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, antidepressant, anticoagulant, immunomodulatory, adaptogenic, 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

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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 μg/mL 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 digestive tract 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-deoxywithastramonolide, 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% confluency) 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 kit[4] (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) supplemented medium for 48 to 72 h, expanded to 70% confluency, and were then treated with i-Extract (24 μg/mL). shRNAs that resulted in the survival of cells (in the presence of i-Extract) were selected and taken through the second round of transfections and confirmation. Cell viability was measured by staining with Crystal Violet and AlamarBlue assay (BioSource International, Camarilo, CA).

Western blotting. The protein sample (20 μg) separated on SDS-polyacrylamide gel was electroblotted onto a nitrocellulose membrane (BA85, Schleicher and Schuell, Dassel, Germany) using a semidry transfer blotter. Immunoassays were done with anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), anti-actin (Chemicon International, Temecula, CA) and anti-$p21^{WAF-1}$ (C-19; Santa Cruz Biotechnology) antibodies. The immunocomplexes formed were visualized with horseradish peroxidase–conjugated anti-rabbit immunoglobulin G (IgG; ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation. Control and i-Extract/i-Factor–treated cells were lysed in Nonidet P-40 lysis buffer. About 400 μg protein was incubated with wild-type p53-specific antibody (Ab-5; Calbiochem, Darmstadf, Germany) at 4°C for 1 to 2 h. Immunocomplexes were separated by incubation with Protein-A/G Sepharose, and Western blotting was done with anti-p53–specific antibody (FL-393; Santa Cruz Biotechnology).

Immunostaining. Immunostaining was done as described earlier (24, 25). Cells were stained with anti-p53 antibody (DO-1; Santa Cruz Biotechnology), a monoclonal anti-mortalin antibody (MA3-028, Affinity Bioreagents, Golden, CO), or a polyclonal anti-mortalin antibody. Immunostaining was visualized by secondary staining with goat anti-rabbit IgG (Alexa-594–conjugated) and anti-mouse IgG (Alexa 488–conjugated; Molecular Probes, Eugene, OR).

Reporter assays. Cells were transfected with the p53-responsive luciferase reporter plasmid, PG-13luc (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Medical Institutions, Baltimore, MD) and pPur plasmid (for puromycin resistance) in a 1:30 ratio. Transfected cells were selected in puromycin (2-3 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 rTdT 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:^{1}H NMR ($CDCl_3$, 500 MHz):d 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, 17-OH, H-4, 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, 5-OH), 3.31 to 3.33 (m, 1H, H-7), 4.59 to 4.63 (m, 1H, H-22), 5.85 (dd, J = 10.1, 2.6 Hz, 1H, H-2), 6.58 to 6.61 (m, 1H, H-3).^{13}C NMR ($CDCl_3$, 125 MHz): d 9.5 (C-21), 12.3 (C-27), 14.7 (C-19), 15.1 (C-18), 20.5 (C-28), 21.6 (C-11), 22.9 (C-15), 32.5 (C-12), 32.8 (C-23), 35.2 (C-9), 36.0 (C-8), 36.7 (C-16), 36.8 (C-4), 42.9 (C-20), 45.9 (C-14), 48.7 (C-13), 51.0 (C-10), 56.3 (C-6), 57.2 (C-7), 73.2 (C-5), 78.7 (C-22), 84.6 (C-17), 121.4 (C-25), 129.0 (C-2), 139.7 (C-3), 150.4 (C-24), 167.1 (C-26), 203.1 (C-1).

Results and Discussion

To examine the tumor-inhibitory effect of the leaf extract (i-Extract), we did nude mice assays using highly malignant human fibrosarcoma, HT1080 (mutant p53 and telomerase positive) cells. Subcutaneous injection of 10^6 cells resulted in the formation of solid tumors within 10 to 15 days (Fig. 1A). Injections of i-Extract, commenced either along with the injection of cells or before the formation of tumor buds or when small tumor buds (5 mm) were formed, resulted in tumor suppression. Of note, mice fed with i-Extract showed significant tumor suppression (Fig. 1B), suggesting that the i-Extract has a strong tumor-suppressive activity *in vivo*. We fractionated the i-Extract into its components by reverse-phase HPLC and identified the presence of withaferin A, withanolide

[4] Cell Proliferation Reagent WST-1, a colorimetric assay for the quantification of cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dihydrogenases in viable cells.

