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Molecular Cancer Therapeutics

Selective Delivery of PEGylated Compounds to Tumor Cells by Anti-PEG Hybrid Antibodies

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Abstract

Polyethylene glycol (PEG) is attached to many peptides, proteins, liposomes, and nanoparticles to reduce their immunogenicity and improve their pharmacokinetic and therapeutic properties. Here, we describe hybrid antibodies that can selectively deliver PEGylated medicines, imaging agents, or nanomedicines to target cells. Human IgG_1 hybrid antibodies αPEG:αHER2 and αPEG:αCD19 were shown by ELISA, FACS, and plasmon resonance to bind to both PEG and HER2 receptors on SK-BR-3 breast adenocarcinoma and BT-474 breast ductal carcinoma cells or CD19 receptors on Ramos and Raji Burkitt's lymphoma cells. In addition, αPEG:αHER2 specifically targeted PEGylated proteins, liposomes, and nanoparticles to SK-BR-3 cells that overexpressed HER2, but not to HER2-negative MCF-7 breast adenocarcinoma cells. Endocytosis of PEGylated nanoparticles into SK-BR-3 cells was induced specifically by the αPEG:αHER2 hybrid antibody, as observed by confocal imaging of the accumulation of Qdots inside SK-BR-3 cells. Treatment of $HER2^+$ SK-BR-3 and BT-474 cancer cells with αPEG:αHER2 and the clinically used chemotherapeutic agent PEGylated liposomal doxorubicin for 3 hours enhanced the *in vitro* effectiveness of PEGylated liposomal doxorubicin by over two orders of magnitude. Hybrid anti-PEG antibodies offer a versatile and simple method to deliver PEGylated compounds to cellular locations and can potentially enhance the therapeutic efficacy of PEGylated medicines. *Mol Cancer Ther; 14(6); 1317–26. ©2015 AACR.*

Introduction

Attachment of polyethylene glycol (PEG), a hydrophilic, nontoxic, and nonantigenic biocompatible polymer, to peptides and proteins can enhance drug stability (1) and bioavailability (2) as well as reduce immunogenicity (3). PEGylation of polymeric nanoparticles, liposomes, and micelles can also greatly extend their circulation time in the body, enhance tumor accumulation, and decrease systemic toxicity, which in turn can improve therapeutic efficacy (4, 5). PEGylation has, therefore, been widely used to improve the properties of diagnostic and therapeutic agents.

Nanocarriers can accumulate in tumors via the enhanced permeability and retention effect due to leakage of nano-sized materials through the tumor vasculature and defects of lymphatic clearance within tumors (6). In addition to the enhanced serum half-life afforded by PEGylation, covalent attachment of targeting ligands or antibodies to PEGylated nanocarriers can further increase their uptake into tumor cells via receptor-mediated endocytosis. For example, PEGylated liposomes covalently linked to anti-HER2 Fab fragments (fragment antigen binding) are more effective than nontargeted liposomes for the treatment of mouse breast cancer xenografts (7), due to increased uptake of the immunoliposomes in tumor cells (8). However, the processes of covalently conjugating antibodies can damage their binding activity, alter the physiologic and pharmacodynamic properties of nanocarriers, and generate new antigenic epitopes (9). Furthermore, covalent attachment of antibodies to nanocarriers can hinder the release of therapeutic payloads inside cells (10), thereby decreasing treatment efficacy. Covalent attachment of antibodies to nanoparticles can also greatly complicate the manufacturing process.

Bispecific antibodies (BsAb) are antibody derivatives that possess two distinct antigen-binding specificities. BsAbs have been developed to simultaneously block two antigens expressed on cancer cells (11), establish crosslinks to activate effector cells at cancer cells (12), and deliver payloads containing drugs, toxins, and imaging reagents to tumors (13). Here, we describe new bispecific (hybrid) antibodies with dual specificities for PEGylated compounds or nanocarriers and tumor antigens (Fig. 1A). We show that a humanized hybrid antibody in an intact IgG_1 format was able to tether PEGylated compounds to $HER2^+$ cells and increase the *in vitro* cytotoxicity of liposomal doxorubicin to $HER2^+$ cancer cells.

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Materials and Methods

Reagents

CH_3O-PEG_{1K}-NH_2, CH_3O-PEG_{2K}-NH_2, NH_2-PEG_{3K}-NH_2, OH-PEG_{5K}-NH_2, CH_3O-PEG_{5K}-NH_2, and CH_3O-PEG_{20K}-NH_2 were purchased from Sigma-Aldrich. PEGASYS (PEG–IFN-alfa-2a) was from Roche and Neulasta (PEGylated granulocyte-colony stimulating factor, G-CSF) was a gift from Amgen. Qtracker655 (Qdot655), a nanocrystal semiconductor coated with multiple linear CH_3O-PEG_{2K} molecules, was purchased from Invitrogen. Alexa Fluor 647-N-hydroxysuccinimide ester (NHS ester) was purchased from Molecular Probes. Doxisome (PEGylated liposomal doxorubicin) was from Taiwan Liposome Company, Ltd. Recombinant HER2 extracellular domain (ECD) was a gift from Dr. An-Suei Yang (Genomics Research Center, Academia Sinica, Taiwan). Anti-HER2 antibody (Herceptin) used for detecting surface HER2 expression on various cell lines via FACS analysis was from Roche. Distearoyl phosphatidylcholine (DSPC) and distearoyl phosphatidylethanolamine-PEG_{2K} (DSPE-PEG_{2K}) were purchased from Avanti Polar Lipids.

Cell lines and culture

SK-BR-3 human breast adenocarcinoma (ATCC; HTB-30), MCF-7 human breast adenocarcinoma (ATCC; HTB-22), Jurkat E6-1 human acute T lymphoma cells (ATCC; TIB-152), Raji human Burkitt's lymphoma cells (ATCC; CCL-86), HT29 human colon colorectal adenocarcinoma cells (ADCC; HTB-38), HCC-36 human hepatocellular carcinoma cells, and SW-480 (ATCC; CCL-228) human colon colorectal adenocarcinoma cell lines were obtained from the American Tissue Culture Collection in 1999 except for SK-BR-3, which was obtained in 2004. Ramos human Burkitt's lymphoma cells (ATCC; CRL-1596) and BT-474 human ductal carcinoma (ATCC; HTB-20) cells were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan) in 2005 and 2009, respectively. E11, 3.3 and 6.3 mouse anti-PEG hybridoma cells were developed in our laboratory (14, 15). Viral transformed human embryonal kidney 293 FT cells were kindly provided by Dr. Ming-Zong Lai (Institute of Molecular Biology, Academia Sinica, Taiwan) in 2012. All cell lines were *Mycoplasma* free and cultured according to the suppliers' recommendations. Hybridoma cells, 293 FT cells, and HCC-36 cells were cultured in DMEM (Sigma-Aldrich) supplemented with 6 g/L HEPES, 3.7 g/L $NaHCO_3$, 10% heat-inactivated FBS (HyClone), penicillin (100 U/mL), and streptomycin (100 μg/mL). SK-BR-3, MCF-7, BT-474, Jurkat, Raji, Ramos, HT29, and SW-480 cells were cultured in RPMI-1640 (Life Technologies) supplemented with 6 g/L HEPES, 2 g/L $NaHCO_3$, 10% heat-inactivated FBS (HyClone), penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. No authentication besides confirming surface expression levels of CD19 or HER2 was performed by the authors.

