(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 May 2022 (19.05.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/103856 A1

(51) International Patent Classification:
A61K 47/64 (2017.01) *A61P 19/08* (2006.01)
A61K 38/39 (2006.01) *A61P 25/28* (2006.01)
C07K 14/78 (2006.01)

(21) International Application Number:
PCT/US2021/058807

(22) International Filing Date:
10 November 2021 (10.11.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/112,039 10 November 2020 (10.11.2020) US

(71) Applicant: **NEW YORK UNIVERSITY** [US/US]; 70 Washington Square South, New York, NY 10012 (US).

(72) Inventors: **RAMIREZ, Craig**; 2933 Wickersham Lane, Austin, TX 78741 (US). **HAUSER, Andrew**; 155 E 34th Street, Apt. 17R, New York, NY 10016 (US). **BEALS, Nathan**; c/o New York University, 522 First Avenue, New York, NY 10016 (US). **BAR-SAGI, Dafna**; c/o New York University, 522 First Avenue, New York, NY 10016 (US). **KOIDE, Akiko**; c/o New York University, 522 First Avenue, New York, NY 10016 (US). **KOIDE, Shohei**; c/o New York University, 522 First Avenue, New York, NY 10016 (US).

(74) Agent: **CHILDS, Carissa, R.**; Troutman Pepper Hamilton Sanders LLP, 70 Linden Oaks, Suite 210, Rochester, NY 14625 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: MACROPINOCYTOSIS SELECTIVE MONOBODY-DRUG CONJUGATES

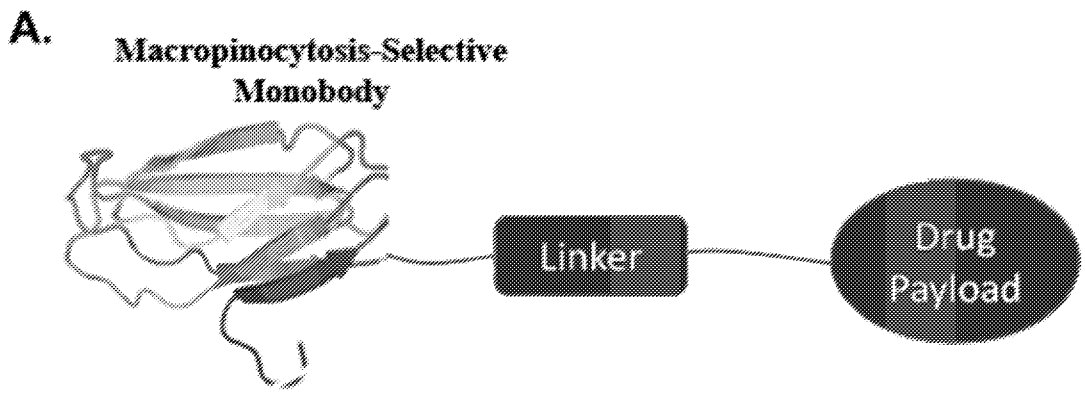


FIG. 1A

(57) Abstract: The present disclosure is directed to pharmaceutical and diagnostic compositions comprising macropinocytosis selective non-binding protein-drug conjugates. These non-binding protein-drug conjugates comprise a non-binding fibronectin type III (FN3) domain coupled to a pharmaceutically active moiety or a diagnostic moiety. The disclosure is also directed to methods of treatment and diagnosis that involve administering the pharmaceutical compositions described herein to a subject in need.

WO 2022/103856 A1

[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

MACROPINOCYTOSIS SELECTIVE MONOBODY-DRUG CONJUGATES

[0001] This application claims the priority benefit of U.S. Provisional Patent Application Serial No. 63/112,039, filed November 10, 2020, which is hereby incorporated by reference in its entirety.

FIELD

[0002] The present disclosure is directed to pharmaceutical and diagnostic compositions comprising macropinocytosis selective non-binding protein-drug conjugates. These non-binding protein-drug conjugates comprise a non-binding fibronectin type III (FN3) domain coupled to a pharmaceutically active moiety or a diagnostic moiety. The disclosure is also directed to methods of treatment and diagnosis that involve administering the pharmaceutical compositions described herein to a subject in need.

BACKGROUND

[0003] It has been known for over 30 years that about one-third of all human cancers arise from mutations in KRas proteins. This includes a high percentage of lung (~25%), pancreatic (~95%), and colon (~45%) cancers. When KRas proteins are mutated, cells grow uncontrollably, evade death signals, and even make cells resistant to a number of available cancer therapies. Although great strides have been made over the past 30 years toward understanding the biology of KRas cancers, current therapies against these cancers remain relatively ineffective. A major obstacle in developing a new drug candidate and gaining FDA approval are the toxicity issues that often arise from off-target effects.

[0004] The present disclosure is directed to overcoming this and related limitations in the art.

SUMMARY

[0005] A first aspect of the present disclosure is directed to a pharmaceutical composition comprising a non-binding protein-drug conjugate and a pharmaceutically acceptable carrier. The non-binding protein-drug conjugate of the pharmaceutical composition comprises a first portion, an amino acid linker, and a second portion. The first portion of the protein-drug conjugate comprises a non-binding fibronectin type III (FN3) domain, and the second portion of the protein-drug conjugate, which is coupled to the first portion via the amino acid linker, is selected from a pharmaceutically active moiety or a diagnostic moiety.

[0006] Another aspect of the present disclosure relates to a method of treating cancer in a subject. This method involves administering to the subject having cancer, a composition

comprising a non-binding protein-drug conjugate as described herein in an amount effective to treat the cancer.

[0007] Another aspect of the present disclosure relates to a method of imaging a tumor in a subject. This method involves selecting a subject having a tumor and administering to the subject a composition comprising a non-binding protein-drug conjugate as described herein.

[0008] Another aspect of the present disclosure relates to a method of modulating a subject's immune response. This method involves administering to a subject having a condition that would benefit from immune system modulation, a composition comprising a non-binding protein-drug conjugate as described herein in an amount effective to modulate the subject's immune response.

[0009] Another aspect of the present disclosure relates to a method of treating a neurodegenerative condition in a subject. This method involves administering to the subject having the neurodegenerative condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the neurodegenerative condition.

[0010] Another aspect of the present disclosure relates to a method of treating an inflammatory condition in a subject. This method involves administering to the subject having the inflammatory condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the inflammatory condition.

[0011] Another aspect of the present disclosure relates to a method of treating a bone condition in a subject. This method involves administering to the subject having the bone condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the bone condition.

[0012] Another aspect of the present disclosure relates to a method of treating an infectious disease or condition in a subject. This method involves administering to the subject having the infectious disease a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the infectious disease.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIGs. 1A–1D show the characterization of MC-small molecule (monobody) conjugation and efficiency. FIG. 1A shows a schematic design of the monobody-small molecule conjugate. FIG. 1B is a gel shift assay showing a shift after conjugation of monomethyl auristatin E (MMAE). Conjugation of MMAE was determined to be >95%. FIG. 1C shows a SDS-PAGE gel imaged at 800nm to confirm conjugation of the Cy5.5 to the monobody. FIG. 1D shows an

Ellman's test to observe the percentage of MMAE conjugation to the monobody. Ellman's reagent measures free available thiols within the solution.

[0014] FIGs. 2A-2C show that the cellular uptake of non-binding protein-drug conjugates as disclosed herein is specifically through macropinocytosis (MP). FIG. 2A and FIG. 2B show the uptake of the fluorescently-tagged monobody and MP marker in human PDAC MIA PaCa and HeLa or HeLa $KRas^{V12}$ cells. Co-localization of the fluorescently-tagged monobody (green) and MP marker (dextran, red) in. DAPI, blue. Boxed region is enlarged, inset. FIG. 2C shows the flow cytometry analysis of monobody (FN-Cy5.5) in oncogenic Ras (HeLa$KRas^{V12}$and MIAPaCa-2) cells.

[0015] FIGs. 3A-3B show that the cellular uptake of non-binding protein-drug conjugates as disclosed herein is specifically through macropinocytosis (MP). FIG. 3A shows the uptake of the fluorescently-tagged monobody and MP marker in mouse PDAC KPC 1203 cells. Co-localization of the fluorescently-tagged monobody (red) and MP marker (dextran, green) in. DAPI, blue. Boxed region is enlarged, inset. FIG. 3B shows the uptake of the fluorescently-tagged monobody and MP marker in WT Ras and mutant Ras human colorectal cancer cells. Co-localization of the fluorescently-tagged monobody (green) and MP marker (dextran, red) with DAPI, blue.

[0016] FIGs. 4A-4C show the enhanced toxicity of monobody-drug conjugates in oncogenic KRas cell lines. FIG. 4A tracks cell viability as monitored by the infrared dye Syto60 in HeLa (-KRas) and HeLa $KRas^{V12}$ (+KRas) cells treated with increasing concentrations of FN-MMAE. FIG. 4B shows the wild type Ras cell lines (above dashed line, open circles) and oncogenic Ras cell lines (below dashed line, solid circles) from different tissues were treated with increasing concentrations of FN-MMAE and MMAE. FN-MMAE IC_{50} values were normalized to MMAE values. FIG. 4C shows the wildtype Ras cell line (BxPC3) and oncogenic Ras cell line (MiaPaca-2) were treated with increasing concentrations of FN-SN38.

[0017] FIGs. 5A-5B show the enhanced toxicity of FN-MMAE (monobody) in oncogenic KRas cell lines *in vivo*. In FIG. 5A, established (100-150mm^3) subcutaneous MiaPaCa and BxPC3 tumors were treated with, PBS (negative control), 0.2 mg/kg of MMAE alone, or 0.2 mg/kg of FN-MMAE non-binding protein conjugate on Days 0, 7, and 14 (yellow arrows). Tumor growth as assessed by volume was slower in FN-MMAE treated animals compared to MMAE treated alone. n=4 per treatment group. Error bars represent SEM of tumor volume. FIG. 5B shows the H&E of *ex vivo* organs of MIA PaCa tumor bearing mice showing no apparent toxicity for FN-MMAE while MMAE alone saw visual liver damage.

[0018] FIGs. 6A-6B show *ex vivo* analysis and mode of action of MMAE. FIG. 6A shows the *ex vivo* analysis of kidneys from MIA PaCa tumor bearing mice treated with PBS,

MMAE, and FN-MMAE with a number of apoptotic markers (*i.e.*, CC3, HMGB1, and cleaved Parp1). Marker expression in these images indicates that FN-MMAE showed little or no difference as compared to PBS, whereas the free MMAE had substantial increase in non-specific toxicity. The images of FIG. 6B show that MMAE's mode of action is through tubulin destabilization. Tubulin staining was drastically reduced in kidneys of MMAE treated mice, whereas FN-MMAE showed no apparent effect.

[0019] FIGs. 7A-7E show tumor specific biodistribution. FIG. 7A shows that biodistribution was detected using IVIS over a time course of 24hr in MIA PaCa tumor bearing mice with PBS, Cy5.5-COOH, or FN-Cy5.5 treatment. FIG. 7B shows biopsied tissues were analyzed *ex vivo* by IVIS imaging at 24hr timepoint. MIA PaCa2 and BxPC3 xenografts were treated with FN-Cy5.5 and ex *vivo* imaging was used to observe the KRas selective uptake of FN-Cy5.5 (FIG. 7C). FIG. 7D shows after 6 hr, mice were sacrificed, and essential organs were isolated and imaged *ex-vivo* by IVIS to analyze tumor uptake and accumulation of the monobody, systemic clearance, and tissues with potential toxicities. FIG. 7E shows tumor sections imaged to depict tumor uptake, retention, and co-localization of Cy5.5 and FN-Cy5.5 with a cancer cell maker (CK8).

[0020] FIGs. 8A-8C show biodistribution of FN-Cy5.5 in the orthotopic implant KPC tumor bearing mice. KPC tumor bearing mice were treated with PBS or 5nmol, in reference to Cy5.5, of FN-Cy5.5, Mouse albumin (MSA)-Cy5.5, and mouse IgG-Cy5.5. Mice were sacrificed at 1hr (FIG. 8A) and 24hr (FIG. 8B) to observe *ex vivo* biodistribution and blood fluorescence (FIG. 8C). Graphs represent normalized values comparing MSA to FN (red) and IgG to FN (blue). At 1hr, FN had drastic 2 fold increases in the kidneys and liver, displaying quick excretion while having similar or increased tumor retention. At 24hr, FN showed reductions in kidneys and liver while have similar tumor values (90% and 100% for MSA and IgG respectively). FN displayed a 3-fold decrease in blood fluoresce at 1hr, again highlighting quick excretion compared to other protein conjugates.

[0021] FIGs. 9A-9C show lymph node analysis of FN-Cy5.5. FIG. 9A provides schematics of Cy5.5-COOH, FN-Cy5.5, and pegylated nanoparticles with Cy5.5 conjugated to the surface that were administered to orthotopic implanted KPC tumor bearing mice. In FIG. 9B, *ex vivo* IVIS imaging showed FN-Cy5.5 had a 2-fold preference for PDAC lymph nodes (PLN) compared to bronchial lymph nodes (BLN). FIG. 9C are graphs showing Cy5.5 uptake in dendritic cells (DCs), i.e., classic dendritic cells (top graphs) and plasmacytoid dendritic cells (bottom graphs). As shown, a 2-fold increase in cellular uptake of FN was observed.

[0022] FIGs. 10A-10D show FN-Cy5.5 uptake in a genetically modified mouse model of PDAC. Autochonous LSL-KPC^{G12V}; $LSLp53^{R172H}$; p48Cre (KPC, 4mo) and wild type

(WT, 4 mo) mice were treated with FN-Cy5.5. *Ex vivo* images of FIG. 10A depict biodistribution in various organs at 1hr and 24hr. Normalized radiant efficiency displays preference for tumor uptake in the autochonous KPC model (FIG. 10B). In FIGs. 10C-10D, *ex vivo* images (6hr) depict tumor uptake, retention, and colocalization with a cancer cell maker (CK8).

[0023] FIG. 11 shows *ex vivo* tumor analysis in autochonous LSL-KPC^{G12V}; $LSLp53^{R172H}$; p48Cre (KPC, 4mo) mice treated with FN-Cy5.5 and albumin-FITC for 2hrs. Mice were sacrificed and *ex vivo* analysis was carried out on tumors displaying albumin-FITC (green), FN-Cy5.5 (purple), tumor cell marker CK8 (orange), and dapi (blue). Visually, FN-Cy5.5 was observed to potentially stain CK8 positive PDAC tumor ducts better than albumin-FITC but this observation did show some variance based on tumor location.

[0024] FIGs. 12A-12C show MRI detection using FN-DOTA-Gd^{3+}. FIG. 12A is a schematic showing a chelator (DOTA) alone (left) or conjugated to the lone cysteine at the C-terminus of a linker molecule (right). In FIG. 12B, DOTA-Gd^{3+} and FN-DOTA-Gd^{3+} were injected into KPC orthotopic tumor bearing mice at 2 μmol/kg. Increased signal and longer retention were observed in the tumor for FN- DOTA-Gd^{3+}. FIG. 12C shows a FN-DOTA-Gd^{3+} that was injected via tail vein in a 4-month-old autochthonous KPC mouse. MRI imaging of FN-DOTA-Gd^{3+}pre-injection(left) and post-injection (right) at 2 anatomical planes across the ventral to dorsal direction (top and bottom, respectively) are shown. Red arrows point to detection of signal in kidneys; red arrowheads point to possible pancreatic tumor. Outlines in top show Ki, kidney; Pa, pancreas; Sp, spleen, St, stomach.

[0025] FIGs. 13A-13B show MRI imaging of KPC orthotopic tumors. MRI imaging of KPC orthotopic implanted tumors with (FIG. 13A) DOTA-Gd^{3+} and (FIG. 13B) FN-DOTA-Gd^{3+}.

[0026] FIGs. 14A-14C show uptake of fluorescently-tagged monobody and MP marker in human multiple myeloma cancer cell lines. FIGs. 14A and 14B are images showing co-localization of the fluorescently-tagged monobody (red) and MP marker (dextran, green) in KMS11 WT Ras, L363 Mutant NRAS, and RPMI 8226 Mutant KRas cells. DAPI, blue. FN-Cy5.5 showed increased uptake in mutant Ras cell lines which was abolished with the addition of macropinocytosis inhibitor EIPA and KH7. FIG. 14C shows the uptake of FN-Cy5.5 in mutant KRas RPMI 8226 cells at 37°C and 4°C.

[0027] FIGs. 15A-15C shows a mutant KRas inducible system that was created in WT Ras KMS11 multiple myeloma cells. FIG. 15A shows a western blot and FIG. 15B shows a membrane ruffling assay confirming mutant KRas expression with the treatment of doxycycline. FIG. 15C shows that with the addition of doxycycline, the KMS11 cells displayed uptake of FN-

Cy5.5 which wasn't observed in the minus doxycycline. This again highlights the selective uptake of FN-Cy5.5 in mutant Ras cells.

[0028] FIG. 16 shows flow cytometry analysis of Cy5.5 and FN-Cy5.5 in oncogenic Ras various multiple myeloma cell lines.

[0029] FIG. 17 shows differential tumor accumulation based on Ras status in multiple myeloma. Biodistribution of FN-Cy5.5 in mice bearing mutant NRas L363 or WT Ras KMS11 tumors. The mutant Ras tumor displayed a significant increase in FN-Cy5.5 uptake as compared to the WT tumor (bottom graph).

DETAILED DESCRIPTION

[0030] A first aspect of the present disclosure is directed to a pharmaceutical composition comprising a non-binding protein-drug conjugate and a pharmaceutically acceptable carrier. The non-binding protein-drug conjugate of the pharmaceutical composition comprises a first portion, an amino acid linker, and a second portion. The first portion of the non-binding protein-drug conjugate comprises a non-binding fibronectin type III (FN3) domain, and the second portion of the non-binding protein-drug conjugate, which is coupled to the first portion via the amino acid linker, is selected from a pharmaceutically active moiety or a diagnostic moiety.

[0031] The FN3 domain is an evolutionary conserved protein domain that is about 100 amino acids in length and possesses a beta sandwich structure. The beta sandwich structure of human FN3 comprises seven beta-strands, referred to as strands A, B, C, D, E, F, G, with six connecting loops, referred to as loops AB, BC, CD, DE, EF, and FG that exhibit structural homology to immunoglobulin binding domains. Three of the six loops, *i.e.*, loops DE, BC, and FG, correspond topologically to the complementarity determining regions of an antibody, *i.e.*, CDR1, CDR2, and CDR3. The remaining three loops are surface exposed in a manner similar to antibody CDR3. While many naturally occurring FN3 domains and modified FN3 domains contain loop and strand regions that enable binding to a particular target protein or target domain of a protein (*i.e.*, monobodies), the FN3 domain of the protein-drug conjugate of the present disclosure is a non-binding FN3 domain. As used herein, a "non-binding FN3 domain" encompasses naturally occurring and modified FN3 domains that do not bind to or interact with any other protein or protein domain, *i.e.*, the domain lacks an RGD binding domain and any other protein interacting region.

[0032] The non-binding FN3 domain of the protein-drug conjugate of the present disclosure can be a FN3 domain derived from any of the wide variety of animal, yeast, plant, and bacterial extracellular proteins containing these domains. In one embodiment, the FN3 domain

is a mammalian FN3 domain or a non-binding variant thereof. Exemplary FN3 domains include, for example and without limitation, any one of the FN3 domains present in human tenascin C, or the FN3 domains present in human fibronectin (FN). In some embodiments, the FN3 domain is a non-binding FN3 domain of human fibronectin (UniProtKB Accession No. P02751) selected from an amino acid sequence of SEQ ID NOs: 6–21 as shown in Table 1 or a non-binding variant or derivative thereof. In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is a variant of the 10th fibronectin type III domain of human fibronectin (*i.e.*, a variant of SEQ ID NO: 1). In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is a non-binding FN3 domain of human tenascin (UniProtKB Accession No. P24821) selected from an amino acid sequence of SEQ ID NOs: 22–36 (Table 1) or a variant or derivative thereof. In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is a non-natural synthetic non-binding FN3 domain, such as those described in U.S. Pat. Publ. No. 2010/0216708 to Jacobs et al., which is hereby incorporated by reference in its entirety. Individual FN3 domains are referred to by domain number and protein name, *e.g.*, the 10th FN3 domain of fibronectin (10FN3).

Table 1. Exemplary FN3 domains from Human Fibronectin and Tenascin

Domain*	SEQ ID NO	Sequence
1st FN3 of Fibronectin	6	GPVEVFITETPSQPNSHPIQWNAPQPSHISKYILRWRPKNSVGRWKEA TIPGHLNSYTIKGLKPGVVYEGQLISIQQYGHQEVTRFDFTTTST
2nd FN3 of Fibronectin	7	VATSESVTEITASSFVVSWVSASDTVSGFRVEYELSEEGDEPQYLDLP STATSVNIPDLLPGRKYIVNVYQISEDGEQSLILSTSQTTAPD
3rd FN3 of Fibronectin	8	APPDTTVDQVDDTSIVVRWSRPQAPITGYRIVYSPSVEGSSTELNLPE TANSVTLSDLQPGVQYNITIYAVEENQESTPVVIQQETTGTPRS
4th FN3 of Fibronectin	9	SPRDLQFVEVTDVKVTIMWTPPESAVTGYRVDVIPVNLPGEHGQRLP ISRNTFAEVTGLSPGVTYYFKVFAVSHGRESKPLTAQQTTKLD
5th FN3 of Fibronectin	10	APTNLQFVNETDSTVLVRWTPPRAQITGYRLTVGLTRRGQPRQYNV GPSVSKYPLRNLQPASEYTVSLVAIKGNQESPKATGVFTTLQPG
6th FN3 of Fibronectin	11	SSIPPYNTEVTETTIVITWTPAPRIGFKLGVRPSQGGEAPREVTSDSGSI VVSGLTPGVEYVYTIQVLRDGQERDAPIVNKVVTPLS
7th FN3 of Fibronectin	12	PPTNLHLEANPDTGVLTVSWERSTTPDITGYRITTTPTNGQQGNSLEE VVHADQSSCTFDNLSPGLEYNVSVYTVKDDKESVPISDTIIPEVPQL
EDB domain of Fibronectin	13	TDLSFVDITDSSIGLRWTPLNSSTIIGYRITVVAAGEGIPIFEDFVDSSV GYYTVTGLEPGIDYDISVITLINGGESAPTTLTQQTAVP
8th FN3 of Fibronectin	14	PPTDLRFTNIGPDTMRVTWAPPPSIDLTNFLVRYSPVKNEEDVAELSI SPSDNAVVLTNLLPGTEYVVSVSSVYEQHESTPLRGRQKTGLDSP
9th FN3 of Fibronectin	15	TGIDFSDITANSFTVHWIAPRATITGYRIRHHPEHFSGRPREDRVPHSR NSITLTNLTPGTEYVVSIVALNGREESPLLIGQQSTVSD
10th FN3 of Fibronectin	1	VPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTV PGSKSTATISGLKPGVDYTITVYAVTGRGDSPASSKPISINYRTEID
11th FN3 of Fibronectin	16	KPSQMQVTDVQDNSISVKWLPSSSPVTGYRVTTTPKNGPGPTKTKTA GPDQTEMTIEGLQPTVEYVVSVYAQNPSGESQPLVQTAVTNIDRP
EDA domain of Fibronectin	17	KGLAFTDVDVDSIKIAWESPQGQVSRYRVTYSSPEDGIHELFPAPDGE EDTAELQGLRPGSEYTVSVVALHDDMESQPLIGTQSTAIP

12th FN3 of Fibronectin	18	APTDLKFTQVTPTSLSAQWTPPNVQLTGYRVRVTPKEKTGPMKEINL APDSSSVVVSGLMVATKYEVSVYALKDTLTSRPAQGVVTTLENVSP P
13th FN3 of Fibronectin	19	RRARVTDATETTITISWRTKTETITGFQVDAVPANGQTPIQRTIKPDV RSYTITGLQPGTDYKIYLYTLNDNARSSPVVIDASTAID
14th FN3 of Fibronectin	20	APSNLRFLATTPNSLLVSWQPPRARITGYIIKYEKPGSPPREVVPRPRP GVTEATITGLEPGTEYTIYVIALKNNQKSEPLIGRKKTDELP
15th FN3 of Fibronectin	21	PGLNPNASTGQEALSQTTISWAPFQDTSEYIISCHPVGTDEEPLQFRVP GTSTSATLTGLTRGATYNVIVEALKDQQRHKVREEVVTVGNSVNEG
1st FN3 of Tenascin	22	PPKDLVVTEVTEETVNLAWDNEMRVTEYLVVYTPTHEGGLEMQFR VPGDQTSTIIQELEPGVEYFIRVFAILENKKSIPVSARVATYLPAP
2nd FN3 of Tenascin	23	EGLKFKSIKETSVEVEWDPLDIAFETWEIIFRNMNKEDEGEITKSLRRP ETSYRQTGLAPGQEYEISLHIVKNNTRGPGLKRVTTTRLD
3rd FN3 of Tenascin	24	APSQIEVKDVTDTTALITWFKPLAEIDGIELTYGIKDVPGDRTTIDLTE DENQYSIGNLKPDTEYEVSLISRRGDMSSNPAKETFTTGLD
4th FN3 of Tenascin	25	APRNLRRVSQTDNSITLEWRNGKAAIDSYRIKYAPISGGDHAEVDVP KSQQATTKTTLTGLRPGTEYGIGVSAVKEDKESNPATINAATELDTP KD
5th FN3 of Tenascin	26	LQVSETAETSLTLLWKTPLAKFDRYRLNYSLPTGQWVGVQLPRNTT SYVLRGLEPGQEYNVLLTAEKGRHKSKPARVKASTEQAP
6th FN3 of Tenascin	27	ELENLTVTEVGWDGLRLNWTAADQAYEHFIIQVQEANKVEAARNL TVPGSLRAVDIPGLKAATPYTVSIYGVIQGYRTPVLSAEASTGET
7th FN3 of Tenascin	28	NLGEVVVAEVGWDALKLNWTAPEGAYEYFFIQVQEADTVEAAQNL TVPGGLRSTDLPGLKAATHYTITIRGVTQDFSTTPLSVEVLTEEV
8th FN3 of Tenascin	29	DMGNLTVTEVSWDALRLNWTTPDGTYDQFTIQVQEADQVEEAHNL TVPGSLRSMEIPGLRAGTPYTVTLHGEVRGHSTRPLAVEVVTEDLPQ L
9th FN3 of Tenascin	30	GDLAVSEVGWDGLRLNWTAADNAYEHFVIQVQEVNKVEAAQNLT LPGSLRAVDIPGLEAATPYRVSIYGVIRGYRTPVLSAEASTAKEP
10th FN3 of Tenascin	31	EIGNLNVSDITPESFNLSWMATDGIFETFTIEIIDSNRLLETVEYNISGA ERTAHISGLPPSTDFIVYLSGLAPSIRTKTISATATTEALPL
11th FN3 of Tenascin	32	ENLTISDINPYGFTVSWMASENAFDSFLVTVVDSGKLLDPQEFTLSGT QRKLELRGLITGIGYEVMVSGFTQGHQTKPLRAEIVTEAEP
12th FN3 of Tenascin	33	EVDNLLVSDATPDGFRLSWTADEGVFDNFVLKIRDTKKQSEPLEITL LAPERTRDITGLREATEYEIELYGISKGRRSQTVSAIATTAMG
13th FN3 of Tenascin	34	SPKEVIFSDITENSATVSWRAPTAQVESFRITYVPITGGTPSMVTVDGT KTQTRLVKLIPGVEYLVSIIAMKGFEESEPVSGSFTTALDG
14th FN3 of Tenascin	35	PSGLVTANITDSEALARWQPAIATVDSYVISYTGEKVPEITRTVSGNT VEYALTDLEPATEYTLRIFAEKGPQKSSTITAKFTTDLD
15 FN3 of Tenascin	36	SPRDLTATEVQSETALLTWRPPRASVTGYLLVYESVDGTVKEVIVGP DTTSYSLADLSPSTHYTAKIQALNGPLRSNMIQTIFTTIGLL

*Domain nomenclature for human fibronectin is the conventional nomenclature as set forth in Potts and Campbell, "Fibronectin Structure and Assembly," *Curr. Opin. Cell Biol.* 6:648-655 (1994). Domain nomenclature for human tenascin is the nomenclature provided in UniProt Accession No. P24821

[0033] In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is a FN3 domain known to have little or no functional binding activity. Suitable non-binding FN3 domains include the 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 11th, 12th, and 13th type III fibronectin domains of human fibronectin (SEQ ID NOs: 6–12, 16, 18, and 19) and the EDB and EDA domains of human fibronectin (SEQ ID NOs: 13 and 17). Suitable FN3 domains also encompass non-binding domains having an amino acid sequence that is at least 80%, 81%, 82%,

83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% similar to an amino acid sequence of SEQ ID NO: 6–12, 16, 18, or 19.

[0034] In some embodiments, a suitable FN3 domain of the non-binding protein-drug conjugate comprises any one of the 1st, 2nd, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, 12th, 13th, 14th, and 15th type III fibronectin domains of human tenascin (SEQ ID NOs: 22, 23, 25–36). Suitable FN3 domains of the non-binding protein-drug conjugate also encompass non-binding domains having an amino acid sequence that is at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% similar to an amino acid sequence of SEQ ID NO: 22, 23, and 25–36.

[0035] In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is a non-binding variant of the 8th, 9th, 10th, 14th and 15th type III fibronectin domains of human fibronectin or a non-binding variant of the 3rd type III fibronectin domain of human tenascin. These non-binding variants contain at least one amino acid residue modification in the amino acid sequence of SEQ ID NOs: 1, 14, 15, 20, 21, and 24. Accordingly, suitable FN3 domains include FN3 domains having an amino acid sequence that is 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% similar in sequence to the amino acid sequence of SEQ ID NO: 1, 14, 15, 20, 21, or 24. Suitable FN3 domains include FN3 domains having an amino acid sequence that is <100% similar in sequence to the amino acid sequence of any one of SEQ ID NO: 1, 14, 15, 20, 21, or 24.

[0036] In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is derived from the 10th FN domain of fibronectin (^{10}FN3). In some embodiments, the FN3 domain of the non-binding protein-drug conjugate is derived from the human ^{10}FN3 domain. The human ^{10}FN3 domain has the amino acid sequence of SEQ ID NO: 1 as shown below. The locations of the BC (residues 24-30), CD (residues 40-45), DE (residues 51-55), and FG (residues 75-86) loops are underlined within the wild-type sequence of SEQ ID NO: 1. In accordance with the present disclosure, the non-binding FN3 domain of the non-binding protein-drug conjugate is a non-binding variant of SEQ ID NO: 1, *i.e.*, it comprises one or more amino acid additions, substitutions, or deletions within the amino acid sequence of SEQ ID NO:1.

