Abstract:

Title: THERAPEUTIC METHODS WITH WITHAFERIN A AND ANALOGS

Compounds of Formulas 1 (withaferin A, also referred to as D004), 2 (viscosalactone B, also referred to as E002), and 3 (22R-5β-formyl-6β,27-dihydroxy-1'-oxo-4-norwith-24-enolide, also referred to as O010), as well as salts, prodrugs, analogs, and derivatives thereof are biologically active for modulating Hsp70, Hsp90, and/or HSFl. Also, these compounds can be included in pharmaceutical compositions for administration to a subject for modulating the expression or activity of Hsp70, Hsp90, and/or HSFl in order to produce a therapeutic effect.
THERAPEUTIC METHODS WITH WITHAFERIN A AND ANALOGS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present invention claims the benefit of U.S. Provisional Application Serial No. 61/112,465 filed November 7, 2008, which application is incorporated herein by specific reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of grant 5P20 RR015563-10 awarded by the National Institutes of Health.

BACKGROUND OF THE INVENTION

Heat shock proteins (HSP) are a class of functionally related proteins whose expression is transcriptionally regulated and is increased when cells are exposed to elevated temperatures or other stresses. HSPs are found in virtually all living organisms, from bacteria to humans. HSPs are named according to their molecular weight. For example, Hsp60, Hsp70 and Hsp90 (the most widely-studied HSPs) refer to families of heat shock proteins on the order of 60, 70 and 90 kilodaltons in size, respectively. Intracellular heat shock proteins are highly expressed in cancerous cells and are essential to the survival of these cell types. The potent Hsp90 inhibitor 17-AAG is currently in clinical trials for the treatment of several types of cancer.

The Hsp70 (heat shock protein 70) is in a family of ubiquitously expressed heat shock proteins. Proteins with similar structure exist in virtually all living organisms. The Hsp70s are an important part of the cell's machinery for protein folding, and help to protect cells from stress. HSP 70 is overexpressed in malignant melanoma, and underexpressed in renal cell cancer. Hsp70 also aids in transmembrane transport of proteins, by stabilizing them in a partially-folded state. In addition to improving overall protein integrity, Hsp70 directly inhibits apoptosis by binding and stabilizing Bcl2 prosurvival proteins.

Hsp90 (heat shock protein 90) is a molecular chaperone and is one of the most abundant proteins expressed in cells. It is a member of the heat shock protein family which is upregulated in response to stress. The function of Hsp90 includes assisting in
protein folding, cell signaling, and delivery of unfolded proteins to proteasomal system. This protein was first isolated by extracting proteins from stressed cells. These cells were stressed by heating, dehydrating or by other means, all of which caused the cell’s proteins to begin to denature. As discussed in more detail below, researchers later realized that Hsp90 has other essential functions in unstressed cells. In unstressed cells, Hsp90 plays a number of important roles, which include assisting in folding, intracellular transport, maintenance, and delivery of unfolded proteins for degradation as well as facilitating cell signaling. Cancerous cells over express a number of proteins, including PI3Kinase and AKT and inhibition of these two proteins triggers apoptosis. Another function of Hsp90 is through molecular association with several kinases is that it stabilizes their biological activity. Furthermore Hsp90 stabilizes the PI3K and AKT proteins. So far more that 300 proteins have been identified that associate with Hsp90. These proteins are called client proteins. Hence inhibition of Hsp90 appears to induce apoptosis through inhibition of the PI3K/AKT signaling pathway among others. Another important role of Hsp90 in cancer is the stabilization of mutant proteins such as v-Src, the fusion oncogene Bcr/Abl, B-RAF(VoOOE), NRAS and p53 that appear during cell transformation. Therefore cancer cells can need the presence of the Hsp90 for the maintenance of properly folded oncoproteins. Cancer cell have been adapted to harsh environment of acidic pH and hypoxic conditions and therefore the requirement for "active" chaperones such Hsp90 is greater compared to normal cells. It appears that Hsp90 can act as a "protector" of less stable proteins produced by DNA mutations. Hsp90 is also required for induction of vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS). Both are important for de novo angiogenesis that is required for tumor growth beyond the limit of diffusion distance of oxygen in tissues. It also promotes the invasion step of metastasis by assisting the matrix metalloproteinase MMP2. Together with its co-chaperones, Hsp90 modulates tumor cell apoptosis "mediated through effects on AKT, tumor necrosis factor receptors (TNFR) and nuclear factor-κB (NF-κB) function." Finally Hsp90 participates in many key processes in oncogenesis such as self-sufficiency in growth signals, stabilization of mutant proteins, angiogenesis, and metastasis.

Heat Shock Factor 1 (HSFl) is a transcription factor that is involved in heat shock protein expression. Recently, it was discovered that HSFl is a powerful multifaceted modifier of carcinogenesis. HSFl knockout mice show significantly decreased incidence of skin tumor after topical application of DMBA (7,12-dimethylbenzanthracene), a mutagen. HSFl exists as an inactive monomer in a complex with Hsp40/Hsp70 and
Hsp90. Upon stress, such as elevated temperature, HSFl is released from the chaperone complex and trimerizes. HSFl is then transported into the nucleus where it is hyperphosphorylated and binds to DNA containing heat shock elements (HSE; NGAAN). HSFl's target genes include major inducible heat shock proteins such as Hsp72, and interestingly, noncoding RNA within Satellite IH repeat regions. Its attenuation phase is initiated as a negative feedback loop with its gene product Hsp70 binding to its transactivation domain. HSFl has been shown to interact with NCOA6, SYMPK, RALBPl, HSF2, CEBPB, and HSPAIA.

It appears that Hsp70, Hsp90, and HSFl are involved in diseases such as cancer and neurodegenerative diseases. Therefore, there is a need for molecules, compositions, and methods for modulating the expression and/or function Hsp70, Hsp90, and HSFl for therapeutic purposes.
SUMMARY

In one embodiment, the present invention includes a method for modulating a heat shock protein. Such a method can include: providing a composition having a compound of one or more of Formulas 1-3, as described below, or analog, derivative, prodrug, or pharmaceutically acceptable salt thereof; and administering the composition such that the heat shock protein is modulated. The heat shock protein can be one of heat shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), or heat shock factor 1 (HSFl). The composition is administered in an effective amount to increase Hsp70, to decrease Hsp90, and/or increase HSFl.

The method can include administering the composition in a therapeutically effective amount sufficient to provide a treatment by sufficiently modulating the heat shock protein. The therapeutically effective amount can be sufficient for use in treating, inhibiting, and/or preventing cancer. Also, the therapeutically effective amount can be sufficient for use in treating, inhibiting, and/or preventing cancer cell propagation. The cancer can be selected from thyroid cancer, papillary thyroid cancer, follicular thyroid cancer, head and neck squamous cell cancer, and melanoma.

In one embodiment, the composition can be administered in a therapeutically effective amount sufficient for use in treating, inhibiting, and/or preventing a neurodegenerative disease. For example, the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing at least one of alcoholism, Alexander's disease, Alper's disease, Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, batten disease (also known as Spielmeyer-Vogt-Sjogren-Batten disease), bovine spongiform encephalopathy, canavan disease, cockayne syndrome, corticobasal degeneration, Creutzfeldt-Jakob disease, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, Lewy body dementia, neuroborreliosis, Machado-Joseph disease (also known as Spino cerebellar ataxia type 3), multiple system atrophy, narcolepsy, Niemann Pick disease, Parkinson's disease, Pelizaeus-Merzbacher disease, Pick's disease, primary lateral sclerosis, prion diseases, progressive supranuclear palsy, Refsum's disease, Sandhoff's disease, Schilder's disease, subacute combined degeneration of spinal cord, spinocerebellar ataxia, spinal muscular atrophy, Steele-Richardson-Olszewski disease, tabes dorsalis, or symptoms thereof.

In one embodiment, the composition can be administered in a therapeutically effective amount sufficient for use in providing a cytoprotective activity to a cell.
In one embodiment, the heat shock protein is in a cell, which cell can be located in a subject.

In one embodiment, the composition includes an extract of a plant.

In one embodiment, the method also includes modulating a protein downstream from one of Hsp70, Hsp90, Hsp40, Hsp105, heat shock co-chaperone (CHOP) and HSFl.

Such a downstream protein can include NF-Kappa B, IKK beta, or 1KB alpha is modulated.

