EFFECTS OF INOTILONE ON INFLAMMATION AND INFLAMMATION-ASSOCIATED TUMORIGENESIS

by

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ABSTRACT OF THE DISSERTATION

Effects of inotilone on inflammation and inflammation-associated tumorigenesis

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Inflammation, a complex process, involving numerous mediators of cellular and plasma origins, is considered to be a critical factor in many human diseases and conditions, including obesity, cardiovascular diseases, diabetes, aging, and cancers. Inotilone, a secondary metabolite recently found in the dietary *Inonotus* mushroom, has been reported as a potent inflammatory inhibitor in test tube. However, its inhibitory effect at cellular level as well as in animal model remain unclear. The anti-inflammatory effects of inotilone were investigated *in vitro* using lipopolysaccharide (LPS)-stimulated murine macrophage. Inotilone was shown to inhibit nitric oxide (NO) and prostaglandin E_2 (PGE_2) production through modulating inducible nitric oxide synthase (iNOS) expression and cyclooxygenase-2 (COX-2) enzyme activity, respectively. It is also found that inotilone can only suppressed the expression of iNOS but not COX-2. This divergence may origin from the differential effect of inotilone on transcription factors, nuclear factor-κB (NF-κB) and CCAAT enhancer-binding protein (C/EBP). However, this differential effect was not found in the *in vivo* study employing 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated mouse skin. This finding suggested that the effects of inotilone on C/EBPβ expression may be cell type- or stimuli-specific.

The inhibitory effects of inotilone were also observed on the activation of phosphatidylinositol 3-kinase/Akt (PI3K/Akt), extracellular signal-regulated protein kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) signaling pathways both *in vitro* and *in vivo*. Furthermore, the ability of inotilone to prevent inflammation-associated tumorigenesis was also evaluated using a classical two-stage mouse skin carcinogenesis model. After initiation of 7,12-dimethylbenz[a]nthracene (DMBA), applying inotilone topically before each TPA treatment was found to reduce the incidence and multiplicity of papillomas at 20^{th} week. Taken together, the results suggest 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.

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Table of Contents

List of Figures

List of Tables

Abbreviations

Akt	Protein kinase B
C/EBP	CCAAT/enhancer-binding protein
COX	Cyclooxygenase
DMBA	7,12-dimethylbenz[a]nthracene
ERK	Extracellular signal-regulated kinase
IκB	IκB kinase
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-κB
NO	Nitric oxide
NOS	Nitric oxide synthase
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TPA	12-*O*-tetradecanoylphorbol-13-acetate
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

The inflammatory response is a defense system that our bodies use to protect ourselves from pathogen infection or tissue injury. Its purpose is to localize and eliminate the injurious agent, remove damage tissue, and initiate tissue repair process. Therefore, the inflammatory response is important for us to remain healthy. However, if this protective response is not well regulated, inflammation can become chronic and leads to many inflammatory diseases, including arthritis, inflammatory bowel disease, and atherosclerosis. Over the past several years, scientists have further found that chronic inflammation may be the root of many human diseases and conditions, including obesity, cardiovascular diseases, type II diabetes, and cancer [1].

Chemoprevention is a relatively new and promising approach to prevent, delay or reverse the occurrence of a disease. Considering for the long-term use, an ideal chemopreventive agent should be nontoxic and can be effective at lower dose. Natural dietary compounds with low toxicity then become a promising group of chemopreventive agent. Indeed, a number of natural dietary compounds are claimed to have the ability to prevent and mitigate chronic diseases. Numerous studies in different cell lines and animal models have demonstrated that various bioactive compounds in fruits and vegetables play a protective role against different types of cancers. Many natural dietary compounds with potent anti-inflammatory properties have been noted as plausible approaches for clinical cancer prevention trials [2].

Inotilone (Figure 1.1), a secondary metabolite recently found in the *Inonotus* mushroom, was reported as a potent inflammatory inhibitor [3]. Like many non-steroidal anti-inflammatory drugs (NSAIDs), inotilone inhibits cyclooxygenase (COX), an enzyme involved in inflammatory response, in the enzyme assay. This potency of anti-inflammatory property makes inotilone an attractive object of chemopreventive agent for a variety of inflammatory diseases and cancer and requires further investigation.

We hypothesize that it is possible inotilone exhibits anti-inflammatory properties both *in vitro* and *in vivo*. Therefore the objectives of this research are to evaluate the following issues in a systematic study: (1) investigate the anti-inflammatory properties of inotilone *in vitro* and the underlying mechanisms using murine macrophage; (2) explore the inhibitory effects of inotilone on the TPA-induced acute inflammation model in mouse skin; (3) evaluate the anti-tumorigenic effects of inotilone in the two-stage mouse skin carcinogenesis model.

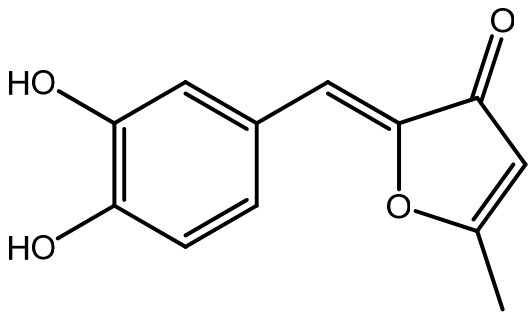


Figure 1.1 Chemical structure of inotilone.

Chapter 2. Literature Review

2.1. Inflammation

Inflammation is a physiological response to microbial pathogen infections, chemical irritation and tissue injury. It can be classified into two forms: acute and chronic inflammation. Acute inflammation normally is a self-limiting and rapidly resolved process. It is characterized by classical signs of pain, heat, redness, swelling and loss of function. The main function of acute inflammation is to initiate pathogen killing as well as tissue repair processes. During the inflammatory process, macrophage and leukocyte are activated by chemokines secreted by local mast cell and invade the affected area. They generate highly reactive species to eliminate foreign pathogens and pro-inflammatory cytokines to recruit a wide range of immune cells to the infected area which amplify the state of inflammation. However, excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by these immune cells such as superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and singlet oxygen ($^1O_2$) also attack normal tissue surrounding the infected area and result in oxidative DNA damage, gene mutation which may alter normal cell function and finally lead to cancer and other inflammatory diseases [4]. Besides, activated immune cells secret a series of enzymes and signaling proteins related to inflammation.

Among the pro-inflammatory enzymes, the inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX) are responsible for increasing levels on NO

and prostaglandin E_2 (PGE_2) which are known to be involved in various chronic diseases, including cancer .

Inducible Nitric Oxide Synthase (iNOS)

Nitric oxide synthase (NOS) is a family of enzymes which catalyze the production of NO from L-arginine. Depending on the location of expression and manner of expression, NOS are classified into subfamilies, constitutive or inducible. Constitutive NOS is found constitutively expressed in neural cells (nNOS) and endothelial cells (eNOS), whereas inducible NOS expresses in various cell types, including macrophages, keratinocytes, hepatocytes, and endothelium, in response to infectious and pro-inflammatory stimuli. A marked high expression of iNOS is commonly identified at sites of inflammation.

NO produced by these NOS is related to a wide range of physiological processes. It is involved in vasodilation, neurotransmission, platelet aggregation and immune regulation. NO is also an anti-bacterial effecter involved in host defense and cytoprotective action. However, sustained and/or excess production of NO, mostly attributed to iNOS expression, and its derivatives, such as peroxynitrite, is often observed in pathogenesis of inflammation-related diseases and cancers [5]. It is reported that NO induce the expression of matrix metalloproteinase (MMP) and vascular endothelial growth factor (VEGF), which in turn activate tumor cell invasion and metastasis [6;7].

Bacterial lipopolysaccharide (LPS) and inflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and interferon-γ (IFN-γ) are known to trigger the expression of iNOS [8;9]. In the region of iNOS promoter, there are a number

of binding sites for transcription factors, including nuclear factor-κB (NF-κB), activator protein-1 (AP-1), interferon regulatory factor 1 (IRF1), and CCAAT/enhancer-binding protein (C/EBP) [10-13]. Different inducers can activate distinct transcription factors to up-regulate iNOS expression through diverse signaling pathways. Among these inducers and transcription factors, the activation of mitogen-activated protein kinase (MAPK) and NF-κB is essential for LPS-activated *iNOS* transcription. The activation of NF-κB will be discussed later.

Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) is an enzyme catalyzing the conversion of arachidonic acid to prostaglandins. At present, three isoforms are found, designated COX-1, COX-2, and COX-3. COX-1 expresses constitutively in most tissues at relatively stable levels and is believed to have some housekeeping functions, such as gastric cytoprotection and platelet aggregation. In contrast to COX-1, COX-2 is an inducible form that has a role in many inflammatory and proliferative reactions. It is only slightly expressed in normal tissue or resting immune cells, but can be induced by specific stimuli. COX-3, a splice variant of COX-1, is now found abundant in human cerebral cortex and heart. However, its precise role remains unclear [14].

Many cell types involved in inflammation, such as macrophages, endothelial cells and fibroblasts, express COX-2 in response to various stimuli such as LPS, growth factors, and inflammatory cytokines. The expression of COX-2 is regulated by several different transcription factors, including NF-κB, C/EBP, AP-1 and cyclicAMP response element binding protein (CREB) [15-18]. Multiple lines of evidence have shown that

overexpression of COX-2 is associated with the tumorigenicity of cells. Prostaglandins derived by COX-2, especially PGE_2, are known to promote the growth of malignant cells by increasing cell proliferation, promoting angiogenesis, decreasing apoptosis, and modifying immunosuppression [19-23]. Furthermore, aberrant up-regulation of COX-2 has often been observed in various premalignant and malignant tissues, including lung, breast and pancreas [24-28].

Nuclear Factor-κB (NF-κB)

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 iNOS and COX-2. NF-κB is a collective term for the homo- and heterodimeric transcription factors of the Rel family [29]. Five NF-κB family members have been identified in mammalian cells, including p50/p105, p52/p100, p65 (Rel A), c-Rel, and Rel B. All of them contain a Rel homology domain in their N-terminus which is responsible for DNA binding and dimerization. Despite obvious structural similarities and their ability to bind related DNA sequences, genetic studies have shown that all NF-κB subunits have distinct and non-overlapping functions.

