Ingenol Mebutate Signals via PKC/MEK/ERK in Keratinocytes and Induces Interleukin Decoy Receptors IL1R2 and IL13RA2

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Abstract

Squamous cell carcinoma (SCC) is the second most common human skin cancer and the second leading cause of skin cancer–related death. Recently, a new compound, ingenol mebutate, was approved for treatment of actinic keratosis, a precursor of SCC. As the mechanism of action is poorly understood, we have further investigated the mechanism of ingenol mebutate–induced cell death. We elucidate direct effects of ingenol mebutate on primary keratinocytes, patient-derived SCC cells, and a SCC cell line. Transcriptional profiling followed by pathway analysis was performed on ingenol mebutate–treated primary keratinocytes and patient-derived SCC cells to find key mediators and identify the mechanism of action. Activation of the resulting pathways was confirmed in cells and human skin explants and supported by a phosphorylation screen of treated primary cells. The necessity of these pathways was demonstrated by inhibition of certain pathway components. Ingenol mebutate inhibited viability and proliferation of all keratinocyte-derived cells in a biphasic manner. Transcriptional profiling identified the involvement of PKC/MEK/ERK signaling in the mechanism of action and inhibition of this signaling pathway rescued ingenol mebutate–induced cell death after treatment with 100 nmol/L ingenol mebutate, the optimal concentration for the first peak of response. We found the interleukin decoy receptors IL1R2 and IL13RA2 induced by ingenol mebutate in a PKC/MEK/ERK–dependent manner. Furthermore, siRNA knockdown of IL1R2 and IL13RA2 partially rescued ingenol mebutate–treated cells. In conclusion, we have shown that ingenol mebutate–induced cell death is mediated through the PKC/MEK/ERK pathway, and we have functionally linked the downstream induction of IL1R2 and IL13RA2 expression to the reduced viability of ingenol mebutate–treated cells. Mol Cancer Ther; 14(9); 1–11. © 2015 AACR.

Introduction

Actinic keratoses are hyperkeratotic lesions on sun-exposed surfaces such as the face, scalp, and lower arms. Actinic keratoses are caused by accumulated UV exposure over lifetime and develop from atypically proliferating keratinocytes (1). In about 8% of cases, actinic keratosis can progress to invasive SCCs (2), the second most common form of skin cancer, and therefore need to be treated. About 50% of SCCs harbor p53 mutations, some of them with the typical UV signature of cyclobutane pyrimidine dimers (3). The risk for organ transplant recipients to develop SCC is highly increased due to their treatment with immunosuppressive drugs (4) such as azathioprine, a photosensitizer to UVA light (5) or cyclosporine A treatment with immunosuppressive drugs (4) such as azathioprine, a photosensitizer to UVA light (5) or cyclosporine A.

The incidence of SCC in the general population continues to increase with high morbidity and low mortality (8). In contrast to basal cell carcinoma (BCC), SCC carries a risk of metastasis. The current treatment options for actinic keratosis range from topical treatments with gels or creams for field cancerization to cryotherapy for single lesions (9), whereas SCC is normally treated by surgical excision. Self-directed treatments are preferred by patients, but are time consuming, as they need to be applied for weeks or months to produce clinical results.

Recently, a new compound, ingenol mebutate, was registered in the United States and Europe for topical treatment of actinic keratosis in two different concentrations depending on the treatment location. Ingenol mebutate showed 42.2% complete clinical clearance compared with placebo (10) and has the great advantage of a short treatment duration (i.e., two to three consecutive days), a short period of local skin reaction and no systemic adverse events (11, 12). Although ingenol mebutate is registered and used in the clinic, its exact mechanism of action is not fully understood. Recent studies suggest a dual mechanism of action with rapid initial necrosis (13) followed by activation of the innate immune system (14). Although several reports investigated the mechanism of action of ingenol mebutate on different cancer cell lines like colon cancer (15), melanoma (16) and leukemia (17), there is only one report studying the effect on epithelial cells (18). In that article, the authors demonstrate acute cytotoxicity of clinical drug.
concentrations on cancer cell lines and keratinocytes with disruption of the mitochondrial network and the involvement of intracellular calcium release.

