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(54) Title: GRP78 TARGETED LIPOSOMAL NANOPARTICLES (TLNPS) FOR THE TREATMENT OF CANCER

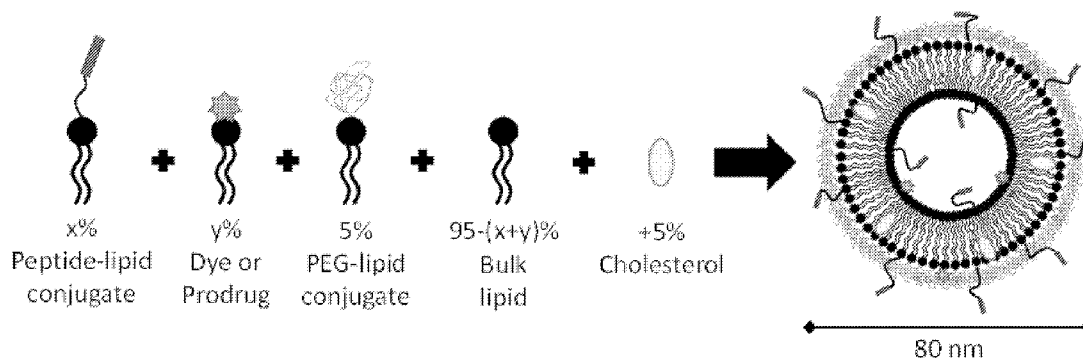


Fig. 8C

(57) Abstract: A nanoparticle generally comprising a targeting peptide-lipid conjugate, wherein a targeting peptide moiety of the targeting peptide-lipid conjugate comprises a GRP78 targeting peptide, a polyethylene glycol (PEG)-lipid conjugate, a drug-lipid conjugate comprising a prodrug moiety, wherein the drug-lipid conjugate comprises one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and a bortezomib (BTZ) prodrug; and wherein the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; cholesterol comprising about 1 mol% to about 10 mol% of the nanoparticle; and distearoylphosphatidylcholine (DSPC).



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GRP78 TARGETED LIPOSOMAL NANOPARTICLES (TLNPS)
FOR THE TREATMENT OF CANCER

RELATED APPLICATIONS

5 This application claims priority under 35 U.S.C. §119(e) to U.S. Provisional Patent Application No. 63/521,609, filed June 16, 2023, which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

10 Despite the recent advances in novel therapies, most cancers, and in particular, metastatic breast cancer (MBC) still remain incurable, comprising the majority of deaths of breast cancer cases with only a ~20% survival rate. More importantly, due to the poor prognosis, approximately 30% of breast cancer cases diagnosed in early stages eventually develop into invasive MBC, resulting in a persistent increase in the number of MBC cases over recent decades. To date, conventional chemotherapy has been commonly used to treat MBC, which has delivered poor clinical outcomes.
15 Conventional approaches have fallen short due to poor overall efficacy. This can be largely attributed to an insufficient delivery of a desired drug to the diseased cells, which is also limited by the need to balance drug dose administration because of the detrimental side effects to the patient. To address this problem, several targeted therapies, such as monoclonal antibody (mAb)-targeted therapy, have been developed to show relative therapeutic effectiveness over conventional chemotherapy. Nevertheless,
20 despite improvements in some cases, these therapies still exhibit significant hurdles that need to be addressed, such as a lack of selectivity for MBC cells over healthy cells, which still result in poor overall clinical outcomes and unintended systemic toxicity.

As an alternative, targeted liposomal nanoparticles (TNPs) have been investigated to improve selective drug efficacy when targeting tumor cells. TNPs benefit from a unique system, such as
25 pegylated surface coating to reduce reticuloendothelial system (RES) clearance and surface functionalization, and a variety of targeting elements to improve drug efficacy via enhanced cellular uptake. Traditionally, TNPs have been mostly used to treat solid tumors, particularly taking advantage of the enhanced permeability and retention (EPR) effect to preferentially accumulate at tumor sites. Therefore, TNPs have been synthesized as small as possible to improve tumor penetration into the
30 depth of tumor sites. However, during metastasis, circulating tumor cells (CTCs) dislodged from the tumor, continuously circulate in the blood until they seed into different organs in the body. When targeting CTCs and early metastatic seeding events, the EPR effect does not need to be taken into consideration. In these cases, TNPs can instead be designed as larger particles, which provide additional benefits due to their typically increased *in vivo* circulation half-life, which improves the
35 probability of TNPs encountering CTCs in the systemic circulation¹⁵. Additionally, during solid tumor targeting, the design of the TNPs needs to take the binding site barrier (BSB) phenomenon into

account. Nevertheless, since there is no concern for BSB effect while targeting CTCs and initial metastatic seeding, TNPs can be designed with higher density of targeting elements to maximize adherence and targeting efficiency, which should result in enhanced efficacy.

Despite TNPs having been recognized as a novel paradigm in cancer therapy decades ago, very few have successfully translated into the clinic. The failure in translation is mainly due to two reasons: poor efficacy in the clinic, and poor batch-to-batch reproducibility of the targeted particles during manufacturing. We and others believe the main reason for poor efficacy is the poor cancer cell selectivity during drug delivery. This is mainly attributed to targeting elements with high monovalent affinity, such as monoclonal antibodies or single chain variable fragments (scFv), that not only bind to the target receptors overexpressed on cancer cells but to the same receptor on healthy cells as well.

SUMMARY OF THE INVENTION

To improve the poor efficacy of targeted liposomal nanoparticles (TNP), we utilized peptides as targeting elements to take advantage of their weak to moderate monovalent affinity. By presenting multiple copies of peptides on liposomes, particles can achieve increased avidity, and therefore, extended residence time, especially for target cancer cells. This provides increased selectivity for targeted cells over healthy cells, and results in reduced off-target effects. In addition to poor efficacy, inconsistent TNP synthesis methods have resulted in significant variation in chemical coupling yields of targeting elements per nanoparticle, leading to poor reproducibility in the clinic.

To address this obstacle, we synthesized and purified peptides as lipid conjugates, and then simultaneously incorporated them with other pure liposome constituents at exact molar ratios to engineer liposome platforms. This strategy allows precise control over the functionalities of TNPs, with high reproducibility in manufacturing between batches, thereby producing consistent and reliable experimental outcomes.

Glucose-regulated protein 78 (GRP78; also known as heat shock 70 kDa protein 5, HSPA5) has been identified as a promising target for achieving selective targeting on cancer cells over healthy cells. GRP78 is a stress inducible endoplasmic reticulum (ER) chaperone protein that is part of the larger heat shock protein superfamily. GRP78 is typically localized in the ER to assist in the protein folding and assembly of membrane or secreted proteins. However, it has been shown to be expressed on the cell surface of several types of cancer cells *in vivo*, including breast cancer, which has correlated to poor prognosis, overall metastasis and aggressiveness. Importantly, in our previous work, we have shown that sGRP78 marks a stem-like population of breast cancer cells that has increased metastatic potential *in vivo*.

The objective of this study was to (1) develop a drug-loaded TNP to selectively target aggressive sGRP78+ cells, and (2) evaluate, using a mouse model to mimic events of MBC, the efficacy of sGRP78+ TNPs *in vivo*. We employed a previously identified linear sGRP78 binding

peptide (SNTRVAP, herein referred to as GRP78_{pep}; (SEQ ID NO: 1)), as the targeting element conjugated on the nanoparticle surface to achieve selective binding and drug delivery (TNP^{GRP78_{pep}}). As the chemotherapeutic agent, Doxorubicin (Dox), which has been approved by the FDA for treatment of MBC, was used in a prodrug form. In this study, initial optimization of the TNP^{GRP78_{pep}} formulation was accomplished by systematically evaluating its design parameters such as peptide hydrophilicity, ethylene glycol (EG) linkers, and peptide-density using *in vitro* cell culture assays. This optimization was further evaluated *in vivo* through analysis of tissue biodistribution in animal models. Finally, we investigated the *in vivo* drug efficacy of TNP^{GRP78_{pep}} by testing its ability to inhibit seeding of metastatic cells in the lung tissue of animals. The results demonstrated significant inhibition of MBC cell seeding into lungs when the targeted nanoparticle formulation was used, whereas non-targeted drug-loaded nanoparticles did not achieve any detectable inhibition. Combined, this study highlights the superiority of sGRP78-targeted drug-loaded nanoparticles that clearly outperform non-targeted drug-loaded nanoparticles in eliminating circulating/seeded metastatic cancer cells. Thus, these novel TNPs provide a selective, and therefore less toxic method, to target the specific more aggressive breast cancer cells while leaving normal healthy cells intact. It is our hope that this approach could enable more efficacious and less toxic MBC treatments.

Accordingly, the disclosure provides nanoparticles comprising a targeting peptide-lipid conjugate, wherein a targeting peptide moiety of the targeting peptide-lipid conjugate comprises a GRP78 targeting peptide; a polyethylene glycol (PEG)-lipid conjugate; a drug-lipid conjugate comprising a prodrug moiety. The drug-lipid conjugate comprises one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and a bortezomib (BTZ) prodrug, or another chemotherapeutic agent described herein in the form of a prodrug, wherein the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond. The nanoparticle can further comprise cholesterol comprising about 1 mol% to about 10 mol% of the nanoparticle; and a bulk lipid, such as distearoylphosphatidylcholine (DSPC).

In some embodiments, the targeting peptide-lipid conjugate comprises Formula I: A-B-C-D-E (I), wherein A is the GRP78 targeting peptide; B is a first ethylene glycol spacer; C is an oligolysine linker; D is a second ethylene glycol linker; E is a C₁₂-C₂₀ fatty acid; wherein Formula I optionally includes an amino acid linker moiety disposed between D and E; wherein the first ethylene glycol spacer has a formula (EG)_n, wherein EG is an ethylene glycol monomer of polyethylene glycol and n is the number of EG moieties, and n is about 1 to about 5; and the second ethylene glycol spacer has a formula (EG)_x, wherein x is the number of EG moieties, and x is about 1 to about 50.

In one embodiment, a nanoparticle comprises a GRP78 protein targeting peptide-lipid conjugate comprising Formula I: A-B-C-D-E-F (I), wherein A is a GRP78 protein targeting peptide moiety having an amino acid sequence of SNTRVAP (SEQ ID NO: 1); B is a first ethylene glycol spacer having a formula (EG)_n, wherein EG is an ethylene glycol monomer of polyethylene glycol and

n is the number of EG moieties, and n is 2; C is an oligolysine linker comprising between 1 and 3 lysine residues; D is a second ethylene glycol spacer having a formula (EG)_x, wherein x is the number of EG moieties, and x is 2, 8, 18, 30, or 45; E an amino acid linker moiety comprising a tryptophan residue; and F is a palmitic acid moiety; a polyethylene glycol (PEG)-lipid conjugate; a drug-lipid conjugate comprising a prodrug moiety, wherein the drug-lipid conjugate comprises: i) one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and bortezomib (BTZ) prodrug; and ii) a lipid moiety comprising 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE), wherein the prodrug moiety is linked to the lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; cholesterol comprising about 5 mol% of the nanoparticle; and distearoylphosphatidylcholine (DSPC) or a similarly functioning bulk lipid.

The disclosure also provides for methods of treating various cancers such as, but not limited to, breast cancer, lung cancer, and ovarian cancer. Methods of treating a cancer may comprise the steps of administering to a subject having the cancer a first nanoparticle population comprising a bulk lipid, a PEG-lipid conjugate, cholesterol, a drug-lipid conjugate, and targeting moiety-lipid conjugate, thereby treating the cancer. Optionally, the methods of treating the cancer also may include administration of a second population of nanoparticles to the subject that do not include a targeting moiety-lipid conjugate (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, and a drug-lipid conjugate). The second population of nanoparticles may be administered concurrently with the first population of nanoparticles, or sequentially.

These and other features and advantages of this invention will be more fully understood from the following detailed description of the invention taken together with the accompanying claims. It is noted that the scope of the claims is defined by the recitations therein and not by the specific discussion of features and advantages set forth in the present description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the specification and are included to further demonstrate certain embodiments or various aspects of the invention. In some instances, embodiments of the invention can be best understood by referring to the accompanying drawings in combination with the detailed description presented herein. The description and accompanying drawings may highlight a certain specific example, or a certain aspect of the invention. However, one skilled in the art will understand that portions of the example or aspect may be used in combination with other examples or aspects of the invention.

FIG. 1A-B. Determination of GRP78pep binding constant for sGRP78+ human breast cancer cells. (A) Chemical structure of GRP78pep. (B) Binding curve of GRP78pep for sGRP78+ human breast cancer cells. In vitro peptide cellular binding assays were performed using fluorescently labeled GRP78pep with BT-474, MCF-7, and MDA-MB-231. iPSCs were used as a control. Background

signals from control experiments using fluorescently labeled scrambled version of GRP78pep were almost negligible and subtracted for each data point. Dissociation constant (K_d) was determined to be $7.4 \pm 1.0 \mu\text{M}$ for all the cell lines. All experiments were performed in triplicate, and data represent means ($\pm\text{SD}$).

5 FIG. 2A-F. Design and preparation of Doxorubicin (Dox) prodrug loaded TNPGRP78pep. (A) Chemical structure of GRP78pep-lipid conjugate. The GRP78pep-lipid conjugate was synthesized using Fmoc-chemistry based solid phase peptide synthesis method. A GRP78pep was conjugated to oligolysines (Km ; where m is the number of lysines) through EG2 linker. EG2 plays a role in a spacer that minimizes interaction between the GRP78pep and oligolysines. Oligolysines help make
10 GRP78pep more hydrophilic to present beyond PEG cloud, leading to more efficient target receptor binding. The GRP78pep-EG2- Km moiety was conjugated to two palmitic acids via an EGn linker. While palmitic acids are embedded into a lipid bilayer, the EGn linker gives GRP78pep flexibility to achieve enhanced target receptor binding. (B) Chemical structure of Dox-lipid (a drug-lipid conjugate comprising a prodrug moiety). A doxorubicin molecule was conjugated to a polar head group of
15 DPPE-GA via an acid-labile hydrazone bond to generate the lipid-conjugated doxorubicin prodrug, Dox-lipid. (C) A thin lipid film method was exploited to formulate Dox prodrug loaded TNPGRP78pep. GRP78pep-lipid and Dox-lipid were incorporated into a liposomal scaffold with all other pure lipid constituents at a desired molar ratio, and completely dried to generate a thin lipid film. The dried lipid film was then rehydrated and sized by extrusion using a polycarbonate membrane. (D)
20 Determination of size of Dox prodrug loaded TNPGRP78pep. All nanoparticles were determined to be $\sim 100 \text{ nm}$ using dynamic light scattering (DLS). Data shown here is the representative size distribution of 1% peptide and 1% Dox prodrug loaded TNPGRP78pep (E) and (F) Loading efficiency of peptide and drug. Actual concentrations of GRP78pep and Dox in the liposomal nanoparticles were measured and compared with their respective intended loading concentrations using RP-HPLC. All
25 data shown are the representative experiment.

FIG. 3A-E. Specific binding of GRP78pep to sGRP78 presenting cells. In vitro nanoparticle uptake competitive inhibition assays were conducted using TNPGRP78pep and soluble free GRP78pep using (A) iPSCs, (B) MCF-7, (C) MDA-MB-231, and (D) BT-474 to determine specificity of GRP78pep to sGRP78. Excess free GRP78pep ($50 \mu\text{M}$) was introduced to the cells 30 min prior to
30 nanoparticle administration for competitive nanoparticle uptake inhibition. (E) Percent inhibition (%) at 0.7% and 1% peptide-density for each cell line. All experiments were done in triplicate, and data shows means ($\pm\text{SD}$).

FIG. 4. Selective uptake of TNPGRP78pep by sGRP78+ breast cancer cells. In vitro nanoparticle uptake assays were performed to investigate selectivity of TNPGRP78pep for sGRP78+
35 breast cancer cells, MCF-7, MDA-MB-231, and BT-474. iPSCs were used as a control. Raji (yellow;

B cell lymphoma) and H929 (orange; multiple myeloma) were employed as a negative control. All experiments were performed in triplicate, and data represent means ($\pm$ SD).

FIG. 5A-C. In vitro optimization of TNPGRP78pep for enhanced sGRP78+ breast cancer cell uptake. The effects of different nanoparticle design parameters including (A) peptide EG linker length, (B) oligolysines, and (C) peptide density, on cellular uptake were evaluated to optimize TNPGRP78pep formulation parameters using MDA-MB-231, MCF-7, and BT-474 cells. All experiments were done in triplicates, and data shows means ($\pm$ SD).

FIG. 6. Evaluation of in vivo nanoparticle tissue biodistribution using an in vivo mouse model to mimic breast cancer lung metastasis. sGRP78+ MCF-7 cells were intracardially injected into Balb/c Rag1^{-/-} mice to form sGRP78+ lung metastases. After 4 days, the mice were intravenously treated with either NP (0% peptide) or 1% peptide loaded TNPGRP78pep (n=4 per treatment group). All nanoparticles were loaded with 0.75% DiD dye for fluorescence quantification. The mice were sacrificed 3 days post-treatment, and major organs were dissected and their images were taken by IVIS Lumina II imager for biodistribution analysis. A student t-test was used for P values (asterisk (*); p < 0.01 and N.S.; not significant).

FIG. 7. Drug-loaded TNPGRP78pep effectively target sGRP78+ breast cancer cells at metastatic sites in vivo. sGRP78+ MCF-7 cells were intracardially injected into Rag1^{-/-} mice to trigger sGRP78+ lung metastases. After 4 days, the mice were treated with 1) NP (0% peptide); 2) 1% peptide loaded TNPGRP78pep; 3) Dox prodrug loaded NP; 4) Dox prodrug and 1% peptide loaded TNPGRP78pep; and 5) Dox prodrug and 1.5% peptide loaded TNPGRP78pep (n=3-4 per treatment group). All Dox prodrug loaded nanoparticles were incorporated with 1% Dox-lipid. Nanoparticle-untreated mice injected with either sGRP78- or sGRP78+ cells were used as controls. To see whether TNPGRP78pep affects GRP78-cell seeding, the mice were treated with 1% peptide loaded TNPGRP78pep. All the mice were sacrificed 3 days post-treatment, and lungs were dissected. DiO+ cells were manually counted using a fluorescent microscope to assess in vivo efficacy. A student t-test was used for P values (asterisk (*); p < 0.001 and N.S.; not significant).

FIG. 8A-D. Cartoon schematics of design and synthesis of targeted nanoparticle (TNPGRP78pep). (A) Structures of GRP78-targeting peptide (GRP78pep; left) and GRP78pep-lipid conjugate (right) are shown. The GRP78pep-lipid conjugate contains the GRP78pep (SNTRVAP) (SEQ ID NO: 1), an EG2 linker, oligolysines (Km), an ethylene glycol linker (EGn), a tryptophan, and two palmitic acid lipid tails. (B) Structures of DM1 (mertansine; left) and the DM1-prodrug (right) are shown. (C) Cartoon schematics of nanoparticle assembly from specified stoichiometric ratios of purified components are shown. These include the GRP78pep-lipid conjugate, PEG, bulk lipid, cholesterol, and lipophilic dye (DiD or DiR) or DM1-prodrug. (D) Dynamic light scattering analysis was used to determine the size of the synthesized nanoparticles. Representative results of non-targeted

(NP) and targeted nanoparticles (0.75% TNPGRP78pep, 1% TNPGRP78pep, or 1.5% TNPGRP78pep) with various peptide density.

FIG. 9A-E. Cellular binding studies of GRP78pep and uptake of targeted nanoparticles (TNPGRP78pep) by ovarian cancer cells. (A) Cellular binding of fluorescein-labeled GRP78pep to cell surface-GRP78 expressing human ovarian cancer cell lines OVCAR5 and OVCAR8 (left), and murine ovarian cancer cell lines ID8, ID8 p53^{-/-}, and ID8 BRCA p53^{-/-} (right) is shown. All ovarian cancer cell lines demonstrated a K_d of approximately 5 μ M for GRP78pep. Raji cell line, which expresses low-to-no cell surface GRP78 receptor (Burkitt lymphoma; GRP78^{low}) was used as a negative control. (B) Nanoparticles presenting GRP78pep (TNPGRP78pep) were prepared with varying EG linker length (EG0-EG45) while holding 1% GRP78pep density and 3 lysines constant, and cellular uptake of TNPGRP78pep was performed with human (left) and murine (right) ovarian cancer cells. PBS and non-targeted nanoparticles (NP) were used as controls. (C) Cellular uptake of TNPGRP78pep, with varying oligolysines (0-3 lysines) while holding 1% GRP78pep density and EG8 linker constant, was performed with human (left) and murine (right) ovarian cancer cells. PBS and NP were used as controls. (D) Cellular uptake of TNPGRP78pep, with varying GRP78pep density from 0 - 1% (while holding EG8 and 3 lysines constant), by ovarian cancer cells is shown. PBS and NP were used as controls. Raji cells were used as a negative control cell line. (E) Cellular binding of TNPGRP78pep, with GRP78pep density varying from 0 - 1.5% (while holding EG8 and 3 lysines constant), was performed with human and murine ovarian cancer cell lines (solid bars). PBS and NP were used as controls. To evaluate specificity of nanoparticle binding, competitive binding experiments were simultaneously performed in the presence of excess soluble GRP78pep (free peptide; dashed bars). In all the experiments, cellular binding and uptake was measured by flow cytometry. All binding assays were performed on ice, while uptake assays were performed at 37°C.

FIG. 10A-C. *In vitro* cytotoxicity of DM1, NP[DM1], and TNPGRP78pep[DM1] against ovarian cancer cells. (A) Cytotoxicity of DM1 against OVCAR5 (circle), OVCAR8 (square), and ID8 p53^{-/-} (triangle) ovarian cancer cell lines was determined at 72 h (left). Standard-of-care chemotherapeutics Paclitaxel (center) and Doxorubicin (right) showed similar order-of-magnitude IC₅₀, compared to DM1, at 72 h. (IC₅₀_{DM1} $\approx$ 50 nM, IC₅₀_{Paclitaxel} $\approx$ 10 nM (on OVCAR5 & OVCAR8), IC₅₀_{paclitaxel} $\approx$ 500 nM (on ID8 p53^{-/-}), and IC₅₀_{Doxorubicin} $\approx$ 50 nM) (B) Cytotoxicity of DM1-prodrug loaded NP (NP[DM1] with 0% GRP78pep density) and DM1-prodrug loaded TNPGRP78pep[DM1] (with 0.75% or 1.5% GRP78pep density) was determined at 48 h (left) and 72 h (right) using ID8 p53^{-/-} cell lines. At 48 h, the IC₅₀s were as follows: IC₅₀DM1 $\approx$ 120 nM, IC₅₀NP $\approx$ 120 nM, IC₅₀_{0.75%TNP} $\approx$ 60 nM, and IC₅₀_{1.5%TNP} $\approx$ 60 nM. At 72 h, the IC₅₀s were: IC₅₀DM1 $\approx$ 110 nM, IC₅₀NP $\approx$ 40 nM, IC₅₀_{0.75%TNP} $\approx$ 40 nM, and IC₅₀_{1.5%TNP} $\approx$ 40 nM. (C) Pulsed cytotoxicity of NP[DM1] and TNPGRP78pep[DM1] (with 0.75% or 1.5% GRP78pep density) against ID8 p53^{-/-} cell lines was determined at 48 h (left) and 72 h (right). At 48 h, the IC₅₀s were as follows: IC₅₀DM1 $\approx$ 550 nM,

IC50_{NP} $\approx$ 2100 nM, IC50_{0.75%TNP} $\approx$ 650 nM, and IC50_{1.5%TNP} $\approx$ 350 nM. At 72 h, the IC50s were: IC50_{DM1} $\approx$ 350 nM, IC50_{NP} $\approx$ 900 nM, IC50_{0.75%TNP} $\approx$ 700 nM, and IC50_{1.5%TNP} $\approx$ 350 nM. For pulse assays, cells were washed after 3 hours of drug treatment, and fresh media was added for the remainder of the 48- or 72-h period.

5 FIG. 11A-F. *In vivo* biodistribution of NP and TNP^{GRP78pep}. (A) Mice were injected i.p. with red fluorescent protein (RFP)-tagged ID8 p53^{-/-} ovarian cancer cells. DiR-tagged nanoparticles (NP, 0.75% TNP^{GRP78pep}, 1.5% TNP^{GRP78pep}, or NP+1.5%TNP^{GRP78pep} combination treatment) were injected i.p. 6 weeks after injection of cancer cells (when sufficient metastatic tumor burden was observed in the peritoneal cavity via live mouse RFP imaging). To determine colocalization and uptake of DiR-

10 tagged nanoparticles by RFP+ metastatic solid tumors and metastatic ascites cells, mice were dissected 24 h post-nanoparticle treatment. (B) To study colocalization of DiR-labeled nanoparticles and metastasized RFP+ solid tumors on the indicated organs, metastatic tumors on peritoneal organs were collected, imaged for RFP and DiR fluorescence, and analyzed with ImageJ. Representative images of organs from a mouse in the combination treatment group are shown. (C) To further analyze if

15 TNP^{GRP78pep} preferentially colocalized to solid tumors, relative to NP, the fluorescence of DiR-labeled nanoparticles (NP and TNP^{GRP78pep}) and RFP+ solid tumors on peritoneal organs was quantified via ImageJ. RFP fluorescence (shown as x-axis) was plotted against DiR fluorescence (shown as y-axis), and linear fit analysis was applied to the data. A greater positive slope indicates enhanced colocalization. Analysis of this quantified colocalization demonstrated that colocalization (indicated

20 by positive slope) occurred with all of the nanoparticle treatment groups to differing degrees. Slope for NP = 1.7; slope for 0.75% TNP^{GRP78pep} = 2.6; slope for 1.5% TNP^{GRP78pep} = 0.59; or slope for combination = 0.88. Slope determined via linear regression. As metastatic tumors did not grow on all studied organs, only organs which demonstrated RFP+ tumor are shown (omentum/pancreas, ovaries/uterus, mesentery, and fat). Triangle = Omentum/pancreas, down solid triangle =

25 ovaries/uterus, diamond = mesentery, down hollow triangle = fat. (D) To demonstrate the varying colocalization trends between nanoparticle formulations, the slopes identified in (C) are shown. (E) To study DiR-labeled nanoparticle uptake by RFP+ metastatic solid tumors, tumors obtained from peritoneal organs were disaggregated, and cellular uptake was detected via flow cytometry (gated for RFP+ cells). (F) To study DiR-labeled nanoparticle uptake by RFP+ metastatic ascites cells, peritoneal

30 lavage (ascites with metastatic cells) was collected, and uptake was detected via flow cytometry (gated for RFP+ cells). Parametric Welch's t-test, * p-value < 0.05.

FIG. 12A-D. *In vivo* efficacy of NP[DM1] and TNP^{GRP78pep}[DM1]. (A) Mice were injected i.p. with RFP-tagged ID8 p53^{-/-} ovarian cancer cells. Nanoparticle treatments with varying GRP78pep density (NP[DM1], 0.75% TNP^{GRP78pep}[DM1], 1.5% TNP^{GRP78pep}[DM1], or a combination treatment

35 with NP[DM1] + 1.5%TNP^{GRP78pep}[DM1]) were administered i.p. on days 16, 20, 23, 27, 30, 34, 37, and 41. PBS was used as a vehicle control. All treatments contained the equivalent of 3 mg DM1-

prodrug per kg mouse weight. Mice were observed for 58 days. (B) The average weight of mice in each treatment group is shown as a marker of systemic toxicity. No statistically significant toxicity (i.e. weight loss) was observed in the treatment groups, relative to the PBS control. (C) At end of study, organs were collected and imaged for RFP to detect metastatic tumors. Final tumor burden was determined via ImageJ quantification of RFP+ tumor fluorescence. The NP[DM1]+1.5%TNP^{GRP78pep}[DM1] combination treatment showed statistically lower tumor burden, relative to the PBS control. (D) Organs were weighed to check for systemic toxicity. Organs from noncancerous healthy mice were also weighed, as an additional control. None of the treatments demonstrated any significant organ toxicity relative to the controls. Nonparametric, unpaired t-tests, * p-value < 0.05.

FIG. 13A-D. *In vivo* effect of NP[DM1] and TNP^{GRP78pep}[DM1] on peritoneal anti-tumorigenic immune cell involvement. The levels of immune cell subsets in the peritoneal cavity fluid were determined for the indicated treatment groups. All nanoparticle treatment groups received the equivalent of 3 mg DM1-prodrug per kg mouse weight. PBS was used as a vehicle control. To determine levels of immune cells in the peritoneal fluid, peritoneal lavage was performed on mice immediately after sacrifice. After processing lavage samples to remove red blood cells, immune cell-specific antibody panels and flow cytometry were used to identify the various immune cells. Overall, results indicated that the DM1-prodrug-loaded nanoparticles did not have an adverse effect on immune cell levels, relative to PBS control. (A) The effect of nanoparticle treatment groups on T cell subsets (T cytotoxic, T helper, NKT, TCR $\gamma\delta$ + T, and T regulatory cells) are shown. T cells were gated based on CD3+ marker, and the subsets were gated on the following markers: cytotoxic T cells based on CD8+, helper T cells on CD4+, NK T cells on CD56+, TCR $\gamma\delta$ T cells on TCR $\gamma\delta$ +, and regulatory T cells on CD25+. Frequencies of the indicated T cell subsets were determined as a percentage of total cells collected from mice peritoneal fluid. (B) The effect of nanoparticle treatment groups on other anti-tumorigenic lymphocytes, B cells (*left*) and NK cells (*right*), are shown as a percentage of cells in peritoneal fluid. B cells were gated based on marker CD19+, and NK cells based on CD56+ marker. (C) To evaluate the impact of nanoparticle treatment on monocyte & granulocyte cell populations, neutrophils/MDSCs (myeloid-derived suppressor cells; *left*), dendritic cells (DCs; *center*), and macrophages (*right*) were studied. Neutrophils/MDSCs were gated on marker Ly6G+ and Ly6C+, DCs on Ly6G- CD11b-/lo CD11c+ markers, and macrophages on CD11b+ CD11c-. (D) To further study macrophage levels, a macrophage-specific panel of antibodies was used to evaluate frequencies of macrophages. Overall, macrophages were determined via F4/80+ marker (*left*), while macrophage subset M1 (anti-tumorigenic; *center-left*) was gated on CD86+CD206-, subset M2 (pro-tumorigenic; *center-right*) gated on CD86-CD206+, and subset M1M2 (transitioning between M1 and M2; *right*) gated on CD86+CD206+, shown as a percentage of cells from peritoneal fluid. Parametric unpaired

Welch's t-test, * p-value < 0.05. Arrow indicates all groups in the direction of the arrow have noted p-value, relative to the group at the arrow's start.

Fig. 14. Competitive inhibition of cellular binding. Soluble GRP78pep competitively inhibited binding of TNP^{GRP78pep}, of varying peptide density, proving specificity of GRP78pep to bind to GRP78+ human (A) and murine (B) ovarian cancer cells. With 1.5% TNP^{GRP78pep}, the following percent of binding was inhibited: >95% (OVCAR5 and OVCAR8), >90% (ID8 p53^{-/-} and ID8 BRCA p53^{-/-}), and 85% (ID8).

Fig. 15. Levels of (A) dendritic cells and (B) macrophages in the peritoneal cavity with various treatment groups compared to non-cancerous, untreated control (Healthy Control).

FIG. 16A-F. Synthetic schematics of preparing prodrug loaded targeted nanoparticle formulations. A) DM1-Prodrug synthesis via conjugating the active drug DM1 with linkage molecule 1a. B) BTZ-Prodrug synthesis via conjugating the active drug BTZ with linkage molecule 1b. C) Structure of GRP78pep-lipid conjugate, containing GRP78pep sequence, EG₂ spacer, oligolysine, EG₈ peptide linker and fatty acid tails. D) Illustration of components and preparations of TNP[DM1] which is a targeted nanoparticle incorporating GRP78pep-lipid conjugate and DM1-Prodrug. E) Illustration of components and preparations TNP[BTZ], a targeted nanoparticle incorporating GRP78pep-lipid conjugate and BTZ-Prodrug. F) Sketch of prodrug loaded non-targeted nanoparticle (NP[DM1] or NP[BTZ]) that does not contain GRP78pep-lipid conjugate.

FIG. 17A-E. Characterization of DM1 and BTZ prodrug loaded liposomal nanoparticles. A) Dynamic light scattering (DLS) analysis of nanoparticles. Non-targeted particle (NP), DM1-Prodrug loaded non-targeted particle (NP[DM1]), BTZ-Prodrug loaded non-targeted particle (NP[BTZ]), GRP78pep-targeted particle (TNP), DM1-Prodrug loaded GRP78pep-targeted particle (TNP[DM1]), and BTZ-Prodrug loaded GRP78pep-targeted particle (TNP[BTZ]), exhibited an average size distribution of ~75 nm. B) Zeta potential of NP, NP[DM1], NP[BTZ], TNP, TNP[DM1], and TNP[BTZ] demonstrated negligible charge of nanoparticle formulations. Each bar represents mean ± S.D. (n=5) C) Transmission electron microscopy (TEM) images of NP, NP[DM1], NP[BTZ], TNP, TNP[DM1], and TNP[BTZ]. Scale bar at the bottom right corner of each image represents 100 nm. D), E) Stability analysis of (d) TNP[DM1] (d) and (e) TNP[BTZ] formulations. Stability studies were performed using phosphate buffer (PB), acetate buffer (AB) and enzyme containing buffer (EB) at 37 °C. PB and AB had pH 7.4 and pH 4.8 respectively; while EB had a pH of 7.4 to facilitate enzymatic reaction. Nanoparticles were analyzed after the indicated incubation period to determine whether prodrug molecules were stable under different pH and reaction condition. Prodrug elution were tracked and detected using a Zorbax C3 semiprep column and a 2-propanol/acetonitrile/water gradient. Area under the curve (AUC) from prodrug elution peaks was calculated for indicated incubation periods. Normalized AUC= (AUC of Prodrug Peak at a given hour/ AUC of Prodrug Peak at 0 h). NP[DM1] and NP[BTZ] showed similar results (results not shown).

FIG. 18A-G. *In vitro* evaluation of cellular binding and uptake of GRP78pep-targeted particles (TNPs) using A549 lung carcinoma cell line. A) Cellular binding assay was performed by using fluorescein-labeled GRP78pep on ice for 1h, and analyzed by flow cytometry. B) Cellular binding of DiO-labeled TNPs containing varying GRP78pep density from 0% to 0.75% is shown. Cells were incubated with the indicated TNPs on ice for 1 h, and analyzed by flow cytometry. C) Cellular uptake of DiO-labeled TNPs containing varying GRP78pep density from 0% to 0.75% is shown. TNPs contained EG₈ peptide linker and were incubated with cells at 37 °C for 4h and 24 h, analyzed by flow cytometry. D) Effect of GRP78pep linker length on cellular uptake was evaluated. DiO-labeled TNPs were incubated with cells at 37 °C for 4 h and 24 h, and analyzed by flow cytometry. Non-targeted particle (NP) was used as control. Unpaired t-test were used for determining P-value (**, p<0.001). E) Fluorescent microscopy images to evaluate cellular internalization of TNPs containing varying GRP78pep density are shown. TNPs were labeled with DiO dyes and cell nuclei were stained with DAPI. Scale bar represents 50 μm. F) Cellular binding of prodrug loaded nanoparticle formulations TNP[DM1], TNP[BTZ], NP[DM1] and NP[BTZ] is shown. No prodrug loaded counterparts were also included for comparison. Nanoparticles were incubated with cells for 1 h on ice, and analyzed by flow cytometry. Prodrug incorporation to nanoparticles had no significant impact on cellular binding. The statistical analysis was performed using One-way ANOVA G) Specificity of TNP binding to lung cancer cells were assessed by using excess monovalent GRP78pep (free GRP78pep) in competitive cellular binding experiment. TNPs were evaluated from low to high nanoparticle concentrations (0 - 1.25 nM) that were added to cells on ice for 1 h. Y-axis represents mean fluorescence binding of TNPs, detected by flow cytometry. Percentages (%) represent binding inhibition of TNPs by free GRP78pep. Fluorescence from cells were minimal and subtracted for the calculation of binding inhibition (%). Each data represents means of triplicate cultures (± S.D.).

FIG. 19A-B. *In vitro* cytotoxicity evaluation of targeted and non-targeted nanoparticle formulations in A549. A) Cells were cultured for 48 h in the presence of equivalent drug concentrations of either NP[DM1], TNP[DM1]), or free DM1. B) Cells were cultured for 48 h in the presence of equivalent drug concentrations of either NP[BTZ], TNP[BTZ]), or free BTZ. Cell viability was assessed by CCK-8 for detecting cellular toxicity. Each data represents means of triplicate cultures (± S.D.).

FIG. 20A-C. *In vivo* biodistribution and uptake of GRP78pep-targeted nanoparticles (TNPs). A) Nanoparticles loaded with DiD fluorescent dye were tested in a subcutaneous xenograft mouse model of lung carcinoma. Mice were injected with A549 cells and once tumors became palpable (>200 mm³), (from left to right in graph) NP and TNPs loaded with 0.25%, 0.5% and 0.75% GRP78pep were injected intravenously. 24 h after nanoparticle injection, mice were sacrificed, and tumor and major organs were imaged with IVIS lumina for nanoparticle accumulation. B) Uptake of DiD loaded nanoparticles by tumor cells was detected. Tumors were dissected 24 h after nanoparticle injection

and were processed using disaggregation solution (containing collagenase and DNase) to get single cell suspension for flow cytometric analysis. C) Hematoxylin and eosin (H&E) staining of major organs and tumor tissue were performed to determine whether nanoparticles exerted any organ toxicity. Scale bar at the bottom right corner of each images represents 25 μm . N= 3~4 for all groups and data represents means ($\pm$ S.D.). Unpaired t-test was used for determining P-value.

FIG. 21A-F. *In vivo* efficacy of GRP78pep-targeted and non-targeted, DM1-Prodrug or BTZ-Prodrug loaded nanoparticle prodrug formulations. Subcutaneous xenograft mouse model was used to test prodrug loaded nanoparticle formulations. Mice were injected with A549 cells and tumors were allowed to grow to a palpable size ($>80 \text{ mm}^3$) prior to treatments. Mice were treated with either PBS (control), NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ], Free DM1 or Free BTZ on day 1, 5, 9, 13 and 17. All TNP formulations contained 0.5% GRP78pep loading. Nanoparticle formulations (NP[DM1] and TNP[DM1]) and Free DM1 were delivered at 2.5 mg/kg and 0.5 mg/kg (the highest possible maximum tolerated dose) concentrations respectively, while all BTZ formulations (NP[BTZ], TNP[BTZ] and Free BTZ) were delivered at 0.75 mg/kg concentration. A), B) Tumor volumes for DM1 (A) and BTZ (B) formulations were measured during the study period. C), D) Systemic toxicity of DM1 (C) and BTZ (D) formulations were detected by measuring body weight of mice. E) Tumor burden were compared for statistical analysis. From left to right, PBS, NP[DM1], TNP[DM1], Free DM1, NP[BTZ], TNP[BTZ], and Free BTZ. F) H&E staining of tumor tissues from all treatment groups and control. $n = 8\sim 9$ for all nanoparticle formulations and $n = 4\sim 6$ for rest of the groups. Data represents means ($\pm$ S.E.). Unpaired t-test was used for determining P-value (***, $p < 0.004$, **, $p < 0.04$; *, $p < 0.05$).

FIG. 22A-B. Organ weight and H&E staining of the major organs and tumor to determine toxicity of NP and TNP formulations. A) Post-dissection major organ weights from treatment groups. From left to right, PBS, NP[DM1], TNP[DM1], Free DM1, NP[BTZ], TNP[BTZ], and Free BTZ. B) Overall, no observable differences in major organs were observed among the treatment groups and PBS (control). Scale bar at the bottom right corner of each images represents 25 μm .

Fig. 23. The comparison of cellular uptake of TNPs in A549 and Jurkat cell line. TNPs were observed to get internalized in an enhanced manner to A549 cells as GRP78pep density was increased. On the other hand, Jurkat cells exhibited minimal cellular uptake of TNPs. The cellular uptake was analyzed via flow cytometry and conducted in triplicate cultures. Data show mean $\pm$ S.D. Asterisk(s) represent a statistically significant uptake of TNP compared to NP (**, $p < 0.0001$; *, $p < 0.001$). Unpaired t-test was used for determining P-value.

Definitions

The following definitions are included to provide a clear and consistent understanding of the specification and claims. As used herein, the recited terms have the following meanings. All other

terms and phrases used in this specification have their ordinary meanings as one of skill in the art would understand. Such ordinary meanings may be obtained by reference to technical dictionaries, such as Hawley's Condensed Chemical Dictionary 14th Edition, by R.J. Lewis, John Wiley & Sons, New York, N.Y., 2001 or Singleton, *et al.*, Dictionary of Microbiology and Molecular Biology, 2d ed., John Wiley and Sons, New York (1994), and Hale & Markham, The Harper Collins Dictionary of Biology. Harper Perennial, N.Y. (1991). General laboratory techniques (DNA extraction, RNA extraction, cloning, cell culturing. etc.) are known in the art and described, for example, in Molecular Cloning: A Laboratory Manual, J. Sambrook *et al.*, 4th edition, Cold Spring Harbor Laboratory Press, 2012; also see the series Ausubel *et al.* eds. (2007) Current Protocols in Molecular Biology; the series Methods in Enzymology (Academic Press, Inc., N.Y.); MacPherson *et al.* (1991) PCR 1: A Practical Approach (IRL Press at Oxford University Press); MacPherson *et al.* (1995) PCR 2: A Practical Approach; Harlow and Lane eds. (1999) Antibodies, A Laboratory Manual; Freshney (2005) Culture of Animal Cells: A Manual of Basic Technique, 5th edition; Gait ed. (1984) Oligonucleotide Synthesis; U.S. Patent No. 4,683,195 (Mullis); Hames and Higgins eds. (1984) Nucleic Acid Hybridization; Anderson (1999) Nucleic Acid Hybridization; Hames and Higgins eds. (1984) Transcription and Translation; Immobilized Cells and Enzymes (IRL Press (1986)); Perbal (1984) A Practical Guide to Molecular Cloning; Miller and Calos eds, (1987) Gene Transfer Vectors for Mammalian Cells (Cold Spring Harbor Laboratory); Makrides ed. (2003) Gene Transfer and Expression in Mammalian Cells; Mayer and Walker eds. (1987) Immunochemical Methods in Cell and Molecular Biology (Academic Press, London); Herzenberg et al. eds (1996) Weir's Handbook of Experimental Immunology; and Hermanson, Greg. (2013). Bioconjugate Techniques: Third Edition. (Academic Press, Inc., N.Y.) 1-1146.

References in the specification to "one embodiment", "an embodiment", etc., indicate that the embodiment described may include a particular aspect, feature, structure, moiety, or characteristic, but not every embodiment necessarily includes that aspect, feature, structure, moiety, or characteristic. Moreover, such phrases may, but do not necessarily, refer to the same embodiment referred to in other portions of the specification. Further, when a particular aspect, feature, structure, moiety, or characteristic is described in connection with an embodiment, it is within the knowledge of one skilled in the art to affect or connect such aspect, feature, structure, moiety, or characteristic with other embodiments, whether or not explicitly described.

The singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a compound" includes a plurality of such compounds, so that a compound X includes a plurality of compounds X. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for the use of exclusive terminology, such as "solely," "only," and the like, in

connection with any element described herein, and/or the recitation of claim elements or use of "negative" limitations.

The term "and/or" means any one of the items, any combination of the items, or all of the items with which this term is associated. The phrases "one or more" and "at least one" are readily understood by one of skill in the art, particularly when read in context of its usage. For example, the phrase can mean one, two, three, four, five, six, ten, 100, or any upper limit approximately 10, 100, or 1000 times higher than a recited lower limit.

As will be understood by the skilled artisan, all numbers, including those expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth, are approximations and are understood as being optionally modified in all instances by the term "about." These values can vary depending upon the desired properties sought to be obtained by those skilled in the art utilizing the teachings of the descriptions herein. It is also understood that such values inherently contain variability necessarily resulting from the standard deviations found in their respective testing measurements. When values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value without the modifier "about" also forms a further aspect.

The terms "about" and "approximately" are used interchangeably. Both terms can refer to a variation of $\pm 5\%$, $\pm 10\%$, $\pm 20\%$, or $\pm 25\%$ of the value specified. For example, "about 50" percent can in some embodiments carry a variation from 45 to 55 percent, or as otherwise defined by a particular claim. For integer ranges, the term "about" can include one or two integers greater than and/or less than a recited integer at each end of the range. Unless indicated otherwise herein, the terms "about" and "approximately" are intended to include values, e.g., weight percentages, proximate to the recited range that are equivalent in terms of the functionality of the individual ingredient, composition, or embodiment. The terms "about" and "approximately" can also modify the endpoints of a recited range as discussed above in this paragraph.

As will be understood by one skilled in the art, for any and all purposes, particularly in terms of providing a written description, all ranges recited herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof, as well as the individual values making up the range, particularly integer values. It is therefore understood that each unit between two particular units is also disclosed. For example, if 10 to 15 is disclosed, then 11, 12, 13, and 14 are also disclosed, individually, and as part of a range. A recited range (e.g., weight percentages or carbon groups) includes each specific value, integer, decimal, or identity within the range. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, or tenths. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art, all language such as "up to", "at least", "greater than", "less

than", "more than", "or more", and the like, include the number recited and such terms refer to ranges that can be subsequently broken down into sub-ranges as discussed above. In the same manner, all ratios recited herein also include all sub-ratios falling within the broader ratio. Accordingly, specific values recited for radicals, substituents, and ranges, are for illustration only; they do not exclude other defined values or other values within defined ranges for radicals and substituents. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

This disclosure provides ranges, limits, and deviations to variables such as volume, mass, percentages, ratios, etc. It is understood by an ordinary person skilled in the art that a range, such as "number 1" to "number 2", implies a continuous range of numbers that includes the whole numbers and fractional numbers. For example, 1 to 10 means 1, 2, 3, 4, 5, ... 9, 10. It also means 1.0, 1.1, 1.2, 1.3, ..., 9.8, 9.9, 10.0, and also means 1.01, 1.02, 1.03, and so on. If the variable disclosed is a number less than "number 10", it implies a continuous range that includes whole numbers and fractional numbers less than number 10, as discussed above. Similarly, if the variable disclosed is a number greater than "number 10", it implies a continuous range that includes whole numbers and fractional numbers greater than number 10. These ranges can be modified by the term "about", whose meaning has been described above.

One skilled in the art will also readily recognize that where members are grouped together in a common manner, such as in a Markush group, the disclosure encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group. Additionally, for all purposes, the disclosure encompasses not only the main group, but also the main group absent one or more of the group members. The disclosure therefore envisages the explicit exclusion of any one or more of members of a recited group. Accordingly, provisos may apply to any of the disclosed categories or embodiments whereby any one or more of the recited elements, species, or embodiments, may be excluded from such categories or embodiments, for example, for use in an explicit negative limitation.

The term "contacting" refers to the act of touching, making contact, or of bringing to immediate or close proximity, including at the cellular or molecular level, for example, to bring about a physiological reaction, a chemical reaction, or a physical change, e.g., in a solution, in a reaction mixture, *in vitro*, or *in vivo*.

An "effective amount" refers to an amount effective to treat a disease, disorder, and/or condition, or to bring about a recited effect. For example, an effective amount can be an amount effective to reduce the progression or severity of the condition or symptoms being treated. Determination of a therapeutically effective amount is well within the capacity of persons skilled in the art, especially in light of the detailed disclosure provided herein. The term "effective amount" is intended to include an amount of a compound described herein, or an amount of a combination of

compounds described herein, e.g., that is effective to treat or prevent a disease or disorder, or to treat the symptoms of the disease or disorder, in a host. Thus, an "effective amount" generally means an amount that provides the desired effect.

Alternatively, the terms "effective amount" or "therapeutically effective amount," as used herein, refer to a sufficient amount of an agent or a composition or combination of compositions being administered which will relieve to some extent one or more of the symptoms of the disease or condition being treated. The result can be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. For example, an "effective amount" for therapeutic uses is the amount of the composition comprising a compound as disclosed herein required to provide a clinically significant decrease in disease symptoms. An appropriate "effective" amount in any individual case may be determined using techniques, such as a dose escalation study. The dose could be administered in one or more administrations. However, the precise determination of what would be considered an effective dose may be based on factors individual to each patient, including, but not limited to, the patient's age, size, type or extent of disease, stage of the disease, route of administration of the compositions, the type or extent of supplemental therapy used, ongoing disease process and type of treatment desired (e.g., aggressive vs. conventional treatment).

The terms "treating", "treat" and "treatment" include (i) preventing a disease, pathologic or medical condition from occurring (e.g., prophylaxis); (ii) inhibiting the disease, pathologic or medical condition or arresting its development; (iii) relieving the disease, pathologic or medical condition; and/or (iv) diminishing symptoms associated with the disease, pathologic or medical condition. Thus, the terms "treat", "treatment", and "treating" can extend to prophylaxis and can include prevent, prevention, preventing, lowering, stopping, or reversing the progression or severity of the condition or symptoms being treated. As such, the term "treatment" can include medical, therapeutic, and/or prophylactic administration, as appropriate.

As used herein, "subject" or "patient" means an individual having symptoms of, or at risk for, a disease or other malignancy. A patient may be human or non-human and may include, for example, animal strains or species used as "model systems" for research purposes, such a mouse model as described herein. Likewise, a patient may include either adults or juveniles (e.g., children). Moreover, patient may mean any living organism, preferably a mammal (e.g., human or non-human) that may benefit from the administration of compositions contemplated herein. Examples of mammals include, but are not limited to, any member of the Mammalian class: humans, non-human primates such as chimpanzees, and other apes and monkey species; farm animals such as cattle, horses, sheep, goats, swine; domestic animals such as rabbits, dogs, and cats; laboratory animals including rodents, such as rats, mice and guinea pigs, and the like. Examples of non-mammals include, but are not limited to, birds, fish, and the like. In one embodiment of the methods provided herein, the mammal is a human.

As used herein, the terms “providing”, “administering”, “introducing,” are used interchangeably herein and refer to the placement of the compositions of the disclosure into a subject by a method or route which results in at least partial localization of the composition to a desired site. The compositions can be administered by any appropriate route which results in delivery to a desired location in the subject. The compositions described herein may be administered with additional compositions to prolong stability and activity of the compositions, or in combination with other therapeutic drugs.

The terms "inhibit", "inhibiting", and "inhibition" refer to the slowing, halting, or reversing the growth or progression of a disease, infection, condition, or group of cells. The inhibition can be greater than about 20%, 40%, 60%, 80%, 90%, 95%, or 99%, for example, compared to the growth or progression that occurs in the absence of the treatment or contacting.

The term “substantially” as used herein, is a broad term and is used in its ordinary sense, including, without limitation, being largely but not necessarily wholly that which is specified. For example, the term could refer to a numerical value that may not be 100% the full numerical value. The full numerical value may be less by about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 15%, or about 20%.

Wherever the term “comprising” is used herein, options are contemplated wherein the terms “consisting of” or “consisting essentially of” are used instead. As used herein, “comprising” is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the aspect element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the aspect. In each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The disclosure illustratively described herein may be suitably practiced in the absence of any element or elements, limitation, or limitations not specifically disclosed herein.

As used herein, a “lipid” or “bulk lipid” is any compatible lipid that has a hydrophilic region and a hydrocarbon tail that can facilitate the incorporation of epitope-lipid conjugate into a lipid membrane. Examples include, but are not limited to, phospholipids, such as 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), and fatty acids, such as palmitic acid.

As used herein, “nanoparticle” refers to any partially or wholly lipid-coated nanostructure having a cross-section length ("diameter") in the range of 1 to 300 nanometers (nm). As used herein, cross-section length refers to the measurement of the longest cross-section length of the nanoparticle (e.g., the longest distance that can be measured between two points of a cross-section of the nanoparticle). In some instances, such particles will have a cross-section length in the range of about 10 nm to about 300 nm, about 10 to about 250 nm, about 10 to about 200 nm, about 10 to about 150

nm, about 50 to 125 nm, about 10 to about 120 nm, about 10 to 115 nm, about 10 to 110 nm, about 10 to 105 nm, about 10 to 100 nanometers, and/or 50 to 110 nm.

