Published in final edited form as:
Cancer Lett. 2013 June 28; 334(1): 101–108. doi:10.1016/j.canlet.2012.08.026.

Withaferin A-induced apoptosis in human breast cancer cells is associated with suppression of inhibitor of apoptosis family protein expression

Eun-Ryeong Hahm and **Shivendra V. Singh***
Department of Pharmacology & Chemical Biology, and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

Abstract

The present study provides novel insight into the mechanism of apoptosis induction by withaferin A (WA), which is a bioactive constituent of an Ayurvedic medicine plant (*Withania somnifera*). Exposure of MDA-MB-231 and MCF-7 human breast cancer cells to WA resulted in suppression of XIAP, cIAP-2, and Survivin protein levels. The WA-induced apoptosis was significantly attenuated by ectopic expression of XIAP, Survivin, and cIAP-2 in both cells. However, the WA-mediated inhibition of MDA-MB-231 xenograft growth *in vivo* was associated with suppression of Survivin protein level only. These results indicate important contribution of Survivin suppression in WA-induced apoptosis.

Keywords

Withaferin A; Breast Cancer; Apoptosis; XIAP; Survivin

1. Introduction

Constituents of Ayurvedic medicine, which has been practiced in India for thousands of years for the treatment of different ailments, continue to gain momentum for the discovery of novel anticancer agents [1,2]. *Withania somnifera* is one such medicinal plant capable of eliciting a variety of pharmacological effects in experimental rodents [3,4]. The known pharmacological effects of *Withania somnifera* include cardioprotection from ischemia reperfusion injury [5], inhibition of 6-hydroxydopamine-induced Parkinsonism in rats [6], suppression of hepatic lipid peroxidation concomitant with an increase in activity of antioxidant enzymes in mice [7], anticancer effects [8–11], and inhibition of angiogenesis [12].

Anticancer effect of *Withania somnifera* is attributed to withanolides including withaferin A (WA) [13–18]. For example, WA was shown to cause destruction of Ehrlich ascites tumor cells *in vivo* by causing immune activation [13]. Treatment of mouse melanoma bearing

*Corresponding author. Address: 2.32A Hillman Cancer Center Research Pavilion, 5117 Centre Avenue, Pittsburgh, PA 15213, USA. Tel: +1 412 623 3263; Fax: +1 412 623 7828. singhs@upmc.edu (S.V. Singh).

Conflict: None

mice with WA (10–60 mg/kg body weight, i.p.) resulted in a dose-dependent delay of tumor growth [14]. Oral administration of WA (20 mg/kg body weight) for 14 weeks completely prevented 7,12-dimethylbenz[a]anthracene-induced oral carcinogenesis in hamsters [15]. The WA-mediated growth inhibition of human cancer cells xenografted in athymic mice has also been documented [16–18]. For example, the growth of MDA-MB-231 human breast cancer cells implanted in female athymic mice was retarded significantly by 5 times/week i.p. injections of 4 mg WA/kg [17]. Intra-tumor injections of WA retarded growth of PC-3 human prostate cancer xenografts in athymic mice [16]. Treatment with WA resulted in inhibition of breast cancer cell invasion and metastasis at sub-cytotoxic doses in association with vimentin disassembly and its serine56 phosphorylation [18].

Mechanisms underlying anticancer effects of WA are not fully understood but known cellular responses to WA in cultured cancer cells include G2 phase and mitotic arrest [19], apoptotic cell death [16,17,20,21], and autophagy induction [22]. Autophagy induction seems dispensable for growth inhibitory and proapoptotic effects of WA [22]. On the other hand, the WA-mediated inhibition of cancer cell growth is associated with apoptosis induction *in vitro* as well as *in vivo* [16,17,20,21]. The mechanism by which WA treatment causes apoptosis is still not fully understood, but reactive oxygen species production is intimately linked to the cell death resulting from this agent [23–25]. Notably a normal human mammary epithelial cell line (HMEC) is resistant to WA-induced production of reactive oxygen species as evidenced by fluorescence microscopy using a chemical probe (MitoSOX Red) and electron paramagnetic resonance spectroscopy [23]. Consistent with these observations, WA treatment fails to trigger apoptotic cell death in HMEC [23]. We have also shown previously that even though WA treatment causes stabilization of the p53 protein and promotes its Ser15 phosphorylation, apoptotic cell death resulting from WA treatment is only marginally attenuated by siRNA knockdown of the p53 tumor suppressor protein at least in the MCF-7 cell line [26]. Furthermore, WA has been shown to suppress multiple oncogenic pathways often hyperactive in human cancers including Akt [20,25], nuclear factor-κB [27], signal transducer and activator of transcription 3 [28], and estrogen receptor-α [26]. Inhibition of tumor proteasome activity and angiogenesis by WA has also been reported [29,30].

Inhibitor of apoptosis (IAP) family proteins play critical role in apoptosis regulation by inhibiting caspases [31,32]. The IAPs are often overexpressed in human cancers and contribute to therapy resistance [31,32]. The present study was undertaken to determine the role of IAP family proteins in regulation of WA-induced apoptosis using cultured MDA-MB-231 (an estrogen-independent cell line with mutant p53) and MCF-7 (an estrogen-responsive cell line with wild-type p53) human breast cancer cells, and MDA-MB-231 xenografts from control and WA-treated female athymic mice as models.

2. Materials and methods

2.1. Reagents

WA (purity ≥ 99%) was purchased from Enzo Life Sciences (Farmingdale, NY), dissolved in dimethyl sulfoxide (DMSO), and diluted with complete media immediately before use. The 4’,6-diamidino-2-phenylindole (DAPI), actinomycin D, lactacystin, cycloheximide,

anti-actin antibody, and anti-α-tubulin antibody were purchased from Sigma-Aldrich (St. Louis, MO). The anti-XIAP (X-linked inhibitor of apoptosis) antibody used for immunoblotting and immunofluorescence microscopy was purchased from BD Biosciences (San Jose, CA). Antibodies specific for detection of cIAP-2 (for western blotting), cleaved caspase-3, and cleaved poly-(ADP-ribose)-polymerase (PARP) were purchased from Cell Signaling Technology (Danvers, MA). The anti-cIAP-2 antibody used for immunofluorescence microscopy, the anti-MDM2 antibody used for western blotting, and an antibody detecting both full-length and cleaved PARP (western blotting) were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Survivin antibody was from Novus Biologicals (Littleton, CO). Antibodies against cIAP (recognizes both cIAP-1 and cIAP-2) and XIAP used for immunohistochemistry were purchased from ProSci (Poway, CA) and Abcam (Cambridge, MA), respectively.

2.2. Cell lines, stable transfection, and western blotting

The MDA-MB-231 and MCF-7 cells were purchased from American Type Culture Collection (Manassas, VA) and maintained as described by us previously [17,22,28]. The MDA-MB-231 or MCF-7 cells were stably transfected with empty pcDNA3.1 vector, empty pCMV6-AC-GFP vector, pcDNA3.1 vector encoding for XIAP or cIAP-2 or pCMV6-AC-GFP vector encoding for Survivin using FuGENE6. Cells stably overexpressing XIAP, cIAP-2, and Survivin were selected by culture in medium supplemented with 800 μg/mL of G418 over a period of 8 weeks. Overexpression of the desired protein was confirmed by western blotting. Procedures for preparation of cell lysates and immunoblotting have been described by us previously [33,34]. Immunoreactive bands were visualized using enhanced chemiluminescence method. In some experiments, MDA-MB-231 cells were plated in 60-mm culture dishes at about 70% confluency, allowed to attach, and then treated with 10 μg/mL cycloheximide in the absence or presence of 2.5 μM WA for 6 h, 12 h or 24 h prior to harvesting and western blotting for cIAP-2, XIAP, and Survivin to determine protein stability.

