Folate Receptor Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple Negative Breast Cancer

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Abstract

Triple negative breast cancer (TNBC) is a recalcitrant malignancy with no available targeted therapy. Off target effects and poor bioavailability of the FDA approved anti-obesity drug orlistat hinder its clinical translation as a repurposed new drug against TNBC. Here we demonstrate a newly engineered drug formulation for packaging orlistat tailored to TNBC treatment. We synthesized TNBC-specific folate receptor targeted micellar nanoparticles (NPs) carrying orlistat, which improved the solubility (70-80 μg/ml) of this water insoluble drug. The targeted NPs also improved the delivery and bioavailability of orlistat to MDA-MB-231 cells in culture and to tumor xenografts in nude mouse model. We prepared HEA-EHA copolymer micellar NPs by copolymerization of 2-hydroxyethylacrylate (HEA) and 2-ethylhexylacrylate (EHA), and functionalized them with folic acid and an imaging dye. Fluorescence activated cell sorting (FACS) analysis of TNBC cells indicated a dose dependent increase in apoptotic populations in cells treated with free orlistat, orlistat NPs, and folate-receptor targeted Fol-HEA-EHA-orlistat NPs in which Fol-HEA-EHA-orlistat NPs showed significantly higher cytotoxicity than free orlistat. In vitro analysis data demonstrated significant apoptosis at nanomolar concentrations in cells activated through caspase 3 and PARP inhibition. In vivo analysis demonstrated significant antitumor effects in living mice after targeted treatment of tumors, and confirmed by fluorescence imaging. Moreover, Folate receptor targeted Fol-DyLight747-orlistat NPs treated mice exhibited significantly higher reduction in tumor volume compared to control group. Taken together, these results indicate that orlistat packaged in HEA-b-EHA micellar NPs is a highly promising new drug formulation for TNBC therapy.
Introduction

Breast cancer is the most frequent cancer in women, affecting approximately 1.5 million women each year worldwide (1, 2). Triple negative breast cancer (TNBC) is a subtype accounting for 15% to 20% of all breast cancers. It is particularly recalcitrant owing to its aggressive nature, treatment failure, and the high incidence of TNBC in younger women. TNBC is negative for the expression of estrogen receptor (ER), progesterone receptor (PR), and HER2/neu, thus making it especially difficult to develop personalized therapies that target ER, PR and Her2 (e.g. tamoxifen, monoclonal antibodies) (3). Palliative chemotherapy is the only option available for treating advanced metastatic TNBC patients, despite the off target toxicity of the drugs used. Indeed, the standard systemic injection of anticancer drugs is generally associated with significant limitations, including rapid degradation and clearance of drugs, their poor bioavailability, low-water solubility, and side effects accrued in healthy cells through non-specific accumulation (4).

Thus, newer strategies to deliver drugs specifically to tumors are imperative to improve the effectiveness of cancer chemotherapy. In this respect, the use of targeted nanoparticles (NPs) is of considerable current interest (5, 6). Several NP based drug delivery platforms are at various stages of clinical trials for cancer therapy (7). Polymer based NPs are simple and convenient for loading a wide spectrum of drugs differing in chemical properties. Since polymers under a certain size limit are eventually cleared by the body, the use of polymer based NPs has the advantage of reducing systemic toxicity. Nanomedicine has witnessed significant recent advances, especially in development of drug delivery strategies for cancer treatment (8-12).
Micelles, in particular, have been shown to increase drug water solubility, reduce non-specific toxicity, enhance permeability through membranes at the sub-100 nm level, and increase bioavailability (4).

Polymeric micelles are colloidal nanoparticles (5-100 nm) that contain a hydrophobic core and a hydrophilic corona, made of multi-block ($n \geq 2$; $n = \text{number of blocks}$) copolymers with amphiphilic properties. We synthesized polymeric micelles from two types of monomers, 2-hydroxyethylacrylate (HEA) and 2-ethylhexyl acrylate (EHA). Both HEA and EHA have been used for drug delivery either independently or in combinations with other polymers (13, 14). Here, we use a novel combination of HEA and EHA block polymers to engineer self-assembled NPs for drug loading. We synthesized these polymers using RAFT (Reversible Addition-Fragmentation chain Transfer) polymerization to allow functionalization of the polymer chain ends. The advantages of using controlled/living radical polymerization (CLRJP) are: the mechanism allows chains to grow uniformly, and it provides the ability to incorporate branching points and specific functionalities such as click chemistry strategies to attach drugs, targeting moieties, and imaging dyes to the polymer backbone (15, 16). By using micellar NPs, not only is it possible to deliver greater amounts of therapeutic drug to tumors without being degraded in the body from early release, but the NPs can also be functionalized by conjugation procedures to minimize drug loss and increase payload.

Drug repurposing is a convenient, speedy, cost effective, and intuitively better approach to discover and reassign drugs for treatment of diseases other than those for which such drugs were initially intended and developed. Orlistat (Xenicale®), also known as tetrahydrolipstatin, is
a US-FDA approved drug used to induce bodyweight loss in diabetic patients (17). Previous studies have demonstrated its antitumor effect in different cancers, but not TNBC (18). Since orlistat is a hydrophobic drug, its solubility in aqueous medium is limited. This property also limits its bioavailability, which is currently reported to be less than 1% (19, 20). The poor bioavailability of this drug is also partially associated with its systemic adsorption and gastrointestinal excretion (21). The antitumor effect of orlistat is linked to its ability to block the lipogenic activity of fatty acid synthase (FAS). FAS is an oncogenic antigen-519 up-regulated in more than 50% of breast cancers, and associated with poor prognosis (18). In contrast, FAS expression is significantly low in all normal tissues, which makes it an attractive target for development of an anticancer therapy.

