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Aminoflavone-Loaded EGFR-Targeted Unimolecular Micelle Nanoparticles Exhibit Anti-Cancer Effects in Triple Negative Breast Cancer

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Abstract

Triple negative breast cancer (TNBC) is an aggressive subtype of breast cancer for which there is no available targeted therapy. TNBC cases contribute disproportionately to breast cancer-related mortality, thus the need for novel and effective therapeutic methods is urgent. We have previously shown that a National Cancer Institute (NCI) investigational drug aminoflavone (AF) exhibits strong growth inhibitory effects in TNBC cells. However, *in vivo* pulmonary toxicity resulted in withdrawal or termination of several human clinical trials for AF. Herein we report the *in vivo* efficacy of a nanoformulation of AF that enhances the therapeutic index of AF in TNBC. We engineered a unique unimolecular micelle nanoparticle (NP) loaded with AF and conjugated with GE11, a 12 amino acid peptide targeting epidermal growth factor receptor (EGFR), since EGFR amplification is frequently observed in TNBC tumors. These unimolecular micelles possessed excellent stability and preferentially released drug payload at endosomal pH levels rather than blood pH levels. Use of the GE11 targeting peptide resulted in enhanced cellular uptake and strong

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growth inhibitory effects in TNBC cells. Further, AF-loaded, GE11-lacking, GE11-conjugated (targeted) unimolecular micelle NPs significantly inhibit orthotopic TNBC tumor growth in a xenograft model, compared to treatment with AF-loaded, GE11-lacking (non-targeted) unimolecular micelle NPs or free AF. Interestingly, the animals treated with AF-loaded, targeted NPs had the highest plasma and tumor level of AF among different treatment groups yet exhibited no increase in plasma aspartate aminotransferase (AST) activity level or observable tissue damage at the time of sacrifice. Together, these results highlight AF-loaded, EGFR-targeted unimolecular micelle NPs as an effective therapeutic option for EGFR-overexpressing TNBC.

Keywords

Aminoflavone; triple negative breast cancer; unimolecular micelle nanoparticles; GE11; targeted drug delivery

1. Introduction

Triple negative breast cancer (TNBC) lacks expression of common therapeutic targets estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and thus there are no targeted therapies available for these patients. TNBC constitutes 15–20% of all breast cancer diagnoses and is clinically aggressive, contributing to a disproportionately high number of metastatic cases and breast-cancer related deaths [1–3]. Current treatment for TNBC patients relies primarily on surgery, non-specific and toxic chemotherapies, and ionizing radiation [4, 5]. While a subset of TNBC is known to be chemotherapy-responsive, these patients represent a minority of TNBC cases, and overall, TNBC exhibits higher rates of recurrence and statistically shorter survival times [2, 6, 7]. Due to the lack of a consensus standard of care for TNBC combined with the aggressiveness of this disease, the development of novel therapies is crucial.

We and others have discovered that aminoflavone (AF; 4H-1-benzopyran-4-one,5-amino-2-(4-amino-3-fluorophenyl)-6,8-difluoro-7-methyl, NSC 686288), a synthetic flavonoid, exhibits significant growth inhibitory effects at low doses in human TNBC *in vitro* models [8, 9]. The anti-cancer effect of AF appears to be complex. Studies have shown that AF metabolites act as DNA damaging agents and promote adduct formation in DNA and other macromolecules [8, 10, 11]. In addition, AF treatment results in cell cycle arrest, apoptosis, and a senescence-like phenotype *in vitro* [8, 9, 12, 13]. Despite its promising anti-cancer effects *in vitro*, *in vivo* studies and clinical trials have revealed that AF may mediate dose-limiting pulmonary toxicity, halting its progress in the clinic [14, 15]. Given its effectiveness at low doses in models of aggressive TNBC, the development of alternative methods for delivering AF to tumors is key for reducing systemic toxicity while improving therapeutic outcome for TNBC.

Nanotechnology-based drug delivery systems may revolutionize cancer treatment by offering a viable method to overcome the off-target toxicities of otherwise promising cancer therapies because they can specifically target their payloads to tumor tissues via both passive and receptor-mediated active tumor targeting abilities, thereby decreasing systemic exposure while enhancing anti-cancer endpoints. The success of nanoparticle (NP)-based therapies to

treat TNBC depends partly on the NP's ability to harbor various moieties, including peptides, aptamers, and antibodies to specifically target TNBC tumors. Multiple studies and clinical trials have attempted to identify the key features of TNBC, including overexpression of epidermal growth factor receptor (EGFR) [16]. It is estimated that over half of all TNBC overexpresses or contains amplifications of EGFR [17, 18]. Because epidermal growth factor (EGF), the endogenous and activating ligand for EGFR, is mitogenic and neoangiogenic, alternative methods for targeting EGFR, including non-mitogenic peptides, are needed [19]. One such peptide is GE11 (amino acid sequence YHWYGYTPQNVI), which binds with good efficiency to EGFR and lacks the growth-promoting effects of EGF [20]. Thus, given the EGFR overexpression associated with TNBC, the ability to target EGFR using the GE11 peptide, and the anti-cancer properties of AF in models of TNBC, we hypothesize that AF-loaded NPs conjugated with GE11 have therapeutic potential in TNBC.

Many types of NPs exist, but polymer micelles are among the most widely studied nanocarriers for hydrophobic drugs [21–23]. Conventional multi-molecular polymer micelles formed by self-assembly of multiple linear amphiphilic block copolymers tend to exhibit insufficient *in vivo* stability. Premature dissociation of the self-assembled multi-molecular polymer micelles during circulation can cause a burst release of drug into the bloodstream, leading to potential systemic toxicity and undermining their tumor-targeting ability, thereby severely limiting their use for *in vivo* applications [23–27]. In contrast, judiciously engineered unimolecular micelles formed by individual multi-arm star amphiphilic block copolymers possess excellent *in vitro* and *in vivo* stability while offering high drug loading capacity [28–34].

In this study, we developed multifunctional unimolecular micelles conjugated with EGFR-binding peptide GE11 to encapsulate and deliver AF to TNBC. The AF-loaded, GE11 conjugated NPs enhance cellular uptake and cytotoxicity of AF *in vitro* and potentiate the *in vivo* efficacy of AF to inhibit tumor growth in TNBC xenografts.

