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Original Article

Targeted delivery of Dbait by an artificial extracellular vesicle for improved radiotherapy sensitivity of esophageal cancer

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Abstract: Intensification of radiotherapy has been shown to be an effective way for improving the therapeutic efficacy of radiation sensitive malignancies such as esophageal cancer (EC). The application of DNA Bait (Dbait), a type of DNA repair inhibitor, is an emerging strategy for radiosensitization. In this study, a Eca-109 cancerous cytomembrane-cloaked biomimetic drug delivery system (DDS), CMEC-Dbait, was designed and successfully fabricated, for targeted delivery of Dbait. Our systematic evaluation demonstrated that the ingenious artificial gastrointestinal extracellular vesicle owns neat spherical structure, proper particle size (154.6±5.5 nm) and surface charge (2.6±0.3 mV), favourable biocompatibility and immunocompatibility, being conducive to *in vivo* drug delivery. Besides, Eca-109 cytomembrane coating endowed CMEC-Dbait with effective targeting ability to homologous EC cells. Owing to these advantages, the biomimetic DDS was proved to be a potent radiosensitizer *in vitro*, indicated by remarkably reduced cell viability and enhanced cellular apoptosis by the combination therapy of radiation and CMEC-Dbait. The result was validated *in vivo* using mouse xenograft models of EC, the results illustrated that radiotherapy plus CMEC-Dbait significantly suppressed tumor growth and prolonged survival of tumor bearing mice. Western blotting results showed that CMEC-Dbait can significantly inhibit DNA damage repair signaling pathways by simulating DNA double-strand breaks both *in* and *ex vivo*. In conclusion, the versatile biomimetic CMEC-Dbait was characterized of low toxicity, excellent biocompatibility and satisfactory drug delivery efficiency, which is confirmed to be an ideal radiosensitizer for homologous cancer and merits further investigation in both pre-clinical and clinical studies.

Keywords: Esophageal cancer, Dbait, artificial gastrointestinal extracellular vesicle, biomimetic drug delivery system, radiosensitivity

Introduction

Esophageal cancer (EC) is the seventh most common malignancy and the sixth most common cause of cancer-related mortality worldwide in 2020 [1]. According to the statistics of the American Cancer Society (ACS), the new cases and cancer-causing deaths of EC in the United States were respectively estimated to be 20,640 and 16,410 in 2022 [2]. More than 90% of EC cases is recorded in Asia and sub-Saharan Africa, with men significantly being affected more than women [3]. China has a high prevalence of EC, accounting for approximately 50% of the global morbidity and mortality [4]. Despite tremendous progresses in the treatment of cancer during the past few decades, patients with locally advanced EC still have a poor prognosis, with a 5-year survival rate of only 25% [5].

Radiotherapy plays indispensable roles in the treatment of radiation sensitive malignancies such as EC, nasopharynx cancer (NC), etc. Previous studies have suggested neoadjuvant chemoradiotherapy to be the standard therapeutics for locally advanced EC [6]. Presently, a large number of clinical evidence shows that

intensive high-does radiotherapy can improve the therapeutic efficacy for malignant cancers, while high radiation dosage also increases undesired adverse toxicity to normal tissues [7-9]. Therefore, alternative approaches that strike a better balance between increased therapeutic efficacy and reduced toxicity are urgently needed.

The primary mechanism of radiation is its ionization, which kills malignant cells by inducing DNA double-strand breaks (DSBs) [10]. However, some malignant cells can escape this lethal effect by enhancing their DNA-repair capability, leading to suboptimal treatment and even radiation resistance [11]. Therefore, drugs that can inhibit DNA DSB repair might sensitize cancer cells to radiotherapy, improving therapeutic efficacy without the need for dose escalation [12].

DNA bait (Dbait) is a new class of DNA DSB repair inhibitors. It is composed of short strand DNA molecules, which can simulate DNA double-strand breaks, triggering false DNA damage signals and inducing excessive activation of DNA-dependent protein kinase (DNA-PK) [12, 13]. Overactivation of DNA-PK promotes the phosphorylation of its targets, such as H2AX and poly (ADP-ribose) polymerase (PARP), thus preventing subsequent detection of true DSBs caused by irradiation [13]. Given that Dbait is a nucleic acid molecule that functions in the nucleus, formulating it in an appropriate drug delivery system (DDS) to prolong its circulation time and provide targeting to malignant tissues and cells would be an efficient way for promoting its *in vivo* efficacy.

In the past decades, a variety of smart biological materials have provided plenty of options for targeted drug delivery [14-16]. The biomimetic strategy making use of natural cytomembrane camouflage is one of the most promising technologies, which has been extensively employed for treating and diagnosing cancer [17, 18]. Numerous studies have shown that in addition to the enhanced permeability and retention (EPR) effect of nanoparticles (NPs), the adhesion molecules expressed on cancer cell cytomembrane (CCM) can also navigate and anchor cancerous cells through receptor-ligand binding, and promote the accumulation of biomimetic NPs in malignant tissues and cells [19, 20]. Besides, CCM-camouflage endows NPs with immunomodulatory markers, providing an ideal platform that mimics the function of their source cells when interacting with surrounding biological components, thus enhancing their anti-phagocytic and extending their cycling time in the blood circulation [21-23].

Inspired by these superiorities, in this study, we successfully designed and constructed an ingenious biomimetic DDS for targeted delivery of Dbait molecules to EC tissues and cells. We hypothesize that applying isotypic CCM of EC cells as a cloak could endow NPs with excellent isotypic targeting capability and favorable immunocompatibility, leading to increased radiosensitivity and prominent EC inhibitory activity both *in vitro* and *in vivo* (**Figure 1**).

Materials and methods

Materials and cells

Dbait molecules were synthesized by Sangon Biotechnology (Shanghai, China). The sequence is: 5'-GCTGTGCCCACAACCCAGCAAACAAGCCTA-GA-(H)-TCTAGGCTTGTTTGCTGGGTTGTGGGCAC-AGC-3', where the letters in bold are phosphorodiamidate nucleosides, and H is a hexaethylene glycol linker [9]. Coomassie brilliant blue, 4% paraformaldehyde, and DAPI nuclear staining solution, was bought from Beyotime Biotechnology Co. LTD. (Shanghai, China). Two human EC cell lines (Eca-109 and KYSE-150), human normal esophageal epithelial cell line (HEEC), human breast cancer cell line (MDA-MB-231), and mouse mononuclear macrophage cell line (J774A.1) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were incubated with DMEM containing 10% (v/v) Fetal Bovine Serum (FBS) (Thermo Fisher Scientific, Massachusetts, U. S. A.) in a humidified incubator at 37°C with 5% CO_2.