Table 1. List of genes and their target sequences of shRNA plasmids used to identify cellular targets of i-Extract

Serial number	Accession number	Gene name	Target sequence
1	**NM_004936**	**CDKN2B**	**AATAGAGGTTGAACTTCAA**
2	NM_004517	ILK	GATCTAAATTTGACGTGAT
3	NM_004517	ILK	GCGGGGATTTCAATGGAGA
4	NM_004064	p27 Kip1	AAGGGAGGGTTTATGTAGA
5	NM_004064	p27 Kip1	GAGGCAGTTGATATTTCAT
6	NM_002611	PDK2	GGTCCAATTCTAAGGTGGA
7	NM_002611	PDK2	AGGTCAACTTGCTTCTCGA
8	NM_002610	PDK1	GCTGGAGCTTAGAAGTCTA
9	NM_002610	PDK1	AATGGGAATTGAATTTCAT
10	NM_002498	NEK3	GCTCCAGTATGGATGGCTA
11	NM_002498	NEK3	GTTTGGGAATTGAATGTAT
12	**NM_002497**	**NEK2**	**GCTGGAATGTTAAACTATA**
13	NM_002497	NEK2	GATAGAAGTTGAGAAGCAA
14	NM_002376	MARK3	GAGGTAAGATTTAAATTTA
15	NM_002376	MARK3	AGTAAGGCCTCGATCTCTA
16	NM_002314	LIMK1	GCTAGAGGATTTATGGTGA
17	NM_002314	LIMK1	ACGAGAGGTTCAATTTCAT
18	NM_001800	CDKN2D	GGTAGAGTATTTAAGGGTA
19	NM_001800	CDKN2D	GGGAGGAATACTTTCTCTT
20	NM_001799	CDK7	GGGTAAAGTGTTATGGGAA
21	NM_001799	CDK7	GGATATAAATTTAATGTTA
22	NM_001798	CDK2	GCTATAATGTTTATAGAGA
23	NM_001798	CDK2	GGGTGAATAATTATGTTTA
24	NM_001262	CDKN2C	GTTCTGATATGTAGTTGCA
25	NM_001262	CDKN2C	ATGTGAACGTTAATGTACA
26	NM_001261	CDK9	GGTCAAATGTGGACAGCTA
27	NM_001261	CDK9	GCTAGAGGGTGGCTTTGAA
28	NM_001260	CDK8	GCTTGAATATTCAACGTTA
29	NM_001260	CDK8	GATAGAATATTTAGTGTAA
30	NM_001259	CDK6	GATTAAGATTTGACCGCTT
31	NM_001259	CDK6	GCGAAGATCTATTTCTGAA
32	NM_001258	CDK3	GCGGTAAGTTCTATATCAA
33	NM_001258	CDK3	GGGAGGGATTGGAAGGGAT
34	NM_000077	CDKN2A	ACTAGAGAGGTTCTGGGAA
35	NM_000077	CDKN2A	ATTGCGGTGTCGCATGGTA
36	**NM_000076**	**CDKN1C**	**GTGTGAAGCTTTGAGGGTA**
37	NM_000076	CDKN1C	GTTCTAAAGTGTAAGGCTT
38	NM_000075	CDK4	GGGTGAGGGTAATCTGGAA
39	NM_000075	CDK4	AGGGTAGTCTTTGTCTTTA
40	NM_138793	SHAPY	GCGGGGGGTTGTAGGGAAA
41	NM_138793	SHAPY	GCGTGGAATATGAGGGCAT
42	NM_000314	PTEN	GGTGGAGCTTGCAATTCTA
43	NM_000314	PTEN	GGGATAATATTGATGGTGT
44	**NM_000546**	**p53**	**GCTAAAGAAGAAATCGCA**
45	NM_000546	p53	GCTGGGAGGGTTAATATCT
46	NM_000077	p16INK4A	GAGTGTGAGAGTCTCTAAT
47	NM_000077	p16INK4A	GATAACTCTGATGTTTGAG
48	NM_015322	FEM	ATTTCATGtTATAAAGGAC
49	NM_015322	FEM	GTCATAAACTTGCACAGAC
50	NM_002156	HSP60	GGTACTAGCTCTTCATTTC
51	NM_002156	HSP60	GCTCGAGTCTTAATGCTTG