Hybrid antibodies

A schematic of hybrid antibody expression constructs is shown in Fig. 1B. The V_H-C_H1 and V_L-C_κ fragments of mouse anti-PEG backbone antibody 6.3 were amplified from the cDNA isolated from 6.3 hybridoma cells and humanized as described (16). To facilitate heterodimer formation of the hybrid antibodies, mutations were introduced by site-directed mutagenesis in the human IgG_1 C_H3 domain (S354C and T366W) to create a knob structure and mutations in a separate IgG_1 C_H3 domain (S349C, T366S, L368A, and Y407V) to create a hole structure (17, 18). The humanized 6.3 V_H-C_H1 domain, assembled with the human IgG_1 Fc domain with knob mutations via assembly PCR (19), was cloned into the pLNCX-hβG-e-B7 vector (20) to acquire the human Ig_κ leader sequence (Ig_κ LS) at the N terminus of the humanized 6.3 (h6.3)-knob heavy-chain expression sequence, whereas an HA tag downstream of the Ig_κ LS was removed. A furin-2A (F2A; ref. 21)–based bicistronic sequence, and the humanized 6.3 V_L-C_κ domain with a Ig_κ LS sequence inserted at the N terminus, were cloned into the pLNCX-Ig_κ LS-h6.3-knob heavy-chain expression vector to form a Ig_κ LS-h6.3-knob heavy-chain F2A-Ig_κ LS-h6.3 light-chain expression cassette. The entire h6.3-knob antibody expression cassette was subsequently cloned into the lentivirus expression vector pLKOAS3w-hyg (RNAi core; Academia Sinica).

The human anti-HER2 antibody sequence was cloned from the pBub-YCMC plasmid kindly provided by Prof. Louis M. Weiner of Fox Chase Cancer Center (Philadelphia, PA; ref. 22). A human anti-CD19 (BU12) antibody sequence (23) was cloned via assembly PCR. The C_H1 and C_κ domains of the HER2 and the CD19 antibodies were individually exchanged, to promote paring of the correct light and heavy chains (24). The V_H-C_κ domains of the anti-HER2 or anti-CD19 antibodies, assembled with the human IgG_1 Fc domain with hole structure mutations (S349C, T366S, L368A, and Y407V) via assembly PCR, were cloned into pLNCX-hβG-e-B7 vector (20) to acquire the human Ig_κ LS and HA tag at the N terminus of the anti–HER2-hole and anti–CD19-hole heavy-chain expression sequence. A F2A bicistronic sequence and the V_L-C_H1 domains of the anti-HER2 or anti-CD19 antibodies with an Ig_κ LS sequence inserted at the N terminus, were cloned into the pLNCX-Ig_κ LS-HA-anti–HER2-hole or pLNCX-Ig_κ LS-HA-anti–CD19-hole heavy-chain expression vector. The entire anti–HER2-hole or anti–CD19-hole antibody expression cassette was cloned into the lentivirus expression vector pLKOAS3w-pur (RNAi core; Academia Sinica).

Production of recombinant proteins

Recombinant lentivirus particles were packaged by cotransfection of pLKOAS3w-hyg αPEG-knob, and either pLKOAS3w-pur αCD19-hole or pLKOAS3w-pur αHER2-hole, together with pCMVΔR8.91 packaging plasmid and pMD.G VSV-G envelope plasmid (RNAi core, Academia Sinica). 293 FT cells were seeded at 15% of confluence in a 6-well plate in DMEM medium containing 10% FBS 24 hours before viral infection. On the next day, the cells were infected with lentivirus particles expressing αPEG-knob and αCD19-hole, or αPEG-knob and αHER2-hole in the presence of 5 μg/mL polybrene (Sigma-Aldrich). Twenty-four hours later, the cells were selected with hygromycin (0.5 μg/mL) and puromycin (3 μg/mL) to generate stable cell lines expressing αPEG:αCD19 or αPEG:αHER2 hybrid antibodies.

Purification of hybrid antibodies

A total of 5×10^7 of selected 293FT cells stably expressing hybrid antibodies in 15 mL culture medium (DMEM with 10% low bovine IgG medium, serum was preabsorbed with protein A Sepharose 4 Fast Flow chromatography; GE Healthcare) were cultured in a CELLine adhere 1,000 two-compartment bioreactor (Integra Biosciences AG). Antibody-containing culture

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medium was harvested every week. The pooled supernatant was centrifuged at 910 × *g* for 15 minutes at 4°C to remove cells and subsequently centrifuged at 21600 × *g* for 30 minutes at 4°C to remove cell debris. The affinity purification process is summarized in Supplementary Fig. S1. Briefly, the clarified supernatant was passed through a G25 column (GE Healthcare) in PBS, and the hybrid antibodies were affinity purified by protein A Sepharose 4 Fast Flow chromatography (GE Healthcare) and eluted in 0.1 mol/L glycine buffer (pH 3.5) to remove mismatched or excess light chains. The eluted products were further purified by cyanogen bromide (CNBr)-CH_3O-PEG_{1K}-NH_2 affinity chromatography (35 mg of CH_3O-PEG_{1K}-NH_2 conjugated per gram of CNBr activated Sepharose 4B) using PBS-T (0.05% Tween-20) as the elution buffer to remove αHER2-hole or αCD19-hole homodimers. Subsequently, the eluted hybrid antibodies were purified by affinity chromatography using Pierce anti-HA agarose (Thermo Scientific) and 3 mol/L SCN as the elution buffer to remove αPEG-knob homodimers. Purified hybrid antibody fractions were pooled and dialyzed against PBS three times after each affinity purification step, concentrated using Amicon Ultra (30 kD cutoff; Millipore) and sterile filtered.

Mass spectrometry

The molecular weight of intact hybrid antibodies was determined on a Voyager DE-STR MALDI-TOF mass spectrometer (AB Sciex). 1.5 μg antibody in 0.5 μL water was spotted onto a polished steel target and mixed directly onto the plate with 0.5 μL of 45 mmol/L sinapinic acid in 30% acetonitrile/0.3% trifluoroacetic acid. The samples were allowed to air dry, and then loaded into the mass spectrometer. The instrument was operated in the linear positive ion mode with accelerating voltage of 25 kV and delayed extraction of 1,700 ns to acquire the molecular weight of intact proteins. To improve the signal-to-noise ratio, 600 shots were averaged for each mass spectrum. An external mass spectrum calibration was performed using the IgG Calibration Standard Kit (AB Sciex).