Cell membrane extraction

EC cell membrane was extracted according to the methods described in our previous studies [16, 18]. Briefly, cells were collected, washed with pre-cooled Phosphate Buffer Saline (PBS), and lysed with cell lysis buffer at 4°C for 2 h. The cell lysates were sonicated at 80-100 W for 10 m, and then centrifuged (20,000 g × 20 m). The supernatant was collected and centri-

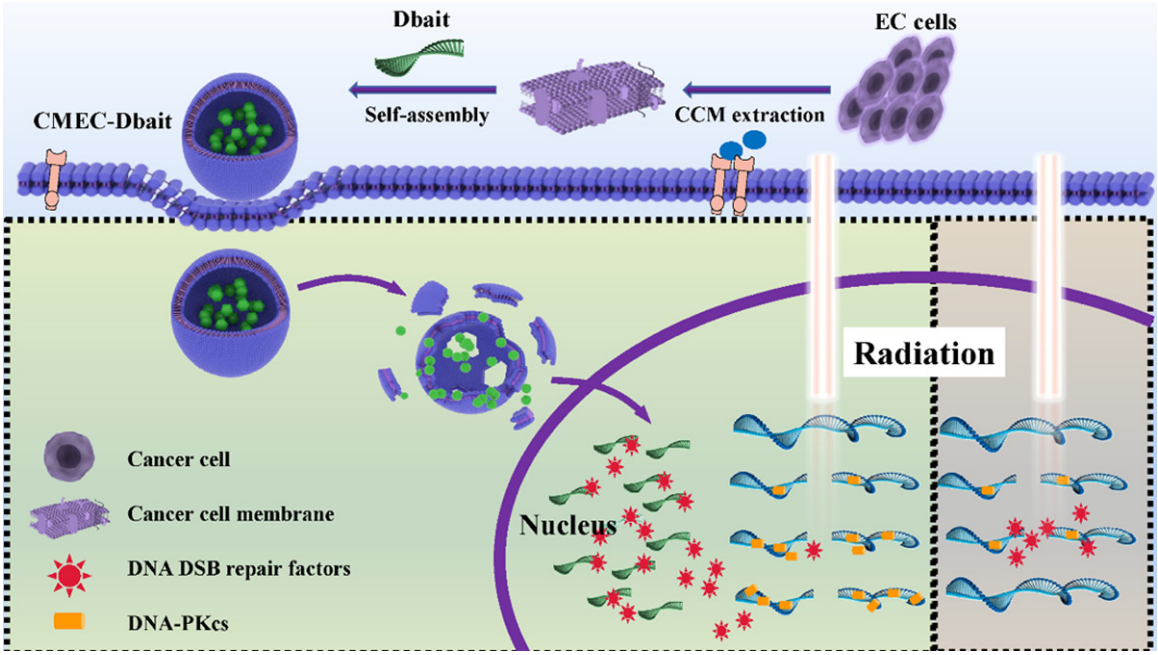

Figure 1. Schematic illustration of CCM-cloaked biomimetic vesicles for enhanced radiosensitivity of homotypic EC cells.

fuged (100,000 g × 30 min), with the precipitates being collected and stored at -80°C for future usage.

Encapsulation of Dbait into the biomimetic DDS

The mixture of Dbait and extracted cytomembrane was extruded through polycarbonate films with pore sizes of 400 nm and 200 nm in turn to obtain artificial vesicles with uniform size. The solution was dialyzed against PBS by a membrane with a molecular weight cut-off (MWCO) of 3 kDa at 4°C overnight to remove unencapsulated Dbaits. Dbaits encapsulated within the artificial vesicles of Eca-109 esophagus cancer cells and MDA-MB-231 breast cancer cells were respectively marked as CMEC-Dbait (CMEC stands for the cytomembrane of esophageal cancer cells) and CMBC-Dbait (CMBC stands for the cytomembrane of breast cancer cells). In order for fluorescence labelling, the quantum dots (QDs) were encapsulated into the biomimetic vesicles in the same way.

Particle characterization

The hydrodynamic diameter and Zata-potential of the biomimetic vesicles were measured using a ZetaSizer (Malvern Instruments, UK). Transmission electron microscopy (TEM, Emesis Veleta G3 transmission electron microscope) was employed to observe the morphology of biomimetic NPs. To prepare specimens for TEM, the droplet containing biomimetic NPs was placed on a Holey Carbon Coated-Copper 200 mesh Grid (Ted Pella, California, USA). Samples were examined on TEM at 80 kV after air-dried. UV/Vis spectrophotometry was used to detect the absorbance of free and wrapped Dbait, respectively.

Membrane protein characterization analysis

The membrane protein characterization of the biomimetic vesicles was validated by SDS-PAGE [24]. Samples were prepared in the loading buffer and heated in a metal bath for 5 m. Equal samples were subjected to SDS-PAGE, with the protein bands being stained by Coomassie blue before imaging.

In vitro serum stability and drug release evaluation

For evaluating the stability of biomimetic vesicles, DMEM containing 50% (v/v) FBS was employed to simulate the *in vivo* serum environment, into which CMEC-Dbait NPs were added,

and the whole system were fixed in a water bath to keep the temperature at 37°C. The size distribution profile was measured by DLS every day. For evaluating the drug release profile, a dialysis bag (MWCO: 3.5 kDa) containing 2 mL CMEC-Dbait was put in a beaker containing 10 mL of PBS with different pH values (Ph=5.0 or 7.4) at 37°C with continuous stirring. At various time points, samples (500 μL) outside the dialysis bag were taken up and the concentration of Dbait was measured. Cumulative release of contained Dbait was calculated according to the following function: $\text{Cumulative release} = \frac{C_{Outside} \times 10\ ml}{C_{Inside} \times 2.0\ ml} \times 100\%$.

Biocompatibility evaluation

Free and CMEC encapsulated Dbaits were added to the suspended red blood cells (RBCs) isolated from mouse orbital blood. Milli-Q water was employed as a positive control. After a 2-hour-incubation, cells were centrifuged (10,000 g × 5 m), with the supernatant being collected. The absorbance at 540 nm (Ab_{540}) of each supernatant was measured and the hemolysis rate was calculated following the function: $\text{Hemolysis rate (\%)} = \frac{Ab_{drug}}{Ab_{positive}} \times 100\%$ [18].

