Published in final edited form as:

Nanomedicine. 2018 June ; 14(4): 1441–1454. doi:10.1016/j.nano.2018.04.004.

CD44 Directed Nanomicellar Payload Delivery Platform for Selective Anticancer Effect and Tumor Specific Imaging of Triple Negative Breast Cancer

Zhaoxian Wang^{a,#}, Samaresh Sau^{a,#}, Hashem O. Alsaab^a, and Arun K. Iyer^{a,b,*}

^aUse-inspired Biomaterials & Integrated Nano Delivery (U-BiND) Systems Laboratory Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, 259 Mack Ave, Wayne State University, Detroit, MI 48201, USA

^bMolecular Imaging Program, Barbara Ann Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, Michigan, 48201, USA

Abstract

Triple negative breast cancer (TNBC) is a highly aggressive tumor subtype, lacking estrogen, progesterone and human epidermal growth factor-2 (HER-2) receptors. Thus, early detection and targeted therapy of TNBC is an urgent need. Herein, we have developed a CD44 targeting Hyaluronic Acid (HA) decorated biocompatible oligomer, containing FDA approved vitamin E TPGS and Styrene Maleic Anhydride (SMA) (HA-SMA-TPGS) for targeting TNBC. The self-assembling HA-SMA-TPGS was encapsulated with poorly water soluble, potent curcumin analogue (CDF) to form nanomicelles (NM), HA-SMA-TPGS-CDF has demonstrated excellent nanoparticle characteristics for parenteral delivery. The targeted NM can selectively kill TNBC cells through CD44 mediated apoptosis pathway. Tumor imaging using phase-2 clinical trial near infrared (NIR)-fluorescent dye (S0456) conjugate, HA-SMA-TPGS-S0456 showed excellent TNBC tumor accumulation with minimum liver and spleen uptake. To our best of knowledge, for the first time, we are reporting a promising platform for CD44 mediated multimodal NIR imaging and cytotoxin delivery to TNBC.

Graphical abstract

This image indicates the development of CD44 selective HA-SMA-TPGS oligomer and encapsulation of CDF to form HA-SMA-TPGS-CDF nano-micelle. The HA-SMA-TPGS oligomer was ligated with near infrared dye for imaging of TNBC. The HA-SMA-TPGS-CDF showed significantly higher tumor cell killing effect as compared to SMA-TPGS or CDF treatment through upregulation of tumoricidal PTEN protein and downregulation of tumorigenic NF-κB

*Corresponding author: Arun K. Iyer, Ph.D., Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, 259 Mack Ave, Room 3601, Wayne State University, Detroit, MI 48201, USA, Phone: 313-577-5875, Fax: 313-577-2033, arun.iyer@wayne.edu (A.K. Iyer).

[#]equal contribution.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosures: There is no conflict of interest and disclosures associated with the manuscript.

protein, resulting in induction of apoptosis. The cell uptake study of HA-SMA-TPGS-rhodamine demonstrates TNBC cell selective endocytosis. Overall the purpose of study is to demonstrate CD44 selective TNBC imaging and pronounced cell killing.

Keywords

Triple negative breast cancer; CD44 targeting; vitamin E-TPGS; nano-micelles; fluorescence imaging of TNBC; apoptosis

1. Introduction

TNBC is among the most commonly diagnosed cancers in females and the second leading cause of death in the USA [1][2]. ‘Triple-negative’ terminology represents the negative expression of three major receptors, such as Estrogen (ER), Progesterone (PR), and Herceptin (HER2) [3] and due to lack of these receptors the targeted chemotherapy of TNBC is challenging in clinical set up [4]. Thus, more investigation is an urgent need for receptor targeted therapy to TNBC. Currently, chemotherapy and immunotherapy are the first line treatment for TNBC patients, although surgery is often used if tumor is detected in early stage[5]. However, the major limitations of chemotherapy are the non-specific side effects and drug resistance. Although immunotherapy, such as check point inhibitors (PD-1 and PD-L1 antibody) has a promising effect of TNBC patients [6], but the cost of treatment is very high. Thus there must be rational approach to develop a low cost but efficient targeted drug delivery agent for a highly metastatic TNBC therapy[7][8].

The nanomedicine strategies are generally classified into passive targeting and active targeting. Nanoparticles can selectively accumulate to the tumor environment through enhanced permeability and retention effect (EPR) [9]. Many studies have shown the ability of nanovesicles such as nanoparticles[10] [11], nanomicelles[12] and liposomes [13] to deliver chemotherapeutic drugs in tumor.

The CD44, a type 1 transmembrane glycoprotein, is highly overexpressed in TNBC epithelial and stem cells[14]. Due to significant overexpression of CD44 (80–90% of TNBC cell lines are CD44 positive) makes it as an excellent target for TNBC therapy [15]. Several approaches have been made to target CD44 receptor and few of them has entered to clinical trial including bivatuzumab mertansine, a CD44v6 targeting antibody drug conjugate was studied in phase I clinical trial for head and neck squamous cell carcinoma[16][17]. However, the study was terminated due to acute side effects with 80% case of skin erythema, blister formation and skin desquamation and maculopapular rash [16]. Hyaluronic acid (HA), a natural, biodegradable and non-immunogenic polymer has high affinity to CD44 receptor [18]. Thus, repurposing HA as a CD44 targeted drug delivery agent becomes a rational approach for TNBC therapy and early diagnosis to overcome multidrug resistance and metastasis.

Polymeric NMs are made with amphiphilic polymers, which possess core-shell hydrophobic domain that is suitable for encapsulating hydrophobic chemotherapeutic agents[19][20]. The nano-sized micelles help to effectively target tumor through EPR effect and their surface can

be decorated with tumor antigen specific ligands for active targeting [21]. Currently, several polymeric NM-based formulations have been moved into clinical trials including genexol-PM [22,23] and (DTXL-TNP) [24].

Our lab has extensively studied on nano-formulation of 3,4-difluorobenzylidene diferuloylmethane (CDF), a stable cytotoxic analogue of curcumin for improving its water solubility and targeted delivery to pancreatic, lung, cervical and ovarian cancers [5,25–27]. The reason of using CDF is that it has higher photo stability and better cytotoxicity compared to its parental compound, curcumin [28]. CDF can down-modulate the tumorigenic signaling pathways consistent with NF- κ B, and up-regulate tumor suppressor gene phosphatase and tensin homolog (PTEN) [29–31]. However, the major limitation of CDF for preclinical and clinical translation is the poor water solubility. Thus, to solve the CDF delivery issue, we successfully formulated various nano-systems such as dendrimer, NMs, and liposomes that significantly improve therapeutic efficacy of CDF [5,25,32].

The styrene maleic anhydride (SMA) polymer has an excellent micelle forming property with high drug loading efficiency, and moreover SMA conjugated proteinaceous anti-cancer agent neocargenostatin (SMANCS) is undergoing in human trial [33,34]. Alongside, D- α -Tocopherol polyethylene glycol 1000 succinate (Vitamin E TPGS) is a derivative of the natural vitamin E (PEGylated vitamin E) and it has been FDA approved as a safe pharmaceutical adjuvant for drug formulations [35]. TPGS functions as solubility and permeation enhancer of hydrophobic drugs that makes it as a promising strategy for drug delivery system [36]. Thus, chemical conjugation of SMA-TPGS is a smart approach to enhance the hydrophobic drug, such as CDF loading efficacy while maintaining biocompatibility for in vivo set up.

