

Research Article

Notch-1 Inhibition by Withaferin-A: A Therapeutic Target against Colon Carcinogenesis

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Abstract

Notch signaling plays a crucial role in the development of colon cancer; targeting the Notch pathway may sensitize colon cancers to various adjuvant agents. The focus of our current study is to identify natural compounds that target Notch signaling and that might be beneficial for the prevention and treatment of colon cancer. Withaferin-A (WA) is a bioactive compound derived from *Withania somnifera*, which inhibits Notch-1 signaling and downregulates prosurvival pathways, such as Akt/NF- κ B/Bcl-2, in three colon cancer cell lines (HCT-116, SW-480, and SW-620). In addition, WA downregulated the expression of mammalian target of rapamycin signaling components, pS6K and p4E-BP1, and activated c-Jun-NH₂-kinase-mediated apoptosis in colon cancer cells. We also established the molecular link between Notch/Akt/mammalian target of rapamycin signaling by complementary approaches (i.e., overexpression of Notch-1 or inhibition of Notch-1 by small interfering RNA). Our results suggest that WA inhibits Notch-mediated prosurvival signaling, which facilitates c-Jun-NH₂-kinase-mediated apoptosis in colon cancer cell lines. These results underscore the anticancer activity of WA, which exhibits potential for further development for targeted chemotherapy and/or chemoprevention strategies in the context of colon cancer. *Mol Cancer Ther*; 9(1); 202–210. ©2010 AACR.

Introduction

Over the past several years, therapeutic options for patients with colon cancer have increased substantially due to earlier diagnosis and more effective chemotherapeutic agents. However, efforts to better understand the biological basis for colon cancer progression and identifying novel agents that target specific signaling pathways may provide a better therapeutic option for patients with colon cancer.

Notch signaling has also been considered as an oncogene involved in the pathogenesis of colorectal cancer (1–3). Previous studies revealed deregulated Notch signaling in several solid human tumors including colon cancers. Thus far, four Notch genes have been identified (*Notch-1*, *Notch-2*, *Notch-3*, and *Notch-4*) and five Notch ligands (Dll-1, Dll-3, Dll-4, Jagged-1, and Jagged-2) have been found in mammals. These molecules play important roles in regulating cell fate decisions (4). Activation of the Notch pathway occurs when specific ligands such as Jagged-1 (JAG-1) or Δ -like-3 (DLL3) bind to four related transmembrane Notch receptors, which bind and activate the γ -secretase protein complex. This complex cleaves the Notch-1 receptor in the transmembrane domain to release the cytoplasmic portion, known as the Notch-1 intracellu-

lar domain (N^{ICD}/Truncated/activated-Notch; refs. 5–7). γ -Secretase is a complex of proteins that has not yet been fully characterized (8) but minimally consists of four subunits: Presenilin-1, Presenilin-2, Nicastrin, and Anterior Pharynx-defective-1 (9). Presenilin-1 and Presenilin-2 catalyze the intramembrane cleavage of integral membrane proteins such as Notch receptors, but the other members of the γ -secretase complex are required for protease activity (8, 9). Activated Notch-1 translocates to the nucleus and forms a ternary complex with a highly conserved transcription factor, CBF1/Suppressor of Hairless/Lag1 and coactivators of the mastermind-like family (5). This complex activates target gene transcription, including Hes-1 and Hey-1 (10, 11). Multiple oncogenic pathways, such as mitogen-activated protein kinase, Akt, NF- κ B, matrix metalloproteinases, and mammalian target of rapamycin (mTOR) signaling have been reported to engage in crosstalk with Notch signaling. Therefore, it is believed that this signaling collectively plays an important role in tumor aggressiveness in colon cancer (12, 13).

Withaferin-A is a bioactive compound isolated from the medicinal plant *Withania somnifera*, which has been safely used for centuries in the practice of Indian Ayurvedic medicine for the treatment of various ailments, including cancer and inflammatory conditions (14, 15). Because Notch-1 is one of the major causative factors of inflammatory diseases (16) and also *Withania somnifera* is a widely used for anti-inflammatory, we investigated whether withaferin-A inhibits Notch and its associated signaling, which might cause growth arrest in colon cancer cells. This study provides the evidence that withaferin-A exerts anticancer effects by downregulating Notch and its crosstalk signaling (Akt/NF- κ B/mTOR), which resulted in the

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inhibition of colon cancer survival. On the other hand, withaferin-A induces c-Jun-NH₂-kinase (JNK)-mediated apoptosis in colon cancer cells without any significant effect on normal colon epithelial (FHC) cells.