These and other embodiments and features of the present invention will become more fully apparent from the following description and appended claims, or may be learned by the practice of the invention as set forth hereinafter.
BRIEF DESCRIPTION OF THE DRAWINGS

To further clarify the above and other advantages and features of the present invention, a more particular description of the invention will be rendered by reference to specific embodiments thereof which are illustrated in the appended drawings. It is appreciated that these drawings depict only illustrated embodiments of the invention and are therefore not to be considered limiting of its scope. The invention will be described and explained with additional specificity and detail through the use of the accompanying drawings in which:

Figures IA-I G show the results of cell viability assays in response to withaferin A treated transiently for up to 8 hr and then withaferin A was washed completely and replaced with media devoid of withaferin A.

Figures I H-I I show the proliferation of melanoma cells in response to increasing concentrations of withaferin A.

Figure U shows the results of trypan blue exclusion percentage in response to increasing concentrations of withaferin A.

Figures 2A and 2B show data related to apoptosis. The data shows the apoptotic events (annexin V/PI staining) in human melanoma cells SKMEL28 in response to increasing concentrations of withaferin A.

Figures 3A-3H show that withaferin A induces cell cycle shift from GO/G1 to G2/M phase in B16F10, SKMEL28, NPA and DRO melanoma cells, and that withaferin A increases subGO in DRO and NPA cells indicating withaferin-mediated fragmentation of DNA in these cells. Figures 3C-3E show cell cycle arrest of withaferin-treated B16F10 melanoma cells. Similarly Figure 3F-3H show cell cycle arrest of SKMEL28 melanoma cells.

Figure 4 shows the effect withaferin A from 0 to 24 hours on activation of apoptotic end product caspases 3 and cleavage of caspase 3 substrate PARP in B16F10 cells.

Figure 5 shows the effect of withaferin A when treated for 24 hours on activation of apoptotic end product caspases 3 and cleavage of caspase 3 substrate PARP in SKMEL28 melanoma cells.

Figure 6 shows the effect withaferin A on activation caspase 3 and cleavage of its substrate PARP after treatment for 24 hr.

Figure 7 displays concentration-dependent effect of withaferin A treatment on pro-survival pathways in B16F10 cells.
Figure 8 shows time dependent modulation of prosurvival pathways on withaferin A treatment.

Figures 9-10 show concentration-dependent modulation of prosurvival pathways by withaferin A in SKMEL28, NPA and DRO melanoma cells.

Figures 1IA-1IB show modulation of heat shock proteins by withaferin A in a concentration-dependent fashion in melanoma cells.

Figure 12 shows the effect on modulation of heat shock proteins of increasing concentrations of withaferin A on NPA and DRO cells.

Figure 13 show the effect on protein expression of increasing concentrations of withaferin A.

Figure 14 shows induced expression of HSP70 by withaferin A analogues.

Figures 15-17 show confocal microscopy of analysis of HSP70 levels of SKMEL cells that were treated with 1.0 uM of withaferin A.

Figure 18 shows the effect of withaferin A on RAF levels.
Generally, the present invention is related to the compounds of Formulas 1 (withaferin A, also referred to as D004), 2 (viscosalactone B, also referred to as E002), and 3 (22R-5β-formyl-6β,27-dihydroxy-1-oxo-4-norwith-24-enolide, also referred to as OO1O), as well as salts, prodrugs, analogs, and derivatives thereof which are biologically active for modulating Hsp70, Hsp90, and/or HSFl. Also, these compounds can be included in pharmaceutical compositions for administration to a subject for modulating the expression or activity of Hsp70, Hsp90, and/or HSFl in order to produce a therapeutic effect.

Formula 1

Formula 2

Formula 3

Previously, analogs of geldanamycin, such as 17-allylamino-17-demethoxygeldanamycin (17 AAG), have been proposed for treatment of cancer;
however, the utility of these drugs has been limited by their hepatotoxicity, poor solubility, and poorly tolerated formulations. Accordingly, compounds of Formulas 1-3 have shown to have better cell viability and less toxicity while also being effective for reducing cancer cell proliferation.

Also, viability assay shows that withaferin A from *Vassobia breviflora* reduces proliferation of cancer cells from various sources such as thyroid cancer (papillary, anaplastic, follicular), head and neck squamous cell carcinoma, and melanoma cells. In addition, withaferin A induced apoptosis in these cells. Withaferin A also modulates cell cycles progression of tumor cells. The compounds of Formulas 2-3 are expected to have similar biological activity with withaferin A.

Additionally, withaferin A can be effective in treating, inhibiting, and/or preventing other diseases such as neurodegenerative diseases, muscular dystrophy, and cell over-production and under-production diseases. In part, the therapeutic benefit of withaferin A can be attributed to the biological activity of decreasing the amount or activity of Hsp90. Western blot has shown that withaferin A can reduce the expression of Hsp90, as shown herein.

Also, it is thought that withaferin A can inhibit Hsp90 activity by disrupting the Hsp90-Cdc37 interaction in pancreatic cancer cells. Withaferin A has been shown to disrupt the Hsp90-Cdc37 interaction to halt Hsp90 chaperoning cycle and induce client protein degradation, which may attribute to the anticancer activity of withaferin A. Co-immunoprecipitation (Co-IP) was carried out to determine Hsp90-Cdc37 interaction, which showed that withaferin A (10 uM) disrupted Hsp90-Cdc37 interaction as early as 1 hour post treatment.

An ATP binding assay tested whether withaferin A blocked ATP binding to Hsp90, which showed that withaferin A (5 uM-20 uM) did not block ATP binding to Hsp90. In contrast, 5 uM 17-AAG completely blocked ATP binding to Hsp90.

The expression levels of Hsp90 client proteins Akt, Cdk-4 and Hsp70 were measured by western blot. By disrupting Hsp90-Cdc37 interaction, withaferin A induced Hsp90 client protein Akt, Cdk-4 degradation and Hsp70 upregulation.

MTS assay was used to quantify the cytotoxicity of withaferin A on pancreatic cancer Panc-1 cells. It was found that withaferin A (IC50=1.1 uM) exhibited potent cytotoxicity against pancreatic cancer cells (Panc-1) in vitro. As such, Annexin V staining was used to detect apoptosis in Panc-1 cells. It was found that withaferin A at 1 uM, 5 uM, and 10 uM induced 18%, 46% and 68% apoptosis in Panc-1 cells. The
cytotoxicity can be attributed to apoptosis rather than general toxicity. Accordingly, withaferin A inhibits Hsp90 chaperoning cycle by disrupting Hsp90-Cdc37 interaction, induces client protein degradation, and leads to apoptosis of pancreatic cancer cell.

The anti-cancer effect of withaferin A has been shown on thyroid anaplastic cancer cell line (DRO), papillary thyroid cancer cell line (NPA), follicular thyroid cancer cell line (FTC 133), head and neck squamous cell carcinoma cell line (MDA 1986 and JAMAR) and melanoma cell B16F10. Withaferin A reduces viability of these cancer cells. The IC50 of withaferin A is about 0.6 to about 1 micromolar. In addition, at concentrations close to IC50 (e.g., 0.3 to about 1.5 micromolar) withaferin A induces apoptosis in these cancer cells. Withaferin A decreases HSP90 in tumor cells, which may contribute to withaferin A inhibiting cancer cell propagation.

In one embodiment, the present invention includes the administration of a withaferin A composition for modulation of Hsp90 such that the amount or activity of Hsp90 is reduced. Hsp90 has an ATPase active site, and for Hsp90 to behave as a chaperone it needs to interact with ATP and as a result it is an energy dependent chaperone. It is thought, without being bound thereto, that withaferin A inhibits the ability of HSP90 to behave as a chaperone and/or interact with ATP. In addition, withaferin A can have an effect on the stability of Hsp90 chaperone complex.

The ability of withaferin A to modulate Hsp90 can be used to inhibit cancer progression because many cancer cells are heavily dependent on chaperones or heat shock proteins in order to get proper folding of mutated proteins and regular proteins. At the same time many cancer issues are due to hypoxia because the cancer cells and other cells are under constant stress. Accordingly, inactivation or inhibition of Hsp90 can help the normal tissue combat the stressful situations that the tissue is under in the presence of a tumor.

