BIO Integration

HER2-targeting Peptide Drug Conjugate with Better Penetrability for Effective Breast Cancer Therapy

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Abstract

Antibody-drug conjugates (ADCs) have the following advantages: target specificity; wide therapeutic index; and prolonged circulation half-life. A key limitation of ADCs, however, is the large size (~150 kDa), which markedly slows diffusion through the interstitium of solid tumors and prevents efficient penetration. To address the size issue of ADCs in targeted drug delivery, we developed a HER2-targeting peptide-mertansine conjugate (HER2-TPMC) and conducted a head-to-head comparison with HER2-targeting antibody-mertansine conjugate (HER2-TAMC) as a possible alternative for high-penetration breast cancer therapeutics. As expected, a pharmacokinetic (PK) assay revealed that HER2-TP had lower levels persisting in the circulation after 1 h (~75%) compared to 85% of HER2-targeting antibody (HER2-TA). The cellular cytotoxic effect of HER2-TPMC was similar to HER2-TAMC in the HER2+ BT474 breast cancer cell line, thus demonstrating similar bioactivity of both conjugates. HER2-TPMC not only revealed higher uptake and specificity in *in vitro* 3D spheroid cultures compared to the parental drug, mertansine, but HER2-TPMC also had a significant retention in the spheroids. This finding was in stark contrast to HER2-TAMC, a large-sized conjugate which was not able to penetrate the spheroid barrier, thus resulting minimal penetration. *In vivo* tumoral uptake in a BT474 orthotopic model indicated increased tumor uptake and penetration of HER2-TP compared to parental drug and HER2-TAMC. To summarize, we successfully developed a HER2-targeting peptide-mertansine conjugate with specific cellular uptake that resulted in longer retention times *in vitro* and *in vivo*. HER2-TPMC (~5 kDa in size) exhibited rapid tissue penetration and enhanced tumoral uptake and retention *in vitro* and *in vivo*. Therefore, HER2-TPMC is a reasonable alternative for HER2-positive cancer chemotherapeutics.

Keywords

Breast cancer, cancer therapeutics, enhanced tumor penetration, HER2, peptide-drug conjugate.

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Introduction

Cytotoxic agent-based chemotherapy serves as the foundation for traditional anti-cancer treatment and is widely used to treat a wide range of cancer types [1]. The non-selective toxicity of chemotherapeutic agents, however, leads to reduced efficacy, systemic toxicity, drug resistance, and a narrow therapeutic window, which have highlighted the need and prompted the development of new strategies for more effective and safer delivery of small molecular drugs [2]. Targeted delivery systems are designed to direct toxic agents to tumor sites by targeting specific tumor surface receptors [3]. This approach can be traced back to the early 1900s when Paul Ehrlich, the founder of chemotherapy, created the "magic bullet concept," according to which drugs go straight to the intended cell structural targets while sparing bystander targets [3]. Subsequently, a novel category of medications (antibody–drug conjugates [ADCs]) was developed. All ADCs have three core components: a humanized monoclonal antibody (mAB) that binds a tumor-associated antigen; a cytotoxic agent (the "payload"); and a connecting linker [4]. The antigen-independent uptake of the cytotoxic payload in antigen-negative cells is limited due to conjugation to a large hydrophilic antibody, contributing to target specificity, wide therapeutic index, and long circulation half-life [5, 6]. Fourteen ADCs have received market approval since the first approval event by the Food and Drug Administration (FDA) in 2000 [7].

Notably, there are some limitations with ADCs serving as carriers. First, an antibody with a high molecular weight (~150 kDa) markedly slows diffusion through the

interstitium of solid tumors. Second, ADCs tend to gather in excretory organs, such as the liver and kidneys, which leads to potential life-threatening side effects by off-target toxicity. Finally, although nearly all pharmaceutical companies now have an ADC program in their pipeline, the manufacturing process of antibodies is both time-consuming and costly [8, 9]. Therefore, it is necessary to establish a proper carrier that has durable targeting, but also addresses the aforementioned problems.

Surprisingly, one-half of the approved ADCs are mainly used against hematologic malignancies and the rest are indicated for treatment of solid tumors, especially breast cancer. Trastuzumab emtansine (T-DM1), which contains a trastuzumab backbone linked through a linker (N-maleimidomethyl) to mertansine (DM1), a potent microtubule inhibitor, was the first FDA-approved ADC for the treatment of advanced human epidermal growth factor receptor 2 (HER2)-positive breast cancer, and more recently approved for adjuvant treatment of early-stage, high-risk patients with residual disease after neoadjuvant taxane and trastuzumab-based therapy [10, 11]. Nevertheless, a recent clinical trial showed that T-DM1 was not superior to trastuzumab plus chemotherapy in prolonging progression-free survival [12]. A key limitation of T-DM1, and other ADCs in general, is the large size, which results in slow, inefficient, and heterogeneous tissue penetration [5, 13, 14]. Angiogenesis, capillary pressure gradients, and stromal tissue components may be highly abnormal in solid tumors, demonstrating an initial barrier for ADC penetration into tumors, which also results in slow, inefficient, and heterogeneous tissue penetration [15, 16]. Evidence from a simple mechanistic model of antibody uptake and retention, which approximates the time course of antibody concentration in solid tumors, suggests that only a small percentage of the administered ADC dose actually reaches tumor cells, again highlighting the importance of size in targeted drug delivery [17]. Consequently, ADCs are generally more suitable for hematologic cancers because of the slow penetration in solid tumors.

In recent years ADC subtypes have been developed using different tumor-homing carriers, such as peptides, which usually consist of < 40 amino acid residues [18]. Peptides have advantages as ADCs, such as lower molecular weight, better permeability, lower immunogenicity, simpler design, and lower synthesis costs, thus making peptides superior to mABs [8]. Peptide-drug conjugates (PDCs) are gaining attention as a new option for anti-cancer treatment [19]. In addition, when compared to the low molecular weight drug, which can diffuse freely and quickly into and out of tumor tissues, PDCs have much higher specificity for tumor cells, resulting in a longer retention time in tumor tissues [20] and a better therapeutic outcome than the parental drug.

