Research Article

Cancer Prevention Research

Effect of Combined Treatment with Ursolic Acid and Resveratrol on Skin Tumor Promotion by 12-*O*-Tetradecanoylphorbol-13-Acetate

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Abstract

In this study, the effects of combining ursolic acid + resveratrol, for possible combined inhibitory effects on skin tumor promotion, were evaluated. Ursolic acid, resveratrol, and the combination of ursolic acid + resveratrol were applied topically prior to 12-*O*-tetracanoylphorbol-13-acetate (TPA) treatment on mouse skin to examine their effect on TPA-induced signaling pathways, epidermal hyperproliferation, skin inflammation, inflammatory gene expression, and skin tumor promotion. The combination of ursolic acid + resveratrol produced a greater inhibition of TPA-induced epidermal hyperproliferation. The combination of ursolic acid + resveratrol inhibited TPA-induced signaling pathways, including EGFR, STAT3, Src, Akt, Cox-2, Fas, NF-κB, p38 MAPK, c-Jun, and JNK1/2 while increasing levels of tumor suppressors, such as p21 and PDCD4, to a greater extent compared with the groups treated with the individual compounds. Ursolic acid + resveratrol also induced a dramatic increase of p-AMPK-α^{Thr172}. Combined treatment with ursolic acid + resveratrol resulted in a greater inhibition of expression of proinflammatory cytokines, including *Il1a*, *Il1b*, and *Il22*. Furthermore, NF-κB, Egr-1, and AP-1 DNA binding activities after TPA treatment were dramatically decreased by the combination of ursolic acid + resveratrol. Treatment with ursolic acid + resveratrol during skin tumor promotion with TPA produced greater inhibition of tumor multiplicity and tumor size than with either agent alone. Collectively, the greater ability of the combination of ursolic acid + resveratrol to inhibit skin tumor promotion was due to the greater inhibitory effects on growth factor and inflammatory signaling, skin inflammation, and epidermal hyperproliferation induced by TPA treatment. *Cancer Prev Res; 8(9); 817–25. ©2015 AACR.*

Introduction

Ursolic acid is a natural pentacyclic triterpenoid carboxylic acid found in many plants, including *P. frutescens* (Japanese basil), rosemary, apples, elder flowers, and many others. Ursolic acid has been shown to have apoptotic, anti-inflammatory, and antitumorigenic effects in various cancer models, including prostate, ovary, stomach, intestine, and skin (1–3). Further studies have revealed that ursolic acid has broad-spectrum anticarcinogenic effects, including prevention of DNA damage, inhibition of EGFR/MAPK signaling, inhibition of angiogenesis, activation of apoptotic pathways, and inhibition of the Akt/mTOR, NF-κB, Cox-2, and STAT3 signaling pathways (1, 4). Although several studies have reported that ursolic acid inhibited carcinogen and 12-O-tetracanoylphorbol-13-acetate (TPA)–induced inflammation, hyperplasia, and tumor promotion in mouse skin (2, 3, 5), its inhibitory mechanism on skin tumor promotion is not fully understood. Recently, several studies reported that ursolic acid has an anti-obesity effect and mimics some of the effects of calorie restriction by modulating the Akt/mTOR signaling pathways (6–8). Ursolic acid has also been shown to activate the LKB1/AMPK pathway for inhibition of adipogenesis (9).

Resveratrol is a phytoalexin and is present in grapes, berries, peanuts, and red wine. Resveratrol has been shown to have cardiovascular benefit and anti-diabetic effects in both mice and humans. In addition, resveratrol was shown to inhibit skin tumor promotion and also inhibit the growth of many cancer cell lines, including breast, prostate, colon, and liver (5, 10–13). Mechanisms associated with the antitumor-promoting effects of resveratrol include inhibition NF-κB, AP-1, and Cox-2 (10, 13, 14). Several reports have suggested that resveratrol also mimics some of the effects of calorie restriction on lifespan in worms and other model organisms, especially by inhibiting inflammation and mTOR signaling (15, 16). Resveratrol also mimics effects of calorie restriction by increasing SirT1 and AMPK activation (17). Boily and colleagues have suggested that the antipromoting effect of resveratrol on mouse skin is at least partially mediated by SirT1 (18).

Emerging evidence suggests that combinations of phytochemicals may be an effective strategy to achieve a greater chemopreventive effect than with single agents (19–21). Several studies have shown that combinations of natural

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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compounds can produce potential synergistic inhibitory effects in various cancers (e.g., resveratrol + grape seed extract and ellagic acid + grape seed extract; refs. 12, 20–24). Recently, Junco and colleagues reported that resveratrol potentiates the growth inhibitory effect of ursolic acid in mouse skin papilloma and carcinoma cell lines (25). Thus, combining agents may provide the most rational and effective approach to cancer chemoprevention. In addition, using combinations of phytochemicals may produce overall effects that more similarly mimic calorie restriction.

In the current study, topical treatment with a combination of ursolic acid + resveratrol produced a greater inhibitory effect on skin tumor promotion by TPA than with either agent alone. Further mechanistic studies revealed that this combination produced a greater inhibition of multiple growth factor and inflammation signaling pathways as well as greater upregulation of tumor suppressor genes, such as p21 and PDCD4. Interestingly, the combination of ursolic acid + resveratrol induced a dramatic increase of p-AMPK-α^{Thr172} and its downstream target p-Ulk1-^{Ser555}. Collectively, the current data suggest that combined treatment of ursolic acid + resveratrol is a more effective inhibitor of skin tumor promotion than either ursolic acid or resveratrol given alone. The mechanism for this greater inhibition appears to be multifaceted with similarities to changes observed with calorie restriction.

Materials and Methods

Animals and diets

For all experiments except the label retaining cell assay (LRC assay), Female Hsd: ICR (CD-1) mice 6 to 7 weeks of age were used and purchased from Harlan Laboratories Inc. For LRC assays, 10-day-old mice were obtained by breeding FVB/N female and male mice (purchased from the NCI, Bethesda, MD). Mice were group housed in a 12-hour dark/12-hour light cycle at 24°C for all experiments. For the short-term experiments, mice were fed a regular chow diet. For tumor experiments, mice received either an overweight control diet (D12450B, 10 Kcal% fat; Research Diets Inc.) or an obesity-inducing diet (D12492, 60 Kcal% fat; Research Diets Inc). All animal experiments were conducted in accordance with both Institutional as well as NIH guidelines under an approved Institutional Animal Care and Use Committee protocol.

