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Original Article

Dual Drug-Loaded Liposomes for Synergistic Efficacy in MCF-7 Breast Cancer Cells and Cancer Stem Cells

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Breast cancer stem cells (BCSCs) in breast cancer cells have self-renewal ability and differentiation potential. They are also resistant to drugs after chemotherapy. To overcome this resistance, we designed negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG)-based liposomes for drug delivery. These liposomes have enhanced the therapeutic effects of a range of antitumor therapies by increasing the cellular uptake and improving drug delivery to targets sites. In this study, we investigated whether DMPG-POPC liposomes, including the neutral lipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholin (POPC), can specifically bind to MCF-7 breast cancer cells and increase cellular uptake compared with that by CHOL-POPC liposomes. We also estimated the cytotoxicity of DMPG-POPC liposomes encapsulated with both metformin (Met) and sodium salicylate (Sod) against breast cancer cells and BCSCs compared with that of the free drugs. Our results demonstrated that these dual drug-encapsulated liposomes significantly enhanced the cytotoxic and anti-colony formation abilities compared with individual drug-encapsulated liposomes or free drugs in BCSCs. Overall, our results suggest that DMPG-POPC liposomes containing two drugs (Met + Sod) show promise for synergistic anti-cancer therapy of breast cancer by increasing drug delivery efficiency into breast cancer cells and BCSCs.

Key Words: Cancer stem cell, Combination chemotherapy, DMPG, Liposomes, Drug delivery, Metformin, Sodium salicylate

INTRODUCTION

Breast cancer is the most common type of cancer in women and the second most common cause of cancer-related death in women (Zhang et al., 2014). Breast cancer-related death is mainly due to the development of metastatic ability of the primary tumor (Minn et al., 2005). Considering the high mortality rate, it is critical to perceive the mechanisms underlying metastasis and verify new targets for breast cancer therapy. In the last few decades, despite various trials on cancer therapy, breast cancer has remained unconquered because of the invasive characteristics (Mukherjee et al., 2014; Kim et al., 2017a). The metastatic nature of breast cancer might be due to the existence of breast cancer stem cells (BCSCs; approximately 1% population) within a large population of breast cancer cells. Breast cancer stem cells (CSCs) are tumor cells that have the abilities of proliferation and self-renewal, and differentiation potential (Li et al., 2008). In addition, several studies have proved that CSCs develop resistance to routine chemotherapy and radiotherapy, leading to cancer recurrence after treatment (Dean

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et al., 2005; Gangemi et al., 2009; Kai et al., 2015; Lee, 2018).

Metformin (Met) is a drug that is generally prescribed for type 2 diabetics. Recently, metformin has attracted attention as a potentially beneficial drug for treating cancer. Several studies have demonstrated that patients with type 2 diabetes taking metformin have a considerably diminished risk of cancer and cancer-related death than those with diabetes not taking metformin (Evans et al., 2005; Bowker et al., 2006; Libby et al., 2009). In patients with breast cancer, especially those with diabetes, it has been implied that metformin improves sensitivity to chemotherapy compared with that in patients not taking metformin (Jiralerspong et al., 2009). Furthermore, recent studies have found that metformin promotes the induction of apoptosis and cell cycle arrest in several cancer cells (Dowling et al., 2007; Zakikhani et al., 2008; Alimova et al., 2009; Liu et al., 2009; Kim et al., 2017b), as well as prevents cellular transformation and CSC growth by inhibiting the associated inflammatory response (Hirsch et al., 2013) or suppressing CSC-specific gene expression (Bao et al., 2012).

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, sodium salicylate, and indomethacin—candidate anticancer drugs—are widely used to treat inflammation (Amann and Peskar, 2002). Aspirin and its natural deacetylated form sodium salicylate (Sod) promote anti-inflammatory activities by restraining the expression of diverse proinflammatory molecules, including prostaglandin (Amann and Peskar, 2002; Ulrich et al., 2006; Flossmann and Rothwell, 2007). These NSAIDs have also been revealed to possess tumor repressive activities by inducing apoptosis and cell cycle arrest in breast, lung, colon cancers, and leukemia (Bellosillo et al., 1998; Klampfer et al., 1999; Law et al., 2000; Dikshit et al., 2006). However, both metformin and sodium salicylate limit the sensitivity to anti-cancer therapies such as common chemotherapy and radiotherapy. Therefore, the development of breast cancer- and BCSC-targeting drug delivery systems is desirable to optimize anti-breast cancer and anti-BCSC therapies.

