Acta Biomaterialia 111 (2020) 386–397

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actbio

Full length article

Bispecific antibody (HER2 × mPEG) enhances anti-cancer effects by precise targeting and accumulation of mPEGylated liposomes

I-Ju Chen[a,b,c,1], Yi-An Cheng[a,b,1], Kai-Wen Ho[a], Wen-Wei Lin[a,b,d,e], Kai-Wen Cheng[b], Yun-Chi Lu[a,b], Yuan-Chin Hsieh[b], Chien-Chiao Huang[c,f], Chih-Hung Chuang[g], Fang-Ming Chen[b,h,i,j], Yu-Cheng Su[k], Steve R. Roffler[a,l,*], Tian-Lu Cheng[a,b,c,e,*]

[a] Graduate Institute of Medicine College of Medicine Kaohsiung Medical University, Kaohsiung, Taiwan
[b] Drug Development and Value Creation Research Center, Kaohsiung Medical University, Kaohsiung, Taiwan
[c] Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung, Taiwan
[d] Department of Laboratory Medicine, School of Medicine College of Medicine Kaohsiung Medical University, Kaohsiung, Taiwan
[e] Department of Medical Research, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[f] Center for Molecular Medicine, China Medical University Hospital, Taichung, Taiwan
[g] Department of Medical Laboratory Science and Biotechnology, College of Health Sciences Kaohsiung Medical University, Kaohsiung, Taiwan
[h] Department of Surgery Faculty of Medicine College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan
[i] Cancer Center, Kaohsiung Municipal Ta-Tung Hospital, Kaohsiung, Taiwan
[j] Division of Breast Surgery Department of Surgery, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan
[k] Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu, Taiwan
[l] Institute of Biomedical Sciences Academia Sinica, Taipei, Taiwan

ARTICLE INFO

Article history:
Received 25 October 2019
Revised 13 April 2020
Accepted 14 April 2020
Available online 15 May 2020

Keywords:
Bispecific antibody
mPEGylated liposomal doxorubicin
HER2
Methoxy poly (ethylene glycol)
Combination therapy
Targeted therapy

ABSTRACT

Targeted antibodies and methoxy-PEGylated nanocarriers have gradually become a mainstream of cancer therapy. To increase the anti-cancer effects of targeted antibodies combined with mPEGylated liposomes (mPEG-liposomes), we describe a bispecific antibody in which an anti-methoxy-polyethylene glycol scFv (αmPEG scFv) was fused to the C-terminus of an anti-HER2 (αHER2) antibody to generate a HER2 × mPEG BsAb that retained the original efficacy of a targeted antibody while actively attracting mPEG-liposomes to accumulate at tumor sites. HER2 ×mPEG BsAb can simultaneously bind to HER2-high expressing MCF7/HER2 tumor cells and mPEG molecules on mPEG-liposomal doxorubicin (Lipo-Dox). Pre-incubation of HER2 × mPEG BsAb with cells increased the endocytosis of Lipo-DiD and enhanced the cytotoxicity of Lipo-Dox to MCF7/HER2 tumor cells. Furthermore, pre-treatment of HER2 × mPEG BsAb enhanced the tumor accumulation and retention of Lipo-DiR 2.2-fold in HER2-high expressing MCF7/HER2 tumors as compared to HER2-low expressing MCF7/neo1 tumors. Importantly, HER2 × mPEG BsAb plus Lipo-Dox significantly suppressed tumor growth as compared to control BsAb plus Lipo-Dox in MCF7/HER2 tumor-bearing mice. These results indicate that HER2 × mPEG BsAb can enhance tumor accumulation of mPEG-liposomes to improve the therapeutic efficacy of combination treatment. Anti-mPEG scFv can be fused to any kind of targeted antibody to generate BsAbs to actively attract mPEG-drugs and improve anti-cancer efficacy.

Statement of Significance

Antibody targeted therapy and PEGylated drugs have gradually become the mainstream of cancer therapy. To enhance the anti-cancer effects of targeted antibodies combined with PEGylated drugs is very important. To this aim, we fused an anti-PEG scFv to the C-terminal of HER2 targeted antibodies to generate a HER2×mPEG bispecific antibody (BsAb) to retain the original efficacy of targeted antibody whilst actively attract mPEG-liposomal drugs to accumulate at tumor sites. The present study demonstrates pre-treatment of HER2×mPEG BsAb can enhance tumor accumulation of mPEG-liposomal drugs to improve the therapeutic efficacy of combination treatment. Anti-mPEG scFv can be fused to any kind of targeted antibody to generate BsAbs to actively attract mPEG-drugs and improve anti-cancer efficacy.

* Corresponding authors.
E-mail addresses: sroff@ibms.sinica.edu.tw (S.R. Roffler), tlcheng@kmu.edu.tw (T.-L. Cheng).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.actbio.2020.04.029
1742-7061/

1. Introduction

Targeted antibodies and nanomedicines have gradually become mainstreams of cancer therapy [1,2]. Targeted antibodies include drugs such as trastuzumab [3], a humanized anti-HER2 antibody that can specifically target human epidermal growth factor receptor 2 (HER2) tumor antigen to abolish tumor cell proliferation [4] and whose Fc region can activate immune-mediated tumor cell killing mechanisms [5] (including, antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)). Trastuzumab has been approved for the treatment of 20-30% of breast cancer patients in whom HER2 is overexpressed [6]. Nanomedicines, like mPEGylated liposomal doxorubicin (PLD, Caelyx and Lipo-Dox) [7], which encapsulates the chemotherapeutic drug doxorubicin in liposomes and attaches methoxy-polyethylene glycol (mPEG) to the surface of the liposomes can improve the pharmacokinetic profile of the drug, as measured by increased drug bioavailability, decreased systemic toxicity, and prolonged circulation time in the blood [8–10]. In recent years, PLD combined with trastuzumab as a combination therapy has been shown to prolong progression-free survival and overall survival in HER2-overexpressed metastatic breast cancer patients [11–13]. However, the liposome drugs passively accumulate in the tumor through the enhanced permeability and retention (EPR) effect [14], which means the combination of liposome drugs and free antibodies does not increase the tumor uptake of liposome drugs as compared with liposome drug monotherapy [15]. Thus, using antibodies to confer active tumor targeting of liposome drugs can further increase liposome drug uptake into tumors to improve the anti-cancer efficacy.

Conjugation of intact targeted antibodies at the surface of liposomes can increase liposome uptake into tumors through receptor-mediated endocytosis, which can exhibit better therapeutic efficacy than a combination of non-targeted liposomes and free antibodies [16]. For instance, Allen et al. reported that intact anti-CD19 antibody-conjugated liposome drugs (mAb-liposomes) were more effective in prolonging survival times than a combination of non-targeted liposome drugs plus free antibodies in a model of human $CD19^+$ B lymphoma [17]. However, they also found the efficacy of mAb-liposomes was lower than anti-CD19 Fab-conjugated liposomes (Fab-liposomes), because mAb-liposomes were cleared rapidly from the circulation due to a high Fc receptor-mediated uptake in the liver and spleen [18]. Short circulation time of mAb-liposomes resulted in insufficient concentration and residence time for accumulation in tumors, thus reducing the anti-tumor efficacy [19]. To prevent the clearance problem, use of Fab or scFv fragments instead of intact antibodies helped immunoliposomes evade the clearance via Fc receptor uptake on macrophages [16,18,20]. For example, the liposomal drug MM302 used an anti-HER2 scFv covalent conjugated-liposomal doxorubicin to potentially improve the anti-cancer efficacy demonstrated by liposomal doxorubicin for HER2-positive cancers and possessed indistinguishable pharmacokinetics (terminal $t_{1/2}$: 13.4 and 13.6 h, respectively) [21,22]. We also previously described mPEG × HER2 BsAb (Fab × scFv format) one-step non-covalent modification of mPEGylated liposomal doxorubicin (Lipo-Dox) for enhancement of the therapeutic efficacy of Lipo-Dox for HER2-positive tumors [23,24]. Likewise, Caruso and colleagues also developed PEG × EGFR BsAb (scFv × scFv format) for modulating targeting of PEGylated particles to EGFR-positive tumors [25,26]. However, loss of the Fc fragment led to loss-of-function of ADCC and CDC of antibodies. Maintaining the immune-mediated activity of antibodies and actively attracting the mPEGylated liposomal drugs to accumulate in tumors may further enhance the therapeutic efficacy of combination therapy.

Here, we describe a bispecific antibody made by fusion of an anti-mPEG scFv (αmPEG scFv) to the C-terminus of an intact anti-HER2 (αHER2) antibody (clone C6ML3-9 [27]) named HER2 × mPEG BsAb (IgG-scFv format). Pre-treatment of tumors with HER2 × mPEG BsAb not only retained the original efficacy of the targeted antibodies but also actively attracted mPEGylated liposomes to accumulate at the tumor sites (Fig. 1) to generate an active attraction-based combination therapy. We determined the bi-functional binding activity of HER2 × mPEG BsAb to HER2-expressing cells and mPEGylated-liposomes. The endocytosis and cytotoxicity of HER2 × mPEG BsAb plus mPEGylated liposomes were investigated and compared with control groups (mPEGylated liposomes alone or HER2 × DNS BsAb plus mPEGylated liposomes) in HER2-expressing cells. We also examined the tumor specific targeting of HER2 × mPEG BsAb plus mPEGylated liposomes to HER2 high-expressing tumors. Finally, the anti-cancer efficacy of HER2 × mPEG BsAb plus mPEGylated liposomes was determined in a HER2-expressing xenograft model. Using this double-attack strategy, we expect that mPEG-binding BsAb may retain the original anti-cancer efficacy (first attack) and actively attract mPEGylated liposomes to tumors (second attack) to generate a more efficacious tumor-killing effect.

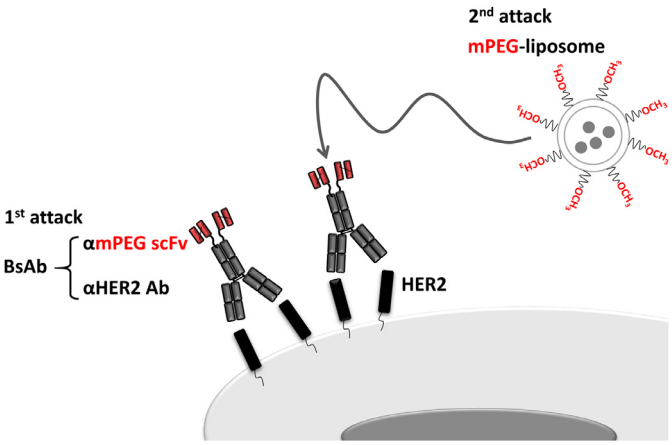

Fig. 1. A BsAb for enhancing anti-cancer effects by precise targeting and accumulation of mPEGylated liposomes to tumors. Schematic representation of enhanced accumulation of mPEGylated liposomes (mPEG-liposomes) at $HER2^+$ tumors by a HER2 × mPEG bispecific antibody (BsAb). The humanized anti-methyl-polyethylene glycol scFv (αmPEG scFv) was fused to the C-terminus of the HER2-targeted antibody (αHER2 Ab) to form an IgG-scFv bispecific antibody. 1st attack: pre-treatment with HER2 × mPEG BsAb can retain the original efficacy of HER2-targeted antibody, and 2nd attack: the anti-mPEG portion of BsAb can actively attract mPEG-liposomes to accumulate at tumor sites thus enhancing the therapeutic efficiency of the combination therapy.

