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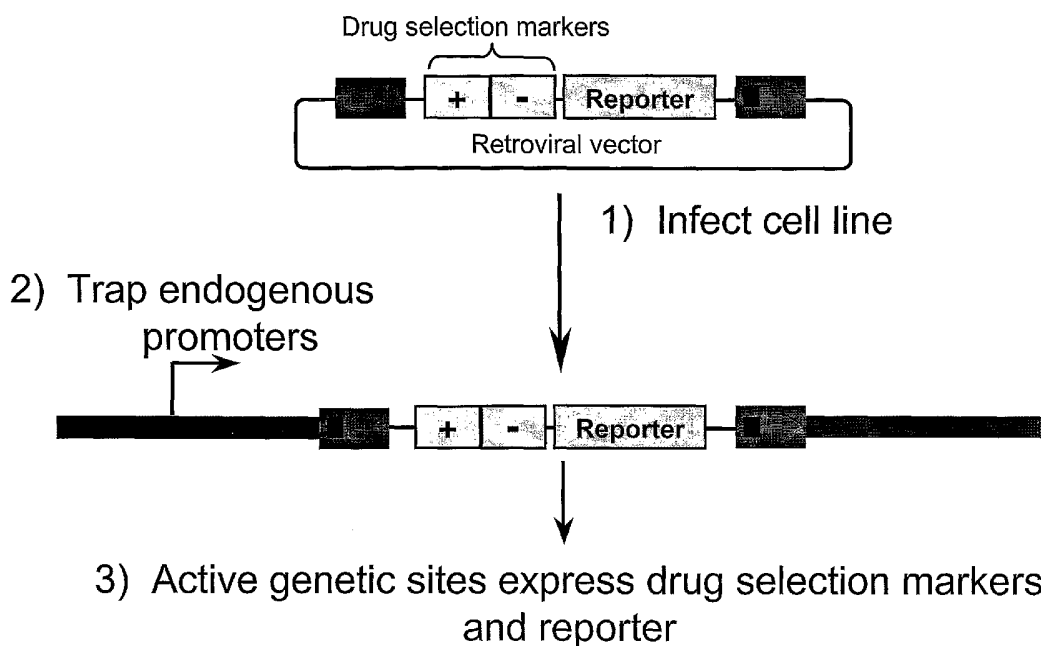
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(54) Title: TREATMENTS OF REFRACTORY CANCERS USING Na^+/K^+ -ATPASE INHIBITORS



(57) Abstract: The reagent, pharmaceutical formulation, kit, and methods of the invention provides a new approach to treat refractory cancers using Na^+/K^+ -ATPase inhibitors, such as cardiac glycosides (e.g. ouabain or proscillaridin, etc.).

TREATMENT OF REFRACTORY CANCERS USING Na^+/K^+ -ATPASE INHIBITORS

REFERENCE TO RELATED APPLICATION

This application claims the benefit of the filing date of U.S. Provisional
5 Application Serial No. 60/606,777, entitled "TREATMENTS OF REFRACTORY
CANCERS USING CARDIAC GLYCOSIDES AND OTHER Na^+/K^+ -ATPASE
INHIBITORS," and filed on September 2, 2004. The teachings of the referenced
application are incorporated herein by reference.

10 BACKGROUND OF THE INVENTION

Clinical drug resistance, either intrinsic or acquired, is a major barrier to
overcome before chemotherapy can become curative for most patients presenting
with cancer. In many common cancers (for example, non-small cell lung, testicular
and ovarian cancers), substantial tumor shrinkage can be expected in more than 50%
15 of cases with conventional chemotherapy. In other cases, response rates are lower;
10–20% of patients with renal cell carcinoma, pancreatic and esophageal cancers
respond to treatment. In almost all cases, drug resistance eventually develops shortly
and is often fatal. If this could be treated, prevented or overcome, the impact would
be substantial.

20 Such resistance or refractory phenotype may be brought about by a variety of
mechanisms. For example, there is (i) p-glycoprotein mediated multi-drug
resistance (MDR); (ii) mutant topoisomerase mediated atypical MDR; (iii) tubulin
mutation mediated resistance to taxanes; and (iv) resistance to cisplatin.

In addition, response of certain tumors to conventional chemotherapy and/or
25 radio therapy may also contribute to refractory cancer by promoting HIF-1
expression. HIF-1 is a transcription factor and is critical to survival in hypoxic
conditions, both in cancer and cardiac cells. HIF-1 is composed of the O_2 - and
growth factor-regulated subunit HIF-1 α , and the constitutively expressed HIF-1 β
subunit (arylhydrocarbon receptor nuclear translocator, ARNT), both of which
30 belong to the basic helix-loop-helix (bHLH)-PAS (PER, ARNT, SIM) protein

family. So far in the human genome 3 isoforms of the subunit of the transcription factor HIF have been identified: HIF-1, HIF-2 (also referred to as EPAS-1, MOP2, HLF, and HRF), and HIF-3 (of which HIF-32 also referred to as IPAS, inhibitory PAS domain).

5 Under normoxic conditions, HIF-1 α is targeted to ubiquitinylation by pVHL and is rapidly degraded by the proteasome. This is triggered through posttranslational HIF- hydroxylation on specific proline residues (proline 402 and 564 in human HIF-1 α protein) within the oxygen dependent degradation domain (ODDD), by specific HIF-prolyl hydroxylases (HPH1-3 also referred to as PHD1-3)
10 in the presence of iron, oxygen, and 2-oxoglutarate. The hydroxylated protein is then recognized by pVHL, which functions as an E3 ubiquitin ligase. The interaction between HIF-1 α and pVHL is further accelerated by acetylation of lysine residue 532 through an N-acetyltransferase (ARD1). Concurrently, hydroxylation of the asparagine residue 803 within the C-TAD also occurs by an asparaginyl hydroxylase
15 (also referred to as FIH-1), which by its turn does not allow the coactivator p300/CBP to bind to HIF-1 α subunit. In hypoxia HIF-1 α remains not hydroxylated and stays away from interaction with pVHL and CBP/p300 (Fig. 6). Following hypoxic stabilization HIF-1 α translocates to the nucleus where it heterodimerizes with HIF-1 β . The resulting activated HIF-1 drives the transcription of over 60 genes
20 important for adaptation and survival under hypoxia including glycolytic enzymes, glucose transporters Glut-1 and Glut-3, endothelin-1 (ET-1), VEGF (vascular endothelial growth factor), tyrosine hydroxylase, transferrin, and erythropoietin (Brahimi-Horn et al., 2001 Trends Cell Biol 11(11): S32-S36.; Beasley et al., 2002 Cancer Res 62(9): 2493-2497; Fukuda et al., 2002 J Biol Chem 277(41): 38205-
25 38211; Maxwell and Ratcliffe, 2002 Semin Cell Dev Biol 13(1): 29-37).

Hypoxia appears to promote tumor growth by promoting cell survival through its induction of angiogenesis and its activation of anaerobic metabolism. The inventors have discovered that certain anti-tumor agents in fact promote an hypoxic stress response in tumor cells, which accordingly should have a direct
30 consequence on clinical and prognostic parameters and create a therapeutic challenge, such as refractory cancer. This hypoxic response includes induction of

HIF-1 dependent transcription. The effect of HIF-1 on tumor growth is complex and involves the activation of several adaptive pathways.

It is an object of the present invention to provide a novel and more effective approach to treat cancers refractory to conventional chemotherapy.

5

SUMMARY OF THE INVENTION

A salient feature of the present invention is the discovery that Na^+/K^+ -ATPase inhibitors, such as cardiac glycosides, can be used to effectively treat at least certain cancers refractory to conventional chemo- or radio-therapy.

10 One aspect of the invention provides a packaged pharmaceutical comprising a Na^+/K^+ -ATPase inhibitor formulated in a pharmaceutically acceptable excipient and suitable for use in humans, and a label or instructions for administering the Na^+/K^+ -ATPase inhibitor as part of a treatment for inhibiting the growth or spread of a refractory cancer.

15 Another aspect of the invention provides a method of inhibiting the growth or spread of a refractory cancer in an individual, comprising administering to the individual an effective amount of a Na^+/K^+ -ATPase inhibitor.

Yet another aspect of the invention provides a method for promoting treatment of an individual suffering from a refractory cancer, comprising packaging,
20 labeling and/or marketing a Na^+/K^+ -ATPase inhibitor to be used as part of a treatment for inhibiting the growth or spread of the refractory cancer.

Still another aspect of the invention provides a method of treating multidrug resistance of refractory tumor cells in a refractory cancer patient in need of such treatment, said method comprising administering, concurrently or sequentially, an
25 effective amount of a Na^+/K^+ -ATPase inhibitor and an antineoplastic agent to said patient.

For any of the different aspects of the invention, the cancer may be refractory to radiation therapy, or refractory to anti-cancer chemotherapy.

The refractory cancer may be a solid tumor, such as a tumor in the pancreas,
30 lung, kidney, ovarian, breast, prostate, gastric, colon, bladder, prostate, brain, skin,

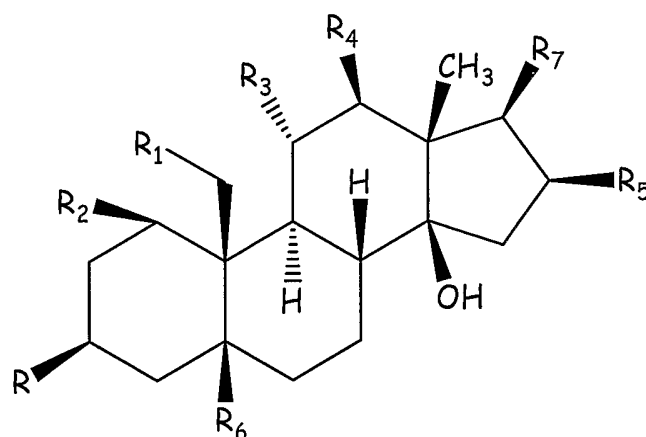
testicles, cervix, or liver. The solid tumor may be a pancreatic tumor refractory to treatment by one or more of: fluorouracil, carmustine (BCNU), temozolomide (TMZ), streptozotocin, and gemcitabine. The solid tumor may be a lung tumor refractory to etoposide or platinum-based therapy. For example, the lung tumor may be refractory
5 small cell lung cancer, or refractory non-small cell lung cancer. The refractory cancer may also be a hematological cancer, such as one selected from: acute lymphoblastic leukemia (ALL), acute lymphoblastic B-cell leukemia, acute lymphoblastic T-cell leukemia, acute nonlymphoblastic leukemia (ANLL), acute myeloblastic leukemia (AML), acute promyelocytic leukemia (APL), acute
10 monoblastic leukemia, acute erythro-leukemic leukemia, acute megakaryoblastic leukemia, chronic myelocytic leukemia (CML), chronic lymphocytic leukemia (CLL), multiple myeloma, myelodysplastic syndrome (MDS), or chronic myelomonocytic leukemia (CMML), wherein MDS may be either refractory anemia with excessive blast (RAEB) or RAEB in transformation to leukemia (RAEB-T).

15 In certain preferred embodiments, the Na^+/K^+ -ATPase inhibitor may be a cardiac glycoside.

For example, the cardiac glycoside may have an IC_{50} for killing one or more different cancer cell lines of 500 nM or less, and even more preferably 200 nM, 100 nM, 10 nM or even 1 nM or less.

20 The cardiac glycoside may comprise a steroid core with either a pyrone substituent at C17 (the "bufadienolides form"), or a butyrolactone substituent at C17 (the "cardenolide" form).

In certain embodiments, the cardiac glycoside is represented by the general formula:

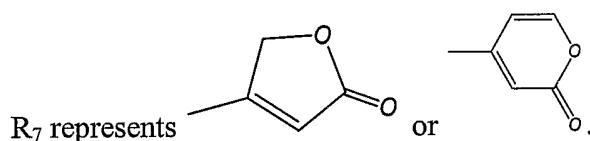


wherein

R represents a glycoside of 1 to 6 sugar residues;

R₁ represents hydrogen, -OH or =O;

5 R₂, R₃, R₄, R₅, and R₆ each independently represents hydrogen or -OH;



The sugar residues may be selected from: L-rhamnose, D-glucose, D-digitoxose, D-digitalose, D-digginose, D-sarmentose, L-vallarose, or D-fructose. These sugars may be in the β -conformation. The sugar residues may be acetylated, e.g., to effect the lipophilic character and the kinetics of the entire glycoside. The glycoside may be 1-4 sugar residues in length.

The cardiac glycoside may be selected from: digitoxigenin, digoxin, lanatoside C, Strophanthin K, uzarigenin, desacetyllanatoside A, acetyl digitoxin, desacetyllanatoside C, strophanthoside, scillaren A, proscillaridin A, digitoxose, gitoxin, strophanthidiol, oleandrin, acovenoside A, strophanthidine digilanobioside, strophanthidin-d-cymaroside, digitoxigenin-L-rhamnoside, digitoxigenin theretoside, strophanthidin, digoxigenin 3,12-diacetate, gitoxigenin, gitoxigenin 3-acetate, gitoxigenin 3,16-diacetate, 16-acetyl gitoxigenin, acetyl strophanthidin, ouabagenin, 3-epigoxigenin, neriifolin, acetylneriifolin cerberin, theventin, somalin, odoroside, honghelin, desacetyl digilanide, calotropin, calotoxin, convallatoxin, oleandrogenin, bufalin, periplocynarin, digoxin (CP 4072), strophanthidin oxime,

strophanthidin semicarbazone, strophanthidinic acid lactone acetate, ernicyrnarin, sannentoside D, sarverogenin, sarmentoside A, sarmentogenin, or a pharmaceutically acceptable salt, ester, amide, or prodrug thereof

In certain embodiments, the cardiac glycoside is ouabain or proscillaridin.

5 Other Na^+/K^+ -ATPase inhibitors are available in the literature. See, for example, U.S. Patent 5240714 which describes a non-digoxin-like Na^+/K^+ -ATPase inhibitory factor. Recent evidence suggests the existence of several endogenous Na^+/K^+ -ATPase inhibitors in mammals and animals. For instance, marinobufagenin (3,5-dihydroxy-14,15-epoxy bufodienolide) may be useful in the current
10 combinatorial therapies.

Those skilled in the art can also rely on screening assays to identify compounds that have Na^+/K^+ -ATPase inhibitory activity. PCT Publications WO00/44931 and WO02/42842, for example, teach high-throughput screening assays for modulators of Na^+/K^+ -ATPases.

15 The Na^+/K^+ -ATPase consists of at least two dissimilar subunits, the large α subunit with all known catalytic functions and the smaller glycosylated β subunit with chaperonic function. In addition there may be a small regulatory, so-called FXYP peptide. Four α peptide isoforms are known and isoform-specific differences in ATP, Na^+ and K^+ affinities and in Ca^{2+} sensitivity have been described. Thus
20 changes in Na^+/K^+ -ATPase isoform distribution in different tissues, as a function of age and development, electrolytes, hormonal conditions etc. may have important physiological implications. Cardiac glycosides like ouabain are specific inhibitors of the Na^+/K^+ -ATPase. The four α peptide isoforms have similar high ouabain affinities with K_d of around 1 nM or less in almost all mammalian species. In certain
25 embodiments, the Na^+/K^+ -ATPase inhibitor is more selective for complexes expressed in non-cardiac tissue, relative to cardiac tissue.

The subject cardiac glycoside may be conjointly administered with an effective amount of one or more anti-tumor agents, such as one selected from the group consisting of: an EGF-receptor antagonist, and arsenic sulfide, adriamycin,
30 cisplatin, carboplatin, cimetidine, carminomycin, mechlorethamine hydrochloride,

pentamethylmelamine, thiotepa, teniposide, cyclophosphamide, chlorambucil, demethoxyhypocrellin A, melphalan, ifosfamide, trofosfamide, Treosulfan, podophyllotoxin or podophyllotoxin derivatives, etoposide phosphate, teniposide, etoposide, leurosine, vindesine, 9-aminocamptothecin, 5 camptothecin, crisnatol, Chlorambucil, megestrol, methoposterin, mitomycin C, ecteinascidin 743, busulfan, carmustine (BCNU), lomustine (CCNU), lovastatin, 1-methyl-4-phenylpyridinium ion, semustine, staurosporine, streptozocin, thiotepa, phthalocyanine, dacarbazine, aminopterin, methotrexate, trimetrexate, thioguanine, mercaptopurine, fludarabine, pentastatin, cladribin, cytarabine (ara C), porfiromycin, 10 5-fluorouracil, 6-mercaptopurine, doxorubicin hydrochloride, leucovorin, mycophenolol acid, daunorubicin, deferoxamine, floxuridine, doxifluridine, ratitrexed, idarubicin, epirubicin, pirarubicin, zorubicin, mitoxantrone, bleomycin sulfate, mitomycin C, actinomycin D, safracins, saframycins, quinocarcins, discodermolides, vincristine, vinblastine, vinorelbine tartrate, vertoporphin, paclitaxel, 15 tamoxifen, raloxifene, tiazofuran, thioguanine, ribavirin, EICAR, estramustine, estramustine phosphate sodium, flutamide, bicalutamide, buserelin, leuprolide, pteridines, diynes, levamisole, aflacon, interferon, interleukins, aldesleukin, filgrastim, sargramostim, rituximab, BCG, tretinoin, irinotecan hydrochloride, betamethosone, gemcitabine hydrochloride, verapamil, VP-16, altretamine, 20 thapsigargin and topotecan.

In certain embodiments, the anti-cancer agent induces HIF-1 α -dependent transcription.

The anti-cancer agent may induce expression of one or more of cyclin G2, IGF2, IGF-BP1, IGF-BP2, IGF-BP3, EGF, WAF-1, TGF- α , TGF- β 3, ADM, EPO, 25 IGF2, EG-VEGF, VEGF, NOS2, LEP, LRP1, HK1, HK2, AMF/GP1, ENO1, GLUT1, GAPDH, LDHA, PFKFB3, PKFL, MIC1, NIP3, NIX and/or RTP801.

The anti-cancer agent may induce mitochondrial dysfunction and/or caspase activation.

The anti-cancer agent may induce cell cycle arrest at G2/M in the absence of 30 the cardiac glycoside.

The anti-cancer agent may be an inhibitor of chromatin function.

The anti-cancer agent may be a DNA topoisomerase inhibitor, such as one selected from: adriamycin, amsacrine, camptothecin, daunorubicin, dactinomycin, doxorubicin, eniposide, epirubicin, etoposide, idarubicin, irinotecan (CPT-11) or
5 mitoxantrone.

The anti-cancer agent may be a microtubule inhibiting drug, such as a taxane, including paclitaxel, docetaxel, vincristin, vinblastin, nocodazole, epothilones and navelbine.

The anti-cancer agent may be a DNA damaging agent, such as actinomycin,
10 amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchloroethamine, mitomycin, mitoxantrone, nitrosourea, plicamycin, procarbazine, taxol, taxotere, teniposide, triethylenethiophosphoramidate or etoposide
15 (VP16).

The anti-cancer agent may be an antimetabolite, such as a folate antagonists, or a nucleoside analog. Exemplary nucleoside analogs include pyrimidine analogs, such as 5-fluorouracil; cytosine arabinoside, and azacitidine. In other embodiments, the nucleoside analog is a purine analog, such as 6-mercaptopurine; azathioprine; 5-
20 iodo-2'-deoxyuridine; 6-thioguanine; 2-deoxycoformycin, cladribine, cytarabine, fludarabine, mercaptopurine, thioguanine, and pentostatin. In certain embodiments, the nucleoside analog is selected from AZT (zidovudine); ACV; valacyclovir; famciclovir; acyclovir; cidofovir; penciclovir; ganciclovir; Ribavirin; ddC; ddI (zalcitabine); lamuvidine; Abacavir; Adefovir; Didanosine; d4T (stavudine); 3TC;
25 BW 1592; PMEA/bis-POM PMEA; ddT, HPMP, HPMPG, HPMPA, PMEA, PMEG, dOTC; DAPD; Ara-AC, pentostatin; dihydro-5-azacytidine; tiazofurin; sangivamycin; Ara-A (vidarabine); 6-MMPR; 5-FUDR (floxuridine); cytarabine (Ara-C; cytosine arabinoside); 5-azacytidine (azacitidine); HBG [9-(4-hydroxybutyl)guanine], (1S,4R)-4-[2-amino-6-cyclopropyl-amino]-9H-purin-9-yl]-
30 2-cyclopentene-1-methanol succinate ("159U89"), uridine; thymidine; idoxuridine;

3-deazauridine; cyclocytidine; dihydro-5-azacytidine; tricitabine, ribavirin, and fludrabine.

In certain embodiments, the nucleoside analog is a phosphate ester selected from the group consisting of: Acyclovir; 1- β -D-arabinofuranosyl-E-5-(2-bromovinyl)uracil; 2'-fluorocarbocyclic-2'-deoxyguanosine; 6'-fluorocarbocyclic-2'-deoxyguanosine; 1-(β -D-arabinofuranosyl)-5(E)-(2-iodovinyl)uracil; {(1 α , 2 β , 3 α)-2-amino-9-(2,3-bis(hydroxymethyl)cyclobutyl)-6H-purin-6-one} Lobucavir; 9H-purin-2-amine, 9-((2-(1-methylethoxy)-1-((1-methylethoxy)methyl)ethoxy)methyl)-(9Cl); trifluorothymidine; 9- $\rightarrow$ (1,3-dihydroxy-2-propoxy)methylguanine (ganciclovir); 5-ethyl-2'-deoxyuridine; E-5-(2-bromovinyl)-2'-deoxyuridine; 5-(2-chloroethyl)-2'-deoxyuridine; buciclovir; 6-deoxyacyclovir; 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; E-5-(2-iodovinyl)-2'-deoxyuridine; 5-vinyl-1- β -D-arabinofuranosyluracil; 1- β -D-arabinofuranosylthymine; 2'-nor-2'-deoxyguanosine; and 1- β -D-arabinofuranosyladenine.

In certain embodiments, the nucleoside analog modulates intracellular CTP and/or dCTP metabolism.

In certain preferred embodiments, the nucleoside analog is gemcitabine.

In certain embodiments, the anti-cancer agent is a DNA synthesis inhibitor, such as a thymidilate synthase inhibitors (such as 5-fluorouracil), a dihydrofolate reductase inhibitor (such as methotrexate), or a DNA polymerase inhibitor (such as fludarabine).

In certain embodiments, the anti-cancer agent is a DNA binding agent, such as an intercalating agent.

In certain embodiments, the anti-cancer agent is a DNA repair inhibitor.

In certain embodiments, the anti-cancer agent is part of a combinatorial therapy selected from ABV, ABVD, AC (Breast), AC (Sarcoma), AC (Neuroblastoma), ACE, ACe, AD, AP, ARAC-DNR, B-CAVe, BCVPP, BEACOPP, BEP, BIP, BOMP, CA, CABO, CAF, CAL-G, CAMP, CAP, CaT, CAV, CAVE ADD, CA-VP16, CC, CDDP/VP-16, CEF, CEPP(B), CEV, CF, CHAP, ChIVPP, CHOP, CHOP-BLEO, CISCA, CLD-BOMP, CMF, CMFP, CMFVP, CMV, CNF, CNOP, COB, CODE, COMLA, COMP, Cooper Regimen,

COP, COPE, COPP, CP - Chronic Lymphocytic Leukemia, CP - Ovarian Cancer,
 CT, CVD, CVI, CVP, CVPP, CYVADIC, DA, DAT, DAV, DCT, DHAP, DI,
 DTIC/Tamoxifen, DVP, EAP, EC, EFP, ELF, EMA 86, EP, EVA, FAC, FAM,
 FAMTX, FAP, F-CL, FEC, FED, FL, FZ, HDMTX, Hexa-CAF, ICE-T, IDMTX/6-
 5 MP, IE, IfoVP, IPA, M-2, MAC-III, MACC, MACOP-B, MAID, m-BACOD,
 MBC, MC, MF, MICE, MINE, mini-BEAM, MOBP, MOP, MOPP, MOPP/ABV,
 MP - multiple myeloma, MP- prostate cancer, MTX/6-MO, MTX/6-MP/VP, MTX-
 CDDPAdr, MV - breast cancer, MV - acute myelocytic leukemia, M-VAC
 Methotrexate, MVP Mitomycin, MVPP, NFL, NOVP, OPA, OPPA, PAC, PAC-I,
 10 PA-CI, PC, PCV, PE, PFL, POC, ProMACE, ProMACE/cytaBOM,
 PRoMACE/MOPP, Pt/VM, PVA, PVB, PVDA, SMF, TAD, TCF, TIP, TTT,
 Topo/CTX, VAB-6, VAC, VACAdr, VAD, VATH, VBAP, VBCMP, VC, VCAP,
 VD, VelP, VIP, VM, VMCP, VP, V-TAD, 5 + 2, 7 + 3, "8 in 1".