In most cells, NF-κB complex is sequestered in the cytosol by a family of inhibitory proteins known as IκB (Figure 2.1). In response to extracellular stimuli, IκB is phosphorylated by the IκB kinase (IKK), ubiquitylated and degraded by proteasome. The liberated NF-κB then can translocate to the nucleus and bind to a common DNA sequence, known as κB elements, within the promoters/enhancers region of target genes to regulate the transcription of the target gene through the recruitment of coactivators and

corepressors. Many studies have implied that the activation of NF-κB can be modulated through MAPK and phosphoinositide 3-kinase (PI3-kinase)/protein kinase B (Akt) signaling pathways [30-34].

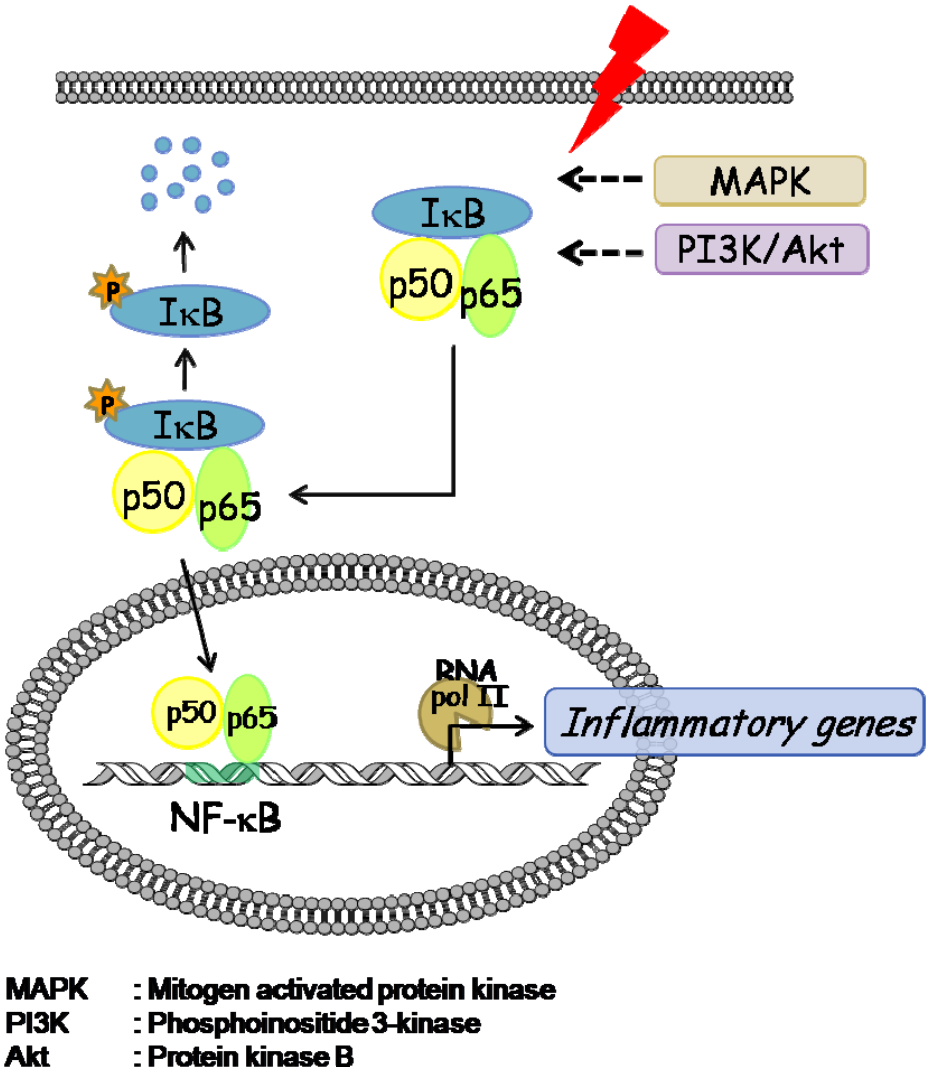


Figure 2.1 Simplified scheme of the activation of the transcription factor NF-κB.

CCAAT-enhancer Binding protein (C/EBP)

The C/EBPs is another family of transcription factors related to inflammation. At least six family members have been identified to date (C/EBPα to C/EBPζ) [35]. They share a common structure with a C-terminus containing a leucine-zipper domain which allows the homo- or heterodimerization of these factors adjacent to a basic DNA binding domain. Studies have shown that the activity and/or expression levels of various C/EBPs are differentially modulated in response to inflammatory stimuli, including LPS and a range of cytokines [36-39]. When expose to stimuli, up-regulation of C/EBPβ and C/EBPδ expressions are seen in a number of cell types, such as hepatocytes, macrophages, and astroglial cells, whereas the level of C/EBPα is quite low.

The binding sites for C/EBPs have been identified in the promoter regions of various genes that are involved in the inflammatory response, including iNOS, COX-2, inflammatory cytokines such as IL-1, IL-6, and TNF-α and their receptors [39;40]. However, due to the complexity of the C/EBP family and the expression of each C/EBP isoforms is quite cell type- and differentiation stage-specific, it is difficult to generalize the functions of C/EBPs in the regulation of these inflammatory genes. Even same C/EBP isoform shows different effects depending on the cell type. For example, Gorgoni *et al.* have demonstrated that C/EBPβ is essential for COX-2 expression in macrophages but not in fibroblasts [41]. The induction of COX-2 expression was greatly impaired in C/EBPβ-deficient macrophages where it was completely normal in C/EBPβ-deficient fibroblasts.

2.2. Inflammation and Cancer

The concept that inflammation is related to cancer is not new. The link between cancer and inflammation was first hypothesized by Virchow in 1858 based on the observations that cancers often occurred at sites of chronic inflammation [42]. He suggested that chronic inflammation could lead to cellular proliferation and promote cancer cell growth. Nowadays, several epidemiological studies have supported this hypothesis [4;43-45]. They showed that chronic inflammation predisposes individual to various types of cancer, including colon, gastric, and prostate cancer. At least 15 % of cancer death is linked to infection and inflammation. Accordingly, treatment with NSAIDs decreases the incidence and mortality of certain cancers.

At site of inflammation, macrophage, together with other leukocytes, generate high levels of reactive oxygen and nitrogen species which cause DNA damage in proliferating epithelial and surrounding cells. Moreover, migration inhibitory factor (MIF) released by macrophages exacerbates the DNA damage by suppressing the transcription of p53 and results in the lack of a DNA-damage-repair response, and consequently causing the accumulation of oncogenic mutations [45]. Other than resulting in permanent genomic alteration, inflammatory cells develop an inflammatory microenvironment to promote tumor cell growth by generating a variety of cytokines and other molecules which not only enhance cell proliferation, angiogenesis, invasion, and metastasis but also inhibit anti-tumor immune responses [46]. For instance, tumor-associated macrophages (TAMs), a component of inflammatory cells in neoplastic tissue, facilitate tumor growth, invasion, and metastasis by releasing VEGF, an angiogenic factors, and matrix metalloproteinases (MMP-2 and MMP-9), which degrade the extracellular matrix and the

basement membrane. TAMs may also produce interleukin-10 (IL-10) and PGE_2, which effectively blunt the anti-tumor response.

2.3. The Concept of Chemoprevention

Chemoprevention, by definition, is to use natural or synthetic agent to prevent, slow down, or reverse the occurrence of a disease. Cancer chemoprevention has appeared to be a promising and cost-effective alternative to both prevention and therapy of cancer. The concept of cancer chemoprevention is based on the idea that cancer development is a multistep process. The three critical steps involved in cancer development are initiation, promotion, and progression (Figure 2.2) [47].

Initiation stage involves mutation of cellular DNA resulting in the activation of oncogenes, inactivation of tumor suppressor genes and DNA repair enzymes. This step is thought to be irreversible and happen rapidly. In the promotion stage, the proliferation of the initiated cells raises a large number of daughter cells containing the mutation created by the initiator. Compare to the initiation step, this step occurs over a long period of time, and is reversible in its early stages. The last step leading to cancer is called progression which involves further genetic damage that results in the conversion of benign tumor into malignant one which is capable of invading adjacent tissues and metastasizing to distant sites.

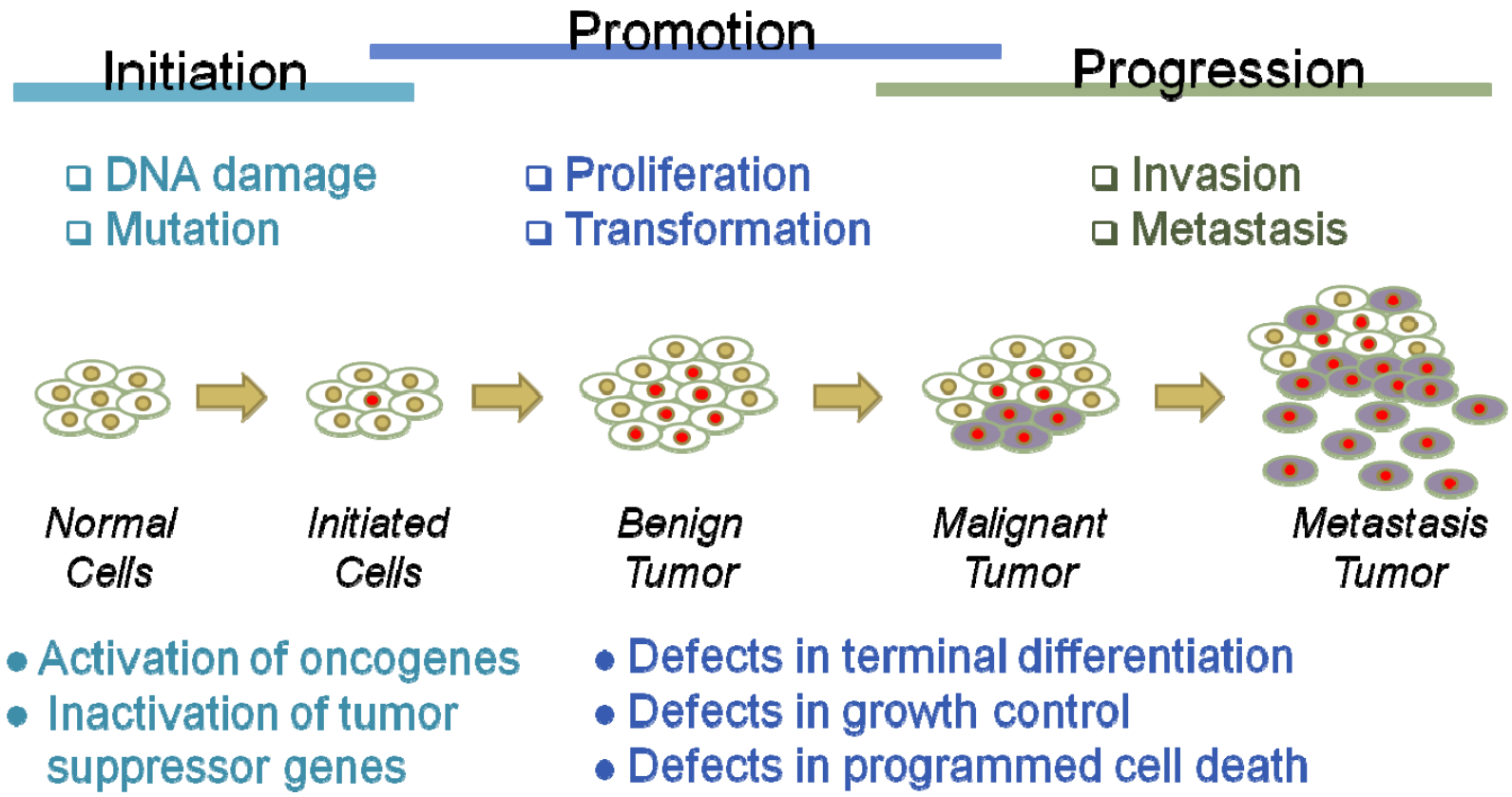


Figure 2.2 The development of cancer is a multistage process. (Modified from Ref.[48])