Cellular uptake

Cellular uptake of the biomimetic vesicles was evaluated by confocal laser scanning microscopy (CLSM) and flow cytometry (FCM). For CLSM, cells were seeded in special culture dishes at a density of 3 × 10^4/well for 12 h. Then, cells were co-cultured with equal concentration of free and CCM encapsulated QDs. After 2 hours, the cells were washed, fixed with 4% paraformaldehyde, stained with DAPI, and subjected to CLSM observation. For FCM, cells co-cultured with free and CCM encapsulated QDs were collected, washed, and the fluorescence signal was quantificationally analyzed by a Flow Cytometer (Beckman Coulter, U. S. A).

In vitro cytotoxicity

The cytotoxicity of CMEC-Dbait in comparison with that of its counterparts was evaluated by Cell Counting Kit-8 (CCK-8) analysis. HEEC cells were seeded into a 96-well-plate at a density of 5 × 10^4/well and incubated with different concentration and formulation of Dbait for 48 h. Then, 10 μl CCK-8 (Beyotime Biotechnology, Shanghai, China) was respectively added to each well for another 2-hour-incubation in the dark. A Multiskan MK3 microplate reader (Thermo Fisher Scientific, Massachusetts, USA) was employed to read the absorbance at 450 nm (Ab_{450}) to calculate the cell viability via the following function: $\text{Cell viability\%} = \frac{(Ab_{Samples} - Ab_{Blank})}{(Ab_{Control} - Ab_{Blank})} \times 100\%$, where Ab_{blank} are the absorbance of the culture medium with no cells.

Cell transfection of Dbait

EC cells (Eca-109 and KYSE-150) were seeded in 6-well plates (3 × 10^5/well) and cultured normally overnight. Then, the original medium was replaced with DMEM supplemented with 2% FBS and 1 μg/ml free and CCM encapsulated Dbait. After incubation for 24 hours, cells were harvested for future experiments.

Radiosensitivity evaluation

The sensitivity of EC cells to radiotherapy was evaluated by CCK-8 assays, live-dead staining, and apoptosis detection. Dbait transfected cells were seeded into a 96 well plate (5 × 10^4/well) and cultured for 12 hours. Then, cells were irradiated with 0, 2, 4, 6 and 8 Gy of X-rays (0.3 Gy/min), respectively. After 48 hours, CCK-8 was employed to evaluate the cell viability. For live-dead staining, cells were washed and stained with LIVE/DEAD™ Fixable Dead Cell Stain Kits (Thermo Fisher Scientific, Massachusetts, U. S. A.) according to the product instructions and observed by CLSM. For apoptosis detection, cells were collected, washed with pre-cooled PBS, incubated with Annexin V-Alexa Fluor® 488 & PI apoptosis detection kit (Thermo Fisher Scientific, Massachusetts, U. S. A.) according to the product instructions in the dark, and analyzed by a Flow Cytometer.

Western blotting

Cells were collected and suspended in cell lysis buffer containing phosphatase and protease inhibitors. After being quantified by a BCA protein detection kit (Beyotime Biotechnology, Shanghai, China), equal amount of protein samples was subjected to SDS-PAGE and immunoblotted with γ-H2AX, DNA-PKcs, PARP and

GAPDH antibodies (Cell Signaling Technology, Massachusetts, U. S. A.) [25].

In vivo studies

Treatment of established tumors in vivo: The *in vivo* experiments were carried out according to the guidelines of Council of Shanghai University on Animal Care. All the experiments on live mice were approved by the Ethics Committee of the Shanghai University. Four-week-old female BAL B/c nude mice were purchased from Slack Laboratory Animals Co., Ltd (Shanghai, China) and housed in specific pathogen free (SPF) environments. Xenograft tumors were inoculated in the right flank of BAL B/c nude mice by subcutaneous injection of Eca-109 cells (1.5×10^7) in PBS (100 μL). When tumors reached approximately 10 mm in length, mice were randomly divided into 5 experimental groups and subjected with different treatments. For NT and CMEC-Dbait groups, mice were respectively injected with 100 μL PBS or CMEC-Dbait via tail vein every other day for 3 times. For IR, IR plus CMBC-Dbait, IR plus CMEC-Dbait groups, tumor bearing mice were respectively injected with 100 μL of PBS, CMBC-Dbait, and CMEC-Dbait (with a Dbait amount of 3 mg/kg), and the tumor regions were radiated with 3 Gy (0.3 Gy/minutes) of X-ray after 12 hours, with the remaining parts being lead-shielded. The above treatments were given every other day for a total of three times. The tumor volume was measured every day and calculated according to the following equation: $\text{Tumor Volume} = \frac{L \times W^2}{2}$ (L: tumor length; W: tumor width). All mice were euthanized when the tumor volume reached 3,000 mm^3. Briefly, the mice were anesthetized with isoflurane at a concentration of 3-5% and euthanized with cervical dislocation after the rapid loss of consciousness. Tumor growth curves during 4 weeks and overall survival (OS) curves of tumor-bearing mice during 10 weeks were recorded and compared among groups.

Statistical analysis

Quantitative data were presented as mean ± standard deviation (SD). Statistical analysis was performed by Student's unpaired *t* test or one-way ANOVA to identify significant differences unless otherwise indicated, and $P < 0.05$ was considered to be statistically significant.

Results and discussion

Characterizations of the biomimetic vesicles

For preparing biomimetic DDS, we firstly extracted the cytomembrane of Eca-109 cells, and then wrapped Dbait molecules by liposome squeezer. TEM images (**Figure 2A**) show that that the artificial extracellular vesicle possessed a neat spherical structure, with diameters of approximately 150 nm. DLS results quantitatively indicate that the average hydrodynamic diameter and Zeta potential of empty vesicles (CMEC) assembled by Eca-109 cytomembrane was respectively 138.2 nm and -9.77 mV, which was separately changed to 154.6 nm and 2.59 mA after the encapsulation of Dbait (**Figure 2B-D**). **Figure 2E** illustrates that the UV/vis spectra of CMEC-Dbait owns remarkable absorption peaks of both Dbait and empty vesicles, confirming the successful package of Dbait in the biomimetic vesicles. Besides, the retention of membrane proteins of the artificial vesicles was evaluated by SDS-PAGE, with the results (**Figure 2F**) showing that similar protein composition exists among cancer cell lysates (I, lane 1-2), extracted cytomembrane (II, lane 3-4), empty CCM vesicles (CMEC, III, lane 5-6), and CMEC-Dbait (IV, lane 7-8).

