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(54) Title: AFFINITY DISPLACEMENT MOLECULAR SEPARATION

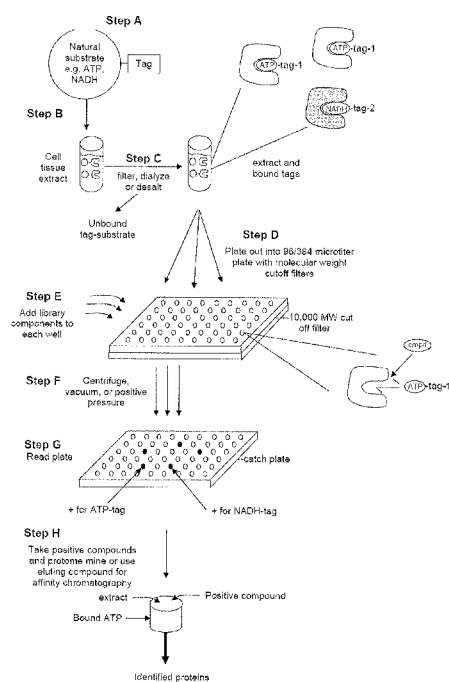


FIG. 1

(57) Abstract: Provided herein are methods for identifying molecules that compete with a ligand for binding to a protein. The method comprises contacting a ligand with a proteome to allow protein members of the proteome to bind with the ligand, followed by the removal of the unbound ligand. At least one test molecule is added to the mixture and after passing the mixture through an ultrafiltration membrane, the filtrate is assayed for the displaced ligand. The presence of the ligand within the filtrate indicates the molecule competes with the ligand for binding to at least one protein member within the proteome.



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AFFINITY DISPLACEMENT MOLECULAR SEPARATION

FIELD OF THE INVENTION

The present invention relates to the field of drug discovery. Specifically, the present invention provides for the identification of molecules that can be further developed as possible therapeutics.

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BACKGROUND OF THE INVENTION

Traditional drug discovery methods begin with a basic research effort to understand the etiology of the disease for which a pharmaceutical treatment is desired and to identify useful drug targets, the majority of which are proteins. A single target protein is then screened against a large library of small molecules to identify molecules that specifically inhibit or activate the protein. Once a series of molecules are identified, the selectivity of the molecule can be determined by counter-screening each molecule against isoforms of the target protein. Compounds passing this test will be chemically iterated upon to improve potency and selectivity, followed by cell-based or other biological assays. Screening molecules against a single protein target has the disadvantage of not providing information concerning the selectivity or potency of the molecule in the context of the entire proteome of a cell or organism. Thus, traditional drug discovery efforts generate multiple molecules that exhibit non-selective behavior, contributing to side effects and toxicities when tested *in vivo*.

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BRIEF SUMMARY OF THE INVENTION

Methods for identifying molecules that compete with a ligand for binding to a protein are provided. The method comprises contacting a ligand with a proteome to allow protein members of the proteome to bind with the ligand. Following removal of unbound ligand, at least one test molecule is added to the mixture to allow the test molecule to displace the ligand bound to the protein member. After passing the mixture through an ultrafiltration membrane, the filtrate is assayed for the displaced ligand. The presence of the ligand within the filtrate indicates the test molecule competes with the ligand for

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binding to at least one protein member within the proteome. Since the ligand interacts with the proteins through a functional binding site, molecules that displace the ligand represent potential therapeutics. The binding profile of the molecule for proteins within a proteome generated through measuring the ability of the test molecule to displace the ligand at increasing concentrations can provide information regarding the selectivity of the molecule. Moreover, the proteins that interact with the test molecule(s) can be identified and analyzed to determine possible sources of side effects or toxicity of the molecule.

To facilitate measurement of the ligand that is displaced by a particular molecule, the ligand may be tagged. In these embodiments, the tag is attached to the ligand such that binding of the ligand to its target protein(s) is not disrupted.

In some embodiments, the assay is performed in a high throughput manner by carrying out the screen in multiwell titer plates equipped with ultrafiltration membranes. In this manner, the ligand bound to protein(s) from a proteome of interest is added to a titer plate and at least one test molecule is added to each well of the multiwell plate, followed by filtration of the molecule/ligand/protein mixture and measurement of the displaced ligand within the filtrate. Thus, the method allows for selective screening of chemical libraries for potential therapeutic candidates.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts the steps of a non-limiting embodiment of the methods described herein. In this example, two ligands, ATP and NADH are each labeled with a unique tag prior to mixing the ligands with a cellular extract comprising a proteome. The extract is then filtered, dialyzed, or desalted to remove unbound ligand. The ligand/protein mixture is aliquoted into a 96-well or 384-well microtiter plate with an ultrafiltration membrane having a molecular weight cut-off of about 10,000 Daltons. At least one molecule from a chemical library is added to each well of the microtiter plate. The plate is centrifuged or a vacuum or positive pressure is applied to the filter to promote filtration of the molecule/ligand/protein mixture. The filtrate collected in the collection plate (catch plate) is analyzed for the presence of each of the tagged ligands. Target proteins of test molecules capable of competing with the ligand can then be identified using proteome mining or other affinity chromatography techniques.

Figures 2A-2C show representative binding curves indicative of an interaction between a test compound and a single protein target in a dose-dependent manner, exhibiting a classic sigmoidal binding curve (Fig. 2A); an interaction between a test

compound and more than one protein, showing a complex binding curve (Fig. 2B); and a non-selective compound (Fig. 2C), exhibiting a linear binding curve. The graphs display the fluorescence intensity, which corresponds to the displaced fluorophore-tagged ligand on the y-axis and the concentration of the compound added to the affinity displacement molecular separation (ADMS) assay presented on the X-axis on a logarithmic scale.

Figure 3 shows a graph depicting results from an ADMS assay, wherein increasing concentrations of geldanamycin (GA) compete with fluorescein-labeled ATP (labeled via the gamma phosphate) for binding to proteins within an ultra-filtered mouse muscle extract.

Figure 4 shows a graph depicting results from an ADMS assay, wherein increasing concentrations of staurosporine (stauro) compete with fluorescein-labeled ATP (labeled via the gamma phosphate) for binding to proteins within an ultra-filtered mouse muscle extract.

Figure 5 shows a graph depicting results from an ADMS assay, wherein increasing concentrations of chloroquine (CQ) compete with fluorescein-labeled primaquine (labeled via its amino side chain) for binding to proteins within a clarified human red blood cell extract.

DETAILED DESCRIPTION OF THE INVENTION

Methods for identifying molecules that compete with ligands for binding to at least one protein member of a proteome are provided. The method comprises mixing a ligand with a proteome to allow the ligand to bind to at least one protein member of the proteome, followed by the removal of any unbound ligand. At least one test molecule is added to the mixture and the mixture is passed through an ultrafiltration membrane. The filtrate is assayed for the ligand and the presence of displaced ligand within the filtrate indicates that at least one of the test molecules can compete with the ligand for binding to at least one protein member of the proteome. The methods can further comprise generating a binding curve to characterize the selectivity of the molecule and in some embodiments, the subsequent identification of the molecule's target proteins, providing further information about the selectivity of the molecule and potential side effects or toxicities.

The presently disclosed methods are referred to herein as "affinity displacement molecular separation" (ADMS), as the methods identify molecules having an affinity for the same proteomic targets as a ligand of interest through molecular separation and

detection of the competitively displaced ligand. ADMS provides an improvement in drug discovery and development, providing a rapid means to obtain highly enriched small molecule libraries that can be used as multiple lead compounds for drug discovery programs. The invention is based on the idea that most drugs are likely to be derived from small molecule inhibitors or activators of a protein that compete for a binding pocket (e.g. active site, allosteric binding site or ligand binding site) that is normally occupied by a natural substrate (e.g. ATP), cofactor (e.g. NADH, co-enzyme A), or agent such as a neurotransmitter, hormone or peptide. In contrast to traditional drug discovery efforts that screen single protein targets for potential drugs, ADMS allows for the screening of molecules in a rapid and efficient manner against many hundreds of enzymes and proteins simultaneously. In doing so, all of the molecules within a chemical library that are likely to compete with a given ligand are identified. Thus, the methods allow for the identification of novel compound-target associations within a chemical library that may not be predictable from individual chemical structures within the library. The methods can be used to identify molecules that show selectivity in binding to a single or small number of proteins as well as molecules that bind to multiple target proteins. Further, the methods can be multiplexed, simultaneously assaying for the ability of a given molecule or group of molecules to compete with more than one ligand. This process greatly increases the probability of identifying molecules within a large chemical library that will have value in drug development programs.

