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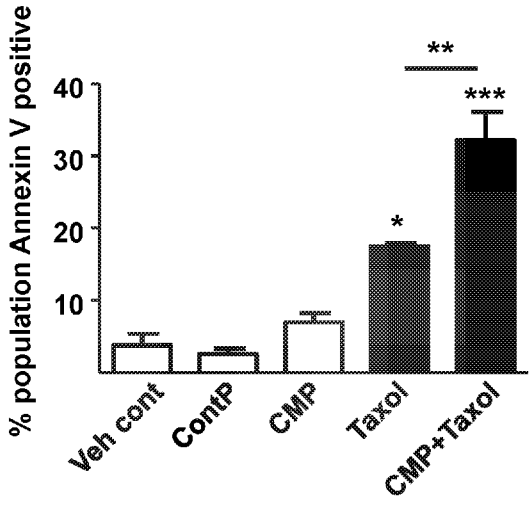

FIG. 9

(57) Abstract: The present invention provides a method of treating cancer in a subject. In certain embodiments, the method comprises administering to the subject a claudin blocking/disrupting agent and at least one additional compound. In other embodiments, the at least one additional compound is a chemotherapeutic agent including but not limited to paclitaxel.

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TITLE OF THE INVENTION

Compositions and Methods for Improving Ovarian Tumor Cell Sensitivity to Drugs

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Patent Application No. 62/713,865, filed August 2, 2018, the contents of which are incorporated by reference herein in their entirety.

BACKGROUND OF THE INVENTION

Chemoresistance is a significant obstacle in successful treatment for ovarian cancer patients. Due to the lack of adequate early detection screening, the majority (> 85%) of patients with epithelial ovarian cancer (EOC) are diagnosed at advanced stages of disease. Delayed detection means that ovarian tumor cells have disseminated beyond their site of origin (fallopian tube or uterus) into the peritoneal cavity. The standard of care for these patients involves surgical debulking of the tumor followed by adjuvant platinum- and taxane- based chemotherapies (cisplatin/carboplatin and paclitaxel/docetaxel). One third of ovarian cancer patients have tumors that do not respond to initial chemotherapy, and approximately 75% of the unresponsive patients will have disease recurrence. Those with recurrent disease succumb to the development of tumor resistance to currently available chemotherapeutic drugs.

There are several proposed mechanisms of chemoresistance that include both *de novo* pathways that inherently make tumor cells resistant and acquired pathways that are upregulated in response to repeated chemotherapeutic insult. These pathways include, but are not limited to, drug transporters (*i.e.* P-glycoprotein, organic anion-transporting polypeptides transporters, and so forth) that either inhibit drug uptake or enhance drug efflux epigenetic changes (*i.e.* MLH1 and Tap73 methylation, miR-214 and miR-376c upregulation) that prevent expression of anti-tumorigenic genes; expression or mutations in proteins that inhibit efficient execution of apoptotic cell death (*i.e.* p53, Bcl-2 family proteins, PTEN); and disruption of cytoskeletal organization that prevent binding of microtubule-targeting agents.

Claudin-4 is a member of a large family of transmembrane proteins, of which there are 27 different subtypes. Claudins are known for their canonical role in tight junctions, providing specific paracellular barrier properties to epithelium. Although claudin-4 has been shown to alter

permeability in epithelial cells in culture, its expression in human and mouse tissue is rarely localized to tight junctions, and instead is observed along basolateral membranes and throughout the cytosol. These observations suggest a non-canonical role for claudin-4. Supporting this notion, claudin-4 knockout (KO) mice are phenotypically normal with only a subclinical increase in permeability to small molecules in lung epithelium, determined by *in vitro* analysis. The claudin-4 KO mice, however, exhibit a reduced capacity for wound healing. Furthermore, claudin-4 is significantly upregulated in multiple epithelia-derived cancer cells that lack traditional tight junction structures. High claudin-4 expression has, in fact, been associated with a more phenotypically aggressive ovarian cancer cell (chemoresistant, highly mobile, and stem-like).

Accordingly, there is a need in the art for novel therapeutics to more effectively prevent chemoresistance in cancer patients, such as those suffering from ovarian cancer. The present invention provides novel methods and compositions for addressing this unmet need.

SUMMARY OF THE INVENTION

In certain embodiments, the present invention provides a method of treating cancer in a subject, the method comprising administering to the subject a claudin blocking/disrupting agent and at least one additional chemotherapeutic agent. In some embodiments, the claudin blocking/disrupting agent is selected from the group consisting of a mimetic peptide, blocking peptide, antibody, peptidomimetic, antisense nucleic acid, ribozyme, or a small molecule chemical compound. In some embodiments, the claudin blocking/disrupting agent mimics or blocks activity of the second extracellular loop of at least one of claudin-3, claudin-4, claudin-7, and claudin-8. In some embodiments, the claudin blocking/disrupting agent comprises a peptide of amino acid sequence DYFNP (SEQ ID NO:1). In some embodiments, the claudin blocking/disrupting agent is a peptide of amino acid sequence of SEQ ID NO:1. In some embodiments, at least one amino acid has D-configuration in the amino acid sequence of SEQ ID NO:1. In some embodiments, each one amino acid has D-configuration in the amino acid sequence of SEQ ID NO:1. In some embodiments, the chemotherapeutic agent is a microtubule-targeting drug. In some embodiments, the microtubule-targeting drug is selected from the group consisting of paclitaxel, taxane, docetaxel, other microtubule-targeting drugs, and any combinations thereof. In some embodiments, the chemotherapeutic agent comprises paclitaxel.

In some embodiments, the claudin blocking/disrupting agent and the at least one additional compound are co-administered at about the same time to the subject.

In some embodiments, the cancer is ovarian cancer. In some embodiments, the cancer is selected from the group consisting of ovarian cancer, breast cancer, and pancreatic cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of selected embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, selected embodiments are shown in the drawings . It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

FIGs. 1A-1C illustrate claudin-4 expression in high grade serous ovarian cancer cells. FIG. 1A depicts CLDN4 gene expression levels in laser capture microdissected specimens of human high grade serous ovarian tumors compared to isolated normal human ovarian surface epithelial cells. Western blot analysis of claudin-4 protein levels in ovarian cancer (FIG. 1B) as well as high grade serous ovarian tumor cell lines (FIG. 1C), using GAPDH as a loading control. Mean ± s.e.m., n=10 HOSE, n=53 HGSOC tumor specimens, ***$p<0.0001$. HGSOC: high grade serous ovarian cancer; OVCAR3, PEO4, OV429, OVCAR5, OVCAR8, OVCAR4, DOV13, ovarian cancer cell lines.

FIGs. 2A-2C depict exemplary results demonstrating that disruption of claudin-4 activity with CMP enhances paclitaxel response. Representative images of immunofluorescence of nuclei (DAPI staining) and activated caspase-3 (antibody directed to cleaved caspase-3) from OVCAR3 (FIG. 2A). Quantification of caspase-3 activation in OVCAR3 (FIG. 2B) and OVCAR8 (FIG. 2C) cells untreated or treated for 24 hours with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 μM Cisplatin (Cis), CMP + Cisplatin, 10 nM paclitaxel (taxol), or CMP + paclitaxel. Percent of cell population positive for caspase-3 activation was calculated using SlideBook software (3i). Mean ± s.e.m., n=3, NS=not significant, ***$p<0.001$ vs. control/drug only.

FIG. 3 depicts exemplary results demonstrating that claudin-4 disruption enhances apoptosis through extrinsic pathway. OVCAR3 cells were treated with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 nM paclitaxel (taxol), CMP + paclitaxel, or 50 μM etoposide

for 24 hours. Fixed cells were treated with fluorescent antibodies directed to cleaved caspase-8 (black bars) or cleaved caspase-9 (gray bars) and DAPI to stain for nuclei. Percent of cell population positive for caspase activation was calculated for each treatment using SlideBook software (3i). Mean ± s.e.m., n=3 per treatment group, NS=not significant, **p<0.01 vs. control/paclitaxel only.

FIGs. 4A-4D depict exemplary results demonstrating that the loss of claudin-4 expression enhances paclitaxel response. Western blot analysis of claudin-4 protein in OVCAR3 (FIG. 4A) and PEO4 (FIG. 4B) cells that were treated with control empty vector shRNA (contKD, gray bars) or claudin-4-targeted shRNA (cld4KD, black bars). GAPDH was used as a loading control. Immunofluorescence analysis of fixed OVCAR3 (FIG. 4C) and PEO4 (FIG. 4D) cells that were treated with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 nM paclitaxel (taxol), or CMP + paclitaxel for 24 hours. Cells were treated with fluorescent antibody directed to cleaved caspase-3 and DAPI (nuclei) and percent of cell population positive for caspase-3 was calculated using SlideBook software (3i). Mean ± s.e.m., n=3 per treatment group, NS=not significant, ***p<0.001 vs. control/paclitaxel only.

FIGs. 5A-5D depict exemplary results demonstrating that overexpression of claudin-4 reduces paclitaxel response. Western blot analysis of claudin-4 protein in OVCAR8 (FIG. 5A) and OVCAR4 (FIG. 5B) cells transduced with GFP only or claudin-4-GFP. These cell lines do not express endogenous claudin-4. GAPDH was used as a loading control. Immunofluorescence analysis of fixed OVCAR8 (FIG. 5C) and OVCAR4 (FIG. 5D) cells that were treated with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 nM paclitaxel (taxol), or CMP + paclitaxel for 24 hours. Cells were treated with fluorescent antibody directed to cleaved caspase-3 and DAPI (nuclei) and percent of cell population positive for caspase-3 was calculated using SlideBook software (3i). Mean ± s.e.m., n=3 per treatment group, NS=not significant, *p<0.05, **p<0.01, ***p<0.001 vs. control/paclitaxel only.

FIGs. 6A-6D depict exemplary results demonstrating that the loss of claudin-4 leads to delayed mitotic progression. FIG. 6A depicts representative images of DAPI (DNA) staining in fixed monolayers of control knockdown (shCTRL) and claudin-4 knockdown (shLDN4_2) OVCAR3 cells. Yellow circles highlight mitotic figures. FIG. 6B illustrates visual counting of mitotic figures from DAPI images and FIG. 6C illustrates colorimetric measurement of phosphorylated H2B (mitotic marker) to quantify population of mitotic cells in shCTRL (white

bars) and shCLDN4_2 (black bars) OVCAR3 cells. FIG. 6D depicts proliferation rates of shCTRL (circles/solid line) and shCLDN4_2 (triangles/dotted line) OVCAR3 cells. Mean±sem, n=3, *p<0.05, ***p<0.001 vs. shCTRL.

FIGs. 7A-7D demonstrate that claudin-4 interacts with tubulin. FIG. 7A depicts exemplary results from a proximity ligation assay using antibodies directed at claudin-4 and α-tubulin or β-tubulin. Red fluorescence indicates protein-protein interaction. Yellow outlined boxes are higher magnification of cell from image. Arrows point to sites of protein-protein interaction. FIG. 7B depicts immunoprecipitation (IP) of claudin-4 (cld-4 IP) and IgG (mouse IgG IP) from OVCAR3 lysates blotted for presence of α-tubulin and claudin-4. FIG. 7C illustrates immunofluorescence of claudin-4 (green) and β-tubulin (red), with DAPI (blue), in mitotic OVCAR3 cell. FIG. 7D depicts results from immunofluorescence analysis of microtubules (β-tubulin, white/red) and nuclei (DAPI, blue) in OVCAR3 ovarian tumor cells expressing claudin-4 (shCTRL) and not expressing claudin-4 (shCLDN4_2).

FIGs. 8A-8E depict exemplary results demonstrating that CMP enhances paclitaxel response (Caspase-3 activation). Caspase-3 activation in ovarian tumor cells was determined from immunofluorescence of fluorescent antibody directed to caspase-3 and DAPI staining of cells untreated or treated for 24 hours with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 μM Cisplatin (Cis), CMP + Cisplatin, 10 nM paclitaxel (taxol), or CMP + paclitaxel. Percent of cell population positive for caspase-3 activation was calculated using SlideBook software (3i). Mean ± s.e.m., n=3, NS=not significant, **p<0.01, ***p<0.001 vs. control/drug only. OVCAR4 and DOV3 cells do not express claudin-4 protein.

