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(54) Title: A PHOSPHATIDYLSERINE TARGETING COMBINATION FOR CANCER IMMUNOTHERAPY

(57) Abstract: A pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents; compositions and sustained-release formulations comprising the same; and their use in cancer immunotherapy; and a method of preparing a sustained-release formulation.

A PHOSPHATIDYLSERINE TARGETING COMBINATION FOR CANCER IMMUNOTHERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. provisional patent application no. 63/543,396, which was filed October 10, 2023, and which is hereby incorporated by reference in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under CA232419 and CA258737 awarded by the National Institute of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[0003] The present disclosure relates to a combination for the treatment of cancer immunotherapy. In particular, it is a combination that targets phosphatidylserine and comprises a phosphatidyl serine blocker and an immune stimulant, thereby enhancing immunotherapy in cancer treatment.

BACKGROUND

[0004] This section introduces aspects that may help facilitate a better understanding of the disclosure. Accordingly, these statements are to be read in this light and are not to be construed as admissions about what is or is not prior art.

[0005] Immunotherapy has significantly improved cancer treatment. However, many tumors remain resistant to current immunotherapy due to the highly immunosuppressive tumor microenvironment (TME). Phosphatidylserine (PS) is identified as one of the significant cellular components that contribute to immunosuppressive TME. For example, tumor cells use PS to escape immune activation against them. PS externalized on the outer leaflet of the apoptotic cell membrane attracts phagocytic cells to remove the dying cells quickly via a process called efferocytosis. Simultaneously, PS turns on anti-inflammatory (tolerogenic) signals to prevent further damage and maintain homeostasis. Tumor cells hijack the latter process to induce immunosuppressive TME. Recent studies show that PS is upregulated in TME and further increased by radiation or chemotherapy, two commonly used cancer treatments. For effective immunotherapy of tumors, PS needs to be antagonized to relieve immunosuppressive TME and sensitize tumors to immune stimulants.

[0006] Thus, there is an unmet need for an effective combination used as a cancer immunotherapy that blocks the PS, relieves the PS-induced immunosuppressive TME, and boosts anti-tumor immune responses in tumors to enhance immunochemotherapy of tumors. It is an object of the present disclosure to provide such an effective combination. This and other objects and advantages, as well as inventive features, will be apparent from the detailed description provided herein.

SUMMARY

[0007] Provided is a pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents.

[0008] The PS blocker can be selected from a dipicolylamine (DPA) or its metal complex, a metal salt, Annexin V, an anti-PS antibody, and a combination of two or more thereof. In some embodiments, the PS blocker is DPA or its metal complex. The metal in the metal complex of DPA is a transition metal. Examples of transition metals include, but are not limited to, zinc (Zn), cobalt (Co), copper (Cu), platinum (Pt), iron (Fe), nickel (Ni), silver (Ag), chromium (Cr), manganese (Mn), titanium (Ti), vanadium (Vn), cadmium (Cd) and scandium (Sc). In some embodiments, the PS blocker is DPA or its metal complex, such as DPA-Zn.

[0009] In some embodiments, the metal salt can be zinc acetate dihydrate, zinc gluconate, zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide, calcium chloride, calcium sulfate, or a combination of two or more thereof. In some embodiments, the PS blocker is Annexin V.

[0010] The immune stimulant can be a granulocyte-macrophage colony-stimulating factor (GM-CSF), a stimulator of interferon genes (STING) agonist, an anti-PD-L1 antibody, an anti-PD-1 antibody, a CpG oligodeoxynucleotide, a polyinosinic-polycytidylic acid (poly (I:C)), a single-stranded RNA (ssRNA), a short double-stranded RNA (dsRNA), a long ds RNA, or a combination of two or more thereof. In some embodiments, the immune stimulant is the STING agonist. In some embodiments, the STING agonist is the CDN or its metal complex. In some embodiments, the metal ion can be selected from Zn, Mn, Cu, Fe, Co, and aluminum (Al).

[0011] In some embodiments, one or more chemotherapeutic agents are doxorubicin, paclitaxel, nab-paclitaxel, docetaxel, cisplatin, carboplatin, oxaliplatin, carfilzomib, bortezomib, mitoxantrone, dacarbazine, temozolomide, vinblastine, or a combination of two or more thereof.

[0012] Provided is a pharmaceutical composition comprising a therapeutically effective amount of (i) a PS blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents of the pharmaceutical combination and a pharmaceutically acceptable carrier, diluent, or excipient.

[0013] Provided is a combination of pharmaceutical compositions comprising (i) a pharmaceutical composition comprising a therapeutically effective amount of the phosphatidylserine blocker, (ii) a pharmaceutical composition comprising a therapeutically effective amount of the immune stimulant, and (iii) a pharmaceutical composition comprising a therapeutically effective amount of one or more chemotherapeutic agents, wherein (i), (ii), and (iii) are independently formulated to be administered by the same or different routes. In some embodiments, (i), (ii), and (iii) are independently formulated to be administered intravenously, subcutaneously, intratumorally, intracranially, intraperitoneally, intrapulmonarilly, intranasally, topically, or orally.

[0014] Provided is a method for treating a patient of cancer, which method comprises administering to the patient (i) a therapeutically effective amount of a phosphatidylserine blocker, (ii) a therapeutically effective amount of an immune stimulant, and (iii) a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer. In some embodiments, (i), (ii), and (iii) are formulated as a single pharmaceutical composition or as separate pharmaceutical compositions, which can be administered simultaneously or sequentially by the same or different routes. In some embodiments, (i), (ii), and (iii) are administered intravenously, subcutaneously, intratumorally, intracranially, intraperitoneally, intrapulmonarilly, intranasally, topically, or orally. In some embodiments, the cancer is melanoma.a

[0015] In some embodiments, the therapeutically effective amount of (i) is from about 2 mg/kg to about 50 mg/kg. In some embodiments, the therapeutically effective amount of (ii) is from about 0.01 mg to about 10 mg. In some embodiments, the therapeutically effective amount of (iii) is from about 5 mg/kg to about 20 mg/kg.

[0016] Further provided is a sustained-release formulation, which formulation comprises: a therapeutically effective amount of a hydrogel comprising a phosphatidylserine blocker, alone or further in combination with a crosslinker, and an immune stimulant, wherein the immune stimulant is optionally encapsulated. The hydrogel can be alginate, pectin, carrageenan, polylactic acid, polyethylene glycol, collagen, fibrin, hyaluronic acid, matrigel, chitosan, silk fiber, or a combination of two or more thereof. In some embodiments, the hydrogel is alginate.

[0017] In some embodiments, the immune stimulant is encapsulated in a liposomal formulation or a nanoparticulate formulation. The crosslinker can be zinc acetate dihydrate, zinc gluconate, zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide, calcium chloride, or calcium sulfate.

[0018] Further provided is a method for treating a patient of cancer, which method comprises administering to the patient (i) a therapeutically effective amount of the sustained-release formulation described above and (ii) a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer. The sustained-release formulation and one or more chemotherapeutic agents can be administered, simultaneously or sequentially, by a same or different routes, intravenously, subcutaneously, intratumorally, intracranially, intraperitoneally, intrapulmonarilly, intranasally, topically, or orally. In some embodiments, the cancer is melanoma.

[0019] Still further provided is a method for preparing a sustained-release formulation, which method comprises:

(i) mixing a solution comprising a hydrogel and a solution comprising a phosphatidylserine (PS) blocker to formulate a hydrogel of PS blocker;

(ii) optionally mixing the hydrogel of step (i) with a suspension comprising a crosslinker to formulate a hydrogel comprising the PS blocker and the crosslinker; and

(iii) mixing the hydrogel of step (i) or step (ii) with a solution comprising an optionally encapsulated immune stimulant,

whereupon the sustained-release formulation is formulated.

[0020] In some embodiments, the immune stimulant is optionally encapsulated in the liposomal or the nanoparticulate formulation.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] The present disclosure will be more readily understood from the detailed description of embodiments presented below considered in conjunction with the attached drawings of which:

[0022] Fig. 1A shows the phosphatidylserine (PS) exposure on B16F10 tumor cells increased after chemotherapy and flow cytometry of B16F10 cells received doxorubicin (DOX) liposomal form (Doxil) for 24 hours.

[0023] Fig. 1B shows PS exposure on B16F10 tumor cells increased after chemotherapy and quantification of PS-positive B16F10 cells after 24 hours of treatment with liposomal doxorubicin (Doxil).

[0024] Fig. 2A is schematics of the study of the anti-inflammatory effect of PS liposome on lipopolysaccharides (LPS)-stimulated macrophages.

[0025] Fig. 2B shows PS liposomes exert immune suppressive effects. PS liposome reduces the response of bone marrow-derived macrophages (BMMs) to LPS. The immune suppressive effect of PS/phosphatidylcholine (PC) liposome containing 50 wt% PS at varying liposome concentrations.

[0026] Fig. 2C shows PS liposomes exert immune suppressive effects. PS liposome reduces the response of BMMs to LPS. The immune suppressive effect of 300 μg/mL PS/PC liposomes with various PS wt% were incubated with BMMs for 24 hours. Subsequently, 10 ng/mL LPS was added to BMMs and further incubated for another 24 hours. The released TNF-α was quantified by ELISA. PS liposomes reduce the NK-κB activation.

[0027] Fig. 2D shows PS liposomes exert immune suppressive effects. PS liposome reduces the response of BMMs to LPS. THP-Xblue™-MD-CD14 reporter cells (monocytes) were treated with LPS (10 ng/mL) for 4 hours, then treated with 300 μg/mL PS liposome (50 wt%) or PC liposome. Activation was detected by Quanti-blue™ at 72 hours.

[0028] Fig. 3A is schematics of the anti-inflammatory effect of PS liposome on LPS-stimulated BMDCs.

[0029] Fig. 3B shows PS reduces bone marrow-derived dendritic cell (BMDC) maturation. It shows BMDC maturation marker CD86 expression after the treatment with 300 μg/mL PS/PC liposomes (50% PS) for 24 hours, further stimulated with 10 ng/mL LPS for another 24 hours.

[0030] Fig. 3C shows PS reduces BMDC maturation. Representative histogram plots of CD86 expression on BMDC. Data is presented as mean ± SD.

[0031] Fig. 4A shows dipicolylamine-Zn (DPA-Zn) selectively binds to PS with high affinity. The binding effects of DPA-Zn with liposomes containing different percentages of PS. Liposome concentration was fixed at 1 mg/mL.

[0032] Fig. 4B shows that DPA-Zn selectively binds to PS with high affinity. Isothermal titration calorimetry (ITC) analysis of the interaction of PS liposome (50% PS) with DPA-Zn.

[0033] Fig. 4C shows that DPA-Zn selectively binds to PS with high affinity and DPA-Zn at 25 °C. Data is presented as mean ± SD.

[0034] Fig. 5 shows LPS-induced tumor necrosis factor-α (TNF-α) production by BMM after 24 hours of incubation with PS liposomes (50% PS) with or without DPA-Zn. Data is presented as mean ± SD.

[0035] Fig. 6 shows the maximum tolerance dose (MTD) of DPA-Zn in mice. Old C57BL/6 mice (> 6 months old) were treated with DPA-Zn solution by intravenous (i.v.) injection at days 0, 1, 3, 4, 6, and 7, with dose at 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg (n=1). Mice weight change after injection was monitored daily.

[0036] Fig. 7A is the schematics of the dosing schedule. C57BL/6 old mice were injected with 10^6 B16F10 cells/mouse subcutaneously. When tumors grew to 50 mm^3 (day 0), mice were treated intravenously with Doxil (equivalent to 2.5 mg/kg DOX) or 5% dextrose (D5W) on days 0 and 3, intratumorally with 10 mg/kg DPA-Zn or D5W on days 1, 2, 4, 5 (n=5 per group).

[0037] Fig. 7B shows the tumor growth curve and mice weight change after treatment.

[0038] Fig. 8 shows the immunophenotyping in tumors 11 days post-first treatment. Data is presented as mean ± SD.

[0039] Fig. 9A is the schematics of the dosing schedule. C57BL/6 mice were injected with 10^6 B16F10 cells/mouse subcutaneously. When tumors grew to 50 mm^3 (day 0), mice were treated intravenously with Doxil (equivalent to 2.5 mg/kg DOX) or 5% dextrose (D5W) on days 0 and 3. On days 1, 2, 4, and 5, mice were intratumorally injected with DPA-Zn or granulocyte-macrophage colony-stimulating factor (GM-CSF) or DPA-Zn + GM-CSF. The dose of DPA-Zn is 10 mg/kg per injection, and GM-CSF is 2.5 μg/mouse per injection (n=5 per group).

[0040] Fig. 9B shows the tumor growth curve. Tumor size data was analyzed by unpaired two-tailed t-test. Data is presented as mean ± SEM.

[0041] Fig. 10 shows immunophenotyping in tumors on day 11 post-first treatment. Data was analyzed by one-way ANOVA. Data is presented as mean ± SD.

[0042] Fig. 11A is the schematics of the dosing schedule. C57BL/6 mice were injected with 10^6 B16F10 cells/mouse subcutaneously. When tumors grew to 100-150 mm^3 (day 0), mice were treated intravenously with Doxil (equ. DOX is 2.5 mg/kg) or 5% dextrose (D5W) on days 0 and 3, intratumorally with 5 mg/kg DPA-Zn and/or cyclic dinucleotides (CDN) (10 μg/mouse) or D5W on days 1, 2, 4, 5 (n=5 per group).

[0043] Fig. 11B shows the tumor growth curve. Data is presented as Mean ± SD.

[0044] Fig. 11C shows the tumor size on day 12. Tumor size data was analyzed by one way ANOVA. Mean ± SD.

[0045] Fig. 11D shows weight changes in mice after treatment.

[0046] Fig. 11E shows the tumor growth curve with individual treatment of Doxil, Doxil and DPA-Zn, Doxil + CDN, and Doxil + CDN + DPA-Zn.

[0047] Fig. 12 shows the transmission electron microscope (TEM) images of CDN-Zn co-ordination NPs (nanoparticles) with varying molar ratios of CDN/Zn^{2+}.

[0048] Fig. 13A-B shows the isothermal titration calorimetry (ITC) result of Zn^{2+} solution titrating into CDN solution.

[0049] Fig. 14A shows CDN liposomes (CDN@lip) characterization. The particle size distribution profiles of CDN@lip.

[0050] Fig. 14B shows TEM images of CDN@lip. Scale bar: left: 200 nm, right: 100 nm.

[0051] Fig. 15 shows *in vitro* CDN release of CDN@lip in PBS at 37 °C for seven days. (n=3).

[0052] Fig. 16 shows 293-dual stimulator of interferon genes (STING) reporter cells, e.g., mSTING, were treated with an equivalent of 5 μg/mL of CDN and incubated for 24 hours and 48 hours (n=3). The activation of the STING pathway was quantified using the Quanti-Blue assay. Data were analyzed by one-way ANOVA. Data is presented as mean ± SD.

[0053] Fig. 17A is the schematics of the dosing schedule. Tumors were treated with DPA-Zn by intratumoral (i.t.) on days 1, 2, 4, and 5 and Doxil by intravenous (i.v.) on days 0 and 3. CDN administration was given either as free CDN on days 1, 2, 4, and 5 with 10 μg per dose or as CDN@lip (equitant to 40 μg CDN) on day 1.

[0054] Fig. 17B shows the tumor growth curve, the survival curve, and body weight changes after treatment (described in **Fig. 17A)**. It shows improved anti-tumor effects of CDN@lip compared to free CDN in the combination of Doxil and DPA-Zn.

[0055] Fig. 18 shows the schematic presentation of the proposed DPA-Zn and CDN@lip loaded into an alginate-based hydrogel.

[0056] Fig. 19A is a schematic representation of the basic steps involved in preparing DPA-Zn in alginate hydrogel.

[0057] Fig. 19B shows the image showing 1% alginate solution and DPA-Zn loaded in 1% alginate in the preparation of DPA-Zn@alginate hydrogel.

[0058] Fig. 19C shows the image showing DPA-Zn loaded in 1% (DPA-Zn@1%alg) or 2% alginate (DPA-Zn@2%alg). The concentration of DPA-Zn in the gel is 10 mg/mL. The hydrogel was injected using a 27-gauge needle. The plus sign (+) indicates injectability, with more plus signs indicating better injectability in the preparation of DPA-Zn@alginate hydrogel.

[0059] Fig. 19D shows DPA-Zn release kinetics in 0.4 μm Transwell against 25 mM HEPES buffer at 37 °C (n=1).

DETAILED DESCRIPTION

[0060] For the purposes of promoting an understanding of the principles of the present disclosure, reference will now be made to the embodiments illustrated in the drawings, and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the claimed invention is thereby intended.

[0061] The terms "chemotherapeutic agent," "anti-cancer drug", and "anti-cancer prodrug" refers to drugs (i.e., chemical compounds) or prodrugs known to, or suspected of being able to treat cancer (i.e., to kill cancer cells, prohibit the proliferation of cancer cells, or treat a symptom related to cancer). In some embodiments, the term "chemotherapeutic agent" refers to molecule used to treat cancer and/or for cytotoxic activity.

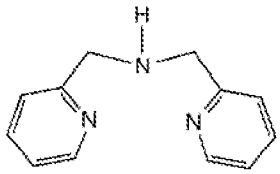
[0062] This disclosure is partly based on the discovery that the effect of cancer immunotherapy is still limited because phosphatidylserine (PS), a potent immunosuppressive molecule, is exposed on tumor cells and tumor blood vessels in the immunosuppressive tumor microenvironment (TME) and further increases after treatment with chemotherapy. The PS in TME prevents antigen-presenting cells from initiating an immune response to tumors. Blocking PS exposure caused by chemotherapy can restore the sensitivity of antigen-presenting cells to immune stimulants.

[0063] In view of the above, the present disclosure provides a combination of compounds that block the PS on tumor and peritumoral vessels to downregulate an immunosuppressive environment and activate immune cells in TME to facilitate the development of anti-tumor immunity.

[0064] Provided is a pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents.

[0065] The PS blocker can be selected from dipicolylamine (DPA) or its metal complex, a metal salt, Annexin V, an anti-PS antibody, and a combination of two or more thereof.

[0066] In some embodiments, the PS blocker is DPA or its metal complex. Dipicolylamine is also known as di-(2-picolyl) amine and is represented by a structure:



PS exposure on the outer membrane of cancer cells significantly increases after the chemotherapy treatment, which causes immunosuppressive effects on antigen-presenting cells (APCs). Thus, blocking of PS is an essential function of APCs in anti-tumor immune response. DPA and its metal complex can block the immunosuppressive effect of PS on APCs, such as, macrophages, and can restore the response of macrophages to lipopolysaccharides (LPS). DPA with or without metal complex can significantly improve the anti-tumor efficacy of chemotherapeutic agents in cancer, such as, for example, melanoma.

[0067] A metal complex can be formed of a compound and a metal ion. The metal ion is a cation having two or more charges. The metal complex is typically formed via the chelation of a metal ion and a compound. The metal complex of DPA can be formed with transition metal ions by forming co-ordination bonds. Certain examples of transition metal ions include, but are not limited to, zinc (Zn), cobalt (Co), copper (Cu), platinum (Pt), iron (Fe), nickel (Ni), silver (Ag), chromium (Cr), manganese (Mn), titanium (Ti), vanadium (Vn), cadmium (Cd), and scandium (Sc).

[0068] In some embodiments, the PS blocker is the complex of DPA with Zn (DPA-Zn). DPA-Zn can bind to PS selectively with high affinity (**Fig. 4A**), thereby overcoming the immunosuppressive effect of PS in TME. DPA-Zn can restore the ability of macrophages to respond to immune stimulants (**Fig. 5**) and can develop immunoactive TME. Thus, DPA-Zn can be an effective PS-blocking agent, which can enhance the anti-tumor efficacy of chemotherapeutic agents.

[0069] In some embodiments, the PS blocker is Annexin V. Annexin V belongs to a family of phospholipid-binding proteins, the Annexins. It binds in the presence of metal ions with high affinity to negatively charged phospholipids like PS.

[0070] In some embodiments, the PS blocker is a metal salt. Any suitable metal salt can be used. The metal salt can be zinc acetate dihydrate, zinc gluconate (Zn(glu)), zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide, calcium chloride, calcium sulfate, or a combination of two or more thereof. The metal ions can bind to PS by forming a PS-metal complex.