The ability to inhibit Hsp90 can be effective in inhibiting cancer cell propagation without substantially or adversely affecting normal tissues and cells. It has been determined that cancerous cells and tissue have Hsp90 in an active complex form, whereas normal cells and tissues have Hsp90 in an inactive form. As such, inhibiting Hsp90 can preferentially target cancer cells where Hsp90 is active. Inhibiting Hsp90 when in an inactive form is likely to be inconsequential to normal cells and tissues. This allows for selective targeting of cancerous cells for inhibiting Hsp90. Thus, one advantage of a withaferin A composition targeting Hsp90 is that the active agent can essentially kill cancer cells without having measurable effect on the normal cells.
Hsp70 is a chaperone which either converts an unfolded protein in the cell to a properly folded protein or delivers the unfolded or improperly folded protein for degradation, and thereby, increasing Hsp70 can inhibit improperly folded proteins. It has been found that withaferin A can be effective by increasing the amount or activity of Hsp70. It has been observed that when treating cancer cell lines with withaferin A, Hsp90 is decreased but Hsp70 expression is increased. It is possible that when Hsp90 is inhibited, then a transcription factor is infused and the cell attempts to increase the level of Hsp90, but Hsp70 is increased.

The compounds of Formulas 1-3 were tested side by side with known inducers of Hsp70 like celastrol and 17AAG and it was determined that withaferin A was the highest inducer of Hsp70. In microarray studies we have shown that withaferin A increases the mRNA level of the Hsp70 close to 250 fold. As such, withaferin A increases Hsp70 mRNA and protein levels. Formula 3 has also been shown to increase Hsp70 similar to withaferin, and it is expected that Formula 2 will also have a similar biological activity. Importance of Formula 3 is in treatment of neurodegenerative diseases since this compound those not display reduced cell viability at 20uM concentration.

In terms of biological activity or the range of functions that Hsp90 performs, Hsp70 is different and does not functionally compensate for Hsp90 or provide the activity or function of Hsp90. The fact that Hsp90 is inactive in normal cells and tissues allows for withaferin A to be used to increase Hsp70 in normal tissues without the consequence of substantial negative side effects. Thus, withaferin A compositions can be used to decrease the production, amount, or function of Hsp90 in cancerous cells, and/or increase the production, amount, or function of Hsp70 in any cells, such as normal cells.

Induction of Hsp70 by withaferin A can be used for the treatment of neurodegenerative diseases where chaperone activity of cells are unable to counter balance the production of unfolded proteins such as beta amyloid in Alzheimer's disease and diabetes. In case of Alzheimer's disease, unfolded beta-amyloid proteins are excreted to extracellular space and aggregate to produce neuronal plaque. These plaques induce strong inflammatory response ultimately leading to apoptosis of nerve cells. Induction of HSP 70 will either restore normal folding of unfolded proteins or deliver them for degradation. It has been recently shown that Hsp70 inhibits alpha-synuclein toxicity in a Drosophelia model of Parkinson's disease, and MPTP-induced nigrostriatal degeneration in the mouse model of Parkinson's disease.
The upregulation of Hsp70 can provide cytoprotective activity, which can be useful as a therapy for treating, inhibiting, and/or preventing neurodegenerative diseases such as Alzheimer's. Examples of neurodegenerative diseases that may be treated, inhibited, or prevented with withaferin A compositions can include any of the following: alcoholism, Alexander's disease, Alper's disease, Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, batten disease (also known as Spielmeyer-Vogt-Sjogren-Batten disease), bovine spongiform encephalopathy, canavan disease, cockayne syndrome, corticobasal degeneration, Creutzfeldt-Jakob disease, frontotemporal lobar degeneration, Huntingdon's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, lewy body dementia, neuroborreliosis, Machado-Joseph disease (also known as Spinocerebellar ataxia type 3), multiple system atrophy, narcolepsy, Niemann Pick disease, Parkinson's disease, Pelizaeus-Merzbacher disease, Pick's disease, primary lateral sclerosis, prion diseases, progressive supranuclear palsy, Refsum's disease, Sandhoff's disease, Schilder's disease, subacute combined degeneration of spinal cord, spinocerebellar ataxia, spinal muscular atrophy, Steele-Richardson-Olszewski disease, tabes dorsalis, and the like.

It has been found that withaferin A modulates (inhibits) IKK beta, and this pathway also results in NFKB being modulated (inhibited). Under normal conditions, IKK beta is activated and then this enzyme phosphorylates a smaller protein called IKB alpha. By inhibiting IKK beta, IKB alpha can also be inhibited. The protein IKB alpha also complexes with NFKB, and once IKB alpha is phosphorylated by IKK Beta, then it is released from NFKB and delivered for proteasomal degradation. NFKB is released to go to nucleus to infuse activation of several genes as a transcription factor. NFKB induces expression of proinflammatory cytokines thereby, by inhibiting IKK beta, withaferin A can modulate these proteins and act as a strong anti-inflammatory drug.

Additionally, it has been found that withaferin A can modulate heat shock factor-1 (HSFl) by deceasing the amount or activity. Data shown herein indicates that withaferin A reduces the expression and function of HSFl.

Withaferin A has also been found to modulate NF-kappa B so as to decrease NF-kappa B activity. Accordingly, one of the potential therapeutic application of a withaferin A composition is for the treatment of muscular dystrophy because it has been shown that NF-kappa B is activated in the dystrophic muscle. It is thought, without being bound thereto, that withaferin A modulates the pathway upstream from NF-kappa B by inhibiting an enzyme called IKK beta. It appears that withaferin A inhibits IKK beta and
as a result modulates NF-κB activity. Under normal conditions, IKK beta is activated and phosphorylates a smaller protein called IKB alpha. Normally, IKB alpha is complexed with NF-κB. Once IKB alpha is phosphorylated by IKK Beta, IKB alpha is released from NF-κB and IKB alpha is delivered for degradation. NF-Kappa B is then released to go to nucleus to induce activation of several genes. Thus, withafenn A by inhibiting IKK beta inhibits the phosphorylation of IKB alpha, and this in turn will lead to inactivation of NF-Kappa B. Activation of NF kappa B induces expression of inflammatory proteins and cytokines which induces and enhances the progression of inflammatory diseases such as arthritis and cancer.

Hsp70 is also behave as a "chaperokme". Chaperokine is coined to more properly explain the dual role of extracellular Hsp70 as both chaperone and cytokine. Stimulation of host cells leads to increased expression and release of Hsp70 to the extracellular space. The intracellular elevation of Hsp70 has clearly been shown to be cytoprotective, activating anti-apoptotic and anti-inflammatory pathways, exerting inhibitory effects on cell cycle progression and suppressing genes important in proliferation and differentiation. In addition, the other hand, Extracellular Hsp70 plays an important function as a cytoprotective protein by enhancing pro-inflammatory cytokine and chemokine synthesis, up-regulate co-stimulatory molecule expression, enhance the maturation of dendritic cells and promote antitumour surveillance.

EXTRACT

Withafenn A has been isolated from Vassobia breviflora from an extract obtained from biomass collected from above ground plant parts. Withafenn A can be extracted from Vassobia breviflora by using organic extraction or CO₂ extraction techniques. Briefly, Vassobia breviflora can be obtained and crushed into smaller plant parts, dust, powder, particles, or the like. The crushed Vassobia breviflora is then placed into an organic solvent, and the organic solvent is partitioned into different portions. A wide range of organic solvents can be used. The portion having withafenn A is then collected and optionally filtered, refined, or otherwise processed. The portion of the extract having withafenn A is then processed through HPLC to obtain substantially pure withafenn A.

The CO₂ extraction can be performed in a similar manner but with CO₂ as the extractant. Examples of some organic solvents can include hexane, benzene, toluene, diethyl ether, chloroform, ethyl acetate, 1,4-dioxane, tetrahydrofuran, dichloromethane, acetone, acetonitrile, dimethylformamide, dimethyl sulfoxide, acetic acid, n-butanol, 2-butanol, 3-
butanol, t-butyl alcohol, carbon tetrachloride, chlorobenzene, isopropanol, n-propanol, 
ethanol, methanol, formic acid, water, cyclohexane, 1,2-dichloroethane, diethyl ether, 
diethylene glycol, diglyme, dimethyl ether, dioxane, ethylene glycol, glycerin, heptane, 
hexamethylphosphoramide, hesamethylphosphorous triamide, hexane, nitromethane, 
pentane, petroleum ether, propanol, pyridine, o-xylene, m-xylene, p-xylene, and the like.