Materials and Methods

Cell Lines and Reagents

Human colon cancer cell lines (HCT-116, SW-480, and SW-620) and a normal colon epithelial cell line (FHC) were purchased from the American Type Culture Collection. HCT-116 and SW-480 cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and antibiotics in the presence of 5% CO₂ at 37°C in an incubator. SW-620 cells were grown in Leibovitz's L-15 Medium (American Type Culture Collection) in the absence of CO₂ (tightly capped) at 37°C in the incubator. The FHC cells were grown in Ham's F12 medium (45%) and DMEM (45%), which contains 25 mmol/L HEPES, 10 ng/mL cholera toxin, 0.005 mg/mL insulin, 0.005 mg/mL transferrin, 100 ng/mL hydrocortisone, and 10% fetal bovine serum. Commercially available high performance liquid chromatography-grade withaferin-A was purchased from the Chromadex. The plasmids pCMV-ICN1-GFP obtained from Addgene contains cleaved (active form) Notch (NICD). The small interfering RNA (siRNA) oligonucleotide duplexes for human Notch-1 or scrambled control (nontargeting siRNA) were obtained from Dharmacon, Inc.

Transient Transfections

HCT-116 and SW-620 cells were transiently transfected with Notch-GFP plasmid using the TransIT transfection reagent from the Mirus. After 48 h of transfection, the cells were treated with either DMSO or withaferin-A for 12 h and whole-cell lysates were extracted for Western blot analysis.

siRNA Knockdown Assay

Notch1 siGENOME SMARTpool (Dharmacon) was used to knock down Notch-1 protein expression in HCT-116 and SW-620 cells. Briefly, cells were grown in six-well dishes and Notch-1 siRNA was transfected using DharmaFECT-1 transfection reagent (Dharmacon) according to the manufacturer's recommendations. As a transfection control, cells were transfected with control siRNA (nontarget) from the Dharmacon reagent. Whole-cell lysates were prepared and subjected to Western blot analysis.

Western Blot Analysis

HCT-116, SW620 (5 μmol/L), and SW 480 (4 μmol/L) cells were treated with withaferin-A based on the IC₅₀ values obtained from our cell viability assays for various time intervals. Whole-cell lysates were obtained and subjected to Western blot analysis using the following antibodies: Presenilin-1, Presenilin-2, and Nicastrin (purchased from GeneScript), Notch-1 (Cleaved or

NID), Hes-1, Hey-1, Akt, pAkt (Ser⁴⁷³), S6K, pS6K (Thr³⁹⁸), 4E-BP1, p4E-BP1 (Thr⁷⁰), c-Jun, p-c-Jun, JNK-1, pMEK-3/6, extracellular signal-regulated kinase (ERK), pERK, IκB kinase (IKK)-α, IκB, Bcl-2, pHistone H3, and p65-NF-κB (from Santa Cruz Biotechnology); poly ADP ribose polymerase and cleaved caspase-3 were from Cell Signaling Technology. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-mouse, and anti-rabbit secondary antibodies were acquired from Santa Cruz Biotechnology.

Cell Viability and Apoptotic Assays

Colon cancer cells (HCT-116, SW-480, and SW-620) were treated with withaferin-A or with a vehicle (DMSO) for 24 h. Trypan blue dye exclusion or MTT assays for cell viability (17) and apoptotic assay (Annexin V-FITC) were performed on HCT-116, SW-480, and SW-620 cell lines as described earlier (18).

Statistical Analysis

All the experiments were performed thrice to ascertain the reproducibility of the results. The data shown are representative of three experiments. The ANOVA was used to calculate statistical significance between samples.

Results

Withaferin-A Negatively Regulates Notch-1 Activation in Colon Cancer Cells

Notch signaling is known to suppress apoptosis and promote cell proliferation/survival pathways in colon cancer cells (13, 19). We explored whether withaferin-A targets Notch-1 signaling in colon cancer cells (HCT-116, SW-480, and SW-620). As depicted in Fig. 1A, we observed a gradual time-dependent decrease of cleaved Notch-1 expression in HCT-116 and SW-620 cells, whereas in SW-480 cells, cleaved Notch-1 was drastically reduced after 3 hours of treatment with withaferin-A (4 μmol/L). Next, we investigated whether inhibition of Notch-1 affects the downstream targets Hes-1 and Hey-1, which were also downregulated after 3 hours of treatment with withaferin-A in all three colon cancer cell lines (Fig. 1A). These results suggest that withaferin-A significantly inhibits Notch signaling in colon cancer cells. Next, we investigated whether withaferin-A inhibits γ-secretase (an activator of Notch-1), which in turn downregulates Notch signaling in colon cancer cells. We analyzed the expression of γ-secretase subunits Presenilin-1, Presenilin-2, and Nicastrin in withaferin-A-treated colon cancer cell lines. Our results suggest that withaferin-A fails to inhibit γ-secretase subunits in all three cell lines, implying that withaferin-A may directly inhibit Notch signaling in colon cancer cells (Fig. 1B). To determine the transcriptional regulation of Notch-1 by withaferin-A, we performed reverse transcription-PCR analysis. Our results suggest that withaferin-A downregulates Notch-1

