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An In Vitro Demonstration of Overcoming Drug Resistance in SKOV3 TR and MCF7 ADR with Targeted Delivery of Polymer Pro-Drug Conjugates

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Abstract

Drug resistance is a common phenomenon that occurs in cancer chemotherapy. Delivery of chemotherapeutic agents as polymer pro-drug conjugates (PPDCs) pretargeted with bispecific antibodies could circumvent drug resistance in cancer cells. To demonstrate this approach to overcome drug resistance, Paclitaxel (Ptxl) resistant SKOV3 TR human ovarian- and doxorubicin (Dox) resistant MCF7 ADR human mammary-carcinoma cell lines were used. Pre-targeting over-expressed biotin or HER2/neu receptors on cancer cells was conducted by biotinylated anti-DTPA or Anti-HER2/neu affibody - anti-DTPA Fab bispecific antibody complexes. The targeting PPDCs are either D-Dox-PGA or D-Ptxl-PGA. Cytotoxicity studies demonstrate that the pretargeted approach increases cytotoxicity of Ptxl or Dox in SKOV3 TR or MCF7 ADR resistant cell lines by 5.4 and 27 times respectively. Epifluorescent microscopy -used to track internalization of D-Dox-PGA and Dox in MCF7 ADR cells - shows that the pretargeted delivery of D-Dox-PGA resulted in a 2 to 4 fold increase in intracellular Dox concentration relative to treatment with free Dox. The mechanism of internalization of PPDCs is consistent with endocytosis. Enhanced drug delivery and intracellular retention following pretargeted delivery of PPDCs resulted in greater tumor cell toxicity in the current *in vitro* studies.

Keywords

Bispecific Antibodies; Targeted Polymer Pro-drug Conjugates; Drug Resistance; MCF7 ADR; SKOV3 TR

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Introduction

Anti-cancer chemotherapeutics are usually delivered by parenteral administration and, therefore, uptake in cancer is dependent on the blood concentration of the chemotherapeutic that encounters the cancer. Two common chemotherapeutics used in treating cancer are Doxorubicin (Dox) and Paclitaxel (Ptxl). Dox diffuses into cancer cells and intercalates DNA (Št'astný *et al* 1999), while Ptxl enters cancer cells and inhibits microtubule function (Horwitz 1994). However, a major limitation with this drug delivery approach is the indiscriminate toxicity affecting healthy cells, tissues, and organs during treatment to eradicate the tumors (Liang *et al* 2010, Plenderleith 1999, Haag and Kratz 2006). This could affect the optimal chemotherapeutic efficacy.

To reduce the non-targeted toxicity, polymer pro-drug conjugates as drug carriers, and bispecific antibodies as pre-targeting agents, have been developed in recent years. Polymer pro-drug conjugates (PPDCs) use biocompatible polymers as carriers of the chemotherapeutic drugs. Such drug conjugation reduces non-target toxicity and enhances the bioavailability of poorly soluble drugs, improves pharmacokinetics of the drug, increases the ability to provide passive or active targeting of the drugs to the sites of action, and is able to carry the payload while preserving the integrity of the drug during circulation and transportation (Duncan 2006, Larson and Ghandehari 2012). Furthermore, polymer drug conjugates improve the therapeutic profile of anti-cancer drugs by increasing the half-life of the anti-cancer drug (Yusuf *et al* 2003, Spanswick *et al* 2002, Xu and Mcleod 2001, Ringsdorf *et al* 1975, Khandare *et al* 2006). Passive targeting of polymer drug conjugates requires the presence of the enhanced permeability and retention (EPR) effect of the tumor vasculature (Greco *et al* 2009).

In order to use PPDCs in active targeting, bispecific antibodies (bsMABCx) may be used for pre-targeting the cancer cells. The pre-targeting approach involves initially targeting the cancer cells by the tumor marker specific arm of the bsMABCx and subsequent capture of the PPDC by the polymer capture arm of the pretargeted bispecific antibody (Cao and Suresh 1998). The specificity for tumors is provided by the targeting arm and the capture of the PPDC is provided by the capture arm (Wadhwa and Mumper 2015, Kontermann 2012). PPDCs, used in conjunction to pre-target with bsMABCx, are capable of enhancing drug delivery, increasing cancer specificity, and reducing off-target toxicity (Patil *et al* 2013, Khaw *et al* 2006).

Multidrug resistance, a mechanism where cancers develop resistance to chemotherapeutic drugs, is another major limitation of chemotherapy (Cao and Suresh 1998). Some cancers become multidrug resistant through the efflux of hydrophobic drugs that enter through diffusion, such as Dox. One mechanism of drug-resistance involves an energy dependent ATP-binding cassette (ABC) transporters. P-glycoprotein (Pgp) is one of these ABC transporters with a broad substrate specificity. Overexpression of this transporter is associated with multidrug resistance (Št'astný *et al* 1999). Certain cancer cell lines such as MCF7 ADR, a Dox resistant human mammary carcinoma cell line, have major vault proteins in the nuclear membrane associated with a similar efflux mechanism that causes Dox to be effluxes out of the nuclei (Hana *et al* 2012). Pre-targeting with bsMABCx and

targeting with PPDCs are promising approaches that may allow overcoming multidrug resistance in different cancers due to targeted delivery and internalization of the PPDCs that lead to subsequent release of the active drugs intracellularly away from the cell membrane associated efflux pumps (Boerman *et al* 2003).

In order to target both multidrug resistant and drug sensitive cancer cells, overexpression of receptors common to both are used. Tumor cells replicating rapidly require high concentrations of vitamins or ligands for growth receptors for cell growth. This requirement is met by overexpression of the vitamin- or growth-receptors. Vitamin receptor for Biotin is over-expressed in many different cancers, including the breast cancer cell line MCF7 (Boerman *et al* 2003, Russell-Jones *et al* 2004). Studies showed that biotin conjugated macromolecules are able to increase specific uptake of the anti-cancer drug-conjugates by these tumor cells (Chen *et al* 2010). Therefore, use of biotinylated anti-DTPA bsMAbCx to pre-target the PPDCs should result in the delivery of greater concentrations of the drug in the PPDC format (Chen *et al* 2015). The growth receptor HER2/neu is also frequently expressed in cancer cells (Khaw *et al* 2014). Affibody specifically for HER2/neu has been used as pre-targeting bispecific antibody complexes for visualization and treatment of HER2/neu receptor positive human mammary carcinoma in the xenografted murine model (Khaw *et al* 2014, Gada 2011), however, the mechanism of internalization, catabolism, and release of the free drug from the bispecific antibody-mediated delivery of the PPDCs have not been clearly elucidated. The current study shows that the entry of PPDCs such as DTPA-Dox-Polyglutamic acid (D-Dox-PGA) or DTPA-Paclitaxel-Polyglutamic acid (D-Ptxl-PGA), into cancer cells is by a mechanism involving the receptor-mediated endocytosis that is different from the intracellular entry of Dox or Ptxl (Thorn *et al* 2006). The red fluoresce emission of Dox on UV light activation is used to track the intracellular entry of Dox by epifluorescent microscopy. The entry of the bispecific antibody complex is tracked by using fluorescein labeled bsMAbCx. Furthermore, the release and delivery of free Dox to the nuclei are also monitored and quantitated.

Methods and Materials

Materials

Monoclonal antibody anti-DTPA (2C31E11C7) is produced in house (Khaw *et al* 2006). Poly-L-glutamic acid (Molecular weight 11,600 Da) (PGA), bicyclic anhydride of Diethylene triamine pentaacetic acid and N-hydroxysuccinimide ester of Bromoacetic acid were purchased from Sigma Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, Penicillin-Streptomycin and Melphalan are purchased from Thermo Fisher Scientific. K-Blue is purchased from Neogen Corporation, KY. Fluorescein Isothiocyanate is purchased from Beckton Dickerson, NJ. Dox hydrochloride and Ptxl are purchased from LC labs (Woburn, MA). MCF7 and SKOV3, cell lines were purchased from ATCC. SKOV3 TR was produced by Dr. Duan Zhenfeng (MGH, Boston MA) (Zhenfeng *et al* 1999). MCF7 ADR was produced by Batist *et al* (1986). Anti-HER2/neu affibody X anti-DTPA Fab bispecific complex was prepared as previously published (Gada *et al* 2012).

