The phytonutrient ursolic acid (UA), present in apples, rosemary, and other plant sources, has anti-cancer properties in a number of systems, including skin cancers. However, few reports have examined upstream mechanisms by which UA may prevent or treat cancer. Recent reports have indicated UA induces death of cancer cell lines via AMP-activated protein kinase (AMPK), an energy-sensing kinase which possesses both pro-metabolic and anti-cancer effects. Other studies have shown UA activates peroxisome proliferator activated receptor α (PPARα) and the glucocorticoid receptor (GR). Here, we found the cytotoxic effect of UA in skin carcinoma cells required AMPK activation. In addition, two inhibitors of PPARα partially reversed the cytotoxic effects of UA, suggesting its effects are at least partially mediated through this receptor. Finally, inhibition of the GR did not reverse the effects of UA nor did this compound bind the GR under the conditions of experiments performed. Overall, studies elucidating the anti-cancer effects of UA may allow for the development of more potent analogues utilizing similar mechanisms. These studies may also reveal the mediators of any possible side effects or resistance mechanisms to UA therapy.

**KEYWORDS**
AMP-activated protein kinases, neoplasms, phytochemicals

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**INTRODUCTION**

The natural phytonutrient ursolic acid (UA) has been shown to have both anti-cancer and anti-diabetic effects in a variety of in vitro and in vivo systems. A number of studies indicated UA activates the energy sensor AMP-activated protein kinase (AMPK), which has both anti-cancer and anti-diabetic properties. AMPK is activated by both exercise and calorie restriction. These lifestyle factors induce a negative energy balance, and the resulting increases in the AMP/ATP ratio allosterically interact with and allow activation of AMPK via phosphorylation at threonine 172 (Thr172) by upstream kinases such as LKB1 and CAMKKβ. Activated AMPK stimulates...
catabolic processes such as fat oxidation and glucose uptake and inhibits anabolic processes such as fatty acid synthesis and gluconeogenesis to restore ATP levels.\textsuperscript{16,17}

AMPK also has anti-tumor properties. In this regard, AMPK loss leads to increased tumor formation in lymphoma-susceptible mice.\textsuperscript{18} LKB1, the upstream kinase of AMPK, also functions as a tumor suppressor.\textsuperscript{19} In addition, recent studies have shown the cytotoxic effects of UA against cancer cells are mediated by AMPK. The AMPK inhibitor Compound C decreased UA-mediated pro-apoptotic signaling in liver cancer cells.\textsuperscript{8} Another report found UA-induced apoptosis of bladder cancer cells was suppressed by knockdown of AMPK.\textsuperscript{7} In addition, inhibitory phosphorylation of AMPK was shown to increase during progression to human SCC,\textsuperscript{20} suggesting a potential role of AMPK as a suppressor of skin carcinoma formation or viability. These studies further suggest that the effects of UA against skin tumor growth in vivo\textsuperscript{3} may be mediated, at least in part, by AMPK activation.

Despite many reports demonstrating the anti-cancer effects of UA, very few studies have attempted to determine which upstream proximal receptor(s) contribute to these effects. The peroxisome proliferator activated receptor \(\alpha\) (PPAR\(\alpha\)) is primarily expressed in the liver, kidney, skeletal muscle, and heart and is important for lipid homeostasis and may have anti-inflammatory properties.\textsuperscript{21} Ligands for PPAR\(\alpha\) have been shown to inhibit skin tumorigenesis in vivo\textsuperscript{22} and activate AMPK.\textsuperscript{23} Also, synthesized derivatives of a UA isomer bound different PPARs\textsuperscript{24} and resulted in downstream anti-inflammatory and anti-skin tumor effects.\textsuperscript{25} In addition, UA enhanced PPAR\(\alpha\) promoter-binding and overall transcriptional activity in HepG2 liver cancer cells. However, UA did not directly bind PPAR\(\alpha\).\textsuperscript{26} Overall, these studies suggest that the anti-cancer effect of UA may also involve activation of PPAR\(\alpha\) through a mechanism independent of ligand binding.