2. Materials And Methods

2.1. Cell lines and animals

Human breast cancer cells MCF7/HER2 (HER2 high; stable HER2-transfected MCF7 human breast cancer cells) and MCF7/neo1 (HER2 low; control plasmid-transfected MCF7 human breast cancer cells) were kindly provided by Dr. M. C. Hung (Department of Molecular and Cellular Oncology, University of Texas, M. D. Anderson Cancer Center, Houston, TX), and were cultured in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12, Gibco, Grand Island, NY, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco Laboratories, Grand Island, NY, USA) in a humidified atmosphere of 5% CO_2 at 37°C. MDA-MB-468 human breast cancer cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated bovine calf serum (BCS, Thermo, Waltham, MA,

USA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Gibco Laboratories, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2. Chinese hamster ovary (CHO)/DG44 cells (Invitrogen), in which the dihydrofolate reductase (*dhfr*) gene locus was deleted were cultured in CD DG44 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 8 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 1.8% Pluronic F-68 (Invitrogen, Carlsbad, CA, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco Laboratories, Grand Island, NY, USA) at 37°C in an incubator with 8% CO_2. Healthy 6- to 8-week-old female BALB/c and BALB/c nude mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan. Animal experiments were performed according to the guidelines of the Kaohsiung Medical University Institutional Animal Care and Use Committee.

2.2. Bispecific antibody plasmid construction

To construct the expression vectors encoding the full-length anti-HER2 antibody (αHER2 Ab, clone C6ML3-9 [27]), a variable fragment of human αHER2 Ab was cloned from the pBub-YCMC plasmid (kindly provided by Prof. Louis M. Weiner of Fox Chase Cancer Center, Philadelphia, PA, USA) [28], then the VH-CH1 and VL-Cκ domains of the αHER2 Ab were constructed via assembly PCR. The VH-CH1 domain of αHER2 Ab was assembled with the human IgG1 Fc domain via PCR to generate the heavy chain of αHER2 Ab. The αHER2 light and heavy chain sequences, joined by a furin-2A (F2A)-based bicistronic sequence [29], was inserted into the lentiviral vector with a DHFR selectable marker (pLKO_AS2-ires-dhfr), by use of NheI and AscI restriction sites to generate pLKO_AS2-αHER2 Ab-ires-dhfr. The VL and VH domains of the anti-mPEG antibody (αmPEG Ab) were amplified from the cDNA isolated from the 15-2b hybridoma and were humanized as described previously [30,31], then the VL and VH domain of the αmPEG Ab were connected with a GGGGS linker to generate αmPEG scFv. The anti-dansyl (αDNS) scFv was cloned from pLNCX-DNS-B7 [32]. The amino acid GGGSGGG flexible linker was placed between the αHER2 Ab and scFv genes to generate two BsAb plasmids, the pLKO_AS2-αHER2 × mPEG-ires-dhfr and pLKO_AS2-αHER2 × DNS-ires-dhfr.

2.3. Stable expression and purification of bispecific antibodies

Pseudotyped lentiviruses were generated by cotransfecting pLKO_AS2-αHER2 Ab-ires-dhfr or pLKO_AS2-αHER2 × mPEG-ires-dhfr or pLKO_AS2-αHER2 × DNS-ires-dhfr with pMD.G and pCMVDR8.91 (Academia Sinica, Taipei, Taiwan), respectively, in phoenix cells by TransIT-LT1 transfection reagent (Mirus Bio Corporation, Madison, WI, USA). Two days after transfection, the lentivirus-containing culture medium was filtered, mixed with 2 μg/mL Polybrene (Sigma-Aldrich, St. Louis, MO, USA), and added to CHO DG44 cells. Following lentiviral transduction, the cells were selected in CD OptiCHO medium (Invitrogen, Carlsbad, CA, USA) supplemented with 8 mM L-glutamine (Invitrogen, Carlsbad, CA, USA) to obtain the CHO DG44/αHER2 Ab, CHO DG44/αHER2 × mPEG or CHO DG44/αHER2 × DNS stably expressed cell lines. The producer cells were cultured under methotrexate (MTX, Sigma-Aldrich, St. Louis, MO, USA) selection conditions in which a stepwise increase in the MTX concentration was employed to obtain Ab or BsAb high productivity cell lines. The high productivity cells were then cultured in a CELLine (INTEGRA Biosciences AG, Zizers, Switzerland) in MTX-containing OptiCHO medium for collecting Ab- or BsAb-containing culture medium. The αHER2 Ab, αHER2 × mPEG BsAb and αHER2 × DNS BsAb were purified by protein A Sepharose 4 Fast Flow chromatography (GE Healthcare, Little Chalfont, UK). The purity of purified αHER2 Ab, αHER2 × mPEG BsAb and αHER2 × DNS BsAb was analyzed by SDS-PAGE in reducing conditions and stained with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO, USA).

2.4. Bi-functional binding activities of bispecific antibodies by cell-based ELISA

HER2-overexpressing MCF7/HER2 cells (10^5 cells/well) were grown overnight in 96-well microtiter plates pre-coated with 40 μg/mL of poly-$_D$-lysine (Corning, New York, USA). After removing the original culture medium from the 96-well plates, the cells were washed with serum-free DMEM three times, and graded concentrations of αHER2 Ab or αHER2 × mPEG or αHER2 × DNS BsAbs were added to the cells (50 μL/well) at room temperature (RT) for 1 hour. After the cells were washed with serum-free DMEM three times to remove unbound antibodies, 1 μg/mL of Lipo-Dox (TTY Biopharm Co., Ltd., Taipei, Taiwan; particle size of Lipo-Dox, 98.8 ± 0.6 nm; polydispersity index value, around 0.2; zeta potential, -43.4 ± 0.6 mA, as described in our previous study [23]) was added to the wells at RT for 30 minutes.. After washing, the cells were sequentially incubated with 5 μg/mL of biotinylated-AGP4 anti-PEG backbone antibody [30] (0.25 μg/well) and 1 μg/mL of horseradish peroxidase (HRP)-conjugated streptavidin (50 ng/well) at RT for 1 hour, respectively. Finally, after washing with serum-free DMEM three times and PBS once, 0.4 mg/mL of ABTS solution (150 μL/well, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (Sigma-Aldrich, St. Louis, MO, USA), 0.01% (v/v) H_2O_2, and 100 mM phosphate citrate, pH 4.0) were added for 30 minutes before optical absorbance values at 405 nm were measured using an ELISA reader (Molecular Devices, Menlo Park, CA, USA). Data are presented as the mean of three independent experiments.

2.5. Confocal microscopy of bispecific antibodies plus PEGylated liposomal DiD

HER2-overexpressing MCF7/HER2 cells (2 × 10^5 cells) and HER2 negative MDA-MB-468 cells (2 × 10^5 cells) were seeded on a 35 mm glass bottom μ-dish (Ibidi, Martinsried, Germany) pre-coated with 40 μg/mL of poly-$_L$-lysine overnight. The cells were incubated with 1 μg/mL Hoechst 33342 (Sigma-Aldrich) to stain nuclei and 0.2 nM Lysotracker green DND-26 (Invitrogen) to stain lysosomes in 1 mL phenol red-free DMEM (Gibco Laboratories) supplemented with 10% FBS for 40 minutes at 37°C in a humidified atmosphere of 5% CO_2. After removing the medium, the cells were stained with 66.7 nM of HER2 × mPEG in 1 mL phenol red-free DMEM growth medium for 30 minutes at RT. For competition experiments, we used anti-HER2 antibody (αHER2 Ab) as competitor and anti-CD20 antibody (αCD20 Ab) as an isotype control. The cells were pre-incubated with 66.7 nM of αHER2 Ab or αCD20 Ab in 1 mL phenol red-free DMEM growth medium at RT for 30 minutes, then cells were incubated with 66.7 nM of HER2 × mPEG BsAbs in 1 mL phenol red-free DMEM growth medium at RT for 30 minutes. Unbound BsAbs were removed by washing with phenol red-free DMEM, and then 5 nM of Lipo-DiD (DiD-labeled mPEGylated liposome, FormuMax, CA, USA) was added to the cells in 1 mL phenol red-free DMEM growth medium. The fluorescence signal was examined by real-time imaging on an Olympus FluoView FV1000 confocal microscope (Olympus Imaging America, Center Valley, PA). The excitation and emission wavelengths were 346 and 460 nm for Hoechst 33342, 504 and 511 nm for Lysotracker green DND-26, and 644 and 665 nm for Lipo-DiD.

2.6. Examination of the expression level of HER2 on the cell surface by flow cytometry

Surface expression of HER2 in human breast cancer cells MCF7/HER2, MCF7/neo1 and MDA-MB-468 was examined by staining the cells with 1 μg/mL of αHER2 Ab in PBS containing 0.05% (v/v) bovine serum albumin (BSA) for 50 minutes at 4°C, followed by 1 μg/mL of FITC-conjugated goat anti-human IgG Fc (Jackson ImmunoResearch Laboratories, West Grove, PA) for 50 minutes at 4°C. After extensive washing in ice-cold PBS containing 0.05% BSA, the surface immunofluorescence of viable cells was measured with a FACScan flow cytometer (Beckman Coulter FC500 Cytometer) and fluorescence intensities were analyzed with CXP software (Beckman Coulter).

2.7. In vitro cytotoxicity of bispecific antibodies plus PEGylated liposomal doxorubicin

MCF7/HER2, MCF7/neo1 or MDA-MB-468 cells (3×10^3 cells/well) were grown overnight in 96-well microtiter plates precoated with 40 μg/mL of poly-$_D$-lysine (Corning, New York, USA). 66.7 nM of αHER2 Ab, HER2 × mPEG BsAb, HER2 × DNS BsAb, or medium only were added to the wells at RT for 30 minutes, then graded concentrations of Lipo-Dox were added at 37°C for 6 hours. The culture medium was replaced with fresh medium and the cells were incubated until the untreated cells reached 90% cell confluence. The cell viability was measured with ATPlite Luminescence Assay System (Perkin-Elmer, Waltham, MA, USA). Results are expressed as percentage cell viability of luminescence as compared with untreated cells according to the following formula: Percentage (%) cell viability = 100 × (treated luminescence / untreated luminescence). Data are presented as the mean of three independent experiments. The half maximal inhibitory concentration (IC_{50}) values were calculated by fitting the data to a log (inhibitor) versus response (variable slope model) with GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

2.8. Serum half-life of bispecific antibodies

BALB/c mice were intravenously injected with 2.5 mg/kg (333 nM in 100 μl PBS per mouse) of αHER2 Ab, HER2 × mPEG BsAb, or HER2 × DNS BsAb and blood samples were periodically collected from the tail veins of the mice. The preparation of plasma was from whole blood by centrifugation (3 minutes, 3000 rpm). The BsAb levels in the plasma were determined by quantitative sandwich ELISA. Ninety-six-well ELISA microplates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 0.5 μg/ml of goat anti-human IgG Fab antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in 0.1 M $NaHCO_3$ (pH 8.3) coating solution at 37°C for 2 hours and then blocked with 200 μL PBS containing 5% (v/v) skim milk for 2 hours at 37°C. Graded concentrations of αHER2 Abs or plasma samples in PBS containing 2% (v/v) skim milk dilution buffer were added to the wells (50 μL/well) at RT for 1 hour. After washing with PBST three times and PBS one time, the wells were stained with 1 μg/mL of HRP-conjugated goat anti-human IgG Fcγ antibody (Jackson ImmunoResearch Laboratories) at RT for 1 hour. Finally, after washing, 0.4 mg/mL of ABTS solution (150 μL/well) was added for 30 minutes before optical absorbance values at 405 nm were measured using an ELISA reader (Molecular Devices, Menlo Park, CA, USA). Data are presented as the mean of four independent experiments. The initial and terminal half-lives of the BsAbs were estimated by fitting the data to a one-phase exponential decay model with GraphPad Prism 6.0 software.