The terms “conjugate” and “conjugated” as used herein can refer to the attachment (e.g., the covalent attachment) of two or more components (e.g., chemical compounds, polymers, biomolecule, particles, etc.) to one another. In some embodiments, a conjugate can comprise monovalent moieties derived from two different chemical compounds covalently linked via a bivalent linker moiety (e.g., an optionally substituted alkylene or arylene). In some embodiments, the linker can contain one or more biodegradable bond, such that one or more bonds in the linker can be broken when the prodrug is exposed to a particular physiological environment or enzyme.

The term “prodrug” as used herein, can refer to a compound that, upon administration to a subject or sample, is capable of providing (directly or indirectly) another compound (i.e., a “parent compound”) having a desired biological activity (e.g., anticancer activity). In some, but not all, embodiments, the prodrug compound has less of the desired biological activity than the parent compound. In some embodiments, the prodrug compound has no measurable biological activity prior to transformation to the parent compound. In some embodiments, the prodrug itself has the desired activity.

Transformation of the prodrug to the parent compound can take place in the presence of particular enzymes (e.g., esterases) or under certain biological conditions (e.g., at a physiologically relevant pH or in the presence of reducing agents present in a physiological environment). In some embodiments, the prodrug is initially transformed into another prodrug, which is then transformed (sometimes much more slowly) into the parent compound. Prodrugs can provide increased bioavailability and/or enhanced delivery to a biological compartment (e.g., a lysosome, the brain or lymphatic system, etc.) relative to a parent compound. In some embodiments, the prodrug can be more compatible with a particular delivery platform or formulation than the parent compound.

The number of molecules of a component in a nanoparticle may also be described in terms of a “mole percentage,” which is calculated by dividing the number of molecules of that component by the number of molecules in the nanoparticle. For example, in a nanoparticle with 100 component molecules, 93 of which are component A, 5 of which are component B, 2 of which are component C, the “molecular ratio” of the components (A:B:C) is 93:5:2, and the mole percentages of the three components are 93%, 5% and 2%, respectively. If not specifically identified, percentages referenced herein are molar percentages (mol %), unless the context specifically indicates otherwise.

As used herein, the terms “encapsulated” and “encapsulated drugs” refer to component of the nanoparticle such as a therapeutic agent localized to the aqueous core of the liposome.

As used herein, the terms “liposome” and “liposomes” refer to a spherical structure having at least one lipid bilayer. A liposome can be used for the administration of therapeutic agents. A liposome can comprise a combination of one or more phospholipids, an optional lipid that is not a phospholipid,

such as cholesterol, pegylated lipids, or a combination thereof. As used herein, a liposome may have a diameter of about 30 nm to about 200 nm. In some embodiments, the diameter of the liposomes is about 75 nm to about 125 nm. In certain embodiments, the liposomes can have diameters of about 110 nm and 125 nm.

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Embodiment of the Invention.

A nanoparticle of the present disclosure generally comprises a targeting peptide-lipid conjugate, wherein a targeting peptide moiety of the targeting peptide-lipid conjugate comprises a GRP78 targeting peptide; a polyethylene glycol (PEG)-lipid conjugate; a drug-lipid conjugate
10 comprising a prodrug moiety, wherein the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; cholesterol comprising about 1 mol% to about 10 mol% of the nanoparticle; and a bulk lipid comprising distearoylphosphatidylcholine (DSPC). In preferred embodiments, the nanoparticle is a liposome or a micelle.

In some embodiments, the targeting peptide-lipid conjugate comprises a GRP78 targeting
15 peptide; a first ethylene glycol spacer; an optional amino acid linker; a second ethylene glycol linker; and the lipid is a C₁₂-C₂₀ fatty acid.

In some embodiments, the targeting moiety of the targeting moiety-lipid conjugate comprises an antibody, an antibody fragment, a peptide, a protein, or a ligand that specifically binds to a GRP78 protein displayed on the surface of a cell, and in particular, a cancerous cell. In some embodiments,
20 the targeting moiety is a peptide that specifically binds to a GRP78 protein. In some embodiments, the targeting peptide comprises the amino acid sequence SNTRVAP (SEQ ID NO: 1). In another embodiment, the targeting peptide consists of the amino acid sequence SNTRVAP (SEQ ID NO: 1).

In some embodiments, the targeting moiety-lipid conjugate (e.g., GRP78 targeting peptide-lipid conjugate) can comprise about 0.1 mol%, about 0.25 mol%, about 0.5 mol%, about 0.75 mol%,
25 about 1 mol%, about 2 mol%, about 3 mol%, about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, or about 20 mol% of the nanoparticle. In some embodiments, the targeting moiety-lipid conjugate can comprise about 0.1 mol% to about 10 mol% or about 0.1 mol% to about 5 mol%, or about 1 mol% to
30 about 5 mol% of a nanoparticle.

In some embodiments, the targeting moiety-lipid conjugate (e.g., GRP78 targeting peptide-lipid conjugate) can comprise one or more linkers disposed between the targeting moiety and the lipid. As used herein, a "linker" can be a sugar, an oligosaccharide, an amino acid, peptides, a polymer, or other molecules that can provide favorable results in targeting peptide display and binding. Examples
35 of a linker include, but are not limited to, ethylene glycol molecules (e.g., polyethylene glycol). In

some embodiments, a linker comprises polyethylene glycol polymers. For example, the PEG linker may comprise about 1 to about 50 ethylene glycol residues.

In other embodiments, the linker can be any moiety that will improve targeting peptide-lipid water solubility profile. The linker increases hydrophilicity and improves targeting peptide display on the nanoparticle surface. Examples include, but are not limited to, charged amino acids such as aspartic acid (D), glutamic acid (E), lysine (K) and arginine (R) or polar amino acids, such as, glutamine (Q), asparagine (N), histidine (H), serine (S), threonine (T), and methionine (M). In some embodiments, an amino acid may comprise one or more amino acids, and in particular, one or more charged amino acids such as poly-lysine (*e.g.*, a monomer, dimer or trimer).

The lipid of the targeting peptide-lipid conjugate (*e.g.*, GRP78 targeting peptide-lipid conjugate) may comprise a (C₁₂-C₂₀) fatty acids or fatty acid esters. A "fatty acid" refers to an alkanolic acid or an alkanolic acid moiety (*i.e.*, the residue left after formal removal of the acid hydrogen), where the fatty acid includes at least about nine or ten carbon atoms. Non-limiting examples of fatty acids include lauric acid (12:0), cis-5-dodecanoic acid (12:1), tridecanoic acid (13:0), myristic acid (14:0), myristoleic acid (cis-9-tetradecenoic acid, 14:1), pentadecanoic acid (15:0), palmitic acid (16:0), palmitoleic acid (cis-9-hexadecenoic acid, 16:1), heptadecanoic acid (17:1), stearic acid (18:0), elaidic acid (trans-9-octadecenoic acid, 18:1), oleic acid (cis-9-octadecanoic acid, 18:1), nonadecanoic acid (19:0), and eicosanoic acid (20:0). In preferred embodiments, the C₁₂-C₂₀ fatty acid is a myristic acid moiety or palmitic acid moiety.

In some embodiments, the targeting peptide-lipid conjugate comprises Formula I:



wherein A is the GRP78 targeting peptide; B is a first ethylene glycol spacer; C is an oligolysine linker; D is a second ethylene glycol linker; E is a C₁₂-C₂₀ fatty acid; wherein Formula I optionally includes an amino acid linker moiety disposed between D and E; wherein the first ethylene glycol spacer has a formula (EG)_n, wherein EG is an ethylene glycol monomer of polyethylene glycol and n is the number of EG moieties; and the second ethylene glycol spacer has a formula (EG)_x, wherein x is the number of EG moieties. In some embodiments, the number n is about 1 to about 20, or about 1 to about 15, or about 1 to about 10, or about 1 to about 5. In some embodiments, the number n is about 1 to about 5. In some embodiments, the number x is about 1 to about 50, or about 2, about 4, about 6, about 8, about 10, about 12, about 14, about 16, about 18, about 20, about 22, about 24, about 26, about 28, about 30, about 32, about 34, about 36, about 38, about 40, about 42, about 44, about 46, about 48, or about 50. In some embodiments of a nanoparticle, n is 2; and x is 2, 8, 18, 30, or 45. In one specific embodiment, n is 2 and x is 18, or n is 2 and x is about 14 to about 22. In some embodiments, the amino acid linker is one more tryptophane residues or one or more lysine residues. In some embodiments, the amino acid linker comprises 1, 2, 3, 4, or 5 lysine or tryptophan residues.

In some embodiments, a nanoparticle may not include a targeting moiety. For example, a nanoparticle may comprising a drug-lipid conjugate, wherein the drug-lipid conjugate comprises: a) a prodrug moiety comprising one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and a bortezomib (BTZ) prodrug; and b) a lipid moiety comprising 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE); wherein the prodrug is linked to the lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; a polyethylene glycol (PEG)-lipid conjugate; cholesterol comprising about 0.1% to about 10% w/w of the nanoparticle; and distearoylphosphatidylcholine (DSPC).

In some embodiments, a nanoparticle may not include a drug-lipid conjugate. For example, a nanoparticle may comprise a targeting moiety-lipid conjugate, a polyethylene glycol (PEG)-lipid conjugate; cholesterol comprising about 0.1% to about 10% w/w of the nanoparticle; and distearoylphosphatidylcholine (DSPC).

In some embodiments, the drug moiety of the drug-lipid conjugate comprises a chemotherapeutic agent covalently conjugated to a lipid to form the prodrug. A "chemotherapeutic agent" is a chemical compound useful in the treatment of cancer, regardless of mechanism of action. Classes of chemotherapeutic agents include, but are not limited to, alkylating agents, antimetabolites, spindle poison plant alkaloids, cytotoxic/antitumor antibiotics, topoisomerase inhibitors, antibodies, photosensitizers, and kinase inhibitors. Examples of chemotherapeutic agents include erlotinib (TARCEVA®, Genentech/OSI Pharm.), docetaxel (TAXOTERE®, Sanofi-Aventis), 5-FU (fluorouracil, 5-fluorouracil, CAS No. 51-21-8), gemcitabine (GEMZAR®, Lilly), PD-0325901 (CAS No. 391210-10-9, Pfizer), cisplatin (cis-diamine,dichloroplatinum(II), CAS No. 15663-27-1), carboplatin (CAS No. 41575-94-4), paclitaxel (TAXOL®, Bristol-Myers Squibb Oncology, Princeton, N.J.), trastuzumab (HERCEPTIN®, Genentech), temozolomide (4-methyl-5-oxo-2,3,4,6,8-pentazabicyclo [4.3.0] nona-2,7,9-triene- 9-carboxamide, CAS No. 85622-93-1, TEMODAR®, TEMODAL®, Schering Plough), tamoxifen ((Z)-2-[4-(1,2-diphenylbut-1-enyl)phenoxy]-N,N-dimethyl-ethanamine, NOLVADEX®, ISTUB AL®, VALODEX®), and doxorubicin (ADRIAMYCIN®), Akti-1/2, HPPD, and rapamycin.

More examples of chemotherapeutic agents include oxaliplatin(ELOXATIN®, Sanofi), bortezomib (VELCADE®, Millennium Pharm.), sunitinib (SUNITINIB®, SUI 1248, Pfizer), letrozole (FEMARA®, Novartis), imatinib mesylate (GLEEVEC®, Novartis), XL-518 (MEK inhibitor, Exelixis, WO 2007/044515), ARRY-886 (Mek inhibitor, AZD6244, Array BioPharma, AstraZeneca), SF-1126 (PI3K inhibitor, Semafore Pharmaceuticals), BEZ-235 (PI3K inhibitor, Novartis), XL-147 (PI3K inhibitor, Exelixis), PTK787/ZK 222584 (Novartis), fulvestrant (FASLODEX®, AstraZeneca), leucovorin (folinic acid), rapamycin (sirolimus, RAPAMUNE®, Wyeth), lapatinib (TYKERB®, GSK572016, Glaxo Smith Kline), lonafarnib (SARASAR™, SCH 66336, Schering Plough), sorafenib (NEXAVAR®, BAY43-9006, Bayer Labs), gefitinib (IRESSA®, AstraZeneca), irinotecan (C

AMPTOS AR®, CPT-11, Pfizer), tipifarnib (ZARNESTRA™, Johnson & Johnson), ABRAXANE™ (Cremophor-free), albumin- engineered nanoparticle formulations of paclitaxel (American Pharmaceutical Partners, Schaumburg, IL), vandetanib (rINN, ZD6474, ZACTIMA®, AstraZeneca), chlorambucil, AG1478, AG1571 (SU 5271; Sugen), temsirolimus (TORISEL®, Wyeth), pazopanib (GlaxoSmithKline), canfosfamide (TELCYTA®, Telik), thiotepa and cyclophosphamide (CYTOXAN®, NEOSAR®); alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, triethylenephosphoramidate, triethylenethiophosphoramidate and trimethylmelamine; acetogenins (especially bullatacin and bullatacinone); a camptothecin (including the synthetic analog topotecan); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogs); cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogs, KW-2189 and CBI-TMI); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, chlorophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosoureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (e.g., calicheamicin, calicheamicin gammall, calicheamicin omegall (*Angew Chem. Intl. Ed. Engl.* (1994) 33:183-186); dynemicin, dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, carminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, porfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogs such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6- mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiothane, testolactone; anti-adrenals such as aminogluthetamide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elfornithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet;

pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, OR); razoxane; rhizoxin; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2"-trichlorotriethylamine; trichothecenes (T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol;

5 pipobroman; gacytosine; arabinoside (Ara-C); cyclophosphamide; thiotepa; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; etoposide (VP- 16); ifosfamide; mitoxantrone; vincristine; vinorelbine (NAVELBINE®); novantrone; teniposide; edatrexate; daunomycin; aminopterin; capecitabine (XELOD A®, Roche); ibandronate; CPT-I 1; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as

10 retinoic acid; and pharmaceutically acceptable salts, acids and derivatives of any of the above. Also included in the definition of "chemotherapeutic agent" are: (i) anti- hormonal agents that act to regulate or inhibit hormone action on tumors such as anti- estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (NOLVADEX®; tamoxifen citrate), raloxifene, droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY1 17018, onapristone, and FARESTON®

15 (toremifme citrate); (ii) aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminoglutethimide, MEGASE® (megestrol acetate), AROMASIN® (exemestane; Pfizer), formestanie, fadrozole, RIVISOR® (vorozole), FEMARA® (letrozole; Novartis), and ARIMIDEX® (anastrozole; AstraZeneca); (iii) anti-androgens such as flutamide, nilutamide, bicalutamide, leuprolide, and

20 goserelin; as well as troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); (iv) protein kinase inhibitors such as MEK inhibitors (WO 2007/044515); (v) lipid kinase inhibitors; (vi) antisense oligonucleotides, particularly those which inhibit expression of genes in signaling pathways implicated in aberrant cell proliferation, for example, PKC-alpha, Raf and H-Ras, such as oblimersen (GENASENSE®, Genta Inc.); (vii) ribozymes such as VEGF expression inhibitors (e.g.,

25 ANGIOZYME®) and HER2 expression inhibitors; (viii) vaccines such as gene therapy vaccines, for example, ALLOVECTIN®, LEUVECTIN®, and VAXID®; PROLEUKIN® rIL-2; topoisomerase 1 inhibitors such as LURTOTECAN®; ABARELIX® rmRH; (ix) anti-angiogenic agents such as bevacizumab (AVASTIN®, Genentech); and pharmaceutically acceptable salts, acids and derivatives of any of the above. Also included in the definition of "chemotherapeutic agent" are therapeutic

30 antibodies such as alemtuzumab (Campath), bevacizumab (AVASTIN®, Genentech); cetuximab (ERBITUX®, Imclone); panitumumab (VECTIB IX®, Amgen), rituximab (RITUXAN®, Genentech/Biogen Idee), pertuzumab (OMNITARG™, 2C4, Genentech), trastuzumab (HERCEPTIN®, Genentech), tositumomab (Bexxar, Corixa), and the antibody drug-conjugate, gemtuzumab ozogamicin (MYLOT ARG®, Wyeth). Humanized monoclonal antibodies with

35 therapeutic potential as chemotherapeutic agents in combination with trastuzumab-MCC-DMI include alemtuzumab, apolizumab, aselizumab, atlizumab, bapineuzumab, bevacizumab, bivatumzumab

mertansine, cantuzumab mertansine, cedelizumab, certolizumab pegol, cidfusituzumab, cidtuzumab, daclizumab, eculizumab, efalizumab, epratuzumab, erlizumab, felvizumab, fontolizumab, gemtuzumab ozogamicin, inotuzumab ozogamicin, ipilimumab, labetuzumab, lintuzumab, matuzumab, mepolizumab, motavizumab, motovizumab, natalizumab, nimotuzumab, nolovizumab, numavizumab, ocrelizumab, omalizumab, palivizumab, pascolizumab, pecfusituzumab, pectuzumab, pertuzumab, pexelizumab, ralivizumab, ranibizumab, reslivizumab, reslizumab, resyvizumab, rovelizumab, ruplizumab, sibrotuzumab, sipilizumab, sontuzumab, tacatuzumab tetraxetan, tadocizumab, talizumab, tefibazumab, tocilizumab, toralizumab, trastuzumab, tucotuzumab celmoleukin, tucosituzumab, umavizumab, urtoxazumab, and visilizumab.

In some embodiments, the prodrug moiety of the drug-lipid conjugate comprises a daunorubicin prodrug, a cytarabine prodrug, an idarubicin prodrug, a cyclophosphamide prodrug, a gemcitabine prodrug, a docetaxel prodrug, a carboplatin prodrug, a cisplatin prodrug, a paclitaxel prodrug, a capecitabine prodrug, a doxorubicin prodrug, a mertansine prodrug, or a bortezomib prodrug. In some embodiments, the prodrug moiety of the drug-lipid conjugate comprises one or more of a mertansine prodrug, a doxorubicin prodrug, and a bortezomib prodrug. Preferably, the prodrug of the drug-lipid conjugate is linked to a lipid moiety of the drug-lipid conjugate via a functional group such as, but not limited to, carboxylic, hydroxyl, amine, phosphate/phosphonate, esters, and carbonyl groups (see, for example, Figs. 2, 8, and 16).

In some embodiments, the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond.

In some embodiments, a nanoparticle comprises only a single species of prodrug. In some embodiments, the prodrug or a free drug (i.e., unconjugated drug) may be encapsulated within the nanoparticle (i.e., a liposome or micelle) such as within an aqueous core of the liposome or micelle. In some embodiments, the nanoparticles (e.g., liposomes or micelles) may include combinations of various encapsulated drugs and/or various drug-lipid conjugates.

A chemotherapeutic agent can be conjugated to a lipid to form a drug-lipid conjugate by various synthetic techniques known in the art. The techniques described in *Bioconjugate Techniques*, 3rd Ed. (2013), Hermanson (Academic Press, Inc., N.Y.) can be used to conjugate various chemotherapeutic agents to a polar head group of a lipid moiety such as DPPE-GA, Linkage Molecule 1a, Linkage Molecule 1b, and similar lipids with an appropriate linking moiety. For example, drug-lipid conjugates of daunorubicin, idarubicin, docetaxel, and paclitaxel can be prepared in a manner similar to the method of preparing the Dox-lipid conjugate described herein, wherein the chemotherapeutic agent is conjugated to a polar head group of DPPE-GA via acid-labile hydrazone bond. See also, the techniques described by Stefanick *et al.*, *Nanoscale* 2019, 11 (10), 4414-4427. As would be readily recognized by one of skill in the art, an available hydroxyl or amine group of chemotherapeutic agents such as capecitabine, cytarabine, cyclophosphamide, gemcitabine,

carboplatin, or cisplatin can be conjugated to a linkage molecule such as Linkage Molecule 1a, using known techniques and/or optionally an alkyl linker comprising a thiol group, to form a corresponding drug-lipid conjugate of a nanoparticle described herein.

In some embodiments, the drug-lipid conjugate can comprise about 0.1 mol%, about 0.25 mol%, about 0.5 mol%, about 0.75 mol%, about 1 mol%, about 2 mol%, about 3 mol%, about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, or about 20 mol% of the nanoparticle. In some embodiments, the drug-lipid conjugate can comprise about 0.1 mol% to about 10 mol% or about 1 mol% to about 5 mol% of a nanoparticle. In other embodiments, the drug-lipid conjugate can comprise about 0.1 mol% to about 1 mol% of a nanoparticle.

The drug-lipid conjugate also may include other compounds or effector molecules in addition to or instead of a prodrug, including detectable substances useful for example in diagnosis. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, radioactive nuclides, positron emitting metals (for use in positron emission tomography), and nonradioactive paramagnetic metal ions. See generally U.S. Pat. No. 4,741,900 for metal ions which can be conjugated to antibodies for use as diagnostics. Suitable enzymes include horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; suitable prosthetic groups include streptavidin, avidin and biotin; suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride and phycoerythrin; suitable luminescent materials include luminol; suitable bioluminescent materials include luciferase, luciferin, and aequorin; and suitable radioactive nuclides include ^{125}I , ^{131}I , ^{111}In and ^{99}Tc . In some embodiments, the effector molecules are conjugated to a lipid moiety or entrapped within a nanoparticle. In some embodiments, the effector molecule can comprise about 0.1 mol% to about 20 mol% of a nanoparticle.

In some embodiments, nanoparticles may include a hydrophilic polymer conjugated to a hydrophobic region of lipid molecule. The polymer can be water-soluble polymer, such as polyethylene glycol (PEG), forming a PEG-lipid conjugate. In some embodiments, the PEG-lipid conjugates, comprises, for example, PEG conjugated diacylglycerols and dialkylglycerols; PEG-conjugated phosphatidylethanolamine and phosphatidic acid; PEG conjugated ceramides; PEG conjugated dialkylamines; PEG conjugated 1,2-diacyloxypropan-3-amines; 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000); and any combinations thereof. In some embodiments, the PEG-lipid conjugate comprises DSPE-PEG2000. The term "DSPE" refers to "1,2-distearoyl-sn-glycero-3-phosphoethanolamine. DSPE can be readily conjugated to poly(ethylene glycol) to provide a pegylated phospholipid (PEG-DSPE) for the preparation of micelles or liposomes. DSPE and PEG-DSPE are commercially available from

suppliers such as Avanti Polar Lipids, Inc.

In some embodiments, the PEG-lipid conjugate can comprise about 0.1 mol%, about 0.25 mol%, about 0.5 mol%, about 0.75 mol%, about 1 mol%, about 2 mol%, about 3 mol%, about 4 mol%, about 5 mol%, about 6 mol%, about 7 mol%, about 8 mol%, about 9 mol%, about 10 mol%, about 11 mol%, about 12 mol%, about 13 mol%, about 14 mol%, about 15 mol%, about 16 mol%, about 17 mol%, about 18 mol%, about 19 mol%, or about 20 mol% of the nanoparticle. In some embodiments, the PEG-lipid conjugate can comprise about 0.1 mol% to about 10 mol% or about 1 mol% to about 5 mol% of a nanoparticle.

In some embodiments, a nanoparticle can include a molecule that can improve stability of the nanoparticle, such as, but is not limited to a sterol such as cholesterol, cholesterol-sulfate, a sterol-ester such as an ester linked fatty acids (C₁₆:0, C₁₈:1, and C₁₈:2) (*e.g.*, cholesterol-palmitate), beta-sitosterol, stigmasterol, campesterol, lanosterol, brassicasterol, fucosterol, lathosterol, spinasterol, desmosterol, and dehydrocholesterol, (*e.g.*, 7-dehydrocholesterol). In some embodiments, the amount of a sterol (*e.g.*, cholesterol) in the nanoparticle in an amount of about 0.1 mol% to about 35 mol% cholesterol, or about 0.1 mol% to about 10 mol%. In some embodiments, the amount of sterol (*e.g.*, cholesterol) in the nanoparticle is about 1 mol% to about 5 mol%, or the amount of sterol (*e.g.*, cholesterol) in the nanoparticle is about 5 mol%. In other embodiments, the nanoparticle does not include a sterol (*e.g.*, cholesterol).

Bulk lipids can include a lipid molecule coupled directly or indirectly to one or more additional molecules, or just lipid molecules. Typically, lipid molecules are amphipathic lipid molecules, each with a polar/hydrophilic region and a non-polar/hydrophobic/hydrocarbon tail. Optionally, some or all of the lipid molecules can be phospholipids or fatty acids or compatible lipids that can facilitate the components incorporation into a lipid membrane.

The term “phospholipid” as used herein refers to a glycerol phosphate with an organic headgroup such as choline, serine, ethanolamine or inositol and zero, one or two (typically one or two) fatty acids esterified to the glycerol backbone. Exemplary phospholipids, include, but are not limited to, phosphatidyl cholines; phosphatidyl cholines with acyl groups having 6 to 22 carbon atoms; phosphatidyl ethanolamines; phosphatidyl inositols; phosphatidic acids; phosphatidyl serines; sphingomyelin; phosphatidyl glycerols; phosphatidylcholine; phosphatidylglycerol; lecithin; β,γ -dipalmitoyl- α -lecithin; sphingomyelin; phosphatidylserine; phosphatidic acid; N-(2,3-di(9-(Z)-octadecenyl-oxy))-prop-1-yl-N,N,N-trimethylammonium chloride; phosphatidylethanolamine; lysolecithin; lysophosphatidylethanolamine; phosphatidylinositol; cephalin; cardiolipin; cerebrosides; dicetylphosphate; dioleoylphosphatidylcholine; dipalmitoylphosphatidylcholine; dipalmitoylphosphatidylglycerol; dioleoylphosphatidylglycerol; palmitoyl-oleoyl-phosphatidylcholine; distearoylphosphatidylcholine; stearoyl-palmitoyl-phosphatidylcholine; di-palmitoylphosphatidylethanolamine; di-stearoyl-phosphatidylethanolamine; di-myristoyl-phosphatidylserine; di-oleyl-

phosphatidylcholine; dimyristoyl phosphatidyl choline (DMPC); dioleoylphosphatidylethanolamine (DOPE); palmitoyloleoylphosphatidylcholine (POPC); egg phosphatidylcholine (EPC); distearoylphosphatidylcholine (DSPC); dioleoylphosphatidylcholine (DOPC); dipalmitoylphosphatidylcholine (DPPC); dioleoylphosphatidylglycerol (DOPG); dipalmitoylphosphatidylglycerol (DPPG); -phosphatidylethanolamine (POPE); dioleoylphosphatidylethanolamine 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (DOPE-mal); L- α -phosphatidylcholine; and any combinations thereof. Exemplary fatty acids include palmitic acid, myristic acid, palmitic acid, and stearic acid. In some embodiments, the bulk lipid comprises 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC).

In some embodiments, the bulk lipid may include about 60 mol%, 61 mol%, about 62 mol%, about 63 mol%, 64 mol%, 65 mol%, about 66 mol%, about 67 mol%, 68 mol%, 69 mol%, about 70 mol%, about 71 mol%, about 72 mol%, about 73 mol%, about 74 mol%, about 75 mol%, about 76 mol%, about 77 mol%, about 78 mol%, about 79 mol%, about 80 mol%, about 81 mol%, about 82 mol%, about 83 mol%, 84 mol%, about 85 mol%, about 86 mol%, about 87 mol%, 88 mol%, about 89 mol%, about 90 mol%, about 91 mol%, about 92 mol%, about 93 mol%, about 94 mol%, about 95 mol%, about 96 mol%, about 97 mol%, or greater than 97 mol% of the nanoparticle.

In some embodiments, the nanoparticle has a diameter of about 10 nm to about 300 nm. In other specific embodiments, the nanoparticle can have a diameter of about 80 nm to about 220 nm, about 100 nm to about 160 nm, or about 100 nm.

In some embodiments, an amount of components of an exemplary a nanoparticle includes about 80 mol% to about 97 mol% bulk lipid, about 0.1 mol% to about 10 mol% PEG-lipid conjugate, about 0.1 mol% to about 10 mol% cholesterol, about 0.1 mol% to about 10 mol% prodrug, and about 0.01% to about 5 mol% targeting moiety-lipid conjugate.

In some embodiments, an amount of components of an exemplary a nanoparticle includes about 85 mol% to about 97 mol% bulk lipid, about 1 mol% to about 7 mol% PEG-lipid conjugate, about 1 mol% to about 7 mol% cholesterol, about 1 mol% to about 7 mol% drug-lipid conjugate, and about 0.01% to about 3 mol% targeting moiety-lipid conjugate.

In one certain embodiment, an amount of components of an exemplary a nanoparticle includes about 85 mol% to about 95 mol% bulk lipid, about 5 mol% PEG-lipid conjugate, about 5 mol% cholesterol, about 0.01 mol% to about 1.5 mol% drug-lipid conjugate, and about 0 mol% to about 4 mol% targeting moiety-lipid conjugate, where the bulk lipid is DSPC, the PEG-lipid conjugate is PEG-DSPE, the prodrug is a DM1-lipid conjugate, a Dox-lipid conjugate, or a BTZ-lipid conjugate, and the targeting moiety-lipid conjugate is a GRP-78 peptide-lipid conjugate.

In one certain embodiment, an amount of components of an exemplary a nanoparticle includes about 92 mol% bulk lipid, about 5 mol% PEG-lipid conjugate, about 5 mol% cholesterol, about 0.1 to about 3 mol% drug-lipid conjugate, and about 0.01 mol% to about 1.5 mol% targeting moiety-lipid

conjugate, where the bulk lipid is DSPC, the PEG-lipid conjugate is PEG-DSPE, the prodrug is DM1-lipid conjugate, a Dox-lipid conjugate, or a BTZ-lipid conjugate, and the targeting moiety-lipid conjugate is a GRP-78 peptide-lipid conjugate.

In another embodiment, an amount of components of an exemplary a nanoparticle includes about 88 mol% to about 93 mol% bulk lipid, about 5 mol% PEG-lipid conjugate, about 0 mol% to about 5 mol% cholesterol, about 0.1 to about 1.5 mol% drug-lipid conjugate, and about 0 mol% to about 1.5 mol% targeting moiety-lipid conjugate, where the bulk lipid is DSPC, the PEG-lipid conjugate is PEG-DSPE, the prodrug is DM1-lipid conjugate, a Dox-lipid conjugate, or a BTZ-lipid conjugate, and the targeting moiety-lipid conjugate is a GRP78 peptide-lipid conjugate.

The disclosure also provides methods of treating a cancer in a subject in need thereof comprising administering to the subject an effective amount of a nanoparticle or a composition as described herein, thereby treating the cancer. For example, a method of treating a cancer in a subject in need thereof comprising administering to the subject an effective amount of a nanoparticle, thereby treating the cancer, wherein the nanoparticle comprises a targeting peptide-lipid conjugate, wherein a targeting peptide moiety of the targeting peptide-lipid conjugate comprises a GRP78 targeting peptide; a polyethylene glycol (PEG)-lipid conjugate; a drug-lipid conjugate comprising a prodrug moiety, wherein the drug-lipid conjugate comprises one or more of a mertansine (DM1) prodrug, a doxorubicin (Dox) prodrug, and a bortezomib (BTZ) prodrug, and wherein the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; cholesterol comprising about 1 mol% to about 10 mol% of the nanoparticle; and distearoylphosphatidylcholine (DSPC).

In some embodiments, the cancer comprises adipose cancer, anogenital cancer, breast cancer, bladder cancer, blood cancer, bone cancer, a brain tumor, central nervous system cancer, colon cancer, colorectal cancer, connective tissue cancer, a gynecological tumor, a head tumor, kidney cancer, lung cancer, lymphoid cancer, a leukemia (e.g., acute myeloid leukemia), mesothelioma, multiple myeloma, a neck tumor, neuroblastoma, pancreatic cancer, prostate cancer, retinal cancer, skin cancer (e.g., melanoma), a soft tissue sarcoma, or stomach cancer. In certain specific embodiments, the cancer is a cancer in which the cancer cells overexpress the GRP78 protein or a fragment thereof. In some embodiments, the cancer is one of breast cancer, ovarian cancer, lung cancer, and acute myeloid leukemia.

In some embodiments, nanoparticles comprising a drug-lipid conjugate having a prodrug moiety of mertansine (DM1). DM1 may be effective in treating, for example, leukemias, sarcomas, ovarian cancer, lung cancer, squamous cell head & neck carcinoma, multiple myeloma, gliomas, colorectal cancer, breast cancer, cervical cancers, Wilms tumor, rhabdomyosarcoma, neuroblastoma, non-Hodgkins lymphoma, pleuropulmonary blastoma, malignant peripheral nerve sheath tumor (MPNST), renal cell carcinoma, pancreatic cancer, and synovial sarcoma.

In some embodiments, nanoparticles comprising a drug-lipid conjugate having a prodrug moiety of doxorubicin (Dox). Dox may be effective in treating, for example, Bladder carcinoma Multiple myeloma, Breast cancer, Prostate cancer, Endocrine carcinoma, leukemias, sarcomas, Thymoma, Ewing's sarcoma, Gastric cancer, Gynecological carcinoma, Head and neck cancer, Hepatic carcinoma, Hepatoma, Kaposi's sarcoma, acute lymphoblastic Leukemia, acute myeloblastic Leukemia, Lung cancer, Lymphoma, Hodgkin's Lymphoma, non-Hodgkin's lymphoma, Neuroblastomas, Osteosarcoma, Pancreatic cancer, Sarcoma, soft tissue, Testicular carcinoma, Thyroid carcinoma, Urothelial carcinoma, and Wilm's tumor.

In some embodiments, nanoparticles comprising a drug-lipid conjugate having a prodrug moiety of bortezomib (Btz). Btz may be effective in treating, for example, multiple myeloma, lymphoma, mantle cell lymphoma, leukemias, and lung cancer.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is daunorubicin, cytarabine, idarubicin, or cyclophosphamide that preferably may be used to treat, for example, leukemias such as acute myeloid leukemia.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is gemcitabine or docetaxel that preferably may be used to treat, for example, pancreatic cancer.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is carboplatin, cyclophosphamide, or doxorubicin that preferably may be used to treat, for example, neuroblastoma.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is cisplatin that preferably may be used to treat, for example, cervical cancer or lung cancer.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is carboplatin or paclitaxel that preferably may be used to treat, for example, ovarian cancer.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is capecitabine that preferably may be used to treat, for example, colon cancer.

In other embodiments, the prodrug moiety of a drug-lipid conjugate is cyclophosphamide that preferably may be used to treat, for example, lymphomas.

In some embodiments, an amount of nanoparticles administered to a subject comprises about 0.1 mg/kg, about 0.2 mg/kg, about 0.3 mg/kg, about 0.4 mg/kg, about 0.5 mg/kg, about 0.6 mg/kg, about 0.7 mg/kg, about 0.8 mg/kg, about 0.9 mg/kg, about 1 mg/kg, about 1.25 mg/kg, about 1.5 mg/kg, about 1.75 mg/kg, about 2 mg/kg, about 2.5 mg/kg, about 3 mg/kg, about 3.5 mg/kg, about 4 mg/kg, about 4.5 mg/kg, about 5 mg/kg, about 5.5 mg/kg, about 6 mg/kg, about 6.5 mg/kg, about 7 mg/kg, about 7.5 mg/kg, about 8 mg/kg, about 8.5 mg/kg, about 9 mg/kg, about 9.5 mg/kg, about 10 mg/kg, about 10.5 mg/kg, about 11 mg/kg, about 11.5 mg/kg, about 12 mg/kg, about 12.5 mg/kg, about 13 mg/kg, about 13.5 mg/kg, about 14 mg/kg, about 14.5 mg/kg, about 15 mg/kg, about 15.5 mg/kg, about 16 mg/kg, about 17 mg/kg, about 18 mg/kg, about 19 mg/kg, about 20 mg/kg, about 22.5 mg/kg, about 25 mg/kg, about 27.5 mg/kg, or about 30 mg/kg. In some embodiments, an amount of

nanoparticles administered to a subject comprises about 0.1 mg/kg to about 50 mg/kg. In some embodiments, the nanoparticles are administered over the course of a defined time period that may be consecutive or non-consecutive days. For example, doses may be administered on non-consecutive days over a time period of 10 days to about 25 days. Dosage conversion between animals and humans are known in the art; see, for example, Nair *et al*, *J Basic Clin Pharma* 2016;7:27-31.

In some embodiments, the route of administration of the nanoparticle or pharmaceutical composition comprising the nanoparticle may include subcutaneous injection, intravenous injection or infusion, intramuscular injection, intraarterial administration, intrathecal administration, oral administration, sublingual administration, nasal administration, inhalation administration, rectal administration, or transdermal administration.

In one embodiment, a method of treating breast cancer comprises administering an effective amount of a first population of nanoparticles (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, a drug-lipid conjugate, and targeting moiety-lipid conjugate) or a composition as described herein to a person in need thereof, thereby treating the breast cancer. In some embodiments of a method of treating breast cancer, the effective amount of nanoparticle or composition comprising nanoparticles is about 0.1mg/kg to about 10 mg/kg. Optionally, the method of treating breast cancer also may include administration of a second population of nanoparticles that do not include a targeting moiety-lipid conjugate (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, and a drug-lipid conjugate). The second population of nanoparticles may be administered concurrently with the first population of nanoparticles, or sequentially.

In one embodiment, a method of treating lung cancer comprises administering an effective amount of a first population of nanoparticles (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, a drug-lipid conjugate, and targeting moiety-lipid conjugate) or a composition as described herein to a person in need thereof, thereby treating the lung cancer. Optionally, the method of treating lung cancer also may include administration of a second population of nanoparticles that do not include a targeting moiety-lipid conjugate (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, and a drug-lipid conjugate). The second population of nanoparticles may be administered concurrently with the first population of nanoparticles, or sequentially. In some embodiments of a method of treating lung cancer, the effective amount of nanoparticle or composition comprising nanoparticles is about 0.1mg/kg to about 10 mg/kg.

In one embodiment, a method of treating ovarian cancer comprises administering an effective amount of a nanoparticle (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, a drug-lipid conjugate, and targeting moiety-lipid conjugate) or a composition as described herein to a person in need thereof, thereby treating the ovarian cancer. Optionally, the method of treating ovarian cancer also may include administration of a second population of nanoparticles that do not include a targeting moiety-lipid conjugate (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, and a drug-

lipid conjugate). The second population of nanoparticles may be administered concurrently with the first population of nanoparticles, or sequentially. In some embodiments of a method of treating ovarian cancer, the effective amount of nanoparticle or composition comprising nanoparticles is about 0.1mg/kg to about 10 mg/kg.

5 In one embodiment, a method of treating acute myeloid leukemia (AML) comprises administering an effective amount of a nanoparticle (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, a drug-lipid conjugate, and targeting moiety-lipid conjugate) or a composition as described herein to a person in need thereof, thereby treating the lung cancer. Optionally, the method of treating AML also may include administration of a second population of nanoparticles that do not
10 include a targeting moiety-lipid conjugate (e.g., comprising bulk lipid, a PEG-lipid conjugate, cholesterol, and a drug-lipid conjugate). The second population of nanoparticles may be administered concurrently with the first population of nanoparticles, or sequentially. In some embodiments of a method of treating lung cancer, the effective amount of nanoparticle or composition comprising nanoparticles is about 0.1mg/kg to about 10 mg/kg.

15 Other embodiments also may include the use of the nanoparticles or a composition of the nanoparticles to manufacture a medicament for the treatment of various diseases such as cancer, and in particular, breast cancer, lung cancer, ovarian cancer, and acute myeloid leukemia.

Results and Discussion.

20 *Determination of GRP78^{pep} Binding Constant for sGRP78+ Human Breast Cancer Cells.* We have previously shown that sGRP78 marks a stem-like population of breast cancer cells that has increased metastatic potential *in vivo*. The SNTRVAP (SEQ ID NO: 1) peptide (here referred to as GRP78^{pep}), was reported to specifically bind GRP78 presented on prostate cancer cell surfaces (Figure 1A) (Mandelin *et al.*, *Proc Natl Acad Sci USA*, 2015, 112 (12), 3776-3781). Therefore, to
25 investigate binding of GRP78^{pep} to GRP78 on breast cancer cells, we performed an *in vitro* peptide cellular binding assay using human breast cancer cell lines: (1) MCF-7 (luminal A), (2) BT-474 (luminal B), and (3) MDA-MB-231 (triple negative). We measured GRP78^{pep} binding to sGRP78 presenting cells using flow cytometry. Control experiments conducted with a scrambled version of the fluorescently labeled GRP78^{pep} demonstrated insignificant binding to cells (data not shown). Induced
30 pluripotent stem cells (iPSCs) were utilized as a positive control, as we have previously shown that GRP78 is homogeneously expressed on the cell surface of iPSCs (Fig. 1B). The results showed that GRP78^{pep} effectively bound to iPSCs and breast cancer cells with an observed K_d of $7.4 \pm 1.0 \mu\text{M}$. While K_d was determined to be the same for all cell types, differences in fluorescence intensity at a given ligand concentration for different cell types were mainly due to variations in sGRP78 expression
35 levels. Overall, these results showed that GRP78^{pep} binds to sGRP78 positive cells with a moderate

monovalent affinity, which in turn can be incorporated into a multivalent targeting strategy for selective drug delivery to aggressive metastatic breast cancer cells.

We next utilized the GRP78pep to develop a drug-loaded nanoparticle to target sGRP78 expressing cells. We engineered a prodrug loaded targeted nanoparticle (TNP) formulation by adopting a design approach for peptide-lipid conjugates that we previously developed. Briefly, A peptide-lipid conjugate was comprised of 5 functional moieties: (1) GRP78pep for targeting, (2) EG₂ linker to separate lysines from the binding sequence, (3) oligolysines (K_m; where m is the number of lysine residues), (4) EG_n linker (n is the repeating unit of ethylene glycol where 0 ≤ n ≤ 45), and (5) two palmitic acid moieties (Fig. 2A). GRP78pep specifically binds to sGRP78 and is connected to K_m via EG₂ linker. EG₂ linker functions as a spacer to separate lysine conjugates from GRP78pep to preserve the peptide sequence and its binding activity. Oligolysines (K_m) are included to improve the solubility characteristics of the targeting elements to result in favorable partitioning of GRP78pep into aqueous phase beyond the polyethylene glycol (PEG) coating, enabling more efficient peptide binding to the target receptor. The GRP78pep-EG₂-K_m moiety was then conjugated to two palmitic acids via an EG_n linker to generate a GRP78pep-lipid conjugate (GRP78pep-K_m-EG_n-lipid). Palmitic acids enable insertion and anchoring of lipid conjugate into lipid bilayer of TNPs. The EG_n linker provides GRP78pep with reach and flexibility for more efficient target receptor binding.

Next, we employed a prodrug, Dox-lipid conjugate described in our former studies, due to two significant advantages it provides (Fig. 2B). Primarily, as a lipid derivative used in liposome preparation, it enables precise control over drug loading with batch-to-batch consistency during nanoparticle formulations. Secondly, owing to its prodrug form with an acid-labile bond, premature release of Dox is minimized during systemic circulation, reducing non-specific toxicity to healthy cells and organs. Meanwhile, Dox is preferentially released upon endocytosis of nanoparticles under acidic environments by breaking the hydrazone bond, enabling selective drug delivery for target cancer cells. Particle preparation, analysis and characterization are detailed in materials and methods section.

To assess whether Dox prodrug loaded TNP^{GRP78pep} preserved precise molar ratios of peptide and drug, we conducted a loading efficiency experiment using RP-HPLC. Prodrug and targeting peptide concentrations in particle formulations were measured and compared to their theoretical concentrations. The results demonstrated that over 98% peptide and drug loading efficiency was achieved, confirming Dox prodrug loaded TNP^{GRP78pep} maintained precision in peptide and drug stoichiometry (Fig. 2 E and F). Therefore, the synthetic approach that was used generated highly and precisely controlled homogeneous TNP^{GRP78pep} particles, confirming Dox prodrug loaded TNP^{GRP78pep} formulations will consistently provide reliable experimental results.

Specificity and Selectivity of Binding/Uptake of TNP^{GRP78pep} by sGRP78+ Breast Cancer Cells.

To corroborate binding specificity of TNP^{GRP78pep} for sGRP78+ breast cancer cells, we performed a competitive nanoparticle uptake inhibition assay using excess soluble GRP78pep *in vitro*. Breast

cancer cells including MCF-7, MDA-MB-231, and BT-474 were incubated with excess soluble GRP78_{pep} (50 μ M) prior to treatment with nanoparticle formulations. iPSCs were used as a positive control. The results showed that iPSC uptake of TNP^{GRP78_{pep}} was significantly inhibited (>80%) by free GRP78_{pep} at both 0.7% and 1% peptide density (Fig. 3A and E). A similar trend was also observed in MCF-7 and MDA-MB-231 cells with over 80% inhibition of cellular uptake of TNP^{GRP78_{pep}} formulations with the same peptide densities (Fig. 3B, C and E). Surprisingly, although cellular uptake inhibition of TNP^{GRP78_{pep}} at 0.7% peptide-density by BT-474 cells was measured at ~80%, the uptake inhibition reduced to half when the peptide density was increased to 1% (Fig. 3D and E). This was presumably due to variations in the expression levels and metabolic activities of different cells resulting in increased endocytosis, thereby effectively overcoming competitive inhibition. Overall, these results demonstrated specific binding/uptake of TNP^{GRP78_{pep}} to sGRP78+ breast cancer cells.

To demonstrate that TNP^{GRP78_{pep}} is selective in binding to sGRP78+ breast cancer cells over sGRP78- cells, we compared cellular uptake by sGRP78+ breast cancer cells to other cell lines that don't present GRP78 on cell surface. For this, NCI-H929 (multiple myeloma) and Raji (B cell lymphoma) cell lines were used as negative controls. iPSCs were also utilized as a positive control (Fig. 4). The results demonstrated that BT-474 cellular uptake of TNP^{GRP78_{pep}} increased as peptide density increased from 0% to 0.7% peptide density and plateaued. MDA-MB-231 cellular uptake of TNP^{GRP78_{pep}} increased with increasing peptide density and reached maximum at 1%. Meanwhile, there was no detectable MCF-7 cellular uptake of TNP^{GRP78_{pep}} at 0.7%, while uptake gradually increased as peptide density increased to 4%. Most importantly, although the cellular uptake of TNP^{GRP78_{pep}} by the negative controls were insignificant, there was an observed gradual increase in the level of cellular uptake by the negative controls beyond 1% peptide density, compromising the selectivity of TNP^{GRP78_{pep}} on sGRP78+ breast cancer cells. Therefore, we rationally determined 1% as the *in vitro* optimized peptide density of TNP^{GRP78_{pep}} to maximize the cellular uptake while preserving the selectivity on sGRP78+ cells.

In Vitro Optimization of TNP^{GRP78_{pep}} to Enhance GRP78+ Breast Cancer Cellular Uptake.

Typically, the formulation parameters that we optimize to maximize selective targeting with minimal side effects include particle size, PEG coating, peptide loading, peptide EG-linker length, and oligolysines. Despite larger particles reportedly having longer circulation half-lives, in general, smaller particles (20-50 nm) are preferred in solid tumor targeting due to their ability to penetrate deeper into a tumor tissue. Nevertheless, in this study, since we are seeking to model eliminating CTCs/early metastatic seeded cells that represent the cells that dislodge from parent solid tumors, larger sized particles (100 nm) with longer circulation times are more suitable. Moreover, despite recent concerns that PEG2000 (~EG45 in length) can potentially trigger immunogenicity in ~15% of patient populations, it is well-established to increase shelf-life as well as the circulation half-life of

nanoparticles. Since Doxil has been FDA approved and in the clinic for over two decades, we chose to use it in our formulations. The remaining design parameters we incorporate in our targeted nanoparticles - peptide density, peptide EG linker length, and oligolysines - needed further studies to optimize the peptide-targeted liposomes for efficient binding/uptake. Thus, we analyzed the individual effects of various design parameters on TNP^{GRP78pep} using *in vitro* cellular uptake studies.

First, the effects of EG linker length on cellular uptake were evaluated using an *in vitro* assay (Fig. 5A). The results showed a general trend where the cellular uptake was improved by increasing linker length and reached a maximum with an EG18 linker, followed by a decrease with linkers that were longer than this, for all breast cancer cell lines. As we explained in earlier reports, this was likely due to longer linkers, such as EG45, adopting a more globular conformation, hindering peptide accessibility for binding. On the other hand, optimal linkers, such as EG18, present the peptide more effectively and raise it above the PEG coating, making it more readily available for binding to target receptors. Consequently, the results established the EG18 to be the most effective linker length in maximizing cellular uptake by breast cancer cells *in vitro*.

Next, we investigated the effects of oligolysines in the cellular uptake of TNP^{GRP78pep} (Fig. 5B). Optimizing targeting element hydrophilicity is also very important for enhancing cellular uptake, since effective presentation of the peptide also requires it to be better partitioned into the solvent. In this experiment, GRP78pep-lipid conjugates with various number of lysine were prepared, and their effects were evaluated by analyzing the TNP^{GRP78pep} (1% peptide density) cellular uptake by sGRP78+ cell lines. The results demonstrated that with zero lysines (K0), there was no detectable cellular uptake of TNP^{GRP78pep} by all the cell lines. Significantly, the addition of a single lysine (K1) as part of the linker increased cellular uptake of TNP^{GRP78pep} by up to ~50-fold enhancement over a Nanoparticle (NP) control that does not contain the targeting peptide. An additional lysine residue (K2) displayed further significant enhancement of cellular uptake by up to ~400-fold higher levels over a NP control. With three lysines, the cellular uptake of TNP^{GRP78pep} reached its maximum, demonstrating that 3 lysines are the optimal lysine residues that result in dramatically improved cellular uptake of TNP^{GRP78pep}.

Finally, we took a closer look at the effects of peptide density in the cellular uptake of TNP^{GRP78pep} (Fig. 5C). Adjusting peptide density allows for fine tuning of the nanoparticle avidity for the target cells, thereby providing selectivity in targeting and promoting receptor-mediated endocytosis. Here, our data confirmed earlier observations where BT-474 cellular uptake increased as the peptide density increased up to 0.7%, where it stabilized and plateaued, presumably owing to particle saturation on the cell surface (Fig. 5C). Similarly, MDA-MB-231 cellular uptake was enhanced as the peptide density increased up to 1% and stabilized. Meanwhile, MCF-7 cellular uptake continued to increase with increasing peptide density throughout the experiment to a final loading of 4% (Fig. 5C). These results clearly demonstrated that the peptide density needs to be optimized for

each breast cancer cell line due to the varying characteristics of target cells, such as differences in metabolic rates, as well as expression levels of the target receptor on these cells. Furthermore, as described in Fig. 4, when fine tuning particle peptide density, it is imperative to identify the optimal peptide density that will not compromise selectivity in cell targeting, while still enhancing the cellular uptake. Taken together, three lysines, EG18 linkers, and 1% peptide density were determined to be the optimal design parameters for TNP^{GRP78pep}, which significantly enhanced the *in vitro* cellular uptake by sGRP78+ breast cancer cells, and more importantly, preserved the selective cell targeting.

Although *in vitro* optimized design parameters can deliver exceptional *in vitro* experimental results, such parameters do not guarantee similar outcomes *in vivo* due to the complexity of whole organisms, such as unpredictable off-target effects, reticuloendothelial system (RES) clearance, etc. Specifically, peptide density and EG linker length have been identified as the two important parameters that significantly affect experimental outcomes within an *in vivo* environment. For example, as we reported in a recent study of TNP drug delivery in HER2⁺ breast cancer, although an EG18 linker was determined to be the optimal linker for *in vitro* cell uptake, an EG8 linker was more effective to achieve *in vivo* tumor cell uptake and tumor inhibition efficacy. Thus, the EG8 linker for the targeting peptide was used when evaluating *in vivo* efficacy, primarily due to a better synthetic yield and lower cost relative to the EG18 linker, as it still delivered ~100-fold enhancement in cellular uptake with the MCF-7 cell line, which was used for *in vivo* studies. Furthermore, even slightly higher than optimal peptide loading proved to be an ineffective strategy in TNP design, because of significant reduction in solid tumor accumulation of the nanoparticle as a result of off-target binding and binding site barrier. These effects typically result in reduced *in vivo* tumor cell uptake and cause significant reduction in the efficacy of treatment. Hence, we identified the optimized design parameters to include EG8 with three lysines as the linker at 1% peptide density for Dox prodrug loaded 100 nm sized TNP^{GRP78pep} for further evaluation for efficacy via *in vivo* animal studies.