In this study, we developed HA decorated oligomer, namely HA-SMA-TPGS for targeting CD44 receptor of TNBC and SMA-TPGS as a non-targeted control. CDF has been self-assembled to form targeted and non-targeted NM respectively. The physicochemical characteristics of the NMs have revealed that they have homogenous spherical shaped particle, low critical micelle concentration (CMC), high drug encapsulation efficacy and sustain drug release property. The cytotoxicity study in MDA-MB-231 and MDA-MB-468 demonstrated that the HA-SMA-TPGS-CDF has higher cell killing effect as compared to SMA-TPGS-CDF or free CDF. The cancer cell killing effect of HA-SMA-TPGS-CDF is CD44 receptor mediated. The HA-targeted micelle acts through induction of apoptosis that is linked with upregulation of tumor suppressing phosphatase and tensin homolog (PTEN) protein and down-regulation of tumorigenic nuclear factor-kappa b (NF- κ b) tumorigenic transcription factor. Further to evaluate TNBC selectivity of HA-SMA-TPGS, we sought to conjugate it with S0456 NIR dye and administered to MDA-MB-231 tumor bearing mice. The HA-SMA-TPGS-S0456 oligomer showed significantly higher accumulation in tumor compared to non-specific liver, spleen, whereas nano-targeted SMA-TPGS-S0456 did not show any noteworthy tumor uptake. Thus, targeting CD44 receptor with biocompatible NMs cater as a new strategy for TNBC theranostic and this can be repurposed for delivery of other poorly water-soluble chemotherapeutics (Figure 1).

2. Methods

2.1 Materials and Reagent

CDF was synthesized as described early[27]. SMA (M=1.6 kDa), N-(3-(dimethylamino) propyl)-N'-ethylcarbodiimide hydrochloride (EDC), 3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) and Sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). Hyaluronic acid (MW = 13 kDa) was purchased from Lifecore Biomedical (Chaska, MN). Vitamin E TPGS was purchased from Antares health products, INC. All the other reagents used were of analytical grade and used without any modification.

2.2 Cell lines

TNBC cells, MDA-MB-231 and MDA-MB-468 were used as the *in vitro* model due to its overexpression of CD44 receptor and obtained Dr. Arun Rishi from John D. Dingell VA Medical Center, Wayne State University, Detroit, MI. All cell lines were cultured in DMEM medium with 10% fetal bovine serum (FBS) and 1 % penicillin-streptomycin in a 37 °C incubator with 5% CO₂ incubator.

2.3 Synthesis and characterization of SMA-TPGS conjugates (Non-targeted) and HA-SMA-TPGS conjugates (Targeted)

60 mg HA, 140 mg TPGS was mixed in deionized water (DI water) at RT, and 1M NaHCO₃ was added to the solution slowly and stirred for several hours. Then the pH was raised to 8.9 and 210 mg SMA was added under stirring until solution became clear. Only difference in non-targeted conjugates is that at beginning HA was not added into the solution. SMA-TPGS and HA-SMA-TPGS conjugates were purified by dialysis bag (MW 2 kDa) for 24 h, lyophilized and characterized by ¹H NMR and FTIR. The CMC of oligomers was determined by a pyrene fluorescence method. In briefly, 12 μM of 100 μL of pyrene solution was added into amber color bottle, and the methanol was evaporated under shaking for overnight at room temperature. 2 ml of the oligomer solution with a series of concentrations was added into each amber bottle. The fluorescence spectrum was recorded using F-2500/F-4500 Fluorescence Spectrophotometer. The CMC value is determined by plotting the fluorescence intensity of excitation wavelengths ratio (I335 nm/I332 nm) as a function of polymer concentration.

2.4 Preparation and characterization of CDF-loaded NMs

SMA-TPGS-CDF and HA-SMA-TPGS-CDF were prepared according to our previous reported method[37,38]. First, 100 mg of conjugates polymer was dissolved in 100 ml of DI water under stirring. Then CDF (30 mg) were dissolved in 1 ml of DMSO and mixed with the polymer solution. After that 40 mg of EDC was added dropwise into solution and pH was kept at 5.0 to stir for 30 min. Then raised pH to 11 and kept for other 30 min. At last, pH was adjusted to 7.8 – 8.0 and the free drug CDF were removed by dialysis bag for 4–5 h (MW 2 kDa). Eventually, the solution was lyophilized to obtain the final NMs. Average hydrodynamic diameter of CDF-loaded NMs were measured using Dynamic Light Scattering (Beckman Coulter Delsa Nano CTM). In addition, the size distribution and morphology were observed by transmission electron microscopy (TEM, H-7500, and

Hitachi Ltd, Tokyo, Japan). For TEM sample preparation, 10 μ l diluted nanomicelles were placed onto the copper grid and left for few minutes until the liquid is dispersed inside the grid[10]. Then particles were visualized using TEM. All procedures were performed in clean bench area.

2.5 Drug encapsulation and loading

The encapsulation efficiency (EE) and drug loading of CDF-loaded NMs were determined by high-performance liquid chromatography (HPLC) analysis in 70% methanol, 29.55% water and 0.45% formic acid (v/v/v) at 447 nm detection wavelength. The injection was triplicated, and data was represented as average of triplicated value. The drug loading was calculated by the following equations:

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Amount of CDF in micelles}}{\text{Amount of CDF used}} \times 100$$

$$\text{Drug Loading efficiency (\%)} = \frac{\text{Amount of CDF in micelles}}{\text{Amount of micelles}} \times 100$$

2.6 In vitro release profile of CDF-loaded NMs

The release of CDF from the NMs was determined in phosphate buffer solution (PBS, pH 7.4). Briefly, NMs with 1 mg of CDF were placed in 5 ml PBS (pH 5.5 or 7.4) containing 0.5% (w/w) tween-80 in a dialysis bag (MWCO = 2K) and incubated in corresponding PBS solution with gentle agitation at 37°C. The PBS solution outside the dialysis solution was collected at designated time and an equivalent volume of fresh PBS was compensated. The collected CDF contained was analyzed by HPLC.

2.7 Cellular uptake study

In-vitro cellular uptake study was done using both CD44 overexpressed TNBC cells line, MDA-MB-231/468. First, 2×10^5 cells were seeded in 24-well plate and then cells were treated with 100 nM Rhodamine B labeled formulations (SMA-TPGS-Rho and HA-SMA-TPGS-Rho) diluted with serum free DMEM medium for 3 h. Then medium was removed, and the cells were washed 3-times with PBS containing 0.1% FBS and fixed for 15 min then stained with Hoechst 33342 (10 μ g/ml) for 15 min, and washed with PBS and visualized in fluorescence microscope.

2.8 In vitro cytotoxicity assay

The cytotoxicity of SMA-TPGS conjugates, HA-SMA-TPGS conjugates, free CDF, targeted and non-targeted CDF NMs was evaluated by MTT assay. MDA-MBA-231 and MDA-MB-468 cells were seeded 3000 cells each well in 96-well plate. After that, the medium was removed, and different concentrations of conjugates and CDF NMs were treated to cells. After 24 h and 48 h, the medium was removed and performed MTT assay at 595 nm wave length in plate reader [39]. The results were calculated as the mean percentages of the viability of treated cells relative to untreated cells.

2.9 CD44 receptor blocking assay

The CD44 receptor blockade assay based on the principle of blocking the CD44 receptor using an excess amount of HA (5 mg/ml), followed by cells were treated with different concentrations of formulations[26]. The cell viability was evaluated by MTT assay again as described above.