The methods of the invention comprise five general steps. The first step involves contacting a ligand with a proteome to produce a ligand/protein mixture and to allow the ligand to bind to at least one protein member of the proteome. The term "proteome," as used herein, refers to a complex protein mixture obtained from a biological sample. As used herein, the phrase "complex protein mixture" refers to a mixture of proteins having at least about 10, at least about 20, more usually at least about 50, and in some embodiments, about 100 or more distinct proteins. In some embodiments, the proteome comprises at least about 5% of the total repertoire of proteins present in a biological sample (e.g., the cells, tissue, organ, or organism from which a lysate is obtained; the serum or plasma, etc.), at least about 10%, at least about 25%, at least about 75%, at least about 90% or more, up to and including the entire repertoire of proteins obtainable from the biological sample.

As used herein, the phrase "biological sample" refers to a sample obtained from or comprising a cell, tissue, organ, or organism. Non-limiting examples of biological samples include cellular organelles, cells (e.g., mammalian cells, bacterial cells, cultured cells), a biological fluid, such as blood, plasma, serum, urine, bile, saliva, tears, cerebrospinal fluid, aqueous or vitreous humor, or any bodily secretion), a transudate or exudate (e.g. fluid obtained from an abscess or other site of infection or inflammation), a fluid obtained from a joint (e.g. a normal joint or a joint affected by disease such as rheumatoid arthritis, osteoarthritis, gout or septic arthritis), or the like, and a lysate or extract of an organelle, cell, tissue, organ, or organism.

Biological samples may be obtained from any organ or tissue (normal or diseased, including a biopsy or autopsy specimen) or may comprise cells or a lysate or extract thereof (including primary cells, passaged or cultured primary cells, cell lines, cells conditioned by a specific medium or grown under a particular set of environmental conditions) or medium conditioned by cells. In some embodiments, the proteome may be obtained from a biological sample (e.g., cell, tissue, organism or extract thereof) after exposure to a hormone or other biological or pharmacological agent. If desired, the biological sample may be subjected to processing, such as lysis, extraction, subcellular fractionation, or other standard biochemical procedures known in the art to solubilize the protein members of the proteome. See, Deutscher (ed.) (1990) *Methods in Enzymology* 182:147-238, which is herein incorporated by reference in its entirety. For example, tissues or cells can be ground and homogenized in buffers appropriate for solubilizing proteins and retaining their native conformations, followed by clarification by centrifugation. Other methods known in the art can be used to process the biological sample to obtain the proteome, including but not limited to osmotic lysis, detergent lysis, sonication, heat, and rapid decompression. In general, methods used to obtain the proteome are performed under non-denaturing conditions, allowing the majority of proteins to retain their native conformations.

Proteins comprising a proteome are referred to herein as protein members of the proteome. While the proteome may be a purified protein mixture, in some embodiments, the proteome will comprise other biological molecules (e.g., nucleic acids, lipids) commonly found in biological samples and extracts thereof. In some embodiments, the proteome comprises whole cells, providing for the identification of compounds that might compete with ligands for binding to cell surface proteins.

The proteome of any biological sample may be used for the methods of the invention, including the proteome obtained from a mammal, human, animal, vertebrate or invertebrate, insect, fungi, plant, prokaryote, protozoan, or subcellular organism, such as a virus or a prion. In some embodiments, the biological sample is obtained from a cell,
5 tissue, organ, or organism that has been genetically modified. For example, the biological sample can be obtained from a cell that has been genetically engineered through molecular biology techniques known in the art to express recombinant polynucleotides or protein(s).

The selection of the proteome to use in the presently disclosed methods will depend upon the problem being addressed or the chemical molecule being pursued. For
10 example, when searching for a molecule useful for the treatment of cancer, a proteome from a cancerous tissue or transformed cell line can be used to screen molecules. As another non-limiting example, if the identification of a herbicide is desired, a plant proteome can be used for the ADMS assay.

In particular embodiments, the proteome of interest can be further processed prior
15 to mixing the proteome with the ligand. For example, certain components of the proteome can be removed prior to contacting the proteome with the ligand, such as by fractionating the proteome by molecular weight, electric charge, and/or hydrophobicity. Alternatively, specific protein members can be removed by immunoprecipitation or affinity chromatography. Such methods can allow for the removal or reduction of proteins that are
20 expressed at high levels in the biological sample used to generate the proteome or for the removal of proteins that are not therapeutically relevant and bind the ligand with a relatively high affinity.

The amount of starting material from which the proteome is obtained is critical, especially in those embodiments wherein the target proteins of a test molecule are
25 identified, and should be based on the expression level of the type of proteins known to interact with the ligand of interest. For example, if the ligand against which test molecules are screened interacts with signal transduction molecules, the expression level (copy number) of these proteins within the biological sample should be taken into account. In general, low copy number proteins (expressed at low levels) require a larger amount of
30 starting material (biological sample), whereas higher copy number proteins require less starting material for obtaining the proteome. Potential losses of protein due to proteolysis or inefficiency of extraction should also be considered.

The proteome is mixed with a ligand to allow the ligand to reversibly bind to at least one protein member of the proteome. As used herein, the term "ligand" refers to any

bioactive molecule capable of reversibly binding a protein. The specific identity of one or all of the target proteins of the ligand may or may not be known. A ligand may or may not be naturally occurring and can be purified from a biological sample or synthetically derived. Non-limiting examples of ligands include substrates, cofactors, hormones, coenzymes, inhibitors, and allosteric activators. The ligand may be a peptide, protein, carbohydrate, lipid, glycoprotein, nucleic acid, or other type of small molecule. Non-naturally occurring ligands may include, for example, drugs or small molecules known to inhibit or activate a protein or class of proteins.

The ligand reversibly binds to the protein through a protein binding site. The specific binding sites through which the ligand interacts with the protein may or may not be known. In some embodiments, the activity of the protein is modulated (e.g., positively or negatively) by binding of the ligand. The activity that can be modulated by the ligand can be any activity associated with the protein, including for example, an enzymatic activity or an interaction with a biological molecule (e.g., protein, nucleic acid, hormone). For example, the ligand can function as an allosteric activator, as a substrate of an enzyme, or as a binding partner necessary for the normal function of the protein or can inhibit any of these activities.

In some embodiments, the ligand is a pharmacological agent or drug. For example, the ligand may be an antifungal, antibacterial, antiviral, or chemotherapeutic agent, or an insecticide or herbicide. The presently disclosed methods allow for the screening of molecules that can compete with the drug for binding to target proteins. In this manner, molecules that might serve as suitable substitutes for known drugs, potentially with more desirable pharmacokinetic or toxicity profiles can be identified.

In some embodiments, the ligand comprises a purine. A purine is a heterocyclic aromatic organic compound comprising a pyrimidine ring fused to an imidazole ring. The term purine encompasses substituted purines and their tautomers. Non-limiting examples of purines include adenine, adenosine, deoxyadenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), deoxyadenosine monophosphate (dAMP), deoxyadenosine diphosphate (dADP), deoxyadenosine triphosphate (dATP), cyclic adenosine monophosphate (cAMP), cyclic adenosine diphosphate ribose (c-ADPR), guanine, guanosine, deoxyguanosine, guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), deoxyguanosine monophosphate (dGMP), deoxyguanosine diphosphate (dGDP), deoxyguanosine triphosphate (dGTP), cyclic guanosine monophosphate (cGMP), (3',5')-

cyclic dimeric guanosine monophosphate (c-di-GMP), nicotinamide adenine dinucleotide (NAD/NADH), nicotinamide adenine dinucleotide phosphate (NADP/NADPH), flavin adenine dinucleotide (FAD/FADH), co-enzyme A, hypoxanthine, caffeine, xanthine, theobromine, uric acid, and isoguanine. In particular embodiments, the ligand comprises ATP.

The purine-binding proteome includes important therapeutic proteins, such as kinases, metabolic enzymes, DNA and RNA binding proteins, dehydrogenases, heat shock proteins, transferases, carboxylases, helicases, formylases, reductases, synthetases, and other proteins critical for normal cellular function. These proteins are associated with disease states such as cancer, diabetes, inflammatory conditions, autoimmune conditions, hypertension, and viral, bacterial and parasitic infections. Thus, molecules identified using the presently disclosed methods that are able to competitively displace purines potentially have wide-ranging therapeutic applications.

According to the presently disclosed methods, the ligand and proteome are contacted to produce a ligand/protein mixture and to allow binding of the ligand to at least one protein member of the proteome. The ligand may be contacted with the proteome through placing the ligand in a solution with the proteome of interest. In some embodiments, the ligand/protein mixture can be allowed to equilibrate in solution or the solution can be mixed through physical means (e.g., turning, rocking, swirling, shaking, vortexing) to facilitate binding of the ligand to at least one protein member of the proteome. The solution may comprise an aqueous solution, including but not limited to a buffered solution. A non-limiting example of a buffered solution useful in the presently disclosed methods and commonly used in the art is phosphate-buffered solution (PBS). In general, the solution will comprise the solution that was used to solubilize or extract the proteome from the biological sample from which it derives. The ligand can first be solubilized in an aqueous solution or an organic solvent (e.g., dimethyl sulfoxide) prior to contacting the ligand with the proteome. The presently disclosed ADMS assay allows for interactions between ligands/test molecules and proteins within a proteome to occur free in solution, as opposed to methods known in the art wherein either the proteins or ligand/test molecules are immobilized on a column or other solid support. Thus, the methods of the invention maximizes the display of surfaces involved in protein/molecule interactions facilitating the discovery of a wide array of molecules capable of competing with a given ligand.

