Co-targeting Bulk Tumor and CSCs in Clinically Translatable TNBC Patient-Derived Xenografts via Combination Nanotherapy

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Abstract

Triple-negative breast cancer (TNBC) accounts disproportionally for the majority of breast cancer–related deaths throughout the world. This is largely attributed to lack of a specific therapy capable of targeting both bulk tumor mass and cancer stem cells (CSC), as well as appropriate animal models to accurately evaluate treatment efficacy for clinical translation. Thus, development of effective and clinically translatable targeted therapies for TNBC is an unmet medical need. We developed a hybrid nanoparticles-based co-delivery platform containing both paclitaxel and verteporfin (PV-NP) to target TNBC patient-derived xenograft (PDX) tumor and CSCs. MRI and IVIS imaging were performed on mice containing PDX tumors to assess tumor vascularity and accumulation of NPs. NF-κB, Wnt, and YAP activities were measured by reporter assays. Mice bearing TNBC PDX tumor were treated with PV-NPs and controls, and tumors progression and CSC subpopulations were analyzed. MRI imaging indicated high vascularization of PDX tumors. IVIS imaging showed accumulation of NPs in PDX tumors. In comparison with control-NPs and free-drug combination, PV-NPs significantly retarded tumor growth of TNBC PDX. PV-NPs simultaneously repressed NF-κB, Wnt, and YAP that have been shown to be crucial for cancer growth, CSC development, and tumorigenesis. In conclusion, NPs containing two clinically used drugs concurrently inhibited NF-κB, Wnt, and YAP pathways and exhibited synergic effects on killing TNBC bulk tumor and CSCs. This combination nanotherapy evaluated with a PDX model may lead to an effective treatment of patients with TNBC.

Introduction

Breast cancer remains a leading cause of death in women worldwide (1). Among the various breast cancer subtypes, triple-negative breast cancer (TNBC, a highly heterogeneous disease) accounts for only one fifth of all breast cancers, but disproportionally accounts for the majority of breast cancer–related deaths. Relapse has been closely associated with cancer stem cells (CSC) and is a major challenge in TNBC wherein approximately one third of patients will experience a distant recurrence within 2.6 years (2). In contrast to other breast cancer subtypes, due to lack of specific targets, conventional chemotherapy is still the clinical standard treatment for TNBC. However, chemotherapy has been shown to promote CSC enrichment after treatment (3).

In TNBC, CSCs have been found to coexist in two distinct but interconvertible subtypes: epithelial ALDH+/CD44-/CD24− subpopulations that are closely associated with chemoresistance, tumor regrowth, and disease relapse (4–6). A recent study revealed that YAP signaling was highly activated in mesenchymal CD44+/CD24− subpopulations while Wnt signaling in epithelial CSCs (7). In addition, it has been found that NF-κB, an essential mediator of the inflammatory response, is a potent signaling modulator in tumor cells and tumor microenvironment. NF-κB stimulates Wnt and other signaling pathways and facilitates the survival of both bulk and CSC populations (8, 9). As such, repression of NF-κB signaling has been considered as one of the most effective approaches in cancer treatment (8, 9). We thus hypothesize that...
therapeutic strategies capable of effectively delivering drugs into tumor to coinhibit NF-kB, Wnt, and YAP signals and evaluating therapeutic efficacy with a clinically translatable model may lead to the effective treatment of TNBC.

Cancer nanomedicines overcome the intrinsic limits of drug delivery and conventional cancer therapies through their uniquely appealing features, such as improved blood circulation, increased tumor accumulation, and reduced off-target toxicities, leading to a higher therapeutic index (10, 11). However, in preclinical research, the therapeutic efficacy of drugs and drugs formulated in NPs is assessed by using cancer cell line–based animal models that do not represent the heterogeneity and complexity of patients’ primary tumors. Even though cell lines originate from patient’s tumors, due to artificial tissue culture conditions, they have adapted for adherence/growth in a monoculture, leading to uniformity in cells and loss of heterogeneity. Most of the cancer cell lines used in preclinical research are genetically and epigenetically divergent from their starting source and real tumors (12, 13). This agglomeration may culminate with artifact discoveries and clinical trial failures for the therapeutic agents including cancer nanomedicines (12, 13). In contrast, patient-derived xenograft (PDX) tumors are obtained from patients and engrafted directly into immunocompromised mice, bypassing extensively in vitro selection. As such, PDX tumors retain the original patient’s tumor heterogeneity, three-dimensional architecture, and microenvironment representing the tumors biology, and resulting in a 92% concordance with patient tumors (14).