2.10 Flow cytometry analysis

MDA-MB-231 and MDA-MB-468 cells (2×10^5 cells/well) were seeded in 6-well plate for overnight. After that, free SMA-TPGS conjugated, HA-SMA-TPGS conjugated, free CDF, SMA-TPGS-CDF and HA-SMA-TPGS-CDF were treated for 12 h, then cells were trypsinized and analyzed for apoptosis assay using Guava Nexin Annexin V assay (EMD Millipore, USA) as per manufacturer protocol and our previously published paper[26].

2.11 Western blot

Control, NMs and CDF treated cells were collected and lysed with lysis buffer containing cocktail protease and phosphatase inhibitors. Total protein content was performed using Bradford assay. An equal amount of protein was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. For detection of specific protein, membrane was incubated with PTEN and NF- κ b primary antibody (Cell Signaling Technology, USA) after blocking in 5% nonfat milk PBS solution and HRP-linked secondary antibodies were used to detect the bands in X-ray film.

2.12 Animal husbandry

6–8 weeks old Athymic nu/nu female mice were purchased from Jackson Laboratories, housed in a sterile environment on a standard 12 h light–dark cycle and maintained on normal rodent diet and water. All animal procedures were approved by the Wayne State Animal Care and IACUC committee in accordance with National Institutes of Health guidelines.

2.13 In vivo NIR imaging and bio-distribution of oligomer

Six-week-old female nu/nu mice were injected subcutaneously with MDA-MB-231 cells in PBS medium (5.0×10^6 cells per mouse). Tumor bearing mice were treated via tail vein injection with 10 nmole/mouse doses of HA-SMA-TPGS-S0456 and SMA-TPGS-S0456 oligomer respectively. Mice were imaged 4 h post-injection using a Carestream In Vivo MS FX PRO, Light Source: 400 W Xenon, Monochrome interlined, fixed lens (10 $\times$), CCD camera (13.8 $\times$ 13.8 cm / 2048 $\times$ 2048 px, 67 μ m $\times$ px, 16 bit), excitation 750 nm, emission 830 nm wavelength for fluorescence, and X-ray images were captured. Both fluorescence and X-ray images of mouse was merged to demonstrate the localization of oligomers.

3. Results

3.1 Synthesis and characterization of TPGS-SMA conjugates (Non-targeted) and HA-SMA-TPGS Conjugates (Targeted)

The TPGS-SMA and HA-SMA-TPGS conjugates were synthesized and characterized by using ^1H NMR and FT-IR spectroscopy. As shown in figure S1A, FTIR spectra SMA-TPGS and HA-SMA-TPGS conjugates showed stretching vibration frequency at 1735 cm^{-1} for carbonyl group of ester group, and peaks at 3100 cm^{-1} indicates the vibration of aromatic hydrogen of SMA (Figure S1A) [20][32]. The similarity of IR-pattern among SMA, TPGS and HA with SMA-TPGS and HA-SMA-TPGS indicates the presence of monomer units into the polymer conjugates. Further the conjugate formation was confirmed by ^1H -NMR (Figure S1B) and the characterized peak of HA in HA-SMA-TPGS was appeared at 3.2–4.1 ppm (for glucoside H), and aromatic H peaks of SMA at 7–7.5 ppm were maintained in both SMA-TPGS and HA-SMA-TPGS, indicating successful conjugation. Retention of characteristic peaks of HA-SMA-TPGS at 4.5 ppm of HA and 3.7 ppm of TPGS (for $\text{CH}_2\text{-O-}$) indicate and confirmed successfully coupling between HA and SMA-TPGS [10,20,32]. The conjugates in an aqueous phase could self-assemble into micelles owing to the hydrophobic part in SMA and TPGS. In figure 2A the plotting of fluorescence intensity of excitation wavelengths ratio (I_{335}/I_{332}) increase from 0.87 to 1.14 with increasing concentration of conjugates (Figure S2). The CMC of both conjugates is less than 0.001 mg/ml which indicate a strong tendency of conjugates towards formation of NMs in water at a lower concentration.

3.2 CDF-loaded NMs demonstrated excellent nanoparticle characteristic and sustain CDF release

The average particles size of TPGS-SMA-CDF showed 167.6 nm and for HA-SMA-TPGS-CDF showed 129.4 nm with narrow polydispersity index (Figure 2A). The morphology of CDF-loaded NMs was characterized by transmission electron microscopy (TEM) (Figure 2B). The TEM data revealed that the diameter of TPGS-SMA-CDF is around 150–180 nm and HA-SMA-TPGS-CDF is around 80–120 nm. Both micelles were spherical in shape with a smooth surface. Smaller hydrodynamic diameter of HA-SMA-TPGS-CDF demonstrates the favorable characteristics of EPR effect and enhanced tumor penetration. The CDF loading for TPGS-SMA-CDF is $14.9 \pm 1.4\%$ and EE is $84.3 \pm 2.4\%$; and HA-SMA-TPGS-CDF is $19.6 \pm 1.8\%$ and the EE is $81.8 \pm 3.7\%$ ($n=3$) respectively. The efficient drug loading capacity of NMs will improve bioavailability of CDF derivatives and reduce amount of polymer.

The CDF release study as illustrated by figure 2C, both targeted and non-targeted NM showed sustain release profiles at various pH conditions. The cumulative release of CDF for SMA-TPGS-CDF and HA-SMA-TPGS-CDF NM at 24 h, at pH 7.4 was $67.56\% \pm 2.87$, $78.65\% \pm 2.58$, at pH 5.5 it was found to be $57.9\% \pm 6.95$ and $69.53\% \pm 4.01$ respectively. After 72h, the cumulative release of the CDF for SMA-TPGS-CDF and HA-SMA-TPGS-CDF micelles at physiological pH was $84.73\% \pm 7.4$ and $86.97\% \pm 5.85$, at acidic pH it was $77.98\% \pm 6.9$ and $84.59\% \pm 6.35$, respectively. The sustain release pattern of CDF favors the feasibility of NMs for selective tumor accumulation.

3.3 Presence of HA enhanced TNBC cell accumulation

As shown in figure 3, the selective accumulation of targeted oligomer in CD44 positive MDA-MB-231 and MDA-MB-468 cells. The cell's nucleus was stained with blue-color Hoechst dye, and red-fluorescence indicates the signal of Rhodamine B. In both cell lines, the HA-targeted oligomer showed highest red-fluorescence intensity which corroborated with faster cellular compared to non-targeted oligomer. Higher accumulation of targeted formulation is probably due to high affinity of HA-ligand to CD44 receptor-mediated endocytosis. The receptor mediated uptake of HA-SMA-TPGS-Rod is a useful feature in inducing cancer cell selective cytotoxicity.

3.4 Targeted NM facilitated TNBC cell killing

For cell killing study, MDA-MB-231 and MDA-MB-468 cells were treated either with free CDF and NMs. As shown in figure 4A, HA-TPGS-SAM-CDF micelles showed a higher cell killing compared with non-targeted NMs and free CDF. In MDA-MB-231, the IC_{50} of CDF for 24 h and 48 h of post treatment is 280.4 nM and 161.8 nM, whereas the IC_{50} of non-targeted NM is 196.1 nM and 96.3 nM respectively. In case of HA-SMA-TPGS-CDF IC_{50} has been reduced to 88.7 nM and 59.3 nM after 24 h and 48 h of post treatment.