2.9. Imaging of bispecific antibodies plus PEGylated liposomal DiR

BALB/c nude mice bearing MCF7/HER2 (HER2 high) and MCF7/neo1 (HER2 low) subcutaneous tumors (~100 mm^3) in their mammary fat pad were intravenously injected with HER2 × mPEG BsAb or HER2 × DNS BsAb (2.5 mg/kg) or PBS, respectively. Forty-eight hours after BsAb injection, the mice were intravenously injected with 25 μL of 0.5 mM of DiR-labeled mPEGylated liposomes (Lipo-DiR). Isoflurane anesthetized mice were imaged with an IVIS spectrum optical imaging system (excitation, 745 nm; emission, 800 nm; PerkineElmer, Waltham, MA, USA) at 24, 72 and 96 hours after injection.

2.10. In vivo therapy with bispecific antibodies plus PEGylated liposomal doxorubicin

BALB/c nude mice were inoculated subcutaneously with 3×10^6 MCF7/HER2 cells in 100μL of 1:1 PBS/Matrigel (BD Biosciences, San Jose, CA, USA) in the mammary fat pad. After tumors reached a volume of 50-70 mm^3, mice were intravenously injected with PBS, HER2 × mPEG BsAb or HER2 × DNS BsAb (2.5 mg/kg). After 48 hours, the mice were intravenously administrated Lipo-Dox (4 mg/kg). Treatment was repeated once after 12 days for a total of two rounds. Tumor sizes were measured every 7 days, and tumor volumes were calculated according the formula: (length × width × high)/2. Mice were weighed every 7 days to examine treatment toxicity.

2.11. Statistical analysis

The results of pharmacokinetics and cytotoxicity assays are presented as mean values ± SD. The results of tumor sizes and body weights are shown as mean values ± SEM. All experiments were repeated at least two times with representative data shown. Differences in tumor volumes between groups were examined for statistical significance using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons; P values < 0.05 were considered statistically significant. No statistical method was used to predetermine sample sizes.

3. Results

3.1. Characterization of HER2 × mPEG BsAb

Fig. 2A shows a schematic representation of the bispecific antibody (BsAb) expression constructs. We selected a humanized anti-HER2 antibody (αHER2 Ab) as the tumor targeting antibody to develop a HER2 × mPEG bispecific antibody (BsAb). The light chain and heavy chains of the αHER2 Ab were linked through a furin-2A-based bicistronic sequence to generate the recombinant αHER2 Ab (Fig. 2A). In addition, the C-terminus of the αHER2 Ab was fused with anti-methoxy-PEG scFv (αmPEG scFv) via a flexible linker (GGGSGGG) to generate the HER2 × mPEG BsAb. We also replaced the αmPEG scFv with an anti-hapten dansyl scFv (αDNS scFv) to generate HER2 × DNS BsAb as a negative control.

All of the recombinant antibodies (αHER2 Ab, HER2 × mPEG BsAb, and HER2 × DNS BsAb) were stably expressed in CHO DG44 cells. After purification, the molecular weights of all the antibodies were confirmed by SDS-PAGE under reducing conditions. Fig. 2B shows that the αHER2 Ab was composed of a 55 kDa heavy chain fragment (HC) and a 30 kDa light chain fragment (LC), and the BsAbs were composed of 85 kDa heavy chain-scFv fragments (HC-scFv) and a 30 kDa LC, which were the expected molecular weights of all the antibodies.

To further examine the bi-functional binding activity of the HER2 × mPEG BsAb, the HER2 overexpressing MCF7 (MCF7/HER2)

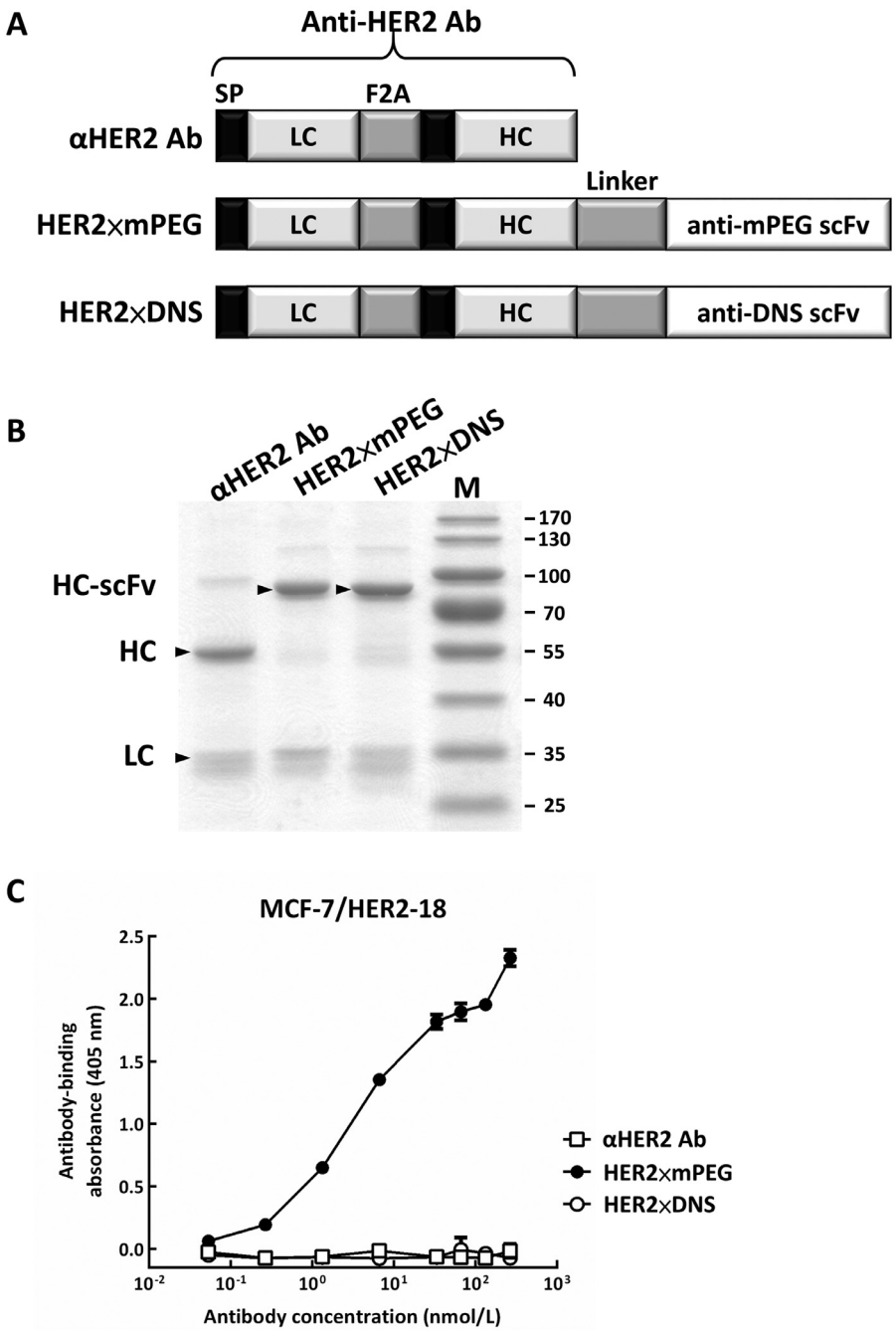

Fig. 2. Characterization of HER2 × mPEG BsAbs. (A) The recombinant anti-HER2 antibody gene encodes an Igκ signal peptide (SP), the anti-HER2 antibody human Igκ light chain (LC), a furin-2A bicistronic element sequence (F2A), and the anti-HER2 antibody human IgG_1 heavy chain (HC). The bispecific antibodies contain an additional GGGSGGG flexible linker peptide (Linker) and anti-mPEG scFv (αHER2×mPEG) or control anti-dansyl scFv (αHER2×DNS). (B) Reducing SDS-PAGE of purified Abs and BsAbs. HC-scFv: heavy chain fused with scFv. HC: heavy chain. LC: light chain. M: PageRuler pre-stained protein ladder (Fermentas). (C) MCF7/HER2 cells were incubated with αHER2 Ab (white squares), HER2×mPEG (black circles), or HER2×DNS (white circles), then incubated with Lipo-Dox (mPEG-liposomes). Binding of Lipo-Dox to MCF7/HER2 cells mediated by HER2×mPEG BsAbs was measured by ELISA. The mean absorbance values (405 nm) of triplicate determinations are shown. The bars indicate SD.

cells were incubated with different concentrations of αHER2 Ab, HER2 × mPEG BsAb, or HER2 × DNS BsAb at room temperature (RT) for 1 hour. After washing, mPEGylated liposomal doxorubicin (Lipo-Dox) was incubated with these cells. The amount of bound Lipo-Dox was detected via biotinylated-AGP4 anti-PEG backbone antibody [30], HRP-conjugated streptavidin and ABTS substrate, then the absorbance was measured at 405 nm. As shown in Fig. 2C, the absorbance could be detected in cells incubated with HER2 × mPEG BsAb but not αHER2 Ab or HER2 × DNS BsAb. These results indicate that HER2 × mPEG BsAb can simultaneously bind to HER2 expressed on the cell surface and mPEG molecules on mPEGylated liposomes.