To better characterize the mechanism of action of ingenol mebutate in epithelial cells, we analyzed the impact of ingenol mebutate on the proliferation and viability of primary keratinocytes, primary patient-derived SCC cells and a SCC cell line. Furthermore, we identified signaling pathways essential for ingenol mebutate action by gene expression analysis and confirmed their contribution in primary cell cultures and human skin explants. We functionally demonstrated the critical importance of several novel genes using functional in vitro assays.

**Materials and Methods**

**Cell culture and skin explants**

The human SCC12 cell line (19) was cultured in Keratinocyte–SFM supplemented with t-glutamine, EGF, and BPE (Gibco). The human SCC13 cell line (19) was cultured in DMEM (Gibco) with 10% FCS. Both cell lines were obtained from Gian-Paolo Dotto (University of Lausanne, Lausanne, Switzerland) in 2011 and were not further tested. Human primary keratinocytes and fibroblasts were isolated from healthy human skin. Human fibroblasts and melanoma cells were cultured in RPMI1640 (Sigma) with 10% FCS. Melanoma cells were isolated from biopsies taken from consenting patients. Normal skin samples were obtained from abdominal and mammary surgery. Human primary SCC cells and melanoma cells were isolated from punch biopsies taken from SCC surgical excisions. Both healthy skin and SCC biopsies were also used as whole explants for experiments. Primary cells were cultured in CnT-07 medium (CellNTec). All cells were cultured at 37°C and 5% CO₂.

International board approval for the use of human tissue was granted; all donors signed written informed consent forms in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (ethical approval number EK647). All samples were obtained from the University Hospital Zurich (Zurich, Switzerland).

**Viability and proliferation assay**

Cells were seeded on 24- or 96-well plates at 50% confluence and treated for 24 hours with ingenol mebutate or DMSO, respectively. When indicated, ingenol mebutate and DMSO were combined with speciﬁc kinase inhibitors. Viability was analyzed by the MTT assay (Sigma). Proliferation was assessed by bromodeoxyuridine (BrdU) incorporation using the BrdU assay Kit (Merck Millipore).

**Gene expression array and analysis**

Gene expression was analyzed in normal keratinocytes and patient-derived SCC cells 24 hours after ingenol mebutate or control treatment. RNA was isolated and cDNA was generated. The SurePrint G3 Human GE v2 8 × 60 K Microarray (Agilent) was used to analyze the samples.

Raw data were processed in the programming environment R using the limma package (20). The data were background corrected and quantile normalized. Pairwise comparison of treatment versus control samples was performed for each cell line. Differentially expressed genes were deemed to be significant if the absolute log-fold change was more than 2 and the FDR-adjusted P value was less than 0.05. Heatmap was generated by hierarchical clustering and Euclidean distance was used for distance metric. Microarray data have been deposited in NCBI’s Gene Expression Omnibus (21) in accordance with MIAME guidelines (GEO Series accession number GSE63308). Pathway analysis of deregulated genes was done using the software GeneGo (Thomson Reuters).

**RNA isolation and qPCR**

RNA from cells and tissue was isolated with TRIzol (Invitrogen) as described in the protocol. Reverse transcription was achieved using the Reverse Transcription Kit from Promega. qPCR was performed on the Viia7 real-time PCR machine (Life Technologies) using SYBR Green Mix (Roche). The following primers from Microsynth were used.

<table>
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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>IL1R2</td>
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<td>DEFB4A</td>
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**Protein detection**

Cells were lysed in RIPA buffer (Cell Signaling Technology, #9806) and protein extracts were analyzed by Western blotting using the following antibodies: anti-pPKCδ T505 (#9374), anti-pPKCδ Y311 (#2055), anti-pPKCα/βII T638/641 (#9375), anti-pPKCα/βII (#2056), anti-pp42/44 (#9101), anti-pp42/44 (#9102), anti-pJNK (#9251), anti-pJNK (#9252), anti-pSPRY2 (#9967), anti-pSPRY2 (#9968), anti-pJNK (#3146), anti-JNK (#9252), anti-pMAPK (#9522), anti-pMAPK (#9522), anti-pERK (#9522), anti-ERK (#9522), anti-rabbit HRP (#7074) all from Cell Signaling Technology, anti-β-actin (sc-47778, Santa Cruz Biotechnologies), Rabbit anti-mouse IgG H&L HRP (ab6728). Proteins were detected by ECL on Hyperfilm (Amersham, GE Healthcare).