Table 1. List of genes and their target sequences of shRNA plasmids used to identify cellular targets of i-Extract (Cont'd)

Serial number	Accession number	Gene name	Target sequence
52	NM_003156	STIM1	GGGAAGACCTCAATTACCA
53	NM_003156	STIM1	GGCCAAGAAGACATTTATA
54	NM_014622	AW551984	GAACAAACTTCTATCTTTA
55	NM_014622	AW551984	AGTCAAACTTGTAAATAGA
56	NM_007215	POLG2	GAAGCAAACTTTACTACAA
57	NM_007215	POLG2	GGGAAAGGAGCCAATAGAA
58	NM_016248	PRKA-2	GTGAAGCTTTATCAAATGC
59	NM_016248	PRKA-2	ACAAACATGGAAAGTCATG
60	NM_005923	MAP3K5	GGGACAGCTTTCAAATTAA
61	NM_005923	MAP3K5	ATCTGTTTCTGTATAGAAT
62	NM_004672	MAP3K6	GTGGGTTGCTTAAGGTTTC
63	NM_004672	MAP3K6	AGGGATCGTTCTTCACAGA
64	NM_145333	MAP3K7	GGGTGAATTTGGATGTTTA
65	NM_145333	MAP3K7	AGGTGTTGCTATTAAATAA
66	NM_005204	MAP3K8	GTAAGCACCTTCACTGGAG
67	NM_005204	MAP3K8	AGGAAGTGATTCATTATGA
68	NM_001880	ATF2	GGGTAGTCCTTTATCTCAC
69	NM_001880	ATF2	AGTTCTGGCTTAAGTGTAG
70	NM_002759	PRKR	AAGATTAATTGTAAATTG
71	NM_002759	PRKR	ATTGGTCGCTAAATTTGCG
72	NM_005157	ABL1	GGTCCTAGCTTTATGCTCG
73	NM_005157	ABL1	GTTCAGCTCTGCTACCTAT
74	NM_001626	AKT2	ATCTCATGTTGGACAAAGG
75	NM_001626	AKT2	GGCTTCGATTATCTCAAAT
76	NM_001166	BIRC2	AGGAGTTACTATAACCCTA
77	NM_001166	BIRC2	GGATATGTCTCTAATGAGA
78	**NM_001165**	**BIRC3**	**ACTTTGACTTTCATTCTAG**
79	NM_001165	BIRC3	AGCTTTAAATGTATAGAAA
80	NM_001167	BIRC4	GTATCATACTGTAACTGAA
81	NM_001167	BIRC4	GTGTTGGACTTTACTACAC
82	NM_001168	BIRC5	ATTGTTGATTCATAGAATG
83	NM_001168	BIRC5	GTAGGTTCCTTGTCTGTCA
84	NM_000633	BCL2α	AGTGTGAATTTAAACTTTA
85	NM_000633	BCL2α	AAGTAAGGCTTTGAATGAT
86	**NM_004935**	**CDK5**	**ATGGTGGCCTTGATCTTGA**
87	NM_004935	CDK5	GGGTGGGGTTGGATGATGA
88	NM_023109	FGFR1	GGTGGAACCTTCACGCCGG
89	NM_023109	FGFR1	ATTTAAACCTGATCACAGA
90	NM_000142	FGFR3	GTGTATATTTCTGTAAATG
91	NM_005248	FGR	GGTCCGGCCTGTATCCTCG
92	NM_005248	FGR	GGGCCCATCTATATCGTGA
93	NM_000875	IGF1R	ATACCATCCTGCATAATAC
94	NM_000875	IGF1R	AGTTATGGTTTAAATTTAA
95	NM_000208	INSR	GGTGAGATTTATATGATTA
96	NM_000208	INSR	GGAATGACTTTCATTCTGG

NOTE: shRNA (1, 12, 36, 44, 78, and 86) that resulted in the abrogation of the effect of i-Extract are indicated in bold.