FIG. 9 depicts exemplary results demonstrating that CMP enhances paclitaxel response (Annexin V binding). Apoptosis in OVCAR3 ovarian tumor cells expressing claudin-4 was determined by Annexin V binding. Cells were untreated or treated with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 nM paclitaxel (taxol), or CMP + paclitaxel for 24 hours before addition of fluorescently conjugated Annexin V and Propidium Iodide (PI). Cells positive for Annexin V binding and/or PI were determined by flow cytometry. Mean ± s.e.m., n=3, *p<0.05, **p<0.01, ***p<0.001 vs. control/drug only.

FIGs. 10A-10D depict exemplary results demonstrating that silencing claudin-4 expression enhances response to paclitaxel. Western blot analysis of claudin-4 protein expression in OVCAR3 (FIG. 10A) and PEO4 (FIG. 10B) cells treated with empty vector control shRNA or

two different shRNAs directed to claudin-4. GAPDH was used as a loading control. FIGs. 10C-10D depict caspase-3 activation in OVCAR3 and PEO4 ovarian tumor cells, respectively, determined from immunofluorescence of fluorescent antibody directed to caspase-3 and DAPI staining of cells untreated or treated for 24 hours with 400 μM inactive control peptide (ContP), 400 μM CMP, 10 nM paclitaxel (taxol), or CMP + paclitaxel. Percent of cell population positive for caspase-3 activation was calculated using SlideBook software (3i). Black bars represent claudin-4 knockdown (cld4KD). Mean ± s.e.m., n=3, NS=not significant, *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. control/drug only.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20%, ±10%, ±5%, ±1%, or ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

As used herein, the term "antibody" refers to an immunoglobulin or antigen-binding fragment thereof, and encompasses any such polypeptide comprising an antigen-binding fragment of an antibody. The term includes but is not limited to polyclonal, monoclonal, monospecific, polyspecific, humanized, human, single-chain, single-domain, chimeric, synthetic, recombinant, hybrid, mutated, grafted, and in vitro generated antibodies. The term "antibody"

also includes antigen-binding fragments of an antibody. Examples of antigen-binding fragments include, but are not limited to, Fab fragments (consisting of the VL, VH, CL and CH1 domains); Fd fragments (consisting of the VH and CH1 domains); Fv fragments (referring to a dimer of one heavy and one light chain variable domain in tight, non-covalent association); dAb fragments (consisting of a VH domain); single domain fragments (VH domain, VL domain, VHH domain, or VNAR domain); isolated CDR regions; (Fab')2 fragments, bivalent fragments (comprising two Fab fragments linked by a disulfide bridge at the hinge region), scFv (referring to a fusion of the VL and VH domains, linked together with a short linker), and other antibody fragments that retain antigen-binding function.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

The present invention provides methods for treating cancer in a subject. In certain embodiments, the subject is administered a claudin blocking/disrupting agent and at least one additional compound selected from the group consisting of one or more paclitaxel-related compounds and one or more additional chemotherapeutic agents. The contents of U.S. Patent No. 8,563,515 B2, including any of the peptides disclosed therein, are incorporated herein their entireties by reference.

In certain embodiments, the cancer is any claudin-driven cancer and/or any cancer with high claudin expression levels, wherein the claudin is claudin-3, claudin-4, claudin-7, and/or claudin-8. In other embodiments, the cancer is ovarian cancer. In other embodiments, the cancer includes, for example, breast cancer, pancreatic cancer, and ovarian cancer (Baumgartner, *et al.*, 2011, Chem. Biol. Drug Des. 77(2):124-36); Baumgartner, *et al.*, 2017, J. Mammary Gland Biol.

Neoplasia 22(2):141-157; proteinatlas *dot* orgENSG00000189143-CLDN4/pathology/tissue/endometrial+cancer). In yet other embodiments, the cancer is a high-claudin expressing epithelial cancer.

In certain embodiments, the cancer is not responsive to the at least one additional compound (*i.e.*, is not treated or prevented by the at least one additional compound in the absence of the claudin blocking/disrupting agent). In other embodiments, the cancer is responsive to the combination of the at least one additional compound and the claudin blocking/disrupting agent.

In certain embodiments, the combination of the at least one additional compound and the claudin blocking/disrupting agent is synergistic.

In certain embodiments, administration of the claudin blocking/disrupting agent allows for administration of a lower dose of the at least one additional compound to the subject as compared to the dose of the at least one additional compound alone that is required to elicit an equivalent biological response in the subject.

In certain embodiments, administration of the claudin blocking/disrupting agent allows for administration of a dose of the at least one additional compound that does not cause significant side effects in the subject as compared to the dose of the at least one additional compound alone that is required to elicit an equivalent biological response in the subject.

In certain embodiments, the claudin blocking/disrupting agent disrupts tight junction assembly and/or signaling. In other embodiments, the claudin blocking/disrupting agent interferes with claudin-claudin interactions. In yet other embodiments, the claudin blocking/disrupting agent interferes with claudin-occludin interactions. In yet other embodiments, the claudin blocking/disrupting agent mimics activity(ies) of at least one extracellular loop of at least one claudin. In yet other embodiments, the claudin blocking/disrupting agent mimics and/or disrupts activity of the DFYNP sequence of the second extracellular loop of at least one claudin. In yet other embodiments, the claudin blocking/disrupting agent blocks or disrupts activity of at least one classic claudin, for example claudin-3, claudin-4, claudin-5, claudin-7, and claudin-8.

In certain embodiments, the claudin blocking/disrupting agent blocks or disrupts activity of at least one claudin selected from the group consisting of claudin-3, claudin-4, claudin-7, and claudin-8. In other embodiments, the claudin blocking/disrupting agent blocks or disrupts

activity of claudin-3 and/or claudin-4. In yet other embodiments, the claudin blocking/disrupting agent comprises a peptide of amino acid sequence DYFNP (SEQ ID NO:1). In yet other embodiments, the claudin blocking/disrupting agent consists essentially of a peptide of amino acid sequence DYFNP (SEQ ID NO:1). In yet other embodiments, the claudin blocking/disrupting agent consists of a peptide of amino acid sequence DYFNP (SEQ ID NO:1).

In certain embodiments, the claudin blocking/disrupting agent comprises a biologically active small peptide or peptide fragment. In other embodiments, the claudin blocking/disrupting agent is at least one of a claudin mimetic peptide, blocking peptide, small molecule compound, protein, peptide, antibody, peptidomimetic, antisense nucleic acid, ribozyme, or the like. In yet other embodiments, the claudin blocking/disrupting agent is soluble. In yet other embodiments, the claudin blocking/disrupting agent includes one or more DNA and/or RNA sequences that code for DFYNP peptide.

In certain embodiments, the claudin blocking/disrupting agent activates apoptotic signaling. In other embodiments, the claudin blocking/disrupting agent activates extrinsic apoptotic signaling. In yet other embodiments, the claudin blocking/disrupting agent modulates cell migration. In yet other embodiments, the claudin blocking/disrupting agent reduces cell migration. In yet other embodiments, the claudin blocking/disrupting agent modulates interactions of at least one claudin with extracellular constituents. In yet other embodiments, the claudin blocking/disrupting agent modulates interactions of at least one claudin with one or more integrins. In yet other embodiments, the claudin blocking/disrupting agent disrupts interactions of at least one claudin with one or more integrins, for example collagen receptor integrins such as $\alpha 1$, $\alpha 2$, $\beta 1$, $\alpha 2\beta 1$, and $\alpha 1\beta 1$, for example. In yet other embodiments, the claudin blocking/disrupting agent disrupts interactions of at least one claudin with extracellular constituents including extracellular matrix constituents. In yet other embodiments, the claudin blocking/disrupting agent disturbs interactions of at least one claudin with extracellular constituents, including, for example, hyaluronan, collagen, fibronectin, fibrin, elastin, gelatin, laminin, glycosaminoglycans, and/or any other extracellular matrix constituents as understood in the art.

In certain embodiments, the at least one additional compound interferes with one or more cellular behaviors, for example motility, proliferation, apoptosis, replication, division, enzymatic activity, and the like.

In certain embodiments, the at least one additional compound is a microtubule-targeting drug, such as but not limited to, paclitaxel, taxane, and/or docetaxel.

In certain embodiments, the subject is administered a claudin blocking/disrupting agent and a chemotherapeutic agent.

In certain embodiments, the claudin blocking/disrupting agent and the at least one additional compound are co-administered simultaneously. In other embodiments, the claudin blocking/disrupting agent is administered first, and then the at least one additional compound is administered after a period of time. In yet other embodiments, the at least one additional compound is administered first, and then the claudin blocking/disrupting agent is administered after a period of time. In yet other embodiments, the period of time includes up to about 1 hour, up to about 2 hours, up to about 8 hours, up to about 12 hours, up to about 24 hours, and/or more than 24 hours. In yet other embodiments, the claudin blocking/disrupting agent is administered every other day, for a period of 4 weeks. In yet other embodiments, the at least one additional compound is administered at least once per week.

Administration/Dosage/Formulations

The regimen of administration may affect what constitutes an effective amount. The therapeutic formulations may be administered to the subject either prior to or after the onset of a cancer. Further, several divided dosages, as well as staggered dosages may be administered daily or sequentially, or the dose may be continuously infused, or may be a bolus injection. Further, the dosages of the therapeutic formulations may be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

Administration of the compositions of the present invention to a patient, preferably a mammal, more preferably a human, may be carried out using known procedures, at dosages and for periods of time effective to treat a cancer in the patient. An effective amount of the therapeutic compound necessary to achieve a therapeutic effect may vary according to factors such as the state of the disease or disorder in the patient; the age, sex, and weight of the patient; and the ability of the therapeutic compound to treat a cancer in the patient. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A non-limiting example of an effective dose range for a

therapeutic compound of the invention is between about 1 mg/kg and 5,000 mg/kg of body weight/per day. For example, an effective does for a therapeutic compound may be about 20 mg/kg to about 200 mg/kg as a single dose or a daily dose over one or more days. One of ordinary skill in the art would be able to study the relevant factors and make the determination regarding the effective amount of the therapeutic compound without undue experimentation.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

In particular, the selected dosage level depends upon a variety of factors including the activity of the particular compound employed, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds or materials used in combination with the compound, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well-known in the medical arts.

A medical doctor, *e.g.*, physician or veterinarian, having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In particular embodiments, it is especially advantageous to formulate the compound in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the patients to be treated; each unit containing a predetermined quantity of therapeutic compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical vehicle. The dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the therapeutic compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding/formulating such a therapeutic compound for the treatment of a cancer in a patient.

The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the

like), suitable mixtures thereof, and vegetable oils.

In certain embodiments, the compositions of the invention are administered to the patient in dosages that range from one to five times per day or more. In other embodiments, the compositions of the invention are administered to the patient in range of dosages that include, but are not limited to, once every day, every two days, every three days to once a week, and once every two weeks. It is readily apparent to one skilled in the art that the frequency of administration of the various combination compositions of the invention varies from individual to individual depending on many factors including, but not limited to, age, disease or disorder to be treated, gender, overall health, and other factors. Thus, the invention should not be construed to be limited to any particular dosage regime and the precise dosage and composition to be administered to any patient is determined by the attending physical taking all other factors about the patient into account.

Compounds of the invention for administration may be in the range of from about 1 μg to about 10,000 mg, about 20 μg to about 9,500 mg, about 40 μg to about 9,000 mg, about 75 μg to about 8,500 mg, about 150 μg to about 7,500 mg, about 200 μg to about 7,000 mg, about 350 μg to about 6,000 mg, about 500 μg to about 5,000 mg, about 750 μg to about 4,000 mg, about 1 mg to about 3,000 mg, about 10 mg to about 2,500 mg, about 20 mg to about 2,000 mg, about 25 mg to about 1,500 mg, about 30 mg to about 1,000 mg, about 40 mg to about 900 mg, about 50 mg to about 800 mg, about 60 mg to about 750 mg, about 70 mg to about 600 mg, about 80 mg to about 500 mg, and any and all whole or partial increments there between.

