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Axl is a novel target of withaferin A in the induction of apoptosis and the suppression of invasion

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ABSTRACT

Withaferin A, a withanolide derived from the medicinal plant *Withania somnifera*, has been reported to exhibit anti-tumorigenic activity against various cancer cells. In this study, we show that withaferin A inhibits the constitutive and recombinant human growth-arrest-specific protein 6 (rhGas6)-induced phosphorylation of Axl and STAT3. In addition, withaferin A also induces the down-regulation of Axl protein expression in a lysosome-dependent manner and inhibits rhGas6-induced wound healing and cell migration. Furthermore, the overexpression of Axl attenuates withaferin A-induced apoptosis. Taken together, the data from the present study indicate that the withaferin A-mediated down-regulation of the Gas6/Axl signaling pathway mediates the inhibition of cell migration and the induction of apoptosis.

1. Introduction

Axl is a member of the TAM (Tyro-Axl-Mer) receptor tyrosine kinase (RTK) family and is identified as a transforming gene in chronic myeloid leukemia [1]. The ligand for Axl, namely growth arrest-specific 6 (Gas6), is a vitamin K-dependent protein that binds Axl with high affinity [2]. The binding of Gas6 and Axl induces activation of the Gas6/Axl signal pathway in multiple cellular functions, including cell proliferation and migration [3,4]. The Axl receptor is overexpressed in cancers, is correlated with multidrug resistance and contributes to tumorigenesis by regulating migration and tumor growth [3–7]. Furthermore, increased levels of Axl protein have been observed in renal cell carcinoma compared with the normal kidney pair [8]. Inhibition of the Axl receptor kinase promotes apoptosis, blocks growth and enhances the chemosensitivity of human non-small cell lung cancer [7]. For these reasons, the identification of novel inhibitory compounds of Axl is an area of active interest in the cancer research community.

Withaferin A exhibits the most anti-cancer activity among structurally divergent withanolides purified from the Indian medicinal plant *Withania somnifera* [9]. Withaferin A induces apoptosis through several different mechanisms, including reactive oxygen species (ROS) generation [10], Par-4 induction [11], proteasome inhibition [12], stabilization of p53 [13], induction of endoplasmic reticulum (ER) stress [14], down-regulation of Akt phosphorylation [15] and p38 MAP kinase activation [16]. We also found that withaferin A induces apoptosis through down-regulation of the JAK/STAT3 signaling pathway [17]. However, the cellular and molecular mechanisms underlying withaferin A-mediated migration and apoptosis are not fully understood.

In this study, we investigated whether withaferin A can modulate the constitutive and rhGas6-inducible Axl pathways in Caki cells, leading to the induction of apoptosis and the suppression of migration. The present study demonstrates that withaferin A-induced apoptosis is associated, at least in part, with the Gas6/Axl-mediated down-regulation of the STAT3 signaling pathway.

2. Materials and methods

2.1. Cells and materials

Caki and A498 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 20 mM HEPES buffer, and 100 μg/ml gentamicin.

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Anti-phospho-STAT3 (Tyr705), anti-STAT3, and anti-Axl antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Axl and anti-PARP antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-actin was purchased from Sigma–Aldrich (St. Louis, MO, USA). Withaferin A was purchased from ENZO (Enzo Biochem Inc., NY, USA). Recombinant Gas6 and the receptor tyrosine kinase array were purchased from R&D Systems (Minneapolis, MN, USA). All of the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Human phospho-receptor tyrosine kinase (RTK) array

Caki cells were plated at 0.2×10^6 cells/ml in 12-well plates and treated with 4 μM withaferin A. After treatment, a human phospho-RTK array was performed according to the manufacturer's instructions. Briefly, the cells were lysed on ice in lysis buffer, and 200 μg of the lysates was incubated overnight with the blocked array membranes. After washing, the cytokines were detected by a chemoluminescence reaction.

2.3. Western blot analysis

The cells were washed with cold PBS and lysed on ice in modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na_3VO_4, and 1 mM NaF) containing protease inhibitors (100 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 mM EDTA). The lysates were centrifuged at 10,000×*g* and 4 °C for 10 min, and the supernatant fractions were collected. The proteins were separated by SDS–PAGE and transferred to an Immobilon-P membrane. The specific proteins were detected using an enhanced chemiluminescence (ECL) Western blotting kit according to the manufacturer's instructions.