In this report, we developed co-delivery NPs containing the conventional chemotherapeutic agent paclitaxel in combination with an FDA-approved porphyrin-based photosensitizer, verteporfin (PV-NP). Paclitaxel is a routinely prescribed chemotherapeutic for the treatment of TNBC. It inhibits the mitotic spindle apparatus, preventing cancer cell division (15). However, resistance to paclitaxel is common in the clinic. Although the mechanism behind this phenomenon is still under investigation, paclitaxel-induced CSC enrichment has been demonstrated to be one of the key players mediating drug resistance and disease relapse (16, 17). Verteporfin is a FDA-approved photosensitizer for photodynamic therapy to eliminate the abnormal blood vessels in the eye such as macular degeneration (18). Verteporfin has been reported to possess potent anticancerous activity in patients with pancreatic and breast cancer (19, 20) and is currently in a clinical trial for the treatment of breast cancer (NCT02939274). However, paclitaxel resistance, CSC enrichment, and drug delivery to tumors remain challenges in cancer treatment. Here, we provided the first demonstration that PV-NPs accumulate within TNBC PDX tumors and potentially inhibit both bulk tumor mass and CSC populations. Furthermore, we showed that PV-NPs suppressed NF-kB, Wnt, and YAP pathways that are crucial for cancer growth, CSC development, and tumorigenesis. These findings suggest that the development of nanoparticle platforms encapsulated with specific drugs to promote synergistic inhibition of bulk tumor and CSCs is an effective and translatable approach for TNBC treatment.

Materials and Methods

Cell culture and reagents

MDA-MB-231 breast cancer cells were purchased from the ATCC and maintained in DMEM-F12 media supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin. Cells were cultured at 37°C in a 5% CO2 incubator. Verteporfin was purchased from Calbiochem, and paclitaxel from Cederlane. PLGA was purchased from LACTEL polymers, Lechitin from Alfa Aesar, and DSPE-PEG2K from Avanti Lipids. Alexa Fluor 750 and Qdot800 were purchased from Thermo Fisher Scientific. Acetonitrile, dry DMF, and acetone were purchased from Fisher Chemicals.

Synthesis and characterization of NPs

Lipid-polymer hybrid NPs were synthesized via previously reported nanoprecipitation method (21). Briefly, lecithin and DSPE-PEG2K in molar ratio of 6.5:1 were dissolved in 4% ethanol aqueous solution (0.02% w/v) and heated for 2–4 minutes at 68°C under gentle stirring. After heating, poly lactic co-glycolic acid (PLGA) and appropriate drugs (10: 1 w/w ratio) in either acetone or acetone (1–2 mg/mL) were added dropwise at 0.6 mL/min rate and stirred at room temperature to form self-assembly of hybrid NPs. NPs were concentrated, and purified by centrifugal filters and analyzed by ZetaView, Malvern Zetasizer Dynamic light scattering (DLS), and transmission electron microscopy (TEM). Stability of NPs in biologically relevant conditions was tested according to literature procedure (22). The amount of paclitaxel encapsulated in NPs was analyzed using HPLC at 204 nm, with H2O:acetonitrile mobile phase with 5%–90% acetonitrile gradient. The amount of verteporfin encapsulated in NPs was quantified by NanoDrop at 430 nm absorbance. Drug release profiles were performed using published procedures (23).

PLGA-Qdot800 conjugate was synthesized according to previously reported literature procedure (22). PLGA-Alexa750 conjugate was synthesized via ester coupling by reacting amine end group of PLGA with Alexa Fluor 750 NHS ester.

DAPI staining and fluorescence microscopy

MDA-MB-231 cells cultured on glass coverslips were treated with nanoparticle-Bodipy FL for 3 hours. After being fixed with 4% paraformaldehyde and stained with 100 ng/mL of DNA-specific fluorophore DAPI for 1 hour at room temperature, the coverslips were mounted on a glass slide for fluorescence microscopy. Fluorescence images were obtained by using a Leica AF6000 deconvolution microscope system equipped with a fully automated microscope (DMi6000B) and a DFC350 FX digital camera (Leica Microsystems). Fluorescence images were acquired under the identical exposure time and instrument settings among different groups, and analyzed using Leica LAS AF6000 software.