2. Materials and Methods

2.1 Reagents

Aminoflavone (AF) was obtained from the Developmental Therapeutics Program Repository of the National Cancer Institute at Frederick (Frederick, MD), and was stored in dimethyl sulfoxide (DMSO) (100 mM). Poly(amidoamine) (PAMAM) dendrimer (ethylenediamine core, generation 4.0), L-lactide (LA), stannous (II) octoate ($\text{Sn}(\text{Oct})_2$), and triethylamine (TEA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). The poly(ethylene glycol) (PEG) derivatives, maleimide-PEG-COOH (Mal-PEG-COOH) (MW = 5 kDa), NH_2 -PEG-COOH (MW = 5 kDa) and OCH_3 -PEG-COOH (Mw = 5 kDa) were acquired from JenKem Technology (Allen, TX, USA). Cy5.5 was purchased from Lumiprobe Corporation (Hallandale Beach, FL, USA). All other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and used as received. Phosphate buffered solutions (PBS, pH 7.4) and acetate buffered solutions (ABS, pH 5.3) were freshly prepared. GE11 peptide (YHWYGYTPQNVIGGGGC) was synthesized by Tufts University Core Facility (Boston, MA, USA). Zileuton [N-(1-benzobthien-2-ylethyl)-N-hydroxyurea], an internal standard used for mass spectrometry, was obtained from Sigma (St. Louis, MO, USA) and was stored

in methanol (10 mg/mL). 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was obtained from VWR (Radnor, PA, USA).

2.2 Synthesis of poly(amidoamine)–polylactide–poly(ethylene glycol)–OCH₃/Cy5.5/GE11 (PAMAM-PLA-PEG-OCH₃/Cy5.5/GE11)

2.2.1 Synthesis of poly(amidoamine)–polylactide–OH (PAMAM–PLA–OH)—

PAMAM–PLA–OH was prepared by ring–opening polymerization of LA monomer using PAMAM–OH as the micro–initiator. A 50 mL two–neck flask equipped with an argon gas inlet was charged with PAMAM–OH (20 mg, 1.4 μ mol) and LA (172 mg, 1.2 mmol), and placed in an oil bath. After the mixture melted at 120 °C, a catalytic amount of Sn(Oct)₂ ([Sn(Oct)₂]/[LA]=1/1000 mol/mol) was added to start the reaction. The reaction was carried out at 120 °C for 24 h. Thereafter, the mixture was rapidly cooled down to the room temperature and dissolved in 2 mL of tetrahydrofuran (THF). The resulting solution was precipitated dropwise into methanol to yield a pale yellow powder. The products were dried under vacuum.

2.2.2 Synthesis of PAMAM–PLA–PEG–OCH₃—PAMAM–PLA–PEG–OCH₃ was

prepared by esterification between PAMAM–PLA–OH and OCH₃–PEG–COOH. In brief, PAMAM–PLA–OH (20 mg, 0.38 μ mol) and OCH₃–PEG–COOH (121 mg, 24.3 μ mol) were dissolved in 10 mL anhydrous dimethyl sulfoxide (DMSO). Dicyclohexylcarbodiimide (DCC; 5.5 mg, 26.7 μ mol) and 4-dimethylaminopyridine (DMAP; 0.65 mg, 5.3 μ mol) were added as catalysts. The reaction was carried out at room temperature for 24 h. After the by–product dicyclohexylcarbodiurea (DCU) was removed by filtration, the solution was precipitated into cold diethyl ether. The precipitate was re-dissolved in deionized water and dialyzed against deionized water for 48 h using cellulose membrane (molecular weight cut–off 15 kDa). The multi-arm star amphiphilic block copolymer PAMAM-PLA-PEG-OCH₃ (used for the preparation of AF-loaded non-targeted unimolecular micelle NP) was obtained after lyophilization.

2.2.3 Synthesis of PAMAM–PLA–PEG–OCH₃/Mal/NH₂—PAMAM–PLA–OH (20

mg, 0.38 μ mol), OCH₃–PEG–COOH (73 mg, 14.6 μ mol), maleimide (Mal)–PEG–COOH (36.5 mg, 7.3 μ mol) and NH₂–PEG–COOH (12 mg, 2.4 μ mol) were dissolved in 10 mL anhydrous DMSO in the presence of DCC (5.5 mg, 26.7 μ mol) and DMAP (0.65 mg, 5.3 μ mol). The reaction was carried out at room temperature for 24 h. After the by–product DCU was removed by filtration, the solution was precipitated into cold diethyl ether. The precipitate was then dialyzed against deionized water for 48 h using cellulose membrane (molecular weight cut–off 15 kDa) and freeze–dried. The multi-arm star amphiphilic block copolymer PAMAM–PLA–PEG–OCH₃/Mal (for the preparation of PAMAM–PLA–PEG–OCH₃/GE11) and PAMAM–PLA–PEG–OCH₃/NH₂ (for the synthesis of PAMAM–PLA–PEG–OCH₃/Cy5.5) were also synthesized with a similar method by using appropriated PEGs.

2.2.4 Synthesis of PAMAM–PLA–PEG–OCH₃/Cy5.5/GE11—PAMAM–PLA–PEG–

OCH₃/Mal/NH₂ (10 mg, 47 nmol) and GE11-SH (360 μ g, 232 nmol) were dissolved in 3 mL anhydrous DMSO. The pH was adjusted between 6.0 and 6.5. After continuous stirring

for 8 h, Cy5.5 (65 μ g, 93 nmol) was added and the pH was adjusted to 8.5. The reaction was further carried out at room temperature for 12 h. The impurities were removed by dialysis against deionized water for 48 h using cellulose membrane (molecular weight cut-off 15 kDa) and the final product was freeze-dried. The PAMAM-PLA-PEG-OCH₃/GE11 (for the preparation of AF-loaded targeted unimolecular micelle NPs) was synthesized by the reaction between PAMAM-PLA-PEG-OCH₃/Mal and GE11, and PAMAM-PLA-PEG-OCH₃/Cy5.5 (without GE11) was prepared using PAMAM-PLA-PEG-OCH₃/NH₂ and Cy5.5.

2.3 Preparation of AF-Loaded Unimolecular Micelles

To prepare AF-loaded non-targeted unimolecular micelles, AF (25 mg) and PAMAM-PLA-PEG-OCH₃ (75 mg) were dissolved in 3 mL dimethylformamide (DMF) at room temperature. Deionized water was added dropwise into the above solution. After 4 h of continuous stirring, DMF and free AF were removed by dialysis against deionized water using a 15 kDa cut-off cellulose membrane. The final product was freeze-dried. The AF-loaded targeted unimolecular micelles were prepared with a similar method using PAMAM-PLA-PEG-OCH₃/GE11 polymer.