VSDVPRDLEVVAATPTSLLISWD$\underline{A_{24}PAVTVR_{30}}YYR_{33}$ITYGET$\underline{G_{40}GNSPV_{45}}$QEFTV$\underline{P_{51}GSKS_{55}}$ TATISGLKPGVDYTITVYA$\underline{V_{75}TGRGDSPASSK_{86}}$PISINYRT (SEQ ID NO: 1)

[0037] In accordance with the present disclosure, the non-binding FN3 domain of the protein-drug conjugate of the present disclosure comprises a variant SEQ ID NO: 1 containing at least one modification within the RGD amino acid sequence of the FG loop of the FN3 domain,

i.e., amino acid residues 75–86 of SEQ ID NO: 1. Suitable modifications include amino acid residue substitutions, insertions, and/or deletions. In some embodiments, at least one of, at least two of, or at least three of the RGD amino acid residues of the FG loop of the FN3 domain are modified to eliminate RGD mediated binding. In some embodiments, the non-binding FN3 domain of the present disclosure comprises a variant of SEQ ID NO:1, wherein the arginine (R) at the position corresponding to position 78 of SEQ ID NO: 1 (R78) is substituted or deleted. Suitable amino acid substitutions include, without limitation, substitution with a residue selected from serine (S), alanine (A), glutamic acid (E), glycine (G), lysine (K), asparagine (N), aspartic acid (D), and glutamine (Q). In some embodiments, the amino acid substitution is an arginine to serine substitution at the amino acid residue corresponding to the arginine at position 78 (R78S) of SEQ ID NO: 1. In some embodiments, the non-binding FN3 domain of the present disclosure comprises a variant of SEQ ID NO:1, wherein the glycine (G) at the position corresponding to position 79 of SEQ ID NO: 1 (G79) is substituted or deleted. Suitable amino acid substitutions include, without limitation, substitution with a residue selected from S, A, E, D, K, N, or Q. In some embodiments, the amino acid substitution is a glycine to serine substitution at the amino acid residue corresponding to the glycine at position 79 (G79S) of SEQ ID NO: 1. In some embodiments, the non-binding FN3 domain of the present disclosure comprises a variant of SEQ ID NO:1, wherein the aspartic acid (D) at the position corresponding to position 80 of SEQ ID NO: 1 (D80) is substituted or deleted. Suitable amino acid substitutions include, without limitation, substitution with a residue selected from S, A, E, G, K, N, or Q. In some embodiments, the amino acid substitution is an aspartic acid to serine substitution at the amino acid residue corresponding to the aspartic acid at position 80 (D80S) of SEQ ID NO: 1.

[0038] In some embodiments, the non-binding FN3 domain of the protein-drug conjugate of the present disclosure comprises one more additional amino acid modifications in the FG loop, *i.e.*, corresponding to amino acid residues 75-86 of SEQ ID NO:1. Suitable amino acid substitutions include, without limitation, substitution with a residue selected from S, A, E, G, K, N, or Q. In some embodiments, the non-binding FN3 domain of the protein-drug conjugate comprises a variant of SEQ ID NO: 1, wherein the FG loop comprises the amino acid sequence of SSSSSSSSS (SEQ ID NO: 4).

[0039] In some embodiments, the non-binding FN3 domain of the protein-drug conjugate, further comprises one or more amino acid substitutions in the amino acid sequence of the BC loop, *i.e.*, amino acid residues 24–30 of SEQ ID NO: 1. Suitable amino acid substitutions include, without limitation, substitution with a residue selected from S, A, E, G, K, N, or Q. In some embodiments, the non-binding FN3 domain of the protein-drug conjugate

comprises a variant of SEQ ID NO: 1, wherein the BC loop comprises the amino acid sequence of SSSSSVS (SEQ ID NO: 5).

[0040] In some embodiments, the non-binding FN3 domain of the protein-drug conjugate comprises an amino acid sequence of SEQ ID NO: 2 as shown below.

VSX_3VPT X_7LEVVAATPTSLLISWD$X_{24}X_{25}$ X_{26} X_{27} X_{28}V X_{30}YYRITYG ETGGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYA X_{75} X_{76} X_{77} X_{78} X_{79} X_{80} X_{81} X_{82} X_{83}SSKPISINYRT (SEQ ID NO: 2)

Wherein,

X_3 is selected from D, S, A, E, G, K, N, and Q

X_7 is selected from D, S, A, E, G, K, N, and Q

X_{24} is selected from S, A, E, G, K, N, D and Q

X_{25} is selected from P, S, A, E, G, K, N, D and Q

X_{26} is selected from A, S, E, G, K, N, D, and Q

X_{27} is selected from V, S, A, E, G, K, N, D, and Q

X_{28} is selected from T, S, A, E, G, K, N, D, and Q

X_{30} is selected from R, S, A, E, G, K, N, D, and Q

X_{75} is selected from V, S, A, E, G, K, N, D, and Q

X_{76} is selected from T, S, A, E, G, K, N, D, and Q

X_{77} is selected from G, S, A, E, K, N, D, and Q

X_{78} is selected from R, S, A, E, G, K, N, D, and Q

X_{79} is selected from G, S, A, E, K, N, D, and Q

X_{80} is selected from D, S, A, E, G, K, N, and Q

X_{81} is selected from S, A, E, G, K, N, D, and Q

X_{82} is selected from P, S, A, E, G, K, N, D, and Q

X_{83} is selected from A, S, E, G, K, N, D, and Q

With the proviso, that the non-binding FN3 domain does not comprise the amino acid sequence of SEQ ID NO: 1.

[0041] In some embodiments, the non-binding FN3 domain of the protein-drug conjugate comprises the amino acid sequence of SEQ ID NO: 3.

VSSVPTKLEVVAATPTSLLISWDASSSSVSYYRITYGETGGNSPVQEFTVPGSKSTATISG LKPGVDYTITVYASSSSSSSSSSSKPISINYRT (SEQ ID NO:3).

[0042] In some embodiments, the first portion of the non-binding protein-drug conjugate comprises two or more non-binding FN3 domains linked together in tandem. In some embodiments, the first portion of the non-binding protein-drug conjugate comprises, two, three, four, five, six, or more non-binding FN3 domains as described herein linked together in tandem.

[0043] In some embodiments, the first portion of the non-binding protein-drug conjugate further comprises one or more tag sequences, *e.g.*, a poly-histidine (His_{6-}) tag, a glutathione-S-transferase (GST–) tag, biotinylation tag, or maltose-binding protein (MBP–) tag for purification/production purposes, and/or one or more protease cleavage sites. The tags and cleavage sites can be incorporated into the amino or carboxy termini of the non-binding FN3 domain.

[0044] The non-binding protein-drug conjugate of the present disclosure further comprises an amino acid linker that couples the first portion of the non-binding protein-drug conjugate to the second portion of the conjugate. In some embodiments, the amino acid linker is a cleavable linker. In some embodiments, the amino acid linker is a non-cleavable linker. Suitable linkers include peptides composed of repetitive modules of one or more of the amino acids, such as glycine and serine or alanine and proline. Exemplary linker peptides include, *e.g.*, $(Gly\text{-}Gly)_n$, $(Gly\text{-}Ser)_n$, $(Gly_3\text{-}Ser)_n$, $(Ala\text{-}Pro)_n$ wherein $_n$ is an integer from 1-25. The length of the linker may be appropriately adjusted as long as it does not affect the function of the non-binding protein-drug conjugate. The standard 15 amino acid $(Gly_4\text{-}Ser)_3$ linker peptide has been well-characterized and has been shown to adopt an unstructured, flexible conformation. In addition, this linker peptide does not interfere with assembly and activity of the domains it connects (Freund et al., "Characterization of the Linker Peptide of the Single-Chain Fv Fragment of an Antibody by NMR Spectroscopy," *FEBS* 320:97 (1993), the disclosure of which is hereby incorporated by reference in its entirety).

[0045] Well known chemical coupling methods may be used to attach the first and second portions using a peptide or other linker to produce non-binding protein-drug conjugates. For example, covalent conjugation of the first and second portions can be accomplished via lysine side chains using an activated ester or isothiocyanate, or via cysteine side chains with a maleimide, haloacetyl derivative or activated disulfide. Site specific conjugation of the first and second portions can also be accomplished by incorporating unnatural amino acids, self-labeling tags (e.g., SNAP or DHFR), or a tag that is recognized and modified specifically by another enzyme such as sortase A, lipoic acid ligase, and formylglycine-generating enzyme. In some embodiments, site specific conjugation of the first and second portions is achieved by the introduction of cysteine residue either at the C-terminus of the non-binding FN3 portion or at a specific site as described by Goldberg et al., "Engineering a Targeted Delivery Platform Using Centyrins," *Protein Engineering, Design & Selection* 29(12):563-572 (2016) and U.S. Patent Application Publication No. 20200325210 to Anderson et al., which are hereby incorporated by reference in their entirety.

[0046] In some embodiments, the second portion of the non-binding protein-drug conjugate as described herein is a pharmaceutically active moiety. Suitable pharmaceutically active moieties include small molecules, nucleic acid molecules, antibodies, proteins or polypeptide fragments thereof, and a proteolysis targeting chimeras (PROTAC).

[0047] In some embodiments, the pharmaceutically active moiety is a cancer therapeutic. Suitable cancer therapeutics include, without limitation, an antimetabolite, an alkaloid, an alkylating agent, an anti-mitotic agent, an antitumor antibiotic, a DNA binding drug, a toxin, an antiproliferative drug, a DNA antagonist, a radionuclide, a thermoablative agent a proteolysis targeting chimera (PROTAC), a nucleic acid inhibitor, and an immune-modulatory agent.

[0048] In some embodiments, the cancer therapeutic is an alkaloid. Suitable alkaloids include, without limitation, duocarmycin, docetaxel, etoposide, irinotecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, vindesine, and analogs and derivatives thereof.

[0049] In some embodiments, the cancer therapeutic is an alkylating agent. Suitable alkylating agents include, without limitation, busulfan, improsulfan, piposulfan, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphorarnide, chlorambucil, chloranaphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide HCl, melphalan, novemebichin, perfosfamide phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, semustine ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, and analogs and derivatives thereof.

[0050] In some embodiments, the cancer therapeutic is an antitumor antibiotic. Suitable antitumor antibiotics include, without limitation, aclacinomycin, actinomycin, anthramycin, azaserine, bleomycin, cactinomycin, calicheamicin, carubicin, carzinophilin, cromomycin, dactinomycin, daunorubicin, 6-diazo-5-oxo-l-norleucine, doxorubicin, epirabicin, idarubicin, menogaril, mitomycin, mycophenolic acid, nogalamycine, olivomycin, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycine, pyrrolobenzodiazepine, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, and analogs and derivatives thereof.

[0051] In some embodiments, the cancer therapeutic is an antimetabolite agent. Suitable antimetabolite agents include, without limitation, SN-38, denopterin, edatrexate, mercaptopurine (6-MP), methotrexate, piritrexim, pteropterin, pentostatin (2'-DCF), tomudex, trimetrexate, cladridine, fludarabine, thiamiprine, ancitabine, azacitidine, 6- azauridine, carmofur, cytarabine, doxifluridine, emitefur, floxuridine, fluorouracil, gemcitabine, tegafur, hydroxyurea, urethane, and analogs and derivatives thereof.

[0052] In some embodiments, the cancer therapeutic is an anti-proliferative drug. Suitable anti-proliferative drugs include, without limitation, aceglatone, amsacrine, bisantrene, camptothecin, defosfamide, demecolcine, diaziquone, diflomotecan, eflornithine, elliptinium acetate, etoglucid, etopside, fenretinide, gallium nitrate, hydroxyurea, lamellarin D, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracrine, pentostatin, phenamet, podophillinic acid 2-ethyl-hydrazide, procarbazine, razoxane, sobuzoxane, spirogermanium, teniposide, tenuazonic acid, triaziquone 2,2' ,2"- trichlorotriethylamine, and analogs and derivatives thereof.

[0053] In some embodiments, the cancer therapeutic is an antimitotic agent. Suitable antimitotic agents include, without limitation, auristatin, a maytansinoid, a dolastatin, a tubulysin, a taxane, a epothilone, a vinca alkaloid, and analogs and derivatives thereof. In some embodiments, the antimitotic agent is an auristatin. In some embodiments, the auristatin is monomethyl auristatin E (MMAE).

[0054] In some embodiments, the cancer therapeutic is a PROTAC. Suitable PROTACs include, without limitation BET degraders, such as that disclosed by Pillow et al., "Antibody Conjugation of a Chimeric BET Degrader Enables *In vivo* Activity," *ChemMedChem* 15(1): 17-25 (2020), which is hereby incorporated by reference in its entirety. Suitable PROTACs also include Ras degraders.

[0055] In some embodiments, the pharmaceutically active moiety of the non-binding protein-drug conjugate as described herein is an immunomodulatory agent. In some embodiments, the immunomodulatory agent is an agent that modifies the phenotype of one or more types of immune cells, *e.g.*, type-1 macrophages, type-2 macrophages, dendritic cells, neutrophils, B cells, and T cells. For example, in some embodiments, the immunomodulatory agent is an agent that modifies the phenotype of an immune cell to result in immune cell activation. In some embodiments, the immunomodulatory agent is an agent that modifies the phenotype of an immune cell to result in immune cell suppression.

[0056] In some embodiments, the immunomodulatory agent is a macrophage type-1 stimulating agent. Suitable macrophage type-1 stimulating agents include, without limitation, paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

[0057] In some embodiments, the macrophage type-1 stimulating agent is a CSF-1 receptor antagonist. Suitable CSF-1 receptor antagonists include, without limitation ABT-869 (Guo et al., "Inhibition of Phosphorylation of the Colony-Stimulating Factor-1 Receptor (c-Fms) Tyrosine Kinase in Transfected Cells by ABT-869 and Other Tyrosine Kinase Inhibitors," *Mol.*

Cancer. Ther. 5(4):1007-1012 (2006), which is hereby incorporated by reference in its entirety), imatinib (Guo et al., "Inhibition of Phosphorylation of the Colony-Stimulating Factor-1 Receptor (c-Fms) Tyrosine Kinase in Transfected Cells by ABT-869 and Other Tyrosine Kinase Inhibitors," *Mol. Cancer. Ther.* 5(4):1007-1012 (2006), which is hereby incorporated by reference in its entirety), PLX3397 (Mok et al., "Inhibition of CSF1 Receptor Improves the Anti-tumor Efficacy of Adoptive Cell Transfer Immunotherapy," *Cancer Res.* 74(1):153-161 (2014), which is hereby incorporated by reference in its entirety), PLX5622 (Dagher et al., "Colony-stimulating Factor 1 Receptor Inhibition Prevents Microglial Plaque Association and Improves Cognition in 3xTg-AD Mice," *J. Neuroinflamm.* 12:139 (2015), which is hereby incorporated by reference in its entirety), DCC-3014 (Deciphera Pharmaceuticals), BLZ945 (Krauser et al., "Phenotypic and Metabolic Investigation of a CSF-1R Kinase Receptor Inhibitor (BLZ945) and its Pharmacologically Active Metabolite," *Xenobiotica* 45(2):107-123 (2015), which is hereby incorporated by reference in its entirety), and GW2580 (Olmos-Alonso et al., "Pharmacological Targeting of CSF1R Inhibits Microglial Proliferation and Prevents the Progression of Alzheimer's-like Pathology," *Brain* 139:891-907 (2016), which is hereby incorporated by reference in its entirety.

[0058] In some embodiments, the macrophage type-1 stimulating agent is an IL-10 receptor antagonist. Suitable IL-10 receptor antagonists include, without limitation peptide antagonists as described in Naiyer et al., "Identification and Characterization of a Human IL-10 Receptor Antagonist," *Hum. Immunol.* 74(1):28-31 (2013), which is hereby incorporated by reference in its entirety, and IL-10 receptor antagonistic antibodies as described in U.S. Patent No. 7,553,932 to Von Herrath et al., which is hereby incorporated by reference in its entirety.

[0059] In some embodiments, the macrophage type-1 stimulating agent is a TLR agonist, *i.e.*, a TLR2, TLR3, TLR4, TLR7, TLR8, or TLR9 agonist. Suitable TLR-2 agonists for use in the methods described herein include Pam3CSK4, a synthetic triacylated lipoprotein, and lipoteichoic acid (LTA) (Brandt et al., "TLR2 Ligands Induce NF-κB Activation from Endosomal Compartments of Human Monocytes" *PLoS One* 8(12):e80743, which is hereby incorporated by reference in its entirety). A suitable TLR-3 agonist includes, without limitation, polyinosinic:polycytidylic acid (poly I:C) (Smole et al., "Delivery System for the Enhanced Efficiency of Immunostimulatory Nucleic Acids," *Innate Immun.* 19(1):53-65 (2013), which is hereby incorporated by reference in its entirety). Suitable TLR-4 agonists include, without limitation, MPL (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety), Glucopyranosyl Lipid-A (Matzner et al., "Perioperative treatment with the new synthetic TLR-4 agonist GLA-SE reduces cancer

metastasis without adverse effects," *Int. J. Cancer* 138(7):1754-64 (2016), which is hereby incorporated by reference in its entirety), and Immunomax® (Ghochikyan et al., "Targeting TLR-4 with a novel pharmaceutical grade plant derived agonist, Immunomax®, as a therapeutic strategy for metastatic breast cancer," *J. Trans. Med.* 12:322 (2014), which is hereby incorporated by reference in its entirety)

[0060] Suitable TLR-7 agonists include, without limitation, uridine/guanidine-rich single-stranded RNA (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety), 852A (Dudek et al., "First in Human Phase I Trial of 852A, a Novel Systemic Toll-like Receptor 7 Agonist, to Activate Innate Immune Responses in Patients With Advanced Cancer," *Clin. Cancer Res.* 13(23):7119-7125 (2007), which is hereby incorporated by reference in its entirety), resiquimod (Chang et al., "Topical resiquimod Promotes Priming of CTL to Parenteral Antigens," *Vaccine* 27(42):5791-5799 (2009), which is hereby incorporated by reference in its entirety), imidazoquinolines (Itoh et al., "The Clathrin-mediated Endocytic Pathway Participates in dsRNA-induced IFN-beta Production," *J. Immunol.* 181:5522-9 (2008), which is hereby incorporated by reference in its entirety), ANA975 (Fletcher et al., "Masked oral Prodrugs of Toll-like Receptor 7 Agonists: a New Approach for the Treatment of Infectious Disease," *Curr. Opin. Investig. Drugs* 7(8):702-708 (2006), which is hereby incorporated by reference in its entirety), and imiquimod (Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety).

[0061] Suitable TLR-8 agonists include, without limitation, resiquimod (Chang et al., "Topical resiquimod Promotes Priming of CTL to Parenteral Antigens," *Vaccine* 27(42):5791-5799 (2009), which is hereby incorporated by reference in its entirety), and imidazoquinolines (Itoh et al., "The Clathrin-mediated Endocytic Pathway Participates in dsRNA-induced IFN-beta Production," *J. Immunol.* 181:5522-9 (2008), which is hereby incorporated by reference in its entirety),

[0062] Suitable TLR-9 agonists include, without limitation, CpG-ODN (Yao et al., "Late Endosome/Lysosome-localized Rab7b Suppresses TLR-9-initiated Proinflammatory Cytokine and Type I IFN Production in Macrophages," *J. Immunol.* 183:1751-8 (2009), which is hereby incorporated by reference in its entirety). Specific CpG-ODNs suitable for use are described in Engel et al., "The Pharmacokinetics of Toll-like Receptor Agonists and the Impact on the Immune System," *Expert Rev. Clin. Pharmacol.* 4(2):275-289 (2011), which is hereby incorporated by reference in its entirety.

[0063] Other agents known in the art to reprogram type-2 macrophages to type-1 macrophages (*i.e.*, macrophage type-1 stimulating agent) include, manganese dioxide nanoparticles (*see e.g.*, Song et al., "Bioconjugated Manganese Dioxide Nanoparticles Enhance Chemotherapy Response by Priming Tumor-Associated Macrophages toward M1-like Phenotype and Attenuating Tumor Hypoxia" *ACS Nano.* 10:633–647 (2016), which is hereby incorporated by reference in its entirety), ferumoxytal nanoparticles (Zanganeh, et al. "Iron oxide nanoparticles inhibit tumour growth by inducing pro-inflammatory macrophage polarization in tumour tissues," *Nat. Nanotechnol.* 11:986–994 (2016), which is hereby incorporated by reference in its entirety), mannosylated nanoparticles encapsulating siRNA against IκBα (Ortega et al. "Manipulating the NF-kappaB pathway in macrophages using mannosylated, siRNA-delivering nanoparticles can induce immunostimulatory and tumor cytotoxic functions," *Int. J. Nanomed.* 2163–2177 (2016), which is hereby incorporated by reference in its entirety). In accordance with the present disclosure, these agents can be coupled to a non-binding FN3 domain described herein to form a non-binding protein-drug conjugate.

[0064] In some embodiments, the immunomodulatory agent is a macrophage type-2 stimulating agent. Suitable macrophage type-2 stimulating agents include, without limitation, IL-33, IL-4 receptor agonists, glucocorticoids, IL-10 receptor agonists, and IL-1 receptor agonists.

[0065] Suitable IL-4 receptor agonists include, without limitation, mutant IL-4 proteins. Exemplary mutant IL-4 proteins include, but are not limited to those described in U.S. Patent No. 5,723,118 to Sebald, which is hereby incorporated by reference in its entirety.

[0066] Glucocorticoids are a class of corticosteroids, which are well known in the art and suitable for inducing a macrophage type-2 phenotype. Exemplary glucocorticoids for incorporation into the non-binding protein-drug conjugate of the present disclosure include, without limitation, cortisol, cortisone, prednisone, prednisolone, methylprednisolone, dexamethasone, betamethasone, triamcinolone, beclomethasone, fludrocortisone, deoxycorticosterone, and aldosterone.

[0067] IL-10 receptor agonists are also capable of inducing a macrophage type-2 phenotype in accordance with the conjugates and methods described herein. Suitable IL-10 receptor agonists include, without limitation, mutant IL-10 proteins as described in U.S. Patent No. 7,749,490 to Sommer et al., which is hereby incorporated by reference in its entirety.

[0068] IL-1 receptor agonists are also capable of inducing a macrophage type-2 phenotype and, therefore, can be incorporated as the drug component of the non-binding protein-conjugates described herein. Suitable IL-1 receptor agonists include, without limitation, IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, and IL-36γ (Palomo et al., "The Interleukin (IL)-1 Cytokine

Family- Balance Between Agonists and Antagonists in Inflammatory Diseases," *Cytokine* 76(1):25-37 (2015), which is hereby incorporated by reference in its entirety).

[0069] In some embodiments, the immunomodulatory agent is a macrophage type-2 depleting agent. Suitable macrophage depleting agents include, without limitation clodronate, zoledronic acid, alendronate, and trabectedin.

[0070] In some embodiments, the immunomodulatory agent is a T cell stimulating agent. Suitable T cell stimulating agents include, without limitation stimulator of interferon genes (STING) agonists. STING agonists include, without limitation, cyclic dinucleotides (CDNs), such as cyclic dimeric guanosine monophosphate (c-di-GMP), cyclic dimeric adenosine monophosphate (c-di-AMP), cyclic GMP-AMP (cGAMP), and dithio-(R_P,R_P)-[cyclic[A(2',5')pA(3',5')p (ADU-S100, Aduro Biotech) and small molecules, such as 5,6-dimethylxanthenone-4-acetic acid (DMXAA) and linked amidobenzimidazole. Other STING agonists under development that are also suitable immunomodulatory agents in accordance with the present disclosure include BMS-986301, E7766, GSK3745417, MK-1454, MK-2118, and SB11285.

[0071] In some embodiments, the immunomodulatory agent is a dendritic cell stimulating agent. Suitable dendritic cell stimulating agents for inclusion in the non-binding protein-drug conjugate as described herein include, without limitation, CpG oligonucleotide, imiquimod, topoisomerase I inhibitors (*e.g.,* camptothecin and derivatives thereof), microtubule depolymerizing drugs (*e.g.*, colchicine, podophyllotoxin, and derivatives thereof).

[0072] In some embodiments, the immunomodulatory agent is a neutrophil stimulating agent. Suitable neutrophil stimulating agents include, without limitation, recombinant granulocyte colony stimulating factor protein (filgrastim) or a pegylated recombinant granulocyte colony stimulating factor protein.

[0073] In some embodiments, the pharmaceutically active moiety of the non-binding protein-drug conjugate of the present disclosure is an oligonucleotide. Suitable oligonucleotides include, without limitation, an siRNA, an aptamer, an miRNA, an immunostimulatory oligonucleotide, a splice-switching oligonucleotide, and guide RNA.

[0074] In some embodiments, the pharmaceutically active moiety of the non-binding protein-drug conjugate of the present disclosure is a wound healing agent. Suitable wound healing agents in accordance with this aspect of the disclosure include, without limitation, an agent that stimulates a proinflammatory phenotype of an immune cell. In some embodiments, the pharmaceutically active moiety for the treatment of wound healing is a macrophage type-1 stimulating agent as described *supra*.

[0075] In some embodiments, the pharmaceutically active moiety of the non-binding protein-drug conjugate of the present disclosure is a therapeutic moiety suitable for treating a neurodegenerative disease. Exemplary neurodegenerative diseases include, without limitation, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, and analogs and derivatives thereof.

[0076] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an ALS therapeutic suitable for treating ALS in a subject. Suitable ALS therapeutics include, without limitation, glutamate blockers (*e.g.* Riluzole, Rilutek, and other derivatives), Endaravone, Radicava, muscle relaxants (*e.g.* Baclofen, Tizanidine, and other derivatives), and analogs and derivatives thereof.

[0077] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is a Parkinson's disease therapeutic to treat Parkinson's disease in a subject. Suitable therapeutics to treat Parkinson's disease include, without limitation, dopamine promoters (*e.g.*, Carbidopa, Levodopa, Carbidopa-levodopa, Entacapone, Cabergoline, Tolcapone, Bromocriptine, Amantadine, and other derivatives), dopamine agonists (*e.g.* pramipexole, Mirapex, ropinirole, Requip, rotigotine, Neupro, apomorphine, Apokyn), cognition-enhancing medication (Rivastigmine, and other derivatives), anti-tremor drugs (*e.g.* Benzotropine, and other derivatives), MAO B inhibitors (selegiline, Zelapar, rasagiline, Azilect, safinamide, Xadago, and other derivatives), catechol O-methyl transferase (COMT) inhibitors (*e.g.* entacapone, Comtan, opicapone, Ongentys, tolcapone, Tasmar, anticholinergics (*e.g.* benzotropine, Cogentin, trihexyphenidyl, and other derivatives), and analogs and combinations thereof.

[0078] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is a Huntington's disease therapeutic to treat Huntington's disease in a subject. Suitable therapeutics to treat symptoms of Huntington's disease include, without limitation, movement controlling drugs (*e.g.* tetrabenazine, Xenazine, deutetrabenazine, Austedo, and other derivatives) antipsychotic drugs (*e.g.* haloperidol, Haldol, fluphenazine, risperidone, Risperdal, olanzapine, Zyprexa, quetiapine, Seroquel, and other derivatives), chorea suppressants (*e.g.* amantadine, Gocovri ER, Osmolex ER, levetiracetam, Keppra, Elepsia XR, Spritam, clonazepam, Klonopin, and other derivatives), and analogs and derivatives thereof.

[0079] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an Alzheimer's disease therapeutic to treat Alzheimer's disease in a subject. Suitable therapeutics to treat Alzheimer's disease include, without limitation, cognition-enhancing medication (*e.g.* memantine, Namenda, and other derivatives), cholinesterase

inhibitors (*e.g.* donepezil, Aricept, galantamine, Razadyne, rivastigmine, Exelon, and other derivatives), aducanumab, Aduhelm, and analogs and derivatives thereof.

[0080] In some embodiments, the pharmaceutically active moiety of the non-binding protein-drug conjugate of the present disclosure is a therapeutic moiety suitable for treating an inflammatory condition. Exemplary inflammatory conditions that can be treated utilizing this non-binding protein-drug conjugate include, without limitation, rheumatoid arthritis, atherosclerosis, macular degeneration, osteoporosis, immune inflammation, non-immune inflammation, renal inflammation, tuberculosis, multiple sclerosis, arthritis, chronic obstructive pulmonary disease (COPD), and Alzheimer's disease,

[0081] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is a nonsteroidal anti-inflammatory drug (NSAID) (*e.g.* ibuprofen, Advil, Motrin IB, naproxen sodium, Aleve, and other derivatives), a corticosteroid medication (*e.g.* prednisone and other derivatives), a conventional disease-modifying antirheumatic drug (DMARDs) (*e.g.* methotrexate, Trexall, Otrexup, leflunomide, Arava, hydroxychloroquine, Plaquenil, sulfasalazine Azulfidine, and other derivatives), a biologic DMARD (abatacept, Orencia, adalimumab, Humira, anakinra, Kineret, certolizumab, Cimzia, etanercept, Enbrel, golimumab, Simponi, infliximab, Remicade, rituximab, Rituxan, sarilumab, Kevzara, tocilizumab, Actemra, and other derivatives), a targeted synthetic DMARD (*e.g.*baricitinib, Olumiant, tofacitinib, Xeljanz, upadacitinib, Rinvoq, and other derivatives), and analogs and derivatives thereof.

[0082] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an anti-inflammatory therapeutic selected from statins (*e.g.* Atorvastatin, Lovastatin, Simvastatin, Pravastatin, and other derivatives) and other cholesterol medications (*e.g.* exetimibe, Zetia, Fenofibrate, Gemfibrozil, and other derivatives), anticoagulants (*e.g.* aspirin and other derivatives), blood thinners, and analogs and derivatives thereof.

[0083] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is a therapeutic is suitable for treating a bone condition. Suitable bone conditions that can be treated using this non-binding protein-drug conjugate include, for example, osteoporosis and Paget's Bone disease.

[0084] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an osteoporosis therapeutic suitable for treating osteoporosis in a subject. Suitable osteoporosis therapeutics include, without limitation, bisphosphonates (*e.g.* Alendronate, Binosto, Fosamax, Ibandronate, Boniva, Risedronate, Actonel, Atelvia, Zoledronic acid, Reclast, Zometa, and other derivatives), denosumab (*e.g.* Prolia, Xgeva, and other

derivatives), hormone-related therapy (*e.g.* estrogen, raloxifene, Evista, testosterone, and other derivatives), bone-building medications (*e.g.* Teriparatide, Bonsity, Forteo, Abaloparatide, Tymlos, Romosozumab, Evenity, and other derivatives), and analogs and derivatives thereof.