In certain embodiments, the anti-cancer agent is selected from altretamine,
 15 aminoglutethimide, amsacrine, anastrozole, asparaginase, bcg, bicalutamide,
 bleomycin, buserelin, busulfan, calcium folinate, camptothecin, capecitabine,
 carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, colchicine,
 crisantaspase, cyclophosphamide, cyproterone, cytarabine, dacarbazine,
 dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin,
 20 epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine,
 fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, genistein,
 goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan,
 ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine,
 mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine,
 25 mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole,
 octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer,
 procarbazine, raltitrexed, rituximab, streptozocin, suramin, tamoxifen,
 temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride,
 topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and
 30 vinorelbine.

In certain embodiments, the anti-cancer agent is selected from tamoxifen, 4-(3-chloro-4-fluorophenylamino)-7-methoxy-6-(3-(4- α -morpholinyl)propoxy)quinazoline, 4-(3-ethynylphenylamino)-6,7-bis(2-methoxyethoxy)quinazoline, hormones, steroids, steroid synthetic analogs, 17a-ethinyloestradiol, diethylstilbestrol, testosterone, prednisone, fluoxymesterone, dromostanolone propionate, testolactone, megestrolacetate, methylprednisolone, methyl-testosterone, prednisolone, triamcinolone, chlorotrianisene, hydroxyprogesterone, aminoglutethimide, estramustine, medroxyprogesteroneacetate, leuprolide, flutamide, toremifene, Zoladex, antiangiogenics, matrix metalloproteinase inhibitors, VEGF inhibitors, ZD6474, SU6668, SU11248, anti-Her-2 antibodies (ZD1839 and OSI774), EGFR inhibitors, EKB-569, Imclone antibody C225, src inhibitors, bicalutamide, epidermal growth factor inhibitors, Her-2 inhibitors, MEK-1 kinase inhibitors, MAPK kinase inhibitors, P13 inhibitors, PDGF inhibitors, combretastatins, MET kinase inhibitors, MAP kinase inhibitors, inhibitors of non-receptor and receptor tyrosine kinases (imatinib), inhibitors of integrin signaling, and inhibitors of insulin-like growth factor receptors.

In certain embodiments, the subject combinations are used to inhibit growth of a tumor cell selected from a pancreatic tumor cell, lung tumor cell, a prostate tumor cell, a breast tumor cell, a colon tumor cell, a liver tumor cell, a brain tumor cell, a kidney tumor cell, a skin tumor cell, an ovarian tumor cell and a leukemic blood cell.

In certain embodiments, the subject combination is used in the treatment of a proliferative disorder selected from renal cell cancer, Kaposi's sarcoma, chronic lymphocytic leukemia, lymphoma, mesothelioma, breast cancer, sarcoma, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer, liver cancer, mammary adenocarcinoma, pharyngeal squamous cell carcinoma, prostate cancer, pancreatic cancer, gastrointestinal cancer, and stomach cancer.

It is contemplated that all embodiments of the invention may be combined with any other embodiment(s) of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

- Figure 1. Schematic diagram of using Sentinel Line promoter-less trap vectors to generate active genetic sites expressing drug selection markers and/or reporters.
- Figure 2. Schematic diagram of creating a Sentinel Line by sequential isolation of cells resistant to positive and negative selection drugs.
- Figure 3. Adaptation of a cancer cell to hypoxia, which leads to activation of multiple survival factors. The HIF family acts as a master switch transcriptionally activating many genes and enabling factors necessary for glycolytic energy metabolism, angiogenesis, cell survival and proliferation, and erythropoiesis. The level of HIF proteins present in the cell is regulated by the rate of their synthesis in response to factors such as hypoxia, growth factors, androgens and others. Degradation of HIF depends in part on levels of reactive oxygen species (ROS) in the cell. ROS leads to ubiquitylation and degradation of HIF.
- Figure 4. FACS Analysis of Sentinel Lines. Sentinel Lines were developed by transfecting A549 (NSCLC lung cancer) and Panc-1 (pancreatic cancer) cell lines with gene-trap vectors containing *E. coli* LacZ-encoded β -galactosidase (β -gal) as the reporter gene. The β -gal activity in Sentinel Lines (green) was measured by flow cytometry using a fluorogenic substrate fluorescein di-beta-D-galactopyranoside (FDG). The autofluorescence of untransfected control cells is shown in purple. The graphs indicate frequency of cells (y-axis) and intensity of fluorescence (x-axis) in log scale. The bar charts on the right depict median fluorescent units of the FACS curves. They indicate a high level of reporter activity at the targeted site.
- Figure 5. Western Blot analysis of HIF1 α expression indicates that cardiac glycoside compounds inhibit HIF1 α expression.
- Figure 6. Demonstrates that BNC1 inhibits HIF1 α synthesis.

- Figure 7. Demonstrates that BNC1 induces ROS production and inhibits HIF-1 α induction in tumor cells.
- Figure 8. Demonstrates that the cardiac glycoside compounds BNC1 and BNC4 directly or indirectly inhibits in tumor cells the secretion of the angiogenesis factor VEGF.
- Figure 9. These four charts show FACS analysis of response of a NSCLC Sentinel Line (A549), when treated 40 hrs with four indicated agents. Control (untreated) is shown in purple. Arrow pointing to the right indicates increase in reporter activity whereas inhibitory effect is indicated by arrow pointing to the left. The results indicate that standard chemotherapy drugs turn on survival response in tumor cells.
- Figure 10. Effect of BNC4 on Gemcitabine-induced stress responses visualized by A549 Sentinel LinesTM.
- Figure 11. Pharmacokinetic analysis of BNC1 delivered by osmotic pumps. Osmotic pumps (Model 2002, Alzet Inc) containing 200 μ l of BNC1 at 50, 30 or 20 mg/ml in 50% DMSO were implanted subcutaneously into nude mice. Mice were sacrificed after 24, 48 or 168 hrs, and plasma was extracted and analyzed for BNC1 by LC-MS. The values shown are average of 3 animals per point.
- Figure 12. Shows effect of BNC1 alone or in combination with standard chemotherapy on growth of xenografted human pancreatic tumors in nude mice.
- Figure 13. Shows anti-tumor activity of BNC1 and Cytosine against Caki-1 human renal cancer xenograft.
- Figure 14. Shows anti-tumor activity of BNC1 alone or in combination with Carboplatin in A549 human non-small-cell-lung carcinoma.
- Figure 15. Titration of BNC1 to determine minimum effective dose effective against Panc-1 human pancreatic xenograft in nude mice. BNC1 (sc, osmotic pumps) was tested at 10, 5 and 2 mg/ml.
- Figure 16. Combination of BNC1 with Gemcitabine is more effective than either drug alone against Panc-1 xenografts.

Figure 17. Combination of BNC1 with 5-FU is more effective than either drug alone against Panc-1 xenografts.

Figure 18. Comparison of BNC1 and BNC4 in inhibiting hypoxia-mediated HIF-1 α induction in human tumor cells (Hep3B cells).

5 Figure 19. Comparison of BNC1 and BNC4 in inhibiting hypoxia-mediated HIF-1 α induction in human tumor cells (Caki-1 and Panc-1 cells).

Figure 20. BNC4 blocks HIF-1 α induction by a prolyl-hydroxylase inhibitor under normoxia.

10 DETAILED DESCRIPTION OF THE INVENTION

I. Overview

The present invention is based in part on the discovery that Na⁺/K⁺-ATPase inhibitors, such as cardiac glycosides, can be used to effectively treat at least certain cancers refractory to conventional chemo- or radio-therapy.

15

II. Definitions

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

20 As used herein, the term "cancer" refers to any neoplastic disorder, including such cellular disorders as, for example, renal cell cancer, Kaposi's sarcoma, chronic leukemia, prostate cancer, breast cancer, sarcoma, pancreatic cancer, ovarian carcinoma, rectal cancer, throat cancer, melanoma, colon cancer, bladder cancer, mastocytoma, lung cancer, mammary adenocarcinoma, myeloma, lymphoma,
25 pharyngeal squamous cell carcinoma, and gastrointestinal or stomach cancer. Preferably, the cancer which is treated in the present invention is melanoma, lung cancer, breast cancer, pancreatic cancer, prostate cancer, colon cancer, or ovarian cancer.

30 The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

As used herein, "hyperproliferative disease" or "hyperproliferative disorder" refers to any disorder which is caused by or is manifested by unwanted proliferation of cells in a patient. Hyperproliferative disorders include but are not limited to cancer, psoriasis, rheumatoid arthritis, lamellar ichthyosis, epidermolytic
5 hyperkeratosis, restenosis, endometriosis, and abnormal wound healing.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "unwanted proliferation" means cell division and growth that is not part of normal cellular turnover, metabolism, growth, or propagation of the
10 whole organism. Unwanted proliferation of cells is seen in tumors and other pathological proliferation of cells, does not serve normal function, and for the most part will continue unbridled at a growth rate exceeding that of cells of a normal tissue in the absence of outside intervention. A pathological state that ensues because of the unwanted proliferation of cells is referred herein as a
15 "hyperproliferative disease" or "hyperproliferative disorder."

As used herein, "transformed cells" refers to cells that have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with
20 respect to their loss of growth control. For purposes of this invention, the terms "transformed phenotype of malignant mammalian cells" and "transformed phenotype" are intended to encompass, but not be limited to, any of the following phenotypic traits associated with cellular transformation of mammalian cells: immortalization, morphological or growth transformation, and tumorigenicity, as
25 detected by prolonged growth in cell culture, growth in semi-solid media, or tumorigenic growth in immuno-incompetent or syngeneic animals.

III. Exemplary Embodiments

Many Na^+/K^+ -ATPase inhibitors are available in the literature. See, for
30 example, U.S. Patent 5,240,714 which describes a non-digoxin-like Na^+/K^+ -ATPase

inhibitory factor. Recent evidence suggests the existence of several endogenous Na^+/K^+ -ATPase inhibitors in mammals and animals. For instance, marinobufagenin (3,5-dihydroxy-14,15-epoxy bufodienolide) may be useful in the current combinatorial therapies.

5 Those skilled in the art can also rely on screening assays to identify compounds that have Na^+/K^+ -ATPase inhibitory activity. PCT Publications WO00/44931 and WO02/42842, for example, teach high-throughput screening assays for modulators of Na^+/K^+ -ATPases.

 The Na^+/K^+ -ATPase consists of at least two dissimilar subunits, the large α subunit with all known catalytic functions and the smaller glycosylated β subunit with chaperonic function. In addition there may be a small regulatory, so-called FXYP-peptide. Four α peptide isoforms are known and isoform-specific differences in ATP, Na^+ and K^+ affinities and in Ca^{2+} sensitivity have been described. Thus changes in Na^+/K^+ -ATPase isoform distribution in different tissues, as a function of age and development, electrolytes, hormonal conditions etc. may have important physiological implications. Cardiac glycosides like ouabain are specific inhibitors of the Na^+/K^+ -ATPase. The four α peptide isoforms have similar high ouabain affinities with K_d of around 1 nM or less in almost all mammalian species. In certain embodiments, the Na^+/K^+ -ATPase inhibitor is more selective for complexes expressed in non-cardiac tissue, relative to cardiac tissue. The following section describes a preferred embodiments of Na^+/K^+ -ATPase inhibitors – cardiac glycosides.

A. Exemplary Cardiac Glycosides

 The subject cardiac glycosides are effective in treating refractory cancers. For example, cardiac glycosides are effective in suppressing EGF, insulin and/or IGF-responsive gene expression in various growth factor responsive cancer cell lines. As another example, the inventors have observed that cardiac glycosides are effective in suppressing HIF-responsive gene expression in cancer cell lines and furthermore, cardiac glycosides are shown to have potent antiproliferative effects in cancer cell lines. Since Hypoxia appears to promote tumor growth by promoting cell

survival through its induction of angiogenesis and its activation of anaerobic metabolism. The inventors have discovered that certain anti-tumor agents in fact promote an hypoxic stress response in tumor cells, which accordingly should have a direct consequence on clinical and prognostic parameters and create a therapeutic
5 challenge. This hypoxic response includes induction of HIF-1 dependent transcription. The effect of HIF-1 on tumor growth is complex and involves the activation of several adaptive pathways. Therefore, hypoxia response of cancer cells in response to certain cancer treatments is at least partially responsible for refractory cancers.

10 The term “cardiac glycoside” or “cardiac steroid” is used in the medical field to refer to a category of compounds tending to have positive inotropic effects on the heart. As a general class of compounds, cardiac glycosides comprise a steroid core with either a pyrone or butenolide substituent at C17 (the “pyrone form” and “butenolide form”). Additionally, cardiac glycosides may optionally be glycosylated
15 at C3. Most cardiac glycosides include one to four sugars attached to the 3 β -OH group. The sugars most commonly used include L-rhamnose, D-glucose, D-digitoxose, D-digitalose, D-digginose, D-sarmentose, L-vallarose, and D-fructose. In general, the sugars affect the pharmacokinetics of a cardiac glycoside with little other effect on biological activity. For this reason, aglycone forms of cardiac
20 glycosides are available and are intended to be encompassed by the term “cardiac glycoside” as used herein. The pharmacokinetics of a cardiac glycoside may be adjusted by adjusting the hydrophobicity of the molecule, with increasing hydrophobicity tending to result in greater absorption and an increased half-life. Sugar moieties may be modified with one or more groups, such as an acetyl group.

25 A large number of cardiac glycosides are known in the art for the purpose of treating cardiovascular disorders. Given the significant number of cardiac glycosides that have proven to have anticancer effects in the assays disclosed herein, it is expected that most or all of the cardiac glycosides used for the treatment of cardiovascular disorders may also be used for treating proliferative disorders.
30 Examples of preferred cardiac glycosides include ouabain, digitoxigenin, digoxin and lanatoside C. Additional examples of cardiac glycosides include: Strophantin K,

uzarigenin, desacetyl lanatoside A, acetyl digitoxin, desacetyl lanatoside C, strophanthoside, scillaren A, proscillaridin A, digitoxose, gitoxin, strophanthidiol, oleandrin, acovenoside A, strophanthidine digilanoside, strophanthidine-d-cymaroside, digitoxigenin-L-rhamnoside, digitoxigenin theretoside, strophanthidin, digoxigenin 3,12-diacetate, gitoxigenin, gitoxigenin 3-acetate, gitoxigenin 3,16-diacetate, 16-acetyl gitoxigenin, acetyl strophanthidin, ouabagenin, 3-epigoxigenin, neriifolin, acetylneriifolin cerberin, theventin, somalin, odoroside, honghelin, desacetyl digilanide, calotropin and calotoxin. Cardiac glycosides may be evaluated for effectiveness in the treatment of cancer by a variety of methods, including, for example: evaluating the effects of a cardiac glycoside on expression of a HIF-responsive gene in a cancer cell line or evaluating the effects of a cardiac glycoside on cancer cell proliferation.

Notably, cardiac glycosides affect proliferation of cancer cell lines at a concentration well below the known toxicity level. The IC_{50} measured for ouabain across several different cancer cell lines ranged from about 15 nM to about 600 nM, or about 80 nM to about 300 nM. The concentration at which a cardiac glycoside is effective as part of an antiproliferative treatment may be further decreased by combination with an additional agent that negatively regulates HIF-responsive genes, such as a redox effector or a steroid signal modulator. For example, as shown herein, the concentration at which a cardiac glycoside (e.g. ouabain or proscillaridin) is effective for inhibiting proliferation of cancer cells is decreased 5-fold by combination with a steroid signal modulator (Casodex). Therefore, in certain embodiments, the invention provides combination therapies of cardiac glycosides with, for example, steroid signal modulators and/or redox effectors. Additionally, cardiac glycosides may be combined with radiation therapy, taking advantage of the radiosensitizing effect that many cardiac glycosides have.

B. Exemplary Anti-cancer Agents

Although the subject Na^+/K^+ -ATPase inhibitors (e.g. cardiac glycosides) can be used alone to treat refractory cancers, they can also be used in combination with

other pharmaceutical agents. The pharmaceutical agents that may be used in the subject combination therapy with Na^+/K^+ -ATPase inhibitors (e.g. cardiac glycosides) include, merely to illustrate: aminoglutethimide, amsacrine, anastrozole, asparaginase, bcr, bicalutamide, bleomycin, buserelin, busulfan, camptothecin, capecitabine, carboplatin, carmustine, chlorambucil, cisplatin, cladribine, clodronate, colchicine, cyclophosphamide, cyproterone, cytarabine, dacarbazine, dactinomycin, daunorubicin, dienestrol, diethylstilbestrol, docetaxel, doxorubicin, epirubicin, estradiol, estramustine, etoposide, exemestane, filgrastim, fludarabine, fludrocortisone, fluorouracil, fluoxymesterone, flutamide, gemcitabine, genistein, goserelin, hydroxyurea, idarubicin, ifosfamide, imatinib, interferon, irinotecan, ironotecan, letrozole, leucovorin, leuprolide, levamisole, lomustine, mechlorethamine, medroxyprogesterone, megestrol, melphalan, mercaptopurine, mesna, methotrexate, mitomycin, mitotane, mitoxantrone, nilutamide, nocodazole, octreotide, oxaliplatin, paclitaxel, pamidronate, pentostatin, plicamycin, porfimer, procarbazine, raltitrexed, rituximab, streptozocin, suramin, tamoxifen, temozolomide, teniposide, testosterone, thioguanine, thiotepa, titanocene dichloride, topotecan, trastuzumab, tretinoin, vinblastine, vincristine, vindesine, and vinorelbine.

These anti-cancer agents may be categorized by their mechanism of action into, for example, following groups: anti-metabolites/anti-cancer agents, such as pyrimidine analogs (5-fluorouracil, floxuridine, capecitabine, gemcitabine and cytarabine) and purine analogs, folate antagonists and related inhibitors (mercaptopurine, thioguanine, pentostatin and 2-chlorodeoxyadenosine (cladribine)); antiproliferative/antimitotic agents including natural products such as vinca alkaloids (vinblastine, vincristine, and vinorelbine), microtubule disruptors such as taxane (paclitaxel, docetaxel), vincristin, vinblastin, nocodazole, epothilones and navelbine, epididodophyllotoxins (teniposide), DNA damaging agents (actinomycin, amsacrine, anthracyclines, bleomycin, busulfan, camptothecin, carboplatin, chlorambucil, cisplatin, cyclophosphamide, cytoxan, dactinomycin, daunorubicin, docetaxel, doxorubicin, epirubicin, hexamethylmelamineoxaliplatin, iphosphamide, melphalan, merchloroethamine, mitomycin, mitoxantrone, nitrosourea, paclitaxel,

plicamycin, procarbazine, teniposide, triethylenethiophosphoramidate and etoposide (VP16)); antibiotics such as dactinomycin (actinomycin D), daunorubicin, doxorubicin (adriamycin), idarubicin, anthracyclines, mitoxantrone, bleomycins, plicamycin (mithramycin) and mitomycin; enzymes (L-asparaginase which
5 systemically metabolizes L-asparagine and deprives cells which do not have the capacity to synthesize their own asparagine); antiplatelet agents; antiproliferative/antimitotic alkylating agents such as nitrogen mustards (mechlorethamine, cyclophosphamide and analogs, melphalan, chlorambucil), ethylenimines and methylmelamines (hexamethylmelamine and thiotepa), alkyl
10 sulfonates-busulfan, nitrosoureas (carmustine (BCNU) and analogs, streptozocin), trazenes - dacarbazine (DTIC); antiproliferative/antimitotic antimetabolites such as folic acid analogs (methotrexate); platinum coordination complexes (cisplatin, carboplatin), procarbazine, hydroxyurea, mitotane, aminoglutethimide; hormones, hormone analogs (estrogen, tamoxifen, goserelin, bicalutamide, nilutamide) and
15 aromatase inhibitors (letrozole, anastrozole); anticoagulants (heparin, synthetic heparin salts and other inhibitors of thrombin); fibrinolytic agents (such as tissue plasminogen activator, streptokinase and urokinase), aspirin, COX-2 inhibitors, dipyridamole, ticlopidine, clopidogrel, abciximab; antimigratory agents; antisecretory agents (breveldin); immunosuppressives (cyclosporine, tacrolimus
20 (FK-506), sirolimus (rapamycin), azathioprine, mycophenolate mofetil); anti-angiogenic compounds (TNP-470, genistein) and growth factor inhibitors (vascular endothelial growth factor (VEGF) inhibitors, fibroblast growth factor (FGF) inhibitors, epidermal growth factor (EGF) inhibitors); angiotensin receptor blocker; nitric oxide donors; anti-sense oligonucleotides; antibodies (trastuzumab); cell cycle
25 inhibitors and differentiation inducers (tretinoin); mTOR inhibitors, topoisomerase inhibitors (doxorubicin (adriamycin), amsacrine, camptothecin, daunorubicin, dactinomycin, eniposide, epirubicin, etoposide, idarubicin, irinotecan (CPT-11) and mitoxantrone, topotecan, irinotecan), corticosteroids (cortisone, dexamethasone, hydrocortisone, methylprednisolone, prednisone, and prednisolone); growth factor
30 signal transduction kinase inhibitors; mitochondrial dysfunction inducers and caspase activators; chromatin disruptors.

These anti-cancer agents are used by itself with an HIF inhibitor, or in combination. Many combinatorial therapies have been developed in prior art, including but not limited to those listed in Table 1.

