Conditional internalization of PEGylated nanomedicines by PEG engagers for triple negative breast cancer therapy

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Triple-negative breast cancer (TNBC) lacks effective treatment options due to the absence of traditional therapeutic targets. The epidermal growth factor receptor (EGFR) has emerged as a promising target for TNBC therapy because it is overexpressed in about 50% of TNBC patients. Here we describe a PEG engager that simultaneously binds polyethylene glycol and EGFR to deliver PEGylated nanomedicines to EGFR⁺ TNBC. The PEG engager displays conditional internalization by remaining on the surface of TNBC cells until contact with PEGylated nanocarriers triggers rapid engulfment of nanocargos. PEG engager enhances the anti-proliferative activity of PEG-liposomal doxorubicin to EGFR⁺ TNBC cells by up to 100-fold with potency dependent on EGFR expression levels. The PEG engager significantly increases retention of fluorescent PEG probes and enhances the antitumour activity of PEGylated liposomal doxorubicin in human TNBC xenografts. PEG engagers with specificity for EGFR are promising for improved treatment of EGFR⁺ TNBC patients.
Breast cancer is the second most common cancer in the world. Triple-negative breast cancer (TNBC), which comprises 11.2–16.3% of breast cancers, lacks expression of oestrogen receptors, progesterone receptors and human epidermal growth factor receptor 2 (HER2). TNBC is typically heterogeneous, aggressive and is associated with poor prognosis with limited treatment options due to the absence of well-defined therapeutic targets. Systemic chemotherapy has been the primary treatment option for TNBC until it was discovered that epidermal growth factor receptor (EGFR) is overexpressed in ~50% of TNBC tumours. EGFR-targeted agents are therefore under development for the treatment of TNBC. However, EGFR-targeted tyrosine kinase inhibitors such as gefitinib and erlotinib display minimal therapeutic efficacy in TNBC patients.

Nanomedicines, including PEGylated liposomal doxorubicin, are currently under investigation for the treatment of TNBC. Nanocarriers are attractive because they can alter the pharmacokinetic profile of drugs, reduce off-target toxicity and improve the therapeutic index. Tumour accumulation of nanomedicines relies on the enhanced permeability and retention (EPR) effect in which the leaky blood vasculature combined with impaired lymphatic drainage can facilitate passive accumulation of nanosized particles in tumours. Lung, breast and ovarian tumours display high enhanced permeability and retention effect-mediated accumulation of nanocarriers, making nanomedicines an attractive treatment alternative for TNBC.

Active targeting by functionalizing the surface of nanocarriers with ligands that bind to endocytic receptors on cancer cells can promote receptor-mediated endocytosis for increased cellular uptake of nanomedicines with concomitant improved antitumour activity. However, many technical and regulatory hurdles must be overcome to produce new nanocarriers with reproducible and homogenous ligand densities and activities. Attachment of targeting ligands can also compromise the stealth features of nanocarriers and hinder tumour uptake.

Pre-targeting strategies can help curtail these problems by separating the production of the targeting moiety and nanocarrier as well as allowing the administration of unmodified highly stealth nanocarriers. Because nanomedicines are commonly grafted with poly (ethylene glycol) (PEG) to decrease uptake and clearance by the reticuloendothelial system, here we develop a general pre-targeting strategy for conditional internalization of PEGylated nanomedicines for improved treatment of EGFR+ TNBC. This is accomplished by generating bispecific PEG-binding antibodies (PEG engagers) for targeted delivery of PEGylated nanomedicines to tumours. Pre-targeting of PEG engagers induce endocytosis of PEGylated nanocarriers into EGFR+ TNBC cancers leading to enhanced antitumour efficacy of PEG-modified therapeutic agents in vitro and in vivo.

Results
Characterization of bispecific PEG engagers. A bispecific PEG engager was generated by genetically fusing a humanized anti-PEG Fab fragment with an anti-EGFR single-chain disulfide-stabilized Fv fragment. The PEG engager was designed to bind to EGFR on TNBC cells but remain dormant until contact and binding to PEG-coated nanocarriers induces rapid internalization into cancer cells (Fig. 1). Briefly, a humanized anti-PEG (6.3) Fab was constructed as a single open reading frame by fusing variable light chain with constant kappa light chain and variable heavy chain with first constant heavy chain domains with an internal ribosome entry site bicistronic expression vector and stable 293FT producer cells were generated. PEG engager purified from the culture medium display the expected molecular sizes as visualized on a reducing and a non-reducing 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2b). The PEG engager CD19 and PEG engagerEGFR have molecular weights of 78 kDa and 79 kDa, respectively, as determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Fig. 2c). Size-exclusion high-performance liquid chromatography analysis showed a major peak corresponding to a monomer with minimal aggregation (Supplementary Fig. 1a). The melting temperatures of PEG engagerCD19 and PEG engagerEGFR (75 °C and 75.8 °C, respectively), as determined by differential scanning calorimetry (Supplementary Fig. 1b), were higher than the typical melting temperatures of Fab fragments (61.9–69.4 °C), indicating that the engagers display good thermal stabilities. Analysis of PEG engager binding to PEG by microscale thermophoresis showed that PEG engagerEGFR (equilibrium dissociation constant, $K_D = 7.6 \pm 1.13 \text{nM}$) and PEG engagerCD19 ($K_D = 7.6 \pm 1.05 \text{nM}$) displayed similar binding affinities (Fig. 2d). The binding affinity ($K_D$) of the PEG engagers to their corresponding tumour antigen targets (EGFR and CD19) was 0.96 ± 0.23 nM and 3.6 ± 0.35 nM for PEG engagerEGFR and PEG engagerCD19, respectively (Fig. 2e).

Specific delivery of PEGylated nanoparticles by PEG engagers. To determine whether PEG engagerEGFR can specifically deliver PEGylated nanoparticles into EGFR-positive cancer cells, we investigated cancer cell lines with different expression levels of EGFR, including MCF7 EGFR-negative non-TNBC breast adenocarcinoma cells, MDA-MB-468 EGFR-positive TNBC cells
Representative microscale thermophoresis data from three independent experiments are shown. 6.3 heavy chain fragment (6.3 VH–CH1), a glycine-serine peptide linker (GGGGS), an antitumour single chain disulfide-stabilized variable fragments H (heavy chain), L (light chain). (Electrophoresis showing Coomassie blue staining of Erbitux, PEG engagerEGFR and PEG engagerCD19. M, PageRuler prestained protein ladder (Fermentas). (dsFv, anti-CD19 or anti-EGFR), and a polyhistidine-tag (His tag). (Fig. 3(i–iv)). We conclude that PEG engagerEGFR can deliver to recombinant CD19 protein (ii) analysed by microscale thermophoresis (Fig. 2(ii)) and PEG engagerCD19 (i) analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mass-to-charge ratio (m/z). (d) Binding affinity of PEG engagerEGFR (i) and PEG engagerCD19 (ii) analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mass-to-charge ratio (m/z). (d) Binding affinity of PEG engagerEGFR (i) and PEG engagerCD19 (ii) to PEGs analysed by microscale thermophoresis (n = 3). (e) Binding affinity of PEG engagerEGFR to recombinant EGFR protein (i) and PEG engagerCD19 to recombinant CD19 protein (ii) analysed by microscale thermophoresis (n = 3). Equilibrium dissociation constant (K_D). Data are shown as mean ± s.d. Representative microscale thermophoresis data from three independent experiments are shown.

