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(54) Title: FULVESTRANT FOR INDUCING IMMUNE-MEDIATED CYTOTOXIC LYSIS OF CANCER CELLS

(57) Abstract: A method of enhancing immune-mediated lysis of mesenchymal cancer cells and a method of sensitizing mesenchymal cancer cells to chemotherapy or immune-mediated lysis are provided. The methods comprise administering fulvestrant to the cancer cells. A method of treating lung cancer comprising administering a combination of fulvestrant and an immune-mediated therapy to a patient also is provided.



FULVESTRANT FOR INDUCING IMMUNE-MEDIATED CYTOTOXIC LYSIS OF CANCER CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This patent application claims the benefit of U.S. Provisional Patent Application No. 62/290,117, filed February 2, 2016, which is incorporated by reference.

SEQUENCE LISTING

[0002] Incorporated by reference in its entirety herein is a nucleotide/amino acid sequence listing submitted concurrently herewith.

BACKGROUND OF THE INVENTION

[0003] One of the recently proposed mechanisms utilized by carcinoma cells to disseminate and to metastasize involves the conversion of tumor cells from an epithelial to a mesenchymal-like phenotype via a process designated as the epithelial-mesenchymal transition (EMT) (Thiery et al., *Cell*, 139: 871-890 (2009); Kalluri et al., *J. Clin. Invest.*, 119: 1420-1428 (2009); and Polyak et al., *Nat. Rev. Cancer*, 9: 265-273 (2009)). In addition to becoming prone to metastasize, carcinoma cells undergoing EMT become resistant to cytotoxic treatments, including chemotherapy (Huang et al., *Cell Death Dis.*, 4: e683 (2013); and Mitra et al., *Oncotarget*, 6: 10697-711 (2015)), radiation (Kurrey et al., *Stem Cells*, 27: 2059-68 (2009)), or small molecule targeted therapies (Thomson et al., *Cancer Res.*, 65: 9455-62 (2005); and Byers et al., *Clin. Cancer Res.*, 19: 279-90 (2013)). Interfering with, or reversing the process of EMT represents an attractive therapeutic modality against tumor dissemination and, perhaps more importantly, to minimize the occurrence of therapeutic resistance (Palena et al., *Exp. Biol. Med. (Maywood)*, 236: 537-45 (2011); Palena et al., *Oncoimmunology*, 3: e27220 (2014); and Davis et al., *Trends Pharmacol. Sci.*, 35: 479-88 (2014)).

[0004] The association between EMT and tumor resistance to therapies has been extended to immunotherapy as mesenchymal-like tumor cells have also been shown to be less susceptible to the cytotoxic effect of adaptive or innate immune effector cells than their epithelial counterparts (Hamilton et al., *Cancer Res.*, 74: 2510-2519 (2014); and Akalay et

al., *Cancer Res.*, 73: 2418-2427 (2013)). Data suggest that tumor EMT could have a negative impact on the various immune-based interventions against cancer that are currently being investigated in the clinic, all of which ultimately rely on the ability of effector immune cells to efficiently lyse cancer cells.

[0005] Therefore, there is a desire for improved cancer treatment methods.

BRIEF SUMMARY OF THE INVENTION

[0006] The invention provides a method of enhancing immune-mediated lysis of mesenchymal cancer cells comprising administering fulvestrant to the cancer cells, thereby enhancing immune-mediated lysis of cancer cells.

[0007] The invention provides a method of sensitizing mesenchymal cancer cells to chemotherapy or immune-mediated lysis comprising administering fulvestrant to the cancer cells, thereby sensitizing cancer cells to chemotherapy or immune-mediated lysis.

[0008] The invention also provides a method of treating lung cancer comprising administering a combination of fulvestrant and an immune-mediated therapy to a patient, thereby treating lung cancer.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0009] Figs. 1A-E are images demonstrating H460-M clone exhibits resistance to cell death. Fig. 1A is a Western blot analysis of indicated proteins expressed by the H460-E and H460-M clones. Figs. 1B-E are graphs showing susceptibility of H460-E vs. H460-M clone to (B) brachyury-specific CD8⁺ T cells, (C) NK effector cells, (D) recombinant TRAIL, and (E) cisplatin. Error bars indicate the standard error of the mean (SEM) of triplicate measurements. [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$].

[0010] Figs. 2A-C are graphs demonstrating that fulvestrant renders H460-M cells more sensitive to TRAIL-mediated lysis. Fig. 2A is a graphical depiction of compounds having measurable activity in the qHTS assay. Large circles represent 53 hits with curves class of 4 when used with PBS, and curves classes -1 or -2 when used with TRAIL. The top three ranked compounds are fulvestrant, selegiline, and midazolam. Fig. 2B are dose response curves of top compounds in combination with TRAIL vs. PBS. Fig. 2C are graphs demonstrating TRAIL lysis of H460 cells treated for 48 hours with indicated concentrations of compounds prior to addition of 30 ng/mL TRAIL.

[0011] Figs. 3A-F are graphs demonstrating that fulvestrant renders mesenchymal cells more sensitive to immune-mediated lysis. Fig. 3A is a series of dose response curves of H460-E and H460-M cells treated with indicated doses of fulvestrant, 4-hydroxytamoxifen or DMSO to TRAIL-mediated lysis. Brachyury (B), fibronectin (C) and estrogen-receptor alpha (D) expression in clonally derived H1703 cells transfected with pCMV vs. pBr. (E) Susceptibility to TRAIL-mediated lysis in cells pre-treated with fulvestrant vs. DMSO. (F) Susceptibility of parental H460 cells treated with fulvestrant vs. DMSO to lysis by NK cells at various effector-to-target ratios. Error bars indicate the standard error of the mean (SEM) of triplicate measurements. [$* p<0.05$, $** p<0.01$, $*** p<0.001$].

[0012] Figs. 4A-I are images demonstrating fulvestrant reverts immune-resistance of chemo-resistant H1703 and H460 cells. Fig 4A is a graph showing fold change in expression levels of indicated mRNA in chemo-resistant vs. control H1703 cells. Figs. 4B and 4C are graphs showing susceptibility of fulvestrant-treated cells to TRAIL (B) or (C) NK cells. Fig. 4D are graphs showing sensitivity of the H1703 pair to a combination of vinorelbine and cisplatin; tumor cells were left untreated (left panel) or treated with fulvestrant prior to exposure to chemotherapy. Figs. 4E and 4F are graphs showing fold-change in expression levels of indicated mRNA in chemo-resistant vs. control H460 cells. Fig. 4G is an image showing immunohistochemical analysis of ESR1 expression in H460 tumor xenografts of mice treated with either HBSS or docetaxel. Fig. 4H is a graph showing susceptibility of parental vs. chemo-resistant H460 treated with fulvestrant vs. DMSO to lysis by MUC1-specific T cells. Fig. 4I is a graph showing the effect of brachyury and ESR1 silencing on the susceptibility of indicated cells to TRAIL-mediated lysis. Error bars indicate the standard error of the mean (SEM) of triplicate measurements. [$* p<0.05$, $** p<0.01$, $*** p<0.001$].

[0013] Figs. 5A-G are graphs demonstrating that the estrogen receptor mediates resistance to immune attack. In Fig. 5A, H460 cells stably transfected with pCMV or a vector encoding the ESR1 gene were assessed for their sensitivity to NK-mediated lysis. In Figs. 5B and 5C, single cell clones of H460 cells with High vs. Low ESR1 expression were evaluated for lysis by TRAIL (B) or (C) NK cells that were either untreated or pre-treated with CMA. Fig. 5D demonstrates the expression of indicated mRNA, relative to GAPDH, in clonal H460 ESR1-High (gray bars) vs. ESR1-Low cells (black bars). Figs. 5E and 5F show ESR1 mRNA (E) and ESR2 mRNA (F) in normal lung vs. lung adenocarcinoma tissues. Shaded areas correspond to the normal range of expression for each gene, calculated as the mean expression in normal lung tissues ($\pm$ two standard deviations). Fig. 5G shows mRNA

expression of the indicated genes in lung samples categorized as either ESR1 Low or High, based on the expression in normal lung tissues. Error bars indicate the standard deviation of the mean. [* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$].

[0014] Figs. 6A-F are images demonstrating that fulvestrant treatment reduces EMT markers and increases sensitivity of lung xenografts to docetaxel. Fig. 6A is a Western blot analysis of brachyury, fibronectin, and vimentin protein levels in H460 cells treated for six days with indicated concentrations of fulvestrant. Fig. 6B is a schematic representation of the brachyury response element (AATTTTCACACCTAGGTGTGAAATT; SEQ ID NO: 1). Fig. 6C is a graph showing brachyury transcriptional activity in H460 cells treated for six days with indicated concentrations of fulvestrant. Fig. 6D is a graph showing brachyury promoter activity in H460 cells treated for three days with indicated concentrations of fulvestrant. Fig. 6E are images showing estrogen receptor 1, brachyury, and fibronectin expression in H460 tumor xenografts five days after a single injection of either HBSS or fulvestrant. Fig. 6F is a series of graphs showing tumor volume of H460 xenografts treated as indicated, with fulvestrant (250 mg/kg) given on days 4 and 11 and docetaxel (20 mg/kg) on days 7 and 10. Error bars indicate the standard error of the mean (SEM) of triplicate measurements. [* $p < 0.05$, ** $p < 0.01$].

DETAILED DESCRIPTION OF THE INVENTION

[0015] Fulvestrant is an FDA-approved, selective estrogen receptor antagonist used in the treatment of hormone receptor-positive breast cancer with well-known pharmacokinetics and pharmacological and toxicity profiles (Kuter et al., *Breast Cancer Res. Treat.*, 133: 237-46 (2012) and Robertson et al., *Clin. Pharmacokinet.*, 43: 529-38 (2004)).

[0016] The invention is predicated, at least in part, on the unexpected discovery that fulvestrant renders mesenchymal-like lung cancer cells significantly more susceptible to immune effector cells and chemotherapy. A robust association between the acquisition of mesenchymal features by lung carcinoma cells and the expression of estrogen receptor 1 (Esr1, ER-alpha) and blockade of estrogen signaling via fulvestrant revert tumor phenotype while significantly augmenting tumor cell susceptibility to NK cells, tumor-reactive cytotoxic T cells, and chemotherapy. Therefore, the efficacy of immune-mediated therapies, such as cancer vaccine approaches, adoptively transferred anti-tumor lymphocytes, or monoclonal antibodies against checkpoint inhibitors or those that mediate antibody-dependent cell cytotoxicity (ADCC), can be improved when used in combination with fulvestrant.

[0017] The invention provides a method of enhancing immune-mediated lysis of mesenchymal cancer cells comprising administering fulvestrant to the cancer cells, thereby enhancing immune-mediated lysis of cancer cells.