Preparation and Characterization of Biotinylated Anti-DTPA Bispecific Antibody

Biotinylation of Anti-DTPA Antibody—An aliquot of 0.4 mg/ml of Anti-DTPA is dialyzed in 0.1M PBS pH 7.4 overnight. 20 mM stock solution of NHS-PEG₄-Biotin is prepared and 20 molar excess is added to the antibody solution (Bayer *et al* 1986). Reaction mixture is incubated for 2 h (hour) on ice, and non-reacted NHS-PEG₄-Biotin is removed by dialysis using a 3,500 Dalton molecular weight cut off dialysis membrane (Spectra/Por Dialysis membrane, Spectrum Laboratories, Rancho Dominguez, CA), overnight in 0.1M PBS pH 7.4.

Characterization of Biotinylated anti-DTPA by ELISA—A 96 well microtiter plate (BD Falcon) is coated with 100 μ L aliquots of serial dilutions (1, 1, 0.1, 0.01, 0.001, 0.0001 μ g/ml) of Biotinylated Bovine Serum Albumin (BSA) in triplicates. Additional wells of the microtiter plate are coated with 100 μ L aliquots of serial dilutions (1, 1, 0.1, 0.01, 0.001 0.0001 μ g/ml) of Biotinylated anti-DTPA in triplicates. The plate is incubated for 2 h in a 37°C water bath, then it is washed 5X with 200 μ L of PBST followed by blocking, as published (Bayer *et al* 1986). Then, 100 μ L aliquots of Streptavidin-HRP (1:4000 or 1:8000 dilutions) are added to each well and the plate is incubated for 1 h at 37°C. After additional washing, 50 μ L aliquots of substrate K-Blue are added to each well. The plate is incubated and the optical density reading is obtained, as described above.

Synthesis and Characterization of PPDCs

Conjugation of DTPA to PGA—A solution of 50 mg/ml of PGA in 0.1 M sodium bicarbonate pH 8.6 is prepared. 3X D-PGA excess of the anhydride of DTPA relative to moles of PGA is dissolved in a minimum quantity of DMSO. The solution is added dropwise to the PGA solution, while mixing vigorously using a vortex mixer. The mixture is incubated at room temperature for 4 h, and then it was dialyzed overnight at 4°C in 4 liters of 0.1M phosphate buffered saline in a dialysis bag with a 3,500 Dalton molecular weight cut off. Modification of the N-terminal amino of PGA is determined by the 2, 4, 6-Trinitrobenzene sulfonic acid assay relative to the absorbance at 420 nm of un-modified PGA (Nitecki *et al* 1967).

Anti-DTPA ELISA for detection of D-PGA—The presence and quantitation of DTPA conjugated to PGA is determined by ELISA using anti-DTPA as the antibody and DTPA-conjugated BSA as the antigen. The ELISA is as described above (Gada *et al* 2012), except quadruplicate (n=4) samples are used.

Conjugation of Dox to DTPA-PGA—DTPA-PGA (D-PGA) and D-Dox-PGA were prepared as previously described by Gada *et al* 2012. Free unconjugated Dox is separated from the D-Dox-PGA conjugate by gel filtration chromatography using Sephadex G-25 columns (35 $\times$ 1 cm column). The concentration of Dox in D-Dox-PGA conjugate is determined using the Dox standard curve obtained at 490nm by the optical density reading (Gada *et al* 2012).

Conjugation of Ptxl to D-PGA—Conjugation of Ptxl to D-PGA is essentially according to Li *et al* (1998). Briefly, an aliquot of 32 mg of PGA is dissolved in 1.5 ml of dry N, N –

dimethylformamide and 11 mg of Ptxl, 15 mg of Dicyclohexylcarbodiimide, and 3 mg of dimethylaminopyridine are added to the solution. The reaction is allowed to proceed overnight at room temperature. Thin Layer Chromatography (silica plate developed in CHCl_3 : methanol (10:1)) is performed and visualized under UV illumination the next day to determine conjugation of Ptxl to the polymer (Li *et al* 1998). The reaction is then stopped by the addition of the reaction mixture into chloroform (5 mL). The resulting precipitate is recovered and dried using a Rotavapor R-210 (Buchi, New Castle, Delaware). The precipitate is then dissolved in 0.5 M sodium bicarbonate buffer (pH 9.6) and dialyzed overnight against 4 liters of 0.1 M sodium bicarbonate buffer (pH 9.6). To the Ptxl-PGA solution, a 20 X moles excess of the anhydride of DTPA dissolved in 200 μL DMSO is added dropwise. The reaction mixture is incubated for 2 h at room temperature and then dialyzed against 0.1 M PBS pH 7.4 overnight at 4°C. Anti-DTPA ELISA is performed to determine conjugation of DTPA to Ptxl-PGA. A standard curve of Ptxl obtained by the optical density reading at 227 nm was used to determine the concentration of Ptxl in the D-Ptxl-PGA conjugate.

Measurement of Zeta Potential of D-Dox-PGA

Zeta potential of the PPDCs is measured using the Zeta Plus (Brookhaven Instruments Corporation) equipped with a palladium electrode with the acrylic support. PPDCs (1mg/ml) were diluted 100 X using deionized water and transferred in disposable transfer cuvettes for measurement of zeta potential. The BIC zetapw32 software is used to determine the Zeta Potential measured at 25°C using the High Precision Mode (Khaw *et al* 2014).

Western Blot Analysis

SKOV3 sensitive, SKOV3 TR, MCF7 and MCF7 ADR cell lines are cultured in 25 mm Petri dishes for preparation of protein extracts. Cells were washed in ice cold PBS, and lysed in Ripa buffer (150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% DOC, 0.1% SDS) supplemented with complete miniprotease inhibitor cocktail tablets (complete Mini, Roche). Protein concentrations are determined using bicinchoninic acid (BCA) protein assay (Pierce Biotechnology) and samples containing equal amount of protein (50 μg) are used for immunoblotting, after addition of 5 $\times$ concentrated sample buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol [DTT], 0.012% bromophenol blue). Samples are then heated for 5 min at 95°C and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresed protein bands are transferred electrophoretically to polyvinylidene fluoride membrane (Amersham HybondTM-P, GE Healthcare). Prestained molecular weight protein markers (Precision Plus Protein Dual Color Standards, BioRad) were included in the SDS-PAGE gels. The membrane is blocked for 1 h at room temperature in PBS containing 0.1% Tween-20 (PBS-T) and 5% BSA. The membrane is incubated with the primary antibody overnight at 4°C (Anti-P-Glycoprotein Mouse monoclonal antibody, 517310 Calbiochem). After washing for 1 h in PBS-T with 1% BSA, the membrane is incubated for 1 h at room temperature with horseradish peroxidase-linked anti-mouse IgG+IgM secondary antibody (NIF1316 from Sigma, 1:10,000 in PBS-T with 1% BSA). Immunoblot is developed using the Enhanced Chemi-Fluorescence system (ECF; GE Healthcare) and a Storm device (Molecular Dynamics, GE Healthcare). The membrane is then re-probed and tested for β -actin immunoreactivity (Anti- β -Actin Mouse

monoclonal antibody, A5441 from Sigma, 1:5000) to confirm that equivalent concentrations of protein extracts were compared.

Cell Viability Assays

CellTiter Blue® (Promega, Madison, WI) is used for the assessment of cell viability according to the manufacturer's instructions. 5000 cells/well are seeded in the 96-well cell culture plates and incubated at 37°C for 24 h in a 5% CO₂ incubator. Cells are then treated with the various formulations (free drug, polymer drug conjugate alone and targeted polymer drug conjugates) in complete DMEM medium for 48 h. For the targeted treatment, SKOV3 cells were treated with anti-HER2/neu affibody X anti-DTPA Fab bispecific complex (10, 20 or 40 µg/ml) and MCF7 cells were treated with Biotinylated anti-DTPA bsMAbCx (10, 20, or 40 µg/ml) for 1 h. After pre-treatment, media is removed and the plate is washed 2X with 200 µL of complete medium. Then, Ptxl or Dox, D-Ptxl-PGA or D-Dox-PGA at 0.001 to 15 µg/ml is added to the medium. The drug treated cells are incubated at 37°C for 48 h. The cells are then washed and incubated with 50 µL of 1:5 dilution of CellTiter Blue® reagent for 2 h. Cell viability is evaluated by measuring the fluorescence (excitation 530 nm, emission 590 nm) using a Synergy HT multi-21 detection microplate reader (Biotek, Winooski, VT). Cells treated with complete media alone are used as a control to calculate 100% cell viability. The studies are performed in triplicates at 3 different occasions.