2.4 Characterization of Polymer Products

The proton nuclear magnetic resonance (¹H NMR) spectra of all polymer products were recorded on a Varian Mercury Plus 300 spectrometer (International Equipment Trading Ltd., USA) using CDCl₃ or DMSO-d₆ as a solvent at 25 °C. Fourier Transform infrared (FT-IR) spectra were recorded on a Bruker Tensor 27 FT-IR spectrometer (Bruker Corporation, USA). Molecular weights (number average molecular weight (M_n) and weight average molecular weight (M_w)) and polydispersity indices (PDI) of all polymers were determined by gel permeation chromatography (GPC; Viscotek, USA) equipped with a refractive index detector, a viscometer detector, and a light scattering detector. DMF with 0.1 mmol/L of LiBr was used as a mobile phase with a flow rate of 1 mL/min. The hydrodynamic size and zeta potential of the unimolecular micelles were studied by dynamic light scattering (DLS, ZetaSizer Nano ZS90, Malvern Instruments, USA). The sizes and morphologies of the unimolecular micelles were determined transmission electron microscopy (TEM, FEI Tecnai G2 F30 TWIN 300 KV, E.A. Fischione Instruments, Inc., USA). The TEM sample was prepared by depositing a drop of the copolymer solution (0.05 mg/ml) containing 1 wt% of phosphotungstic acid onto a 200 mesh copper grid coated with carbon. The AF drug loading level, defined as the weight percentage of AF in the AF-loaded unimolecular micelles, was measured by UV-Vis spectrometer (Varian Cary 300 Bio UV-Vis spectrophotometer, Agilent Technologies, USA) at 357 nm.

2.5 In Vitro Drug Release

AF-loaded unimolecular micelles (5 mL, 1 mg/mL) were enclosed in a cellulose membrane dialysis bag (molecular weight cut-off, 15 kDa) and incubated in 50 mL PBS (pH 7.4) or ABS (pH 5.3) under mild agitation at 37 °C. At predetermined time intervals, 3 mL samples were collected and replaced with the same volume of fresh media. The amount of AF in the collected samples was measured by UV-Vis spectrometer at 357 nm.

2.6 Human Breast Cancer Cell Lines

Human MDA-MB-468 and BT474 cells were purchased from ATCC (Manassas, VA), and maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY) at 37 °C and 5% CO₂. MDA-MB-468 are epithelial mammary adenocarcinoma cells from a pleural effusion. BT474 are epithelial ductal carcinoma cells. MDA-MB-468 is ER/PR/HER2 negative, while BT474 is ER positive, PR+/- and HER2 positive. Relating to this study, MDA-MB-468 harbors a genetic amplification of EGFR, and BT474 expresses a relatively low level of EGFR. Cells were passaged for fewer than six months after receipt or resuscitation, and ATCC characterizes cell lines using short tandem repeat profiling. We routinely performed mycoplasma analysis of all cultured cell lines to ensure no contamination.

2.7 Cellular Uptake Studies

Cellular uptake of the unimolecular micelle NPs conjugated to or lacking GE11 was qualitatively examined using fluorescent microscopy based on Cy5.5 dye conjugated to the NPs. 2.5×10^4 MDA-MB-468 or BT474 cells were seeded in each well of an eight-well chamber slide system (Nunc ® Lab-Tek®, Sigma Alrich, St. Louis, USA) treated with control media (DMEM + 2% FBS), media containing 100 µg/mL of GE11-lacking NPs (non-targeted NPs; NT), or media containing 100 µg/mL GE11-conjugated NPs (targeted NPs; T) in duplicate wells per treatment. Cells were treated for 15 or 120 minutes at 37 °C and 5% CO₂, washed once with 1× PBS (Gibco, Grand Island, NY, USA), fixed with 4% paraformaldehyde in 1× PBS for ten minutes at room temperature, and mounted with ProLong Gold Antifade containing 4',6-diamidino-2-phenylindole (DAPI) mountant (Invitrogen, Carlsbad, CA, USA). Images were acquired on a three-channel multi-purpose confocal microscope (Prairie Technologies/Bruker Nano Fluorescence Microscopy, Middleton, WI). Cellular uptake was quantitatively examined using flow cytometry. 2.0×10^5 MDA-MB-468 or BT474 cells were seeded in each well of a six-well plate and treated with control media (DMEM + 2% FBS), media containing 100 µg/mL non-targeted NPs, or media containing 100 µg/mL targeted NPs in triplicate wells per treatment. Cells were treated for 15 or 120 minutes at 37 °C and 5% CO₂, washed twice with 1× PBS and harvested with 0.25% trypsin (Gibco, Grand Island, NY, USA). Cells were collected by centrifugation at 1,000 rpm/179 × g for four minutes, washed twice with 1× PBS, and resuspended in 500 µL 1× PBS for analysis. Cellular uptake of the NPs was examined by quantifying Cy5.5 fluorescence intensity using a FACSCalibur instrument (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software. Data are representative of triplicate experiments.

2.8 Measuring Cell Viability Using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) Assay

To determine the effects of unimolecular micelle treatment on cell viability, MTT assays were performed. MDA-MB-468 or BT474 cells were seeded at a density of 5,000 cells per well in DMEM +10% FBS in clear 96-well plates. Cells were treated with control media (0.1% DMSO in DMEM + 2% FBS), media containing free AF, AF-loaded GE11-conjugated targeted (AF-T) or AF-loaded GE11-lacking non-targeted (AF-NT) unimolecular

micelles at a final concentration of 0.01, 0.05, or 0.1 $\mu\text{g/mL}$ AF, or equivalent concentrations of empty (i.e., no AF encapsulation) targeted (T) or non-targeted (NT) micelles. Each treatment condition contained at least three replicate wells. Cells were incubated with treatment for 24 hours at 37 °C and 5% CO_2 . MTT treatment and absorbance measurement was performed as described elsewhere [9]. Data are representative of triplicate experiments and is normalized as a percentage of the 0.1% DMSO-treated control samples.

2.9 *In Vivo* Analysis of Tumor Growth

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. MDA-MB-468 cells were injected bilaterally into the inguinal mammary fat pads of 5 to 6 week old athymic nude-Foxn1^{nu} mice (Harlan Laboratories, Indianapolis, IN, USA) (1×10^6 cells/site) and allowed to form palpable tumors (average approximate volume = 500 mm³) for approximately eight weeks. Treatment (saline control, free AF, AF loaded into either targeted (AF-T) or non-targeted (AF-NT) unimolecular micelles at equivalent concentration of 7 mg AF/kg•body weight (BW)) was intravenously administered to mice via the retro-orbital venous sinus under brief exposure to isofluorane anesthetic every fourth day for a total of five administrations. Beginning the day prior to the first administration and every other day thereafter, each tumor was measured using an electronic caliper and tumor volume was approximated using a modified ellipsoid formula [35]. To account for variability in initial tumor volume, normalized tumor volume (volume at time x $[V_x]$ divided by volume at time zero $[V_0]$) was calculated. Animals were sacrificed at day 46 due to tumor burden in the saline control group.