Figure 2 | Production and analysis of recombinant PEG engagers. (a) Schematic representation of PEG engager constructs, which code for a murine immunoglobulin kappa chain leader sequence (Igk), a humanized 6.3 light chain (6.3 VL–Ck), an internal ribosome entry site (IRES) sequence, a humanized 6.3 heavy chain fragment (6.3 VH–CH1), a glycine-serine peptide linker (GGGGS), an antitumour single chain disulfide-stabilized variable fragments (dsFv, anti-CD19 or anti-EGFR), and a polyhistidine-tag (His tag). (b) Reducing (i) and non-reducing (ii) sodium dodecyl sulfate polyacrylamide gel electrophoresis showing Coomassie blue staining of Erbitux, PEG engagerEGFR and PEG engagerCD19. M, PageRuler prestained protein ladder (Fermentas). H (heavy chain), L (light chain). (c) Precise molecular weight of PEG engagerEGFR (i) and PEG engagerCD19 (ii) analysed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mass-to-charge ratio (m/z). (d) Binding affinity of PEG engagerEGFR (i) and PEG engagerCD19 (ii) to PEGs analysed by microscale thermophoresis (n = 3). (e) Binding affinity of PEG engagerEGFR to recombinant EGFR protein (i) and PEG engagerCD19 to recombinant CD19 protein (ii) analysed by microscale thermophoresis (n = 3). Equilibrium dissociation constant (K_D). Data are shown as mean ± s.d. Representative microscale thermophoresis data from three independent experiments are shown.

and A431 EGFR-positive epidermoid carcinoma cells. Cancer cell-specific uptake of PEGylated nanoparticles mediated by PEG engagers was examined by real-time confocal microscopy cell imaging of EGFR-negative MCF7 cells or EGFR-positive MDA-MB-468 and A431 cells treated stepwise with PEG engagerEGFR or PEG engagerCD19 and then fluorescent PEGylated Qtracker 655 non-targeted quantum dots (PEG-Qdot655). Both MDA-MB-468 TNBC and A431 cells express EGFR but not CD19. MCF7 cells express neither EGFR nor CD19. PEG engagerEGFR-mediated rapid accumulation of PEG-Qdot655 in both MDA-MB-468 and A431 cells (Fig. 3a,b(iii–iii)), but not in MCF7 cells as shown in Fig. 3c(i–iii). By contrast, no uptake of PEG-Qdot655 was observed in MDA-MB-468, A431 and MCF7 cells treated with control PEG engagerCD19 as shown in Fig. 3(iv–vi). We conclude that PEG engagerEGFR can deliver PEGylated nanoparticles into cancer cells that express EGFR.

Conditional internalization of PEGylated nanoparticles. We examined whether PEG engagerEGFR could trigger conditional receptor-mediated internalization dependent on the presence of PEGylated nanoparticles. Alexa Fluor 647-labelled PEG engagerEGFR was first added to live cancer cells for 1 h. Confocal imaging revealed that the engager remained on the plasma membrane of MDA-MB-468 (Fig. 4a(i–iii) and Supplementary Fig. 3a) and BT-20 (Supplementary Fig. 2i–iv) cells at 37 °C for 1 h with almost no internalization (Alexa Fluor 647 appears in green pseudo color). PEG engagerEGFR displayed limited endocytosis in MDA-MB-468 cells even after 9 h (Supplementary Fig. 3b). PEG-Qdot655 added to the cells, however, bound to PEG engagerEGFR on the cell membrane and were rapidly internalized into the cells (Fig. 4a(ii–xiv, Supplementary Fig. 2ii–xiv and Supplementary Fig. 3iii–vii, red colour). Co-localization of PEG engagerEGFR and PEG-Qdot655 or lysosomes (Fig. 4a, Supplementary Figs 2 and 3b) verified that PEG engagerEGFR can conditionally stimulate endocytosis of PEGylated nanoparticles and then localize in lysosomes (Fig. 4b,c, and Supplementary Fig. 3c).

Efficacy of PEG engager-directed liposomal drugs in vitro. We next investigated whether PEG engagerEGFR can enhance the anti-proliferation activity of a drug-loaded nanocarrier in different type of cancer cells that express either wild-type EGFR or mutated EGFR. MDA-MB-231, MDA-MB-468 and BT-20 are TNBC cancer cell lines that express wild-type EGFR. SKBR3 is a...
confocal images from three independent experiments are shown.

with a digital confocal microscope. Scale bar, 10 or MCF7 (Hoechst 33342 (blue) were incubated with MDA-MB-468 (Doxisome) or PEG-liposomal vinorelbine. PEG engager EGFR or vinorelbine), empty liposomes, PEG-liposomal doxorubicin treated with graded concentrations of free drug (doxorubicin 4

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significantly enhanced the anti-proliferation activity of Doxisome

or vinorelbine), empty liposomes, PEG-liposomal doxorubicin

targeted Doxisome, respectively (Fig. 5d). Neither PEG engagerEGFR nor PEG engagerCD19 altered the sensitivity of HepG2 cells (EGFR negative) to Doxisome (Supplementary Fig. 4c). In contrast to wild-type BT-20 cells, PEG engagerEGFR did not enhance the anti-proliferation activity of Doxisome in BT-20/shEGFR cancer cells (BT-20 cells treated with short hairpin RNA to knockdown the expression of EGFR) as compared with drug-loaded nanocarrier alone or drug-loaded nanocarrier plus PEG engagerCD19 (Supplementary Fig. 6), further showing that PEG engagerEGFR mediates endocytosis of PEGylated nanocarriers via the EGFR internalization pathway. We conclude that PEG engagerEGFR can increase the anticancer activity of PEGylated medicines (Doxisome and PEG-liposomal vinorelbine) to EGFR + cancer cells.

