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(54) PHARMACODYNAMIC ASSAYS USING FLOW CYTOMETRY.

PHARMAKODYNAMISCHE TESTS MIT DURCHFLUSSZYTOMETRIE

ESSAIS PHARMACODYNAMIQUES À CYTOMÉTRIE DE FLUX

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Description

Field of the Invention

[0001] The invention relates to fast, simple assays for detecting the pharmacodynamic effects of drugs in small samples of mixed populations of cells, for example, in small blood samples.

Background of the Invention

[0002] Initial screening for the pharmacodynamic effects of drugs typically involves western analysis and/or immunocytochemical observation of the drug response in a selected number of relevant cell types or biological samples. However, such procedures are labor intensive and provide limited information on only one or two variables that relate to the pharmacodynamic effects of the drug. Moreover, the effects of drug combinations cannot easily be understood by examination of western blots or by viewing a limited number of cells through a microscope. Hence, new procedures are needed that allow analysis of multiple pharmacodynamic markers in multiple cells at once. Such procedures would better reflect the overall response of multiple cell types to the drug(s).

[0003] Pharmacodynamic drug effects are also better understood when large number of samples from different people are tested. However, collection, storage and testing of such large numbers of samples can be burdensome, particularly if the samples must be extensively purified or manipulated before the actual test is performed. For example, researchers frequently study the effects of drugs on lymphocytes. However, separation of lymphocytes from whole blood typically is done by Ficoll gradient separation, which requires technical expertise and expensive equipment. For example, Jaboin and coworkers (J.Jaboin, et. al., Cancer Research, 2002, Vol. 62, pages 6108-6115) describe the effect of MS-275 on the acetylation of histones which is measured in solid tumour cell lines by using anti-acetylated H3 antibodies but employing Western analysis or immunocytochemistry. They do not use mixed cell samples nor employ flow cytrometry in their analysis.

[0004] In EP-A-1403639 (an Article 54(3) EPC document) the use of anti-acetylated-histone antibodies (T52) for monitoring the effect of deacetylase inhibitors (trichostatin A) on acetylation of histones in human U-937 cells and in isolated mononuclear cells using flow cytometry is disclosed. Mononuclear cells are first isolated by Ficoll procedure followed by flow cytrometric analysis. Hence, screening procedures are needed that do not require extensive manipulation or purification of samples prior to testing.

Summary of the Invention

[0005] The invention provides pharmacodynamic assay methods for easily screening large numbers of mixed cell samples. Several pharmacodynamic parameters and/or the effects of combinations of drugs can be monitored at once. Only small sample volumes of mixed cell populations are needed for the present methods. For example, volumes of whole blood samples as small as about fifty microliters can readily be tested by the methods of the invention. No purification of the different cell types within the sample is required, first, because it is desirable to observe the effect of the drug (s) on multiple cell types and, second, because the present methods can simultaneously be used to identify different cell types and observe how they are responding to the drug (s). The inventive methods are therefore useful for quickly screening large numbers of blood samples to identify useful drugs and their pharmacodynamic effects upon various cell types.

[0006] The object of the present invention is solved by the teaching of the independent claims. Further advantageous features and preferred embodiments of the invention are evident from the dependent claims. Methods and embodiments mentioned in description of the present application which are not claimed only have describing character but do not belong to the invention as such.

[0007] In some embodiments, the invention provides methods for detecting and quantifying protein acetylation levels within the eukaryotic cells. According to the invention, the degree of acetylation in such a sample is one measure of whether a drug (e. g. a deacetylase inhibitor) can influence acetylation in the subject from which the sample was obtained. Thus, one aspect of the invention is a method of monitoring protein acetylation in whole blood, bone marrow or cerebrospiral fluid exposed to a deacetylase inhibitor. The method involves: (a) providing a sample of whole blood, bone marrow or cerebrospinal fluid exposed *in vitro* or *in vivo* to the deacetylase inhibitor to form a first test mixture; (b)contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by flow cytometry; wherein the sample has a volume ranging from 25 microliters to 150 microliters.

[0008] Quantifying the protein acetylation of the cells can include calculating what proportion of cells in the mixed population exhibit the protein acetylation. Alternatively, quantifying the protein acetylation of the cells to the drug can involve calculating an increase or decrease in fluorescence signal during flow cytometry relative to one or more suitable controls. One example of a suitable control is a sample of the same mixed population of cells subjected to the method without exposure to the drug. Another example of a suitable control is a sample of the same mixed population of cells subjected to the method after being exposed to a drug that is known to produce the response. The mixed population of cells can, for example, be human blood, animal blood or other cells samples including cell lines available in the art. Only small volumes are needed to perform the methods of the in-

vention, for example, volumes ranging from 25 microliters to 150 microliters.

[0009] Reagents that can detect protein acetylation include, for example, antibody preparations that can bind to acetylated protein, where the antibodies have a detectable label directly linked thereto or where the antibodies indirectly associate with a detectable label, for example, by binding to a secondary antibody that is labeled.

[0010] The drug can, for example, be a deacetylase inhibitor. Examples of deacetylase inhibitors whose pharmacodynamic responses can be monitored include MS-275, trichostatin A, trapoxin, sodium butyrate, apicidin, sodium phenylbutyrate, phenylacetate, depsipeptide, 3-bromopropionate, valproic acid, tributyrin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydoxamic acid (CBHA), oxamflatin, pyroxamide, CHAP, depsipeptide (FK228), NVP-LAQ824, CI-994, PXD101, apicidin-derived quinolone derivatives and combinations thereof.

[0011] Another embodiment relates to acetylated tubulin. In this case the drug can, for example, be an anticancer drug.

[0012] In some embodiments, the mixed population of cells can be exposed to more than one drug and the effects of all such drugs can be monitored simulaneously.

[0013] The methods of the invention can readily be adapted to include observing which cell types exhibit protein acetylation, observing in what cell cycle stage the cells exhibit protein acetylation, observing whether some of the cells are undergoing apoptosis, or a combination thereof.

[0014] Another aspect of the invention is a method of identifying whether a test agent modulates protein acetylation in whole blood, bone marrow or cerebrospinal fluid. This method involves: (a) providing a sample of whole blood, bone marrow or cerebrospinal fluid that has been exposed in vitro or in vivo to a test agent to form a first test mixture; (b) contacting the first test mixture with a reagent that can detect protein acetylation to thereby form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by flow cytometry wherein the sample has a volume ranging from 25 microliters to 150 microliters.

[0015] Further described and not being part of the present invention is a method of monitoring deacetylation in whole blood, bone marrow or cerebrospinal fluid exposed to a deacetylase inhibitor. This method involves: (a) providing a sample of whole blood, bone marrow or cerebrospinal fluid exposed *in vitro* or *in vivo* to the deacetylase inhibitor to form a first test mixture; (b) contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by flow cytometry.

[0016] Further described and not being part of the present invention is a method of monitoring deacetylation in a small sample of whole blood exposed to a deacetylase inhibitor. This method involves: (a) providing a small sample of whole blood exposed *in vitro* or *in vivo* to the deacetylase inhibitor to form a first test mixture; (b) contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by flow cytometry.

[0017] Further described and not being part of the present invention is a method of monitoring deacetylation in a small sample of whole blood exposed to MS-275. This method involves: (a) providing a small sample of whole blood exposed *in vitro or in vivo* to MS-275 to form a first test mixture; (b) contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by flow cytometry.

[0018] Further described and not being part of the present invention is a method of monitoring deacetylation in whole blood, bone marrow or cerebrospinal fluid exposed to a deacetylase inhibitor. This method involves: (a) providing said sample exposed in *vitro* or *in vivo* to the deacetylase inhibitor to form a first test mixture; (b) contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and (c) quantifying protein acetylation in the second test mixture by fluorimetry.

[0019] Another aspect of the invention is a method of quantifying protein acetylation in a small sample of whole blood. This method involves: (a) fixing cells from the whole blood with 0.4% paraformaldehyde in phosphate buffered saline to generate fixed cells; (b) incubating the fixed cells with 0.4% Triton X-100 in phosphate buffered saline to generate permeabilized cells; (c) reacting the permeabilized cells with an anti-acetylated lysine antibody preparation to form a complex between the permeabilized cells and the anti-acetylated lysine antibody; and (d) quantifying protein acetylation using flow cytometry by observing a signal from a label associated with the anti-acetylated lysine antibody wherein the sample has a volume ranging from 25 microliters to 150 microliters.

[0020] In most instances no purification of specific cell types from the small sample of whole blood need be performed.

[0021] Sample sizes are small samples of blood of 25 to 150 microliters.

Description of the Drawings

[0022] FIG. 1A-E illustrate that concentration-dependent protein acetylation occurs in healthy donor peripheral blood mononuclear cells that were incubated *in vitro* with the histone deacetylase inhibitor MS-275. The MS-275 compound is N-(2-aminophenyl)-4-[N-(pyridin-3-ylmethoxycarbonyl)aminomethyl] benzamide. See, Saito et al., Proc. Natl. Acad. Sci. USA 96, 4592-4597 (1999). As shown in FIG. 1A, the peak of fluorescence reflecting acetylated lysine levels observed for cells treated with no MS-275 is centered over a lower fluorescence reading

than the peak of cells treated with 10 nM MS-275 (FIG. 1B) and especially the peak of cells treated with 1.0 μM MS-275 (FIG. 1C). FIG. 1D provides a negative control showing the fluorescence of cells treated with normal rabbit antibodies. FIG. 1E provides a merged graph showing the amount of acetylated lysine in populations of control cells (left-most peak), of cells treated with 10 nM MS-275 (middle peak) and of cells treated with 1.0 μM MS-275 (right-most peak).

[0023] FIG. 2A-E show that *in vivo* administration of the histone deacetylase inhibitor MS-275 gives rise to concentration-dependent protein acetylation in peripheral blood mononuclear cells. Whole blood was obtained before MS-275 administration and then 24 hours after MS-275 treatment. FIG. 2A-B show the fluorescence detected from CD3 labeled cells on the y-axis and the fluorescence detected from anti-acetylated lysine residues on the x-axis. The fluorescence pattern for cells obtained before MS-275 treatment (FIG. 2A) was low and diffuse. However, as shown in FIG. 2B, acetylated lysine fluorescence increases after MS-275 treatment. Moreover, FIG. 2A-B indicate that there are positive and negative populations of CD3-positive cells: those that express CD-3 are T cells while non-T cells express no CD3 and form a smaller population of cells nearer the x-axis. FIG. 2D further illustrates the amount of acetylated lysine detected in cells isolated after MS-275 treatment is greater than that detected before treatment (FIG. 2C). FIG. 2E provides a graph showing fluorescence from both pre-treatment (left peak) and post-treatment (right peak) cells.

[0024] FIG. 3A-C illustrate that concentration-dependent protein acetylation occurs in bone marrow aspirates of leukemia patients treated *in vivo* with the histone deacetylase inhibitor MS-275. The acetylation of bone marrow aspirates is shown by flow cytometry analysis prior to treatment (FIG. 3A) and after treatment with MS-275 (FIG. 3B). FIG. 3C provides a graph showing fluorescence from both pre-treatment (unshaded peak) and post-treatment (shaded peak) cells. These data illustrate that the assay can be used for detection of a drug response in bone marrow aspirates.

[0025] FIG. 4A and 4B illustrate that the methods of the invention can be used not only for quantifying total acetylation but also for correlating the level of such acetylation with the presence or absence of cell-type specific markers.

[0026] FIG. 4A1-5 illustrate that a large variety of cells types can be detected in samples by the present methods, as shown by the results of a five-color, seven parameter flow cytometric analysis. This assay was performed by incubating peripheral blood with antibody preparations directed against different markers and then detecting the presence of those markers using flow cytometric procedures. The markers employed were the B cell-specific CD19 marker (using a PE-Cy5 label), the T cell-specific CD3 marker (using a PE label), the granulocyte/monocyte CD15 marker (using a FITC label) and the monocyte-specific CD14 marker (using an APC-Cy7 label). A scatter gram is provided in FIG. 4A1, showing the forward (FSC-A) and side (SSC-A) light scattering of this mixed population of cells. FIG. 4A2 shows the fluorescence colors associated with the fluorophore types on antibody preparations used to detect CD19, CD3, CD15 and CD14. FIG. 4A3 shows the fluorescence of cells displaying the CD15 marker along the x-axis and fluorescence of cells displaying the CD3 marker along the y-axis. The CD15 marker is most visible in the group of cells at the lower right of FIG. 4A3 (blue in the original). FIG. 4A4 provides a graph showing fluorescence of cells displaying the CD3 marker along the x-axis and fluorescence of cells displaying the CD19 marker along the y-axis. CD19 cells (red in original) are much more predominant on the left, whereas CD3 cells (boxed in cells; pink in original) are much more predominant on the right. FIG. 4A5 is a graph showing fluorescence of cells displaying the CD15 marker along the x-axis and fluorescence of cells displaying the CD 14 marker along the y-axis. CD15 cells (R4 circled cells; blue in original) are much more predominant on the lower right, whereas CD14 cells (R2 circled cells; green in original) are much more predominant on the right.

[0027] FIG. 4B1-4 illustrate the effects of the deacetylase inhibitor MS-275 upon the various cell types, as observed by a five-color, seven parameter flow cytometric analysis of protein acetylation using flow cytometric procedures. The cells were stained with cell-specific markers as described for FIG. 4A1-5 and simultaneously stained for acetylated lysine. FIG. 4B3 and 4B4 show that cells expressing both low and higher levels of the granulocyte/monocyte CD15 marker exhibit increased acetylation after treatment with the MS-275 deacetylase inhibitor (FIG. 4B4) compared to cells that did not receive MS-275 (FIG. 4B3). The CD15-expressing cells are found mostly within the upper right quadrant of FIG. 4B3-4. FIG. 4B1 and 4B2 show that cells positive and negative for expression of the T cell-specific CD3 marker exhibit increased acetylation after treatment with the MS-275 deacetylase inhibitor (FIG. 4B2) compared to cells that did not receive MS-275 (FIG. 4B 1). The CD3-expressing cells are most visible in the lower right quadrant of FIG. 4B1, and after MS-275 treatment shift upward into part of the upper right quadrant of FIG. 4B2.

[0028] FIG. 5A-B illustrate that the pharmacodynamic effects of different drugs can be separately monitored using the methods of the invention. The drugs employed were the anti-cancer drug 17-allylaminogeldanamycin (17-AAG) and the deacetylase inhibitor trichostatin A (TSA). The effects of 17-AAG can be monitored by observing whether the levels of Hsp70 change - increased Hsp70 levels indicate that the 17-AAG drug is having an effect upon the cells. As shown in FIG. 5A, increased levels of Hsp70 were detected using the methods of the invention after treatment of the cells with 17-AAG. The effect of TSA on leukemia cells can be seen in FIG. 5B. While TSA is a generalized deacetylase inhibitor, the effect of TSA in this study was assessed by observing

whether a change in the levels of tubulin acetylation (using anti-acetylated tubulin antibodies) occurred. FIG. 5B shows that increased levels of acetylated tubulin were apparent after treatment of the cells with TSA.