Chemopreventive agents can be categorized broadly as blocking agents or suppressing agents based on the stage of carcinogenesis they target at [49]. Blocking agents aim at the initiation stage. The blocking mechanisms include preventing the formation of reactive carcinogens, inducing rapid deactivation and clearance of carcinogens, and inhibiting the interaction of carcinogens with vital cellular macromolecules. Suppressing agents, on the other hand, inhibit or arrest the promotion and progression stages by reducing proliferation of initiated cells, restoring apoptosis to normal levels to prevent the accumulation of mutated cells, or by preventing the transformation of precancerous cells into malignant ones.

2.4. Chemopreventive Effects of Natural Dietary Compounds

The use of natural dietary bioactive compounds as chemopreventive agents is gaining increasing attention because of their low toxicity and relative safety. Epidemiological studies have provided convincing evidence that dietary patterns are closely associated with the risk of several types of cancer [50]. People who consume relatively larger amount of fruits and vegetables have a significantly lower cancer incidence. A number of bioactive compounds in fruits and vegetables have been identified exhibiting chemoprevention properties [49;51-53]. The chemical structures of representative bioactive compounds that have been known to possess chemopreventive potential and their dietary source are listed in Table 2.1 along with their proposed chemopreventive mechanisms. Many mechanisms have been shown to account for the chemopreventive effects of dietary compounds on inflammation and cancers. The following sections summarize some of the potential mechanisms of action.

Table 2.1 Representative dietary chemopreventive compounds and their prevention mechanisms.

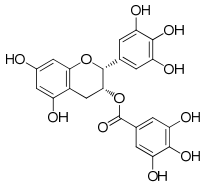
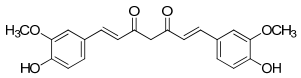
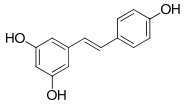
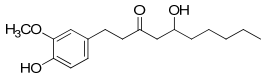
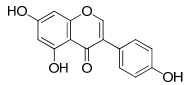
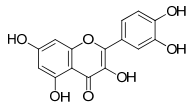
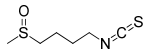
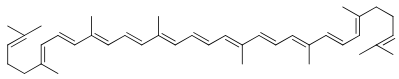
Compound	Structure	Dietary source	Proposed mechanisms
EGCG		Tea	1. Modulates Nrf2-mediated antioxidant and detoxifying enzymes [54] 2. Inhibits cell transformation by reducing the activation of AP-1 [55] 3. Inhibit VEGF production and angiogenesis through inhibiting NF-κB and STAT3 signaling pathway [56]
Curcumin		Tumeric	1. Modulates phase I and phase II enzyme expressions in benzo[a]pyrene-treated mice [57] 2. Inhibits TPA-induced and tumor promotion through inhibition of ERK and NF-κB activation in mouse skin [58]. Suppresses NF-κB and STAT3 signaling pathway and induces apoptosis [59]
Resveratrol		Grapes, red wine	1. Modulates TPA-induced activation of NF-κB, and AP-1in mouse skin [60] 2. Induces antioxidant enzymes by activation of Nrf2 [61] 3. Induces autophagy in ovarian cancer cells [62]
[6]-Gingerol		Ginger	1. Inhibits COX-2 expression by blocking the activation of p38 and NF-κB in TPA-induced mouse skin [63] 2. Inhibits metastasis of MDA-MB-231 human breast cancer cell by decreasing the activity of MMP-2 and MMP-9 [64] 3. Induces apoptosis *via* modulating p53 and mitochondrial signaling pathway in benzo[a]pyrene-induced carcinogenesis [65]

Table 2.1 Representative dietary chemopreventive compounds and their prevention mechanisms. (continued)

Compound	Structure	Dietary source	Proposed mechanisms
Genistein		Soybean	1. Inhibit H_2O_2-induced DNA damage through up-regulation of anti-oxidant proteins in LAPC-4 prostate cancer cell.[66] 2. Inhibits UVB-induced activation of AP-1 in mouse skin[67] 3. Induces apoptosis by activation ASK-1/p38 signaling pathway [68]
Quercetin		Onion	1. Modulates phase I and phase II enzyme expressions in hepatocytes [69] 2. Induces apoptosis via PI3K/Akt and ERK pathways in human hepatoma cell line [70] 3. Suppresses tumor growth through regulating MMP-2 and angiogenic process [71]
Sulforaphane		Cruciferous vegetables	1. Induces expression of phase II detoxification genes through the Nrf2/ARE pathway [72] 2. Induces cell cycle arrest and apoptosis in human colon cancer cell[73] 3. Inhibits angiogenesis by regulating HIF-1α and c-Myc [74]
lycopene		Tomato, watermelon, papaya and orange	1. Reduces lipid peroxidation and oxidative DNA damage [75] 2. Inhibits lung squamous metaplasia and induces apoptosis in smoke-exposed ferrets [76] 3. Interferes with cell cycle progression and IGF-1 signaling in mammary cancer cells [77]

Antioxidant Activity

The generation of ROS is unavoidable during normal metabolism or inflammatory responses. Under normal circumstances, an effective defense system is used to neutralize the toxicity of ROS. This system includes antioxidative enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, and antioxidants, for instance, glutathione and albumin. Imbalance between the generation of ROS and their removal results in accumulation of ROS which can cause potential cell damage, especially DNA damage. Thus, dietary compounds with antioxidant activities may prevent DNA damage caused by free radicals and block the initiation step of carcinogenesis. Studies have shown that dietary antioxidants can either act as direct scavengers for ROS or modulate the antioxidative enzymes to remove ROS.

Effects on Phase I and Phase II Enzymes

Metabolism of xenobiotics is generally divided into phase I and phase II metabolism which sometimes, but not always, occur sequentially. Phase I metabolism involves process such as oxidation, reduction, and hydrolysis to modify the structure of a compound. Products of phase I may be either pharmacologically active or inactive and usually represent substrates for phase II enzymes. Phase II metabolism involves conjugation process such as glucorunidation, sulfation, acetylation, and methylation to increase the polarity of the compound, hence facilitate its clearance. Several dietary compounds have shown to alter the procarcinogenic metabolism by inhibiting the metabolic activation of carcinogens by phase I cytochrome P450 enzymes or enhance the detoxification by phase II enzymes through increasing their activities or modulating their expressions.

Modulation of Cell Signaling Pathways and Inflammation

During all three stages of carcinogenesis, many of the molecular alternation occur in cell signaling pathways. Results of such changes include activation of oncogenes, inhibition of tumor suppressor genes, induction of the inflammatory response, induction of angiogenesis and up-regulation of matrix metalloproteinases. Some of the best characterized signaling pathways changed by carcinognes are the MAPKs. Abnormal or improper activation of the MAPK pathway and its downstream transcription factors can induce changes that direct cells toward uncontrolled cell proliferation and malignancy. Several dietary compounds have been implicated to prevent, control, or reverse these changes caused by carcinogens. Other than MAPKs, cell signaling kinases such as protein kinase C (PKC) and PI3K are also important targets of certain chemopreventive agents.

2.5. Inotilone

Mushrooms have attracted a great interest because of their nutritional value and their biopharmaceutical properties for a long time [78]. Extracts of *Inonotus* mushroom have been reported to exhibit several therapeutic effects, such as anti-inflammatory [79], anti-hyperglycemic and anti-lipid peroxidative [80], anti-cancer [81], and anti-tumor activities [82]. Recently, Hertweck and co-workers reported several new phenolic compounds extracted from the fruiting body of *Inonotus* mushroom [3]. Among them, inotilone (Figure 1.1) showed significant selective inhibitory effects against COX with IC_{50} of COX-2 at 0.03 μM, COX-1 at 0.36 μM, and 0.08 for COX-2/COX-1 in enzyme assay. This selective inhibitory property makes inotilone a possible chemoprevention

agent. However, the inhibitory effects of inotilone at cellular level and in animal model remain unclear. In this study, the anti-inflammatory effects of inotilone were evaluated both *in vitro* and *in vivo*. In addition, its anti-tumor promotion effects were explored using two-stage mouse skin carcinogenesis model.

Chapter 3. Inhibitory Effects of Inotilone in LPS-activated Murine Macrophage[1]

3.1. Introduction

LPS, a major component of the outer membrane of Gram-negative bacteria, is known to induce the production of many inflammatory mediators, including oxygen and nitrogen intermediates, PGs, and cytokines in macrophages [83]. By binding to its receptor, LPS activates several intracellular signaling pathways, including MAPKs, PI3K/Akt, and IKK/NF-κB pathways. These signaling pathways in turn activate a variety of transcription factors which coordinate the transcription of many genes encoding inflammatory mediators.

In this study, LPS was used to stimulate the inflammatory response in murine macrophage RAW 264.7. The cells were cotreated with inotilone to evaluate its anti-inflammatory properties. The effects of inotilone on the expression of iNOS and COX-2 induced by LPS were examined and the inhibitory mechanisms were further investigated.

[1] Parts of this chapter have been published in Mol Nutr. Food Res. as Differential inhibitory effects of inotilone on inflammatory mediators, inducible nitric oxide synthase and cyclooxygenase-2, in LPS-stimulated murine macrophage.

