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(54) Title: METHOD FOR PREDICTING CANCER SENSITIVITY

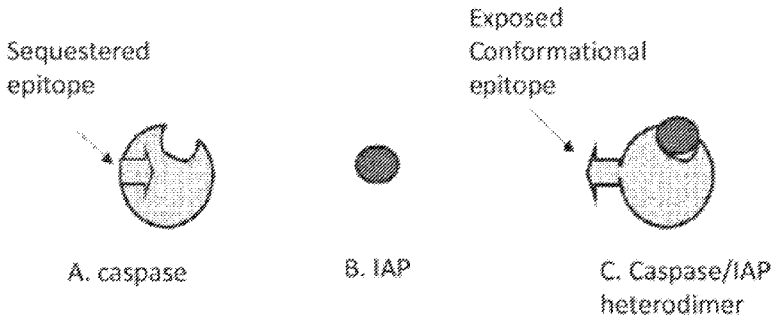


Figure 1

(57) Abstract: The present invention relates to methods of determining cancer cell sensitivity to treatment by using antibodies to detect the presence of heterodimers comprising Bcl-2 proteins or caspase-IAP proteins in the cell. The invention also provides a method of predicting therapeutic efficacy in a cancer patient.

METHOD FOR PREDICTING CANCER SENSITIVITY

PRIORITY

[0001] This application claims the benefit of U.S. Provisional Application No. 61/861,009 filed August 1, 2013 which is hereby incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[0002] The present disclosure relates to methods that are useful in evaluating tumors in human samples.

BACKGROUND

[0003] The use of predictive and prognostic biomarkers paired with targeted cancer therapies may hold the key to reducing drug development time, improving drug efficacy, and guiding clinical decision making. While there are advances in cancer treatment, chemotherapy remains largely inefficient and ineffective. One reason for the generally poor performance of chemotherapy is that the selected treatment is often not closely matched to the individual patient's disease. A personalized medicine approach that couples precision diagnostics with therapeutics, especially targeted therapeutics, is considered a highly promising method for enhancement of the effectiveness of current and future drugs. Biomarkers can facilitate the development and use of such targeted therapeutics as well as standard of care therapies.

[0004] To date there are only a handful of biomarkers that have added value to clinical oncology practice. In part this is because perceived markers often are correlative but not causal to drug mechanism. Even when the "biomarker" biology does line up with the pharmacology of the companion therapy there is still significant challenge to predicting how a drug will work in a patient. Beyond this, the path to clinical development requires the participation of physician-scientists who see the value of the test and believe it can bring benefit to their patients.

[0005] Chemotherapy used in the treatment of cancers can induce apoptosis of the tumor cells. Apoptosis is a process of programmed cell death mediated by a number of signaling pathways that converge at the mitochondria and is effected by caspases, a group of cytosolic proteins. These proteins are activated through a series of biochemical events and the terminal caspase activating event can be blocked by proteins called the inhibitors of apoptosis (IAPs) which can prevent apoptosis and block drug response in cancer patients. Inhibitor of apoptosis proteins (IAPs) suppresse apoptosis through binding and inhibiting active caspases-3, -7 and -9 via its baculoviral IAP repeat (BIR) domains.

Caspase inhibition by IAPs can be negatively regulated by a mitochondrial protein second mitochondrial-derived activator of caspase (SMAC). SMAC physically interacts with multiple IAPs and relieves their inhibitory effect on caspases-3, -7 and -9. A new class of treatment that mimics the function of the protein SMAC, perturbs the IAP function and activates the otherwise blocked caspase, thereby allowing apopotosis to be induced in a cell.

[0006] Further, apoptosis can be regulated by the Bcl-2 proteins, a group of mitochondrial proteins. The response to the Bcl-2 family members in a cell is in part regulated by dimerization domains within this family. More specifically, pro-apoptotic and anti-apoptotic Bcl-2 proteins form heterodimers with their cognate regulating Bcl-2 proteins (i.e., the BH3-only Bcl-2 proteins), thereby executing cell death or survival signals. For example, the ability of Bcl-2 to inhibit apoptosis is blocked by the formation of a heterodimer with Bax (Yang and Korsmeyer, 1996).

[0007] Essentially all effective cancer drugs induce apoptosis in target cancer cells. However, different cancer cells respond to an apoptosis-inducing drug in different manners. This can be due to the presence of different heterodimers between the caspases and the IAPs or the Bcl-2 heterodimers with their cognates. Determining the presence of these heterodimers in a cancer patient can then help in assessing that patient's responsiveness to an apoptosis-inducing cancer drug.

SUMMARY OF THE INVENTION

[0008] Here we provide methods for detecting the presence of a heterodimer complex that will provide a predictive tool to identify patients likely to respond to drugs that perturb heterodimer binding and induce apoptosis in a cancer cell. In one aspect of the invention, caspase-IAP heterodimers are detected. In a further aspect of the invention, Bcl-2 heterodimers are detected. The presence or absence of a particular heterodimer can be correlated to a patient's responsiveness to a particular treatment, thereby guiding the treatment regimen administered to the patient.

[0009] In one aspect, the invention provides a method for detecting a heterodimer in a patient sample, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to the heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; and d) determining the presence of the heterodimer based on the intensity of the signal.

[0010] Another aspect of this invention is a method for detecting the presence of a heterodimer of the Bcl-2 family using any of the antibodies described above. This method includes (i) providing a tissue sample suspected of having a heterodimer of the Bcl-2 family, (ii) contacting the sample with the antibody, (iii) detecting a signal indicative of binding of the antibody to the heterodimer, and (iv)

determining the presence of the heterodimer in the sample based on the intensity of the signal. Examples of the heterodimer include Bim/Mcl-1 and Bim/Bcl-2. The tissue sample examined in this method can be a peripheral blood sample, a lymph-node sample, a bone marrow sample, or an organ tissue sample. Preferably, the specimen is a mitochondrial fraction.

[0011] In one aspect, the invention provides a method for determining a cancer treatment for a patient, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; d) determining the presence of the heterodimer based on the intensity of the signal; e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and f) classifying the patient for likelihood of clinical response to one or more cancer treatments, wherein the presence of a heterodimer correlates with treatment efficacy.

[0012] In one aspect, the invention provides a method for predicting cancer sensitivity to treatment, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; d) determining the presence of the heterodimer based on the intensity of the signal; e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and f) classifying the patient for likelihood of clinical response to one or more cancer treatments, wherein the presence of a heterodimer correlates with treatment efficacy.

[0013] In one embodiment, the heterodimer comprises a caspase and an inhibitor of apoptosis protein (IAP). In another embodiment, the heterodimer comprises an IAP and TRAF-2. In another embodiment, the caspase is selected from the group consisting of caspase 2, caspase 3, caspase 5, caspase 7, caspase 8, and caspase 9. In a further embodiment, the IAP is selected from the group of XIAP, IAP-1, cIAP-2, nIAP, and survivin.

[0014] In one embodiment, the heterodimer comprises different members of the Bcl-2 family. In another embodiment, the heterodimer of Bcl-2 family contains a first member of the Bcl-2 family selected from the group consisting of Bim, Bid, Bad, Puma, Noxa, Bak, Hrk, Bax, Bmf, and Mule, and a second member of the Bcl-2 family selected from the group consisting of Mcl-1, Bcl-2, Bcl-XL, Bfl-1, and Bcl-w. In another embodiment, the first member of the Bcl-2 family is Bim and the second member of the Bcl-2 family is Mcl-1, Bcl-XL, or Bcl-2.

[0015] In one embodiment, the heterodimer is an anti-apoptotic heterodimer and its presence indicates that the patient is sensitive to the drug. In another embodiment, the heterodimer is a pro-apoptotic heterodimer and its presence indicates that the patient is responsive to the drug.

[0016] In one embodiment of the invention, the cancer is a hematologic cancer. In another embodiment, the hematologic cancer is selected from acute myelogenous leukemia (AML), multiple myeloma, follicular lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, and non-Hodgkin's lymphoma.

[0017] In one embodiment, the cancer is a solid tumor cancer. In a further embodiment, the solid tumor cancer is selected from non-small lung cell carcinoma, ovarian cancer, and melanoma.

[0018] In one embodiment, the cancer treatment is one or more of anti-cancer drugs, chemotherapy, antagonist of an anti-apoptotic protein, surgery, adjuvant therapy, and neoadjuvant therapy. In a further embodiment, the cancer treatment is one or more of a SMAC mimetic, BH3 mimetic, proteasome inhibitor, histone deacetylase inhibitor, glucocorticoid, steroid, monoclonal antibody, antibody-drug conjugate, or thalidomide derivative. In one embodiment, the treatment blocks formation of the particular heterodimer detected. In one embodiment, the treatment perturbs formation of the particular heterodimer detected.

[0019] In one embodiment, the specimen is a biopsy selected from a tissue sample, frozen tumor tissue specimen, cultured cells, circulating tumor cells, and a formalin-fixed paraffin-embedded tumor tissue specimen. In a further embodiment, the tissue sample is a peripheral blood sample, a lymph-node sample, a bone marrow sample, or an organ tissue sample. In one embodiment, the sample is a mitochondrial fraction. In a further embodiment, the specimen is a human tumor-derived cell line. In another embodiment, the specimen is a cancer stem cell. In one embodiment, the specimen is derived from the biopsy of a non-solid tumor. In another embodiment, the specimen is derived from the biopsy of a patient with multiple myeloma, acute myelogenous leukemia, acute lymphocytic leukemia, chronic lymphogenous leukemia, mantle cell lymphoma, diffuse large B-cell lymphoma, and non-Hodgkin's lymphoma. In a further embodiment, the specimen is derived from a circulating tumor cell. In another embodiment, the specimen is derived from the biopsy of a solid tumor cancer. In a further embodiment, the specimen is derived from the biopsy of a patient with non-small lung cell carcinoma, ovarian cancer, and melanoma.

[0020] In one embodiment, the method further comprises determining one or more clinical factors of the patient. In another embodiment, the clinical factor is one or more of age, cytogenetic status, performance, histological subclass, gender, and disease stage.

[0021] In one embodiment, the method further comprises predicting a clinical response in the patient.

[0022] In another aspect, the invention provides an isolated antibody that specifically binds to the heterodimer. In one embodiment, the heterodimer comprises a caspase and an inhibitor of apoptosis protein (IAP). The caspase family proteins are found in inactive and active forms. In some cases IAP proteins bind to the inactive caspase (i.e., an xIAP inactive caspase 9 heterodimer). In other cases IAP proteins bind to and inactivate active caspases, (i.e., a caspase 7 and cIAP-1 heterodimer). In

another embodiment, the heterodimer comprises an IAP and TRAF-2. In another embodiment, the caspase is selected from the group consisting of is selected from the group consisting of caspase 2, caspase 3, caspase 5, caspase 7, caspase 8, and caspase 9. In a further embodiment, the IAP is selected from the group of XIAP, IAP-1, cIAP-2, nIAP, and survivin. In one embodiment, the heterodimer comprises different members of the Bcl-2 family. In another embodiment, the heterodimer of Bcl-2 family contains a first member of the Bcl-2 family selected from the group consisting of Bim, Bid, Bad, Puma, Noxa, Bak, Hrk, Bax, Bmf, and Mule, and a second member of the Bcl-2 family selected from the group consisting of Mcl-1, Bcl-2, Bcl-XL, Bfl-1, and Bcl-w. In another embodiment, the first member of the Bcl-2 family is Bim and the second member of the Bcl-2 family is Mcl-1, Bcl-XL, or Bcl-2. In one embodiment, the heterodimer is an anti-apoptotic heterodimer and its presence indicates that the patient is sensitive to the drug. In another embodiment, the heterodimer is a pro-apoptotic heterodimer and its presence indicates that the patient is responsive to the drug.

[0023] The details of one or more examples of the invention are set forth in the description below. Other features or advantages of the present invention will be apparent from the following drawings, detailed description of several examples, and also from the appended claims. The details of the invention are set forth in the accompanying description below. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, illustrative methods and materials are now described. Other features, objects, and advantages of the invention will be apparent from the description and from the claims. In the specification and the appended claims, the singular forms also include the plural unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

BRIEF DESCRIPTION OF THE FIGURES

[0024] **FIG. 1** is a schematic illustration depicting the conformational change of a caspase protein following binding of an IAP protein.

[0025] **FIG. 2** is a schematic illustration depicting the process of selecting antibodies specific to caspase-IAP heterodimers via an immunoassay. Panel A: antibodies binding to a caspase-IAP heterodimer being positively selected. Panel B: antibodies binding to non-dimerized members of the heterodimer being negatively selected. Panel C: illustrate the symbols in Panels A and B.

[0026] **FIG. 3** is a schematic illustration depicting an immunoassay for profiling caspase-IAP heterodimers in cancer cells using the antibody of this invention, i.e., an antibody specifically recognizes caspase-IAP heterodimers.

[0027] **FIG. 4** is a schematic illustration showing substitution of an aromatic amino acid in the BIR domain of IAP protein and covalent binding of this peptide to the caspase to make the covalent heterodimer for antibody production.

[0028] **FIG. 5** is a schematic illustration depicting the structure of the IAP proteins and the sequences of the BIR domains.

[0029] **FIG. 6** shows the hydrophobic groove of BCL-XL formed by BH1-3. Panel A is a ribbon representation of BCL-XL with BH1 colored pink, BH2 colored yellow, and BH3 colored red. Panel B is a surface representation of BCL-XL bound to BIM BH3 peptide, shown in ribbon.

[0030] **FIG. 7** shows a schematic illustration depicting the coupling of photactivatable benzophenyl alanine modified Bim-BH3 peptide. This peptide is allowed to complex with Bcl-xL-GST while exposed to UV light. The covalent heterodimer is purified and assessed for function before being used to immunize mice.

[0031] **FIG. 8** shows the results of an ELISA of various dilutions of hybridoma clones 7146, 7197, and 7198.

[0032] **FIG. 9** is a schematic illustration depicting the screen/counterscreen of monoclonal antibodies derived from fusions. Eight 96-well plates = 768 clones tested in ELISA assay plates coated with Bcl-XL-GST/Bim BH3 peptide conjugate, and counter screened against the Bcl-XL GST or Bim-BH3 peptide coated plates. From this, 39 selectively binding clones, were advanced for testing and subcloning.

[0033] **FIG. 10A-B** shows an assay in which covalent heterodimer was bound to Glutathione-coated ELISA plates and tested for binding of fusion clones to GST-Bcl-XL-BIM heterodimer. Panel A shows the set-up of reagents. Panel B shows the results for two dilutions of the heterodimer.

[0034] **FIG. 11A-D** shows the heterodimer binding affinity ranked for all 31 IgG clones tested in this ELISA assay.

[0035] **FIG. 12** shows an alternative strategy in which biotinylated peptide was bound to Streptavidin-coated plates and then incubated with the GST-Bcl-XL fusion proteins.

[0036] **FIG. 13A-D** shows the heterodimer binding affinity ranked for all 31 IgG clones tested in this ELISA assay.

[0037] **FIG. 14** shows the selective binding of Bim versus other peptides. GST-Bcl-XL fusion protein was added to Glutathione coated plates first, and subsequently clones were screened for specificity by adding non-modified pro-apoptotic BH3-only subfamily domain peptides. This figure shows an example of five different clones that showed specificity for BIM, but no specificity for BID peptide.

[0038] **FIG. 15** shows the selective inhibition of HSBXB binding to heterodimer Bcl-XL/BIM-BH3 with BH3 mimetic Abt-263: In this assay, non-covalent Bcl-XL-GST/BIM-BH3 heterodimer was bound to Glutathione-coated ELISA plates and treated with ABT-263 (Navitoclax), a potent BCL2/Bcl-XL targeted compound. The compound was added to the ELISA plates after addition of peptides and before adding the monoclonal antibody. The Bib BH3 domain peptide was added as a negative control.

[0100] **FIG. 16A-B** shows immunofluorescence microscopy for 6 clones selected for subcloning based on the combined ELISA results. Melanoma AUCC903N cells were either fixed with Methanol (Panel A) or with 4% paraformaldehyde and permeabilized with 0.2% TritonX100 (Panel B) and incubated with subclone #32. The cells were incubated with an Alexa488-conjugated goat anti-mouse antibody. Panel B shows an overlay with DAPI nuclear DNA stain. The mitochondrial staining is visible.

[0101] **FIG 17A-B** shows HSBXB binding of Bcl-XL/BIM heterodimer in cells incubated with different concentrations of ABT-263. IRDye 800CW goat anti-mouse antibody was used for detecting the heterodimer specific mouse monoclonal antibody and IRDye 800CW Goat anti-rabbit antibody was used to detect the commercial Bcl-XL rabbit monoclonal antibody. Panel A is a bar graph, and Panel B is a line graph showing the percent heterodimer specific signal in relation to ABT-263 concentration.

[0102] **FIG. 18** shows a FACS readout in JEKO-1 cells which were treated with ABT263, no treatment or BIM peptide for 3 hours is represented positive signal is percentage of signal generated by the isotype (negative control).

DETAILED DESCRIPTION OF THE INVENTION

[0039] It should be understood that singular forms such as "a," "an," and "the" are used throughout this application for convenience, however, except where context or an explicit statement indicates otherwise, the singular forms are intended to include the plural. Further, it should be understood that every journal article, patent, patent application, publication, and the like that is mentioned herein is hereby incorporated by reference in its entirety and for all purposes. All numerical ranges should be understood to include each and every numerical point within the numerical range, and should be interpreted as reciting each and every numerical point individually. The endpoints of all ranges directed to the same component or property are inclusive, and intended to be independently combinable.

[0040] "About" includes all values having substantially the same effect, or providing substantially the same result, as the reference value. Thus, the range encompassed by the term "about" will vary

depending on context in which the term is used, for instance the parameter that the reference value is associated with. Thus, depending on context, "about" can mean, for example, ±15%, ±10%, ±5%, ±4%, ±3%, ±2%, ±1%, or ± less than 1%. Importantly, all recitations of a reference value preceded by the term "about" are intended to also be a recitation of the reference value alone. Notwithstanding the preceding, in this application the term "about" has a special meaning with regard to pharmacokinetic parameters, such as area under the curve (including AUC, AUC_t, and AUC_∞) C_{max}, T_{max}, and the like. When used in relationship to a value for a pharmacokinetic parameter, the term "about" means from 85% to 115% of the reference parameter.

[0041] As used herein, the word "include," and its variants, is intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this technology. Similarly, the terms "can" and "may" and their variants are intended to be non-limiting, such that recitation that an embodiment can or may comprise certain elements or features does not exclude other embodiments of the present technology that do not contain those elements or features. Although the open-ended term "comprising," as a synonym of terms such as including, containing, or having, is used herein to describe and claim the invention, the present technology, or embodiments thereof, may alternatively be described using more limiting terms such as "consisting of" or "consisting essentially of" the recited ingredients.

[0042] Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patents, and patent publications cited are incorporated by reference herein in their entirety for all purposes.

[0043] Apoptosis occurs through two main pathways: the extrinsic or cytoplasmic pathway, triggered through the Fas death receptor, a member of the tumor necrosis factor (TNF) receptor superfamily; and the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway involving the activation of a cascade of caspases, a family of proteases that cleave regulatory and structural molecules, culminating in the death of the cell.

[0044] Cancer cells, without wishing to be bound by theory, exhibit abnormalities, such as DNA damage, genetic instability, abnormal growth factor signaling, and abnormal or missing matrix interactions, any of which should typically induce apoptosis through the intrinsic (mitochondrial) apoptosis pathway. However, rather than respond to these apoptosis signals some cancer cells survive.

Often, in doing so, these cells become highly dependent on selected blocks to chronic apoptosis signals. The formation of certain heterodimers can block the apoptotic signals.

