

Selective cytotoxicity of non-small cell lung cancer cells by the withaferin A-fortified root extract of Ashwagandha involves differential cell-cycle arrest and apoptosis

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Abstract

Withaferin A (WFA) isolated from the root and leaf extracts of *Withania somnifera* (Ashwagandha, ASH) has been shown to exhibit promising anti-tumor properties. Even though WFA is cytotoxic to a variety of cancer cell types, it is also toxic to normal cells. To selectively chemosensitize cancer cells to WFA, we have utilized a novel approach of fortifying the crude root ASH extract with WFA (FASH) and compared the effects of WFA and FASH on normal and non small cell lung cancer (NSCLC) cells. WFA-induced cytostatic and apoptotic effects on normal and NSCLC cells were accompanied by an increased oxidative stress induced lipid peroxidation and GSSG/GSH ratio. While FASH potentiated the cytotoxic effects of WFA on NSCLC cells, they were significantly inhibited in normal cells. The protective effects of FASH on normal cells were associated with the up regulation of antioxidant enzymes, alteration in the expression and functions of cell cycle, and apoptosis related proteins. The anti-apoptotic effects of FASH on normal cells were exerted via the activation and phosphorylation of AKT, ERK1/2 and BAD. Overall, we demonstrate that FASH selectively chemo sensitizes NSCLC through a differential modulation of oxidative stress induced signaling. These findings provide a novel strategy to target a variety of cancers.

Keywords: Ayurveda, Ashwagandha, *Withania somnifera*, withaferin A, lipid peroxidation, antioxidant

Introduction

Withania somnifera Dunal, commonly known as Ashwagandha (ASH) is a plant belonging to the Solanacea family. It has been safely used for centuries in Indian Ayurvedic medicine for the treatment of a variety of ailments (Mishra, 2000). Intensive research has now confirmed its antioxidant, anti-inflammatory, immune-modulating, and anti-stress prop-

erty in extracts obtained from the whole plant as well as different parts like the roots, leaves etc (Jayaprakasam et al., 2003; Matusuda et al., 2001; Mishra, 2000). Out of the several withanolides isolated from crude ASH, withaferin A (WFA), a steroidal lactone is the major prototype most actively pursued for its important anti-cancer properties (Devi et al., 1995; Devi and Kamath., 2003; Fuska et al., 1984; Malik et al., 2007; Shohat and Joshua., 1971; Shohat et al., 1978). Studies demonstrating the WFA-induced inhibition in cancer growth, cytotoxic, apoptotic and inhibition of angiogenesis (Koduru et al., 2010; Mohan et al., 2004; Mandal et al., 2008; Oh et al., 2008; Srinivasan et al., 2007) in different *in vitro* and animal models of cancer have attributed these effects to the induction of reactive oxygen species (ROS) (Malik et al., 2007). WFA therefore, appears to have great potential for its use as a novel complementary therapy for integrative oncology care. However, as observed with several chemotherapeutic agents either synthetic or purified from natural products, WFA also exhibits toxic effects on normal healthy cells that can lead to serious side-effects. Strategies are therefore required to overcome this impediment as well as improve its selectivity for cancer cells.

Recent studies have shown that the alcoholic leaf extract of ASH (i-extract) can selectively kill tumor cells by activation of the tumor suppressor protein p53. An inhibitory factor (i-factor) identified as withanone in the 'i-extract' has been suggested to exhibit differential cytotoxic effects on normal and cancer cells (Widodo et al., 2007; Widodo et al., 2009). In the light of these findings, we hypothesized that the toxic effects of WFA on normal cells may be mitigated by the fortification of the crude root extract of ASH with optimized concentrations of WFA and formulated the fortified ASH extract as 'FASH'. Furthermore, we believe that the WFA-induced cytotoxic, cytostatic and apoptotic effects maybe differentially modulated by FASH through cellular redox signaling in normal and cancer cells. During present studies, we have examined the efficacy of purified WFA alone as well as in its fortified optimized formulation-'FASH', for their varied effects on the NSCLC (H358 and H460), normal human bronchial epithelial (BEAS 2B) and human embryonic kidney (HEK293) cell lines as respective models of cancer and normal cells. With the help of biochemical and molecular approaches, we provide evidence to show that FASH selectively attenuates WFA-induced cytotoxicity, growth arrest, and apoptosis in normal BEAS 2B and HEK 293 cells. Overall, our results suggest that FASH elicits an enhanced antioxidant response derived from the other constituents in the ASH root extract ensuing a therapeutic protective response in normal cells. Based on this scientific basis, we propose that FASH can be utilized to target a variety of cancer cell types with little or no effect on normal cells.

Materials and Methods

Chemicals and reagents

RPMI-1640 medium, penicillin-streptomycin solution (P/S), phosphate buffered saline (PBS), fetal bovine serum (FBS), HEPES, and, trypsin were purchased from Gibco (Grand Island, NY). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotting were purchased from Bio-Rad (Hercules, CA). RIPA lysis buffer was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Western blot stripping buffer was from Pierce (Rockford, IL). All other chemicals and reagents were

obtained from Sigma-Aldrich (St. Louis, MO). Withaferin A (WFA) and ASH root powder were obtained from Chromadex Inc. (Santa Anna, CA).

Cell culture

The human NSCLC cell lines H358 and H460, primary bronchial epithelial (BEAS-2B) and human embryonic kidney HEK 293 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). NSCLC cells were cultured in RPMI-1640 supplemented with 10% FBS, 1% of a stock solution containing 10,000 IU/ml penicillin and 10 mg/ml streptomycin. BEAS-2B, an adenovirus-12 SV40 hybrid virus transformed, immortalized human bronchial epithelial cell line was cultured in tissue culture flasks/plates coated with collagen in bronchial epithelial growth medium supplemented with growth factors, all-trans retinoic acid, and hormones (Lonza Rockland Inc. Rockland, ME). Cells were kept in an incubator at 37°C under a humidified atmosphere containing 5% CO₂. HEK 293 cells were grown in MEM supplemented with 10% FBS and 1% penicillin/streptomycin.

ASH root extract

1g of powdered root of ASH was extracted with 25ml of methanol and water mixture (80:20). The methanolic extract was centrifuged and concentrated in a stream of nitrogen. The methanolic extract of ASH was completely dried under nitrogen and the brown powder thus obtained was weighed.