Two-stage skin carcinogenesis assays

Female ICR mice (n = 30/group) 7 to 8 weeks of age were shaved on the dorsal skin and then 48 hours later initiated with a single topical application of 25 nmol of 7, 12-dimethylbenz[a]anthracene (DMBA) (Sigma-Aldrich) in 0.2 mL acetone or acetone vehicle. Two weeks after initiation, mice were randomized to receive one of the two experimental diets (overweight control and obesity-inducing diet) for 6 weeks before starting treatment with the tumor promoter, TPA. During tumor promotion, mice received 2 μmol of ursolic acid (Sabinsa Corporation) and 2 μmol of resveratrol (Orchid Chemicals & Pharmaceuticals Ltd.) 15 minutes prior to each TPA application. For the combination, resveratrol was given 30 minutes and ursolic acid was given 15 minutes prior to each 6.8 nmol dose of TPA (LC Laboratories) to allow time for absorption of each compound prior to TPA application. All other aspects of the tumor experiments were as previously described (26–28).

Short-term treatment protocol

For a number of experiments, mice were treated using a short-term treatment protocol involving 4 applications of TPA. For this protocol, groups of mice (7–8 weeks of age) were shaved on the dorsal skin and then 2 days later treated twice weekly for two weeks with 0.2 mL acetone vehicle, ursolic acid (2 μmol), or resveratrol (2 μmol) 15 minutes prior to each 6.8 nmol of TPA treatment. For the combination, mice received ursolic acid (2 μmol) and resveratrol (2 μmol) 15 minutes and 30 minutes prior to TPA treatment, respectively. Mice were then sacrificed at various times thereafter for collection of epidermal tissue.

Label retaining cell assay

For these experiments, 10-day old FVB/N mice were injected with bromodeoxyuridine (BrdUrd; 50 μg/g body weight) intraperitoneally every 12 hours over 2 days. Seventy days later, mice were shaved on the dorsal skin and then treated with the short-term protocol. Mice were sacrificed 48 hours after the last treatment and dorsal skin samples were prepared and analyzed as previously described (28).

Histologic analyses

For analysis of epidermal thickness and labeling index as well as the number of dermal inflammatory cells, mice were shaved on the dorsal skin and then treated with the short-term protocol. All procedures for these analyses were as previously described (26–28).

Preparation of epidermal protein lysates, cytosolic fractions, nuclear fractions, and RNA

Groups of mice were treated with the short-term protocol and then sacrificed 6 hours after the last TPA treatment. After sacrifice, epidermal protein lysates were collected as previously described (26). For electrophoretic mobility shift assays (EMSA), the epidermal cytosolic and nuclear fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific Inc). The protein lysates and nuclear/cytosolic fractions were used immediately or stored at −80°C until used. Epidermal RNA samples were isolated as previously described (29, 30) and subjected to quantitative real-time PCR (qRT-PCR) analysis.

Western blot analysis

Western blot analyses were performed as previously described (26). Antibodies used are listed in Supplementary Table S1.

EMSA

EMSA was performed using a DNA-protein binding detection kit according to the manufacturer's protocol (Thermo Scientific Inc.). See Supplementary Table S2 for the sequences of the NF-κB, Egr-1, and AP-1 oligos used.

qRT-PCR analysis

qRT-PCR analyses were performed as previously described (29, 30). cDNA (150 ng) was mixed with 2× TaqMan gene expression master mix (AB Applied Biosciences), 20× primer sets (*Il1a, Il1b, Il22, Rn18s*), and nuclease-free water in a total volume of 10 μL. For qPCR of Cox-2 mRNA, 2X iTaq Universal SYBR Green Supermix (Bio-Rad), 1 μmol/L primers (*Cox-2* and *Gapdh*), and nuclease-free water were added to cDNA (150 ng).

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The mixtures were then subjected to qRT-PCR using ViiA 7 real-time instrument and analysis software.

Statistical analysis

For comparisons of quantitative protein expression, gene expression, epidermal thickness, labeling index, the number of infiltrated inflammatory cells, transcriptional activities, and tumor multiplicity and tumor size, the Mann–Whitney *U* test was used. A one-tailed Fisher exact test and a Mantel–Cox test were used for comparisons of tumor incidence and tumor latency, respectively. Significance in all cases was set at $P \leq 0.05$.

Results

Effect of ursolic acid + resveratrol on skin tumor promotion by TPA

The ability of a combination of ursolic acid + resveratrol to inhibit skin tumor promotion by TPA was evaluated in ICR mice maintained on either an overweight control diet or a diet-induced obesity diet (DIO diet). After completion of the tumor experiment, the tumor responses in both diet groups were similar for all groups (see Supplementary Fig. S1A and S1B). Therefore, the data for the corresponding treatment groups on each diet were combined as presented in Fig. 1. As shown in Fig. 1, pretreatment with ursolic acid or resveratrol alone inhibited tumor multiplicity by 38.6% and 20.8%, respectively. The reduction in tumor multiplicity with ursolic acid was statistically significant ($P < 0.05$; Mann–Whitney *U* test) compared with the group treated with TPA alone. Pretreatment with the combination of ursolic acid + resveratrol resulted in 56% reduction in tumor multiplicity that was significantly lower when compared with both resveratrol + TPA and ursolic acid + TPA groups ($P < 0.05$; Mann–Whitney *U* test). Furthermore, the incidence of papillomas in the mice treated with ursolic acid + resveratrol was significantly lower than that observed in the TPA and resveratrol + TPA–treated groups ($P < 0.05$; Fisher exact test) but not the ursolic acid + TPA group. An effect on tumor latency was also observed as shown in Fig. 1C. In this regard, the percent of tumor-free mice treated with the combination of ursolic acid + resveratrol was significantly higher than that of the TPA-only