Similarly, in MDA-MB-468 cells, in 24 h and 48 h the IC_{50} of CDF is 468.8 nM and 151.4 nM and for non-targeted NMs 10.9 nM and 65.6 nM respectively. With the targeted NMs the IC_{50} has been decreased to 99.1 nM and 59.9 nM. Results from figure 4 A suggest the need of HA functionalization to NMs for enhancing the TNBC cell killing effect. The higher cytotoxicity of HA-micelles is attributed to the active targeting of cancer cells followed by receptor mediated endocytosis. In figure 4B, we found that there is no significant cell killing effect in empty oligomer treated cells, indicating the cytotoxicity effect of CDF encapsulated NMs is due to presence of CDF in HA-SMA-TPGS-CDF or SMA-TPGS-CDF formulations. Comparison of IC_{50} values from table 1 clearly indicate the effectiveness of HA-SMA-TPGS-CDF for cancer cell killing.

3.5 Cell killing effect of HA-targeted NM is CD44 receptor selective

Receptor blocking assay was performed to further verify whether cell killing effect of HA-targeted NM is followed through CD44 receptor mediated. The cells were pretreated with excess amount of HA (5 mg/ml) followed by treatment of NMs. In MDA-MB-231 cells (Table 1), it was observed that before CD44 receptor blockade the IC_{50} values at 24 h of post-treatment with free CDF, non-targeted micelles and HA-targeted micelles were 280.4 nM, 196.1 nM, 88.7 nM respectively. But after the receptor blockade, the IC_{50} value (figure 5A) of free CDF and non-targeted micelles remained unchanged of 279.3 nM, 200.2 nM respectively. Contrastingly the IC_{50} of HA-SMA-TPGS-CDF has been increased to 194.2 nM at 24 h post treatment. Similarly, for 48h treatment of MDA-MB-231 the IC_{50} value of targeted micelles were changed from 59.3 nM (figure 4A) to 94.2 nM (Figure 5A, Table 1). One can also observe the increment of cell viability for HA-targeted formulation in HA-blocked MDA-MB-468 cell line (Figure 5B). The summary of IC_{50} values in normal and HA-blockade cell culture conditions are illustrated in Table 1. The increasing IC_{50} values of targeted formulation clearly indicate that HA-targeted NM induces CD44 receptor mediated

TNBC killing. This observation opens a new avenue for targeted drug delivery of most challenging and limited biomarker abundant TNBC tumor.

3.6 Apoptosis mediated cell killing effect of targeted NM

In order to evaluate the pathway of TNBC cell killing, we performed apoptosis assay using dual apoptosis and cell viability marker using Annexin-V and 7-AAD respectively. The dual staining discriminates among unaffected cells (annexin-V negative, 7-AAD negative), early apoptotic cells (annexin-V positive, 7-AAD negative), late apoptotic cells (annexin-V positive, 7-AAD positive), and necrotic cells (annexin V negative, 7-AAD positive). As shown in figure 6, MDA-MB-231 and MDA-MB-468 cells were treated with free-CDF, targeted and non-targeted and empty micelles. As expected, HA-SMA-TPGS-CDF induced more apoptosis in both MDA-MB-231 and MDA-MB-468 cells line than control and non-targeted formulation. The increment of % early apoptosis as represented in bar graph and dot plot indicates the resurrection of apoptosis in HA-NMs treated cells. These results demonstrate that higher cell killing of HA-targeted NMs is due to selective induction of early and late stage apoptosis and efficient CDF delivery to TNBC cytoplasm.

To evaluate caspase-3/7 activity, cells were cultured and treated with DMEM medium containing CDF, SMA-TPGS-CDF or HA-SMA-TPGS-CDF with 100 nM CDF for 24h. Subsequently, cells were incubated with Caspase-Glo 3/7 assay solution (Promega kit) and analyzed for chemiluminescence using Promega microplate reader, according to the manufacturer's protocol.

3.7 Down-modulation of tumorigenic transcription factor and upregulation pro-apoptotic proteins

As HA-SMA-TPGS-CDF can significantly induce apoptosis to TNBC, we were keen to find mechanistic pathway of cell killing. Previously our lab has identified that CDF can cause inactivation of oncogenic signaling pathways through down-regulation of NF- κ B and up-regulation of PTEN expression level [26]. Thus to find the effect of HA-SMA-TPGS-CDF formulation, western blot was performed to detect PTEN and NF- κ B protein level after treating with free drug, targeted and non-targeted formulation. As shown in figure 7, both MDA-MB-231 and MDA-MB-468 cells showed a significant down-regulation of NF- κ B and up-regulation of PTEN in HA-SMA-TPGS-CDF treatment group in comparison with free CDF and non-targeted CDF formulation. Quantification of β -actin normalized PTEN protein bands of HA-targeted micelles treatment in MDA-MB-231 and MDA-MB-468 indicated 2-3-fold increase in comparison with control and free CDF treatment respectively. Concordantly, the NF- κ B level of HA-SMA-TPGS-CDF treatment in MDA-MB-231 and MDA-MB-468 decreased by 2–3 fold as compared to control and free CDF treatment respectively. The upregulation of PTEN and downregulation of NF- κ B of HA-SMA-TPGS-CDF treatment is directly associated with reactivation of anti-cancer signalling pathway, leading to induction of apoptosis.

3.8 NIR Image guided TNBC tumor detection with minimum liver uptake

NIR imaging is a cost effective, less toxic, advanced diagnostic tool for tumor detection. As TNBC grows asymptomatic and has high metastatic tendency, thus the early detection of

tumor mass is one of unmet need for surgical resection. To determine the in vivo TNBC targeting efficacy of HA-SMA-TPGS oligomer, we conjugated the clinically used, high contrast, structurally symmetrical near infrared dye (S0456) to arrive at HA-SMA-TPGS-S0456 and SMA-TPGS-S0456 was used as non-targeted control[40][41]. MDA-MB-231 tumor bearing mice were injected with HA-SMA-TPGS-S0456 and SMA-TPGS-S0456 and whole-body imaging was captured after 4 h post I.V. injection. As shown in figure 8 (A, B), HA-targeted conjugate was predominantly accumulated in CD44 positive MDA-MB-231 tumor. Whereas non-targeted (E, F) conjugate did not show any significant tumor uptake and it was non-specifically distributed to lung, skin and other healthy organs. To better understand the targetability in tumor and healthy tissues, major organs/tissues were removed and examined for NIR fluorescence bio-distribution at 4 h post-injection. As shown in figure 8C, fluorescence intensity of HA-conjugate is significantly higher in tumor than other healthy tissues and higher NIR-dye uptake in tumor with respect liver (figure 8D) indicates receptor targetability of HA-SMA-TPGS conjugate. Contrastingly, the bio-distribution of NIR-dye in SMA-TPGS treated mice was majorly accumulated in non-specific lung, liver with low accumulation in tumor (Figure G, H). Thus, CD44-targeting oligomer has potency to distinguish the tumor lesion from the healthy organs and it can be further developed for image guided TNBC surgery [41].