[0045] One of the hallmarks of apoptosis is mitochondrial outer membrane permeabilization (MOMP), a process regulated by the Bcl-2 family of proteins. The activity of this family of proteins is linked to the onset of lymphoid and several solid tumor cancers and is believed in many cancers to be a key mediator of resistance to chemotherapy. Bcl-2 proteins are regulated by distinct protein-protein interactions between pro-survival (anti-apoptotic) and pro-apoptotic members. These interactions occur primarily through BH3 (Bcl-2 homology domain-3) mediated binding. Apoptosis-initiating signaling occurs for the most part upstream of the mitochondria and causes the translocation of short, BH3-only, Bcl-2 family members to the mitochondria where they either activate or sensitize MOMP. The activator BH3 only proteins, Bim and Bid, bind to and directly activate the effector, pro-apoptotic proteins Bax and Bak, and also bind to and inhibit the anti-apoptotic Bcl-2 family proteins, Bcl-2, Mcl-1, Bfl-1, Bcl-w and Bcl-xL. The sensitizer BH3 proteins, Bad, Bik, Noxa, Hrk, Bmf and Puma, bind only to the anti-apoptotic Bcl-2 family proteins, Bcl-2, Mcl-1, Bfl-1, Bcl-w and Bcl-xL, blocking their anti-apoptotic functions. Without wishing to be bound by theory, each sensitizer protein has a unique specificity profile. For example, Noxa (A and B) bind with high affinity to Mcl-1, Bad binds to Bcl-xL and Bcl-2 but only weakly to Mcl-1, and Puma binds well to all three targets. An anti-apoptotic function of these proteins is the sequestering of the activator BH3 protein Bim and Bid by binding to form heterodimers. Displacement of these activators by sensitizer peptides or treatments results in Bax/Bak-mediated apoptotic commitment. These interactions can have various outcomes, including, without limitation, homeostasis, cell death, sensitization to apoptosis, and blockade of apoptosis.

[0046] A feature of cancer cells in which apoptotic signaling is blocked is an accumulation of the BH3 only activator proteins at the mitochondrial surface, which results from these proteins being sequestered by the anti-apoptotic proteins. This accumulation and proximity to their effector target proteins accounts for increased sensitivity to antagonism of Bcl-2 family proteins in the "BH3 primed" state.

[0047] The value of Bcl-2 as a target in anti-tumor therapy has been well established. Briefly, without wishing to be bound by theory, as a result of aberrant phenotypes, cancer cells develop blocks in apoptosis pathways. These blocks make cancer cells both resistant to some therapies, and, surprisingly, make some cancer cells sensitive to other therapies. Bcl-2 promotes cell survival and normal cell growth, and is expressed in many types of cells including lymphocytes, neurons, and self-renewing cells, such as basal epithelial cells and hematopoietic progenitor cells in the bone marrow. Researchers have recognized that proteins in the Bcl-2 family regulate apoptosis and are key effectors of tumorigenesis (Reed, (2002) Nat Rev. Drug Discov. 1(2): 111-21). It has also been reported that Mcl-1 is a target in treating NHL, CLL, and acute mylogenous leukemia (AML) (Derenne, *et al.*

(2002) Blood, 100:: 194-99; Kitada, *et al.* (2004) J. Nat. Canc. Inst. 96: 642-43; Petlickovski, *et al.* (3018) Blood 105: 4820-28).

[0048] In many cancers, anti-apoptotic Bcl-2 proteins, block the sensitivity of tumor cells to cytostatic or apoptosis inducing drugs, and these proteins have become targets for anti-tumor therapy. BH3 mimetic compounds comprise a recently described class of small molecules that inhibits Bcl-2 family proteins are the (*reviewed in* Bajwa, *et al.* (2013) Expert Opin Ther Pat. 2012 January; 22(1): 37–55) These compounds function by inhibiting BH3 mediated protein/protein interactions among the Bcl-2 family proteins. Several studies have described BH3 mimetic small molecules that function as Bcl-2 inhibitors by blocking BH3 binding (reviewed in Billard, (2013) Mol Cancer Ther. 12(9):1691-700). Compounds with BH3 mimic function include HA-14-1 (Wang, *et al.* (2000) Proc. Natl. Acad. Sci. USA 97: 7124-9), Antimycin-A (Tzung, *et al.* (2001) Nat. Cell. Biol. 3: 183-191), BH3I-1 and BH3I-2 (Degterev, *et al.* (2001) Nat. Cell. Biol. 3: 173-82), and seven un-named compounds (Enyedy, *et al.* (2001) J. Med Chem 44: 4313-24), as well as a series of terphenyl derivatives (Kutzki, *et al.* (2002) J. Am. Chem. Soc. 124: 11838-9), and two new classes of molecules (Rosenberg, *et al.* (2004) Anal. Biochem. 328: 131-8). Compounds with selective BH3 mimic function include Bcl-2 selective activity (Ng (2014) Clin Adv Hematol Oncol. 12(4):224-9)—as well as selective Mcl-1 activity (Richard, et al. (2013) Bioorg Med Chem. 21(21):6642-9) and are in various stages of clinical development. More recently, a BH3 mimic compound has been tested in a mouse tumor model (Oltersdorf, *et al.* (2005) Nature 435: 677-81).

[0049] Regardless of the initiating event or the path taken, the common final portion of the apoptotic program involves the activation of effector caspases which cause cell death. There may be an element of cross talk between death receptor-induced apoptotic signalling and the intrinsic apoptotic program. Evidence suggests that activated caspase-8 can cleave Bid (a pro-apoptotic BH3-only Bcl-2 family member) to a truncated form, which is then able to activate the intrinsic pathway and thus amplify the apoptotic program (Luo et al. Cell. 1998;94:481–90; Li et al. Cell. 1998;94:491–501; Gross et al. J Biol Chem. 1999;274:1156–63). Bid-deficient mice show some resistance to Fas-induced hepatocyte apoptosis but their lymphocytes are normal and remain sensitive to Fas-induced killing (Yin et al. Nature. 1999;400:886–91). Thus, Bid may play a role in amplifying the death receptor signal through the intrinsic Bcl-2 apoptotic pathway in some but not all cells. Indeed, since Bid can also be cleaved by caspases other than caspase-8 (Luo et al. Cell. 1998;94:481–90; Li et al. Cell. 1998;94:491–501; Yin et al. Nature. 1999;400:886–91), it may play a more general role as an amplifier in apoptosis signalling.

[0050] Caspases are the central components of the execution phase of apoptosis. Caspases may interact with members of the TNF receptor superfamily which activates the caspases to effect cell death. For example, cell death signals, such as Fas ligand and tumor necrosis factor -2 can be specifically recognized by their corresponding receptors (e.g. Fas or TNFR-1) in the plasma

membrane. This binding activates the death receptors which induces oligomerization of procaspases on the cytosolic side of the plasma membrane and activates them. These active caspases start a cascade resulting in cell death (see Fan et al. Acta Biochimica et Biophys Sinica, 37:719-727 (2005).

[0051] The activation and inactivation of caspases are regulated by various proteins, ions and other factors, such as IAP, Bcl-2 family proteins, calpain, Ca2+, Gran B and cytokine response modifier A (Crm A). In humans, the IAP family includes cIAP1, cIAP2, XIAP (X-linked mammalian inhibitor of apoptosis protein), NAIP (neuronal apoptosis inhibitory protein), survivin and livin. All members of the family contain 1−3 N-terminal baculovirus IAP repeat (BIR) domains and one conservative C-terminal RING (really interesting new gene) domain. The BIR domains are zinc finger-like structures that can chelate zinc ions. These zinc fingers can bind to the surface of caspases so that the amino acid sequences, or linkers, between BIR domains can block the catalyzing grooves of caspases. As a result, IAPs can protect a cell from apoptosis by inhibiting the activity of caspases. The activity of IAP can be inhibited by SMAC released from mitochondria, which can recognize and bind to the caspase-binding site of the IAP, thereby inactivating the IAP, and inhibiting its effect on caspases.

[0052] While the promise for using BH3 or SMAC mimetic compounds as anti-tumor therapeutics has been recognized, to date there are no conclusive clinical reports on the efficacy of any anti-cancer drug with these modes of action. For example, while pharmacological manipulation of the Bcl-2 family proteins is a feasible approach to achieving therapeutic benefit for cancer patients, the complexity of the network of proteins that comprise this family makes this prospect difficult. Therefore, with the large unmet medical need for treating hematological malignancies, new approaches to assessing and utilizing the detailed activity of the BH3 mimetic molecules will have value in developing this class of therapeutics.

[0053] Cetain methods disclosed herein involve the coupling of an oncology therapy and unique companion diagnostic test that is used to predict likely response to treatment. This information can be used to determine the appropriateness of administering a given treatment, and to then guide alternative treatment if required.

[0054] The heterodimer detection assays described herein provide a predictive test for cancer treatments that work through the apoptosis pathway. These assays detect the presence of heterodimers that are indicative of a cell's readiness to undergo apoptosis when exposed to an apoptotic-inducing compound or treatment. For example, some, not all, cancer cells are "pre-set" to undergo drug-induced apoptosis, which is induced by exposure to certain BH3 peptides, chemotherapeutics, or SMAC mimetics. The determination of the presence or absence of Bcl-2 or caspase-IAP heterodimers allows a determination of the cell or specimen's particular chemoresistance or chemosensitivity, and provides insight into the likelihood of a cancer cell to respond to treatment.

[0055] A critical area of focus in cancer treatment is understanding, detecting, and controlling cellular function in response to drugs and other treatments. Events occurring in the cell determine the ability of the cancer cell to respond to apoptosis-inducing cancer therapy. Cells can be evaluated to determine a cell's state using antibodies that bind to heterodimers comprising Bcl-2 proteins and their cognates and/or caspases and IAPs.

Bcl-2 heterodimers

[0056] The present invention uses the determination of a cancer cell's predisposition to undergo apoptosis to elucidate the cancer's susceptibility to a particular treatment. One way this can be done is by using antibodies that bind to Bcl-2 heterodimers which regulate apoptosis. Formation of a heterodimer induces conformational changes in both members of the heterodimer, resulting in exposure of antigenic epitopes that are sequestered in both members before dimerization. The isolated antibody of this invention specifically recognizes such an epitope and only binds to a heterodimer of the Bcl-2 family, not to either non-dimerized member.

[0057] Bcl-2 proteins, found in mitochondria, are major regulators of the commitment to programmed cell death and executioners of death/survival signals. See Reed, Natural Clinical Practice Oncology, 3:388-398 (2006), Green et al., Cancer Cell 1:19-30 (2002), and Adams et al., Cold Spring Harb. Symp. Quant. Biol. 70:469-477 (2005). There are four sub-groups of Bcl-2 proteins: (i) multi-domain anti-apoptotic Bcl-2 proteins, (ii) multi-domain pro-apoptotic Bcl-2 proteins, (iii) activator BH3-only Bcl-2 proteins, and (iv) sensitizer BH3-only Bcl-2 proteins. Table 1 below lists major human Bcl-2 proteins and their GenBank® accession numbers:

Table 1

Bcl-2 Proteins		GenBank Accession Numbers
Multi-domain Anti-Apoptotic Bcl-2 Proteins	Bcl-2	AAH27258 (July 15, 2006)
	Bcl-XL	AAH19307 (July 15, 2006)
	Mcl-1	AAF64255 (July 17, 2000)
	BCL-w	AAB09055 (September 29, 1996)
	BFL-1	Q16548 (March 3, 2009)
Multi-domain Pro-Apoptotic Bcl-2 Proteins	BAX	Q07812 (April 14, 2009)
	BAK	Q16611 (April 14, 2009)
Sensitizer BH3-only Bcl-2 Proteins	BAD	CAG46757 (June 29, 2004)
	BIK	CAG30276 (October 16, 2008)
	NOXA	Q13794 (March 3, 2009)
	HRK	AAC34931 (September 9, 1998)
	BMF	AAH69328 (August 19, 2004); AAH60783 (January 27, 2004)
	PUMA	Q9BXH1 (April 14, 2009)
	Mule	Q7Z6Z7 (April 14, 2009)
Activator BH3-only Bcl-2 Proteins	BID	P55957 (March 3, 2009)
	BIM	O43521 (April 14, 2009)

[0058] Other Bcl-2 proteins, can be identified by homologous search using the amino acid sequence of a known Bcl-2 protein as a query. Polypeptides can be identified based on homology to the BH3 domain, and polypeptides can possess at least about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 96%, about 97%, about 98%, about 99% sequence homology to the amino acid sequences of the polypeptides disclosed in Table 1. Preferred variants are those that have conservative amino acid substitutions made at one or more predicted non-essential amino acid residues. For example, a "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. In a further embodiment, the BH3 domain peptide is an activator or a sensitizer of apoptosis. In a preferred embodiment, the BH3 domain peptide is a sensitizer.

[0059] In one embodiment, the heterodimer comprises different members of the Bcl-2 family. In another embodiment, the heterodimer of Bcl-2 family contains a first member of the Bcl-2 family selected from the group consisting of Bim, Bid, Bad, Puma, Noxa, Bak, Hrk, Bax, Bmf, and Mule, and a second member of the Bcl-2 family selected from the group consisting of Mcl-1, Bcl-2, Bcl-XL, Bfl-1, and Bcl-w. In another embodiment, the first member of the Bcl-2 family is Bim and the second member of the Bcl-2 family is Mcl-1, Bcl-XL, or Bcl-2. In one embodiment, the heterodimer comprises Bcl-XL and Bim. In another embodiment, the heterodimer comprises Bim and Mcl-1. In

another embodiment, the heterodimer comprises Bim and Bcl-2. In another embodiment, the heterodimer comprises Bid and Bcl-2.

[0060] If a cell is pre-set to undergo drug-induced apoptosis (e.g. the cell is dependent on Bcl-2 polypeptide activity for survival), the antibodies of the invention can be used to identify the specific Bcl-2 proteins that are responsible for apoptotic block.

Caspase-IAP heterodimers

[0061] The present invention also provides an isolated antibody specific to a caspase-IAP heterodimer, i.e., a naturally-occurring heterodimer formed between any one of the caspases and any one of the IAP proteins.

[0062] Caspases, or cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases are a family of cysteine proteases that play essential roles in apoptosis (programmed cell death), necrosis, and inflammation. (Alnemri ES, Emad S; et al. (1996). Cell 87 (2): 171.) The inhibitor of apoptosis (IAP) proteins, found in cytosol of cells, are regulators of the commitment to programmed cell death and execution of death/survival signals. (Eckelman and Salvese, J. Biol. Chem. 2006, 281:3254-3260). They function to inhibit the activity of caspases by binding to a caspase polypeptide and forming a heterodimer, thereby preventing the caspase from effecting apoptosis.

[0063] Table 2 below lists major human caspases and the IAP proteins xIAP, Ciap1, cIAP2 and surviven, and their GenBank® accession numbers:

Table 2

Polypeptide	**Accession Number**
XIAP	NM_001167.3
CIAP1	NM_001166.4
CIAP2	NM_001165.4
Survivin	U75285.1
caspase 2	NM_032982.3
caspase 3	NM_004346.3
caspase 7	NM_001227.4

caspase 8	AB038985.2
caspase 9	AB019205.2

[0064] IAPs comprise BIR domains which belong to the zinc-finger domain family and characteristically have a number of invariant amino acid residues, including 3 conserved cysteines and one conserved histidine, which coordinates a zinc ion. BIR domains are typically composed of 4-5 alpha helices and a three-stranded beta sheet and are approximately 70 amino acids in length. These domains bind to the caspases at the IAP binding motifs and are essential for the anti-apoptotic function of these proteins. The sequences within the BIR domains required for caspase binding have been identified. (Eckelman and Guy, J. Biol. Chem. 2006, 281:3254-3260). **FIG. 5** shows the structure of the IAP proteins and the sequences of the BIR domains.

[0065] When IAPs and caspases bind to form heterodimers, conformational changes in both members of the heterodimer are induced, resulting in exposure of unique antigenic epitopes that are sequestered in both members before dimerization (**FIG. 1**). Antibodies that bind specifically to heterodimers of the caspase and IAP proteins, but not non-dimerized proteins, may be used to identify and measure heterodimers. In one embodiment, the disclosure provides antibodies that bind specifically to heterodimers of the caspase and IAP proteins, but not non-dimerized proteins.

[0066] Examples of the caspase-IAP heterodimer include caspase 2, 3, 5, 7, 8, or 9 binding with XIAP, IAP-1, cIAP-2, nIAP, or survivin.

[0067] Caspase-IAP heterodimer profiling (**FIG. 3**) can also be used to predict responsiveness to drugs targeting the apoptotic pathway in patients suffering from apoptosis-related diseases, e.g., autoimmune disease (see Adams et al., Cold Spring Harb Symp Quant Biol. 70:469-477; 2005) and/or cancer.

[0068] In one embodiment, the presence of a particular caspase-IAP heterodimer in a patient indicates that patient's responsiveness to a drug that blocks formation of the particular heterodimer and inhibits its function. In one embodiment, the presence of a particular caspase-IAP heterodimer in a cancer patient indicates that this patient is sensitive to a drug that interferes with formation of this anti-apoptotic IAP/caspase heterodimer.

[0069] Another aspect of this invention is a method for assessing whether a patient is sensitive or resistant to drug that works through the TNF receptor or other of the death domain family of receptors. The protein TNF receptor associated factors (TRAF1 and TRAF2) are required for TNF-alpha-mediated activation of MAPK8/JNK and NF-κB. The protein complex formed by TRAF2 and TRAF1 interacts with the IAP family members cIAP1 and cIAP2, and functions as a mediator of the

anti-apoptotic signals from TNF receptors. The interaction of this protein with TRADD, a TNF receptor associated apoptotic signal transducer, ensures the recruitment of IAPs for the direct inhibition of caspase activation. Song and Donner (Biochem J. 309 (Pt 3): 825–9. 1995).

Antibodies

[0070] One aspect of this invention features an isolated antibody that specifically binds to a heterodimer of the Bcl-2 family (i.e., a Bcl-2 heterodimer). The Bcl-2 family includes both Bcl-2 proteins (monomers) and naturally-occurring heterodimers formed between two Bcl-2 proteins. The heterodimer contains a first Bcl-2 protein (e.g., Bim, Bid, Bad, Puma, Noxa, Bak, Hrk, Bax, or Mule) and a second Bcl-2 protein (e.g., Mcl-1, Bcl-2, Bcl-XL, Bfl-1 or Bcl-w). One aspect of this invention features an isolated antibody that specifically binds to a caspase-IAP heterodimer. Examples of the caspase-IAP heterodimer include caspase 2, 3, 5, 7, 8, or 9 binding with XIAP, IAP-1, cIAP-2, nIAP, or survivin.

[0071] In various embodiments, antibodies include whole antibodies and/or any antigen binding fragment (*e.g.*, an antigen-binding portion) and/or single chains of these (*e.g.* an antibody comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, an Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; a $F(ab)_2$ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; a Fd fragment consisting of the V_H and CH1 domains; a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; and the like). In various embodiments, polyclonal and monoclonal antibodies are useful, as are chimeric antibodies, isolated human or humanized antibodies, or functional fragments thereof. The term "isolated antibody" used herein refers to an antibody substantially free from naturally associated molecules, i.e., the naturally associated molecules constituting at most 20% by dry weight of a preparation containing the antibody.

[0072] The antibodies of the invention can be prepared by conventional methods. See, for example, Harlow and Lane, (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York. In general, a heterodimer can be prepared by producing its two members separately by recombinant technology and then incubating both members under suitable conditions to allow formation of the heterodimer. To produce antibodies against the heterodimer, the heterodimer, optionally coupled to a carrier protein (e.g., KLH), can be mixed with an adjuvant, and injected into a host animal. Antibodies produced in the animal can then be purified by heterodimer affinity chromatography. Commonly employed host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants that can be used to increase the immunological response depend on the host species and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, CpG, surface-active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil

emulsions, keyhole limpet hemocyanin, and dinitrophenol. Useful human adjuvants include BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Polyclonal antibodies, i.e., heterogeneous populations of antibody molecules, are present in the sera of the immunized animal.

[0073] Monoclonal antibodies, i.e., homogeneous populations of antibody molecules, can be prepared using standard hybridoma technology (see, for example, Kohler et al. (1975) Nature 256, 495; Kohler et al. (1976) Eur. J. Immunol. 6, 511; Kohler et al. (1976) Eur J Immunol 6, 292; and Hammerling et al. (1981) Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y.). In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al. (1975) Nature 256, 495 and U.S. Pat. No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al. (1983) Immunol Today 4, 72; Cole et al. (1983) Proc. Natl. Acad. Sci. USA 80, 2026, and the EBV-hybridoma technique (Cole et al. (1983) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the monoclonal antibodies of the invention may be cultivated in vitro or in vivo. The ability to produce high titers of monoclonal antibodies in vivo makes it a particularly useful method of production.

[0074] In addition, techniques developed for the production of "chimeric antibodies" can be used. See, e.g., Morrison et al. (1984) Proc. Natl. Acad. Sci. USA 81, 6851; Neuberger et al. (1984) Nature 312, 604; and Takeda et al. (1984) Nature 314:452. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. Nos. 4,946,778 and 4,704,692) can be adapted to produce a phage or yeast library of scFv antibodies. scFv antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge.