Preparation of WFA and FASH and treatment of cells

Stock solution of WFA was prepared in DMSO that was further diluted either in PBS or growth medium to get its desired concentrations. While treating the cells, final concentration of DMSO was kept at 0.1% or less. 40mg of root extract was dissolved in PBS (1 ml) and the soluble material was fortified with pure WFA to achieve different stock solutions of 0.25-5 mM WFA. For the treatment of cells, the dilutions of FASH were prepared in growth medium so as to vary the concentration of WFA while keeping the ASH extract concentration fixed for all the experiments.

Cytotoxicity assays

The sensitivity of the NSCLC and normal cells to WFA, and FASH was measured by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay as described by Mosmann.(1983). The absorbance of formazan at 562 nm was measured using a microplate reader (Elx 808, BioTek Instruments, Inc). A dose response curve was plotted and the concentration of WFA or FASH resulting in a 50% decrease in formazan formation was calculated as the IC₅₀ value of WFA/FASH.

Exposure of cells to WFA and FASH for signaling studies

NSCLC and normal cells were plated at a density of 5x10⁵ cells per 100 mm petri dish. When the cell density reached to 70-80% confluency, the NSCLC culture medium was replaced with the medium supplemented with 0.1% FBS for several hours after which the

two types of cells were treated with WFA(0.5 μ M) alone and in combination with ASH (FASH: combination of 40mg/ml ASH and 0.5 μ M WFA) for 24h. Control and treated cells were harvested, washed with cold PBS and incubated in 100 μ l of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5; 1% NP-40; 150 mM NaCl; 1 mg ml⁻¹ aprotinin; 1 mg ml⁻¹ leupeptin; 1 mM Na₃VO₄; 1 mM NaF) at 4°C for 30 min. RIPA insoluble cellular material was removed by centrifugation at 14,000 g for 10 min at 4°C. Protein concentrations in the supernatants were determined by Bradford assay (Bradford, 1976) as described in standard protocol.

Western blot analysis

Cell extracts prepared as described above were separated on SDS polyacrylamide gels (4-20%), and transferred onto nitrocellulose (Bio-Rad). Membranes were blocked with 5% fat-free milk at room temperature for 60 min, and incubated overnight at 4°C with the appropriate primary antibody in 5% milk in Tris-buffered saline (TBS). After 3X washing with T-TBS (Tris-buffered saline containing 0.05% Tween 20), the membrane was incubated with the appropriate secondary antibody at room temperature for 2 h. After washing again with T-TBS, the membrane was treated with Super signal 'West Pico' chemiluminescence reagent (Pierce, Rockford, IL) as per manufacturer's instructions, and exposed to Hyperfilm ECL film (Amersham) at room temperature.

Determination of cellular GSH and GSSG levels

Reduced GSH and its oxidized form (GSSG) were analyzed spectrophotometrically by using a GSH/GSSG measurement kit (Abcam, San Francisco, CA) based on the recycling method of Teitze. (1969).

Determination of LPO levels

The extent of lipid peroxidation (LPO) in the extracts of control and treated cells were analyzed by colorimetric method using LPO586 kit (Oxis International, Beverly Hills, CA) as per the manufacturer's instructions.

Measurement of antioxidant enzyme activities

The effect of WFA and FASH on the activities of glutathione peroxidase (GPX) (Mannervik., 1985), glutathione reductase (GR) (Carlberg and Mannervik., 1985) and γ -glutamyl cysteine synthase (γ -GCS) (Seelig and Meister., 1984) were analyzed in control and treated NSCLC and normal cells using previously published methods.

FACS analysis

Cells (2×10^5 in a 100mm dish) were treated with WFA (0.5 μ M) and FASH (0.5 WFA and 20mg/ml ASH) for 24h at 37 °C. Appropriate controls were also set up. Care was taken to assure that the cells were no more than 50% confluent on the day of the treatment. After treatment, floating and adherent cells were collected, washed with PBS two times by centrifugation at 300 g for 5 min at 4 °C, and fixed with 70% ethanol. On the day of flow analysis, cell suspensions were centrifuged, counted and approximately 600,000 cells were

resuspended in 500 µl PBS in flow cytometry tubes. Cells were incubated with 2.5 µl RNase (20 mg/ml) and incubated at 37°C for 30 min after which they were treated with 5 µl of propidium iodide (1mg/ml) solution and incubated at room temperature for 30 min in the dark. The stained cells were analysed using the Beckman Coulter Cytomics FC500, Flow Cytometry Analyzer. CXP2.2 analysis software from Beckman Coulter was used to deconvolute the cellular DNA content histograms to obtain quantitation of the percentage of cells in the respective phases (G1, S and G2/M) of the cell cycle. Appearance of the sub-G0/G1 peak indicates the cells undergoing apoptosis.

Fluorescence analysis of apoptosis by confocal microscopy

NSCLC and BEAS-2B cells were seeded onto glass cover slips in 12-well plates. After reaching 50% confluency, they were exposed to WFA (0.5 µM) and FASH (0.5µM WFA +40mg/ml ASH). Cells treated with vehicle (PBS containing 0.1% DMSO final concentration) remained as controls. After completion of treatment for 24h, cells were washed twice with ice-cold PBS (pH 7.4), fixed with 4% paraformaldehyde for 30 min. After washing with ice cold PBS, the cover slips were mounted on glass slides with 20 µL of VectaShield medium containing DAPI (1.5µg/mL) (Vector Laboratories, Inc., USA). Slides were examined using a LSM 510 Meta confocal system equipped with an inverted microscope (Axio Observer Z1, Carl Zeiss).

Statistical analysis:

Values are expressed as mean± SD. Data was analyzed for significance by the standard student's t test and p-value less than 0.05 was considered significant.