As used herein, the term “mixture” refers to a composition comprising two or more chemically distinct substances that have been physically combined. The substances within a mixture may or may not be chemically bound to one another. A mixture can refer to a solution, such as an aqueous solution or a solution comprising an organic solvent (e.g., dimethyl sulfoxide). In some embodiments, the concentration of the ligand within the ligand/protein solution is between about 10 nmol/ml to about 1 μ mol/ml or higher, including but not limited to about 10 nmol/ml, 20 nmol/ml, 30 nmol/ml, 40 nmol/ml, 50 nmol/ml, 60 nmol/ml, 70 nmol/ml, 80 nmol/ml, 90 nmol/ml, 100 nmol/ml, 200 nmol/ml, 300 nmol/ml, 400 nmol/ml, 500 nmol/ml, 600 nmol/ml, 700 nmol/ml, 800 nmol/ml, 900 nmol/ml, and 1 μ mol/ml.

The second step of the method of the invention comprises removing unbound ligand not specifically bound to protein members of the proteome from the ligand/protein mixture. Unbound ligand can be separated from ligand that is bound to at least one protein member of the proteome using any method known in the art, including gel filtration, dialysis, ultrafiltration, tangential flow methods or precipitation under non-denaturing conditions (e.g., with ammonium sulfate). In some embodiments, the removal of the unbound ligand comprises passing the ligand/protein mixture through an ultrafiltration membrane.

As used herein, the term “ultrafiltration membrane” refers to a semi-permeable membrane comprising pores having a size of about 0.001 micron to about 0.1 micron, including but not limited to about 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, 0.007, 0.008, 0.009, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1 micron. Ultrafiltration membranes can be used to selectively fractionate components of a solution on the basis of size. The term “filtrate” refers to the portion of the solution that has passed through the membrane, whereas the term “retentate” refers to the portion that is unable to pass through the membrane and is retained due to size. Ultrafiltration membranes are known in the art and can be made of any material that is able to selectively fractionate molecules, including but not limited to polysulfone, polyethersulfone, cellulose acetate, cellulose diacetate, and cellulose triacetate. For the presently disclosed methods, ultrafiltration membranes that are composed of materials that do not exhibit an affinity for proteins (e.g., low protein-binding) are especially useful.

An ultrafiltration membrane can be classified based on its molecular weight cut-off, that is, its ability to retain at least 90 percent (including, but not limited to, about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, and higher) of molecules that are

equal to or greater than a specified molecular weight. For example, an ultrafiltration membrane having a molecular weight cut-off of about 5,000 Da is able to retain (i.e., is not permeable to) at least 90% of all molecules having a molecular weight of about 5,000 Da or above. In some embodiments, the presently disclosed methods make use of
5 ultrafiltration membranes having a molecular weight cut-off of at least 500 Daltons, including but not limited to, about 1,000 Da, 2,000 Da, 3,000 Da, 4,000 Da, 5,000 Da, 10,000 Da, 20,000 Da, 30,000 Da, 40,000 Da, 50,000 Da, 60,000 Da, 70,000 Da, 80,000 Da, 90,000 Da, 100,000 Da, or greater. In some embodiments, the ultrafiltration membranes useful in the methods of the invention have a molecular weight cut-off of
10 about 5,000 Da. In other embodiments, the ultrafiltration membranes have a molecular weight cut-off of about 10,000 Da.

While filtration through the membrane can occur via passive diffusion, filtration can be facilitated through the application of positive pressure to the side of the membrane containing the solution that is to be filtered. For example, positive hydrostatic pressure
15 can be applied through the addition of a larger volume of the solution onto the membrane. Alternatively, negative pressure (e.g., a vacuum) may be applied to the opposite side of the membrane to draw the solution through the membrane. Centrifugal force can also be used to promote filtration of the solution. In these embodiments, centrifugal filter tubes, such as those commercially available from Amicon (Millipore Corp., Billerica, Massachusetts),
20 can be used. In addition, multiwell microtiter plates that are commonly used to filter fluids using either vacuum or centrifugal force (for example, Millipore Multiscreen® filter plate with Ultracel®-PPB membrane, Millipore Corp.; Pall AcroPrep™ ultrafiltration filter plate, Pall Corp., East Hills, New York) can be used in the methods of the invention. Centrifuges adapted with rotors and carriers for the multiwell microtiter plates are very
25 common laboratory equipment.

Ultrafiltration membranes can also be in the form of a bag for filtration of solutions via dialysis. Dialysis involves the immersion of a bag comprising an ultrafiltration membrane surrounding a solution to be filtered within a bath solution. Filtration of the solution within the dialysis bag occurs through passive diffusion of molecules below the
30 molecular weight cut-off of the ultrafiltration membrane into the bath solution. Dialysis generally requires relatively large volumes of the bath solution.

According to the presently disclosed methods, an ultrafiltration membrane with a molecular weight cut-off that is higher than the molecular weight of the free (i.e., unbound) ligand and lower or equal to the molecular weight of the ligand/target protein

complex should be chosen. When removing the ligand from the ligand/protein mixture by passing the mixture through an ultrafiltration membrane, the unbound ligand will be found in the filtrate and the ligand/protein mixture will be retained in the retentate. The retentate comprising the ligand/protein mixture is collected for further processing.

5 In those embodiments wherein the unbound ligand is separated from ligand bound to at least one protein member of a proteome by ultrafiltration, the process can comprise diafiltration, wherein additional solution is added to the membrane or retentate following the filtration of the ligand/protein mixture or molecule/ligand/protein mixture. The additional solution can comprise a buffered solution (e.g., PBS) or other non-denaturing
10 solution. A series of these washing steps with additional solution effectively results in buffer exchange and promotes complete or essentially complete removal of the unbound ligand from the ligand/protein mixture.

 According to the presently disclosed methods, following the removal of the unbound ligand, at least one test molecule is added to the ligand-protein mixture to
15 determine if the molecule is able to competitively displace the ligand. As used herein, a “molecule” refers to any chemical molecule or compound, including but not limited to, oligonucleotides, peptides, proteins, carbohydrates, lipids, glycoproteins, and other small molecules. The term “test molecule” refers to the molecule that is being assayed using the presently disclosed methods to determine if the test molecule is capable of competitively
20 displacing ligand(s) of interest. Test molecules that are determined to be capable of competing with a ligand for a binding site on a protein member of a proteome using the methods of the invention can be referred to as a competing molecule. The test molecule can be naturally-occurring or non-naturally occurring and can be purified from a biological sample or synthetically derived. The structure of the test molecule may be
25 known or unknown. The test molecule need not have a known biological activity or a known ability to interact with proteins. The molecule may be solubilized in a solution, such as an aqueous solution or an organic solvent (e.g., dimethyl sulfoxide) prior to addition of the molecule to the ligand/protein mixture. Considering the methods of the invention are useful for identifying molecules that compete with a given ligand for binding
30 to at least one protein member of a proteome, prior to the assay, the test molecule is not known to compete with the ligand for binding to proteins found within the particular proteome chosen for the assay.

In some embodiments, the test molecule is a member of a chemical library. A chemical library refers to a plurality of molecules. The components of the chemical library can be well-defined, containing known mixtures of molecules. For example, each molecule of a well-defined chemical library can be catalogued. Alternatively, the components of the library can be poorly defined, as is often the case with combinatorial libraries. Likewise, the structures of the molecules within the chemical library can be known or unknown.

In particular embodiments, the test molecule is a member of a combinatorial chemical library. A combinatorial chemical library is a plurality of molecules or compounds which are formed by selectively combining a particular set of chemical building blocks. Combinatorial libraries can be constructed according to methods familiar to those skilled in the art. For example, see Rapoport *et al.*, (1995) *Immunology Today* 16:43-49; Sepetov, N. F. *et al.*, (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92:5426-5430; Gallop, M. A. *et al.*, (1994) *J. Med. Chem.* 9:1233-1251; Gordon, E. M. *et al.*, (1994) *J. Med. Chem.* 37:1385-1401; Stankova, M. *et al.*, (1994) *Peptide Res.* 7:292-298; Erb, E. *et al.*, (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:11422-11426; DeWitt, S. H. *et al.*, (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909-6913; Barbas, C. F. *et al.*, (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:4457-4461; Brenner, S. *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:5381-5383; Lam, K. S. *et al.*, (1991) *Nature* 354:82-84; Devlin, J. J. *et al.*, (1990) *Science* 245:404-406; Cwirla, S. E. *et al.*, (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:6378-6382; Scott, J. K. *et al.*, (1990) *Science* 249:386-390, and U.S. Pat. No. 5,463,564, each of which is herein incorporated by reference in its entirety.