2.3. Immunofluorescence microscopy

Cells treated for 24 h with desired concentration of WA or DMSO (control) were fixed with paraformaldehyde followed by permeabilization with Triton X-100. Next, the cells were treated with phosphate-buffered saline containing 0.5% bovine serum albumin and 0.15% glycine for 1 h and incubated with anti-XIAP, anti-cIAP-2, or anti-Survivin antibody overnight at 4°C. The cells were incubated with Alexa Fluor 488-conjugated secondary antibody for 1 h at room temperature, and then incubated with anti-α-tubulin antibody for 2 h at room temperature. After washing, the cells were incubated with Alexa Fluor 568-conjugated secondary antibody for 1 h at room temperature, counterstained with DAPI, and examined under a Leica DC 300F fluorescence microscope.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was done to determine the effect of WA treatment on mRNA levels of *cIAP-2*, *XIAP*, and *Survivin*. Total RNA was extracted using RNeasy mini kit and by following the manufacturer's instructions (Qiagen, Valencia, CA). Complementary DNA (cDNA) was

synthesized from 1 μg of total RNA with the use of reverse transcriptase and oligo$(dT)_{20}$. PCR reaction was carried out using GoTaq® qPCR Master Mix (Promega, Madison, WI), gene-specific primers, and cDNA. PCR products were resolved by 1.5% agarose gel electrophoresis pre-stained with ethidium bromide. Bands were visualized using UV illuminator. Primers for *cIAP-2*, *XIAP*, *Survivin*, and *GAPDH* were obtained from Invitrogen (Carlsbad, CA). PCR primers and amplification conditions were as follows: *GAPDH* (25 cycles): forward- 5′-TGATGACATCAAGAAGGTGGTGAAG-3′, reverse- 5′-TCCTTGGAGGCCATGTGGGCCAT-3′, 95°C for 30s, 55°C for 30s, 72°C for 30s; *XIAP* (25 cycles): forward-5′-AAGAGAAGATGACTTTTAACAG-3′, reverse- 5′-TGCTGAGTCTCCATATTGCC-3′, 94°C for 45 s, 48 or 49°C for 45 s, 72°C for 45 s; *cIAP-2* (30 cycles for MDA-MB-231 and 55 cycles for MCF-7 cells): forward- 5′-AAGTTCCTACCACTGTGCAATG-3′, reverse- 5′-CAAGTAGATGAGGGTAACTGGC-3′, 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; *Survivin* (30 cycles): forward- 5′-AGAACTGGCCCTTCTTGGAGG-3′, reverse- 5′-CTTTTTATGTTCCTCTATGGGGTC-3′, 94°C for 15 s, 60°C for 20 s, 72°C for 15 s. In some experiments, the MDA-MB-231 cells were plated in 60-mm culture dishes at about 70% confluency, allowed to attach, and then treated with 1 μg/mL actinomycin D (a non-toxic concentration) in the absence or presence of 2.5 μM WA for 6 h, 12 h or 24 h prior to harvesting and RT-PCR analysis for *cIAP-2*, *XIAP*, and *Survivin* to determine stability of the corresponding mRNA..

2.5. Detection of apoptosis

Apoptosis induction was assessed by DAPI assay or quantitation of histone-associated DNA fragment release into the cytosol using an ELISA kit (Roche Applied Science, Indianapolis, IN).

2.6. Immunohistochemistry

The MDA-MB-231 xenografts archived from our previously published study [17] were used for immunohistochemistry to determine the *in vivo* effect of WA administration on expression of cIAP, XIAP, and Survivin proteins. Immunohistochemistry was performed essentially as described by us previously for other proteins [34]. Briefly, tumor sections (3–4 μm) were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then probed with the desired primary antibody (anti-cIAP, anti-XIAP, or anti-Survivin antibody), washed with Tris-buffered saline (25 mM Tris containing 150 mM NaCl and 2 mM KCl, pH 7.4), and incubated with an appropriate secondary antibody. Characteristic brown color was developed by treatment with 3,3′-diaminobenzidine. The sections were counterstained with Mayer's hematoxylin, and examined under a Leica microscope. The images were analyzed using Image ProPlus 5.0 software (Media Cybernetics, Bethesda, MD) as previously described [34].

2.7. Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Bonferroni's test or by two-sided Student's *t*-test. Data are shown as mean ± SD and $P < 0.05$ was considered

statistically significant. All statistical analyses were performed using GraphPad Prism v.4.03 software (GraphPad Software, La Jolla, CA).

3. Results

3.1. Effect of WA treatment on expression of IAP family proteins

We used a pair of well-characterized human breast cancer cell lines (MDA-MB-231 and MCF-7) to determine the effect of WA treatment on expression of IAP family proteins by western blotting (Fig. 1A). Levels of XIAP, cIAP-2, and Survivin proteins were decreased markedly after treatment with 2.5 and 5 μM WA in both cell lines (Fig. 1A). The WA-mediated decrease in levels of XIAP and cIAP-2 proteins was evident as early as 6h post-treatment at the 2.5 μM dose but suppressive effect on Survivin protein was observed at the 5 μM concentration at 6 h (Fig. 1A). The WA-mediated decrease in IAP proteins was sustainable for the duration of the experiment (Fig. 1A). Suppression of IAP family protein expression resulting from WA treatment was confirmed by immunofluorescence microscopy (Fig. 1B). We have shown previously that the IC_{50} for WA is about 2 μM in MDA-MB-231 and MCF-7 cells following 24h treatment [17]. This IC_{50} concentration is close to the peak plasma achievable concentration in mice following a single i.p. injection of 4 mg WA/kg body weight [18]. Notably, the 4 mg WA/kg body weight dose (i.p., five times/week) effectively inhibits growth of MDA-MB-231 xenografts in athymic mice [17]. However, we have not determined the effect of 5 μM WA on viability of cultured MDA-MB-231 and MCF-7 cells. Nevertheless, these results indicated dose-dependent downregulation of XIAP, cIAP-2, and Survivin protein expression in WA-treated breast cancer cells regardless of the p53 status or estrogen responsiveness.

RT-PCR was performed to gain insight into mechanism by which WA treatment caused suppression of IAP family protein expression. The MDA-MB-231 and MCF-7 cells exhibited a marked decrease in *cIAP-2* and *Survivin* mRNA levels after 24h treatment with WA when compared with DMSO-treated control (Fig. 2A). Interestingly, the WA-mediated suppression of *Survivin* was relatively more pronounced at the lower dose compared with 5 μM concentration in both cell lines (Fig. 2A). On the other hand, expression of *XIAP* mRNA was only modestly decreased (20% decrease compared with DMSO-treated control) after treatment with WA in both MDA-MB-231 and MCF-7 cells (Fig. 2A). However, the stability of *cIAP-2*, *XIAP or Survivin* mRNA was not decreased after treatment with WA as evidenced by RT-PCR in cells after treatment (6 h, 12 h, and 24 h) with 1 μg/mL actinomycin D and/or 2.5 μM WA (results not shown). These results indicated that WA treatment caused transcriptional repression of *cIAP-2*and *Survivin*.

We used a proteasomal inhibitor (MG132) to test an alternate possibility that WA-mediated decrease in levels of XIAP protein was due to its proteasomal degradation. The results were inconclusive in MDA-MB-231 cells as MG132 was highly toxic to these cells even at 1 μM concentration (Fig. 2B). In MCF-7 cells, presence of MG132 (10 μM) conferred only partial protection against degradation of XIAP protein at the 2.5 μM WA dose but this protective effect was more or less abolished at the 5 μM concentration (Fig. 2B). Because the results with MG132 were inconclusive in the MDA-MB-231 cell line, we used a different proteasomal inhibitor (lactacystin) to further probe into this question. Similar to MG132,

lactacystin (7.5 μM) conferred only partial protection against WA-mediated decrease in XIAP protein level at the 2.5 μM dose (Fig. 2C). Interestingly, both MG132 (Fig. 2B) and lactacystin treatment alone (Fig. 2C) resulted in a marked decrease in protein expression of both cIAP-2 and Survivin. Reasons for these intriguing observations are not clear, but similar effects were observed even at a lower (5 μM) dose of lactacystin (results not shown). Nevertheless, stability of XIAP or cIAP-2 protein was not affected by WA treatment as evidenced by western blotting experiments using lysates from MDA-MB-231 cells treated for 6 h, 12 h, and 24 h with cycloheximide in the absence or presence of 2.5 μM WA (results not shown).

