Withanone Rich Combination of Ashwagandha Withanolides

Restricts Metastasis And Angiogenesis Through hnRNP-K

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Running title: Ashwagandha inhibits Metastasis and Angiogenesis

Key words: Ashwagandha, Withanone, Withaferin A, combination, metastasis inhibition

Abbreviations:

hnRNP-K, heterogeneous nuclear ribonucleoprotein-K; i-Extract, alcoholic extract; WA, Withaferin A; WiNA, Withanone and Withaferin A; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cells; MMP, matrix metalloproteinase
Financial Information:

This work was partly supported by grants from the Ministry of Knowledge Economy, Korea (10030051) and the Korea Science and Engineering Foundation (2013K1A1A2A02050188, 2013M3A9D3045879, 2010-0029220) to C-O Yun and AIST, Japan Special Budget to S. C. Kaul and R. Wadhwa.

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Disclosure of Potential Conflicts of Interest:

No potential conflicts of interest were disclosed.

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Abstract

Ashwagandha is an important herb used in Indian system of traditional home medicine, Ayurveda. Alcoholic extract (i-Extract) from its leaves and its component, Withanone, were previously shown to possess anticancer activity. In the present study, we developed a combination of Withanone and Withaferin A, major withanolides in the i-Extract, that retained the selective cancer cell killing activity, and found that it also has significant anti-migration, -invasion and -angiogenic activities, both in \textit{in vitro} and \textit{in vivo} assays. Using bioinformatics and biochemical approaches, we demonstrate that these phytochemicals caused downregulation of migration-promoting proteins: hnRNP-K, VEGF and metalloproteases, and hence are candidate natural drugs for metastatic cancer therapy.

Introduction

Metastasis is a complex and multi-step process of cancer cell movement from their primary to a secondary site within the body through bloodstream or the lymph system. Cancer itself is extremely complex to understand and treat; metastasis poses additional hurdles in its therapy. Multiple factors that determine metastasis include origin, type and stage of the cancer, and in turn determine the type and outcome of the treatment. The process of metastasis requires (i) an acquisition of migratory characteristics by tumor cells to leave the primary site, (ii) capacity to proteolytically
digest the surrounding connective tissues, (iii) characteristics to enter the lymphatic or blood vessels through which they travel to distant sites of the body and (iv) ability to proliferate at the new site to establish into tumor. The choice of cancer treatment (either surgery, chemotherapy, radiation therapy, hormone therapy, laser-immunotherapy or a combination) depends on the type of primary cancer, size and location of the metastasis. However, current options to cure metastatic cancers are very limited. There is a substantial need to understand the phenomenon of metastasis, uncover the factors that influence cellular migration and adhesion characteristics, and formulate new strategies and reagents for safe and effective cancer treatment.

Recently, there has been renewed interest in herbal medicines because of the safety and economic issues on one hand and the traditional history of wide use on the other. According to the World Health Organization, 80 percent of the world population do not have access to advanced medical care and use herbs for primary health and therapeutic purposes. Ashwagandha (*Withania somnifera*) is often categorized amongst the world’s most renowned herbs. It is classified in GRAS (generally regarded as safe) family as a non-toxic edible herb, and is also called as Indian ginseng and Queen of Ayurveda (Indian traditional home medicine). The main active constituents of Ashwagandha are alkaloids and steroidal lactones, commonly known as withanolides. Withaferin A (WA) is a major chemical constituent of *Withania somnifera*. It has been shown to possess anti-tumor potentials, induces apoptosis by activation of caspase-3 and inhibits JNK, Akt, pERK and IL-6 signal pathways (1-3). Compared to treatment with either Withaferin A or radiation alone, the combination of both resulted in a significant enhancement of apoptosis in human renal cancer cells, and hence was suggested as an effective radiosensitizer in cancer therapy (4). Withaferin A was shown to induce depolymerization of vimentin (5) and
cause reregulation of Notch1 signaling (6). We have earlier shown that the high dose of Withaferin A, but not Withanone, was cytotoxic to normal cells (7). Furthermore, bioinformatics and experimental data suggested the differential binding efficacies of Withaferin A and Withanone to cellular targets including mortalin, p53, p21 and NRF2 (8). We had earlier reported that the low dose of alcoholic extract of Ashwagandha leaves (i-Extract) and its components, Withanone and Withaferin A, were non-toxic instead induced differentiation in glioma cells (9). Hence, in low dose, they were proposed as useful in differentiation based milder and effective glioma therapy. We also found that Withanone, when added along with Withaferin A, was able to decrease the toxicity of the latter in normal cells (7). Based on these findings, we undertook the present study to formulate potent combination of Withanone and Withaferin A that could have stronger anti-metastatic and anti-angiogenic activities than the i-Extract. The combination, rich in Withanone, was non-toxic to normal cells and showed potent anti-metastatic and anti-angiogenesis activities in in vitro and in vivo assays. We demonstrate that such activities are mediated through inactivation of multifunctional RNA binding protein, heterogeneous nuclear ribonucleoprotein K (hnRNP-K) and its downstream effectors, matrix metalloproteinases (MMPs), pERK and vascular endothelial growth factor (VEGF).

