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Kelland L.R. et al., J. Natl. Cancer Inst., 1999, Vol. 91, No. 22, pages 1940-1949
Marcu M.G. et al., J. Biol. Chem., 2000, Vol. 275, No. 47, pages 37181-37186.
Chiosis G. et al., Chemistry & Biology, 2001, Vol. 8, pages 289-299

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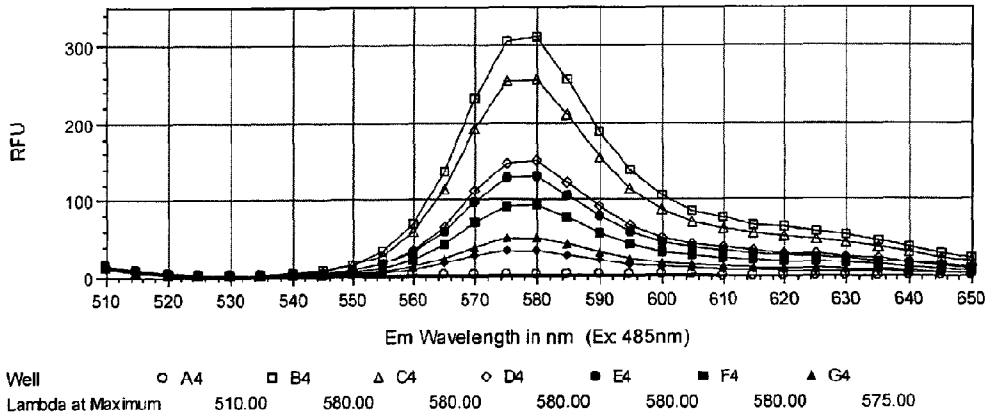
(54) Title: ASSAYS AND IMPLEMENTS FOR DETERMINING AND MODULATING HSP90 BINDING ACTIVITY



(57) Abstract: Ligand binding assays as applied to HSP90s as receptors or ligands, and reagents useful therefore, are described and claimed, as are methods of assaying for HSP90 modulators and methods of using the resulting products identified thereby.

WO 03/050295 A2

ASSAYS AND IMPLEMENTS FOR DETERMINING AND MODULATING HSP90 BINDING ACTIVITY

RELATED APPLICATIONS

This application claims priority to and herein incorporates by reference in its entirety Kamal et al., United States Provisional Patent Application Ser. No. 60/340,762, filed December 12, 2002, and entitled ASSAYS FOR DETERMINING HSP90 BINDING ACTIVITY.

FIELD OF INVENTION

The invention relates generally to assays for assessing ligand binding and binding affinity, and more specifically to heat shock protein 90 ("HSP90") binding assays.

BACKGROUND

The following description includes information that may be useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed inventions, or that any publication specifically or implicitly referenced is prior art.

17-allylamino-geldanamycin (17-AAG) is a synthetic analog of geldanamycin (GDM). Both molecules belong to a broad class of antibiotic molecules known as ansamycins. GDM, as first isolated from the microorganism *Streptomyces hygroscopicus*, was originally identified as a potent inhibitor of certain kinases, and was later shown to act by stimulating kinase degradation, specifically by targeting "molecular chaperones," e.g., heat shock protein 90s (HSP90s). Subsequently, various other ansamyins have demonstrated more or less such activity, with 17-AAG being among the most promising and the subject of intensive clinical studies currently being conducted by the National Cancer Institute (NCI). *See, e.g.*, Federal Register, 66(129): 35443-35444; Erlichman *et al.*, Proc. AACR (2001), 42, abstract 4474.

HSP90s 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. Researchers have reported that HSP90 chaperone proteins are associated with important signaling proteins, such as steroid hormone receptors and protein kinases, including, e.g., Raf-1, EGFR, v-Src family kinases, Cdk4, and ErbB-2 (Buchner J., 1999, *TIBS*, 24:136-141; Stepanova, L. *et al.*, 1996, *Genes Dev.* 10:1491-502; Dai, K. *et al.*, 1996, *J. Biol. Chem.* 271:22030-4). Studies further indicate that certain co-chaperones, e.g., 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).

Ansamycin antibiotics, e.g., herbimycin A (HA), geldanamycin (GM), and 17-AAG are thought to exert their anticancerous effects by tight binding of the N-terminus pocket of HSP90 (Stebbins, C. *et al.*, 1997, *Cell*, 89:239-250). This pocket is highly conserved and has weak homology to the ATP-binding site of DNA gyrase (Stebbins, C. *et al.*, *supra*; Grenert, J.P. *et al.*, 1997, *J. Biol. Chem.*, 272:23843-50). Further, ATP and ADP have both been shown to bind this pocket with low affinity and to have weak ATPase activity (Proromou, C. *et al.*, 1997, *Cell*, 90: 65-75; Panaretou, B. *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, T., H. *et al.*, 1999, *Proc. Natl. Acad. Sci. U S A* 96:1297-302; Schulte, T. W. *et al.*, 1995, *J. Biol. Chem.* 270:24585-8; Whitesell, L., *et al.*, 1994, *Proc. Natl. Acad. Sci. U S A* 91:8324-8328). Ansamycins have also been demonstrated to inhibit the ATP-dependent release of chaperone-associated protein substrates (Schneider, C., L. *et al.*, 1996, *Proc. Natl. Acad. Sci. U S A*, 93:14536-41; Sepp-Lorenzino *et al.*, 1995, *J. Biol. Chem.* 270:16580-16587). In either event, the substrates are degraded by a ubiquitin-dependent process in the proteasome (Schneider, C., L., *supra*; Sepp-Lorenzino, L., *et al.*, 1995, *J. Biol. Chem.*, 270:16580-16587; Whitesell, L. *et al.*, 1994, *Proc. Natl. Acad. Sci. USA*, 91: 8324-8328).

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, *e.g.*, Raf (Schulte, T. W. *et al.*, 1997, *Biochem. Biophys. Res. Commun.* 239:655-9; Schulte, T. W., *et al.*, 1995, *J. Biol. Chem.* 270:24585-8), nuclear steroid receptors (Segnitz, B., and U. Gehring. 1997, *J. Biol. Chem.* 272:18694-18701; Smith, D. F. *et al.*, 1995, *Mol. Cell. Biol.* 15:6804-12), *v-src* (Whitesell, L., *et al.*, 1994, *Proc. Natl. Acad. Sci. U S A* 91:8324-8328) and certain transmembrane tyrosine kinases (Sepp-Lorenzino, L. *et al.*, 1995, *J. Biol. Chem.* 270:16580-16587) such as EGF receptor (EGFR) and Her2/Neu (Hartmann, F., *et al.*, 1997, *Int. J. Cancer* 70:221-9; Miller, P. *et al.*, 1994, *Cancer Res.* 54:2724-2730; Mimnaugh, E. G., *et al.*, 1996, *J. Biol. Chem.* 271:22796-801; Schnur, R. *et al.*, 1995, *J. Med. Chem.* 38:3806-3812), CDK4, and mutant p53. Erlichman *et al.*, Proc. AACR (2001), 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, R. C. *et al.*, 1998, *J. Biol. Chem.* 273:29864-72), and apoptsosis, and/or differentiation of cells so treated (Vasilevskaya, A. et al., 1999, *Cancer Res.*, 59:3935-40). Ansamycins and HSP90 ligands in general thus hold great promise for the treatment and/or prevention of many types of cancers and proliferative disorders.

In addition to anti-cancer and antitumorgenic 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.*, Rosen et al., WO 02/09696; PCT/US01/23640; Degranco et al., WO 99/51223; PCT/US99/07242; Gold, U.S. Patent 6,210,974 B1). There are reports in the literature that fibrogenetic disorders including but not limited to scleroderma, polymyositis, systemic lupus, rheumatoid arthritis, liver cirrhosis, keloid formation, interstitial nephritis, and pulmonary fibrosis may be treatable. (Strehlow, WO 02/02123; PCT/US01/20578). Still further HSP90 modulation, modulators, and uses thereof are reported in PCT/US98/09805, PCT/US00/09512, PCT/US01/09512, PCT/US01/23640, PCT/US01/46303, PCT/US01/46304, PCT/US02/06518, PCT/US02/29715, PCT/US02/35069, PCT/US02/35938, 60/293,246, 60/371,668, 60/331,893, 60/335,391, 06/128,593, 60/337,919, 60/340,762, 60/355,275, 60,367,055 and 60/359,484.

Recently, Nicchitta et al., WO 01/72779 (PCT/US01/09512), demonstrated that HSP90 can assume a different conformation upon heat shock and/or binding by the fluorophore bis-ANS. Specifically, Nicchitta et al. demonstrated that this induced conformation exhibits a higher affinity for certain HSP90 ligands than for a different form of HSP90 that predominates in normal cells.

A fundamental step in identifying and evaluating HSP90 ligands is to be able to conveniently assay their binding affinity for HSP90. A variety of nonisotopic procedures, e.g., colorimetric, enzymatic, and densitometric, afford sufficient sensitivity in other contexts where they are preferred over isotopic procedures for health and disposal reasons and Chiosis *et al.*, Chemistry and Biology 8:289-299 (2001), recently described a procedure for evaluating HSP90 ligand ability. The Chiosis procedure, however, is cumbersome and time-consuming from the standpoint of requiring gels to be run, blotted, and probed with antibody. The Chiosis assay is further limited in its ability to conveniently support high-throughput screening. Further, it appears that Chiosis employed a standard form of HSP90 that is characteristic of normal, healthy cells.

Alternative and preferably simplified assays are therefore needed that facilitate high throughput screening for, and evaluation of, compounds that bind HSP90s. Also needed, e.g., for proof in clinical trial studies, are forms of HSP90 that more closely resemble or mimic those found in abnormal cells, e.g., cancer or tumor cells.

Summary of the Invention

The invention features convenient binding assays and reagents for identifying and/or evaluating HSP90 ligands. The ligands identified thereby can then be used to treat or prevent various HSP90 mediated diseases.

According to the invention there is provided a method for modulating a high affinity form of HSP90, having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, that is found in cancer or tumor cells, comprising contacting said high affinity form with an HSP90 modulator to thereby modulate said high affinity form of HSP90.

According to the invention there is also provided a method for degrading HSP90 client proteins by specifically modulating a high affinity form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, that is found in cancer or tumor cells.

According to the invention there is also provided a method of screening for HSP90 modulators, comprising:

providing a high affinity form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less that is found in cancer or tumor cells;

contacting said HSP90 form with a compound of interest; and measuring or evaluating the ability of said compound to modulate said HSP90 form.

According to the invention there is also provided a purified or isolated preparation or complex of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, taken from tumor or cancer cells exhibiting high affinity binding for an HSP90 modulator.

According to the invention there is also provided an assay that measures the binding of a compound of interest to a particular form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells.

According to the invention there is also provided a method of evaluating the ability of a compound of interest to bind an HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells comprising the steps:

providing an HSP90 member having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells, an HSP90 ligand member, and a compound of interest member together on a solid support under conditions sufficient for one or the other of said HSP90 ligand member and said compound of interest member to complex with said HSP90 member and be retained on said solid support; one of said members comprising a label;

removing members that are not complexed on or otherwise conjugated to said solid support; and

assaying said solid support for the presence of said label as a measure of the ability of said compound of interest member to bind said HSP90 member.

According to the invention there is also provided a composition comprising a labeled ansamycin.

According to the invention there is also provided a complex comprising a biotinylated ansamycin bound to an HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells, said HSP90 optionally bound to a solid support.

In a first aspect, the invention features an assay that is preferably, although not necessarily, a competitive binding assay. The assay features a first HSP90 ligand that is

labeled and that in practice is more or less displaced by or displaces a second HSP90 ligand when in complex form with HSP90. The second ligand may either be unlabeled or differently labeled from the first ligand. At least one of the ligands is preferably known in advance to be an HSP90 ligand and its ligand ability for HSP90 is preferably not substantially affected by the presence of the label. In embodiments where the second

ligand is also labeled, its binding ability to HSP90 preferably also is not substantially affected by the presence of the label attached to it. The assay may be conveniently streamlined by allowing for HSP90:ligand complex retention and detection on a solid support matrix by assaying for the presence of the label(s). Such a system lends well to high throughput screening and can assume a variety of configurations, as will be appreciate by those of skill in the art. The relative amount of label present or absent determines ligand binding ability. This can be a diagnostic, qualitative approach to determine whether or not a compound of interest is a suitable ligand or not and/or can be a quantitative approach designed to determine precise and/or relative binding affinities for one or more ligands of interest.

