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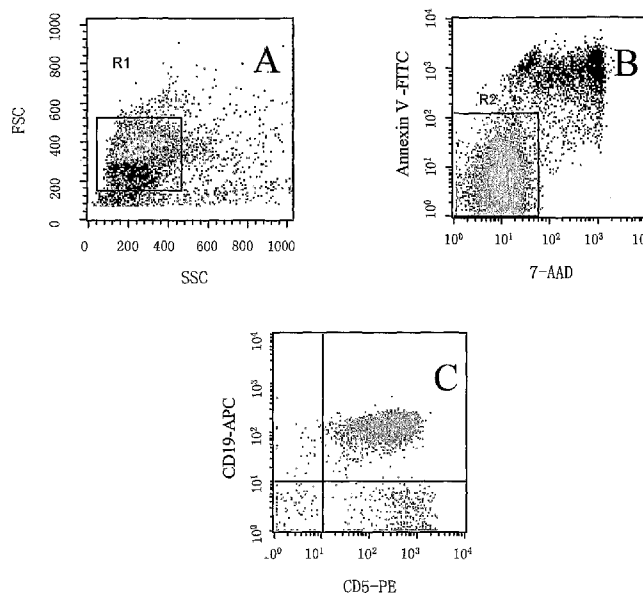
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(54) Title: MULTIPARAMETER FLOW CYTOMETRIC CYTOTOXICITY SYSTEMS, METHODS, COMPOSITIONS AND KITS FOR EVALUATING THE SUSCEPTIBILITY OF CANCER CELLS TO A TREATMENT MODALITY



(57) Abstract: Provided are multiparameter flow cytometric cytotoxicity systems, methods, compositions, and kits for assessing whether one or more cancer cell(s) in a patient sample are susceptible to a chemotherapeutic or other cancer treatment modality. The present invention will find utility in the development of personalized therapeutic strategies for selection of appropriate chemotherapeutic or other treatment modalities based on the drug sensitivity and resistance profiles of cancer cells from individual patients.



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**MULTIPARAMETER FLOW CYTOMETRIC CYTOTOXICITY
SYSTEMS, METHODS, COMPOSITIONS AND KITS FOR EVALUATING THE
SUSCEPTIBILITY OF CANCER CELLS TO A TREATMENT MODALITY**

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CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to co-pending U.S. Provisional Patent Application No. 60/702,438, filed July 25, 2005, which application is hereby incorporated by reference in its entirety.

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BACKGROUND OF THE INVENTION

Technical Field of the Invention

The present invention relates to the fields of oncology, cell biology, and flow cytometry. More specifically, provided herein are multiparameter flow cytometric cytotoxicity systems, methods, compositions, and kits for assessing, in a patient sample, whether one or more cancer cell(s) is susceptible to a chemotherapeutic or other cancer treatment modality.

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Brief Description of the Related Art

Current practices in cancer treatment have been around since the 1940s. New drugs have been developed but the 'miracle' cure or prevention for cancer remains elusive to modern medicine. Given the anticipation for new diagnosis tools, new prevention methods, or even hopes of a cancer cure, there has been little emphasis on improving chemotherapy -- the current highly toxic treatment of choice.

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Once a patient presents with symptoms, a blood or biopsy sample of bone marrow, lymph node, or other tissue is sent to the pathology lab to determine whether or not cancerous cells are present. Current diagnosis technologies are very sensitive and can detect cancer cells even when present at very low levels. There are as many types of cancer as there are individuals. Since cancer cells multiply quickly, gene mutations or chromosomal abnormalities are frequently introduced during disease progression. Thus, cancers are typically comprised of multiple clonal cell populations. Cancer cells from one patient may differ in biology and, consequently, in response to a therapeutic regimen as compared to cancer cells from another patient afflicted with the same disease.

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5 In the United States, blood cancers account for 7% of all cancers for a total of
100,000 new cases per year. B-cell chronic lymphocytic leukemia (B-CLL) is the most
common form of leukemia in North America and Europe. The clinical course for CLL is
quite variable. Montserrat, *Hematol J.* 5 Suppl. 1:S2-9 (2004); Wendtner *et al.*, *Semin.*
Hematol. 41:224-233 (2004); and Leporrier, *Hematol. J.* 5 Suppl 1:S10-19 (2004). Some
10 patients have an indolent clinical course while for other patients treatment is indicated.
For those patients requiring treatment, however, clinical responses are quite variable;
many patients show no response or only partial response.

A diagnosis of CLL leads to three possible treatment options: (1) "watch and
wait", (2) chemotherapy, and (3) bone marrow transplantation. Watch and wait is often
15 chosen for non-aggressive types of cancer, largely because the available treatment
regimens are more toxic than the disease itself. Chemotherapy is chosen for patients
whose cancer is growing and poses a significant risk to a patient's short-term health.
Bone marrow transplantation is commonly performed upon recurrence of disease
following a course of chemotherapy, typically due to out-growth of residual drug resistant
20 cancer cells.

A limitation in current cancer treatment regimens lies in the vast number of
choices of chemotherapy drugs and the high incidence of resistance to these drugs.
Currently there are over 50 drugs available for the treatment of blood cancers. Usually a
patient is given 1 to 3 chemotherapy drugs for treatment. An oncologist prescribes the
25 drugs that work based upon the previous responses of patients with similar cancers.

Currently the selection of appropriate drugs for the treatment of CLL remains
empirical. The prediction of clinical response to a particular drug, or regimen for a given
patient, remains a great challenge to clinicians. Although some biological factors,
especially cytogenetic abnormalities, have been shown of prognostic value for survival, it
30 is not clear whether these biological factors are directly involved in drug sensitivity or
resistance.

If a patient's cancer cells are resistant to a prescribed chemotherapy, the patient
will undergo an extremely toxic regimen yet realize little or no therapeutic benefit. If the
drug(s) administered kills the majority of cancer cells, with residual disease unaffected
35 and undetected, the cancer will likely recur. And, after an initial round of chemotherapy,
a patient may be too compromised to undergo subsequent rounds with another therapeutic
modality.

5 Fludarabine is a first line drug for B-cell chronic lymphocytic leukemia (CLL). About 10 to 20% of patients, however, do not respond to Fludarabine therapy attributing to the poor prognosis for those patients. Assessment of Fludarabine sensitivity or resistance prior to therapy would be predictive of therapeutic outcome and would prevent patients from unnecessarily receiving expensive and ineffective chemotherapy.

10 There remains an unmet need in the art for systems, methods, and associated compositions and kits for identifying patients within a patient population that are afflicted with a disease state comprising one or more cells that are susceptible to treatment with one or more candidate chemotherapy and/or other treatment modalities.

15 SUMMARY OF THE INVENTION

The present invention addresses these and other related needs by providing, *inter alia*, multiparameter flow cytometric cytotoxicity systems, methods, compositions, and kits that are suitable for assessing, prior to the initiation of treatment, whether a patient sample has one or more cancer cell(s) that is susceptible to one or more candidate
20 chemotherapeutic or other cancer treatment modality. A tissue sample comprising one or more cancer cell(s) is collected from a patient and incubated *in vitro* with one or more treatment modality of interest. Cancer cells and non cancer cells within the tissue sample are identified based upon the presence of cell population specific surface markers (cell-surface markers; typically antigens) using a label such as a protein ligand, antibody,
25 and/or antibody fragment that specifically binds to a cell population surface-marker within the tissue sample. Drug-induced cell death (cytotoxicity) in cancer cells as well as non-cancerous cells is subsequently detected and quantified without interference from healthy cells within the tissue sample. Cytotoxicity of one or more cancer cells within the cell population indicates that the drug or agent is effective in a treatment regimen against
30 those one or more cancer cells. In contrast, absence of cytotoxicity of one or more cancer cells within the cell population indicates that the drug or agent is ineffective in a treatment regimen against those one or more cancer cell(s). Cytotoxicity of non-cancerous cells within the same sample indicates an undesired toxic effect of the treatment regimen.

35 Thus, within certain aspects, the present invention provides methods for assessing the suitability of a cytotoxic drug and/or treatment regimen comprising a cytotoxic drug for the treatment of a cancer patient. By such methods, the susceptibility to drug-induced toxicity of one or more normal cell in a patient sample is compared to the susceptibility to

5 drug-induced toxicity of one or more cancer cell in the same patient sample. Thus, by these methods, the susceptibility of the cancer cell(s) is compared to the susceptibility of the normal cell(s) and the relative susceptibility is calculated and a corresponding *in vitro* therapeutic index is determined. Such methods will find utility in assessing whether a given cytotoxic drug and/or treatment regimen is suitable for one or more individual
10 cancer patient(s) wherein a high degree of susceptibility to a cytotoxic drug of one or more normal cell(s) as compared to one or more cancer cell(s) indicates that the cytotoxic drug is toxic the patient and consequently is not indicated for treatment of the patient whereas a low degree of susceptibility to a cytotoxic drug of one or more normal cell(s) as compared to one or more cancer cell(s) indicates that the cytotoxic drug is not toxic to
15 the patient and consequently may be indicated for treatment of the patient without an expectation of *in vivo* toxicity.

The methods disclosed herein can be completed in two days or less, with early detection of cancer cell death, and are sensitive at a single cell level. Thus, the assay is suitable for determining the best chemotherapeutic or other treatment modality for
20 individual cancer patients. The assay is suitable for all clinical samples including those with only a small amount of residual disease. The samples can be either freshly harvested from the patient or cryopreserved.

Patient tissue samples that may be subjected to the multiparameter flow cytometric cytotoxicity systems and methods disclosed herein include, but are not limited
25 to, blood, bone marrow, and biopsy samples of solid tumors. Cancer cells within such patient samples that may be assayed for susceptibility to one or more chemotherapy or other treatment modality include, but are not limited to, cells associated with a wide variety of hematopoietic cancers including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous
30 leukemia (CML), hairy cell leukemia (HCL), non-Hodgkin's lymphoma (either T-cell, NK-cell, or B-cell lymphoma), and multiple myeloma. Other suitable patient samples include biopsy samples from solid tumors such as soft tissue sarcomas and cancers of the brain, esophagus, skin, uterine cervix, bone, lung, liver, endometrium, bladder, breast, larynx, colon/rectum, stomach, ovary, pancreas, adrenal gland, and prostate. It will be
35 understood that other patient samples may also be tested by the systems and methods presented herein.

Candidate drugs or agents that may be tested for efficacy, individually or in combination, against one or more cancer cells from a patient sample include, without

5 limitation, one or more known chemotherapy drug(s) and other small molecules as well as one or more therapeutic biomolecule such as, for example, an antibody or fragment thereof and/or a protein ligand or soluble receptor.

Suitable chemotherapy drugs that may be tested for efficacy in the present systems and methods include, but are not limited to, Altretamine, Arsenic Trioxide, Asparaginase,
10 Bleomycin, Busulfan, Capecitabine, Carboplatin, Carmustine, Chlorambucil, Cisplatin, Cladribine, Cyclophosphamide, Cytarabine, Dacarbazine, Dactinomycin, Daunorubicin, Docetaxel, Doxorubicin, Epirubicin, Estramustine, Etoposide, Floxuridine, Fludarabine, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Ifosfamide, Irinotecan, Isotretinoin, Lomustine, Mechlorethamine, Melphalan, Mercaptopurine, Methotrexate, Mitomycin,
15 Mitoxantrone, Oxaliplatin, Paclitaxel, Pegaspargase, Pemetrexed, Prednisolone, Procarbazine, Streptozocin, Temozolomide, Teniposide, Thioguanine, Thiotepa, Thiotepa, Topotecan, Tretinoin, Vinblastine, Vincristine, and Vinorelbine. Further exemplified herein are systems, methods, compositions, and kits that employ the chemotherapy drugs Fludarabine, Chlorambucil, Cladribine, and Prednisolone.