Pre-existing anti-PEG antibodies in healthy donors might negatively impact PEG engager targeting of PEGylated medicines by blocking engager binding to PEG on the nanomedicines

We measured anti-PEG antibody concentrations in healthy human plasma samples using an anti-PEG enzyme-linked immunosorbent assay (ELISA) developed in our lab (Supplementary Fig. 7a). The pre-existing anti-PEG IgG concentrations ranged from 0.3 to 237.5 μg ml −1 with a mean concentration of 5.75 ± 16.0 μg ml −1 in 386 anti-PEG positive samples. We selected a human serum sample containing a relatively high concentration of anti-PEG IgG (51.4 μg ml −1). However, PEG engagerEGFR plus Doxisome in 20% human serum that was positive for anti-PEG IgG antibodies or in control human serum displayed similar EC50 values against MDA-MB-468 cells (Supplementary Fig. 7b). These results suggest that pre-existing anti-PEG antibodies in patients do not effectively compete with PEG engagerEGFR, presumably due to the high anti-PEG affinity of the PEG engager and abundant PEG chains on Doxisome.

**Figure 3 | Dual antigen-binding activity of PEG engagers.** PEG engagerEGFR (i–iii) and PEG engagerCD19 (iv–vi) supplemented with Hoechst 33342 (blue) were incubated with MDA-MB-468 (a), A431 (b) or MCF7 (c) cells followed by PEG-Qdot655 (red) and observed in real time with a digital confocal microscope. Scale bar, 10 μm. Representative confocal images from three independent experiments are shown.

non-TNBC breast adenocarcinoma cell line that expresses wild-type EGFR and PC9 is non-small cell lung cancer cell line that expresses EGFR with a delta E746-A750 deletion in the tyrosine kinase domain. These cells were incubated with PEG engagerEGFR or PEG engagerCD19 as a negative control and subsequently treated with graded concentrations of free drug (doxorubicin or vinorelbine), empty liposomes, PEG-liposomal doxorubicin (Doxisome) or PEG-liposomal vinorelbine. PEG engagerEGFR significantly enhanced the anti-proliferation activity of Doxisome (Fig. 5 and Supplementary Fig. 4) and PEG-liposomal vinorelbine (Supplementary Fig. 5) against EGFR-positive cancer cells as compared with drug-loaded nanocarrier alone, drug-loaded nanocarrier plus PEG engagerCD19 or empty liposomes with PEG engagerEGFR. The half maximal effective concentration (EC50) values for PEG engagerEGFR targeted Doxisome in BT-20, MDA-MB-468 and MDA-MB-231 cells were 101-fold, 74-fold and 107-fold lower than control PEG engagerCD19 targeted Doxisome, respectively (Fig. 5d).

**Pharmacokinetics and tumour targeting of the PEG engager.**

Pre-targeting of PEG engagers to tumours may allow for subsequent accumulation and endocytosis of PEGylated nanocarriers in cancer cells. The in vivo pharmacokinetics of PEG engagers was examined to determine a reasonable time point for administration of PEGylated nanocarriers after administration of PEG engager. The half-lives of the PEG engagers were ~2.1 h (PEG engagerEGFR) and 2.2 h (PEG engagerCD19) (Fig. 6a) after intravenous administration of 150 μg of PEG engager. Almost 90% of the PEG engagers were cleared from the circulation by 5 h after injection (Fig. 6a).

To visualize whether pre-targeting can facilitate the uptake and retention of PEGylated compounds in tumours, mice bearing established high EGFR expression levels (MDA-MB-468 and A431) or low EGFR expression levels (HepG2) tumours were intravenously injected with 6 mg kg −1 PEG engager. The half-lives of the PEG engagers was 2.7-fold, 2.1-fold and 2.8-fold greater than in the control PEG engager and then pharmacokinetics of PEG engagers

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engagerCD19-treated tumours, respectively (Fig. 6c). Neither PEG engagerEGFR nor PEG engagerCD19 enhanced the fluorescence signal in HepG2 (low EGFR expression levels) tumour-bearing mice (Supplementary Fig. 9).

Antitumour activity of pre-targeted PEG engager. To investigate whether PEG engagerEGFR can inhibit EGFR signalling, EGFR-positive A431 cells were stimulated with or without epidermal growth factor and then co-incubated with PEG engagers or control antibodies. Both Erbitux (monoclonal anti-EGFR IgG) and PEG engagerEGFR inhibited the phosphorylation of EGFR and Erk as compared with negative control Herceptin (anti-HER2 IgG) and PEG engagerCD19 (Supplementary Fig. 10). Mice bearing human MDA-MB-468 or MDA-MB-231 TNBC xenografts were intravenously injected with phosphate-buffered saline (PBS), free doxorubicin (3 mg kg\(^{-1}\)) or PEG engagerCD19 or PEG engagerEGFR (6 or 18 mg kg\(^{-1}\)) 5 h before Doxisome treatment (1 or 3 mg kg\(^{-1}\)) on days 1, 8, 15 and 22. Although engagerEGFR inhibited EGFR signalling in vitro, mice treated only with PEG

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**Figure 4 | PEG engagerEGFR conditional internalization.** (a) Fluorescent-labelled PEG engagerEGFR on the cell membrane of MDA-MB-468 cells after 1 h at 37°C (green, iv) was real-time imaged 5, 30 and 60 min after incubating without (i–iii) or with (v–vii) PEG-Qdot655. Hoechst 33342 (blue), PEG-Qdot655 (red, xiii–xiv) and LysoTracker Red DND-99 (purple pseudo colour, xv–xxi) are also shown for nucleus, Qdots and lysosome staining, respectively. Merged (xxii–xxviii) and enlarged (xxix–xxxv) images are also shown. Scale bar, 10 μm. (b) Percentage of the PEG engagerEGFR that internalized into cells complexed with (white bars) or without (red bars) PEG-Qdot655 at different times was quantified from confocal images of individual cells (n = 15). (c) Percentage of PEG engagerEGFR that co-localized with LysoTracker Red DND-99 (lysosomes) at different times was quantified from confocal images of individual cells (n = 15). Representative confocal images from two independent experiments are shown. Data are shown as mean ± s.d. Significant differences in percentage of PEG engagerEGFR internalization or lysosome co-localization with and without addition of PEG-Qdot655 are indicated: **P ≤ 0.001, ***P ≤ 0.0001 (two-way analysis of variance). NS, not significant.
**Figure 5 | PEG engager**<sup>EGFR</sup> **enhances nanomedicine anti-proliferative activity.** BT-20 (a), MDA-MB-468 (b) and MDA-MB-231 cells (c) were incubated with PEG engager<sup>EGFR</sup> for 30 min followed by addition of serial dilutions of Doxisome (white circles) or empty liposomes (white squares) in triplicate for 4 h. The cells were also incubated with culture medium or PEG engager<sup>CD19</sup> for 30 min followed by addition of serial dilutions of Doxisome (black circles and red squares, respectively). Serial dilutions of free doxorubicin (white triangles) or PEG engager<sup>EGFR</sup> alone (white diamonds) were also added to cells for 4 h. The incorporation of <sup>3</sup>H-thymidine into cellular DNA was measured 72 h later. The data are representative of three independent experiments.