Table 1: Exemplary conventional combination cancer chemotherapy

Name	Therapeutic agents
ABV	Doxorubicin, Bleomycin, Vinblastine
ABVD	Doxorubicin, Bleomycin, Vinblastine, Dacarbazine
AC (Breast)	Doxorubicin, Cyclophosphamide
AC (Sarcoma)	Doxorubicin, Cisplatin
AC (Neuroblastoma)	Cyclophosphamide, Doxorubicin
ACE	Cyclophosphamide, Doxorubicin, Etoposide
ACe	Cyclophosphamide, Doxorubicin
AD	Doxorubicin, Dacarbazine
AP	Doxorubicin, Cisplatin
ARAC-DNR	Cytarabine, Daunorubicin
B-CAVe	Bleomycin, Lomustine, Doxorubicin, Vinblastine
BCVPP	Carmustine, Cyclophosphamide, Vinblastine, Procarbazine, Prednisone
BEACOPP	Bleomycin, Etoposide, Doxorubicin, Cyclophosphamide, Vincristine, Procarbazine, Prednisone, Filgrastim
BEP	Bleomycin, Etoposide, Cisplatin
BIP	Bleomycin, Cisplatin, Ifosfamide, Mesna
BOMP	Bleomycin, Vincristine, Cisplatin, Mitomycin
CA	Cytarabine, Asparaginase
CABO	Cisplatin, Methotrexate, Bleomycin, Vincristine
CAF	Cyclophosphamide, Doxorubicin, Fluorouracil
CAL-G	Cyclophosphamide, Daunorubicin, Vincristine, Prednisone, Asparaginase
CAMP	Cyclophosphamide, Doxorubicin, Methotrexate, Procarbazine
CAP	Cyclophosphamide, Doxorubicin, Cisplatin
CaT	Carboplatin, Paclitaxel
CAV	Cyclophosphamide, Doxorubicin, Vincristine
CAVE ADD	CAV and Etoposide
CA-VP16	Cyclophosphamide, Doxorubicin, Etoposide
CC	Cyclophosphamide, Carboplatin
CDDP/VP-16	Cisplatin, Etoposide
CEF	Cyclophosphamide, Epirubicin, Fluorouracil
CEPP(B)	Cyclophosphamide, Etoposide, Prednisone, with or without/ Bleomycin
CEV	Cyclophosphamide, Etoposide, Vincristine
CF	Cisplatin, Fluorouracil or Carboplatin Fluorouracil

CHAP	Cyclophosphamide or Cyclophosphamide, Altretamine, Doxorubicin, Cisplatin
ChIVPP	Chlorambucil, Vinblastine, Procarbazine, Prednisone
CHOP	Cyclophosphamide, Doxorubicin, Vincristine, Prednisone
CHOP-BLEO	Add Bleomycin to CHOP
CISCA	Cyclophosphamide, Doxorubicin, Cisplatin
CLD-BOMP	Bleomycin, Cisplatin, Vincristine, Mitomycin
CMF	Methotrexate, Fluorouracil, Cyclophosphamide
CMFP	Cyclophosphamide, Methotrexate, Fluorouracil, Prednisone
CMFVP	Cyclophosphamide, Methotrexate, Fluorouracil, Vincristine, Prednisone
CMV	Cisplatin, Methotrexate, Vinblastine
CNF	Cyclophosphamide, Mitoxantrone, Fluorouracil
CNOP	Cyclophosphamide, Mitoxantrone, Vincristine, Prednisone
COB	Cisplatin, Vincristine, Bleomycin
CODE	Cisplatin, Vincristine, Doxorubicin, Etoposide
COMLA	Cyclophosphamide, Vincristine, Methotrexate, Leucovorin, Cytarabine
COMP	Cyclophosphamide, Vincristine, Methotrexate, Prednisone
Cooper Regimen	Cyclophosphamide, Methotrexate, Fluorouracil, Vincristine, Prednisone
COP	Cyclophosphamide, Vincristine, Prednisone
COPE	Cyclophosphamide, Vincristine, Cisplatin, Etoposide
COPP	Cyclophosphamide, Vincristine, Procarbazine, Prednisone
CP(Chronic lymphocytic leukemia)	Chlorambucil, Prednisone
CP (Ovarian Cancer)	Cyclophosphamide, Cisplatin
CT	Cisplatin, Paclitaxel
CVD	Cisplatin, Vinblastine, Dacarbazine
CVI	Carboplatin, Etoposide, Ifosfamide, Mesna
CVP	Cyclophosphamide, Vincristine, Prednisone
CVPP	Lomustine, Procarbazine, Prednisone
CYVADIC	Cyclophosphamide, Vincristine, Doxorubicin, Dacarbazine
DA	Daunorubicin, Cytarabine
DAT	Daunorubicin, Cytarabine, Thioguanine
DAV	Daunorubicin, Cytarabine, Etoposide
DCT	Daunorubicin, Cytarabine, Thioguanine

DHAP	Cisplatin, Cytarabine, Dexamethasone
DI	Doxorubicin, Ifosfamide
DTIC/Tamoxifen	Dacarbazine, Tamoxifen
DVP	Daunorubicin, Vincristine, Prednisone
EAP	Etoposide, Doxorubicin, Cisplatin
EC	Etoposide, Carboplatin
EFP	Etoposide, Fluorouracil, Cisplatin
ELF	Etoposide, Leucovorin, Fluorouracil
EMA 86	Mitoxantrone, Etoposide, Cytarabine
EP	Etoposide, Cisplatin
EVA	Etoposide, Vinblastine
FAC	Fluorouracil, Doxorubicin, Cyclophosphamide
FAM	Fluorouracil, Doxorubicin, Mitomycin
FAMTX	Methotrexate, Leucovorin, Doxorubicin
FAP	Fluorouracil, Doxorubicin, Cisplatin
F-CL	Fluorouracil, Leucovorin
FEC	Fluorouracil, Cyclophosphamide, Epirubicin
FED	Fluorouracil, Etoposide, Cisplatin
FL	Flutamide, Leuprolide
FZ	Flutamide, Goserelin acetate implant
HDMTX	Methotrexate, Leucovorin
Hexa-CAF	Altretamine, Cyclophosphamide, Methotrexate, Fluorouracil
ICE-T	Ifosfamide, Carboplatin, Etoposide, Paclitaxel, Mesna
IDMTX/6-MP	Methotrexate, Mercaptopurine, Leucovorin
IE	Ifosfamide, Etoposide, Mesna
IfoVP	Ifosfamide, Etoposide, Mesna
IPA	Ifosfamide, Cisplatin, Doxorubicin
M-2	Vincristine, Carmustine, Cyclophosphamide, Prednisone, Melphalan
MAC-III	Methotrexate, Leucovorin, Dactinomycin, Cyclophosphamide
MACC	Methotrexate, Doxorubicin, Cyclophosphamide, Lomustine
MACOP-B	Methotrexate, Leucovorin, Doxorubicin, Cyclophosphamide, Vincristine, Bleomycin, Prednisone
MAID	Mesna, Doxorubicin, Ifosfamide, Dacarbazine
m-BACOD	Bleomycin, Doxorubicin, Cyclophosphamide, Vincristine, Dexamethasone, Methotrexate, Leucovorin
MBC	Methotrexate, Bleomycin, Cisplatin
MC	Mitoxantrone, Cytarabine
MF	Methotrexate, Fluorouracil, Leucovorin
MICE	Ifosfamide, Carboplatin, Etoposide, Mesna
MINE	Mesna, Ifosfamide, Mitoxantrone, Etoposide

mini-BEAM	Carmustine, Etoposide, Cytarabine, Melphalan
MOBP	Bleomycin, Vincristine, Cisplatin, Mitomycin
MOP	Mechlorethamine, Vincristine, Procarbazine
MOPP	Mechlorethamine, Vincristine, Procarbazine, Prednisone
MOPP/ABV	Mechlorethamine, Vincristine, Procarbazine, Prednisone, Doxorubicin, Bleomycin, Vinblastine
MP (multiple myeloma)	Melphalan, Prednisone
MP (prostate cancer)	Mitoxantrone, Prednisone
MTX/6-MO	Methotrexate, Mercaptopurine
MTX/6-MP/VP	Methotrexate, Mercaptopurine, Vincristine, Prednisone
MTX-CDDPAdr	Methotrexate, Leucovorin, Cisplatin, Doxorubicin
MV (breast cancer)	Mitomycin, Vinblastine
MV (acute myelocytic leukemia)	Mitoxantrone, Etoposide
M-VAC Methotrexate	Vinblastine, Doxorubicin, Cisplatin
MVP Mitomycin	Vinblastine, Cisplatin
MVPP	Mechlorethamine, Vinblastine, Procarbazine, Prednisone
NFL	Mitoxantrone, Fluorouracil, Leucovorin
NOVP	Mitoxantrone, Vinblastine, Vincristine
OPA	Vincristine, Prednisone, Doxorubicin
OPPA	Add Procarbazine to OPA.
PAC	Cisplatin, Doxorubicin
PAC-I	Cisplatin, Doxorubicin, Cyclophosphamide
PA-CI	Cisplatin, Doxorubicin
PC	Paclitaxel, Carboplatin or Paclitaxel, Cisplatin
PCV	Lomustine, Procarbazine, Vincristine
PE	Paclitaxel, Estramustine
PFL	Cisplatin, Fluorouracil, Leucovorin
POC	Prednisone, Vincristine, Lomustine
ProMACE	Prednisone, Methotrexate, Leucovorin, Doxorubicin, Cyclophosphamide, Etoposide
ProMACE/cytaBOM	Prednisone, Doxorubicin, Cyclophosphamide, Etoposide, Cytarabine, Bleomycin, Vincristine, Methotrexate, Leucovorin, Cotrimoxazole
PRoMACE/MOPP	Prednisone, Doxorubicin, Cyclophosphamide, Etoposide, Mechlorethamine, Vincristine, Procarbazine, Methotrexate, Leucovorin
Pt/VM	Cisplatin, Teniposide
PVA	Prednisone, Vincristine, Asparaginase
PVB	Cisplatin, Vinblastine, Bleomycin
PVDA	Prednisone, Vincristine, Daunorubicin, Asparaginase

SMF	Streptozocin, Mitomycin, Fluorouracil
TAD	Mechlorethamine, Doxorubicin, Vinblastine, Vincristine, Bleomycin, Etoposide, Prednisone
TCF	Paclitaxel, Cisplatin, Fluorouracil
TIP	Paclitaxel, Ifosfamide, Mesna, Cisplatin
TTT	Methotrexate, Cytarabine, Hydrocortisone
Topo/CTX	Cyclophosphamide, Topotecan, Mesna
VAB-6	Cyclophosphamide, Dactinomycin, Vinblastine, Cisplatin, Bleomycin
VAC	Vincristine, Dactinomycin, Cyclophosphamide
VACAdr	Vincristine, Cyclophosphamide, Doxorubicin, Dactinomycin, Vincristine
VAD	Vincristine, Doxorubicin, Dexamethasone
VATH	Vinblastine, Doxorubicin, Thiotepa, Flouxymesterone
VBAP	Vincristine, Carmustine, Doxorubicin, Prednisone
VBCMP	Vincristine, Carmustine, Melphalan, Cyclophosphamide, Prednisone
VC	Vinorelbine, Cisplatin
VCAP	Vincristine, Cyclophosphamide, Doxorubicin, Prednisone
VD	Vinorelbine, Doxorubicin
VeIP	Vinblastine, Cisplatin, Ifosfamide, Mesna
VIP	Etoposide, Cisplatin, Ifosfamide, Mesna
VM	Mitomycin, Vinblastine
VMCP	Vincristine, Melphalan, Cyclophosphamide, Prednisone
VP	Etoposide, Cisplatin
V-TAD	Etoposide, Thioguanine, Daunorubicin, Cytarabine
5 + 2	Cytarabine, Daunorubicin, Mitoxantrone
7 + 3	Cytarabine with/, Daunorubicin or Idarubicin or Mitoxantrone
"8 in 1"	Methylprednisolone, Vincristine, Lomustine, Procarbazine, Hydroxyurea, Cisplatin, Cytarabine, Dacarbazine

In addition to conventional anti-cancer agents, the agent of the subject method can also be compounds and antisense RNA, RNAi or other polynucleotides to inhibit the expression of the cellular components that contribute to unwanted cellular proliferation that are targets of conventional chemotherapy. Such targets are, merely to illustrate, growth factors, growth factor receptors, cell cycle regulatory proteins, transcription factors, or signal transduction kinases.

The method of present invention is advantageous over combination therapies known in the art because it allows conventional anti-cancer agent to exert greater effect at lower dosage. In preferred embodiment of the present invention, the effective dose (ED₅₀) for a anti-cancer agent or combination of conventional anti-cancer agents when used in combination with a cardiac glycoside is at least 5 fold less than the ED₅₀ for the anti-cancer agent alone. Conversely, the therapeutic index (TI) for such anti-cancer agent or combination of such anti-cancer agent when used in combination with a cardiac glycoside is at least 5 fold greater than the TI for conventional anti-cancer agent regimen alone.

10

C. Refractory Tumors Treatable by Na⁺/K⁺-ATPase Inhibitors

Cancers or tumors that are resistant or refractory to treatment of a variety of therapeutic agents may benefit from treatment with the methods of the present invention. Preferred tumors are those resistant to chemotherapeutic agents other than the subject compounds disclosed herein. In certain embodiments of the instant invention, the subject compounds may be useful in treating tumors that are refractory to platinum-based chemotherapeutic agents, including carboplatin, cisplatin, oxaliplatin, iproplatin, tetraplatin, lobaplatin, DCP, PLD-147, JM118, JM216, JM335, and satraplatin. Such platinum-based chemotherapeutic agents also include the platinum complexes disclosed in EP 0147926, US 5072011, US 5244919, 5519155, 6503943 (LA-12/PLD-147), 6350737, and WO 01/064696 (DCP). Resistance to these platinum-based compounds can be tested and verified using the methods described in U.S.S.N. 60/546097.

Suitable agents for which the subject compounds are not cross-resistant are described in the following sections, which may be taken as non-limiting examples of "anti-cancer therapeutic agents."

1. Taxanes

Resistance to taxanes like paclitaxel and docetaxol is a major problem for all chemotherapeutic regimens utilizing these drugs. Taxanes exert their cytotoxic effect by binding to tubulin, thereby causing the formation of unusually stable

30

microtubules. The ensuing mitotic arrest triggers the mitotic spindle checkpoint and results in apoptosis. Other mechanisms that mediate apoptosis through pathways independent of microtubule dysfunction have been described as well, including molecular events triggered by the activation of Cell Division Control-2 (cdc-2)
5 Kinase, phosphorylation of BCL-2 and the induction of interleukin 1 β (IL-1 β) and tumor necrosis factor- α (TNF- α). Furthermore, taxanes have been shown to also exert anti-tumor activity via other mechanisms than the direct activation of the apoptotic cascade. These mechanisms include decreased production of metalloproteinases and the inhibition of endothelial cell proliferation and motility,
10 with consequent inhibition of angiogenesis.

Thus, one embodiment of the present invention relates to methods of treating patients with tumors resistant to taxanes by administering a subject compound.

By the term "taxane", it is meant to include any member of the family of terpenes, including, but not limited to paclitaxel (Taxol) and docetaxel (Taxotere),
15 which were derived primarily from the Pacific yew tree, Taxus brevifolia, and which have activity against certain tumors, particularly breast, lung and ovarian tumors (See, for example, Pazdur et al. Cancer Treat Res. 1993.19:3 5 1; Bissery et al. Cancer Res. 1991 51:4845). In the methods and packaged pharmaceuticals of the present invention, preferred taxanes are paclitaxel, docetaxel, deoxygenated
20 paclitaxel, TL-139 and their derivatives. See Annu. Rev. Med. 48:353-374 (1997).

The term "paclitaxel" includes both naturally derived and related forms and chemically synthesized compounds or derivatives thereof with antineoplastic properties including deoxygenated paclitaxel compounds such as those described in U.S. Pat. No. 5,440,056, U.S. Patent No. 4942184, which are herein incorporated by
25 reference, and that sold as TAXOL[®] by Bristol-Myers Oncology. Paclitaxel has been approved for clinical use in the treatment of refractory ovarian cancer in the United States (Markman et al., Yale Journal of Biology and Medicine, 64:583, 1991; McGuire et al., Ann. Intern. Med., 111:273, 1989). It is effective for chemotherapy for several types of neoplasms including breast (Holmes et al., J. Nat. Cancer Inst.,
30 83:1797, 1991) and has been approved for treatment of breast cancer as well. It is a

potential candidate for treatment of neoplasms in the skin (Einzig et al., Proc. Am. Soc. Clin. Oncol., 20:46) and head and neck carcinomas (Forastire et al. Sem. Oncol., 20:56, 1990). The compound also shows potential for the treatment of polycystic kidney disease (Woo et al, Nature, 368:750, 1994), lung cancer and malaria. Docetaxel (N-debenzoyl-N-tert-butoxycarbonyl-10-deacetyl paclitaxel) is produced under the trademark TAXOTERE[®] by Aventis. In addition, other taxanes are described in "Synthesis and Anticancer Activity of Taxol other Derivatives," D. G. I. Kingston et al., Studies in Organic Chemistry, vol. 26, entitled "New Trends in Natural Products Chemistry" (1986), Atta-urRabman, P. W. le Quesne, Eds. (Elvesier, Amsterdam 1986), pp 219-235 are incorporated herein. Various taxanes are also described in U.S. 6380405, the entirety of which is incorporated herein.

Methods and packaged pharmaceuticals of the present invention are applicable for treating tumors resistant to treatment by any taxane, regardless of the resistance mechanism. Known mechanisms that confer taxane resistance include, for example, molecular changes in the target molecules, i.e., α -tubulin and/or β -tubulin, up-regulation of P-glycoprotein (multidrug resistance gene MDR-1), changes in apoptotic regulatory and mitosis checkpoint proteins, changes in cell membranes, overexpression of interleukin 6 (IL-6; Clin Cancer Res (1999) 5, 3445-3453; Cytokine (2002) 17, 234-242), the overexpression of interleukin 8 (IL-8; Clin Cancer Res (1999) 5, 3445-3453; Cancer Res (1996) 56, 1303-1308) or the overexpression of monocyte chemotactic protein-1 (MCP-1; (MCP-1; Clin Cancer Res (1999) 5, 3445-3453), changes in the levels of acidic and basic fibroblast growth factors, transmembrane factors, such as p185 (HER2; Oncogene (1996) 13, 1359-1365) or EGFR (Oncogene (2000) 19, 6550-6565; Bioessays (2000) 22, 673-680), changes in adhesion molecules, such as β 1 integrin (Oncogene (2001) 20, 4995-5004), changes in house keeping molecules, such as glutathione-S-transferase and/or glutathione peroxidase (Jpn J Clin Oncol (1996) 26, 1-5), changes in molecules involved in cell signaling, such as interferon response factor 9, molecules involved in NF- κ B signaling, molecules involved in the PI-3 kinase/AKT survival pathway, RAF-1 kinase activity, PKC α/β or PKC β/β 2 and via nuclear proteins, such as

nuclear annexin IV, the methylation controlled J protein of the DNA J family of proteins, thymidylate synthetase or c-jun.

Another known mechanism that confers taxane resistance is, for example, changes in apoptotic regulatory and mitosis checkpoint proteins. Such changes in
5 apoptotic regulatory and mitosis checkpoint proteins include the over-expression of Bcl-2 (Cancer Chemother Pharmacol (2000) 46, 329-337; Leukemia (1997) 11, 253-257) and the over-expression of Bcl-xL (Cancer Res (1997) 57, 1109-1115; Leukemia (1997) 11, 253-257). Over-expression of Bcl-2 may be effected by estradiol (Breast Cancer Res Treat (1997) 42, 73-81).

10 Taxane resistance may also be conferred via changes in the cell membrane. Such changes include the change of the ratio of fatty acid methylene:methyl (Cancer Res (1996) 56, 3461-3467), the change of the ratio of choline:methyl (Cancer Res (1996) 56, 3461-3467) and a change of the permeability of the cell membrane (J Cell Biol (1986) 102, 1522-1531).

15 A further known mechanism that confers taxane resistance is via changes in acidic and basic fibroblast growth factors (Proc Natl Acad Sci USA (2000) 97, 8658-8663), via molecules involved in cell signaling, such as interferon response factor 9 (Cancer Res (2001) 61, 6540-6547), molecules involved in NF- κ B signaling (Surgery (2991) 130, 143-150), molecules involved in the PI-3 kinase/AKT survival
20 pathway (Oncogene (2001) 20, 4995-5004), RAF-1 kinase activity (Anticancer Drugs (2000) 11, 439-443; Chemotherapy (2000) 46, 327-334), PKC α/β (Int J Cancer (1993) 54, 302-308) or PKC $\beta/\beta 2$ (Int J Cancer (2001) 93, 179-184, Anticancer Drugs (1997) 8, 189-198).

25 Taxane resistance may also be conferred via changes nuclear proteins, such as nuclear annexin IV (Br J Cancer (2000) 83, 83-88), the methylation controlled J protein of the DNA J family of proteins (Cancer Res (2001) 61, 4258-4265), thymidylate synthetase (Anticancer Drugs (1997) 8, 189-198) or c-jun (Anticancer Drugs (1997) 8, 189-198), via paracrine factors, such as LPS (J Leukoc Biol (1996) 59, 280-286), HIF-1 (Mech Dev (1998) 73, 117-123), VEGF (Mech Dev (1998) 73,

117-123) and the lack of decline in bcl-XL in spheroid cultures (Cancer Res (1997) 57, 2388-2393).

2. Indole Alkaloid

5 Thus, one embodiment of the present invention relates to methods of treating patients with tumors resistant to an indole alkaloid by administering a subject compound.

Exemplary indole alkaloids include bis-indole alkaloids, such as vincristine, vinblastine and 5'-nor-anhydrovinblastine (hereinafter: 5'-nor- vinblastine). It is
10 known that bis-indole compounds (alkaloids), and particularly vincristine and vinblastine of natural origin as well as the recently synthetically prepared 5'-nor-vinblastine play an important role in the antitumor therapy. These compounds were commercialized or described, respectively in the various pharmacopoeias as salts (mainly as sulfates or difumarates, respectively).

15 Preferred indole alkaloids are camptothecin and its derivatives and analogues. Camptothecin is a plant alkaloid found in wood, bark, and fruit of the Asian tree *Camptotheca acuminata*. Camptothecin derivatives are now standard components in the treatment of several malignancies. See Pizzolato and Saltz, 2003. Studies have established that CPT inhibited both DNA and RNA synthesis. Recent
20 research has demonstrated that CPT and CPT analogues interfere with the mechanism of action of the cellular enzyme topoisomerase I, which is important in a number of cellular processes (e.g., DNA replication and recombination, RNA transcription, chromosome decondensation, etc.). Without being bound to theory, camptothecin is thought to reversibly induce single-strand breaks, thereby affecting
25 the cell's capacity to replicate. Camptothecin stabilizes the so-called cleavable complex between topoisomerase I and DNA. These stabilized breaks are fully reversible and non-lethal. However, when a DNA replication fork collides with the cleavable complex, single-strand breaks are converted to irreversible double-strand breaks. Apoptotic cell death is then mediated by caspase activation. Inhibition of
30 caspase activation shifts the cells from apoptosis to transient G1 arrest followed by cell necrosis. Thus, the mechanisms of cell death need active DNA replication to be

happening, resulting in cytotoxic effects from camptothecin that is S-phase-specific. Indeed, cells in S-phase in vitro have been shown to be 100–1000 times more sensitive to camptothecin than cells in G1 or G2.

Camptothecin analogues and derivatives include, for example, irinotecan
5 (Camptosar, CPT-11), topotecan (Hycamptin), BAY 38-3441, 9-nitrocamptothecin (Orethecin, rubitecan), exatecan (DX-8951), lurtotecan (GI-147211C), gimatecan, homocamptothecins diflomotecan (BN-80915) and 9-aminocamptothecin (IDEC-13'). See Pizzolato and Saltz, *The Lancet*, 361:2235-42 (2003); and Ulukan and Swaan, *Drug* 62: 2039-57 (2002). Additional Camptothecin analogues and
10 derivatives include, SN-38 (this is the active compound of the prodrug irinotecan; conversion is catalyzed by cellular carboxylesterases), ST1481, karanitecin (BNP1350), indolocarbazoles, such as NB-506, protoberberines, intoplicines, idenoisoquinolones, benzo-phenazines and NB-506. More camptothecin derivatives are described in WO03101998: NITROGEN-BASED HOMO-CAMPTOTHECIN
15 DERIVATIVES; US6100273: Water Soluble Camptothecin Derivatives, US 5587673, Camptothecin Derivatives.

The methods and pharmaceutical compositions of the present invention are useful for treating tumors resistant to any one or more of above-listed drugs.

20 3. *Platinum-based therapeutic agents*

In an alternative embodiment, the methods, packaged pharmaceuticals and pharmaceutical compositions of the present invention are useful for treating tumors resistant to platinum-based chemotherapeutic agents.

Such platinum-based chemotherapeutic agents may include: carboplatin,
25 cisplatin, oxaliplatin, iproplatin, tetraplatin, lobaplatin, DCP, PLD-147, JM118, JM216, JM335, and satraplatin. Such platinum-based chemotherapeutic agents also include the platinum complexes disclosed in EP 0147926, US 5072011, US 5244919, 5519155, 6503943 (LA-12/PLD-147), 6350737, and WO 01/064696 (DCP).

30 As is understood in the art, the platinum-based chemotherapeutic agents, or platinum coordination complexes, typified by cisplatin [cis-

diamminedichloroplatinum (II)] (Reed, 1993, in Cancer, Principles and Practice of Oncology, pp. 390-4001), have been described as "the most important group of agents now in use for cancer treatment". These agents, used as a part of combination chemotherapy regimens, have been shown to be curative for testicular and ovarian
5 cancers and beneficial for the treatment of lung, bladder and head and neck cancers. DNA damage is believed to be the major determinant of cisplatin cytotoxicity, though this drug also induces other types of cellular damage.

In addition to cisplatin, this group of drugs includes carboplatin, which like cisplatin is used clinically, and other platinum-containing drugs that are under
10 development. These compounds are believed to act by the same or very similar mechanisms, so that conclusions drawn from the study of the bases of cisplatin sensitivity and resistance are expected to be valid for other platinum-containing drugs.

Cisplatin is known to form adducts with DNA and to induce interstrand
15 crosslinks. Adduct formation, through an as yet unknown signaling mechanism, is believed to activate some presently unknown cellular enzymes involved in programmed cell death (apoptosis), the process which is believed to be ultimately responsible for cisplatin cytotoxicity (see Eastman, 1990, Cancer Cells 2: 275-2802).

Applicants have demonstrated that the subject compounds are effective in
20 treating resistant tumors in which resistance is mediated through at least one of the following three mechanisms: multidrug resistance, tubulins and topoisomerase I. This section describes these three resistance mechanisms and therapeutic agents for which resistance arises through at least one of these mechanisms. One of skill in the art will understand that tumor cells may be resistant to a chemotherapeutic agent
25 through more than one mechanism. For example, the resistance of tumor cells to paclitaxel may be mediated through via multidrug resistance, or alternatively, via tubulin mutation(s).

In a preferred embodiment, the methods and pharmaceutical compositions of the present invention are useful for treating tumors resistant to certain
30 chemotherapeutic agents.

a. Resistance mediated through tubulins

Microtubules are intracellular filamentous structures present in all eukaryotic cells. As components of different organelles such as mitotic spindles, centrioles, basal bodies, cilia, flagella, axopodia and the cytoskeleton, microtubules are involved in many cellular functions including chromosome movement during mitosis, cell motility, organelle transport, cytokinesis, cell plate formation, maintenance of cell shape and orientation of cell microfibril deposition in developing plant cell walls. The major component of microtubules is tubulin, a protein composed of two subunits called alpha and beta. An important property of tubulin in cells is the ability to undergo polymerization to form microtubules or to depolymerize under appropriate conditions. This process can also occur in vitro using isolated tubulin.

Microtubules play a critical role in cell division as components of the mitotic spindle, an organelle which is involved in distributing chromosomes within the dividing cell precisely between the two daughter nuclei. Various drugs prevent cell division by binding to tubulin or to microtubules. Anticancer drugs acting by this mechanism include the alkaloids vincristine and vinblastine, and the taxane-based compounds paclitaxel and docetaxel {see, for example, E. K. Rowinsky and R. C. Donehower, *Pharmacology and Therapeutics*, 52, 35-84 (1991)}. Other antitubulin compounds active against mammalian cells include benzimidazoles such as nocodazole and natural products such as colchicine, podophyllotoxin, epithilones, and the combretastatins.