Anti-PEG antibody ELISA

Maxisorp 96-well microplates (Nalge-Nunc International) were coated with 10 μg/mL CH_3O-PEG_{2K}-NH_2, NH_2-PEG_{3K}-NH_2, OH-PEG_{5K}-NH_2, or CH_3O-PEG_{20K}-NH_2 in 50 μL per well 0.1 mol/L $NaHCO_3/Na_2CO_3$ (adjusted to pH 8.0 with HCl) buffer for 3 hours at 37°C and then blocked with 200 μL per well dilution buffer (5% skim milk in PBS) at 4°C overnight. Graded concentrations of anti-PEG antibodies E11, 3.3 or 6.3 in 50 μL 2% skim milk in PBS were added to the plates at room temperature (RT) for 1 hour. The plates were washed with PBS containing 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; Sigma-Aldrich) three times. Horseradish peroxidase (HRP)–conjugated goat anti-human IgG fragment crystallizable region (Fc) antibody (Sigma-Aldrich) in 50 μL dilution buffer were used for detecting bound anti-PEG antibodies on the microplates. After incubating for 1 hour at RT, the plates were washed as described above. The bound peroxidase activity was measured by adding 100 μL per well ABTS solution [0.4 mg/mL, 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H_2O_2, and 100 mmol/L phosphate–citrate, pH 4.0) at RT. The absorbance (405 nm) of wells was measured in a microplate reader (Molecular Device).

Cell-based ELISA

To measure hybrid antibody binding to cancer cells, 96-well microplates were coated with 2 μg per well poly-L-lysine (40 μg/mL) in PBS for 30 minutes at 37°C, washed twice with PBS and then coated with 2×10^5 SK-BR-3 ($HER2^+$) or MCF-7 ($HER2^-$) cells per well. The cells were centrifuged at 910 × *g* for 5 minutes and then fixed with 4% para-formaldehyde for 20 minutes at RT. The plates were washed with PBS four times and blocked with 200 μL per well 2% skimmed milk in PBS at 4°C overnight. The plates were then incubated with graded concentrations of hybrid antibodies at RT for 1 hour, then washed with PBS-T (0.05% Tween-20) five times and incubated with HRP-conjugated goat anti-human IgG Fc (Sigma-Aldrich) for 1 hour at RT. The plates were washed with PBS-T three times and PBS twice. A 100 μL per well ABTS solution, 0.003% H_2O_2, 100 mmol/L phosphate citrate, pH 4.0) was added for 40 minutes at RT, and the absorbance of the wells at 405 nm was measured on a microplate reader.

Flow cytometer analysis

To test the dual binding activity of purified hybrid antibodies to cancer cells and PEGylated nanoparticles, 2.5×10^5 SK-BR-3 ($HER2^+$), BT-474 ($HER2^+$) or MCF-7 breast cancer cells ($HER2^-$), $CD19^-$ Jurkat T-cell lymphoma cells ($CD19^-$), or Raji ($CD19^+$) or Ramos Burkitt's ($CD19^+$) lymphoma cells were incubated with 10 μg/mL purified hybrid antibodies in RPMI medium for 30 minutes at 4°C. After washing with cold 0.05% BSA/PBS twice, the cells were incubated with 10 nmol/L Qdot655 in RPMI medium for 30 minutes at 4°C. The cells were washed with cold 0.05% BSA/PBS twice. Afterward, the cells were stained with SYTOX Blue cell viability dye (Invitrogen), and the surface fluorescence of Qdot655 on 10^4 viable cells was measured on a LSR II (BD Biosciences). Fluorescence intensities were analyzed with Flowjo (Tree Star Inc.)

PEGylated protein preparation

CH_3O-PEG_{2K}-succinimidyl propionic acid (SPA; Shearwater) dissolved in DMSO at 2 mg/mL was mixed with BSA (10 mg/mL) or β-glucuronidase (10 mg/mL) at a molar ratio of PEG to protein of 10 for 2 hours at RT to produce PEGylated BSA or PEGylated β-glucuronidase. One-tenth volume of 1 mol/L glycine solution was added to stop the reaction. PEGylated BSA was purified via gel filtration using Sephacryl S-300 HR (GE Healthcare) and PEGylated β-glucuronidase was purified via ion exchange using DEAE Sephadex A-50 (Pharmacia).

Liposome preparation

For generation of PEG-liposomes, DSPC and DSPE-PEG_{2K} and cholesterol were dissolved in chloroform at a 65:5:30 molar ratio, respectively. Naked liposomes were prepared using DSPC:cholesterol at a molar ratio of 70:30. A dried lipid film was formed at 65°C by rotary evaporation (Büchi, Rotavapor RII) and rehydrated in Tris buffered saline (50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4) at 65°C to a final lipid concentration of 10 mg/mL. The liposomal suspension was submitted to 5 freeze/thaw cycles using liquid nitrogen followed by extrusion 11 times at 70°C through 400, 200, and 80 nm polycarbonate membranes each using a mini-extruder (Avanti Polar Lipids, Inc.). Final lipid concentrations were measure by Bartlett's assay (25).

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Cell-based sandwich ELISA

Cells were coated in 96-well microplates as previously described and fixed. After adding graded concentrations of hybrid antibodies in PBS for 1 hour, the plates were washed and then incubated with fixed concentrations of naked liposomes (1.4 μg/mL lipid), PEG-liposomes (1.4 μg/mL lipid), *E.coli* β-glucuronidase (60 nmol/L), *E.coli* β-glucuronidase-PEG_{2K} (60 nmol/L), BSA (60 nmol/L), BSA-PEG_{2K} (60 nmol/L), PEGASYS (40 nmol/L), Neulasta (20 nmol/L), or Qdot655 (3 nmol/L) for 1 hour. After washing with PBS, the plates were further incubated with biotinylated 15-2b antibody (anti–methoxy-PEG; ref. 26) for 1 h to detect bound conjugates. The plates were washed with PBS and further incubated with streptavidin-HRP (Jackson ImmunoResearch) for 1 hour. The plates were washed with PBS and 100 μL per well ABTS solution and 0.003% H_2O_2 was added for 40 minutes at RT. The absorbance of the wells at 405 nm was measured on a microplate reader.

