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RESEARCH ARTICLE

Inotilone suppresses phorbol ester-induced inflammation and tumor promotion in mouse skin

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Scope: Chemoprevention is one of the most feasible approaches to reduce the risk of cancer. Over the past decades, scientists have realized that chronic inflammation is a critical component of cancer development. Inotilone, existing in *Inonotus* mushroom has been reported to exhibit anti-inflammatory properties in vitro. Hence, we investigated the effects of inotilone on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-mediated acute inflammation and tumor promotion in mouse skin and the underlying molecular mechanisms.

Methods and results: Inotilone was topically applied to mouse skin 30 min prior to TPA treatment. The results have shown that inotilone inhibited the production of inflammatory mediators by attenuating the activation of nuclear factor-κB (NF-κB) and the expression of CCAAT/enhancer binding protein β (C/EBPβ). Furthermore, the ability of inotilone to prevent tumorigenesis at promotion stage was evaluated using a classical two-stage mouse skin carcinogenesis model. After initiation of 7,12-dimethylbenz[*a*]anthracene (DMBA), applying inotilone topically before each TPA treatment was found to reduce the tumor incidence and tumor multiplicity of papillomas.

Conclusion: Based on the results, we concluded that inotilone has potential to be developed into an effective chemopreventive agent for the treatment of a variety of inflammatory diseases, especially the prevention and treatment of epithelial skin cancer.

Keywords:
COX-2 / Inflammation / iNOS / Inotilone / Skin cancer

1 Introduction

Cancer is a growing health problem and continues to be the leading cause of death in the world. Despite its high incidence and mortality, cancer should be a preventable disease. One of the most promising approaches to reduce the risk of cancer is chemoprevention based on the idea that cancer development is a multistep process with sequential stages of initiation, promotion, and progression [1]. Considering for the long-term use, natural dietary compounds with low toxicity become an attractive group of chemopreventive agent.

Accumulating evidence has shown that inflammation plays a crucial role in cancer development [2, 3]. Aberrant upregulation of proinflammatory mediators has often been observed in various premalignant and malignant tissues. Therefore, the suppression of the overproduction of proinflammatory mediators and the normalization of abnormally activated inflammatory signaling cascades may be a rational strategy for achieving chemoprevention. Indeed, a number of natural dietary compounds with potent anti-inflammatory properties have shown to modify cancer development process

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Abbreviations: **C/EBP**, CCAAT/enhancer-binding protein; **COX-2**, cycoloxygenase-2; **DMBA**, 7,12-dimethylbenz[*a*]anthracene; **ERK1/2**, extracellular signal-regulated kinases 1/2; **IκB**, inhibitor κB; **iNOS**, inducible nitric oxide synthase; **MAPK**, mitogen-activated protein kinase; **MMP-9**, matrix metallopeptidase-9; **NF-κB**, nuclear factor-κB; **PGE_2**, prostaglandin E_2; **PI3K**, phosphatidylinositol 3-kinase; **TPA**, 12-*O*-tetradecanoylphorbol-13-acetate; **VEGF**, vascular endothelial growth factor

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through interfering with intracellular signaling network and the production of inflammatory mediators [4, 5].

NF-κB is one of the most important transcription factors that regulate the inflammatory responses. Activation of NF-κB often facilitates transcription of numerous genes related to inflammation, including adhesion molecules, cytokines, chemokines, matrix metalloproteases, and inducible enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) [6]. NF-κB has been shown to be essential for promoting inflammation-associated cancer [7]. Constitutively activated NF-κB has been observed in many types of cancer. Therefore, dietary compounds that can inhibit the activation of NF-κB may be used as chemopreventive agents to prevent cancer.

The CCAAT/enhancer binding proteins (C/EPBs) are a family of basic leucine-zipper (bZIP) transcription factors and are involved in biological processes such as cell proliferation, differentiation, and inflammation that are thought to be crucial in tumorigenesis [8]. At least six family members have been identified to date (C/EBPα–C/EBPζ). Despite the high degree of homology between the family members in the bZIP domain, mice deficient in any of them each has a unique phenotype. Among them, the null mutation in C/EBPβ has the most phenotypes by affecting diverse cell types and a multitude of functions in many different organs. Moreover, recent studies revealed that C/EBPβ is implicated in tumor development in several cell types/ tissues. Increased expression of C/EBPβ is associated with human breast, colorectal, and ovarian tumorigenesis [9, 10]. Accordingly, C/EBPβ may be another potential target for chemopreventive agents.

Inotilone, a secondary metabolite found in the dietary *Inonotus* mushroom, has been reported to as a potent inflammatory inhibitor based on an enzyme assay [11]. Our previous study has also shown that inotilone suppresses lipopolysaccharide (LPS)-stimulated inflammatory response in RAW 264.7 macrophages by decreasing the production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) [12]. However, inotilone with the differential inhibitory effect on NF-κB and C/EBPβ can only repress the expression of iNOS but not COX-2. The reduction of PGE_2 was attributed to the inhibition of the enzyme activity of COX-2. In the present study, the effects of inotilone on the inflammatory response induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter, in mouse skin and the possible molecular mechanisms underlying these effects were investigated. To further evaluate the chemopreventive potential of inotilone, a classical two-stage mouse skin carcinogenesis model was applied to study the antitumor promotional effect of inotilone.