Results

Effect of WFA, ASH and FASH on cell viability

WFA, ASH and FASH were examined separately for their effects on the viability of normal and NSCLC cells using the simple and sensitive MTT assay. The IC₅₀ values (Table 1) calculated on the basis of cell viability curves presented in Figure 1 A-D for WFA and FASH indicated that as compared to normal cells, NSCLC cells were relatively more resistant to WFA. Surprisingly, the p53 null H358 NSCLC cells were more sensitive to WFA (IC₅₀ 2.0

Table 1. IC₅₀ values of WFA and FASH in NSCLC and normal cells

Cell Line	IC ₅₀ (µM) (Mean ± SD)*	
	WFA	FASH
H460	2.45 ± 0.20	1.70 ± 0.17 ^a
H358	2.0 ± 0. 11	1.50 ± 0.08 ^b
BEAS 2B	0.75 ± 0.026	1.26 ± 0.15 ^c
HEK 293	1.62 ± 0.12	2.0 ± 0.23 ^d

* Viability of cells at different doses of WFA and FASH was analyzed by MTT assay (Mossman T., 1983) described in Methods section. a: p < 0.001; b: p < 0.05; c: p < 0.05; d: p <0.05.

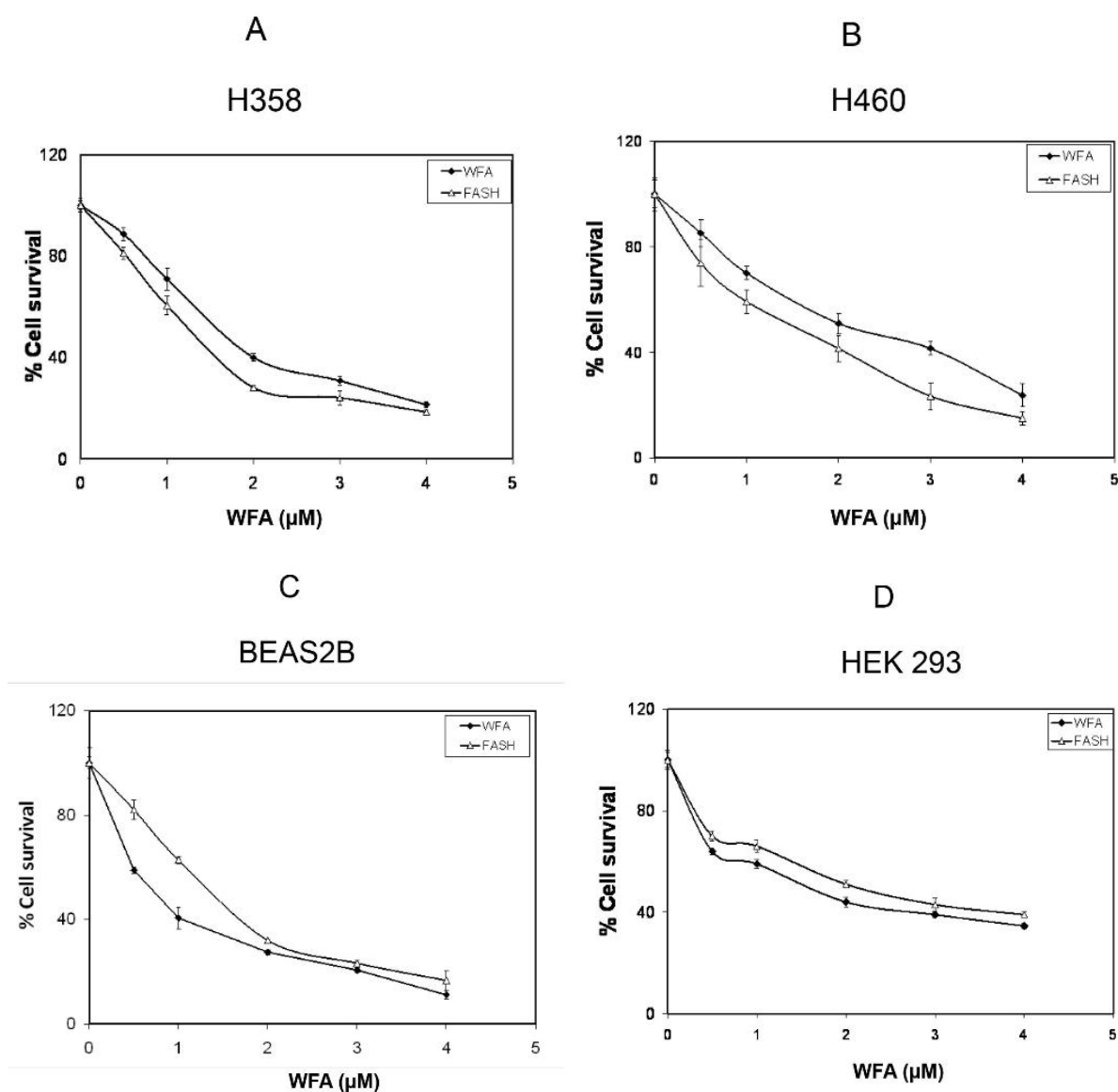


Figure 1. Viability of NSCLC and normal cells treated with different concentrations of WFA and FASH: H358 (A), H460 (B), BEAS 2B (C) and HEK 293 (D) cells (2×10^4) were plated in 96 well plates and were allowed to attach overnight. Cells were then treated with WFA (0-5 μ M) and FASH (containing 0-5 μ M WFA) and incubated for 24h at 37°C in CO₂ incubator. MTT assay was performed after completion of treatment period as described in the Methods section. Percent cell viability (mean \pm SD of 3 separate experiments done in quadruples) plotted against different doses are shown in the curves.

μ M) as compared to the wild type p53 expressing H460 cells (IC₅₀ 2.45 μ M). On the other hand, BEAS 2B were relatively more susceptible to WFA as compared to HEK293 cells (IC₅₀ 0.75 vs 1.62 μ M). While the root extract of ASH (2-200 μ g/ml) did not affect the viability of both normal and NSCLC cells, FASH significantly attenuated the WFA-induced cytotoxicity in normal cells, and potentiated NSCLC cell killing thereby indicating that FASH exhibited a differential cytotoxic response in normal and cancer cells.