3.2. Material and Methods

All chemicals used here were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless specified.

3.2.1. Cell Culture

The murine monocyte/macrophage cell line, RAW 264.7, was obtained from American Type Culture Collection (Manassas, VA). RAW 264.7 cells were cultured in DMEM supplemented with 10 % endotoxin-free, heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. All cells were grown in a humidified incubator containing 5 % CO_2 at 37 °C. After the cells were planted at a density of 1×10^6 cells/mL overnight, the culture media were changed to serum-free DMEM without phenol red. The cells were activated by incubation in medium containing *E. coli* LPS 100 ng/mL. Inotilone of various concentration was dissolved in dimethylsulfoxide (DMSO) and was added to cell right before LPS.

3.2.2. Nitrite Assay

The nitrite concentration in the culture medium was measured as an indicator of NO production using the Griess test [84]. 100 μL of the culture medium was mixed with 50 μL of the Griess reagent A (1 % sulfanilamide in 5 % phosphoric acid) and 50 μL of the Griess reagent B (0.1 % naphthylethylenediamine dihydrochloride in 5 % phosphoric acid). The absorbance at 540 nm of the mixture was measured with a Thermo Labsystems Multiskan Ascent Photometric plate reader (Thermo Fisher Scientific Inc., Waltham,

MA). The concentration of nitrite was calculated using a standard curve prepared with sodium nitrite.

3.2.3. Determination of Prostaglandin E_2 (PGE_2)

The level of PGE_2 released into the culture medium was quantified using a PGE_2 EIA kit (Assay Designs, Ann Arbor, MI) according to the manufacturer's instruction.

3.2.4. Western Blotting

The cells were washed with PBS and lysed in an ice-cold 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] on ice for 1 h, followed by centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant was collected and total protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Inc. Hercules, CA). Equal amount of total cellular protein (50 μg) were resolved by 8-15 % SDS-PAGE and electrotransferred onto polyvinylidene fluoride (PVDF) 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 location as listed below: for COX-2 and iNOS were from BD Transduction Laboratories (San Jose, CA), for C/EBPβ and C/EBPδ were from Santa Cruz Biotechnology (Santa Cruz, CA), for IkBα, p65, and phospho-IkBα (Ser 32) were from New England Biolabs (Ipwich, MA), for phospho-Akt (Ser 473), phospho-p65 (Ser 536), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), ERK, p38, and Akt antibodies were from Cell Signaling Technology, Inc. (Danvers, MA).

3.2.5. Reverse Transcription

Total RNA was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. 2 μg of total RNA was transcribed into cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μL. RT reactions were performed at 50 °C for 60 min and 70 °C for 15 min in GeneAmp® PCR System 9700 (Applied Biosystems Inc., Foster City, CA).

3.2.6. Semiquantitative PCR

PCR reactions were initiated at 95 °C for 1 min followed by 30-35 cycles of amplification (94 °C for 30 s, 58 °C for 25 s, and 72 °C for 1 min), and extension at 72 C

for 3 min. The products were analyzed by agarose gel electrophoresis and visualized using ethidium bromide staining. Amplification of β-actin was used as a control for sample loading and integrity. Primers used for amplification are listed in Table 3.1.

3.2.7. Quantitative Real-time PCR (SYBR Green)

Real-time PCR was performed with LightCycler® FastStart DNA Master SYBR Green I kit and LightCycler®1.5 System (Roche, Indianapolis, IN) according to the manufacturer's instructions. Primers used in this experiment are listed in Table 3.2. 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. Specificity of the amplified PCR product was assessed by performing a Melting Curve analysis. The expression levels of the specific genes in each sample were calculated with the LightCycler software and normalized with a housekeeping control (β-actin).

Table 3.1 Primers used for semiquantitative PCR.

Primer	Orientation	Sequence
iNOS	Sense	5′-CCC TTC CGA AGT TTC TGG CAG CAG C-3′
	Antisense	5′-GGC TGT CAG AGA GCC TCG TGG CTT TGG-3′
COX-2	Sense	5′-GGA GAG ACT ATC AAG ATA GTG ATC-3′
	Antisense	5′-ATG GTC AGT AGA CTT TTA CAG CTC-3′
Actin	Sense	5′-ACC AAC TGG GAC GAT ATG GAG AAG A-3′
	Antisense	5′-TAC GAC CAG AGG CAT ACA GGG ACA A-3′

Table 3.2 Primers used for quantitative real-time PCR (SYBR Green).

Primer	Orientation	Sequence
iNOS	Sense	5′-CTT TGC CAC GGA CGA GAC-3′
	Antisense	5′-TCA TTG TAC TCT GAG GGC TGA C-3′
COX-2	Sense	5′-CAC CTC TGC GAT GCT CTT C-3′
	Antisense	5′-TGG ATT GGA ACA GCA AGG AT-3′
Actin	Sense	5′-CCA ACC GTG AAA AGA TGA CC-3′
	Antisense	5′-ACC AGA GGC ATA CAG GGA CA-3′

3.2.8. Transient Transfection and Luciferase Assay

The luciferase assay was performed as described by George et al. [85] with some modifications. The cells were seeded in a 60-mm dish. When the cells reached confluence, the medium was replaced with serum-free Opti-MEM (Invitrogen, Carlsbad, CA). The cells were then transfected with the pNFκB-Luc plasmid reporter gene (Stratagene, La Jolla, CA) using Lipofectamine™ reagent (Invitrogen, Carlsbad, CA). After 24 h of incubation, the medium was replaced with complete medium. After another 24 h, the cells were trypsinized and equal numbers of cells were plated in 12-well tissue culture plates for 6 h. The cells were then treated with 100 ng/mL LPS and inotilone for 6 h. Luciferase activity was assayed by means of the britelite™ plus reporter gene assays kit (PerkinElmer Inc, Waltham, MA). Luminescence was measured on a Top Counter Microplate Scintillation and Luminescence Counter (Packard 9912 V) in single photon counting mode for 0.1 min/well.

3.2.9. Extraction of Nucleus and Cytosolic Protein

The cells were suspended in hypotonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5 mM EDTA, 1 mM PMSF, 1 % NP-40, and 5 mM $MgCl_2$) for 10 min on ice followed by centrifugation at 4,000 g for 15 min at 4 °C. The supernatant containing cytosolic protein was collected. The pellet containing nuclei was resuspended in hypertonic buffer (10 mM NaH_2PO_4, 10 mM NaF, 5 mM EDTA, 1 mM PMSF, 1 % NP-40, and 5 mM $MgCl_2$, and 2 % NaCl) for 3 h on ice followed by centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant containing nucleus protein was collected for further analysis.

3.2.10. 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.

3.3. Results

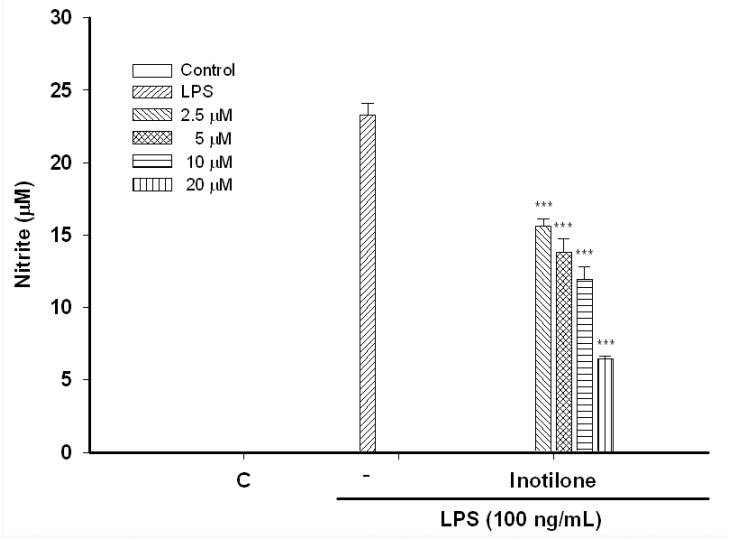
3.3.1. Effects of Inotilone on LPS-Induced Nitrite and PGE_2 Production

To investigate the anti-inflammatory effects of inotilone, the levels of nitrite and PGE_2 in the culture media were determined 24 h after co-treatment with 100 ng/mL LPS and various concentrations of inotilone. As shown in Figure 3.1A, inotilone inhibited LPS-induced nitrite production in a dose dependent manner with IC_{50} at 11.2 μM. Similar inhibition effect of inotilone can also be seen in PGE_2 production (Figure 3.1B).

3.3.2. Effect of Inotilone on the Enzyme Activity of INOS

As inotilone has been previously reported to inhibit the enzyme activity of COX-2 [3], indirect nitrite assay were performed to assess the possibility that it inhibits the intrinsic activity of iNOS and as a result of NO reduction. Cells were stimulated with LPS for 12 h first to induce iNOS expression and washed with PBS to remove LPS. The cells were then treated with different doses of inotilone and the nitrite in the media was determined after another 12 h. As can be seen in Figure 3.2, inotilone was unable to inhibit the nitrite production when iNOS is already expressed. The result indicating that inotilone is unable to inhibit the enzyme activity of iNOS.

(A)



(B)

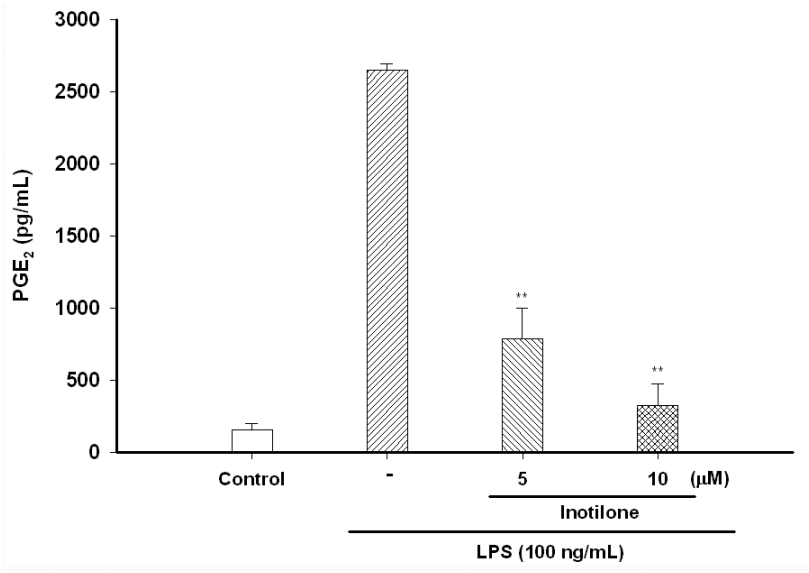


Figure 3.1 Effects of inotilone on LPS-induced NO and PGE_2 production in RAW 264.7.