In some embodiments, the chemical library is biologically synthesized and is constructed using molecular biology techniques. These library components can be expressed using bacteria or viruses. For example, U.S. Pat. Nos. 5,270,170 and 5,338,665 to Schatz (each of which are herein incorporated by reference in its entirety) describe the construction of a recombinant plasmid encoding a fusion protein created through the use of random oligonucleotides inserted into a cloning site of the plasmid. Similarly, bacteriophage display libraries have been constructed through cloning random oligonucleotides into a portion of a gene encoding one or more of the phage coat or pili proteins. Such phage expression libraries are described in, for example, Sawyer *et al.* (1991) *Protein Engineering* 4:947-953, which is herein incorporated by reference in its entirety.

Another approach to generating molecularly diverse combinatorial libraries has been the use of large numbers of very small derivatized beads, which are divided into as many equal portions as there are different building blocks. In the first step of the synthesis, each of these portions is reacted with a different building block. The beads are then thoroughly mixed and again divided into the same number of equal portions. In the second step of the synthesis, each portion, now theoretically containing equal amounts of each building block linked to a bead, is reacted with a different building block. The beads are again mixed and separated, and the process is repeated as desired to yield a large number of different molecules, with each bead containing only one type of molecule. This methodology, termed the "one-bead, one-compound" method, yields a mixture of beads with each bead potentially bearing a different compound. The compounds displayed on the surface of each bead can be tested for the ability to compete for binding with a ligand to protein members of a proteome.

To determine if a given test molecule can compete with a ligand for a binding site on at least one protein of a proteome, the molecule/ligand/protein mixture is passed through an ultrafiltration membrane to produce a filtrate and a retentate, and the filtrate is assayed for the displaced ligand.

In some embodiments, a plurality of molecules of a chemical library or an entire chemical library is added to the ligand/protein mixture to assay multiple test molecules simultaneously for the ability to displace at least one ligand. The chemical library can be fractionated and the steps of the method of the invention repeated with the fractions, with each fraction representing a certain percentage of the molecules within the complete chemical library. Fractions that comprise molecule(s) able to displace the ligand of interest can then be further fractionated (sub-fractionated) and assayed or single molecules from the positive fractions can then be assayed to identify the molecule(s) within the library or fraction that are capable of competing with the ligand(s) of interest. An entire chemical library can also be screened by assaying each compound individually without performing screens of fractions of the library. Molecules of a chemical library able to compete with a ligand of interest, wherein the structures of the molecules are unknown can then be further characterized and the structure of the molecule determined using methods known in the art.

In other embodiments wherein a chemical library or a fraction thereof has been determined using the methods of the invention to comprise at least one molecule capable of competing with ligand(s) of interest, the competing molecule can be isolated and

identified using methods known in the art. For example, the target protein(s) of the test molecule can be identified using methods known in the art, including those described elsewhere herein (e.g., proteome mining). The target protein(s) can then be produced recombinantly and immobilized on a solid support. The library or fraction thereof
5 comprising the competing test molecule can then be contacted with the immobilized target protein(s), followed by a series of washing steps (e.g., with low and/or high ionic buffers) to remove those molecules within the library or fraction thereof binding non-specifically to the immobilized target protein(s). The captured test molecules can then be non-specifically dissociated from the immobilized target protein(s) and the structure and
10 identity of the compound(s) determined using methods such as mass spectrometry.

Following the addition of the test molecule(s) to the protein/ligand mixture, the resulting molecule/ligand/protein mixture is passed through an ultrafiltration membrane. In accordance with the presently disclosed methods, the ultrafiltration membrane is permeable to the free, unbound ligand, but retains the ligand bound to its protein binding
15 partner. That is, the membrane has a molecular weight cut-off that is higher than the molecular weight of the ligand of interest and lower than or equal to the molecular weight of the ligand bound to a protein member. In this step of the method, the filtrate is collected and assayed for the ligand. The presence of the ligand within the filtrate indicates that the test molecule was able to compete with the ligand for a binding site on at
20 least one protein member of the proteome.

By “assay” is intended the use of any method known in the art to detect the ligand, including, but not limited to, immunoblotting, polymerase chain reaction (PCR, in those embodiments wherein the ligand of interest comprises a nucleic acid), or mass spectrometry.

25 In some embodiments, the ligand is attached to a tag to facilitate detection and quantitation of the ligand. As used herein, the terms “tag” or “label” refers to any chemical moiety or molecule that enables detection of the ligand to which the tag is attached. The tag may be attached to the ligand via adsorption, electrostatic interactions, or conjugation through a covalent bond. Non-limiting examples of tags include a
30 fluorophore, radiolabel, non-radiolabeled isotopes (e.g., molecular weight isotopic tags that would allow discrimination in a mass spectrometer), chromophores, dyes, nanoparticles, and quantum dots. In some embodiments, the tag comprises a chemical moiety or molecule that is soluble in an aqueous or organic solvent. Thus, in some

embodiments, the term “tag” does not refer to solvent-insoluble molecules. According to the methods of the invention, the tag must be attached to the ligand such that binding of the ligand to at least one protein partner is not inhibited.

A variety of fluorophores can be used as tags including, for example, fluorescein and fluorescein derivatives, rhodamine and rhodamine derivatives, naphthylamine and naphthylamine derivatives, benzamidizoles, ethidiums, propidiums, anthracyclines, mithramycins, acridines, actinomycins, merocyanines, coumarins, pyrenes, chrysenes, stilbenes, anthracenes, naphthalenes, salicylic acids, benz-2-oxa-1-diazoles (also called benzofurazans), fluorescamines and bodipy dyes. Specific, non-limiting examples of fluorophores that can be used as tags include thioinosine, and N-ethenoadenosine, formycin, dansyl derivatives, 6-propionyl-2-dimethylamino)-naphthalene (PRODAN), 2-anilinonaphthalene, and N-arylamine-naphthalene sulfonate derivatives such as 1-anilinonaphthalene-8-sulfonate (1,8-ANS), 2-anilinonaphthalene-6-sulfonate (2,6-ANS), 2-aminonaphthalene-6-sulfonate, N,N-dimethyl-2-aminonaphthalene-6-sulfonate, N-phenyl-2-aminonaphthalene, N-cyclohexyl-2-aminonaphthalene-6-sulfonate, N-phenyl-2-aminonaphthalene-6-sulfonate, N-phenyl-N-methyl-2-aminonaphthalene-6-sulfonate, N-(o-toluidyl)-2-aminonaphthalene-6-sulfonate, N-(m-toluidyl)-2-aminonaphthalene-6-sulfonate, N-(p-toluidyl)-2-aminonaphthalene-6-sulfonate, 2-(p-toluidinyl)-naphthalene-6-sulfonic acid (2,6-TNS), 4-(dicyanovinyl) julolidine (DCVJ), 6-dodecanoyl-2-dimethylaminonaphthalene (LAURDAN), 6-hexadecanoyl-2-(((2-trimethylammonium-ethyl)methyl)amino)naphthalenechloride (PATMAN), Nile red, N-phenyl-1-naphthylamine, 1,1-dicyano-2-[6-(dimethylamino) naphthalen-2-yl]propene (DDNP), 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS), and DAPOXYL™ derivatives. A variety of appropriate fluorescent dyes are commercially available from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes, Inc. (Eugene, OR). In certain embodiments, the ligand is conjugated to fluorescein or a derivative thereof. In those embodiments wherein the ligand is tagged with a fluorophore, the fluorophore is detected through the illumination of the filtrate with a light source having a wavelength capable of exciting the particular fluorophore. The light that is emitted from the excited fluorophore is then measured with a fluorimeter.

Other molecules that can be used as tags to enable detection of a ligand of interest attached thereto include quantum dots (Gao *et al.* (2002) *J. Biomed. Opt.* 7:532-537, which is herein incorporated by reference in its entirety). Quantum dots are constructed of a semiconductor core material and are highly photostable. Quantum dots are known in the

art and commercially available (see, for example Qdot® from Invitrogen, Carlsbad, CA) and can be covalently coupled to ligands using methods known in the art (see, for example, Chan and Nie (1998) *Science* 281:2016-2018, which is herein incorporated by reference).

5 Radiolabels can serve as tags for the detection of ligands of interest. Suitable radiolabels are well known in the field of biochemistry and include, but are not limited to, P^{32} , S^{35} , and I^{125} . The filtrate can be analyzed for the presence of a radiolabel with a Geiger counter, scintillation counter, or autoradiography.