The selective overexpression of folate receptors in breast cancer is emerging as a new biomarker, especially for TNBC, since folate receptor is overexpressed in ER and PR negative breast cancers. Moreover, its presence is independent of Her2 status (22, 23), thus making it more specific for the TNBC subtype. The folate receptor is also overexpressed in metastases found in advanced TNBC patients. Here, we develop and validate novel telechelic HEA-b-EHA diblock polymer micellar NPs for delivery of the hydrophobic anti-obesity drug orlistat as a repurposed anticancer agent against TNBC. Since the folate receptor is specific to TNBC, we use folic acid conjugation of these micellar NPs to selectively target TNBC cells. Orlistat specifically acts on tumor cells through the FAS pathway, which is upregulated in TNBC tumors (24, 25).

We evaluate the anti-proliferative and apoptotic action of orlistat-loaded micellar NPs (NP-Orlistat) and orlistat loaded folate receptor targeted micellar NPs (Fol-NP-Orlistat) in two
phenotypically different TNBC (MBA-MB-231 and SkBr3) cell lines in vitro to test the efficacy of our orlistat loaded NPs in different cells. We also test the effects of this drug on PARP and caspase 3 protein levels and cleavage to further investigate the mechanisms of its action. Next, we evaluate the antitumor effects of NP-Orlistat in tumor xenografts in nude mice model by molecular imaging. For this we use micelles conjugated to a near infrared (NIR) dye as an imaging agent, to test the micellar NP distribution and tumor specific accumulation using optical imaging. We demonstrate an efficient apoptotic effect of orlistat, and the folate receptor targeted micellar NPs improve orlistat solubility and show enhanced therapeutic efficiency by induction of apoptosis in MDA-MB-231 TNBC cells. We found significant tumor reduction in animals receiving orlistat delivered by folate receptor targeted NPs compared with the drug delivered by NPs prepared from polymers without conjugated folate, or free circulating orlistat.

Material and Methods

Materials, cell lines

All chemical reagents used for the study were of analytical grade or above, purchased from commercial suppliers and used without further purification unless otherwise mentioned. Orlistat (≥98%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 4-dimethylaminopyridine (DMAP) and diisopropylethylamine (DIPEA), 2-hydroxyethylacrylate (HEA), 2-ethylhexyl acrylate (EHA), Azobisisobutyronitrile (AIBN), copper(I) bromide, triethyl amine (Et₃N), 1,4-dioxane and RAFT-agent were obtained from Sigma Aldrich (St. Louis, MO, USA). Cell culture media, fetal bovine serum (FBS), antibiotics streptomycin and penicillin (PS) were purchased from Invitrogen (Carlsbad, CA, USA). MDA-MB-231 and SkBR3 breast cancer cell lines, and HeLa ovarian cancer cells were
purchased from American type culture collection (ATCC) (Manassas, VA, USA) between 2012 and 2013. MCF10A breast fibroblast cells were purchased American type culture collection (ATCC) (Manassas, VA, USA) in 2015. All cell lines were authenticated by short tandem repeat DNA profiling by Genetica-DNA laboratories (Labcorp, Burlington, NC). The cell lines were used for less than 40 passages. Cell lines were routinely tested for mycoplasma contamination.

**Synthesis of (HEA-b-EHA) diblock copolymer**

We synthesized the HEA-b-EHA copolymer according to a previously published report(26). We added monomer HEA-TMS (1.412 g, 7.5 mmol), RAFT agent (0.024 g, 0.075 mmol), AIBN (1.724 mg, 0.0105 mmol) and anhydrous 1,4-dioxane (3.0 mL) to a 10 mL MW reaction vessel with an appropriate stir bar. We degassed the reaction vessel by purging the solution with N2 gas for 30 min while the reaction vessel was immersed in an ice-water bath. We then transferred the vessel to a microwave reactor and heated it at 70°C for 3 h under fast stirring conditions. The resulting polymer was precipitated from cold hexanes multiple times and dried under vacuum to yield 0.950 g of HEA polymer (Mn = 19.7 kg/mol, PDI = 1.15).

This polymer formed the hydrophilic block and it was chain extended with EHA to form the diblock copolymer. As a representative example where the monomer ratio was 150, we added macroRAFT agent pHEA-TMS (0.800 g, 0.044 mmol), AIBN (1.022 mg, 6.22 µmol), EHA (1.229 g, 6.67 mmol) and anhydrous 1,4-dioxane (3.0 mL) to a 10 mL microwave reaction vessel with an appropriate stir bar. We degassed the reaction vessel by purging the solution for 30 min with N2 gas while the vessel was immersed in an ice-water bath. We then transferred the vessel to a microwave reactor and heated it at 70°C for 1.5 h under fast stirring conditions. The
resulting polymer was precipitated from cold MeOH multiple times and then dried under vacuum to yield 1.2 g of HEA-b-EHA polymer (Mn = 30.8 kg/mol, PDI = 1.25)

**Synthesis of amine functionalized Polymer (2)**

To a 42 mM solution of dithioester-terminated HEA-EHA polymer (1) (500 mg, 0.017 mmol) in Chloroform (397 µl), we first added 20 equivalents of S-(2-aminoethyl) methanesulfonothioate hydrobromide (79 mg, 0.333 mmol), followed by 50 equivalents of propan-1-amine (68.5 µl, 0.833 mmol) under vigorous stirring. The reaction mixture was stirred overnight at room temperature, turning first orange and then yellow within a few hours. We purified the resulting polymer by several consecutive precipitations in cold ether to yield the amine functional HEA-EHA block polymer. This polymer was later reacted with a few drops of Acetic acid in (THF + MeOH) to deprotect the hydroxyl groups and then dialyzed with millipore water to obtain polymer (2) devoid of silyl and propyl amine moieties (Supplementary Scheme 1).