2 Material and methods

2.1 Chemicals

Inotilone was synthesized according to the method of Shamshina and Snowden [13]. TPA, 7,12-dimethylbenz[*a*]anthracene (DMBA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). All other chemicals used were in the purest form commercially available.

2.2 Animal treatment

Female Institute of Cancer Research (ICR) mice at 5–6 wk-old were obtained from the BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan). All animals were housed in a controlled atmosphere (25 ± 1°C at 50% relative humidity) with a 12 h/12 h light/dark cycles and free access to water and food. The dorsal skin of each mouse was shaved with electric clippers before application of tested compound. Inotilone and TPA were dissolved in dimethyl sulfoxide (DMSO)/acetone (10:90, v/v). Control animals were treated with 10% DMSO in acetone as vehicle in all experiments. All materials were applied topically to the shaved dorsal skin of mice. All animal experimental protocol used in this study was approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU).

2.3 Western blot analysis

Inotilone (1 or 5 μmol) was treated to the shaved dorsal skin 30 min before application of TPA (10 nmol). Control animals were treated with acetone instead of TPA. Depending on the maximum expression of the response markers, mice were sacrificed at various time intervals after treated with TPA. Dorsal skins of mice were excised and heated to 58°C for 15 s in water bath and then immersed in ice water to separate epidermis and dermal fractions. The epidermis was gently removed using a scalpel on cold iron block and homogenized in 200 μL lysis buffer (10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM $Na_4P_2O_7$, 20 mM Tris buffer [pH 7.9], 100 μM β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 50 mL). The homogenates were incubated on ice for 1 h, followed by centrifugation at 12 000 rpm for 30 min at 4°C. The supernatants were collected as total protein. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc. Hercules, CA). Equal amount of total cellular protein (50 μg) was resolved by 8–15% SDS-PAGE and electrotransferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membrane was then blocked at room temperature for 1 h with blocking solution (20 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.2% Tween 20, 1% bovine serum albumin, and 0.1 % NaN_3) followed by incubation with the primary antibody overnight at 4°C. The membrane was then washed with 0.2% TPBS (0.2% Tween-20/PBS) and subsequently probed with anti-mouse, anti-rabbit, or anti-goat IgG antibody conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The

bound antibody was visualized with VisGlow™ Chemiluminescent Substrate, HRP (Visual Protein, Taipei, Taiwan) and X-ray film (Super RX, Fujifilm, Japan). Relative band intensities were determined by a densitometer (Alphalmager™ 2200, Alpha Innotech Corp., San Leandro, CA). Primary antibodies of specific protein were purchased from various locations as listed below: for COX-2 were from BD Transduction Laboratories (San Jose, CA), for iNOS, C/EBPβ, p50, phospho-PI3K(Tyr 508), and lamin B were from Santa Cruz Biotechnology (Santa Cruz, CA), for IκBα, p65, and phospho-IκBα (Ser 32) were from New England Biolabs (Ipwich, MA), for phospho-Akt (Ser 473), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), ERK, p38, Akt, and GAPDH were from Cell Signaling Technology, Inc. (Danvers, MA), and for β-actin was from Oncogene Science Inc. (Uniondale, NJ).

2.4 Quantitative real-time PCR (TaqMan)

The collected epidermal sample was homogenized in 500 μL TRIzol® according to the manufacturer's instructions. Two micrograms of total RNA were transcribed into cDNA using SuperScript™ II Reverse Transcriptase in a final volume of 20 μL. RT reactions were performed at 50°C for 60 min and 70°C for 15 min. Real-time PCR was performed with LightCycler® TaqMan® Master kit and LightCycler®1.5 System (Roche) according to the manufacturer's instructions. Primers and TaqMan probes used in this experiment are: iNOS, sense 5′-ACC CTA AGA GTC ACC AAA ATG G-3′, antisense 5′-CCA GGG ATT CTG GAA CAT TCT-3′, Universal ProbeLibrary (UPL) probe no. 17; COX-2, sense 5′-GGG AGT CTG GAA CAT TGT GAA-3′, antisense 5′-GCA CAT TGT AAG TAG GTG GAC TGT-3′, UPL probe no. 4; actin, sense 5′-CCA ACC GTG AAA AGA TGA CC-3′, antisense 5′-ACC AGA GGC ATA CAG GGA CA-3′, UPL probe no. 64. The thermal cycling conditions were 10 min at 95°C, followed by 45 cycles of 95°C for 10 s, 55°C for 5 s, and 72°C for 15 s. The expression levels of the specific genes in each sample were calculated with the LightCycler software and normalized with a housekeeping control (β-actin).