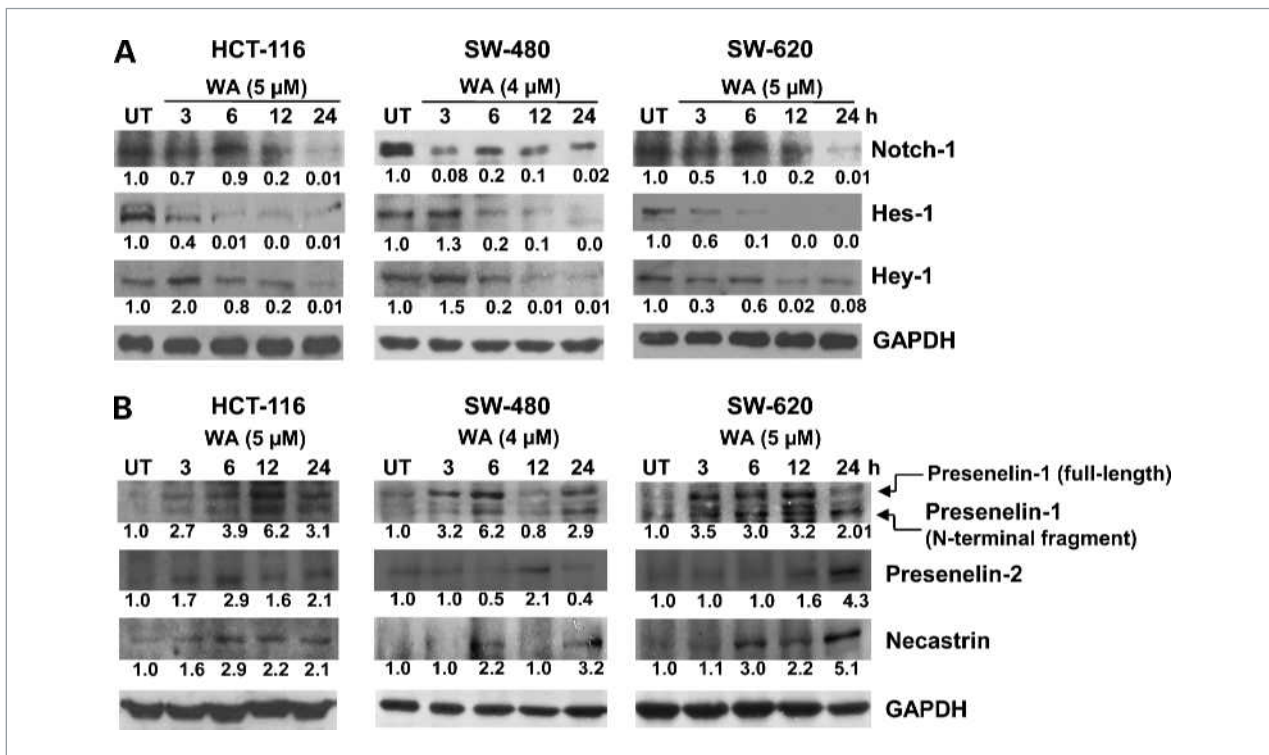


Figure 1. Withaferin-A inhibits Notch signaling in colon cancer cells. HCT-116, SW-480, and SW-620 cells were treated with either vehicle control (DMSO) or withaferin-A (WA) for varying time intervals. Cell lysates were subjected to Western blot analysis using (A) Notch-1 (cleaved), Hey-1, and Hes-1 antibodies, and (B) γ -secretase components. GAPDH was used as the internal loading control.

mRNA expression in all three cell lines in a time-dependent manner (data not shown).

Inhibition of Akt Signaling by Withaferin-A in Colon Cancer Cells

It has been shown that activated Notch induces Akt expression in many cancer cell types (20); hence, we investigated whether withaferin-A inhibits Akt phosphorylation in colon cancer cells. We noted a time-dependent inhibition of pAkt, yet did not observe any alterations in total Akt protein levels in HCT-116, SW-480, or SW-620 cells (Fig. 2A). Activated Akt regulates NF- κ B signaling (21); on the other hand, the cross-talk during activation of Notch and NF- κ B in colon cancer cells is well established (22). Because withaferin-A inhibits Notch and Akt activation, we examined whether inhibition of both signaling pathways negates NF- κ B activation in colon cancer cells. We observed that expression of the NF- κ B p65 subunit was downregulated by withaferin-A in a time-dependent manner in HCT-116, SW-480, and SW-620 cells (Fig. 2B). On the other hand, total I κ B- α protein level increased in a time-dependent manner with withaferin-A treatment (Fig. 2B), suggesting that withaferin-A is capable of maintaining I κ B- α in the unphosphorylated form, thereby retaining the active NF- κ B dimers in the cytosol. Furthermore, when withaferin-A modulates upstream IKK- α activity, this phosphorylates I κ B- α and releases NF- κ B subunits. As depicted in Fig. 2B, treatment

with withaferin-A downregulated IKK- α in all three colon cancer cell lines. Finally, we examined whether inhibition of Notch-1, Akt, and NF- κ B affects major survival factor Bcl-2 in colon cancer cells. As expected, withaferin-A downregulated Bcl-2 levels (Fig. 2C), suggesting that withaferin-A significantly inhibits prosurvival molecules in colon cancer cells.