In some embodiments, the dose of a compound of the invention is from about 1 mg and about 2,500 mg. In some embodiments, a dose of a compound of the invention used in compositions described herein is less than about 10,000 mg, or less than about 8,000 mg, or less than about 6,000 mg, or less than about 5,000 mg, or less than about 3,000 mg, or less than about 2,000 mg, or less than about 1,000 mg, or less than about 500 mg, or less than about 200 mg, or less than about 50 mg. Similarly, in some embodiments, a dose of a second compound as described herein is less than about 1,000 mg, or less than about 800 mg, or less than about 600 mg, or less than about 500 mg, or less than about 400 mg, or less than about 300 mg, or less than about 200 mg, or less than about 100 mg, or less than about 50 mg, or less than about 40 mg, or less than about 30 mg, or less than about 25 mg, or less than about 20 mg, or less than about 15 mg, or less than about 10 mg, or less than about 5 mg, or less than about 2 mg, or less than about

1 mg, or less than about 0.5 mg, and any and all whole or partial increments thereof.

In certain embodiments, the present invention is directed to a packaged pharmaceutical composition comprising a container holding a therapeutically effective amount of a compound of the invention, alone or in combination with a second pharmaceutical agent; and instructions for using the compound to treat, prevent, or reduce one or more symptoms of a cancer in a patient.

Formulations may be employed in admixtures with conventional excipients, *i.e.*, pharmaceutically acceptable organic or inorganic carrier substances suitable for oral, parenteral, nasal, intravenous, subcutaneous, enteral, or any other suitable mode of administration, known to the art. The pharmaceutical preparations may be sterilized and if desired mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure buffers, coloring, flavoring and/or aromatic substances and the like. They may also be combined where desired with other active agents, *e.g.*, other analgesic agents.

Routes of administration of any of the compositions of the invention include nasal, rectal, intravaginal, intra peritoneal, parenteral, buccal, sublingual or topical. The compounds for use in the invention may be formulated for administration by any suitable route, such as for parenteral, for example, transdermal, transmucosal (*e.g.*, sublingual, lingual, (trans)buccal, (trans)urethral, vaginal (*e.g.*, trans- and perivaginally), (intra)nasal and (trans)rectal), intravesical, intrapulmonary, intraduodenal, intragastrical, intrathecal, subcutaneous, intramuscular, intradermal, intra-arterial, intravenous, intrabronchial, inhalation, and topical administration.

In certain embodiments, the administration is intraperitoneal and/or intravenous. In other embodiments, oral dosing of compounds of the invention can lead to interactions with claudin-4 expressed in intestinal epithelial cells.

Suitable compositions and dosage forms include, for example, tablets, capsules, caplets, pills, gel caps, troches, dispersions, suspensions, solutions, syrups, granules, beads, transdermal patches, gels, powders, pellets, magmas, lozenges, creams, pastes, plasters, lotions, discs, suppositories, liquid sprays for nasal administration, dry powder or aerosolized formulations for inhalation, compositions and formulations for intravesical administration and the like. It should be understood that the formulations and compositions that would be useful in the present invention are not limited to the particular formulations and compositions that are described herein.

Parenteral Administration

For parenteral administration, the compounds of the invention may be formulated for injection or infusion, for example, intravenous, intraperitoneal, intramuscular or subcutaneous injection or infusion, or for administration in a bolus dose and/or continuous infusion. Suspensions, solutions or emulsions in an oily or aqueous vehicle, optionally containing other formulatory agents such as suspending, stabilizing and/or dispersing agents may be used.

Additional Administration Forms

Additional dosage forms of this invention include dosage forms as described in U.S. Patents Nos. 6,340,475; 6,488,962; 6,451,808; 5,972,389; 5,582,837; and 5,007,790. Additional dosage forms of this invention also include dosage forms as described in U.S. Patent Applications Nos. 20030147952; 20030104062; 20030104053; 20030044466; 20030039688; and 20020051820. Additional dosage forms of this invention also include dosage forms as described in PCT Applications Nos. WO 03/35041; WO 03/35040; WO 03/35029; WO 03/35177; WO 03/35039; WO 02/96404; WO 02/32416; WO 01/97783; WO 01/56544; WO 01/32217; WO 98/55107; WO 98/11879; WO 97/47285; WO 93/18755; and WO 90/11757.

Controlled Release Formulations and Drug Delivery Systems

In certain embodiments, the formulations of the present invention may be, but are not limited to, short-term, rapid-offset, as well as controlled, for example, sustained release, delayed release and pulsatile release formulations.

The term sustained release is used in its conventional sense to refer to a drug formulation that provides for gradual release of a drug over an extended period of time, and that may, although not necessarily, result in substantially constant blood levels of a drug over an extended time period. The period of time may be as long as a month or more and should be a release which is longer that the same amount of agent administered in bolus form.

For sustained release, the compounds may be formulated with a suitable polymer or hydrophobic material which provides sustained release properties to the compounds. As such, the compounds for use the method of the invention may be administered in the form of microparticles, for example, by injection or in the form of wafers or discs by implantation.

In one embodiment of the invention, the compounds of the invention are administered to a patient, alone or in combination with another pharmaceutical agent, using a sustained release formulation.

The term delayed release is used herein in its conventional sense to refer to a drug

formulation that provides for an initial release of the drug after some delay following drug administration and that may, although not necessarily, include a delay of from about 10 minutes up to about 12 hours.

The term pulsatile release is used herein in its conventional sense to refer to a drug formulation that provides release of the drug in such a way as to produce pulsed plasma profiles of the drug after drug administration.

The term immediate release is used in its conventional sense to refer to a drug formulation that provides for release of the drug immediately after drug administration.

As used herein, short-term refers to any period of time up to and including about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1 hour, about 40 minutes, about 20 minutes, or about 10 minutes and any or all whole or partial increments thereof after drug administration after drug administration.

As used herein, rapid-offset refers to any period of time up to and including about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1 hour, about 40 minutes, about 20 minutes, or about 10 minutes, and any and all whole or partial increments thereof after drug administration.

Dosing

The therapeutically effective amount or dose of a compound of the present invention depends on the age, sex and weight of the patient, the current medical condition of the patient and the progression of a cancer in the patient being treated. The skilled practitioner is able to determine appropriate dosages depending on these and other factors.

A suitable dose of a compound of the present invention may be in the range of from about 0.01 mg to about 10,000 mg per day, such as from about 0.1 mg to about 1,000 mg, for example, from about 1 mg to about 500 mg, such as about 5 mg to about 250 mg per day. The dose may be administered in a single dosage or in multiple dosages, for example from 1 to 4 or more times per day. When multiple dosages are used, the amount of each dosage may be the same or different. For example, a dose of 1 mg per day may be administered as two 0.5 mg doses, with about a 12-hour interval between doses. The dose may be administered based on patient weight. For example, the dose may be in the range of from about 0.1 mg/kg to about 10 mg/kg, such as, for example, between about 1 mg/kg and about 50 mg/kg, between about 5 mg/kg and about 30 mg/kg, between about 10 mg/kg and about 20 mg/kg and the like. The dose

may be administered as a single compound and/or combination of one or more compounds administered at the same dose or different doses and at the same time or a different times.

It is understood that the amount of compound dosed per day may be administered, in non-limiting examples, every day, every other day, every 2 days, every 3 days, every 4 days, or every 5 days. For example, with every other day administration, a 5 mg per day dose may be initiated on Monday with a first subsequent 5 mg per day dose administered on Wednesday, a second subsequent 5 mg per day dose administered on Friday, and so on.

In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the inhibitor of the invention is optionally given continuously; alternatively, the dose of drug being administered is temporarily reduced or temporarily suspended for a certain length of time (*i.e.*, a "drug holiday"). The length of the drug holiday optionally varies between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday includes from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, is reduced, as a function of the viral load, to a level at which the improved disease is retained. In certain embodiments, patients require intermittent treatment on a long-term basis upon any recurrence of symptoms and/or infection.

The compounds for use in the method of the invention may be formulated in unit dosage form. The term "unit dosage form" refers to physically discrete units suitable as unitary dosage for patients undergoing treatment, with each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, optionally in association with a suitable pharmaceutical carrier. The unit dosage form may be for a single daily dose or one of multiple daily doses (*e.g.*, about 1 to 4 or more times per day). When multiple daily doses are used, the unit dosage form may be the same or different for each dose.

Toxicity and therapeutic efficacy of such therapeutic regimens are optionally determined in cell cultures or experimental animals, including, but not limited to, the determination of the

LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio between LD_{50} and ED_{50}. The data obtained from cell culture assays and animal studies are optionally used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with minimal toxicity. The dosage optionally varies within this range depending upon the dosage form employed and the route of administration utilized.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents were considered to be within the scope of this invention and covered by the claims appended hereto. For example, it should be understood, that modifications in protocols with art-recognized alternatives and using no more than routine experimentation, are within the scope of the present application.

It is to be understood that wherever values and ranges are provided herein, all values and ranges encompassed by these values and ranges, are meant to be encompassed within the scope of the present invention. Moreover, all values that fall within these ranges, as well as the upper or lower limits of a range of values, are also contemplated by the present application.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Methods

Cell culture

Human-derived OVCAR3, OVCAR4, OVCAR5, OVCAR8, PEO4, OV429, and DOV13 ovarian tumor cells were cultured in RPMI-1640 medium (Gibco, Thermo Fisher Scientific, Grand Island, NY, USA) plus 10% heat-inactivated fetal bovine serum (Access Cell Culture, Vista, CA, USA) and 1% penicillin/streptomycin (Gibco, Thermo Fisher Scientific) at 37°C and 5% CO_2. Cells were trypsinized (0.25% trypsin, EDTA, Mediatech) and plated 1:3 every 3-4 days. All cell lines were authenticated at the beginning of this study by short tandem repeat profiling, as described previously. Cells were plated at $1x10^4$ cells/well into type I collagen-coated 8-well chamber slides (Lab-Tek, NUNC, Rochester, NY, USA) for treatment and analysis. Upon 80-90% confluence, cells were treated with either 400 μM control peptide (NH_2-GDGYNPG-OH, D-amino acid conformation), 400 μM claudin mimic peptide (CMP, NH_2-GDFYNPG-OH, D-amino acid conformation), 10 nM paclitaxel (Sigma-Aldrich, St Louis, MO, USA), and/or 10 μM cisplatin (Sigma-Aldrich).

Ovarian Tumor Samples

De-identified patient tumor samples were collected from the University of Colorado Gynecologic Tissue and Fluid Bank under Institutional Review Board approved protocol. Each tumor sample was flash frozen in liquid nitrogen and stored in -80°C until processed for protein. Frozen tissue was placed in lysis buffer (30 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 0.57 mM PMSF, 1X cOmplete™ Protease Inhibitor Cocktail) and homogenized using a Polytron tissue homogenizer (Brinkmann Instruments, Fisher Scientific). Lysates were then spun at 13,000 rpm for 10 minutes and supernatant collected for protein analysis.

Western blot analysis

To analyze levels of claudin-4 protein expression, tumor cells were scraped from culture plates in presence of lysis buffer (30 mM Tris HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 0.57 mM PMSF, 1X cOmplete™ Protease Inhibitor Cocktail), placed on a shaker for 10 minutes and spun at 13,000 rpm for 10 minutes. Supernatant was collected, and 20 μg of total protein was denatured, resolved on 10% SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Bio Rad, Hercules, CA, USA).

Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for one hour at room temperature (RT) before treatment with either rabbit anti-human claudin-4 (1:500, Invitrogen), mouse anti-human claudin-4 (1:500; Invitrogen), or rabbit anti-human GAPDH (1:10,000; Sigma) overnight at 4°C. Membranes were then washed 4 times with TBST for 15 minutes before treatment with horseradish peroxidase-conjugated goat anti-rabbit (1:10,000; GE Healthcare, Buckinghamshire, UK) or goat anti-mouse (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) antibodies for 1 hour at room temperature. Membranes were washed with TBST as described above and then visualized using an ECL Prime Western Blotting Detection Reagent (GE Healthcare) and X-ray film (CL-XPosure Film, Thermo Scientific, Rockford, IL, USA).