The *Vassobia breviflora* extract was now been found to have withaferin A, and 
withaferin A can be extracted or purified from *Vassobia breviflora*. This allows for the 
extract of *Vassobia breviflora* to be used in the methods in place of purified withaferin A. 
Accordingly, the methods of treating, inhibiting, and or preventing cancer or other disease 
(e.g., neurodegenerative diseases, muscular dystrophy, and cell over and under production 
diseases) with withaferin A in accordance with the present invention can be practiced 
with withaferin A in a pure or substantially pure form, non-pure withaferin A, 
compositions that include withaferin A, an extract of *Vassobia breviflora*, or a *Vassobia 
breviflora* composition that has a sufficient amount of active agent (e.g., withaferin A) to 
function in the methods of treatments described herein.

It has been found that an extract of *Vassobia breviflora* can include both 
withaferin A and viscosalactone B, and thereby the extract can be used in an effective 
amount for treatment, inhibition, and/or prevention of cancer or cancer propagation. 
Additionally, the present invention relates to viscosalactone B and the therapeutic uses 
thereof. Viscosalactone B can also be obtained from *Vassobia breviflora*. Viscosalacton 
B has been found to have significant anticancer activity, and it is thought that since the 
structure of viscosalactone B is similar to withaferin A, that these two structures have 
similar activity on Hsp90, Hsp70, HSFl and the like. Thus, viscosalacton B can be used 
with or in place of withaferin A for treatment, inhibition, and/or prevention of cancer or 
cancer propagation as well as neurodegenerative conditions.

Additionally, the compound of Formula 3 was purified from an extract that also 
included withaferin A, and functioned similarly by increasing HSP70. As such, Formula 
3 can be prepared into compositions and methods of use as described with respect to 
withaferin A.

**PHARMACEUTICAL COMPOSITIONS AND METHODS**

Generally, the pharmaceutical compositions can be used for providing a 
compound in an effective amount for the modulation of Hsp90, Hsp70, HSFl, or proteins 
that are downstream in the biological pathways. As such, pharmaceutical compositions 
can be prepared for being administered for the treatment of a disease, disorder, condition
or other physical condition that is responsive to Hsp90 and HSFl being reduced and/or Hsp70 being increased, such as for the treatment of cancer or neurological diseases.

The compounds of the present invention can be formulated into a pharmaceutically acceptable formulation. Such a composition can be useful to prevent, alleviate, eliminate, inhibit or delay the onset of a disease, disorder, and/or condition related thereto. Accordingly pharmaceutical compositions can be used as a prophylactic or treatment for a disease, disorder, and/or condition.

In embodiments of the present invention, the pharmaceutical composition comprises at least one active component and inactive components. The active components are an Hsp90, Hsp70, and HSFl modulation compound as described herein and their derivatives/analalogues, salts, and prodrugs thereof. The inactive components are selected from the group consisting of excipients, carriers, solvents, diluents, stabilizers, enhancers, additives, adhesives, and combinations thereof.

The amount of the compound in a formulation can vary within the full range employed by those skilled in the art. Typically, the formulation will contain, on a weight percent basis, from about 0.01-99.99 weight percent of the compounds of the present invention based on the total formulation, with the balance being one or more suitable pharmaceutical excipients. Preferably, the compounds are present at a level of about 1-80 weight percent.

Pharmaceutical preparations include sterile aqueous or non-aqueous solutions, suspensions and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil such as olive oil, injectable organic esters such as ethyloliate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, antioxidants, chelating agents and inert gases and the like. Those of skill in the art can readily determine the various parameters for preparing these pharmaceutical compositions without resort to undue experimentation.

Pharmacological compositions may be prepared from water-insoluble compounds, or salts thereof, such as aqueous base emulsions. In such embodiments, the pharmacological composition will typically contain a sufficient amount of
pharmaceutically acceptable emulsifying agent to emulsify the desired amount of the pharmacological agent. Useful emulsifying agents include, but are not limited to, phosphatidyl cholines, lecithin, and the like.

Additionally, the compositions may contain other additives, such as pH-adjusting additives. In particular, useful pH-adjusting agents include acids, such as hydrochloric acid, bases or buffers, such as sodium lactate, sodium acetate, sodium phosphate, sodium citrate, sodium borate, or sodium gluconate. Furthermore, pharmacological agent compositions may, though not always, contain microbial preservatives. Microbial preservatives that may be employed include, but are not limited to, methylparaben, propylparaben, and benzyl alcohol. The microbial preservative may be employed when the pharmacological agent formulation is placed in a vial designed for multi-dose use.

Pharmacological agent compositions for use in practicing the subject methods may be lyophilized using techniques well known in the art.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Examples of suitable excipients can include, but are not limited to, the following: acidulents, such as lactic acid, hydrochloric acid, and tartaric acid; solubilizing components, such as non-ionic, cationic, and anionic surfactants; absorbents, such as bentonite, cellulose, and kaolin; alkalizing components, such as diethanolamine, potassium citrate, and sodium bicarbonate; anticaking components, such as calcium phosphate tribasic, magnesium trisilicate, and talc; antimicrobial components, such as benzoic acid, sorbic acid, benzyl alcohol, benzethonium chloride, bronopol, alkyl parabens, cetrimide, phenol, phenylmercuric acetate, thimerosal, and phenoxyethanol; antioxidants, such as ascorbic acid, alpha tocopherol, propyl gallate, and sodium metabisulfite; binders, such as acacia, aiginic acid, carboxymethyl cellulose, hydroxyethyl cellulose; dextrin, gelatin, guar gum, magnesium aluminum silicate, maltodextrin, povidone, starch, vegetable oil, and zein; buffering components, such as sodium phosphate, malic acid, and potassium citrate; chelating components, such as EDTA, malic acid, and maltol; coating components, such as adjunct sugar, cetyl alcohol, polyvinyl alcohol, carnauba wax, lactose maltitol, titanium dioxide; controlled release vehicles, such as microcrystalline wax, white wax, and yellow wax; desiccants, such as calcium sulfate; detergents, such as sodium lauryl sulfate; diluents, such as calcium phosphate, sorbitol, starch, talc, lactitol, polymethacrylates, sodium chloride, and glyceryl palmitostearate; disintegrants, such as colloidal silicon dioxide, croscarmellose sodium, magnesium aluminum silicate, potassium polacriliα and sodium
starch glycolate; dispersing components, such as poloxamer 386, and polyoxyethylene fatty esters (polysorbates); emollients, such as cetearyl alcohol, lanolin, mineral oil, petrolatum, cholesterol, isopropyl myristate, and lecithin; emulsifying components, such as anionic emulsifying wax, monoethanolamine, and medium chain triglycerides; flavoring components, such as ethyl maltol, ethyl vanillin, fumaric acid, malic acid, maltol, and menthol; humectants, such as glycerin, propylene glycol, sorbitol, and triacetin; lubricants, such as calcium stearate, canola oil, glyceryl palmitostearate, magnesium oxide, poloxamer, sodium benzoate, stearic acid, and zinc stearate; solvents, such as alcohols, benzyl phenylformate, vegetable oils, diethyl phthalate, ethyl oleate, glycerol, glycofurol, for indigo carmine, polyethylene glycol, for sunset yellow, for tartazine, triacetin; stabilizing components, such as cyclodextrins, albumin, xanthan gum; and tonicity components, such as glycerol, dextrose, potassium chloride, and sodium chloride; and mixture thereof. Excipients include those that alter the rate of absorption, bioavailability, or other pharmacokinetic properties of pharmaceuticals, dietary supplements, alternative medicines, or nutraceuticals.


In general, pharmaceutically acceptable carriers for are well-known to those of ordinary skill in the art. This carrier can be a solid or liquid and the type is generally chosen based on the type of administration being used. Suitable pharmaceutical carriers are, in particular, fillers, such as sugars, for example lactose, sucrose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, for example tricalcium phosphate or calcium hydrogen phosphate, furthermore, binders such as starch paste, using, for example, corn, wheat, rice or potato starch, gelatin, tragacanth, methylcellulose and/or polyvinylpyrrolidone, if desired, disintegrants, such as the above mentioned starches; furthermore carboxymethyl starch, crosslinked polyvinylpyrrolidone, agar, alginic acid or a salt thereof, such as sodium alginate; auxiliaries are primarily glidants, flow-regulators and lubricants, for example silicic acid, talc, stearic acid or salts thereof, such as magnesium or calcium stearate, and/or polyethylene glycol. Sugar-coated tablet cores are provided with suitable coatings which, if desired, are resistant to gastric juice, using, inter alia, concentrated sugar solutions which, if desired, contain gum arabic, talc,
polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, coating solutions in
suitable organic solvents or solvent mixtures or, for the preparation of gastric juice-
resistant coatings, solutions of suitable cellulose preparations, such as acetylcellulose
phthate or hydroxypropylmethylcellulose phthalate. Colorants or pigments, for
example, to identify or to indicate different doses of active ingredient, may be added to
the tablets or sugar-coated tablet coatings.