2.5 Preparation of cytosolic and nuclear extracts from mouse skin

The collected epidermal sample was homogenized in 200 μL of hypotonic buffer (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, and 0.1 mM PMSF). The homogenate was incubated on ice with gentle shaking for 15 min and centrifuged at 14 800×g for 2 min. The supernatant was collected as cytosolic fraction. The pellet was washed with 500 μL of hypotonic buffer plus 40 μL of 10% NP-40, centrifuged and resuspended in 200 μL of hypertonic buffer (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, and 20% glycerol). It was kept on ice for 1 h followed by centrifugation at 12 000 rpm for 30 min at 4°C. The supernatant was collected as nuclear fraction.

2.6 Measurement of epidermal hyperplasia and infiltrated inflammatory cells

In the epidermal thickness study, skin samples from different treatment groups were fixed in 10% formalin and embedded in paraffin for histological examinations. Sections (4 μm in thickness) of the skin samples were cut and mounted on polylysin-coated slides. Each section was deparaffinized in xylene, rehydrated through a series of graded alcohols, and subjected to stain with hematoxylin and eosin. The thickness of the epidermis (μm) was measured using a Nikon light microscope (Japan) equipped with an ocular micrometer at a 400 × magnification in 15 fields per section. The number of dermal infiltrated inflammatory cells was determined by counting the stained cells at five different areas.

2.7 Two-stage mouse skin tumorigenesis

Female ICR mice were randomly divided into four groups, each consisting of 12 animals. Six-week-old mice were treated on their shaved back with a single dose of 200 nmol DMBA. Mice in control group received 200 μL of acetone alone. One week after initiation, the mice were topically treated with acetone or 5 nmol TPA twice a week for 19 weeks. For the other two groups, the mice were treated with inotilone (1 or 5 μmol) 30 min before each TPA treatment. Tumors of at least 1 mm^2 diameter measured by an electronic digital caliper were counted and the diameters of skin tumors were recorded. The results were expressed as the average number of tumors per mouse (tumor multiplicity) and the percentage of tumor-bearing mice (tumor incidence).

2.8 Statistical analysis

Data are presented as the means ± SD of at least three independent experiments. The statistical significance was evaluated by Student's *t*-test and a *p*-value < 0.05 was considered to be statistically significant.

3 Results

3.1 Inhibitory effects of inotilone on the expression of inflammatory mediators induced by TPA

The effects of inotilone on the expression of inflammatory mediators were used to demonstrate the anti-inflammatory

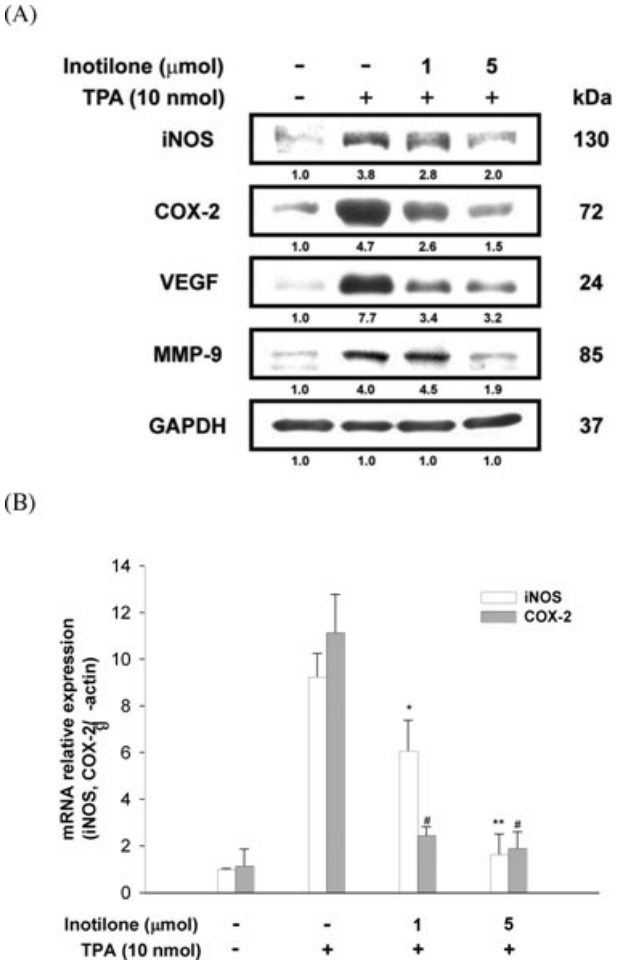

Figure 1. Effects of inotilone on TPA-induced inflammatory mediators expressions. Animals were treated as described in Material and Methods and sacrificed after indicated time periods. (A) The total epidermal protein extract was analyzed for iNOS (2 h), COX-2 (4 h), VEGF (4 h), and MMP-9 (4 h) expressions by Western blotting. (B) Animals were sacrificed after 1 and 2 h to prepare complementary DNA to analyze iNOS and COX-2 mRNA expression, respectively. Data are presented as mean ± SE of triplicate tests. $^{*}p < 0.05$, $^{**}p < 0.01$ (for iNOS), and $^{***}p < 0.05$ (for COX-2) were versus TPA alone.