Previous studies have also shown that MDM2 is involved in post-transcriptional regulation of XIAP protein [35]. As shown in Fig. 2D the WA treatment resulted in a dose- and time-dependent decrease in protein levels of MDM2 in the MDA-MB-231 cell line. On the other hand, level of MDM2 protein was increased after treatment with WA in the MCF-7 cell line especially at the 2.5 μM concentration, which may be attributed to expression of wild-type p53 in this cell line. In summary, these results not only indicated involvement of both transcriptional and post-transcriptional mechanisms in WA-mediated suppression cIAP-2, XIAP, and Survivin proteins but also highlighted cell line-specific mechanistic complexity underlying downregulation of IAPs by WA.

3.2. XIAP overexpression conferred protection against WA-induced apoptosis

We next proceeded to determine the functional significance of XIAP protein downregulation in WA-induced apoptosis. Level of XIAP protein was 2.5-fold higher in MDA-MB-231 cells stably transfected with the XIAP plasmid in comparison with empty pcDNA3.1 vector transfected control cells (Fig. 3A). The WA-induced release of histone-associated DNA fragments into the cytosol, which is a well-accepted method for quantitation of apoptotic cells, was fully attenuated by XIAP overexpression in MDA-MB-231 cells (Fig. 3B). Similarly, a 6.4-fold overexpression of XIAP protein in MCF-7 cells (Fig. 3C) conferred significant protection against WA-mediated release of histone-associated DNA fragments into the cytosol (Fig. 3D). This protection was partial at the 5 μM WA concentration (Fig. 3D). These results indicated that XIAP overexpression significantly attenuated WA-induced apoptosis in both MDA-MB-231 and MCF-7 cells.

3.3. WA-induced apoptosis was inhibited by overexpression of Survivin and cIAP-2

Expression of Survivin protein was higher by 21-fold and 6.1-fold, respectively, in MDA-MB-231 and MCF-7 cells stably transfected with the pCMV6-AC-GFP (pCMV6) vector encoding for Survivin when compared with the cells transfected with the empty vector (Fig. 4A). Similar to XIAP (Fig. 3), ectopic expression of Survivin conferred partial but significant protection against WA-induced apoptosis in both cell lines as revealed by DAPI assay or DNA fragment release assay (Fig. 4B). Consistent with these observations, WA-mediated cleavage of PARP and caspase-3 was relatively more pronounced in the empty vector-transfected cells than in Survivin overexpressing cell line (Fig. 4C). It is important to note that the MCF-7 cell line lacks caspase-3.

Fig. 5A shows western blotting for cIAP-2 protein in MDA-MB-231 and MCF-7 cells stably transfected with the empty pcDNA3.1 vector and the same vector encoding for cIAP-2. Overexpression of cIAP-2 was achieved in both cell lines (Fig. 5A). Stable overexpression of cIAP-2 was also protective against WA-induced apoptosis as judged by DAPI assay or DNA fragment release assay (Fig. 5B), and western blotting for cleaved PARP and cleaved caspase-3 (Fig. 5C). Collectively, these results indicated protection against WA-induced apoptosis by ectopic expression of both Survivin and cIAP-2, and this effect was not a cell line-specific phenomenon.

3.4 WA administration decreased Survivin protein level in MDA-MB-231 xenografts

We have shown previously that WA administration inhibits growth of MDA-MB-231 xenografts in female athymic mice in association with apoptosis induction [17]. We used archived tumor sections from the same study to determine the effect of WA administration on expression of IAP proteins. Expression of cIAP (Fig. 6A) or XIAP (Fig. 6C) did not differ between tumors from control and WA-treated mice. On the other hand, expression of Survivin protein was modestly but significantly lower in tumor sections from WA-treated mice in comparison with control (Fig. 6B). These results demonstrated WA-mediated suppression of Survivin protein levels *in vivo*.

4. Discussion

The objective of the present study was to determine the role of IAP family proteins in regulation of proapoptotic response to WA. The IAP family proteins have emerged as critical regulators of apoptosis by different stimuli [31,32,36,37]. For example, the IAPs play an important role in apoptosis resulting from death receptor activation, growth factor withdrawal, radiation exposure, and genotoxic insults to name a few [31,32,36,37]. Furthermore, the IAP family protein is implicated in adaptive response to cellular stress, differentiation, motility, and immune response [37]. The IAP proteins are structurally characterized by baculovirus IAP repeat (BIR) domains [37,38]. The present study reveals that WA treatment decreases protein expression of XIAP, cIAP-2 and Survivin in breast cancer cells in culture. Moreover, overexpression of all the three IAP family members confers statistically significant protection against WA-mediated apoptosis in both MDA-MB-231 and MCF-7 cells.

The present study also reveals mechanistic complexity underlying WA-mediated downregulation of IAPs. The WA treatment suppresses mRNA levels of *Survivin* and *cIAP-2*, but only marginally affects *XIAP* mRNA level. Stability of *cIAP-2*, *XIAP*, *or Survivin* mRNA is not reduced after treatment with WA at least in the MDA-MB-231 cell line. Several possibilities exist to explain these results. For example, Survivin and XIAP proteins have functional resemblance of caspase inhibitory activity [31,32,36], but their structures are quite different [31,32]. The XIAP protein is structurally characterized by 3 BIR domains (BIR1, BIR2, and BIR3) and a RING-finger domain. The RING-finger domain functions as an E3 ubiquitin ligase [37,38]. On the other hand, Survivin contains a single BIR domain (BIR1) and an extended C-terminal helical coiled-coil domain, but lacks the RING-finger domain [37,38]. Consequently, XIAP is susceptible to auto-ubiquitination and

proteasomal degradation [38,39]. The WA-mediated suppression of XIAP protein is partially reversible in the presence of proteasomal inhibitors at the lower dose only. Previous studies have also shown that MDM2 physically interacts with the internal ribosome entry segment of the 5'-untranslated region of XIAP, and positively regulates XIAP internal ribosome entry segment activity [39]. It is possible that WA treatment affects translational of XIAP at least in the MDA-MB-231 cells as evidenced by downregulation of the MDM2 protein in WA-treated cells. However, additional work is needed to experimentally validate this possibility as expression of the MDM2 protein is increased in the MCF-7 cell line especially at the 2.5 μM dose.

Recent studies have pointed towards important roles of Survivin in both cell cycle regulation and apoptosis control [40]. Survivin expression is very low or undetectable in most terminally differentiated normal tissues, but this protein is overexpressed in different tumor types [40,41]. Survivin overexpression in tumors correlates with aggressive disease and treatment resistance [40,42]. The present study reveals that WA-induced apoptosis is significantly attenuated by Survivin overexpression in both MDA-MB-231 and MCF-7 cells. Furthermore, the WA-mediated inhibition of MDA-MB-231 xenograft growth *in vivo* is accompanied by a significant decrease in the levels of Survivin protein consistent with cellular *in vitro* data. However, WA administration does not cause a decrease in protein levels of cIAP or XIAP *in vivo*. The reasons for discrepancy in cellular data and *in vivo* results concerning effect on XIAP and cIAP-2 are not clear.

In conclusion, the present study indicates that the WA-induced apoptosis in breast cancer cells is associated with suppression of Survivin protein irrespective of the p53 or the estrogen receptor status. We also conclude that Survivin protein may be a viable biomarker to assess WA exposure and possibly response in human clinical investigations.

Acknowledgments

Funding Source: This study was supported by the USPHS grant RO1 CA142604-03, awarded by the National Cancer Institute. The funder had no role in the design of the experiments, data analysis, or preparation and submission of the manuscript.