[0071] The immune stimulant can be selected from a granulocyte-macrophage colony-stimulating factor (GM-CSF), a stimulator of interferon genes (STING) agonist, anti-PD-L1 antibody, anti-PD-1 antibody, CpG oligodeoxynucleotides, polyinosinic-polycytidylic acid (poly (I:C)), single-stranded RNA (ssRNA), short double-strandedRNA (dsRNA), long ds-RNA and a combination of two or more thereof. In some embodiments, the immune stimulant is the STING agonist. The STING agonist can trigger the innate immune response in eukaryotic cells through the STING signaling pathway thus, they are potential immunostimulators and novel molecular adjuvants for induction of systemic and mucosal innate and adaptive immune responses. STING agonist can be a cyclic dinucleotide (CDN) or its metal complex.

[0072] The metal complex of cyclic nucleotide can be formed with the metal ion selected from Zn, Mn, Cu, Fe, Co, and aluminum (Al). Certain examples of the CDNs include, but are not limited to, cyclic GMP-AMP (cGAMP), cyclic di-GMP (c-di-GMP or CDG), cyclic di-AMP (c-di-AMP or CDA), or a combination of two or more thereof. Chemically synthesized CDN 2′3′-c-di-AM(PS)2 (Rp, Rp), an analogue of c-di-AMP (ADU-S100), can be used. It has a higher affinity to STING than c-di-AMP.

[0073] The anti-PD1 antibody can be nivolumab, pembrolizumab, camrelizumab, cemiplimab, sintilimab, toripalimab, or a combination of two or more thereof.

[0074] In some embodiments, one or more chemotherapeutic agents are doxorubicin, paclitaxel, nab-paclitaxel, docetaxel, cisplatin, carboplatin, oxaliplatin, carfilzomib, bortezomib, mitoxantrone, dacarbazine, temozolomide, vinblastine, or a combination of two or more thereof. In some embodiments, the chemotherapeutic agent is doxorubicin.

[0075] Doxorubicin, an anthracycline antibiotic, is the treatment of choice as first-line chemotherapy for many cancer patients not previously treated with anthracyclines. Doxorubicin can be used to treat soft tissue and bone sarcomas and cancers of the breast, ovary, bladder, and

thyroid. It can also be used to treat acute lymphoblastic leukemia, acute myeloblastic leukemia, Hodgkin lymphoma, and small-cell lung cancer. However, it is ineffective against melanoma cells due to the frequent development of resistance.

[0076] Provided is a pharmaceutical composition comprising (i) a therapeutically effective amount of PS blocker, (ii) a therapeutically effective amount of an immune stimulant, and (iii) a therapeutically effective amount of one or more chemotherapeutic agents, and one or more pharmaceutically acceptable carrier, diluent, or excipient.

[0077] Provided is a combination of pharmaceutical compositions comprising (i) a pharmaceutical composition comprising a therapeutically effective amount of the phosphatidylserine (PS) blocker, (ii) a pharmaceutical composition comprising a therapeutically effective amount of the immune stimulant, and (iii) a pharmaceutical composition comprising a therapeutically effective amount of one or more chemotherapeutic agents, wherein (i), (ii), and (iii) are independently formulated to be administered by the same or different routes.

[0078] In some embodiments, the (i) PS blocker and (ii) immune stimulant can be administered simultaneously as a single composition or two separate compositions, in either order by the same or different routes, whereas one or more chemotherapeutic agents can be administered simultaneously with (i) and (ii) or sequentially, in either order by the same or different routes.

[0079] The pharmaceutical combination or the pharmaceutical composition can be administered by any suitable route. In some embodiments, the suitable route can be intravenous, subcutaneous, intratumoral, topical, intracranial, intraperitoneal, intrapulmonary, intranasal, or oral.

[0080] Provided is a method for treating a patient of cancer, which method comprises administering to the patient (i) a therapeutically effective amount of a phosphatidylserine (PS) blocker, (ii) a therapeutically effective amount of an immune stimulant, and (iii) a therapeutically effective amount of one or more chemotherapeutic agents, optionally as a pharmaceutical composition comprising (i), (ii) and (iii) and pharmaceutically acceptable carrier, diluent or excipient whereupon the patient is treated for cancer.

[0081] The method can comprise administering a therapeutically effective amount of (i), (ii), and (iii) as neat compounds or as a pharmaceutical composition. The compounds or a pharmaceutical

composition can be administered during or after the onset of the disease or condition. The compounds can be administered intravenously, subcutaneously, intratumorally, intracranially, intraperitoneally, intrapulmonarilly, intranasally, topically, or orally.

[0082] In some embodiments, (i), (ii), and (iii) can be formulated as a single pharmaceutical composition or as separate pharmaceutical compositions, which can be administered simultaneously or sequentially by the same or different routes.

[0083] Examples of cancer include, but are not limited to, melanoma, breast cancer, head and neck cancer, oral cavity cancer, ovarian cancer, prostate cancer, lung cancer, colon cancer, liver cancer, cervical cancer, brain cancer, colorectal cancer, bladder cancer, and gastrointestinal cancer. In some embodiments, the cancer is melanoma.

[0084] The PS blocker can be administered in a dose range of about 2.0 mg/kg to about 50 mg/kg, such as about 2.0 mg/kg to 50 mg/kg, 2.0 mg/kg to about 50 mg/kg, or 2.0 mg/kg to 50 mg/kg. In some embodiments, the dose is 2.0 mg/kg. In some embodiments, the dose is 2.5 mg/kg. In some embodiments, the dose is 5 mg/kg. In some embodiments, the dose is 10 mg/kg. In some embodiments, the dose is 15 mg/kg. In some embodiments, the dose is 20 mg/kg. In some embodiments, the dose is 30 mg/kg. In some embodiments, the dose is 40 mg/kg.

[0085] The immune stimulant can be administered in a dose range of about 0.01 mg to about 10 mg. In some embodiments, the dose is 0.02 mg. In some embodiments, the dose is 0.04 mg. In some embodiments, the dose is 0.06 mg. In some embodiments, the dose is 0.08 mg. In some embodiments, the dose is 0.1 mg. In some embodiments, the dose is 0.3 mg. In some embodiments, the dose is 0.5 mg. In some embodiments, the dose is 0.8 mg. In some embodiments, the dose is 1 mg. In some embodiments, the dose is 3 mg. In some embodiments, the dose is 5 mg. In some embodiments, the dose is 8 mg. In some embodiments, the dose is 10 mg.

[0086] The chemotherapeutic agent can be administered in the dose range of about 5 mg/kg to about 20 mg/kg, such as about 5 mg/kg to 20 mg/kg, 5 mg/kg to about 20 mg/kg, or 5 mg/kg to 20 mg/kg.

[0087] Further provided is a sustained-release formulation of a combination disclosed herein that can prolong local retention of the combination in the tumor and maximize the therapeutic efficacy

of the compounds. The PS blocker and immune stimulant can be provided in delivery vehicles, such as, for example, liposomes, nanoparticles, hydrogel, implants, and microspheres.

[0088] The sustained-release formulation comprises: a therapeutically effective amount of a hydrogel comprising a PS blocker, alone or in further combination with a crosslinker, and an immune stimulant, wherein the immune stimulant is optionally encapsulated.

[0089] In some embodiments, the sustained-release formulation can be a hydrogel formulation, which formulation comprises the PS blocker, such as DPA or DPA-Zn, and the crosslinker, such as zinc gluconate, in which a formulation of immune stimulant, such as CDN or CDN-Zn can be encapsulated. This hydrogel formulation can extend the retention of compounds in the tumor. The hydrogel formulation can be administered in combination with one or more chemotherapeutic agents.

[0090] Any suitable natural or synthetic polymers, as known in the art, can be used for hydrogel formulation. The polymer used for hydrogel formulation can be a natural polymer. The natural polymer can be selected from alginate, pectin, carrageenan, polylactic acid, polyethylene glycol, collagen, fibrin, hyaluronic acid, matrigel, chitosan, silk fiber, and a combination of two or more of the foregoing.

[0091] In some embodiments, the natural polymer is alginate. Alginate is biocompatible and non-immunogenic. It has a shear-thinning property and contains many carboxylic groups that can electronically interact with cations. The alginate used for hydrogel formulation can be about 1% w/v to about 4% w/v, such as about 1% w/v to 4% w/v or 1% w/v to about 4% w/v or 1% w/v to 4% w/v.

[0092] The crosslinker can be any suitable metal salt. The metal salt can be selected from zinc acetate dihydrate, zinc gluconate, zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide, calcium sulfate, and calcium cloride. The crosslinker can be selected based on its solubility in water. Slowly dissolving crosslinker can maintain a constant supply of the crosslinker in the formulation. It can improve the stability of the alginate hydrogel. The sustained-presence of the crosslinker, such as Zn^{2+} in alginate, can keep alginate crosslinked for a prolonged period, allowing for sustained PS blocker, such as DPA-Zn release, and also serving as a PS blocker.

[0093] In some embodiments, the immune stimulant is encapsulated in a liposomal formulation or a nanoparticulate formulation.

[0094] The liposomal formulation comprises (a) an immune stimulant, (b) a cationic lipid, (c) a cholesterol, and (d) a phospholipid. The liposomal formulation can be prepared using a thin-film hydration method.

[0095] Provided is a method for preparing a liposomal formulation comprising an immune stimulant, which method comprises:

(i) mixing one or more cationic lipids, cholesterol, and one or more phospholipids in an organic solvent;
(ii) evaporating the organic solvent to obtain a lipid film;
(iii) mixing a solution of an immune stimulant to the lipid film of step (ii) and hydrating the mixture to obtain a suspension; and
(iv) extruding the solution of step (iii), whereupon the liposomal formulation is formulated.

[0096] Suitable cationic lipids, as known in the art, can be used. The cationic lipid can be selected from 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1,2-di-O-octadecenyl-3-trimethylammonium-propane (DOTMA), 1,2-dioleoyl-sn-glycero-3- phosphoethanolamine (DOPE), 1,2-dimyristyloxy-propyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dimyristoyl-3-trimethylammonium-propane (DMTAP). In some embodiments, the cationic lipid is DOTAP. Cholesterol can stabilize the lipid membrane and control the fluidity.

[0097] Suitable phospholipids, as known in the art, can be used. They can prevent aggregate formation. The phospholipid can be selected from polyethyleneglycol (PEG), phosphatidylcholine, distearoylphosphatidylcholine- PEG2000 or 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethyleneglycol)-2000] (DSPE-PEG2000), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS) and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC).

[0098] The immune stimulant used can be a STING agonist. In some embodiments, the STING agonist is CDN or its metal complex. Liposome formulation can be prepared by varying the molar ratio of the DOTAP, DSPE-PEG, and cholesterol mixture and the total lipid amount. The CDN loading efficiency can be improved with the adjustment of the lipid feed, STING agonist concentration, hydration volume, and the lipid/CDN ratio. The diameter of the liposome formed can be from about 100 nm to about 500 nm, such as about 100 nm to 500 nm, or 100 nm to about 500 nm or 100 nm to 500 nm. In some embodiments, the diameter is from about 150 nm to about 450 nm, such as about 150 nm to 450 nm, or 150 nm to about 450 nm or 150 nm to 450 nm. In some embodiments, the diameter is 180 nm. In some embodiments, the diameter is 200 nm. In some embodiments, the diameter is 400 nm.

[0099] Further provided is a method for preparing a nanoparticulate formulation, which method comprises:

(i) mixing a solution comprising a metal salt with a solution comprising an immune stimulant with stirring;

(ii) agitating the mixture of step (i); and

(iii) centrifugating the agitated mixture whereupon the nanoparticulate formulation is collected.

[0100] Still further provided is a method for preparing sustained-release formulation, which method comprises:

(i) mixing a solution comprising a hydrogel and a solution comprising phosphatidylserine (PS) blocker to obtain a hydrogel of PS blocker;

(ii) optionally mixing the hydrogel of step (i) with a suspension comprising a crosslinker to formulate a hydrogel comprising the PS blocker and the crosslinker; and

(iii) mixing the hydrogel of step (i) or step (ii) with a solution comprising an optionally encapsulated immune stimulant,

whereupon the sustained-release formulation is formulated.

[0101] In some embodiments, the immune stimulant is encapsulated in the liposomal or nanoparticulate formulation described herein.

[0102] The sustained-release formulation can be administered by any suitable route. In some embodiments, the suitable route can be subcutaneous, intratumoral, intravenous, topical, intracranial, intraperitoneal, intrapulmonary, intranasal, or oral.

[0103] The dosage levels of compounds used in the compositions and sustained-release formulations can be varied to administer an amount of the composition and the formulation that is effective in achieving the desired effect for a particular patient. The selected dosage level can depend upon the activity of the composition, formulation, and route of administration.

[0104] Further, provided is a method for treating cancer in a patient, which method comprises administering to the patient a therapeutically effective amount of a sustained-release formulation described herein and one or more therapeutic agents.

[0105] In some embodiments, the pharmaceutical combination used in the chemoimmuno combination therapy can have an additive or a synergistic effect. In combination therapy, each component plays a distinct role in providing additive or synergistic effects. A chemotherapeutic agent can reduce tumor burden and expose tumor antigens. PS blocking agent covers the PS exposure caused by chemotherapy, restoring the sensitivity of innate immune cells to immune stimulants. Immune stimulant energizes the immune system by inducing activation or increasing activity of its components, for example, STING stimulators can activate the STING pathway to stimulate innate immune response.

[0106] The methods of combination therapy disclosed herein can result in an additive or a synergistic effect, wherein the effect of a combination of compounds or other therapeutic agents is equal or greater than the sum of the effects resulting from the administration of any of the compounds or other therapeutic agents as single agents. An additive or a synergistic effect may also be an effect that cannot be achieved by the administration of any of the compounds or other therapeutic agents as single agents. The additive or synergistic effect may include, but is not limited to, an effect of treating cancer by reducing tumor size, inhibiting tumor growth, or increasing the survival of the subject. It may also include reducing cancer cell viability, inducing cancer cell death, and inhibiting or delaying cancer cell growth.

[0107] The term "therapeutically effective amount" or "therapeutically effective dose" refers to an amount of the active ingredient(s) that is(are) sufficient, when administered, to deliver efficaciously the active ingredient(s) for the treatment of a disease or condition of interest to a subject in need thereof. The prophylactically or therapeutically effective amount of such combination will vary depending upon the patient and the disease or condition being treated, the weight and age of the patient, the severity of the disease or condition, the manner of administration, and the like, which can readily be determined by one of ordinary skill in the art. In the case of a cancer or other proliferative disorder, the prophylactically or therapeutically effective amount of the agent may reduce (*i.e.*, inhibit to some extent or stop) unwanted cellular proliferation; reduce the number of cancer cells; reduce the tumor size; inhibit (or stop) cancer cell infiltration into peripheral organs; inhibit (or stop) tumor metastasis; inhibit, *e.g.*, to some extent, tumor growth; and/or relieve, to some extent, one or more of the signs or symptoms associated with the cancer. To the extent the administered compound or composition prevents growth and/or kills existing cancer cells, it may be cytostatic and/or cytotoxic.

[0108] For any compound therapeutically effective amount can be initially determined from animal models. A therapeutically effective dose can also be determined from human data for compounds which have been tested in humans and for compounds which are known to exhibit similar pharmacological activities, such as other related active agents. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound. Adjusting the dose to achieve maximal efficacy based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

[0109] The terms "treat," "treating," "treatment," and the like refer to eliminating, reducing, or ameliorating a disease or condition, and/or symptoms associated therewith. Although not precluded, treating a disease or condition does not require that the disease, condition, or symptoms associated therewith be completely eliminated. The term "treat" and synonyms contemplate administering a prophylactic or therapeutically effective amount of a combination or composition described herein to a subject in need of such treatment. The treatment can be orientated symptomatically, for example, to suppress symptoms. It can be effective over a short period, be oriented over a medium term, or can be a long-term treatment, for example within the context of maintenance therapy.

[0110] Generally, daily oral doses of a compound are from about 0.01 milligrams/kg per day to 1,000 milligrams/kg per day. Oral doses in the range of 0.5 to 50 milligrams/kg, in one or more administrations per day, can yield therapeutic results. Dosage can be adjusted appropriately to achieve desired drug level, local or systemic, depending upon the mode of administration. For example, intravenous administration can vary from one order to several orders of magnitude lower dose per day. If the response in a subject is insufficient at such doses, even higher doses (or effective higher doses by a different, more localized delivery route) can be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of the compound.

[0111] The compounds can be typically administered in admixture with a pharmaceutical carrier to give a pharmaceutical composition selected with regard to the intended route of administration and standard pharmaceutical practice. Pharmaceutical compositions can be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and/or auxiliaries that facilitate the processing of the compound. The pharmaceutical compositions can be manufactured, for example, by conventional mixing, dissolving, granulating, dragee-making, emulsifying, encapsulating, entrapping, or lyophilizing processes. Proper formulation is dependent upon the route of administration chosen. When a therapeutically effective amount of a compound described herein is administered orally, the composition typically is in the form of a tablet, capsule, powder, solution, or elixir. When administered in tablet form, the composition additionally can contain a solid carrier, such as a gelatin or an adjuvant. The tablet, capsule, and powder can contain about 0.01% to about 95%, and preferably from about 1% to about 50%, of the combination of compounds. When administered in liquid form, a liquid carrier can be added, such as water, petroleum, or oils of animal or plant origin. The liquid form of the composition can further contain the physiological saline solution, dextrose or other saccharide solutions, or glycols. When administered in liquid form, the composition contains about 0.1% to about 90%, and preferably about 1% to about 50%, by weight, of the combination of compounds.

[0112] For oral administration, the compounds can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers, excipients, or diluents well-known in the art. Such carriers, excipients, or diluents enable the compounds to be formulated as tablets, pills, powders, dragees, capsules, liquids, gels, syrups, slurries, suspensions, solutions, and the like for oral ingestion by a subject to be treated.

[0113] The exact formulation, route of administration, and dosage of a pharmaceutical composition comprising an effective amount of the compound are determined by an individual physician in view of the diagnosed condition or disease. The dosage amount and interval can be adjusted individually to provide levels of the compound that are sufficient to maintain a prophylactic or therapeutic effect.

[0114] Toxicity and therapeutic efficacy of the combination can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the maximum tolerated dose (MTD) of a compound, which is defined as the highest dose that causes no toxicity in animals. The therapeutic index is the dose ratio between the maximum tolerated dose and therapeutic effects (*e.g.*, inhibition of tumor growth). The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. The determination of a therapeutically effective amount is well within the capability of those ordinarily skilled in the art, especially in light of the detailed disclosure provided herein.

[0115] As stated above, a combination or composition described herein can be administered in with one or more other prophylactically or therapeutically active agents.

[0116] It will be appreciated by persons skilled in the art that the present disclosure is not limited by what has been particularly shown and described herein above. Rather the scope of the present disclosure includes both combinations and sub-combinations of the various features described hereinabove as well as variations and modifications which would occur to persons skilled in the art upon reading the specification and which are not in the prior art.

EXAMPLES

[0117] The following examples serve to illustrate the present disclosure. The examples are not intended to limit the scope of the claimed invention in any way.

Doxorubicin used as a liposomal doxorubicin which is a doxorubicin HCl liposome or liposomal doxorubicin

[0118] Annexin V and Caspase 3/7 staining of tumor cells:

B16F10 melanoma cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 5% CO_2, 37 °C. 10^5 B16F10 cells were seeded into 24-well plates and treated with liposomal doxorubicin in concentrations equivalent to doxorubicin (DOX) ranging from 1.56 to 200 μg/mL for 24 hours. After treatment, cells were collected and pelleted by centrifugation at 1000 rpm for 3 minutes. Next, cells were incubated in 100 μL of cell complete medium containing 5 μM caspase 3/7 for 30 min at 37 °C. Following the incubation, cells were rinsed with Annexin V binding buffer and pelleted by centrifugation. The cell pellet was incubated with 100 μL annexin V binding buffer containing 5 μL Annexin V-PE antibody for 20 min in the dark at room temperature. Cells were rinsed twice with 200 μL annexin V binding buffer and analyzed by flow cytometry.

[0119] Preparation and characterization of phosphatidylserine (PS) liposome:

Liposomes composed of different PS contents (PS wt%: 0, 20, 50, and 80) were prepared by the extrusion method. Specifically, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine sodium salt (POPS) were dissolved in a mixture of organic solvents (chloroform/methanol = 3/1, v/v) in a round-bottomed flask. The organic solvent was then evaporated by rotary evaporator and formed a thin film. Next, deionized water was added to hydrate the membrane, resulting in a final lipid concentration of approximately 10 mg/mL. The suspension of the hydrated membrane was sonicated in a water bath and extruded through 0.2 μm filter for 15-30 cycles. The hydrodynamic diameter and surface charge of the final liposomes were characterized by dynamic light scattering.