(d) The half maximal effective concentration (EC<sub>50</sub>) values of BT-20, MDA-MB-468 and MDA-MB-231 cells treated with PEG engager<sup>CD19</sup> plus Doxisome or PEG engager<sup>EGFR</sup> plus Doxisome were analysed (n = 3). Data are shown as mean ± s.d. Significant differences in mean EC<sub>50</sub> values are indicated: **P ≤ 0.001, ***P ≤ 0.0001 (two-way analysis of variance).

PEG engager<sup>EGFR</sup> displayed similar tumour growth as mice treated with PBS (Fig. 7a,c). Free doxorubicin suppressed tumour growth as compared to treatment of mice with PBS (Fig. 7a, P < 0.05). PEG engager<sup>CD19</sup> combined with 1 mg kg<sup>−1</sup> Doxisome or 1 mg kg<sup>−1</sup> Doxisome alone displayed similar and better suppression of tumour growth as compared to treatment of mice with free doxorubicin or PBS vehicle (Fig. 7a, P < 0.0001 and Fig. 7c, P < 0.001). PEG engager<sup>EGFR</sup> plus Doxisome significantly suppressed TNBC tumour growth as compared to treated with Doxisome alone (Fig. 7a, P < 0.0005 and Fig. 7c, P = 0.0012). The maximum-tolerated dose of doxorubicin in severe combined immunodeficiency (SCID) mice is around 2.5−3 mg kg<sup>−1</sup> due to defective DNA repair in these mice<sup>28</sup>. Increasing the dose of Doxisome to 3 mg kg<sup>−1</sup> did not provide better therapeutic activity because the mice experienced significant body weight loss and early deaths (Fig. 7b). Our results demonstrate that pre-targeting PEG engager<sup>EGFR</sup> to EGFR overexpressing TNBC tumours can markedly enhance the therapeutic efficacy of PEGylated liposomal doxorubicin (Doxisome) with limited side effects as shown by body weight loss analysis (Fig. 7b).

We further investigated whether pre-docking of PEGylated nanoparticles with PEG engagers could enhance their therapeutic efficacy. We used a molar ratio of PEG engager and PEG-lipid on Doxisome of 1:55 (Supplementary Fig. 11a). Mice were administrated with a mixture of PEG engager and Doxisome (pre-incubated at 4°C for 1 h) and blood samples were then periodically collected from the tail vein of the mice. The half-lives of the PEG engagers as determined by quantitative ELISA were ~3.5 h (PEG engager<sup>EGFR</sup>) and 3.8 h (PEG engager<sup>CD19</sup>) (Supplementary Fig. 11b) after intravenous administration of PEG engager-docked Doxisome (containing 30 μg PEG engager).

To examine the therapeutic activity of PEG engager-docked Doxisome, non-obese diabetic-severe combined immunodeficiency (NOD SCID) mice bearing human MDA-MB-468 TNBC xenografts were intravenously injected with PBS, 3 mg kg<sup>−1</sup> free doxorubicin, 6 mg kg<sup>−1</sup> PEG engager<sup>EGFR</sup> alone, PEG engager<sup>CD19</sup>-decorated Doxisome or PEG engager<sup>EGFR</sup>-decorated Doxisome (1 mg kg<sup>−1</sup> of doxorubicin) on days 1, 8, 15 and 22. Doxorubicin slightly inhibited tumour growth while significantly better antitumour activity was observed in mice treated with Doxisome (1 mg kg<sup>−1</sup>) or PEG engager<sup>CD19</sup>-decorated Doxisome as compared to mice treated with PBS vehicle (Supplementary Fig. 11c, P < 0.005). A higher dose (3 mg kg<sup>−1</sup>) of Doxisome was toxic to the mice (Fig. 7b and Supplementary Fig. 11d). PEG engager<sup>EGFR</sup>-decorated Doxisome significantly suppressed TNBC tumour growth as compared to mice treated with Doxisome alone (Supplementary Fig. 11c, P < 0.005). Thus, pre-docking PEG engager<sup>EGFR</sup> on Doxisome markedly enhanced therapeutic efficacy with minimal side effects as measured by body weight loss (Supplementary Fig. 11d).

**Off-target effects of PEG engager-mediated therapy.** EGFR is expressed at low levels on normal cells of epithelial, mesenchymal and neuronal origin. We hypothesized that the density of EGFRs on cells might be an important factor for conditional internalization of PEGylated nanocarriers by PEG engager<sup>EGFR</sup> (Fig. 8a). Indeed, a linear correlation was observed between the logarithm of EGFR expression levels on cancer cell lines and the logarithm of the anti-proliferative activity (EC<sub>50</sub> values) of PEG
indicated: *P < 0.01, **P < 0.001 (two-way analysis of variance). NS, not significant.

Figure 6 | Pharmacokinetics and imaging of PEG engagers in mice. (a) NSG mice were intravenously injected with 6 mg kg\(^{-1}\) PEG engager\(^{\text{EGFR}}\) (white circles) or PEG engager\(^{\text{CD19}}\) (red squares). Mean plasma concentrations of the PEG engagers were measured by sandwich ELISA (n = 3 mice). (b) Five hours before intravenous administration of 4armPEG\(_{10k}\)-NIR-797 probe (5 mg kg\(^{-1}\)), NSG mice bearing subcutaneous MDA-MB-468 tumours were intravenously injected with 6 mg kg\(^{-1}\) PEG engager\(^{\text{EGFR}}\) or PEG engager\(^{\text{CD19}}\) and the whole-body imaging were sequentially imaged at 24, 48 and 72 h with an IVIS Spectrum imaging system. (c) The uptake of PEG-NIR797 in MDA-MB-468 tumours was determined by measuring fluorescence intensities (n = 3). Data are shown as mean ± s.d. Significant differences in mean fluorescent intensity between PEG engager\(^{\text{EGFR}}\) and PEG engager\(^{\text{CD19}}\) groups are indicated: *P < 0.01, **P < 0.001 (two-way analysis of variance). NS, not significant.