Herein we synthesized a HER2-targeting peptide-mertansine conjugate (HER2-TPMC) with less cytotoxicity and higher specificity than mertansine, as well as the ability to reduce systemic toxicity and side effects. With a smaller size compared to ADCs, HER2-TPMC showed rapid tissue penetration at a lower concentration and good tumoral uptake *in vivo* after tail vein injection. These results indicate the feasibility of using HER2-TPMC as an alternative for effective breast cancer therapy.

Materials and methods

Materials

HER2-targeting peptide (HER2-TP, sequence: CDTFPYLGWWNPNEYRY [21]) was custom-synthesized at the MIT (Cambridge, MA, USA). HER2-targeting antibody (HER2-TA [trastuzumab]) was acquired from Abcam (ab134182; Cambridge, Cambridgeshire, UK). Mertansine was purchased from Selleckchem (No. S6773; Houston, TX, USA). Cyanine5.5 (Cy5.5)-NHS ester and Cy5.5-maleimide were obtained from Lumiprobe (27020 and 27080, respectively; Tallahassee, FL, USA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (ab211091) was purchased from Abcam. A Sepharose G-10 pre-packed column was purchased from GE Healthcare Life Sciences (now Cytiva; Uppsala, Uppland, Sweden) and used according to the instructions. Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (227056 and 276855, respectively; St. Louis, MO, USA) and used as received. Fetal bovine serum (FBS), penicillin-streptomycin, Roswell Park Memorial Institute (RPMI)-1640 medium, Dulbecco's modified Eagle medium (DMEM), and trypsin were obtained from Invitrogen Corp. (Carlsbad, CA, USA). Phosphate-buffered saline (PBS) was obtained from Solarbio (P1020; Beijing, China). All solvents and reagents were of analytical grade and used directly.

Synthesis

Peptide-cy5.5 conjugate

For synthesizing peptide-cy5.5 conjugate, the peptide and Cy5.5 were dissolved in DMSO and mixed at a 1:1 ratio. Triethylamine was added to the reaction mixture and stirred overnight at room temperature. The obtained peptide-cy5.5 conjugate was then freeze-dried into a powder.

Peptide-mertansine conjugate

The synthesis of peptide-mertansine conjugate has been previously reported [22]. We followed the methods described in that literature to prepare the HER2-targeting peptide-mertansine conjugate. Mertansine and 1-hydroxybenzotriazole (HOBt) were dissolved in DMF and activated by the addition of diisopropylcarbodiimide (DIC). Then, the mixture was reacted with peptide for at least 12 h. After filtering, anhydrous ether was added to the filtrate, then crude conjugate was obtained by centrifugation.

Antibody-cy5.5 conjugate

Antibodies were diluted in elution buffer and Cy5.5 was dissolved in DMSO to synthesize the antibody-cy5.5 conjugate. The dissolved Cy5.5 was added to the antibody and vortexed to mix. The antibody-cy5.5 conjugate was allowed to react in the dark at room temperature for 12 h.

Antibody-mertansine conjugate

The synthesis of antibody-mertansine conjugate has been previously reported [23, 24]. HER2-targeting antibodies were reduced with dithiothreitol (DTT [1mM]) at a 1:3 molar ratio. The reduced antibody was reacted with mertansine dissolved in DMF at a 1:6 molar ratio. For coupling, the two solutions were mixed and allowed to react at room temperature. The antibody-mertansine conjugate prepared by this method had an average of 3.56 drugs per antibody.

Pharmacokinetics study

Normal BALB/c-nude female mice were randomly divided into 3 groups (n=3) and treated with Cy5.5, HER2-TP-cy5.5, or HER2-TA-cy5.5 (1 ug/mL of Cy5.5) via lateral tail vein injection. At various pre-determined time intervals, 20 µL of blood was collected from the orbital vein in a capillary tube containing heparin. The fluorescence intensity of Cy5.5 in the blood was determined using a fluorescence microplate reader.

2D cell culture

Human breast cancer cells (BT474 and MCF-7) were cultured in RPMI-1640 medium and DMEM, each containing 10% FBS, respectively, in cell culture incubator at 37 °C in a humidified atmosphere containing 5% CO_2.

MTT assay

The MTT cell proliferation assay kit (Abcam, Cambridge, Cambridgeshire, UK) was composed of two liquid materials (MTT reagent and MTT solvent), which was used directly as supplied. The MTT assay was used to evaluate cell viability. Cells were seeded into 96-well plates with 5000 cells per well and incubated overnight at 37 °C in 5% CO_2. Different concentrations (0, 0.006, 0.0125, 0.025, 0.05, and 0.1 µM) of mertansine, HER2-targeting antibody-mertansine, and HER2-targeting peptide-mertansine were used to treat the cells for 4 h. Two scenarios were established: a) the MTT assay was performed immediately after treatment with specific mertansine formulations; and b) specific mertansine formulations were thrice-washed using 1X 0.01 M PBS (pH 7.2−7.4), cultured for an additional 48 h, then the MTT assay was performed. MTT reagent (100 µL) was added to each well and incubated at 37 °C with 5% CO_2 for 2 h. Following incubation, 150 µL of MTT solvent was added to each well and incubated at room temperature on an orbital shaker for 15 min before measuring the absorbance at OD=590 nm.

Western blot

BT474 and MCF7 cells were lysed with RIPA lysis buffer (P0013B; Beyotime, Nanjing, China) containing phosphatase (CW2383; Cwbio, Guangzhou, China) and protease inhibitor cocktails (CW2200; Cwbio) to extract total protein. Protein concentrations were quantified with the BCA protein assay kit (CW0014; Cwbio). Total protein samples (30 µg) were then separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (IPVH00010; Millipore, Darmstadt, Hesse, Germany). The primary antibodies (anti-HER2, ab134182; Abcam and anti-GAPDH no.60004-1-Ig; Proteintech, Rockford, IL, USA) and secondary antibody (HRP-conjugated anti-rabbit IgG, NA934; Cell Signaling Technology, Danvers, MA, USA) were used for incubation. Protein bands were visualized using ECL Western blotting substrate (321096; Thermo Fisher, Wilmington, MA, USA) and imaged on Invitrogen iBright CL1000 (Thermo Fisher Scientific, Wilmington, MA, USA).