[0120] Bone marrow cells collection and induction:

Bone marrow cells were retrieved from female or male C57BL/6 mice. Animals were sacrificed by CO_2 asphyxiation followed by cervical dislocation. The femur and tibia were separated and flushed through the bone cavity with RPMI 1640 via a 25 gauge needle. The obtained cells were passed through 40 μm cell strainer and centrifuged at 500 g for 10 minutes. Cell pellets were treated with ACK lysis buffer for 3 minutes to remove red blood cells, supplemented with additional phosphate-buffered saline (PBS), and centrifuged at 500 g for 10 minutes. The collected cells were suspended in Alpha minimum essential medium (MEM-Alpha, 4 mM L-glutamine, 1 mM sodium pyruvate, ribonucleotides, deoxyribonucleotides) supplemented with 20% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, 30 ng/mL murine macrophage colony-stimulating factor (M-CSF), and 10 mM 2-mercaptoethanol. Cells were seeded into a non-tissue pretreated petri-dish for 5-7 days. The floating cells were collected by centrifugation and identified

as bone marrow-derived dendritic cells (BMDC). The adherent cells were identified as bone marrow-derived macrophages (BMM).

[0121] Effects of PS liposome on BMMs and BMDCs:

BMMs were plated at 2×10^5 per well in a 96-well plate and incubated with PS liposomes. After 24 hours of incubation, LPS was added to challenge BMMs and further incubated for another 24 hours. Similarly, BMDCs were plated at 5×10^5 per well in 24 well-plate and incubated with PS liposomes (50%) in varying concentrations. After 24 hours of incubation, cells were collected, resuspended in staining buffer, and incubated with Fc-blocking antibody (anti-CD16/32 antibody) for 15 min at 4 °C. Cells were further stained with anti-CD11c, anti-CD86 antibodies for 20 min at 4 °C and analyzed by the flow cytometer. FACS data was analyzed using FlowJo™ (version 10, Tree Star Inc.).

[0122] Preparation of DPA-Zn:

DPA-Zn was prepared by mixing DPA solution with zinc solution. Specifically, 180 µL DPA liquid was added to 1 mL $ZnSO_4$ (1M) solution, vortexed for 1 min, and further agitated for > 2 hours.

[0123] Binding affinity of DPA-Zn to PS:

Different PS liposomes were incubated with a fixed concentration of DPA-Zn to test the selective binding of DPA-Zn to PS in the liposome. Specifically, 0.5 mL of 2 mg/mL PS-liposomes with varying PS contents (PS% in PS/PC liposome at 0%, 20%, 50%, and 80%) were incubated with 0.5 mL of 1M DPA-Zn for 20 minutes. The liposomes were then centrifuged at 305,000 g for 1 hour. DPA-Zn concentration in the supernatant was measured at the wavelength of 245 nm.

[0124] Isothermal titration calorimetry (ITC) of DPA-Zn to PS liposome:

The binding affinity (K_d) was measured by isothermal titration calorimetry. For each measurement, 350 µL of PS liposome (contains 6.83 µM PS) was loaded in the ITC cell, and 100 µL DPA-Zn solution (50 µM) was loaded into the titration syringe. The temperature was kept at 25°C, and DPA-Zn solution was slowly titrated into the liposome solution in the ITC cell by 10 µL per time.

[0125] DPA-Zn blockade of PS in BMM:

BMMs were seeded in 96-well plates at a density of 10^5 cells per well with 0.2 mL of culture medium and incubated overnight until cells adhered to the well. Cells were incubated with PS

liposomes ± varying concentrations of DPA-Zn for 24 h. Subsequently, BMMs were incubated with 10 ng/mL lipopolysaccharide (LPS) for an additional 24 h. TNF-α secretion in the supernatant was measured by ELISA.

[0126] Animal experiments:

Unless stated otherwise, male C57BL/6 mice, aged 5-6 weeks, were obtained from Envigo (Indianapolis, IN, USA) and acclimatized for at least one week prior to the procedure.

[0127] Maximum tolerance dose (MTD) of DPA-Zn by intravenous injection:

To determine the maximum tolerance dose (MTD) of DPA-Zn, mice were intravenously injected with DPA-Zn solution on day 0, 1, 4, 5, 8, and 9. The doses used were 2.5 mg/kg, 5 mg/kg, 10 mg/kg, and 20 mg/kg, with two mice assigned per each dose group. Mice were monitored for two weeks for any signs of toxicity, such as weight loss exceeding 20%, changes in behaviors (lethargy, low body temperature), etc.

[0128] Anti-tumor effect of liposomal doxorubicin+DPA-Zn in B16F10 tumor model:

A total volume of 0.1 mL containing about 10^6 B16F10 tumor cells was subcutaneously injected into the right flank of a wild-type C57BL/6 mouse (6-8 weeks old). Treatment was initiated when tumors were palpable and reached a volume of 50-100 mm^3. Mice were injected with 10^6 B16F10 cells per mouse subcutaneously. When tumors grew to 50 mm^3 (day 0), mice were treated with 10 mg/kg DOX intraperitoneally (i.p.) or liposomal doxorubicin equivalent to 2.5 mg/kg DOX intratumorally (i.t.). Mice were treated intratumorally (i.t.) with 10 mg/kg DPA-Zn on days 1, 2, 4, 5. Mice receiving 5% dextrose (D5W) as the same administration route served as control group. Tumor size was assessed over time using a digital caliper. Tumor size was calculated based on the equation: Volume = length × $width^2$ × 0.5. On day 10-12, animals were sacrificed, and tumors, tumor-draining lymph nodes (TDLNs), and spleens were extracted. The projected spleen area was calculated by ImageJ software (National Institute of Health, Bethesda, MD).

[0129] Anti-tumor efficacy of the inclusion of an immunostimulant in liposomal doxorubicin + DPA-Zn:

A total volume of 0.1 mL containing approximately 10^6 B16F10 tumor cells was subcutaneously injected into the right flank of wild-type C57BL/6 mice (6-8 weeks old). When tumors reached a target size for each study, mice were randomized into different groups and treated as described below.

[0130] To assess the anti-tumor efficacy of DPA-Zn with or without GM-CSF, tumors were grown to 50 mm^3, and mice were treated intravenously with liposomal doxorubicin (equivalent to DOX at 2.5 mg/kg) or 5% dextrose (D5W) on days 0 and 3, followed by intratumoral injection of 10 mg/kg DPA-Zn and/or rmGM-CSF (2.5 μg/mouse) or D5W on days 1, 2, 4, 5 (n=5 per group). Tumor size was assessed over time using a digital caliper. Animals were sacrificed at day 10-12 post first injection, and tumor, tumor-draining lymph nodes (TDLNs), and spleens were extracted for immunophenotyping.

[0131] For the comparison of combinations with different immunostimulants, tumors were grown to 50 mm^3, and mice were intravenously treated with liposomal doxorubicin (equivalent to DOX at 2.5 mg/kg) on day 0, 3, followed by DPA-Zn (10 mg/kg) with one of the following on day 1,2, 4, and 5: D5W, rmGM-CSF (2.5 μg/mouse), anti-PD-L1 antibody (150 μg/mouse), or CDN (10 μg/mouse) per day. Tumor size was assessed over time using a digital caliper. Mice were sacrificed when the tumor reached 2000 mm^3 or when they became moribund with severe weight loss or ulceration.

[0132] To assess the anti-tumor efficacy of DPA-Zn with or without cyclic dinucleotides (CDN), tumors were grown to 100-150 mm^3, and mice were treated intravenously with liposomal doxorubicin (equivalent to DOX at 2.5 mg/kg) or 5% dextrose (D5W) on days 0 and 3, followed by intratumoral injection of 5 mg/kg DPA-Zn and/or CDN (10 μg/mouse) on days 1, 2, 4, 5 (n=5 per group). Animals were sacrificed at days 10-12 from the first treatment, and tumors, TDLNs, and spleens were extracted for phenotyping.

[0133] Immunophenotyping by flow cytometry:

Tumors were excised, finely minced and blended with 2 mg/mL collagenase type IV, 0.2 mg/mL DNase I, and 0.2 mg/mL hyaluronidase for 2h at 37°C, according to manufacturer's instruction. The dissociated tumors, TDLNs, and spleens were crushed with the rubber end of a syringe plunger and filtered through 70 μm and 40 μm cell strainers, sequentially to obtain single cell suspension. The dissociated tumor cells were further incubated with ACK lysis buffer to lyse red blood cells. Cells were then incubated with Zombie dye and anti-mouse CD16/CD32 antibody for 20 min at room temperature. Afterward, cells were stained with a cocktail of antibodies for 1h at 4°C. Cells were fixed and permeabilized by the FoxP3/transcription factor staining buffer set, following the manufacture's guidelines. Next, cells were washed, resuspended in PBS, and analyzed using flow cytometry. Compensation was performed manually. All FACS analysis was performed by the FlowJo™ V10 software and GraphPad prism 9.

[0134] Preparation of CDN-Zn nanoparticle (NP):

CDN-Zn coordination NPs were produced by mixing $ZnCl_2$ solution with CDN solution under vigorous stirring. Specifically, a 100 mM $ZnCl_2$ solution was added to 1 mg/mL CDN solution, vortexed vigorously, and sonicated for 2 minutes. The mixture was placed in a shaker and agitated at room temperature for 1hour. CDN-Zn NP was collected by ultracentrifugation at 20,000 g for 10 minutes and then dispersed in deionized water.

[0135] Isothermal titration calorimetry (ITC) of CDN-Zn:

The dissociation constant (K_d) and the stoichiometry of CDN-Zn reaction was measured using isothermal titration calorimetry. The sample cell in the ITC was filled with 350 μL of CDN solution (1.36 mM). The titration syringe was loaded with 100 μL of $ZnCl_2$ solution (10 mM). A preliminary injection of 0.4 μL was performed to ensure proper mixing, followed by a series of 19 injections, with each injection consisting of 5 μL of $ZnCl_2$ solution. The injections were spaced 150 seconds apart to allow for complete equilibration between injections. The stirring speed was maintained at 1000 rpm, and the temperature was kept at 25°C throughout the experiment. The temperature was kept at 25°C. The raw ITC data was integrated using the Origin software provided by the manufacturer.

[0136] Preparation of liposomal CDN (CDN@lip):

Liposomal CDN was prepared using thin-film hydration method. Specifically, a mixture of lipids, including 1,2-Dioleoyl-3-trimethylammonium (DOTA), cholesterol, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[carboxy(polyethylene glycol)-2000] sodium salt (DSPE-PEG200), at a molar ratio of 1:1:0.2, was dissolved in chloroform in a round-bottom flask. The solvent was evaporated using a rotary evaporator to form a thin lipid film on the walls of the flask. Next, 1 mL of CDN solution (1 mg/mL) was added to hydrate the lipid membrane, and the mixture was bath sonicated for 1-5 minutes. The resulting suspension was then extruded through a polycarbonate filter with a pore size of 0.2 μm using a mini-extruder for 20-30 cycles to obtain liposomal CDN. To determine the loading efficiency (LE) and encapsulation efficiency (EE) of CDN in the liposomes, the amount of CDN in liposome solution (total CDN) and the amount of unencapsulated CDN were measured by a UV spectrophotometer. The liposomes were first lyophilized and then accurately weighed before dissolution in ethanol. The total amount of CDN in the liposomes was calculated based on the measured CDN content. The amount of unencapsulated CDN was measured by quantifying the CDN in the supernatant after

ultracentrifugation at 256,000g at 4°C for 50 minutes. The LE and EE were then calculated using the following equations. Of note, the EE*% = EE% × (liposome yield).

$$EE^{*}\% = \frac{\text{Total CDN} - \text{Unecapsulated}}{\text{weight of feeding CDN}} \times 100\%$$

$$LE\% = \frac{\text{Total CDN} - \text{Unencapsulated CDN}}{\text{weight of lyophilized liposome}} \times 100\%$$

[0137] Particle characterization (DLS, TEM):

This Hydrodynamic diameter and surface charge of the final liposomes were characterized by dynamic light scattering. Z-average was measured by dispersing particles in DI water. Zeta potential was measured by dispersing particles in 1 mM phosphate buffer (pH 7.4). Their morphology was examined by a FEI Tecnai T20 transmission electron microscope (Hillsboro, OR) after negative staining with 1% uranyl acetate (CDN-Zn NP) or 1% phosphotungstic acid (CDN@lip).

[0138] *In vitro* release CDN from CDN@lip:

The release kinetics of CDN from CDN@lip were assessed using a dialysis device with a molecular weight cutoff of 8-10 kDa. Specifically, 0.2 mL CDN@lip (equivalent to 0.91 mg/mL CDN) was suspended in 0.8 mL of phosphate-buffered saline (PBS, pH 7.4) and placed in the 1 mL dialysis tube. The dialysis tube was then immersed in 9 mL of fresh PBS at 37 °C under constant agitation. At predetermined time points, 1 mL of release medium was collected and replaced with an equal volume of fresh PBS. The concentration of CDN in sampled release medium was determined by HPLC.

[0139] *In vitro* evaluation of CDN activity:

293-Dual™ mSTING Cells (ISG-SEAP/KI-[IFN-β]Lucia) is a mSTING reporter cells derived from human embryonic kidney 293 cells that can be used to monitor the activation of interferon regulatory factor (IRF) and its binding to ISRE (IFN-stimulated response elements) and/or the expression of IFN-β in STING pathway. CDN stimulation can be assessed by monitoring ISRE-induced SEAP (secreted embryonic alkaline phosphatase) production. To test the activation of STING pathway, 293-Dual™ mSTING cells were seeded in a 96 well-plate at a cell density of 50,000 cells per well. Cells were treated with 20 μL of CDN or CDN@lip and cultured for 24 to 48 h. After incubation, cell supernatant was collected and measured at 620 nm by QUANTI-Blue™ (InvivoGen) assay.

[0140] Skin irritation of CDN@lip:

To assess the local response to CDN@lip, male C57BL/6 mice were subcutaneously injected once with either 100 μL of 5% dextrose solution (DW5) or CDN@lip containing an equivalent dose of 40 or 80 μg of CDN using a 27-gauge insulin syringe on the dorsal skin. Prior to the injection, the mice were shaved to help visualize the injection site. After dosing, the injection site was monitored daily for signs of irritation or inflammation, including erythema and edema, for 14 days. The severity of skin reactions was scored using the Draize scale scoring system (see **Table 1).** D5W was used as a control to establish the baseline.

Table 1 Standard for skin irritation study (Draize scale scoring)

Skin responses		Score
Erythema and eschar formation	No erythema	0
	Very slight erythema (barely perceptible)	1
	Well-defined erythema	2
	Moderate to severe erythema	3
	Severe erythema (beet-redness) to slight eschar formation (injuries in depth)	4
Edema formation	No edema	0
	Very slight edema (barely perceptible)	1
	Slight erythema (edges of area well-defined by definite raising)	2
	Moderate edema (raised approximately 1.0 mm)	3
	Severe erythema (raised more than 1.0 mm and extending beyond exposure area)	4
Total possible score for irritation		8

[0141] Anti-tumor efficacy of CDN@lip:

Male C57BL/6 mice, aged 5-6 weeks, were obtained from Envigo (Indianapolis, IN, USA) and acclimatized for at least one week prior to the procedure. B16F10 melanoma was inoculated. Treatments were applied when tumors reached a volume of 50-150 mm^3. Mice were treated with liposomal doxorubicin (equivalent to 2.5 mg/kg DOX) by intravenous injection, followed by either one dose of CDN@lip (equivalent to CDN 40mg/mouse on day 1) or four doses of CDN solution (equivalent to CDN 10 mg/mouse/injection on day 1, 2, 4, and 5) by intratumoral injection. Simultaneously, DPA-Zn solution (10 ug/mL) was given at day 1, 2, 4 and 5 intratumorally. D5W treated mice served as the control group.

[0142] Quantitative analysis of CDN and DPA-Zn:

To determine the maxima absorption wavelength of DPA-Zn and CDN, DPA-Zn solution and CDN solution was scanned from 200 to 400 nm by spectrophotometry. CDN and DPA-Zn were quantified by reverse phase-high performance liquid chromatography (RP-HPLC). The mobile phase consisted of a mixture of solvent A (methanol) and solvent B (10 mM pH 4.6 ammonium acetate/0.1 % acetic acid buffer). To separate DPA-Zn and CDN, the mobile phase was run in gradient: started with 20% A and maintained for 7 min, increased %A to 50% at 7 min and maintained for 3 min. A% was decreased back to 20% at 12 min and maintained until 15 min. The flow rate was set at 0.8 mL/min at room temperature. Both DPA-Zn and CDN were detected at 260 nm. The injection volume was 20 µL. For analysis of CDN alone, spectrophotometry or RP-HPLC was used. The mobile phase was a mixture of methanol and 10 mM pH 4.6 ammonium acetate/0.1 % acetic acid buffer and flowed at 0.8 mL/min at room temperature. CDN was detected at 256 nm with a retention time of 9 min.

[0143] DPA-Zn@alg preparation:

A 2% (w/v) alginate solution was prepared by dissolving alginate solution in deionized water under magnetic stirring overnight at room temperature. The alginate hydrogel preparation was modified from a routine protocol. Briefly, hydrogels were prepared by mixing the alginate solution with a specified crosslinker and other encapsulated reagents using two Luerlock syringes connected via a Luerlock connector.

[0144] DPA-Zn@alg hydrogel preparation:

One milliliter of alginate solution (2%) was loaded in syringe 1 and the bubble was removed. One milliliter of of DPA-Zn solution was loaded in syringe 2. A Luerlock adapter was attached between syringe 1 and syringe 2, and the two parts were mixed 50 times.

[0145] DPA-Zn@Zn(glu)/alg hydrogel preparation:

DPA-Zn@alg hydrogel was first prepared as described above. The entire hydrogel was then pushed into syringe 1, and the device was disconnected. Zinc gluconate (Zn(glu)) was weighed and suspended in glycerol using an Ultra-Sonicator (SONICS Vibra-Cell ultrasonic liquid processor, Newtown, CT) run at 30% amplitude with 1 second on/off cycle for 30 seconds. The Zn(glu) suspension was loaded in syringe 2, and the two parts were mixed 20 times.

[0146] [DPA-Zn+CDN or CDN@lip]@Zn(glu)/alg hydrogel preparation:

DPA-Zn@alg hydrogel was first prepared as described above. The entire hydrogel was pushed into syringe 1, and the device was disconnected. Then, CDN or CDN@lip solution was loaded into syringe 2 and mixed 20 times. Next, the hydrogel was pushed into syringe 1 and the device was disconnected. Zn(glu) suspension was loaded into syringe 2, and the two parts were mixed 20 times.

[0147] Stability of alginate hydrogels:

To identify the most effective zinc salt in keeping alginate hydrogel crosslinked, the hydrogel was incubated in DMEM, and the weight change was monitored. Briefly, 1 mL of 2% alginate solution was mixed with 1 mL of solution or suspension of different zinc salts via two 1 mL syringes connected by a Luerlock connector. The two solutions were thoroughly mixed by plunging two syringes back and forth 50 times. Next, 0.5 mL of formed hydrogel was injected into a 2 mL microcentrifuge tube and kept for 5-10 min until the gel settled on the bottom in the tube. One milliliter of DMEM was added without disturbing the gel. The tubes were placed on a shaker at 200 rpm in 37 °C. At 1, 2, 4, 8, 24, and 48h, the supernatant was discarded, the gel was blotted with tissue paper to absorb the extra liquid in the tube, and the tube was weighed. The tube was replenished with 1 mL of fresh DMEM. The mass of hydrogel was calculated by subtracting the mass of an empty tube from the mass of tube with the hydrogel.

[0148] *In vitro* release of DPA-Zn or CDN from hydrogels:

The release of DPA-Zn or CDN from hydrogels was performed using dialysis. Briefly, 0.5 mL of hydrogel containing drug was placed in mini dialysis devices (MWCO: 20K) and then immersed in 14 mL of Dulbecco's phosphate-buffered saline (DPBS) at pH 7.4. The entire assembly was placed in a shaking incubator at 200 rpm at 37 °C. At predetermined time points, 1 mL of the release medium was collected and replaced with fresh PBS. The concentration of the released drug was measured by HPLC. In a pilot study, the release kinetics of DPA-Zn@alg with 1% or 2% was performed in 0.4 μm Transwell against 25 mM HEPES buffer at 37 °C.

[0149] Anti-tumor efficacy of hydrogel formulation:

B16F10 tumor reached 100-150 mm^3, all mice were treated by tail vein injection of liposomal doxorubicin (equivalent to DOX at 2.5 mg/kg) or 5% dextrose (D5W) on days 0 and 3. In the non-hydrogel group, mice were intratumorally injected with 10 mg/kg DPA-Zn or D5W on day 1, 2, 4, and 5, or one dose of CDN@lip (equivalent to 40 μg CDN) on day 1. For the hydrogel group,

mice were intratumorally injected with one dose of hydrogel containing 40 mg/kg DPA-Zn a 40 μg CDN in liposome (CDN@lip) on day 1. Tumor size was assessed over time using a digital caliper. Mice were sacrificed when the tumor reached 2000 mm^3 or exhibited moribund symptoms such as severe weight loss (more than 20%), ulceration, bleeding, hunched posture, or lethargy.