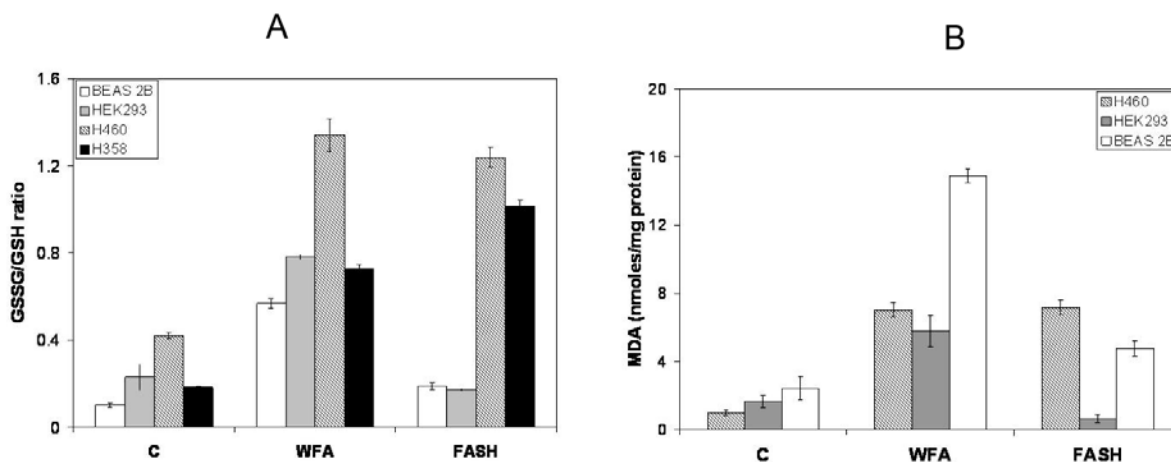


Figure 2. Effect of WFA and FASH on GSSG/GSH ratio (A) and LPO (B) in NSCLC and normal cells. H358, H460, BEAS 2B and HEK293 cells (5×10^5) were plated in 150 mm petri-dishes and allowed to grow in complete growth medium. When confluent (60-80%), cells were treated with WFA (0-0.5 μ M) and FASH (0- 0.5 μ M WFA +40mg/ml ASH) and incubated for 24h after which they were harvested using cell scrapers, centrifuged and washed with PBS (2x). The cell pellet was then resuspended in 10mM phosphate buffer, pH 7.0 containing 5mM BHT and sonicated on ice (3x15W, 10sec). After centrifuging again at 14,000 rpm for 10min at 4⁰C, the supernatants were collected and used for the analyses of GSH, GSSG (A) and malondialdehyde (MDA) (B) as described in the Methods section. Values presented in the bar charts are Mean \pm SD of two separate experiments done in triplicate.

Effect of WFA and FASH on GSH and GSSG levels

The non-protein thiol, GSH plays an important role in the reduction of peroxides, the detoxification and efflux of xenobiotics, and maintenance of cellular redox homeostasis (Meister and Anderson., 1983). Basal GSH levels were found to be lower in BEAS 2B and HEK 293 (15-17 nmol/mg protein) when compared to H358 and H460 (30-35 nmoles/mg protein) cells and are consistent with previous findings (Estrela et al., 2006). Treatment with WFA in both NSCLC and normal cells significantly attenuated GSH levels (>50%). More importantly, while there was a significant recovery in the WFA-induced depletion of GSH in FASH-treated BEAS 2B and HEK 293 cells, this treatment caused a further decline of GSH levels in H460 cells. Since GSSG/GSH ratios are important indicators of oxidative insult, we analyzed this ratio in control, WFA- and FASH-treated cells. As shown in Figure 2A, treatment with WFA enhanced the GSSG/GSH ratio in normal and NSCLC cells thereby confirming its pro-oxidant effect. In the presence of FASH, however, WFA-induced increase in the GSSG/GSH ratio was attenuated in normal cells but remained either unaltered or was potentiated in NSCLC cells. Taken together, these results suggested that FASH exerted its antioxidant effects selectively on normal cells.

FASH selectively enhanced LPO in NSCLC cells

Generation of ROS and accumulation of LPO products in cells upon exposure to various physicochemical and cancer chemopreventive agents is an important indicator of oxidative stress (Halliwell and Gutteridge., 1990; Sharma et al., 2010, 2011). Therefore, we analyzed and compared the levels of MDA in WFA and FASH-treated H460, HEK 293 and

BEAS 2B cells. Results presented in Figure 2B indicated that WFA-induced accumulation of MDA was selectively enhanced in FASH-treated NSCLC cells further supporting the data on the differential modulation of the redox status (GSSG/GSH ratio) by FASH in normal and cancer cells.

Phase II antioxidant enzymes are potentiated in FASH-treated normal cells

Cellular redox homeostasis is maintained by several major enzymes involved in GSH synthesis and metabolism (Dickinson and Forman, 2002a; Dickinson and Forman, 2002b). While GPx mediates GSH-dependent reduction of hydrogen peroxide and hydroperoxides (Mannervik, 1985), GR catalyzes the NADPH-dependent reduction of GSSG for the recycling of GSH (Carlberg and Mannervik, 1985). Enzymes involved in GSH-synthesis including the γ -GCS further contribute in maintaining cellular levels of GSH (Seelig and Meister, 1984). Therefore, we compared the activities of these enzymes in WFA- and FASH-treated normal and cancer cells. Results presented in Figure 3 (A-C) revealed that treatment with WFA caused a decline in the activities of GPx, GR and γ -GCS in both the cell types. On the other hand, while treatment with FASH significantly enhanced the activities of these antioxidant enzymes in the normal HEK293 cells, WFA-induced decline in enzyme-activity remained unaltered in FASH-treated H460 cells thereby indicating a differential effect of FASH on the functions of antioxidant enzymes in normal and NSCLC cells.

FASH enhanced WFA-induced cell cycle arrest selectively in NSCLC cells

Previous studies have shown that WFA inhibits the proliferation of various cancer cell types by inducing the arrest of cells in the G2/M phase of the cell cycle (Stan et al. 2008a, 2008b). Effects of WFA and FASH on the cell cycle of HEK293 and H460 were therefore compared by FACS analysis. Results of these analyses presented in Figure 4A indicated that WFA-induced arrest of H460 and HEK 293 cells in the G2/M phase after 24h of treatment. However, the percentage of H460 cells arresting in G2/M phase was less as compared to that of H358 cells, thereby supporting our cytotoxicity data which showed that the H460 cells were relatively more resistant to WFA. On the other hand, while treatment of NSCLC cells with FASH resulted in a further increase of G2/M arrest (Figure 4A) along with an increased population of cells in sub G0/G1 phase indicative of apoptosis, FASH-treated HEK 293 cells displayed an attenuated G2/M arrest and apoptosis. Taken together, these results revealed that FASH selectively inhibited the cell cycle progression of cancer cells rendering them more sensitive to WFA-induced cytotoxicity. Since G1-S and G2-M phase transitions are regulated by the universal inhibitor of the cyclin-dependent kinases (Cdks), p21, which belongs to the Cip/Kip family of inhibitors of Cdk's (Cdkis) (Jung et al. 2010; Satyanarayana and Kaldis, 2009), we next analyzed the expression of p21 by Western blotting in RIPA lysates of WFA- and FASH-treated H460 and BEAS 2B cells.