Additional pharmaceutically acceptable carriers that may be used in these
pharmaceutical compositions include, but are not limited to, ion exchangers, alumina,
aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer
substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride
mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as prolamine
sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride,
zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based
substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes,
polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

Additional formulations for use in the present invention can be found in
Remington's Pharmaceutical Sciences (Mack Publishing Company, Philadelphia, Pa,
17th ed. (1985)), which is incorporated herein by reference. Moreover, for a brief review
of methods for drug delivery, see, Langer, Science 249:1527-1533 (1990), which is
incorporated herein by reference. The pharmaceutical compositions described herein can
be manufactured in a manner that is known to those of skill in the art, i.e., by means of
conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying,
encapsulating, entrapping, or lyophilizing processes. Other examples of suitable
pharmaceuticals are listed in 2000 Med Ad News 19:56-60 and The Physicians Desk
Reference, 53rd edition, 792-796, Medical Economics Company (1999), both of which
are incorporated herein by reference.

In general, compounds of this invention can be administered as pharmaceutical
compositions by any one of the following routes: oral, systemic (e.g., transdermal,
intrasal or by suppository), or parenteral (e.g., intramuscular, intravenous or
subcutaneous) administration. One manner of administration is oral using a convenient
daily dosage regimen which can be adjusted according to the degree of affliction.
Compositions can take the form of tablets, pills, capsules, semisolids, powders, sustained
release formulations, solutions, suspensions, elixirs, aerosols, or any other appropriate
compositions. Another manner for administering compounds of this invention is
According to the methods of the present invention, the compositions of the invention can be administered by injection by gradual infusion over time or by any other medically acceptable mode. Any medically acceptable method may be used to administer the composition to the patient. The particular mode selected will depend of course, upon factors such as the particular drug selected, the severity of the state of the subject being treated, or the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active composition without causing clinically unacceptable adverse effects.

The administration may be localized (i.e., to a particular region, physiological system, tissue, organ, or cell type) or systemic. For example, the composition may be administered through parental injection, implantation, orally, vaginally, rectally, buccally, pulmonary, topically, nasally, transdermally, surgical administration, or any other method of administration where access to the target by the composition is achieved. In one example, the administration is directly into the brain or brain cavity. Examples of parenteral modalities that can be used with the invention include intravenous, intradermal, subcutaneous, intracavity, intramuscular, intraperitoneal, epidural, or intrathecal. Examples of implantation modalities include any implantable or injectable drug delivery system. Oral administration may be used for some treatments because of the convenience to the patient as well as the dosing schedule. Compositions suitable for oral administration may be presented as discrete units such as capsules, pills, cachettes, tables, or lozenges, each containing a predetermined amount of the active compound. Other oral compositions include suspensions in aqueous or non-aqueous liquids such as a syrup, an elixir, or an emulsion.

The compounds can be encapsulated in a vehicle such as liposomes that facilitates transfer of the bioactive molecules into the targeted tissue, as described, for example, in U.S. Pat. No. 5,879,713 to Roth et al. and Woodle, et al., U.S. Pat. No. 5,013,556, the contents of which are hereby incorporated by reference. The compounds can be targeted by selecting an encapsulating medium of an appropriate size such that the medium delivers the molecules to a particular target. For example, encapsulating the compounds within microparticles, preferably biocompatible and/or biodegradable microparticles, which are appropriate sized to infiltrate, but remain trapped within, the capillary beds and alveoli of the lungs can be used for targeted delivery to these regions of the body.
following administration to a patient by infusion or injection.

Microparticles can be fabricated from different polymers using a variety of different methods known to those skilled in the art. The solvent evaporation technique is described, for example, in E. Mathiowitz, et al., J. Scanning Microscopy, 4, 329 (1990); L. R. Beck, et al., Fertil. Steril., 31, 545 (1979); and S. Benita, et al., J. Pharm. Sci., 73, 1721 (1984). The hot-melt microencapsulation technique is described by E. Mathiowitz, et al., Reactive Polymers, 6, 275 (1987). The spray drying technique is also well known to those of skill in the art. Spray drying involves dissolving a suitable polymer in an appropriate solvent. A known amount of the compound is suspended (insoluble drugs) or co-dissolved (soluble drugs) in the polymer solution. The solution or the dispersion is then spray-dried. Microparticles ranging between 1-10 microns are obtained with a morphology which depends on the type of polymer used.

Embodiments may also include administration of at least one pharmacological agent using a pharmacological delivery device or system such as, but not limited to, pumps (implantable or external devices), epidural injectors, syringes or other injection apparatus, catheter and/or reservoir operatively associated with a catheter, injection, and the like. For example, in certain embodiments a delivery device employed to deliver at least one pharmacological agent to a subject may be a pump, syringe, catheter or reservoir operably associated with a connecting device such as a catheter, tubing, or the like. Containers suitable for delivery of at least one pharmacological agent to a pharmacological agent administration device include instruments of containment that may be used to deliver, place, attach, and/or insert at least one pharmacological agent into the delivery device for administration of the pharmacological agent to a subject and include, but are not limited to, vials, ampules, tubes, capsules, bottles, syringes and bags. In one embodiment, a catheter can be used to direct the composition directly to the brain or other location in the body for systemic delivery.

The compositions of the present invention may be given in dosages, generally at the maximum amount while avoiding or minimizing any potentially detrimental side effects. The compositions can be administered in effective amounts, alone or in a cocktail with other compounds, for example, other compounds that can be used to treat, inhibit, or prevent drug addiction or drug-seeking behavior.

In one embodiment of the present invention, therapeutically effective amounts of compounds of the present invention may range from approximately 0.05 to 50 mg per kilogram body weight of the recipient per day; preferably about 0.01-25 mg/kg/day, more
preferably from about 0.5 to 10 mg/kg/day. Thus, for administration to a 70 kg person, the dosage range would most preferably be about 35-70 mg per day.

In another embodiment of the present invention, dosages may be estimated based on the results of experimental models, optionally in combination with the results of assays of the present invention. Generally, daily oral doses of active compounds will be from about 0.01 mg/kg per day to 2000 mg/kg per day. Oral doses in the range of 10 to 500 mg/kg, in one or several administrations per day, may yield suitable results. In the event that the response of a particular subject is insufficient at such doses, even higher doses (or effective higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are also contemplated in some cases to achieve appropriate systemic levels of the composition.

Use of a long-term release implant may be particularly suitable in some cases. "Long-term release," as used herein, means that the implant is constructed and arranged to deliver therapeutic levels of the composition for at least 30 or 45 days, and preferably at least 60 or 90 days, or even longer in some cases. Long-term release implants are well known to those of ordinary skill in the art, and include some of the release systems described above.

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound which are sufficient to maintain therapeutic effect. Preferably, therapeutically effective serum levels will be achieved by administering multiple doses each day. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration. One having skill in the art will be able to optimize therapeutically effective local dosages without undue experimentation.

A. Definitions

As used herein, the terms "an effective amount", "therapeutic effective amount", or "therapeutically effective amount" shall mean an amount or concentration of a compound according to the present invention which is effective within the context of its administration or use. Thus, the term "effective amount" is used throughout the specification to describe concentrations or amounts of compounds according to the present invention which may be used to produce a favorable change in the disease or condition treated, inhibited, or prevented, whether that change is a remission, a decrease in a symptom, a favorable physiological result, or the like, depending upon the disease or condition treated.
As used herein, the term "pharmaceutically acceptable excipient" means an excipient that is useful in preparing a pharmaceutical composition that is generally safe, non-toxic and neither biologically nor otherwise undesirable, and includes an excipient that is acceptable for veterinary use as well as human pharmaceutical use. A "pharmaceutically acceptable excipient" as used in the specification and claims includes both one and more than one such excipient.

As used herein, the term "coadministration" or "combination therapy" is used to describe a therapy in which at least two active compounds in effective amounts are used for the treatment, inhibition, and/or prevention of a condition.