Inhibition of endocytosis of pretargeted D-Ptxl-PGA by chlorpromazine

Chlorpromazine, a known inhibitor of endocytosis (Wiranowska *et al* 2011), is used to demonstrate that endocytosis is the process of intracellular delivery of D-Ptxl-PGA associated with enhanced cytotoxicity in SKV3 TR cells. SKOV3-TR cells (5,000) are pre-incubated with 10 µg/mL of chlorpromazine for 30 min before incubation with bispecific antibody followed by incubation with D-Ptxl-PGA. Incubation of the cells and assessment of cell viability are as described above.

Visualization of intracellular internalization of D-Dox-PGA

Coverslips are placed in 24 well plates and sterilized with UV light for 30 min. MCF7 ADR cells are trypsinized and counted. Aliquots of 40,000 cells are added to each coverslip and allowed to attach overnight. For the pretargeted approach, media is removed from the plates and replaced with fresh media consisting of 10 µg/ml of biotinylated anti-DTPA and is incubated for 1 h at 37° C. After 1 h, the medium is removed and replaced with medium containing 20 µg/ml of DTPA-Dox-PGA, and incubated with D-Dox-PGA for various time-points (5, 10, 15, 30 min, 1, 2, 5, and 6 h). The coverslips for the D-DOX-PGA alone are incubated with 20 µg/ml D-Dox-PGA for the time-points as described above. The coverslips for the Dox group had the coverslips treated with 20 µg/ml of Dox as described before. One set of coverslips were incubated with the bsMAbCx and treated with 20 µg/ml of DTPA-Dox-PGA for 1 h. Then, the medium is removed and fresh medium is added for 4 h of incubation. This is repeated for the Dox and D-Dox-PGA alone. Three groups of coverslips are treated with 10 µg/mL Chlorpromazine for 30 min. The coverslips are then treated with either bsMAbCx and targeted treatment or treatment with Dox or D-Dox-PGA alone. Coverslips with cells alone with no treatment are used as a control. After incubation with the DTPA-Dox-PGA, at various time-points the medium is removed and washed once with

chilled PBS. Cells are then fixed with 4% paraformaldehyde for 15 min at room temperature. The coverslips are washed 2 times each with PBS-T and PBS for 5 min each. Cells are counterstained with 1 µg/ml of Hoechst to stain the nuclei. Coverslips are then washed as before. After the final PBS wash, coverslips are washed with deionized water. The coverslips are mounted on a clean microscope slides with the mounting medium Fluoromount-G. The coverslips are sealed and stored in -20 °C freezer until epifluorescent microscopic examination (Nikon Eclipse E400) was performed. The excitation wavelength of 528–553nm, DM 565nm and emission wave length of (BA) 600–660nm are set for epifluorescent microscopy. Ocular CFI 10X22M and the objective Plan 40X 0.40 PH1 DL were used. Digital micrographs were acquired at an exposure time of 800ms and Gain is set at 1. White Balance was set at 1.013, 1, 1.126. The image size is 1600 X 1200 pixels. Bit Depth is 24 bpp (RGB) Sensor Clear Mode: Continuous Chip Area: Full Chip. In order to visualize the internalization of Dox, red fluorescence is tracked through the MCF7 ADR cells. Images are taken using a Nikon Eclipse E400. Exposure of all images is enhanced by 5.20 times using Adobe Photoshop. The brightness of the Hoechst stain images is increased by 150 in all images. The superimposition images are created in Adobe Photoshop by layering the red fluorescence image and the Hoechst stain image.

Similarly, the bsMAbCx, biotinylated anti-DTPA was fluoresceinated to demonstrate internalization of the biotinylated anti-DTPA in the process of PPDC delivery. A 1 mL aliquot of 100 molar excess solution of fluorescein isothiocyanate (FITC) in DMSO is added dropwise to 2 mL of 400 µg/mL of Biotinylated anti-DTPA. The reaction mixture is incubated for 2 h at 37°C. Free unconjugated FITC is separated from the FITC labeled biotinylated anti-DTPA by Sephadex G-25 columns (10×1 cm column) centrifugation at 2000 rpm (Fisher Scientific™ Centrifuge Model 225 Benchtop Centrifuge, Fisher Scientific, Waltham) (Anderson *et al* 1982).

Data Analysis

Data is generated in triplicates and reported as mean ± Standard Deviation for cytotoxicity assays. Student's t-test is used to determine statistical significance. A P-value of less than 0.05 is considered statistically significant. Two-way ANOVA with post-hoc Bonferroni multiple comparison test is used to determine statistical difference between different treatment groups. NIH ImageJ software is used to measure the corrected total cell fluorescence (CTCF = Integrated Density – [Area of selected cell X Mean fluorescence. of background readings]).

Results

Characterization of Biotinylated Anti-DTPA Bispecific Antibody

The presence of biotin on anti-DTPA is determined by ELISA and compared to binding to unmodified anti-DTPA MAb. Figure 1A shows the presence of biotin on biotinylated anti-DTPA. Biotinylated BSA is prepared as described above for preparation of biotinylated anti-DTPA MAb. Utilizing the TNBS assay, biotinylated BSA is assessed to have 18 moles of biotin per mole of BSA. Figure 1B shows the comparison of binding of Biotinylated anti-DTPA to Strep Avidin relative to Biotinylated BSA. The binding curves were used to

estimate the number of moles of biotin per mole of MAb to be 2:1. The difference in the maximum absorbance in Figure 1A and B is due to the dilution of 1:4000 and 1:8000 of the secondary antibody, goat anti-murine antibody-HRP used. The 1:8000 dilution of the secondary antibody is used in Figure 1B because absorbance at 630 nm is above 1:00 for Biotinylated-BSA.

Characterization of PPDCs

Purification and Characterization of D-DOX-PGA complex—Sephadex G25 size exclusion column is used for the separation of the D-Dox-PGA complex from free Dox. Blue dextran exclusion was used to determine the void volume. The column is approximately 35×1 cm. Figure 2A shows the elution profile of the blue dextran (■) and D-Dox-PGA (◆). D-Dox-PGA eluted in the void volume due to its high molecular weight compared to free Dox. Fraction numbers 17–21 are pooled and are dialyzed against 4L 0.1M PBS (pH7.4) buffer overnight. A standard curve of Dox at O.D._{490 nm} is generated using serial dilutions of free Dox (Figure 2C). The concentration of Dox in D-Dox-PGA is then extrapolated using the formula of the standard curve ($y = 0.046x + 0.0074$, $R^2 = 0.997$). The concentration of Dox in D-Dox-PGA is calculated to be 438.98 µg/ml (approximately 12.3 moles Dox/mole PGA).

Purification and Characterization of D-Ptxl-PGA Conjugate—Thin Layer chromatography is performed with the overnight reaction mixture of PGA and Ptxl to confirm conjugation of Ptxl to PGA. Figure 2B shows that the R_f value of Ptxl is 0.70 as compared to that of Ptxl-PGA ($R_f \approx 0.09$) and PGA ($R_f \approx 0.0$). Ptxl-PGA complex is then precipitated and DTPA is conjugated to the complex to obtain D-Ptxl-PGA. A standard curve of Ptxl (Figure 2D) at O.D._{227nm} is then used to determine the concentration of Ptxl in D-Ptxl-PGA complex. The concentration of Ptxl in the conjugate is calculated using the formula of the standard curve ($y = 0.0241x - 0.0052$, $R^2 = 0.9982$) to be 475 Ptxl µg/ml. Thus, the D-Ptxl-PGA conjugate prepared contained 20 moles of Ptxl per mole of PGA.