Nanocarriers are regarded as a useful platform for chemotherapeutic drug delivery, allowing therapeutic drugs to specifically target cancer cells by minimizing toxicity to normal cells (De Jong and Borm, 2008). Liposomes among various nanocarriers have been used as the most standard vehicle for targeted drug delivery. They consist of amphiphilic phospholipid bilayers, which can be encapsulated with both hydrophilic and hydrophobic drugs. Liposomes are also ideal nanocarriers for anti-breast cancer and anti-BCSCs therapeutics owing to their desirable characteristics such as biocompatibility, ease of surface modification, and prolonged blood circulation (Song et al., 2015; Jeong et al., 2016). However, it is still challenging to specifically deliver liposome-encapsulated drugs to cancer cells. This challenge has been overcome by designing a new class of lipids for liposomes. The new phenomenon of surface modification of liposomes can lead to the formulation of advanced drug-delivery systems using polyethylene glycol (PEG)-coated liposome modified with 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) (Nag et al., 2013). According to recent studies, the improved activity of liposomal formulation is attributable to intra-liposomal activity by reaction with DMPG. The liposomal composition with DMPG and phosphatidylcholine (PC) lipids facilitate higher entrapment of a drug and flexibility in the selection of formulation, which delivers a higher proportion of drugs to the tumor (Zou et al., 1996). Thus, it is essential to use the DMPG-based liposomal delivery system that can deliver anticancer agents to breast cancer cells and BCSCs selectively. The aim of this study was to increase the therapeutic efficacy of a combination of metformin and sodium salicylate using the DMPG-POPC liposome delivery system.

MATERIALS AND METHODS

Materials

1,2-dimyristoyl-*sn*-glycero-3-phosphorylglycerol sodium salt (DMPG), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(po-lyethylene glycol)-2000 ammonium salt ($PEG_{2000}PE$), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholin (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl] (Rho-DOPE), and cholesterol (CHOL) were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA). Metformin and sodium salicylate were procured from Sigma-Aldrich (St. Louis,

MO, USA). Human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

MCF-7 breast cancer cell culture

MCF-7 cells were maintained in Eagle's minimum essential medium (EMEM) (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GE healthcare, Chicago, IL, USA) and 1% penicillin and streptomycin (GE healthcare, Chicago, IL, USA). The cell lines were cultured in an incubator with 5% CO_2 at 37℃.

Breast cancer stem cells culture

Mammosphere culture has been widely used to enrich CSCs (Ji et al., 2016). Therefore, for mammosphere culture in this study, single-cell suspensions of MCF-7 cells were seeded at 20,000 cells per well in six-well ultralow adherence plates (Corning, NY, USA) with DMEM/F-12 (Corning, NY, USA) containing 5 mg/mL insulin, 2% B27 (Invitrogen Ltd., Paisley, Scotland), 20 ng/mL epidermal growth factor (EGF), and 20 ng/mL basic fibroblast growth factor (FGF) (2 mL per well). The cultures were passaged every seven days. When passaged, BCSCs were harvested and washed in 5 mL of DPBS. After centrifugation at 1,000 rpm for 3 min, the harvested cells were gently suspended in trypsin and serum-free DMEM/F12 medium at a 1:1 ratio, and dispersed by pipetting with a 23-gauge needle. After confirming the dissociation of cells, the cells were pelleted and suspended in BCSCs culture medium at a concentration of 20,000 cells per well before placing in ultralow adherence six-well plates.

Isolation of BCSCs with magnetic beads

$CD44^+/CD24^-$ are the expression biomarkers of interest in MCF-7-derived mammospheres. After continuous culture for two weeks, BCSCs were gathered for identification of MCF-7 BCSCs phenotype and purity. The gathered BCSCs were separated by pipetting with a 23-gauge needle and washed in DPBS two times. BCSCs enriched with $CD44^+/CD24^-$ were sorted from MCF-7 mammospheres using a MagCellect $CD24^-/CD44^+$ breast Cancer Stem Cell Isolation Kit (R&D System, Minneapolis, MN, USA) according to their protocol. Initially, $CD24^+$ cells were labeled and removed magnetically. Thereafter, from the $CD24^-$ population, $CD44^+$ cells were sorted magnetically using a biotinylated human antibody and streptavidin-conjugated magnetic beads as a positive selection model. The efficiency of sorting was assessed by staining the recovered cells with fluorochrome-conjugated anti-human $CD44^+$ and $CD24^-$ antibodies. Isolated $CD44^+/CD24^-$ cells were later identified via FACS (ACEA Biosci-ences Inc, San Diego, CA, USA) analysis.

Preparation of liposomes loaded with metformin and sodium salicylate formulations (dual drug-loaded liposomes)

CHOL-POPC liposomes composed of PEG-DSPE, CHOL, and POPC (2:33:65, %mol) and DMPG-POPC liposomes consisting of PEG-DSPE, DMPG, and POPC (2:33:65, %mol) were prepared. All lipids were mixed with chloroform -methanol mixture (2:1, v/v). The chloroform-methanol mixture was evaporated with nitrogen gas, and then dried using a vacuum pump for 1 h to form a thin film of lipid. After drying, the lipid film was hydrated with 1 mL of Met and Sod solution for 1 h at 60℃ in water bath. The solution was sonicated for 5 min at 60℃. To prepare unilamellar vesicles from multilamellar vesicles, the liposome solution was extruded 10 times via Whatman nuclepore polycarbonate membrane filters (GE Healthcare, Chicago, IL, USA) of pore size ranging from 800 to 100 nm on an Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL, USA). The uncapsulated drugs (Met or sodium salicylate) were separated from the liposome formulation by CL-4B gel-filtration. The total amount of encapsulated Met was determined by high performance liquid chromatography after disrupting the vesicles with Triton X-100. The drug content was analyzed at 235 nm using a liquid chromatograph Alliance 2690 (Waters). The column used was a C18 (60 Å, 4 μm; Waters) and the mobile phase was PBS at pH 6, delivered at a flow rate of 0.8 mg/mL.

