(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 August 2007 (09.08.2007)

PCT

(10) International Publication Number
WO 2007/090032 A2

(51) International Patent Classification:
G01N 33/50 (2006.01)

(21) International Application Number:
PCT/US2007/061060

(22) International Filing Date: 25 January 2007 (25.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/762,755 27 January 2006 (27.01.2006) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/090032 A2

(54) Title: RIBOSOMAL PROTEIN S6 AS A PHARMACODYNAMIC MARKER FOR HSP90 INHIBITION

(57) Abstract: The present invention provides methods for identifying inhibitors of heat shock protein 90 (Hsp90), for identifying clinical conditions responsive to Hsp90 inhibitors, and for monitoring clinical efficacy of Hsp90 inhibitors. Methods for identifying Hsp90 inhibitors comprise comparing the level of phosphorylation of ribosomal protein S6 (RPS6) in a sample contacted with a potential Hsp90 inhibitor to the level of phosphorylation of RPS6 in a control sample not exposed to the potential inhibitor. In some embodiments, a test cell is contacted with a putative Hsp90 inhibitor and the level of phosphorylation of RPS6 in the cell is compared to the level of phosphorylation of RPS6 in a control cell not exposed to the putative inhibitor, where a decrease in the level of phosphorylation of RPS6 in the contacted test cell is indicative of inhibition of Hsp90 activity. The invention further provides methods of predicting whether a subject with a tumor or other cellular proliferative disorder will respond to an agent that inhibits Hsp90 activity. Such predictions are based on a comparison of the levels of phosphorylation of RPS6 in test tumor cells or other abnormally proliferating cells contacted with the Hsp90 inhibitor to the level of phosphorylation of RPS6 in control cells not treated with the inhibitor, where a decrease in the level of phosphorylation of RPS6 in the test cells contacted with the Hsp90 inhibitor is predictive of a positive response to the Hsp90 inhibitor. The methods of the invention find use in monitoring clinical efficacy of Hsp90 inhibitors.

RIBOSOMAL PROTEIN S6 AS A PHARMACODYNAMIC MARKER FOR HSP90 INHIBITION

FIELD OF THE INVENTION

The invention relates to the field of diagnostic and prognostic medicine, more particularly to methods of identifying inhibitors of heat shock protein 90 (Hsp90), and methods for predicting efficacy of Hsp90 inhibitors in treatment of cancers and other diseases that are associated with overexpression of Hsp90 or any of its client proteins.

BACKGROUND OF THE INVENTION

Heat shock proteins (HSPs) are ubiquitous chaperone proteins that are involved in folding, activation, and assembly of a wide range of proteins, including key proteins involved in signal transduction, cell cycle control, and transcriptional regulation. A number of multigene families of HSPs exist, with individual gene products varying in cellular expression, function, and localization. They are classified according to their molecular weight, for example, heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), and heat shock protein 27 (Hsp27).

Researchers have reported that Hsp90 chaperone proteins are associated with important signaling proteins, such as steroid hormone receptors and protein kinases, including, for example, Raf-1, EGFR, v-Src family kinases, Cdk4, and ErbB-2 (Buchner J. (1999) *TIBS* 24:136-41; Stepanova *et al.* (1996) *Genes Dev.* 10:1491-502; Dai *et al.* (1996) *J. Biol. Chem.* 271:22030-34). Studies further indicate that certain co-chaperones, for example, Hsp70, p60/Hop/Sti1, Hip, Bag1, Hsp40/Hdj2/Hsj1, immunophilins, p23, and p50, may assist Hsp90 in its function (see, *e.g.*, Caplan, A. (1999) *Trends in Cell Biol.* 9:262-68). Hsp90 has also been implicated in various cellular proliferative disorders, affected cells of which appear to be hypersensitive to Hsp90 inhibitors relative to normal cells.

Various small molecule compounds, including ansamycin antibiotics derived from *Streptomyces hygroscopicus*, are known to inhibit Hsp90. These antibiotics,

such as herbimycin A and geldanamycin, as well as other Hsp90 inhibitors such as radicicol, bind tightly to an N-terminus pocket in Hsp90 (Stebbins *et al.* (1997) *Cell* 89:239-50). This pocket is highly conserved and has weak homology to the ATP-binding site of DNA gyrase (Stebbins *et al.* (1997) *Cell* 89:239-50; Grenert *et al.* (1997) *J. Biol. Chem.* 272:23843-50). Additionally, ATP and ADP have both been shown to bind this pocket with low affinity (Proromou *et al.* (1997) *Cell* 90:65-75; Panaretou *et al.* (1998) *EMBO J.* 17:4829-36). *In vitro* and *in vivo* studies have demonstrated that occupancy of this N-terminal pocket by ansamycins and other Hsp90 inhibitors alters Hsp90 function and inhibits protein folding. At high concentrations, ansamycins and other Hsp90 inhibitors have been shown to prevent binding of protein substrates to Hsp90 (Scheibel *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:1297-302; Schulte *et al.* (1995) *J. Biol. Chem.* 270:24585-88; Whitesell *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8324-28). Ansamycins have also been demonstrated to inhibit the ATP-dependent release of chaperone-associated protein substrates (Schneider *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14536-41; Sepp-Lorenzino *et al.* (1995) *J. Biol. Chem.* 270:16580-87). In either event, the substrates are degraded by a ubiquitin-dependent process in the proteasome (Schneider *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:14536-41; Sepp-Lorenzino *et al.* (1995) *J. Biol. Chem.* 270:16580-87; Whitesell *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8324-28).

This substrate destabilization occurs in tumor and non-transformed cells alike and has been shown to be especially effective on a subset of signaling regulators, such as Raf (Schulte *et al.* (1997) *Biochem. Biophys. Res. Commun.* 239:655-59; Schulte *et al.* (1995) *J. Biol. Chem.* 270:24585-88), nuclear steroid receptors (Segnitz and Gehring (1997) *J. Biol. Chem.* 272:18694-701; Smith *et al.* (1995) *Mol. Cell. Biol.* 15:6804-12), v-Src (Whitesell *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8324-28), and certain transmembrane tyrosine kinases, including EGF receptor and Her2/Neu (Hartmann *et al.* (1997) *Int. J. Cancer* 70:221-29; Miller *et al.* (1994) *Cancer Res.* 54:2724-30; Mimnaugh *et al.* (1996) *J. Biol. Chem.* 271:22796-801; Schnur *et al.* (1995) *J. Med. Chem.* 38:3806-12), CDK4, and mutant p53 (Erlichman *et al.* (2001) *Proc. AACR* 42, abstract 4474). The ansamycin-induced loss of these proteins leads to the selective disruption of certain regulatory pathways and results in growth arrest at specific phases of the cell cycle (Muise-Heimericks *et al.* (1998) *J. Biol. Chem.* 273:29864-72), apoptosis, and/or differentiation of cells so treated (Vasilevskaya *et al.* (1999) *Cancer Res.* 59:3935-40).

In addition to anti-cancer and anti-tumorgenic activity, Hsp90 inhibitors have also been implicated in a wide variety of other utilities, including use as anti-inflammation agents, anti-infectious disease agents, agents for treating autoimmunity, agents for treating ischemia, and agents useful in promoting nerve regeneration (see, *e.g.*, published PCT Applications Nos. WO 02/09696 and WO 99/51223, and U.S. Patent No. 6,210,974). Hsp90 inhibitors thus hold great promise for the treatment and/or prevention of many types of cancers and other disorders.

Accordingly, there remains a need for improved methods of identifying inhibitors of Hsp90 and for monitoring efficacy of treatment with Hsp90 inhibitors.

