Selective Killing of Cancer Cells by Leaf Extract of Ashwagandha: Identification of a Tumor-Inhibitory Factor and the First Molecular Insights to Its Effect

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Abstract

Purpose: Ashwagandha is regarded as a wonder shrub of India and is commonly used in Ayurvedic medicine and health tonics that claim its variety of health-promoting effects. Surprisingly, these claims are not well supported by adequate studies, and the molecular mechanisms of its action remain largely unexplored to date. We undertook a study to identify and characterize the antitumor activity of the leaf extract of ashwagandha.

Experimental Design: Selective tumor-inhibitory activity of the leaf extract (i-Extract) was identified by in vivo tumor formation assays in nude mice and by in vitro growth assays of normal and human transformed cells. To investigate the cellular targets of i-Extract, we adopted a gene silencing approach using a selected small hairpin RNA library and found that p53 is required for the killing activity of i-Extract.

Results: By molecular analysis of p53 function in normal and a variety of tumor cells, we found that it is selectively activated in tumor cells, causing either their growth arrest or apoptosis. By fractionation, purification, and structural analysis of the i-Extract constituents, we have identified its p53-activating tumor-inhibiting factor as withanone.

Conclusion: We provide the first molecular evidence that the leaf extract of ashwagandha selectively kills tumor cells and, thus, is a natural source for safe anticancer medicine.

Ashwagandha (Withania somnifera, an evergreen shrub commonly found in the drier parts of the Indian subcontinent) is widely used in Indian natural medicine, Ayurveda. Extracts from different parts of ashwagandha have been claimed to promote physical and mental health due to its effects, ranging from antistress, antiinflammatory, antioxidant, antipyretic, analgesic, antiarthritic, cardioprotective, rejuvenating, and regenerating properties (1–13). Few reports have characterized the activities of the root extract of ashwagandha and include an induction of nitric oxide synthase–inducible protein expression (4, 14), down-regulation of p34cdc2 expression (15), and its antioxidant, free radical–scavenging, and detoxifying properties (16–19). However, the mechanistic aspects of its effects, including tumor suppression and isolation of active components, have largely remained unexplored. Hence, the use of ashwagandha has not been developed to a systemic medicine.

Although ashwagandha roots are most commonly used in Indian Ayurvedic medicine, we undertook a study to examine the effects of its leaf extract because of the easy accessibility and abundant availability. We earlier reported that the leaf extract obtained by a series of extractions (20) has an antimutagenic effect (21). In the present study, we examined the effect of the leaf extract on human normal and cancer cells and found that it selectively kills tumor cells. Fractionation of the tumor-inhibitory extract (i-Extract) and characterization of its constituents led to the identification of a tumor-inhibitory factor (i-Factor). Nuclear magnetic resonance (NMR) spectra revealed its identity as withanone. By employing small hairpin RNA (shRNA) library and molecular analysis, we report for the first time that the selective killing of tumor cells by i-Extract and i-Factor involves an activation of the wild-type p53 function.

Materials and Methods

Preparation of leaf extract from ashwagandha from field-raised plants. Ashwagandha (W. somnifera) leaf extracts were prepared as described earlier (20–22). The leaves were air dried, ground to a fine powder, and subjected to extraction with methanol (60°C) in Soxhlet apparatus for 4 to 5 days. The methanol extracts were further extracted with hexane to remove chlorophyll and other pigments and then with diethyl ether that was evaporated to obtain the ether extract. Ether extract solubilized in DMSO was used for the present studies.

Nude mice assay. BALB/c nude mice (4 weeks old, female) were bought from Nihon Clea (Japan). Mice were fed on standard food pellet and water ad libitum, acclimatized to our laboratory condition at

Adenine is a nitrogenous base that is a component of RNA and DNA. It is also used in various biochemical reactions and pharmaceutical applications.

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a temperature of 24 ± 2°C, relative humidity of 55% to 65%, and 12-h light/dark cycle, for 3 days. Fibrosarcoma (HT1080) cells (1 × 10^6 suspended in 0.5 mL of growth medium) were injected s.c. into the flank of nude mice (one site per mouse). I-Extract injections were commenced at three different times, i.e., (a) mixed with cells at the time of injection, (b) injection before the formation of tumor buds, and (c) injection when small tumor buds (5 mm) were formed. In each case, s.c. injection (0.3 mL of 24% i-Extract in the cell growth medium) was given. Tumor formation was monitored during the next 15 to 20 days, with local injection of i-Extract to the tumor site every third day. For oral feeding, i-Extract or its components were suspended in 2% sterile carboxymethyl cellulose (a vehicle) and injected into the stomach of mice using a flexible Teflon needle on alternate days.