Abbreviations

WA	withaferin A
IAP	inhibitor of apoptosis
XIAP	X-linked inhibitor of apoptosis
DMSO	dimethyl sulfoxide
DAPI	4',6-diamidino-2-phenylindole
PARP	poly-(ADP-ribose)-polymerase

References

1. Garodia P, Ichikawa H, Malani N, Sethi G, Aggarwal BB. From ancient medicine to modern medicine: Ayurvedic concepts of health and their role in inflammation and cancer. J. Soc. Integr. Oncol. 2007; 5(1):25–37. [PubMed: 17309811]
2. Baliga MS. *Triphala*, Ayurvedic formulation for treating and preventing cancer: A review. J. Altern. Complement. Med. 2010; 16(12):1301–1308. [PubMed: 21138390]
3. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of *Withania somnifera* (ashwagandha): A review. Altern. Med. Rev. 2000; 5(4):334–346. [PubMed: 10956379]
4. Winters M. Ancient medicine, modern use: *Withania somnifera* and its potential role in integrative oncology. Altern. Med. Rev. 2006; 11(4):269–277. [PubMed: 17176166]
5. Gupta SK, Mohanty I, Talwar KK, Dinda A, Joshi S, Bansal P, Saxena A, Arya DS. Cardioprotection from ischemia and reperfusion injury by *Withania somnifera*: A hemodynamic, biochemical and histopathological assessment. Mol. Cell. Biochem. 2004; 260(1–2):39–47. [PubMed: 15228084]
6. Ahmad M, Saleem S, Ahmad AS, Ansari MA, Yousuf S, Hoda MN, Islam F. Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. Hum. Exp. Toxicol. 2005; 24(3):137–147. [PubMed: 15901053]
7. Panda S, Kar A. Changes in thyroid hormone concentrations after administration of ashwagandha root extract to adult male mice. J. Pharm. Pharmacol. 1998; 50(9):1065–1068. [PubMed: 9811169]
8. Devi PU, Sharada AC, Solomon FE. Antitumor and radiosensitizing effects of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumor, Sarcoma-180. Indian J. Exp. Biol. 1993; 31(7): 607–611. [PubMed: 8225418]
9. Leyon PV, Kuttan G. Effect of *Withania somnifera* on B16F-10 melanoma induced metastasis in mice. Phytother. Res. 2004; 18(2):118–122. [PubMed: 15022162]
10. Padmavathi B, Rath PC, Rao AR, Singh RP. Roots of *Withania somnifera* inhibit forestomach and skin carcinogenesis in mice. Evid. Based Complement Alternat. Med. 2005; 2(1):99–105. [PubMed: 15841284]
11. Widodo N, Kaur K, Shrestha BG, Takagi Y, Ishii T, Wadhwa R, Kaul SC. Selective killing of cancer cells by leaf extract of Ashwagandha: Identification of a tumor-inhibitory factor and the first molecular insights to its effect. Clin. Cancer Res. 2007; 13(7):2298–2306. [PubMed: 17404115]
12. Mathur R, Gupta SK, Singh N, Mathur S, Kochupillai V, Velpandian T. Evaluation of the effect of *Withania somnifera* root extracts on cell cycle and angiogenesis. J. Ethnopharmacol. 2006; 105(3): 336–341. [PubMed: 16412596]
13. Shohat B, Joshua H. Effect of withaferin A on ehrlich ascites tumor cells. II. Target tumor cell destruction in vivo by immune activation. Int. J. Cancer. 1971; 8(3):487–496. [PubMed: 5002873]
14. Devi PU, Kamath R, Rao BS. Radiosensitization of a mouse melanoma by withaferin A: in vivo studies. Indian J. Exp. Biol. 2000; 38(5):432–437. [PubMed: 11272405]
15. Manoharan S, Panjamurthy K, Menon VP, Balakrishnan S, Alias LM. Protective effect of Withaferin-A on tumour formation in 7,12-dimethylbenz[a]anthracene induced oral carcinogenesis in hamsters. Indian J. Exp. Biol. 2009; 47(1):16–23. [PubMed: 19317347]
16. Srinivasan S, Ranga RS, Burikhanov R, Han SS, Chendil D. Par-4-dependent apoptosis by the dietary compound withaferin A in prostate cancer cells. Cancer Res. 2007; 67(1):246–253. [PubMed: 17185378]
17. Stan SD, Hahm ER, Warin R, Singh SV. Withaferin A causes FOXO3a- and Bim-dependent apoptosis and inhibits growth of human breast cancer cells *in vivo*. Cancer Res. 2008; 68(18): 7661–7669. [PubMed: 18794155]
18. Thaiparambil JT, Bender L, Ganesh T, Kline E, Patel P, Liu Y, Tighiouart M, Vertino PM, Harvey RD, Garcia A, Marcus AI. Withaferin A inhibits breast cancer invasion and metastasis at sub-cytotoxic doses by inducing vimentin disassembly and serine 56 phosphorylation. Int. J. Cancer. 2011; 129(11):2744–2755. [PubMed: 21538350]

19. Stan SD, Zeng Y, Singh SV. Ayurvedic medicine constituent withaferin A causes G2 and M phase cell cycle arrest in human breast cancer cells. Nutr. Cancer. 2008; 60(S1):51–60. [PubMed: 19003581]
20. Oh JH, Lee TJ, Kim SH, Choi YH, Lee SH, Lee JM, Kim YH, Park JW, Kwon TK. Induction of apoptosis by withaferin A in human leukemia U937 cells through down-regulation of Akt phosphorylation. Apoptosis. 2008; 13(12):1494–1504. [PubMed: 19002588]
21. Mandal C, Dutta A, Mallick A, Chandra S, Misra L, Sangwan RS, Mandal C. Withaferin A induces apoptosis by activating p38 mitogen-activated protein kinase signaling cascade in leukemic cells of lymphoid and myeloid origin through mitochondrial death cascade. Apoptosis. 2008; 13(12):1450–1464. [PubMed: 18987975]
22. Hahm ER, Singh SV. Autophagy fails to alter withaferin A-mediated lethality in human breast cancer cells. Curr. Cancer Drug Targets. 2012 In Press.
23. Hahm ER, Moura MB, Kelley EE, Van Houten B, Shiva S, Singh SV. Withaferin A-induced apoptosis in human breast cancer cells is mediated by reactive oxygen species. PLoS One. 2011; 6(8):e23354. [PubMed: 21853114]
24. Mayola E, Gallerne C, Esposti DD, Martel C, Pervaiz S, Larue L, Debuire B, Lemoine A, Brenner C, Lemaire C. Withaferin A induces apoptosis in human melanoma cells through generation of reactive oxygen species and down-regulation of Bcl-2. Apoptosis. 2011; 16(10):1014–1027. [PubMed: 21710254]
25. Yang ES, Choi MJ, Kim JH, Choi KS, Kwon TK. Withaferin A enhances radiation-induced apoptosis in Caki cells through induction of reactive oxygen species, Bcl-2 downregulation and Akt inhibition. Chem. Biol. Interact. 2011; 190(1):9–15. [PubMed: 21256832]
26. Hahm ER, Lee J, Huang Y, Singh SV. Withaferin A suppresses estrogen receptor-α expression in human breast cancer cells. Mol. Carcinog. 2011; 50(8):614–624. [PubMed: 21432907]
27. Ichikawa H, Takada Y, Shishodia S, Jayaprakasam B, Nair MG, Aggarwal BB. Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor-κB (NF-κB) activation and NF-κB-regulated gene expression. Mol. Cancer Ther. 2006; 5(6):1434–1445. [PubMed: 16818501]
28. Lee J, Hahm ER, Singh SV. Withaferin A inhibits activation of signal transducer and activator of transcription 3 in human breast cancer cells. Carcinogenesis. 2010; 31(11):1991–1998. [PubMed: 20724373]
29. Yang H, Shi G, Dou QP. The tumor proteasome is a primary target for the natural anticancer compound Withaferin A isolated from "Indian winter cherry". Mol. Pharmacol. 2007; 71(2):426–437. [PubMed: 17093135]
30. Mohan R, Hammers HJ, Bargagna-Mohan P, Zhan XH, Herbstritt CJ, Ruiz A, Zhang L, Hanson AD, Conner BP, Rougas J, Pribluda VS. Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis. 2004; 7(2):115–122. [PubMed: 15516832]
31. LaCasse EC, Baird S, Korneluk RG, MacKenzie AE. The inhibitors of apoptosis (IAPs) and their emerging role in cancer. 1998; Oncogene17(25):3247–3259.
32. Deveraux QL, Reed JC. IAP family proteins- suppressors of apoptosis. Genes Dev. 1999; 13(3): 239–252. [PubMed: 9990849]
33. Xiao D, Srivastava SK, Lew KL, Zeng Y, Hershberger P, Johnson CS, Trump DL, Singh SV. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G_2/M arrest and inducing apoptosis. Carcinogenesis. 2003; 24(5):891–897. [PubMed: 12771033]
34. Powolny AA, Bommareddy A, Hahm ER, Normolle DP, Beumer JH, Nelson JB, Singh SV. Chemopreventative potential of the cruciferous vegetable constituent phenethyl isothiocyanate in a mouse model of prostate cancer. J. Natl. Cancer Inst. 2011; 103(7):571–584. [PubMed: 21330634]
35. Galbán S, Duckett CS. XIAP as a ubiquitin ligase in cellular signaling. Cell Death Differ. 2010; 17(1):54–60. [PubMed: 19590513]
36. Srinivasula SM, Ashwell JD. IAPs: What's in a Name? Mol. Cell. 2008; 30(2):123–135. [PubMed: 18439892]
37. Dubrez-Daloz L, Dupoux A, Cartier J. IAPs: more than just inhibitors of apoptosis proteins. Cell Cycle. 2008; 7(8):1036–1046. [PubMed: 18414036]