BRIEF SUMMARY OF THE INVENTION

The present invention provides methods for identifying inhibitors of Hsp90, for identifying clinical conditions responsive to Hsp90 inhibitors, and for monitoring efficacy of Hsp90 inhibitors in treatment of various clinical conditions, including cancers and other cellular proliferative disorders such as inflammatory/autoimmune disorders. The Hsp90 inhibitors identified by the methods of the invention inhibit one or more activities of Hsp90, and thus find use in treating cancer and other diseases. The methods rely on the finding that phospho-ribosomal protein S6 (p-RPS6) is a pharmacodynamic marker for Hsp90 inhibition, and Hsp90 inhibitors are identified based on their ability to block phosphorylation of ribosomal protein S6 (RPS6). Also provided are methods of predicting whether a subject with a tumor or other disorder associated with overexpression of Hsp90 will respond to an agent that inhibits Hsp90 activity. Such predictions are based on a comparison of the levels of phosphorylation of RPS6 in biological samples, for example, samples comprising tumor cells or other proliferative cells, treated with the Hsp90 inhibitor to the level of phosphorylation of RPS6 in respective control biological samples not treated with the inhibitor, where a decrease in the level of phosphorylation of RPS6 is predictive of a positive response to the Hsp90 inhibitor.

In one embodiment, the present invention provides a method of measuring the ability of an agent to inhibit Hsp90 activity. The method comprises determining the level of phosphorylation of RPS6 in a sample in the absence of the agent and in the presence of the agent and then comparing the level of phosphorylation of RPS6 from

the two samples, where a decrease in the level of phosphorylation of RPS6 in the presence of the agent is indicative of the ability of the agent to inhibit Hsp90 activity.

In another embodiment, a method of identifying an agent that inhibits Hsp90 activity is provided. The method comprises contacting a test cell with the agent and comparing the level of phosphorylation of RPS6 in the test cell to the level of phosphorylation of RPS6 in a control cell not exposed to the agent, where a decrease in the level of phosphorylation of RPS6 in the test cell is indicative of an agent that inhibits Hsp90 activity. In a specific, non-limiting example, both the test cell and the control cell are peripheral blood mononuclear cells (PBMCs), and the method further comprises contacting both the test cell and the control cell with a stimulatory compound prior to comparing the level of phosphorylation of RPS6.

In yet another embodiment, the present invention provides a method of predicting whether a tumor in a subject will respond to treatment with an Hsp90 inhibitor. The method comprises contacting an isolated tumor cell obtained from the subject with the inhibitor and comparing the level of phosphorylation of RPS6 in the contacted tumor cell to the level of phosphorylation of RPS6 in a control tumor cell not exposed to the inhibitor, where a decrease in the level of phosphorylation of RPS6 in the contacted tumor cell is predictive of a positive response of the tumor to treatment with the Hsp90 inhibitor. Where a tumor is predicted to respond to treatment with an Hsp90 inhibitor, the subject having this tumor can beneficially be treated with the Hsp90 inhibitor to obtain a positive therapeutic response.

A method for predicting the responsiveness of other cellular proliferative disorders in a subject to an Hsp90 inhibitor is also provided. The method comprises contacting a sample from said subject with the inhibitor, wherein the sample comprises cells exhibiting abnormal cell proliferation, and comparing the level of phosphorylation of RPS6 in the contacted sample to the level of phosphorylation of RPS6 in a control sample not exposed to the inhibitor, where a decrease in the level of phosphorylation of RPS6 in the contacted sample is predictive of a positive response of the cellular proliferative disorder to the Hsp90 inhibitor. Where a cellular proliferative disorder is predicted to respond to treatment with an Hsp90 inhibitor, the subject having this disorder can beneficially be treated with the Hsp90 inhibitor to obtain a positive therapeutic response.

Methods for monitoring the efficacy of an Hsp90 inhibitor in treatment of a subject for a tumor or other cellular proliferative disorder are also provided. The

methods comprise obtaining a baseline biological sample from the subject prior to administering a dose of the Hsp90 inhibitor, where the baseline biological sample comprises tumor cells or abnormally proliferating cells, detecting the level of phosphorylation of RPS6 in the baseline biological sample, administering the Hsp90 inhibitor to the subject, obtaining from the subject at least one subsequent biological sample, detecting the level of phosphorylation of RPS6 in the subsequent sample(s), and

comparing the level of phosphorylation of RPS6 in the subsequent sample(s) with the level of phosphorylation of RPS6 in the baseline biological sample, and correlating a change in the level of phosphorylation of RPS6 with treatment efficacy. These methods can be used to monitor efficacy of treatment with an Hsp90 inhibitor, where the inhibitor is administered as a single dose or as multiple doses.

In a further embodiment, the present invention provides methods for monitoring dosage of an Hsp90 inhibitor in treatment of a subject for a tumor or other cellular proliferative disorder. The methods comprise obtaining a baseline biological sample from the subject prior to administering a dose of the Hsp90 inhibitor, where the baseline biological sample consists of PBMCs, contacting the PBMCs with a stimulatory compound, detecting the level of p-RPS6 in the PBMCs, administering the Hsp90 inhibitor to the subject, obtaining from the subject at least one subsequent biological sample consisting of PBMCs, contacting the subsequent sample(s) of PBMCs with a stimulatory compound, detecting the level of p-RPS6 in the subsequent sample(s) of PBMCs, and correlating a change in the level of p-RPS6 with dosage of the Hsp90 inhibitor. These methods can be used to monitor dosage with an Hsp90 inhibitor, where the inhibitor is administered as a single dose or as multiple doses.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a composition of several Western blots illustrating the effects of various Hsp90 inhibitors on Hsp90 client proteins and downstream effectors of Hsp90. Nude mice with subcutaneous HT29 human colon carcinoma xenograft tumors were dosed with various Hsp90 inhibitors as described in the Experimental section. The animals were sacrificed six hours following the second dose, tumor samples were excised, flash frozen and homogenized. Homogenate extracts were loaded onto an

SDS-PAGE gel for separation and then transferred to nitrocellulose for Western blot analysis.

Figure 2 is a composition of several Western blots illustrating the effects of various Hsp90 inhibitors on Hsp90 client proteins and downstream effectors of Hsp90 *in vitro*. A375 human amelanotic melanoma cells were seeded in culture plates as described in the Experimental section and treated with or without various Hsp90 inhibitors for two, four, six, eight, or twenty-four hours. The cells were then lysed and the extracts resolved on an SDS-PAGE gel and transferred to nitrocellulose for Western blot analysis.

Figure 3 illustrates the effects of various stimulatory compounds on *in vitro* induction of RPS6 phosphorylation. Human PBMCs were incubated for sixteen hours with or without 17-AAG, then stimulated for fifteen minutes with phytohemagglutinin (PHA), fetal bovine serum (FBS), lipopolysaccharide (LPS), interleukin-2 (IL-2), or phorbol 12-myristate 13-acetate (PMA). The cells were then lysed and the extracts resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose for Western blot analysis.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods directed to identification of Hsp90 inhibitors, for identifying clinical conditions responsive to Hsp90 inhibitors, and for monitoring efficacy of Hsp90 inhibitors in treatment of various clinical conditions, including cancers and other cellular proliferative disorders such as inflammatory/autoimmune disorders. Generally, the Hsp90 inhibitor identification methods include the step of comparing the level of phosphorylation of RPS6 in a sample (*e.g.*, a biological sample such as a cell) treated with a potential Hsp90 inhibitor to the level of phosphorylation of RPS6 in a control sample not exposed to the potential Hsp90 inhibitor. Ribosomal protein S6 assembles onto the 45S rRNA precursor in the nucleolus and is located at the small head region of the cytosolic 40S ribosomal subunit (Nygärd and Nilsson (1990) *Eur. J. Biochem.* 191:1-17). Ribosomal protein S6 can be directly cross-linked to mRNA, tRNA, and initiation factors, confirming its location in a region involved in the initiation of translation. Ribosomal protein S6, a 30 kDa protein, is the major phosphoprotein of eukaryotic ribosomes.