**Characterization of i-Extract by column chromatography.** Ether extract of the leaves was subjected to reversed-phase high-performance liquid chromatography (HPLC) analysis on a C-18 column (5 mm, 150 × 4.6 mm internal diameter; Waters, Milford, MA or YMC, Kyoto, Japan) at 40°C or 50°C using 1% methanol/H_2O (solution A) and methanol/ethanol/isopropanol (52:25:45;30:2:45; solution B) for elution. Elution was done with a gradient of 35% to 45% solution B in 25 min at a flow rate of 1 mL/min. The detection was done at 220 nm. Withaferin A, 12-deoxywithastramoline, and withanolide D were used as standards for comparison.

**Human cell culture and treatments.** Normal diploid fibroblasts (TIG-1 and WI-38), osteogenic sarcoma (U2OS and Saos-2), breast carcinoma (MCF7, HS578T, and SK-BR3), fibrosarcoma (HT1080), colon carcinoma (HCT116), and lung carcinoma (PC14) cells were cultured in DMEM (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum in a humidified incubator (37°C and 5% CO_2). Cells (~50-60% confluence) were treated with i-Extract (6-36 μg/mL) for time periods as indicated.

**Growth assays.** Equal number of cells, counted by Neubauer hemocytometer, was plated in six-well dishes for control and treatment wells. Cells were harvested every 24 h up to 96 h, counted, and plotted as growth curves. Viability was monitored by WST-based cell proliferation kit4 (Roche, Mannheim, Germany).

**Preparation and use of shRNAs.** shRNAs for the genes listed in Table 1 were cloned in a U6-driven expression vector as described earlier (23). Two target sites per gene were used; the sequences for each of the target site are listed in Table 1. Cells were plated in 96-well plates and were transfected at ~70% confluency with 50 ng of the plasmid DNA. At 24 h posttransfection, cells were selected in puromycin (2 μg/mL) and were then treated with i-Extract (24 μg/mL)–supplemented medium for 48 to 96 h and were then treated with i-Extract (24 μg/mL) for 48 h. Cells were lysed, and luciferase activity was measured by Dual-Luciferase reporter assay system (Promega, Madison, WI). Luciferase activity was calculated per microgram of protein and presented as the percent relative activity normalized against untreated cells. Results presented are from three independent experiments.

**Terminal nucleotidyl transferase–mediated nick end labeling assays.** Terminal nucleotidyl transferase (TdT)–mediated nick end labeling (TUNEL) assay was done using DeadEnd Fluorometric TUNEL System (Promega). Cells were grown on coverslips and treated with i-Extract for 48 h, after which they were fixed with 4% formaldehyde solution in PBS at 4°C for 20 min. Cells were washed with PBS and permeabilized with 0.2% Triton X-100 solution in PBS for 5 min and were then incubated with 100 μL equilibration buffer for 10 min followed by the addition of 50 μL TdT incubation buffer for tailing reaction (37°C for 60 min in a humidified chamber). The reaction was terminated by incubation in 2× SSC for 15 min at room temperature. Cells were washed with PBS, stained with propidium iodide (1 μg/mL in PBS), followed by washing with water and examined under a fluorescence microscope.

**NMR analysis of i-Factor.** One-dimensional NMR:^3H NMR (CDCl_3, 500 MHz) δ 0.86 (S, 3H, H-18), 1.04 (d, J = 7 Hz, 3H, H-21), 1.18 (s, 3H, H-19), 1.26 to 1.40 (m, 3H, H-11, 15), 1.53 to 1.62 (m, 2H, H-9, H-12), 1.67 to 1.80 (m, 2H, H-8, H-12), 1.88 (S, 3H, H-27), 1.94 (S, 3H, H-28), 1.88 to 1.95 (m, 2H, H-16), 1.98 to 2.07 (m, 1H, H-14), 2.30 to 2.35 (m, 1H, H-20), 2.42 to 2.56 (m, 4H, 1H, H-17, H-23), 2.67 to 2.71 (m, 1H, H-4), 2.80 to 2.84 (m, 1H, H-11), 3.05 (d, J = 3.7 Hz, 1H, H-6), 3.15 to 3.16 (m, 1H, H-5, O-H), 3.31 to 3.33 (m, 1H, H-7), 4.59 to 4.63 (m, 1H, H-22), 5.85 (dd, J = 10.1, 1.2 Hz, 1H, H-1), 6.52 to 6.61 (m, 1H, H-3).^13C NMR (CDCl_3, 125 MHz): δ 22.3 (C-2), 24.7 (C-3), 25.7 (C-4), 26.8 (C-5), 27.8 (C-6), 28.4 (C-7), 28.7 (C-8), 29.0 (C-9), 29.2 (C-10), 31.7 (C-11), 33.0 (C-12), 48.7 (C-13), 51.0 (C-14), 56.3 (C-15), 57.2 (C-16), 73.2 (C-17), 78.7 (C-18), 116.4 (C-19), 121.4 (C-20), 139.7 (C-21), 140.0 (C-22), 144.3 (C-23), 150.4 (C-24), 167.1 (C-25), 203.1 (C-26).
We next examined the effect of i-Factor in it as i-Factor because of its tumor-inhibitory activity (Fig. 2B). We purified this ingredient of interest from i-Extract and named it as i-Factor showed tumor suppressor activity, similar to i-Extract, and was not toxic to mice. On the other hand, when mice were given with either withaferin A or 12-deoxywithastramonolide, they lost weight and looked sick (data not shown). Most interestingly, in \textit{in vitro} assays, (a) both i-Extract and i-Factor selectively killed human tumor cells and (b) i-Factor, when added along with withaferin A, partially neutralized the toxicity of the latter in normal human cells (Fig. 2D). The antitumor activity of i-Extract was supported by treating a large variety of tumor assays as described below. As shown in Fig. 2C, i-Factor showed tumor suppressor activity, similar to i-Extract, and was not toxic to mice. 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human transformed (including MCF7, U2OS, Saos-2, SKBR3, HS578T, PC-14, HCT116, HeLa, HT1080) cells, whereas normal (MRC-5, TIG-1, and WI-38) cells were least affected (data not shown). Whereas withaferin A was toxic to both cancer and normal cells, withanolide A and 12-deoxywithastramonolide did not show any effect on either of the cell types (data not shown).