To evaluate drug efficacy and potential side-effects of TNP^{GRP78pep}, the first step is to assess its biodistribution and accumulation at different organs to predict its potential off-target effects. Therefore, we first analyzed *in vivo* nanoparticle tissue distribution by assessing the distribution of the aggressive sGRP78+ breast cancer cells by mimicking conditions that lead to breast cancer lung metastasis in a mouse model. Briefly, sGRP78+ MCF-7 cells were sorted and were injected intracardially into Balb/c Rag1^{-/-} mice to allow seeding onto lungs. 4 days after cell injection, the mice were intravenously injected with two different nanoparticle formulations (n=4 per group): non-targeted NP and TNP^{GRP78pep} with 1% peptide density. All nanoparticles were loaded with DiD fluorescent dye for tracking and quantifying purposes. Finally, 3 days after nanoparticle injection (i.e. 7 days after cancer cells were injected), the mice were sacrificed, and organ tissues were harvested for a closer biodistribution analysis.

The results demonstrated that both nanoparticle formulations were mostly accumulated at the spleen, indicating clearance of the nanoparticles by spleen, regardless of the presence of the targeting GRP78pep on the nanoparticles (Fig. 6). Nevertheless, there was a slight difference between NP and TNP^{GRP78pep} accumulation at kidneys, which potentially may be attributed to modest increase in excretion of TNP^{GRP78pep} over NP. More importantly, NP and TNP^{GRP78pep} did not show a significant difference in accumulation at other organs. These results suggest that loading 1% peptide density on TNP^{GRP78pep} does not result in potential unintended off-target systemic toxicity, in agreement with the fact that somatic cells do not express GRP78 at their cell surface *in vivo*.

Next, to assess the efficacy of TNP^{GRP78pep} to inhibit metastatic seeding of sGRP78+ breast cancer cells, we performed an *in vivo* efficacy study using the animal model described earlier (Fig. 7). We had previously shown that sGRP78+ breast cancer cells aggressively seed to the lungs, in contrast to sGRP78- cells²². In agreement with our previous findings, our results here confirmed that any considerable seeding of cells to the lungs was not observed in the mice groups injected with sGRP78- MCF-7 cells, and significant metastatic seeding only took place with the mice groups injected with sGRP78+ MCF-7 cells (Fig. 7). Treatments of the sGRP78+ injected mice included one of the 5 nanoparticle formulations: (1) NP; (2) 1% peptide loaded TNP^{GRP78pep} (1% TNP^{GRP78pep}); (3) Dox prodrug loaded NP (NP_{Dox}); (4) Dox prodrug and 1% peptide loaded TNP^{GRP78pep} (1% TNP^{GRP78pep} [Dox]); and (5) Dox prodrug and 1.5% peptide loaded TNP^{GRP78pep} (1.5% TNP^{GRP78pep} [Dox]). Each drug loaded formulation (NP_{Dox}, 1% TNP^{GRP78pep} [Dox], and 1.5% TNP^{GRP78pep} [Dox]) had 1 mole percent of Dox prodrug (Dox-lipid) in the particles. The nanoparticle formulations were injected 4 days past the intracardiac injection of the MCF-7 cancer cells. At 3 days post nanoparticle-treatment, the mice were sacrificed, and lungs were collected to be analyzed for *in vivo* efficacy.

The results showed a remarkable difference in the ability of TNP^{GRP78pep} to specifically inhibit sGRP78+ MCF-7 injected animals. Treating the sGRP78- group with TNP^{GRP78pep} had no noticeable effect on metastatic seeding at the lung compared to the sGRP78-group that was untreated, demonstrating that 1% TNP^{GRP78pep} nanoparticles had no positive or negative impact on the metastatic propensity of sGRP78- MCF-7 cells. Furthermore, in comparison to the untreated sGRP78+ control group, other control groups treated with nanoparticles without the pro-drug (NP and 1% TNP^{GRP78pep}) also had similar numbers of seeding at the lungs, indicating that the particles alone did not inhibit the sGRP78+ MCF-7 cell seeding. Most notably, NP_{Dox} treatment resulted in almost identical numbers of sGRP78+ MCF-7 cells seeded at the lungs as the control nanoparticle formulation groups without the drug loading. This validates that non-targeted NP_{Dox} was ineffective in delivering drug to the GRP78+ MCF-7 cells in the absence of the targeting GRP78pep peptide. Most importantly, complete inhibition of sGRP78+ cell seeding in the lungs relative to negative control levels was accomplished, by both 1% TNP^{GRP78pep} [Dox] and 1.5% TNP^{GRP78pep} [Dox] formulations. Combined, these striking results

clearly demonstrated that Dox prodrug loaded TNP^{GRP78pep} effectively inhibited sGRP78+ lung metastatic seeding via rationally engineered peptide-targeted nanoparticle formulation.

In this study, we demonstrated how liposomal nanoparticles can be used beyond their typical purpose to treat solid tumors, to also be used to target and eliminate cancer cells that are temporarily in the systemic circulation and have recently seeded to an organ to initiate metastasis. We accomplished this by rationally engineering the nanoparticle system through optimization of the critical design parameters, such as particle size, linker length, peptide hydrophilicity, and peptide density, which in combination impact the overall valency and avidity of the targeted particles. Importantly, the drug-loaded TNP^{GRP78pep} were effective towards multiple types of breast cancer lines irrespective of their hormone receptor status, including triple negative breast cancer. Ultimately, we optimized the sGRP78 targeted Dox prodrug loaded TNP^{GRP78pep} formulation so that it exhibited distinct advantages over nontargeted Dox prodrug loaded NP in achieving efficacy, which resulted in powerful inhibition of seeding mimicking lung metastatic events *in vivo*.

The study also demonstrated an unconventional utility for nanoparticles. Nanomedicine has been traditionally studied in targeting solid tumors due to its reported accumulation at tumor sites via passive targeting, known as the enhanced permeability and retention (EPR) effect. Nevertheless, its potential in targeting recently reseeded circulating cancer cells that have critical roles in metastasis has not been strategically evaluated. As demonstrated in this study, the multivalent platform that liposomal nanoparticles provide can be rationally engineered to have selective avidity for aggressive (and in our case stem-like) cancer cells, and extended dissociation time for the effective delivery of the drug payload. Nanoparticle design considerations for targeting cancer cells recently adhered to an organ, or still in circulation, are fundamentally different from those that aim to treat solid tumors since angiogenesis isn't yet underway. Properties relating to solid tumors - including changes in vascularization, microenvironment and osmotic pressure at tumor tissue - impact factors such as the EPR effect and binding-site barrier become irrelevant when targeting aggressive/metastatic cancer cells that are in circulation or at this early stage of adhesion to a tissue. In contrast, nanoparticles can be designed to take advantage of increased *in vivo* circulation half-life of which larger sized nanoparticles are capable. This improves the probability for a particle to encounter a cancer cell before it gets cleared, which increases the overall efficiency for delivering the drug load. Furthermore, without concern for binding site barrier, nanoparticles can be designed with increased valency (achieved by both increased peptide loading and particle size) to improve efficacy.

One final consideration relates to the timing of the particle injections and how it can be planned to improve efficacy even further. During our *in vivo* studies, the nanoparticle treatment was administered 4 days after the intracardiac injection of the cancer cells. Despite the reported half-life of circulation for average CTCs to be around only a few hours, this scheduling with extended incubation was designed to increase the probability of metastasis in all groups to provide a clear

comparison between treated and control groups. Nevertheless, in a clinical setting, the timing of the treatment should be planned to improve the efficacy even further. Currently, surgery is the primary approach for most breast cancer treatments. However, it is not a standard approach for metastatic breast cancer due to the difficulty in effectively eradicating tumor cells that have developed across multiple organs/sites, including those where surgical intervention may not be possible. Furthermore, even when a surgical approach is feasible, additional tumor relapses may still occur, as additional CTCs/dislodged cancer cells can leak into the systemic circulation and eventually seed at healthy organs to initiate new tumors. Conventional approaches address these concerns using chemotherapy and/or radiation following surgical intervention with the goal of inhibiting metastasis and preventing relapse. We believe that the results described here clearly demonstrate the potential of utilizing TNP^{GRP78_{pep}} to replace conventional post-surgical treatments, and/or to target early metastatic seeded cells, to selectively deliver toxic chemotherapeutic drug payloads *in vivo* with high accuracy and efficiency for optimal therapeutic efficacy. These results provide a potential positive translational impact by providing a selective, and thus less toxic approach, to target the specific breast cancer cells that lead to poor patient outcome, that is applicable to a variety of breast cancers.

Here, we described rationally engineered TNP^{GRP78_{pep}} that effectively inhibited lung metastasis *in vivo*. The results established that the optimized Dox prodrug-loaded TNP^{GRP78_{pep}} formulation outperformed NP_{Dox} significantly by eradicating seeded tumor cells in the lungs. The exact formulation for the best performing targeted nanoparticles was identified through optimization of the individual nanoparticle design parameters. Specifically, these parameters include peptide density, linker length, and peptide hydrophilicity, which, combined, maximized the nanoparticle cellular uptake while maintaining selectivity for the target cancer cells. Furthermore, this study confirmed cell surface expressed GRP78 as a promising target receptor to differentiate aggressive subpopulation of stem-like breast cancer cells that exhibit tumor metastasis *in vivo*. To further improve the outcome of TNP^{GRP78_{pep}} treatment, the injection schedule, dosage, and number of administrations can be optimized to increase its therapeutic potential as a metastatic cancer treatment in the clinic.

The following Examples are intended to illustrate the above invention and should not be construed as to narrow its scope. One skilled in the art will readily recognize that the Examples suggest many other ways in which the invention could be practiced. It should be understood that numerous variations and modifications may be made while remaining within the scope of the invention.

EXAMPLES

Example 1. Material and methods.

Materials were obtained from NovaPEG Rink Amide Low Loading Resin, all Fmoc-protected amino acids, and 2-(1H-benzotriazol-1-yl)-1,1,3,3 tetramethyluronium-hexafluorophosphate (HBTU) from EMD Millipore. We purchased N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA),

triisopropylsilane (TIS), dimethylformamide (DMF), dichloromethane (DCM), 2-propanol (IPA), acetonitrile (AcN), ethanol, Kaiser test reagents, cholesterol, *N,N'*-diisopropylcarbodiimide (DIC), hydrazine, chloroform, doxorubicin hydrochloride, and DNase I from Sigma-Aldrich. 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine-*N*-(glutaryl) (DPPE-GA), 1,2-distearoyl-sn-glycero-3-phosphocholine (sodium salt) (DSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (mPEG2000-DSPE) were purchased from Avanti Polar Lipids. 3*H*-Indolium, 2-(5-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2*H*-indol-2-ylidene)-1,3-pentadienyl)-3,3-dimethyl-1-octadecyl-, perchlorate (DiD fluorescent dye) was obtained from Invitrogen. All Fmoc protected ethylene glycol (EG) linkers (Fmoc-EG_n-OH; where n is the repeating unit of ethylene glycol) were purchased from Quanta Biodesign. Fluorescein 5-isothiocyanate (FITC) was purchased from Toronto Research Chemicals (Toronto, Canada).

Synthesis and Characterization of GRP78pep and GRP78pep-Lipid Conjugates. A GRP78 binding peptide with FITC conjugate (GRP78pep-FITC) and GRP78pep-lipid (GRP78pep-K_m-EG_n-lipid: where m is the number of lysines and n is the repeating units of ethylene glycol, respectively) conjugates were synthesized by solid phase peptide synthesis method using Fmoc chemistry as described in Stefanick *et al.*, *Nanoscale* 2019, 11 (10), 4414-4427. The peptide sequence is NH₂-SNTRVAP-COOH (SEQ ID NO: 1). The GRP78pep-FITC conjugate and the GRP78pep-lipid conjugates were purified using Agilent 1200 Reverse Phase High Performance Liquid Chromatography (RP-HPLC). AcN/H₂O mixture was used for purification of the GRP78pep-FITC with a Zorbax C18 semi-preparative column at a flow rate of 4 mL/min. Meanwhile, IPA/AcN/H₂O mixture was employed for purification of GRP78pep-lipids by using a Zorbax C3 semi-preparative column at a flow rate of 3 mL/min. The products were characterized by microTof-Q II and their purities were determined to be >98% using analytical RP-HPLC.

Synthesis and Characterization of Dox-lipid conjugate. Doxorubicin-lipid (Dox-lipid) conjugate was synthesized as described in Stefanick *et al.*, *Nanoscale* 2019, 11 (10), 4414-4427. Briefly, Dox was conjugated to a polar head group of DPPE-GA via acid-labile hydrazone bond. The Dox-lipid was purified by chloroform extraction and characterized by microTof-Q II. Purity was determined to >98% using RP-HPLC analytical injections using a Zorbax C3 semi-preparative column.

2,2'-(Hexadecylazanediy)ldiacetic acid and BTZ were reacted at a molar ratio of 1:1 in toluene at 125 °C for 2 h under reflux. After removing the reaction solvent, the prodrug molecules were purified via RP-HPLC using C3 semiprep column. The products were characterized by MALDI ultraflex, and their purities (>95%) were determined by the RP-HPLC analytical injections using a Zorbax C3 semi-preparative column.

The DM1-prodrug was synthesized as described in Khan *et al.*, *Biomaterials* 292, 121913 (2023). Mertansine (MedChemExpress, NJ, USA) was reacted overnight with a DSPE-maleimide lipid

tail (NanoSoft Polymers, NC, USA) at room temperature in DMF. The reaction was stopped by removal of DMF (via rotary evaporation), and the prodrug was purified via RP-HPLC and characterized via mass spectrometry (Bruker microTof-Q II).

Preparation of Liposomal Nanoparticles. TNP^{GRP78pep} was prepared using a thin film method as described in Stefanick *et al.*, *Nanoscale* 2019, 11 (10), 4414-4427. TNP^{GRP78pep} was prepared at the following molar ratios: (95-x-y)%/5%/x%/y% of DSPC/mPEG2000-DSPE/GRP78pep-K_m-EG_n-lipid/DiD dye, where x is mol % of GRP78pep-K_m-EG_n-lipid (0≤x≤4) and y is mol % of DiD dye (0.1≤y≤0.75), respectively. The lipid film was then hydrated and extruded through a polycarbonate membrane to generate unilamellar liposomes. DSPC plays a role in bulk lipid of liposome. While DSPE lipid is embedded into lipid bilayer, mPEG2000 (~EG45) forms PEG cloud on the liposomal surface to prevent opsonization and reticuloendothelial system (RES) clearance. 0.1% and 0.75% DiD dye were incorporated into the TNP^{GRP78pep} and non-targeted nanoparticle (NP; 0% peptide loading) for *in vitro* cellular uptake studies and *in vivo* nanoparticle biodistribution studies, respectively. For *in vivo* tumor cell growth inhibition efficacy study, 1% Dox-lipid was loaded to all nanoparticles instead of the DiD dye, and Dox prodrug loaded non-targeted nanoparticles (NP_{Dox}; 0% peptide-density) was also prepared as a control.

Other nanoparticles including a mixture of DSPC / mPEG2000-DSPE / GRP78pep-lipid / DiD / cholesterol was prepared and dried with nitrogen gas to make a thin lipid film. After overnight incubation in a desiccator, the film was rehydrated with PBS (pH 7.4) at 70°C for 10 min and extruded through a 0.05 μm polycarbonate filter membrane with the Avanti Polar Lipid extruder set to size the nanoparticles (Stefanick *et al.*, *ACS Nano* 7, 8115–8127 (2013); Stefanick *et al.*, *ACS Nano* 7, 2935–2947 (2013)). The nanoparticle formulation in molar ratio is as follows: 1) (94.9 - x)% / 5% / x% / 0.1% of DSPC / mPEG2000-DSPE / GRP78pep-lipid conjugates / DiD, where x was varied from 0% to 1.5%, for *in vitro* cellular uptake and competitive binding assays, 2) (94.25-x)% / 5% / x% / 0.75% of DSPC / mPEG2000-DSPE / GRP78pep-lipid conjugates / DiR, where x varied 0 - 1.5%, for *in vivo* biodistribution assays, or 3) (92-x)% / 5% / x% / 2% of DSPC / mPEG2000-DSPE / GRP78pep-lipid conjugates / DM1-prodrug, where x varied 0 - 1.5%, for *in vivo* efficacy assays. Cholesterol was included as an additional 5 mol% of the total phospholipid concentration in nanoparticles throughout the entire study.

Other liposomal nanoparticles were prepared by dry film hydration and extrusion. Briefly, lipids were mixed in chloroform, at specific stoichiometry by applying the formula (95-x):5:5:5:x which indicated ratios of DSPC:mPEG-DSPE:Cholesterol:DM1-Prodrug:GRP78pep-lipid, where x denotes the molar ratio of GRP78pep-lipid conjugate present on the surface of the nanoparticle. Later, lipid mixtures were dried to form a thin film using nitrogen gas, and then placed under vacuum overnight to remove residual solvent. The lipid films were hydrated with PBS (pH 7.4) at 65 °C for 7 min by gentle agitation and extruded at 65 °C through a polycarbonate membrane using Avanti Polar

Lipid extruder set. For preparing BTZ-Prodrug loaded nanoparticles, the BTZ-Prodrug was post-inserted into the particles at the molar ratios of 95:5:1.5:x DSPC:mPEG-DSPE:BTZ prodrug:GRP78pep-lipid after extrusion.

Particle Sizing. Nanoparticle size measurements were performed using dynamic light scattering (DLS), NanoBrook Omni Particle Size Analyzer (Brookhaven Instruments Corp.) as described in Stefanick *et al.*, *Nanoscale* 2019, 11 (10), 4414-4427. All nanoparticles prepared were characterized by DLS, showing a narrow polydispersity with ~100 nm size (Fig. 2).

Peptide and Drug Loading Efficiency. Dox prodrug loaded TNP^{GRP78pep} was prepared and purified using liposome extruder purification (LEP) method as described in Panopoulos *et al.*, *PLoS One* 2011, 6 (5), e19743. To determine peptide and drug loading efficiency, actual GRP78pep and Dox concentrations in the nanoparticle were measured using RP-HPLC at 220 nm and 485 nm, respectively. This was followed by comparison with their respect intended sGRP78pep and Dox loading concentrations measured by RP-HPLC at the same wavelengths.

Cell Culture. Induced pluripotent stem cells (iPSCs) were derived from human umbilical vein endothelial cells. BT-474, MDA-MB-231, MCF-7, Raji, and NCI-H929 cells were purchased from ATCC (Rockville, MD). iPSCs were cultured in mTeSR-1 (Stem Cell Technologies) on Matrigel (Corning) as previously as described in Panopoulos *et al.*, *PLoS One* 2011, 6 (5), e19743. BT-474, MDA-MB-231, and MCF-7 cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% Benchmark fetal bovine serum (Gemini), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). NCI-H929 cells were cultured in RPMI 1640 media supplemented with 20% fetal bovine serum, 2 mM L-glutamine (Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin, and 55 µM 2-mercaptoethanol. Raji cells were cultured in RPMI 1640 media (Gibco) supplemented with 10% Hyclone fetal bovine serum (Gemini), 2 mM L-glutamine (Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco).

In Vitro Peptide Cellular Binding Assay. *In vitro* peptide cellular binding assay was performed as described in Stefanick *et al.*, *ACS Nano* 2013, 7 (9), 8115-8127. Briefly, 1×10^5 cells were incubated in a 24-well plate overnight. After 16 hours, the cells were pre-treated with 1.5% BSA in PBS on ice for 30 min, followed by administration of GRP78pep-FITC conjugate for 1 hour on ice. The cell fluorescence was detected by Guava easyCyte 8HT flow cytometer.

In Vitro Nanoparticle Cellular Uptake Assay. *In vitro* nanoparticle cellular uptake assay was conducted as previously described²⁹. Briefly, 1×10^5 cells were incubated in a 24-well plate overnight. The cells were then treated with nanoparticles (22.5 µM total phospholipid concentration) for 3 hours, followed by trypsinization for removal of cell surface associated nanoparticles. For nanoparticle uptake inhibition, soluble GRP78pep (50 µM) was introduced to the cells 30 min prior to nanoparticle administrations. The cells were wash with PBS buffer (pH 7.4) twice and the cell fluorescence was

detected by Guava easyCyte 8HT flow cytometer. 0.1% DiD dye was incorporated into the nanoparticles for fluorescence quantification.

Animal Model. All animal experiments were performed with protocols approved by the University of Notre Dame Institutional Animal Care and Use Committee guidelines. Balb/c Rag1^{-/-} mice (C.129S7(B6)-Rag1^{tm1Mom}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice used in this study were maintained under pathogen-free conditions in the University of Notre Dame Freimann Life Sciences animal facility.

Fluorescence-Activated Cell Sorting (FACS). sGRP78⁺ and sGRP78⁻ MCF-7 cells were sorted as previously described²². Briefly, MCF-7 cells (1 x 10⁶ cells per sample) were incubated with rabbit anti-human GRP78 ET-21 antibody (5 µg/sample; Sigma) for 1 hour at 4°C. The cells were then stained with anti-rabbit 488 secondary antibody (Invitrogen) for an additional 1 hour at 4°C. Cells stained with both an IgG isotype antibody (Sigma) and the secondary antibody or single-stained controls (APC/FITC) were used as controls. Both sGRP78⁺ and sGRP78⁻ MCF-7 cells were sorted using BD FACSAria Cell Sorter, and all cells were then labeled with a DiO dye (3,3'-Diocadecyloxacarbocyanine Perchlorate; Invitrogen) according to the manufacturer's instruction.

In Vivo Breast Cancer Lung Metastasis Animal Experiment. A breast cancer lung metastasis animal model was employed for *in vivo* studies as described in Conner *et al.*, *Sci Rep* 2020, 10 (1), 3474. sGRP78⁺ MCF-7 cells (2 x 10⁴ cells per mouse) were injected into the left cardiac ventricle of Balb/c Rag1^{-/-} mice. Mice injected with sGRP78⁻ MCF-7 cells were used as a negative control. After 4 days of tumor cell seeding, the mice were intravenously treated with either DiD dye-labeled non-targeted nanoparticles (NP) or TNP^{GRP78^{pep}} for nanoparticle tissue biodistribution studies. For tumor cell growth inhibition efficacy study, mice were intravenously injected with treatments of either Dox prodrug loaded NP or TNP^{GRP78^{pep}}. Mice treated with either the DiD loaded NP or TNP^{GRP78^{pep}} were used as controls to compare the cytotoxic effect of drug payload with the dye molecule. After 3 days of treatments, all mice were sacrificed by cardiac perfusion while anesthetized using isoflurane to get rid of remaining nanoparticles in systemic circulation. Major organs were dissected and collected to read fluorescent nanoparticles at each organ using IVIS Lumina II imager (PerkinElmer) at an emission wavelength of 640 nm. The images were analyzed for nanoparticle tissue biodistribution using Image J. For metastatic tumor cell growth inhibition efficacy study, lungs were fixed in 4% paraformaldehyde overnight, and DiO labeled cells were counted manually using a fluorescent microscope.

In vivo biodistribution study. Following a previously described MOC mouse model, ID8 p53^{-/-} red fluorescent protein (RFP)-labeled cells (1x10⁶ cells per mouse) were injected via i.p. into mice on day (Raiter *et al.*, *Oncotarget* 2014, 5 (22), 11452-11463). Each following week, fur was removed from the mouse abdomen prior to fluorescent imaging of RFP due to cancer progression (IVIS Lumina II, Perkin Elmer). To observe tumor burden progression, images of tumor bearing mice were compared

to imaging control mice (no cells injected), after standardizing the images in ImageJ (NIH) via spectral unmixing (Mandelin *et al. Proc Natl Acad Sci USA* 2015, 112 (12), 3776-3781). Mouse health was also checked weekly by weighing mice and checking for ascites accumulation in the peritoneal cavity.

When significant tumor burden existed (6 weeks), the mice were injected via i.p. with nanoparticles (1.25 mM lipid per g mouse weight) containing 0.75% DiR. 24 hours post-injection of particles, mice were sacrificed, peritoneal lavage was collected, and the mice were dissected. Biodistribution of particles was measured using fluorescent imaging. Colocalization of cancer (i.e. RFP) and nanoparticles (i.e. DiR) were evaluated using ImageJ (NIH). Organs were collected from each mouse and imaged both with tumors *in situ* and tumors removed from the organs, then weighed. Separated tumor from each mouse was cut and disaggregated (rocked for 45 min at 37 °C in 1 mg/mL Collagenase Type IV and 0.03 mg/mL DNase in PBS pH 7.4), then washed with PBS. Peritoneal lavage samples were subject to red blood cell lysis (ACK lysis buffer) before the cells were washed, counted, and resuspended in PBS at 1.5×10^6 cells/mL. A Guava easyCyte 8HT flow cytometer was used to identify the RFP and DiR fluorescence of the disaggregated tumor cells and peritoneal lavage cells. Flow cytometry results were then analyzed using FlowJo (BD Life Sciences, Ashland, OR) to determine the colocalization of nanoparticles to RFP+ cells.

In vivo efficacy study. On day 0, mice were injected (i.p.) with ID8 p53^{-/-} RFP-labeled cells (1×10^6 cells per mouse). Each following week, fur was removed from the mouse abdomen prior to fluorescent imaging of RFP due to cancer progression (IVIS Lumina II, Perkin Elmer). To observe tumor burden progression, images of tumor bearing mice were compared to imaging control mice (no cells injected), after standardizing the images in ImageJ (NIH) via spectral unmixing. Mouse health was also checked weekly by weighing mice and checking for ascites accumulation in the peritoneal cavity.

After 2.5 weeks, mice began treatment with PBS (vehicle) or nanoparticles loaded with 3 mg/kg of DM1-prodrug. The nanoparticles were loaded with varying amounts of targeting element presented (0% [NP], 0.75%, 1.5%), and a combination treatment was prepared containing an equal parts NP and 1.5% TNP^{GRP78^{pep}} post extrusion (NP&1.5%TNP^{GRP78^{pep}}). Treatments continued two times per week for 3.5 weeks, for a total of 8 treatments. After the end of treatments, mice were monitored for an additional 2.5 weeks. Then, the mice were sacrificed, peritoneal lavage was collected, and the mice were dissected and imaged.

Peritoneal lavage samples were collected from each mouse immediately after sacrifice. To collect the samples, 7 mL of PBS was injected i.p. after sacrifice and recovered from the peritoneal cavity via a needle and syringe. The samples were kept on ice throughout processing. Samples were spun to retain the cell pellet, then ACK lysis buffer was added to lyse red blood cells. The lysis buffer was neutralized with PBS before the cells were spun and resuspended in FACS buffer (10% FBS in PBS). The cells were then counted and washed with PBS before resuspension at 1×10^7 cells/mL. The

cells from the peritoneal lavage were then stained with live/dead yellow dye (ThermoFisher), then blocked with a blocking antibody (BioLegend) in a 96-well V-bottom plate. Then antibody cocktails were prepared and added to the cells for either a general immune cell antibody panel or a macrophage antibody panel. The immune cell antibody panel consisted of antibodies specific to the following:

5 CD3, CD4, CD8, CD19, CD25, NK 1.1 (CD56), TCR $\gamma\delta$, CD127, CD117, CD16 (Fc γ RIII), Ly6C, Ly6G, CD11b, and CD11c. The macrophage antibody panel consisted of antibodies specific to: CD45, F4/80, CD86, and CD206. The cells were washed and resuspended in FACS buffer (10% FBS in PBS) before measurement of binding via a Northern Lights Cytex flow cytometer. Flow cytometry was analyzed using FlowJo (BD Life Sciences, Ashland, OR) to determine the quantity of the above

10 immune cells in peritoneal lavage samples.

Synergy calculation. The below equations were used to determine if the NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment provided synergistic efficacy, relative to its components, based on accepted synergy calculation methods. To prove synergy, R must be > 1, where:

$$R = Y_{\text{obs}}/Y_{\text{exp}}$$

15 with Y_{obs} as the observed decrease in tumor burden and Y_{exp} as the expected decrease in tumor burden if NP[DM1] and 1.5% TNP^{GRP78pep}[DM1] treatments occurred sequentially.

$$Y_{\text{obs}} = 1 - (M_{\text{combination}}/M_{\text{PBS}})$$

$$Y_{\text{exp}} = (1 - (M_{\text{NP}}/M_{\text{PBS}})) + (M_{\text{NP}}/M_{\text{PBS}})(1 - (M_{1.5\% \text{TNP}}/M_{\text{PBS}}))$$

All M_n values are based on the mean tumor burden of the treatment groups (e.g., M_{PBS} is the mean tumor burden of the PBS vehicle control; $M_{\text{combination}}$ is the mean tumor burden of the combination NP[DM1]+TNP^{GRP78pep}[DM1] treatment group; etc.).

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Synthesis and characterization of fluorescein-GRP78pep and GRP78-lipid conjugates. Fluorescein-labeled GRP78pep and GRP78-lipid conjugates were synthesized using solid-phase peptide synthesis via Fmoc chemistry on Rink Amide resin, as previously described (Omstead *et al.*, *J Hematol Oncol* 2020, 13 (1), 145; Kim *et al.*, *Nanoscale* 2020, 12 (21), 11672-11683; Murthy *et al.*, *Int J Nanomedicine* 2007, 2 (2), 129-141). Residues were activated and conjugated with HBTU/DIEA in DMF and the Kaiser test was performed to monitor the coupling. Fmoc was deprotected with 20% piperidine in DMF. After completing the synthesis, peptides were cleaved with a solution of 95 / 2.5 / 2.5 TFA / H₂O / TIS twice for 45 min each time. Then, the molecules were purified by reverse phase

25 high performance liquid chromatography (RP-HPLC) on an Agilent (Santa Clara, CA) 1200 series system using either a semi-preparative Zorbax C18 or C3 column with ACN/H₂O or IPA/ACN/H₂O, respectively, in the mobile phase. After purification, the molecules were characterized using a Bruker microTof-Q II, and their purities were evaluated by RP-HPLC analytical injections.

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Example 2. Combination non-targeted and sGRP78-targeted lipid nanoparticle drug delivery synergistically outperforms either component in treatment of metastatic ovarian cancer

To engineer GRP78pep-targeted nanoparticles (TNP^{GRP78pep}), first we synthesized the GRP78-targeting peptide (GRP78pep) sequence SNTRVAP (SEQ ID NO: 1), as a peptide-lipid conjugate to be incorporated into our targeted nanoparticles. The peptide-lipid conjugate was composed of five functional moieties: 1) GRP78 binding peptide, 2) EG₂ linker, 3) oligolysines (K_m, where 'm' indicates the number of lysines), 4) EG_n linker, where n is the repeating unit of ethylene glycol, and 5) two palmitic acid lipid tails (Fig. 8A). The EG₂ functions as a spacer to separate the GRP78pep sequence from the lysines, the oligolysines (K_m) improve hydrophilicity of the targeting peptide, the EG_n linker provides efficient reach and flexibility of the lipid conjugated peptides to improve receptor-peptide interactions, and the two palmitic acids enable anchoring of lipid conjugates into lipid bilayer of nanoparticles. The DM1-prodrug was synthesized by conjugating DM1 to a phospholipid for incorporation into the liposomal nanoparticles (Fig. 8B). Nanoparticles were synthesized from the pure components using precise stoichiometric ratios to ensure consistent targeting peptide density and DM1-prodrug (or DiD lipophilic dye) loading across batches (Fig. 8C). This nanoparticle synthetic approach produces a homogeneous population of nanoparticles that preserve precise control over targeting elements at specific molar ratios, thereby resulting in consistent and reproducible experimental results. The lipid components of the nanoparticles were extruded through 50 nm membranes to create unilamellar liposomes. Nanoparticle sizing was confirmed using dynamic light scattering (DLS) (Fig. 8D). The loading efficiency of the GRP78pep-lipid conjugates and DM1-prodrug in the nanoparticles was evaluated with RP-HPLC analysis; their concentrations in the nanoparticles were measured and compared to the theoretical concentrations. The results showed 87±4% GRP78pep and 89±4% prodrug loading efficiency in the nanoparticles, confirming the precise control over the peptide and drug stoichiometry. Non-targeted (NP) nanoparticles were also prepared and used as controls.

Evaluation of GRP78pep binding on GRP78+ ovarian cancer cells. To evaluate the binding of GRP78pep on ovarian cancer cell lines with expression of cell surface GRP78 (GRP78+), *in vitro* cellular binding assays were performed with human (OVCAR5 and OVCAR8) and murine (ID8, ID8 p53 -/-, and ID8 BRCA p53 -/-) ovarian cancer cell lines. Raji (Burkitt lymphoma; GRP78^{low}) was used as a negative control cell line, due to reported low-to-no surface expression of GRP78. All cell lines were treated, on ice, with increasing concentrations of fluorescein-conjugated GRP78pep for 1 hr, and fluorescence was quantified by flow cytometry to analyze the binding of GRP78pep on each cell line. The results showed that GRP78pep effectively bound to both GRP78+ human cells (Fig. 9A *left*) and GRP78+ murine cells (Fig. 9A *right*) with a K_d of approximately 5 μ M. GRP78pep demonstrated minimal binding to Raji negative control cells.

Nanoparticle optimization for selective uptake of TNP^{GRP78pep} on GRP78+ ovarian cancer cells. After establishing that GRP78pep binds efficiently to GRP78+ ovarian cancer cells, we next evaluated uptake of TNP^{GRP78pep} by human and murine ovarian cancer cell lines. We predicted that cellular uptake of targeted nanoparticles would depend on optimal nanoparticle formulation, including the EG_n linker length, oligolysines (K_m), and GRP78pep density (Fig. 8A *left*). To identify the optimal EG_n linker length, TNP^{GRP78pep} were prepared with 1% loading of GRP78pep-lipid with EG linkers varying between EG₀ to EG₄₅ (Fig. 9B). NP and PBS were used as controls. Cells were incubated with nanoparticles at 37°C for 3 h, and cellular uptake was analyzed by flow cytometry. Both human and murine ovarian cancer cells showed maximal uptake with the mid-range linker length, typically EG₈-EG₃₀. With shorter linker lengths, the peptide is expected to be less accessible for binding, as it is buried in the PEG coating. With longer linkers, such as EG₄₅, the linker is known to fold up on itself, resulting in less consistently presented targeting peptides.

To determine the optimal number of oligolysines (K_m) for uptake of the TNP^{GRP78pep}, the GRP78pep-conjugate was synthesized with an EG₈ linker and varying numbers of lysines (0 to 3). TNP^{GRP78pep} were prepared with 1% density of GRP78pep-lipid, and cellular uptake studies were performed (Fig. 9C). NP and PBS were used as controls. Our results demonstrated that three lysines (K_m = 3) delivered optimal nanoparticle uptake. Accordingly, EG₈ linker and 3 lysines were used in the nanoparticle formulations for subsequent experiments.

To evaluate the effect of peptide density on selective uptake of TNP^{GRP78pep} by ovarian cancer cells, TNP^{GRP78pep} were prepared at various peptide densities (0.25% to 1%), with an EG₈ linker and 3 lysines. NP and PBS were used as controls. Raji (Burkitt lymphoma; GRP78^{low}) was also used as a negative control cell line. Both human and murine GRP78+ cells showed increased cellular uptake of the TNP^{GRP78pep} with increasing peptide density, while NP showed negligible uptake by the csGRP78+ cells (Fig. 9D). At 1% GRP78pep density, the cellular uptake of TNP^{GRP78pep} was enhanced 56- and 51-fold over NP and PBS controls in OVCAR5 and OVCAR8, and 23-, 18-, and 37-fold enhancement over controls in ID8, ID8 p53 -/-, and ID8 BRCA, p53 -/-, respectively. Importantly, the uptake by the negative control cell line, Raji, was negligible, demonstrating the maintained selectivity of the TNP^{GRP78pep}.

Next, we assessed the binding specificity of the optimized TNP^{GRP78pep} on GRP78+ ovarian cancer cells, by performing an *in vitro* competitive binding assay with excess soluble GRP78pep. NP and PBS were used as controls. Human and murine ovarian cancer cells were incubated on ice with excess concentration of soluble GRP78pep (100 μM) prior to nanoparticle treatments. Cellular binding was analyzed by flow cytometry (Fig. 9E), and the percent inhibition of binding was calculated. Our results demonstrated that cellular binding of TNP^{GRP78pep} was dramatically inhibited (~90%) by soluble GRP78pep on GRP78+ cells, even with TNP^{GRP78pep} presenting the highest peptide density (1.5%

TNP^{GRP78pep}) (Fig. 14). Combined, these results confirmed that TNP^{GRP78pep} specifically and selectively targeted GRP78+ ovarian cancer cells.

DM1 provides similar in vitro cytotoxicity to standard-of-care chemotherapeutics in ovarian cancer cells. The *in vitro* cytotoxicity of DM1 was evaluated compared to the ovarian cancer standard-of-care paclitaxel and second-line chemotherapeutic doxorubicin, in human ovarian cancer lines (OVCAR5 and OVCAR8) and the murine line (ID8 p53-/-). For doxorubicin and DM1, IC₅₀ was not significantly different between cell lines, at ~50 nM for both doxorubicin (Fig. 10A, right) and DM1 (Fig. 10A, left). Paclitaxel demonstrated an IC₅₀ of 10 nM (OVCAR lines) to 500 nM (ID8 p53-/-) (Fig. 10A, center). Overall, these results indicated that DM1 had similar cytotoxicity (i.e., same order of magnitude IC₅₀) to the standard-of-care chemotherapeutics in ovarian cancer cell lines.

DM1-prodrug-loaded nanoparticles are cytotoxic against ovarian cancer cells. DM1-prodrug-loaded nontargeted (NP[DM1]) and targeted (TNP^{GRP78pep}[DM1]) nanoparticles were prepared, and their cytotoxic effects were compared to free DM1, in ID8 p53-/- murine ovarian cancer cells. The ID8 p53-/- cells were incubated with free DM1, NP[DM1], or two formulations of TNP^{GRP78pep}[DM1] – one with 0.75% GRP78pep density (0.75% TNP^{GRP78pep}[DM1]) and one with 1.5% GRP78pep density (1.5% TNP^{GRP78pep}[DM1]) – for 48 h (Fig. 10B, left) or 72 h (Fig. 10B, right). Cell viability was assessed via CCK8 assay. At 48 h, both TNP^{GRP78pep}[DM1] formulations showed IC₅₀ ≅ 60 nM; while the free DM1 and NP[DM1] showed IC₅₀ ≅ 120 nM. By 72 h, the IC₅₀ of all the nanoparticles shifted down to ~40 nM, while the free DM1 IC₅₀ remained approximately the same. Thus, the prodrug-loaded nanoparticles were determined to be at least equally cytotoxic as the free DM1.

To evaluate the cytotoxic effects of the NP[DM1] and TNP^{GRP78pep}[DM1] in more physiologically relevant conditions, pulse cytotoxicity assays were also performed. ID8 p53-/- cells were incubated with free DM1 or nanoparticle formulations for 3 h, then cells were washed and incubated in fresh media for the remainder of the 48 h or 72 h period. At 48 h, the 1.5% TNP^{GRP78pep}[DM1] showed the lowest IC₅₀ ≅ 350 nM; the free DM1 and 0.75% TNP^{GRP78pep}[DM1] showed similar IC₅₀ of around 550 nM and 650 nM, respectively. The NP[DM1] demonstrated the highest IC₅₀ ≅ 2.1 μM, at 48 h (Fig. 10C, left). By 72 h, the free DM1 and 1.5% TNP^{GRP78pep}[DM1] demonstrated similar IC₅₀ ≅ 350 nM; the 0.75% TNP^{GRP78pep}[DM1] remained at ~700 nM; and the NP[DM1] dropped to IC₅₀ ≅ 1.1 μM (Fig. 10C, right). These assays demonstrated the TNP^{GRP78pep}[DM1] to be equivalent in cytotoxicity to the free DM1, while the NP[DM1] had reduced efficacy likely due to slower endocytosis during the 3 hour pulsed dosing.

Next, we evaluated if the DM1-prodrug loaded nanoparticles (NP[DM1] and 1.5% TNP[DM1]) induced cell death in the ovarian cancer cells, via similar mechanism to free DM1. For this we analyzed the cleavage of caspase 3, caspase 8, and caspase 9, indicators of the intrinsic, mitochondrial apoptotic pathway. (Bates & Eastman, 2017; Deng et al., 2022; Gamage et al., 2019)

Western blot analysis of caspase 3, caspase 8, and caspase 9 showed significant cleavage of caspases in cells treated with NP[DM1] and 1.5% TNP[DM1], as achieved with free DM1 (Fig. 10D).

TNP^{GRP78pep} preferentially and efficiently target unadhered metastatic ascites cancer cells while both NP and TNP^{GRP78pep} moderately home to the solid tumors in vivo. MOC treatment can be more effective when injected intraperitoneally (i.p.), however, chemotherapies administered via i.p. tend to result in higher rates of toxicity to the intraperitoneal organs (e.g., gastrointestinal toxicity).^{31,32} Hence, TNPGRP78pep[DM1] formulation could achieve reduced toxicity to the intraperitoneal organs and improved efficacy over free drug via the same method of administration. Here, the *in vivo* biodistribution and uptake study of the NP and TNP^{GRP78pep} were performed using a syngeneic mouse model of metastatic ovarian cancer, where red fluorescent protein (RFP)-tagged ID8 p53^{-/-} ovarian cancer cells were injected intraperitoneally (i.p.) into Black6 mice. Ascites burden, which is an indication of cancer progression, was detected by visually analyzing the abdominal area (including for discoloration and distention of the abdomen). In addition, metastatic solid tumor progression on organs in the peritoneal cavity was monitored via weekly live mouse RFP imaging. When sufficient metastatic tumor burden was observed (44 days after injection of cancer cells) via RFP imaging, DiR-tagged nanoparticles (NP, 0.75% TNP^{GRP78pep}, 1.5% TNP^{GRP78pep}, or NP+1.5%TNP^{GRP78pep} combination treatment) were prepared and injected i.p into mice (Fig. 11A). To determine colocalization and uptake of DiR-tagged nanoparticles by RFP+ metastatic solid tumors and metastatic ascites cells, mice were dissected 24 h post-nanoparticle injection.

To evaluate the colocalization of DiR-labeled nanoparticles by RFP+ metastatic solid tumors, the following organs were collected and imaged for overlapping fluorescence of RFP+ solid tumors and DiR-labeled nanoparticles: liver, stomach, omentum/pancreas, diaphragm, peritoneum (left and right sides), small intestine, spleen, ovaries/uterus, colon/large intestine, mesentery, fat packs, kidneys, heart, and lungs (Fig. 11B). Our results demonstrated colocalization of all types of nanoparticles with RFP+ tumors on organs. To further analyze if TNP^{GRP78pep}, in comparison to NP, preferentially colocalized to solid tumors, the fluorescence of DiR-labeled nanoparticles (NP and TNP^{GRP78pep}) and RFP+ solid tumors on peritoneal organs was quantified via ImageJ (Fig. 11C). RFP fluorescence (shown as x-axis) was plotted against DiR fluorescence (shown as y-axis), and linear fit analysis was applied to the data. A greater positive slope indicates enhanced colocalization. Linear regression analysis of the quantified fluorescence indicated a positive correlation (positive slope) between RFP and DiR for all nanoparticle formulations to differing degrees. The correlation for each nanoparticle formulation was as follows: slope for NP = 1.7; slope for 0.75% TNP^{GRP78pep} = 2.6; slope for 1.5% TNP^{GRP78pep} = 0.59; or slope for combination = 0.88 (Fig. 11C & D). According to this analysis, we observed highest levels of colocalization with 0.75% TNP^{GRP78pep} (slope = 2.6), followed by NP (slope = 1.7). For the NP+1.5%TNP^{GRP78pep} combination treatment (slope = 0.88), the levels of colocalization

fell in between that of NP (slope = 1.7) and 1.5% TNP^{GRP78^{pep}} (slope = 0.59), which was in accordance with our expectations.

After colocalization studies, to analyze DiR-labeled nanoparticle uptake by RFP+ metastatic solid tumors, tumor tissue was dissected from organs and disaggregated, and then uptake of nanoparticles by tumor cells were analyzed via flow cytometry (gated for RFP+ cells) (Fig. 11E). Analysis of DiR-labeled nanoparticle uptake by RFP+ solid tumor cells showed that at 24 hours post-injection of nanoparticles, neither the NP nor TNP^{GRP78^{pep}} were taken up by the solid tumor cells at substantial levels, with similar results across all treatment groups. The colocalization studies presented in Fig. 11B-D, combined with the uptake studies presented in Fig. 11E, revealed that even though the nanoparticles were not taken up at significant levels by the metastatic solid tumor cells, the nanoparticles efficiently accumulated at the tumor site.

As the lack of nanoparticle uptake by the metastatic solid tumors was unexpected, we next studied if the nanoparticles were instead taken up by metastatic ascites cells. For this purpose, peritoneal lavage samples were collected from mice, and uptake of DiR-labeled nanoparticles by RFP+ metastatic ascites cells was analyzed via flow cytometry (gated for RFP+ cells). Analysis of the RFP+ metastatic ascites cells showed significant uptake with differing levels based on the type of nanoparticle treatments (Fig. 11F). Both the 1.5% TNP^{GRP78^{pep}} and the NP+1.5%TNP^{GRP78^{pep}} combination groups demonstrated a 3-fold higher uptake compared to the NP alone, and 2-fold higher relative to 0.75% TNP^{GRP78^{pep}} (Fig. 11F). These results demonstrated the effectiveness of the 1.5% TNP^{GRP78^{pep}} in targeting the unadhered metastatic ascites cells in the peritoneal fluid when delivered alone or in combination with NP.

Overall, the biodistribution and uptake studies performed with metastatic solid tumor cells and metastatic ascites cells indicated that the 1.5% TNP^{GRP78^{pep}} preferentially targeted the metastatic ascites cells, while the NP more effectively reached the solid tumor site. Importantly, the decreased colocalization of 1.5% TNP and combination observed in Fig. 11D could be attributed to the temporal sequence of the 1.5% TNP first reaching the metastatic ascites cells and being efficiently taken up before it has a chance to reach the solid tumor sites.

NP[DM1]+TNP^{GRP78^{pep}}[DM1] combination treatment inhibits tumor growth without systemic toxicity. We next evaluated the *in vivo* efficacy of the DM1-prodrug-loaded nanoparticles (NP[DM1], 0.75% TNP^{GRP78^{pep}}[DM1], 1.5% TNP^{GRP78^{pep}}[DM1], and combination treatment NP[DM1]+1.5%TNP^{GRP78^{pep}}[DM1]) via the previously described metastatic ovarian cancer mouse model.²⁸ Mice were injected i.p. with RFP-tagged ID8 p53-/- ovarian cancer cells, and cells were given 2.5 weeks to seed solid tumor sites. (Asem *et al.*, *Cancer Res* **80**, 1156–1170 (2020); Asem *et al.*, *Sci Rep* **10**, 11913 (2020)). Mice were then treated i.p. with nanoparticle treatment groups on days 16, 20, 23, 27, 30, 34, 37, and 41 with each nanoparticle formulation containing a total of 3 mg/kg DM1-prodrug equivalent or PBS vehicle control (Fig. 12A). Mice were weighed every other day throughout

the study. Limited weight loss in treated groups demonstrated minimal systemic toxicity of the prodrug-loaded nanoparticles (Fig. 12B). It is noteworthy that our results showed lethal toxicity in mice with free DM1 doses over 0.5 mg/kg (Khan *et al.*, *Biomaterials* 292, 121913 (2023)).

To quantify total tumor burden, on day 58, when the study ended, mice were sacrificed. The RFP-tagged cancer cells that formed solid tumors on organs in the peritoneal cavity were collected, and total tumor burden was quantified via ImageJ analysis of RFP fluorescence. Our results showed the NP[DM1]+1.5%TNP^{GRP78^{pep}}[DM1] combination treatment to be most efficacious for reducing tumor burden (Fig. 12C). Importantly, 50% of mice in NP[DM1]+1.5%TNP^{GRP78^{pep}}[DM1] combination group had no detectable tumor burden at the end of study. Further, synergy calculations showed the NP[DM1]+1.5%TNP^{GRP78^{pep}}[DM1] combination group to have synergy ($R > 1$), compared to either of its components alone (NP[DM1] or 1.5% TNP^{GRP78^{pep}}[DM1]). In addition, peritoneal cavity mouse organs were weighed to determine off-target toxicity of NP[DM1] and TNP^{GRP78^{pep}}[DM1]. Individual organ weights were largely consistent between treatment groups (Fig. 12D). None of the treatment groups demonstrated statistically significant drops in organ weight, relative to the controls, implying no organ toxicity by the NP[DM1] or TNP^{GRP78^{pep}}[DM1]. Overall, our results demonstrated that the NP[DM1]+1.5%TNP^{GRP78^{pep}}[DM1] combination treatment significantly reduced tumor burden at end of study, relative to PBS control, while also demonstrating no systemic or organ-specific toxicity.

NP[DM1]+TNP^{GRP78^{pep}}[DM1] combination treatment does not cause immune system toxicity, to the contrary, it promotes anti-tumorigenic immune cell involvement. Traditional chemotherapy, including DM1, has been known to cause toxic effects on the immune system, significantly reducing total leukocyte numbers. As we designed our DM1-prodrug-loaded nanoparticles with the goal of reducing off-target toxicity, next we evaluated if NP[DM1] and TNP^{GRP78^{pep}}[DM1] could overcome the toxic effects of DM1 on immune system cells. For this analysis, at the end of the tumor inhibition study, cells from the peritoneal lavage were collected from each mouse and analyzed via flow cytometry with two antibody panels. (ref) The first antibody panel was used to identify subsets of lymphoid- and myeloid-cell lineage. A second antibody panel was used to further focus on macrophages to evaluate the potential anti-tumorigenic immunomodulatory properties of NP[DM1] and TNP^{GRP78^{pep}}[DM1]. Based on these two panels, the overall analysis showed no apparent toxicity of NP[DM1] or TNP^{GRP78^{pep}}[DM1] on the immune system cells analyzed, relative to the PBS vehicle control (Fig. 13).

When we focused specifically on T cells, we observed that T cell counts in the DM1- loaded nanoparticle treatment groups were equivalent to or higher than counts in PBS vehicle (Fig. 13A). In addition, known anti-tumorigenic T cells, such as CD8+ cytotoxic T cells, CD4+ helper T cells, NK T cells, and TCR $\gamma\delta$ + T, were typically higher in the NP[DM1]+TNP^{GRP78^{pep}}[DM1] combination treatment group relative to PBS control. Regulatory (CD25+) T cells, known to regulate immune

responses, were significantly higher in NP[DM1], 0.75% TNP^{GRP78pep}[DM1], and NP[DM1]+TNP^{GRP78pep}[DM1] combination groups relative to the PBS vehicle control, as expected. Overall, all nanoparticle treatment groups, including NP[DM1], TNP[DM1], and NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment, were well tolerated by T cells. Interestingly, the NP[DM1]+TNP^{GRP78pep}[DM1] combination group showed further elevated levels of all the anti-tumorigenic T cell subpopulations, indicating possible immunomodulatory effects of the combination group.

Next, we analyzed the effect of nanoparticle treatment on the NK and B cell populations (Fig. 13B). Neither the NK nor B-cell numbers showed any significant differences when compared to PBS control. Overall, the nanoparticle treatment groups had no detectable effects on NK or B cell levels when compared to PBS control, indicating lack of off-target toxicity on the immune system.

Next, we analyzed the effect of DM1-prodrug-loaded nanoparticle treatments on cells of myeloid lineage, including neutrophils/myeloid-derived suppressor cells (MDSCs), dendritic cells (DCs), and macrophages (Fig. 13C). The levels of neutrophils/MDSCs (Ly6G+ Ly6C+) showed no statistical difference in any of the treatment groups, compared to the PBS vehicle control, indicating no off-target toxicity on the immune system. DCs (Ly6G- CD11b-/lo CD11c+) were significantly lower in all nanoparticle treatment groups relative to the PBS vehicle control. As DCs bridge the transition from an innate to adaptive immune response, the lower levels of DCs in treatment groups at the end of study, relative to PBS control, may be indicative of the lessened tumor burden and a return to healthier condition in the nanoparticle treatment groups (Fig. 15). Macrophages (CD11b+CD11c-) were significantly increased in all TNP^{GRP78pep}[DM1] groups (0.75%, 1.5%, and NP[DM1]+TNP^{GRP78pep}[DM1] combination), relative to the PBS vehicle control. The NP[DM1] group also appeared to have increased macrophages but not statistically different from controls. The fact that NP[DM1]+TNP^{GRP78pep}[DM1] combination and both TNP^{GRP78pep}[DM1] groups demonstrated elevated levels of macrophages implies the possibility of an anti-tumorigenic immunomodulatory response to the TNP^{GRP78pep}[DM1], which needs to be further explored. Combined, these results demonstrated that the DM1-loaded nanoparticle treatments did not cause immunotoxicity against the cells of myeloid-lineage studied.