2.10 Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS) Analysis of Plasma AF Concentration

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Post administration of treatment in the aforementioned 46 day *in vivo* study (one hour post injection #1, four hours post injection #2, eight hours post injection #3, and 24 hours post injection #4), approximately 100–200 μL of whole blood was collected from the maxillary vein of each animal into heparin-coated tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged at 4 °C for ten minutes at $1,300 \times g$, and the plasma supernatant was stored at -80 °C until analysis. Prior to analysis, frozen samples were thawed at ambient temperature, and 25 μL of sample was utilized for each extraction. The extraction method was modified from previous work [36]. AF concentrations were analyzed by high-performance liquid chromatography (Agilent 1100, Santa Clara, CA, USA) coupled with tandem mass spectrometric detection (5500 QTRAP, AB/SCIEX, Framingham, MA, USA) using Turbo Ion SprayTM positive mode ionization. Aliquots of 10 μL of reconstituted sample were injected onto a 2.6 μm , 100A, 4.6×100 mm Phenomenex Kinetex C₁₈ Column (Phenomenex, Torrance, CA, USA) and separated by isocratic elution at 0.4 mL/min flow rate using a 12 minute run time (solvent A = 0.1% Formic acid, solvent B = methanol). Specific experimental conditions are described in the supplemental information. Data were analyzed using SCIEX Analyst v1.5.1 software.

2.11 LC/MS/MS Analysis of Intratumoral AF Concentration

All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison. Using the aforementioned orthotopic xenograft model, mice were treated with saline control, free AF, AF loaded into either targeted (AF-T) or non-targeted (AF-NT) unimolecular micelles at equivalent concentration of 7 mg AF/kg•BW retroorbitally under brief exposure to isoflurane anesthetic. Animals were sacrificed four hours after treatment and tumors were harvested and stored at -80°C . After thawing, tumors were cut into samples of equal wet weight and homogenized using a Pressure BioSciences shredder SG3 (Pressure BioSciences, Inc., South Easton, MA, USA) in the presence of extraction reagent ethyl acetate. Homogenizer tubes were rinsed three times with equal volumes of ethyl acetate, and samples were subjected to LC/MS/MS. Specific experimental conditions are described in the supplemental information.

2.12 Aspartate Aminotransferase (AST) Activity Assay

Per manufacturer protocol, mouse plasma collected via the maxillary vein at two time points during the 46 day *in vivo* study (days 4 and 13) was subjected to a colorimetric assay to measure aspartate aminotransferase (AST) activity (Sigma, MAK055, St. Louis, MO). Briefly, 10 μL of plasma was added per well in duplicate in a clear 96-well plate and brought to a volume of 50 μL with the provided AST assay buffer. A glutamate standard curve, blank, and positive control were also added to the plate in duplicate. Then, 100 μL of master mix, generated using the provided AST assay buffer, AST enzyme mix, AST developer, and AST substrate was added to each well. The plate was incubated at 37°C for 2–3 minutes, and then absorbance at 450 nm was read on a Perkin Elmer Victor X5 plate reader (Perkin Elmer, Waltham, MA, USA). The plate was again incubated at 37°C and absorbance was read every five minutes until the most active sample saturated the values seen in the highest standard. Absorbance values were corrected for background and the change in absorbance from the initial read to the final read was calculated. Using the standard curve, the amount of glutamate generated in the plasma samples was calculated, and this value was used to determine AST assay in milliunits/mL.

2.13 Histologic Analysis

Mouse lung, liver and kidney samples collected after the 46 day *in vivo* study were fixed in 10% formalin for 48 hours then placed in two changes of 70% ethanol for storage. Samples were then embedded in paraffin and cut into sections of between 5–7 μm . Rehydration was performed, followed by staining with hematoxylin, differentiation in acidic alcohol (0.1%), staining with eosin counterstain, dehydration with 100% ethanol, a xylene rinse, and finally mounting with Cytoseal™ XYL (Thermo Scientific, Waltham, MA, USA). Samples were imaged using a Leica DM 5000B upright microscope using the Leica Applications Suite software (Leica, Buffalo Grove, IL, USA).

2.14 Statistical Analysis

MTT data are displayed $\pm$ standard deviation. Two-tailed, unpaired Student's T Tests were performed for statistical analysis of MTT data using Microsoft Excel, where ** is $p < 0.005$

compared to DMSO control. Tumor volume data are also displayed $\pm$ standard deviation. Tumor volume was modeled using a mixed repeated measures model in SAS (SAS Institute, Inc. SAS OnlineDocR 9.1.4. Cary, NC, USA). Akaike's Information Criteria was used to choose the most suitable covariance structure for the overall experimental error (mouse over time); this was the heterogeneous autoregressive covariance structure. F-tests from this model were used to generate p-values was performed to determine differences between treatment arms at various time points; a Tukey adjustment was done at each time to adjust for multiple comparisons. For tumor volume data, *** indicates $p < 0.001$. AF plasma concentration data are displayed $\pm$ standard deviation, and two-tailed unpaired Student's T Tests were applied, where * is $p < 0.05$, ** is $p < 0.005$, and *** is $p < 0.001$.

3. Results

3.1 Preparation of the Multi-arm Star Amphiphilic Block Copolymer PAMAM-PLA-PEG-OCH₃/Cy5.5/GE11

The multi-arm star amphiphilic block copolymer PAMAM-PLA-PEG-OCH₃/Cy5.5/GE11 (Figure 1A) was synthesized following the scheme described in Figure 1B. Briefly, PAMAM-PLA-OH was synthesized by ring opening polymerization of LA monomer, using a dendrimer PAMAM-OH (G4; 64 terminal -OH groups) as the micro-initiator. The chemical structure of PAMAM-PLA-OH was confirmed by ¹H NMR (Figure S1A). The peaks at (a) 5.16 and (b) 1.54 ppm are ascribed to the protons of methane and methyl groups in the PLA main chains, respectively. The signal at (c) 4.35 ppm corresponding to the terminal methane protons of PLA can also be identified. The peaks located at 2.75, 2.83 and 3.48 ppm, indicated by arrows in the Figure S1A, were assigned to PAMAM core. FT-IR spectrum, shown in Figure S1B, further confirmed the successful formation of PAMAM-PLA. The peaks at 1723 cm⁻¹ is assigned to ester bond of PLA arms. The peaks at 1652 cm⁻¹ and 1540 cm⁻¹ are ascribed to C=O stretching and N-H bending of the amide groups of PAMAM core. According to the ¹H NMR spectrum, the repeat units in PLA arms were determined to be 16, based on the relative intensity ratio of the peaks at (a) 5.16 ppm and (c) 4.35 ppm. The average number of arms (# arm) per PAMAM-PLA was estimated by comparing the molecular weights of PAMAM-OH and PAMAM-PLA determined by GPC, as shown in Table S1. The # arm per PAMAM-PLA was estimated to be 33 according to Eq. 1.