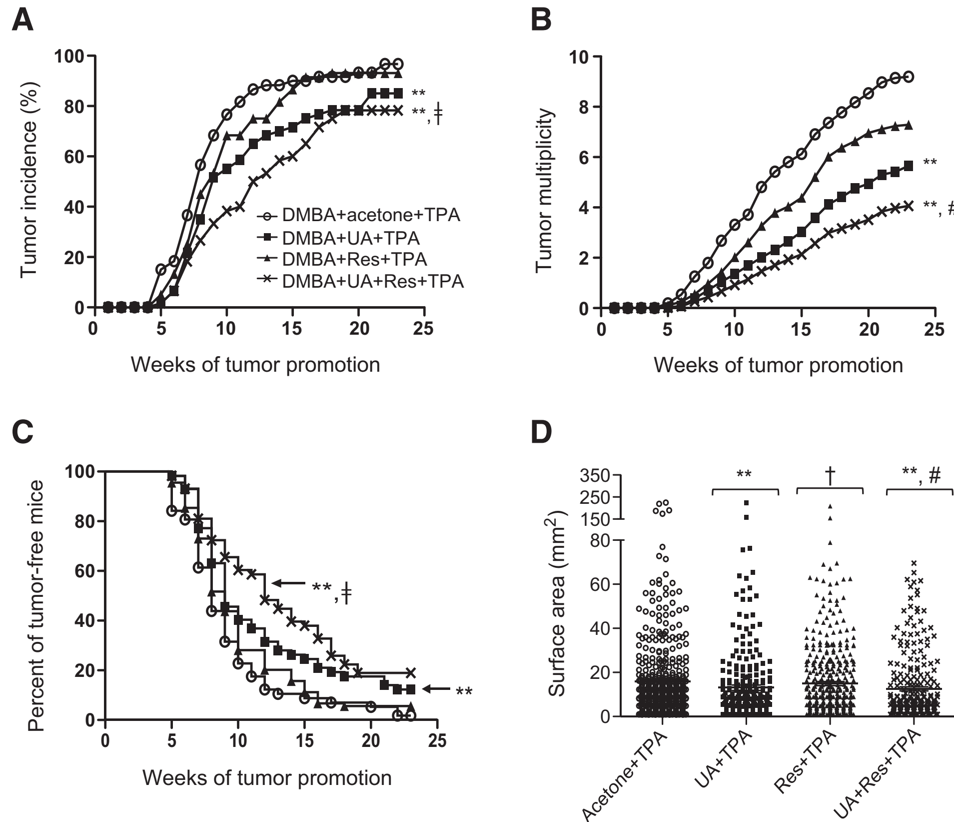

Figure 1.

Effect of ursolic acid (UA) + resveratrol (Res) on skin tumor promotion in ICR mice. A, incidence of tumors (percentage of mice with papillomas). The percentage of mice with papillomas in the group treated with ursolic acid + resveratrol +TPA was significantly lower than TPA (**) or resveratrol + TPA (‡) treated groups ($P < 0.05$; Fisher exact test). B, tumor multiplicity (average number of papillomas per mouse). Both the ursolic acid and the ursolic acid + resveratrol–pretreated groups had significantly reduced tumor multiplicity compared with the TPA-treated group (**, $P < 0.05$). The tumor multiplicity in the ursolic acid + resveratrol + TPA-treated (#, $P < 0.05$; Mann–Whitney *U* test) was also significantly lower than both ursolic acid + TPA and resveratrol + TPA-treated groups. C, tumor latency (tumor-free survival). Significant differences were observed between the ursolic acid + TPA and ursolic acid + resveratrol + TPA compared with the TPA-only group (**, $P < 0.05$; Mantel–Cox test). Percent of tumor free mice in the combination group (‡, $P < 0.05$; Mantel–Cox test) was greater than the resveratrol + TPA group. D, tumor size. The surface area of papillomas was measured at the 23rd week. **, $P < 0.05$ when compared with the TPA group; †, $P < 0.05$ when compared with the ursolic acid + TPA group; and #, $P < 0.05$ when compared with both the resveratrol + TPA and ursolic acid + TPA groups. The Mann–Whitney *U* test was used for all statistical comparisons of tumor size.

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and resveratrol + TPA groups over the 23-week observation period ($P < 0.05$; Mantel–Cox test) but not the ursolic acid + TPA-treated group.

As shown in Fig. 1D, the combination of ursolic acid + resveratrol significantly reduced the size of papillomas compared with the TPA-only group as well as both the ursolic acid + TPA and resveratrol + TPA groups ($P < 0.05$; Mann–Whitney U test). Thus, the combination was more effective at reducing both the number and size of papillomas when compared with either agent given alone.

Body weight gain for the 23-week experiment for each group is shown in Supplementary Fig. S1C and S1D. As expected, there were significant differences in body weight between untreated mice on the overweight control and DIO diets (41.57 ± 2.49 g and 56.57 ± 2.39 g, respectively; $P < 0.05$; Mann–Whitney U test). No significant differences were observed in body weight between the treated groups in mice on either the control diet or DIO diet. Overall, these data suggest that the combined treatment of ursolic acid + resveratrol, at the doses used, had a greater inhibitory effect on skin tumor promotion compared with the groups pretreated with either of the compounds alone and with no apparent toxicity.

Effect of ursolic acid + resveratrol treatment on TPA-induced epidermal hyperproliferation and LRCs

As shown in Fig. 2A and B, ursolic acid or resveratrol alone significantly reduced BrdUrd incorporation and epidermal thickness following TPA treatment ($P < 0.05$; Mann–Whitney U test). However, treatment with the combination of ursolic acid + resveratrol produced a greater inhibition of BrdUrd incorporation and epidermal thickness induced by TPA compared with that observed with either of the compounds given alone with TPA ($P < 0.05$; Mann–Whitney U test).

As shown in Supplementary Fig. S2, LRCs in acetone-treated mice were confined to the hair follicle bulge-region as expected on the basis of previous studies (28, 30). However, after a 2-week treatment regimen with TPA, the LRCs can be seen moving up and out of the hair follicle into the interfollicular epidermis (Supplementary Fig. S2A). Pretreatment with ursolic acid or resveratrol partially inhibited the effect of TPA on proliferation and migration of LRCs. However, treatment with the combination of ursolic acid + resveratrol prior to application of TPA produced a greater inhibitory effect on the proliferation and migration of these cells compared with the ursolic acid- or resveratrol-only treated groups (Supplementary Fig. S2B; $P < 0.05$; Mann–Whitney U test).