The cells were treated with 100 ng/mL LPS only or with different concentrations of inotilone. After incubation for 24 h, 100 μL of culture media were collected for (A) nitrite assay, (B) PGE_2.The values are expressed as means ± SE of triplicate tests. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate statistically significant differences from the LPS-treated group.

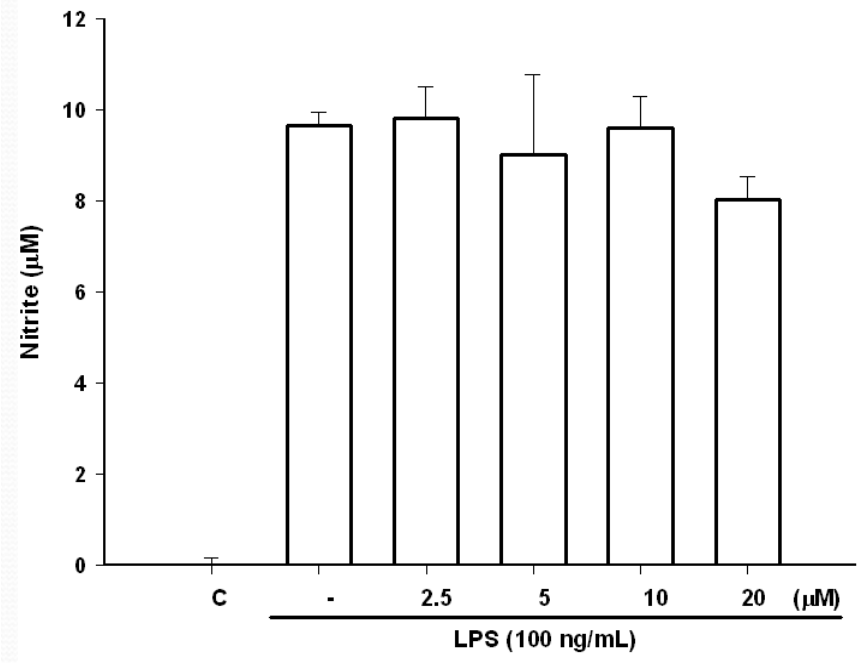


Figure 3.2 Effect of inotilone on the activity of NOS enzyme in RAW 264.7. The cells were stimulated with 100 ng/mL LPS for 12 h, and were washed with PBS to remove LPS before treated with indicated concentration of inotilone for another 12 h. The culture media were then collected for nitrite assay.

3.3.3. Effects of Inotilone on LPS-inducible INOS and COX-2 Expressions

Since iNOS and COX-2 are the key enzymes for the production of nitrite and PGE_2, respectively, the effects of inotilone on LPS-induced iNOS and COX-2 protein expressions were studied by western blotting. As presented in Figure 3.3, LPS treatment significantly increased iNOS and COX-2 protein levels, whereas co-treatment with inotilone suppressed the induction of iNOS but not COX-2.

RT-PCR and real-time PCR analyses were used to assess the effect of inotilone on *iNOS* and *COX-2* mRNA expression. Consistent with western blotting, LPS-stimulated gene expression of *iNOS* was restrained by inotilone whereas that of *COX-2* remained the same (Figure 3.4). These data suggest that transcription of LPS-induced iNOS and COX-2 can be regulated differently by inotilone.

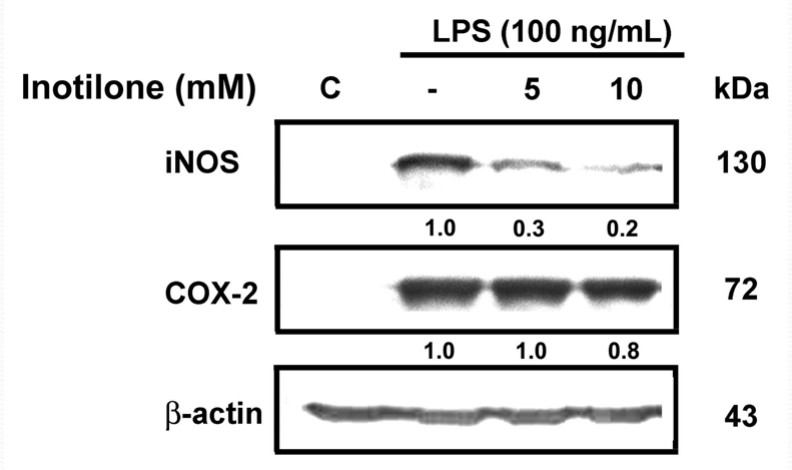
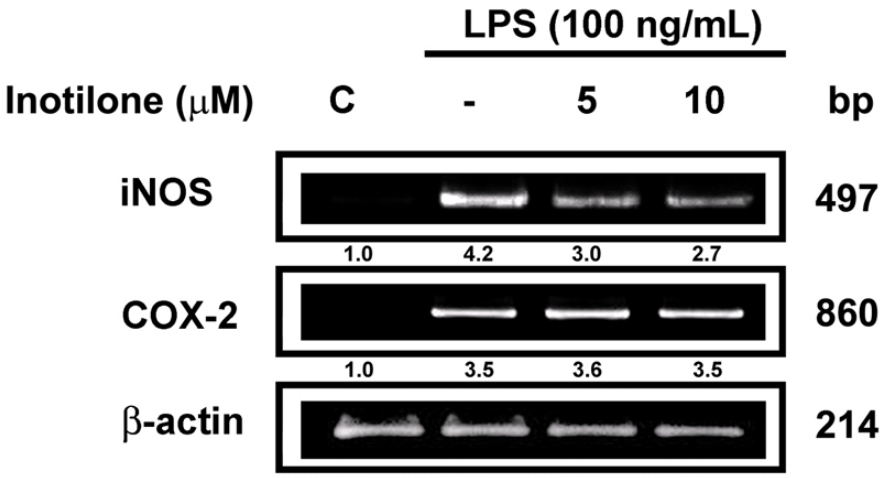
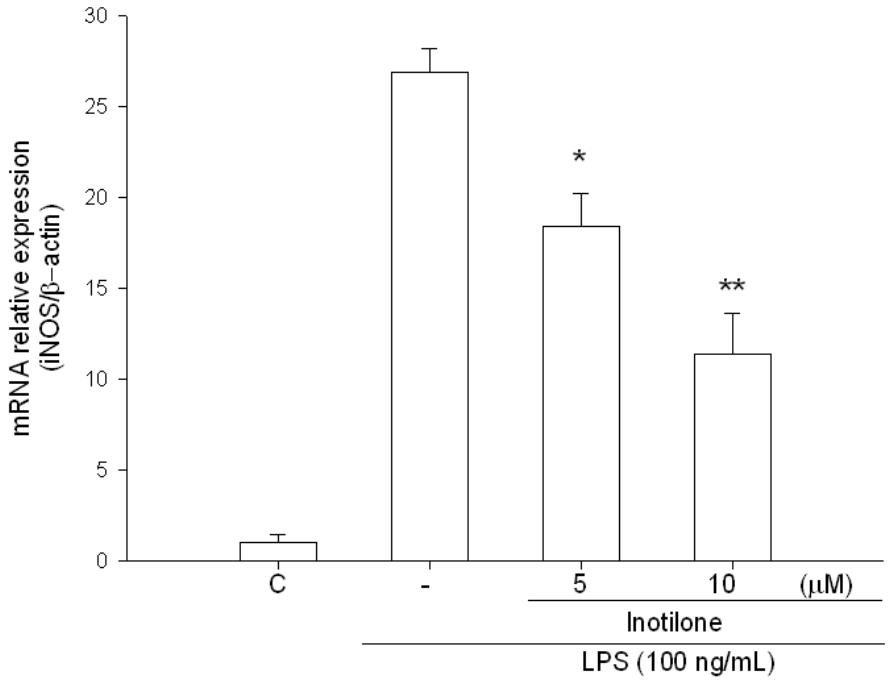


Figure 3.3 Effects of inotilone on LPS-induced iNOS and COX-2 protein expressions in RAW 264.7.
The cells were treated with 100 ng/mL LPS only or with inotilone (5 and 10 μM) for 24 h. The levels of iNOS or COX-2 in lysates were analyzed by western blotting. β-actin was used as a loading control.

(A)


LPS (100 ng/mL)
Inotilone (μM)
C
-
5
10
bp
iNOS
497
1.0
4.2
3.0
2.7
COX-2
860
1.0
3.5
3.6
3.5
β-actin
214

(B)


mRNA relative expression
(iNOS/β−actin)
30
25
20
15
10
5
0
*
**
C
-
5
10
(μM)
Inotilone
LPS (100 ng/mL)

(C)

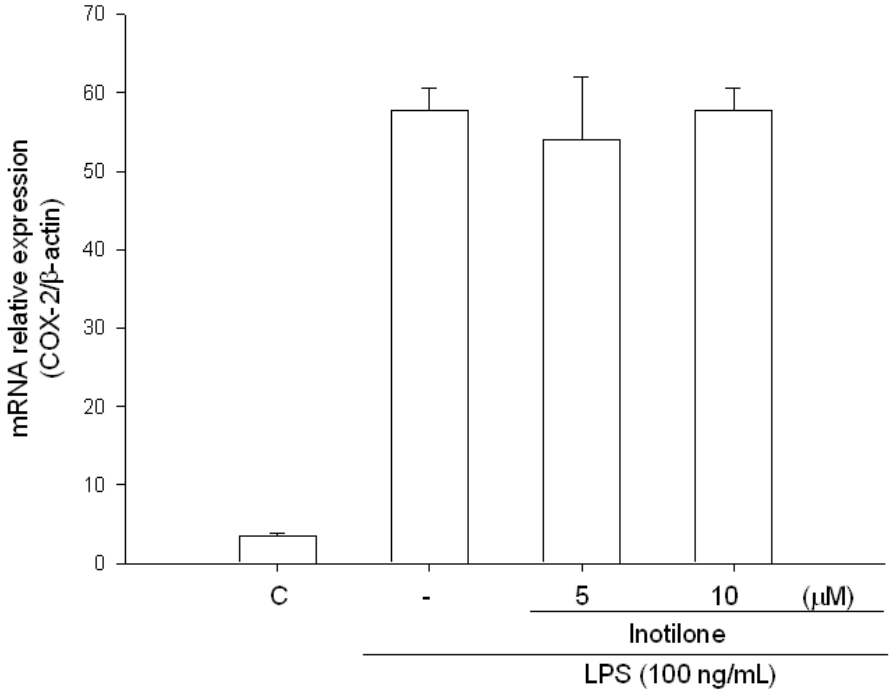


Figure 3.4 Effects of inotilone on LPS-stimulated *iNOS* and *COX-2* gene expression in RAW 264.7.