2.4. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

The total RNA was isolated as previously described using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA). Single-strand cDNA was synthesized from 2 μg of total RNA using MMLV (Moloney Murine leukemia virus) reverse transcriptase (Gibco-BRL, Gaithersburg, MD, USA). The cDNAs for Axl and actin were amplified by PCR with specific primers. The sequences of the forward and reverse primers were the following: Axl (forward) 5′-TGA ACA GCT TGG GCA TCA GT-3′ and (reverse) 5′-TTC ATG CAG ACC GCT TCA CT-3′; actin (forward) 5′-GGC ATC GTC ACC AAC TGG GAC-3′ and (reverse) 5′-CGA TTT CCC GCT CGG CCG TGG-3′. The PCR amplification was conducted using the following cycling conditions: 94 °C for 3 min, followed by 17 (actin) and 30 (Axl) cycles of 94 °C for 45 s, 55 °C for 40 s, and 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplified products were separated by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide.

2.5. Wound healing assay

A wound healing assay was performed to detect Caki cell migration. Briefly, Caki cells were grown to full confluency in 12-well plates and incubated overnight in starvation medium. The cell monolayers were wounded with a sterile 100-μl pipette tip and washed with starvation medium to remove the detached cells from the plates. The cells were left either untreated or treated with rhGas6 in the presence or absence of withaferin A and maintained in a CO_2 incubator for 24 h. After 24 h, the medium was replaced with phosphate-buffered saline (PBS) buffer, the wound gap was observed, and the cells were photographed using a Carl Zeiss microscope. The area of the wound was quantified using the public domain JAVA image-processing program ImageJ (http://rsb.info.nih.gov/ij) with the polygon selection mode. The migration of the cells was expressed as the percentage of wound closure [18].

2.6. Migration assay

The migration capacity of Caki cells was determined *in vitro* using Boyden chambers (6.5-mm diameter filters, 8-μm pore size). Briefly, Caki cells (1×10^5 cells/200 μl of serum-reduced medium) were placed in the upper chamber of Boyden chambers. The test agents were added to the lower chamber (200 μl), and the upper chamber contained the medium alone. The chambers were assembled and maintained in an incubator for 24 h. At the desired time point, the cells from the upper surface of the Millipore membranes were removed with gentle swabbing, and the migrant cells on the lower surface of the membranes were fixed and stained with crystal violet. The membranes were then washed and mounted onto glass slides. The membranes were examined microscopically, and the cellular migration per sample was determined by counting the number of stained cells in at least four to five randomly selected fields visualized with a Carl Zeiss microscope (Jena, Germany). The data are presented as the means of the migrating cells ± SD/microscopic field per sample. Each cell migration experiment was repeated at least three times.

2.7. Flow cytometry analysis

The cells were suspended in 100 μl of phosphate-buffered saline (PBS), and 200 μl of 95% ethanol was added during a vortexing step. The cells were incubated at 4 °C for 1 h, washed with PBS and resuspended in 250 μl of 1.12% sodium citrate buffer (pH 8.4) with 12.5 μg of RNase. The incubation was continued at 37 °C for 30 min. The cellular DNA was then stained by applying 250 μl of propidium iodide (50 μg/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting on a FACScan flow cytometer to determine the relative DNA content based on red fluorescence.

2.8. Transfection

Caki cells were seeded at a density of 0.2×10^6 cells/well in six-well culture plates the day before transfection to achieve 50–60% confluence. The Axl plasmid or an empty vector was transfected into cells using the Lipofectamine 2000 reagent. Axl (plasmid number: 20428), which was deposited by Thomas M. Roberts, was purchased from Addgene (Cambridge, MA, USA) [19].