Discussion

We report bispecific PEG engagers that simultaneously bind to PEG on nanomedicines and EGFR on cancer cells for selective delivery of PEGylated stealth nanocarriers to EGFR\(^{+}\) TNBC cells. PEG engager\(^{\text{EGFR}}\) bound to EGFRs on TNBC cells but remained in a dormant state on the plasma membrane until contact with PEGylated nanocarriers triggered rapid endocytosis of both the engager and PEGylated nanocarrier. PEG engager\(^{\text{EGFR}}\) markedly increased the anticancer activity of PEG-liposomal doxorubicin (Doxisome) against EGFR\(^{+}\) TNBC in vitro and in vivo. The PEG engager tolerated the presence of pre-existing anti-PEG antibodies and effectiveness correlated to the EGFR expression levels on cells. This simple and flexible strategy appears promising for enhanced delivery of PEGylated medicines for improved therapy of TNBC patients.

TNBC is highly aggressive and metastatic with high rates of recurrence\(^{5}\). About 50% of TNBC tumours overexpress EGFR, which is correlated with poor prognosis in TNBC patients\(^{5}\). Overexpressed EGFR can induce cell proliferation, promote angiogenesis, increase cell migration and enhance chemoresistance\(^{30}\). Therefore, EGFR has been considered as a therapeutic target for the treatment of TNBC. However, several receptor tyrosine kinase inhibitors (RTKIs) including gefitinib and erlotinib did not benefit TNBC patients\(^{5,6}\). Compared to wild-type EGFR, EGFRs with activating mutations in non-small cell lung cancers display higher binding to RTKIs, leading to 100-fold greater sensitivity to RTKIs\(^{31}\). Thus, the poor efficacy of RTKIs in TNBC may be due to the low occurrence of activating EGFR mutations in TNBC (3–11%)\(^{32,33}\) as compared to non-small cell lung cancers (10–35%)\(^{34,35}\). In contrast to RTKIs that target activated intracellular EGFR kinase domains, antibodies that recognize the extracellular domain of the EGFR can target both wild-type and mutated EGFRs (Fig. 5 and Supplementary Fig. 4). Therapeutic monoclonal antibodies that recognize extracellular domains of EGFR, such as cetuximab, have been applied for TNBC therapy. However, the response rate of cetuximab alone or combined with carboplatin for TNBC treatment was not promising\(^{36}\). These EGFR-targeted antibodies and RTKIs focus on the blockage of EGFR signalling pathways, which involves complicated signalling networks. For instance, temsirolimus can inhibit the EGFR-PI3K-AKT-mTOR pathway resulting in cancer cell death. However, MDA-MB-231 TNBC cells, which possess normal phosphatase and tensin homologue function (a tumour suppressor gene that inactivates AKT) are resistant to temsirolimus, whereas MDA-MB-468 TNBC cells (with loss of phosphatase and tensin homologue function) were 8,000-fold more sensitive to this drug\(^{37}\). Alternatively, delivery of anticancer drugs to TNBC cells by targeting EGFRs may bypass the drawbacks of EGFR signalling inhibition. Thus, PEG engager\(^{\text{EGFR}}\) may be useful for selective delivery of PEGylated nanomedicines to EGFR overexpressing TNBC.

Active targeting of nanomedicines to tumours by functionalizing nanocarriers with targeting ligands is attractive to improve tumour targeting and intracellular delivery of nanocargos\(^{13,14}\). However, the complexity involved in production of targeted nanomedicines is time-consuming, technically challenging and expensive. The technical challenges include difficulty in controlling ligand conjugation stoichiometry, aggregation of
ligands on nanocarrier surfaces, intercarrier variations in ligand density, loss of ligand bioactivity, deattachment of targeting ligands from nanocarriers in vivo and poor scalability or reproducibility of the conjugation process\textsuperscript{17}. The resulting batch-to-batch variations can hinder clinical translation and commercialization of targeted nanocarriers. To help overcome these problems, Brinkmann and colleagues developed bispecific digoxigenin-binding antibodies for delivery of digoxigenin-modified nanocarriers to disease sites\textsuperscript{38,39}. Likewise, we previously described bispecific PEG-binding antibodies for delivery of PEGylated compounds\textsuperscript{40,41}. However, direct attachment of targeting ligands to nanocarriers can reduce the stealth feature of the nanocarriers, resulting in accelerated clearance and reduced uptake into tumours\textsuperscript{19}. In addition, attachment of targeting ligands can increase nanocarrier size, further impeding tumour uptake\textsuperscript{18}.

Pre-targeting strategies may help translate targeted nanomedicines to the clinic by decreasing nanocarrier complexity, maintaining the stealth properties of nanocarriers and facilitating personalized combinations of targeting ligands and nanomedicines. For example, monovalent ligands can be targeted to high density receptors on cancer cells for subsequent internalization of nanocarriers modified with biontogonal click-chemistry agents\textsuperscript{42,43}. However, both the targeting ligand and the nanocarrier require chemical modification and the kinetics of the biontogonal click-chemistry reactions may limit nanocarrier capture in vivo\textsuperscript{44}. By contrast, PEG engagers can be used with any nanomedicine that is coated with PEG, which is commonly used to decrease nanocarrier uptake in macrophages and increase nanocarrier circulation time. Thus, even ‘off-the-shelf’ nanomedicines, such as Doxil, can be targeted to EGFR\textsuperscript{+} TNBC tumours by this approach. Antibody–antigen interactions also rapidly occur in vivo, promoting effective capture and internalization of stealth nanocarriers.

Figure 7 | Therapeutic efficiency of PEG engager-directed Doxisome. (a) Groups of eight SCID mice bearing MDA-MB-468 tumours were intravenously injected with 6 mg kg\textsuperscript{-1} PEG engager\textsuperscript{CD19} (red squares), 6 mg kg\textsuperscript{-1} PEG engager\textsuperscript{EGFR} (white circles) or 18 mg kg\textsuperscript{-1} PEG engager\textsuperscript{EGFR} (white squares) 5 h before intravenous injection of 1 mg kg\textsuperscript{-1} Doxisome. Groups of eight mice were also intravenously injected with 6 mg kg\textsuperscript{-1} free doxorubicin (white triangles), 3 mg kg\textsuperscript{-1} Doxisome (black diamonds), PBS (black circles) or 18 mg kg\textsuperscript{-1} PEG engager\textsuperscript{EGFR} (white diamonds). Treatment was performed once a week for 4 weeks (arrows). Results show mean tumour sizes ± s.d. (b) Mean body weights of treated MDA-MB-468 mice (n = 8). (c) Groups of six SCID mice bearing MDA-MB-231 tumours were intravenously injected with 6 mg kg\textsuperscript{-1} PEG engager\textsuperscript{EGFR} (white circles) or 6 mg kg\textsuperscript{-1} PEG engager\textsuperscript{CD19} (red squares) 5 h before intravenous injection of 1 mg kg\textsuperscript{-1} Doxisome. Groups of six mice were also intravenously injected with PBS (black circles), PEG engager\textsuperscript{EGFR} alone (white diamonds) or 1 mg kg\textsuperscript{-1} Doxisome (black triangles). Treatment was performed once a week for 4 weeks. Results show mean tumour sizes at 43 days post first treatment. Statistical analysis of the differences in tumour volumes between treatment and control groups was performed by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons. *P ≤ 0.05, **P ≤ 0.005.
that pre-decoration of Doxisome with PEG engager-EGFR could enhance the half-life of the engagers and improve the therapeutic index of Doxisome treatment against EGFR+ human tumour xenografts, indicating that both pre-mixing and pre-targeting of PEG engagers are promising therapeutic approaches.

