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Curcuminoids Activate p38 Map Kinases and Promote UVB-Dependent Signaling in Keratinocytes

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Abstract

Curcuminoids exhibit anti-proliferative properties in many cell lines by modulating signaling pathways to inhibit cell growth. However, the specific effects of curcuminoids on human keratinocytes are not well defined, and this situation impairs mechanistic thinking regarding potential therapeutic uses. We hypothesized that curcuminoids would modulate key growth regulatory pathways in keratinocytes to inhibit cell proliferation. To test this hypothesis, the effects of curcumin and tetrahydrocurcumin (THC) on MAP kinase signaling in keratinoctyes were determined.

Primary human keratinocytes treated with curcumin or THC demonstrated decreased activation of p44/42 MAP kinases but increased levels of activated p38 MAP kinases. These data suggest that curcuminoids specifically activate stress-induced MAP kinases while inhibiting mitogen-induced MAP kinases. Curcuminoids also promote the phosphorylation of p53 on serine 15 in a dose-dependent and p38-dependent manner, suggesting that these compounds may activate p53. The effects of curcuminoids on keratinocytes mirrored some aspects of UVB and could be inhibited by N-acetylcysteine, suggesting that these compounds activate p38 through a mechanism that involves glutathione depletion. Both curcuminoids induced G2/M block and inhibited keratinocyte growth, and THC increased cellular levels of p21, a known p53 transcriptional target.

These data demonstrate that curcuminoids can differentially regulate MAP kinases to inhibit keratinocyte growth while inducing p21. Curcuminoids also synergize with UVB to enhance p53 phosphorylation. The findings provide a rationale for testing curcuminoids in disorders associated with impaired p53 function or in which UVB-treatment is efficacious.

Keywords

Curcumin; keratinocytes; MAP kinases; p53; UVB

Introduction

The polyphenol curcumin (aka diferuloylmethane or 1,7-bis-(-4-hydroxy-3-methoxiphenyl)-1,6-heptadiene-3,5-dione) is derived from the plant Curcuma longa, which is widely cultivated in tropical areas of Asia and Central America. Tumeric, the powdered

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form of the rhizome, has been used for over 2000 years as a food additive and as a medicinal agent for treating a variety of diseases (1).

Numerous studies have shown that curcumin and the hydro-alcoholic extract of the Curcuma rhizome exhibit anti-inflammatory and anti-proliferative properties; these findings raise the hypothesis that curcuminoids may have therapeutic potential in treating inflammatory and neoplastic skin diseases (2). Some of the anti-tumorigenic effects of curcumin appear to be related to the induction of apoptosis in cancer cells, which, in some models, is associated with increased p53 expression during the G2 phase of the cell cycle (3, 4). Additional anti-tumorgenic properties of curcumin include the inhibition of arachidonic acid metabolism (5), protein kinase activity (6), redox regulation in cells (7), and ornithine decarboxylase activity, while promoting glutathione production (3),(8),(9). Despite the broad literature regarding the anti-proliferative effects of curcumin on various cell lines, little is known about its effects on cutaneous keratinocytes.

Cutaneous squamous cell carcinoma (SCC) is a common form of non-melanoma skin cancer in the United States with over 250,000 cases annually, resulting in approximately 2,500 deaths (10, 11). Most cutaneous SCCs arise from an actinic keratosis (AK), and it has been estimated that 60% of all individuals over the age of 40 will develop an AK that requires treatment (12). Such data demonstrate the clinical importance of treating AKs effectively.

AKs have aberrant p53 immunohistochemical staining patterns, p53 mutations, and increased loss of heterozygosity at a variety of genetic loci (13-15). This p53 functional deficit in AKs promotes acquisition of additional DNA mutations leading to the generation of an aberrant keratinocyte clone with a growth advantage (13, 14, 16, 17). Therefore, small molecules that enhance the functional effectiveness of the p53 may have chemopreventive efficacy in treating AKs. The small molecule CP-31398 enhances p53 function and has shown efficacy in inhibiting cutaneous SCCs in murine models (18).

Curcumin is yellow, and its topical application to skin leads to discoloration, hence limiting its therapeutic utility. To circumvent this issue, white derivatives of curcumin such as, tetrahydrocurcumin (THC), have been developed. However, it is not known if the biological activity of THC upon keratinocytes mimics that of curcumin.

Based on these data, we hypothesized that curcuminoids would modulate key cell signaling pathways, such as MAP kinases, to inhibit keratinocyte growth. MAP kinases are highly conserved molecules that regulate cell proliferation (19). In this study, the data demonstrate that curcumin and THC suppress p44/42 MAP kinases while persistently activating p38 MAP kinases. Persistent activation of p38 promotes phosphorylation of p53 at serine 15 which enhances its transcriptional activity; this finding correlates with curcuminoids inducing p21 expression in PHKs. Curcuminoid treatment also promoted G2/M block and decreased keratinocyte growth. The effects of curcuminoids on keratinocyte signaling are similar to those of UVB, and pre-treatment of PHKs with curcumin or THC can promote the UVB-dependent activation of p38 MAP kinases and p53 phosphorylation. Together, these data define a signaling mechanism through which curcuminoids can inhibit keratinocyte growth; the data also provide a rationale for testing these compounds in cutaneous disorders associated with p53 functional deficits and in which UVB-treatment is efficacious.