Effect of ursolic acid + resveratrol on TPA-induced epidermal signaling pathways

As shown in the Fig. 3A and B, ursolic acid + resveratrol significantly inhibited TPA-activated p-STAT3^{Tyr705}, p-Akt^{Thr308}, p-NF-κB p65^{Ser536}, p-JNK1/2$^{Thr183/Tyr185}$, p-c-Jun^{Ser73}, and p-p38 MAPK$^{Thr180/Tyr182}$, whereas ursolic acid or resveratrol alone either produced no significant effects or a moderate inhibition of phosphorylation of these proteins relative to the combination. Cox-2 induction by TPA was not significantly decreased by pretreatment with either ursolic acid or resveratrol alone; however, the combination of ursolic acid + resveratrol produced a statistically significant inhibition of Cox-2 induction by TPA. The levels

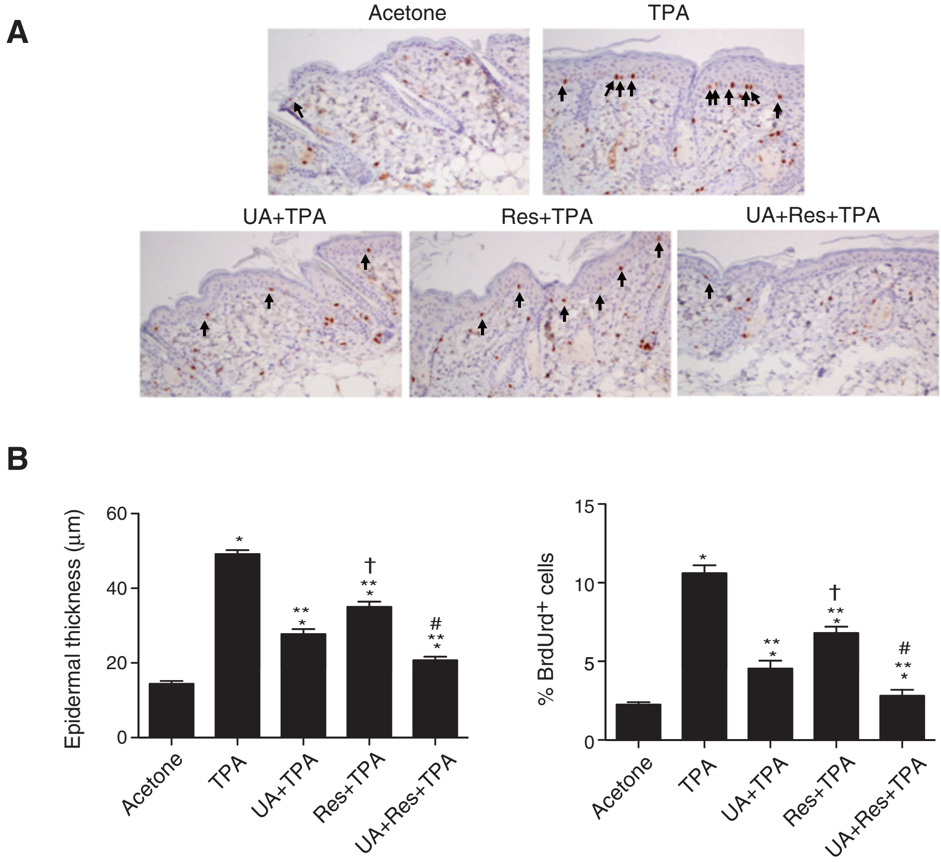

Figure 2.
Effect of ursolic acid (UA) + resveratrol (Res) on TPA-induced epidermal hyperproliferation in ICR mice. Female ICR mice at 7 to 8 weeks of age maintained on standard chow diet were treated topically with the short-term protocol. Dorsal skin was collected at 48 hours after the last treatment for histologic evaluation. Sections were stained with hematoxylin (H&E) and for BrdUrd incorporation. A, representative sections of BrdUrd stained skin. Arrows indicate BrdUrd-positive cells. Magnification, 20×. B, quantitative analysis of the effects of ursolic acid, resveratrol, or ursolic acid + resveratrol on TPA-induced epidermal thickness and labeling index (% BrdUrd-positive cells). The values in B represent the means ± SEM. *, $P < 0.05$ when compared with the acetone-treated group; **, $P < 0.05$ when compared with the TPA-treated group; †, $P < 0.05$ when compared with the ursolic acid + TPA group; #, $P < 0.05$ when compared with the ursolic acid + TPA and resveratrol + TPA group. All statistical analyses were performed using the Mann–Whitney U test.

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of several tumor suppressors were also evaluated (see again Fig. 3A and B). The combination of ursolic acid + resveratrol significantly reversed the effect of TPA on PDCD4 and p21 levels while pretreatment with either compound alone had no effect. In contrast, none of the treatments reversed the effects of TPA treatment on p27 levels.

The phosphorylation of both EGFR^{Tyr1086} and Src^{Tyr416} was also significantly inhibited by the combination of ursolic acid + resveratrol at the doses and time point examined, whereas neither ursolic acid nor resveratrol alone significantly inhibited phosphorylation of these proteins. On the other hand, the increased level of Fas induced by TPA was decreased by treatment with resveratrol alone and the combination of ursolic acid + resveratrol but not with ursolic acid. Again, the combination was the most effective at inhibiting the increase in Fas seen following treatment with TPA.

