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Tuning polypeptide-based micellar carrier for efficient combination therapy of ErbB2-positive breast cancer

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Graphical Abstract

1. Introduction

Combination therapy has become the mainstay of cancer treatment, given the aggressive nature of the disease and development of resistance to monotherapy. While it is expected that delivering two drugs at their maximum tolerated dose (MTD) would result in a greater therapeutic response, often times this is not the case. Examples abound in the literature where drugs have been found to have a beneficial effect only at certain dose ratios.

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Moreover, when two drugs are administered systemically even at their synergistic ratio, the actual ratio presented to the tumor can vary drastically due to differences in their pharmacokinetic parameters like circulation half-life and clearance [1]. However, when such drugs are administered via a single nanocarrier system, the probability of maintaining the same ratio till they actually reach the tumor increases tremendously. Furthermore, incorporation of low molecular mass drugs into delivery carrier alters their pharmacokinetics and biodistribution in the body as well as reduces side effects. Altogether, these advantages justify the development of nanocarriers tailored to meet the demands of ratiometric delivery. Of equal importance with enhanced therapeutic efficacy is the avoidance of toxicity. Although encapsulation of cytotoxic agents into nanocarriers has shown to reduce the toxicities associated with free drugs, one can take advantage of the high level of synergy to use the therapeutic agents at doses much lower than their MTDs that drastically reduces the overall clearance burden on the body for these drugs and the synergy of action ensures that the net effect remains much higher than the sum of individual effects to maintain the desired therapeutic effect.

Breast cancer is no exception to the combination therapy approach. The present study is focused on human epidermal growth factor 2 positive (ErbB2+) breast cancer, accounting for about 20% of all breast cancer cases diagnosed. Oncogenic ErbB2 signaling results in hyperproliferation of the cancer cells, increases cell survival, promotes invasion and early metastasis, and is a relatively poor prognosis for the patient [2,3]. Inclusion of targeted therapy with humanized antibody trastuzumab (Herceptin) that interferes with ErbB2 signaling into conventional chemotherapy regimen significantly improved response rates, time of progression, and survival, and has become the mainstay of treatment for patients with this aggressive breast cancer subtype [4–7]. However, a substantial number of patients that do respond initially still relapse and develop resistance to anti-ErbB2 therapy. Therefore, the new and more effective treatment regimens combining trastuzumab with cytotoxic and other agents that target downstream signaling are being actively investigated in the preclinical and clinical setting [8]. The HSP90 inhibitor, 17-allylaminodemethoxygeldanamycin (17-AAG), which induces ErbB2 degradation and attenuates downstream oncogenic signaling, is one such agent that showed significant anticancer activity in early phase I/II clinical trials when given in combination with trastuzumab to patients with ErbB2+, metastatic breast cancer previously progressing on trastuzumab [9]. It has also been reported that 17-AAG sensitizes tumor cells to paclitaxel, one of the most active cytotoxic agents for the treatment of breast cancer [10,11]. These data suggest that combination chemotherapy using co-administration of 17-AAG with a paclitaxel-based regimen can be a potential therapy for ErbB2+ breast cancer. However, the low water solubility of both, paclitaxel and 17-AAG, potential toxicities and undesirable side effects observed in patients, partly due to the formulation excipients [12,13], may represent the hurdles in the advancement of this promising combination into clinical use.

Recent advances in cancer nanomedicines provide unique opportunities not only to tailor the unfavorable physicochemical properties of chemotherapeutics but also to modify the toxicity profile of the cargo therapeutics and increase their delivery to tumor. Combining drugs cargos with synergistic activities in one delivery carrier emerged as an attractive strategy for the development of treatment modalities with enhanced therapeutic efficacy [14]. In this

respect, polymeric micelles with a capacity to load multiple hydrophobic drugs into their cores have been widely studied in pre-clinical drug development and drug targeting [15–17]. Here, we developed a formulation of PTX and 17-AAG in a single polymeric micelle based on amphiphilic block copolymer composed of polyethylene glycol (PEG) as the hydrophilic, stealth imparting block and poly-leucine (PLEu) as the hydrophobic part and the initiator of micelle formation in aqueous environment [18,19]. In general, the advantage of using polypeptide-based polymers, apart from being biodegradable, is their tendency to form ordered conformations such as α -helices or β -strands, that may provide an additional means to control nanoscale structure formation through intermolecular hydrogen bonding [20]. Tuning the secondary structure of polypeptide block by using different enantiomeric forms of the amino acids can offer leverage to optimize the polymer's self-assembly for desired attributes like better stability and drug holding capacity [21]. The variables explored in the current study were the length ratio of the constituent blocks as well as chirality of PLEu block and the temperature of solvent used for preparation of micelles. The impact of all these variables on the thermodynamic stability and the ability of the micelles to encapsulate a combination of hydrophobic drugs into their core are also described. Our *in vitro* and *in vivo* results indicate that the developed pharmacologically synergistic combination of 17-AAG and PTX loaded into PEG-PLeu micelles exert remarkable antitumor efficacy at sub-therapeutic doses of both drugs. Furthermore, we demonstrated that combined therapeutic action and active molecular recognition of trastuzumab antibody conjugated to the surface of these micelles led to tumor regression in orthotopic xenografts of ErbB2+ breast cancer.

2. Materials and Methods

2.1 Materials

α -Amino- ω -methoxy poly(ethylene glycol) (mPEG-NH₂, M_w = 10,000 g mol⁻¹, M_w/M_n = 1.05) and Fmoc-NH-PEG-NH₂, M_w = 10,000 g mol⁻¹ and mPEG-NH₂, M_w = 5,000 g mol⁻¹ were purchased from JenKem Technology (TX, USA). L-leucine and D, L-leucine (Leu), 1, 2-ethylenediamine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), paclitaxel, and other chemicals were purchased from Sigma-Aldrich (St Louis, MO) and were used without further purification. Trastuzumab was obtained from UNMC pharmacy. Fetal bovine serum (FBS), DMEM and RPMI 1640 medium, penicillin, streptomycin, Trypsin–ethylenediaminetetraacetic acid (EDTA) (0.05% trypsin, 5.3 mM EDTA tetra-sodium) and other chemicals were purchased from Invitrogen (Carlsbad, CA, USA). MTT reagent (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was purchased from Research Products International (Prospect, IL). Cyanine3 (Cy3) carboxylic acid was purchased from Lumiprobe Life Science Solutions. All other chemicals were of reagent grade and used without further purification.

2.2 Methods

2.2.1 N-carboxyanhydride (NCA) of Leu—D, L or L-Leu (0.015 mol) and anhydrous tetrahydrofuran (THF) were added into a dried glass reactor in inert (nitrogen) atmosphere to form a suspension. Triphosgen (0.017 mol) was likewise separately dissolved in fresh anhydrous THF and injected dropwise into reaction mixture. Nitrogen was bubbled through the mixture during the reaction. The mixture was heated at about 55°C with constant stirring

till it became transparent. The product was precipitated by addition of 5 times excess of n-hexane and then stored at -20°C overnight in order to allow complete precipitation of D,L or L-N-carboxyanhydride (Leu-NCA). The obtained product was purified further by repeated precipitation with n-hexane, dried under vacuum for 24 h and characterized by proton nuclear magnetic resonance (^1H -NMR).