[0075] Moreover, antibody fragments can be generated by known techniques. For example, such fragments include, but are not limited to, F(ab')2 fragments that can be produced by pepsin digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of F(ab')2 fragments. Antibodies can also be humanized by methods known in the art. For example, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; and Oxford Molecular, Palo Alto, Calif.). Fully human antibodies, such as those expressed in transgenic animals are also features of the invention (see, e.g., Green et al. (1994) Nature Genetics 7, 13; and U.S. Pat. Nos. 5,545,806 and 5,569,825).

[0076] The antibodies prepared by any of the methods described above are confirmed for their binding to a caspase-IAP heterodimer or a Bcl-2 heterodimer. They are further subjected to a negative

selection to exclude those that also bind to either non-dimerized member of the heterodimer. For example, each of the two members, i.e., monomer A and monomer B, is labeled with a distinct fluorescent dye, i.e., dye x and dye y, respectively. Dyes x and y have different optimal emission wavelengths. The antibody is first incubated with labeled monomer A, labeled monomer B, or the A/B heterodimer (double labeled) for a suitable period and then captured by GamaBind Sepharose beads. Whether the antibody is capable of binding to either monomer or to the heterodimer can be determined based on the fluorescent signal released from the captured antibody. Antibodies that bind to the heterodimer and not to either non-dimerized member are selected.

[0077] In one embodiment, the antibodies that bind to Bcl-2 heterodimers are those disclosed in US Patent No. 8,168,755 and US 2012-0225794, the contents of which are incorporated by reference in its entirety for all purposes.

Heterodimer Binding Assay

[0078] In one aspect, the invention provides a method for detecting a heterodimer in a patient sample, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to the heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; and d) determining the presence of the heterodimer based on the intensity of the signal.

[0079] The assay comprises detecting the presence or absence of a Bcl-2 or caspase-IAP heterodimer in a sample, and associating the presence or absence of one or more of these heterodimers with patient classification (e.g. responder/non-responder). The heterodimers can be detected through any means commonly known in the art, including, but not limited to ELISA (as described for example in Certo *et al.* Cancer Cell 9(5):351-365 (2006), immunofluorescence microscopy, immunohistochemical staining, western blotting, in cell western, immunofluorescent staining, ELISA, and fluorescent activating cell sorting (FACS), bioluminescence, or fluorescent marker detection.

[0080] Displacement of the components of the heterodimers may be assayed by first detecting whether such heterodimers are produced in the cell or sample of interest, treating with a therapeutic, compound, or treatment, and then assaying for the presence of the heterodimers in said sample. If the chosen treatment successfully disrupts the formation of heterodimers in the cell or sample, the number of heterodimers will decrease (as measured, for example, by amounts of fluorescent signal). A decrease in heterodimers in a sample after treatment indicates the cell or sample tested is sensitive to said tested treatment. Alternatively, if a decrease in heterodimers is not observed, this may be an indication that the cell or sample will not respond to said tested treatment, which may guide the decision to chose an alternative treatment for the patient from whom the sample was obtained.

[0081] Alternatively, sensitivity to a particular treatment may be measured by determining the predisposition of the cell to undergo apoptosis. In one embodiment, this can be determined by measuring the mitochondrial outer membrane permeabilization (MOMP), which increases when a cell is about to undergo apoptosis. Mitochondrial outer membrane permeabilization can be measured for example, using the potentiometric dye JC-1 or dihydrorhodamine. MOMP can be measured using standard techniques known in the art, including those described in Bogenberger et al. (Leukemia et al. (2014) which is herein incorporated by reference in its entirety). In a non-limiting example, cells are permeabilized and incubated with a mitochondrial dye (e.g. JC-1 or dihydrorhodamine 123) and BH3 peptides with dimethyl sulfoxide or carbonyl cyanide m-chlorophenyl hydrazone (CCCP) and the degree of staining is measured. In one embodiment, the predisposition of a cell to undergo apoptosis is determined by measuring the amount of cytochrome C released from the mitochondria. This can be measured using standard techniques known in the art (See for example, Current Protocols in Molecular Biology, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, MA, 1993).

[0082] In another embodiment, the method comprises conducting the heterodimer binding assay on a cell that comprising one or more of a cell surface marker CD33, a cell surface marker CD34, a FLT3 mutation status, a p53 mutation status, a phosphorylation state of MEK-1 kinase, and phosphorylation of serine at position 70 of Bcl-2; and correlating to efficacy in treating cancer patients with chemotherapy.

[0083] In one embodiment, the heterodimer binding assay is performed on patient samples taken before treatment begins (time "0"). In another embodiment, the heterodimer binding assay is performed on patient samples taken during the course of treatment. In a further embodiment, the heterodimer binding assay is performed on the patient's cell or sample taken before and at various time points during treatment. In another embodiment, the heterodimer binding assay is performed on the patient's cell or sample taken at various time points during treatment. In one embodiment, the decision to perform a subsequent heterodimer binding assay in a patient is made when the patient stops responding to a current course of treatment. In another embodiment, the decision to perform a subsequent heterodimer binding assay is made independently of the patient's response to treatment.

[0084] In one aspect, the heterodimer binding assay is performed *in vitro*. Standard assays to evaluate the binding ability of the antibodies toward the target of various species are known in the art, including for example, ELISAs, western blots and RIAs. The binding kinetics (*e.g.*, binding affinity) of antibodies also can be assessed by standard assays known in the art, such as by Biacore analysis.

Patient Evaluation and Treatment

[0085] In some embodiments, the methods described herein are useful in the evaluation of a patient, for example, for evaluating diagnosis, prognosis, and response to treatment. In various aspects, the

present invention comprises evaluating a tumor or hematological cancer. In various embodiments, the evaluation may be selected from diagnosis, prognosis, and response to treatment.

[0086] Diagnosis refers to the process of attempting to determine or identify a possible disease or disorder, such as, for example, cancer. Prognosis refers to predicting a likely outcome of a disease or disorder, such as, for example, cancer. A complete prognosis often includes the expected duration, the function, and a description of the course of the disease, such as progressive decline, intermittent crisis, or sudden, unpredictable crisis. Response to treatment is a prediction of a patient's medical outcome when receiving a treatment. Responses to treatment can be, by way of non-limiting example, pathological complete response, survival, and progression free survival, time to progression, probability of recurrence.

[0087] In various embodiments, the invention predicts the efficacy of a cancer treatment which can include one or more of anti-cancer drugs, chemotherapy, surgery, adjuvant therapy, and neoadjuvant therapy. In an exemplary embodiment, the present method will indicate a likelihood of response to a specific treatment. For example, in some embodiments, the present methods indicate a high or low likelihood of response to a pro-apoptotic agent and/or an agent that operates via apoptosis and/or an agent that operates via apoptosis driven by direct protein modulation. In an exemplary embodiment, the present method will indicate whether a patient is to receive a pro-apoptotic agent or an agent that operates via apoptosis for cancer treatment. In another exemplary embodiment, the present method will indicate whether a patient is to receive an agent that does not operate via apoptosis. In another exemplary embodiment, the present invention predicts a cancer patient's likelihood of response to chemotherapy and comprises an evaluation of the heterodimer binding, age profile and cytogenetic factors of the patient.

[0088] As used herein, the term "neoadjuvant therapy" refers to treatment given as a first step to shrink a tumor before the main treatment, which is usually surgery, is given. Examples of neoadjuvant therapy include chemotherapy, radiation therapy, and hormone therapy. In some embodiments, the present methods direct a patient's treatment to include neoadjuvant therapy. For example, a patient that is scored to be responsive to a specific treatment may receive such treatment as neoadjuvant therapy. In some embodiments, neoadjuvant therapy means chemotherapy administered to cancer patients prior to surgery. In some embodiments, neoadjuvant therapy means an agent, including those described herein, administered to cancer patients prior to surgery. Further, the present methods may direct the identity of a neoadjuvant therapy, by way of non-limiting example, as a treatment that induces and/or operates in a pro-apoptotic manner or one that does not. In one embodiment, the present methods may indicate that a patient will not be or will be less responsive to a specific treatment and therefore such a patient may not receive such treatment as neoadjuvant therapy. Accordingly, in some embodiments, the present methods provide for providing or withholding

neoadjuvant therapy according to a patient's likely response. In this way, a patient's quality of life, and the cost of case, may be improved.

[0089] As used herein, the term "adjuvant therapy" refers to additional cancer treatment given after the primary treatment to lower the risk that the cancer will come back. Adjuvant therapy may include chemotherapy, radiation therapy, hormone therapy, targeted therapy, or biological therapy. In some embodiments, the present methods direct a patient's treatment to include adjuvant therapy. For example, a patient that is scored to be responsive to a specific treatment may receive such treatment as adjuvant therapy. Further, the present methods may direct the identity of an adjuvant therapy, by way of non-limiting example, as a treatment that induces and/or operates in a pro-apoptotic manner or one that does not. In one embodiment, the present methods may indicate that a patient will not be or will be less responsive to a specific treatment and therefore such a patient may not receive such treatment as adjuvant therapy. Accordingly, in some embodiments, the present methods provide for providing or withholding adjuvant therapy according to a patient's likely response. In this way, a patient's quality of life, and the cost of care, may be improved.

[0090] In various embodiments, the present methods direct a clinical decision regarding whether a patient is to receive a specific treatment. In one embodiment, the present methods are predictive of a positive response to neoadjuvant and/or adjuvant chemotherapy or non-responsiveness to neoadjuvant and/or adjuvant chemotherapy. In one embodiment, the present methods are predictive of a positive response to a pro-apoptotic agent or an agent that operates via apoptosis and/or an agent that does not operate via apoptosis or a non-responsiveness to apoptotic effector agent and/or an agent that does not operate via apoptosis. In various embodiments, the present invention directs the treatment of a cancer patient, including, for example, what type of treatment should be administered or withheld.

[0091] In some embodiments, the method comprises analysis of a patient's clinical factor. In various embodiments, the clinical factor is one or more of age, cytogenetic status, performance, histological subclass, gender, and disease stage. In another embodiment, the method further comprises a measurement of an additional biomarker selected from mutational status, single nucleotide polymorphisms, steady state protein levels, and dynamic protein levels, which can add further specificity and/or sensitivity to the test. In another embodiment, the method further comprises predicting a clinical response in the patient. In another embodiment, the clinical response is at least about 1, about 2, about 3, or about 5 year progression/event-free survival.

[0092] In one embodiment, the determination of the sensitivity or resistance of a patient's cancer cell to a particular therapeutic is used to classify the patient into a treatment or prognosis group. In some non-limiting examples, patients are classified into groups designated as cure, relapse, no complete response, complete response, refractory to initial therapy, responder, non-responder, high likelihood of response, or low likelihood of response. In further embodiments, analysis of the heterodimer

binding and patient classification direct a clinical decision regarding treatment, such as, for example, switching from one therapeutic to another, a change in dose of therapeutic, or administration of a different type of treatment (e.g. surgery, radiation, allogenic bone marrow or stem cell transplant). In a further embodiment, the clinical decision is directed by the analysis of a change in cancer sensitivity, classification, and consideration of clinical factors, such as age and/or cytogenetic status. In various embodiments, a cancer treatment is administered or withheld based on the methods described herein. Exemplary treatments include surgical resection, radiation therapy (including the use of the compounds as described herein as, or in combination with, radiosensitizing agents), chemotherapy, pharmacodynamic therapy, targeted therapy, immunotherapy, and supportive therapy (*e.g.*, painkillers, diuretics, antidiuretics, antivirals, antibiotics, nutritional supplements, anemia therapeutics, blood clotting therapeutics, bone therapeutics, and psychiatric and psychological therapeutics).

[0093] In one embodiment, a comparison of the data generated in the heterodimer binding assay performed at various time points during treatment shows a change heterodimer production indicating a change in the cancer's sensitivity to a particular treatment. In one embodiment, the determination of a cancer's change in sensitivity to a particular treatment is used to re-classify the patient and to guide the course of future treatment.

Cancers

[0103] In some embodiments the invention provides a method for determining a cancer treatment and/or comprises a patient's tumor or cancer cell specimen. A cancer or tumor refers to an uncontrolled growth of cells and/or abnormal increased cell survival and/or inhibition of apoptosis which interferes with the normal functioning of the bodily organs and systems. A subject that has a cancer or a tumor is a subject having objectively measurable cancer cells present in the subject's body. Included in this invention are benign and malignant cancers, as well as dormant tumors or micrometastatses. Cancers which migrate from their original location and seed vital organs can eventually lead to the death of the subject through the functional deterioration of the affected organs.

[0104] In one aspect, the invention provides a method for determining a cancer treatment for a patient, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; d) determining the presence of the heterodimer based on the intensity of the signal; e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and f) classifying the patient for likelihood of clinical response to one or more cancer treatments, wherein the presence of a heterodimer correlates with treatment efficacy.

[0105] In one aspect, the invention provides a method for predicting cancer sensitivity to treatment, comprising: a) isolating a cancer cell or specimen from said patient; b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer; c) detecting a signal that indicates binding of the antibody to the heterodimer; d) determining the presence of the heterodimer based on the intensity of the signal; e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and f) classifying the patient for likelihood of clinical response to one or more cancer treatments, wherein the presence of a heterodimer correlates with treatment efficacy.

[0106] In various embodiments, the invention is applicable to pre-metastatic cancer, or metastatic cancer. Metastasis refers to the spread of cancer from its primary site to other places in the body. Cancer cells can break away from a primary tumor, penetrate into lymphatic and blood vessels, circulate through the bloodstream, and grow in a distant focus (metastasize) in normal tissues elsewhere in the body. Metastasis can be local or distant. Metastasis is a sequential process, contingent on tumor cells breaking off from the primary tumor, traveling through the bloodstream, and stopping at a distant site. At the new site, the cells establish a blood supply and can grow to form a life-threatening mass. Both stimulatory and inhibitory molecular pathways within the tumor cell regulate this behavior, and interactions between the tumor cell and host cells in the distant site are also significant. Metastases are often detected through the sole or combined use of magnetic resonance imaging (MRI) scans, computed tomography (CT) scans, blood and platelet counts, liver function studies, chest X-rays and bone scans in addition to the monitoring of specific symptoms.

[0107] The methods described herein are directed toward the prognosis of cancer, diagnosis of cancer, treatment of cancer, and/or the diagnosis, prognosis, treatment, prevention or amelioration of growth, progression, and/or metastases of malignancies and proliferative disorders associated with increased cell survival, or the inhibition of apoptosis. In some embodiments, the cancer is a hematologic cancer, including, but not limited to, acute myelogenous leukemia (AML), multiple myeloma, follicular lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, and non-Hodgkin's lymphoma including, but not limited to, mantle cell lymphoma and diffuse large B-cell lymphoma. In some embodiments, the cancer is a solid tumor, including, but not limited to, non-small lung cell carcinoma, ovarian cancer, and melanoma.

[0108] In some embodiments, the invention relates to one or more of the following cancers: acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), adrenocortical carcinoma, AIDS-related cancers, anal cancer, appendix cancer, astrocytoma (*e.g.* childhood cerebellar or cerebral), basal-cell carcinoma, bile duct cancer, bladder cancer, bone tumor (*e.g.* osteosarcoma, malignant fibrous histiocytoma), brainstem glioma, brain cancer, brain tumors (*e.g.* cerebellar astrocytoma, cerebral astrocytoma/malignant glioma, ependymoma, medulloblastoma, supratentorial primitive neuroectodermal tumors, visual pathway and hypothalamic glioma), breast cancer, bronchial

adenomas/carcinoids, Burkitt's lymphoma, carcinoid tumors, central nervous system lymphomas,cerebellar astrocytoma, cervical cancer, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), chronic myeloproliferative disorders, colon cancer, cutaneous t-cell lymphoma, desmoplastic small round cell tumor, endometrial cancer, ependymoma, esophageal cancer, Ewing's sarcoma, extracranial germ cell tumor, extragonadal germ cell tumor, extrahepatic bile duct cancer, eye cancer, gallbladder cancer, gastric (stomach) cancer, gastrointestinal stromal tumor (GIST), germ cell tumor (*e.g.* extracranial, extragonadal, ovarian), gestational trophoblastic tumor, gliomas (*e.g.* brain stem, cerebral astrocytoma, visual pathway and hypothalamic), gastric carcinoid, head and neck cancer, heart cancer, hepatocellular (liver) cancer, hypopharyngeal cancer, hypothalamic and visual pathway glioma, intraocular melanoma, islet cell carcinoma (endocrine pancreas), kidney cancer (renal cell cancer), laryngeal cancer, leukemias (*e.g.* acute lymphocytic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, chronic myeloid leukemia, hairy cell), lip and oral cavity cancer, liposarcoma, liver cancer, lung cancer (*e.g.* non-small cell, small cell), lymphoma (*e.g.* AIDS-related, Burkitt, cutaneous T-cell Hodgkin, non-Hodgkin, primary central nervous system), medulloblastoma, melanoma, Merkel cell carcinoma, mesothelioma, metastatic squamous neck cancer, mouth cancer, multiple endocrine neoplasia syndrome, multiple myeloma, mycosis fungoides, myelodysplastic syndromes, myelodysplastic/myeloproliferative diseases, myelogenous leukemia, myeloid leukemia, myeloid leukemia, myeloproliferative disorders, chronic, nasal cavity and paranasal sinus cancer, nasopharyngeal carcinoma, neuroblastoma, non-Hodgkin lymphoma, non-small cell lung cancer, oral cancer, oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, pancreatic cancer, paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pharyngeal cancer, pheochromocytoma, pineal astrocytoma and/or germinoma, pineoblastoma and supratentorial primitive neuroectodermal tumors, pituitary adenoma, plasma cell neoplasia/multiple myeloma, pleuropulmonary blastoma, primary central nervous system lymphoma, prostate cancer, rectal cancer, renal cell carcinoma (kidney cancer), renal pelvis and ureter, retinoblastoma, rhabdomyosarcoma, salivary gland cancer, sarcoma (*e.g.* Ewing family, Kaposi, soft tissue, uterine), Sézary syndrome, skin cancer (*e.g.* nonmelanoma, melanoma, merkel cell), small cell lung cancer, small intestine cancer, soft tissue sarcoma, squamous cell carcinoma, squamous neck cancer, stomach cancer, supratentorial primitive neuroectodermal tumor, t-cell lymphoma, testicular cancer, throat cancer, thymoma and thymic carcinoma, thyroid cancer, trophoblastic tumors, ureter and renal pelvis cancers, urethral cancer, uterine cancer, uterine sarcoma, vaginal cancer, visual pathway and hypothalamic glioma, vulvar cancer, Waldenström macroglobulinemia, and Wilms tumor.

[0109] In one embodiment, the cancer is multiple myeloma (MM). In one embodiment, the cancer is AML. AML is the second most common leukemia, with approximately 13,000 newly diagnosed cases and 9,000 deaths annually in the US. Although approved therapies exist, the prognosis of many

leukemia patients is poor and the likelihood of successful treatment is low. The current standard of care for AML is induction cytosine arabinoside (ara-C) in combination with an anthracycline agent (such as, for example, daunarubicin, idarubicine or mitoxantrone). This therapeutic regimen is typically followed by administration of high dose cytarabine and/or stem cell transplantation. These treatments have improved outcome in young patients. Progress has also been made in the treatment of acute promyelocytic leukemia, where targeted therapy with all-trans retinoic acid (ATRA) or arsenic trioxide have resulted in excellent survival rates. However, patients over 60, a population which represents the vast majority of AML cases, remain a therapeutic enigma. Although 65-85% of patients initially respond to existing treatments, 65% of such responders undergo relapse, and many patients succumb to the disease. For at least this reason and because the afore-mentioned treatments may have severe side effects, the inventive predictive test can guide use of the treatment that mitigates these litigations. In some embodiments, the present invention improves the likelihood of successful treatment by matching the right patient to the right treatment. Further, there are currently no tests to predict AML patient response to treatment.

[0110] The term subject, as used herein unless otherwise defined, is a mammal, *e.g.*, a human, mouse, rat, hamster, guinea pig, dog, cat, horse, cow, goat, sheep, pig, or non-human primate, such as a monkey, chimpanzee, or baboon. The terms "subject" and "patient" are used interchangeably.

