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Acetyl-CoA Carboxylase 1 Up-Regulation and Skin Tumor Formation in a Skin Carcinogenesis Mouse Model are Suppressed by Withaferin A.

Sonu Sharma[1], Shilpa Mamgain[2], Jyoti Gupta[3]

[1]Galgotias College of Pharmacy, Greater Noida, U.P., India
Sonusharmapharma[at]gmail.com

[2]Galgotias College of Pharmacy, Greater Noida, U.P. , India
mamgain.shilpa[at]gmail.com

[3]Galgotias College of Pharmacy, Greater Noida, U.P. , India
jyogupta21[at]gmail.com

Abstract: ***Traditional oriental treatments have employed withaferin A (WA), a natural substance derived from Withania somnifera, to treat neurological diseases. A clinical trial has been started to test WA in treating melanoma in light of recent studies that suggest this molecule may have the capacity to treat cancer. Here, the chemo preventive potential of WA was examined in a mouse model of chemically induced skin carcinogenesis. Pathological analyses showed that WA greatly reduced the development of skin tumours. According to morphological analyses of the skin tissues, WA prevented cell division rather than causing apoptosis during the development of skin cancer. A skin cell transformation model and antibody Micro array analysis both showed that WA prevented acetyl-CoA carboxylase 1 (ACC1) from being up-regulated by carcinogens. Transformable skin cells' anchorage-independent proliferation and oncogene activation were increased by ACC1 overexpression, whereas they were reduced by ACC1 knockdown. Additional research revealed that WA prevented activator protein 1 from becoming activated, which prevented tumour promotor-induced ACC1 gene transcription. WA was similarly effective at reducing ACC1 expression in melanoma cells. The up-regulation of ACC1 in tumours compared to nearby normal tissues was lastly validated by research employing human skin cancer tissues. In conclusion, our findings imply that withaferin A may be useful in chemoprevention and that ACC1 may be an important target of WA. Wiley Periodicals, Inc. 2015***

Keywords: withaferin A; skin carcinogenesis; ACC1; cell proliferation; apoptosis

1. Introduction

The main bioactive component of Withania somnifera, Ashwagandha, which has been used in traditional Indian medicine to treat neurological and sexual issues, is withaferin A (WA) [1]. Withanolides are a class of naturally occurring C28 steroids that include a number of biologically active substances, including WA. Current research reveals that WA inhibits the growth of human breast cancer [2-4], prostate cancer [5], colon cancer [6], pancreatic cancer [7], glioma [8], renal cancer [9], and leukaemia [10] either in vitro or in vivo. This is because WA has an anti-tumor potential. Additionally, WA is being tested in a clinical setting to treat metastatic melanoma [11]. That Withania somnifera is a major priority shouldn't come as a surprise. It is hardly surprising that Withania somnifera is on the National Center for on by tumour promoters [[12], which offers a justification and a potential unique mechanism for WA's use as a chemopreventive drug. Here, we use established chemically induced skin carcinogenesis models to investigate the mechanism of action of WA in chemoprevention. The rate-limiting enzyme in the creation of new fatty acids is called acetyl-CoA carboxylase (ACC), which generates malonyl-CoA through the ATP-dependent carboxylation of acetyl-CoA. One of the two ACC isoforms isACC1. Human malignancies such as breast [13], prostate [14], and liver carcinoma [15] have been shown to up-regulate ACC1. In order to address the needs of cancer cells for quick growth and proliferation, ACC1 over expression encourages lipogenesis. It is not shocking that ACC1 small-molecule inhibitors have been created, because pharmacological inhibition of ACC1 selectively causes growth arrest and cytotoxicity in cancer cells [16]. Because of this, ACC1 may be a useful target for cancer intervention, and the inhibitors created for the treatment of metabolic illnesses may be useful therapeutic agents for the treatment of cancer [17]. This study examines the chemo-preventive activity of WA as well as if ACC1 is up-regulated in human skin cancer and whether WA controls ACC1 during skin carcinogenesis. Herein, we aim to study the mechanism of action of WA in chemoprevention using the well-developed chemically induced skin carcinogenesis models.

Acetyl-CoA carboxylase (ACC) is the rate-limiting enzyme in de novo fatty acid synthesis, producing malonyl-CoA via ATP-dependent carboxylation of acetyl-CoA. ACC1 is one of the two ACC isoforms. ACC1 up-regulation has been found in human cancers, including breast [13], prostate [14], and liver carcinoma [15]. ACC1 over expression promotes lipogenesis to meet the need of cancer cells for rapid growth and proliferation. Not surprising, small- molecule inhibitors of ACC1 have been developed and chemical inhibition of ACC1 induces growth arrest and cytotoxicity selectively in cancer cells [16].Therefore, ACC1 might be effective as a potent target for cancer intervention, and the inhibitors developed for the treatment of metabolic diseases would be potential therapeutic agents for cancer therapy [17]. In this study, in addition to test WA’s chemo- preventive activity,

whether ACC1 is up-regulated in human skin cancer, and whether WA modulatesACC1 during skin carcinogenesis, are also studied.