[0029] FIG. 6A-D illustrate that the pharmacodynamic effects of different drugs can be simultaneously monitored using the methods of the invention. The drugs employed were the anti-cancer drug 17-allylaminogeldanamycin (17-AAG) and the deacetylase inhibitor trichostatin A (TSA). As described above, the effects of 17-AAG were monitored by observing whether the levels of Hsp70 increased and the effects of TSA in this study were assessed by observing whether increased levels of tubulin acetylation occurred. As shown in FIG. 6C-D, both Hsp70 and acetylated tubulin levels, respectively, increased in cells treated with 17-AAG and TSA. The dot blots shown in FIG. 6A-B confirm that the population of cells exhibited increased fluorescence for both the Hsp70 and acetylated tubulin markers, respectively.

[0030] FIG. 7A-D illustrate that the pharmacodynamic effects of different drugs can be simultaneously monitored using the methods of the invention. The drugs employed were the anti-cancer drug 17-allylaminogeldanamycin (17-AAG) and the deacetylase inhibitor MS-275. As described above, the effects of 17-AAG were monitored by observing whether the levels of Hsp70 increased. The effects of MS-275 were assessed by observing whether increased levels of overall protein acetylation occurred. As shown in FIG. 7C-D, both Hsp70 and acetylated protein levels, respectively, increased in cells treated with 17-AAG and MS-275. The three-dimensional graph shown in FIG. 7B confirms that cells treated with 17-AAG and MS-275 exhibit increased fluorescence for both the Hsp70 and acetylated proteins compared to the non-treated cells shown in FIG. 7A.

[0031] FIG. 8A-D provide an immunocytochemical analysis of protein acetylation. Healthy donor unfractionated buffy coats were treated with carrier only (FIG. 8A) or 1 μM MS-275 (FIG. 8B) for 24 hours, labeled with anti-acetylated lysine antibody, and nuclei were counterstained with DAPI. FIG. 8C-D illustrates the subcellular localization of acetylated proteins in cells treated and stained as in FIG. 8B. FIG. 8C shows a cell with predominantly nuclear staining, whereas FIG. 8D shows a cell with predominantly cytoplasmic staining.

[0032] FIG. 9A-D illustrate that apoptosis and protein acetylation can be monitored simultaneously in cells treated with MS-275 (deacetylase inhibitor) and/or the anti-cancer agent imatinib. K562 cells were incubated with vehicle alone (FIG. 9A), 1 μM imatinib (FIG. 9B), 1 μM MS-275 (FIG. 9C), or both (FIG. 9D) for 48 h, and analyzed by multiparameter flow cytometry after reaction with antibodies directed against caspase3 and acetylated lysine. Dot plots display acetylated lysine on the x-axis and activated caspase 3 on the y-axis.

[0033] FIG. 10A-B illustrate p21 expression *versus* acetylated lysine in bone marrow aspirates in response to MS-275 *in vivo.* Bone marrow aspirates from a leukemia patient treated on a MS-275 protocol were analyzed by flow cytometry for expression of p21 *versus* protein hyperacetylation, pre-treatment (FIG. 10A) and post-treatment (FIG. 10B) with MS-275.

Detailed Description of the Invention

[0034] The invention provides methods for screening mixed cell samples for protein acetylation as the pharmacodynamic response to one or more drugs. In one embodiment, the invention provides methods for screening mixed cell samples for the degree of protein acetylation in the cell samples. As little as 25 to 150 microliters of whole blood (e.g., obtained by a finger prick) can be quickly screened to determine and/or to quantify the pharmacodynamic response. Cell samples exposed *in vivo* or *in vitro* to one or more drugs can effectively be tested for their pharmacodynamic response thereto using the methods of the invention. No separation of cell types in the whole blood samples prior to detecting the pharmacodynamic response is needed, or is generally desirable, for the practice of the invention.

[0035] The methods of the invention are simple. Many of the steps require little or no technical expertise or expensive equipment. Hence, the methods of the invention can be used for large scale screening procedures where many samples can be collected in the field and then processed at a convenient location such as a hospital or clinical laboratory.

Assay Methods

[0036] The invention provides methods for detecting and/or quantifying the pharmacodynamic response of mixed cell populations to one or more drugs. The effects of several drugs on a population of cells can readily be observed at once. The methods of the invention generally involve providing a cell sample, fixing the cells, permeabilizing the cells, reacting the cells with one or more reagents that reflect the pharmacodynamic response of the cells to the selected test agent(s) or drug(s) and using cell cytometry to observe and/or quantify the pharmacodynamic response of the cells to the test agent(s) or drug (s).

[0037] In one embodiment, the methods of the invention are used for detecting and/or quantifying protein acetylation in mixed populations of cells. The pharmacodynamic effect and/or the degree of protein acetylation can be correlated with other factors such as the cell cycle, cell differentiation, cell type or apoptosis, simply by staining the cells during the present methods using available markers for various stages of the cell cycle, cell differentiation, different cell types or for apoptosis.

[0038] The methods of the invention can be performed on many different cellular samples, for example, blood, bone marrow aspirates, isolated cell lines, tissue biopsies, cerebrospinal fluid, lymph, skin scrapings, tumor biopsy samples, fluids extracted from physiological tis-

sues and the like. However, in many embodiments, the sample collected and tested is whole blood. Whole blood is preferably used for several reasons. First, whole blood has a variety of cell types which, according to the invention, reflect the physiological state of the donor and his or her response to drugs or to the environment. Second, the inventors have determined that only small amounts (e.g. 25-150 microliters) of whole blood are needed for accurate assessment of pharmacodynamic responses. Third, whole blood is easily obtained. No sophisticated equipment or technical expertise is required to collect the small amounts needed. No purification (e.g. no Ficoll gradient separation) of different cell types is typically performed. Numerous small blood samples can quickly be collected in the field for testing later at a more convenient location.

[0039] Samples from mammals and birds may be obtained for use the methods of the invention. Such mammals and birds include humans, mice, rats, dogs, cats, horses, cattle, sheep, goats, chickens, turkeys and the like. Animals are contemplated for initial testing or screening studies such as toxicology studies, dosage testing and other studies that facilitate drug development.

[0040] Small samples of mixed cell populations can be collected using standard procedures for collecting biological samples. Because only small amounts of cell samples are needed, a finger prick can provide sufficient whole blood for practice of the invention. Blood samples from the finger, arm, leg or any other site can be used. Animal blood samples are collected by procedures available in the art. If bone marrow aspirates, biopsies or tissue samples are to be tested, these samples are also obtained by standard procedures. Again, because only small numbers of cells are needed, just a small proportion of the total bone marrow aspirate, biopsy or tissue sample may be needed for performing the present methods. The rest can be reserved for other types of testing or for any other purpose contemplated by one of skill in the art.

[0041] After collection of the samples, the cells should be stabilized by fixation. In some instances one of skill in the art may choose to remove extracellular materials from the cells prior to fixation. However, such removal may not be necessary and factors loosely associated with the cell surface may be lost. Hence, one of skill in the art may frequently choose to skip such a cell washing step. If one of skill in the art chooses to wash the cells, for example, because only intracellular pharmacodynamic markers are of interest, washing can be performed by standard procedures such as by centrifuging the cells in an appropriately buffered saline solution. Bovine serum albumin (BSA), or other stabilizing material, can be added to the buffered saline solution during such a washing procedure. Washing the cells generally involves suspending the cells in the buffered saline solution, centrifuging the cells into a pellet, removing the supernatant and re-suspending the cells in the buffered saline solution. Several rounds of such washing can be performed if one of skill in the art chooses.

[0042] The cells are gently fixed in an available fixative for a time and under conditions sufficient to stabilize the cells. Fixative solutions generally contain a fixative in an appropriately buffered saline solution without any BSA or other such materials. Fixatives that can be used include dilute solutions of paraformaldehyde, for example, solutions of about 0.1% to about 4% paraformaldehyde. In some embodiments, the fixative solution is 0.4% paraformaldehyde in phosphate buffered saline. Generally, only short periods of time are required for fixation, for example, fixation can be for about 2 minutes to about 20 minutes. Fixation is done at mild temperatures, for example, at about 4 °C to about 42 °C. When cooler temperatures are employed, longer fixation times are required; shorter fixation times are used when higher temperatures are employed. In some embodiments, fixation is at 37 °C for about 5 minutes to about 10 minutes. The cells are then washed in buffered saline solution as described above. After fixation, the cells can be stored at various temperatures, including room temperature or temperatures of about 4 °C, until it is convenient for one of skill in the art to assess a pharmacodynamic response in the cells.

[0043] Cells can be gently permeabilized prior to reaction with many available reagents that detect the pharmacodynamic response. In general, such permeabilization is performed using a mild detergent in a buffered saline solution for a time and under appropriate conditions for gently permeabilizing the cells. For example, the permeablization solution can include small amounts of Triton X-100 in phosphate buffered saline. Amounts ranging from about 0.1 % to 1% Triton X-100 can be used. In some embodiments, the permeabilization solution is a solution of 0.4% Triton X-100 in phosphate buffered saline. Permeabilization is for short periods.of time at mild temperatures. For example, permeabilization can be performed for about 2 minutes to about 10 minutes at temperatures ranging from about 10 °C to about 37 °C. In some embodiments, permeabilization is performed for about 5 minutes at room temperature. After permeabilization, the cells are washed in buffered saline as described above. Small amounts of BSA (e.g. 0.1% BSA) can be included in the wash solution at this stage.

[0044] Cells are exposed to a selected reagent that can detect the pharmacodynamic response. Such a reagent is any reagent that can selectively detect the pharmacodynamic marker known to one of skill in the art The reagent can be antibody, an enzyme, an enzyme substrate, an mRNA or other detectable substance. Examples of the pharmacodynamic markers that can detect the pharmacodynamic response include protein acetylation and, tubulin acetylation.

[0045] In one embodiment, the reagent that can detect the pharmacodynamic response can detect protein acetylation. In many embodiments, the acetylation detection reagent can generally detect acetylation of lysine residues in substantially all types of proteins. The use of a reagent that detects acetylation of lysine residues in

substantially all types of proteins permits detection of the spectrum of nuclear and cytoplasmic proteins that can be acetylated. Over forty proteins can be acetylated in eukaryotic cells, including histones, p53, tubulin, c-jun and the like. Many of these proteins perform crucial functions. For example, transcriptionally silenced chromatin, such as heterochromatin and inactivated mammalian X chromosomes, are associated with hypoacetylated histones. In contrast, transcriptionally active domains in euchromatin are often associated with histone hyperacetylation. According to the invention, the acetylation levels of such a spectrum of potentially acetylated proteins provides a measure of the sample donor's physiological state, response to drugs, disease progression and the like. Hence, the assay methods of the invention can be used for monitoring a patient's physiological state, disease progress and/or drug response over time by monitoring the acetylation of a spectrum of proteins.

[0046] Hence, any reagent that can detect acetylation in substantially any protein can be employed in the methods of the invention. One example of a reagent that can detect acetylation of lysine residues within proteins is an anti-acetylated lysine antibody preparation. Such anti-acetylated lysine antibodies are available to one of skill in the art. For example, such anti-acetylated lysine antibodies can be obtained from Cell Signaling Technology (Beverly, MA), Upstate Cell Signaling Solutions (Charlottesville, VA), Novus (Littleton, NY), Abcam (Cambridge, MA) or New England Biolabs (Beverly, MA). If one of skill in the art wishes to ascertain which protein is acetylated, a number of antibodies to specific acetylated proteins are available, including antibodies to specific acetylated histones, to acetylated tubulin (a marker for Taxol pharmacodynamic responses), and the like. Such specific antibodies can also be used in the inventive procedures.

[0047] The cells are exposed to the reagent that can detect the pharmacodynamic response for a time and under conditions sufficient for reaction between the reagent and the pharmacodynamic marker. Hence, cells can be suspended in a small volume of buffered saline, which can contain 0.1 % BSA, and then mixed with an appropriate amount of the reagent. The cells are then incubated at mild temperatures for several minutes to several hours. For example, the cells can be incubated with anti-acetylated lysine antibodies at temperatures ranging from about 4 °C to about 37 °C for about 10 minutes to about 24 hours. In some embodiments, the cells are incubated with anti- acetylated lysine antibodies for about 1 hour at about room temperature. The c ells are then washed as described above.

[0048] Many antibodies are directly attached to a detectable label so no further labeling reagents or secondary antibodies are needed. If a secondary reagent is desired or needed for visualization of the reagent that can detect a pharmacodynamic response, the cells are then reacted with this reagent. For example, the anti-acetylated lysine antibodies that are bound to acetylated lysine residues can be detected by use of an anti-mouse secondary antibody that binds to monoclonal anti-acetylated antibodies from mice. The secondary antibody can have a detectable label, such as a fluorescent dye, that can be followed and observed.

[0049] A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions.

[0050] After reaction with the selected reagents, the cells are analyzed by convenient methods, for example, by fluorimetry or flow cytometry. In some embodiments, detection of overall increases or decreases in signal from a selected label may be quantified by simple spectrophotometric or fluorometric means. However, for many embodiments, including those involving detection and/or quantification of multiple markers, flow cytometry is used. Flow cytometry, cell sorting and cell analysis methods are available and are described in, for example, The Handbook of Experimental Immunology, Volumes 1 to 4, (D. N. Weir, editor) and Flow Cytometry and Cell Sorting (A. Radbruch, editor, Springer Verlag, 1992).

[0051] In general, cells are analyzed and sorted on a flow sorter based on the cells' tendency to scatter light forward (FSC) and to the side (SSC). Such cell signals reflect the cell type and may be detected and quantified. In each experiment, parameters are empirically established regarding the forward and side scatter properties. In general, the gain on the photomultiplier tubes detecting the forward-scattered light and the side-scattered light in each dimension is adjusted to distribute the array of signals from the cells across the channels available for analysis in a manner known to one skilled in the art. Under these circumstances a characteristic pattern is observed.

[0052] Pharmacodynamic response patterns can be further analyzed by staining the cells with labeled antibodies or other reagents that bind to a variety of markers. Markers that may be examined include cell-type specific markers, cell cycle staging markers, differentiation markers, markers that indicate the cell may undergo apoptosis and the like. Thus, the assay procedures of the invention can be adapted to include a step for staining the cells with selected antibodies or other reagents that provide information as to cell type, differentiation, stage of the cell cycle and the like. In general, detection of such markers can be performed by adding the relevant antibody or other reagent to the cell samples before or after fixation. The reagent that detects a selected marker can be reacted with the cells before, after or during reaction of the cells with the reagent that detects the pharmacodynamic response. The various markers and different cell types can be detected using flow cytometry. Hence, parameters such as the type of cell that exhibits the pharmacodynamic response, the stage in the cell cycle of that cell, the differentiation stage, the likelihood of that cell to undergo apoptosis and the existence of primary pharmacodynamic markers can be assessed simultaneously.

[0053] Where the assay is a binding assay, one or more of the antibodies or other reagents that bind to a variety of pharmacodynamic markers may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, particles, e.g. magnetic particles, and the like. Such labels include pairs of molecules that can bind to each other, such as biotin and streptavidin, digoxin and antidigoxin, and the like. One member of such a pair of molecules can be attached to a label that permits detection of the pair, and any pharmacodynamic or other marker to which they are attached.