$$\#arm = (Mn_{PAMAM-PLA-OH} - Mn_{PAMAM-OH}) / Mn_{PLA} \quad \text{Eq. 1}$$

In the next step, three types of PEG, OCH₃-PEG-COOH, Mal-PEG-COOH and NH₂-PEG-COOH, were conjugated onto the PAMAM-PLA-OH via ester linkages to form the multi-arm star amphiphilic block copolymer PAMAM-PLA-PEG-OCH₃/Mal/NH₂. The feed molar ratio of OCH₃-PEG-COOH, Mal-PEG-COOH and NH₂-PEG-COOH was set at 6:3:1. The maleimide terminal groups were used for GE11 peptide conjugation and NH₂ terminal groups for Cy5.5 conjugation. In the ¹H NMR spectrum of PAMAM-PLA-PEG-OCH₃/Mal/NH₂ (Figure S1C), in addition to the peaks from PLA blocks, the peaks at 3.67 ppm and 3.37 ppm were observed due to the methylene protons of oxyethylene units and

methoxyl protons of PEG, respectively. The peak at 6.67 ppm was ascribed to the proton of the maleimide functional group. The molar ratio of the maleimide functional group was calculated to be 20%, which is based on the relative intensity ratio of the peaks at 6.68 ppm and 3.67 ppm corresponding to the proton of the maleimide group and the methylene protons in the PEG main chains. Thus, the ^1H NMR spectra clearly demonstrated the formation of the multi-arm star amphiphilic block copolymer PAMAM-PLA-PEG-OCH₃/Cy5.5/GE11, which is further supported by the molecular weight measure shown in Supplemental Table S1. The # arm per PAMAM-PLA was estimated to be 33 according to Eq. 2.

$$\# \text{arm} = (\text{Mn}_{\text{PAMAM-PLA-PEG}} - \text{Mn}_{\text{PAMAM-PLA-OH}}) / \text{Mn}_{\text{PEG}} \quad \text{Eq. 2}$$

Lastly, GE11 peptide was conjugated by the maleimide-thiol reaction. Cy5.5 dye was also conjugated onto the ends of the PEG for visualization and detection of the unimolecular micelles. The molar ratio of PAMAM-PLA-PEG-OCH₃/Mal/NH₂, GE11 and Cy5.5 was set at 1: 4: 2 (approximately 4 GE11 peptide molecules and 2 Cy5.5 dye molecules per unimolecular micelle). The new peaks between 6.5 and 7.0 ppm attributed to GE11 and Cy5.5 were observed in Figure S1D.

3.2 Unimolecular Micelles are Stable and Relatively Uniform in Size

Single multi-arm star amphiphilic block copolymers such as PAMAM-PLA-PEG with a sufficient number of arms and proper hydrophobic to hydrophilic ratios can form a stable unimolecular micelle in an aqueous solution due to their globular structure [28, 30]. For drug nanocarriers, size distribution and aqueous stability are two key parameters that influence *in vivo* performance. To test the size of unimolecular micelles, DLS and TEM analyses were performed. The size distribution histogram of the micelles in an aqueous solution determined by DLS is shown in Figure 2A. The hydrodynamic diameter of the micelles ranged from 20 to 100 nm with an average diameter of 58 nm (polydispersity index = 0.12). TEM images of the dried unimolecular micelles (Figure 2B) confirmed that they were spherical with an average diameter around 32 nm. The stability of the unimolecular micelles was also studied. The size distribution of the micelles stored at 4°C in an aqueous solution for over one month were measured and compared with that of the freshly prepared micelles (Supplemental Figure S2). It was found that there were no obvious changes in the appearance (e.g., no precipitation) and the size of the micelle over one month, demonstrating its excellent stability.

3.3 AF-Loaded Unimolecular Micelles Show pH-Sensitive Drug Release Behavior *In Vitro*

The AF loading level of AF-loaded micelles was 16.7 wt%. The AF-loaded unimolecular micelles are taken up by endocytosis where acidic endocytotic organelles (i.e., endosomes/lysosomes) are involved. Therefore, *in vitro* drug release was carried out at 37 °C in PBS (pH 7.4) and ABS (pH 5.3), which mimics physiological conditions (e.g., the bloodstream, intracellular cytosol and extracellular space) and acidic endocytotic compartments, respectively. Drug release was measured in both AF-loaded, GE11-conjugated (Targeted) and AF-loaded GE11-lacking (Non-Targeted) unimolecular micelles. As shown in Figure

2C, AF was released from the Non-Targeted micelles to a greater extent at pH 5.3 than at pH 7.4. At pH 5.3, 40 % of the total AF was released after 24 h, and 65 % after 144 h. However, at pH 7.4, only 12 % of the AF was released after 24 h and 26 % was released after 144 h. Similar percentages of drug release were observed for the Targeted micelles (Figure 2C). These results clearly demonstrate that AF-loaded micelles exhibit a pH-dependent *in vitro* release behavior regardless of GE11 conjugation status.

3.4 GE11 Conjugation Enhances the Cellular Uptake of Unimolecular Micelles in EGFR-overexpressing MDA-MB-468 Human TNBC Cells

To evaluate the effectiveness of GE11 as an agent to target EGFR *in vitro*, cellular uptake of GE11-conjugated (Targeted) or GE11-lacking (Non-Targeted) unimolecular micelles conjugated with fluorescent dye Cy5.5 was examined in EGFR-overexpressing human TNBC cell line MDA-MB-468 and EGFR low human breast cancer cell line BT474. Relative EGFR protein levels in these two cell lines and other breast cancer cell lines, as well as the associated protocol, can be found in the supplemental information (Supplemental Figure S3). As demonstrated in Figure 3A and 3B, targeted micelles accumulated to a greater extent in the cytoplasm of MDA-MB-468 cells than the non-targeted micelles at both the 15 and 120 minute time points. Furthermore, EGFR low BT474 human breast cancer cells treated with targeted and non-targeted micelles did not exhibit a qualitative difference in Cy5.5 fluorescence at either time point (Supplemental Figure S4). These data suggest that GE11 can effectively target overexpressed EGFR in MDA-MB-468, but has no effect on cellular uptake in models that lack EGFR overexpression. This finding was validated by another quantitative method, flow cytometry, in MDA-MB-468 and BT474 cells. The analysis showed that MDA-MB-468 cells treated with targeted micelles exhibited greater Cy5.5 fluorescence intensity compared to cells treated with non-targeted micelles, as indicated by a right shift in fluorescence intensity of Cy5.5 at both the 15 minute and 120 minute time points (Figure 3C and 3D). Unlike EGFR-overexpressing MDA-MB-468 cells, BT474 cells did not show any difference in fluorescence intensity between cells treated with targeted and non-targeted micelles (Figure 3E and 3F). Combined, these data indicate that conjugation of the EGFR targeting peptide, GE11, to unimolecular micelles results in enhanced cellular uptake to an EGFR-overexpressing cell line model, but no differential cellular uptake in an EGFR low cell line model, suggesting that GE11 can effectively target the micelles to EGFR-overexpressing TNBC.