Thus, in one embodiment, the present invention pertains to a method of measuring the ability of an agent to inhibit Hsp90 activity. The method comprises determining the level of phosphorylation of RPS6 in a sample under conditions in which, (i) the agent is absent from the sample, or (ii) the agent is present in the sample, and comparing the level of phosphorylation of RPS6 from (i) to the level of phosphorylation of RPS6 from (ii). A decrease in the level of phosphorylation of RPS6 in the sample that has been exposed to the agent (*i.e.*, wherein the agent is present in the sample or the sample has been contacted with the agent) is indicative that the agent is an inhibitor of Hsp90 activity.

By "sample" is intended a portion, piece, or segment that is representative of the whole from which the sample is obtained. This term encompasses any material, including for instance samples obtained from an animal, such as a mammal, and may also be referred to as a "clinical specimen." As used herein, a sample includes all material useful for assaying the phosphorylation level of RPS6 in subjects, including, but not limited to, biological samples such as cells, tissues, and bodily fluids, such as blood, and biopsied or surgically removed tissue, including tissues that are, for example, unfixed, frozen, fixed in formalin, and/or embedded in paraffin.

As used herein, "inhibit" (or "interfering with" or "inhibiting") in reference to Hsp90 activity refers to the ability of an agent to measurably reduce the activity of Hsp90, as shown by a decrease in the level of phosphorylation of RPS6. It is understood that the term is relative, and does not require absolute suppression of Hsp90 activity. Thus, in certain embodiments, interfering with or inhibiting Hsp90 activity requires that, following the contacting of the sample with the agent of interest, Hsp90 activity within the sample is at least 5% less than that measured at baseline (*i.e.*, prior to the exposure of the sample to the agent), such as at least 10% less, at least 15% less, at least 20% less, at least 25% less, or even more reduced (as measured by progressively greater decreases in RPS6 phosphorylation levels). Thus, in some particular embodiments, contacting a sample with an agent reduces Hsp90 activity in the sample by about 30%, about 40%, about 50%, about 60%, or more. In specific examples, where the agent is particularly effective as an Hsp90 inhibitor, Hsp90 activity in a sample contacted with the agent is reduced by 70%, 80%, 85%, 90%, 95%, or even more, including by 100% (*i.e.*, complete suppression).

Likewise, "decrease," in reference to the level of phosphorylation of RPS6, is a relative term. Accordingly, in certain embodiments, interfering with or inhibiting

Hsp90 activity in a sample results in at least a 5% decrease in RPS6 phosphorylation within the sample following the contacting of the sample with the inhibiting agent, such as at least 10%, at least 15%, at least 20%, at least 25%, or an even greater decrease. Thus, in some particular embodiments, contacting a sample with an agent that inhibits Hsp90 activity decreases RPS6 phosphorylation as measured within the sample by about 30%, about 40%, about 50%, about 60%, or more. In specific examples, where the agent is particularly effective as an Hsp90 inhibitor, RPS6 phosphorylation within a sample contacted with the agent is reduced by 70%, 80%, 85%, 90%, 95%, or even more.

Candidate agents that inhibit Hsp90 activity as measured by a decrease in the level of phosphorylation of RPS6 may be derived from almost any source of chemical libraries, naturally occurring compounds, or mixtures of compounds. Exemplary sources of candidate inhibitors include, but are not limited to, libraries of peptides, peptoids, and small organic molecules. Any agent that is an inhibitor or antagonist of Hsp90 activity as measured by a decrease in the level of phosphorylation of RPS6 can be identified using the screening methods of the present invention. The inhibitor of Hsp90 activity can be a peptide antagonist, a peptoid antagonist, or a small organic molecule antagonist. Analogs of peptides as used herein include peptides having one or more peptide mimics, for example peptoids that possess protein-like activity. Included within the definition are, for example, peptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids), peptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and not naturally occurring.

The term "small molecule" includes any chemical or other moiety that can act to affect biological processes. Small molecules can include any number of therapeutic agents presently known and used, or can be small molecules synthesized in a library of such molecules for the purpose of screening for function. Small molecules are distinguished from polymers and macromolecules by size and lack of polymerization. Small molecules can include peptides, peptoids, and small organic molecules.

The candidate inhibitors of Hsp90 activity and libraries of candidate inhibitors for screening by the assays disclosed herein can be derived from any of the various possible sources of candidate inhibitors, such as for example, libraries of peptides, peptoids, and small molecules.

Thus, the invention provides a method (also referred to herein as a "screening assay") for identifying inhibitors, that is, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small organic molecules, or other drugs) that inhibit Hsp90 activity as measured by a decrease in the level of phosphorylation of RPS6. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including biological libraries, spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the "one-bead one-compound" library method, and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, nonpeptide oligomer, or small molecule libraries of compounds (Lam (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Bio/Techniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), ribosomes (Hudson and Souriau (2001) *Expert. Opin. Biol. Ther.* 1(5):845-855), bacteria (U.S. Patent No. 5,223,409), spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:1865-1869), phage (Scott and Smith (1990) *Science* 249:386-390; Devlin (1990) *Science* 249:404-406; Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382; and Felici (1991) *J. Mol. Biol.* 222:301-310), and yeast (Hudson and Souriau (2001) *Expert. Opin. Biol. Ther.* 1(5):845-855).

In some embodiments, the candidate agents to be tested as inhibitors of Hsp90 activity are identified using proteome mining. See, for example, WO 00/63694; and copending U.S. Patent Application No. 09/958,787; the contents of both of which are herein incorporated by reference in their entirety.

Detection of phosphorylated RPS6 may be performed by methods well known in the art, including, but not limited to, Western blotting, immunoprecipitation,

immunofluorescence, immunocytochemistry, immunohistochemistry, immunomagnetic assays, ELISA, agglutination assays, and flocculation assays. The Western blotting technique (Sambrook *et al.* (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989)) is a convenient method of detecting phosphorylated RPS6. Briefly, total cellular protein is extracted from tissue (*e.g.*, human tissue or mouse tissue) or cells (*e.g.*, mammalian cells such as tumor cells or peripheral blood mononuclear cells) and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. The proteins are then transferred to a membrane (*e.g.*, nitrocellulose) by Western blotting, and a phosphospecific antibody (*e.g.*, anti-phospho RPS6) preparation is incubated with the membrane. After washing the membrane to remove non-specifically bound antibodies, the presence of specifically bound antibodies is detected by the use of a secondary antibody, which is conjugated to an enzyme (*e.g.*, alkaline phosphatase) or fluorescently labeled (*e.g.*, FITC). Anti-phospho RPS6 antibodies are known in the art and are commercially available, for example, from Cell Signaling (Danvers, MA).

Phosphorylation of RPS6 can also be detected via direct binding of phosphospecific antibodies (*e.g.*, anti-phospho RPS6) or by measuring displacement of a phosphospecific antibody from a competitor (see, *e.g.*, Parker *et al.* (2000) *J. Biomolec. Screening* 5:77-88). Fluorescence methods, such as fluorescence resonance energy transfer (FRET) or fluorescence polarization (FP), can be used to detect the specific phosphoprotein-antibody complexes. These methods have the advantage that they employ "homogeneous" detection that is not dependent on isolation of the bound species, but rather depends on changes in fluorescence that occur owing to specific binding in solution.