Flow cytometry analysis

Cancer cells or PDX tumor cells were dissociated and filtered through a 40-μm strainer and suspended in PBS supplemented with 2% FBS and 2 mmol/L EDTA. One microliter of mouse IgG (1 mg/mL) was then added and incubated at 4°C for 10 minutes. Afterwards, the cells were resuspended in 1× binding buffer (eBioscience) and cell apoptosis was determined using Annexin-V-V450 Apoptosis Detection Kit (BD Bioscience). Afterwards, cells were incubated with the different reagents as described below at 4°C for 30 minutes in ALDEFLUOR Assay Buffer. Anti-CD44 (APC) and anti-CD24 (PE; BD Pharmingen) antibodies were added according to the manufacturer's instructions as described previously (6). ALDH activity was determined
using ALDEFLUOR (Stem Cell Technology) with a DEAB control according to the manufacturer's instructions. Finally, cells were washed twice and 7-aminoactinomycin D (7-AAD, eBioscience) was added to exclude dead cells. Flow cytometry was performed on the BD LSRII Fortessa. Data were analyzed with FlowJo Software (Ashland). To analyze cell uptake of nanoparticles in different organs versus TNBC PDX tumors, mice were injected Qdot800-conjugated lipid-hybrid nanoparticles 3 hours before euthanization. Different organs and TNBC PDX tumors were harvested, dissociated into single-cell suspensions, and washed three times with PBS before analysis with the BD LSRII Fortessa. FlowJo software was used for data analysis.

Cell viability assays
Cells were seeded into 12-well plates (1.5 × 10⁴ cells/well). After 120 hours of treatment, viability analysis was performed by incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, 1 mg/mL) for 4 hours. Absorbance was measured at 570 nm.

HCl-002 PDX TNBC tumor fragments were incubated in 24-well plates (i.e., organotypic slice culture). After 120 hours of treatment, Alamar blue viability assay was performed via incubation with 10% Alamar blue solution (Thermo Fisher Scientific) for 4 hours, followed by measurement of fluorescence at 560 nm excitation and 590 nm emission as performed previously (24).

Luciferase assay
MDA-MB-231 TNBC cells were seeded into 12-well plates and transfected with 1,000 ng of a NF-kB reporter p1242 3xKB-L (Addgene Plasmid #26699, a gift from Dr. Bill Sugden, McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, Wisconsin, USA; ref. 25), or YAP reporter 8xGTHIC-luciferase (Addgene Plasmid #34615, a gift from Dr. Stefano Piccolo, Department of Histology, Microbiology and Medical Biotechnologies, University of Padua School of Medicine, viale Colombo 3, 35131 Padua, Italy; ref. 26), or Wnt reporter M50 Super 8xTOPFlash (Addgene Plasmid #12456, a gift from Dr. Randall Moon, Howard Hughes Medical Institute, Department of Pharmacology, Center for Developmental Biology, Seattle, WA 98195 USA; ref. 27) constructs in conjunction with 1,000 ng Renilla pRL-SV40P (Addgene Plasmid #27163, a gift from Dr. Ron Prywes, Department of Biological Sciences, Columbia University, New York, NY 10027; ref. 28) construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 18 hours, cells were treated with either empty (vehicle) lipid-hybrid nanoparticles or lipid-hybrid nanoparticles with paclitaxel (10 nmol/L), verteporfin (500 nmol/L), or both for 24 hours, after which cells were lysed and both Firefly and Renilla luciferase activity was quantified using a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's instructions.

Xenograft tumor growth
All protocols described throughout this article regarding animal studies were performed in strict pathogen-free conditions and in accordance with ethical guidelines as approved by The Ottawa Hospital Research Ethics Board (Protocol #20120559-01H). TNBC PDX HCI-002 tumor chunks (2 mm × 4 mm) were transplanted into the mammary fat pad of athymic nude mice (purchased from Charles River). After the tumors reached a mean diameter of 3 mm, drug treatment was initiated. Mice were randomly divided into three groups and treated with either vehicle (empty) lipid-polymer hybrid nanoparticles, free drugs (1 mg/kg of paclitaxel and 9 mg/kg of verteporfin), or PV-NPs loaded with 0.5 mg/kg of paclitaxel and 3.2 mg/kg of verteporfin every other day for 20 days (n = 5 mice for each group). Tumor growth was monitored every other day using a caliper and tumor volume was determined using the formula: V = 1/2(tumor length × tumor width²). After the completion of the treatment, mice were humanely euthanized and tumors were weighed and photographed. For flow cytometry analysis, tumors were mechanically minced and then enzymatically digested into single-cell suspension using 1× collagenase/hyaluronidase in DMEM (Stem Cell Technology).