**Phosphoprotein array**

PathScan Intracellular Signaling Array Kit from Cell Signaling Technology (#7323) was used according to the manufacturer's instructions.

**Drugs and inhibitors**

Ingenol mebutate (Supplementary Fig. S1A) was kindly provided by LEO Pharma A/S. Ingenol mebutate was either dissolved in DMSO for cell culture treatment or suspended in commercial vehicle for treatment of organ cultures. Cells and organ cultures were treated with different drug concentrations for varying durations as indicated. For PKC inhibition, AE8071 (Selleckchem) was used at concentrations between 500 nmol/L and 10 μmol/L. The ERK inhibitor SCH772984 (ChemScene, CS-1421) and the MEK
Ingenol Mebutate’s Mechanism in Keratinocytes

Ingenol mebutate affects viability and proliferation of keratinocytes and SCC cells. Primary keratinocytes and patient-derived SCC cells of three different donors and the human squamous cell carcinoma cell lines SCC12 and SCC13 were treated with the indicated concentrations of ingenol mebutate for 24, 48, or 72 hours. A, cell viability measured by the MTT assay. B, cell proliferation measured by BrdU incorporation. All values were normalized to DMSO-treated cells. Graphs represent mean and SD of three independent experiments.

Results

Ingenol mebutate inhibits viability and proliferation of keratinocytes and SCC cells

To investigate the impact of ingenol mebutate on epithelial cell viability and proliferation and to determine a working concentration for further experiments, we treated primary keratinocytes, patient-derived SCC cells, and SCC cell lines for 24, 48, and 72 hours with ingenol mebutate in a range from 1 nmol/L to 10^5 nmol/L. All cell types showed a biphasic decrease in viability (Fig. 1A) and proliferation (Fig. 1B) upon drug treatment. Interestingly, all cells exhibited a drop in viability and proliferation around a concentration of 100 nmol/L, while cells appeared healthier at lower (1–10 nmol/L) and higher (10^2–10^3 nmol/L) concentrations (Fig. 1A and B). At a concentration of 10^5 nmol/L, cells died to a great extent due to cytotoxicity. For further experiments, we used a concentration of 100 nmol/L, as this concentration had the largest effect at the first peak of the biphasic response.

To find out whether the biphasic effect was cell type–specific, we additionally treated fibroblasts and melanoma cells with similar concentrations of ingenol mebutate for 24 hours. We found no biphasic effect in fibroblasts, as they were resistant to the highest concentration used (10^5 nmol/L). Melanoma cells did not show a biphasic effect either, as they were almost unaffected at low concentrations, whereas viability was 50% decreased at the highest concentration used in the experiment (10^5 nmol/L; Supplementary Fig. S3A). Furthermore, we analyzed the viability of the SCC13 cell line after 24-hour treatment with several concentrations of the tumor promoting TPA, a drug structurally related to the anticancer drug ingenol mebutate, both being phorbol esters

Knock down

For knock down of PKCβ, PKCa, IL1R2, and IL13RA2, cells were transfected with INTERFERin (Polyplus transfections) and specific siRNAs (QIagen).

siCtrl: target sequence 5'-AATTCGCCGAACGTGTCACGT-3'
siPKCβ #3: target sequence 5'-CAAGACGCCTTTCGAACTCCAA-3'
siPKCβ #5: target sequence 5'-AACCATGCCTCACCACCTAA-3'
siPKCβ #1: target sequence 5'-CCCGGACACTATATTCCAGAA-3'
siPKCβ #7: target sequence 5'-AACCITACGCCACGCTTT-3'
siPKCβ #11: target sequence 5'-CACGACGCAAGCTGCAACATCAA-3'
siIL1R2 #2: target sequence 5'-CACGCCAGGAATTATCCAGAAA-3'
siIL1R2 #4: target sequence 5'-AAGACGACAATTCCGCTTAA-3'
siIL13RA2 #1: target sequence 5'-CAAGATATGGATGCTATAT-3'
siIL13RA2 #5: target sequence 5'-AAGGTGAAGACCTATC-

Cells were incubated for 30 hours with the transfection mix before they were treated with the drug for 24 hours. Knock down of each gene was confirmed (Supplementary Fig. S2).