2. Materials and Methods

Treatment, reagents, and cell lines

To investigate tumour promotion, murine skin epidermal JB6 Cl-41 (P) cells were employed (purchased from American Type Culture Collection [ATCC]). A 378°C incubator with 5% CO2 was used to culture the cells in EMEM medium (Lonza, Rockland, ME) with 4% foetal bovine serum (FBS, Gemini, West Sacramento, CA), 2 mM L-glutamine, 2.5 mg/ml penicillin, and 2.5 mg/ml streptomycin. In DMEM media (Lonza) augmented with 10% FBS, murine melanoma B16F10 cells (also bought from American Type Culture Collection) were grown. The ATCC has authenticated these two cell lines through testing. Using a MycoAlert Mycoplasma Detection Kit obtained from Lonza, the levels of mycoplasma were frequently (once every three months) tested in these cells for cell culture, and the results were consistently negative.

Purchased from ChromaDex (ASB-00023250), withaferin A (WA) was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO). Additionally, DMSO was used to prepare the chemical carcinogen dimethylbenz[a]anthracene (DMBA, Sigma) and the tumour promoter 12-O-tetradecanoylphorbol-13- acetate (TPA, Sigma).OriGene sold empty (pCMV6-based) vectors and human ACC1 expression vectors (Rock- ville, MD, RC218293). X-tremeGENE Transfection Reagent (Roche, Indianapolis, IN) was used to transfect JB6 P cells with these two vectors, and stable transfectants were obtained after selection with 400 mg/ml G418 for two weeks. Ten pairs of skin cancer patient specimens—ten skin tumour and surrounding normal tissue pairs—were collected from the Louisiana State University Health Sciences Center's Feist-Weiller Cancer Center's Tissue and Serum Repository in Shreveport. We did not collect data through direct or indirect contact with real people, nor did we have access to personally identifiable information. The Institutional Review Board at our institution gave its approval to this study.

Chemically-Induced Mouse Skin Carcinogenesis

The Institutional Animal Care and Use Committee of the LSU Health Sciences Center in Shreveport approved the animal protocol, and the animal study was carried out in accordance with it. The Jackson Laboratory in Indianapolis, IN, separated 40 female DBA/2 mice, who are 6–8 weeks old and relatively susceptible to skin carcinogenesis, into four groups: DMSO, DMBA/TPA, WA, and WA DMBA/TPA. The DMBA/TPA group (five mice) received a single topical administration of 200 nmol DMBA for two weeks, followed by topical applications of 4 mg TPA once per day, five times per week for 14 weeks. The DMSO group (five mice) received DMSO treatment as the vehicle control. The WA group received topical application of 20 mg WA following the same schedule for DMBA/TPA treatments. The identical treatment regimen for DMBA/TPA treatments was followed for the WA group, who received topical application of 20 mg WA. The TPA therapy was administered to the WA DMBA/TPA group after 30 minutes of WA treatment. An outline of these treatment methods was shown in a schematic picture (Supplementary Figure S1).Mice were euthanized by intraperitoneal injection of pentobarbital (150 mg/kg bodyweight) at the conclusion of the skin carcinogenesis research. The skin samples were obtained and handled according to the procedures outlined in our earlier research [18, 19].

Histological Examination of Mouse Skin Tissues

Skin epidermal tissues were embedded in paraffin, fixed in 4% buffered formaldehyde, and subjected to hematoxylin and eosin (H&E) staining. Light microscopy analysis was used to count mitotic and apoptotic cells. Apoptosis was distinguished from other morphological processes by the appearance of cytoplasmic vacuoles, nuclear condensation, and shrinking of the cell. A licenced pathologist oversaw the morphologic analysis (XG). The two carcinogen-treated groups' papillomas were likewise H&E stained, and similarly to what has previously been described [20], TUNEL (Roche, #11684817910) and Ki-67 (Abcam, ab16667) staining were used to identify apoptosis and cell proliferation, respectively.

Preparation of Whole Cell Lysate

Collected skin cells were suspended in 150 ml of RIPA buffer containing a proteinase inhibitor cocktail (Calbiochem, La Jolla, CA). Cells were sonicated on ice for two strokes (10 s per stroke) using a Fisher Sonic Dismembrator (Model 100, Scale 2). The whole cell lysate was obtained by centrifuging the cell lysate at 14,000 g for 15 minutes after it had been incubated on ice for 30 minutes.