Another potential candidate receptor for UA is the glucocorticoid receptor (GR), which has anti-inflammatory properties both through transrepressive interactions with pro-inflammatory transcription factors and through transactivation of promoters leading to synthesis of anti-inflammatory proteins.\textsuperscript{27} The structure of UA strongly resembles that of typical glucocorticoids, which also inhibit skin tumor promotion.\textsuperscript{28,29} UA also enhanced GR nuclear translocation\textsuperscript{20,31} and UA-mediated decreases in MMP-9 expression are reversed by GR antagonist RU486 in fibrosarcoma cells.\textsuperscript{30} Finally, UA has been shown to slightly bind the GR in breast cancer cells, even though UA did not affect GR-dependent transcription.\textsuperscript{31} Although these studies indicate UA does not impact the transcriptional activity of the GR, UA could still exert beneficial effects by mediating transrepressive protein-protein interactions between the GR and oncogenic transcription factors,\textsuperscript{32,33} which are suggested to mediate the anti-skin cancer effects of GR.\textsuperscript{34}

In the current studies, we used pharmacological inhibitors of AMPK, PPAR\(\alpha\), and GR to determine if these pathways contribute to the cytotoxic effects of UA in skin cancer cells. An accurate characterization of the anti-cancer effects of UA will assist with future clinical aspects, such as explanation of potential side effects, identification of resistance mechanisms, and the possibility of encountering target polymorphisms among non-melanoma skin cancer patients.

\textbf{FIGURE 1} UA-mediated AMPK activation was reversed with Compound C in Ca3/7 cells. A, Compound C reversed UA-mediated AMPK activation in Ca3/7 cells (average of four independent experiments, \(* P < 0.05\), \(** P < 0.01\)). B, Representative Western blot shown
2 | MATERIALS AND METHODS

2.1 | Reagents

GW6471 and MK 886 were purchased from Tocris Bioscience (Bristol, UK). UA, Compound C, RU486, dexamethasone, cortisol, corticosterone, and thiazolyl blue tetrazolium bromide (MTT reagent) were obtained from Sigma (St. Louis, MO). A kit for assaying lactate dehydrogenase (LDH) was purchased from Roche (Indianapolis, IN). [3H]dexamethasone was obtained from American Radiolabeled Chemicals (St. Louis, MO). Human recombinant GR was purchased from Invitrogen (Grand Island, NY). Primary antibodies against phosphorylated AMPK (Thr172) and AMPK were obtained from Cell Signaling Technology (Danvers, MA), while primary antibody against β-actin was purchased from Abcam (Cambridge, MA).

2.2 | Cell culture

A mouse squamous cell carcinoma cell line (Ca3/7) and a mouse skin papilloma cell line (MT1/2) were used for the current studies. The cells were maintained in Joklik MEM supplemented with 8% FBS, 50 U/mL penicillin, 50 ng/mL streptomycin, 10 μg/mL transferrin, 50 μg/mL gentamicin sulfate, 5 μg/mL insulin, 5 ng/mL EGF, 10 μM o-phosphorylethanolamine, and 10 μM 2-aminoethanol. The immortalized human keratinocyte cell line, HaCaT, were maintained in high glucose DMEM + GlutaMAX supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 ng/mL streptomycin. All cells were grown in an incubator at 5% CO2 and 37°C. The murine papilloma cell line MT1/2 was generated from papilloma from the MNNG/TPA skin carcinogenesis protocol. The squamous cell carcinoma cell line Ca3/7 was created with carcinoma from mice treated with the DMBA/TPA skin carcinogenesis protocol. MT1/2 and Ca3/7 cells were obtained from the University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX. Ca3/7 and HaCaT cells were verified free of mycoplasma the year before experiments were performed. The cell culture working environment was demonstrated to be mycoplasma-free after concluding all experiments. Each vial of cells was used within 2 months of thawing.