Methods

Cell Culture and Treatment - PHKs were isolated from foreskins, and cultured in MCDB153 medium, as previously described (20). Cells were used before passage 4. PHKs were treated with 20μM curcumin (Fluka #28260, 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin, at least 97% percent curcuminoids) or the indicated concentrations

of tetrahydrocurcumin (Sabiwhite tetrahydrocurcumin not less than 95% curcuminoids by HPLC, mean percent composition by HPLC: THC-75%, Demethoxy THC-20%, Bisdemethoxy THC-4.5%., Sabinsa Corporation, Piscataway NJ) in DMSO at approximately 80% confluency for the indicated times. Where indicated, PHKs were treated with 10 mM N-acetylcysteine (Sigma) for 45 minutes prior to curcumin exposure. Experiments utilized at least two independent lines. UVB exposure – UV calibration was performed in minimal layers of PBS or MCDB medium using doses from 25 to 250 mJ/cm^2. PHKs irradiated in PBS or MCDB demonstrated no detectable difference in p38 MAP kinase activation, which concurs with prior studies (21). PHKs were exposed to varying doses of UVB/A supplied by FS20 T12 lamps filtered with cellulose triacetate to remove all wavelengths below 290 nm. UVB doses were calibrated using a National Biological Corporation 500-c UVB meter (National Biological Corportation, Twinsburg, OH). Spectral output was 290-340 nm, max 305 nm, 70% UVB and 30% UVA; a total dose of 25 mJ/cm^2 irradiation would correspond with 18 mJ/cm^2 UVB. Sham irradiation controls were subjected to all steps except UVB exposure.

Antibodies - α-p44/42 MAP kinase antibody and α-phospho-p44/42 MAP kinase antibody (9101, 9102, Cell Signaling Technology) 1:1000 to detect total and phosphorylated p44/42 MAP kinase proteins. α-p38 antibody, α-phospho-p38 antibody (9212, 9211, Cell Signaling Technology), 1:1000 to detect total and phosphorylated p38 MAP kinase proteins. α-p21 mouse mAb(F-5), sc-6246, at 1:500 (Santa Cruz Biotechnology). α-p53 mouse mAb (1C12), 2524, Cell Signaling at 1:1000. α-phospho-p53 antibody (serine 15) 9284, Cell Signaling, at 1:500. α-β-actin (Abcam) antibody was used at 1:5000.

Immunoblotting: Transfected cells were washed twice with cold PBS, cell lysates were prepared using RIPA lysis buffer, and protein concentrations were determined as previously described (22). Western blotting was performed with the indicated antibodies and developed using Lumilight Plus (Boehringer Mannheim) according to the manufacturer's indications as previously described (20).

Cell cycle analysis: PHKs were incubated with 20μM curcumin, 200 μM tetrahydrocurcumin or DMSO for the indicated times. Cells were processed as previously described (20). DNA content was analyzed using a Becton Dickinson FACScan and Cell Quest Software, plotting at least 20,000 events per sample; the cell populations were sorted into G0/G1, G2/M, and S phase according to the manufacturer's specifications. Subsequent data analysis was performed using ModFit LT 3.1 Software (Verity Software House) to determine cell cycle fractions.

Cell Growth Assay: PHKs were plated at 2.2×10^5 cells per 35 mm well in MCDB medium. Approximately 16 hours later, the cells were exposed to fresh medium, medium + DMSO (0.1%), 20 μM curcumin, or 200 μM THC. Cell numbers were determined 72 hours later in triplicate. Percent growth represents: (cell number at 72 hours- 2.2×10^5)/ 2.2×10^5 +/- standard deviation expressed as a percent. p values were determined using the Student's T test. Image Acquistion and Statistical Analysis: Developed films of western blots were scanned and quantitated using a Canon Scanner and Scion densitometry imaging software (Frederick, MD). Scanned images were cropped using Adobe Photoshop CS and converted to JPEG files. Figures were made using Adobe Illustrator CS. Densitometric values were background corrected and expressed as a mean ± standard deviation of at least triplicate samples. Flow cytometric values were processed in a similar manner. Statistical significance was evaluated using an independent group T-test for means.

Results

Curcumin and THC decrease the S-phase and promote G2/M block in PHKs

Although curcumin increases p21 levels and promotes G2/M block in melanoma, bladder carcinoma, glioma, and immortalized endothelial cell lines, the effect of curcuminoids on the cell cycle of primary keratinocytes is unknown. (23-26). To determine the effects of curcumin and THC on the cell cycle of PHKs, flow cytometric quantitation of DNA content was performed. PHKs were stimulated with 20μM curcumin for various times and compared to controls. Curcumin treatment of PHKs caused a decrease in the percentage of PHKs in G1 and S phases, with a concomitant increase in cells in the G2/M phase (Figs. 1A and B). The increase in the G2/M fraction was gradual starting after 2 h of stimulation and was maximal and statistically significant by 24 h ($p < 0.05$) (Fig. 1B). These results indicate that curcumin exerts an “anti-proliferative” effect on PHKs by blocking cells in the G2/M phase of the cell cycle. Treatment of PHKs with THC exhibits similar effects by promoting the accumulation of cells in the G2/M phase of the cell cycle within 24 hours (Fig. 1C).