[0018] The invention provides a method of sensitizing mesenchymal cancer cells to chemotherapy or immune-mediated lysis comprising administering fulvestrant to the cancer cells, thereby sensitizing mesenchymal cancer cells to chemotherapy or immune-mediated lysis.

[0019] In one embodiment, the immune-mediated lysis is cytotoxic T-cell (CTL) mediated killing. In another embodiment, the immune-mediated lysis is natural killer (NK) cell mediated killing.

[0020] Although not wishing to be bound by any particular theory, fulvestrant treatment of mesenchymal-like carcinoma (e.g., lung carcinoma) cells increase immune-mediated cell death by repairing defective apoptotic mechanisms driven by the epithelial-mesenchymal transition (EMT). Treatment with fulvestrant reconstitutes sensitivity of tumor cells to chemotherapy and improved lysis by immune effector mechanisms including NK cells and antigen-specific T cells.

[0021] Non-limiting examples of specific types of cancer cells include cancer cells of the head and neck, eye, skin, mouth, throat, esophagus, chest, bone, lung, colon, sigmoid, rectum, stomach, prostate, breast, ovaries, kidney, liver, pancreas, brain, intestine, heart or adrenals. More particularly, cancer cells include cells from solid tumors, sarcoma, carcinomas, fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, Kaposi's sarcoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, menangioma, melanoma, neuroblastoma, retinoblastoma, a blood-born tumor, acute lymphoblastic leukemia, acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute

myeloblastic leukemia, acute promyelocytic leukemia, acute monoblastic leukemia, acute erythroleukemic leukemia, acute megakaryoblastic leukemia, acute myelomonocytic leukemia, acutenonlymphocytic leukemia, acute undifferentiated leukemia, chronic myelocytic leukemia, chronic lymphocytic leukemia, hairy cell leukemia, or multiple myeloma. See, e.g., *Harrison's Principles of Internal Medicine*, Eugene Braunwald et al., eds., pp. 491-762 (15th ed. 2001). In one embodiment, the cancer cells are lung cancer cells (e.g., mesenchymal lung cancer cells).

[0022] The invention also provides a method of treating lung cancer comprising administering a combination of fulvestrant and an immune-mediated therapy to a patient, thereby treating lung cancer. For example, the combination of fulvestrant and an immune-mediated therapy can be used for the management of advanced lung cancer patients.

[0023] The term “immune-mediated therapy” or “immunotherapy,” as used herein refers to the treatment of a disease by inducing, enhancing, or suppressing an immune response. Immunotherapies designed to elicit or enhance an immune response are referred to as activation immunotherapies, while immunotherapies designed to suppress an immune response are referred to as suppression immunotherapies. Types of immunotherapies include, but are not limited to, checkpoint inhibitors, immunomodulators, cell-based immunotherapies, monoclonal antibodies, radiopharmaceuticals, and vaccines. Immunotherapy strategies for cancer are described in, for example, Waldmann, T.A., *Nature Medicine*, 9: 269-277 (2003).

[0024] Immunomodulators can be recombinant, synthetic, or natural substances that include, but are not limited to, cytokines (e.g., TNF- α , IL-6, GM-CSF, IL-2, and interferons), co-stimulatory molecules (e.g., B7-1 and B7-2), chemokines (e.g., CCL3, CCL26, CXCL7), glucans, and oligodeoxynucleotides.

[0025] Cell-based immunotherapies typically involve removal of immune cells (e.g., cytotoxic T-cells, natural killer cells, or antigen presenting cells (APCs)) from a subject, modification (e.g., activation) of immune cells, and return of the modified immune cells to the patient (e.g., adoptively transferred anti-tumor lymphocytes). In the context of the inventive method, the cell-based immunotherapy desirably is Sipuleucel-T (PROVENGE™), which is an autologous active cellular immunotherapy used in the treatment of asymptomatic or minimally symptomatic CRPC (Plosker, G.L., *Drugs*, 71(1): 101-108 (2011); and Kantoff et al., *New Engl. J. Med.*, 363: 411-422 (2010)).

[0026] Several monoclonal antibodies have been approved for the treatment of cancer, including naked antibodies and antibody-drug conjugates based on human, humanized, or chimeric antibodies (Scott et al., *Nat Rev Cancer*, 12(4): 278-87 (2012); Harding et al., *MAbs*, 2(3): 256-65 (2010); and Weiner et al., *Nature Rev. Immunol.*, 10(5): 317-327 (2010)). In one embodiment, the inventive method comprises treating cancer cells with any suitable monoclonal antibody known in the art. Such monoclonal antibodies include, for example, ipilumimab (YERVOY™), which is a fully human antibody that binds to CTLA-4 and is indicated for the treatment of melanoma. Antibodies that target the interaction of programmed death receptor-1 (PD-1) with its ligands PD-L1 and PD-L2, also can be used in the invention (see, e.g., Weber, *Semin. Oncol.*, 37(5): 430-4309 (2010); and Tang et al., *Current Oncology Reports*, 15(2): 98-104 (2013)). Antibodies that inhibit PD-1 signaling include, for example nivolumab (also known as BMS-936558 or MDX1106; see, e.g., ClinicalTrials.gov Identifier NCT00730639), sipuleucel-T CT-011, pembrolizumab, atezolizumab, and MK-3575 (see, e.g., Patnaik et al., 2012 *American Society of Clinical Oncology (ASCO) Annual Meeting*, Abstract # 2512). Monoclonal antibodies that specifically target prostate cancer are under development and also can be used in the invention (see, e.g., Jakobovits, A., *Handb. Exp. Pharmacol.*, 181: 237-56 (2008); and Ross et al., *Cancer Metastasis Rev.*, 24(4): 521-37 (2005)). Monoclonal antibodies suitable for treatment of breast cancer include, for example, trastuzumab (HERCEPTIN™), pertuzumab (PERJETA™), and the antibody-drug conjugate ado-trastuzumab emtansine (KADCYLA™). Cetuximab is an anti-EGFR antibody that is suitable for treatment of colorectal, non-small cell lung cancer, and squamous cell carcinoma of the head and neck.

[0027] Radiopharmaceuticals are radioactive drugs which are currently used to treat and diagnose a variety of diseases, including cancer. For example, radionuclides can be targeted to antibodies (i.e., radioimmunotherapy) to treat blood-derived cancers (Sharkey, R.M. and Goldenberg, D.M., *Immunotherapy*, 3(3): 349-70 (2011)). Several radioisotopes have been approved to treat cancer, including iodine-125, iodine-131, and radium-223 (marketed as XOFIGO™). Radium-223 has been approved as a radiopharmaceutical to treat metastatic bone cancer and CRPC. In CRPC, radium-223 also has been shown to enhance the anti-tumor immune response.

[0028] Vaccines represent another strategy to prevent and treat cancer. Many different cancer vaccine platforms are currently being evaluated in phase II and/or phase III clinical trials, including, for example, yeast-based vaccines, peptide-based vaccines, recombinant

viral vectors, killed tumor cells, or protein-activated dendritic cells (see, e.g., Schlom, J., *J. Natl. Cancer. Inst.*, 104: 599-613 (2012)). Any suitable vaccine can be used in the inventive method.

[0029] In one embodiment, the vaccine is a yeast-based vaccine or a virus-based vaccine, such as a poxviral-based or adenoviral-based vaccine. For example, the vaccine can be the PSA/TRICOM vaccine (PROSTVAC™), which is a cancer vaccine composed of a series of poxviral vectors engineered to express PSA and a triad of human T-cell costimulatory molecules (see, e.g., Madan et al., *Expert Opin. Investigational Drugs*, 18(7): 1001-1011 (2009); and U.S. Patents 4,547,773; 6,045,802; 6,165,460; 6,548,068; 6,946,133; 7,247,615; 7,368,116; 7,598,225; 7,662,395; 7,871,986; and 8,178,508). The vaccine also can be a MUC-1/CEA vaccine (e.g., PANVAC), which is composed of a series of poxviral vectors (e.g., recombinant vaccinia and recombinant fowlpox) engineered to express MUC-1 and CEA and optionally human T-cell costimulatory molecules (e.g., TRICOM) (see, e.g., Madan et al., *Expert Opin Biol Ther.*, 7(4): 543-54; International Patent Application Publications WO 2005/046622, WO 2005/046614, and WO 2015/061415); and U.S. Patents 5,698,530; 6,001,349; 6,319,496; 6,969,609; 7,211,432; 7,368,116; 7,410,644; 7,771,715; 7,999,071; and 8,609,395). Alternatively, the cancer vaccine can comprise poxviral vectors (e.g., MVA and/or fowlpox) that have been genetically modified to express CEA and TRICOM (e.g., MVA/rF-CEA/TRICOM). The vaccine also can be a yeast MUC-1 immunotherapeutic, such as those described in, e.g., U.S. Patent Application Publication 2013/0315941 and International Patent Application Publication WO 2012/103658.

[0030] In another embodiment, the vaccine can be a Brachyury vaccine, which comprises recombinant yeast or poxvirus that has been genetically modified to express the Brachyury transcription factor and optionally TRICOM (see, e.g., International Patent Application Publications WO 2014/043518 and WO 2014/043535; U.S. Patents 8,188,214 and 8,613,933; Heery et al., *Cancer Immunol. Res.*, DOI:10.1158/2326-6066.CIR-15-0119 (2015); Hamilton et al., *Oncotarget*, 4: 1777-90 (2013); and www.clinicaltrials.gov/ct2/show/NCT02179515, Safety and Tolerability of a Modified Vaccinia Ankara (MVA)-Based Vaccine Modified to Express Brachyury and T-cell and Costimulatory Molecules (MVA-Brachyury-TRICOM) (2014)).

[0031] In another embodiment, the vaccine (e.g., yeast-based vaccine or a virus-based vaccine) comprises at least one (e.g., one two, three, four, five, or more) cancer antigen selected from the group consisting of CEA, MUC (e.g., MUC-1, MUC-2, MUC-3, MUC-4,

MUC-5AC, MUC-5B, MUC-6, MUC-7, MUC-11, and MUC-12), PSA, HER2, NY-ESO (e.g., NY-ESO-1), Brachyury, MAGE (e.g., MAGE-3, MAGE-6, and MAGE D), p53, GM-CSF, ras (e.g., k-ras and h-ras), gastrin, PANC1A, PANC1B, neoantigens, modified versions thereof (e.g., CEA(6D), and fragments thereof (e.g., mini-mucin).

[0032] The cancer cells (e.g., lung cancer cells) can be *in vivo* or *in vitro*. The term “*in vivo*” refers to a method that is conducted within living organisms in their normal, intact state, while an “*in vitro*” method is conducted using components of an organism that have been isolated from its usual biological context (e.g., isolating and culturing cells obtained from an organism). Preferably, the cancer cells are *in vivo*. For example, when the cancer cells are lung cancer cells, preferably the lung cancer cells exist within a human male or female lung cancer. When the cancer cells (e.g., lung cancer cells) are *in vivo*, i.e., in a human, the inventive methods induce a therapeutic effect in the cancer patient and treat the cancer (e.g., lung cancer).