Determination of Withaferin A in Skin Tissue Samples by HPLC

Six female DBA/2 mice aged 6 to 8 weeks were equally divided into two groups. Mice in the WA group received a single application of WA (20 mg) topically for 24 hours before being put to death. Mice in the DMSO group received a DMSO treatment. Whole Cell Lysate was created from stripped skin epidermal cells as previously reported and combined in each treatment group. Cold acetonitrile (2/1, v/v) was added to the pooled samples to extract them, and the mixture was vortexed for three minutes. Samples were centrifuged at 10,000 rpm for 5 minutes after being allowed to stand in ice for 10 minutes Shimadzu's high-performance liquid chromatography (HPLC) system, which included a binary, high-pressure gradient solvent delivery pump (model LC 20AB), an autosampler with a cooling sample device (model SIL-20AC HT), a UV-visible absorbance detector (model SPD-20A), and data processing software, received an aliquot of 20 ml from the supernatant (LCsolution Version 1.23).

Sample vials were placed in a tray section that was chilled to 48C. A Guard column (4.6 12.5 mm, 80) and an XDB-C18 column (4.6 250 mm, 80) were used to separate withaferin A. Water (A) and acetonitrile (B) made up the mobile phase, which was used in the gradient elution described below. The mobile-phase composition changed from 70A:30B to 60A:40B over 10 minutes at 1.0 ml/min flow rate. e. From the tenth minute through the fifteenth minute at 1.5 ml/mi, the ratio returned to 70A:30B following the elution of WA. n.

For a total run time of 20 minutes, the composition was kept at 70A:30B for an additional 5 min at 1.0 ml/min to equilibrate the column. The detection was observed for 20 minutes at a wavelength of 225 nm. WA has a retention time of 11.414 minutes.

Antibody Microarray Analysis

The Phospho Explorer Array Assay kit, available from Full Moon BioSystems, Inc. under the catalogue number KAS02, was utilised to identify and evaluate hundreds of proteins during phosphorylation events. The 1,324 highly specific antibodies used in the array kit were spotted in duplicate on a high-quality glass surface and coated with unique 3-D polymer materials. Each slide consists of an array of well-characterized antibodies. Each slide has six copies of a variety of well-characterized antibodies, numerous positive indicators for Cy3, and BSA is used as a negative control. The background in data analysis can be made up entirely of empty spaces. On the website of Full Moon Biosystems, you can find the list of arrayed antibodies. Randomly chosen from the DMBA/TPA and WA DMBA/TPA groups, five samples (Whole Cell Lysate) from each group were pooled together, and the experiment was carried out in accordance with the guidelines given by the manufacturer.

Western Blot Analysis

Whole Cell Lysate was used for the assay. Anti- bodies against ACC1 (04–322) and p-c-Jun (9164, ser73) were purchased from EMD Millipore (Billerica, MA); antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sc-32233), b-actin (sc- 47778), succinate dehydrogenase subunit b (SDHB, sc-25851), and Fra-1 (sc-605) were purchased from Santa Cruz Biotechnology.

ACC1 siRNA Transfections

JB6 P or B16F10 cells were sown in six-well tissue culture plates at a density of 3 105 cells per well, and they were then incubated at 378°C in a 5% CO2 incubator until they reached a confluence of 70–80%. For each transfection, 100 ml of siRNA transfection medium (sc-36869, Santa Cruz Biotechnology) was mixed with 1 mg of the ACC1 siRNA duplex (sc-40313, Santa Cruz Biotechnology), which was then labelled as Solution A. Solution B contained 100 ml of siRNA transfection medium and 8 ml of sc-29528, a transfection reagent from Santa Cruz Biotechnology. Gently combining Solution A and B, the mixture was incubated for 30 minutes at room temperature. Once with 2 ml of siRNA transfection medium, cells were washed. After that, each tube containing the solution A/B mixture received 0.8 ml of siRNA transfection medium, which was combined and then immediately added to the washed cells. 6 hours were spent incubating cells at 378C with 5% CO2incubator.The cells containing the transfection mixture were then given 1 ml of 2 EMEM medium right away. The medium was changed to 1 x regular medium after the cells had been cultured for an additional 24 hours. The cells underwent Western blot and soft agar analyses after a 24-hour incubation period.

Anchorage-Independent Growth Assay in Soft Agar

In six-well plates, a soft agar-based cell transformation experiment was conducted. The bottom agar was prepared with 1.25 percent agar, 2 EMEM medium, 10% FBS, PBS, glutamine, and penicillin, and it was incubated in a water bath at 508 degrees Celsius. Then, several treatment reagents were divided into the mixture (0.5% agar). Two fractions of the 0.5% agar combinations mentioned above and one fraction of 1 104 JB6 or B16F10 cells were present in the top agar mixture. The agar was allowed to set, incubated for 10 days at 378°C with 5% CO2, and stained for 24 hours with 0.25 mg/ml Neutral Red.RNA Extraction and Real-Time PCR. Total RNA was isolated from cells using TRIzol reagent (Thermo Fisher Scientific) following the manufacturer's instructions. 2 mg of the purified RNA was reverse transcribed using RT2 HT First Strand kit (QIAGEN, Catalog Number 330411) according to the manufacturer's instructions. The mRNA levels of ACC1 were determined by quantitative real-time PCR analysis using an RT2 SYBR Green qPCR Mastermix kit (QIAGEN, Catalog Number 330502). ACC1 primer: GAGAGTTCACCCAGCAGAATAA (Sense), CTGATC-CACCTCACAGTTGAC (Antisense). The levels of b-Actin expression were measured in all samples to normalize ACC1 gene expression. Each sample was analyzed in triplicate, and the expression levels were compared according to the 2—DDCt method.