Curcumin and THC inhibit keratinocyte growth

The accumulation of PHKs in the G2/M phase of the cell cycle could represent a transient anti-proliferative effect or a DNA-repair stall, rather than growth inhibition. Therefore, the effects of curcumin and THC on keratinocyte growth were evaluated. Equivalent numbers of PHKs were plated and exposed to medium alone, 0.1% DMSO, curcumin, and THC for three days, and then the total cell number was determined. The percent of cell growth was 327 +/- 15% for untreated cells and 310 +/- 27% for DMSO-treated cells (Fig. 1D). The percent of cell growth was 60 +/- 6% for curcumin-treated cells and 10 +/-3% for THC-treated cells. The growth-inhibition for curcumin- and THC-treated cells compared to DMSO-treated cells was statistically significant to $p < 0.001$. These data demonstrate that curcumin and THC inhibit keratinocyte growth raising a question regarding the mechanism of growth inhibition.

Curcumin and tetrahydrocurcumin promote transient activation and subsequent suppression of p44/42 MAP kinases

Curcumin has demonstrated anti-proliferative properties in a variety tumor cell lines at concentrations ranging from 10-150 μM (23, 24, 27, 28). If curcumin and THC suppress the growth of human keratinocytes, then these compounds may inhibit p44/42 MAP kinases, which promote keratinocytic neoplasia and hyperplasia (29, 30). The effects of curcumin upon p44/42 MAP kinases were studied in PHKs subjected to 20μM curcumin over a range of times, followed by western blot analysis to detect levels of phosphorylated (activated) p44/42 MAP kinases or total cellular p44/42. Activation of p44/42 MAP kinases was rapid and detected after five minutes of curcumin treatment; however, the levels of activated p44/42 MAP kinases decreased abruptly and returned to background levels by 45 minutes (Fig. 2A). For exposure times ranging from 90 minutes to eight hours, p44/42 MAP kinase activation levels were lower than control cells (Figs. 2A and B). By 24 hours, levels of p44/42 MAP kinase activation were similar to controls. The mechanism of p44/42 activation/deactivation by curcumin likely depends on MEK1/2 activation as pharmacologic inhibition of the MEK 1/2 kinases results in a loss of the curcumin-induced p44/42 phosphorylation (data not shown). Treatment of PHKs with 200 μM THC resulted in p44/42 phosphorylation within 5 minutes followed by a rapid decrease in activity by 20 minutes (Fig. 2C). The suppression of p44/42 MAP kinases by curcumin and THC may inhibit keratinocyte growth.

Curcumin and tetrahydrocurcumin promote sustained activation of p38 MAP kinases

Curcuminoids have been indirectly implicated in regulating the activity of p38 MAP kinases; however, published data are not clear regarding the effects of curcuminoids on p38 MAP kinase activity in PHKs (31, 32). Curcumin activated p38 MAP kinases in PHKs within 10 minutes (Fig. 2D). After 30 minutes of curcumin treatment, p38 MAP kinases were markedly elevated compared with controls. The curcumin-induced activation of p38 MAP kinases persisted at a high level for at least 24 hours (Fig. 2E). These data demonstrate that curcumin induces sustained p38 activation in PHKs.

The relative potency of curcumin and tetrahydrocurcumin in inducing p38 MAP kinase activity was compared. PHKs treated for two hours with 20 μM curcumin prominently activated p38 MAP kinases while 50 μM tetrahydrocurcumin (THC) weakly activated p38 MAP kinases (Fig. 2F). PHKs exposed to 200 μM THC exhibit comparable levels of p38 MAP kinase activation as 20 μM curcumin. Therefore, in PHKs, THC appears to be about ten-fold less potent than curcumin in promoting p38 MAP kinase activation.

Curcuminoids enhance UVB-induced activation of p38 MAP kinases

PHKs respond to UVB by activating p38 MAP kinases as part of a stress response (33, 34). Therefore, the ability of curcuminoids to enhance UVB-induced p38 MAP kinase activation was tested. PHKs treated with 20 μM curcumin or 200 μM THC for two hours demonstrated activation of p38 MAP kinases (Fig. 3A and B). PHKs exposed to UVB and lysed two hours later also demonstrated low-level activation of p38 MAP kinases. Cells treated with curcumin or THC, then immediately exposed to UVB, and lysed two hours later demonstrated higher levels of activated p38 MAP kinases than seen with either stimulus alone (Fig. 3A and 2B). The level of p38 activation in PHKs treated with UVB and curcumin or UVB and THC is significantly higher than that seen in cells exposed to UVB alone with $p = 0.048$ and $p < 0.01$, respectively (Fig. 3B). These data demonstrate that curcumin and THC can enhance UVB-induced p38 MAP kinase activation in PHKs, raising the possibility that curcuminoids may enhance UVB-induced responses in keratinocytes.