Caspase activation assay

Apoptosis was measured by caspase-3 activation. After treatment, cells were washed with phosphate buffered saline (PBS; Gibco, Thermo Fisher Scientific) and fixed with 10% phosphate buffered formalin (Fisher Scientific, Pittsburg, PA, USA) at room temperature (RT) for 15 minutes. Cells were washed twice with PBS before cell membrane permeabilization with 0.5% Triton X-100 (IBI Scientific, Peosta, IA, USA) for 5 minutes and washed again with PBS. Cells were treated with blocking buffer (2% bovine serum albumin; Sigma-Aldrich) for 1 hour before application of primary antibody directed to cleaved caspase-3 (1:400; rabbit anti-human cleaved caspase-3, Cell Signaling, Danvers, MA, USA), cleaved caspase-8 [1:100; rabbit anti-human cleaved caspase-8 (D391), Cell Signaling], or cleaved caspase-9 [1:100, rabbit anti-human cleaved caspase-9 (Asp315), Thermo Scientific] overnight at 4°C. Cells were then washed with PBS five times before application of secondary antibody conjugated to CY3 (1:100; donkey anti-rabbit, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 5 µg/ml 4',6-diamidina-2-phenylindole (DAPI; Sigma) for 45 minutes at RT followed by five washings with PBS. After removal of PBS, o-phenylenediamine dihydrochloride (20 mg/ml; OPDA) in 1 M Tris, pH 8.5 was added to the slides to preserve fluorescence and coverslip mounted. The Olympus FV1000/RICS confocal microscope (University of Colorado AMC Light Microscopy Core) was used to image fluorescence. Images were analyzed for percent of cell population positive for active caspase using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

shRNA knockdown

OVCAR3 and PEO4 cells were plated 3.2 x10^4 in a 96-well plate and incubated at 37°C for 24 hours. When cells reached 70% confluence, 10 µl of claudin-4 shRNA (TRC#: TRCN0000116627 or TRCN0000116628) or control shRNA (SHC001, pLK0.1-puro Empty Vector) lentiviral suspension (Sigma-Aldrich MISSION® shRNA, University of Colorado Functional Genomics Facility, Aurora, CO, USA) was added to the cells and incubated overnight at 37°C. Fresh medium was added to remove lentivirus and cells were allowed to recover for 24 hours before being treated with 0.5 µg/ml puromycin for selection and expansion of transduced cells. Western blot analysis was performed to confirm loss of claudin-4 expression.

Overexpression of claudin-4

GFP-tagged claudin-4 and a GFP only control (pLenti-C-mGFP vector; OriGene, Rockville, MD, USA) were transduced into OVCAR8 and OVCAR4 cells via lentiviral suspension. Cells expressing GFP were flow sorted and expanded.

Mitotic Assays

To examine the number of cells that were in mitosis, images of DAPI fluorescence were examined in OVCAR3 cells cultured in 8-well chamber slides. Cells that had clearly condensed chromatin that aligned along a single plane were counted as mitotic cells and percentage of total number of nuclei in a field of view was determined. Additionally, a Mitotic Assay Kit (Active Motif, Carlsbad, CA, USA) was used per manufacturer's instructions. Briefly, cells were cultured in 96-well plates, fixed, and treated with a phospho-histone H3 (Ser 28) monoclonal primary antibody followed by an HRP-conjugated secondary antibody. Absorbance at 450 nm was read after 20 minute incubation with the Developing Solution. Crystal Violet was used to normalize for total cell number.

Proliferation Assay

Ten thousand cells were plated in each well of 12-well culture plates. Cells were trypsinized (0.25% trypsin, EDTA) and counted by a Countess™ Automated Cell Counter (Invitrogen) after 24, 48, and 72 hours in culture.

Proximity Ligation Assay

Claudin-4 interaction with tubulin was tested using DuoLink® Proximity Ligation Assay Kit (Sigma), following manufacturer's protocol. Briefly, cells were plated in 8-well chamber slides and cultured to 70% confluence before being fixed, permeabilized, and blocked as described above (caspase-3 immunofluorescence). Cells were then treated with antibodies directed to claudin-4 (mouse anti-human claudin-4; 1:200; Invitrogen) and α-tubulin (rabbit anti-human α-tubulin; 1:100; Abcam, Cambridge, MA, USA) or β-tubulin (rabbit anti-human β-tubulin; 1:100; Abcam) overnight at 4°C. After washing with PBS, cells were treated with PLA Probes (1:5; anti-mouse PLUS, anti-rabbit MINUS) for 1 hour at 37°C in a pre-heated humidity chamber. After washing, Ligation Solution was added for 30 min at 37°C followed by washing and addition of Amplification-Polymerase Solution for 1.5 hours at 37°C in a humidity chamber. Slides were washed and dried before adding mounting medium and placing coverslip. Fluorescence was imaged using an Olympus FV1000/RICS confocal microscope (University of Colorado AMC Light Microscopy Core).

Immunoprecipitation

Claudin-4 (and interacting proteins) was pulled from cell lysates with antibody directed to claudin-4 (mouse anti-human claudin-4, Invitrogen) bound to Protein A/G Dynabeads® Magnetic beads, using the Pierce™ Crosslink IP Kit (ThermoFisher). Beads were incubated with lysate (~500 μg protein) for 1 hour at room temperature and then collected using a Magnetic Separation Rack (New England BioLabs, Ipswich, MA, USA). Proteins were eluted from beads per manufacturer's protocol. Eluted proteins were run on 10% SDS-PAGE and Western blot analysis was performed, using antibodies directed to claudin-4 (rabbit anti-human claudin-4; 1:500) and tubulin (rabbit anti-human α-tubulin or β-tubulin; 1:1000).

Annexin V Apoptosis Assay

Following the manufacturer's protocol, the Alexa Fluor™ 488 Annexin V/Dead Cell Apoptosis Kit (ThermoFisher) was used to treat cells with fluorescently conjugated Annexin V and Propidium Iodide for the detection of apoptotic and necrotic cells, respectively. Cells were analyzed by flow cytometry (Beckman Coulter Gallios, University of Colorado Flow Cytometry

Core Facility), measuring fluorescence emission at 530 nm (FL1) and >575 nm (FL3). Percent of total population positive for Annexin V binding was plotted.

Statistics

Data are presented as mean ± standard error of the mean (s.e.m.). An unpaired Student t test was used for statistical comparison between control and treatment groups. A one-way ANOVA was used to determine variance among multiple gestational groups, with a Bonferroni Multiple Comparison post-test to determine significance between individual groups. A p value of < 0.05 was considered significant.

Example 1:

To investigate the non-canonical role of claudin-4 in both normal and tumor cell biology, a small claudin mimic peptide (CMP) was previously designed that interferes with the DFYNP sequence in the second extracellular loop of claudin-4. CMP, synthesized in the D-amino acid configuration to increase stability, was recently used to demonstrate that claudin-4 expression by ovarian cancer cells has a functional role in tumor progression. Disrupting claudin-4 activity with CMP increased tumor cell response to the potent apoptotic inducer staurosporine and inhibited cell migration. Additionally, injection of CMP into mice bearing EOC cell line-derived xenograft tumors significantly reduced tumor burden. To determine the role that claudin-4 plays in apoptotic response to standard chemotherapeutics, gain- and loss-of function claudin-4 EOC cell lines was evaluated in parallel with CMP. Caspase activation was examined in response to cisplatin and paclitaxel. Claudin-4 expression led to reduced tumor cell sensitivity to apoptosis induced by paclitaxel that can be restored by co-treatment with the CMP or silencing claudin-4 expression. Additionally, a non-canonical role of claudin-4 was shown in facilitating cell cycle progression as well as an interaction of claudin-4 with microtubules that may provide a mechanism by which claudin-4 could be driving these phenotypes. Overall, the studies disclosed herein demonstrate that ovarian tumor cell expression of claudin-4 reduces apoptotic response to paclitaxel.

Example 2: Claudin-4 is highly expressed in high grade serous epithelial ovarian tumor cells

To identify changes in claudin-4 mRNA expression levels in human ovarian cancer patient tumors compared to normal human ovarian surface epithelium, the Gene Expression Omnibus (GEO) database was queried. Expression was analyzed in the GSE18521 (ID: 200018521) dataset that contained 10 normal human ovarian surface epithelium (HOSE) and 53 high grade serous epithelial ovarian cancer (HGSOC) samples. A significant ($p = 0.0002$) difference in claudin-4 expression was determined, with 1.8- to 18-fold higher expression in the HGSOC compared to HOSE (FIG. 1A). Next, claudin-4 protein expression was examined in patient tumor samples from the University of Colorado Gynecologic Tissue and Fluid Bank (FIG. 1B). The majority of the tumor samples expressed claudin-4, with a benign tumor not expressing claudin-4 and all of the high grade serous ovarian cancer (HGSOC) samples expressing claudin-4. Claudin-4 protein levels were then examined in various ovarian tumor cell lines that have been identified as having originated from high grade serous epithelial ovarian tumors (CITE). In agreement with previous findings from other investigators, OVCAR3, PEO4, OV429, and OVCAR5 expressed high levels of claudin-4 protein and OVCAR8, OVCAR4, and DOV-13 cells expressed very low/no claudin-4 protein (FIG. 1C).

Example 3: Claudin-4-expressing cells exhibit reduced apoptotic response to paclitaxel

To determine whether claudin-4 expression in tumor cells alters response to standard-of-care chemotherapies, activation of caspase-3 in response to cisplatin and paclitaxel was examined. Claudin-4 expressing OVCAR3 cells exhibited an increase in apoptosis (as measured by percent cleaved caspase-3 positive) in response to the claudin-4-disrupting peptide (CMP), cisplatin, and paclitaxel (FIG 2., Panels A and B). Increase in cleaved-caspase 3 cells was not observed following the combined treatment of cisplatin and CMP compared to cisplatin alone ($p = 0.3602$). However, there was a significant enhancement of apoptosis when cells were co-treated with CMP and paclitaxel compared to paclitaxel alone ($p = 0.0002$). Similar to OVCAR3 cells, claudin-4-expressing PEO4, OV429 and OVCAR5 cells showed an enhanced apoptotic response to paclitaxel when claudin-4 activity was simultaneously disrupted (FIGs. 8A-8C). In contrast, the OVCAR8 (lacking claudin-4 expression) cells were found to be more sensitive to both cisplatin ($p = 0.0032$) and paclitaxel ($p = 0.0063$) compared to OVCAR3 cells. The claudin-4-disrupting CMP peptide did not enhance apoptotic response to cisplatin or paclitaxel in OVCAR8 cells, which would be expected with the lack of claudin-4 (FIG. 2). These studies

suggest specificity in claudin-4 activity that interferes with the tumor cell's response to paclitaxel that may involve the second extracellular loop interactions of claudin-4 (the target of the CMP peptide).

Analysis of additional EOC cell lines (FIGs. 8B-8E) revealed a consistent enhancement of apoptotic response to paclitaxel when co-treated with CMP only in claudin-4 positive lines (OV429 and OVCAR5) compared to no enhancement in claudin-4 deficient lines (OVCAR4 and DOV-13). Additionally, enhanced apoptotic response to paclitaxel with co-treatment with CMP was seen when measuring apoptosis in claudin-4-expressing OVCAR3 cells by flow cytometry of Annexin V binding (FIG. 9).

Example 4: Loss of claudin-4 activity enhances apoptosis through the extrinsic apoptotic pathway

It was next evaluated whether claudin-4 plays a role in the death receptor-mediated extrinsic apoptotic pathway and/or the mitochondria-mediated intrinsic apoptotic pathway. To determine specific claudin-4 dependent apoptosis the activation of caspase-8 (extrinsic) and caspase-9 (intrinsic) was examined in OVCAR3 cells treated with 400 μM inactive control peptide, 400 μM CMP, 10 nM paclitaxel or CMP plus paclitaxel. As a positive control, 50 μM etoposide led to a significant increase in the caspase-9 mediated intrinsic pathway. CMP and paclitaxel alone promoted the activation of the extrinsic caspase-8 pathway. In addition, co-treatment of tumor cells with CMP and paclitaxel induced an enhanced activation to caspase-8, with no activation of caspase-9 (FIG. 3). These results suggest that enhancement of paclitaxel-induced apoptosis mediated by claudin-4 involves the activation of the extrinsic apoptotic pathway.