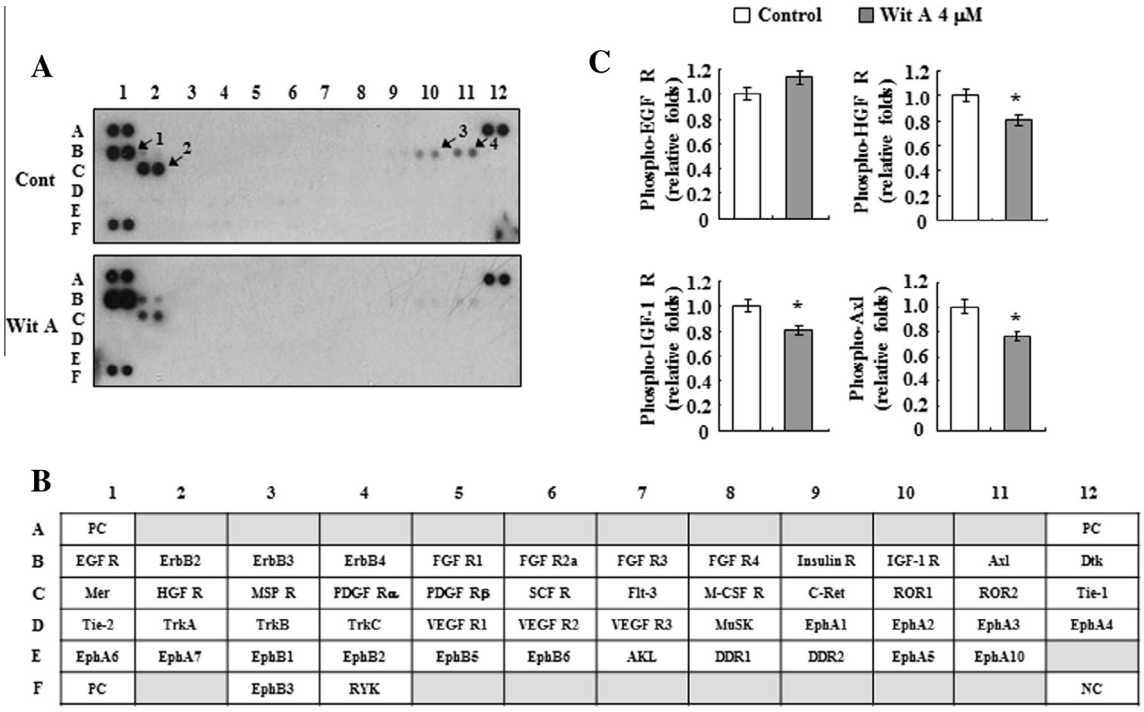
2.9. Statistical analysis

The data were analyzed by one-way ANOVA followed by post hoc comparisons (Student–Newman–Keuls) using the Statistical Package for Social Sciences 8.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Effect of withaferin A on the phosphorylation levels of receptor tyrosine kinases in Caki cells

We previously reported that withaferin A inhibits STAT3 signaling and induces apoptosis [17]. To identify the factors associated with STAT3 phosphorylation, we determined the inhibitory levels of receptor tyrosine kinase in withaferin A-treated cells using a phospho-receptor tyrosine kinase array kit (Fig. 1A and B). The phosphorylation levels of HGFR, IGF-1R and Axl were inhibited



B

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC											PC
B	EGF R	ErbB2	ErbB3	ErbB4	FGF R1	FGF R2a	FGF R3	FGF R4	Insulin R	IGF-1 R	Axl	Dtk
C	Mer	HGF R	MSP R	PDGF Rα	PDGF Rβ	SCF R	Flt-3	M-CSF R	C-Ret	ROR1	ROR2	Tie-1
D	Tie-2	TrkA	TrkB	TrkC	VEGF R1	VEGF R2	VEGF R3	MuSK	EphA1	EphA2	EphA3	EphA4
E	EphA6	EphA7	EphB1	EphB2	EphB5	EphB6	AKL	DDR1	DDR2	EphA5	EphA10	
F	PC		EphB3	RYK								NC

Fig. 1. Effects of withaferin A on levels of phosphorylated receptor tyrosine kinase in Caki cells. (A) Caki cells were treated with or without 4 μM withaferin A for 30 min. A receptor tyrosine kinase array performed as described in Section 2. The signals were detected with HRP-conjugated streptavidin and ECL. (B) The map of the receptor tyrosine kinase array kit can be used to identify individual receptor tyrosine kinases. (C) The represented graph shows the actual pixel densities measured using the Image J program. The values for the pixels are the means ± S.D. $^{*}p < 0.05$ compared with the control. The data represent three independent experiments.