3.5 GE11 Conjugation Enhances the Cytotoxic Effects of AF-Loaded Unimolecular Micelles in EGFR-Overexpressing MDA-MB-468 Human TNBC Cells

After confirming that active targeting conferred by GE11 resulted in enhanced cellular uptake in EGFR-overexpressing human TNBC cell line MDA-MB-468, we examined whether AF-loaded targeted micelles elicited cytotoxic effects. In order to assess alterations in cell viability, MDA-MB-468 and BT474 were treated with control media, various concentrations of AF (either in free form, or encapsulated in targeted (AF-T), or non-targeted (AF-NT) micelles), or the corresponding amounts of empty (i.e., no AF encapsulation) targeted (T) or non-targeted NPs (NT) micelles for 24 hours and then subjected to MTT assays. As shown in Figure 4A, a statistically significant decrease in cell viability was observed when MDA-MB-468 cells were treated with AF-T micelles compared

to AF-NT micelles at all tested concentrations of AF. No dose-dependent or statistically significant difference in cell viability was seen in cells treated with empty T or NT micelles, suggesting that the micelles were not inherently cytotoxic. In addition, while cell viability was decreased in EGFR low BT474 cells treated with free AF, BT474 cells subjected to equivalent NP treatment did not exhibit any statistically significant differences in cell viability (Figure 4B). These data suggest that the targeted micelles not only exhibited enhanced cellular uptake in EGFR-overexpressing cells, but when loaded with AF, they elicited stronger cytotoxic effects compared to AF-loaded non-targeted micelles. This effect appears to be specific to and dependent on the interaction between GE11 and EGFR, as AF-loaded targeted and non-targeted micelles do not exhibit differential ability to decrease cell viability in EGFR low BT474 cells. Overall, these data serve as *in vitro* proof of concept that AF-loaded, GE11-conjugated targeted micelles may be an effective means to induce cytotoxic effects on TNBC which overexpress EGFR.

3.6 AF-Loaded GE11-Conjugated Unimolecular Micelles Mediate a Decrease in Xenograft Tumor Volume

To study the effects of AF-loaded targeted (AF-T) and AF-loaded non-targeted (AF-NT) micelles in an *in vivo* context, an orthotopic xenograft model of human TNBC and EGFR-overexpressing MDA-MB-468 cells engrafted into the inguinal mammary fat pads of athymic Foxn1^{nu} mice was utilized. Animals bearing palpable xenografts were treated with saline control, or different AF formulations (either in free form, AF-T, or AF-NT) at an equivalent concentration of 7 mg AF/kg•BW every four days for a total of five treatments (Figure 5A). Tumor volume was approximated every other day for the duration of the treatment schedule and subsequent to the treatment schedule (Figure 5B). Statistical analysis indicated that normalized tumor volume in AF-T treated mice was significantly lower than normalized tumor volume in AF-NT treated mice after day eight ($p=0.01$ on day eight, $p=0.002$ at day ten and $p<0.001$ on day 12 and after) (Figure 5B). These data suggest that conjugation of the EGFR-binding GE11 peptide to the micelles enhanced the tumor-targeting capability of the AF-loaded micelles in the EGFR-overexpressing xenograft animal, resulting in significant tumor shrinkage over time. Normalized tumor volumes in the AF-T treated group were also compared to the free AF treated group. Again, we observed that normalized tumor volume in AF-T treated mice was significantly lower than that in free AF treated mice after day eight ($p<0.001$ at day eight and after), suggesting that targeted micelles conferred therapeutic benefits over free drug treatment (Figure 5B). Together, these data demonstrated that intravenous administration of AF-loaded GE11-conjugated targeted micelles successfully decreased MDA-MB-468 xenograft tumor volume *in vivo*, and that the anticancer efficacy of the AF-loaded targeted micelles were significantly higher than that of AF-loaded non-targeted micelles and free AF. Taken together, these results suggest that AF-loaded targeted micelles may be a viable therapeutic option to decrease tumor burden in EGFR-overexpressing TNBC.

3.7 AF Concentration in Plasma and Tumor Tissue is Greatest Using AF-Loaded Targeted Micelles

To determine whether the therapeutic formulations had an effect on plasma drug concentration, whole blood samples were collected from the maxillary vein of mice in the

aforementioned *in vivo* study. Given constraints on plasma volume required for analysis and the volume which can be obtained from a single animal, we constructed a time course with plasma collected one hour post injection #1, four hours post injection #2, eight hours post injection #3, and 24 hours post injection #4. As shown in Figure 6A, plasma AF levels were the highest in animals treated with AF-T. Statistically significant differences in plasma [AF] between AF-T and AF-NT treated animals as well as between AF-T and free AF-treated animals were observed at all time points. These data suggest that AF-loaded targeted micelles may have a longer circulation time than both AF-loaded non-targeted micelles and free AF formulations. AF-loaded targeted micelles were found to have a slightly more negative zeta potential than AF-loaded non-targeted micelles, with readings of -9.64 mV and 0.85 mV, respectively, which could potentially explain greater circulation time, and thus, higher plasma AF concentration [37]. We further performed mass spectrometry analysis of AF concentration on tumor tissue from animals exposed to short-term treatment (i.e., four hours) in order to directly assess the effectiveness of drug delivery. As shown in Figure 6B, the concentration of AF in the tumors from animals treated with AF-loaded targeted micelles was 10 and 72 fold greater than the concentration of AF in the tumors from mice treated with AF-loaded non-targeted micelles and free AF, respectively. The much higher AF accumulation observed in the tumors of the mice treated with targeted micelles may be attributed to two factors. First, the targeted micelles exhibited a longer circulation time as aforementioned, leading to a higher tumor accumulation of the micelles via the enhanced permeability and retention (EPR) effect. Second, once the targeted micelles reached the tumor tissue, they were taken up by the cancer cells much more effectively than non-targeted micelles through receptor (i.e. EGFR)-mediated endocytosis as supported by the *in vitro* cellular uptake studies discussed earlier, which can help retain the micelles entrapped in the tumor tissue.

3.8 Aspartate Aminotransferase (AST) Activity and Organ Histology is Similar Between Treatment Arms

In order to assess potential systemic toxicity during the treatment course, plasma collected from animals four hours post injection #2 (day 4) and 24 hours post injection #4 (day 13) was subjected to an AST activity assay. As shown in Figure 7A, AST activity levels in any of the treatment arms at 7 mg AF/kg•BW do not exhibit statistically significant differences from control animals at the time points examined. This suggests that compared to control animals, AF treatment in the form of free AF, AF-T, or AF-NT at an equivalent concentration of 7 mg AF/kg•BW, does not result in systemic toxicity using AST activity as a toxicity indicator. In addition to measuring AST activity during the course of treatment, lung, liver, and kidney tissue were harvested from control, free AF, AF-T, and AF-NT treated mice following completion of the *in vivo* experiment. It is important to note that these tissues were gathered after a period of just over four weeks where no treatments were administered. Histological analyses of tissues gathered from free AF, AF-T, and AF-NT treated animals compared to tissues gathered from saline treated animals revealed no obvious differences (Figure 7B). Lung tissue in all groups contained small amounts of inflammatory infiltrates (neutrophils and macrophages), but none of the groups exhibited indications of pulmonary toxicity above controls. Minimal perivascular damage in kidney and slightly increased mitotic rate and necrosis in liver samples was also observed, but

again, no indication of organ toxicity above what was present in saline-treated animals. These data suggest that treatment with free AF, AF-T, and AF-NT (all at equivalent doses of 7 mg AF/kg•BW) did not result in significant organ damage or toxicity approximately four weeks subsequent to the last administration of AF.