Favorable serum stability and controlled drug release

In was anticipated that the artificial biomimetic DDS could be an outstanding drug delivery tool benefits from its stable and natural structure. Given the intended use by intravenous administration (I. V.), favorable serum stability is one of the necessary features for high drug delivery efficiency. Herein, the DMEM containing 50% FBS was employed as an *in vitro* serum model to check the stability of the artificial extracellular vesicles following our previous publications [26]. As displayed in **Figure 3A**, CMEC-Dbait owns excellent stability in the serum model, indicated by no obvious alteration of average particle size during the experimental period of a week. Also, the encapsulation efficiency (EE) of CMEC-Dbait was calculated to be 53±3.04%. **Figure 3B** demonstrates that CMEC-Dbait biomimetic DDS exhibits a slow and gradual drug release in neutral (pH=7.4) environment in the course of 72 hours, which was remarkably accelerated in acid conditions (pH=5.0), with

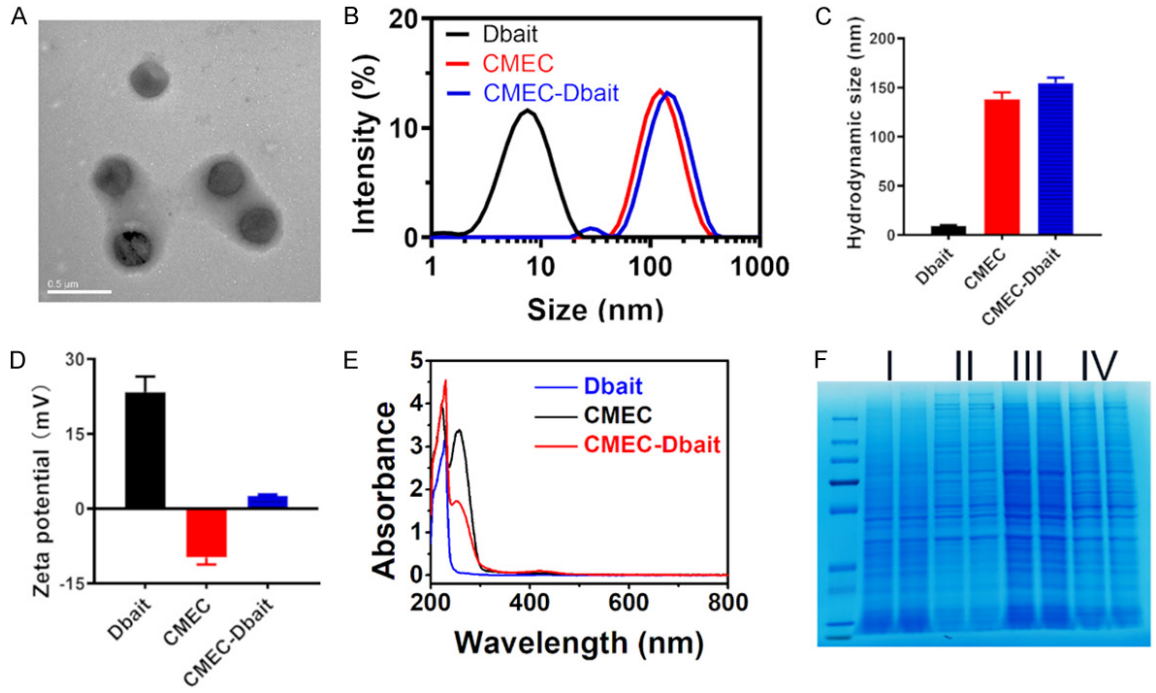

Figure 2. Characterization of the biomimetic vesicles (CMEC-Dbait). (A) TEM morphology of CMEC-Dbait NPs. Scale bar: 500 nm. (B-D) Size distribution (B), Hydrodynamic diameters (C), and Zeta potential (D) of the artificial extracellular vesicles (CMEC-Dbait) and counterparts. (E) UV-vis absorption of CMEC-Dbait and counterparts. (F) SDS-PAGE for different samples (I: Eca-109 cell lysate, II: Eca-109 cell membrane, III: empty CMEC vehicles IV: CMEC-Dbait).

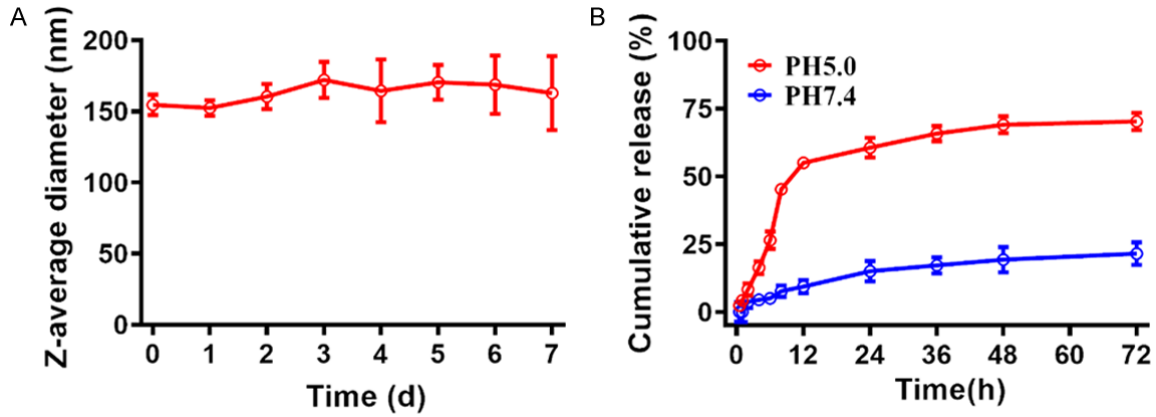

Figure 3. Serum stability and controlled drug release profiles of CMEC-Dbait. A. Excellent *in vitro* serum stability of CMEC-Dbait. B. Drug release profile of Dbait from biomimetic DDS at different conditions.

approximately 60% wrapped Dbait molecules being released within 24 h. This is because that acid environment increases the extent of protonated phosphatidic acid (PA) headgroups on membrane bilayer lipids, PA-rich heterogeneities, and leading to molecular packing defects originating at the phase boundaries [27]. It seems that the biomimetic CMEC-Dbait could maintain stability in the blood circulation with a neutral pH value. However, the acidic tumor microenvironment (TME) can promote the release of containing Dbaits, giving rise to their accumulation in malignant tissues as desired.