Among all immune system cells, macrophages, particularly, have been shown to be the predominant immune cells present in the tumor microenvironment. Hence, we further analyzed the M1 and M2 subsets of macrophages. While M1 subset is known to be anti-tumorigenic, M2 subset has been shown to have pro-tumorigenic effects. Furthermore, these two subsets have been shown to have plasticity to transition from one subtype to another. Tumor-associated macrophages are often polarized to the M2 subset which has pro-tumorigenic effects. Total number of macrophages (identified with F4/80 marker) demonstrated no reduction in macrophage levels in the nanoparticle treatment groups, relative to PBS control, indicating no off-target toxicity (Fig. 13D *left*). Further,

macrophage levels were significantly elevated in all cancerous groups (including PBS control and nanoparticle treatment groups), relative to non-cancerous healthy control, indicating increased macrophage presence associated with cancer microenvironment (Fig. 15). The M1 (antitumorigenic, CD86+) macrophages, however, were significantly elevated in both the TNP^{GRP78pep}[DM1] (0.75% and 1.5%) and NP[DM1]+TNP^{GRP78pep}[DM1] combination groups, relative to the PBS vehicle control (Fig. 13D *center-left*). This further indicates the anti-tumorigenic immunomodulatory effects of the targeted nanoparticles on M1 macrophages. No significant effects were observed on M2 (pro-tumorigenic; CD206+) macrophages (Fig. 13D *center-right*) nor in the macrophages transitioning between M1 and M2 subsets (M1M2; CD86+CD206+; Fig. 13D *right*), relative to the PBS control. Overall, the macrophage-specific panel confirmed that the nanoparticle treatments did not show toxicity against the macrophages. Further, the TNP^{GRP78pep}[DM1] groups and NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment induced antitumorigenic M1 macrophage involvement.

In this study, we used a novel synthetic strategy to prepare DM1-prodrug loaded non-targeted (NP[DM1]) and GRP78-targeted (TNP^{GRP78pep}[DM1]) nanoparticles, which allowed us to strategically dissect the nanoparticle parameters for optimal delivery to the complex metastatic ovarian cancer (MOC) disease. The precision of our approach to nanoparticle design and its impact on biological endpoints enabled us to demonstrate, for the first time, that a combination therapy of non-targeted and targeted nanoparticles, specifically NP[DM1]+1.5%TNP^{GRP78pep}[DM1], provided highly efficacious tumor inhibition and immunomodulatory effects against MOC.

First, *in vitro* binding, uptake, and cytotoxicity studies were used to optimize the selectivity of the TNP^{GRP78pep}[DM1] for MOC cells by strategically analyzing, one at a time, the GRP78pep linker length, number of lysines, and GRP78pep density. While the *in vitro* studies enabled us to optimize the design of nanoparticle formulations, our *in vivo* studies revealed that the NP[DM1]+1.5%TNP^{GRP78pep}[DM1] combination treatment provided the most efficacious therapy against MOC, compared to TNP^{GRP78pep}[DM1] or NP[DM1] alone. Most literature reports support that targeted nanoparticles are more efficacious than non-targeted nanoparticles, however, our study showed that the combination treatment resulted in the least tumor burden in the MOC disease. Furthermore, there was no detectable tumor in 50% of the NP[DM1]+1.5%TNP^{GRP78pep}[DM1] combination treatment group at the end of study. Importantly, both NP[DM1] and TNP^{GRP78pep}[DM1] significantly improved the toxicity profile reported with free DM1, which has historically hindered the use of free DM1 as a chemotherapeutic option in the clinic.

Metastatic ovarian cancer (MOC) is a complex disease, mainly confined to the peritoneal cavity, but comprising two subpopulations of cells. On one hand, metastatic solid tumors are produced on peritoneal organs when low flow areas of the peritoneal cavity trap metastasized cells (metastasized solid tumors). On the other hand, unadhered metastasized cells make up a second subpopulation of

MOC cells, circulating in the peritoneal cavity (metastasized ascites cancer cells). The unique bimodal subpopulations of MOC cells could potentially be a major reason for the effectiveness of the NP[DM1]+1.5% TNP^{GRP78pep}[DM1] combination treatment. Supporting this hypothesis, analysis of the *in vivo* biodistribution, tumor growth inhibition, and immune cell involvement studies indicated that the NP[DM1] and 1.5% TNP^{GRP78pep}[DM1] worked via different mechanisms against the two subpopulations.

Focusing on the *in vivo* biodistribution and uptake data, our results demonstrated that the TNP^{GRP78pep}[DM1] formulation primarily targeted the unadhered metastatic ascites cells in the peritoneal cavity, possibly before having a chance to reach the metastatic solid tumor sites. This is similar to a phenomenon that has been described in literature as the binding site barrier. Namely, the metastatic ascites cells in the MOC model could be acting as a sink for the TNP^{GRP78pep}[DM1], leaving fewer TNP^{GRP78pep}[DM1] available to target the metastatic solid tumors. On the other hand, according to colocalization and uptake analysis, the NP[DM1] preferentially accumulated at the metastatic solid tumor sites and likely provided sustained release of the active drug in the periphery of the solid tumor for enhanced efficacy. Combined, these results support the differential mechanisms of action of NP[DM1] versus TNP^{GRP78pep}[DM1] for the two subpopulations of cells in the MOC disease, explaining why the NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment was most efficacious compared to TNP^{GRP78pep}[DM1] or NP[DM1] alone. While the TNP^{GRP78pep}[DM1] preferentially targeted the metastatic ascites cells, potentially inhibiting generation of new metastatic solid tumor foci, the NP[DM1] reached the pre-existing metastatic solid tumor sites for enhanced efficacy. Therefore, the two mechanisms working in synergy allowed the NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment to achieve the highest reduction of tumor burden.

Analysis of the effect of the nanoparticles on the immune system cells in the peritoneal cavity demonstrated that the TNP^{GRP78pep}[DM1] and NP[DM1] did not show any detectable immunotoxicity. To the contrary, our analysis of macrophages and various T cell subsets indicated that NP[DM1]+TNP^{GRP78pep}[DM1] combination treatment exerted immunomodulatory effects by inducing antitumorigenic effector functions. It is well established that M1 macrophages target tumor cells both directly (e.g. phagocytosis) and indirectly (e.g. activation of T cells and NK cells), while M2 macrophages promote tumor growth in the TME. The NP[DM1]+1.5% TNP^{GRP78pep}[DM1] combination treatment showed increased M1 macrophage involvement with no detectable changes in M2 macrophages, presenting a favorable M1:M2 ratio, which is a desirable outcome with any cancer treatment. The NP[DM1]+1.5% TNP^{GRP78pep}[DM1] combination treatment also showed increased levels of several antitumorigenic T cell subsets, including cytotoxic, helper, and NK T cells, which is again a favorable outcome. Regulatory T cells (T_{reg}) were also slightly increased in the NP[DM1]+1.5% TNP^{GRP78pep}[DM1] combination treatment. T_{reg} are known to aid in self-tolerance and protection from autoimmune diseases. Hence, the upregulation of T_{reg} might be indicative of a

regulated anti-tumorigenic immune response, preventing excessive inflammation. Combined, these results suggest the anti-tumorigenic immune effector functions induced by the NP[DM1] and TNP^{GRP78pep}[DM1]. A full elucidation of the immunomodulatory effects requires further study.

This study highlights the significance of systematic design and analysis of nanoparticle parameters, specifically tailored to the particular disease pathology for optimal clinical outcomes. Our NP[DM1]+1.5%TNP^{GRP78pep}[DM1] combination treatment is well-tolerated, inhibits tumor growth, and induces immunomodulatory effects, while allowing for delivery of otherwise potentially lethal doses of DM1. Taken together, these data provide the preclinical rationale for the evaluation of NP[DM1]+1.5%TNP^{GRP78pep}[DM1] to improve patient outcomes in the treatment of MOC and also of other cancers that are metastatic or particular to the peritoneal cavity.

Example 3. Tailoring GRP78pep-targeted lipid nanoparticles for DM1 delivery in aggressive lung cancer treatment achieved outstanding tumor inhibition due to stable phosphodiester prodrug chemistry.

Synthesis of prodrug loaded target nanoparticle formulations. To synthesize GRP78pep targeted DM1 (TNP[DM1]) or BTZ (TNP[BTZ]) loaded nanoparticles, we first synthesized the two prodrug molecules: DM1-Prodrug and BTZ-Prodrug. The DM1-Prodrug was synthesized by conjugating the active DM1 with linkage molecule 1a that contained phosphodiester functional group and C18 hydrophobic tails (Fig. 16A, details in method). Similarly, the BTZ-Prodrug was formed by conjugating BTZ with linkage molecule 2b that contained boronic ester chemistry and C14 aliphatic lipid tails (Fig. 16B, details in method). Both prodrug compounds, DM1-Prodrug and BTZ-Prodrug, were designed to have hydrophobic lipid tails in order to facilitate their tethering to lipid bilayer of nanoparticles without destabilizing the nanoparticles or affecting cellular uptake.

As the targeting ligand, we utilized a GRP78 receptor targeting peptide (GRP78pep), identified by Mandelin *et al.* from *in vivo* phage display, to target aggressive lung cancer cells. In order to synthesize the GRP78pep-targeted nanoparticles (TNP) and anchor the targeting peptide (GRP78pep), we designed GRP78pep-lipid with five essential components according to our previous studies (Fig. 16C). The GRP78pep-lipid conjugate consists of (i) targeting peptide (GRP78pep), (ii) an EG₂ spacer, repeating unit of ethylene glycol, to minimize the interaction between the ligand and lipid tails, (iii) a short oligolysine chain with a lysine residue to improve peptide solubility and availability by partitioning into solvent, (iv) an EG₈ peptide-linker to present the targeting peptide above the liposomal polyethylene glycol (PEG), (v) two palmitic acid lipid tails for association with the lipid bilayer of the nanoparticles (Fig. 16C). By using these design elements, it allows to fine tune the availability of targeting moiety above PEG2000 coating of nanoparticles, and eventually leads to significantly improved and selective cellular binding and uptake compared to traditional targeting strategies. A shorter linker, such as EG₈, enables more efficient binding to the target receptors than a

longer linker because of their ability to present peptides beyond PEG coating with linear conformation. We designed GRP78pep-lipid conjugate containing either EG₈ or EG₂₄ to compare cellular/tumor uptake of GRP78pep-targeted nanoparticles (TNP). The GRP78pep-lipid was synthesized on a solid support using standard Fmoc chemistry (details in method). We also synthesized the GRP78pep sequence as free GRP78pep to evaluate monovalent cellular binding experiment.

Prodrug loaded targeted nanoparticles (TNP[DM1] or TNP[BTZ]) were prepared using specific stoichiometric quantities of DSPC, mPEG-DSPE, Cholesterol, GRP78pep-lipid, and DM1-Prodrug/BTZ-Prodrug (Figure 16D; details in methods section). Both PEGylated lipids and cholesterol were included in the formulation since they provide extended circulation half-life and improve particle stability both in experimental and clinical research. DM1-Prodrug loaded non-targeted particle (NP[DM1]) and BTZ-Prodrug loaded non-targeted particle (NP[BTZ]) without GRP78pep were also prepared as controls (Fig. 16E). Prodrug loaded nanoparticles formulations contained either 5% DM1-Prodrug for TNP[DM1] and NP[DM1] or 1.5% BTZ-Prodrug for TNP[BTZ] and NP[BTZ]. Additionally, we prepared targeted nanoparticles (TNPs) containing various GRP78pep densities (0.25%, 0.5%, 0.75%, 1%) to examine the relationship of peptide valency and tumor targeting. Hydrophobic fluorescent dye (DiD or DiO) was incorporated in nanoparticle formulations for cellular binding and uptake experiments. Nanoparticles were prepared via hydration and extrusion method and sized using polycarbonate membrane for uniform size distribution. This multifaceted synthetic strategy enables precise control over stoichiometric surface functionalities and eliminates batch-to-batch variability.

The size of nanoparticle formulations was measured using dynamic light scattering (DLS) analysis. All nanoparticle formulations had a size of ~75 nm with a narrow distribution at physiological condition (pH 7.4) (Fig. 17A). Incorporation of prodrug molecules and targeting peptide did not affect the size of nanoparticles drastically, as shown by the small polydispersity (PDI) values (Fig. 17A). Additionally, utilizing different GRP78pep densities (0.25%, 0.5%, 0.75%) and ethylene glycol (EG) linkers (EG₈ and EG₂₄) did not alter the nanoparticle sizes. The zeta potential of non-targeted nanoparticles, such as NP, NP[DM1], and NP[BTZ] were measured -8.99, -14.68, and -7.12 mV, respectively (Fig. 17B). Interestingly, the addition of the GRP78pep to prepare targeted nanoparticles, TNP[DM1] or TNP[BTZ], resulted in negligible change in zeta potential. The zeta potential for TNP, TNP[DM1], and TNP[BTZ] were -4.24, -2.35, and -4.61 mV. Consistent with the DLS results, transmission electron microscopy (TEM) images further confirmed that all nanoparticle formulations had spherical structures with uniform size (Fig. 17C).

Next, we evaluated whether prodrug molecules (DM1-Prodrug or BTZ-Prodrug) in nanoparticle formulations were stable under different pH and reaction conditions. We observed that the TNP[DM1] were able to retain >85% of DM1-Prodrug at 37 °C, at pH 7.4 (Fig. 17D) and 4.8 (Fig. 17D) over extended period of time (up to 48 h). This sort of stable retention is advantageous in

nanomedicine-based drug delivery because it prevents premature leakage of the drug prior to reaching the target site and ensures controlled delivery of the active drug at the tumor site after endocytosis. Supporting this, we determined DM1-Prodrug is released in a controlled manner over the period of 48 h in enzyme containing buffer (EB) (Fig. 17D) mimicking the conditions of endocytic compartments.

To the contrary, BTZ-Prodrug containing nanoparticles (TNP[BTZ]) showed rapid degradation of the prodrug within 6 h regardless of the incubation conditions (pH 7.4 and 4.8, as well as enzyme containing buffer (EB)) (Fig. 17E). This type of rapid release could possibly result in premature leaking of the active drug and insufficient drug delivery at the target site ultimately. The difference in release profile of DM1 (slow release) and BTZ (rapid release) could emphasize whether GRP78pep-targeted nanoparticles (TNPs) can improve selective delivery to enhance efficacy over free drug.

In vitro evaluation of cellular binding and uptake of GRP78pep-targeted particles (TNPs) using A549 lung carcinoma cell line. The GRP78 receptor targeting peptide (GRP78pep), identified by Mandelin *et al.* from *in vivo* phage display, was utilized as targeting ligand to evaluate cellular binding and uptake of GRP78 expressing lung cancer cell line. We selected A549 lung cancer cell line since it is known to express high level of GRP78 surface receptors. First, we determined the GRP78pep binding to the GRP78 receptor on A549 human lung carcinoma cells using flow cytometry. The results showed that the fluorescein-labeled GRP78pep binds to GRP78-expressing A549 cells with a K_d of $\sim 3 \mu\text{M}$ (Fig. 18A). Next, we prepared GRP78pep targeted nanoparticles (TNPs) with various density (0.25%, 0.5%, 0.75%) to determine the optimal loading of the GRP78pep for cellular binding of nanoparticles using A549 cells. DiO fluorescent dye was incorporated within nanoparticles for cellular quantification via flow cytometry. The GRP78pep-targeted nanoparticles (TNPs) exhibited approximately 4, 30, and 100-fold improved binding for GRP78pep densities of 0.25%, 0.5%, and 0.75%, respectively, compared to non-targeted control (NP) (Fig. 18B). Similarly, significant cellular uptake of TNPs was observed with ~ 2 , ~ 5 , and ~ 10 -fold enhancement for same GRP78pep densities (0.25%, 0.5%, and 0.75%) compared to NP control, at both 4 h and 24 h incubation duration (Figure 18c). Then, we evaluated the effect of ethylene glycol (EG) linker length (EG₈ or EG₂₄) used in the TNPs formulations in cellular uptake using flow cytometer at both 4 h and 24 h incubation duration. The TNPs utilizing a shorter EG₈ linker showed increased uptake over those using a moderately longer EG₂₄ linker during 4 h and 24 h incubation time (Fig. 18D). This observation suggested that the shorter EG₈ linker used in nanoparticle formulations is an optimal length compared to the longer linker for efficient cellular uptake *in vitro*. We presumed a shorter linker, EG₈, enables more efficient uptake of TNPs via target receptors than a longer linker because of their ability to present peptides with linear conformation beyond PEG coating of nanoparticles.

We further validated the uptake results by fluorescent microscopy imaging. We observed significant accumulation of TNPs into A549 lung cancer cells for GRP78pep density 0.5% and 0.75%, whereas no uptake was detected by NP control (Figure 18e). Additionally, Jurkat cells which have low

expression of GRP78 receptors, were used as negative control that did not show any significant uptake with TNP. This result indicated the selective binding and uptake of TNPs to the GRP78 receptor on A549 lung carcinoma cell line. To make sure the incorporation of prodrugs would not interfere with the binding of GRP78pep to the GRP78 receptors, we repeated same experiment with prodrug loaded nanoparticles, namely, TNP[DM1] and TNP[BTZ]. Since both targeting peptides and prodrugs are exposed on the liposomal nanoparticles, there is a possibility prodrug might interfere with cellular binding of TNPs. To confirm the possible hindrance, we performed a binding assay with prodrug loaded NPs (NP[DM1], NP[BTZ]) and prodrug loaded TNPs (TNP[DM1], TNP[BTZ]) and compared to non-loaded versions (NP, TNP). The DM1-Prodrug and BTZ-Prodrug loading did not alter the binding of either TNP[DM1] or TNP[BTZ] when compared to non-drug loaded TNPs (Fig. 18F). To further verify the specificity of GRP78pep towards A549 cells, a competitive binding inhibition assay was performed using an excess amount of free GRP78pep. When introduced with 0.25 nM and 0.5 nM concentration of TNPs, free GRP78pep significantly inhibited the cellular binding of TNPs containing 0.5% density by $\geq 92\%$ (Fig. 18G). This result indicated that GRP78pep is highly specific to the GRP78 receptor found on A549 cells. Taken together, we determined GRP78pep targeted nanoparticles (TNPs) has greater potential to improve the delivery of DM1 or BTZ in GRP78 receptor expressing lung cancer cells and to minimize toxic side-effects of free drug.

In vitro cytotoxicity evaluation of targeted and non-targeted nanoparticle formulations in A549 lung cancer cell line. Next, we evaluated the potency of prodrug loaded nanoparticle formulations (NP[DM1], TNP[DM1], NP[BTZ] and TNP[BTZ]) against A549 lung carcinoma cells *in vitro*. For this, cells were incubated with NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ], free DM1 or free drug for 48 h and later cell viability was determined using cell counting kit-8 (CCK-8) reagent. All TNP formulations contained 0.5% GRP78pep density. Nanoparticles containing DM1-Prodrug (NP[DM1], TNP[DM1]) demonstrated a slight reduction in cytotoxic effects with an IC_{50} of 100 nM compared to free DM1 (Fig. 19A). Both TNP[BTZ] and NP[BTZ] exhibited IC_{50} values ~ 24 nM at 48 h, similar as free BTZ (Fig. 19B). The cytotoxicity results indicate that we were able to induce the cytotoxic effects *in vitro* using prodrug loaded nanoparticle formulations of DM1 (NP[DM1], TNP[DM1]) and BTZ (NP[BTZ] and TNP[BTZ]) as their free drug counterpart.

In vivo biodistribution and uptake of GRP78pep-targeted nanoparticles (TNPs). *In vivo* biodistribution studies of GRP78pep-targeted nanoparticles (TNPs) were conducted using a subcutaneous xenograft mouse model of lung carcinoma. NOD–SCID mice were injected with A549 cells. Nanoparticles were prepared with a range of GRP78pep densities (0.35%, 0.50% and 0.70%), along with incorporation of fluorescent label DiD. Nanoparticles were administered intravenously once tumors reached 200 mm³ size. 24 h after nanoparticle injections, mice were sacrificed and dissected, and major organs and tumors were collected for imaging to determine nanoparticle accumulation. Non-targeted nanoparticles (NP) were used as control. All TNPs (containing 0.35%,

0.5%, and 0.75 % GRP78pep density), as well as NP control accumulated efficiently at the tumor site (Figure 20a). However, TNPs prepared with higher GRP78pep densities (0.5% and 0.7%) demonstrated slightly reduced tumor accumulation when compared to TNP with 0.35% GRP78pep density and NP control. Vehicle control experiments done with PBS had negligible fluorescent signal and were subtracted from all groups during data analysis. The slightly reduced accumulation of TNPs with 0.5% and 0.7% GRP78pep density is most likely due to off-target binding to other tissues expressing GRP78 receptor, caused by high avidity GRP78pep ligands. Importantly, overall accumulation of TNPs showed no increased fluorescence in other organs relative to NP group, demonstrating a lack of off-target accumulation of TNPs in any organs.

Next, we studied *in vivo* cellular uptake of the TNPs (containing 0.35%, 0.5%, and 0.75 % GRP78pep density) by A549 lung cancer cells. 24h after nanoparticle injection intravenously (IV), tumor tissues were dissected, disaggregated, and the nanoparticle uptake was analyzed via flow cytometry. Significant levels of tumor cell uptake were detected both with TNPs and NP control (Fig. 20B). Vehicle control experiments done with PBS did not show any fluorescent signal (results not shown).

Furthermore, we evaluated whether these nanoparticles had toxic effects on major organs *in vivo*. 24h after nanoparticle injection intravenously (IV), tissues from major organs were dissected, and slides containing tissues were prepared using microtome. We performed H&E staining of tissues from major organs that were imaged using high resolution light microscope (Fig. 20C). No noticeable differences in major organs were observed among the nanoparticle groups, demonstrating nanoparticle formulations had no toxic effects.

In vivo efficacy of GRP78pep-targeted and non-targeted, DM1-Prodrug or BTZ-Prodrug loaded nanoparticles. To evaluate the therapeutic potential of DM1-Prodrug or BTZ-Prodrug loaded nanoparticle formulations (NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ]), mice were injected with A549 cells and tumors were allowed to grow to a palpable size ($>80 \text{ mm}^3$) prior to treatments. Mice were randomized into different groups and were treated intravenously with either PBS (control), NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ], Free DM1 or Free BTZ on day 1, 5, 9, 13 and 17. All TNP formulations contained 0.5% GRP78pep density. Prodrug loaded nanoparticles formulations contained either 5% DM1-Prodrug for TNP[DM1] and NP[DM] or 1.5% BTZ-Prodrug for TNP[BTZ] and NP[BTZ]. DM1-Prodrug loaded nanoparticle formulations (NP[DM1] and TNP[DM1]) at 2.5 mg/kg equivalent DM1 concentration and Free DM1 were delivered at 0.5 mg/kg (the highest possible maximum tolerated dose above which free DM1 becomes lethal to mice). All BTZ formulations (NP[BTZ], TNP[BTZ] and Free BTZ) were delivered at 0.75 mg/kg equivalent BTZ concentration. Mice were monitored for tumor growth inhibition and systemic toxicity for 32 days.

NP[DM1], TNP[DM1], and free DM1 all demonstrated significant tumor growth inhibition when compared to the PBS control (Fig. 21A). Importantly, TNP[DM1] demonstrated statistically

significantly higher enhancement in tumor growth inhibition relative to NP[DM1] (with a p-value <0.04). Specifically, TNP[DM1] and NP[DM1] achieved ~81% and ~41% tumor growth inhibition respectively when compared to PBS control by day 32. The improved efficacy of TNP[DM1] when compared to NP[DM1] can be attributed to the GRP78 receptor targeting enabled by multivalent display of GRP78pep on TNP[DM1]. With TNP[DM1], we were able to fully harness the advantages of ligand targeted nanoparticle approach, presumably due to the stable phosphodiester bond of the DM1-Prodrug, which prevents premature release of DM1 from nanoparticle at pH 7.4 (Fig.17D). The stability of the phosphodiester bond at pH 7.4 allows TNP[DM1] to first accumulate at the tumor site via GRP78 ligand-receptor interactions, and then allows for controlled release of DM1 predominantly after endocytosis by the lung cancer cells. While NP[DM1] also has the phosphodiester bond, it does not have GRP78 targeting ligands, hence lacks the advantage of enhanced uptake at the tumor site.

Our studies also demonstrated that NP[DM1] and TNP[DM1] did not exhibit any systemic toxicity based on overall weight of mice during the duration of study (Figure 21b). The Free DM1 also did not show any systemic toxicity, however, the free DM1 concentration (0.5 mg/kg) in this study was much lower compared to the DM1-Prodrug concentration in NP and TNP (2.5 mg/kg equivalent DM1 concentration). This is because our former study demonstrated free DM1 is lethal to mice at dosages above 0.5 mg/kg. Combined, the toxicity study showed that TNP[DM1] and NP[DM1] can be delivered at 5 times higher dosage than free DM1, thereby dramatically eliminating the toxicity issues associated with free DM1.

Studies performed with NP[BTZ], TNP[BTZ], and free BTZ demonstrated that developing nanoparticle formulations of BTZ did not provide any enhancement in the efficacy of BTZ in the A549 subcutaneous lung cancer model. While the TNP[BTZ] showed to some extent enhanced tumor killing than NP[BTZ], neither nanoparticle provided any enhancement over free BTZ (Figure 21c). Similarly, nanoparticle formulations of BTZ did not improve the toxicity profile of BTZ (Fig. 21D). The results we observed with BTZ-Prodrug loaded nanoparticles were unexpected. This could be due to the instability of the boronic ester bond that was used to synthesize the BTZ-Prodrug, which might have caused premature and uncontrolled release of BTZ from nanoparticle in physiological pH (Fig. 21E).

To further validate these results, at the end of study, we dissected and weighed the tumors and performed H&E staining to assess tumor cell death. Consistent with the results present in Fig. 21A, weight of post-dissection tumor burden reflects that TNP[DM1] by far was most efficacious in reducing tumor mass, closely followed by NP[DM1] (Fig. 21E). Both TNP[DM1] and NP[DM1] demonstrated enhanced efficacy compared to free DM1. With BTZ, our results were also consistent shown in Figure 21c, demonstrating no significant changes in tumor burden between free BTZ, TNP[BTZ], NP[BTZ]. These results were further validated by H&E staining of the tumor tissue (Fig. 21F), where we observe most tumor cell death (less nucleated cells) in TNP[DM1] treatment group compared to control and other treatment groups.

Organ toxicity studies of NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ]. Next, we evaluated the effect of NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ] on organ toxicity. At the end of the study, major organs were dissected, weighed and H&E staining was performed. There was no detectable change in any of the major organ weights when they were treated by the nanoparticles (Fig. 22A). We also performed histology experiments to qualitatively determine whether nanoparticles had any organ toxicity. As can be seen in Figure 22b, H&E staining revealed that there were no observable differences in tissues from major organs between the treatment groups and the control (PBS) group. Taken together these results demonstrated the NP[DM1], TNP[DM1], NP[BTZ], TNP[BTZ] were all well tolerated in mice.

Finding efficacious therapeutic strategies are still underway for treating aggressive lung cancer. We hypothesized that since targeted delivery typically increases efficacy while reducing systemic toxicity, developing and incorporating a prodrug formulation of either DM1 or BTZ as part of a peptide-targeted nanoparticle platform to treat lung cancer could offer a potent alternative to existing treatments in the clinic. Ideally, prodrug formulation for effective treatment of cancers should possess favorable characteristics such as, resilient to premature degradation prior to reaching target cells, and rapid conversion to active form upon reaching the target. To demonstrate the combined impact of targeting with peptides and prodrug chemistry, we assessed the prodrug compounds using a GRP78pep-targeted nanocarrier. Evaluations started with *in vitro* experiments of cellular binding, uptake and cytotoxicity for different formulations prior to moving forward with *in vivo* efficacy.

The multifaceted synthetic approach developed in our lab previously, enabled precise control of surface functionalities of nanoparticle formulations (TNP[DM1], NP[DM1], TNP[BTZ], NP[BTZ]) as well as eliminated potential pitfalls related to conventional approach of particle synthesis, such as particle instability, inconsistent loading of targeting ligand and prodrug, batch-to-batch variability. The particle sizing and TEM analysis demonstrated uniform and homogeneous distribution of ~70 nm nanoparticle formulations. By comparing the stability of prodrugs, evaluated in the form of nanoparticle formulations, we identified the DM1-Prodrug was least susceptible to hydrolysis under different buffer conditions (with the exception of enzyme cocktail) (Fig. 17D). Interestingly, the BTZ-Prodrug was observed to rapidly degrade in those conditions (Fig. 17E). This relative bond stability demonstration suggested that despite being delivered via TNP formulations, BTZ-Prodrug may leak before reaching the tumor tissue during systemic circulation. Nevertheless, the DM1-Prodrug in nanoparticle formulations (TNP[DM1], NP[DM1]) showed modest potency (IC₅₀ of ~100 nM) similar as the Free DM1 (Figure 19a). Nanoparticle formulations of BTZ-Prodrug (TNP[BTZ], NP[BTZ]) exhibited outstanding potency (IC₅₀ of ~24 nM) similar to that of free BTZ (Fig. 19B). Based on relative bond stability and *in vitro* cytotoxicity evaluations, it was challenging to choose the tradeoff between potency and stability of prodrug formulations and predict efficacious formulation prior to *in vivo* experiments.

All nanoparticle formulations were safe in animals as determined by toxicity and histology data (Fig. 20C, 21B, 21D, 22A, 22B). For *in vivo* efficacy experiment, both DM1 and BTZ drug dosages in nanoparticle formulations were delivered at below maximum tolerated dose, for performing several rounds of treatment to obtain tumor growth inhibition in an aggressive tumor model. The TNP[DM1] appeared to be most efficacious and was able to suppress tumor growth by ~81% compared to PBS control at the end of the study and statistically better than NP[DM1] treatment (Fig. 21A). Importantly, the stable phosphodiester prodrug chemistry in physiological pH and the nanoparticle formulation using our multifaceted synthetic approach, combinedly enabled 5 times higher equivalent DM1 delivery in a controlled manner, compared to free DM1 in animals and demonstrated dramatic improvement in therapeutic efficacy.

Surprisingly, the nanoparticle formulations of BTZ (TNP[BTZ] and NP[BTZ]) did not provide enhancement/improvement *in vivo* over free BTZ treatment in terms of efficacy and toxicity profile, presumably due to instability of boronic ester prodrug chemistry in physiological pH. The efficacy study performed here strongly demonstrated the importance of combining the strategy of utilizing GRP78 receptor targeting and rational/stable prodrug design in nanoparticle drug delivery systems for treating aggressive lung cancer. We demonstrated the rational optimization of the nanoparticle delivery system by fine tuning design parameters, such as peptide linker length and peptide density, that impact overall valency and avidity of GRP78pep targeted nanoparticles (TNPs). Most importantly, we accomplished improved efficacy and controlled release of active DM1 from TNP[DM1] by utilizing stable prodrug chemistry as part of the formulation. Simply optimizing only targeting ligands or choosing easily hydrolysable functional linkers in designing prodrugs alone cannot achieve the best possible efficacy when it comes to treating complex diseases. A finely tuned design along with proper targeting elements can overcome non-selective uptake of therapeutic payloads by healthy cells and maximize delivery of active drug in tumor tissue.

While specific embodiments have been described above with reference to the disclosed embodiments and examples, such embodiments are only illustrative and do not limit the scope of the invention. Changes and modifications can be made in accordance with ordinary skill in the art without departing from the invention in its broader aspects as defined in the following claims.

All listed publications, patents, and patent documents are incorporated by reference herein, as though individually incorporated by reference, and in particular, U.S. Patent Publication Nos. 2018/0177810 to Bilgicer *et al.*; 2018/0263909 to Bilgicer *et al.*; and U.S. patent No.10,342,846 to Bilgicer *et al.*; PCT Publication No. WO 2024/108173 to Bilgicer *et al.*; Omstead *et al.*, *J Hematol Oncol* 13, (2020); Mejia *et al.*, *Nanoscale* 14, 1226–1240 (2022); Shin *et al.*, *Nanoscale*, 15(32), 13322–13334 (2023); Farshbaf *et al.*, *Journal of Controlled Release*, Volume 328, 10 December 2020, Pages 932–941; Conner *et al.*, *Sci Rep.* 2020 Feb 26;10(1):3474; Stefanick *et al.*, *ACS Nano* 7, 8115–

8127 (2013); and Stefanick *et al.*, *ACS Nano* 7, 2935–2947 (2013). No limitations inconsistent with this disclosure are to be understood therefrom. The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

5

CLAIMS

What is claimed is:

1. A nanoparticle comprising:
 - a targeting peptide-lipid conjugate, wherein a targeting peptide moiety of the targeting peptide-lipid conjugate comprises a GRP78 targeting peptide;
 - a polyethylene glycol (PEG)-lipid conjugate;
 - a drug-lipid conjugate comprising a prodrug moiety, wherein the drug-lipid conjugate comprises one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and a bortezomib (BTZ) prodrug, and wherein the prodrug is linked to a lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond;
 - cholesterol comprising about 1 mol% to about 10 mol% of the nanoparticle; and
 - distearoylphosphatidylcholine (DSPC).
2. The nanoparticle of claim 1, wherein the targeting peptide-lipid conjugate comprises Formula I:

A-B-C-D-E (I),

 - wherein A is the GRP78 targeting peptide; B is a first ethylene glycol spacer; C is an oligolysine linker; D is a second ethylene glycol linker; E is a C₁₂-C₂₀ fatty acid; wherein Formula I optionally includes an amino acid linker moiety disposed between D and E;
 - wherein the first ethylene glycol spacer has a formula (EG)_n, wherein EG is an ethylene glycol monomer of polyethylene glycol and n is the number of EG moieties, and n is about 1 to about 5; and
 - the second ethylene glycol spacer has a formula (EG)_x, wherein x is the number of EG moieties, and x is about 1 to about 50.
3. The nanoparticle of claim 2, wherein n is 2; and x is 2, 8, 18, 30, or 45.
4. The nanoparticle of claim 2, wherein the C₁₂-C₂₀ fatty acid is a palmitic acid moiety.
5. The nanoparticle of claim 1, wherein the GRP78 targeting peptide comprises an amino acid sequence of SNTRVAP (SEQ ID NO: 1).
6. The nanoparticle of claim 1, wherein the nanoparticle comprises about 0.1 mol% to about 10 mol% of the targeting peptide-lipid conjugate.
7. The nanoparticle of claim 6, wherein the nanoparticle comprises about 0.1 mol% to about 5 mol% of the targeting peptide-lipid conjugate.

8. The nanoparticle of claim 1, wherein the nanoparticle comprises about 0.1 mol% to about 10 mol% of the drug-lipid conjugate.
9. The nanoparticle of claim 8, wherein the nanoparticle comprises about 0.1 mol% to about 5 mol% of the drug-lipid conjugate.
10. The nanoparticle of claim 1, wherein the nanoparticle comprises about 5 mol% cholesterol.
11. The nanoparticle of claim 1, wherein the nanoparticle comprises about 90 mol% to about 98 mol% of DSPC.
12. The nanoparticle of claim 1, wherein the nanoparticle comprises about 0.1 mol% to about 10 mol% of the PEG-lipid conjugate.
13. The nanoparticle of claim 1, wherein the lipid moiety of the drug-lipid conjugate is a 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE) moiety.
14. The nanoparticle of claim 1, wherein the nanoparticle comprises an average diameter of about 50 nm to about 125 nm.
15. A nanoparticle comprising:
 - a GRP78 protein targeting peptide-lipid conjugate comprising Formula I:

A-B-C-D-E-F (I), wherein

A is a GRP78 protein targeting peptide moiety having an amino acid sequence of SNTRVAP (SEQ ID NO: 1);

B is a first ethylene glycol spacer having a formula (EG)_n, wherein EG is an ethylene glycol monomer of polyethylene glycol and n is the number of EG moieties, and n is 2;

C is an oligolysine linker comprising between 1 and 3 lysine residues;

D is a second ethylene glycol spacer having a formula (EG)_x, wherein x is the number of EG moieties, and x is 2, 8, 18, 30, or 45;

E an amino acid linker moiety comprising a tryptophan residue; and

F is a palmitic acid moiety;
 - a polyethylene glycol (PEG)-lipid conjugate;
 - a drug-lipid conjugate comprising a prodrug moiety, wherein the drug-lipid

conjugate comprises:

- i) one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and bortezomib (BTZ) prodrug; and
- ii) a lipid moiety comprising 1,2-distearoyl-sn-glycero-3-phosphoryl-ethanolamine (DSPE), wherein the prodrug moiety is linked to the lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond; cholesterol comprising about 5% w/w of the nanoparticle; and distearoylphosphatidylcholine (DSPC).

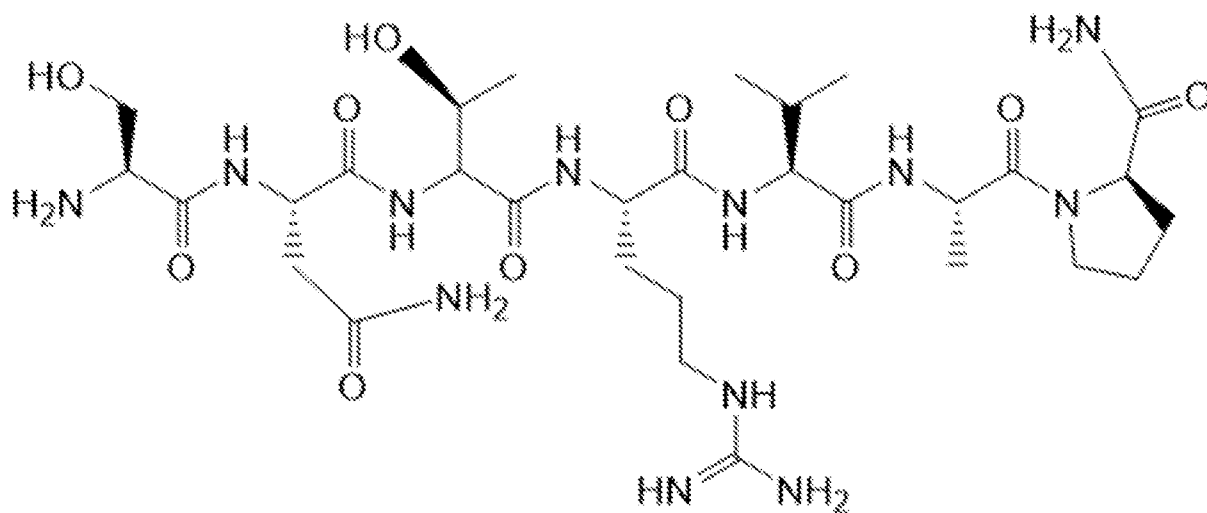
16. A composition comprising the nanoparticle of claim 1 and a pharmaceutically acceptable excipient, carrier, or diluent.

17. The composition of claim 16, further comprising a second nanoparticle comprising:
a drug-lipid conjugate, wherein the drug-lipid conjugate comprises:
a) a prodrug moiety comprising one or more of a mertansine (DM1) prodrug, a doxorubicin prodrug, and a bortezomib (BTZ) prodrug; and
b) a lipid moiety comprising 1,2-distearoyl-sn-glycero-3-phosphorylethanolamine (DSPE);
wherein the prodrug is linked to the lipid moiety of the drug-lipid conjugate via a phosphodiester bond or a boron ester bond;
a polyethylene glycol (PEG)-lipid conjugate;
cholesterol comprising about 1% to about 10% w/w of the nanoparticle; and
distearoylphosphatidylcholine (DSPC).

18. A method of treating a cancer in a subject in need thereof comprising administering to the subject an effective amount of the nanoparticle of any one of claims 1-15 or the composition of claim 16 or 17, thereby treating the cancer.

19. The method of claim 18, wherein the cancer is breast cancer, lung cancer, ovarian cancer, acute myeloid leukemia, acute lymphoblastic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, neuroblastoma, bone sarcomas, thyroid cancer, stomach cancer, kidney cancer, and multiple myeloma.

20. The method of claim 18, wherein the effective amount of the nanoparticles or the composition is about 0.1 mg/kg to about 5 mg/kg.

A

GRP78pep

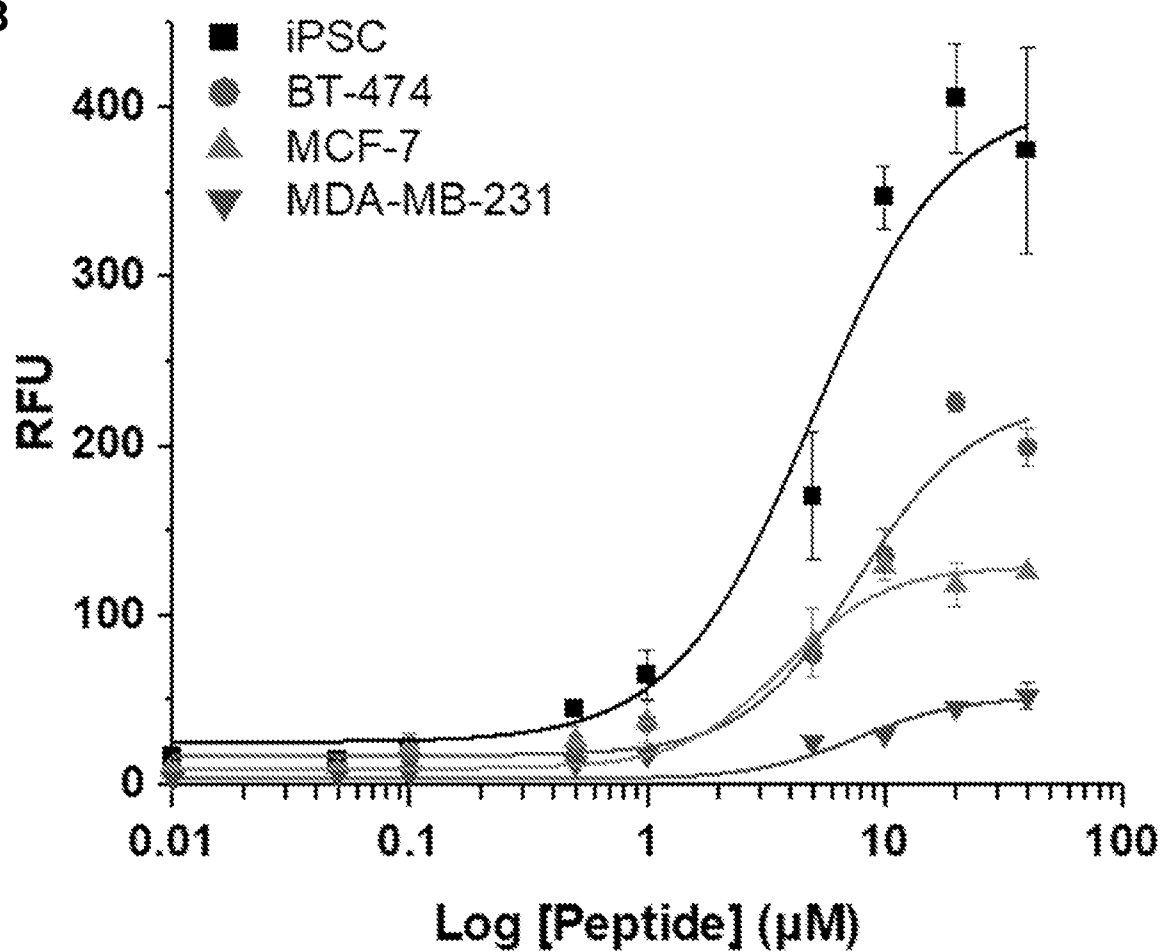
B

Fig. 1

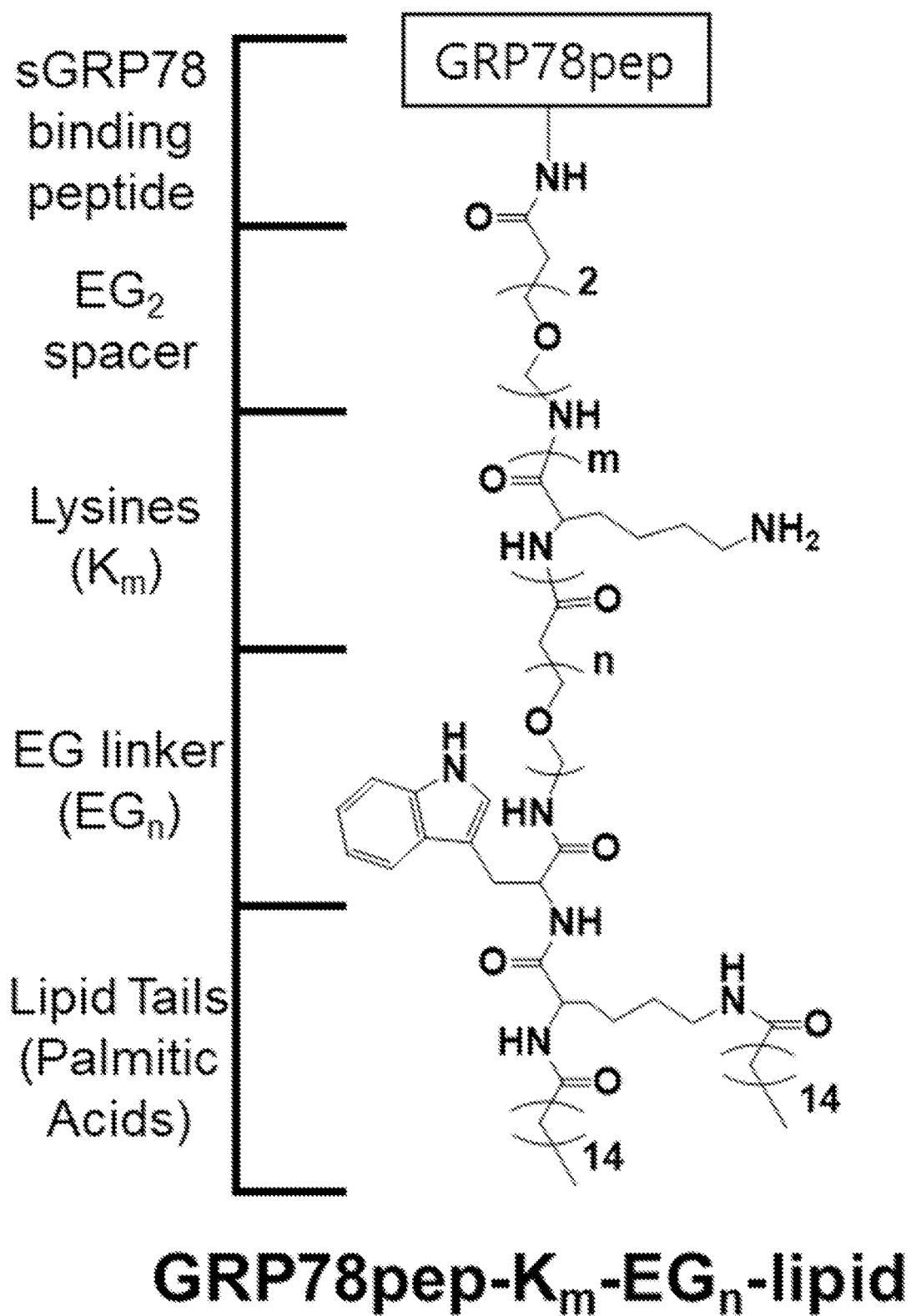
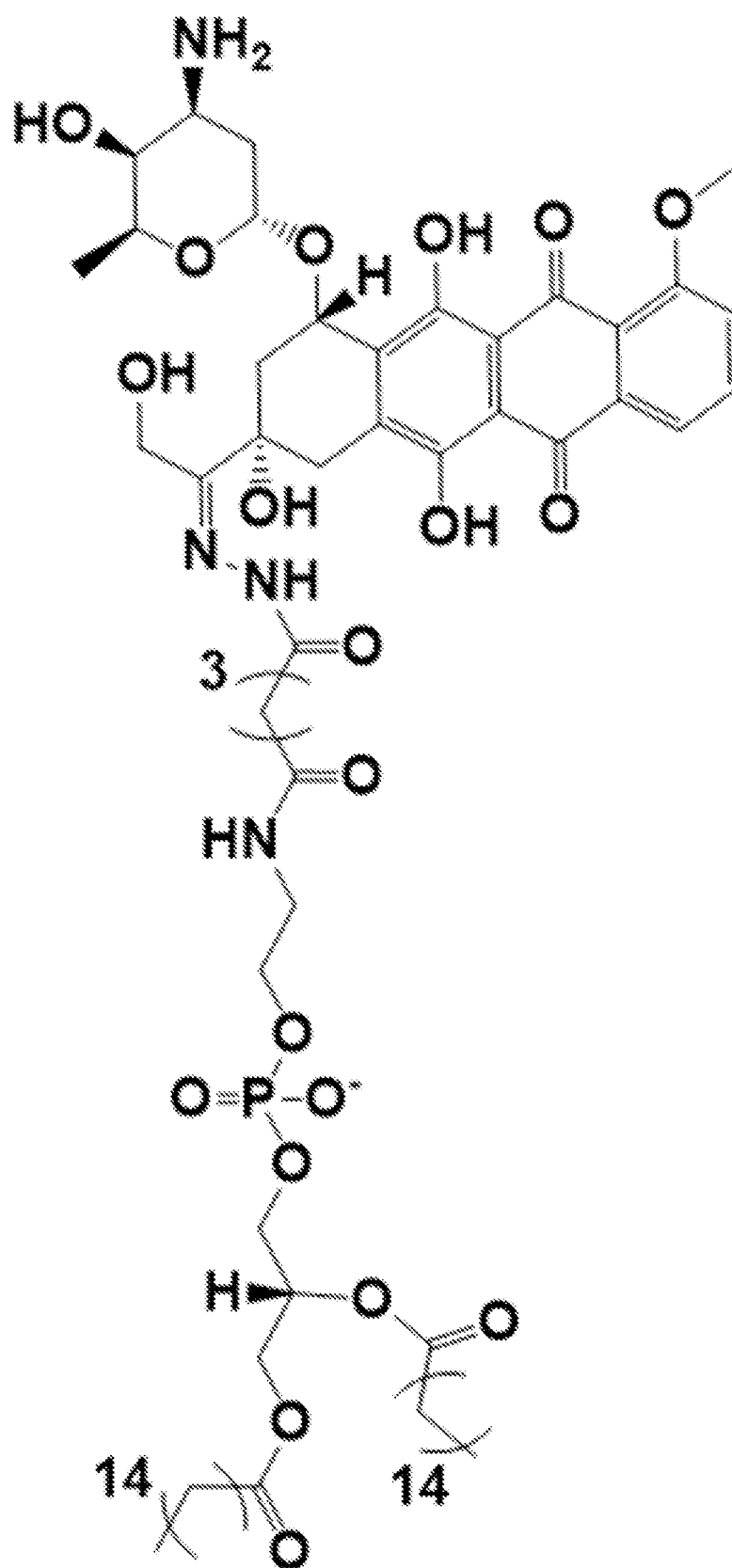