**Synthesis of NIR-DyLight747-B1 and Folate functionalized HEA-EHA block copolymer**

We first dissolved amine functionalized HEA-EHA block copolymer (2) (100 mg, 0.00333 mmol, 1 equiv) in Acetone-d6 (50 µL) and then diluted with DMSO-d6 (400.0 µL). We added triethylamine (11.62 mg, 0.115 mmol, 34.4 equiv) to the mixture and then degassed this by
freeze pump thaw. To this frozen mixture, we added NIR Dye DyLight 747-B1 NHS ester (1.0 mg, 0.001235 mmol, 0.37 equiv) using 100 μL DMSO-d6. We heated this oxygen free mixture at 42°C for 8 h to yield the dye conjugated polymer (3a) and then added 2,5-dioxopyrrolidin-1-yl acetate (0.495 mg, 0.00315 mmol, 0.945 equiv) (Model NHS) to ensure reaction of all remaining free -NH₂ groups, rendering them non-hydrophilic. This reaction was carried out at 42°C for an additional 14 h to ensure complete conversion to achieve 3b. At this point we took the reaction off heating and cooled it to room temperature. We added the Folate Azide coupling mixture to the frozen reaction mixture using 100 μL of DMSO-d6. This coupling mixture comprised of Folate Azide (2.04 mg, 0.004 mmol, 1.2 equiv), PMDETA (0.693 mg, 0.004 mmol, 1.2 equiv) and copper(I) bromide (0.956 mg, 0.00667 mmol, 2 equiv) for carrying out a copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction. This reaction was carried out at 30°C for 20 h after which we transferred the reaction mixture into a 3500 Da dialysis bag and dialyzed with MeOH to give the desired products (4a-b, 90 mg) (Supplementary Scheme 1).

**Synthesis and optimization of Orlistat loading in HEA-b-EHA micellar nanoparticles**

Solvent optimization and concentration of drug loading must be well characterized before deciphering the effects of drug treatment. In the first experiment, we mixed 1 mg of polymer (0.5 ml, from a 2 mg/mL stock) with 0, 100, 200, and 400 μg of orlistat dissolved in DMF (10 mg/mL stock). The volume was made up to 1 mL using DMF. We prepared 0, 10, 20, and 40% orlistat to polymer concentrations for drug loading into dialysis tubes (10 kDa, Sigma Aldrich, St Louis, MI) and dialysis was conducted in a 1 L beaker containing sterile deionized H₂O that was changed every 6 h for 36 h. Orlistat (200 μg) without any polymer in 1 mL of DMF was dialyzed under similar conditions as a control (Supplementary Scheme 2). We developed a standard graph using a UV-Vis Spectrophotometer by measuring the optical absorbance of orlistat diluted in H₂O (OD-A₂₅₄). After 36 h, the micellar NPs collected from the dialysis tubing were filtered.
through a 0.2 μm membrane filter, and we measured encapsulation efficiency, loading efficiency, particle size, charge, and polydispersity index by DLS (Malvern Instruments, U.K.).

**Characterization of micelles**

We synthesized the EHA-b-HEA polymers through several steps, and each step of the synthesis was characterized using ¹H-NMR. The number of EHA groups was determined by ¹H-NMR. Critical micelle concentration (CMC) was measured for the minimum concentration of polymer needed for micellization using pyrene as an extrinsic probe, and is based on the solvent dependence of vibrational band intensities in the fluorescence emissions(26). We used Dynamic light scattering (DLS) to calculate size (nm), polydispersity index, and zeta-potential using a Zetasizer-90 (Malvern Instruments, U.K.) and a Brookhaven Research Goniometer and Laser Light Scattering System at 25°C and 90° scattering angle.

**Transmission electron microscopy (TEM)**

We obtained transmission electron microscopy (TEM) images of micellar NPs using a FEI TITAN 80-300 kV environmental transmission electron microscope (ETEM) at the Stanford Nanocharacterization Laboratory, by operating at 80 kV using negatively stained micelles. In brief, a drop of HEA-b-EHA micellar NPs were incubated with a drop of 1% phosphotungstic acid (PTA) (pH of PTA was adjusted to 7.5 with 1 N NaOH) for 3 min. Then we plated a drop of PTA stained micellar NPs onto a carbon film coated copper grid, and excess solution was drained off after 3 min. We then air dried the grid and observed it under TEM. We performed size analysis using IMAGEJ software.

**In vitro orlistat dose evaluation for apoptotic induction in SkBr3 and MDA-MB-231 cells**
SkBr3 and MDA-MB-231 cells were cultured in DMEM high glucose medium supplemented with 10% FBS and 1% Penicillin and Streptomycin. We maintained the cells at 37°C with 5% CO₂ in a humid incubator. We treated the cells plated in triplicate in 6-well plates at 1×10⁵ cells in normal medium with different concentrations of orlistat or NPs loaded with orlistat in DMEM with 2% FBS. Both dead and live cells were trypsinized, collected and fixed in 70% ice cold ethanol, after 48 h of incubation at 37°C with 5% CO₂. We stored the samples in a -20°C freezer for 24 h before they were processed for FACS analysis. We washed the samples once in PBS and stained with 0.5 mL of PBS containing 0.5 µg/mL propidium iodide, 10 µg/mL RNAse A, and 0.1% TritonX-100. After 15 min of incubation at room temperature in the dark, cells were subjected to FACS analysis (BD FACSARia™ III), and we analyzed the generated data by FlowJo 8.8.6 software for the quantification of live and dead cells.

**In vitro anti-proliferative effect of orlistat loaded micellar NPs in MDA-MB-231 breast cancer cells**

We used MTT assay to determine the anti-proliferative effect of orlistat and orlistat loaded micellar NPs in MDA-MB-231 cells by assaying for cell viability. We treated the MDA-MB-231 cells (5×10⁵ cells/well in 96-well plated in regular DMEM with 10% FBS) with various concentrations of orlistat or orlistat loaded in micellar NPs with and without being conjugated to folic acid in DMEM with 2% FBS for 24, 48, and 72 h at 37°C with 5% CO₂. After respective treatment times, we carefully removed the media and added 50 µL of phenol red free DMEM with 2% FBS containing 0.5 mg/mL of MTT reagent. We incubated the cells further for 2 h and dissolved the violet formazan crystals formed in the cells in 100 µL of DMSO by incubating at 37°C for 30 min in the dark. We measured the soluble MTT-formazan derivative using a
TECAN-Safire UV-Vis spectrophotometer at 540 nm. We plotted the results, and cell viabilities were compared to those of the control (untreated) samples.