[0085] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is a Paget's bone disease therapeutic suitable for treating Paget's bone disease a subject. Suitable therapeutics for Paget's Bone disease include, without limitation, bisphosphonates (*e.g.* Zoledronic acid, Reclast, Zometa, Pamidronate, Aredia, Ibandronate, Boniva, and other derivatives), and oral bisphosphonates (*e.g.* Alendronate, Binosto, Risedronate, Actonel, Atelvia, and other derivatives), and analogs and derivatives thereof.

[0086] In any embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an anti-viral therapeutic suitable for treating infectious disease in a subject. Suitable anti-viral therapeutics include, without limitation, oseltamivir, zanamivir, peramivir, baloxavir, penciclovir, Abacavir, Acyclovir, Adefovir, Amantadine, Amprenavir, Umifenovir, Atazanavir, Atripla, Baloxavir marboxil, Biktarvy, Boceprevir, Bulevirtide, Cidofovir, Combivir, Daclatasvir, Darunavir, Delavirdine, Descovy, Docosanol, Dolutegravir, Ibacitabine, Idoxuridine, Imiquimod, Imunovir, Letermovir, Lopinavir, Maraviroc, Methisazone, Moroxydine, Nelfinavir, Nitazoxanide, Oseltamivir, Remdesivir, Ribavirin, Rimantadine, Ritonavir, Saquinavir, Sofosbuvir, Tipranavir, Valaciclovir, Vicriviroc, and Zanamivir,

[0087] In any embodiments, the subject having the infectious disease has a filovirus. In some embodiments, the filovirus is ebola virus or Marburg virus. Ebola and other filoviruses attach and enter a host cell via endocytosis. The internalized virus is localized in late endosomes/lysosomes and is cleaved by cysteine proteases. In accordance with this embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an anti-viral therapeutic suitable for treating filovirus in a subject. Suitable anti-viral therapeutics include, without limitation, remdesivir, mAb114 (Kugelman et al. "Emergence of Ebola virus escape variants in infected nonhuman primates treated with the MB-003 antibody cocktail," *Cell Rep* 2015; 12: 2111–20, which is hereby incorporated by reference in its entirety), REGN-EB3 (Pascal et al. "Development of clinical-stage human monoclonal antibodies that treat advanced Ebola virus disease in nonhuman primates," *J Infect Dis* 2018; 218 (suppl 5): S612–26, which is hereby incorporated by reference in its entirety), and ZMapp (Qiu et al. "Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp," *Nature* 2014; 514: 47–53, which is hereby incorporated by reference in its entirety)

[0088] In some embodiments, the subject having the infectious disease has a coronavirus. In some embodiments, the coronavirus is Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) or Middle East Respiratory Syndrome Coronavirus (MERS-

CoV). In accordance with this embodiment, the pharmaceutically active moiety of the non-binding protein-drug conjugate is an anti-viral therapeutic suitable for treating coronavirus in a subject. Suitable anti-viral therapeutics include, without limitation, chloroquine, hydroxychloroquine, ivermectin, remdesivir, baricitinib,and paxlovid.

[0089] Accordingly, the NPC1 binding molecules described herein can be administered to a subject that has or is at risk of having a coronavirus infection as a therapeutic means of inhibiting infection, inhibiting the progression of infection, and/or decreasing infection in the subject.

[0090] In some embodiments, the second portion of the non-binding protein-drug conjugate of the present disclosure is a diagnostic moiety. Suitable diagnostic moieties are those that facilitate the detection, quantitation, separation, and/or purification of the non-binding protein-drug conjugate. Suitable diagnostic moieties include, without limitation, purification tags (e.g., poly-histidine (His_{6}–), glutathione-S-transferase (GST–), maltose-binding protein (MBP–)), fluorescent dyes or tags (e.g., chelates (europium chelates), fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red, an enzymatic tag, a radioisotope or radioactive label (e.g., ^{4}C, ^{11}C, ^{14}N, ^{35}S, ^{3}H, ^{32}P, ^{99m}Tc, ^{111}In, $^{62/64}Cu$, ^{125}I, ^{18}F, $^{67/68}Ga$, ^{90}Y, ^{177}Lu and $^{186/188}Re$), a radionucleotide with chelator (*e.g.*, MAG3, DTPA, and DOTA, *see also*, Liu S., "Bifunctional Coupling Agents for Radiolabeling of Biomolecules and Target Specific Delivery of Metallic Radionuclides," *Adv. Drug Deli. Ref.* 60(12):1347-1370 (2008), which is hereby incorporated by reference in its entirety), a microbubble (Abou-Elkacem et al., "Ultrasound molecular imaging of the breast cancer neovasculature using engineered fibronectin scaffold ligands: A novel class of targeted contrast ultrasound agent," *Theranostics* 6:1740–1752 (2016), which is hereby incorporated by reference in its entirety), a contrast agent suitable for imaging, or a photosensitize.

[0091] In some embodiments, the diagnostic moiety is a radiolabel, radionuclide or radioisotope bound to a chelating agent. Particularly useful diagnostic radiolabels, radionuclides, or radioisotopes that can be bound to a chelating agent include, without limitation ^{110}In, ^{m}In, ^{177}Lu, ^{18}F, ^{52}Fe, ^{62}Cu, ^{64}Cu, ^{67}Cu, ^{67}Ga, ^{68}Ga, ^{86}Y, ^{9}V, ^{89}Zr, ^{94}Tc, ^{94}Tc, ^{99m}Tc, ^{120}I, ^{123}I, ^{124}I, ^{125}I, ^{131}I, 154Gd, ^{158}Gd, ^{32}P, ^{n}C, ^{13}N, ^{15}O, ^{186}Re, ^{188}Re, ^{51}Mn, ^{52m}Mn, ^{55}Co, ^{72}As, ^{75}Br, ^{76}Br, ^{82m}Rb, ^{83}Sr, or other gamma-, beta-, or positron-emitters. The diagnostic radiolabels include a decay energy in the range of 25 to 10,000 keV, more preferably in the range of 25 to 4,000 keV, and even more preferably in the range of 20 to 1 ,000 keV, and still more preferably in the range of 70 to 700 keV. Total decay energies of useful positron- emitting radionuclides are preferably <2,000 keV, more preferably under 1,000 keV, and most preferably <700 keV.

[0092] Chelators such as NOTA (1, 4, 7-triaza-cyclononane-N,N',N"-triacetic acid), DOTA (1, 4, 7, 10-tetraazacyclododecane-l, 4, 7, 10-tetraacetic acid), DTP A (1, 1, 4, 7, 7-Diethylenetriaminepentaacetic acid), TETA (p-bromoacetamido-benzyl-tetraethylaminetetraacetic acid), and Df (desferrioxamine B) are of use with a variety of radiolabels, radionuclides, radioisotopes, metals and radiometals. DOTA-type chelators, where the ligand includes hard base chelating functions such as carboxylate or amine groups, are most effective for chelating hard acid cations. Such metal-chelate complexes can be made very stable by tailoring the ring size to the metal of interest. Also, more than one type of chelator may be conjugated to the targetable construct to bind multiple metal ions, e.g., diagnostic radionuclides and/or therapeutic radionuclides.

[0093] Chelators are covalently bound to the non-binding FN3 domain of the conjugate using standard methods of bioconjugation. Amine containing residues (*e.g.*, lysine) in the FN3 domain undergo amide bond formation with a chelator containing an activated ester (e.g., an N-hydroxysuccinimidyl ester). Sulfur containing residues (*e.g.*, cysteine) undergo conjugation with chelators containing an activated ester or maleimide moiety. Alternatively, bioconjugates are formed when activated carboxylate residues of the FN3 domain undergo amide or thoiester formation with amine or thiol groups, respectively, on the chelator. Bifunctional linkers, such as, for example, PEG-maleimide (PEG-Mal), succinimidyl-4-(N-maleimidomethyl)cyclohexane-l -carboxylate (SMCC) or N-succinimidyl 3-(2-pyridylthio)propionate (SPDP) can be alternatively used.

[0094] In some embodiments, the non-binding protein-drug conjugate of the present disclosure further comprises a third portion. In some embodiments, the third portion of the protein-drug conjugate of the present disclosure comprises a half-life extending moiety. Exemplary half-life extending moieties include, without limitation, albumin, albumin variants (*see e.g.*, U.S. Patent Nos. 8,822,417 to Andersen et al., U.S. Patent No. 8,314,156 to Desai et al., and U.S. Patent No. 8,748,380 to Plumridge et al., which are hereby incorporated by reference in their entirety), albumin-binding proteins and/or domains, transferrin and fragments and analogues thereof (*see e.g.*, U.S. Patent No. 7,176,278 to Prior et al., which are hereby incorporated by reference in their entirety), Fc regions and variant Fc regions (*see e.g.*, U.S. Patent No. 8,546,543 to Lazar et al., U.S. Patent Publication No. 20150125444 to Tsui, and U.S. Patent No. 8,722,615 to Seehra et al., which are hereby incorporated by reference in their entirety).

[0095] Other half-life extending moieties of the non-binding protein-drug conjugate include, without limitation, polyethylene glycol (PEG) molecules, such as PEG5000 or PEG20,000, fatty acids and fatty acid esters of different chain lengths, for example laurate,

myristate, stearate, arachidate, behenate, oleate, arachidonate, octanedioic acid, tetradecanedioic acid, octadecanedioic acid, docosanedioic acid, and the like, polylysine, octane, carbohydrates (dextran, cellulose, oligo- or polysaccharides) for desired properties. A pegyl moiety may for example be added to the first portion, *i.e.*, the non-binding FN3 portion, by adding a cysteine residue to the C-terminus of the molecule and attaching a pegyl group to the cysteine using methods well known in the art.

[0096] For therapeutic or diagnostic use, the non-binding protein-drug conjugates as described herein are prepared as pharmaceutical or diagnostic compositions containing an effective amount of the protein-drug conjugate as an active ingredient in a pharmaceutically acceptable carrier. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the active compound is administered. Such vehicles can be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. For example, 0.4% saline and 0.3% glycine can be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating and coloring agents, etc. The concentration of non-binding protein-drug conjugate as described herein in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on required dose, fluid volumes, viscosities, etc., according to the particular mode of administration selected. Suitable vehicles and formulations, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in e.g. REMINGTON: THE SCIENCE AND PRACTICE OF PHARMACY, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, *see* especially pp. 958-989, which is hereby incorporated by reference in its entirety.

[0097] The non-binding protein-drug conjugates described herein can be used in non-isolated or isolated form. Furthermore, the non-binding protein-drug conjugates described herein can be used alone or in a mixture comprising at least one other non-binding protein-drug conjugate as described herein. In other words, the non-binding protein-drug conjugates can be used in combination, e.g., as a pharmaceutical composition comprising two or more non-binding protein-drug conjugates. For example, non-binding protein-drug conjugates having different, but complementary activities can be combined in a single therapy to achieve a desired therapeutic effect, but alternatively, non-binding protein-drug conjugates having identical activities can also

be combined in a single therapy to achieve a desired therapeutic or diagnostic effect. Optionally, the mixture further comprises at least one other therapeutic agent.

[0098] Another aspect of the present disclosure relates to a method of treating cancer in a subject. This method involves administering to the subject having cancer a non-binding protein-drug conjugate as described herein or pharmaceutical composition comprising the non-binding protein-drug conjugate to the subject in an amount effective to treat the cancer.

[0099] In accordance with all of the methods described herein a "subject" refers to any animal. In some embodiments, the subject is a mammal. Exemplary mammalian subjects include, without limitation, humans, non-human primates, dogs, cats, rodents (*e.g.*, mouse, rat, guinea pig), horses, cattle and cows, sheep, and pigs. In some embodiments, the subject is a human.

[0100] In some embodiments, the subject has a type of cancer that is characterized by cancerous cells having enhanced macropinocytosis relative to their corresponding non-cancerous cells. In some embodiments, the cancer is characterized by cancerous cells having an oncogenic mutation in ras, *i.e.*, an oncogenic mutation in H-ras, N-ras, or K-ras. In some embodiments, the subject has a cancer selected from pancreatic cancer, lung cancer, breast cancer, colon cancer, glioma, solid tumor, melanoma, glioblastoma multiforme, leukemia, renal cell carcinoma, hepatocellular carcinoma, prostate cancer, and myeloma.

[0101] In some embodiments the subject has a type of cancer that is or has become resistant to primary cancer therapeutic treatment, e.g., resistant to chemotherapy treatment, prior to administering the non-binding protein-drug conjugate or pharmaceutical composition comprising the same. Administering the non-binding protein-drug conjugate comprising a cancer therapeutic or pharmaceutical composition comprising the same is carried out in an amount effective to directly target and kill cancerous cells. Suitable non-binding protein-drug conjugates comprising a cancer therapeutic, e.g., an antimetabolite, an alkaloid, an alkylating agent, an anti-mitotic agent, an antitumor antibiotic, a DNA binding drug, a microtubule targeting drug, a toxin, an antiproliferative drug, a DNA antagonist, radionuclide, a thermoablative agent or a PROTAC are described *supra.*

[0102] In some embodiments, the subject has a type of cancer that is or has become immune tolerant. Administering the non-binding protein-drug conjugate comprising an immunomodulatory agent or pharmaceutical composition comprising the same is carried out in an amount effective to enhance the antitumor immune response. Suitable non-binding protein-drug conjugates comprising an immunomodulatory agent, e.g., a macrophage type-1 stimulating agent, a macrophage type-2 depleting agent, a T cell stimulating agent, a dendritic cell stimulating agent, and/or a neutrophil stimulating agent are described *supra.*

[0103] In some embodiments, the method of treating a subject having cancer further involves administering an additional cancer therapeutic in conjunction with the non-binding protein-drug conjugate, or pharmaceutical composition comprising the same. Suitable cancer therapeutics that can be administered in combination with the non-binding protein-drug conjugates described herein as a combination therapy include, for example and without limitation, chemotherapeutic agents. Suitable chemotherapeutics include, without limitation, alkylating agents (*e.g.*, chlorambucil, cyclophophamide, CCNU, melphalan, procarbazine, thiotepa, BCNU, and busulfan), antimetabolites (*e.g.*, methotraxate, 6-mercaptopurine, and 5-fluorouracil), anthracyclines (daunorubicin, doxorubicin, idarubicin, epirubicin, and mitoxantrone), antitumor antibiotics (*e.g.*, bleomycin, monoclonal antibodies (*e.g.*, Alemtuzumab, Bevacizumab, Cetuximab, Gemtuzumab, Ibritumomab, Panitumumab, Rituximab, Tositumomab, and Trastuxmab), platiniums (*e.g.*, cisplatin and oxaliplatin) or plant alkaloids (*e.g.*, topoisomerase inhibitors, vinca alkaloids, taxanes (*e.g.* paclitaxel), and epipodophyllotoxins). In some embodiments, the cancer chemotherapeutic is selected from cyclophosphamide, gemcitabine, vorinostat, temozolomide, bortezomib, carmustine, and paclitaxel.

[0104] In accordance with the methods described herein, administration of the non-binding protein-drug conjugates or pharmaceutical composition comprising the same, alone or in combination with one or more additional cancer therapeutics, is carried out by systemic or local administration. Suitable modes of systemic administration of the protein-drug conjugates and/or combination therapeutics disclosed herein include, without limitation, orally, topically, transdermally, parenterally, intradermally, intrapulmonary, intramuscularly, intraperitoneally, intravenously, subcutaneously, or by intranasal instillation, by intracavitary or intravesical instillation, intraocularly, intra-arterially, intralesionally, or by application to mucous membranes. In certain embodiments, the therapeutic agents of the methods described herein are delivered orally. Suitable modes of local administration of the therapeutic agents and/or combinations disclosed herein include, without limitation, catheterization, implantation, direct injection, dermal/transdermal application, or portal vein administration to relevant tissues, or by any other local administration technique, method or procedure generally known in the art. The mode of affecting delivery of agent will vary depending on the type of cancer therapeutic being delivered and the type of cancer to be treated.

[0105] A therapeutically effective amount of the non-binding protein-drug conjugate or a pharmaceutical composition comprising the same, alone or in combination with an additional cancer therapeutic, in the methods disclosed herein is an amount that, when administered over a particular time interval, results in achievement of one or more therapeutic

benchmarks (*e.g.*, slowing or halting of tumor growth, tumor regression, cessation of symptoms, etc.). The non-binding protein-drug conjugate or a pharmaceutical composition comprising the same for use in the presently disclosed methods may be administered to a subject one time or multiple times. In those embodiments where the therapeutic composition is administered multiple times, it may be administered at a set interval, *e.g.*, daily, every other day, weekly, or monthly. Alternatively, it can be administered at an irregular interval, for example on an as-needed basis based on symptoms, patient health, and the like. For example, a therapeutically effective amount may be administered once a day (q.d.) for one day, at least 2 days, at least 3 days, at least 4 days, at least 5 days, at least 6 days, at least 7 days, at least 10 days, or at least 15 days. Optionally, the status of the cancer or the regression of the cancer is monitored during or after the treatment, for example, by a multiparametric ultrasound (mpUS), multiparametric magnetic resonance imaging (mpMRI), and nuclear imaging (positron emission tomography [PET]) of the subject. The dosage of the non-binding protein-drug conjugate or combination therapy administered to the subject can be increased or decreased depending on the status of the cancer or the regression of the cancer detected.

[0106] The skilled artisan can readily determine this amount, on either an individual subject basis (*e.g.*, the amount of a non-binding protein-drug conjugate necessary to achieve a particular therapeutic benchmark in the subject being treated) or a population basis (*e.g.*, the amount of non-binding protein-drug conjugate necessary to achieve a particular therapeutic benchmark in the average subject from a given population). Ideally, the therapeutically effective amount does not exceed the maximum tolerated dosage at which 50% or more of treated subjects experience side effects that prevent further drug administrations.

[0107] A therapeutically effective amount may vary for a subject depending on a variety of factors, including variety and extent of the symptoms, sex, age, body weight, or general health of the subject, administration mode and salt or solvate type, variation in susceptibility to the drug, the specific type of the disease, and the like.

[0108] Another aspect of the present disclosure is directed to a method of modulating a subject's immune response. This method involves administering to the subject having a condition that would benefit from immune system modulation, a non-binding protein-drug conjugate as described herein in an amount effective to modulate the subject's immune response. In accordance with this aspect of the disclosure, suitable non-binding protein-drug conjugates include those conjugates comprising an immunomodulatory agent as the second portion of the conjugate. Suitable immunomodulatory agents, *e.g.*, type-1 macrophage stimulating agents, type-2 macrophage stimulating agents, T cell stimulating agents, dendritic cell stimulating agents, and neutrophil stimulating agents, are all described *supra*.

[0109] Modulating or modifying a subject's immune response in accordance with this aspect of the disclosure is carried out for the purpose of treating, preventing, or slowing the progression of a disease or condition that is caused or exacerbated, at least in part, by the immune response and/or cells of the immune system, e.g., type-1 macrophages, type-2 macrophages, T cells, B cells, dendritic cells, neutrophils. For example, inflammatory diseases and conditions, including but not limited to macular degeneration, atherosclerosis, osteoporosis, immune inflammation, non-immune inflammation, renal inflammation, tuberculosis, multiple sclerosis, arthritis, chronic obstructive pulmonary disease (COPD), and Alzheimer's disease, involve the undesired actions of type-1 macrophages. Employing the methods of the present invention to induce a macrophage type-2 phenotypic change in the type-1 pro-inflammatory macrophages that are involved in or contributing to these disease processes alleviates one or more symptoms or causes of the disease. Accordingly, in one embodiment, the administering is carried out *in vivo* or *ex vivo* to a population of type-1 macrophages in or from a subject having an inflammatory or autoimmune condition, including, but not limited to any of those enumerated above. Administering a type-2 macrophage stimulating agent to a population of type-1 macrophages in this context will induce a type-2 phenotypic change, thereby reducing the undesired actions of the type-1 macrophages associated with the disease.

[0110] Modulating or modifying immune cell phenotype is also therapeutically beneficial in context of treating various forms of cancer. Recent studies indicate that tumor-associated macrophages (TAMs) exhibit a macrophage type-2-like phenotype. These type-2 macrophages are important tumor-infiltrating cells and play pivotal roles in tumor growth and metastasis. In most solid tumors, the existence of TAMs is advantageous for tumor growth and metastasis. These TAMs produce interleukin IL-10 and transforming growth factor (TGF) β to suppress general antitumor immune responses. Meanwhile, TAMs promote tumor neo-angiogenesis by the secretion of pro-angiogenic factors and define the invasive microenvironment to facilitate tumor metastasis and dissemination. Therefore, employing the methods of the present disclosure to administer a non-binding protein-drug conjugate comprising a macrophage type-1 stimulating agent to induce a type-1 phenotypic change in the TAMs to enhance anti-tumor immunity will significantly alter the progression of the cancer. Cancers that typically have a type-2 macrophage-related component include, without limitation, pancreatic cancer, breast cancer, and non-small cell lung cancer. Alternatively, or in conjunction with administering a non-binding protein-drug conjugate comprising a macrophage type-1 stimulating agent, a non-binding protein-drug conjugate comprising a T cell stimulating agent, a dendritic cell simulating agent, or a neutrophil stimulating agent can be administered to activate or enhance the antitumor immune response.

[0111] In some embodiments, the subject in need of immune system modulation is a subject suffering from an interferonopathy. As referred to herein, an interferonopathy is a condition involving the enhanced expression of type I interferons, *e.g.*, IFN-α, IFN-β, and IFN-Ω. Interferonopathies that can be treated in accordance with the present disclosure include, without limitation, Aicardi-Goutieres syndrome, Cree encephalitis, systemic lupus erythematosus, rheumatoid arthritis, Sjögrens syndrome, dermatomyositis, multiple sclerosis, spondyloenchondrodysplasia with immune dysregulation, stimulator of interferon genes (STING)-associated vasculopathy with onset in infancy (SAVI), Japanese autoinflammatory syndrome with lipodystrophy (JASL), ubiquitin-specific peptidase 18 deficiency, chronic atypical neutrophilic dermatitis with lipodystrophy, DNA II deficiency, Singleton-Merten syndrome, and chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature (CANDLE). Non-binding protein-drug conjugates according to the present disclosure suitable for treating an interferonopathy comprise the non-binding FN3 domain coupled to a type I interferon antagonist. In some embodiments, the type I interferon antagonist is a type I interferon receptor antagonist, such as a janus kinase (JAK) inhibitor. Suitable JAK inhibitors include, JAK1/JAK2 inhibitors, such as and without limitation baricitinib (CAS No. 1187594-09-7), tofacitinib (CAS No. 477600-75-2), ruxolitinib (941678-49-5), AG490 (Tyrphostin family) (CAS No. 133550-30-8), Lestaurtinib (CEP-701; CAS No. 111358-88-4), WP-1034 (Tyrphostin family; CAS No. 857064- 42-7), BMS-911543 (CAS No. 1271022-90-2), Fedratinib (TG101348; CAS No. 936091-26-8), Parcritinib (SB1518; CAS No. 937272-79-2), and Momelotinib (CYT387; CAS No. 1056634-68-4).

[0112] In some embodiments, the subject in need of immune system modulation is a subject having a wound or undergoing wound healing. In accordance with this embodiment, the subject having a wound or in need of wound healing is administered a non-binding protein-drug conjugate comprising a macrophage type-1 stimulating agent. Suitable type-1 stimulating agents, e.g., paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist, are described *supra*.

[0113] In some embodiments, the subject in need of immune system modulation is a subject having an inflammatory condition and the non-binding protein-drug conjugate comprises a macrophage depleting agent. Suitable inflammatory conditions that can be treated with a macrophage depleting agent, e.g., clodronate, zoledronic acid, and trabectedin, include, without limitation, rheumatoid arthritis, obesity and obesity related complications, endometriosis, inflammatory conditions of the lung (*e.g.*, chronic obstructive pulmonary disease and pulmonary tuberculosis).

[0114] Another aspect of the present disclosure relates to a method of treating a neurodegenerative condition in a subject. This method involves administering to the subject having the neurodegenerative condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the neurodegenerative condition. Exemplary neurodegenerative diseases (*e.g.*, amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease) and non-binding protein-drug conjugates suitable for treating these conditions are disclosed *supra*.

[0115] Another aspect of the present disclosure relates to a method of treating an inflammatory condition in a subject. This method involves administering to the subject having the inflammatory condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the inflammatory condition. Exemplary inflammatory conditions that can be treated in accordance with this method include, without limitation, rheumatoid arthritis, atherosclerosis, macular degeneration, osteoporosis, immune inflammation, non-immune inflammation, renal inflammation, tuberculosis, multiple sclerosis, arthritis, chronic obstructive pulmonary disease (COPD), and Alzheimer's disease. Suitable non-binding protein-drug conjugates comprising pharmaceutically active moieties for treating these conditions are disclosed *supra*.

[0116] Another aspect of the present disclosure relates to a method of treating a bone condition in a subject. This method involves administering to the subject having the bone condition a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the bone condition. Exemplary bone conditions include, without limitation, osteoporosis and Paget's bone disease, and non-binding protein-drug conjugates suitable for treating these conditions are disclosed *supra*.

[0117] Another aspect of the present disclosure relates to a method of treating an infectious disease or condition in a subject. This method involves administering to the subject having the infectious disease a non-binding protein-drug conjugate as described herein comprising a pharmaceutically active moiety suitable for treating the infectious disease. Exemplary infectious diseases (filoviral infections and coronavirus infections) and non-binding protein-drug conjugates suitable for treating these conditions are disclosed *supra*.

[0118] Another aspect of the present disclosure is directed to a method of imaging a tumor in a subject. This method involves selecting a subject having a tumor and administering to said subject a composition comprising a non-binding protein-drug conjugate. In accordance with this aspect of the disclosure, the second portion of the non-binding protein-drug conjugate comprises a diagnostic moiety. Suitable diagnostic moieties are described *supra*, e.g.,

fluorescent dyes, radioisotopes, radionuclides, radioisotopes, microbubbles, a contrast agent suitable for imaging, and a photosensitizer.

[0119] In accordance with this aspect of the disclosure, the tumor to be imaged is characterized by cancerous cells having enhanced macropinocytosis relative to their corresponding non-cancerous cells. In some embodiments, the tumor to be imaged is characterized by cancerous cells having an oncogenic mutation in H-ras, N-ras, or K-ras. In some embodiments, the tumor to be imaged is a pancreatic tumor, lung tumor, breast tumor, colon tumor, glioma, solid tumor, melanoma, glioblastoma multiforme, leukemia, renal cell carcinoma, hepatocellular carcinoma, prostate tumor, and myeloma.

[0120] Detecting the presence of a tumor in a subject using the diagnostic non-binding protein-drug conjugate of the present disclosure is achieved using *in vivo* imaging techniques. *In vivo* imaging involves administering to the subject the non-binding protein-diagnostic moiety conjugate described herein, and detecting the tumor cell macropinocytotic-mediated uptake of the conjugate *in vivo*.

[0121] In accordance with this aspect of the disclosure, diagnostic non-binding protein conjugates are administered by intravenous injection into the body of the subject, or directly into the tumor. The dosage of non-binding protein-diagnostic moiety conjugate should be within the same ranges as for treatment methods. In accordance with this embodiment, the diagnostic moiety conjugated to the non-binding FN3 domain is an imaging agent that facilitates *in vivo* imaging. Suitable imaging agents are described *supra* and include, without limitation, single photon emission computed tomography (SPECT) agents, positron emission tomography (PET) agents, magnetic resonance imaging (MRI) agents, nuclear magnetic resonance imaging (NMR) agents, x-ray agents, optical agents (*e.g.*, fluorophores, bioluminescent probes, near infrared dyes, quantum dots), ultrasound agents and neutron capture therapy agents, computer assisted tomography agents, two photon fluorescence microscopy imaging agents, and multi-photon microscopy imaging agents. Exemplary detectable markers include radioisotypes (*e.g.*, ^{18}F, ^{11}C, ^{13}N, ^{64}Cu, ^{124}I, ^{76}Br, ^{82}Rb, ^{68}Ga ^{99m}Tc, ^{111}In, ^{201}Tl or ^{15}O, which are suitable for PET and/or SPECT use) and ultra-small superparamagnetic particles of iron oxide (USPIO) which are suitable for MRI.

[0122] Imaging of a tumor in a subject is performed by detecting the number, size, and/or intensity of detected non-binding protein-diagnostic moiety conjugates in the subject. In some embodiments, the level of detected diagnostic conjugate is compared to a corresponding baseline value. An appropriate baseline value can be the average level of non-binding protein diagnostic conjugate found within cells in a population of undiseased individuals. Alternatively,

an appropriate baseline value may be the level of non-binding protein diagnostic conjugate found within cells of the same subject determined at an earlier time.

[0123] The diagnostic imaging methods described herein can also be used to monitor a subject's response to therapy. In this embodiment, detection of the non-binding protein diagnostic conjugate in the subject is determined prior to the commencement of treatment. The level of non-binding protein diagnostic conjugate in the subject at this time point is used as a baseline value. At various times during the course of treatment administration and detection of the non-binding protein diagnostic conjugate is repeated, and the measured values thereafter compared with the baseline values. A decrease in values relative to baseline signals a positive response to treatment.

EXAMPLES

[0124] The following examples are provided to illustrate embodiments of the present disclosure but are by no means intended to limit its scope

Example 1 – Production of Non-Binding Protein Drug Conjugate

[0125] As an initial study, a non-binding FN domain (comprising the amino acid sequence of SEQ ID NO: 3 with the addition of a C-terminal serine and cysteine residues for conjugation) was conjugated to monomethyl auristatin E (MMAE) (*see* schematic of FIG. 1A). MMAE is a highly toxic agent commonly used in antibody drug conjugates (ADC). The MMAE molecule was attached to the non-binding FN domain with a linker that contains a cathepsin-cleavable linker and a maleimide group for conjugation to the C-terminal free cysteine on the non-binding FN domain protein (FN-MMAE). Maleimide conjugation was performed with a two-step process and 1.5 molar excess of drug. Conjugation was verified by gel shift assay (FIG. 1B), Ellman's test and spectroscopic analysis (FIG. 1D). For *in vitro* and *in vivo* imaging, Cy5.5 replaced the drug molecule to monitor pharmacokinetics of the newly designed complex (FIG. 1C).