Prominent in vitro biocompatibility and immunocompatibility

Figure 4A illustrates that either free or CCM encapsulated Dbaits exhibit moderate hemolysis rate (less than 6%) in concentrations of up

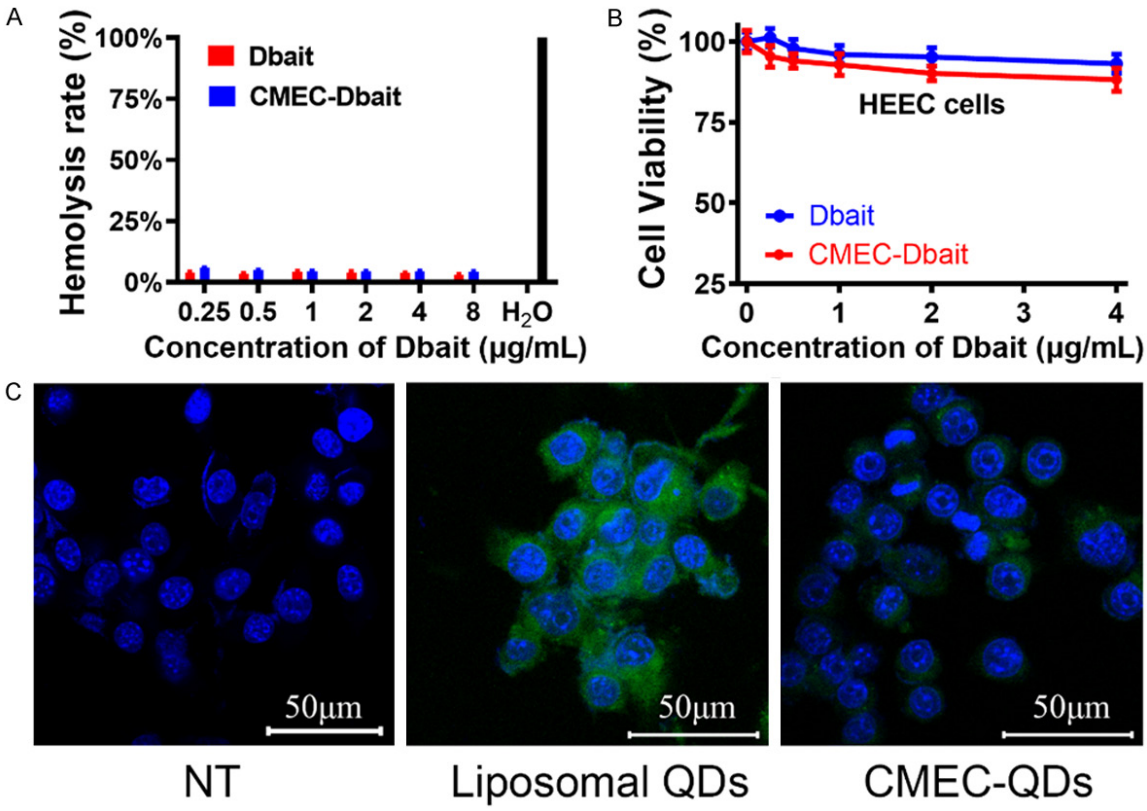

Figure 4. *In vitro* biocompatibility of the biomimetic DDS. A. Hemolysis quantification of RBC incubated with different formulations of Dbait. B. Cytotoxicity of free and CMEC encapsulated Dbaits. C. CLSM images of J774A.1 macrophages incubated with different formulations of QDs. Green and blue fluorescence respectively indicates QDs and nucleus. Scale bar: 50 μm.

to 8 μg/mL. Besides, both formulations of Dbaits (0.125-4 μg/mL) demonstrate moderate-cytotoxicity to normal esophageal epithelial cells (HEEC) (**Figure 4B**). The above-mentioned results clearly reflect that the biomimetic vesicles own prominent biocompatibility.

The recognition and elimination of most nanoparticles by the reticuloendothelial system (RES) remains the principal obstacles for clinical transformation of nanomaterials [28]. However, cancer cells, the cytomembrane of which was used for particle camouflage in this study, are capable of immune escape, providing an effective technology to clear away the above-mentioned obstacle [29]. In order to evaluate the immune escape ability (immunocompatibility), the swallow of CMEC encapsulated QDs and counterparts by J774A.1 macrophages was thus determined and compared. As shown in **Figure 4C**, cancer cell membrane encapsulated QDs are less prone than liposomal ones to be recognized and ingested by macrophages, indicated by decreased green fluorescence in CMEC-QD treated J774A.1 macrophages.

Enhanced targeting delivery of encapsulated agents to homologous cancer cells

The cellular uptake of the CMEC extracellular vesicles and counterparts was compared in both Eca-109 and KYSE-150 cells. The results of FCM displayed in **Figure 5A**, **5B** revealed that encapsulating QDs in the cytomembrane of a nonhomologous breast cancer cell line (CMBC-QDs) can significantly promote the intracellular uptake, being indicated by enhanced mean fluorescence intensity (MFI) (**$P < 0.01$). On the other hand, cells incubated with homologous CCM encapsulated vesicles (CMEC-QDs) showed the highest MFI among all the experimental groups. Similar results were confirmed by CLSM shown **Figure 5C**. As we can see, both EC cells treated with homologous vesicles (CMEC-QDs) exhibited stronger green fluores-