ELISA for the incorporation of DTPA in different PPDCs—Indirect anti-DTPA ELISA is undertaken to determine the presence of DTPA on PGA. DTPA-BSA is used as a standard positive control. The control DTPA-BSA has at least 46 moles of DTPA per mole of BSA as determined by TNBS assay (Gada *et al* 2012). Aliquots of D-Dox-PGA, and D-Ptxl-PGA showed equivalent binding to anti-DTPA antibody (at 10 µg/ml) (Figure 2E). PGA has only one N-terminal amino group for modification, therefore, binding to anti-DTPA antibody is as expected for one mole of DTPA per mole of PGA.

Zeta Potential values of various PPDCs—Table 1 shows the negative zeta potentials of different PPDCs. Presence of the negative charge reduces non-specific interaction of these conjugates with the negatively charged cell membranes surface structures by ionic repulsion (Khaw *et al* 2014).

Western blot analysis

The Western blot shows that the Ptxl and Dox resistant SKOV3 TR and MCF7 ADR cell lines respectively expressed significantly greater concentrations of Pgp (~170 kDa) (Figure

3, Lanes B and C). SKOV3 and MCF7 drug sensitive cell lines show minimal Pgp expression (lanes A and D). The internal standard β -actin shows that equivalent concentrations of protein extract are loaded in each electrophoretic lane.

Cell Viability Assays using PPDCs

Figure 4A shows that Ptxl is highly effective in inducing cell death in SKOV3 sensitive cells. Cytotoxicity of Ptxl in SKOV3 sensitive ovarian cancer cells is similar to cell death of cells pretargeted and targeted with D-Ptxl-PGA. Even at high concentrations of 40 μ g/mL of bsMAbCx there was no significant difference in tumor toxicity between free Ptxl, and targeted D-Ptxl-PGA in Ptxl sensitive SKOV3 cells ($p = \text{NS}$).

Figure 4B shows the reversal of multidrug resistance in SKOV3 TR cells pretargeted for 1 h with 20 μ g/mL of bsMAbCx complex and targeted with serial dilutions of D-Ptxl-PGA relative to treatment with free Ptxl after 48 h ($p = 0.04$). Increasing the concentration of the targeting bispecific antibody complex (40 μ g/mL) resulted in higher cell cytotoxicity ($p < 0.01$).

Treatment of SKOV3 TR cell lines with 10 μ g/ml of chlorpromazine, an inhibitor of endocytosis (Wiranowska *et al* 2011) for 30 min before pre-incubation with bsMAbCx abrogated the cytotoxicity of the targeted D-Ptxl-PGA (Figure 4C).

Table 2, shows the IC_{50} values of Ptxl and targeted D-Ptxl-PGA in SKOV3 and SKOV3 TR cell lines. IC_{50} value of Ptxl for SKOV3 TR relative to SKOV3 sensitive cell line was approximately 10.5 times greater. However, the IC_{50} of SKOV3 TR cells pretargeted and targeted with D-Ptxl-PGA (0.172 μ g/ml) is 5.4 times less than that of free Ptxl (0.936 μ g/ml).

Pretargeting with bsMAbCx and targeting with D-Dox-PGA in Dox resistant MCF7 ADR and Dox sensitive MCF7 cells show similar results to those of SKOV3 cells treated with Ptxl. Dox is highly effective in inducing cell death in MCF7 sensitive cells (Figure 5A). There is no difference in cytotoxicity between free Dox and pretargeted D-Dox-PGA treatment at 20 μ g/ml bsMAbCx pretargeting dose in the sensitive cell line. However, in MCF7 ADR cells, the pretargeted approach induced significantly greater cell death than treatment with free Dox (Figure 5B & C) ($p < 0.001$). However, cytotoxicity in MCF7 ADR cells pretargeted with 40 or 60 μ g/ml bsMAbCx doses, is not enhanced indicative of saturation of the biotin receptors at 40 μ g/ml bsMAbCx concentration. MCF7 ADR cells pretargeted with 40 μ g/ml of bsMAbCx with a combination of D-Dox-PGA and D-Ptxl-PGA enhances cell death compared to single drug treatment (Figure 5C, $p < 0.05$). Table 3 shows the IC_{50} of Dox and pretargeted D-Dox-PGA in MCF7 sensitive (0.0088 and 0.0261 μ g/ml respectively) and resistant (11.9 and 0.442 μ g/ml [pretargeted with 40 μ g/ml bsMAbCx] respectively) breast cancer cells. IC_{50} of pretargeted followed by targeted D-Ptxl-PGA delivery is approximately 27 times less than that of free Dox.

Fluorescence Microscopy Studies

Figure 6 shows the increase in internalization of D-Dox-PGA in Dox resistant MCF-7 ADR cells after pre-targeting with biotinylated anti-DTPA antibody (20 μ g/ml) from 5 min to 6 h

of incubation with media containing D-Dox-PGA. Increase in intracellular fluorescence with increasing incubation time is observed. However, non-pretargeted D-Dox-PGA accumulation over the same period of incubation shows significantly less fluorescence indicative of minimal non-specific sequestration of D-Dox-PGA in Dox resistant MCF7 ADR cells (Figure 7). Dox treatment alone in MCF7 ADR resistant cells (Figure 8), leads to higher intracellular localization of Dox than in cells treated with non-pretargeted D-Dox-PGA. However, intracellular accumulation of Dox in these cells is less than that seen in MCF7 ADR cells pretargeted and targeted with D-Dox-PGA (Figure 6). Figure 9A&B represents the quantitation of fluorescent intensity reflective of Dox concentration in MCF7 ADR cells and the nuclei of these cells. A major difference in Dox concentration is seen in the total cell assessment (Figure 9A, and C) and in the nuclei (Figure 9B and D). Greater differential uptake is seen in cells incubated for 1 h in either pretargeted D-Dox-PGA or free Dox followed by incubation in drug free media for 4 h (ratio 3.9:1) relative to Dox concentrations determined immediately after 1h of incubation (1.7:1) (Figure 9C). However, this difference is less dramatic in the nuclei (1.4:1 and 1.9:1 respectively) (Figure 9D). Approximately 4 times higher Dox concentration with pretargeted D-Dox-PGA after washing indicates that more Dox in the form of D-Dox-PGA or released as free Dox, is retained in whole cells.

To show that the biotinylated anti-DTPA bsMAbCx and the D-Dox-PGA are both internalized intracellularly, but only Dox released from the digestion of D-Dox-PGA is sequestered in the nuclei, cells pre-treated with biotinylated-Anti-DTPA-FITC and treated with D-Dox-PGA are viewed for the green and red fluorescence by epifluorescent microscopy and compared to the corresponding nuclear stain. Figure 10 shows that the green fluorescence remained in the cytoplasm (Figure 10A, b, c and f), whereas red fluorescence of Dox was seen to sequester primarily in the nuclei (Figure 10A, b, d and e). Figure 10A, g represents superimposition of Figure 10 A c and d and figure 10A, h represents superimposition of Figure 10A, b, c and d. No specific fluorescence is seen in the untreated control cells (Figure 10B).

When MCF7 ADR cells are pretargeted with 40 µg/ml bsMAbCx and targeted with 20 µg/ml of Dox equivalent in D-Dox-PGA, followed by washing and incubation for 4 h in drug free media, there was significantly greater retention of Dox fluorescence (Figure 11A) than in cells treated with free Dox under the same wash and incubation conditions (Figure 11B). Loss of the intracellular Dox fluorescence intensity is observed. Similar studies in MCF7 ADR cells with pretargeted approach led to higher intracellular retention of Dox by CTCF analyses (Figure 9A and C, P values <0.01). Non-pretargeted D-Dox-PGA showed no cellular activity in the MCF7 ADR cells treated under the same washing and incubation conditions (Figure 11C).

When the MCF7 ADR cells are pre-incubated with Chlorpromazine, an inhibitor of endocytosis, no D-Dox-PGA is internalized in the cells pretargeted with Biotinylated-Anti-DTPA bsMAbCx (Figure 12A), similar to that of MCF ADR cells treated with D-Dox-PGA without pre-targeting with bsMAbCx (Figure 12B) or that of the untreated control (Figure 12D). However, internalization of free Dox was not affected by pre-incubation with Chlorpromazine (Figure 12C).