Materials and Methods

Cell culture and colony forming assays

Human glioblastoma (A172 and YKG1), osteosarcoma (U20S), fibrosarcoma (HT1080), neuroblastoma (IMR32); rat glioblastoma (C6) and mouse immortal fibroblasts (NIH 3T3) were used for this study. Cells were purchased from JCRB (Japanese Collection of Research Bioresources) Cell Bank, National Institute of
Biomedical Innovation, Japan, and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen)-supplemented with 10% fetal bovine serum in a humidified incubator (37°C and 5% CO2). Cells were used within 10-15 passages of the original stocks and hence no additional authentications were performed. Human umbilical vein endothelial cells (HUVEC) were cultured in M199 medium (Invitrogen, Carlsbad, CA) containing 20% FBS, penicillin-streptomycin (100 IU/ml), 3 ng/ml basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY), and 5 U/ml heparin. HUVECs were used for experiments in passages 2 through 7. Cells (40-60% confluency) were treated with Withanone (i-Factor) (5 µg/ml; 10.6 µM), Withaferin A (0.25 µg/ml; 0.531 µM), and their combination WiNA 20-1 for about 48 h, and were harvested for molecular assays as described below. For colony forming assay, 1000 cells were plated in 10-cm dish, in triplicates. Cells were allowed to expand and make colonies during the next two weeks with regular replacement of culture medium (either control or phytochemical-supplemented, as mentioned) with the fresh medium every alternate day. Colonies were fixed in methanol : acetone (1:1), stained with Crystal violet, destained with running tap water, and were then counted. The plates were scanned using Epson GT-9800F scanner. Statistical significance of the data was calculated from three independent experiments.

**Cell cycle analysis**

For cell cycle analysis, cells (1 X 10^5) treated with indicated drugs for 48 h were harvested with trypsin, washed twice with phosphate-buffered saline (PBS) and fixed with 70% ethanol at 4°C for 12 h. The fixed cells were centrifuged (2000 rpm for 10 min), washed twice with cold PBS and re-suspended in 0.25 ml PBS. RNA was removed by RNAse A treatment (5 µl of 10 mg/ml RNAse was added to 250 µl
of the cell suspension and incubated at 37°C for 1 h. The cell suspension was stained with propidium iodide (PI) (10 μl of PI, 1 mg/ml) at 4°C in dark for 30 min. The cell cycle analysis was done using Guava cell cycle flow cytometer (Millipore) following the manufacturer’s protocol.

**Wound-scratch assay**

Cell motility was examined using the Wound-scratch assay. Cell monolayers were wounded by uniformly scratching the surface with a needle (20 gauge). Movement of the control and treated cells in the scratched area were serially monitored under a phase contrast microscope with a 10 X phase objective. Migration capacity was calculated by measuring the percent of open area in 6-10 randomly captured images.

**Cell invasion assays**

Invasion assays were carried out in Boyden chambers (pore size of 8 μm: Corning Inc., Corning, NY) using Matrigel following the manufacturer’s instructions. For fluorometric determination of Cell Invasion, QCM™ Cell invasion assay kit (Millipore) was used. It was also performed using xCELLigence System (Roche) that used dual chamber Matrigel plates equipped with sensor. Automatic scan and the data were acquired for 48 h following the manufacturer’s instructions.