The labels can assume a variety of forms. They may be direct or indirectly attached to the ligand(s) and, depending on the specific embodiment, also to the HSP90 (receptor). An example of a direct approach is where, for example, a fluor, a dye, an enzyme, or a radioisotope is covalently bound to the ligand or receptor and affords a label. An example of an indirect approach is a biotin:avidin or biotin:streptavidin linkage in which the known control ligand or receptor is biotinylated and a separate avidin/streptavidin component, while technically on another molecule, is brought in proximity to the biotinylated compound to thereby provide for a label. Labels can be radioactive, fluorescent, colorimetric, enzymatic, densitometric, and/or anything else capable of distinguishing one ligand or ligand:receptor complex for another ligand or ligand:receptor complex. A variety of devices for performing or assisting in such detections are well known in the art and include, e.g., spectrofluorometers, spectrophotometers, mass spectrometers and light scattering devices, densitometers, fluorescence activated cell sorters (FACS), cameras and digital or nondigital imaging devices having appropriate color filters, scintillation counters, luminometers, etc.

In embodiments utilizing a solid support matrix, one of the component members, ligand or receptor, is made to adhere to the solid support in such a way that it retains adherence even when complexed with its corresponding binding member (receptor or ligand, depending on which member is adhered). The adhesion and exact solid support composition and configuration can vary according to many well known techniques in the art. An analogous example for one embodiment may be drawn to Enzyme Linked

Immunosorbant Assays (ELISAs) in which an antibody molecule is fixed to a solid support and used to screen for antigens, or vice-versa.

It is possible for both the control (known) ligand and the receptor (HSP90) to be labeled with the same label, and complexes discriminated from noncomplexes by adjusting the detection device or means for threshold label intensity and/or by subtracting a base intensity supplied by a noncomplexed but labeled and adhered/conjugated component. Appropriate positive and/or negative controls facilitate this and may be included in the assay methods of the invention.

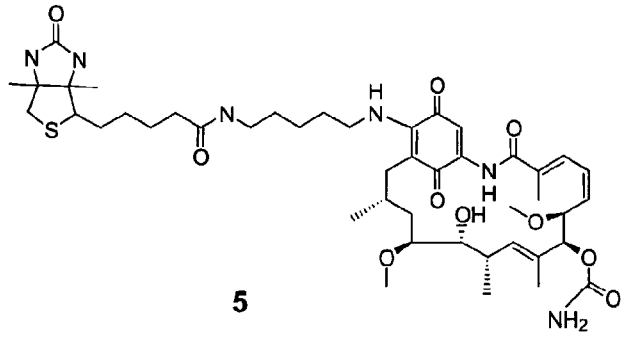
Control (standard/known) HSP90 ligands are preferably those that attach to the N-terminus ATP binding pocket of HSP90 (Stebbins, C. *et al.*, 1997, *Cell*, 89:239-250), and include but are not limited to such molecules as ansamycins and purines. Examples of the first include, e.g., geldanamycin, and examples of the second include, e.g., PU3 (*see, e.g.*, Chiosis et al., *supra*). One of skill will appreciate that numerous other ansamcyins and purines exist that can be substituted in lieu of geldanamycin and PU3.

The assays of the invention can be in vitro assays in which all of the individual components are present outside of a live cell. Alternatively, the assays can be made to be in vivo wherein the receptors and ligands are contacted with or inherently present in a live cell. Cells, as is well known in the art, can also be made to adhere to a variety of solid support matrices. So can isolated HSP90 molecules or complexes that exist in lysates or that are purified.

In another aspect, the invention features a method of evaluating the ability of a compound of interest to bind an HSP90. The method features contacting three members (an HSP90, a known HSP90 ligand, and a second possible HSP90 ligand) on a solid support such that one or more receptor:ligand complexes are formed and retained thereon. Retention on the solid support is afforded, e.g., by one of the members, e.g., the HSP90 member, being adhered to or conjugated to the support in such a way that it can still be bound by at least one of the other members and form a complex. Nonconjugated (nonadhered) and noncomplexed components can be conveniently washed away from the support leaving only complexes and adhered/conjugated components on the support, which are then evaluated for the presence of label. The amount of label present

determines how good or bad a particular HSP90 ligand is. Comparisons and hierarchical rankings between different known and unknown HSP90 ligands are also contemplated for some embodiments. Although the claims recite singular members, it is clear that homogenous populations of such members can be present therein, and that the label detected is representative of the total number of labeled species of the population retained on the solid support. Further, the claim term "removing" does not necessarily connote complete 100% removal.

In one preferred embodiment, the HSP90 member is conjugated/adhered to the solid support and the HSP90 control ligand member is labeled and the compound to be tested or evaluated for ligand ability (compound of interest) is unlabeled or differently labeled such that it can be differentiated from the other. The control HSP90 ligand member in some preferred embodiments is biotinylated and comprises a structure of formula 5:



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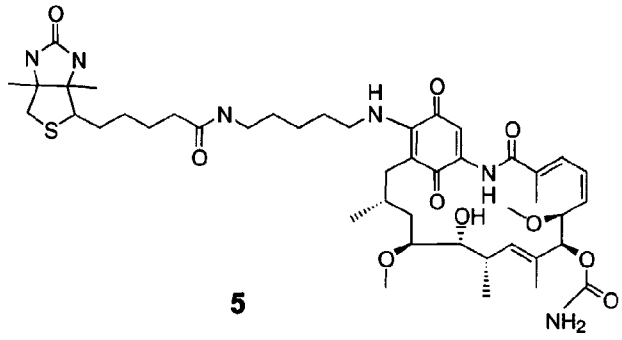
wherein the label in practice further comprises an independent avidin or streptavidin component electrostatically bound to said structure.

In some embodiments, the solid support is a multiwell plate suitable for high throughput screening. A variety of compositions and configurations of solid supports exist that can be exploited by one or ordinary skill in the art for high throughput screening purposes or for non high throughput screening purposes. In some embodiments having multiwell plates, there are different concentrations of one or more of the binding members as between two or more of the different wells on the plate.

In another aspect, the invention features novel reagents useful in performing the assay methods of the previous aspects. Such reagents can include labeled ansamycins,

purines, other HSP90 ligands, and one or more HSP90s. In some preferred embodiments, the label is afforded indirectly by ways of a biotinylation and the addition of a separated avidin or streptavidin complex. In other embodiments, the label is directly affixed. In some preferred embodiments, the label is afforded by a biotinylated ansamycin, more preferably a biotinylated geldanamycin, preferably but not necessarily one of formula 5



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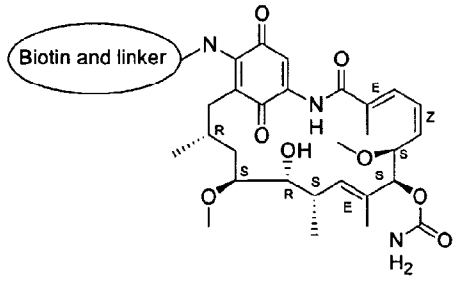
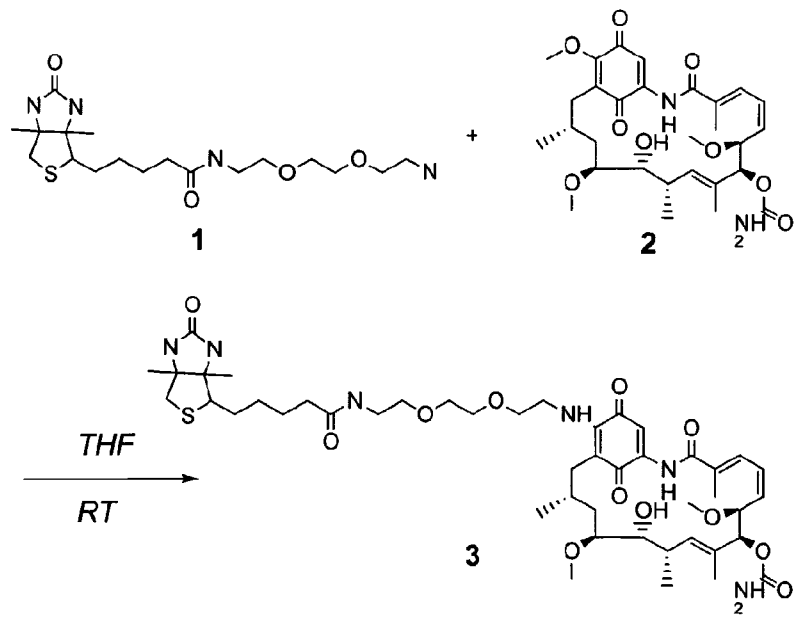
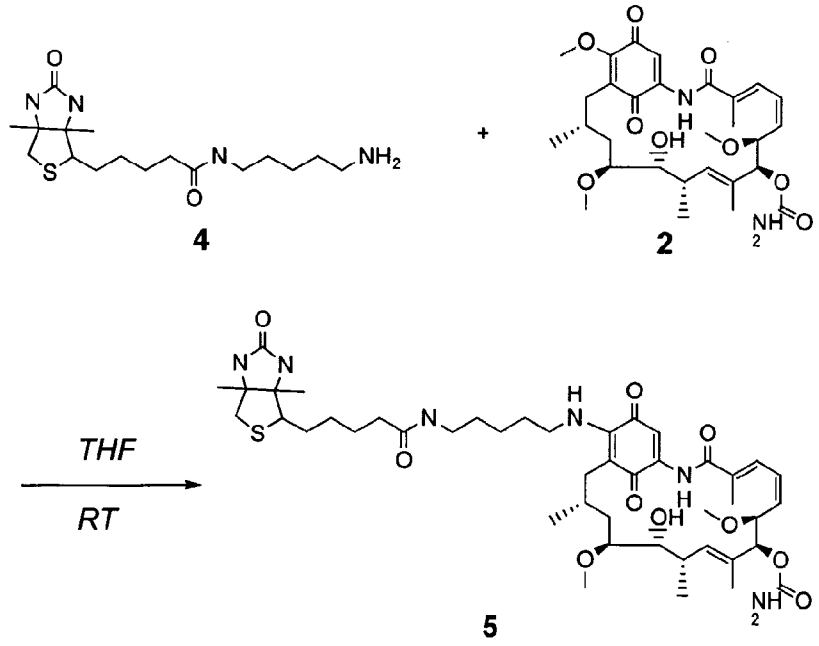
One of skill will appreciate that biotin labeling can occur at different positions on a given ligand or receptor. The above ligand, geldanamycin, is shown biotinylated at the carbon 17 position, and other derivatives can also be fashioned at this particular position on the molecule, e.g., a pyrine can be used in place of the biotin by reacting, e.g., a 1-pyrene butyl amine (Pierce Biochemical) at this position on geldanamycin. Other "direct" and "indirect" labels can similarly be tethered to this and other positions. In addition, the labels may be separated from the base ligand, here geldanamycin, by a variety of different linkers as known in the art. For geldanamycin/biotin embodiments, this may be illustrated as follows.



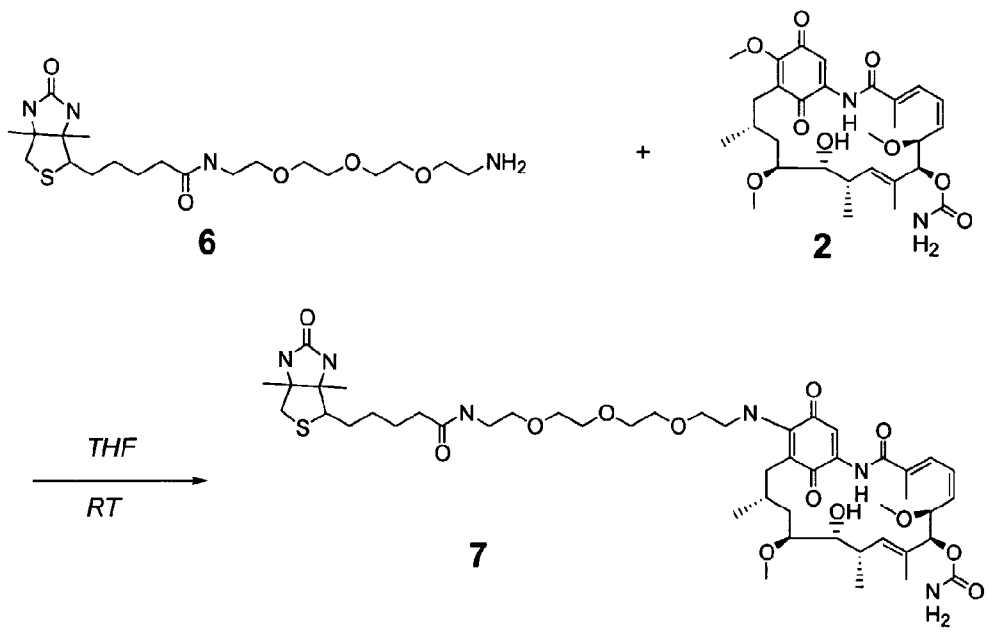
In another aspect, the invention features different methods of making a biotinylated geldanamycin derivative useful in the preceding inventive assay methods. One embodiment features the following scheme for synthesizing such a reagent:



Another features the following synthetic scheme:



Still another features the following synthetic scheme:



In another aspect, the invention features a purified isolated, or simulated conformation, preparation or complex of HSP90 as found in tumor or cancer cells. Applicants have determined that such HSP90 forms are different than those found in normal cells. Applicants have found, for example, that HSP90 conformations or complexes as found in tumor cells, cancer cells, or lysates thereof exhibit a relatively higher affinity for the known HSP90 modulator, 17-allylaminogeldanamycin (17-AAG), than HSP90 conformations found in normal cells, than conformations produced by heat-shocking normal cells, and conformations produced by binding normal cell HSP90s with bis-ANS as reported by Nicchitta. Specifically, Applicants have found that certain tumor and cancer cell conformations are bound at their ATP binding site by modulators approximately 5x better than heat-shock conformations and approximately 10x better than HSP90/bis-ANS conformations. This translates to the ability to more readily identify high-affinity modulators of HSP90, especially of those HSP90 conformations that exist in abnormal cells. One of skill in the art can titrate effective amounts of such compounds relative to such cell-types, effectively targeting those cells and cell-types preferentially while minimizing the effect on normal cells. In some embodiments, the abnormal cells are melanoma, breast cancer, or lung cancer cells. In some embodiments, the high affinity conformation is purified to from between about 0.01% and 99.9% relative to how they

exist in abnormal cells. In some embodiments, the conformation is present as a crude cellular lystate. In some embodiments, the HSP90 is recombinant HSP90, i.e., has been introduced to a cell line using recombinant DNA techniques. In some embodiments featuring HSP90's isolated or purified from cancer, tumor, or recombinant cells, the cells are also heat shocked. In some embodiments, the HSP90s are bound to another compound covalently or non-covalently such that the overall complex binds HSP90 modulators more readily or avidly. Such "additional" compounds may take the form of one or more HSP90 client proteins or co-chaperone proteins as known in the art and be supplied, e.g., using biochemical extracts purified or taken from other cells or cell lines, or even the same line(s). In some embodiments, such complexes and conformations are present on solid support such as a microtiter dish, well or plate, resinous bead, or other solid support form as known in the art.

