan in past decades mainly focused on pharmacokinetics in solid tumor models, including lymph node uptake in metastasis animal models and plasma clearance.[24,25] Another research area was the efficacy of heat-sensitive liposomal melphalan combined with local hyperthermia in animal models of melanoma.[26,29] Recently, Sambamoorthy et al. constituted vitamin E oil-incorporated liposomal melphalan particles and showed that the particles exhibited cytotoxicity to MM cells in vitro.[20] Compared with previous studies of liposomal melphalan, our work is novel and valid. First, we used EPC/CHO/mPEG-DSPE to constitute a liposomal capsule, which is different from previous liposomal MEL formulations. It is valid to test different formulas for liposomal encapsulation and find an appropriate one for further investigation. Second, we focused on MM. We performed in vitro and in vivo experiments to characterize the liposomal MEL, not only its physicochemical features but

also its anti-MM activity and toxicity in vitro and in vivo. Very few similar studies have performed in vivo experiments in animal models. However, in vivo assays provided more reliable results to address the organ toxicity of liposomal MEL. Our data suggested that liposomal encapsulation conferred particle bone marrow enrichment in vivo. Liposomal MEL had potent anti-MM activity, similar to that of free melphalan. Furthermore, liposomal MEL reduced systemic organ toxicity in vivo in a human MM mouse model. Overall, our data provide a preliminary landscape of liposomal MEL usage in MM treatment.

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Notes

The authors declare no competing financial interest.

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