We next set out to characterize the molecular mechanism of i-Extract–induced growth arrest of cancer cells. To get an insight to the signaling pathways that are involved in i-Extract–induced death of cancer cells, we prepared 96 shRNA expression plasmids to target 48 genes (most of them were targeted to kinases; Table 1) as described in Materials and Methods. Six shRNAs that had target sites for (a) cyclin-dependent kinase inhibitor 2B (CDKN2B), (b) never in mitosis arrest–related kinase 2, (c) cyclin-dependent kinase 8 (CDK8), (d) tumor suppressor protein (p53), (e) inhibitor of apoptosis protein 1/baculoviral inhibitor of apoptosis repeat–containing 3 (BIRC3), and (f) CDK5 resulted in the abrogation of cell death induced by i-Extract. In the present study, we analyzed the involvement of p53 in i-Extract– and i-Factor–induced selective death of cancer cells as described below.

Normal (TIG-1) and tumor-derived (U2OS with wild-type p53 protein) cells were treated with i-Extract or i-Factor. The

![Fig. 1. Tumor suppression by i-Extract in nude mice assays.](image-url)
Fig. 2. Fractionation of i-Extract by reverse-phase HPLC. A, the identified components included withaferin A, withanolide A, and 12-deoxywithastramonolide. In addition, a component that eluted at 22 min was identified in all the i-Extract preparations, and its amount correlated with the tumor suppressor activity (TSA) of i-Extract, shown by strong (+++), moderate (++), and low (−). B, purification of peak 22 component. C, tumor suppression by i-Extract (0.3 mL of 24 μg/mL) and i-Factor (0.3 mL of 10 μg/mL), but not by withaferin A (0.3 mL of 10 μg/mL). Columns, average tumor size (mm) in control and treated mice. D, selective killing of tumor cells by i-Extract (24 μg/mL) and i-Factor (10 μg/mL). Whereas withaferin A (0.1 μmol/L) was toxic to normal cells also, withanolide A (1 μmol/L) and 12-deoxywithastramonolide (1 μmol/L) did not show any activity. i-Factor (10 μg/mL) reduced the toxicity of withaferin A in normal cells.
level of p53 protein and its transcriptional activation function (p21WAF-1 level) increased in U2OS, but not in normal (TIG-1) cells (Fig. 3A). Interestingly, i-Factor–treated cells showed a moderate decrease in p53 level and its transcriptional activation function only in normal cells (Fig. 3A and data not shown). In contrast, withaferin A led to an induction of the p53 function in normal cells, and i-Factor partially neutralized its effect (Fig. 3A). The activity of wild-type p53 is regulated by multiple factors, including its interactions with binding partners. In transformed cells, mortalin, a hsp70 family member, interacts and inactivates p53 by sequestering it in the cytoplasm (24–28). Pancytoplasmic subcellular distribution of mortalin has been shown as a marker for normal cells; tumor cells show perinuclear staining pattern (29). Interestingly, a shift in perinuclear mortalin staining pattern, typical of tumor cells, to pancytoplasmic pattern was observed in i-Extract–treated tumor cells. Tumor cells that were induced to undergo senescence-like growth arrest in response to treatment with a rhodacyanine dye (MKT-077) or with bromodeoxyuridine also showed a shift in perinuclear staining to the pancytoplasmic type (24, 30). Taken together, these data suggested that the i-Extract abrogated mortalin-p53 interactions and induced senescence-like growth arrest in cancer cells through an activation of wild-type p53 function. Similar to i-Extract, i-Factor induced a shift in the staining pattern of mortalin and nuclear translocation of p53 in cancer cells (Fig. 3B). The induction of senescence was also supported by positive senescence-associated β-gal staining in i-Extract– and i-Factor–treated cells (data not shown).