Statistical Analysis

Data were presented as mean standard deviation (S.D.) in all of the Tables or mean standard error (S.E.) in all of the figures. One-way ANOVA was used to compare the body weight and tumor numbers among groups. The Tukey–Kramer method was used to adjust for multiple comparisons. Paired student's t- test was used to compare ACC1 expression levels between human skin tumor and normal tissues. All P values less than 0.05 were considered as statistically significant.

3. Results

WA Suppressed Skin Tumor Formation

At first, the amount of WA penetrated into the epidermal layer of the skin was determined by HPLC. The result revealed that approximately 50% (or10.4 mg) of the input (20 mg) of WA was present in the whole epidermal layer of the skin 24 h after the application. The chemically induced two-stage skin carcinogen- esis was next performed and at the end of the study, animal body weights were measured. The data (DMSO Group: 24.23.1 g; DMBA/TPA Group: 23.72.6 g;WA Group: 22.61.4 g; WA DMBA/TPA Group: 21.6 0.9 g) indicate that neither WA nor TPA alone caused a significant loss in animal's body weight; and the WA DMBA/TPA treatment cause a 10% loss in body weight compared with the DMSO Group, which is not statistically significant. Tumor pathology was next analyzed; representative histological images of the skin tissues/tumors in all the treatment groups were shown in Supplementary Figure S2, and the results were summarized in Table 1. Since this is a 14 wk study, all of the tumors were benign papillomas, and the tumor incidences were 100% in both of the TPA-treated groups. Pretreatment with WA reduced tumor multiplicity by 58%, which is statistically significant. To detect whether WA induces apoptosis and/or suppresses cell proliferation in the skin epidermal layer, apoptotic and mitotic cells were counted and the results were summarized in Table 2, and Ki-67 and TUNEL staining of skin epidermal tissues were shown in Supplementary Figure S3. Similarly to

what have been observed in our earlier studies [21, 22], TPA treatment induced increases in both mitosis and apoptosis in the skin epidermal cells. WA alone did not show significant effects on both events; however, it suppressed TPA-induced mitosis, which is consistent with the tumor pathology result.

Table 1: Papilloma Formation in the Multistage Carcinogenesis Model

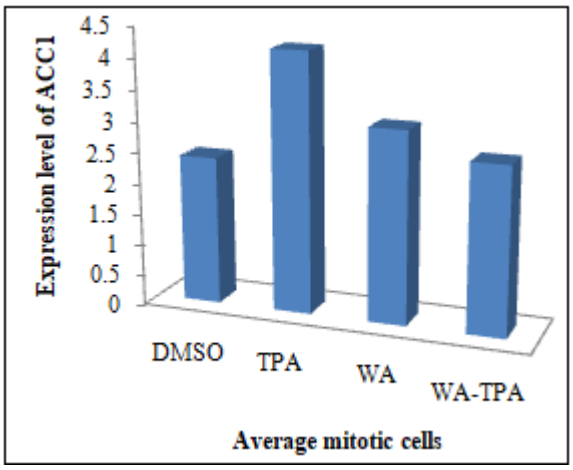
Treatment	Number of mice	Tumor incidence (%)	Papillomas/ mouse	Total papillomas
DMSO	5	0	0±0	0
TPA	15	93	9.8 ± 3.2	159
WA	5	0	0 ± 0	0
WA-TPA	15	93	4.5 ± 1..7	65

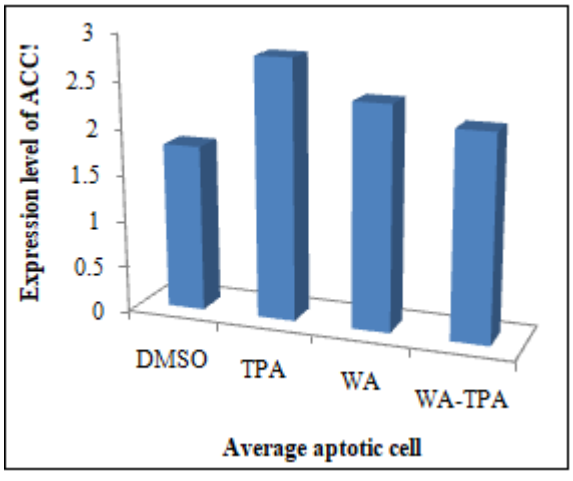
All of the TPA groups received a single application of DMBA.
ω P < 0.01 compared with the TPA alone group.