Photoactivation of curcumin by UVA in PHKs and HaCaT cells has been reported (35). Therefore, to determine if our UV source with approximately 30% UVA photoactivates curcumin to enhance p38 MAP kinase activity, the effects of curcumin on p38 MAP kinase activity were evaluated before or after UV exposure. The level of p38 MAP kinase activation was comparable when curcumin and UV were given simultaneously, curcumin was given before UV, and curcumin was given after UV (Fig. 3C). Each combination of curcumin and UV produced a higher level of p38 MAP kinase activation than curcumin or UV alone. Therefore, curcumin activates p38 MAP kinases via a mechanism that does not involve UVA-dependent photoactivation.

Curcumin-dependent p38 activation is regulated by N-acetylcysteine

Activation of p38 MAP kinases occurs when glutathione levels decrease in the setting of cellular stress (36). Since curcumin may lower intracellular glutathione by forming covalent biproducts and directly inhibiting glutathione-S-transferase activity, it is reasonable to hypothesize that the mechanism of curcumin-dependent p38 activation may involve short-term cellular glutathione depletion resulting in oxidative stress (37, 38). To test this hypothesis, PHKs were pre-incubated with N-acetylcysteine (NAC), to enhance cellular glutathione levels, before curcumin treatment (Fig. 3D). The ability of curcumin to activate p38 MAP kinases at doses below 20 μM is proportional to dose (Fig. 3C, left panel). Pre-treatment of PHKs with NAC followed by 5 μM curcumin resulted in mildly increased p38 MAP kinase activation compared to 5 μM curcumin alone. Therefore, 5 μM curcumin activates p38 MAP kinases in a manner that is not inhibited by NAC pre-treatment.

However, PHKs pretreated with N-acetylcysteine and then treated with 20 μM curcumin showed lower levels of p38 MAP kinase activation than untreated cells (Fig. 3D). Therefore, in this experimental system, a component of curcumin-induced p38 MAP kinase activation appears to involve a cellular response that can be inhibited by raising glutathione levels.

Curcumin and tetrahydrocurcumin promote phosphorylation of p53

In PHKs, activation of p38 MAP kinases by UVB has been linked to phosphorylation of p53 at serine 15 which stabilizes the protein (33, 39, 40). Since curcumin and THC induce p38 MAP kinase activation in PHKs, the ability of these compounds to promote p53 phosphorylation at serine 15 was tested. PHKs were exposed to curcumin or increasing doses of THC for 2 hours; PHKs exposed to curcumin exhibited mildly increased levels of phosphorylated p53, and cells exposed to THC demonstrated significantly higher levels of phosphorylated p53 (Fig. 4A). The effect of THC was tested on PHKs over a broader range of timepoints. PHKs exposed to THC from two-to-eight hours demonstrated higher levels of phosphorylated p53 compared to controls (Fig. 4B). Exposure of PHKs to curcumin and THC followed by UVB resulted in higher levels of p53 phosphorylation than UVB, curcumin, or THC alone (Fig 4C and D). These data demonstrate that curcumin and THC promote p53 phosphorylation at serine 15 and enhance the level of UVB-induced phosphorylation at this site.

THC increases p21 levels in PHKs

Increased p53 transcriptional activity is associated with elevated p21 levels, a negative regulator of the cell cycle (41). PHKs treated with THC demonstrated a prominent increase in p21 that persisted for at least 24 hours (Fig. 4E). The time course of THC-induced p21 levels correlates with the THC-dependent activation of p38 and p53, suggesting that THC activates a p38-p53-p21 signaling module that could regulate the proliferation of PHKs.

Inhibition of p38 MAP kinases blocks THC-induced p53 phosphorylation

The data above suggest that curcumin and THC promote p53 phosphorylation, in part, through a p38 MAP kinase-dependent mechanism. To further clarify a mechanistic link between between curcuminoids, p38 MAP kinases, and p53, levels of p53 phosphorylation were assessed in THC-treated PHKs subjected to pharmacologic inhibition of p38 MAP kinases. In control cells, THC activates p38 MAP kinases and promotes p53 phosphorylation at serine 15 (Fig. 5). In addition, THC-treatment of PHKs potentiates the UVB-induced activation of p38 MAP kinases and phosphorylation of p53. A parallel set of PHKs exposed to SB202190, an inhibitor of p38 MAP kinases, for 30 minutes prior to lysis, resulted in markedly diminished levels of p38 MAP kinase activity and p53 phosphorylation, while treatment with a control compound maintained levels of phosphorylated p38 and p53. These data suggest that THC promotes p53 phosphorylation in PHKs through a p38 MAP kinase-dependent pathway; however, the incomplete inhibition of p53 phosphorylation by SB202190 raises the possibility that p38-independent mechanisms may contribute a minor component to this process.

Discussion

In this manuscript, the anti-proliferative effects of curcuminoids on PHKs are more clearly delineated at the molecular level. The data show that curcumin and THC inhibit p44/42 MAP kinases and activate a p38/p53/p21 signaling axis which alters cell growth in PHKs. The observation that curcuminoids inhibit p44/42 MAP kinases in human keratinocytes is supported by a recent publication demonstrating that curcumin inhibits p44/42 activation in PC12 cells (42). However, the inhibition of p44/42 phosphorylation by curcumin may be cell type dependent, as this type of inhibition is not detected in melanoma cell lines (43).