[0054] For example, apoptosis can be assayed by detecting TUNEL (TdT-mediated dUTP nick-end labeling) labeling of the 3'-OH free end of DNA fragments produced during apoptosis (Gavrieli et al. (1992) J. Cell Biol. 119:493). TUNEL assays generally consist of catalytically adding a nucleotide, which has been conjugated to a chromogen system or to a fluorescent tag, to the 3'-OH end of the 180-bp (base pair) oligomer DNA fragments in order to detect the fragments. The presence of a DNA ladder of 180-bp oligomers is indicative of apoptosis. Procedures to detect cell death based on the TUNEL method are available commercially, e.g., from Boehringer Mannheim (Cell Death Kit) and Oncor (Apoptag Plus).

[0055] Another apoptosis marker that is currently available is annexin, sold under the trademark APOPTEST™. The annexin marker is used in the "Apoptosis Detection Kit," which is also commercially available, for example, from R&D Systems. During apoptosis, a cell membrane's phospholipid asymmetry changes such that the phospholipids are exposed on the outer membrane. Annexins are a homologous group of proteins that bind phospholipids in the presence of calcium. A second reagent can be used in conjunction with the reagent that detect annexin, propidium iodide (PI), which is a DNA binding fluorochrome. When a cell population is exposed to both reagents, apoptotic cells stain positive for annexin and negative for PI, necrotic cells stain positive for both, while live cells stain negative for both. Other methods of testing for apoptosis are known in the art and can be used in the methods of the invention.

Applications

[0056] The present invention provides assays involving methods to detect the pharmacodynamic response patterns of mixed populations of cells. These assay methods can be used to detect and monitor a drug response in the individual from which the cells were obtained. Moreover, the assay methods of the invention can be used to detect and monitor drug responses in many people at once, or in a population of individuals over time. Because the sample size required for testing by the present methods is very small, the methods of the invention can be used for screening studies where the pharmacodynamic response patterns in many, many samples is quickly quantified. Hence, the methods of the invention have utility for clinical trials of drugs, for example, for phase I, II, III and IV clinical trials performed to obtain regulatory approval of a drug or a combination of drugs.

[0057] The methods of the invention can also be used to identify new drugs that elicit a desired pharmacodynamic response. The desired pharmacodynamic response can be any cellular response that is correlated with administration of a selected class of drugs. For example, in one embodiment, the screening methods of the invention can be used to identify agents that modulate a level of generalized protein acetylation in cells, the level of histone deacetylase enzymatic activity in cells or the level of tubulin acetylation in cells. Anti-acetylated lysine antibodies, anti-acetylated histone antibodies, anti-acetylated tubulin antibodies and the like can be used in such methods. In addition the screening methods have been described to identify test agents that modulate a level of Hsp70 expression, because certain anti-cancer drugs are known to increase Hsp70 expression. Hence, a test agent that increases Hsp70 expression is a candidate for further testing to ascertain whether that test agent has anti-cancer activity. Many such pharmacodynamic responses and pharmacodynamic markers are known to those of skill in the art.

[0058] Thus, the invention provides methods for identifying test agents that modulate the pharmacodynamic response in a eukaryotic cell. The term "modulate" encompasses an increase or a decrease in the measured pharmacodynamic response when compared to a suitable control. The method generally involves:

(a) contacting a mixed population of cells with a test agent to form a first test mixture;
(b) contacting the first test mixture with a reagent that can detect a pharmacodynamic response to form a second test mixture;
(c) subjecting the second test mixture to flow cytometry; and
(d) observing whether the cells exhibit the pharmacodynamic response. An increase or a decrease in the pharmacodynamic response relative to a suitable control (e.g., a sample of the same mixed population of cells subjected to the method without exposure to the test agent) is an indication that the substance modulates a pharmacodynamic response. Another control could be, for example, a sample of the same mixed population of cells subjected to the method after being exposed to a drug that is known to produce the desired phannacodynamic response. Test agents that increase or decrease a pharmacodynamic response to a desired extent may be selected for further study, and assessed for cellular cytotoxicity, biocompatibility, etc.

[0059] The terms "agent", "test agent", "substance" and "compound" are used interchangeably herein. Test agents encompass numerous chemical classes, typically synthetic, semi-synthetic, or naturally-occurring inorgan-

ic or organic molecules. Test agents may be small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Test agents may comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and may include at least an amine, carbonyl, hydroxyl or carboxyl group, and may contain at least two of the functional chemical groups. The test agents may comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Test agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[0060] Test agents can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acetylation, acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

[0061] In another embodiment, the effects of known, approved drugs on patients can be monitored by the methods of the invention. For example, deacetylase inhibitors are administered to treat cancer and other diseases in some patients, including children. The effects of such deacetylase inhibitors upon the patient can be monitored using the present methods by detecting general acetylation levels, tubulin acetylation levels or histone acetylation levels using the present methods. For example, the methods of the invention can be used to monitor the effects of deacetylase inhibitors such as MS-275, trichostatin A, trapoxin, sodium butyrate, apicidin, sodium phenylbutyrate, phenylacetate, depsipeptide, 3-bromopropionate, valproic acid, tributyrin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydoxamic acid (CBHA), oxamflatin, pyroxamide, CHAP, depsipeptide (FR901228 or more recently FK228), NVP-LAQ824, CI-994, PXD101 or apicidin-derived quinolone derivatives.

[0062] Similarly, the methods of the invention can be used to monitor the effects of known anti-cancer agents such 17-allylaminogeldanamycin (17-AAG) or imatinib (also called Gleevec).

[0063] Geldanamycin is an antibiotic that binds to Hsp90 and inhibits its adenosine triphosphate binding and activity as a chaperone. A derivative of geldanamycin is the Hsp90 inhibitor 17-allylaminogeldanamycin, which preferentially kills tumor cells and has been in phase I clinical trials. When 17-allylaminogeldanamycin regulates Hsp90 activity, the cell responds by increasing the levels of Hsp70. Hence, Hsp70 is a pharmacodynamic marker for the activity of 17-allylaminogeldanamycin. As illustrated herein, the pharmacodynamic response of cells to 17-allylaminogeldanamycin can be observed by observing the levels of Hsp70 using the methods of the invention. Such pharmacodynamic monitoring of 17-allylaminogeldanamycin can be performed with or without monitoring of other pharmacodynamic markers. For example, as shown herein, the levels of tubulin acetylation and/or overall cellular protein acetylation can be monitored simultaneously with the pharmacodynamic effects of 17-allylaminogeldanamycin.

[0064] Gleevec (imatinib mesylate) is approved to treat a rare cancer called Chronic Myeloid Leukemia (CML). Imatinib mesylate is a protein-tyrosine kinase inhibitor that inhibits the Bcr-Abl protein tyrosine kinase, which is made by the abnormal Philadelphia chromosome in chronic myeloid leukemia. The Bcr-Abl protein tyrosine kinase carries messages to the cell telling it to divide and grow. By blocking this message, imatinib mesylate prevents the cancer cells from making more cells and causes them to die by apoptosis. The chemical name for Gleevec (imatinib mesylate) is 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)- 2-yrimidinyl]amino]-phenyl]benzamide methanesulfonate.

[0065] Hence, the invention provides a method of monitoring the pharmacodynamic response of a mixed population of eukaryotic cells to a selected drug. The method generally comprises:

(a) contacting a mixed population of cells with a drug to form a first test mixture;

(b) contacting the first test mixture with a reagent that can detect a pharmacodynamic response to the drug to form a second test mixture;

(c) subjecting the second test mixture to flow cytometry; and

(d) observing whether the cells exhibit the pharmacodynamic response. The method can further include quantifying the pharmacodynamic response of the cells to the drug. Such quantification can include calculating a proportionate increase or decrease in the pharmacodynamic response. For example, an increase or decrease in fluorescent signal during flow cytometry relative to one or more suitable controls can be used as a quantitative measure of the pharmacodynamic response. Such quantification can also include calculating what proportion of cells in the mixed population tested exhibit the pharmacodynamic response.

[0066] A suitable control can be, for example, a sample of the same mixed population of cells subjected to the method without exposure to the drug. Another control could be, for example, a sample of the same mixed population of cells subjected to the method after being ex-

posed to a drug or test agent that is known to produce the desired pharmacodynamic response.

[0067] In another embodiment, the invention provides a method of monitoring the pharmacodynamic response of a mixed population of eukaryotic cells that have already been exposed to a selected drug, for example, in a patient receiving the drug as a result of treatment or during a clinical trial. The method generally comprises:

(a) obtaining a mixed population of cells that have been exposed to a drug;
(b) contacting the mixed population of cells with a reagent that can detect a pharmacodynamic response to the drug to form a second test mixture;
(c) subjecting the second test mixture to flow cytometry; and
(d) observing whether the cells exhibit the pharmacodynamic response.

[0068] In another embodiment, the assays of the invention are used to detect histone, p53 or tubulin acetylation as a marker for cancer development, cancer regression or cancer progression. Accordingly, the invention further provides methods of identifying a cancerous cell in a sample constituting a mixed population of cells, where the mixed population of cells is suspected of containing cancerous cells and non-cancerous cells. Of particular interest in some embodiments is the detection of tumors of lymphoid origin including, but are not limited to, hematological malignancies, such as childhood acute leukemia, non-Hodgkin's lymphomas, chronic lymphocytic leukemia, malignant cutaneous T-cells, mycosis fungoides, non-MF cutaneous T-cell lymphoma, lymphomatoid papulosis, T-cell rich cutaneous lymphoid hyperplasia, bullous pemphigoid, discoid lupus erythematosus, lichen planus, thymomas, and the like.

[0069] Hence, the disclosure provides a method of monitoring the progression or regression of cancer in a mixed population of eukaryotic cells. The method generally comprises:

(a) obtaining a mixed population of cells from a patient;
(b) contacting the mixed population of cells with a reagent that can detect acetylation of histones, tubulin or p53 to form a test mixture;
(c) subjecting the test mixture to flow cytometry; and
(d) observing whether the cells have increased or decreased levels of histone, tubulin or p53 acetylation. The method can further include quantifying the levels of acetylation over time. Such quantification can include calculating a proportionate increase or decrease in acetylation relative to previously observed levels in the patient or in patients having known cancers or known cancer stages. For example, an increase or decrease in fluorescent signal during flow cytometry relative to one or more suitable controls can be used as a quantitative measure of the pharmacodynamic response. Such quantification can also include calculating what proportion of cells in the mixed population tested exhibit the increases or decreases in acetylation.

[0070] The disclosure provides methods for identifying whether a specific test agent or drug that modulate a pharmacodynamic response in a particular eukaryotic cell type. This method permits evaluation of effects of the test agent or the drug upon specific cell types. In this method, selected cell types or cell lines are tested for their response to the test agent or the drug. Such cell types can be purified from a mixed population of cells. Cell lines of a particular cell type can be obtained from cell depositories, for example, from the American Type Culture Collection (10801 University Blvd., Manassas, Va., 20110-2209 USA (ATCC)). The method generally involves:

(a) contacting a population of cells of a selected cell type with a test agent or drug to form a first test mixture;
(b) contacting the first test mixture with a reagent that can detect a pharmacodynamic response to form a second test mixture;
(c) subjecting the second test mixture to flow cytometry; and
(d) observing whether the cells exhibit the pharmacodynamic response. An increase or a decrease in the pharmacodynamic response relative to a suitable control (e.g., a sample of the same population of cells subjected to the method without exposure to the test agent) is an indication that the substance modulates a pharmacodynamic response. Another control could be, for example, a sample of the same population of cells subjected to the method after being exposed to a drug that is known to produce the desired or expected pharmacodynamic response. Test agents that increase or decrease a pharmacodynamic response to a desired extent may be selected for further study, and assessed for cellular cytotoxicity, biocompatibility, etc.

[0071] The disclosure provides assays for identifying whether a subject has or may develop an autoimmune disease. Histone deacetylase enzymes such as HDAC7 are expressed during T cell development at a time when T cells learn to distinguish self from non-self (thymic negative selection). Inappropriate HDAC7 activity could lead to selective dysregulation of the immune system such as autoimmune diseases or immune deficiencies. In the case of autoimmune diseases, such diagnostic assay is useful for diseases such as juvenile diabetes, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis and other related disorders. Hence, the invention provides assays for identifying whether a subject has or may develop an autoimmune disease. Such methods involve detecting whether histone deacetylase activity is

elevated in immune cells.

Kits

[0072] The disclosure provides a kit for assaying cell samples according to the methods of the invention. The kit can have a reagent for detecting a pharmacodynamic response and instructions for using the reagent to detect and/or quantify the pharmacodynamic response in a mixed cell sample (e.g. blood). For example, the kit can have an anti-acetylated lysine antibody preparation for detecting protein acetylation. The kit can have anti-Hsp70 antibodies for detecting a pharmacodynamic response to an anti-cancer agent such as 17-AAG or imatinib mesylate. The kit can have anti-tubulin antibodies for detecting a pharmacodynamic response to taxol. The kit can have reagents for detecting apoptosis, for example, antibodies reactive with factors involved in the apoptosis pathway. Such apoptosis factors include, for example, poly(ADP-ribose)polymerase (PARP) and of capases 6, 7, 8 and 9. Hence, antibodies or other reagents reactive with these apoptosis factors can be used in the kits of the invention.

[0073] The kits can also have a container and a means for collecting samples. For example, the kits can have alcohol swabs, a syringe, a sharp object for pricking the skin and/or a capillary tube, vacutainer or other means for collecting blood from the finger, arm or other site. The kits can also have containers of solutions for fixing and permeabilizing cells within collected samples.

[0074] The present disclosure further describes a kit for collecting and stabilizing samples to be tested using the methods of the invention. The kit has a container and a means for collecting samples as described above, along with instructions for using the collecting means and the container for collecting samples. The kit can also contain a fixation solution for stabilizing the cells in the collected samples. This kit may be used in the field for collecting and stabilizing samples that will be tested by the methods of the invention at a convenient location.

[0075] The invention will be further described by reference to the following detailed examples, which are given for illustration of the invention.

EXAMPLE 1: Detection of Acetylation in Whole Blood

[0076] This Example illustrates that acetylation of proteins in whole blood cells changes upon exposure of the cells to an acetylation inhibitor in *vitro* or upon administration of the deacetylase inhibitor *in vivo*. This assay can be used for large screening studies such as clinical trials because this assay requires only small amounts of blood, no purification of specific cell types is needed and the assay procedure is simple.

Materials and Methods

[0077] Peripheral whole blood samples of approximately 50-100 microliters in size were collected. After collection, whole blood samples were exposed to the deacetylase inhibitor MS-275 at concentrations varying from 0 to 1 micromolar. The MS-275 compound is N-(2-aminophenyl)- 4-[N-(pyridin- 3- ylmethoxycarbonyl) aminomethyl] benzamide. See, Saito et al., Proc. Natl. Acad. Sci. USA 96, 4592-4597 (1999). Blood cells were washed in wash buffer (phosphate buffered saline (PBS) containing 0.1% BSA).

[0078] In another series of experiments, the blood samples were obtained from a patient treated with the deacetylase inhibitor MS-275 at a dosage of 12 mg/m^2. Whole blood samples from this patient were obtained before MS-275 administration and then 24 hours after MS-275 treatment. Blood cells were washed in wash buffer (phosphate buffered saline (PBS) containing 0.1% BSA).