Observation of binding of CHOL-POPC and DMPG-POPC liposomes to cells

The cellular binding affinity of CHOL-POPC and DMPG-POPC liposomes was evaluated in MCF-7 cells (5×10^5 cells/well) with an Axio Zeiss A1 Imager compound micro-

scope (CarlZeiss, Oberkochen, Germany). The cells were grown in four-well-chamber slides (Nalgene Nunc International Corp., Naperville, IL, USA) for 24 h. Rhodamine-labeled CHOL-POPC and DMPG-POPC liposomes were added to MCF-7 cells, and then the treated cells were incubated for 5 min at 37℃. After washing three times with DPBS, the cells were immediately investigated using an Axio Zeiss A1 Imager compound microscope (×40).

Cytotoxicity assay

MCF-7 cells were seeded at a concentration of 40,000 cells per well in 96-well plates. After 24 h, the medium was removed, and the cells were treated with Met (100, 200, and 300 mM), Sod (40, 80, and 160 mM), or DMPG-POPC liposome loaded with these drugs. The free drugs or drug-loaded DMPG-POPC liposomes were removed after 4 h of incubation at 37℃, and then 100 μL of serum-containing medium was added to each well. After another 24 h, 10 μL of EZ-CyTox reagent (Daeil Lab Service, Seoul, South Korea) was added to each well and incubated for 1 h at 37℃. The absorbance of the samples was measured at 450 nm using an ELISA microplate reader. Mean optical density at each drug concentration was calculated after discarding the highest and lowest values. The anti-tumor effects of respective drugs (Met or Sod) for each MCF-7 cell line were shown in terms of inhibitory concentration at 25% (IC_{25}) or 50% (IC_{50}) for Met or Sod, which was determined by plotting the graph of percentage of cell growth inhibition (Y-axis) versus drug concentration (X-axis). IC_{25} and IC_{50} values were expressed as mean and standard errors (SE). The assays were repeated more than three times.

To determine the combination effects of Met and Sod in BCSCs, the isolated BCSCs were seeded in 96-well ultralow adherence plates (Corning, NY, USA) (5,000 cells/well) and incubated for 2 h. The cells were treated with doxorubicin (Dox), Met, Sod, or a combination of free drugs [Met and Sod] and drug-loaded DMPG-POPC [Met and Sod] (Combination), compared with $CD44^{-/low}/CD24^{+}$ population, and then analyzed using the WST-1 method after 48 h. The absorbance of the samples was measured at 450 nm using an ELISA microplate reader.

Colony formation assay

Breast cancer stem cells were seeded in a six-well plate at 5,000 cells/well concentration. After 2 h, the cells were treated with control, Lipo-Empty, free drugs (Dox, Met, Sod, and Met+Sod), and drug-loaded CHOL-POPC and DMPG-POPC liposomes (Dox, Met, Sod, and Met+Sod) to each well. After incubation for 14 days, the visible colonies were stained with 1 mL of crystal violet for 10 min, and then washed three times with DPBS after each step. After drying, the colonies were observed and scored under an inverted microscope. The percentage of colony formation was calculated using the following equation:

$$\text{Colony formation (\% of control)} = \frac{\text{Number of colonies after treatment}}{\text{Number of colonies of control}} \times 100$$

Statistical analysis

The experiment results are represented as mean ± SD (standard deviation). Statistical significance was determined by two-tailed Student's *t*-test. The results with $P < 0.05$ or $P < 0.01$ were regarded statistically significant.

RESULTS

Characterization of breast cancer stem cells

Breast cancer stem cells originated from MCF-7 cells grew as non-adherent mammospheres. The mammospheres of BCSCs were formed after culture for one week, and their diameter increased for three weeks (Fig. 1A). To compare the phenotypes of MCF-7 cells and BCSCs, BCSCs were isolated, and then mixed with anti-CD44-PE and anti-CD24-APC depending on the property of BCSCs, including $CD44^{+}/CD24^{-}$/low surface markers. The proportion of $CD44^{+}CD24^{-}$/low cells in BCSCs at 7, 14, and 21 days after culture and that in MCF-7 cells was 20.38%, 40.34%, 21.51%, and 6.51%, respectively, determined by flow cytometry (Fig. 1B). Compared with MCF-7 cells, BCSCs separated from MCF-7 cells inhibited normal CSC-like nature. Therefore, these results verified the successful isolation of BCSCs from MCF-7 cells.