activity of inotilone. Previous study had shown that single topical application of TPA induced the expression of iNOS, COX-2, vascular endothelial growth factor (VEGF), and matrix metallopeptidase-9 (MMP-9) in mouse skin [14]. As illustrated in Fig. 1A, topical application of inotilone 30 min prior to TPA treatment greatly reduced the levels of iNOS, COX-2, VEGF, and MMP-9 protein expressions in a concentration dependent manner. Real-time PCR analyses were also performed to examine the effects of inotilone on *iNOS* and *COX-2* mRNA induction caused by TPA. As shown in Fig. 1B, pretreatment with inotilone greatly attenuated *iNOS* and *COX-2* mRNA expressions in a dose-dependent manner that was in concordance with the results obtained from Western blotting.

3.2 Inhibitory effect of inotilone on TPA-induced activation of NF-κB and C/EBPβ expression

To further identify the molecular targets of inotilone for suppressing the inflammatory mediators in TPA-treated mouse skin, the effects of inotilone on the activation of NF-κB induced by TPA was first investigated. One of the most critical steps for the activation of NF-κB is its dissociation from inhibitor κB (IκB) α, which is mediated through the phosphorylation and subsequent proteolytic degradation of IκBα. Accordingly, the effects of inotilone on the phosphorylation and cytoplasmic levels of IκB induced by TPA were studied by Western blotting. As presented in Fig. 2A, pretreatment with inotilone diminished the phosphorylation and subsequent degradation of IκBα induced by TPA. Cytosolic and nuclear extract were also prepared to identify the nuclear translocation of NF-κB. As shown in Fig. 2B, TPA evoked the nuclear translocation of both the subunits of NF-κB, p50 and p65, whereas pretreatment with inotilone suppressed their translocation. Lamin B, a nuclear protein and β-actin, a cytosolic protein, were used as controls to confirm there was no contamination during the extraction of each fraction.

Besides NF-κB, C/EBPβ is another transcription factor that has also been reported to be involved in inflammation and tumorigenesis [15]. Therefore, the effect of inotilone on the expression of C/EBPβ was evaluated. Western blot analysis had shown that TPA induced the expression of C/EBPβ in mouse skin (Fig. 3). Topical application of inotilone prior to TPA application dose dependently inhibited TPA-induced C/EBPβ expression.

3.3 Inhibitory effect of inotilone on TPA-induced activation of p38, ERK1/2 MAPK, and PI3K/Akt signaling pathways

Studies have indicated that p38, ERK1/2 MAPK, and phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways are involved in the activation of NF-κB in TPA-treated mouse skin [16, 17]. All of these kinases need to be phosphorylated to become activated. Therefore, the effects of inotilone on TPA-induced phosphorylation of p38, ERK1/2, PI3K, and Akt were studied by Western blotting. As shown in Fig. 4, p38 and ERK MAPK in mouse skin were phosphorylated in response to TPA treatment. Pretreatment with inotilone attenuated TPA-induced phosphorylation of p38 but slightly decreased phosphorylated ERK1/2. The inhibitory effect of inotilone was also found in PI3K/Akt pathway. Upon TPA treatment, the phosphorylation of PI3K and its downstream kinase, Akt, increased and were blocked by pretreatment with inotilone.