Fig. 2A

*Fig. 2B*

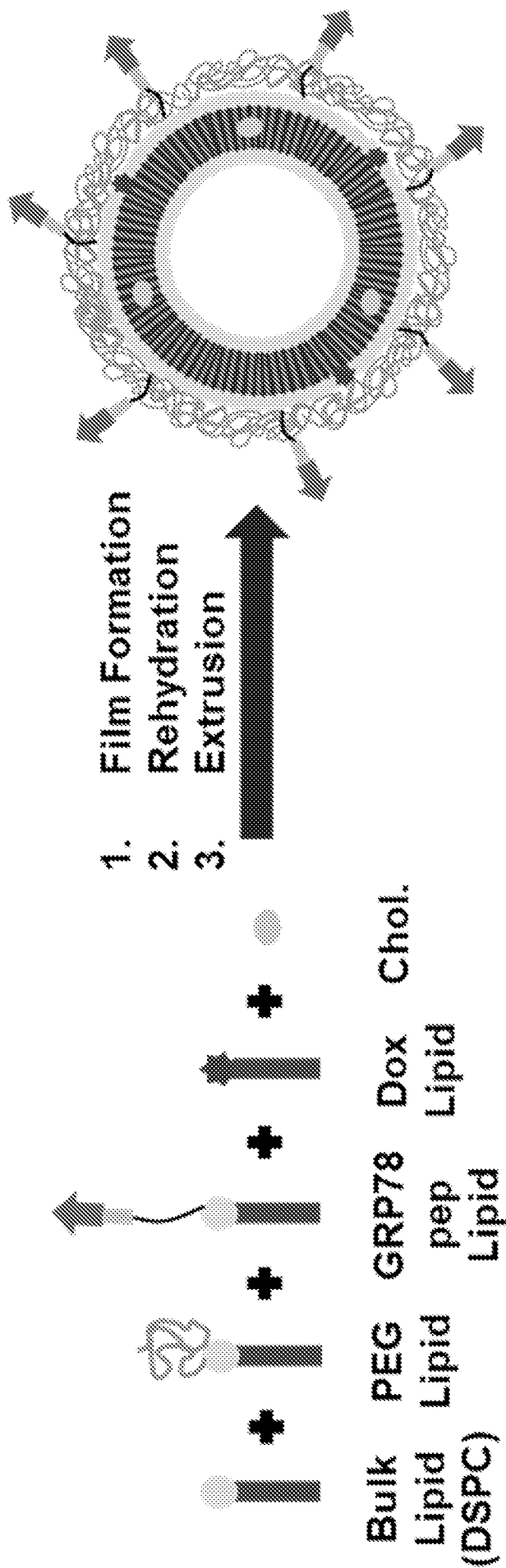


Fig. 2C

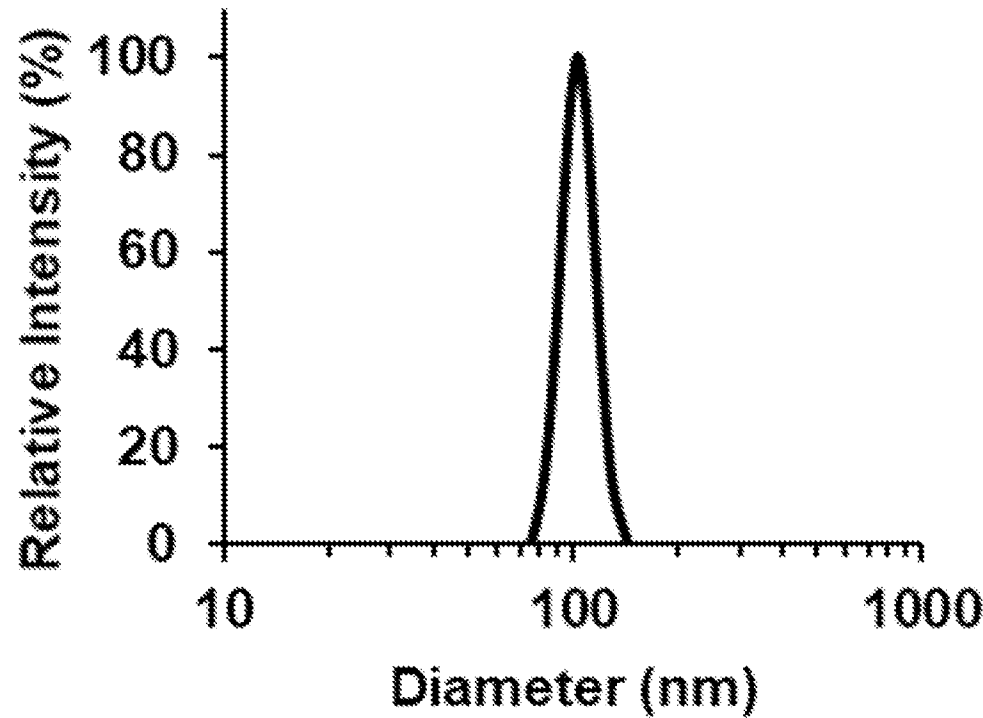


Fig. 2D

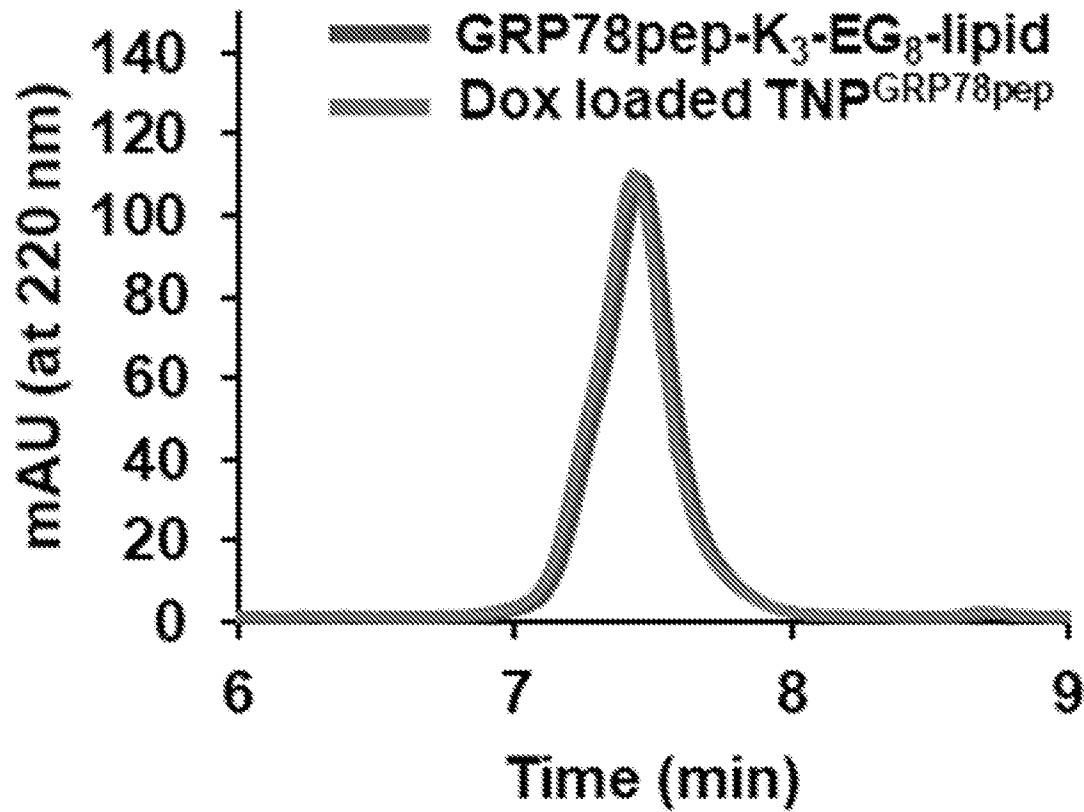
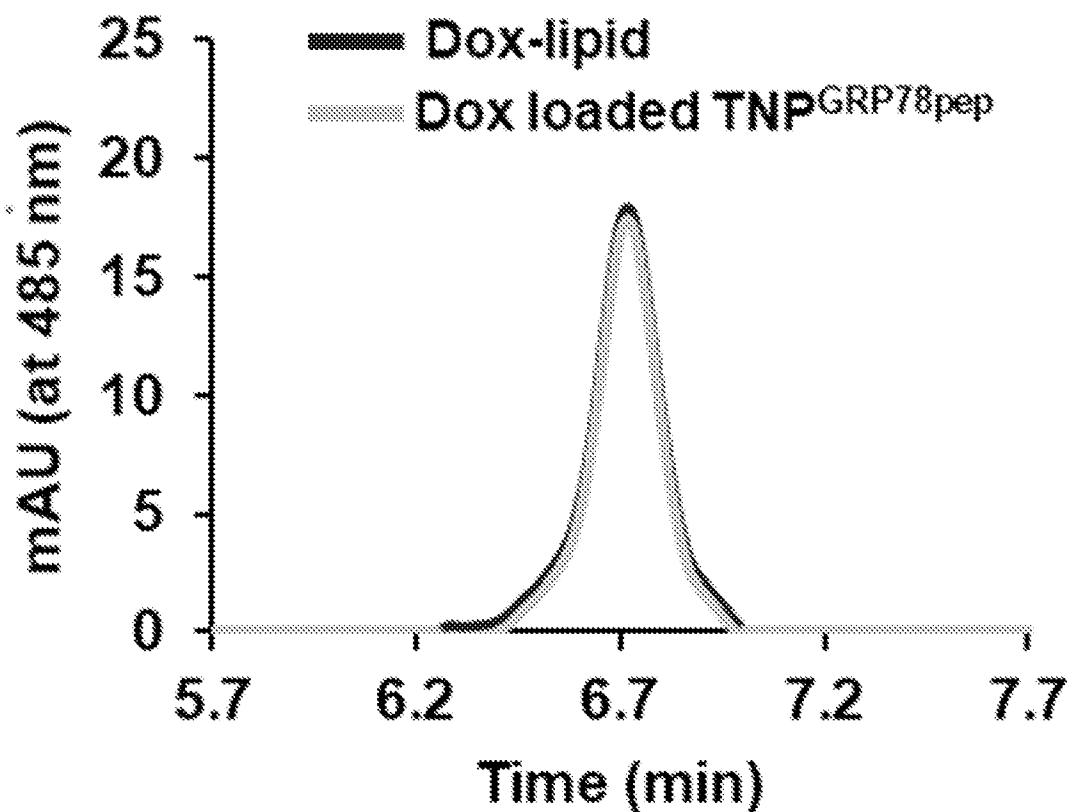
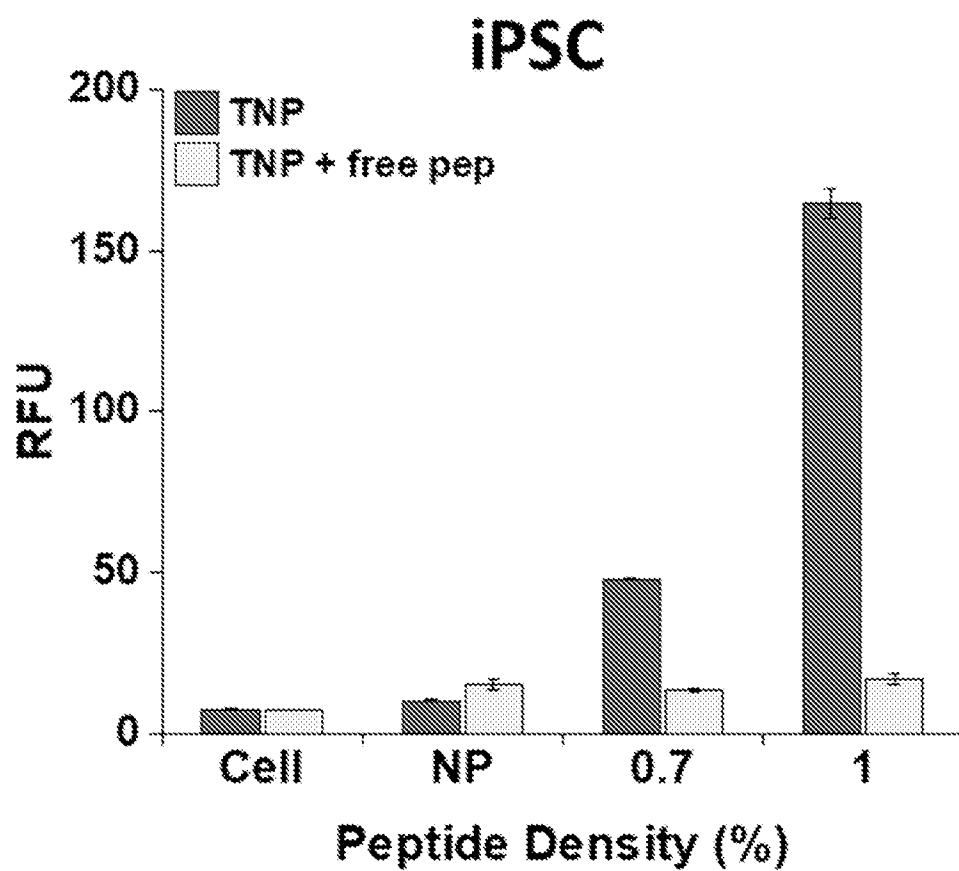
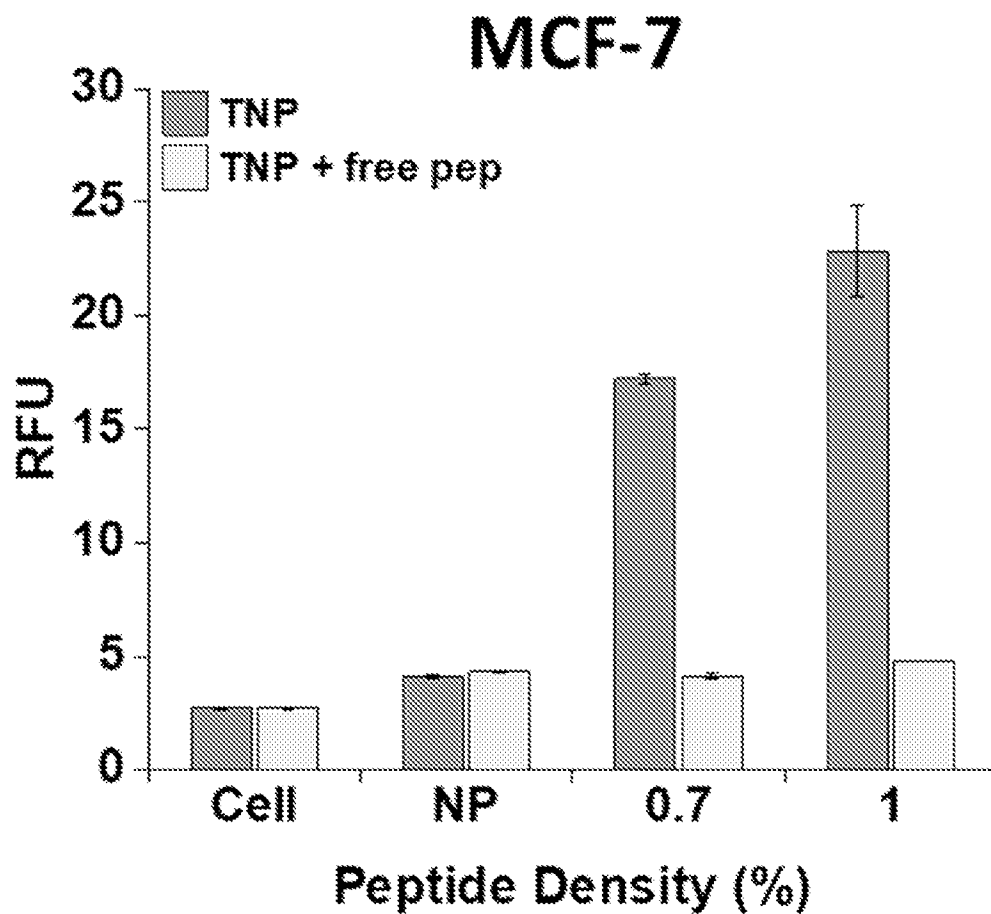
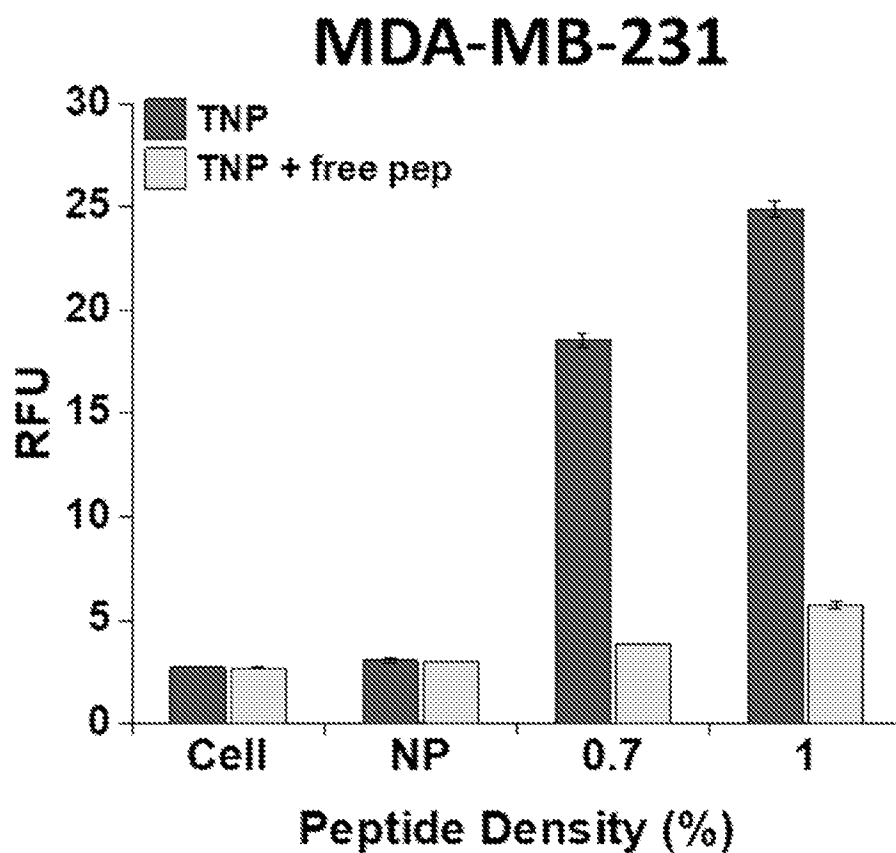


Fig. 2E

*Fig. 2F**Fig. 3A*

*Fig. 3B**Fig. 3C*

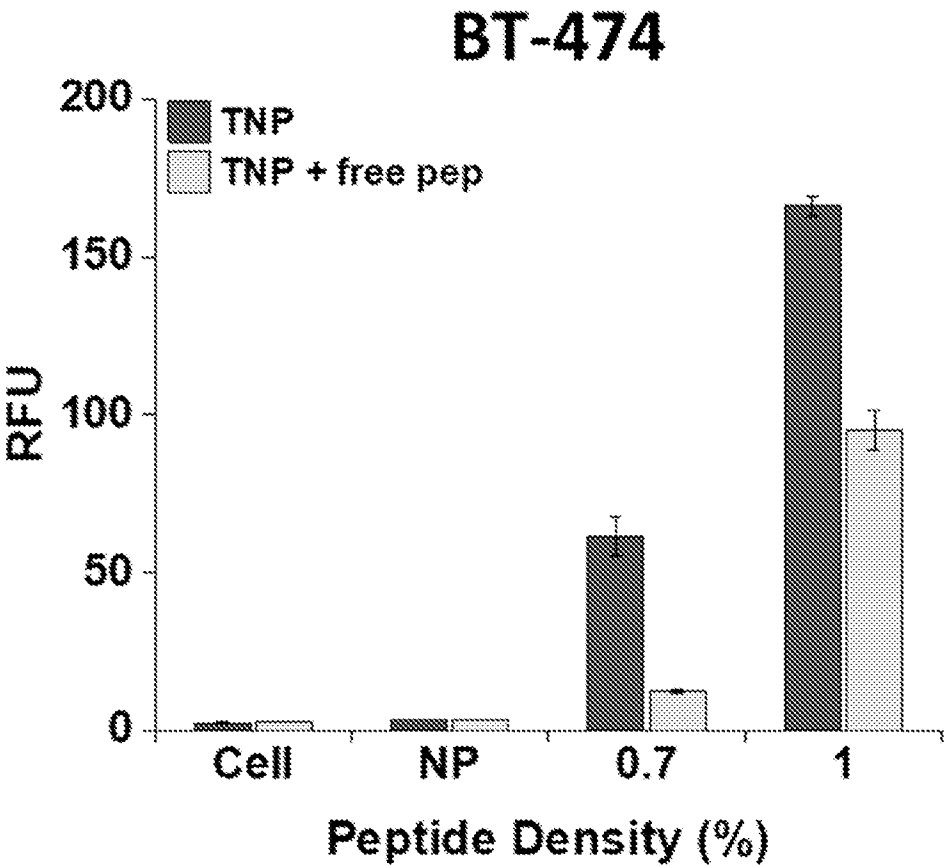


Fig. 3D

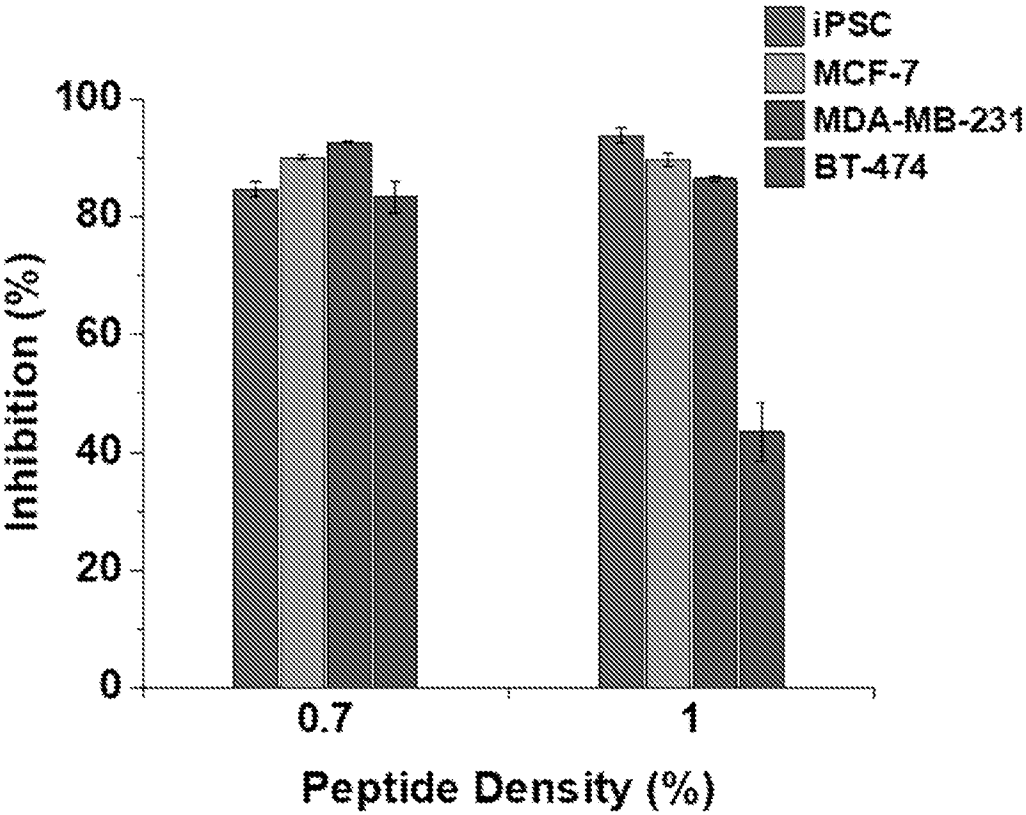


Fig. 3E

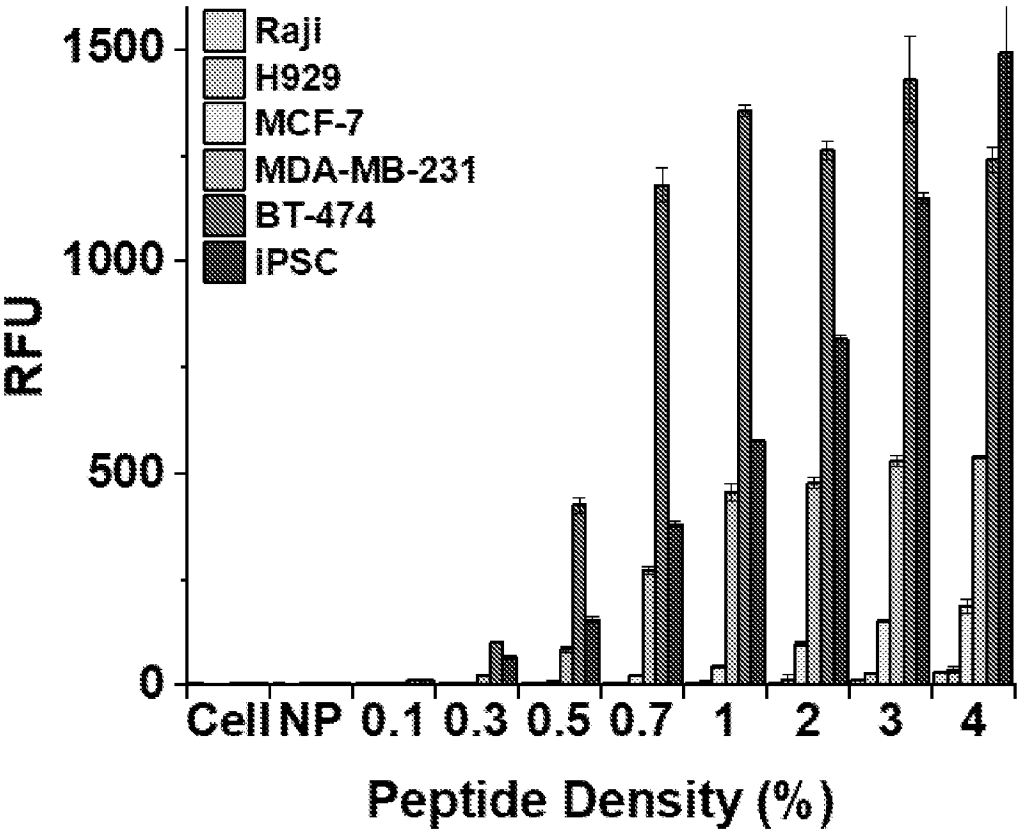


Fig. 4

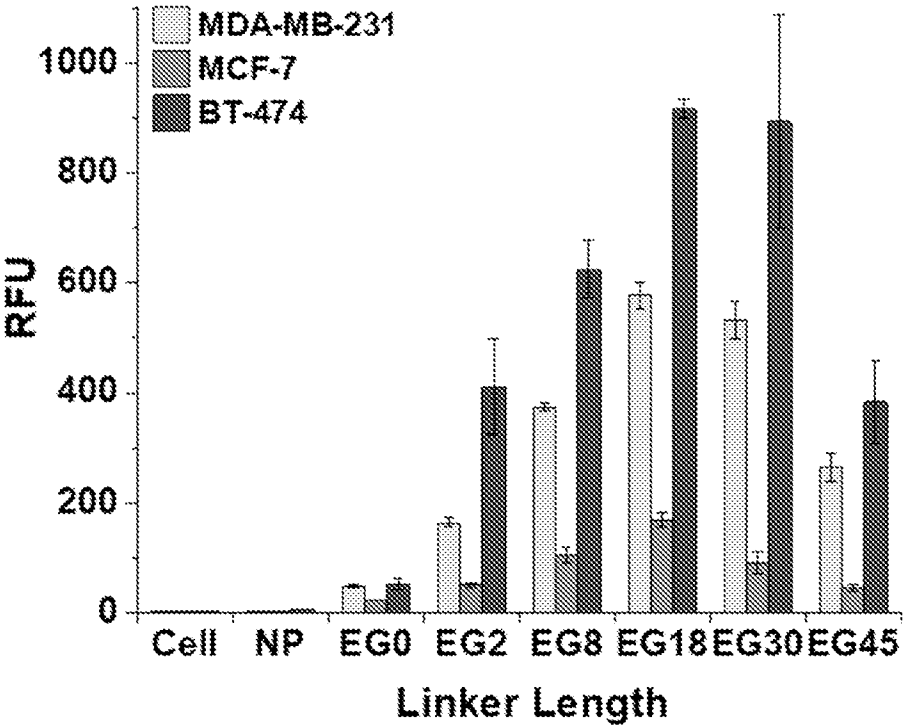


Fig. 5A

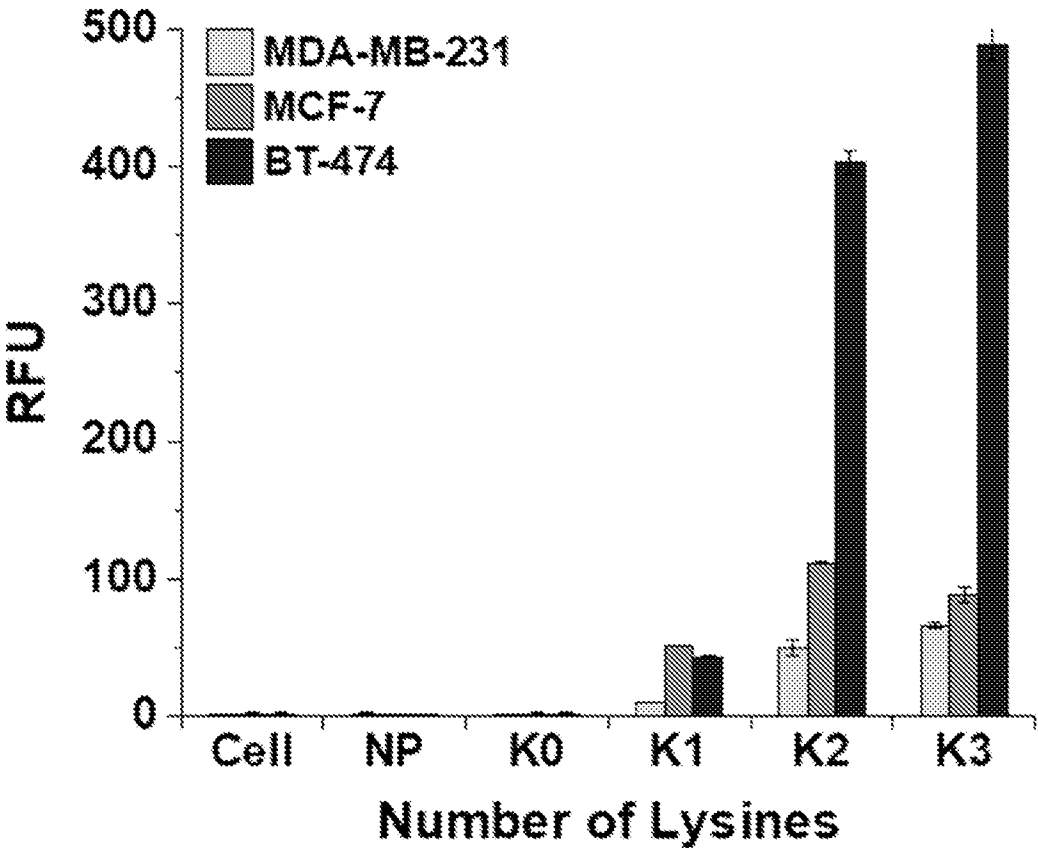


Fig. 5B

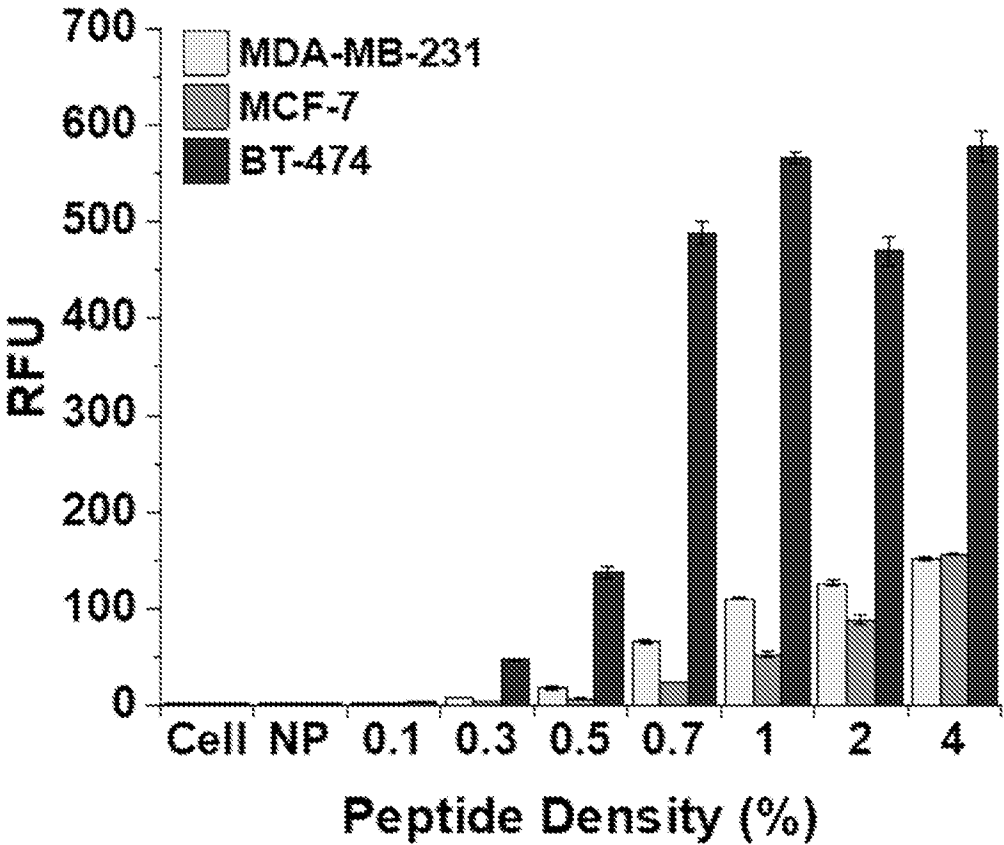


Fig. 5C

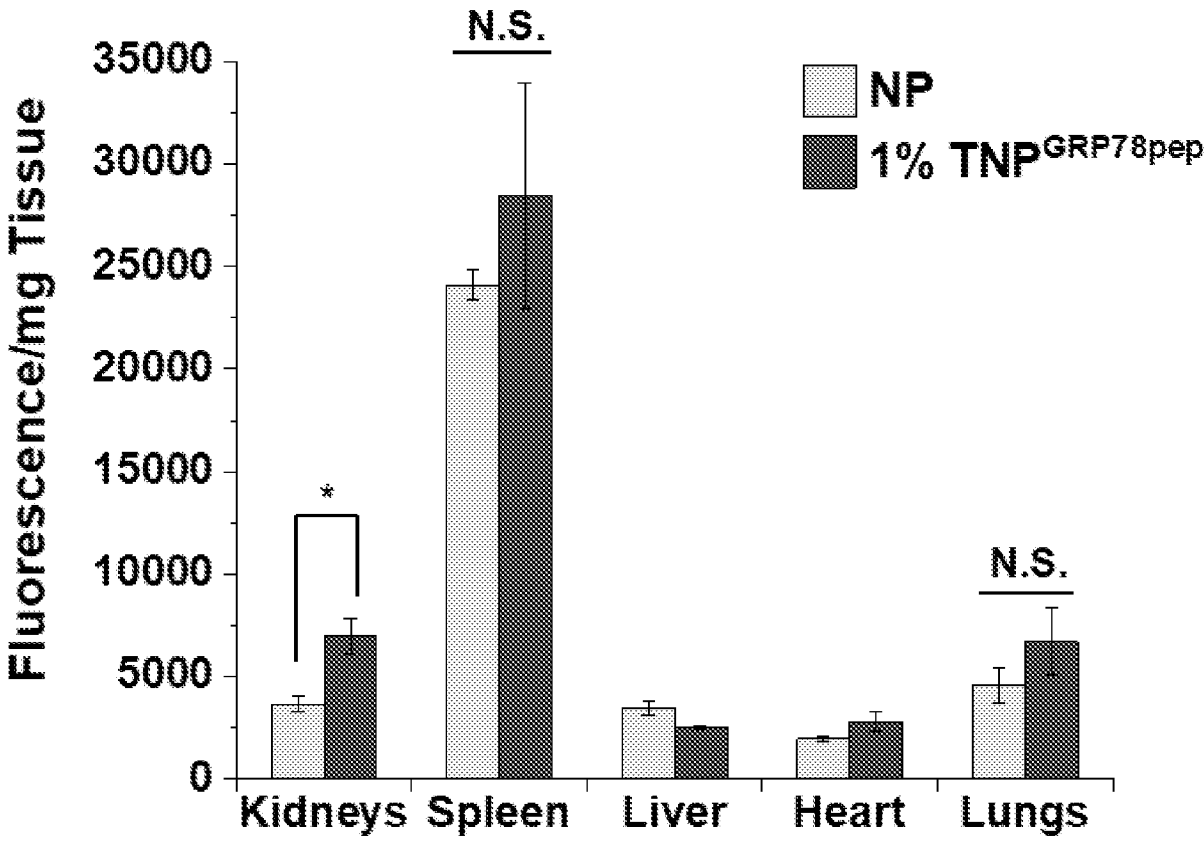


Fig. 6

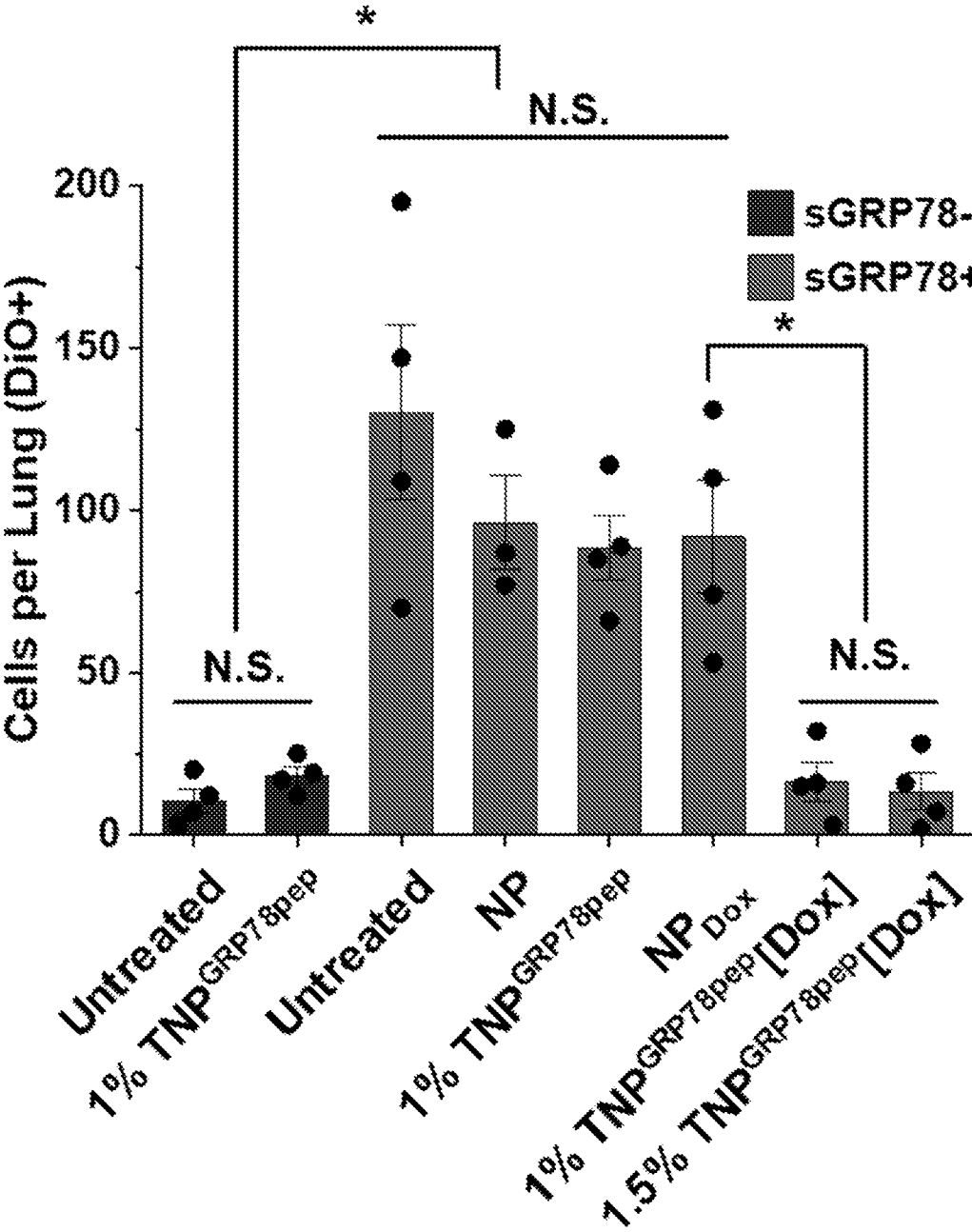


Fig. 7

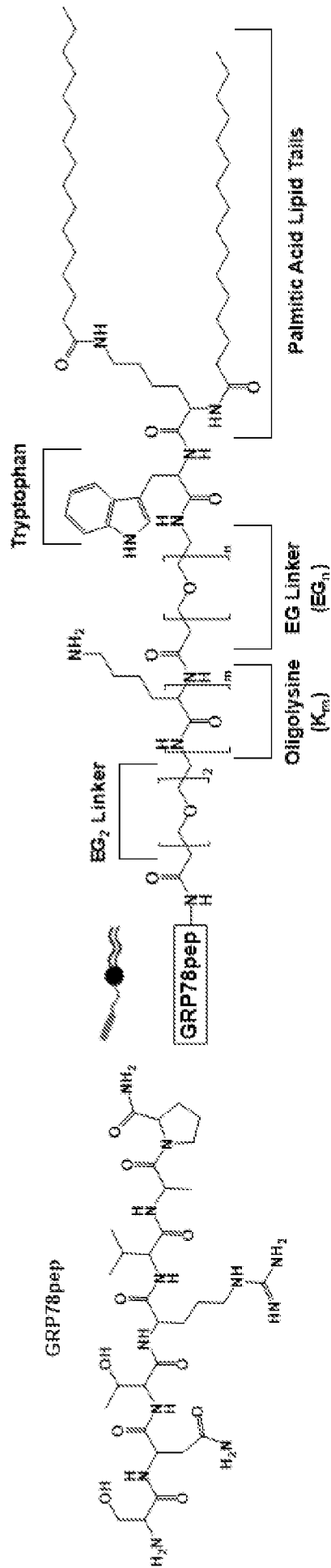


Fig. 8A

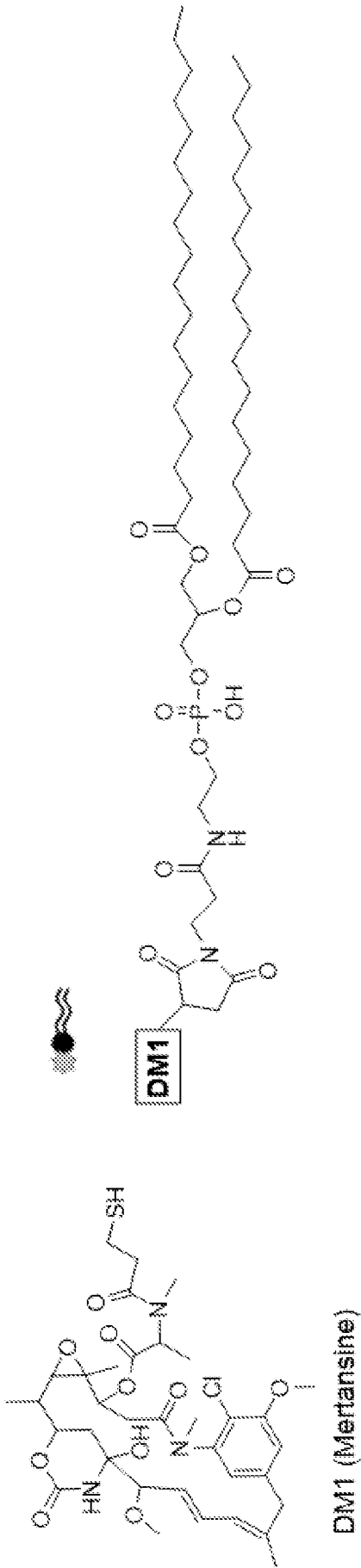


Fig. 8B

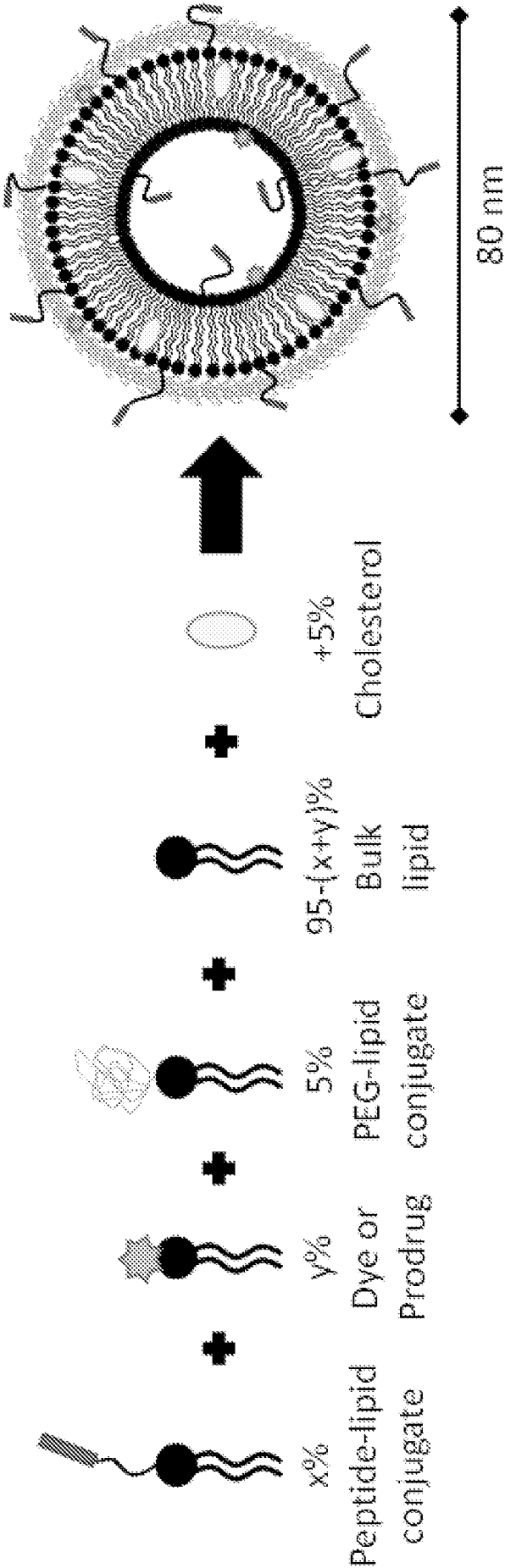
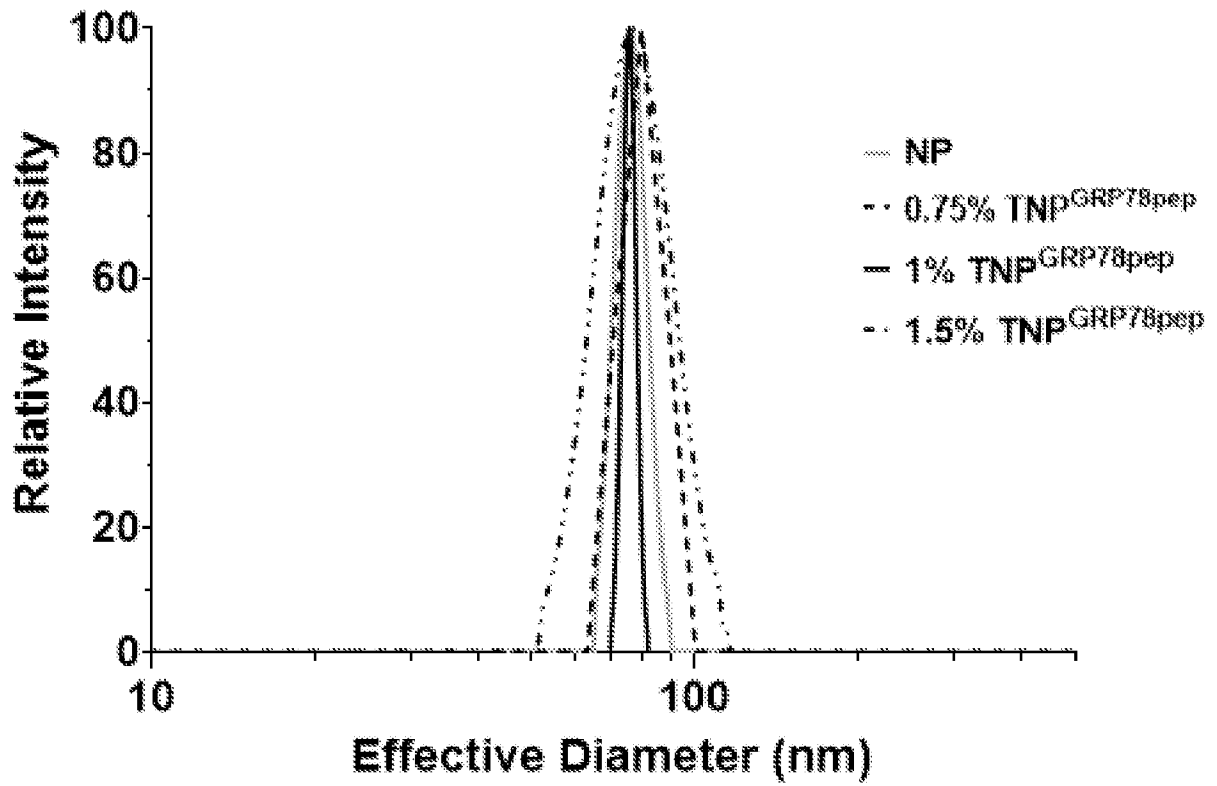