The screening assays of the invention can be used to assay for HSP90 modulators, which can take the form of inhibitors or activators, antagonists or agonists. Such modulators can assume any form, e.g., they can be small molecule, peptide, cyclic, organic, inorganic, etc. Particularly preferred types of compounds that can be screened for HSP90 binding or modulating activity include purines or purine analogs, ansamycins, radicicol, zearalanols, ATP analogs, indoles, chalcones, and benzimidazoles, which compound types are well known in the art. In some embodiments, the assays are in vitro assays, e.g., where the HSP90 modulators are supplied outside a live cell in lysates or purified form. Other assays are in vivo assays, e.g., ones that use live cells, whether isolated or still present in a tissue or multicellular host organism.

In another aspect, the invention features a method of treating or preventing an HSP90-mediated disease by administering to a subject a pharmaceutically effective amount of a compound or pharmaceutically acceptable salt thereof which modulates a higher affinity form of HSP90. Preferred agents are selective for the high affinity form over the lower affinity form found in normal cells, and can be identified according to the screening methods of any of the preceding aspects. In some preferred embodiments, it is a tumor or cancer that is to be treated or prevented. In other embodiments, it is viral or bacterial infection. In still other embodiments it is used to combat or treat ischemia, or other disorders. Some cancer cells, e.g., certain breast cancer cells are known to express

supranormal levels of Her-2 transcript or protein, and are targeted in some method embodiments of the invention. Modes of treatment or prevention can include, for example, oral, parenteral, topical, or in situ administration formats. The treated subject is preferably a mammal, more preferably a mouse or rat, and most preferably a human. Some embodiments feature a combinatorial method or chemotherapy regimen that further includes administration of one or more members selected from the group consisting of radioisotopes, antibodies, recombinant products, small molecules, antineoplastic agents, Herceptin, taxol, taxanes and taxane derivatives, gleevec, alkylating agents, anti-metabolites; epidophyllotoxin; an antineoplastic enzyme; a topoisomerase inhibitor; procarbazine; mitoxantrone; platinum coordination complexes; biological response modifiers/growth inhibitors; hormonal/anti-hormonal therapeutic agents and haematopoietic growth factors, anthracycline drugs, vinca drugs, mitomycins, bleomycins, cytotoxic nucleosides, tepothilones, discodermolide, pteridine drugs, diynenes, podophyllotoxins, carminomycin, daunorubicin, aminopterin, methotrexate, methopterin, dichloromethotrexate, mitomycin C, porfiromycin, 5-fluorouracil, 6-mercaptopurine, gemcitabine, cytosine arabinoside, podophyllotoxin, podo-phyllotoxin derivatives, etoposide, etoposide phosphate or teniposide, melphalan, vinblastine, vincristine, leurosidine, vindesine, leurosine, paclitaxel, estramustine, carboplatin, cyclophosphamide, bleomycin, gemcitibine, ifosamide, melphalan, hexamethyl melamine, thiotepa, cytarabin, idatrexate, trimetrexate, dacarbazine, L-asparaginase, camptothecin, CPT-11, topotecan, ara-C, bicalutamide, flutamide, leuprolide, pyridobenzoindole derivatives, interferons and interleukins.

In another aspect, the invention features a diagnostic kit comprising one or more members selected from the group consisting of: (a) the isolated, purified, or simulated preparation of HSP90 as discussed above, and (b) a compound that binds HSP90 with an IC50 of less than 20 nM, preferably less than about 10 nM. The kit can include isolated, purified or simulated preparations of HSP90 that may take the form of whole cells, cell lysates, or purified extracts. In addition, known HSP90 activators, inhibitors, antagonists and/or agonists can be supplied. Lower affinity forms of HSP90 can also be supplied, e.g., for use as negative controls. Still further, the kits can feature one or more members selected from the group consisting of a resin, a bead, a lysis buffer, a labeled HSP90

ligand, and a protocol. In some embodiments, the HSP90 ligand is labeled, e.g., with biotin-geldanamycin conjugate as described herein.

One of skill will appreciate that various embodiments and aspects of the invention as discussed above can be combined, where appropriate.

The assay methods and reagents of the invention save time, labor, and/or materials over existing HSP90 binding assays, e.g., that described by Chiosis *et al*, *supra*, and, further, they promote high throughput screening and the identification of ligands that bind to the higher affinity form of HSP90 that is associated with various disease states, and more particularly, diseased cells. This bodes excellent utility in future clinical trial studies. Other advantages, aspects, and embodiments of the invention will be apparent from the figures, the detailed description, and claims to follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows competitive binding of geldanamycin and biotinylated geldanamycin for HSP90.

Figure 2 shows competitive binding of 17-allyl amino geldanamycin (17-AAG) and biotinylated geldanamycin for HSP90.

Figure 3 shows competitive binding of free geldanamycin, 17-AAG , and biotinylated geldanamycin for HSP90 .

Figure 4 shows that 17-AAG (CF7) has a higher apparent binding affinity for HSP90 from tumor cells (BT474) than normal cells (fibroblasts, RPTEC) or purified HSP90 alone, as determined using methods described herein.

Figure 5 shows that 17-AAG (CF7) has a higher apparent binding affinity for HSP90 from the specific high Her2 expressing cells, SKOV-3, SKBR-3, and N87, than from normal cells, heat-shocked HSP90, or bis-ANS treated HSP90.

Figure 6 shows the results of various test compounds used in certain assay embodiments of the invention. The cell line used was MCF7. Synthesis and use of the modulators shown is described in US Ser. No. 60/367,055 and/or PCT/US02/29715.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The terms used in the claims all have well known meanings in the pertinent art.

A "pharmaceutically acceptable salt," for example, may be prepared for any compound of the appropriate aspect of the invention having a functionality capable of forming a salt, for example an acid or base functionality. Pharmaceutically acceptable salts may be derived from organic or inorganic acids and bases. Compounds of the invention that contain one or more basic functional groups, e.g., amino or alkylamino, are capable of forming pharmaceutically acceptable salts with pharmaceutically acceptable organic and inorganic acids. These salts can be prepared in situ during the final isolation and purification of the compounds of the invention, or by separately reacting a purified compound of the invention in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed. Examples of suitable acids include hydrochloric, hydrobromic, sulfuric, nitric, perchloric, fumaric, maleic, phosphoric, glycolic, gluconic, lactic, salicylic, succinic, toluene-p-sulfonic, tartaric, acetic, citric, methanesulfonic, formic, benzoic, malonic, naphthalene-2-sulfonic, benzenesulfonic, 1,2 ethanesulfonic acid (edisylate), galactosyl-d-gluconic acid, and the like. Other acids, such as oxalic acid, while not themselves pharmaceutically acceptable, may be employed in the preparation of salts useful as intermediates in obtaining the compounds of this invention and their pharmaceutically acceptable acid addition salts. See, e.g., Berge et al. "Pharmaceutical Salts", J. Pharm. Sci. 66:1-19 (1977).

Compounds of the present invention that contain one or more acidic functional groups are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of compounds of the present invention. These salts can likewise be prepared in situ during the final isolation and purification of the compounds, or by separately reacting the purified compound in its free acid form with a suitable base, such as the hydroxide, carbonate or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary or tertiary amine. Representative alkali or alkaline

earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Illustrative examples of some of the bases that can be used include sodium hydroxide, potassium hydroxide, choline hydroxide, sodium carbonate, and the like. Representative organic amines useful for the formation of base addition salts include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. See, for example, Berge et al., supra.

A "pharmacological composition" refers to a mixture of one or more of the compounds described herein, or pharmaceutically acceptable salts thereof, with other chemical components, such as pharmaceutically acceptable carriers and/or excipients. The purpose of a pharmacological composition is to facilitate administration of a compound to an organism.

The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations. A physiologically acceptable carrier should not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound.

An "excipient" refers to an inert substance added to a pharmacological composition to further facilitate administration of a compound. Examples of excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

A "pharmaceutically effective amount" means an amount which is capable of providing a therapeutic and/or prophylactic effect. The specific dose of compound administered according to this invention to obtain therapeutic and/or prophylactic effect will, of course, be determined by the particular circumstances surrounding the case, including, for example, the specific compound administered, the route of administration, the condition being treated, and the individual being treated. A typical daily dose (administered in single or divided doses) will contain a dosage level of from about 0.01 mg/kg to about 50-100 mg/kg of body weight of an active compound of the invention. Preferred daily doses generally will be from about 0.05 mg/kg to about 20 mg/kg and ideally from about 0.1 mg/kg to about 10 mg/kg. Factors such as clearance rate and half-life and maximum tolerated dose (MTD) have yet to be determined but one of ordinary skill in the art can determine these using standard procedures.

In some method embodiments, the preferred therapeutic effect is the inhibition, to some extent, of the growth of cells characteristic of a proliferative disorder, e.g., breast cancer. A therapeutic effect will also normally, but need not, relieve to some extent one or more of the symptoms other than cell growth or size of cell mass. A therapeutic effect may include, for example, one or more of 1) a reduction in the number of cells; 2) a reduction in cell size; 3) inhibition (i.e., slowing to some extent, preferably stopping) of cell infiltration into peripheral organs, e.g., in the instance of cancer metastasis; 3) inhibition (i.e., slowing to some extent, preferably stopping) of tumor metastasis; 4) inhibition, to some extent, of cell growth; and/or 5) relieving to some extent one or more of the symptoms associated with the disorder.

In some method embodiments of the invention, the "IC_{50}" value of a compound of the invention can be greater for normal cells than for cells exhibiting a proliferative disorder, e.g., breast cancer cells. The value depends on the assay used.

By a "standard" is meant a positive or negative control. A negative control in the context of HER-2 expression levels is, e.g., a sample possessing an amount of HER-2 protein that correlates with a normal cell. A negative control may also include a sample that contains no HER-2 protein. By contrast, a positive control does contain HER-2 protein, preferably of an amount that correlates with overexpression as found in proliferative disorders, e.g., breast cancers.. The controls may be from cell or tissue samples, or else contain purified ligand (or absent ligand), immobilized or otherwise. In some embodiments, one or more of the controls may be in the form of a diagnostic "dipstick."

By "selectively targeting" is meant affecting one type of cell to a greater extent than another, e.g., in the case of cells with high as opposed to relatively low or normal Her-2 levels.

The invention features, in some aspects, assays for identifying and/or evaluating HSP90 ligands and reagents useful in such assays. In one aspect, an HSP90 is contacted with a known HSP90 ligand, e.g., an ansamycin such as geldanamycin or 17-AAG. The ligand is "labeled" to permit detection of its binding with HSP90. During the assay, the labeled ligand's ability to bind to, or remain bound to, HSP90, is potentially competed with by the co-presence of a compound of interest suspected of having, or being screened for, HSP90 binding ability. Binding ability and affinity of the compound of interest is based on the amount of signal present by way of the competing ligand. Label is preferably detected on a solid support, e.g., a multiwell dish or plate, which detection may be aided by the use of various commercially available detection devices well known in the art.