As used herein, the term "antibody" includes intact immunoglobulins as well as a number of well-characterized fragments. For instance, Fabs, Fvs, and single-chain Fvs (SCFvs) that bind to target protein (or epitope within a protein or fusion protein) would also be specific binding agents for that protein (or epitope). These antibody fragments are as follows: (1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule;

(3) $F(ab')_2$, the fragment of an antibody obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; $F(ab')_2$ is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, a genetically engineered fragment containing the variable region of a light chain and the variable region of a heavy chain expressed as two chains; and (5) single chain antibody (SCA), a genetically engineered molecule containing the variable region of a light chain and the variable region of a heavy chain, linked by a suitable polypeptide linker, as a genetically fused single chain molecule. Methods of making these fragments are routine (see, *e.g.*, Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999).

Antibodies for use in the methods of this invention can be monoclonal or polyclonal, for example, monoclonal or polyclonal anti-phospho RPS6 antibodies. Merely by way of example, monoclonal antibodies can be prepared from murine hybridomas according to the classical method of Kohler and Milstein (*Nature* 256:495-97, 1975) or derivative methods thereof. Methods for producing antibodies, including phosphospecific antibodies, are well known in the art (see, *e.g.*, Harlow and Lane, *Using Antibodies: A Laboratory Manual*, CSHL, New York, 1999).

In some embodiments, RPS6 occurs naturally in a host cell, for example a mammalian cell, such as a tumor cell or a peripheral blood mononuclear cell (PBMC). By "host cell" is intended a cell from a human or veterinary subject. The term "tumor cell" as used herein refers to a neoplastic cell (*i.e.*, an abnormal cell in which growth is uncontrolled and progressive) that may be either malignant or non-malignant (benign), and includes cells derived from both solid and non-solid tumors (such as hematologic malignancies). In a specific, non-limiting example, a tumor cell includes A375 cells, a human amelanotic melanoma cell line that has been used for a variety of studies in cell biology (Giard *et al.* (1973) *J. Natl. Cancer Inst.* 51:1417-23).

By "peripheral blood mononuclear cell" is intended a mononuclear cell, such as a lymphocyte or a monocyte, isolated from the peripheral blood. Peripheral blood mononuclear cells can be isolated from peripheral blood by methods well known in the art. For example, they may be isolated by density centrifugation over a step gradient consisting of a mixture of the carbohydrate polymer Ficoll™ and the dense iodine-containing compound metrizamide. This yields a population of mononuclear cells at the interface that has been depleted of red blood cells and most

polymorphonuclear leukocytes and granulocytes. The resulting PBMCs consist mainly of lymphocytes and monocytes.

In some embodiments, the present invention provides for the partial purification of RPS6 prior to analysis of its phosphorylation state. Such purification can also include the isolation of RPS6. Ribosomal protein S6 that has been "partially purified" or "isolated" has been separated, produced apart from, or purified away from at least one other biological component in the cell of the organism in which it naturally occurs. Therefore, isolated RPS6 can be contained in a subcellular fraction or extract prepared from cells containing RPS6, such as a cytoplasmic lysate, a membrane preparation, a nuclear extract, or a crude or purified protein preparation. The term "isolated" or "partially purified" does not require absolute purity; rather, it is intended as a relative term. Thus, for example, partial purification of RPS6 results in its enrichment, relative to its level within a cell. A sample containing RPS6 can be prepared by methods known in the art suitable for the particular format of the detection method. For example, biochemical methods such as precipitation and immunoaffinity methods can be used to isolate RPS6 from a cell. Procedures for preparing subcellular fractions, such as nuclear fractions and cell lysates, are well known to those of skill in the art, and include, for example, cell disruption followed by separation methods such as gradient centrifugation and biochemical purification methods.

In further embodiments, the present invention provides for a qualitative determination or a quantitative determination of the phosphorylation level of RPS6. By "qualitative" determination is intended the presence or absence of phosphorylated RPS6, irrespective of the level of phosphorylation of RPS6. In contrast, by "quantitative" determination is intended a measurement of the level of phosphorylation of RPS6. The methods described herein above for the detection of phosphorylated RPS6 are amenable to both qualitative and quantitative determinations of the phosphorylation level of RPS6, using methodologies well known in the art.

In another embodiment, the present invention pertains to a method of identifying an agent that inhibits Hsp90 activity, comprising contacting a test cell with the agent, and comparing the level of phosphorylation of RPS6 in the contacted test cell to the level of phosphorylation of RPS6 in a control cell not exposed to the agent, where a decrease in the level of phosphorylation of RPS6 in the contacted test cell is

indicative of an agent that inhibits Hsp90 activity. By "control cell" is intended a cell derived from the same sample source as the cell contacted with the agent, but lacking exposure to the agent. In a specific, non-limiting example, both the test cell and the control cell are PBMCs, and the method further comprises contacting both the test cell and the control cell with a stimulatory compound prior to comparing the levels of phosphorylated RPS6.

The phosphorylation state of RPS6 in PBMCs isolated from healthy subjects can be low, providing marginal signal-to-noise activity. However, phosphorylation of RPS6 can be induced in *in vitro* populations of isolated PBMCs using a stimulatory compound. This increases the signal-to-noise ratio, allowing the isolated PBMCs to be used to measure the ability of an agent to inhibit Hsp90 activity, by determining the level of phosphorylation of RPS6 in the presence and absence of the agent. By "stimulatory compound" is intended a substance that promotes, specifically or non-specifically, biochemical changes in a cell. Exemplary stimulatory compounds include lectins, lipopolysaccharides, phorbol esters, and interleukins.

By "lectin" is intended a polypeptide, particularly one obtained from the seeds of leguminous plants, having binding sites for specific mono- or oligosaccharides. Exemplary lectins include, but are not limited to, phytohemagglutinin (PHA), concanavalin A, and wheat germ agglutinin. By "lipopolysaccharide" is intended lipid-containing polysaccharides derived from the cell walls of gram-negative bacteria. A lipopolysaccharide molecule consists of three parts, lipid, a core polysaccharide, and o-specific chains. Lipopolysaccharides are highly immunogenic and stimulate the production of multiple cellular factors. By "phorbol ester" is intended a polycyclic compound in which two hydroxyl groups on neighboring carbon atoms are esterified to fatty acids. Phorbol esters were originally isolated from croton oil, and are potent tumor promoters. An exemplary phorbol ester is phorbol 12-myristate 13-acetate (PMA).

As used herein, "interleukin" is used as a generic name for a diverse group of soluble proteins and peptides that act as humoral regulators at nano- to picomolar concentrations and which, either under normal or pathological conditions, modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. An exemplary interleukin is interleukin-2 (IL-2), a protein

of 133 amino acids (15.4 kDa) with a slightly basic pI that does not display sequence homology to any other factors.

Any acceptable protocol to contact the test cell with a candidate Hsp90 inhibitory agent can be used in the screening methods of the invention. Factors to be considered include, but are not limited to, the concentration of the candidate agent to be contacted with the test cell; the incubation time of the candidate agent with the test cell; where applicable, the concentration of a stimulatory compound, for example, a lectin such as phytohemagglutinin, to be contacted with the test cell; and, where applicable, the incubation time of the stimulatory compound with the test cell. Determination of such factors can be accomplished by those skilled in the art based on variables such as the type of cell being tested, size of the holding container, the volume of liquid in the container, and the chemical composition of the candidate agent (*i.e.*, size, charge, and the like) being tested.

In one embodiment, a test sample or subsample thereof comprising a suitable number of test cells is added to a 96-well tissue culture dish. The suitable number of test cells is a number of cells that enables one to detect a change in the level of phosphorylation of RPS6 using one or more of the detection methods described elsewhere herein. In some embodiments, the suitable number of test cells is between about 100 and about $1x10^6$ cells per well of a 96-well tissue culture dish (*e.g.*, from about 1,000 to about 10,000 cells per well). Following addition of the test cells to the tissue culture dish, the test cells can be preincubated between about 0 to about 96 hours before contacting the test cells with the candidate agent. In some embodiments, the test cells are preincubated with a stimulatory compound as noted herein above.