38. Dean EJ, Ranson M, Blackhall F, Dive C. X-linked inhibitor of apoptosis protein as a therapeutic target. Expert Opin. Ther. Targets. 2007; 11(11):1459–1471. [PubMed: 18028010]

39. Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. Science. 2000; 288(5467): 874–877. [PubMed: 10797013]

40. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Curr. Opin. Cell Biol. 2006; 18(6):609–615. [PubMed: 16934447]

41. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, *survivin*, expressed in cancer and lymphoma. Nat. Med. 1997; 3(8):917–921. [PubMed: 9256286]

42. Pennati M, Folini M, Zaffaroni N. Targeting survivin in cancer therapy: fulfilled promises and open questions. Carcinogenesis. 2007; 28(6):1133–1139. [PubMed: 17341657]

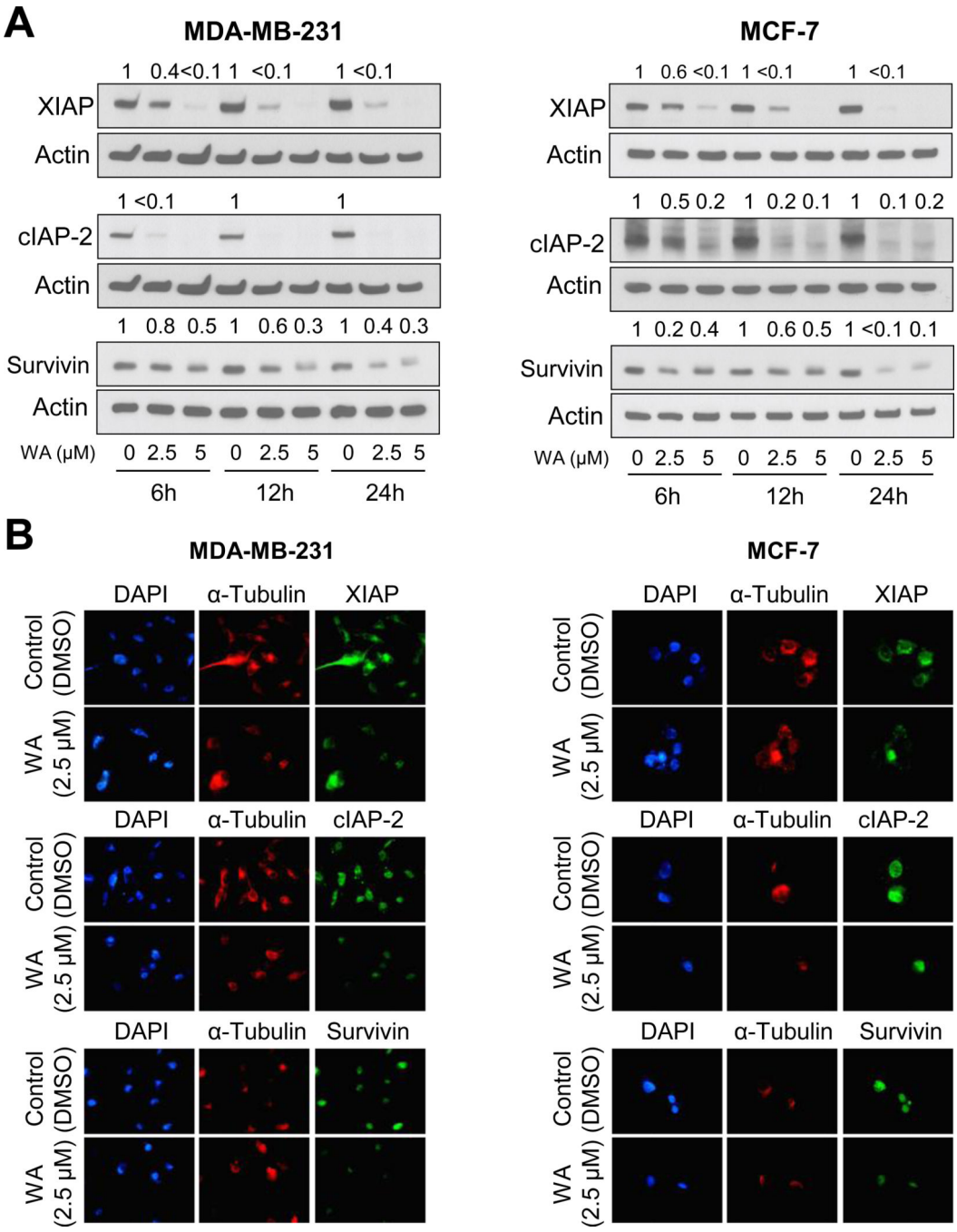

Fig. 1. Withaferin A (WA) treatment inhibits the expression of inhibitor of apoptosis proteins (IAP) in human breast cancer cells

(A) Immunoblotting for IAP family proteins using lysates from MDA-MB-231 and MCF-7 cells treated with DMSO (control) and WA for indicated times. Results (number on the top of each band) are shown as fold changes in expression level relative to corresponding control. (B) Immunofluorescence microscopy for IAP family proteins (green fluorescence) using MDA-MB-231 and MCF-7 cells following 24-h treatment with DMSO or 2.5 μM WA. DAPI and α-Tubulin staining is indicated by blue and red fluorescence, respectively (100× objective magnification).