[0079] The different cell types were then fixed in fixation solution (0.4% paraformaldehyde in PBS), incubated at 37 °C for 5-10 minutes and washed with wash buffer. The fixed cells were then resuspended in permeabilization solution (0.4 % Triton X-100 in wash buffer) and incubated at room temperature for 5 minutes. After washing with wash buffer, the fixed and permeabilized cells were resuspended in 100 microliters of wash buffer and incubated with anti-acetylated lysine antibodies for 1 hour at room temperature. Cells were then washed with wash buffer and incubated simultaneously with anti-CD3 antibodies conjugated with PE and secondary antibodies (FITC-labeled anti-mouse antibodies) for 1 hour at room temperature, then washed again in wash buffer. Fluorescence associated with the cells was detected and quantified by flow cytometry.

Results

[0080] Flow cytometry patterns for whole blood samples treated with MS-275 *in vitro* are provided in FIG. 1A-E. Comparison of FIG. 1A-E shows that treatment of blood cells with the MS-275 deacetylase inhibitor gives rise to a peak of cells that have increased acetylation. In other words, the fluorescence detected for the population of cells generally increases, indicating that more FITC-labeled acetylated lysine residues are present in samples that were treated with the deacetylase inhibitor. A shoulder can be seen on the peak in FIG. 1C, showing flow cytometer results for cells treated with 1 micromolar MS-275. This shoulder likely represents a sub-population of cells that responds differently to the deacetylase inhibitor. Alternatively, this shoulder may represent a sub-population of cells in a different part of the cell cycle or a sub-population of cells undergoing apoptosis. The procedures described in Example 2 that involve multi-parameter analyses can be used to analyze what types of cells exist in this shoulder and/or what types of cellular events

are happening to cells in this shoulder.

[0081] Flow cytometry patterns for whole blood samples obtained from a patient treated with MS-275 *in vivo* are provided in FIG. 2. As shown FIG. 2C-E, *in vivo* treatment of this patient with the MS-275 deacetylase inhibitor gives rise to a distinct peak of cells that have increased acetylation. Compared to the diffuse peak shown in FIG. 2C for non-treated cells, the post-treatment cells shown in FIG. 2D exhibit increased amounts of acetylated lysine. FIG. 2A-B show the fluorescence detected from CD3 labeled cells on the y-axis and the fluorescence detected from anti-acetylated lysine residues on the x-axis. As shown, there are positive and negative populations of CD3-positive cells: those that express CD-3 are T cells while non-T cells express no CD3 and form a smaller population of cells nearer the x-axis. Upon treatment with the MS-275 deacetylase inhibitor, the fluorescence for both populations of cells shifts to the right, indicating that both types of cells have increased acetylation. Hence, both T cells and non-T cells respond to the MS-275 deacetylase inhibitor.

EXAMPLE 2: Protein Acetylation Patterns in Bone Marrow Aspirates

[0082] This Example illustrates that acetylation of proteins in bone marrow aspirates changes upon exposure of leukemia patients to a deacetylase inhibitor *in vivo*.

Materials and Methods

[0083] Bone marrow samples from a leukemia patient were collected before and 24 hours after administration of the MS-275 deacetylase inhibitor. Cells were washed in wash buffer (phosphate buffered saline (PBS) containing 0.1% BSA). The washed cells were resuspended in fixation solution (0.4% paraformaldehyde in PBS), incubated at 37 °C for 5-10 minutes and washed with wash buffer. The fixed cells were then resuspended in permeabilization solution (0.4 % Triton X-100 in wash buffer) and incubated at room temperature for 5 minutes. After washing with wash buffer, the fixed and permeabilized cells were resuspended in 100 microliters of wash buffer and incubated with anti-acetylated lysine antibodies for 1 hour at room temperature. Cells were then washed with wash buffer and incubated with secondary antibody (FITC-labeled anti-rabbit antibodies) for 1 hour at room temperature, then washed in wash buffer. Fluorescence associated with the cells was detected and quantified by flow cytometry.

Results

[0084] Flow cytometry patterns for bone marrow samples obtained from a leukemia patient treated with MS-275 *in vivo* are provided in FIG. 3. As shown in FIG. 3A, prior to *in vivo* treatment with MS-275, bone marrow cells comprise a broad peak of acetylated cells. However, after administration of the MS-275 deacetylase inhibitor, the bone marrow samples separate into two distinct peaks of cells (FIG. 3B). This is further illustrated by FIG. 3C, which shows both pre-treatment and post-treatment peaks. The presence of two post-treatment peaks may indicate that the treated sample is heterogeneous in some respect. For example, the tumor cells may be undergoing apoptosis as a result of treatment with the MS-275 deacetylase inhibitor. This hypothesis can readily be tested by labeling the cells with a marker for apoptosis and then observing whether the apoptosis marker associates with one or the other of the cell peaks detected after treatment with MS-275.

EXAMPLE 3: Detection of Acetylation Simultaneously

with Several Cell-Type Specific Markers

[0085] This Example illustrates that the procedures of the invention can be adapted to detect cell type specific markers in addition to acetylation of proteins in whole blood cells. These studies permit correlations to be made between the degree of acetylation and the cell type, the stage of the cell cycle, apoptosis or other factors.

Materials and Methods

[0086] Peripheral whole blood samples of approximately 50-100 microliters in size were collected and buffy coats were prepared by centrifugation of the anticoagulated whole blood. Aliquots of these buffy coat samples were exposed to 1 micromolar MS-275 deacetylase inhibitor for 18 hour. Control aliquots of the buffy coat samples received no MS-275 deacetylase inhibitor. The cells were washed to remove the deacetylase inhibitor and resuspended in fixation solution (0.4% paraformaldehyde in PBS), incubated at 37 °C for 5-10 minutes and washed with wash buffer (PBS with 0.1 % BSA). The washed cells were then resuspended in permeabilization solution (0.4 % Triton X-100 in wash buffer) and incubated at room temperature for 5 minutes. After washing with wash buffer, the fixed and permeabilized cells were resuspended in 100 microliters of wash buffer and simultaneously incubated with antibodies to various cell type specific markers as well as anti-acetylated lysine antibodies for 1 hour at room temperature. The antibody markers employed were the B cell-specific CD19 marker (using a PE-Cy5 label), the T cell-specific CD3 marker (using a PE label), the granulocyte/monocyte CD15 marker (using a FITC label) and the monocyte-specific CD 14 marker (using an APC-Cy7 label). Cells were then washed with wash buffer and incubated with secondary antibody (APC-labeled anti-rabbit antibodies) for 1 hour at room temperature, then washed in wash buffer. Fluorescence associated with the cells was detected and quantified by flow cytometry.

Results

[0087] Flow cytometry patterns for samples that received no MS-275 are provided in FIG. 4A1-5. A scatter gram showing the forward (FSC-A) and side (SSC-A) light scattering of this population of cells is provided in FIG. 4A1. Each cell type exhibits a characteristic forward and side scatter pattern and the flow cytometer can be gated to detect and/or sort specific cell types by their scattering patterns. FIG. 4A2 shows the fluorescence colors associated with the fluorophore types on antibody preparations used to detect CD19, CD3, CD15 and CD14. FIG. 4A3-5 illustrate that the blood samples collected contain a variety of cell types that express different types of surface markers. As shown in FIG. 4A3, cells expressing the CD3 marker (darker shade at the top right; pink in the original) can be distinguished from those expressing the CD15 marker (lighter shade at the lower right; blue in the original). As shown in FIG. 4A5, cells expressing the CD14 marker (circled lighter shade at the top; green in the original) can be distinguished from those expressing the CD15 marker (circled darker shade at the lower right; blue in the original). The sample populations contained a significant proportion of T cells, as shown by detection of the CD3 marker, and a significant proportion of granulocytes and monocytes, as shown by detection of the CD14 and CD15 markers. None-the-less, the buffy coat samples collected contained a large number of different cell types.

[0088] Flow cytometric patterns for cell samples that received MS-275 treatment indicated that all cell types had increased acetylation after MS-275 treatment (see, FIG. 4B1-4). For example, comparison of FIG. 4B1 and 4B3, with FIG. 4B2 and 4B4 shows that the fluorescence due to acetylated lysine for essentially all cell types shifted upward, indicating that these cells had increased acetylation. Hence, essentially all of the blood cell types present in the samples collected responded to the MS-275 deacetylase inhibitor and exhibited increased acetylation. Therefore, samples collected from patients to test for drug effects need not be extensively purified before detection of the marker that identifies the drug effect.

EXAMPLE 4: The Pharmacodynamics of Anti-Cancer Drugs and Deacetylase Inhibitors Can Be Monitored Simultaneously

[0089] This Example illustrates that the procedures of the invention can be adapted to simultaneously detect the effects of two or more drugs on their pharmacodynamic markers. Previous methods relied upon western blot analysis, ELISA or immunocytochemical analysis. However, such procedures are cumbersome, time-consuming and cannot easily detect two or more events in a large population of cells. This Example illustrates that such multi-variable analysis can readily be performed using flow cytometry of small samples of blood.

Materials and Methods

[0090] Leukemia K562 cell line samples containing approximately $5x10^6$ to $1x10^7$ cells were exposed to either the anti-cancer drug 17-allylaminogeldanamycin (17-AAG) or one of the deacetylase inhibitors MS-275 or trichostatin A (TSA). Some samples received both 17-AAG and TSA or both 17-AAG and MS-275. Control samples received no drug. Administration of the 17-AAG anti-cancer drug led to functional changes in Hsp90 and increased expression of Hsp70. Hence, the pharmacodynamic effect of 17-AAG can be detected by observing whether Hsp70 expression increases. TSA is a deacetylase inhibitor that can affect acetylation of numerous proteins. In this study, the effect of TSA on tubulin acetylation was observed using an antibody that specifically binds to acetylated tubulin.

[0091] After treatment with the various drugs, the cells were resuspended in fixation solution (0.4% paraformaldehyde in PBS), incubated at 37 °C for 5-10 minutes and washed with wash buffer. The fixed cells were then resuspended in permeabilization solution (4 % Triton X-100 in wash buffer) and incubated at room temperature for 5 minutes. Cells were incubated for 1 hour at room temperature with antibodies to the various pharmacodynamic markers. Several cell samples were incubated with antibodies to several markers at once. These antibody preparations included antibodies to Hsp70 to detect the pharmacodynamic effect of 17-AAG and/or with anti-acetylated tubulin antibodies to detect the pharmacodynamic effect of the deacetylase inhibitors on tubulin acetylation and/or with anti-acetylated lysine antibodies to detect the general effect of deacetylase inhibitors on protein acetylation. The cells were then washed with wash buffer (PBS with 0.1% BSA) and incubated with secondary antibodies for 1 hour at room temperature, then washed in wash buffer. Fluorescence associated with the cells was detected and quantified by flow cytometry.

Results

[0092] Flow cytometry patterns for samples that received 17-AAG or TSA are provided in FIG. 5A-B. As shown in FIG. 5A, cells receiving 17-AAG had increased levels of Hsp70 relative to control cells that received no 17-AAG. These results indicate that the cells are responding to the 17-AAG anti-cancer drug by increasing the synthesis of Hsp70. As shown in FIG. 5B, cells receiving TSA had increased levels of acetylated tubulin, indicating that the TSA deacetylase inhibitor has inhibited deacetylation of tubulin.

[0093] Flow cytometric results for the dual pharmacodynamic testing of the effects of both 17-AAG and TSA are shown in FIG. 6A-D. As shown in FIG. 6C-D, the levels of Hsp70 (C) and acetylated tubulin (D) both increased when these drugs were simultaneously administered. The dot plots in FIG. 6A-B show that only low

levels of Hsp70 and acetylated tubulin are detected before drug administration (FIG. 6A). However, after exposure to 17-AAG and TSA, the levels of both Hsp70 and acetylated tubulin increase substantially (FIG. 6B). Hence, the pharmacodynamics of two drugs in a mixed population of cells were readily observed.

[0094] Flow cytometric patterns for the dual pharmacodynamic testing of the effects of both 17-AAG and MS-275 are shown in FIG. 7A-D. As shown in FIG. 7C-D, the levels of both Hsp70 (C) and acetylated lysine (D) increased when these drugs were simultaneously administered. The three dimensional maps shown in FIG. 7A-B show that only low levels of Hsp70 and acetylated lysine are detected before drug administration (FIG. 7A). However, after exposure to 17-AAG and MS-275, the levels of both Hsp70 and acetylated lysine increase substantially (FIG. 7B-D). Hence, the pharmacodynamics of two drugs in a mixed population of cells were readily observed.

EXAMPLE 5: Immunocytochemical analysis of protein acetylation

[0095] This Example uses deconvolution microscopy to illustrate that the staining procedure used in the flow assay can detect hyperacetylation of both nuclear and cytoplasmic proteins.

Materials and Methods

[0096] Cells were pelleted onto glass slides by cytocentrifugation, stained as described above for flow cytometric analysis, counterstained with the fluorescent DNA dye DAPI, and viewed using a Leica DM IRB fluorescence microscope equipped with a Z-axis motor (Ludl Electronics, Hawthorne, NY). Stacks of images (between 13 and 19 optical sections at a step size of 0.3 μm) were taken with a digital camera (Hamamatsu) and processed using Openlab Volume Deconvolution software (Improvision, Lexington, MA).

Results

[0097] To determine whether an antibody to acetylated lysine can be used to assess the response to HDAC inhibitors, and to assess if the response can be observed in both nuclear and cytoplasmic compartments, unfractionated buffy coats of healthy donors were incubated with the HDAC inhibitor MS-275 and examined for protein acetylation by immunocytochemistry. Untreated cells showed a variable level of acetylation that ranged from undetectable to moderate (Fig 8A). In the majority of cells treated with MS-275 (1 μM, 20 hours), protein acetylation was markedly increased (Fig 8B). Examination of MS-275-treated cells by optical sectioning demonstrated that both cytoplasmic and nuclear staining could be visualized, with considerable cell-to-cell heterogeneity in the localization of acetylated proteins. FIG. 8C displays a cell with predominantly nuclear signal and FIG. 8D shows a cell with predominantly cytoplasmic signal.

EXAMPLE 6: Flow cytometric analysis of apoptosis *versus* protein acetylation

[0098] This Example illustrates that the multiparameter flow approach can be used to detect the correlation, at the single cell level, of protein hyperacetylation and the induction of tumor cell apoptosis in response to anticancer drug treatment.

Materials and Methods

[0099] K562 chronic myelogenous leukemia cells were incubated with vehicle alone, 1 μM imatinib (the anticancer drug also known as Gleevec), 1 μM MS-275, or both for 48 hours. The cells were then stained for acetylated lysine as described above and co-stained with antibody to activated caspase 3 as an indicator of cells undergoing apoptosis. Dot plots display acetylated lysine on the x-axis and activated caspase 3 on the y-axis.

Results

[0100] The multiparameter flow cytometric assay is a powerful tool to monitor pharmacodynamic changes induced by anticancer drugs used in monotherapy or combination therapy protocols. HDAC inhibitors have been reported to promote imatinib-mediated apoptosis in Bcr/Abl+ human myeloid leukemia cells, including imatinib-resistant cells. The effect of MS-275 and imatinib were therefore examined, alone and in combination, on apoptosis in the Bcr/Abl+ cell line K562. Because both MS-275 and imatinib have been reported to induce apoptosis associated with caspase 3 activation, an antibody was used that specifically recognizes activated caspase 3 in a flow assay. This caspase 3 flow assay effectively monitors drug-induced apoptosis. When combined detection of acetylated lysine, this flow assay permitted simultaneous monitoring of apoptosis (caspase 3) and acetylated lysine.

