Withanone-Rich Combination of Ashwagandha Withanolides Restricts Metastasis and Angiogenesis through hnRNP-K

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Abstract
Ashwagandha is an important herb used in the 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 antimigratory, -invasive, and -angiogenic activities, in both in vitro and 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. Mol Cancer Ther; 13(12); 2930–40. ©2014 AACR.

Introduction
Metastasis is a complex and multistep 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 tumor. The choice of cancer treatment (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% of the world population does not have access to advanced medical care and hence uses 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 nontoxic edible herb and is also called 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 is a major chemical constituent of W. somnifera. It has been shown to possess antitumor potentials, induces apoptosis by activation of caspase-3, and inhibits JNK, Akt, pERK, and IL6 signal pathways (1–3). Compared with 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 deregulation 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 nontoxic and 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). On the basis of these findings, we undertook the present study to formulate potent combination of withanone and withaferin A that could have stronger anti-metastatic and antiangiogenic activities than the i-Extract. The combination, rich in withanone, was nontoxic 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 (MMP), pERK, and VEGF.

Materials and Methods

Cell culture and colony-forming assays

Human glioblastoma (A172 and YKG1), osteosarcoma (U2OS), 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 DMEM (Invitrogen) supplemented with 10% FBS in a humified incubator (37°C and 5% CO2). Cells were used within 10 to 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) containing 20% FBS, penicillin/streptomycin (100 IU/mL), 3 ng/mL basic FGF (Upstate Biotechnology), 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 μmol/L), withaferin A (0.25 μg/mL; 0.531 μmol/L), and their combination WiNA 20-1 for about 48 hours and were harvested for molecular assays as described below. For colony-forming assay, 1,000 cells were plated in 10-cm dish in triplicates. Cells were allowed to expand and make colonies during the next 2 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 then counted. The plates were scanned using Epson GT-9800F scanner. Statistical significance of the data was calculated from 3 independent experiments.

Cell-cycle analysis

For cell-cycle analysis, cells (1 × 10⁶) treated with indicated drugs for 48 hours were harvested with trypsin, washed twice with PBS, and fixed with 70% ethanol at 4°C for 12 hours. The fixed cells were centrifuged (2,000 rpm for 10 min), washed twice with cold PBS, and resuspended 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 hour). The cell suspension was stained with propidium iodide (PI; 10 μL, 1 mg/mL) at 4°C in dark for 30 minutes. 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 was serially monitored under a phase contrast microscope with a 10× phase objective. Migration capacity was calculated by measuring the percentage of open area in 6 to 10 randomly captured images.

Cell invasion assays

Invasion assays were carried out in Boyden chambers (pore size of 8 μm; Corning Inc.) using Matrigel following the manufacturer’s instructions. For fluorometric determination of Cell Inversion, 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 hours 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), -MMP2 (Santa Cruz Biotech); and -actin (Chemicon International) 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 Bio tech), 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) at 37°C for 30 minutes. HUVECs were seeded on the Matrigel bed (1.5 × 10⁵ cells per 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 hours. 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).

**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) 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 withanolone or ashwagandha regents for 30 minutes at room temperature and then seeded into the lower wells. HUVECs, incubated for 4 hours in M199 medium containing 1% FBS, were harvested by trypsinization and loaded into the upper wells (1 x 10⁵ cells per well). The chamber was then incubated at 37°C for 4 hours. Chemotaxis was quantified by counting the migrated cells under an optical microscope (Olympus IX 71; Olympus) in 10 random fields.