by withaferin A treatment. Unexpectedly, the phosphorylation levels of EGFR were increased in withaferin A-treated cells (Fig. 1C). We selected the Axl receptor tyrosine kinase for further studies because it has been previously reported that Gas6 (growth-arrest-specific protein 6; Axl ligand)-mediated STAT3 activation plays a pivotal role in mesangial cell proliferation [20]. To determine whether withaferin A inhibits Axl activation, we incubated Caki cells with 4 μM withaferin A for the indicated time points. We examined the activation of Axl by Western blot analysis using an antibody that recognizes the phosphorylation of Axl at tyrosine 702. As shown in Fig. 2A and B, withaferin A inhibited constitutive Axl and STAT3 phosphorylation of Caki and A498 cells in a time- dependent manner. In addition, to determine whether withaferin A inhibits inducible Axl and STAT3 activation, Caki cells were starved for 24 h and then treated with 200 ng/ml rhGas6 in the presence or absence of withaferin A (4 μM). Gas6 treatment induced an increase in the phosphorylated Axl and STAT3 levels. Withaferin A decreased the levels of Gas6-induced phosphorylation of Axl and STAT3 (Fig. 2C). These results demonstrate that withaferin A inhibits both constitutive and rhGas6-induced phosphorylation of Axl and STAT3.

3.2. Withaferin A also induces the down-regulation of Axl expression at the post-translational level

Although withaferin A inhibited Axl phosphorylation within 15 min (Fig. 2), we found that Caki cells treated with withaferin A for 24 h exhibited the down-regulation of Axl expression in a dose-dependent manner (Fig. 3A). Therefore, we investigated how withaferin A inhibits Axl protein expression. As shown in Fig. 3A, the Axl mRNA levels remained constant in the presence of withaferin A. Therefore, these data suggest that the withaferin A-mediated down-regulation of Axl is regulated at the post-transcriptional levels. To further clarify the underlying mechanisms of the

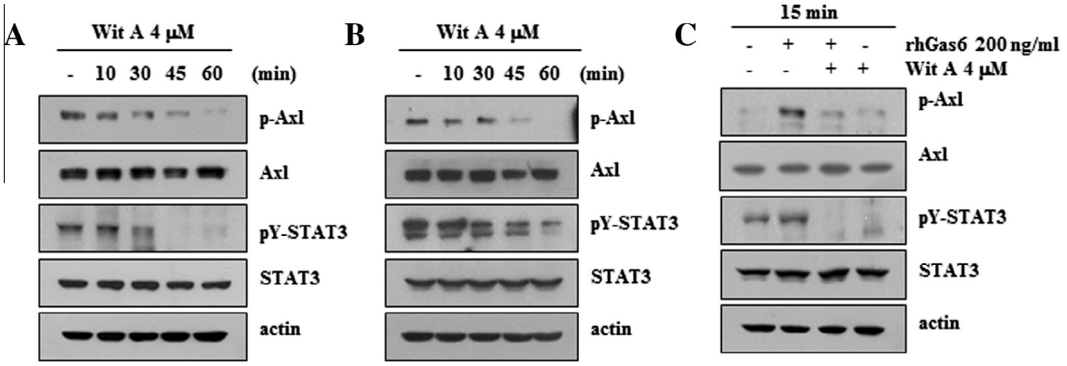


Fig. 2. Withaferin A inhibits phosphorylation of Axl and STAT3. (A and B) Caki and A498 cells were treated with 4 μM withaferin A for the indicated time points. (C) Caki cells were treated with rhGas6 (200 ng/ml) in the presence or absence of withaferin A for 15 min. Equal amounts of cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot to determine the levels of phospho-Axl (Tyr702), phospho-STAT3 (Tyr705), Axl, and STAT3 using actin as a control for protein loading.