4. Discussion

TNBC is one of the most lethal and highly refractory among all malignant aggressive breast cancer. TNBC remains a major clinical challenge to physicians, researchers and patients even though it contributes to a smaller percentage of breast cancer subtype [42]. The potential cellular molecules including Epidermal growth factor receptor (EGFR)[43–45], Insulin growth factor 1 receptor (IGF-1R)[46], Folate receptor- α/β (FA)[47–49], Mucin 1 (MUC1)[50], and CD44 receptor[51,52] has been used for TNBC targeted therapy[15][52]. Among all this surface specific markers, CD44 has been extensively studied for selective nanoparticle drug delivery to tumor epithelial and cancer stem cell (CSC) of various solid tumors, including TNBC. This attributes to its signature features such as (i) important role in tumor proliferation, migration and tumor extravasation by interacting with cell-cell and cell-tumor stromal matrix[53]; (ii) greater than 85% of MDA-MB-231 subpopulation of cells overexpressed CD44 protein whereas healthy breast ductal tissue has very low expression[54]; (iii) high affinity to naturally abundant HA-polymer[55]. Due to presence of –COOH and primary –OH functional group and disaccharide backbone in HA makes it convenient for chemical conjugation with SMA-TPGS hydrophobic oligomer, resulting in formation of stable and low CMC featured nano-sized CDF micelles. Higher drug loading efficiency is an important criterion for developing clinically translatable nano-carrier. Our synthesized targeted and non-targeted NMs have shown higher CDF loading efficiency and this is due to the influence of SMA-TPGS interior hydrophobic cavities. Drug release kinetics of CDF from targeted and non-targeted NMs in acidic (pH 5.5) condition demonstrated the feasibility of HA-SMA-TPGS-CDF formulation in cancer cell cytoplasmic drug release after CD44 receptor mediated endocytosis. Utilization of the EPR effect coupled with active cell surface receptor targeting of epithelial cells, stromal fibroblasts, and immune cells is likely to overcome bottle neck barriers of tumors and facilitate more

effective drug delivery. For accessing these advantages, herein we report, HA-SMA-TPGS-CDF NMs for efficient drug delivery to CD44 receptor overexpressing MDA-MB-231 and MDA-MB-468 TNBC cell lines, also addressing the poor aqueous solubility issue of CDF. In order to improve of TNBC selective drug delivery, HA is an essential component of SMA-TPGS micelles as we have demonstrated that rhodamine uptake is significantly higher in HA-conjugated NMs as compared to non-targeted SMA-TPGS micelles. The improvement of IC₅₀ value for targeted NM compared to non-targeted NM in both TNBC cells is attributed to the presence of HA-targeting. The enhancement of fluorescence uptake supports an indication of receptor mediated endocytosis of HA-SMA-TPGS that was also confirmed by increment of IC₅₀ value in HA-pretreated CD44 receptor competition cell viability assay. The resurrection of apoptosis is a principal step of chemotherapy associated cytotoxicity and failure to activate apoptotic signaling cascade is a well-recognized mechanism of drug resistance[10,56,57]. Herein, we have demonstrated that HA-SMA-TPGS-CDF is highly potent on inducing early phase of apoptosis in TNBC cells, resulting programmed cell death and down-regulation of NF- κ B and up-regulation of P53 protein. The upregulation of pro-apoptotic caspase 3/7 protein in HA-SMA-TPGS-CDF treated MDA-MB-231 cells (compared to untreated control, free CDF and non-targeted formulation) indicates the apoptosis mediated cell killing of TNBC.

Recently FDA approved Poly ADP-ribose polymerase (PARP) inhibitor, Olaparib has been used as an orally administrable chemotherapeutic for BRCA-1/2 mutated TNBC patients and it functions as an activator of apoptosis. But the progression-free survival rate of Olaparib has shown little benefit in TNBC populations, 5.7 months as compared with 3.8 months for control cohort[58][59]. Thus, newer therapy with feasibility of i.v administration is a rational approach. Toward this HA-SMA-TPGS-CDF nanomedicine platform represents as a potent activator of apoptosis in TNBC cells, thus supporting our logical approach of developing i.v administrable chemotherapeutic for TNBC.

In clinic there is significant progress in development of ligand-targeted NIR imaging agent that bind to folate receptor for ovarian cancer (clinical trial: NCT02317705), and PSMA for prostate cancer (clinical trial: NCT02048150) [60]. As per our knowledge, there is no TNBC targeting NIR dye for clinical investigation. Thus, our tumor imaging approach using HA-SMA-TPGS-S0456 oligomer opens an opportunity for further developing more selective NIR imaging agent for image-guided intraoperative TNBC surgery. In our attempt we showed targeted NIR-dye is more selective to tumor as compared with non-targeted dye. In summary the current study demonstrates the dual approach of TNBC selective NIR-imaging in preclinical tumor xenograft models and evaluating cell killing effect of CD44 receptor targeted CDF-loaded NMs, warranting further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial Support Information: AKI would like to acknowledge partial support from Wayne State University start-up funding and American Cancer Society (ACS-IRG #4493533) funding to perform the studies. We

acknowledge NIH center grant (#P30 CA22453) to Karmanos Cancer Institute, Wayne State University, and the Perinatology Research Branch for the services of Microscopy, Imaging and Cytometry Resources (MICR) core. TEM analysis in JEOL 2010 was supported by an NSF Award#0216084.

References

- Carey L, Winer E, Viale G, Cameron D, Gianni L. Triple-negative breast cancer: disease entity or title of convenience? *Nat Rev Clin Oncol*. 72010; :683–692. DOI: 10.1038/nrclinonc.2010.154 [PubMed: 20877296]
- Desantis C, Siegel R, Jemal A. Breast Cancer Facts & Figures 2015–2016. *Surveill Heal Serv Res Progr*. 2015; doi: 10.1016/B978-1-4377-1757-0.00028-7
- Carey LA, Dees EC, Sawyer L, Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F, Ollila DW, Sartor CI, Graham ML, Perou CM. The Triple Negative Paradox: Primary Tumor Chemosensitivity of Breast Cancer Subtypes riple Negative Paradox: Primary Tumor Chemosensitivity of Breast Cancer Subtypes. 2007; :2329–2334. DOI: 10.1158/1078-0432.CCR-06-1109
- Foulkes WD, Smith IE, Reis-Filho JS. Triple-negative breast cancer. *N Engl J Med*. 3632010; : 1938–1948. DOI: 10.1056/NEJMra1001389 [PubMed: 21067385]
- Sau S, Alsaab HO, Kashaw SK, Tatiparti K, Iyer AK. Advances in antibody?drug conjugates: a new era of targeted cancer therapy. *Drug Discov Today*. 2017; doi: 10.1016/j.drudis.2017.05.011
- Sau S, Alsaab HO, Bhise K, Alzhrani R, Nabil G, Iyer AK. Multifunctional nanoparticles for cancer immunotherapy: A groundbreaking approach for reprogramming malfunctioned tumor environment. *J Control Release*. 2018; doi: 10.1016/j.jconrel.2018.01.028
- Sau S, Alsaab HO, Kashaw SK, Tatiparti K, Iyer AK. Advances in antibody–drug conjugates: A new era of targeted cancer therapy. *Drug Discov Today*. 222017; :1547–1556. DOI: 10.1016/j.drudis.2017.05.011 [PubMed: 28627385]
- Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov*. 52006; :219–234. DOI: 10.1038/nrd1984 [PubMed: 16518375]
- Bhise K, Kashaw SK, Sau S, Iyer AK. Nanostructured Lipid Carriers Employing Polyphenols as Promising Anticancer Agents: Quality by Design (QbD) approach. *Int J Pharm*. 2017
- Cheriyian VT, Alsaab HO, Sekhar S, Stieber C, Iyer AK, Rishi AK. A CARP-1 functional mimetic loaded vitamin E-TPGS micellar nano-formulation for inhibition of renal cell carcinoma, (2017).
- Sau S, Agarwalla P, Mukherjee S, Bag I, Sreedhar B, Pal-Bhadra M, Patra CR, Banerjee R. Cancer cell-selective promoter recognition accompanies antitumor effect by glucocorticoid receptor-targeted gold nanoparticle. *Nanoscale*. 62014; :6745–54. DOI: 10.1039/c4nr00974f [PubMed: 24824564]
- Ganesh S, Iyer AK, Morrissey DV, Amiji MM. Hyaluronic acid based self-assembling nanosystems for CD44 target mediated siRNA delivery to solid tumors. *Biomaterials*. 342013; : 3489–3502. DOI: 10.1016/j.biomaterials.2013.01.077 [PubMed: 23410679]
- Iyer AK, Su Y, Feng J, Lan X, Zhu X, Liu Y, Gao D, Seo Y, VanBrocklin HF, Broaddus VC, et al. The effect of internalizing human single chain antibody fragment on liposome targeting to epithelioid and sarcomatoid mesothelioma. *Biomaterials*. 322011; :2605–2613. [PubMed: 21255833]
- Morath I, Hartmann TN, Orian-Rousseau V. CD44: More than a mere stem cell marker. *Int J Biochem Cell Biol*. 812016; :166–173. DOI: 10.1016/j.biocel.2016.09.009 [PubMed: 27640754]
- Agrawal S, Dwivedi M, Ahmad H, Chadchan SB, Arya A, Sikandar R, Kaushik S, Mitra K, Jha RK, Dwivedi AK. CD44 targeting hyaluronic acid coated lapatinib nanocrystals foster the efficacy against triple-negative breast cancer. *Nanomedicine Nanotechnology. Biol Med*. 142018; :327–337. DOI: 10.1016/j.nano.2017.10.010
- Riechelmann H, Sauter A, Golze W, Hanft G, Schroen C, Hoermann K, Erhardt T, Gronau S. Phase I trial with the CD44v6-targeting immunoconjugate bivatuzumab mertansine in head and neck squamous cell carcinoma. *Oral Oncol*. 442008; :823–829. DOI: 10.1016/j.oraloncology.2007.10.009 [PubMed: 18203652]