Table 2: Mitotic and Apoptotic Cell Counts at the End of Skin Carcinogenesis Study

	Average mitotic cells (per 100 cells)	Average apoptotic cells (per 100 cells)
DMSO	2.4± 0.8	1.8± 1.2
TPA	4.2± 2.0*	2.8 ± 1.2
WA	3.1± 0.9	2.4± 0.9
WA-TPA	2.7± 1.2#	2.2± 1.4

All of the TPA groups received a single application of DMBA.
*P<0.05 compared with the DMSO group
#P<0.05 compared with the TPA alone group





Identification of Target Proteins Regulated by WA

Antibody microarray analysis was performed to identify the potential targets of WA using pooled Whole Cell Lysate from each treatment group (five samples randomly selected from each group). Proteins that were up-regulated (at least 1.6-fold) by TPA treatment but maintained at or lower than the Control (DMSO treatment) levels when WA was present were selected and summarized in Supplementary Table S1. These identified proteins were grouped into seven pathways: apoptosis (p53 [p-ser9 and p-ser392], BAD [p-ser112], and WAVE1); cell cycle and mitosis(Chk1 [p-Ser317] and stathmin 1 [p- Ser37]); protein kinases (ASK1 [p-Ser83], BTK [p-Tyr550], EGFR [p-Tyr1069], MAPKAPK2 [p-Thr222],and PAK4/5/6); lipid metabolism (ACC1 andPLCG1); protein modifier (HDAC5); transcription factors (MITF[p-Ser73], NFkB[p-Ser893], andTFII-I[p-Tyr248]); and (VAV2). Most of these identified targets have been well studied in skin carcinogenesis except a few. Since WA is a steroidal lactone, the lipid metabolic enzyme ACC1 (acetyl-CoA carboxylase 1) was selected and studied further.

To detect if ACC1 is up-regulated in human skin cancer tissues, 10 pairs of human skin tumor and adjacent normal tissues samples were obtained and Western blot analysis was performed. Squamous (five pairs) and basal cell carcinoma (three pairs), atypical fibroxanthoma (one pair), and nodular melanoma (one pair) are among the histology of this group. As shown in Supplementary Figure S4, the overall ACC1/b-actin ratios in the normal and tumor samples were: 0.22, 0.07 and 0.41 0.21, respectively (P < 0.05). Among the five pairs of the squamous cell carcinoma samples, the overall ACC1/b-actin ratios in the normal and tumor samples in these five pairs were: 0.22 0.05, and 0.41 0.28, respectively (P > 0.05).

Over expression of ACC1 Enhanced Whereas Knockdown of ACC1 Suppressed Skin Cell Promotion

As shown in Figure 1A, the ability of WA inhibiting carcinogen-induced ACC1 up-regulation was con- firmed in skin epidermal tissues collected at the end of the skin carcinogenesis study. To study if ACC1 is up- regulated during tumor promotion, JB6 P cells, the only characterized skin epidermal cells to study tumor promotion [23], were treated with TPA and WA. As shown in Figure 1B, a time-course study revealed that ACC1 expression was up-regulated 30 min after TPA treatment and reached a peak at the 6 and 24 h time- point. Next, JB6 cells were treated with WA (0.625 mM, has been used in our previous study and skin cell transformation can be inhibited at this concentration [12]. A lower concentration of WA [0.125 mM] has also been tested and cell transformation was not inhibited (data not shown) 30 min before TPA treatment (24 h). The results in Figure 1C indicate that WA suppressed TPA-induced up-regulation of ACC1.

To detect how up-regulation of ACC1 contributes to tumor promotion, human ACC1, and empty vector stably transfected JB6 Pþ cells were used for anchorage-independent growth assays. As shown in Figure 2A, overexpression of ACC1 in the transfected cells was confirmed by Western blot analysis. Tumor promoter TPA induced colony formation in soft agar, whereas the number of colonies was increased by 75% in ACC1 stably

transfected cells (Figure 2B).

TPA treatment is known to induce the activation of activator protein one (AP-1) during skin carcinogenesis and Fra-1, as a subunit of AP-1, contributes to AP-1 DNA binding activity in these models [12, 24]. The results (Figure 2C) show that ACC1 over- expression increased the protein expression levels of Fra-1, and TPA treatment further enhanced this increase. Next, the expression levels of ACC1 were suppressed via a siRNA approach. As shown in Figure 2D, knockdown of ACC1 (confirmed by Western blot shown in the left panel) suppressed anchorage-independent growth of the transform- able JB6 cells (right panel).

Can WA suppress skin cell transformation even ACC1 is overexpressed? ACC1 transfected JB6 cells were treated with WA and the results in Figure 3 demonstrate that WA suppressed TPA-induced in- creases in Fra-1 expression (Figure 3A) and colony formation in soft agar (Figure 3B).