As used herein, the term "treating" or "treatment" of a disease, cancer or neurodegenerative disease, includes: (a) preventing the disease, i.e. causing the clinical symptoms of the disease not to develop in a mammal that may be exposed to or predisposed to the disease but does not yet experience or display symptoms of the disease; (b) inhibiting the disease, i.e., arresting or reducing the development of the disease or its clinical symptoms; or (c) relieving the disease, i.e., causing regression of the disease or its clinical symptoms.

As used herein, the term "unit dosage form," refers to physically discrete units suitable as unitary dosages for human and animal subjects, each unit containing a predetermined quantity of pharmacological agent calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier or vehicle.

As used herein, a "subject" or a "patient" refers to any animal (preferably, a human), and preferably a mammal. Examples of a subject or patient include a human, a non-human primate, a cow, a horse, a pig, a sheep, a goat, a dog, a cat or a rodent such as a mouse, a rat, a hamster, or a guinea pig. Generally, the invention is directed toward use with humans.

In some embodiments, the compounds in the compositions may be present as a pharmaceutically acceptable salt. The pharmaceutically acceptable salts includes salts of the active agent or components of the composition, prepared, for example, with acids or bases, depending on the particular substituents found within the composition and the treatment modality desired. Pharmaceutically acceptable salts can be prepared as alkaline metal salts, such as lithium, sodium, or potassium salts; or as alkaline earth salts, such as beryllium, magnesium or calcium salts. Examples of suitable bases that may be used to form salts include ammonium, or mineral bases such as sodium hydroxide, lithium
hydroxide, potassium hydroxide, calcium hydroxide, magnesium hydroxide, and the like. Examples of suitable acids that may be used to form salts include inorganic or mineral acids such as hydrochloric, hydrobromic, hydroiodic, hydrofluoric, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, phosphorous acids and the like. Other suitable acids include organic acids, for example, hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as trifluoroacetic, acetic, propionic, glycolic, pyruvic, oxalic, maleic, malonic, succinic, fumaric, tartaric, citric, benzoic, cinnamic, mandelic, methanesulfonic, ethanesulfonic, p-toluensulfonic, salicylic, isobutyric, suberic, phthalic, benzenesulfonic, p-tolylsulfonic, salicylic, formic, naphthalene-2-sulfonic, and the like. Still other suitable acids include amino acids such as arginate, aspartate, glutamate, and the like.

Additionally, the compounds can be prepared to be prodrugs that include a cleavable linker between the base analog and the prodrug portion. Phosphate groups that cleave to leave hydroxyl groups are one example of produg moieties.

As used herein, the term "analogue" or the like is meant to refer to a structurally related compound or compounds with a common scaffold that different functional groups or substituents. For example, the different functional groups or hydrogens in Formulas 1-3 can be prepared into analogs by changing one or more groups or hydrogens. Altering the number of backbone atoms in a ring can be considered analogs.

The substitutions of the functional groups or hydrogens can be replaced with any one of: hydrogen, halogen, hydroxyl, straight or branched substituted or unsubstituted alkoxy, amine, straight or branched substituted or unsubstituted alkyl, substituted or unsubstituted cycloalkyl, substituted or unsubstituted aryl, branched or unbranched or cyclic substituted or unsubstituted arylalkyl, or combinations thereof, where the alkyls can be C1-C20, C1-C10, C1-C6, or C1-C4, where the rings can be 3, 4, 5, 6, 7, or 8 membered, and any can include chain atoms having hetero atoms selected from N, O, S, or P.

As used herein, the term "derivative" or the like is meant to refer to a change or substitution of an atom with another atom or group. For example, when a hydrogen is replaced with a halogen or a hydroxyl group, such a change produces a derivative.

The compounds described herein can be prepared into racemic mixtures, or the individual enantiomers thereof. Each compound can also be present as an individual
enantiomer that is separate from its other enantiomers. It is thought that an individual enantiomer may have enhanced biological activity over its other enantiomers.

The present invention may be embodied in other specific forms without departing from its spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive. The scope of the invention is, therefore, indicated by the appended claims rather than by the foregoing description. All changes which come within the meaning and range of equivalency of the claims are to be embraced within their scope. All references recited herein are incorporated herein in their entirety by specific reference.
EXPERIMENTAL

Withaferin A (Formula 1) and viscosalactone B (Formula 2) can be isolated from the aerial parts of *Vassobia breviflora* (Sendtn.) Hunz. (Solanaceae). Briefly, Aerial parts *V. breviflora* were collected from Formosa, Argentina. 100 g of dried, ground biomass were exhaustively extracted with a 1:1 mixture of DCM and MeOH. Crude extract was fractionated by normal phase solid phase extraction (SPE) to yield eight fractions of increasing polarity (hexane-ethyl acetate-methanol), which were subjected to bioassay. The remaining crude extract was defatted with hexane and then taken up in water and washed with ethyl acetate followed by butanol. The ethyl acetate portion correlated with the active SPE fraction (B007) by TLC and was subsequently fractionated by vacuum liquid chromatography (VLC) with DCM and increasing amounts of MeOH. VLC fraction 3 (95:5 DCM:MeOH; 820.3 mg) was further fractionated by normal phase SPE to yield [1] (350.7 mg), while VLC fractions 5 and 6 (90:10 and 80:20 DCM:MeOH; 190.6 mg) were combined and fractionated by reversed phase SPE to yield [2] (71.1 mg). Withanolides were identified by a combination of 1D and 2D NMR techniques (Bruker Avance 400 MHz NMR spectrometer) and the chemical shifts were found to be in accordance with those in the literature. Withanolides are well documented constituents of the Solanaceae and have been previously isolated from *V. lorentzii*.

Extraction and isolation procedures were performed to obtain withaferin A (Formula 1, D004), viscosalactone B (Formula 2, E002), and (22R)-β-formyl-6β,27-dihydroxyl-l-oxo-4-norwith-24-enolide (Formula 3, O010). Dried, ground biomass (100 g) of *V. breviflora* was extracted at room temperature with CH$_2$Cl$_2$-MeOH 1:1 (3X). The concentrated extract (9.8 g) was dissolved in MeOH-H$_2$O 10:90 followed by partition with r-t-hexane, EtOAc and n-butanol. The EtOAc extract (2.65 g) was subjected to silica gel column chromatography (32-60 μm, 5 x 60 cm, Sorbent Technologies) and eluted with a mixture OfCH$_2$Cl$_2$-MeOH (100:0, 98:2, 95:5, 93:7, 90:10, 80:20, 50:50 and 0:100) in order of increasing polarity. A total of 182 fractions were collected: fractions 49-54 were combined as fraction C (830.3 mg, CH$_2$Cl$_2$-MeOH 93:7); fractions 58-68 were combined as fraction E (190.6 mg, CH$_2$Cl$_2$-MeOH 93:7). Fraction C was further purified by normal phase SPE (20 g, 60 ml, Phenoment strata si) using a step gradient of CH$_2$Cl$_2$-MeOH (90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 50:50 and 0:100). Among nine collected fractions, 50 mg withaferin A (compound D004) were obtained in the most active fractions (CH$_2$Cl$_2$-MeOH 70:30). Fraction E was separated on a reverse phase SPE (5 g, 20 ml, Phenoment strata C-18), eluted with a step gradient OfH$_2$O-MeOH (30:70,
40:60, 50:50, 60:40, 70:30, 80:20 and 90:10), and viscosalactone B (compound E002) was identified in faction 3 (71.1 mg, H$_2$O-MeOH 50:50).

The compounds can also be extracted from *Withania somnifera* (Dunal). Briefly, the organic extract of *W. somnifera* was purchased from a commercial vendor (PhytoMyco Research Corporation). The organic extract (189 g) was dissolved in 2 L of water to form a suspension. The mixture was defatted with hexane (2 L x 3), and the aqueous phase was extracted with EtOAc (2 L x 6) to obtain 32.9g of an EtOAc extract. This extract was then subjected to passage over a silical gel column (32-60 µm, 600g, Sorbent Technologies), eluted with acetone-DCM (from 100% DCM to 100% acetone, then 100% MeOH), to give eight major fractions (Fraction A-H). Fraction E (6g, DCM-acetone 9:1) was chromatographed on a silica gel column (12-26 µm, 5 x 60 cm Sorbent Technologies), eluted with DCM-acetone (10:1), to collect twelve fractions (E1-E12). Fraction E6 was identified as withaferin A (compound D004). Fraction E12 (12mg) was separated over a C-18 reverse phase SPE column (5 g, 20 ml, Phenomen strata C-18 ), eluted with MeOH-H$_2$O (1:1), to give a mixture of compounds (22R)-5β-formyl-6β,27-dihydroxy-l-oxo-4-norwith-24-enolide (compound OOIO) and 2,3-dihydrowithaferin A. Further separation of this mixture on HPLC (Acetonitrile- water 45:55) produced both pure compounds.