Confocal live cell imaging

$HER2^+$ SK-BR-3 breast cancer cell cells (2×10^5) and $HER2^-$ MCF-7 control cells (2×10^5) cultured in RPMI medium supplemented with 10% FBS were seeded on a glass slide precoated with 2 μg per well poly-L-lysine (40 μg/mL) overnight. The cells were incubated with 1 μg/mL Hoechst 33342 (Invitrogen) to stain nuclei and 0.1 μmol/L LysoTracker green DND-26 in RPMI (Sigma-Aldrich) to stain lysosomes for 40 minutes at 37°C in an atmosphere of air containing 5% CO_2. After washing with RPMI growth medium supplemented with 10% heat-inactivated FBS, the cells were stained with 10 μg/mL αPEG:αHER2 or αPEG:αCD19 hybrid antibodies in 500 μL RPMI growth medium supplemented with 10% heat-inactivated FBS for 30 minutes. After washing the cells, 10 nmol/L Qdot655 in 500 μL RPMI growth medium was added to the cells and further incubated for 5 hours at 37°C before the fluorescence signals were detected on a Zeiss LSM780 laser-scanning microscope (Carl Zeiss AG).

^{3}H-thymidine incorporation assay

10^4 SK-BR3 ($HER2^+$), BT-474 ($HER2^+$) or 5×10^3 MCF-7 ($HER2^-$) cells were seeded in 96-well plates overnight. The next day, 2 or 10 μg/mL αPEG:αHER2 or 10 μg/mL control αPEG:αCD19 hybrid antibodies and graded concentrations of doxisome were added in triplicate to the wells for 3 hours at 37°C. After removing the medium and adding fresh medium, the cells were incubated for 48 hours and then pulsed with ^{3}H-thymidine (1 μCi/well, specific activity 25.0 Ci/mmol; Moravek Biochemicals) for 18 hours. The cells were harvested and the radioactivity was measured on a TopCount microplate scintillation counter (Packard). Results are expressed as the percentage of ^{3}H-thymidine incorporation compared with untreated cells by the following formula:

The percentage of ^{3}H-thymidine incorporation = 100 × (average sample ^{3}H-thymidine cpm/average untreated control ^{3}H-thymidine cpm). IC_{50} values were calculated by fitting the data to a log (inhibitor) versus responses (variable slopes model) with *Prism 5* software (GraphPad Software).

Statistical analysis

Statistical significance of differences between mean values was estimated with Excel (Microsoft) using the independent Student *t* test for unequal variances. *P* values <0.05 were considered statistically significant.

Results

Anti-PEG monoclonal antibody 6.3 binds PEG with high affinity

We have generated several hybridoma cells (E11, 3.3 and 6.3) that secrete IgG monoclonal antibodies with specificity for the repeating ethylene oxide subunits in the PEG backbone (15, 27). The anti-PEG antibodies were compared for binding to amino-PEG coated in 96-well ELISA plates (Supplementary Methods and Supplementary Fig. S2). Amino-PEG was used because the terminal amine group allows stable attachment of the PEG molecules to the microplates. 6.3 bound to wells coated with PEG_{5K} with an apparent affinity that was 14-fold greater than 3.3, and 34-fold greater than E11 anti-PEG antibodies (Supplementary Table S1). Likewise, 6.3 displayed superior binding to short methylated PEG (CH_3O-PEG_{2K}) or long methylated PEG (CH_3O-PEG_{20K}) molecules as compared with 3.3 and E11 antibodies (Supplementary Fig. S2B and S2C). We therefore chose to use 6.3 to construct hybrid antibodies due to the higher affinity of 6.3 as compared with our previous best IgG anti-PEG antibody 3.3 (15).

Expression and purification of hybrid antibodies

We developed two hybrid antibodies: αPEG:αHER2, which targets the well-known tumor antigen HER2/neu that is overexpressed in 20% to 30% of human breast and other adenocarcinomas and αPEG:αCD19, which targets CD19, a surface coreceptor overexpressed in the majority of B cell tumors. Recombinant DNA technology was used to create hybrid antibodies derived from the cDNA coding regions of the V_H and V_L domain of either humanized anti-HER2 or anti-CD19 antibodies, and humanized 6.3 anti-PEG monoclonal antibody using the "knobs-into-holes" strategy (17) and the immunoglobulin domain crossover approach (24) for heterodimer formation and correct antibody heavy- and light-chain assembly. The construction map of αPEG:αCD19 and αPEG:αHER2 hybrid antibodies is shown in Fig. 1B. The heavy-chain and light-chain domains of both half antibodies were linked with a F2A bicistronic sequence and cloned into lentivirus expression vectors. The constructs were used to produce lentivirus particles, which were subsequently used for generation of stable 293 FT producer cells. The secreted hybrid antibodies (αPEG:αCD19 and αPEG:αHER2) in the culture medium were sequentially affinity purified on protein A resin, CNBr-PEG_{1K} beads and anti-HA Sepharose (Supplementary Fig. S1). SDSPAGE analysis showed that purified hybrid antibodies were composed of two heavy chains (~55 kDa) and two light chains (~25 kDa) under reducing conditions (Fig. 1C). The molecular weights of αPEG:αHER2 and αPEG:αCD19 hybrid antibodies were 153.8 and 152.4 kDa, respectively, as determined by MALDI-TOF.

Functional characterization of hybrid antibodies

The purified hybrid antibodies were first analyzed for binding to different PEG molecules by direct ELISA. αPEG:αHER2 and αPEG:αCD19 showed similar binding to ELISA microplates coated with methoxy PEG (Fig. 2A), diamine PEG (Fig. 2B), and PEG (Fig. 2C), whereas no binding was observed in wells coated with human fibronectin (Fig. 2D) or BSA (Fig. 2E). These results show that the hybrid antibodies retained PEG-binding activity.

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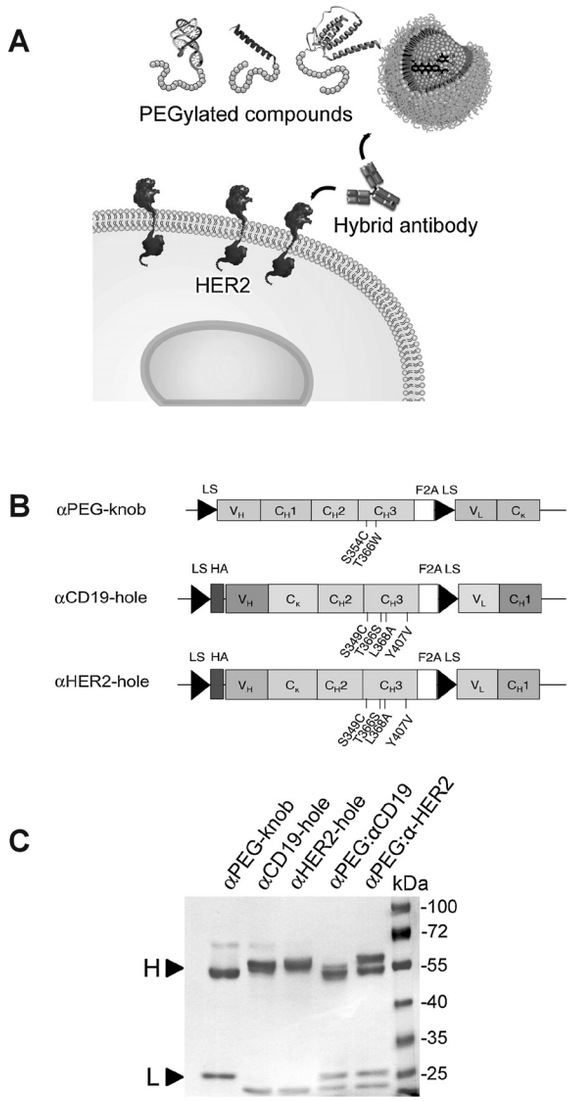