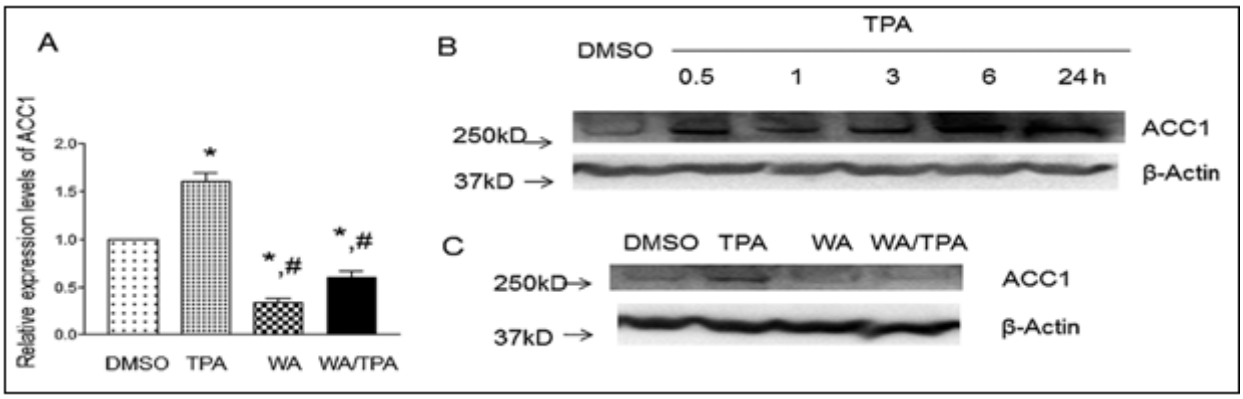


Figure 1: (A) Withaferin A (WA) suppressed increases in ACC1 in mouse skin carcinogenesis. Quantified results were shown from the antibody array analysis. (B) A time-course study showing the expression levels of ACC1 after TPA (5 nM) treatment in JB6 P cells. GAPDH served as the loading control. (C) Withaferin A (WA) suppressed TPA-induced up-regulation of ACC1 in JB6 P cells. Cells were treated with TPA and/or WA for 24 h. ωP< 0.05 compared with its own DMSO group; #P < 0.05 compared with the TPA group.

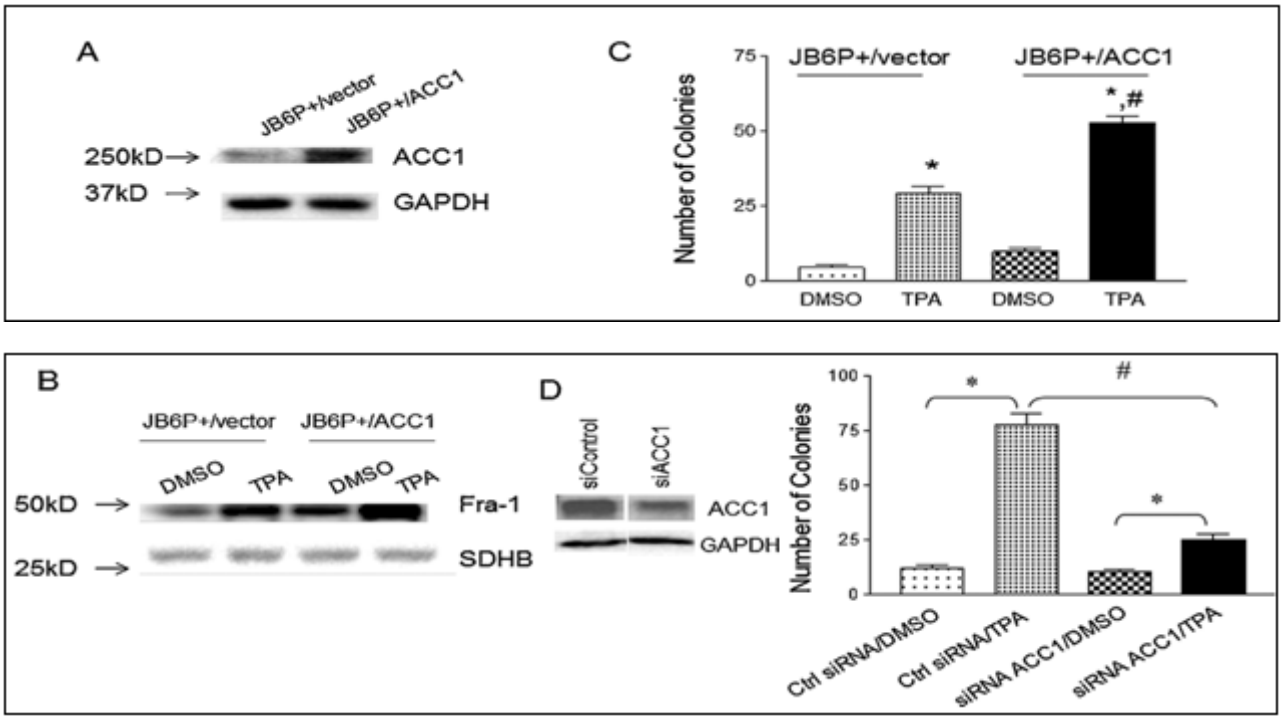


Figure 2: Up-regulation of ACC1 enhanced skin cell transformation.