**Immunoblot analysis for FASN, PARP, Pro-Caspase 3, Caspase 3, Bax, and tubulin levels to confirm the mechanism of apoptotic induction by orlistat delivered by micellar NPs in MDA-MB-231 cells**

We grew MDA-MB-231 cells treated with different concentrations of orlistat loaded micellar NPs (1.25, 2.5, 5 and 10 μM orlistat equivalent) for 24 h and assessed for the expression of PARP, cleaved PARP, Pro-Caspase 3, Caspase-3, Bax and tubulin levels using immunoblot analysis. Cells treated with micellar NP without orlistat, and without any treatment served as controls. Other cells treated with Staurosporine (1 μM) served as positive control to detect the cleavage of PARP enzyme. To obtain total cell lysate after treatment, we collected cells and re-suspended them in 100 μL of lysis buffer [50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, protease inhibitors (Roche, Pleasanton, CA)] and then lysed them by keeping on ice for 30 min with intermittent mixing. We resolved 30 μg of total protein from the cell lysates in 4-12% gradient polyacrylamide gel by electrophoresis, and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) of 0.2 μm pore size, with subsequent exposure of the membrane to rabbit anti-PARP antibody (1:1000 dilution, Cell Signaling, Boston, MA) for 12 h in TBS/0.05% Tween 20 and 3% dry milk. The membrane was washed and probed with anti-rabbit HRP conjugated antibody (1:10,000 dilution, Sigma Aldrich, St. Louis, MI). We detected the signal using an ECL system (Thermo Scientific, San Jose, CA). The membrane was stripped and probed for Caspase and Pro-Caspase 3 (anti-mouse, 1:200
dilution, Santa Cruz Biotechnologies, Santa Cruz, CA) followed by tubulin (1:1000 dilution, Sigma Aldrich, St. Louis, MI).

**Construction of MDA-MB-231 cells stably expressing Firefly Luciferase-Enhanced Green Fluorescent Protein (FLuc-eGFP) fusion reporter for in vivo therapeutic evaluation of Fol-HEA-b-EHA micellar NPs in living mice.**

To evaluate the therapeutic efficiency of Fol-HEA-b-EHA micellar NPs in tumor xenografts of MDA-MB-231 cells in living mice by *in vivo* imaging, we constructed MDA-MB-231 cells stably expressing FLuc-EGFP reporter fusion gene by lentiviral transduction. We expanded the cells, after FACS sorting for clonal selection, and tested them for their response to orlistat loaded-NPs before they were used for animal experiments. We treated the cells, plated at 150×10^3/well in 6-well plates, with 1.25, 2.5, 5 and 10 μM orlistat equivalent of micellar NPs in DMEM with 2% FBS for 48 h, and we tested for induced apoptosis by PI staining based FACS analysis as described above.

**Animal Experiments**

We conducted all animal experiments under the guidance of the Administrative Panel on Laboratory Animal Care (APLAC), Stanford University. The Institute animal research committees at Stanford approved all animals handling. We purchased all animals (CD1, mice, nude, females) from Charles River laboratories (Wilmington, MA).

**In vivo anti-tumor evaluation of orlistat loaded Fol-HEA-b-EHA micellar NPs in MDA-MB-231 tumor xenografts in mouse model by optical fluorescence and bioluminescence imaging**
We used the MDA-MB-231 cells stably expressing FLuc-eGFP reporter gene to generate tumor xenografts in nude mice. We implanted 30 mice with \(5 \times 10^6\) MDA-MB-231-FLuc-eGFP cells in the left and right lower flanks (n=60 tumors) by using 50% (vol/vol) Matrigel (100 \(\mu\)L total volume injected). The tumors were allowed to grow to reach about 150-200 mm\(^3\), and the mice were randomized to 5 animals (mice) per group (n=10 tumors, 5 animals each bearing two tumors each) into 6 groups (Group 1: Solvent control, Group 2: Free Orlistat, Group 3: DyLight747-NP, Group 4: DyLight747-NP-Orlistat, Group 5: Fol-DyLight747-NP, and Group 6: Fol-DyLight747-NP-Orlistat). We treated the animals once a week with their respective regimens by intravenous (IV) injections, twice in the entire study (Dose 1: two weeks after tumor implantation; Dose 2: three weeks after tumor implantation). We optically imaged the animals for expressed FLuc signal after injecting 3 mg of D-Luciferin in 100 \(\mu\)l of PBS by intraperitoneal injection. We used a Bruker Xtreme in vivo molecular imaging system to image the animals, and measured tumor sizes on every experimental day using electronic calipers. Similarly, we measured the animals for NIR-fluorescence using an excitation filter at 730 nm and an emission at 790 nm. We quantified the signals by Bruker analysis software.

**Ex-vivo analysis**

**H&E staining**

On Day 14 after treatment initiation, we sacrificed the animals and tumors were excised and then frozen in OCT cryoprotective fixing medium. Tumor xenografts were sliced at 10 \(\mu m\) in a Leica cryomicrotome. We stained tumor sections in undiluted hematoxylene (Sigma-Aldrich, USA) for 2 min, rinsed in running water, and differentiated in 1% HCl acid/alcohol for 30 sec. We then washed and immersed these sections in Bluing solution (Fisher, USA) for 1 min, washed in
running water and rinsed in 10 dips of 95% alcohol. After this, we counterstained slides in eosin by dipping into 1:5 ethanol-diluted Eosin solution (Fisher) for a total of less than 30 sec, then dehydrated through 95% alcohol, absolute alcohol, and xylene for 5 min each. We mounted slides with xylene based mounting medium (Permount, Sigma) and imaged them using a Nanozoomer (Hamamatsu, Japan).