Effect of Withaferin-A on mTOR Signaling in Colon Cancer Cells

Interestingly, mTOR has recently been shown to mediate Notch in survival signaling (23). The relationship between Notch and mTOR signaling has not yet been fully established. On the other hand, Akt-mediated mTOR regulation in colon cancer cells is well established (24). We noted that treatment with withaferin-A downregulated the phosphorylation of p70-S6K in SW-480 cells when compared with HCT-116 and SW-620 cells (Fig. 3A); however, withaferin-A significantly downregulated phosphorylation of 4E-BP1 in HCT-116 and SW-620 when compared with SW480 (Fig. 3A). These results suggest that withaferin-A inhibits Notch and its cross-talk with mTOR signaling in colon cancer cells.

Activation of c-Jun and JNK Signaling in Colon Cancer Cells

Cross-talk among intracellular signaling pathways is important for the regulation of cell fate decisions and

cellular responses to extracellular signals. Both Notch and mitogen-activated protein kinase pathways play important roles in many biological processes, and the Notch pathway has been shown to interact with the ERK and JNK pathways (25, 26). Activated Notch-1 negatively regulates c-Jun and JNK in many cancer types, thereby inhibiting apoptosis (26). Therefore, we sought to determine the role of withaferin-A in JNK signaling in withaferin-A-treated colon cancer cells. As seen in Fig. 3B, significant increases in phosphorylated c-Jun (active) and JNK were observed in HCT-116, SW-480, and SW-620 cells. Next, we determined the effect of withaferin-A on ERK signaling in colon cancer cells. Phosphorylated ERK1/2 was upregulated in a timely manner in all three cell lines, without alterations to total ERK protein levels (data not shown). We also observed an induction of apoptotic markers, such as caspase-3 and poly ADP ribose polymerase cleavage in HCT-116, SW-480, and SW-620 cells, suggesting that JNK activation would have induced apoptosis in colon cancer cells (Fig. 3C).

Notch-1 Regulates Akt/mTOR Signaling in Colon Cancer Cells

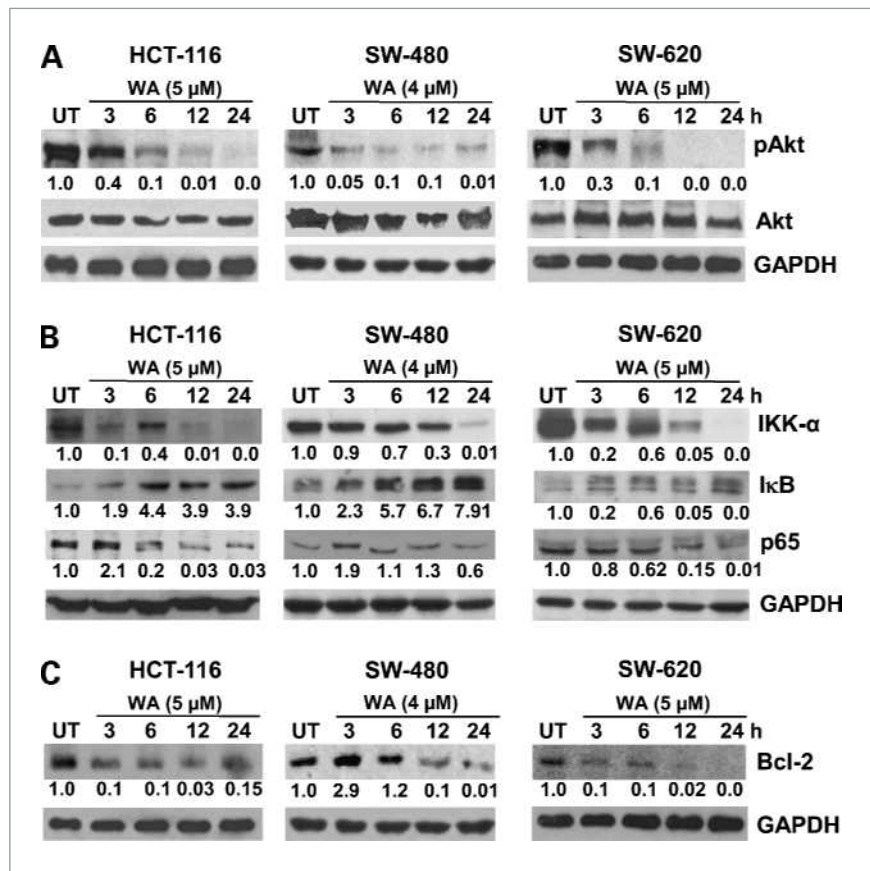
To establish whether Notch-1 regulates Akt and mTOR signaling in colon cancer cells, we either overexpressed