The cells were treated with 100 ng/mL LPS only or with inotilone (5 and 10 μM) for 5 h and total RNA were isolated. (A) The mRNA expressions of *iNOS* and *COX-2* were determined by semiquantitative RT-PCR. This experiment was repeated three times with similar results. (B) The mRNA levels of *iNOS*, and (C) *COX-2*, were quantified by LightCycler System using SYBR Green I. Data are presented as mean ± SE of triplicate tests. $*P < 0.05$ and $**P < 0.01$ indicate statistically differences from the LPS-treated group.

3.3.4. Differential Effect of Inotilone on C/EBP and NF-κB in LPS-activated Macrophages

The promoter regions of *iNOS* and *COX-2* both contain the binding sites of C/EBP and NF-κB. Therefore, to investigate the differential effects of inotilone on iNOS and COX-2 expression, the effects of inotilone on these two transcription factors were determined by western blotting. As shown in Figure 3.5, cell treated with LPS increased the expression of both C/EBPβ and C/EBPδ, whereas concurrent inotilone treatment greatly inhibited the induction of C/EBPδ but not C/EBPβ. As for NF-κB, Figure 3.6A shows that inotilone inhibited the nuclear translocation of both of the subunits of NF-κB, p50 and p65. Furthermore, the phosphorylation of p65 at serine 536 in LPS-mediated induction was also inhibited by inotilone in a dose dependent manner. Reporter gene assay was also performed to confirm if inotilone inhibited NF-κB transcriptional activity. Macrophages transiently transfected with an NF-κB-dependent luciferase reporter plasmid (pNF-κB-Luc) were treated with LPS alone or with inotilone. As shown in Figure 3.6B, LPS-induced transcriptional activity of NF-κB was strongly reduced by inotilone.

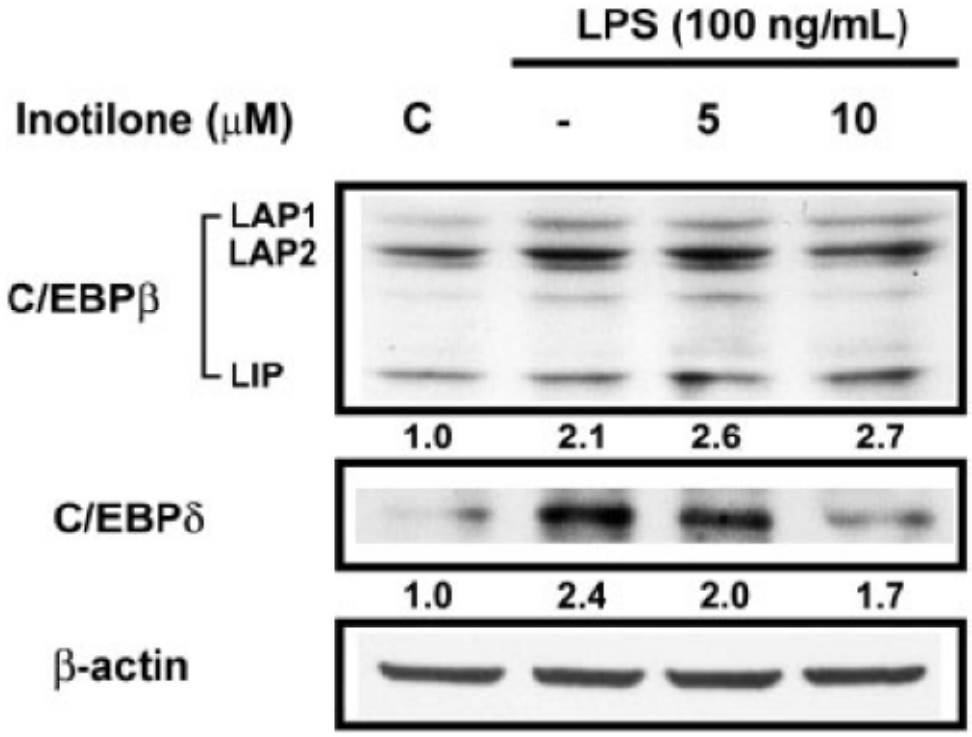
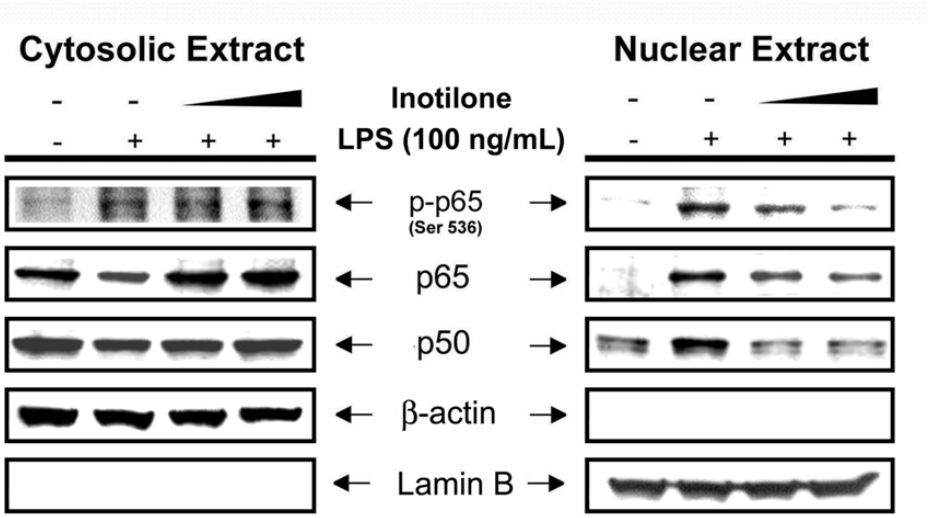


Figure 3.5 Effects of inotilone on LPS-induced C/EBPβ and C/EBPδ protein levels in RAW 264.7.
The cells were treated with 100 ng/mL LPS with or without inotilone (5 or 10 μM) for 30 min. Total cell lysates were then prepared for western blot analysis.

(A)



(B)

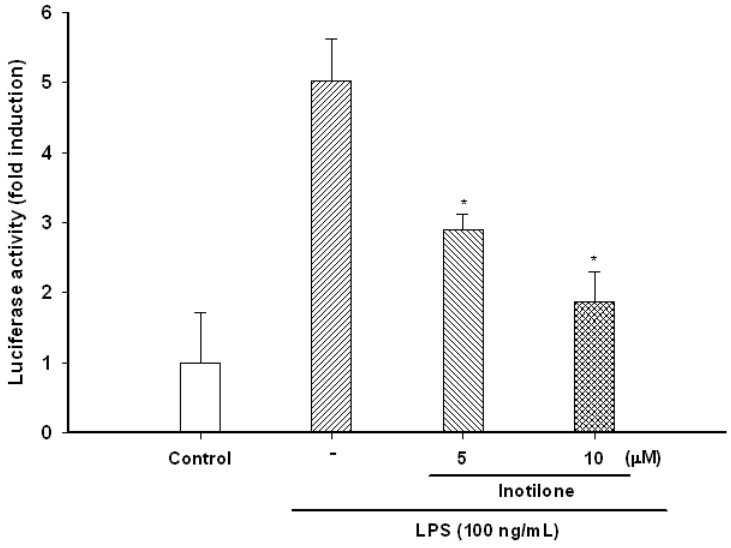
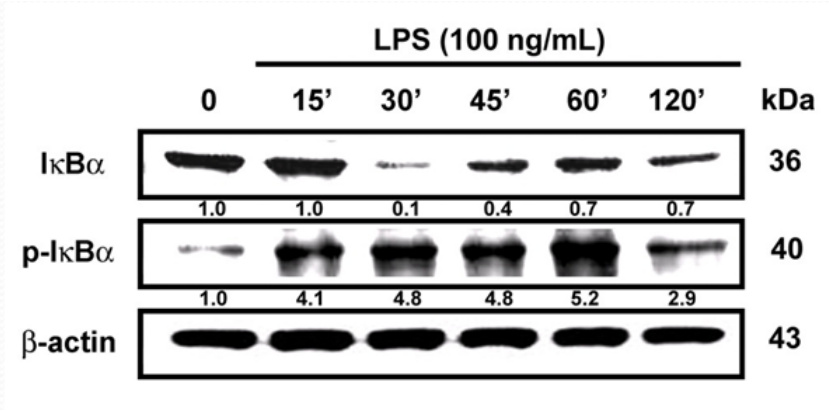


Figure 3.6 Effects of inotilone on LPS-induced activation of NF-κB in RAW 264.7. (A) The cells were treated with 100 ng/mL LPS alone or with inotilone (5 and 10 μM) for 1 h. Cytosolic and nuclear fractions were prepared and analyzed by western blotting. (B) The cells were transiently transfected with pNF-κB-Luc reporter gene and then treated with 100 ng/mL LPS in the presence or absence of inotilone (5 and 10 μM) for 6 h. The results are expressed as means ± SE of triplicate tests. *$P < 0.05$ indicates statistically differences from the LPS-treated group.

3.3.5. Blockade of Inotilone on LPS-induced Phosphorylation and Degradation of IκBα

LPS-mediated translocation of NF-κB to the nucleus is preceded by proteolytic degradation of IκBα. To explore whether inotilone could affect IκBα in macrophages, the phosphorylation and protein level of IκBα were analyzed by western blotting in a time-course study. As shown in Figure 3.7A, LPS exposure increased the phosphorylation of IκBα protein after 15 min and peaked at 120 min whereas the degradation occurred after 30 min and gradually recovered after 45-120 min. In Figure 3.7B, treatment with inotilone effectively attenuated the increased phosphorylation of IκBα and sustained the IκBα protein content. These results suggest that blocking the phosphorylation and the degradation of IκBα protein can prevent the activation and translocation of NF-κB to the nucleus and further inhibit the downstream transcriptional activity.