**Immunoblotting**

Cells were lysed in RIPA lysis buffer. The protein (20 μg) was immunoblotted with anti-phospho Rb, -phospho ERK, -phospho p38 (Cell Signalling), -NCAM (AbCys SA, Paris, France), -MMP-2 (Santa Cruz Biotech) and -
Actin (Chemicon International, Temecula, CA) antibodies by standard Western blotting as described earlier (7).

**Immunostaining**

Cells were cultured and treated on glass coverslips placed in 12-well culture dish. The cells were stained with anti-VEGF and -hnRNP-K antibodies (Santa Cruz Biotech), as described previously (7, 9).

**Endothelial cell tube formation assay**

The formation of HUVEC capillary like structures on a basement membrane matrix was used to assess the anti-angiogenic activity of i-Extract and its constituent phytochemicals. The 16-mm diameter tissue culture plates were coated with 250 μl growth factor-reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) at 37°C for 30 min. HUVECs were seeded on the Matrigel bed (1.5 × 10^5 cells/well) and cultured in M199 medium containing either Avastin or Ashwagandha extract or phytochemical combination in the presence of VEGF165 (10 ng/ml) for 20 h. M199 medium containing VEGF165 alone served as a control. Capillary networks were photographed, and the area covered by the tube network was quantified by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

**Endothelial cell chemotactic migration assay**

The effect of Ashwagandha on the chemotactic motility of HUVECs responding to VEGF165 was assessed using transwell migration chambers (Corning Costar, Cambridge, MA) with 6.5-mm diameter polycarbonate filters (8 μm pore size). Cells in M199 medium containing 1% FBS were stimulated with 10 ng/ml VEGF165
and treated with Avastin or Ashwagandha regents for 30 min at room temperature, and then seeded into the lower wells. HUVECs, incubated for 4 h in M199 medium containing 1% FBS were harvested by trypsination and loaded into the upper wells (1 × 10^5 cells/well). The chamber was then incubated at 37 °C for 4 h. Chemotaxis was quantified by counting the migrated cells under an optical microscope (Olympus IX 71; Olympus, Melville, NY) in 10 random fields.

**VEGF ELISA**

Human VEGF-A was quantified in cell supernatants using the human VEGF Quantikine Immunoassay Kit (R&D Systems, Minneapolis, MN), following the manufacturer’s protocol.

**In vivo study**

Nude mice were obtained from Charles River, Japan. HT1080 (1 X 10^6) cells were injected subcutaneously into the abdomen. Intraperitoneal (IP) injections of WiNA (Withanone, 1 mg/kg and Withaferin A, 0.5 mg/kg) were started when small tumor buds were formed in about 7 days. These concentrations were determined based on independent experiments that involved testing of different ratios of Withaferin A and Withanone for intraperitoneal injections. The concentrations higher than 1 mg/kg Withanone and 0.5 mg/kg Withaferin A in 100 μl volume of 0.5% DMSO showed precipitation, and hence were considered inappropriate. Injections were continued every alternate day and the mice were monitored for tumor size until 3-4 weeks. For metastasis assay, cells were injected into the tail vein. After 7 days, WiNA injections were performed. Mice were sacrificed and the lungs were examined for the presence of tumors.
Molecular docking and dynamics simulations

Virtual molecular docking of KH3 domain of hnRNP-K protein with Withaferin A and Withanone was executed using Autodock suite 4.2 (10). KH3 domain of hnRNP-K (KH3_hnRNP-K) protein was downloaded from Protein Data Bank (PDBID: 1ZZI) and PubChem Compound database was used to retrieve the structure of Withaferin A [PubChem: 265237] and Withanone [PubChem: 21679027]. Structure files of both the ligands were prepared for molecular docking by defining the number of torsion angles, addition of hydrogen atoms and conversion into software specific file format (pdbqt). Similarly, KH3_hnRNP-K protein was also prepared by removal of ssDNA, removing bad contacts, addition of hydrogen atoms, removal of needless water molecules, and conversion of file format into pdbqt. ssDNA binding site on KH3_hnRNP-K was defined as the site of ligand binding over KH3_hnRNP-K. First, prepared ligands were virtually docked against KH3_hnRNP-K protein blindly. Further, both the ligands were docked at the defined binding site using Lamarckian Genetic Algorithm of Autodock 4.2. Top scoring conformations were inspected against binding at defined binding site.