Results presented in Figure 4B indicated that WFA-induced activation of p21 was selectively enhanced in FASH-treated H460 cells. Consistent with this, the expression of cyclin B1 and Cdk1/2 was also down regulated in WFA and FASH-treated H460 cells thereby supporting the FACS analyses data that FASH selectively inhibited the cell cycle progression of NSCLC cells.

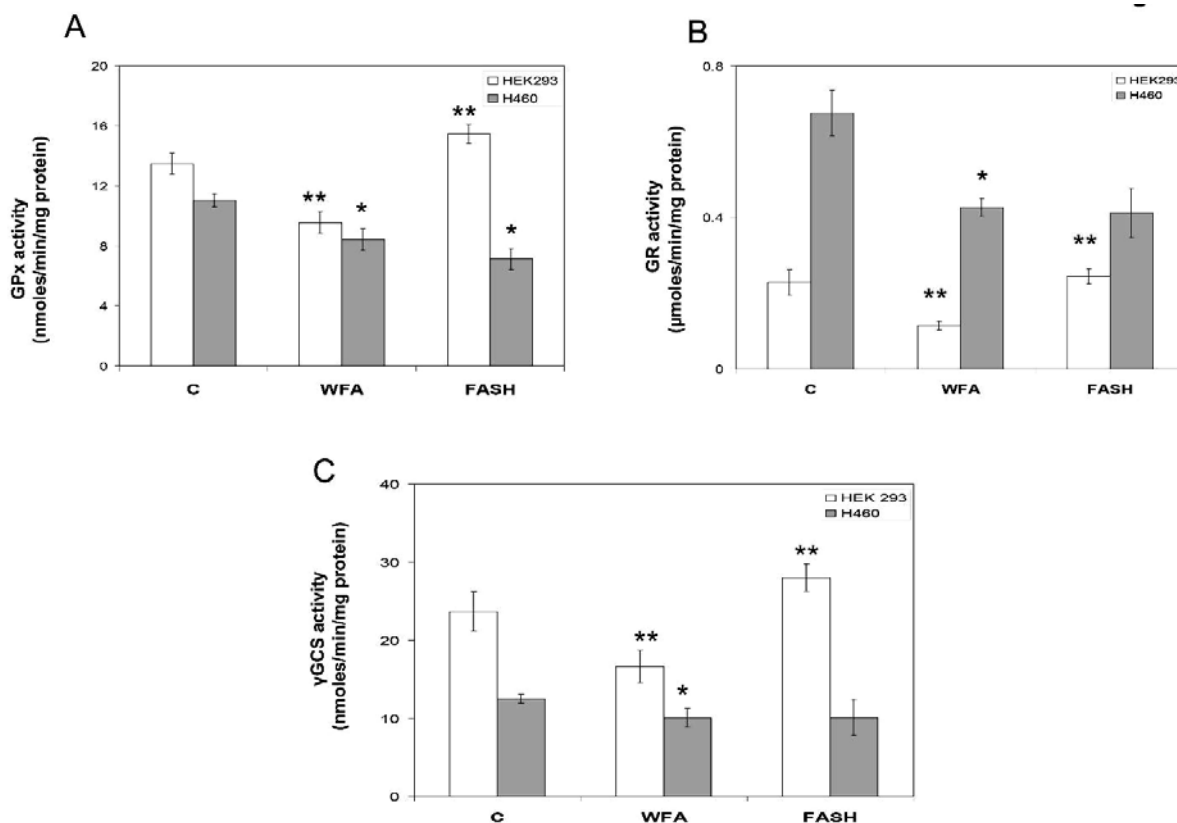


Figure 3. Effect of WFA and FASH on the activities of GSH-linked enzymes in NSCLC and normal cells: HEK293 and H460 cells were treated with WFA (0-0.5 μ M) and FASH (0- 0.5 μ M WFA +40 mg/ml ASH) for 24h. Cells were harvested and their extracts were prepared in 10 mM phosphate buffer, pH 7.4 containing 1.4mM β -mercaptoethanol. Enzyme activities of GPx (A), GR (B) and γ -GCS (C) were analyzed by using previously published methods. Bar charts (show activities of different enzymes in control and treated cells as marked on the figures (Mean \pm SD of two separate experiments done in triplicate. * and ** show significant differences in the activities of enzymes in controls and treated H460 and HEK 293 cells respectively ($p < 0.05$)).

WFA-induced apoptosis is increased selectively in FASH-treated NSCLC cells

Anti-tumor properties of WFA have been attributed to its ability to induce apoptosis in cancer cells (Devi et al., 1995; Devi and Kamath., 2003; Fuska et al., 1984; Malik et al., 2007). Results of present studies revealed that both normal and NSCLC cells were susceptible to WFA-induced apoptosis in a dose- and time-dependent manner. Next, we compared the effects of FASH on the extent of apoptosis induced in WFA-treated normal and cancer cells. Confocal microscopy of the DAPI-stained nuclei of WFA and FASH-treated BEAS 2B and H460 cells indicated nuclear condensation in the WFA-treated cell-types (Figure 5A) that was significantly suppressed in FASH-treated BEAS 2B cells indicating its differential apoptotic effect on normal and cancer cells. One of the hallmarks of apoptosis is the cleavage of poly ADP-ribose polymerase (PARP) (116 kDa protein) to the 89kDa fragment in response to DNA damage under a variety of toxic insults (Soldani and Scovassi, 2002). WFA has been shown to cause the cleavage of PARP at sub-lethal doses in different