The mechanism of p44/42 deactivation could involve decreased MEK1/2 activation as inhibition of these kinases decreases transient curcumin-induced p44/42 phosphorylation (data not shown). Inhibition of p44/42 MAP kinases by curcuminoids likely represents a component of the anti-proliferative effects of these compounds on keratinocytes. Since increased signaling of p44/42 kinases are associated with cutaneous SCC formation, use of curcuminoids to inhibit these kinases in human skin may be a logical step in treating cutaneous neoplasia (19, 44)

To further delineate a potential mechanism for the curcuminoid-dependent activation of p38 MAP kinases, lysates from treated cells were assessed for the levels of MKK3/6 activation using a phospho-specific antibody; curcuminoids did not significantly stimulate levels of phosphorylated MKK 3/6 in PHKs suggesting that activation of p38 MAP kinases by curcuminoids occurs via a different mechanism (data not shown). These data correlate with prior studies on various tumor cell lines showing that curcumin promotes the expression of p21, p27, and p53, which promote anti-oncogenic properties (45). However, activation of p38 MAP kinases by 20 μM curcumin was inhibited by pre-treating PHKs with N-acetylcysteine; this results implies that curcumin activates p38 MAP kinases through a cellular stress response triggered by gluthathione depletion. This new mechanistic data provides insights into potential therapeutic use of curcuminoids.

AKs are the most common precancerous lesions and approximately 35% of them spontaneously regress within one year (14, 46). AKs do contain UV-signature p53 mutations, however, these mutations may not be common as some studies report an incidence of about 10% of AKs (15). Therefore, enhancing p53 signaling in keratinocytes may be an effective means of AK chemoprevention. Phosphorylation of p53 at serine 15 typically occurs in response to DNA damage, and stabilizes the molecule, likely enhancing its function (39, 47). The activation of p38 MAP kinases by curcumin and THC temporally correlated with phosphorylation of p53 at serine 15, a known UVB-induced phosphorylation site in keratinocytes (33). Since curcumin and THC promote the phosphorylation of p53 at serine 15, these molecules potentially could serve as small molecule enhancers of p53 function in conditions with one dysfunctional allele, as in actinic keratoses. The data presented provide a novel molecular mechanism by which curcuminoids can enhance p53 function through activation of p38 MAP kinases.

One likely limitation of using curcumin as a topical agent is yellow skin discoloration. Furthermore, curcumin has poor and variable oral bioavailability which would limit its effectiveness in AK chemoprevention (48). However, given that THC induces parallel effects on PHKs as curcumin, albeit at a higher dose, this white compound may better tolerated as a topical agent.

Given that THC and curcumin enhance phosphorylation of p53 that promotes stabilization, cellular levels of p21 were assessed because it is a well characterized p53 transcriptional target (41). PHKs treated with THC demonstrate markedly increased levels of p21, consistent with increased p53 transcriptional activity. The increased p21 expression in THC-treated keratinocytes also correlates with curcuminoid-induced G2/M block. These data concur with findings from previous studies performed in cancer cell lines and with various types of primary cells, which showed that curcumin treatment (24-96 hours) induced the accumulation of cells in the G2/M phase, including oral keratinocytes and SCC lines (49)-(50). Our findings support these previous studies and the hypothesis that curcumin has anti-proliferative effects on both cancer cell lines and primary cells, now including PHKs.

The reason for the reduced potentcy of THC remains unclear. Since THC has fewer unsaturated bonds, THC may be less effective at inducing oxidative stress and reducing

glutathione levels than curcumin. However, the precise mechanistic details pertaining to these observations remain to be defined.

The data presented in this study provide new mechanistic insights into how curcumin modulates signaling pathways in PHKs important for regulation of cell growth (Fig. 6). Curcumin and THC rapidly activate stress-induced p38 MAP kinases while inhibiting growth-factor activated p44/42 MAP kinases. The anti-proliferative effects of curcumin and THC on PHKs appear to correlate with the downregulation of p44/42 MAP kinase phosphorylation and activation of the p38/p53/p21 signaling axis. The biological effects of curcumin and THC on primary keratinocytes described in this study plausibly indicate that these compounds may represent potential therapeutic options for treating hyperproliferative epidermal disorders, such as AKs. Additional studies evaluating the safety and efficacy of topical THC in the treatment and/or prevention of AKs and SCCs may be warranted.

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Abbreviations used are

BPE	bovine pituitary extract
DMSO	dimethyl sulfoxide
PHKs	primary human keratinocyte
MAP	mitogen-activated protein

MEK	mitogen-activated protein kinase kinase
NAC	N-acetylcysteine
PBS	phosphate-buffered saline
RIPA	radioimmunoprecipitation
MKP	MAP kinase phosphatase
THC	tetrahydrocurcumin