[0033] The cancer cells can cancer cells (e.g., lung cancer cells) that have become resistant to other standard treatment regimens. For example, the cancer cells can be resistant to chemotherapy and/or radiation therapy.

[0034] The patient can be any suitable patient, such as a mammal (e.g., mouse, rat, guinea pig, hamster, rabbit, cat, dog, pig, goat, cow, horse, or primate (e.g., human)).

[0035] As used herein, the terms “treatment,” “treating,” and the like refer to obtaining a desired pharmacologic and/or physiologic effect. Preferably, the effect is therapeutic, i.e., the effect partially or completely cures a disease and/or adverse symptom attributable to the disease. To this end, the inventive method comprises administering a “therapeutically effective amount” of fulvestrant, immunotherapy, and/or compositions thereof. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve a desired therapeutic result. The therapeutically effective amount may vary according to factors such as the disease state, age, and weight of the individual, and the ability of the fulvestrant, immunotherapy, and/or compositions thereof to elicit a desired response in the individual.

[0036] A combination of fulvestrant and immunotherapeutic agent (e.g., cancer vaccine) can be administered sequentially or simultaneously. In certain embodiments, fulvestrant is administered in combination with one or more (e.g., 2, 3, 4, or 5) immunotherapeutic agents (e.g., cancer vaccines). In additional embodiments, the combination of a fulvestrant and immunotherapeutic agent can be administered with one or more (e.g., 2, 3, 4, or 5) additional

therapeutic agents (e.g., chemotherapy, small molecule inhibitors (e.g., erlotinib, gefitinib, afatinib, osimertinib, bevacizumab, crizotinib, and ceritinib), endocrine deprivation therapy, androgen deprivation therapy (e.g., enzalutamide), a histone deacetylase (HDAC) inhibitor, and/or cabozantinib).

[0037] The term “androgen deprivation therapy” (ADT), as used herein, refers to a treatment for cancer in which the level of androgen hormones, such as testosterone, in a patient are reduced, typically by pharmaceutical or surgical methods (see, e.g., Perlmutter and Lepor, *Rev. Urol.*, 9 (Suppl 1): S3-8 (2007)). Surgical approaches to ADT include surgical castration. Pharmaceutical approaches to ADT include androgen inhibitors (antiandrogens) and chemical castration. ADT also is referred to in the art as androgen suppression therapy. Androgen inhibitors used in prostate cancer can be steroidal or non-steroidal (also referred to as “pure” antiandrogens). Steroidal androgen inhibitors include, for example, e.g., megestrol (MEGACE™), cyproterone acetate, abiraterone, and abiraterone acetate (ZYTIGA™). Non-steroidal androgen inhibitors include, for example, bicalutamide (CASODEX™), flutamide (EULEXIN™), nilutamide (ANANDRON™ and NILANDRON™), and enzalutamide (XTANDI™).

[0038] In one embodiment, the androgen deprivation therapy is enzalutamide. Enzalutamide (marketed as XTANDI™ by Medivation and Astellas and formally known as MDV3100) is an oral non-steroidal small molecule androgen receptor inhibitor that prolongs survival in men with metastatic castration resistant prostate cancer in whom the disease has progressed after chemotherapy. Preclinical studies also suggest that enzalutamide also inhibits breast cancer cell growth (see, e.g., Cochrane et al., *Cancer Research*, 72(24 Suppl): Abstract nr P2-14-02 (2012)).

[0039] Immunogenic modulation by enzalutamide has been described in murine prostate carcinomas (see, e.g., Ardiani et al., *Clinical Cancer Res.*, 19(22): 6205-6218 (2013)), where enzalutamide up-regulated MHC-I and Fas on the surface of tumor cells, thus improving the cells’ sensitivity to T-cell killing. In these studies, treatment with enzalutamide did not alter the number or function of T-cells. Enzalutamide-mediated immunogenic modulation increased the efficacy of a therapeutic cancer vaccine in TRAMP mice with spontaneous prostate tumors, which subsequently translated to significant improvements in overall survival (Ardiani et al., *supra*).

[0040] In another embodiment, the androgen deprivation therapy is abiraterone, which is formulated as abiraterone acetate and marketed as ZYTIGA™ by Janssen Biotech, Inc.

Abiraterone inhibits CYP17A1, a rate-limiting enzyme in androgen biosynthesis. Inhibition of CYP17A1 subsequently blocks the production of androgen in all endocrine organs, including the testes, adrenal glands, and in prostate tumors (Harris et al., *Nature Clinical Practice Urology*, 6(2): 76-85(2009)). In a phase III study in patients with CRPC previously treated with docetaxel, abiraterone was shown to improve overall survival by 3.9 months compared to placebo (de Bono et al., *New England J. Med.*, 364(21): 1995-2005(2011)). Abiraterone is indicated for use in combination with prednisone to treat CRPC.

[0041] The term “endocrine deprivation therapy” (also referred to as “hormonal therapy”), as used herein, refers to a treatment for breast cancer in which the level of endocrine hormones, such as estrogen and/or testosterone, in a patient are reduced, typically by pharmaceutical or surgical methods (see, e.g., Angelopoulos et al., *Endocr. Relat. Cancer*, 11: 523-535 (2004); Dhingra, K., *Invest. New Drugs*, 17(3): 285-311 (1999); and Garay, J.P. and Park, B.H., *Am. J. Cancer Res.*, 2(4): 434-445 (2012)). Surgical approaches to endocrine deprivation include oophorectomy. Pharmaceutical approaches to endocrine deprivation therapy include estrogen inhibitors and androgen inhibitors. In one embodiment, the endocrine deprivation therapy is an androgen inhibitor such as, for example, cyproterone acetate, abiraterone, abiraterone acetate (ZYTIGA™), or enzalutamide (XTANDI™). The androgen inhibitor preferably is abiraterone or enzalutamide. Alternatively or additionally, the endocrine deprivation therapy is an estrogen inhibitor, such as, for example, megestrol (MEGACE™), an aromatase inhibitor (e.g., anastrozole), a selective estrogen receptor down-regulator (SERD) (e.g., fulvestrant), a gonadotropin-releasing hormone (GnRH) analogue, or a selective estrogen receptor modulator (SERM) (e.g., tamoxifen or raloxifene). The estrogen inhibitor preferably is tamoxifen.

[0042] Tamoxifen is a selective estrogen receptor modulator (SERM) which is indicated for the treatment of metastatic breast cancer in women and men and ductal carcinoma *in situ*. Tamoxifen a nonsteroidal agent that binds to estrogen receptors (ER), inducing a conformational change in the receptor, which results in a blockage or change in the expression of estrogen-dependent genes. Prolonged binding of tamoxifen to the nuclear chromatin of estrogen-dependent genes results in reduced DNA polymerase activity, impaired thymidine utilization, blockade of estradiol uptake, and decreased estrogen response. Like most SERMs, tamoxifen is antiestrogenic in breast tissue, but is estrogenic in the uterus and bone. Tamoxifen is described in detail in, for example, Jordan, V.C., *Br J Pharmacol.*, 147 (Suppl 1): S269-76 (2006); and U.S. Patent 4,536,516.

[0043] The invention includes a prime and boost protocol. In particular, the protocol includes an initial “prime” with a composition comprising fulvestrant and optionally one or more immunotherapeutic agents (e.g., cancer vaccines) followed by one or preferably multiple (e.g., two, three, four, five, six, or more) “boosts” with a composition containing one or more immunotherapeutic agents (e.g., cancer vaccines) and optionally fulvestrant.

Alternatively, the protocol includes a prime with a composition comprising one or more immunotherapeutic agents (e.g., cancer vaccines) and optionally fulvestrant followed by one or multiple boosts with a composition comprising fulvestrant.

[0044] When fulvestrant is administered with one or more immunotherapeutic agents (e.g., vaccines, such as cancer vaccines), the fulvestrant and one or more immunotherapeutic agents (e.g., cancer vaccines) can be coadministered to the mammal. By “coadministering” is meant administering one or more immunotherapeutic agents (e.g., cancer vaccines) and the fulvestrant sufficiently close in time such that the fulvestrant can enhance the effect of the one or more immunotherapeutic agents (e.g., cancer vaccines). In this regard, the fulvestrant can be administered first and the one or more immunotherapeutic agents (e.g., cancer vaccines) can be administered second, or *vice versa*. Alternatively, the fulvestrant and the one or more immunotherapeutic agents (e.g., cancer vaccines) can be administered simultaneously.

[0045] In one embodiment, fulvestrant and an anti-EGFR therapy (e.g., erlotinib, gefitinib, afatinib, osimertinib, and/or cetuximab) are administered to a subject.

[0046] The fulvestrant, an immunotherapeutic agent, and/or compositions thereof can be administered to a subject by various routes including, but not limited to, subcutaneous, intramuscular, intradermal, intraperitoneal, intravenous, and intratumoral. When multiple administrations are given, the administrations can be at one or more sites in a subject.

[0047] Administration of fulvestrant, an immunotherapeutic agent, and/or compositions thereof can be “prophylactic” or “therapeutic.” When provided prophylactically, the fulvestrant, an immunotherapeutic agent, and/or compositions thereof is provided in advance of tumor formation to allow the host’s immune system to fight against a tumor that the host is susceptible of developing. For example, hosts with hereditary cancer susceptibility are a preferred group of patients treated with such prophylactic immunization. The prophylactic administration of fulvestrant, an immunotherapeutic agent, and/or compositions thereof (e.g., including a vaccine) prevents, ameliorates, or delays cancer. When provided therapeutically, the fulvestrant, an immunotherapeutic agent, and/or compositions thereof is provided at or

after the diagnosis of cancer. When the host has already been diagnosed with cancer (e.g., metastatic cancer), the fulvestrant, an immunotherapeutic agent, and/or compositions thereof can be administered in conjunction with other therapeutic treatments such as chemotherapy or radiation.

[0048] The following formulations for oral, aerosol, parenteral (e.g., subcutaneous, intravenous, intraarterial, intramuscular, intradermal, interperitoneal, and intrathecal), rectal, and vaginal administration are merely exemplary and are in no way limiting.