Confocal laser scanning microscopy (CLSM)

BT474 and MCF-7 cells (50,000 cells) were seeded in confocal dishes and incubated in 2 mL of CM for 24 h, and 10 nM HER2-TP or HER2-TA was added to the cells. After incubation for 2 h at 37°C, the cells were thrice-washed with 1X 0.01M PBS (pH 7.2-7.4), fixed with 4% paraformaldehyde solution, the nuclei were stained with Hoechst 33342, then the cells were visualized under a CLSM (ZEISS 800; Tutzing, Bavaria, Germany).

3D spheroid cultures

BT474 and MCF-7 cells were planted in 24-well ultra-low attachment plates (3473; Corning, Corning, NY, USA) at 10,000 cells per well in 1 mL of RPMI-1640 and DMEM medium supplemented with 10% FBS and incubated at 37 °C in 5% CO_2. For optimum spheroid growth, the culture medium was refreshed every 2–3 days. Spheroids were observed with an optical microscope (Ti−U; Nikon, Tokyo, Japan) on days 5, 6, and 7.

Animals

Healthy BALB/c-nude female mice (3−5 weeks old) were purchased from the Sun Yat-sen University Experimental Animal Center (Guangzhou, China). All *in vivo* studies were performed in a designated animal facility in accordance with the rules and regulations of the Institutional Animal Care and Use Committee at Sun Yat-sen University (SYSU-IACUC-2020-B0167).

In vivo tumor uptake

In vivo tumor uptake was determined using an orthotopic breast cancer mouse model. BT474 cells (2×10^6 cells) were mixed with Matrigel in a 1:1 volume ratio, then injected into the mammary fat pads of BALB/c-nude female mice. The mice bearing BT474 orthotopic breast cancers were randomized into 4 groups (n=4): (i) PBS; (ii) Cy5.5; (iii) HER2-TA-cy5.5; or (iv) HER2-TP-cy5.5 at a dose of 1 µg/mL of Cy5.5 per mouse. Twenty-four hours after the injection,

the mice were sacrificed and the tumors were collected and observed using an IVIS Lumina III imaging system (PerkinElmer, city, state, country).

Tumor slice staining

The tumor specimens were embedded in paraffin and sliced. After dewaxing and antigen repair, the slices were blocked with bovine serum albumin (BSA) and incubated overnight at 4 °C with anti-HER2 antibody. Then, the tumor specimens were incubated with Alexa Fluor® 488-conjugated goat anti-rabbit IgG [H+L] (GB25303; Servicebio, city, China) for 1 h at room temperature in the dark. The slides were observed and imaged under CLSM (Alexa Fluor® 488 excitation wavelength, 493 nm; emission wavelength, 519 nm).

Statistical analysis

GraphPad Software Prism 8.0 was used to perform statistical analyses and graph drawing. Data are presented as the
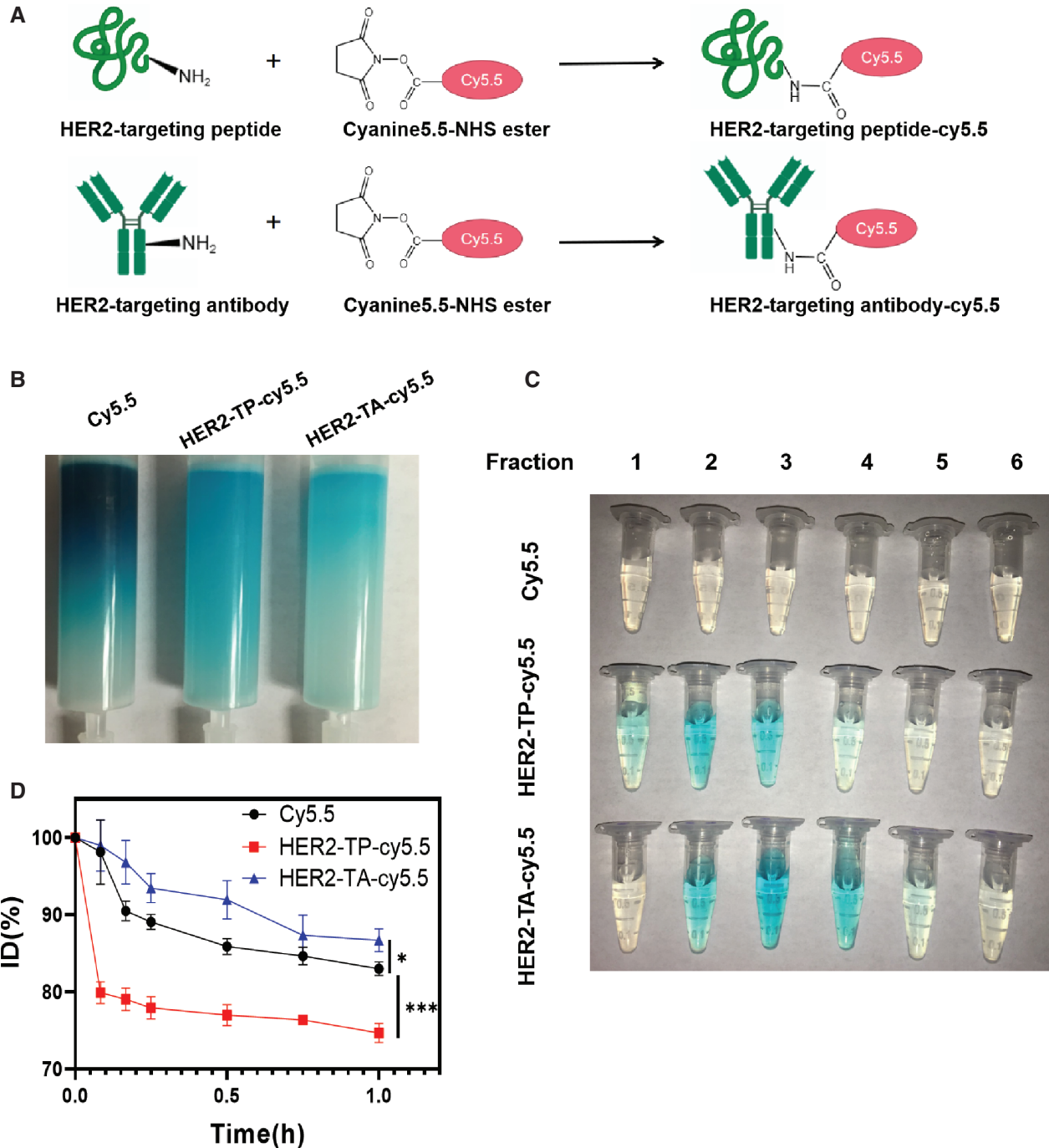
Figure 1 Schematic diagram and pharmacokinetics of HER2-TP-cy5.5 and HER-TA-cy5.5 conjugate synthesis. (A) Schematic representation of the utilization of HER2-targeting peptide/antibody and Cyanine5.5-NHS ester (Cy5.5) to form conjugates. (B) Purification of the conjugates, (C) Collection of the purified components. (D) The circulation time of free Cy5.5 and Cy5.5-labeled peptide/antibody after intravenous injection in mice via the tail vein. mean±SD; n=3; *$p < 0.05$, ***$p < 0.001$. HER2-TP-cy5.5: HER2-targeting peptide-cy5.5 conjugate; HER2-TA-cy5.5: HER2-targeting antibody-cy5.5 conjugate.