A, and 12-deoxywithastramonolide (Fig. 2A). These components, however, did not show any antitumor activity either by local injections into the tumor sites or by oral feeding (Fig. 1B and data not shown). Upon HPLC analysis of different batches of i-Extract that were used for nude mice assays, we noticed that the content of its component that elutes at 22 min in HPLC correlated positively with its tumor suppressor activity (Fig. 2A). We purified this ingredient of interest from i-Extract and named it as i-Factor because of its tumor-inhibitory activity (Fig. 2B). We next examined the effect of i-Factor in *in vivo* and *in vitro* 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. 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 *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

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

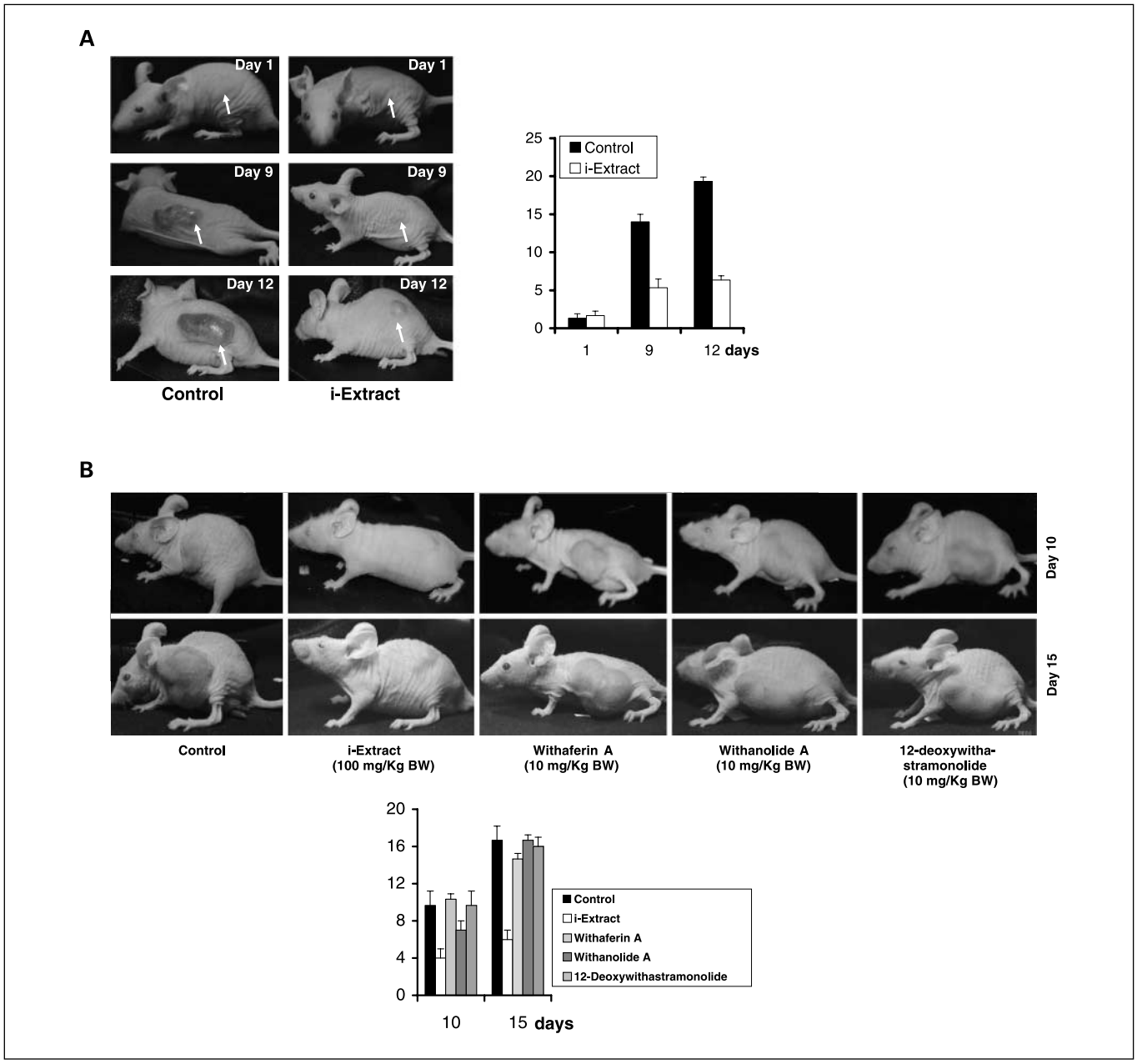

Fig. 1. Tumor suppression by i-Extract in nude mice assays. *A, in vivo* tumor suppression by i-Extract in nude mice. Control HT1080 cells formed a large tumor in 6 to 15 d. Local injections of i-Extract (0.3 mL of 24 μg/mL) to tumor sites significantly inhibited their growth. Injections were repeated every third day. Columns, average tumor size (mm) in control and i-Extract – injected mice. *B,* oral feeding of i-Extract (100 mg/kg body weight), but not of withaferin A (10 mg/kg body weight), withanolide A (10 mg/kg body weight), or 12-deoxywithastramonolide (10 mg/kg body weight), resulted in tumor suppression. Columns, average tumor size (mm) in control and treated mice.