Immunohistochemistry

After the treatment, samples were fixed in 4% formalin for 2 days, followed by dehydration and embedding in paraffin. Seven micron sections were made and stained with H&E or for pERK with the antibody pp42/44 (Cell Signaling Technology, #9101).
and known potent PKC activators (22). This was done to investigate whether the effect observed with ingenol mebutate was drug-specific. Unlike ingenol mebutate, we did not detect any biphasic effect upon TPA treatment (Supplementary Fig. S3B).

Gene expression analysis reveals involvement of PKC and MAPK signaling

To identify genes and signaling pathways mediating ingenol mebutate activity, we performed gene expression analysis on primary keratinocytes and patient-derived SCC cells treated for 24 hours with either DMSO or different concentrations of ingenol mebutate (1 nmol/L, 100 nmol/L, 10^4 nmol/L). In concordance with the results from the viability and proliferation assays, most genes were differentially expressed in the presence of 100 nmol/L ingenol mebutate (Fig. 2A). Fewer changes were detected at a concentration of 10^4 nmol/L while 1 nmol/L of ingenol mebutate had almost no effect (Supplementary Fig. S4A). At a concentration of 100 nmol/L, we found 1,227 genes upregulated in keratinocytes and 795 in SCC cells. Three-hundred and ninety of these genes overlap between the two cell types. Furthermore, we found 1,887 downregulated genes in keratinocytes and 996 in SCC cells with 713 genes overlapping (Fig. 2B). Hierarchical clustering of the microarray data showed common clusters of gene expression patterns in the keratinocytes and SCC cells upon drug treatment, as compared with vehicle-treated cells. Pathway analysis of downregulated genes using the software GeneGo identified mostly cell cycle-associated pathways and DNA damage repair pathways (Supplementary Fig. S4B). Upregulated genes following 100 nmol/L ingenol mebutate treatment mostly affected pathways such as ERK1/2 and PKC signaling in keratinocytes and ERK1/2 signaling in SCC cells (Supplementary Fig. S4C).

To identify key mediators of the ingenol mebutate effect, we took the top 5 upregulated genes in SCC cells that were also upregulated in keratinocytes. DEFB4A, ZP4, IL13RA2, CCL5, and IL1R2 were thus selected. We did not focus on C15orf48, which was also was among the top five upregulated genes, since we were interested in genes that are upregulated in both healthy and cancerous keratinocytes and C15orf48 was only found to be upregulated in SCC cells. Furthermore, we could not confirm the upregulation of C15orf48 in SCC tissue (data not shown). From literature research, we found DEFB4A, IL1R2, and IL13RA2 to be the most interesting ones. IL1R2 and IL13RA2 are decoy receptors that were thought to have no

Figure 2.
Gene expression analysis of ingenol mebutate (IM)-treated primary keratinocytes and patient-derived SCC cells. A, heatmap showing triplicates of differentially expressed genes between DMSO-treated and ingenol mebutate-treated primary keratinocytes and patient-derived SCC cells. Cells were treated with either DMSO or 100 nmol/L ingenol mebutate for 24 hours followed by RNA extraction and performance of the microarray. B, number of differentially expressed genes in primary keratinocytes and patient-derived SCC cells after treatment with 100 nmol/L ingenol mebutate. Upregulated genes are displayed in red and downregulated genes are displayed in blue.
Figure 3.
PKCδ is phosphorylated after ingenol mebutate treatment and is essential for the performance of ingenol mebutate. A, primary keratinocytes and SCC13 cells were treated with 100 nmol/L ingenol mebutate for 10, 30, or 45 minutes followed by protein extraction. Phosphorylation of PKCδ Y311 and PKCα/βII T638/641 was detected by Western blotting. Total PKCδ or PKCα was used as loading control. B, primary keratinocytes, patient-derived SCC cells, and SCC13 cells were treated for 24 hours with 100 nmol/L ingenol mebutate alone or in combination with the PKC inhibitor AEB071 followed by protein extraction. Phosphorylation of PKCδ T505 was detected by Western blotting. Total PKCδ was used as loading control. C, primary keratinocytes, patient-derived SCC cells, and SCC13 cells were treated for 24 hours with 100 nmol/L ingenol mebutate alone or in combination with the PKC inhibitor AEB071 followed by assessing cell viability by the MTT assay. Graphs represent mean and SD of three independent experiments. Data were analyzed by one-way ANOVA followed by the Dunnett multiple-comparison test. ***, P < 0.001; ****, P < 0.0001. D, SCC13 cells were transfected with two different siRNA against PKCδ (left) or PKCα (right) followed by 24-hour treatment with 100 nmol/L ingenol mebutate and followed by assessing cell viability by the MTT assay. Graphs represent mean and SD of three independent experiments. Data were analyzed by the Student t test. ***, P < 0.001; ****, P < 0.0001.
signaling function. However, a recent article demonstrated a signaling ability of IL13RA2 (23). In addition, IL13AR2 was reported to slow down or even prevent tumor growth in mice when overexpressed in pancreatic or breast cancer cells (24). Furthermore, IL1β was shown to enhance proliferation of oral keratinocytes (25) and therefore the upregulation of its decay receptor by ingenol mebutate may play a crucial role in the mechanism of action of ingenol mebutate. DEFB4A was shown to be differentially expressed in SCC (26). We then confirmed the upregulation of these genes by qPCR in keratinocytes, patient-derived SCC cells, two SCC cell lines, human epidermis, and human SCC explants after 24 hours of ingenol mebutate treatment. We found IL1R2 and IL13RA2 consistently and significantly upregulated in all tested cell types and explant tissues after treatment with ingenol mebutate, while DEFB4A upregulation could not be confirmed in SCC cell lines and SCC explants (Supplementary Fig. S4D; Supplementary Table S1).