3.2. HER2 × mPEG BsAb enhances the internalization of Lipo-DiD by receptor-mediated endocytosis

To investigate whether HER2 × mPEG BsAb can specifically enhance internalization of mPEGylated particles into tumor cells by HER2-mediated endocytosis, MCF7/HER2 (HER2 overexpressing) cells and MDA-MB-468 (HER2 negative) cells were incubated

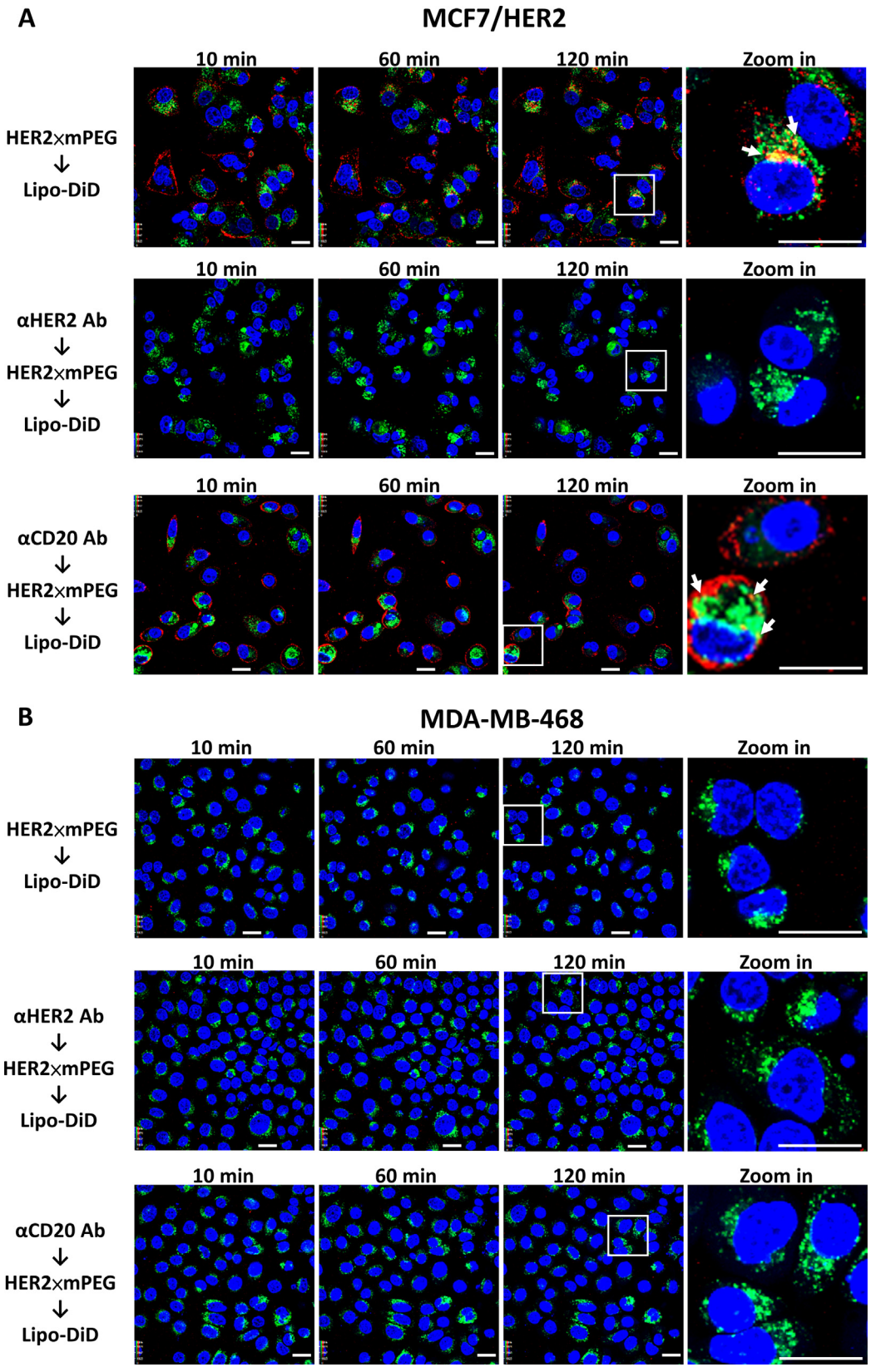

Fig. 3. *In vitro* confocal microscope imaging of the enhanced internalization of Lipo-DiD mediated by pre-treatment with HER2 × mPEG BsAb. (A) MCF7/HER2 cells and (B) MDA-MB-468 cells were incubated with medium only (top row), αHER2 Ab (middle row) and αCD20 Ab (bottom row) supplemented with Hoechst 33342 and LysoTracker Green DND-26 for 30 min at room temperature, then incubated with HER2 × mPEG for 30 min at room temperature, followed by Lipo-DiD and observed in real time with a confocal microscope. Hoechst 33342 (blue), LysoTracker Green DND-26 (green) and Lipo-DiD (red) stain for the nuclei, lysosomes and Lipo-DiD, respectively. The red fluorescence signal (Lipo-DiD) were gradually internalized from the plasma membrane into the cytoplasm with the passage of time and co-localized with green fluorescence signals (lysosomes) showing co-localization color (yellow). Arrows represent co-localization yellow color. White scale bars represent 20 μm.

with HER2 × mPEG BsAb at RT for 30 minutes. We also examined the effect of competition by adding anti-HER2 antibody (αHER2 Ab) or anti-CD20 antibody (αCD20 Ab) as an isotype control to MCF7/HER2 or MDA-MB-468 cancer cells at RT for 30 minutes before the cells were incubated with HER2 × mPEG BsAb at RT for 30 minutes. After washing, Lipo-DiD was added to these cells, and then the fluorescence signal of Lipo-DiD was detected by real-time confocal microscopy imaging. Hoechst 33342 and Lysotracker Green were used to stain the nuclei and lysosomes, respectively. Fig. 3 shows that red fluorescence signals (Lipo-DiD) were detected in MCF7/HER2 cells (Fig. 3A, top) but not in MDA-MB-468 cells (Fig. 3B, top) when they were directly incubated with HER2 × mPEG BsAb and Lipo-DiD, indicating that HER2 × mPEG BsAb could specifically attract Lipo-DiD to the MCF7/HER2 cells and enhance the internalization of Lipo-DiD. However, the HER2 × mPEG BsAb-enhanced internalization of Lipo-DiD was not observed in MCF7/HER2 cells when pre-incubated with competitor αHER2 Ab (Fig. 3A, middle), indicating the function of HER2 × mPEG BsAb could be competed with αHER2 Ab. By contrast, the HER2 × mPEG BsAb-enhanced internalization of Lipo-DiD could be observed in MCF7/HER2 cells when pre-incubated with isotype control αCD20 Ab (Fig. 3A, bottom) as well as directly incubated with HER2 × mPEG BsAb (Fig. 3A, top). HER2 × mPEG BsAb-enhanced binding and internalization of Lipo-DiD was not observed in HER2 negative MDA-MB-468 cells (Fig. 3B). These results suggested that HER2 × mPEG BsAb can mediate selective binding and enhance the internalization of mPEG-liposomes (Lipo-DiD) through HER2-mediated endocytosis, which is important to increase the cellular uptake of nanoparticles.

3.3. HER2 × mPEG BsAb enhances the cytotoxicity of Lipo-Dox to HER2-expressing cancer cells

We next analyzed whether the HER2 × mPEG BsAb can enhance the cytotoxicity of Lipo-Dox to breast cancer cells. We used three breast cancer cell lines with varying levels of HER2 (supplementary Figure 1), the HER2-high expressing cells (MCF7/HER2), HER2-low expressing cells (MCF7/neo1) or HER2 negative cells (MDA-MB-468) were incubated with or without αHER2 Ab, HER2 × mPEG BsAb or HER2 × DNS BsAb at RT for 30 minutes. After removing the antibody-containing medium, all of the groups were subsequently treated with graded concentrations of Lipo-Dox. Cell viability were measured by ATPlite luminescence assay, and the results are shown as percentage inhibition of luminescence as compared with untreated groups. The half maximal inhibitory concentration (IC_{50}) values of MCF7/HER2, MCF7/neo1 and MDA-MB-468 cells treated with each group were analyzed ($n = 3$). As shown in Fig. 4A, the HER2 × mPEG BsAb enhanced the cytotoxicity of Lipo-Dox (IC_{50}: 0.04 μg/mL) against HER2-high expressing MCF7/HER2 cancer cells as compared with HER2 × DNS BsAb plus Lipo-Dox (IC_{50}: 4.3 μg/mL), αHER2 Ab plus Lipo-Dox (IC_{50}: 3.1 μg/mL) and Lipo-Dox alone (IC_{50}: 3.9 μg/mL). In the HER2-low expressing MCF7/neo1 cancer cells (Fig. 4B), the HER2 × mPEG BsAb also enhanced the cytotoxicity of Lipo-Dox (IC_{50}: 0.7 μg/mL) as compared with HER2 × DNS BsAb plus Lipo-Dox (IC_{50}: 4.4 μg/mL), αHER2 Ab plus Lipo-Dox (IC_{50}: 4.6 μg/mL) and Lipo-Dox alone (IC_{50}: 6.7 μg/mL). The results indicate that the HER2 × mPEG BsAb can potentially promote the anti-cancer efficacy of Lipo-Dox to $HER2^+$ cancer cells as compared with HER2 × DNS control BsAb. By contrast, HER2 × mPEG, HER2 × DNS or αHER2 Ab did not improve the cytotoxic effect of Lipo-Dox to HER2 negative MDA-MB-468 cancer cells (Fig. 4C) as compared with Lipo-Dox alone, the IC_{50} was 1.3 μg/mL, 1.2 μg/mL, 1.4 μg/mL and 1.4 μg/mL, respectively. The IC_{50} of HER2 × mPEG BsAb plus Lipo-Dox on MCF7/HER2, MCF7/neo1 and MDA-MB-468 cells were 107.5-fold, 6.3-fold and 0.9-fold lower than HER2 × DNS BsAb plus Lipo-Dox, respectively. We found the cytotoxic effect of HER2 × mPEG BsAb plus Lipo-Dox was more efficient against MCF7/HER2 cells than MCF7/neo1 cells but had no effect on MDA-MB-468 cells. Our observations are consistent with the HER2 antigen-dependent levels on the surfaces of these cells, indicating that the αHER2 recombi-