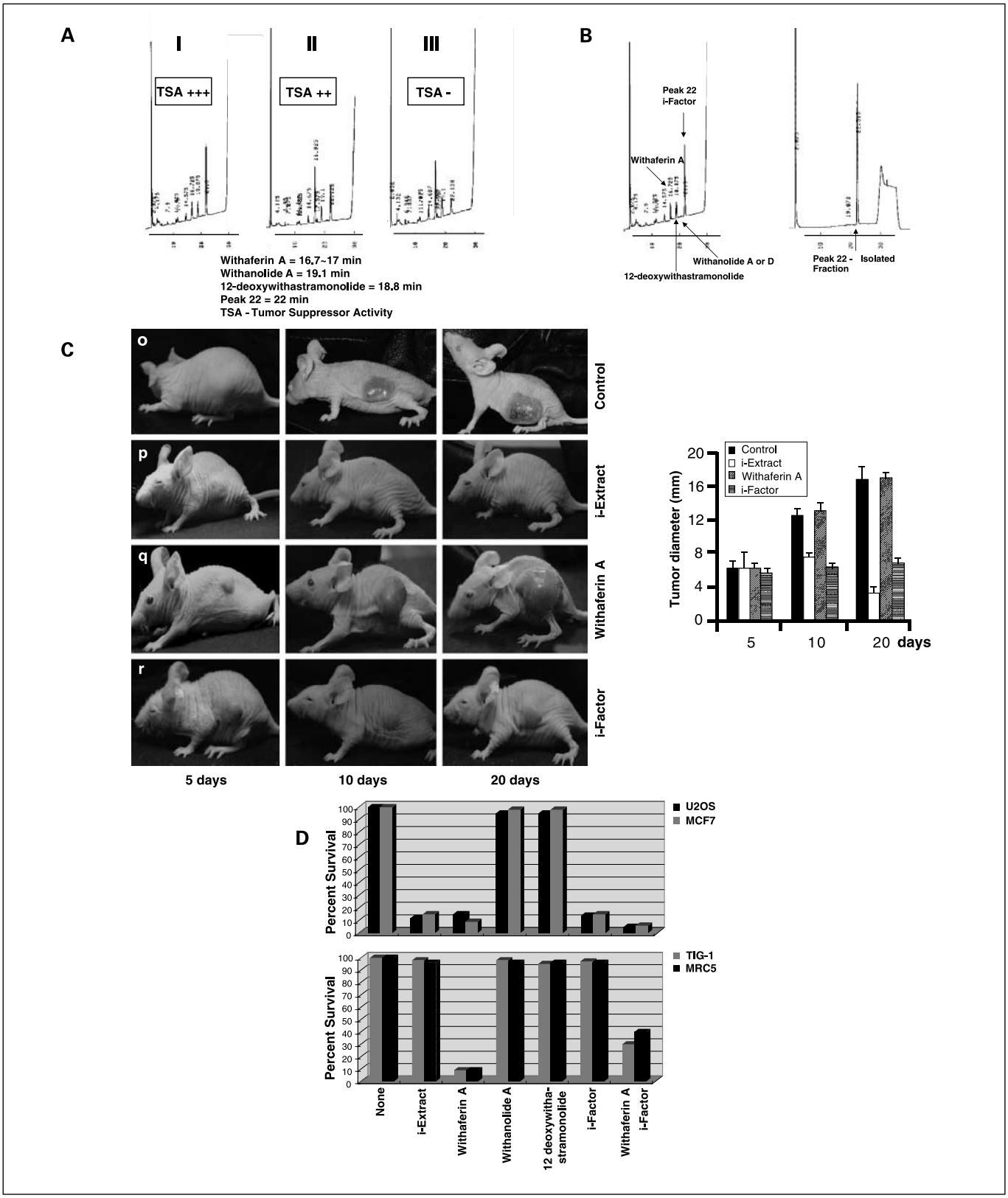

Fig. 2. Fractionation of i-Extract by reverse-phase HPLC. *A,* the identified components included withaferin A, withanolide A, and 12-deoxywithastramonolide. In addition, a component that eluted at 22 min was identified in all the i-Extract preparations, and its amount correlated with the tumor suppressor 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 ($p21^{WAF-1}$ 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