The GPU accelerated Amber Molecular Dynamics suite with Amber ff99SB protein force field was used to perform all atoms explicit molecular dynamics simulations (MD simulations) of protein-ligand complexes http://ambermd.org/#Amber12 (11-13). Protein-ligand complex molecules were solvated with TIP3P water model in a cubic periodic boundary box to generate required systems for MD simulations and systems were neutralized using appropriate number of counterions. The distance between box wall and protein complex was set to greater than 10Å to avoid direct interaction with its own periodic image. Neutralized system was then minimized, heated up to 300 K temperature and
equilibrated until the pressure and energies of systems were stabilized. Finally, equilibrated systems were used to run 60 ns long MD simulations.

During the MD simulations, H-bond fluctuations of ligand with protein were calculated using VMD software (14). Molecular interaction diagrams were generated using Maestro, version 9.4, Schrödinger LLC, New York, NY, 2013. All simulation studies were performed on Intel Core 2 Duo CPU @ 3 GHz of HP origin with 1 GB DDR RAM and DELL T3600 workstation with 8 GB DDR RAM and NVIDIA GeForce GTX TITAN 6 GB GDDR5 Graphics Card.

Results

Effect of Withanone and Withaferin A combination on cancer cell proliferation

in vitro

Alcoholic extract of Ashwagandha leaves was earlier shown to possess Withanone and Withaferin A as major, and Withanolide A, Withanolide D, 12-deoxywita-stramonolide as minor withanolides. The ratio of these constituents in leaves varies depending on their source and stages. Whereas Withanone and Withaferin A were found to be toxic to cancer cell, Withanolone rich i-Extract was nontoxic to normal human cells (7). Based on these findings, we first generated various combinations of Withanone and Withaferin A, and investigated their effect on cancer and normal cell viability in culture. We found that the combination of Withanone (5 µg/ml; 10.6 µM) and Withaferin A (0.25 µg/ml; 0.53 µM), at a ratio of Withanone to Withaferin A as 20:1, called WiNA 20-1 selectively killed cancer cells. Normal cells remained unaffected (Fig. 1A). In contrary, the combination of Withanone and Withaferin A either at 5:1 (WiNA 5-1) or 3:1 (WiNA 3-1) were toxic to normal cells also (Fig. 1A). Furthermore, the combination WiNA 20-1 was cytotoxic to a variety
of human cancer cells including osteosarcoma (U20S), breast carcinoma (MCF7),
glioblastoma, (A172 and YKG1), fibrosarcoma (HT1080), neuroblastoma (IMR32);
rat glioblastoma (C6) and mouse immortal fibroblasts (NIH 3T3) (Fig. 1A and data not
shown). Therefore, in the present study, 20:1 was determined as the optimum ratio of
Withanone and Withaferin A in the mixture to selectively kill cancer cells in culture.

We first examined the effect of Withanone and Withaferin A, and their
combination WiNA 20-1 on cancer cell proliferation by colony forming assay using
highly metastatic human fibrosarcoma, HT1080. As shown in Figs. 1B and 1C,
treatment with WiNA 20-1 caused reduction in colony number and size as compared
to either the control or the cells treated with individual purified components. The
effect of WiNA 20-1 was stronger and statistically significant; 35%, 55% and 70%
reduction in colony formation in the presence of Withaferin A, Withanone and WiNA
20-1, respectively (Fig. 1C). The data was supported by cell cycle analysis (Fig. 1D).
Number of cells in G2 increased from 51.33% to 67.38%, to 77.08% and to 85.37% in
control, Withaferin A, Withanone and WiNA 20-1 treated cells, respectively. Cell
number at S phase decreased from control (27.64%) to Withaferin A treated (14.53%)
to Withanone treated (11.06%) and WiNA 20-1 treated (6.06%) cells.

Effect of Withanone and Withaferin A combination on in vitro cancer cell
migration

Wound-scratch assays revealed that the cells treated with either Withanone or
Withaferin A moved slowly to the scratched area as compared to the control.
Furthermore, the migration of cells treated with WiNA 20-1 combination was highly
reduced (Figs. 2A and 2B). Real time measure of cell migration also showed that the
treatment of cells with (i) either Wi-A or Wi-N reduced their migration capacity and
(ii) WiNA 20-1 combination the effect was ore pronounced (Fig. 2C). Furthermore, quantitative assays (Figs. 2D and 2E) revealed that WiNA 20-1 treated cells showed strongest reduction both in their invasion and migration capacity.