[0150] Maximum tolerated dose (MTD) of DPA-Zn by subcutaneous administration:
Male C57BL/6 mice (aged 5-6 weeks) were administered subcutaneously with DPA-Zn with a standard dose escalation protocol to determine the MTD of DPA-Zn subcutaneously. Briefly, mice were randomly assigned to 5 groups, with 2 mice in each group. Each mouse received one single dose of 30 μL different concentration of DPA-Zn by subcutaneous injection via an insulin syringe with a 30-gauge needle. The injected DPA-Zn dose were 40, 20, 10, 5, and 2.5 mg/kg. The injection site was closely monitored for any signs of irritation or inflammation, including erythema and edema daily for 14 days after dosing. The security of skin reactions was scored using the Draize scale scoring system. The MTD was decided by the presence of severe skin irritation or toxic effects.

[0151] Statistical analysis:
Data were analyzed by one-way ANOVA test to determine the statistical difference. A p value of < 0.05 was considered statistically significant. Quantitative data were presented as mean ± standard deviation (SD). The Log-rank/Mantel-Cox test was performed to determine the difference among indicated groups in the Kaplan-Meier survival curve.

[0152] As used herein, the following terms and phrases shall have the meanings set forth below. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art.

[0153] The term "about" can allow for a degree of variability in a value or range, for example, within 10%, within 5%, or within 1% of a stated value or of a stated limit of a range.

[0154] The term "substantially" can allow for a degree of variability in a value or range, for example, within 90%, within 95%, or within 99% of a stated value or of a stated limit of a range.

[0155] The terms "a," "an," or "the" are used to include one or more than one unless the context clearly dictates otherwise. The term "or" is used to refer to a nonexclusive "or" unless otherwise indicated. In addition, the phraseology or terminology employed herein, and not otherwise defined, is for the purpose of description only and not of limitation. Any use of section headings is intended to aid the reading of the document and is not to be interpreted as limiting. Further, information that is relevant to a section heading may occur within or outside of that particular section. The terms "including" and "having" are defined as comprising (i.e., open language).

[0156] All patents, patent application publications, journal articles, textbooks, and other publications mentioned in the specification are indicative of the level of skill of those in the art to which the disclosure pertains. All such publications are incorporated herein by reference to the same extent as if each individual publication were specifically and individually indicated to be incorporated by reference.

WE CLAIM:

1. A pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents.

2. The pharmaceutical combination of claim 1, wherein the PS blocker is selected from a dipicolylamine (DPA) or its metal complex, a metal salt, Annexin V, an anti-PS antibody or a combination of two or more thereof.

3. The pharmaceutical combination of claim 2, wherein the PS blocker is DPA or its metal complex.

4. The pharmaceutical combination of claim 3, wherein the metal in the metal complex of DPA is a transition metal.

5. The pharmaceutical combination of claim 4, wherein the transition metal is zinc (Zn), cobalt (Co), copper (Cu), platinum (Pt), iron (Fe), nickel (Ni), silver (Ag), chromium (Cr), manganese (Mn), titanium (Ti), vanadium (Vn), cadmium (Cd) or scandium (Sc).

6. The pharmaceutical combination of claim 1, 2, 3, 4 or 5, wherein the PS blocker is DPA or DPA-Zn.

7. The pharmaceutical combination of claim 2, wherein the metal salt is zinc acetate dihydrate, zinc gluconate, zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide, calcium chloride, calcium sulfate or a combination of two or more thereof.

8. The pharmaceutical combination of claim 2, wherein the PS blocker is Annexin V.

9. The pharmaceutical combination of claim 1, wherein the immune stimulant is a granulocyte macrophage colony-stimulating factor (GM-CSF), a stimulator of interferon genes (STING) agonist, an anti-PD-L1 antibody, an anti-PD-1 antibody, a CpG oligodeoxynucleotide, a polyinosinic-polycytidylic acid (poly (I:C)), a single-stranded RNA (ssRNA), a short double-strandedRNA (dsRNA), a long dsRNA, or a combination of two or more thereof.

10. The pharmaceutical combination of claim 9, wherein the STING agonist is a CDN or its metal complex.

11. The pharmaceutical combination of claim 10, wherein the metal in the metal complex of the CDN is Zn, Mn, Cu, Fe, Co, and aluminum (Al).

12. The pharmaceutical combination of claim 1, wherein one or more chemotherapeutic agents are doxorubicin, paclitaxel, nab-paclitaxel, docetaxel, cisplatin, carboplatin, oxaliplatin, carfilzomib, bortezomib, mitoxantrone, dacarbazine, temozolomide, vinblastine, or a combination of two or more thereof.

13. A pharmaceutical composition comprising therapeutically effective amounts of (i), (ii), and (iii) of the pharmaceutical combination of any one of claims 1-12 and a pharmaceutically acceptable carrier, diluent, or excipient.

14. A combination of pharmaceutical compositions comprising (i) a pharmaceutical composition comprising a therapeutically effective amount of the phosphatidylserine blocker (PS) of any one of claims 1-8, (ii) a pharmaceutical composition comprising a therapeutically effective amount of the immune stimulant of any one of claims 1 and 9-11 and (iii) a pharmaceutical composition comprising a therapeutically effective amount of one or more chemotherapeutic agents, wherein (i), (ii) and (iii) are independently formulated to be administered by the same or different routes.

15. A method for treating a patient with cancer, which method comprises administering to the patient (i) a therapeutically effective amount of a phosphatidylserine blocker, (ii) a therapeutically effective amount of an immune stimulant, and (iii) a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer.

16. The method of claim 15, wherein (i), (ii), and (iii) are formulated as a single pharmaceutical composition or as separate pharmaceutical compositions, which can be administered simultaneously or sequentially by the same or different routes.

17. The method of claim 15 or 16, wherein the cancer is selected from melanoma.

18. The method of any one of claims 15-17, wherein the therapeutically effective amount of (i) is from about 2 mg/kg to about 50 mg/kg.

19. The method of any one of claims 15-17, wherein the therapeutically effective amount of (ii) is from about 0.01 mg to about 10 mg.

20. The method of any one of claims 15-17, wherein the therapeutically effective amount of (iii) is from about 5 mg/kg to about 20 mg/kg.

21. A sustained-release formulation, which formulation comprises: a therapeutically effective amount of a hydrogel comprising a phosphatidylserine blocker, alone or further in combination with a crosslinker, and an immune stimulant, wherein the immune stimulant is optionally encapsulated.

22. The sustained-release formulation of claim 21, wherein the hydrogel comprises alginate, pectin, carrageenan, polylactic acid, polyethylene glycol, collagen, fibrin, hyaluronic acid, matrigel, chitosan, silk fiber, or a combination of two or more of the foregoing.

23. The sustained-release formulation claim 21, wherein the immune stimulant is encapsulated in a liposomal formulation or a nanoparticulate formulation.

24. The sustained-release formulation claim 21, wherein the crosslinker is zinc acetate dihydrate, zinc gluconate, zinc chloride, zinc picolinate, zinc phosphate, zinc acetate, zinc sulfate, zinc oxide calcium chloride, calcium sulfate or a combination of two or more thereof.

25. A method for treating a patient with cancer, which method comprises administering to the patient (i) a therapeutically effective amount of the sustained-release formulation of claim 21 and, simultaneously or sequentially, by a same or different routes, (ii) a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer.

26. The method of claim 25, wherein the cancer is melanoma.

27. A method of preparing a sustained-release formulation, which method comprises:

(i) mixing a solution comprising a hydrogel and a solution comprising a phosphatidylserine (PS) blocker to formulate a hydrogel of PS blocker;

(ii) optionally mixing the hydrogel of step (i) with a suspension comprising a crosslinker to formulate a hydrogel comprising the PS blocker and the crosslinker; and

(iii) mixing the hydrogel of step (i) or step (ii) with a solution comprising an optionally encapsulated immune stimulant, whereupon the sustained-release formulation is formulated.

28. The method claim 27, wherein the formulation of the immune stimulant is a liposomal formulation or a nanoparticulate formulation.

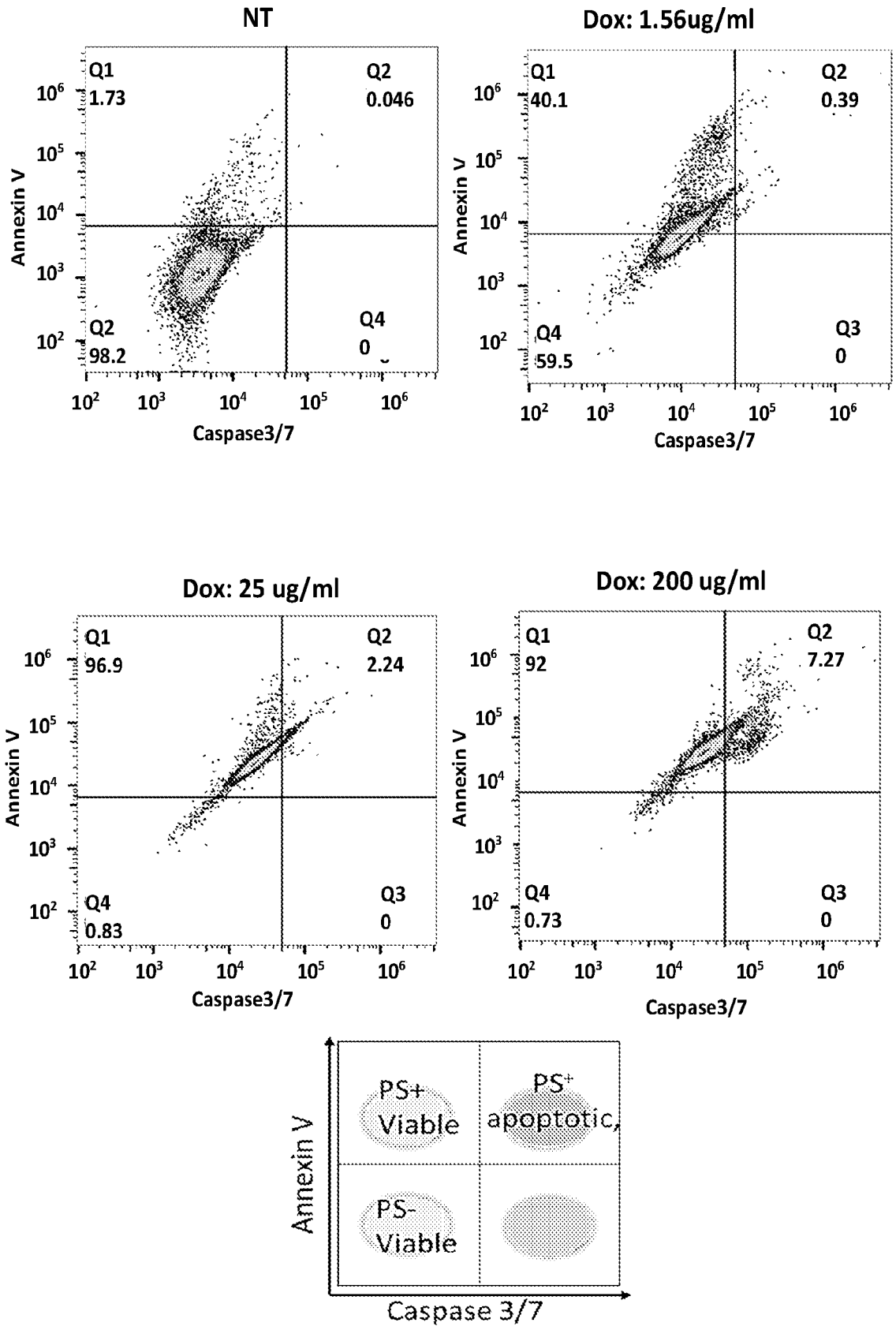
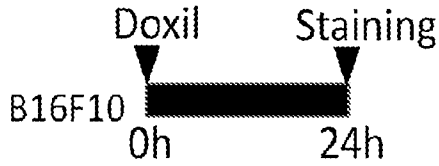
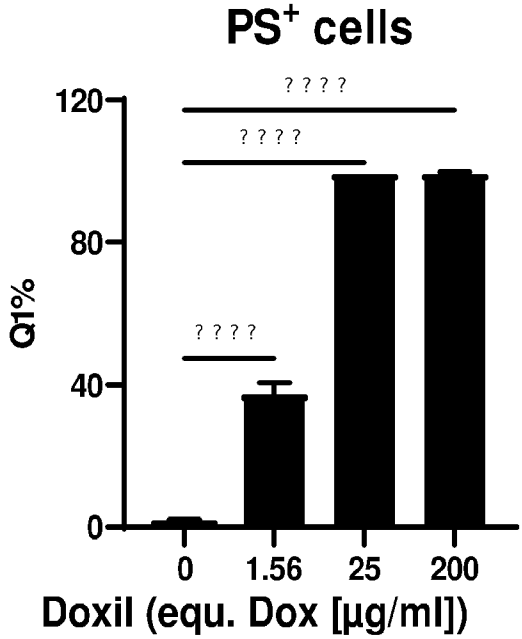

NT
Q1
1.73
Q2
0.046
Q2
98.2
Q4
0
Annexin V
Caspase3/7
Dox: 1.56ug/ml
Q1
40.1
Q2
0.39
Q4
59.5
Q3
0
Dox: 25 ug/ml
Q1
96.9
Q2
2.24
Q4
0.83
Q3
0
Dox: 200 ug/ml
Q1
92
Q2
7.27
Q4
0.73
Q3
0
PS+
Viable
PS+
apoptotic
PS-
Viable

Fig. 1A


Doxil
Staining
B16F10
0h
24h


PS+ cells
Q1%
120
80
40
0
? ? ? ?
? ? ? ?
? ? ? ?
0
1.56
25
200
Doxil (equ. Dox [µg/ml])

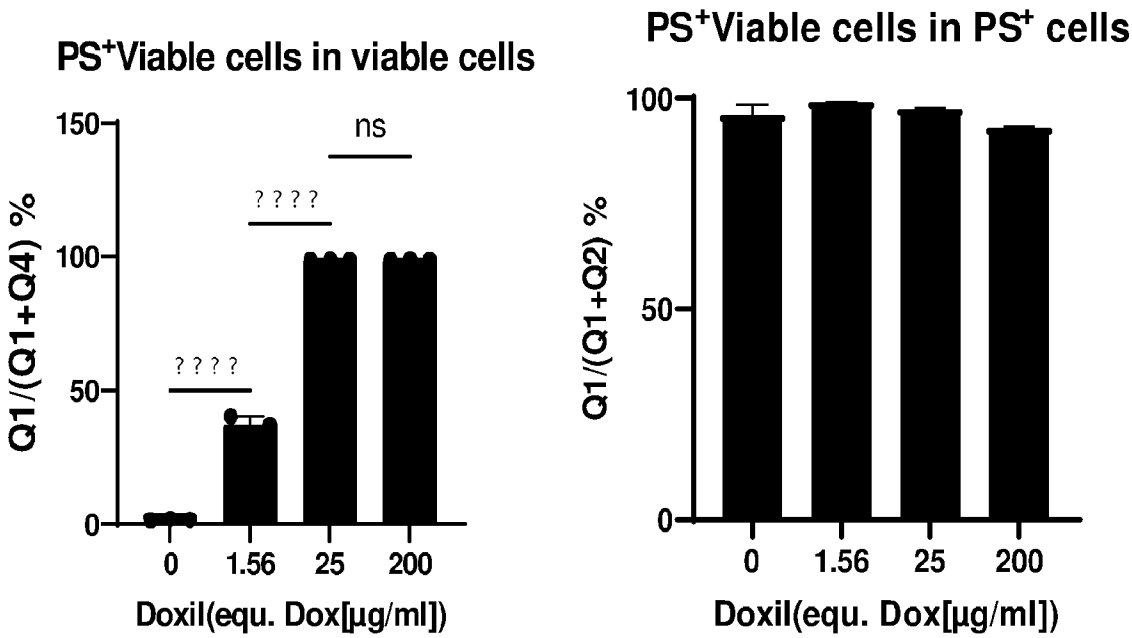

PS+Viable cells in viable cells
Q1/(Q1+Q4) %
150
100
50
0
ns
? ? ? ?
? ? ? ?
0
1.56
25
200
Doxil(equ. Dox[µg/ml])
PS+Viable cells in PS+ cells
Q1/(Q1+Q2) %
100
50
0
0
1.56
25
200
Doxil(equ. Dox[µg/ml])

Fig. 1B

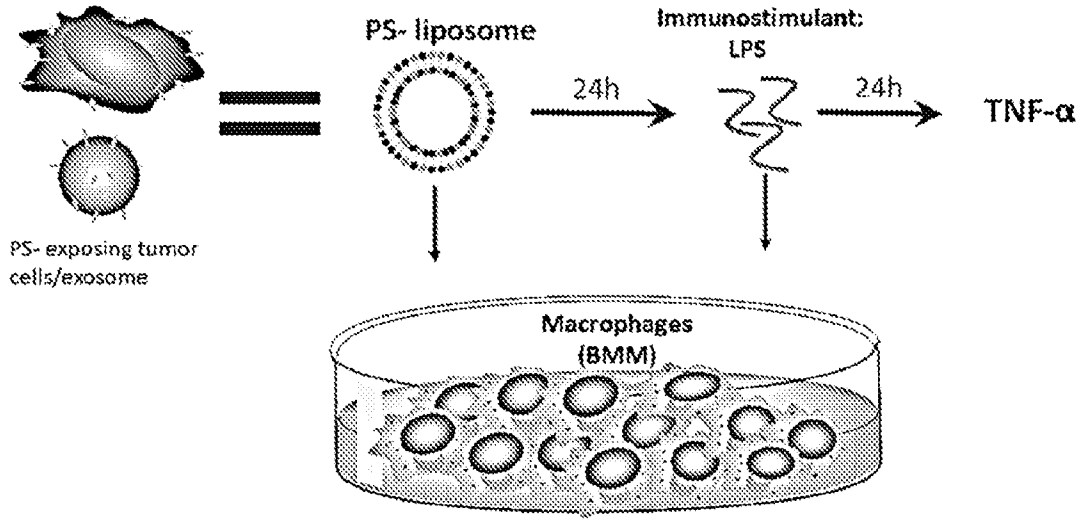


Fig. 2A

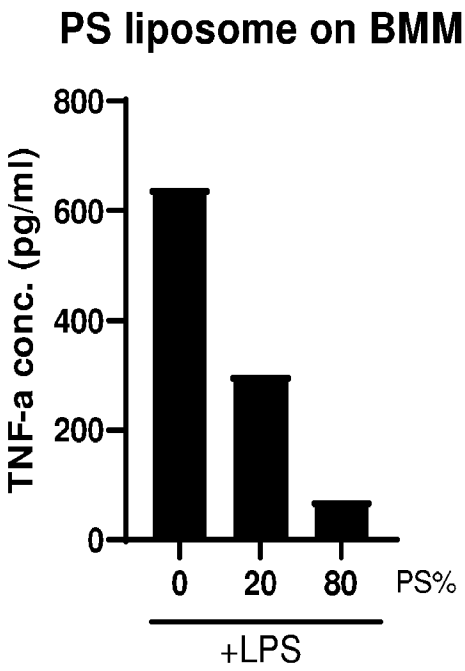


Fig. 2B

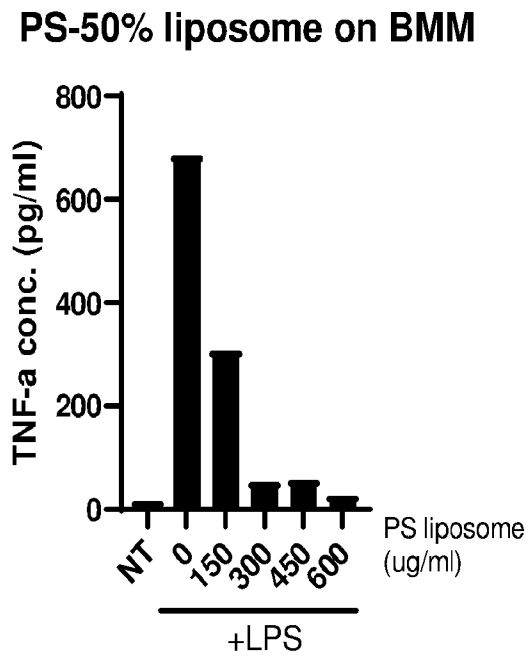


Fig. 2C

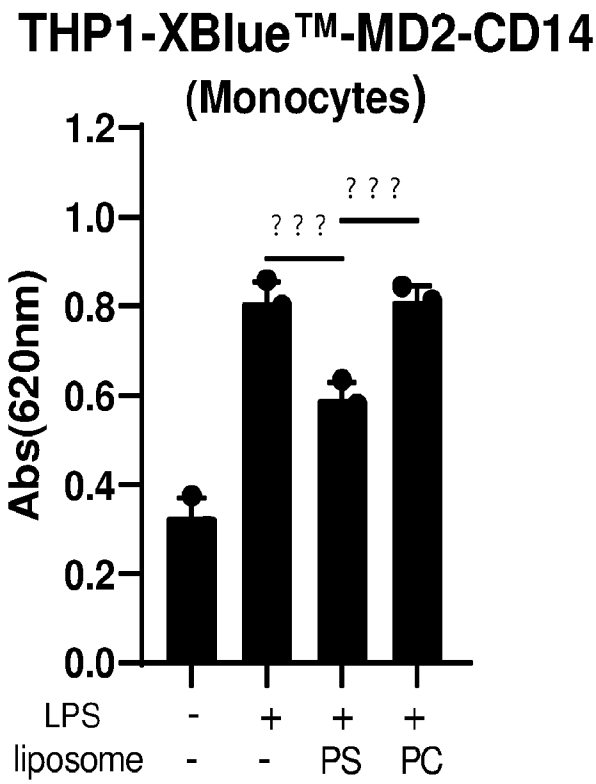

THP1-XBlue™-MD2-CD14
(Monocytes)
Abs(620nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
? ? ?
? ? ?
LPS - + + +
liposome - - PS PC