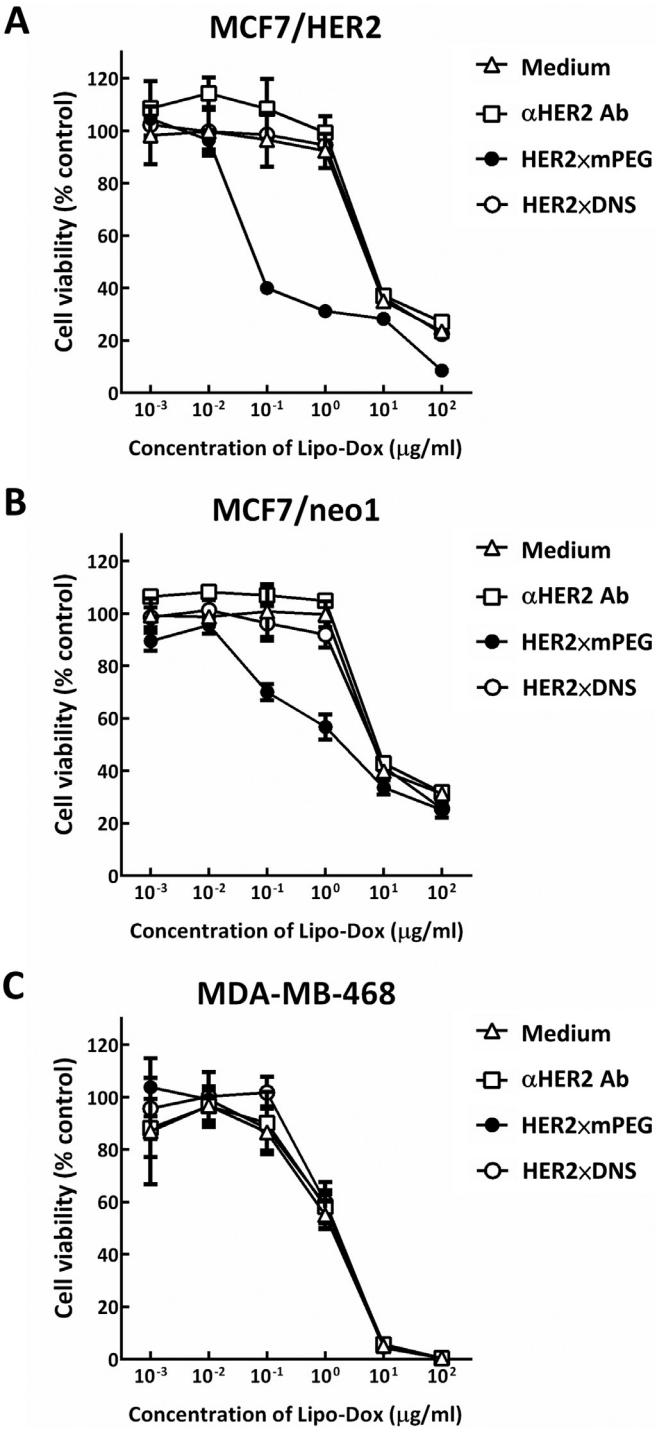

Fig. 4. HER2 × mPEG BsAb enhances the cytotoxicity of Lipo-Dox. Cell viability of (A) MCF7/HER2 (HER2 high), (B) MCF7/neo1 (HER2 low) and (C) MDA-MB-468 (HER2 negative) cells treated with culture medium (white triangles), αHER2 Ab (white squares), HER2 × mPEG (black circles) or HER2 × DNS (white circles) for 30 min, respectively, followed by addition of serial dilutions of Lipo-Dox in triplicate for 6 h. The cell viability was measured by ATPlite analysis 96 h later. Results show mean cell viability compared to untreated control cells ($n = 3$). The bars indicate the SD.

nant antibodies indeed exhibit specificity to the HER2 antigen expressed on these cells.

3.4. Serum half-life and specific targeting of HER2 × mPEG BsAb

The serum half-lives of αHER2 Ab, HER2 × mPEG BsAb and HER2 × DNS BsAb were evaluated to determine a reasonable time point for administration of mPEG-liposomes after administration of BsAbs *in vivo*. The 333 nM recombinant antibody samples were injected intravenously into BALB/c mice, then serum samples were taken at various times and the concentration of recombinant antibodies were determined by sandwich ELISA. The half-lives of the αHER2 Ab, HER2 × mPEG BsAb and HER2 × DNS BsAb were 4.3, 2.7 and 2.6 days, respectively (Fig. 5A).

We further examined the different time intervals between injection of BsAb and mPEG-liposomes to find the optimal time point to effectively accumulate mPEG-liposomes in tumors (Supplementary Figure 2). We chose 24 h, 48 h and 72 h post-injection of HER2 × mPEG BsAb or HER2 × DNS BsAb for the injection of Lipo-DiR into the tumor-bearing mice. Then, we used an *in vivo* imaging system to image mice to detect DiR fluorescence signals. The results show that the DiR fluorescence signals detected at MCF7/HER2 tumors (HER2 high) were highest in the mice in which the injection time interval was 48 h between HER2 × mPEG BsAbs and Lipo-DiR injection, followed by the 72 h time interval and finally the 24 h time interval. On the other hand, with administration of HER2 × DNS BsAb plus Lipo-DiR at different time intervals, the DiR fluorescence signals detected at MCF7/HER2 tumors were similar to those with administration of Lipo-DiR alone. Therefore, we chose 48 h as the optimal time interval between BsAb and mPEG-liposome administration in subsequent studies.

To examine whether the accumulation rate and retention time of mPEG-liposomes were increased by pre-treating HER2-expressing tumors with HER2 × mPEG BsAbs, BALB/c nude mice bearing established HER2-high expressing MCF7/HER2 (right side) and HER2-low expressing MCF7/neo1 (left side) tumors in their fourth pair of mammary fat pads, respectively, were intravenously injected with HER2 × mPEG BsAb or HER2 × DNS BsAb. After 48 hours, they were intravenously injected with Lipo-DiR (DiR-labeled mPEGylated liposomes). These mice were optically imaged using an *in vivo* imaging system at 24, 72 and 96 hours after Lipo-DiR injection. After pre-treatment with HER2 × mPEG BsAb, DiR fluorescence signals were obviously detected in MCF7/HER2 (HER2 high) tumors as compared with the MCF7/neo1 (HER2 low) tumors from 24 to 96 hours after Lipo-DiR injection (Fig. 5B, bottom). The DiR fluorescence intensity of HER2 × mPEG BsAb plus Lipo-DiR in MCF7/HER2 (HER2 high) tumors was 2.2-fold greater than in MCF7/neo1 (HER2 low) tumors at 96 hours. By contrast, with administration of Lipo-DiR alone (Fig. 5B, top) or HER2 × DNS BsAb plus Lipo-DiR (Fig. 5B, middle), the DiR fluorescence signals in MCF7/HER2 (HER2 high) tumors and MCF7/neo1 (HER2 low) tumors were 1.3-fold and 1.0-fold higher, respectively. These results indicate that the specific targeting of HER2 × mPEG BsAb but not HER2 × DNS BsAb can increase the accumulation and retention time of Lipo-DiR to HER2-high expressing MCF7/HER2 tumors as compared with administration of Lipo-DiR alone.

3.5. HER2 × mPEG BsAb improves the antitumor activity of Lipo-Dox in HER2-high expressing tumors

To examine whether HER2 × mPEG BsAb could enhance the anti-cancer efficacy of Lipo-Dox in HER2-expressing tumors, BALB/c nude mice bearing established MCF7/HER2 tumors were divided into five groups: phosphate-buffered saline (PBS), Lipo-Dox alone, HER2 × mPEG BsAb alone, HER2 × mPEG BsAb plus Lipo-Dox and HER2 × DNS BsAb plus Lipo-Dox. The BsAbs were intravenously injected into mice 48 hours before Lipo-Dox treatment on day 1 and day 13. Fig. 6A shows that the combination treatment of HER2 × DNS BsAb plus Lipo-Dox significantly inhibited MCF7/HER2 tumor growth as compared to the mice treated with Lipo-Dox alone or HER2 × mPEG BsAb alone ($P < 0.0001$). Importantly, HER2 × mPEG BsAb plus Lipo-Dox significantly suppressed MCF7/HER2 tumor growth as compared to the mice treated with HER2 × DNS BsAb plus Lipo-Dox ($P = 0.0025$) and without obvious toxicity as assessed by mouse body weight (Fig. 6B). Our results demonstrated that HER2 × mPEG BsAb greatly enhances the therapeutic efficacy of Lipo-Dox to HER2-high expressing tumors, which is attributed to the HER2 × mPEG BsAb being able to actively attract mPEGylated liposomes to tumors, while HER2 × DNS BsAb cannot. We conclude that the active attraction-based combination therapy of HER2 × mPEG BsAb plus Lipo-Dox is more effective than combination therapy of HER2 × DNS BsAb plus Lipo-Dox against HER2-expressing tumors.

4. Discussion

We successfully used pre-treatment with HER2 × mPEG BsAb to effectively attract mPEGylated products (Lipo-Dox) to $HER2^+$ tumor cells, and facilitate the internalization and cytotoxicity of mPEGylated products. Importantly, HER2 × mPEG BsAb can prolong the retention time of Lipo-DiR and thereby greatly improve the therapeutic effect of Lipo-Dox in $HER2^+$ tumors. This active attraction-based combination therapy is a much more efficient therapy than traditional combination therapy because mPEG-binding BsAb retains the anti-tumor activity of the targeted antibody and can actively attract and accumulate any mPEGylated drug (such as liposomes, micelles, proteins, etc) in tumors. We believe that the mPEG-binding BsAb possesses a more powerful ability to enhance the efficacy of mPEGylated drugs available in the clinic than traditional antibodies, and potentially have extensive clinical applications.

It is important to increase the tumor accumulation of hapten-modified anti-cancer drugs for effective therapeutic efficacy of cancer, because hapten-modified drugs generally lack tumor specificity. Active targeting has been one approach to enhance tumor specific targeting and cellular uptake of drug payloads [33]. Several strategies have been successfully designed which use BsAb for delivering hapten-modified payloads to target sites [23,34–36]. For example, Brinkmann and colleagues developed digoxigenin (dig)-binding bispecific antibodies (HER2 × dig, IGF1R × dig, CD22 × dig or LeY × dig) that could effectively increase accumulation of digoxigeninylated compounds (dig-siRNAs, dig-Doxorubicin, dig-Cy5) to tumor antigen-expressing tumors [37,38]; Goldenberg and colleagues also described a CEA × HSG BsAb that could deliver histamine-succinyl-glycine (HSG) hapten-modified radiopeptide (^{111}In-labeled HSG peptide) to CEA-expressing GW-39 human colon tumors [39]. However, these methods need additional chemical conjugation of hapten (dig and HSG) to the payloads, and the hapten moiety modified on the payload is not approved by the Food and Drug Administration (FDA), thus these methods may have limited clinical applications. Here, we generated a HER2 × mPEG BsAb that can simultaneously target $HER2^+$ tumors and actively attract mPEGylated products (Lipo-Dox, Lipo-DiD, Lipo-DiR) to target sites for enhancement of tumor imaging and therapy. PEG molecules have been approved by the FDA for drug development [40], and many PEGylated drugs are already used in the clinical setting [41,42], such as PEGylated liposomal drugs (e.g., Caelyx and Onivyde) [7,43], PEGylated nanoparticles (e.g., CALLA 01) [44], PEGylated micelles (e.g., Genexol-PM and Nanoplatin) [45,46], PEGylated proteins/enzymes (e.g., Sylatron, Oncaspar, and Pegasys) [47–49], and PEGylated low molecular weight drugs (e.g., PEG-