Example 2 – Macropinocytic Uptake of Non-Binding Protein Drug Conjugate

[0126] To determine if non-binding FN domains could be internalized, a well-established doxycycline-inducible mutant $KRas^{V12}$ HeLa cell system (hereafter, HeLa $KRas^{V12}$) was used in which macropinocytosis is monitored by high-molecular-weight (70kDa) tetramethyl-rhodamine labeled (TMR-) dextran. As shown in FIG. 2B, fluorescently labeled non-binding FN domain (FN-Cy5.5) co-localized with TMR-dextran. Importantly, the internalization was dependent on oncogenic Ras, since cells in which doxycycline was not added

(HeLa) did not have detectable non-binding FN domain uptake. Further, the macropinocytosis inhibitor, EIPA blocked both non-binding FN domain and dextran uptake into HeLa $KRas^{V12}$ cells (FIG. 2B). Similarly, the mutant KRas PDAC cell line MIA PaCa-2 (human, FIG. 2A) and KPC 1203 (mouse, FIG. 3A) showed co-localization occurring between dextran and FN-Cy5.5; whereas EIPA abolished uptake of both macromolecules (FIGs. 2A and 3A). In FIG. 3B, human colorectal cancer line HCT116 with Ras mutation showed co-localization occurring between dextran and FN-Cy5.5 but no uptake was seen in HCA7 cells with WT Ras. Analysis by flow cytometry of non-binding FN domain treated HeLa KTO +$KRas^{V12}$ and MIA PaCa-2 were positive for FN-Cy5.5 uptake (FIG. 2C).

[0127] The differential uptake was established not only in cell lines of solid tumors, but also in blood cancer such as multiple myeloma. The wildtype Ras cell line (KMS11) displays little to no macropinocytosis compared to mutant NRas (L363) or mutant KRas (RPMI-8226) multiple myeloma cell lines (FIGs. 14 and 16). Using a similar system to the HeLa $KRasV^{12}$ inducible system, we created an inducible $KRas^{V12}$ cell system in wild type KRas KMS11 multiple myeloma cells. As seen in the HeLa system, $KRas^{V12}$ expression in KMS11 cells was evident with doxycycline treatment and dependent for FN-Cy5.5 cellular uptake (FIG. 15).

Example 3 – Differential Cytotoxicity of Non-Binding Protein Drug Conjugate

[0128] By creating a cell impermeable protein complex, the ability of the non-binding FN domain to enter cells without macropinocytosis is hampered, creating a selective cellular uptake pathway. To investigate if Ras mutational status confers differential cytotoxicity, increasing concentrations of FN-MMAE conjugate on cell viability in HeLa and HeLa $KRasV^{12}$ cells were tested. Indeed, a 16-fold decrease in IC50 in HeLa $KRasV^{12}$ cells compared to HeLa cells was detected (FIG. 4A). FN-MMAE was also highly effective in mutant Ras pancreatic, multiple myeloma, lung, and colon cancer cell lines (FIG. 4B). After normalizing IC50 values of FN-MMAE to free MMAE to control for cellular differences, a significant decrease in cytotoxicity for cell lines without mutant Ras (macropinocytosis-negative) compared to cell lines with mutant Ras (macropinocytosis-positive) was observed, suggesting a macropinocytosis dependency.

[0129] A unique attribute to the non-binding FN domain platform is the flexibility of cargo conjugation. This was tested in FIG. 4C where SN-38, an irinotecan metabolite was conjugated to FN. Similar to FN-MMAE, a mutant Ras specific cytotoxicity was observed in the PDAC cell lines treated.

[0130] To test the effects *in vivo*, a MIA PaCa-2 subcutaneous tumor model was used to compare the efficacy of PBS, MMAE, and FN-MMAE (FIG. 5A). After tumors were

established, three injections of 0.2 mg/kg drug with respect to MMAE concentration was administered every 7 days. Compared to MMAE, the FN-MMAE conjugate more effectively reduced tumor growth by extending the time to reach the tumor size end point by 47 days (2.2-fold increase). FN-MMAE also showed reduced non-specific toxicities, specifically reduced cleaved caspase 3, HMGB1, and increased tubulin staining (MMAE mode of cytotoxicity is as a tubulin destabilizer) in both the kidneys and liver (FIGs. 5B, 6A, and 6B). This highlights the specificity of reduced uptake by non-macropinocytic cells in relation to free drug, which contributes to the selective effect of the non-binding FN domain-drug conjugate.

Example 4 – Biodistribution of Non-Binding Protein Drug Conjugate

[0131] The size of the non-binding FN domain confers a very short half-life in the serum and clearance is subjected to renal excretion with high collection in the kidneys and liver. To test whether non-binding FN domain biodistribution was altered, a MIA PaCa-2 xenograft mouse model was used. After injecting mice harboring established tumors with FN-Cy5.5 or Cy5.5, an IVIS time course uncovered an altered pharmacokinetic profile of the non-binding FN domain compared to the free Cy5.5 (FIGs. 7A and 7B). Next, MIA PaCa-2 and BxPC3 xenograft mice were treated on the same time course to see if mutational status affected retention (FIG. 7C). At 1, 4, and 24hr time points, there was over a doubling in uptake in the MIA-PaCa-2 tumors. Next, KPC cells were orthotopically injected into the pancreas of mice to assess the treatment and biodistribution of PBS, Cy5.5-COOH or FN-Cy5.5. *Ex vivo* imaging of whole tumors and tumor slices were analyzed at the 6hr timepoint (FIGs. 7D and 7E) displaying selective uptake for the tumorigenic pancreas compared to wild pancreas, which was not seen in the small molecule treatment.

[0132] In FIG. 8, biodistribution of PBS, FN-Cy5.5, Mouse albumin (MSA)-Cy5.5, or mouse IgG-Cy5.5 was observed in orthotopic implanted KPC tumor bearing mice at 1hr and 24hr after IP injection. Mice were sacrificed at 1hr (FIG. 8A) and 24hr (FIG. 8B) to observe *ex vivo* biodistribution and blood fluorescence (FIG. 8C). At 1hr, FN-Cy5.5 had drastic 2-fold increases in the kidneys and liver compared to other protein conjugates, displaying quick excretion while having similar or the increased tumor retention. At 24hr, FN-Cy5.5 showed reductions in kidneys and liver while have similar tumor values (90% and 100% for MSA and IgG respectively). FN-Cy5.5 displayed a 3-fold decrease in blood fluoresce at 1hr, again highlighting quick excretion compared to other protein conjugates.

[0133] In FIG. 9, the uptake of the non-binding protein drug conjugate in pancreatic draining lymph nodes was examined. There is a susceptibility for metastases within the pancreatic draining lymph node in PDAC patients, while also being a target tissue for a number

of encouraging tumor immunomodulator therapies currently being developed. To assess if there is selective uptake in this tissue, and more specifically dendritic cells, orthotopic implanted KPC tumor bearing mice were treated with PBS, Cy5.5-COOH, and FN-Cy5.5. *Ex vivo* IVIS imaging showed FN-Cy5.5 localized within PDAC draining lymph nodes (FIG. 9A) and had a 2 fold preference for PDAC lymph nodes compared to bronchial lymph nodes. Additionally, lymph nodes were digested and flow cytometry was used to observe Cy5.5 positive cells (FIG. 9B and 9C). There was over a 2-fold increase in positive Cy5.5 cells for FN conjugate as compared to free drug, signifying an improved dendritic cell uptake with the material delivery. Together this data indicates that not only is the FN platform selectively retained in pancreatic lymph nodes, but also the FN conjugate can significantly improve drug uptake.

[0134] Further analysis using a genetically modified mouse model, *KrasG12D;Trp53R172H;p48Cre (KPC),* which recapitulates human PDAC progression from pancreatic intraepithelial neoplasm (PanIN) lesions to PDAC and metastasis, also displayed enhanced tumor selective uptake in the pancreas (FIGs. 10A–C). Importantly, less uptake was observed in the pancreata of wild type mice (FIGs. 10A–C). Consistent Cy5.5 staining throughout the tumor was observed, indicating good penetration and colocalization with a cancer cell marker (CK8) (FIG. 10D). Importantly, it was found that the FN-Cy5.5 complex reached the tumor and was internalized by the tumor cells. Additionally, similar experimentation was carried out with co-delivery of an albumin bound FITC and FN-Cy5.5. Albumin based therapy is a current standard of care in PDAC treatment. Albumin-FITC and FN-Cy5.5 both colocalized within the established pancreatic tumors in mice (FIG. 11). The *ex vivo* analysis showed FN-Cy5.5 had a larger propensity to be up taken in the PanINs, as seen by colocalization with CK8 staining, while albumin-FITC had larger accumulation in the extracellular tumor microenvironment.

[0135] The differential tumor accumulation was established not only in solid tumors, but also in blood cancer such as multiple myeloma. The mutant NRas xenograft (L363) displays heightened tumor accumulation of FN-Cy5.5 compared to the wildtype Ras xenograft (KMS11) (FIG. 17).

[0136] The pharmacokinetic profile exhibited indicates non-binding FN domain treatment will be a superior asset for both chemotherapy and imaging. Creating a short half-life while maintaining tumor retention and enhanced penetration will allow for larger dose regiments with limited toxicities, making treatment potentially more effective.

Example 5 – Imaging Using the Non-Binding Protein Conjugate

[0137] Current imaging methods have created modest solutions to detect early stages of PDAC. Specifically, current MRI imaging systems have poor spatial resolution with moderate ability to detect lesions. Investigations into traditional PET agents (^{18}FDG) have reported mixed results. ^{18}FDG also can detect inflammatory responses, making differentiation between pancreatitis and PDAC challenging. In preliminary studies using an orthotopic implanted KPC tumor (FIGs. 12B and 13) and an autochthonous KPC mouse model (FIGs. 12C and 13), FN-DOTA-Gd^{3+} follows the same biodistribution as the fluorescent probes (FIG. 10A), with localization in the kidneys within 10 minutes post injection (FIGs. 12C and 13) and detection of tumor mass (FIG. 7B and FIG. 7C bottom-red arrowheads).

[0138] Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

What is claimed is:

1. A pharmaceutical composition comprising:

a non-binding protein-drug conjugate, wherein said non-binding protein-drug conjugate comprises:

a first portion, said first portion comprising a non-binding fibronectin type III (FN3) domain;

an amino acid linker within or coupled to the first portion; and

a second portion coupled to said first portion via the amino acid linker, said second portion selected from a pharmaceutically active moiety or a diagnostic moiety; and

a pharmaceutically acceptable carrier.

2. The composition of claim 1, wherein the FN3 domain of the non-binding protein-drug conjugate comprises one or more amino acid substitutions in its RGD amino acid sequence of its FG loop.

3. The composition of claim 1 or claim 2, wherein the FN3 domain of the non-binding protein-drug conjugate comprises one or more amino acid substitutions in the amino acid sequence of the BC loop.

4. The composition of claim 1, wherein the FN3 domain of the non-binding protein-drug conjugate is a non-binding human FN3 domain.

5. The composition of any one of claims 1–4, wherein the FN3 domain of the non-binding protein-drug conjugate is a non-binding variant of the 10^{th} type III fibronectin domain of human fibronectin (^{10}Fn3).

6. The composition of any one of claims 1–5, wherein the first portion of the non-binding protein-drug conjugate comprises an amino acid sequence of SEQ ID NO: 2.

7. The composition of any one of claims 1–6, wherein the FN3 domain of the non-binding protein-drug conjugate comprises a FG loop amino acid sequence of SEQ ID NO: 4.

8. The composition of any one of claims 3–7, wherein the FN3 domain of the non-binding protein-drug conjugate comprises a BC loop amino acid sequence of SEQ ID NO: 5.

9. The composition of claim 1, wherein the first portion of the non-binding protein-drug conjugate comprises an amino acid sequence of SEQ ID NO: 3.

10. The composition of any one of claims 1–9, wherein the amino acid linker comprises a cysteine residue substitution within the FN3 domain of the first portion of the protein-drug conjugate.

11. The composition of any one of claims 1–10, wherein the amino acid linker comprises a cysteine residue addition at the FN3 domain C-terminus.

12. The composition of any one of claims 1–11, wherein the amino acid linker is a cleavable linker.

13. The composition of any one of claims 1–11, wherein the amino acid linker is a non-cleavable linker.

14. The composition of any one of claims 1–11, wherein the first portion of the non-binding protein-drug conjugate comprises two or more FN3 domains linked together in tandem.

15. The composition of any one of claims 1–14, wherein the second portion of the non-binding protein-drug conjugate is a pharmaceutically active moiety.

16. The composition of claim 15, wherein the pharmaceutically active moiety is a cancer therapeutic.

17. The composition of claim 16, wherein the cancer therapeutic is selected from an antimetabolite, an alkaloid, an alkylating agent, an anti-mitotic agent, an antitumor antibiotic, a DNA binding drug, a toxin, an antiproliferative drug, a DNA antagonist, a radionuclide, a thermoablative agent a proteolysis targeting chimera (PROTAC), a nucleic acid inhibitor, and an immune-modulatory agent.

18. The composition of claim 16, wherein the alkaloid is selected from the group consisting of duocarmycin, docetaxel, etoposide, irinotecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, vindesine and analogs and derivatives thereof.

19. The composition of claim 16, wherein the alkylating agent is selected from the group consisting of busulfan, improsulfan, piposulfan, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphorarnide, chlorambucil, chloranaphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide HCl, melphalan, novemebichin, perfosfamide phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, semustine ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, and analogs and derivatives thereof.

20. The composition of claim 16, wherein the antitumor antibiotic is selected from the group consisting of aclacinomycin, actinomycin, anthramycin, azaserine, bleomycin, cactinomycin, calicheamicin, carubicin, carzinophilin, cromomycin, dactinomycin, daunorubicin, 6-diazo-5-oxo-l-norleucine, doxorubicin, epirabicin, idarubicin, menogaril, mitomycin, mycophenolic acid, nogalamycine, olivomycin, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycine, pyrrolobenzodiazepine, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, and analogs and derivatives thereof.

21. The composition of claim 16, wherein the antimetabolite is selected from the group consisting of from SN-38, denopterin, edatrexate, mercaptopurine (6-MP), methotrexate, piritrexim, pteropterin, pentostatin (2'-DCF), tomudex, trimetrexate, cladridine, fludarabine, thiamiprine, ancitabine, azacitidine, 6- azauridine, carmofur, cytarabine, doxifluridine, emitefur, floxuridine, fluorouracil, gemcitabine, tegafur, hydroxyurea, urethane, and analogs and derivatives thereof.

22. The composition of claim 16, wherein the anti-proliferative drug selected from the group consisting of aceglatone, amsacrine, bisantrene, camptothecin, defosfamide, demecolcine, diaziquone, diflomotecan, eflornithine, elliptinium acetate, etoglucid, etopside, fenretinide, gallium nitrate, hydroxyurea, lamellarin D, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracrine, pentostatin, phenamet, podophillinic acid 2-ethyl-

hydrazide, procarbazine, razoxane, sobuzoxane, spirogermanium, teniposide, tenuazonic acid, triaziquone 2,2' ,2"- trichlorotriethylamine, and analogs and derivatives thereof.

23. The composition of claim 16, wherein the antimitotic agent is selected from the group consisting of an auristatin, a maytansinoid, a dolastatin, a tubulysin, a taxane, a epothilone, a vinca alkaloid, and analogs and derivatives thereof.

24. The composition of claim 15, wherein the pharmaceutically active moiety is an immunomodulatory agent.

25. The composition of claim 24, wherein the immunomodulatory agent is a macrophage type-1 stimulating agent.

26. The composition of claim 25, wherein the macrophage type-1 stimulating agent is selected from the group consisting of paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

27. The composition of claim 24, wherein the immunomodulatory agent is a macrophage type-2 stimulating agent.

28. The composition of claim 25, wherein the macrophage type-2 stimulating agent is selected from the group consisting of IL-33, IL-4 receptor agonists, glucocorticoids, IL-10 receptor agonist, IL-1 receptor agonist.

29. The composition of claim 24, wherein the immunomodulatory agent is an T cell stimulating agent.

30. The composition of claim 29, wherein the T cell stimulating agent is a stimulator of interferon genes (STING) agonist.

31. The composition of claim 24, wherein the immunomodulatory agent is a dendritic cell stimulating agent.

32. The composition of claim 31, wherein the dendritic cell stimulating agent is selected from the group consisting of CpG oligonucleotide, imiquimod, camptothecin, colchicine, podophyllotoxin, and derivatives thereof.

33. The composition of claim 24, wherein the immunomodulatory agent is a neutrophil stimulating agent.

34. The composition of claim 33, wherein the neutrophil stimulating agent is a recombinant granulocyte colony stimulating factor protein (filgrastim) or a pegylated recombinant granulocyte colony stimulating factor protein.

35. The composition of claim 15, wherein the pharmaceutically active moiety is an oligonucleotide.

36. The composition of claim 35, wherein the oligonucleotide is selected from the group consisting of an siRNA, an aptamer, an miRNA, an immunostimulatory oligonucleotide, a splice-switching oligonucleotide, and guide RNA.

37. The composition of claim 15, wherein the pharmaceutically active moiety is a wound healing agent.

38. The composition of any one of claims 1–14, wherein the second portion of the non-binding protein-drug conjugate is a diagnostic moiety.

39. The composition of claim 38, wherein the diagnostic moiety is selected from the group consisting of a fluorescent dye, a radioisotope, a contrast agent suitable for imaging, a radionucleotide with a chelator, and a photosensitizer.

40. A method of treating cancer in a subject, said method comprising:
administering, to the subject having cancer, a composition of any one of claims 1–35 in an amount effective to treat the cancer.

41. The method of claim 40, wherein the cancer is characterized by cancerous cells having enhanced macropinocytosis relative to their corresponding non-cancerous cells.

42. The method of claim 40, wherein the cancer is characterized by cancerous cells having an oncogenic mutation in H-ras, N-ras, or K-ras.

43. The method of any one of claims 40–42, wherein the second portion of the non-binding protein-drug conjugate of the composition is a pharmaceutically active moiety and said pharmaceutically active moiety is a cancer therapeutic.

44. The method of claim 43, wherein the cancer therapeutic is an antimetabolite, an alkaloid, an alkylating agent, an anti-mitotic agent, an antitumor antibiotic, a DNA binding drug, a microtubule targeting drug, a toxin, an antiproliferative drug, a DNA antagonist, radionuclide, a thermoablative agent and a PROTAC.

45. The method of claim 44, wherein the alkaloid is selected from the group consisting of duocarmycin, docetaxel, etoposide, irinotecan, paclitaxel, teniposide, topotecan, vinblastine, vincristine, vindesine, and analogs and derivatives thereof.

46. The method of claim 44, wherein the alkylating agent is selected from the group consisting of busulfan, improsulfan, piposulfan, benzodepa, carboquone, meturedepa, uredepa, altretamine, triethylenemelamine, triethylenephosphoramide, triethylenethiophosphorarnide, chlorambucil, chloranaphazine, cyclophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide HCl, melphalan, novemebichin, perfosfamide phenesterine, prednimustine, trofosfamide, uracil mustard, carmustine, chlorozotocin, fotemustine, lomustine, nimustine, semustine ranimustine, dacarbazine, mannomustine, mitobronitol, mitolactol, pipobroman, temozolomide, and analogs and derivatives thereof.

47. The method of claim 44, wherein the antitumor antibiotic is selected from the group consisting of aclacinomycin, actinomycin, anthramycin, azaserine, bleomycin, cactinomycin, calicheamicin, carubicin, carzinophilin, cromomycin, dactinomycin, daunorubicin, 6-diazo-5-oxo-l-norleucine, doxorubicin, epirabicin, idarubicin, menogaril, mitomycin, mycophenolic acid, nogalamycine, olivomycin, peplomycin, pirarubicin, plicamycin, porfiromycin, puromycine, pyrrolobenzodiazepine, streptonigrin, streptozocin, tubercidin, zinostatin, zorubicin, and analogs and derivatives thereof.

48. The method of claim 44, wherein the antimetabolite is selected from the group consisting of from SN-38, denopterin, edatrexate, mercaptopurine (6-MP), methotrexate, piritrexim, pteropterin, pentostatin (2'-DCF), tomudex, trimetrexate, cladridine, fludarabine, thiamiprine, ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, emitefur, floxuridine, fluorouracil, gemcitabine, tegafur, hydroxyurea, urethane, and analogs and derivatives thereof.

49. The method of claim 44, wherein the anti-proliferative drug selected from the group consisting of aceglatone, amsacrine, bisantrene, camptothecin, defosfamide, demecolcine, diaziquone, diflomotecan, eflornithine, elliptinium acetate, etoglucid, etopside, fenretinide, gallium nitrate, hydroxyurea, lamellarin D, lonidamine, miltefosine, mitoguazone, mitoxantrone, mopidamol, nitracrine, pentostatin, phenamet, podophillinic acid 2-ethyl-hydrazide, procarbazine, razoxane, sobuzoxane, spirogermanium, teniposide, tenuazonic acid, triaziquone 2,2' ,2"- trichlorotriethylamine, and analogs and derivatives thereof.

50. The method of claim 44, wherein the antimitotic agent is selected from the group consisting of an auristatin, a maytansinoid, a dolastatin, a tubulysin, a taxane, a epothilone, a vinca alkaloid, and analogs and derivatives thereof.

51. The method of claim 44, wherein the cancer therapeutic is an immunomodulatory agent.

52. The method of claim 51, wherein the immunomodulatory agent is selected from a macrophage type-1 stimulating agent, a macrophage type-2 depleting agent, a T cell stimulating agent, a dendritic cell stimulating agent, and a neutrophil stimulating agent.

53. The method of claim 52, wherein the macrophage type-1 stimulating agent is selected from the group consisting of paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

54. The method of claim 52, wherein the T cell stimulating agent is a stimulator of interferon genes (STING) agonist.

55. The method of claim 52, wherein the dendritic cell stimulating agent is selected from the group consisting of CpG oligonucleotide, imiquimod, camptothecin, colchicine, podophyllotoxin, and derivatives thereof.

56. The method of claim 52, wherein the neutrophil stimulating agent is a recombinant granulocyte colony stimulating factor protein (filgrastim) or a pegylated recombinant granulocyte colony stimulating factor protein.

57. The method of claim 52, wherein the macrophage type-2 depleting agent is selected from the group consisting of clodronate, zoledronic acid, alendronate, and trabectedin.

58. A method of imaging a tumor in a subject, said method comprising:
selecting a subject having a tumor and
administering to said subject a composition of claim 38.

59. The method of claim 58, wherein the diagnostic moiety of the non-binding protein-drug conjugate is selected from the group consisting of a fluorescent dye, a radioisotope, a contrast agent suitable for imaging, a radionucleotide with chelator, and a photosensitizer

60. The method of claim 58, wherein the tumor is characterized by cancerous cells having enhanced macropinocytosis relative to their corresponding non-cancerous cells.

61. The method of claim 58, wherein the tumor is characterized by cancerous cells having an oncogenic mutation in H-ras, N-ras, or K-ras.

62. A method of modulating a subject's immune response, said method comprising:
administering, to a subject having a condition that would benefit from immune system modulation, and a composition of any one of claims 24–36 in an amount effective to modulate the subject's immune response.

63. The method of claim 62, wherein the immunomodulatory agent is a macrophage type-1 stimulating agent.

64. The method of claim 63, wherein the macrophage type-1 stimulating agent is selected from the group consisting of paclitaxel, a colony stimulating factor -1 (CSF-1) receptor antagonist, an IL-10 receptor antagonist, a Toll-like receptor (TLR)-2 agonist, a TLR-3 agonist, a TLR-4 agonist, a TLR-7 agonist, a TLR-8 agonist, and a TLR-9 agonist.

65. The method of claim 62, wherein the immunomodulatory agent is a macrophage type-2 stimulating agent.

66. The method of claim 65, wherein the macrophage type-2 stimulating agent is selected from the group consisting of IL-33, IL-4 receptor agonists, glucocorticoids, IL-10 receptor agonist, IL-1 receptor agonist.

67. The method of claim 62, wherein the immunomodulatory agent is an T cell stimulating agent.

68. The method of claim 67, wherein the T cell stimulating agent is a stimulator of interferon genes (STING) agonist.

69. The method of claim 62, wherein the immunomodulatory agent is a dendritic cell stimulating agent.

70. The method of claim 69, wherein the dendritic cell stimulating agent is selected from the group consisting of CpG oligonucleotide, imiquimod, camptothecin, colchicine, podophyllotoxin, and derivatives thereof.

71. The method of claim 62, wherein the immunomodulatory agent is a neutrophil stimulating agent.

72. The method of claim 71, wherein the neutrophil stimulating agent is a recombinant granulocyte colony stimulating factor protein (filgrastim) or a pegylated recombinant granulocyte colony stimulating factor protein.

73. The method of claim 62, wherein the immunomodulatory agent is an oligonucleotide.

74. The method of claim 73, wherein the oligonucleotide is selected from the group consisting of an siRNA, an aptamer, an miRNA, an immunostimulatory oligonucleotide, and a splice-switching oligonucleotide.

75. The method of claim 62, wherein subject has an interferonopathy and the immunomodulatory agent is type-1 interferon antagonist.

76. The method of claim 62, wherein the subject has a wound and the immunomodulatory agent is a type-1 macrophage stimulating agent.

77. The method of claim 62, wherein the subject has an immune tolerant form of cancer, and the immunomodulatory agent is selected from a type-1 macrophage stimulating agent, a T cell stimulating agent, a neutrophil stimulating agent, and a dendritic cell stimulating agent.

78. The method of any one of claims 40–77, wherein the subject is a human.

79. The method of any one of claims 40–77, wherein said administering is intravenous administration.

80. A method of treating a neurodegenerative condition in a subject, said method comprising:

administering, to the subject having the neurodegenerative condition, a non-binding protein-drug conjugate comprising a pharmaceutically active moiety suitable for treating the neurodegenerative condition.

81. A method of treating an inflammatory condition in a subject, said method comprising:

administering, to the subject having the inflammatory condition, a non-binding protein-drug conjugate comprising a pharmaceutically active moiety suitable for treating the inflammatory condition.

82. A method of treating a bone condition in a subject, said method comprising:

administering, to the subject having the bone condition, a non-binding protein-drug conjugate comprising a pharmaceutically active moiety suitable for treating the bone condition.

83. A method of treating an infectious disease or condition in a subject, said method comprising:

administering, to the subject having the infectious disease, a non-binding protein-drug conjugate comprising a pharmaceutically active moiety suitable for treating the infectious disease.

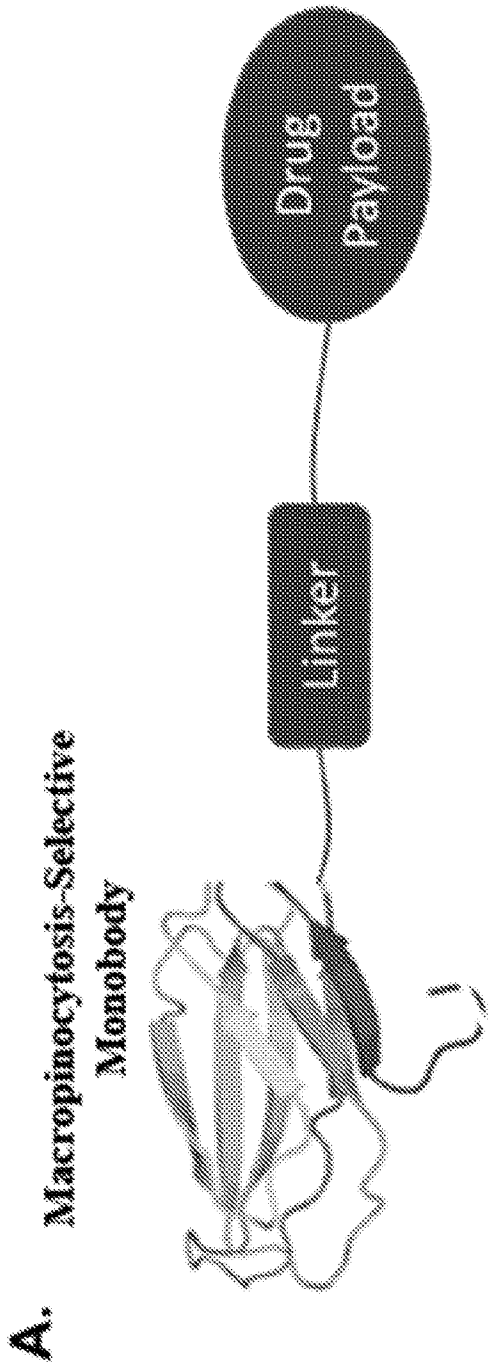
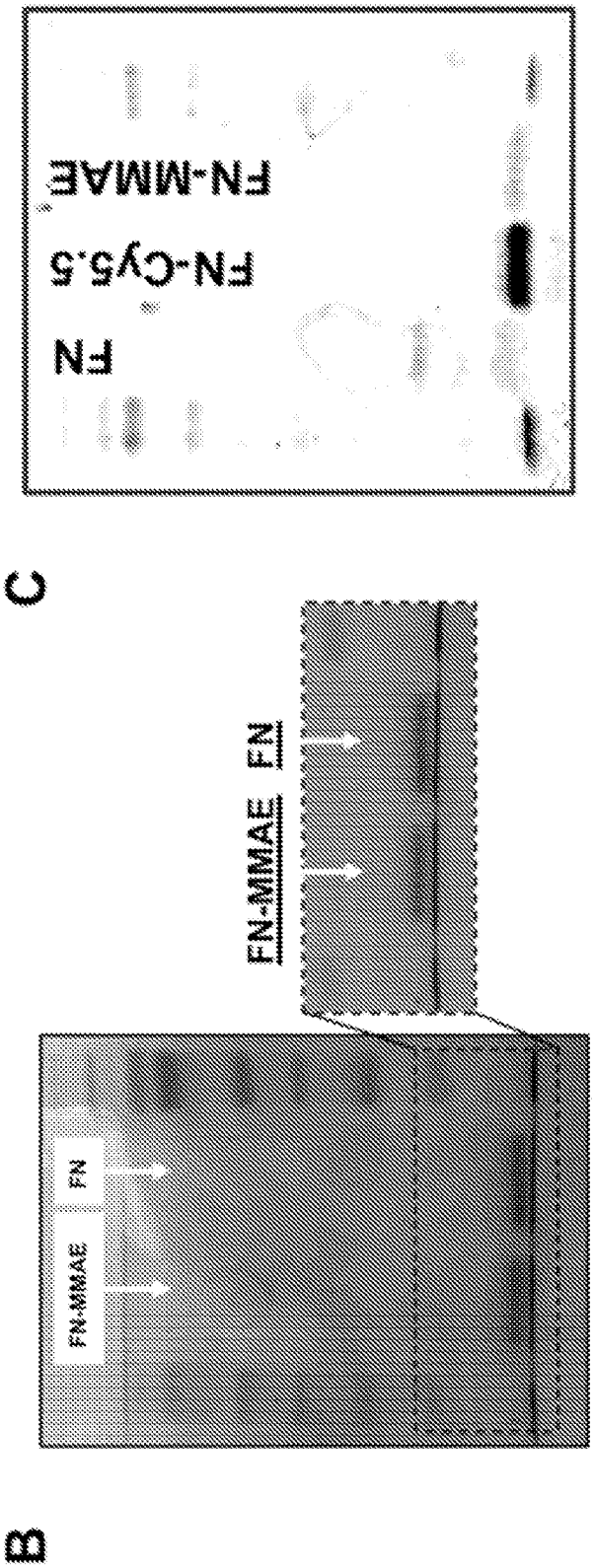