Certain therapeutic agents may exert their activities by, for example, binding to α -tubulin, β -tubulin or both, and/or stabilizing microtubules by preventing their depolymerization. Other modes of activity may include, down regulation of the expression of such tubulin proteins, or binding to and modification of the activity of other proteins involved in the control of expression, activity or function of tubulin.

In one embodiment, the resistance of tumor cells to a therapeutic agent is mediated through tubulin. By "mediated through tubulin", it is meant to include direct and indirect involvement of tubulin. For example, resistance may arise due to

tubulin mutation, a direct involvement of tubulin in the resistance. Alternatively, resistance may arise due to alterations elsewhere in the cell that affect tubulin and/or microtubules. These alterations may be mutations in genes affecting the expression level or pattern of tubulin, or mutations in genes affecting microtubule assembly in general. Mammals express 6 α - and 6 β -tubulin genes, each of which may mediate drug resistance.

Specifically, tubulin-mediated tumor resistance to a therapeutic agent may be conferred via molecular changes in the tubulin molecules. For example, molecular changes include mutations, such as point mutations, deletions or insertions, splice variants or other changes at the gene, message or protein level. In particular embodiments, such molecular changes may reside in amino acids 250-300 of β -tubulin, or may affect nucleotides 810 and/or 1092 of the β -tubulin gene. For example, and without wishing to be limited, the paclitaxel-resistant human ovarian carcinoma cell line 1A9-PTX10 is mutated at amino acid residues β 270 and β 364 of β -tubulin (see Giannakakou et al., 1997). For another example, two epothilone-resistant human cancer cell lines has acquired β -tubulin mutations at amino acid residues β 274 and β 282, respectively (See Giannakakou et al., 2000). These mutations are thought to affect the binding of the drugs to tubulins. Alternatively, mutations in tubulins that confer drug resistance may also be alterations that affect microtubule assembly. This change in microtubule assembly has been demonstrated to compensate for the effect of drugs by having diminished microtubule assembly compared to wild-type controls (Minotti, A. M., Barlow, S. B., and Cabral, F. (1991) J Biol Chem 266, 3987-3994). It will also be understood by a person skilled in the art that molecular changes in α -tubulin may also confer resistance to certain compounds. WO 00/71752 describes a wide range of molecular changes to tubulin molecules and the resistance to certain chemotherapeutic compounds that such molecular changes may confer on a cell. WO 00/71752, and all references therein, are incorporated in their entirety herein.

Tubulin-mediated tumor resistance to therapeutic agents may also be conferred via alterations of the expression pattern of either α -tubulin or the β -

tubulin, or both. For example, several laboratories have provided evidence that changes in the expression of specific β -tubulin genes are associated with paclitaxel resistance in cultured tumor cell lines (Haber, M., Burkhart, C. A., Regl, D. L., Madafiglio, J., Norris, M. D., and Horwitz, S. B. (1995) *J Biol Chem.* 270, 31269-75; Jaffrezou, J. P., Durnontet, C., Deny, W. B., Duran, G., Chen, G., Tsuchiya, E., Wilson, L., Jordan, M. A., and Sikic, B. 1. (1995) *Oncology Res.* 7, 517-27; Kavallaris, M., Kuo, D. Y. S., Burkhart, C. A., Regl, D. L., Norris, M. D., Haber, M., and Horwitz, S. B. (1997) *J Clin. Invest.* 100, 1282-93; and Ranganathan, S., Dexter, D. W., Benetatos, C. A., and Hudes, G. R. (1998) *Biochim. Biophys. Acta* 1395, 237-245).

Tubulin-mediated tumor resistance to therapeutic agents may also be conferred via an increase of the total tubulin content of the cell, an increase in the α -tubulin content or the expression of different electrophoretic variants of α -tubulin. Furthermore, resistance may be conferred via alterations in the electrophoretic mobility of β -tubulin subunits, overexpression of the H β 2 tubulin gene, overexpression of the H β 3 tubulin gene, overexpression of the H β 4 tubulin gene, overexpression of the H β 4a tubulin gene or overexpression of the H β 5 tubulin gene.

Tubulin-mediated tumor resistance to therapeutic agents may also be conferred via post-translational modification of tubulin, such as increased acetylation of α -tubulin (*Jpn J Cancer Res* (85) 290-297), via proteins that regulate microtubule dynamics by interacting with tubulin dimmers or polymerized microtubules. Such proteins include but are not limited to stathmin (*Mol Cell Biol* (1999) 19, 2242-2250) and MAP4 (*Biochem Pharmacol* (2001) 62, 1469-1480).

Exemplary chemotherapeutic agents for which resistance is at least partly mediated through tubulin include, taxanes (paclitaxel, docetaxel and Taxol derivatives), vinca alkaloids (vinblastine, vincristine, vindesine and vinorelbine), epothilones (epothilone A, epothilone B and discodermolide), nocodazole, colchicin, colchicines derivatives, allocolchicine, Halichondrin B, dolstatin 10, maytansine, rhizoxin, thiocolchicine, trityl cysterin, estramustine and nocodazole. See WO 03/099210 and Giannakakou et al., 2000. Additional exemplary chemotherapeutic

agents for which resistance is at least partly mediated through tubulin include, colchicine, curacin, combretastatins, cryptophycins, dolastatin, auristatin PHE, symplostatin 1, eleutherobin, halichondrin B, halimide, hemiasterlins, laulimalide, maytansinoids, PC-SPES, peloruside A, resveratrol, S-allylmercaptocysteine (SAMC), spongistatins, taxanes, vitilevuamide, 2-methoxyestradiol (2-ME2), A-289099, A-293620/A-318315, ABT-751/E7010, ANG 600 series, anhydrovinblastine (AVLB), AVE806, bivatuzumab mertansine, BMS-247550, BMS-310705, cantuzumab mertansine, combretastatin, combretastatin A-4 prodrug (CA4P), CP248/CP461, D-24851/D-64131, dolastatin 10, E7389, EPO906, FR182877, HMN-214, huN901-DM1/BB-10901TAP, ILX-651, KOS-862, LY355703, mebendazole, MLN591DM1, My9-6-DM1, NPI-2352 and NPI-2358, Oxi-4503, R440, SB-715992, SDX-103, T67/T607, trastuzumab-DM1, TZT-1027, vinflunine, ZD6126, ZK-EPO.

Resistance to these and other compounds can be tested and verified using the methods described in the Examples. The methods and pharmaceutical compositions of the present invention are useful for treating tumors resistant to any one or more of above-listed agents.

Preferred chemotherapeutic agents for which resistance is at least partly mediated through tubulin are taxanes, including, but not limited to paclitaxel and docetaxel (Taxotere), which were derived primarily from the Pacific yew tree, *Taxus brevifolia*, and which have activity against certain tumors, particularly breast and ovarian tumors (See, for example, Pazdur et al. Cancer Treat Res. 1993.19:3 5 1; Bissery et al. Cancer Res. 1991 51:4845).

b. Resistance mediated through multidrug resistance

In another embodiment, the resistance of tumor cells to a therapeutic agent is mediated through multidrug resistance. The term "multidrug resistance (MDR)", as used herein, refers to a specific mechanism that limits the ability of a broad class of hydrophobic, weakly cationic compounds to accumulate in the cell. These

compounds have diverse structures and mechanisms of action yet all are affected by this mechanism.

Experimental models demonstrate that multidrug resistance can be caused by increased expression of ATP-binding cassette (ABC) transporters, which function as ATP-dependent efflux pumps. These pumps actively transport a wide array of anti-cancer and cytotoxic drugs out of the cell, in particular natural hydrophobic drugs. In mammals, the superfamily of ABC transporters includes P-glycoprotein (P-gp) transporters (MDR1 and MDR3 genes in human), the MRP subfamily (already composed of six members), and bile salt export protein (ABCB11; Cancer Res (1998) 58, 4160-4167), MDR-3 (Nature Rev Cancer (2002) 2, 48-58), lung resistance protein (LRP) and breast cancer resistant protein (BCRP). See Kondratov et al., 2001 and references therein; Cancer Res (1993) 53, 747-754; J Biol Chem (1995) 270, 31269-31275; Leukemia (1994) 8, 465-475; Biochem Pharmacol (1997) 53, 461-470; Leonard et al (2003), The Oncologist 8:411-424). These proteins can recognize and efflux numerous substrates with diverged chemical structure, including many anticancer drugs. Overexpression of P-gp is the most common cause for MDR. Other causes of MDR have been attributed to changes in topoisomerase II, protein kinase C and specific glutathione transferase enzymes. See Endicott and Ling, 1989.

The methods of the present invention are useful for treating tumors resistant to a therapeutic agent, in which resistance is at least partially due to MDR. In a preferred embodiment, the drug resistance of the tumor is mediated through overexpression of an ABC transporter. In a further preferred embodiment, the drug resistance of the tumor is mediated through the overexpression of P-gp. Numerous mechanisms can lead to overexpression of P-gp, including amplification of the MDR-1 gene (Anticancer Res (2002) 22, 2199-2203), increased transcription of the MDR-1 gene (J Clin Invest (1995) 95, 2205-2214; Cancer Lett (1999) 146, 195-199; Clin Cancer Res (1999) 5, 3445-3453; Anticancer Res (2002) 22, 2199-2203), which may be mediated by transcription factors such as RGP8.5 (Nat Genet 2001 (27), 23-29), mechanisms involving changes in MDR-1 translational efficiency (Anticancer Res (2002) 22, 2199-2203), mutations in the MDR-1 gene (Cell (1988) 53, 519-529;

Proc Natl Acad Sci USA (1991) 88, 7289-7293; Proc Natl Acad Sci USA (1992) 89, 4564-4568) and chromosomal rearrangements involving the MDR-1 gene and resulting in the formation of hybrid genes (J Clin Invest (1997) 99, 1947-1957).

In other embodiments, the methods of the present invention are useful for
5 treating tumors resistant to a therapeutic agent, in which resistance is due to other causes that lead to MDR, including, for example, changes in topoisomerase II, protein kinase C and specific glutathione transferase enzyme.

Therapeutic agents to which resistance is conferred via the action of P-gp include, but is not limited to: vinca alkaloids (e.g., vinblastine), the anthracyclines
10 (e.g., adriamycin, doxorubicin), the epipodophyllotoxins (e.g., etoposide), taxanes (e.g., paclitaxel, docetaxel), antibiotics (e.g., actinomycin D and gramicidin D), antimicrotubule drugs (e.g., colchicine), protein synthesis inhibitors (e.g., puromycin), toxic peptides (e.g., valinomycin), topoisomerase Inhibitors (e.g., topotecan), DNA intercalators (e.g., ethidium bromide) and anti-mitotics. See WO
15 99/20791. The methods and pharmaceutical compositions of the present invention are useful for treating tumors resistant to any one or more of above-listed drugs.

c. Resistance mediated through topoisomerase I

In a further embodiment, the resistance of tumor cells to a therapeutic agent
20 is mediated through topoisomerase. Exemplary therapeutic agents that belong to this category include those that target topoisomerase, either directly or indirectly.

DNA normally exists as a supercoiled double helix. During replication, it unwinds, with single strands serving as a template for synthesis of new strands. To relieve the torsional stress that develops ahead of the replication fork, transient
25 cleavage of one or both strands of DNA is needed. Without wishing to be bound to any mechanism, it is believed that Topoisomerases facilitate this process as follows: Topoisomerase II causes transient double-stranded breaks, whereas topoisomerase I causes single-strand breaks. This action allows for rotation of the broken strand around the intact strand. Topoisomerase I then re-ligates the broken strand to restore
30 integrity of double-stranded DNA.

In one embodiment, resistance of tumor cells to a therapeutic agent is mediated through topoisomerase. By “mediated through topoisomerase”, it is meant to include direct and indirect involvement of topoisomerase. For example, resistance may arise due to topoisomerase mutation, a direct involvement of topoisomerase in the resistance. Alternatively, resistance may arise due to alterations elsewhere in the cell that affect topoisomerase. These alterations may be mutations in genes affecting the expression level or pattern of topoisomerase, or mutations in genes affecting topoisomerase function or activity in general. In preferred embodiments said topoisomerase is topoisomerase I. In other embodiments said topoisomerase is
10 Topoisomerase II.

Without being bound by theory, compounds that act on topoisomerase I bind to the topoisomerase I-DNA complex in a manner that prevents the relegation of DNA. Topoisomerase I initially covalently interacts with DNA. Topoisomerase I then cleaves a single strand of DNA and forms a covalent intermediate via a
15 phosphodiester linkage between tyrosine-273 of topoisomerase I and the 3'-phosphate group of the scissile strand of DNA. The intact strand of DNA is then passed through the break and then topoisomerase I religates the DNA and releases the complex. Drugs such as camptothecins bind to the covalent complex in a manner that prevents DNA relegation. The persistent DNA breaks induce apoptosis, likely
20 via collisions between these lesions and or replication or transcription complexes.

Preferred therapeutic agents to which resistance is mediated through topoisomerase I include camptothecin and its derivatives and analogues, such as 9-nitrocamptothecin (IDEC-132), exatecan (DX-8951f), rubitecan (9-nitrocamptothecin), lurtotecan (GI-147211C), the homocamptothecins such as
25 diflomotecan (BN-80915) and BN-80927, topotecan, NB-506, J107088, pyrazolo [1,5-a] indole derivatives, such as GS-5, lamellarin D, SN-38, 9-aminocamptothecin, ST1481 and karanitecin (BNP1350) and irinotecan (CPT-11). Other related camptothecins can be found in *The Camptothecins: Unfolding Their Anticancer Potential*, Annals of the New York Academy of Science, Volume 922 (ISBN 1-
30 57331-291-6).

Without wishing to be bound by any particular theory, it is believed that camptothecins inhibit topoisomerase I by blocking the rejoining step of the cleavage/religation reaction of topoisomerase I, resulting in accumulation of a covalent reaction intermediate, the cleavable complex. Specifically, topoisomerase I-mediated tumor resistance to therapeutic agents may be conferred via molecular changes in the topoisomerase I molecules. For example, molecular changes include mutations, such as point mutations, deletions or insertions, splice variants or other changes at the gene, message or protein level.

In particular embodiments, such molecular changes reside near the catalytic tyrosine residue at amino acid position 723. Residues at which such molecular changes may occur include but are not limited to amino acid positions 717, 722, 723, 725, 726, 727, 729, 736 and 737 (see *Oncogene* (2003) 22, 7296-7304 for a review).

In equally preferred embodiments, such molecular changes reside between amino acids 361 and 364. Residues at which such molecular changes may occur include but are not limited to amino acid positions 361, 363 and 364.

In other equally preferred embodiments, such molecular changes reside near amino acid 533. Residues at which such molecular changes may occur include but are not limited to amino acid positions 503 and 533.

In other equally preferred embodiments, such molecular changes may also reside in other amino acids of the topoisomerase I protein. Residues at which such molecular changes may occur include but are not limited to amino acid positions 418 and 503.

In other embodiments, such molecular changes may be a duplication. In one embodiment such a duplication may reside in the nucleotides corresponding to amino acids 20-609 of the topoisomerase I protein.

In other embodiments, topoisomerase I-mediated tumor resistance may also be conferred via cellular proteins that interact with topoisomerase-1. Proteins that are able to do so include, but are not limited to, nucleolin.

In particular embodiments, such molecular changes may reside in amino acids 370 and/or 723. For example, and without wishing to be limited, the camptothecin-resistant human leukemia cell line CEM/C2 (ATCC No. CRL-2264)

carries two amino acid substitution at positions 370 (Met→Thr) and 722 (Asn→Ser) (Cancer Res (1995) 55, 1339-1346). The camptothecin resistant CEM/C2 cells were derived from the T lymphoblastoid leukemia cell line CCRF/CEM by selection in the presence of camptothecin *in vitro* (Kapoor *et al.*, 1995. Oncology Research 7; 83-95, ATCC). The CEM/C2 resistant cells display atypical multi-drug resistance and express a form of topoisomerase I that is less sensitive to the inhibitory action of camptothecin than that from CCRF/CEM cells at a reduced level relative to the parental cells. In addition to resistance to camptothecin, the CEM/C2 cells exhibit cross resistance to etoposide, dactinomycin, bleomycin, mitoxantrone, daunorubicin, doxorubicin and 4'-(9-acridinylamino)methanesulfon-m-anisidide.

In other embodiments, topoisomerase I-mediated tumor resistance to therapeutic agents may also be conferred via alterations of the expression pattern the topoisomerase I gene (Oncol Res (1995) 7, 83-95). In further embodiments, topoisomerase I-mediated tumor resistance may also be conferred via altered metabolism of the drug. In yet further embodiments, topoisomerase I-mediated tumor resistance may also be conferred via inadequate and/or reduced accumulation of drug in the tumor, alterations in the structure or location of topoisomerase 1, alterations in the cellular response to the topoisomerase I-drug interaction or alterations in the cellular response to drug-DNA-ternary complex formation (Oncogene (2003) 22, 7296-7304; Ann N Y Acad Sci (2000) 922, 46-55).

Topoisomerase I is believed to move rapidly from the nucleolus to the nucleus or even cytoplasm after cellular exposure to camptothecins.

In one embodiment topoisomerase I-mediated tumor resistance is mediated through factors involved in the relocation of topoisomerase I from the nucleolus to the nucleus and/or the cytoplasm, such as factors involved in the ubiquitin/26S proteasome pathway or SUMO.

In other embodiments topoisomerase I-mediated tumor resistance is mediated through factors involved in DNA replication, DNA checkpoint control and DNA repair.

Factors of the DNA checkpoint control include proteins of the S-checkpoint control, such as Chk1, ATR, ATM, and the DNA-PK multimer.

In other embodiments topoisomerase I-mediated tumor resistance is mediated via factors of apoptosis pathways or other cell death pathways. This includes, but is not limited to, the overexpression of bcl-2 and the overexpression of p21^{Waf1/Cip1}.

5 In other embodiments topoisomerase I-mediated tumor resistance is mediated via post-translational modifications of topoisomerase I. Such post-translational modifications are ubiquitination and sumoylation. Furthermore, such post-translational modifications may involve other cellular proteins, such as Ubp11, DOA4 and topor.

10 Therapeutic agents to which resistance is mediated through topoisomerase II include epipodophyllotoxins, such as VP16 and VM26, [1,5-a], pyrazolo [1,5-a] indole derivatives, such as GS-2, GS-3, GS-4 and GS-5.

d. Resistance to Mitoxanthrone

15 In another embodiment, tumor cells resistant to Mitoxanthrone can be treated using the subject compounds.

Resistance to the anticancer drug mitoxanthrone has been associated with several mechanisms, including drug accumulation defects and reduction in its target proteins topoisomerase II α and β 4. Recently, overexpression of the breast cancer
20 resistance half transporter protein (BCRP1) was found to be responsible for the occurrence of mitoxanthrone resistance in a number of cell lines (Ross et al., J. Natl. Cancer Inst. 91: 429-433, 1999; Miyake et al., Cancer Res. 59: 8-13, 1999; Litman et al., J. Cell Sci. 113(Pt 11): 2011-2021, 2000; Doyle et al., Proc. Natl. Acad. Sci. U.S.A. 95: 15665-15670, 1998). However, not all mitoxanthrone resistant cell lines
25 express BCRP1 (Hazlehurst et al., Cancer Res. 59: 1021-1028, 1999; Nielsen et al., Biochem. Pharmacol. 60: 363-370, 2000). The efflux pump responsible for the mitoxanthrone resistance in these cell lines is less clear. Boonstra et al. (Br J Cancer. 2004 May 18 [Epub ahead of print]) report that overexpression of the ABC transporter ABCA2 may lead to the efflux of mitoxanthrone by exploring

estramustine, which is able to block mitoxantrone efflux in the mitoxantrone resistant GLC4 sub line GLC4-MITO (does not overexpress BCRP1).

The methods of the present invention are useful for treating tumors resistant to a mitoxanthrone.

5

D. Other treatment methods

In yet other embodiments, the subject method combines a Na^+/K^+ -ATPase inhibitor (e.g. cardiac glycoside) with radiation therapies, including ionizing radiation, gamma radiation, or particle beams.

10

E. Administration

The Na^+/K^+ -ATPase inhibitor (e.g. cardiac glycoside), or a combination containing a Na^+/K^+ -ATPase inhibitor (e.g. cardiac glycoside) may be administered orally, parenterally by intravenous injection, transdermally, by pulmonary
15 inhalation, by intravaginal or intrarectal insertion, by subcutaneous implantation, intramuscular injection or by injection directly into an affected tissue, as for example by injection into a tumor site. In some instances the materials may be applied topically at the time surgery is carried out. In another instance the topical administration may be ophthalmic, with direct application of the therapeutic
20 composition to the eye.

In a preferred embodiment, the subject Na^+/K^+ -ATPase inhibitors (e.g. cardiac glycosides) are administered to a patient by using osmotic pumps, such as Alzet[®] Model 2002 osmotic pump. Osmotic pumps provides continuous delivery of test agents, thereby eliminating the need for frequent, round-the-clock injections.
25 With sizes small enough even for use in mice or young rats, these implantable pumps have proven invaluable in predictably sustaining compounds at therapeutic levels, avoiding potentially toxic or misleading side effects.

To meet different therapeutic needs, ALZET's osmotic pumps are available in a variety of sizes, pumping rates, and durations. At present, at least ten different
30 pump models are available in three sizes (corresponding to reservoir volumes of 100

μL, 200 μL and 2 mL) with delivery rates between 0.25 μL/hr and 10 μL/hr and durations between one day to four weeks.

While the pumping rate of each commercial model is fixed at manufacture, the dose of agent delivered can be adjusted by varying the concentration of agent with which each pump is filled. Provided that the animal is of sufficient size, multiple pumps may be implanted simultaneously to achieve higher delivery rates than are attainable with a single pump. For more prolonged delivery, pumps may be serially implanted with no ill effects. Alternatively, larger pumps for larger patients, including human and other non-human mammals may be custom manufactured by scaling up the smaller models.

The materials are formulated to suit the desired route of administration. The formulation may comprise suitable excipients include pharmaceutically acceptable buffers, stabilizers, local anesthetics, and the like that are well known in the art. For parenteral administration, an exemplary formulation may be a sterile solution or suspension; For oral dosage, a syrup, tablet or palatable solution; for topical application, a lotion, cream, spray or ointment; for administration by inhalation, a microcrystalline powder or a solution suitable for nebulization; for intravaginal or intrarectal administration, pessaries, suppositories, creams or foams. Preferably, the route of administration is parenteral, more preferably intravenous.

EXEMPLIFICATION

The following examples are for illustrative purpose only, and should in no way be construed to be limiting in any respect of the claimed invention.

The exemplary cardiac glycosides used in following studies are referred to as BNC1 and BNC4.

BNC1 is ouabain or g-Strophanthin (STRODIVAL[®]), which has been used for treating myocardial infarction. It is a colorless crystal with predicted IC₅₀ of about 0.009-0.35 μg/mL and max. plasma concentration of about 0.03 μg/mL. According to the literature, its plasma half-life in human is about 20 hours, with a

range of between 5-50 hours. Its common formulation is injectable. The typical dose for current indication (i.v.) is about 0.25 mg, up to 0.5 mg /day.

BNC4 is proscillaridin (TALUSIN[®]), which has been approved for treating chronic cardiac insufficiency in Europe. It is a colorless crystal with predicted IC₅₀ of about 0.002-0.008 µg/mL and max. plasma concentration of about 0.001 µg/mL. According to the literature, its plasma half-life in human is about 40 hours. Its common available formulation is a tablet of 0.25 or 0.5 mg. The typical dose for current indication (p.o.) is about 1.5 mg /day.

10

Example I. Sentinel Line Plasmid Construction and Virus Preparation

Figure 1 is a schematic drawing of the Sentinel Line promoter trap system, and its use in identifying regulated genetic sites and in reporting pathway activity. Briefly, the promoter-less selection markers (either positive or negative selection markers, or both) and reporter genes (such as beta-gal) are put in a retroviral vector (or other suitable vectors), which can be used to infect target cells. The randomly inserted retroviral vectors may be so positioned that an active upstream heterologous promoter may initiate the transcription and translation of the selectable markers and reporter gene(s). The expression of such selectable markers and/or reporter genes is indicative of active genetic sites in the particular host cell.