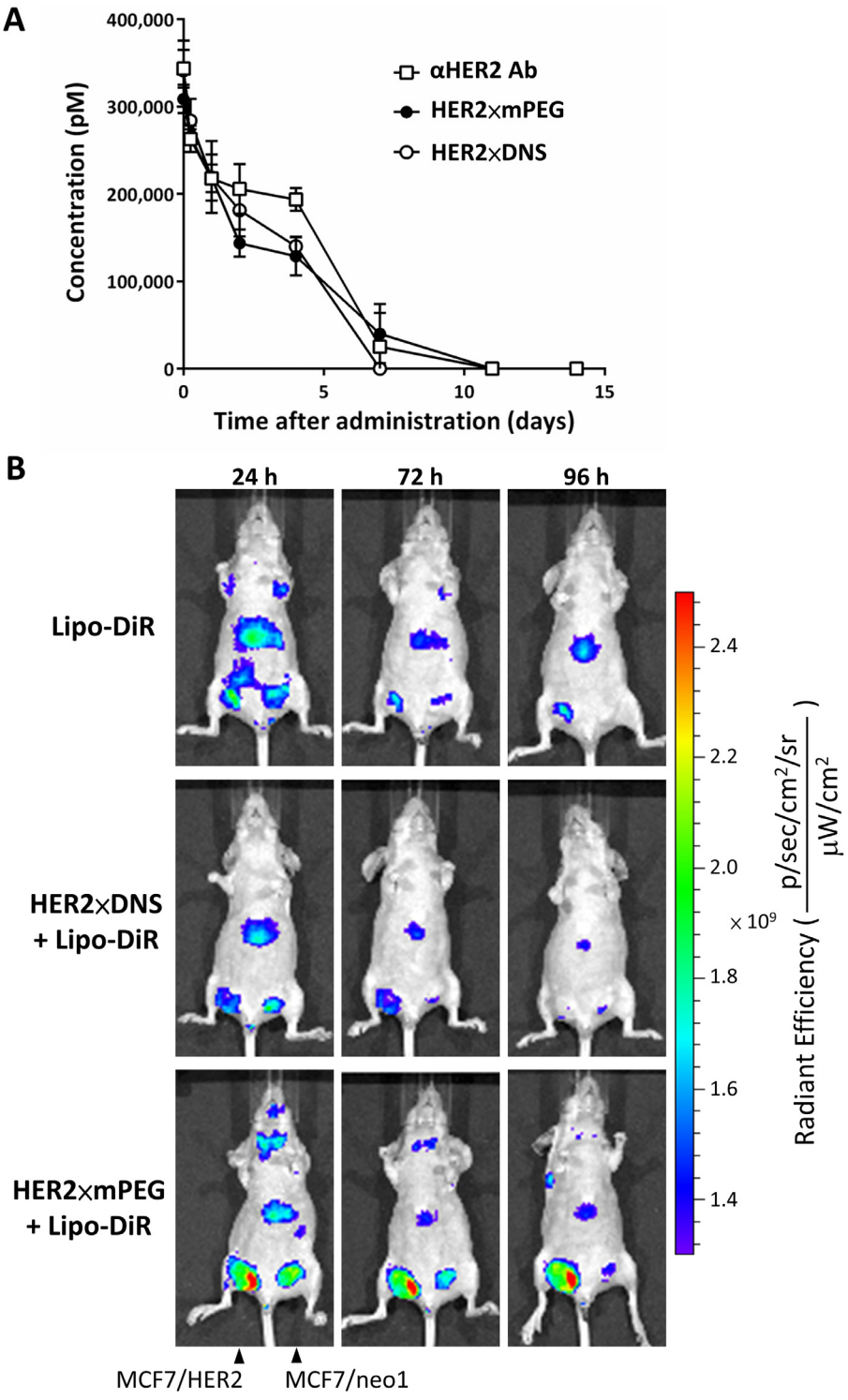

Fig. 5. Imaging of Lipo-DiR accumulation in HER2-high expressing tumors with HER2 × mPEG BsAb pre-treatment. (A) Pharmacokinetics of αHER2 Ab (white squares), HER2 × mPEG (black circles) or HER2 × DNS (white circles). The 333 nM Abs or BsAbs were injected intravenously into BALB/c mice and then serum samples were taken at various times. Mean concentration of recombinant antibodies were measured by sandwich ELISA ($n = 4$). (B) *In vivo* imaging of Lipo-DiR in BsAb pre-treated tumor-bearing mice. HER2-high expressing MCF7/HER2 (right mammary fat pad) and HER2-low expressing MCF7/neo1 (left mammary fat pad) tumor-bearing nude mice were intravenously injected with PBS (top row), HER2 × DNS (middle row) or HER2 × mPEG (bottom row). After 48 h, each of the mice received intravenous injection of Lipo-DiR. Mice were sequentially imaged at 24 h, 72 h and 96 h with an IVIS spectrum optical imaging system.

irinotecan) [50]. The aforementioned mPEGylated drugs may be attracted by HER2 × mPEG BsAb to accumulate at target sites. We believe that the HER2 × mPEG BsAb is a promising tool for combining currently available mPEGylated drugs or probes that can actively attract these clinical agents to target sites to enhance the therapeutic or diagnostic efficacy.

The concept of a BsAb that can simultaneously target tumors and directly attract therapeutic agents to tumors may greatly increase the therapeutic efficacy of antibodies for cancer. For example, in recent decades, the anti-CD3 antibodies (αCD3 Abs) has been paired with various tumor-targeted antibodies to generate tumor marker × CD3 BsAbs to direct $CD3^+$ T cells to eliminate

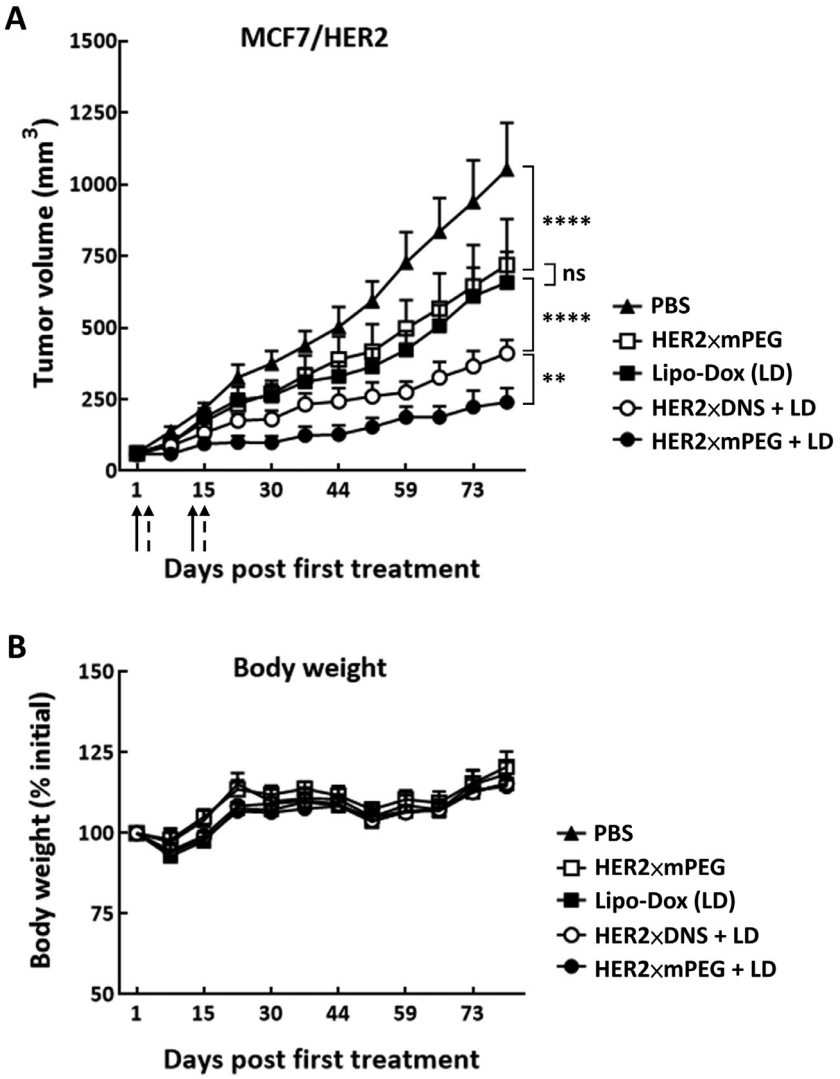

Fig. 6. Therapeutic efficacy of active attraction-based combination therapy (HER2 × mPEG BsAb plus Lipo-Dox) against HER2-expressing tumors. BALB/c nude mice bearing MCF7/HER2 tumors were intravenously injected with PBS (black triangles), HER2 × mPEG BsAb alone (white squares), Lipo-Dox alone (black squares), HER2 × DNS BsAb plus Lipo-Dox (white circles) and HER2 × mPEG BsAb plus Lipo-Dox (black circles). The 2.5 mg/kg of BsAbs were intravenously injected into mice on day 1 and day 13 (solid arrows). After 48 hours, the mice were intravenously administrated with 4 mg/kg of Lipo-Dox (dotted arrows). (A) Tumor volume was estimated once a week. Results show mean tumor sizes ± SEM; $n = 4$ mice per group in two independent experiments; ****, $P < 0.0001$, **, $P = 0.0025$ on day 80 analyzed by two-way ANOVA; ns, not significant. (B) Mean body weights of treated MCF7/HER2-bearing mice ($n = 4$, in duplicates).

tumors. Lindhofer and colleagues found that the therapeutic efficacy of EpCAM × CD3 BsAb (Catumaxomab) was 2.3-fold higher than the combination of the two parental antibodies [51]; they also showed that after changing the αEpCAM arm of the BsAb with an αCD20 Ab, the CD20 × CD3 BsAb (Lymphomun) displayed 2-fold higher tumor elimination than αCD20 Ab [52]. The CD3 BsAb enhanced the anti-cancer efficacy of the parental antibody via CD3 T cell recruitment. Similarly, in our study, the HER2 × mPEG BsAb can simultaneously target $HER2^+$ breast tumors and attract the mPEGylated drug Lipo-Dox to target sites to significantly enhance the therapeutic efficacy of Lipo-Dox against $HER2^+$ tumors as compared with HER2 × DNS control BsAb or αHER2 Ab *in vitro* and *in vivo*. These results indicate that the efficacy of antibody plus mPEGylated drugs can be improved by fusing a novel mPEG-specific scFv to the Fc terminus of an αHER2 Ab. Notably, the mPEG-specific scFv is not limited to HER2-targeted antibodies, but may be further expanded for other tumor-targeted antibodies [1], such as αEGFR Ab, αPSMA Ab and αCD20 Ab to generate various BsAb that can attract mPEGylated drugs to enhance anticancer activity. Changeable tumor-targeted antibodies can be tailor-made to suit patients' tumor-associated antigen profiling at the tumor sites to achieve precision medicine. Therefore, the mPEG-specific scFv is a flexible element that can be extensively fused to various disease-targeted antibodies to develop next generation BsAbs to directly attract mPEGylated drugs to tumors to enhance their therapeutic efficacy.