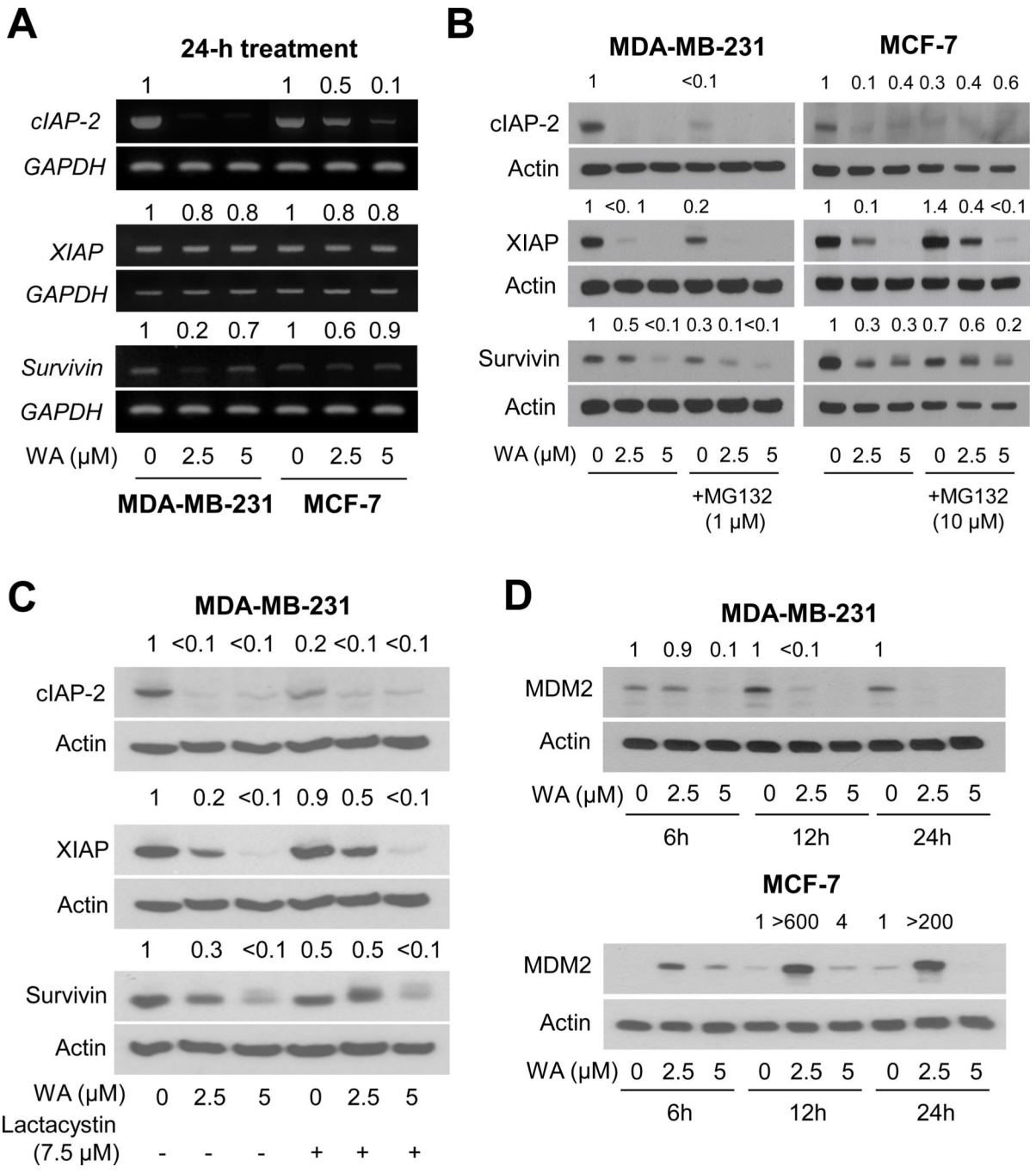

Fig. 2. Withaferin A (WA) treatment suppresses *Survivin* and *cIAP-2* mRNA levels in breast cancer cells

(A) RT-PCR analysis for mRNA expression of IAP using MDA-MB-231 and MCF-7 cells following 24-h treatment with DMSO or the indicated concentrations of WA. Each gene was amplified from cDNA with the use of gene-specific primers and PCR product was normalized to GAPDH level. Numbers on the top of each band are fold changes in mRNA levels relative to the corresponding control. (B) Western blotting for IAP family proteins using lysates from MDA-MB-231 and MCF-7 cells after 24-h treatment with WA and/or MG132 (2-h pre-treatment). Numbers on the top of each band are fold changes in expression

level relative to the corresponding DMSO-treated control. (C) Western blotting for IAP family proteins using lysates from MDA-MB-231 cells after 24-h treatment with WA and/or Lactacystin (1-h pretreatment). Numbers on the top of each band are fold changes in protein expression levels relative to the corresponding control. (D) Immunoblotting for MDM2 using lysates from MDA-MB-231 and MCF-7 cells treated with DMSO (control) and WA for indicated times. Numbers on the top of each band are fold changes in expression levels relative to the corresponding control.

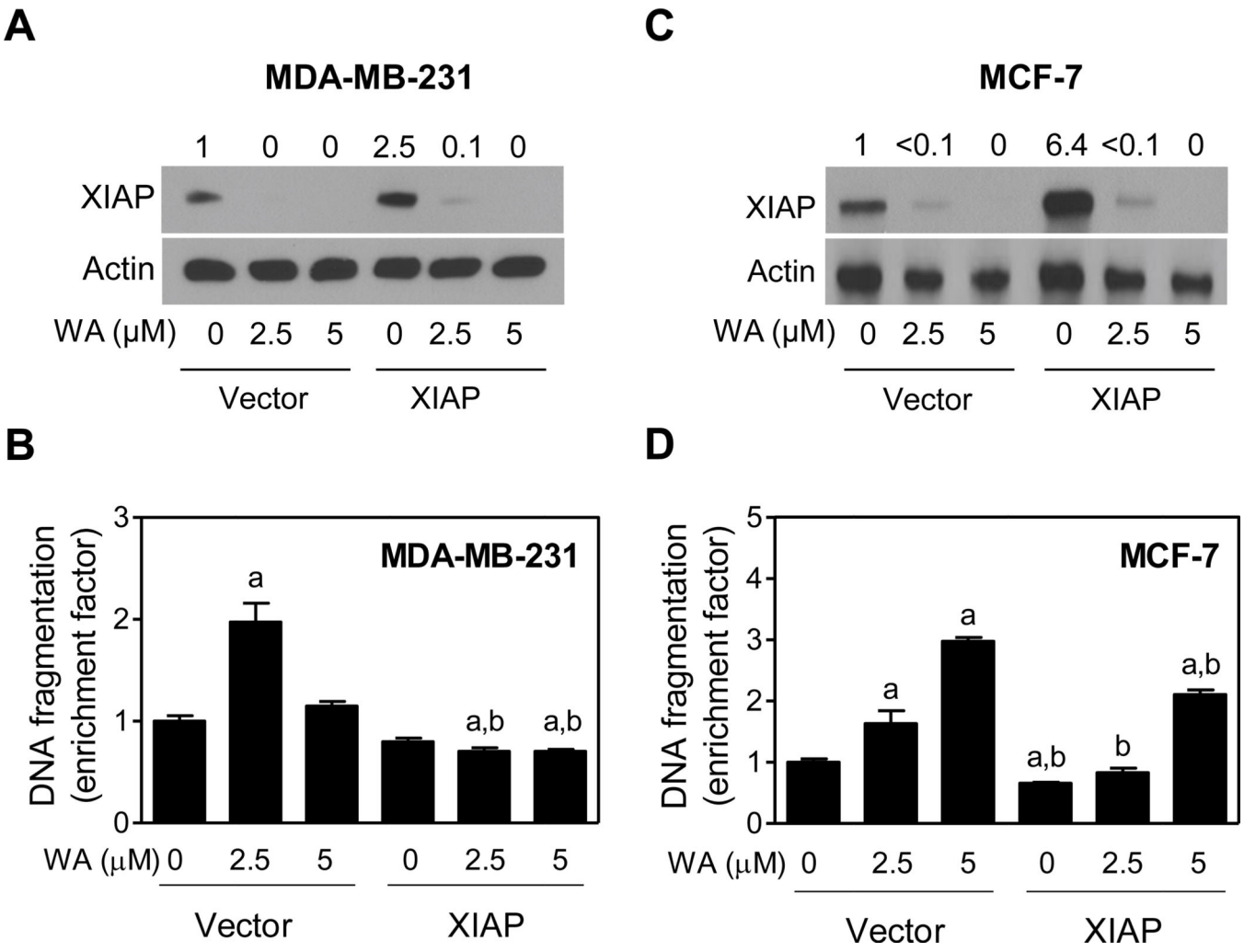

Fig. 3. Overexpression of XIAP inhibits WA-induced apoptosis in MDA-MB-231 and MCF-7 cells

(A,C) Immunoblotting for XIAP using lysates from MDA-MB-231 and MCF-7 cells stably transfected with empty vector (pcDNA3.1) or the same vector encoding for XIAP and treated for 24 h with DMSO or WA. The number above the immunoreactive band represents change in protein level relative to empty vector transfected cells treated with DMSO (first lane from the left). (B,D) Histone-associated DNA fragment release into the cytosol in empty vector-transfected control cells and XIAP-overexpressing cells after 24-h treatment with DMSO or WA. Results shown are mean ± SD (n = 3). Significantly different (P<0.05) compared with [a]DMSO-treated empty vector-transfected cells and [b]between groups at each dose by one-way ANOVA followed by Bonferroni's test.