An effective amount of a candidate agent is added to a test cell, or cells of a test sample, to provide for regulation of the activity of interest (*i.e.*, inhibition of Hsp90 activity as detected by a decrease in the level of phosphorylation of RPS6) such that the regulation is detectable using one or more detection methods disclosed elsewhere herein. The effective amount will of course be dependent upon the candidate agent being tested. Generally, where cells of a test sample or subsample thereof are to be contacted, an effective amount of a candidate agent is between about 0.1 nM to about 10 mM of the agent (*e.g.*, about 1 nM to about 100 μM) per well of a 96-well plate where the test cells have been seeded at between about 100 and about $1x10^6$ cells per well (*e.g.*, from about 1,000 to about 10,000 cells per well). The test cell or cells within the test sample or subsample thereof are allowed to incubate for a

suitable length of time to allow the candidate agent to interact with the cell(s) and generate one or more biological responses. In some embodiments, the incubation time between the candidate agent and the test cell or cells of the test sample or subsample thereof is between about 30 minutes to about 48 hours. In other embodiments, the incubation time is about 1 hour, about 2 hours, about 4 hours, about 8 hours, about 12 hours, about 20 hours, or about 24 hours.

In a further embodiment, a method of predicting whether a tumor in a subject will respond to a known Hsp90 inhibitor is provided. The method comprises contacting an isolated tumor cell from the subject with the inhibitor, and comparing the level of phosphorylation of RPS6 in the tumor cell to the level of phosphorylation of RPS6 in a control tumor cell not exposed to the inhibitor, where a decrease in the level of phosphorylation of RPS6 in the contacted tumor cell is predictive of a positive response of the tumor to the inhibitor. The term "tumor" as used herein refers to a neoplasm that may be either malignant or non-malignant (benign) and includes both solid and non-solid tumors (such as hematologic malignancies). By "neoplasm" is intended an abnormal growth of cells or tissue, particularly a new growth of cells or tissue in which the growth is uncontrolled and progressive. A tumor is an example of a neoplasm. A tumor cell can be isolated from a histologic section of a specimen obtained by biopsy, from cells obtained from body fluids, or from cells that are placed in or adapted to tissue culture.

By "known Hsp90 inhibitor" is intended an agent known to inhibit one or more activities of Hsp90. Exemplary known Hsp90 inhibitors include, but are not limited to, ansamycin antibiotics, such as geldanamycin, herbimycin A, radicicol, 17-allylamino-17-demethoxygeldanamycin (17-AAG), 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), and radicicol oximes (Stebbins *et al.* (1997) *Cell* 89:239-50; Prodromou *et al.* (1997) *Cell* 90:65-75; Roe *et al.* (1999) *J. Med. Chem.* 42:260-66); bacterial gyrase inhibitors (*e.g.*, novobiocin) (Marcu *et al.* (2000) *J. Biol. Chem.* 275:37181-86); cytotoxic DNA damaging agents (*e.g.*, cisplatin) (Itoh *et al.* (1999) *Biochem. J.* 343:697-703); and purine based inhibitors (*e.g.*, PU3) (Chiosis *et al.* (2002) *Bioorg. Med. Chem.* 10:3555-64).

Any living, multicellular, vertebrate organism capable of developing a tumor is contemplated as a subject for the disclosed method of predicting whether the tumor will respond to a known Hsp90 inhibitor. Thus, in particular examples, a subject of the disclosed method is a human or veterinary subject. Non-limiting examples of

tumors include tumors of the skin, such as squamous cell carcinoma, basal cell carcinoma, melanoma, skin appendage tumors, papilloma, cutaneous T-cell lymphoma (mycosis fungoides), apocrine carcinoma of the skin, or Merkel cell carcinoma, breast carcinomas, for example, lobular and duct carcinomas and other solid tumors, sarcomas and carcinomas of the lung, such as small cell carcinoma, large cell carcinoma, squamous carcinoma, adenocarcinoma, and mesothelioma of the lung, colorectal adenocarcinoma, stomach carcinoma, prostatic adenocarcinoma, ovarian carcinoma, such as serous cystadenocarcinoma and mucinous cystadenocarcinoma, and ovarian germ cell tumors, testicular carcinomas, germ cell tumors, pancreatic adenocarcinoma, biliary adenocarcinoma, hepatocellular carcinoma, bladder carcinoma, including transitional cell carcinoma, adenocarcinoma and squamous carcinoma, renal cell adenocarcinoma, endometrial carcinomas, including adenocarcinomas and mixed Mullerian tumors (carcinosarcomas), carcinomas of the endocervix, ectocervix and vagina, such as adenocarcinoma and squamous carcinoma, esophageal carcinoma, carcinomas of the nasopharynx and oropharynx, including squamous carcinoma and adenocarcinomas, salivary gland carcinomas, brain and central nervous system tumors, including tumors of glial, neuronal and meningeal origin, tumors of peripheral nerve, soft tissue sarcomas and sarcomas of bone and cartilage, and non-solid hematopoietic tumors, such as leukemias.

Where a tumor is predicted to respond to treatment with an Hsp90 inhibitor, the subject having this tumor can beneficially be treated with the Hsp90 inhibitor to obtain a positive therapeutic response. By "positive therapeutic response" with respect to treatment of a tumor or cancer is intended an improvement in the disease in association with the anti-tumor activity of the Hsp90 inhibitor and/or an improvement in the symptoms associated with the disease of interest. That is, an anti-proliferative effect, the prevention of further tumor outgrowths, a reduction in tumor size, a reduction in the number of cancer cells, and/or a decrease in one or more symptoms of the disease can be observed. Thus, for example, a positive therapeutic response would refer to one or more of the following improvements in the disease: (1) a reduction in tumor size; (2) a reduction in the number of cancer (*i.e.*, neoplastic) cells; (3) inhibition of neoplastic cell growth; (4) inhibition (*i.e.*, slowing to some extent, preferably halting) of tumor growth; (5) inhibition (*i.e.*, slowing to some extent, preferably halting) of cancer cell infiltration into peripheral organs; (6)

inhibition (*i.e.*, slowing to some extent, preferably halting) of tumor metastasis; (7) the prevention of further tumor outgrowths; (8) an increased patient survival rate; and (9) some extent of relief from one or more symptoms associated with the cancer. Positive therapeutic responses in any given malignancy can be determined by standardized response criteria specific to that malignancy. Tumor response can be assessed for changes in tumor morphology (*i.e.*, overall tumor burden, tumor size, and the like) using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, bone scan imaging, endoscopy, and tumor biopsy sampling including bone marrow aspiration (BMA) and counting of tumor cells in the circulation. In addition to these positive therapeutic responses, the subject undergoing therapy with the Hsp90 inhibitor may experience the beneficial effect of an improvement in the symptoms associated with the disease.

In further embodiments, the present invention provides a method of predicting the responsiveness of a cellular proliferative disorder, other than a neoplasm, in a subject or other disorder associated with aberrant Hsp90 activity, will respond to a known Hsp90 inhibitor. By "cellular proliferative disorder" is intended any pathological condition in which there is an abnormal increase in cell proliferation, including, for example, inflammatory diseases, hyperplasias and autoimmune diseases. The method comprises contacting a sample from the subject with the inhibitor, wherein the sample comprises cells exhibiting abnormal cell proliferation. The level of phosphorylation of RPS6 in the sample is then compared to the level of phosphorylation of RPS6 in a control sample not exposed to the inhibitor. A decrease in the level of phosphorylation of RPS6 in the contacted sample is predictive of a positive response of the cellular proliferative disorder to the Hsp90 inhibitor.