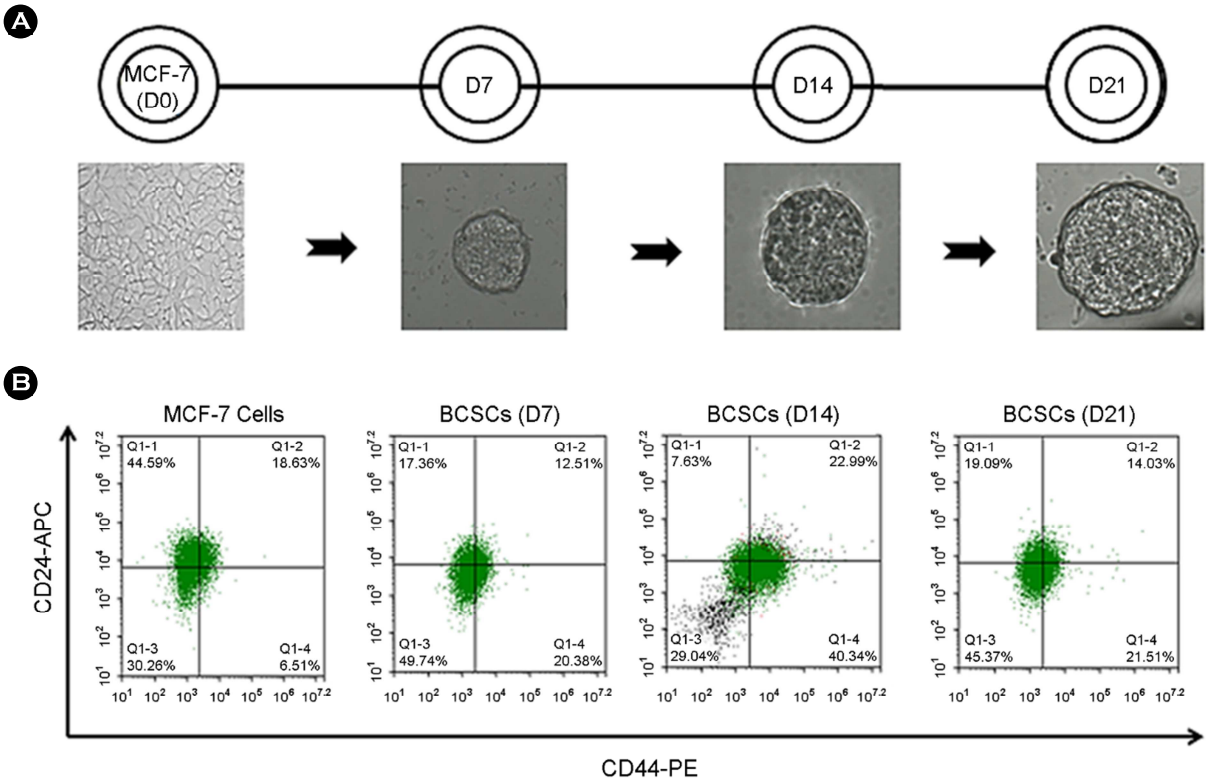

Fig. 1. Isolation and characterization of breast cancer stem cells (BCSCs). (A) Morphology of BCSCs from MCF-7 cells at 7, 14 and 21 days of culture, observed using the microscope. MCF-7 cells were capable of forming BCSCs of about 50 mm in diameter under BCSCs culture condition for 1 week. (B) BCSCs and MCF-7 cells were analyzed using fluorescence activated cell sorting (FACS) and showed cell surface proteins. BCSCs at 14 days showed higher levels of $CD44^{+}$ $CD24^{-}$/low populations than MCF-7 cells or BCSCs at 7 and 21 days cells.