20 Suitable antibody therapeutics that may be tested for efficacy in the present systems and methods include, but are not limited to, Alemtuzumab, Bevacizumab, Cetuximab, Gemtuzumab ozogamicin, Trastuzumab, and Rituximab.

Typically, labels that specifically bind to a cancer cell bind to a cell-surface marker such that cancer cells can be distinguished from admixed non-cancer cells. Cell-
25 surface markers include, for example, proteins, such as enzymes, hormones, oncofetal antigens, and blood-group antigens; and cell-surface displayed carbohydrates, including glycoproteins and glycolipids. Labels that may be suitably employed for specifically labeling one or more cancer cells include protein ligands, antibodies, and fragments thereof. Exemplary cell-surface protein markers include, but are not limited to, Alpha-
30 fetal Protein (AFP), Human Chorionic Gonadotrophin (hCG), Calcitonin, CA27.29, CA15-3, CA19.9, CA125, CA72-4, Carcinoembryonic Antigen (CEA), CD22, CD3, CD33, CD13, CDw65, Lactoferrin (LF), CD68, CD15, CD15s, CD79a (MB-1), CD79b (B29), CD87 (uPA-R), and CD117 (c-kit). Exemplary cell-surface carbohydrate markers include, but are not limited to, N-linked and O-linked mucin-like glycoproteins; cell
35 membrane-bound mucin-like carbohydrate structures such as Tn, sialyl-Tn, and T; and blood group-related carbohydrate structures such as Le(x), sialyl-Le(x), ABH, and Le(y). An exemplary cell-surface glycolipid is CA19.9.

5 In the case of cancers of the blood, including leukemias and lymphomas, one or more of the following cell-surface markers may be advantageously employed: (1) T-cell markers such as CD1a, CD2, CD3, CD4, CD5, CD7, CD8, TCR alpha-beta, and TCR gamma-delta; (2) B-cell markers such as CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79b, CD103, Kappa, Lambda, IgG, IgM, IgD, IgA, and FMC-7; and (3)
10 Myeloid/Monocytic-cell markers such as CD13, CD14, CD15, CD33, CD11b, CD117, and myeloperoxidase. One or more other marker(s) may also be used such as, for example, CD11c, CD25, CD34, CD45, HLA-Dr, Tdt, CD16, CD30, CD38, CD41, CD42b, CD56, CD57, CD61, and glycophorin. In the specific case of B-CLL, exemplified herein, leukemic cells may be identified with a label comprising antibodies
15 that specifically bind to CD5 and antibodies that specifically bind to CD19.

In order to achieve detection by the multiparameter flow cytometric cytotoxicity methods of the present invention, labels typically include one or more fluorescent dye such as, for example, allophycocyanin (APC), phycoerythrin (PE), and fluorescein-isothiocyanate (FITC). Labels may also include a tag such as biotin. Within related
20 aspects, the label may be a first antibody that does not otherwise include a fluorescent dye or other tag. In such cases, a second antibody may be employed wherein the second antibody is capable of specifically binding to the first antibody. Within such aspects, the second antibody typically includes one or more fluorescent dye or other detectable tag.

A number of assay systems that are available in the art may be employed for
25 measuring the cytotoxicity of a specifically labeled cancer cell treated with a candidate chemotherapeutic or other treatment modality. As exemplified herein, cytotoxicity may be measured by staining with annexin, anti-phosphatidylserine, and/or 7-AAD. Other assay systems may also be employed.

These and other aspects of the present invention will become apparent upon
30 reference to the following detailed description. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1C present a gating strategy for measuring drug-induced cytotoxicity
35 in B-CLL cells. Lymphocytes are gated based on forward and side scatter in region R (Fig. 1A). Viable cells, without binding of either 7-AAD or Annexin V, are gated in R2 (Fig. 1B). The surviving B-CLL cells are those viable cells that express both CD5 and CD19 shown in the upper right quadrant (Fig. 1C).

5 Figure 2 presents the variability of *in vitro* drug sensitivity profiles of 43 samples of B-CLL. The drug concentrations for Fludarabine, Chlorambucil, Cladribine and Prednisolone are at 2.5 mg/ml, 2.5 mg/ml, 0.5 mg/ml, and 25 mg/ml, respectively. Leukemic survival indices are displayed as a mean $\pm$ SE of triplicate wells.

10 Figures 3A-3F present the correlation of *in vitro* drug sensitivity to Fludarabine, Chlorambucil, Cladribine, and Prednisolone. A good correlation is observed between Fludarabine and Chlorambucil (Fig. 3A), between Fludarabine and Cladribine (Fig. 3B), and between Chlorambucil and Cladribine (Fig. 3C). A poor correlation is seen between Prednisolone and Fludarabine (Fig. 3D), between Prednisolone and Chlorambucil (Fig. 3E), and between Prednisolone and Cladribine (Fig. 3F).

15 Figures 4A-4B present differences of *in vitro* drug sensitivity among different cytogenetic groups. Abbreviations: del = deletion; +12 = trisomy 12; and -13q = 13q deletion. Leukemic survival indices are displayed in mean $\pm$ SE. Fludarabine *in vitro* resistance is significantly higher in cases with a p53 deletion (>10%) than other groups ($p < 0.05$) (Fig. 4A). Prednisolone *in vitro* resistance is not associated with particular
20 cytogenetic group of B-CLL (Fig. 4B).

 Figures 5A-5B present *in vitro* drug sensitivity of B-CLL cells from patients at different clinical stages of disease. B-CLL cells from patients with early disease are more sensitive to Fludarabine than those from patients with advanced disease (Fig. 5A). No significant difference is seen in B-CLL cells from patients with early and advanced stage
25 exposed to Prednisolone (Fig. 5B).

DETAILED DESCRIPTION OF THE INVENTION

 As disclosed above, and as described in greater detail herein below, the present invention provides multiparameter flow cytometric cytotoxicity systems, methods,
30 compositions, and kits for assessing a clinical response to one or more drug and/or agent either alone or in combination. By the present systems and methods, a patient sample comprising one or more cancer cells is collected from a patient and contacted *in vitro* with one or more treatment modality. Cancer cells are specifically identified in the patient sample by contacting the cell population with a label that specifically binds to one
35 or more cancer cell(s) but does not specifically bind normal cells within the cell population. Efficacy of the one or more candidate drug or agent is assessed by assaying for drug-induced cancer cell death (*i.e.* cytotoxicity) in the specifically labeled cancer cell population. Cytotoxicity of one or more cancer cells within the cell population indicates

5 that the drug or agent is effective in a treatment regimen against those one or more cancer cells. In contrast, absence of cytotoxicity of one or more cancer cells within the cell population indicates that the drug or agent is ineffective in a treatment regimen against those one or more cancer cells.

The multiparameter flow cytometric cytotoxicity systems, methods, compositions,
10 and kits of the present invention are exemplified herein by their application to the evaluation of the efficacy of four commonly used chemotherapeutic agents (*i.e.* Fludarabine, Chlorambucil, Cladribine, and Prednisolone) on chronic lymphocytic leukemia (CLL) cells from 43 patients. Specific drug-induced cytotoxicity of leukemic cells was measured after subjecting a patient sample to treatment with Fludarabine,
15 Chlorambucil, Cladribine, and/or Prednisolone. Leukemic cells were identified by staining with antibodies against CD5 and CD19. Cytotoxicity was assessed with Annexin V and 7-AAD. Leukemic cell survival indices were calculated by expressing viable leukemic cells in test culture as percent of viable leukemic cells in control cultures.

The data presented herein demonstrated a marked variability in *in vitro* drug
20 sensitivity on leukemic cells after *in vitro* incubation with each of these four chemotherapeutic agents. Without wishing to be limited to any specific cytogenetic characteristics and/or mode of action for any of these chemotherapeutic agents, it is believed that *in vitro* resistance seen in B-CLL cells is correlative with deletions of p53 or ataxia telangiectasia-mutated gene (ATM), cytogenetic abnormalities that are associated
25 with a poor *in vivo* therapeutic response to each of these agents.

The present methods can be advantageously completed in two days or less, with early detection of cell death, and are sensitive at a single cell level. The results achieved by these methods yield drug sensitivity profiles that will find utility in guiding the selection of a drug having efficacy against a given disease. The present methods also
30 permit the identification of an appropriate drug for residual disease that exhibits resistance to an initial therapeutic regimen. Thus, within certain embodiments, the present invention permits a determination of a drug modality having efficacy as a second line of chemotherapy to kill the remaining cancerous cells.

As used in this specification and the appended claims, the singular forms "a," "an"
35 and "the" include plural references unless the content clearly dictates otherwise.

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of flow cytometry, cell biology, immunology, molecular biology, oncology, and protein chemistry within the skill of the art, many of

5 which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Shapiro, "Practical Flow Cytometry" (4th Ed. Wiley-Liss, 2003); Longobardi Givan, "Flow Cytometry : First Principles" (2nd Ed. Wiley-Liss, 2001); Watson, "Introduction to Flow Cytometry" (Cambridge University Press, 2004); Nguyen et al., "Flow Cytometry in Hematopathology: A Visual Approach to Data
10 Analysis and Interpretation" (Humana Press, 2002); Hawley and Hawley, "Methods in Molecular Biology: Flow Cytometry Protocols" V. 263. (2nd Ed., Humana Press, 2004); Radbruch, "Flow Cytometry and Cell Sorting" (2nd Ed. Springer, 1999); Sambrook, *et al.*, "Molecular Cloning: A Laboratory Manual" (2nd Edition, 1989); Maniatis *et al.*, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: A Practical
15 Approach, vol. I & II" (D. Glover, *ed.*); "Animal Cell Culture" (R. Freshney, *ed.*, 1986); Perbal, "A Practical Guide to Molecular Cloning" (1984); Ausubel *et al.*, "Current protocols in Molecular Biology" (New York, John Wiley and Sons, 1987); Bonifacino *et al.*, "Current Protocols in Cell Biology" (New York, John Wiley & Sons, 1999); Coligan *et al.*, "Current Protocols in Immunology" (New York, John Wiley & Sons, 1999); and
20 Harlow and Lane *Antibodies: a Laboratory Manual* Cold Spring Harbor Laboratory (1988).

All publications, patents, and patent applications cited herein, whether *supra* or *infra*, are hereby incorporated by reference in their entirety. The present invention will be better understood through the detailed description of the specific embodiments, each of
25 which is described in detail herein below.

Patient Samples Comprising Cancer Cells

Patient samples that may be subjected to the multiparameter flow cytometric cytotoxicity methods disclosed herein include, but are not limited to, blood, bone marrow,
30 and biopsy samples of solid tumors. Cancer cells within such patient samples that may be assayed for susceptibility to one or more chemotherapy or other treatment modality include, but are not limited to, cells associated with a wide variety of hematopoietic cancers including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), hairy cell
35 leukemia (HCL), non-Hodgkin's lymphoma (either T-cell, NK-cell, or B-cell lymphoma), and multiple myeloma. Other suitable patient samples include biopsy samples from solid tumors such as soft tissue sarcomas and cancers of the brain, eye, skin, liver, esophagus,

5 uterine cervix, bone, lung, endometrium, bladder, breast, larynx, colon/rectum, stomach, ovary, pancreas, adrenal gland, and prostate. (See, also, Table 2).

Methodologies for the isolation of blood, bone marrow, and solid tumor samples are well known in the art. Typically, blood is drawn from a vein (venipuncture) and bone marrow is aspirated from the posterior iliac crest. Specimens are typically collected in
10 the presence of an anticoagulant such as, for example, ethylenediaminetetraacetic acid (EDTA) or heparin. Red blood cells in blood or bone marrow samples are, generally, removed by a lysis procedure or by ficoll gradient centrifugation, by standard methods. Mononuclear cells typically containing cancer cells are either used fresh or cryopreserved.