Where a cellular proliferative disorder is predicted to respond to treatment with an Hsp90 inhibitor, the subject having this disorder can beneficially be treated with the Hsp90 inhibitor to obtain a positive therapeutic response. For example, a "positive therapeutic response" with respect to an autoimmune disease and/or inflammatory disease would include an improvement in the disease in association with the inhibitory action of the Hsp90 inhibitor and/or an improvement in the symptoms associated with the disease. That is, an anti-proliferative effect as measured by decreased levels of phosphorylated RPS6, the prevention of further proliferation of the Hsp90-expressing cells, a reduction in the inflammatory response, combinations thereof, and the like can be observed.

Clinical response can be assessed using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, flow cytometry or fluorescence-activated cell sorter (FACS) analysis, histology, gross pathology, and blood chemistry, including but not limited to changes detectable by ELISA, RIA, chromatography, and the like. In addition to these positive therapeutic responses, the subject undergoing therapy with the Hsp90 inhibitor may experience the beneficial effect of an improvement in the symptoms associated with the disease.

The phosphorylation state of RPS6 can be used as a pharmacodynamic marker to optimize the dosage and the regimen of an Hsp90 inhibitor in a clinical setting by monitoring RPS6 in a subject's biological sample(s), and dosing with the Hsp90 inhibitor to achieve a desirable level of down regulation of RPS6 phosphorylation. In one embodiment, a baseline biological sample is obtained from the subject prior to administering a dose of the Hsp90 inhibitor, where the baseline biological sample consists of PBMCs. The PBMCs are contacted with a stimulatory compound, following which the level of p-RPS6 in the PBMCs is detected. The Hsp90 inhibitor is then administered to the subject, following which at least one subsequent biological sample consisting of PBMCs is obtained from the subject. The subsequent sample(s) of PBMCs are contacted with a stimulatory compound, and the level of p-RPS6 in the subsequent sample(s) of PBMCs is detected. A change in the level of p-RPS6 with dosage of the Hsp90 inhibitor is then correlated. The inhibitor can be administered as a single dose or as multiple doses.

Accordingly, the method of the present invention can be used to monitor the therapeutic efficacy of an Hsp90 inhibitor and/or to find a therapeutically effective amount or dosage regimen for the selected Hsp90 inhibitor, thereby individually selecting and optimizing a therapy for a subject. Factors for consideration in this context include the particular condition being treated, the particular subject being treated, the clinical condition of the subject, the site of delivery of the Hsp90 inhibitor, the method of administration, the scheduling of administration, and other factors known to medical practitioners skilled in the art. The therapeutically effective amount of an Hsp90 inhibitor to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the tumor or other cellular proliferative disorder.

The present invention also encompasses kits for carrying out the assays of the present invention. For example, the kit can contain a labeled compound or agent capable of detecting p-RPS6 in a biological sample (*e.g.*, an antibody that binds to the p-RPS6) and means for determining the amount of the p-RPS6 in the sample following incubation of the sample with the labeled compound or agent. Kits can be packaged to allow for detection of other molecules of interest in addition to detection of p-RPS6. Kits can also include instructions for treating a subject when the prognostic assay generates a result that is indicative of a positive treatment outcome with the Hsp90 inhibitor.

For antibody-based kits, the kit can include, for example: (1) a first antibody (*e.g.*, attached to a solid support) that binds to p-RPS6; and, optionally, (2) a second, different antibody that binds to the p-RPS6 or the first antibody and is conjugated to a detectable agent (*e.g.*, alkaline phosphatase or a fluorescent label). The kit can also include, for example, a buffering agent, a preservative, or a protein stabilizing agent. The kit can also include components necessary for detecting the detectable agent (*e.g.*, an enzyme or a substrate). The kit can further contain a control sample or a series of control samples that can be assayed and compared to the test sample contained. Each component of the kit is usually enclosed within an individual container, and all of the various containers are within a single package along with instructions for observing whether the tested subject is a candidate for treatment with the Hsp90 inhibitor.

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

EXPERIMENTAL

Example 1: Effects of Hsp90 Inhibitors on Hsp90 Client Proteins and Downstream Effectors of Hsp90 *in vivo*

Nude mice (NCr nu/nu mice) with subcutaneous HT29 human colon carcinoma xenograft tumors were dosed with Serenex Hsp90 inhibitors or vehicle alone, either per os (PO) or intraperitoneally (IP); dosing was a single daily dose for two consecutive days. The animals were sacrificed six hours following the second dose and tumor samples were excised and flash frozen in liquid nitrogen. The frozen tumor tissue was homogenized in 3x volume of Buffer 1 (25mM HEPES pH 7.4, 150mM NaCl, 50mM NaF, 0.25% NP-40, 1mM vanadate, and a protease inhibitor

cocktail). The homogenate was diluted in 3 volumes Buffer 2 (25mM HEPES pH 7.4, 150mM NaCl, 50mM NaF, 1mM vanadate, and a protease inhibitor cocktail) and centrifuged at 20,000 RPM for 10 minutes in a Beckman JA25.5 rotor. The resulting extract was loaded onto an SDS-PAGE gel for separation and transferred to nitrocellulose for Western blot analysis (Figure 1). Primary antibodies to Her2, AKT, B-Raf, and PDGFR were from Upstate Biotech (Lake Placid, NY). Anti-phospho ERK and anti-phospho RPS6 antibodies were from Cell Signaling (Danvers, MA). The anti-Hsp70 antibody was from StressGen (Ann Arbor, MI). Secondary goat anti-mouse-AP conjugate and goat anti-rabbit-AP conjugate were from BioRad (Hercules, CA).

Example 2: Effects of Hsp90 Inhibitors on Hsp90 Client Proteins and Downstream Effectors of Hsp90 *in vitro*

A375 human amelanotic melanoma cells were seeded in 24-well culture plates and grown to 50% confluence. Cells were treated with or without the Hsp90 inhibitors 17-AAG, SNX1807, or SNX2112 for two, four, six, eight, or twenty-four hours. The cells were then lysed in 200ul SDS sample buffer and sonicated for six seconds. The resulting extracts were resolved on 10% SDS-PAGE gels and transferred to nitrocellulose for Western blot analysis (Figure 2). Primary antibodies to Her2 and B-Raf were from Upstate Biotech (Lake Placid, NY). Anti-phospho-ERK and anti-phospho RPS6 antibodies were from Cell Signaling (Danvers, MA). The anti-Hsp70 antibody was from StressGen (Ann Arbor, MI).

Example 3: Effect of the Hsp90 Inhibitor 17-AAG on RPS6 Phosphorylation *in vitro*

A375 human amelanotic melanoma cells were seeded into black walled viewplates96 and allowed to adhere to the plates overnight. Cells were then treated for twenty-four hours with 17-AAG or DMSO control. After treatment, the cell media was removed, cells were fixed, permeabilized, and probed with anti-phospho RPS6 antibody (Cell Signaling, Danvers, MA). The primary antibody was detected with a FITC-labeled goat secondary antibody (Molecular Probes, Eugene, OR). DNA was stained with Hoechst 33342 nuclear dye (Invitrogen, Carlsbad, CA). DMSO-treated control cells were positive for p-RPS6, while 17-AAG-treated cells exhibited only nuclear staining (data not shown).

Example 4: Effects of Stimulatory Compounds on *in vitro* Induction of RPS6 phosphorylation

Human PBMCs were isolated from healthy volunteers, seeded into culture plates ($1x10^6$ cells per test condition) and incubated for sixteen hours, with or without 17-AAG. The PBMCs were then stimulated for fifteen minutes with phytohemagglutinin (PHA), fetal bovine serum (FBS), lipopolysaccharide (LPS), interleukin-2 (IL-2), or phorbol 12-myristate 13-acetate (PMA), and lysed by the addition of SDS sample buffer. Cell lysates were sonicated for six seconds and clarified by centrifugation at 14,000xg for ten minutes. Samples were resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose for Western blot analysis (Figure 3). Anti-phospho RPS6 antibody was from Cell Signaling (Danvers, MA), and the secondary goat anti-rabbit-AP conjugate antibody was a from BioRad (Hercules, CA).