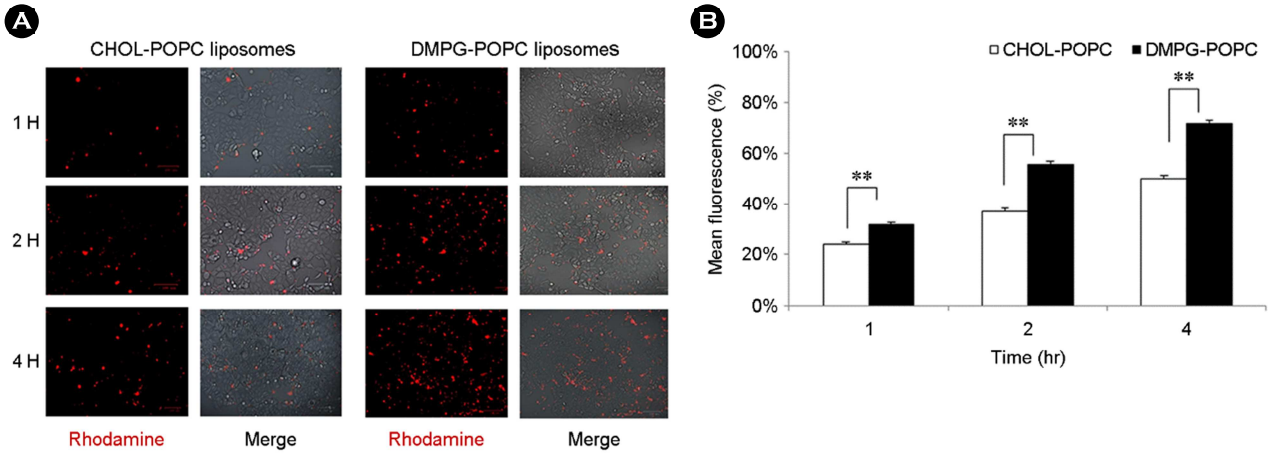

Fig. 2. Cell uptake of CHOL-POPC or DMPG-POPC liposomes to MCF-7 breast cancer cells. (A) MCF-7 cells treated with CHOL-POPC and DMPG-POPC liposomes were incubated in time dependent (1, 2, and 4 hrs). MCF-7 cells were washed with DPBS buffer three times and were observed using fluorescence microscope. The treated concentration of rhodamine labeled liposome was fixed at 20 μg in all experiments and showed red color in each cells. (B) Red fluorescence of each rhodamine-labeled liposome on MCF-7 cells evaluated using Tail® image based cytometer. Error bars represent standard deviation of three independent experiments (n=3, $^{**}P<0.01$ when compared to DMPG-POPC with CHOL-POPC liposome).

Binding of CHOL-POPC or DMPG-POPC liposomes to MCF-7 breast cancer cells

To compare cellular binding of CHOL-POPC and DMPG-POPC liposomes, MCF-7 cells were made to take up CHOL-POPC and DMPG-POPC liposomes for different intervals (1, 2, and 4 h). As shown in Fig. 2B, cell surface binding of DMPG-POPC liposome was 32%, 57%, and 71% on MCF-7 cells at 1, 2, and 4 h. Whereas, CHOL-POPC liposome bound to MCF-7 cells showed relatively low uptake of 24%, 37%, and 50% at 1, 2, and 4 h, respectively. The cell binding of these DMPC-POPC liposomes increases at least 20% ($P<0.01$) on the surface of MCF-7 cells at 4 h when compared to CHOL-POPC liposomes. Fluorescence images in Fig. 2A verified these results. Furthermore, DMPG-POPC liposomes bound rapidly to the cells at 1~4 h, and a strong signal was observed by fluorescence microscopy. These results demonstrated that DMPG-POPC liposome nanoparticles can cross the cell membrane barrier better than CHOL-POPC liposomes. Hence, we used DMPG-POPC liposomes to validate the antitumor effect of drug-encapsulated liposomes in comparison with that of free drugs on breast cancer