[0049] Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the compound dissolved in diluents, such as water, saline, or orange juice; (b) capsules, sachets, tablets, lozenges, and troches, each containing a predetermined amount of the active ingredient, as solids or granules; (c) powders; (d) suspensions in an appropriate liquid; and (e) suitable emulsions. Liquid formulations may include diluents, such as water and alcohols, for example, ethanol, benzyl alcohol, and the polyethylene alcohols, either with or without the addition of a pharmaceutically acceptable surfactant, suspending agent, or emulsifying agent. Capsule forms can be of the ordinary hard- or soft-shelled gelatin type containing, for example, surfactants, lubricants, and inert fillers, such as lactose, sucrose, calcium phosphate, and cornstarch. Tablet forms can include one or more of lactose, sucrose, mannitol, corn starch, potato starch, alginic acid, microcrystalline cellulose, acacia, gelatin, guar gum, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, calcium stearate, zinc stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, disintegrating agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia, emulsions, gels, and the like containing, in addition to the active ingredient, such carriers as are known in the art.

[0050] Fulvestrant, a immunotherapeutic agent, and/or compositions thereof can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like. They also may be formulated as pharmaceuticals for non-pressured preparations, such as in a nebulizer or an atomizer.

[0051] Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers,

bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Fulvestrant, immunotherapeutic agent, and/or compositions thereof can be administered in a physiologically acceptable diluent in a pharmaceutical carrier, such as a sterile liquid or mixture of liquids, including water, saline, aqueous dextrose and related sugar solutions, an alcohol, such as ethanol, isopropanol, or hexadecyl alcohol, glycols, such as propylene glycol or polyethylene glycol, glycerol ketals, such as 2,2-dimethyl-1,3-dioxolane-4-methanol, ethers, such as poly(ethyleneglycol) 400, an oil, a fatty acid, a fatty acid ester or glyceride, or an acetylated fatty acid glyceride with or without the addition of a pharmaceutically acceptable surfactant, such as a soap or a detergent, suspending agent, such as pectin, carbomers, methylcellulose, hydroxypropylmethylcellulose, or carboxymethylcellulose, or emulsifying agents and other pharmaceutical adjuvants.

[0052] Oils, which can be used in parenteral formulations include petroleum, animal, vegetable, or synthetic oils. Specific examples of oils include peanut, soybean, sesame, cottonseed, corn, olive, petrolatum, and mineral. Suitable fatty acids for use in parenteral formulations include oleic acid, stearic acid, and isostearic acid. Ethyl oleate and isopropyl myristate are examples of suitable fatty acid esters.

[0053] Suitable soaps for use in parenteral formulations include fatty alkali metal, ammonium, and triethanolamine salts, and suitable detergents include (a) cationic detergents such as, for example, dimethyl dialkyl ammonium halides, and alkyl pyridinium halides, (b) anionic detergents such as, for example, alkyl, aryl, and olefin sulfonates, alkyl, olefin, ether, and monoglyceride sulfates, and sulfosuccinates, (c) nonionic detergents such as, for example, fatty amine oxides, fatty acid alkanolamides, and polyoxyethylene-polypropylene copolymers, (d) amphoteric detergents such as, for example, alkyl-beta-aminopropionates, and 2-alkyl-imidazoline quaternary ammonium salts, and (3) mixtures thereof.

[0054] Suitable preservatives and buffers can be used in such formulations. In order to minimize or eliminate irritation at the site of injection, such compositions may contain one or more nonionic surfactants having a hydrophile-lipophile balance (HLB) of from about 12 to about 17. The quantity of surfactant in such formulations ranges from about 5% to about 15% by weight. Suitable surfactants include polyethylene sorbitan fatty acid esters, such as sorbitan monooleate and the high molecular weight adducts of ethylene oxide with a hydrophobic base, formed by the condensation of propylene oxide with propylene glycol.

The parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampoules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets.

[0055] Fulvestrant, an immunotherapeutic agent, and/or compositions thereof can be administered as an injectable formulation. The requirements for effective pharmaceutical carriers for injectable compositions are well known to those of ordinary skill in the art. See *Pharmaceutics and Pharmacy Practice*, J. B. Lippincott Co., Philadelphia, Pa., Banker and Chalmers, eds., pages 238-250 (1982), and *ASHP Handbook on Injectable Drugs*, Toissel, 4th ed., pages 622-630 (1986).

[0056] Topical formulations, including those that are useful for transdermal drug release, are well known to those of skill in the art and are suitable in the context of the invention for application to skin.

[0057] The fulvestrant, immunotherapeutic agent, and/or compositions thereof can be administered as a suppository by mixing with a variety of bases, such as emulsifying bases or water-soluble bases. Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams, or spray formulas containing, in addition to the active ingredient, such carriers as are known in the art to be appropriate.

[0058] Methods for preparing administrable (e.g., parenterally administrable) fulvestrant, immunotherapeutic agents, and/or compositions thereof are known or apparent to those skilled in the art and are described in more detail in, for example, *Remington's Pharmaceutical Science* (17th ed., Mack Publishing Company, Easton, PA, 1985).

[0059] In addition to the aforescribed pharmaceutical compositions, the fulvestrant, immunotherapeutic agent, and/or compositions thereof can be formulated as inclusion complexes, such as cyclodextrin inclusion complexes, or liposomes. Liposomes can serve to target fulvestrant, the immunotherapeutic agent, and/or compositions thereof to a particular tissue. Liposomes also can be used to increase the half-life of fulvestrant, the immunotherapeutic agent, and/or compositions thereof. Many methods are available for preparing liposomes, as described in, for example, Szoka et al., *Ann. Rev. Biophys. Bioeng.*, 9, 467 (1980) and U.S. Patents 4,235,871, 4,501,728, 4,837,028, and 5,019,369.

[0060] The invention further provides a kit that contains fulvestrant and immunotherapeutic agent (e.g., in one or more compositions with a pharmaceutically

acceptable carrier). The kit further provides containers, injection needles, and instructions on how to use the kit.

[0061] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLE 1

[0062] This example provides the materials and methods for the following examples.

[0063] ***Cell lines and culture conditions***

[0064] H460 and H1703 cells were originally purchased from the American Type Culture Collection (ATCC) and propagated as recommended. The cells lines were authenticated by short tandem repeat (STR) analysis (Bio-Synthesis Inc. or IDEXX BioResearch) in Jan 2013, May 2014 and Dec 2015. Two single cell-derived clonal populations of H460 cells, designated as H460-M and H460-E were expanded from the parental H460 cell line. Cells were not Chemoresistant H1703-Cis/Vin cells were generated by repeated (4-6 cycles) weekly exposure of H1703 cells to culture medium containing 500 ng/mL cisplatin (APP Pharmaceuticals) and 40 ng/mL vinorelbine (Tocris) for six hours. Chemoresistant H460-Cis/Vin cells were generated by continuous growth in the presence of 10 ng/mL cisplatin and 1 ng/mL vinorelbine.

[0065] ***Compound library***

[0066] The NPC collection, consisting of 2816 small molecule compounds, was assembled as described in Huang et al., *Science Translational Medicine*, 3:80ps16 (2011). Approximately 50% of compounds in the collection are approved for human or animal use by the United States Food and Drug Administration (FDA).

[0067] ***1536-well microplate cell viability assay***

[0068] H460-M cells were dispensed in two sets of 1536-well plates (Greiner Bio-One) at 1000 cells/well in 5 μ L of phenol red-free RPMI-1640 medium supplemented with 5% FBS, using a Multidrop Combi Reagent dispenser and a small pin cassette (Thermo Scientific). After overnight incubation, 23 nL of compounds in DMSO were transferred using a Kalypsys pin tool. Plates were covered with stainless steel Kalypsys lids and placed at 37 °C, with 5% CO₂ and 95% relative humidity. After 48 hours, each set of plates received 1 μ L of PBS or recombinant TRAIL (30 ng/mL final concentration, Enzo Life Sciences, Farmingdale, NY, USA) dispensed with a Multidrop Combi Reagent dispenser and a small pin cassette. Cell viability was assessed at four hours post-TRAIL addition (PBS for vehicle set) by dispensing

3 μ L of CellTiter-Glo reagent (Promega, Madison, WI, USA) with a BioRAPTR® (Beckman Coulter, Indianapolis, IN, USA). Plates were incubated for 30 minutes at room temperature, spun at 1000 rpm and relative luciferase units (RLU) were quantified using a ViewLux (PerkinElmer, Waltham, MA, USA).

[0069] *Quantitative High-Throughput Screen (qHTS)*

[0070] For the screen, the NPC library of compounds were transferred to columns 5-48 and controls were added in columns 1-4 of the 1536-well assay plate. Columns 1 and 2 contained DMSO and PBS or TRAIL, respectively; columns 3 and 4 contained proteasome inhibitor bortezomib (10 μ mol/L final concentration) and PBS or TRAIL, respectively. Compounds were tested as dose responses starting at a stock concentration of 10 mmol/L (final compound concentration of 46 μ mol/L) in DMSO, and diluted 3-fold with DMSO. The library was tested at 4 compound concentrations for quantitative HTS (qHTS) analysis as described below. RLU for each well were normalized to the median RLU from the DMSO control wells as 0% activity, and median RLU from control wells with bortezomib only as - 100% activity.

[0071] *qHTS data analysis*

[0072] Activity of the hits from the qHTS screen was analyzed using the Curve Response Class (CRC) classification from dose response HTS, in which normalized data is fitted to a 4-parameter dose response curves using a custom grid-based algorithm to generate curve response class (CRC) score for each compound dose response (see Inglese et al., *Proc. Natl. Acad. Sci. USA*, 103: 11473-8 (2006); and Wang et al., *Curr. Chem. Genomics*, 4: 57-66 (2010)). CRC values of -1.1, -1.2, -2.1 and -2.2 are considered highest quality hits; CRC values of -1.3, -1.4, -2.3, -2.4 and -3 are inconclusive hits; and CRC values of 4 are inactive compounds. Additional parameters obtained from qHTS and used for hit selection were the Maximum Response, which is the % activity at the maximum concentration of compound tested (46 μ mol/L) and the AC₅₀, which is obtained from the curve fitting obtained using the CRC algorithm.

[0073] *Cytotoxic assays*

[0074] Peripheral blood from healthy donors and cancer patients was obtained under appropriate Institutional Review Board approval and informed consent. Immune-mediated lytic assays were performed as described in Hamilton et al. (*Cancer Res.*, 74: 2510-9 (2014); and Jochems et al., *Cancer Immunology, Immunotherapy: CII*, 63: 161-74 (2014)). Tumor cells were incubated with compounds (Sigma) vs. DMSO for 48-72 hours prior to the

cytotoxic assays. Cultures also were treated with 1 $\mu\text{mol/L}$ β -estradiol (Sigma, St. Louis, MO, USA) 24 hours prior to the addition of fulvestrant. For chemotherapy-mediated cytotoxicity, similarly treated cultures were exposed to cisplatin and vinorelbine for six hours; media was replaced and cells were allowed to grow for three days, followed by cell survival analysis by MTT assay. Survival for treated wells was calculated as a percentage of the values representing wells of untreated cells.