In one embodiment, the invention features an assay in which the label is a fluorescent molecule or fluor capable of excitation and measurement, e.g., phycoerythrin. In one embodiment, a strepavidin-phycoerythrin molecule is attached to a biotinylated geldanamycin compound which serves as the control HSP90 ligand; geldanamycin is known to bind HSP90. The ability of these molecules to bind one another (complex) is challenged with a nonlabeled or differently labeled compound of interest that can displace or be displaced by (to more or less degree) the compound of interest being tested for HSP90 binding ability. In this way, binding ability and affinity can be determined as a function of remaining ligand label. There may be a direct or reciprocal correlation of label

to compound of interest binding affinity, depending on how the assay is configured. In embodiments where the complexes are affixed to or otherwise created on a solid support matrix, e.g., a multiwell plate, the amount of labeled complex can be detected to give an indication of the binding ability of, and/or measured affinity for, HSP90 by the compound of interest.

Another aspect of the invention features a labeled, e.g., biotinylated, HSP90 ligand, the utility of which is clear in light of the foregoing aspect. In yet another aspect, the invention features HSP90:labeled ligand complexes.

Details of the various assay components and methodologies that can be used, as well as specific working examples of some, follows. Those of ordinary skill in the art can put the different possibilities into use without undue experimentation.

Ansamycins

As described, various ansamycins are known to bind and inhibit HSP90 function. The term "ansamycin" as used herein is well-known in the art and refers to a broad class of structures characterized by aliphatic rings of various length and constitution bridging opposite ends of aromatic ring structures and their reduced equivalents. Subsumed within this broad class is the sub-class, benzoquinone ansamycins. A "benzoquinone ansamycinin" as used herein possesses a benzoquinone as the aromatic ring structure and includes any benzoquinone ansamycinin known in the art having an alkoxy moiety on the benzoquinone portion of the molecule, preferably a methoxy, and preferably at the 17 position, that can be replaced by a nucleophile. The result of the reaction is the formation of a "benzoquinone ansamycin derivative." Ansamycins and benzoquinone ansamycins according to the invention may be synthetic, naturally-occurring, or a combination of the two, i.e., "semi-synthetic." Exemplary benzoquinone ansamycins useful in the processes of the invention and their methods of preparation include but are not limited to those described, e.g., in U.S. Patents 3,595,955 (describing the preparation of geldanamycin), 4,261,989, 5,387,584, and 5,932,566. Geldanamycin is also commercially available, e.g., from CN Biosciences, an Affiliate of Merck KGaA, Darmstadt, Germany, and headquartered in San Diego, California, USA (cat. no. 345805. The biochemical purification of 4,5-Dihydrogeldanamycin and its hydroquinone from cultures of

Streptomyces hygroscopicus (ATCC 55256) are described in International Application Number PCT/US92/10189, assigned to Pfizer Inc., published as WO 93/14215 on July 22, 1993, and listing Cullen *et al.* as inventors; an alternative method of synthesis for 4,5-Dihydrogeldanamycin by catalytic hydrogenation of geldanamycin is also known. *See, e.g.*, Progress in the Chemistry of Organic Natural Products, *Chemistry of the Ansamycin Antibiotics*, 33 1976, p. 278. Applicants recently described the preparation of numerous other ansamycin-type compounds in co-pending applications 60/367,055 and PCT/US02/29715.

HSP90s

HSP90 proteins are ubiquitous cellular proteins that are highly conserved in nature. The term "an HSP90" or "an HSP90 member" as claimed herein includes but is not limited to the following: NCBI accession #'s P07900 and XM 004515 (human α and β HSP90, respectively), P11499 (mouse), AAB2369 (rat), P46633 (chinese hamster), JC1468 (chicken), AAF69019 (flesh fly), AAC21566 (zebrafish), AAD30275 (salmon), O02075 (pig), NP 015084 (yeast), and CAC29071 (frog). Further included in the definition are any variations of such proteins that may exist in nature or that are man-made. All are expected to have more or less utility in connection with the methods, assays, and ligands of the invention, i.e., in identifying and/or quantifying binding affinities of various HSP90 ligands, and thereby identifying and/or prioritizing new drug candidates for clinical trials. One aspect of the invention exploits Applicants' finding that cancer and tumor cells possess a more sensitive form of HSP90s than do normal cells. Ligands bind HSP90s found in cancer or tumor cells much more avidly despite the protein itself having an identical amino acid constituency. Without being limiting of the invention, this is thought to be a consequence of a different tertiary or quarternary configuration of the protein that is present in such cells, possibly being afforded by co-chaperone proteins or client proteins that are bound to the HSP90 to make it behave as such.

The following discussion sections concerning labeling and solid supports and high throughput screening are borrowed largely from United States Patents 6,203,989, 6,153,442, 6,096,508, 5,846,537, and 5,585,241. The procedures described therein and

below can be assimilated, adapted, and/or otherwise implemented in furtherance of the novel and unobvious features of the present invention.

Labels and Labeling

Biotin:(Strept)Avidin Labels. Preferred embodiments of the invention exploit the natural high affinity of streptavidin for biotin. Streptavidin is related to avidin, a 67 kilodalton (kD) glycoprotein found in egg whites and which has an exceptionally high binding affinity (K.sub.d =10.sup.-15 M) for biotin. Avidin consists of four subunits, each capable of binding one biotin molecule. Streptavidin is produced by Streptomyces avidinii and shares significant conformation and amino acid composition with avidin, as well as high affinity for biotin and stability. Streptavidin is also not glycosylated and reportedly exhibits less non-specific binding than avidin, making it the superior choice of the two for most biotin-based applications.

Biotin, a member of the B-complex vitamins, is found naturally in nature, and is essential for amino acid and fatty acid degradation, gluconeogenesis, and fatty acid synthesis. The binding interactions between biotin and the biotin binding site of avidin and streptavidin are the result of noncovalent hydrogen bonding and van der Waals interactions between biotin and avidin, together with the ordering of surface polypeptide loops that bury the biotin in the protein interior. Biotin has previously been chemically or enzymatically coupled extensively to probe biomolecules in ways that minimize interference with target recognition, and the results described herein for geldanamycin provide further examples of how this may be done.

Reagents for labeling streptavidin or avidin with a fluorescent tag are commercially available. For example, the reagents, 5(6)-Carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), 7-amino-4-methyl-coumarin-3-acetic acid-N'-hydroxysuccinimide ester (AMCA, acitvated) and fluorescein isothiocyanate (FITC) are available from Boehringer Mannheim, Indianapolis, Ind. Methods for fluorescently labeling proteins with fluorescent labels, and methods for detection of the fluorescent labels, are described in Howard, G., Labeling Proteins with Fluorochromes, in "Methods in Nonradioactive Detection,", G. Howard, Ed., Appleton and Lange, Norwalk, Conn. 1993, pp. 39-68, the disclosure of which is incorporated herein. Additionally, there are a variety of commercially available labeled streptavidin and avidin molecules. Examples

include streptavidin-gold, streptavidin-fluorochrome, streptavidin-AMCA, streptavidin-fluorescein, streptavidin-phycoerythrin (STPE), streptavidin-sulforhodamine 101, avidin-FITC and avidin-Texas red.RTM., which are commercially available from Boehringer Mannheim, Indianapolis, Ind. A working example of the use of streptavidin-phycoerythrin in the methods and reagents of the invention is described below.

Alternative labeling systems. Applicants anticipate that other labels or label complexes can also be used to generate a detectable signal to relate the amount of bound and/or unbound label. The label can be any molecule that produces or can be induced to produce a signal, and may be, for example, a fluorescer, radio-label, enzyme, chemiluminescer or photosensitizer. Thus, the signal, depending on the label embodiment, can be detected and/or measured by detecting enzyme activity, luminescence, light absorbance or radioactivity as the case may be. As described previously, nonradioactive applications are preferred but radioactive applications should not be read out of the claims where not specifically so stated in the claims.

Specific labels that can be used illustratively include, e.g., enzymes such as alkaline phosphatase, glucose-6-phosphate dehydrogenase ("G6PDH") and horseradish peroxidase; dyes; fluorescers such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde, and fluorescamine; chemiluminescers such as isoluminol; sensitizers; coenzymes; enzyme substrates; radiolabels such as .sup.125 I, .sup.131 I, .sup.14 C, .sup.3 H, .sup.57 Co and .sup.75 Se; particles such as latex or carbon particles; metal sol; crystallite; liposomes; cells, etc., which may be further labeled with a dye, catalyst or other detectable group. Other suitable enzymes and coenzymes are disclosed in Litman, et al., U.S. Pat. No. 4,275,149, columns 19-28, and Boguslaski, et al., U.S. Pat. No. 4,318,980, columns 10-14; suitable fluorescers and chemiluminescers are disclosed in Litman, et al., U.S. Pat. No. 4,275,149, at columns 30 and 31; which disclosure are herein incorporated by reference.

The label used may directly produce a signal. Alternatively, the label may indirectly produce a signal and therefore require additional reagents and/or physical stimulation, e.g., bombardment with electromagnetic energy or the addition of a chemical substrate or co-factor. In the instacce of fluorescers, for example, these are able to absorb ultraviolet and visible light where the light absorption transfers energy to these molecules and elevates them to an excited energy state, which absorbed energy is then dissipated by

emission of light at a second wavelength. By contrast, labels that directly produce a signal include, e.g., radioactive isotopes and dyes.

Examples of labels that need other reagent components to produce a signal include, e.g.. substrates and coenzymes (for enzyme labels), substances that react with enzymic products, catalysts, activators, cofactors, inhibitors, scavengers, metal ions, and a specific binding substance required for binding of signal generating substances. Additional discussion of suitable labeling systems can be found in Ullman, et al. U.S. Pat. No. 5,185,243, columns 11-13, which disclosure is herein incorporated by reference.

Solid Supports and High Throughput Screening

A solid support according to the invention can include a porous or non-porous water insoluble material that can have any one of a number of shapes, such as strip, rod, plate, well, particle or bead. A wide variety of suitable supports are disclosed in Ullman, et al. U.S. Pat. No. 5,185,243, columns 10-11, Kurn, et al., U.S. Pat. No. 4,868,104, column 6, lines 21-42 and Milburn, et al., U.S. Pat. No. 4,959,303, column 6, lines 14-31, which are incorporated herein by reference.

The solid support surface can be hydrophilic or capable of being rendered hydrophilic, e.g., by the addition of inorganic powders such as silica, magnesium sulfate, and alumina; natural polymeric materials, particularly cellulosic materials and materials derived from cellulose, such as fiber containing papers, e.g., filter paper, chromatographic paper, etc.; synthetic or modified naturally occurring polymers, such as nitrocellulose, cellulose acetate, poly (vinyl chloride), polyacrylamide, cross linked dextran, agarose, polyacrylate, polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), nylon, poly(vinyl butyrate), etc.; either used by themselves or in conjunction with other materials; glass, ceramics, metals, and the like. Binding of a member of the invention to such a support may be accomplished by well-known techniques commonly available in the literature, e.g., as described in "Immobilized Enzymes," Ichiro Chibata, Halsted Press, New York (1978) and Cuatrecasas, J. Biol. Chem., 245:3059 (1970).

Preferred solid supports include any materials that are used as affinity matrices or supports for chemical and biological molecule syntheses and analyses, such as, but not limited to: polystyrene, polycarbonate, polypropylene, nylon, glass, dextran, chitin, sand,

pumice, polytetrafluoroethylene, agarose, polysaccharides, dendrimers, buckyballs, polyacrylamide, Kieselguhr-polyacrylamide non-covalent composite, polystyrene-polyacrylamide covalent composite, polystyrene-PEG [polyethyleneglycol] composite, silicon, rubber, and other materials used as supports for solid phase syntheses, affinity separations and purifications, hybridization reactions, immunoassays and other such applications. The material of such a support may be particulate or may be in the form of a continuous surface, such as a microtiter dish or well, a glass slide, a silicon chip with a surface adapted for linking of biological particles or molecules, a nitrocellulose sheet, nylon mesh, or other such materials.

The material used should be compatible with the specific embodiment members and assay reagents and will depend on the identity and nature of the specific members employed. The surface (usually a solid) can be any of a variety of organic or inorganic materials or combinations thereof, including, merely by way of example, plastics such as polypropylene or polystyrene; ceramic; silicon; (fused) silica, quartz or glass, which can have the thickness of, for example, a glass microscope slide or a glass cover slip; paper, such as filter paper; diazotized cellulose; nitrocellulose filters; nylon membrane; or polyacrylamide or other type of gel pad, e.g., an aeropad or aerobead, made of an aerogel, which is, e.g., a highly porous solid, including a film, which is prepared by drying of a wet gel by any of a variety of routine, conventional methods. Substrates that are transparent to light are useful when the method of performing an assay involves optical detection. In a preferred embodiment, the surface is the plastic surface of a multiwell, e.g., tissue culture dish, for example a 24-, 96-, 256-, 384-, 864- or 1536-well plate (e.g., a modified plate such as a Corning Costar plate). Anchors can be associated, e.g., bound, directly with a surface, or can be associated with one type of surface, e.g., glass, which in turn is placed in contact with a second surface, e.g., within a plastic "well" in a microtiter dish. The shape of the surface, again, is not critical. It can, for example, be a flat surface such as a square, rectangle, or circle; a curved surface; or a three dimensional surface such as a bead, particle, strand, precipitate, tube, sphere; etc.