**Treatment with Withanone and Withaferin A limits migration, invasion and angiogenic potential of human endothelial cells**

Based on the above data, we next investigated the effect of WiNA 20-1 on migration, invasion and tube formation capacity of human umbilical vein endothelial cells (HUVEC). Cells were treated with VEGF (10 ng/ml) for induction of migration, on basement membrane matrix constituted of Matrigel. Avastin (humanized monoclonal antibody that inhibits vascular endothelial growth factor A, VEGF-A), an approved inhibitor of angiogenesis was used as a positive control. As shown in Fig. 3A, whereas VEGF induced the tube formation, Avastin showed clear inhibition. Of note, both i-Extract and WiNA 20-1 strongly inhibited the tube formation even at lower doses (1/5<sup>th</sup> of the concentration used for cytotoxicity assays in HT1080 and YKG-1 cells as described in Fig. 1). We found that the i-Extract and WiNA 20-1 also limited the migration and invasion capacity of VEGF-stimulated HUVEC cells, as shown in Figs. 3B and 3C. Most interestingly, the efficacy of inhibition of migration and invasion was comparable to Avastin (50 μg/ml) suggesting that the i-Extract and WiNA 20-1 possess highly potent anti-metastatic activities. We next performed VEGF ELISA in control and treated cells to investigate whether this effect was due to the direct inhibition of VEGF by i-Extract and WiNA 20-1. As shown in Fig. 3D, we found that the treatment with i-Extract and WiNA 20-1 both resulted in substantial downregulation of VEGF in the conditional medium. Western blotting and
immunostaining of VEGF also showed strong decrease in WiNA 20-1 treated cells (Figs. 3E and 3F).

**Molecular mechanism of anti-migratory activity of Withanone, Withaferin A and WiNA 20-1: hnRNP-K as a target**

As shown in Fig. 3, anti-migration and anti-angiogenesis activities of WiNA 20-1 seemed to be mediated by downregulation of VEGF, an established player of metastasis and angiogenesis, and is regulated by hnRNP-K (15). We next examined the expression of hnRNP-K in control and treated cells. Two other proteins, mortalin (a member of HSP70 stress chaperone family) and ezrin (a member of Ezrin-Radixin-Moesin (ERM) family), reported to be associated with cancer cell metastasis (16-18), were also examined. As shown in Fig. 4, the three proteins were downregulated in WiNA 20-1 treated cells, suggesting their relationship with decreased migration of cells, as shown in Figs. 2 and 3. Mortalin was earlier shown to be a target of Withaferin A and Withanone (8). Examination of hnRNP-K by immunocytochemistry showed that the number of cells with bright nuclear staining was less in treated cells suggesting an inhibition of its transcriptional activation function (Figs. 4C and 4D).

Based on the above data, we hypothesized that Withaferin A and Withanone may bind to hnRNP-K and result in an inhibition of its metastatic signaling, as shown in Fig. 5A. We, therefore, investigated the binding by molecular dynamics simulations. The crystal structure of KH3_hnRNP-K was available as DNA-protein complex with ssDNA in PDB. The analysis of ssDNA and KH3_hnRNP-K complex revealed the binding site of ssDNA/RNA over KH3_hnRNP-K. ssDNA was found to interact with residues Lys22, Asp23, Ala25, Ile29, Lys31, Arg40, Lys47, Ile48,
Asp50, Tyr84, and Ser85 of KH3_hnRNP-K protein (Fig. 5B). Before docking to ssDNA/RNA binding site, Withaferin A and Withanone were docked randomly over KH3_hnRNP-K protein to identify most preferable binding site of ligands over protein. We found that the binding sites of both the ligands were coinciding with ssDNA/RNA binding site of the protein suggesting that both Withaferin A and Withanone have tendency to hinder the binding of ssDNA/RNA at KH3_hnRNP-K protein.