In one exemplary embodiment, the promoter trap vector BV7 was derived from retrovirus vector pQCXIX (BD Biosciences Clontech) by replacing sequence in between packaging signal (Psi⁺) and 3' LTR with a cassette in an opposite orientation, which contains a splice acceptor sequence derived from mouse engrailed 2 gene (SA/en2), an internal ribosomal entry site (IRES), a LacZ gene, a second IRES, and fusion gene TK:Sh encoding herpes virus thymidine kinase (HSV-tk) and phleomycin followed by a SV40 polyadenylation site. BV7 was constructed by a three-way ligation of three equal molar DNA fragments. Fragment 1 was a 5 kb vector backbone derived from pQCXIX by cutting plasmid DNA extracted from a Dam⁻ bacterial strain with Xho I and Cla I (Dam⁻ bacterial strain was needed here

30

because Cla I is blocked by overlapping Dam methylation). Fragment 2 was a 2.5 kb fragment containing an IRES and a TK:Sh fusion gene derived from plasmid pIREStksh by cutting Dam⁻ plasmid DNA with Cla I and Mlu I. pIREStksh was constructed by cloning TK:Sh fragment from pMODtksh (InvivoGen) into pIRES
5 (BD Biosciences Clontech). Fragment 3 was a 5.8 kb SA/en2-IRES-LacZ fragment derived from plasmid pBSen2IRESLacZ by cutting with BssH II (compatible end to Mlu I) and Xho I. pBSen2IRESLacZ was constructed by cloning IRES fragment from pIRES and LacZ fragment from pMODLacZ (InvivoGen) into plasmid pBSen2.

10 To prepare virus, packaging cell line 293T was co-transfected with three plasmids BV7, pVSV-G (BD Biosciences Clontech) and pGag-Pol (BD Biosciences Clontech) in equal molar concentrations by using Lipofectamine 2000 (InvitroGen) according to manufacturer's protocol. First virus "soup" (supernatant) was collected 48 hours after transfection, second virus "soup" was collected 24 hours later. Virus
15 particles were pelleted by centrifuging at 25,000 rpm for 2 hours at 4°C. Virus pellets were re-dissolved into DMEM/10% FBS by shaking overnight. Concentrated virus solution was aliquot and used freshly or frozen at -80 °C.

Example II. Sentinel Line Generation

20 Target cells were plated in 150 mm tissue culture dishes at a density of about 1×10^6 / plate. The following morning cells were infected with 250 µl of Bionaut Virus #7 (BV7) as prepared in Example I, and after 48 hr incubation, 20 µg/ml of phleomycin was added. 4 days later, media was changed to a reduced serum (2%FBS) DMEM to allow the cells to rest. 48h later, ganciclovir (GCV) (0.4µM,
25 sigma) was added for 4 days (media was refreshed on day 2). One more round of phleomycin selection followed (20 µg/ml phleomycin for 3 days). Upon completion, media was changed to 20%FBS DMEM to facilitate the outgrowths of the clones. 10 days later, clones were picked and expanded for further analysis and screening.

30 Using this method, several Sentinel Lines were generated to report activity of genetic sites activated by hypoxia pathways (Figure 4). These Sentinel lines were

generated by transfecting A549 (NSCLC lung cancer) and Panc-1 (pancreatic cancer) cell lines with the subject gene-trap vectors containing *E. coli* LacZ-encoded β -galactosidase (β -gal) as the reporter gene (Figure 4). The β -gal activity in Sentinel Lines (green) was measured by flow cytometry using a fluorogenic substrate fluorescein di-beta-D-galactopyranoside (FDG). The autofluorescence of untransfected control cells is shown in purple. The graphs indicate frequency of cells (y-axis) and intensity of fluorescence (x-axis) in log scale. The bar charts on the right depict median fluorescent units of the FACS curves. They indicate a high level of reporter activity at the targeted site.

10

Example III. Cell Culture and Hypoxic Conditions

All cell lines can be purchased from ATCC, or obtained from other sources.

A549 (CCL-185) and Panc-1 (CRL-1469) were cultured in Dulbecco's Modified Eagle's Medium (DMEM), Caki-1 (HTB-46) in McCoy's 5a modified medium, Hep3B (HB-8064) in MEM-Eagle medium in humidified atmosphere containing 5% CO₂ at 37°C. Media was supplemented with 10% FBS (Hyclone; SH30070.03), 100 µg/ml penicillin and 50 µg/ml streptomycin (Hyclone).

To induce hypoxia conditions, cells were placed in a Billups-Rothenberg modular incubator chamber and flushed with artificial atmosphere gas mixture (5% CO₂, 1% O₂, and balance N₂). The hypoxia chamber was then placed in a 37°C incubator. L-mimosine (Sigma, M-0253) was used to induce hypoxia-like HIF1- α expression. Proteasome inhibitor, MG132 (Calbiochem, 474791), was used to protect the degradation of HIF1- α . Cycloheximide (Sigma, 4859) was used to inhibit new protein synthesis of HIF1- α . Catalase (Sigma, C3515) was used to inhibit reactive oxygen species (ROS) production.

25

Example IV. Identification of Trapped Genes

Once a Sentinel Line with a desired characteristics was established, it might be helpful to determine the active promoter under which control the markers / reporter genes are expressed. To do so, total RNAs were extracted from cultured

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Sentinel Line cells by using, for example, RNA-Bee RNA Isolation Reagent (TEL-TEST, Inc.) according to the manufacturer's instructions. Five prime ends of the genes that were disrupted by the trap vector BV7 were amplified by using BD SMART RACE cDNA Amplification Kit (BD Biosciences Clontech) according to the manufacturer's protocol. Briefly, 1 µg total RNA prepared above was reverse-transcribed and extended by using BD PowerScriptase with 5' CDS primer and BD SMART II Oligo both provided by the kit. PCR amplification were carried out by using BD Advantage 2 Polymerase Mix with Universal Primer A Mix provided by the kit and BV7 specific primer 5'Rsa/ires (gacgcggatcttcgggtaccgagctcc, 28 mer).

5'Rsa/ires located in the junction of SA/en2 and IRES with the first 7 nucleotides matching the last 7 nucleotides of SA/en2 in complementary strand. 5' RACE products were cloned into the TA cloning vector pCR2.1 (InvitroGen) and sequenced. The sequences of the RACE products were analyzed by using the BLAST program to search for homologous sequences in the database of GenBank.

Only those hits which contained the transcript part of SA/en2 were considered as trapped genes.

Using this method, the upstream promoters of several Sentinel Lines generated in Example II were identified (see below). The identity of these trapped genes validate the clinical relevance of these Sentinel Lines™, and can be used as biomarkers and surrogate endpoints in clinical trials.

Sentinel Lines	Genetic Sites	Gene Profile
A7N1C1	Essential Antioxidant	Tumor cell-specific gene, over expressed in lung tumor cells
A7N1C6	Chr. 3, BAC, map to 3p	novel
A7I1C1	Pyruvate Kinase (PKM 2), Chr. 15	Described biomarker for NSCLC
A6E2A4	6q14.2-16.1	Potent angiogenic activity
A7I1D1	Chr. 7, BAC	novel

Example V. Western Blots

For HIF1-alpha Western blots, Hep3B cells were seeded in growth medium at a density of 7×10^6 cells per 100 mm dish. Following 24-hour incubation, cells
5 were subjected to hypoxic conditions for 4 hours to induce HIF1-alpha expression together with an agent such as 1 μ M BNC1. The cells were harvested and lysed using the Mammalian Cell Lysis kit (Sigma, M-0253). The lysates were centrifuged to clear insoluble debris, and total protein contents were analyzed with BCA protein assay kit (Pierce, 23225). Samples were fractionated on 3-8% Tris-Acetate gel
10 (Invitrogen NUPAGE system) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. HIF1-alpha protein was detected with anti-HIF1-alpha monoclonal antibody (BD Transduction Lab, 610959) at a 1:500 dilution with an overnight incubation at 4°C in Tris-buffered solution-0.1% Tween 20 (TBST) containing 5% dry non-fat milk. Anti-Beta-actin
15 monoclonal antibody (Abcam, ab6276-100) was used at a 1:5000 dilution with a 30-minute incubation at room temperature. Immunoreactive proteins were detected with stabilized goat-anti mouse HRP conjugated antibody (Pierce, 1858413) at a 1:10,000 dilution. The signal was developed using the West Femto substrate (Pierce, 34095).

We examined the inhibitory effect of BNC1 on HIF-1 alpha synthesis. 24
20 hours prior to treatment, Hep3B cells were seeded in growth medium. To show that BNC1 inhibits HIF1-alpha expression in a concentration dependent manner, cells were treated with 1 μ M BNC1 together with the indicated amount of MG132 under hypoxic conditions for 4 hours. To understand specifically the impact of BNC1 on HIF-1 alpha synthesis, Hep3B cells were treated with MG132 and 1 μ M BNC under
25 normoxic conditions for the indicated time points. The observed expression is accounted by protein synthesis.

We examined the role of BNC1 on the degradation rate of HIF-1 alpha. 24
hours prior to treatment, Hep3B cells were seeded in growth medium. The cells were placed in hypoxic conditions for 4 hours for HIF1-alpha accumulation. The protein

synthesis inhibitor, cycloheximide (100 μ M) together with 1 μ M BNC1 were added to the cells and kept in hypoxic conditions for the indicate time points.

To induce HIF1- α expression using an iron chelator, L-mimosine was added to Hep3B cells, seeded 24 hours prior, and placed under normoxic conditions
5 for 24 hours.

Example VI. Sentinel Line Reporter Assays

The expression level of beta-galactosidase gene in sentinel lines was determined by using a fluorescent substrate fluorescein di-B-D-Galactopyranside
10 (FDG, Marker Gene Tech, #M0250) introduced into cells by hypotonic shock. Cleavage by beta-galactosidase results in the production of free fluorescein, which is unable to cross the plasma membrane and is trapped inside the beta-gal positive cells. Briefly, the cells to be analyzed are trypsinized, and resuspended in PBS containing 2 mM FDG (diluted from a 10mM stock prepared in 8:1:1 mixture of
15 water: ethanol: DMSO). The cells were then shocked for 4 minutes at 37°C and transferred to FACS tubes containing cold 1 $\times$ PBS on ice. Samples were kept on ice for 30 minutes and analyzed by FACS in FL1 channel.

Example VII. Testing Standard Chemotherapeutic Agents

20 Sentinel Line cells with beta-galactosidase reporter gene were plated at 1 $\times$ 10⁵ cells / 10 cm dish. After overnight incubation, the cells were treated with standard chemotherapeutic agents, such as mitoxantrone (8 nM), paclitaxel (1.5 nM), carboplatin (15 μ M), gemcitabine (2.5 nM), in combination with one or more BNC compounds, such as BNC1 (10 nM), BNC2 (2 μ M), BNC3 (100 μ M) and
25 BNC4 (10 nM), or a targeted drug, Iressa (4 μ M). After 40 hrs, the cells were trypsinized and the expression level of reporter gene was determined by FDG loading.

When tested in the Sentinel Lines, mitoxanthrone, paclitaxel, and carboplatin each showed increases in cell death and reporter activity (see Figure 9). No effect
30 had been expected from the cytotoxic agents because of their nonspecific

mechanisms of action (MOA), making their increased reporter activity in HIF-sensitive cell lines surprising. These results provide a previously unexplored link between the development of chemotherapy resistance and induction of the hypoxia response in cells treated with anti-neoplastic agents. Iressa, on the other hand, a known blocker of EGFR-mediated HIF-1 induction, showed a reduction in reporter activity when tested. The Sentinel Lines thus provide a means to differentiate between a cytotoxic agent and a targeted drug.

Example VIII. Pharmacokinetic (PK) Analysis

The following protocol can be used to conduct pharmacokinetic analysis of any compounds of the invention. To illustrate, BNC1 is used as an example.

Nude mice were dosed *i.p.* with 1, 2, or 4 mg/kg of BNC1. Venous blood samples were collected by cardiac puncture at the following 8 time points: 5 min, 15 min, 30 min, 45 min, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr. For continuous BNC1 treatment, osmotic pumps (such as Alzet[®] Model 2002) were implanted *s.c.* between the shoulder blades of each mouse. Blood was collected at 24 hr, 48 hr and 72 hr. Triplicate samples per dose (i.e. three mice per time point per dose) were collected for this experiment.

Approximately 0.100 mL of plasma was collected from each mouse using lithium heparin as anticoagulant. The blood samples were processed for plasma as individual samples (no pooling). The samples were frozen at -70°C ($\pm 10^\circ\text{C}$) and transferred on dry ice for analysis by HPLC.

For PK analysis plasma concentrations for each compound at each dose were analyzed by non-compartmental analysis using the software program WinNonlin[®]. The area under the concentration vs time curve AUC (0-Tf) from time zero to the time of the final quantifiable sample (Tf) was calculated using the linear trapezoid method. AUC is the area under the plasma drug concentration-time curve and is used for the calculation of other PK parameters. The AUC was extrapolated to infinity (0-Inf) by dividing the last measured concentration by the terminal rate constant (k), which was calculated as the slope of the log-linear terminal portion of

the plasma concentrations curve using linear regression. The terminal phase half-life ($t_{1/2}$) was calculated as $0.693/k$ and systemic clearance (Cl) was calculated as the dose(mg/kg)/AUC(Inf). The volume of distribution at steady-state (V_{ss}) was calculated from the formula:

5

$$V_{ss} = dose(AUMC)/(AUC)^2$$

where $AUMC$ is the area under the first moment curve (concentration multiplied by time versus time plot) and AUC is the area under the concentration
10 versus time curve. The observed maximum plasma concentration (C_{max}) was obtained by inspection of the concentration curve, and T_{max} is the time at when the maximum concentration occurred.

Figure 11 shows the result of a representative pharmacokinetic analysis of BNC1 delivered by osmotic pumps. Osmotic pumps (Model 2002, Alzet Inc)
15 containing 200 μ l of BNC1 at 50, 30 or 20 mg/ml in 50% DMSO were implanted subcutaneously into nude mice. Mice were sacrificed after 24, 48 or 168 hrs, and plasma was extracted and analyzed for BNC1 by LC-MS. The values shown are average of 3 animals per point.

20 **Example IX. Human Tumor Xenograft Models**

Female nude mice (nu/nu) between 5 and 6 weeks of age weighing approximately 20 g were implanted subcutaneously (s.c.) by trocar with fragments of human tumors harvested from s.c. grown tumors in nude mice hosts. When the tumors were approximately 60-75 mg in size (about 10-15 days following
25 inoculation), the animals were pair-matched into treatment and control groups. Each group contains 8-10 mice, each of which was ear tagged and followed throughout the experiment.

The administration of drugs or controls began the day the animals were pair-matched (Day 1). Pumps (Alzet® Model 2002) with a flow rate of 0.5 μ l/hr were
30 implanted s.c. between the shoulder blades of each mice. Mice were weighed and

tumor measurements were obtained using calipers twice weekly, starting Day 1. These tumor measurements were converted to mg tumor weight by standard formula, $(W^2 \times L)/2$. The experiment is terminated when the control group tumor size reached an average of about 1 gram. Upon termination, the mice were weighed, sacrificed and their tumors excised. The tumors were weighed and the mean tumor weight per group was calculated. The change in mean treated tumor weight/the change in mean control tumor weight $\times 100$ (dT/dC) is subtracted from 100% to give the tumor growth inhibition (TGI) for each group.

10 **Example X. Cardiac Glycoside Compounds Inhibits HIF-1 α Expression**

Cardiac glycoside compounds of the invention targets and inhibits the expression of HIF 1 α based on Western Blot analysis using antibodies specific for HIF1 α (Figure 5).

Hep3B or A549 cells were cultured in complete growth medium for 24 hours and treated for 4 hrs with the indicated cardiac glycoside compounds or controls under normoxia (N) or hypoxia (H) conditions. The cells were lysed and proteins were resolved by SDS-PAGE and transferred to a nylon membrane. The membrane was immunoblotted with anti-HIF1 α and anti-HIF1 β MAb, and anti-beta-actin antibodies.

20 In Hep3B cells, various effective concentrations of BNC compounds (cardiac glycoside compounds of the invention) inhibits the expression of HIF-1 α , but not HIF-1 β . The basic observation is the same, with the exception of BNC2 at 1 μ M concentration.

To study the mechanism of HIF-1 α inhibition by the subject cardiac glycoside compounds, Hep3B cells were exposed to normoxia or hypoxia for 4 hrs in the presence or absence of: an antioxidant enzyme and reactive oxygen species (ROS) scavenger catalase (1000 U), prolyl-hydroxylase (PHD) inhibitor L-mimosine, or proteasome inhibitor MG132 as indicated. HIF1 α and β -actin protein level was determined by western blotting.

Figure 6 indicates that the cardiac glycoside compound BNC1 may inhibits steady state HIF-1 α level through inhibiting the synthesis of HIF-1 α .

In a related study, tumor cell line A549(ROS) were incubated in normoxia in the absence (control) or presence of different amounts of BNC1 for 4 hrs. Thirty
5 minutes prior to the termination of incubation period, 2,7-dichlorofluorescein diacetate (CFH-DA, 10 mM) was added to the cells and incubated for the last 30 min at 37°C. The ROS levels were determined by FACS analysis. HIF1 α protein accumulation in Caki-1 and Panc-1 cells was determined by western blotting after incubating the cells for 4 hrs in normoxia (21% O₂) or hypoxia (1% O₂) in the
10 presence or absence of BNC1. Figure 7 indicates that BNC1 induces ROS production (at least as evidenced by the A549(ROS) Sentinel Lines), and inhibits HIF1 α protein accumulation in the test cells.

Figure 8 also demonstrates that the cardiac glycoside compounds BNC1 and BNC4 directly or indirectly inhibits in tumor cells the secretion of the angiogenesis
15 factor VEGF, which is a downstream effector of HIF-1 α (see Figure 3). In contrast, other non-cardiac glycoside compounds, BNC2, BNC3 and BNC5, do not inhibit, and in fact greatly enhances VEGF secretion.

Figures 18 and 19 compared the ability of BNC1 and BNC4 in inhibiting hypoxia-mediated HIF1 α induction in human tumor cells. The figures show result of
20 immunoblotting for HIF-1 α , HIF-1 β and β -actin (control) expression, in Hep3B, Caki-1 or Panc-1 cells treated with BNC1 or BNC4 under hypoxia. The results indicate that BNC4 is even more potent (about 10-times more potent) than BNC1 in inhibiting HIF-1 α expression.

Thus, while not wishing to be bound by any particular theory, the ability of
25 the subject compounds to treat refractory cancer may be at least partially related to their ability to inhibit HIF-1 α expression.

Example XI. Neutralization of Gemcitabine-induced Stress Response as Measured in A549 Sentinel Line

The cardiac glycoside compounds of the invention were found to be able to neutralize Gemcitabine-induced stress response in tumor cells, as measured in A549
5 Sentinel Lines.

In experiments of Figure 10, the A549 sentinel line was incubated with Gemcitabine in the presence or absence of indicated Bionaut compounds (including the cardiac glycoside compound BNC4) for 40 hrs. The reporter activity was measured by FACS analysis.

10 It is evident that at least BNC4 can significantly shift the reporter activity to the left, such that Gemcitabine and BNC4-treated cells had the same reporter activity as that of the control cells. In contrast, cells treated with only Gemcitabine showed elevated reporter activity.

15 Example XII. Effect of BNC1 Alone or in Combination with Standard Chemotherapy on Growth of Xenografted Human Pancreatic Tumors in Nude Mice

To test the ability of BNC1 to inhibit xenographic tumor growth in nude mice, either along or in combination with a standard chemotherapeutic agent, such
20 as Gemcitabine, Panc-1 tumors were injected subcutaneously (sc) into the flanks of male nude mice. After the tumors reached 80 mg in size, osmotic pumps (model 2002, Alzet Inc., flow rate 0.5 μ l/hr) containing 20 mg/ml of BNC1 were implanted sc on the opposite sides of the mice. The control animals received pumps containing vehicle (50% DMSO in DMEM). The mice treated with standard chemotherapy
25 agent received intra-peritoneal injections of Gemcitabine at 40 mg/kg every 3 days for 4 treatments (q3d $\times$ 4). Each data point represent average tumor weight (n = 8) and error bars indicate SEM.

Figure 12 indicates that, at the dosage tested, BNC1 alone can significantly reduce tumor growth in this model. This anti-tumor activity is additive when BNC1
30 is co-administered with a standard chemotherapeutic agent Gemcitabine. Results of the experiment is listed below:

Group (Animal No.)	Dose / Route	Final weight (Mean)	Tumor Day 25 SEM	%TGI
Control (8)	Vehicle / i.v.	1120.2	161.7	-
BNC1 (8)	20 mg/ml; s.c.; C.I.	200	17.9	82.15
Gemcitabine (8)	40 mg/kg; q3d×4	701.3	72.9	37.40
BNC1 + Gem (8)	Combine both	140.8	21.1	87.43

Similarly, in the experiment of Figure 13, BNC1 (20 mg/ml) was delivered by sc osmotic pumps (model 2002, Alzet Inc.) at 0.5 μ l/hr throughout the study.

- 5 Cytoxan (q1d×1) was injected at 100 mg/kg (Cyt 100) or 300 mg/kg (Cyt 300). The results again shows that BNC1 is a potent anti-tumor agent when used alone, and its effect is additive with other chemotherapeutic agents such as Cytoxan. The result of this study is listed in the table below:

Group (Animal No.)	Dose / Route	Final weight (Mean)	Tumor Day 22 SEM	%TGI
Control (10)	Vehicle / i.v.	1697.6	255.8	-
BNC1 (10)	20 mg/ml; s.c.; C.I.	314.9	67.6	81.45
Cytosan300 (10)	300 mg/ml; ip; qd×1	93.7	24.2	94.48
Cytosan100 (10)	100 mg/ml; ip; qd×2	769	103.2	54.70
BNC1 + Cytosan100 (10)	Combine both	167	39.2	90.16

In yet another experiment, the anti-tumor activity of BNC1 alone or in combination with Carboplatin was tested in A549 human non-small-cell-lung carcinoma. In this experiment, BNC1 (20 mg/ml) was delivered by sc osmotic pumps (model 2002, Alzet Inc.) at 0.5 μ l/hr throughout the study. Carboplatin (q1d $\times$ 1) was injected at 100 mg/kg (Carb).

Figure 14 confirms that either BNC1 alone or in combination with Carboplatin has potent anti-tumor activity in this tumor model. The detailed results of the experiment is listed in the table below:

Group (Animal No.)	Dose / Route	% Weight Change at Day 38	Final Tumor weight Day 38 (Mean)	SEM	%TGI
Control (8)	Vehicle / i.v.	21%	842.6	278.1	-
BNC1 (8)	20 mg/ml; s.c.; C.I.	21%	0.0	0.0	100.00
Carboplatin (8)	100 mg/kg; ip; qd $\times$ 1	16%	509.75	90.3	39.50
BNC1 + Carb (8)	Combine both	0%	0.0	0.0	100.00

Notably, in both the BNC1 and BNC1 / Carb treatment group, none of the experimental animals showed any signs of tumor at the end of the experiment, while all 8 experimental animals in control and Carb only treatment groups developed tumors of significant sizes.

Thus the cardiac glycoside compounds of the invention (e.g. BNC1) either alone or in combination with many commonly used chemotherapeutic agents (e.g. Carboplatin, Gem, Cytosan, etc.) has potent anti-tumor activities in various xenographic animal models of pancreatic cancer, renal cancer, hepatic, and non-small cell lung carcinoma.

Example XIII. Determining Minimum Effective Dose

Given the additive effect of the subject cardiac glycosides with the traditional chemotherapeutic agents, it is desirable to explore the minimal effective doses of the subject cardiac glycosides for use in conjoint therapy with the other anti-tumor agents.

Figure 15 shows the titration of the exemplary cardiac glycoside BNC1 to determine its minimum effective dose, effective against Panc-1 human pancreatic xenograft in nude mice. BNC1 (sc, osmotic pumps) was first tested at 10, 5 and 2 mg/ml. Gem was also included in the experiment as a comparison.

Figure 16 shows that combination therapy using both Gem and BNC1 produces a combination effect, such that sub-optimal doses of both Gem and BNC1, when used together, produce the maximal effect only achieved by higher doses of individual agents alone.

A similar experiment was conducted using BNC1 and 5-FU, and the same combination effect was seen (see Figure 17).

Similar results are also obtained for other compounds (e.g. BNC2) that are not cardiac glycoside compounds (data not shown).

Example XIV. BNC4 Inhibits HIF-1 α Induced under Normoxia by PHD Inhibitor

As an attempt to study the mechanism of BNC4 inhibition of HIF-1 α , we tested the ability of BNC1 and BNC4 to inhibit HIF-1 α expression induced by a PHD inhibitor, L-mimosone (see Figure 6), under normoxia condition.

In the experiment represented in Figure 20, Hep3B cells were grown under normoxia, but were also treated as indicated with 200 μ M L-mimosone for 18h in the presence or absence of BNC1 or BNC4. Abundance of HIF1 α and β -actin was determined by western blotting.

The results indicate that L-mimosone induced HIF-1 α accumulation under normoxia condition, and addition of BNC4 eliminated HIF-1 α accumulation by L-

mimosone. At the low concentration tested, BNC1 did not appear to have an effect on HIF-1 α accumulation in this experiment. While not wishing to be bound by any particular theory, the fact that BNC4 can inhibit HIF-1 α induced under normoxia by PHD inhibitor indicates that the site of action by BNC4 probably lies down stream
5 of prolyl-hydroxylation.