Pre-treatment with BsAb followed by administration of mPEGylated drugs can increase the tumor accumulation of drugs. This non-attached administration may help to maintain the natural properties of both treatments. Conjugation of cytotoxic drugs to a targeted antibody is a well-known method to increase the tumor accumulation of drugs. For example, antibody-drug conjugates (ADCs) consist of antibodies conjugated with cytotoxic drugs via a biodegradable linker to increase the internalization of cytotoxic drugs into tumor cells [53]. However, figuring out the optimal drug-to-antibody ratio (DAR) which does not affect the basic properties of antibodies and cytotoxic drugs takes a lot of time and is expensive. Furthermore, a DAR>4 can diminish ADC solubility and influence stability, thus, ADC are limited by the low DAR of 2-4 [54]. Our previous studies described one-step mixing of mPEG × HER2 BsAb (Fab × scFv format) and mPEG-liposomes to form targeted-liposomes followed by direct dosing of breast tumor-

bearing mice [23,24]. However, formulation of targeted-liposomes may produce heterogeneous products and might alter the *in vivo* behavior of the liposomes in unpredictable ways [55]. In our study, pre-treatment of HER2 × mPEG BsAb followed by administration of Lipo-Dox exhibited more efficient anti-cancer activity than Lipo-Dox single treatment or HER2 × DNS control BsAb plus Lipo-Dox against antigen$^+$ tumors *in vitro* and *in vivo*, suggesting the HER2 × mPEG BsAb could enhance tumor specificity and therapeutic effects of Lipo-Dox in two-step administration. There is no chemical modification between the BsAbs and liposomes in this method, thus simplifying the manufacturing process, and it will be helpful to maintain the natural properties of BsAbs and liposomes. In addition, two-step administration provides high flexibility to choose a wider range of liposomal drugs, and each liposome can encapsulate ~10,000 drug molecules [7] that may greatly increase the therapeutic efficacy of drugs. However, two-step administration has a limitation, i.e., a reasonable time point for administration of mPEGylated-liposomes following BsAbs must be rigorously evaluated to confirm its suitability for clinical applications. This limitation might be solved by further assessing the half-life of BsAbs in the systemic circulation of patients and determining the proper interval between administration of BsAbs and mPEG-liposomes to effectively accumulate mPEG-liposomes in tumors. In addition, pretargeted BsAb may be endocytosed into cancer cells before liposomes are administered. However, endocytosed receptors may be recycled back to the cell surface after cellular internalization [56], thus the recycled receptors may be retargeted by BsAbs to continuously attract mPEG-liposomes to the cell. Therefore, mPEG-binding BsAb can increase the tumor accumulation of mPEGylated drugs without the influence of chemical modification, meaning that it has development potential and possible clinical application.

In summary, our results demonstrate that HER2 × mPEG BsAb can simultaneously bind tumor antigens and mPEG molecules, and actively attract mPEGylated products, such as Lipo-Dox, Lipo-DiD and Lipo-DiR to tumor sites. Pre-treatment of HER2 × mPEG BsAb greatly enhanced the therapeutic efficacy of Lipo-Dox against HER2$^+$ tumors. Moreover, the strategy of mPEG-binding BsAb plus mPEGylated drugs has following advantages: (1) enhancement of the therapeutic efficacy of traditional combination therapies (targeted antibody plus mPEGylated drugs); (2) the BsAb can actively attract any mPEGylated drugs used in the clinic; (3) the mPEG-specific scFv can be fused to any kind of disease-targeted antibody, therefore it is flexible and has potentially wide clinical applications; (4) two-step administration may help to maintain the natural properties of BsAb and mPEGylated drugs as compared with chemical conjugation; (5) more simple manufacturing processes as chemical conjugation steps are not required. Additionally, (6) in retaining the intact IgG format it is expected that the immune-mediated function will be retained, which is a desirable add-on for therapeutic antibodies, such as ADCC and CDC. According to these advantages, we anticipate the mPEG-specific scFv can serve as a versatile bridging element to generate next-generation mPEG-binding BsAbs for connecting disease-targeted antibodies and mPEGylated drugs.

Declaration of Competing Interest

The authors declare no potential conflicts of interest or personal 764 relationships that could have appeared to influence the work re- 765 ported in this paper.

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology, Taipei, Taiwan (MOST 107-2320-B-037-024 -MY3, MOST 107-2320-B-037-028-MY2 and MOST 108-2311-B-037-001-MY2); the National Health Research Institutes, Taiwan (NHRI-EX108-10729EI); Academia Sinica, Taiwan (AS-TP-107-L11); the Ministry of Education, Taiwan (108RSB0029); the Program for Translational Innovation of Biopharmaceutical Development-Technology Supporting Platform Axis, Academia Sinica, Taiwan (Grant No. AS-KPQ-106-TSPA); and the KMU-KMUH Co-Project of Key Research (KMU-DK109001) and Research Foundation (KMU-DK109004) from Kaohsiung Medical University, Taiwan. This study is also supported partially by Kaohsiung Medical University Research Center Grant (Drug Development and Value Creation Research Center) (KMU-TC108A03). We also thank the Center for Research Resources and Development of Kaohsiung Medical University for providing the service of Olympus FV1000 system.

Author Contributions Statement

I.J. Chen designed and performed experiments in the manuscript, analyzed data and wrote the manuscript; Y.A. Cheng helped with experiments, data analysis and contributed to manuscript editing; K.W. Ho helped with experiments and contributed to manuscript editing; W.W. Lin, K.W. Cheng, Y.C. Lu, Y.C. Hsieh, C.C. Huang, C.H. Chuang helped with data analysis and contributed to manuscript editing; F.M. Chen, Y.C. Su helped with data analysis; S.R. Roffler provided the concept, experimental design and contributed to manuscript writing and editing; T.L. Cheng provided the concept, experimental design, assisted in eliminating problems during experiments and contributed to manuscript writing and editing.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actbio.2020.04.029.

References

[1] A.M. Scott, J.D. Wolchok, L.J. Old, Antibody therapy of cancer, Nat Rev Cancer 12 (4) (2012) 278–287.
[2] L. Sercombe, T. Veerati, F. Moheimani, S.Y. Wu, A.K. Sood, S. Hua, Advances and Challenges of Liposome Assisted Drug Delivery, Front Pharmacol 6 (2015) 286.
[3] C.A. Hudis, Trastuzumab–mechanism of action and use in clinical practice, N Engl J Med 357 (1) (2007) 39–51.
[4] R. Kumar, M. Mandal, R. Vadlamudi, New insights into anti-HER-2 receptor monoclonal antibody research, Semin Oncol 27 (6 Suppl 11) (2000) 84–91 discussion 92-100.
[5] A. Musolino, N. Naldi, B. Bortesi, D. Pezzuolo, M. Capelletti, G. Missale, D. Laccabue, A. Zerbini, R. Camisa, G. Bisagni, T.M. Neri, A. Ardizzoni, Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer, J Clin Oncol 26 (11) (2008) 1789–1796.
[6] D.J. Slamon, G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, W.L. McGuire, Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene, Science 235 (4785) (1987) 177–182.
[7] Y. Barenholz, Doxil(R)–the first FDA-approved nano-drug: lessons learned, J Control Release 160 (2) (2012) 117–134.
[8] M.E. O'Brien, N. Wigler, M. Inbar, R. Rosso, E. Grischke, A. Santoro, R. Catane, D.G. Kieback, P. Tomczak, S.P. Ackland, F. Orlandi, L. Mellars, L. Alland, C. Tendler, C.B.C.S. Group, Reduced cardiotoxicity and comparable efficacy in a phase III trial of pegylated liposomal doxorubicin HCl (CAELYX/Doxil) versus conventional doxorubicin for first-line treatment of metastatic breast cancer, Ann Oncol 15 (3) (2004) 440–449.
[9] A.A. Gabizon, Y. Barenholz, M. Bialer, Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs, Pharm Res 10 (5) (1993) 703–708.
[10] S.M. Moghimi, J. Szebeni, Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties, Prog Lipid Res 42 (6) (2003) 463–478.
[11] S. Chia, M. Clemons, L.A. Martin, A. Rodgers, K. Gelmon, G.R. Pond, L. Panasci, Pegylated liposomal doxorubicin and trastuzumab in HER-2 overexpressing metastatic breast cancer: a multicenter phase II trial, J Clin Oncol 24 (18) (2006) 2773–2778.