[0075] *Plasmids*

[0076] The plasmids encoding the full-length human brachyury and ESR1 along with empty vectors were purchased from Origene Technologies (Rockville, MD, USA). Brachyury and GAPDH promoter reporter plasmids were purchased from SwitchGear genomics. Brachyury promoter activity was normalized to GAPDH promoter activity. The protein expression laboratory, NCI-Frederick, produced the plasmid encoding the brachyury response element.

[0077] *RNA interference*

[0078] ON-TARGET plus SMART pool siRNA for Brachyury (L-011399-00-0020), ESR1 (L-003401-00-0005) and non-targeting control siRNA and DharmaFECT-2 transfecting reagent were purchased from GE Dharmacon (Lafayette, CO, USA). Cells were transfected with 25 nmole/L siRNA constructs using the manufacture's recommended protocol. Assays were performed 72 hours post-transfection.

[0079] *RNA expression*

[0080] RNA isolation and real time PCR assays were performed as described in Hamilton et al. (*Seminars in Oncology*, 39: 358-66 (2012)) utilizing recommended probes (Life Technologies, Grand Island, NY, USA). Estrogen signaling qPCR array was purchased from SA Biosciences (Valencia CA, USA). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). ESR1/2 expression in association with various markers of EMT in lung cancer was assessed using a TCGA dataset containing data from 490 lung adenocarcinomas and 58 histologically normal lung tissues (<http://cancergenome.nih.gov/>; downloaded April 2014). Data were analyzed utilizing the Nexus Expression 3 analysis software package (BioDiscovery, Hawthorne, CA, USA); classification of samples in high vs. low ESR1 groups was performed by comparison to the mean expression level observed in normal tissues plus or minus two standard deviations.

[0081] *Western blot*

[0082] Western blots were performed as described in Hamilton et al. (*Cancer Res.*, 74: 2510-9 (2014)) using the following antibodies: pan-actin (clone Ab-5, Thermo Scientific), fibronectin, VIM, ZO-1 (BD Biosciences, San Jose, CA, USA) and brachyury (MAb 54-1).

[0083] *Tumor xenografts*

[0084] Studies involving the use of animals were carried out in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines, and under the approval of the NIH intramural animal care and use committee. Five-week old female immune-compromised mice were implanted subcutaneously with 2×10^6 H460 cells; when tumors became palpable, mice were treated with intraperitoneal injection of either HBSS or 20 mg/kg docetaxel every three days for three cycles. Fulvestrant-treated animals were given a single dose of 250 mg/kg fulvestrant s.c. five days prior to tumor collection. In the combination study, animals were implanted subcutaneously with 1×10^6 H460 cells. Fulvestrant (250 mg/kg) was given on days 4 and 11 of tumor growth, while docetaxel (20 mg/kg) was given on days 7 and 11 of tumor growth. Tumors were stained using primary antibodies against ESR1 (Abcam), brachyury (MAb 54-1) and fibronectin (GeneTex), and sections were counterstained with haematoxylin.

EXAMPLE 2

[0085] This example demonstrates the identification of compounds that enhance immune-mediated lysis via qHTS.

[0086] Acquisition of mesenchymal features by carcinoma cells imparts tumor resistance to immune-mediated attack previously was demonstrated (Hamilton et al., *Cancer Res.*, 74: 2510-9 (2014)). In the present study, homogenous populations of epithelial vs. mesenchymal-like cancer cells were generated by single cell-derived culture of lung carcinoma H460 cells. H460-E cells were characterized by low levels of expression of mesenchymal brachyury and fibronectin and high levels of epithelial ZO-1 (Fig. 1A). In contrast, clone H460-M was considered mesenchymal-like, with high levels of brachyury and fibronectin and very low levels of ZO-1 (Fig. 1A).

[0087] H460-M was significantly less sensitive than the epithelial counterpart clone (H460-E) to the cytotoxic effect of both brachyury-specific CD8⁺ cytotoxic T cells and effector NK cells, at all effector-to-target (E:T) ratios evaluated (Fig. 1B and C, respectively). In addition, H460-M cells exhibited a marked resistance to a range of concentrations of the

immune-mediator TRAIL (Fig. 1D) or the chemotherapeutic cisplatin (Fig. 1E) compared to H460-E cells.

[0088] Utilizing the H460-M clone as a model and a qHTS assay, the NCGC Pharmacological Collection was screened to identify clinically-relevant compounds that could enhance the susceptibility of resistant lung cancer cells to immune-mediated lysis. The screen was aimed at identifying compounds that were cytotoxic for TRAIL-treated cells but were devoid of cell toxicity when used alone. Using these selection criteria, 53 hits were identified corresponding to 51 unique compounds (Fig. 2A, larger dots).

[0089] These 51 hits were subsequently ranked based on 1) $\Delta\%$ MaxResponse = $[(\text{MaxResponse}_{\text{TRAIL+compound}}) - (\text{MaxResponse}_{\text{PBS+compound}})] < -50\%$; 2) $\text{AC}_{50} < 20 \mu\text{mol/L}$ for TRAIL-treated cells; and because the objective of the screen was to identify drugs that could be rapidly translated into clinical studies, the focus was on compounds that are 3) approved and available for clinical use in the US; 4) have well-known pharmacological, pharmacokinetic and toxicity profiles; and 5) show activity within a range of concentrations attainable in vivo.

[0090] Based on these criteria, fulvestrant, selegiline, and midazolam were selected for further analysis (Fig. 2A and Fig. 2B). As midazolam has been replaced in the clinic by newer generations of benzodiazepines, clonazepam, diazepam, and lorazepam were further evaluated in secondary assays. As shown in Fig. 2C, only fulvestrant was confirmed to enhance susceptibility to TRAIL with parental H460 cells, thus being chosen as the lead compound for further studies.

EXAMPLE 3

[0091] This example demonstrates that fulvestrant enhances immune cytotoxicity of mesenchymal-like tumor cells.

[0092] Unlike tamoxifen, a widely used estrogen receptor blocker that retains agonistic activity in certain tissues, fulvestrant is a pure estrogen receptor antagonist that induces receptor degradation. To assess whether the ability of fulvestrant to enhance the sensitivity of mesenchymal-like tumor cells to TRAIL-mediated lysis might be a consequence of its ability to downregulate estrogen receptor levels, its activity was compared with that of 4-hydroxy-tamoxifen, the active metabolite of tamoxifen.

[0093] H460-E and H460-M cells were pre-treated for 3 days with various concentrations of fulvestrant vs. 4-hydroxy-tamoxifen prior to the addition of TRAIL. Intriguingly, both

antagonists failed to modify the cytotoxic response of the epithelial H460-E cells, while fulvestrant (and not 4-hydroxy-tamoxifen) was able to significantly augment the susceptibility of the mesenchymal H460-M cells to TRAIL-mediated lysis (Fig. 3A), thus suggesting that the effect observed with fulvestrant might be due to receptor downregulation. Fulvestrant alone (PBS), however, had no direct toxic effect on these cells.

[0094] To confirm these observations, isogenic H1703 lung carcinoma lines stably transfected with either a control (pCMV) or a brachyury expressing (pBr) vector were generated from which two clonally-derived cell populations characterized by low (pBr-Cl1) or high (pBr-Cl2) levels of brachyury were generated (Fig. 3B). pBr-Cl2 with the highest expression of brachyury also exhibited mesenchymal features, including high expression of fibronectin (Fig. 3C) and, surprisingly, high ESR1 mRNA levels (Fig. 3D).

[0095] When cytotoxicity was evaluated, only H1703 cells with mesenchymal features (pBr-Cl2) exhibited resistance to TRAIL (Fig. 3E, black bars), a phenomenon that could be alleviated by tumor pre-treatment with fulvestrant (Fig 3E, gray bars).

[0096] These results demonstrated that fulvestrant treatment of mesenchymal-like (and not epithelial) lung carcinoma cells could increase immune-mediated lysis potentially by repairing defective cell death mechanisms driven by the EMT.

[0097] In addition to TRAIL, the above observations were extended to include NK cells. As shown in Fig. 3F, H460 lung carcinoma cells pre-treated with 50 or 500 nmol/L fulvestrant were significantly lysed by NK effector cells compared to untreated H460 cells. As the effect of fulvestrant was similar with both doses, all subsequent experiments were conducted with 50 nmol/L fulvestrant, otherwise indicated, which is comparable to the plasma Cmax (~40 nmol/L) for multiple dose steady state observed in patients treated with the drug (Kuter et al., *Breast Cancer Res. Treat.*, 133: 237-46 (2012)). These observations suggested that estrogen signaling might play an important role in protecting mesenchymal-like lung carcinoma cells to immune-mediated attack.

EXAMPLE 4

[0098] This example demonstrates upregulation of ESR1 signaling in chemo-resistant lung cancer cells.

[0099] Several studies have shown that in vitro or in vivo exposure of carcinoma cells to chemotherapeutic agents can select for a population of chemo-resistant cells with mesenchymal-like features (Huang et al., *Cell Death Dis.*, 4: e682 (2013)). As shown in Fig.

4A, H1703 cells selected in vitro in the presence of a combination of cisplatin and vinorelbine exhibited enhanced expression of T, SNAI2, FN1 and OCLN mRNA (encoding brachyury, slug, fibronectin, and occludin protein, respectively), and had a 672-fold increase in ESR1 mRNA levels, compared to control H1703 cells. These chemo-resistant cells also were highly resistant to immune-effector mechanisms, including TRAIL (Fig. 4B) and effector NK cells (Fig. 4C). However, pre-treatment with fulvestrant effectively restored their TRAIL or NK-mediated lysis to levels observed with control H1703 cells. Interestingly, the sensitivity of the H1703 chemo-resistant cells to a combination of cisplatin and vinorelbine was also reconstituted when the tumor cells were exposed to fulvestrant prior to, and during the cytotoxic assay (Fig 4D).

[00100] Similar results were observed with H460 cells selected in vitro for cisplatin and vinorelbine resistance, which also demonstrated upregulation of T, SNAI2, FN1, and OCLN mRNA and showed an eight-fold increase in the expression of ESR1 mRNA (Fig. 4E) compared to control H460 cells. Further analysis of an array of 84 genes involved in estrogen receptor activation and response demonstrated that estrogenic signaling is active in these cells, as the expression of 20 out of the 84 genes analyzed was upregulated ≥ 2 -fold (Fig. 4F) in chemo-resistant vs. parental cells. Noteworthy, upregulation of ESR1 but not ESR2 mRNA was observed in these cells. In vivo, the upregulation of ESR1 in response to chemotherapy was confirmed with xenografts of H460 cells in mice treated with repeated doses of docetaxel, which markedly increased the expression of ESR1 protein as compared to tumors of mice treated with HBSS (Fig. 4G). As shown in Fig. 4H, the ability of MUC1-specific CD8⁺ T cells to lyse H460 chemo-resistant cells was markedly reduced compared to control cells, but their lysis was fully reconstituted by pre-treatment with fulvestrant prior to the cytotoxic assay.