[0101] As can be seen in FIG. 9B-C, both MS-275 and imatinib increased the percent of apoptotic cells, and MS-275 strongly upregulated acetylation in over 50% of the cells. One population of cells lost acetylation when treated with imatinib (FIG. 9B) and these cells were positive for activated caspase 3. Furthermore, a population of non-apoptotic cells was present after treatment with either MS-275 or imatinib alone (cells clustered near the bottom of the FIG. 9B-C), and this population was almost eliminated by treatment with both MS-275 and imatinib.

[0102] These results demonstrate that hyperacetylation combined with the tyrosine kinase inhibitor imatinib is a highly effective treatment for chronic myelogenous leukemia cells.

Example 7: Detection of p21 expression *versus* acetylated lysine in leukemia patient bone marrow aspirates in response to MS-275 *in vivo*

[0103] This Example demonstrates that the multi-parameter flow assay can be used to monitor changes in protein expression in response to anticancer drug treatment, and that the effect of this treatment on protein expression can be correlated at the single cell level to treatment-induced protein hyperacetylation.

Materials and Methods

[0104] Bone marrow aspirates were obtained and stained for acetylated lysine as described above. The cells were co-stained for the expression of the cyclin-dependent kinase inhibitor p21. The samples were then analyzed by multiparameter flow cytometry.

Results

[0105] Histone deacetylase inhibitors can modulate the pattern of gene expression in tumor cells, and this modulation of gene expression may be critical to histone deacetylase inhibitor anti-tumor activity. One of the most important genes induced by HDAC inhibitors is the cyclin-dependent kinase inhibitor p21. As shown in FIG. 10A-B, there is a low level of protein acetylation and p21 expression prior to treatment of the patient with MS-275 (FIG. 10A). However, the level of both protein acetylation and p21 were clearly increased in response to treatment with MS-275 (FIG. 10B).

Claims

1. A method of monitoring protein acetylation in whole blood, bone marrow or cerebrospinal fluid exposed to a deacetylase inhibitor, comprising:

   (a) providing a sample of whole blood, bone marrow or cerebrospinal fluid exposed *in vitro* or *in vivo* to the deacetylase inhibitor to form a first test mixture;
   (b) contacting the first test mixture with a reagent that can detect protein acetylation to form a second test mixture; and
   (c) quantifying protein acetylation in the second test mixture by flow cytometry; wherein the sample has a volume ranging from 25 microliters to 150 microliters.

2. The method of claim 1, wherein quantifying protein acetylation comprises calculating an increase or decrease in fluorescence signal during flow cytometry relative to one or more controls.

3. The method of claim 2, wherein one control is a sample subjected to the method without exposure to the deacetylase inhibitor.

4. The method of claim 2, wherein one control is a sample subjected to the method after being exposed to a drug that is known to inhibit or increase protein acetylation.

5. The method of claim 1, wherein the sample is human whole blood.

6. The method of claim 1, wherein the deacetylase inhibitor is MS-275, trichostatin A, trapoxin, sodium butyrate, apicidin, sodium phenylbutyrate, phenylacetate, depsipeptide, 3-bromopropionate, valproic acid, tributyrin, suberoylanilide hydroxamic acid (SAHA), m-carboxycinnamic acid bishydoxamic acid, oxamflatin, pyroxamide, CHAP, depsipeptide (FK228), NVP-LAQ824, CI-994, PXD101, apicidin-derived quinolone derivatives or a combination thereof.

7. The method of claim 1, wherein the deacetylase inhibitor is MS-275.

8. The method of claim 1, wherein the reagent that can detect protein acetylation is an antibody that can bind to an acetylated protein.

9. The method of claim 8, wherein the acetylated protein is acetylated tubulin.

10. The method of claim 1, wherein the method further comprises observing which cell types exhibit protein acetylation.

11. The method of claim 1, wherein the method further comprises observing in what cell cycle stage the cells exhibit protein acetylation.

12. The method of claim 1, wherein the method further comprises observing whether some of the cells are undergoing apoptosis.

13. The method of claim 1, wherein the sample has been exposed to a drug other than the deacetylase inhibitor.

14. The method of claim 1, wherein the deacetylase inhibitor is MS-275, and the sample is whole blood.

15. A method of identifying whether a test agent modulates protein acetylation in whole blood, bone marrow or cerebrospinal fluid, comprising:

   (a) providing a sample of whole blood, bone marrow or cerebrospinal fluid that has been exposed *in vitro* or *in vivo* to a test agent to form a first

test mixture;
(b) contacting the first test mixture with a reagent that can detect protein acetylation to thereby form a second test mixture; and
(c) quantifying protein acetylation in the second test mixture by flow cytometry; wherein the sample has a volume ranging from 25 microliters to 150 microliters.

16. A method of quantifying protein acetylation in a sample of whole blood, comprising:

(a) fixing cells from the whole blood with 0.4% paraformaldehyde in phosphate buffered saline to generate fixed cells;
(b) incubating the fixed cells with 0.4% Triton X-100 in phosphate buffered saline to generate permeabilized cells;
(c) reacting the permeabilized cells with an anti-acetylated lysine antibody preparation to form a complex between the permeabilized cells and the anti-acetylated lysine antibody; and
(d) quantifying protein acetylation using flow cytometry by observing a signal from a label associated with the anti-acetylated lysine antibody; wherein the sample has a volume ranging from 25 microliters to 150 microliters.

17. The method of claim 16, wherein no purification of specific cell types from the sample of whole blood is performed.

Patentansprüche

1. Verfahren zur Überwachung der Proteinacetylierung in Vollblut, Knochenmark oder Gehirn-Rückenmarks-Flüssigkeit, das bzw. die einem Deacetylase-Inhibitor ausgesetzt wurde, umfassend die Schritte, dass:

(a) eine Probe von Vollblut, Knochenmark oder Gehirn-Rückenmarks-Flüssigkeit vorgesehen wird, die *in vitro* oder *in vivo* dem Deacetylase-Inhibitor ausgesetzt wurde, um ein erstes Testgemisch zu bilden;
(b) das erste Testgemisch mit einem Reagens in Kontakt gebracht wird, das eine Proteinacetylierung nachweisen kann, um ein zweites Testgemisch zu bilden; und
(c) die Proteinacetylierung in dem zweiten Testgemisch durch Durchflusszytometrie quantifiziert wird; wobei die Probe ein Volumen im Bereich von 25 Mikroliter bis 150 Mikroliter aufweist.

2. Verfahren nach Anspruch 1, wobei die Quantifizierung der Proteinacetylierung die Berechnung einer Zunahme oder Abnahme eines Fluoreszenzsignals relativ zu einer oder mehreren Kontrolle(n) während der Durchflusszytometrie umfasst.

3. Verfahren nach Anspruch 2, wobei eine Kontrolle eine Probe ist, die dem Verfahren unterzogen wird, ohne zuvor dem Deacetylase-Inhibitor ausgesetzt zu werden.

4. Verfahren nach Anspruch 2, wobei eine Kontrolle eine Probe ist, die dem Verfahren unterzogen wird, nachdem sie einem Arzneistoff ausgesetzt wurde, der bekanntermaßen die Proteinacetylierung hemmt oder steigert.

5. Verfahren nach Anspruch 1, wobei die Probe menschliches Vollblut ist.

6. Verfahren nach Anspruch 1, wobei der Deacetylase-Inhibitor MS-275, Trichostatin A, Trapoxin, Natriumbutyrat, Apicidin, Natriumphenylbutyrat, Phenylacetat, Depsipeptid, 3-Brompropionat, Valproinsäure, Tributyrin, Suberoylanilid-Hydroxamsäure (SAHA), m- Carboxy- Cinnaminsäure- Bishydroxamsäure, Oxamflatin, Pyroxamid, CHAP, Depsipeptid (FK228), NVP-LAQ824, CI-994, PXD101, von Apicidin abgeleitete Quinolonderivate oder eine Kombination daraus ist.

7. Verfahren nach Anspruch 1, wobei der Deacetylase-Inhibitor MS-275 ist.

8. Verfahren nach Anspruch 1, wobei das Reagens, das eine Proteinacetylierung nachweisen kann, ein Antikörper ist, der an acetyliertes Protein binden kann.

9. Verfahren nach Anspruch 8, wobei das acetylierte Protein acetyliertes Tubulin ist.

10. Verfahren nach Anspruch 1, wobei das Verfahren des Weiteren den Schritt umfasst, dass beobachtet wird, welche Zelltypen eine Proteinacetylierung zeigen.

11. Verfahren nach Anspruch 1, wobei das Verfahren des Weiteren den Schritt umfasst, dass beobachtet wird, in welchem Stadium des Zellzyklus die Zellen eine Proteinacetylierung zeigen.

12. Verfahren nach Anspruch 1, wobei das Verfahren des Weiteren den Schritt umfasst, dass beobachtet wird, ob einige der Zellen der Apoptose unterliegen.

13. Verfahren nach Anspruch 1, wobei die Probe einem anderen Arzneistoff als dem Deacetylase-Inhibitor ausgesetzt wurde.

14. Verfahren nach Anspruch 1, wobei der Deacetylase-Inhibitor MS-275 ist und die Probe Vollblut.

15. Verfahren zur Feststellung, ob ein Testwirkstoff die Proteinacetylierung in Vollblut, Knochenmark oder Gehirn-Rückenmarks-Flüssigkeit moduliert, umfassend die Schritte, dass:

(a) eine Probe von Vollblut, Knochenmark oder Gehirn-Rückenmarks-Flüssigkeit vorgesehen wird, die *in vitro* oder *in vivo* einem Testwirkstoff ausgesetzt wurde, um ein erstes Testgemisch zu bilden;
(b) das erste Testgemisch mit einem Reagens in Kontakt gebracht wird, das eine Proteinacetylierung nachweisen kann, um **dadurch** ein zweites Testgemisch zu bilden; und
(c) die Proteinacetylierung in dem zweiten Testgemisch durch Durchflusszytometrie quantifiziert wird; wobei die Probe ein Volumen im Bereich von 25 Mikroliter bis 150 Mikroliter aufweist.

16. Verfahren zur Quantifizierung der Proteinacetylierung in einer Vollblut-Probe, umfassend die Schritte, dass:

(a) Zellen aus dem Vollblut mit 0,4 % Paraformaldehyd in phosphatgepufferter Kochsalzlösung fixiert werden, um fixierte Zellen zu erzeugen;
(b) die fixierten Zellen mit 0,4 % Triton X-100 in phosphatgepufferter Kochsalzlösung inkubiert werden, um permeabilisierte Zellen zu erzeugen;
(c) die permeabilisierten Zellen mit einer Zubereitung eines Antikörpers gegen acetyliertes Lysin umgesetzt werden, um einen Komplex aus den permeabilisierten Zellen und dem Antikörper gegen acetyliertes Lysin zu bilden; und
(d) die Proteinacetylierung unter Verwendung der Durchflusszytometrie quantifiziert wird, indem ein Signal von einem mit dem Antikörper gegen acetyliertes Lysin verbundenen Marker beobachtet wird; wobei die Probe ein Volumen im Bereich von 25 Mikroliter bis 150 Mikroliter aufweist.

17. Verfahren nach Anspruch 16, wobei keine Aufreinigung spezifischer Zelltypen aus der Vollblut-Probe durchgeführt wird.

Revendications

1. Procédé de contrôle de l'acétylation de protéines dans le sang complet, la moelle osseuse ou le liquide céphalorachidien exposé à un inhibiteur de désacétylase, comprenant les étapes consistant à :

(a) fournir un échantillon de sang complet, moelle osseuse ou liquide céphalorachidien exposé *in vitro* ou *in vivo* à l'inhibiteur de désacétylase pour former un premier mélange test ;
(b) mettre en contact le premier mélange test avec un réactif qui peut détecter l'acétylation de protéines pour former un deuxième mélange test ; et
(c) quantifier l'acétylation de protéines dans le deuxième mélange test par cytométrie de flux ; dans lequel l'échantillon présente un volume allant de 25 microlitres à 150 microlitres.

2. Procédé selon la revendication 1, dans lequel la quantification de l'acétylation de protéines comprend le calcul d'une augmentation ou diminution du signal de fluorescence au cours de la cytométrie de flux par rapport à un ou plusieurs témoins.

3. Procédé selon la revendication 2, dans lequel un témoin est un échantillon soumis au procédé sans exposition à l'inhibiteur de désacétylase.

4. Procédé selon la revendication 2, dans lequel un témoin est un échantillon soumis au procédé après avoir été exposé à un médicament qui est connu comme inhibant ou augmentant l'acétylation de protéines.

5. Procédé selon la revendication 1, dans lequel l'échantillon est du sang complet humain.

6. Procédé selon la revendication 1, dans lequel l'inhibiteur de désacétylase est MS-275, trichostatine A, trapoxine, butyrate de sodium, apicidine, phénylbutyrate de sodium, phénylacétate, depsipeptide, 3-bromopropionate, acide valproïque, tributyrine, acide suberoylanilide hydroxamique (SAHA), acide bishydroxamique d'acide m-carboxycinnamique, oxamflatine, pyroxamide, CHAP, depsipeptide (FK228), NVP-LAQ824, CI-994, PXD101, dérivés de quinolone dérivés de l'apicidine ou une combinaison de ceux-ci.

7. Procédé selon la revendication 1, dans lequel l'inhibiteur de désacétylase est MS-275.

8. Procédé selon la revendication 1, dans lequel le réactif qui peut détecter l'acétylation de protéines est un anticorps pouvant se lier à une protéine acétylée.

9. Procédé selon la revendication 8, dans lequel la protéine acétylée est de la tubuline acétylée.

10. Procédé selon la revendication 1, dans lequel le procédé comprend en outre l'étape consistant à observer quels types de cellules présentent l'acétylation de protéines.

11. Procédé selon la revendication 1, dans lequel le procédé comprend en outre l'étape consistant à observer à quel stade du cycle cellulaire les cellules présentent l'acétylation de protéines.

12. Procédé selon la revendication 1, dans lequel le procédé comprend en outre l'étape consistant à observer si certaines des cellules sont en apoptose.

13. Procédé selon la revendication 1, dans lequel l'échantillon a été exposé à un médicament autre que l'inhibiteur de désacétylase.

14. Procédé selon la revendication 1, dans lequel l'inhibiteur de désacétylase est MS-275, et l'échantillon est du sang complet.

15. Procédé consistant à identifier si un agent test module l'acétylation de protéines dans le sang complet, la moelle osseuse ou le liquide céphalorachidien, comprenant les étapes consistant à :

(a) fournir un échantillon de sang complet, moelle osseuse ou liquide céphalorachidien qui a été exposé *in vitro* ou *in vivo* à un agent test pour former un premier mélange test ;
(b) mettre en contact le premier mélange test avec un réactif qui peut détecter l'acétylation de protéines pour former ainsi un deuxième mélange test ; et
(c) quantifier l'acétylation de protéines dans le deuxième mélange test par cytométrie de flux ; dans lequel l'échantillon présente un volume allant de 25 microlitres à 150 microlitres.