Example 5: Screening a Subject With a Cellular Proliferative Disorder For Treatment With an Hsp90 Inhibitor

Subjects with a cellular proliferative disorder, such as a neoplasm, an inflammatory disease or an autoimmune disease, are screened to determine whether treatment with an Hsp90 inhibitor will be therapeutically effective in treating or inhibiting the disorder. A sample exhibiting abnormal cell proliferation from the subject, such as a cell or a biopsied or surgically removed tissue, is contacted with the Hsp90 inhibitor, following which the level of phosphorylation of RPS6 in the sample is compared to the level of phosphorylation of RPS6 in a control sample not exposed to the inhibitor. A decrease in the level of phosphorylation of RPS6 in the treated sample is predictive of a positive response in the subject to the Hsp90 inhibitor.

The article "a" and "an" are used herein to refer to one or more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element.

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.

THAT WHICH IS CLAIMED:

1. A method of measuring the ability of an agent to inhibit heat shock protein 90 (Hsp90) activity, comprising determining the level of phosphorylation of ribosomal protein S6 (RPS6) in a sample under conditions in which:

(a) said agent is absent from said sample;

(b) said agent is present in said sample; and

comparing the level of phosphorylation of RPS6 in said sample of (a) to the level of phosphorylation of RPS6 in said sample of (b), wherein a decrease in the level of phosphorylation of RPS6 in said sample of (b) is indicative of inhibition of Hsp90 activity.

2. The method of claim 1, wherein said RPS6 occurs naturally in a host cell.

3. The method of claim 2, wherein said host cell is a mammalian cell.

4. The method of claim 3, wherein said host cell is a tumor cell.

5. The method of claim 3, wherein said host cell is a peripheral blood mononuclear cell.

6. The method of claim 2, wherein said RPS6 is extracted and partially purified from said host cell.

7. The method of claim 1, wherein the determination of said level of phosphorylation of RPS6 is qualitative.

8. The method of claim 1, wherein the determination of said level of phosphorylation of RPS6 is quantitative.

9. The method of claim 1, wherein determining said level of phosphorylation of RPS6 comprises the use of an anti-phospho RPS6 antibody.

10. A method of identifying an agent that inhibits heat shock protein 90 (Hsp90) activity, comprising:

(a) contacting a test cell with said agent; and

(b) comparing the level of phosphorylation of ribosomal protein S6 (RPS6) in said test cell to the level of phosphorylation of RPS6 in a control cell not exposed to said agent;

wherein a decrease in the level of phosphorylation of RPS6 in said test cell contacted with said agent is indicative that said agent inhibits Hsp90 activity.

11. The method of claim 10, wherein both said test cell and said control cell are mammalian cells.

12. The method of claim 10, wherein both said test cell and said control cell are tumor cells.

13. The method of claim 12, wherein said tumor cells are A375 human amelanotic melanoma cells.

14. The method of claim 10, wherein both said test cell and said control cell are peripheral blood mononuclear cells (PBMCs), and wherein the method further comprises contacting both said test cell and said control cell with a stimulatory compound prior to comparing the level of phosphorylation of RPS6.

15. The method of claim 14, wherein said stimulatory compound is selected from the group consisting of phytohemagglutinin, interleukin-2, a phorbol ester, and combinations thereof.

16. The method of claim 10, wherein said RPS6 is extracted and partially purified from said test cell and said control cell.

17. The method of claim 10, wherein the comparison of said level of phosphorylation of RPS6 is qualitative.

18. The method of claim 10, wherein the comparison of said level of phosphorylation of RPS6 is quantitative.

19. The method of claim 10, wherein the comparison of said level of phosphorylation of RPS6 comprises the use of an anti-phospho RPS6 antibody.

20. The method of claim 10, wherein contacting a test cell with said agent, comprises administering said agent to a subject.

21. A method of predicting whether a tumor in a subject will respond to a known heat shock protein 90 (Hsp90) inhibitor, comprising:

(a) contacting a test tumor cell from said subject with said inhibitor; and

(b) comparing the level of phosphorylation of ribosomal protein S6 (RPS6) in said test tumor cell to the level of phosphorylation of RPS6 in a control tumor cell not exposed to said inhibitor;

wherein a decrease in the level of phosphorylation of RPS6 in said test tumor cell contacted with said inhibitor relative to the level of phosphorylation of RPS6 in said control tumor cell is predictive of a positive response of said tumor to said inhibitor.

22. The method of claim 21, wherein said tumor is a hematological tumor.

23. The method of claim 21, wherein said tumor is a solid tumor.

24. A method of treating a subject with a tumor that is responsive to therapy with a heat shock protein 90 (Hsp90) inhibitor, comprising screening said tumor of said subject with a method according to any one of claims 21 through 23, and then treating said subject with said Hsp90 inhibitor when said method according to any one of claims 21 through 23 generates a result that is indicative of a positive treatment outcome with said Hsp90 inhibitor.

25. A method of predicting the responsiveness of a cellular proliferative disorder in a subject to a known heat shock protein 90 (Hsp90) inhibitor, comprising:

(a) contacting a test sample from said subject with said inhibitor, wherein said test sample comprises cells exhibiting abnormal cell proliferation; and

(b) comparing the level of phosphorylation of ribosomal protein S6 (RPS6) in said test sample to the level of phosphorylation of RPS6 in a control sample not exposed to said inhibitor;

wherein a decrease in the level of phosphorylation of RPS6 in said test sample contacted with said inhibitor relative the level of phosphorylation of RPS6 in said control sample is predictive of a positive response of said cellular proliferative disorder to said Hsp90 inhibitor.

26. The method of claim 25, wherein said cellular proliferative disorder is an inflammatory or autoimmune disorder.

27. A method of treating a subject with a cellular proliferative disorder that is responsive to therapy with a heat shock protein 90 (Hsp90) inhibitor, said method comprising screening said cellular proliferative disorder of said subject with a method according to claim 25 or claim 26, and then treating said subject with said Hsp90 inhibitor when said method according to claim 25 or claim 26 generates a result that is indicative of a positive treatment outcome with said Hsp90 inhibitor.

28. A method for monitoring efficacy of a known heat shock protein 90 (Hsp90) inhibitor in treatment of a subject for a tumor or other cellular proliferative disorder, said method comprising:

a) obtaining a baseline biological sample from said subject prior to administering a dose of said Hsp90 inhibitor, wherein said baseline biological sample comprises tumor cells or cells exhibiting abnormal cell proliferation;

b) detecting the level of phosphorylated ribosomal protein S6 (p-RPS6) in said baseline biological sample;

c) administering said Hsp90 inhibitor to said subject;

d) obtaining from said subject at least one subsequent biological sample;

e) detecting the level of said p-RPS6 in said at least one subsequent sample; and

f) comparing the level of said p-RPS6 in said at least one subsequent sample with the level of said p-RPS6 in said baseline biological sample, and correlating a change in the level of said p-RPS6 with treatment efficacy.

29. The method of claim 28, wherein a single dose of said Hsp90 inhibitor is administered to said subject.

30. The method of claim 28, wherein multiple doses of said Hsp90 inhibitor are administered to said subject.

31. A method for monitoring dosage of a known heat shock protein 90 (Hsp90) inhibitor in treatment of a subject for a tumor or other cellular proliferative disorder, said method comprising:

a) obtaining a baseline biological sample from said subject prior to administering a dose of said Hsp90 inhibitor, wherein said baseline biological sample consists of peripheral blood mononuclear cells (PBMCs);

b) contacting said PBMCs with a stimulatory compound;

c) detecting the level of phosphorylated ribosomal protein S6 (p-RPS6) in said PBMCs;

d) administering said Hsp90 inhibitor to said subject;

e) obtaining from said subject at least one subsequent biological sample consisting of PBMCs;

f) contacting said at least one subsequent sample of PBMCs with a stimulatory compound;

g) detecting the level of said p-RPS6 in said at least one subsequent sample of PBMCs; and

h) comparing the level of said p-RPS6 in said at least one subsequent sample of PBMCs with the level of said p-RPS6 in said baseline biological sample of PBMCs, and correlating a change in the level of said p-RPS6 with dosage of said Hsp90 inhibitor.