[00101] To ascertain a role for brachyury and estrogen receptor-alpha in mediating this increased resistance, each gene was silenced using specific siRNA pools in both control and chemo-resistant H460 cells. While silencing of brachyury (T) resulted in a modest but significant increase of cell death in response to TRAIL, silencing of ESR1 was able to fully reconstitute the susceptibility of the chemo-resistant cells to TRAIL-mediated lysis (Fig. 4I), confirming the central role of ESR1 signaling in the resistant phenotype of these cells.

EXAMPLE 5

[00102] This example demonstrates overexpression of ESR1 drives resistance to immune-mediated cytotoxicity.

[0100] To ascertain whether ESR1 could have a direct role in the phenomenon of resistance to immune attack exhibited by mesenchymal-like lung cancer cells, H460 cells were stably modified to overexpress ESR1. As shown in Fig. 5A, high expression of ESR1 significantly decreased the response of H460 cells to NK effector cells. Moreover, single clonal populations of H460 selected based on the expression of ESR1 demonstrated a direct association between ESR1 level and resistance to immune-mediated lysis. As shown in Fig. 5B, an H460 ESR1-High clone was completely resistant to the effect of a range of concentrations of TRAIL compared to an H460 ESR1-Low clone. Similar results were observed in response to NK effector cells where the H460 ESR1-Low clone was lysed more efficiently than the ESR1-High clone, an effect that was exacerbated when using NK effector cells devoid of perforin/granzyme activity (Fig. 5C).

[0101] As induction of EMT was shown to associate with expression of ESR1 in lung cancer cells, whether mesenchymal markers were differentially expressed in clonal H460 cells with High vs. Low levels of ESR1 was investigated. The ESR1-High clone (Fig. 5D) had significantly higher levels of expression of mesenchymal SNAI1, SNAI2, T, FN1 and VIM mRNA (encoding for snail, slug, brachyury, fibronectin and vimentin, respectively) as compared with the ESR1-Low clone. These results prompted the analysis of whether the association between estrogen receptor expression and markers of EMT also is present in lung tumor tissues. An initial analysis of mRNA data from the lung adenocarcinoma TCGA dataset demonstrated over-expression of ESR1 and ESR2 mRNA in 18% (88/490) and 11% (53/490) of tumors, respectively, compared to normal lung tissues (Figs. 5E and 5F). Further analysis of tumor samples segregated into Low vs. High ESR1 groups demonstrated statistically significant higher levels of mRNA for the mesenchymal markers FN1, VIM, ZEB1, ZEB2, SNAI2, and T in the High vs. Low ESR1 group, while the expression of the epithelial marker JUP mRNA (encoding for plakoglobin) was higher in the ESR1 Low vs. High group (Fig. 5G). No correlation, however, was observed between the levels of ESR2 and mesenchymal or epithelial markers (data not shown).

EXAMPLE 6

[0102] This example demonstrates the association of estrogen signaling and EMT of lung carcinomas.

[0103] The role of fulvestrant in EMT modulation was first evaluated with H460 cells treated with fulvestrant *in vitro*. As shown in Fig. 6A, fulvestrant markedly reduced the expression of the mesenchymal proteins brachyury, fibronectin and vimentin in H460 cells in a dose-dependent manner. To more directly assess the effects of fulvestrant treatment on the transcriptional activity of the brachyury protein, a luciferase reporter vector was generated (Fig. 6B) containing a promoter with a synthetic brachyury response element consisting of a single brachyury palindromic binding site (Chaffer et al., *Science*, 331: 1559-64 (2011)). This construct was transfected into the H460 cell line, and the effect of fulvestrant treatment on brachyury transcriptional activity was measured. A dose-dependent decrease in brachyury activity was observed in response to fulvestrant treatment (Fig. 6C). Further, fulvestrant also was able to reduce, on a dose-dependent fashion, the activity of a brachyury promoter reporter construct (Fig. 6D), thus demonstrating that estrogen signaling directly or indirectly regulates the transcription of the EMT transcription factor brachyury in lung cancer cells.

[0104] In subsequent experiments, the effect of fulvestrant was evaluated *in vivo* by administration of a single dose fulvestrant to mice bearing lung H460 xenografts. To assess changes on tumor phenotype, expression of estrogen receptor 1, brachyury, and fibronectin were evaluated by immunohistochemistry (Fig 6E). Fulvestrant was able to decrease the levels of all three proteins in tumor cells with the most significant reductions of fibronectin and brachyury taking place in tumors where the highest decrease of ESR1 levels (tumors T-4 and T-6, Fig. 6E).

[0105] Furthermore, the potential effect of fulvestrant treatment *in vivo* on tumor sensitivity to cytotoxic treatment was evaluated in mice bearing H460 xenografts treated with either docetaxel or fulvestrant alone, or a combination of both. As shown in Fig. 6F, neither treatment alone had any measurable impact on tumor growth. However, when fulvestrant was administered three days prior to docetaxel, a marked reduction of tumor volume was observed compared to single treatments, and almost complete tumor control was achieved in 2/4 treated mice (Fig. 6F).

[0106] These results thus demonstrated that fulvestrant can reduce mesenchymal tumor features in lung carcinoma cells, both *in vitro* and *in vivo*, and sensitize tumor cells *in vivo* to the cytotoxic effect of chemotherapy.

[0107] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0108] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0109] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible

variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

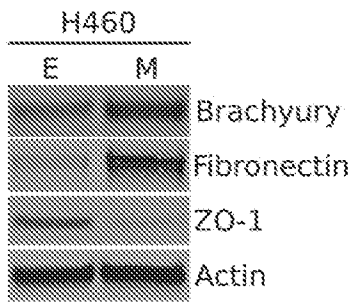
CLAIMS:

1. A method of enhancing immune-mediated lysis of mesenchymal cancer cells comprising administering fulvestrant to the cancer cells, thereby enhancing immune-mediated lysis of cancer cells.
2. A method of sensitizing mesenchymal cancer cells to chemotherapy comprising administering fulvestrant to the cancer cells, thereby sensitizing cancer cells to chemotherapy.
3. A method of sensitizing mesenchymal cancer cells to immune-mediated lysis comprising administering fulvestrant to the cancer cells, thereby sensitizing cancer cells.
4. The method of any one of claims 1-3, wherein the cancer is lung cancer.
5. A method of treating lung cancer comprising administering a combination of fulvestrant and an immune-mediated therapy to a patient, thereby treating lung cancer.
6. The method of claim 5, wherein the immune-mediated therapy is selected from the group consisting of a vaccine, a monoclonal antibody, a checkpoint inhibitor, an immunomodulator, a cell-based immunotherapy, and a radiopharmaceutical.
7. The method of claim 6, wherein the monoclonal antibody is cetuximab.
8. The method of claim 6, wherein the vaccine is a yeast-based vaccine.
9. The method of claim 6, wherein the vaccine is a virus-based vaccine.
10. The method of claim 9, wherein the virus-based vaccine is a poxviral-based vaccine.
11. The method of claim 9, wherein the virus-based vaccine is an adenoviral-based vaccine.
12. The method of any one of claims 6-11, wherein the vaccine comprises at least one cancer antigen.
13. The method of claim 12, wherein the vaccine is a Brachyury vaccine.

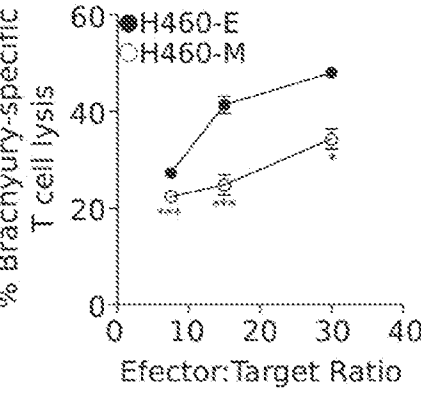
14. The method of claim 12, wherein the vaccine is a MUC-1 vaccine.
15. The method of claim 12, wherein the vaccine is a CEA vaccine.
16. The method of claim 12, wherein the vaccine comprises at least two cancer antigens.
17. The method of claim 12, wherein the vaccine comprises at least three cancer antigens.
18. The method of claim 6, wherein the immunotherapy is Sipuleucel-T (PROVENGE™), ipilumimab, nivolumab, radium-223 (XOFIGO™), a yeast-MUC-1 immunotherapeutic, or trastuzumab (HERCEPTIN™).
19. The method of any one of claims 1-18, further comprising treating the cancer cells with one or more additional therapeutic agents.
20. The method of claim 19, wherein the one or more additional therapeutic agents are selected from the group consisting of chemotherapy, a small molecule inhibitor, endocrine deprivation therapy, androgen deprivation therapy, a histone deacetylase (HDAC) inhibitor, and/or cabozantinib.
21. The method of claim 19 or 20, wherein the one or more additional therapeutic agents is selected from the group consisting of enzalutamide, erlotinib, gefitinib, afatinib, and osimertinib.
22. The method of claim 19 or 20, wherein the one or more additional therapeutic agents is chemotherapy.
23. The method of any one of claims 1-22, wherein the cancer cells are *in vivo*.
24. The method of claim 23, wherein the cancer cells are in a human.
25. The method of any one of claims 1-22, wherein the cancer cells are *in vitro*.