full-length Notch-1 or specifically inhibited Notch-1 by siRNA in colon cancer cells. Our results showed that an overexpression of Notch-1 increased the expression levels of its downstream targets, Hey-1 and Hes-1, in both colon cancer cell lines (Fig. 4A). In addition, there was also an increase in the expression of pAkt and mTOR (p-p70S6K and p-4E-BP1) in both HCT-116 and SW-620 (Fig. 4A). On the other hand, inhibition of Notch using siRNA Notch-1 significantly reduced the expression of Hey-1, Hes-1, pAkt, and mTOR (p-p70-S6K and p4E-BP1) in both HCT-116 and SW-620 cells (Fig. 4B). These results imply that Notch-1 regulates Akt/mTOR signaling in colon cancer cells. As seen in Fig. 4A, we also observed that withaferin-A overcomes Notch-mediated resistance by downregulating the expression of pAkt, p-p70-S6K, and p4E-BP1 in colon cancer cells overexpressing Notch-1. These results establish the link between Notch-Akt-mTOR signaling in colon cancer cells.

Withaferin-A Inhibits Cell Viability and Induces Apoptosis in Colon Cancer Cells *In vitro*

Molecular kinetic studies revealed that withaferin-A downregulates cell proliferation/survival signaling; therefore, we determined the effect of withaferin-A treatment on colon cancer cell survival/proliferation

Figure 2. Withaferin-A inhibits Akt/NF- κ B/ Bcl-2 signaling in colon cancer cells. **A**, **B**, and **C**, HCT-116, SW-480, and SW-620 cells were treated with either vehicle control (DMSO) or withaferin-A for varying time intervals, and cell lysates were subjected to Western blot analysis using the indicated antibodies. GAPDH was used as the internal loading control.



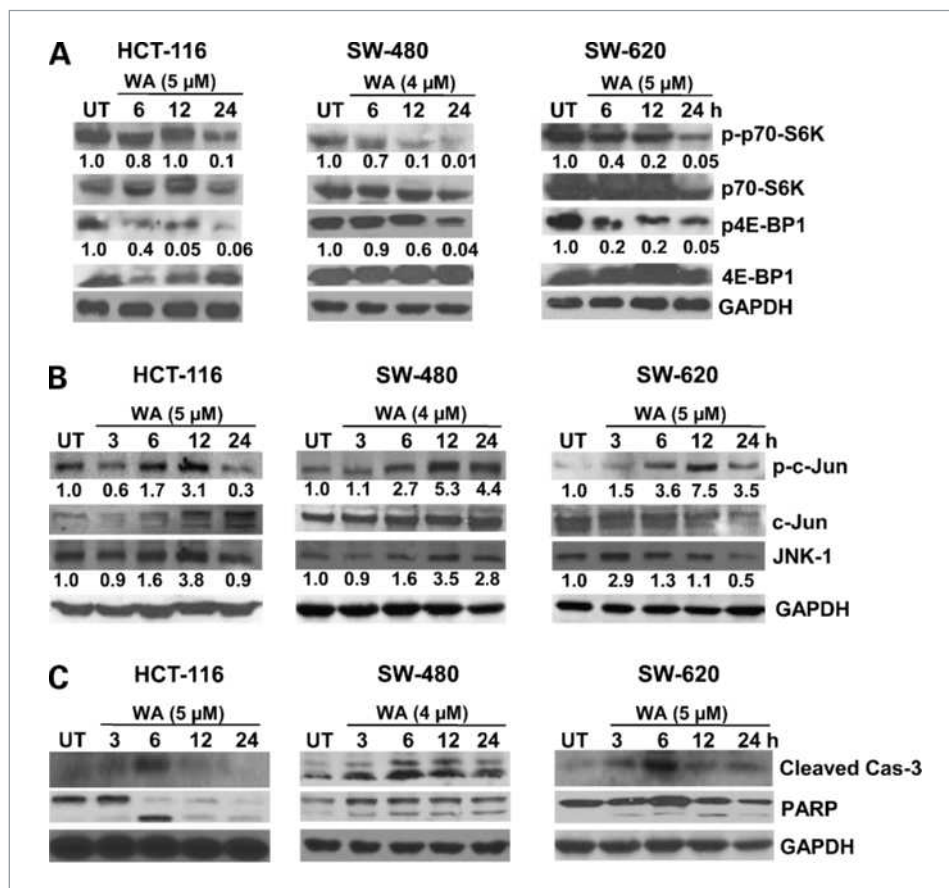


Figure 3. Withaferin-A regulates mTOR, ERK, and JNK pathways in colon cancer cells, leading to the induction of apoptosis. **A**, **B**, and **C**, HCT-116, SW-480, and SW-620 cells were treated with either vehicle control (DMSO) or withaferin-A for varying time intervals, and cell lysates were subjected to Western blot analysis using the indicated antibodies. GAPDH was used as loading control.