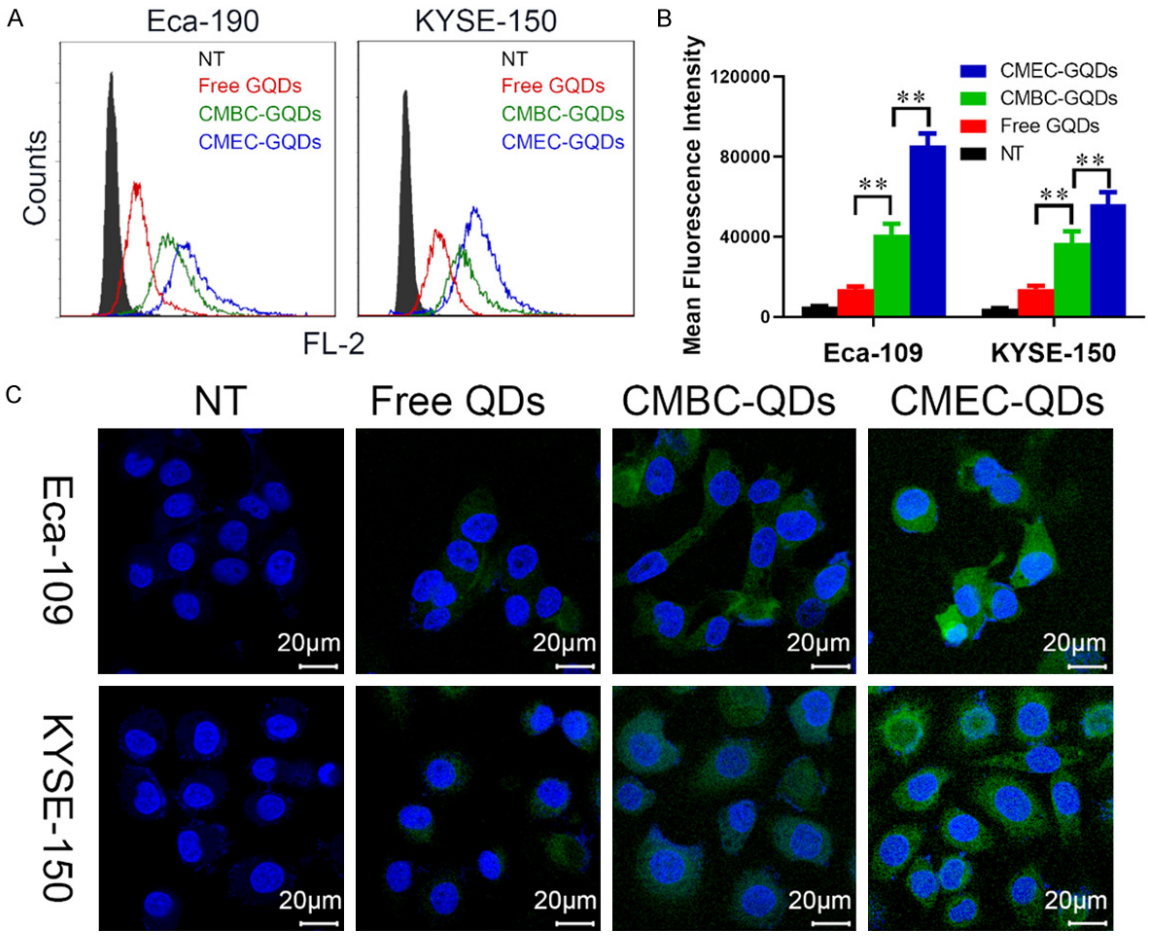

Figure 5. Cellular uptake of the biomimetic DDS. A. Effects of CCM camouflaging on intracellular uptake of QDs in homologous cells (Eca-109 and KYSE-150) by FCM. B. Quantitative analysis of FCM results. MFI was counted using FlowJo software. Data are mean ± SD (n=3), **P < 0.01. C. Effects of CCM camouflaging on the intracellular uptake of QDs in homologous cells by CLSM. Green: QD; blue: nucleus. Scale bar: 20 μm.

cence than cells treated with free QDs or non-homologous vesicles (CMBC-Dbaits). These results indicate that CCM coverage can significantly promote endocytosis of cancer cells, and better homologous membrane camouflage than nonhomologous membrane in the promotion.

CMEC-Dbait improved in vitro radiosensitivity of EC cells

The influence of the biomimetic vesicles on the radiosensitivity of EC cells was firstly analyzed by CCK-8 assay. Radiation survival curves were generated according to the survival fractions of both EC cells under different radiation doses. As illustrated in **Figure 6A**, **6B**, the cell viability of both cells significantly decreased following radiation in a dose dependent manner. Free Dbait transfected cells demonstrate similar radiosensitivity to wild type cells. Encapsulated Dbait transfection can remarkably promote the radiosensitivity of both cells indicated by the downshift of survival curves when CMEC-Dbait or CMBC-Dbait was given with radiotherapy. In line with our expectations, the homologous vesicles (CMEC-Dbait) were found to be better radiosensitizers than the non-homologous ones (CMBC-Dbait) in either Eca-109 or KYSE-150 cells. CMEC-Dbait's role as a potent radiosensitizer was also validated by apoptosis detection. **Figure 6C**, **6D** illustrates that 4 Gy radiation can obviously enhance the percentage of apoptotic cells. In accordance with the CCK-8 results, the radiosensitivity of both cells was significant promoted by encapsulated Dbaits, and the homologous vesicle (CMEC-Dbait) was found to be the most effective radio-