15 An excisional biopsy procedure is often performed to remove a cancer sample of an involved lymph node or other tissue. The cells are disaggregated, single cells are prepared, and they are either used fresh or cryopreserved.

Candidate Chemotherapeutic Modalities

20 Candidate drugs or agents that may be tested for efficacy against one or more cancer cells from a patient sample include, without limitation, one or more known chemotherapy drugs and other small molecules as well as one or more therapeutic biomolecule such as, for example, an antibody or fragment thereof and/or a protein ligand or soluble receptor.

25 Suitable chemotherapy drugs that may be tested for efficacy in the present systems and methods include, but are not limited to, Altretamine, Arsenic Trioxide, Asparaginase, Bleomycin, Busulfan, Capecitabine, Carboplatin, Carmustine, Chlorambucil, Cisplatin, Cladribine, Cyclophosphamide, Cytarabine, Dacarbazine, Dactinomycin, Daunorubicin, Docetaxel, Doxorubicin, Epirubicin, Estramustine, Etoposide, Floxuridine, Fludarabine,
30 Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Ifosfamide, Irinotecan, Isotretinoin, Lomustine, Mechlorethamine, Melphalan, Mercaptopurine, Methotrexate, Mitomycin, Mitoxantrone, Oxaliplatin, Paclitaxel, Pegaspargase, Pemetrexed, Prednisolone, Procarbazine, Streptozocin, Temozolomide, Teniposide, Thioguanine, Thiotepa, Thiotepa, Topotecan, Tretinoin, Vinblastine, Vincristine, and Vinorelbine. Further
35 exemplified herein are systems, methods, compositions, and kits that employ the chemotherapy drugs Fludarabine, Chlorambucil, Cladribine, and Prednisolone.

5 Suitable antibody therapeutics that may be tested for efficacy in the present systems and methods include, but are not limited to, Alemtuzumab, Bevacizumab, Cetuximab, Gemtuzumab ozogamicin, Trastuzumab, and Rituximab.

Each of the above mentioned treatment modalities has been shown to be effective in at least one type of cancer or other disease as summarized in Table 1.

10

Table 1
Cancers and Other Diseases that are Currently being Treated by
Existing Candidate Treatment Modalities

Chemotherapeutic Drug	
Treatment Modality	Cancers and Other Conditions Treated
Altretamine	Ovarian cancer
Arsenic Trioxide	Acute promyelocytic leukemia (APL) Multiple myeloma Chronic myelogenous leukemia Acute myelogenous leukemia.
Asparaginase	Acute lymphocytic leukemia (ALL)
Bleomycin	Squamous cell cancers Melanoma Sarcoma Testicular cancer Hodgkin's lymphoma Non-Hodgkin's lymphoma
Busulfan	Chronic myelogenous leukemia (CML). Polycythemia vera Myeloid metaplasia
Capecitabine	Metastatic colon or rectal cancer Metastatic breast cancer
Carboplatin	Ovarian, lung, head and neck, endometrial, esophageal, bladder, breast, and cervical cancers Central nervous system or germ cell tumors Osteogenic sarcoma
Carmustine	Brain tumors including glioblastoma, brainstem glioma, medulloblastoma, astrocytoma, ependymoma and metastatic brain tumors Multiple myeloma Hodgkin's disease Non-Hodgkin's lymphoma Melanoma Lung cancer Colon cancer
Chlorambucil	Chronic lymphocytic leukemia (CLL) Hodgkin's disease

	Non-Hodgkin's lymphoma Breast cancer Ovarian cancer Testicular cancer Waldenstrom's macroglobulinemia Thrombocythemia Choriocarcinoma
Cisplatin	Testicular cancer Ovarian cancer Bladder cancer Head and neck cancer Esophageal cancer Small and non-small cell lung cancer Breast cancer Cervical cancer Stomach cancer Prostate cancer Hodgkin's lymphoma Non-Hodgkin's lymphoma Neuroblastoma Sarcoma Multiple myeloma Melanoma Mesothelioma
Cladribine	Hairy cell leukemia Chronic lymphocytic leukemia (CLL) Non-Hodgkin's lymphoma
Cyclophosphamide	Hodgkin's lymphoma Non-Hodgkin's lymphoma Burkitt's lymphoma Chronic lymphocytic leukemia (CLL) Chronic myelocytic leukemia (CML) Acute myelocytic leukemia (AML) Acute lymphocytic leukemia (ALL) T-cell lymphoma (mycosis fungoides) Multiple myeloma Neuroblastoma Retinoblastoma Rhabdomyosarcoma Ewing's sarcoma Breast cancer Testicular cancer Endometrial cancer Ovarian cancer Lung cancer
Cytarabine	Acute myelogenous leukemia (AML) Chronic myelogenous leukemia (CML) Acute lymphocytic leukemia (ALL) Meningeal leukemia and lymphoma
Cytarabine liposomal	Lymphomatous meningitis

Dacarbazine	Metastatic malignant melanoma Hodgkin's disease Soft tissue sarcoma Neuroblastoma Fibrosarcoma Rhabdomyosarcoma Islet cell carcinoma Medullary carcinoma of the thyroid
Dactinomycin	Wilms' tumor Rhabdomyosarcoma Germ cell tumors Gestational trophoblastic disease Ewing's sarcoma Testicular cancer Melanoma Choriocarcinoma Neuroblastoma Retinoblastoma Uterine sarcomas Kaposi's sarcoma Sarcoma botryoides Soft tissue sarcoma
Daunorubicin	Acute myelogenous leukemia (AML) Acute lymphoblastic leukemia (ALL)
Docetaxel	Breast cancer Non-small cell lung cancer Small cell lung cancer Metastatic prostate cancer Head and neck cancer Stomach cancer Ovarian cancer Bladder cancer Pancreatic cancer Soft tissue sarcoma Melanoma
Doxorubicin	Bladder cancer Breast cancer Head and neck cancer Leukemia Liver Lung cancer Lymphoma Mesothelioma Multiple myeloma Neuroblastoma Ovarian cancer Pancreatic cancer Prostate cancer Sarcoma Stomach cancer

	Testicular cancer (germ cell) Thyroid cancer Uterine cancer
Epirubicin	Breast cancer
Estramustine	Prostate cancer
Etoposide	Testicular cancer Bladder cancer Prostate cancer Lung cancer Stomach cancer Uterine cancer Hodgkin's lymphoma Non-Hodgkin's lymphoma Mycosis fungoides Kaposi's sarcoma Wilm's tumor Rhabdomyosarcoma Ewing's sarcoma Neuroblastoma Brain tumor
Floxuridine	Colon cancer Kidney cancer Stomach cancer
Fludarabine	Chronic lymphocytic leukemia (CLL) Non-Hodgkin's lymphoma Acute leukemias
Fluorouracil	Colon cancer Rectal cancer Breast cancer Gastrointestinal cancers including: anal, esophageal, pancreas and gastric cancers Head and neck cancer Liver cancer (Hepatoma) Ovarian cancer
Gemcitabine	Pancreas cancer Non-small cell lung cancer Bladder cancer Soft-tissue sarcoma Metastatic breast cancer
Hydroxyurea	Chronic myeloid leukemia (CML) Essential thrombocytosis and polycythemia vera Head and neck cancer Melanoma Ovarian cancer (refractory)
Idarubicin	Acute myelogenous leukemia (AML) Acute lymphoblastic leukemia (ALL) Chronic myelogenous leukemia (CML; in blast crisis) Myelodysplastic syndromes

Ifosfamide	Testicular cancer (recurrent) Germ cell tumors (recurrent) Sarcomas (soft-tissue, osteogenic sarcoma, Ewing's sarcoma) Non-Hodgkin's lymphoma Hodgkin's disease Non-small cell and small cell lung cancer Bladder cancer Head and neck cancer Cervix cancer
Irinotecan	Metastatic colon or rectal cancer
Isotretinoin	Being investigated for use treating various different cancers
Lomustine	Brain tumors Hodgkin's disease Non-Hodgkin's lymphoma Melanoma Lung cancer Colon cancer
Mechlorethamine	Hodgkin's disease Non-Hodgkin's lymphoma Lung cancer Breast cancer Mycosis fungoides (cutaneous T-cell lymphoma)
Melphalan	Multiple myeloma Ovarian cancer Neuroblastoma Rhabdomyosarcoma Breast cancer
Mercaptopurine	Acute lymphoblastic leukemia (ALL)
Methotrexate	Breast cancer Head and neck cancer Lung cancer Stomach cancer Esophageal cancer Acute lymphoblastic leukemia (ALL) Sarcoma Non-Hodgkin's lymphoma (NHL) Gestational trophoblastic cancer Mycosis fungoides (cutaneous T-cell lymphoma)
Mitomycin	Adenocarcinoma of the stomach or pancreas Anal cancer Bladder cancer Breast cancer Cervical cancer Colorectal Head and neck cancer

	Non-small cell lung cancer
Mitoxantrone	Prostate cancer (advanced; not responding to hormone treatment) Acute myelogenous leukemia (AML) Breast cancer Non- Hodgkin's lymphoma
Oxaliplatin	Colon cancer (metastatic) Rectal cancer (metastatic) [often given in combination with fluorouracil and/or leucovorin]
Paclitaxel	Breast cancer Ovarian cancer Lung cancer Bladder cancer Prostate cancer Melanoma Esophageal cancer Kaposi's sarcoma
Pegaspargase	Acute lymphocytic leukemia (ALL) Non-Hodgkin's lymphoma
Pemetrexed	Malignant mesothelioma Non-small cell lung cancer (after prior chemotherapy)
Procarbazine	Hodgkin's disease Non-Hodgkin's lymphoma Brain tumors Melanoma Lung cancer Multiple myeloma
Streptozocin	Islet cell cancer of the pancreas Carcinoid tumor and syndrome
Temozolomide	Anaplastic astrocytoma Metastatic malignant melanoma
Teniposide	Acute lymphocytic leukemia (particularly in children)
Thioguanine	Acute myelogenous leukemia Chronic myelogenous leukemia
Thiotepa	Breast cancer Ovarian cancer Hodgkin's lymphoma Non-Hodgkin's lymphoma Superficial tumors of the bladder
Topotecan	Ovarian cancer Small cell lung cancer
Tretinoin	Acute promyelocytic leukemia (APL, APML; in patients non-responsive to other treatments)
Vinblastine	Hodgkin's disease Non-Hodgkin's lymphoma

	Testicular cancer Breast cancer Lung cancer Head and neck cancer Bladder cancer Kaposi's sarcoma Mycosis fungoides (T-cell lymphoma) Choriocarcinoma Histiocytosis Idiopathic thrombocytopenia purpura (ITP)
Vincristine	Acute leukemias Hodgkin's lymphoma Non-Hodgkin's lymphoma Neuroblastoma Rhabdomyosarcoma Ewing's sarcoma Wilms' tumor Multiple myeloma Chronic leukemias Thyroid cancer Brain tumors
Vinorelbine	Non-small cell lung cancer Breast cancer Ovarian cancer Hodgkin's disease
Monoclonal Antibodies	
Treatment Modality	Cancers and Other Conditions Treated
Alemtuzumab	B-cell chronic lymphocytic leukemia (B-CLL)
Bevacizumab	Metastatic colon or rectal cancer
Cetuximab	Metastatic colorectal cancer (may be effective in colorectal cancers that over-expresses the epidermal growth factor receptor (EGFR))
Gemtuzumab ozogamicin	CD33 positive acute myeloid leukemia (AML) (in first relapse, 60 years of age or older, and for whom other chemotherapy is not recommended)
Trastuzumab	Metastatic breast cancer (may be effective against tumors that overexpress the HER2/neu protein) [It is not known whether or not trastuzumab may be effective in other cancers that may also have this HER-2/neu protein, including ovarian, stomach, colon, endometrial, lung, bladder, prostate, and salivary gland tumors]
Rituximab	Non-Hodgkin's lymphoma

5 Specific Labeling of Cancer Cells within a Patient Population

Typically, labels that specifically bind to a cancer cell bind to a cell-surface marker. Cell-surface markers include, for example, proteins, such as enzymes, hormones, oncofetal antigens, and blood-group antigens; and cell-surface displayed carbohydrates, including glycoproteins and glycolipids. Labels that may be suitably employed for specifically labeling one or more cancer cells include protein ligands, antibodies, and
10 fragments thereof.