**TUNEL assay**

We used the tumor xenografts of MDA-MB-231-Fluc-eGFP cells to assess the therapeutic effects of orlistat by measuring the apoptosis levels. We performed a terminal deoxynucleotidyl-transferase (TdT) nick-end labeling (TUNEL) assay using a Trevigen TACS 2 TdT-DAB *in situ* Apoptosis Detection Kit (TREVIGEN, Gaithersburg, MD). A portion of the tumor fixed in OCT (TissueTek) was sectioned at 10 µm in a Cryomicrotome (Leica CM1850, Wetzlar, Germany). We processed the tumor slices and assessed the apoptosis level by following the manufacturer protocol. After staining, we scanned the slides using a Nanozoomer 2.0 RS (Hamamatsu, Japan) digital scanner and viewed for Diaminobenzidine (DAB) staining of apoptotic cells using a Nanozoomer Digital Pathology Software.

**Ki67 staining to monitor cell proliferation in the tumors**

For immunofluorescence staining of Ki67 proliferation marker, we fixed 10 µm tumor OCT sections in acetone for 2 minutes, and washed OCT sections in PBS thrice for 5 min each. We then incubated the slides in 0.03% hydrogen peroxide for 5 min, blocked in 5% NGS/PBS for 1 h and incubated with mouse anti-Ki67 antibody (1:200 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA) overnight at 4°C. We then washed the sections three times in PBS, and incubated with
anti-mouse Alexa Fluor-594 secondary antibody (1:5,000 dilution, Invitrogen, Carlsbad, CA) for 1 h, followed by nuclear counterstaining with DAPI for 2 min. After washing the slides we mounted them in mounting medium and imaged using a Zeiss Observer Z1 fluorescent microscope.

**Statistical Analysis**

The experimental data were presented as mean values with standard deviations. We performed statistical analysis using a Student's *t*-test. Statistically significant differences were set at *p*<0.05 or 95% confidence levels.

**Results**

We successfully synthesized HEA-b EHA polymer micelles and demonstrated their favorable characteristics, including orlistat loading efficiency, hydrodynamic size, polydispersity index and zeta potential (See Supplementary Results, Supplementary Schemes 1-2, Supplementary Table 1 and Supplementary Fig. 1-3).

Orlistat has been functionally linked with FAS. Therefore we initially confirmed the presence of FAS overexpression in three different cancer cell lines (MDA-MB-231, SkBr3 and HeLa cells) as well as MCF10A normal breast epithelial cells (Figure 1a). We then evaluated the cytotoxic effects of free orlistat alone on SkBr3 and MDA-MB-231-TNBC cells and the results are shown in Fig. 1b-1d and Supplementary Fig. 4. In brief, in SkBr3 cells, the basal level apoptosis of 2.34 ± 0.23% increased to 12 ± 1.47% when treated with 40 μM of orlistat. In contrast, in MDA-
MB-231 cells, the basal level apoptosis of $3.89 \pm 0.21\%$ rose to $82.9 \pm 2.13\%$ at $40 \mu M$ of orlistat treatment.

**Cytotoxic effect of orlistat delivered by folate receptor targeted HEA-\(b\)-EHA micellar nanoparticles in SkBr3 and MDA-MB231 cells**

Orlistat is a hydrophobic drug and its bioavailability in the current anti-obesity therapeutic regimen of 60 mg and 120 mg daily dose has been estimated to be less than 1%. We therefore hypothesized that by increasing orlistat solubility after loading it in NPs (Fig 2a-2b), it would be possible to enhance its bioavailability, and, it may in turn reduce the orlistat dose needed for both anti-obesity and repurposed anti-cancer therapeutic applications. We found that the loading of orlistat in HEA-\(b\)-EHA polymeric micellar NPs improved its solubility by up to 20% of drug to polymer ratio in water (Supplementary Fig. 1). We then tested the cytotoxic effect of this drug formulation in both SkBr3 and MDA-MB-231 cells with free Orlistat serving as a positive control (Fig 2c-2d). We treated the cells with 0, 2.5, 5, 10 and 20 $\mu M$ of free orlistat, or orlistat loaded in HEA-\(b\)-EHA micellar NPs either with or without folic acid conjugation to its hydrophobic corona (Orlistat, NP-Orlistat and Fol-NP-Orlistat) as a targeting moiety. We then analyzed the cells with FACS after staining with PI for assessing the level of induced apoptosis. We found a dose dependent increase in apoptotic populations in both SkBr3 and MDA-MB-231 cells treated with both free orlistat and orlistat loaded in micellar-NPs. Of note, and as observed in the previous dose study, a significantly higher level of response ($p<0.01$) to treatment was observed in MDA-MB-231 cells when compared to SkBr3 cells. The SkBr3 cells treated with folate receptor targeted micellar NP-loaded orlistat induced twice as many apoptotic cells at 20
μM orlistat concentration compared to respective cells treated with either free orlistat or orlistat loaded in micellar NP (Orlistat: 10.22 ± 0.46%; NP-Orlistat: 10.83 ± 0.25% and Fol-NP-Orlistat: 20.33 ± 0.8%) (Fig. 2c, Supplementary Fig. 5a-5c). Owing to the targeting effect, the MDA-MB-231 cells showed significant response (p<0.01) at much lower concentrations of orlistat treatment when compared to the SkBr3 cells (Fig. 2d, Supplementary Fig. 5d-5h). The cells treated with NPs alone showed no toxic effects.