Stepwise accumulation of mutations can lead to distinct cancer cell subpopulations within the same tumour. For example, discordant HER2 expression has been observed in the same biopsy from cancer patients. This could be a major obstacle for conventional single-ligand targeted cancer therapy since subpopulations lacking expression of target receptors may repopulate the tumour after treatment. To overcome this problem, multi-ligand nanocarriers can be employed to improve drug delivery to heterogeneous tumours. A cocktail of PEG engagers with specificity to different tumour-associated antigens (for example, insulin-like growth factor 1 receptor, HER2, HER3, HER4 and c-Met) might offer a flexible yet effective treatment option for heterogeneous tumours.

Antibody-drug conjugates (ADC) are one of the most promising targeted drug delivery systems. ADCs are composed of monoclonal antibodies conjugated with cytotoxic drug molecules via a biodegradable linker to allow drug release after ADC internalization into cancer cells. Low drug-to-antibody ratios of two–four are required to prevent alteration of antibody properties and maintain efficient cancer therapy, thereby requiring attachment of highly cytotoxic drugs such as monomethyl auristatin E and maytansine. EGFR is also a potential target for developing ADCs as two EGFR-targeting ADCs are in clinical trials (ABT-414/Abbvie and IMGN289/ImmuGen). However, off-target uptake of ADCs in normal cells which express low levels of the target receptors can cause severe side effects. We determined that EGFR expression density is critical for efficient PEG engager-EGFR-mediated endocytosis of PEGylated liposomal doxorubicin, suggesting that PEG engagers may possess lower off-target toxicity.

This may expand the range of targetable tumour antigens as well as allow delivery of a broader range of therapeutic cancer drugs since each nanocarrier can encapsulate up to 10,000 drug molecules. On the other hand, PEG engagers might be less effective for treatment of cancers that express low levels of target antigens.

Covalent attachment of PEG to peptides, proteins, nucleic acids and nanoparticles can improve their pharmacokinetic properties and stability. Although PEG is thought to be non-immunogenic, several studies have detected pre-existing anti-PEG antibodies in normal donors and in normal sera of patients treated with PEGylated medicines. However, we found that human serum that contained high levels of anti-PEG antibodies did not interfere with PEG engager-mediated delivery of Doxisome into TNBC cells. This may be because PEG engagers display high affinity to PEG (KD = 7.55 nM) and are not easily competed by naturally occurring anti-PEG antibodies.

In conclusion, the humanized PEG engager generated in this study is anticipated to help overcome major bottlenecks in targeted nanomedicines by reducing manufacturing complexity and providing flexibility in choosing the appropriate disease targets by simply changing the targeting portion of these molecules. Flexible targeting coupled with the capability to directly deliver nanocarriers into target cells may expand the range of therapeutic agents available for therapy.

**Methods**

**Cell lines and animals.** Human 293FT cells (Thermo Fisher Scientific, San Jose, CA) were cultured in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% heat-inactivated foetal calf serum (HyClone, Logan, Utah), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 37°C in an atmosphere of 5% CO₂ in air. Human hepatocytes were purchased from BD Biosciences (San Jose, CA) EGFR-mutated PC9 (EGFR exon19del E746-A750) cell line was kindly provided by Dr Pan-Chyr Yang (President of the National Taiwan...
University, Taiwan). PC3, SKBR3, SK-MES-1, Hut125, Caski, HT29, H2170, LS174T, HepG2, SW480, MCF7 and TNBC cell lines (MDA-MB-231, MDA-MB-468 and BT-20 cells) were obtained from American Type Culture Collection (Manassas, VA) and were maintained in RPMI-1640 containing the same supplements. Knockdown of EGFR in BT20 cells is described in Supplementary Methods. Except for BT-20 cells, the cell lines used in this study are not authenticated by our laboratory. All cell lines were tested for mycoplasma and propagated for less than 6 months after resuscitation. Female NSG mice (non-obese diabetic. Cg-Prkdc<sup>scid</sup>Il2rg^<sup>tm1Wjl/Siz</sup>, 6–8 week old), NOD SCID mice (NOD.CB17-Prkdc<sup>scid</sup>NcrCrl, 6–8 week old) and BALB/c nude mice (BALB/cAnN.Cg-Foxn<sup>1</sup>nu/CgNarl) were obtained from the National Laboratory Animal Center, Taipei, Taiwan and were maintained under specific pathogen-free conditions. All animal experiments were performed in accordance with institutional guidelines and ethically approved by the Laboratory Animal Facility and Pathology Core Committee of IBM, Academia Sinica. Mice were randomly assigned to treatment groups but the investigators were not blinded to the treatments.

**DNA plasmid construction.** pAS3w.Ppuro, pMD.DG (VSV-G envelope plasmid) and pCMVR8.91 (packaging plasmid) vectors were obtained from the National RNAi Core Facility (Institute of Molecular Biology/Genomic Research Center, Academia Sinica, Taiwan)79. To generate the anti-PEG Fab-based bispecific PEG engager antibodies, 6.3 VH and 6.3 VL domains were assembled from the CDRs of the 6.3 antibody that was isolated from human peripheral blood mononuclear cells CD146<sup>+</sup> (http://www.ncbi.nlm.nih.gov/igblast/). The human germline VH IGHV7-4<sup>-</sup>1*02 and VL IGKV4-1*01 exons were selected based on the framework homology. The complementarity-determining regions of mouse 6.3 VH and 6.3 VL domains were then grafted onto human VH<sup>I</sup>IGHV7-4<sup>-</sup>1*02 and VL<sup>I</sup>IGKV4-1*01 genes using assembly PCR. Human immunoglobulin G1 (IgG<sub>1</sub>) C<sub>1</sub> and C<sub>2</sub> constant domains were cloned from human peripheral blood mononuclear cells CD146<sup>+</sup> (http://www.ncbi.nlm.nih.gov/igblast/). The humanized 6.3 VH–CH<sub>1</sub> and 6.3 VS<sub>1</sub>–CH<sub>3</sub> domains were assembled by overlap polymerase chain reaction from humanized 6.3 VH and 6.3 VS<sub>1</sub>–CH<sub>3</sub> humanized by first aligning the VH<sub>I</sub> and VL<sub>I</sub> sequences of the mouse 6.3 antibody and human immunoglobulin germline sequences using the IgBLAST program (http://www.ncbi.nlm.nih.gov/igblast/). The human germline VH<sup>I</sup>IGHV7-4<sup>-</sup>1*02 and VL<sup>I</sup>IGKV4-1*01 genes using assembly PCR. Human immunoglobulin G1 (IgG<sub>1</sub>) C<sub>1</sub> and C<sub>2</sub> constant domains were cloned from human peripheral blood mononuclear cells CD146<sup>+</sup> (http://www.ncbi.nlm.nih.gov/igblast/).