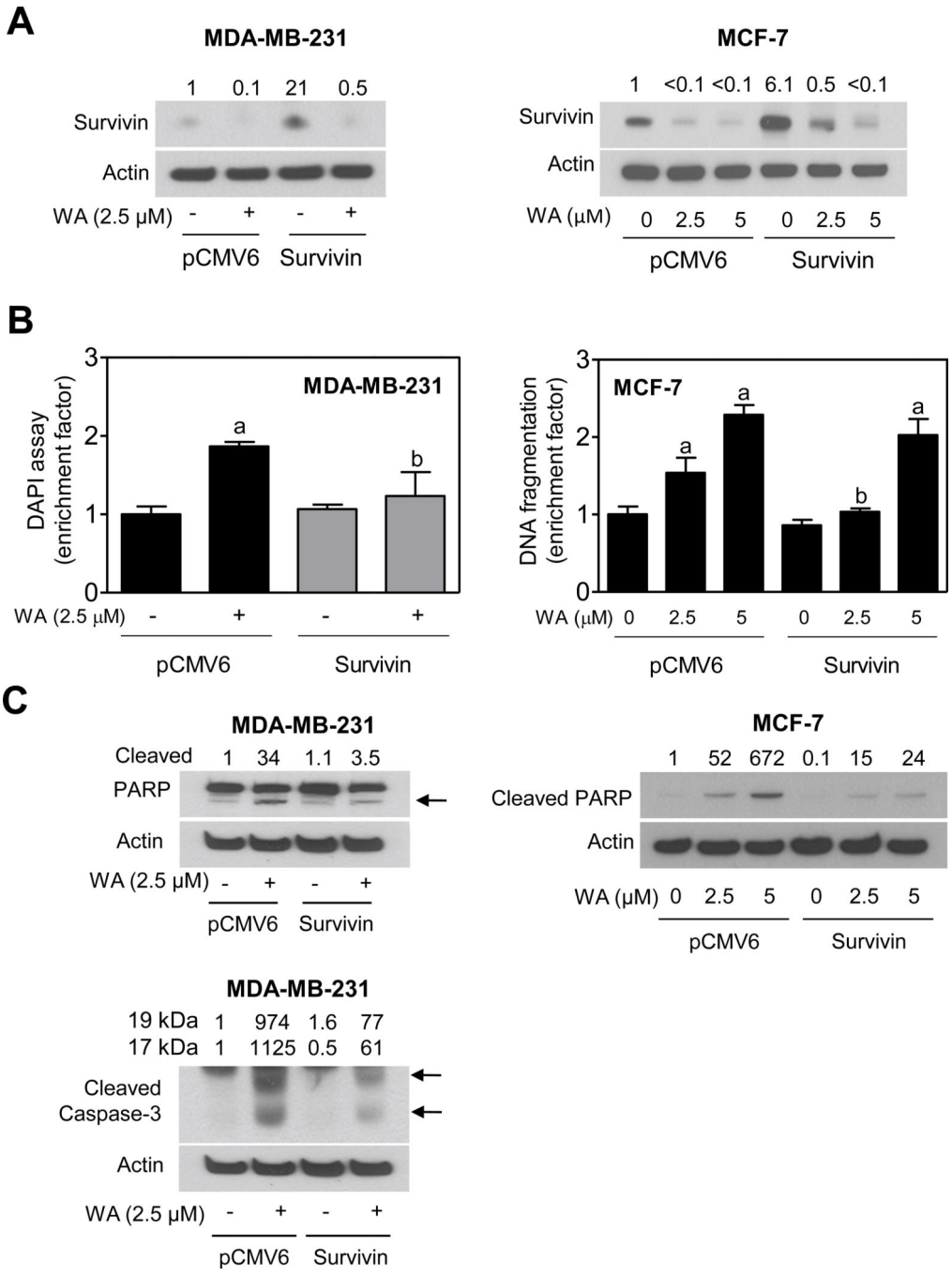

Fig. 4. Overexpression of Survivin inhibits WA-induced apoptosis in MDA-MB-231 and MCF-7 cells

(A) Immunoblotting for Survivin using lysates from MDA-MB-231 and MCF-7 cells stably transfected with empty vector (pCMV6-AC-GFP) or the same vector encoding for Survivin and treated for 24 h with DMSO or WA. The number above the immunoreactive band represents change in protein level relative to empty vector-transfected cells treated with DMSO (first lane from the left). (B) Quantitation of apoptosis by DAPI assay (MDA-MB-231 cells) or histone-associated DNA fragment release into the cytosol assay (MCF-7) in empty vector-transfected control cells and Survivin-overexpressing cells after 24-h

treatment with DMSO or WA. Results shown are mean ± SD (n = 3). Significantly different (P<0.05) compared with [a]DMSO-treated empty vector-transfected cells and [b]between groups at each dose by one-way ANOVA followed by Bonferroni's test. (C) Immunoblotting for PARP cleavage and cleaved caspase-3 using lysates from cells stably transfected with empty vector or vector encoding for Survivin and treated for 24 h with DMSO or WA. Quantitation relative to empty vector-transfected cells treated with DMSO is shown. In MDA-MB-231 cells, the antibody used recognized both full-length and cleaved forms of the PARP protein. An antibody specific for detection of cleaved PARP only was used for the immunoblotting using lysates from MCF-7 cells. The arrows indicate the cleaved form of PARP or caspase-3.