2.2.2 PEG-*b*-PLeu block copolymer—Monoaminomethoxypoly(ethylene glycol) (mPEG-NH₂) (0.03 mmol) was dissolved under stirring in 25 mL of anhydrous dimethylformamide (DMF) in nitrogen atmosphere at 30°C . Leu-NCA (0.75 mmol, the feed molar ratio of mPEG-NH₂ to Leu-NCA was 1: 25) dissolved in 5 mL of anhydrous DMF was added dropwise and the solution was stirred for 5 days. The aliquot of the reaction mixture was precipitated using excess of diethyl ether, dried under vacuum, and the composition of PEG-PBGlu diblock copolymer was determined by ^1H -NMR from the peak intensity ratios of the methylene protons of PEG and the methyl protons of the isopropyl groups of Leu (500 MHz in DMSO- d_6 , 80°C : ^1H -NMR [$\delta = 3.53$ (-CH₂-CH₂-O of PEG), 1.43 – 1.72 {-CH₂-CH-(CH₃)₂}, 0.8 – 0.97 {CH₂-CH-(CH₃)₂} for Leu. The product (PEG-*b*-Leu) was precipitated by diethyl ether, purified by repeated precipitation in diethyl ether and dried under vacuum. By varying the feed molar ratio of mPEG-NH₂ to Leu-NCA, copolymers with different lengths of the PLeu block were synthesized. Self-assembly behavior of PEG-*b*-PLeu copolymers was examined using pyrene as a hydrophobic fluorescence probe [22]. The biodegradability of the copolymer was studied upon incubation with Cathepsin B as reported previously [23]. To introduce reactive group on PEG terminus for conjugation with antibody, copolymer was synthesized using Fmoc-NH-PEG-NH₂ as the initiator as described above. When the polymerization reaction was complete, 10% v/v piperidine in DMF for 1 h was used to deprotect Fmoc group. The polymer was precipitated and washed with diethyl ether, redissolved in anhydrous DMF and reacted with 4 eq of succinic anhydride overnight. The polymer was purified by reprecipitation and washing with ether, dissolved in 70% v/v ethanol in water and subjected to exhaustive dialysis against deionized water, followed by lyophilization. For the synthesis of Cy3 labeled polymer, Cy3 carboxylic acid was conjugated to polymer in DMF prior to deprotection step and processed further as described above.

2.2.3 Preparation of polymeric micelles—Micelles were prepared by film rehydration method. PEG-*b*-PLeu copolymer was dissolved in acetonitrile (1mg/mL) followed by evaporation of the organic solvent under reduced pressure to form a thin film. The film was then rehydrated using hot water (60°C) and the solution was stirred for additional 1h at 60°C and then cooled gradually to room temperature.

2.2.4 Physicochemical and biophysical methods of characterization—The ^1H NMR spectra for the monomer and copolymers were acquired in DMSO- d_6 at 25°C and 80°C respectively using a Bruker Avance III HD NMR spectrometer (500 MHz). Effective hydrodynamic diameters (D_{eff}) of micelles were determined by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK). All measurements were performed in automatic mode at 25°C . Software provided by the manufacturer was used to calculate the size, polydispersity indices (PDI), and ζ -potential of

micelles. All measurements were performed at least in triplicate to calculate mean values $\pm$ SD. The CD spectra were recorded by using an Aviv circular dichroism spectrometer (model 202SF, Aviv Associates Inc., Lakewood, NJ). The spectra were measured at 25 °C and 60 °C, respectively, using a 1 cm path length cell over a wavelength range from 190 to 260 nm in deionized water. Data were collected at 1 nm intervals with a scan rate of 15 nm/min. All spectra were acquired in triplicate and averaged. The spectrum of an appropriate control sample was then subtracted from each of the sample spectra. The final spectral data were converted to mean molar ellipticities. The polymer concentrations were 0.02 mM.

Differential Scanning Calorimetry was used to measure the heat capacity of temperature-induced transitions in the dispersion of micelles relative to water using a VP-DSC from Microcal (Malvern, Northampton, MA). A temperature ramp of 0.5 °C/min and polypeptide concentration of 0.167 mM were used. Standard thermodynamic profiles were determined using the following equations: $G_T = H_{cal} (1 - T/T_m)$ and $G_T = H_{cal} - T \Delta S$, where $T = 298$ K. The H_{VH} 's were determined from analysis of the shape of the temperature-integrated experimental curves using procedures described earlier [24].

Molecular weight of micelles was determined using Agilent 1260 Infinity LC system equipped with a miniDAWN TREOS multi-angle light scattering (MALS) detector and a Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA) operated in an offline batch mode at a flow rate of 0.5 mL/min using DI water as the mobile phase. All analysis was carried out using the software Astra 6.1 (Wyatt Technology), and the dn/dc value of PEG (0.162 mL/g) was used for calculations.

2.2.5 Drug loading, conjugation of mAb and drug release—17-AAG and/or PTX-loaded micelles were prepared by film rehydration method. Briefly, polymer and either single drug or both drugs (drug/polymer ratio – 0.8:1 for 17-AAG and 0.6:1 for PTX) were dissolved in acetonitrile and the solvent was evaporated to form a thin film. This film was rehydrated with hot water (60 °C) and the solution was stirred for additional 1h at 60 °C and then cooled gradually to room temperature. Precipitates of unloaded drugs were removed by centrifugation. Drug content was determined by high performance liquid chromatography (HPLC) analysis under isocratic conditions using an Agilent 1200 HPLC system and a diode array detector set at 227 nm (PTX) and 334 nm (17-AAG). A Nucleosil C18 column was used as stationary phase (250 mm $\times$ 4.6 mm), and mobile phase comprised of acetonitrile/water mixture (55/45, v/v) at a flow rate of 1 mL/min.

Drug-loaded micelles were prepared from carboxyl-functionalized PEG-P(D,L-Leu) in the same manner. The terminal carboxylic acid groups of micelles were activated by excess of EDC (1.5 molar excess), then trastuzumab antibody was added to activated micelles (PBS, pH 7.4) and allowed to react for 2 hours. Trast-modified micelles were purified from unconjugated antibody using size exclusion chromatography (Ä KTA Fast Protein Liquid Chromatography system). The amount of conjugated antibody was determined by Bradford method using blank micelles as control.

Drug release from the micelles was examined in PBS (pH 7.4) or 10% FBS in PBS containing 0.1% sodium azide by dialysis method using a membrane with 3,500 Da cutoff.

The concentrations of PTX and 17-AAG released were determined by HPLC and expressed as a percentage of the total PTX or 17-AAG available *vs.* time.

The unbound fraction of PTX and 17-AAG in human whole blood (*fu*) was determined by an erythrocyte *vs.* plasma or buffer partitioning method [25–27]. Plasma was separated from erythrocytes in whole human blood (Innovative Research, MI) by centrifugation at 2500 g for 10 min. After removal of the plasma, the erythrocytes were washed in an equal volume of PBS, followed by centrifugation at 2500 g for 10 min, for a total of 3 times. Plasma or PBS was added to the tubes containing erythrocytes to a final concentration of 40% (HCT) (*n*=3) and vortexed to get a suspension. PTX(7.5 µg)/17-AAG(15 µg) solution in DMSO or drug-loaded micellar formulations were added to the blood suspension and incubated at 37°C for 2 hrs. Aliquots (150 µL) were taken for C_B or C_{B^*} measurement and centrifuged at 2500 g for 10 min. Another aliquot (150 µL) from the supernatant was used for C_p or C_b measurement. Acetonitrile (850 µL) was added to all the aliquots, followed by sonication and vortexing each sample thoroughly. Samples were then centrifuged at 5000 g for 10 min, supernatant was collected from each tube and solvent was evaporated. Acetonitrile (200 µL) was again added to all the tubes, followed by sonication and vortexing thoroughly. Drug content was determined by HPLC. Spiking solution (drug solution in DMSO) was added to erythrocyte-plasma, plasma, erythrocyte-buffer, buffer and acetonitrile solution. Acetonitrile was added into the tubes, and processed in the same way as the samples and drug content was determined by HPLC.

The concentration of drug associated with erythrocytes in the erythrocyte-plasma samples (C_E) and erythrocyte-buffer samples (C_{E^*}) were determined by:

$$C_E = \frac{C_B - C_p(1-HCT)}{HCT} \text{ and } C_{E^*} = \frac{C_{B^*} - C_b(1-HCT)}{HCT}$$

Where C_B and C_{B^*} were the concentration of drug in the erythrocyte-plasma and erythrocyte-buffer suspensions and C_p and C_b were the concentration of drug in the plasma and buffer.

The partition coefficients for erythrocyte-plasma (P_p) and erythrocyte-buffer (P_b), and *fu* (%) were determined by:

$$P_p = \frac{C_E}{C_p}, P_b = \frac{C_{E^*}}{C_b} \text{ and } fu (\%) = \frac{P_p}{P_b} \times 100, \text{ respectively.}$$

Average *fu* ± SD (*n*=3) were compared by One-Way ANOVA with Bonferroni's Multiple Comparison Test.