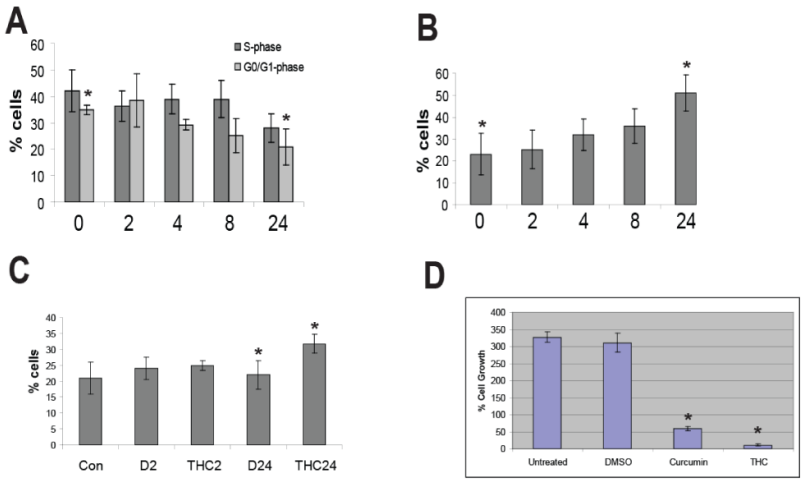

Figure. 1. Curcuminoids promote G2/M block, and inhibit growth in PHKs

A) PHKs were cultured for the indicated times in the presence or absence of 20μM curcumin, harvested, and stained with PI. Labeled keratinocytes were analyzed by flow cytometry to assess DNA content. Incubation times are indicated on the x-axis. The percentage of cells in the S-phase and G0/G1-phase is indicated on the y-axis **B)** The percentage of curcumin-treated cells from A in the G2/M-phase. N=3 **C)** PHKs were treated with 200μM THC for 2 or 24 hours or with DMSO (D) and analyzed as in B to determine the percentage of cells in G2/M N=3 Standard deviation indicated by error bars. In A, B, and C, asterisks indicate differences associated with p values ≤ 0.05. **D)** PHKs were treated with nothing, 0.1% DMSO, 20μM curcumin, or 200μM THC. After three days, total cell counts were determined in triplicate. Asterisks indicate differences with p values < 0.001 compared to DMSO condition. N=2

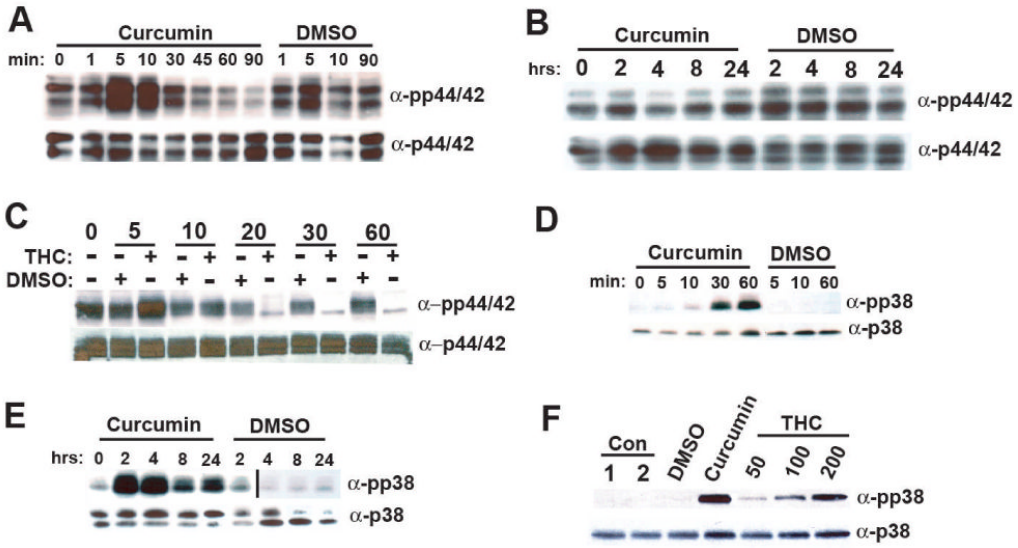

Figure 2. Curcuminoids inhibit p44/42 MAP kinases and activate p38 MAP kinases in PHKs

A), PHKs were treated with 20μM curcumin or DMSO for the indicated times. Lysates were subjected to SDS-PAGE followed by western blotting to detect levels of phosphorylated p44/42 MAP kinases (α-pp44/42, upper panels) and total p44/42 MAP kinases (lower panels). 0=baseline control. N=3. **B)** PHKs were treated with 20μM curcumin or DMSO for the indicated times, and processed as in A. N=2. **C)** PHKs were treated with 200μM THC or DMSO for the indicated times, and processed as in A. N=2. PHKs were treated with 20μM curcumin or DMSO for short **(D)** or long **(E)** time courses. Cells were lysed, and subjected to SDS-PAGE followed by western blotting to detect levels of activated p38 MAP kinases (pp38) and total p38 MAP kinases. N=3. **F**) PHKs were treated for two hours with 20μM curcumin or tetrahydrocurcumin (THC) at 50, 100, 200 μM. Control 1 lysed at the beginning of the time course, 2-at the end. Cells analyzed as in D. N=2.