Discussion

Pretargeting strategies were initially developed for radio-immuno-imaging and radio-immuno-therapy. Pretargeting with bispecific antibodies and targeting with mono- and divalent haptens enabled achievement of high target to background activity as well as very low non-target sequestration (Boerman *et al* 2003). Bombesin bispecific antibody and anti-HER2-affibody-anti-DTPA Fab (BAAC) bispecific antibody complexes were used to demonstrate targeted delivery of PPDCs and radiolabeled polymers for cancer therapy and *in vivo* diagnostic imaging (Patil *et al* 2013, Khaw *et al* 2014). This pre-targeting approach has now been extended to overcome multidrug resistance in cancer. Biotin of the biotinylated anti-DTPA bsMAbCx serves as the targeting arm for selectively binding cancer cells via the biotin receptors. The anti-DTPA monoclonal antibody arm of the bsMAbCx serves as the capture arm for localization of the PPDCs.

One of the mechanisms responsible for multidrug resistance in cancer is over expression of membrane transporter P-glycoprotein (Pgp). Pgp are ATP dependent proteins that are responsible for the continuous efflux of drugs from the cells. Therefore, maintaining high intracellular level of the therapeutic drugs in Pgp over expressing cancer cells becomes a major challenge. To enable maintenance of effective intracellular concentration of drugs in drug resistant cancer cells, various approaches have been developed. These approaches include use of: 1) MDR transporter inhibitors (Didziapetris *et al* 2003, Kathawala *et al* 2015), 2) microRNA and RNA interference for inactivation of MDR associated genes (Li *et al* 2012), and 3) nanoparticles such as dendrimers, quantum dots, polymers, liposomes and micelles for loading anticancer drugs (Shapira *et al* 2012, Cho *et al* 2008, Kim *et al* 2009). Nanoparticles have also been used for combination therapy by encapsulation of anticancer drugs together with MDR inhibitors and RNAi molecules to overcome drug resistance (Kang *et al* 2015, Zhang *et al* 2011). Western blot analysis in Figure 3 confirms overexpression of Pgp receptors in SKOV3 TR and MCF7 ADR cell lines. Therefore our data is consistent with the concept that enhanced cytotoxicity in drug resistant SKOV3 TR and MCF7 ADR cells after pre-targeting with bispecific antibody complexes and targeting with PPDCs may be due to the potential to by-pass or reduce the efflux of the therapeutic drugs by the Pgp.

Doxorubicin and Paclitaxel are two frontline chemotherapeutic agents for cancer therapy. Their use is limited due to the toxicity to normal cells and poor solubility respectively. PGA is chosen as the polymeric drug carrier because of its biocompatibility and presence of large number of carboxylic residues that can be modified for conjugation with either Dox or Ptxl. Conjugation of these drugs to polymers increases the solubility of hydrophobic drugs as well as provides a high specific activity pro-drug delivery mechanism with increased bioavailability. These 2 PPDCs of PGA i.e. DTPA-Dox-PGA and DTPA-Ptxl-PGA, have negative zeta potentials. Khaw *et al* (2006) have shown that use of negatively charged PPDCs reduced non-specific ionic interaction between the negatively charged cell surfaces and the PPDCs.

Non-targeted PPDCs had no cytotoxic effect on either sensitive or resistant ovarian cancer or MCF7 human mammary cancer cell lines. This is probably due to the pro-drug state of Ptxl

and Dox after conjugation to PGA. Studies by Patil *et al* 2013, showed a decrease in cytotoxicity of D-Dox-PGA compared to free Dox in normal cardiomyocytes. In tumor cells, toxicity was enhanced after pre-targeting with bispecific antibody complexes. In both the SKOV3 and MCF7 drug sensitive cell lines, the pre-targeting approach followed by targeting with either D-Ptxl-PGA or D-Dox-PGA showed no significant increase in tumor cytotoxicity relative to treatment with the free drugs. In drug resistant cell lines however, higher tumor toxicity was observed with pretargeted therapy relative to free drugs alone. The tumor toxicity of targeted PPDCs depended on the concentration of the pre-targeting bsMAbCx used. Higher concentration of the pre-targeting bsMAbCx led to higher tumor cytotoxicity (Figure 5B) indicating that all receptors on the tumor cells have not been saturated with lower doses of the bsMAbCx. These receptors were subsequently saturated when 40 and 60 µg/ml of biotinylated anti-DTPA complex were used (Figure 5C).

The pretargeted approach (Figure 6) shows a prolonged ability to deliver the PPDCs over 6 h. At 5 min there was initiation of binding of D-Dox-PGA to the extracellular surface of the cells followed by increase in the intensity of intracellular fluorescence with time. Quantitatively, the whole cell intensity representing localization of D-Dox-PGA increases as time of incubation increases and reached corrected total cell fluorescence (CTCF) values of approximately one million by 6 h incubation relative to approximately 500,000 CTCF of cells incubated for the same period of time with Dox (Figure 9A).

The CTCF values following Dox treatment for 1 h was only about $500,700 \pm 105,700$ as compared to that of the pretargeted D-Dox-PGA in MCF7 ADR cells ($822,300 \pm 246,600$ CTCF) indicating that more Dox in the form of D-DOX-PGA or newly released Dox, is sequestered and retained by the MFC7 ADR cells. Therefore, delivery of PPDCs after pre-targeting resulted in approximately two times the drug concentration obtained with Dox alone (Figure 9C, $P < 0.001$). In the drug retention studies with MCF7 ADR cells, where the medium is replaced with Dox free medium after 1h incubation with either Dox or pretargeted D-Dox-PGA and further incubated for 4 h, nearly 80% of Dox is retained in the pretargeted MCF7 ADR cells relative to approximately 35% for Dox (Figure 11 and 9C). These results combined with Dox uptake studies in Figures 6, 8 and 9A, are consistent with our hypothesis that pretargeted delivery of D-Dox-PGA leads to higher uptake and retention of Dox in MCF ADR resistant cell lines. Even though the pretargeted approach does not directly inhibit the activity of the Pgp, it helps the PPDCs to bypass the efflux action of Pgp receptors (Št'astný *et al* 1999, Hana *et al* 2012).

Nuclei of the cells are targets of Dox. Our study showed that very high Dox fluorescence intensity was associated with the nuclei in the pretargeted cells relative to treatment of the same cancer cells with free Dox (Table 9 B, D). Figures 6 and 8 substantiate the superiority of the pretargeted approach to achieve higher nuclear localization of Dox in MCF7 ADR resistant cells. In addition, the use of FITC labeled biotinylated anti-DTPA permitted tracking of the pre-targeting bsMAbCx in relations to localization of the PPDCs. The green fluorescence of the FITC labeled bsMAbCx is internalized into the cytoplasm but is not observed to sequester to the nuclei of the treated cells (Figure 10Ac and e), whereas the red fluorescence of Dox is initially internalized along with the FITC-bsMAbCx and subsequently, is localized in the nuclei, consistent with the concept that following

internalization of D-Dox-PGA, Dox is released which then is sequestered to the nuclei (Figure 10Ad).

Furthermore, the mechanism of internalization of the pretargeted PPDCs is consistent with endocytosis. Inhibition of endocytosis with pretreatment of cancer cells with chlorpromazine (Figure 12) resulted in the inhibition of uptake of D-Dox-PGA after pre-targeting with bsMAbCx. This observation further support the proposal that uptake of PPDCs after pretargeting the biomarker receptors is via endocytosis and that the cell cytotoxicity is abrogated after preincubation of the cancer cells with chlorpromazine (Figure 4C, 12A). No inhibition of cytotoxicity is observed in the MCF7 ADR cells pretreated with chlorpromazine and treated with Dox, suggesting that the mechanisms of internalization of Dox and pretargeted D-Dox-PGA are not the same. Internalization of antibodies by the targeted cells has been reported to be via clathrin-mediated endocytosis (Št'astný *et al* 1999). Our results are similar to those of Minko *et al* 1998, who reported that HPMA-copolymer Adriamycin conjugates entered the cancer cells via endocytosis circumventing the Pgp efflux pumps relative to free drug entry by diffusion and efflux of the free drug.