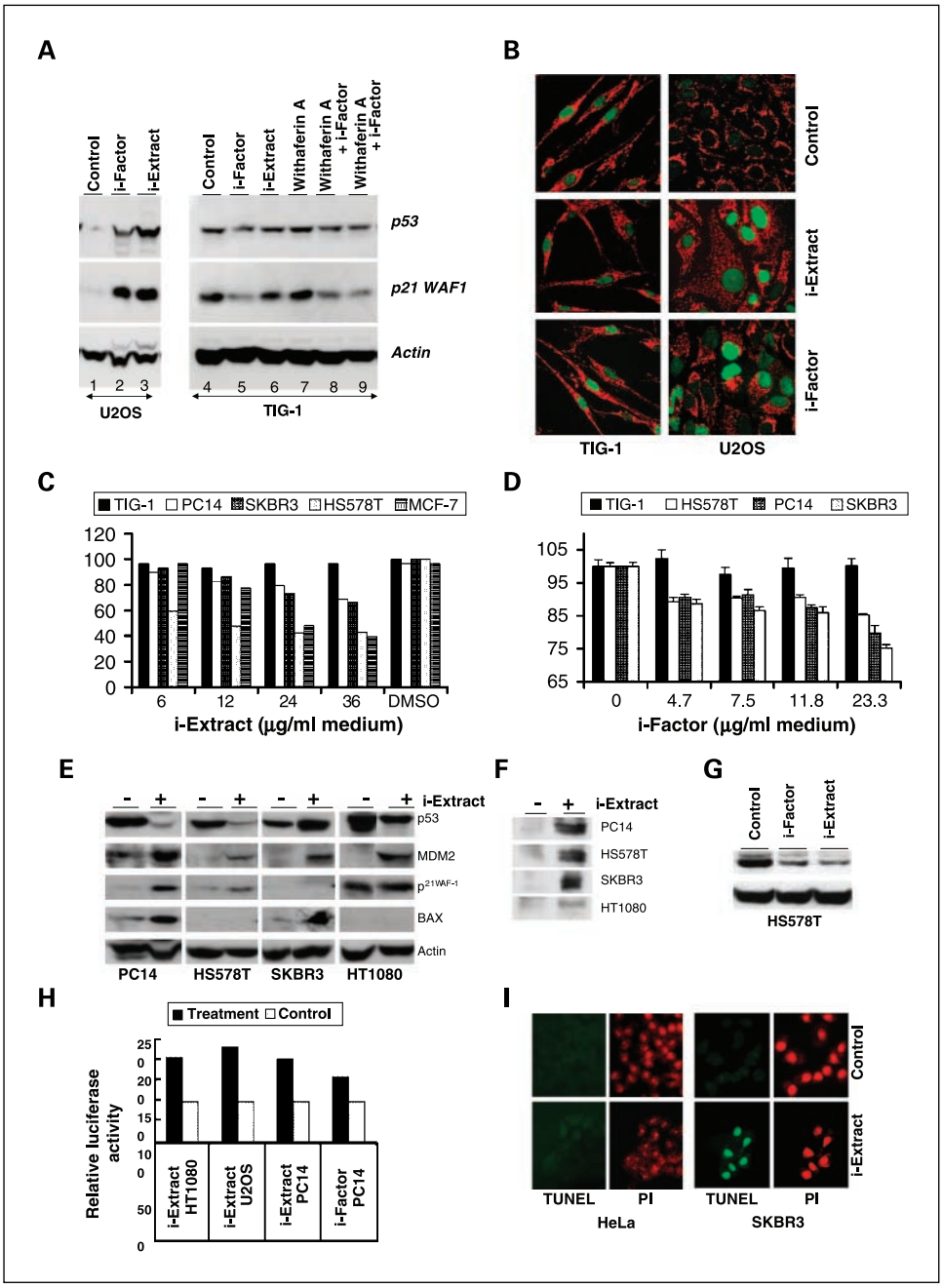

Fig. 3. i-Extract and i-Factor activate wild-type tumor suppressor p53 in cancer cells. *A,* Western blot analysis showing the activation of p53 and its downstream effector $p21^{WAF-1}$ by i-Extract (24 μg/mL) and i-Factor (10 μg/mL) in osteosarcoma (U2OS), but not in normal human skin fibroblasts (TIG-1). Withaferin A (0.1 μmol/L) activated the wild-type p53 function both in cancer and normal cells. Actin was used as a loading control. *B,* i-Extract caused a shift in the staining pattern of mortalin (*red*) in cancer (*U2OS*) cells. Consistent with the shift of mortalin staining pattern (from perinuclear to pancytoplasmic), p53 was translocated into the nucleus of cancer cells (*green nuclear staining*). *C* and *D,* cell viability assay of normal and tumor-derived human cells grown in normal, i-Extract, or i-Factor–supplemented culture medium. All the cancer cells were growth arrested; normal cells remained unaffected. *E,* i-Extract restored the wild-type p53 characteristics and function to the mutant p53 as detected by a decrease in the level of p53 and increased level of $p21^{WAF-1}$, MDM2, and Bax. *F,* immnoprecipitation of wild-type p53 in i-Extract–treated mutant p53-possessing cells. *G,* similar to i-Extract, i-Factor led to a decrease in the level of p53 protein in mutant p53 cells. *H,* detection of wild-type p53-specific reporter activity in i-Extract–treated cells. p53-dependent luciferase activity in i-Extract/i-Factor–treated cells is plotted as percent relative activity to that of the untreated control cells (U2OS, wild-type; HT1080, wild-type and mutant p53; and PC14, mutant p53). *I,* i-Factor triggered apoptosis as detected by TUNEL staining in mutant p53 containing (*SKBR3*) cells; no apoptosis was detected in HeLa and U2OS cells.