and apoptosis induction. The colon cancer cells were treated with various (2–10 μ mol/L) concentrations of withaferin-A for 24 hours, and cell viability was quantified by trypan blue exclusion/MTT assays. Our results indicate that withaferin-A inhibited cell viability in all three colon cancer cell lines in a dose-dependent manner. However, SW-480 (IC₅₀, 3.56 μ mol/L) cells were more sensitive to withaferin-A treatment when compared with SW-620 (IC₅₀, 5.0 μ mol/L) and HCT-116 (IC₅₀, 5.33 μ mol/L) cell lines. In addition, we also tested the toxicity of withaferin-A on normal colon epithelial cells (FHC), which shows no significant effect on FHC cells (Fig. 5A). Similarly, apoptotic assays (using Annexin V-FITC by flow cytometric analysis) were done in colon cancer cell lines following treatment with withaferin-A (4, 6, and 8 μ mol/L). Our results suggest that withaferin-A induced a significant amount of apoptosis in all three colon cancer cell lines (Fig. 5B). These results suggest that withaferin-A is a potent anticancer drug, which we can use as a therapeutic option for the treatment of colon cancer.

Discussion

Notch-1 is overexpressed during colon cancer progression and governs many important functions includ-

ing cell proliferation/differentiation and survival. A recent study reported the overexpression of Notch-1 (77%) and its downstream targets Hey-1 and Hes-1 in human colon cancer (27, 28). There are several groups working on γ -secretase inhibitors that inhibit Notch-1 activation, which represents an effective treatment for sarcoma (29), medulloblastoma (30), breast cancer (31), and colon cancer (32). In our studies, we show for the first time that withaferin-A, a dietary compound, targets and inhibits Notch signaling without altering upstream events, such as activity mediated by the γ -secretase family of proteins (Presenilin-1, Presenilin-2, and Nicastrin), which induces apoptosis in three colon cancer cell lines. Our results showed that withaferin-A inhibits Notch cleavage and downstream activation mediated by Hey-1 and Hes-1 in the HCT-116, SW-480, and SW-620 cell lines. However, it is not clear whether withaferin-A binds to the Notch receptor, which in turn reduces the γ -secretase binding efficiency in colon cancer cells. More studies are required to dissect these molecular events in colon cancer cells.

Activation of Akt by Notch has been shown in many cancer models (23, 33, 34), and Akt activation plays a crucial role in the initiation and progression of colon cancer metastasis (35). In the present study, withaferin-A inhibited Akt activation in colon cancer cells, suggesting it

might be due to the inhibition of Notch-1. To establish the link between Notch and Akt, we either overexpressed Notch-1 or inhibited Notch-1 using Notch siRNA in colon cancer cells. Ectopic expression of Notch-1 induced pAkt expression; on the other hand, inhibition of Notch-1 downregulates Akt activation, which implies that Notch-1 regulates Akt activation in colon cancer cells. Recently, Meurette et al. (36) showed that Notch induced an autocrine signaling loop that activated Akt in breast epithelial cells. Inhibition of Notch-1 caused Akt inhibition, resulting in the induction of apoptosis in breast epithelial cells (36). These results corroborate with our results that Notch-1 may regulate Akt activation and inhibition of Notch-1 may also inhibit Akt-mediated signaling in colon cancer cells.