Conclusion

The entry of PPDCs such as D-Dox-PGA in the pretargeted approach is by the endocytic route, resulting in the release of free drug intracellularly following endolysosomal degradation of the biodegradable polymers, such as Polyglutamic acid as well as the bsMAbCx. Cellular entry of Dox is by diffusion and is susceptible to the efflux pumps in drug resistant cancer cells. The pretargeted approach enhances the specificity of chemotherapy by targeting overexpressed cancer associated biomarkers and reduces the no-target toxicity associated with the conventional chemotherapeutics. The pretargeted approach also increases retention of the therapeutic drugs in drug-resistant cells, increases specificity, and prolongs delivery of the PPDCs as compared to treatment with free drugs. Additional in vivo investigation will be needed to substantiate the potential of the pretargeted delivery approach to overcome multi-drug resistance.

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References

1. Andersen K, Vaughn M. Gel centrifugation chromatography for macromolecular separations. *Journal of Chromatography*. 1982; 240(1):1–8.
2. Batist G, Tulpule A, Sinha BK, et al. Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J Biol Chem*. 1986 Nov 25; 261(33):15544–9. [PubMed: 3782078]
3. Bayer EA, Ben-Hur H, Wilchek M. A sensitive enzyme assay for biotin, avidin, and streptavidin. *Anal Biochem*. 1986 Apr; 154(1):367–70. [PubMed: 3706735]

4. Boerman OC, van Schaijk FG, Oyen WJ, Corstens FH. Pretargeted radioimmunotherapy of cancer: progress step by step. *J Nucl Med.* 2003; 44:400–411. [PubMed: 12621007]
5. Cao Y, Suresh MR. Bispecific Antibodies as Novel Bioconjugates. *Bioconjug Chem.* 1998; 9:635–644. [PubMed: 9815155]
6. Chen S, Zhao X, Chen J, et al. Mechanism-Based Tumor-Targeting Drug Delivery System. Validation of Efficient Vitamin Receptor-Mediated Endocytosis and Drug Release. *Bioconjug Chem.* 2010; 21:979–98. [PubMed: 20429547]
7. Chen Y, Li N, Yang Y, Liu Y. A dual targeting cyclodextrin/gold nanoparticle conjugate as a scaffold for solubilization and delivery of Ptxl. *RSC Adv.* 2015; 5:8938–8941.
8. Cho K, Wang X, Nie S, Chen ZG, Shin DM. Therapeutic nanoparticles for drug delivery in cancer. *Clin Cancer Res.* 2008; 14:1310–1316. [PubMed: 18316549]
9. Didziapetris R, Japertas P, Avdeef A, Petrauskas A. Classification analysis of P-glycoprotein substrate specificity. *J Drug Target.* 2003; 11:391–406. [PubMed: 15203928]
10. Duncan R. Polymer conjugates as anticancer nanomedicines. *Nature reviews Cancer.* 2006; 6(9): 688–701. [PubMed: 16900224]
11. Gada, K. dissertation. Northeastern University; 2011. Targeted Delivery of Radioisotope or chemotherapeutic Loaded Pro-drug-Polymers after Pre-targeting Tumors with Bispecific Antibody Complex.
12. Gada K, Patil V, Panwar R, et al. Companion targeted diagnosis and therapy: Bispecific HER2 affibody-anti-DTPA-Fab for target-specific delivery of Tc-99m-labeled-polymers or DTPA-Dox-conjugated-polymers. *J Nucl Med.* 2012; 53:115.
13. Greco F, Vincent MJ. Combination Therapy: Opportunities and challenges for polymer-drug conjugates as anticancer nanomedicine. *Advanced drug delivery reviews.* 2009; 61(13):1203–13. [PubMed: 19699247]
14. Haag R, Kratz F. Polymer therapeutics: concepts and applications. *Angewandte Chemie.* 2006; 45(8):1198–215. [PubMed: 16444775]
15. Hana M, Lva Q, Tanga X, et al. Overcoming drug resistance of MCF-7/ADR cells by altering intracellular distribution of Dox via MVP knockdown with a novel siRNA polyamidoamine-hyaluronic acid complex. *Journal of controlled release.* 2012; 163(2):136–144. [PubMed: 22940126]
16. Horwitz SB. Taxol (Ptxl): mechanisms of action. *Ann Oncol.* 1994; 5(Suppl 6):S3–6.
17. Kang L, Gao Z, Huang W, Jin M, Wang Q. Nanocarrier-mediated co-delivery of chemotherapeutic drugs and gene agents for cancer treatment. *Acta Pharm Sinica B.* 2015; 5:169–175.
18. Kathawala RJ, Gupta P, Ashby CR Jr, Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Update.* 2015; 18:1–17.
19. Khandare J, Minko T. Polymer–drug conjugates: Progress in polymeric pro-drugs. *Prog Polym Sci.* 2006; 31:359–397.
20. Khaw B, Gada K, Patil V, et al. Bispecific antibody complex pre-targeting and targeted delivery of polymer drug conjugates for imaging and therapy in dual human mammary cancer xenografts: targeted polymer drug conjugates for cancer diagnosis and therapy. *Eur J Nucl Med Mol Imaging.* 2014 Aug; 41(8):1603–16. [PubMed: 24643779]
21. Khaw B, Tekabe Y, Johnson LL. Imaging experimental atherosclerotic lesions in ApoE knockout mice: enhanced targeting with Z2D3-anti-DTPA bispecific antibody and 99mTc-labeled negatively charged polymers. *J Nucl Med.* 2006; 47:868–876. [PubMed: 16644758]
22. Kim CK, Ghosh P, Pagliuca C, Zhu ZJ, Menichetti S, Rotello VM. Entrapment of hydrophobic drugs in nanoparticle monolayers with efficient release into cancer cells. *J Am Chem Soc.* 2009; 131:1360–1361. [PubMed: 19133720]
23. Kontermann R. Dual targeting strategies with bispecific antibodies. *mAbs.* 2012; 4:182–197. [PubMed: 22453100]
24. Larson N, Ghandehari H. Polymeric conjugates for drug delivery. *Chem Mater.* 2012 Mar; 24(5): 840–853. [PubMed: 22707853]
25. Li C, Yu D, Newman RA, et al. Complete regression of well-established tumors using a novel water-soluble poly (L-glutamic acid)-paclitaxel conjugate. *Cancer Res.* 1998; 58:2404–2409. [PubMed: 9622081]