All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

10

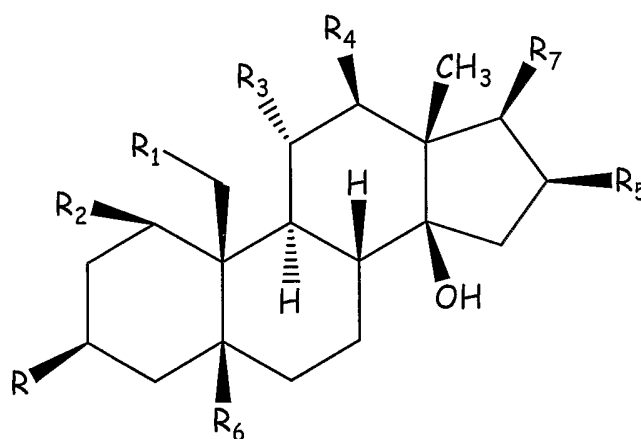
EQUIVALENTS:

While specific embodiments of the subject inventions are explicitly disclosed herein, the above specification is illustrative and not restrictive. Many variations of the inventions will become apparent to those skilled in the art upon review of this
15 specification and the claims below. The full scope of the inventions should be determined by reference to the claims, along with their full scope of equivalents, and the specification, along with such variations.

CLAIMS:

1. A method of inhibiting the growth or spread of a refractory cancer in an individual, comprising administering to the individual an effective amount of a Na^+/K^+ -ATPase inhibitor.
- 5 2. A method for promoting treatment of an individual suffering from a refractory cancer, comprising packaging, labeling and/or marketing a Na^+/K^+ -ATPase inhibitor to be used as part of a treatment for inhibiting the growth or spread of the refractory cancer.
- 3 A method of treating multidrug resistance of refractory tumor cells in a
10 refractory cancer patient in need of such treatment, said method comprising administering, concurrently or sequentially, an effective amount of a Na^+/K^+ -ATPase inhibitor and an antineoplastic agent to said patient.
4. The method of claim 1, wherein the cancer is refractory to radiation therapy.
5. The method of claim 1, wherein the cancer is refractory to anti-cancer
15 chemotherapy.
6. The method of claim 1, wherein the refractory cancer is a solid tumor.
7. The method of claim 6, wherein the solid tumor is a tumor in the pancreas, lung, kidney, ovarian, breast, prostate, gastric, colon, bladder, prostate, brain, skin, testicles, cervix, or liver.
- 20 8. The method of claim 7, wherein the solid tumor is a pancreatic tumor refractory to treatment by one or more of: fluorouracil, carmustine (BCNU), temozolomide (TMZ), streptozotocin, and gemcitabine.
9. The method of claim 7, wherein the solid tumor is a lung tumor refractory to etoposide or platinum-based therapy.
- 25 10. The method of claim 9, wherein the lung tumor is refractory small cell lung cancer.
11. The method of claim 9, wherein the lung tumor is refractory non-small cell lung cancer.

12. The method of claim 1, wherein the refractory cancer is a hematological cancer.
13. The method of claim 1, wherein the Na^+/K^+ -ATPase inhibitor is a cardiac glycoside.
- 5 14. The method of claim 13, wherein the cardiac glycoside has an IC_{50} for killing one or more different cancer cell lines of 500 nM or less.
15. The method of claim 13, wherein the cardiac glycoside is represented by the general formula:

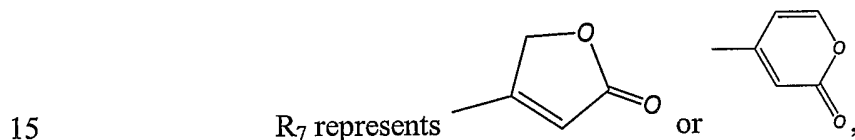


10 wherein

R represents a glycoside of 1 to 6 sugar residues;

R_1 represents hydrogen, $-\text{OH}$ or $=\text{O}$;

R_2 , R_3 , R_4 , R_5 , and R_6 each independently represents hydrogen or $-\text{OH}$; and



which cardiac glycoside has an IC_{50} for killing one or more different cancer cell lines of 500 nM or less.

16. The method of claim 13, wherein the cardiac glycoside comprises a steroid core with either a pyrone substituent at C17 (the “bufadienolides form”) or a butyrolactone substituent at C17 (the “cardenolide” form).
- 20

17. The method of claim 13, wherein the cardiac glycoside is ouabain or proscillaridin.
18. The method of claim 13, wherein the cardiac glycoside is conjointly administered with an effective amount of one or more anti-tumor agents selected from the group consisting of: an EGF-receptor antagonist, and arsenic sulfide, adriamycin, cisplatin, carboplatin, cimetidine, carminomycin, mechlorethamine hydrochloride, pentamethylmelamine, thiotepa, teniposide, cyclophosphamide, chlorambucil, demethoxyhypocrellin A, melphalan, ifosfamide, trofosfamide, Treosulfan, podophyllotoxin or podophyllotoxin derivatives, etoposide phosphate, teniposide, etoposide, leurosine, leurosine, vindesine, 9-aminocamptothecin, camptothecin, crisnatol, Chloroambucil, megestrol, methopterin, mitomycin C, ecteinascidin 743, busulfan, carmustine (BCNU), lomustine (CCNU), lovastatin, 1-methyl-4-phenylpyridinium ion, semustine, staurosporine, streptozocin, thiotepa, phthalocyanine, dacarbazine, aminopterin, methotrexate, trimetrexate, thioguanine, mercaptopurine, fludarabine, pentastatin, cladribin, cytarabine (ara C), porfiromycin, 5-fluorouracil, 6-mercaptopurine, doxorubicin hydrochloride, leucovorin, mycophenolol acid, daunorubicin, deferoxamine, floxuridine, doxifluridine, ratitrexed, idarubicin, epirubicin, pirarubicin, zorubicin, mitoxantrone, bleomycin sulfate, mitomycin C, actinomycin D, safracins, saframycins, quinocarcins, discodermolides, vincristine, vinblastine, vinorelbine tartrate, vertoporphin, paclitaxel, tamoxifen, raloxifene, tiazofuran, thioguanine, ribavirin, EICAR, estramustine, estramustine phosphate sodium, flutamide, bicalutamide, buserelin, leuprolide, pteridines, diyneses, levamisole, aflacon, interferon, interleukins, aldesleukin, filgrastim, sargramostim, rituximab, BCG, tretinoin, irinotecan hydrochloride, betamethosone, gemcitabine hydrochloride, verapamil, VP-16, altretamine, thapsigargin, and topotecan.
19. The method of claim 13, wherein the resistance of the refractory cancer to a therapeutic agent is mediated through tubulin.

20. The method of claim 13, wherein the resistance of the refractory cancer to a therapeutic agent is mediated through multidrug resistance.
21. The method of claim 20, wherein the multidrug resistance is caused by increased expression of ATP-binding cassette (ABC) transporters;
5 overexpression of P-gp; or changes in topoisomerase II, protein kinase C or specific glutathione transferase enzymes.
22. The method of claim 13, wherein the resistance of the refractory cancer to a therapeutic agent is mediated through topoisomerase.
23. The method of claim 13, wherein the resistance of the refractory cancer to a
10 therapeutic agent is mediated through Mitoxantrone.
24. A packaged pharmaceutical comprising a Na^+/K^+ -ATPase inhibitor formulated in a pharmaceutically acceptable excipient and suitable for use in humans, and a label or instructions for administering the Na^+/K^+ -ATPase inhibitor as part of a treatment for inhibiting the growth or spread of a
15 refractory cancer.

Figure 1

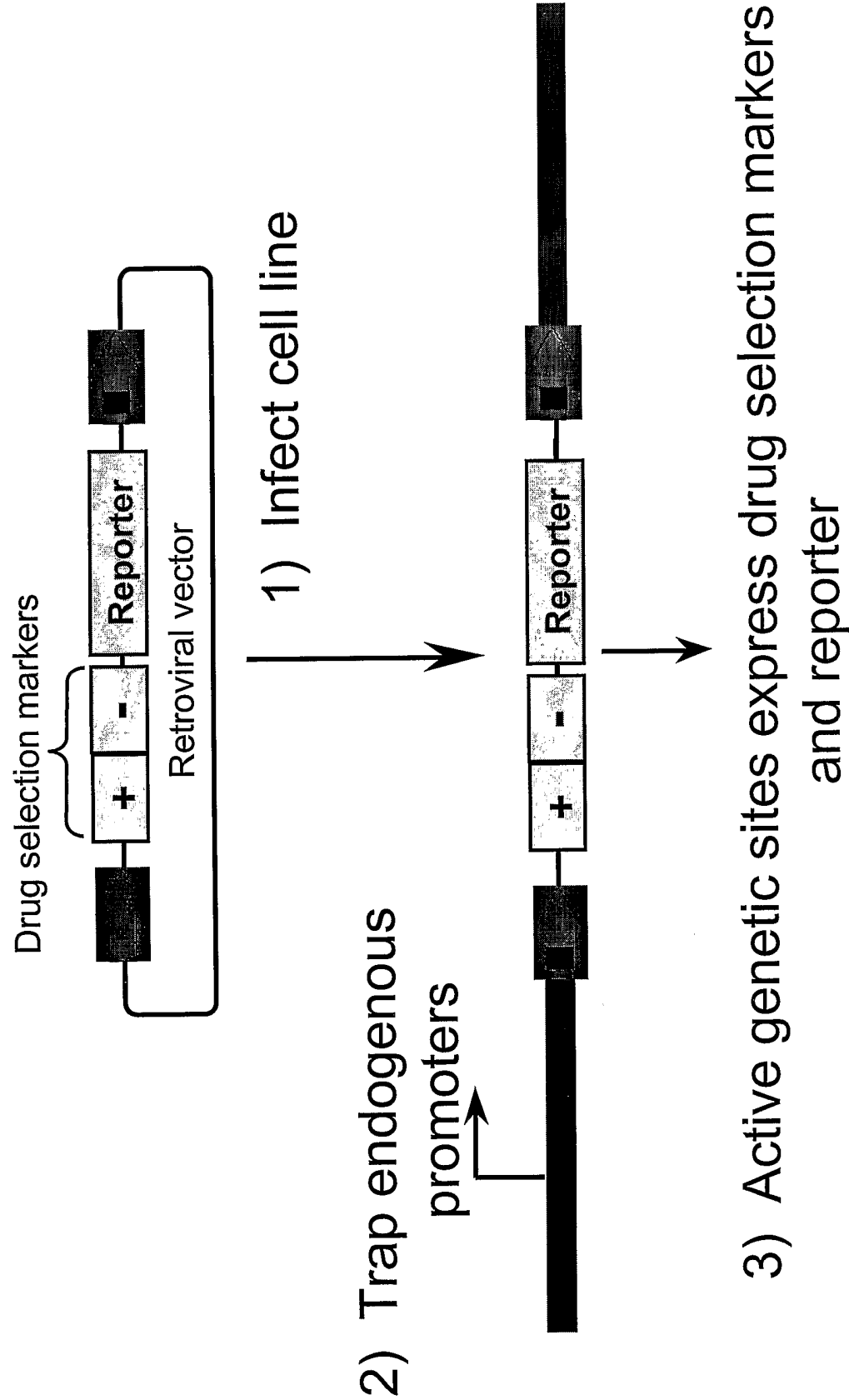


Figure 2

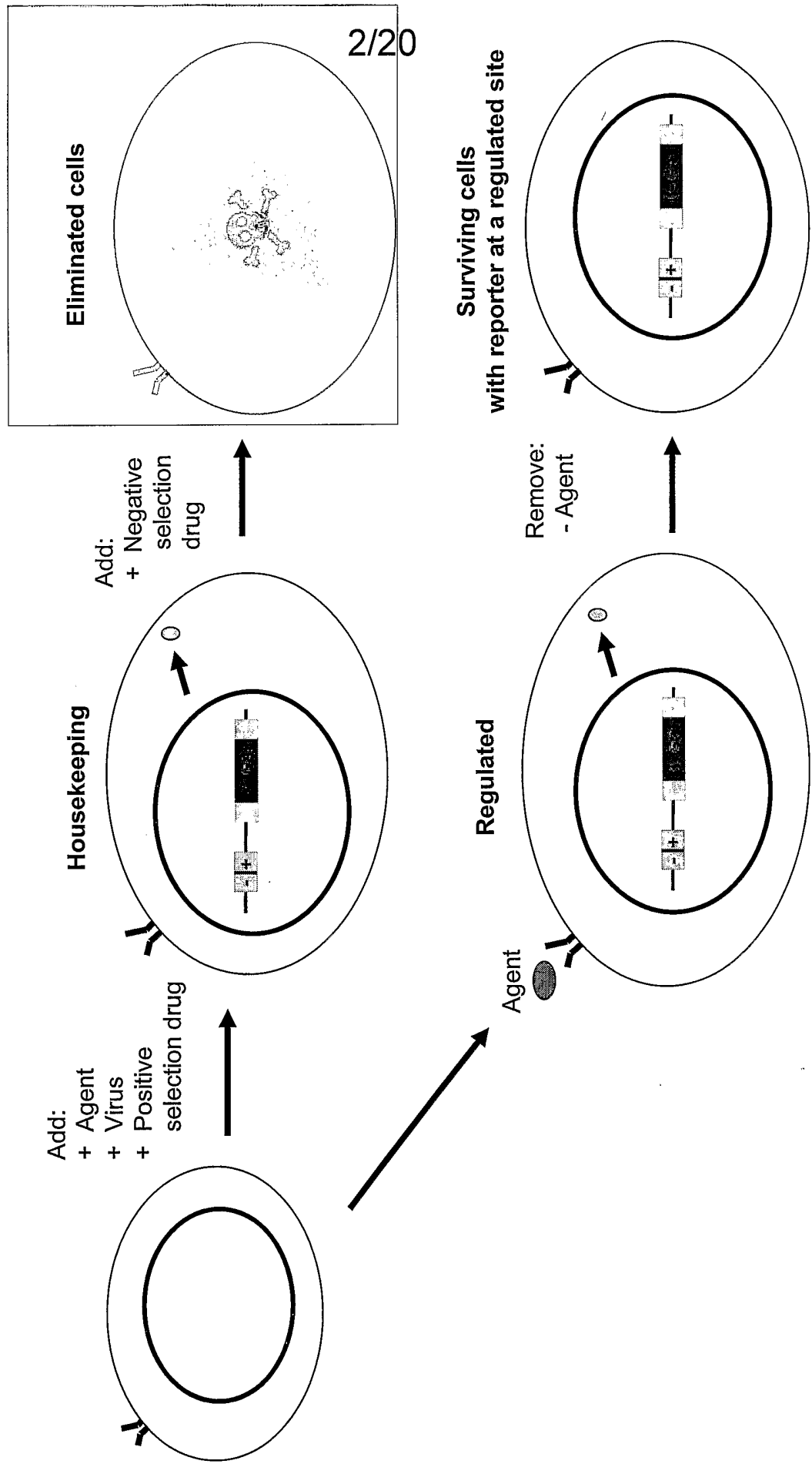
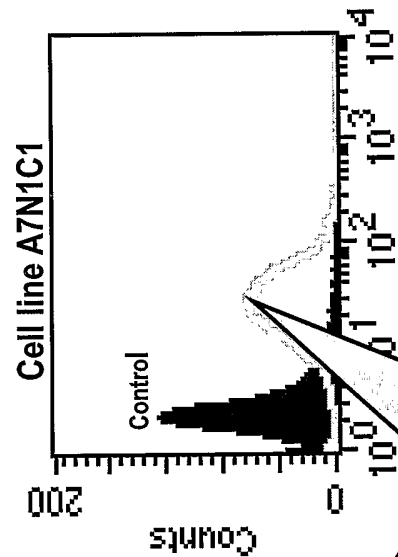
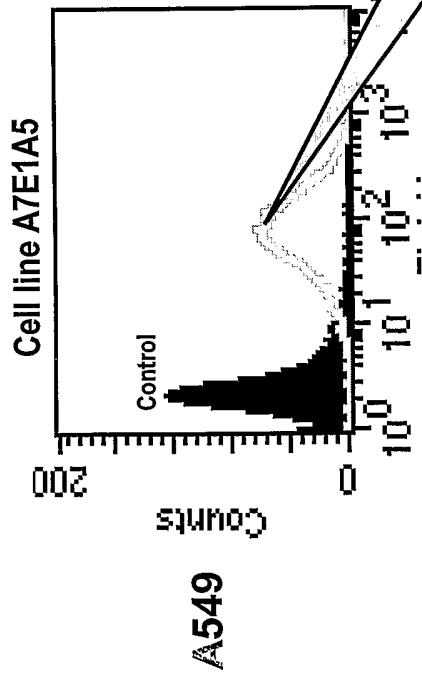
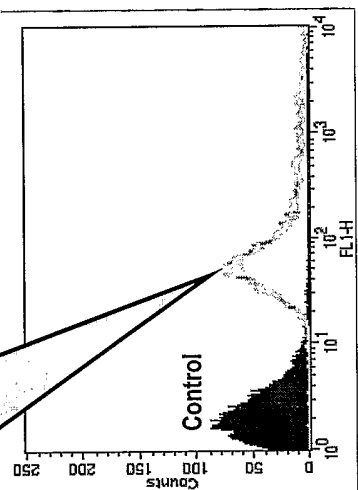
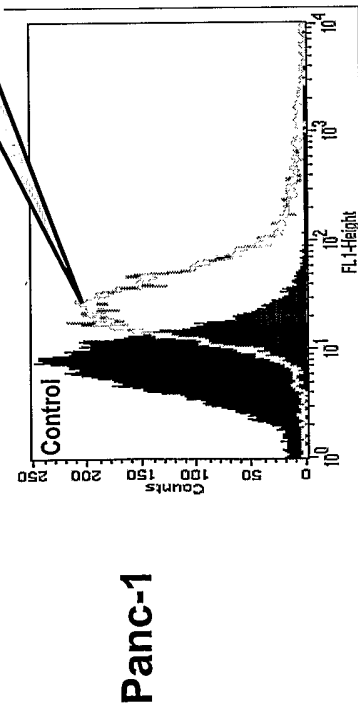


Figure 4

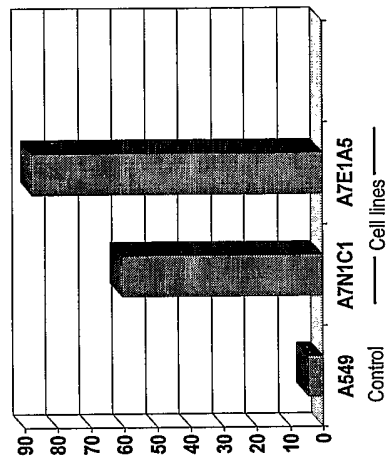
NSCLC Lung Cancer



Pancreatic Cancer

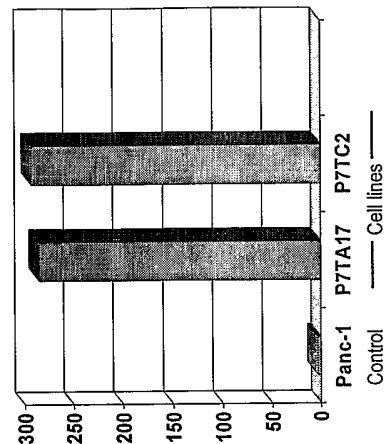


Median FLI



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Median FLI



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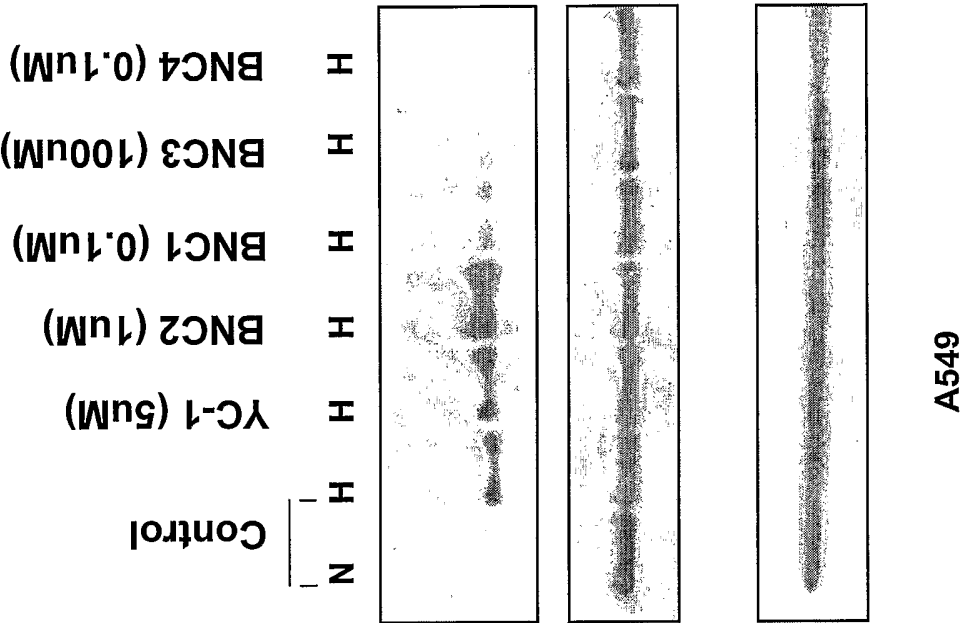
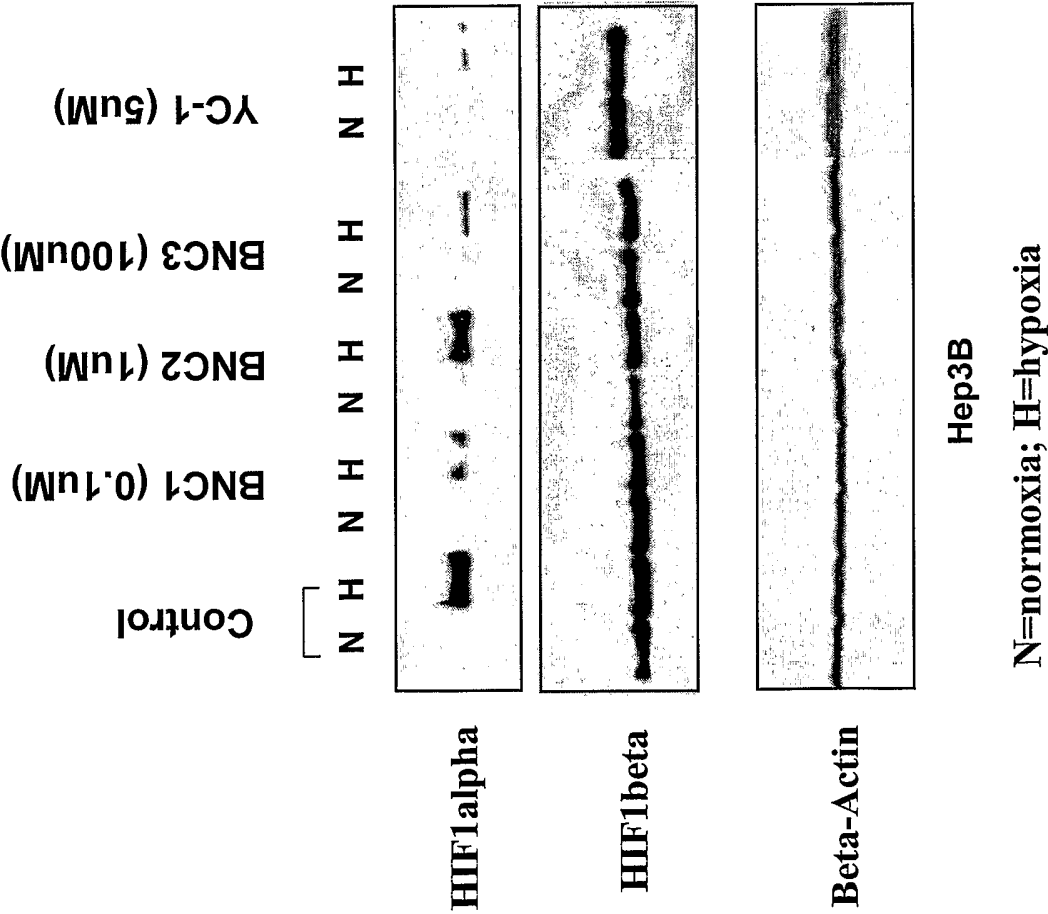
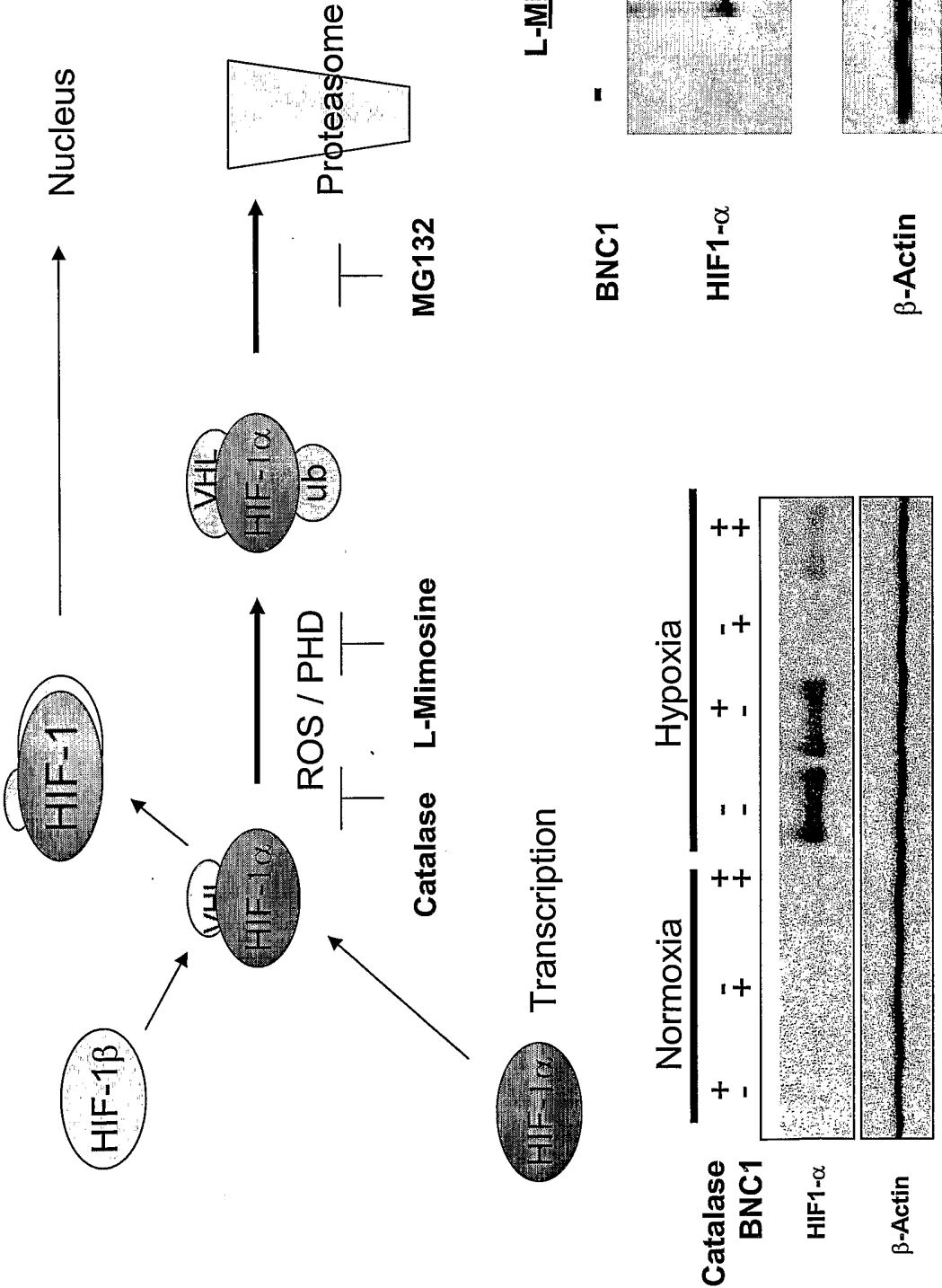