Fig. 8C

*Fig. 8D*

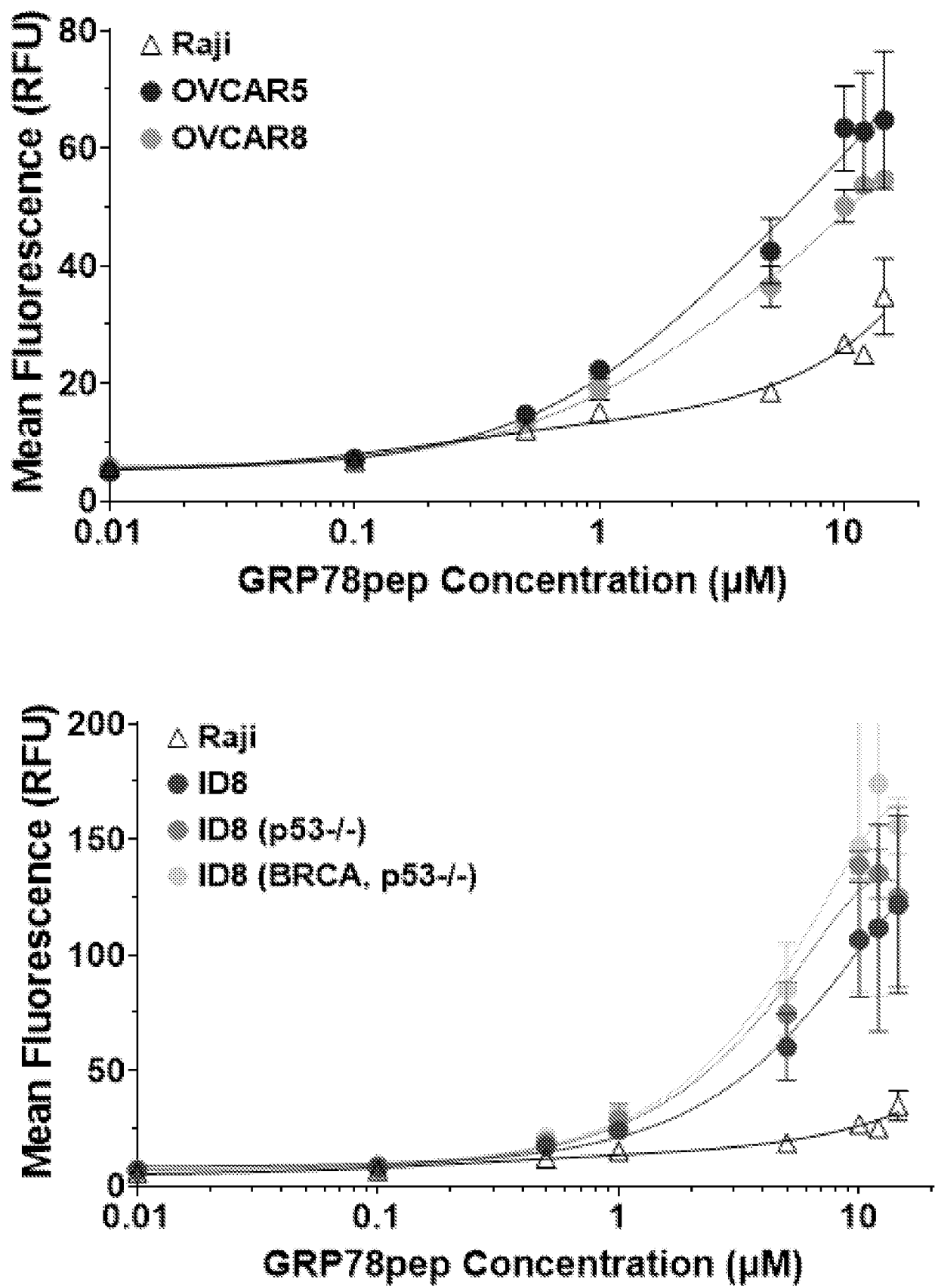


Fig. 9A

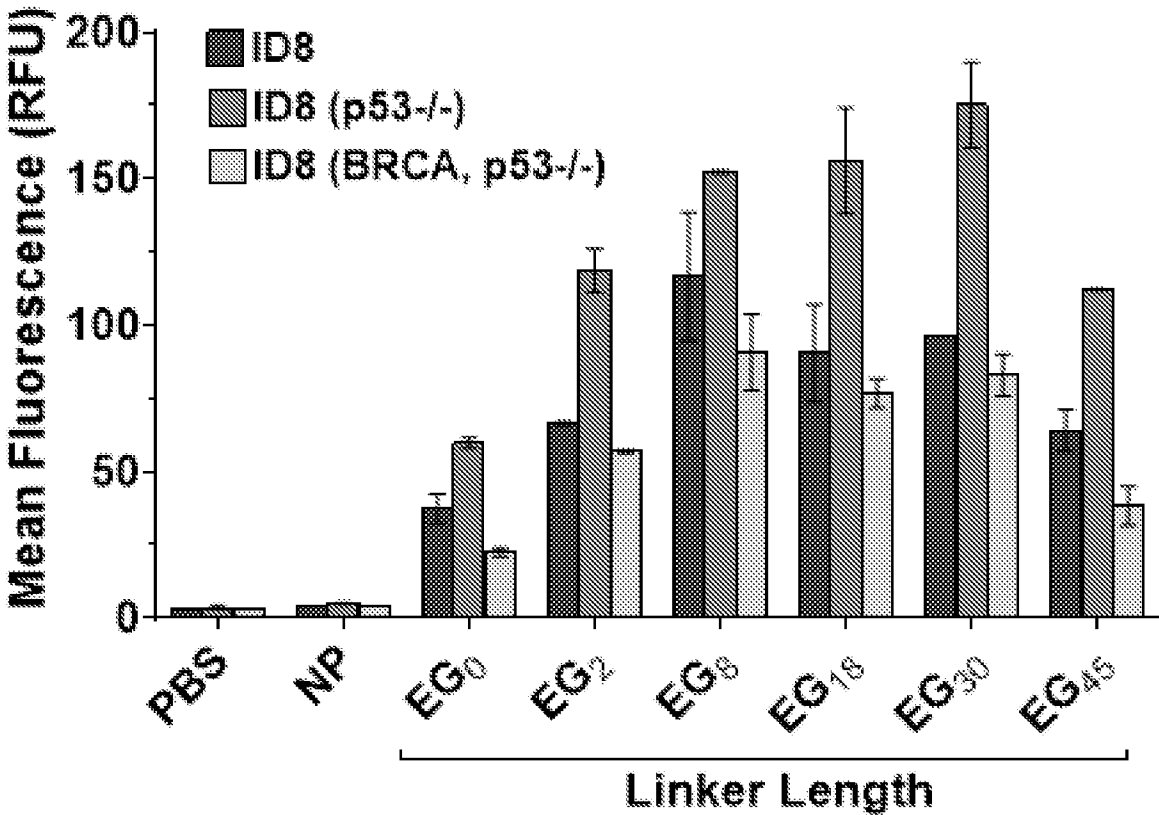
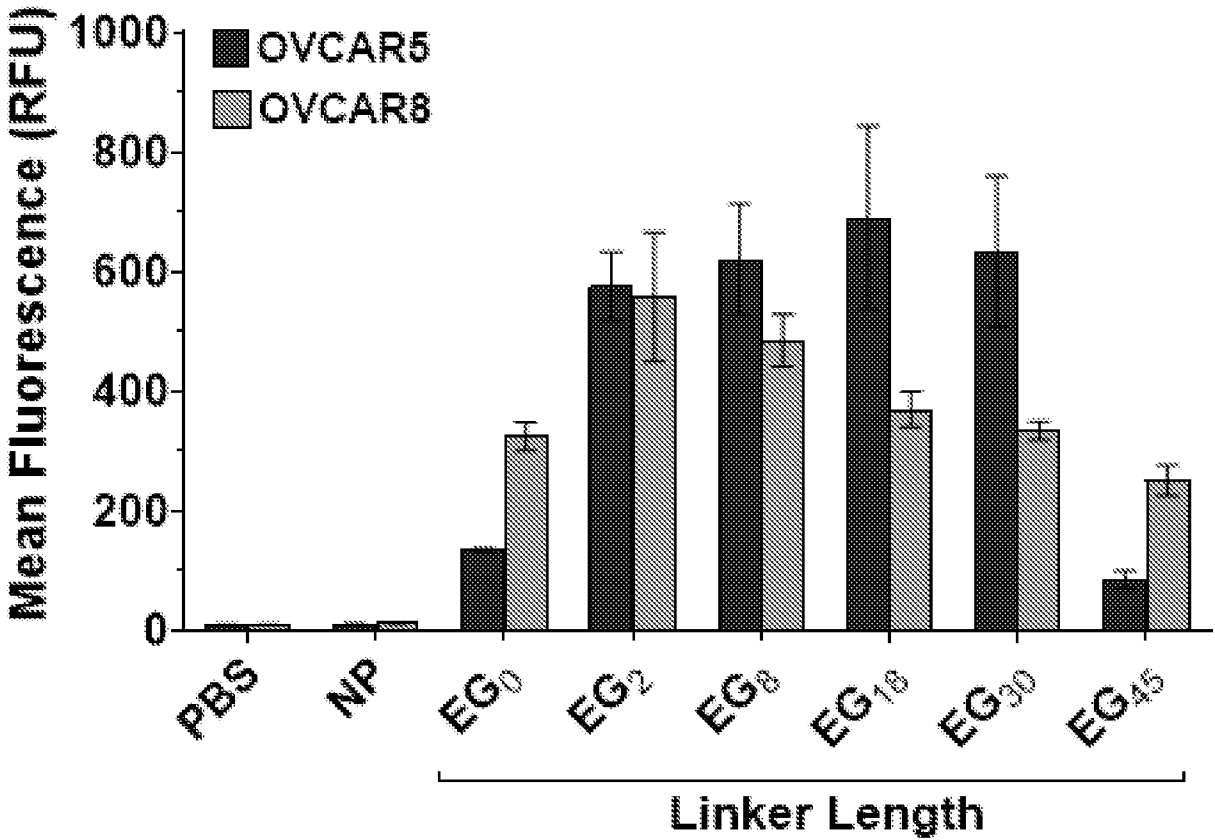


Fig. 9B

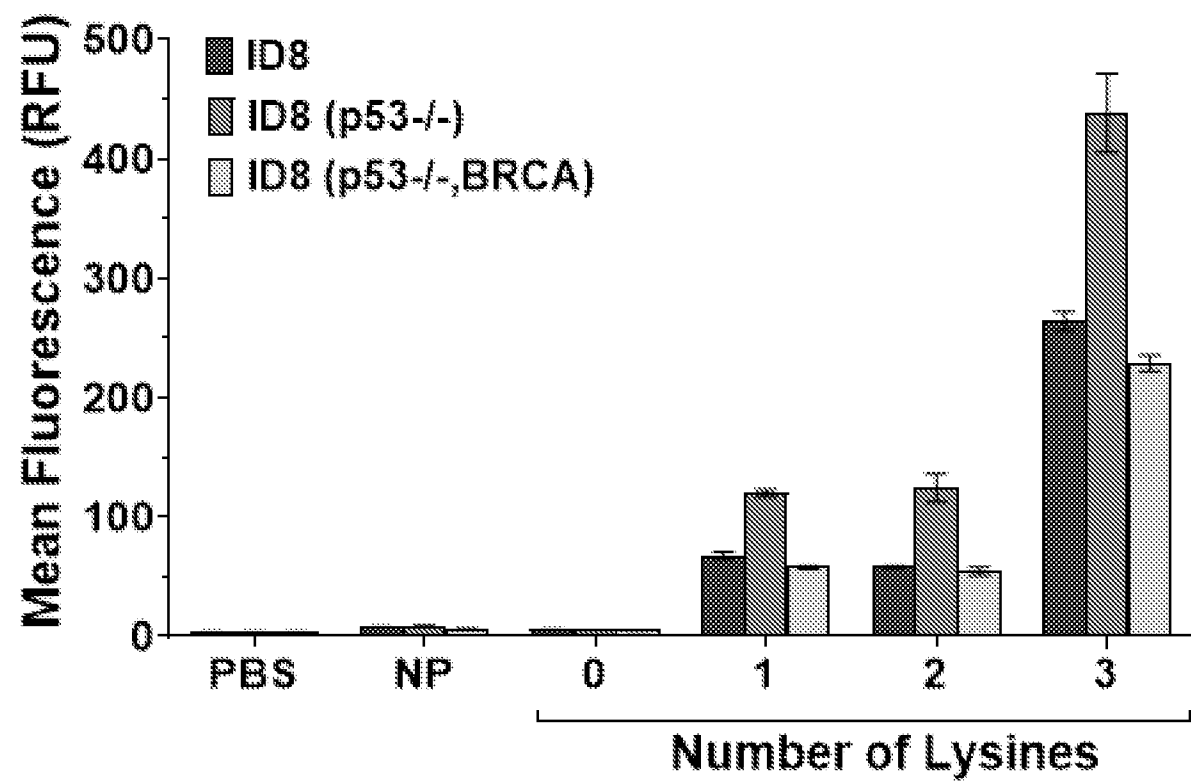
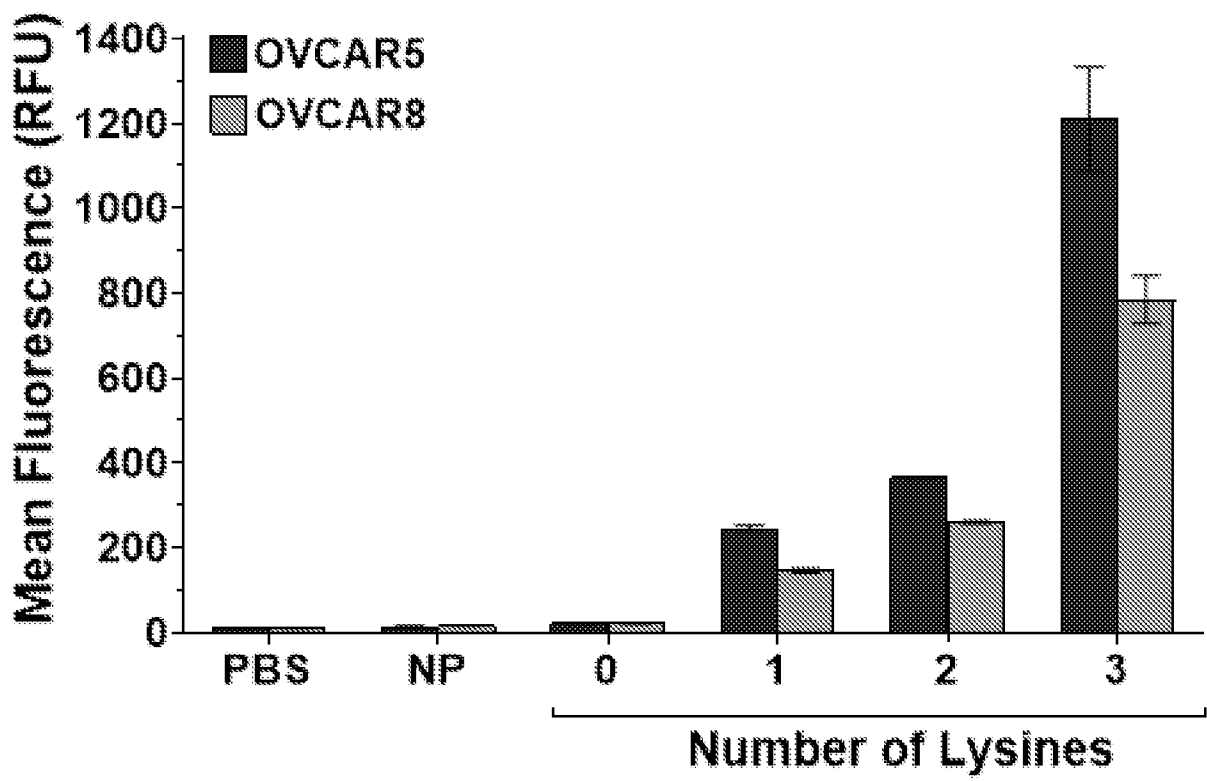


Fig. 9C

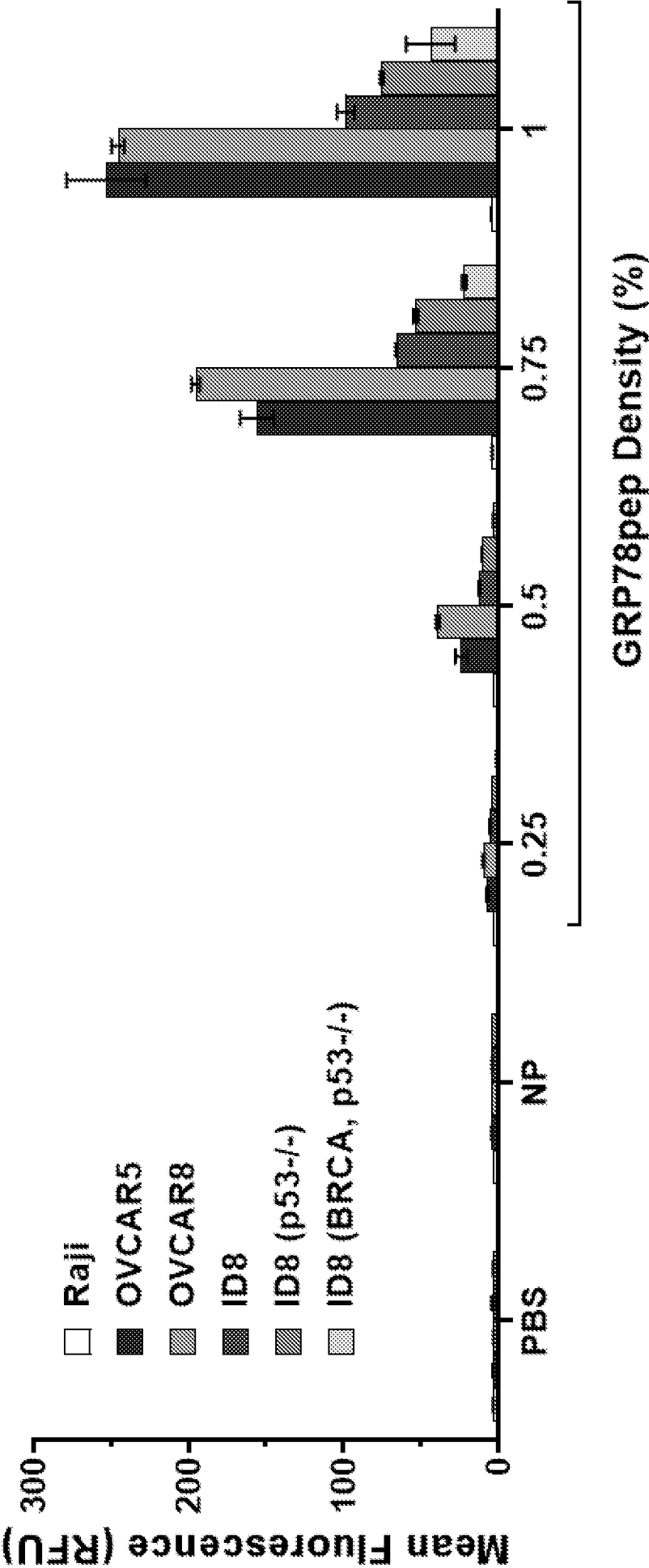


Fig. 9D

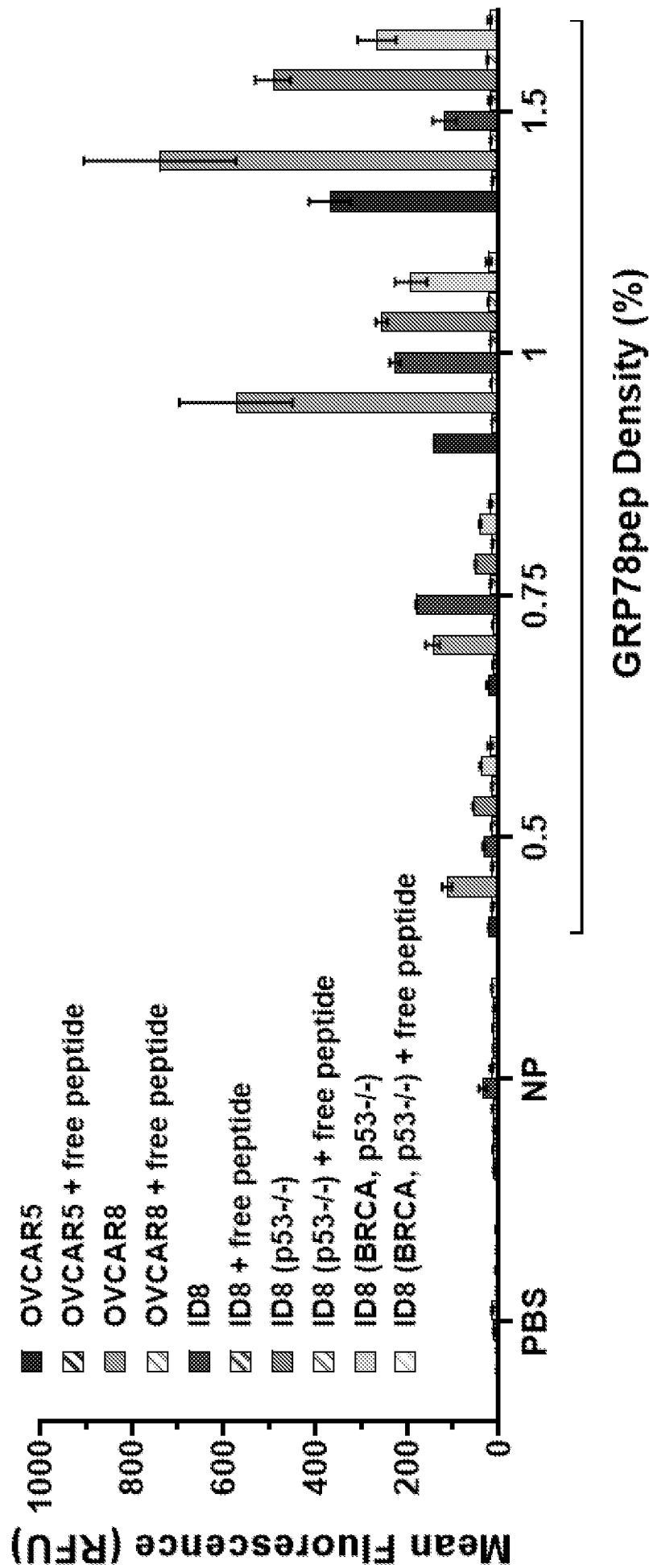


Fig. 9E

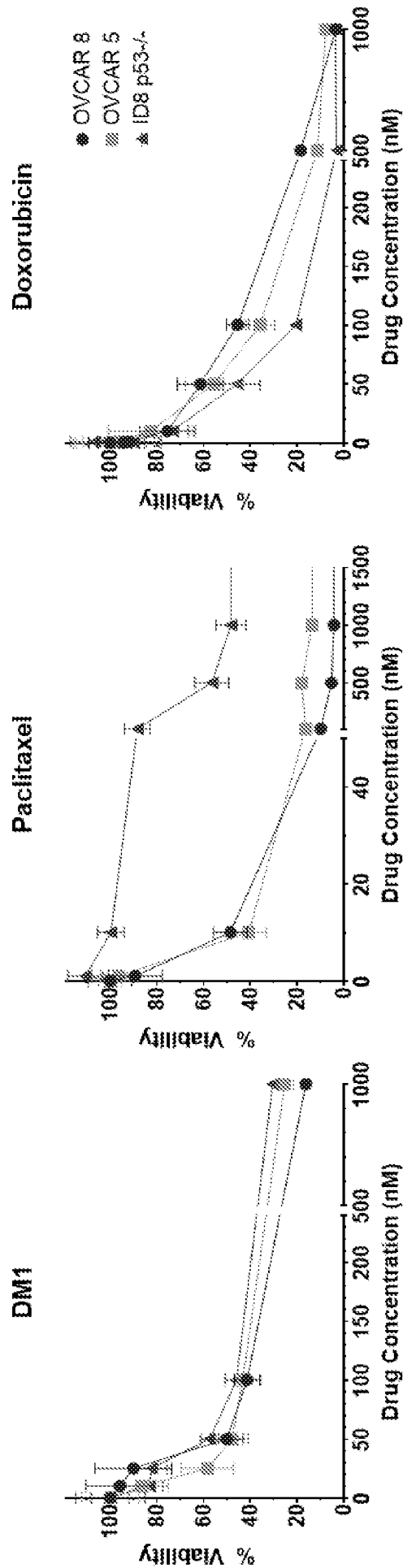


Fig. 10A

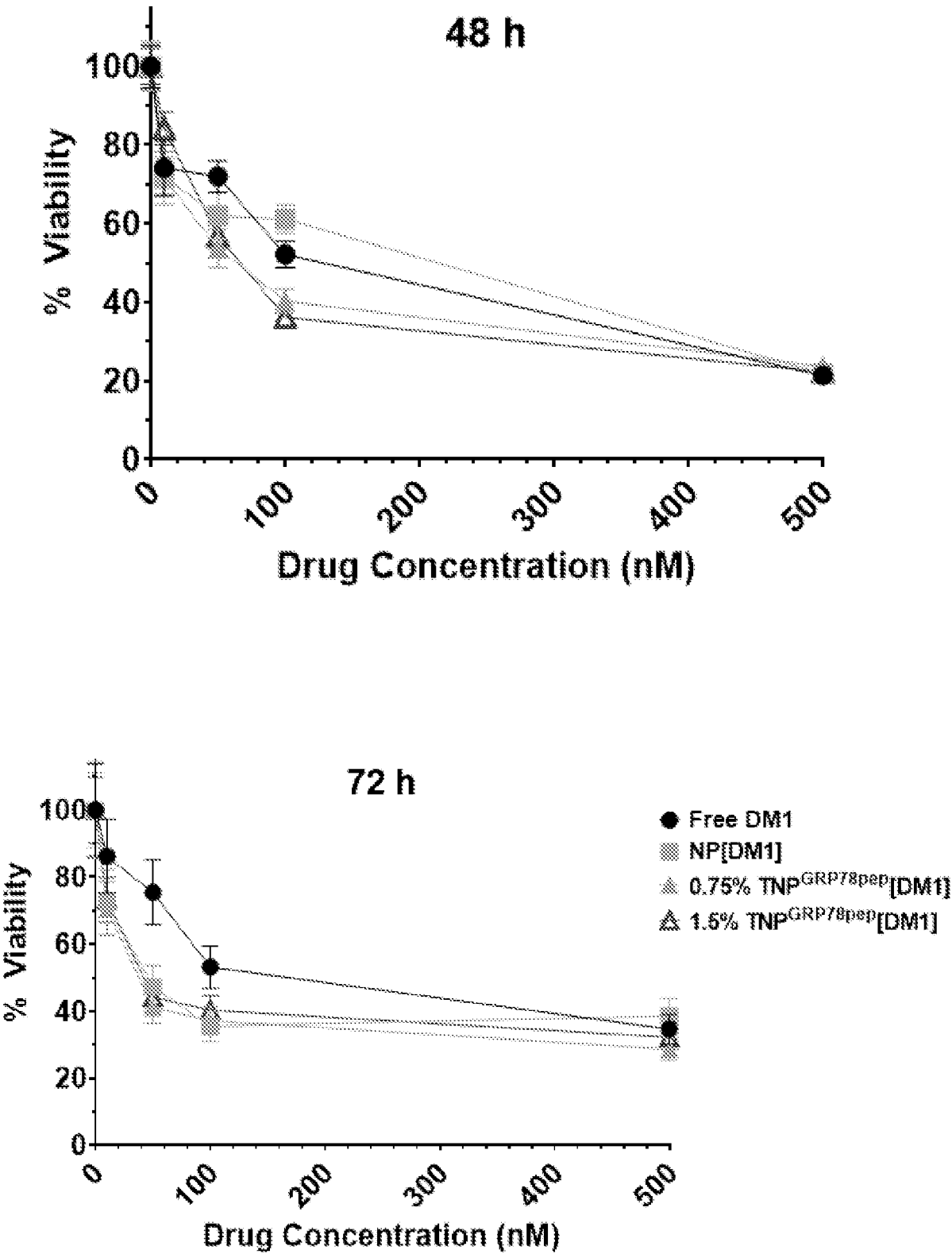
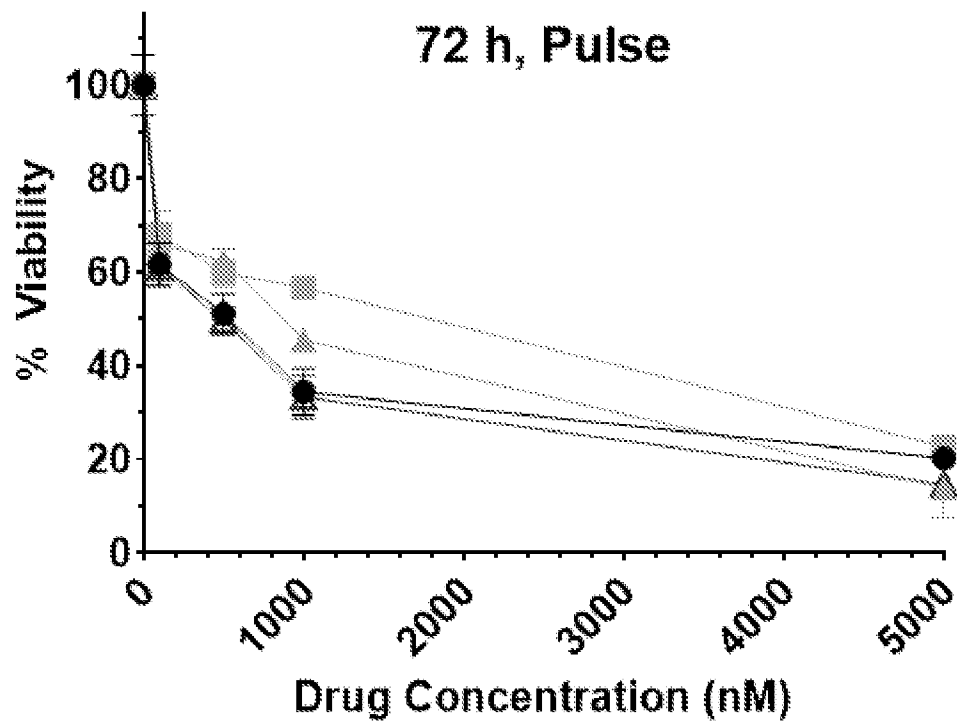
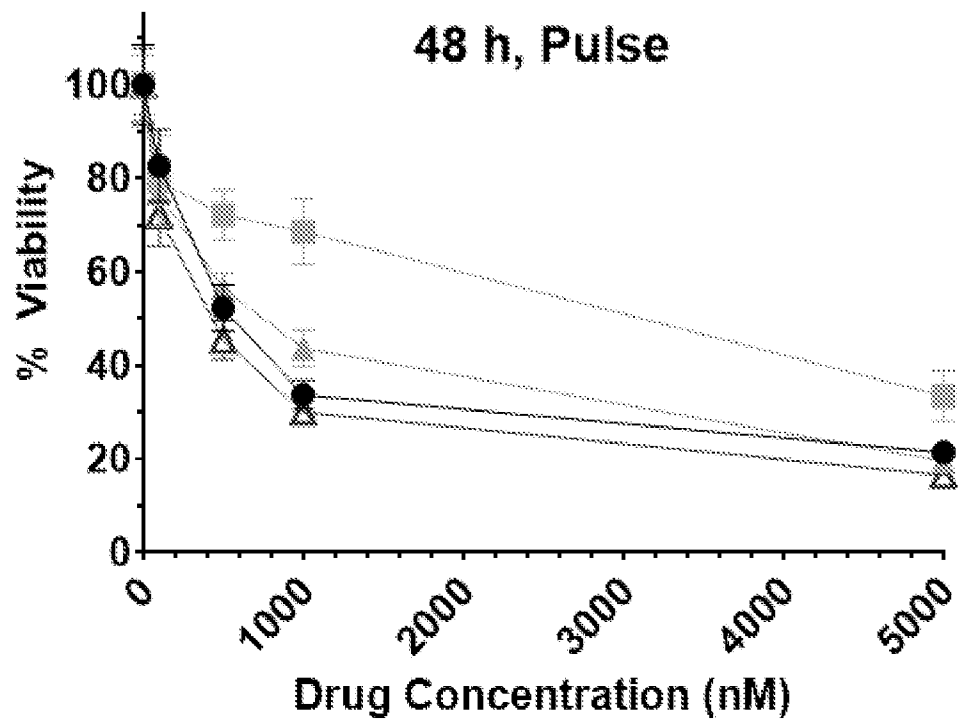
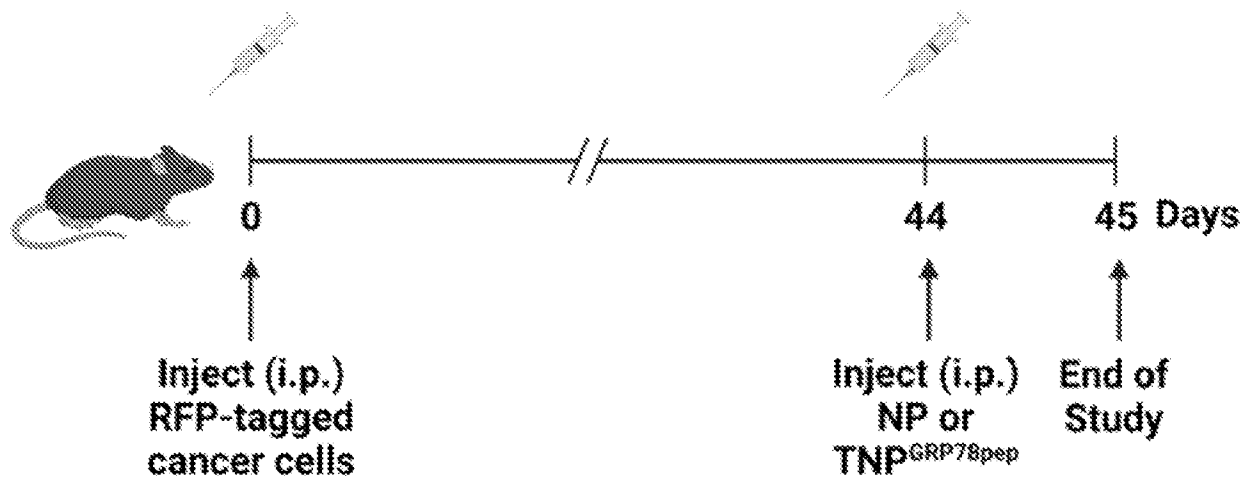


Fig. 10B

*Fig. 10C*

*Fig. 11A*

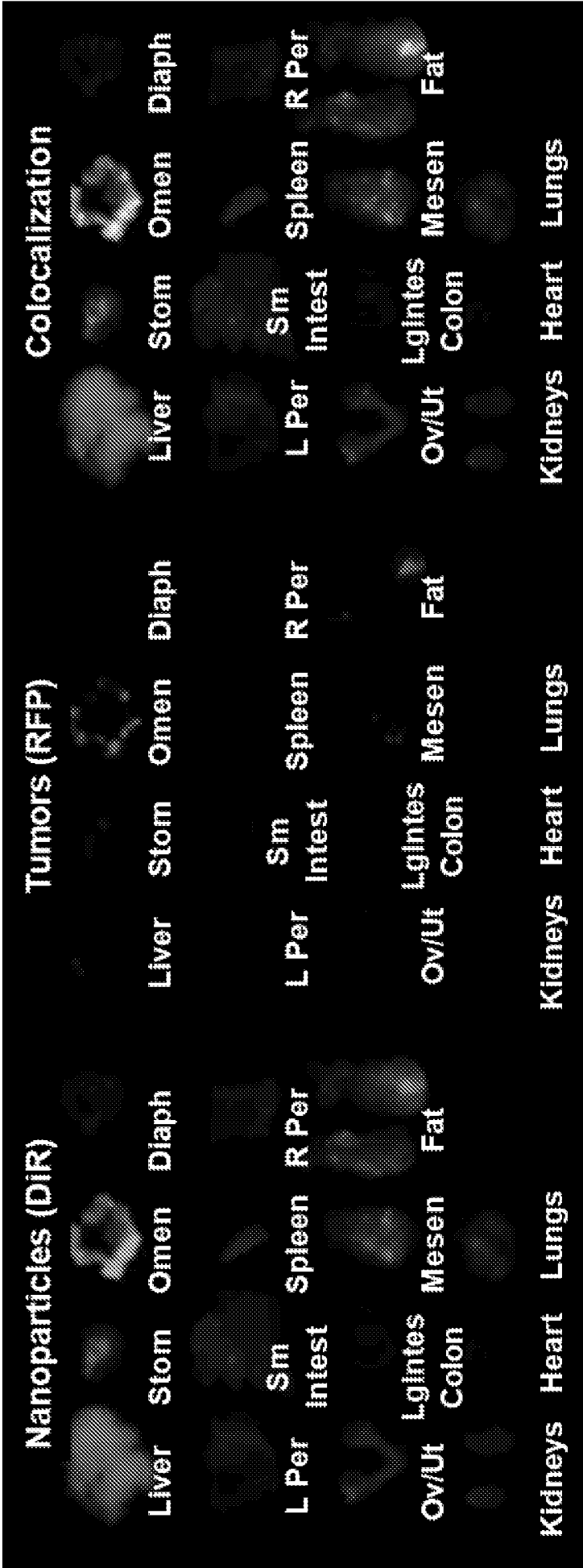


Fig. 11B

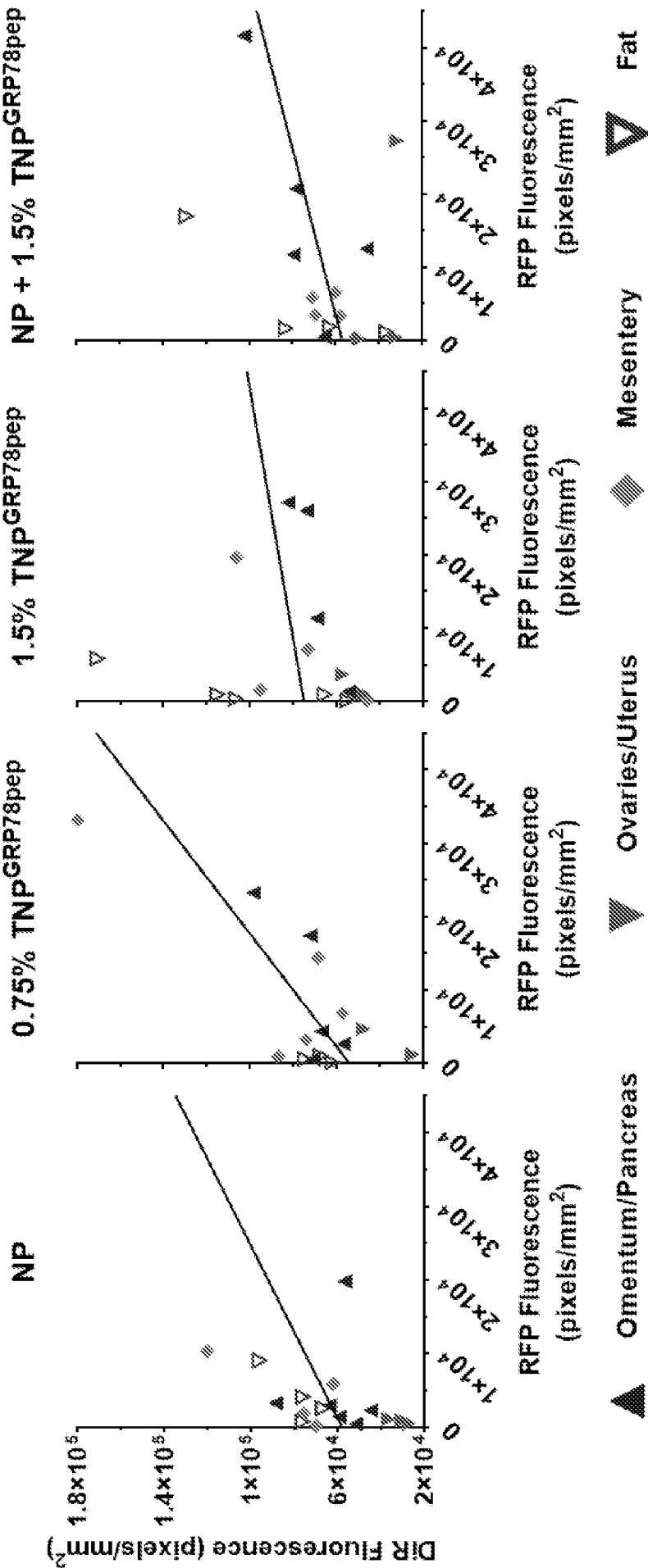


Fig. 11C

Colocalization to RFP+ Solid Tumors

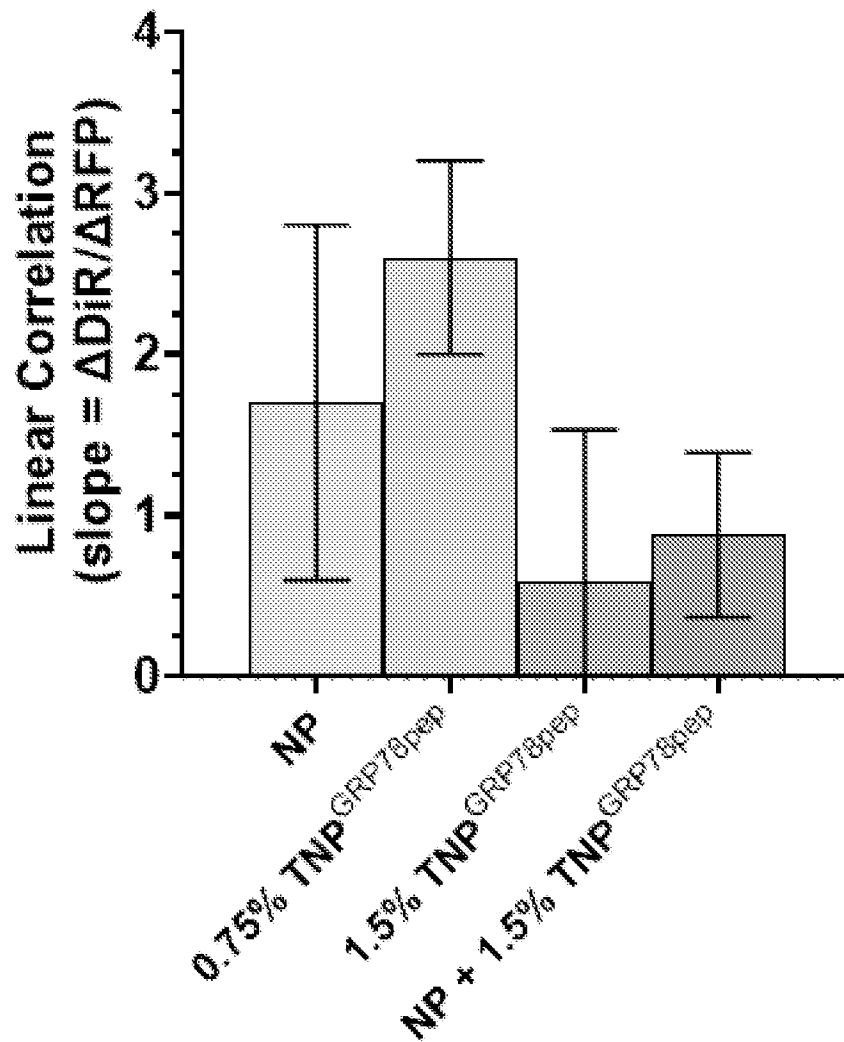


Fig. 11D

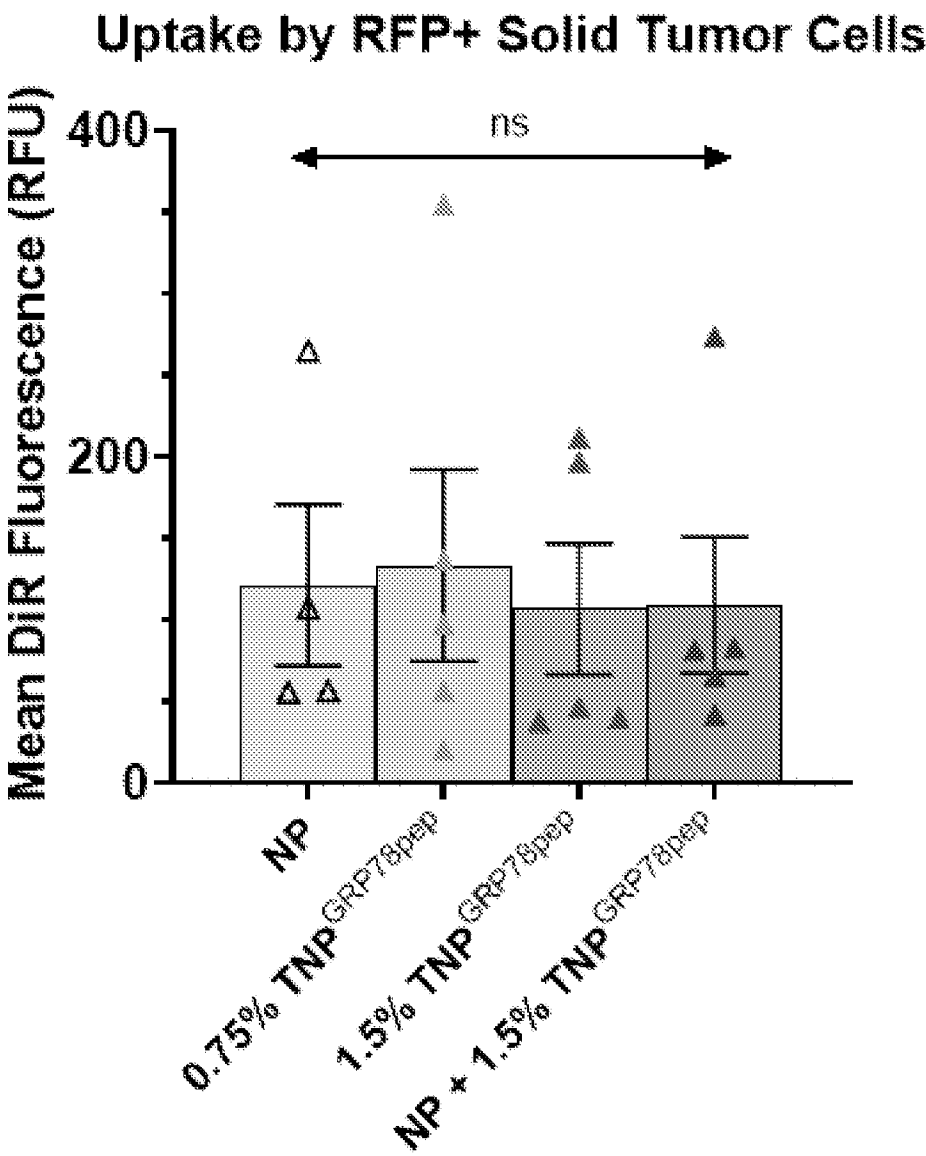


Fig. 11E

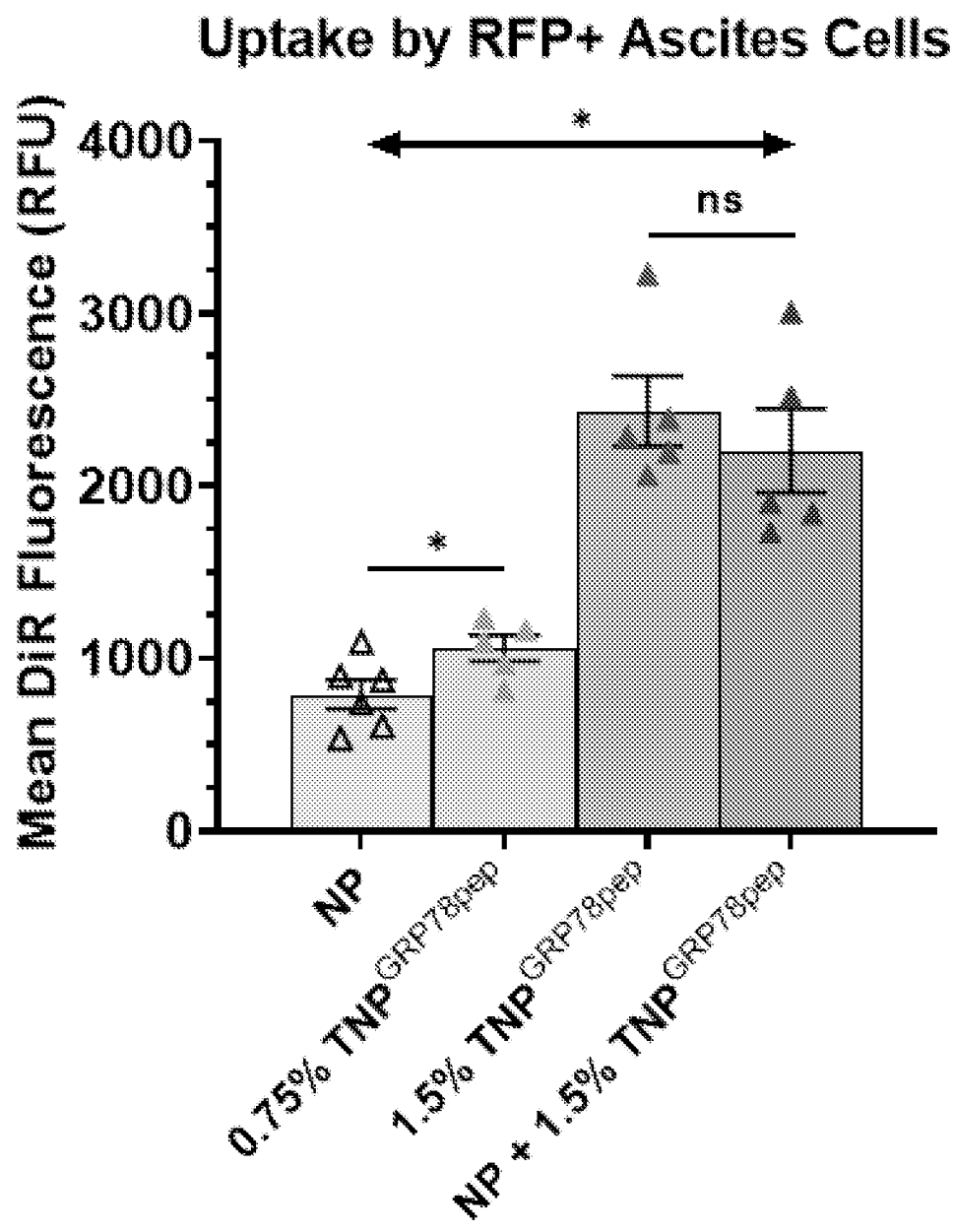


Fig. 11F

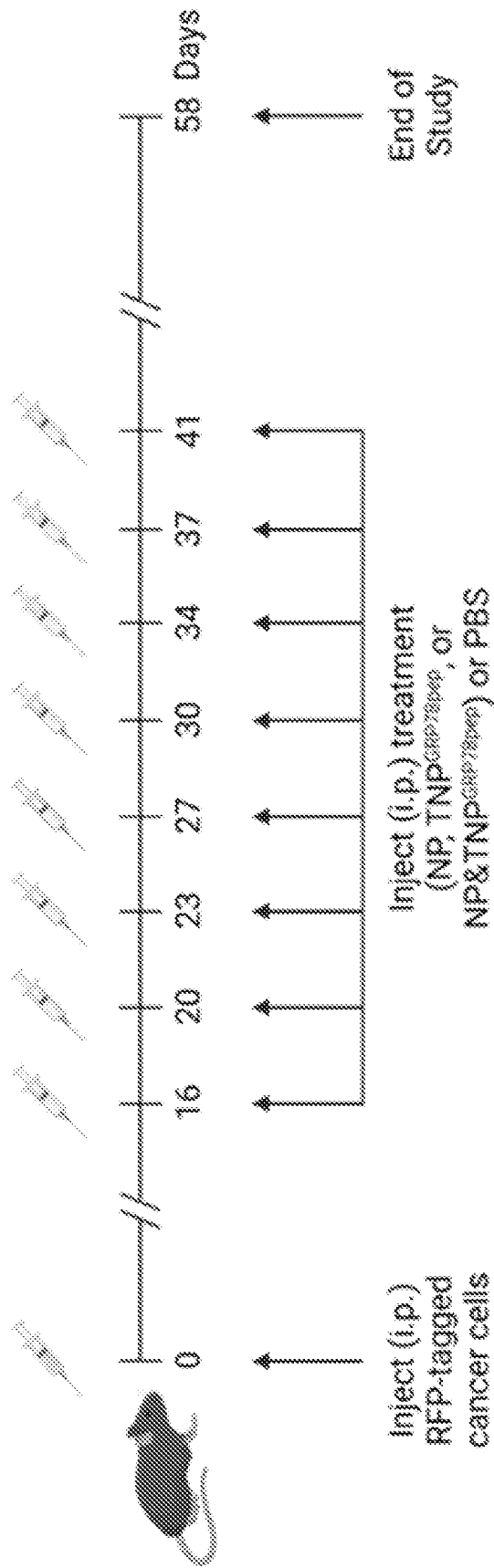
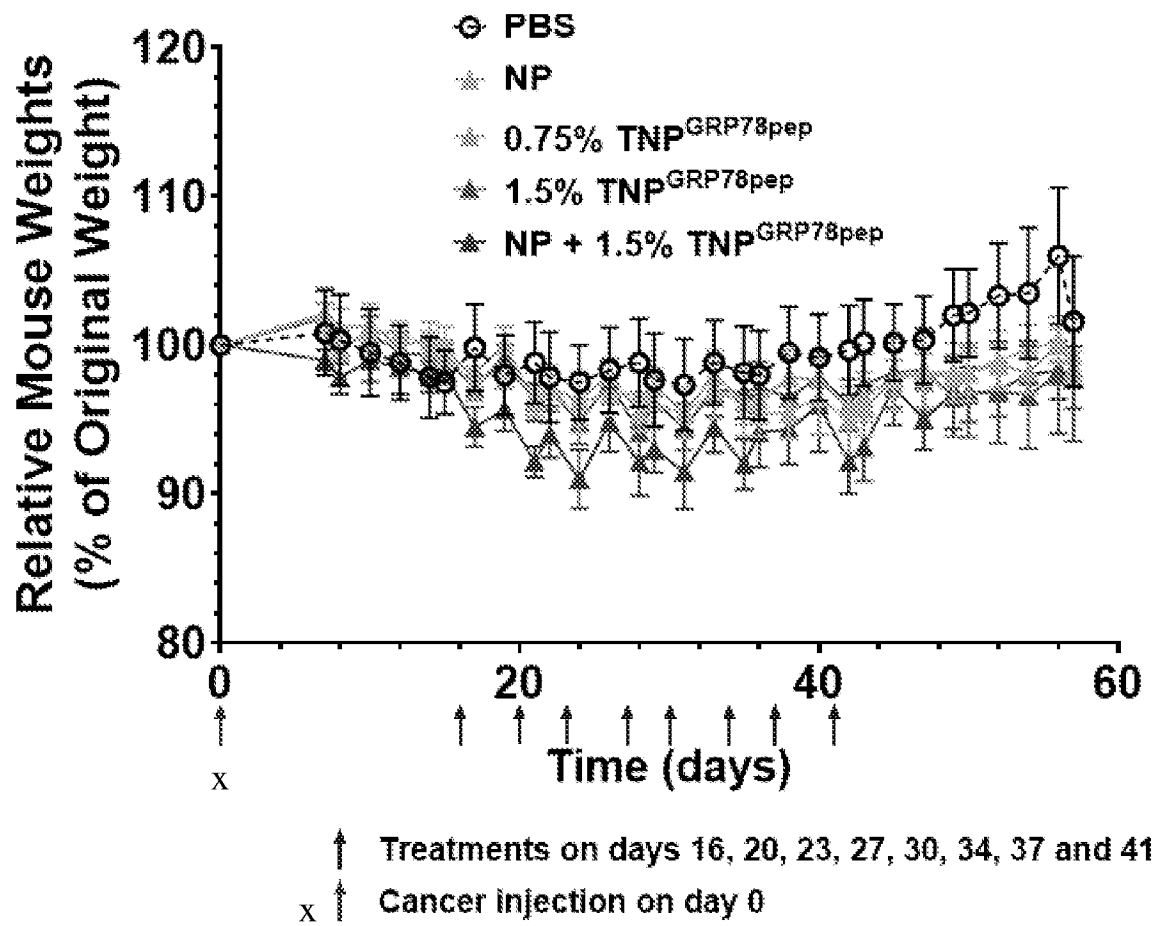