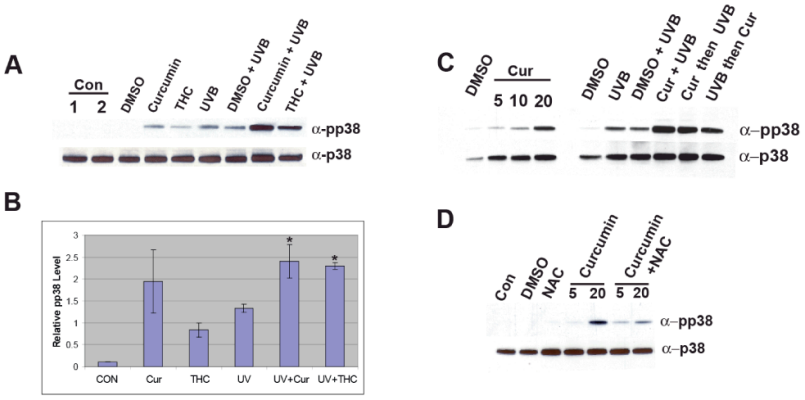

Figure. 3. Curcuminoids enhance UVB-induced activation of p38 MAP kinases

A) PHKs were not stimulated or stimulated with 20μM curcumin, 200μM tetrahydrocurcumin (THC), or DMSO for two hours and lysed. Where indicated, cells were treated with curcumin, THC, or DMSO then subsequently irradiated with UVB (18 mJ/cm^2). Two hours post-irradiation, cells were lysed and subjected to SDS-PAGE followed by western blotting to detect phosphorylated p38 and total p38. N=2 **B)** Densitometric analysis of p38 activation in A. Curcumin and THC pre-treatment enhance the ability of UVB to activate p38 MAP kinases. Standard deviation indicated by error bars. Asterisks indicate differences with UVB condition associated with a $p < 0.05$. N=2 **C). Left panel:** PHKs were either treated with DMSO, 5, 10, or 20 μM curcumin for two hours, lysed, and analyzed as in A. N=2. **Right panel:** PHKs were treated with DMSO, UVB (18 mJ/cm^2), UVB and DMSO, or Curcumin 20 μM followed immediately by UVB exposure and lysed two hours later. Cur then UVB: PHKs were treated with 20μM curcumin for 1 hour then exposed to UVB and incubated for an additional 1 hour, and then lysed. UVB then Curcumin: PHKs were exposed to UVB then treated with 20μM curcumin for two hours before lysis. Lysates were analyzed as above. N=2 **D)** PHKs were treated with 5 μM or 20 μM curcumin, no stimulus, DMSO, or 10 mM N-acetylcysteine (NAC) for two hours. Some cells were pre-incubated with 10 mM NAC for 45 minutes before curcumin treatment. Cells were lysed, and analyzed as in A. N=2

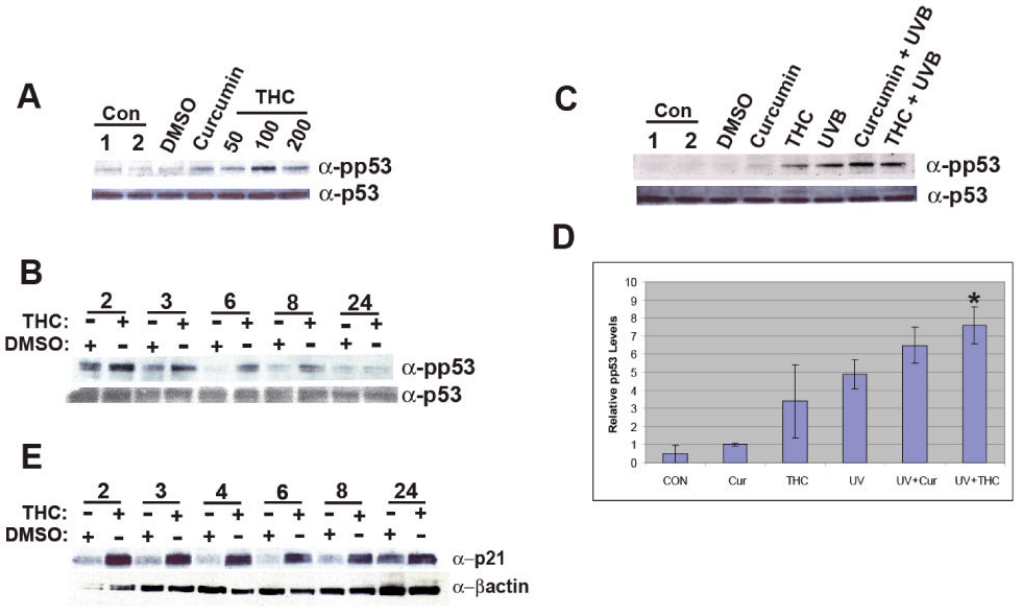

Figure. 4. Curcuminoids promote p53 phosphorylation, enhance UVB-induced p53 phosphorylation, and induce p21 levels