2.3 | Western blotting

Ca3/7 cells were incubated with 10 μM AMPK inhibitor Compound C or 0.1% DMSO vehicle for 4 h followed by incubation with UA or 0.1% DMSO vehicle for 6 h. Cells were rinsed twice with PBS and lysed in buffer containing 1% Triton X-100, 0.5% IGEPAL, 0.05 M TrisHCl and 0.1 M NaCl as well as protease/phosphatase inhibitors and 5 mM

FIGURE 2  The cytotoxic effect of UA on Ca3/7 cells was reversed by AMPK inhibition. A, UA-mediated decrease in Ca3/7 viability is reversed by AMPK inhibitor Compound C (IC50 reversal calculated from average of six independent experiments, n = 3/exp, representative experiment shown). B, UA-mediated increase in LDH release in Ca3/7 is reversed by Compound C (LD50 increase calculated from average of three independent experiments, n = 5/exp, representative experiment shown). For all experiments, **P < 0.01, ****P < 0.001.
EDTA. Proteins were extracted by centrifugation and quantified by Bradford. Proteins were denatured in XT Sample Buffer containing XT reducing agent. Proteins were separated on 4-12% gradient gels and transferred onto PVDF membranes, which were blocked in 5% bovine serum albumin or 5% milk in TBST for 1 h and incubated overnight with primary antibody. Blots were rinsed, incubated with secondary antibody for 1 h and developed with Pierce ECL2 Western blotting substrate.

2.4 | MTT assay for cell viability

Ca3/7 cells were pretreated with indicated doses of Compound C or 0.1% DMSO vehicle (4 h) or GW6471, MK 886, or 0.1% DMSO vehicle (1 h) followed by UA or 0.1% DMSO for 24 h. In the figures, “vehicle” refers to groups treated with UA but without the AMPK inhibitor Compound C, the PPARα inhibitors GW6471 and MK 866, or the GR inhibitor RU486. MTT reagent (0.5 mg/mL) was added, and cells were incubated for an additional 3 h. Plates were centrifuged at 100 g for 5 min, media was removed, and formazan crystals were solubilized with 100 μL DMSO. Plates were measured at 570 nm with a background subtraction at 650 nm, using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT).

2.5 | LDH assay for cell death

Ca3/7 cells were pretreated with 10 μM Compound C or 0.1% DMSO vehicle for 4 h followed by indicated doses of UA or 0.1% DMSO vehicle for 24 h. For positive control wells, 2% Triton X-100 was added. Cell plates were centrifuged at 100 g for 5 min, and 50 μL supernatant/well was transferred to a 96 well plates containing the LDH reagent prepared according to the manufacturer’s instructions. Plates were incubated for 20 min in the dark, then read at 490 nm with background subtraction at 650 nm using a Biotek Synergy HT spectrophotometer.

2.6 | Radioligand binding assays

HaCaT cells were plated at 4.0 × 10⁵ cells per well in 12-well plates in high glucose DMEM with 10% Chelex-treated FBS. Cells were treated with up to 25 μM UA or control glucocorticoids dexamethasone, cortisol, or corticosterone for 24 h. DMSO was used as the vehicle control for UA and ethanol was used for all other compounds. Cells were incubated in media containing the same concentrations of compounds as well as 10 nM [³H]dexamethasone for 2 h. Cells were rinsed three times with PBS and checked under the microscope to verify no cell loss. Cells were lysed for 1 h at 4°C with 0.5 mL 1 M NaOH. [³H] radioactivity in lysates was counted with a Multi-Purpose Scintillation Counter from Beckman Coulter. Background radioactivity from similarly-manipulated cell-free wells was subtracted from each reading.

In order to confirm the binding assay results obtained in HaCaT cells, a cell-free system utilizing human recombinant GR was employed. The reaction was conducted in a binding buffer consisting of 10 mM potassium phosphate, 0.1 mM EDTA, 10 mM sodium molybdate, and 5 mM DTT. A 30 nM [³H]dexamethasone, 30 nM human recombinant GR, and indicated doses of UA or control glucocorticoids were combined in binding buffer and incubated for 2 h at 4°C. A 5% dextran-coated charcoal was added to each tube to chelate unbound ligands for removal. Samples were centrifuged and [³H] in each clear supernatant was counted with a Multi-Purpose Scintillation Counter from Beckman Coulter.

2.7 | siRNA transient transfection

Ca3/7 cells (1 x 10⁶) were transiently transfected with mouse AMPK siRNA with the use of AmiMax Nucleofector Technology according to the manufacturer’s protocol (Lonza Cologne GmbH, 50829 Köln, Germany). AMPK siRNA (sc-45312), purchased from Santa Cruz Biotechnology, Inc., (Dallas, TX), consisted of pools of three to five target-specific 19-25 nt siRNAs designed to knockdown gene expression. The doses of AMPK siRNA were 100 and 200 nM. The scrambled siRNA was used at 200 nM. The effect of AMPK siRNA on cell proliferation was tested in transfected cells by MTT assay at a cell density of 3000 cells/well. Knockdown of AMPK protein expression was confirmed by Western blot analysis.