Since orlistat is a hydrophobic drug, it produces self-segregated particles of 300-500 nM when dialyzed against water. In the presence of HEA-b-EHA polymer, we also observed self-aggregation when the drug to polymer ratio was increased beyond 20%. Despite filtering out all NP preparations using 0.2 μM pore size filters to remove the self-aggregated particles before we used them for various therapeutic experiments, we nonetheless also tested the cytotoxic effect of the orlistat aggregates in MDA-MB-231 cells. The experiments were conducted in parallel by treating the cells with Fol-NP-Orlistat at similar concentrations for comparison. Although self-aggregates of orlistat showed significant cytotoxic effects, the percentage of apoptotic cells induced by orlistat aggregates was significantly (p<0.001) lower than that in the cells treated with the respective concentrations of orlistat loaded in Fol-NPs (Fig. 2e, Supplementary Fig. 6a-6d). This clearly indicates the importance of micelles in enhancing orlistat solubility and intracellular functional delivery to bring about its therapeutic effect.

**Evaluation of cell viability and anti-proliferative effects in MDA-MB-231 cells treated with orlistat delivered by folate receptor targeted HEA-b-EHA micellar NPs**
Most anticancer drugs not only induce cell death, but can also show a cytostatic effect by blocking cell growth. Hence, we also studied the effects of orlistat on cell proliferation and cell death by measuring cell viability using MTT assay. We assessed cell viability at different concentrations (0.625 to 40 μM) of free orlistat or orlistat loaded in micellar NPs with and without folic acid conjugate (NP-Orlistat and Fol-NP-Orlistat) in MDA-MB-231 cells at 24 h, 48 h and 72 h post treatment. We treated the cells with similar concentrations of NPs (polymer equivalent) containing no drug as control. We demonstrated dose dependent reduction in cell viability of MDA-MB-231 cells treated with orlistat, NP-Orlistat and Fol-NP-Orlistat (Fig. 3). The cells treated with orlistat loaded in folate receptor targeted NPs showed significant reduction in cell viability at 24 h post treatment, while other treatment conditions reached a similar level of cytotoxic effect after 48 h. The cells treated with control NPs exhibited no significant cytotoxic effect.

To test the effect of orlistat on normal cells, we used MCF10A breast fibroblast cells, and treated them with free orlistat or orlistat loaded HEA-b-EHA micelles (Fol-NP), with orlistat concentrations similar to those used for treating MDA-MB-231 cells (1.25 μM to 20 μM). We assessed the cells for induced apoptosis 48 h after treatment. The results showed no significant effect even at the highest concentration of orlistat used for treatment (Fig. 4a, Supplementary Fig. 7a-7d). Treatment of MDA-MB-231 cells with orlistat at 20 μM induced approximately 85% to 90% of cells to apoptose in 48 h, but caused only 8% to 10% apoptosis in MCF10A cells. This result clearly shows the selective nature of orlistat in inducing cell death solely in cancer cells.
To further evaluate the downstream pathways through which orlistat induces apoptosis, we performed immunoblot analysis for PARP, Caspase-3, and Bax proteins. Since orlistat was previously reported to activate PARP, we tested for PARP cleavage. The result showed an orlistat dose dependent cleavage of PARP protein. Similarly the results of Pro-caspase 3 and Bax protein levels showed an orlistat concentration dependent decrease in protein levels (Fig. 4b, 4c). Bax cleavage by caspase-3 has been shown to induce enhanced apoptosis (27). We found that treatment of MDA-MB-231 cells with orlistat induced apoptosis through inactivating PARP enzyme function while inducing caspase-3 and Bax activation. Since MDA-MB-231 cells demonstrate higher folate receptor expression level compared to SkBr3 cells (28), we have chosen MDA-MB-231 cells over SkBr3 cells for further studies with folic acid conjugated NPs in vivo in mice model.

Evaluation of anti-tumor effect of Folate-HEA-b-EHA micellar NPs loaded orlistat in MDA-MB-231 tumor xenografts in mice using molecular imaging

We further tested the antitumor effect of orlistat loaded in micelles conjugated with folic acid for targeting, as well as DyLight747-B1 NIR dye for imaging. There were no significant changes observed in nanoparticle CMC value and other physical properties, such as size, zeta potential and PDI, upon DyLight747-B1 NIR dye conjugation (Supplementary Table 1). After orlistat loading, we tested the polymer micelles for DyLight747-B1 fluorescence using an IVIS in vivo imaging system (Perkin Elmer) with an excitation 760 nm filter and an 800 nm emission filter after spotting known polymer equivalent concentrations of micelles in a nitrocellulose membrane. The results showed concentration dependent NIR-fluorescence signal, and no
significant change in the level of fluorescence signal between polymers before and after prepared micelles loaded with orlistat (Fig. 5a).

We used subcutaneous tumor xenografts of MDA-MB-231 cells stably expressing Fluc-eGFP fusion protein to measure tumor volume by bioluminescence imaging in mice. We established tumor xenografts by subcutaneously implanting $5 \times 10^6$ cells. Two weeks later when the tumor sizes reached 200 mm$^3$ to 300 mm$^3$ we randomized the animals (mice) and used them for treatments. We treated the animals with two doses of the different NPs by intravenous injections on Day 0 and Day 4, and imaged for fluorescence and bioluminescence using a Bruker Xtreme optical imaging system (Bruker), as well as planar X-rays for anatomical co-registration of bioluminescence images. We repeatedly performed the imaging and tumor volume measurements immediately after NPs injection, and after 5 h, 24 h, Day 3, 4, 6, and 14, for the two doses of treatment. Since control nanoparticles lack orlistat and shows no toxicity. We observed significant tumor specific accumulation of NIR-fluorescence signal from NPs targeted to folate receptor as early as 5 h and peaked 3 days after initial treatment, while other NPs showing much higher signals in other parts of the body, especially in the liver (Fig. 5b). We expected a large difference in the biodistribution between NPs with and without folic acid. Interestingly, we observed even a difference between NPs-loaded with orlistat and control NPs. The results in Figure 5c indicated 70% reduction of the tumors treated with folic acid-conjugated orlistat loaded NPs compared to control NPs without orlistat. The fluorescence signal dropped 10 days (Day 14) after two doses of NP administration. Tumor volume measurements clearly indicated that the animals receiving Fol-DyLight747-NP-Orlistat showed 70% reduction in
tumor volume compared to control, and 50% and 40% reduction compared to DyLight747-NP and solvent control groups respectively, 14 days after two doses of treatment (Fig. 5c). The bioluminescence signal matched the tumor volume, and showed significant levels of hypoxia-associated heterogeneous signal from tumors of control groups as well as animals treated with free Orlistat and Fol-DyLight747-NP on the Day 14 images compared to respective animals imaged before the start of treatment (Fig. 5d).