16. Procédé de quantification de l'acétylation de protéines dans un échantillon de sang complet, comprenant les étapes consistant à :

(a) fixer les cellules issues du sang complet avec 0,4 % de paraformaldéhyde dans une solution saline tamponnée de phosphate pour générer des cellules fixes ;
(b) incuber les cellules fixes avec 0,4 % de Triton X-100 dans une solution saline tamponnée de phosphate pour générer des cellules perméabilisées ;
(c) faire réagir les cellules perméabilisées avec une préparation d'anticorps anti-lysine acétylée pour former un complexe entre les cellules perméabilisées et l'anticorps anti-lysine acétylée ; et
(d) quantifier l'acétylation de protéines par cytométrie de flux en observant un signal provenant d'un traceur associé à l'anticorps anti-lysine acétylée ; dans lequel l'échantillon présente un volume allant de 25 microlitres à 150 microlitres.

17. Procédé selon la revendication 16, dans lequel aucune purification de types de cellules spécifiques provenant de l'échantillon de sang complet n'est effectuée.

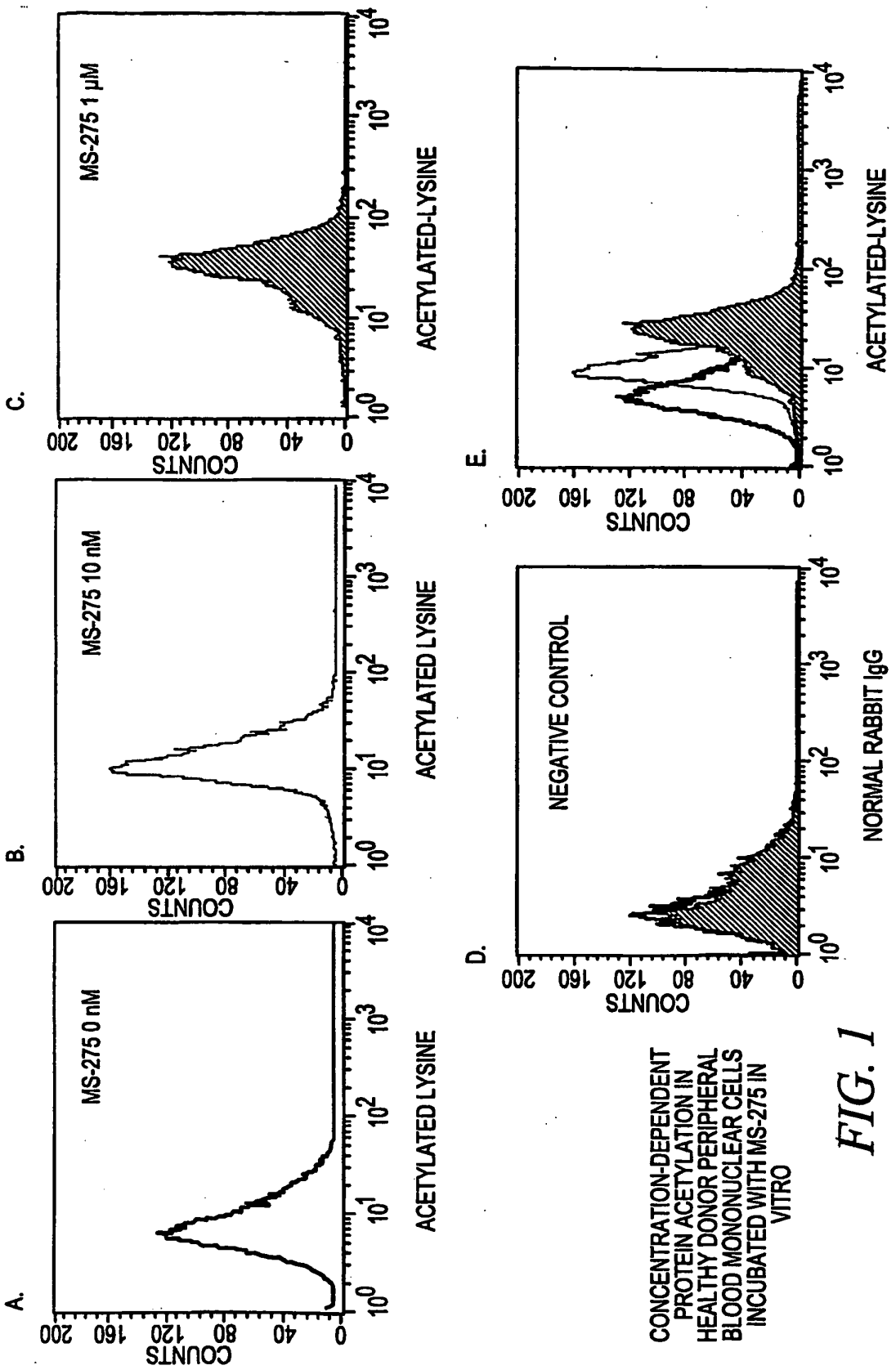
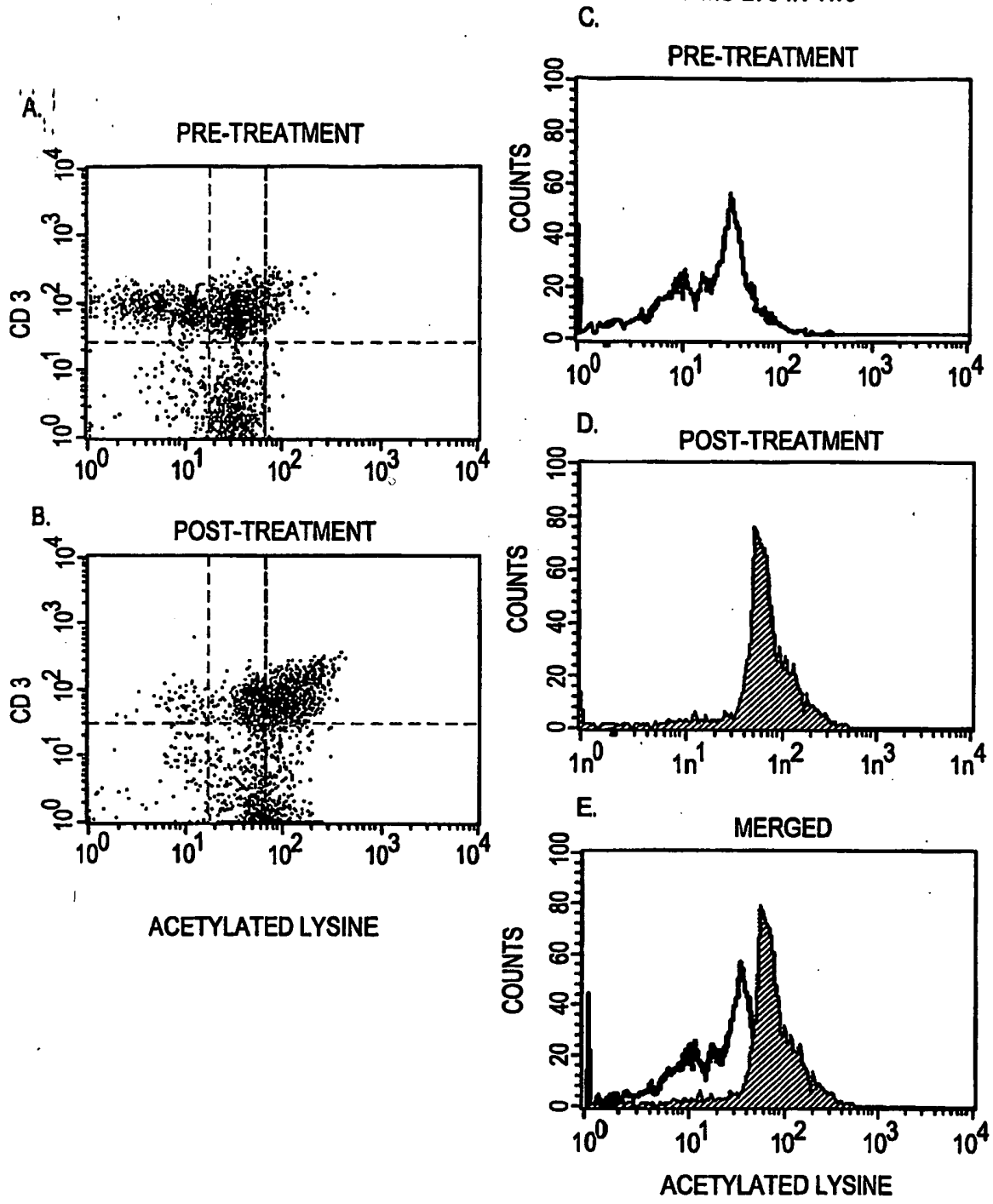
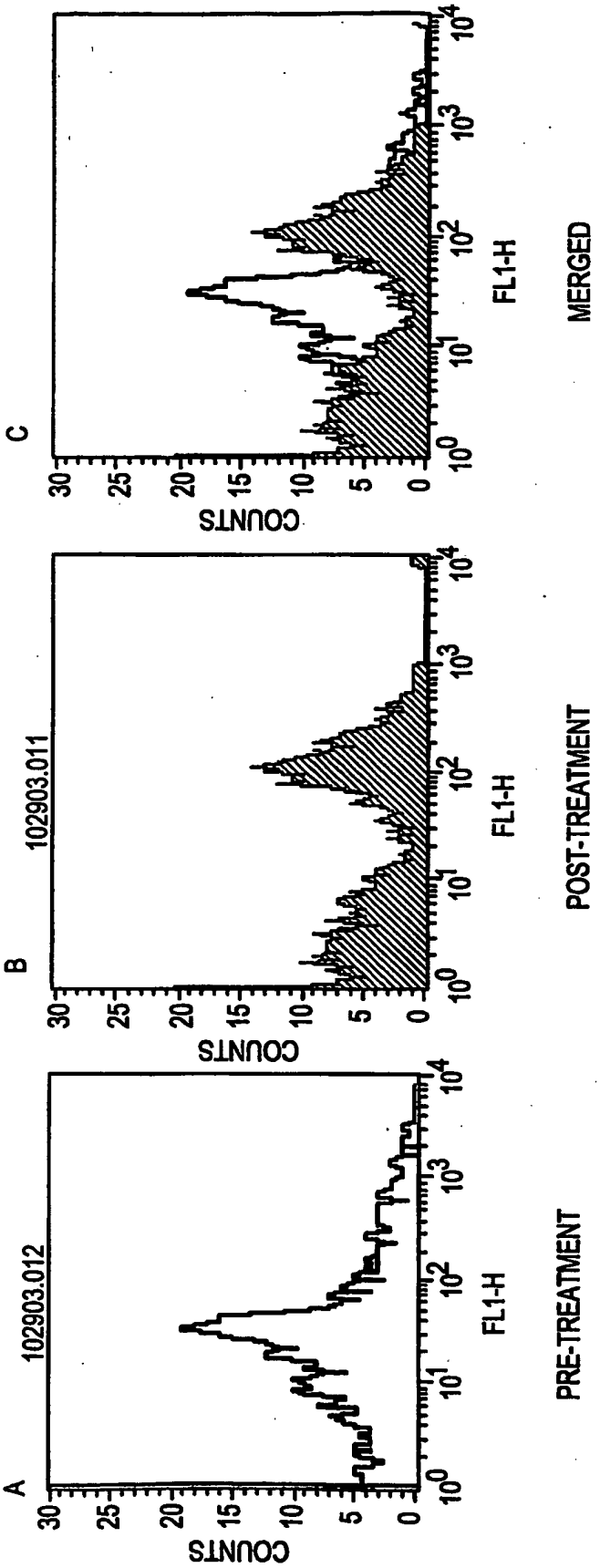

A.
MS-275 0 nM
B.
MS-275 10 nM
C.
MS-275 1 µM
D.
NEGATIVE CONTROL
E.
COUNTS
ACETYLATED LYSINE
ACETYLATED-LYSINE
NORMAL RABBIT IgG
CONCENTRATION-DEPENDENT PROTEIN ACETYLATION IN HEALTHY DONOR PERIPHERAL BLOOD MONONUCLEAR CELLS INCUBATED WITH MS-275 IN VITRO

FIG. 1



WHOLE BLOOD WAS OBTAINED PRE- AND 24 h POST-TREATMENT WITH MS-275, GATED ON LYMPHOCYTES

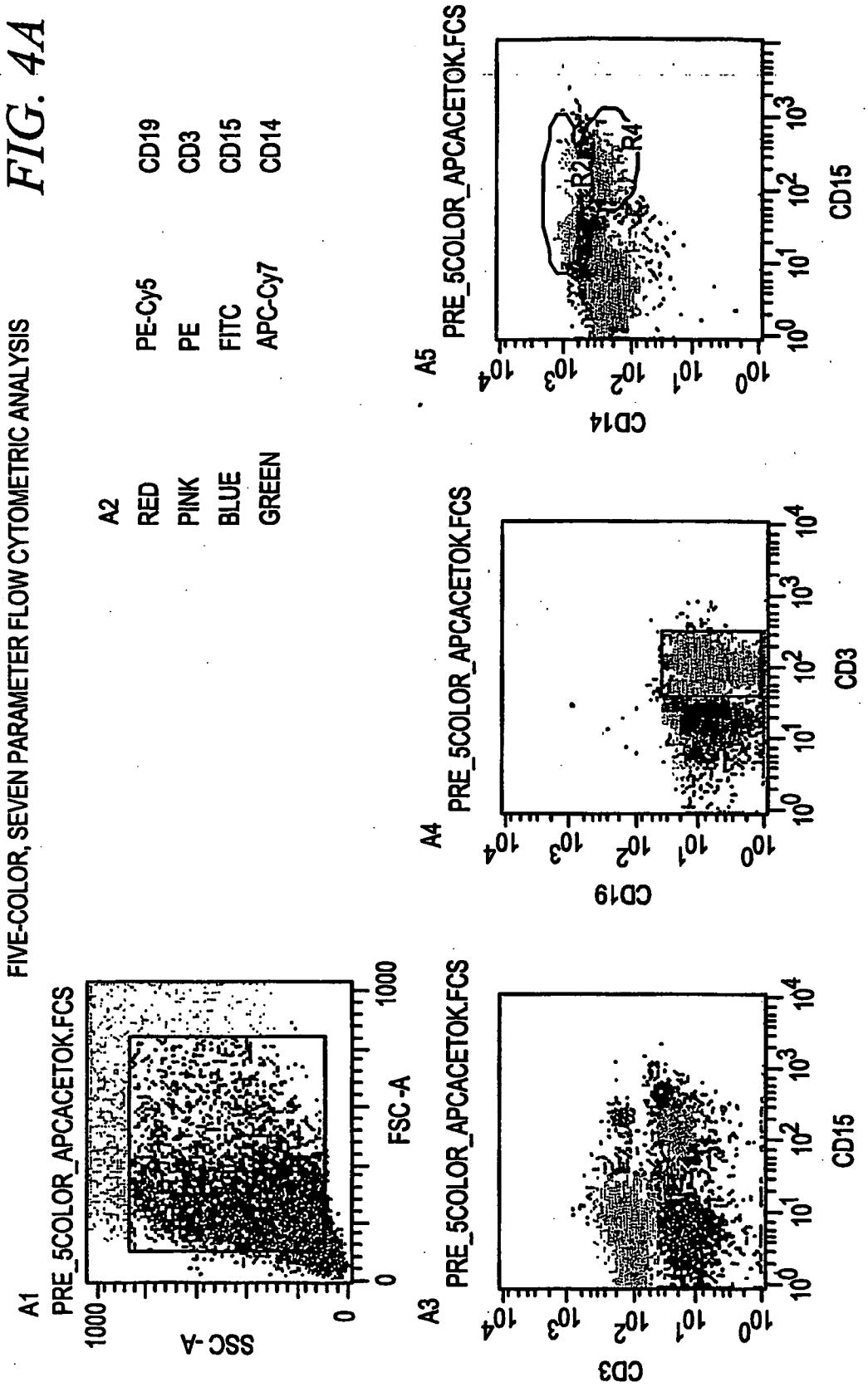
FIG. 2



FLOW CYTOMETRIC ANALYSIS OF PROTIEN ACETYLATION IN A BONE MARROW ASPIRATE OF A LEUKEMIA PATIENT PRE- AND POST-TREATMENT WITH MS-275