The surface can have regions which are spatially discrete and addressable or identifiable. Each region comprises a set of anchors or attachment sites. How the regions are separated, their physical characteristics, and their relative orientation to one another are not critical for some embodiments, and in others are. In one embodiment, the regions

can be separated from one another by any physical barrier which is resistant to the passage of liquids. For example, in a preferred embodiment, the regions can be wells of a multiwell (e.g., tissue culture) dish, for example a 24-, 96-, 256-, 384-, 864- or 1536-well plate. Alternatively, a surface such as a glass surface can be etched out to have, for example, 864 or 1536 discrete, shallow wells. Alternatively, a surface can comprise regions with no separations or wells, for example a flat surface, e.g., a piece of plastic, glass or paper, and individual regions can further be defined by overlaying a structure (e.g., a piece of plastic or glass) which delineates the separate regions. Optionally, a surface can already comprise one or more arrays of anchors, or anchors associated with linkers, before the individual regions are delineated. In another embodiment, arrays of anchors within each region can be separated from one another by blank spaces on the surface in which there are no anchors, or by chemical boundaries such as wax or silicones to prevent spreading of droplets. In yet another embodiment, the regions can be defined as tubes or fluid control channels, e.g., designed for flow-through assays, as disclosed, for example, in Beattie et al (1995). Clin. Chem. 4, 700-706. Tubes can be of any size, e.g., capillaries or wider bore tubes; can allow the flow of liquids; or can be partially or completely filled with a gel, e.g., agarose or polyacrylamide, through which compounds can be transported (passed through, flowed through, pumped through), e.g., by electrophoresis.

The assay methods of the invention may be conveniently performed on a solid support, such as in multi-well plates for an ELISA or on any solid support for high density or chip array analysis. For example, in an ELISA type format, the ligand or receptor molecule is adsorbed to a solid support such as the wells of a 96-well plate. The corresponding complement (receptor or ligand, whichever the case may be), is/are added to the wells and incubated. Alternatively, the complexes may first be formed and then adhered to the solid support, to be competed with later by another ligand or compound of interest. In yet another permutation of the invention, multiple ligands, at least one of which is known and labeled, are mixed together in the presence of an HSP90 and, by whatever means, noncomplexed and nonadhered species (in solid support embodiments) are removed. The amount of label is then assessed using a detection device. Removal of noncomplexed and nonadhered species can be done by wash steps, and in some

embodiments, centrifugation. Unbound ligand/receptor is washed away and the presence of labeled complex is then detected. Many variations are possible.

Detection hardware devices that can be used in connection with various embodiments of the present invention are well known in the art and include but are not limited to, e.g., densitometers, mass spectrometers, fluorometers, scintillation counters, spectrophotometers, luminometers, cameras,and other imaging or detection devices.

Assays to Determine HSP90 Binding and Downstream Effect

In addition to the innovations described herein, a variety of in vitro and in vivo assays are available to test the effect of the compounds of the invention on HSP90. HSP90 competitive binding assays and functional assays can be performed as known in the art substituting in the compounds of the invention. Chiosis et al., Chemistry & Biology 8:289-299 (2001), describe some of the known ways in which this can be done. For example, competition binding assays using, e.g., geldanamycin or 17-AAG as a competitive binding inhibitor of HSP90 can be used to determine relative HSP90 affinity of the compounds of the invention by immobilizing the compound of interest or other competitive inhibitor on a gel or solid matrix, preincubating HSP90 with the other inhibitor, passing the preincubated mix over the gel or matrix, and then measuring the amount of HSP90 that sticks or does not stick to the gel or matrix.

Downstream effects can also be evaluated based on the known effect of HSP90 inhibition on function and stability of various steroid receptors and signaling proteins including, .e.g., Raf1 and Her2. Compounds of the present invention induce dose-dependent degradation of these molecules, which can be measured using standard techniques. Inhibition of HSP90 also results in up-regulation of HSP90 and related chaperone proteins that can similarly be measured. Antiproliferative activity on various cancer cell lines can also be measured, as can morphological and functional differentiation related to HSP90 inhibition. For example, the

Many different types of methods are known in the art for determining protein concentrations and measuring or predicting the level of proteins within cells and in fluid samples. Indirect techniques include nucleic acid hybridization and amplification using, e.g., polymerase chain reaction (PCR). These techniques are known to the person of skill

and are discussed, e.g., in Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., Ausubel, et al., Current Protocols in Molecular Biology, John Wiley & Sons, NY, 1994, and, as specifically applied to the quantification, detection, and relative activity of Her-2/neu in patient samples, e.g., in U.S. Patents 4,699,877, 4,918,162, 4,968,603, and 5,846,749. A brief discussion of two generic techniques that can be used follows.

The determination of whether cells overexpress or contain elevated levels of HER-2 can be determined using well known antibody techniques such as immunoblotting, radioimmunoassays, western blotting, immunoprecipitation, enzyme-linked immunosorbant assays (ELISA), and derivative techniques that make use of antibodies directed against HER-2. As an example, HER-2 expression in breast cancer cells can be determined with the use of an immunohistochemical assay, such as the Dako Hercep™ test (Dako Corp., Carpinteria, CA). The Hercep™ test is an antibody staining assay designed to detect HER-2 overexpression in tumor tissue specimens. This particular assay grades HER-2 expression into four levels: 0, 1, 2, and 3, with level 3 representing the highest level of HER-2 expression. Accurate quantitation can be enhanced by employing an Automated Cellular Imaging System (ACIS) as described, e.g., by Press, M, et al , **2000**, Modern Pathology 13:225A.

Antibodies, polyclonal or monoclonal, can be purchased from a variety of commercial suppliers, or may be manufactured using well-known methods, e.g., as described in Harlow et al., Antibodies: A Laboratory Manual, 2nd Ed; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (**1988**).

HER-2 overexpression can also be determined at the nucleic acid level since there is a reported high correlation between overexpression of the HER-2 protein and amplification of the gene that codes for it. One way to test this is by using RT-PCR. The genomic and cDNA sequences for HER-2 are known. Specific DNA primers can be generated using standard, well-known techniques, and can then be used to amplify template already present in the cell. An example of this is described in Kurokawa, H et al, Cancer Res. 60: 5887-5894 (**2000**). PCR can be standardized such that quantitative differences are observed as between normal and abnormal cells, e.g., cancerous and

noncancerous cells. Well known methods employing, e.g., densitometry, can be used to quantitate and/or compare nucleic acid levels amplified using PCR.

Similarly, fluorescent in situ hybridization (FISH) assays and other assays can be used, e.g., Northern and/or Southern blotting. These rely on nucleic acid hybridization between the HER-2 gene or mRNA and a corresponding nucleic acid probe that can be designed in the same or a similar way as for PCR primers, above. See, e.g., Mitchell MS, and Press MF., 1999, Semin. Oncol., Suppl. 12:108-16. For FISH, this nucleic acid probe can be conjugated to a fluorescent molecule, e.g., fluorescein and/or rhodamine, that preferably does not interfere with hybridization, and which fluorescence can later be measured following hybridization. See, e.g., Kurokawa, H et al, Cancer Res. 60: 5887-5894 (2000) (describing a specific nucleic acid probe having sequence 5'-FAM-NucleicAcid-TAMRA-p-3' sequence). ACIS-based approaches as described above can be employed to make the assay more quantitative (de la Torre-Bueno, J, et al , 2000, Modern Pathology 13:221A).

Immuno and nucleic acid detection can also be directed against proteins other than HSP90 and Her-2, which proteins are nevertheless affected in response to HSP90 inhibition.

Pharmaceutical Compositions, Dosaging and Modes of Administration

Compounds identified as promising using the assays of the invention can in turn be formulated into pharmaceutical compositions and then administered to subjects.

Those of ordinary skill in the art are familiar with formulation and administration techniques that can be employed with the compounds and methods of the invention, e.g., as discussed in Goodman and Gilman's, *The Pharmacological Basis of Therapeutics*, current edition; Pergamon Press; and Remington's Pharmaceutical Sciences (current edition.) Mack Publishing Co., Easton, Pa.

The compounds utilized in the methods of the instant invention may be administered either alone or in combination with pharmaceutically acceptable carriers, excipients or diluents, in a pharmaceutical composition, according to standard pharmaceutical practice. The compounds can be administered orally or parenterally,

including the intraventous, intramuscular, intraperitoneal, subcutaneous, rectal and topical routes of administration.

For example, the therapeutic or pharmaceutical compositions of the invention can be administered locally to the area in need of treatment. This may be achieved by, for example, but not limited to, local infusion during surgery, topical application, e.g., cream, ointment, injection, catheter, or implant, said implant made, e.g., out of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. The administration can also be by direct injection at the site (or former site) of a tumor or neoplastic or pre-neoplastic tissue.

Still further, the compounds or compositions of the invention can be delivered in a vesicle, e.g., a liposome (see, for example, Langer, **1990**, Science, *249*:1527-1533; Treat et al., **1989**, Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Bernstein and Fidler (eds.), Liss, N.Y., pp. 353-365).

The compounds and pharmaceutical compositions used in the methods of the present invention can also be delivered in a controlled release system. In one embodiment, a pump may be used (see, Sefton, **1987**, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., **1980**, Surgery, 88:507; Saudek et al., **1989**, N. Engl. J. Med., 321:574). Additionally, a controlled release system can be placed in proximity of the therapeutic target. (see, Goodson, **1984**, Medical Applications of Controlled Release, Vol. 2, pp. 115-138).

The pharmaceutical compositions used in the methods of the instant invention can also contain the active ingredient in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsions, hard or soft capsules, or syrups or elixirs. Compositions intended for oral use may be prepared according to any method known to the art for the manufacture of pharmaceutical compositions, and such compositions may contain one or more agents selected from the group consisting of sweetening agents, flavoring agents, coloring agents and preserving agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients which are suitable for the manufacture of tablets.

These excipients may be, for example, inert diluents, such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, such as microcrystalline cellulose, sodium crosscarmellose, corn starch, or alginic acid; binding agents, for example starch, gelatin, polyvinyl-pyrrolidone or acacia, and lubricating agents, for example, magnesium stearate, stearic acid or talc. The tablets may be un-coated or coated by known techniques to mask the taste of the drug or delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a water soluble taste masking material such as hydroxypropylmethyl-cellulose or hydroxypropylcellulose, or a time delay material such as ethyl cellulose, or cellulose acetate butyrate may be employed as appropriate.

Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water soluble carrier such as polyethyleneglycol or an oil medium, for example peanut oil, liquid paraffin, or olive oil.

Aqueous suspensions contain the active material in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethyl-cellulose, sodium alginate, polyvinyl-pyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents may be a naturally-occurring phosphatide, for example lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethylene-oxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions may also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose, saccharin or aspartame.

Oily suspensions may be formulated by suspending the active ingredient in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in mineral oil such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavoring agents may be added to provide a palatable oral preparation. These compositions may be preserved by the addition of an anti-oxidant such as butylated hydroxyanisol or alpha-tocopherol.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, may also be present. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid.

The compounds and pharmaceutical compositions used in the methods of the instant invention may also be in the form of an oil-in-water emulsions. The oily phase may be a vegetable oil, for example olive oil or arachis oil, or a mineral oil, for example liquid paraffin or mixtures of these. Suitable emulsifying agents may be naturally-occurring phosphatides, for example soy bean lecithin, and esters or partial esters derived from fatty acids and hexitol anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions may also contain sweetening agents, flavoring agents, preservatives and antioxidants.

Syrups and elixirs may be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain a demulcent, a preservative, flavoring and coloring agents and antioxidant.

The pharmaceutical compositions may be in the form of a sterile injectable aqueous solutions. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and isotonic sodium chloride solution.

The sterile injectable preparation may also be a sterile injectable oil-in-water microemulsion where the active ingredient is dissolved in the oily phase. For example, the active ingredient may be first dissolved in a mixture of soybean oil and lecithin. The oil solution then introduced into a water and glycerol mixture and processed to form a microemulation.

The injectable solutions or microemulsions may be introduced into a patient's blood-stream by local bolus injection. Alternatively, it may be advantageous to administer the solution or microemulsion in such a way as to maintain a constant circulating concentration of the instant compound. In order to maintain such a constant concentration, a continuous intravenous delivery device may be utilized. An example of such a device is the Deltec CADD-PLUS™ model 5400 intravenous pump.

The pharmaceutical compositions may be in the form of a sterile injectable aqueous or oleagenous suspension for intramuscular and subcutaneous administration. This suspension may be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents which have been mentioned above. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example as a solution in 1,3-butane diol. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The Compounds of the present invention used in the methods of the present invention may also be administered in the form of suppositories for rectal administration of the drug. These compositions can be prepared by mixing the inhibitors with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter, glycerinated gelatin, hydrogenated vegetable oils, mixtures of polyethylene glycols of various molecular weights and fatty acid esters of polyethylene glycol.

For topical use, creams, ointments, jellies, solutions or suspensions, etc., containing an compound or composition of the invention can be used. As used herein, topical application can include mouth washes and gargles.