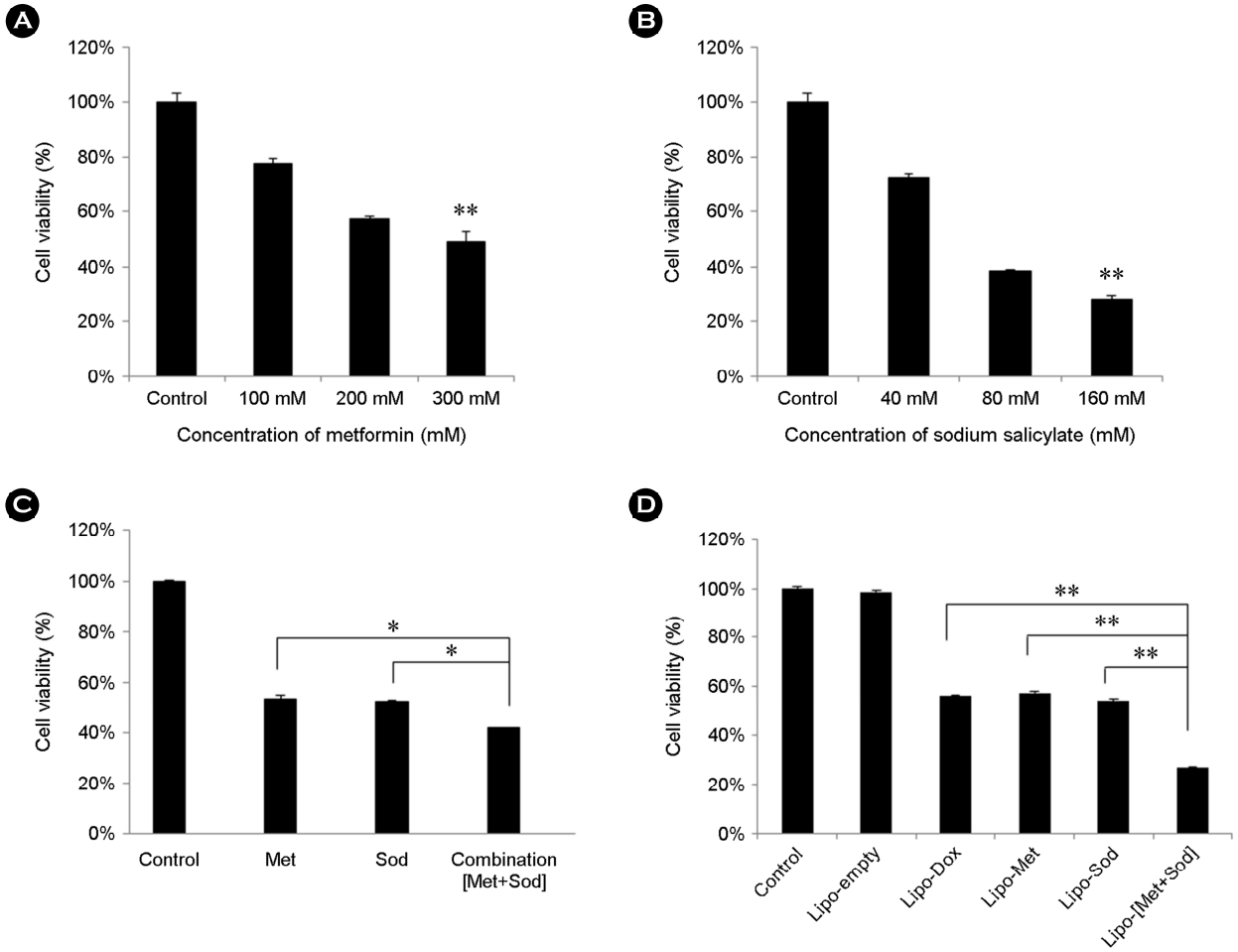

Fig. 3. Cytotoxicity of the dual drug-loaded liposomes in MCF-7 cells. The all control groups were treated with saline. (A and B) To evaluate of IC_{50} for each two-drug, MCF-7 cells were incubated with various concentration of metformin or sodium salicylate. Cell viavility was each monitored by WST assay method using EZ-CyTox reagents after 48 hrs. (C and D) To measure the cytotoxicity senergic effect of the dual drug-loaded liposomes, MCF-7 cells were treated with the combined the free dual drugs (combination) or the dual drug-loaded liposomes (Lipo-[Met+Sod]) for 48 hrs. Data are represented as the mean ± standard deviation (n=3). A; $^{**}P<0.01$ compared with the control, B; $^{**}P<0.01$ compared with the control, C; $^{*}P<0.05$, compared with metformin or sodium salicylate respectively, D; $^{**}P<0.01$ compared with Lipo-Dox, Lipo-Met or Lipo-Sod respectively.