PKCδ plays a fundamental role in the mechanism of action of ingenol mebutate

As our pathway analysis agreed with earlier studies (16, 22) that indicated a role for PKC signaling following ingenol mebutate exposure, we further investigated this pathway as a possible mechanism of action in the loss of epithelial cell viability and proliferation. We incubated primary keratinocytes, patient-derived SCC cells and the SCC13 cell line with 100 nmol/L ingenol mebutate for up to 45 minutes and found PKCδ to be highly phosphorylated in the hinge region (Y311) in all cell types while PKCoα is not phosphorylated (Fig. 3A). Y311 was previously reported to be required for PKCδ activation and furthermore enhances autophosphorylation of PKCδ on T505 (27). Accordingly, we found the activation loop (T505) of PKCδ to be more phosphorylated in all three cell types after 24-hour drug treatment, whereas this phosphorylation could be blocked by the PKC inhibitor AEB071 (Fig. 3B). Although PKCδ can function without being phosphorylated at T505, this phosphorylation increases the catalytic activity of PKCδ (27). Furthermore, T505 phosphorylation influences the substrate specificity of PKCδ and is essential for the activation of the AP1 family of transcription factors (28).

To further investigate the role of PKCδ, we used ingenol mebutate alone or in combination with the PKC inhibitor AEB071 and measured cell viability of primary keratinocytes, SCC patient cells and the SCC13 cell line after 24 hours of treatment. We found cell viability to be partially rescued by PKC inhibition when treated with ingenol mebutate (Fig. 3C). As the PKC inhibitor is not entirely specific for single isoforms, we further validated the role of PKCδ using siRNA to knockdown PKCδ in SCC13 cells followed by analysis of cell viability after 24 hours ingenol mebutate treatment. Similar to our findings with the PKC inhibitor, we detected a rescue of cell viability upon ingenol mebutate treatment when PKCδ was knocked down. A knockdown PKCoα followed by ingenol mebutate treatment led only to a partial rescue, while PKCδ knockdown could rescue the ingenol mebutate–treated cells completely (Fig. 3D).

ERK is activated by ingenol mebutate treatment in a PKC-dependent manner and mediates ingenol mebutate–dependent effects on cell viability