**Flow cytometer analysis.** Human hepatocytes, for further analytical characterization, the antibodies were dialysed into water for 5 h. Lentiviral particles were suspended in culture medium and centrifuged for 1.5 h (500 *g*). Lentiviral particles were collected and then 5 × 10<sup>5</sup> MDA-MB-468 (SW480, MDA-MB-231, MDA-MB-468 and BT-20 cells) were collected by ultracentrifugation (Beckman SW 41 Ti Ultracentrifuge Swing dish (90% confluency). After 48 h, lentiviral particles were collected and 10<sup>7.5</sup> cells were added to each well of 6-well plates (1 × 10<sup>6</sup> cells per well) 1 day before viral infection. Lentivirus containing medium was added to the cells and then centrifuged for 1.5 h (500g, 32°C). The cells were selected in puromycin (5 µg/ml) to generate stable cell lines. 5 × 10<sup>5</sup> 293FT/PEG engager<sup>CD19</sup> or 293FT/PEG engager<sup>EGFR</sup> cells in 15 ml DMEM culture medium were cultured in CELLline adhered 1000 bioreactors (INTEGRA Biosciences AG) and the medium was collected from every 7–10 days. Polyhistidine-tagged bispecific antibodies were purified on a Co<sup>2+</sup>-TALON column (GE Healthcare Life Sciences). Protein concentrations were determined by the bichinchoninic acid protein assay (Thermo Fisher Scientific).

**Characterization of PEG engager.** Three microgram of Eributis, purified PEG engager<sup>CD19</sup> or PEG engager<sup>EGFR</sup> were electrophoresed in a 10% SDS–PAGE gel under reducing or non-reducing conditions and then stained by Coomassie blue. For further analytical characterization, the antibodies were dialysed into water and analysed by matrix-assisted laser desorption/ionization/time-of-flight mass spectrometry (New ultraLXtreme, Bruker). Additional characterization of PEG engagers is described in Supplementary Methods. 10<sup>6</sup> of 10-µl fold excess of Alexa Fluor 647 succinimidyl esters (Thermo Fisher Scientific) (in dimethyl sulfoxide) for 2 h at room temperature to produce Alexa Fluor 647-conjugated PEG engager<sup>CD19</sup> or PEG engager<sup>EGFR</sup> respectively. One-tenth volume of 1 M glycine solution (pH 8.0) was added to stop the reaction. The PEG engagers were dialysed (molecular weight cutoff ~12,000–14,000 daltons) against PBS to remove free Alexa Fluor 647, sterile filtered and stored at −80°C.

**Microscale thermophoresis–binding analysis.** HEPS buffered saline/CHAPS buffer (10 mM HEPS, 150 mM NaCl, 3 mM EDTA, 0.05% CHAPS, pH = 7.4) was used for sample preparation. To determine the PEG-binding affinity of PEG engagers, 5 nM of Cy5-conjugated methoxy PEG<sub>2000</sub> (Naonco) was mixed at a 1:1 volume ratio with graded concentrations (0.24–500 nM) of PEG engager<sup>CD19</sup> or PEG engager<sup>EGFR</sup> antibodies. To analyse the tumour antigen-binding affinity of PEG engagers, 2 nM of Alexa Fluor 647-conjugated PEG engager<sup>CD19</sup> or PEG engager<sup>EGFR</sup> were mixed at a 1:1.2 volume ratio with graded concentrations (0.02–50 nM) of recombinant (E. coli) or E. coli expressed (His tagged) PEG engagers. The samples were incubated for 5 min at room temperature and loaded into standard capillaries and heated at 5% LED and 40% laser power for 30 s, cooled for 10 s and measured on a NanoTemper Monolith NT.115 instrument (NanoTemper Technologies GmbH). All experiments were performed with three replicates.

**Conflon microscopy.** Coverslips (30 mm) in cell cultivation PCOmin (perfusion, open and closed) chambers (PeCon GmbH) were coated with 10 µg/ml poly-l-lysine (Sigma-Aldrich) in PBS for 30 min at room temperature. The coverslips were washed twice with PBS and then 5 × 10<sup>5</sup> MDA-MB-468 (SW480, MDA-MB-231, MDA-MB-468 and BT-20 cells) were counted by staining the cells with 10 µg/ml of PEG engager<sup>CD19</sup> or PEG engager<sup>EGFR</sup> antibodies at 37°C for 30 min in medium containing 1 µg/ml of Hoechst 33342 (Thermo Fisher Scientific). Unbound PEG engagers were removed by washing with cold PBS twice and the surface fluorescence of 10<sup>4</sup> viable cells was measured by FACScalibert flow cytometer (Becton Dickinson) and analysed with Flowjo (Tree Star Inc.).

**Ethical statement.** The studies were approved by the Institutional Review Boards and Ethics Committees of Academia Sinica in Taiwan. Written informed consent was obtained from the subjects in accordance with institutional requirements and Declaration of Helsinki principles.

**Sample plasma collection.** Plasma samples of healthy subjects were enrolled from a prior project that had been collected, centrifuged and stored at the National Center for Genome Medicine, Academia Sinica. All subjects of this study agreed to the following centrifugal plasma samples for other research by treatment de-link. Details of anti-PEG antibody detection and their effects on PEG engagers is described in Supplementary Methods.

**PEGylated liposome preparation.** Distearoyl phosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG<sub>2000</sub>) and cholesterol (Avanti Polar Lipids, Inc.) were dissolved in distearoyl phosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000 (DSPE-PEG<sub>2000</sub>) and cholesterol (Avanti Polar Lipids, Inc.) were dissolved in DMSO (Sigma-Aldrich) and then rotary evaporation (Eppendorf RII) and rehydrated in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) at 65°C to a final lipid concentration of 20 ng/ml. The liposomal suspension was...
submitted to 10 freeze/thaw cycles in liquid nitrogen and a heated water bath at 80 °C, followed by 21 extractions at 75 °C through 400, 200 and 100 nm poly-carbonate membranes each using a mini-extruder (Avanti Polar Lipids, Inc.). The final lipid concentration was measured by Bartlett’s assay and adjusted to 13.9 μmol ml⁻¹ with TBS before use.