Specimens

[0111] In some embodiments, the present invention includes the measurement of a tumor specimen, including biopsy or surgical specimen samples. In some embodiments, the biopsy is a human biopsy. In various embodiments, the biopsy is any one of a tissue sample, a frozen tumor tissue specimen, cultured cells, circulating tumor cells, and a formalin-fixed paraffin-embedded tumor tissue specimen. In some embodiments the tissue sample is a peripheral blood sample, a lymph-node sample, a bone marrow sample, or an organ tissue sample. In another embodiment, the specimen is a mitochondrial fraction.

[0112] In some embodiments, the tumor specimen may be a biopsy sample, such as a frozen tumor tissue (cryosection) specimen. As is known in the art, a cryosection may employ a cryostat, which comprises a microtome inside a freezer. The surgical specimen is placed on a metal tissue disc which is then secured in a chuck and frozen rapidly to about -20°C to about -30°C. The specimen is embedded in a gel like medium consisting of, for example, poly ethylene glycol and polyvinyl alcohol. The frozen tissue is cut frozen with the microtome portion of the cryostat, and the section is optionally picked up on a glass slide and stained.

[0113] In some embodiments, the tumor specimen may be a biopsy sample, such as cultured cells. These cells may be processed using the usual cell culture techniques that are known in the art. These cells may be circulating tumor cells.

[0114] In some embodiments, the tumor specimen may be a biopsy sample, such as a formalin-fixed paraffin-embedded (FFPE) tumor tissue specimen. As is known in the art, a biopsy specimen may be placed in a container with formalin (a mixture of water and formaldehyde) or some other fluid to preserve it. The tissue sample may be placed into a mold with hot paraffin wax. The wax cools to form a solid block that protects the tissue. This paraffin wax block with the embedded tissue is placed on a microtome, which cuts very thin slices of the tissue.

[0115] In certain embodiments, the tumor specimen (or biopsy) contains less than 100 mg of tissue, or in certain embodiments, contains about 50 mg of tissue or less. The tumor specimen (or biopsy) may contain from about 20 mg to about 50 mg of tissue, such as about 35 mg of tissue.

[0116] The tissue may be obtained, for example, as one or more (*e.g.*, 1, 2, 3, 4, or 5) needle biopsies (*e.g.*, using a 14-gauge needle or other suitable size). In some embodiments, the biopsy is a fine-needle aspiration in which a long, thin needle is inserted into a suspicious area and a syringe is used to draw out fluid and cells for analysis. In some embodiments, the biopsy is a core needle biopsy in which a large needle with a cutting tip is used during core needle biopsy to draw a column of tissue out of a suspicious area. In some embodiments, the biopsy is a vacuum-assisted biopsy in which a suction device increases the amount of fluid and cells that is extracted through the needle. In some embodiments, the biopsy is an image-guided biopsy in which a needle biopsy is combined with an imaging procedure, such as, for example, X ray, computerized tomography (CT), magnetic resonance imaging (MRI) or ultrasound. In other embodiments, the sample may be obtained via a device such as the MAMMOTOME® biopsy system, which is a laser guided, vacuum-assisted biopsy system for breast biopsy.

[0117] In certain embodiments, the specimen is a human tumor-derived cell line. In certain embodiments, the specimen is a cancer stem cell. In other embodiments, the specimen is derived from the biopsy of a solid tumor, such as, for example, a biopsy of a colorectal, breast, prostate, lung, pancreatic, renal, or ovarian primary tumor.

[0118] In certain embodiments, the specimen is of epithelial origin. In some embodiments, the epithelial specimen is enriched by selection from a biopsy sample with an anti-epithelial cell adhesion molecule (EpCAM) or other epithelial cell binding antibody bound to solid matrix or bead.

[0119] In certain embodiments, the specimen is of mesenchymal origin. In some embodiments, the mesenchymal specimen is enriched by selection from a biopsy sample with a neural cell adhesion molecule (N-CAM) or neuropilin or other mesenchymal cell binding antibody bound to a solid matrix or bead.

[0120] In certain embodiments, the specimen is derived from the biopsy of a solid tumor. In certain embodiments, the specimen is derived from the biopsy of a non-solid tumor, such as, for example, any of the cancer described herein. In specific embodiments, the specimen is derived from the biopsy of a patient with multiple myeloma, acute myelogenous leukemia, acute lymphocytic leukemia, chronic lymphogenous leukemia, mantle cell lymphoma, diffuse large B-cell lymphoma, and non-Hodgkin's lymphoma. In a specific embodiment, the specimen is a multiple myeloma cell that is enriched by selection from a biopsy sample with an anti-CD138 antibody bound to a solid matrix or bead. In a specific embodiment, the specimen is an acute myelogenous leukemia cell that is enriched by binding to a CD45-directed antibody. In a specific embodiment, the specimen is a chronic lymphogenous leukemia or diffuse large B-cell lymphoma that is enriched by non-B cell depletion. In some embodiments, the specimen is derived from a circulating tumor cell.

Treatments

[0094] Also within the scope of this invention is a method for assessing whether a patient is sensitive or resistance to a drug that interferes with formation of a heterodimer based on the presence of that heterodimer in the patient. A cancer patient is sensitive to an apoptosis inducer that blocks formation of an anti-apoptotic heterodimer if this heterodimer is present in that patient. A neurodegenerative disease or cardiovascular disease patient, on the other hand, is responsive to an apoptosis inhibitor that blocks formation of a pro-apoptotic heterodimer if this heterodimer is present in that patient.

[0095] In exemplary embodiments, the invention selects a treatment agent. Examples of such agents include, but are not limited to, one or more of anti-cancer drugs, chemotherapy, surgery, adjuvant therapy, and neoadjuvant therapy.

[0096] In various embodiments, the invention pertains to cancer treatments including, without limitation, one or more of alkylating agents such as thiotepa and CYTOXAN cyclosphosphamide; kinesin-spindle protein stabilizing agent; proteasome inhibitor; modulator of cell cycle regulation (by way of non-limiting example, a cyclin dependent kinase inhibitor); a modulator of cellular epigenetic mechanistic (by way of non-limiting example, one or more of a histone deacetylase (HDAC) (*e.g.* one or more of vorinostat or entinostat), azacytidine, decitabine); a glucocorticoid; a steroid; a monoclonal antibody; an antibody-drug conjugate; a thalidomide derivative; an inhibitor of MCL1; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethiylenethiophosphoramide and trimethylolomelamine; acetogenins (*e.g.*, bullatacin and bullatacinone); a camptothecin (including the synthetic analogue topotecan); bryostatin; cally statin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); cryptophycins (*e.g.*, cryptophycin 1 and cryptophycin 8);

dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB 1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimnustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammall and calicheamicin omegall (*see, e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; bisphosphonates, such as clodronate; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antibiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabicin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, ADRIAMYCIN doxorubicin (including morpholino- doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin and deoxy doxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; an anthracycline or anthracenedione; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, a cytarabine-based chemotherapy, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as minoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; demecolcine; diaziquone; elformithine; elliptinium acetate; an epothilone; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSK polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (*e.g.*, T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxoids, *e.g.*, TAXOL paclitaxel (Bristol-Myers Squibb Oncology, Princeton, N.J.), ABRAXANE Cremophor-free, albumin-engineered nanoparticle formulation of paclitaxel (American Pharmaceutical Partners, Schaumberg, 111.), and TAXOTERE doxetaxel (Rhone-Poulenc Rorer, Antony, France); chloranbucil; GEMZAR gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin, oxaliplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine; NAVELBINE.

vinorelbine; novantrone; teniposide; edatrexate; daunomycin; aminopterin; xeloda; ibandronate; irinotecan (Camptosar, CPT-11) (including the treatment regimen of irinotecan with 5-FU and leucovorin); topoisomerase inhibitor e.g. RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; capecitabine; combretastatin; leucovorin (LV); oxaliplatin, including the oxaliplatin treatment regimen (FOLFOX); lapatinib (Tykerb); inhibitors of PKC-α, Raf, H-Ras, EGFR (*e.g.*, erlotinib (Tarceva)) and VEGF-A that reduce cell proliferation, dacogen, velcade, and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[0097] In various embodiments, cancer treatments include, without limitation, one or more BH3 mimetics. BH3 mimetics or analogs thereof, that may be used include, but are not limited to, Gossypol and its analogs (e.g. Ideker et al. Genome Res. 2008), ABT-199, ABT-737 (e.g. Petros et al. Protein Sci. 2000), ABT-263 (e.g. Letai et al. Cancer Cell 2002) and their analogues (e.g. WO2005049593, US7,767,684, US 7,906,505), Obatoclax (e.g. WO2004106328, WO2005117908, US 7,425,553), EU-5148, EU-5346, EU-4030, EU-51aa48 (Eutropics), compounds that selectively inhibit Mcl-1 (e.g. WO2008131000, WO2008130970, Richard, et al. (2013) Bioorg Med Chem. 21(21):6642-9)), HA-14-1 (e.g. Wang, *et al.* (2000) Proc. Natl. Acad. Sci. USA 97: 7124-9), Antimycin-A (e.g. Tzung, *et al.* (2001) Nat. Cell. Biol. 3: 183-191), BH3I-1 and BH3I-2 (e.g. Degterev, *et al.* (2001) Nat. Cell. Biol. 3: 173-82), terphenyl derivatives (e.g. Kutzki, *et al.* (2002) J. Am. Chem. Soc. 124: 11838-9), and compounds with selective BH3 mimic function (e.g. Ng (2014) Clin Adv Hematol Oncol. 12(4):224-9.

[0098] In various embodiments, cancer treatments include, without limitation, one or more SMAC mimetics or analogs thereof. SMAC mimetics mimetics or analogs thereof, that may be used include, but are not limited to, small molecule inhibitors, Smac-mimic (Li et al., Science 305: 1471–1474 (2004)), LBW242 (Petrucci et al. PLoS ONE 7(4): e35073 (2012), TL32711 (TetraLogic Pharmaceuticals), LCL161 (Novartis), GDC-0917 (Genentech), AEG40826/HGS1029 (Aegera), AT-406 (Ascenta), and the SMAC mimetics disclosed in US Patent No. 7,807,699.

[0099] In various embodiments, the invention pertains to cancer treatments including, without limitation, those described in US Patent Publication No. US 2012-0225851 and International Patent Publication No. WO 2012/122370, the contents of which are hereby incorporated by reference in their entireties.

Clinical Factors and Additional Biomarkers

[0121] In some embodiments, the invention comprises the evaluation of clinical factors. In some embodiments, the invention comprises an evaluation of heterodimer binding and/or clinical factors to assess a patient response. In some embodiments, a clinical factor that provides patient response

information in combination with a heterodimer binding study may not be linked to apoptosis. In some embodiments, a clinical factor is non-apoptosis affecting.

[0122] In one embodiment, the clinical factor is one or more of age, cytogenetic status, performance, histological subclass, gender, and disease stage

[0123] In one embodiment, the clinical factor is age. In one embodiment, the patient age profile is classified as over about 10, or over about 20, or over about 30, or over about 40, or over about 50, or over about 60, or over about 70, or over about 80 years old.

[0124] In one embodiment, the clinical factor is cytogenetic status. In some cancers, such as Wilms tumor and retinoblastoma, for example, gene deletions or inactivations are responsible for initiating cancer progression, as chromosomal regions associated with tumor suppressors are commonly deleted or mutated. For example, deletions, inversions, and translocations are commonly detected in chromosome region 9p21 in gliomas, non-small-cell lung cancers, leukemias, and melanomas. Without wishing to be bound by theory, these chromosomal changes may inactivate the tumor suppressor cyclin-dependent kinase inhibitor 2A. Along with these deletions of specific genes, large portions of chromosomes can also be lost. For instance, chromosomes 1p and 16q are commonly lost in solid tumor cells. Gene duplications and increases in gene copy numbers can also contribute to cancer and can be detected with transcriptional analysis or copy number variation arrays. For example, the chromosomal region 12q13-q14 is amplified in many sarcomas. This chromosomal region encodes a binding protein called MDM2, which is known to bind to a tumor suppressor called p53. When MDM2 is amplified, it prevents p53 from regulating cell growth, which can result in tumor formation. Further, certain breast cancers are associated with overexpression and increases in copy number of the *ERBB2* gene, which codes for human epidermal growth factor receptor 2. Also, gains in chromosomal number, such as chromosomes 1q and 3q, are also associated with increased cancer risk.

[0125] Cytogenetic status can be measured in a variety of manners known in the art. For example, FISH, traditional karyotyping, and virtual karyotyping (*e.g.* comparative genomic hybridization arrays, CGH and single nucleotide polymorphism arrays) may be used. For example, FISH may be used to assess chromosome rearrangement at specific loci and these phenomenon are associated with disease risk status. In some embodiments, the cytogenetic status is favorable, intermediate, or unfavorable.

[0126] In one embodiment, the clinical factor is performance. Performance status can be quantified using any system and methods for scoring a patient's performance status are known in the art. The measure is often used to determine whether a patient can receive chemotherapy, adjustment of dose adjustment, and to determine intensity of palliative care. There are various scoring systems, including the Karnofsky score and the Zubrod score. Parallel scoring systems include the Global Assessment of

Functioning (GAF) score, which has been incorporated as the fifth axis of the Diagnostic and Statistical Manual (DSM) of psychiatry. Higher performance status (*e.g.*, at least 80%, or at least 70% using the Karnofsky scoring system) may indicate treatment to prevent progression of the disease state, and enhance the patient's ability to accept chemotherapy and/or radiation treatment. For example, in these embodiments, the patient is ambulatory and capable of self care. In other embodiments, the evaluation is indicative of a patient with a low performance status (*e.g.*, less than 50%, less than 30%, or less than 20% using the Karnofsky scoring system), so as to allow conventional radiotherapy and/or chemotherapy to be tolerated. In these embodiments, the patient is largely confined to bed or chair and is disabled even for self-care.

[0127] The Karnofsky score runs from 100 to 0, where 100 is "perfect" health and 0 is death. The score may be employed at intervals of 10, where: 100% is normal, no complaints, no signs of disease; 90% is capable of normal activity, few symptoms or signs of disease, 80% is normal activity with some difficulty, some symptoms or signs; 70% is caring for self, not capable of normal activity or work; 60% is requiring some help, can take care of most personal requirements; 50% requires help often, requires frequent medical care; 40% is disabled, requires special care and help; 30% is severely disabled, hospital admission indicated but no risk of death; 20% is very ill, urgently requiring admission, requires supportive measures or treatment; and 10% is moribund, rapidly progressive fatal disease processes.

[0128] The Zubrod scoring system for performance status includes: 0, fully active, able to carry on all pre-disease performance without restriction; 1, restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, *e.g.*, light house work, office work; 2, ambulatory and capable of all self-care but unable to carry out any work activities, up and about more than 50% of waking hours; 3, capable of only limited self-care, confined to bed or chair more than 50% of waking hours; 4, completely disabled, cannot carry on any self-care, totally confined to bed or chair; 5, dead.

[0129] In one embodiment, the clinical factor is histological subclass. In some embodiments, histological samples of tumors are graded according to Elston & Ellis, Histopathology, 1991, 19:403-10, the contents of which are hereby incorporated by reference in their entirety.

[0130] In one embodiment, the clinical factor is gender. In one embodiment, the gender is male. In another embodiment the gender is female.

[0131] In one embodiment, the clinical factor is disease stage. By way of non-limiting example, using the overall stage grouping, Stage I cancers are localized to one part of the body; Stage II cancers are locally advanced, as are Stage III cancers. Whether a cancer is designated as Stage II or Stage III can depend on the specific type of cancer. In one non-limiting example, Hodgkin's disease, Stage II indicates affected lymph nodes on only one side of the diaphragm, whereas Stage III indicates

affected lymph nodes above and below the diaphragm. The specific criteria for Stages II and III therefore differ according to diagnosis. Stage IV cancers have often metastasized, or spread to other organs or throughout the body.

[0132] In some embodiments, the clinical factor is the French–American–British (FAB) classification system for hematologic diseases (*e.g.* indicating the presence of dysmyelopoiesis and the quantification of myeloblasts and erythroblasts). In one embodiment, the FAB for acute lymphoblastic leukemias is L1-L3, or for acute myeloid leukemias is M0-M7.

[0133] In another embodiment, the method further comprises a measurement of an additional biomarker selected from mutational status, single nucleotide polymorphisms, steady state protein levels, and dynamic protein levels. In another embodiment, the method further comprises predicting a clinical response in the patient. In another embodiment, the clinical response is about 1, about 2, about 3, or about 5 year progression/event-free survival.

[0134] A variety of clinical factors have been identified, such as age profile and performance status. A number of static measurements of diagnosis have also been utilized, such as cytogenetics and molecular events including, without limitation, mutations in the genes MLL, AML/ETO, Flt3-ITD, NPM1 (NPMc+), CEBPα, IDH1, IDH2, RUNX1, ras, and WT1 and in the epigenetic modifying genes TET2 and ASXL, as well as changes in the cell signaling protein profile.

[0135] Further, in some embodiments, the any one of the following clinical factors may be useful in the methods described herein: gender; genetic risk factors; family history; personal history; race and ethnicity; features of the certain tissues; various benign conditions (*e.g.* non-proliferative lesions); previous chest radiation; carcinogen exposure and the like.

[0136] Further still, in some embodiments, the any one of the following clinical factors may be useful in the methods described herein: one or more of a cell surface marker CD33, a cell surface marker CD34, a FLT3 mutation status, a p53 mutation status, a phosphorylation state of MEK-1 kinase, and phosphorylation of serine at position 70 of Bcl-2.

[0137] In some embodiments, the clinical factor is expression levels of the cytokines, including, without limitation, interleukin-6. In some embodiments, interleukin-6 levels will correlate with likelihood of response in MM patients, including a poor patient prognosis or a good patient prognosis.

[0138] In another embodiment, the method comprises measuring the heterodimer binding of a cell expressing one or more of a cell surface marker CD33, a cell surface marker CD34, a FLT3 mutation status, a p53 mutation status, a phosphorylation state of MEK-1 kinase, and phosphorylation of serine at position 70 of Bcl-2; and correlating to efficacy in treating cancer patients with chemotherapy.

[0139] In still another embodiment, the cancer is AML and/or MM and the clinical factor is age profile and/or cytogenetic status; or the cancer is AML and/or MM and the cancer treatment is

cytarabine or cytarabine-based chemotherapy and/or azacytidine, or the cancer treatment is cytarabine or cytarabine-based chemotherapy and/or azacytidine and the clinical factor is age profile and/or cytogenetic status, or the cancer treatment is cytarabine or cytarabine-based chemotherapy and/or azacytidine; the cancer is AML and/or MM; and the clinical factor is age profile and/or cytogenetic status.

[0140] The invention also provides kits that can simplify the evaluation of tumor or cancer cell specimens. A typical kit of the invention comprises various reagents including, for example, one or more agents to detect a BH3 peptide. A kit may also comprise one or more of reagents for detection, including those useful in various detection methods, such as, for example, antibodies. The kit can further comprise materials necessary for the evaluation, including welled plates, syringes, and the like. The kit can further comprise a label or printed instructions instructing the use of described reagents. The kit can further comprise a treatment to be tested.

Detection Methods

[00100] In various embodiments, the present methods comprise evaluating the cytogenetic status of a cell (e.g. evaluating a presence, absence, or level of a protein and/or a nucleic acid). In various embodiments, the present methods comprise evaluating a presence, absence, or level of a protein and/or a nucleic acid which can enhance the specificity and/or sensitivity of heterodimer binding. In some embodiments, the evaluating is of a marker for patient response. In some embodiments, the present methods comprise measurement using one or more of immunohistochemical staining, western blotting, in cell western, immunofluorescent staining, ELISA, and fluorescent activating cell sorting (FACS), bioluminescence, fluorescent marker detection, or any other method described herein or known in the art. The present methods may comprise contacting an antibody with a tumor specimen (*e.g.* biopsy or tissue or body fluid) to identify an epitope that is specific to the tissue or body fluid and that is indicative of a state of a cancer.

[00101] There are generally two strategies used for detection of epitopes on antigens in body fluids or tissues, direct methods and indirect methods. The direct method comprises a one-step staining, and may involve a labeled antibody (*e.g.* FITC conjugated antiserum) reacting directly with the antigen in a body fluid or tissue sample. The indirect method comprises an unlabeled primary antibody that reacts with the body fluid or tissue antigen, and a labeled secondary antibody that reacts with the primary antibody. Labels can include radioactive labels, fluorescent labels, hapten labels such as, biotin, or an enzyme such as horse radish peroxidase or alkaline phosphatase. Methods of conducting these assays are well known in the art. *See, e.g.*, Harlow *et al.* (Antibodies, Cold Spring Harbor Laboratory, NY, 1988), Harlow *et al.* (Using Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, NY, 1999), Virella (Medical Immunology, 6th edition, Informa HealthCare, New York,

2007), and Diamandis *et al.* (Immunoassays, Academic Press, Inc., New York, 1996). Kits for conducting these assays are commercially available from, for example, Clontech Laboratories, LLC. (Mountain View, CA).