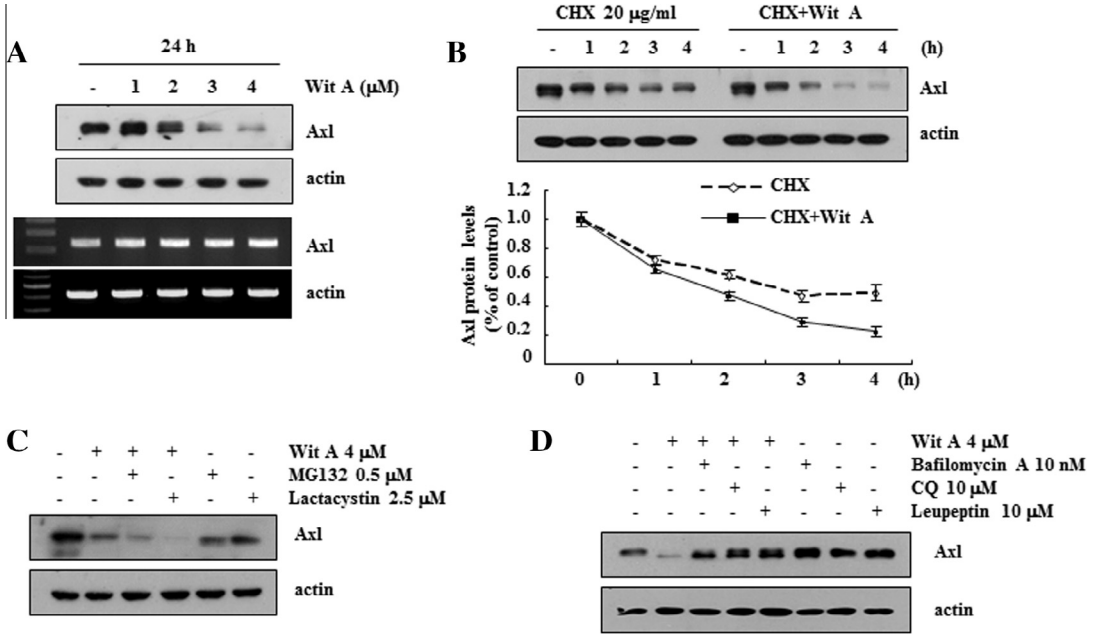


Fig. 3. Withaferin A induces the down-regulation of Axl protein expression in a lysosome-dependent manner. (A) Caki cells were treated for 24 h with the indicated concentrations of withaferin A. The protein and mRNA expression levels of Axl were determined by Western blotting and RT-PCR, respectively. (B) Caki cells were treated with 20 μg/ml cycloheximide (CHX) in the presence or absence of 4 μM withaferin A for the indicated time points. A Western blot analysis was performed as described in Section 2. The band intensities reflecting the level of Axl protein expression were measured using the public domain JAVA image-processing program ImageJ (http://rsb.info.nih.gov/ij). (C) Caki cells were treated with MG132 (0.5 μM) or lactacystin (2.5 μM) in the presence or absence of 4 μM withaferin A for 24 h. (D) Caki cells were treated with chloroquine (10 μM), bafilomycin A (10 nM) or leupeptin (10 μM) in the presence or absence of 4 μM withaferin A for 24 h. Equal amounts of the cell lysates (40 μg) were subjected to electrophoresis and analyzed by Western blot to determine the Axl level using actin as a control for protein loading (C and D).

down-regulation of Axl in withaferin A-treated cells, we performed a Axl protein stability assay. Caki cells were treated with cyclohexamide (CHX), an inhibitor of de novo protein synthesis, or CHX plus withaferin A. We found that the degradation of Axl protein was facilitated by withaferin A treatment (Fig. 3B). We then examined whether the withaferin A-induced Axl down-regulation is associated with the proteasome–ubiquitin system. As shown in Fig. 3C, proteasome inhibitors (MG132 and lactacystin) did not block the down-regulation of Axl expression. The delivery of proteins from the plasma membrane to the lysosome for degradation is critical to normal cellular function [21]. To investigate the effect of lysosomal degradation on withaferin A-induced Axl down-regulation, we used an inhibitor of lysosome function or lysosomal enzyme. As shown in Fig. 3D, chloroquine (CQ; a lysosomotropic agent), bafilomycin A (vacuolar ATPase inhibitor) and leupeptin (lysosomal enzyme cathepsin inhibitor) blocked the down-regulation of Axl. These results suggest that the down-regulation of Axl expression due to the induction of lysosomal degradation.

3.3. Withaferin A inhibits Gas6/Axl-mediated tumor cell migration and apoptosis

Gas6/Axl signaling is well known to enhance migration in various tumor cells [4]. We examined whether withaferin A suppresses the stimulatory effect of rhGas6 on Caki cell migration using a wound healing assay. As shown in Fig. 4A and B, rhGas6 increased the migration of Caki cells, and their migration capacity was markedly reduced by withaferin A pre-treatment. We have further confirmed the inhibitory effect of withaferin A on rhGas6-induced Caki cell migration through a Boyden chamber assay.