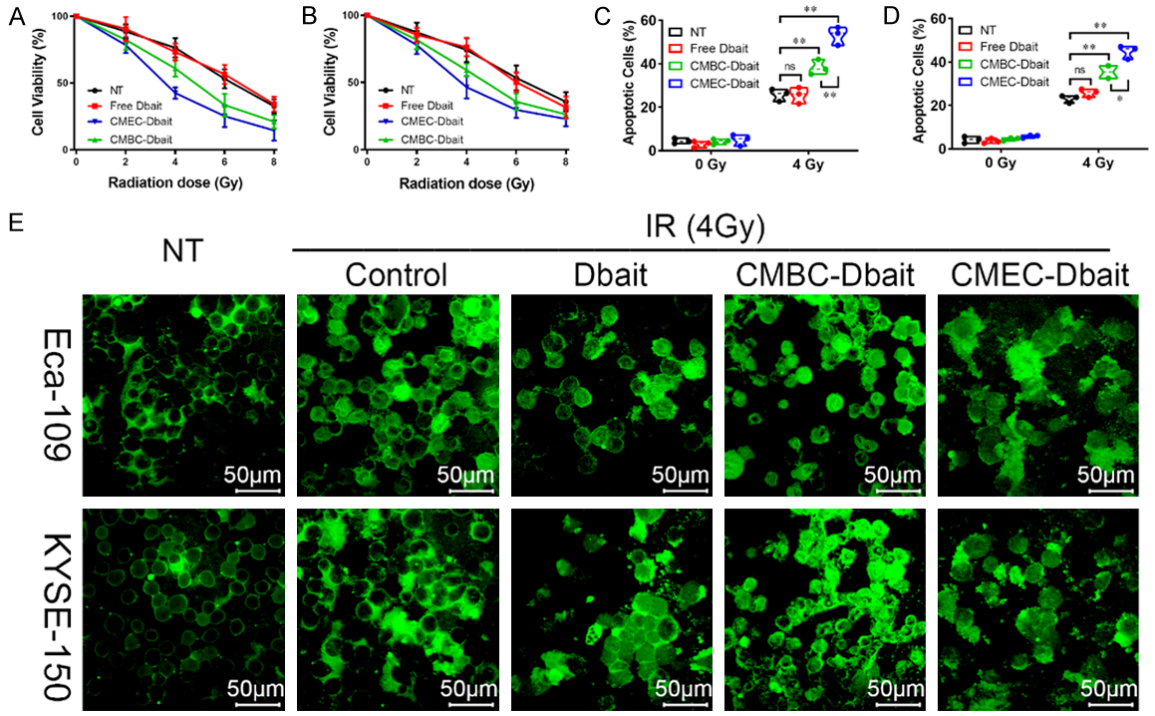

Figure 6. CMEC-Dbait improved *in vitro* radiosensitization of EC cells. (A, B) Cell viability of Eca-109 (A) and KYSE-150 (B) cells after treatment with different formulations of Dbait and radiation (0-8Gy) by CCK-8 analysis. (C, D) Apoptosis of Eca-109 (A) and KYSE-150 (B) cells after different treatment analyzed by Annexin V/PI staining. (E) Live/dead staining of Eca-109 and KYSE-150 cells with different treatments. For necrotic cells, the reactive dye can permeate the compromised membranes and react with free amines both in the interior and on the cell surface. In contrast, only the cell-surface amines of viable cells are available to react with the dye, resulting in relatively dim staining. Scale bar: 50 μm. Data expressed as means ± SD. $*P < 0.05$; $**P < 0.01$. Scale bar: 50 μm.

sensitizer among groups, indicated by more than 40% of cells underwent apoptosis after 4 Gy radiation. All these results were validated by live-dead staining with the CLSM photos showing in **Figure 6E**.

CMEC-Dbait inhibits the repair of radiation-induced DNA damage in vitro

In order to evaluate the underlying radiation sensitizing mechanisms of the artificial extracellular vesicles, we firstly investigated whether free and encapsulated Dbait would lead to increased H2AX phosphorylation in target cells. It should be noted that the presence of phosphorylated H2AX at Ser139 (γ-H2AX) is usually formed at the sites of DSB damage and resolved when the damage repair completed [30, 31]. As illustrated in **Figure 7**, 4 Gy radiation significantly upregulated γ-H2AX levels in both EC cells, which was further upregulated by encapsulated but not free Dbait. Among all the experimental groups, CMEC-Dbait treated cells demonstrated the highest γ-H2AX levels. The effects of CMEC-Dbait on other DNA repair factors, such as DNA-PKcs and PARP, were also evaluated. As we can see, CMEC-Dbait plus radiation also enhanced their expression more remarkable than other treatments, in keeping with the variation tendency of γ-H2AX. All these results demonstrate that the biomimetic DDS can effectively improve the radiosensitivity of target cells by simulating DNA DSB and inhibiting DNA damage repair factors.

CMEC-Dbait is an effective radiosensitizer in vivo

The subcutaneous tumor model was established to evaluate the efficacy of the biomimetic DDS as a radiosensitizer *in vivo*. BAL B/c nude mice bearing flank Eca-109-xenografts were given different formulations of Dbait plus radiation or not. **Figure 8A**, **8B** indicates that mice treated with CMEC-Dbait (without radiation) exhibit rapid tumor growth and short survival, being similar to those treated with PBS. Radiotherapy slightly slowed the tumor growth

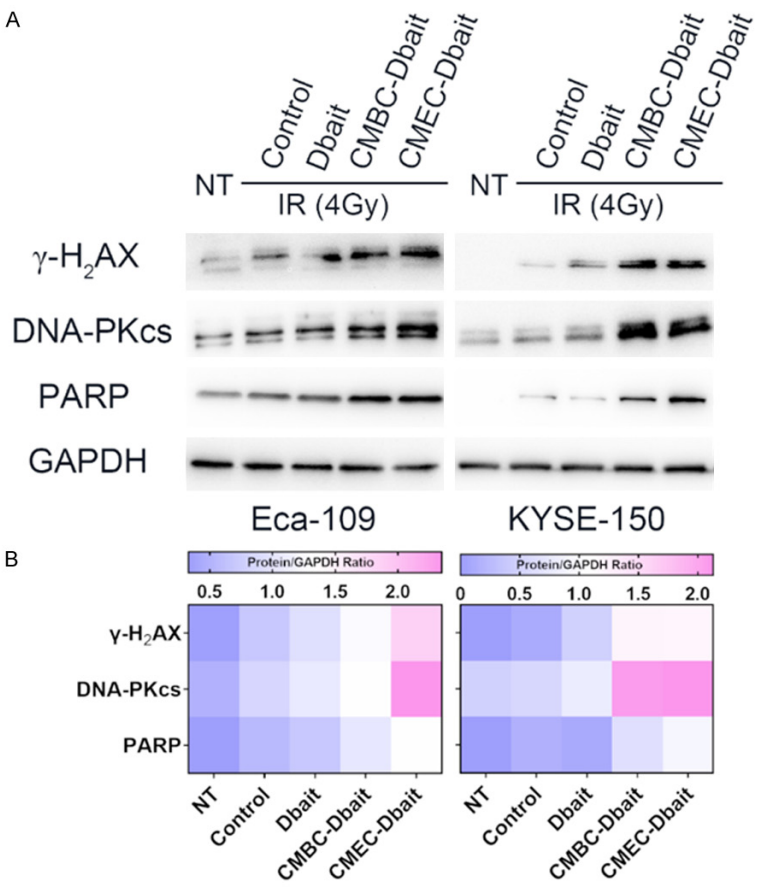

Figure 7. A. Western Blotting analysis of γ-H2AX, DNA-PKcs and PARP in Eca-109 and KYSE-150 cells being treated with radiation (4 Gy) plus different formulations of Dbait. B. Gray value of WB protein bands analyzed by Quality One software (Version 4.62) displayed in heat map.

and prolonged the median survival time (MST) of tumor-bearing mice from 36 to 45 days. An obviously enhanced radiosensitivity of subcutaneous tumor was found for CCM encapsulated Dbaits, with homologous (CMEC-Dbait) being better than un-homologous DDS. As we can see, combination therapy of CMEM-Dbait plus radiation led to the most outstanding tumor suppressing activity, being indicated by more than 80% reduction of average tumor volume (VS PBS control group) and 2/5 mice's survival at the end of survival analysis (70 days post treatment).

In order to evaluate the inhibitory effects on DNA DSB repair of the biomimetic vesicles, representative Eca-109 xenograft tumors were removed 48 h after the third time of radiation, and the tumor tissues were analyzed for γ-H2AX, DNA-PKcs or PARP by WB. Consistently, tumor tissues treated with combination therapy of CMEC-Dbait plus radiation exhibit the highest levels of γ-H2AX, DNA-PKcs and PARP (**Figure 8C**, **8D**), confirming that CMEC-Dbait can effectively inhibit DNA damage repair *in vivo*.