**Cell lines and cell culture:** The human melanoma cancer cell (NPA), and DRO were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1% L-glutamine, and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA), squamous cell carcinoma of head and neck (MDA 1986, JMAR) and melanoma cells lines B16F10, SKMEL28 were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 1X MEM-essential amino acid, 1X MEM-vitamin, and penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humid environment of 5% CO$_2$ at 37°C.

**In vitro proliferation assay:** Cells were cultured in 96 well plates at a concentration of 1-2x10$^4$ cells/mL. Cell viability was assessed with the nonradioactive cell proliferation MTS assay using Cell Titer 96AQueous One Solution Reagent (Promega, Madison, WI, USA), according to the manufacturer's instruction. After 72 hr treatment with the plant extracts and pure compounds, 20 uL of Cell Titer 96AQueous One Solution Reagent were added to each well and incubated for 3hr at 37°C 5% CO$_2$. 
and formazan absorbance was measured at 490nm. Each measurement was made in triplicates.

Figures IA-I C show the results of the cell viability assay. Cell Viability assay was performed on two hundred plant extracts, fractions or purified compounds from 14 medicinal plants were screened for activity against human melanoma cancer cell lines DRO and NPA and HNSCC cell lines MDA 1986 and JMAR. Five plants [ including *curcuma longa* (tumeric), one plant from the *Solanaceae* family, and *Gutteria recurviseplala*] including several extracts and pure compounds derived from each plant show significant antiproliferative activity against all melanoma cancer and HNSCC cell lines tested by MTS assay, with IC50 values ranging from 20 nM for triptolide to 5.3 uM for curcumin. Curcumin extracts at IC50 concentrations had moderate co-staining with Annexin-v-FITC with 30-40% of cells gated to apoptosis by flow cytometry (data not shown). Cell viability of cancer cells treated with pure withaferin A (D004) and viscosalactone B (E002) from *Solanaceae* decreased with IC50 ranging 0.95 to 1.4 uM for withaferin A (D004) and IC50 for viscosalactone B (E002) ranged from 1.97 to 2.18 uM.

Figure ID shows that withaferin A reduces cell viability of melanoma cells, such as B16F10, SKMEL28, NPA and DRO. Also, withaferin A reduced cell proliferation of the melanoma cells.

Figure IE shows the viability of melanoma cells were decreased upon treatment with withaferin A; IC50 for B16F10, SKMEL28, NPA and DRO cells were 1.45, 3.85, 1.264 and 1.367 respectively in a concentration-dependent manner.

Figure IF shows that when B16F10 cells were treated with different concentrations of withaferin A for up to 72 hr. Also, withaferin A decreases cell viability in a time-dependent manner.

Figure IG shows that the cell viability when melanoma cells were treated for indicated times [3.0 uM] and then cells were washed three times with PBS and fresh media were added without withaferin A. As it is shown, as little as 4 hours of treatment commits B16F10 cells to cell-death even in the absence of withaferin A, indicating the withaferin A irreversibly commits cells to cell death at early stages and apoptotic events are not stopped once withaferin A is removed.

Figured 1H-II also shows that withaferin A reduces cell viability in melanoma cancer cells. The melanoma cell lines were treated with different concentration of withaferin A as shown. Accordingly, withaferin A reduces cell proliferation of murine
melanoma cells B16F10 and human melanoma cell line SKMEL28 in a concentration-dependent manner. In addition these data shows that B16F10 are more sensitive to withaferin A antiproliferative effects.

Figure 1J shows that treatment of melanoma cells with withaferin A induced cell death by the trypan blue exclusion experiments show that withaferin A induces apoptosis in B16F10 and SKMEL28 cells.

Flow cytometry: Apoptosis was confirmed using annexin-V-FITC staining (BD Bioscience), according to manufacturer's instruction. Cell cycle fractions were determined by propidium iodide (PI) nuclear staining. Briefly, cells were treated with plant extracts and pure compounds for the designated time points and were harvested, washed in phosphate-buffered saline, fixed with 70% ethanol, and incubated with PI for 30 minutes at 37°C. Data were collected and analyzed on a Becton Dickinson FACSCalibur flow cytometer CellQuestPro (BD Biosciences, San Jose, CA, USA).

Figures 2A-2B show data related to apoptosis. Study of induction of apoptosis in melanoma cell lines B16F10, SKMEL28, DRO and NPA cells by withaferin A (D004) shows that all cancer cells tested underwent apoptosis detected by staining of annexin V. Treatment with up to 2X of IC50 of withaferin A (D004) for 24 hr induced a large degree of apoptosis in these cells. At these concentration very little necrosis was detected in these cancer cells. The cells were treated with 3.0 μM of withaferin A for 24 hr, and treatment of cells with withaferin A increases annexin V staining.

Figures 3A-3H show that withaferin A induces cell cycle shift from G0/G1 to G2/M phase in B16F10, SKMEL28, NPA and DRO cells. In addition, withaferin A increases subGO in DRO and NPA cells indicating fragmentation of DNA in these cells. The cell cycle analysis of thyroid cancer cells and HNSCC indicate that withaferin A induces apoptosis which was detected by increased subGO levels in DRO and NPA cells. When DRO cells were treated with withaferin A (D004) the levels of subGO cells increased 6.4% to 13.3% and similarly NPA cells display increase from 1.4% to 14.5%, which is almost a 10-fold increase. Treatment of HNSCC cells with withaferin A (D004) caused G2/M cell cycle arrest. Accordingly, withaferin A induces cell cycle shift from GO/G1 to G2/M phase in B16F10, SKMEL28, NPA and DRO cells. In addition, withaferin A increases subGO in DRO and NPA cells indicating fragmentation of DNA in these cells.

Western blot analysis: After treatment with withaferin A, cells were washed with ice cold PBS and harvested and suspended in ice-cold lysis buffer (50 mM Tris-HCl, pH
The suspension was kept on ice for 20 min, and passed through 25" gauge syringe and centrifuged at 14,000 xg for 15 min at 4°C. 25-50 ug of protein was applied to 10-12% SDS-PAGE and proteins were electrotransfered to cellulose acetate membrane. After immunoblotting, bands were detected by chemiluminesence detection.

Also, strong antiproliferative activity in all melanoma and HNSCC cell lines was observed after treatment with the compounds of the invention. This mechanism in several compounds is likely due to induction of apoptosis with withaferin A (D004) also demonstrating cell cycle arrest in G2/M phase in HNSCC. Withaferin A (D004) is also a Hsp90 inhibitor as well as a strong inducer of Hsp70 and GRP94 in melanoma cells. These data suggest encouraging evidence for future in vivo analysis to determine if these natural plant compounds have translational application as future cancer therapies.

To confirm annexin V data, melanoma cells were treated with 3.0 uM of withaferin A for 24 hr and cell lysates were prepared for immunoblotting against caspase 3 and PARP. Figures 4-6 shows that the results confirm by cytometric analysis of annexin V stained cells that withaferin A induced activation of caspase 3 which is evident from conversion of procaspase 3 to active caspase 3 and cleavage of cellular caspase 3 substrate PARP in a concentration and time-dependent manner.

Figures 7-10 show that withaferin A inhibits activation of AKT, p38 MAPK, and JNK in a concentration-dependent and time-dependent manner. In addition, withaferin A reduces the total levels of AKT, p38 MAPK and JNK, whereas phosphorylated levels of ERK1/2 and the total levels of ERK are constant. Since these melanoma cells including B16F10, SKMEL28, NPA and DRO carry a BRAF(V600E) mutation and therefore this mutation creates a constitutively active RAF-RAS-MEK-ERK pathway, withaferin A at 10% fetal bovine serum containing media does not change the ERK phosphorylation levels while all of these cells undergo apoptosis. Cancer cells display increased proliferative signal transduction pathways which are responsible for increased growth and viability. The effect of withaferin A on prosurvival pathways was examined in melanoma cancer cells.

Figure 11A-I 1B and 13 shows the concentrical effects of withaferin A on various proteins. It was found that withaferin A "modulates heat shock proteins in melanoma cells. Treatment of B16F10 and SKMEL28 cells in a concentration dependent manner increased Hsp72 levels. Treatment with higher levels (e.g., 10 micromolar) of
withafe π n A reduced the Hsp72 levels (Hsp72 can be equivalent to Hsp70). This is a new and unexpected effect of withafenn A in mammalian cells. In addition to treatment of cancer cells, withafe π n A may have potential therapeutic effect in prevention and treatment of neurodegenerative diseases.