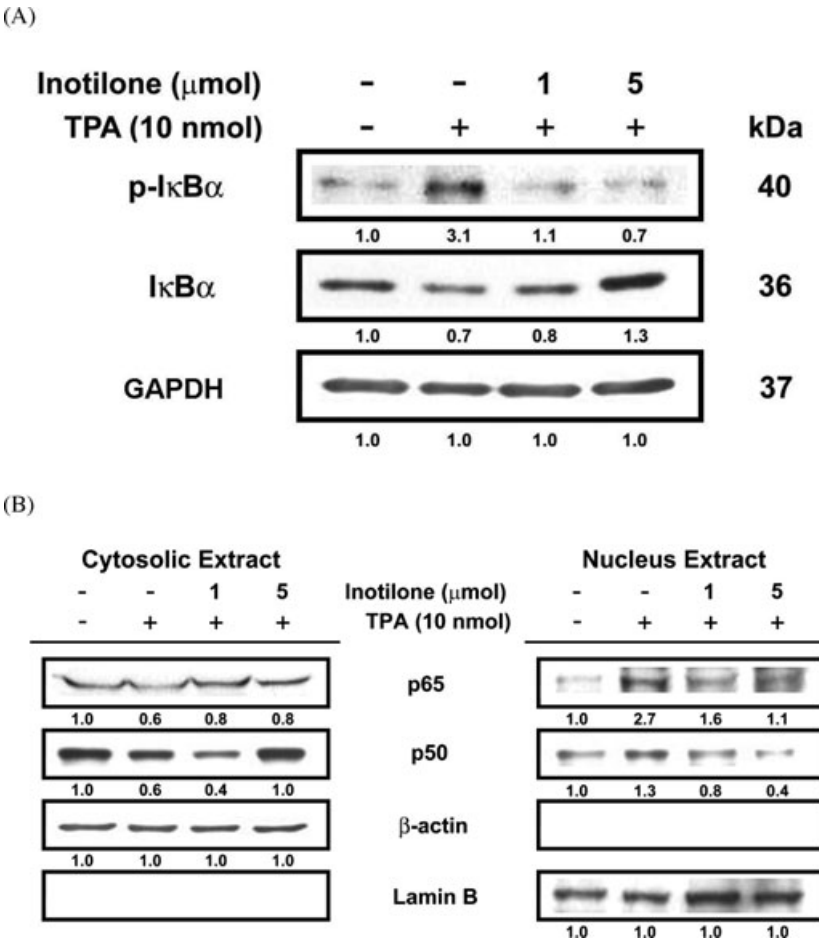

Figure 2. Effects of inotilone on TPA-induced activation and nuclear translocation of NF-κB. Animals were treated as described in Material and Methods and sacrificed after 1 h of TPA treatment. (A) Total extract were analyzed for the phosphorylated and total IκBα. (B) Cytosolic and nuclear fractions were analyzed for p50 and p65. Lamin B, a nuclear protein and β-actin, a cytosolic protein, were used as controls to confirm there was no contamination during the extraction of each fraction.

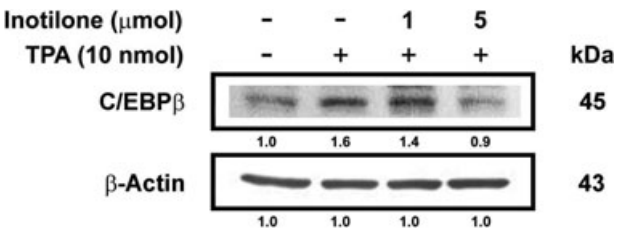

Figure 3. Effect of inotilone on the expression of C/EBPβ in TPA-treated mouse skin. Animals were treated as described in material and methods. The epidermal lysates wre analyzed for C/EBPβ expression by Western blotting.

3.4 Effects of inotilone on epidermal hyperplasia and infiltration of inflammatory cells induced by TPA in mouse skin

The anti-inflammatory activities of inotilone were also evaluated by its effects on TPA-mediated induction of epidermal hyperplasia and inflammatory cells infiltration. As summarized in Table 1, topical application of TPA at a dose of 10 nmol resulted in a significant increase in epidermal thickness as compared to the control animals (6.8 ± 1.2 μm in group 1 versus 20.2 ± 1.2 μm in group 2, $p < 0.001$). Pretreatment with inotilone prior to TPA application greatly suppressed TPA-mediated hyperplastic response. The epidermal thickness of TPA-treated mouse skins were reduced to 7.5 ± 1.5 μm and 6.1 ± 1.3 μm with inotilone pretreatment at 1 and 5 μmol, respectively.

The inhibitory effects of inotilone on the induction of inflammatory cell infiltration were also presented in Table 1. A greater number of inflammatory cells in dermis were observed upon TPA treatment as compared to the control animals. After 24 h of TPA treatment, the numbers of infiltrating inflammatory cells were increased from 23.0 ± 4.8/mm^2 to 77.7 ± 8.7/mm^2 ($p < 0.001$). Pretreatment the skin with 1 and 5 μmol inotilone inhibited the filtration by 71% and 91%, respectively.

3.5 Suppression of DMBA-initiated and TPA-promoted mouse skin tumorigenesis

The antitumor promotion activity of inotilone was evaluated on TPA-induced tumor formation in DMBA-initiated mouse skin. Hematoxylin and eosin analysis showed that