a) ACC1 was over expressed in stably transfected cells. GAPDH served as the loading control.
b) Overexpression of ACC1 enhanced TPA-induced up- regulation of Fra-1. SDHB (succinate dehydrogenase subunit b) served as the loading control. Cells were treated with TPA (5 nM) for 24 h.
c) ACC1 over expression enhanced TPA-induced colony formation in soft agar. Soft agar contains TPA (5 nM) and cells were incubated by 10 d.
d) Knockdown of ACC1 suppressed colony formation. ω P < 0.05 compared with its own DMSO group; #P < 0.05 compared with the TPA group.

WA Inhibited AP-1 Activation and ACC1 Gene Expression

To examine whether WA regulates ACC1 expression at the transcription level, real-time PCR analysis was performed. The results demonstrated that although WA alone did not inhibit ACC1 gene transcription, it suppressed tumor promoter TPA- induced ACC1 upregulation (Figure 4A). TPA is known to activation AP-1, and AP-1 can also induce ACC1 gene expression [25]. To study the effect of WA on AP-1 activation, p-c-Jun (ser73) was detected and the results showed that TPA induced p-c-Jun expression, which was inhibited by WA (Figure 4B). These data suggest that withaferin A could inhibit ACC1 gene transcription by suppressing AP-1 activation.

WA Suppressed ACC1 Expression and Melanoma Cell Growth

Since WA was in trial for treating melanoma [11], whether WA has an effect on ACC1expression in melanoma cells was examined. As shown in Figure 5A, WA suppressed the expression levels of ACC1 at the same concentration (0.625 mm) used in the JB6 cell study. At this concentration, anchor- age-independent growth of B16F10 cells was also suppressed (Figure 5B).

4. Discussion

In this study, the chemoprevntive activity of withaferin A was detected using the chemically- induced skin carcinogenesis mouse model and the potential mechanism of action was also investigated. The most studied topicin WA is testing its cancer therapeutic effects, and the mechanisms of action have been demonstrated in human cancer cells.

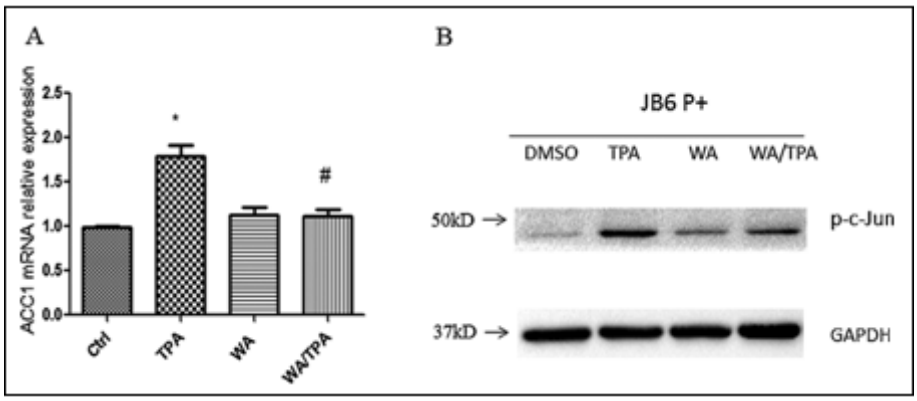


Figure 3: WA suppressed skin cell transformation in ACC1 over expressed JB6 cells. (A) WA suppressed the up-regulation of Fra-1 upon TPA (5 nM, 24 h) treatment. (B) WA suppressed anchorage-independent grow of ACC1 overexpressed JB6 cells. ωP< 0.05 compared with the DMSO group; #P<0.05 compared with the TPA group.

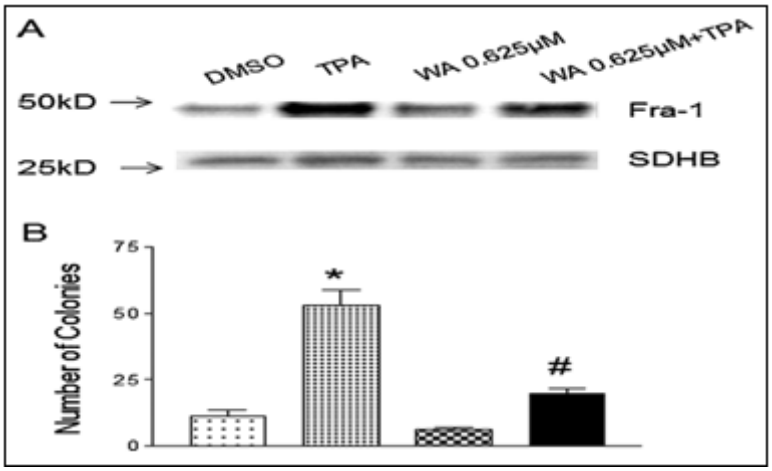


Figure 4: (A) Detection of ACC1 gene transcription using real-time PCR. JB6 P cells were treated with TPA (5 nM) and WA (0.625 mM) for 1 h. ωP< 0.05 compared with the DMSO group; # P < 0.05compared with the TPA group. (B) WA suppressed tumor promoter TPA-induced phosphorylation of c-Jun (Ser73). JB6 P cells were treated with TPA (5 nM) and WA (0.625 mM) for 24 h.