Fig. 1

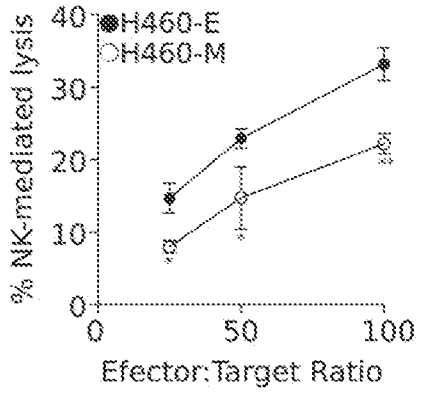
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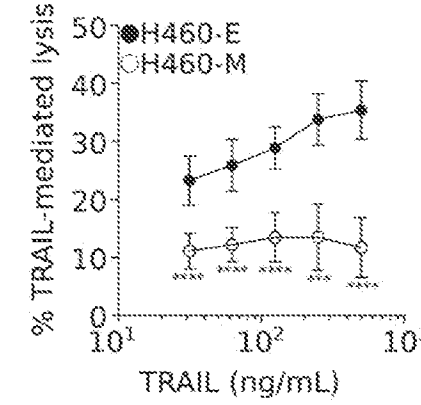
B



C



D



E

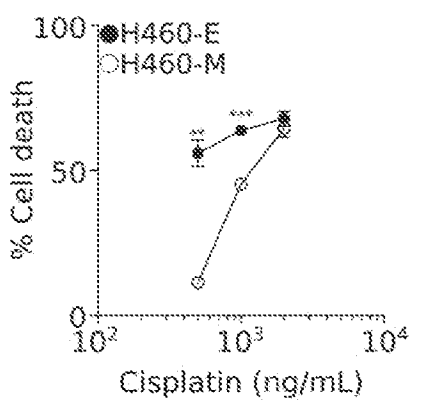


Fig. 2

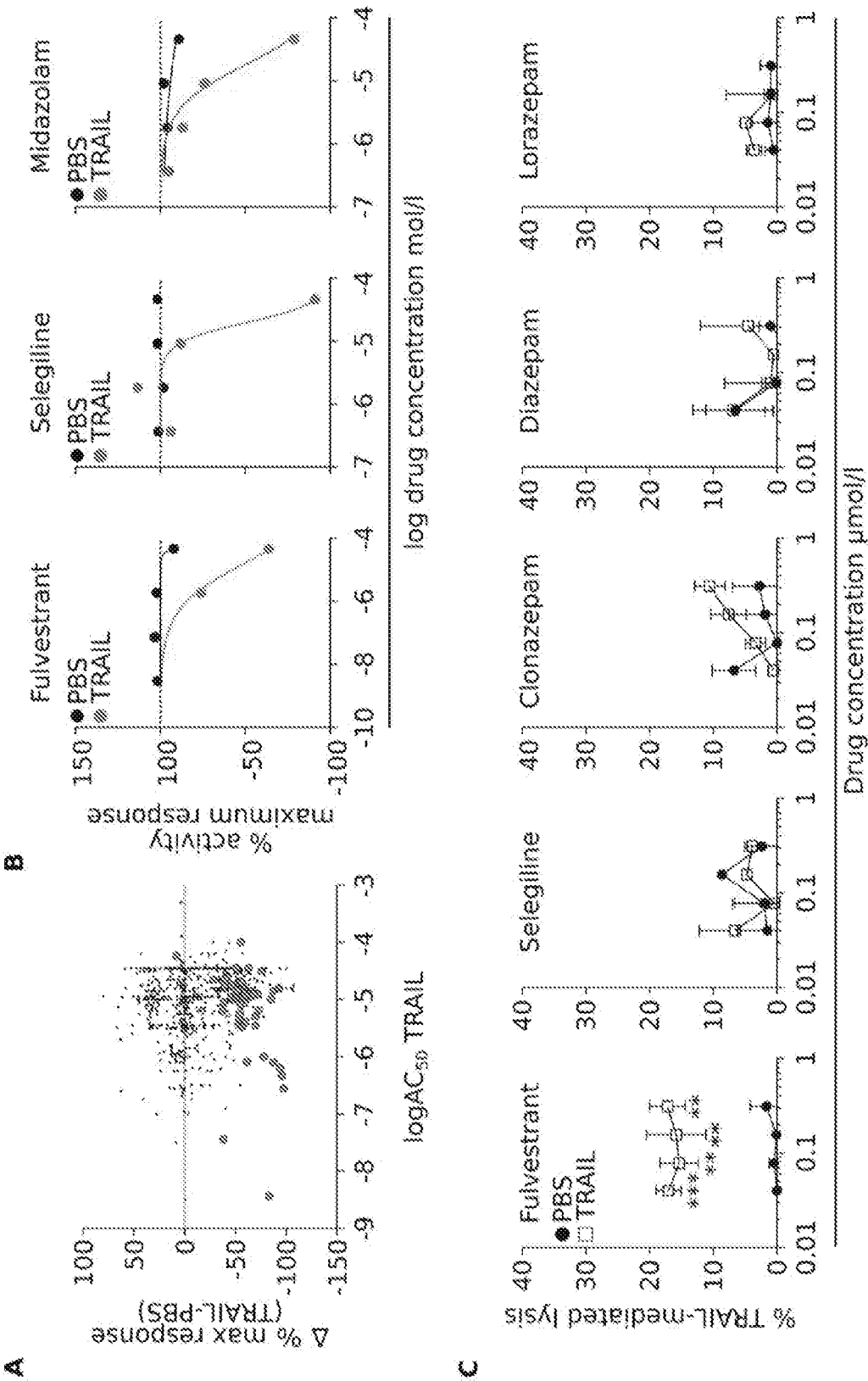


Fig. 3

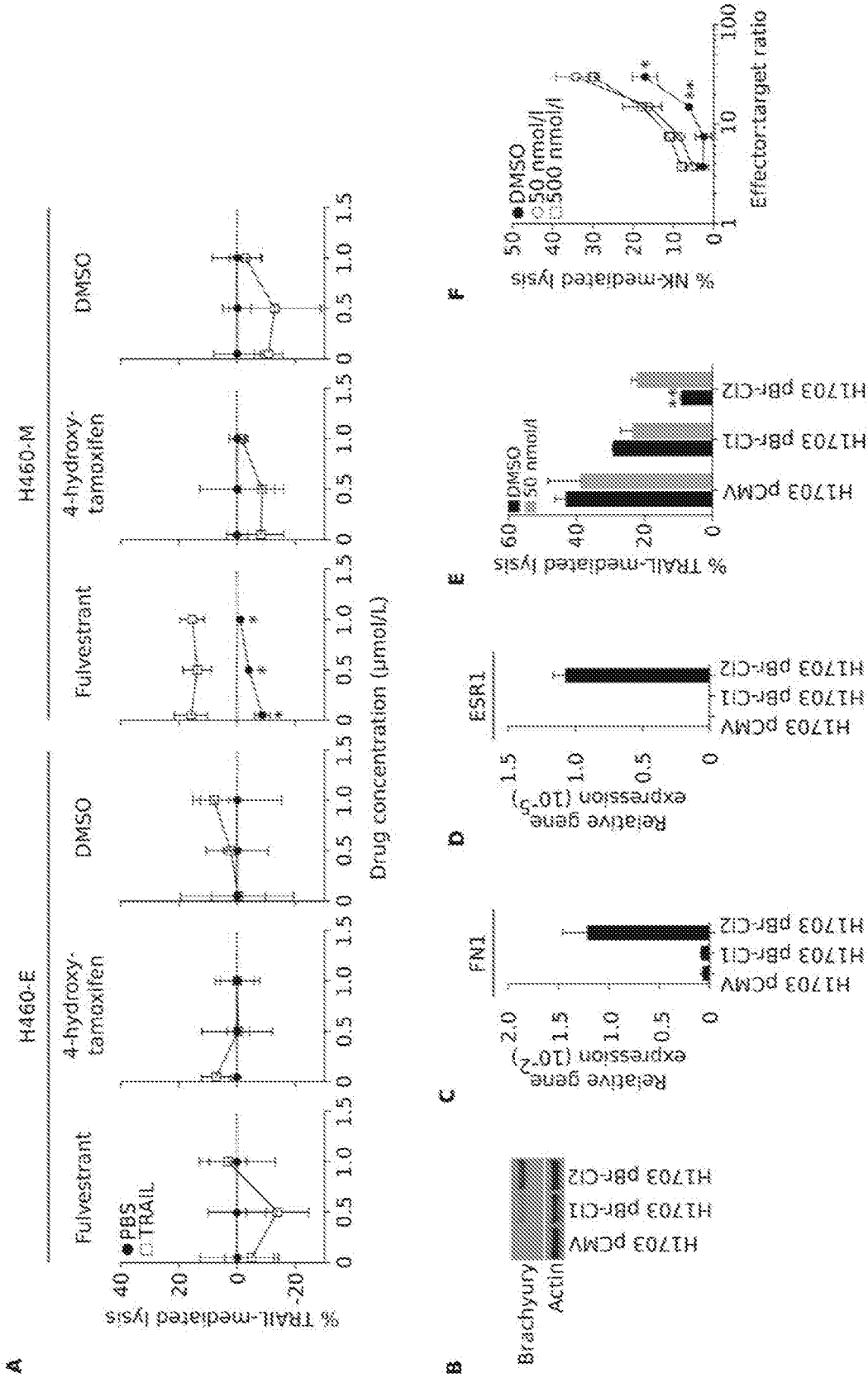


Fig. 4

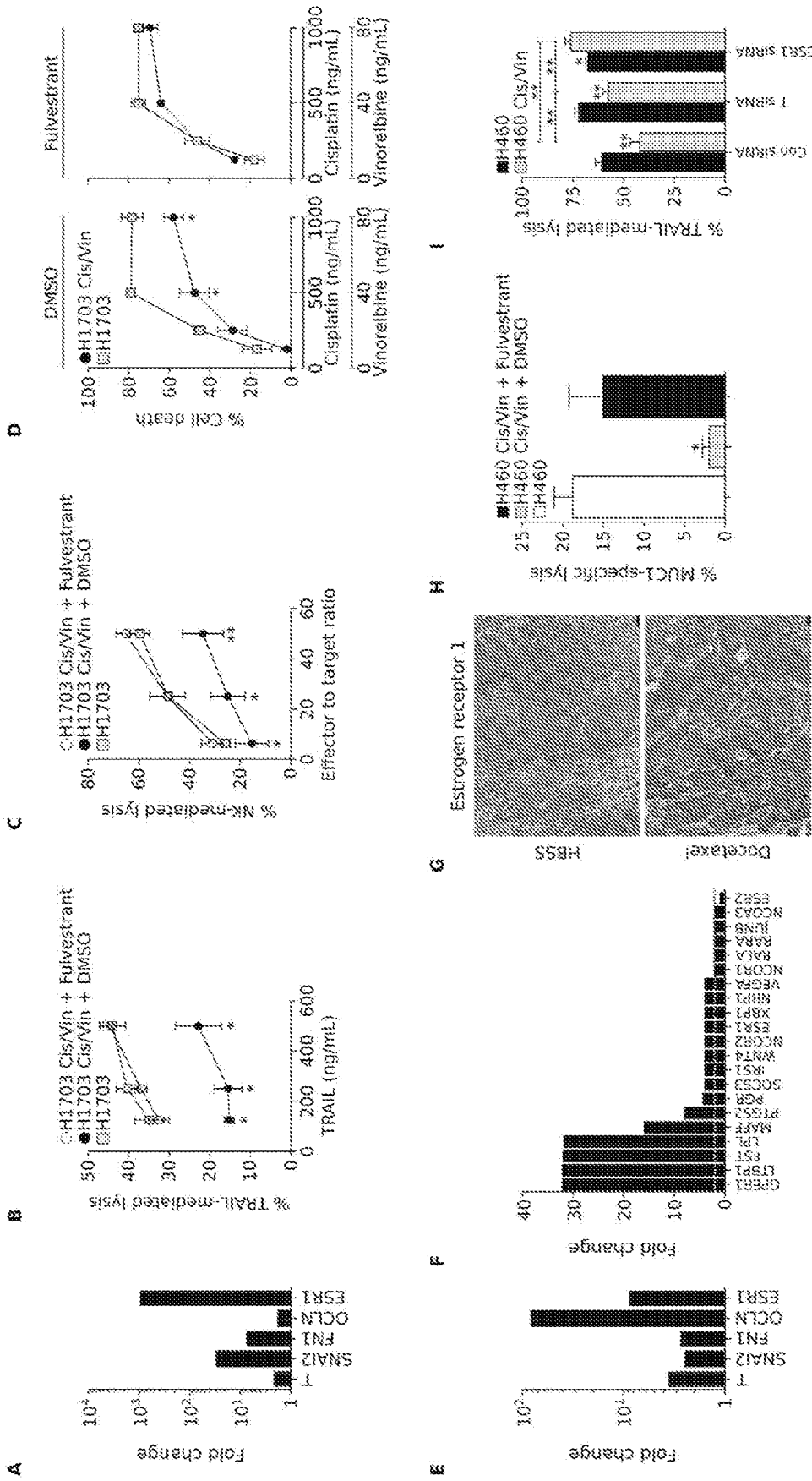


Fig. 5

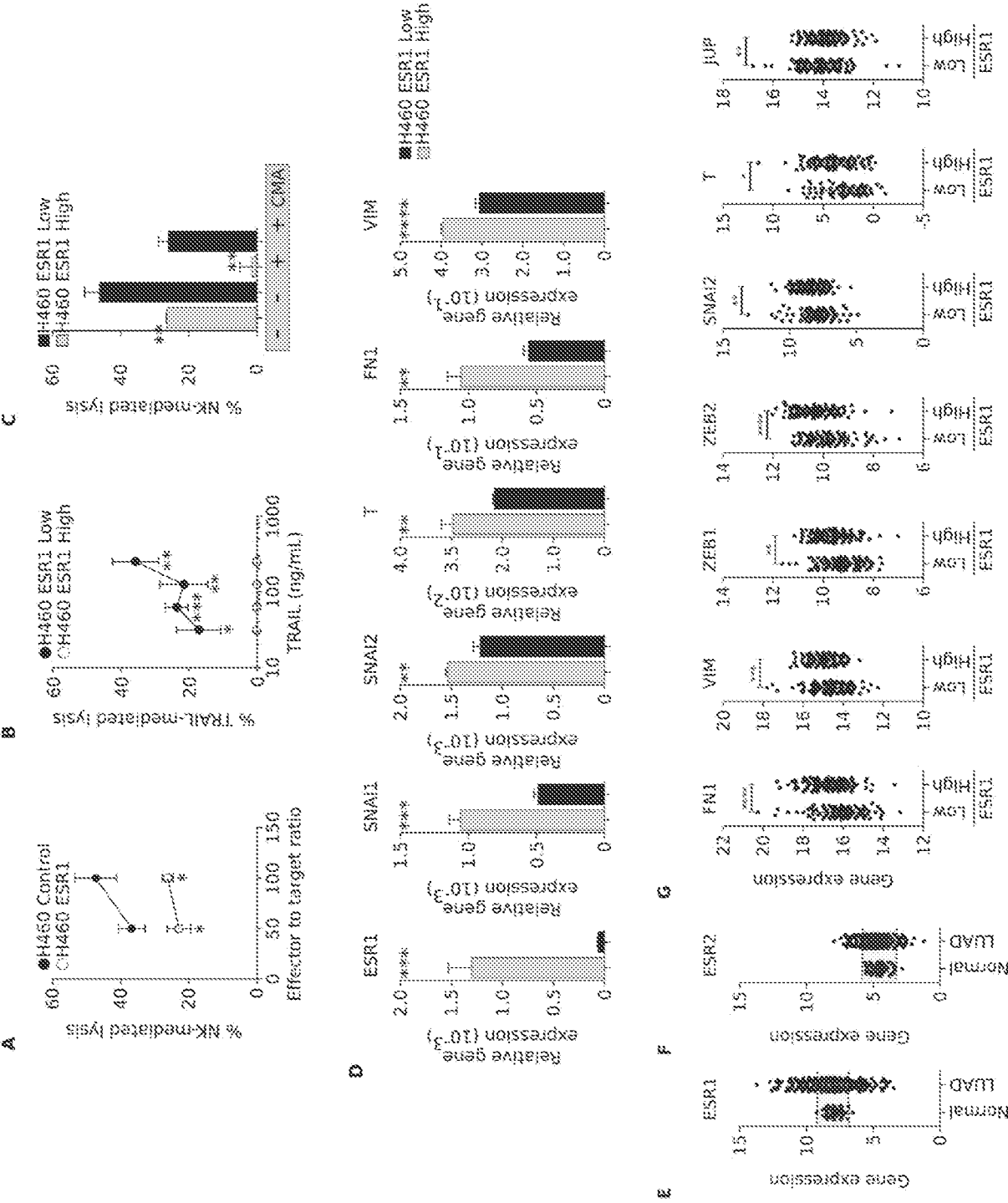
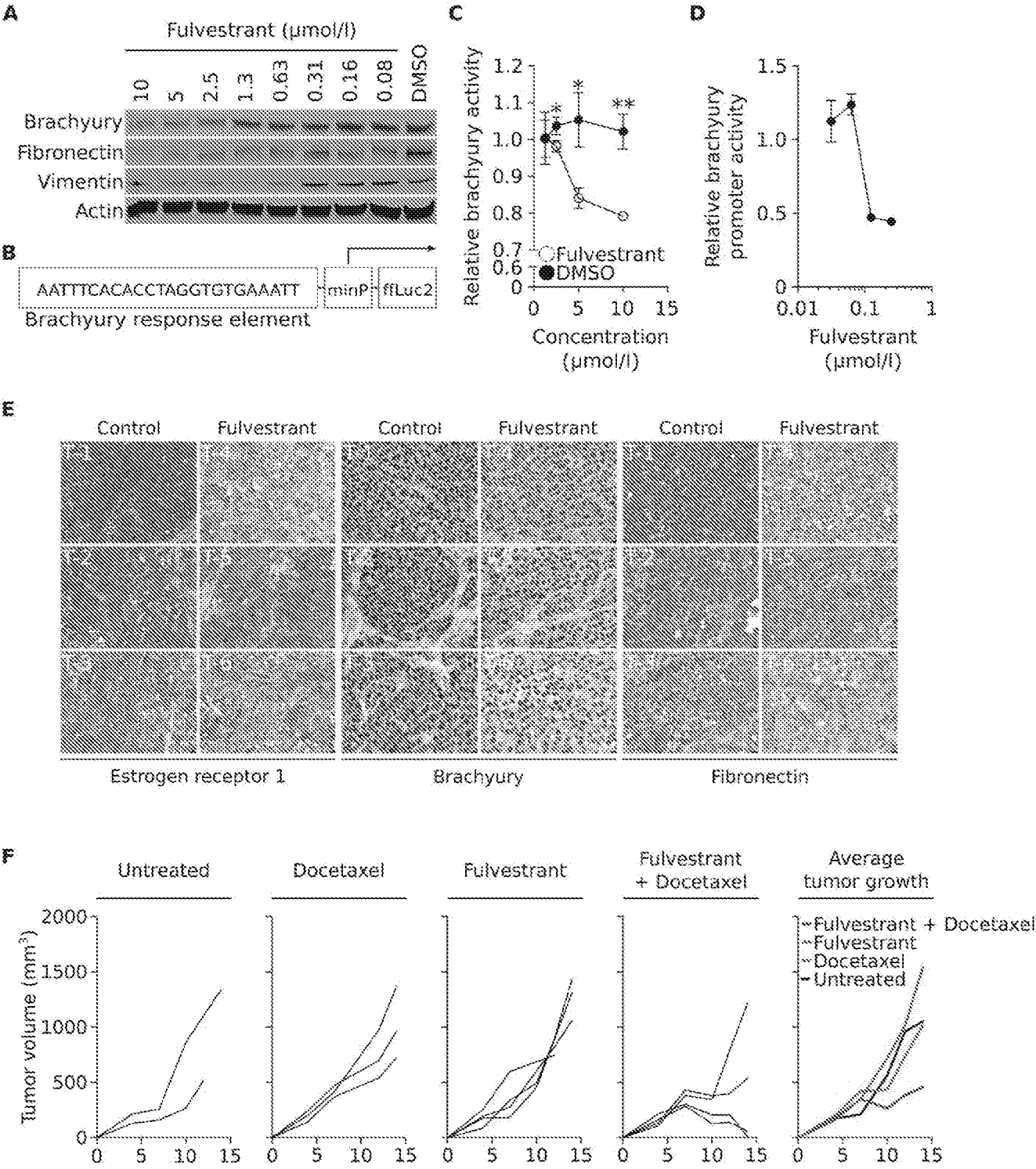


Fig. 6



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/015829A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/565
ADD.

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)
A61K

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

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

EPO-Internal, EMBASE, BIOSIS, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROLFO CHRISTIAN ET AL: "Novel therapeutic strategies for patients with NSCLC that do not respond to treatment with EGFR inhibitors", CANCER TREATMENT REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 40, no. 8, 6 June 2014 (2014-06-06), pages 990-1004, XP029006659, ISSN: 0305-7372, DOI: 10.1016/J.CTRV.2014.05.009 page 998, column 2, paragraph 2 ----- -/--	1-5, 19-25



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

"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

10 April 2017

Date of mailing of the international search report

20/04/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Bonzano, Camilla