32. The method of claim 31, wherein a single dose of said Hsp90 inhibitor is administered to said subject.

33. The method of claim 31, wherein multiple doses of said Hsp90 inhibitor are administered to said subject.

34. The method of claim 31, wherein said stimulatory compound is selected from the group consisting of phytohemagglutinin, interleukin-2, a phorbol ester, and combinations thereof.

35. A kit comprising a stimulatory compound, an anti-phospho ribosomal protein S6 (RPS6) antibody, and instructions for use.

36. The kit of claim 35, wherein said stimulatory compound is selected from the group consisting of phytohemagglutinin, interleukin-2, a phorbol ester, and combinations thereof.

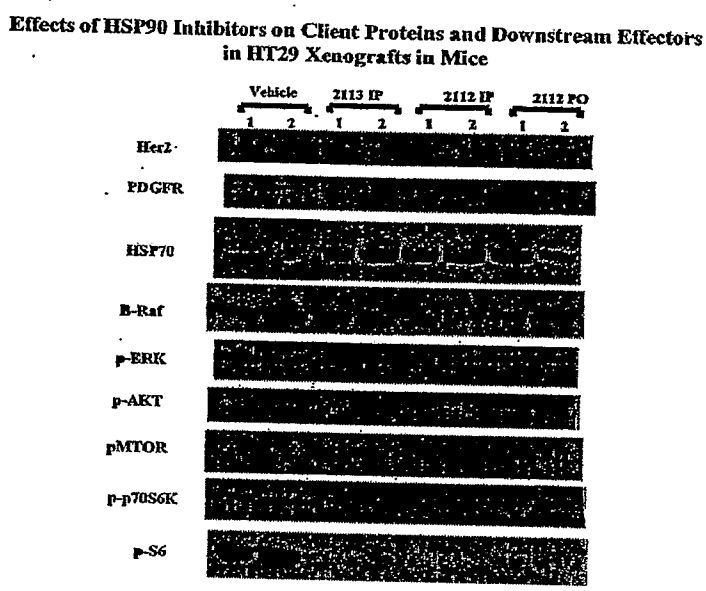
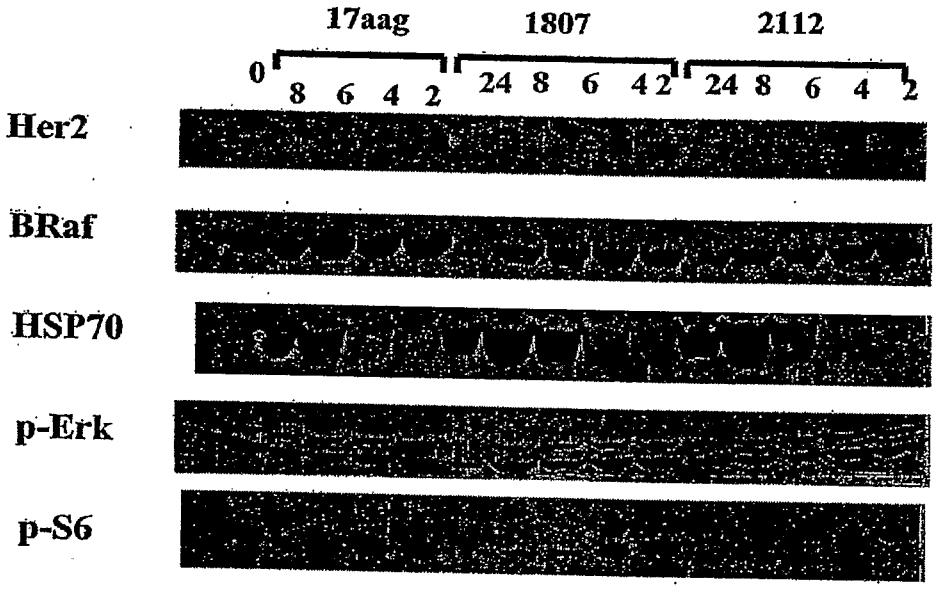

Effects of HSP90 Inhibitors on Client Proteins and Downstream Effectors in HT29 Xenografts in Mice
Vehicle
2113 IP
2112 IP
2112 PO
1 2 1 2 1 2 1 2
Her2
PDGFR
HSP70
B-Raf
p-ERK
p-AKT
pMTOR
p-p70S6K
p-S6

FIG. 1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1807
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0
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24 8 6 4 2
24 8 6 4 2
Her2
BRaf
HSP70
p-Erk
p-S6

FIG. 2

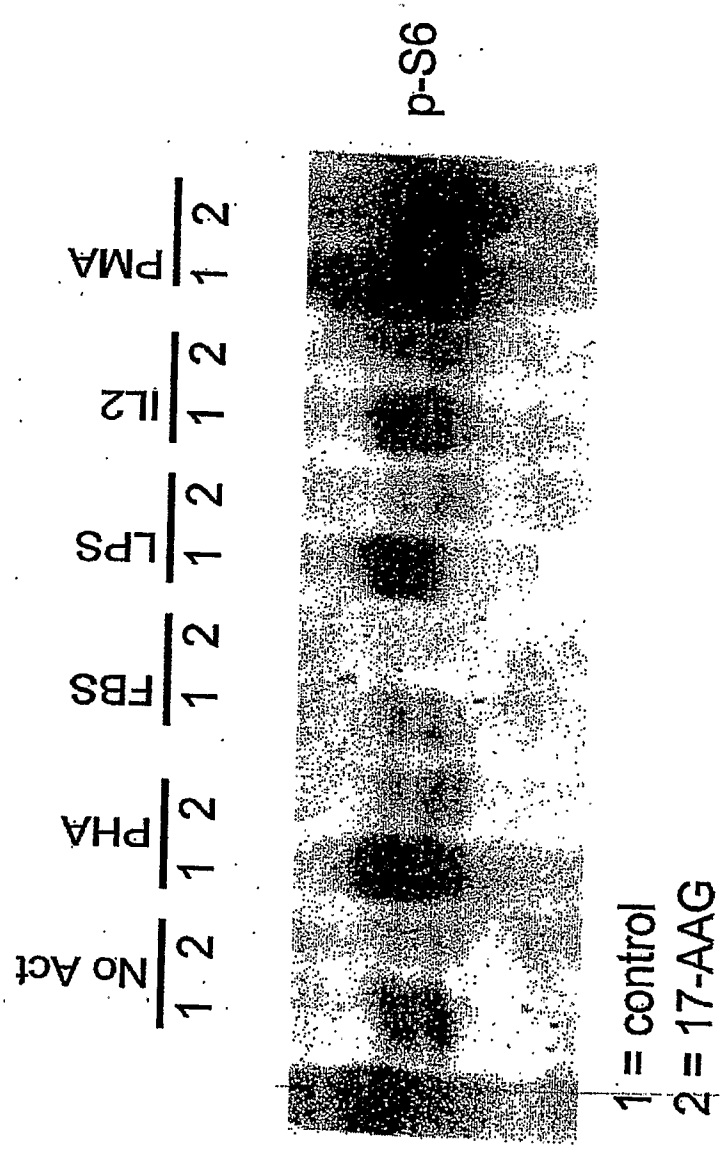

FIG. 3