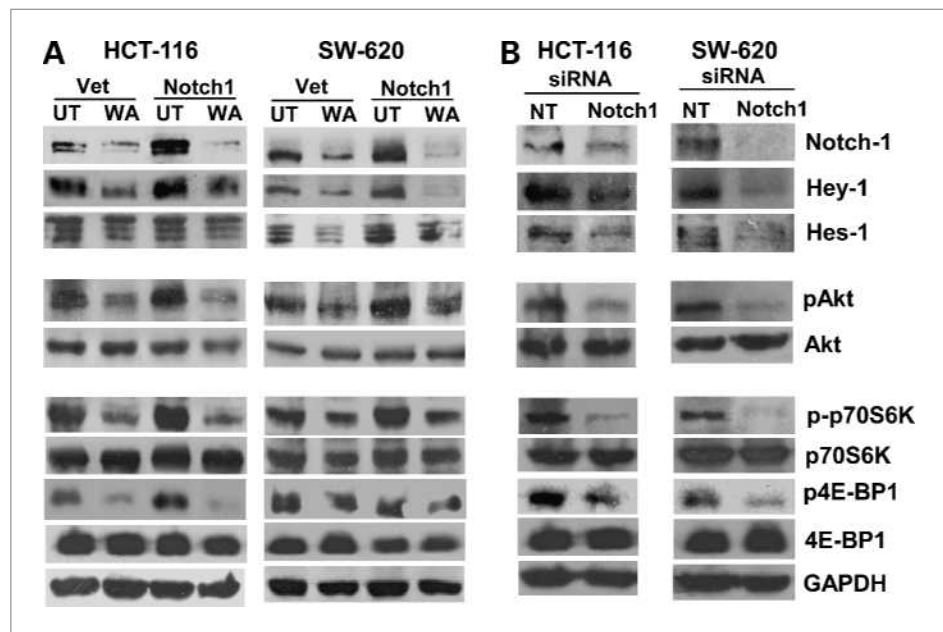
The transcription factor NF- κ B, downstream of Akt, is activated in colon cancer cells. Inhibition of NF- κ B markedly sensitizes colon cancer cells to apoptosis (37). On the other hand, Notch inhibits NF- κ B activation by modulating the recombination signal binding protein J κ (38). So inhibition of both Notch-1 and Akt may inhibit NF- κ B signaling in colon cancer cells. To confirm this hypothesis, we examined NF- κ B signaling. Our results suggest inhibition of IKK- α and upregulation of I κ B result in reduced nuclear translocation of p65 protein in colon cancer cells. IKK is a protein kinase complex responsible for I κ B phosphorylation in response to proinflammatory stimuli, resulting in ubiquitination and degradation. IKK is a multisubunit complex that contains two catalytic subunits, IKK- α and IKK- β , and a regulatory subunit, IKK- γ /NEMO (NF- κ B essential modulator). Recent published results indicate that withaferin-A might inhibit tumor necrosis factor-induced NF- κ B activation by blocking the activity of IKK- β kinase *in vitro* (39). On

the other hand, IKK- α and IKK- β are known to regulate mTOR activation (40, 41) in an Akt-dependent and Akt-independent fashion in many cancer types. In our studies, withaferin-A seems to inhibit activation of both IKK- α and IKK- β , resulting in decreased NF- κ B activity in many cell types including colon cancer cells. In addition, downregulation of Bcl-2 expression in all three colon cancer cell lines and the consequent overexpression imparts survival advantages to cancer cells (42). Downregulation of Bcl-2 may increase the sensitivity of the cell to chemotherapeutic drugs and radiation (43, 44). Thus, therapeutic strategies directed toward inhibition of NF- κ B and Bcl-2 activation may have great clinical importance. We found that withaferin-A downregulates Notch-1/Akt/NF- κ B/Bcl-2 protein expression in all three colon cancer cell lines, suggesting that withaferin-A may prove to be a potent therapeutic agent for colon cancer.

We observed the inhibition of mTOR components pS6K and p4E-BP1 in all three colon cancer cell lines. Although the molecular link between Notch and mTOR remains to be clarified, few published reports suggest that Notch regulates mTOR in both an Akt-dependent and Akt-independent manner in T-ALL. For example, γ -secretase inhibitor (GSI) treatment effectively suppresses mTOR activation in T-ALL, suggesting a possible molecular link between these two pathways (45). Withaferin-A inactivates Notch-1 and Akt, which might result in the inhibition of mTOR in colon cancer cells. The expression pattern of mTOR in colon cancer specimens is not known; however, it is well established that mTOR is overexpressed or is aberrant in other cancer types. Furthermore, inhibition of mTOR by rapamycin significantly inhibited tumor growth in prostate, breast, and T-ALL (46–48). The chemotherapeutic efficacy of rapamycin is

Figure 4. Knockdown or overexpression of Notch-1 significantly modulates Akt/mTOR signaling in colon cancer cells.

A, full-length Notch-1 was overexpressed in HCT-116 and SW-620 cells followed by treatment with vehicle or DMSO, and cell lysates were subjected to Western blot analysis using the indicated antibodies. **B**, HCT-116 and SW-620 cells were transfected with siRNA and cell lysates were subjected to Western blot analysis; GAPDH was used as a loading control.