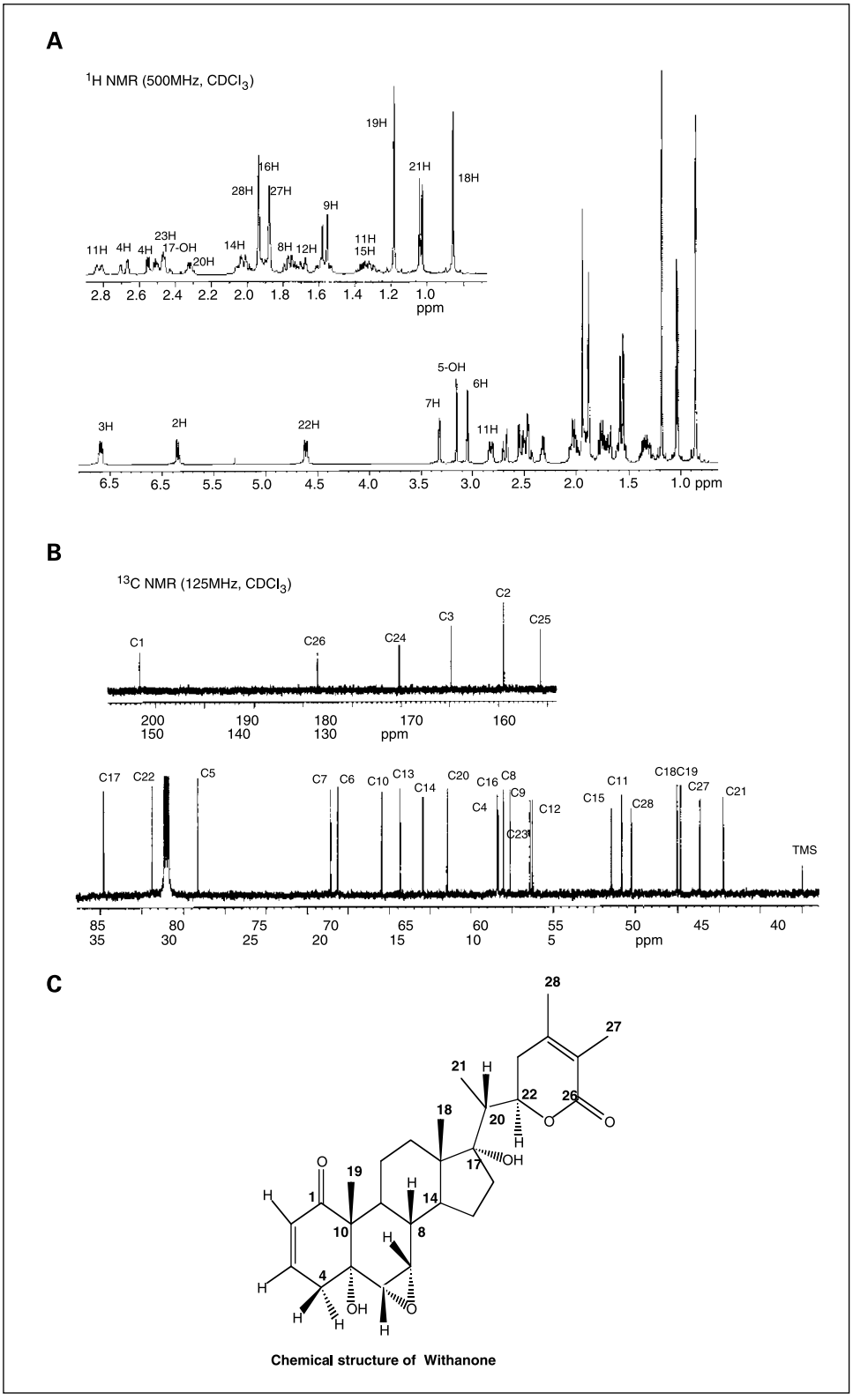

Fig. 4. One-dimensional NMR spectra of i-Factor and a structure of withanone. One-dimensional NMR of i-Factor was done in $CDCl_3$. *A,* ^1H NMR spectrum. *B,* ^{13}C NMR spectrum. *C,* withanone.

(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, HS578T, 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 HS578T 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, HS578T, 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 (HS578T, 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, ^{1}H NMR and ^{13}C 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 sources for safe antitumor medicine.

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