Fig. 2D

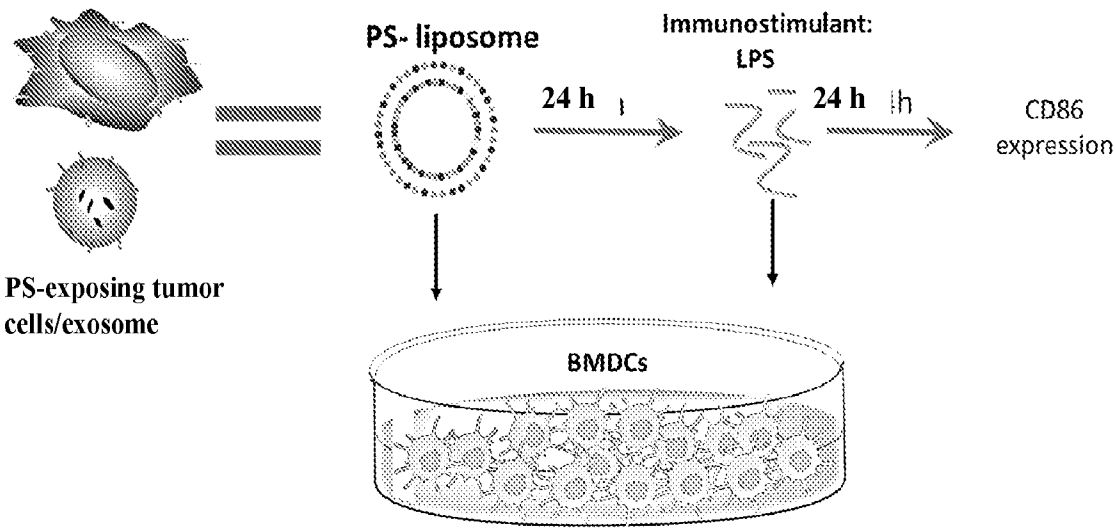

PS- liposome
Immunostimulant:
LPS
24 h
24 h
CD86
expression
PS-exposing tumor
cells/exosome
BMDCs

Fig. 3A

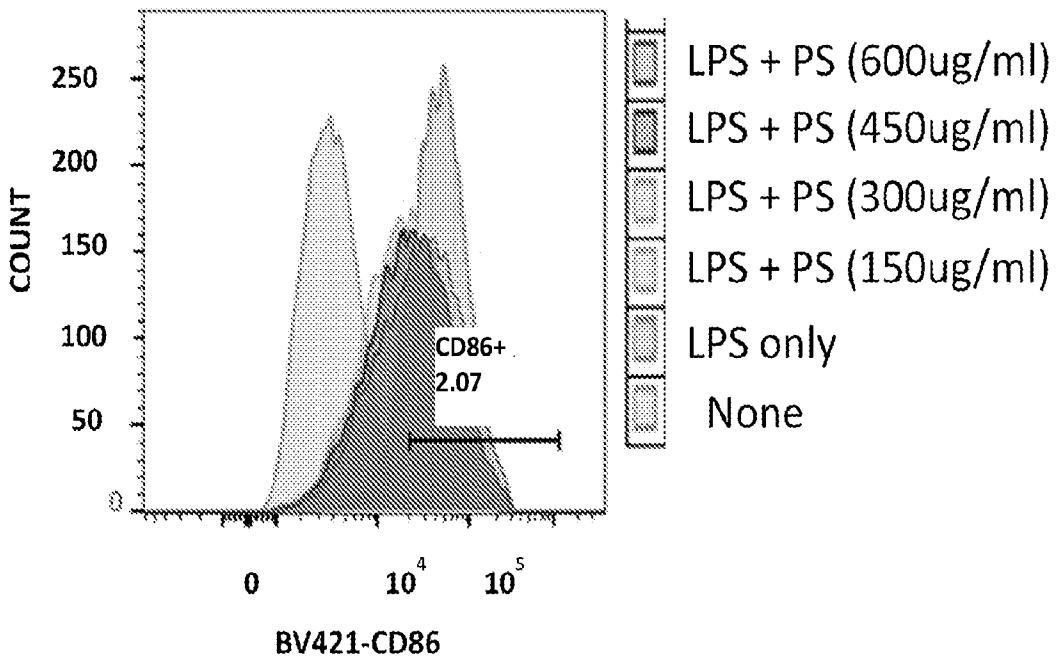

250
200
150
100
50
0
COUNT
CD86+
2.07
0
10^4
10^5
BV421-CD86
LPS + PS (600ug/ml)
LPS + PS (450ug/ml)
LPS + PS (300ug/ml)
LPS + PS (150ug/ml)
LPS only
None

Fig. 3B

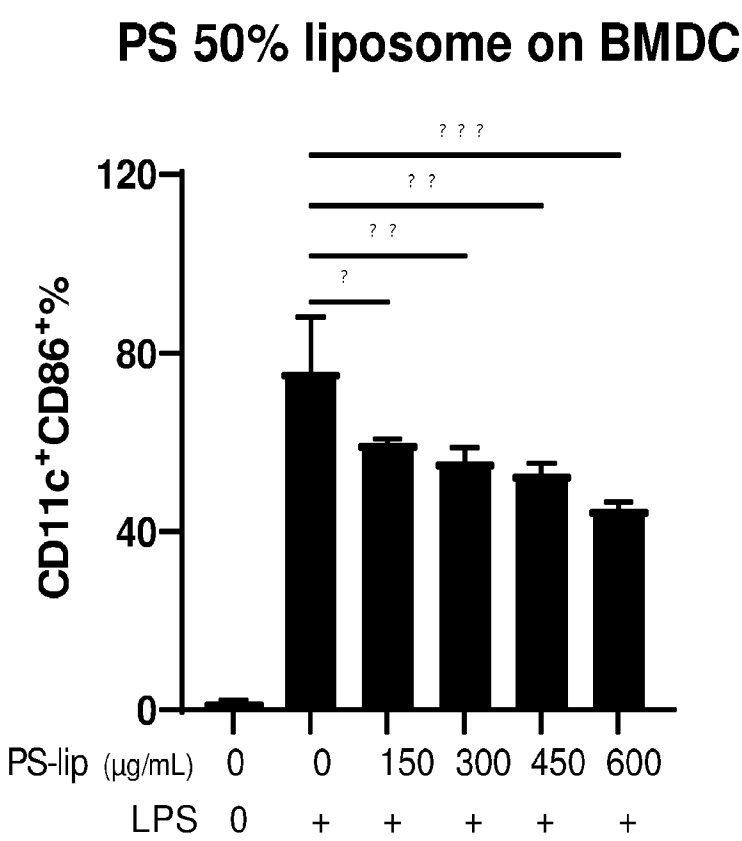

PS 50% liposome on BMDC
? ? ?
? ?
? ?
?
120
80
40
0
CD11c+CD86+%
PS-lip (μg/mL) 0 0 150 300 450 600
LPS 0 + + + + +

Fig. 3C

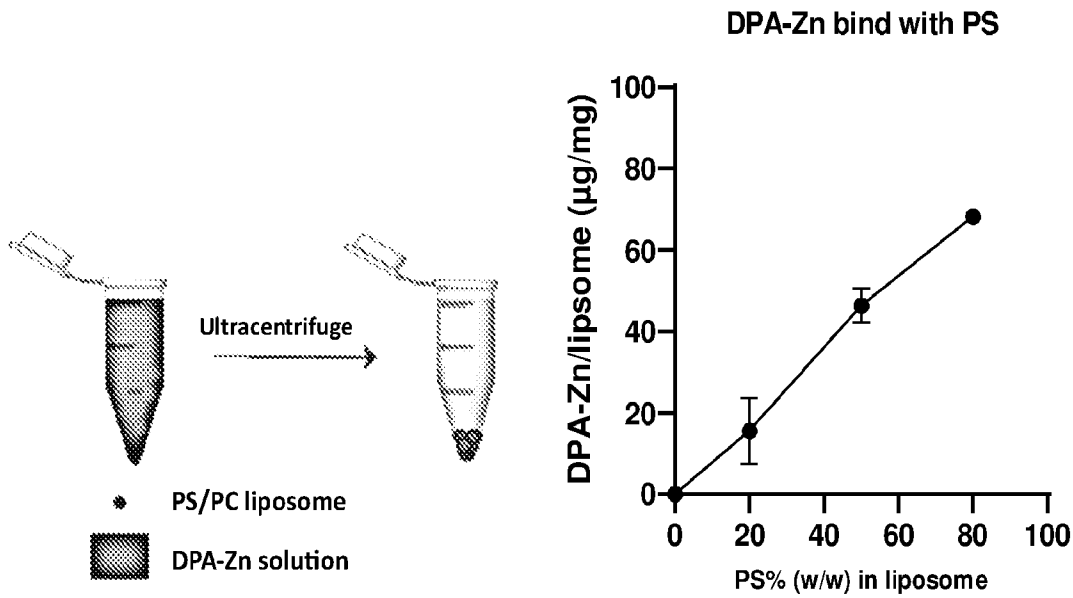

Ultracentrifuge
PS/PC liposome
DPA-Zn solution
DPA-Zn bind with PS
DPA-Zn/lipsome (µg/mg)
PS% (w/w) in liposome
0
20
40
60
80
100

Fig. 4A

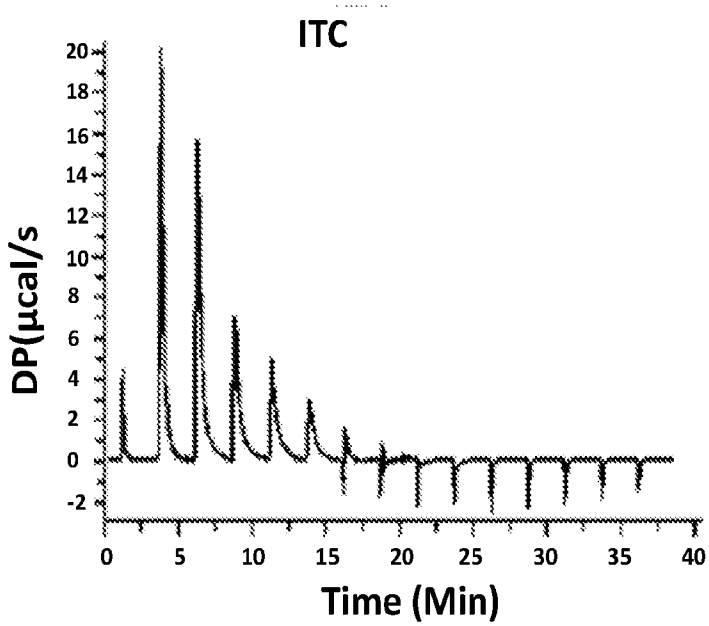

ITC
DP(µcal/s
Time (Min)
20
18
16
14
12
10
8
6
4
2
0
-2
0
5
10
15
20
25
30
35
40

Fig. 4B

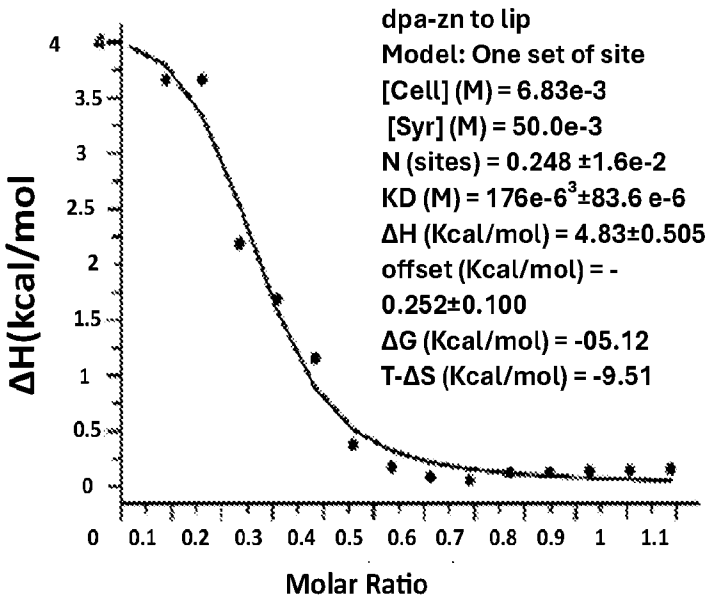

dpa-zn to lip
Model: One set of site
[Cell] (M) = 6.83e-3
[Syr] (M) = 50.0e-3
N (sites) = 0.248 ±1.6e-2
KD (M) = 176e-6^3±83.6 e-6
ΔH (Kcal/mol) = 4.83±0.505
offset (Kcal/mol) = -0.252±0.100
ΔG (Kcal/mol) = -05.12
T-ΔS (Kcal/mol) = -9.51
ΔH(kcal/mol)
Molar Ratio

Fig. 4C

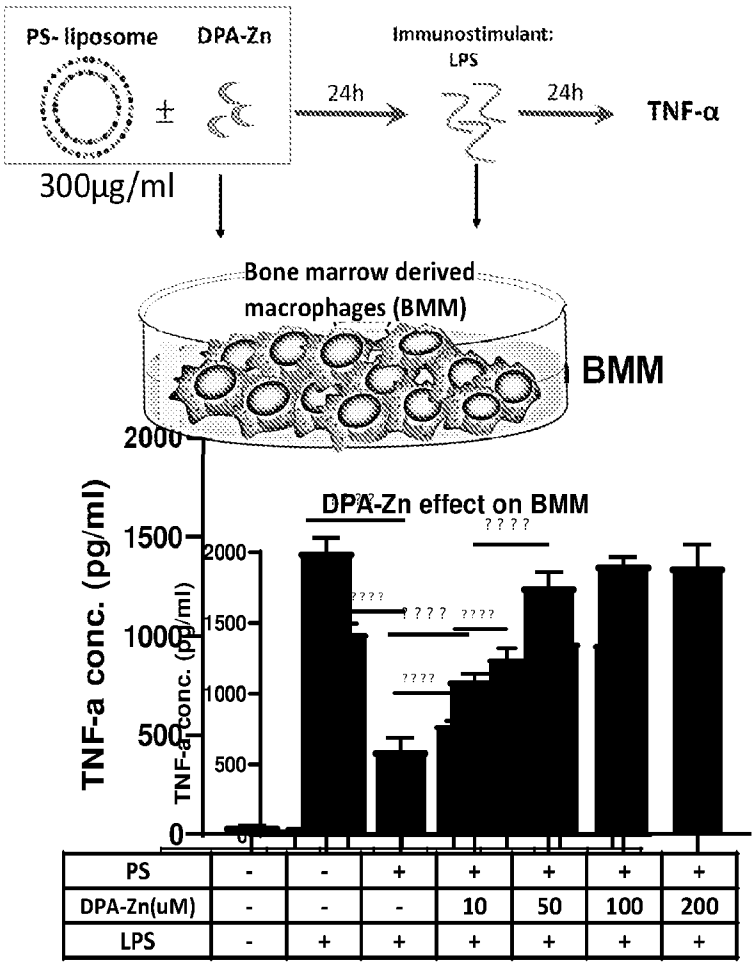

PS- liposome
DPA-Zn
300µg/ml
Immunostimulant: LPS
24h
24h
TNF-α
Bone marrow derived macrophages (BMM)
BMM
DPA-Zn effect on BMM
TNF-a conc. (pg/ml)
PS - - + + + + +
DPA-Zn(uM) - - - 10 50 100 200
LPS - + + + + + +

Fig. 5

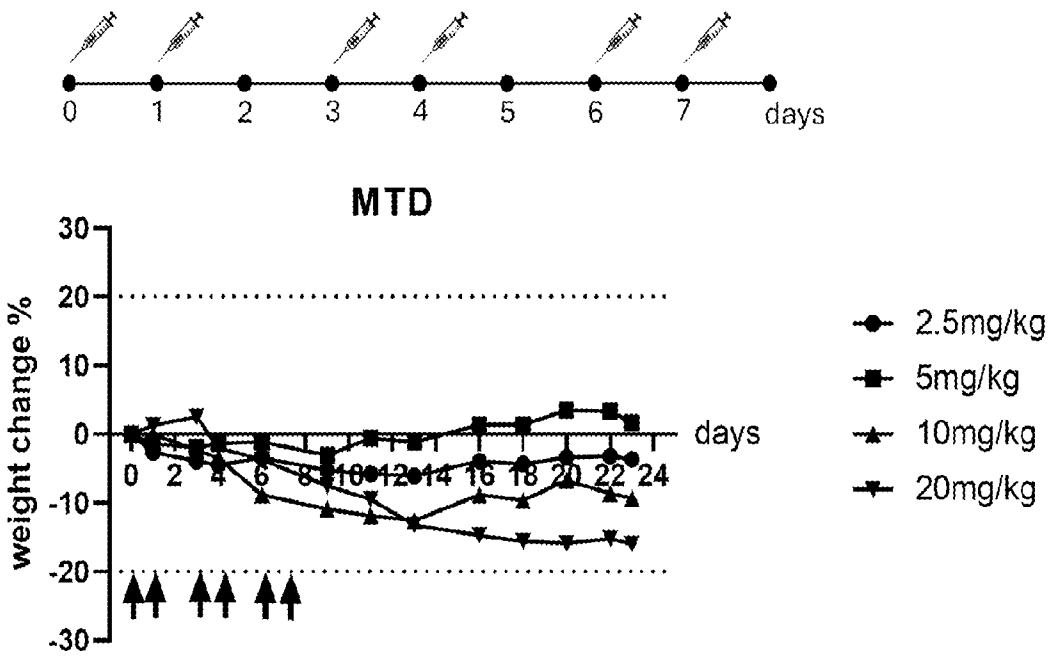

0 1 2 3 4 5 6 7 days
MTD
weight change %
30
20
10
0
-10
-20
-30
0 2 4 6 8 10 12 14 16 18 20 22 24
days
2.5mg/kg
5mg/kg
10mg/kg
20mg/kg