**FIGURE 3** The cytotoxic effect of UA on Ca3/7 cells was reversed by siRNA knockdown of AMPK. A, Western blot showing knockdown of AMPK. B, siRNA knockdown of AMPK makes Ca3/7 cells resistant to cytotoxicity from 10 μM UA. (n = 6, ****P < 0.001)
2.8 Statistical analyses

For siRNA knockdown experiments, differences between the two groups were determined by two-tailed Student’s t-test. Statistically significant differences between treatment groups in other experiments were determined by using ANOVA. IC_{50} and LD_{50} values were calculated using GraphPad Prism.

3 RESULTS

3.1 UA decreased carcinoma cell viability via AMPK activation

In these experiments, we first used the AMPK inhibitor, Compound C, to determine whether UA toxicity in Ca3/7 skin carcinoma cells is mediated by AMPK activity. Western blotting experiments revealed UA enhanced AMPK activity at cytotoxic doses, and AMPK phosphorylation was significantly prevented by Compound C (Figure 1). Cell viability (MTT assay) and cell death (LDH assay) analyses showed Compound C significantly prevented the effects of UA in Ca3/7 skin carcinoma cells (Figure 2). Finally, siRNA knockdown of AMPK led to a reversal of UA-mediated decreases in Ca3/7 cell viability (Figure 3). These results reveal AMPK activation plays a role in the ability of UA to inhibit the viability of Ca3/7 skin carcinoma cells.

3.2 PPARα partially mediated the cytotoxic effects of UA

As noted in the Introduction, a number of studies have shown UA can activate PPARα, which is also activated by other anti-skin cancer compounds.22 We performed experiments to determine whether PPARα contributes to UA-mediated death of skin cancer cells. Mouse skin papilloma cells (MT1/2) or Ca3/7 cells were pretreated with the PPARα inhibitors GW6471 or MK 886 for 1 h prior to adding UA at increasing doses. Cells were harvested 24 h later. GW6471 slightly but significantly increased the IC_{50} of UA on MT1/2 (Figure 4A) and Ca3/7 (Figure 4B) cell viability. In order to confirm these findings, we repeated these experiments with another PPARα inhibitor, MK 886, which is less potent than GW6471. Similar effects were observed, as MK 886 significantly increased the IC_{50} of UA in both MT1/2 (Figure 4C) and Ca3/7 (Figure 4D) cell lines. Overall, these results indicate that UA-mediated cytotoxicity in skin cancer cells is at least partially mediated by PPARα.

3.3 GR did not mediate the cytotoxic effects of UA

A number of studies have shown UA may modulate the GR, which mediates the effects of skin cancer-suppressing glucocorticoids.28–30,34 We found the GR inhibitor RU486, which inhibits both the transactivation and transrepression properties of GR,39 did not suppress UA-mediated decreases in Ca3/7 cell viability (Figure 5A). On the contrary,
RU486 significantly enhanced decreases in Ca3/7 viability in response to UA. This indicates that GR activity may mediate a cytoprotective resistance to UA treatment, or RU486 may enhance the effects of UA through a GR activity-independent mechanism.

UA has been demonstrated to have GR-activating properties, and the structure of UA resembles that of typical glucocorticoids. In order to determine if UA functions as a typical glucocorticoid, we performed radioligand binding assays. These assays determine if UA can displace radiolabeled dexamethasone, which should be bound to the traditional ligand-binding site. In initial experiments, we employed the human keratinocyte cell line HaCaT, in which UA is less cytotoxic. Non-toxic doses of UA up to 25 μM did not displace [3H]dexamethasone, while control glucocorticoids dexamethasone, cortisol, and corticosterone all competed with [3H]dexamethasone for binding (Figure 5B). In other experiments we incubated UA with [3H]dexamethasone over a series of time points (1-8 h), all of which showed no decrease in [3H] dexamethasone in cell lysates (data not shown). These results also suggest UA does not interact with GR as a traditional glucocorticoid in cell culture. In order to confirm the lack of UA binding to GR in HaCaT cells was not due to cellular manipulations such as efflux, metabolism, or sequestration of UA, we employed a simple cell-free system using human recombinant GR. In this study, we also used EtOH as the vehicle for UA, to confirm the use of different vehicles did not complicate the findings. Similar to the cell culture studies, UA did not displace [3H]
dexamethasone from human recombinant GR, while dexamethasone, cortisol, and corticosterone all competed for binding (Figure 5C).