The treatment of Caki cells with rhGas6 resulted in a marked increase in cell migration compared with cells that were not treated with rhGas6. The rhGas6-induced migration was significantly prevented by 81.4% ($P < 0.05$) after treatment with withaferin A (2 μM) for 24 h (Fig. 4C). In addition, withaferin A inhibited the basal migration capacity of A498 cells by 35.4% ($P < 0.05$) (Fig. 4D). These results suggest that withaferin A has the ability to inhibit migration.

To investigate the relationship between Axl expression and apoptosis, we assessed whether the overexpression of Axl could rescue withaferin A-induced apoptosis. Caki cells were transfected with the Axl plasmid for 24 h. The cells were then treated with withaferin A for 18 h, and we examined the sub-G1 population. As shown in Fig. 4E, the overexpression of Axl markedly reduced the withaferin A-induced sub-G1 phase population. We then measured the expression levels of Axl and PARP cleavage. PARP cleavage was inhibited by the ectopic expression of Axl (Fig. 4F). Taken together, these results indicate that the Axl pathway plays a role in regulating the withaferin A-induced apoptosis of Caki cells.

4. Discussion

We have previously shown that withaferin A inhibits JAK/STAT3 signaling and induces apoptosis [17]. However, the RTK (receptor tyrosine kinase) signal pathway of STAT3 activation involved in withaferin A-induced apoptosis is not well established. In this study, we provide important evidence that suggests that withaferin A inhibits the Gas6/Axl pathway and induces apoptosis in Caki cells. This view is supported by the following findings: (a) withaferin A inhibits both constitutive and inducible Axl activation; (b) withaferin A effectively down-regulates the expression levels of Axl in a lysosome-dependent manner; and (c) withaferin A suppresses rhGas6-induced Caki cell migration and induces apoptosis.

The up-regulation of the Gas6/Axl pathway is evident in a number of chronic pathological conditions [4]. Furthermore, the activation of Axl protects cells from apoptosis and increases migration and angiogenesis through multiple signaling pathways