Figure 1.
Illustration of the dual-binding function of hybrid antibodies, construction, expression, and purification of hybrid antibodies. A, illustration of selective delivery of PEGylated compounds to $HER2^+$ cancer cells by an anti-PEG hybrid antibody. The hybrid IgG antibody possesses two antigen-binding sites, one for PEG and one for HER2 on cancer cells. The hybrid antibody also possesses an Fc domain that can potentially bind to Fc receptors on accessory immune cells. The PEGylated compounds represent PEGylated DNA, PEGylated peptide, PEGylated protein, and PEG-liposome (left to right). B, schematic of hybrid antibody expression constructs. αPEG-knob codes for a human Ig_κ signal peptide, the humanized V_H domain of anti-PEG antibody 6.3, a human IgG_1 heavy chain with knob mutations (S354C, T366W), followed by a F2A bicistronic sequence, the humanized V_L domain of 6.3 and a human C_κ domain. αCD19-hole and αHER2-hole code for a human Ig_κ signal peptide, a HA tag, the humanized V_H domain of an anti-CD19 or anti-HER2 antibody, a human C_κ domain, a human IgG_1 heavy-chain Fc domain with hole mutations (S349C, T366S, L368A, and Y407V), followed by a F2A bicistronic sequence, a human Ig_κ signal peptide, the humanized V_L domain of the anti-CD19 or anti-HER2 antibody and a human IgG_1 C_H1 domain. C_H, heavy-chain constant domain; V_H, heavy chain variable domain; V_L, light chain variable domain; C_κ, κ light chain constant domain; LS, leader sequence; HA, human influenza hemagglutinin epitope tag. C, reducing SDSPAGE showing the purified hybrid antibodies and half antibodies separately expressed by different 293 FT cell clones; H, heavy chain; L, light chain.

FACS analysis using an anti-HER2 (Herceptin) antibody was used to determine HER2 levels on the surface of live HT29 and SW-480 human colon colorectal adenocarcinoma cells, HCC-36 human hepatocellular carcinoma cells, as well as on SK-BR-3, BT-474, and MCF-7 human breast cancer cells (Supplementary Methods). SK-BR-3 cells expressed the highest levels of HER2, followed by BT-474 cells whereas essentially background levels of HER2 were detected on MCF-7, HT-29, SW-480, and HCC-36 cells (Supplementary Fig. S3). Binding of the hybrid antibodies to selected cancer cells was then examined by cell-based ELISA. αPEG:αHER2 bound to $HER2^+$ SK-BR-3 cells but not to $HER2^-$ MCF-7 cells (Fig. 3A). As expected, αPEG:αCD19 did not bind to either SK-BR-3 or MCF-7 cells (Fig. 3A). These results indicate that αPEG:αHER2 could selectively bind to cells that express HER2 on their surface.

The affinity of αPEG:αHER2 to HER2 ECD was determined by plasmon resonance measurement in a Biacore T200 (GE Healthcare; Supplementary Methods). Different concentrations of αPEG:αHER2 (8 nmol/L to 128 nmol/L) were flowed through the cell on a CM5 chip immobilized with HER2 ECD proteins and plasmon resonance was measured. αPEG:αHER2 displayed a dissociation constant (K_D) value of approximately 5×10^{-9} mol/L for binding to HER2 (Supplementary Table S2). The binding affinities of the hybrid antibodies to PEG molecules were determined by microscale thermophoresis (Supplementary Methods). αPEG:αHER2 and αPEG:αCD19 displayed similar binding affinity to fluorescently labeled $CH_3O\text{-}PEG_{5K}$ with K_D values of approximately 17×10^{-9} and 30×10^{-9} mol/L, respectively (Supplementary Table S2).

Specific delivery of PEGylated nanoparticles (Qdot655) to live cancer cells mediated by the hybrid antibodies was examined by FACS analysis. αPEG:αHER2 mediated strong binding of Qdot655 to $HER2^+$ SK-BR-3 and BT-474 breast cancer cells, but not to the $HER2^-$ MCF-7 breast cancer cells or Ramos and Raji Burkitt's lymphoma cells (Fig. 3B, solid lines). On the other hand, αPEG:αCD19 promoted the binding of Qdot655 to $CD19^+$ Ramos and Raji cells but not to the $CD19^-$ cells (Fig. 3B, dashed lines). We conclude that the hybrid antibodies can selectively target a PEGylated nanoparticle to cells that express the corresponding antigen on their surface.

The ability of αPEG:αHER2 to mediate delivery of various PEGylated compounds to cells that expresses HER2 was investigated by cell-based sandwich ELISA. αPEG:αHER2 could tether PEGylated proteins and liposomes to $HER2^+$ SK-BR-3 cells (Fig. 3C). Binding required PEG because naked liposomes or proteins were not retained at $HER2^+$ cells (Fig. 3C). αPEG:αHER2 also mediated binding of commercially available PEGylated nanoparticles (Qdot655) and medicines (PEGASYS and Neulasta) to SK-BR-3 cells (Fig. 3D). By contrast, αPEG:αCD19 did not promote the binding of PEGylated compounds to $HER2^+$ SK-BR3 cells (Fig. 3E) and αPEG:αHER2 did not mediate binding of PEGylated compounds to $HER2^-$ MCF-7 cells (Fig. 3F). Taken together, these results demonstrate that αPEG:αHER2 could mediate specific delivery of diverse PEGylated entities to cancer cells that express HER2 on their surface.