Besides PKC signaling, our pathway analysis suggested ERK1/2 to be involved in mediating the effect of ingenol mebutate. To confirm this and to exclude other pathways involved in MAPK signaling (e.g., p38, JNK) we performed a phosphorylation array experiment with primary keratinocytes and SCC cells from three different donors and found ERK to be phosphorylated upon ingenol mebutate treatment, while other pathways of MAPK signaling (p38 and JNK) showed no or only weak phosphorylation (Supplementary Fig. S5A). Weak or non-existent phosphorylation of JNK and p38 was further validated on cells from more donors by conventional Western blotting (Fig. 4A). To test the effect of ingenol mebutate in a more natural model of skin, we treated human skin explants in three independent experiments with either vehicle or ingenol mebutate gel 0.05%. Assessment of ERK phosphorylation after 24 hours of treatment revealed a sustained activation of ERK compared to vehicle-treated samples (Fig. 4B). To further demonstrate activation of ERK, we analyzed the expression of known ERK target genes (EGR1, SPRY2, GFO; ref. 29) in SCC13 cells, primary keratinocytes, and patient-derived SCC cells after 24 hours of treatment by qPCR and found them to be upregulated upon ingenol mebutate treatment, while the upregulation of the ERK target genes was abolished by an ERK inhibitor (SCH772984) and a MEK inhibitor (GSK1120212B) (Supplementary Fig. S5B). In addition, we used either the PKC inhibitor AEB071 or a knockdown of PKCδ, either alone or in combination with ingenol mebutate and analyzed the expression of ERK target genes. Either inhibition of PKC or knockdown of PKCδ could prevent the upregulation of ERK target gene expression after ingenol mebutate treatment (Fig. 4C). Furthermore, we analyzed the phosphorylation of ERK by Western blotting after short-term ingenol mebutate treatment in cells treated with the PKC inhibitor and in cells with PKCδ knockdown. The drug alone induced phosphorylation of ERK, which was blocked by addition of the PKC inhibitor (Fig. 4D, left) or by knockdown of PKCδ (Fig. 4D, right). This was confirmed in human skin explants preincubated in either DMSO or PKC inhibitor for 2 hours and treated with either vehicle or ingenol mebutate gel 0.015%. After 18 hours of incubation, we analyzed pERK status by immunohistochemistry and confirmed sustained ERK phosphorylation after drug treatment, while this phosphorylation was abolished by the PKC inhibitor (Fig. 4E). Taken together, these results indicate that ERK is rapidly phosphorylated by ingenol mebutate in a PKC-dependent manner upon drug treatment. To further investigate these pathways in the mechanism of action of ingenol mebutate, we analyzed the viability of primary keratinocytes, patient-derived SCC cells and the SCC13 cell line after 24 hours treatment with either the drug alone or in combination with the ERK inhibitor (SCH772984) or the MEK inhibitor (GSK1120212B). As seen in previous experiments, cell viability dropped below 50% upon ingenol mebutate treatment. However, viability was partially rescued when cells were treated in combination with the MEK or ERK inhibitor but not in combination with the JNK inhibitor SP600125 or the p38 inhibitor SB203580 (Fig. 4F).

IL1R2 and IL13RA2 partially mediate the mechanism of action of ingenol mebutate in SCC

To further investigate the role of IL1R2 and IL13RA2, we first confirmed their upregulation upon 24-hour ingenol mebutate treatment in keratinocytes, patient-derived SCC cells, the SCC12 and SCC13 cell lines and epidermis from human...
ERK is activated by ingenol mebutate treatment in a PKC-dependent manner. A, phosphorylation of JNK and p38 was analyzed in total protein extracts of primary keratinocytes and patient-derived SCC cells after 10 and 30 minutes of ingenol mebutate treatment. B, immunohistochemical staining for ERK is activated by ingenol mebutate treatment in a PKC-dependent manner. A, phosphorylation of JNK and p38 was analyzed in total protein extracts of primary keratinocytes and patient-derived SCC cells after 10 and 30 minutes of ingenol mebutate treatment. B, immunohistochemical staining for ERK is activated by ingenol mebutate treatment in a PKC-dependent manner.