FIG. 3

FIG. 4A


FIVE-COLOR, SEVEN PARAMETER FLOW CYTOMETRIC ANALYSIS
A1
PRE_5COLOR_APCACETOK.FCS
SSC-A
FSC-A
A2
RED PE-Cy5 CD19
PINK PE CD3
BLUE FITC CD15
GREEN APC-Cy7 CD14
A3
CD3
CD15
A4
CD19
CD3
A5
CD14
CD15
R2
R4
ASSAY OF DONOR BUFFY COATS WITHOUT FICO11GRADIENT CENTRIFUGATION
ANALYSIS ON BD LSR II

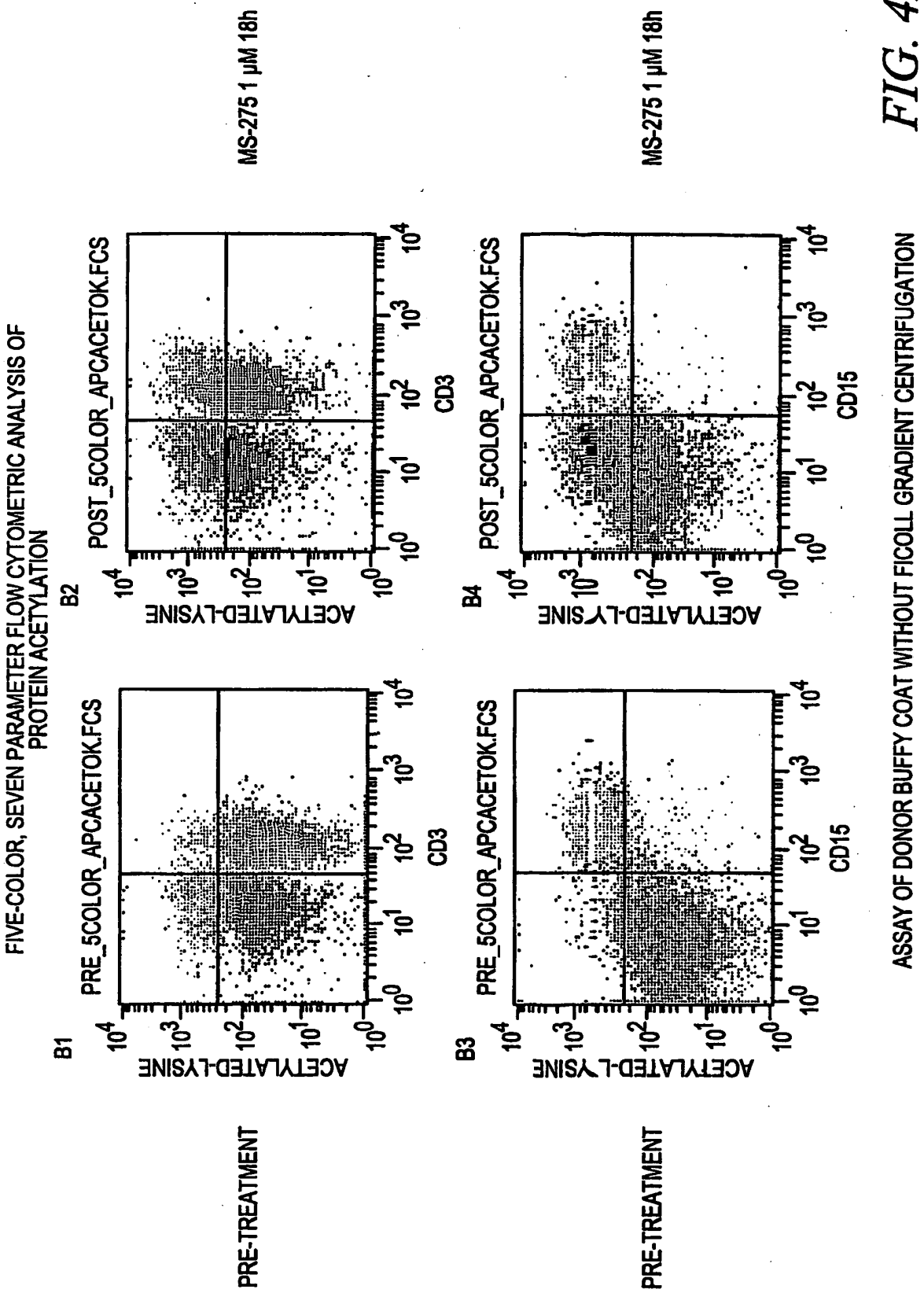

FIVE-COLOR, SEVEN PARAMETER FLOW CYTOMETRIC ANALYSIS OF PROTEIN ACETYLATION
ASSAY OF DONOR BUFFY COAT WITHOUT FICOLL GRADIENT CENTRIFUGATION
B1
PRE_5COLOR_APCACETOK.FCS
ACETYLATED-LYSINE
CD3
PRE-TREATMENT
B2
POST_5COLOR_APCACETOK.FCS
ACETYLATED-LYSINE
CD3
MS-275 1 µM 18h
B3
PRE_5COLOR_APCACETOK.FCS
ACETYLATED-LYSINE
CD15
PRE-TREATMENT
B4
POST_5COLOR_APCACETOK.FCS
ACETYLATED-LYSINE
CD15
MS-275 1 µM 18h

FIG. 4B

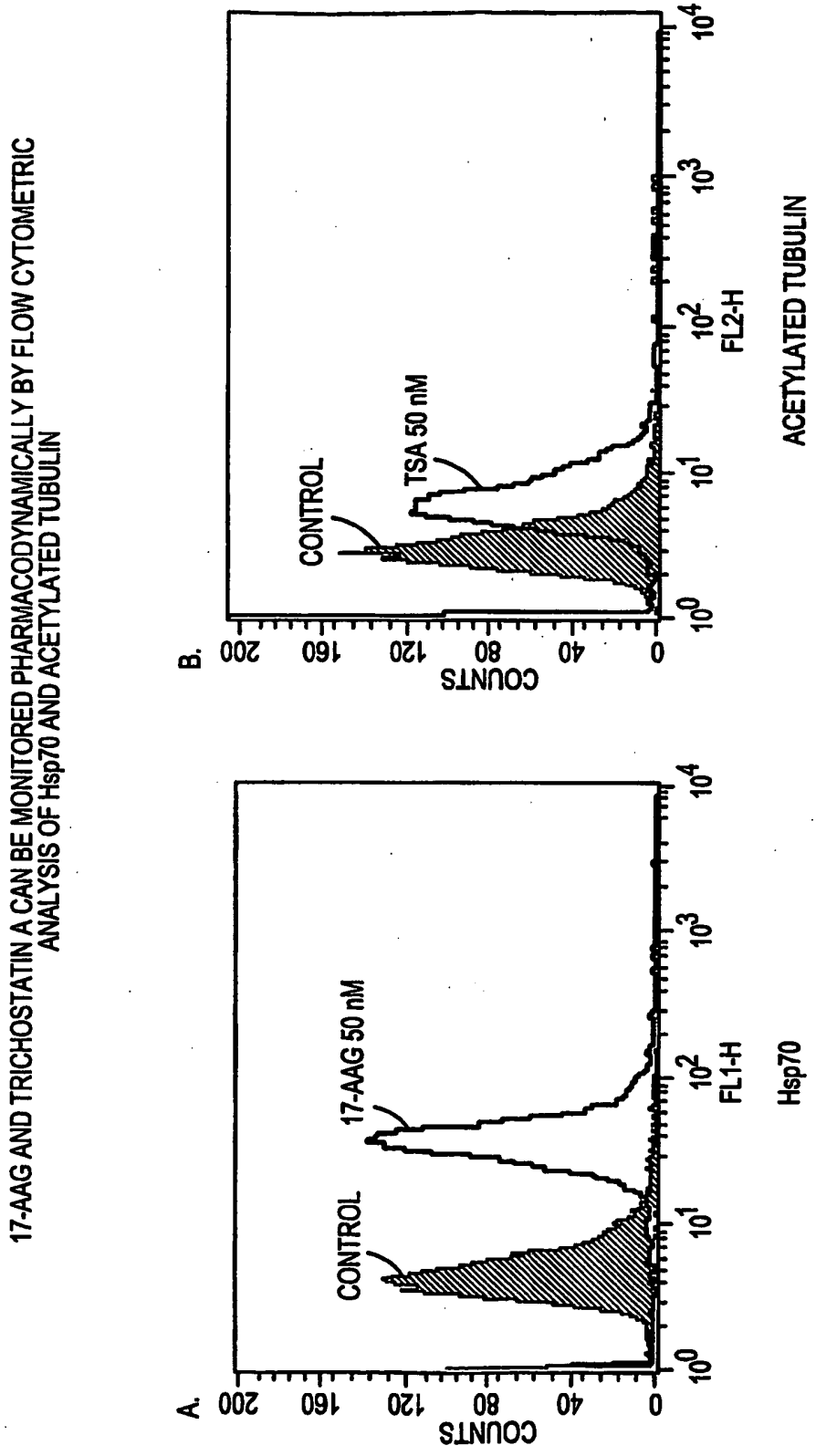

17-AAG AND TRICHOSTATIN A CAN BE MONITORED PHARMACODYNAMICALLY BY FLOW CYTOMETRIC ANALYSIS OF Hsp70 AND ACETYLATED TUBULIN
A.
COUNTS
CONTROL
17-AAG 50 nM
FL1-H
Hsp70
B.
COUNTS
CONTROL
TSA 50 nM
FL2-H
ACETYLATED TUBULIN
TREAT WITH ONE DRUG AND MEASURE THE RESPONSE, I.E. TREAT WITH 17-AAG AND MEASURE HSP70; TREAT WITH TRICHOSTATIN A (TSA) AND MEASURE ACETYLATED TUBULIN

FIG. 5

DUAL PHARMACODYNAMIC RESPONSE OBSERVATION AND MONITORING OF TWO DRUGS (17-AAG AND TSA)