17. Tijink BM, Buter J, De Bree R, Giaccone G, Lang MS, Staab A, Leemans CR, Van Dongen GAMS. A phase I dose escalation study with anti-CD44v6 bivatuzumab mertansine in patients with incurable squamous cell carcinoma of the head and neck or esophagus. *Clin Cancer Res.* 122006; :6064–6072. DOI: 10.1158/1078-0432.CCR-06-0910 [PubMed: 17062682]
18. West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. *Science.* 2281985; :1324–6. DOI: 10.1126/science.2408340 [PubMed: 2408340]
19. Xu H, Yang P, Ma H, Yin W, Wu X, Wang H, Xu D, Zhang X. Amphiphilic block copolymers-based mixed micelles for noninvasive drug delivery. *Drug Deliv.* 75442016; :1–9. DOI: 10.3109/10717544.2016.1149743
20. Alsaab H, Alzhrani R, Kesharwani P, Sau S, Boddu S, Iyer A. Folate Decorated Nanomicelles Loaded with a Potent Curcumin Analogue for Targeting Retinoblastoma. *Pharmaceutics.* 92017; :15.doi: 10.3390/pharmaceutics9020015
21. Cagel M, Tesan FC, Bernabeu E, Salgueiro MJ, Zubillaga MB, Moretton MA, Chiappetta DA. Polymeric mixed micelles as nanomedicines: Achievements and perspectives. *Eur J Pharm Biopharm.* 1132017; :211–228. DOI: 10.1016/j.ejpb.2016.12.019 [PubMed: 28087380]
22. Kim TY, Kim DW, Chung JY, Shin SG, Kim SC, Heo DS, Kim NK, Bang YJ. Phase I and pharmacokinetic study of Genexol-PM, a Cremophor-free, polymeric micelle-formulated paclitaxel, in patients with advanced malignancies. *Clin Cancer Res.* 102004; :3708–3716. DOI: 10.1158/1078-0432.CCR-03-0655 [PubMed: 15173077]
23. Ahn HK, Jung M, Sym SJ, Shin DB, Kang SM, Kyung SY, Park JW, Jeong SH, Cho EK. A phase II trial of Cremophor EL-free paclitaxel (Genexol-PM) and gemcitabine in patients with advanced non-small cell lung cancer. *Cancer Chemother Pharmacol.* 742014; :277–282. DOI: 10.1007/s00280-014-2498-5 [PubMed: 24906423]
24. Lu Y, Park K. Polymeric micelles and alternative nanonized delivery vehicles for poorly soluble drugs. *Int J Pharm.* 4532013; :198–214. DOI: 10.1016/j.ijpharm.2012.08.04 [PubMed: 22944304]
25. Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, Iyer AK. PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome. *Front Pharmacol.* 82017; :1–15. DOI: 10.3389/fphar.2017.00561 [PubMed: 28149278]
26. Luong D, Sau S, Kesharwani P, Iyer AK. Polyvalent Folate-Dendrimer-Coated Iron Oxide Theranostic Nanoparticles for Simultaneous Magnetic Resonance Imaging and Precise Cancer Cell Targeting. *Biomacromolecules.* 2017; doi: 10.1021/acs.biomac.6b01885
27. Gawde KA, Kesharwani P, Sau S, Sarkar FH, Padhye S, Kashaw SK, Iyer AK. Synthesis and characterization of folate decorated albumin bio-conjugate nanoparticles loaded with a synthetic curcumin difluorinated analogue. *J Colloid Interface Sci.* 4962017; :290–299. [PubMed: 28236692]
28. Padhye S, Banerjee S, Chavan D, Pandye S, Swamy KV, Ali S, Li J, Dou QP, Sarkar FH. Fluorocurcumin as cyclooxygenase-2 inhibitor: Molecular docking, pharmacokinetics and tissue distribution in mice. *Pharm Res.* 262009; :2438–2445. DOI: 10.1007/s11095-009-9955-6 [PubMed: 19714451]
29. Bao B, Ali S, Kong D, Sarkar SH, Wang Z, Banerjee S, Aboukameel A, Padhye S, Philip PA, Sarkar FH. Anti-tumor activity of a novel compound-CDF is mediated by regulating miR-21, miR-200, and pten in pancreatic cancer. *PLoS One.* 62011; :1–12. DOI: 10.1371/journal.pone.0017850
30. Bao B, Ali S, Banerjee S, Wang Z, Logna F, Azmi AS, Kong D, Ahmad A, Li Y, Padhye S, Sarkar FH. Curcumin analogue CDF inhibits pancreatic tumor growth by switching on suppressor microRNAs and attenuating EZH2 expression. *Cancer Res.* 722012; :335–345. DOI: 10.1158/0008-5472.CAN-11-2182 [PubMed: 22108826]
31. Vyas A, Dandawate P, Padhye S, Ahmad A, Sarkar F. Perspectives on new synthetic curcumin analogs and their potential anticancer properties. *Curr Pharm Des.* 192013; :2047–69. DOI: 10.1016/j.surg.2006.10.010.Use [PubMed: 23116312]
32. Luong D, Kesharwani P, Alsaab HO, Sau S, Padhye S, Sarkar FH, Iyer AK. Folic acid conjugated polymeric micelles loaded with a curcumin difluorinated analog for targeting cervical and ovarian cancers. *Colloids Surfaces B Biointerfaces.* 2017