Fig. 6

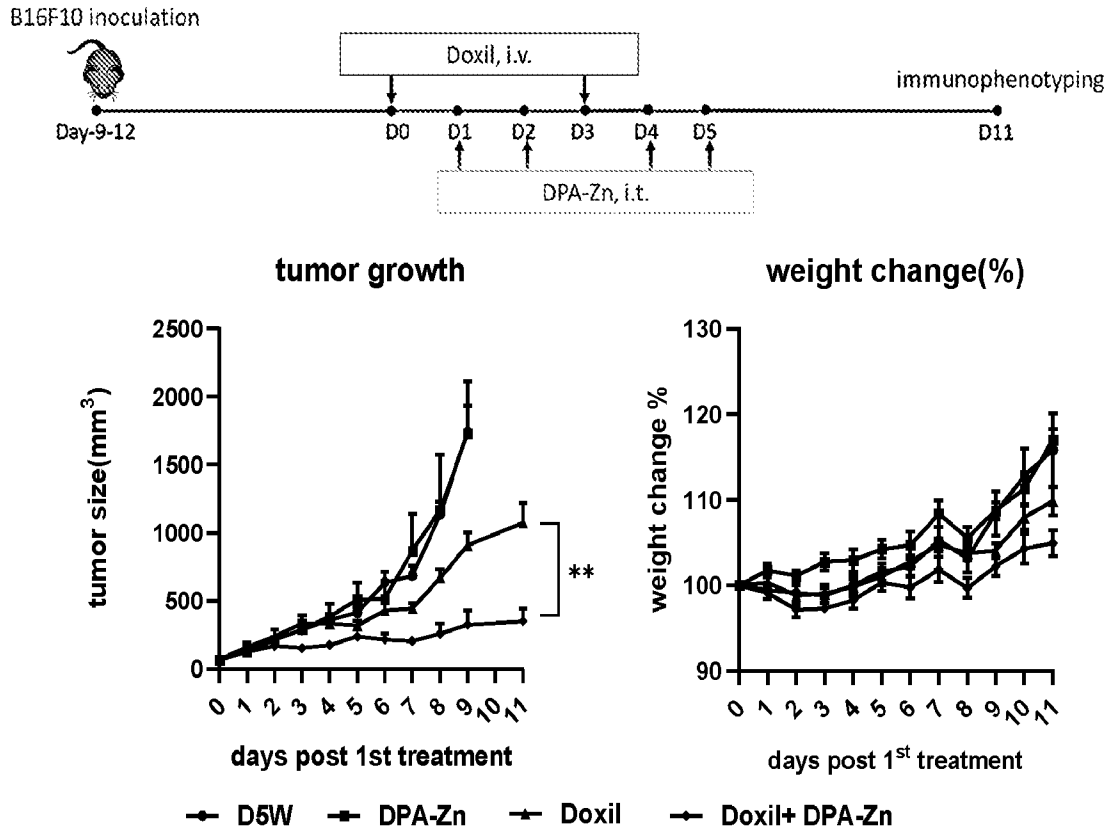

B16F10 inoculation
Doxil, i.v.
immunophenotyping
Day-9-12
D0 D1 D2 D3 D4 D5 D11
DPA-Zn, i.t.
tumor growth
weight change(%)
tumor size(mm3)
2500
2000
1500
1000
500
0
**
days post 1st treatment
weight change %
130
120
110
100
90
days post 1st treatment
D5W
DPA-Zn
Doxil
Doxil+ DPA-Zn

Fig. 7

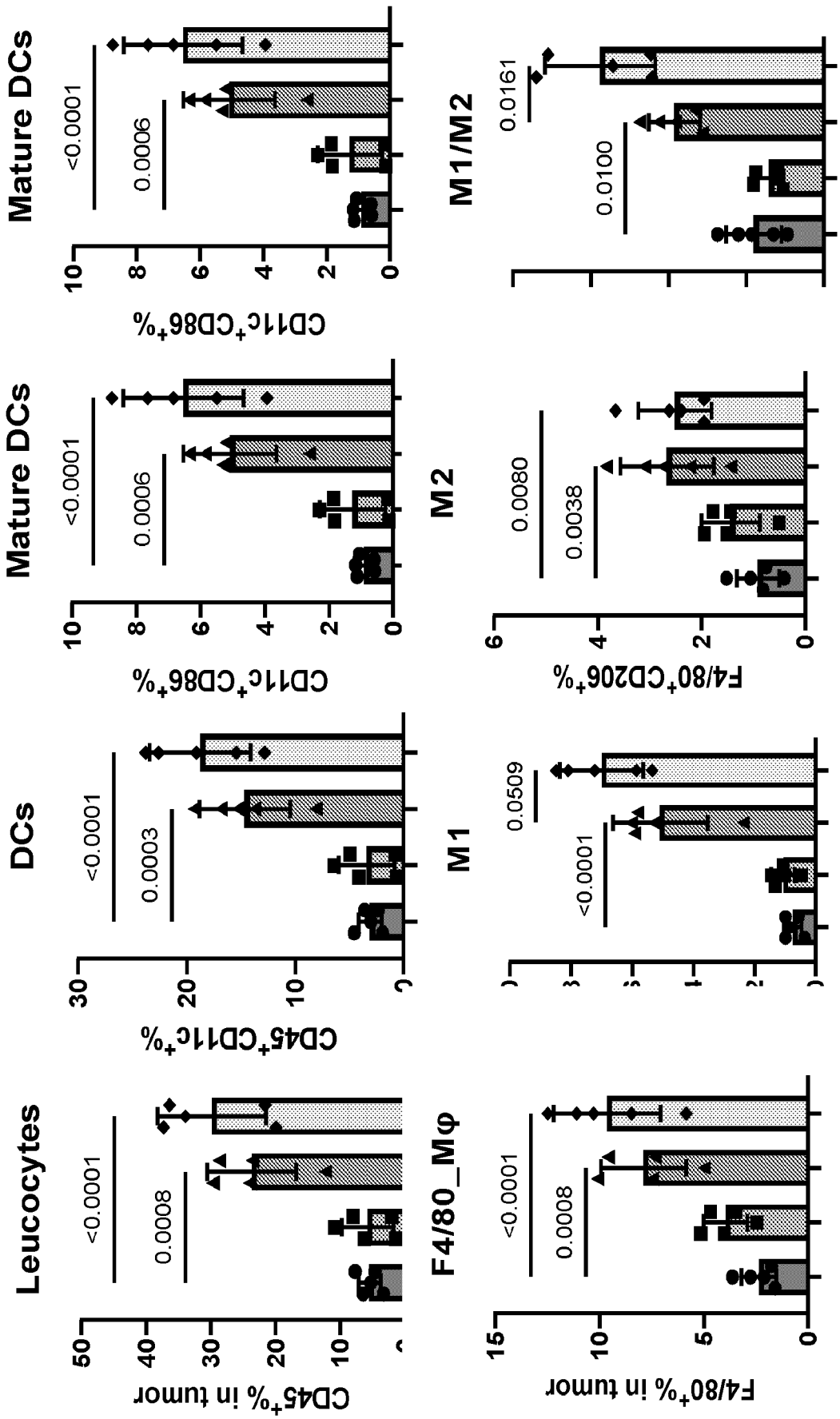


Fig. 8 continued

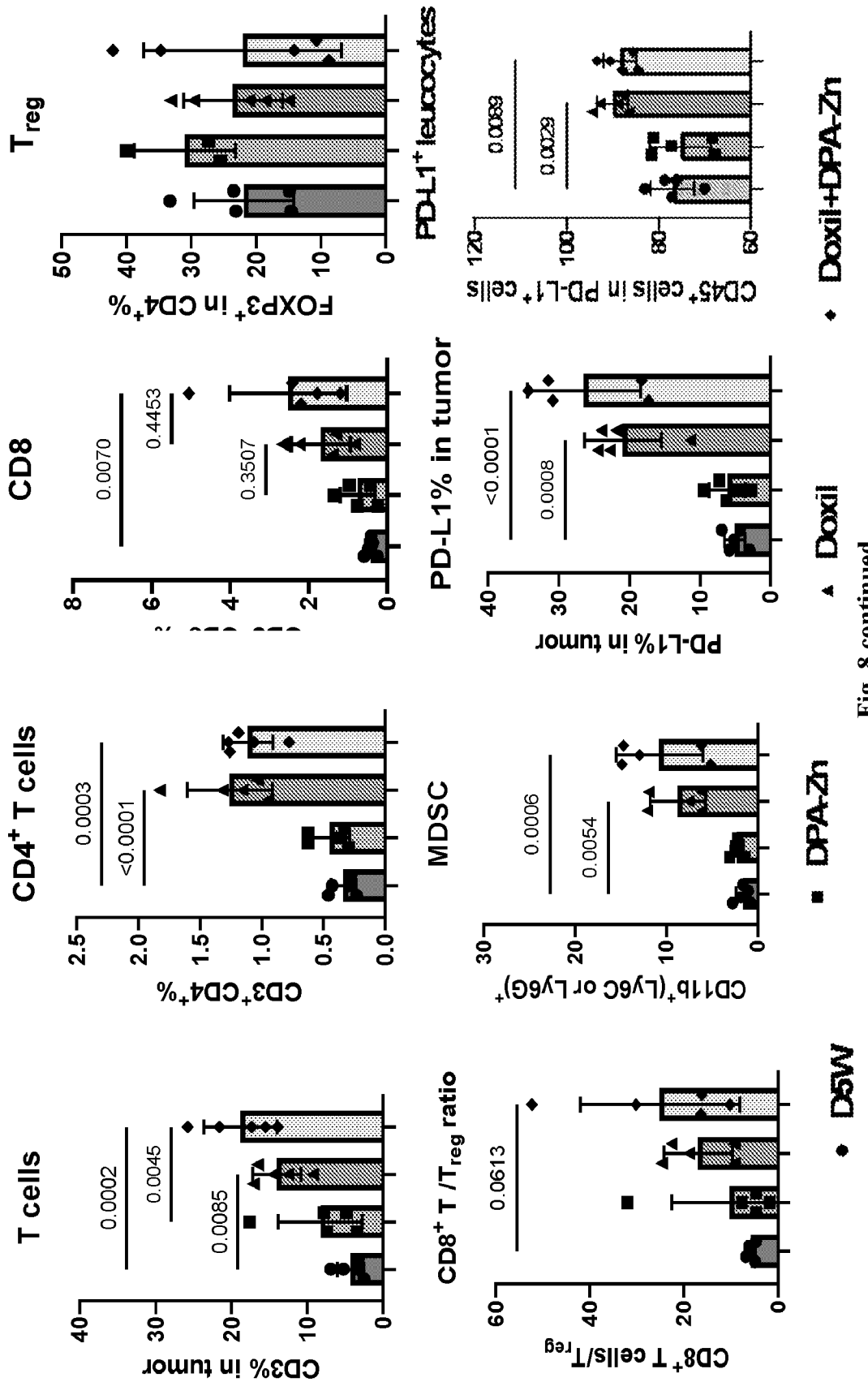


Fig. 8 continued

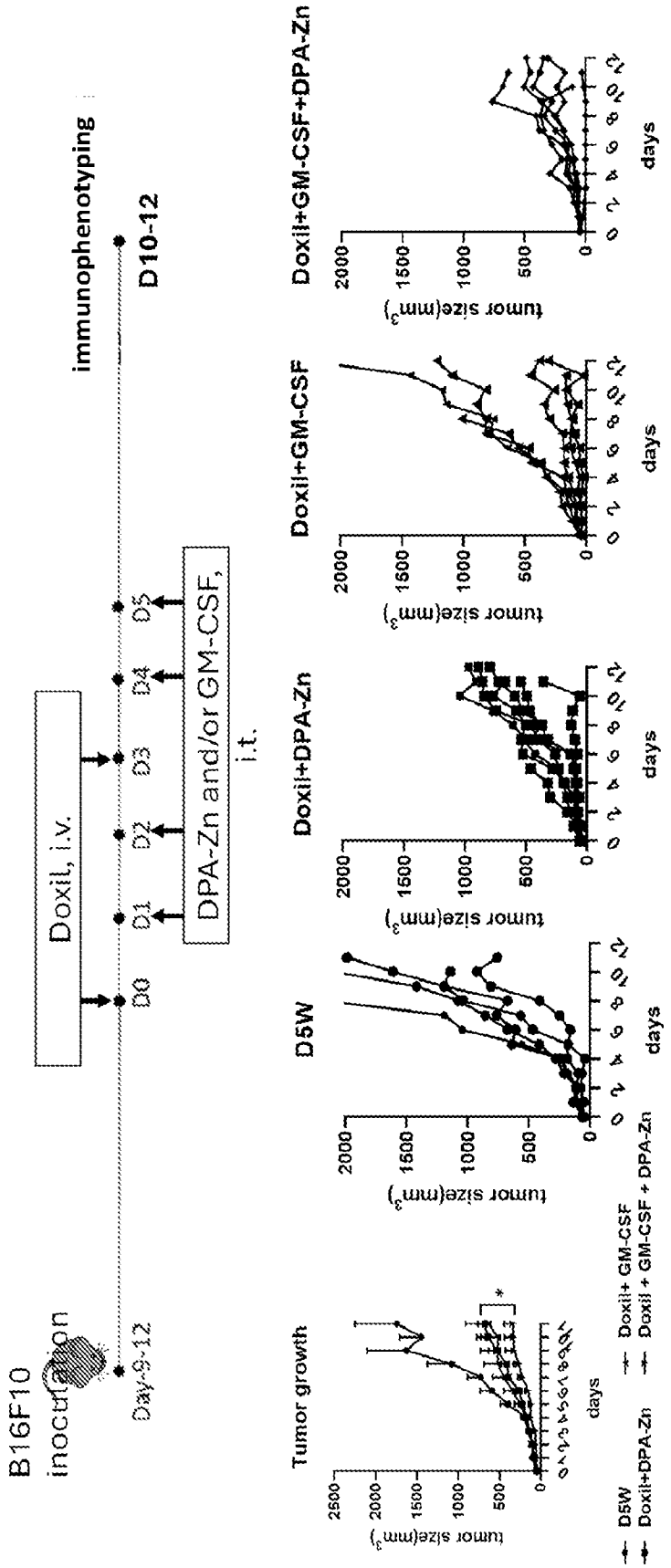

B16F10
inoculation
Day-9~12
Doxil, i.v.
DPA-Zn and/or GM-CSF, i.t.
D0
D1
D2
D3
D4
D5
immunophenotyping
D10-12
Tumor growth
tumor size(mm³)
days
D5W
Doxil+DPA-Zn
Doxil+GM-CSF
Doxil+GM-CSF+DPA-Zn
D5W
Doxil+DPA-Zn
Doxil+ GM-CSF
Doxil + GM-CSF + DPA-Zn

Fig. 9

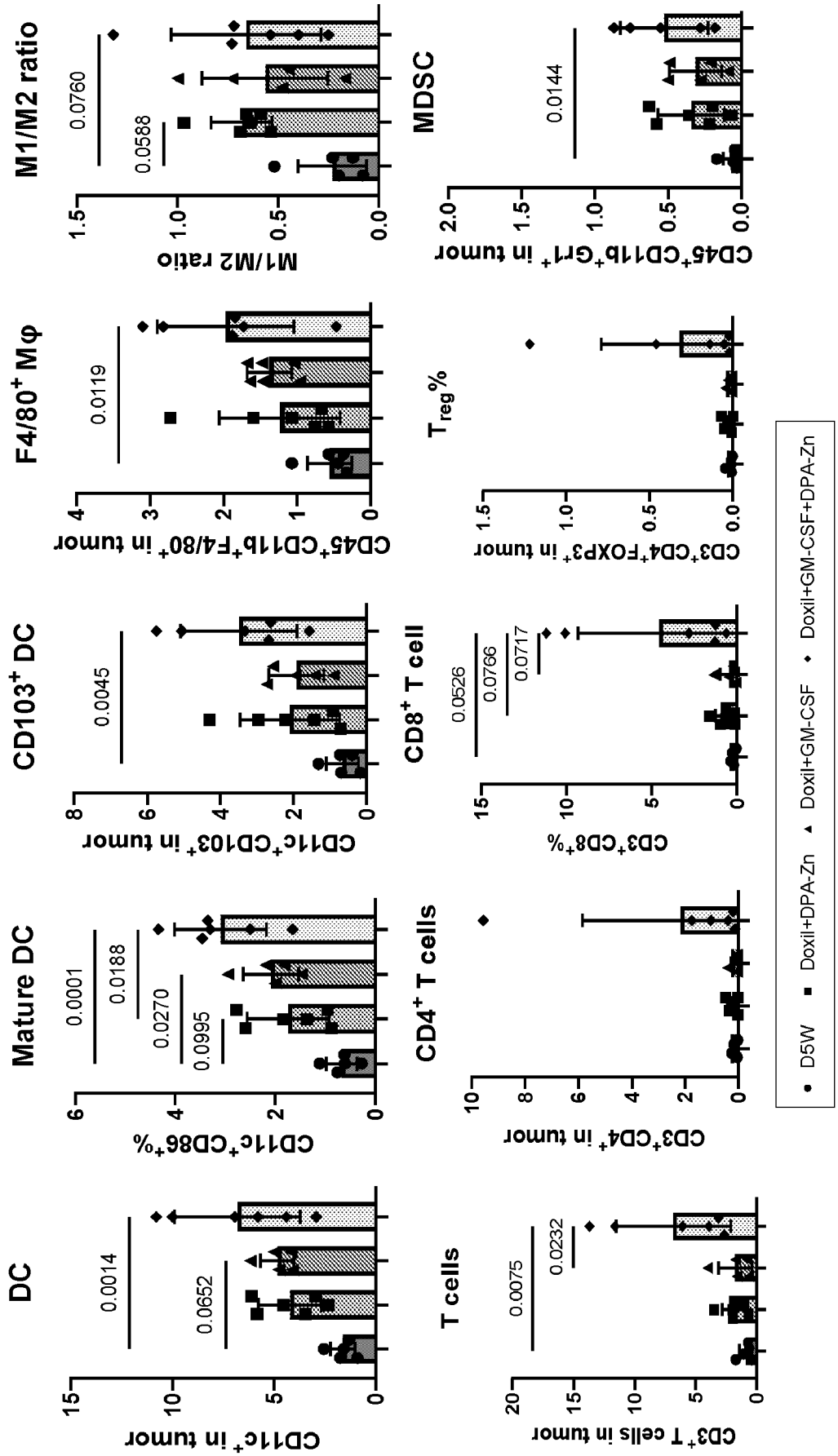

DC
CD11c+ in tumor
0.0014
0.0652
Mature DC
CD11c+CD86+%
0.0001
0.0188
0.0270
0.0995
CD103+ DC
CD11c+CD103+ in tumor
0.0045
F4/80+ Mφ
CD45+CD11b+F4/80+ in tumor
0.0119
M1/M2 ratio
M1/M2 ratio
0.0760
0.0588
T cells
CD3+ T cells in tumor
0.0075
0.0232
CD4+ T cells
CD3+CD4+ in tumor
CD8+ T cell
CD3+CD8+%
0.0526
0.0766
0.0717
Treg%
CD3+CD4+FOXP3+ in tumor
MDSC
CD45+CD11b+Gr1+ in tumor
0.0144
D5W
Doxil+DPA-Zn
Doxil+GM-CSF
Doxil+GM-CSF+DPA-Zn