The compounds used in the methods of the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles and delivery devices, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in the art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

The methods, compounds and compositions of the instant invention may also be used in conjunction with other well known therapeutic agents that are selected for their particular usefulness against the condition that is being treated. For example, the instant compounds may be useful in combination with known anti-cancer and cytotoxic agents. Further, the instant methods and compounds may also be useful in combination with other inhibitors of parts of the signaling pathway that links cell surface growth factor receptors to nuclear signals initiating cellular proliferation.

The methods of the present invention may also be useful with other agents that inhibit angiogenesis and thereby inhibit the growth and invasiveness of tumor cells, including, but not limited to VEGF receptor inhibitors, including ribozymes and antisense targeted to VEGF receptors, angiostatin and endostatin.

Examples of antineoplastic agents that can be used in combination with the compounds and methods of the present invention include, in general, and as appropriate, alkylating agents, anti-metabolites; epidophyllotoxin; an antineoplastic enzyme; a topoisomerase inhibitor; procarbazine; mitoxantrone; platinum coordination complexes; biological response modifiers and growth inhibitors; hormonal/anti-hormonal therapeutic agents and haematopoietic growth factors. Exemplary classes of antineoplastics include the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the epothilones, discodermolide, the pteridine family of drugs, diynenes and the podophyllotoxins. Particularly useful members of those classes include, for example, carminomycin, daunorubicin, aminopterin, methotrexate, methopterin,

dichloromethotrexate, mitomycin C, porfiromycin, 5-fluorouracil, 6-mercaptopurine, gemcitabine, cytosine arabinoside, podophyllotoxin or podo-phyllotoxin derivatives such as etoposide, etoposide phosphate or teniposide, melphalan, vinblastine, vincristine, leurosidine, vindesine, leurosine, paclitaxel and the like. Other useful antineoplastic agents include estramustine, carboplatin, cyclophosphamide, bleomycin, gemcitibine, ifosamide, melphalan, hexamethyl melamine, thiotepa, cytarabin, idatrexate, trimetrexate, dacarbazine, L-asparaginase, camptothecin, CPT-11, topotecan, ara-C, bicalutamide, flutamide, leuprolide, pyridobenzoindole derivatives, interferons and interleukins.

When a compound or composition of the invention is administered into a human subject, the daily dosage will normally be determined by the prescribing physician with the dosage generally varying according to the age, weight, and response of the individual patient, as well as the severity of the patient's symptoms.

In one exemplary application, a suitable amount of compound is administered to a mammal undergoing treatment for cancer, for example, breast cancer. Administration typically occurs in an amount of between about 0.01 mg/kg of body weight to about 100 mg/kg of body weight per day (administered in single or divided doses), more preferably at least about 0.1 mg/kg of body weight per day. A particular therapeutic dosage can include, e.g., from about 0.01 mg to about 1000 mg of compound, and preferably includes, e.g., from about 1 mg to about 1000 mg. The quantity of active compound in a unit dose of preparation may be varied or adjusted from about 0.1 mg to 1000 mg, preferably from about 1 mg to 300 mg, more preferably 10 mg to 200 mg, according to the particular application. The amount administered will vary depending on the particular IC50 value of the compound used and the judgment of the attending clinician taking into consideration factors such as health, weight, and age. In combinational applications in which the compound is not the sole active ingredient, it may be possible to administer lesser amounts of compound and still have therapeutic or prophylactic effect.

Preferably, the pharmaceutical preparation is in unit dosage form. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component, e.g., an effective amount to achieve the desired purpose.

The actual dosage employed may be varied depending upon the requirements of the patient and the severity of the condition being treated. Determination of the proper dosage for a particular situation is within the skill of the art. Generally, treatment is initiated with smaller dosages which are less than the optimum dose of the compound. Thereafter, the dosage is increased by small amounts until the optimum effect under the circumstances is reached. For convenience, the total daily dosage may be divided and administered in portions during the day if desired.

The amount and frequency of administration of the compounds and compositions of the present invention used in the methods of the present invention, and if applicable other chemotherapeutic agents and/or radiation therapy, will be regulated according to the judgment of the attending clinician (physician) considering such factors as age, condition and size of the patient as well as severity of the disease being treated.

The chemotherapeutic agent and/or radiation therapy can be administered according to therapeutic protocols well known in the art. It will be apparent to those skilled in the art that the administration of the chemotherapeutic agent and/or radiation therapy can be varied depending on the disease being treated and the known effects of the chemotherapeutic agent and/or radiation therapy on that disease. Also, in accordance with the knowledge of the skilled clinician, the therapeutic protocols (e.g., dosage amounts and times of administration) can be varied in view of the observed effects of the administered therapeutic agents (i.e., antineoplastic agent or radiation) on the patient, and in view of the observed responses of the disease to the administered therapeutic agents.

Also, in general, the compounds of the invention need not be administered in the same pharmaceutical composition as a chemotherapeutic agent, and may, because of different physical and chemical characteristics, be administered by a different route. For example, the compounds/compositions may be administered orally to generate and maintain good blood levels thereof, while the chemotherapeutic agent may be administered intravenously. The determination of the mode of administration and the advisability of administration, where possible, in the same pharmaceutical composition, is well within the knowledge of the skilled clinician. The initial administration can be made according to established protocols known in the art, and then, based upon the observed

effects, the dosage, modes of administration and times of administration can be modified by the skilled clinician.

The particular choice of compound (and where appropriate, chemotherapeutic agent and/or radiation) will depend upon the diagnosis of the attending physicians and their judgment of the condition of the patient and the appropriate treatment protocol.

The compounds/compositions of the invention (and where appropriate chemotherapeutic agent and/or radiation) may be administered concurrently (e.g., simultaneously, essentially simultaneously or within the same treatment protocol) or sequentially, depending upon the nature of the proliferative disease, the condition of the patient, and the actual choice of chemotherapeutic agent and/or radiation to be administered in conjunction (i.e., within a single treatment protocol) with the compound/composition.

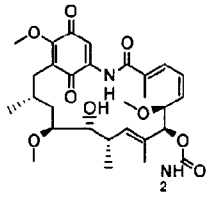
In combinational applications and uses, f the compound/composition and the chemotherapeutic agent and/or radiation are not administered simultaneously or essentially simultaneously, then the initial order of administration of the compound/composition, and the chemotherapeutic agent and/or radiation, may not be important. Thus, the compounds/compositions of the invention may be administered first followed by the administration of the chemotherapeutic agent and/or radiation; or the chemotherapeutic agent and/or radiation may be administered first followed by the administration of the compounds/compositions of the invention. This alternate administration may be repeated during a single treatment protocol. The determination of the order of administration, and the number of repetitions of administration of each therapeutic agent during a treatment protocol, is well within the knowledge of the skilled physician after evaluation of the disease being treated and the condition of the patient. For example, the chemotherapeutic agent and/or radiation may be administered first, especially if it is a cytotoxic agent, and then the treatment continued with the administration of the compounds/compositions of the invention followed, where determined advantageous, by the administration of the chemotherapeutic agent and/or radiation, and so on until the treatment protocol is complete.

Thus, in accordance with experience and knowledge, the practicing physician can modify each protocol for the administration of a compound/composition for treatment according to the individual patient's needs, as the treatment proceeds.

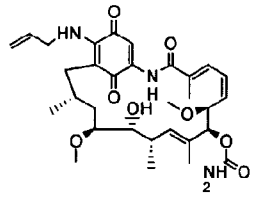
The attending clinician, in judging whether treatment is effective at the dosage administered, will consider the general well-being of the patient as well as more definite signs such as relief of disease-related symptoms, inhibition of tumor growth, actual shrinkage of the tumor, or inhibition of metastasis. Size of the tumor can be measured by standard methods such as radiological studies, e.g., CAT or MRI scan, and successive measurements can be used to judge whether or not growth of the tumor has been retarded or even reversed. Relief of disease-related symptoms such as pain, and improvement in overall condition can also be used to help judge effectiveness of treatment.

EXAMPLES

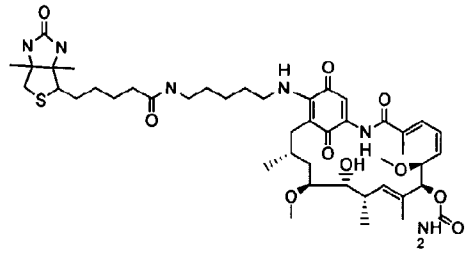
The following examples are offered by way of illustration only and not by way of limitation. Examples 1-3 illustrate alternative methods of producing a biotinylated geldanamycin of formula/compound 5. Example 4 illustrates that such a compound is useful in competitive binding assays with other HSP90 ligands, e.g., other ansamycins such as 2 GM and 17-AAG. 2 GM, 17-AAG, and one embodiment of a biotinylated geldanamycin are structurally illustrated as follows:



2 (GM)



17-AAG



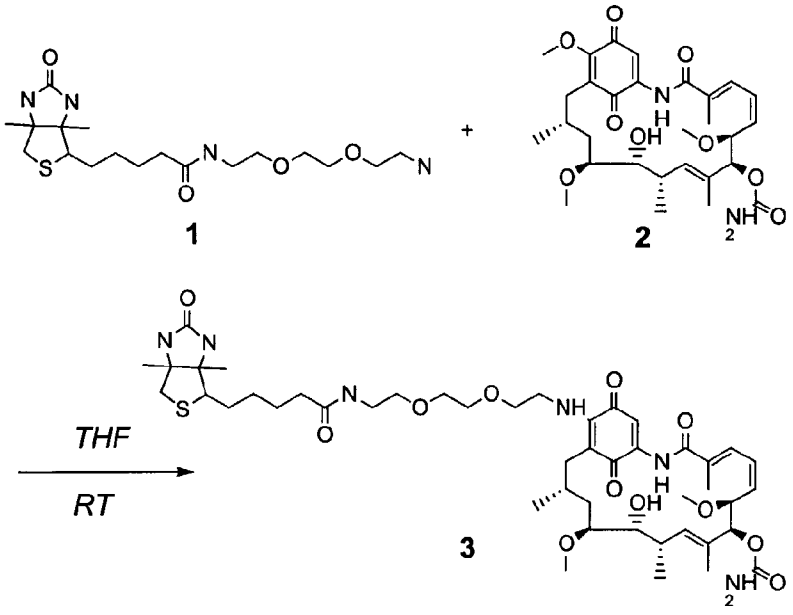
5

Examples

Example 1

Synthesis of biotinylated ansamycins useful for competition binding studies with HSP90

This example follows the scheme:



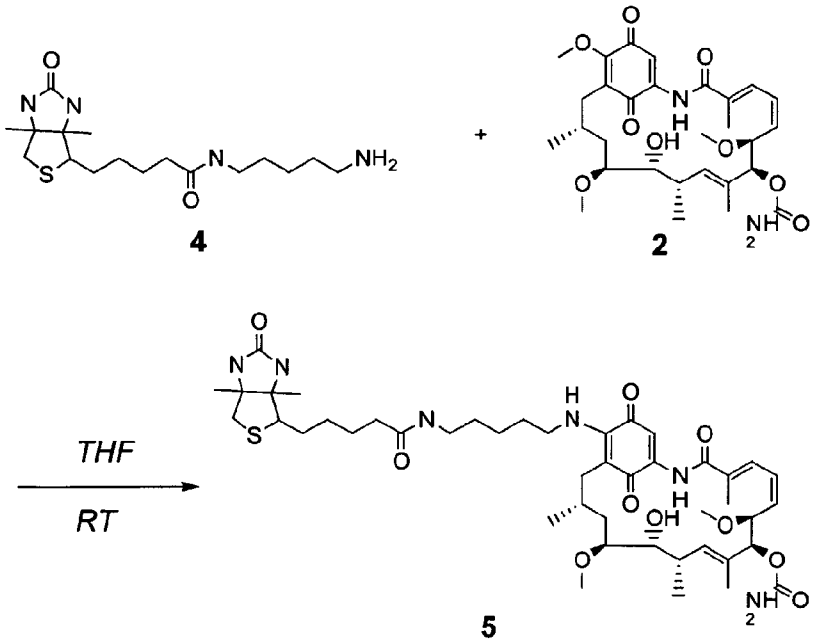
wherein the compound numbers are denoted beneath the corresponding structures, and wherein the details of the synthesis are as follows.

To 50 mg (0.134 mmol) of (+)-biotinyl-3,6-dioxaoctanediamine 1 in 3 mL of 15:1 THF-H2O was added 29.9 (0.053 mmol) of geldanamycin 2 at room temperature. The reaction was stirred overnight, quenched with water (50 mL) and extracted with 2 x 50 mL of EtOAc. The EtOAc extracts were combined, washed with 2 x 50 mL of H2O, 1 x 50 mL of brine, dried (MgSO4) and purified by silica gel flash chromatography to give 88 mg (0.095 mmol) in 71%yield of 3. MP 113-117 oC. MS 926 (M + Na).