Fig. 12A

**Fig. 12B**

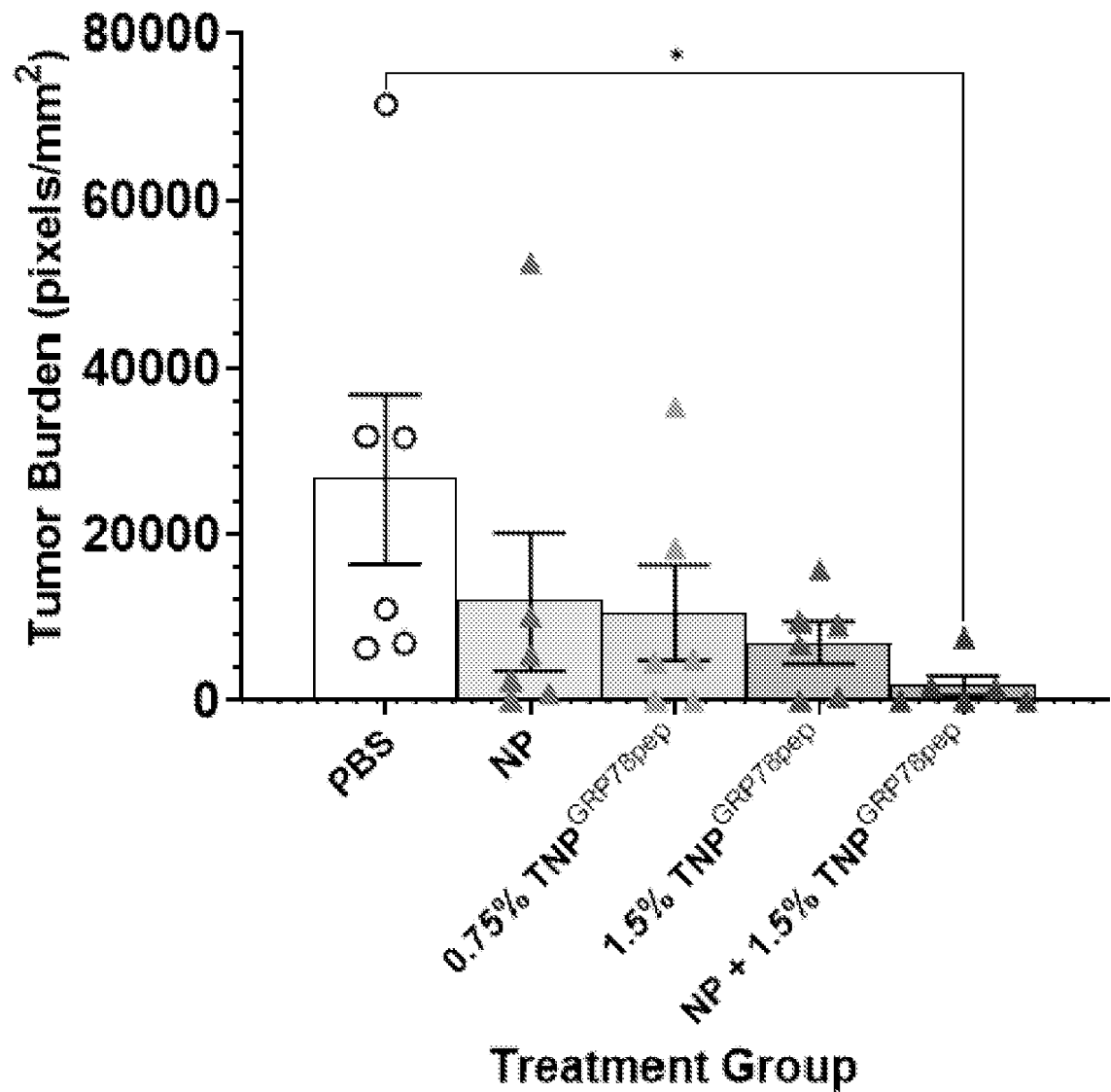


Fig. 12C

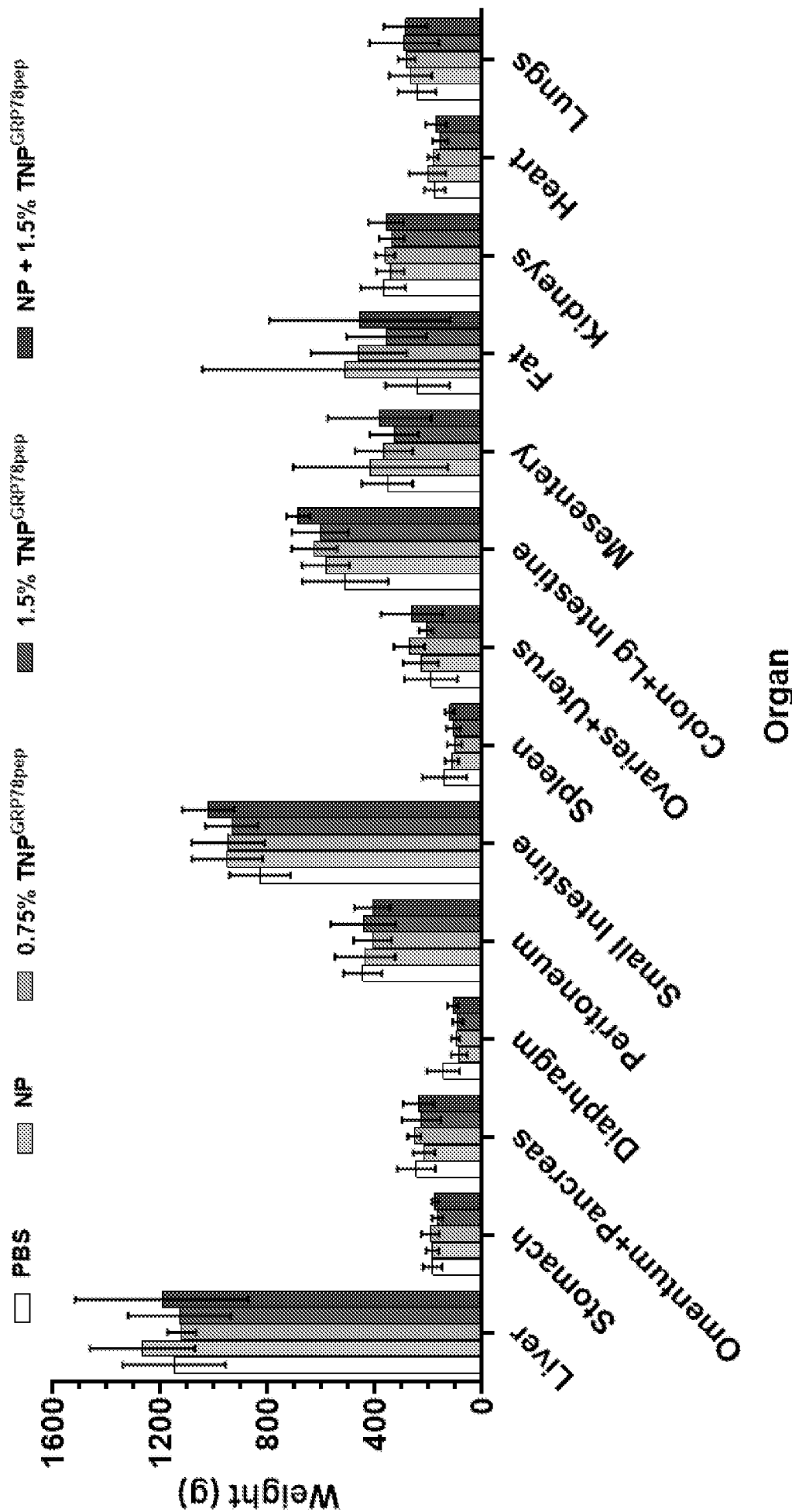


Fig. 12D

Anti-Tumorigenic T Cells

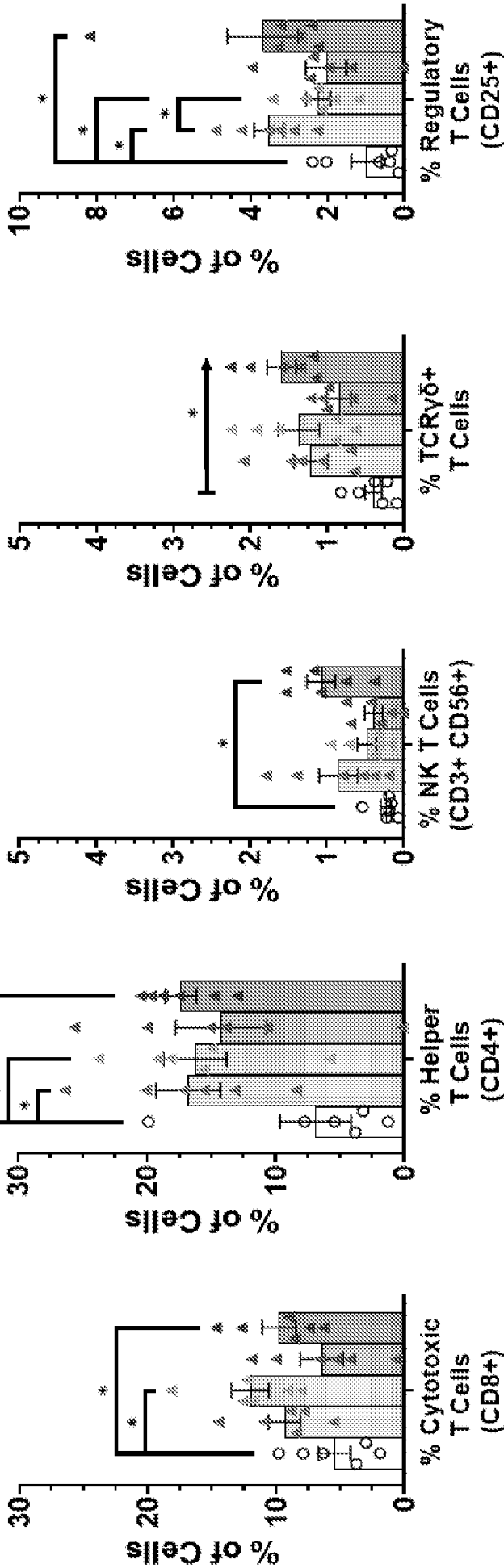


Fig. 13A

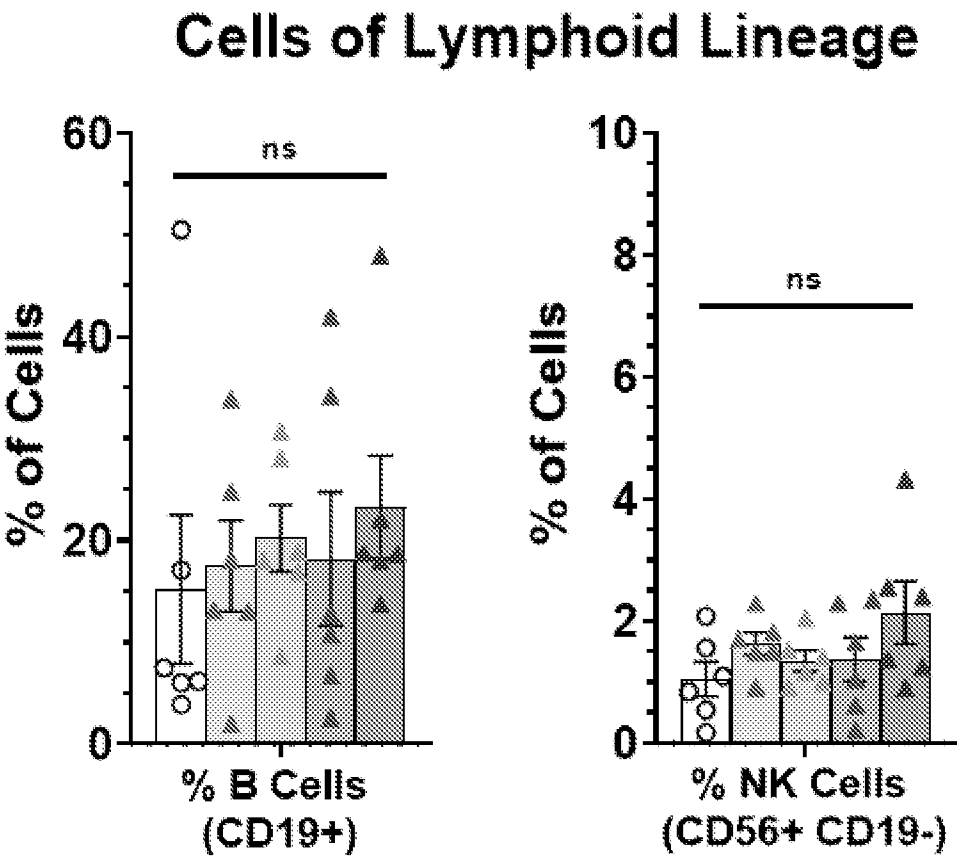


Fig. 13B

Anti-Tumorigenic Cells of Myeloid Lineage

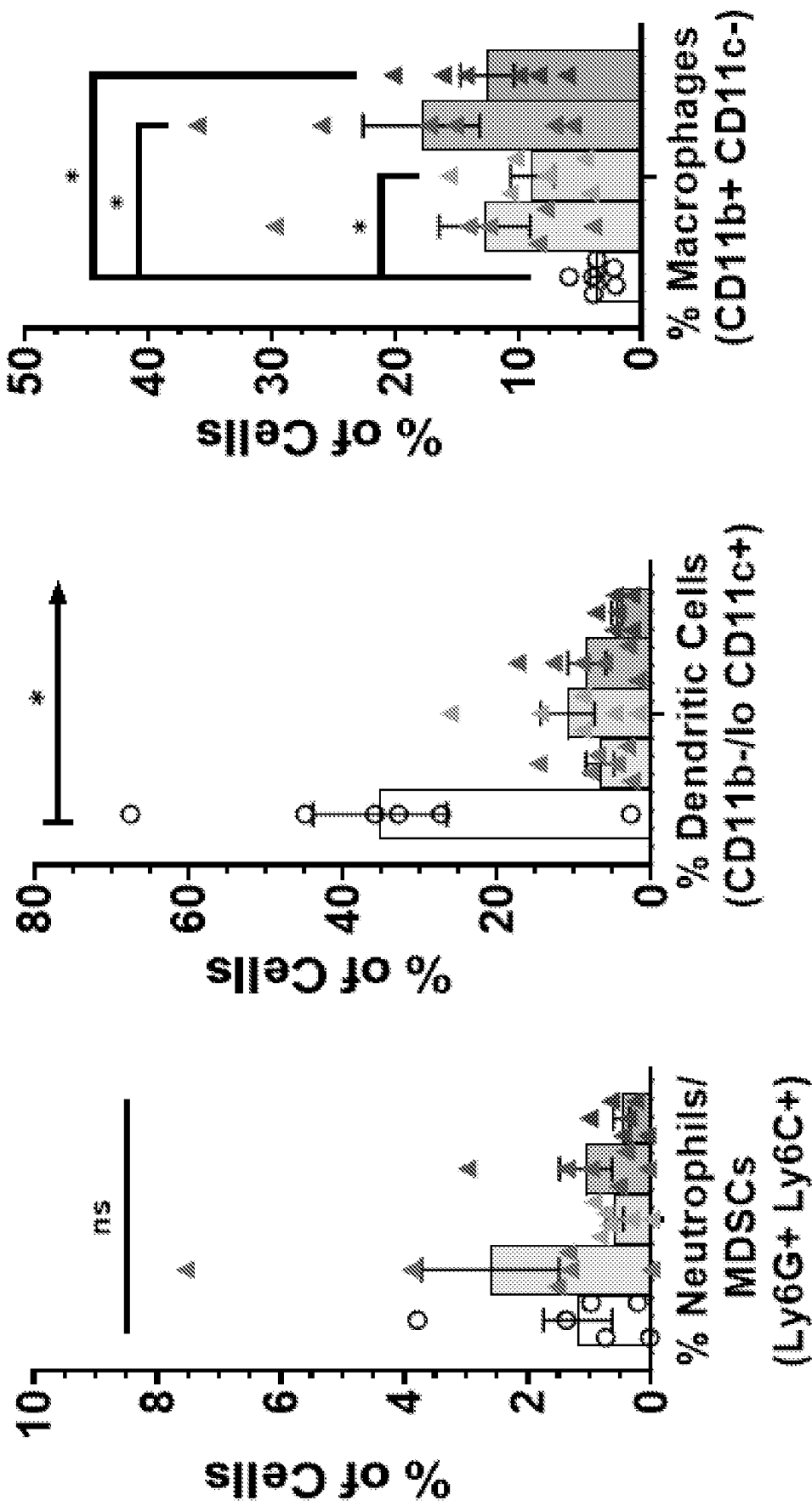


Fig. 13C

Anti-Tumorigenic Macrophages

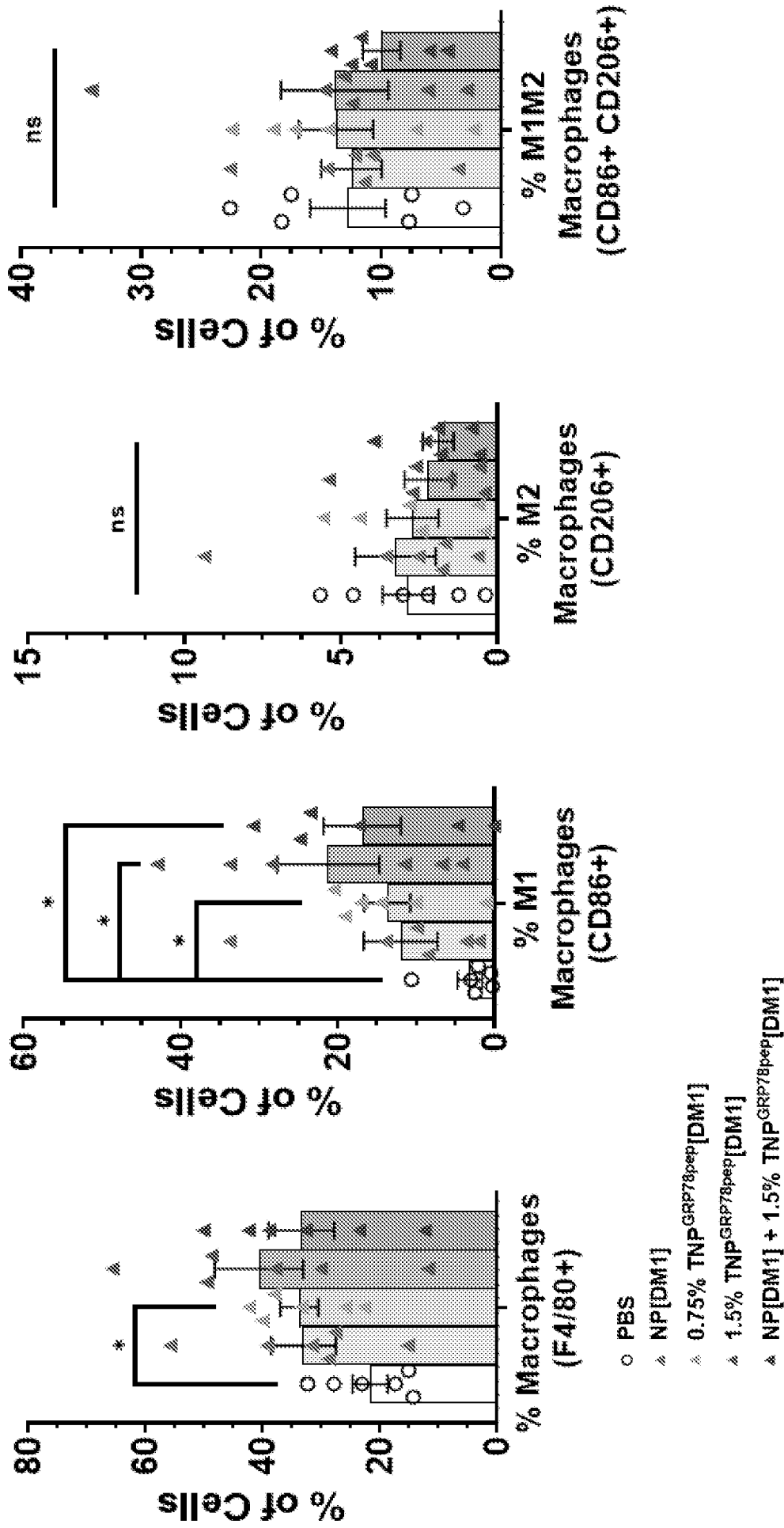


Fig. 13D

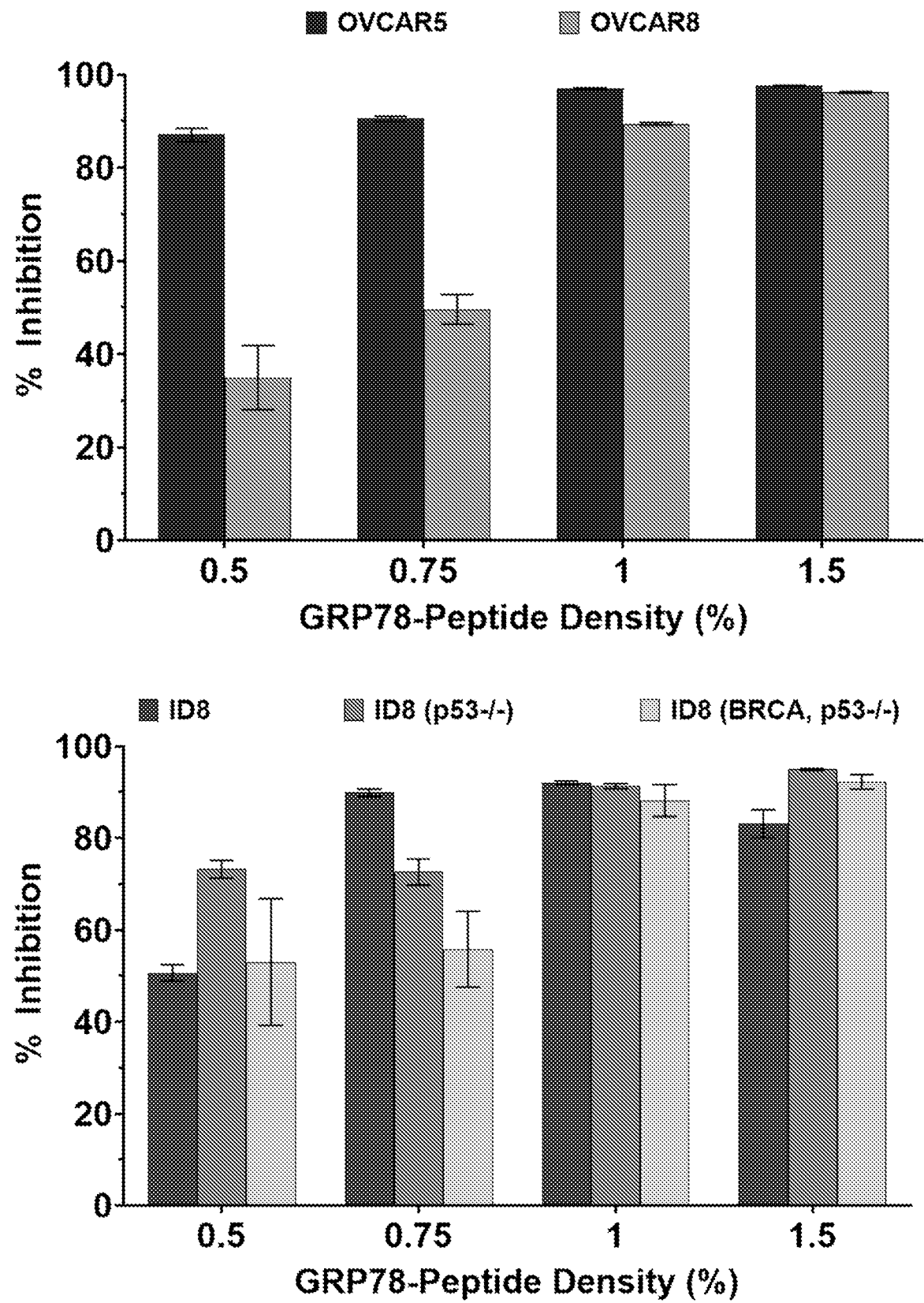


Fig. 14

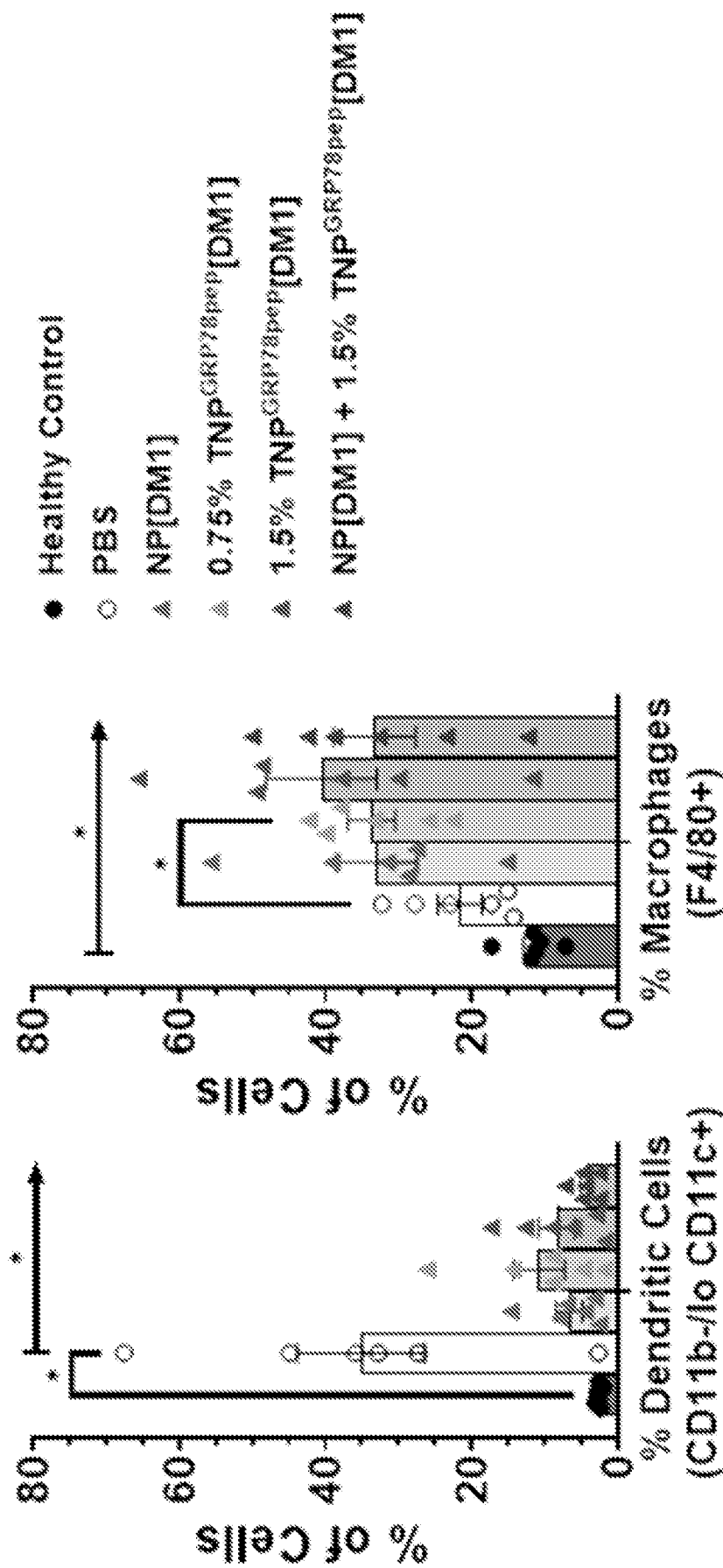
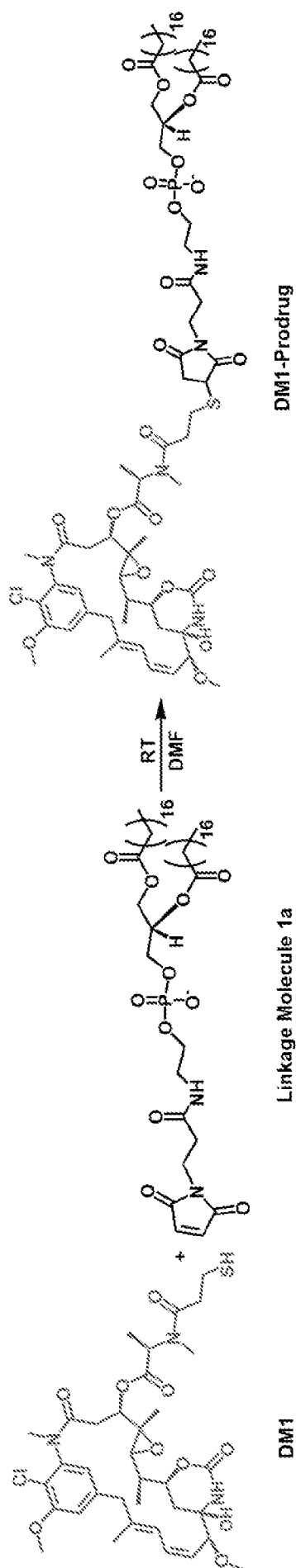
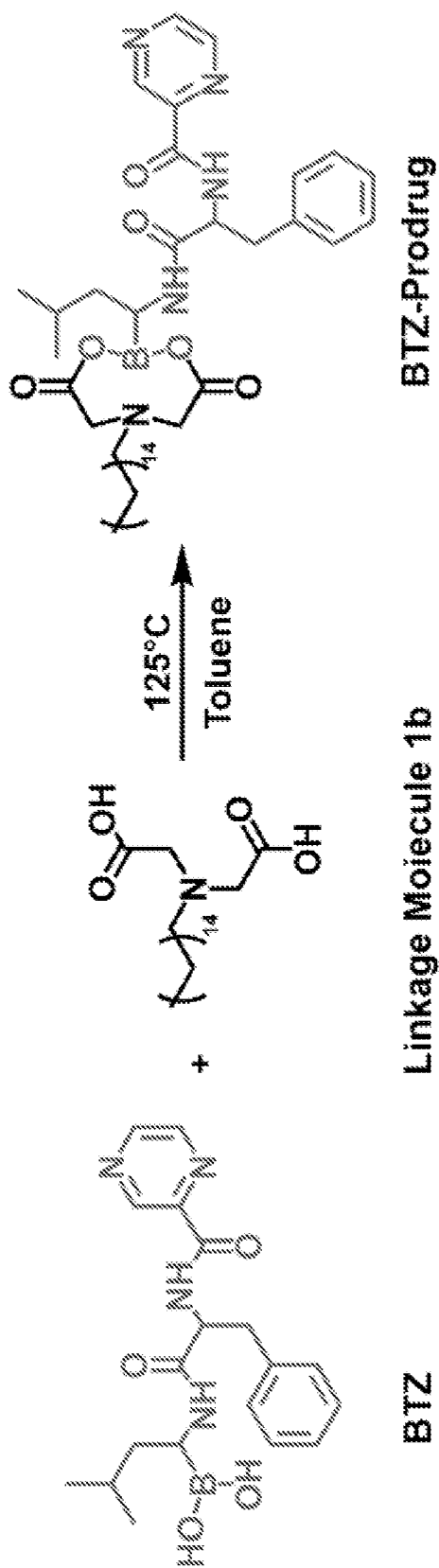