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mean ± SD of three independent experiments. The statistical significance was determined using one-way ANOVA analysis, with a p value < 0.05 considered statistically significant (*p < 0.05, **p < 0.01, and ***p < 0.001).

Results and Discussion

Synthesis of HER2-targeting peptide (HER2-TA-cy5.5) and HER2-antibody-cy5.5 (HER2-TA-cy5.5)

The aim of this study was to develop a drug conjugate with better targeting and penetrability for effective anti-cancer therapy. We conjugated HER2-targeting peptide and antibody with Cy5.5, a fluorescent dye, to visualize the targeting and permeability *in vitro* and *in vivo* (**Figure 1A**). Then, the conjugates were purified with disposable columns prepacked with Sepharose (**Figure 1B**). The drops (1 mL) were collected from the tube before changing to the next tube. Due to affinity exchange, unreacted Cy5.5 bound tightly to the beads, while only conjugated HER2-TP-cy5.5 and HER2-TA-cy5.5 were eluted from the column (**Figure 1C**).

After combining all tubes, both HER2-TP-cy5.5 and HER2-TA-cy5.5 were snap-frozen and lyophilized to obtain pure conjugates (**Figure 1C**).

Pharmacokinetics of HER2-TP-cy5.5 and HER2-TA-cy5.5

We determined the pharmacokinetics of the conjugates to evaluate the circulation stability of the conjugates. The HER2-TP-cy5.5 conjugate was cleared faster from the circulation (~75% within 1 h), while ~85% of the antibody conjugate remained (**Figure 1D**). This was an expected and interesting phenomenon. The significant majority (~98%) of the total ADCs was comprised of the antibody component and the pharmacokinetics of the ADCs were influenced by the properties of the antibody backbone. Antibody properties governing ADC pharmacokinetics include target-specific binding, neonatal Fc receptor-dependent recycling, and Fc effector functions [25]. ADCs exhibit a long half-life property associated with unconjugated antibodies [25]. We speculate that the antibody conjugate high molecular weight might be another possible explanation accounting for this observation. Peptide-drug conjugates allow peptide modification, such as chemical
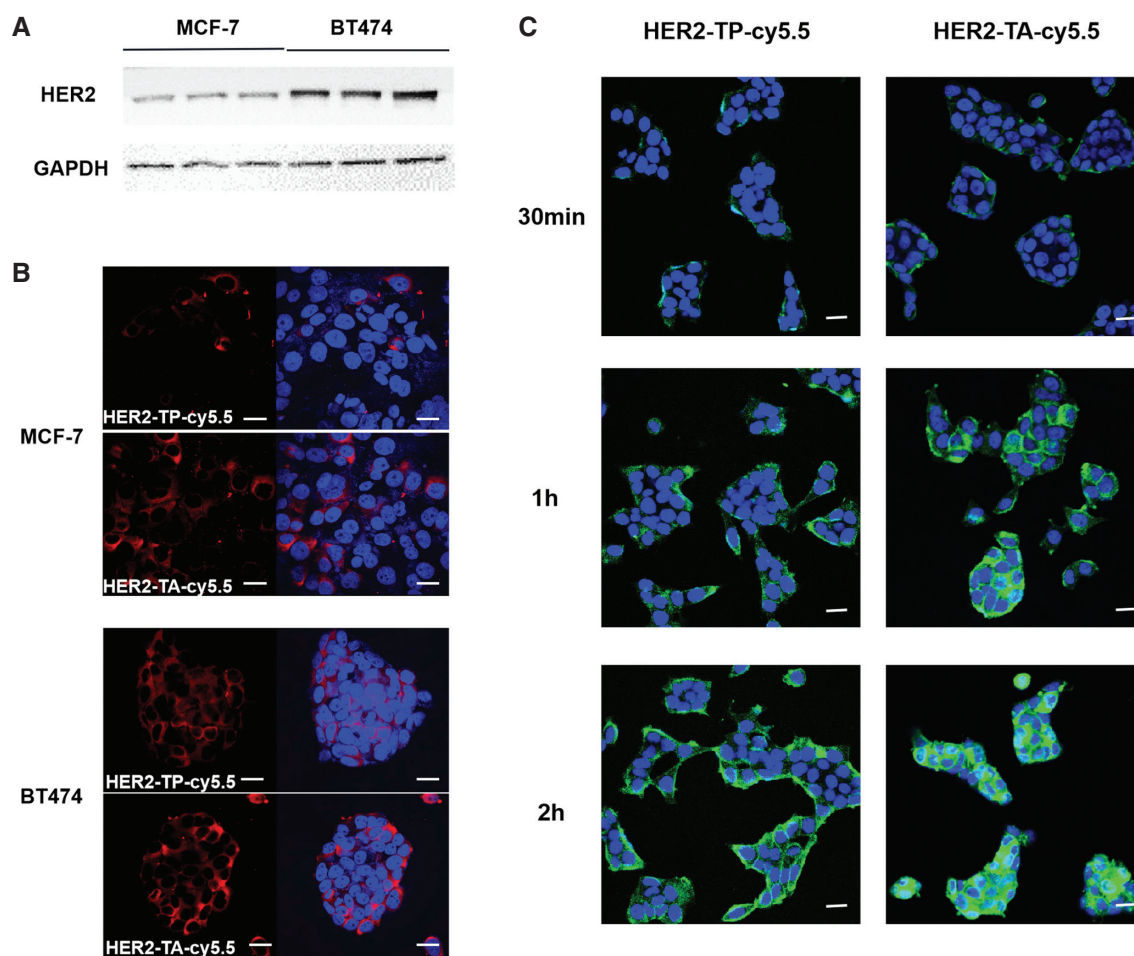
Figure 2 HER2-TP-cy5.5 and HER2-TA-cy5.5 conjugate cellular uptake. (A) Expression of HER2 in MCF-7 and BT474 was detected by Western blot. (B) The uptake of two conjugates in MCF-7 and BT 474 in 2D culture at 1 h. (C) Time-dependent uptake of both conjugates in HER2+ BT474. Nuclei were stained with Hoechst 33342 (blue fluorescence). Scale bar, 30 μm.