4 | DISCUSSION

The current results indicate that AMPK activity at least partially mediates the effects of UA on viability of Ca3/7 skin carcinoma cells. In other studies, the anti-cancer effects of UA have also been shown to depend on AMPK in liver cancer cells and bladder cancer cells. In addition, studies using PPARα inhibitors GW6471 and MK 886 showed that the UA-mediated decrease in the viability of MT1/2 and Ca3/7 cells was also at least partially dependent on PPARα activity. A number of interactions between PPARα and AMPK have been demonstrated. AMPK contributes to phosphorylation and transcriptional activity of PPARα as well as to PPARα expression. Also, PPARα ligands induce activation of AMPK, and AMPK is required for the downstream effects of PPARα ligands. A positive feedback loop between AMPK and PPARα could explain how the induction of cancer cells is reversed by both AMPK and PPARα inhibitors as seen in the current experiments.

Experiments exploring the possible role of the GR showed that RU486 unexpectedly enhanced the anti-cancer effects of UA in the skin carcinoma cell line Ca3/7. Considering the GR-activating effects of UA in other systems and the anti-cancer effects of different glucocorticoids, we expected the anti-cancer effects of UA to be at least partially prevented with the GR antagonist. Interestingly, dexamethasone has been shown to enhance expression of p-glycoprotein, which was reversed with RU486. P-glycoprotein is a drug-effluxing pump which contributes to chemoresistance to a wide variety of xenobiotics, including anti-cancer compounds. In addition, our recent studies show that the cytotoxic effects of UA are enhanced with inhibition of p-glycoprotein. In our system, RU486 may decrease the basal expression of p-glycoprotein resulting in increased intracellular UA and enhanced cytotoxicity. Regardless, our GR binding assays indicate that despite the steroid-like structure of UA and similar downstream effects to other glucocorticoids, UA is not a direct ligand for the GR, at least not at the characteristic binding site for glucocorticoids. UA may interact with and impact the GR through an allosteric interaction, although future experiments are needed to confirm this in our system.

Recent studies from our laboratories using an in vivo model of skin tumor promotion have shown that UA and related triterpenes given topically can activate AMPK in the epidermis of mice treated with TPA. AMPK activation correlated with increased phosphorylation of ULK1 (Ser555) suggesting that AMPK activation by UA and related triterpenes may lead to induction of autophagic cell death. Recent studies have also shown that PPARα activation can regulate autophagy. Autophagic cell death may play an important role in the reduced survival seen in skin cancer cells observed with UA. Our future studies will explore the possible interactions between UA and AMPK- and PPARα-mediated autophagic death pathways in both premalignant and malignant skin cancer cells.

In summary, both the AMPK and PPARα pathways contribute significantly to the cytotoxic effects of UA in skin cancer cells. Furthermore, the effects of UA on cell survival were not dependent on the GR. In addition, UA did not function as a typical GR ligand in the cell lines used. Future studies utilizing in vivo models of skin cancer may further confirm the mechanism of action of UA. This would involve the generation of mouse models lacking AMPK or PPARα in the skin, perhaps through crossing skin-specific Cre recombinase mice with mice with floxed AMPK or PPARα. These mice could then be treated with the DMBA/TPA skin carcinogenesis protocol and UA to determine if AMPK or PPARα mediate the ability of UA to reduce skin cancer formation in vivo. These results delineating the mechanism of action of UA may allow for the development of related anti-skin cancer compounds which operate through similar means. Full characterization of UA may also provide molecular explanations in case any side effects or resistance mechanisms arise during UA therapy, especially in non-melanoma skin cancer patients with certain polymorphisms in the targets for this compound.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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