2.2.6 Cell culture, cellular association and cytotoxicity assay—BT-474 and MCF-7 cell lines were obtained from the American Type Culture Collection (ATCC) and cultured as described previously [28,29]. Cells were harvested with trypsin-EDTA (Life Technologies) after 80% confluence. Cells seeded in 96-well plates (5,000 cells/well) 24 h prior the experiment were exposed to various doses (0–100 ng/mL on 17-AAG or PTX basis) of 17-AAG alone, polymeric micelles alone, 17-AAG-loaded micelles, PTX-loaded

micelles and (17-AAG+PTX)-loaded micelles for 72 h at 37 °C. Cytotoxicity of drug-loaded *cl*-micelles was assessed by a standard MTT assay [30] and the IC₅₀ values were calculated using GraphPad Prism software. Combination index (CI) analysis based on Chou and Talalay method [31] was performed using CompuSyn software for 17-AAG and PTX combinations, determining synergistic, additive, or antagonistic cytotoxic effects against the breast cancer cells. Values of CI < 1 demonstrate synergism while CI = 1 and CI > 1 values represent additive and antagonistic effects of drug combination, respectively. For flow cytometry, cells (50,000 per well) were grown in a 24-well plate 24 hours prior the start of the experiment and exposed to Cy3-labeled Trast- or IgG-micelles (25 or 50 µg protein per mg polymer) at 37 °C, for 1 or 2 h, washed 3 times with PBS, trypsinized, centrifuged (1200 rpm, 3 min) and resuspended in PBS. The % gated cells were analyzed using Becton Dickinson FACStarPlus flow cytometer and FACSDiva software (Version 8.0, Becton Dickinson, San Jose, CA). At least 5000 events were acquired in linear mode, gated to exclude debris and dead cells, and visualized in logarithmic mode.

2.2.7 Animal studies—Animal model was developed as previously reported [28,29]. All studies were performed in strict accordance with the guidelines of University of Nebraska Medical Center Institutional Animal Care and Use Committee (IACUC). 4–6 weeks old female athymic NCr-nu/nu mice (Taconic Biosciences, NY) implanted with 17-β-Estradiol pellets (0.72mg/pellet; 60 day release; Innovative Research of America, Sarasota, FL) were implanted subcutaneously on the lateral side of the neck of the mice three days prior to the injection of tumor cells. 10⁷ BT-474 cells (an ErbB2 overexpressing human breast cancer cell line) reconstituted in 50% Matrigel (BD Biosciences, California) in media were injected directly into the mammary fat pad. The animals were randomized into treatment groups (n=10) when tumor size reached 100–200 mm³. Drug amount was calculated based on the average animal body weight. Treatments were administered by tail vein injection, comprising of various treatments as indicated in each specific section of results at the same drug ratio at a dose of 10 mg/kg 17-AAG and 5 mg/kg PTX and 6.5 mg/kg Trast. Free PTX or free 17-AAG + PTX were formulated using Cremophor-EL: propylene glycol: ethanol (2:3:5 by volume) mixture for injections as a component of the free drug combination. Tumor dimensions were measured using electronic calipers and tumor volume was calculated using the formula $V = 0.5 \times L \times W^2$ where L: longer dimension and W: shorter dimension. Tumor growth inhibition ratio (T/C values) were calculated according to the formula: $T/C = (V_t - V_o)_{\text{treated group}} / (V_t - V_o)_{\text{control group}}$, where V_t is tumor volume at the day of evaluation and V₀ is tumor volume on day 0. Animals were sacrificed when tumor volume was close to 2000 mm³, greatest tumor dimension was close to 20 mm or tumor became necrotic or animal exhibited body weight loss of 20%.

2.2.8 Apoptosis and proliferation—Tumors from mice that received different treatments were excised on day 13 (2–3 mice per group). The tumors were dissected and fixed in 10% neutral buffered formalin. Then, the tissues were processed routinely into paraffin, sectioned at a thickness of 4 µm. Proliferating and apoptotic cells were detected using an antibody against Ki-67 and caspase-3, respectively. Visualization was done by incubation with DAB+ (brown, for Ki-67) and Permanent red (for caspase-3) (DAKO) for 2 min. After being rinsed with distilled water, the sections were counterstained with

hematoxylin. For quantification of Ki-67 and caspase-3 expression, the area of positive cells was determined (Image J) in 3 random high power fields (10X magnification) and divided by the total area of cells for each field of slice.

2.2.9 Sample preparation and measurement of drug content in tissues—Tissue samples harvested from sacrificed animals were frozen and stored at -80°C until further use. Known weights of thawed tissues were homogenized with 3 volumes of HPLC grade water using TissueLyser (Qiagen). Calibration curves were built by spiking tissue samples from control animals to achieve the final concentration of 5 – 2000 ng/mL. Docetaxel was used as an internal standard at a final concentration of 500 ng/mL. Homogenates were vortexed for 30 sec, left for 5 mins, then extracted using excess (1 mL) of ice-cold acetonitrile, vortexed for 3 min and put on a shaker for additional 15 mins. All samples were then centrifuged at $16,000 \times g$ for 10 mins and the clear supernatant was separated into clean vials and evaporated to dryness followed by reconstitution with 100 μL of 70% acetonitrile in water (HPLC grade). These samples were run on LC-MS/MS system using a Nucleosil C18 column (250 mm $\times$ 4.6 mm) and a mobile phase of 70% acetonitrile in water was applied under isocratic conditions at a flow rate of 0.5 mL/min. A LC-MS/MS system equipped with triple quadrupole (QTRAP® 6500, Sciex) coupled to HPLC system (Nexera X2, Shimadzu) was used in multiple reaction monitoring (MRM) mode as follows; m/z 853.995 286 for PTX, 584.07 541 for 17-AAG and 808.08 527 for docetaxel.

2.2.10 Blood chemistry and histopathology—Blood from the sacrificed animals was collected in heparin tubes and analyzed for blood cell count and liver and kidney function markers (VetScan Comprehensive Diagnostic Profile) using Vetscan VS (Abaxis). Fixed tissues were processed, sectioned, inserted into tissue cassettes, dehydrated in 70% ethanol overnight, and paraffin embedded (UNMC Tissue Sciences Facility, Omaha, NE). Serial 5 μm sections were stained with either hematoxylin or eosin (H&E) or by immunohistochemistry (IHC). For histopathological diagnosis, H&E-stained slides were examined by light microscopy and photomicrographs were taken using a Nikon camera mounted on a Nikon Eclipse 600 microscope (both Nikon Instruments, Melville, NY) with Adobe Elements 3.0 software (Adobe Systems, San Jose, CA).

2.2.11 Statistical analysis—Statistical comparisons except animal studies were carried out using Student t-test. For the antitumor study and toxicity studies, group means for tumor volume and body weights were evaluated using repeated measures analysis of variance with the Bonferroni posttest. Survival was estimated using Kaplan–Meier analysis and compared using log-rank test. P values less than 0.05 were considered significant. Analysis of variance with Bonferroni test and Kaplan–Meier analysis with log-rank test were performed using GraphPad Prism 5 (GraphPad Software, Inc.).

3. Results and Discussion

3.1 Synthesis of PEG-b-PLeu block copolymers

The primary objective of this study was to optimize a carrier system for the delivery of hydrophobic drugs and to use it in cancer therapy. Its purpose is to serve as a safer substitute

to toxic cosolvents that are currently commonplace in clinical practice for such agents. To this end, we chose polypeptide-based block copolymers composed of hydrophilic poly(ethylene glycol), PEG, and hydrophobic polyleucine segments to develop micellar carriers. This micellar carrier was used for delivering a combination of hydrophobic drugs, such as 17-AAG and PTX. It is a well-established fact that the lengths of both hydrophilic and hydrophobic blocks play a crucial role in the self-assembly behavior of amphiphilic copolymers and that a certain length of the hydrophilic block is needed to provide stabilization of the micelles in the aqueous media [32–35]. Therefore, a set of diblock copolymers with different lengths of PEG and P(D,L-Leu) segments were synthesized by ring-opening polymerization of Leu-NCA using the amino-terminated PEG as a macroinitiator (Fig. S1, Supporting Information). The length of the PLeu block was controlled by feed ratio of PEG to D,L-Leu-NCA (1:25 and 1:15). The composition of the final copolymers was confirmed by $^1\text{H-NMR}$ (Table S1, Fig. S2). Gel permeation chromatography confirmed the formation of narrowly distributed copolymers (Fig. S3, Table S1).

3.2 Preparation and Characterization of Copolymer Micelles

Micelles were prepared from all the polymers using film rehydration technique [36,37]. Two different conditions were chosen for rehydration: room temperature (RT) and elevated temperature of 60 °C, which is well below the lower critical solution temperature of PEG in water [38]. Physicochemical characteristics of the micelles prepared under both conditions are listed in Table 1.