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2017/015829

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STABILE L P ET AL: "Human non-small cell lung tumors and cells derived from normal lung express both estrogen receptor [alpha] and [beta] and show biological responses to estrogen", CANCER RESEARCH 20020401 US, vol. 62, no. 7, 1 April 2002 (2002-04-01), pages 2141-2150, XP8183963, ISSN: 0008-5472 page 2145, column 2, paragraph 3 - page 2146, column 1, paragraph 1 figures 4,7 page 2148, column 1, paragraph 1 - paragraph 2 -----	1-5, 19-25
X,P	HAMILTON D H ET AL: "Targeting estrogen receptor signaling with fulvestrant enhances immune and chemotherapy-mediated cytotoxicity of human lung cancer", CLINICAL CANCER RESEARCH, THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, US, vol. 22, no. 24, 15 December 2016 (2016-12-15), pages 6204-6216, XP008183962, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-15-3059 abstract -----	1-5
X	L. A. BYERS ET AL: "An Epithelial-Mesenchymal Transition Gene Signature Predicts Resistance to EGFR and PI3K Inhibitors and Identifies Axl as a Therapeutic Target for Overcoming EGFR Inhibitor Resistance", CLINICAL CANCER RESEARCH, vol. 19, no. 1, 22 October 2012 (2012-10-22), pages 279-290, XP55197075, ISSN: 1078-0432, DOI: 10.1158/1078-0432.CCR-12-1558 abstract -----	1-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2017/015829

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: