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(54) Title: A METHOD OF TREATING CANCER

(57) Abstract: Use of a combination of at least one chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist to treat cancer cells. Preferably, the chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist is an a-9 acetylcholine receptor (nAChR) antagonist. The combination of at least one chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist may also be in a pharmaceutically acceptable composition.



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A METHOD OF TREATING CANCER

Field of the Invention

[0001] The present invention generally relates to the treatment of cancer, and in particular, relates to the use of nicotinic acetylcholine receptor antagonists in a method of treating cancer.

Background of Invention

[0002] Cancer medicine faces a continuous challenge of improving response rates to anticancer therapy. The first efforts of systemic treatment development gave rise to single agent chemotherapeutics such as cyclophosphamide (and other alkylating agents) as well as the anti-folates (aminopterin and methotrexate), which showed some efficacy in treating various tumor types. Whereas these agents generally cause temporary remission, many tumors still progress or recur suggesting that they were unable to provide durable cure when used as single agents. This observation led to the development of combination chemotherapy. However, this strategy is still not always successful, especially in the context of advanced disease.

[0003] In an attempt to improve response to treatment, recent work has focused on identifying molecular features of tumors that can be used to predict response to commonly used chemotherapeutic regimens. In this fashion, it is possible to select the most appropriate chemotherapeutic regimen to achieve maximal pathological response. For example, in the absence of patient selection, the use of trastuzumab is clinically beneficial in slightly less than 10% of breast cancer patients. However, when patients are selected for trastuzumab therapy based on ERBB2/HER2 amplification, the response rates rise to ~50%. Another alternative for improving treatment response involves the use of sensitizing compounds to overcome chemotherapeutic resistance. Indeed, this is the rationale underpinning the development of PARP inhibitors, which have shown significant efficacy for breast cancer treatment.

[0004] Doxorubicin (Adriamycin®, A) and docetaxel (Taxotere®, T) are among the most potent anticancer drugs used to treat breast cancer as single agents, and their use as a combinatorial regimen is highly effective as a first-line treatment of patients with advanced breast cancer. However, combinatorial AT therapy still rarely results in complete clinical

response (only ~20% of cases), and provides long-term disease free survival for only ~60% of patients.

[0005] In view of the foregoing, it would be desirable to provide improved cancer treatment methods.

Summary of the Invention

[0006] A novel method of treating cancer has now been developed using a selected chemotherapy in combination with a nicotinic acetylcholine receptor (nAChR) antagonist which functions as a chemo-sensitizing compound effective to enhance the activity of chemotherapeutic agents.

[0007] Thus, in one aspect of the invention, a method of treating cancerous cells is provided comprising the step of administering to the cells a chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist.

[0008] In another aspect, there is provided a composition comprising a chemotherapeutic agent and chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist.

[0009] In another aspect, a method of screening compounds to identify a compound that sensitizes cancer cells to a chemotherapy is provided comprising the steps of: incubating the cells with a candidate compound and at least one chemotherapeutic agent, and determining the effect of the compound on cell viability, wherein a decrease in cell viability in comparison to the viability of cells treated with the chemotherapy only indicates that the candidate compound may sensitize cells to the chemotherapy.

[0010] In a further aspect, a method of screening compounds to identify a compound that sensitizes cancer cells to a chemotherapy is provided comprising the steps of: identifying in a cancer cell genes that are associated with resistance to the chemotherapy and genes that are associated with sensitivity to the chemotherapy, identifying candidate compounds that reduce the expression of genes associated with resistance and/or increase the expression of genes associated with sensitivity to the chemotherapy, exposing the cells resistant to the chemotherapy to a candidate compound and to the chemotherapy, and determining the effect of the compound on the viability of the cells, wherein a decrease in the viability of the cells in

comparison to the viability of cells treated with the chemotherapy alone indicates that the candidate compound sensitizes the cancer cells to the chemotherapy.

[0011] These and other aspects of the invention will become apparent from the following detailed description by reference to the figures.

Brief Description Of The Drawings:

[0012] Figure 1 is a graph demonstrating the sensitivity cut-off value to discriminate between single drug-sensitive or resistant cell lines;

[0013] Figure 2 is a graph demonstrating the relative resistance or sensitivity of several breast cancer cell lines to a combination chemotherapeutic drug regimen (Doxorubicin and Docetaxel);

[0014] Figure 3 graphically illustrates the correlation between predicted response and actual cell response (A), and classification of cell line sensitivity and resistance (B), to Doxorubicin and Docetaxel (AT);

[0015] Figure 4 graphically compares predicted and actual cell viability in multiple AT resistant breast cell lines treated with AT and adiphenine (Mean $\pm$ SE, 3 biological replicates, * $p < 0.05$ in each line, ANOVA, tukey's posthoc test) (A) and a lung cancer cell line (B);

[0016] Figure 5 graphically illustrates growth curve analysis of the HCC1954 and BT549 cell lines treated with either adiphenine, AT, or both (Mean $\pm$ SE, 3 biological replicates, ** $p < 0.05$, t-test [AT compared to adiphenine + AT])(A); and cell viability measurements of cells treated with decreased doses of AT (Mean $\pm$ SE, 3 biological replicates)(B);

[0017] Figure 6 graphically illustrates the predicted and actual cell viability in multiple AT resistant breast cell lines treated with adiphenine in combination with either of Doxorubicin or Docetaxel;

[0018] Figure 7 graphically illustrates dose response curves for i) adiphenine and ii) proadifen in sphere forming assays (A), and sphere forming efficiency for a single dose of either i) adiphenine or ii) proadifen in a variety of cell lines (B); and

[0019] Figure 8 illustrates the amino acid sequence of α -Bungarotoxin.

Detailed Description of the Invention

[0020] A method of treating cancerous cells is provided comprising the step of administering to the cells at least one chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist.

[0021] As used herein, the term “cancer cells” or “cancerous cells” refers to cancer cells both *in vitro*, e.g. in culture, and *in vivo* within a mammal, including both human and non-human mammals. Cells are cancerous when they exhibit multiple characteristics selected from the group consisting of uncontrolled proliferation, evading growth suppressors, avoiding cell death, limitless proliferative capacity (immortality), metastatic capacity and genetic instability. Details of cancer cell properties are described in Hanahan et al. Cell (2011) 144: 646-674, the contents of which are incorporated herein by reference.

[0022] The term “chemotherapeutic agent” is meant to encompass a drug or combination of drugs that have an adverse effect on the viability of cancer cells, e.g. that kill cancer cells.

[0023] The term “chemo-sensitizing” as it is used herein with respect to a compound is meant to refer to a compound that enhances the effect of a chemotherapeutic agent to kill cancer cells, but which itself may or may not exhibit any significant effect on cancer cell viability.

[0024] In the present method, cancerous cells are treated with a chemo-sensitizing nicotinic acetylcholine receptor antagonist to sensitize the cells for chemotherapy. Examples of suitable nicotinic acetylcholine receptor (nAChR) antagonists for use as a chemo-sensitizer include, but are not limited to, Lobeline (2-((2*R*,6*S*)-6-((*S*)-2-hydroxy-2-phenylethyl)-1-methylpiperidin-2-yl)-1-phenylethanone), Dihydro- β -Erythroidine, NDNI (N-n-Decylnicotinium iodide hydriodide), adiphenine (diphenylacetic acid 2-(diethylamino)ethyl ester), proadifen (2-Diethylaminoethyl 2,2-diphenylpentanoate), and α -Bungarotoxin (α -BTX, a 74 amino acid neurotoxin as shown in Figure 8). In one embodiment, the nAChR antagonist is a α -9 nicotinic acetylcholine receptor (nAChR) antagonist; however, suitable nAChR antagonists may also be directed to other receptors. It will be understood by one of skill in the art that pharmaceutically acceptable functionally equivalent analogues, prodrugs,

salts and solvates of a chemo-sensitizing nicotinic acetylcholine receptor antagonist is also encompassed, as well as combinations of nicotinic acetylcholine receptor antagonists. The term "functionally equivalent" as it used herein with respect to analogues, prodrugs, salts and solvates of nicotinic acetylcholine receptor antagonist refers to an analogue, prodrug, salt and solvate that has chemo-sensitizing activity.

[0025] The term "analogue" refers to compound derived from a nicotinic acetylcholine receptor antagonist, e.g. by substitution or modification of an atom, group or portion of the antagonist with a functionally similar atom, group or portion, such as substitution of a heteroatom with a different heteroatom or modification of an alkyl group with a hydroxyl group, halogen atom and the like; or modification by addition or deletion of an atom, group or portion that does not abolish activity such as an alkyl group. For example, analogues of adiphenine include proadifen (which includes addition of a propyl group on adiphenine) and dicyclomine (in which the phenyl rings of adiphenine are substituted with cyclohexyl rings).

[0026] The term "prodrug" refers to a compound (e.g. a drug precursor) that is transformed *in vivo* to yield a compound having the structure of a nicotinic acetylcholine receptor antagonist or a pharmaceutically acceptable analogue, salt, hydrate or solvate thereof. The transformation may occur by various mechanisms (e.g. by metabolic or chemical processes), for example, through hydrolysis in blood. The term "salt(s)", as employed herein, denotes acidic salts formed with inorganic and/or organic acids, e.g. hydrochloride salts, iodide salts and the like, as well as basic salts formed with inorganic and/or organic bases. A "solvate" is formed by admixture of a nicotinic acetylcholine receptor antagonist or an analogue thereof in a solvent.

[0027] The present method encompasses the treatment of cancer cells in a mammal. The terms "treat", "treating" and "treatment" are used broadly herein to denote methods that inhibit cancer cells by decreasing cancer cell viability, including methods that moderate or reverse the progression of, reduce the severity of, prevent, or cure disease resulting from the cancer cells.

[0028] Generally, the cells are exposed to the antagonist for a period of time sufficient to result in chemo-sensitization of the cells. The cells may be exposed to the chemo-sensitizer prior to, at the same time as, or subsequent to a chemotherapeutic treatment.

The chemosensitizing antagonist is used in a chemo-sensitizing amount, e.g. an amount that will increase the effect of the chemotherapeutic treatment on the cancer cells being treated. The chemosensitizing amount will vary from antagonist to antagonist, and will also depend on other factors such as the chemotherapeutic treatment, and the cancer cells being treated; however, it may be dose that results in a plasma level of nAChR antagonist in a range of about 0.001-1000 μ M. However, as one of skill in the art will appreciate, an amount of antagonist that is chemosensitizing can readily be determined using appropriately controlled trials.

[0029] Following sensitization of the cancer cells, the cells are treated with a chemotherapeutic agent. Although the present method is not restricted to use with any particular chemotherapeutic agent, examples of chemotherapeutic agents that may be used in the present method, include Doxorubicin (Adriamycin®, A), Docetaxel (Taxotere®, T), Duanorubicin, Epirubicin (10-(4-amino-5-hydroxy-6-methyl-oxan-2-yl)oxy-6,8,11-trihydroxy-8-(2-hydroxyacetyl)-1-methoxy-9,10-dihydro-7H-tetracene-5,12-dione), Paclitaxel ((2 α ,4 α ,5 β ,7 β ,10 β ,13 α)-4,10-bis(acetyloxy)-13-{[(2R,3S)-3-(benzoylamino)-2-hydroxy-3-phenylpropanoyl]oxy}-1,7-dihydroxy-9-oxo-5,20-epoxytax-11-en-2-yl benzoate), 5-Fluoruracil, Cyclophosphamide ((RS)-N,N-bis(2-chloroethyl)-1,3,2-oxazaphosphinan-2-amine 2-oxide), Methotrexate ((2S)-2-[(4-{[(2,4-diaminopteridin-6-yl)methyl](methyl)amino}benzoyl)amino]pentanedioic acid), Cisplatin ((SP-4-2)-diamminedichloridoplatinum), and combination therapies in which two or more chemotherapeutic agents are utilized in the treatment.

[0030] In one embodiment of the present invention, the chemotherapeutic agent is Doxorubicin (hydroxydaunirubicin), the chemical name of which is (7S,9S)-7-[(2R,4S,5S,6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6,9,11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8,10-dihydro-7H-tetracene-5,12-dione), Docetaxel, the chemical name of which is 1,7 β ,10 β -trihydroxy-9-oxo-5 β ,20-epoxytax-11-ene-2 α ,4,13 α -triyl 4-acetate 2-benzoate 13-{(2R,3S)-3-[(*tert*-butoxycarbonyl)amino]-2-hydroxy-3-phenylpropanoate}, or a combination treatment in which both Doxorubicin and Docetaxel are utilized.

[0031] The chemotherapeutic agent may be administered to the sensitized cancer cells using standard established protocols for its use, including standard dosages. While not wishing to be bound by any particular mode of action, treatment of the sensitized cells with the chemotherapeutic agent results in an enhanced effect of the chemotherapeutic agent on

the viability of the cancer cells, e.g. the chemo-sensitized cancer cells exhibit significantly reduced viability on treatment with a chemotherapeutic agent in comparison to the viability of non-chemosensitized cancer cells treated with the same chemotherapeutic agent. For example, an increased reduction in cancer cell viability of at least about 10%, preferably at least about 20%, and more preferably at least about 25% or greater, results in cancer cells treated with a chemosensitizing nicotinic acetylcholine receptor antagonist in combination with a chemotherapeutic agent, as opposed to treatment with the chemotherapeutic agent alone. The effect of the chemosensitizing agent in combination with the chemotherapeutic agent on the cancer cells is greater than the expected additive independent effect of each agent. Accordingly, the combination of the chemosensitizing agent and the chemotherapeutic agent is a synergistic combination.

[0032] The present method may be used to treat various cancers, including carcinoma such as bladder, breast, colon, kidney, liver, lung, including small cell lung cancer, esophagus, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage including leukaemia, acute lymphocytic leukaemia, acute lymphoblastic leukaemia, B-cell lymphoma, T-cell- lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkitt's lymphoma; hematopoietic tumors of myeloid lineage, including acute and chronic myelogenous leukemias, myelodysplastic syndrome and promyelocytic leukaemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; tumors of the central and peripheral nervous system, including astrocytoma neuroblastoma, glioma and schwannomas; other tumors, including melanoma, seminoma, teratocarcinoma, osteosarcoma, xeroderma pigmentosum, keratoxanthoma, thyroid follicular cancer, Kaposi's sarcoma, and cancers that over-express nicotinic acetylcholine receptor (nAChR).

[0033] In one embodiment, the method is used in the treatment of a breast cancer. The breast cancer may be any molecular subtype of breast cancer as represented by breast cancer cell lines such as HC1954 (Basal A subtype), MCF7 (Luminal), HS578T (Basal B), SKBR3 (Luminal), MDA-MB-231 (Basal B), BT549 (Basal B), HCC38 (Basal B), MDA-MB-453 (Luminal), MDA-MB-361 (Luminal) and HCC1569 (Basal A). In this embodiment, a chemotherapy appropriate for treating a breast cancer is used, e.g. Doxorubicin, Docetaxel, Paclitaxel, Cyclophosphamide, Methotrexate, and 5-Fluorouracil (5-FU). Combination therapies may also be used including, but not limited to, Doxorubicin/Docetaxel,

Docetaxel/Paclitaxel, Docetaxel/5FU/doxorubicin/cyclophosphamide (T/FAC) and Cyclophosphamide/ Methotrexate/5-FU (CMF). Dosages of the chemotherapeutic agent in accordance with accepted treatment regimens are utilized. Treatment with a selected chemo-sensitizing nAChR antagonist may be effected before, after or during chemotherapy.

[0034] The chemo-sensitizing and chemotherapeutic agent(s) may be administered in accordance with methods of the invention alone or combined together in a composition, and may also be combined with one or more pharmaceutically acceptable adjuvants or carriers. The expression "pharmaceutically acceptable" means acceptable for use in the pharmaceutical arts, i.e. not being unacceptably toxic, or otherwise unsuitable for administration to a mammal. Examples of pharmaceutically acceptable adjuvants include, but are not limited to, diluents, excipients and the like. Reference may be made to "Remington's: The Science and Practice of Pharmacy", 21st Ed., Lippincott Williams & Wilkins, 2005, for guidance on drug formulations generally. The selection of adjuvant depends on the intended mode of administration of the composition. In one embodiment of the invention, the compounds are formulated for administration by infusion, or by injection either subcutaneously or intravenously, and are accordingly utilized as aqueous solutions in sterile and pyrogen-free form and optionally buffered or made isotonic. Thus, the compounds may be administered in distilled water or, more desirably, in saline, phosphate-buffered saline or 5% dextrose solution. Compositions for oral administration via tablet, capsule, lozenge, solution or suspension in an aqueous or non-aqueous liquid, an oil-in-water or water-in-oil liquid emulsion, an elixir or syrup are prepared using adjuvants including sugars, such as lactose, glucose and sucrose; starches such as corn starch and potato starch; cellulose and derivatives thereof, including sodium carboxymethylcellulose, ethylcellulose and cellulose acetates; powdered tragacanth; malt; gelatin; talc; stearic acids; magnesium stearate; calcium sulfate; vegetable oils, such as peanut oils, cotton seed oil, sesame oil, olive oil and corn oil; polyols such as propylene glycol, glycerine, sorbitol, mannitol and polyethylene glycol; agar; alginic acids; water; isotonic saline and phosphate buffer solutions. Wetting agents, lubricants such as sodium lauryl sulfate, stabilizers, tableting agents, disintegrating agents, anti-oxidants, preservatives, colouring agents and flavouring agents may also be present. In another embodiment, the composition may be formulated for application topically as a cream, lotion or ointment. For such topical application, the composition may include an appropriate base such as a triglyceride base. Such creams, lotions and ointments may also contain a surface-active agent and other cosmetic additives such as skin softeners and the like

as well as fragrance. Aerosol formulations, for example, for nasal delivery, may also be prepared in which suitable propellant adjuvants are used. Compositions of the present invention may also be administered as a bolus, electuary, or paste. Compositions for mucosal administration are also encompassed, including oral, nasal, rectal or vaginal administration for the treatment of infections, which affect these areas. Such compositions generally include one or more suitable non-irritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax, a salicylate or other suitable carriers. Other adjuvants may also be added to the composition regardless of how it is to be administered, which, for example, may aid to extend the shelf-life thereof.

[0035] In accordance with the present method, chemotherapeutic agents and chemosensitizing nAChR antagonists may be administered using an appropriate route of administration including, but not limited to, oral, subcutaneous, intravenous, intraperitoneal, intranasal, enteral, topical, sublingual, intramuscular, intra-arterial, intramedullary, intrathecal, inhalation, ocular, transdermal, vaginal or rectal means. Depending on the route of administration, the chemotherapeutic agents and/or antagonist compounds may be coated or encased in a protective material to prevent undesirable degradation thereof by enzymes, acids or by other conditions that may affect the therapeutic activity thereof

[0036] The methods and compositions of the present invention advantageously provide an improved treatment of cancer cells. The synergistic combination of nAChR antagonists with one or more chemotherapeutic agents provides a treatment protocol in which the effect on viability of cancer cells is increased without a concomitant increase in the adverse effects associated with cancer treatments, e.g. an increase in toxicity to surrounding non-cancerous cells that would result from an increase in dose of a chemotherapeutic regimen to achieve the same effect on cancer cell viability.

[0037] In another aspect, a method of screening compounds to identify a compound that sensitizes cancer cells to a chemotherapy is provided. The method of screening comprises the steps of: incubating the cells with a candidate compound and at least one chemotherapeutic agent, and determining the effect of the compound on cell viability, wherein a decrease in cell viability in comparison to the viability of cells treated with the chemotherapy only indicates that the candidate compound may sensitize cells to the chemotherapy. Cell viability may be determined using well-established techniques such as those described in the specific examples that follow.

[0038] In order to prescreen candidate compounds prior to determining the effect of candidate compounds on cancer cell viability, the following initial steps may be conducted. In a cancer cell line of interest, genes that are associated with resistance to the chemotherapy and genes that are associated with sensitivity to the chemotherapy may be identified. Then, candidate compounds that reduce the expression of the genes associated with resistance to chemotherapy, and/or increase the expression of the genes associated with sensitivity to the chemotherapy, may be identified. These candidate compounds may then be administered to cancer cells, e.g. cells resistant to chemotherapy, in combination with the chemotherapy of interest, and the effect of the compound on the viability of the cells is determined. Determination of a decrease in the viability of the cells treated with the combination of the candidate compound with the chemotherapy in comparison to cells treated with the chemotherapy alone indicates that the candidate compound sensitizes the cancer cells to the chemotherapy.

[0039] Embodiments of the invention are described by reference to the following specific examples which are not to be construed as limiting.

Example 1

Methods

[0040] **Cell viability screens:** Human cell lines were purchased from the ATCC and passaged according to their protocols. All screening was completed using a Biomek3000 (Beckman Coulter) and a DTX880 plate reader (Beckman Coulter). When cells had reached 80-90% confluency, they were trypsinized, washed and then seeded at 50,000 cells/ml into the wells of an opaque 384-well plate (Nunc) (50µl/well) in quadruplicate. Cells were allowed to adhere for 4 hours, followed by compound addition (50X concentration, 1µl/well). Parent stocks of Doxorubicin (Adriamycin®, A) and docetaxel (Taxotere®, T) were dissolved from powder (Sigma) into DMSO and were diluted to 50X assay stocks the day of the assay. Positive (DMSO only) and negative controls (medium only) were also included in the plate. After 24 hours, 5µl of alamarBlue was added to every well, and alamarBlue reduction was read (530nm excitation / 590nm emission) after an additional 24 hours. Raw intensity values were converted to residual activity using the following formula:

$$\% \text{ residual activity} = \frac{\text{Sample reading} - \text{Negative control}}{\text{Positive control} - \text{Negative control}} * 100$$

The inhibitory concentration resulting in 50% reduction in cell viability (IC_{50}) for each cell line was plotted by fitting unconstrained non-linear regression using GraphPad Prism 5TM.

[0041] **Training and validation of an AT resistance gene signature:** Cell lines representing extremes of resistance and sensitivity were used as a training set to generate a gene signature predicting AT resistance. Cell line expression profiles were downloaded from ArrayExpress (E-TABM-157) and normalized using RMA as described in Irizarry et al. Biostatistics 4, 249-264 (2003). Feature selection was performed using a previously described coarse graining technique and scoring algorithm (Hallett et al. (2010) J Exp Clin Cancer Res 29, 120), resulting in 100 Affymetrix probes representative of 89 genes being selected as a predictor of response. An additional 6 cell lines were used to confirm the predictive ability of the gene signature. All associated statistical tests were performed using GraphPad Prism 5.

[0042] **Connectivity Mapping:** The gene signature was divided into resistance probes (high expression was related to resistance), and sensitive probes (high expression was related to sensitivity), and used to search the Connectivity Map (Broad Institute) for compounds which reduced the expression of resistance probes and increased expression of sensitivity probes.

[0043] **Connectivity Map compound screen:** Compounds identified by Connectivity Mapping were purchased from Sigma and screened in a similar fashion as described above. Parent and assay stocks were prepared as described above. Compounds were added to cells treated with or without AT and cell viability was measured using alamarBlue.

Results

[0044] **Gene signature predictor of breast tumor cell line response to doxorubicin and docetaxel :** To develop a gene signature predictor of breast tumor cell line response to the chemotherapeutic drugs doxorubicin (Adriamycin, A) and docetaxel (Taxotere, T), a panel of 10 breast cancer cell lines, which were representative of the multiple molecular subtypes of breast cancer was assembled, and the response of each cell line to single agent treatment with either A or T was determined. Cell lines tested were the following: HCC1954 (Basal A subtype), MCF7 (Luminal), HS578T (Basal B), SKBR3 (Luminal), MDA-MB-231 (Basal B), BT549 (Basal B), HCC38 (Basal B), MDA-MB-453 (Luminal), MDA-MB-361 (Luminal), HCC1569 (Basal A). An IC_{50} (the dose required to achieve 50% reduction in cell

viability) sensitivity cut-off value was assigned (Figure 1) to discriminate between drug-sensitive or resistant cell lines. Because breast cancer patients are generally treated with combinations of chemotherapeutic drugs, sensitive and resistant cell lines to a combination of A and T (AT) were identified by combining these two drugs at their individual IC50 sensitivity cut-offs (0.1 μ M A and, 100 μ M T) (Fig. 2). Assuming Bliss independence between A and T, a combination between the IC50 sensitivity cutoffs would result in >75% reduction of cell viability for sensitive cell lines, whereas resistant cell lines should experience <75% reduction in cell viability.

[0045] Using the most and least resistant cell lines as well as their associated gene expression profiles, genes whose expression was related to AT response were identified. In this fashion, 100 probes were identified as being maximally predictive at classifying cell lines on the basis of their response to AT. An additional 6 cell lines were used as an independent data set for further validation of the AT response predictor. Significant correlation ($p=0.0001$, F-test) between the predicted and actual cell line response to AT was observed as well as accurate classification of cell line sensitivity and resistance ($p=0.0013$, t-test), confirming the capacity of the AT predictor to model AT response in cell lines (Fig. 3 A, B).

[0046] **Discovery of small molecules that induce sensitivity to doxorubicin and docetaxel:** Patients whose tumors show resistance to front-line chemotherapy are generally given second- or third line therapy or recruited into clinical trials for experimental drugs. Furthermore, many patients who initially respond to front-line therapy suffer relapse, highlighting that additional therapeutic options are required. To identify additional therapeutics that might increase response to chemotherapy, small molecule candidates that might perturb the gene expression of AT resistant cell lines to induce sensitivity to AT were sought. For this approach the Connectivity Map was used to search for molecules that reduce expression of resistance associated probes and increase the expression of sensitivity associated probes as determined by the AT response predictor. The latter analysis revealed compounds with a highly statistically significant ($*p<0.05$) positive mean score.

[0047] To determine whether any of the compounds induced AT sensitivity, the dose response of 10 AT-resistant cell lines to each of the compounds in the absence and presence of a constant dose of AT (0.1 μ M A, 100 μ M T) was measured. This analysis revealed that whereas all of the compounds displayed chemo-sensitizing activity in one or more of the breast tumor cell lines, adiphenine and AT reproducibly resulted in significant cell death in

excess of that observed with either adiphénine or AT alone for each of the cell lines tested (Fig.4a. Mean $\pm$ SE, 3 biological replicates, $*p < 0.05$, ANOVA, tukey's posthoc test).

[0048] The capacity of adiphénine to sensitize lung tumor cells to the cytotoxic effects of AT was also tested in the same manner. Notably, adiphénine sensitized A549 cells (lung tumor cell line) to AT at the same dose that was found to sensitize breast tumor cell lines (Figure 4b, Mean $\pm$ SE, $*p < 0.05$, ANOVA, tukey's posthoc test). The highest concentration of adiphénine sufficient for chemo-sensitizing activity without any effect on cell viability was 250 μ M.

[0049] To directly determine whether adiphénine (with or without AT) affected cell proliferation, growth curves with two of the AT-resistant cell lines were performed. These experiments revealed that adiphénine (250 μ M) alone did not affect cell proliferation compared to the vehicle (0.5% DMSO). Whereas AT alone reduced cell proliferation this effect was substantially augmented by adiphénine (Fig.5a). AT reduced cell number by $\sim 50\%$, whereas fewer than $\sim 10\%$ of the cells exposed to adiphénine and AT remained viable during the time course of the experiment (60 hours). It was also determined whether or not adiphénine could sensitize one of the breast tumor cell lines (HCC1954) to lower concentrations of AT than that used previously. Adiphénine's sensitization effect was evident at 4-fold lower doses of AT, and adiphénine combined with half the original dose of AT was as effective as adiphénine combined with the full AT dose (Fig.5b). The reduction in cell viability after exposure of the cell lines to the AT plus adiphénine regimen was in excess of that predicted by Bliss independence in each AT resistant cell line (Fig. 5b) and was statistically significant ($*p < 0.05$, t-test) suggesting that adiphénine and AT synergized when used in combination.

[0050] These findings demonstrate the capacity of the AT response predictor to identify chemo-sensitizing agents for the AT chemotherapeutic regimen. The results also show that adiphénine induced sensitivity to AT in all 10 AT-resistant human breast tumor cell lines that were tested and the chemo-sensitizing activity of adiphénine was manifest at lower doses of AT commonly required to halt tumor cell proliferation in vitro or achieve tumor cell death in vivo.

[0051] Collectively these findings suggest that adiphénine enhances the efficacy of AT chemotherapy in patients and, thus, would permit lower doses of AT chemotherapy to

achieve the same reduction in cell viability, thereby limiting the toxicity of these chemotherapies.

Example 2 – Antagonists of Nicotinic Acetylcholine Receptors as Chemosensitizers

[0052] To determine whether or not the chemo-sensitizing effect of adiphenine was mediated by its interaction with nAChRs and to gain insight into the nature of the relevant receptor, the capacity of a number of muscarinic (mAChRs) and nAChR antagonists to sensitize AT-resistant human breast tumor cell lines to AT were surveyed. Whereas only one of the mAChR antagonists functioned as a chemo-sensitizer (dicyclomine, an analogue of adiphenine and proadifen), many of the nAChR antagonists did (Table 1).

Table 1.

ANTAGONIST	Concentration	Receptor Type	Chemosensitizer
Lobeline	230uM	Nicotinic	Yes
Dihydro- β -Erythroidine	230uM	Nicotinic	Yes
NDNI	6.25uM	Nicotinic	Yes
α -Bungarotoxin	5uM	Nicotinic	Yes
Adiphenine	250uM	Nicotinic	Yes
Proadifen	25uM	Nicotinic	Yes

[0053] A muscarinic cholinergic receptor antagonist, dicyclomine (an analogue of adiphenine and proadifen), also exhibited sensitizing activity at 100 μ M.

[0054] To determine whether or not the nAChR antagonists sensitized breast tumor cell lines to either A or T when used as single agents, the capacity of proadifen to potentiate the cytotoxic effects these drugs was tested in a similar manner. Notably, proadifen sensitized breast tumor cells (HCC1954s) to both A and T when these drugs were used as single agents. Importantly, these data suggest the nAChR antagonists may act as general chemotherapy sensitizers, rather than specific sensitizers to the AT combination regimen (Fig. 6).

[0055] Adiphenine was also identified using Connectivity Mapping with a gene expression signature derived from transcripts differentially expressed between breast cancer stem cell (BCSC) enriched CD44+/CD24- human breast tumor cell populations, and BCSC-depleted, non-CD44+/CD24-fractions. The latter finding coupled with the observation that

BCSCs are chemotherapy resistant prompted us to determine whether adiphénine and other nAChR antagonists affected sphere formation, an *in vitro* surrogate assay for stem cell activity. It was hypothesized that nAChR antagonists targeted BCSC, whereas AT chemotherapy targeted their rapidly dividing descendants thus accounting for the effect of the nAChR antagonists in chemo-sensitization assays. If the latter were true, then these antagonists might affect sphere-formation independent of AT. Human breast tumor cell lines were used for these experiments including chemotherapy-resistant basal cell lines and chemotherapy-sensitive luminal cell lines that had been established as sphere cultures, including BT474, MCF7, HCC1954, BT549 and MDA-MB-157. Both adiphénine and its more potent analogue, proadifen, reduced sphere-formation by all the cell lines; however, the basal cell lines seemed more sensitive to the antagonists compared to the luminal cell lines (Fig.7a and b). Interestingly, basal cell lines comprise a higher fraction of CD44+ cells compared to luminal cell lines, and this may account for the increased sensitivity of the basal cell lines to the chemosensitizers. Assay of other chemosensitizers in sphere-forming assays revealed that all the compounds that were chemosensitizers (Table 1) also inhibited sphere-formation (Table 2).

Table 2.

Subtype	Cell line	Adiphénine IC50	Proadifen IC50	NDNI IC50	Lobeline IC50
Lu	MCF7	124 μ M	9.8 μ M	2.7 μ M	197 μ M
Lu	BT474	226 μ M	5.8 μ M	0.8 μ M	89.1 μ M
Ba/B	BT549	36 μ M	0.9 μ M	0.21 μ M	19.2 μ M
BaB	MDA-MB-157	-	1.4 μ M	0.12 μ M	17.4 μ M
BaA; Her2+	HCC1954	29 μ M	2.1 μ M	0.09 μ M	41.4 μ M

The contents of all references referred to herein are incorporated by reference.

CLAIMS

We Claim:

1. A method of treating cancer cells comprising the step of administering to the cells at least one chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor antagonist.
2. The method as defined in claim 1, wherein the chemo-sensitizing nicotinic acetylcholine receptor antagonist is selected from the group consisting of Lobeline, Dihydro- β -Erythroidine, NDNI, adiphenine, proadifen α -Bungarotoxin, dicylcomine, functionally equivalent analogues, salts, prodrugs, solvates, hydrates and combinations thereof.
3. The method as defined in claim 1, wherein the chemo-sensitizing nicotinic acetylcholine receptor antagonist is an α -9 sensitizing nicotinic acetylcholine receptor antagonist.
4. The method as defined in claim 1 wherein the cancer cells are selected from the group consisting of bladder, breast, colon, kidney, liver, lung, including small cell lung cancer, esophagus, gall-bladder, ovary, pancreas, stomach, cervix, thyroid, prostate, and skin, including squamous cell carcinoma; hematopoietic tumors of lymphoid lineage including leukaemia, acute lymphocytic leukaemia, acute lymphoblastic leukaemia, B-cell lymphoma, T-cell- lymphoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, hairy cell lymphoma and Burkitt's lymphoma; hematopoietic tumors of myeloid lineage, acute and chronic myelogenous leukemias, myelodysplastic syndrome and promyelocytic leukaemia; tumors of mesenchymal origin, including fibrosarcoma and rhabdomyosarcoma; tumors of the central and peripheral nervous system, astrocytoma neuroblastoma, glioma and schwannomas; melanoma, seminoma, teratocarcinoma, osteosarcoma, xeroderma pigmentosum, keratoxanthoma, thyroid follicular cancer and Kaposi's sarcoma.
5. The method of claim 1, wherein the cancer is one in which the cancer cells over-express nicotinic acetylcholine receptor (nAChR).
6. The method as defined in claim 4 wherein the cancer is breast carcinoma.

7. The method as defined in claim 1, wherein the chemotherapeutic agent is selected from the group consisting of Doxorubicin, Docetaxel, Duanorubicin, Epirubicin, Paclitaxel, Cyclophosphamide, Methotrexate, Cisplatin, 5-Fluorouracil and combinations thereof.
8. The method of claim 7, wherein a combination of chemotherapeutic agents is administered.
9. The method of claim 1, wherein the cells are treated with the nicotinic acetylcholine receptor (nAChR) antagonist prior to, at the same time as or following chemotherapy.
10. A composition comprising a chemotherapeutic agent and chemo-sensitizing nicotinic acetylcholine receptor (nAChR) antagonist.
11. The composition as defined in claim 10, wherein the chemo-sensitizing nicotinic acetylcholine receptor antagonist is selected from the group consisting of Lobeline, Dihydro- β -Erythroidine, NDNI, adiphenine, proadifen α -Bungarotoxin, dicylcomine, functionally equivalent analogues, salts, prodrugs, solvates, hydrates and combinations thereof.
12. The composition as defined in claim 10, wherein the chemotherapeutic agent is selected from the group consisting of Doxorubicin, Docetaxel, Duanorubicin, Epirubicin, Paclitaxel, Cyclophosphamide, Methotrexate, Cisplatin, 5-Fluorouracil and combinations thereof.
13. The composition as defined in claim 10, wherein the chemo-sensitizing nicotinic acetylcholine receptor antagonist is an α -9 sensitizing nicotinic acetylcholine receptor antagonist.
14. A method of screening compounds to identify a compound that sensitizes cancer cells to a chemotherapy comprising the steps of: incubating the cells with a candidate compound and at least one chemotherapeutic agent, and determining the effect of the compound on cell viability, wherein a decrease in cell viability in comparison to the viability of cells treated with the chemotherapy only indicates that the candidate compound may sensitize cells to the chemotherapy.

15. The method of claim 14, wherein the chemotherapeutic agent is selected from the group consisting of Doxorubicin, Docetaxel, Paclitaxel, Cyclophosphamide, Methotrexate, and 5-Fluorouracil (5-FU).
16. The method of claim 14, wherein two or more chemotherapeutic agents are used.
17. The method of claim 14, additionally comprising the initial steps of: identifying in a cancer cell genes that are associated with resistance to the chemotherapy and genes that are associated with sensitivity to the chemotherapy, identifying candidate compounds that reduce the expression of genes associated with resistance and/or increase the expression of genes associated with sensitivity to the chemotherapy, and exposing one of said candidate compounds to cells resistant to the chemotherapy to determine the effect on cell viability.

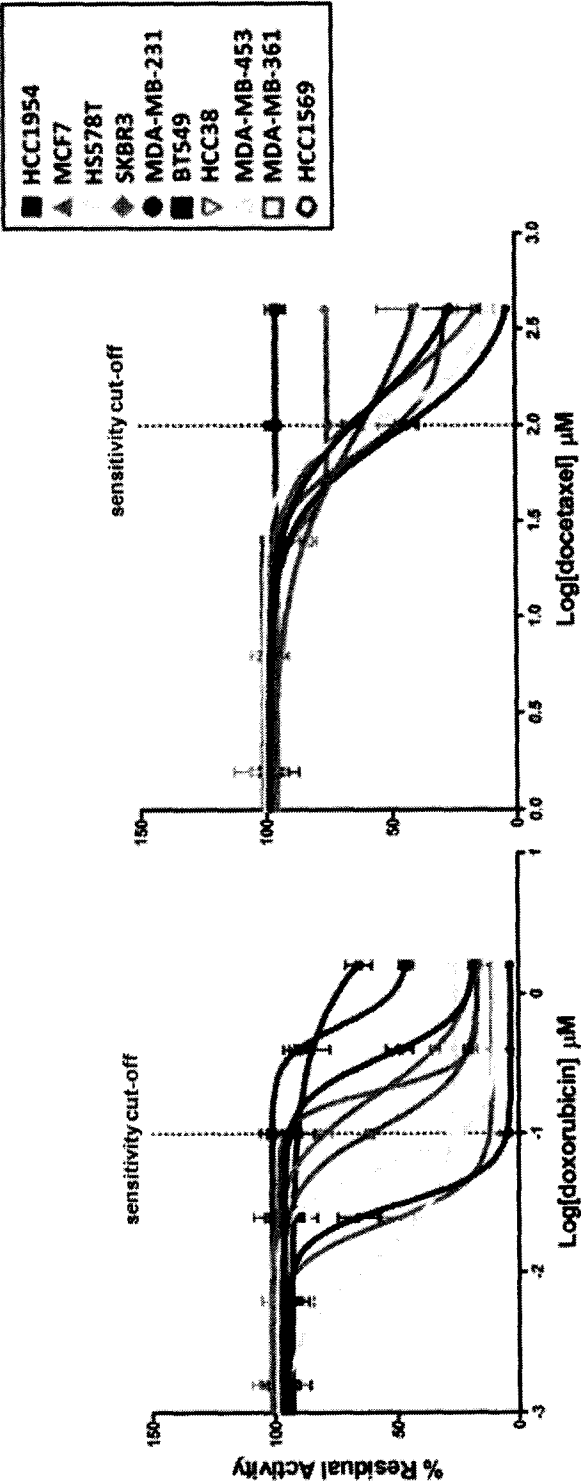


FIGURE 1

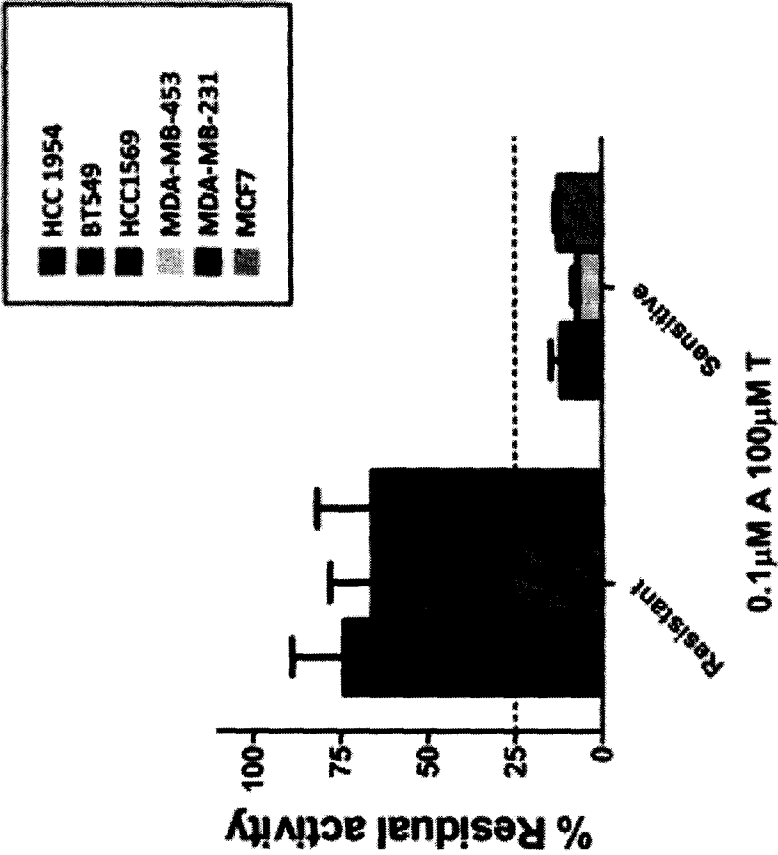


FIGURE 2



FIGURE 3

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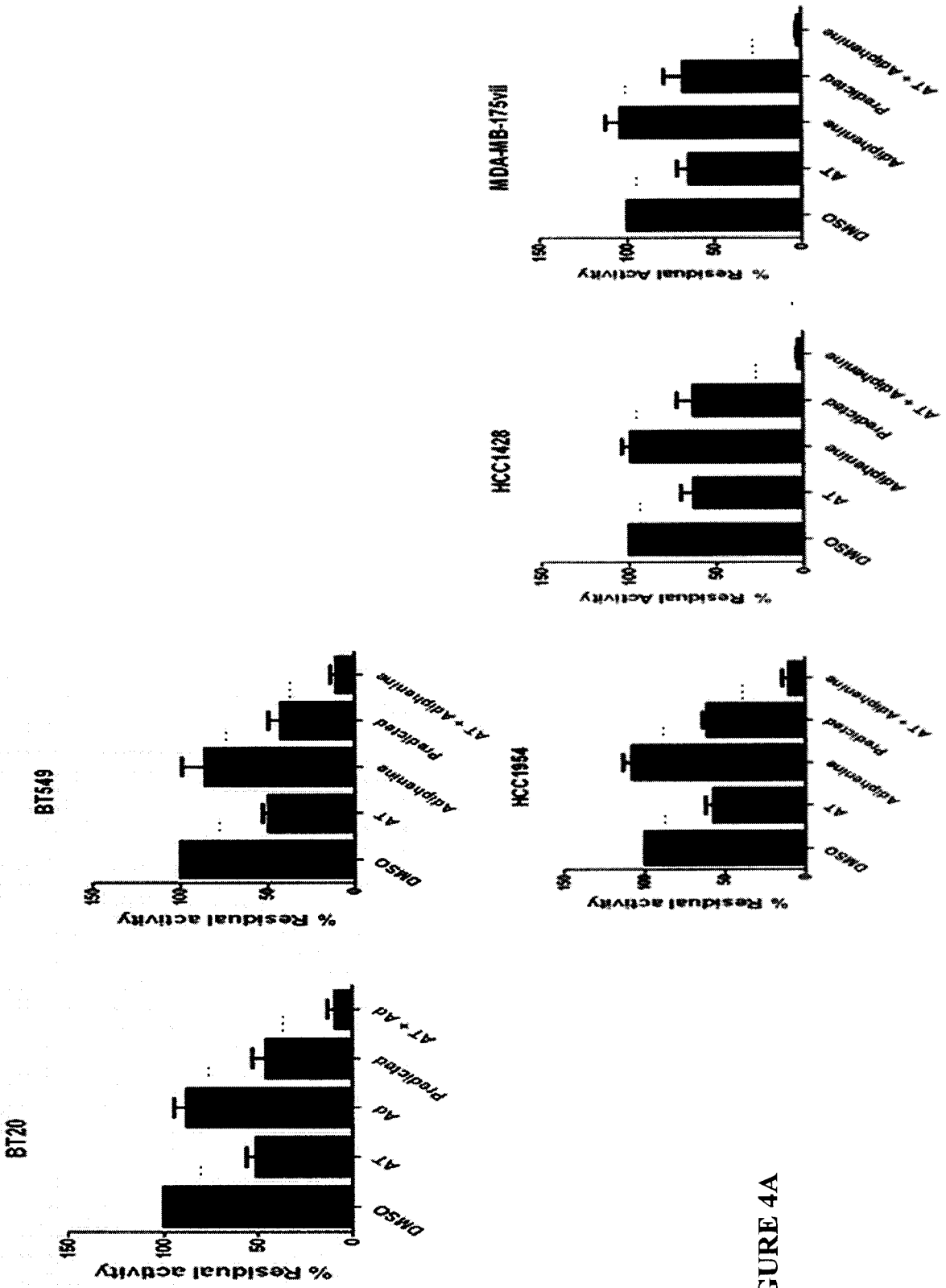


FIGURE 4A

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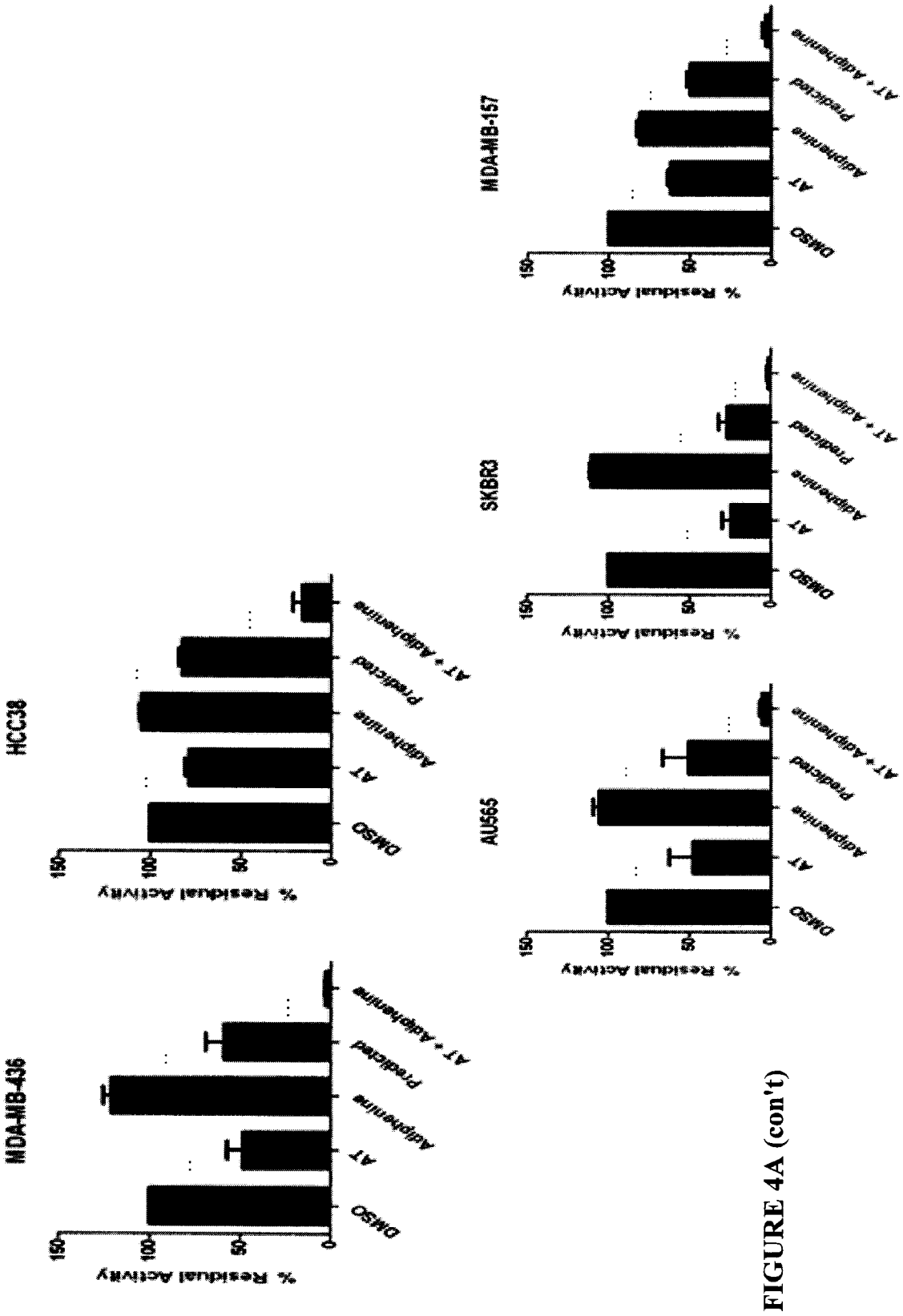


FIGURE 4A (con't)

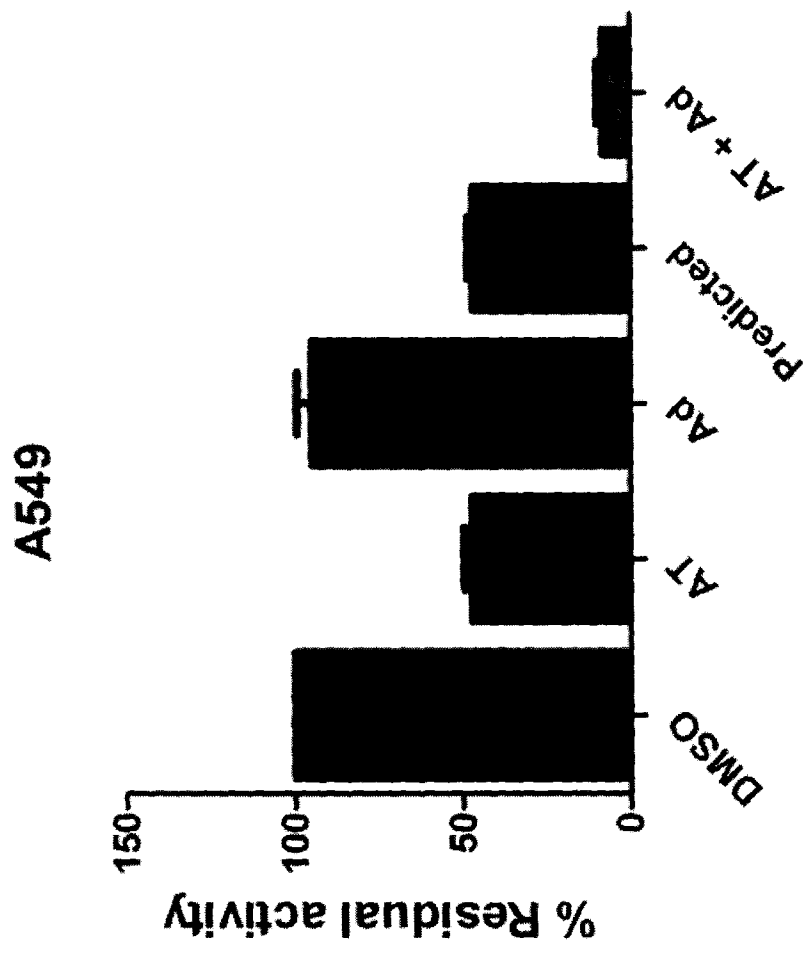


FIGURE 4B

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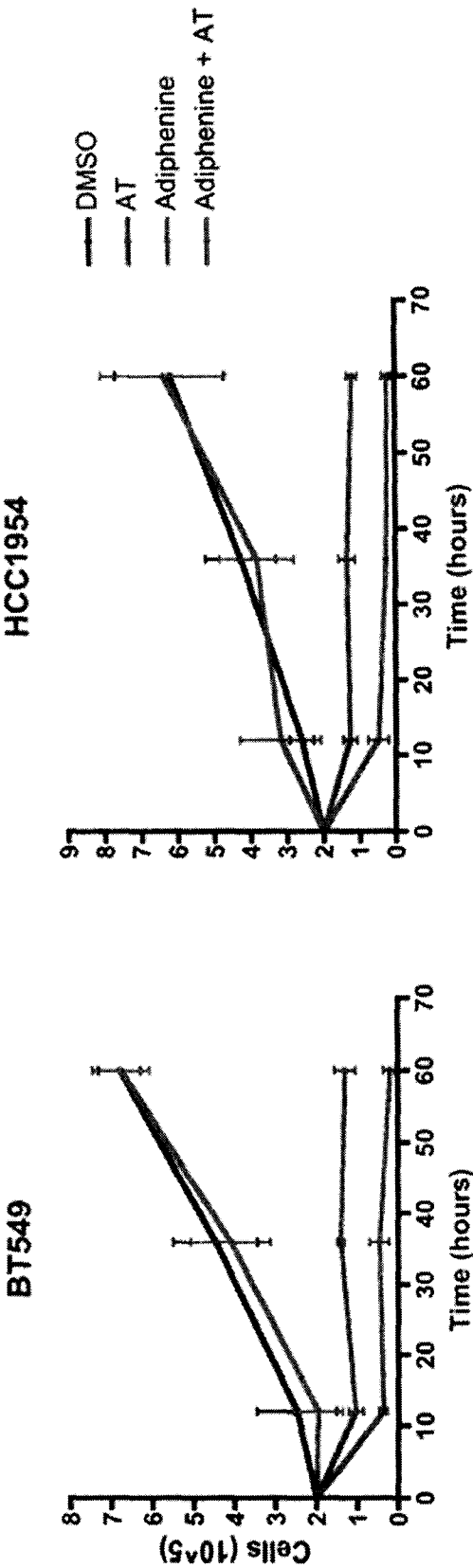


FIGURE 5A

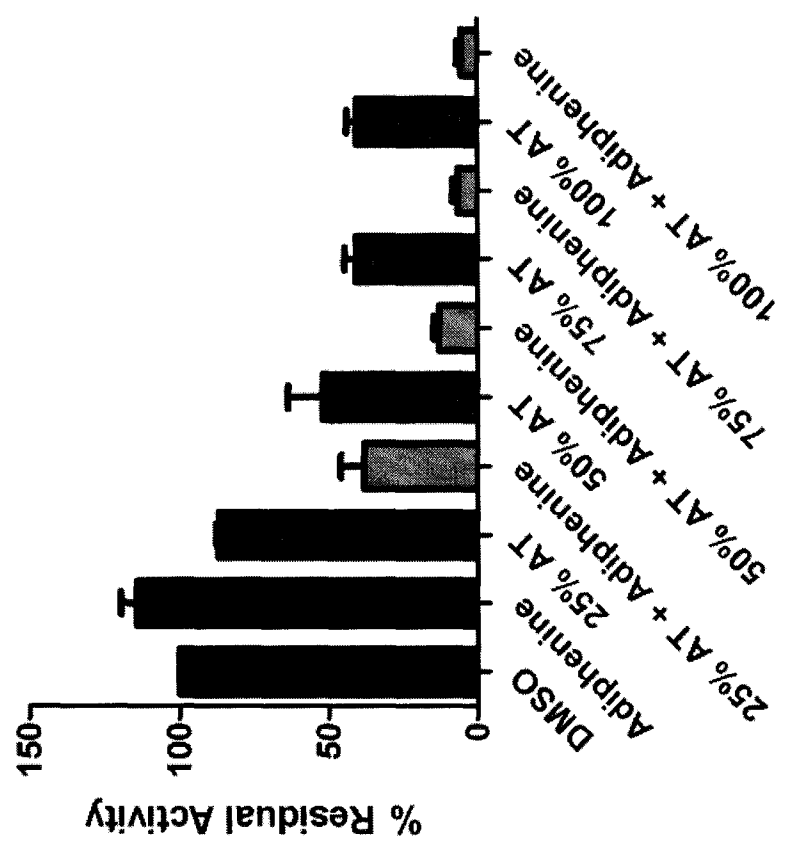


FIGURE 5B

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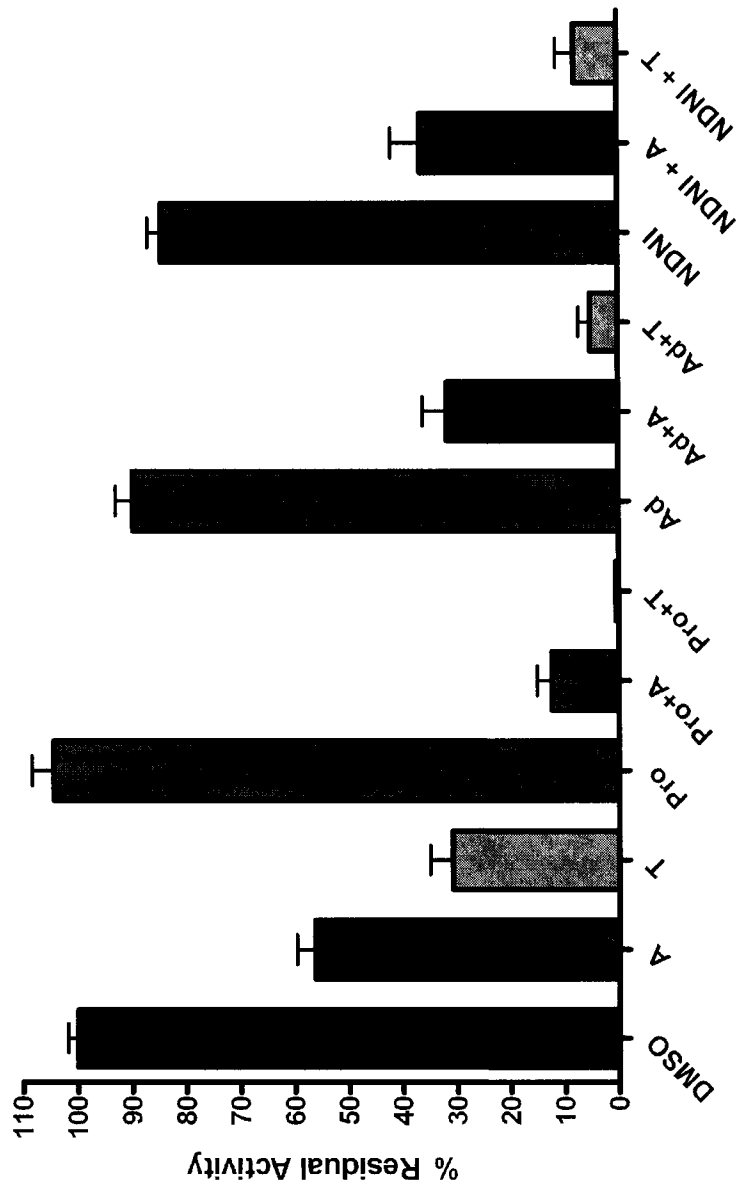


FIGURE 6

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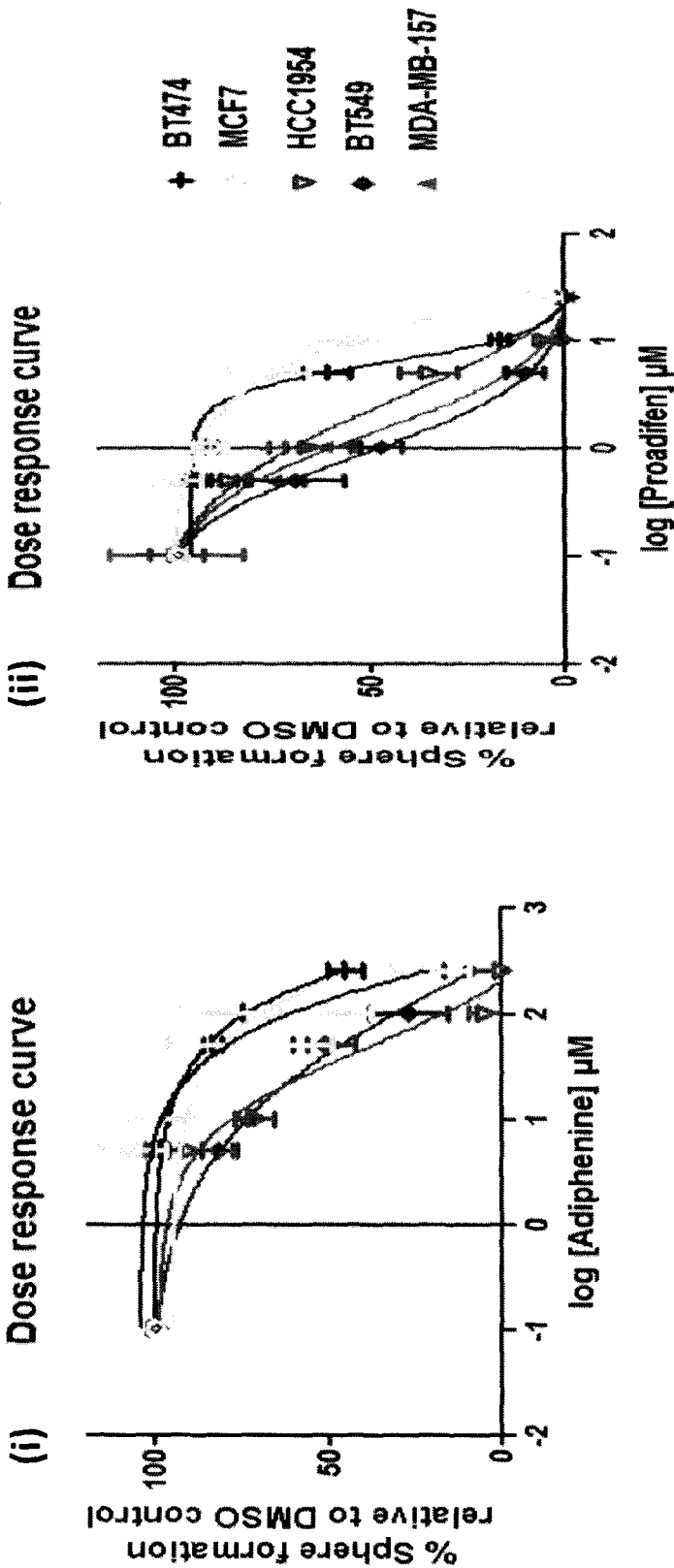
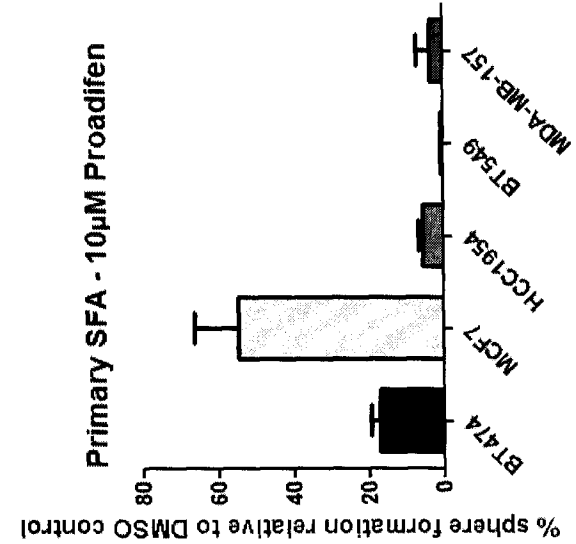
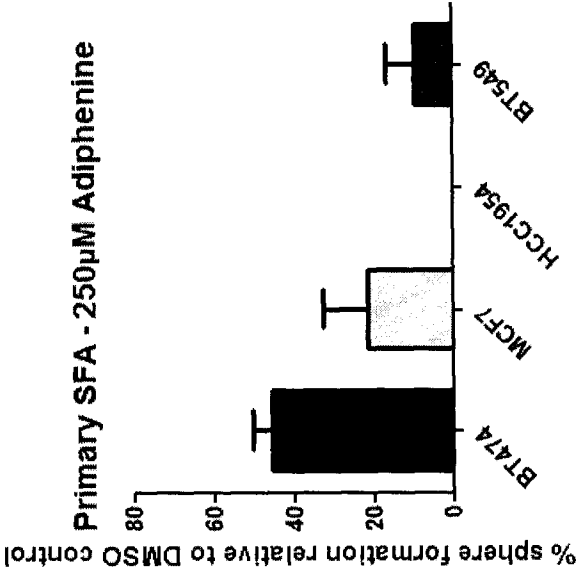


FIGURE 7A

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(ii)



(i)

FIGURE 7B

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IVCHTTATSP ISAVTCPPGE NLCYRKMWCD AFCSSRGKVV ELGCAATCPS KKPYYEVTCC
STDKCNPHPK QRPG

FIGURE 8

WAT_LAW\ 544892\2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000131

A. CLASSIFICATION OF SUBJECT MATTER IPC: A61K 31/216 (2006.01) , A61K 31/337 (2006.01) , A61K 31/704 (2006.01) , A61P 35/00 (2006.01) 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 31				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Epoque (Epodoc, English Full-txt, Medline), Scopus (nicotinic acetylcholine receptor, nAChR, adiphene, proadifen, bungarotoxin, lobeline, dicylcomine, NDNI dihydro-beta-erythroidine, cancer, breast cancer, lung cancer, leukemia, sarcoma, carcinoma tumour, lymphoma and related terms)				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	WO 03/068208 A1 (Cooke, John et al) 21 August 2003 (21-08-2003) see abstract; pages 2-4, 6-8, 11-14; and claims 1-9	1-13		
X	US 2005/0288333 (Kem, W.R.) 29 December 2005 (29-12-2005) see abstract; paragraphs [0010]-[0011], [0020]-[0022], [0059], [0103]-[0153], [0200]-[0216]; and claims 37-48	1-13		
X	WO 2010/057088 A2 (Gant, T.G. et al) 20 May 2010 (20-05-2010) see abstract; pages 1-2, 6-8, 12, 16-20, 26	1-13		
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.				
<table border="0"> <tr> <td> * 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 </td> <td> "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 </td> </tr> </table>			* 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
* 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 14 May 2012 (14-05-2012)		Date of mailing of the international search report 23 May 2012 (23-05-2012)		
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476		Authorized officer Tania Nish (819) 934-3592		

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000131

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIH-HSIN TU et al., "Tea polyphenol (-)-epigallocatechin-3-gallate inhibits nicotine- and estrogen-induced α 9-nicotinic acetylcholine receptor upregulation in human breast cancer cells", 2011, 55(3): 455-466, first published online 28 October 2010 (http://onlinelibrary.wiley.com/doi/10.1002/mnfr.201000254/full) see entire document	1-13
A	GUILLERMO SPITZMAUL et al., "The local anaesthetics proadifen and adiphenine inhibit nicotinic receptors by different molecular mechanisms", 2009, 157: 804-817. see entire document	1-13
P,A	CHIH-HSIUNG WU et al., "Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy", online publication 28 March 2011, 17: 3533-3541. see entire document	1-13
P,A	YUAN-SOON HO et al., "The alpha 9-nicotinic acetylcholine receptor serves as a molecular target for breast cancer therapy", 2011, 3(6): 246-251. see entire document	1-13

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CA2012/000131**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. ☒ Claim Nos. : 1-9
because they relate to subject matter not required to be searched by this Authority, namely :

Claims 1-9 is directed to a method for treatment of the human or animal body by surgery or therapy which the International Search Authority is not required to search (Rule 39.1 of PCT). However, this Authority has carried out a search based on the alleged effect of the combination as defined in claims 1-9.
2. ☐ Claim 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. ☐ Claim 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 :

Continued in Supplemental Box.

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 claim 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 claim Nos. : 1-13

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.

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-13 are directed to composition comprising at least one chemotherapeutic agent and a chemo-sensitizing nicotinic acetylcholine receptor antagonist and the use thereof in treating cancer cells.

Group B - Claims 14-17 are directed to a screening method to identify compounds that sensitize cancer cells to a chemotherapy.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

The technical feature of Group B resides in observing if a compound sensitizes a cancer cell to a chemotherapeutic agent. This method is for any compound in relation to any chemotherapeutic agent. This is not the same technical feature as found in Group A. The technical feature of Group A is the use of chemo-sensitizing nicotinic acetylcholine receptor antagonists to sensitize cancer cells to chemotherapeutic agents. Since there is no single general concept that links the screening method of Group B to the compositions and their use of Group A, there lacks unity of invention between Groups A and B.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CA2012/000131

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
WO03068208A1	21 August 2003 (21-08-2003)	AT478661T	15 September 2010 (15-09-2010)
		AU2003214991A1	04 September 2003 (04-09-2003)
		AU2003214991B2	21 June 2007 (21-06-2007)
		CA2472920A1	21 August 2003 (21-08-2003)
		DE60333901D1	07 October 2010 (07-10-2010)
		DK1480627T3	08 November 2010 (08-11-2010)
		EP1480627A1	01 December 2004 (01-12-2004)
		EP1480627A4	06 April 2005 (06-04-2005)
		EP1480627B1	25 August 2010 (25-08-2010)
		ES2349244T3	29 December 2010 (29-12-2010)
		JP2005521683A	21 July 2005 (21-07-2005)
		JP4494793B2	30 June 2010 (30-06-2010)
		JP2010143935A	01 July 2010 (01-07-2010)
		US2003216314A1	20 November 2003 (20-11-2003)
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		US7652038B2	26 January 2010 (26-01-2010)
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		WO2005123075A2	29 December 2005 (29-12-2005)
		WO2005123075A3	23 February 2006 (23-02-2006)
WO2010057088A2	20 May 2010 (20-05-2010)	US2010125094A1	20 May 2010 (20-05-2010)
		WO2010057088A3	14 October 2010 (14-10-2010)