In summary, WA suppresses tumor growth by inhibiting cell proliferation, cell cycle, inflammation, and angiogenesis; or by promoting apoptosis and oxidative stress. In particular, WA inhibits Notch-1 signaling and down-regulates prosurvival pathways, such as Akt/NF-kB/Bcl-2, in three colon cancer cell lines (HCT-116, SW-480, and SW- 620) [6]. In addition, WA down-regulates the expression of mammalian target of rapamycin signaling components, pS6K and p4EBP1, and activates JNK-mediated apoptosis in colon cancer cells [6]. WA also targets heat shock protein 90 in pancreatic cancer cells [7]; represses IL-6 gene transcription upon dual inhibition of NF-kB and AP-1/Fra-1 transcription

factors in metastatic breast cancer cells [3]; sensitizes TRAIL-induced apoptosis through reactive oxygen species-mediated up regulation of death receptor five and down-regulation of c-FLIP in human renal cancer cells [9]; causes G2 and M phase cell cycle arrest in human breast cancer cells [26]; induces apoptosis in human leukemia U937 cells [27], leukemic cells [10], prostate cancer cells [5], and human breast cancer cells in vivo [2]. The antiangiogenic effect of WA relies on targeting the intermediate filament protein vimentin [28].WA has also been shown to have a radiosensitizing effect on mouse fibrosarcoma and melanoma cells [29].

In our study, WA suppresses chemically-induced skin carcinogenesis via inhibiting cell proliferation rather than inducing cell death. Among WA's target proteins, ACC1 has been found to be up-regulated in human breast [13], prostate [14], and liver [15] cancers, and the inhibitors for ACC1 may serve as potential therapeutic agents for cancer therapy [16]. Data from this study also reveal that ACC1 is up-regulated in human skin cancer samples. Considering WA in a clinical trial for melano- ma therapy, it will be interesting to study further whether ACC1 can be a target of WA for treatment of non-melanoma skin cancer.

ACC1 catalyzes the de novo synthesis of fatty acids. Fatty acids are a major energy source and important constituents of membrane lipids, and they can also serve as cellular signaling molecules that play an important role in the etiology of the metabolic syndrome [30]. Since acetyl-CoA, the substrate of ACC1, is a key intermediate linking carbohydrate, amino acid, and lipid metabolism, the activity of ACC1 is often regulated during these metabolic events. It will be interesting to perform metabolomic studies, especially lipid metabolism in WA-treated skin epidermal samples and identify potential "onco- metabolites" which mediates ACC1 signaling. Next, whether these "on cometabolites" may activate Fra-1/ AP-1, a key player in skin tumorigenesis will be studied, as over expression of ACC1 by itself can induce the expression of Fra-1 (Figure 2B). Our previous studies have shown that the tumor promoter

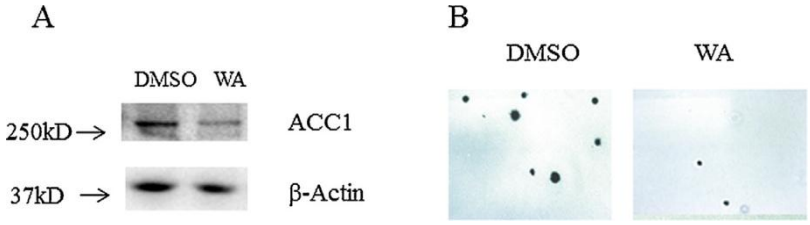


Figure 5: WA suppressed ACC1 expression and the growth of melanoma B16F10 cells. (A) Western blot analysis of ACC1 expression after WA treatment. WA (0.625 mM) was treated for 24 h. (B) Images of the colonies formed in soft agar (magnification: 10x).

TPA inhibits the activity of AMPK by decreasing the expression levels of phosphor-AMPK [31]. AMP- activated kinase (AMPK), a key monitor of cellular energy levels, can inactivate ACC1 via phosphorylation. This may serve as another mechanism of ACC1 activation in our skin carcinogenesis model.

In summary, this study demonstrates that withaferin A suppresses chemically-induced skin carcinogenesis and ACC1 could serve as an important target of withaferin A. These findings could potentially be expanded to melanoma since withaferin A also inhibits ACC1 expression in melanoma cells. Future studies will focus on how withaferin A regulates ACC1 and how ACC1 promotes cancer development.

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