Fig. 15

**Fig. 16A****Fig. 16B**

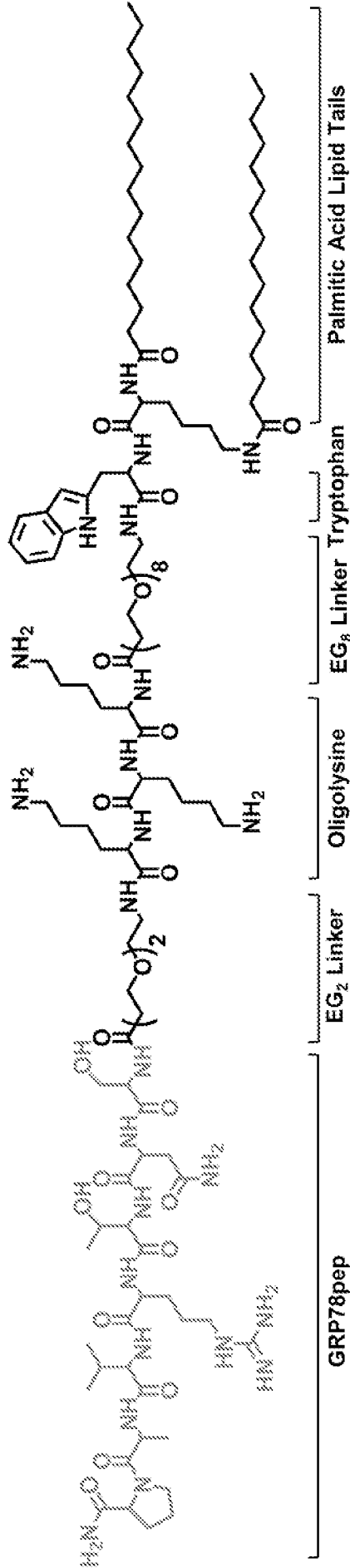


Fig. 16C

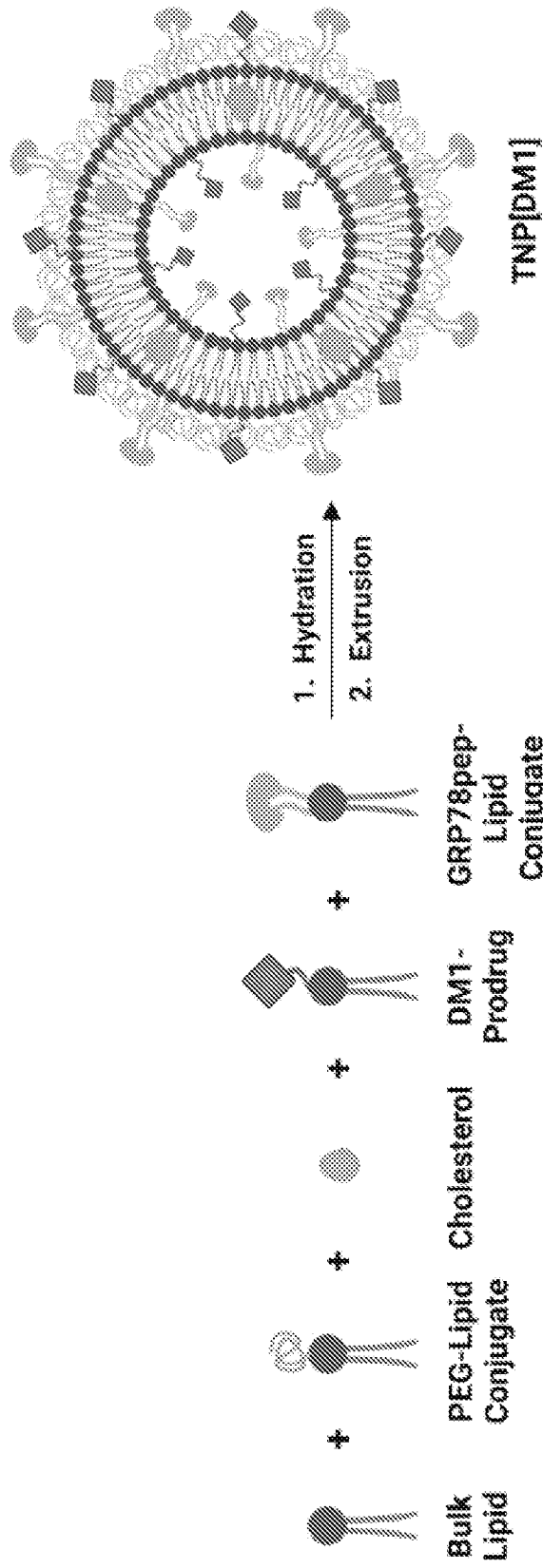


Fig. 16D

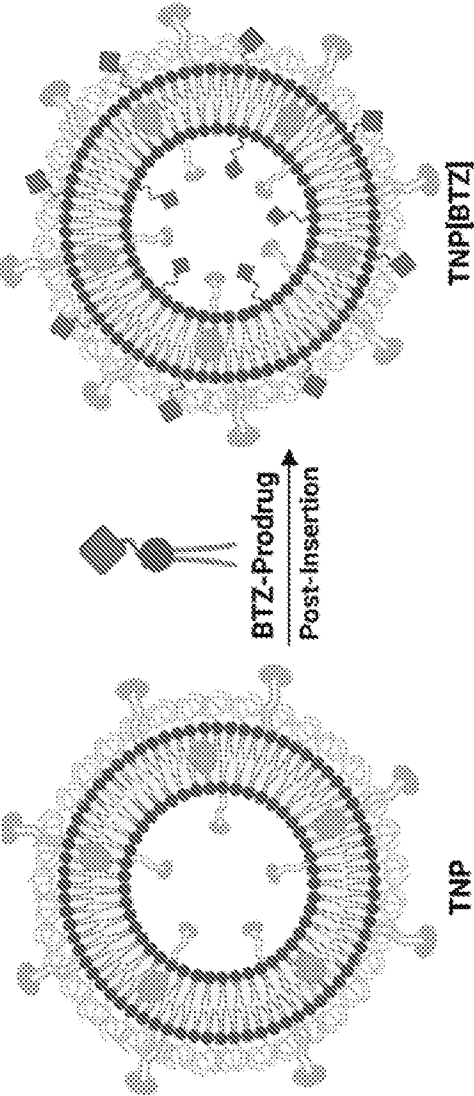


Fig. 16E

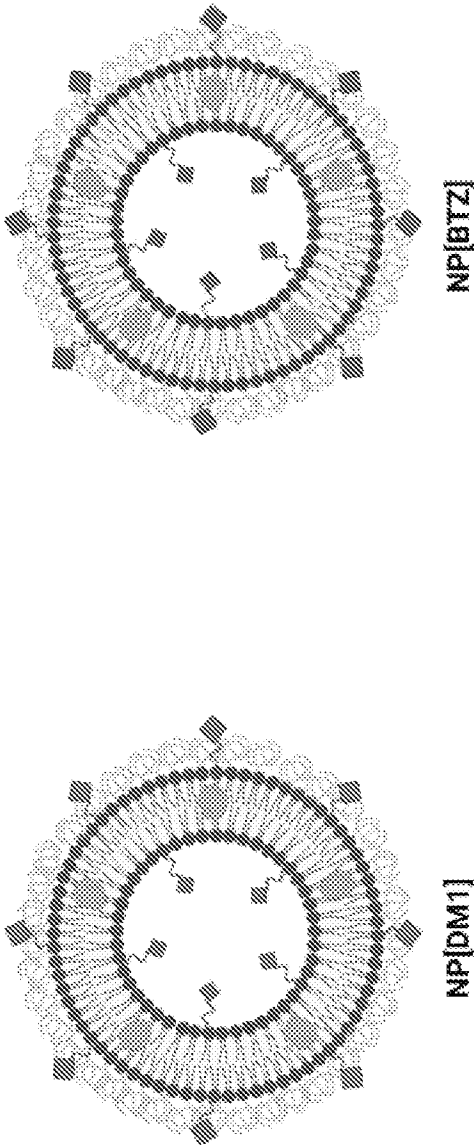


Fig. 16F

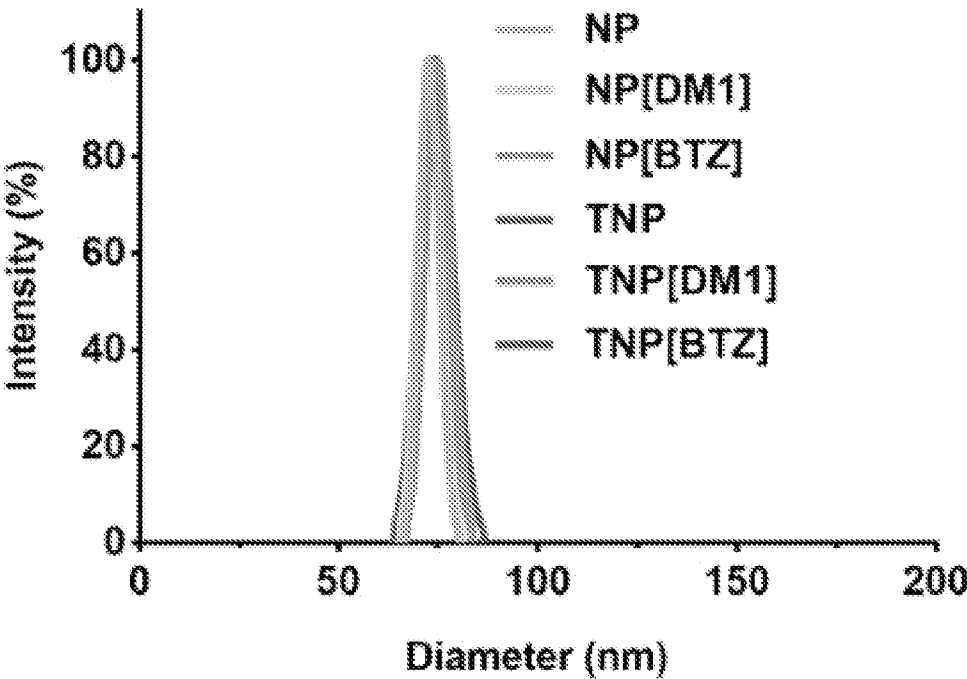


Fig. 17A

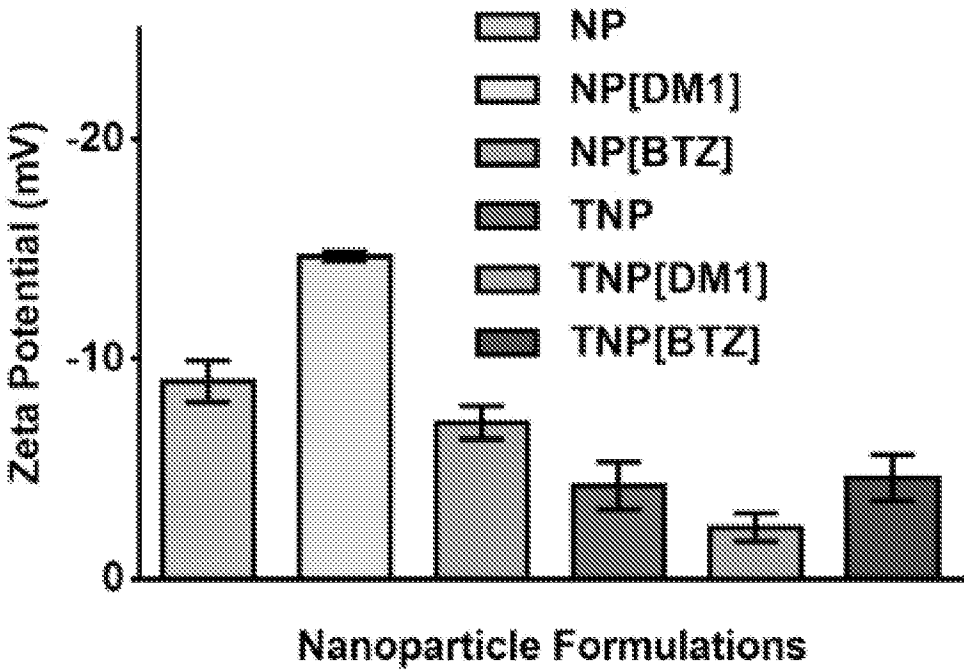


Fig. 17B

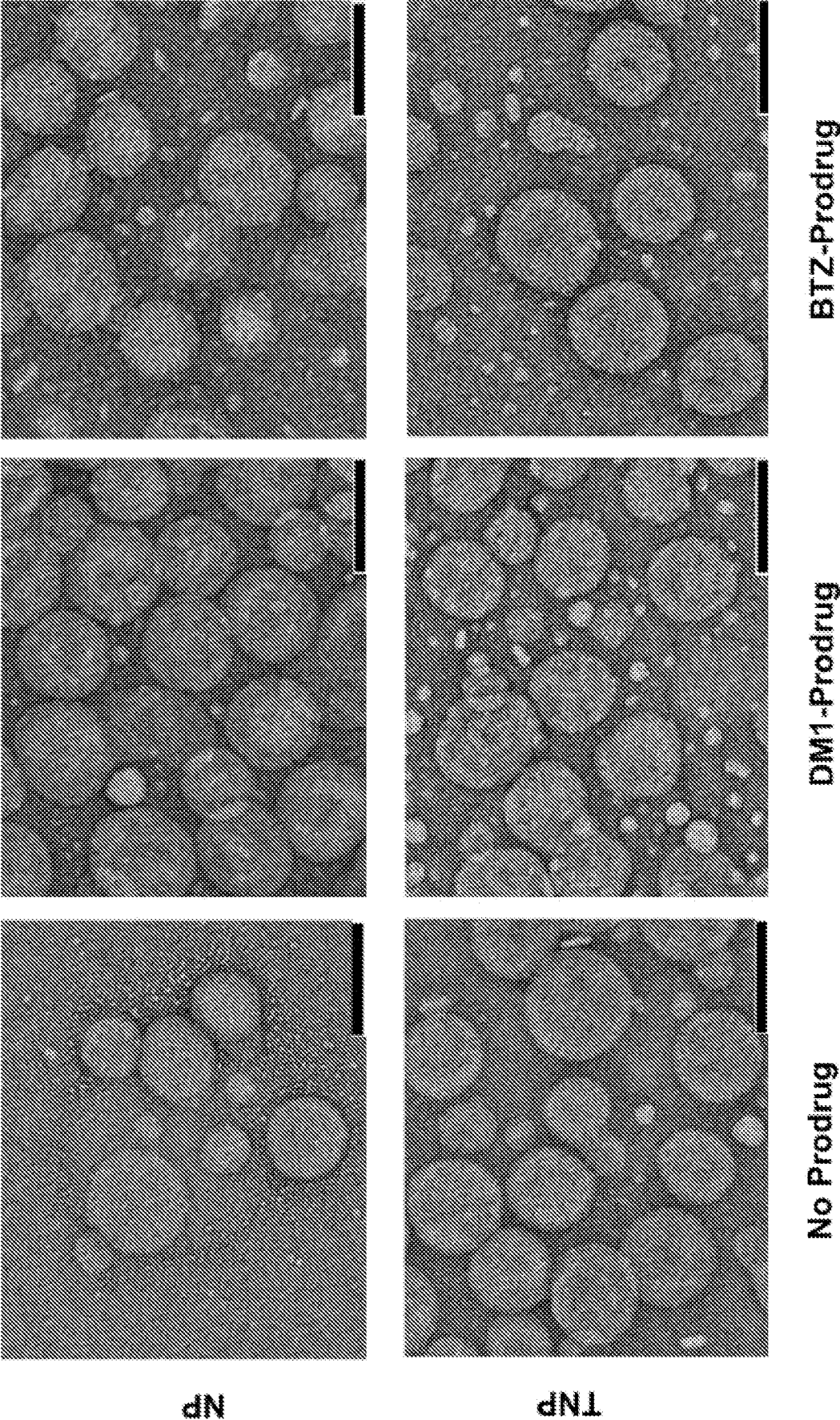


Fig. 17C

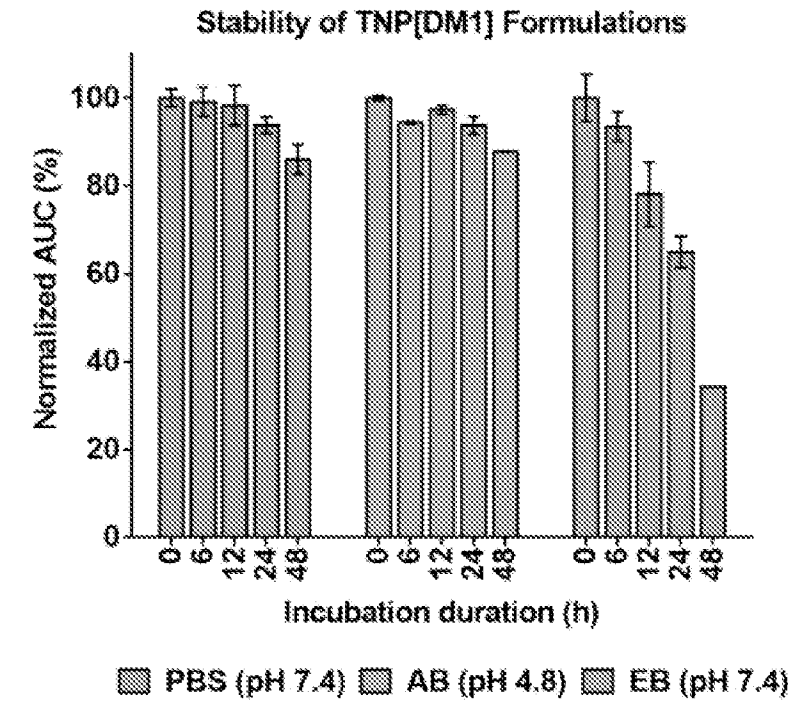


Fig. 17D

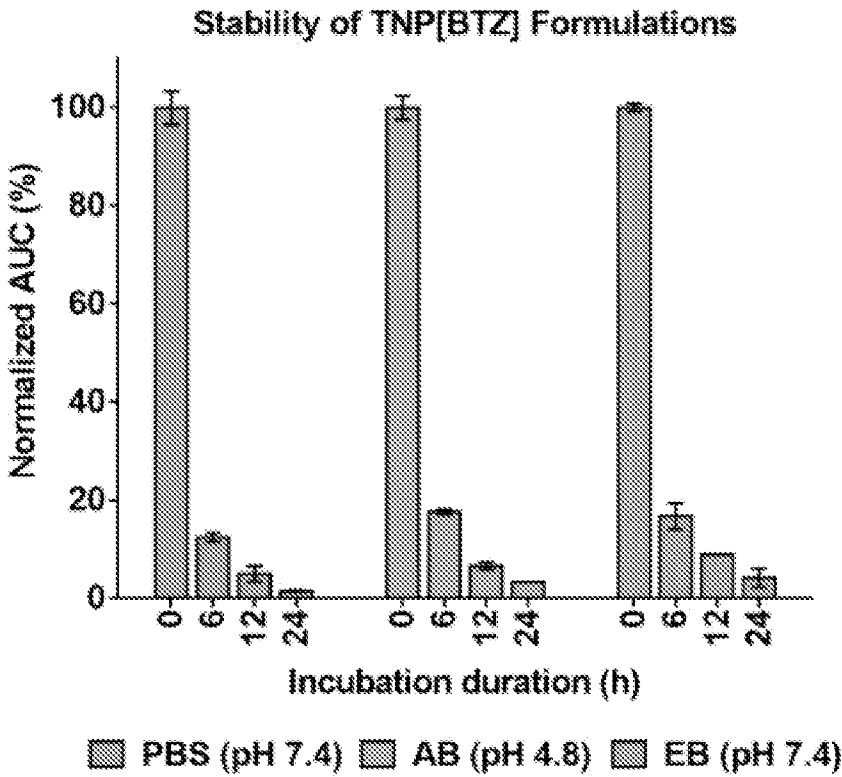
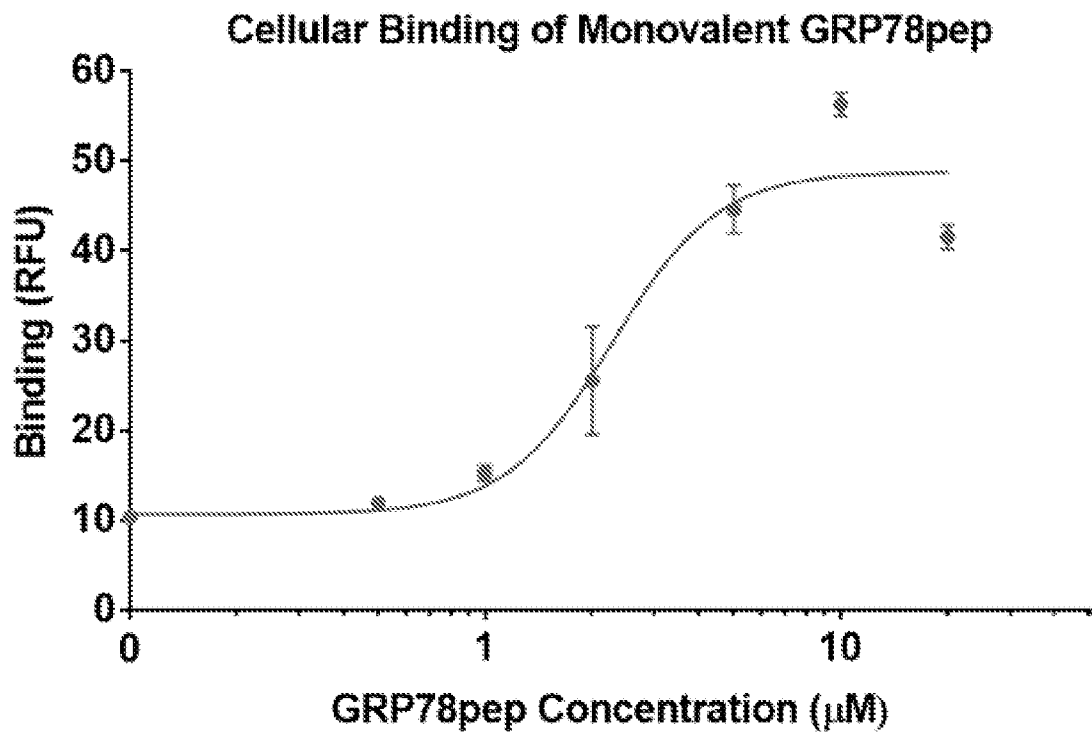
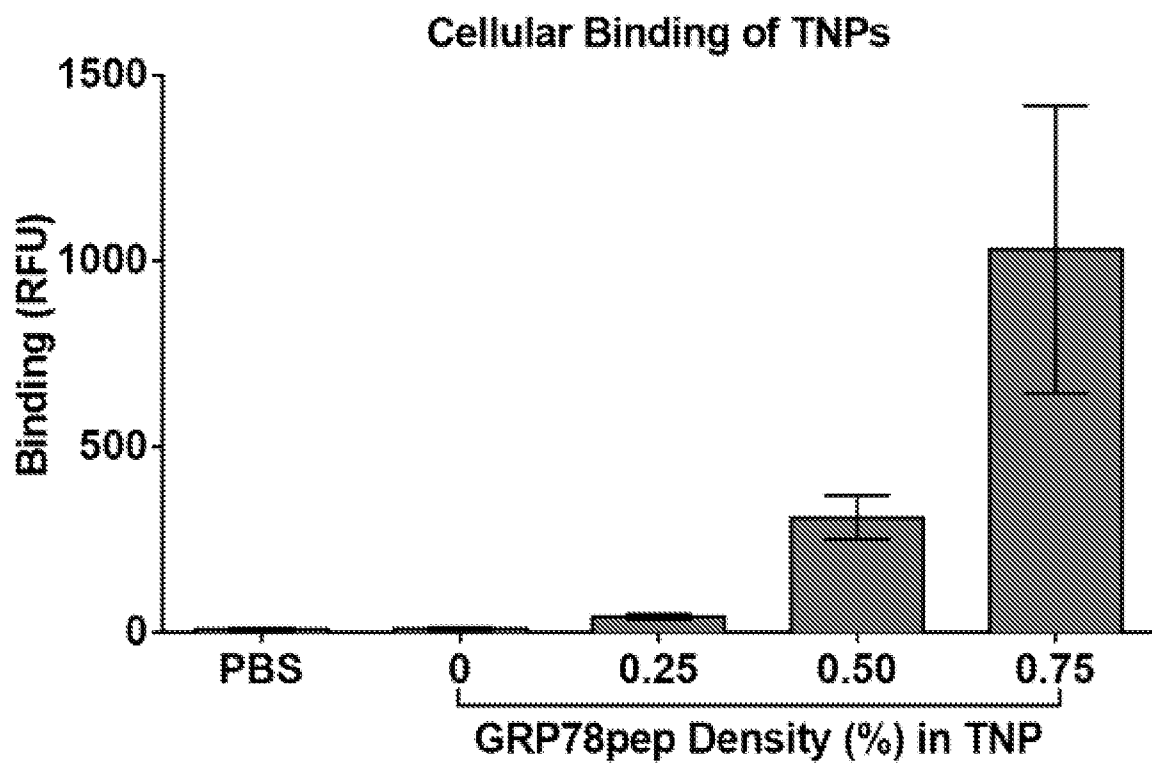
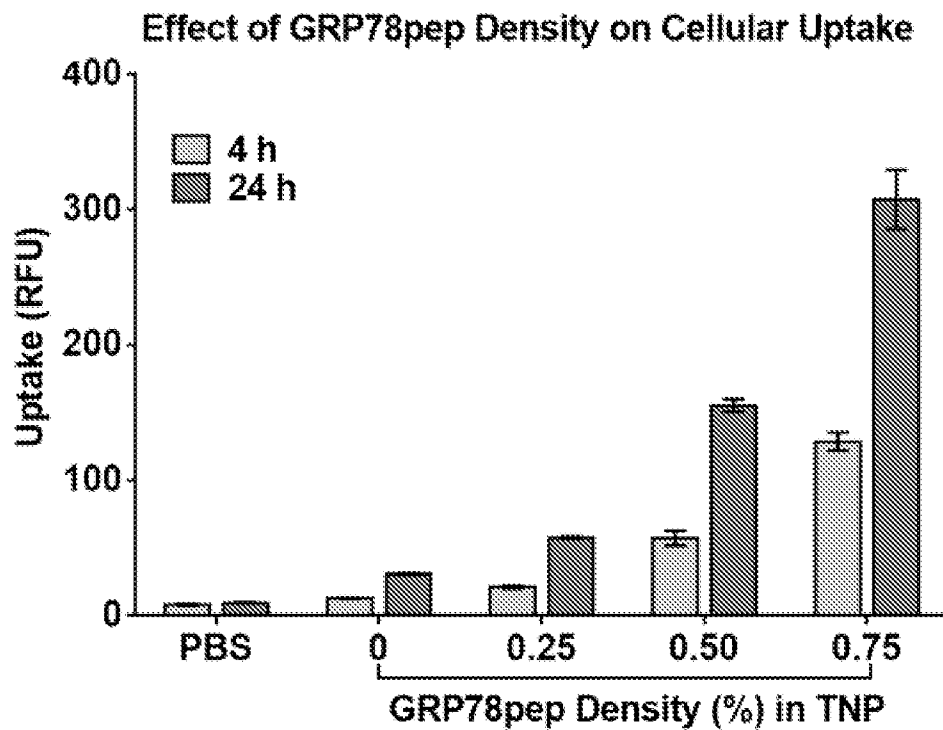
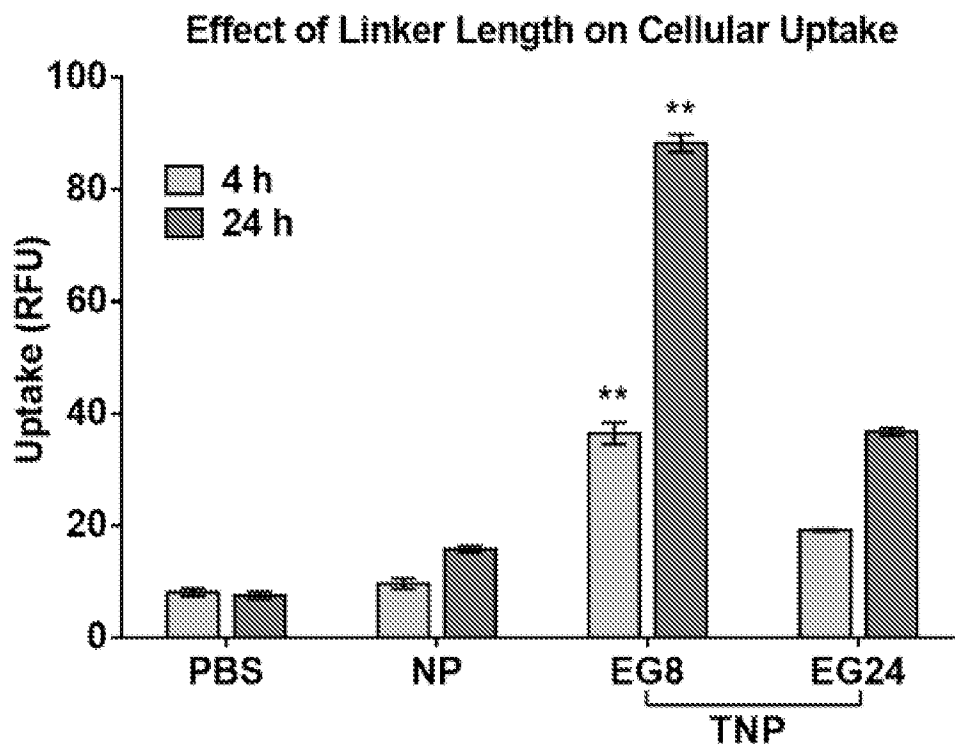


Fig. 17E

*Fig. 18A**Fig. 18B*

*Fig. 18C**Fig. 18D*

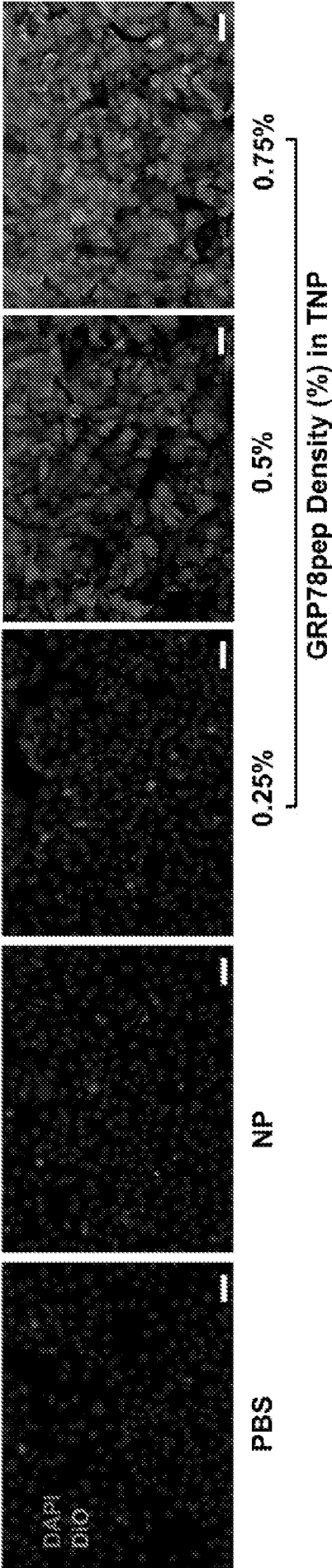
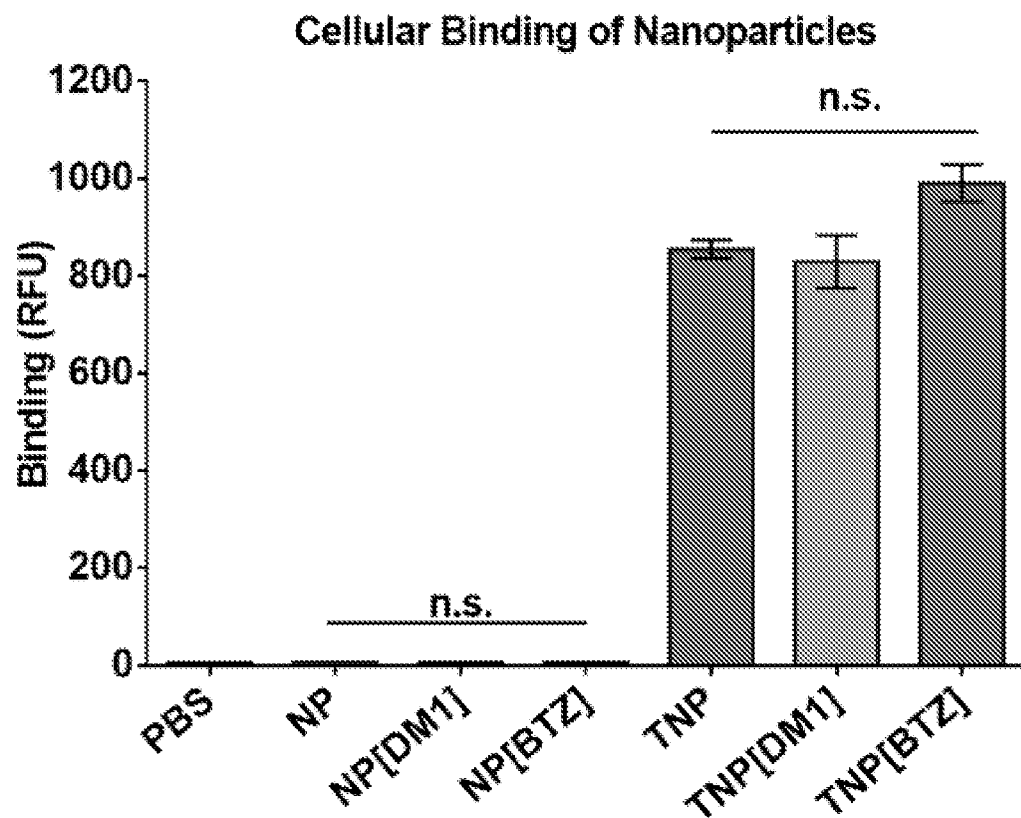
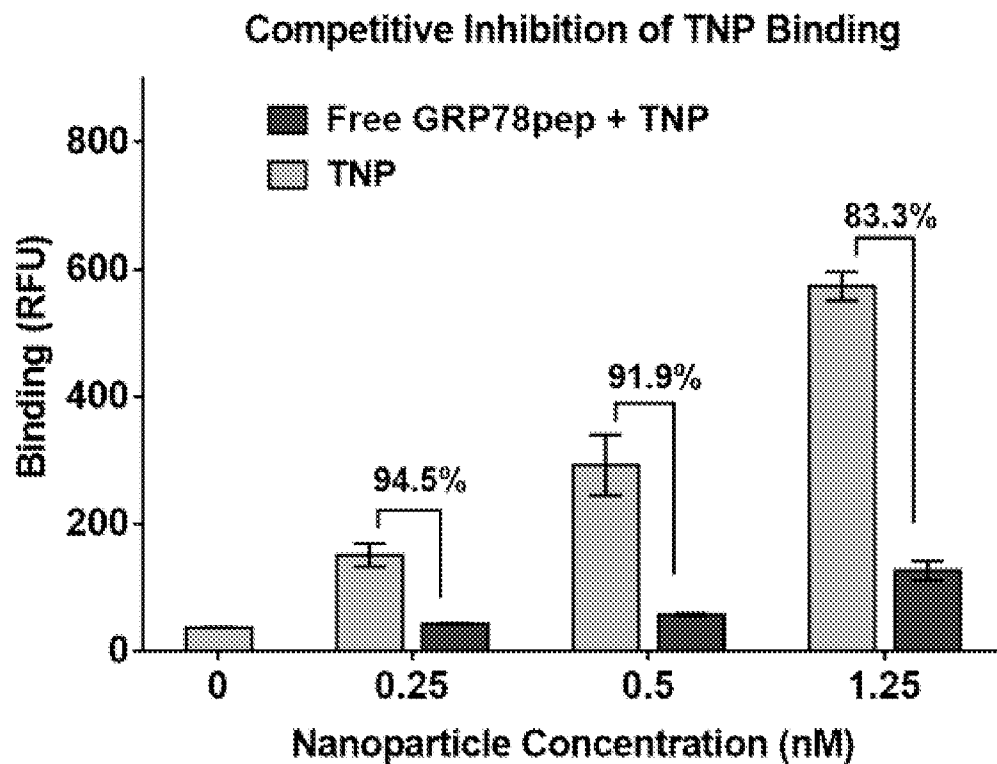


Fig. 18E

*Fig. 18F**Fig. 18G*

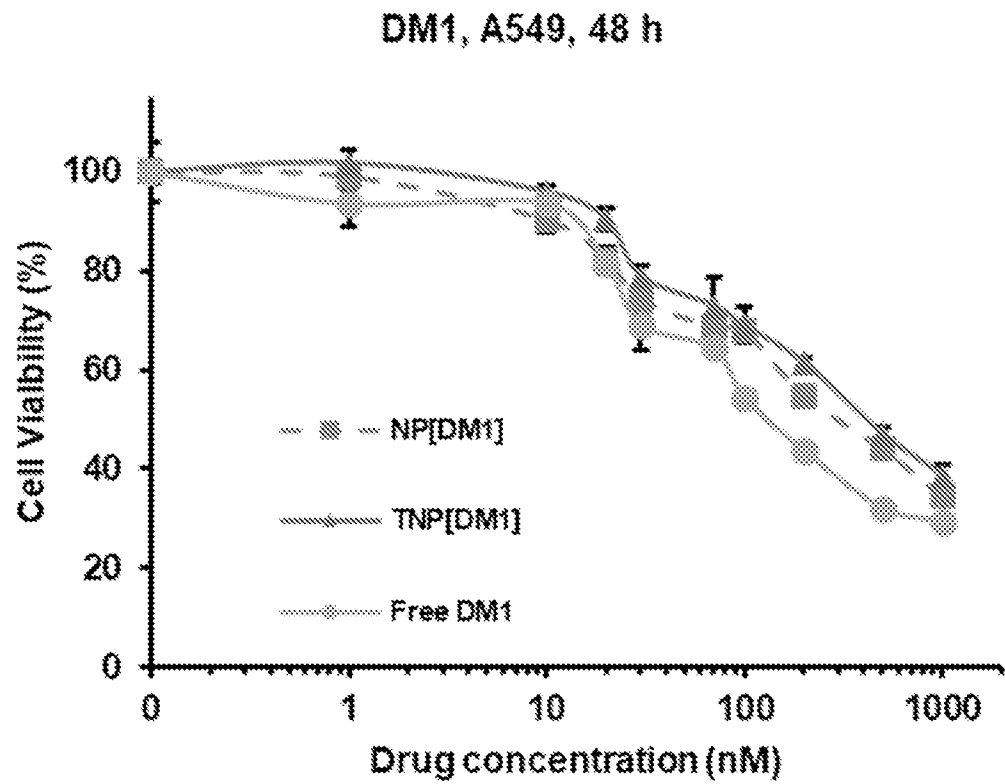


Fig. 19A

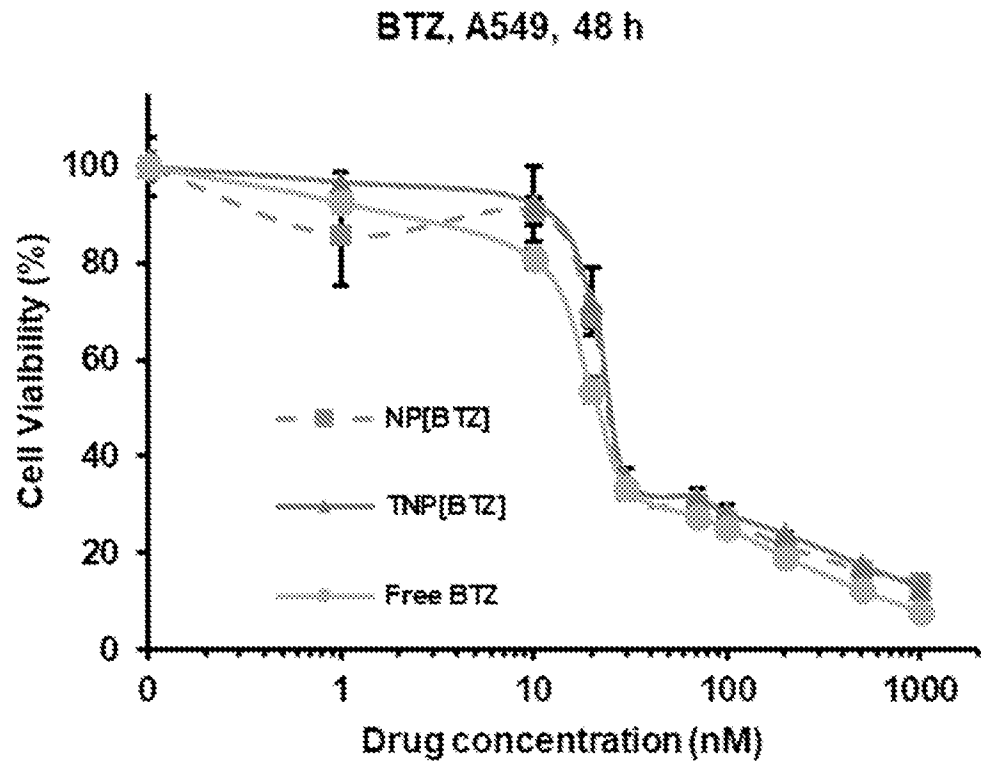


Fig. 19B

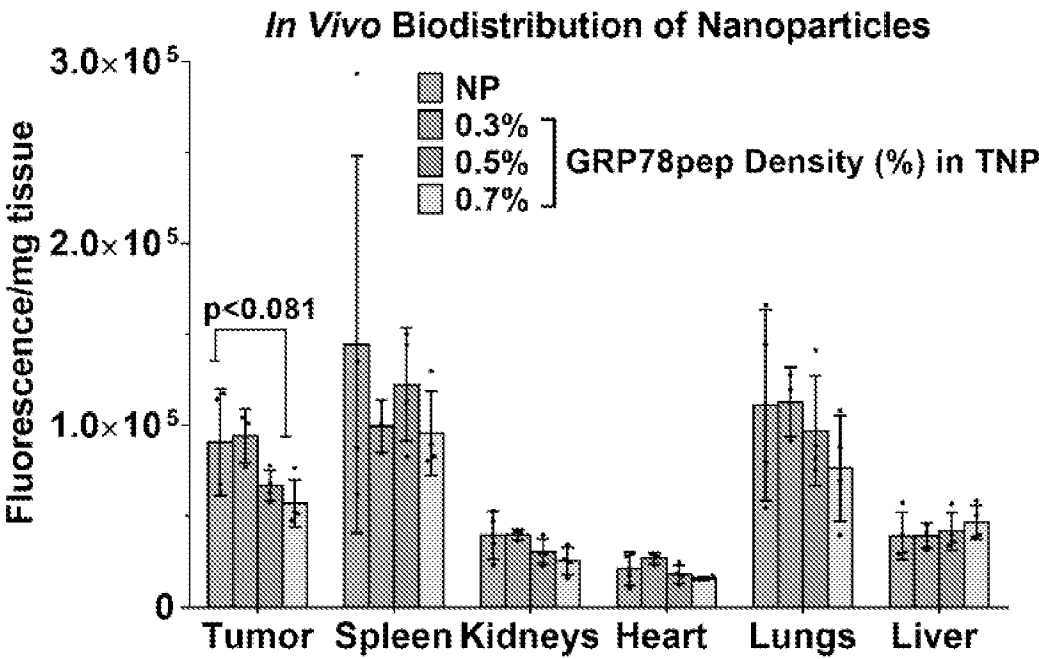


Fig. 20A

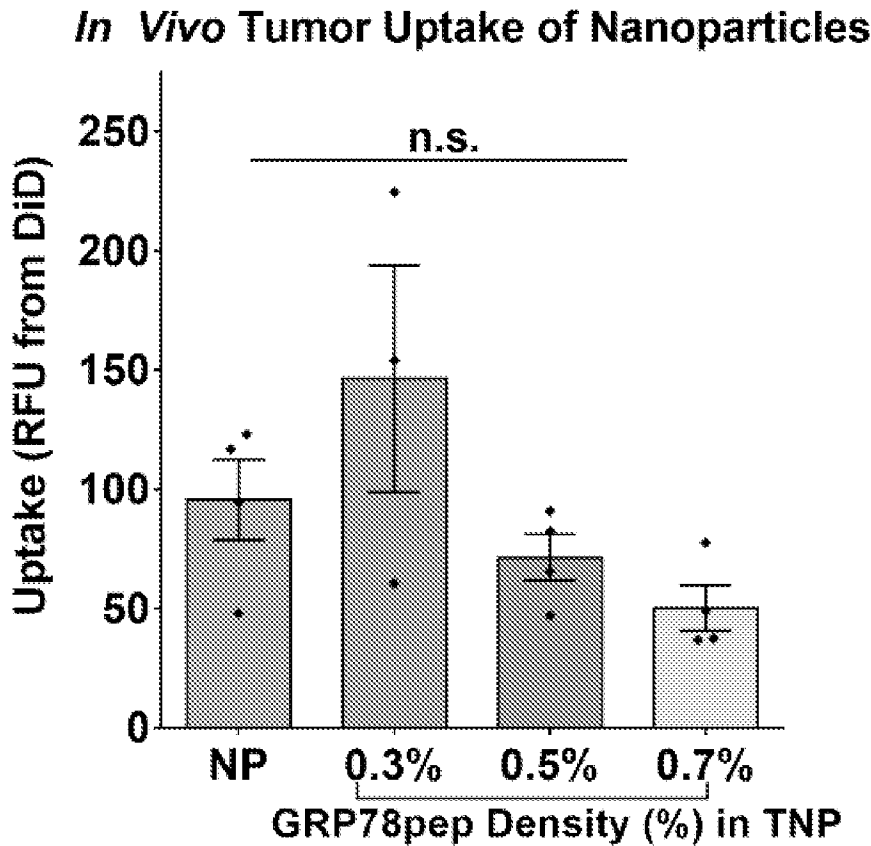


Fig. 20B

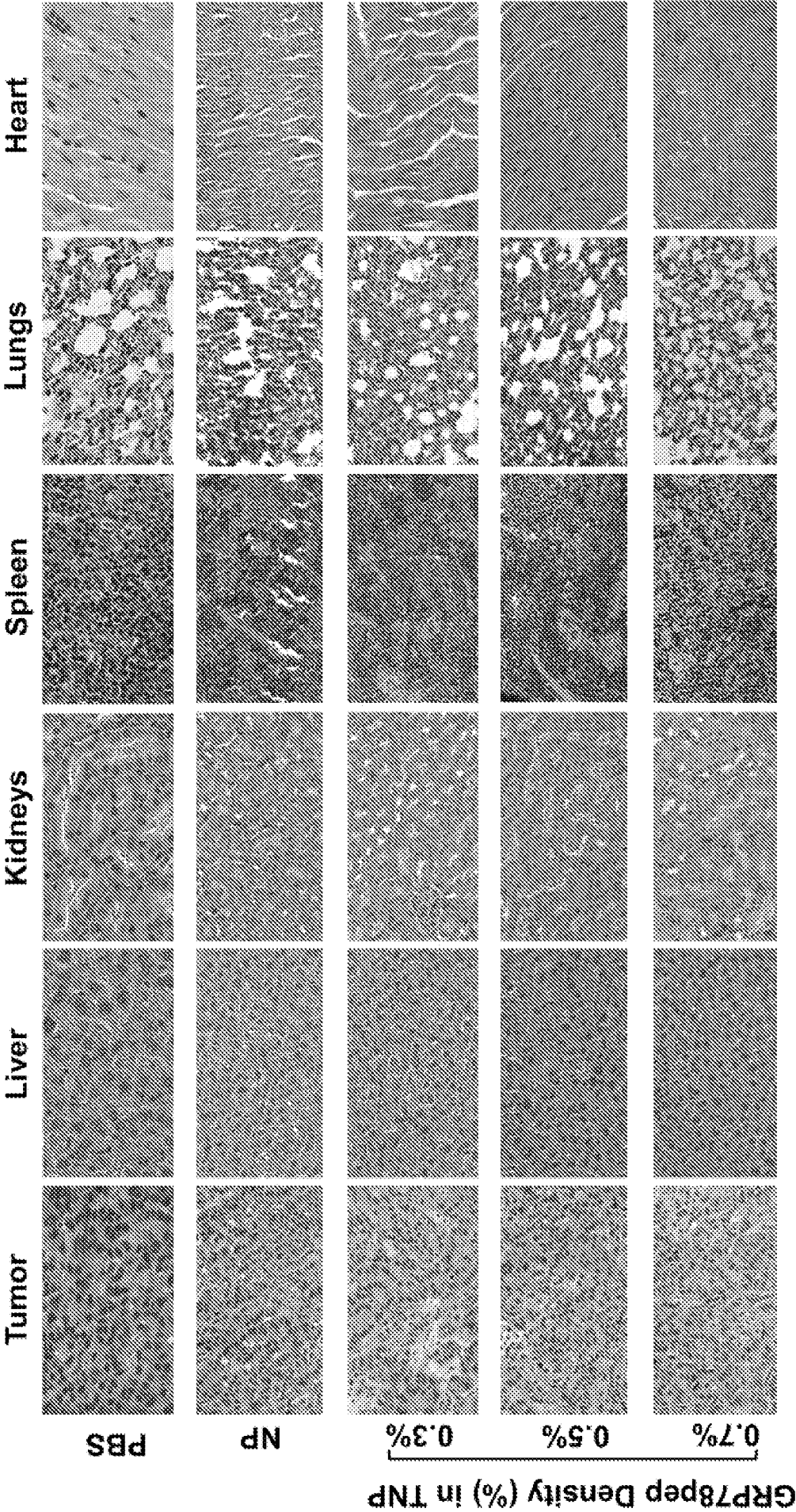


Fig. 20C

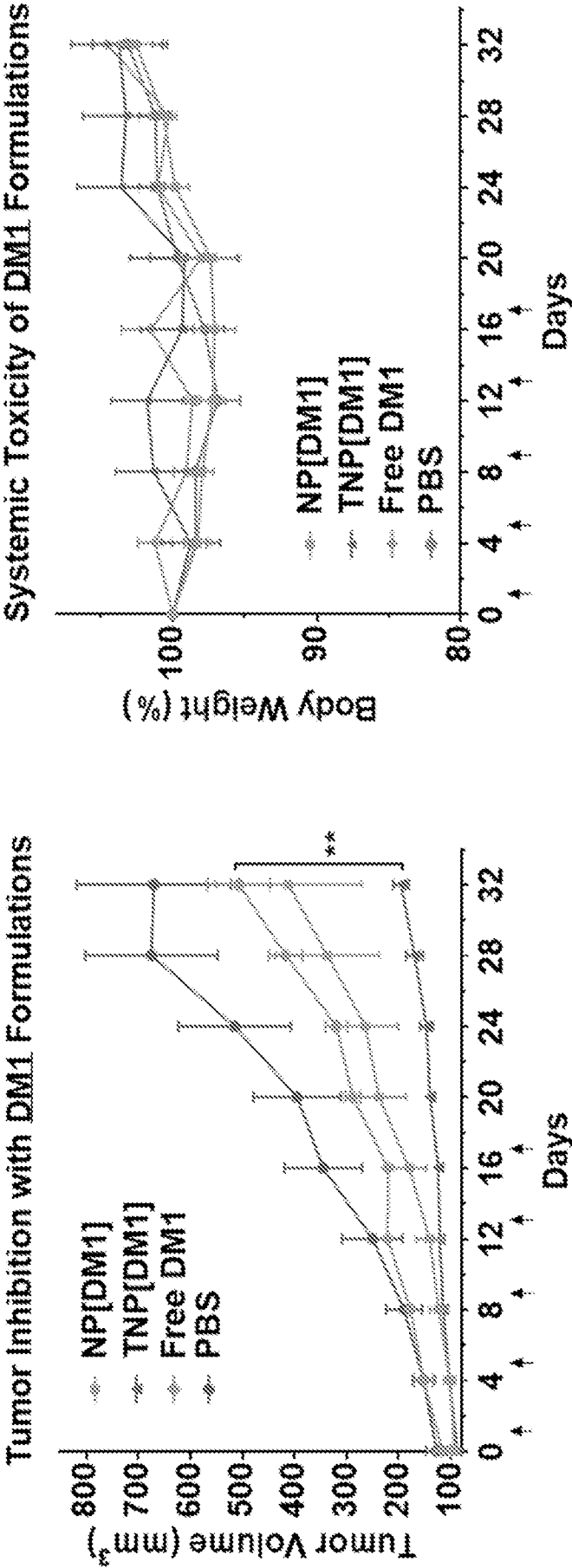


Fig. 21A

Fig. 21B

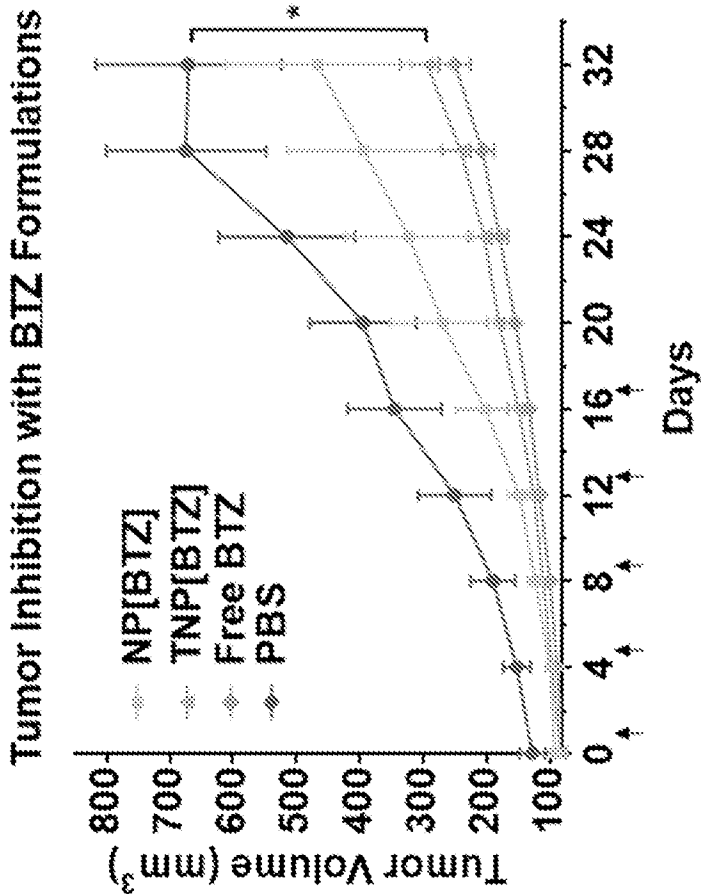


Fig. 21C

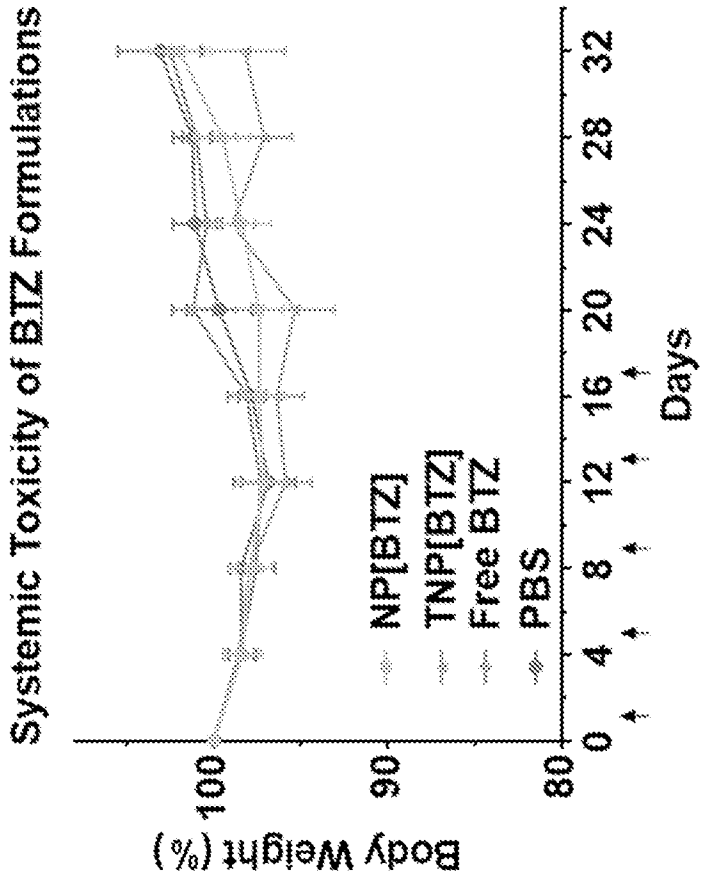


Fig. 21D

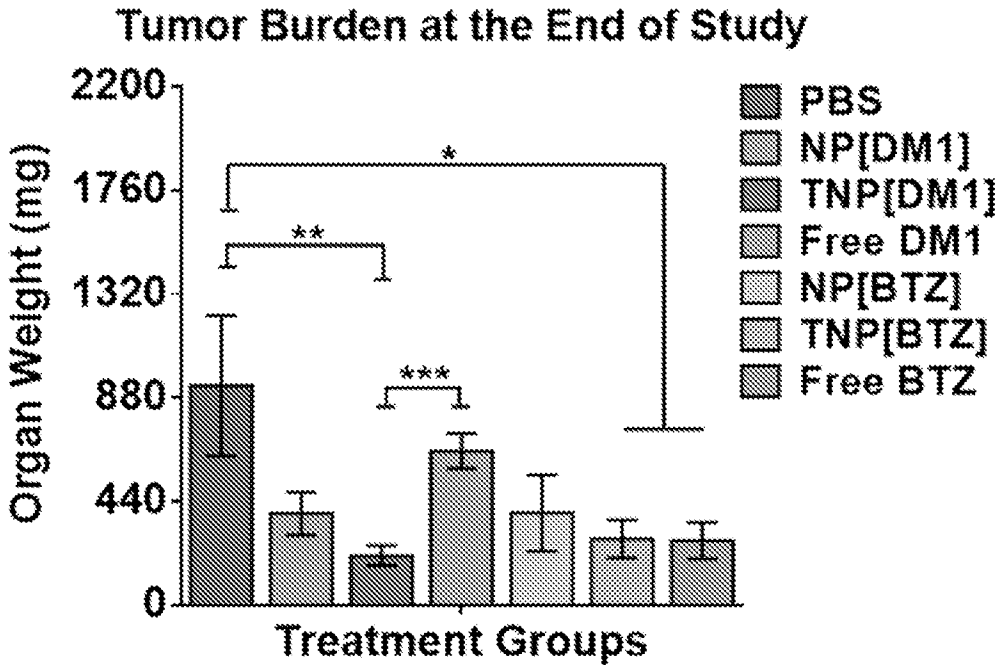


Fig. 21E

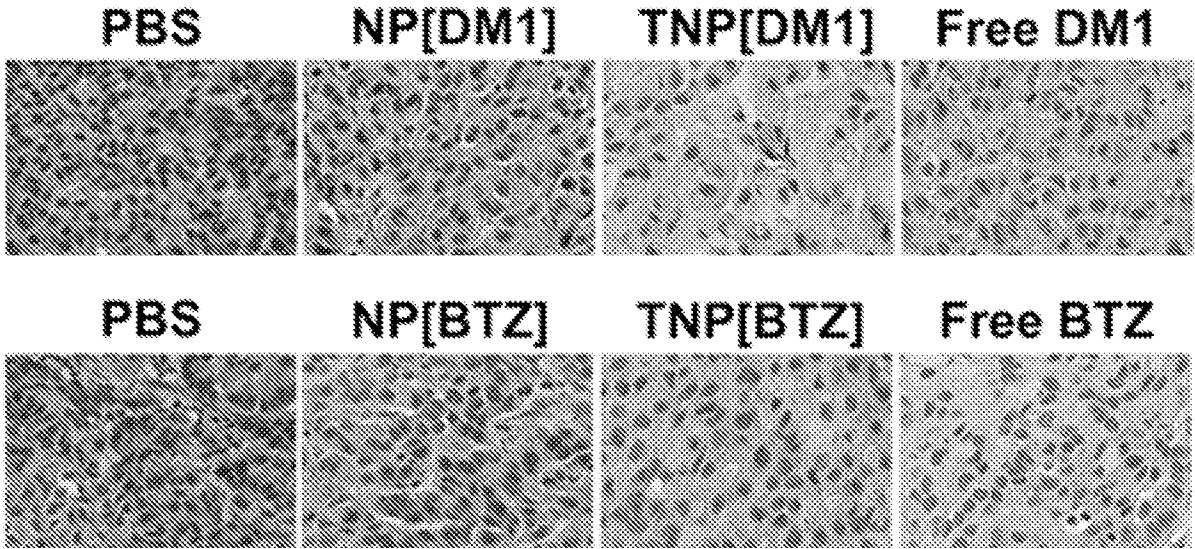


Fig. 21F

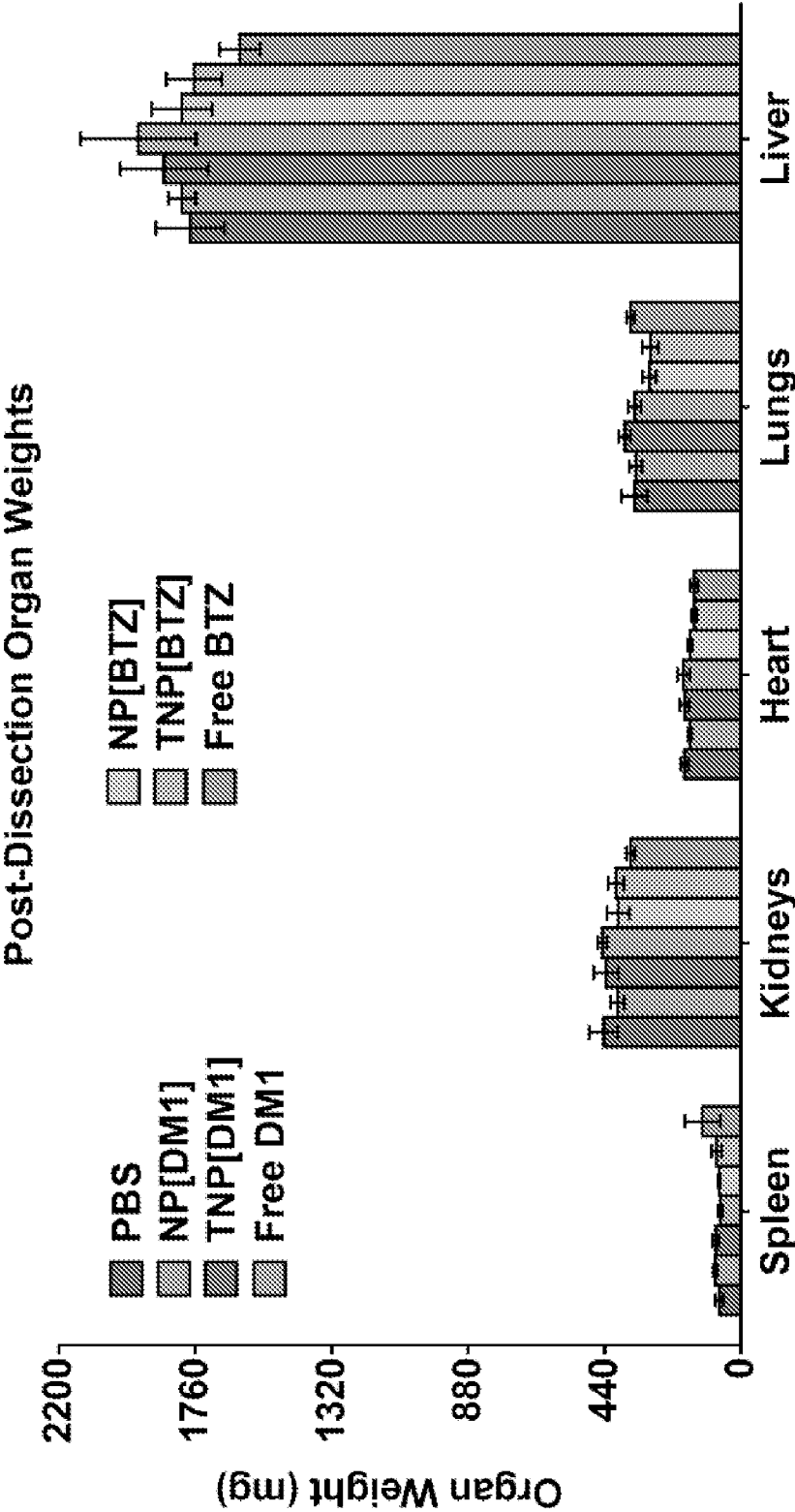


Fig. 22A

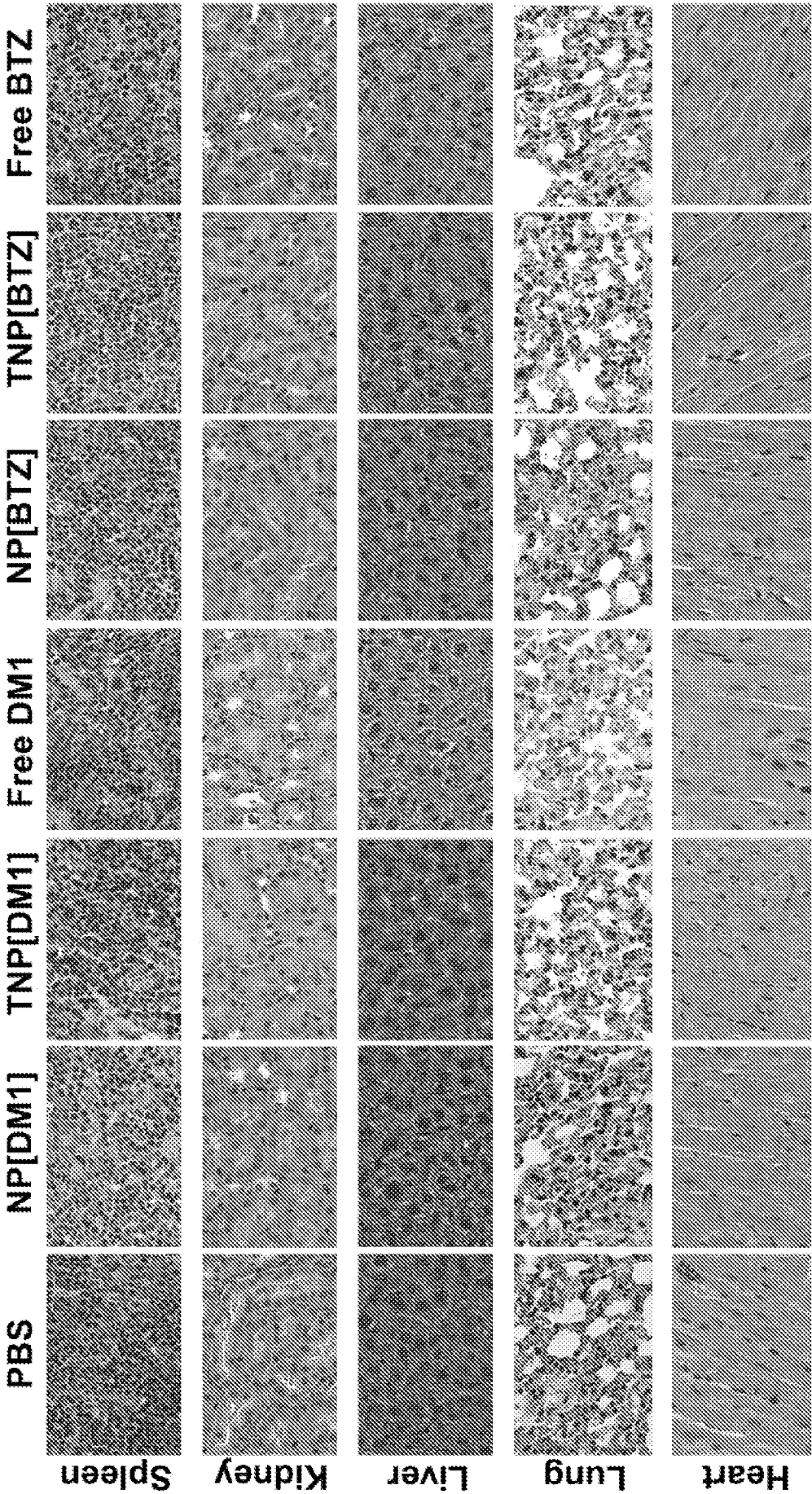
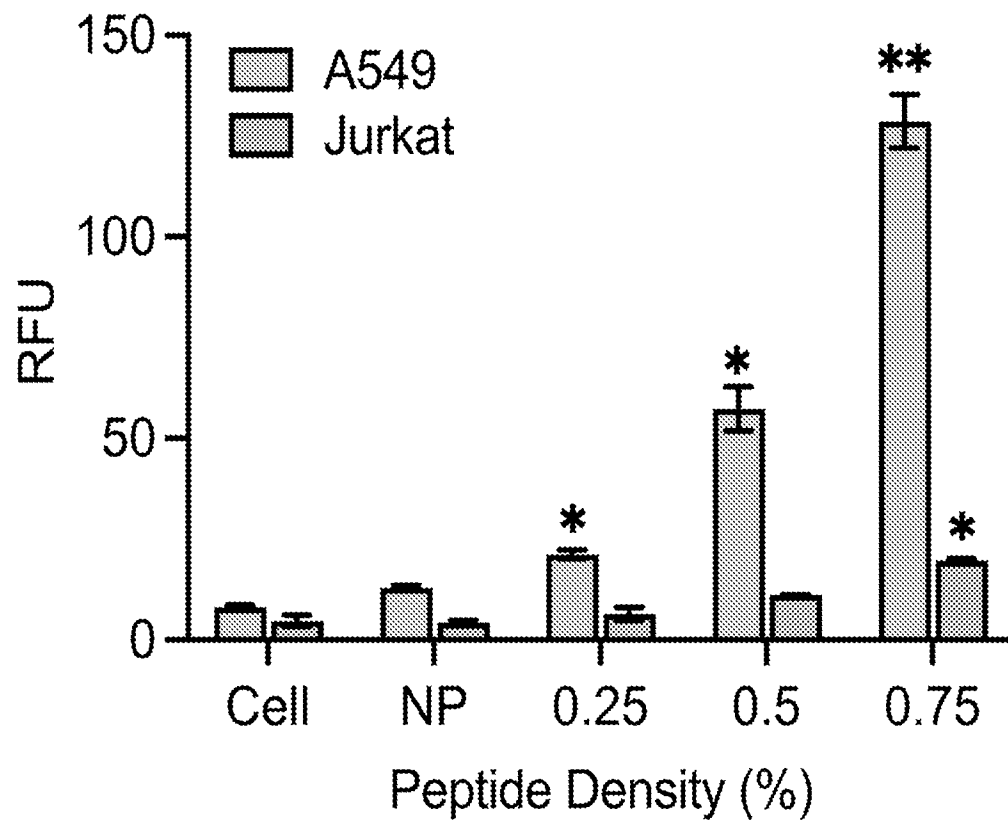


Fig. 22B

**Fig. 23**