As shown in Fig. 4A and B, treatment with TPA alone produced an approximately 2.5-fold increase in p-AMPK-α^{Thr172} compared with the acetone-treated control group. Both ursolic acid and resveratrol when given with TPA further increased p-AMPK-α^{Thr172}, while the combination of ursolic acid + resveratrol together with TPA produced an even greater activation of AMPK-α that was significantly greater than with either ursolic acid or resveratrol given alone ($P < 0.05$). The level of SirT1 was not changed by treatment with TPA or pretreatment with any of the compounds given together with TPA, including the combination of ursolic acid + resveratrol (again see Fig. 4A and B). TPA treatment reduced the level of p-LKB1^{Ser428} compared with the acetone group; however, neither ursolic acid nor resveratrol had any further effect. In contrast, the level of p-LKB1^{Ser428} was further decreased when the combination of ursolic acid + resveratrol was given before TPA treatment. TPA treatment led to activation of

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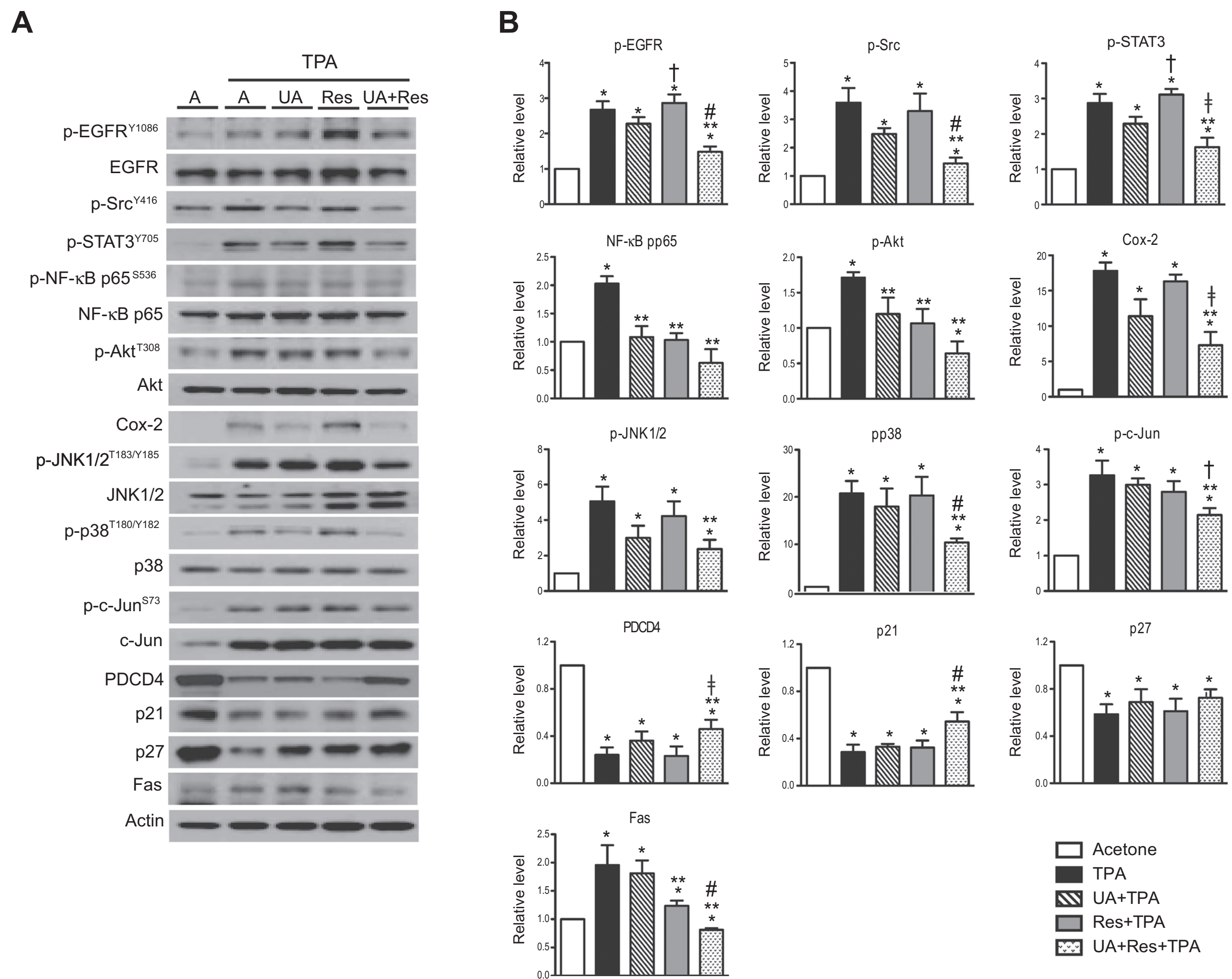

Figure 3.
Effect of ursolic acid (UA) + resveratrol (Res) on TPA-induced signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice ($n = 4$–5/group) receiving treatment with the short-term protocol. A, representative Western blot analyses of multiple signaling pathways. B, quantitative evaluation of Western blot data. Values represent means ± SEM from at least three independent experiments. *, $P < 0.05$ when compared with the acetone-treated group; **, $P < 0.05$ when compared with the TPA-treated group; †, $P < 0.05$ when compared with the ursolic acid + TPA-treated group; ‡, $P < 0.05$ when compared with the resveratrol + TPA-treated group; and #, $P < 0.05$ when compared with the ursolic acid + TPA and resveratrol + TPA-treated groups. The Mann–Whitney U test was used for all statistical comparisons.