Example 5: Loss of claudin-4 expression improves tumor cell apoptotic response to paclitaxel

Using a small-hairpin RNA (shRNA) specific to claudin-4, expression was knocked down in OVCAR3 and PEO4 cells ("cld4KD", Figure 4 A&B). A control, empty vector, shRNA did not impact expression of the claudin-4 protein ("_contKD"). Similar to the parental OVCAR3 cells, contKD cells showed increased apoptosis with CMP or paclitaxel and a significantly enhanced apoptotic response to paclitaxel in the presence of CMP compared to

paclitaxel alone ($p < 0.0001$, FIG. 4C). OVCAR3 cld4KD cells were unable to induce an apoptotic response to treatment with CMP alone and CMP co-treatment did not enhance paclitaxel-induced apoptosis ($p = 0.8890$ and 0.8851, respectively). Response to paclitaxel alone was significantly ($p < 0.0001$) higher in the cld4KD cells compared to contKD cells, suggesting that targeting claudin-4 activity or expression can increase apoptotic response to paclitaxel. Similar results were seen in the PEO4 cell line (FIG. 4D), with the disruption of claudin-4 activity with CMP or loss of claudin-4 expression significantly enhancing tumor cell response to paclitaxel ($p < 0.0001$). Both OVCAR3 and PEO4 cells were transduced with another claudin-4 shRNA; however, this shRNA did not successfully knock down claudin-4 at the protein level ("_28KD", FIGs. 10A-10D). These cells that maintained claudin-4 expression behaved as both the contKD and the parental claudin-4-expressing cells lines, with CMP significantly enhancing paclitaxel response ($p = 0.0002$ and 0.0003, respectively).

Example 6: Forced expression of claudin-4 reduces tumor cell response to paclitaxel

The impact of overexpressing claudin-4 was then evaluated. OVCAR8 and OVCAR4 cells have no/low expression of claudin-4 protein (FIG. 1). Both cell lines were transfected with GFP-tagged claudin-4 or a control GFP vector. Western blot analysis showed strong expression of the claudin-4-GFP in both cell lines (FIGs. 5A-5B). GFP control cells did not respond to CMP treatment, with no induction of apoptosis with CMP alone or enhanced apoptosis with paclitaxel. However, cells with forced claudin-4-GFP expression had a decreased apoptotic response to paclitaxel and did respond to CMP, with induction of apoptosis with CMP alone and enhanced apoptotic response to paclitaxel in the presence of CMP (FIGs. 5C-5D). These responses were more significant in OVCAR8 cells compared to OVCAR4, likely due to OVCAR4 cells expressing low levels of endogenous claudin-4.

Example 7: Claudin-4 expression results in increased mitotic fidelity

Claudin-4 has been shown to diminish paclitaxel response. Such mechanism was investigated. Upon examination of nuclei from the OVCAR3 cell line significantly more mitotic figures were observed in the shCLDN4_2 cells compared to the claudin-4 expressing shCTRL cells ($p < 0.0001$, FIGs. 6A-6B). To confirm these observations a colorimetric mitotic assay kit that utilizes a phospho-histone H3 (Ser28) monoclonal antibody with HRP-conjugated secondary

antibody was used to detect the level of mitosis. The loss of claudin-4 expression significantly increased the number of cells in mitosis (FIG 6, Panel C, $p < 0.0001$). To determine whether this indicated an increase in proliferation, cell number was measured over time in shCTRL and shCLDN4_2 cell lines. The claudin-4 knockdown cells proliferated significantly slower than the control cells ($p = 0.0453$, FIG. 6D). These results suggest that the increase in mitotic figures in the knockdown cells is not the result of increased proliferation, but reflects an inhibition in G2/M progression.

Microtubules are known to play an important role in both paclitaxel response and cell cycle progression. Therefore, a potential interaction of claudin-4 with microtubules was examined. Proximity ligation assays were performed on OVCAR3 cells with antibodies directed to claudin-4 and either α-tubulin or β-tubulin. Claudin-4 was found to be within interacting distance of both α-tubulin and β-tubulin (FIG. 7A). Immunoprecipitation (IP) of claudin-4 confirmed interaction with α-tubulin (FIG. 7B), but it was not possible to confirm interaction with β tubulin via Western blot. However, immunofluorescence assays reveled that claudin-4 was co-localizing with β-tubulin at the mitotic spindle in tumor cells undergoing mitosis (FIG. 7C). Additionally, images of microtubules in claudin-4 expressing and non-expressing OVCAR3 cells revealed significant changes in microtubule structure with claudin-4 expression (FIG. 7D).

Claudin-4 has been demonstrated to be playing an important role in tumor cell resistance to paclitaxel. Therefore, expression of claudin-4 by tumor cells can be a biomarker for patient response to paclitaxel. Additionally, the development of a claudin-4 therapeutic (e.g. CMP) provides a novel way to improve patient response to paclitaxel.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of treating cancer in a subject, the method comprising administering to the subject a claudin blocking/disrupting agent and at least one additional chemotherapeutic agent.

2. The method of claim 1, wherein the claudin blocking/disrupting agent is selected from the group consisting of a mimetic peptide, blocking peptide, antibody, peptidomimetic, antisense nucleic acid, ribozyme, or a small molecule chemical compound.

3. The method of claim 1, wherein the claudin blocking/disrupting agent mimics or blocks activity of the second extracellular loop of at least one of claudin-3, claudin-4, claudin-7, and claudin-8.

4. The method of claim 3, wherein the claudin blocking/disrupting agent comprises a peptide of amino acid sequence DYFNP (SEQ ID NO:1).

5. The method of claim 4, wherein the claudin blocking/disrupting agent is a peptide of amino acid sequence of SEQ ID NO:1.

6. The method of claim 4, wherein at least one amino acid has D-configuration in the amino acid sequence of SEQ ID NO:1.

7. The method of claim 4, wherein each one amino acid has D-configuration in the amino acid sequence of SEQ ID NO:1.

8. The method of claim 1, wherein the chemotherapeutic agent is a microtubule-targeting drug.

9. The method of claim 8, wherein the microtubule-targeting drug is selected from the group consisting of paclitaxel, taxane, docetaxel, other microtubule-targeting drugs, and any combinations thereof.

10. The method of claim 9, wherein the chemotherapeutic agent comprises paclitaxel.

11. The method of claim 1, wherein the claudin blocking/disrupting agent and the at least one additional compound are co-administered at about the same time to the subject.

12. The method of claim 1, wherein the cancer is ovarian cancer.

13. The method of claim 1, wherein the cancer is selected from the group consisting of ovarian cancer, breast cancer, and pancreatic cancer.

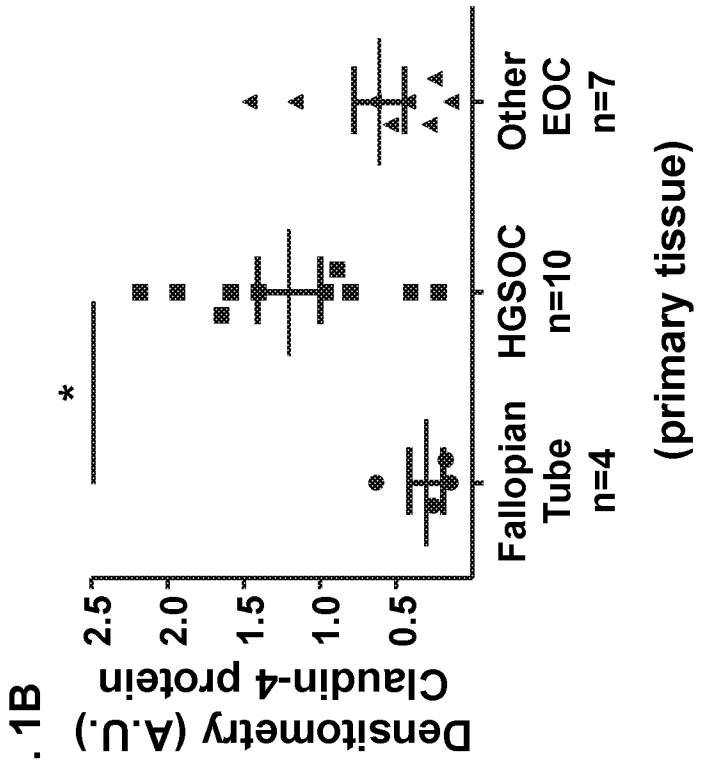


FIG. 1B
Densitometry (A.U.)
Claudin-4 protein
2.5
2.0
1.5
1.0
0.5
*
Fallopian Tube n=4
HGSOC n=10
Other EOC n=7
(primary tissue)