αPEG:αHER2 can induce endocytosis of PEGylated nanoparticles into $HER2^+$ cancer cells

We investigated whether αPEG:αHER2 could promote the internalization of Qdot655 PEGylated nanoparticles into tumor cells. Confocal microscopy demonstrated that αPEG:αHER2

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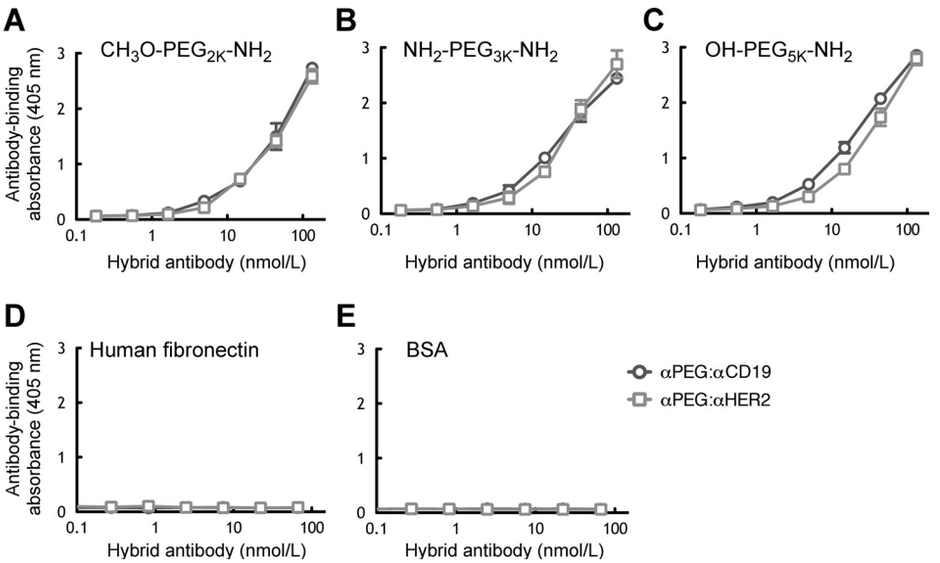

Figure 2.
ELISA analysis of hybrid antibody binding to PEG molecules. The binding of αPEG:αCD19 or αPEG:αHER2 hybrid antibodies to plates precoated with CH_3O-PEG_{2K}-NH_2 (A), NH_2-PEG_{3K}-NH_2 (B), OH-PEG_{5K}-NH_2 (C), human fibronectin (D), or BSA (E) were measured by direct ELISA; mean ± SD; $n = 3$.

could specifically deliver Qdot655 to SK-BR-3 cells, accompanied with accumulation of Qdot655 inside cells after 5 hours of incubation at 37°C (Fig. 4A, top). By contrast, the uptake of Qdot655 was not observed in SK-BR-3 cells incubated with control αPEG:αCD19 hybrid antibody and Qdot655 (Fig. 4A, bottom). Binding and uptake of Qdot655 was also not observed in HER2$^-$ MCF-7 cells treated with αPEG:αHER2 (Fig. 4B). We conclude that αPEG:αHER2 can mediate selective binding and internalization of PEGylated nanoparticles into HER2$^+$ cancer cells.

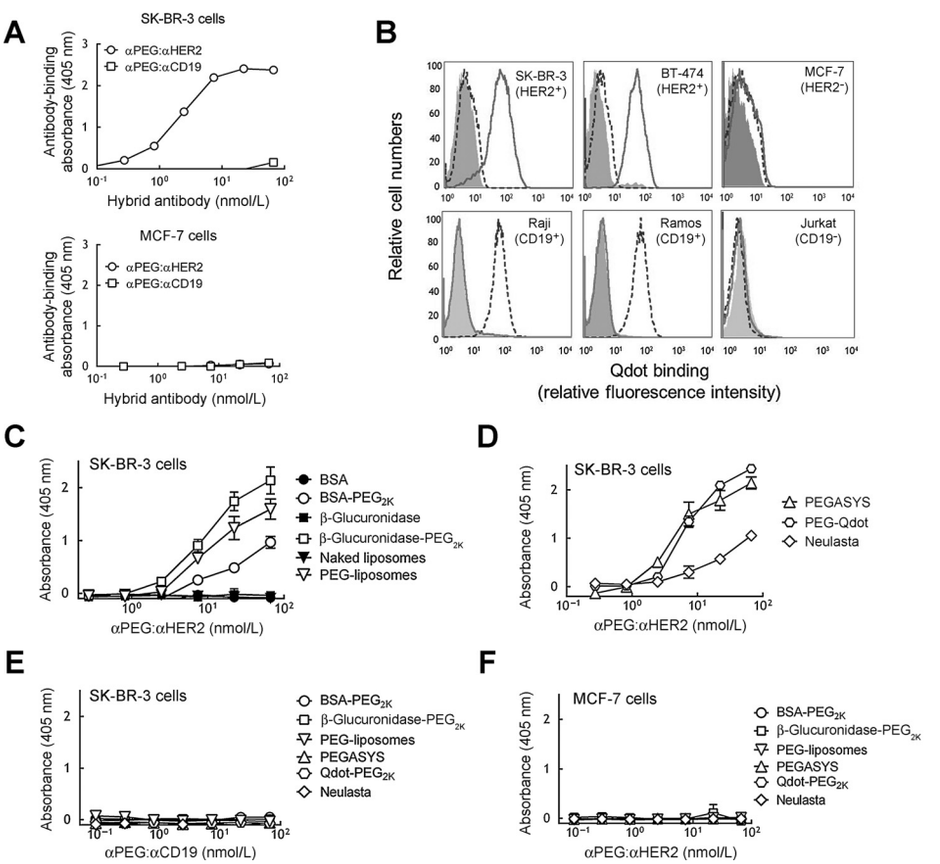

Figure 3.
Hybrid antibodies can target PEGylated compounds to cancer cells. A, binding of hybrid antibodies to SK-BR-3 and MCF-7 cells was determined by direct ELISA; $n = 2$; bars, SD. B, FACS analysis of the dual binding of αPEG:αHER2 (solid lines) or αPEG:αCD19 (dashed lines) hybrid antibodies to cancer cells and PEGylated Qdot655. Shaded curves show the FACs of unstained cells. C, ELISA measurement of the binding of the indicated compounds to SK-BR-3 cells mediated by αPEG:αHER2. D, ELISA measurement of the binding of commercial PEGylated compounds to SK-BR-3 cells mediated by αPEG:αHER2. E, ELISA measurement of the binding of commercial PEGylated compounds to SK-BR-3 cells mediated by αPEG:αCD19. F, ELISA measurement of the binding of compounds to MCF-7 cells mediated by αPEG:αHER2; mean ± SD; $n = 3$.