33. Seymour LW, Olliff SP, Poole CJ, De Takats PG, Orme R, Ferry DR, Maeda H, Konno T, Kerr DJ. *Int J Oncol.* 121998; :1217–23. [PubMed: 9592177]
34. Maeda H. SMANCS and polymer-conjugated macromolecular drugs: Advantages in cancer chemotherapy. *Adv Drug Deliv Rev.* 462001; :169–185. DOI: 10.1016/S0169-409X(00)00134-4 [PubMed: 11259839]
35. Varma MVS, Panchagnula R. Enhanced oral paclitaxel absorption with vitamin E-TPGS: Effect on solubility and permeability in vitro, in situ and in vivo. *Eur J Pharm Sci.* 252005; :445–453. DOI: 10.1016/j.ejps.2005.04.003 [PubMed: 15890503]
36. Zhang Z, Tan S, Feng SS. Vitamin E TPGS as a molecular biomaterial for drug delivery. *Biomaterials.* 332012; :4889–4906. DOI: 10.1016/j.biomaterials.2012.03.046 [PubMed: 22498300]
37. Kesharwani P, Banerjee S, Padhye S, Sarkar FH, Iyer AK. Parenterally administrable nano-micelles of 3,4-difluorobenzylidene curcumin for treating pancreatic cancer. *Colloids Surfaces B Biointerfaces.* 1322015; :138–145. DOI: 10.1016/j.colsurfb.2015.05.007 [PubMed: 26037703]
38. Kesharwani P, Banerjee S, Padhye S, Sarkar FH, Iyer AK. Hyaluronic Acid Engineered Nanomicelles Loaded with 3,4-Difluorobenzylidene Curcumin for Targeted Killing of CD44+ Stem-Like Pancreatic Cancer Cells. *Biomacromolecules.* 162015; :3042–3053. DOI: 10.1021/acs.biomac.5b00941 [PubMed: 26302089]
39. Anjibabu R, Sau S, Reddy BJM, Banerjee R, Reddy BVS. Heteropoly acid catalyzed synthesis of 8-methyl-2-aryl/alkyl-3-oxabicyclo[3.3.1]non-7-ene derivatives through (3,5)-oxonium-ene reaction. *Tetrahedron Lett.* 542013; :7160–7163.
40. Shum CF, Bahler CD, Low PS, Ratliff TL, Kheifets SV, Natarajan JP, Sandusky GE, Sundaram CP. Novel Use of Folate-Targeted Intraoperative Fluorescence, OTL38, in Robot-Assisted Laparoscopic Partial Nephrectomy: Report of the First Three Cases. *J Endourol Case Reports.* 22016; :189–197. DOI: 10.1089/cren.2016.0104
41. Lv PC, Roy J, Putt KS, Low PS. Evaluation of a Carbonic Anhydrase IX-Targeted Near-Infrared Dye for Fluorescence-Guided Surgery of Hypoxic Tumors. *Mol Pharm.* 132016; :1618–1625. DOI: 10.1021/acs.molpharmaceut.6b00065 [PubMed: 27043317]
42. Sharma S, Barry M, Gallagher DJ, Kell M, Sacchini V. An overview of triple negative breast cancer for surgical oncologists. *Surg Oncol.* 242015; :276–283. DOI: 10.1016/j.suronc.2015.06.007 [PubMed: 26092709]
43. Viale G, Rotmensz N, Maisonneuve P, Bottiglieri L, Montagna E, Luini A, Veronesi P, Intra M, Torrisi R, Cardillo A, Campagnoli E, Goldhirsch A, Colleoni M. Invasive ductal carcinoma of the breast with the “triple-negative” phenotype: prognostic implications of EGFR immunoreactivity. *Breast Cancer Res Treat.* 1162009; :317–328. DOI: 10.1007/s10549-008-0206-z [PubMed: 18839307]
44. Baselga J, Gómez P, Greil R, Braga S, Climent MA, Wardley AM, Kaufman B, Stemmer SM, Pêgo A, Chan A, Goeminne JC, Graas MP, Kennedy MJ, Ciruelos Gil EM, Schneeweiss A, Zube A, Groos J, Melezínková H, Awada A. Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. *J Clin Oncol.* 312013; :2586–2592. DOI: 10.1200/JCO.2012.46.2408 [PubMed: 23733761]
45. Masuda H, Zhang D, Bartholomeusz C, Doihara H, Hortobagyi GN, Ueno NT. Role of epidermal growth factor receptor in breast cancer. *Breast Cancer Res Treat.* 1362012; :331–345. DOI: 10.1007/s10549-012-2289-9 [PubMed: 23073759]
46. Yerushalmi R, Gelmon KA, Leung S, Gao D, Cheang M, Pollak M, Turashvili G, Gilks BC, Kennecke H. Insulin-like growth factor receptor (IGF-1R) in breast cancer subtypes. *Breast Cancer Res Treat.* 1322012; :131–142. DOI: 10.1007/s10549-011-1529-8 [PubMed: 21574055]
47. Paulmurugan R, Bhethanabotla R, Mishra K, Devulapally R, Foygel K, Sekar TV, Ananta JS, Massoud TF, Joy A. Folate Receptor-Targeted Polymeric Micellar Nanocarriers for Delivery of Orlistat as a Repurposed Drug against Triple-Negative Breast Cancer. *Mol Cancer Ther.* 152016; :221–31. DOI: 10.1158/1535-7163.MCT-15-0579 [PubMed: 26553061]
48. Necela BM, Crozier JA, Andorfer CA, Lewis-Tuffin L, Kachergus JM, Geiger XJ, Kalari KR, Serie DJ, Sun Z, Aspita AM, O'Shannessy DJ, Maltzman JD, McCullough AE, Pockaj BA, Cunliffe HE,