During the clinical application of anti-cancer drugs, the main side effects are due to their dose-dependent hematotoxicity, hepatotoxicity and nephrotoxicity. Thus, blood routine and biochemical indexes in plasma taken from tumor bearing mice were measured 24 h after the tail vein injection of different formulations of Dbait. As we can see, the counts of white blood cell (WBC), RBC, platelet (PLT), urea nitrogen (BUN) and creatinine (CREA) experienced no obvious alteration. Although blood aspartate transaminase (AST) and alanine transaminase (ALT) slightly increased in mice treated with free and encapsulated Dbaits, the results of which are all within normal limits (**Figure 8E-K**), indicating minimal systematic toxicity of the biomimetic vesicles *in vivo*.

Conclusions

Radiosensitization, the application of agents for improving the sensitivity of malignant to radiotherapy, has been an important concept in cancer treatment [32, 33]. In this study, we aimed to improve the radiosensitivity of EC by directly targeting the DNA damage repair pathways. Based on this hypothesis, a novel nanosized biomimetic DDS, CMEC-Dbait, was designed and successfully fabricated by using an artificial gastrointestinal extracellular vesicle.

Our systematic evaluation demonstrates that the uniform particle structure, proper particle size and surface charge, and favorable biocompatibility & immunocompatibility, are conducive to *in vivo* Dbait delivery. Eca-109 CCM coating endowed CMEC-Dbait with effective targeting ability to homologous cells. Owing to these advantages, the biomimetic vesicles could

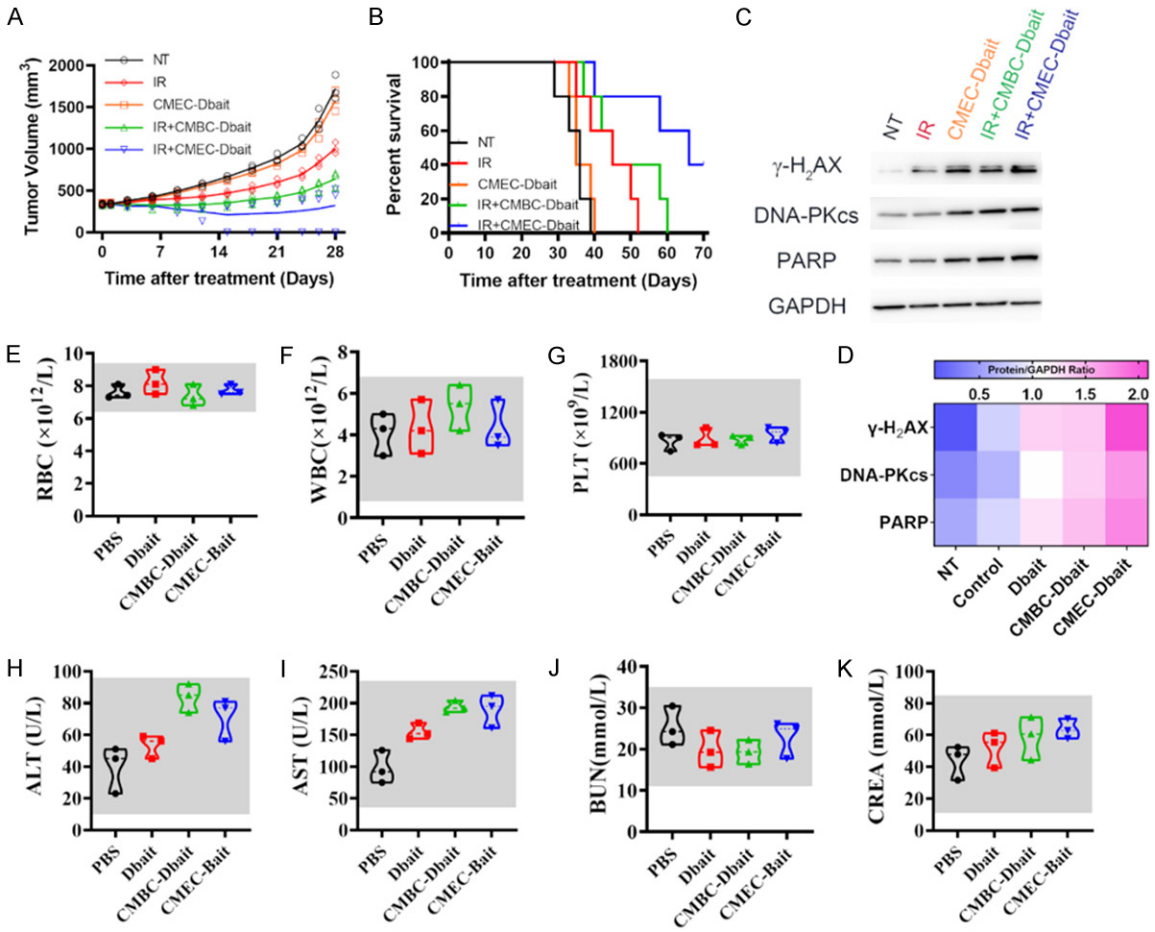

Figure 8. CMEC-Dbait is an effective radiosensitizer *in vivo*. (A, B) Mice bearing Eca-109 xenograft tumors were injected with different formulations of Dbait or PBS, followed by radiotherapy or not. Tumor growth curves (n=3) (A) and survival curves (n=5) (B) were generated. Data expressed as means ± SD. (C) WB analysis of γ-H2AX, DNA-PKcs and PARP in xenograft tumors being treated with radiation plus different formulations of Dbait. (D) Gray value of WB protein bands analyzed by Quality One software (Version 4.62) displayed in heat map. (E-G) Blood routine analysis of tumor-bearing mice after different treatment. (H-K) Biochemical indexes in plasma taken from tumor bearing mice after treatment. ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; BUN: Blood Urea Nitrogen; CREA: serum Creatinine. Data expressed as violin plot.

deliver Dbait, a type of DNA damage repair inhibitor, to EC cells with high efficiency, thus enhancing their sensitivity to radiation both in *in* and *ex vivo* studies. In conclusion, the versatile biomimetic DDS is characterized of low toxicity, excellent biocompatibility & immunocompatibility, and satisfactory drug delivery efficiency, which is confirmed to be an ideal radiosensitizer for homologous EC and merits further investigation in both pre-clinical and clinical studies in the near future.

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Disclosure of conflict of interest

None.

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