10 Non-radiolabeled stable isotopes can be used as tags and detected using mass spectrometry or nuclear magnetic resonance (NMR). Stable isotopes commonly used for this purpose include deuterium, carbon-13, nitrogen-15, and oxygen-18.

In those embodiments wherein the ligand comprises adenosine triphosphate (ATP), the tag can be conjugated to ATP via the gamma phosphate, as described in U.S. Patent No. 5,536,822, which is herein incorporated by reference in its entirety. As demonstrated
15 within U.S. Patent No. 5,536,822, gamma phosphate-conjugated ATP retains the ability to bind protein kinases, a major group of therapeutically relevant ATP-binding proteins. In some embodiments, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) coupling is used to activate the gamma phosphate of ATP to prepare it for attachment to a tag (see, for example Hermanson (1996) *Bioconjugate Techniques*. Academic Press, San Diego, for
20 further discussion of EDC-mediated modifications, which is herein incorporated by reference in its entirety). Other methods known in the art can be used to attach a tag to ATP. These include, but are not limited to, coupling through the N6 amino group on the purine ring or hydroxyl groups of the ribose moiety (Trayer *et al.* (1974) *Biochem. J.* 139:609-623; Jeno and Thomas (1991) *Methods Enzymology* 200:178-187, each of which
25 are herein incorporated by reference in its entirety).

The presence of the ligand within the filtrate indicates the test molecule can compete with the ligand for binding to at least one protein within the proteome. As used herein, the term “presence” can be a relative term, particularly considering trace amounts of the ligand may be present within the filtrate even in the absence of a competing test
30 molecule. Thus, a ligand may be considered “present” in the filtrate if the level of ligand in the filtrate exceeds background levels, such as those found in the absence of a competing test molecule.

In addition to detecting the presence of a ligand in the filtrate, the relative amount of ligand in a filtrate sample can be quantitated using the presently described assays and

tags. Generation of and comparison to a standard curve of known concentrations of the ligand can be used to determine the amount of ligand that has been displaced, which can be compared to the amount of ligand that was added to the assay (input) or the amount that is bound to the proteins of the proteome (bound ligand). The amount of ligand that is bound to the proteins of the proteome can be determined by measuring the amount of unbound ligand that was removed from the ligand/protein mixture and subtracting this amount from the input.

The presently disclosed methods can be multiplexed, wherein more than one ligand can be contacted with a proteome and molecules can be simultaneously screened for the ability to compete with more than one ligand for binding to protein members of the proteome. In some of these embodiments, each ligand is attached to a unique tag, wherein the presence of each displaced ligand in a filtrate can be assayed and thus, the ability of a molecule to compete with each ligand can be determined by assaying for the particular tag attached to each ligand. For example, an assay can be performed wherein a first ligand (e.g., ATP) is conjugated to a first fluorophore (e.g., fluorescein) and a second ligand (e.g., NAD) is conjugated to a second fluorophore (e.g., rhodamine). One can separately detect the first and second ligands through the detection of the first and second fluorophores, respectively, provided the fluorophores do not exhibit overlapping excitation and/or emission spectra. Thus, a single assay or series of assays can provide information concerning the binding of a test molecule or group of molecules to various types of proteins and the type of proteins with which the test molecule interacts without having to sequence or directly identify any of the interacting proteins. In the example provided above, ATP-binding proteins span a wide range of protein classes, including kinases, dehydrogenases, heat shock proteins, RNA and DNA binding proteins, sulfotransferases, carboxylases, helicases, and formylases. The ability of a test molecule to release both ATP and NAD, which can be determined in a single assay using the presently disclosed methods, would suggest the molecule is binding a dehydrogenase, as opposed to the other classes of ATP-binding proteins.

In an alternative embodiment of the invention, the assay can essentially be reversed, wherein a test molecule (which may or may not comprise a tag) can be contacted by a proteome, followed by the removal of unbound test molecule. A ligand of interest with a known bioactivity can then be added to the test molecule/protein mixture. The ligand/molecule/protein mixture can then be passed through an ultrafiltration membrane and the filtrate can be assayed for the displaced test molecule to determine if the ligand

can compete with the test molecule for binding to at least one protein member of the proteome. In those embodiments wherein the test molecule comprises a tag, the tag may be attached to the test molecule at multiple sites to minimize the disruption of interactions between the test molecule and proteins of the proteome. Multiple test molecules can be simultaneously tested in this manner. In some of the embodiments wherein multiple test molecules are assayed, each test molecule can comprise an identical tag or a unique tag.

A binding curve can provide information concerning the number of target proteins within a given proteome with which a particular test molecule competes for binding with a ligand. The binding curve can be generated from the measurement of the amount of ligand that is displaced by a given test molecule over a range of molecule concentrations. The binding curve can be displayed on a graph, wherein the y-axis displays the quantity of the displaced ligand, which in some embodiments, is measured via the detectable tag attached to the ligand, and the x-axis presents the concentration of the test molecule that was added to the ligand/protein mixture. The x-axis is often displayed on a logarithmic scale. In general, a binding curve can have any one of three types of characteristics. A molecule that is selective for one protein (or group of proteins) will exhibit a binding curve over a range of concentrations that has a sigmoidal shape, such as the curve presented in Figure 2A. Alternatively, if the test molecule interacts with more than one target protein (or groups of proteins) within a proteome, the binding curve will be complex, displaying multiple sigmoidal binding curves (see, for example Figure 2B) or a highly disordered or linear binding curve (see, for example, Figure 2C). A highly disordered or linear binding curve is indicative of a test molecule that is able to bind to multiple proteins and can indicate the molecule is non-selective. Thus, the binding curve generated from assays performed over a range of concentrations can provide information regarding the selectivity of the test molecule for proteins within the proteome of interest. Additionally, if the binding curve is non-complex, one can extrapolate the relative affinity of the test molecule for a target protein within the proteome. The relative affinity can be expressed as the EC_{50} or the concentration of the test molecule sufficient to produce 50% of the maximal effect (e.g., displacement of the ligand). Therefore, the presently disclosed ADMS assay allows for the rapid determination of the selectivity and affinity of a particular test molecule for proteins within a proteome without having to analyze the number or identity of the interacting proteins through protein sequencing techniques or mass spectrometry.

The methods of the invention can be performed within any type of assay vessel or chamber (e.g., test tubes, microtubes, vials, microtiter plates, etc.). In certain embodiments, the ligand is contacted with the proteome in a first container and the ligand/protein mixture is then aliquoted into wells of a multiwell microtiter plate, following removal of unbound ligand. A chemical library or a fraction thereof or a unique test molecule can be added to each well of the microtiter plate to generate the molecule/ligand/protein mixture. In some of these embodiments, the multiwell microtiter plates comprise an ultrafiltration membrane on the bottom of each well and a separate multiwell collection plate. The microtiter plate comprising the filters and separate collection plate are collectively referred to herein as the plate apparatus. Multiwell microtiter plates comprising an ultrafiltration membrane are known in the art and available commercially (for example, Millipore Multiscreen® filter plate with Ultracel®-PPB membrane, Millipore Corp., Billerica, Massachusetts; or the Pall AcroPrep™ ultrafiltration filter plate, Pall Corp., East Hills, New York). Centrifugation of the plate apparatus or the application of positive pressure to the top of the plate or a vacuum to the bottom of the plate allows the molecule/ligand/protein mixture to pass through the ultrafiltration membrane. The filtrate that is collected in the wells of the collection plate can then be assayed for the displaced ligand. In this manner, the methods of the invention are amenable to highthroughput screening of large chemical libraries of molecules. The plate apparatus can comprise any number of wells, including but not limited to about 6, 12, 24, 96, 384, 864, 1536, 3456, and 9600 wells.

The methods of the invention can be performed at any temperature that is amenable to retaining protein structure and ligand-protein interactions. In some embodiments, the assays are performed at about 2°C to about 56°C, including, but not limited to about 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, and 56°C.

Molecules that are able to compete with a given ligand within a proteome of interest, including an enriched pool of molecules from a given chemical library, can be further screened for therapeutic utility using various types of assays, the nature of which will depend on the type of molecule that is desired. These include comparative screens in cell-based or other biological assays (e.g. screening compounds for those that selectively

inhibit the growth of transformed cells). A therapeutically relevant molecule discovered using the presently disclosed methods may mimic the activity of the ligand or may inhibit the activity of a naturally-occurring ligand in these therapeutic assays. The respective protein targets of those molecules with the highest activity in these secondary screens can then be identified. Protein targets of molecules identified in the ADMS assays can also be characterized directly, bypassing these additional therapeutic or cell-based assays.