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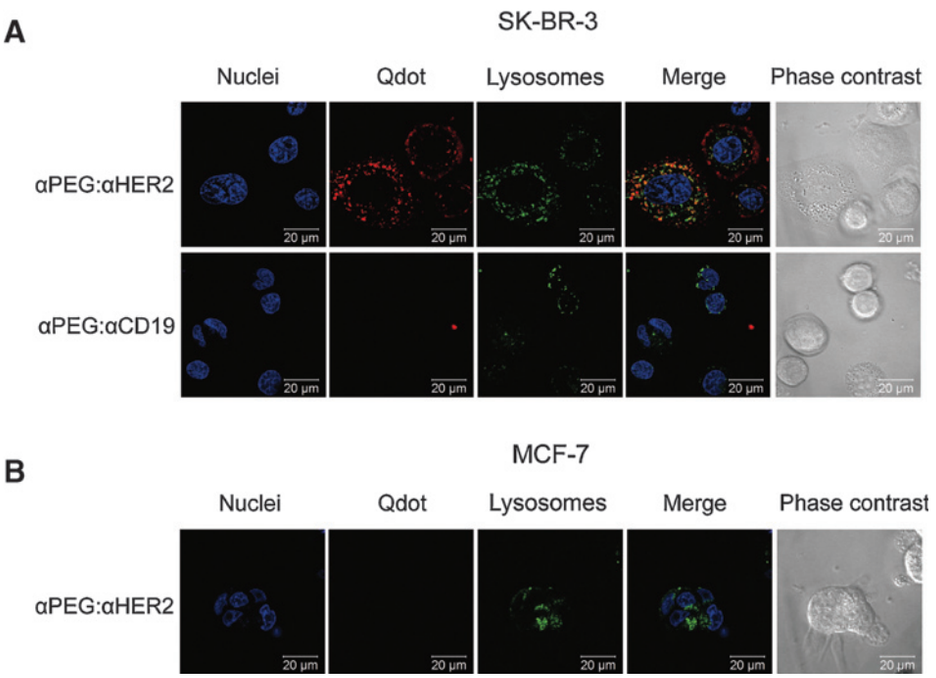

Figure 4.
αPEG:αHER2 can induce the binding and internalization of Qdot655 into HER2$^+$ cancer cells. SK-BR-3 (HER2$^+$; A) cells and MCF-7 (HER2$^-$; B) cells were incubated with Hoechst 33342 (blue) and LysoTracker Green DND-26 (green) before addition of hybrid antibodies on ice for 30 minutes. Qdot655 (red) were added and the cells were cultured for 5 hours at 37°C before confocal images were taken.

αPEG:αHER2 can enhance the cytotoxicity of doxisome to HER2$^+$ cancer cells

Because αPEG:αHER2 triggered the endocytosis of a PEGylated nanoparticle in HER2$^+$ cancer cells, we investigated whether it could enhance the cytotoxicity of PEGylated liposomal doxorubicin (doxisome) in cancer cells. After 3 hours exposure of the cells to hybrid antibodies and doxisome or to doxisome alone, the cells were refreshed with new medium and cultured for 48 hours to allow time for the doxorubicin to kill the cancer cells. We then assessed the proliferation of the remaining cells by adding ^{3}H-thymidine for 18 hours and measuring the incorporation of radiolabeled ^{3}H-thymidine in newly synthesized DNA. This assay measures cell proliferation and gives similar results as the tetrazolium MTT assay (28, 29). αPEG:αHER2 but not αPEG:αCD19 significantly enhanced the cytotoxicity of doxisome to SK-BR-3 (Fig. 5A) and BT-474 (Fig. 5B) HER2$^+$ cancer cells as compared with treatment with doxisome alone. The similar cytotoxicity observed with doxisome and both 10 and 2 μg/mL αPEG:αHER2 suggests that sufficient hybrid antibody was present to saturate the HER2 receptors on SK-BR-3 cells (Fig. 5A). The IC_{50} values of doxisome in combination with αPEG:αHER2 were over two orders of magnitude lower than with doxisome alone (Table 1). Neither αPEG:αHER2 or αPEG:αCD19 altered the sensitivity of HER2$^-$ MCF-7 cells to doxisome (Fig. 5C), showing

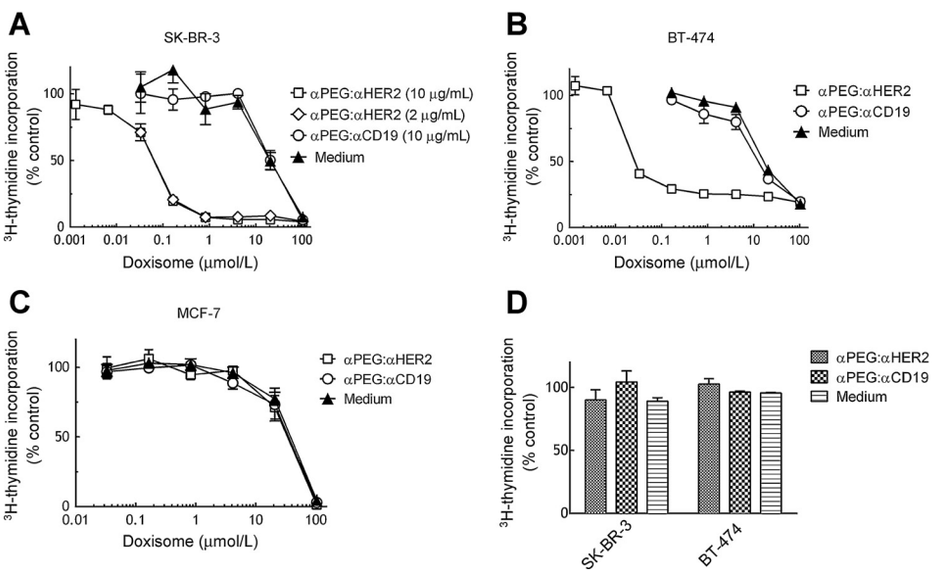

Figure 5.
Enhancement of doxisome cytotoxicity by hybrid antibodies. Proliferation of SK-BR-3 (A), BT-474 (B), and MCF-7 (C) cells treated with doxisome alone or doxisome combined with αPEG:αHER2 or control αPEG:αCD19 hybrid antibodies. D, proliferation of SK-BR-3 and BT-474 cells incubated with hybrid antibodies or medium; mean ± SD; $n = 3$.

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Table 1. Cytotoxicity of doxisome or doxisome combined with 10 μg/mL αPEG:αHER2 or αPEG:αCD19 hybrid antibodies

Cells	Doxisome (μmol/L)	αPEG:αHER2 + doxisome (μmol/L)	αPEG:αCD19 + doxisome (μmol/L)	Fold enhancement[a]
SK-BR-3	34.0 ± 8.0	0.07 ± 0.0**	20.6 ± 1.7	519 ± 176*
BT-474	14.0 ± 0.2	0.02 ± 0.0***	10.8 ± 1.5	715 ± 173**

NOTE: Concentrations of doxisome that produce 50% inhibition of ^{3}H-thymidine incorporation into cellular DNA are indicated in μmol/L (IC_{50}). Results represent mean values of triplicate determinations ± SD. Significant differences relative to doxisome are indicated; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
[a]Fold enhancement of αPEG:αHER2 + doxisome versus doxisome alone.

that the hybrid antibodies displayed specificity for cells that expressed target ligand. The hybrid antibodies alone did not induce cytotoxicity in SK-BR-3 and BT-474 cells (Fig. 5D).