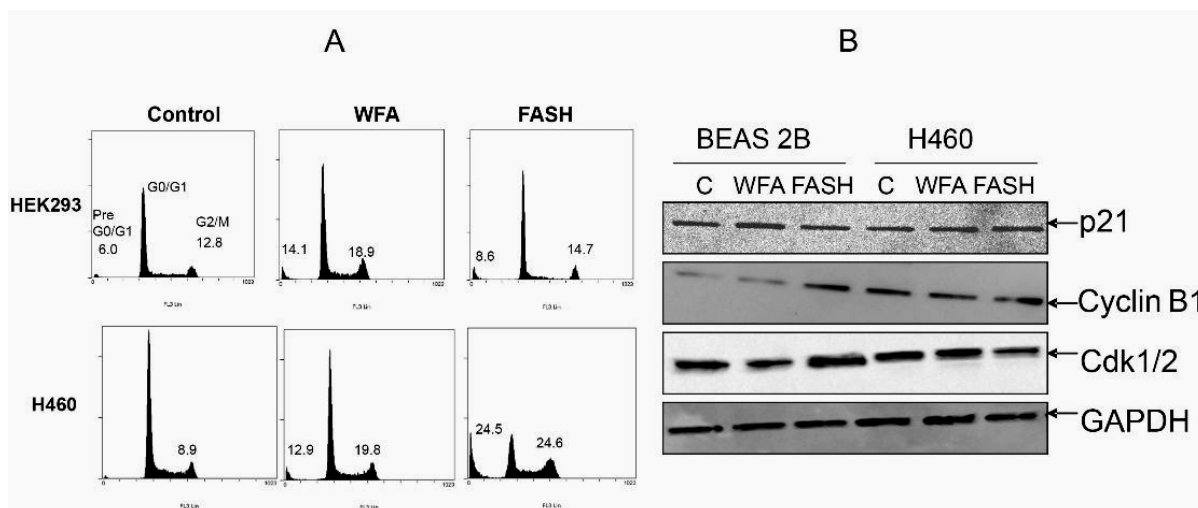


Figure 4. (A) FACS analysis of HEK 293 and H460 cells showing the effect of WFA and FASH on cell cycle: 1×10^5 cells were plated in 150mm petri plates and incubated overnight at 37°C . Cells were treated with WFA ($0.5 \mu\text{M}$) and FASH ($0.5 \mu\text{M}$ WFA + 40mg/ml ASH) for 24h, and then subjected to flow cytometry analysis using propidium iodide as a fluorescent DNA stain (see Methods section for details). Representative histograms of flow analysis of two separate experiments giving similar results are shown. (B) Western blots showing the effects of WFA and FASH on the expression of cell cycle related proteins in NSCLC and normal cells. H460 and BEAS 2B cells were treated with WFA ($0.5 \mu\text{M}$) and FASH ($0.5 \mu\text{M}$ WFA+ASH 40mg/ml) for 24h. After completion of treatment, cells were washed with cold PBS (2x), scraped and centrifuged. Cell pellets were suspended in cold RIPA buffer containing protease inhibitor cocktail and briefly sonicated on ice. After incubating for 30 min on ice, the RIPA lysates were centrifuged at 12000 rpm for 10min and supernatants were collected. Supernatants containing $50\mu\text{g}$ of protein were resolved on 4-20% gels and immunoblotted on nitrocellulose membrane. After washing with TBS and blocking in 5% milk, the immunoblots were incubated separately with primary antibodies (1:500) against p21, cyclin B1, and Cdk1/2 overnight. Blots were then washed and incubated with their respective HRP-conjugated secondary antibodies (1: 1000) for 2h. The bands on immunoblots were detected by Chemiluminescence reagent. GAPDH was used as protein loading control.

cell types (Koduru et al., 2010; Malik et al., 2007; Mandal et al. 2008; Srinivansan et al., 2007). Consistent with these studies, Western blot analysis data presented in Figure 5B indicated PARP cleavage in WFA-treated normal and cancer cells. While there was no significant difference in the cleavage product of PARP in WFA- and FASH-treated H460 cells, these effects were not observed in FASH-treated BEAS 2B cells. Furthermore, in the event of toxic insult by pro-oxidants, the proapoptotic BH3-only protein Bim activates Bax leading to an increase in the mitochondrial membrane permeability and release of cytochrome C which ultimately induces the activation of caspase3 (Stan et al., 2008a). Bim has been shown to be expressed in cells mainly in three subunits Bim-EL (25kDa), Bim-L (20kDa) and Bim-s (15kDa). Earlier studies (Stan et al., 2008b) have shown that the WFA-induced apoptosis of MCF-7 cells is accompanied by the induction of Bim-s and Bim-L isoforms. Results of the Western blot analyses presented in Figure 5B indicated that while treatment with WFA and FASH in H460 cells significantly activated the Bim-L and Bim-s subunits, expression of these subunits was inhibited in FASH-treated BEAS 2B cells. Consistent with this, treatment with FASH also upregulated the expression of the anti-apoptotic protein MCI-1, a member of the BCL2 family of proteins (Adams and Cory, 2007), as well as inhibited the release of cyto- chrome c in normal cells (Figure 5C and 5 D). Togat-