[12] E. Stickeler, M. Klar, D. Watermann, A. Geibel, M. Foldi, A. Hasenburg, G. Gitsch, Pegylated liposomal doxorubicin and trastuzumab as 1st and 2nd line therapy in her2/neu positive metastatic breast cancer: a multicenter phase II trial, Breast Cancer Res Treat 117 (3) (2009) 591–598.
[13] M. Fiegl, B. Mlineritsch, M. Hubalek, R. Bartsch, U. Pluschnig, G.G. Steger, Single-agent pegylated liposomal doxorubicin (PLD) in the treatment of metastatic breast cancer: results of an Austrian observational trial, BMC Cancer 11 (2011) 373.
[14] H. Nehoff, N.N. Parayath, L. Domanovitch, S. Taurin, K. Greish, Nanomedicine for drug targeting: strategies beyond the enhanced permeability and retention effect, Int J Nanomedicine 9 (2014) 2539–2555.
[15] J. Connor, S. Sullivan, L. Huang, Monoclonal antibody and liposomes, Pharmacol Ther 28 (3) (1985) 341–365.
[16] F. Pastorino, C. Brignole, D. Marimpietri, P. Sapra, E.H. Moase, T.M. Allen, M. Ponzoni, Doxorubicin-loaded Fab' fragments of anti-disialoganglioside immunoliposomes selectively inhibit the growth and dissemination of human neuroblastoma in nude mice, Cancer Res 63 (1) (2003) 86–92.
[17] P. Sapra, T.M. Allen, Improved outcome when B-cell lymphoma is treated with combinations of immunoliposomal anticancer drugs targeted to both the CD19 and CD20 epitopes, Clin Cancer Res 10 (7) (2004) 2530–2537.
[18] W.W. Cheng, T.M. Allen, Targeted delivery of anti-CD19 liposomal doxorubicin in B-cell lymphoma: a comparison of whole monoclonal antibody, Fab' fragments and single chain Fv, J Control Release 126 (1) (2008) 50–58.
[19] K. Maruyama, N. Takahashi, T. Tagawa, K. Nagaike, M. Iwatsuru, Immunoliposomes bearing polyethyleneglycol-coupled Fab' fragment show prolonged circulation time and high extravasation into targeted solid tumors in vivo, FEBS Lett 413 (1) (1997) 177–180.
[20] P. Sapra, P. Tyagi, T.M. Allen, Ligand-targeted liposomes for cancer treatment, Curr Drug Deliv 2 (4) (2005) 369–381.
[21] J.W. Park, K. Hong, D.B. Kirpotin, G. Colbern, R. Shalaby, J. Baselga, Y. Shao, U.B. Nielsen, J.D. Marks, D. Moore, D. Papahadjopoulos, C.C. Benz, Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery, Clin Cancer Res 8 (4) (2002) 1172–1181.
[22] J.G. Reynolds, E. Geretti, B.S. Hendriks, H. Lee, S.C. Leonard, S.G. Klinz, C.O. Noble, P.B. Lucker, P.W. Zandstra, D.C. Drummond, K.J. Olivier Jr., U.B. Nielsen, C. Niyikiza, S.V. Agresta, T.J. Wickham, HER2-targeted liposomal doxorubicin displays enhanced anti-tumorigenic effects without associated cardiotoxicity, Toxicol Appl Pharmacol 262 (1) (2012) 1–10.
[23] C.H. Kao, J.Y. Wang, K.H. Chuang, C.H. Chuang, T.C. Cheng, Y.C. Hsieh, Y.L. Tseng, B.M. Chen, S.R. Roffler, T.L. Cheng, One-step mixing with humanized anti-mPEG bispecific antibody enhances tumor accumulation and therapeutic efficacy of mPEGylated nanoparticles, Biomaterials 35 (37) (2014) 9930–9940.
[24] Y.A. Cheng, I.J. Chen, Y.C. Su, K.W. Cheng, Y.C. Lu, W.W. Lin, Y.C. Hsieh, C.H. Kao, F.M. Chen, S.R. Roffler, T.L. Cheng, Enhanced drug internalization and therapeutic efficacy of PEGylated nanoparticles by one-step formulation with anti-mPEG bispecific antibody in intrinsic drug-resistant breast cancer, Biomater Sci 7 (8) (2019) 3404–3417.
[25] J. Cui, Y. Ju, Z.H. Houston, J.J. Glass, N.L. Fletcher, S. Alcantara, Q. Dai, C.B. Howard, S.M. Mahler, A.K. Wheatley, R. De Rose, P.T. Brannon, B.M. Paterson, P.S. Donnelly, K.J. Thurecht, F. Caruso, S.J. Kent, Modulating Targeting of Poly(ethylene glycol) Particles to Tumor Cells Using Bispecific Antibodies, Adv Healthc Mater 8 (9) (2019) e1801607.
[26] D. Song, J. Cui, Y. Ju, M. Faria, H. Sun, C.B. Howard, K.J. Thurecht, F. Caruso, Cellular Targeting of Bispecific Antibody-Functionalized Poly(ethylene glycol) Capsules: Do Shape and Size Matter? ACS Appl Mater Interfaces 11 (32) (2019) 28720–28731.
[27] S.I. Rudnick, J. Lou, C.C. Shaller, Y. Tang, A.J. Klein-Szanto, L.M. Weiner, J.D. Marks, G.P. Adams, Influence of affinity and antigen internalization on the uptake and penetration of Anti-HER2 antibodies in solid tumors, Cancer Res 71 (6) (2011) 2250–2259.
[28] L.S. Shahied, Y. Tang, R.K. Alpaugh, R. Somer, D. Greenspon, L.M. Weiner, Bispecific minibodies targeting HER2/neu and CD16 exhibit improved tumor lysis when placed in a divalent tumor antigen binding format, J Biol Chem 279 (52) (2004) 53907–53914.
[29] J. Fang, J.J. Qian, S. Yi, T.C. Harding, G.H. Tu, M. VanRoey, K. Jooss, Stable antibody expression at therapeutic levels using the 2A peptide, Nat Biotechnol 23 (5) (2005) 584–590.
[30] Y.C. Su, B.M. Chen, K.H. Chuang, T.L. Cheng, S.R. Roffler, Sensitive quantification of PEGylated compounds by second-generation anti-poly(ethylene glycol) monoclonal antibodies, Bioconjug Chem 21 (7) (2010) 1264–1270.
[31] K.H. Chuang, H.E. Wang, T.C. Cheng, S.C. Tzou, W.L. Tseng, W.C. Hung, M.H. Tai, T.K. Chang, S.R. Roffler, T.L. Cheng, Development of a universal anti-polyethylene glycol reporter gene for noninvasive imaging of PEGylated probes, J Nucl Med 51 (6) (2010) 933–941.
[32] K.H. Chuang, C.M. Cheng, S.R. Roffler, Y.L. Lu, S.R. Lin, J.Y. Wang, W.S. Tzou, Y.C. Su, B.M. Chen, T.L. Cheng, Combination cancer therapy by hapten-targeted prodrug-activating enzymes and cytokines, Bioconjug Chem 17 (3) (2006) 707–714.
[33] J. Cui, K. Alt, Y. Ju, S.T. Gunawan, J.A. Braunger, T.Y. Wang, Y. Dai, Q. Dai, J.J. Richardson, J. Guo, M. Bjornmalm, C.E. Hagemeyer, F. Caruso, Ligand-Functionalized Poly(ethylene glycol) Particles for Tumor Targeting and Intracellular Uptake, Biomacromolecules 20 (9) (2019) 3592–3600.
[34] F.G. van Schaijk, E. Oosterwijk, A.C. Soede, M. Broekema, C. Frielink, W.J. McBride, D.M. Goldenberg, F.H. Corstens, O.C. Boerman, Pretargeting of carcinoembryonic antigen-expressing tumors with a biologically produced bispecific anticarcinoembryonic antigen x anti-indium-labeled diethylenetriaminepentaacetic acid antibody, Clin Cancer Res 11 (19 Pt 2) (2005) 7130s-7136s.
[35] H.Y. Tung, Y.C. Su, B.M. Chen, P.A. Burnouf, W.C. Huang, K.H. Chuang, Y.T. Yan, T.L. Cheng, S.R. Roffler, Selective Delivery of PEGylated Compounds to Tumor Cells by Anti-PEG Hybrid Antibodies, Mol Cancer Ther 14 (6) (2015) 1317–1326.
[36] Y.C. Su, P.A. Burnouf, K.H. Chuang, B.M. Chen, T.L. Cheng, S.R. Roffler, Conditional internalization of PEGylated nanomedicines by PEG engagers for triple negative breast cancer therapy, Nat Commun 8 (2017) 15507.
[37] K. Mayer, A.L. Baumann, M. Grote, S. Seeber, H. Kettenberger, S. Breuer, T. Killian, W. Schafer, U. Brinkmann, TriFabs–Trivalent IgG-Shaped Bispecific Antibody Derivatives: Design, Generation, Characterization and Application for Targeted Payload Delivery, Int J Mol Sci 16 (11) (2015) 27497–27507.
[38] B. Schneider, M. Grote, M. John, A. Haas, B. Bramlage, L.M. Ickenstein, K. Jahn-Hofmann, F. Bauss, W. Cheng, R. Croasdale, K. Daub, S. Dill, E. Hoffmann, W. Lau, H. Burtscher, J.L. Ludtke, S. Metz, O. Mundigl, Z.C. Neal, W. Scheuer, J. Stracke, H. Herweijer, U. Brinkmann, Targeted siRNA Delivery and mRNA Knockdown Mediated by Bispecific Digoxigenin-binding Antibodies, Mol Ther Nucleic Acids 1 (2012) e46.
[39] E.A. Rossi, C.H. Chang, M.J. Losman, R.M. Sharkey, H. Karacay, W. McBride, T.M. Cardillo, H.J. Hansen, Z. Qu, I.D. Horak, D.M. Goldenberg, Pretargeting of carcinoembryonic antigen-expressing cancers with a trivalent bispecific fusion protein produced in myeloma cells, Clin Cancer Res 11 (19 Pt 2) (2005) 7122s-7129s.
[40] T.L. Cheng, K.H. Chuang, B.M. Chen, S.R. Roffler, Analytical measurement of PEGylated molecules, Bioconjug Chem 23 (5) (2012) 881–899.
[41] P. Mishra, B. Nayak, R.K. Dey, PEGylation in anti-cancer therapy: An overview, Asian J Pharm Sci 11 (3) (2016) 337–348.
[42] P.L. Turecek, M.J. Bossard, F. Schoetens, I.A. Ivens, PEGylation of Biopharmaceuticals: A Review of Chemistry and Nonclinical Safety Information of Approved Drugs, J Pharm Sci 105 (2) (2016) 460–475.
[43] F.C. Passero Jr., D. Grapsa, K.N. Syrigos, M.W. Saif, The safety and efficacy of Onivyde (irinotecan liposome injection) for the treatment of metastatic pancreatic cancer following gemcitabine-based therapy, Expert Rev Anticancer Ther 16 (7) (2016) 697–703.
[44] M.E. Davis, J.E. Zuckerman, C.H. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas, Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles, Nature 464 (7291) (2010) 1067–1070.
[45] K.S. Lee, H.C. Chung, S.A. Im, Y.H. Park, C.S. Kim, S.B. Kim, S.Y. Rha, M.Y. Lee, J. Ro, Multicenter phase II trial of Genexol-PM, a Cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer, Breast Cancer Res Treat 108 (2) (2008) 241–250.
[46] Y. Matsumura, Polymeric micellar delivery systems in oncology, Jpn J Clin Oncol 38 (12) (2008) 793–802.
[47] T.M. Herndon, S.G. Demko, X. Jiang, K. He, J.E. Gootenberg, M.H. Cohen, P. Keegan, R. Pazdur, U.S. Food and Drug Administration Approval: peginterferon-alfa-2b for the adjuvant treatment of patients with melanoma, Oncologist 17 (10) (2012) 1323–1328.
[48] P.A. Dinndorf, J. Gootenberg, M.H. Cohen, P. Keegan, R. Pazdur, FDA drug approval summary: pegaspargase (oncaspar) for the first-line treatment of children with acute lymphoblastic leukemia (ALL), Oncologist 12 (8) (2007) 991–998.
[49] J.H. Lipton, N. Khoroshko, A. Golenkov, K. Abdulkadyrov, K. Nair, D. Raghunadharao, T. Brummendorf, K. Yoo, B. Bergstrom, C.M.L.S.G. Pegasys, Phase II, randomized, multicenter, comparative study of peginterferon-alpha-2a (40 kD) (Pegasys) versus interferon alpha-2a (Roferon-A) in patients with treatment–naive, chronic-phase chronic myelogenous leukemia, Leuk Lymphoma 48 (3) (2007) 497–505.
[50] A. Awada, A.A. Garcia, S. Chan, G.H. Jerusalem, R.E. Coleman, M.T. Huizing, A. Mehdi, S.M. O'Reilly, J.T. Hamm, P.J. Barrett-Lee, V. Cocquyt, K. Sideras, D.E. Young, C. Zhao, Y.L. Chia, U. Hoch, A.L. Hannah, E.A. Perez, N.-S. Group, Two schedules of etirinotecan pegol (NKTR-102) in patients with previously treated metastatic breast cancer: a randomised phase 2 study, Lancet Oncol 14 (12) (2013) 1216–1225.
[51] R. Zeidler, G. Reisbach, B. Wollenberg, S. Lang, S. Chaubal, B. Schmitt, H. Lindhofer, Simultaneous activation of T cells and accessory cells by a new class of intact bispecific antibody results in efficient tumor cell killing, J Immunol 163 (3) (1999) 1246–1252.
[52] M. Stanglmaier, M. Faltin, P. Ruf, A. Bodenhausen, P. Schroder, H. Lindhofer, Bi20 (fBTA05), a novel trifunctional bispecific antibody (anti-CD20 x anti-CD3), mediates efficient killing of B-cell lymphoma cells even with very low CD20 expression levels, Int J Cancer 123 (5) (2008) 1181–1189.
[53] H.L. Perez, P.M. Cardarelli, S. Deshpande, S. Gangwar, G.M. Schroeder, G.D. Vite, R.M. Borzilleri, Antibody-drug conjugates: current status and future directions, Drug Discov Today 19 (7) (2014) 869–881.
[54] K.J. Hamblett, P.D. Senter, D.F. Chace, M.M. Sun, J. Lenox, C.G. Cerveny, K.M. Kissler, S.X. Bernhardt, A.K. Kopcha, R.F. Zabinski, D.L. Meyer, J.A. Francisco, Effects of drug loading on the antitumor activity of a monoclonal antibody drug conjugate, Clin Cancer Res 10 (20) (2004) 7063–7070.
[55] N. Kamaly, Z. Xiao, P.M. Valencia, A.F. Radovic-Moreno, O.C. Farokhzad, Targeted polymeric therapeutic nanoparticles: design, development and clinical translation, Chem Soc Rev 41 (7) (2012) 2971–3010.
[56] M. Ritchie, L. Tchistiakova, N. Scott, Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates, MAbs 5 (1) (2013) 13–21.