[00102] In another embodiment, the measurement comprises evaluating a presence, absence, or level of a nucleic acid. A person skilled in the art will appreciate that a number of methods can be used to detect or quantify the DNA/RNA levels of appropriate markers.

[00103] Gene expression can be measured using, for example, low-to-mid-plex techniques, including but not limited to reporter gene assays, Northern blot, fluorescent *in situ* hybridization (FISH), and reverse transcription PCR (RT-PCR). Gene expression can also be measured using, for example, higher-plex techniques, including but not limited, serial analysis of gene expression (SAGE), DNA microarrays. Tiling array, RNA-Seq/whole transcriptome shotgun sequencing (WTSS), high-throughput sequencing, multiplex PCR, multiplex ligation-dependent probe amplification (MLPA), DNA sequencing by ligation, and Luminex/XMAP. A person skilled in the art will appreciate that a number of methods can be used to detect or quantify the level of RNA products of the biomarkers within a sample, including arrays, such as microarrays, RT-PCR (including quantitative PCR), nuclease protection assays and Northern blot analyses.

[0141] This invention is further illustrated by the following non-limiting examples.

EXAMPLES

Example 1: Preparation of Monoclonal Antibodies Specific to caspase-IAP Heterodimers

[0142] Genes encoding human caspase, 9, 7 and 3 minus the caspase recruitment domain are cloned and expressed as described in *Curr Protoc Protein Sci.* 2003 Feb; Chapter 21:Unit 21.13. *Expression, purification, and characterization of caspases. Denault JB, Salvesen GS.*)

[0143] All of the DNA constructs are introduced into BL21 E. coli cells. Positive transformants are cultured in a suitable medium and expression of the fusion proteins are induced with isopropyl-1-thio-β-D-galactopyranoside. The expressed fusion proteins are purified using Amersham Hitrap Glutathione column on the ACTA-FPLC (Amersham) and accurately quantified using spectrophotometry.

[0144] Peptides comprising the BIR 1 and 3 domains of xIAP and the BIR-2 domains of xIAP, cIAP1, or cIAP2, as described in table 3 are made with one aromatic amino acid residue replaced with benzol phenylalanine (Bpa) during synthesis. The Bpa modified BIR-3 domain peptides are checked for binding to caspase 3, 7 and 9 using fluorescence polarization, in this case by inhibiting the bonding of wild type peptides are labeled with FITC to caspases (Eckelman and Salvesen, *J. Biol. Chem.* 2006, 281:3254-3260).

[0145] After binding kinetics are determined at equamolar amounts in PBS and exposed to UV light to catalyze covalent attachment of the Bpa residue to the caspase protein. The mixture is stirred on ice for 12 hours to allow formation of heterodimers. The heterodimers are separated from unbound caspase proteins using a BIR 2, BIR-1 or BIR3 peptide coupled sepharose 12 column on a ACTA-FPLC, following the method described in Zue *et al.*, Protein Science 6: 781-788 (2007).

[0146] Each of the heterodimers (1mg) is suspended in monophosphoryl lipid A plus trehalose dicorynomycolate adjuvant. The mixtures thus formed are injected into Balb/c mice at each hind foot pad once every 3-4 days for 14 times. Three days after the final injection, spleen cells are removed from the mice and a single cell suspension is prepared in a DMEM medium supplemented with 1% penicillin-streptomycin. The spleen cells are fused with murine myeloma cells P3X63AgU.1 (ATCC® CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates.

[0147] Hybridomas are selected in super DMEM [DMEM supplemented with 10% fetal calf serum FCS, 100 mM pyruvate, 100 U/ml insulin, 100 mM oxaloacetic acid, 2 mM glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 µg/ml streptomycin] containing 100 µM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine (HAT).

[0148] Hybridoma cells are fed with 200 µl of super DMEM containing 10% FCS and antibiotics. Ten days after the fusion, supernatants of the hybridoma cultures are collected and screened for the presence of antibodies that bind to the cognate heterodimer protein and/or to either member of the heterodimer (as negative controls) in a capture ELISA as described in Certo *et al.*, Cancer Cell., 9(5):351-365 (2006).

[0149] Briefly, 96-well microtiter plates are coated with 50 µl (1 µg/ml) of a heterodimer or a member of the heterodimer at 4° C overnight. The plates are then washed three times with PBS containing 0.05% TWEEN 20™ (PBST) and blocked with 50 µl PBS containing 2.0% bovine serum albumin (BSA) at room temperature for 1 hour. The plates are then washed again three times with PBST. Afterwards, 100 µl of a hybridoma supernatant is added to designated wells. The plates are incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer. Next, 50 µl HRP-conjugated goat anti-mouse IgG Fc, diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% % TWEEN 20™, 0.01% Thimersol in PBS), is added to each well. The plates are then incubated for 1 hour at room temperature on a shaker apparatus and washed three times with wash buffer, followed by addition of 50 µl of substrate DACO and incubation at room temperature for 10 minutes. 50 µl diethyl glycol were added to each well to stop the reaction and absorbance at 450 nm in each well is read in a microtiter plate reader.

[0150] Hybridoma cells producing antibodies that bind to a heterodimer but not to either member of the heterodimer are selected. These positive hybridoma cells are cloned twice and the specificity of

the antibodies produced thereby are retested. The isotypes of the antibodies having the desired specificity are determined by conventional methods, e.g., using isotype specific goat anti-mouse Igs.

Example 2: Preparation of Polyclonal Antibodies Specific to caspase-IAP Heterodimers

[0151] New Zealand rabbits are immunized on the back and proximal limbs of the rabbits with 0.1 ml of a caspase-IAP heterodimer (50 µg/ml) prepared following the method described in Example 1. The heterodimer is pre-mixed with 50% Freund's complete adjuvant. The immunization is repeated 28th days later. On day 35, 0.5 ml of blood is obtained from each of the immunized rabbits and antibody titers in the blood samples are determined by ELISA. Anti-sera are collected from the arterial carotid of rabbits having high antibody titers.

[0152] The specificity of the antibodies in each antiserum is examined by conventional methods, e.g., the immunoprecipitation and FACS assays described in Examples 4 and 5 below.

Example 3: Screening for scFv Antibodies Specific to caspase-IAP Heterodimers Using A Yeast scFv Library

[0153] A nonimmune human scFv yeast library (using expression vector pYD1) is obtained from Pacific Northwest National Laboratories. In this library, a scFv antibody, in which the heavy and light chains are connected by a flexible polypeptide linker, is fused to the adhesion subunit of the yeast agglutinin protein Aga2p and the HA-tag. Upon expression, the scFv is located on the surface of a yeast host cell via binding of Aga2P to Aga1P, a cell surface protein. See **FIG. 4.** Each yeast cell typically displays 1 x 10^5 to 1 x 10^6 copies of the scFv and the surface expression of the scFv. Variations in surface expression can be measured through immunofluorescence labeling of the HA-tag flanking the scFv region.

[0154] The scFv library described above is introduced into yeast strain EBY100 (Invitrogen™) and scFv antibodies having the desired specificity are identified as follows. The EBY yeast cells are first grown overnight in 1 liter of SDCAA medium (containing 20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto casamino acids, 5.4 g Na2HPO4 and 8.56 g NaH2PO4H2O). 1 x 10^{10} yeast cells from the overnight culture are precipitated by centrifugation at 2,500 g for 5 minutes and resuspended in SGCAA medium (a medium identical to SDACC except that it contains galactose instead of dextrose) to an absorbance of about 0.5-1 at 600 nm. The yeast cells are then cultured at 20° C for 36 h to allow expression of scFv antibodies. Afterwards, the cells are collected by centrifugation at 2,500 g for 5 min. The cell pellet is washed with 25 ml PBS.

[0155] Yeast cells expressing scFv antibodies are sorted by flow cytometry. Briefly, about 1 x 10^6 to 1 x 10_7 yeast cells prepared as described above are collected via centrifugation at 14,000 g for 30

seconds, washed with 1 ml PBS buffer, and mixed with 2 µl of 10 µg/ml anti-HA phycoerythrin monoclonal antibody (SIGMA-ALDRICH™) and caspase-IAP heterodimer, in which the anti-capase antibody is labeled with FITC and the IAP antibody is labeled with Texas Red®. After being incubated at room temperature for 1 hour, the mixture is centrifuged at 12,000 g for 30 seconds to precipitate yeast cells. The cell pellet thus formed is resuspended in 500 µl 10 mM Tris (final cell density about 10^6/ml) and subjected to cell sorting by flow cytometry as follows.

[0156] A flow cytometry protocol is pre-determined using EBY100 yeast cells mixed with the anti-HA phycoerythrin antibody as a positive control and EBY100 yeast cells mixed with the double-labeled heterodimer as a negative control. Compensation is performed to reject crosstalk between the FITC, Texas Red®, and phycoerythrin channels of the fluorescence detector. The labeled yeast cells are loaded into a FACSAria Cell-Sorter (Becton Dickinson, Mountain View, Calif.) and gated on forward- and side scatter channels. An appropriate sort gate in the FITC/Texas red/phycoerythrin positive quadrant is drawn and the top 5% triple positive yeast cells are collected in 1 ml SDCAA media. If necessary, the top 0.1% triple-positive yeast cells are collected to ensure that only cells having high affinity to caspase IAP heterodimer is sorted.

[0157] The triple-positive cells thus identified are suspended in 10 ml SDCAA and grown over night at 30° C. These cells are then subjected to two rounds of negative selection to exclude cells expressing scFv antibodies that also bind to caspase monomer or IAP monomer. More specifically, the cells are incubated with FITC-labeled caspase and Texas red-labeled IAP and following the same procedure described above, FITC and Texas Red® double negative cells are sorted. The cells thus collected are labeled with the double-labeled caspase IAP heterodimer to confirm their binding to the heterodimer.

[0158] The yeast cell thus identified are diluted and plated to allow formation of individual clones. Plasmid DNAs are isolated from these clones using a Zymoprep kit (Zymo Research, Orange, Calif.) as described in Weaver-Feldhaus et al., Protein Engineering, Design & Selection vol. 18, no. 11, pp 527-536 (2005). The scFv sequence included in each plasmid DNA is determined following the method described in Chao et al., Nature Protocols 1:755-768 (2006).

[0159] The scFv antibodies thus identified are analyzed by ELISA and FACS to confirm their specificity to caspase-IAP heterodimer. They can subject to mutagenesis to select for scFv antibodies having higher affinity and specificity to caspase IAP heterodimer.

Example 4: Select Antibodies Specific to caspase IAP Heterodimers by Immunoprecipitation

[0160] An immunoprecipitation assay, as illustrated in **FIG. 2**, is performed to ensure that the antibodies obtained in Example 1 above are specific to caspase IAP heterodimer. The two members of a caspase IAP heterodimer are conjugated with two fluorescent probes that have distinct emission

spectra, i.e., one labeled with fluorescein isothiocyanate (FITC; which emits at 488 nm) and the other labeled with Texas red (which emits at 590 nm). The labeled members are incubated together to allow formation of the caspase IAP heterodimer, following the method described in Example 1 above. 0.1 µg of the heterodimer thus formed is incubated with 50 µL of supernatant from a hydridoma clone that produces an antibody of interest in 0.5 mL PBS containing 0.05% tween-20. The non-dimerized labeled members of the heterodimer are used as negative controls. The mixtures are incubated for 1 hour on ice to allow formation of antibody-antigen complexes and 10 µl of GammaBing-G sepharose beads (GE Healthcare™, Piscataway, N.Y.) are added to the mixture. After being incubated on ice for 30 minutes on ice with rotation, the mixtures are centrifuged at 10,000 g for 30 seconds. The pellet beads, to which the antibody-antigen complexes are attached, are washed several times and measured for optical density at 488 nm (OD488) and 590 nm (OD590). The specificity of the antibody is determined based on the values of OD488 and 590 nm OD590.

Example 5: Detecting caspase-IAP Heterodimers in Fixed Cells

[0161] Cells care characterized for having a prevalent caspase IAP heterodimer. These cells, placed on cover slips, are fixed with 2-4% formaldehyde (Formaldehyde, 16%, methanol free) in PBS for 15 minutes at room temperature. The cell-containing cover slips are rinsed with PBS three times, 5 minutes for each. The slips are then soaked in a blocking buffer (TBST/5% normal goat serum: to 5 ml 1 x TBST add 250 µl normal goat serum) for 60 minutes. After the blocking buffer is aspirated, an antibody specific to either caspase 3, 7 or 9 or cIAP1, cIAP2, xIAP heterodimer (0.1 to 15 mg/ml) is added to the slips. After being incubated at 4° C. overnight, the slips are rinsed three times with PBS, 5 minutes each time. A fluorochrome-conjugated secondary antibody, diluted in a dilution buffer, is then added. After being incubated for 1-2 hours at room temperature in dark, the slips are rinsed with PBS three times, 2 minutes each time, and subsequently treated with Prolong Gold Antifade Reagent (Invitrogen™). The slips are then sealed by painting around edges of the slips with nail polish and observed under an inverted fluorescent microscope.

Example 6: Detecting caspase-IAP Heterodimers in Fixed Tissue Samples

[0162] Paraffin embedded and frozen thin section tissue samples from cancer patients and healthy subjects are purchased from Imgenex™. These samples are spotted on microarray chips (4 mm x 4 mm spots that are 4 mm thick). The adjacent normal tissues from the same patients/healthy subjects are also spotted on the array chips.

[0163] The microarray chips mentioned above are washed in turn with xylene three times, 5 minutes each time, 100% ethanol twice, 10 minutes each time, 95% ethanol, twice, 10 minutes each time, and

finally dH2O twice, 5 minutes each time. The chips are then soaked in 1 mM EDTA, pH 8.0, heated to boiling, and then kept at a sub-boiling temperature for 15 minutes.

[0164] If the tissue samples on the microarray chips are fixed with formalin, the chips are washed in turn with 100%, 95%, 80% ethanol 3 times each, 3 minutes each time, followed by two washes with dH2O, 3 minutes each. The chips are then soaked in 0.01M sodium citrate. pH 6.0 for 20 minutes.

[0165] The chips are then washed with dH_2O three times, 5 minutes each time, incubated in 3% hydrogen peroxide for 10 minutes (this step is not needed for formalin fixed samples), and washed again with dH2O twice, 5 minutes each time.

[0166] Next, the chips are subjected to immunostaining using the antibodies prepared in Example 1 or an anti-caspase antibody as a control. The chips are soaked in a wash buffer for 5 minutes and then in 100-400 µl of a blocking buffer (TBST containing 5% normal goat serum) for one hour. After decanting the blocking solution, the chips are incubated with 100-400 µl of an anti-caspase/IAP-heterodimer antibody (primary antibody), diluted to 0.1 to 15 µg/ml for each chip, overnight at 4µ C. Afterwards, the chips are washed with the wash buffer three times, 5 minutes each time, and then incubated with 100-400 µl of a biotinylated goat anti-mouse Ig antibody (the secondary antibody), which is diluted in TBST following the manufacturer's protocol, for 30 minutes at room temperature. The chips are then washed with the wash buffer three times, 5 minutes each time, and incubated with 100-400 µl ABC reagent (Vectastain ABC Kit™), which is prepared following the manufacturer's instructions, for 30 minutes at room temperature. After being washed for three times with the wash buffer, the chips are incubated with 100-400 µl DAB for signal development. The chips are immersed in dH_2O immediately after a color has developed thereon. When necessary, the chips are counterstained with hematoxylin and DAPI following manufacturer's instructions.

[0167] The stained chips are dehydrated by incubation sequentially in 95% ethanol two times, 10 seconds each, in 100% ethanol two times, 10 seconds each, and finally in xylene two times, 10 seconds each. The chips are then mounted with cover slips and examined using Fluorescence and UV microscopy for staining patterns. The staining patterns obtained from cancer tissue samples are compared with those obtained from adjacent normal tissues.

Example 7: Antibodies that Bind Bim-BH3 domain peptide

Preparation of Heterodimer Immunogen

[0168] We prepared an immunogen consisting of a Bcl-xL-GST fusion protein bound with Bim-BH3 domain peptide. (**FIG. 6**). Though there is tight binding between the peptide and the protein it seemed likely that only a covalently attached peptide would remain bound during the immunization process. To make such a covalent heterodimer immunogen we prepared a series of Bim BH3 domain

peptides with 4-benzoylpheylalanine (BPA)residues with replacing each of the sterically similar aromatic amino acids in the peptide one at a time. (**FIG. 7**). A series of such peptides were tested for binding affinities for Bcl-xL using fluorescence polarization and compared to the non-modified Bim BH3 peptide. The peptide that demonstrated the most similar binding affinity to the non-modified Bim BH3 peptide was chosen for covalent linking.

[0169] Coupling was performed by adding a 2 fold molar excess of BPA-Bim-BH3 to Bcl-xL GST and exposing to UV light for 8 hours. Following UV activation each of the different Bcl-xL Bim-BPA-BH3 covalent complexes were tested for physical features by gel electrophoresis, mass spectroscopy analysis. Unbound Bcl-xL GST was removed from the solution by passing over a Biotinylated-Bim BH3, Streptavidin-bead column. The flow through was prepared for immunization.

Monoclonal antibody development

[0170] HTP™ Mice (Abpro, Lexington MA) have been genetically engineered to produce a more sensitive immune response than mouse models. Due the broader epitope diversity of this response, it generates high affinity antibodies to the most traditionally difficult targets. MAbs were generated in mice using a rapid immunization protocol. Using a modified rapid immunization at multiple sites (RIMMS) protocol with the soluble GST-Bcl-xL/BIM, the immunized mice developed high levels of polyclonal IgG to the immunogen within 17 days of the first immunization. The lymph node cells isolated from the immunized animals were then fused with mouse myeloma cells for hybridoma generation. Use of an efficient hybridoma cloning protocol in combination with an ELISA screening procedure (see **FIG. 8**) allowed for early identification of stable hybridomas secreting anti-Bcl-xL/BIM IgG.

[0171] Mice were immunized with 100ug GST-Bcl-xL/BIM protein and Complete Freund's Adjuvant (CFA). Subsequent injections every two or three days were with 100ug immunogen and Incomplete Freund's Adjuvant (IFA). Immunized mice were titer tested for reactivity by ELISA. Mouse lymph cells were fused with murine myeloma cell lines and hybridomas were selected in HAT media.

Screening and selection of monoclonal antibodies

[0172] Fusion hybridoma supernatants were screened for specific reactivity by ELISA. ELISA positive clones were subcloned to obtain monoclonal hybridomas of interest. Clones were ranked by relative affinity. Results were validated using purified GST-Bcl-xL fusion protein in an ELISA-based assay (**FIG. 9**). Eight 96-wells plates or 768 clones were tested in an ELISA assay

[0173] Several identified MAbs specifically reacted with the Bcl-xL/BIM heterodimer protein without binding to protein or peptide alone. As evaluated by ELISA analysis, some MAbs displayed

high affinities to heterodimer. Fifty clones were selected and 39 clones were still viable and positive in the pre-subclone screen.

[0174] A sandwich ELISA was used to determine the antibody concentration in the fusion clone supernatants. Thirty-one of the 39 clones turned out to be IgG class. All following assays were normalized to IgG concentration.

Covalent heterodimer assay

[0175] Supernatants from clones were tested binding activity by EILISA. Protein concentrations were normalized and a concentration series was tested. A representative experiment is shown in **FIG. 10A-B**. Briefly, a covalent heterodimer was bound to Glutathione-coated ELISA plates and tested for binding of fusion clones to GST-Bcl-XL-BIM heterodimer. **FIG. 11A-D** shows the heterodimer binding affinity ranked for all 31 IgG clones tested in this ELISA based assay.

[0176] **FIG. 12** shows a schematic of an alternative strategy where biotinylated peptide was bound to Streptavidin-coated plates and then incubated with the GST-Bcl-XL fusion proteins. **FIG. 13A-D** shows the heterodimer binding affinity ranked for all 31 IgG clones tested in this ELISA based assay.