After 14 days, we excised the tumors from different animals and ex vivo imaged for fluorescence signal, and histologically analyzed for tumor architecture using H&E staining, TUNEL staining for induced apoptotic cells, and Ki67 staining for cell proliferation. We analyzed the organs (kidney, liver and spleen) excised from different treatment groups for the presence of any treatment-associated toxicity. The results showed significant fluorescence signal in the livers and kidneys of animals injected with Fol-DyLight747-B1-NP, DyLight747-B1-NP-Orlistat and Fol-DyLight747-B1-NP-Orlistat (2 mg/kg BW Orlistat; 15mg/kg BW Polymer), while observing no signals from solvent controls, orlistat alone, and NP-without dye (Fig. 5b). Ex vivo fluorescence imaging of different organs from various treatment groups showed significant levels of NIR fluorescence signal in liver, kidney, spleen, pancreas and tumors of animals injected with Fol-DyLight747-NP and Fol-DyLight747-NP-Orlistat (Fig. 5e). Tissues from the solvent control group and animals treated with free orlistat showed no fluorescence. Similarly, ex vivo histological analysis of tumor tissues using TUNEL staining and staining for Ki67 cell proliferation marker showed significant levels of apoptotic cells in the tumors of animals treated with free orlistat and Fol-DyLight747-B1-NP-Orlistat, compared to tumors from control and Fol-DyLight747-B1-NP groups (Fig. 6a-6c). Ki67 staining showed significantly
lower levels of positive cells in tumors of animals treated with Fol-DyLight747-B1-NP-Orlistat compared to all other groups (Fig. 6c). H&E staining of spleen, kidney and liver tissues showed no toxicity associated cell death, or accumulation of red blood cells or recruitment of macrophages, in all groups, demonstrating no visible toxicity associated with treatment using NPs or NPs loaded with orlistat (Fig. 6d)

Discussion

There is no available drug targeted therapy for TNBC. A search for potential biomarkers to aid the development of more effective targeted therapies using new drugs or drug formulations is needed for greater success in management of this prevalent and challenging disease. Here we demonstrate several innovations that collectively point to a significantly promising new drug formulation for treatment of TNBC, namely: use of the approved anti-obesity drug orlistat as a new and effective repurposed drug against TNBC; a newly engineered polymeric micellar nanoparticle for packaging and delivery of orlistat to TNBC cells; and use of folic acid functionalization of these nanocarriers for biological targeting of the orlistat payload specifically to TNBC cells.

In the current study, we successfully developed HEA-\(b\)-EHA polymers synthesized by RAFT polymerization (15, 16, 29) for the engineering of novel micelles showing high loading efficiency for the hydrophobic drug orlistat (Supplementary scheme 1-2). This improved the solubility and bioavailability of the drug, and showed improved therapeutic efficiency in FAS overexpressing MDA-MB-231 and SkBr3 TNBC cells in culture, and MDA-MB-231 tumor xenografts in mice. Moreover, the HEA-\(b\)-EHA polymer also facilitated the simultaneous
conjugation of both a NIR dye for imaging and folic acid for tumor targeting (Figure 2a-2b). Cytotoxicity analysis of TNBC cells treated with different NPs by FACS indicated a dose dependent increase in apoptotic populations in cells treated with free Orlistat, Orlistat loaded NPs, and folate-Orlistat loaded NPs, in which folate receptor targeted NPs produced significantly more cytotoxicity than free Orlistat (Figure 2). Moreover, treatment with folate-receptor targeted Orlistat NPs induced almost 90% apoptosis in MDA-MB-231 cells after 48 h at 20 μM concentration, whereas same NPs induced just 8% to 10% apoptosis in MCF10A normal breast cells (Fig 4a, Supplementary Fig. 7a-7d). This clearly indicates selective nature of orlistat induced cell death in cancer cells. An outstanding observation is that Fol-DyLight747-Orlistat NPs treated tumors showed 70% reduction in tumor volume compared to control group (Fig. 5c). To study active mechanism of apoptotic induction (treatment effect) without significant contribution from nutrients depletion associated natural cell deaths, 48 h is the most appropriate condition for the experiments. Hence, we chose 48 h as a primary time point for most of our experiments.

Importantly, and for the first time, we identified the anti-obesity drug orlistat as an alternative novel repurposed drug for treatment of the recalcitrant and highly challenging TNBC subtype of breast cancer. We also demonstrated the importance of improving the bioavailability of this hydrophobic drug by encapsulating it within micellar NPs, while identifying folic acid as an efficient targeting moiety for improving TNBC therapy. A particularly attractive feature of the novel and rigorously characterized strategy that we developed is its generalizability and potential for far-reaching applications in targeted treatment of many diseases; it can certainly be adapted to a variety of cancers by selecting appropriate drugs and suitable cell surface specific targets. In
future studies we will aim to increase the multifunctionality of derivatives of these HEA-b-EHA micelles concurrently with multiple tumor specific targets that will enhance \textit{in vivo} tumor targeting, such as the folate receptor as well as the urokinase plasminogen activator receptor, when attached to the diblock copolymer to allow binding to overly expressed receptors on cancer cells (10, 30) (31, 32).