Gene expression was assessed by qPCR. Graphs represent mean and SD of three independent experiments. Data were analyzed by one-way ANOVA followed by the Dunnett multiple-comparison test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. D, phosphorylation of ERK after ingenol mebutate treatment can be blocked by the PKC inhibitor or by knockdown of PKCα. Primary keratinocytes, patient-derived SCC cells, and SCC13 cells were treated for 10 minutes with ingenol mebutate alone or in combination with the PKC inhibitor followed by protein extraction. (Continued on the following page.)
IL13RA2 and IL1R2 are involved in the mechanism of action of ingenol mebutate. A, IL13RA2 and IL1R2 gene expression is upregulated by ingenol mebutate treatment in a PKC/MEK/ERK–dependent manner. Cells were treated either with ingenol mebutate alone or in combination with the indicated kinase inhibitors for 24 hours followed by RNA isolation and gene expression analysis by qPCR. Data of at least three independent experiments were analyzed by one-way ANOVA followed by the Dunnett multiple-comparison test. **, P < 0.01; *** P < 0.001. B, IL13RA2 and IL1R2 are PKCδ-dependent. SCC13 cells were transfected with two different siRNA against IL13RA2 or IL1R2 followed by 24 hours of ingenol mebutate treatment. Viability was assessed by the MTT assay. Graphs show mean and SD of three independent experiments in primary keratinocytes and epidermis from explants by qPCR (Supplementary Fig. S4D). C15orf48 was not further investigated due to the lack of a commercially available antibody and the fact that we could not confirm its upregulation in SCC tissue. As we saw an upregulation of IL1R2 and IL13RA2 after 24 hours of treatment, we performed time course experiments in primary keratinocytes and epidermis from...
human skin explants to better define the timescale of gene regulation. We found both genes to be upregulated after 2 to 6 hours of treatment (Supplementary Fig. S6).

Furthermore, we treated cells with ingenol mebutate alone or in combination with the PKC-, the MEK- or the ERK inhibitor, respectively, and found that the drug-dependent upregulation of both genes was partially abolished. Ingenol mebutate in combination with JNK inhibition or p38 inhibition had almost no repressive effect on the expression of the two genes (Fig. 5A). In addition, we knocked down PKCβ by siRNA, which also prevented the drug-dependent upregulation of IL1R2 and IL13RA2 mRNA (Fig. 5B). To find out whether IL1R2 or IL13RA2 play an essential role in the mechanism of action ingenol mebutate, we knocked down these two genes with siRNA and analyzed cell viability upon drug treatment. Interestingly, both IL1R2 and IL13RA2 knock down partially rescued the viability of drug-treated SCC13 cells (Fig. 5C).

Discussion

Ingenol mebutate is approved for the topical treatment of actinic keratosis, the precursor of squamous cell carcinoma (SCC). However, its mechanism of action is not completely understood. Several studies investigated ingenol mebutate in the context of different cancers (15–17) and suggest a role of the innate immune system (14) but there is only one study that focuses on the effect on the actual target, the malignant keratinocytes (18). While that study used high ingenol mebutate concentrations (100–400 μmol/L) and short incubation times, leading to cytotoxicity involving mitochondrial network disruption, we investigated the mechanism of action of ingenol mebutate in healthy keratinocytes, SCC cells, and skin explants at lower concentrations (100 nmol/L) and up to 72 hours. We showed that ingenol mebutate induced a direct effect on viability and proliferation on cells of different origin. Previous studies on different mouse and human cancer cell lines, like melanoma or breast carcinoma, determined the lethal dose (LD₉₀) of ingenol mebutate for these cells to be between 180 and 220 μmol/L after 24 hours (13). Benhadji and colleagues assessed growth inhibition by the MTT assay and calculated the IC₅₀ of colon cancer cell lines, finding great variations from 3 μmol/L in Colo-205 cells to more than 300 μmol/L in HCT116 and HCC2998 cells (30). Overall the concentrations leading to cell death strongly vary depending on the cell line tested and our results fit within this range showing most cells reaching a lethal affect at 100 μmol/L ingenol mebutate. In line with other reports (16), we found fibroblasts to be relatively resistant to ingenol mebutate while melanoma cells were affected, but at higher concentrations than the keratinocytes. In contrast to melanoma cells and fibroblasts, we found a biphasic effect on viability and proliferation on keratinocytes and SCC cells. This determines that ingenol mebutate induces cell death in two ways, depending on concentration and cell type. Furthermore, our results on SCC13 cells indicated that the biphasic effect was ingenol mebutate–specific as compared with another PKC activator, TPA. Our microarray performed with various ingenol mebutate concentrations showed the highest number of genes differentially expressed at 100 nmol/L whereas effects were less pronounced at lower and higher concentrations, consistent with the impact of 100 nmol/L ingenol mebutate on cell viability and proliferation. This pronounced effect at 100 nmol/L again points to the existence of two different concentration-dependent mechanisms of action for ingenol mebutate, whereas the highest concentration used leads to cell death due to direct cytotoxicity. The lower concentration of 100 nmol/L, however, may trigger intracellular pathways that lead to the reduction of cell viability. These potential signaling pathways were subject to our further investigations.