IVIS analysis
Athymic mice were injected via tail vein with lipid-polymer hybrid nanoparticles conjugated with Alexa fluoro750. The fluorescence of Alexa fluor750 was measured at 3, 6, and 24 hours using the Perkin Elmer IVIS Spectrum In Vivo Imaging System.

MRI analysis
To determine TNBC PDX vascularity for potential nanoparticle delivery, whole-body T1-weighted MRI was conducted using a small-animal MRI machine (7T GE/Agilent MR901). After the first scanning, athymic mice were retrieved and injected with Gadovist via tail vein at a concentration of 0.1 mmol/kg (Bayer). Immediately after injection, the mice were rescanned using the 7T GE/Agilent MR901.

Results
Dual-drug delivery PV-NP platforms
In TNBC therapy, conventional paclitaxel treatment has been shown to upregulate NF-kB, YAP, and Wnt pathways, thereby enriching CSCs that are detrimental for long-term disease-free prognosis in patients (17, 29–31). We sought to define an agent capable of co-inhibiting these pathways to prevent subsequent CSC enrichment. After performing some initial in vitro experiments, we found that verteporfin inhibits Wnt, YAP, and NF-kB signaling, thus we theorized that it may be a suitable agent to abolish paclitaxel-induced CSC enrichment as illustrated in the schematic (Fig. 1), and developed a codelivery nanoplatform for its delivery. Codelivered paclitaxel and verteporfin-loaded PV-NPs (1:7.5 molar ratios), paclitaxel-loaded P-NPs, and verteporfin-encapsulated V-NPs were synthesized via self-assembly using a modified nanoprecipitation method. All NPs had slightly negative surface charge without significant difference (~−2−3 mV; Fig. 2A). As expected, there was a small increase in hydrodynamic size of dual-drug containing PV-NPs in comparison with single-drug loaded P-NPs and V-NPs, due to the accommodation of both drugs in single NP (Fig. 2A; Supplementary Fig. S1; ref. 32). TEM imaging showed the spherical structures for all NPs (Fig. 2B), and size range of 80–100 nm (Fig. 2C; Supplementary Figs. S1 and S2) that were matched with hydrodynamic radius measured with DLS and Zetaview (Fig. 2A). Drug encapsulation and loading efficiencies for single- and dual-drug-loaded NPs were within acceptable range. Encapsulation efficiency (EE) of verteporfin from V-NPs to PV-NPs decreased from approximately 73% to 67%, whereas paclitaxel encapsulation improved from P-NPs to PV-NPs (60%−75% EE). This might
be due to the presence of verteporfin in the NPs that increased overall hydrophobicity of NP core or π–π interactions between phenyl groups of paclitaxel and π conjugate system in verteporfin. Drug-loading efficiencies for paclitaxel in P-NP and PV-NPs were 2.8% and 0.56%, whereas for verteporfin-loading efficiencies in V-NPs and PV-NPs drug were 4% and 3.4%, respectively. In vitro serum stability studies for all NPs showed no significant changes in hydrodynamic size and polydispersity, highlighting the excellent stability of NPs under biologically relevant conditions (Supplementary Fig. S1). Drug release profiles of PV-NPs have typical initial burst release followed by slow release for both drugs (Supplementary Fig. S3). In addition, in vitro microscopy studies showed efficient uptake of NPs by MDA-MB-231 TNBC cells after 3 hours of incubation (Fig. 2D).