Spontaneous formation of micelles with relatively large sizes and broad particle size distribution was detected in aqueous dispersions of all synthesized copolymers at RT. Only the polymer PEG₂₂₈-*b*-P(D,L-Leu)₂₀ showed a favorable temperature-dependent change in self-assembly behavior that was reflected in nearly 3-fold reduction of the hydrodynamic diameter of the micelles prepared by film rehydration at elevated temperature of 60 °C. However, the same trend was not observed for the micelles composed of PEG₁₁₄-*b*-P(D,L-Leu)₁₀ and PEG₂₂₈-*b*-P(D,L-Leu)₁₀ copolymers. We rationalized that shortening the length of P(D,L-Leu) block was insufficient to drive proper assembly of the resultant micelles even at the higher temperature. In fact, increasing the length of P(D,L-Leu) block from 10 units to 20 units brought down the critical micelle concentration (CMC) of the PEG₂₂₈-*b*-P(D,L-Leu) copolymers from 1000 mg/L to a mere 15.6 mg/L as determined by pyrene fluorescence method. Such trend is typical for polymeric amphiphiles when longer hydrophobic blocks provide stronger drive for the copolymer assembly into micelles [39–41]. Based on these results, micelles formed by the PEG₂₂₈-*b*-P(D,L-Leu)₂₀ copolymer were chosen for further evaluation. Considering the dependence of block copolymer micellization on preparation method [42–45], we also explored other methods for preparation of PEG₂₂₈-*b*-P(D,L-Leu)₂₀ micelles (i.e. direct dissolution and various co-solvent aided procedures). Among the all explored procedures, film rehydration method at elevated temperature of 60 °C led to the formation of small sized micelles with relatively narrow particle size distribution (Table S2).

The apparent averaged molecular weight and aggregation number (N_{agg}) of the PEG₂₂₈-*b*-P(D,L-Leu)₂₀ micelles assembled at different temperatures was estimated by static light scattering technique and data are summarized in Table 2. Inspection of Table 2 indicates the micelles prepared at 60 °C had an order of magnitude lower values of apparent molecular weight as well as N_{agg} than those micelles prepared at RT. These findings are consistent with the observed difference in their sizes. It is understood that the kinetics of polymeric micelles is generally very slow [46,47], and therefore polymer chain exchange most likely was not fast enough at RT, leading to formation of relatively large and ill-defined micellar aggregates. Upon annealing at 60 °C, chain exchange or other associative mechanisms become possible, favoring the formation of the micelles that are close to, or at, equilibrium. Additionally, these micelles are notably less dispersed, which also might be an indication of an equilibrium state being reached. Interestingly, independently of the method of preparation, once formed these micelles were not further influenced by temperature variations. As shown in Figure 1A and 1B no significant changes were observed in the D_{eff} as well as PDI of the micelles upon heating from 10 to 65 °C. Therefore, it appeared that the structures of PEG₂₂₈-*b*-P(D,L-Leu)₂₀ micelles formed in solutions are locked. The observed differences in the structure of the micelles prepared at two temperatures may be due to the actual temperature difference (40 degrees) upon micellization, enhancing the overall kinetic effects and diminishes slightly the hydrophobic effect with small hydration changes. However, this temperature difference is not enough to overcome the energy barrier required for rearrangement of the formed micelle structure despite of the increased inter-micelle chain mobility at elevated temperatures.

It has been previously shown that pure enantiomer forms of PLeu when sufficiently large (ca. 20 residues or more) can adopt rod-like α -helical conformations that cause strong interchain associations and poor solubility in water and common organic solvents [48]. This can have a profound effect on the manner in which a polymer assembles and its micellar physicochemical properties, such as its hydrodynamic diameter and drug loading capacity. Therefore, this is an important parameter of optimization for polypeptide-based polymers. Once the lengths of the constitutive blocks as well as the method of micelle preparation were optimized, the next step was to compare the effect of chirality of hydrophobic block on the properties of micelles. To this end, a control polymer with P(L-Leu) as the hydrophobic block and similar block length as its racemic counterpart (20 units of Leu) was synthesized via ring-opening polymerization (Table S1, Fig. S3). Micelles prepared from PEG₂₂₈-*b*-P(L-Leu)₂₀ using film rehydration method at 60 °C had a narrow PDI of 0.14 ± 0.03 and hydrodynamic size of 82 ± 2 nm, which was slightly larger than that of PEG₂₂₈-*b*-P(D,L-Leu)₂₀/m (58 ± 1 nm) prepared in a similar manner. Similar to micelles based on racemic copolymer, PEG₂₂₈-*b*-P(L-Leu)₂₀ micelles retained their structural stability at the elevated temperatures.

We further address the thermal and thermodynamic stability of the preformed micelles using differential scanning calorimetry (DSC). The DSC thermograms (heat capacity as a function of temperature) for all samples show broad monophasic transitions that were reversible (data not shown). The temperature integration of the resulting thermograms are shown in Fig. 1C. Standard thermodynamic profiles for the temperature-transition of each micelle are presented in Table 3. The comparison of these data revealed similar transition temperatures

and quite different thermodynamic profiles for PEG-P(D,L-Leu)/m and PEG-P(L-Leu)/m, despite both micelles being prepared in a similar way at the annealing temperature of 60 °C. Inspection of Fig. 1C shows a sharper transition for PEG-P(D,L-Leu)/m that is also characterized by a 2-fold higher enthalpy, second column of Table 3. The PEG-P(D,L-Leu)/m prepared at RT show lower T_m and less endothermic enthalpy. Data shown in Fig. 1A, B indicate that all of the melting transitions occur within individual micelles, since heating preformed micelles to 65°C practically did not change their sizes or particle size distributions. Thus, the observed enthalpy changes can be attributed to the “melting” of the PLeu domains accompanied by the changes in the conformation of hydrophobes in the core of the micelles. The opposite signs of the measured thermodynamic parameters of Table 3 indicate that the favorable interactions of the PLeu segments result from the typical compensation of a favorable enthalpy term with an unfavorable entropy contribution. Importantly, PEG-P(L-Leu)/m and PEG-P(D,L-Leu)/m prepared at RT yielded less favorable Gibbs free energy (ΔG_{25}). Specifically, the ΔG_{25} terms of Table 3 indicate PEG-P(D,L-Leu)/m are slightly more stable than PEG-P(L-Leu)/m by ca. 0.5 kcal, suggesting that the propensity of P(L-Leu) to adopt α -helical conformation in the core of the micelles decreases favorable packing of the hydrophobic segments. Indeed, the CD spectrum of preformed PEG-P(L-Leu)/m exhibited a double minima at 208 and 222 nm along with a positive ellipticity at 195 nm, features that are typical of right-handed α -helices (Fig. 1D) whereas racemic PEG-P(D,L-Leu)/m exhibited as expected disordered conformation (Fig. 1D). Furthermore, no changes were observed in the CD spectra for both types of micelles with the increase in temperature. This suggests that, once the micelles were formed, the PLeu domains retained their conformations in the core of the micelles.

Van't Hoff enthalpies, H_{vH} , corresponding to the number of units melting cooperatively or simultaneously, were obtained from analysis of the shape of the integrated thermograms of Fig 1C. This exercise yielded H_{vH} s of 22–25 kcal/mol for the three reported micelles. The H_{vH}/H_{cal} ratio of one for PEG-P(D,L-Leu)/m-60°C suggests a two-state transition for this micelle [24,49]. On the other hand, we obtained H_{vH}/H_{cal} ratios of 2.5 and 1.7 for the other two types of micelles, which indicates the temperature-induced transition of these micelles is more disorganized. It appeared that formation of thermodynamically more stable PEG-PLeu micelles is tied to conformationally random P(D,L-Leu) segment and higher temperatures. Although hydrophobic PLeu segments are identical in composition, amphiphilicity, and size, the rigidity of P(L-Leu) α -helices may prevent close alignment of the polymer chains even at high temperature and thus lead to the formation of less compact micelles than PEG-P(D,L-Leu)/m which is consistent with the lower magnitude of the enthalpy-entropy compensation (Table 3). Similarly, less compact PEG-P(D,L-Leu) micelles are formed at RT when the mobility of the PLeu chains is limited. Importantly, PEG-P(D,L-Leu) micelles were also prepared by rehydration and subsequent heating at 40 °C, a temperature close to T_m yielding micelles with ~ 114 nm size and a broad particle size distribution (PDI = 0.29). This observation further suggests that there is a kinetic energy barrier to be overcome in order to form micellar structures with the lowest free energy.