We next used tumor-derived cell lines [breast carcinomas HS578T (V157F), SK-BR3 (H175V), and MDA-MB-436...
(H273V); lung carcinoma PC14 (Q248V); and fibrosarcoma HT1080 (mutant p53) that harbor full-length p53 protein with point mutations resulting in one amino acid change (as indicated in parenthesis) in the core domain that recognizes p53 DNA-binding sites. Growth of all these cells was also inhibited by i-Extract and i-Factor (Fig. 3C and D), very similar to the ones that contain wild-type but inactivated p53. We analyzed the function of p53 in these tumor cells with mutant p53. Noticeably, i-Extract restored the wild-type p53 function, at least in part, in these cells. In response to i-Extract treatment, the level
of p53 decreased (typical of wild-type p53 degradation) in three of the four (PC14, HSS578T, and HT1080) cell lines. This was accompanied by the enhanced expression of, at least, two of the three downstream effectors of wild-type p53 (p21^{WAF-1}, MDM2, and Bax; Fig. 3E). Although SKBR3 cells did not show a decrease in p53 level subsequent to the i-Extract treatment, the increase in MDM2 and Bax supported the presence of wild-type activity. It was further confirmed by immunoprecipitation with the wild-type p53-specific antibody (Fig. 3F). On the similar line, Bax was not detected in HT1080 and HSS578T cells. These data indicate the cell line specific response, operative cellular factors, and pathways that warrant further molecular analysis. Immunoprecipitation with wild-type p53-specific antibody revealed the existence of wild-type p53 protein only in i-Extract–treated PC14, HSS578T, SK-BR3, and HT1080 cells (Fig. 3F). Of note, the induction of wild-type p53 in HT1080 was not as significant as in the other three cell lines (Fig. 3E). Consistent with this, the p21^{WAF-1} level in HT1080 cells showed only a small increase (Fig. 3D). Similar to i-Extract, i-Factor–treated tumor cells also showed a decrease in the level of p53 (Fig. 3G) and the wild-type p53-dependent reporter activity (Fig. 3H). We next examined the induction of apoptosis by i-Extract in tumor cells with variable (wild-type: functional or nonfunctional; and mutant type) p53 status. We found that the i-Extract induced apoptosis in tumor cells with mutant p53 (HSS578T, SK-BR3, and PC14). The tumor cells (U2OS, MCF7, and HeLa) with wild-type p53 (functional or nonfunctional) exhibited growth arrest (Fig. 3I). These data were similar to another study that reported the induction of apoptosis by wild-type p53 protein expression in HT29A4 (possess mutant p53) cells (31). The data showed that i-Extract restored the wild-type function (transcriptional activation) of the mutant p53. Such an ability to endow wild-type p53 function (reactivation of the growth arrest or the apoptotic pathway) to mutated p53 protein is regarded to be the most beneficial approach for cancer therapeutics (32, 33). Similar reactivation of p53 was achieved by low-molecular-weight peptides, including PRIMA-1 (34–37) and by global suppressor motif approach (38). Although the molecular mechanism of induction of p53 function by i-Extract and i-Factor warrants further studies, our data showed that they could serve as very useful natural tools for such reactivation of p53. We therefore determined the structure of i-Factor by NMR analysis. As shown in Fig. 4, 1H NMR and 13C NMR data of isolated i-Factor were consistent with published values for withanone (39, 40). The assignment and its structure were also confirmed by 2D/NMR: $^{1}H-^{1}H$ correlated spectroscopy, heteronuclear multiple quantum coherence, heteronuclear multiple bond coherence, and homonuclear Hartman Hahn (data not shown). Electrospray-mass spectrometry of i-Factor showed m/z 493.2 [$M + Na$] that was also consistent with a theoretical value of withanone (C$_{28}$H$_{36}$O$_{6}$; M, 470.58). Based on these data, we concluded that i-Factor is withanone and is a potential anticancer drug.

Identification of novel natural anticancer compounds is a highly demanding avenue of cancer therapeutics. In Indian traditional Ayurvedic medicine, ashwagandha is used to treat several illnesses, including tumors, inflammations, conjunctivitis, and tuberculosis. We have shown here, for the first time, that ashwagandha leaves have inhibitory activity selective for tumor cells that works, at least in part, through the activation of wild-type p53 activity. i-Extract and i-Factor, thus, are valuable natural tools for such reactivation of p53. We therefore thank Nippon Shinyaku Co., Ltd. (Japan) for help in the partial characterization and purification of i-Extract and i-Factor.

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