modification, including cyclization, stapling, and addition of amino acid side chains, to reduce the renal clearance rate, thus prolonging the half-life [26].

In vitro uptake of HER2-TP-cy5.5 and HER2-TA-cy5.5

To assess the cellular binding and uptake of both conjugates, we first rectified the level of HER2 expression in two human breast cancer cells *in vitro*. As shown in the Western blot analysis (**Figure 2A**), HER2 was highly expressed in BT474 cells as compared to MCF7 cells, and a significantly lower level of HER2. Therefore, BT474 cells were used as the HER2+ cell line and MCF7 served as the negative control. When treated with HER2-TP-cy5.5 and HER2-TA-cy5.5 in BT474 and MCF7 cells, respectively, both conjugates had higher uptake in BT474 cells compared to MCF7 cells, indicating HER2-specific binding (**Figure 2B**). In both cells, HER2-TA-cy5.5 also showed higher fluorescence signals compared to HER2-TP-cy5.5 This finding could be due to the multivalency and lower Kd value of antibody, which could lead to the above phenomenon. In a time-dependent manner uptake analysis, both HER2-TP-cy5.5 and HER2-TA-cy5.5 exhibited a linear increment of uptake, indicating successful binding to HER2 receptor and endocytosis (**Figure 2C**).

In vitro cytotoxicity of HER2-TP-mertansine (HER2-TPMC) and HER2-TA-mertansine (HER2-TAMC)

To assess the *in vitro* cytotoxicity of both conjugates, we conjugated HER2-targeting peptide and antibody with mertansine in this study to produce HER2-TPMC and HER2-TAMC (**Figure 3A**). **Figure 3** presents the results showing the effect of concentration and culture time on the viability of BT474 cells treated with mertansine, HER2-TPMC, or the HER2-targeting antibody-mertansine conjugate, HER2-TAMC. In the first experiment, cells were treated with formulations for 4 h before being washed out, then the MTT assay was performed (**Figure 3B**). As shown in **Figure 3B**, all three mertansine formulations showed similar cytotoxicity at different concentrations, indicating that HER2-TPMC retained mertansine bioactivity even after conjugation. In another experiment, after the same treatment as above, the excess drug was washed from the cells using 1X 0.01M PBS (pH 7.2-7.4), and a 48-h post-incubation was completed before the MTT assay was performed (**Figure 3C**). With the additional post-incubation period, the cytotoxicity of 2 drug conjugates was slightly lower than parental mertansine at concentrations of 0.006, 0.0125, and 0.025 μM, which might be attributed to the ligand-mediated endocytosis of the conjugate rather than non-specific uptake of mertansine.
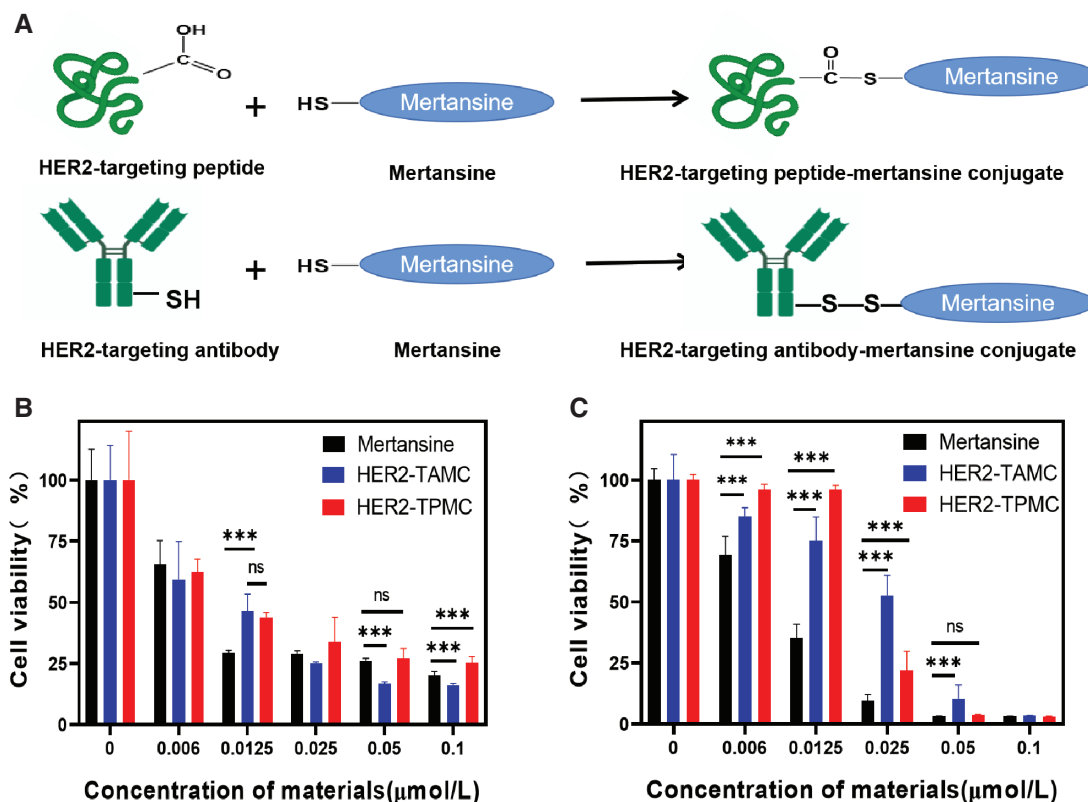

Figure 3 Cytotoxicity of BT474 cells treated with distinct mertansine formulations. (A) Schematic representation of the utilization of HER2-targeting peptide/antibody and mertansine to form HER2-TAMC and HER2-TPMC conjugates, respectively. (B) All formulations (parental mertansine, HER2-TAMC, and HER2-TPMC) were continuously incubated for 4 h with BT474 cells, washed out to carry out cytotoxicity assay. (C) The same experiment in (B), but with a post-incubation of 48 h after washing out the formulations. HER2-TAMC: HER2-targeting antibody-mertansine conjugate; HER2-TPMC: HER2-targeting peptide-mertansine conjugate; ns, no significance; ***$p < 0.001$.