PV-NPs are capable of simultaneously inhibiting NF-κB, YAP, and Wnt signaling activities and concurrently suppressing both mesenchymal and epithelial CSCs in TNBC

Next, we sought to elucidate the effects of P-NPs, V-NPs, and PV-NPs on NF-κB, YAP, and Wnt pathways, which have been shown to be essential for tumor regrowth and CSC development. We transfected MDA-MB-231 cells with pRL-CMV together with the p1242 3x-KB-L luciferase NF-κB reporter, the M50 Super 8x TOPFlash-luciferase Wnt reporter, or the 8xGTHIC-luciferase YAP reporter (25–27). After 24 hours, the cells were exposed to P-NPs, V-NPs, or combinations of both for an additional 24 hours and luciferase activity was determined using the Dual-Glo luciferase assay system. It was found that V-NPs simultaneously inhibited NF-κB, Wnt, and YAP, and abrogated P-NPs–induced upregulation of NF-κB, Wnt, and YAP signaling (Fig. 3A–C). In addition, combination treatment with P-NPs and V-NPs elicited a reduction in TNBC cell viability (Fig. 3D; Supplementary Fig. S4). For effective drug delivery in vivo, we co-encapsulated both paclitaxel and verteporfin in single lipid-hybrid nanoparticles (PV-NPs), which exhibited the same efficacy as combination treatment with individually encapsulated P-NPs plus V-NPs in killing TNBC cells (Supplementary Fig. S4). Significantly, CD44+/CD24− mesenchymal CSCs and ALDH+ epithelial CSCs were enriched after exposure to P-NPs but were diminished after exposure to PV-NPs (Fig. 3E).

Organotypic slice culture of PDX has been shown to faithfully represent PDX tumors and primary patient tumors in drug screening experiments (33). We plated PDX organotypic slices and treated them over 120 hours with PV-NPs and free drugs. We observed similar results to those of TNBC cell line. In comparison with other treatment groups, P-NPs + V-NPs and PV-NPs effectively decreased viability (Supplementary Fig. S5) and paclitaxel-induced CSC enrichment (Supplementary Fig. S6), while promoting apoptosis (Supplementary Fig. S7) in cultured PDX organotypic slices. Together, these data indicate that the encapsulation of drugs within PV-NPs maintains drug function in vitro efficacy, is capable of coinhibiting both subtypes of CSCs, and concurrently suppresses NF-κB, Wnt, and YAP signaling crucial for CSC development. In addition, while verteporfin is frequently used as a photosensitizer (34), it showed no photochemical effects on cellular functions at our experimental conditions (36).

Figure 1.
A schematic representation of PV-NPs effects on TNBC PDX tumors. Upon systemic administration, NPs accumulate and release drugs in PDX tumors via the EPR effect. Paclitaxel promotes bulk cell death via inhibition of the mitotic spindle apparatus; however, paclitaxel also promotes CSC enrichment due to upregulation of NF-κB, Wnt, and YAP signals, whereas verteporfin coinhibits NF-κB, Wnt, and YAP signals, preventing CSC enrichment and increasing the overall efficacy of combination nanotherapy, although it does not potently induce bulk tumor death. MSA, mitotic spindle apparatus.

Figure 2D.
TNBC PDX vasculature is enhanced permeability and retention–active and PDX tumors accumulate NPs

Following in vitro studies, we explored whether our results would be translated in the highly clinically relevant TNBC PDX animal model. A simplified interpretation of enhanced permeability and retention (EPR) effect is based on an assumption that macromolecules such as NPs accumulate more in solid tumors due to leaky vasculature and poor lymphatic drainage. EPR-driven NP accumulation in tumors is a complex multistep biological process influenced by several factors including angiogenesis, vascular permeability, heterogeneities in genetic profile and tumor microenvironments, tumor tissue penetration, tumor cell internalization, and NPs physicochemical properties (36).

Unlike tumors generated from cancer cell lines, PDX tumors retain the patient’s original tumor heterogeneity, microenvironment, intratumoral vasculature, and three-dimensional architecture. Currently, EPR-driven nanomedicines accumulation within PDX tumor models has not been fully described. To determine whether PDX tumors are EPR active, we first performed MRI and IVIS experiments to study vascularity and NPs accumulation in the tumors (35–38). To this end, we surgically engrafted athymic mice with the TNBC PDX tumor fragments within the mammary fat pad. To allow blood vessel growth to detectable size, we waited until tumors reached 100 mm³. We then performed T1-weighted MRI before and 8 minutes after tail-vein injection of Gadovist (0.1 mmol/L/kg, a clinically used contrast agent in angiography). MRI showed marked contrast enhancement, indicating abundant vascularity within PDX tumors (Fig. 4A). To confirm whether this tumor vasculature exhibits EPR effects, fluorescently labeled NPs (Alexa 750) were administrated via tail vein and IVIS imaging was performed at 0, 3, 6, and 24 hours to determine NP accumulation inside the tumors. IVIS analysis showed high levels of NP accumulation within the PDX tumor area and the upper abdomen area for 6 hours (Fig. 4B).