Fig. 10

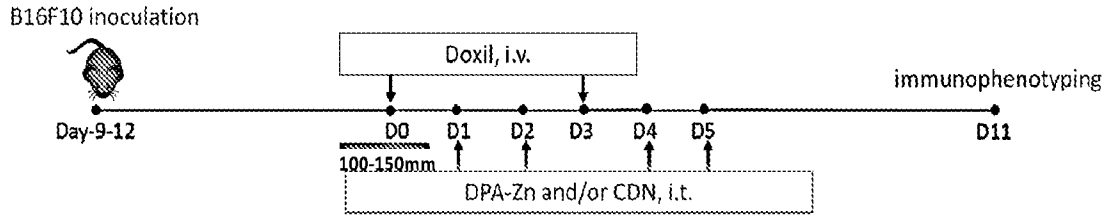


Fig. 11A

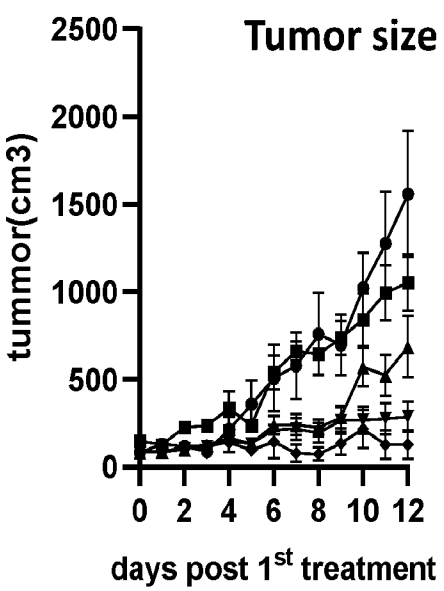


Fig. 11B

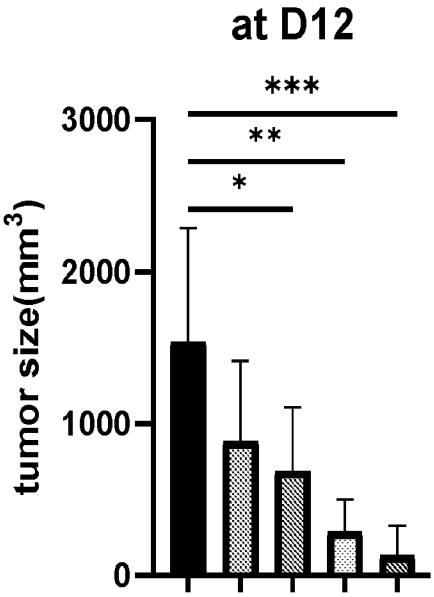


Fig. 11C

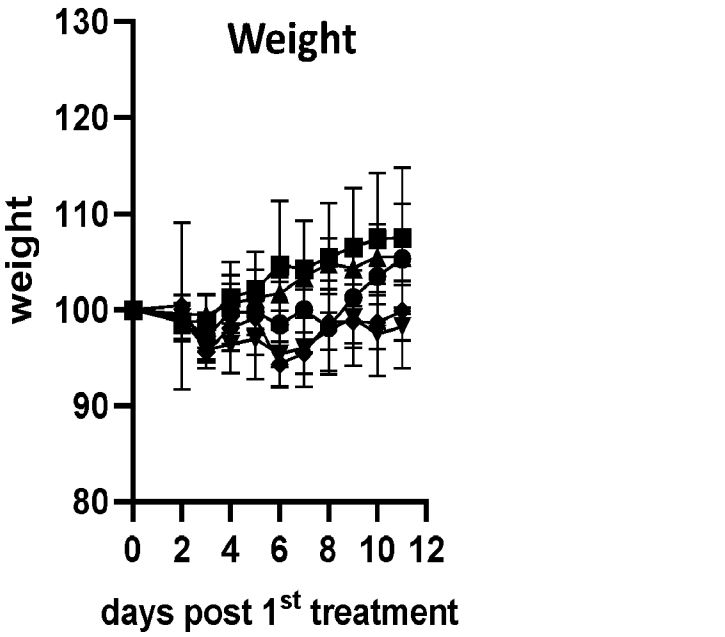


Fig. 11D

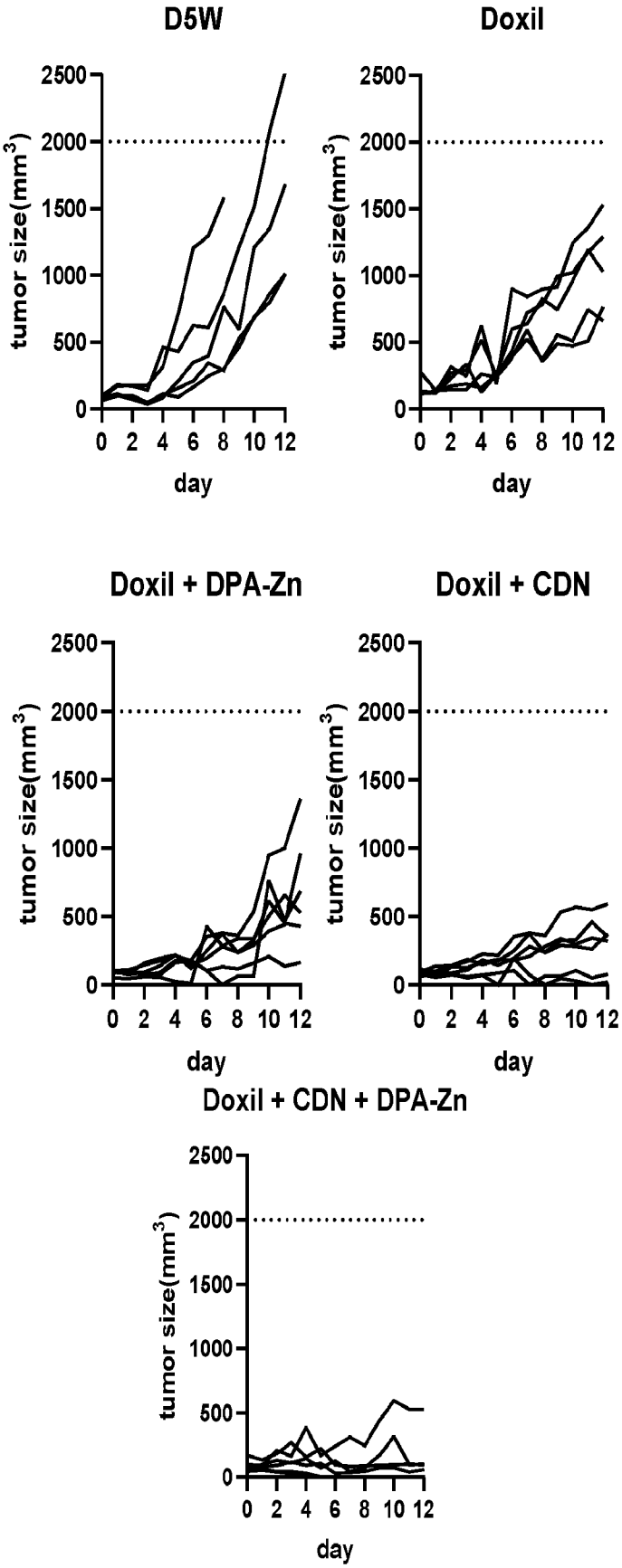

D5W
Doxil
Doxil + DPA-Zn
Doxil + CDN
Doxil + CDN + DPA-Zn
tumor size(mm3)
day
0
500
1000
1500
2000
2500
0 2 4 6 8 10 12

Fig. 11E

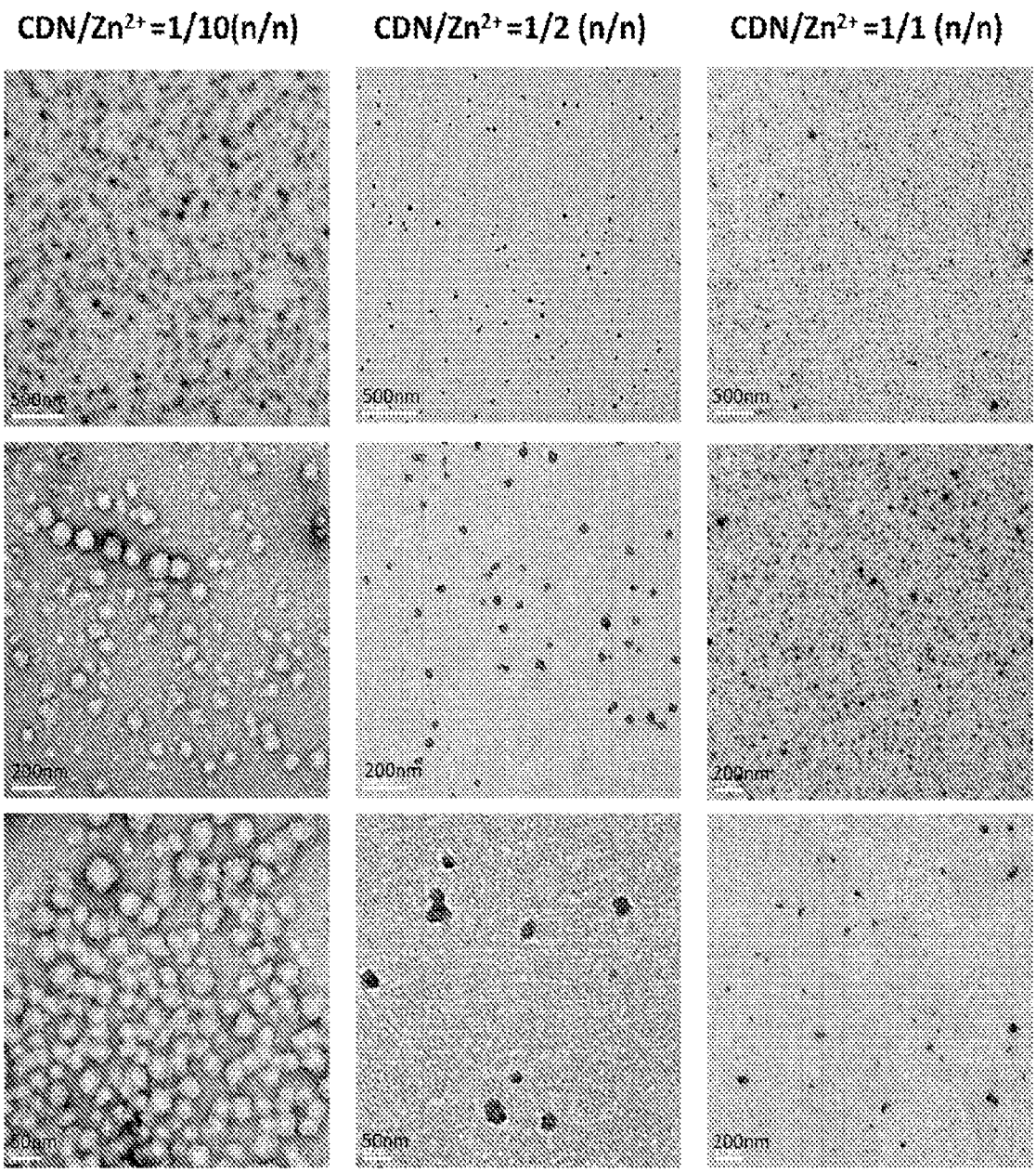

CDN/Zn2+=1/10(n/n)
CDN/Zn2+=1/2 (n/n)
CDN/Zn2+=1/1 (n/n)
500nm
500nm
500nm
200nm
200nm
200nm
50nm
50nm
200nm

Fig. 12

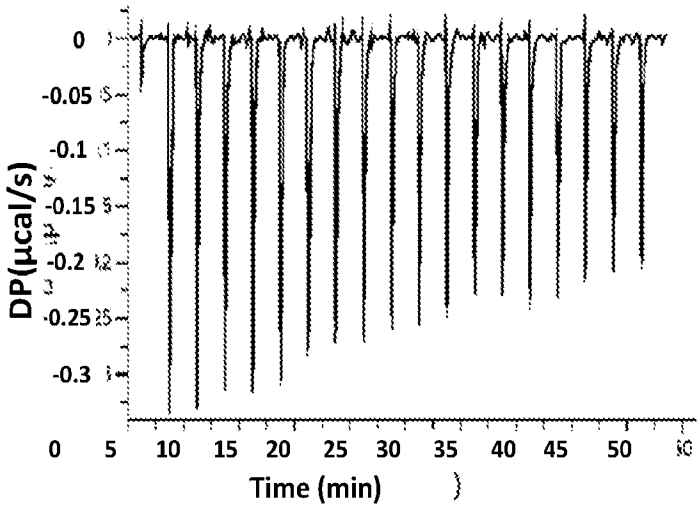


Fig. 13A

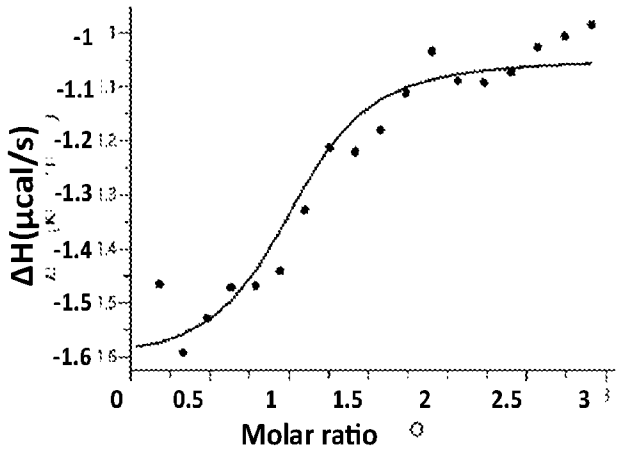


Fig. 13B

CDN@lip

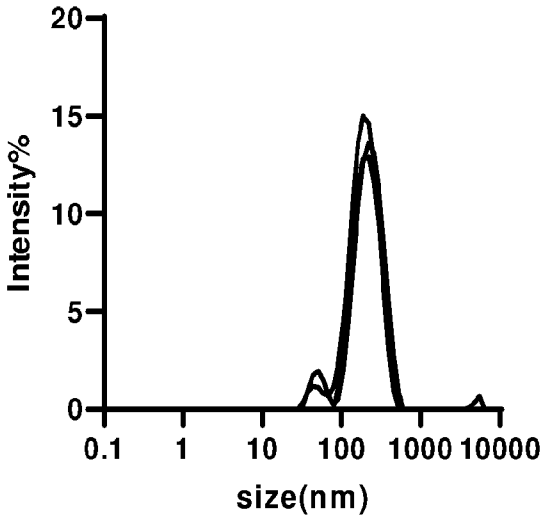


Fig. 14A

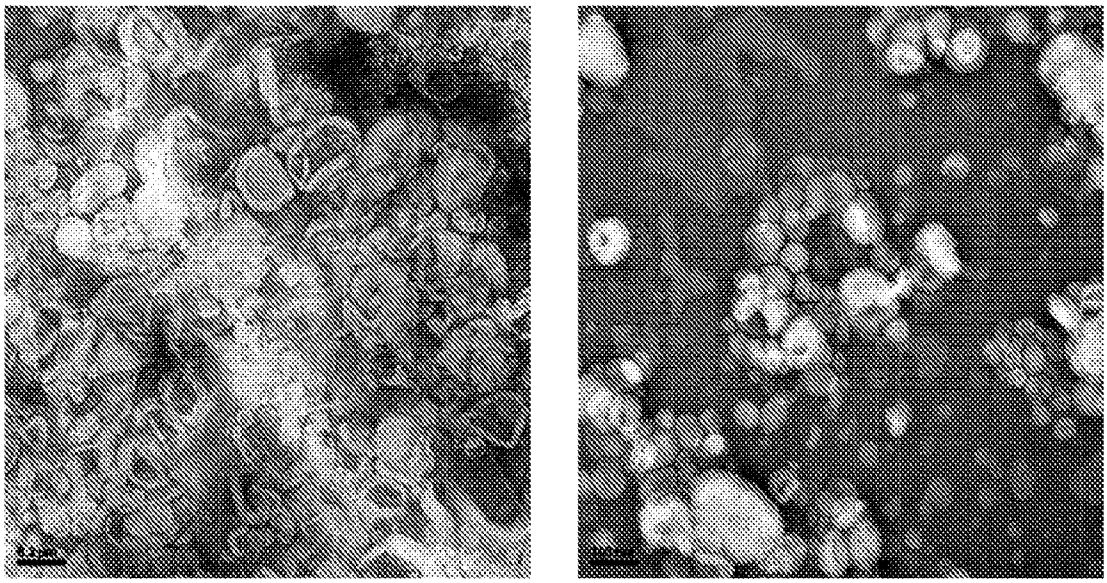


Fig. 14B

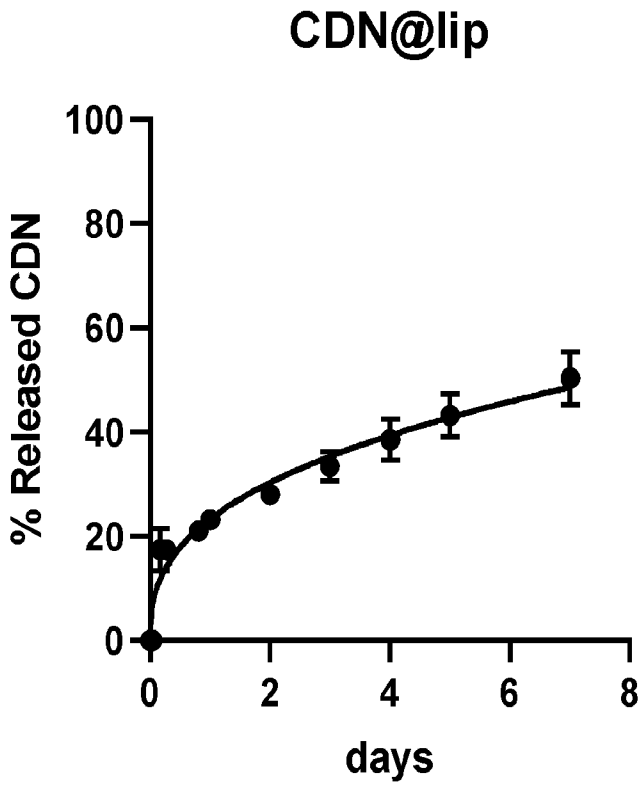


Fig. 15

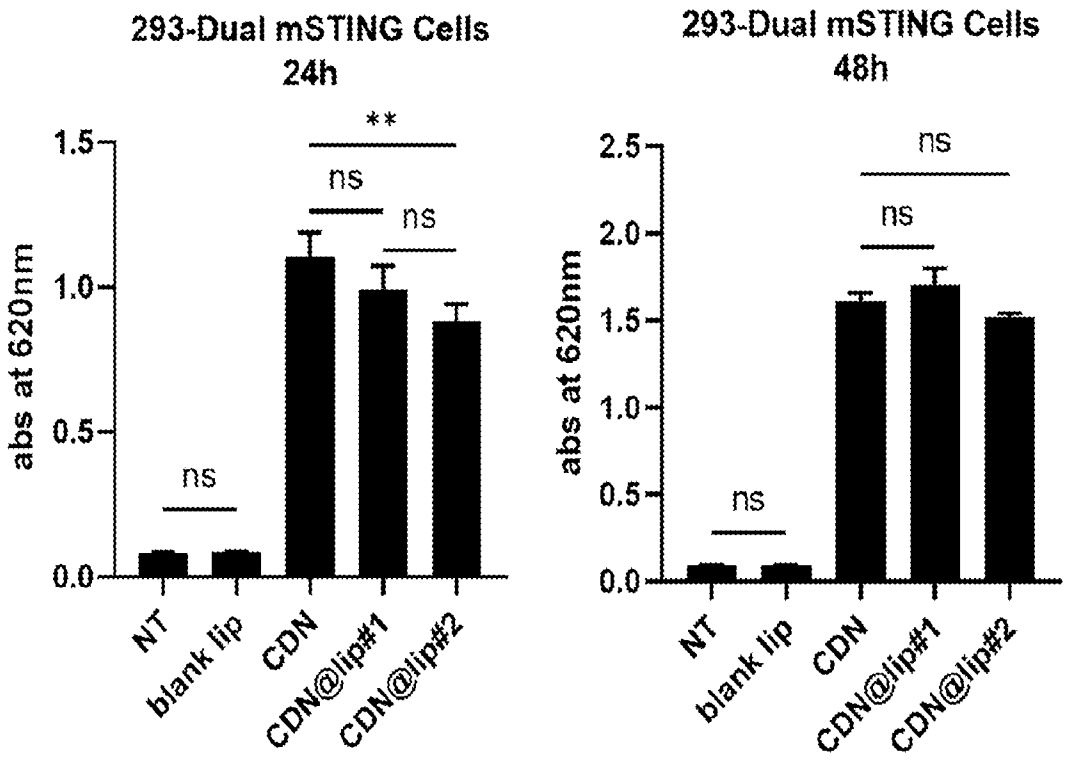


Fig. 16

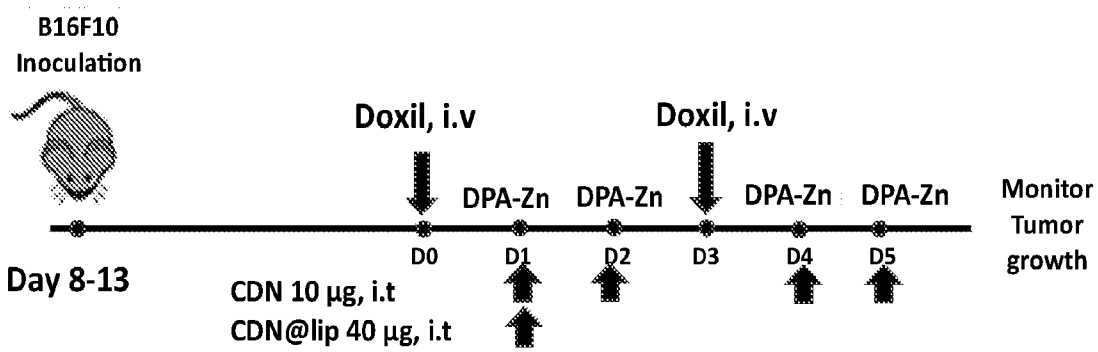


Fig. 17A

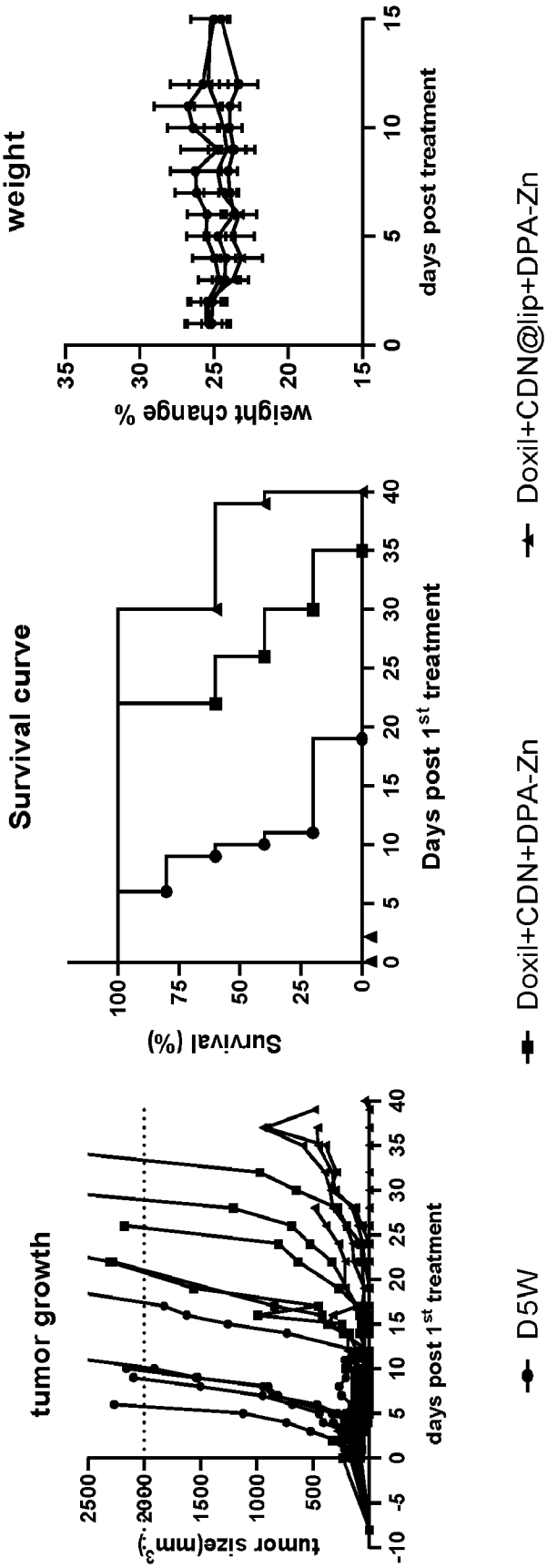

weight
weight change %
days post treatment
Survival curve
Survival (%)
Days post 1st treatment
tumor growth
tumor size(mm3)
days post 1st treatment
D5W
Doxil+CDN+DPA-Zn
Doxil+CDN@lip+DPA-Zn