Protein targets of competing molecules identified in the ADMS screens can be isolated and characterized using any method known in the art, including, but not limited to, proteome mining as described by Haystead (2002) *Mol Pharmacol* 62:1364-1372 and International Application Publication No. WO00/63694, each of which are herein incorporated by reference in its entirety, or other affinity chromatography methods. In proteome mining, the ligand that competes with the test molecule for binding to at least one protein is immobilized to a solid support. The solid support may be a well of a microtiter plate, the interior wall of a tube or vessel, or particles or beads comprised of an acrylamide derivative, agarose, cellulose, nylon, silica, polystyrene divinylbenzene, methacrylate, polymethacrylate, or a magnetic material. The ligand must be immobilized in such a way as to allow the ligand to retain the ability to bind to its protein targets. In those embodiments wherein the ligand is tagged for detection in the ADMS assay, the same reaction chemistry or chemical moiety that was utilized for tagging the ligand can be used for immobilization of the ligand for proteome mining. In some embodiments, a plurality of the ligand is simultaneously immobilized through different chemical moieties to enhance the probability that a protein reaction surface will be available. While the ligand may be attached to the solid support via adsorption or electrostatic interactions, in most embodiments, ligands are immobilized to solid supports through covalent attachments. In these embodiments, the solid support can be functionalized using methods known in the art to promote covalent attachment of the ligand to the solid support. The ligand can be attached to the solid support via amine, carboxylic acid, thiol, hydroxyl, aldehyde, or phosphate linkages, for example.

The immobilized ligand is contacted with the proteome comprising at least one protein member able to bind the test molecule and ligand to allow binding of the protein(s) to the immobilized ligand. The immobilized ligand is washed with a buffered solution to remove any non-specifically associated proteins. In some embodiments, the buffered solution is a high ionic solution, while in other embodiments, the buffered solution comprises a low ionic solution. In certain embodiments, the immobilized ligand is washed

with both a low ionic buffered solution and a high ionic buffered solution. In some embodiments, the buffered solution comprises PBS. The test molecule able to compete with the ligand for binding to at least one protein member of the proteome is added to release proteins bound to the immobilized ligand. The released proteins are collected,
5 characterized, and identified using any method known to one of skill in the art, including, but not limited to, SDS-PAGE (e.g., two-dimensional), mass spectrometry (e.g., MALDI-TOF mass spectrometry, liquid chromatography electrospray ionization tandem mass spectrometry, ICAT mass spectrometry), immunoblotting, and protein sequencing (for example, as described in Damer *et al.* (1998) *J. Biol. Chem.* 273:24396-24405; Alms *et al.*, (1999) *EMBO J.* 18:4157-4168, each of which are herein incorporated by reference in
10 their entirety). The sequences of the proteins can be compared to existing protein databases, such as Entrez Protein, which encompasses data from SwissProt, Entrez, PIR, PRF, PDB, GenBank and RefSeq translations, and is available through the NCBI web portal (ncbi.nih.gov).

15 In those embodiments wherein the solid support to which the ligand is attached is a bead (e.g., sepharose, agarose), the beads can be combined to form a column. After the proteome is applied to the column, the proteins can be allowed to percolate throughout the column based on gravity or additional force can be applied to speed the flow of the proteins through the column; for example, the column can be spun in a centrifuge to
20 enhance flow through the column. After the column has been washed to remove non-specifically associated proteins, the competing molecule(s) can be added to the immobilized ligand/protein mixture on the column to elute proteins. In other embodiments, the beads can be removed and placed in equal amounts into wells of a multiwell microtiter plate (e.g., 96-well, 384-well plate) for specific elution by a test
25 molecule. Maintaining the beads in a column for the elution step has the advantage of potentially recovering more protein per test molecule; however aliquoting the bead suspension into wells of a microtiter plate provides for a more highthroughput analysis of multiple test molecules. Application of the beads and subsequent addition of the test molecules to the multiwell plates can be automated using commercially available robotics.

30 In those embodiments wherein the beads are aliquoted into a microtiter plate prior to elution, an additional step is required to isolate the eluted proteins. Following incubation with the test molecule, the beads are allowed to settle or the bead suspension is centrifuged briefly (e.g., 300 x g) to pellet the beads. In accordance with one embodiment

of the invention, the beads can be magnetized beads, and application of a magnetic field to the bottom of the plate is used to pellet the beads. The supernatant is collected for further analysis.

5 Gel electrophoresis (e.g., SDS-PAGE) can measure the purity of the protein eluate and quantitate the amount of recovered proteins. The electrophoretically separated proteins can be visualized with silver, Coomassie blue, or colloidal gold (for sequencing by mass spectrometry) or transferred to polyvinyl membrane (for mixed peptide sequencing).

10 For mixed peptide sequencing, the proteins on the polyvinyl membrane (PVM) are stained and then excised (see Damer *et al.* (1998) *J. Biol. Chem.* 273:24396-24405 and Alms *et al.* (1999) *EMBO J.* 18:4157-4168, each of which are herein incorporated by reference in their entirety). The membrane pieces are digested briefly with CnBr, washed and placed directly into an automated Edman sequenator. Mass spectrometry can also be used for sequencing analysis. In the case of mixed peptide sequencing, between 6 and 12
15 rounds of Edman sequencing is carried out and the mixed peptide sequences that are generated are sorted out and matched against databases with the FASTF (protein databases) and TFASTF algorithms (DNA data bases). The biological significance of the identified protein is then assessed.

20 Alternatively, protein members that bind to a particular test molecule can be identified through more conventional affinity approaches, wherein the test molecule of interest is immobilized to a solid support (e.g., sepharose bead) and contacted with the proteome of interest. The test molecule can be immobilized through multiple chemical moieties to enhance the probability that the protein interaction site will be displayed and available for binding. Following a washing step with a buffered solution to remove non-
25 specifically associated proteins, the bound proteins can be dissociated from the test molecule non-specifically (e.g., through the use of a chaotropic agent, including but not limited to detergents, such as SDS, Triton X, sarkosyl, denaturants such as urea or chelators, such as EGTA or EDTA) or specifically eluted from the immobilized test
30 molecule prior to analysis, through the addition of the ligand that shares protein binding sites with the test molecule. The eluted proteins can then be identified using methods known in the art, including but not limited to, SDS-PAGE, mass spectrometry, and protein sequencing.

Data from this analysis can be used to create lists of molecule-target associations. The list of molecule-target associations can be used to make decisions on how to proceed with drug discovery efforts. For example, the list can be sorted into molecules that target proteins that are considered valuable or are validated drug targets that can be used to treat human disease. In this instance, one begins a drug discovery effort with a molecule that not only targets a protein of interest, but one also has an idea of its selectivity profile within the entire cellular milieu of a tissue, organ, organism, etc. In instances wherein a molecule interacts with several proteins as determined by proteome mining or other conventional affinity methods, iterative chemistry can be used to reduce the affinity of the molecule for undesirable targets.

It is to be noted that the term “a” or “an” entity refers to one or more of that entity; for example, “a protein” is understood to represent one or more proteins. As such, the terms “a” (or “an”), “one or more,” and “at least one” can be used interchangeably herein.

Throughout this specification and the embodiments, the words “comprise,” “comprises,” and “comprising” are used in a non-exclusive sense, except where the context requires otherwise.

As used herein, the term “about,” when referring to a value is meant to encompass variations of, in some embodiments $\pm 50\%$, in some embodiments $\pm 40\%$, in some embodiments $\pm 30\%$, in some embodiments $\pm 20\%$, in some embodiments $\pm 10\%$, in some embodiments $\pm 5\%$, in some embodiments $\pm 1\%$, in some embodiments $\pm 0.5\%$, and in some embodiments $\pm 0.1\%$ from the specified amount, as such variations are appropriate to perform the disclosed methods or employ the disclosed compositions.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1. Selective and Non-Selective Competitive Inhibitors of a Naturally-Occurring Ligand

ATP linked to fluorescein via the gamma phosphate [100 μ M] was added to 100 μ l aliquots of clarified (100,000 x g 30 min), ultra-filtered (22 μ m) mouse muscle extract in 4 ml Amicon centrifugal filter tubes (5,000 Da cut off). After incubation for 5 minutes, the tubes were centrifuged at 4000 rpm (in an Eppendorf 5801R) for 15 minutes to reduce the volume to ~10 μ l. Phosphate buffered saline (1 ml) was added and the tubes recentrifuged for 30 minutes, reducing the volume back to 100 μ l. The washing step was repeated a second time. A small amount of fluorescence was detected in the low molecular weight flowthrough and subsequently used as background (non-specific) or a baseline signal. The indicated increasing concentrations of geldanamycin (GA) were added to each tube (Figure 3). After 5 minutes, the extracts were recentrifuged for 15 minutes. The low molecular weight eluates (less than 5,000 Da in size) were collected and measured for fluorescence in a fluorimeter. Figure 3 shows a classical sigmoidal dose response curve consistent with competitive binding between geldanamycin and ATP with the known target of this drug, HSP90. A dissociation constant (K_d) was determined based upon the concentration of free ATP derivative (100 μ M) in the extract and determined to be approximately 6nM. This value is close to the reported values for the affinity of geldanamycin for HSP90. Figure 3 contrasts dramatically with Figure 4 in which the experiment was repeated with the non-specific inhibitor staurosporin. Staurosporin is widely recognized as a broadly-acting inhibitor of purine-utilizing enzymes. As shown in Figure 4, rather than producing a sigmoidal curve consistent with a drug competing with a single protein target (such as geldanamycin and HSP90), staurosporin produces a general non-specific increase in fluorescence across the dose response range that is non-saturatable up to the highest concentration tested (10 μ M). These results are consistent with a non-specific inhibitor competing with multiple ATP-binding proteins simultaneously.