A.
Macropinocytosis-Selective
Monobody
Linker
Drug
Payload

FIG. 1A



FIGs. 1B-1C

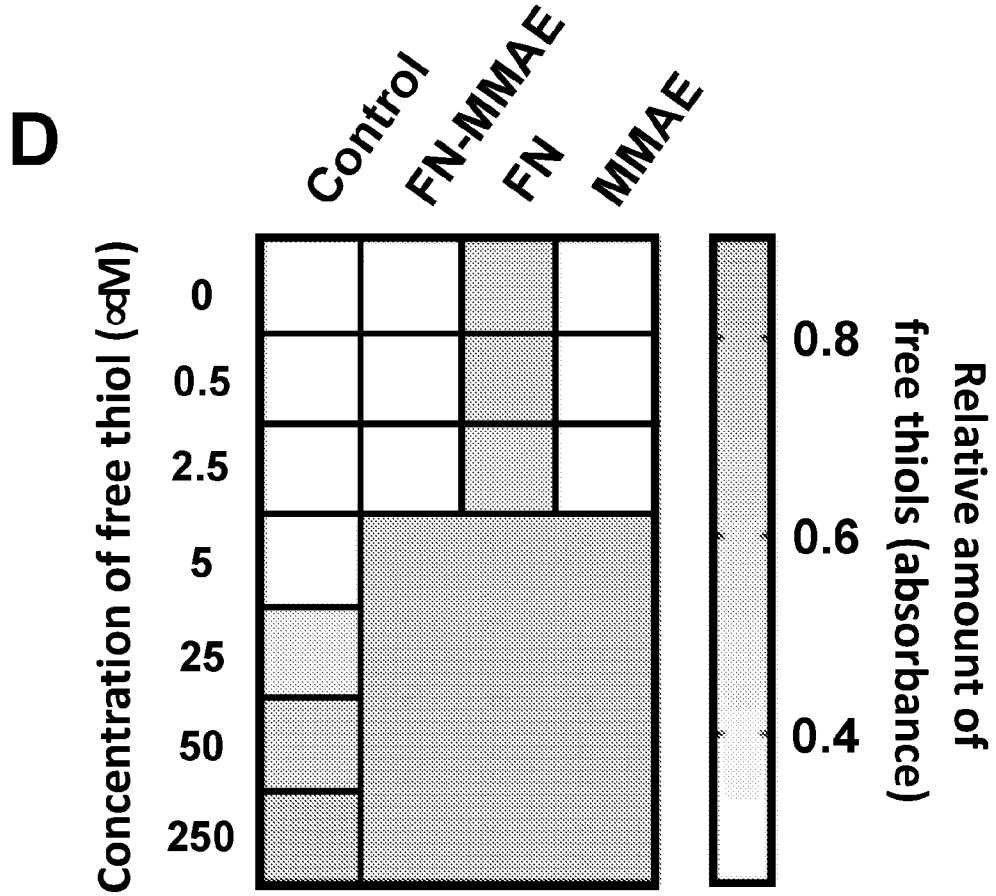

D
Control
FN-MMAE
FN
MMAE
Concentration of free thiol (αM)
0
0.5
2.5
5
25
50
250
0.8
0.6
0.4
Relative amount of free thiols (absorbance)

FIG. 1D

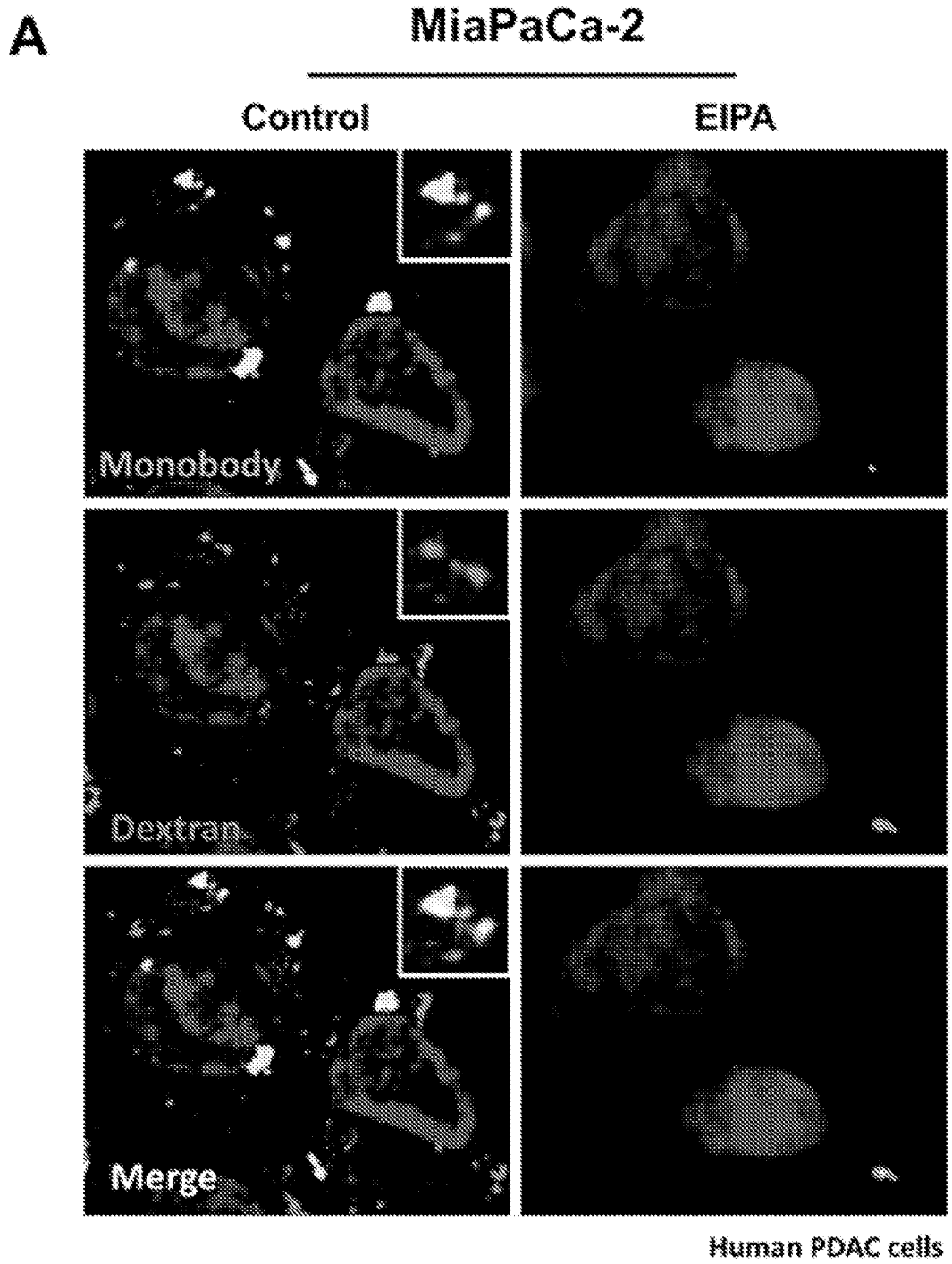

A
MiaPaCa-2
Control
EIPA
Monobody
Dextran
Merge
Human PDAC cells

FIG. 2A

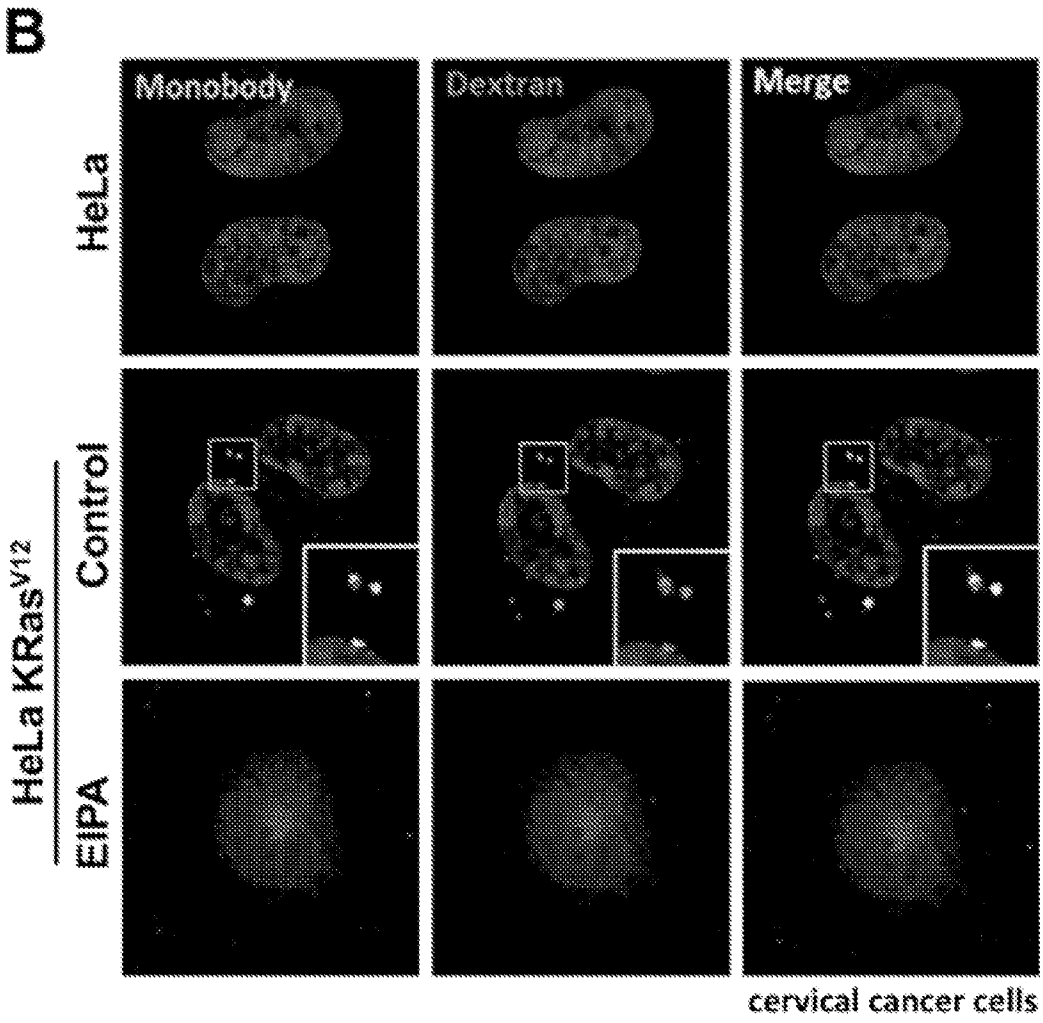

B
Monobody
Dextran
Merge
HeLa
Control
EIPA
HeLa KRas^{V12}
cervical cancer cells

FIG. 2B

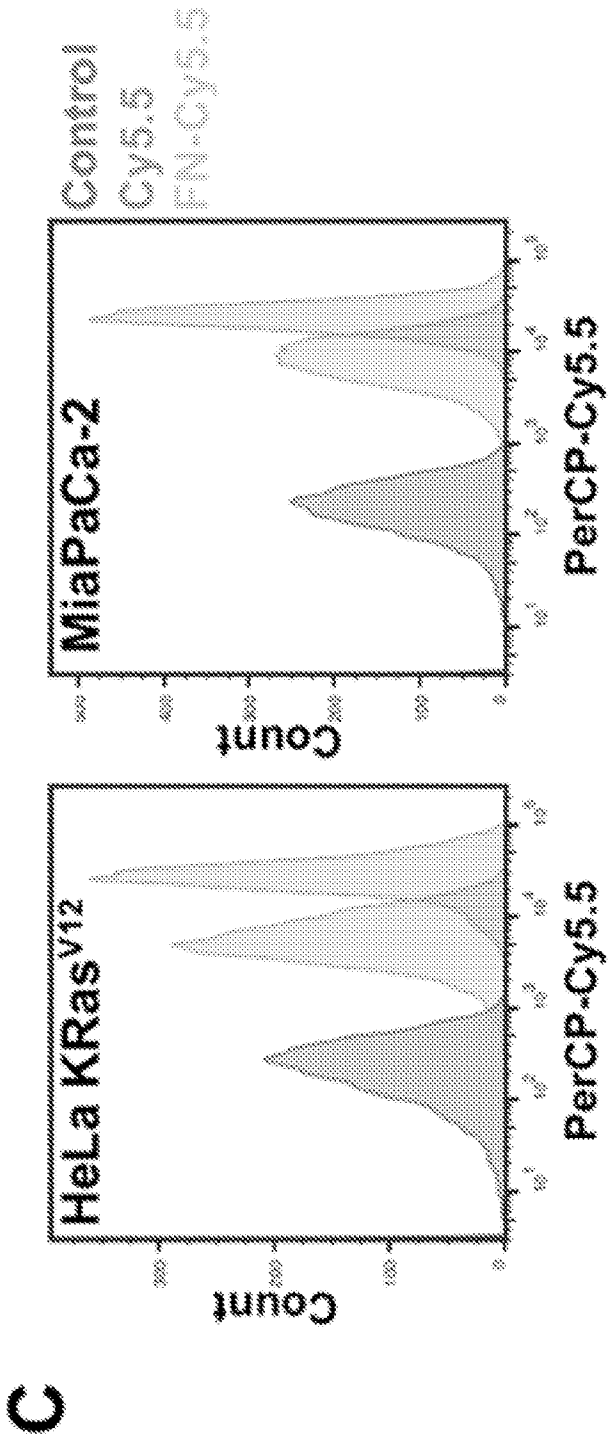

C
HeLa KRas^{V12}
MiaPaCa-2
Count
PerCP-Cy5.5
Control
Cy5.5
FN-Cy5.5

FIG. 2C

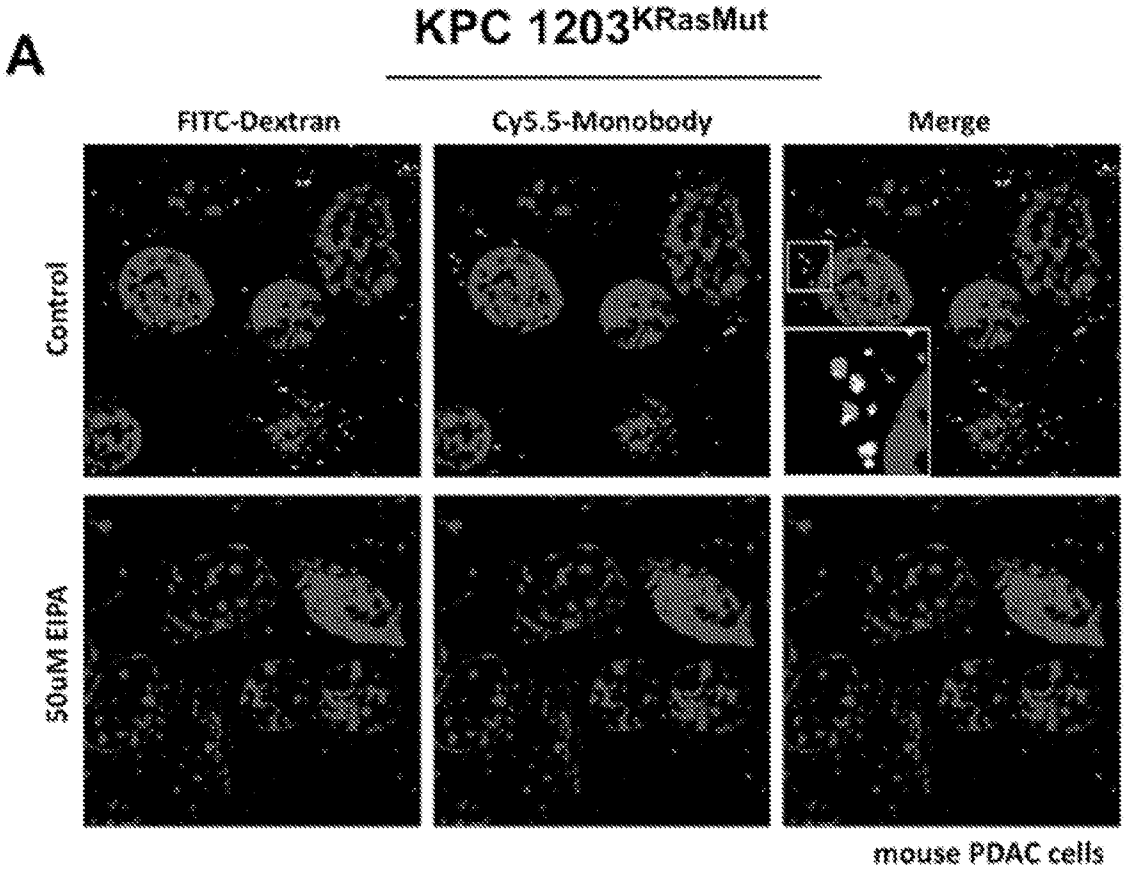

A
KPC 1203KRasMut
FITC-Dextran
Cy5.5-Monobody
Merge
Control
50uM EIPA
mouse PDAC cells

FIG. 3A

B

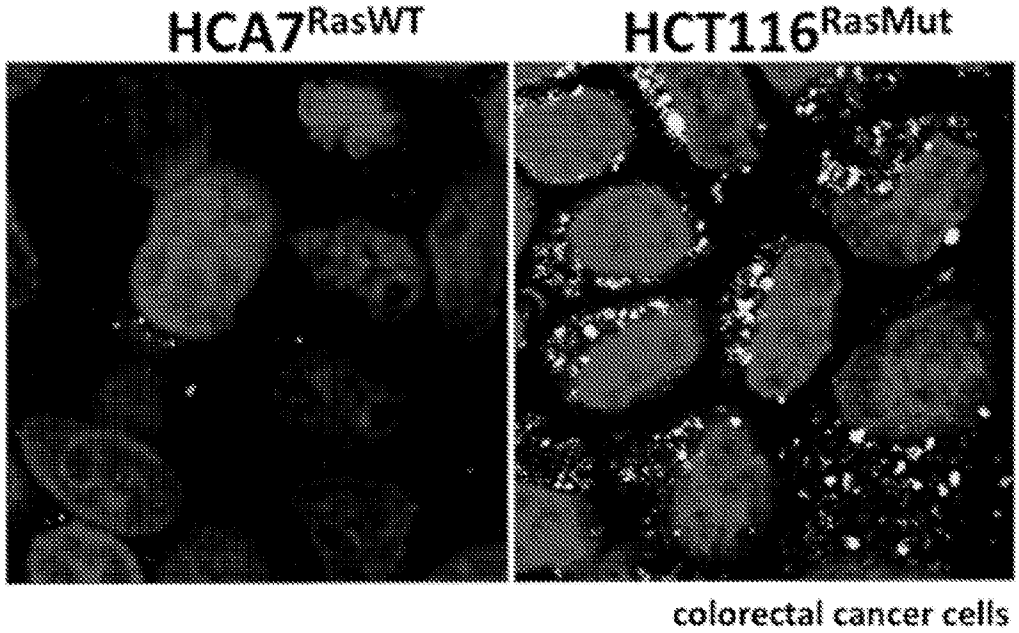
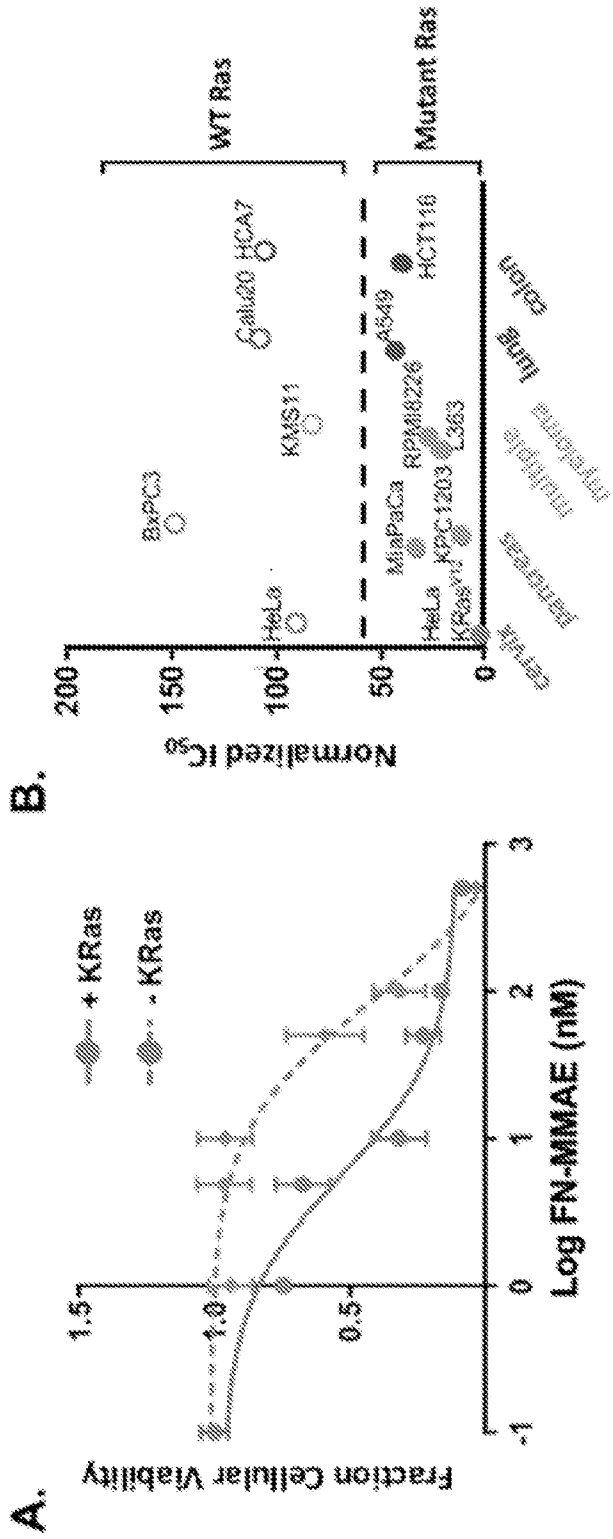


FIG. 3B


A.
Fraction Cellular Viability
+ KRas
- KRas
1.5
1.0
0.5
-1
0
1
2
3
Log FN-MMAE (nM)
B.
Normalized IC50
200
150
100
50
0
WT Ras
Mutant Ras
BxPC3
HeLa
KMS11
Colo205
HCA7
A549
HCT116
MiaPaCa
RPMI8226
KPC 1203
L363
HeLa
KRas-/-
Colon
Lung
Multiple Myeloma
Pancreas
Cervix

FIGs. 4A-4B

C

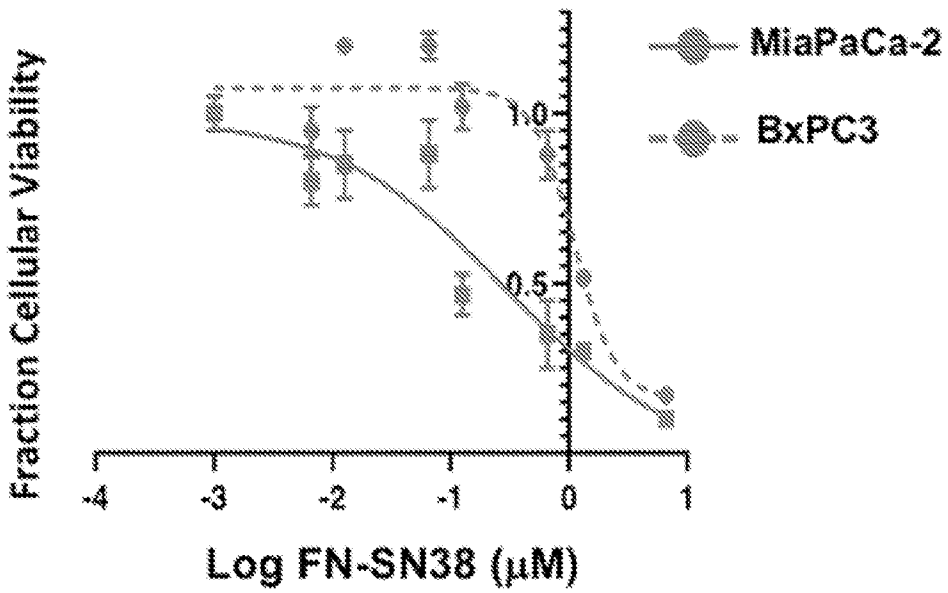

MiaPaCa-2
BxPC3
Fraction Cellular Viability
1.0
0.5
-4
-3
-2
-1
0
1
Log FN-SN38 (μM)

FIG. 4C

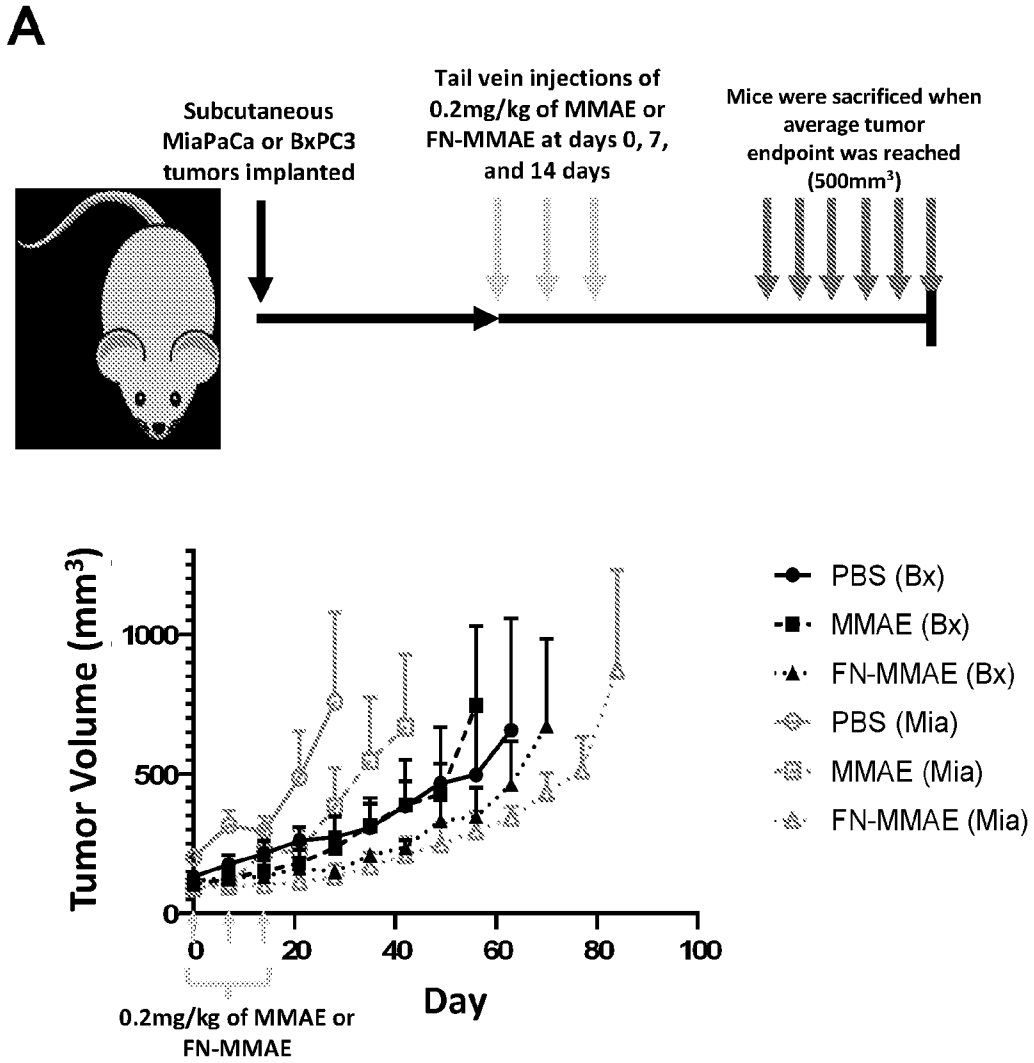

A
Subcutaneous MiaPaCa or BxPC3 tumors implanted
Tail vein injections of 0.2mg/kg of MMAE or FN-MMAE at days 0, 7, and 14 days
Mice were sacrificed when average tumor endpoint was reached (500mm3)
Tumor Volume (mm3)
1000
500
0
0
20
40
60
80
100
Day
0.2mg/kg of MMAE or FN-MMAE
PBS (Bx)
MMAE (Bx)
FN-MMAE (Bx)
PBS (Mia)
MMAE (Mia)
FN-MMAE (Mia)