Cell proliferation assay. PC3, SKBR3, SK-MES-1, Hut125, Caski, HT29, H2170, LS174T, HepG2, SW480 MDA-MB-231, MDA-MB-468, BT-20 and P9C cells (10,000 cells per well) were seeded in 96-well plates overnight. Serial dilutions of free doxorubicin or doxorubicin-loaded liposomes were directly added to the cells as positive controls. Fifteen microgram per ml of PEG engagerCD19 or PEG engagerEGFR antibodies were added to the cells for 30 min at 37 °C followed by addition of graded concentrations of PEGylated liposomal doxorubicin (Doxiśmie, 13.9 μmol ml⁻¹ lipid concentration, Taiwan Liposome Company Ltd., Taipei, Taiwan) or liposomal vinorelbine (provided by Dr. Han-Chieh Hung Wu, Research Fellow, Institute of Cellular and Organismic Biology, Academia Sinica, Taiwan) or empty liposomes to the cells in triplicate at 37 °C for 4 h. The cells were subsequently washed once and incubated for an additional 72 h in fresh culture medium and then pulsed for 18 h with [3H]-thymidine (1 μCi per well). Results are expressed as per cent inhibition of [3H]-thymidine incorporation into cellular DNA in comparison to untreated cells.

In vivo pharmacokinetics. NSG mice were intravenously injected with 150 μg PEG engagerCD19 or PEG engagerEGFR and blood samples were periodically collected from the tail vein of the mice. Plasma was prepared by centrifugation (5 min, 12,000 g). The PEG engager levels in plasma were determined by quantitative sandwich ELISA. Maxisorp 96-well microplates were coated with 50 μl per well of anti-6,8-antibody conjugated to horseradish peroxidase (anti-6,8-antibody (6 μg ml⁻¹) in bicarbonate buffer, pH 7.4) for 2 h at 37 °C and then at 4 °C overnight. The plates were blocked with 200 μl per well 5% skim milk in PBS for 2 h at room temperature and then washed with PBS three times. Graded concentrations of PEG engagerCD19, PEG engagerEGFR or plasma samples in dilution buffer (2% skim milk in PBS) were added to the wells for 2 h at room temperature. After washing with PBS four times, the plates were stained with 50 μl per well horseradish peroxidase-conjugated anti-human IgG Fab antibody (Jackson ImmunoResearch Laboratories) (5 μg ml⁻¹). The plates were washed with PBS six times and 100 μl per well ABTS solution (0.4 mg ml⁻¹ 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H₂O₂, 100 mM phosphate citrate, pH 4.0) was added for 30 min at room temperature. The absorbance of the wells at 405 nm was measured on a microplate reader. The initial and terminal half-lives of the PEG engagers were estimated by fitting the data to a two-phase exponential decay model with Prism 5 software (Graphpad Software).

Synthesis of PEGylated near-infrared probes. 4-arm-PEG10k-NH₂ (Laysan Bio) dissolved in dimethyl sulfoxide at 2 mg ml⁻¹ was mixed with a sixfold molar excess of NIR-797 isothiocyanate (Santa Cruz Biotechnology) (in dimethyl sulfoxide) for 2 h at room temperature to produce 4-arm-PEG10k-NIR-797 probes. These compounds were diluted in a fivefold volume of ddH₂O and dialysed (molecular weight cutoff ~12,000–14,000 daltons) against ddH₂O to remove free doxorubicin or vinorelbine. Samples of free doxorubicin or vinorelbine were directly added to the cells as positive controls. Repeated at least two times with representative data shown. Animal sample size was eight (8) tumours on their right flank were intravenously injected with graded concentrations of PEG engagerCD19 or PEG engagerEGFR (6 mg kg⁻¹) in bicarbonate buffer, pH 8.0 for 4 h at 37 °C and then at 4 °C overnight. The plates were stained with 200 μl per well 5% skim milk in PBS for 2 h at room temperature and then washed with PBS three times. Graded concentrations of PEG engagerCD19, PEG engagerEGFR or plasma samples in dilution buffer (2% skim milk in PBS) were added to the wells for 2 h at room temperature. After washing with PBS four times, the plates were stained with 50 μl per well horseradish peroxidase-conjugated anti-human IgG Fab antibody (Jackson ImmunoResearch Laboratories) (5 μg ml⁻¹). The plates were washed with PBS six times and 100 μl per well ABTS solution (0.4 mg ml⁻¹ 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid), 0.003% H₂O₂, 100 mM phosphate citrate, pH 4.0) was added for 30 min at room temperature. The absorbance of the wells at 405 nm was measured on a microplate reader. The initial and terminal half-lives of the PEG engagers were estimated by fitting the data to a two-phase exponential decay model with Prism 5 software (Graphpad Software).

**In vivo imaging.** BALB/c nude or NOD SCID mice bearing 100 mm³ subcutaneous MDA-MB-468, A431 or HepG2 xenografts were intravenously injected with PEG engagerCD19 or PEG engagerEGFR (6 mg kg⁻¹), respectively. Five hours after PEG engager injection, the mice were intravenously administrated with 4-arm-PEG10k-NIR-797 probes (5 mg kg⁻¹). Pentobarbital anaesthetised mice were imaged with an IVIS Spectrum imaging system (excitation, 745 nm; emission, 840 nm; PerkinElmer) at 24, 48 and 72 h after injection.

**Pre-docked PEG engagers.** Characterization, in vivo blood half-life and in vivo therapy of pre-docked PEG engagers is described in Supplementary Methods.

**In vivo antitumour therapy.** Groups of NSG or NOD SCID mice bearing 4.47 ± 1.77 mm³ MDA-MB-468 (n = 10), 4.3 mm³ A431 (n = 11) or 4.3 mm³ subcutaneous MDA-MB-468 (n = 8) tumours on their right flank were intravenously injected with PBS or 6 or 18 mg kg⁻¹ PEG engagers. After 5 h, the mice were intravenously administrated with free doxorubicin (3 mg kg⁻¹) or Doxissome (1 or 3 mg kg⁻¹). Treatment was repeated once a week for a total of 4 weeks. Tumour sizes were measured every 7 days. Tumour volumes were calculated according the formula: length × width × height ÷ 2.05.

**Statistical analysis.** Results are presented as the mean ± s.d. All experiments were repeated at least two times with representative data shown. Animal sample size was chosen based on similar well-characterized literature. Statistical analyses were examined using the two-way analysis of variance. Differences in tumour volumes between groups were examined for statistical significance using one-way analysis of variance followed by Dunnett’s multiple comparisons; a probability value < 0.05 was considered statistically significant. No statistical method was used to predetermine sample sizes.
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