A) PHKs were treated for two hours with 20μM curcumin or tetrahydrocurcumin (THC) at 50, 100, 200 μM. Control 1 was lysed at the beginning of the time course, 2-at the end. Cells subjected to western blot analysis to detect phosphorylated p53 (serine 15) and total p53. N=3 **B)** PHKs were treated with DMSO or 200μM tetrahydrocurcumin (THC) for the indicated times (hours). Cells were analyzed as in A. N=2. **C)** PHKs were not stimulated or stimulated with 20μM curcumin, 200μM tetrahydrocurcumin (THC), or DMSO. Where indicated, cells were irradiated with UVB (18 mJ/cm^2). Two hours post-irradiation, cells were lysed and subjected to analysis as in A. N=2 **D)** Densitometric analysis of p53 activation in experiment C. Curcuminoids promote UVB-induced phosphorylation of p53. Error bars indicate standard deviation. Asterisk indicates a p=0.09 compared to UV alone. **E)** PHKs were treated with 200μM tetrahydrocurcumin (THC) or DMSO for the indicated times (hours). Cell lysates were analyzed by western blotting to detect levels of p21 and β-actin. N=2

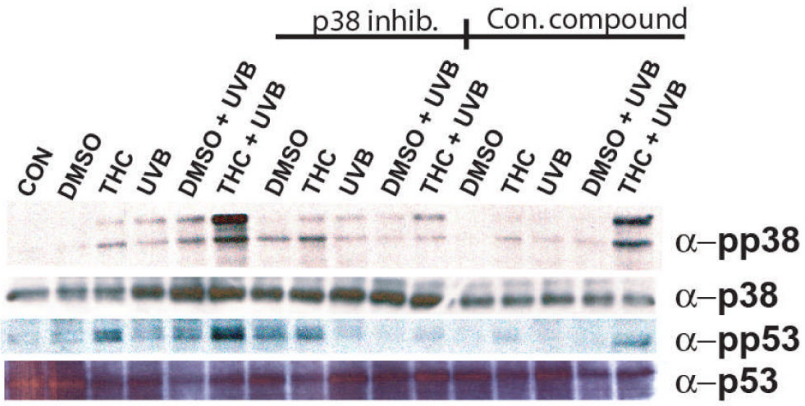

Figure. 5. p38 MAP kinase inhibition decreases curcuminoid-induced p53 phosphorylation

PHKs were not stimulated or stimulated with DMSO or 200μM tetrahydrocurcumin (THC). Where indicated, cells were irradiated subsequently with UVB (18 mJ/cm^2). Duplicate cell sets were exposed to the p38 MAP kinase inhibitor SB202190 or its control compound SB202474 for 30 minutes prior to lysis. Two hours post-irradiation, cells were lysed and subjected to SDS-PAGE followed by western blotting to detect phosphorylated p38, total p38, phosphorylated p53 (serine 15), and total p53. N=3.

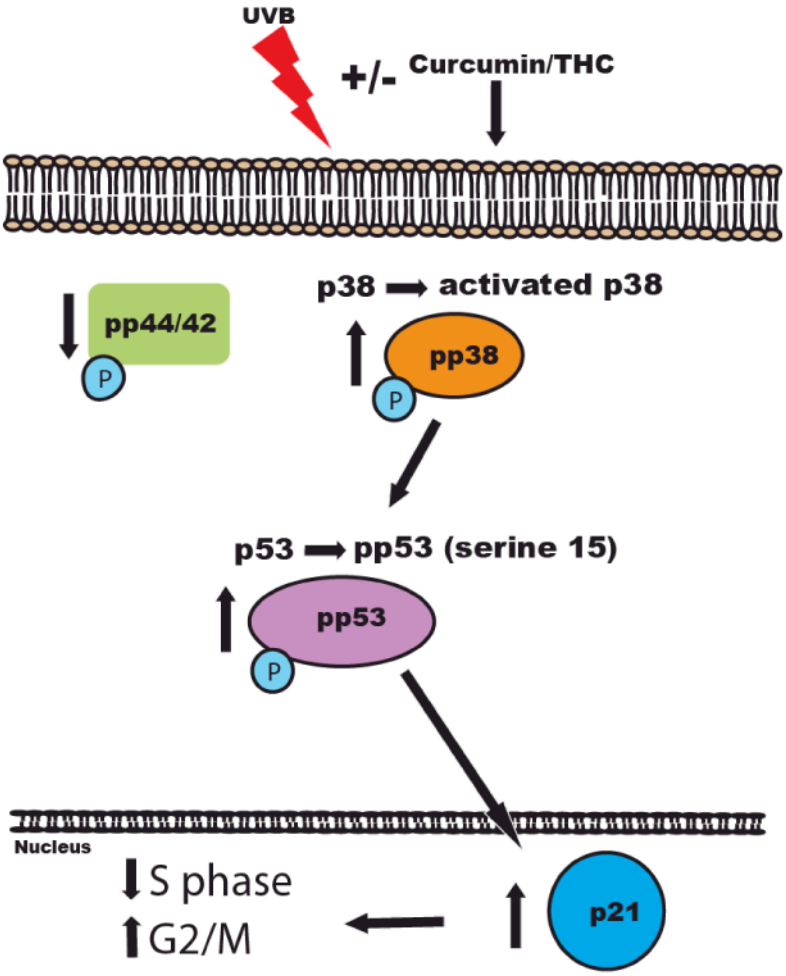

Figure 6. Schematic diagram of the curcuminoid-induced effects on PHKs

Curcuminoids differentially regulate p44/42 and p38 MAP kinases. Activation of p38 leads to p53 phosphorylation, and increased p21 levels. The result of these cellular events is a block in G2/M and decreased S-phase. Curcuminoids enhance p53 function in PHKs.