FIG. 5A

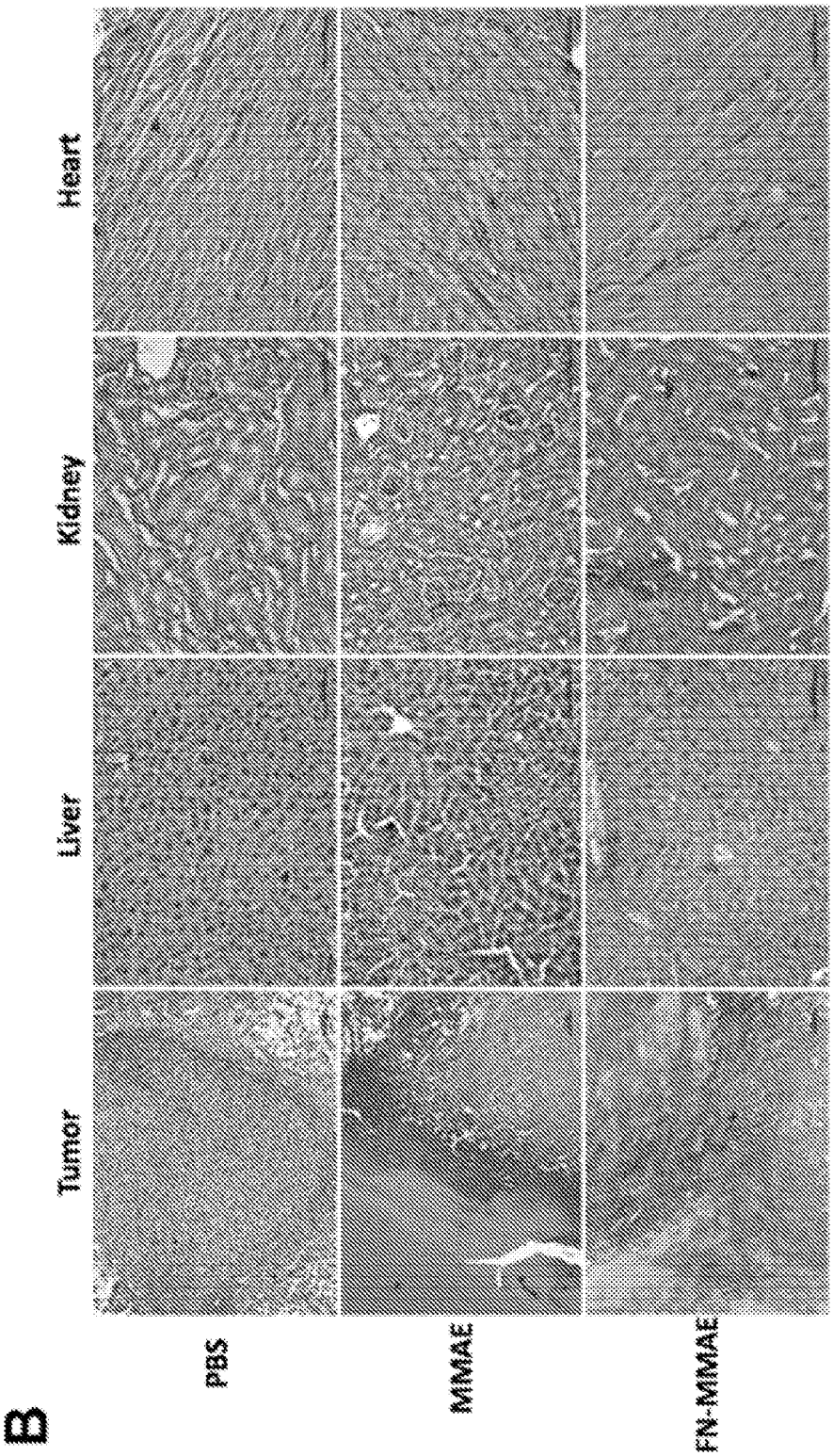


FIG. 5B

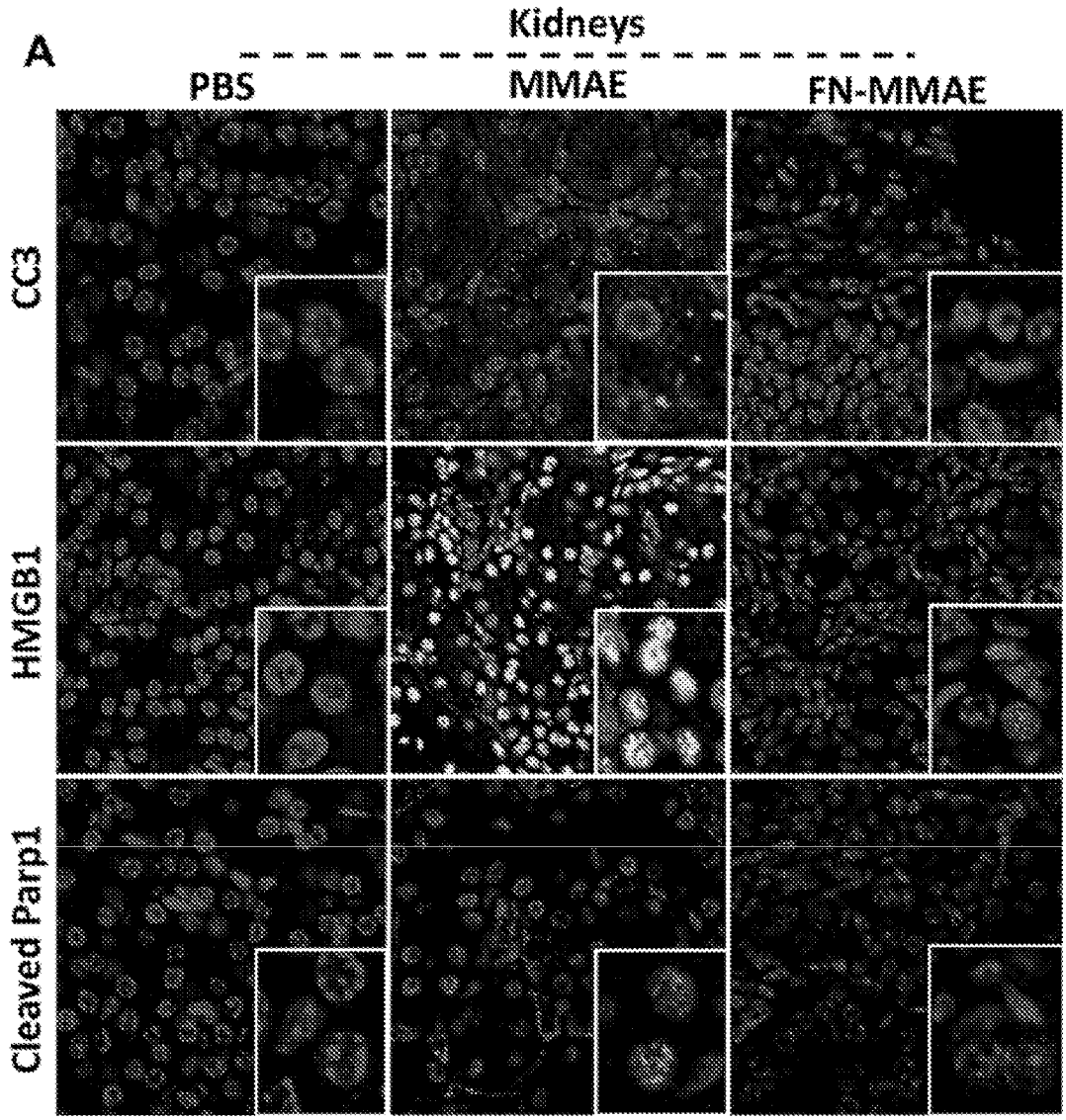


FIG. 6A

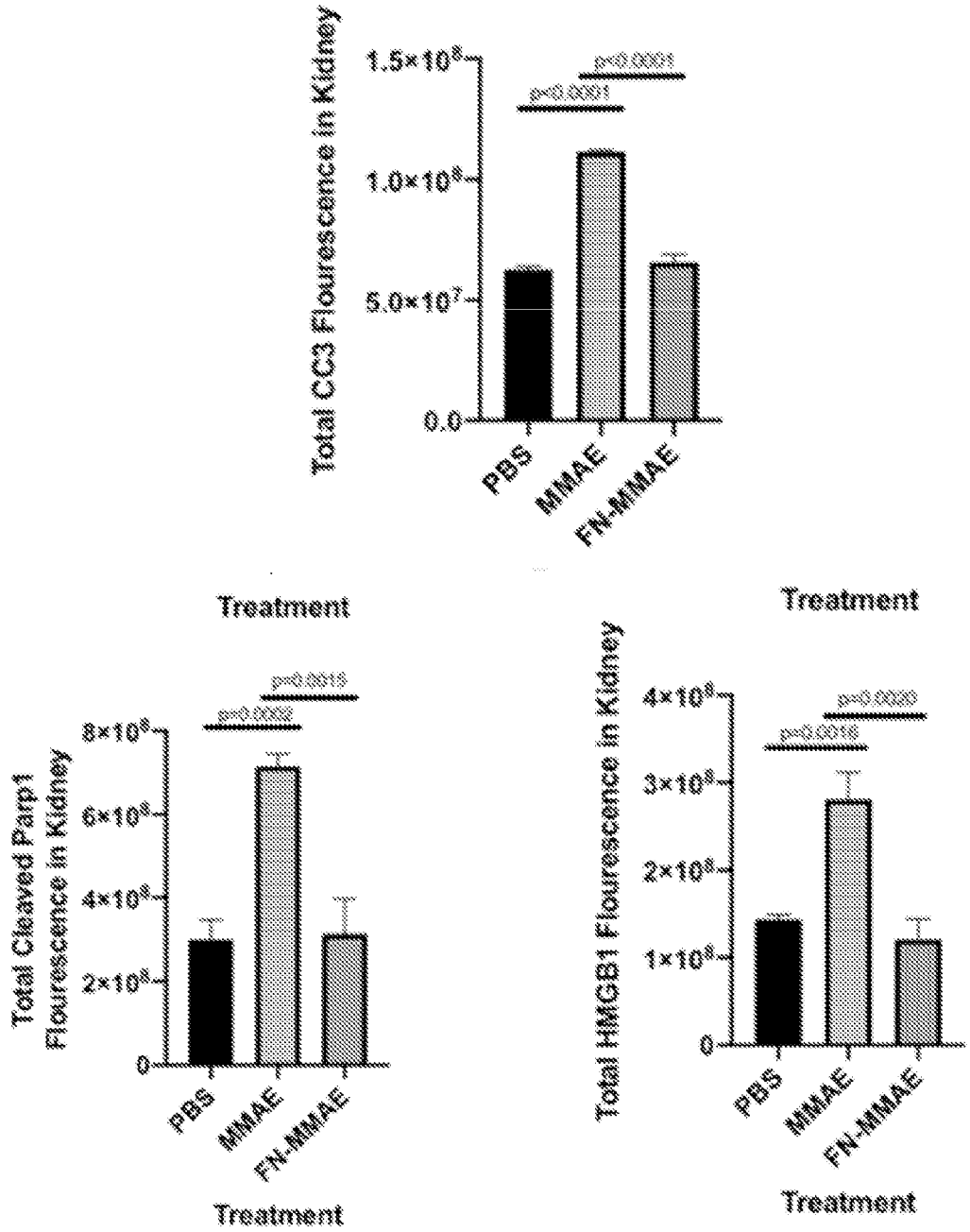

Total CC3 Flourescence in Kidney
1.5×10^8
1.0×10^8
5.0×10^7
0.0
p<0.0001
p<0.0001
PBS
MMAE
FN-MMAE
Treatment
Total Cleaved Parp1 Flourescence in Kidney
8×10^8
6×10^8
4×10^8
2×10^8
0
p<0.0015
p<0.0022
PBS
MMAE
FN-MMAE
Treatment
Total HMGB1 Flourescence in Kidney
4×10^8
3×10^8
2×10^8
1×10^8
0
p<0.0020
p<0.0016
PBS
MMAE
FN-MMAE
Treatment

FIG. 6A (cont.)

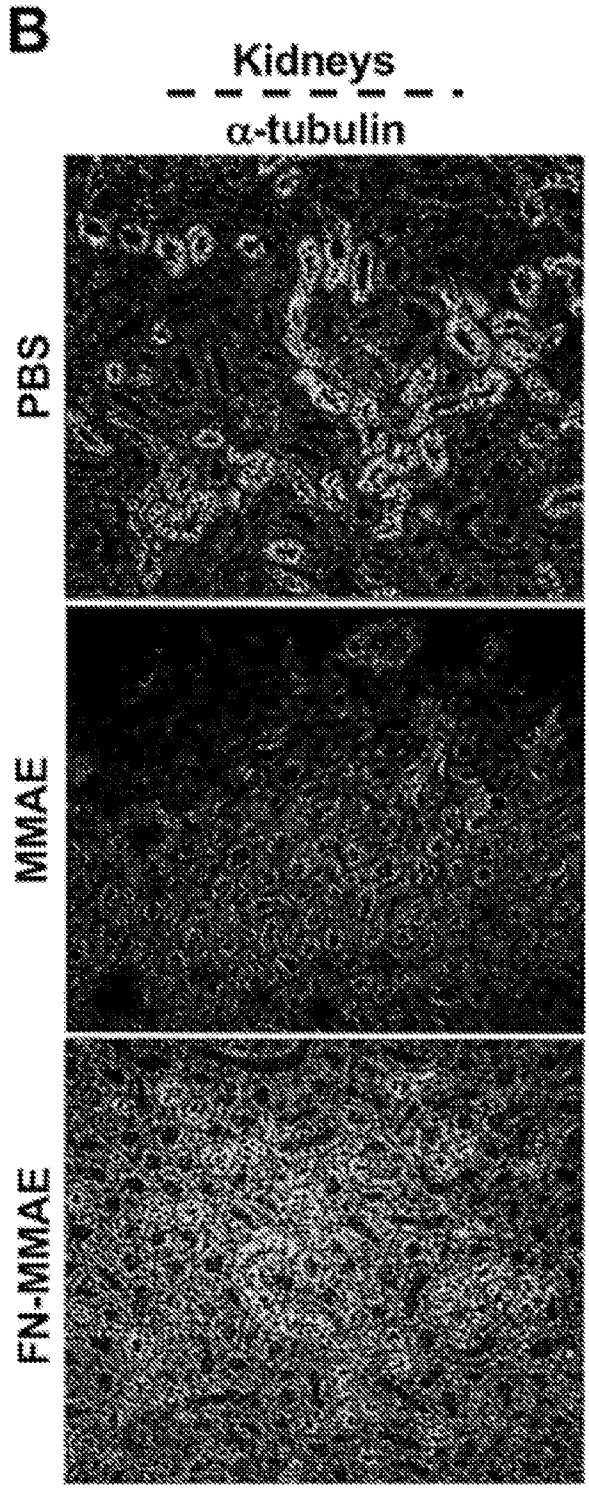
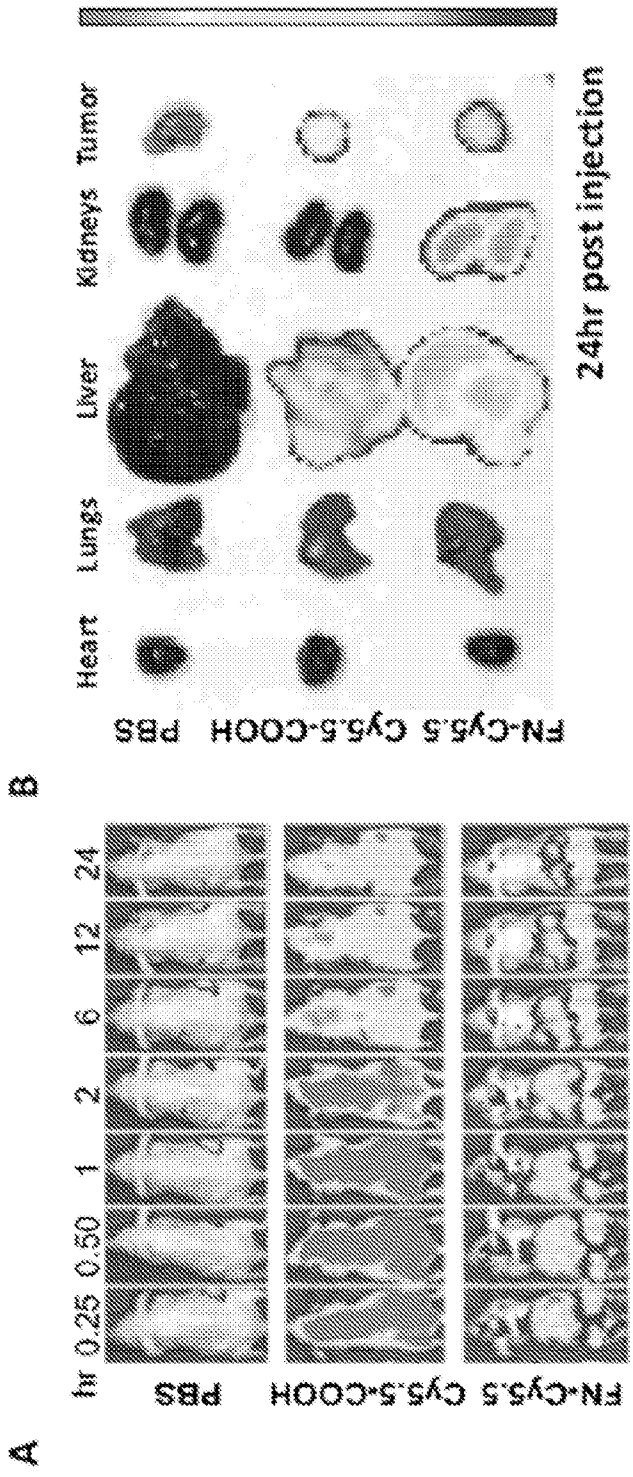


FIG. 6B


A
hr 0.25 0.50 1 2 6 12 24
PBS
Cy5.5-COOH
FN-Cy5.5
B
Heart Lungs Liver Kidneys Tumor
PBS Cy5.5-COOH FN-Cy5.5
24hr post injection

FIGs. 7A-7B

C.

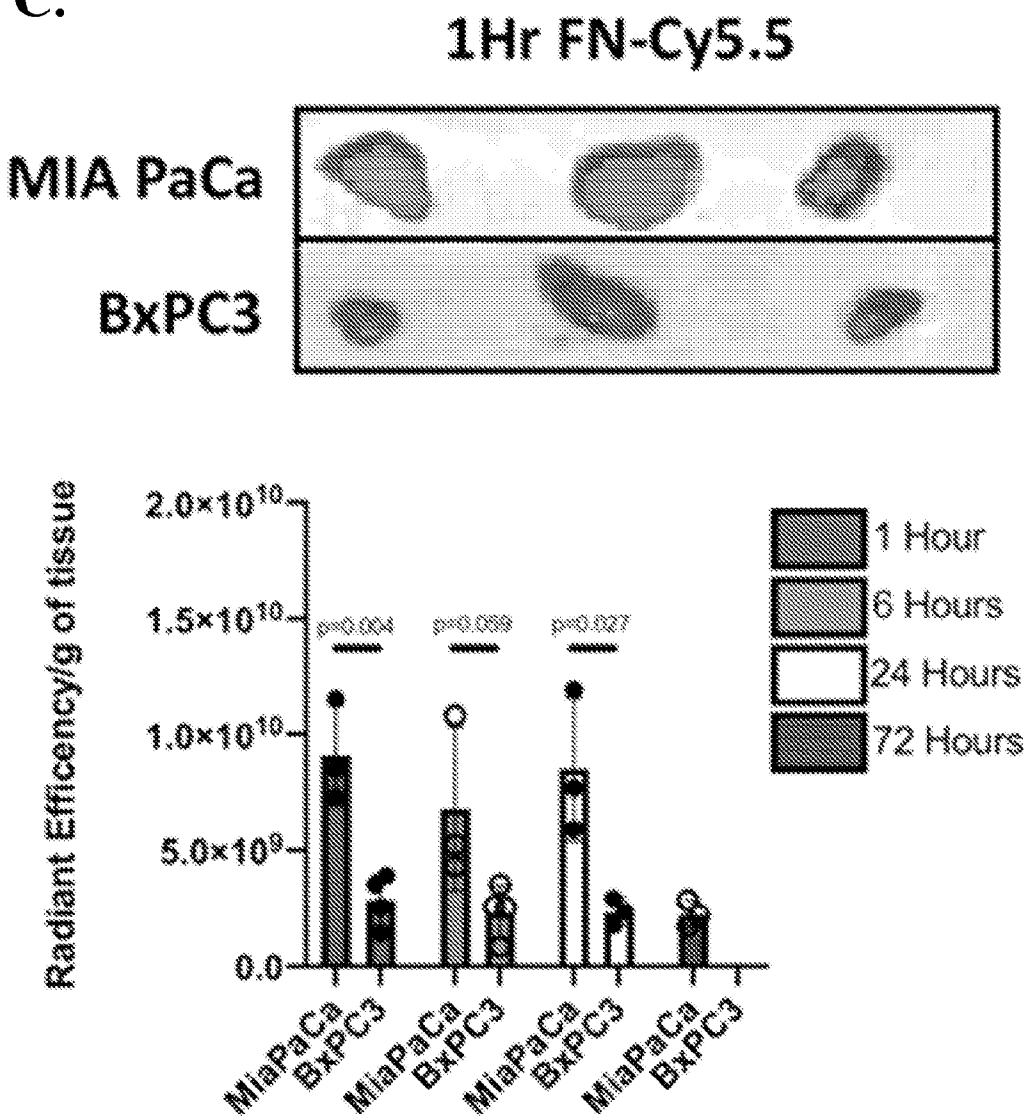
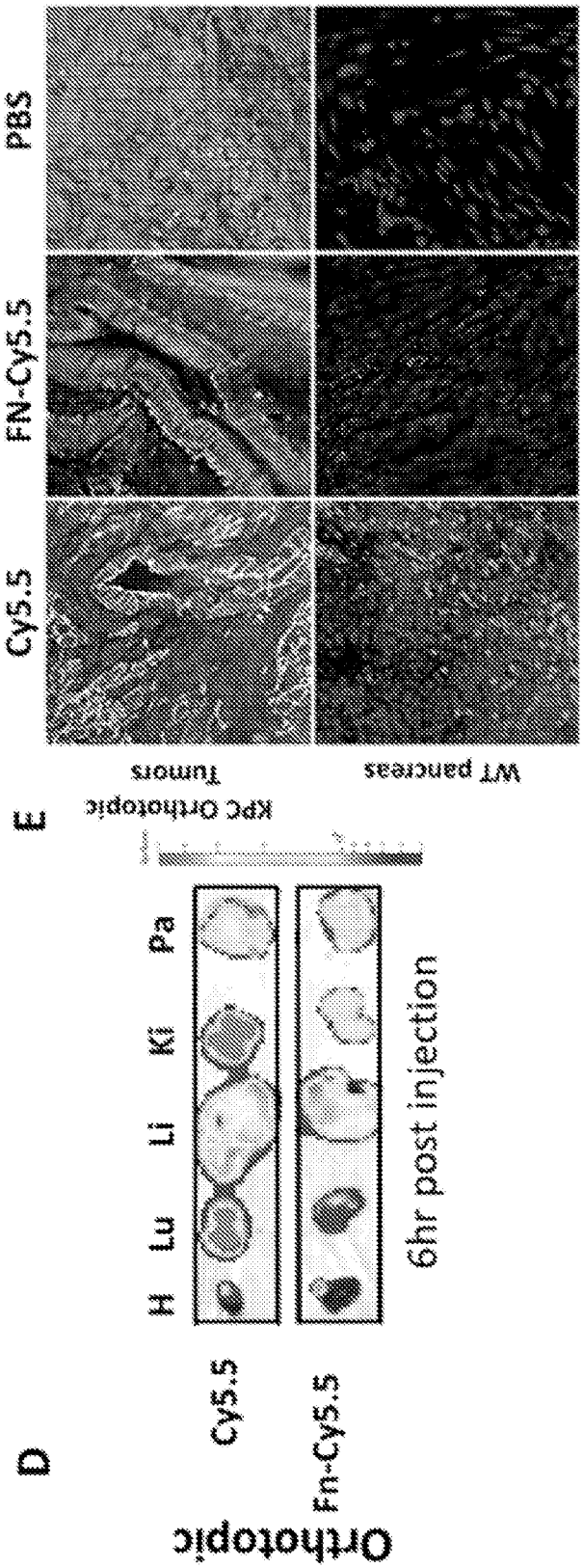
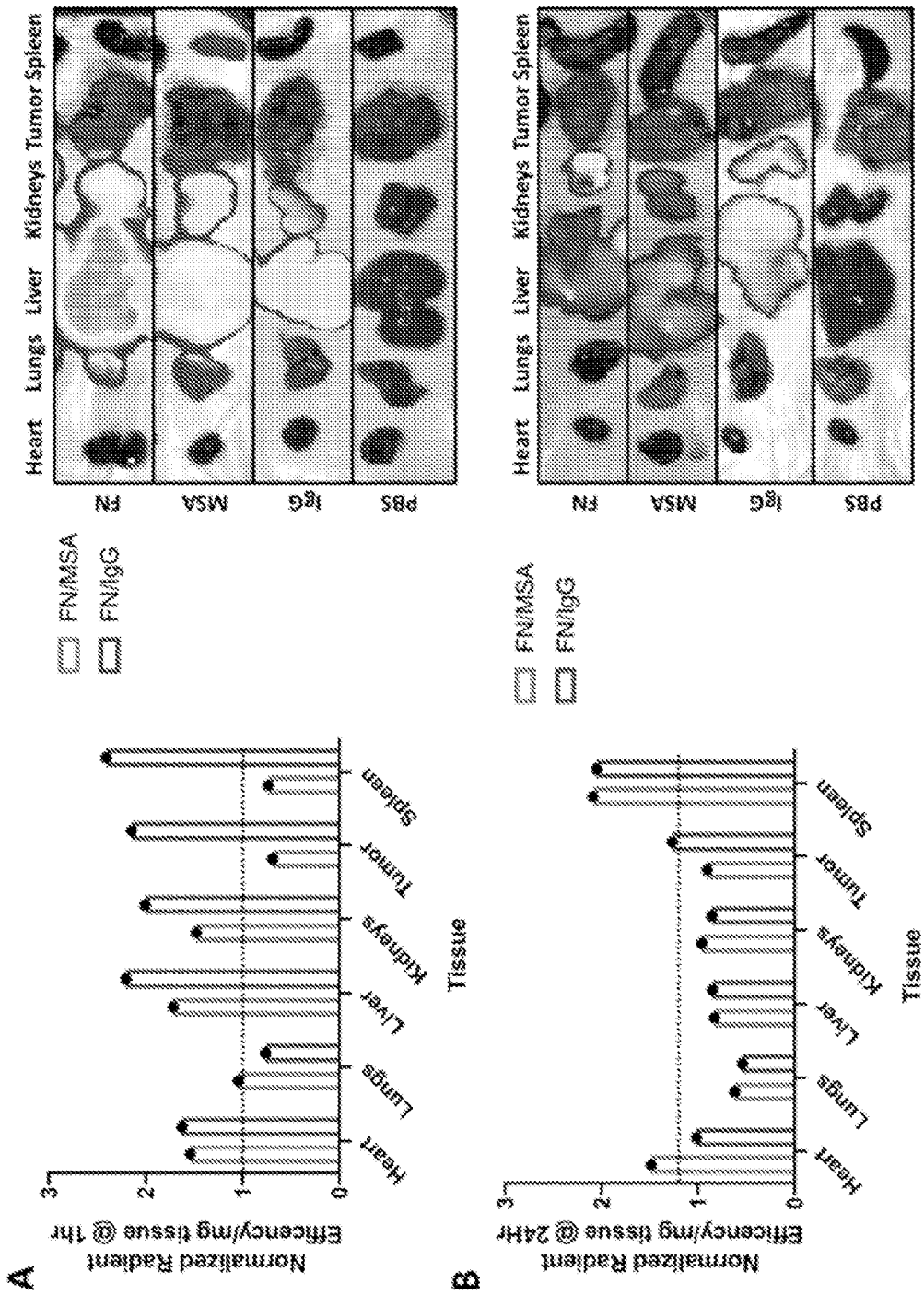

1Hr FN-Cy5.5
MIA PaCa
BxPC3
Radiant Efficency/g of tissue
2.0×10^10
1.5×10^10
1.0×10^10
5.0×10^9
0.0
p=0.004
p=0.009
p=0.027
1 Hour
6 Hours
24 Hours
72 Hours
MiaPaCa
BxPC3
MiaPaCa
BxPC3
MiaPaCa
BxPC3
MiaPaCa
BxPC3

FIG. 7C


D
Orthotopic
Cy5.5
Fn-Cy5.5
H
Lu
Li
Ki
Pa
6hr post injection
E
KPC Orthotopic Tumors
WT pancreas
Cy5.5
FN-Cy5.5
PBS

FIGs. 7D-7E


A
Normalized Radient Efficency/mg tissue @ 1hr
Heart
Lungs
Liver
Kidneys
Tumor
Spleen
Tissue
FN/MSA
FN/IgG
FN
MSA
IgG
PBS
B
Normalized Radient Efficency/mg tissue @ 24hr
Heart
Lungs
Liver
Kidneys
Tumor
Spleen
Tissue
FN/MSA
FN/IgG
FN
MSA
IgG
PBS

FIGs. 8A-8B

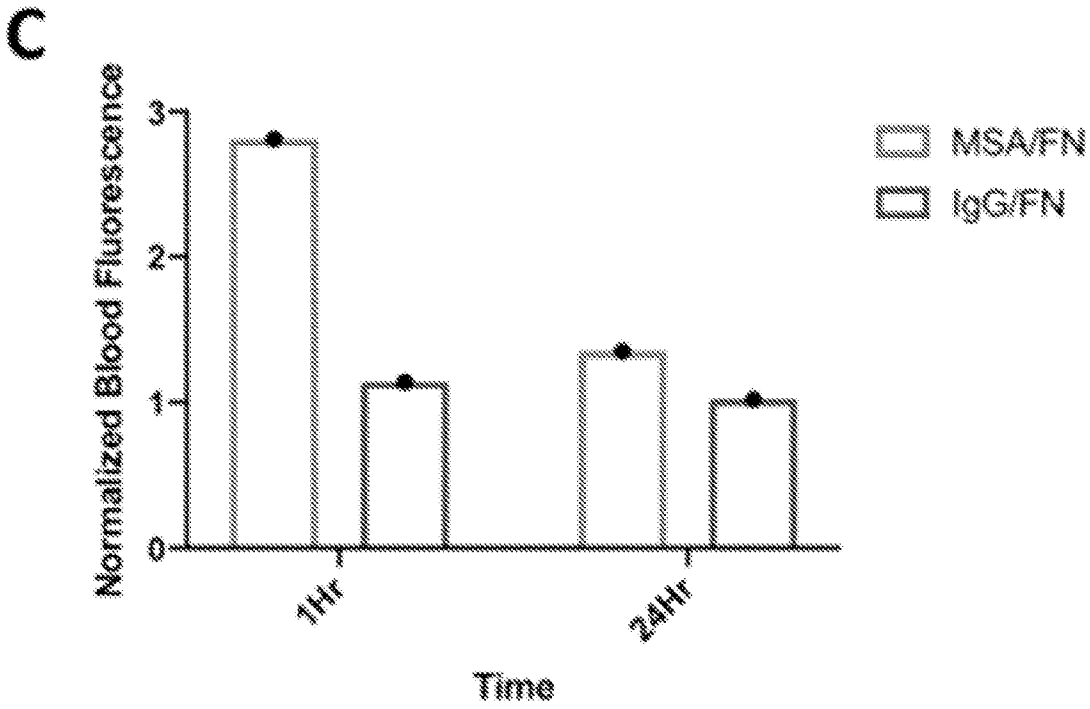

C
Normalized Blood Fluorescence
0
1
2
3
1Hr
24Hr
Time
MSA/FN
IgG/FN

FIG. 8C

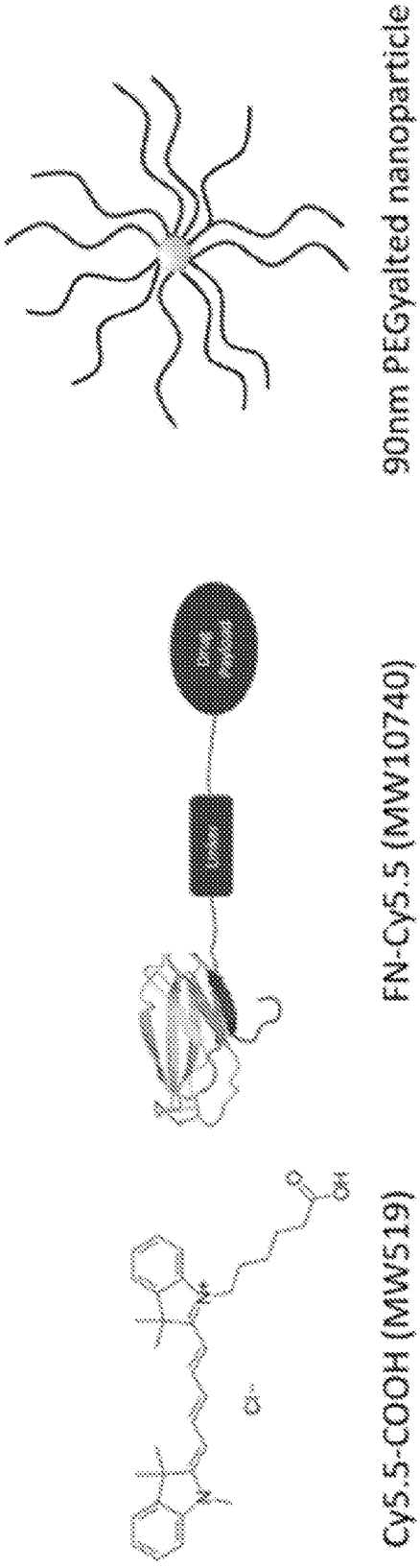

90nm PEGylated nanoparticle
FN-Cy5.5 (MW10740)
Cy5.5-COOH (MW519)