As used herein, the term “specifically bind” as used in the context of the binding of a label to a cell-surface marker refers to binding to the cell-surface marker at a detectable level (within, for example, an ELISA assay) and absence of detectable binding with unrelated polypeptides under similar conditions. Specific binding, as used in this
15 context, generally refers to the non-covalent interactions of the type that occur between an antibody and an antigen for which the antibody is specific. The strength, or affinity of antibody-target antigen binding interactions can be expressed in terms of the dissociation constant (K_d) of the interaction, wherein a smaller K_d represents a greater affinity. Specific binding properties of target-specific antibodies can be quantified using methods well known in the art. One such method entails measuring the rates of target-specific antibody/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the “on rate
25 constant” (K_{on}) and the “off rate constant” (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off}/K_{on} enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant K_d . See, generally, Davies *et al.*, *Annual Rev. Biochem.* 59:439-473 (1990). By “specifically bind” herein is meant that the label binds to a cell-surface
30 antigen, whether a protein, carbohydrate, glycoprotein, lipid, or glycolipid, with a dissociation constant in the range of at least 10^{-6} - 10^{-9} M, more commonly at least 10^{-7} - 10^{-9} M.

Exemplary cell-surface cell-surface protein markers include, but are not limited to, Alpha-fetal Protein (AFP), Human Chorionic Gonadotrophin (hCG), Calcitonin, CA27.29, CA15-3, CA19.9, CA125, CA72-4, Carcinoembryonic Antigen (CEA), CD22,
35 CD3, CD33, CD13, CDw65, Lactoferrin (LF), CD68, CD15, CD15s, CD79a (MB-1), CD79b (B29), CD87 (uPA-R), and CD117 (c-kit). Exemplary cell-surface carbohydrate markers include, but are not limited to, N-linked and O-linked mucin-like glycoproteins;

- 5 cell membrane-bound mucin-like carbohydrate structures such as Tn, sialyl-Tn, and T; and blood group-related carbohydrate structures such as Le(x), sialyl-Le(x), ABH, and Le(y). An exemplary cell-surface glycolipid is CA19.9.

In the case of cancers of the blood, including leukemias and lymphomas, one or more of the following cell-surface markers may be advantageously employed: (1) T-cell markers -- CD1a, CD2, CD3, CD4, CD5, CD7, CD8, TCR alpha-beta, TCR gamma-delta; (2) B-cell markers -- CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79b, CD103, Total Ig, Kappa, Lambda, IgG, IgM, IgD, IgA, FMC-7; and (3) Myeloid/Monocytic-cell markers -- CD13, CD14, CD15, CD33, CD11b, CD117, myeloperoxidase. One or more other marker(s) may also be used such as, for example, CD11c, CD25, CD34, CD45, HLA-Dr, Tdt, CD16, CD30, CD38, CD41, CD42b, CD56, CD57, CD61, glycophorin. Table 2, below, further identifies specific cell-surface antigens and their hematopoietic disease associations. In the specific case of CLL, exemplified herein, leukemic cells may be identified with a label comprising antibodies that specifically bind to CD5 and antibodies that specifically bind to CD19.

20

Table 2
Cell-surface Tumor Markers for Hematopoietic Malignancies

Cell-surface Antigen	Disease Association
CD1	• T-cell lymphoblastic lymphoma • T-cell ALL • Langerhan's cell histiocytosis
CD2	• T-cell neoplasms
CD3	• Mature T-cell neoplasms • Immature T-cell neoplasms may have cytoplasmic CD3, without cell-surface CD3
TCR - $\alpha\beta$	• Most T-cell neoplasms that express CD3
TCR - $\gamma\delta$	• Rare mature T-cell neoplasms • Some lymphoblastic lymphomas
CD4	• Some mature T-cell neoplasms, including most cases of T-cell CLL/prolymphocytic leukemia, peripheral • T-cell lymphoma, adult T-cell leukemia/lymphoma • Sézary syndrome/mycosis fungoides
CD5	• B-cell CLL • Nodal small lymphocytic lymphoma • Mantle cell lymphoma • May be absent in peripheral T-cell lymphoma and plasmacytoid lymphoma • Most T-cell neoplasms

CD7	• Lymphoblastic lymphoma • T-cell ALL • May be absent in peripheral T-cell lymphoma, adult T-cell leukemia/lymphoma, and mycosis fungoides • Some AML
CD8	• Some mature T-cell neoplasms • Large granular lymphocytosis
CD9	• B-cell ALL • Some AML • Some CLL
CD10	• Precursor B-cell ALL • Follicular lymphoma • Small noncleaved lymphoma • Immature T-cell neoplasms
CD11a	• May be absent in B-cell neoplasms including: • Burkitts lymphoma • Hairy cell leukemia
CD11b	• Some AML
CD11c	• Hairy cell leukemia • Marginal zone lymphoma • Some other B-cell neoplasms • Some AML
CD13	• AML
CD14	• Some AML
CD15	• Some AML • Reed-Sternberg cells
CD16	• Large granular lymphocytosis • Absent on neutrophils in PNH
CD18	• Some B-cell neoplasms • Absent in leukocyte adhesion deficiency (LAD)
CD19	• B-cell ALL • Mature B-cell neoplasms • Usually absent in plasma cell neoplasms
CD20	• Some B-cell ALL • Most mature B-cell neoplasms • Usually absent in plasma cell neoplasms
CD21	• Some mature B-cell neoplasms, including: B-cell CLL and Follicular lymphoma
CD22	• Some B-cell ALL • Most mature B-cell neoplasms
CD23	• B-cell CLL • Nodal small lymphocytic lymphoma • Usually absent in mantle cell lymphoma
CD24	• Most B-cell ALL • Most mature B-cell neoplasms • Acute monocytoid leukemias
CD25	• Adult T-cell leukemia/lymphoma • Hairy cell leukemia • Some T and B-cell lymphomas, including large cell anaplastic

	lymphomas • Reed-Sternberg cells
CD26	
CD29	
CD30	• Reed-Sternberg cells • Large cell anaplastic lymphoma
CD33	• AML
CD34	• Many acute leukemias • Not lineage specific
CD38	• Plasmacytic neoplasms • Peripheral T-cell lymphomas • Some B-cell lymphomas
CD41	• AML M7 • Absent in Glanzmann's thrombasthenia
CD42b	• AML M7 • Reduced in Bernard-Soulier syndrome
CD45	• May be reduced/absent in: ALL, Lymphoblastic lymphoma, Large cell anaplastic lymphoma, Immunoblastic lymphoma, and Plasma cell proliferations
CD45RA	• Some T-cell lymphomas • Some mature B-cell lymphomas
CD45RO	• T-cell lymphomas
CD55	• PNH caused by stem cell clones with decreased CD55 on RBCs
CD56	• Large granular lymphocytosis • Angiocentric lymphomas
CD57	• Large granular lymphocytosis
CD59	• PNH caused by stem cell clones with decreased CD59 on RBCs
CD61	• AML M7
CD69	
CD71	• Acute leukemias • Intermediate and high grade lymphomas • Reed-Sternberg cells
CD103	• Hairy cell leukemia • Some splenic lymphomas
CD117	• AML • Rare ALL
HLA-DR	• B-cell leukemias/lymphomas • Most AML (except M3) • Reed-Sternberg cells • Peripheral T-cell lymphoma
FMC-7	• Prolymphocytic leukemia • Hairy cell leukemia • Splenic lymphoma
Glycophorin	• AML M6
TdT	• Lymphoblastic lymphoma • B-cell ALL • T-cell ALL
Modified from Wittwer <i>et al.</i> , "ARUP's Guide to Clinical Laboratory Testing" http://www.arup-lab.com/guides/clt/tests/clt_fro8.jsp	

5

In order to achieve detection with the multiparameter flow cytometric cytotoxicity methods of the present invention, labels typically include one or more fluorescent dye such as, for example, allophycocyanin (APC), phycoerythrin (PE), and fluorescein-isothiocyanate (FITC). Labels may also include a tag such as biotin. Within related
10 aspects, the label may be a first antibody that does not otherwise include a fluorescent dye or other tag. In such cases, a second antibody may be employed wherein the second antibody is capable of specifically binding to the first antibody. Within such aspects, the second antibody typically includes one or more fluorescent dye or other detectable tag.

A wide variety of antibodies that specifically bind to the cell-surface antigens
15 disclosed herein and that may be suitably employed in the systems and methods of the present invention are readily available from commercial sources. For example, Bioprocessing, Inc. (Scarborough, ME) provides antibodies to Ca15-3, Ca27.29, breast tumor antigens, Ca125, ovarian tumor antigen, Ca19-9, GI tumor antigen, CEA, carcinoembryonic antigen, AFP, alpha fetoprotein, Ca72.4, Tag-72, NSE, neuron specific
20 enolase, Cyfra21-1, cytokeratin-19, PSA, prostate specific antigen using cell culture technologies; Diatec (Oslo, Norway) provides monoclonal antibodies including unconjugated antibodies and antibodies conjugated with FITC, PE, biotin, and APC against markers for leukemias and lymphomas; EMD Biosciences (San Diego, CA) and EXBIO Praha (Vestec, Czech Republic) provide monoclonal antibodies to human CD
25 proteins; Euroclone (Pero, Italy) provides monoclonal antibodies to human CD antigens, cytokines, and apoptosis markers; ImmunoTools (Friesoythe, Germany) provides antibodies to cell markers for flow cytometric applications, anti-cytokine antibodies; Insight Biotechnology (Middlesex, UK) provides antibodies directed against a number of cytokines, CDs as well as secondary antibodies; Jackson ImmunoResearch Laboratories,
30 Inc. (Bar Harbor, ME) provides a wide selection of secondary antibodies; Invitrogen/Molecular Probes (Eugene, OR) provides primary and secondary antibodies, including fluorescently tagged antibodies, for flow cytometry; QED Biosciences, Inc. (San Diego, CA) provides antibodies directed to antibodies for cancer markers and apoptosis; R&D Systems (Minneapolis, MN) provides antibodies against cytokine
35 receptors; Vector Laboratories (Burlingame, CA) provides a wide range of primary and secondary antibodies including primary antibodies for flow cytometry and against various tumor markers. Immunostaining procedures for labeling cell-surface markers are readily available in the art. For example, see Ormerod, "Flow Cytometry, A Practical Approach"

- 5 (3rd Ed., Oxford University Press), which is incorporated herein by reference, describing the selection of fluorochromes, methodology for the preparation of single cell suspensions, and techniques for achieving the immunofluorescence of surface markers.

Methodology for Assessing Cancer Cell Cytotoxicity

- 10 The present invention provides multiparameter flow cytometry systems and methods to permit the rapid and simultaneous measurement of individual cells within the context of heterogenous cell populations. Typical flow cytometers comprise three major systems: fluidic, optical, and electronic. The fluidics system pumps a suspension of cells through a nozzle, creating a stream with laminar flow properties. The optical system
15 focuses one or more laser beams on the stream and collects scattered and fluoresced light from cells as they pass through the laser beam. The electronics measure and digitize these light emissions so that they can be analyzed on a desktop computer.