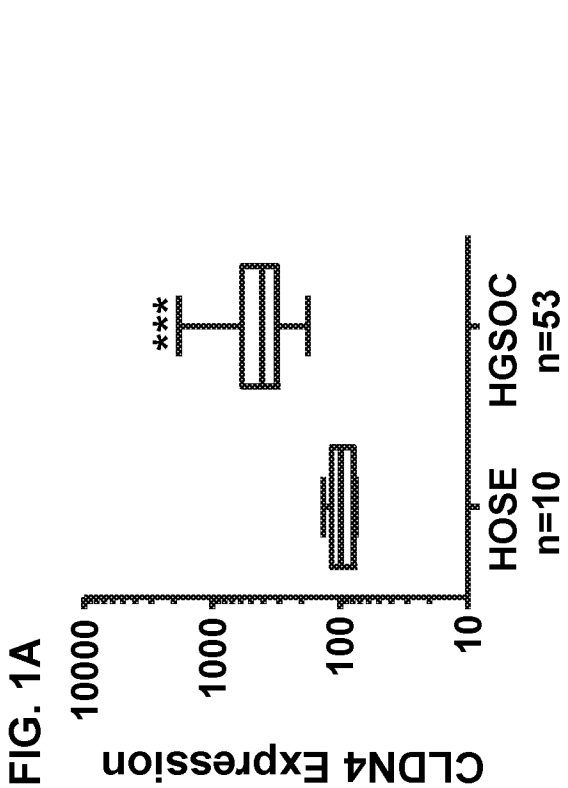


FIG. 1A
CLDN4 Expression
10000
1000
100
10
***
HOSE n=10
HGSOC n=53

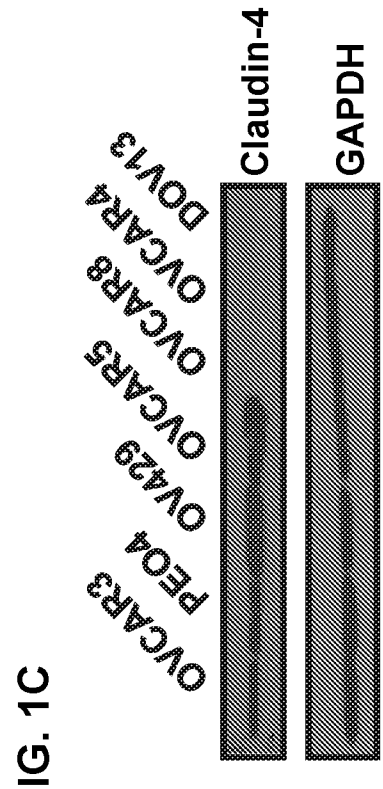


FIG. 1C
OVCAR3
PEO4
OV429
OVCAR5
OVCAR8
OVCAR4
DOV13
Claudin-4
GAPDH

FIG. 2A

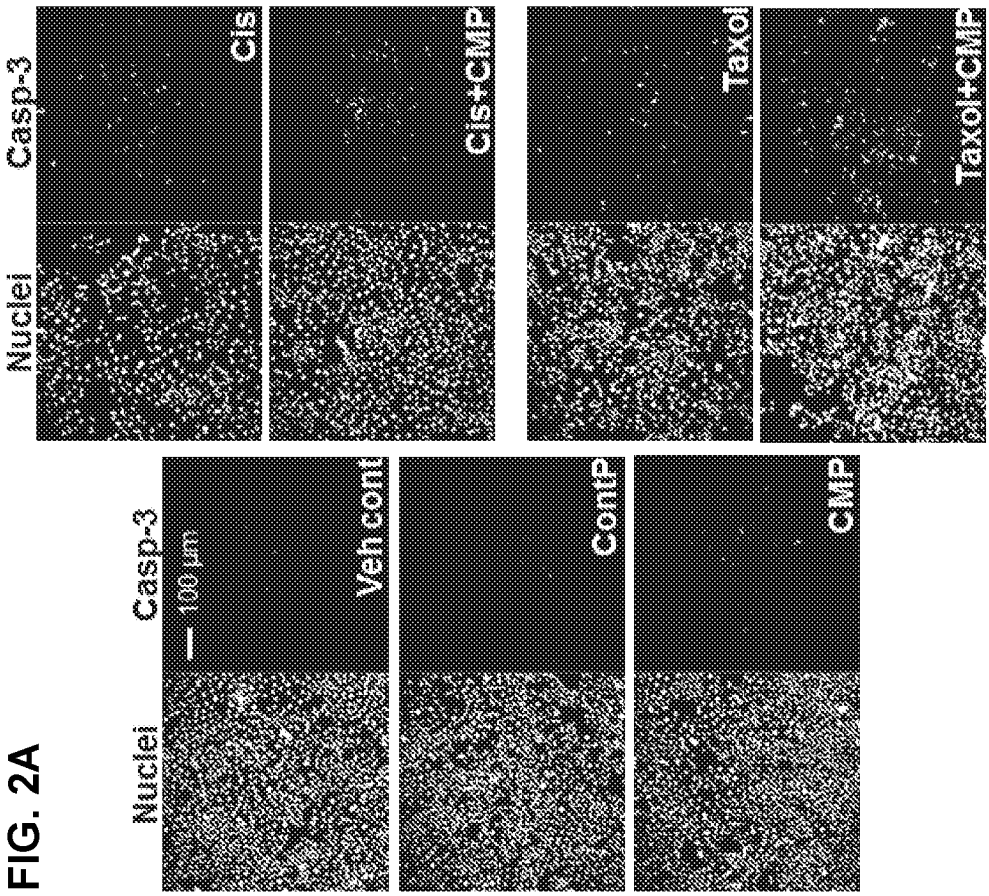


FIG. 2B

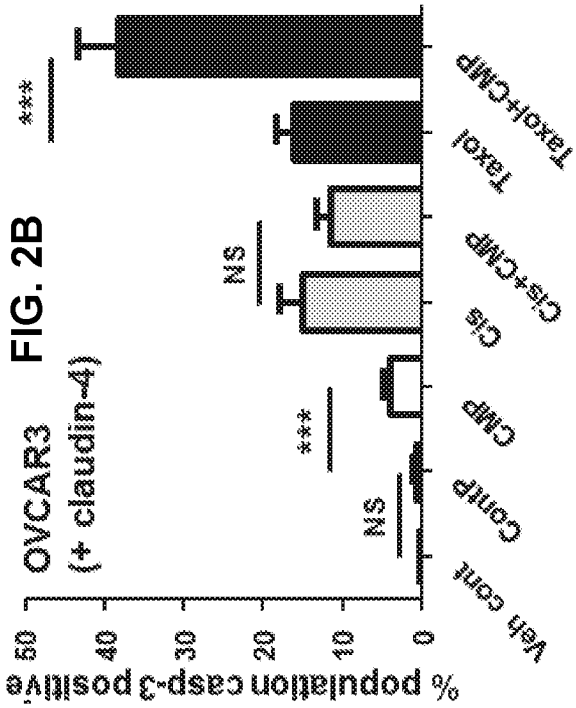
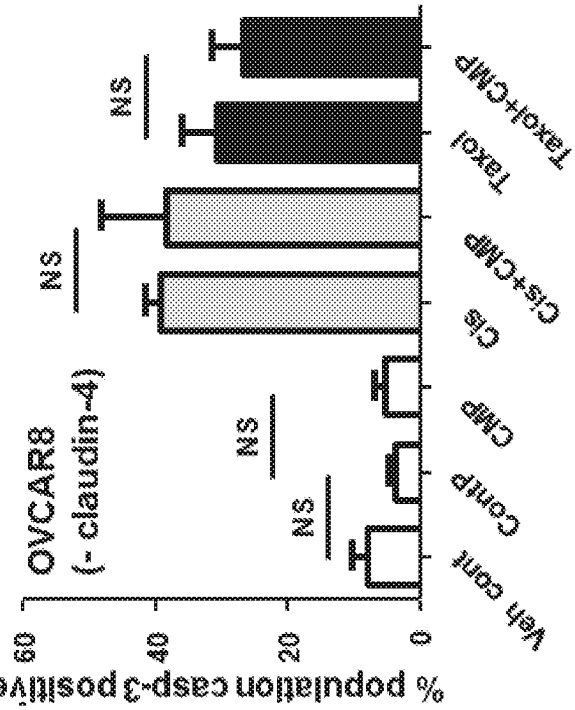


FIG. 2C



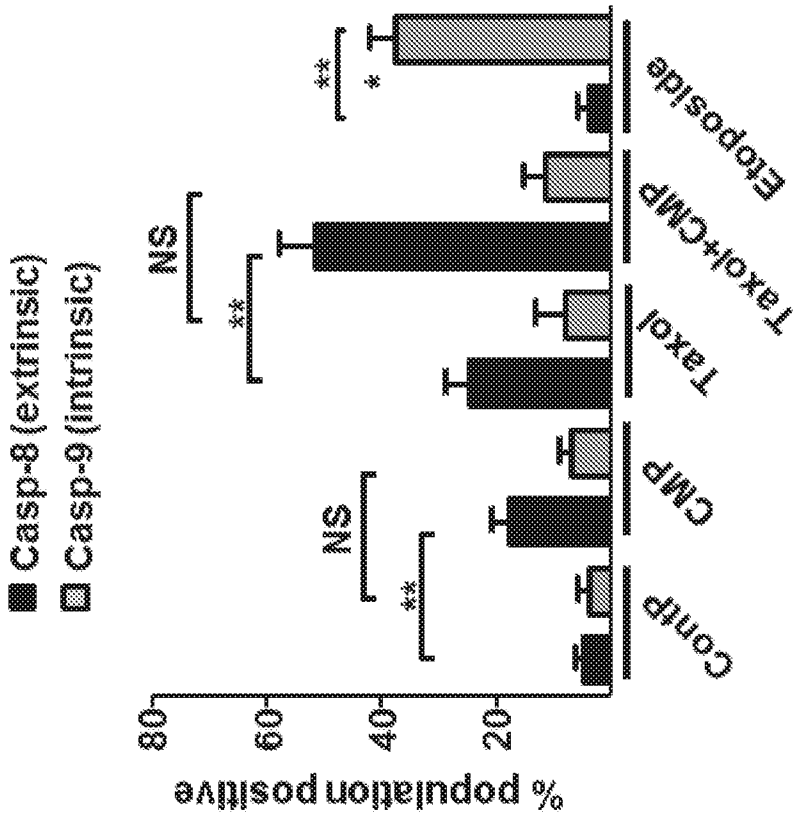

Casp-8 (extrinsic)
Casp-9 (intrinsic)
% population positive
80
60
40
20
NS
**
NS
**
**
*
Contp
CMP
Taxol
Taxol+CMP
Etoposide