\textbf{Acknowledgements}

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\textbf{Footnotes}

\textbf{Note:} Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

\textbf{References}


Figure captions:

**Figure 1**: Cytotoxicity evaluation of orlistat in different cells. (a) Immunoblot analysis for FAS expression in MCF10A, SkBr3, HeLa and MDA-MB231 cells. PI staining based FACS analysis measures apoptotic cells induced in response to the treatment of different concentrations of orlistat for 48 h in SkBr3 (b) and MDA-MB231 (c) cells. (d) Fluorescent microscopic images of MDA-MB231-Fluc-eGFP cells treated with different concentrations of orlistat for 48 h and stained by DAPI.
Figure 2: Orlistat loaded HEA-b-EHA polymer micelles characterization and cytotoxicity evaluation of orlistat-loaded HEA-b-EHA polymer micelles in cells. (a) Schematic illustration of HEA-b-EHA polymer micelles loaded with orlistat and different functional moieties for targeting (Folic acid) and imaging (DyLight747-B1-NIR Dye). (b) TEM image of PTA stained orlistat loaded folic acid and DyLight747 functionalized HEA-b-EHA polymer micelles. (c-d) PI-staining based FACS analysis of apoptotic cells induced in response to the treatment of different concentrations of orlistat-loaded HEA-b-EHA polymer micelles with and without folic acid functionalization for 48 h in SkBr3 (c) and MDA-MB-231 (d) cells. (e) Cytotoxic effect of folic acid functionalized orlistat-loaded HEA-b-EHA polymer micelles and self-aggregates of orlistat at different concentrations (0-20 μM) in MDA-MB231 cells treated for 48 h.

Figure 3: Antiproliferative effect of orlistat-loaded HEA-b-EHA polymer micelles in MDA-MB231 cells assessed by MTT assay. MDA-MB231 cells treated with different concentrations of orlistat-loaded HEA-b-EHA polymer micelles (orlistat equivalent of 0-20 μM) at 24 h (a), 48 h (b) and 72 h (c) post treatment.

Figure 4: (a) Antiproliferative effect of free orlistat and orlistat-loaded HEA-b-EHA polymer micelles, in MCF10A-normal breast fibroblast cells assessed by MTT assay. (b) Immunoblot analysis of MDA-MB231 cells treated with different concentration of orlistat-loaded HEA-b-EHA polymer micelles for 36 h for PARP enzyme cleavage, Pro-caspase 3 and Bax proteins levels. Detection of β-actin served as internal loading control. The cells without any treatment and treated with control NPs served as negative controls. The cells treated with staurosporine (1
μM) for 3 h were used as positive control identifying PARP cleavage. (c) Immunoblot analysis of MDA-MB231 cells treated with Fol-Orlistat-NP for 36 h for Caspase 3 level.

**Figure 5:** (a) DyLight747 dye conjugated orlistat-loaded HEA-b-EHA polymer micelles tested for NIR fluorescence demonstrated by optical CCD camera (IVIS) imaging after loading different concentrations of polymer equivalent NPs on a nitrocellulose membrane (DyLight747-NP, Fol-DyLight747-NP, Fol-DyLight747-NP-Orlistat, and DyLight747-NP-Orlistat). a-i, NIR-Fluorescence image of different DyLight747-NPs. a-ii, Quantitative results of image shown in ‘a-i’. (b) NIR fluorescence images of animals (mice) systemically injected with different HEA-b-EHA polymer micelles (DyLight747-NP, Fol-DyLight747-NP, Fol-DyLight747-NP-Orlistat, and DyLight747-NP-Orlistat) acquired at different time points post treatment. (c) MDA-MB231 cell xenograft tumor volumes (mm$^3$) measured in animals after two doses of different treatment conditions (Solvent control, Free Orlistat, DyLight747-NP-Orlistat, Fol-DyLight747-NP-Orlistat) for 14 days. (d) Bioluminescence images of MDA-MB231 cell tumor xenografts in animals before and 14 days after two doses of treatment with different regimens (Solvent control, Free Orlistat, DyLight747-NP-Orlistat, Fol-DyLight747-NP-Orlistat). (e) NIR-Fluorescence images of organs and tumor tissues of animals collected 14 days after two doses of different treatment conditions.

**Figure 6:** *Ex vivo* histological analyses of different organs and tumor tissues extracted from animals bearing tumor xenografts of MDA-MB231 cells treated with different regimens (Solvent control, Orlistat, DyLight747-NP-Orlistat and Fol-DyLight747-NP-Orlistat). (a) Hematoxylin & Eosin staining of tumor tissues. (b) TUNEL staining of tumor tissues. (c) Ki67 staining of tumor
tissues. (d) Hematoxylin & Eosin staining of kidney, liver and spleen tissues collected from the representative animals of four different treatment groups.
Figure 4

(a) Graph showing % apoptotic cells versus Orlistat concentration (μM) for Orlistat and Fol-NP-Orlistat.

(b) Western blot analysis with heat map indicating high and low expression of PARP, Cleaved-PARP, Pro-Caspase 3, Bax, and α-Tubulin.

(c) Western blot for Fol-NP-Orlistat (μM) with bands for Caspase-3 and Tubulin.
a NIR-Imaging of Free Polymers and Polymer Micelles after loading Orlistat

i Concentration polymer (μg)

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ii Concentration of Polymer (μg)

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iii Tumor Volume (mm³)

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iv Liver, Spleen, Kidney, Lungs, Pancreas, Intestine, Tumor

Pre-Treatment

Post-Treatment

p/s/mm²

2.2 x 10⁷

3.0 x 10⁸

1.0 x 10⁸

1x10⁷
Figure 6

H&E Staining of Tumor Tissues

<table>
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<tr>
<th>Control</th>
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TUNEL Staining of Tumor Tissues

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Ki67 Staining

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H&E Staining of Kidney, Liver and Spleen

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Molecular Cancer Therapeutics

Folate Receptor Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple Negative Breast Cancer

Ramasamy Paulmurugan, Rohith Bhethanabotla, Kaushik Mishra, et al.

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