4. Discussion

The development of methods to treat TNBC is crucial given the “orphan” status of this targeted-therapy lacking disease. Multiple potential therapeutic targets have been explored in TNBC, including EGFR [38]. The EGFR signaling pathway supports proliferation, cell survival, and migration, thus, it is unsurprising that EGFR expression correlates with poorer prognosis in breast carcinomas [39]. Interfering with this pathway using monoclonal antibodies or tyrosine kinase inhibitors have been extensively examined, but clinical trials with these drugs have been less than promising. However, the overexpression of EGFR in various cancers is also being exploited as a method to deliver therapeutics via NP-based platforms [40–43]. Multiple non-targeted nanotherapeutics have been clinically examined for the treatment of TNBC, including paclitaxel-embedded liposomes and IT-101 (a camptothecin-conjugated cyclodextrin polymeric NP) [44]. In this study, we report the use of a non-mitogenic and non-neoangiogenic peptide, GE11, which binds to EGFR, as a targeting moiety for unimolecular micelles made of multi-arm star amphiphilic block copolymers PAMAM-PLA-PEG (which exhibits superior *in vivo* stability compared to other multi-molecular polymeric micelle models) and loaded with the anti-cancer drug AF as a potential therapeutic option for TNBC [20, 29–33].

As a free drug formulation, AF exhibits growth inhibitory effects in TNBC *in vitro* [8, 9]. Despite promising effects, AF is also associated with dose-limiting pulmonary toxicity. This and other drugs that display negative off-target effects can be re-evaluated using NP-based delivery systems that can deliver drug more specifically to targeted tumor tissues due to their passive (i.e., via the EPR effect) and active (i.e., receptor-mediated) tumor-targeting abilities [45, 46]. Drug nanocarriers can potentially increase the therapeutic index of the drug while reducing undesirable off-target effects and systemic toxicity [47]. In this case, active TNBC tumor-targeting ability was achieved by conjugating the EGFR-specific peptide GE11 onto the unimolecular micelles. In addition to their tumor-targeting abilities, the AF-loaded micelles also show a pH-dependent AF release behavior. This may be due to the protonation of the aniline group of AF in acidic conditions and this salt form of AF is readily soluble in water and thus can be easily released. This is similar to the pH-dependent release behavior of doxorubicin reported previously [30]. The faster degradation of the micelle core (PLA) at a lower pH may have also contributed to the faster AF release from the micelles. As such, the pH-sensitive drug release behavior can reduce the amount of the AF released during circulation in the blood stream (pH 7.4), and can provide a desirable level of drug to effectively kill cancer cells once the micelles are internalized into the endocytotic organelles, acting as another mechanism to reduce systemic drug exposure. Moreover, it can enhance the anti-proliferation efficacy of the AF-loaded micelles once they are taken into the acidic endocytotic organelles of the tumor cells.

Through fluorescent microscopy and flow cytometry analyses, we have demonstrated that GE11-conjugated micelles were taken up to a greater extent than non-targeted micelles by EGFR amplified human TNBC cell line MDA-MB-468. In contrast, differential cellular uptake was not observed in human BT474 breast cancer cells, which express relatively low levels of EGFR, suggesting that GE11 is an efficient means to target micelles to EGFR overexpressing cells and tissues. Further, we showed that use of the GE11 peptide on AF-loaded NPs allowed for AF to mediate its growth inhibitory effects in MDA-MB-468 to a significantly greater extent compared to identical treatment with non-targeted micelles. Enhanced cell death using AF-loaded GE11-conjugated micelles was not observed in EGFR low BT474 cells, which still exhibited sensitivity to the parent compound with a GI_{50} (growth inhibition) value around 50 nM (data not shown). Taken together, these data suggest that GE11 peptide can bind to EGFR efficiently and enable the micelles, as well as their payloads, to achieve active targeting to EGFR-overexpressing TNBC. In addition to its ability as a targeting ligand, GE11 does not exhibit the mitogenic and neo-angiogenic effects mediated by the endogenous ligand for EGFR [20]. In the context of cancer treatment, activation of the EGFR signaling pathway has a disadvantageous effect, so the usage of an EGFR-binding peptide that lacks these proliferative properties is desirable [19]. Another potentially disadvantageous aspect of using EGFR as a therapeutic target is that it is a single member of a multi-protein family which plays numerous roles in normal development and biology [48]. However, given the extent to which EGFR is overexpressed in breast cancers compared to other normal tissues, significant off-target effects are hypothesized to be unlikely (Supplemental Figure S5) [49]. Overall, given the limited clinical success of EGFR inhibition, use of EGFR-targeted micelles may provide an alternative method to take advantage of EGFR overexpression in a therapeutic context in TNBC.

In addition to highlighting the ability of AF-loaded GE11-conjugated micelles to effectively target and deliver AF to TNBC and mediate growth inhibitory effects *in vitro*, this study aims to examine anti-tumor effects in female athymic nude mice bearing xenografts of the proof of concept cell line used for *in vitro* studies, MDA-MB-468. We have shown that use of 7 mg AF/kg•BW encapsulated in targeted micelles mediates anti-tumor effects in a statistically significant manner compared to saline-treated animals and animals treated with free AF or AF-loaded non-targeted micelles, with no animals exhibiting weight loss. While AF-loaded non-targeted micelles also resulted in a decrease in normalized tumor volume compared to saline-treated animals and free AF treated animals at some but not all time points, it is clear that animals treated with AF-loaded, GE11-conjugated micelles (AF-T) exhibit the most drastic loss of tumor burden. These data indicate that AF-T treatment clearly reduced tumor volume to a greater extent than free AF or AF-NT at an equivalent concentration of 7 mg AF/kg•BW, and therapeutic effects may be achieved at even lower dosage. Furthermore, processed plasma samples from animals in this group contained the highest concentration of parental AF with a peak concentration one hour post injection. One possible explanation for this is the slightly negative zeta potential of the micelles upon conjugation with the GE11 peptide, which has been shown to reduce liver uptake and increase NP circulation time [37]. The increased circulation time would prolong the passive targeting ability of micelles through the EPR effect and once the micelles pass through the leaky tumor vasculature, the targeting ligand, GE11 would further enhance the tumor uptake

of micelles, which potentially explains the best anti-cancer efficacy of AF-T treatment. Despite the higher circulating level and tumor accumulation of AF in the AF-T treatment group, analysis of AST activity during the course of treatment as an indicator of potential systemic toxicity does not reveal differences among treatment groups. Further, lung, liver, and kidney tissue collected upon harvest and H&E stained gave no indication of differential toxicity between treatment groups and control. The data presented suggest that acute treatment-related toxicity as well as chronic, long-term organ toxicity/damage is negligible compared to control, and that use of AF-loaded GE11-conjugated micelles is an effective means of decreasing TNBC xenograft tumor burden *in vivo*.