To accurately quantify NP uptake by tumor cells in comparison with other organ cells, we injected the mice with Qdot 800–labeled NPs. Three hours post-injection, we euthanized the mice, harvested organs and tumors, dissociated them into a single-cell suspension, and analyzed them using flow cytometry. As shown in Fig. 4C, our data support that NPs were preferentially located within PDX TNBC tumors in comparison with heart, liver, and kidney, although NPs were also highly accumulated in nonvital spleen (Fig. 4C). Taken together, our data suggest that TNBC PDX vasculature is EPR-active, and NPs accumulate within PDX tumors.

PV-NPs retard TNBC PDX tumor growth and suppress CSC populations

Finally, we determined whether coencapsulated PV-NPs could inhibit the growth of TNBC tumors and abrogate the enrichment of CSCs in a highly clinically relevant PDX mouse model. We again surgically engrafted TNBC PDX tumors into athymic mice. When the tumors reached a mean diameter of 3 mm, mice were randomized and treated with vehicle-NPs (empty NPs), free-drug combination (1 mg/kg of paclitaxel and 9 mg/kg verteporfin), or PV-NPs (NPs containing 0.5 mg/kg of paclitaxel and 3.2 mg/kg of verteporfin) every other day for 20 days via tail-vein injection (n = 5 for each group). On the basis of our in vitro results using TNBC PDX organotypic slice culture, we did not include P-NPs, V-NPs, and P-NPs + V-NPs treatments in our in vivo study.
Given the heterogeneity, composition, and variability in each engrafted PDX tumor fragment, it is expected to see differential growth rate for PDX tumors. Indeed, we observed variable tumor growth rate in vehicle-NPs- and free-drug–treated groups. However, despite this variability, PV-NPs treatment (even containing lower dose of drugs than free-drug control, only 50% paclitaxel and 32% verteporfin) significantly retarded PDX tumor growth in comparison with the free-drug and vehicle-NP control groups (Fig. 5A), highlighting the efficacy of PV-NPs treatment. Consistently, mice treated with PV-NPs showed significantly reduced tumor size and tumor weight (Fig. 5B). While PV-NPs treatment effectively diminished TNBC PDX tumor growth, mice body

Figure 3.
NP-encapsulated verteporfin is capable of simultaneously suppressing NF-κB, Wnt, and YAP as well as concurrently inhibiting both mesenchymal and epithelial CSC subpopulations in vitro. A–C, Luciferase reporter activity of NF-κB, YAP, and Wnt activities in MDA MB-231 cells treated with Veh (vehicle-NP), P-NP (containing 25 nmol/L paclitaxel), P-NP (containing 500 nmol/L verteporfin), or both (PV-NP). D, Viability analysis after 120 hours of incubation with vehicle (vehicle-NP), P-NP (5 nmol/L), V-NP (100 nmol/L), or PV-NP. E, Flow cytometric analysis of CD44+CD24− CSCs and ALDH+ CSCs after 120 hours of treatments. Data represent means ± SD; n = 3 for all figures; *, P < 0.05; **, P < 0.01; ***, P < 0.001, in comparison with the indicated groups or vehicle-NP control.
weight remained constant throughout treatment (Supplementary Fig. S8), suggesting the specificity and tolerability of the PV-NP treatment. In contrast to PV-NPs, free-drug did not show statistical difference in comparison with vehicle-NP control. This is likely due to inadequate tumor accumulation and/or retention of free-drug, highlighting the necessity for NPs induced overlap in pharmacologic profiles of dual drugs (10) and NP delivery and accumulation in tumors. Furthermore, in agreement with in vitro cell line results, PV-NPs reduced both mesenchymal CD44+/CD24- CSCs and epithelial ALDH+ CSCs in PDX tumors after treatment, while the free-drug combination was unable to significantly suppress CD44+/CD24- CSC subpopulation when compared with vehicle-NP control (Fig. 5C and D; Supplementary Fig. S9).