FIG. 9A

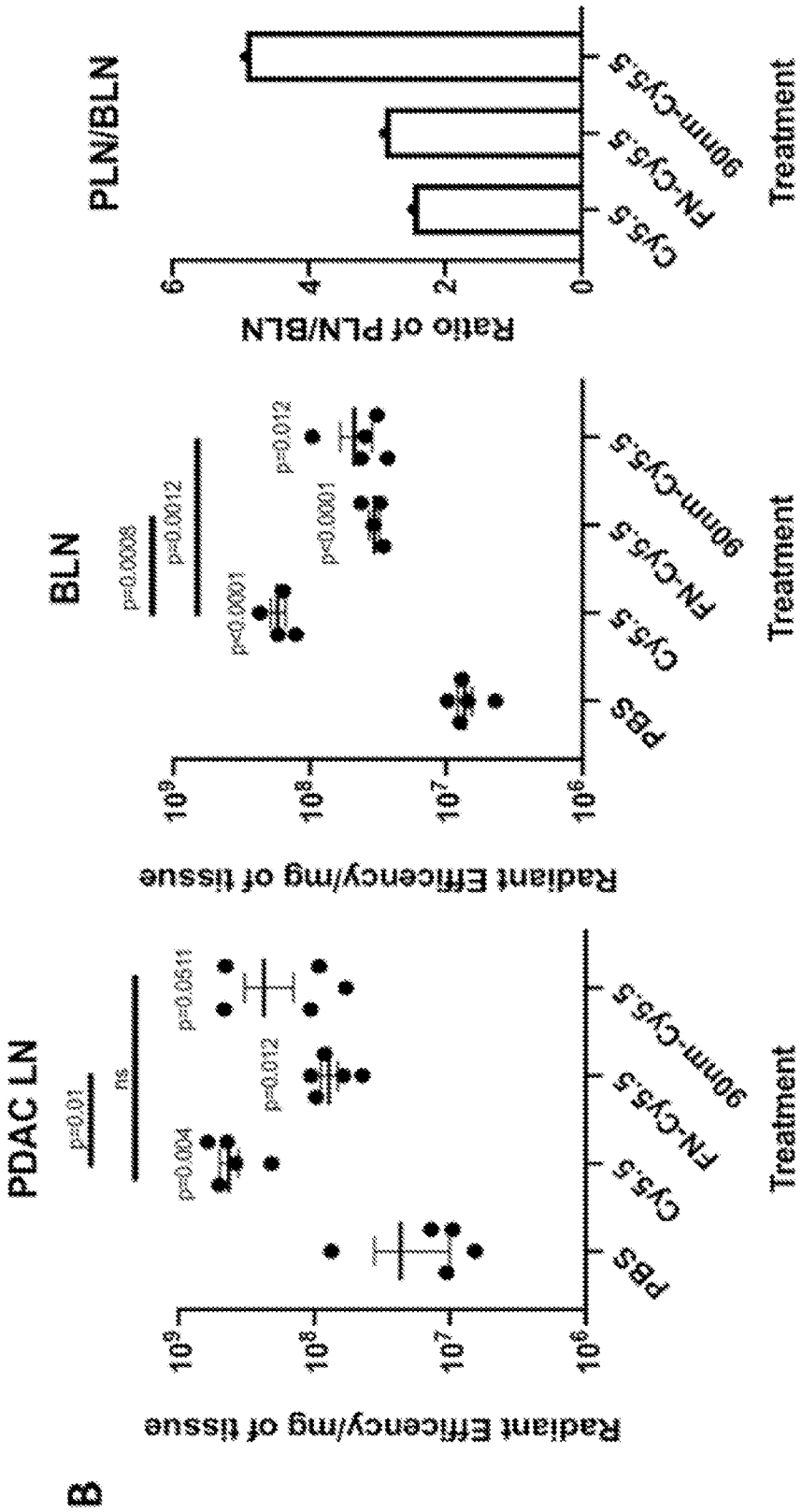

B
PDAC LN
p=0.03
ns
p=0.0004
p=0.0611
p=0.012
Radiant Efficency/mg of tissue
Treatment
PBS
Cy5.5
FN-Cy5.5
90nm-Cy5.5
BLN
p=0.0008
p=0.0012
p<0.0001
p<0.0001
p=0.012
PLN/BLN
Ratio of PLN/BLN

FIG. 9B

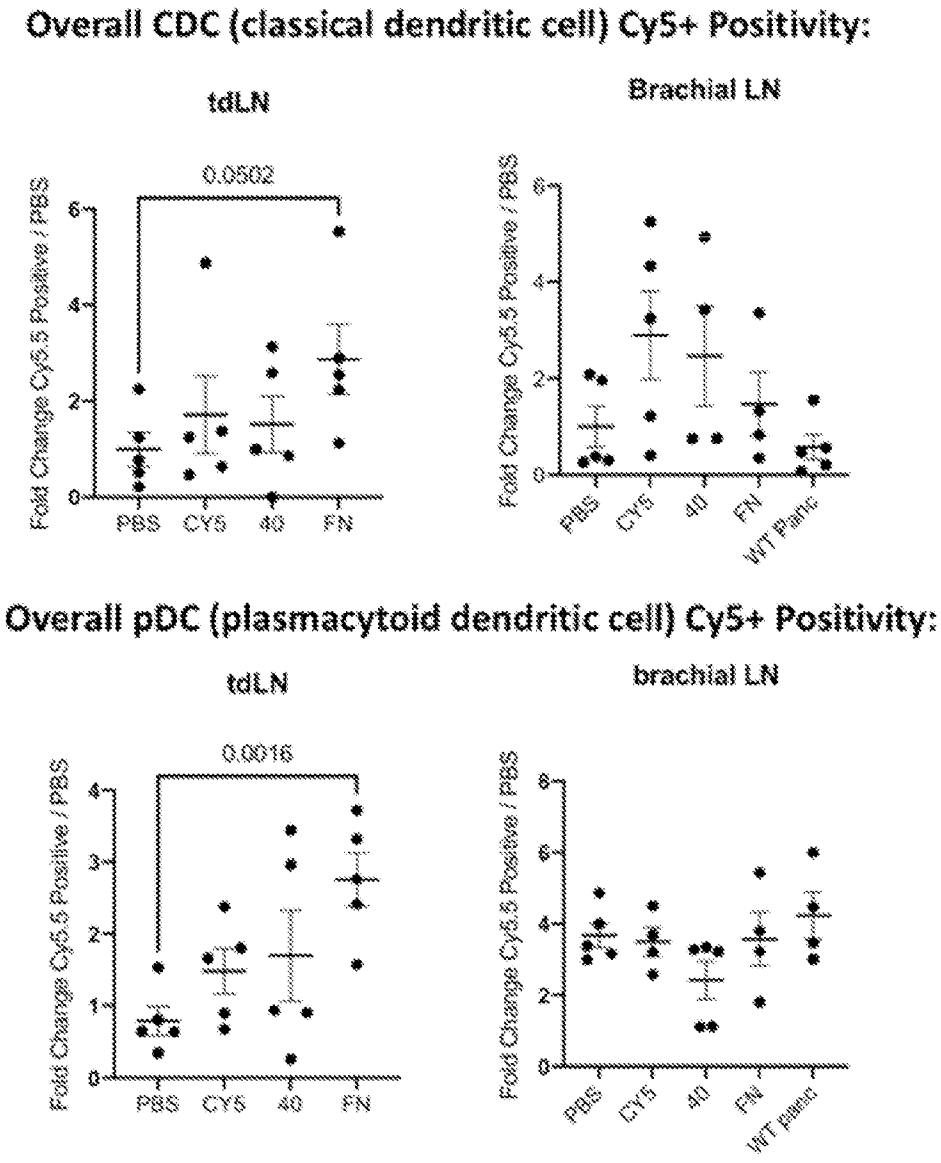

Overall CDC (classical dendritic cell) Cy5+ Positivity:
tdLN
Brachial LN
0.0502
Fold Change Cy5.5 Positive / PBS
PBS
CY5
40
FN
WT Panc
Overall pDC (plasmacytoid dendritic cell) Cy5+ Positivity:
tdLN
brachial LN
0.0016
Fold Change Cy5.5 Positive / PBS
PBS
CY5
40
FN
WT panc

FIG. 9C

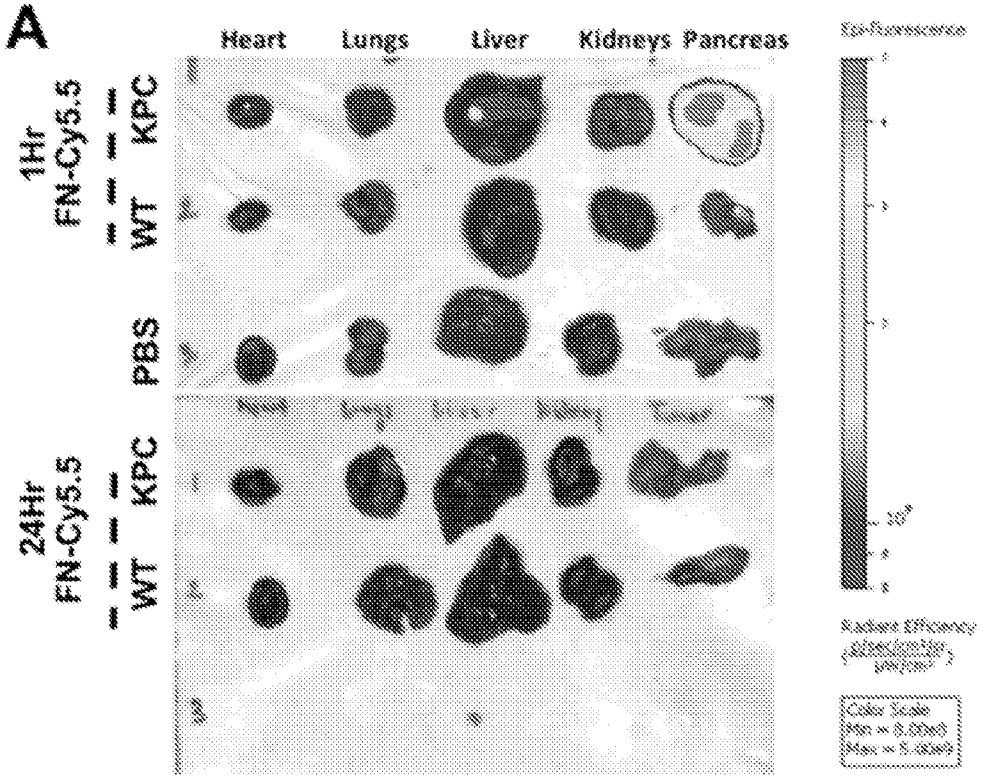

A
Heart
Lungs
Liver
Kidneys
Pancreas
1Hr
FN-Cy5.5
KPC
WT
PBS
24Hr
FN-Cy5.5
KPC
WT

FIG. 10A

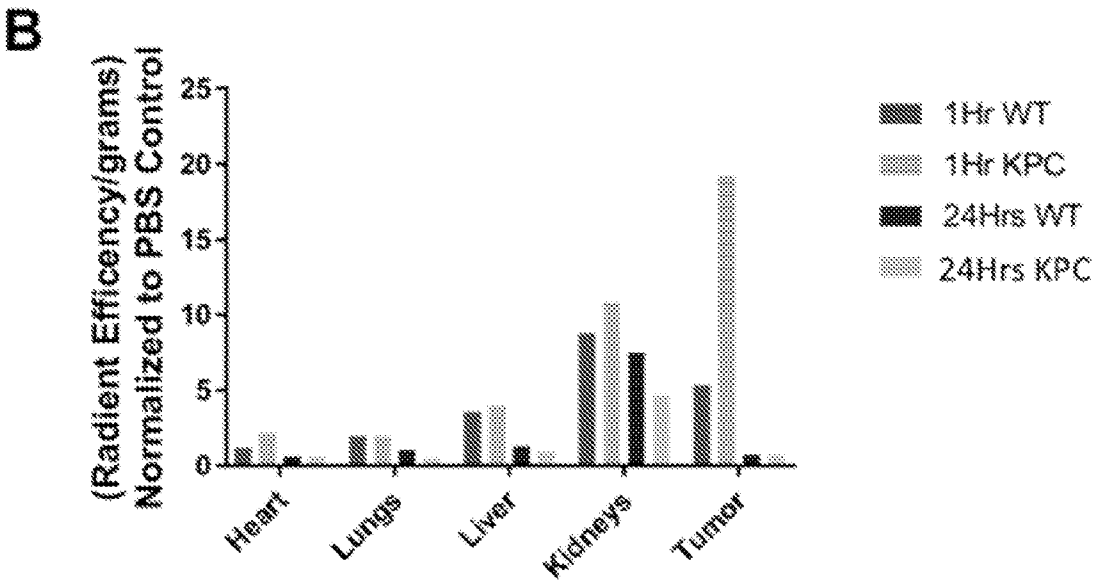
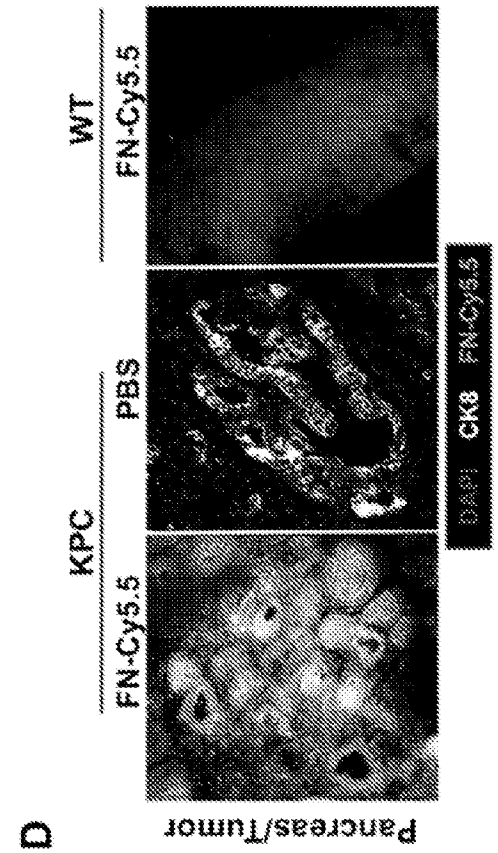
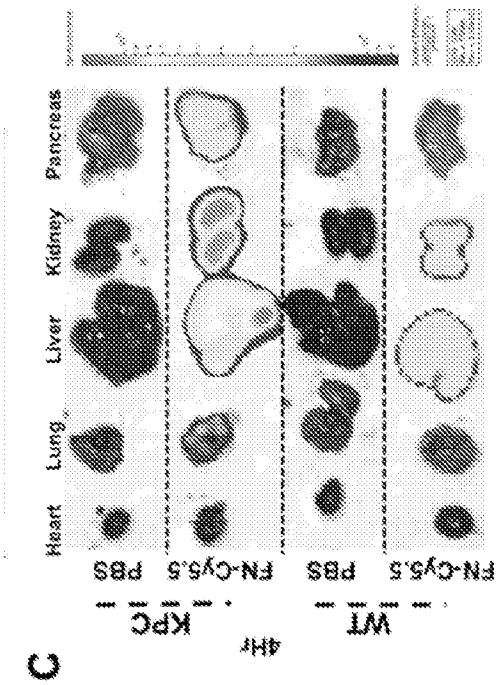

B
(Radient Efficency/grams)
Normalized to PBS Control
25
20
15
10
5
0
Heart
Lungs
Liver
Kidneys
Tumor
1Hr WT
1Hr KPC
24Hrs WT
24Hrs KPC

FIG. 10B


D
Pancreas/Tumor
KPC
FN-Cy5.5
PBS
WT
FN-Cy5.5
DAPI CK8 FN-Cy5.5


C
4Hr
KPC
WT
PBS
FN-Cy5.5
PBS
FN-Cy5.5
Heart
Lung
Liver
Kidney
Pancreas

FIGs. 10C-10D

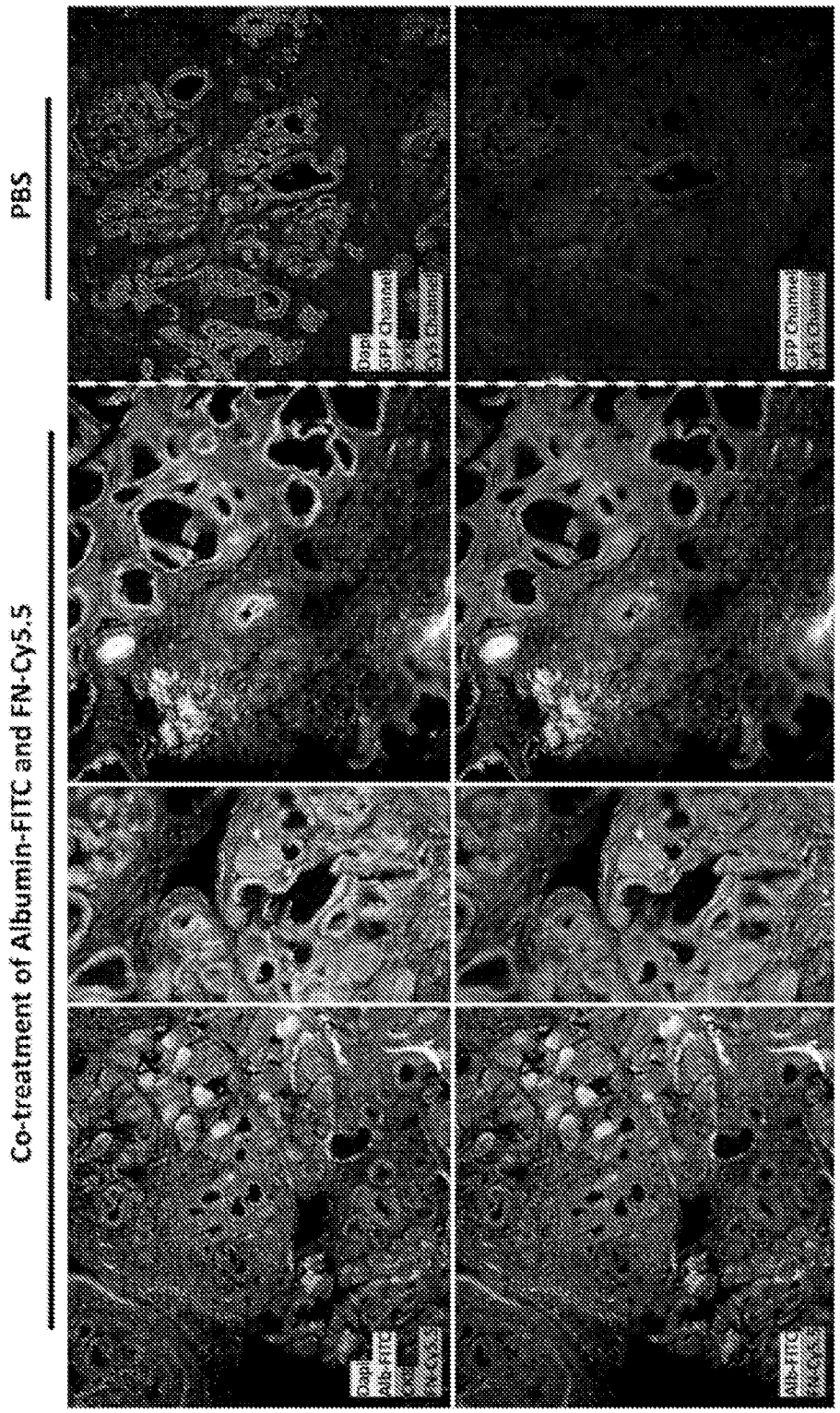

PBS
Co-treatment of Albumin-FITC and FN-Cy5.5
Dapi
GFP Channel
Cy5 Channel
Alb-FITC

FIG. 11

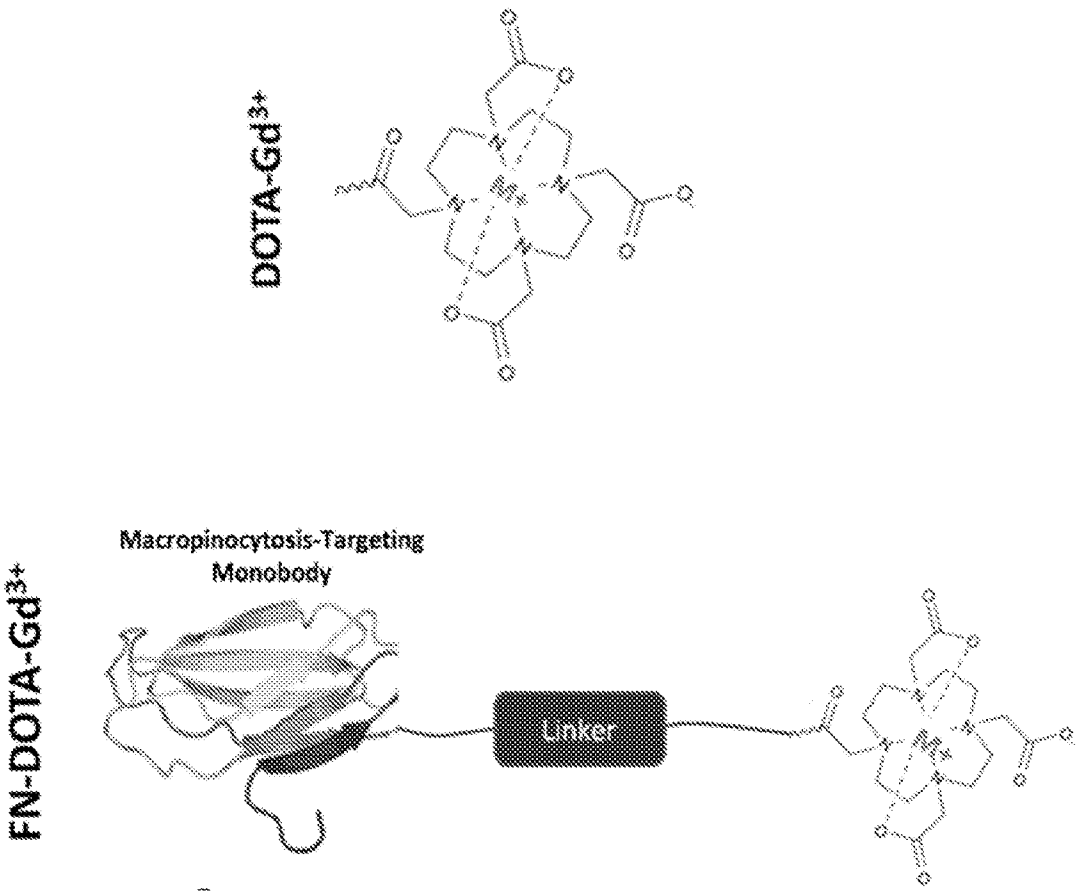

DOTA-Gd3+
FN-DOTA-Gd3+
Macropinocytosis-Targeting Monobody
Linker

FIG. 12A

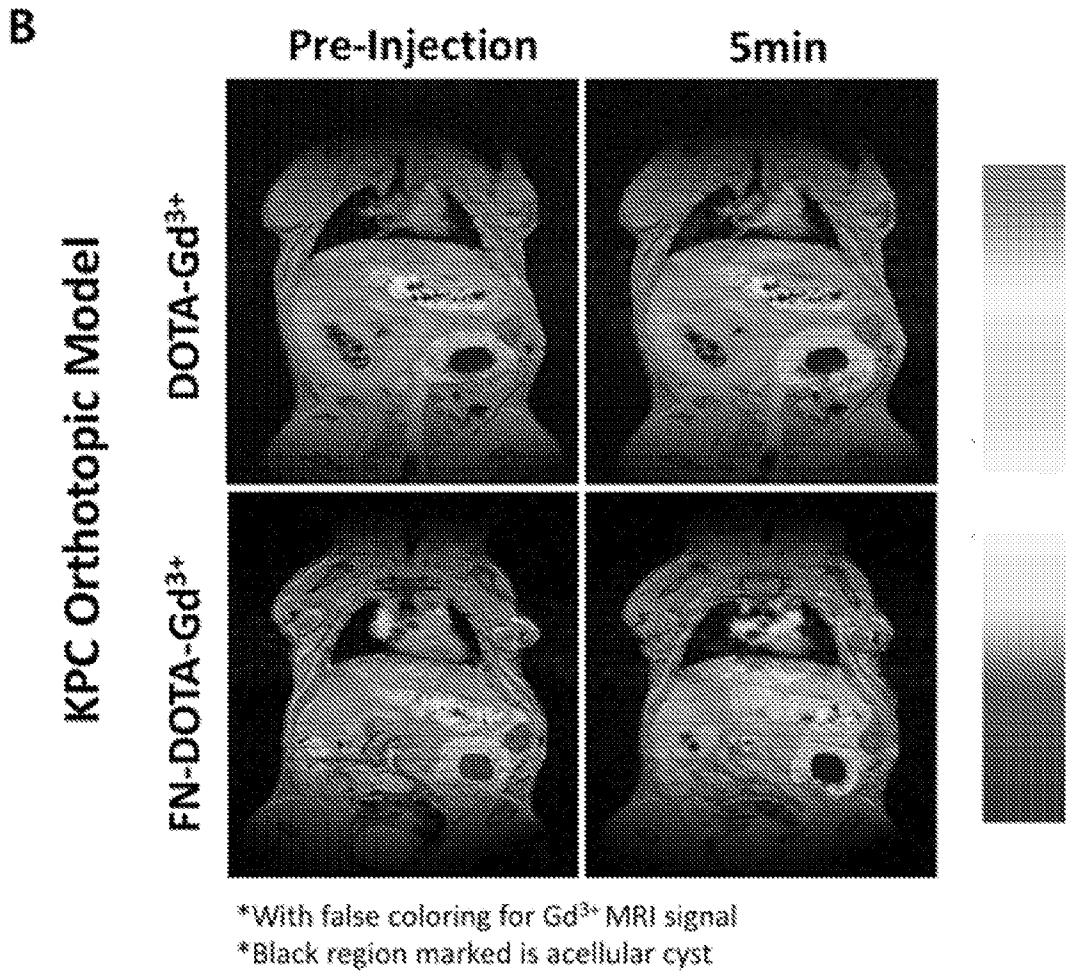

B
Pre-Injection
5min
KPC Orthotopic Model
DOTA-Gd3+
FN-DOTA-Gd3+
*With false coloring for Gd3+ MRI signal
*Black region marked is acellular cyst