3.3 Loading and release profile of hydrophobic drugs

The elevated temperature used for rehydration not only leads to increased mobility of the polymer chains in the cores but also may facilitate its interaction with the hydrophobic drugs and their incorporation into the micelles. Thus, drug loading into the micelles was carried out by the same method of film rehydration at 60°C used for preparing empty micelles. Rehydration of the film consisting of polymer and each drug alone or drug mixture (17-AAG and PTX) led to micelle formation and simultaneous encapsulation of the drugs into the hydrophobic core of the micelles. The incorporation of drugs in micelle dispersion was quantified by HPLC (Table 4). Our attempts at loading drugs alone in PEG-P(D,L-Leu) micelles at a feed ratio of 10 wt % drug *versus* polymer resulted in drug loading of 4 wt % for 17-AAG and 3 wt % for PTX, respectively. Noteworthy, the loading capacity of 17-AAG was nearly doubled (6.6 wt%) when it was co-loaded with PTX in PEG-P(D,L-Leu)/m as has been previously reported with other polymer micelles incorporating hydrophobic drugs[15]. An increase in drug feed ratio up to 20 wt% did not translate into an increase in loading capacity of the micelles either for single drugs or for combination suggesting that we reached maximal loading capacity of the micelles for these hydrophobic drugs. We found that the sizes of the drug-loaded micelles were comparable to those of empty micelles. In spite of incorporation of almost 10% by weight of hydrophobic drugs, the resultant micelles showed no increase in PDI and remained stable in solution for up to 2 months without signs of aggregation. We would like to point out that other explored methods of drug loading produced much larger (~ 100 nm) micelles with lower or comparable drug loading (Table S3).

On the other hand, all of the above attributes were absent for drug-loaded micelles prepared from PEG-P(L-Leu). The loading capacities for individual drugs as well as for their combination remained much lower than for corresponding PEG-P(D,L-Leu)/m. The observed difference in drug loading between these two types of micelles can be attributed to the unfavorable packing of rigid helical P(L-Leu) segments once the micelle cores are formed that compromises the loading capacity of the micelles. CD data confirmed that incorporation of the drugs did not alter the secondary structure of the cores of PEG-P(L-Leu)/m (Fig. S4). Moreover, we can also hypothesize that H-bonding between exposed amide groups of the disordered polypeptide chains and drug molecules in the core of PEG-P(D,L-Leu) micelles would favor micellar drug solubilization in addition to other interaction forces. Our observations are in accordance with the behavior of micellar carriers with conformationally rigid hydrophobic P(L-Leu) segments in the cores where decreased cargo loading was observed [2,19,50]. Drug release data from PEG-P(D,L-Leu)/m showed that the individual drug release rates practically did not change for binary drug formulations compared to the single drug-loaded micelles: over 90% of drugs were released within 24 hours at physiological pH, both in the presence as well as absence of FBS (Fig. S5). We next utilized an indirect approach to determine whether encapsulation of drug combination into the micelles decreases premature release of loaded drugs in whole blood by the erythrocyte vs. buffer or plasma partitioning method [26]. This method was designed to determine the unbound fraction (*fu*) of a drug in blood and was shown to provide a good correlation with in vivo pharmacokinetic results predicting the stability of micellar formulations [26,27]. A decrease in the *fu* value of loaded drug vs. unencapsulated drug indicates that a higher

proportion of drug remains within the polymer micelles. We compared the *fu* values of PTX and 17-AAG loaded in PEG-P(D,L-Leu) micelles and drug combination alone in human whole blood at 37°C. (17-AAG+PTX)/m formulation significantly decreased unbound drug fraction compared to free drug combination ($14.4 \pm 1.6\%$ vs. $40.9 \pm 1.0\%$ for PTX and $10.5 \pm 0.9\%$ vs. $38.4 \pm 3.7\%$ for 17-AAG, $p < 0.001$) (Fig. S6). These data imply that micellar formulation can decrease the premature release of the drugs in whole blood and, consequently, likely to increase bioavailability and subsequent potency after parenteral administration. In addition, enzymatic degradability of PEG-P(D,L-Leu) would be advantageous due to specific intracellular drug release triggered by disassembly of the delivery carrier and reduced risk of polymer accumulation inside the cells (Fig. S7). Altogether, micelles prepared from PEG-P(D,L-Leu) exhibited greater capacity for drug loading and would be better carriers for hydrophobic drugs rather than those prepared from PEG-P(L-Leu). Therefore, we selected PEG-P(D,L-Leu) micelles for further evaluation as potential tumor-targeting delivery system for drug combinations.

3.4 In vitro cytotoxicity of the drug-loaded micelles

In vitro cytotoxicity of free drugs and drug-loaded PEG-P(D,L-Leu) micelles was tested on two different breast cancer cell lines with differential ErbB2 expression using MTT assay. Calculated IC₅₀ values are summarized in Table 5. PTX is a known microtubule stabilizer and the standard chemotherapeutic agent used in ErbB2+ breast cancer in combination with Trastuzumab therapy [4]. Herein we studied the synergy of PTX and 17-AAG, a heat shock protein 90 (HSP90) inhibitor. Among other functions, HSP90 acts as a chaperone protein for ErbB2 and inhibition of HSP90 leads to enhanced lysosomal degradation of ErbB2 [28]. Indeed, in the ErbB2 overexpressing BT-474 cells, we observed a 1000-fold reduction in the IC₅₀ value of PTX when administered in combination with 17-AAG. Combination index analysis using the Chou and Talalay method [31] showed a strong synergy between the two drugs at a 2:1 17-AAG/PTX ratio, both in the form of mixture of free drugs (CI = 0.09) and binary combination loaded in micelles (CI = 0.08). When both drugs were administered in the form of a cocktail of single drug-loaded micelles at equivalent doses, the synergy of action was still retained and the effect was the same as that seen in case of combination drug-loaded micelles. The negligible difference between these treatments can be attributed to the fact that cells under static *in vitro* conditions are evenly exposed to the drug-loaded micelles even when they are given as two separate formulations. Moreover, both drugs have a similar type of interaction with the hydrophobic core of the micelles and therefore exhibit comparable release pattern, making the availability of drugs similar for both (17-AAG + PTX)/m and cocktail of 17-AAG/m + PTX/m. Notably, in similar experiments conducted on the ErbB2-low MCF-7 cells the CI values were close to 1, which is indicative of a mere additive effect. These data suggest that 17-AAG plus PTX combination can induce synergistic anti-proliferative effects selectively in ErbB2-overexpressing breast cancer cells.

3.5 Antitumor activity of single vs combination drug-loaded micelles

Based upon our *in vitro* data, we hypothesized that combined administration of 17-AAG and PTX *in vivo* would allow an anti-tumor therapeutic effect to be reached at lower doses of the drugs, thereby reducing general toxicity. To test this hypothesis, mice bearing orthotopic ErbB2-driven BT-474 tumors were intravenously injected four times at 4-days interval with