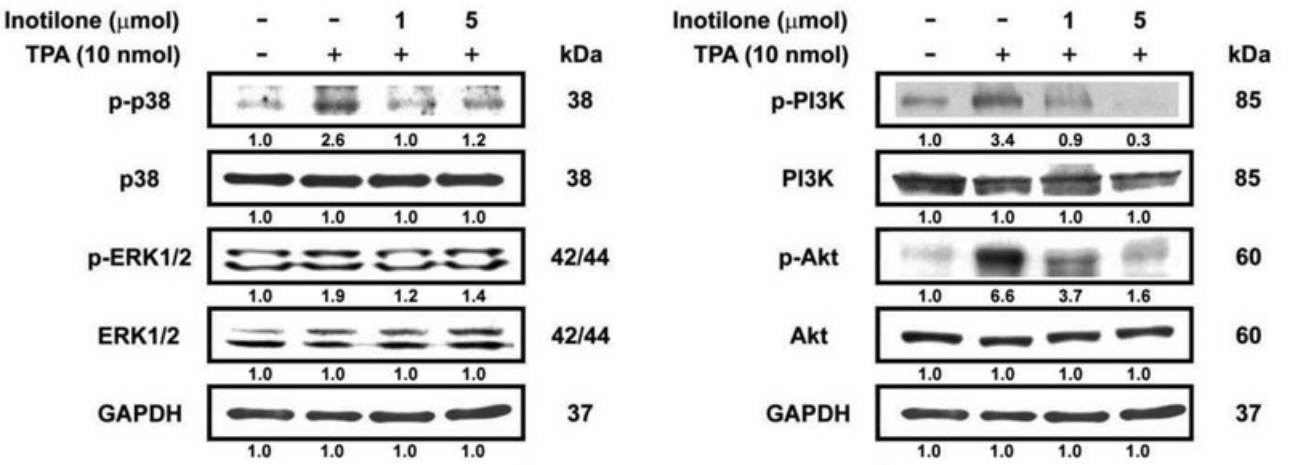

Figure 4. Effects of inotilone on TPA-induced activation of ERK, p38 MAPK, and PI3K/Akt signaling pathways. Animals were treated as described in Material and Methods and sacrificed after 1 h after TPA treatment. The epidermal lysates were analyzed for the phosphorylated and total p38, ERK, PI3K, and Akt.

pretreatment with inotilone significantly inhibited epidermal thickness promoted by TPA for 10 weeks in DMBA-initiated mouse epidermis (Fig. 5A and B). Furthermore, mouse skin treated 5 μmol inotilone alone for 10 weeks did not cause significant epidermal thickness increased and affect the morphology of skin epidermis (Fig. 5A).

At the end of 20 weeks, TPA-induced promotion experiment, no statistically significant difference in body or organ weight was observed between the mice treated with and without inotilone indicating that the topical application of inotilone did not cause any toxicity (data not shown). As presented in Fig. 5C and D, TPA treatment on DMBA-initiated dorsal skin resulted in a tumor incidence of 100% with an average of 24 ± 1.4 papillomas per mouse at the 20th week. Pretreatment with 5 μmol of inotilone 30 min prior to each application of TPA lowered the tumor incidence to 50% with 6 ± 2.1 papillomas per mouse. The inhibition effect of inotilone on tumor promotion was also analyzed in terms of size distribution of papillomas observed (Table 2). The size distribution of papillomas of TPA treatment group was 21 ± 3.6 at 1–3 mm^2, 2.5 ± 1.2 at 3–5 mm^2, and 1 ± 0.1 at >5 mm^2. Pretreatment with 5 μmol inotilone caused a significant shift in the distribution of papillomas size. There were fewer papillomas with size ≥3 mm^2 (0.3 ± 0.1) and more with size <3 mm^2 (6.5 ± 2.3) as compared with TPA treatment group. Papillomas with size >5 mm^2 were completely inhibited.

4 Discussion

Cancer chemoprevention appears to be one of the most feasible approaches to prevent, delay, or reverse carcinogenesis. While the chemoprevention approach targeting at the initiation stage is most desirable, the interference in the promotion stage may be more appropriate and practical. The major reason for this is that tumor promotion is a reversible event, at least in its early stages, and requires repeated and prolonged exposure of a promoting agent [18]. Therefore, agents that have the ability to block or reverse deleterious changes in cellular signaling involved in the process of tumor promotion may become potential candidates for cancer chemoprevention. In addition, it has been known that tumor promotion is closely linked to inflammation, and it is likely that agents with anti-inflammatory properties act as antitumor promoters as well. In the present study, the effects of inotilone on TPA-mediated inflammatory and tumor-promoting responses in mouse skin were investigated.

TPA is a strong inducer of inflammatory reactions [19]. Topical application of TPA to mouse skin is known to result in a number of biochemical alternations, changes in cellular functions, and histological changes leading to skin tumor promotion. Western blotting and real-time PCR analyses have shown that preapplication of inotilone significantly inhibited the expression of inflammatory mediators induced by TPA in a dose-dependent manner, including iNOS, COX-2, VEGF, and MMP-9 (Fig. 1). Table 2 has also shown that inotilone inhibited TPA-induced biological and histological changes related to inflammation. PGE_2 derived by COX-2 is a well-known mediator to increase vascular permeability and cell proliferation [20]. In addition, overexpression of VEGF in skin has been reported to induce vascular hyperpermeability [21]. Although the effect of inotilone on vascular permeability was not evaluated, the reduction of epidermal hyperplasia and inflammatory cells infiltration by inotilone may be partly attributed to the suppression of iNOS, VEGF, and COX-2 production.