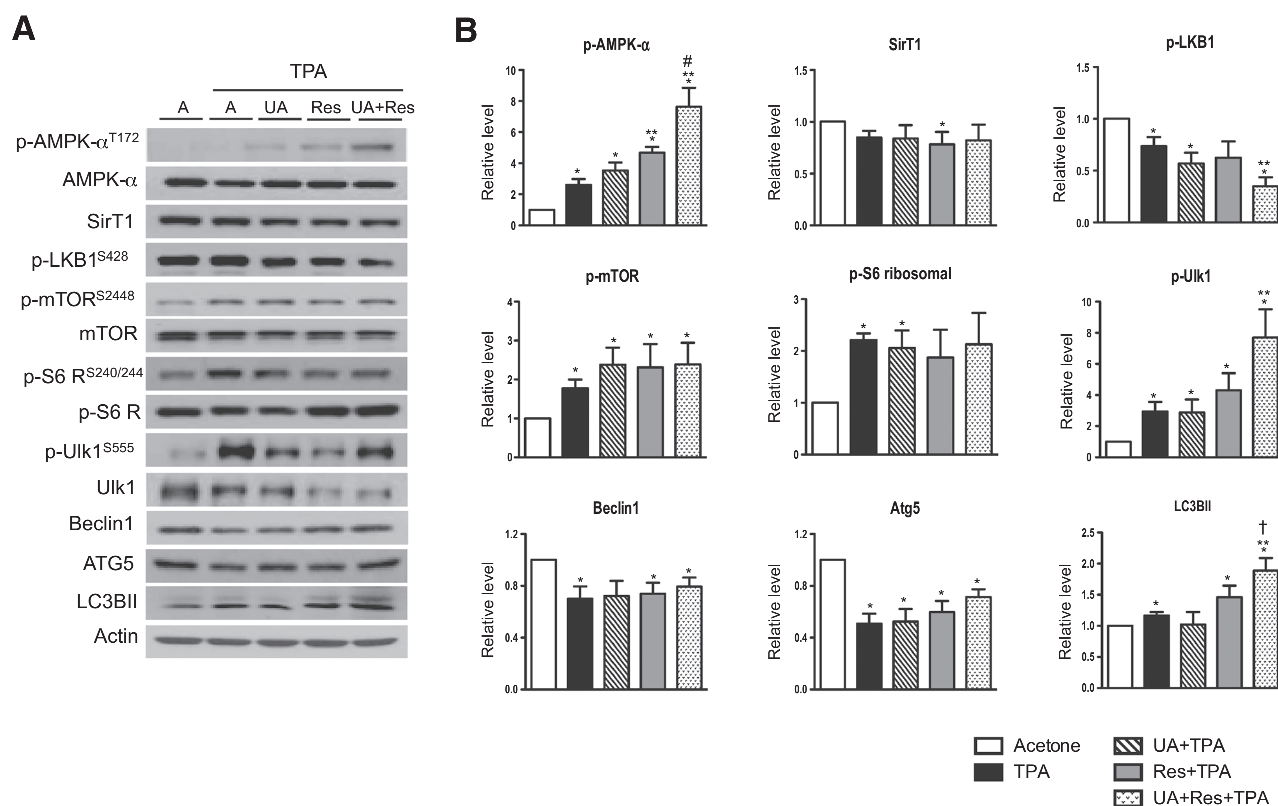

Figure 4.
Effect of ursolic acid (UA) + resveratrol (Res) on TPA-induced AMPK and mTORC1 signaling pathways in epidermis of female ICR mice. Western blot analyses were performed using pooled epidermal protein lysates from mice (n = 4–5/group) that received multiple treatments with short-term protocol. A, representative Western blot analyses. B, quantitative evaluation of the effect of ursolic acid, resveratrol, or ursolic acid + resveratrol on the TPA-induced AMPK-α signaling pathway. Values represent means ± SEM from at least three independent experiments. *, $P < 0.05$ when compared with the acetone group; **, $P < 0.05$ when compared with the TPA group; †, $P < 0.05$ when compared with ursolic acid + TPA group; ‡, $P < 0.05$ when compared with the resveratrol + TPA-treated group; and #, $P < 0.05$ when compared with ursolic acid + TPA and resveratrol + TPA group. The Mann–Whitney U test was used for statistical comparisons.

mTORC1 signaling as previously reported (26, 28, 31); however, the levels of p-mTORC1^{Ser2448} and its downstream target, p-S6-ribosomal protein$^{Ser240/244}$, were not affected by treatment with ursolic acid, resveratrol, or the combination of ursolic acid + resveratrol. Notably, the level of p-Ulk1^{Ser555} was significantly increased when the combination of ursolic acid + resveratrol was given together with TPA. The combination of ursolic acid + resveratrol when given together with TPA significantly increased the level of LC3IIB compared with the acetone, TPA, and ursolic acid + TPA groups while the levels of ATG5 and Beclin1 that were reduced by TPA treatment were not significantly altered further by any of the treatments.

Effect of ursolic acid + resveratrol on TPA-induced inflammation and inflammatory gene expression

As shown in Fig. 5A, the levels of *Il1a*, *Il1b*, *Il22*, and *Cox-2* mRNA were increased following treatment with TPA (given twice weekly for 2 weeks) and significantly decreased in the ursolic acid + resveratrol pretreated group ($P < 0.05$; Mann–Whitney U test). With the exception of ursolic acid pretreatment on Cox-2 mRNA, neither ursolic acid nor resveratrol pretreatment significantly reduced the mRNA levels of these inflammatory genes. As shown in Supplementary Fig. S3 and Fig. 5B and C, pretreatment with ursolic acid and resveratrol decreased the number of mast cells in the dermis seen following TPA treatment; however, an additional decrease in the number of dermal mast cells was observed after treatment with ursolic acid + resveratrol + TPA ($P < 0.05$; Mann–Whitney U test). Ursolic acid alone and ursolic acid + resveratrol also produced a significant decrease in the number of CD45$^+$ cells in dermis ($P < 0.05$). Again, the combination of ursolic acid + resveratrol produced the greatest reduction in the numbers of both mast cells and CD45$^+$ cells.

Effect of ursolic acid + resveratrol on NF-κB, Egr-1, and AP-1 DNA-binding activities induced by TPA

Treatment with TPA significantly increased the amount of NF-κB, Egr-1, and AP-1 bound to their consensus DNA-binding oligos (Supplementary Figs. S4A–S4C and S5A–S5C). The TPA-induced increase in DNA-binding activity of all three transcription factors was significantly reduced by pretreatment with the combination of ursolic acid + resveratrol. Pretreatment with ursolic acid or resveratrol alone significantly reduced binding of NF-κB and ursolic acid pretreatment significantly reduced binding of AP-1. Thus, the combination of ursolic acid + resveratrol was highly effective at inhibiting the activation of all three of these transcription factors by TPA. In addition, the nuclear translocation of NF-κB, Egr-1, and AP-1 induced by TPA was also significantly inhibited by ursolic acid + resveratrol to a greater extent than