either combination of drug-loaded (17-AAG + PTX)/m or cocktail of single drug-loaded 17-AAG/m + PTX/m or free PTX or 5% dextrose as control. We chose to use the 10mg/kg and 5mg/kg doses of 17-AAG and PTX, respectively, which are sub-therapeutic doses for each agent (reported effective antitumor i.v. doses of 17-AAG and PTX are 40 and 20 mg/kg body weight, respectively [51,52]). As shown in Fig. 2, only 2-drug regimens were effective in suppressing tumor growth. Free PTX showed negligible antitumor effect, which is an expected outcome for the sub-therapeutic dose used. The strong synergy of the 2-drug regimen seen *in vitro* was also seen *in vivo*, and binary combination (17-AAG + PTX)/m as well as the cocktail of single drug-loaded micelles 17-AAG/m + PTX/m showed significant inhibition of tumor growth compared to control or free PTX groups. Of great interest, however, was the difference in activity of the (17-AAG + PTX)/m vs cocktail of single drug-loaded 17-AAG/m + PTX/m at the same dose ratio: inhibition of tumor growth was significantly enhanced in animals treated with (17-AAG + PTX)/m compared to those treated with the 17-AAG/m + PTX/m cocktail ($p < 0.05$). The lower therapeutic efficacy of a cocktail of single drug-loaded micelles observed *in vivo* is likely to be related to the altered pharmacokinetics and biodistribution of the mixture of micelles, which led to an altered ratio of the drugs reaching the tumor. Analysis of drug content in tumors collected on the next day after final dosing revealed 1.4-fold higher total drug levels accumulated in the tumors of animals treated with (17-AAG + PTX)/m compared to 17-AAG/m + PTX/m group. Moreover, the average 17-AAG/PTX ratio in the tumors of animals treated with (17-AAG + PTX)/m was determined to be 1.8 ± 0.2 (Fig. 2B and Table S4), which is close to the initially administered drug loading ratio of 2:1 in the micelles. However, in the group receiving 17-AAG/m + PTX/m cocktail, this ratio was found to be 4 ± 0.5 . When BT-474 cells were treated with drug combination at a 17-AAG/PTX ratio of 4:1 *in vitro*, it was found that there was only 10-fold reduction in the IC_{50} of PTX in combination as opposed to the 1000-fold reduction seen when the cells were treated with drugs at a 2:1 ratio and this was also reflected in less pronounced synergistic activity of drug combination (the CI value at IC_{50} increased from 0.09 to 0.5, Table 5 and Table S5). This further reinforces the importance of spatial-temporal synchronization of delivery of the drugs at a defined ratio to retain synergy of action, as was reported in our previous work [53].

Animals treated with (17-AAG + PTX)/m had the longest survival times, with only 2 out of 7 animals reaching the point of termination in the span of 85 days (Fig. 2C). On the other hand, median survival for animals treated with the 17-AAG/m + PTX/m cocktail (48 days) was only slightly better than free PTX group (42 days) or the untreated control (40 days). Excised tumor tissue sections were stained for apoptosis and proliferation markers (cleaved caspase 3 and Ki67) and the results are summarized in Fig. 2D and E. The number of proliferating cells were significantly lower in the tumors of animals treated with (17-AAG + PTX)/m as compared to animals treated with 17-AAG/m + PTX/m cocktail ($p < 0.05$) or free PTX ($p < 0.01$). This could explain the continued inhibition of tumor growth seen in animals treated with (17-AAG + PTX)/m even after 2 weeks post treatment, while the tumors of animals treated with the cocktail of single drug-loaded micelles showed a rather fast rate of disease progression. Overall, these data evidenced a strong synergy of anticancer action of the combination of 17-AAG and PTX in a ratio-specific manner.

Furthermore, micelle-based binary drug combination treatments were well tolerated. Analysis of blood samples collected after final dosing (day 13) did not show any significant changes in the clinical parameters for hepatic, renal and hematopoietic functions (Table S6). The counts of RBCs, WBCs or platelets also remained within normal range. No changes in the morphology of the organs at the tissue level were observed (Fig. S8), except extramedullary hematopoiesis, which was observed in spleens of control as well as treated animals and therefore was of no health concern. The only sign of side effects was loss in body weight of animals treated with free PTX, which is a common effect observed due to the toxic cosolvent Cremophor EL[®] used for solubilization of PTX. Body weights of animals treated with all micellar formulations remained normal throughout the duration of the study (Fig. S9). Collectively, these data demonstrate the utility of PEG-P(D,L-Leu)/m nanocarriers as platform for the simultaneous delivery of PTX and 17-AAG in ErbB2+ tumors to achieve anticancer efficacy without formulation related side effects. The potency of this drug combination can be attributed to marked induction of tumor cell apoptosis and associated reduction in tumor cell proliferation. These data are consistent with the previous reports that concurrent exposure to 17-AAG and paclitaxel is required for the synergistic activity of the two [10,11,54]. Thus, the significant tumor growth delay observed for (17-AAG + PTX)/m treatment confirms our hypothesis that co-delivery of both drugs via the same carrier is essential to enhance the efficacy of combination chemotherapy.

3.6 Development and antitumor efficacy of ErbB2-targeted drug-loaded micelles

We next explored whether combining (17-AAG+PTX)/m with ErbB2-targeting agent, trastuzumab antibody, can further potentiate the tumor-specific delivery and efficacy of drug combination. One of the feasible approaches to achieve active cancer targeting of nanocarrier is its surface functionalization with ligands that can recognize and bind receptors on cancer cell. Ligand incorporation helps in increasing the mean residence time of the drug carrier at the tumor site and facilitates the entry of nanoparticles to cancer cells via receptor-mediated endocytosis. In this context, trastuzumab antibody is an excellent candidate ligand because it combines both therapeutic activity and specific affinity for the ErbB2 receptor for targeting. ErbB2-targeted formulations (Trast/m) were prepared by conjugation of trastuzumab onto preformed carboxyl-functionalized drug-loaded micelles obtained from carboxyl-functionalized PEG-P(D,L-Leu) using carbodiimide-mediated amidation. IgG-linked micelles (IgG/m) were prepared using similar procedure and used as non-targeted control (Fig S10, S11, S12). The hydrodynamic diameter of drug-loaded micelles increased by approximately 20–25 nm after modifying the surface with either Trast or IgG, while the polydispersity indices still remained relatively low ($PDI < 0.2$). The loading capacities for 17-AAG and PTX, alone or in combination, also remained relatively the same as for bare micelles (Table 4 and Table S7).

Micelle association with ErbB2+ BT-474 cells was determined by flow cytometry using fluorescence Cy3-labeled Trast/m or IgG/m with two different targeting ligand loading of 25 and 50 μg ligand per mg of the polymer. As expected, the cellular association of Trast/m with ErbB2+ BT-474 cells greatly exceeded that of the non-targeted IgG/m (Fig. 3A). The uptake of IgG/m, irrespective of the surface density of IgG, remained practically the same over time (Fig. 3A). On the other hand, Trast/m showed increased uptake by BT-474 cells in

a time- and dose-dependent manner, indicating that trastuzumab retained its binding affinity to ErbB2 receptor even after conjugation to micelles. Furthermore, a surface density as low as 25 µg Trast per mg polymer was sufficient to enhance the association and uptake of Trast/m in BT-474 cells (Fig. 3A).

As shown in Table 6, attachment of trastuzumab ligands resulted in significant increase ($p < 0.01$) of cytotoxicity of drug-loaded micelles compared to non-targeted formulation. To maintain the equivalent dose of therapeutic antibody in all treatment groups, the trastuzumab loading for both single drug-loaded micelles in a cocktail formulation was kept at 25 µg ligand/mg polymer, while Trast-(17-AAG + PTX)/m had a loading of 50 µg Trast per mg polymer. Since the doses of all three therapeutic components (Trast, 17-AAG and PTX) were held the same, cocktail of Trast-(17-AAG)/m + Trast-(PTX)/m was nearly as active as Trast-(17-AAG + PTX)/m in ErbB2-overexpressing BT474 cells. Notably, there was no significant difference in cytotoxic activity between Trast-targeted and non-targeted formulations in the ErbB2-low MCF-7 cells. These data indicate that trastuzumab antibody attached to the surface of the drug-loaded micelles had both therapeutic as well as targeting effect that was mediated specifically via its target, ErbB2 receptor.

The targeting and therapeutic effect of drug-loaded Trast/m was further validated in mice bearing orthotropic BT-474 tumors. Animals were administered a total of 4 injections, once every 4th day intravenously via the tail vein. The treatment consisted of 5% dextrose or Trast/m or free 17-AAG + PTX in Cremophor or IgG-(17-AAG + PTX)/m or Trast-(17-AAG + PTX)/m or Trast-(17-AAG)/m + Trast-(PTX)/m at equivalent doses of 6.5 mg Trast, 10 mg 17-AAG and 5 mg PTX per kg body weight. The changes in relative tumor volume and animal lifespan are shown in Fig. 3B, C. Data are representative of 6 animals in each group and curves terminated when the first animal in the group reached the point of sacrifice. The median survival times for each treatment group are listed in Table S8. As expected, combination of low doses of free 17-AAG and PTX formulated in Cremophor had negligible effect on tumor growth inhibition (Fig. 3B), and the disease progression as well as survival (Fig. 3C) remained similar to untreated animals. In contrast, all micelle-based treatments significantly retarded tumor growth with respect to untreated animals or animals treated with free drugs. Therapy with Trast/m without 17-AAG and PTX at the trastuzumab dose within the therapeutic window (3–10 mg/kg [55]) was also effective in inhibiting tumor growth as compared to untreated control ($p < 0.05$), suggesting that conjugation of antibody to micelles does not prevent their binding to ErbB2+ cells *in vivo*. Response to treatment with drug combination loaded non-targeted IgG-(17-AAG + PTX)/m was only marginally better than that for cocktail of Trast-(17-AAG)/m + Trast-(PTX)/m group in terms of tumor progression (not statistically significant) while the survival was significantly better than the latter group ($p < 0.05$). However, the best therapeutic effect was seen in animals treated with Trast-(17-AAG + PTX)/m that outperformed non-targeted IgG-(17-AAG + PTX)/m in terms of tumor progression ($p < 0.001$) and survival ($p < 0.01$). We further calculated tumor growth inhibition ratio (T/C) values defined as the ratio of the average tumor volume for the treated vs. control group. The optimal value is the minimal T/C ratio reflecting the maximal tumor growth inhibition achieved. T/C ratio thus can serve as additional means of comparison of effectiveness of different treatment regimens in a more quantitative fashion (Table S9). Animals receiving targeted therapy of Trast-(17-AAG + PTX)/m showed T/C