Discussion

We developed and investigated hybrid antibodies consisting of PEG-binding and tumor antigen-binding half antibodies. The hybrid antibodies are capable of delivering diverse PEGylated compounds and nanoparticles to cancer cells that express surface antigens recognized by the tumor antigen-binding half antibody. We found that a αPEG:αHER2 hybrid antibody facilitated the uptake of nanoparticles into $HER2^+$ cancer cells and greatly enhanced the cytotoxicity of doxisome to $HER2^+$ cancer cells. We anticipate that anti-PEG hybrid antibodies may serve as a versatile approach for targeted delivery of PEGylated compounds (therapeutic proteins, liposomal drugs, gene delivery carriers, or imaging agents) to tumors and other cellular locations, and may further enhance therapeutic and diagnostic efficacy due to the enhanced cellular uptake of PEGylated compounds.

We previously described anti-PEG IgG antibodies (3.3 and E11) with well-characterized binding to PEG (14, 15, 30). However, we were unable to achieve good tumor targeting using E11 or 3.3 anti-PEG antibodies, supposedly due to insufficient binding affinity to PEG (unpublished results). We therefore developed a new anti-PEG monoclonal antibody, 6.3, which displayed about one order of magnitude higher apparent affinity to PEG molecules as compared with our previous high-affinity anti-PEG IgG monoclonal antibody 3.3 (Supplementary Fig. S2 and Supplementary Table S1). These results suggest that high affinity is required for the anti-PEG arm of the hybrid antibodies for good delivery of PEGylated compounds to cellular targets.

Although PEGylation can extend the half-lives and stability of nanoparticles, the steric hindrance provided by PEG molecules can interfere with desired interactions with target cells. For example, PEG molecules on nanocarriers designed for delivery of DNA or siRNA were found to hamper cellular uptake (31), which is known as the "PEG dilemma" (32). DNA or siRNA must be transported across the cell plasma membrane to enter cells, but the interactions between the gene carriers and cell surface are blocked by the aqueous phase surrounding the PEG moieties. Therefore, although PEGylation can extend the stability and half-lives of nanoparticles, a trade-off exists as the interactions between the PEGylated compounds and their targets might also be hampered by PEGylation.

Active targeting has been one approach to enhance cellular uptake of PEGylated compounds. Target ligands recognizing receptors specifically overexpressed on cancer cells have been covalently linked to PEGylated nanoparticles, which in turn promote selective binding and uptake of the nanoparticles by target cells. Proteins, peptides containing specific motifs such as arginine-glycine-aspartic acid (RGD; ref. 33), folate (34), antibodies and antibody fragments (immunoliposomes), nucleic acids (35), and transferrin (36) have been conjugated to PEGylated nanoparticles to facilitate active targeting.

Anti-PEG hybrid antibodies may possess some advantages compared with other targeting technologies. Chemical conjugation is complicated by potential damage to the targeting ligand and cargo (10, 37) as well as by generation of immunogenic epitopes. Interactions between cellular receptors and conjugated ligands can also be hampered by the steric hindrance provided by PEG moieties (38). By contrast, well-characterized hybrid antibodies specific to PEG molecules and cellular receptors may exert consistent binding of both target cells and PEGylated compounds under physiologic conditions, omitting complexities resulted from conjugation reactions. Moreover, payloads noncovalently bound by antibodies may be a more efficient alternative for cargo release, as targeting ligands may require degradation or complicated cleavage processes to release the active cargos (10). Importantly, separation of antibody development and nanoparticles or drug development may allow for simpler manufacturing processes. Other BsAbs have been developed to deliver nanocargos to cellular targets. For example, BsAbs recognizing both a chemical hapten and a tumor antigen were shown to successfully target hapten-conjugated vehicles to specific cells (13, 39). However, chemical conjugation of haptens to the payloads is required. PEGylation of hapten-conjugated nanoparticles can also hinder binding of anti-hapten BsAbs (40). We overcame this limitation by creating BsAbs that directly bind to PEG molecules already present on many nanaomedicines.

In contrast with our previously described anti–methoxy-PEG BsAb fragments (26), here, we investigated intact IgG_1 hybrid antibodies that bind to the ethylene oxide backbone of PEG. One potential advantage of human IgG_1 hybrid antibodies is the opportunity for both targeted delivery of therapeutic agents and induction of anticancer immune responses. The Fc domain of human IgG_1 when bound to the surface of cancer cells can activate complement and trigger antibody-dependent cellular cytotoxicity (ADCC) by immune effector cells (natural killer cells and monocytes), which contribute to effective antibody therapies (41). It will be of interest to determine whether there are any synergistic effects between ADCC and hybrid antibody delivery of nanomedicines. Immunostimulatory PEGylated cytokines such as IL2, IFNα, IFNγ, and IL12 (42) may also represent rational targets for selective tumor delivery by anti-PEG hybrid antibodies.

In conclusion, our study suggests that anti-PEG hybrid antibodies offer a simple and versatile method to selectively deliver PEGylated compounds the target cells. Examples of clinically used PEGylated proteins include PEG-asparaginase (43), PEG_{40K}–IFNα 2a (PEGASYS; ref. 44). PEG_{12K}–IFNα 2b (PEG-Intron; ref. 45), PEG_{20K}–filgrastim (Neulasta; ref. 46), and PEGylated epoietin (47). PEGylated nanomedicines include such examples as polyplex, a nanoparticle inhalation gene delivery system for gene therapy (48), stealth radiolabeled PEG_{2K}-liposomes and

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PEG_{2K}-liposomes encapsulating drugs, including Doxil (49) and DaunoXome (50), and other PEGylated compounds, including Qdot (51), PEG-aptamer pegaptanib sodium (Macugen, Pfizer), PEG small organic molecules and PEG-antibody fragments ertolizumab pegol (Cimzia, UCB; refs. 5, 52). These and new PEGylated drugs, nanomedicines, and imaging agents under development represent potential sources for targeted delivery by hybrid anti-PEG antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H.-Y. Tung, Y.-C. Su, T.-L. Cheng, S.R. Roffler
Development of methodology: H.-Y. Tung, Y.-C. Su, K.-H. Chuang, T.-L. Cheng, S.R. Roffler
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.-Y. Tung, B.-M. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-Y. Tung, Y.-C. Su
Writing, review, and/or revision of the manuscript: H.-Y. Tung, Y.-T. Yan, S.R. Roffler
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-Y. Tung, P.-A. Burnouf, W.-C. Huang, T.-L. Cheng
Study supervision: Y.-T. Yan, S.R. Roffler

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Molecular Cancer Therapeutics

Selective Delivery of PEGylated Compounds to Tumor Cells by Anti-PEG Hybrid Antibodies

Hsin-Yi Tung, Yu-Cheng Su, Bing-Mae Chen, et al.

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