- The following definitions are commonly used to describe flow cytometer parameters. As used herein, the terms "forward Scatter", "FSC", "FS" and "FALS" are
20 used interchangeably to refer to the parameter that is a rough indicator of a cell's size. The terms "side scatter", "SSC", and "SS" are used interchangeably to refer to the parameter that is a rough indicator of cellular granularity, membrane complexity, and number of organelles. Taken together, the two scatter parameters provide a morphological fingerprint of the cells passing through the flow cytometer.

- 25 Cell-surface markers, such as proteins, carbohydrates, and lipids (see, above) are detected with one or more label, typically an antibody, that has been conjugated to a fluorescent molecule such as, for example, FITC, PE, Texas Red, and APC. DNA content can be measured with propidium iodide (PI), 7-AAD, the Hoechst dyes, or DAPI. BrdU incorporation may be employed for measuring cell proliferation. Apoptosis can be
30 measured in a number of ways. The TUNEL technique identifies DNA strand breaks. Annexin V labeling detects changes in the plasma membrane asymmetry. Nuclear condensation and DNA loss are demonstrated by hypodiploid peaks in DNA content experiments. Uptake of Hoechst 33258 also increases with apoptotic changes.

- A number of other markers and assay systems have been described for assessing
35 the following cellular functions: (1) mitochondrial function using Rh123 or DiOC6; (2) oxidative burst using HE, DCFH-DA, or DHR; (3) reporter genes using GFP and LacZ, along with the substrate FDG; (4) glutathione/reductive reserve using MCB; (5) Ca⁺⁺ flux using Indo-1; (6) enzyme activities using fluorescent substrates; (7) pH using

- 5 SNARF; (8) cell division and conjugation using fluorescein, calcein, or PKH26; (9) total protein content using SR101 or fluorescein)

Assay systems that are available in the art may be employed for measuring the cytotoxicity of a specifically labeled cancer cell treated with a candidate chemotherapy modality and/or biomolecule. As exemplified herein, cytotoxicity may be measured by staining with annexin, anti-phosphatidylserine, and/or 7-AAD.

For example, Cell Technology, Inc. (Mountain View, CA) offers an assay system for measuring cell-mediated cytotoxicity/antigen-dependent cytotoxicity (CMC/ADCC). By this methodology, a cell tracking dye CFSE is utilized to label a patient cell population. De Clerck *et al.*, *J. Immunol. Meth.* 172:115 (1994) and Bronner-Fraser, *J. Cell Biol.* 101:610 (1985). Cell death is measured by addition of 7AAD (live/dead). Rabinovitch *et al.*, *J. Immunol.* 136:2769 (1986) and Su, *J. Immunol.* 156:4198 (1996). 7AAD only enters membrane-compromised cells and binds to DNA. Flow cytometry is utilized to gate on the target cells and measure 7AAD negative vs. 7AAD positive cells. Percent cytotoxicity is calculated by the following equation:

$$20 \quad \text{7AAD positive (upper right quadrant)} = \frac{\text{7AAD Positive (upper right quadrant)}}{\text{7AAD negative (lower right quadrant)}} \times 100$$

Assay systems for measuring apoptosis, such as those based on two-color flow cytometry, may also be employed in the systems and methods presented herein. After a candidate therapeutic modality is incubated with a patient sample comprising one or more cancer, the cancer cells are stained, as described above, with a target cell-specific label such as, for example, a PE-conjugated mAb. Annexin V-FITC, which binds to cells expressing phosphatidylserine (an early marker of apoptosis) on the cell-surface, is subsequently added. Cancer cells are gated upon and quantified with respect to their annexin V positivity. The shift from annexin Vneg to annexin Vhi is a discrete event such that all cancer cells fall within discernible populations with respect to annexin V. This methodology permits the analysis of single cancer cells.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. These examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

5

EXAMPLESExample 1Fludarabine Resistance in Chronic Lymphocytic Leukemia (CLL) Detected by an *In vitro* Multiparameter Flow Cytometric Cytotoxicity Assay

10

This Example discloses an *in vitro* multiparameter flow cytometry-based drug sensitivity and resistance assay for routine analysis of clinical samples containing a heterogeneous population of both leukemic and normal hematopoietic cells.

43 clinical specimens of peripheral blood or bone marrow were isolated from patients with B-cell CLL diagnosed at the Oregon Health & Science University. Clinical characteristics of the corresponding patients are presented in Table 3.

Table 3Clinical and Cytogenetic Features of B-cells from 43 Patients with CLL

20

Male/Female	25/18
Median Age (Range)	70(33-93)
Rai Stage	Number of Patients
0-3	19
3-4	8
Unknown	16
Total Patients	43
Interphase FISH	Number of Patients
Del p53, High (>10%)	7
Del p53, Low (<10%)	3
-11q	4
+12	3
-13q	7
Normal	13
Unknown	6
Total Patients	43

Red cells were removed from each patient sample by Ficoll gradient centrifugation by standard methods. Flow cytometric analysis was performed with fresh samples for diagnostic classification. The remaining cells were cryopreserved in liquid nitrogen with RPMI 1640 culture medium (GIBCO, Grand Island, NY) containing 20% heat-inactivated fetal bovine serum (FBS) (GIBCO, Grand Island, NY) and 10% DMSO (Sigma, St. Louis, MO).

Cryopreserved cells were thawed in a water bath at 37°C and transferred to RPMI 1640 tissue culture medium containing 2.5 mM MgCl₂ and 200 units/ml DNAase I. After

5 incubation at room temperature for 10 minutes, cells were washed twice with phosphate buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 20% FBS, 2 mM glutamine, 100 µg/ml streptomycin, and 100 µg/ml penicillin. Cell viability was determined by trypan blue dye exclusion. Cell concentration was adjusted to $1.5\text{--}3.0 \times 10^6$ viable cells/ml.

10 150 µl of each cell suspension was added to each well of a 96-well microtiter plate containing 50 µl of either Fludarabine (Sigma, St. Louis, MO), Chlorambucil (Sigma, St. Louis, MO), Cladribine (Sigma, St. Louis, MO), or Prednisolone (Sigma, St. Louis, MO), at 4x their respective empirically derived cut-off concentrations (EDCC), which produced the largest scatter of B-cell CLL cell survival seen among 12 representative samples with
15 test protocols. Each drug was tested in triplicate for each treatment group. The culture plates were incubated at 37°C in humidified atmosphere containing 95% air and 5% CO₂ for 48 hours. At the end of the incubation period, the culture plates were centrifuged at 1500 rpm for 5 minutes and the supernatants discarded.

The cells in each well were resuspended with 100 µl of RPMI 1640 supplemented
20 with 10% FBS. 2.5 µl of CD19-APC (BD Biosciences, San Diego, CA) and 2 µl of CD5-PE (BD Biosciences, San Diego, CA) were added to each well. Plates were incubated on ice for 30 minutes in the dark. After one wash with 250 µl 1x binding buffer (BD Biosciences, San Diego, CA), 100 µl of 1x binding buffer was added. The cells in each well were resuspended and transferred to 120 x 75 mm tubes.

25 2 µl of Annexin V-FITC (BD Biosciences, San Diego, CA) and 1 µl of 1 mg/ml 7-AAD (Sigma, St. Louis, MO) were added to each tube and incubated at room temperature in the dark for 10 minutes. 300 µl of 1x binding buffer was added to each tube.

Analysis was performed on a Becton Dickinson FACSCalibur flow cytometer. Results were analyzed using CellQuest Pro software. The specific strategy used is
30 illustrated in Fig. 1. Cells that were CD5 positive, CD19 positive, 7-AAD negative, and Annexin V negative were counted as surviving leukemic cells. A leukemic cell survival index (LCSI) was defined as the average number of viable leukemic cells in drug-treated cultures divided by the average number of viable leukemic cells in the untreated control cultures expressed as a percentage.

35 Statistical analyses were performed using statistic software GraphPad InStat (GraphPad Software, San Diego, CA). The difference of LCSI among different groups was determined by means of one-way analysis of variance or Student's *t*-test. Correlation

5 of drug effect between different drugs was measured by the Pearson correlation coefficient. Differences were considered to be statistically significant when p values were <0.05 .

Exposure to drugs for 48 hours yielded detectable cytotoxicity. The optimal drug concentrations that produced the greatest survival of B-CLL cells were 2.5, 2.5, 0.5, and
10 25 $\mu\text{g/ml}$ for Fludarabine, Chlorambucil, Cladribine, and Prednisolone, respectively, when representative samples were tested. Although spontaneous apoptosis was significant and variable, it did not interfere with the measurement of drug induced cytotoxicity. Viable cells were separated from cells showing toxic effects including cells with early apoptosis (Annexin V positive but 7-ADD negative) and cells with complete
15 loss of membrane integrity (Annexin V positive and 7-ADD positive). Marked variability was seen in the drug-induced cytotoxicity in leukemic cells of 43 cases of B-CLL tested. As shown in Fig. 2, LCSI after drug exposure in culture ranged from less than 10% to 189% among these samples.

A good correlation was observed among responses to Fludarabine, Chlorambucil,
20 and Cladribine in a given B-CLL specimen. See, Figs. 4A-4C. Cross-resistance amongst these 3 drugs was not, however, always observed for individual samples. No correlation was observed for drug-induced cytotoxicity between Prednisolone and the other 3 drugs. See, Figs. 4D-4F. In a majority of the B-CLL samples, there was at least an additive effect of Prednisolone with Cladribine and Chlorambucil. No or very little additive drug
25 effect was observed among a few samples with leukemic cells highly resistant to individual drugs. Occasionally, individual B-CLL samples showed resistance to all drugs and drug combinations tested.

The clinical features were retrospectively reviewed and correlated with the results of the assay for drug resistance. Of 29 patients with stage information available, there
30 was a significant difference between the B-CLL cells from patients at early stages (0-II) and advanced stages (III/IV) (Fig. 5A). B-CLL cells from patients with advanced disease were less sensitive to Fludarabine, Chlorambucil, and Cladribine than were B-CLL cells from patients with early disease. No difference was observed, however, for Prednisolone. Fig. 6B. Of 25 patients with clinical follow-up information, 18 patients were treated with
35 variable regimens and seven patients were on the "watch and wait" list because of indolent disease that does not meet the clinical criteria of treatment. B-CLL cells from patients who were not yet treated generally had greater sensitivity to Fludarabine,

- 5 Chlorambucil, and Cladribine as compared to patients who had more aggressive disease requiring treatment.

These data demonstrate that *in vitro* drug sensitivity assays are predictive of clinical response and prognosis and to monitor the development of drug resistance during treatment and to identify possible new therapeutic strategies in B-CLL patients. See, also,
10 Bousanquet, *Lancet* 337:711-714 (1991); Larsson *et al.*, *Int. J. Cancer* 50:177-185 (1992); Morabito *et al.*, *Br. J. Haematol.* 102:528-531 (1998); Callea *et al.*, *Haematologica* 84:863-864 (1999); Bousanquet *et al.*, *Br. J. Haematol.* 106:71-77 (1999); Sturm *et al.*, *Cell Death Differ.* 10:477-484 (2003); Bousanquet and Bell, *Blood* 87:1962-1971 (1996); Bousanquet and Bell, *Leuk. Res.* 20:143-153 (1996); and Bousanquet *et al.*,
15 *Br. J. Cancer* 76:511-518 (1997).

The presently described assay system is relatively robust and could be adapted to routine clinical laboratory, superior to other *in vitro* assays such as the MTT assay (Twentyman *et al.*, *Br. J. Haematol.* 71:19-24 (1989)), the DISC assay (Bousanquet, *Lancet* 337:711-714 (1991)), and FCMA (Larsson *et al.*, *Int. J. Cancer* 50:177-185
20 (1992)), requiring a relatively homogeneous population of leukemic cells in clinical samples typically seen in advanced stage only.