Example 2. Selective Competitive Inhibitors of a Drug

Primaquine was cross-linked to fluorescein via its amino side chain. Previously, it was shown that primaquine can be linked via its side chain to sepharose beads while retaining its ability to selectively bind two of its targets, quinone reductase 2 (QR2) and aldehyde dehydrogenase 1 (ALDH) (Graves *et al.* (2002) *Mol Pharmacol.* 62(6):1364-72,

herein incorporated by reference in its entirety). The primaquine-fluorescein (PQ-FL) derivative was introduced to a clarified human red blood cell extract at 100 μ M in a 4 ml Amicon centrifugal filter tube. The extract was then subjected to rounds of washing to remove any unbound probe as described for the ATP derivative in Example 1. The extract
5 was restored to 100 μ l by the addition of PBS and the indicated concentrations of chloroquine were added to the extract. Following centrifugation, the low molecular eluates were collected and measured for fluorescence. Figure 5 shows that chloroquine (CQ) selectively competes with PQ-FL. These results are consistent with previous data showing that chloroquine and primaquine are competitive inhibitors of QR2 and ALDH
10 (Graves *et al.* (2002) *Mol Pharmacol.* 62(6):1364-72).

THAT WHICH IS CLAIMED:

1. A method for identifying a molecule that competes with a ligand for a binding site on a protein member of a proteome, said method comprising the steps of:

5 a) contacting a ligand with a proteome to produce a ligand/protein mixture and to allow at least one protein member of said proteome to bind with said ligand;

b) removing unbound ligand from said ligand/protein mixture;

10 c) adding at least one test molecule to said ligand/protein mixture to produce a molecule/ligand/protein mixture;

d) passing said molecule/ligand/protein mixture through an ultrafiltration membrane to generate a filtrate and a retentate; and

15 e) assaying said filtrate for said ligand, wherein the presence of said ligand within said filtrate indicates said at least one test molecule is a molecule that competes with said ligand for a binding site on at least one protein member of said proteome.

2. The method of claim 1 wherein said ligand is selected from the group consisting of a natural substrate, a cofactor, a neurotransmitter, a hormone, a peptide, a drug, an inhibitor, or an allosteric activator.

3. The method of claim 1, wherein said ligand comprises a purine.

4. The method of claim 3, wherein said purine comprises ATP or NADH.

25 5. The method of claim 3, wherein said protein member comprises a purine-binding protein.

6. The method of any one of claims 1-5, wherein the proteome is a human proteome.

30 7. The method of any one of claims 1-6, wherein said ligand comprises a tag such that binding of the ligand to at least one protein partner is not inhibited.

8. The method of claim 7, wherein said ligand is covalently attached to said tag.

9. The method of claim 7 or claim 8, wherein said tag is selected from the group consisting of a fluorophore, a radiolabel, a nonradiolabeled isotope, a chromophore, a dye, a nanoparticle, and a quantum dot.

10. The method of claim 9, wherein said fluorophore comprises fluorescein or a derivative thereof.

11. The method of claim 1, wherein said removing unbound ligand from said ligand/protein mixture comprises desalting, precipitating, dialyzing, or filtering through an ultrafiltration membrane.

12. The method of claim 1, wherein passing said molecule/ligand/protein mixture through an ultrafiltration membrane comprises application of positive hydrostatic pressure or vacuum.

13. The method of claim 1, wherein passing said molecule/ligand/protein mixture through an ultrafiltration membrane comprises centrifuging.

14. The method of claim 1, wherein said ultrafiltration membrane has a molecular weight cut-off of at least about 5,000 Da.

15. The method of claim 1, wherein said ultrafiltration membrane has a molecular weight cut-off of at least about 10,000 Da.

16. The method of any one of claims 7-10, wherein said assaying for said ligand comprises assaying for the presence of said tag.

17. The method of claim 1, wherein said ligand comprises a plurality of ligands, and wherein said filtrate is assayed for each ligand.

18. The method of any one of claims 1-17, wherein said at least one test molecule comprises a chemical library or a portion thereof.

19. The method of any one of claims 1-18, wherein said steps (c)-(e) occur in a multiwell microtiter plate.

20. The method of claim 19, wherein each well of said multiwell microtiter plate comprises a unique test molecule.

21. The method of any one of claims 1-20, further comprising the step of repeating steps a) through e) with a range of concentrations of said test molecule and generating a binding curve for said test molecule to determine the selectivity of said test molecule for protein members of said proteome.

22. The method of claim 21, further comprising the step of analyzing said binding curve to determine the affinity of said test molecule for said at least one protein member, wherein said binding curve is not complex.

23. The method of any one of claims 1-22, wherein said method further comprises identifying proteins that bind to said molecule.

24. The method of claim 23, wherein identifying proteins that bind to said molecule comprises the steps of:

- a) immobilizing said ligand on a solid support;
- b) contacting said immobilized ligand with said proteome to allow binding of protein members of said proteome to said immobilized ligand;
- c) washing said immobilized ligand with a buffered solution;
- d) adding said molecule identified in claim 1 that competes with said ligand for a binding site on a protein member of said proteome to release bound proteins;
- e) identifying said released proteins.

25. The method of claim 23, wherein identifying proteins that bind to said molecule comprises the steps of:

- a) immobilizing said molecule identified in claim 1 that competes with ligand for a binding site on a protein member of said proteome on a solid support;
- 5 b) contacting said immobilized molecule with said proteome to allow binding of protein members of said proteome to said immobilized molecule;
- c) releasing bound proteins; and
- d) identifying said released proteins.

10 26. The method of claim 25, wherein said releasing bound proteins comprises adding said ligand that competes with the molecule for a binding site on a protein member of said proteome.

15 27. The method of any one of claims 24-26, wherein said identifying said released proteins comprises performing mass spectrometry or protein sequencing.

28. The method of any one of claims 24-26, wherein said solid support comprises an acrylamide derivative, sepharose, agarose, cellulose, nylon, silica, or magnetized particles.