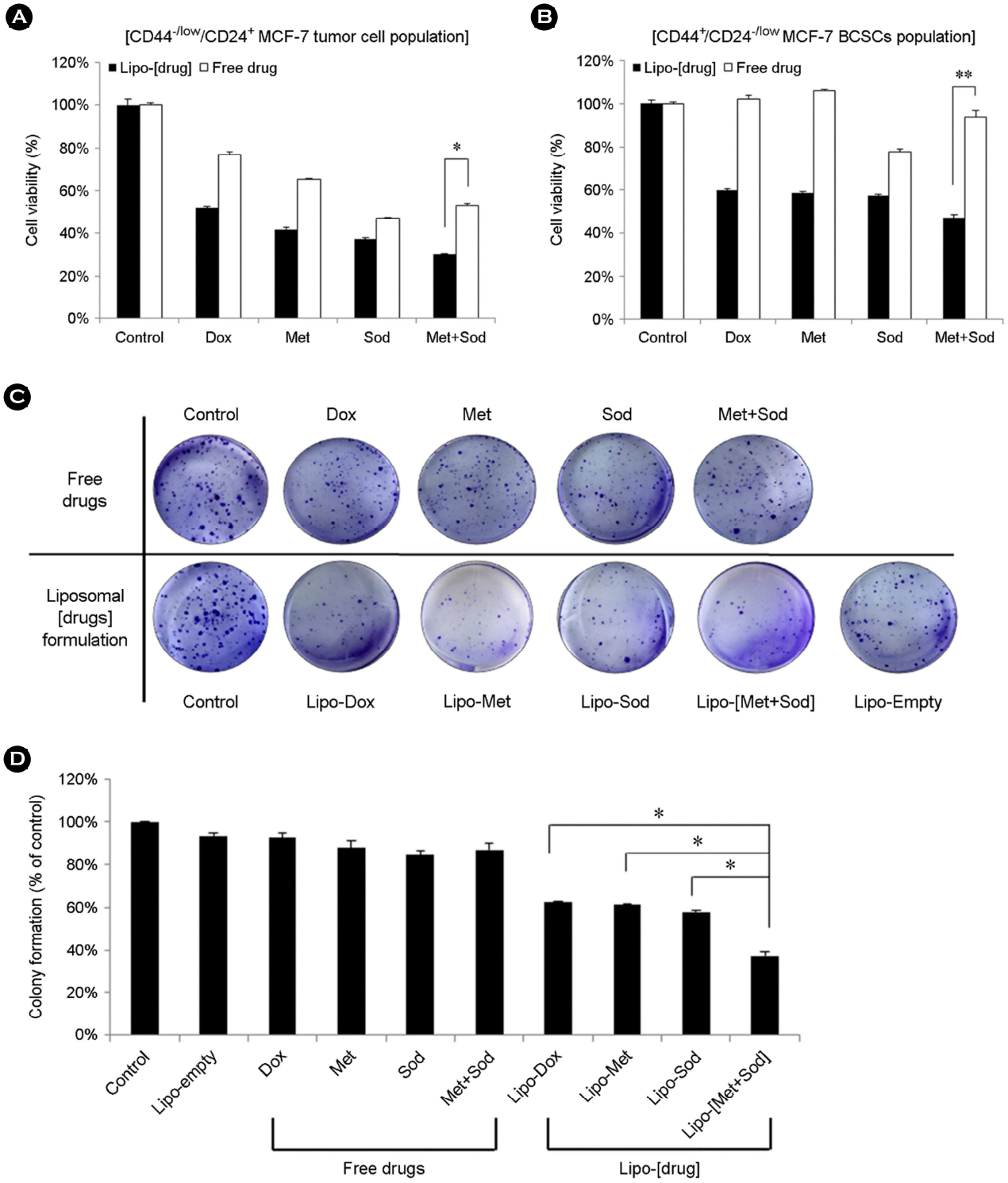

Fig. 4. Anti-proliferation effect of dual drug-loaded liposomes on BCSCs. The growth of the isolated $CD44^{-/low}/CD24^{+}$ MCF-7 tumor cells (A) and the isolated $CD44^{+}/CD24^{-/low}$ MCF-7 BCSCs (B) treated with free drugs (Dox, Met, Sod, and Met+Sod) or liposomal encapsulated drugs (Lipo-Dox, Lipo-Met, Lipo-Sod, and Lipo-[Met+Sod]) for 48 hours was determined by WST-1 assay. The data were caculated as percentage of contol (DPBS). The colonies formed in BCSCs-seeded 6-well plate after treatment of various free drugs or liposomal encapsulated drugs for 14 days were stained with crystal violet (0.5% w/v) and obserbved by a bright-field microscope (C) and quantfied (D). Data shown represent the mean ± SD of three experiments (*P<0.05, **P<0.01). **Abbreviations:** BCSCs, breast cancer stem cells; DPBS, Dulbecco's phosphate buffered saline; Dox, doxorubicin; Met, metformin; Sod, sodium salicylate, Lipo-, liposomal encapsulated; SD, standard deviation.

cells and BCSs.

In vitro cytotoxicity of dual drug-loaded DMPG-POPC liposomes in MCF-7 cancer cells

To compare the combination effect on cytotoxicity between free dual drugs (Met and Sod) and dual drug-loaded DMPG-POPC liposomes, we evaluated their efficacy in MCF-7 breast cancer cells *in vitro*. First, the cytotoxicity of the free drugs was compared with that of the combined free drugs. Combined free drugs [Met+Sod] (IC_{25} of Met (150 mM) and IC_{25} of Sod (30 mM); Fig. 3C) exhibited approximately 15% increase ($P<0.05$) in cell death relative to that of each free drug (IC_{50} of Met (300 mM) and IC_{50} of Sod (60 mM); Fig. 3A and B). Next, the cytotoxicity of dual drug-loaded DMPG-POPC liposomes, Lipo-[Met+Sod] (IC_{25} of Met (150 mM) and IC_{25} of Sod (30 mM)), was more cytotoxic than single drug-loaded liposomes (Fig. 3D). The results demonstrated that dual drug-loaded DMPG-POPC liposomes group (Lipo-[Met+Sod]) was taken up more into MCF-7 cells than free combined drugs, and they acted more efficiently to suppress cell proliferation that that of free drug group.

In vitro evaluation of dual drug-loaded DMPG-POPC liposomes in MCF-7 BCSCs

To evaluate the synergic effect against cell proliferation between single free drug (Met or Sod) and the dual drug-loaded DMPG-POPC liposomes, we conducted the WST-1 assay in BCSCs. The isolated $CD44^{+}/CD24^{-/low}$ MCF-7 BCSC population and $CD44^{-/low}/CD24^{+}$ MCF-7 tumor cell population were treated with the same concentrations of drugs (Fig. 3 C and D). In the $CD44^{+}/CD24^{-/low}$ population, there was no difference in the cytotoxic effect among each free drug group (Fig. 4B). In contrast, compared with the combined free drug (Met+Sod)-treated BCSCs, dual drug [Met+Sod]-DMPG-POPC liposome-treated cells showed significant cell growth inhibition or cell death (Fig. 4B). However, a more growth-inhibitory effect was observed in BCSCs than in MCF-7 cells. Collectively, these data showed that the DMPG-POPC-based liposome delivery system displayed higher decrease in chemo-resistance and increase in sensitivity to anti-cancer therapy of BCSCs than free drugs.