FIG. 12B

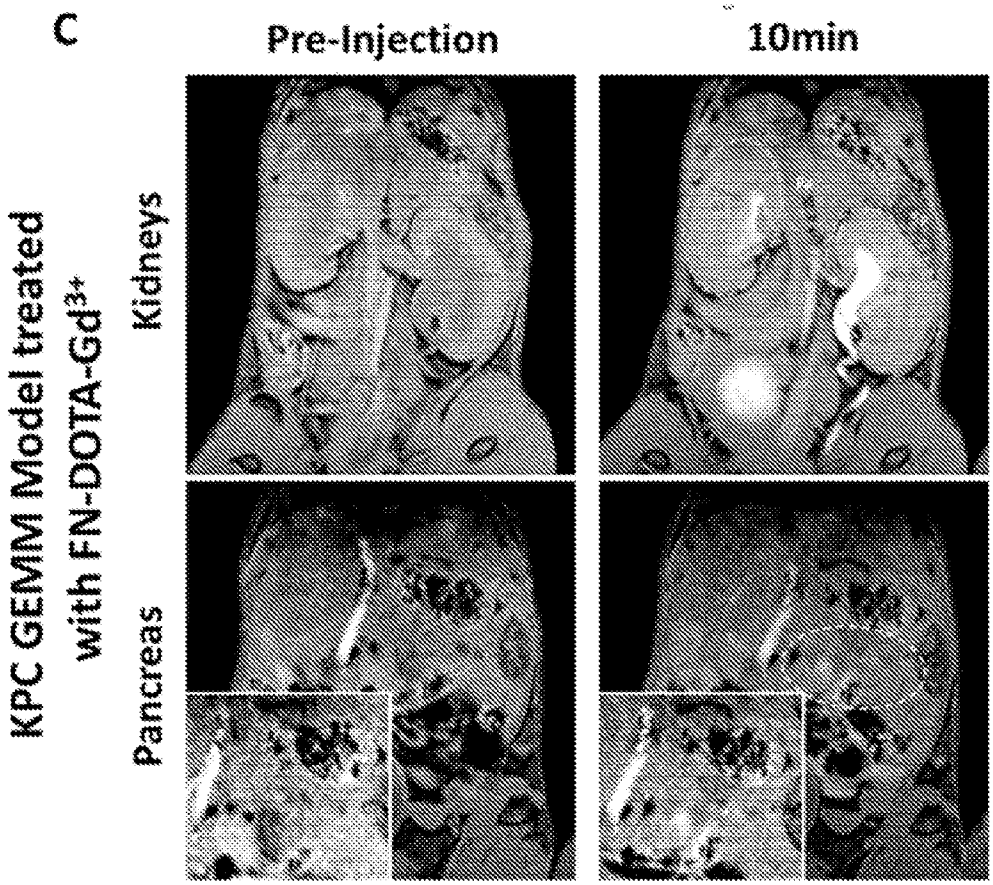

C
Pre-Injection
10min
KPC GEMM Model treated with FN-DOTA-Gd3+
Kidneys
Pancreas

FIG. 12C

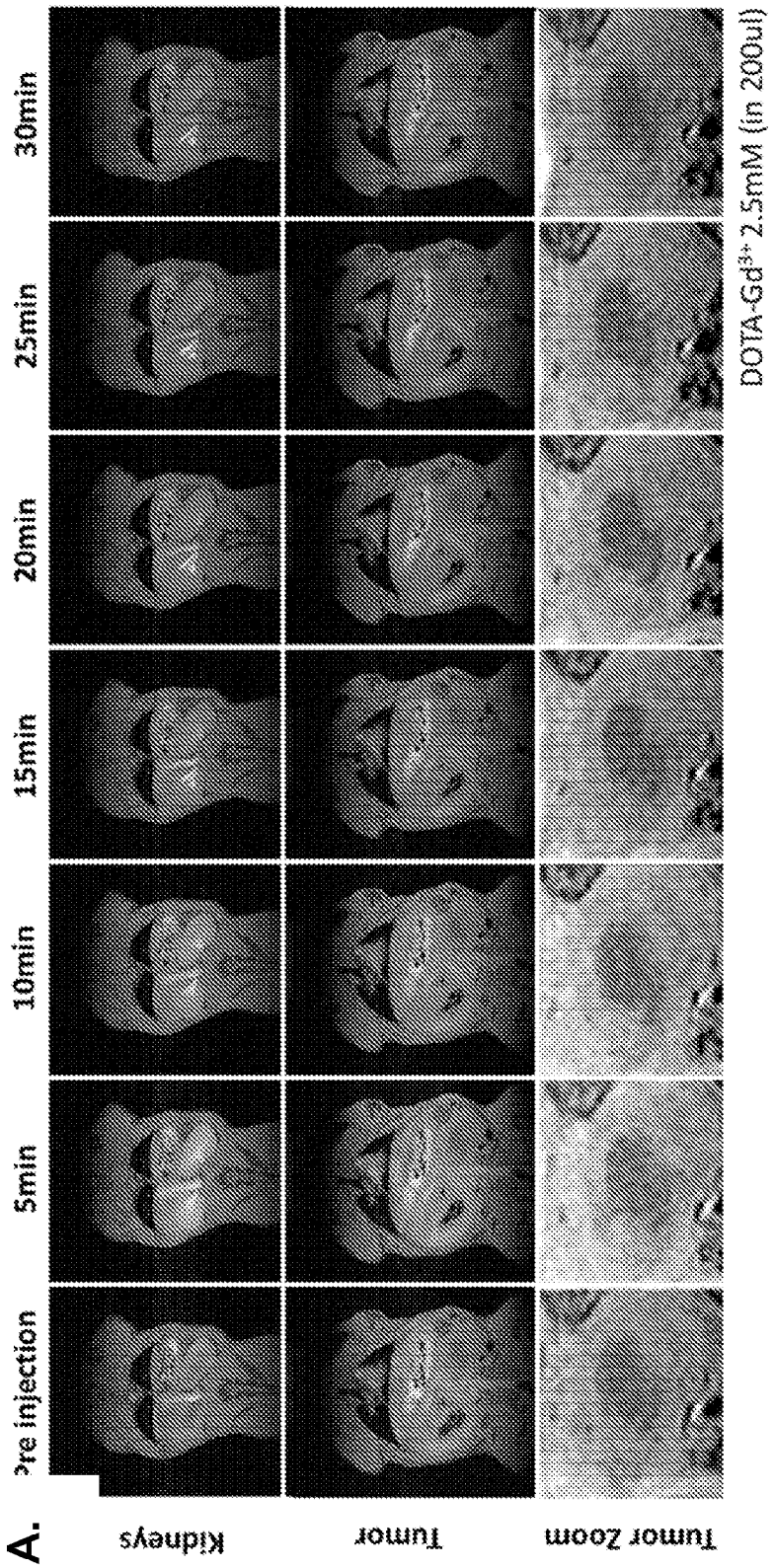

A.
Kidneys
Tumor
Tumor Zoom
Pre injection
5min
10min
15min
20min
25min
30min
DOTA-Gd3+ 2.5mM (in 200ul)

FIG. 13A

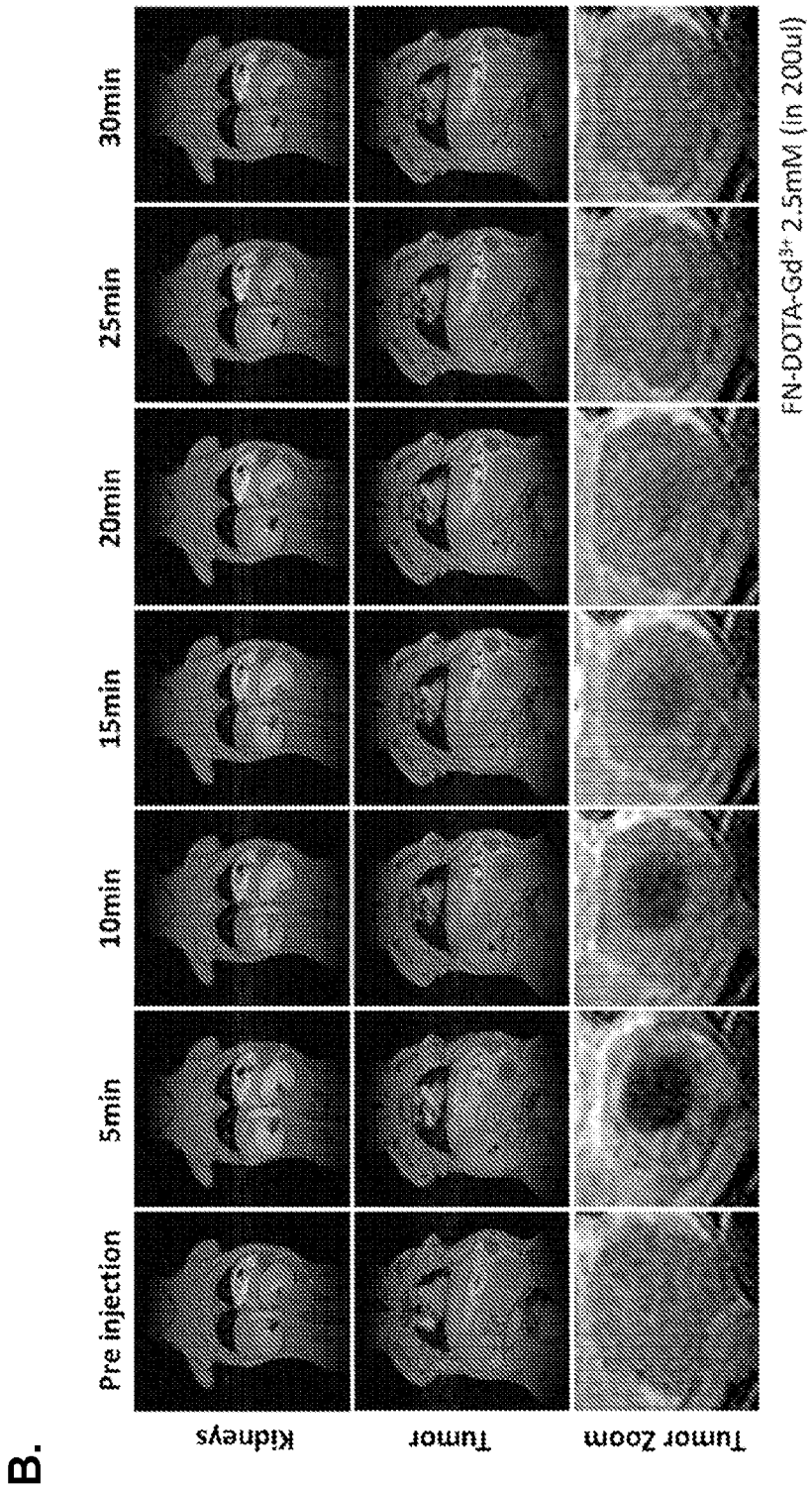

B.
Pre injection
5min
10min
15min
20min
25min
30min
Kidneys
Tumor
Tumor Zoom
FN-DOTA-Gd3+ 2.5mM (in 200ul)

FIG. 13B

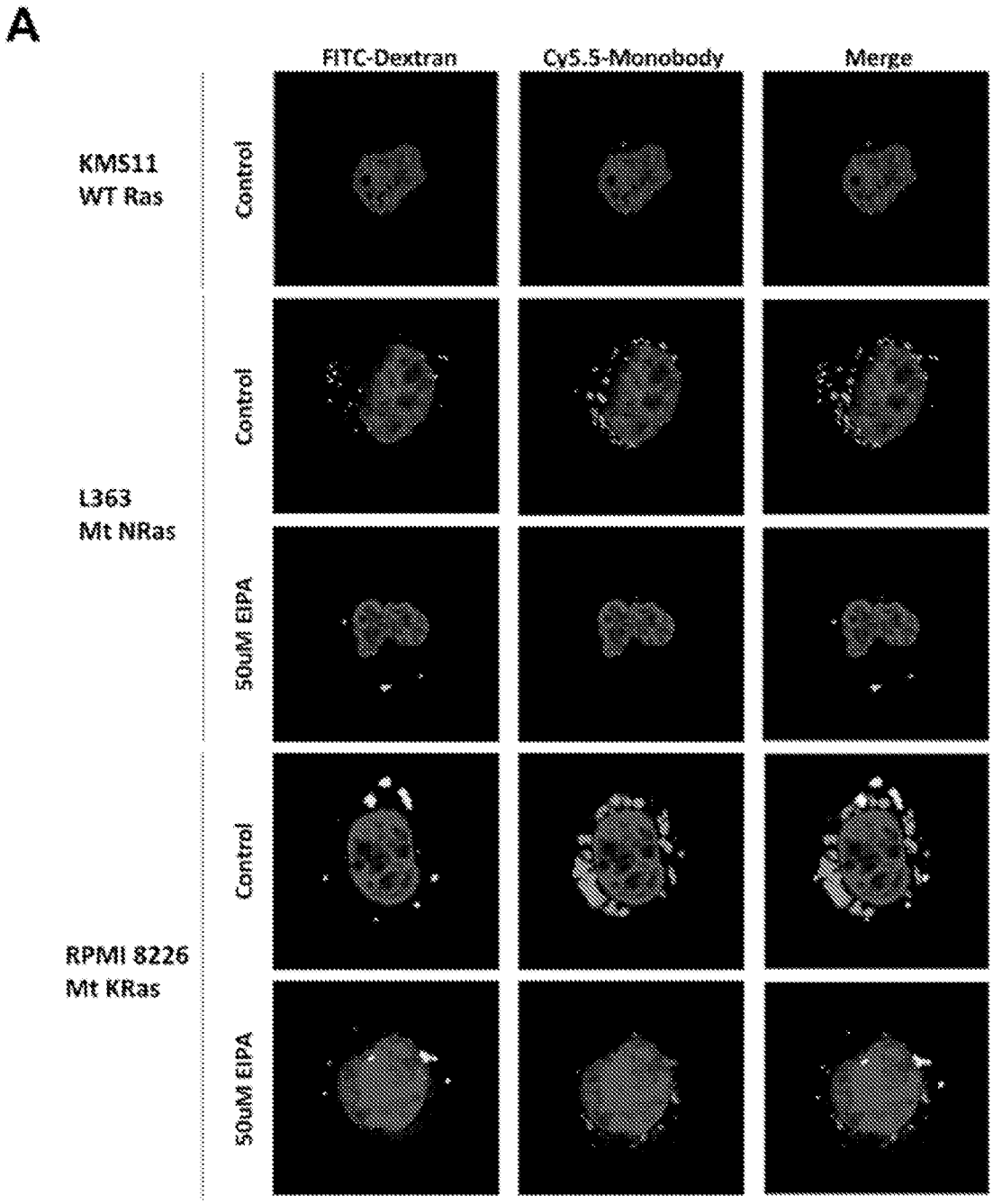
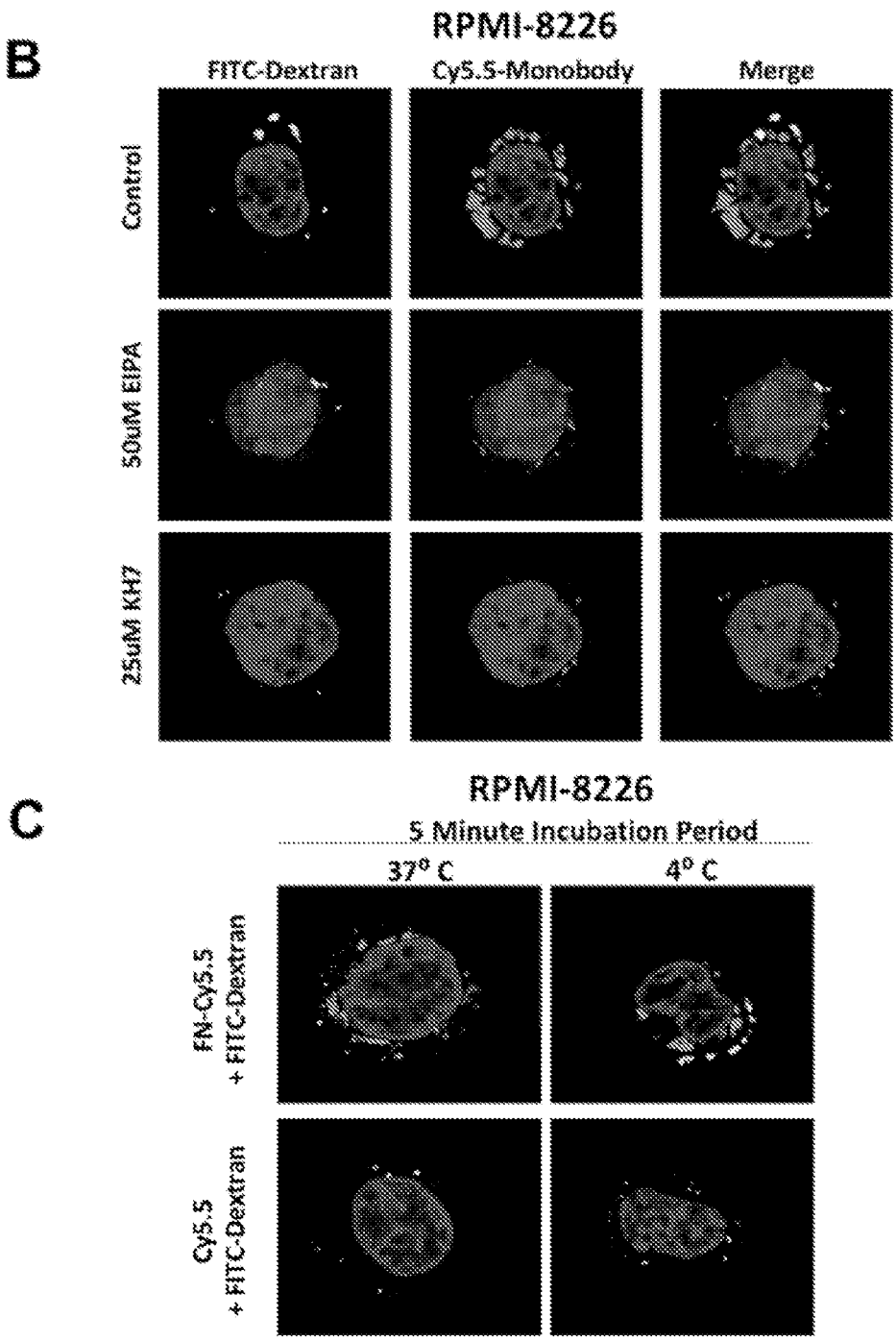

A
FITC-Dextran
Cy5.5-Monobody
Merge
KMS11
WT Ras
Control
L363
Mt NRas
Control
50uM EIPA
RPMI 8226
Mt KRas
Control
50uM EIPA

FIG. 14A


B
RPMI-8226
FITC-Dextran
Cy5.5-Monobody
Merge
Control
50uM EIPA
25uM KH7
C
RPMI-8226
5 Minute Incubation Period
37° C
4° C
FN-Cy5.5
+ FITC-Dextran
Cy5.5
+ FITC-Dextran

FIGs. 14B-14C

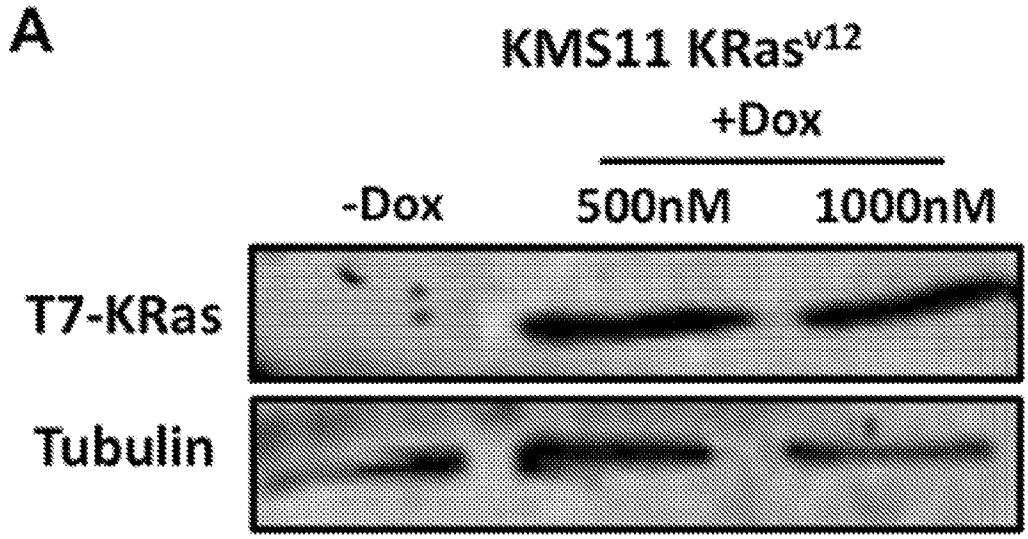


FIG. 15A

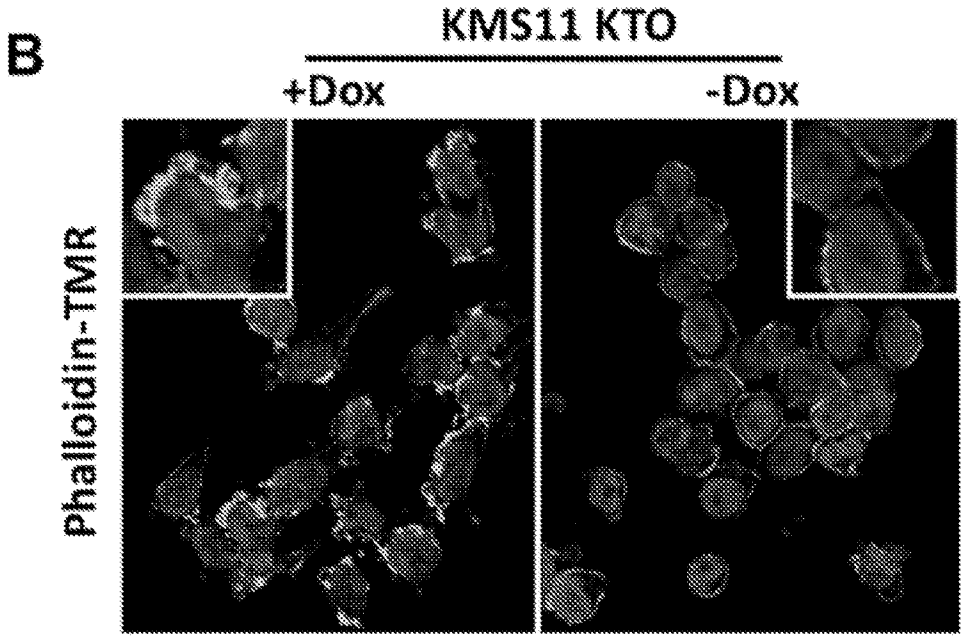


FIG. 15B

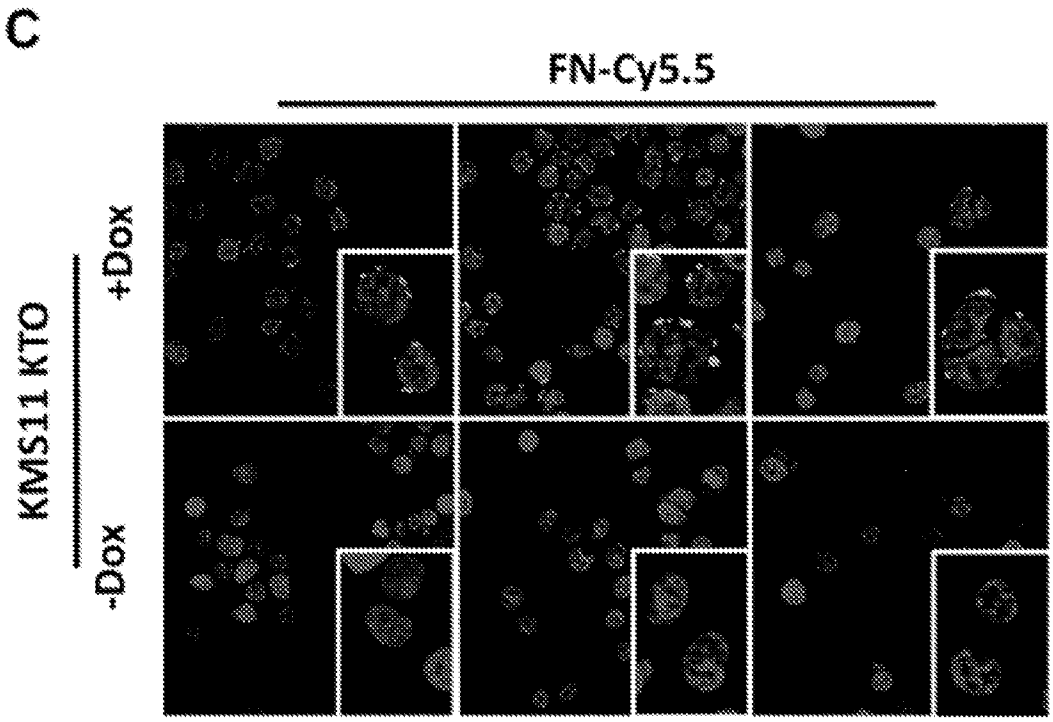


FIG. 15C

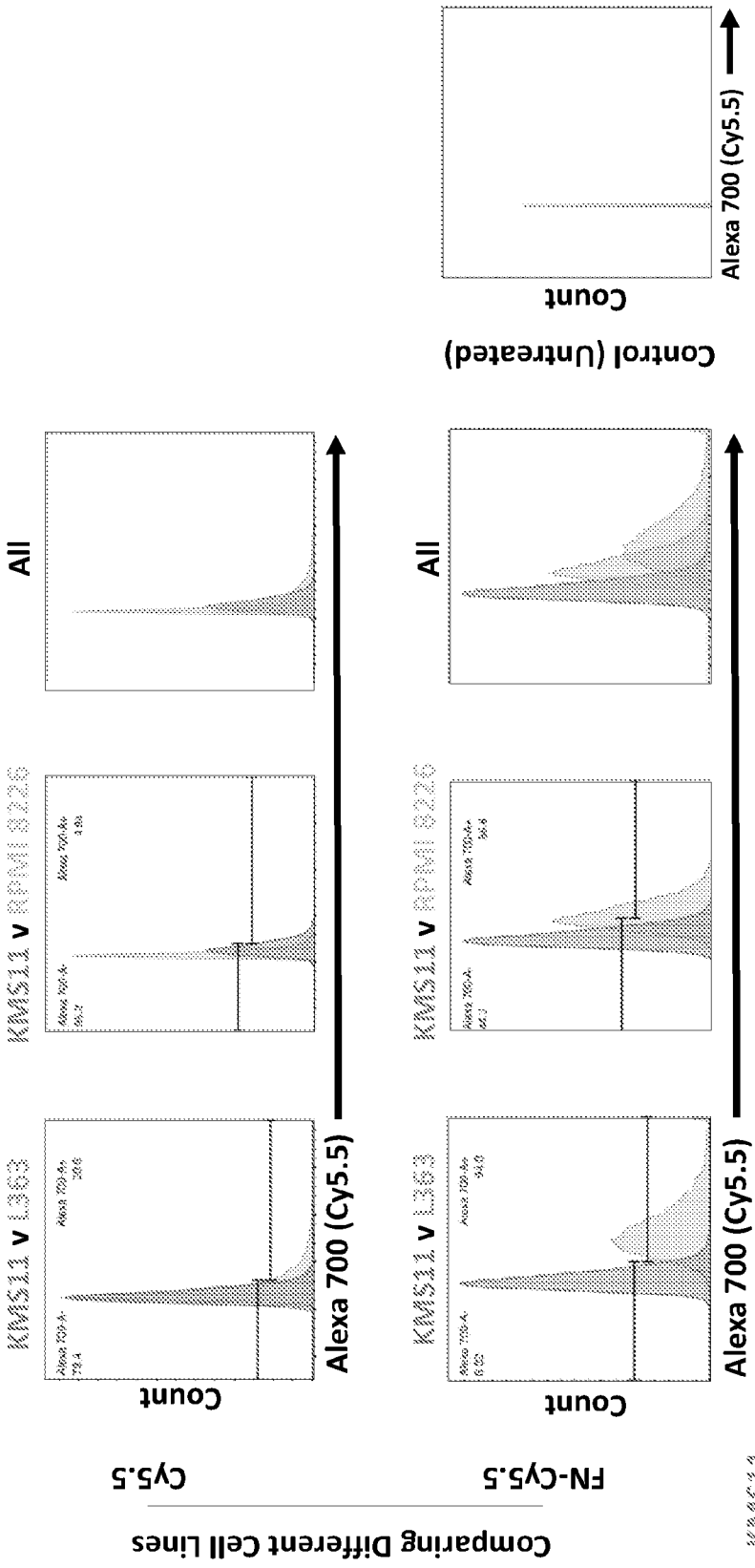
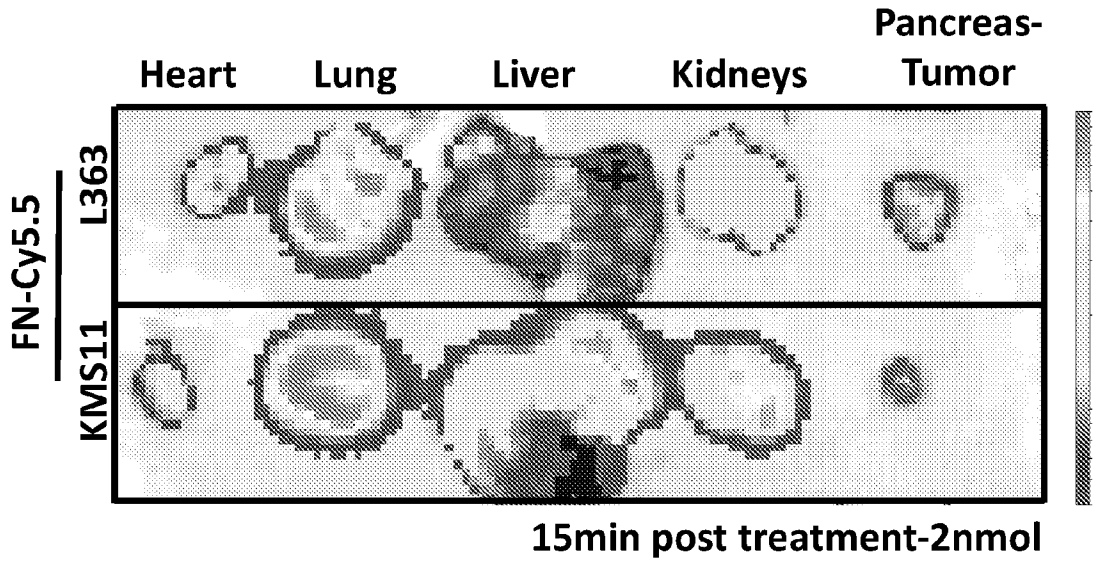

Comparing Different Cell Lines
Cy5.5
FN-Cy5.5
KMS11
L363
RPMI 8226
KMS11 v L363
KMS11 v RPMI 8226
All
Count
Alexa 700 (Cy5.5)
Control (Untreated)

FIG. 16


Heart
Lung
Liver
Kidneys
Pancreas-Tumor
FN-Cy5.5
L363
KMS11
15min post treatment-2nmol

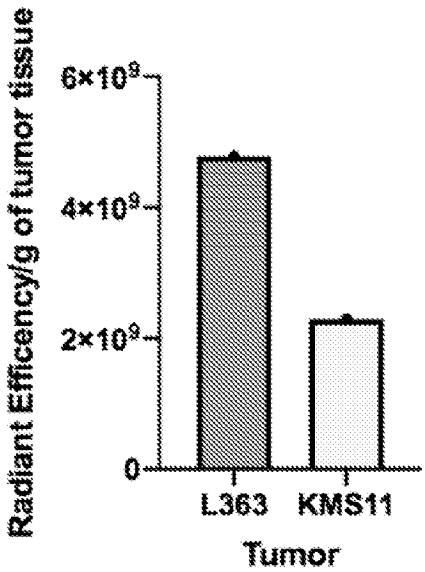

Radiant Efficency/g of tumor tissue
6×10⁹
4×10⁹
2×10⁹
0
L363
KMS11
Tumor

FIG. 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US21/58807

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 47/64; A61K 38/39; C07K 14/78; A61P 19/08; A61P 25/28 (2021.01)

CPC - A61K 47/6435; C07K 14/78; A61K 47/64; A61K 38/39; A61P 19/08; A61P 25/28

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.
X	US 2011/0059076 A1 (MCDONAGH, C ET AL.) 10 March 2011; paragraphs [0095], [0138], [0189]-[0191]	80-81
X --- A	WO 2010/096394 A2 (REDWOOD BIOSCIENCES, INC., ET AL.) 26 August 2010; paragraphs [0076], [00152]	82 --- 1-4, 9
X	US 2018/0334491 A1 (ALBUMEDIX LTD) 22 November 2018; paragraphs [0029], [0046], [0056], [0154], [0157]	83
A	US 2018/0127485 A1 (NOVARTIS AG) 10 May 2018; [0083], [0093], [0095], [0207]	1-4, 9
A	(WOJICK, J ET AL.). "A potent and highly specific FN3 monobody inhibitor of the Abl SH2 domain." pages 519-527. Nature Structural and Molecular Biology. Vol. 17, No. 4. April 2010; page 3, paragraph 4; DOI: 10.1038/nsmb.1793	1-4, 9

☐ 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
14 March 2022 (14.03.2022)	MAR 24 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US21/58807

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US21/58807

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.: 5-8, 10-79
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:

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.:

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.