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3D spheroid uptake and penetration of HER2-TMPC and HER2-TAMC

Spheroid uptake and retention of mertansine-cy5.5 versus HER2-TP-cy5.5

To overcome the limitations of a 2D culture environment, including the lack of interaction between cells and cell matrix, the space communication between cells, heterogeneity, and structural complexity, a 3D culture system [27] was utilized in this study to better observe whether there was any difference in uptake and permeability between the conjugates. To visualize the uptake of mertansine by cells, we first conjugated Cy5.5 to mertansine via a thiol-maleimide bond (**Figure 4A**). Although we used a commercially-available, low-adsorption culture plate to grow 3D spheroids, only BT474 cells can form uniform, clearly marked spheroid barriers, whereas MCF7 failed to generate any spheroids to be used for further study. BT474 spheroids appeared on day 5 and matured on day 7 (**Figure 4B**). The BT474 tumor spheroids were then incubated separately
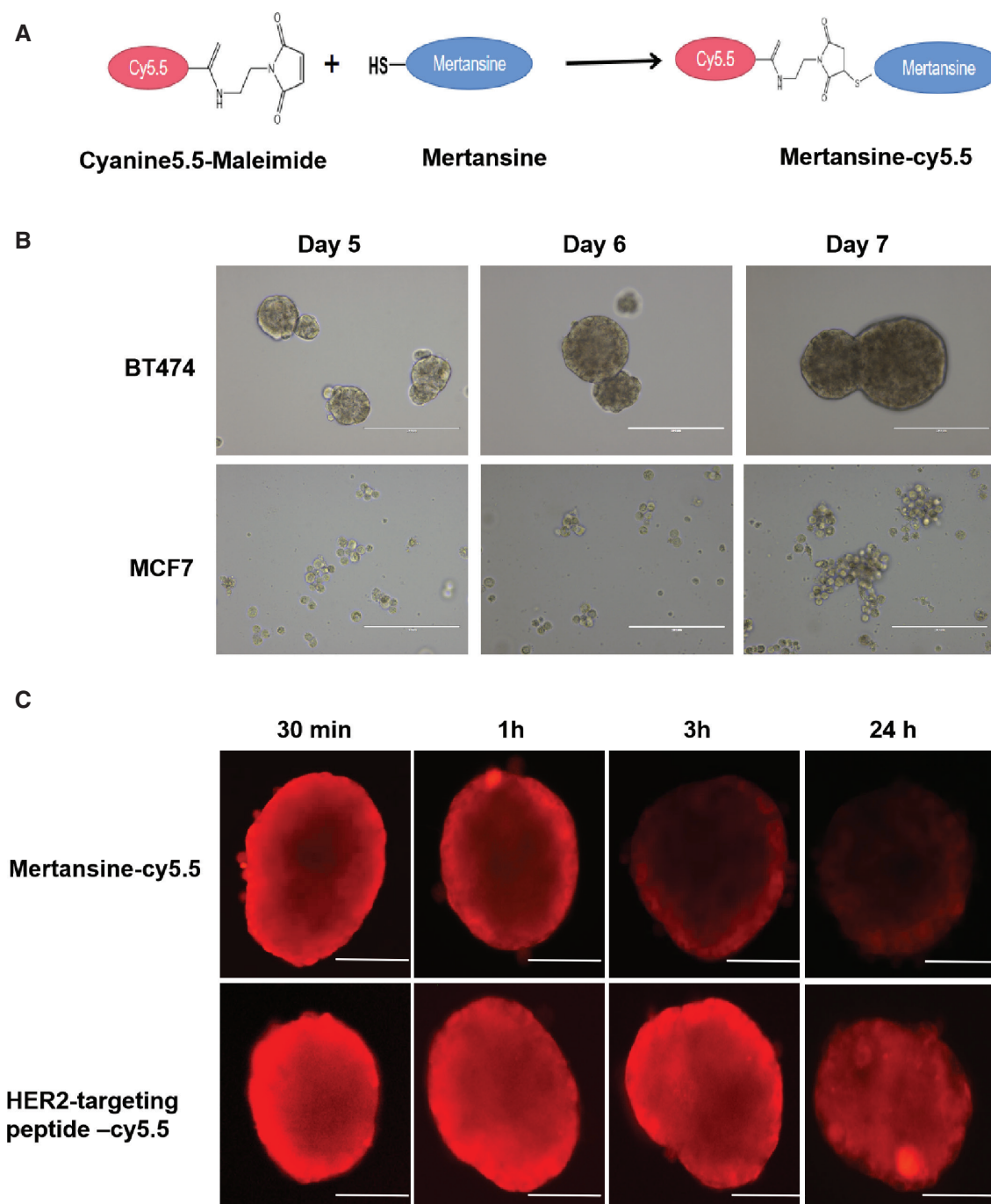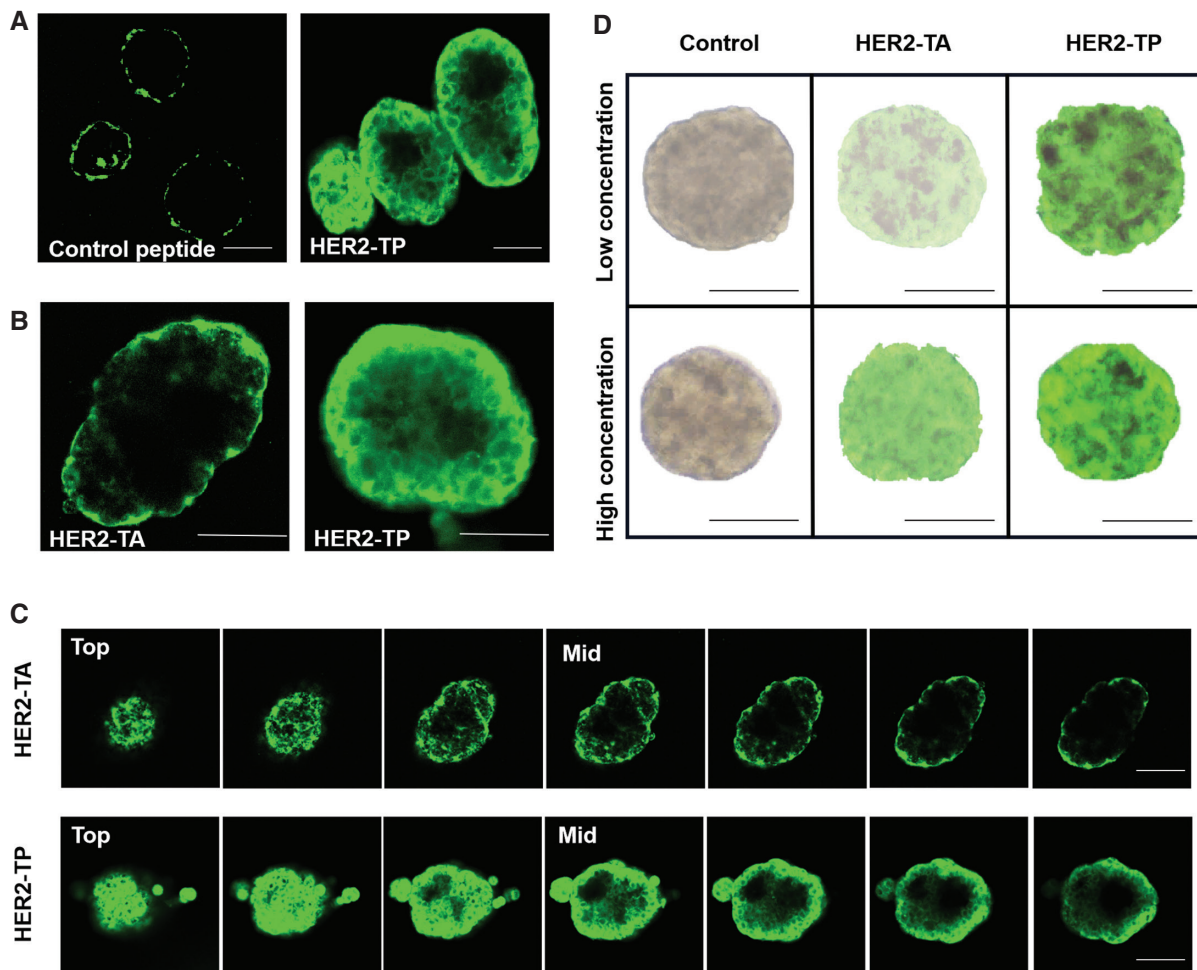