Example 2

Synthesis of biotinylated ansamycins useful for competition binding studies with HSP90

This example follows the scheme:



4 + 2 → 5

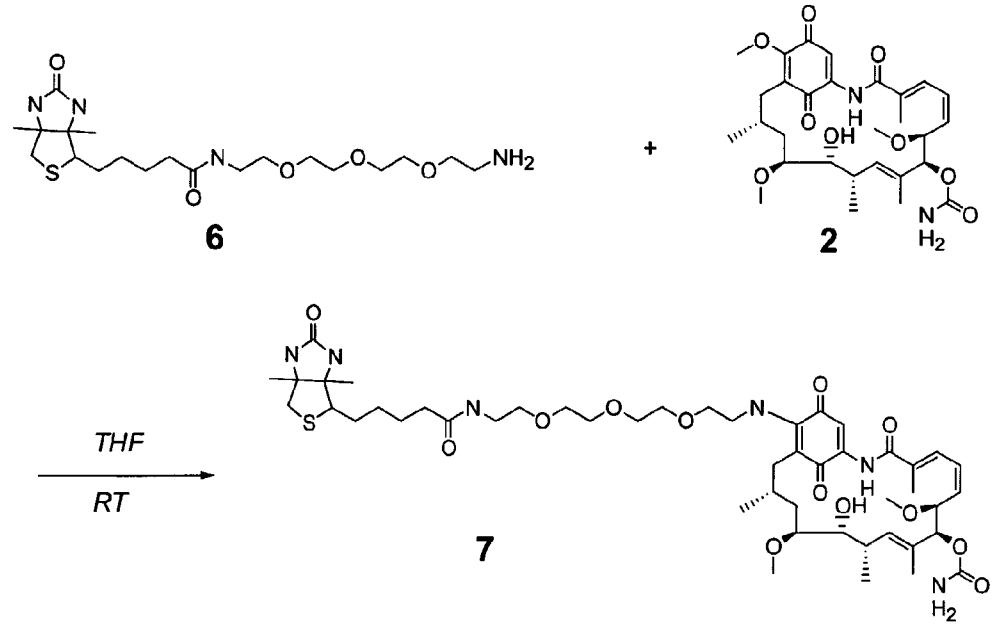
wherein the compound numbers are denoted beneath the corresponding structures, and wherein the details of the synthesis are as follows.

To 50 mg (0.152 mmol) of 5-(biotinamido)pentylamine 4 in 3 mL of 15:1 THF-H2O was added 28 mg (0.050 mmol) of geldanamycin 2 at room temperature. The reaction was stirred overnight, quenched with water (50 mL) and extracted with 2 x 50 mL of EtOAc. The EtOAc extracts were combined, washed with 2 x 50 mL of H2O, 1 x 50 mL of brine, dried (MgSO4) and purified by silica gel flash chromatography to give 100 mg (0.114 mmol) of 5 in 75 % yield. MP 143-147 oC. MS 880 (M + Na).

Example 3

Synthesis of biotinylated ansamycins useful for competition binding studies with HSP90

This example follows the scheme:



wherein the compound numbers are denoted beneath the corresponding structures and wherein the details of the synthesis are as follows.

To 50 mg (0.119 mmol) of (+)-biotinyl-3,6,9-trioxaundecanediamine 6 in 3 mL of 15:1 THF-H2O was added 26.8 mg (0.048 mmol) of geldanamycin 2 at room temperature. The reaction was stirred overnight, quenched with water (50 mL) and extracted with 2 x 50 mL of EtOAc. The EtOAc extracts were combined, washed with 2 x 50 mL of H2O, 1 x 50 mL of brine, dried (MgSO4) and purified by silica gel flash chromatography to give 84 mg (0.087 mmol) of 7 in 73% yield. MP 103-104 oC. MS 970 (M + Na).

Example 4

HSP90 Binding Assay Utilizing A Biotinylated Ansamycin

Purified native HSP90 protein was coated onto 96-well plates by incubating for 1 hr at 37 °C. Uncoated HSP90 was removed and the wells washed twice in 1 x PBS (phosphate-buffered saline) buffer. Compound 5 (biotinylated geldanamycin) was then added to the wells, and the reaction was further incubated for 1hr 37 °C. The wells were washed twice with 1 x PBS, before the addition of 20ug/ml streptavidin-phycoerythrin, and incubated for 1hr at 37 °C. The wells were again washed twice with 1 x PBS. The fluorescence was then measured in a Gemini spectrofluorometer (Molecular Devices) using an excitation of 485 nm and emission of 580 nm.

Figures 1-3 show assay embodiments employing different HSP90 ligands. Details of the assays are as follows:

Figure 1. Competition of biotinylated-GM (compound 5) binding by free geldanamycin (GM).. Native Hsp90 that was coated onto 96-well plates was pre-incubated with increasing concentrations of 0 nM (B3), 100 nM (C3), 300 nM (D3), 1000 nM (E3), 3000 nM (F3), 10,000 nM (G3), and 100,000 nM (closed diamonds) of geldanamycin and then 5 was added. Binding of 5 was detected by measuring the fluorescence of streptavidin-phycoerythrin (excitation: 485 nm; emission: 510-650 nm). The background fluorescence without any Hsp90 present (A3) was minimal. Increasing concentrations of GM inhibits the peak fluorescence at 580 nm.

Figure 2. Competition of biotinylated-GM (compound 5) binding by 17-allyl amino geldanamycin (17-AAG). Native Hsp90 that was coated onto 96-well plates was pre-incubated with increasing concentrations of 0 nM (B4), 100 nM (C4), 300 nM (D4), 1000 nM (E4), 3000 nM (F4), 10,000 nM (G4), and 100,000 nM (closed diamonds) of 17-AAG and then 5 was added. Binding of 5 was detected by measuring the fluorescence of streptavidin-phycoerythrin (excitation: 485nm; emission: 510-650 nm). The background fluorescence without any Hsp90 present (A4) was minimal. Increasing concentrations of 17-AAG inhibits the peak fluorescence at 580 nm

Figure 3. Competition of biotinylated-GM (compound 5) binding by geldanamycin (GM) and 17-allyl amino geldanamycin (17-AAG). Native Hsp90 that was coated onto 96-well plates was pre-incubated with increasing concentrations of 0-100,000 nM of either GM or 17-AAG and then 5 was added Binding of 5 was detected by measuring the fluorescence of streptavidin-phycoerythrin (excitation: 485 nm; emission: 580 nm). The background fluorescence without any Hsp90 present (no Hsp90) was minimal. Increasing concentrations of GM or 17-AAG inhibits the peak fluorescence at 580 nm.

Example 5

HSP90s taken from tumor cell lines more avidly bind known HSP90 modulators

Purified native HSP90 protein (Stressgen) or cell lysates prepared in lysis buffer (20mM Hepes, pH 7.3, 1mM EDTA, 5mM MgCl2, 100mM KCl) were incubated in the absence or presence of CF7 (17-AAG) or test compound for 15min at 4°C. Biotin-geldanamycin (biotin-GM) was then added to the mixture as discussed previously, and the reaction was further incubated by rotating for 1hr at 4°C. BioMag™ streptavidin magnetic beads were then added to the mixture, and the reaction was incubated by rotating for another 1hr at 4°C. Tubes were placed on a magnetic rack, and the unbound supernatant removed. The magnetic beads were washed three times in the lysis buffer, and the washes discarded. SDS-PAGE sample buffer was added to the beads and boiled for 5 min at 95°C. Samples were analyzed on 10% SDS protein gels (Novex), and then Western blots using anti-HSP90 monoclonal antibody (Stressgen SPA-830). The bands in the Western Blots were quantitated using the Bio-rad Fluor-S Imager, and the % inhibition of binding of CF7 or test compound calculated. The IC50 reported is the concentration of the compound needed to cause half-maximal inhibition of binding. For experiments that utilized heat-shocked Hsp90, the purified HSP90 native protein was incubated for 15min at 50°C. For experiments that utilized bis-ANS treated HSP90, the purified HSP90 protein was incubated with bis-ANS (Molecular Probes) for 30 min at 37°C. The results are shown in Figures 4-6.

* * *

The foregoing examples are not limiting and are merely representative of various aspects and embodiments of the present invention. All documents cited are indicative of the levels of skill in the art to which the invention pertains. The reagents used, other than those novel reagents of the invention, are commercially available and/or readily synthesized or acquired by one or ordinary skill in the art without undue effort. The disclosure of each document is incorporated by reference herein to the same extent as if each had been incorporated by reference in its entirety individually, although none of the documents is admitted to be prior art.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described illustrate preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Certain modifications and other uses will occur to those skilled in the art, and are encompassed within the spirit of the invention, as defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the invention and the following claims.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, while the terms "comprising", "consisting essentially of" and "consisting of," each carries a different meaning as a transition phrase, each such phrase may be used in lieu of the others to demonstrate a different aspect or embodiment of the invention. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described, or portions thereof. It is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modifications and variations of the concepts herein disclosed may be resorted to by those skilled in the art,

and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group, and exclusions of individual members as appropriate.

Other embodiments are within the following claims.

The claims defining the invention are as follows:

1. A method for modulating a high affinity form of HSP90, having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, that is found in cancer or tumor cells, comprising contacting said high affinity form with an HSP90 modulator to thereby modulate said high affinity form of HSP90.

2. The method of claim 1 wherein said method is selective for said high affinity form of HSP90 over a lower affinity form of HSP90.

3. The method of claim 2 wherein said method is at least 2x, or at least 10x, or at least 50x, or at least 100x, or at least 500x more selective for said high affinity form versus a low affinity form of HSP90.

4. The method of claim 2 wherein said method is between about 30x and 500x more selective for said high affinity form versus a low affinity form of HSP90.

5. A method for degrading HSP90 client proteins by specifically modulating a high affinity form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, that is found in cancer or tumor cells.

6. A method of screening for HSP90 modulators, comprising:

providing a high affinity form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less that is found in cancer or tumor cells;

contacting said HSP90 form with a compound of interest; and

measuring or evaluating the ability of said compound to modulate said HSP90 form.

7. A method of treating or preventing an HSP90-mediated disease, comprising administering to a subject a pharmaceutically effective amount of a compound or pharmaceutically acceptable salt thereof according to the method of any of claims 1 to 4.

8. A method of treating or preventing an HSP90-mediated disease, comprising administering to a subject a pharmaceutically effective amount of a compound or pharmaceutically acceptable salt thereof identified according to the method of any of claims 1 to 4.

9. A purified or isolated preparation or complex of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, taken from tumor or cancer cells exhibiting high affinity binding for an HSP90 modulator.

10. A diagnostic kit comprising one or more members selected from the group consisting of: (a) the preparation or complex of HSP90 of claim 9 and (b) a compound that binds said HSP90.

11. An assay that measures the binding of a compound of interest to a particular form of HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells.

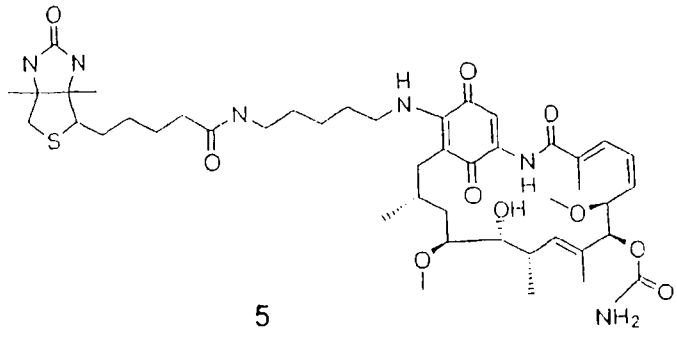
12. The assay of claim 11 that is a competition binding assay.

13. The assay of claim 12 wherein said competition binding assay further makes use of a labeled HSP90 ligand member to compete for binding with said compound of interest.

14. The assay of claim 13 wherein said HSP90 ligand member is biotinylated.

15. The assay of claim 14 wherein said HSP90 ligand member is selected from the group consisting of purines or purine analogs, ansamycins, radicicol, zearalanols, ATP analogs, indoles, chalcones and benzimidazoles.

16. The assay of claim 14 or 15 wherein said HSP90 ligand is an ansamycin of the following structure:



17. A method of evaluating the ability of a compound of interest to bind an HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells comprising the steps:

providing an HSP90 member having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells, an HSP90 ligand member, and a compound of interest member together on a solid support under conditions sufficient for one or the other of said HSP90 ligand member and said compound of interest member to complex with said HSP90 member and be retained on said solid support; one of said members comprising a label;

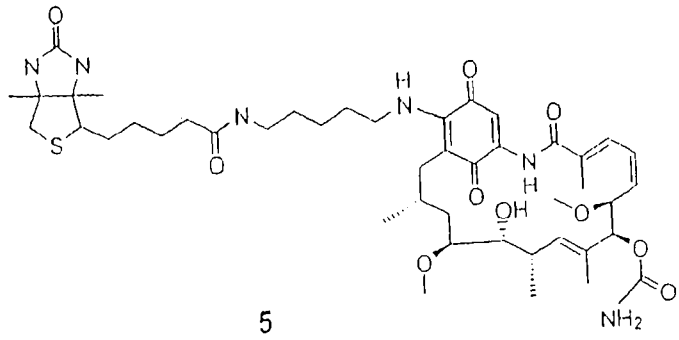
removing members that are not complexed on or otherwise conjugated to said solid support; and

assaying said solid support for the presence of said label as a measure of the ability of said compound of interest member to bind said HSP90 member.