**VEGF ELISA**

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

**In vivo study**

Nude mice were obtained from Charles River. HT1080 (1 x 10⁶) cells were injected subcutaneously into the abdomen. Intraperitoneal injections of WiNA (withanolone, 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 on the basis of independent experiments that involved testing of different ratios of withaferin A and withanolone for intraperitoneal injections. The concentrations higher than 1 mg/kg withanolone 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 to 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 withanolone 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 structures of withaferin A (PubChem: 265237) and withanolone (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 single-stranded DNA (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. Furthermore, 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 (MD) simulations of protein–ligand complexes (http://ambermd.org/#Amber12; refs. 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 10 Å 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. All simulation studies were performed on Intel Core 2 Duto 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 withanolone and withaferin A combination on cancer cell proliferation in vitro**

Alcoholic extract of ashwagandha leaves was earlier shown to possess withanolone and withaferin A as major and withanolide A, withanolide D, 12-deoxywitha-stramonolide as minor withanolides. The ratio of these constituents in leaves varies depending on their source and stages. Whereas withanolone and withaferin A were found to be toxic to cancer cell, withanolone-rich i-Extract was nontoxic to normal human cells (7). On the basis of these findings, we first generated various combinations of withanolone and withaferin A and investigated their effect on cancer and normal cell viability in culture. We found that the combination of withanolone (5 µg/mL; 10.6 µmol/L) and withaferin A (0.25 µg/mL; 0.53 µmol/L), at a ratio of withanolone 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 withanolone 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.
Withanone and withaferin A (WiNA 20-1) selectively inhibit the proliferation and migration of various cancer cell lines, including breast carcinoma (MCF7), glioblastoma (A172 and YKG1), fibrosarcoma (HT1080), neuroblastoma (IMR32), rat glioblastoma (C6), and mouse immortal fibroblasts (NIH3T3; Fig. 1A and data not shown). Therefore, in the present study, 20:1 was determined as the optimum ratio of withanone (Wi-N) and withaferin A (Wi-A) in the ratio of 20:1 to selectively inhibit cancer cell proliferation.

We first examined the effect of withanone and withaferin A in the mixture to selectively kill cancer cells in culture. We found that the combination of WiNA 20-1 was selectively cytotoxic to cancer 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).

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 with the control. Furthermore, the migration of cells treated with WiNA 20-1 was reduced (Fig. 2A and B). 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) the effect of WiNA 20-1 combination was more pronounced (Fig. 2C). Furthermore, quantitative assays (Fig. 2D and E) revealed that WiNA 20-1–treated cells showed strongest reduction both in their invasion and migration capacities.

Treatment with withanone and withaferin A limits migration, invasion, and angiogenic potential of human endothelial cells

On the basis of the above data, we next investigated the effect of WiNA 20-1 on migration, invasion, and tube formation capacity of HUVECs. 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 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 (one fifth 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 Fig. 3B and C. Most interestingly, the
efficacy of inhibition of migration and invasion was comparable with Avastin (50 μg/mL) suggesting that the i-Extract and WiNA 20-1 possess highly potent ant 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 (Fig. 3E and F).

Molecular mechanism of antimigratory activity of withanone, withaferin A, and WiNA 20-1: hnRNP-K as a target

As shown in Fig. 3, antimigratory and antiangiogenic activities of WiNA 20-1 seemed to be mediated by down-regulation 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 3 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 (Fig. 4C and D).

On the basis of 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 MD 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 (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 scores of −8.92 and −9.19. Withaferin A was in contact with residues Gly26, Ser27, Gly30, Lys31, Glu34, Glu83, and
Figure 3. Withanone and withaferin A combination (WINA 20-1) has antiangiogenic activity. Tube formation capacity (A), migration (B), and invasion (C) of control and treated HUVECs. Quantitation from three independent experiments is shown. /C3/C3/C3, P < 0.001 compared with VEGF165.