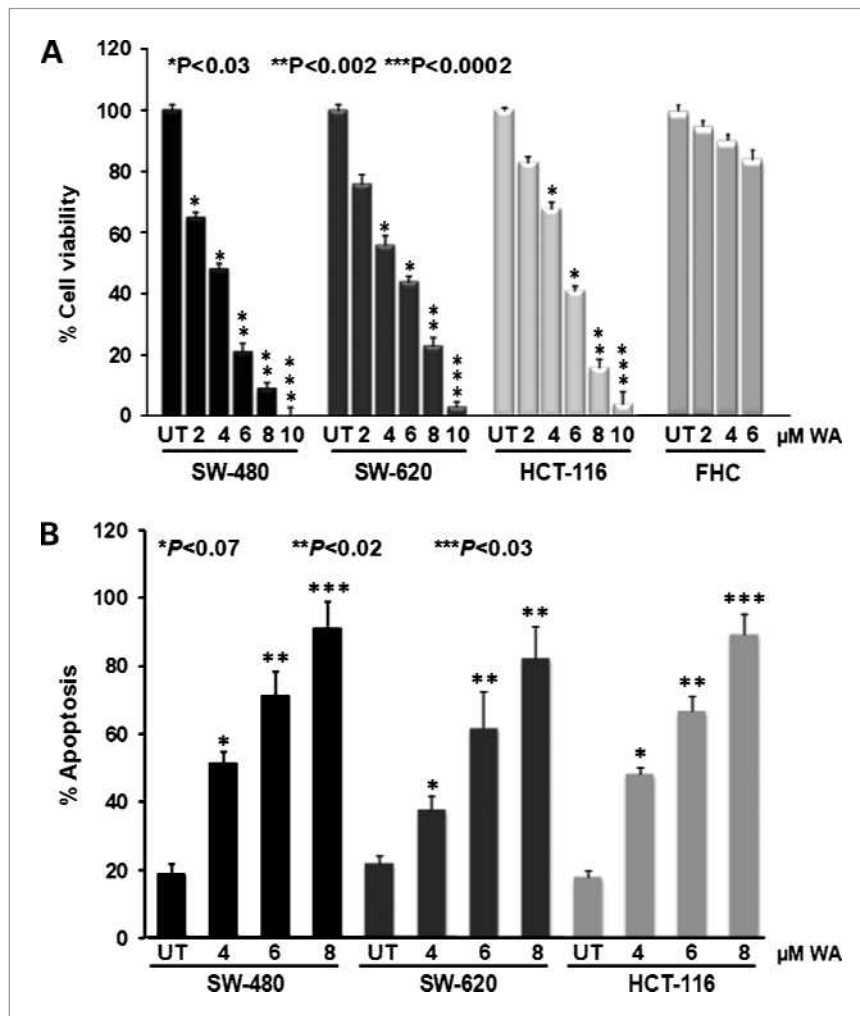


Figure 5. Withaferin-A inhibits cell viability and induces apoptosis in colon cancer cells. **A**, HCT-116, SW-480, SW-620, and FHC cells were treated with varying concentrations of withaferin-A for 24 h. Trypan blue exclusion assay was performed. *Columns*, mean of four wells from three independent experiments; *bars*, SEM. **B**, apoptotic assays were done using Annexin V-FITC staining. *Columns*, mean from three independent experiments; *bars*, SEM.

controversial, as there are reports that rapamycin-treated cells showed increased Akt expression, as mediated by a feedback mechanism. Thus, some studies have concluded that simultaneous inhibition of both Akt and mTOR is essential for an effective therapeutic strategy (18, 49). We believe that withaferin-A could be a better therapeutic compound because it inhibits Notch/Akt/NF- κ B/mTOR-mediated prosurvival signaling in colon cancer cells.

To elucidate the mechanisms by which withaferin-A induces apoptosis, we examined the potential contributions of ERK and JNK signaling in colon cancer cells. ERKs and JNKs (stress-activated protein kinases) are members of the mitogen-activated protein kinase super family. In general, JNKs are activated by stress and inflammatory signals, which induce apoptosis and inhibit cell growth (50). Our results revealed an induction of pJNK and pc-Jun in all three colon cancer cell lines, suggesting the possible role of JNK-mediated proapoptotic signaling induced by withaferin-A. In addition, apoptotic

markers such as cleaved caspase-3 and poly ADP ribose polymerase cleavage confirmed the induction of withaferin-A-mediated apoptosis in our cell lines. Furthermore, our cell viability and apoptotic studies suggest that withaferin-A exerts a potent chemotherapeutic effect on colon cancer cells. Interestingly, no significant toxicities were observed in normal colon epithelial cells (FHC), which might suggest that withaferin-A targets only cancer cells. We believe that the inhibition of Notch-mediated prosurvival signaling could play a major role in the induction of apoptosis in colon cancer cells in response to withaferin-A treatment.

In summary, this study shows that direct modulation of Notch/Akt/NF- κ B signaling activity by withaferin-A could provide the molecular basis for apoptosis induction in colon cancer cells. Considering the pivotal role of Notch/Akt signaling in the pathogenesis of human colon cancer, these findings may have significant clinical relevance, and withaferin-A could be developed as an agent for the management of colon cancer. However, further

studies are warranted to fully dissect the mechanism of action of withaferin-A in colon cancer models, as well as to validate our *in vitro* findings in an *in vivo* xenograft model.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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