A majority of clinical samples from CLL patients have leukemic cells admixed with a variable number of normal lymphocytes and other normal hematopoietic cells. CLL cells have morphological features indistinguishable from admixed normal
25 lymphocytes that are almost always sensitive to chemotherapeutic drugs. Our protocol is suitable to all routine clinical samples regardless of the percentages of B-CLL cells in the samples.

Taking the advantage of multiparameter flow cytometry, cell population surface markers and drug-induced cytotoxicity are measured simultaneously. In addition, the
30 present methods eliminate possible interference of spontaneous apoptosis that may overshadow the actual drug effect on the B-CLL cells seen in other assays. Therefore, the present assay system is suitable for both fresh and cryopreserved samples. This assay detects a remarkable variability in sensitivity to various drugs and their combinations in B-CLL cells, which is consistent with the reported results of other *in vitro* chemotherapy
35 sensitivity assays. Morabito *et al.*, *Br. J. Haematol.* 102:528-531 (1998); Callea *et al.*, *Haematologica* 84:863-864 (1999); Bousanquet *et al.*, *Br. J. Haematol.* 106:71-77 (1999); Bousanquet *et al.*, *Br. J. Haematol.* 106:474-476 (1999).

5 The present data also demonstrate that cross-resistance among fludarabine, chlorambucil and cladribine was quite common. The difference of drug sensitivity among different cytogenetic groups of B-CLL provides additional validation for the reliability of *in vitro* drug effect as measured by our multiparameter flow cytometric cytotoxicity assay and supports the utility of personalization of therapy for B-CLL
10 patients with different cytogenetic abnormalities and *in vitro* drug sensitivity profiles. The methodology disclosed herein permit the assessment of drug sensitivity and resistance in drug discovery, clinical trials, and routine clinically practice and offers a cost effective approach to help clinicians identify optimal therapeutic regimens for biologically heterogeneous diseases.

15

Example 2

In vitro Resistance to Fludarabine Correlates with Deletion of p53 or ATM

This Example demonstrates that deletion of p53 and ATM genes is correlative of B-CLL resistance to Fludarabine.

20 The prognostic significance of cytogenetic abnormalities has been well established for B-CLL over the last decade. Fenaux *et al.*, *Leukemia* 6:246-250 (1992); el Rouby *et al.*, *Blood* 82:3452-3459 (1993); Wattel *et al.*, *Blood* 84:3148-3157 (1994); Dohner *et al.*, *Blood* 85:1580-1589 (1995); Dohner *et al.*, *Leukemia* 11 Suppl 2:S19-24 (1997); Shaw and Kronberger, *Cancer Genet Cytogenet.* 119:146-154 (2000); Oscier *et al.*, *Blood* 100:1177-1184 (2002); and Stilgenbauer and Dohner, *Hematol. Oncol. Clin. North Am.* 18:827-848 (2004).
25

Deletions of p53 or ATM are associated with an aggressive clinical course and poor survival. In addition, B-CLL patients with a p53 deletion have been found to be almost invariably resistant to therapy with Fludarabine, currently the first line drug. It is
30 believed that such drug resistance, at least in part, results from a defect in activation of p53-dependent pro-apoptotic responses after DNA damage due to a loss of the p53 gene located on 17p. Rosenwald *et al.*, *Blood* 104:1428-1434 (2004) and Stankovic *et al.*, *Blood* 103:291-300 (2004).

Previous studies examining the association between p53 mutation and *in vitro*
35 drug sensitivity to purine analogs or alkylating agents, have yielded inconsistent and sometimes conflicting results. Thomas *et al.*, *Oncogene* 12:1055-1062 (1996); Johnston *et al.*, *Leuk. Lymphoma* 26:435-449 (1997); Bromidge *et al.*, *Leukemia* 12:1230-1235 (1998); Pettitt *et al.*, *Br. J. Haematol.* 106:1049-1051 (1999); and Sturm *et al.*, *Cell Death*

- 5 *Differ.* 10:477-484 (2003). Differences in *in vitro* drug sensitivity profiles among different cytogenetic subgroups of B-CLL has not been previously studied.

To correlate *in vitro* drug sensitivity/resistance results with cytogenetic abnormalities, each of the 43 cases described in Example 1 was further studied by interphase FISH in order to identify and characterize common cytogenetic abnormalities in B-CLL, including trisomy 12, deletion of p53, ATM, or 13q. Interphase FISH was performed with fresh slides made from the fixed cell pellets of untreated cells. Hybridization was performed using commercially available probes (Vysis, Downers Grove, IL) to detect genomic losses in 13q, ATM (11q22.3), and p53 (17p13.1), and to detect trisomy 12 (CEP12). 200 interphase nuclei were scored for hybridization signals for each probe. On the basis of the results in healthy controls, chromosomal losses and gains were interpreted as negative if they occurred in 5% or fewer of the nuclei.

The FISH results for 37 B-CLL cases are summarized in Table 2. 10 cases of B-CLL with p53 deletion were further divided into 2 subsets, based on the percentages of cells with p53 deletions as defined by FISH. As shown in Fig. 5A, 7 cases with high p53 deletion (*i.e.* > 10% of cells in the sample) were most resistant to Fludarabine-induced cytotoxicity, among these 5 different cytogenetic groups. Cases with ATM deletions had moderate resistance to Fludarabine. Cases with low p53 deletion (<10% of cells), were sensitive to Fludarabine similar to that of cases with normal cytogenetics, indicating that p53 deletion with in the 5-10% range is most likely false positive due to a variation of background.

No definitive dose effect was detected for p53 deletion. In case 1, four specimens collected at different times over a two-year period were compared. Similar drug resistance was demonstrated in B-CLL cells from peripheral blood, bone marrow, and lymph node. There were no significant changes in drug resistance (data not shown). The presence of additional cytogenetic abnormalities had no significant impact on drug resistance associated with p53 deletion.

Although there was cross-resistance between Fludarabine, Chlorambucil, and Cladribine, in general, *in vitro* sensitivities to Chlorambucil and Cladribine were less well predicted by the cytogenetic abnormalities of individual cases. Occasionally, leukemic cells with p53 deletions had moderate sensitivity to Chlorambucil and Cladribine. There was no association between cytogenetic abnormalities and *in vitro* sensitivity to prednisolone. Fig. 5B.

5 Table 4 lists individual correlations between interphase FISH cytogenetic abnormalities and clinical responses available in follow-up. The seven patients (cases 1 - 7) having the highest B-CLL cells survival indices to Fludarabine had p53 deletions and belonged to a high-risk group clinically. Three of these 7 patients (*i.e.* cases 4-6) were highly resistant to multiple chemotherapeutic drugs, including Fludarabine. The clinical
10 *in vivo* effectiveness of Fludarabine could not be evaluated in the other patients because it had either not been used or was used in combination with other drugs. One specimen (*i.e.* case 7) from a patient with a p53 deletion who was clinically resistant to Fludarabine but responsive to Chlorambucil showed an identical response pattern in the *in vitro* assay.

15

Table 4
Summary of *In vitro* Response, Clinical Response, and Clinical Follow-up

Case ID	Age/sex	Rai	Clinical Course	FISH*	Flu **	Chl **	Cla **	Pre **	Clinical treatment and Response
1	52/F	1	Progressive	Del p53 (75)	160	106	166	56	FCR x 3, PR; BMT, CR
2	33/F	4	Progressive	Del p53 (13)	167	97	60	25	Watch and wait
3	71/F	4	Progressive	Del p53 (100)	113	141	189	104	FCR x 6, CR
4	74/F	3	Progressive	Del p53 (89)	95	79	87	64	Chl, NR; Flu NR; Rit-PR
5	70/M	4	progressive	Del p53 (87) +12 (63)	88	73	63	63	Flu x 4, NR; FC x 2, NR;
6	68/F	4	Progressive	Del p53 (79) - 13q (70)	81	70	64	26	Flu x 1, Rit+Pen x 3 NR; Rit+Pent+Cyt, NR; FCR x 5, PR
7	72/F	1	Stable	Del p53 (74) - 13q (70)	80	46	26	45	Chl, no progression
8	54/F	2	Progressive	Del p53 (5)	36	15	19	13	Chl x 3, PR
9	70/M	1	Progressive	Del p53 (6)	35	21	52	50	Chl+Pre x 3, NR; FCR x 3, PR
10	81/F	1	Stable	Del ATM (80)	77	88	71	31	FCR x 2, PR
11	74/M	4	Progressive	Del ATM (84)	71	NA	75	37	FCR x 1, PR
12	56/M	3	Progressive	Del ATM -(48)	50	NA	78	31	CHOP + DHAP X5, NR, Campath-1 Rit x 1, NR; Cam, NR; Cam+Flu, NR
13	67/M	1	Progressive	Del ATM (80) -13q(29)	58	NA	44	70	Chl+Pre x 3, NR; CHOP x 1, NR;
14	77/F	1	Progressive	+12(25)	27	NA	NA	34	Chl+Pre x 3, NR; Flu x 2, PR; FCR x 2, CR
15	75/M	0	Stable	+12(61)	78	68	81	77	Watch and wait
16	41/M	1	Progressive	-13q(88)	74	85	86	46	CHOP x 5, PR; BMT, CR
17	63/M	2	Progressive	-13q (85)	52	19	22	19	FCR x 6, CR
18	50/M	0	Stable	-13q (32)	32	51	42	39	Watch and wait
19	55/M	2	Progressive	Normal	54	NA	57	30	FCR x 1, PR
20	56/M	0	Stable	Normal	75	56	53	50	Watch and wait
21	60/M	0	Stable	Normal	61	14	46	12	Watch and wait
22	36/M	0	Stable	Normal	41	NA	47	74	Watch and wait
23	80/M	1	Stable	Normal	13	NA	18	40	Watch and wait

Abbreviations: Del, deletion; Flu, fludarabine; Chl, chlorambucil; Cla, cladribine; Rit, rituxan; Pen, pentostatin; Pre, prednisolone; FCR, fludarabine, cyclophosphamide and rituxan; CHOP, cyclophosphamide, hydroxydaunomycin, vincristine and prednisone; BMT, bone marrow transplantation; CR, complete remission; PR, partial response; NR, no response; NA, not available. *Data show as detectable abnormalities and % of abnormal cells. ** data shown as leukemic cell survival index (%).

5

These data demonstrate that high resistance *in vitro* Fludarabine cytotoxicity in B-CLL cells correlates with p53 deletion. Moderate resistance was associated with ATM deletion, and no significant resistance to Fludarabine was associated with other cytogenetic abnormalities. We also found that leukemic cells from patients with p53 deletion were frequently resistant to Chlorambucil and Cladribine but only occasionally resistant to Prednisolone. These findings strongly support the concept that a loss of the function of the p53-dependent apoptotic signaling is a major contribution to drug resistance to DNA damaging agents in B-CLL patients. We therefore provide convincing evidence that B-CLL patients with p53 deletion would not benefit from Fludarabine therapy. The *in vitro* drug resistance seen in B-CLL cells with deletion of ATM is also likely related to the defect in activation of the p53 pathway which is partially dependent on ATM. Pettitt *et al.*, *Blood* 98:814-822 (2001).

With the increasing therapeutic options available for B-CLL, an *in vitro* assay for drug sensitivity may have broad application in the clinical management. For cases with p53 or ATM deletions detected by FISH, this assay could be used to confirm the drug resistance to purine analogues or alkylating agents since occasional B-CLL with monoallelic loss or inactivation of the p53 gene may still have moderate Fludarabine sensitivity. In addition, the assay may be used for identification of drugs that are potentially more effective than Fludarabine since B-CLL cells with Fludarabine resistance *in vitro* are not always resistant to Chlorambucil or Cladribine. See, Case 7 in Table 2. An alternative effective therapy should be sought early for these patients given that they almost invariably have an aggressive course. In cases without p53 or ATM deletions, *in vitro* drug sensitivity are also variable, suggesting additional biological factors may affect the drug effect in B-CLL cells.