FIG. 3

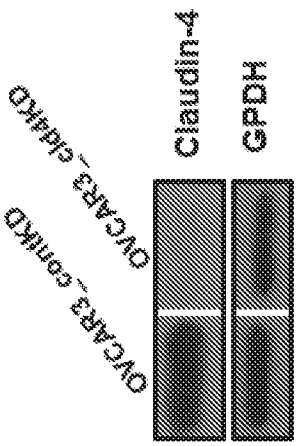


FIG. 4A

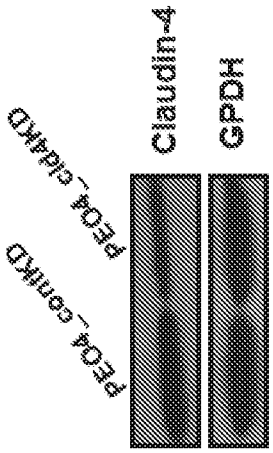


FIG. 4B

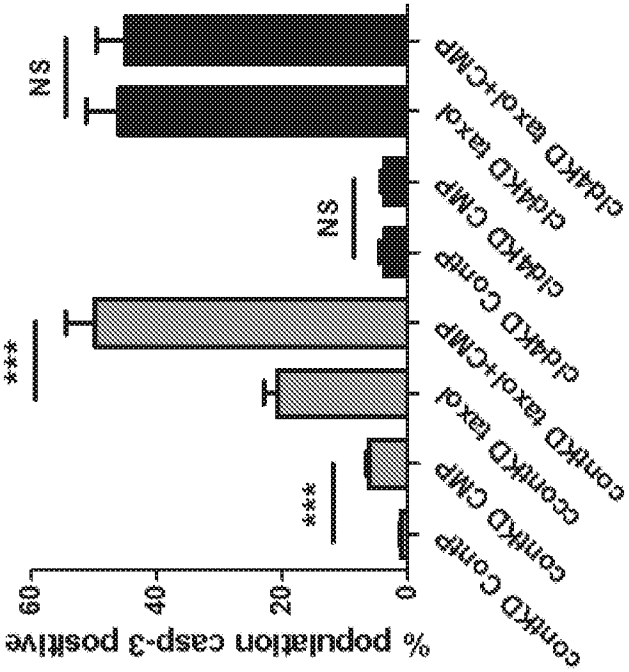


FIG. 4C

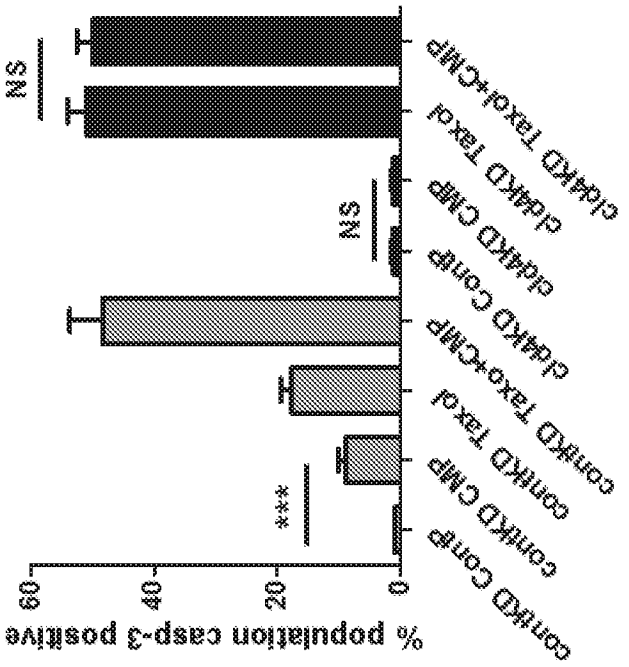


FIG. 4D

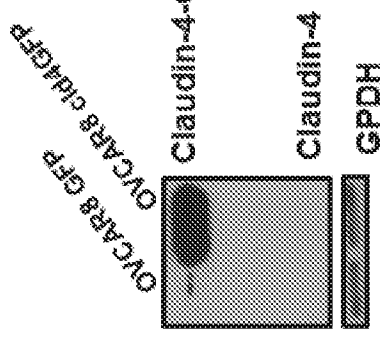


FIG. 5A

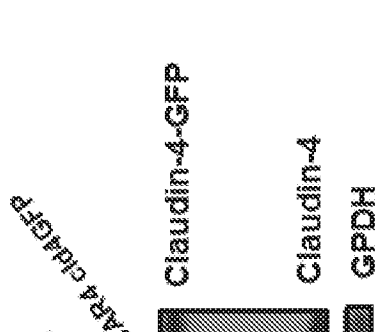


FIG. 5C

FIG. 5B

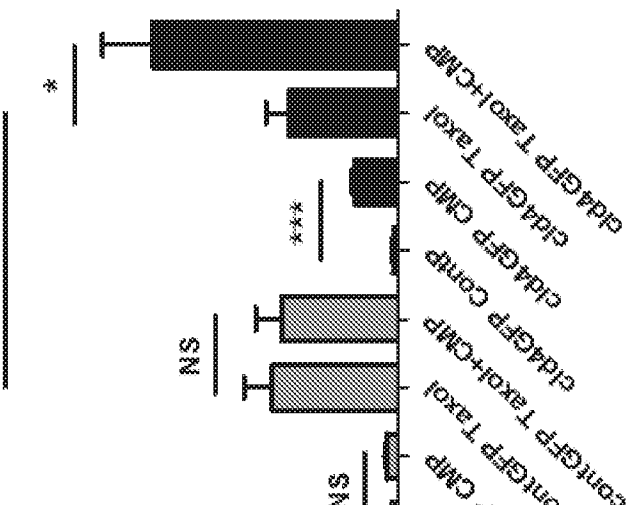


FIG. 5D

FIG. 6A

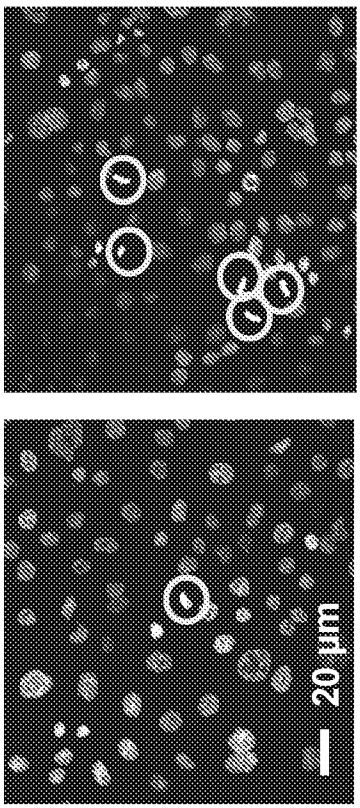


FIG. 6B

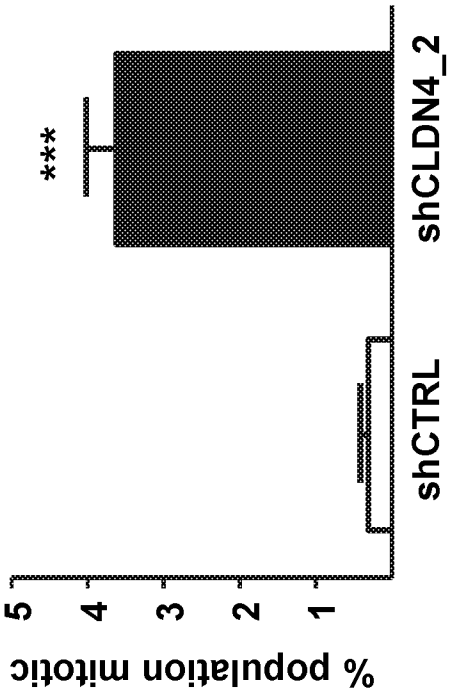


FIG. 6C

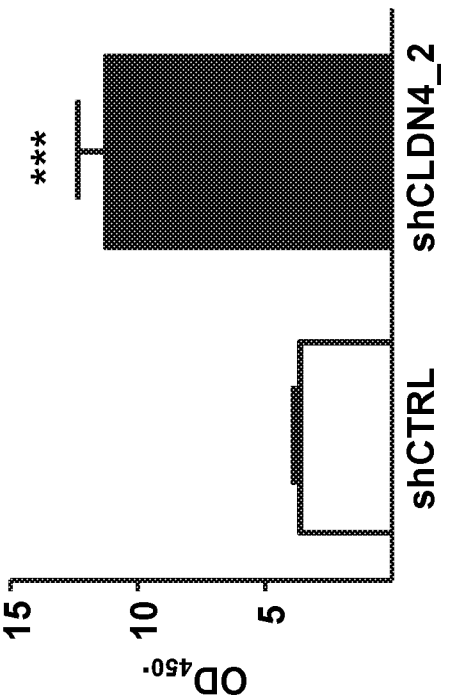
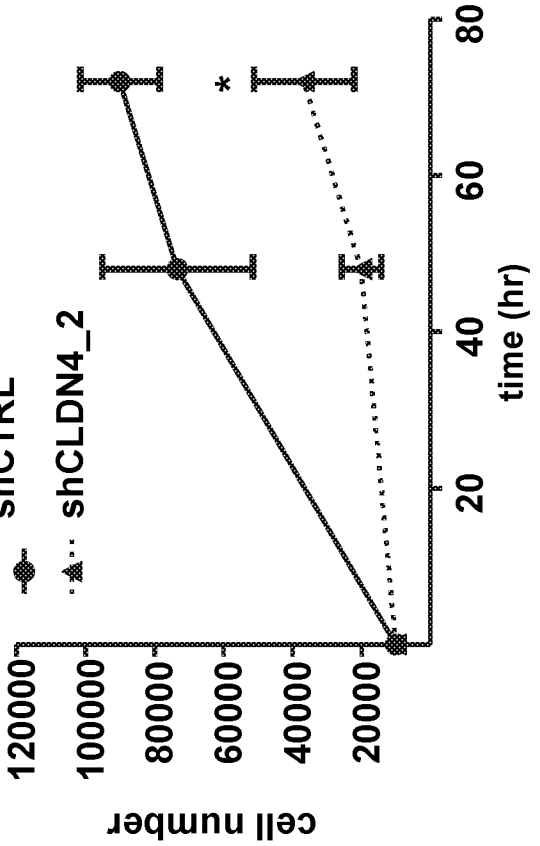


FIG. 6D



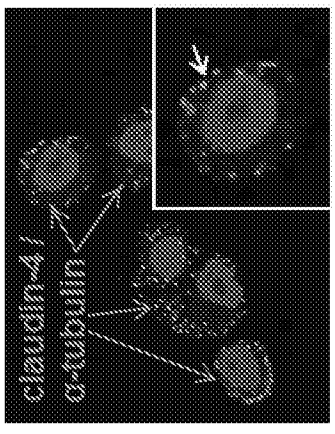
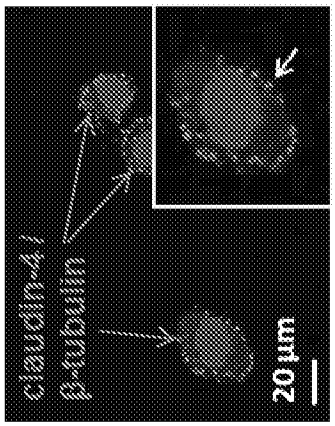


FIG. 7A



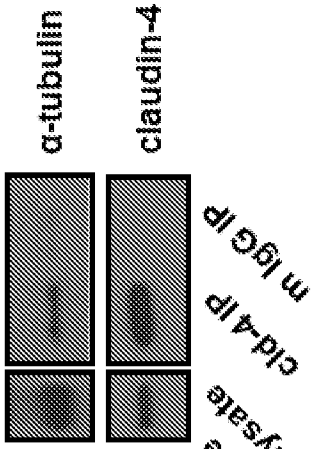
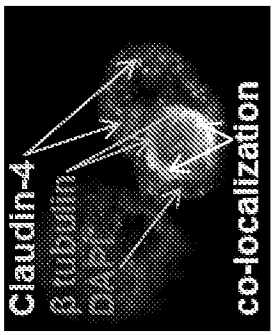
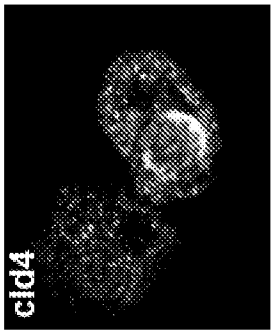
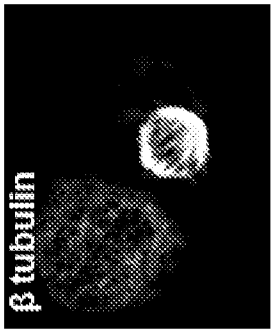


FIG. 7B







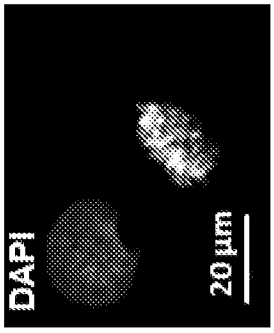
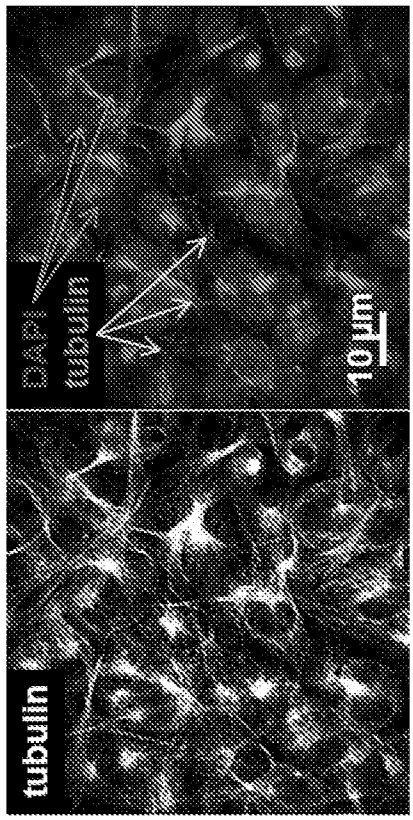


FIG. 7C



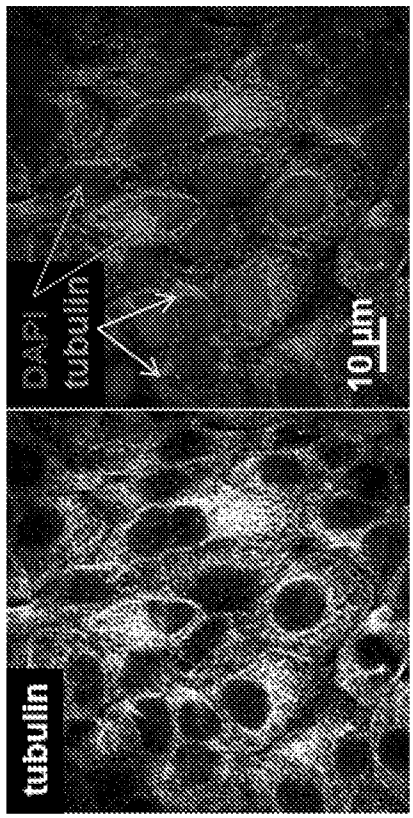
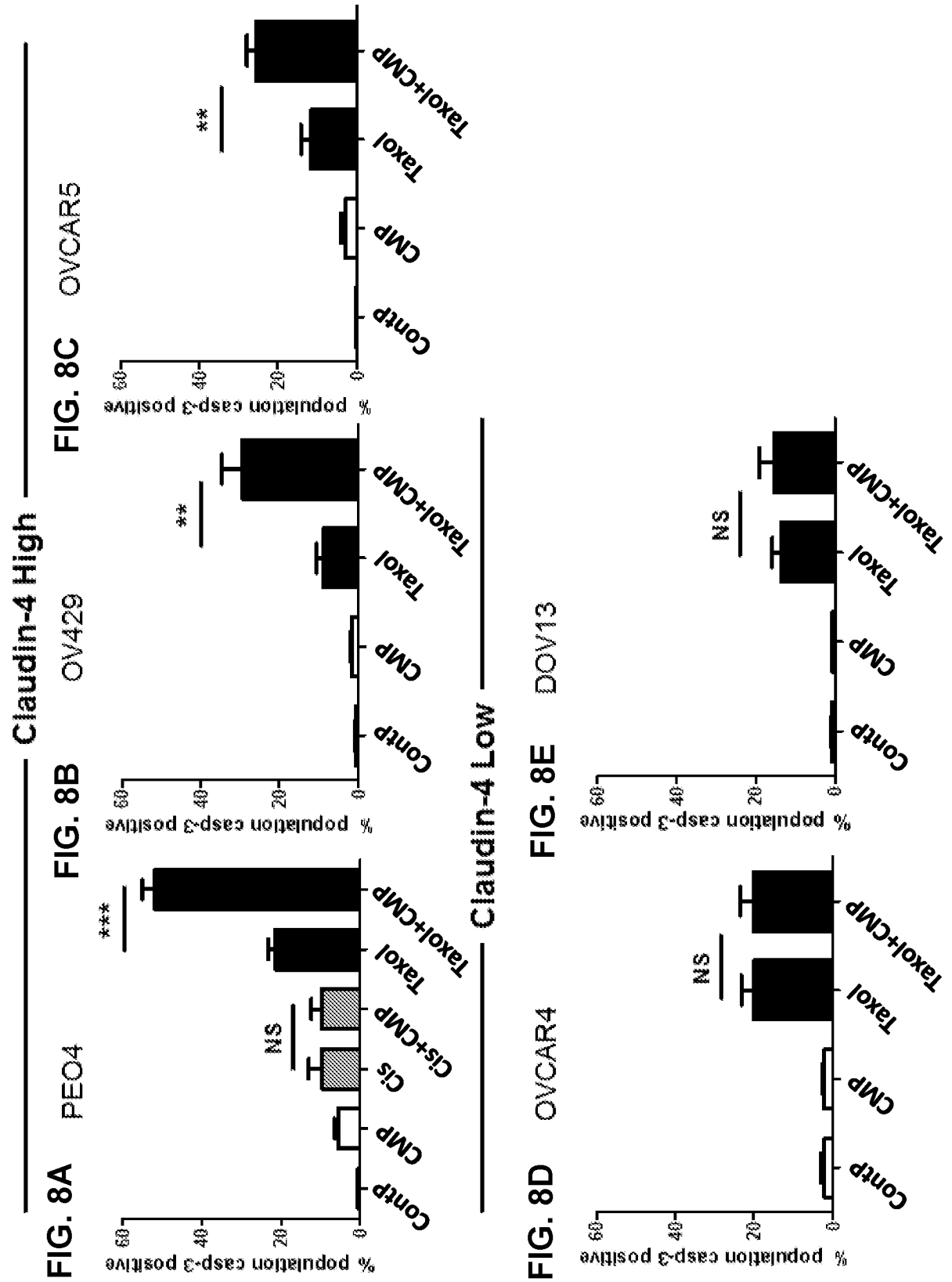