Establishing selective recognition of BIM BH3 induced epitope

[0177] The results from the titrations of 31 clones were confirmed by binding to heterodimers formed by non-covalent interactions. In addition, this experiment examined the binding of clone supernatants to other BH3 only protein peptides, Bid, Puma, and Noxa as well as the BPA-Bim BH3 peptide, the native BIM BH3 peptide, the native BIM BH3 peptide with several flanking amino acids. As shown in **FIG. 14**, several clones demonstrated selective binging to the Bim-BH3 peptide over the Bid, Puma and Noxa peptides. Of these we preferred those that bound to each of the BIM BH3 peptides and we selected clone 32, now called Heterodimer, Bcl-xL Specific to Bim (hence forth referred to as HBXSB) as the parent clone for further study.

Establishing selective inhibition of BIM BH3 induced epitope

[0178] ABT-263 is a BH3 domain mimetic that competitively inhibits BH3 domain mediated binding. ABT-263 disrupts Bcl-2/Bcl-xL interactions with pro-death proteins (e.g., Bim), leading to the initiation of apoptosis within 2 hours post treatment (Tse et al., 2008). A dose-dependent inhibition of heterodimer antibody signal was observed in heterodimers formed with the BIM peptide. BID peptide or no peptide served as negative controls confirming a heterodimer specificity of the monoclonal antibody. The data in **FIG. 15** demonstrates that displacement of the Bcl-xL bound BIM BH3 peptide is detected by HSBXB. A dose dependent inhibition of heterodimer antibody signal was observed with BIM peptide. BID peptide, or no peptide, served as negative controls confirming a heterodimer specificity of the monoclonal antibody.

Application of HSBXB to Fixed Cells

[0179] To demonstrate the utility of HSBXB as biomarker that could be used in fixed archived tumor samples we used immunofluorescence microscopy to test 6 of the clones (**FIG. 16A-B**). Melanoma AUCC903N cells were fixed with either methanol (Panel A) or 4% paraformaldehyde (Panel B), permeabilized with 0.2% TritonX100 and incubated with a subclone of HSBSX (#32). Then cells were incubated with an Alexa488-conjugated goat anti-mouse antibody.

Inhibition of HSBXB binding with ABT 263 and detection in fixed cells

[0180] We have determined that the our novel imaging system would be well suited for quantitative signal analysis in fixed cells and solid tumor thin sections. The system provides several advantages over microscopy or high throughput western blotting. The detection system uses near-infrared (IR) fluorophores (670-1100 nm) that have a distinct advantage over visible dyes, in that very low background fluorescence at longer wavelengths provides an excellent signal- to-noise ratio. Common visible fluorophores cannot be used effectively for direct protein detection on membranes and in plastic plates because of their high background fluorescence in the visible range. In this system antibodies labeled with IR dyes at different wavelengths are used for detection of multiple targets. The imager simultaneously detects two distinct wavelengths. A scanning optical assembly carries two laser diodes that generate excitation light at 680 and 780 nm, as well as two avalanche photodiodes, which detect emitted fluorescence at 720 and 820 nm.

[0181] Using this system we have demonstrated that displacement of the Bcl-xL bound BIM BH3 peptide is detected by HSBXB in paraformaldehyde fixed cells. **FIG. 17A-B** demonstrates HSBXB binding of Bcl-XL/BIM heterodimer in cells incubated with different concentrations of ABT-263 to shows quantitative measurements of heterodimer in response to ABT-263 in SKBR3 cells. IRDye 800CW goat anti-mouse antibody was used for detecting the heterodimer specific mouse monoclonal antibody and IRDye 800CW Goat anti-rabbit antibody was used to detect the commercial Bcl-XL rabbit monoclonal antibody.

Inhibition and enhancement of HSBXB and detection by flow cytometry

[0182] We have established a method for intracellular staining with the Bcl-xL and the HSBXB antibodies and used that in several leukemia cell lines. As a positive control we pretreated with the Bim BH3 peptide at a concentration that achieve saturated binding to the endogenous Bcl-XL. This treatment is routinely used as a positive control for complete mitochondrial priming in our mitochondrial profiling AML test. As a negative control we pretreated with ABT 263 to displace Bim from Bcl-xL by the BH3, as we have established above this treatment diminished binding in in vitro assays and in our novel platform. The displacement of Bim by ABT263 results in MOMP as measured by the mitochondrial profiling assay (unpublished data). **FIG. 18** shows that this displacement in measurable by flow cytometry using the HSBXB antibody. To enhance staining and to establish a positive control we added saturating amount of the Bim BH3 peptide to partially lysed cells. As a

negative control we pretreated with BH3 mimetic compound, ABT 263 to displace Bim from Bcl-xL. In this experiment 5x10e6 JEKO 1 cells were suspended in Newmeyer buffer (Ryan et al Proc Natl Acad Sci USA 2010;107:12895-900), digitonin (Sigma-Aldrich, St Louis MO) and treated with 100 uM Bim BH3 peptide; or ABT263 compound at 10uM; or not treated. Cells were incubated on ice for 3 hours, and then washed and treated with clone 32 at 10ug/ml for 20 minutes, washed again and stained with secondary goat anti- mouse IgG alexa-488. An IgG-2A isotype control was also prepared and run in parallel. Samples are analyzed on a FACS Canto II (BD Biosciences, San Jose CA) using the BD FACS Diva software.

EQUIVALENTS

[0183] The detailed description herein describes various aspects and embodiments of the invention, however, unless otherwise specified, none of those are intended to be limiting. Indeed, a person of skill in the art, having read this disclosure, will envision variations, alterations, and adjustments that can be made without departing from the scope and spirit of the invention, all of which should be considered to be part of the invention unless otherwise specified. Applicants thus envision that the invention described herein will be limited only by the appended claims.

[0184] Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

INCORPORATION BY REFERENCE

[0185] All patents and publications referenced herein are hereby incorporated by reference in their entireties.

[0186] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

CLAIMS

What is claimed is:

1. A method for detecting a heterodimer in a patient sample, comprising:

a) isolating a cancer cell or specimen from said patient;

b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to the heterodimer;

c) detecting a signal that indicates binding of the antibody to the heterodimer; and

d) determining the presence of the heterodimer based on the intensity of the signal.

2. A method for determining a cancer treatment for a patient, comprising:

a) isolating a cancer cell or specimen from said patient;

b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer;

c) detecting a signal that indicates binding of the antibody to the heterodimer;

d) determining the presence of the heterodimer based on the intensity of the signal;

e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and

f) classifying the patient for likelihood of clinical response to one or more cancer treatments,

wherein the presence of a heterodimer correlates with treatment efficacy.

3. A method for predicting cancer sensitivity to treatment, comprising:

a) isolating a cancer cell or specimen from said patient;

b) contacting said cancer cell or specimen with one or more antibodies that specifically bind to a heterodimer;

c) detecting a signal that indicates binding of the antibody to the heterodimer;

d) determining the presence of the heterodimer based on the intensity of the signal;

e) determining a correlation between the antibody binding to a heterodimer said cancer cell or specimen and the sensitivity of said cell or specimen to said treatment; and

f) classifying the patient for likelihood of clinical response to one or more cancer treatments,

wherein the presence of a heterodimer correlates with treatment efficacy.

4. The method of claims 1-3, wherein the heterodimer comprises a caspase and an inhibitor of apoptosis protein (IAP).

5. The method of claim 4, wherein the caspase is selected from the group consisting of caspase 2, caspase 3, caspase 5, caspase 7, caspase 8, and caspase 9.

6. The method of claim 4, wherein the IAP is selected from the group of XIAP, IAP-1, cIAP-2, nIAP, and survivin.

7. The method of claims 1-3, wherein the heterodimer comprises different members of the Bcl-2 family.

8. The method of claim 7, wherein the heterodimer of Bcl-2 family contains a first member of the Bcl-2 family selected from the group consisting of Bim, Bid, Bad, Puma, Noxa, Bak, Hrk, Bax, Bmf, and Mule, and a second member of the Bcl-2 family selected from the group consisting of Mcl-1, Bcl-2, Bcl-XL, Bfl-1, and Bcl-w.

9. The method of claim 6, wherein the first member of the Bcl-2 family is Bim and the second member of the Bcl-2 family is Mcl-1, Bcl-XL, or Bcl-2.

10. The method of claims 1-3, wherein the heterodimer is an anti-apoptotic heterodimer and its presence indicates that the patient is sensitive to the drug.

11. The method of claims 1-3, wherein the heterodimer is a pro-apoptotic heterodimer and its presence indicates that the patient is responsive to the drug.

12. The method of claims 1-3, wherein the cancer is a hematologic cancer.

13. The method of claim 12, wherein the hematologic cancer is selected from acute myelogenous leukemia (AML), multiple myeloma, follicular lymphoma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia, and non-Hodgkin's lymphoma.

14. The method of claims 1-3, wherein the cancer is a solid tumor cancer.

15. The method of claim 14, wherein the solid tumor cancer is selected from non-small lung cell carcinoma, ovarian cancer, and melanoma.

16. The method of claims 1-3, wherein the cancer treatment is one or more of anti-cancer drugs, chemotherapy, antagonist of an anti-apoptotic protein, surgery, adjuvant therapy, and neoadjuvant therapy.

17. The method of claim 16, wherein the cancer treatment is one or more of a SMAC mimetic, BH3 mimetic, proteasome inhibitor, histone deacetylase inhibitor, glucocorticoid, steroid, monoclonal antibody, antibody-drug conjugate, or thalidomide derivative.

18. The method of claim 17, wherein the treatment blocks formation of the particular heterodimer detected.

19. The method of claim 17, wherein the treatment perturbs formation of the particular heterodimer detected.

20. The method of claims 1-3, wherein the specimen is a biopsy selected from a frozen tumor tissue specimen, cultured cells, circulating tumor cells, and a formalin-fixed paraffin-embedded tumor tissue specimen.

21. The method of claims 1-3, wherein the specimen is a human tumor-derived cell line.

22. The method of claims 1-3, wherein the specimen is a cancer stem cell.

23. The method of claims 1-3, wherein the specimen is derived from the biopsy of a non-solid tumor.

24. The method of claim 23, wherein the specimen is derived from the biopsy of a patient with multiple myeloma, acute myelogenous leukemia, acute lymphocytic leukemia, chronic lymphogenous leukemia, mantle cell lymphoma, diffuse large B-cell lymphoma, and non-Hodgkin's lymphoma.

25. The method of claims 1-3, wherein the specimen is derived from a circulating tumor cell.

26. The method of claim 1-3, wherein the specimen is derived from the biopsy of a solid tumor cancer.

27. The method of claim 26, wherein the specimen is derived from the biopsy of a patient with non-small lung cell carcinoma, ovarian cancer, and melanoma.

28. The method of claims 1-3, further comprising determining one or more clinical factors of the patient.

29. The method of claim 28, wherein the clinical factor is one or more of age, cytogenetic status, performance, histological subclass, gender, and disease stage.

30. The method of claims 1-3, wherein the method further comprises predicting a clinical response in the patient.

31. An isolated antibody that specifically binds to the heterodimer of claims 4 to 11.

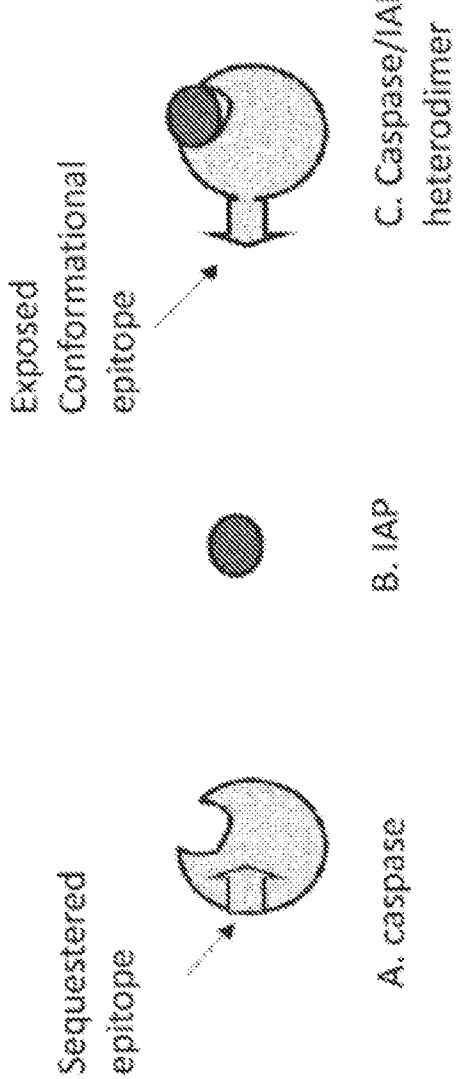
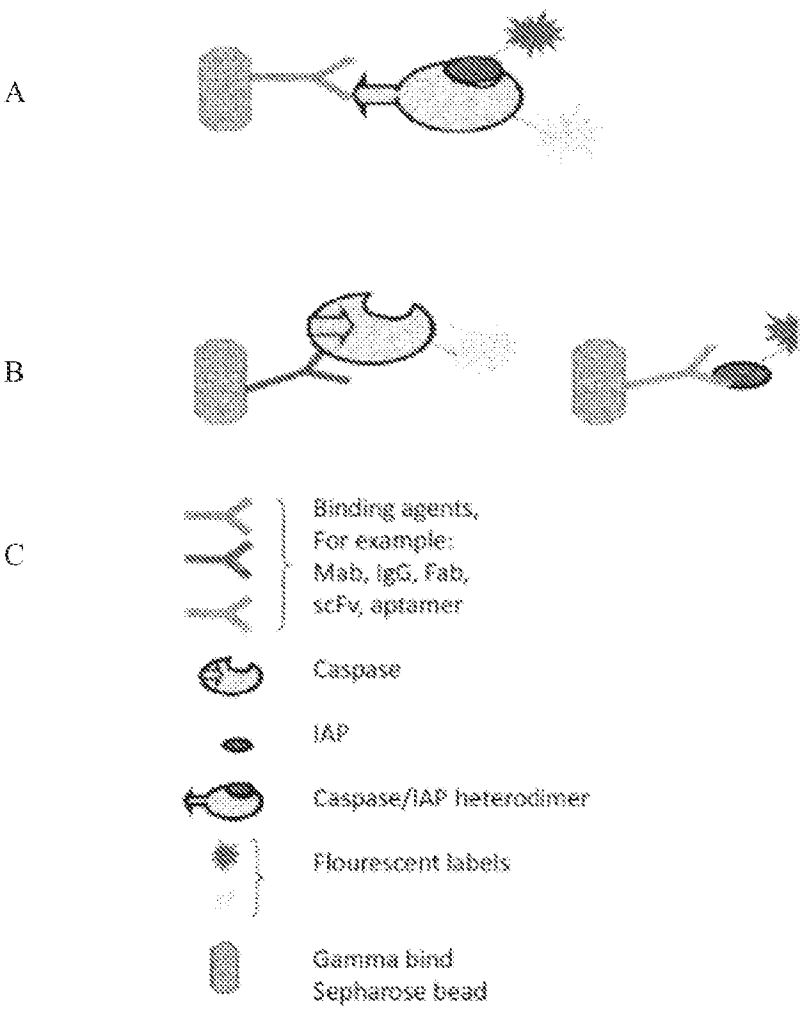

Sequestered
epitope
A. caspase
B. IAP
C. Caspase/IAP
heterodimer
Exposed
Conformational
epitope

Figure 1

Figure 2


A
B
C
Binding agents,
For example:
Mab, IgG, Fab,
scFv, aptamer
Caspase
IAP
Caspase/IAP heterodimer
Flourescent labels
Gamma bind
Sepharose bead

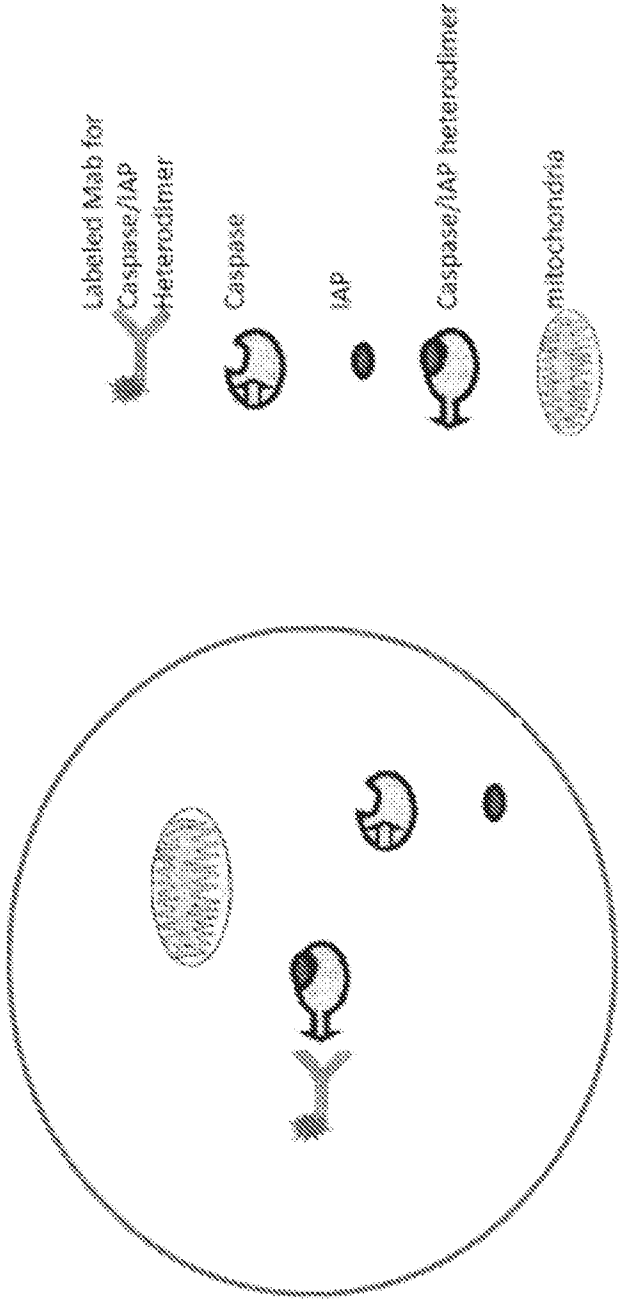

Labeled Mab for Caspase/IAP Heterodimer
Caspase
IAP
Caspase/IAP heterodimer
mitochondria
Cell

Figure 3

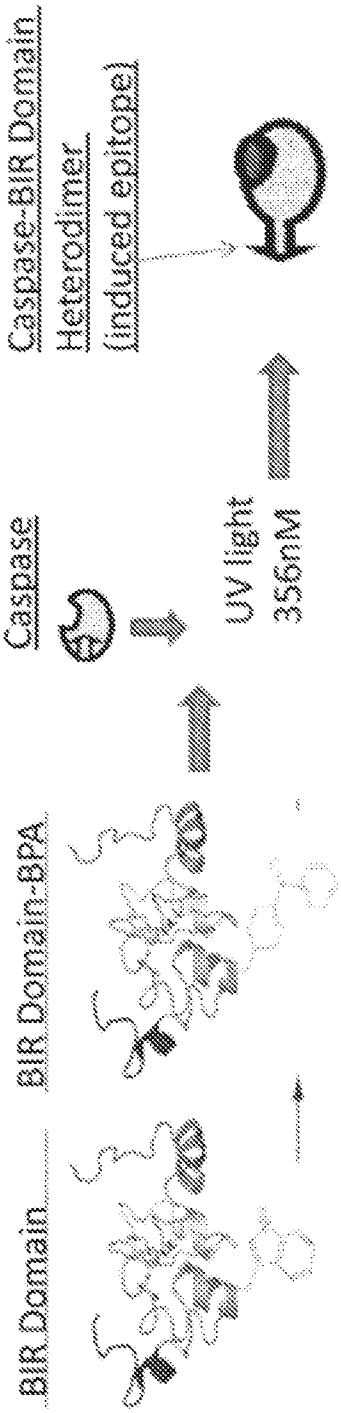
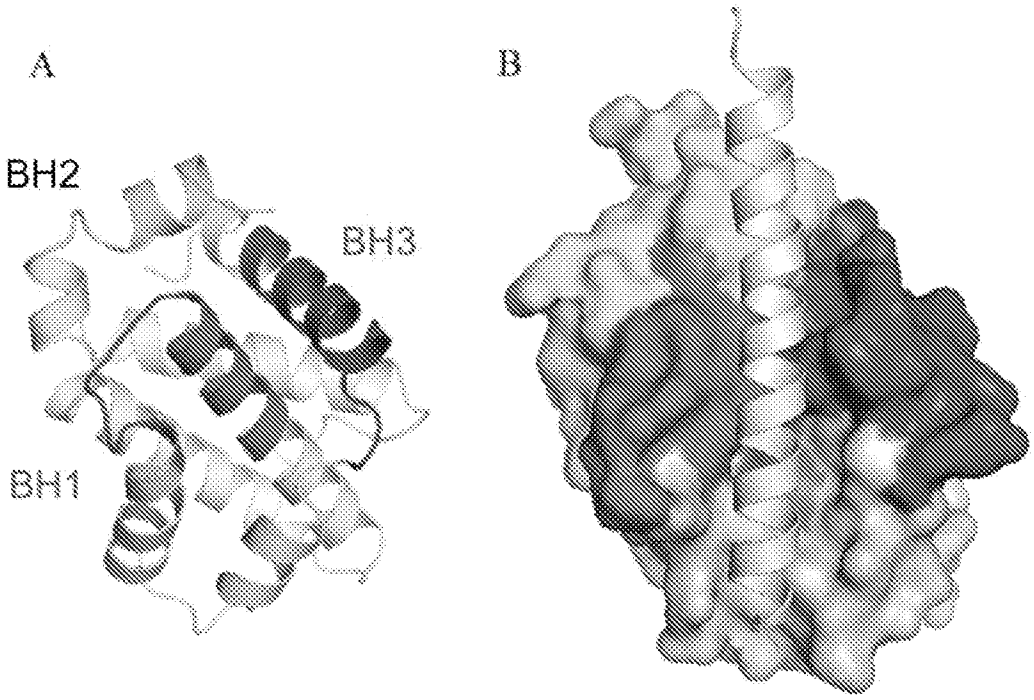