value of 0.06 on day 28, which is about 3 times lower than non-targeted treatment regimens ($T/C = 0.18$ for 17-AAG + PTX)/m and $T/C = 0.21$ for IgG-(17-AAG + PTX)/m). T/C values for animals treated with either free PTX or combination of free drugs were close to 1 (0.72 and 0.78 respectively), indicating that free drug regimens had negligible therapeutic effects. Remarkably, no palpable tumors were present in the animals treated with Trast-(17-AAG + PTX)/m by the end of treatment (day 13) followed by a significant delay in tumor growth when tumors reached a palpable size on day 45 after start of the treatment. Consequently, this group had the best survival outcome, wherein all six animals were alive until the last day of survival monitoring (day 92). This shows that all the three components, trastuzumab, 17-AAG and PTX, have a strong synergy of action, and that trastuzumab antibody has both a therapeutic as well as targeting effect, which would have potentially resulted in highest accumulation of drugs in the tumor. Simultaneous delivery of 17-AAG and PTX within one carrier has also played a crucial role in this effect, as was evident from the lower therapeutic response when animals were treated with cocktail of Trast-(17-AAG)/m + Trast-(PTX)/m, which had all the therapeutic elements at equivalent doses.

Drug content in tumor and vital organs was determined post 24 and 72 hours of the last injection (day 13 and 15 of the treatment) using LC-MS/MS method ($n = 3$ per each treatment group). Results are summarized in Fig. 3D and Fig. S13. In animals treated with Trast-(17-AAG + PTX)/m, tumors were practically nonexistent and thus no tumor drug content data from this group could be obtained. In general, administration of both 17-AAG and PTX encapsulated in micelles led to significantly higher accumulation ($p < 0.001$) of the both drugs in the tumor as compared to treatment with free drugs. Notably, in the case of cocktail of targeted micelles the presence of specific anti-ErbB2 antibody had limited effect on increasing drug accumulation in the tumor compared to that exhibited by non-targeted IgG-(17-AAG + PTX)/m (Fig. 3D). While the initial dose ratio for 17-AAG/PTX was 2:1 in all the cases, the ratio in which these drugs accumulated into the tumor tissue varied widely (Table S10). For free drugs, the ratio was random and variable in all the three animals tested (1.9 ± 0.5). The same trend was observed in animals treated with Trast-(17-AAG)/m + Trast-(PTX)/m with a ratio of 1.6 ± 0.5 . However, in group treated with IgG-(17-AAG + PTX)/m, where both the drugs were co-loaded in the same carrier, the 17-AAG/PTX ratio was determined to be 1.8 ± 0.2 , which is much closer to the initial dose ratio of 2:1. As a result, the antitumor efficacy of Trast-(17-AAG)/m + Trast-(PTX)/m was not better than that of non-targeted IgG-(17-AAG + PTX)/m, indicating that the ratio-specific synergy of action might have been mitigated due to the randomness of delivery of the drug cargo. Both drugs were cleared from the tumor across all treatment groups over time (Fig. 3D and Fig. S13A). However, the overall levels of the drugs at 72 h post last injection were still higher in animals treated with micellar formulations, which explains the higher therapeutic efficacy in terms of retardation of tumor growth that was seen in these groups as compared to free drugs.

Antibody-functionalized micellar formulations exhibited higher levels of drug accumulation in organs of mononuclear phagocyte system (liver and spleen), compared to those for free drugs, which gradually decreased over the period of 72 h after final dosing (Fig S13). This trend was found to be similar across all treatment groups, irrespective of the presence of Trast or IgG on the micellar surface. Despite much higher exposure of these organs to the

cytotoxic drugs, toxicity profiles remained favorable, with the exception of Trast-(17-AAG)/m + Trast-(PTX)/m, wherein the livers of 2 out of 3 animals showed signs of chronic inflammation upon microscopic examination of H & E stained liver sections. This was consistent with the elevated levels of serum ALP and ALT in these animals as compared to other groups. However, hematocrit of blood samples across all treatment groups remained normal and no other vital organs like the spleen, kidney or heart showed signs of toxicity, indicating that the therapy was well-tolerated (Table S11).

4. Conclusion

We engineered an actively-targeted nanocarrier based on biodegradable polypeptide-based micelles that can simultaneously deliver and release encapsulated combination of two hydrophobic drugs, 17-AAG and PTX. The present study highlights the importance of biophysical characteristics of core-forming polyleucine block for the preparation of stable micelles. The self-assembly behavior of PEG-PLeu block copolymer was dictated not only by the lengths of both constituent blocks but also can be tuned by conditions of preparing these micelles. Thin film rehydration at elevated temperature of 60 °C lead to the formation of small sized micelles with relatively narrow particle size distribution. Furthermore, the nature of the polyleucine block was also an important parameter for consideration; racemic PEG-P(D, L-Leu) micelles had a smaller hydrodynamic diameter and much higher capacity for encapsulation of hydrophobic drugs as compared to pure enantiomer counterpart. We have found that PEG-P(D, L-Leu) micelles were able to encapsulate both 17-AAG and PTX at a ratio that exhibited a strong synergy and was effective both *in vitro* and *in vivo*. We have further confirmed that this drug combination exerts the maximal antitumor efficacy in ErbB2+ orthotopic breast cancer xenografts when delivered *via* the same micellar carrier as compared to a cocktail of individual drug-loaded micelles administered at the same drug ratio. Most importantly, marked antitumor effect was seen at sub-therapeutic doses of both 17-AAG and PTX, which lead to much better tolerability of the therapy and lack of signs of acute toxicity. Ultimately, fusion with active targeting modality via conjugation of Trastuzumab antibody to the surface of drug-loaded micelles led to a further significantly enhanced antitumor efficacy. Our studies therefore provide the rationale for development of combinatorial receptor targeted nanomedicine to maximize therapeutic efficiency and improve outcomes of therapy of ErbB2+ breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Changes in (A) hydrodynamic diameter (D_{eff}) and (B) PDI as a function of temperature as measured by DLS after every 0.5 °C change and (C) changes in enthalpy as a function of temperature as measured by DSC for PEG-P(D,L-Leu)/m - 60 °C (red), PEG-P(D,L-Leu)/m - RT (green) and PEG-P(L-Leu)/m - 60 °C (blue). All samples were heated starting from 10° – 65°C, at the rate of 0.5 °C/min. (D) Changes in secondary structure as measured by CD spectroscopy at different temperatures; micelles were prepared by film rehydration at 60 °C from PEG-P(L-Leu) and measured at 60 °C (orange) or RT (blue) and from PEG-P(D,L-Leu), measured at 60 °C (olive green) or RT (red). No significant changes in secondary structure were observed for micelles prepared from either polymer when measured at different temperatures.

Figure 2.