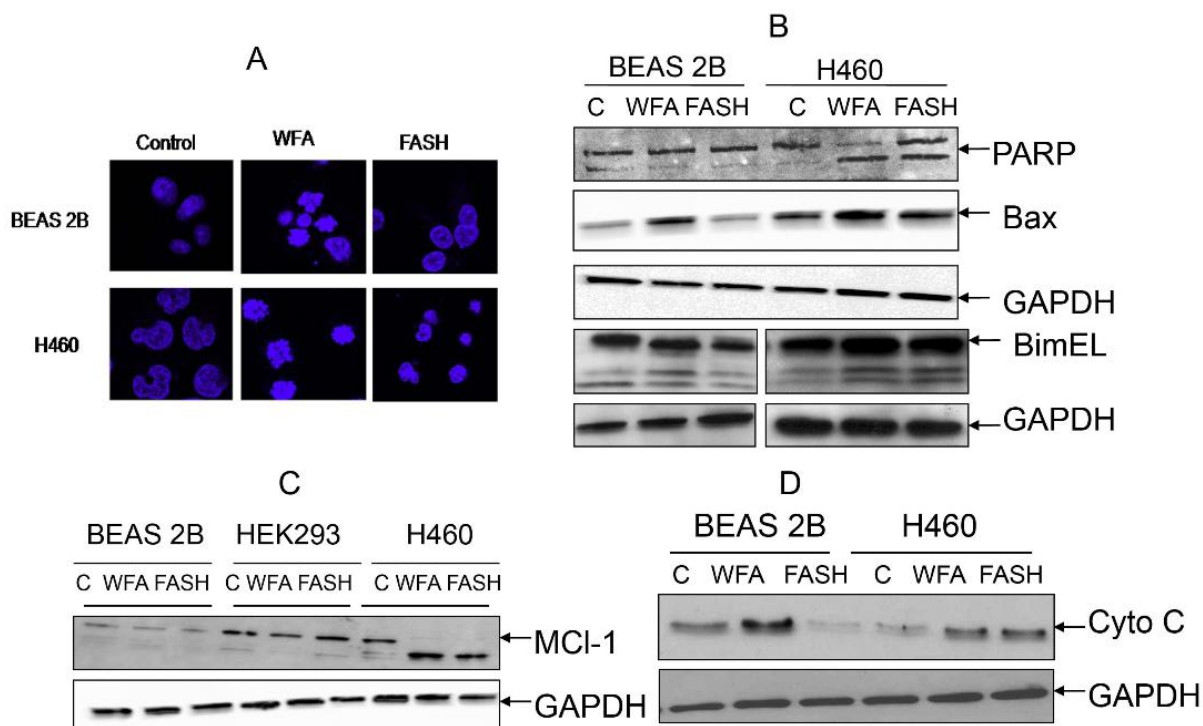


Figure 5. (A) Analyses of apoptosis in WFA and FASH treated NSCLC and normal cells by confocal microscopy: H460 and BEAS 2B cells were grown on cover slips and treated with WFA (0.5 μ M) and FASH (0.5 μ M WFA +40mg/ml ASH) for 24h. Cells were fixed with 4% paraformaldehyde for 20min. washed with PBS (2x) and cover slips were mounted with Vectashield medium containing DAPI nuclear stain. Confocal microscopic examination of the slides was carried out as described in the Methods section. (B-D) Western blot analyses of apoptosis related proteins in WFA and FASH treated NSCLC and normal cells: H460, BEAS 2B and HEK 293 cells were treated with WFA (0.5 μ M) and FASH (0.5 μ M WFA +40mg/ml ASH) and processed in a similar manner as described in the Figure legend 4B. Immunoblots were incubated separately with primary antibodies against PARP (1:500), Bax (1:1000), Bim (1:500), Mcl-1 (1:500) cytochrome C (1:1000) overnight. Blots were washed with TBS and then incubated separately with respective HRP-conjugated secondary antibodies (1:1000) for 2h. The bands on immunoblots were detected by Chemiluminescence reagent. GAPDH was used as protein loading control.

her, these findings support our conclusions that FASH differentially affected the expression and functions of proteins involved in apoptotic signaling in normal and cancer cells.

FASH selectively promotes survival of normal cells via activation of EGFR and its downstream anti-apoptotic proteins AKT/ERK

One of the suggested protective signaling mechanisms from the harmful effects of ROS and ensuing LPO have been shown to be mediated by the activation and phosphorylation of the epidermal growth factor receptor (EGFR) and its downstream signaling proteins such as protein kinase B (AKT/PKB) and the mitogen activated protein kinase (MAPK), extracellular regulatory kinases1/2 (ERK1/2) (Chen et al., 2001; Klein and Levitzki, 2009; Lonardo et al., 2002). To examine the mechanisms of the differential effects

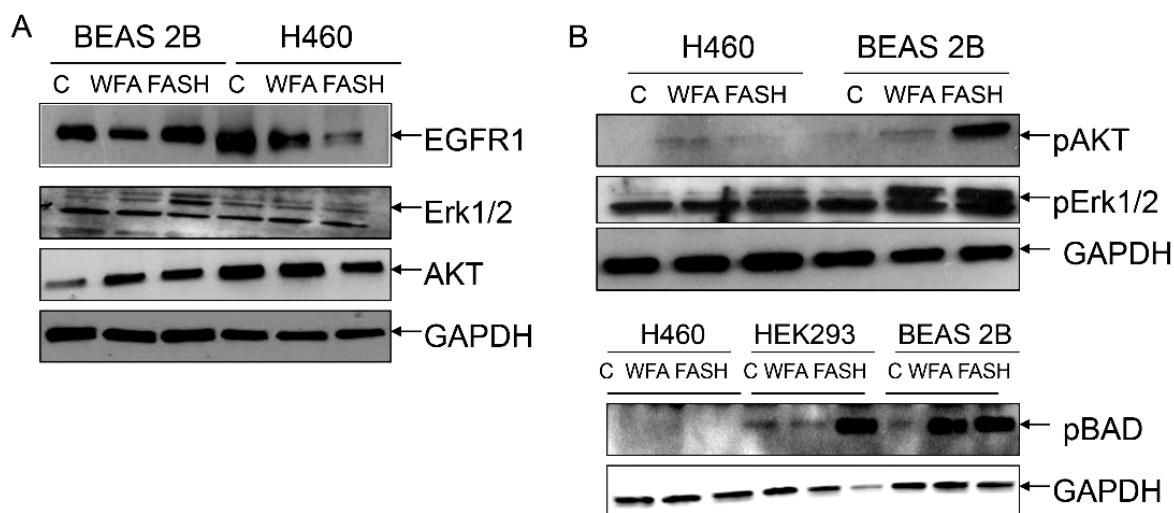


Figure 6. Effect of WFA and FASH on the expression of EGFR, AKT, ERK1/2 (A), and phosphorylation of AKT, ERK1/2 and BAD (B) in NSCLC and normal cells: H460, BEAS 2B and HEK 293 cells were treated and processed for the Western blot analyses as described in the legend for Figure 4B. Immunoblots were probed separately for the expression of EGFR, AKT, and ERK1/2 by using specific commercially procured antibodies against these proteins. Phosphorylation status of AKT, ERK1/2, and BAD was analyzed in control and treated cell extracts by probing the immunoblots separately with phospho protein specific antibodies procured for each protein, incubated overnight. Blots were washed with TBS and then incubated separately with respective HRP-conjugated secondary antibodies (1:1000) for 2h. The bands on immunoblots were detected by Chemiluminescence reagent. GAPDH was used as protein loading control.