Topical application of TPA on mouse skin has been reported to induce the production of inflammatory mediators through regulating the transcriptional activity of NF-κB. In this study, inotilone was found to inhibit TPA-induced

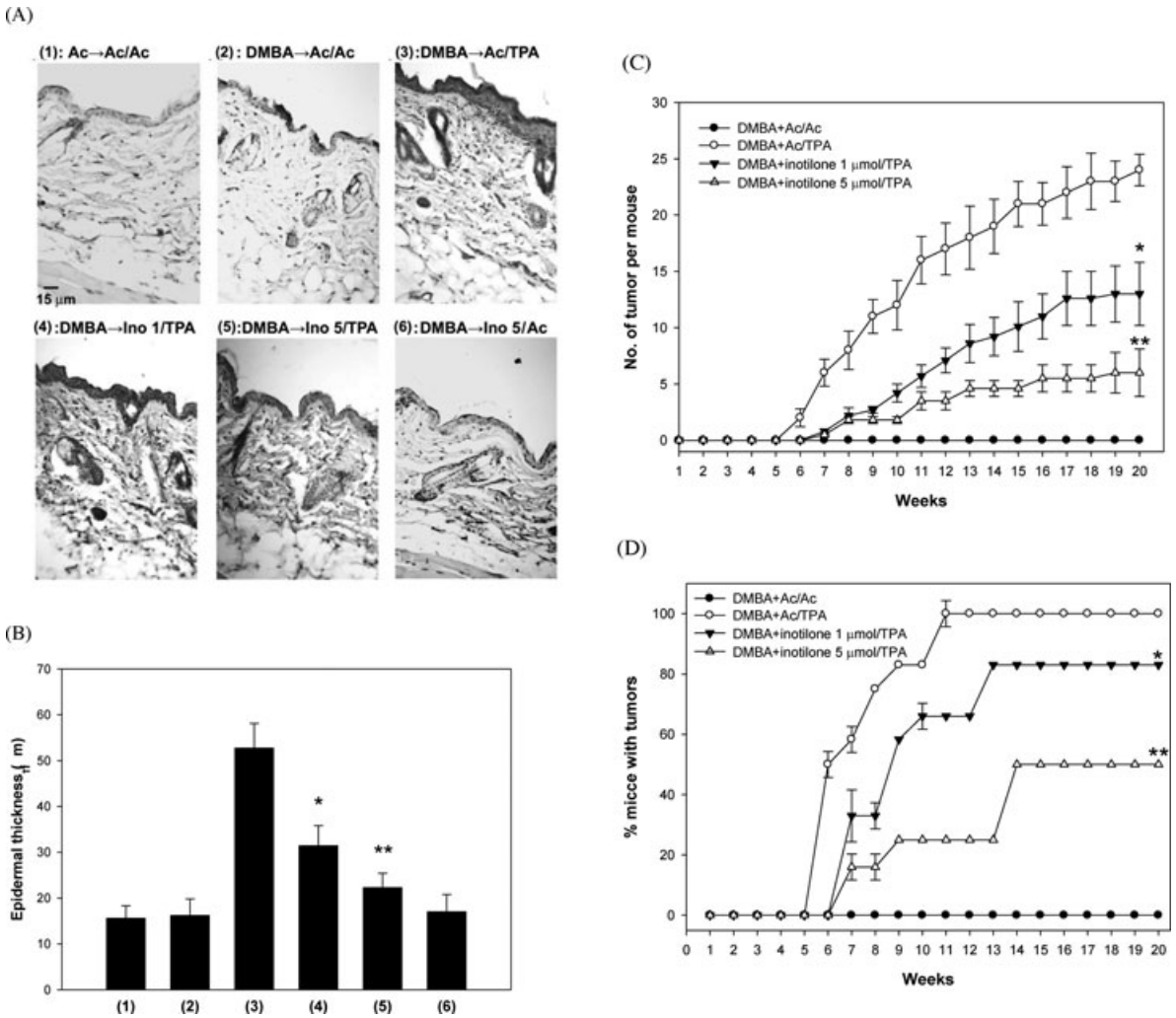

Figure 5. Antitumor promotion effects of inotilone on DMBA/TPA-induced skin tumorigenesis in ICR female mice. Tumor promotion in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 week after initiation. Inotilone (1 and 5 μmol) were dissolved in 0.2 mL acetone and topically applied 30 min prior to each TPA treatment. After 10 weeks, mice were killed for (A) HE staining and (B) epidermal thickness (μm) analysis. Three mice were used in one experimental group. $^{*}p < 0.01$ and $^{**}p < 0.001$ were versus group 3. (C) Average number of tumors per mouse (tumor multiplicity). (D) Percentage of tumor-bearing mice (tumor incidence). Tumors of at least 1 mm in diameter were counted and recorded weekly. $^{*}p < 0.01$ and $^{**}p < 0.001$ were versus DMBA+Ac/TPA group. Statistical analysis was done by Student's *t*-test.