BIR Domain
BIR Domain-BPA
Aromatic Residue
(e.g. Trp)
Benzophenone
Caspase
UV light
356nM
Caspase-BIR Domain
Heterodimer
(induced epitope)

Figure 4

XIAP
497
cIAP1
604
NAIP
1403
BIR
RING
CARD
NACHT
LRR
RING Zinc binding
CARD Capase recruitment domain
NACHT Neuronal apoptosis binding
LRR Leucine Rich Region
Type I
XIAP-BIR1
cIAP1-BIR1
cIAP2-BIR1
Type II
XIAP-BIR2
cIAP1-BIR2
cIAP2-BIR2
Survivin-BIR
Type III
XIAP-BIR3
cIAP1-BIR3
cIAP2-BIR3
NAIP-BIR2
Type X
NAIP-BIR1
NAIP-BIR3

Figure 5

Figure 6



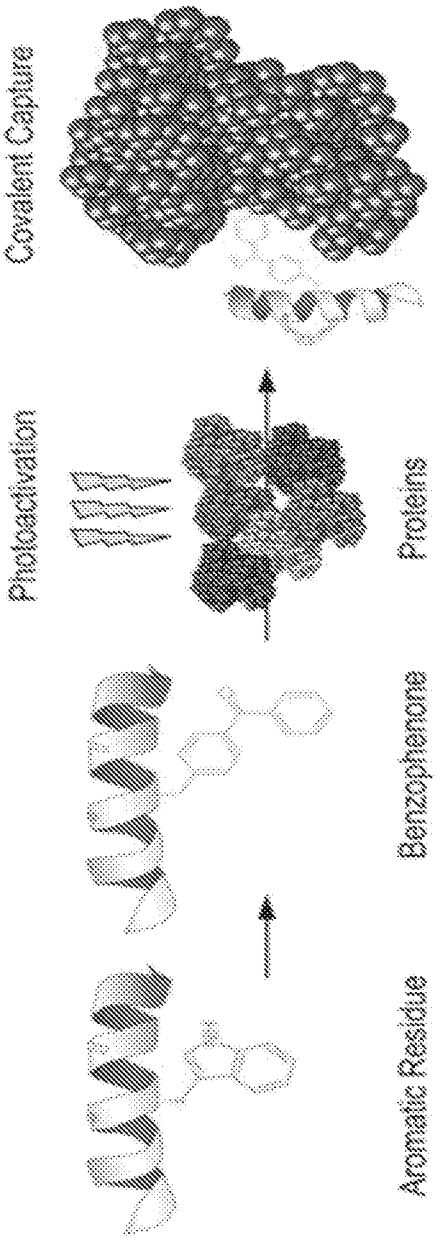
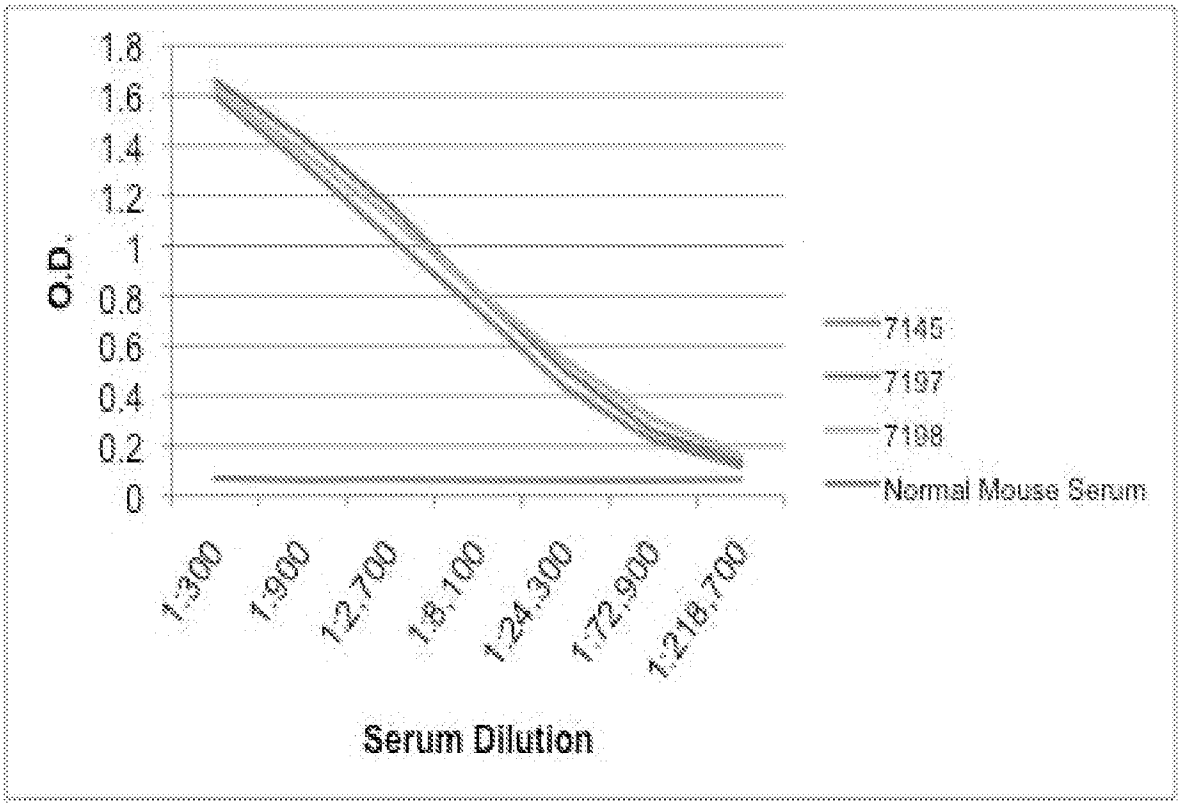

Covalent Capture
Photoactivation
Proteins
Benzophenone
Aromatic Residue

Figure 7

Figure 8

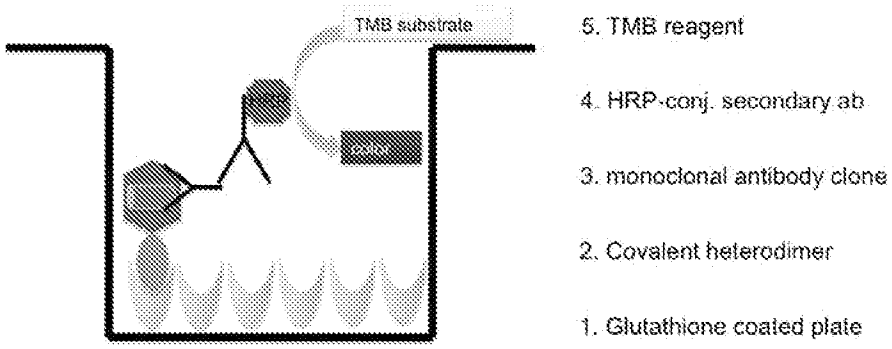

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
O.D.
7145
7197
7198
Normal Mouse Serum
1:300
1:900
1:2,700
1:8,100
1:24,300
1:72,900
1:218,700
Serum Dilution

plates coated with
GST-BCL-XL-Bim
Immunogen
GST-BCL-XL
Counterscreen1
Bim
Counterscreen 2
example: plate 5
selected clones
5G2 = clone #28

Figure 9

Figure 10

A


TMB substrate
5. TMB reagent
4. HRP-conj. secondary ab
3. monoclonal antibody clone
2. Covalent heterodimer
1. Glutathione coated plate

B

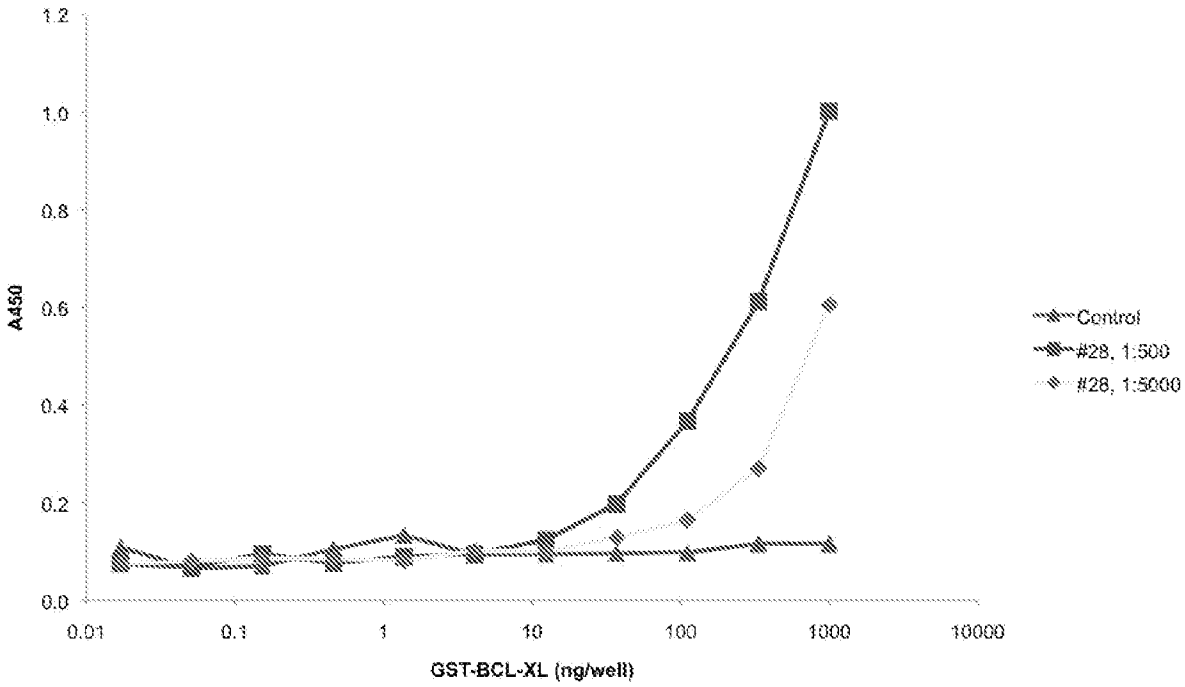

A450
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.01
0.1
1
10
100
1000
10000
GST-BCL-XL (ng/well)
Control
#28, 1:500
#28, 1:5000

Figure 11

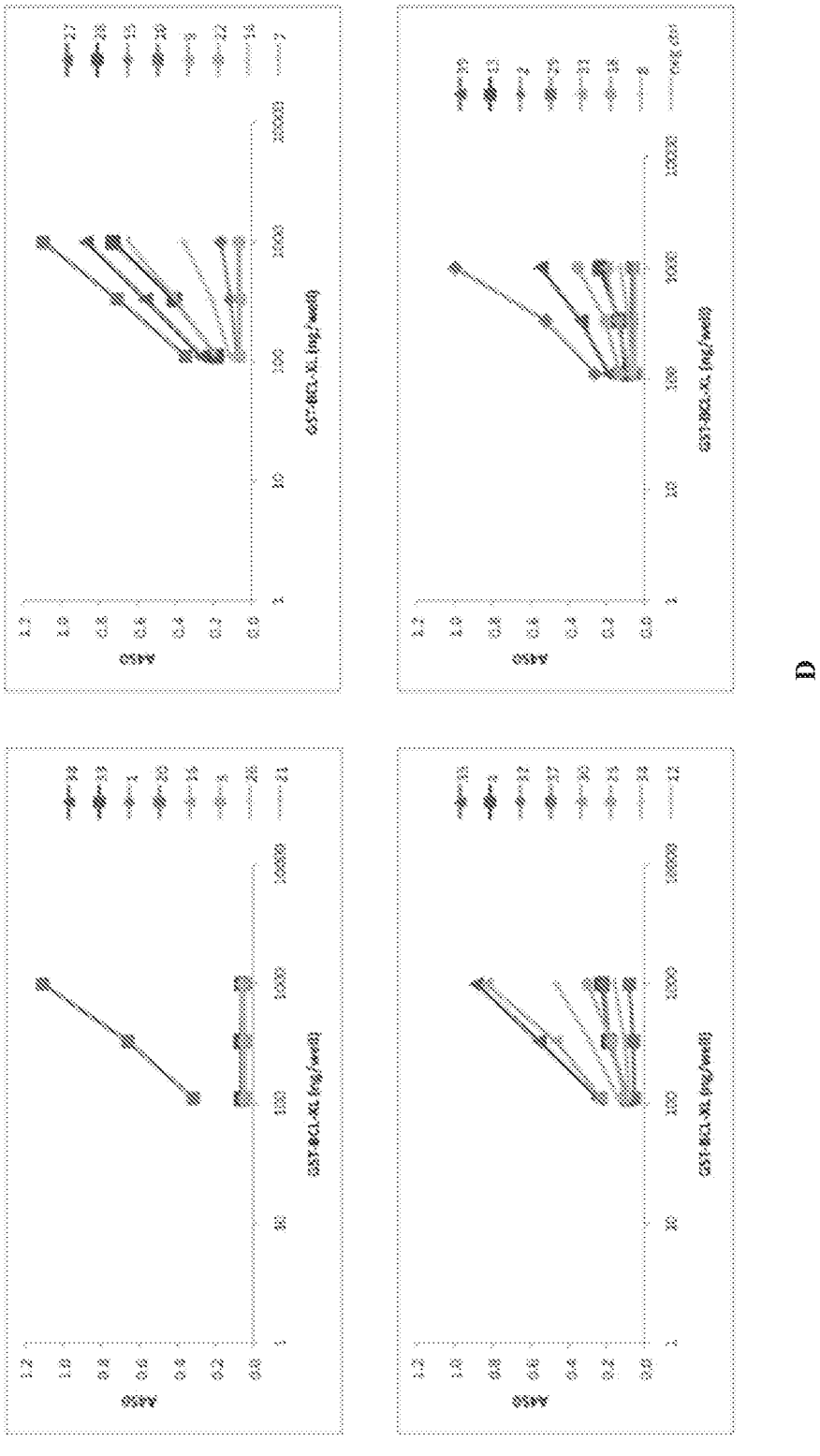
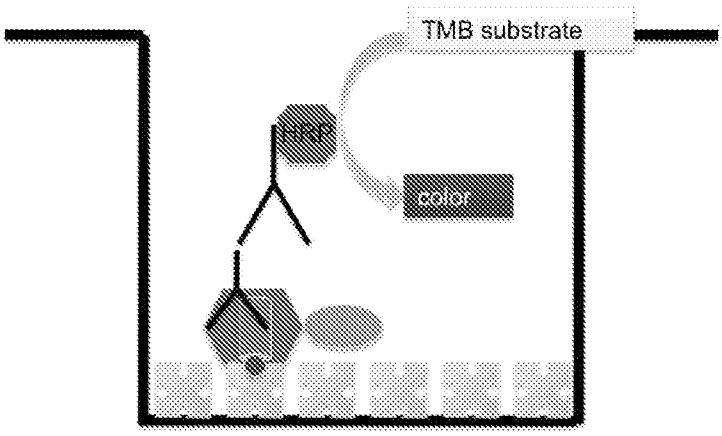


Figure 12



6. TMB reagent

5. HRP-conj. secondary ab

4. monoclonal antibody clone

3. GST-Bcl-XL

2. Biotinylated peptide

1. Streptavidin coated plate

Figure 13

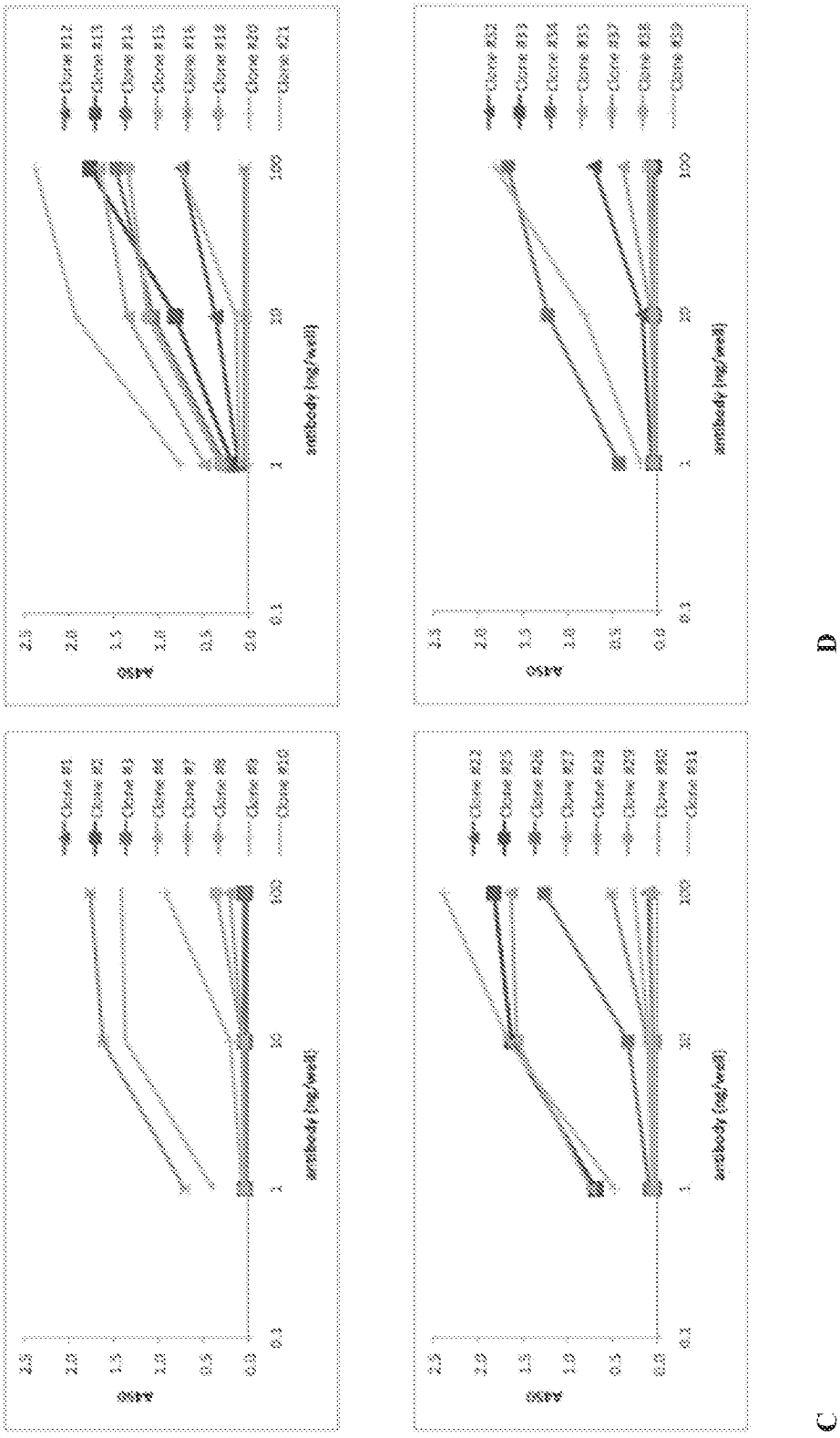

A
B
C
D
OD450
antibody (ng/well)
Clone #1
Clone #2
Clone #3
Clone #4
Clone #5
Clone #6
Clone #7
Clone #8
Clone #9
Clone #10
Clone #11
Clone #12
Clone #13
Clone #14
Clone #15
Clone #16
Clone #17
Clone #18
Clone #19
Clone #20
Clone #21
Clone #22
Clone #23
Clone #24
Clone #25
Clone #26
Clone #27
Clone #28
Clone #29
Clone #30
Clone #31