Fig. 17 B

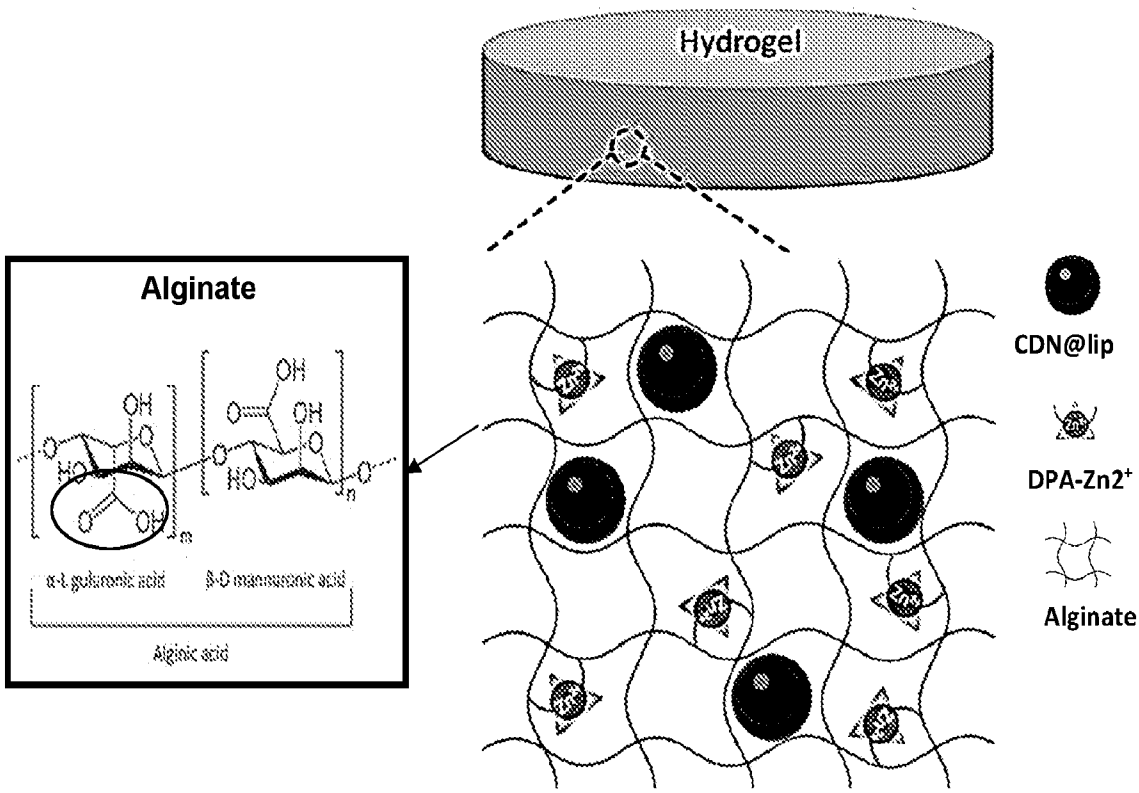


Fig. 18

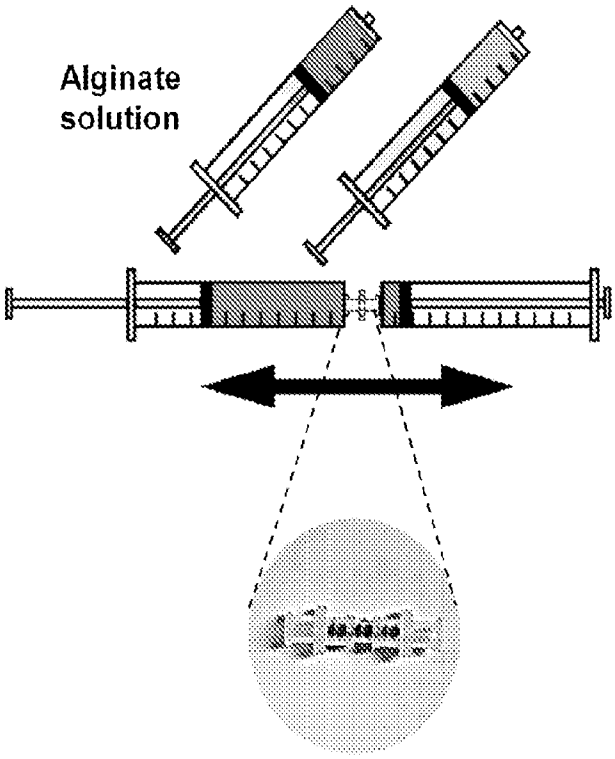
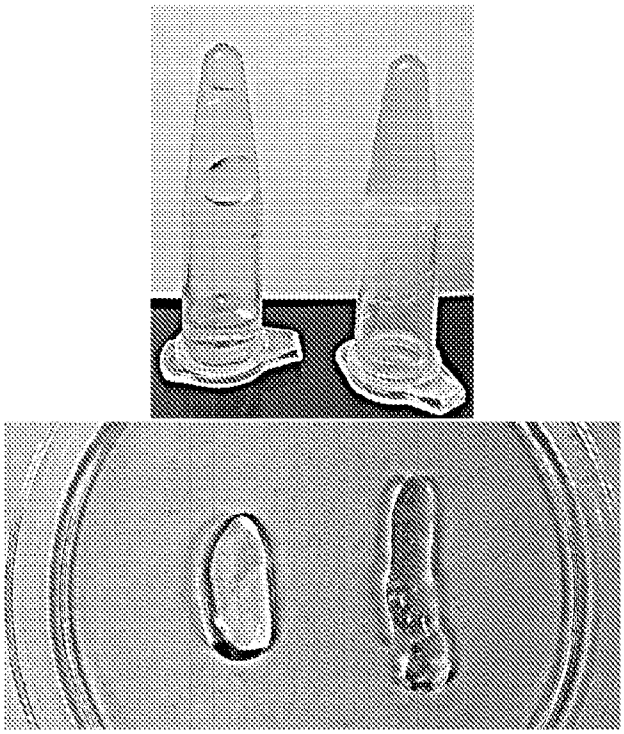
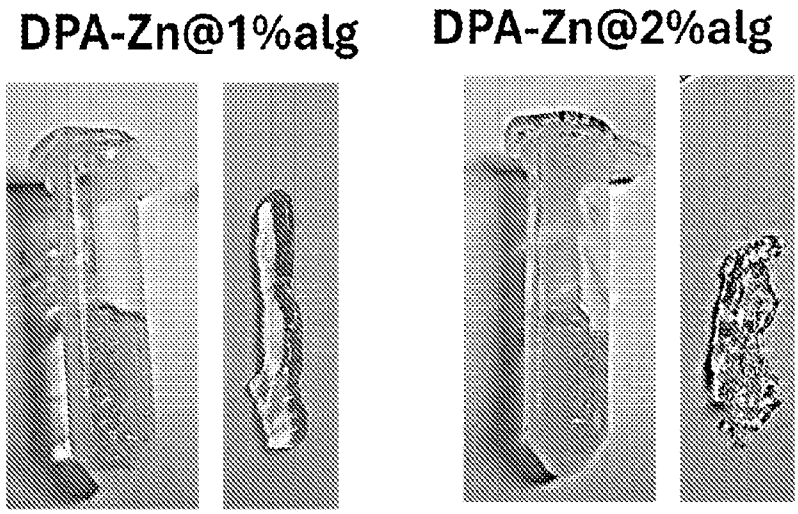


Fig. 19A



1% alg 1% alg + DPA-Zn

Fig. 19B



	injectability via 27G1/2 needle
1% alg	+++
2% alg	+

Fig. 19C

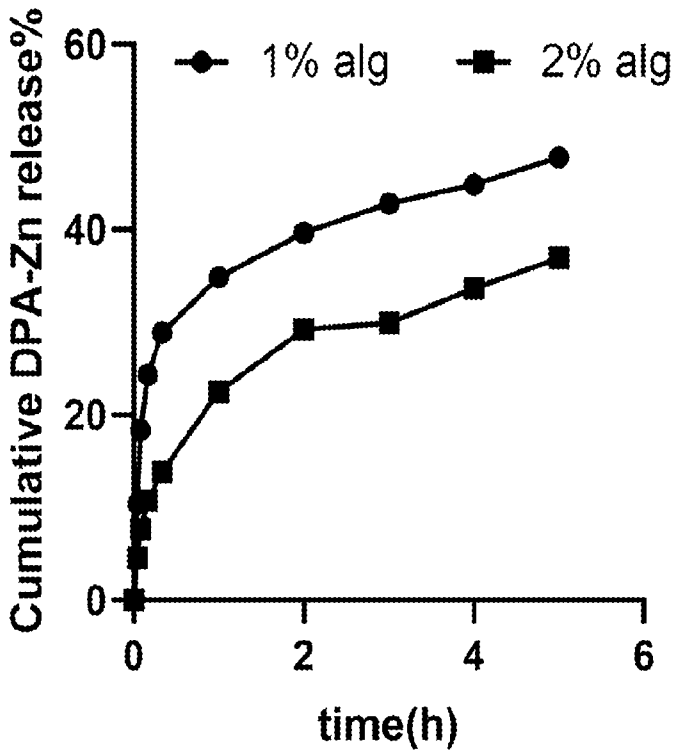


Fig. 19D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/045085

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***A61K 31/4402*** (2024.01); ***A61K 38/19*** (2024.01); ***A61K 33/24*** (2024.01); ***A61K 45/06*** (2024.01); *A61K 35/00* (2024.01)

CPC: ***A61K 31/4402; A61K 38/193; A61K 33/24; A61K 45/06;*** *A61K 35/00*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	US 2023/0203104 A1 (THE JOHNS HOPKINS UNIVERSITY) 29 June 2023 (29.06.2023)	
X	para [0006], [0048], [0051], [0133]-[0136], [0140], [0214], [0216]	1, 12
Y	para [0006], [0048], [0051], [0133]-[0136], [0140], [0214], [0216]	2-3, 6, 9
	US 2020/0138828 A1 (TEMPLE UNIVERSITY-OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION ET AL.) 07 May 2020 (07.05.2020)	
Y	para [0006], [0011], [0072], [0128]	2-3, 6
	US 2019/0038713 A1 (MULTIVIR INC.) 07 February 2019 (07.02.2019)	
Y	para [0007], [0018]-[0020]	9
	WANG, Phosphatidylserine Targeting for Enhancing Chemoimmunotherapy Of Cancer. 19 July 2024 [online]. [Retrieved on 26 December 2024]. Retrieved from the internet	

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 December 2024 (26.12.2024)	**13 January 2025 (13.01.2025)**

Name and mailing address of the ISA/US	Authorized officer
COMMISSIONER FOR PATENTS **MAIL STOP PCT, ATTN: ISA/US** **P.O. Box 1450** **Alexandria, VA 22313-1450** **UNITED STATES OF AMERICA**	**KARI RODRIQUEZ**
Facsimile No. **571-273-8300**	Telephone No. **PCT Help Desk: 571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/045085

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☑ Claims Nos.: **13-14, 18-20**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+: Claims 1-12, directed to a pharmaceutical combination comprising a phosphatidylserine blocker, an immune stimulant, and one or more chemotherapeutic agents. The pharmaceutical combination will be searched to the extent that the phosphatidylserine blocker encompasses dipicolylamine (DPA), the immune stimulant encompasses granulocyte macrophage colony-stimulating factor (GM-CSF), and the chemotherapeutic agent(s) encompass doxorubicin. The first named invention was determined based on the first phosphatidylserine blocker, immune stimulant, and chemotherapeutic agent(s) alternatives listed in claims 2, 9, and 12. This first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. It is believed that claims 1-3, 6(in-part), 9, 12 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass DPA, GM-CSF, and doxorubicin. Additional phosphatidylserine blockers, immune stimulants, and/or chemotherapeutic agents will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected phosphatidylserine blockers, immune stimulants, and/or chemotherapeutic agents. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be where the phosphatidylserine blocker encompasses DPA-Zn, the immune stimulant encompasses GM-CSF, and the chemotherapeutic agent(s) encompass doxorubicin (claims 1-6, 9, 12).

Group II: Claims 15-17, directed to a method of treating a patient with cancer.

Group III: Claims 21-24, directed to a sustained-release formulation comprising a hydrogel comprising a phosphatidylserine blocker, alone or further in combination with a crosslinker, and an immune stimulant.

Group IV: Claims 25-26, directed to a method for treating a patient with cancer comprising administering a sustained-release formulation and one or more chemotherapeutic agents.

Group V: Claims 27-28, directed to a method of preparing a sustained-release formulation comprising mixing a solution comprising a hydrogel and a solution comprising a PS blocker to formulate a hydrogel of PS blocker; and mixing the hydrogel with a solution.

The inventions listed as Groups I+, II, III, IV, and V do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

Special Technical Features

Group I+ requires a composition of matter comprising or consisting of a pharmaceutical combination comprising a phosphatidylserine blocker, an immune stimulant, and one or more chemotherapeutic agents, not required by Groups II-V.

The technical feature of each of the inventions listed as Group I+ is the specific combination of phosphatidylserine blocker, immune stimulant, and chemotherapeutic agent(s) recited therein. Each invention requires a different combination of molecules, not required by any of the other inventions.

Group II requires a method for treating a patient with cancer comprising administering to the patient a phosphatidylserine blocker, an immune stimulant, and one or more chemotherapeutic agents, whereupon the patient is treated for cancer, not required by Groups I+ or III-V.

Group III requires a composition of matter comprising or consisting of a sustained-release formulation comprising a hydrogel comprising a phosphatidylserine blocker and an immune stimulant, not required by Groups I+, II, or IV-V.

Group IV requires a method for treating a patient with cancer comprising administering a sustained-release formulation and one or more chemotherapeutic agents, not required by Groups I+, II-III, or V.

Group V requires a method of preparing a sustained-release formulation comprising mixing a solution comprising a hydrogel and a solution comprising a PS blocker to formulate a hydrogel of PS blocker; and mixing the hydrogel with a solution, not required by Groups I+ or II-IV.

Common Technical Features

The feature shared by Groups I+, II, III, IV, and V is: a PS blocker and an immune stimulant.

The feature shared by Groups I+ and II is: a PS blocker, an immune stimulant, and one or more chemotherapeutic agents.

The feature shared by Groups I+, II, and IV is: a one or more chemotherapeutic agents.

The feature shared by Groups II and IV is: a method for treating a patient with cancer comprising administering to the patient a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer.

The feature shared by Groups III, IV, and V is: a sustained-release formulation.

The feature shared by Groups III and V is: a sustained-release formulation comprising a hydrogel comprising a PS blocker.

Additionally, the feature shard by Groups I+ is: a pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant, and (iii) one or more chemotherapeutic agents.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2023/0203104 A1 to the Johns Hopkins University (hereinafter 'JohnsHopkinsUniv').

JohnsHopkinsUniv discloses (claim 1) a pharmaceutical combination comprising (i) a phosphatidylserine (PS) blocker, (ii) an immune stimulant (para [0133]-[0136] - "In a further embodiment, the compositions and methods of the present invention can be used in combination with one or more additional therapeutically active agents which are known to be capable of treating conditions or diseases discussed above. For example, the compositions of the present invention could be used in combination with one or more known therapeutically active agents, to treat a proliferative disease. Non-limiting examples of other therapeutically active agents that can be readily combined in a pharmaceutical composition with the compositions and methods of the present invention are... antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions. In accordance with a further embodiment, the inventive methods further comprises administering to the subject a pharmaceutical composition comprising an effective amount of one or more additional checkpoint inhibitors. In some embodiments, the checkpoint inhibitor is a composition the blocks the checkpoint protein, programmed cell death protein 1 (PD-1). Examples of such inhibitors include, pembrolizumab, nivolumab, MPDL3280A, MEDI4736, AMP-514, MSB0010718C, anti-TGF-ß, anti-PD-1, anti-PD-L1, or anti-TIM-3, a depleting anti-CTLA-4 antibody... In some embodiments, the one or more checkpoint inhibitors are administered with the comprises an annexin V protein, or a functional

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

portion or fragment or variant thereof"; Note, Annexin V is a PS blocker and anti-PD-L1 and anti-PD-1 antibodies are immune stimulants elected by the instant application, see instant claims 8-9, respectively), and (iii) one or more chemotherapeutic agents (para [0048] - "The present invention also comprises the use of annexin V and annexin V fused in novel chimeric synthetic polypeptides as checkpoint inhibitors in conjunction with chemotherapeutic treatment of various cancers"; para [0140] - "In some embodiments, the subject suffering from a hyperproliferative disease is treated with one or more biologically active agents, such as chemotherapeutic agents, for example, cisplatin, doxorubicin, and/or cyclophosphamide."; para [0141] - "In some embodiments the one or more biologically active agents, or chemotherapeutic agents are given intratumorally or intravenously to the subject, 1 to 10 days prior to treating the subject with an annexin V protein, or a functional portion or fragment or variant thereof, conjugated to an antigen, or a functional portion or fragment or variant thereof.").

JohnsHopkinsUniv discloses a method for treating a patient with cancer comprising administering to the patient a therapeutically effective amount of one or more chemotherapeutic agents, whereupon the patient is treated for cancer (para [0140]-[0141] - "In some embodiments, the subject suffering from a hyperproliferative disease is treated with one or more biologically active agents, such as chemotherapeutic agents, for example, cisplatin, doxorubicin, and/or cyclophosphamide. In some embodiments the one or more biologically active agents, or chemotherapeutic agents are given intratumorally or intravenously to the subject, 1 to 10 days prior to treating the subject with an annexin V protein, or a functional portion or fragment or variant thereof, conjugated to an antigen, or a functional portion or fragment or variant thereof.").

JohnsHopkinsUniv discloses a sustained-release formulation comprising a hydrogel comprising a PS blocker (para [0107] - " a method for treating a tumor in a subject comprising administering to the subject an effective amount of a composition comprising a synthetic polypeptide comprising an annexin V protein, or a functional portion or fragment or variant thereof, conjugated to a HPV 16 E7 tumor antigen, or a functional portion or fragment or variant thereof."; para [0114] - " compositions of the invention can be administered parenterally by injection, infusion or implantation"; para [0120]-[0121] - "The composition may be in the form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation... a therapeutic of the invention is provided within an implant, such as an osmotic pump, or in a graft comprising appropriately transformed cells... Various slow release polymeric devices have been developed and tested for the controlled delivery of drugs, including proteinacious biopharmaceuticals. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant for the sustained release of a bioactive factor at a particular target site.").

As the technical features were known in the art, they cannot be considered special technical features that would otherwise unify the groups.

Groups I+, II, III, IV, and V therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/045085

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-3, 6(in-part), 9, 12, limited to DPA, GM-CSF, and doxorubicin**

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.