PKCβ is a key mediator in cell differentiation and inhibition of proliferation in various tissues (31–34). A direct binding of ingenol mebutate to PKC isoforms has been observed (22). Ingenol mebutate treatment leads to phosphorylation of PKCβ in the Colo-205 cancer cell line (30). In other studies, PKCβ was reported to drive keratinocyte differentiation and inhibit proliferation of the immortalized keratinocyte line HaCaT, which could be reversed by PKCβ inhibition (32). Our data from gene expression to functional experiments in primary cell cultures underline a critical role for PKCβ in mediating the effect of ingenol mebutate in keratinocytes and verify such previous observations correspondingly for ingenol mebutate while PKCα played only a minor role in our setting.

Downstream of PKC signaling, our pathway analysis revealed an involvement of ERK1/2 in the mechanism of action of ingenol mebutate. Previous work on colon cancer cells suggests involvement of MAPK pathways, including JNK and p38 (30, 35), by showing phosphorylation of these proteins after ingenol mebutate treatment. We confirmed activation of ERK1/2 while we were able to exclude a major role of other MAPK pathway components like JNK and p38 in keratinocytes and SCC cells. Similar to previous studies on melanoma cells (16), our data on keratinocytes clearly link PKCβ to the activation of MAPK signaling. Both MEK and ERK are essential for the effect of ingenol mebutate in our assays. Although the activation of the MEK/ERK pathway is generally considered to promote tumor cell growth (36), ERK activation seems to contribute favorably to the mechanism of action of ingenol mebutate, leading to reduced cell viability and proliferation. In line with this assumption, ERK activation was found to suppress tumors by promotion of selective protein degradation (37). Moreover, high ERK activation can serve as a marker of improved outcome in breast cancer patients (38).

We identified and validated a role for several novel genes as critical players in the mechanism of action of ingenol mebutate. Our analysis focused on IL13RA2 and IL1R2, which were both induced by ingenol mebutate in a PKCβ/MEK/ERK–dependent manner. Both genes encode for interleukin receptors that are commonly known to function as decoy receptors without signaling ability (39, 40). Nevertheless, a recent publication attributed an active role to IL13RA2 in signaling, thus inducing TGFβ-mediated fibrosis (23). However, in the case for ingenol mebutate–induced effects, induction of fibrosis is unlikely as patients treated with ingenol mebutate do not show (fibrosis-related) scarring and have a good cosmetic outcome post-treatment with ingenol mebutate gel (11). Moreover, a recent study revealed a role of IL13RA2 in tumor suppression, as IL13RA2-overexpressing breast and pancreatic cancer cells showed reduced or no tumor growth when injected into mice (24).

Similarly, IL1R2 recently showed activity against ectopic tissue growth and endometriosis progression and could down-regulate the antiapoptotic protein Bcl2 (41), which could explain that the cell death mechanism by ingenol mebutate

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could be attributed by IL1R2 signaling. As IL1β was shown to promote proliferation of oral keratinocytes (25), one could speculate whether IL1R2 is upregulated by ingenol mebutate to capture IL1β and prevent this effect. Further studies are needed to clarify the role of IL13RA2 and IL1R2 in the mechanism of action of ingenol mebutate.

In summary, we report a mechanism of action for ingenol mebutate in proliferating normal and malignant keratinocytes through specific activation of PKCδ leading to activation of MEK/ERK signaling, resulting in decreased viability. This was dependent on concentration and cell type, resulting in a unique biphasic induced loss of viability through ingenol mebutate. A set of genes responsive to ingenol mebutate treatment, mainly IL13RA2 and IL1R2, showed that they partially mediate the function of ingenol mebutate on SCC, suggesting a function for them apart from their role in the immune system. However, our data do not allow us to conclude whether IL13RA2 and IL1R2 exert their effect primarily in keratinocytes treated by ingenol mebutate or whether they are instrumental in orchestrating the accompanying immune response. Further projects are needed to shed more light on this matter.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
Ingenol Mebutate's Mechanism in Keratinocytes


Ingenol Mebutate Signals via PKC/MEK/ERK in Keratinocytes and Induces Interleukin Decoy Receptors IL1R2 and IL13RA2

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