Docking of Withaferin A and Withanone specifically at ssDNA/RNA binding site generated the bound conformation of ligands within the protein with docking score of -8.92 and -9.19. Withaferin A was in contact with residues Gly26, Ser27, Gly30, Lys31, Gln34, Gln83, and Ser85 of KH3_hnRNPK via Hydrogen bonds (H-bonds) and hydrophobic interactions (Figs. 5C and 5D). Molecular docking of Withanone with KH3_hnRNP-K resulted in two high affinity conformations (docking score -9.1 and -8.9) that were docking at minutely different positions within same binding site. A comparison of Withanone of docking score -9.1 (Withanone_c1) and Withanone of docking score -8.92 (Withanone_c2) with ssDNA bound conformation revealed that Withanone_c1 was binding completely at ssDNA binding site but Withanone_c2 was only partially interfering the binding of ssDNA (data not shown). Because both the conformations were interfering with the binding of hnRNP-K to ssDNA, we confirmed the binding characteristics of Withanone, using its two conformations by MD simulations. Significance of binding of Withaferin A and Withanone was revealed by the fact that the second nucleotide Thymine (DT) of ssDNA also interacts with residues Tyr84 and Ser85. Binding of any of the ligands at these amino acid residues of KH3_hnRNP-K is expected to hinder its binding to ssDNA/RNA (Figs. 5C and 5D). It may either decrease the binding affinity of
RNA/ssDNA to hnRNP-K or may make the interaction of RNA/ssDNA and hnRNP-K completely non-functional because of the involvement of first few nucleotides that play key role in its transcription activation/deactivation function.

Stability of the protein-ligand complex was further verified by long MD simulations. Withaferin A was found highly stable at its place during 60 ns MD simulations with little or no fluctuation. All the interactions of Withaferin A with KH3_hnRNP-K were conserved during and after the 60 ns MD simulations and involved the ssDNA binding site of hnRNP-K (Figs. 5C and 5D). Withanone_c1 showed a slight shift in its binding position within ssDNA binding site, attaining a more stable conformation. Stabilized Withanone_c1 was found interacting with residues Gly26, Ser27, Ile29, Gly30, Lys31, Arg35, Ser84, Tyr85, and Lys87 of KH3_hnRNP-K protein, strongly hindering the binding of the protein to ssDNA (Figs. 5C and 5D). Withanone_c2 was also found stable at its binding site during MD simulation. Based on higher binding efficacy of Withanone_c1 and binding site on the ssDNA, Withanone_c1 conformation appeared as a highly efficacy ligand (Fig. 5D). hnRNP-K was earlier shown to regulate Erkp44/42 and MMP2 signaling (19). Western blotting revealed significant decrease in the expression of MMPs and phospho-Erkp44 in WiNA 20-1 treated cells, as compared to the ones treated with either Withanone or Withaferin A (Fig. 5E). On the other hand, the cell adhesion protein, NCAM, showed maximum increase in WiNA 20-1 treated cells (Fig. 5E)).

In vivo validation of anti-metastasis activity of WiNA 20-1 and hnRNP-K as a target

We next determined the effect of Withaferin A, Withanone and their combination on cancer cell proliferation and migration in in vivo using nude mice
HT1080 subcutaneous xenograft and lung metastasis models. Toxicity as a result of intraperitoneal (IP) injections of either Withanone (1 mg/kg), Withaferin A (0.5 mg/kg) or their combination (WiNA) in 100 μl of 0.5% DMSO was first tested by visual observations and body weight measurements of mice. The combination with constituents higher than 1 mg/kg Withanone and 0.5 mg/kg Withaferin A in 100 μl of injection volume showed precipitation, and hence were considered inappropriate for \textit{in vivo} study. There was no significant difference in the body weight of Withaferin A, Withanone and WiNA-injected mice as compared to the un-injected control (data not shown). Hence the three reagents, at the doses used, were considered non-toxic \textit{in vivo}. Mice with small HT1080 tumor buds (7 days post-injection of cells) were given the intraperitoneal injections of either Withaferin A, Withanone or combination on every alternate day. As shown in Figs. 6\textit{A} and 6\textit{B}, WiNA injected mice showed strong suppression of subcutaneous HT1080 tumor-xenografts. In the lung metastasis model, big tumors were detected only in control mice (Fig. 6\textit{C}). Taken together, these data suggested that WiNA has significant anti-cancer and anti-metastasis activities \textit{in vivo}. We also performed Western blotting of tumor excised from the control and treated mice, and found that hnRNP-K was decreased in WiNA treated small tumors as compared to the control big tumors. Furthermore, in agreement with the \textit{in vitro} data (Figs. 4 and 5), small tumors showed decrease in hnRNP-K downstream effectors VEGF, Erkp44/42 and MMP2.