Figure 12 shows Western blot data related to the treatment of melanoma cells with withafe π n A (D004), which reduced the levels of Hsp90 isoform beta at 250 nM concentration. Levels of other heat shock proteins including HS27, Grap94 and Trapl are increased upon withafe π n treatment. In addition, HSP70 levels are marked increased.

Additionally, heat shock factor 1 (HSFl) levels were reduced upon withafe π n A treatment in a concentration and time-dependent manner. It is interesting that withafe π n A reduces HSFl levels three fold below IC50 for SKMEL cells (0.5 μM vs 3.85 μM). This is a novel and unexpected effect of withafenn A in downregulation of HSFl. This effect of withafe π n A has a great potential for treatment of cancer since almost all tumor cells are highly dependent on the robust expression of heat shock proteins, and results show that withafe π n A reduces cellular levels of HSFl and correspondingly reduces the levels of HSP70 levels in the cancer cells. The data show the levels of Hsp70 initially increases upon withafenn A treatment, however, withafenn A reduced the level of HSFl which can prevent prolonged expression of HSP72. The Hsp90 levels remained unchanged upon withafe π n A treatment.

Figure 14 shows that the ability of some natural analogues of withafe π n A capacity to modulate Hsp72 (Hsp70). It was found that compound OOIO (Formula 3) was capable of upregulating the expression of Hsp70 in B16F10 cells. It is interesting to note that compound OOIO (Formula 3) does not affect the cell viability of B16F10 cells. Accordingly, compound OOIO (Formula 3) may be capable of being used for the treat of neurodegenerative diseases including Alzheimer's, Parkinson, Huntington, and macular degeneration diseases among others.

Figures 15-17 show pictures of SKMEL cells that were treated with 10 μM of withafenn A. The cellular localization of Hsp72 was identified by confocal microscopy. The data show that Hsp72 is overexpressed in the withafe π n A treated cells, and they migrate to the cellular nucleus and cells membrane. The cell membrane localization of Hsp72 and possible release of Hsp72 to extracellular matrix have been shown to activate immune response and modulate inflammation. Therefore, membrane localization of Hsp72 is new and unexpected finding of withafe π n A with may modulate immune response.
Figure 18 shows the effect of withaferin A reduces the expression of RAF in melanoma cells. This is an unexpected and significant finding since melanoma cells are highly dependent on RAF proteins for their survival. Decrease in the level of RAF protein is a novel and unexpected finding of the effect of withaferin A and is very important for the treatment of melanoma cancer.
CLAIMS

1. A method for modulating a heat shock protein, the method comprising:
providing a composition having a compound of one or more of Formulas 1-3 or 
analog, derivative, prodrug, or pharmaceutically acceptable salt thereof; and 
administering the composition such that the heat shock protein is modulated:

Formula 1;

Formula 2; and

Formula 3.

2. A method as in claim 1, wherein the heat shock protein is one of heat 
shock protein 70 (Hsp70), heat shock protein 90 (Hsp90), or heat shock factor 1 (HSF1).
3. A method as in claim 2, wherein the composition is administered in an effective amount to increase Hsp70.

4. A method as in claim 2, wherein the composition is administered in an effective amount to decrease Hsp90.

5. A method as in claim 2, wherein the composition is administered in an effective amount to increase HSFl.

6. A method as in claim 1, wherein the compound is of Formula 1.

7. A method as in claim 1, wherein the compound is of Formula 2.

8. A method as in claim 1, wherein the compound is of Formula 3.

9. A method as in claim 1, wherein the compound is present in a therapeutically effective amount to provide a treatment by sufficiently modulating the heat shock protein.

10. A method as in claim 10, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing cancer.

11. A method as in claim 10, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing cancer cell propagation.

12. A method as in claim 10, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing a cancer selected from thyroid cancer, papillary thyroid cancer, follicular thyroid cancer, head and neck squamous cell cancer, and melanoma.

13. A method as in claim 9, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing a neurodegenerative disease.

14. A method as in claim 13, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing at least one of alcoholism, Alexander's disease, Alper's disease, Alzheimer's disease, amyotrophic lateral sclerosis, ataxia telangiectasia, batten disease (also known as Spielmeyer-Vogt-Sjogren-Batten disease), bovine spongiform encephalopathy, canavan disease, cockayne syndrome, corticobasal degeneration, Creutzfeldt-Jakob disease, frontotemporal lobar degeneration, Huntington's disease, HIV-associated dementia, Kennedy's disease, Krabbe's disease, lewy body dementia, neuroborreliosis, Machado-Joseph disease (also known as Spino cerebellar ataxia type 3), multiple system atrophy, narcolepsy, Niemann Pick disease, Parkinson's disease, Pelizaeus-Merzbacher disease, Pick's disease, primary lateral sclerosis, prion diseases, progressive supranuclear palsy, Refsum's disease, Sandhoff's disease, Schilder's disease, subacute combined degeneration of spinal cord.
spino-cerebellar ataxia, spinal muscular atrophy, Steele-Richardson-Olszewski disease, tabes dorsalis, or symptoms thereof.

15. A method as in claim 14, wherein the therapeutically effective amount is sufficient for use in treating, inhibiting, and/or preventing Alzheimer's disease or symptom thereof.

16. A method as in claim 9, wherein the therapeutically effective amount is sufficient for use in providing a cytoprotective activity to a cell.

17. A method as in claim 1, wherein the heat shock protein is in a subject.

18. A method as in claim 17, wherein the subject is human.

19. A method as in claim 1, wherein the composition includes an extract of a plant.

20. A method as in claim 1, further comprising a protein downstream from one of Hsp70, Hsp90, and HSF1.

21. A method as in claim 20, wherein NF-Kappa B is modulated.

22. A method as in claim 61, wherein IKK beta is modulated.

23. A method as in claim 61, wherein IKB alpha is modulated.
FIG. 1A

FIG. 1B
**FIG. 1C**

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**FIG. 1D**

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<td>0.6737</td>
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FIG. 1G

FIG. 1H
FIG. 2B
FIG. 3B

Control

Withaferin A

DRO

NPA

subG0 6.4  
G0/G1 61.2  
S 13.1  
G2M 19.3

subG0 13.3  
G0/G1 35.2  
S 20.4  
G2M 31.1

subG0 1.4  
G0/G1 54.9  
S 19.2  
G2M 24.0

subG0 14.5  
G0/G1 31.5  
S 24.7  
G2M 27.3
FIG. 3C

FIG. 3D

FIG. 3E

[Graphs showing cell cycle distribution with labels G0/G1, S, G2/M for DMSO and Withaferin A treated groups.]
FIGURE 7

B16F10 Withaferin A[3.0 μM]

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<th>Time</th>
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<th>30 min</th>
<th>1 hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>9 hr</th>
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FIGURE 8
**FIG. 9**

**SKMEL28 Withaferin A**

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**FIG. 10**

**Withaferin A [mM]**

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<th>Concentration [μM]</th>
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<th>NPA</th>
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<td>pERK2</td>
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<tr>
<td>total ERK</td>
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<tr>
<td>BRAF</td>
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<tr>
<td>β - Actin</td>
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</tbody>
</table>

**Protein Bands**

- **Akt**: 60 KDa
- **pERK2**: 42
- **total ERK**: 42
- **BRAF**: 75
- **β - Actin**: 43
**FIG. 11A**

B16F10 Withaferin A

<table>
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<tr>
<th>[µM]</th>
<th>0</th>
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**FIG. 11B**

SKMEL28

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**Fig. 13**

<table>
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**Fig. 14**

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</tbody>
</table>

**Helix in the diagram**

- HSP90
- HSP70
- HSP27
- Grp94
- Trap1
- HSF-1
- β-Actin

**Figures**

- Fig. 13: Graph showing the effect of Withaferin A on various proteins (HSP90, HSP70, HSP27, Grp94, Trap1, HSF-1, β-Actin) in DRO and NPA conditions.
- Fig. 14: Graph showing the effect of different treatments (Control, WA, C004 to C010) on HSP72 and β-Actin.
FIG. 17

<table>
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<tr>
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<tr>
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</table>

- Total Akt
- CRAF
- HSP90
- β-actin

FIG. 18