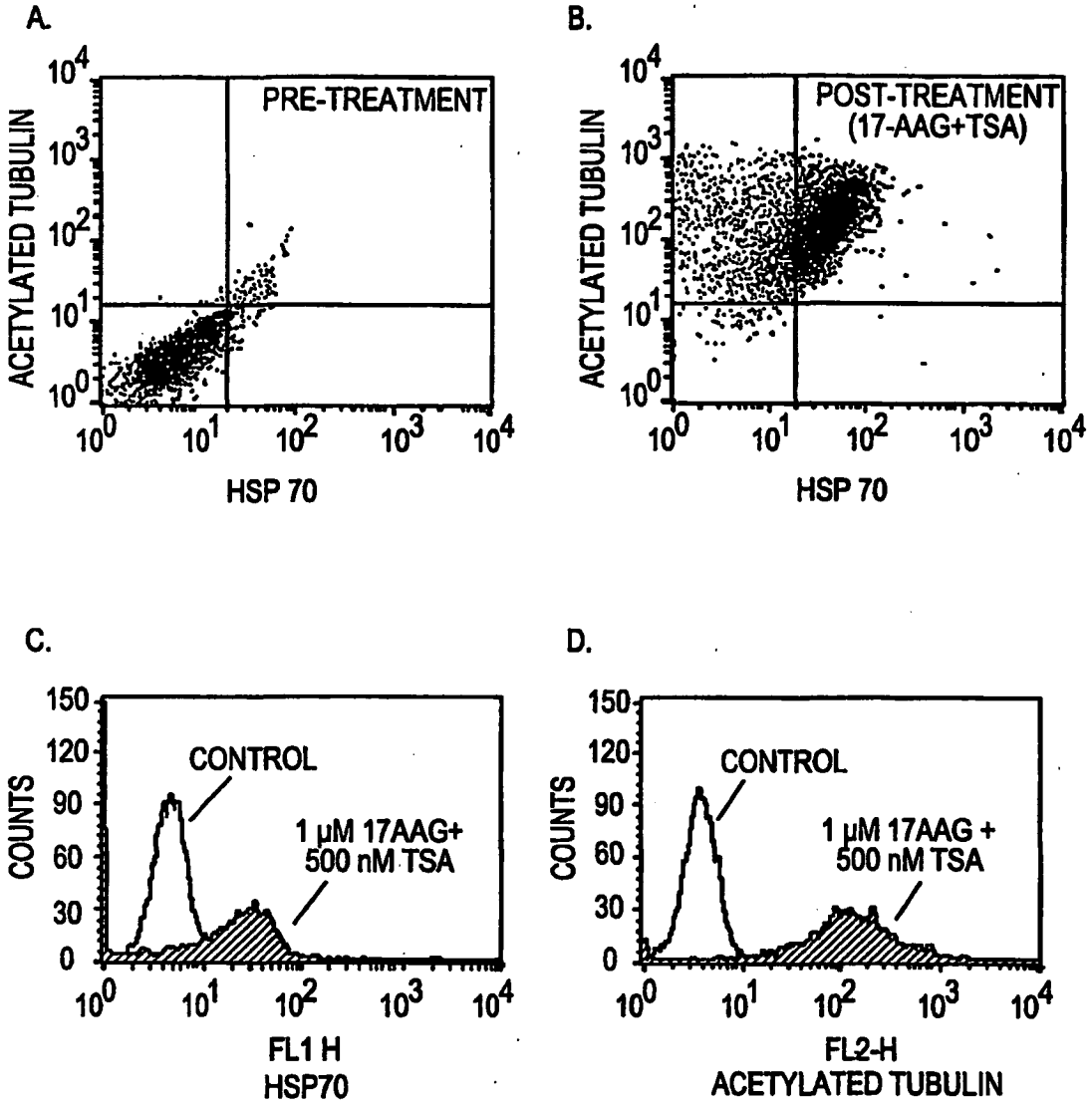


FIG.6

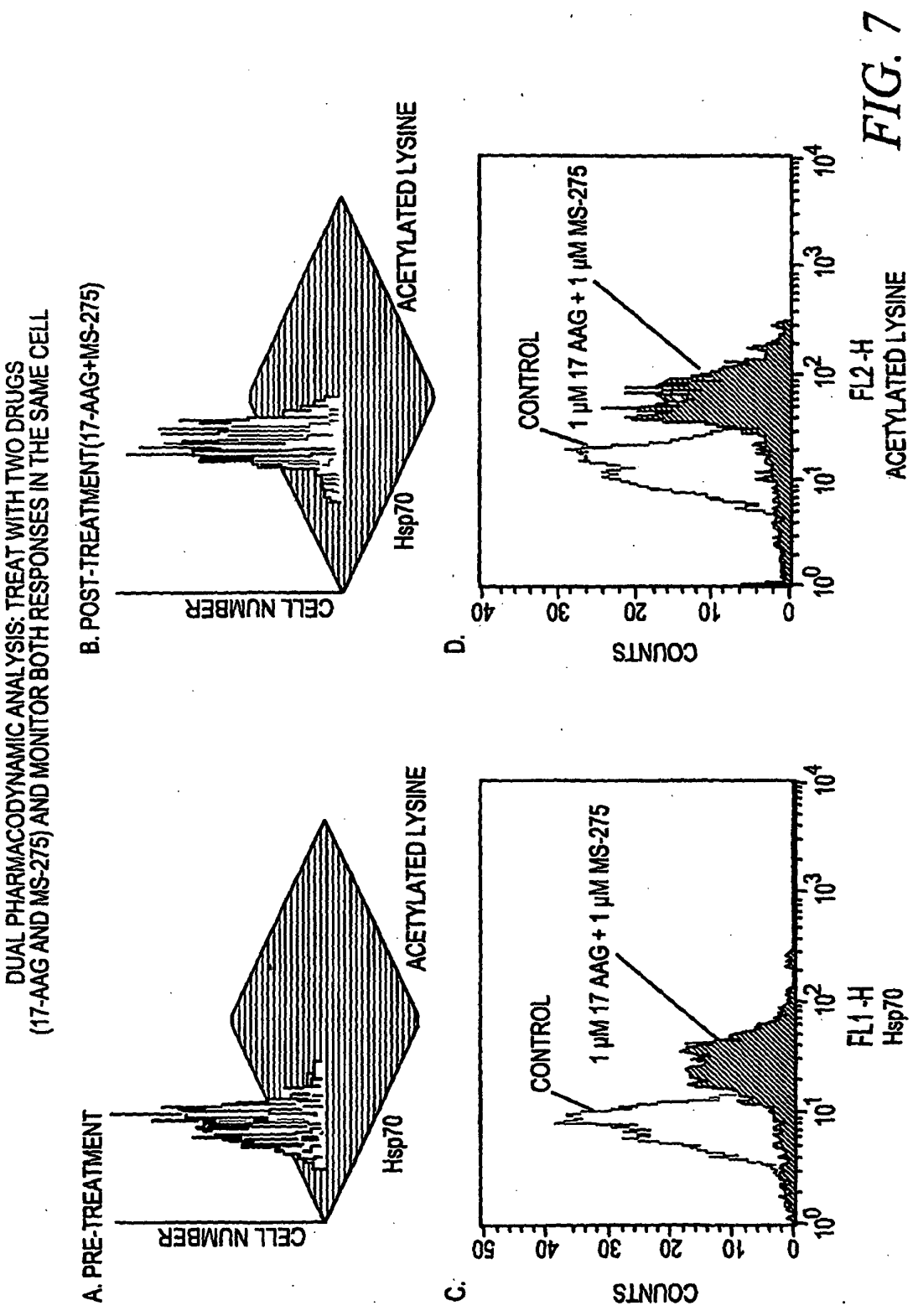

DUAL PHARMACODYNAMIC ANALYSIS: TREAT WITH TWO DRUGS
(17-AAG AND MS-275) AND MONITOR BOTH RESPONSES IN THE SAME CELL
A. PRE-TREATMENT
B. POST-TREATMENT(17-AAG+MS-275)
CELL NUMBER
Hsp70
ACETYLATED LYSINE
C.
D.
COUNTS
CONTROL
1 μM 17 AAG + 1 μM MS-275
FL1-H
Hsp70
FL2-H
ACETYLATED LYSINE

FIG. 7

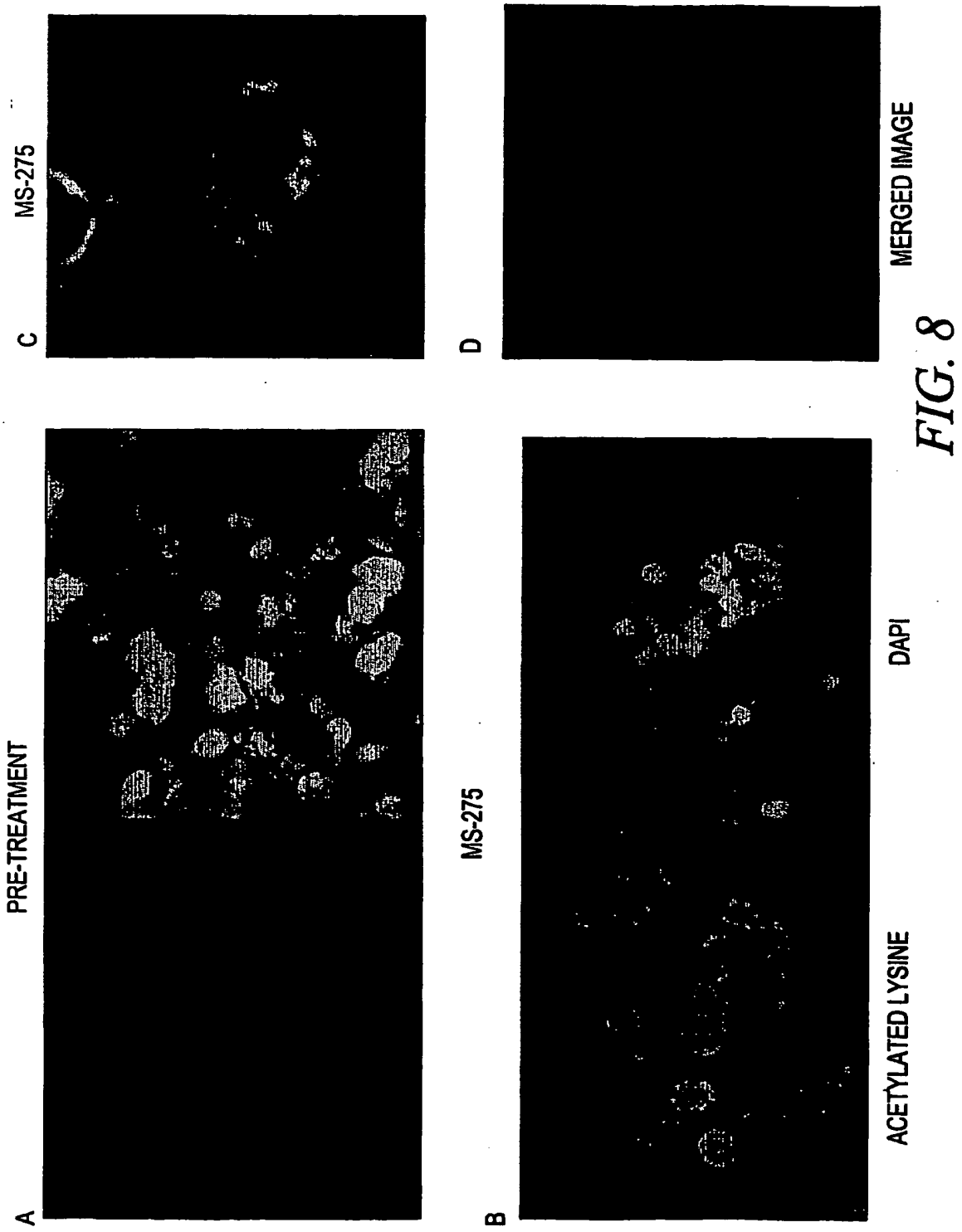

A
PRE-TREATMENT
B
MS-275
ACETYLATED LYSINE
C
MS-275
DAPI
D
MERGED IMAGE

FIG. 8

FLOW ANALYSIS OF APOPTOSIS VERSUS PROTEIN ACETYLATION IN K562 CELLS TREATED WITH MS-275 AND IMATINIB

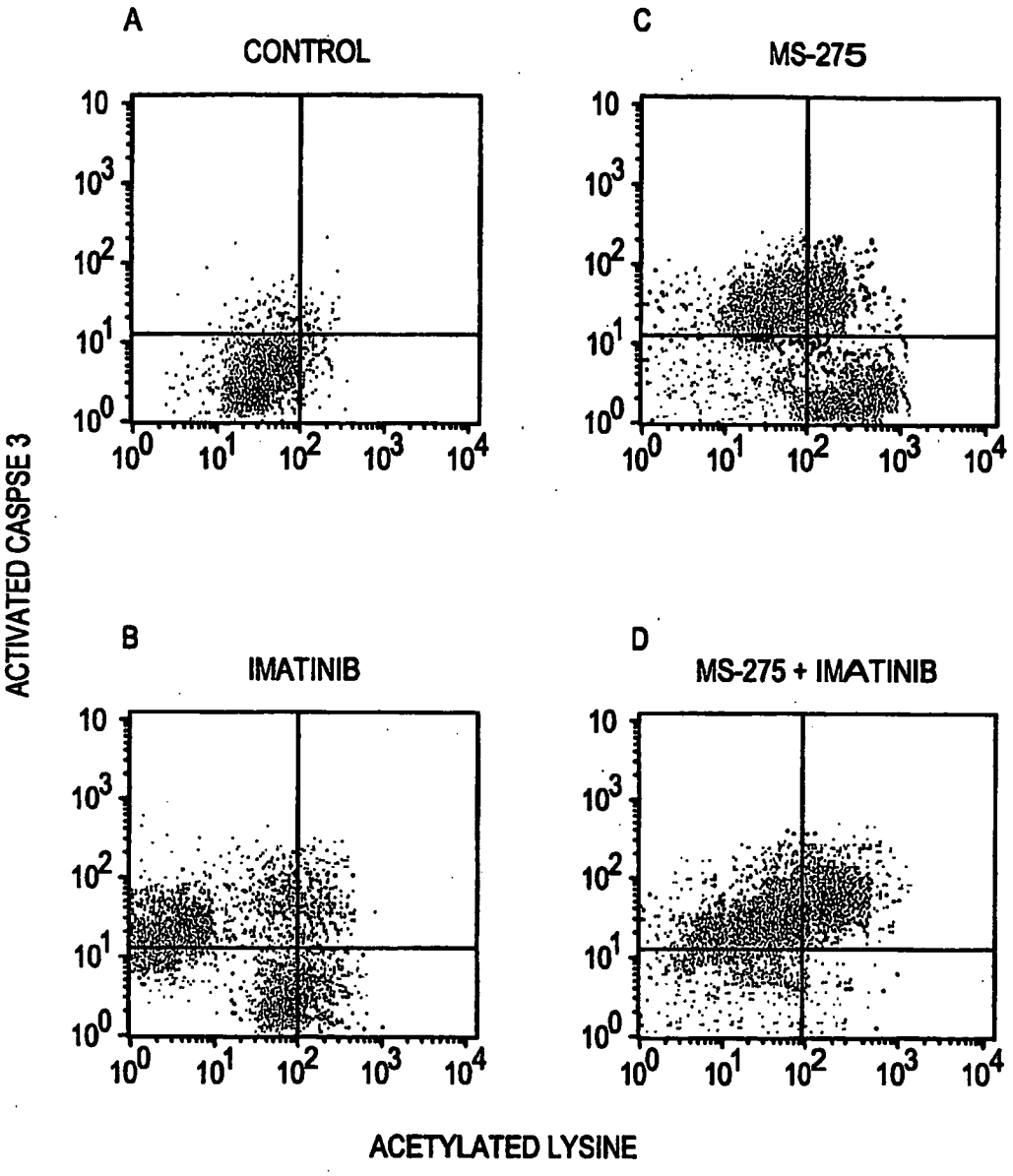


FIG. 9

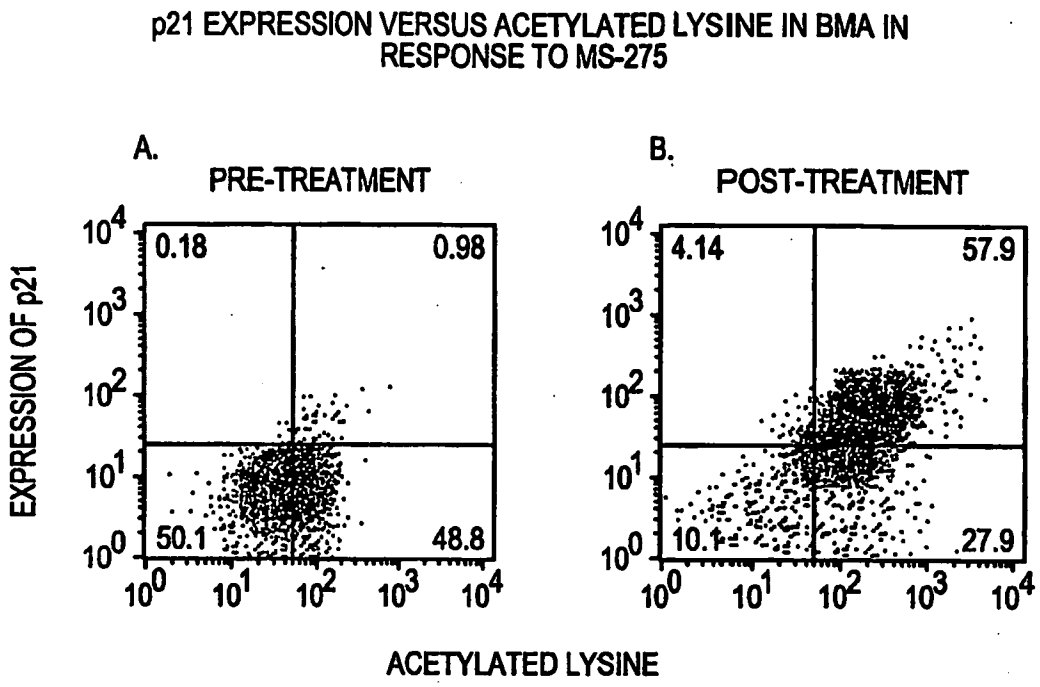

p21 EXPRESSION VERSUS ACETYLATED LYSINE IN BMA IN RESPONSE TO MS-275
A.
PRE-TREATMENT
B.
POST-TREATMENT
EXPRESSION OF p21
ACETYLATED LYSINE
0.18
0.98
50.1
48.8
4.14
57.9
10.1
27.9
10^0
10^1
10^2
10^3
10^4

FIG. 10

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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Non-patent literature cited in the description

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专利名称(译)	使用流式细胞术进行药效学测定。		
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申请(专利权)人(译)	美利坚合众国政府作为代表的健康和人类服务部部长		
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外部链接	Espacenet		

摘要(译)

本发明提供了快速和容易地筛选混合细胞样品以获得对药物或测试剂的药效学效果的方法。

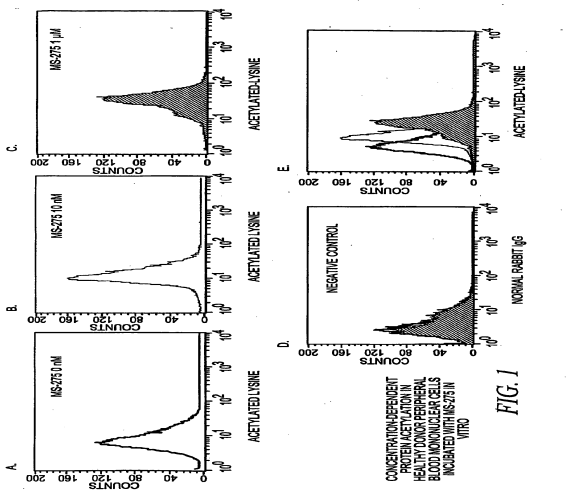

FIG. 1