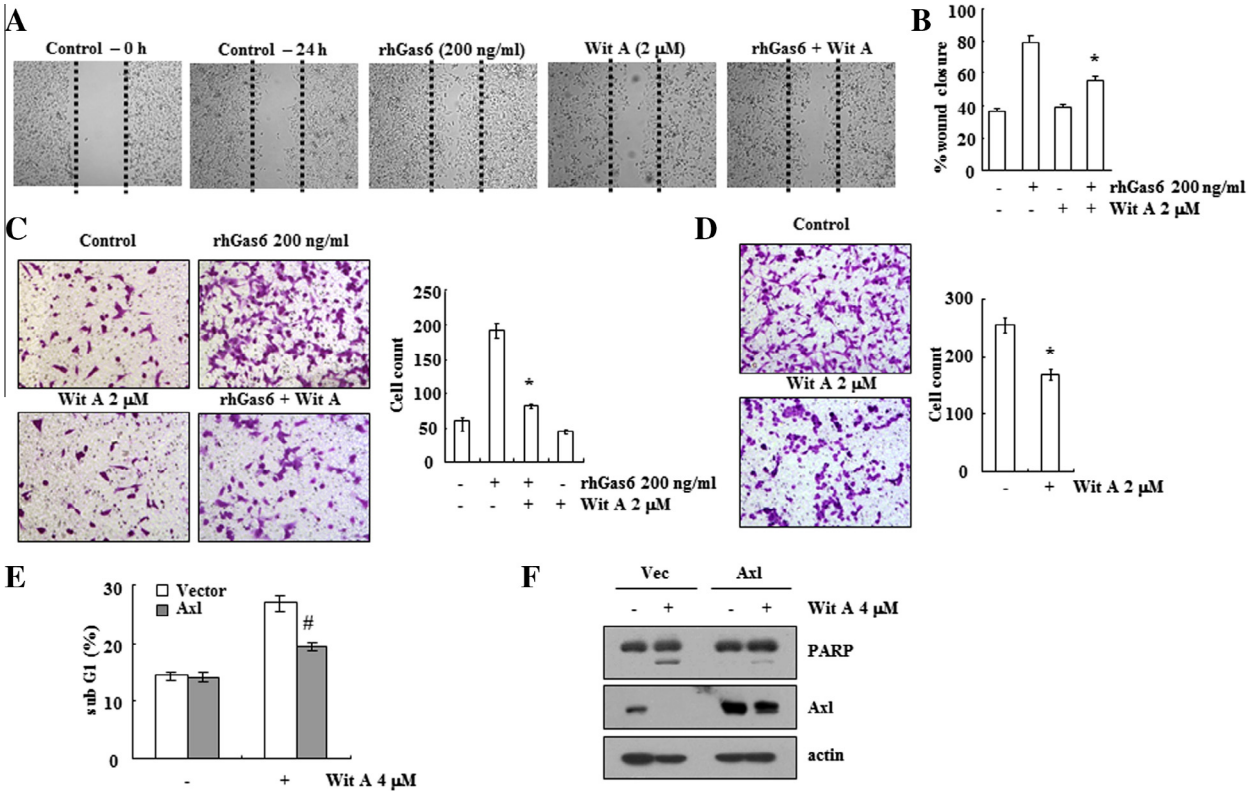


Fig. 4. Withaferin A inhibits cell migration and induces apoptosis through down-regulation of Gas6/Axl signaling. (A and B) A wound healing assay was performed to assess the effect of withaferin A on the migration of Caki cells. Caki cells were pretreated with withaferin A (2 μM) 30 min prior to treatment with rhGas6 (200 ng/ml), and the assay was performed 24 h after rhGas6 treatment. The assay was repeated three times, and representative pictures are shown (A). The effects of withaferin A on rhGAS6-induced migration are represented as percentages of wound closure. The results are expressed as the means ± SD (B). (C) Caki cells were pretreated with withaferin A (2 μM) for 30 min and then treated with rhGas6 (200 ng/ml) for 24 h, and the migrating cells were counted. The cell migration was determined using a Boyden Chamber. The migrating cells were counted, and the results are expressed as the mean numbers of migratory cells ± SD/microscopic field. (D) A498 cells were treated with withaferin A (2 μM) for 24 h, and the migrating cells were counted. The cell migration was determined using a Boyden Chamber. The migrating cells were counted, and the results are expressed as the mean numbers of migratory cells ± SD/microscopic field. (E and F) Caki cells were transfected with the Axl expression plasmid. Twenty-four hours after transfection, the cells were treated with 4 μM withaferin A for 18 h. The level of apoptosis was analyzed using the sub-G1 fraction measured by FACS (E). Equal amounts of the cell lysates (40 μg) were resolved by SDS–PAGE gel electrophoresis, transferred to a membrane and probed with specific anti-PARP, anti-Axl or anti-actin antibodies using actin as the loading control (F).

[4,7]. To investigate the mechanisms of withaferin A-induced RTK inhibition in Caki cells, we utilized a RTK array. Interestingly, the withaferin A-induced inhibition of tyrosine phosphorylation was only observed for HGFR, IGF-1R and Axl (Fig. 1C). We focus on the Axl receptor tyrosine kinase in our further studies. The activation of the Axl kinase was recently identified as one mechanism through which lung cancer can cause resistance to EGFR-target therapy [22]. Yanagita et al. reported that Gas6 stimulates the activation of STAT3 through the Axl receptor tyrosine kinase signaling system [20]. In addition, STAT3 is a key signaling molecule in Gas6-mediated mesangial cell proliferation *in vitro* and *in vivo* [20]. Our results indicate that withaferin A inhibits both constitutive and inducible Axl activation (Fig. 2). The inhibition of Axl activation and the down-regulation of Axl likely contribute to the ability of withaferin A to inhibit STAT3 phosphorylation at Tyr705. We previously reported that withaferin A induces apoptosis via down-regulation of the STAT3 signaling pathway. The down-regulation of STAT3-regulated gene products (Bcl-2, survivin, and Bcl-xL) likely contributes to the ability of withaferin A to induce apoptosis [23,24]. The Gas6/Axl pathway promotes tumor invasion through the epithelial-mesenchymal transition (EMT)-inducing transcription factor Slug. The down-regulation of Axl by siRNA reduces cell migration and invasion in HA227 hepatocellular carcinoma [25]. Furthermore, the rhGas6-induced cell migration was markedly inhibited in withaferin A-treated Caki cells (Fig. 4C).

In conclusion, this study demonstrated that withaferin A induces apoptosis and inhibits cell migration via down-regulation of the Gas6/Axl signaling pathway. A better understanding of the function of withaferin A in the Gas6/Axl/STAT3 pathway may provide a candidate for a cancer chemopreventative or chemotherapeutic agent.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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