Figure 4 The uptake of HER2-TP-cy5.5 conjugates in a 3D tumor model. (A) Schematic representation of preparation of the mertansine-cy5.5 conjugates. (B) 3D spheroid culture of BT474 and MCF-7 cells, in which only BT474 cells successfully produced a 3D spheroid culture. (C) The uptake of BT474 tumor spheroid treated with mertansine or HER2-TP, indicating high retention rate of HER2-TMPC compared to parental drug. Scale bar, 200 μm.

with Cy5.5-labeled mertansine and HER2-targeting peptide, and the fluorescence was analyzed using a confocal microscope. As seen in **Figure 4C**, the uptake of mertansine-cy5.5 and HER2-TP-cy5.5 was similar at early time points (< 1 h). As time increases, mertansine-cy5.5, a free drug, quickly diffuses out of the spheroid and cannot be contained and retained for a long period of time. In contrast, HER2-TP-cy5.5 showed consistently prolonged retention in the BT474 tumor spheroids up to 24 h, indicating that active targeting is vital for small biologic drug conjugates (SBDCs) to not only increase penetration into the tumor core, but also to retain a long retention time within the tumor core.

## Spheroid penetration ability of HER2-TPMC versus HER2-TAMC

To evaluate the targeting ability of HER2-TPMC in 3D tumor spheroids, we first compared the uptake of HER2-TPMC and control peptide-(CP-TPMC) in BT474 tumor spheroids. As shown in **Figure 5A**, only HER2-targeting peptide conjugate were able to highly bind to HER2-overexpressed BT474, again indicating the selectivity and specificity of our HER2-TPMC conjugate. Next, BT474 tumor spheroids were incubated with HER2-TP-cy5.5 and HER2-TA-cy5.5. **Figure 5B** shows that HER2-TP-cy5.5 had significantly higher uptake and penetration into the spheroid core compared to HER2-TA-cy5.5, again affirming our hypothesis that smaller sized peptide-drug conjugates have higher penetration ability in the solid tumor, where the interstitial space is too narrow for large ADCs to penetrate. In a series of multiple Z-section images generated by confocal microscopy, we acquired a whole BT474 spheroid scanning. As shown in **Figure 5C**, HER2-TP-cy5.5 showed stronger and deeper fluorescence intensity in the top-to-middle cross sections of tumor spheroids compared to minimal penetration of HER2-TA-cy5.5. The same experiment performed under fluorescence microscopy to detect the full fluorescence intensity. A significantly higher level of fluorescence was observed with HER2-TP- compared to HER2-TA-cy5.5-treated spheroids (**Figure 5D**). Moreover, HER2-TP achieved full penetration of tumor spheroids at a low concentration, suggesting the possibility of using a lower drug concentration to achieve similar effects with parental drugs or ADCs. This interesting observation could facilitate the use of HER2-TPMC as

Figure 5 Permeability of HER2-TA-cy5.5 and HER2-TP-cy5.5 conjugates in a 3D tumor model. (A) The permeability of non-targeting peptide and HER2-TP in BT474 tumor spheroids. (B) The permeability of HER2-TA and HER2-TP in BT474 tumor spheroids. (C) Multiple Z-section images of BT474 tumor spheroids treated with HER2-TA and HER2-TP individually. (D) The permeability of HER2-TA and HER2-TP in BT474 tumor spheroids with different concentrations. Scale bar, 100 μm.