CLAIMSWhat is claimed is:

1. A multiparameter flow cytometric cytotoxicity method for assessing whether a cancer cell in a patient sample is susceptible to treatment by a candidate treatment modality, said method comprising the steps of:

(a) collecting from a patient a tissue sample comprising one or more cancer cell(s);

(b) treating said tissue sample *in vitro* with one or more candidate therapeutic modality;

(c) contacting said treated tissue sample with a label that specifically binds to a cell-surface marker on said cancer cells;

(d) contacting said labeled and treated cells with one or more reagent for assaying cytotoxicity; and

(e) analyzing the cytotoxicity of said labeled and treated cells with a flow cytometer gated on said labeled cells,

wherein cytotoxicity of said labeled and treated cells indicates that said cancer cells are susceptible to treatment by said candidate treatment modality.

2. The method of claim 1 wherein said tissue sample is selected from the group consisting of a blood sample, a bone marrow sample, and a solid tumor sample.

3. The method of claim 2 wherein said tissue sample is a blood sample or a bone marrow sample and wherein said one or more cancer cell is associated with a hematopoietic cancer selected from the group consisting of acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), hairy cell leukemia (HCL), non-Hodgkin's lymphoma, and multiple myeloma.

4. The method of claim 2 wherein said tissue sample is a solid tumor sample selected from the group consisting of a cancer of the brain, skin, eye, liver, esophagus, uterine cervix, bone, lung, endometrium, bladder, breast, larynx, colon/rectum, stomach, ovary, pancreas, adrenal gland, prostate, and a soft tissue sarcoma.

5. The method of claim 1 wherein said candidate therapeutic modality is selected from the group consisting of a chemotherapy drug selected from the group consisting of Altretamine, Arsenic Trioxide, Asparaginase, Bleomycin, Busulfan, Capecitabine, Carboplatin, Carmustine, Chlorambucil, Cisplatin, Cladribine, Cyclophosphamide, Cytarabine, Dacarbazine, Dactinomycin, Daunorubicin, Docetaxel, Doxorubicin, Epirubicin, Estramustine, Etoposide, Floxuridine, Fludarabine, Fluorouracil, Gemcitabine, Hydroxyurea, Idarubicin, Ifosfamide, Irinotecan, Isotretinoin, Lomustine, Mechlorethamine, Melphalan, Mercaptopurine, Methotrexate, Mitomycin, Mitoxantrone, Oxaliplatin, Paclitaxel, Pegaspargase, Pemetrexed, Prednisolone, Procarbazine, Streptozocin, Temozolomide, Teniposide, Thioguanine, Thiotepa, Thiotepa, Topotecan, Tretinoin, Vinblastine, Vincristine, and Vinorelbine.

6. The method of claim 1 wherein said candidate therapeutic modality is selected from the group consisting of an antibody selected from the group consisting of Alemtuzumab, Bevacizumab, Cetuximab, Gemtuzumab ozogamicin, Trastuzumab, and Rituximab.

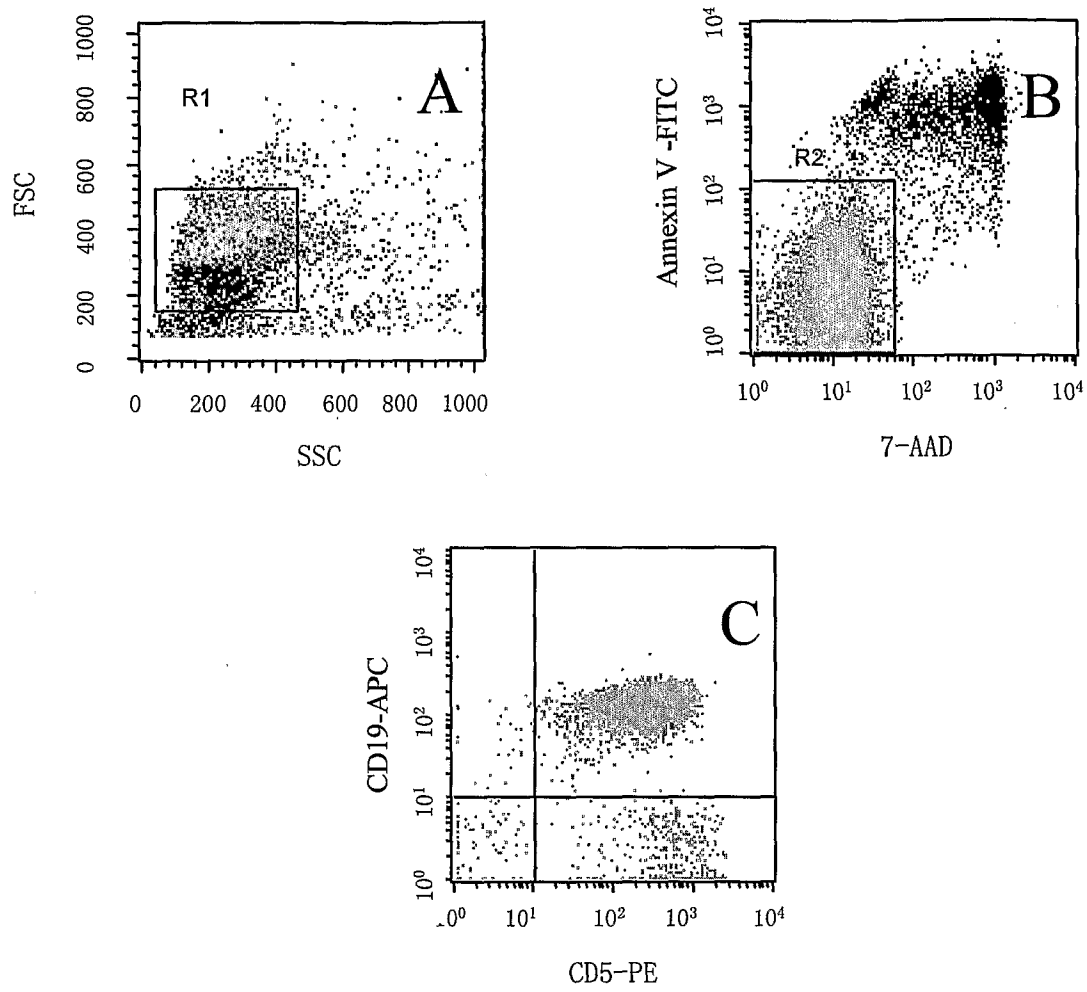
7. The method of claim 1 wherein said cell-surface marker is selected from the group consisting of Alpha-fetal Protein (AFP), Human Chorionic Gonadotrophin (hCG), Calcitonin, CA27.29, CA15-3, CA19.9, CA125, CA72-4, Carcinoembryonic Antigen (CEA), and CA19.9.

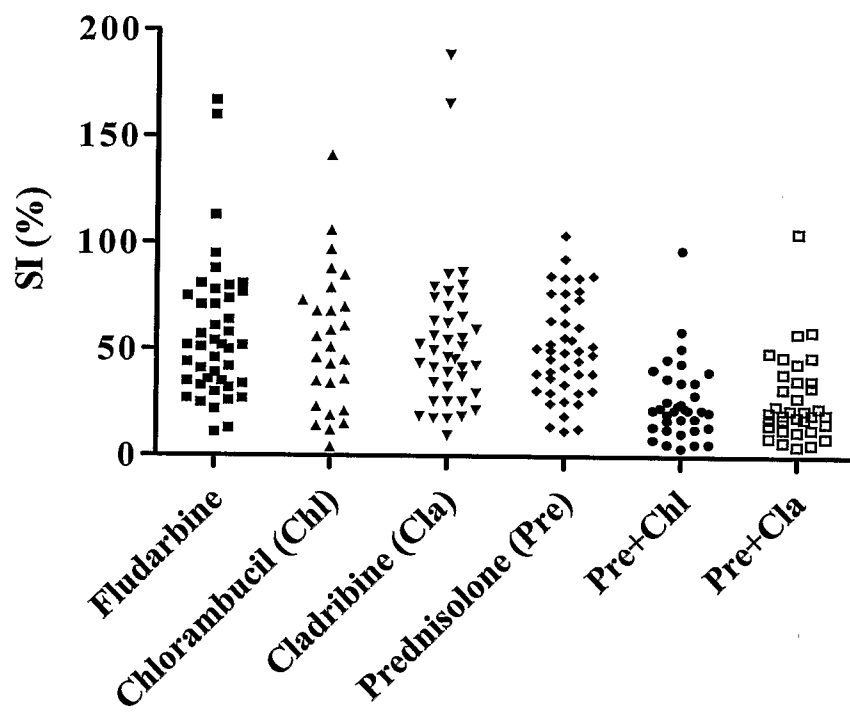
8. The method of claim 1 wherein said cell-surface marker is selected from the group consisting of CD1a, CD2, CD3, CD4, CD5, CD7, CD8, TCR alpha-beta, TCR gamma-delta, CD10, CD19, CD20, CD21, CD22, CD23, CD24, CD79b, CD103, Kappa, Lambda, IgG, IgM, IgD, IgA, FMC-7, CD13, CD14, CD15, CD33, CD11b, CD34, CD117, CDw65, Lactoferrin (LF), CD68, CD15, CD15s, CD79a (MB-1), CD79b (B29), CD87 (uPA-R), CD117 (c-kit),

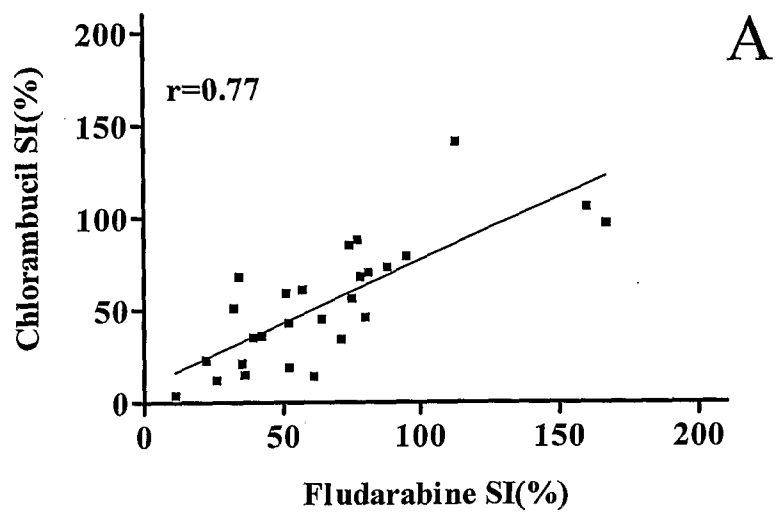
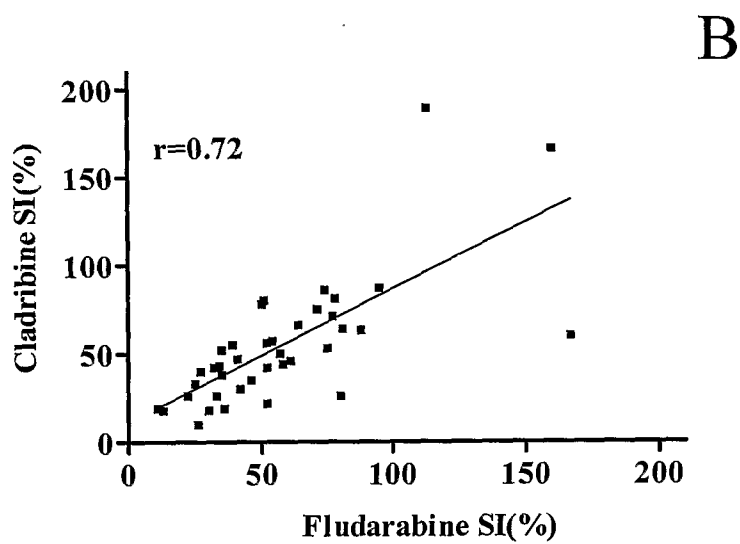
9. The method of claim 1 wherein said cytotoxicity is measured by staining with annexin, anti-phosphatidylserine, and/or 7-AAD.