\textbf{Discussion}

Tumor metastasis involves dissociation of cancer cells from the primary tumor site, followed by migration, invasion, adhesion and proliferation at a distant site. Matrix metalloproteinases (MMPs), critical regulators of extracellular matrix,
metastasis and angiogenesis (20), are regulated by cytokines, growth factors via interlinked signaling pathways (19, 21, 22). hnRNP-K is a multifunctional protein that regulates ERK1/2, MMPs and VEGF, and contribute to cell migration, invasion and ascites formation (19, 23-25). ERK1/2 and VEGF have been connected by positive autocrine feedback loop (26-28).

Several studies have shown that the alcoholic extract of Ashwagandha leaves, and its components, Withaferin A and Withanone, are cytotoxic to cancer cells, and possess radio-sensitizing, immunomodulatory, anti-inflammatory, anti-metastasis and anti-angiogenic properties, suggesting their potential as anticancer drug (1-6, 29-31). Mechanisms of these activities are only beginning to be resolved. It was shown that Withaferin A inhibits pro-metastatic intermediate filament protein - vimentin, an EMT signaling protein (5, 32), pAkt signaling pathway and MMP-9 (33), STAT3 and its downstream effectors Bcl-xL, Bel-2, cyclin D1 (34), pAkt and pERK signaling (2), oncogenic transcription factor STAT3 (35) and NF-kappa B (36). It was shown to induce extracellular pro-apoptotic tumor suppressor protein, Par-4 (2), oxidative stress to cancer cells (37-39). In spite of these beneficial anticancer effects, cytotoxicity of Withaferin A, in high dose, to normal human cells has been a concern (7, 8, 40). A closely related withanolide, Withanone, on the other hand, caused selective cytotoxicity to human cancer cells (7, 40). In view of this, we investigated the cytotoxicity of Withanone and Withaferin A in various combinations in normal and cancer cells. The combination WiNA 20-1 (Withanone -10.6 µM and Withaferin A - 0.53 µM) was selectively toxic to cancer cells and showed potent anti-migratory and anti-angiogenic activities in vitro. These activities were supported by molecular analysis of marker proteins including MMPs that play crucial role in the process of cancer invasion and metastasis. In an earlier study, we had reported that compared to
Withanone, Withaferin A possess stronger affinity for target proteins including mortalin, p53, p21 and Nrf2, and that might account for its toxicity to normal cells (8). In the present study, we demonstrate that Withaferin A targets hnRNP-K, an upstream regulator of MMPs, Erk-p44/42 and VEGF (Fig. 5A), and inhibits its metastasis signaling. Immunofluorescence analysis revealed decrease in the number of cells with bright nuclear hnRNP-K, suggesting its compromised transcriptional function resulting in decreased levels of MMPs, ERK, VEGF, as shown in Figs. 3-6. The latter might also be due to, at least in part, decreased level of ezrin and mortalin proteins that are enriched in cancer cells (16, 41-46) (Figs. 5A and 5B), associated with tumor metastasis as discussed above. Whereas the mechanism(s) of effect of these phytochemicals on ezrin warrant further studies, mortalin-p53 complex has been shown to be targeted by Withaferin A and Withanone resulting in activation of p53 function (41). Furthermore, Withanone was also shown to target TPX2 oncogene, a prime regulator of Aurora A kinase that plays a critical role during mitosis and cytokinesis (47). These mechanisms are also expected to contribute to the anticancer and anti-metastasis activities of Withaferin A, Withanone and WiNA 20-1. In in vivo tumor formation assays, injections of Withanone-rich combination of Withanone and Withaferin A (1 mg and 0.5 mg/kg BW, respectively) showed significant inhibition of tumor growth and metastasis. Taken together, we report that (i) Withanone-rich combination of Withanone and Withaferin A limits cancer cell growth, migration and angiogenesis in vitro and in vivo (ii) the anti-metastasis activity is mediated by targeting multifunctional RNA binding protein, hnRNP-K.