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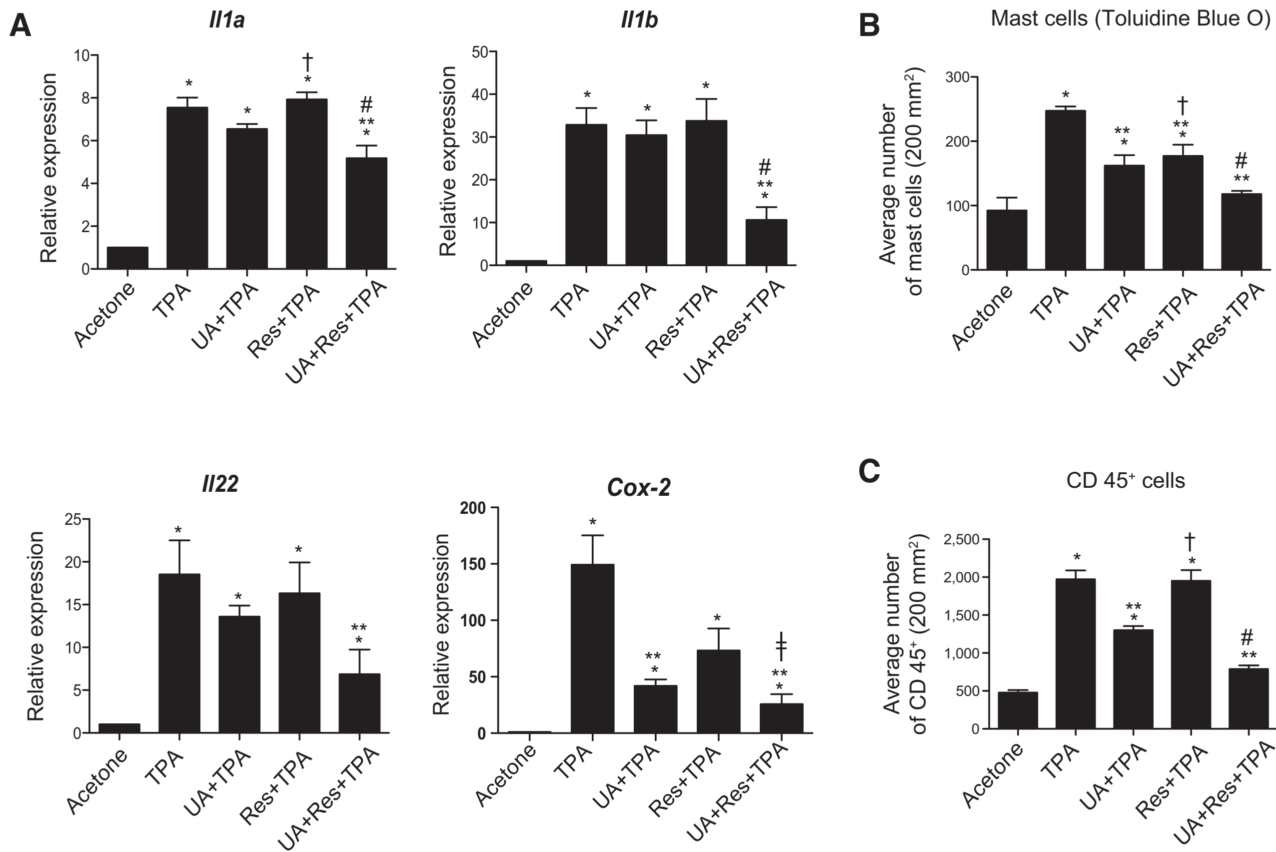

Figure 5.
Effect of ursolic acid (UA) + resveratrol (Res) on TPA-induced inflammatory gene expression and inflammatory cell infiltration. Epidermal RNA samples were prepared from groups of female ICR mice ($n = 4$–5/group) treated using the short-term protocol. RNA samples were then subjected to qRT-PCR analysis as described in Materials and Methods. A, qRT-PCR analysis of *Il1a*, *Il1b*, *Il22*, and *Cox-2*. mRNA levels of *Il1a*, *Il1b*, and *Il22* were normalized to *Rn18s* and the mRNA level of Cox-2 was normalized to GAPDH. B, quantitative evaluation of the effect of ursolic acid, resveratrol, and ursolic acid + resveratrol on the number of mast cells in the dermis 48 hours after the last TPA treatment. Positive cells were counted per 200 mm^2. C, quantitative analysis of the effect of ursolic acid, resveratrol and ursolic acid + resveratrol on the number of CD45-positive cells in the dermis 48 hours after the last TPA treat. Positive cells were counted per 200 mm^2. The graphs in all cases represent means ± SEM of at least three independent experiments. *, $P < 0.05$ when compared with the acetone group; **, $P < 0.05$ when compared with the TPA group; †, $P < 0.05$ when compared with ursolic acid + TPA group; ‡, $P < 0.05$ when compared with the resveratrol + TPA group; and #, $P < 0.05$ when compared with the ursolic acid + TPA and resveratrol + TPA group. The Mann–Whitney *U* test was used for statistical comparisons.

pretreatment with either ursolic acid or resveratrol (Supplementary Figs. S4G–S4I and S5D–S5F).

Discussion

In the current study, topical application of ursolic acid + resveratrol followed by TPA treatment inhibited skin tumor promotion to a greater extent when compared with the groups treated with either resveratrol + TPA or ursolic acid + TPA alone. Further analyses revealed that the greater ability of the combination to inhibit skin tumor promotion correlated with a greater ability to inhibit epidermal proliferation induced by TPA. In addition, combined treatment with ursolic acid + resveratrol produced greater inhibitory effects on TPA-induced epidermal signaling pathways, including EGFR, STAT3, Fas, Src, Akt, Cox-2, NF-κB, p38 MAPK, and JNK1/2. Notably, treatment with the combination also increased the levels of the tumor suppressor proteins p21 and PDCD4 compared with the groups treated with the individual compounds. Both ursolic acid and resveratrol treatment followed by TPA increased AMPK-α activation; however, the combination of ursolic acid + resveratrol together with TPA produced an even greater activation of AMPK-α. Further studies revealed that the activation of NF-κB, Egr-1, and AP-1 (DNA-binding activity and nuclear translocation) were significantly inhibited by the combination of ursolic acid + resveratrol compared with the groups treated with either ursolic acid or resveratrol alone. The combination also produced greater effect on TPA-induced inflammation and inflammatory gene expression. Overall, the current data indicate that combined treatment with ursolic acid + resveratrol led to a greater inhibitory effect on skin tumor promotion than either compound alone via effects on multiple events and pathways critical to the process of skin tumor promotion.