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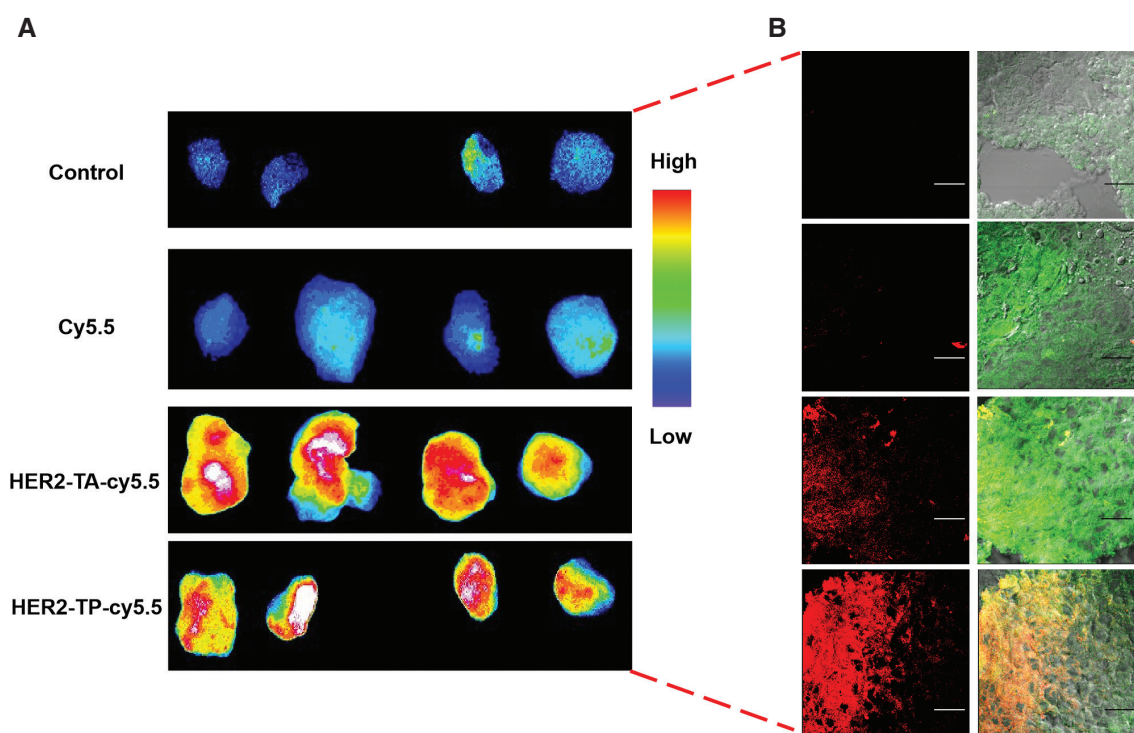
Figure 6 The *in vivo* tumoral uptake of HER2-TP-cy5.5 and HER2-TA-cy5.5 conjugate. (A) BT474 orthotopic model was used to carry out *in vivo* tumoral uptake experiment. We set up four groups with four mice in each group, and injected PBS, Cy5.5, HER2-TA-cy5.5, and HER2-TP-cy5.5 into tail vein. The red fluorescent signal was obtained by an IVIS Lumina III imaging system, indicating the accumulation effects in the tumor after 24 hours injection. (B) The tumor slide staining analysis in (A), indicating higher penetrability of HER2-TP-cy5.5 compared to other formulations. Left panel: Cy5.5 fluorescence signal. Right panel: the expression of HER2 (green fluorescence signal) was superimposed with Cy5.5 fluorescence signal. Scale bar, 100 μm.

a new alternative to drug conjugate for anti-cancer therapy with improved efficacy and reduced side effects.

In vivo tumoral uptake of HER2-TP-cy5.5 and HER2-TA-cy5.5 conjugates

Finally, the *in vivo* tumoral uptake experiment in the BT474 orthotopic model was carried out (4 groups with 4 mice each) with administration of the following via the tail vein: (i) PBS; (ii) Cy5.5; (iii) HER2-TA-cy5.5; and (iv) HER2-TP-cy5.5. As shown in **Figure 6A**, the fluorescent signals collected using an *in vivo* imaging system demonstrated a more significant accumulation of HER2-TA-cy5.5 and HER2-TP-cy5.5 in tumor tissues compared to free Cy5.5, indicating the importance of targeting characteristics in drug delivery systems for anti-cancer therapy. These tumor tissues were collected and prepared into slices. Anti-HER2 antibody and Alexa Fluor® 488-conjugated goat anti-rabbit IgG were used to indicate the HER2 signal. The result of tumor slide staining analysis revealed not only an increased level of red fluorescent signals in the HER2-TP-cy5.5 treatment group compared to the HER2-TA-cy5.5 group, but the depth of HER2-TP-cy5.5 penetration was significantly higher than HER2-TA-cy5.5 (**Figure 6B**). This result indicates that not only HER2-TP-cy5.5 could be successfully taken up by tumor *in vivo*, the specificity of HER2 targeting was visible,

while the small size of HER2-TP-cy5.5 allowed deep penetration and retention into the tumor core.

Conclusion

We have successfully developed a HER2-specific targeting peptide-mertansine conjugate (HER2-TPMC) as an effective small biologic drug conjugate for the treatment of HER2-positive cancer. HER2-TPMC offered specific cellular uptake, and resulted in a longer retention time in BT474 breast tumor spheroids. With the smaller size compared to ADCs, HER2-TPMC showed rapid tissue penetration and good tumoral uptake *in vivo*, while significantly increasing the solid tumor penetration and retention time. Taken together, HER2-TPMC could be an option for active targeting, high tumor specificity, rapid tumor uptake, and deep tumor penetration, which is a combination that is lacking in chemotherapeutic drugs or ADC-based drug delivery.

Funding

Conflict of Interest

None.

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