Figure 5



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Figure 6



Normoxia

Hypoxia

Catalase

BNC1

HIF1-α

β-Actin

L-Mimosine

MG132

Nucleus

Proteasome

Transcription

HIF-1α

HIF-1β

VHL

ub

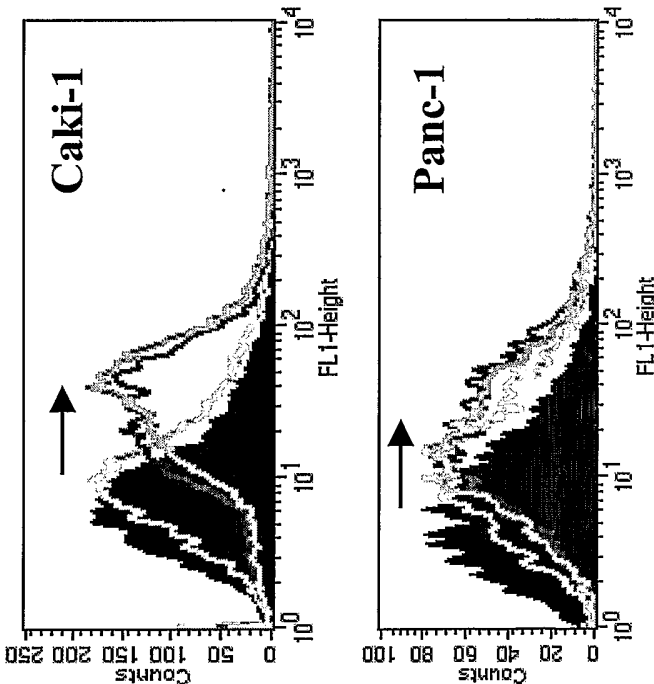
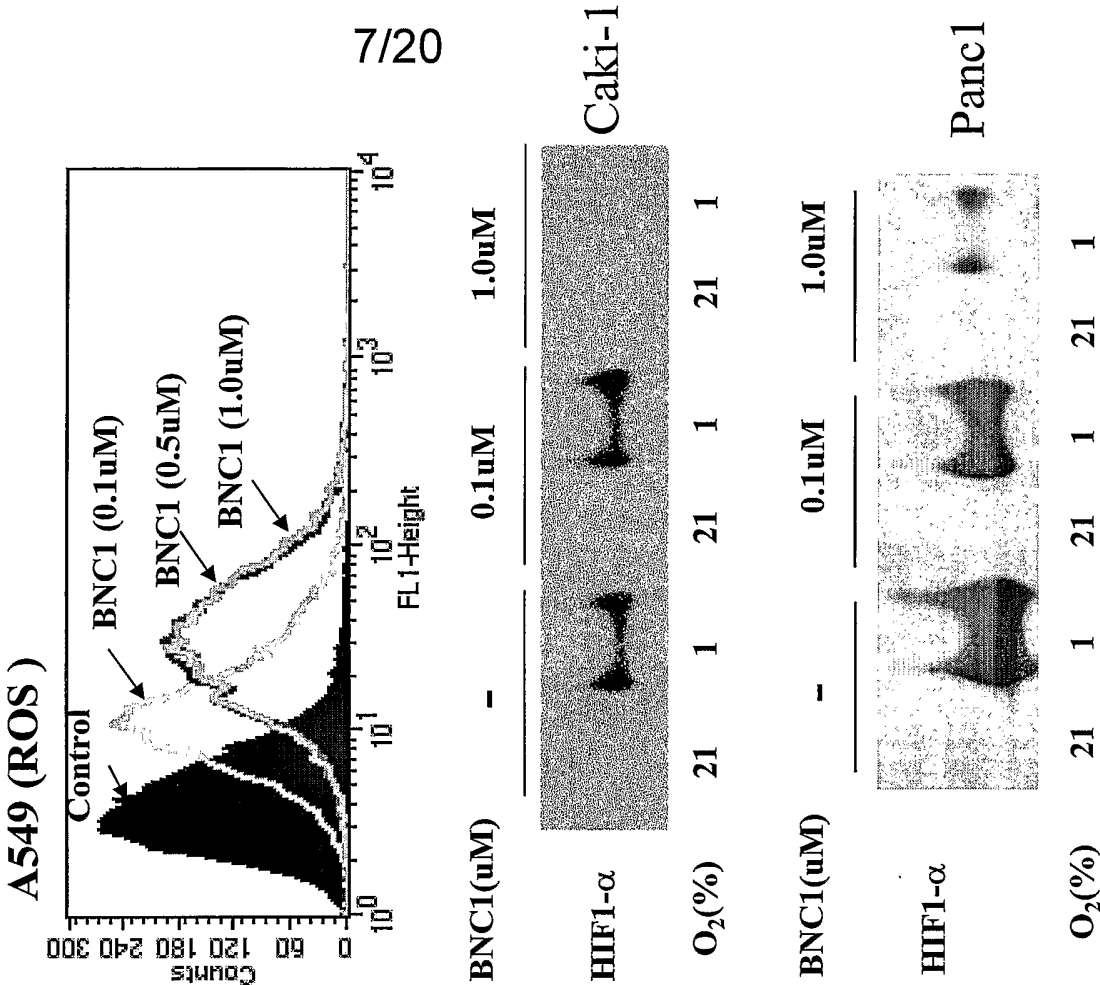
ROS / PHD

Catalase

L-Mimosine

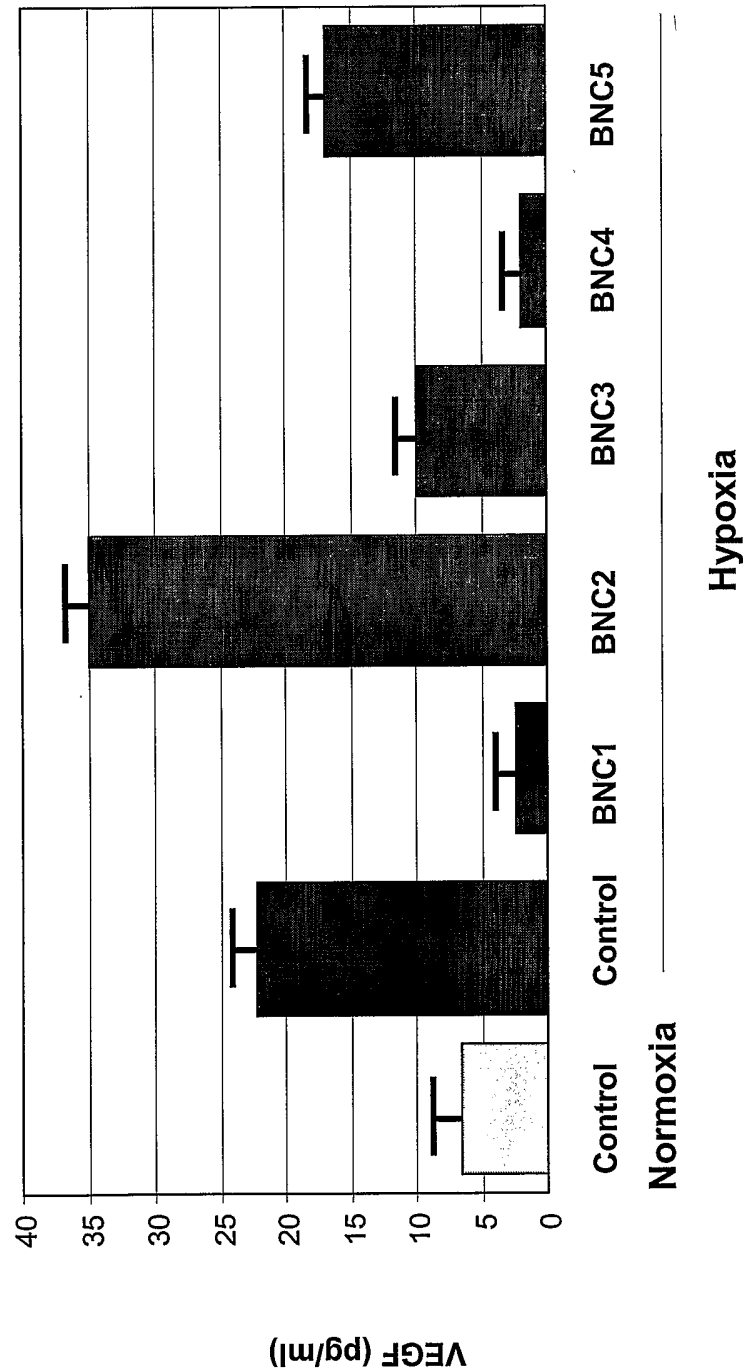
MG132

Figure 7



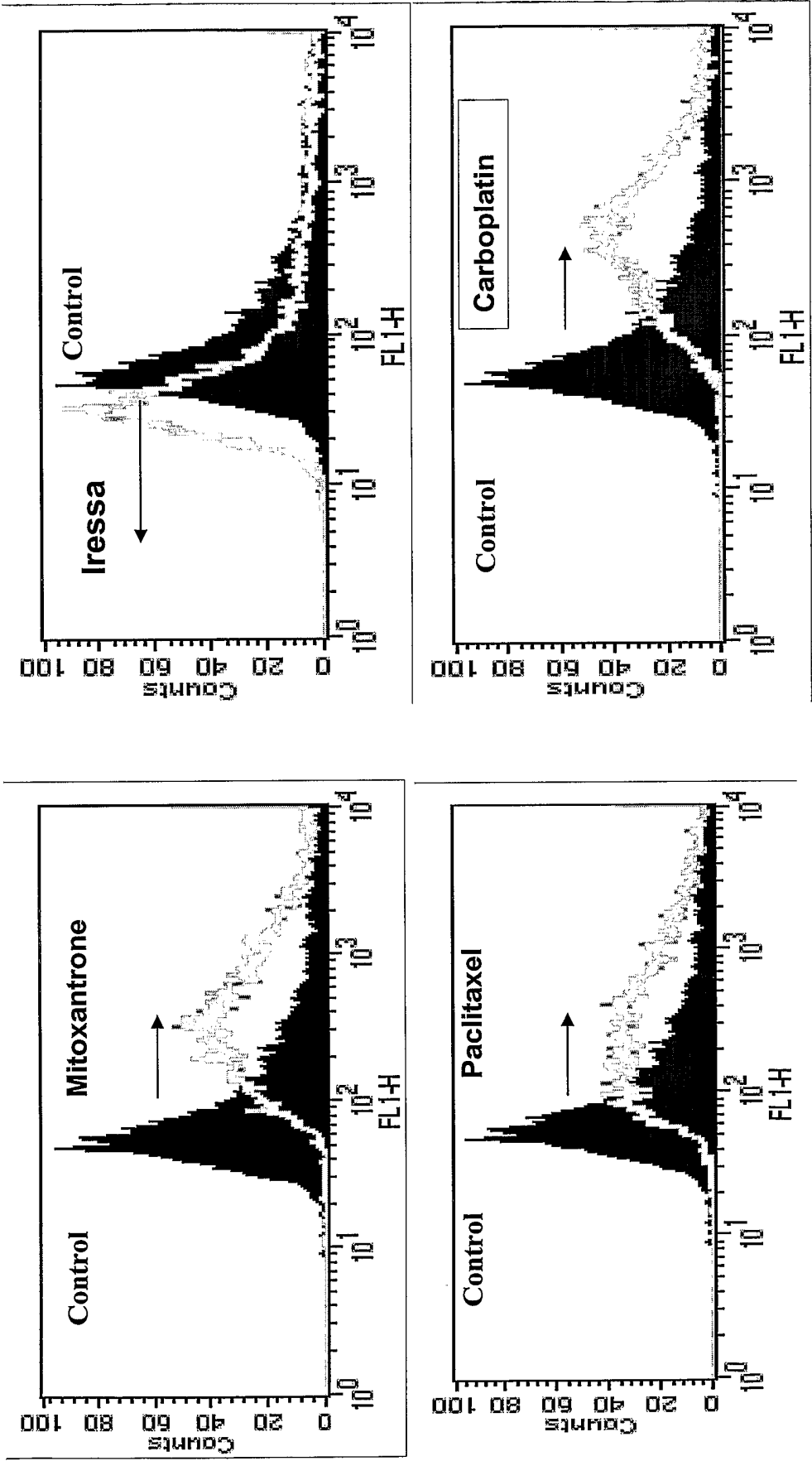
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Figure 8