of FASH on NSCLC and normal cells, we analyzed the expression of EGFR2 (a predominantly expressed isoform of EGFR family of receptors in lung cells), AKT and ERK in WFA- and FASH-treated H460, BEAS 2B and HEK293 cells. Western blot analysis data in Figure 6A and B showed induction of EGFR2, activation and phosphorylation of AKT (pAKT473) in FASH-treated normal BEAS 2B and HEK293 cells. Recently, it has been shown that pro-survival signaling mediated by AKT/PKB is associated with the phosphorylation of proapoptotic BAD protein at Ser136 by AKT resulting in the inhibition of its binding with the BCL2 family of pro-apoptotic proteins (Datta et al., 1991). Therefore, we next analyzed the phosphorylation of BAD in WFA- and FASH-treated H460, HEK293 and BEAS 2B cells. Immunoblot data presented in Figure 6B indicated that similar to FASH-induced phosphorylation of AKT, there was a robust phosphorylation of BAD in HEK293 and BEAS 2B cells. Furthermore, exposure of normal cells to FASH also caused the activation and phosphorylation of ERK1/2 (Figure 6B), a downstream target of EGFR-PI3K-AKT pathway. These results indicated that FASH selectively promoted EGFR/AKT/ERK1/2 mediated anti-apoptotic signaling in normal cells.

Discussion

WFA isolated from the root and leaf extracts of ASH has shown promising anti-tumor properties against a variety of cancer cell types (Devi et al., 1995; Devi and Kamath., 2003; Fuska et al., 1984; Jayaprakasam et al., 2003; Malik et al., 2007; Matsuda et al., 2001; Shohat and Joshua., 1971; Shohat et al., 1978). However, as observed with several other cancer

chemotherapeutic agents, purified WFA is also potentially toxic to the normal, healthy cells. In contrast, there have been no reported incidents of toxicity (acute or chronic) when patients were treated with the crude ASH extract. This suggests that the toxic effects of WFA on normal cells may perhaps be inhibited by other protective constituents of the extract that appear to exhibit significant antioxidant effects. In view of this, during present studies, we tested the hypothesis that the toxicity of WFA on normal cells can be alleviated by using the ASH extract fortified with specific optimized proportions of WFA (FASH) that will selectively sensitize cancer cells through a differential modulation of redox signaling.

Results showing the potentiation of cytotoxic and cytostatic effects of WFA in FASH-treated NSCLC cells, and the attenuation of these effects in normal cells supported our hypothesis that FASH should selectively chemosensitize NSCLC with little or no toxic effect on normal cells. Recent findings demonstrating the selective antitumor effects of the alcoholic ASH leaf extract and the identification of an inhibitory factor (i-factor) withanone in the ASH extract also supported these results (Widodo et al., 2007; Widodo et al., 2009).

Irrespective of the cause, an increased generation of ROS is accompanied by cellular toxicity, ensuing LPO and significant changes in the redox status of the treated cells (Malik et al., 2007). Compared to the normal counterpart, tumor tissues generally have elevated levels of GSH and/or GSH-linked antioxidant enzymes associated with their intrinsic radiation and drug resistance that plays a major role in determining the susceptibility of normal and cancer cells to different therapeutic agents (Meister and Anderson., 1983). Consistent with this, the cytotoxic effects of WFA on normal and NSCLC were found to be associated with an enhanced LPO, depletion of GSH and alteration in the activities of several detoxification enzymes like GPx, GR and γ -GCS. In contrast to this, FASH treatment significantly attenuated these effects of WFA in the normal cells indicating a differential antioxidant response of FASH in normal and NSCLC cells.

At the molecular level, cellular systems respond to oxidative stress-induced DNA damage by 1) inhibiting cell cycle progression, 2) inducing cell apoptosis when damage is irreparable, 3) strengthening the cellular antioxidant scavenging systems by activating the antioxidant response element (ARE)-driven genes by the transcription factor, Nrf2 (Kensler et al., 2007; Mc Walter et al., 2004). Results of the present studies provide evidence that while WFA caused G2/M arrest in both HEK293 and H460 cells, FASH treatment significantly attenuated this effect of WFA in HEK293. FASH also differentially affected the expression of the cell cycle related proteins, cyclin B1, cdk1/2 and p21 in BEAS 2B and H460 cells indicating a selective cytostatic effect of FASH on NSCLC. Furthermore, consistent with the previous studies (Mandal et al., 2008; Oh et al., 2008; Srinivasan et al., 2007) WFA induced apoptosis in NSCLC and normal cells via the mitochondrial- mediated intrinsic apoptotic pathway involving the activation of Bim and Bax, release of cytochrome C and PARP cleavage. However, these apoptotic effects of WFA were significantly inhibited in FASH-treated normal cells indicating a differential effect of FASH on the apoptotic signaling cascade of normal and cancer cells.

Several studies have indicated the importance of EGFR-mediated proliferative signaling in lung cancer (Klein and Levitzki, 2009; Lonardo et al., 2002). To further ascertain the mechanisms of differential effects of FASH on normal and cancer cells, the effects of

WFA and FASH on the EGFR-signaling pathway were analyzed in NSCLC and normal cells. Generally, in response to external stimulus, EGFR is activated through binding to its ligand EGF and transmits signals to the downstream anti-apoptotic proteins, AKT and ERK1/2 (Chen et al., 2001; Mitsiades et al., 2004). AKT enhances the survival of cells by blocking the function of pro-apoptotic proteins and processes. Upon phosphorylation, AKT directly phosphorylates and inhibits the BH3-only pro-apoptotic protein BAD (Datta et al., 1991). One of the important findings of our present investigations is the increased phosphorylation of AKT and BAD at S136 (serine 136) in FASH-treated normal BEAS 2B and HEK 293 cells when compared to similarly treated H460 cells. These effects of FASH on BEAS 2B cells were also found to be associated with the activation of EGFR and enhanced phosphorylation of ERK1/2. Together, these results indicated that the EGFR-mediated pro survival signaling pathway was selectively activated in FASH-treated normal cells. In summary, to the best of our knowledge, this is the first report demonstrating that the toxic effects of WFA on normal cells can be ameliorated by using a WFA-based combination regimen of ASH to selectively achieve synergistic antitumor activities in NSCLC cells with little or no untoward effect on normal cells. We are currently pursuing further studies to analyze the active principles of the ASH extract exhibiting differential effects on normal and cancer cells and believe that these findings will be relevant to improve the design and efficacy of targeted herb-based therapies.

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