(A)



(B)

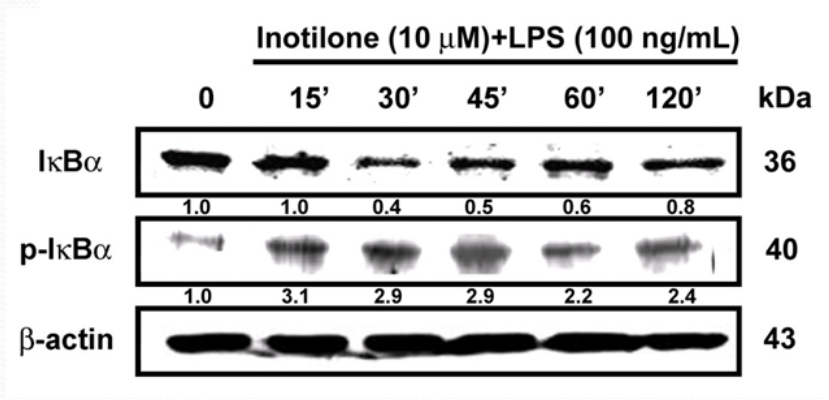


Figure 3.7 Effects of inotilone on LPS-stimulated phosphorylation and degradation of IκBα in RAW 264.7.
The cells were treated with (A) 100 ng/mL LPS alone or (B) co-treated with LPS and inotilone for different times. Total cell lysates were prepared for western blot analysis. These experiments were repeated three times with similar results.

3.3.6. Effects of Inotilone on Activation of PI3K/ Akt, P38, and ERK1/2 MAPK

Several signaling cascades have been reported to be involved in the phosphorylation and activation of NF-κB [86-88]. To determine whether inotilone can modulate these upstream signaling pathways, the potential involvement of the PI3K/Akt and MAPK pathways were evaluated. The inhibition effect of inotilone was found in inhibiting PI3K activity and its downstream target, Akt (Figure 3.8). After cells treated with inotilone and LPS for 1 h, inotilone treatment decreased the phosphorylation of Akt and PI3K compared with LPS alone. Activation of MAPK requires phosphorylation of threonine and tyrosine residues. Anti-phospho-specific antibody was used in western blotting of the activation of ERK1/2 and p38 MAPK. When the cells were co-treated with LPS and inotilone for 1 h, LPS-induced activation of ERK1/2 and p38 MAPK was inhibited in a dose dependent manner. These results of western blotting suggested that inotilone might block the LPS-induced NF-κB activation by inhibiting ERK1/2, p38 MAPK and PI3K/Akt /IKK pathway, which interrupted the degradation of IκBα.

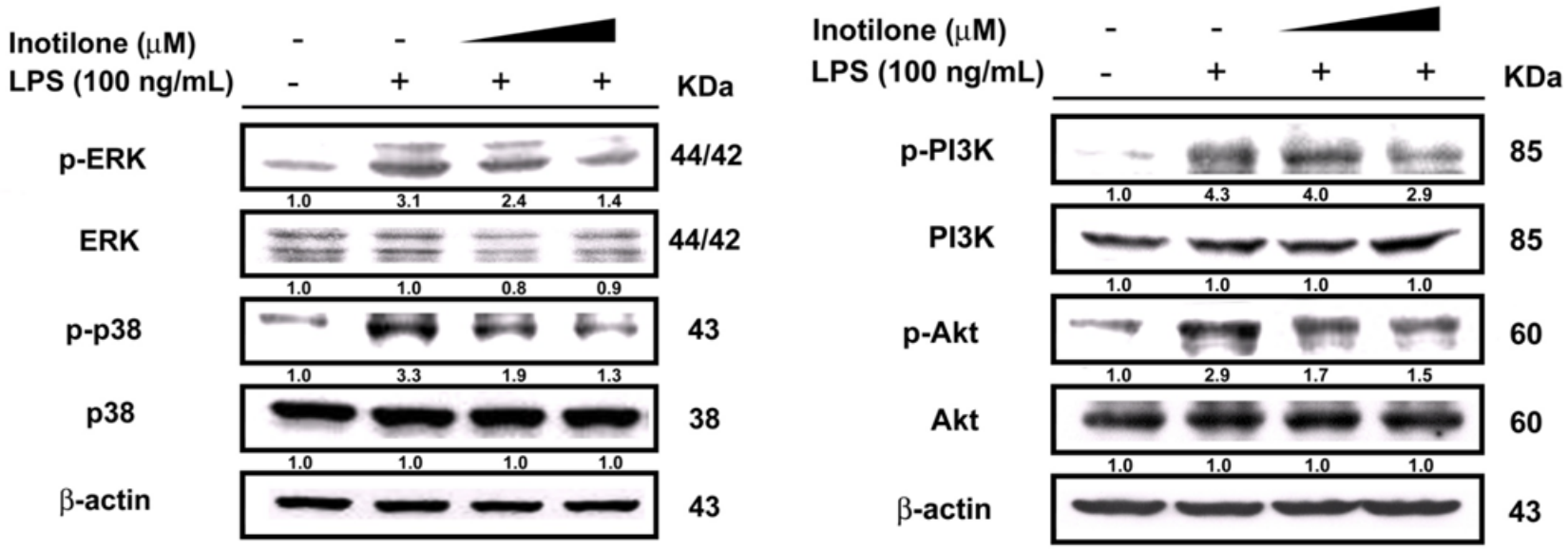


Figure 3.8 Inhibition of PI3K/Akt, ERK1/2, and p38 MAPK by inotilone in LPS-activated RAW 264.7.
The cells were treated with 100 ng/mL LPS with or without inotilone (5 or 10 μM) for 1 h. Total cell lysates were then prepared for western blot analysis. These experiments were repeated three times with similar results.

3.4. Discussion

The anti-inflammatory activity of inotilone was evaluated using LPS-stimulated murine macrophage. The results demonstrated that inotilone attenuated LPS-induced NO and PGE_2 production in RAW 264.7. The effects and mechanisms of inotilone on the expression of related enzymes, iNOS and COX-2, were further explored both at protein and mRNA levels. Inotilone strongly inhibited iNOS expression in LPS-stimulated macrophages and subsequently inhibited the NO production, whereas it decreased the enzyme activity of COX-2 instead of its expression to reduce PGE_2 production. Although the promoter regions of iNOS and COX-2 contain binding sites for same transcription factors, the transcriptional regulation of LPS-stimulated iNOS and COX-2 can be distinguished [89]. Our data suggest that inotilone repressed iNOS but not COX-2 expression may come from the differential inhibition of NF-κB and C/EBPβ. From the reporter assay reported previously, a mutation in C/EBPβ or NF-κB binding sites of COX-2 promoter inhibits LPS-induced reporter activity. Moreover, mutation at NF-κB sites shows less inhibitory effect than that of C/EBPβ [90]. Inotilone with the inhibitor effect on NF-κB but not C/EBPβ is therefore unable to decrease LPS-induced COX-2 expression. These results further confirmed that a diverse signal pathway indeed occurs in the LPS-induced iNOS and COX-2 transcription activity. Comparing the two transcription factor, C/EBPβ is more dominant in regulating transcription of COX-2 while NF-κB is more dominant in iNOS. Based on the different effects of inotilone on *iNOS* and *COX-2* gene expression, inotilone provides an interesting and important

evidence to confide the issue of uncoupled transcriptional regulation. However, the detail mechanism remains to be further elucidated.

The activation of NF-κB induced by LPS is through a cascade of events leading to the activation of inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB and leads to degradation. NF-κB is then translocation to the nucleus and regulates the transcription of target genes. Our results show that inotilone reduces iNOS expression by blocking transcription of its gene, a conclusion supported by the observation that it reduced the steady state of iNOS mRNA levels, and promoter activity (as assessed by luciferase activity assay). It is also found that co-treatment of inotilone blocked the activation of PI3K/Akt, ERK1/2 and p38 MAPK. This suggests that inotilone suppresses LPS-induced NF-κB translocation by inhibiting the activation of these intracellular signaling cascades and subsequently decreases the protein level of iNOS.

In summary, this study demonstrated that inotilone inhibits LPS-induced NO and PGE_2 production through modulating iNOS expression and COX-2 enzyme activity, respectively. Moreover, the differential effect of inotilone on NF-κB and C/EBPβ leads to the inhibition of iNOS but not COX-2 expression. Inotilone as an inhibitor of NF-κB signaling pathway in murine macrophage is believed to be accompanied by the suppression of PI3K/Akt/IKK, ERK1/2, and p38 MAPK. Based on these findings, inotilone shows great potential as a novel chemopreventive agent for the treatment of a variety of inflammatory diseases.

Chapter 4. Inhibitory Effects of Inotilone on 12-*O*-tetradecanoylphorbol-13-acetate-induced Skin Inflammation and Tumor Promotion in Mouse Skin

4.1. Introduction

Multi-stage mouse skin carcinogenesis is one of the best-established and convenient models for the study of the sequential and stepwise development of tumors [91]. This model has the advantage that the initiation and promotion stages of tumor formation can be distinctly separated operationally. In the two-stage model (Figure 4.1), the initiation stage is accomplished by topical application of a single sub-carcinogenic dose of skin carcinogen (initiator) and the promotion stage is achieved by the repeated treatment with non-carcinogenic promoter to induce tumor growth effectively. As the tumor response induced by this two-stage model is highly reproducible, a great number of cancer chemopreventive agents have employed this model to assess their anti-skin tumor effects [92-96].

7,12-dimethylbenz[a]nthracene (DMBA) (Figure 4.2) is one of the most frequently used initiator. Treatment of DMBA causes irreversible DNA damage, resulting in mutation of Ha-*ras* oncogene in epidermal cells [97]. However, no visible tumor will appear until repeated application of a promoter. It is also important to note that exposure to a promoter before an initiator does not lead to formation of tumor. Likewise, after exposure to an initiator, if the interval between the treatment of promoter is too long, tumor will not form.