26. Li X, Gao L, Cui Q, Gary BD, Dyess DL, Taylor WL, Shevde-Samant R, et al. Sulindac inhibits tumor cell invasion by suppressing NF- κ B mediated transcription of microRNAs. *Oncogene*. 2012; 31:4979–4986. [PubMed: 22286762]
27. Liang XJ, Chen C, Zhao Y, Wang PC. Circumventing tumor resistance to chemotherapy by nanotechnology. *Methods in molecular biology*. 2010; 596:467–88. [PubMed: 19949937]
28. Minko T, Kopecková P, Pozharov V, Kopecek J. HPMA copolymer bound adriamycin overcomes MDR1 gene encoded resistance in a human ovarian carcinoma cell line. *J Control Release*. 1998; 54:223–233. [PubMed: 9724909]
29. Nitecki D, Stoltenberg I, Goodman J. Qualitative and quantitative determination of mixtures of amino acids using 2, 4, ,6-trinitrobenzenesulfonic acid. *Anal Biochem*. 1967; 19(2):344–350. [PubMed: 6048702]
30. Patil V, Gada K, Panwar R, et al. In vitro demonstration of enhanced prostate cancer toxicity: pretargeting with Bombesin bispecific complexes and targeting with polymer-drug-conjugates. *Journal of Drug Delivery*. 2013; 21(10):1012–1021.
31. Plenderleith I. Treating the Treatment: Toxicity of Cancer Chemotherapy. *Can Fam Physician*. 1999 Oct;36:1827–1830.
32. Ringsdorf H. Structure and properties of pharmacologically active polymers. *J Polym Sci Polym Symp*. 1975; 51:135–153.
33. Russell-Jones G, McTavish K, et al. Vitamin-mediated targeting as a potential mechanism to increase drug uptake by tumors. *J Inorg Biochem*. 2004 Oct; 98(10):1625–33. [PubMed: 15458825]
34. Shapira A, Davidson I, Avni N, Assaraf YG, Livney YD. β -Casein nanoparticle-based oral drug delivery system for potential treatment of gastric carcinoma: stability, target-activated release and cytotoxicity. *Eur J Pharm Biopharm*. 2012; 80:298–305. [PubMed: 22085654]
35. Spanswick VJ, Craddock C, Sekhar M, et al. Repair of DNA interstrand crosslinks as a mechanism of clinical resistance to melphalan in multiple myeloma. *Blood*. 2002; 100:224–229. [PubMed: 12070031]
36. Št'astný M, Strohalm J, Plocova D, et al. A possibility to overcome P-glycoprotein (PGP)-mediated multidrug resistance by antibody-targeted drugs conjugated to N-(2-hydroxypropyl) methacrylamide (HPMA) copolymer carrier. *Eur J Cancer*. 1999; 35:459–466. [PubMed: 10448300]
37. Thorn C, Oshiro C, Marsh S, et al. Dox pathways: pharmacodynamics and adverse effects. *Pharmacogenet Genomics*. 2012; 21(7):440–446.
38. Wadhwa S, Mumper R. Polymer-Drug Conjugates for Anticancer Drug Delivery. *Crit Rev Ther Drug Carrier Syst*. 2015; 32(3):215–45. [PubMed: 26080809]
39. Wiranowska M, Colina LO, Johnson JO. Clathrin-mediated entry and cellular localization of chlorotoxin in human glioma. *Cancer Cell Int*. 2011; 11:27. [PubMed: 21838899]
40. Xu G, McLeod HL. Strategies for enzyme/pro-drug cancer therapy. *Clin Cancer Res*. 2001; 7:3314–3324. [PubMed: 11705842]
41. Yusuf RZ, Duan Z, Lamendola DE, et al. Ptxl resistance: molecular mechanisms and pharmacologic manipulation. *Curr Cancer Drug Targets*. 2003; 3:1–19. [PubMed: 12570657]
42. Zhang M, Akbulut M. Adsorption, desorption, and removal of polymeric nanomedicine on and from cellulose surfaces: effect of size. *Langmuir*. 2011; 27:12550–12559. [PubMed: 21879763]
43. Zhenfeng D, Fellar A, Penson R, et al. Discovery of Differentially Expressed Genes Associated with Paclitaxel Resistance Using cDNA Array Technology: Analysis of Interleukin (IL) 6, IL-8, and Monocyte Chemotactic Protein 1 in the Paclitaxel-resistant Phenotype. *Clin Cancer Res*. 1999 Nov;5:3445. [PubMed: 10589757]

A

B

Figure 1.

A) Determination for the presence of Biotin on Biotinylated anti-DTPA by ELISA ($\blacktriangle$ = Biotinylated anti-DTPA, and $\blacksquare$ = unmodified anti-DTPA) 1:4000 dilution of Streptavidin-HRP was used **B)** Quantitation of Biotin on biotinylated anti-DTPA by comparison to Biotinylated BSA standard ($\blacklozenge$ = Biotinylated BSA, and $\blacktriangle$ = biotinylated anti-DTPA) 1:8000 dilution of Streptavidin-HRP was used.

E **Figure 2.**

A) Sephadex G25 column (35 × 1 cm) chromatography elution profile of Blue Dextran (squares) and D-Dox-PGA (diamonds). **B)** Thin Layer Chromatography of Ptxl conjugated to PGA relative to free Ptxl and PGA. **C)** Standard curve for determination of the concentration of Dox in D-Dox-PGA. **D)** Standard Curve for determination of the concentration of Ptxl in D-Ptxl-PGA. PGA showed no significant absorbance at 227 nm. **E)** Determination of the presence of DTPA on PGA by ELISA using DTPA-BSA as standard (◆ = DTPA-BSA, ● = D-Dox-PGA, and ▲ = D-Ptxl-PGA).

Figure 3. Western blot showing expression of Pgp protein in: **A)** SKOV3 sensitive, **B)** SKOV3 TR (resistant), **C)** MCF7 ADR (resistant) and **D)** MCF7 sensitive cell lines. β -actin bands showed that equal concentrations of cellular protein extracts were added to each lane.

Figure 4.

A) Cytotoxicity of targeted D-Ptxl-PGA after pre-targeting with 20 or 40 µg/ml of bispecific complex compared to free Ptxl in SKOV3 (sensitive) cells. ● Ptxl ($y = 0.9203\ln(x)$)

+ 91.669, $R^2 = 0.3842$), ■ 20µg/ml of bsMAbCx + Targeted D-Ptxl-PGA ($y = -11.77\ln(x) + 29.965$, $R^2 = 0.98305$), ▲ 40µg/ml of bsMAbCx + Targeted D-Ptxl-PGA ($y = -11.04\ln(x) + 24.667$, $R^2 = 0.97501$), □ Non-targeted D-Ptxl-PGA ($y = -11.08\ln(x) + 20.254$, $R^2 = 0.97822$)

B) Cytotoxicity of targeted D-Ptxl-PGA after pre-targeting with 20 or 40 µg/ml of bispecific complex and targeting with D-Ptxl-PGA relative to free Ptxl treatment in SKOV3 TR (Resistant) cells. ● Ptxl ($y = -12.53\ln(x) + 49.178$, $R^2 = 0.95531$), ■ 20µg/ml of bsMAbCx + Targeted D-Ptxl-PGA ($y = -12.73\ln(x) + 38.605$, $R^2 = 0.95554$), ▲ 40µg/ml of bsMAbCx + Targeted D-Ptxl-PGA ($y = -10.11\ln(x) + 29.166$, $R^2 = 0.97261$), □ Non-targeted D-Ptxl-PGA ($y = -1.55\ln(x) + 93.253$, $R^2 = 0.92284$). * $P < 0.05$ Ptxl vs 20µg/ml of bsMAbCx + Targeted D-Ptxl-PGA, ** $P < 0.01$ Ptxl vs 40µg/ml of bsMAbCx + Targeted D-Ptxl-PGA

C) Cytotoxicity of targeted D-Ptxl-PGA after pretreatment with 10 mg/ml of Chlorpromazine then pre-targeting with 40 µg/ml of bispecific complex and targeting with D-Ptxl-PGA and free Ptxl in SKOV3 TR (Resistant) cell line after 48 h. ● Ptxl ($y = -12.53\ln(x) + 49.178$, $R^2 = 0.95531$), 10µg/ml of Chlorpromazine + Targeted D-Ptxl-PGA ($y = -0.793\ln(x) + 87.967$, $R^2 = 0.25383$), □ Non-targeted D-Ptxl-PGA ($y = 0.9203\ln(x) + 91.669$, $R^2 = 0.38415$)

Figure 5.