18. The method of claim 17 wherein said HSP90 member is conjugated to said solid support and wherein said HSP90 ligand member is labeled.

19. The method of claim 18 wherein said HSP90 member is selected from the group consisting of ansamycins and purines.

20. The method of claim 19 wherein said HSP90 ligand member is biotinylated and comprises a structure of formula 5:



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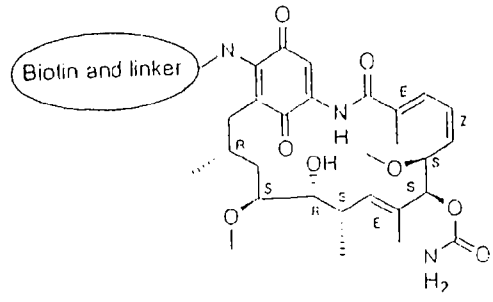
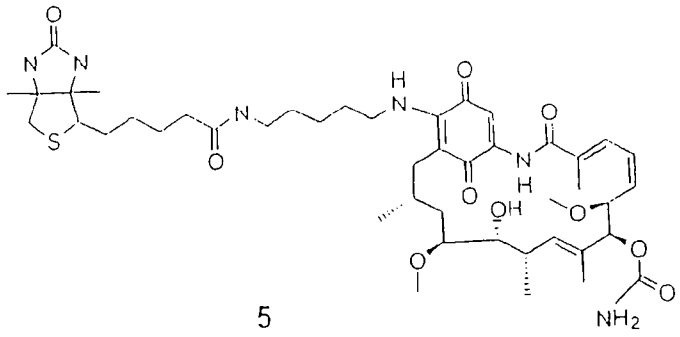
and wherein said label in practice further comprises an avidin or streptavidin component associated with said structure.

21. A complex comprising a biotinylated ansamycin bound to an HSP90 having a 17-AAG IC50 binding value of about 30nm, preferably of about 10nm or less, found in tumor or cancer cells, said HSP90 optionally bound to a solid support.

22. The complex of claim 21 wherein said biotinylated ansamycin has structure



23. The complex of claim 21 wherein said biotinylated ansamycin comprises formula



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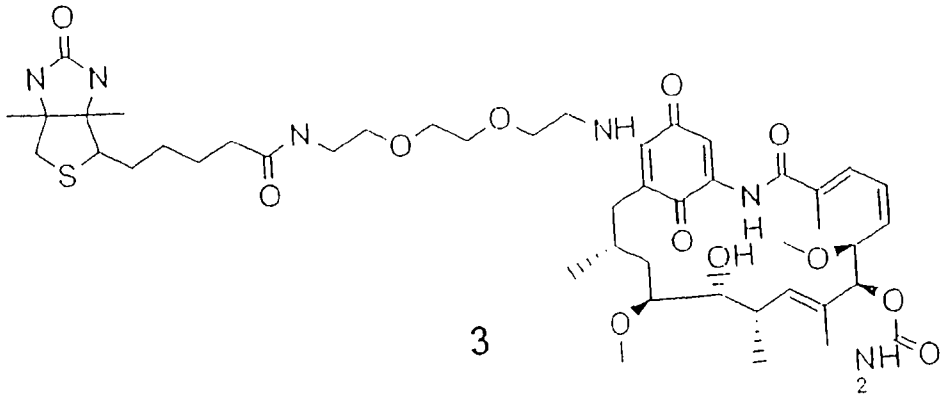
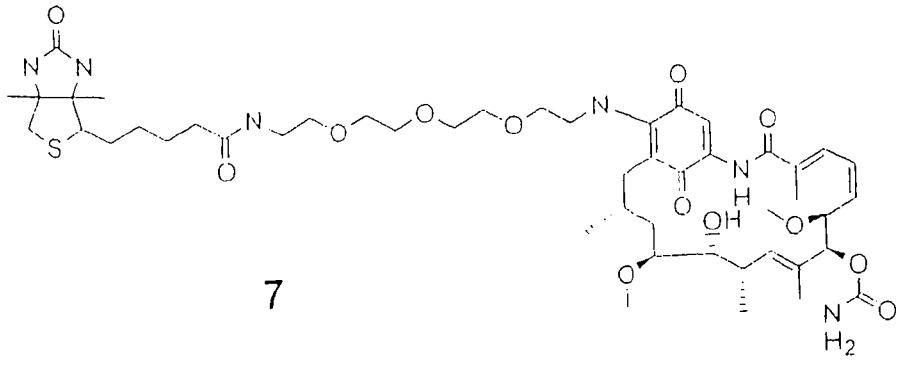
24. The assay of claim 14 or 15 wherein said HSP90 ligand is an ansamycin of formula 3



3

25. The assay of claim 14 or 15 wherein said HSP90 ligand is an ansamycin of formula 7



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Dated 9 March, 2009

Conforma Therapeutics Corporation

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

Figure 1

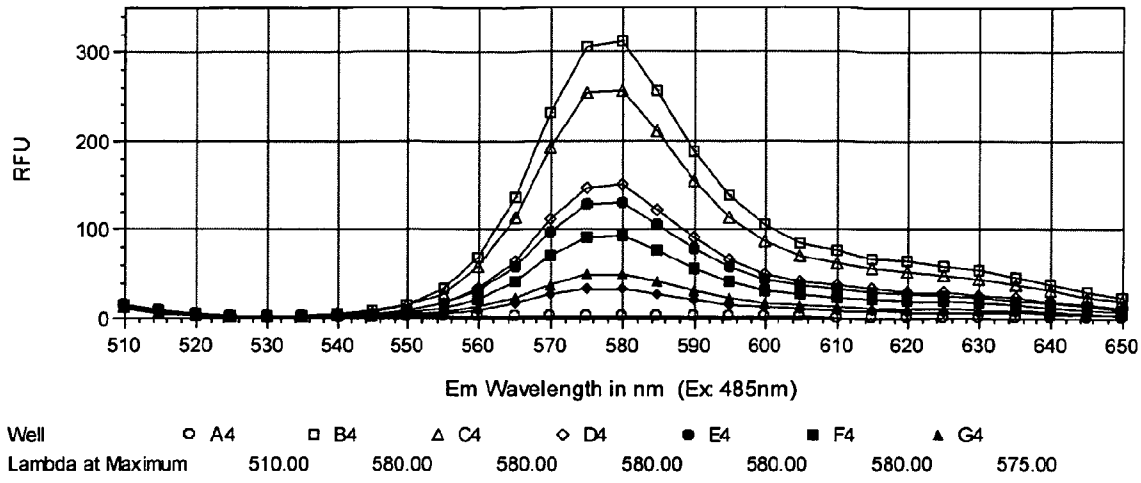


Figure 2

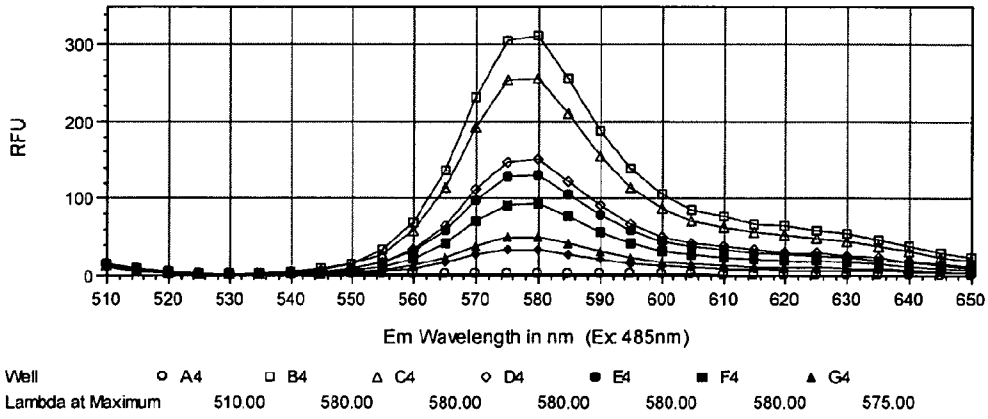


Figure 3

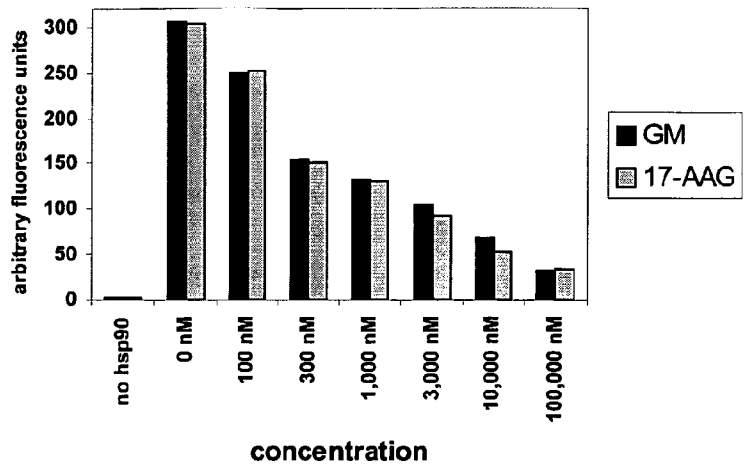
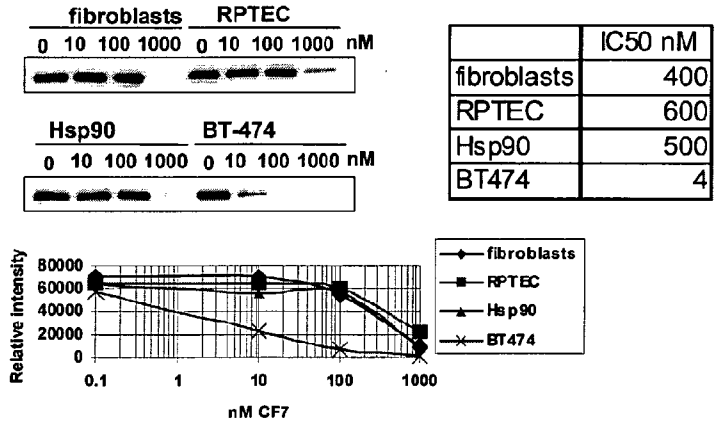
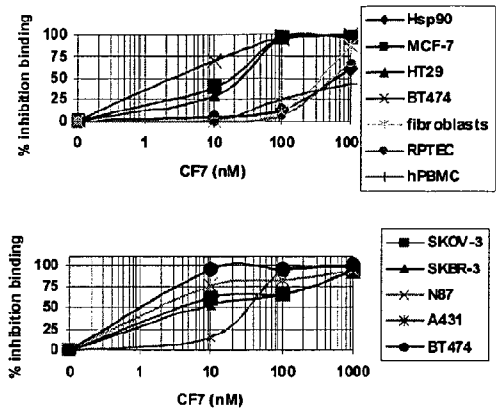


Figure 4



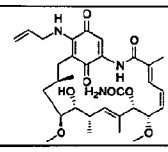
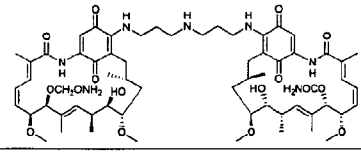
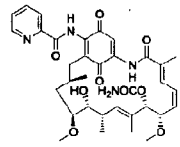
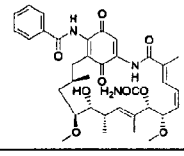
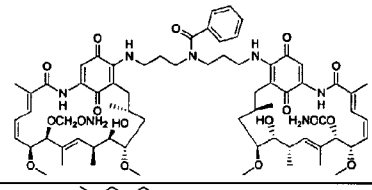
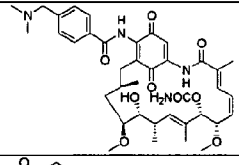
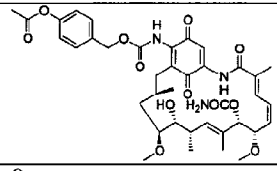
	IC50 nM
fibroblasts	400
RPTEC	600
Hsp90	500
BT474	4

Figure 5



	IC50 (nM)
Hsp90	600
MCF7	20
HT29	25
BT474	3
Fibroblasts	400
RPTEC	600
hPBMC	>1000
SKOV-3	6
SKBR-3	7
N87	4
A431	30
$Hsp90^{bis\text{-}ANS}$	40
$Hsp90^{heat\text{-}shock}$	20

Figure 6

lysate IC50 nM	hsp90 IC50 nM	ratio	EC number	Structure
20	700	35	EC1	
30	2000	67	EC3	
15	1000	67	EC20	
50	4500	90	EC21	
10	1000	100	EC23	
8	2000	250	EC24	
9	1500	167	EC26	
4	2000	500	EC60