Table 1. Inhibitory effects of inotilone on TPA-induced skin thickness and infiltrated inflammatory cells in mouse skin

Group	Epidermal thickness (μm)	Infiltrated inflammatory cells (No. per mm^2)
(1) Acetone/Acetone	6.8 ± 1.2	23.0 ± 4.8
(2) Acetone/TPA	20.2 ± 3.0[a)]	77.7 ± 8.7[a)]
(3) Inotilone 1 μmol/ TPA	7.5 ± 1.5[b)]	38.7 ± 7.6[b)]
(4) Inotilone 5 μmol/TPA	6.1 ± 1.3[b)]	24.5 ± 3.9[b)]

Statistical analysis was done by Student's *t*-test.
a) $p < 0.001$ were versus group 1 and
b) $p < 0.001$ were versus group 2.

nuclear translocation of p50 and p65 subunits of NF-κB by suppressing the phosphorylation and subsequent proteolytic degradation of IκBα (Fig. 2). Members of the MAPK families are known to regulate the activation of NF-κB via this phosphorlayion-dependent degradation of IκBα. Among them, it is indicated that the activation of NF-κB induced by TPA in mouse skin is modulated by ERK1/2 and p38 MAPKs [22]. Topical application of inotilone inhibited TPA-induced activation of p38 MAPK but with minor effect of ERK1/2 signaling, suggesting that inotilone attenuated TPA-induced NF-κB activation possibly through inhibition of p38 MAPK, and ERK1/2 seems less involved. The inhibitory effect of inotilone on another signaling pathway, PI3K/Akt, was also observed. Activation of PI3K leads to phosphorylation of

Table 2. Effects of inotilone on the size distribution of papillomas induced by TPA in DMBA-initiated mouse skin.

Treatment	No. of mice	1–< 3 mm^2 (%)	3–< 5 mm^2 (%)	>5 mm^2 (%)
(1) Acetone/Acetone	12	-	-	-
(2) Acetone/TPA	11	21 ± 3.6	2.5 ± 1.2	1 ± 0.1
(3) Inotilone 1 μmol/ TPA	12	11 ± 2.8[a)]	1.3 ± 0.7	0.3 ± 0.1
(4) Inotilone 5 μmol/TPA	12	6.5 ± 2.3[b)]	0.3 ± 0.1	-

The diameters of skin tumors were measured by an electronic digital caliper and the tumor size was recorded as length × width (mm^2) per mouse. Statistical analysis was done by Student's *t*-test.
a) $p < 0.05$, and
b) $p < 0.01$ were versus group 2.

phosphatidylinositides, which then phosphorylate Akt, which plays a pivotal role in several cell signaling networks involved in carcinogenesis, including cell proliferation, differentiation, survival, invasion, and metastasis [23]. In addition, it has also been suggested that PI3K/Akt pathway can contribute to activation of NF-κB. Therefore, the inhibition of TPA-induced activation of PI3K and Akt by inotilone may also participate in interfering with TPA-induced NF-κB activation, eventually leading to suppression of iNOS, COX-2, and other mediators in mouse skin.

Besides NF-κB, C/EBPβ has been shown to involve in regulating the expression of COX-2 in TPA-treated mouse skin [24]. Moreover, Zhu et al. have also shown that C/EBPβ-deficient mice are completely resistant to chemically induced skin carcinogenesis [25]. Unlike our previous in vitro study that inotilone failed to attenuate C/EBPβ expression in LPS-stimulated macrophages, treatment with inotilone prior to TPA application to mouse skin resulted in the reduction of C/EBPβ expression [26]. The distinct effects of inotilone on C/EBPβ in our studies suggest that the inhibitory effect of inotilone on C/EBPβ may be cell type- or stimuli-specific.

Our study has also shown that the inhibitory effect of inotilone on inflammatory mediators is valuable for not only alleviating the inflammatory response, but also for prevention of tumor promotion. The antitumor-promoting effects of inotilone were assessed by employing a classical DMBA/TPA two-stage mouse skin carcinogenesis model. Topical application of inotilone before TPA treatment during the promotion process significantly lowered the number and the size of papillomas in DMBA-initiated mouse skin. The ability of inotilone to prevent papillomas formation may be the combinative outcome of its inhibitory effect on the production of various inflammatory mediators, the activation of NF-κB and C/EBPβ, and the activation of the p38 MAPK and PI3K/Akt signaling pathways.

In summary, the results have shown that pretreatment with inotilone inhibited TPA-mediated acute inflammation by reducing the levels of inflammatory mediators, iNOS, COX-2, VEGF, and MMP-9, through regulating the signaling pathway, particularly in the activation of PI3K/Akt and p38 MAPK, the degradation and phosphorylation of IκB, the nuclear translocation of NF–κB, and the expression of C/EBPβ. Moreover, inotilone treatment significantly attenuated the incidence and the multiplicity of papillomas in DMBA-initiated and TPA-promoted mouse skin. Based on these findings, inotilone is suggested as a potential chemopreventive agent to be used in the treatment of inflammation-associated tumorigenesis, especially in the prevention and treatment of epithelial skin cancer.

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