In summary, we showed that conjugation of EGFR-specific peptide, GE11, to the unimolecular micelles enhanced the cellular uptake and growth inhibition of the AF-loaded micelles *in vitro* in a proof of concept human TNBC model that harbors a genetic amplification of EGFR, MDA-MB-468. When MDA-MB-468 cells were orthotopically xenografted in mice, treatment with AF-loaded, GE11-conjugated micelles mediated a significantly greater decrease in tumor volume and greater AF concentration in processed plasma compared to treatment with free AF or AF encapsulated in non-targeted micelles, with no indication of toxicity based on AST activity and negligible organ damage based on histologic analysis. Given the clinical aggressiveness and rate of recurrence for patients who have been diagnosed with this form of breast cancer, developing effective and safe treatments is crucial. Our results suggest that with optimization, this GE11-conjugated unimolecular micelle drug delivery system may be able to provide a much-needed targeted therapeutic option for TNBC overexpressing EGFR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2. DLS and TEM characterization of unimolecular micelles and pH-sensitive AF release from AF-loaded unimolecular micelle *in vitro*
A. DLS analysis and **B.** TEM image of the unimolecular micelles. **C.** *In vitro* drug release of AF from AF-loaded targeted and non-targeted unimolecular micelles at pH 7.4 and pH 5.3.

Figure 3. GE11-conjugated unimolecular micelles exhibited greater cellular uptake in EGFR-overexpressing MDA-MB-468 TNBC cells

Representative fluorescent microscopy taken at 100X (10X objective $\times$ 10X eyepiece) of MDA-MB-468 cells treated with control media, GE11-lacking (i.e., Non-Targeted) or GE11-conjugated (i.e., Targeted) unimolecular micelles for **A.** 15 minutes and **B.** 120 minutes. **C.** Representative fluorescence intensity measured via flow cytometry for Cy5.5 for MDA-MB-468 cells treated with control media, non-targeted or targeted unimolecular micelles for **C.** 15 minutes and **D.** 120 minutes. **E.** Representative fluorescence intensity measured via flow cytometry for Cy5.5 for BT474 cells treated with control media, Non-Targeted or Targeted unimolecular micelles for **E.** 15 minutes and **F.** 120 minutes. The concentration for all micelle-related treatment was 100 $\mu\text{g/mL}$.

Figure 4. AF-loaded GE11-conjugated unimolecular micelles mediated greater cell death than AF-loaded GE11-lacking micelles in EGFR-overexpressing MDA-MB-468 TNBC cells

MTT assay for **A.** MDA-MB-468 cells and **B.** BT474 cells treated with 0.01, 0.05, or 0.1 µg/mL of free AF, AF-loaded GE11-conjugated targeted micelles (AF-T), AF-loaded non-targeted micelles (AF-NT) or the equivalent concentration of empty (i.e., no AF encapsulation) targeted (T) or non-targeted (NT) micelles. Cells were incubated with treatment for 24 hours. Data are displayed as the MTT absorbance as a percentage of the control (relative cell viability) $\pm$ standard deviation with $n = 3$ and $**p < 0.005$.

Figure 5. Treatment with AF-loaded GE11-conjugated unimolecular micelles mediates a decrease in normalized MDA-MB-468 xenograft tumor volume compared to treatment with AF-loaded GE11-lacking nanoparticles *in vivo*

A. Schematic of xenograft and nanoparticle treatment schedule. MDA-MB-468 cells (1.0×10^6) were injected bilaterally into each mammary fat pad of nude mice and allowed to form palpable tumors. Initial tumor measurements were taken using an electronic caliper prior to the treatment schedule. Beginning on Day 0, animals ($n = 4$) were retro-orbitally injected with saline control, free AF, AF encapsulated in targeted (AF-T) or non-targeted (AF-NT) micelles at an equivalent concentration of 7 mg AF/kg•BW every four days for a total of five administrations. Mice were released from treatment after Day 16. At Day 0 and after, tumor volume was measured using an electronic caliper every other day. **B.** Calculated tumor volume at each time point was plotted as normalized initial tumor volume (V_X/V_0) + one standard deviation. For statistical comparison between AF-T and AF-NT, *** $p < 0.001$ at Day 12 and after ($p=0.01$ on Day 8, $p=0.002$ on day 10). For statistical comparison between AF-T and Free AF, *** $p < 0.001$ at Day 8 and after.

Figure 6. Treatment with AF-loaded GE11-conjugated unimolecular micelles *in vivo* results in greater plasma and intratumoral AF concentration

A. Graphical representation of AF concentration in the plasma of nude mice treated with saline control, free AF, AF encapsulated in GE11-conjugated targeted (AF-T) or non-targeted (AF-NT) micelles as determined by LC/MS/MS. The concentration for all treatment was set at 7 mg AF/kg•BW. Blood samples were collected one hour post injection #1, four hours post injection #2, eight hours post injection #3, and 24 hours post injection #4, and plasma was obtained and subjected to extraction for AF. n=1 sample per animal per time point, with n=4 per treatment group. Data are shown ± standard deviation, and *p 0.05, **p 0.005, ***p 0.001. **B.** Graphical representation of AF concentration in the tumors of nude mice treated with saline control, free AF, AF encapsulated in GE11-conjugated targeted (AF-T) or non-targeted (AF-NT) micelles as determined by LC/MS/MS. The concentration for all treatment was set at 7 mg AF/kg•BW. N = 4 tumors per treatment arm.

Figure 7. AST activity and histologic analysis of mouse lung, liver, and kidney tissue

A. AST activity during the course of 7 mg AF/kg•BW treatment in the form of free AF (AF), targeted AF-loaded, GE11-conjugated (AF-T) micelles, or non-targeted AF-loaded, GE11-lacking (AF-NT) micelles. Three plasma samples per treatment arm were analyzed in duplicate, and data are represented as average AST activity $\pm$ S.D. **B.** Representative bright field images of lung, liver, and kidney tissue samples from nude mice treated with saline control, free AF, AF encapsulated in targeted (AF-T) or non-targeted (AF-NT) micelles. Tissue samples were collected upon sacrifice at Day 46. The concentration for all treatment was set at 7 mg AF/kg•BW. Scale bar = 100 μ M.