**Discussion**

There is limited data showing EPR effects in patients due to several limitations. One of the main challenges is to achieve meaningful biodistribution data in patients. An indirect method to analyze EPR effects in treatments is to compare treatment responses within patient groups. However, patients’ tumors are heterogeneous and their tumor’s biology, microenvironment, vasculature, drug efflux, and drug response rates vary significantly. In addition, tumor heterogeneity leads to dissimilar NP accumulation within tumors and dissimilar treatment responses. This might be one of the reasons for the poor outcomes in phase II clinical trials of BIND014 and CAALA01. Recent studies have used companion imaging NPs to quantify the EPR effect to identify patients for better nanomedicine therapeutic response (35, 39, 40).

Of note, the majority of preclinical experiments studying NP uptake via EPR have employed syngeneic mouse or human cell line xenograft tumors, which develop over days, are comprised of a homogeneous cellular population, and possess immature vasculature in addition to a malformed tumor microenvironment (38, 41). In contrast, PDX tumors develop over longer periods of time, are comprised of multiple cellular populations, primary tumor microenvironment, architecture, and vasculature, similar to primary patient tumors (42, 43).
similarities translate into the success of PDX emulation of patient tumor response upon exposure to chemotherapies. Hence, increasing numbers of mouse-clinical trials are using PDX tumors for drug development, identification, and clinical translation (44–46).

In this study, we have developed a NP platform for the delivery of our combination therapy to TNBC PDX tumors. TNBC PDX tumors were highly vascularized as indicated by the MRI imaging with Gadovist and showed EPR-related nanoparticle accumulation as determined by IVIS imaging (Fig. 4A and B). Flow cytometry analysis show tumor cell uptake of fluorophore-NPs, supporting NP tumor delivery of the payload (Fig. 4C). We also observed NP accumulation in the spleen. This is likely due to macrophage uptake and the spleen red pulp (47). In spleen red pulp, red blood cells are sieved by splenic sinuses before reentering circulation. Nanoparticles 100–200 nm in size have been shown to difficultly traverse through the red pulp, resulting in accumulation within the red pulp as well as uptake by macrophages (48).

In vivo TNBC PDX studies demonstrated that the efficacy of NP-delivered paclitaxel and verteporfin for inhibiting PDX tumor growth and preventing CD44+/CD24−/CD105− and ALDH+ CSCs enrichment (Fig. 5). To our best knowledge, this is the first report showing the treatment efficacy of drug-NPs using a highly clinically relevant TNBC PDX mouse model. It is also the first demonstration of verteporfin’s capability of simultaneously inhibiting NF-κB, Wnt, and YAP signaling (Fig. 3A–C) as well as repressing both mesenchymal CD44+/CD24− and epithelial

Figure 5.
Efficacy of paclitaxel- and verteporfin-coloaded NPs in the treatment of TNBC PDX tumors. A, TNBC PDX tumors were surgically engrafted into the mammary fat pads of athymic nude mice and treated with vehicle-NPs (empty NPs), free-drugs (FD, 1 mg/kg of paclitaxel and 9 mg/kg verteporfin), or PV-NP (0.5 mg/kg of paclitaxel- and 3.2 mg/kg verteporfin-coloaded NPs) every other day for 20 days. Tumor volumes were determined every 2 days. B, Representative tumor photos after treatments with means of tumor weights shown below. C and D, Flow cytometric analysis of CD44+/CD24− and ALDH+ CSCs within TNBC PDX tumor after completion of in vivo treatment. Data represent means ± SD; n = 5 for mice and n = 3 for flow cytometric analysis (*, P < 0.05; **, P < 0.01).
ALDH<sup>+</sup> TNBC CSC subpopulations (Fig. 3E). Recent reports have demonstrated that paclitaxel-mediated CSC enrichment is due to co-regulation of NF-κB and Wnt pathways and the important role of YAP signaling in CSC development (3, 7, 16). Thus, concurrent inhibition of these three signals might be an important mechanism underlying the effective treatment of PV-NPs. Given that the two drugs are commonly used in clinic and their synergic effects on killing TNBC bulk and CSCs, such a combination nanotherapy, may lead to an effective treatment of patients with TNBC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Chambers, A. Phan, G.O. Cron, D. Figeys, S. Gadde
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Sulaiman, S. McGarry, L. Li, G.O. Cron, T. Alain, S. Gadde
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