As noted in the Introduction, ursolic acid was previously shown to have inhibitory effects on TPA-induced skin inflammation as well as skin carcinogenesis (2, 5). Resveratrol was also shown to be as an effective inhibitor on skin tumor promotion (5, 10, 13, 32) along with inhibitory effects on breast, colorectal, hepatic,

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pancreatic, and prostate cancers (11). Recently, several studies have shown potential combinatorial chemopreventive effects with these agents in preclinical models of cancer. For example, melatonin was shown to potentiate the inhibitory effect of ursolic acid on proliferation and apoptosis in colon cancer cells by modulating multiple signaling pathways, including caspases, PARP, NF-κB, and Cox-2 (33). Kowalczyk and colleagues (5) tested a combination of 2% calcium D-glucarate (CG) given in the diet, with either 2.5 μmol of resveratrol or 1 μmol of ursolic acid applied topically in two-stage skin carcinogenesis model. In this study, ursolic acid applied alone and in combination with CG showed inhibitory effects on skin tumor incidence and multiplicity.

Combinations of resveratrol with other phytochemicals have been shown to have a greater inhibitory effect in several tumor models. For example, combined dietary administration of resveratrol, quercetin, and catechin (combinations at 0.5, 5, or 25 mg/kg) reduced primary tumor growth of breast cancer xenografts in a nude mouse model (34). Resveratrol + black tea polyphenol inhibited mouse skin tumor growth by modulating MAPKs and p53 (32). In other studies, resveratrol + curcumin produced a better chemopreventive effect by maintaining adequate zinc and regulating p21 and Cox-2 level during lung carcinogenesis (35). Genistein + resveratrol also reduced the most severe grade of prostate cancer in the SV-40 tag rat (23).

As shown in the current study, the combination of ursolic acid + resveratrol was a more effective inhibitor of skin tumor promotion by TPA in ICR mice than either agent alone at the dose used and this was true for both tumor multiplicity and tumor size (see again Fig. 1). Although we did not design the current studies to analyze the development of squamous cell carcinomas (SCC), papillomas are considered premalignant tumors and previous studies have shown that reductions in numbers of papillomas leads to reductions in SCCs (27, 36, 37). As noted above, previous studies have shown that ursolic acid broadly inhibited a number of signaling pathways, including EGFR, MAPK, Akt/mTOR, NF-κB, Cox-2, and STAT3, in a variety of cell types, including mouse epidermis *in vivo* (1, 4). Furthermore, mechanisms associated with the antitumor promoting effects of resveratrol include modulation of NF-κB, Cox-2, mTORC1, and SirT1 (10, 13, 17, 18). In our current study, we evaluated a number of oncogenic signaling molecules, including EGFR, Src, STAT3, Fas, NF-κB, Akt, p38, JNK1/2, c-Jun, and mTOR, as well as the tumor suppressors p27, p21, PDCD4, and AMPK. As shown in Figs. 3 and 4, we found that the combination of ursolic acid + resveratrol was more effective at altering these pathways during tumor promotion than either ursolic acid or resveratrol given alone. In particular, the combination was significantly more effective at inhibiting TPA-induced activation (phosphorylation) of EGFR, Src, and p38 MAPK and at altering the levels of Fas (decrease) and p-AMPK-α^{Thr172} and p21 (increase) compared with either ursolic acid or resveratrol given alone. In addition, the combination of ursolic acid + resveratrol produced the greatest inhibition of NF-κB, Egr-1, and AP-1 DNA-binding activities and nuclear translocation (Supplementary Figs. S4 and S5). All three of these transcription factors are known to be upregulated during skin tumor promotion and skin carcinogenesis (38, 39). Thus, the combination of ursolic acid + resveratrol produced a more global and robust inhibition of epidermal signaling pathways compared with either ursolic acid or resveratrol given alone.

TPA-induced epidermal hyperproliferation is required for its tumor-promoting activity (37, 39, 40). Previous studies have shown that topical treatment of both 1 μmol of ursolic acid and 2 μmol of resveratrol reduced TPA-induced BrdUrd incorporation in SENCAR mouse skin (5). In the current study, we observed that the combination of ursolic acid + resveratrol together with TPA at a dose of 2 μmol each inhibited epidermal hyperproliferation to a greater extent than with either compound alone at the same dose. The greater inhibition of TPA-induced epidermal hyperproliferation with the combination was likely due to the greater effects observed on the multiple signaling pathways noted above. Skin inflammation is also known to be an important component of the process of skin tumor promotion by TPA involving the production of proinflammatory cytokines and infiltration of inflammatory cells (39, 41, 42). Again, as seen with the analyses of epidermal proliferation, the combination produced greater inhibition of inflammation. In this regard, the combination of ursolic acid + resveratrol inhibited to a greater extent the increased expression of *Il1a*, *Il1b*, *Il22*, and *Cox-2* seen following treatment with TPA. In addition, as shown in Fig. 5B, the number of mast cells was decreased by either ursolic acid or resveratrol treatment followed by TPA; however, the combination gave rise to an even greater inhibitory effect. The number of lymphocytes, monocytes, and leukocytes ($CD45^+$ cells) in the dermis were also significantly inhibited by the combination of ursolic acid + resveratrol compared with the individual compounds alone. The greater inhibition of inflammation by the combination of ursolic acid + resveratrol was likely due to the greater inhibition of inflammatory signaling pathways and to a greater reduction in NF-κB DNA-binding activity.

In conclusion, the current study shows for the first time the efficacy of a combination of ursolic acid + resveratrol for inhibition of tumor promotion in mouse skin. This combination of ursolic acid + resveratrol produced a greater inhibition of skin tumor promotion by TPA compared with ursolic acid or resveratrol alone. In addition, the combination targeted multiple TPA-induced signaling pathways involved in both epidermal proliferation and inflammation and produced effects on a number of these pathways greater than either compound alone. For the current experiments, we choose to apply the compounds via the topical route. An important goal for future studies will be to examine the efficacy of this and other combinations, when given in the diet. Combining phytochemicals such as ursolic acid and resveratrol appears to produce a calorie restriction mimetic type of effect by targeting multiple signaling pathways and should be explored further for potential cancer chemopreventive efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Cho, T.J. Slaga, J. DiGiovanni
Development of methodology: J. Cho, O. Rho, J. DiGiovanni
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Cho, J.J. Junco, D. Siegel, J. DiGiovanni
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Cho, O. Rho, T.J. Slaga, J. DiGiovanni
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Carbajal, J. DiGiovanni
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