**Acknowledgements**
Authors thank Roche for providing the xCELLigence System and Masumi Maruyama for technical help.

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Legends to the Figures

Figure 1. Withanone and Withaferin A combination (WiNA 20-1) is selectively toxic to cancer cells. (A) Cell morphology (B and C) colony forming efficacy (D) cell cycle distribution of control and treated cells showing that the combination of Withanone (Wi-N) and Withaferin A (Wi-A) in the ratio of 20:1 was selectively cytotoxic to cancer cells. The combinations in the ratio of 5:1 and 3:1 were toxic to normal cells. The differences, between Withaferin A and WiNA 20-1 as well as Withanone and WiNA 20-1 in (C) and (D), were statistically significant (C, as shown; and D, p<0.05).

Figure 2. Withanone and Withaferin A combination (WiNA 20-1) has anti-migration activity. (A) Wound scratch assay showing migration of control and treated HT1080 cells. Open area of the wound revealed that the treated cells moved slowly as compared to the control cells. (B) Quantitation of the open area from three independent experiments. (C) Real time measure of the migration capacity of cells and (D and E) Quantitative cell invasion assays showing strong inhibition by WiNA 20-1.

Figure 3. Withanone and Withaferin A combination (WiNA 20-1) has anti-angiogenic activity. (A) Tube formation capacity, (B) migration, and (C) invasion of control and treated HUVECs. Quantitation from three independent experiments is shown. *P < 0.05, **P < 0.01, ***P < 0.001 compared with VEGF165. VEGF expression, as determined by ELISA (D), Western blotting (E) and immunostaining (F), is shown. Quantitation from three independent experiments was performed using
Imaging J. Actin was used as an internal control (E). An unpaired Student t test was used to determine the statistical significance of the data.

Figure 4. Treatment with Withanone and Withaferin A combination (WiNA 20-1) downregulates the level of ezrin, hnRNP-K and mortalin. (A) Western blotting showing decrease in ezrin, hnRNP-K and mortalin in the treated cells. Actin was used as an internal control. (B) Quantitation of the signals on Western blots was performed from three independent experiments using Imaging J. (C) Immunostaining showing decrease in the number of cells with bright nuclear hnRNP-K in treated cells (D). An unpaired Student t test was used to determine the statistical significance of the data.

Figure 5. Withanone and Withaferin A target hnRNP-K. (A) A schematic diagram showing the interaction of hnRNP-K with DNA and its downstream effectors involved in cancer cell migration. Model also shows the abrogation of hnRNP-K and DNA complex and inhibition of cell migration by Withaferin (WA) or Withanone (WN). (B) Interaction diagram of KH3 domain of hnRNP-K protein with ssDNA is shown. Amino acid residues of KH3 domain of hnRNP-K protein, such as Gly26, Ser27, Gly30, Ile36, Lys37, Tyr84, and Ser85 were seen to interact with ssDNA. (C) Interactions of Withaferin A with amino acid residues of KH3_hnRNP-K protein are shown (left). Interactions of stabilized Withanone after 60 ns MD simulation with KH3_hnRNP-K protein are shown (right). In both the cases, most of the residues were the ones involved in interaction of the protein with ssDNA. (D) Binding conformations of Withaferin A and Withanone with KH3_hnRNP-K are shown. Hindrance in interaction of hnRNP-K with ssDNA is shown in the superimposed
structures of Withanone-KH3_hnRNP-K complex over ssDNA-KH3_hnRNP-K complex. (E) Western blot showing decrease in the level of phospho-ERKp44/42 and MMP2, and increase in the cell adhesion protein NCAM in WiNA 20-1 treated HT1080.

Figure 6. *In vivo* anti-metastasis activity of Withaferin A, Withanone and WiNA 20-1. (A) Nude mice showing the suppression of tumor growth in response to WiNA 20-1 treatment. (B) Quantitation of tumor volume from six mice each. (C) Suppression of lung tumors in WiNA 20-1 injected mice as compared to control. (D) Western blot showing decrease in the level of hnRNP-K, VEGF, ERKp44/42 and MMP2 expression in small tumors excised from the treated mice as compared to the large tumors excised from the control mice.
Fig. 1

A

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Fig. 2

A. HT1080 cells treated with different compounds for 0 h and 8 h.  
B. Percentage of open area in each condition.  
C. Cell index over time for different treatments.  
D. Invasion rate of the cells under different treatments.  
E. Representative images of cell invasion for each condition.
Fig. 3
Fig. 4
Fig. 6