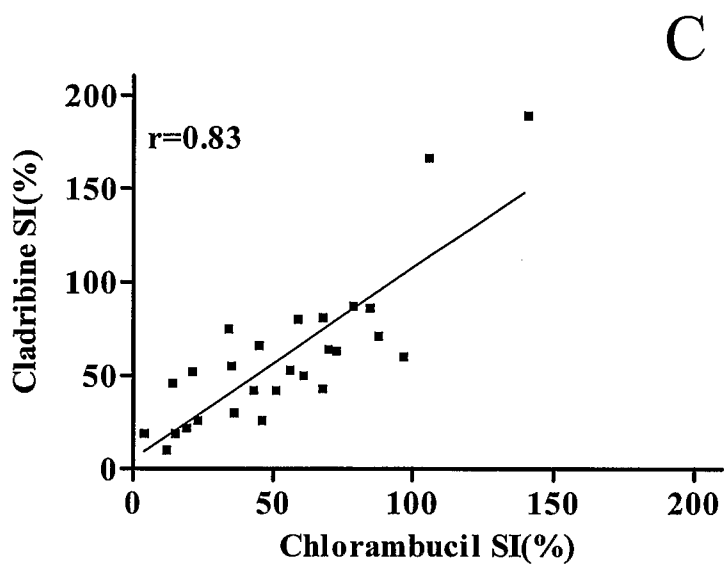
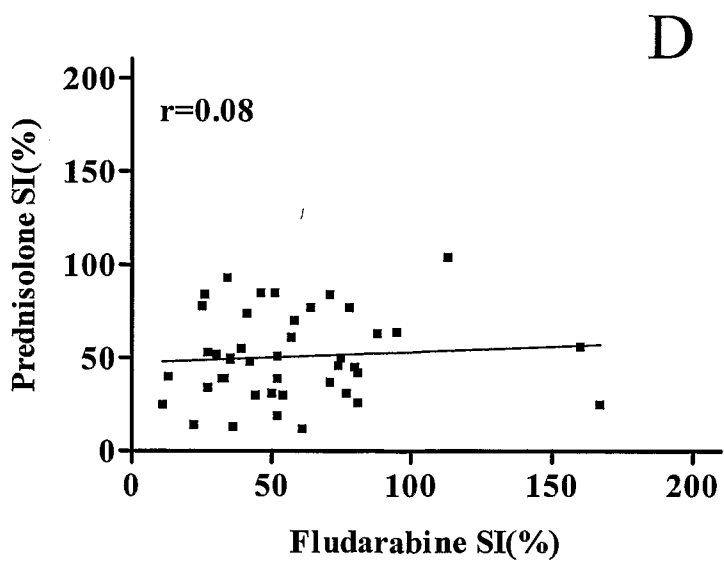
10. The method of claim 3 wherein said cancer cell cancer cell is associated with

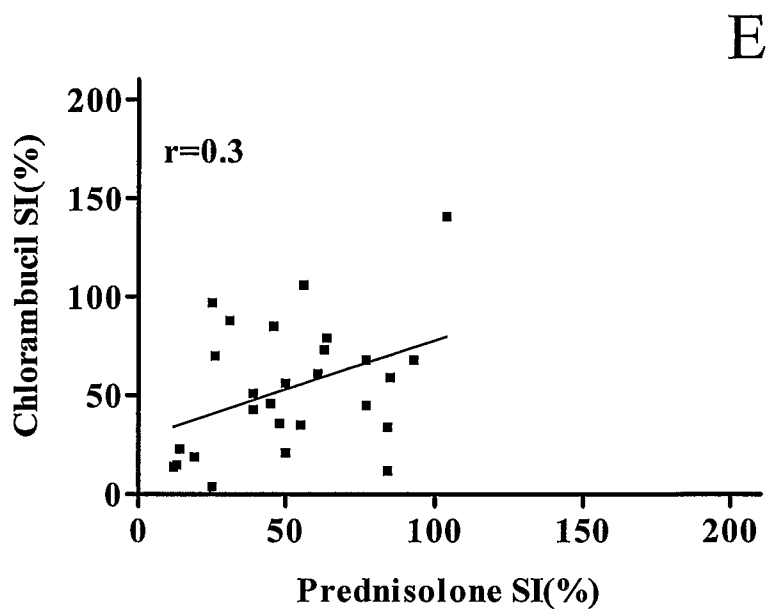
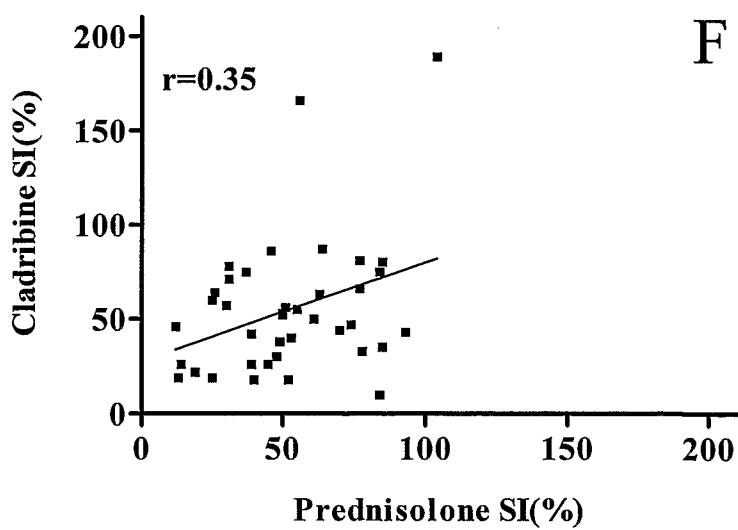
B-cell chronic lymphocytic leukemia (B-CLL), wherein said candidate treatment modality is Fludarabine, and wherein said cell-surface marker is CD5 and CD19.

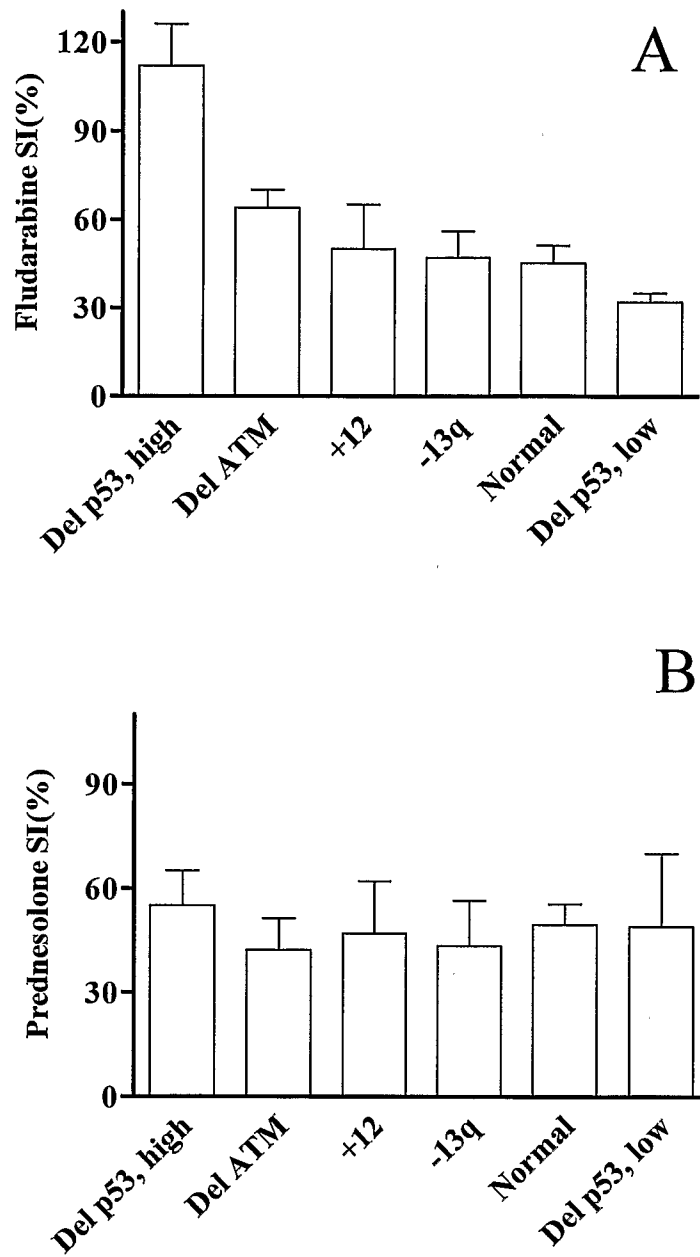
**Figure 1**

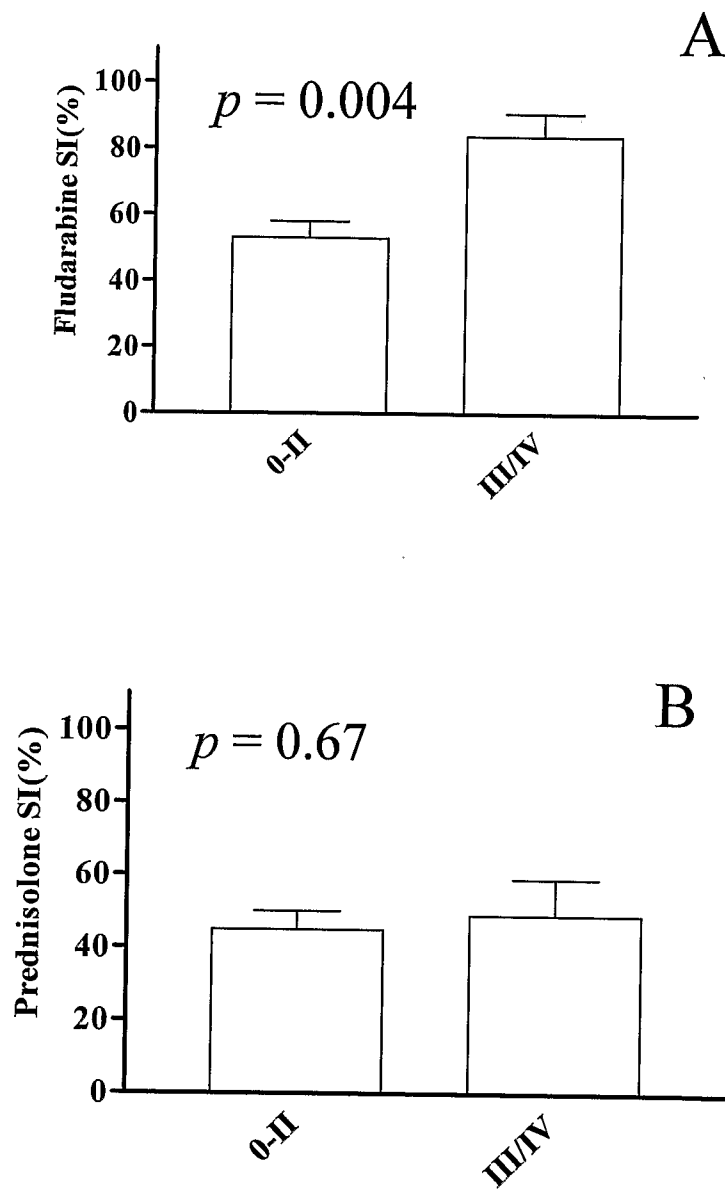
**Figure 2**

**Figure 3A****Figure 3B**

**Figure 3C****Figure 3D**

**Figure 3E****Figure 3F**

**Figure 4**

**Figure 5**