A) Cytotoxicity of targeted D-Dox-PGA after pre-targeting with 10 µg/ml of Biotinylated anti-DTPA bispecific antibody complex compared to free Dox in MCF7 (sensitive) cells. Δ Dox ($y = -6.2899x + 19.478$ $R^2 = 0.73416$), $\bullet$ 10µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -14.057x + 34.656$ $R^2 = 0.96741$), $\blacksquare$ 20µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -13.801x + 27.636$ $R^2 = 0.95997$), $\circ$ Non-targeted D-Dox-PGA ($y = 1.7629x + 93.943$ $R^2 = 0.11468$)

B) Cytotoxicity of targeted D-Dox-PGA after pre-targeting with 20, 40 and 60 µg/ml of bispecific complex and targeting with D-Dox-PGA relative to free Dox in MCF7 ADR resistant cells. Δ Dox ($y = -25.308x + 77.247$ $R^2 = 0.97371$), $\blacklozenge$ 20µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -26.017x + 55.939$ $R^2 = 0.98314$), $\blacksquare$ 40µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -23.324x + 41.726$ $R^2 = 0.92246$), $\blacktriangle$ 60µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -22.258x + 37.718$ $R^2 = 0.9476$), $\circ$ Non-targeted D-Dox-PGA ($y = 7.3759x + 97.36$ $R^2 = 0.87833$). ** $P < 0.01$ Dox vs 20µg/ml of bsMAbCx + Targeted

D-Dox-PGA), * P<0.001 Dox vs 40µg/ml of bsMAbCx + Targeted D-Dox-PGA, P<0.001 Dox vs 60µg/ml of bsMAbCx + Targeted D-Dox-PGA**

C) Cytotoxicity of targeted D-Dox-PGA or combination of D-Dox-PGA and D-Ptxl-PGA after pre-targeting with 40 µg/ml of bispecific complex relative to free Dox in MCF-7 ADR (Resistant) cell line after 48 h. Δ Dox ($y = -25.308x + 77.247$ $R^2 = 0.97371$), $\blacksquare$ 40µg/ml of bsMAbCx + Targeted D-Dox-PGA ($y = -23.324x + 41.726$ $R^2 = 0.92246$), $\blacklozenge$ 40µg/ml of bsMAbCx + Targeted D-Dox-PGA and D-Ptxl-PGA ($y = -19.18x + 29.801$ $R^2 = 0.95206$), $\bigcirc$ Non-targeted D-Dox-PGA ($y = 7.3759x + 97.36$ $R^2 = 0.87833$). *** P<0.001 Dox vs 40µg/ml of bsMAbCx + Targeted D-Dox-PGA, * P<0.05 40µg/ml of bsMAbCx + Targeted D-Dox-PGA vs 40µg/ml of bsMAbCx + Targeted D-Dox-PGA and D-Ptxl-PGA.

Figure 6.

Epifluorescent micrographs of MCF7 ADR cell after 1 h pre-targeting with Biotinylated anti-DTPA bispecific antibody followed by incubation with D-Dox-PGA for: **A.** 5 min, **B.** 10 min, **C.** 15 min, **D.** 30 min, **E.** 60 min, **F.** 120 min, **G.** 300 min, and **H.** 360 min. (a: bright field, b: Dox fluorescence, c: Hoechst stain, d: superposition of b and c). Magnification = 40X.

Figure 7. Epifluorescent micrographs of MCF7 ADR after incubation with D-Dox-PGA without pre-targeting with Biotinylated anti-DTPA bispecific antibody for: **A.** 5 min, **B.** 10 min, **C.** 15 min, **D.** 30 min, **E.** 60 min, **F.** 120 min, **G.** 300 min, and **H.** 360 min (a: bright field, b: Dox fluorescence, c: Hoechst stain, d: superposition of images b and c). Magnification = 40X.

Figure 8. Epifluorescent micrographs of MCF7 ADR cells after incubation with Dox for: **A.** 5 min, **B.** 10 min, **C.** 15 min, **D.** 30 min, **E.** 60 min, **F.** 120 min, **G.** 300 min, and **H.** 360 min. (a: bright field, b: Dox fluorescence, c: Hoechst stain, d: superposition of b and c). Magnification = 40X.

A **B**

C

D

Figure 9.

A. Corrected total cell fluorescence (CTCF) values of MCF7 ADR cells after various treatments. ● Biotinylated-anti-DTPA-bsAbCx and D-Dox-PGA ($y = 213492\ln(x) - 178747$, $R^2 = 0.9318$), Dox ($y = 65660\ln(x) + 226552$, $R^2 = 0.64409$), and D-Dox-PGA

alone ($y = 42987\ln(x) - 58086$, $R^2 = 0.66613$). * $P < 0.05$ Dox vs Biotinylated-anti-DTPA-bsAbCx and D-Dox-PGA

B. CTCF values of the nuclei of MCF7 ADR cells after various treatments. ● **Biotinylated-anti-DTPA-bsAbCx and D-Dox-PGA** ($y = 84364\ln(x) - 54087$, $R^2 = 0.8759$), **Dox** ($y = 26516\ln(x) + 181318$, $R^2 = 0.37921$), and **D-Dox-PGA alone** ($y = 25917\ln(x) - 22318$, $R^2 = 0.64279$). **Ns Dox vs Biotinylated-anti-DTPA-bsAbCx and D-Dox-PGA**

C. CTCF values for MCF7 ADR cells after treatment with either Dox or pre-targeted D-DOX-PGA for 1 h followed by washing and replacing with Dox free medium compared to 1 h treatment without the washing step. ** $P < 0.01$, *** $P < 0.0001$).

D. CTCF values for the nuclei of MCF7 ADR cells after treatment with either Dox or pre-targeted D-DOX-PGA for 1 h followed by washing and replacing with Dox free medium compared to 1 h treatment without the washing step (* $P < 0.05$, ** $P < 0.01$).

Figure 10.

A. Micrographs of MCF7-ADR cells pretreated with biotinylated anti-DTPA-FITC bsAbCx for 1 h and then incubation with D-Dox-PGA for 1 h relative to **(B)** un-treated control cells. a) bright field micrographs, b) Hoechst nuclear stain, c) FITC green fluoromicrograph of the same cells, d) Dox-fluorescence micrograph, e) superimposition of b) and d), f) superimposition of b) and c), g) superimposition of c) and d), and h) superimposition of b), c), and d) to demonstrate nuclear localization of the released Dox. **B)** Same sequence of the micrographs as above in untreated control MCF7-ADR cells.

Figure 11.

Fluorescent Images of MCF7 ADR cells after: **A.** 1 h incubation with biotinylated anti-DTPA, followed by treatment with D-Dox-PGA for 1 h, followed by washing of the cells and incubation for 4 h in D-Dox-PGA free medium. **B.** Treatment with Dox for 1 h, followed by washing and incubation for 4 h in Dox free medium. **C.** Treatment with D-Dox-PGA alone for 1 h and the cells are treated as in A. (a: bright field, b: dox fluorescence, c: Hoechst stain, d: superposition of images b and c). Magnification = 40X.

Figure 12.

Fluorescent Images of MCF7 ADR cells after: **A.** 30 min pre-treatment with 10 $\mu\text{g/mL}$ of Chlorpromazine, followed by pre-targeting with Biotinylated anti-DTPA antibody for 1 h and 1 h incubation with D-Dox-PGA. **B.** 30 min pre-treatment with 10 $\mu\text{g/mL}$ of Chlorpromazine and then 1 h incubation with D-Dox-PGA. **C.** 30 min pre-treatment with 10 $\mu\text{g/mL}$ of Chlorpromazine followed by 1 h incubation with Dox. **D.** Cells alone. (a: bright field, b: Dox fluorescence, c: Hoechst stain, d: superposition of images b and c). Magnification = 40X.

Table 1

Zeta potential of PGA, D-Dox-PGA and D-Ptxl-PGA.

PPDC	Mean Zeta Potential	Standard Deviation
PGA	-21.42	2.34
D-Dox-PGA	-17.25	1.25
D-Ptxl-PGA	-15.75	2.13

Table 2IC₅₀ Values for SKOV3 Resistant and Sensitive cell lines

Cell Line	Treatment	IC ₅₀ (µg/ml)	Standard Deviation
SKOV3 (Sensitive)	Ptxl alone	0.089	0.048
	40 µg/ml bispecific antibody complex +D-Ptxl-PGA	0.069	0.01565
SKOV3 TR (Resistant)	Ptxl alone	0.936	0.1803
	40 µg/ml bispecific antibody complex +D-Ptxl-PGA	0.172	0.58

Table 3IC₅₀ Values for MCF7 Resistant and Sensitive cell lines

Cell line	Treatment	IC ₅₀ (µg/ml)	Standard Deviation
MCF 7 Sensitive	Dox Alone	0.00875	0.00157
	10 µg/ml Biotinaylted-AntiDTPA + D-Dox-PGA	0.0261	0.0094
MCF 7 ADR (Resistant)	Dox Alone	11.9	0.05743
	10 µg/ml Biotinaylted-AntiDTPA + D-Dox-PGA	3.499	0.454
	20 µg/ml Biotinaylted-AntiDTPA + D-Dox-PGA	1.392	0.652
	40 µg/ml Biotinaylted-AntiDTPA + D-Dox-PGA	0.442	0.0892