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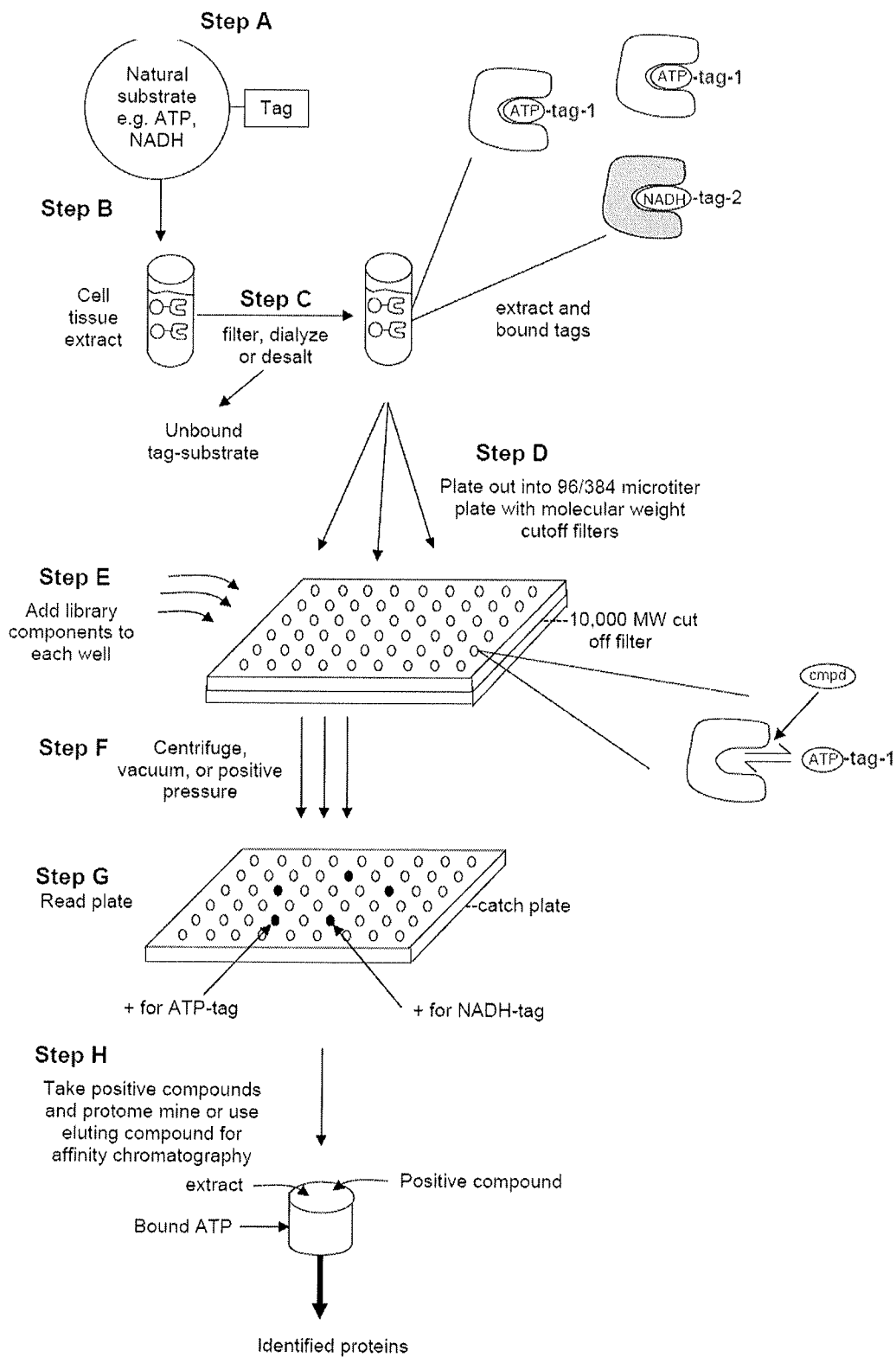
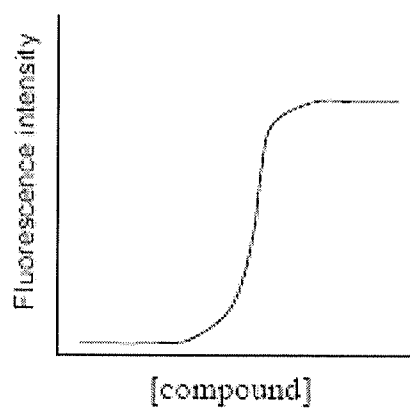
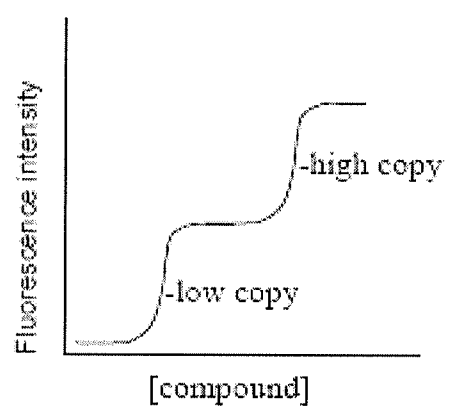
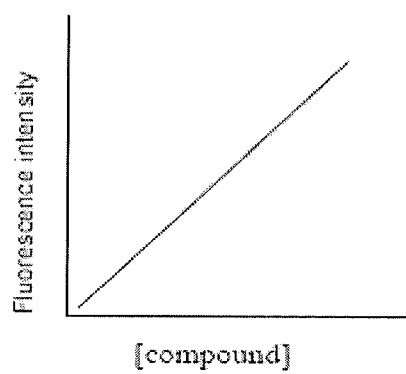


FIG. 1

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**FIG. 2A****FIG. 2B****FIG. 2C**

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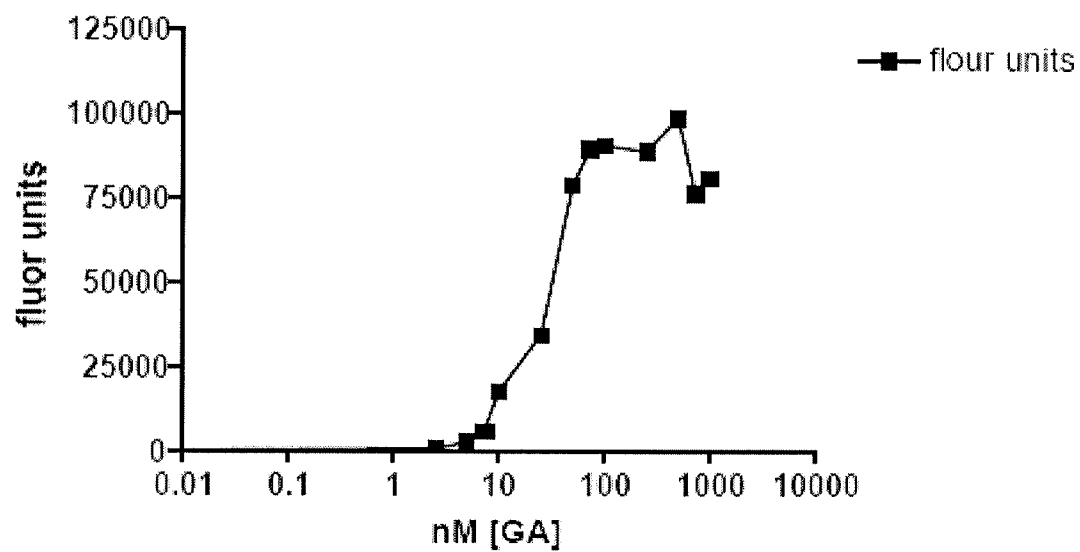


FIG. 3

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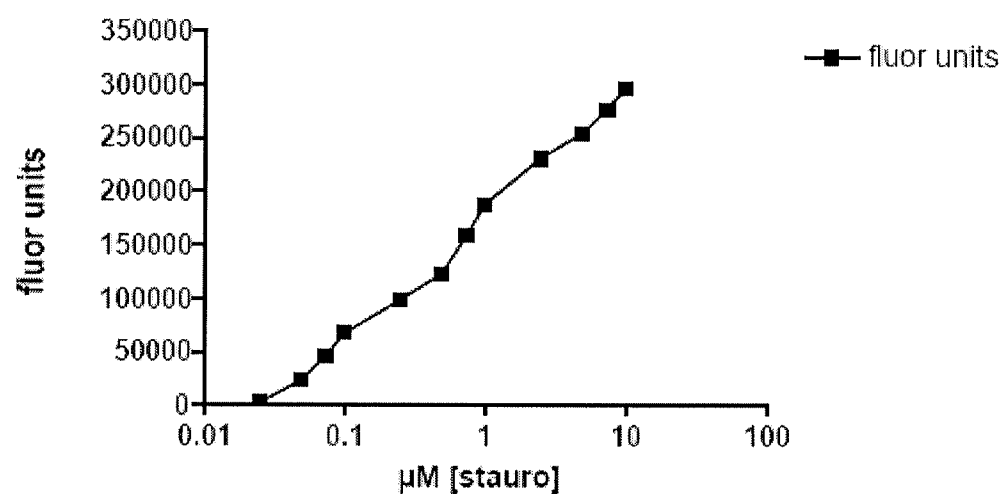


FIG. 4

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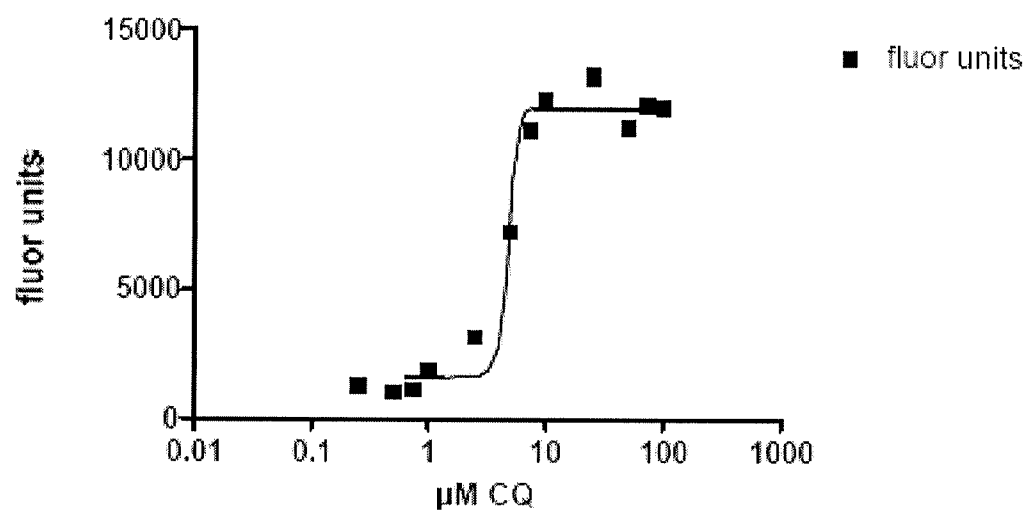


FIG. 5