Colony formation assay

Long-term cytotoxicity assaying on 14 days after the treat ment of free drugs (Met or/and Sod) or dual drug [Met+Sod]-DMPG-POPC liposomes on BCSCs was analyzed using a colony formation assay. Fig. 4 (C and D) shows that the colony formation ability of Lipo-Empty (95%) group or free drug (average 91%) group was similar to that of the control (100%) group. On the contrary, the rate of colony formation was diminished when BCSCs were treated with drug-loaded DMPG-POPC liposomes (Lipo-Dox, Met, and Sod). The Lipo-[Met+Sod] group was the most effective in decreasing colony formation to 36% ($P<0.05$).

DISCUSSION

In recent years, liposome-based drug delivery systems have gained remarkable interest, as they improve the efficacy of traditional therapeutics through controlled release, enhanced tumor accumulation, and reduced systemic toxicities (Allen and Cullis, 2013). However, a major problem in the current cancer therapy is drug resistance of metastatic cancers, resulting in 90% drug failures (Longley and Johnston, 2005). This drug resistance is believed to be because CSCs are exposed to small amounts drugs, increasing the risk of recurrence of cancer, which rapidly grows and resists chemotherapy or radiotherapy (Vinogradov and Wei, 2012). Thus, it is important to develop a novel platform that overcomes drug resistance and accumulates anti-cancer drugs at the tumor site.

In this study, we designed two liposomal formulations, DMPG-POPC- and CHOL-POPC-based liposomes, to choose a liposomal formulation that can increase their uptake by target cells effectively. Negatively charged DMPG tends to inhibit aggregation and enhances liposome stability in suspension during storage, preventing the leakage of reagents from the liposomal formulation compared with that by positively or neutrally charged liposomes (Alhariri et al., 2017). Therefore, in our design, the liposomes were selected owing to their advantage of possessing negatively charged DMPG lipid. Our results demonstrated that DMPG-POPC-based liposomes have improved cellular-binding efficacy *in vitro*

compared with that of the CHOL-POPC-based liposomes in MCF-7 cells in a time-dependent manner (Fig. 2). Furthermore, with DMPG-POPC liposomes, we could increase the loading of two drugs with relatively higher efficiency to increase the effectiveness of liposomal nanoparticles reaching the tumor site.

The cytotoxicity assay revealed that dual drug-loaded DMPG-POPC liposomes (Lipo-[Met+Sod]) is an effective means to deliver Met and Sod to MCF-7 breast cancer cells at their optimal synergistic ratio (IC_{25} of Met (150 mM) + IC_{25} of Sod (30 mM)) for improved therapeutic effect. Our results demonstrated that Lipo-[Met+Sod] had improved efficacy *in vitro* compared with that of the free drug combination (Fig. 3). Furthermore, MCF-7 cells treated with DMPG-POPC liposomes demonstrated higher cytotoxicity rate than that of cells treated with CHOL-POPC liposomes (data not shown). These results of the cell viability assay suggest that DMPG-POPC liposomes enhance the interaction with membranes of MCF-7 cells and accumulate a high amount of drugs into cells.

Although its application for BCSC-targeted therapy shows a limited therapeutic efficacy, Met has been proven to be an effective drug against breast cancer in an animal study (Han and Crowe, 2009). To improve the inhibitory capacity of Met against BCSCs, in this study, for the first time, we demonstrated the synergy between Met and Sod and their incorporation into DMPG-POPC-based liposomes for improved therapeutic effect. The cell viability assay in BCSCs revealed that $CD44^{+}/CD24^{-/low}$ MCF-7 population treated with free Met and Sod had no cytotoxic effect (Fig. 4B). This result suggested that $CD44^{+}/CD24^{-/low}$ populations (BCSCs) had a property of drug resistance. However, $CD44^{+}/CD24^{-/low}$ MCF-7-treated with the dual drug-loaded DMPG-POPC liposomes showed enhanced growth-inhibitory effect in both short-term cytotoxicity assay on 24 hours by anti-proliferation assay (Fig. 4B) and long-term cytotoxicity assay on 14 days by a colony formation assay (Fig. 4C and D). This could be attributed to the high affinity of DMPG-based liposomes to BCSCs, enhancing the intracellular accumulation of Met and Sod and mediating endocytosis.

This delivery system enhanced the delivery of Met and Sod to breast cancer cells and BCSCs compared with that of these free drugs alone, thus promoting effective regression of both cancer cells and BCSCs. On the basis of these results, by combining the enhanced drug delivery capability of DMPG-POPC liposomes with successful combination therapies, significant advances in medicine could be made with propound positive effect in clinical settings. Furthermore, the DMPG-POPC liposome delivery system can minimize the disadvantages of Met and Sod that restrict their effective applications *in vivo*, such as poor solubility, off-target effects, instability, short circulation half-life, undesirable biodistribution, and low therapeutic indices. Overall, our results suggest that the negative charge-based DMPG-POPC liposomal formulation has a high potential to be used as a promising delivery system for dual drug (Metformin and sodium salicylate) treatment in breast cancer therapy.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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