Figure 14

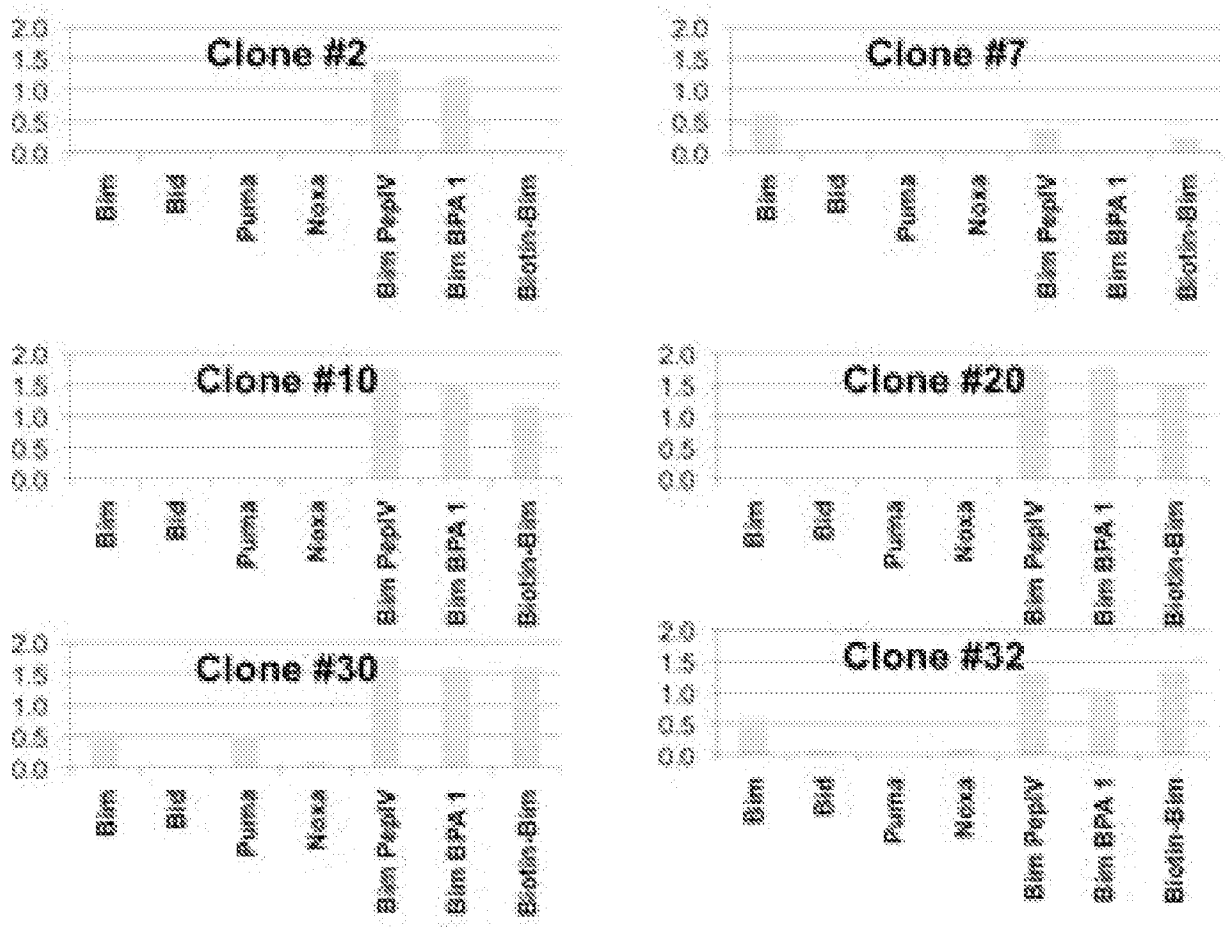

Clone #2
Clone #7
Clone #10
Clone #20
Clone #30
Clone #32
2.0
1.5
1.0
0.5
0.0
Bim
Bid
Puma
Noxa
Bim PepIV
Bim BPA 1
Biotin-Bim

Figure 15

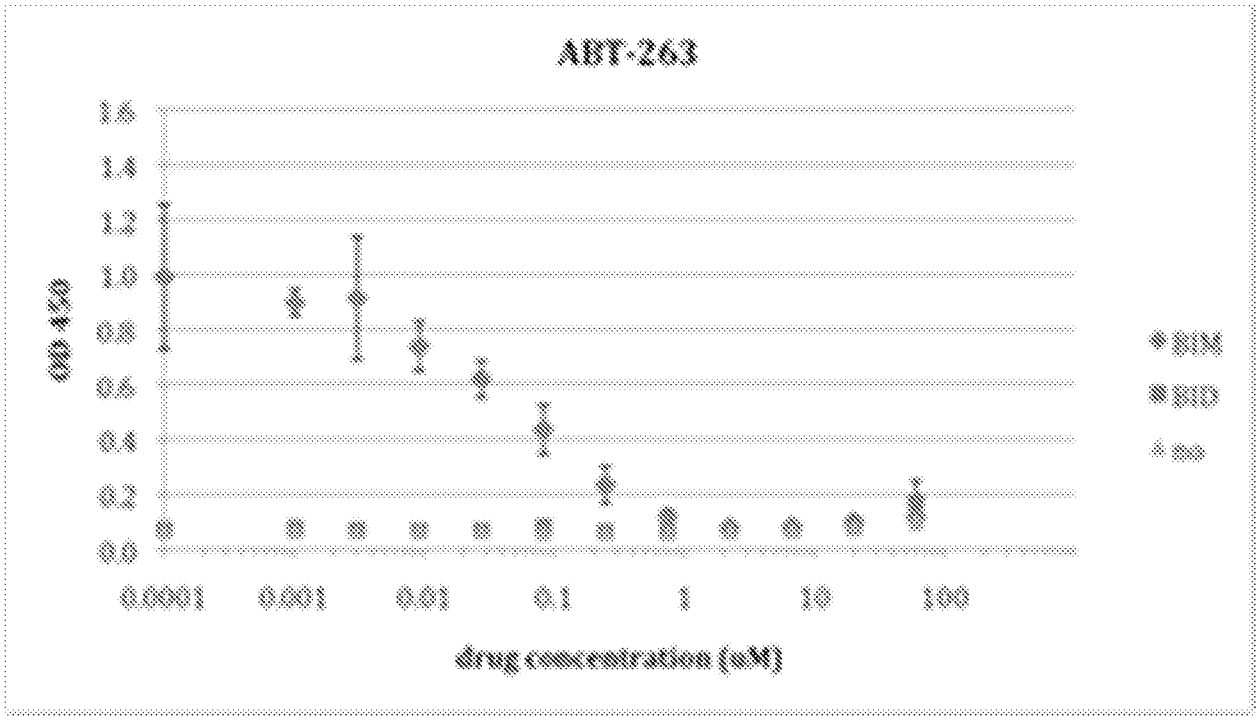
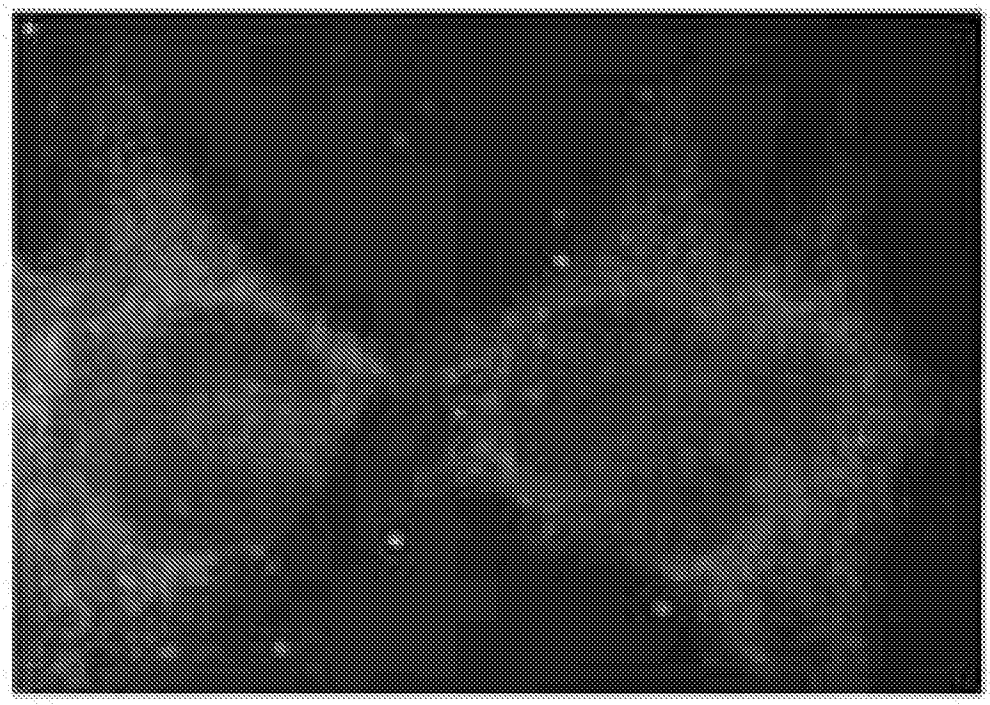

ABT-263
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
drug concentration (nM)
EM
ED
ec

Figure 16

A



B

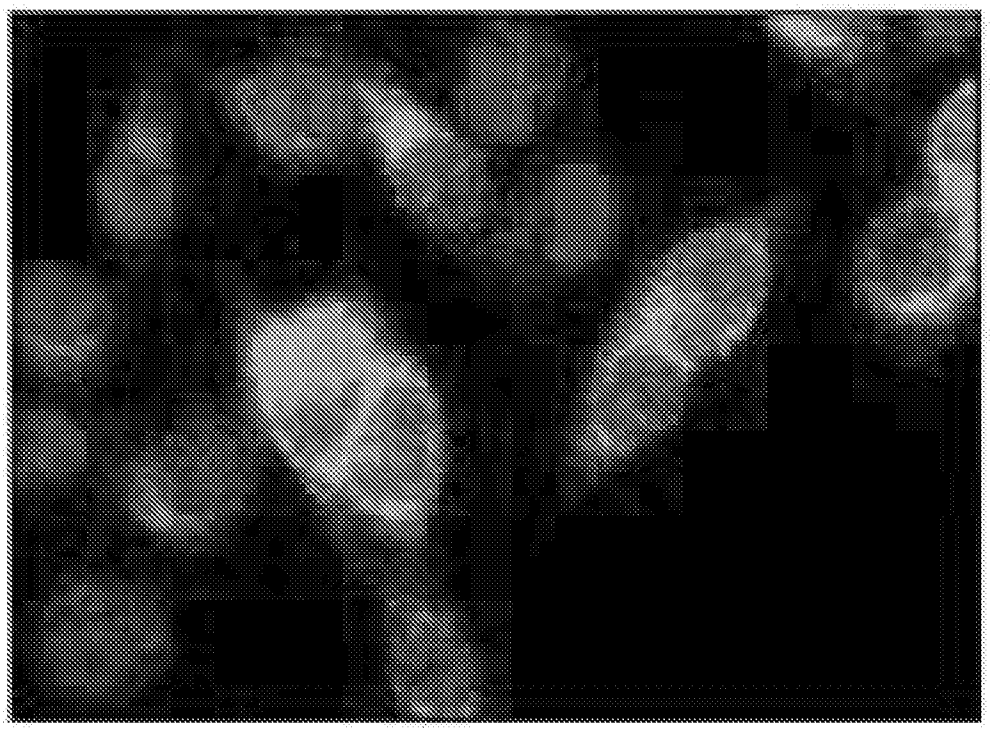
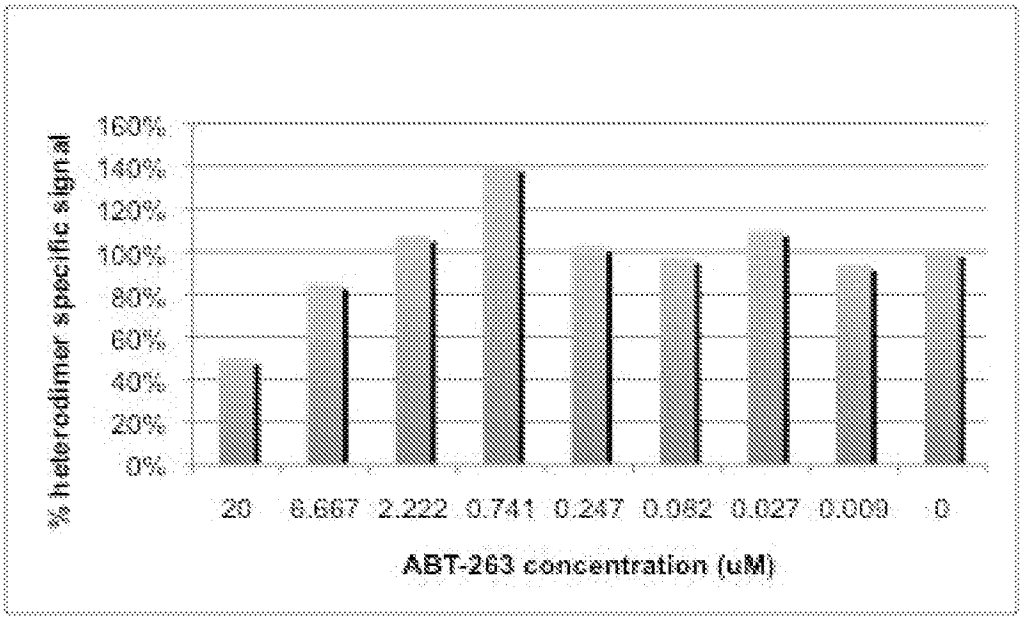


Figure 17

A


% heterodimer specific signal
160%
140%
120%
100%
80%
60%
40%
20%
0%
20
6.667
2.222
0.741
0.247
0.082
0.027
0.009
0
ABT-263 concentration (uM)

B

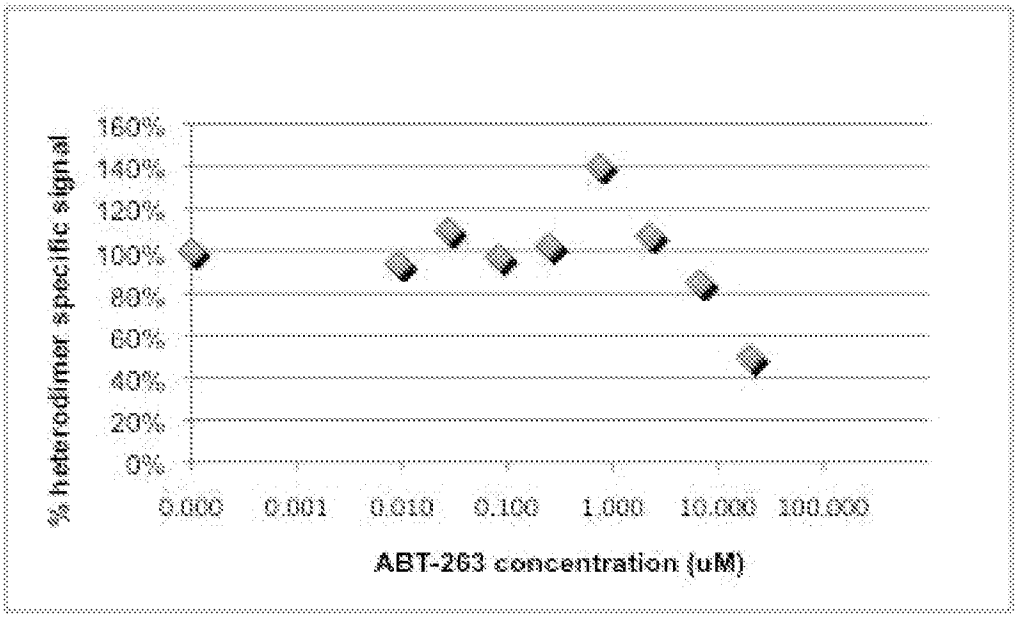
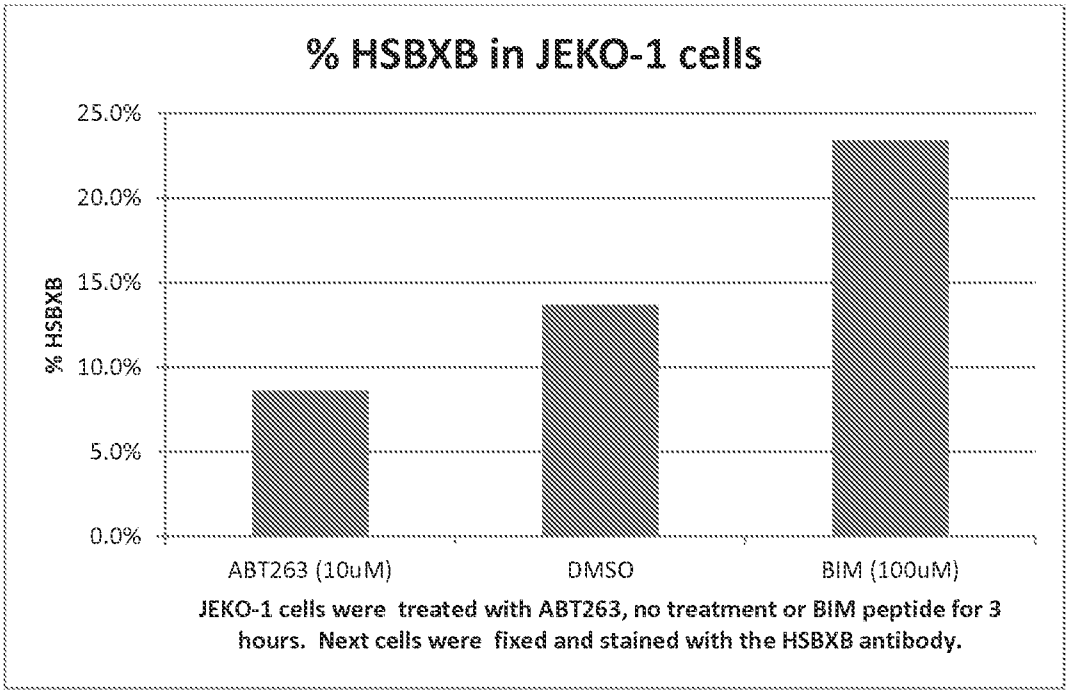

% heterodimer specific signal
160%
140%
120%
100%
80%
60%
40%
20%
0%
0.000
0.001
0.010
0.100
1.000
10.000
100.000
ABT-263 concentration (uM)

Figure 18


% HSBXB in JEKO-1 cells
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
% HSBXB
ABT263 (10uM)
DMSO
BIM (100uM)
JEKO-1 cells were treated with ABT263, no treatment or BIM peptide for 3 hours. Next cells were fixed and stained with the HSBXB antibody.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/049420

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/574 (2014.01)
CPC - G01N 33/574 (2014.11)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - A61P 35/00; C07K16/30; G01N 33/574 (2014.01)
USPC - 435/7.1, 7.23; 506/9; 530/388.8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C07K 16/18, 16/30; G01N 33/50, 33/53, 33/574, 33/5079, 33/57484, 33/57492, 2510/00, 2800/52 (2014.11) (keyword delimited)

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

Search terms used: cancer tumor heterodimer apoptosis sensitivity treatment bcl-2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/0225794 A1 (CARDONE et al) 06 September 2012 (06.09.2012) entire document	1-3
A	WO 2012/122370 A2 (CARDONE et al) 13 September 2012 (13.09.2012) entire document	1-3
A	US 2012/0225851 A1 (CARDONE et al) 06 September 2012 (06.09.2012) entire document	1-3
A	US 2011/0071042 A1 (KIM et al) 24 March 2011 (24.03.2012) entire document	1-3
A	US 2010/0015058 A1 (LI et al) 21 January 2010 (21.01.2010) entire document	1-3

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 November 2014	11 DEC 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/049420

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-31
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.