In vivo antitumor efficacy of different nanoformulations in BT-474 human breast cancer xenograft-bearing female nude mice. **(A)** Relative changes in tumor volume measured following intravenous administration of (17-AAG + PTX)/m (●) or free PTX (■) or 17-AAG/m + PTX/m (▲) or 5% dextrose (▼) at 10 mg 17-AAG or 5 mg PTX equivalents/kg body weight. Drug formulations were injected in 100 µL 4 times at 4-day intervals. Tumor volume is normalized with respect to tumor volume at day 0 (V_t/V_0). Values indicated are means $\pm$ SEM ($n = 7$). **(B)** Drug content in tumor tissue in various treatment groups collected on day 13. Solid bars represent PTX and empty bars represent 17-AAG. Data are

represented as mean $\pm$ SD (n=3). (C) Kaplan–Meier analysis of overall survival in 5% dextrose group (1) or free PTX group (2) or 17-AAG/m + PTX/m group (3) or (17-AAG +PTX)/m group (4). Ki-67-caspase-3 apoptosis assay. Quantification of (D) Ki67 positive and (E) caspase-3 positive cells in tumor tissue from mice from various groups. Data are presented as mean $\pm$ SD (n = 3, 2000 cells in random microscopic fields for each tumor slice). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

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Figure 3.

(A) Cellular association of Cy3-labeled Trast/m and IgG/m at two different targeting ligand loading of 50 μg and 25 μg protein per mg polymer. Cells were treated with Trast/m or IgG/m at 0.5 mg/mL polymer. Data are represented as mean $\pm$ SD. (B-D) *In vivo* antitumor efficacy of Trast-(17-AAG+PTX)/micelles in BT-474 xenograft-bearing female nude mice. (B) Relative changes in tumor volume measured following intravenous administration of 5% dextrose (●) or free 17-AAG + PTX (▲) or Trast/m (■) or Trast-(17-AAG)/m + Trast-(PTX)/m (x) or IgG-(17-AAG + PTX)/m (▼) or Trast-(17-AAG + PTX)/m (◆) at 10 mg 17-AAG or 5 mg PTX or 6.5 mg Trast equivalents/kg body weight. Drug formulations were injected in 100 μL 4 times at 4-day intervals. Values indicated are means $\pm$ SEM (n = 6). (C) Kaplan-Meier analysis of overall survival in 5% dextrose group (1) or free 17-AAG + PTX group (2) Trast/m group (3) or Trast-(17-AAG)/m + Trast-(PTX)/m group (4) or IgG-(17-AAG + PTX)/m (5) or Trast-(17-AAG + PTX)/m (6). (D) Tumor accumulation of 17-AAG and PTX in different treatment groups as determined by LC-MS/MS. Mice were sacrificed and tumors were harvested post 24 h of the last injection (day 13). Solid bars represent PTX and empty bars represent 17-AAG. Values indicated are mean $\pm$ SD (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 1

Physicochemical characteristics of micelles prepared by film rehydration at RT and 60°C^a

Sample	D _{eff} (nm)		PDI	
	RT	60 °C	RT	60 °C
PEG ₁₁₄ - <i>b</i> -P(D,L -Leu) ₁₀ /m	141 ± 4	107 ± 3	0.27 ± 0.06	0.28 ± 0.04
PEG ₂₂₈ - <i>b</i> -P(D,L -Leu) ₁₀ /m	112 ± 2	158 ± 2	0.22 ± 0.06	0.29 ± 0.05
PEG ₂₂₈ - <i>b</i> -P(D,L -Leu) ₂₀ /m	142 ± 4	58 ± 1	0.26 ± 0.04	0.12 ± 0.01

^aHydrodynamic diameter (D_{eff}) and polydispersity index (PDI) were measured by dynamic light scattering (DLS) in water (pH 6.5) at 25°C. Data are presented as average ± SD from three experiments.

Table 2
Structural characteristics of PEG₂₂₈-*b*-P(D,L-Leu)₂₀ micelles prepared at different temperatures.

Sample	D _{eff} ^d (nm)	PDI ^a	M _{w,app} ^b	M _{n,app} ^b	M _w /M _n	N _{agg} ^c
PEG ₂₂₈ - <i>b</i> -P(D,L-Leu) ₂₀ /m - 60 °C	58	0.12	3.3 × 10 ⁶	2.4 × 10 ⁶	1.39	267
PEG ₂₂₈ - <i>b</i> -P(D,L-Leu) ₂₀ /m - RT	142	0.26	4.6 × 10 ⁷	3.3 × 10 ⁷	1.42	2,653

^aHydrodynamic diameter (D_{eff}) and PDI were measured by DLS (water, pH 6.5, 25°C).
^bThe apparent molecular weight (M_{w,app}, M_{n,app}) of the micelles were estimated by static light scattering.
^cNumber of copolymer chains associated in a micelle (N_{agg}) was calculated as N_{agg} = (M_n of micelles)/(M_n of polymer).

Table 3

Thermodynamic profiles for the temperature-induced transition of PEG₂₂₈-*b*-PLeu₂₀ micelles at 25 °C.^a

Sample	T_m (°C)	H_{cal} (kcal/mol)	G_{25} (kcal/mol)	$T \Delta S$ kcal/mol
PEG-P(D,L-Leu)/m – 60 °C	40.8	20.3	–1.0	–19.3
PEG-P(L-Leu)/m – 60 °C	40.8	9.5	–0.5	–9.0
PEG-P(D,L-Leu)/m – RT	37.1	13.0	–0.5	–12.5

^aAll parameters were determined in water at pH 6.5. The T_m values are within 0.5°C, H_{cal} ($\pm 3\%$) and G_{25} (5%).

Table 4Physicochemical characteristics of drug-loaded micelles^a

Micelle	Sample	D _{eff} (nm)	PDI	LC (%w/w) ^b
PEG-P(D,L-Leu)/m	Empty micelles	58 ± 1	0.15 ± 0.01	-
	17-AAG/m	57 ± 2	0.17 ± 0.03	4.0
	PTX/m	52 ± 1	0.11 ± 0.02	3.0
	(17-AAG + PTX)/m	47 ± 1	0.15 ± 0.02	6.6 (17-AAG) 3.0 (PTX)
	Empty micelles	92 ± 2	0.14 ± 0.03	-
PEG-P(L-Leu)/m	17-AAG/m	85 ± 1	0.18 ± 0.05	2.3
	PTX/m	83 ± 2	0.18 ± 0.01	0.8
	(17-AAG + PTX)/m	80 ± 2	0.16 ± 0.01	2.6 (17-AAG) 1.0 (PTX)

^aHydrodynamic diameters (D_{eff}) and polydispersity index (PDI) were determined in water (pH 6.5) by DLS. Data are expressed as mean ± SD (n = 3).

^bLoading capacity (LC) is expressed as mass of incorporated drug per mass of drug-loaded micelles (w/w). Drugs content were determined by HPLC.

Table 5

Comparison of IC₅₀ values for drug-loaded PEG-P(D,L-Leu) micelles and free drugs against breast cancer cell lines^a

Sample	IC ₅₀ (nM)	
	BT-474 (ErbB2-high)	MCF-7(ErbB2-low)
17-AAG	8 ± 0.4	85 ± 1.2
PTX	14 ± 0.3	28 ± 0.6
*17-AAG + PTX	0.043 ± 0.01 (CI = 0.09)	62 ± 0.6 (CI = 0.79)
17-AAG/m	16 ± 1.3	116 ± 1.0
PTX/m	31 ± 1.0	45 ± 0.8
*(17-AAG+PTX)/m	0.12 ± 0.03 (CI = 0.08)	92 ± 0.7 (CI = 0.9)
17-AAG/m + PTX/m	0.18 ± 0.12 (CI = 0.09)	94 ± 1.0 (CI = 0.9)

^aIC₅₀ values were calculated with respect to 17-AAG and represent mean ± SD of at least three independent experiments.

*Combination of 17-AAG and PTX was examined at a 2:1 w/w ratio. CI - combination index, calculated at IC₅₀. Each of the drug combinations groups differs significantly from the free drug or single-drug loaded micelles ($p < 0.001$).

Table 6

IC₅₀ values for targeted and non-targeted drug-loaded micelles against breast cancer cell lines^a

Sample	IC ₅₀ (nM)	
	BT-474 (ErbB2 high)	MCF-7 (ErbB2 low)
IgG-(17-AAG + PTX)/m	0.14 ± 0.03	97 ± 6
Trast-(17-AAG + PTX)/m ^{**}	0.03 ± 0.02	85 ± 9
Trast-(17-AAG) + Trast-(PTX)/m	0.05 ± 0.03	102 ± 11

^aThe IC₅₀ values were calculated with respect to 17-AAG and represent mean ± SD of at least three independent experiments. Combination of 17-AAG and PTX was examined at a 2:1 w/w ratio.

^{**}
 $p < 0.01$ vs. IgG-(17-AAG + PTX)/m