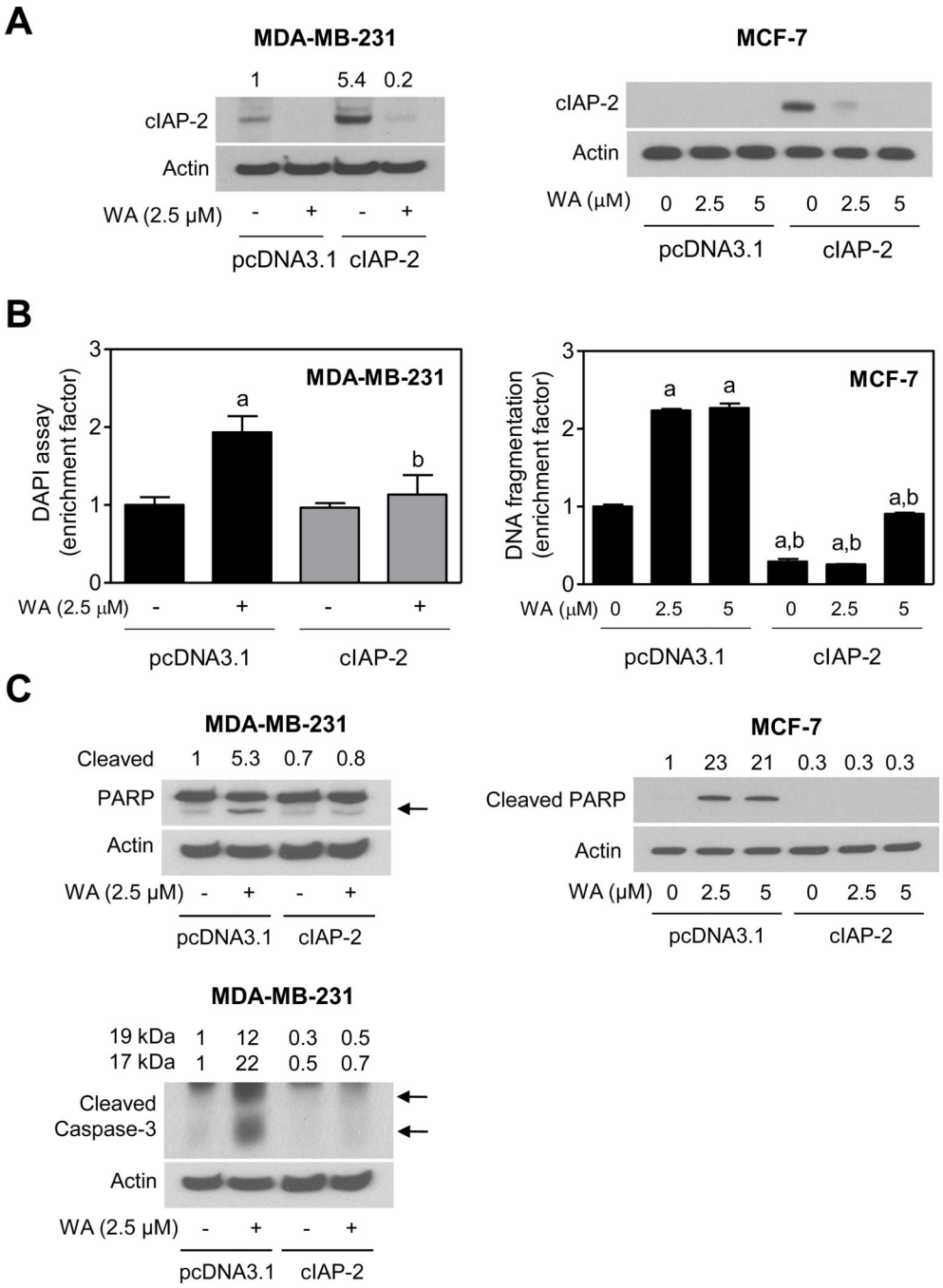

Fig. 5. Ectopic expression of cIAP-2 confers protection against WA-induced apoptosis in MDA-MB-231 and MCF-7 cells

(A) Immunoblotting for cIAP-2 using lysates from MDA-MB-231 and MCF-7 cells stably transfected with empty vector (pcDNA3.1) or the same vector encoding for cIAP-2 and treated for 24 h with DMSO or WA. The number above the immunoreactive band represents change in protein level relative to empty vector-transfected cells treated with DMSO (first lane from the left). (B) Quantitation of apoptosis by DAPI assay (MDA-MB-231 cells) or histone-associated DNA fragment release into the cytosol assay (MCF-7) in empty vector transfected control cells and cIAP-2-overexpressing cells after 24-h treatment with DMSO

or WA. Results shown are mean ± SD (n = 3). Significantly different (P<0.05) compared with [a]DMSO-treated empty vector-transfected cells and [b]between groups at each dose by one-way ANOVA followed by Bonferroni's test. (C) Immunoblotting for PARP cleavage and cleaved caspase-3 using lysates from cells stably transfected with empty vector or vector encoding for cIAP-2 and treated for 24 h with DMSO or WA. Quantitation relative to empty vector-transfected cells treated with DMSO is shown. In MDA-MB-231 cells, the antibody used recognized both full-length and cleaved forms of the PARP protein. An antibody specific for detection of cleaved PARP only was used for the immunoblotting using lysates from MCF-7 cells. The arrows indicate the cleaved form of PARP or caspase-3.

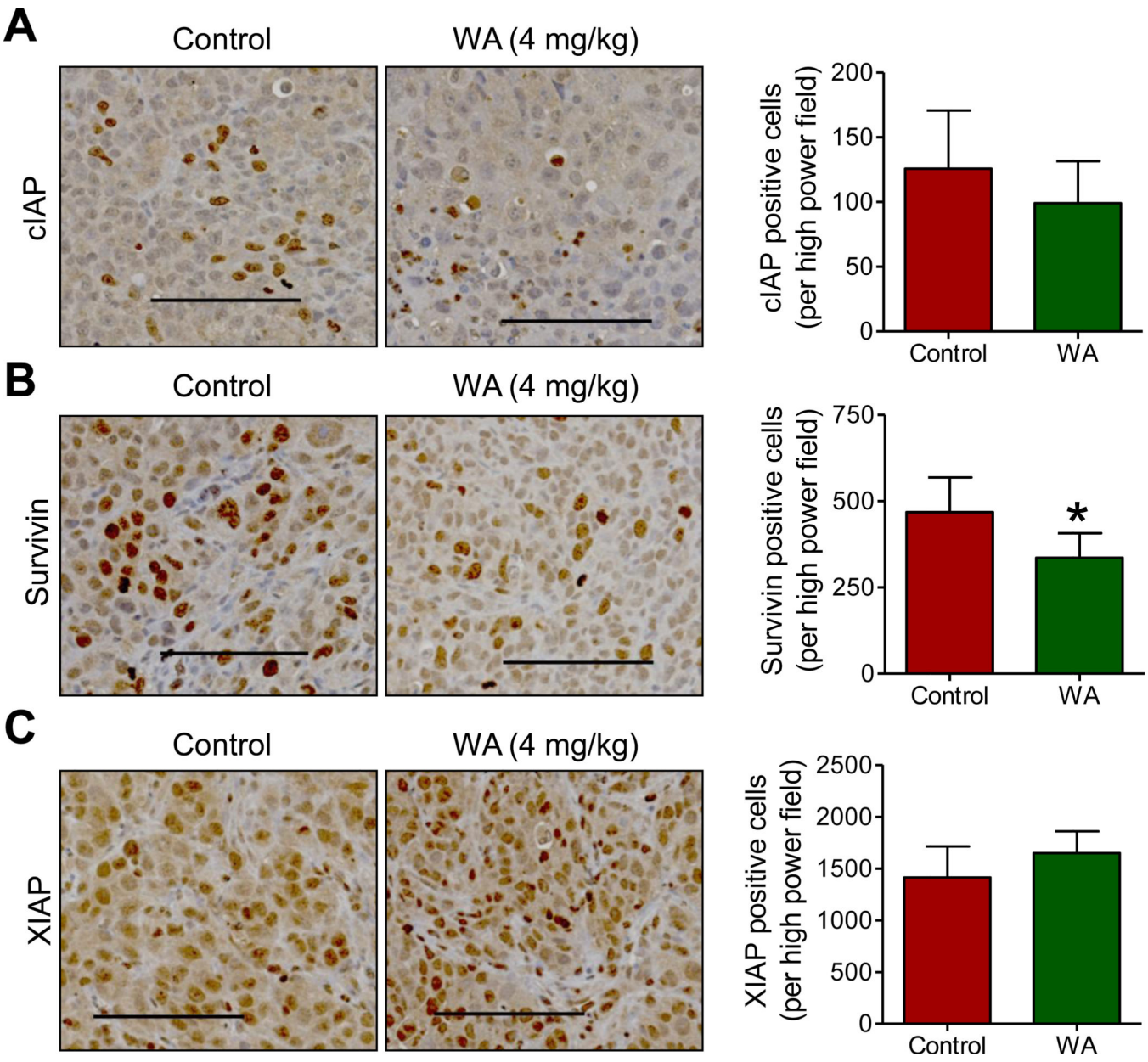

Fig. 6. Withaferin A (WA) administration suppresses Survivin protein expression in MDAMB-231 xenografts *in vivo*

Immunohistochemical analysis for cIAP (A), Survivin (B), and XIAP (C) expression in MDA-MB-231 xenografts from control and WA-treated mice (Scale bar = 100 μm; 20× objective magnification). The bar graph represents mean expression of protein with standard deviation (error bars) (n = 6 for control group and n = 7 for 4 mg/kg WA treatment group). Statistically significant (*P <0.05) compared with control by unpaired Student's t-test.