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Figure 9



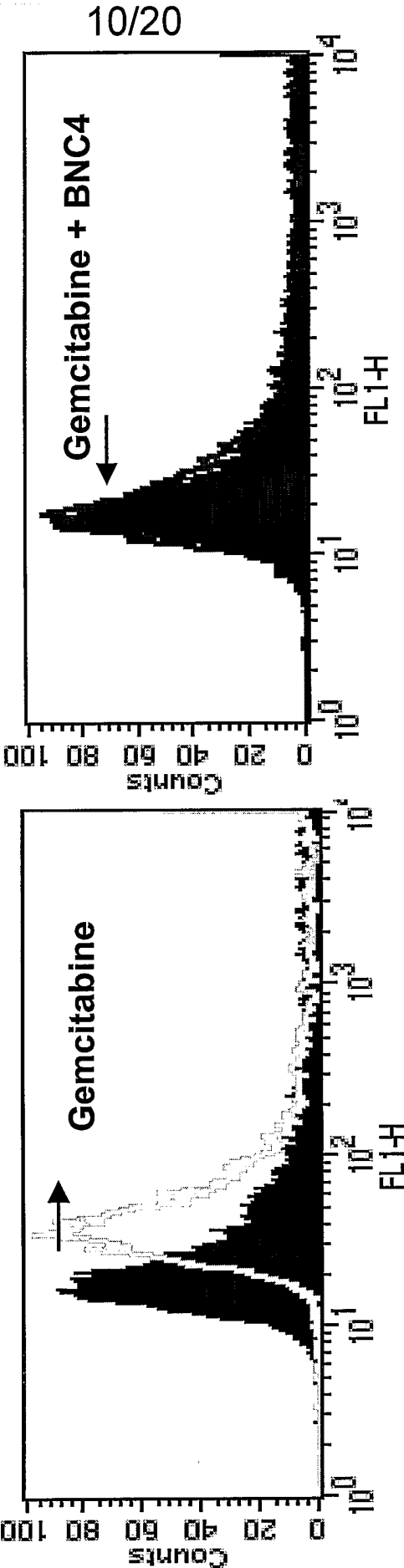


Figure 10

Figure 11

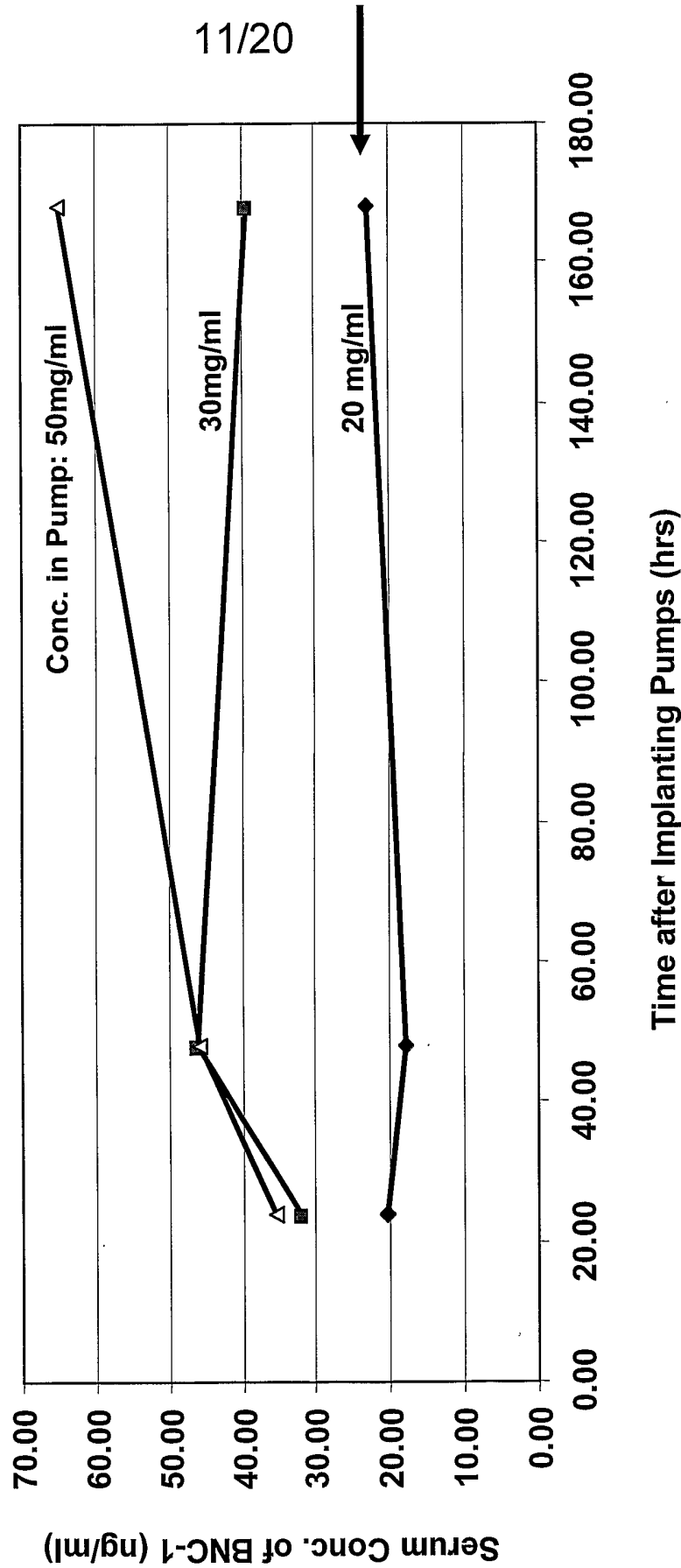


Figure 12

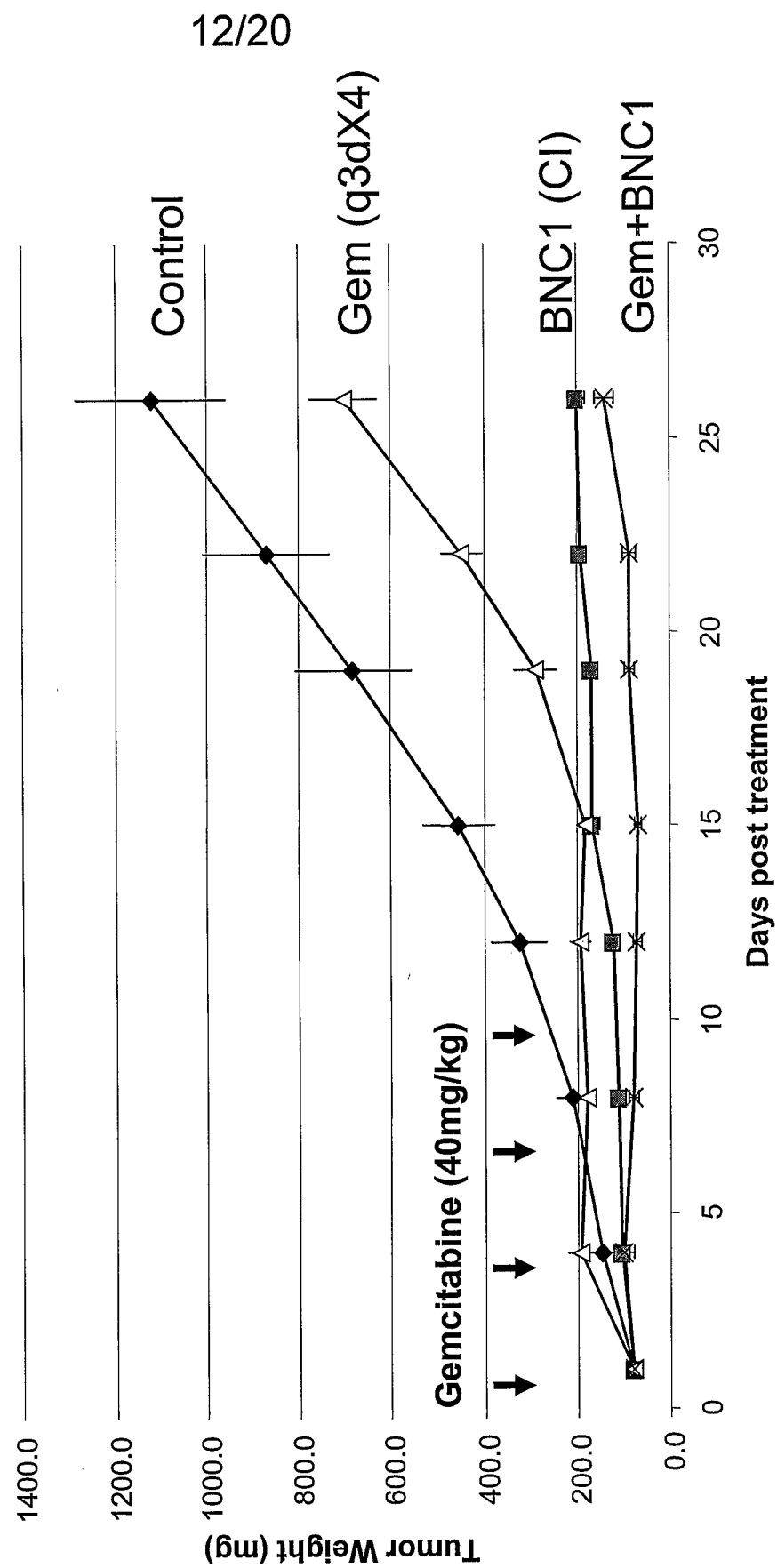


Figure 13

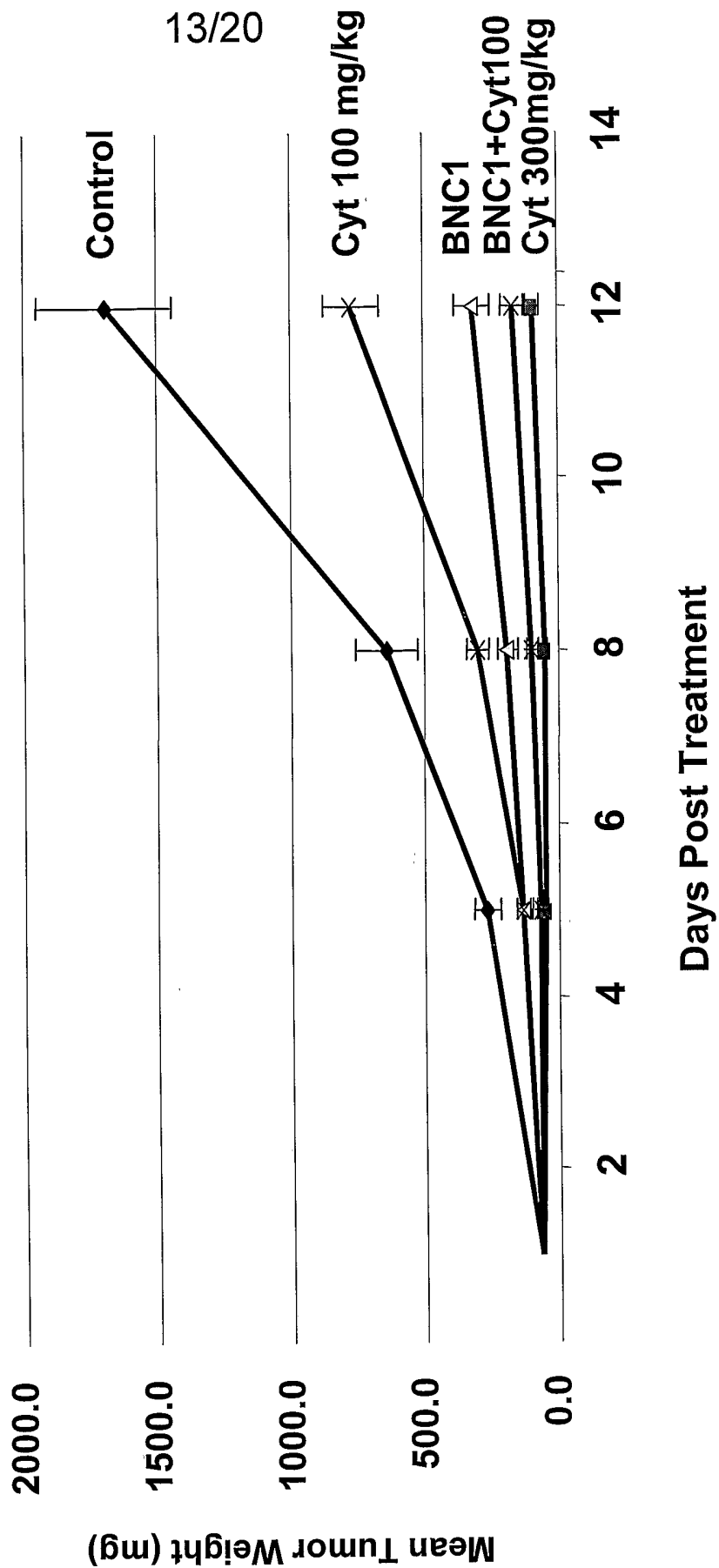


Figure 14

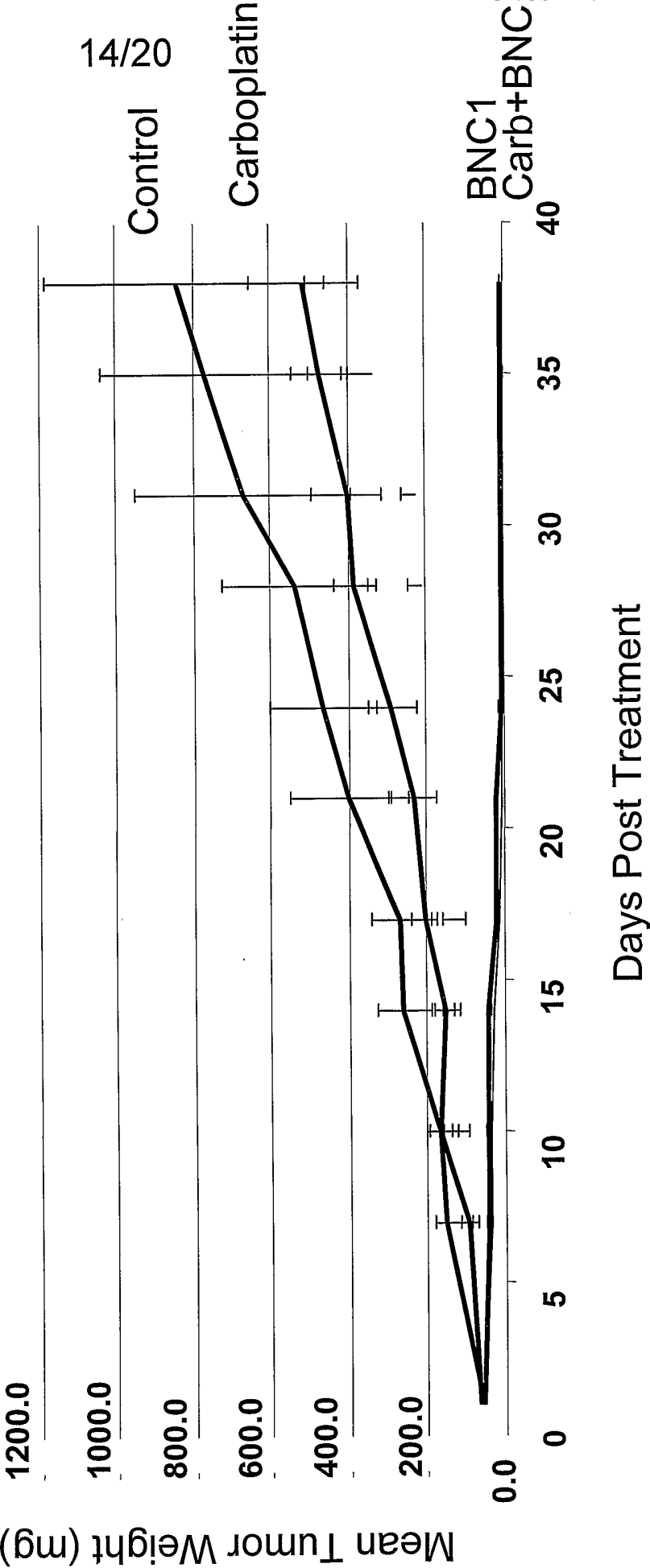


Figure 15

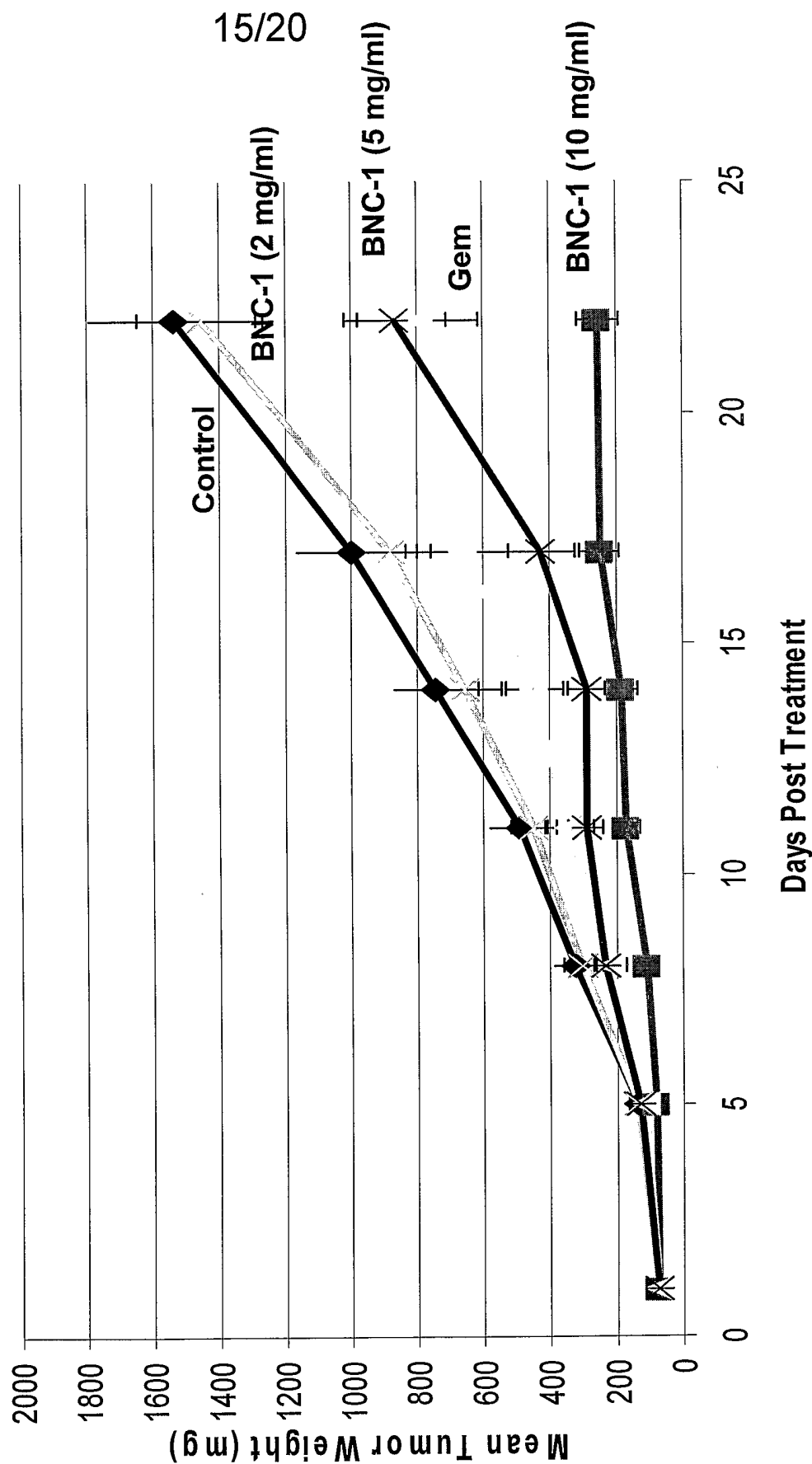
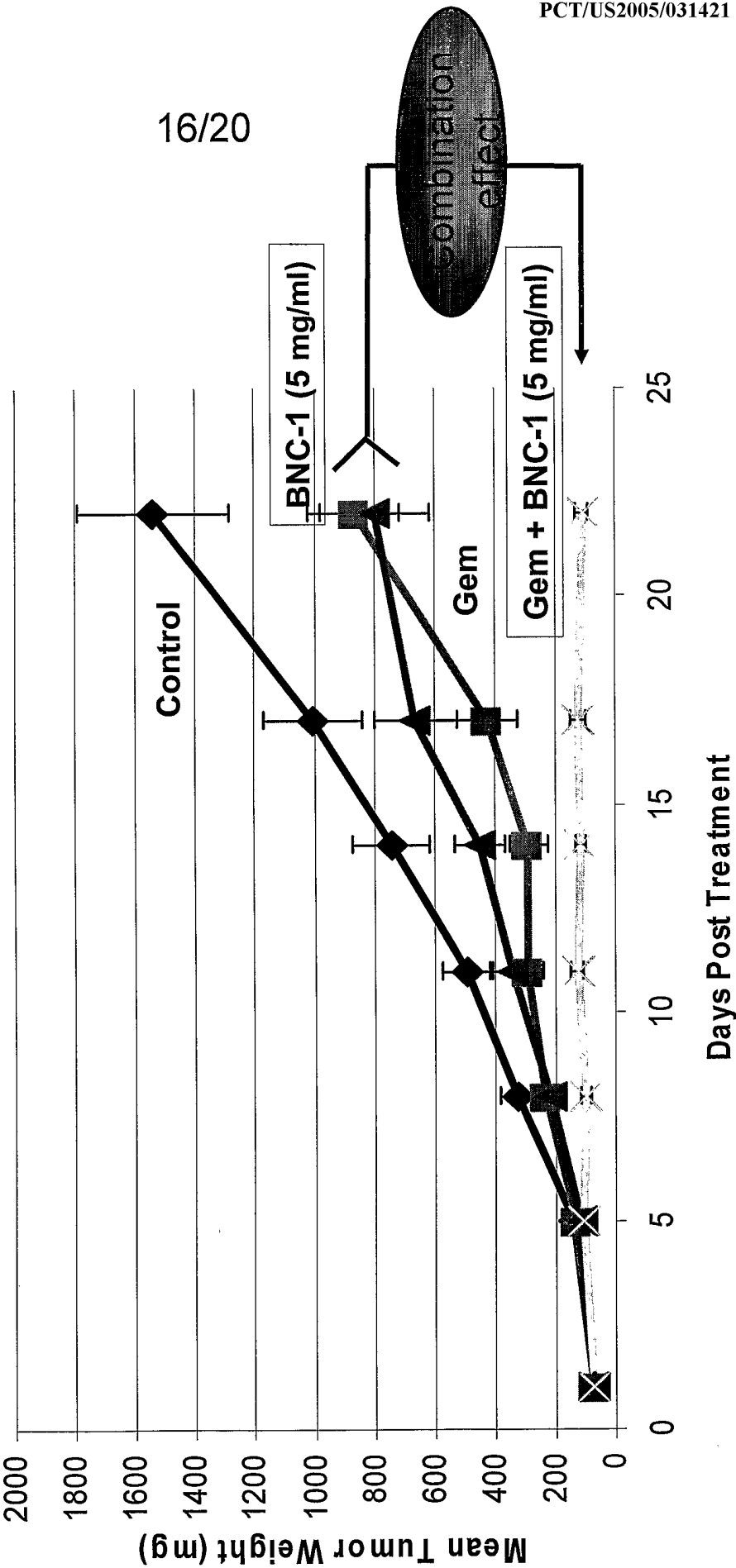
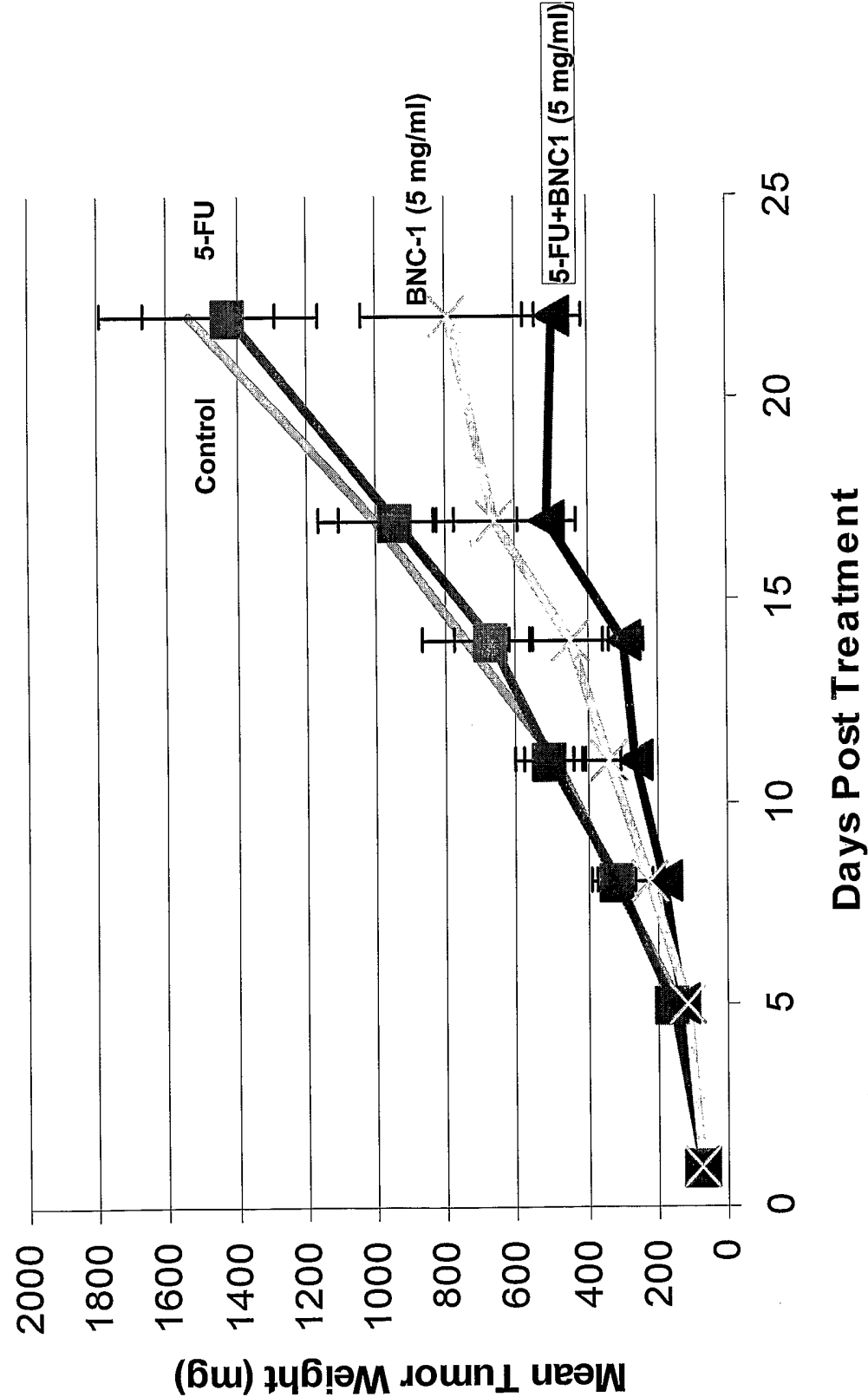


Figure 16



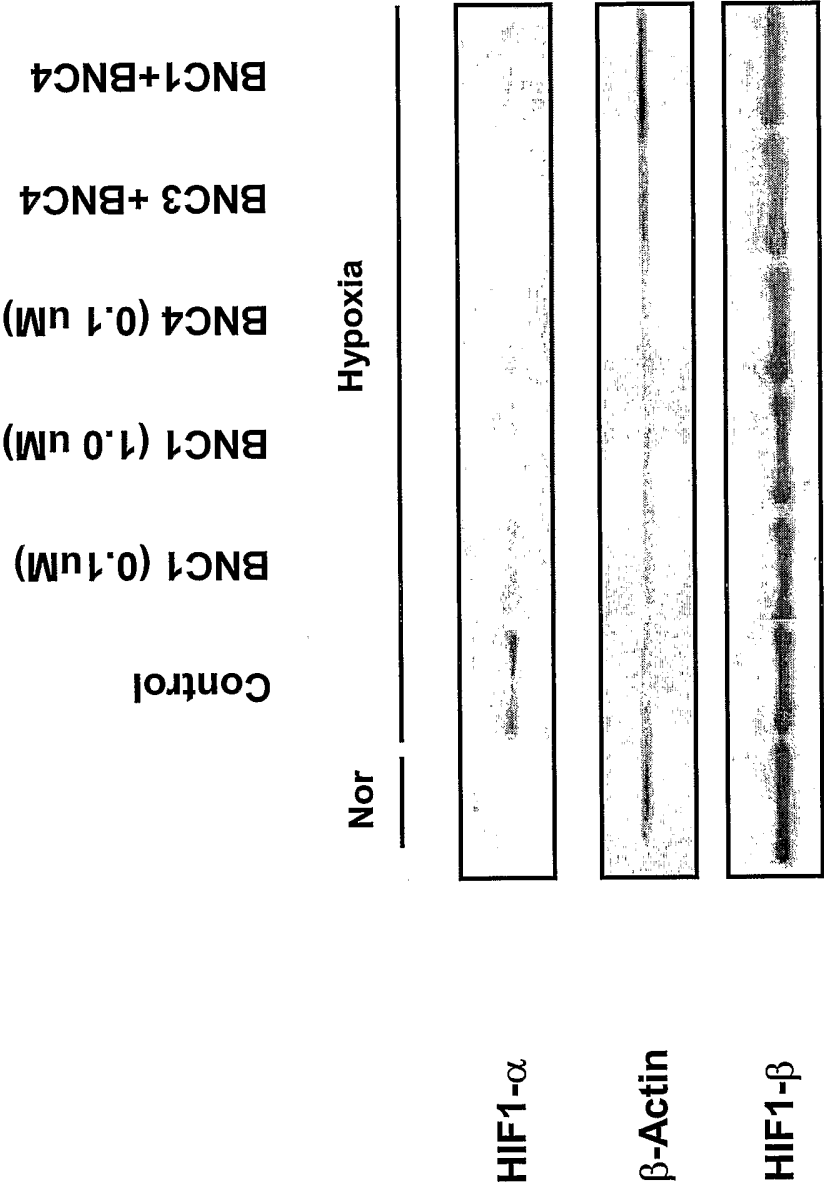
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Figure 17



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Figure 18



Western Blot in Hep3B cells

Figure 19

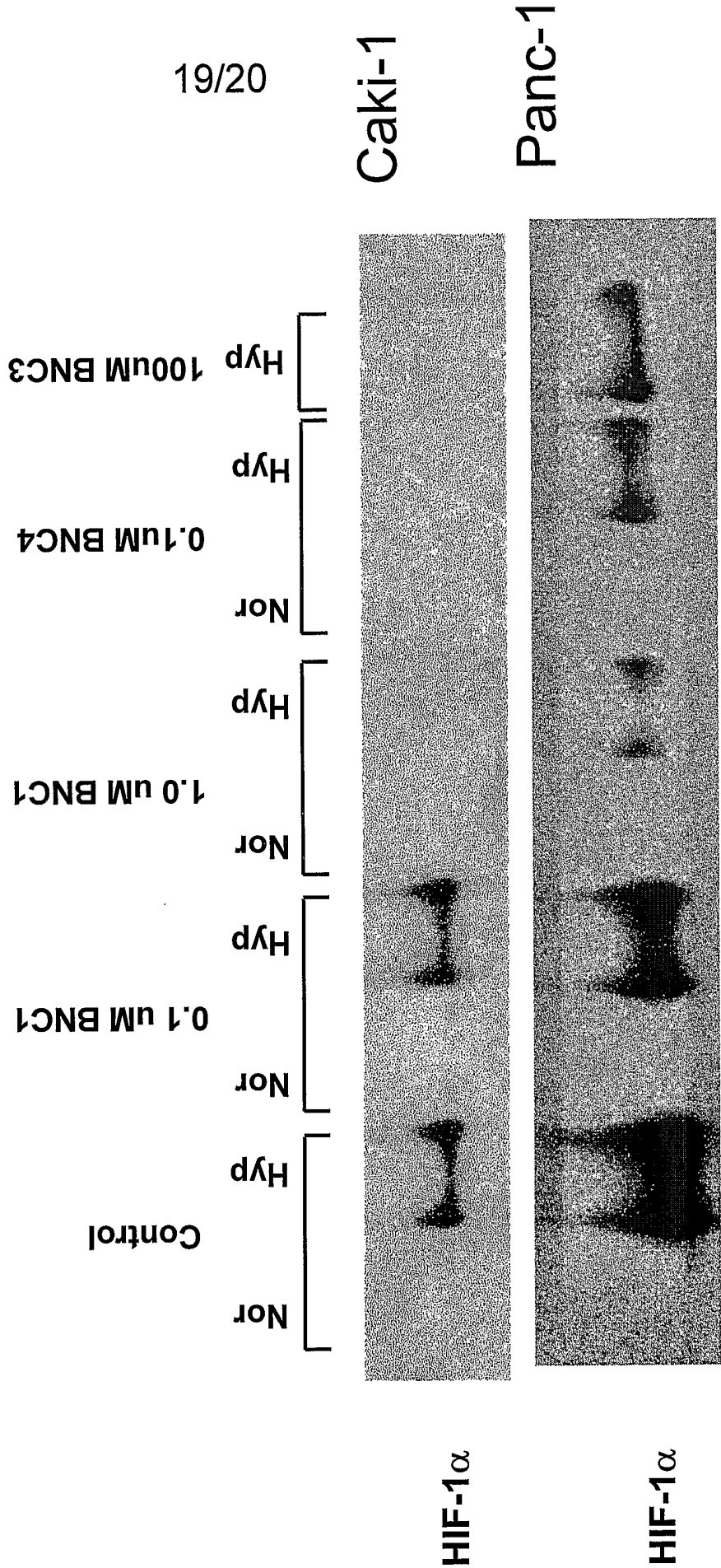


Figure 20