- Ballman KV, Thompson EA, Perez EA. Folate receptor- α (FOLR1) expression and function in triple negative tumors. *PLoS One*. 102015; doi: 10.1371/journal.pone.0122209
49. Zhang Z, Wang J, Tacha DE, Li P, Bremer RE, Chen H, Wei B, Xiao X, Da J, Skinner K, Hicks DG, Bu H, Tang P. Folate receptor α associated with triple-negative breast cancer and poor prognosis. *Arch Pathol Lab Med*. 1382014; :890–5. DOI: 10.5858/arpa.2013-0309-OA [PubMed: 24028341]
 50. Siroy A, Abdul-Karim FW, Miedler J, Fong N, Fu P, Gilmore H, Baar J. MUC1 is expressed at high frequency in early-stage basal-like triple-negative breast cancer. *Hum Pathol*. 442013; :2159–2166. DOI: 10.1016/j.humpath.2013.04.010 [PubMed: 23845471]
 51. Idowu MO, Kmiecik M, Dumur C, Burton RS, Grimes MM, Powers CN, Manjili MH. CD44(+)/CD24(–/low) cancer stem/progenitor cells are more abundant in triple-negative invasive breast carcinoma phenotype and are associated with poor outcome. *Hum Pathol*. 432012; :364–73. DOI: 10.1016/j.humpath.2011.05.005 [PubMed: 21835433]
 52. Wang S, Zhang J, Wang Y, Chen M. Hyaluronic acid-coated PEI-PLGA nanoparticles mediated co-delivery of doxorubicin and miR-542-3p for triple negative breast cancer therapy. *Nanomedicine Nanotechnology. Biol Med*. 122016; :411–420. DOI: 10.1016/j.nano.2015.09.014
 53. Jordan AR, Racine RR, Hennig MJP, Lokeshwar VB. The role of CD44 in disease pathophysiology and targeted treatment. *Front Immunol*. 62015; doi: 10.3389/fimmu.2015.00182
 54. Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, Goulet R, Badve S, Nakshatri H. CD44+/CD24– breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res*. 82006; :59.doi: 10.1186/bcr1610
 55. Wickens JM, Alsaab HO, Kesharwani P, Bhise K, Amin MCIM, Tekade RK, Gupta U, Iyer AK. Recent advances in hyaluronic acid-decorated nanocarriers for targeted cancer therapy. *Drug Discov Today*. 222017; :665–680. DOI: 10.1016/j.drudis.2016.12.009 [PubMed: 28017836]
 56. Almansour AI, Arumugam N, Kumar R Suresh, Mahalingam SM, Sau S, Bianchini G, Menéndez JC, Altaf M, Ghabbour HA. Design, synthesis and antiproliferative activity of decarbonyl luotonin analogues. *Eur J Med Chem*. 1382017; :932–941. DOI: 10.1016/j.ejmech.2017.07.027 [PubMed: 28753517]
 57. Sau S, Banerjee R. Cationic lipid-conjugated dexamethasone as a selective antitumor agent. *Eur J Med Chem*. 832014; :433–447. [PubMed: 24992071]
 58. Audeh MW, Carmichael J, Penson RT, Friedlander M, Powell B, Bell-McGuinn KM, Scott C, Weitzel JN, Oaknin A, Loman N, Lu K, Schmutzler RK, Matulonis U, Wickens M, Tutt A. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and recurrent ovarian cancer: A proof-of-concept trial. *Lancet*. 3762010; :245–251. DOI: 10.1016/S0140-6736(10)60893-8 [PubMed: 20609468]
 59. O'Reilly EA, Gubbins L, Sharma S, Tully R, Guang MHZ, Weiner-Gorzel K, McCaffrey J, Harrison M, Furlong F, Kell M, McCann A. The fate of chemoresistance in triple negative breast cancer (TNBC). *BBA Clin*. 32015; :257–275. DOI: 10.1016/j.bbacli.2015.03.003 [PubMed: 26676166]
 60. Zhang RR, Schroeder AB, Grudzinski JJ, Rosenthal EL, Warram JM, Pinchuk AN, Eliceiri KW, Kuo JS, Weichert JP. Beyond the margins: real-time detection of cancer using targeted fluorophores. *Nat Rev Clin Oncol*. 142017; :347–364. DOI: 10.1038/nrclinonc.2016.212 [PubMed: 28094261]

Figure 1. Schematic illustration of HA-SMA-TPGS conjugate synthesis and nano-micelle (NM) formation with CDF, namely HA-SMA-TPGS-CDF. The HA-SMA-TPGS can selectively home into CD44 overexpressing TNBC, and can be utilized as a strategy for tumor NIR imaging for TNBC diagnosis.

Figure 2. Physico-chemical analysis

(A) Hydrodynamic size of SMA-TPGS-CDF NMs and HA-SMA-TPGS NMs by DLS are shown. (B) The morphology of SMA-TPGS-CDF NMs and HA-SMA-TPGS-CDF NMs characterized by TEM are shown. Scale bar = 500 nm. (C) *In vitro* drug release of SMA-TPGS-CDF NMs and HA-SMA-TPGS-CDF NMs incubated in PBS at pH 5.5 and 7.4, 37°C, respectively are shown. Data are presented as mean $\pm$ SD, n=3.

Figure 3. CD44 receptor mediated selectivity delivery of HA-oligomer

Cancer cell selective uptake of Rhodamine B labeled HA targeted oligomer in comparison to non-targeted NMs in (A) MDA-MB-231, and (B) MDA-MB-468 cells after 3 h treatment. Blue and red fluorescence indicate cell nuclei and Rhodamine B, respectively.

Figure 4. TNBC cell killing effect of CDF loaded NMs

Viability assay of MDA-MB-231 and MDA-MB-468 cells after 24 h and 48 h treated with (A) free CDF, SMA-TPGS-CDF NMs and HA-SMA-TPGS-CDF NMs at various CDF drug concentrations and (B) vehicle control HA, SMA, TPGS, SMA-TPGS and SMA-TPGS oligomers. Data represent mean $\pm$ SD, n=6.

Figure 5. Receptor blockade assay

Improvement of cell viability at 24 h and 48h after CD44 receptor blockade by pretreating HA followed by treatment with free CDF, SMA-TPGS-CDF NMs and HA-SMA-TPGS-CDF NMs on MDA-MB-231 (A) and MDA-MB-468 cells (B) at various CDF concentrations as shown. Data represent mean $\pm$ SD, n=6.

Figure 6. Apoptosis study

Free CDF, SMA-TPGS-CDF NMs and HA-SMA-TPGS-CDF NMs with an increasing apoptosis measured by FACS using staining of Annexin V and 7-AAD in (A) MDA-MB-231 cells and (B) MDA-MB-468 cells are shown. SMA-TPGS copolymer, HA-SMA-TPGS copolymer are used as negative controls. Data represent mean $\pm$ SD, n=3 per group, **p<0.01 vs. control.

Figure 7. Apoptosis signaling analysis

Western blot showing the downregulation of PTEN level and upregulation of NF- κ B level at protein levels in (A) MDA-MB-231 and (B) MDA-MB-468 cells after treating with the free CDF, SMA-TPGS-CDF and HA-SMA-TPGS-CDF NMs are shown. (C) Upregulation of Caspase 3/7 protein in MDA-MB-231 cells treated with HA-SMA-TPGS-CDF compared to control, non-targeted and free CDF treatment supports the TNBC cell killing of targeted formulation is apoptosis pathway mediated. Data represent mean $\pm$ SD, n=3 per group, *p<0.05 vs. control. **p<0.01 vs. control.

Figure 8. TNBC imaging of targeted and non-targeted oligomer

HA-SMA-TPGS-S0456 (left panel) and SMA-TPGS-S0456 (right panel) were injected via tail-vein into mice bearing MDA-MB-231 tumor. Targeted oligomer (left) showed highly selective tumor uptake (A, B) and favorable tumor selective bio-distribution (C, D). Whereas non-targeted oligomer (right) has very less tumor uptake (E, F) with non-specific organ bio-distribution (G, H). Whole body fluorescence and X-ray merged images were acquired at 4 h post-injection.

IC₅₀ comparison of CDF, SMA-TPGS and HA-SMA-TPGS treated cells in either normal cell culture condition or HA-pretreated (CD44-blockade) condition for 24 h or 48 h are shown.

Table 1

Course of treatment	CDF (IC ₅₀)	SMA-TPGS-CDF (IC ₅₀)	HA-SMA-TPGS-CDF (IC ₅₀)	Cell lines	Condition
24 h	280.4 nM	196.1 nM	88.7 nM	MDA-MB-231	Normal
48 h	161.8 nM	96.3 nM	59.3 nM	MDA-MB-231	Normal
24 h	468.8 nM	110.9 nM	99.1 nM	MDA-MB-468	Normal
48 h	151.4 nM	65.6 nM	59.9 nM	MDA-MB-468	Normal
24 h	279.3 nM	202.2 nM	194.2 nM	MDA-MB-231	HA-pretreated
48 h			94.2 nM	MDA-MB-231	HA-pretreated