VEGF expression, as determined by ELISA (D), Western blotting (E), and immunostaining (F), is shown. Quantitation from 3 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.
Ser85 of KH3 hnRNPK via hydrogen bonds (H-bonds) and hydrophobic interactions (Fig. 5C and D). MD of withanone with KH3 hnRNPK resulted in 2 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 hnRNPK to ssDNA, we confirmed the binding characteristics of withanone, using its 2 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 hnRNPK is expected to hinder its binding to ssDNA/RNA (Fig. 5C and D). It may either decrease the binding affinity of RNA/ssDNA to hnRNPK or may make the interaction of RNA/ssDNA and hnRNPK completely nonfunctional 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 hnRNPK were conserved during and after the 60 ns MD simulations and involved the ssDNA-binding site of hnRNPK (Fig. 5C and D). 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 hnRNPK protein, strongly hindering the binding of the protein to ssDNA (Fig. 5C and D). Withanone_c2 was also found stable at its binding site during MD simulation. On the basis of higher binding efficacy of Withanone_c1 and binding site on the ssDNA, Withanone_c1 conformation appeared as a highly efficacy ligand (Fig. 5D). hnRNPK was earlier shown to regulate Erk44/42 and MMP2 signaling (19). Western blotting revealed significant decrease in the expression of MMPs and phospho-Erk44 in WiNA 20-1–treated cells, as
Figure 5. Withanolone and withaferin A target hnRNPK. A, schematic diagram showing the interaction of hnRNPK with DNA and its downstream effectors involved in cancer cell migration. Model also shows the abrogation of hnRNPK and DNA complex and inhibition of cell migration by withaferin (WA) or withanolone (WN). B, interaction diagram of KH3 domain of hnRNPK protein with ssDNA is shown. Amino acid residues of KH3 domain of hnRNPK 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_hnRNPK protein are shown (left). Interactions of stabilized withanolone after 60 ns MD simulation with KH3_hnRNPK 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 withanolone with KH3_hnRNPK are shown. Hindrance in interaction of hnRNPK with ssDNA is shown in the superimposed structures of withanolone–KH3_hnRNPK complex over ssDNA–KH3_hnRNPK complex. E, Western blotting showing decrease in the level of phospho-ERKp44/42 and MMP2 and increase in the cell adhesion protein NCAM in WN 20-1–treated HT1080.
compared with 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 antimetastatic 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 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 was considered inappropriate for *in vivo* study. There was no significant difference in the body weight of withaferin A, withanone, and WiNA-injected mice as compared with the uninjected control (data not shown). Hence, the 3 reagents, at the doses used, were considered nontoxic *in vivo*. Mice with small HT1080 tumor buds (7 days postinjection of cells) were given the intraperitoneal injections of either withaferin A, withanone, and WiNA-injected mice as compared with the un.injected control (data not shown). Hence, the 3 reagents, at the doses used, were considered nontoxic *in vivo*. Mice with small HT1080 tumor buds (7 days postinjection of cells) were given the intraperitoneal injections of either withaferin A, withanone, or combination on every alternate day. As shown in Fig. 6A and 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. 6C). Taken together, these data suggested that WiNA has significant anticancer and antimetastatic activities *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 with the control big tumors. Furthermore, in agreement with the *in vitro* data (Figs. 4 and 5), small tumors showed decrease in hnRNP-K downstream effectors VEGF, Erk44/42, and MMP2.

**Discussion**

Tumor metastasis involves dissociation of cancer cells from the primary tumor site, followed by migration, invasion, adhesion, and proliferation at a distant site. 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 contributes 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 radiosensitizing, immunomodulatory, anti-inflammatory, antimetastatic, and antiangiogenic properties, suggesting their potential as anticancer drugs (1–6, 29–31). Mechanisms of these activities are only beginning to be
resolved. It was shown that withaferin A inhibits prometastatic intermediate filament protein—vimentin, an epithelial-to-mesenchymal transition (EMT) signaling protein (5, 32), pAkt signaling pathway and MMP9 (33), STAT3 and its downstream effectors Bcl-xL, Bcl-2, cyclin D1 (34), pAkt and pERK signaling (2), oncopgenic transcription factor STAT3 (35), and NF-kB (36). It was shown to induce extracellular proapoptotic tumor suppressor protein, Par-4 (2), oxidative stress to cancer cells (37–39). Despite 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 μmol/L and withaferin A, 0.53 μmol/L) was selectively toxic to cancer cells and showed potent antimigratory and antiangiogenic 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 with 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. Immunofluorescent 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 (refs. 16, 41–46; Fig. 5A and B), 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 ant metastatic activities of withaferin A, withanone, and WiNA 20-1. In vivo tumor formation assays, injections of withanone-rich combination of withanone and withaferin A (1 and 0.5 mg/kg body weight, 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 and (ii) the ant metastatic activity is mediated by targeting multifunctional RNA-binding protein, hnRNP-K.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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