FIG. 7D


Claudin-4 High
Claudin-4 Low
FIG. 8A PEO4
FIG. 8B OV429
FIG. 8C OVCAR5
FIG. 8D OVCAR4
FIG. 8E DOV13
% population casp-3 positive
0
20
40
60
Contp
CMP
Cis
Cis+CMP
Taxol
Taxol+CMP
***
**
NS

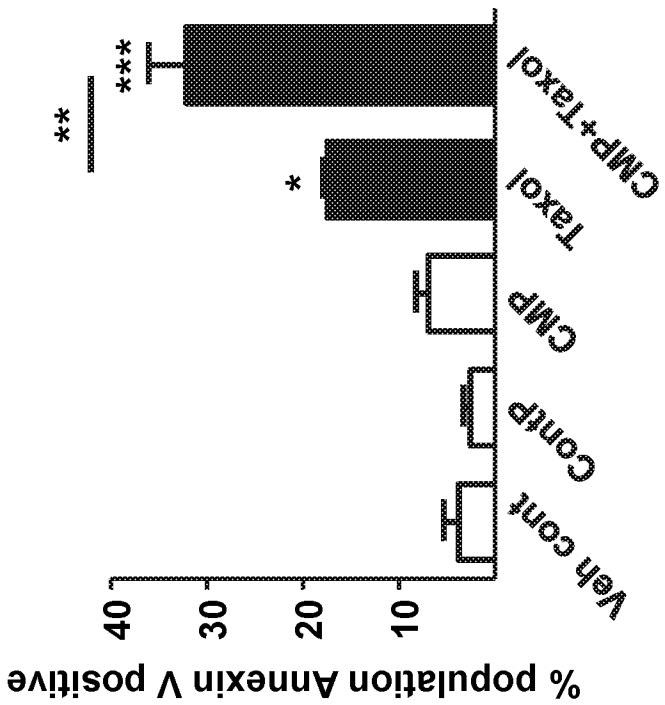

% population Annexin V positive
40
30
20
10
Veh cont
Contp
CMP
Taxol
CMP+Taxol
*
**
***

FIG. 9

FIG. 10A

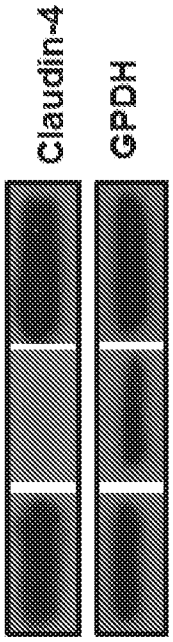


FIG. 10B

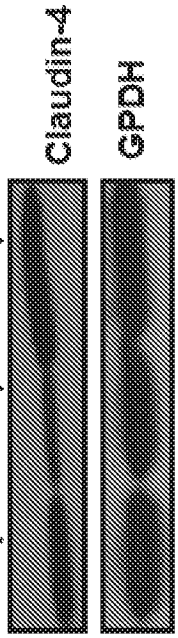


FIG. 10C

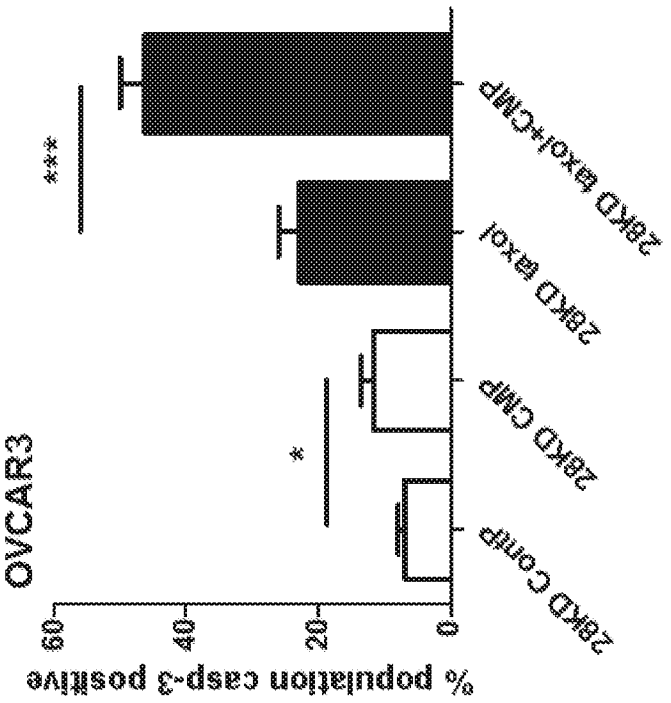
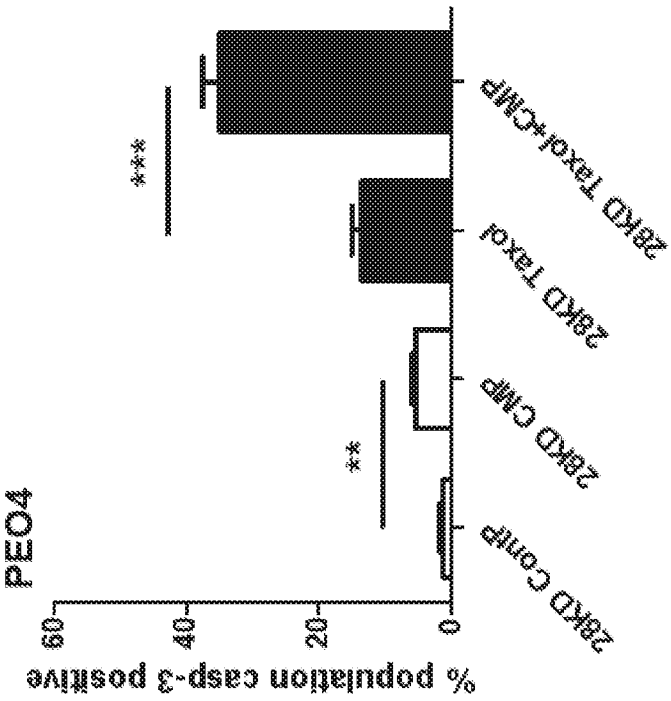


FIG. 10D



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/44928

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/US 19/44928

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.:
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:
----Go to Extra Sheet for continuation------

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 8-13, limited to one D-amino acid in SEQ ID NO: 1, paclitaxel, and ovarian cancer

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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/44928

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 7/06; A61K 38/04; A61P 35/00 (2019.01)

CPC - C07K 7/06; A61K 38/177; A61P 35/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	US 2014/0045768 A1 (The Regents of the University of Colorado) 13 February 2014 (13.02.2014). Especially para [0003], [0015], [0084], sheet 1 fig 1A, claims 1, 5-7	1-6, 11-13 ---------- 8-10
Y	US 2008/0064634 A1 (Markland et al.) 13 March 2008 (13.03.2008). Especially claims 1, 14-16	8-10

☐ 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
18 November 2019	10 DEC 2019
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 Lee Young Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US 19/44928

Continuation of Box III: Observations where Unity of Invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I+: Claims 1-13, drawn to a method of treating cancer in a subject.
The method of treating cancer will be searched to the extent that the first named variant of DYFNP (SEQ ID NO: 1) comprises one amino acid in the D-configuration (claim 6), the additional chemotherapeutic agent is the first named, paclitaxel (claim 9), and the cancer is the first named cancer, ovarian cancer (claim 12). It is believed that claims 1-6, 8-13 read on this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 1 comprising one D-amino acid, the additional chemotherapeutic agent is paclitaxel, and the cancer is ovarian cancer. Additional D- or L- configurations of SEQ ID NO: 1, additional chemotherapeutic agents, and types of cancer will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional D-or L- configurations of SEQ ID NO: 1, additional therapeutic agents and types of cancer. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: each amino acid of SEQ ID NO: 1 has a D- configuration, the additional chemotherapeutic agent is docetaxel, and the cancer is pancreatic cancer (claims 1-13).

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

Special Technical Features:

Among the inventions listed as Groups I+ are the specific D- or L- amino acid configurations of SEQ ID NO: 1, different additional chemotherapeutic agents and the type of cancer treated. Each invention requires a specific D- or L- amino acid species in SEQ ID NO: 1, chemotherapeutic agent and type of cancer treated not required by any other inventions.

Common Technical Feature:

Common Technical Features:

1. Group I+ inventions share the common technical feature of claim 1.

2. Group I+ inventions share the common technical feature that the additional chemotherapeutic agent is a microtubule targeting drug (claim 8).

However, said common technical feature does not represent a contribution over the prior art, and is disclosed by US 2011/0263511 A1 to Neville et al. (hereinafter "Neville"), in view of US 2008/0064634 A1 to Markland et al. (hereinafter "Markland").

As to common technical feature #1, Neville discloses a method of treating cancer in a subject, the method comprising administering to the subject a claudin blocking/disrupting agent (claims 1-2; "1. A method for modulating apoptosis of epithelial cells of a subject comprising administering to the subject a therapeutically effective amount of a tight junction protein modulator. 2. The method of claim 1, wherein the tight junction protein modulator causes internalization of the tight junction protein occludin or claudin") and at least one additional chemotherapeutic agent (para [0086]; "Furthermore, compounds of the invention can be administered in combination with other pharmaceutically active compound").

As to common technical feature #2, Markland discloses the additional chemotherapeutic agent is a microtubule targeting drug (claims 1, 14-16: "1. A method of treating an individual suffering from cancer, said method comprising administering to said individual an effective amount of a disintegrin and at least one microtubule stabilizing agent. 14. The method of claim 1 wherein said microtubule stabilizing agent is a taxane. 15. The method of claim 14 wherein said taxane is docetaxel. 16. The method of claim 14 wherein said taxane is paclitaxel"). An artisan of ordinary skill in the art would have recognized that it was well-known in the art that combination chemotherapy was an effective means to treat a cancer, where microtubule targeting agents were commonly used as one of the agents.

As the common technical features were known in the art at the time of the invention, theu cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Group I+ inventions lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.