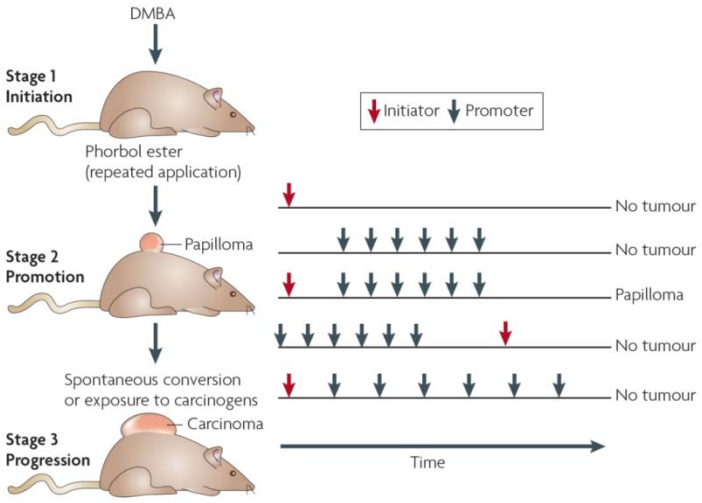


Figure 4.1 Two-stage mouse skin carcinogenesis model.
(Adopted from Ref. [98])

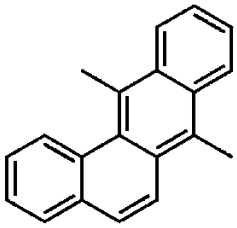


Figure 4.2 Chemical structure of 7,12-dimethylbenz[a]anthracene (DMBA).

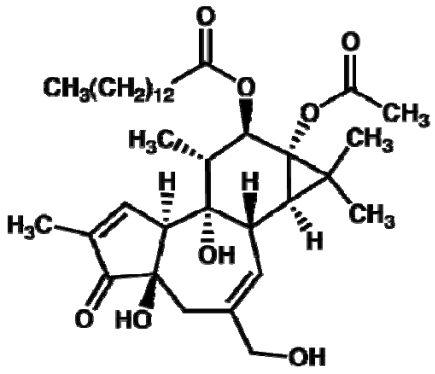


Figure 4.3 Chemical structure of 12-O-tetradecanoylphorbol-13-acetate (TPA).

12-O-tetradecanoylphorbol-13-acetate (TPA) (Figure 4.3) is one of the most potent and widely used promoters in mouse skin carcinogenic model. The tumor promoting activity of TPA comes from its binding and activation of protein kinase C (PKC), which plays a critical role in signal transduction pathway and regulates cell growth and differentiation [98]. A single application of TPA to mouse skin results in an inflammatory response by increasing the levels of proinflammatory mediators through activation of MAPKs and NF-κB signaling pathway [60;94].

To study the anti-inflammatory effects of inotilone *in vivo*, inotilone was pretreated to the shaved dorsal skin before treated with TPA. The effects of inotilone on the expression of TPA-induced iNOS, COX-2 and other inflammatory mediators were investigated, as well as the underlying mechanisms. Furthermore, the anti-skin tumor promoting effects of inotilone were evaluated using the two-stage mouse skin carcinogenesis model. After initiated with DMBA, the mice were treated with inotilone before each TPA treatment.

4.2. Material and Methods

All chemicals used here were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless specify.

4.2.1. Animal Treatment

Female ICR mice at 5–6 week 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. All materials were dissolved in 200 μL of acetone and applied topically to the shaved dorsal skin of mice.

4.2.2. TPA-induced Acute Inflammation on Mouse Skin

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 and their dorsal skins would be excised for further analysis.

4.2.3. Epidermal Collection

The excised dorsal skins were 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 ice 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.

4.2.4. Quantitative Real-time RT-PCR (TaqMan)

The collected epidermal sample was homogenized in 500 μL TRIzol® according to the manufacturer's instructions. 2 μg of total RNA was 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, Indianapolis, IN) according to the manufacturer's instructions. Primers and TaqMan probes used in this experiment are listed in Table 4.1. 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).

Table 4.1 Primers and probes used for quantitative real-time PCR (TaqMan).

Primer	Orientation	Sequence	UPL Probe
iNOS	Sense	5′-ACC CTA AGA GTC ACC AAA ATG G-3′	# 17
	Antisense	5′-CCA GGG ATT CTG GAA CAT TCT-3′	
COX-2	Sense	5′-GGG AGT CTG GAA CAT TGT GAA-3′	# 4
	Antisense	5′-GCA CAT TGT AAG TAG GTG GAC TGT-3′	
Actin	Sense	5′-CCA ACC GTG AAA AGA TGA CC-3′	# 64
	Antisense	5′-ACC AGA GGC ATA CAG GGA CA-3′	

4.2.5. 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 phenylmethylsulfonylfluoride (PMSF)]. The homogenate was incubated on ice with gentle shaking for 15 min and centrifuged at 14800 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.

4.2.6. Measurement of epidermal hyperplasia and leukocytes infiltration

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 400X magnification in 15 fields per section. The number of dermal infiltrating leukocytes was determined by counting the stained cells at five different areas.

4.2.7. Two-stage Mouse Skin Tumorigenesis

Female ICR mice were randomly divided into four groups, each consisting of 12 animals. 6 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 of 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).

4.2.8. Statistical Analysis

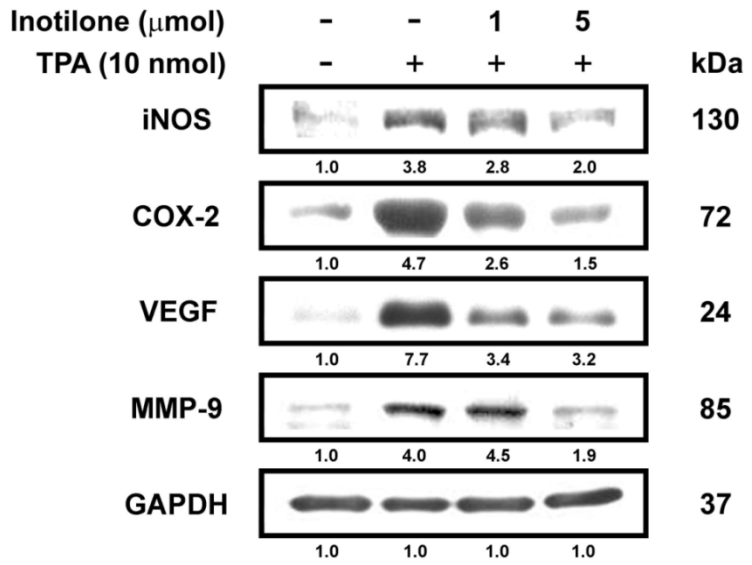
Data are presented as the means ± SD of at least three independent experiments. The statistical significance was evaluated by Student's *t*-test and a *P*-value < 0.05 was considered to be statistically significant.

4.3. Results

4.3.1. 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 activity of inotilone. Previous study has shown that single topical application of TPA induced the expression of iNOS, COX-2, VEGF, and MMP-9 in mouse skin [99]. As illustrated in Figure 4.4A, 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 Figure 4.4B, pretreatment with inotilone greatly attenuated *iNOS* and *COX-2* mRNA expressions in a dose dependent manner which was in concordance with the results obtained from western blotting.

(A)



(B)

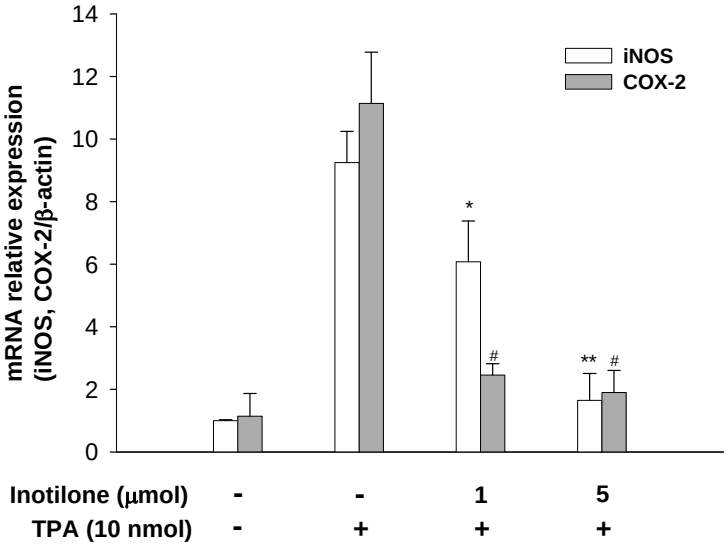


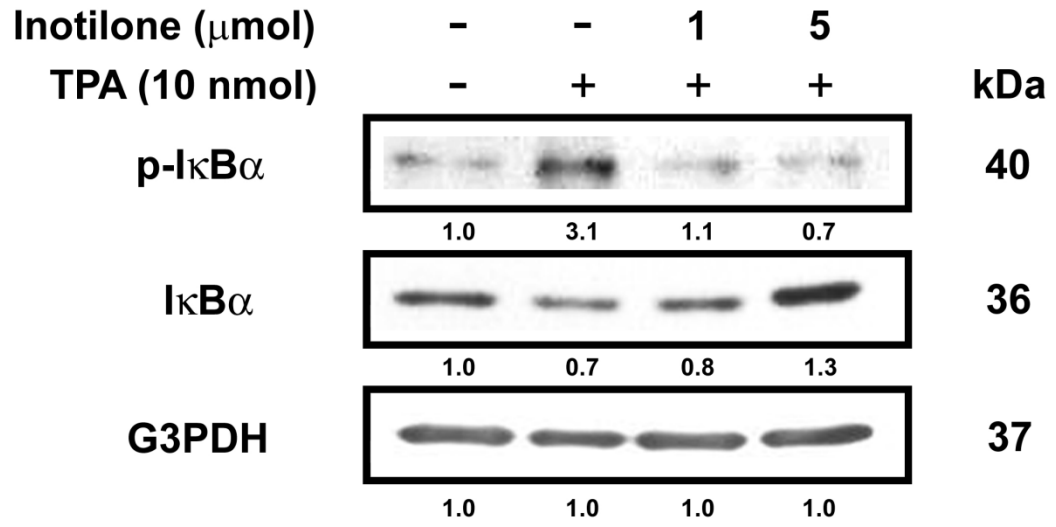
Figure 4.4 Effects of inotilone on TPA-induced inflammatory mediators expressions. Animals were treated as described in material and methods and sacrificed after indicated time periods. (A) The total epidermal protein extract was analyzed for iNOS (2 h), COX-2 (4 h), VEGF (4 h), and MMP-9 (4 h) expressions by western blotting. DAA (B) Animals were sacrificed after 1 h 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.

4.3.2. 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 IκBα, 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 Figure 4.5A, 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 Figure 4.5B, TPA evoked the nuclear translocation of both the subunits of NF-κB, p50 and p65, whereas pretreatment with inotilone could suppress 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 [35]. Therefore, the effect of inotilone on the expression of C/EBPβ was evaluated. Western blot analysis has shown that TPA induced the expression of C/EBPβ in mouse skin (Figure 4.6). Topical application of inotilone prior to TPA application dose-dependently inhibited TPA-induced C/EBPβ expression.

(A)



(B)

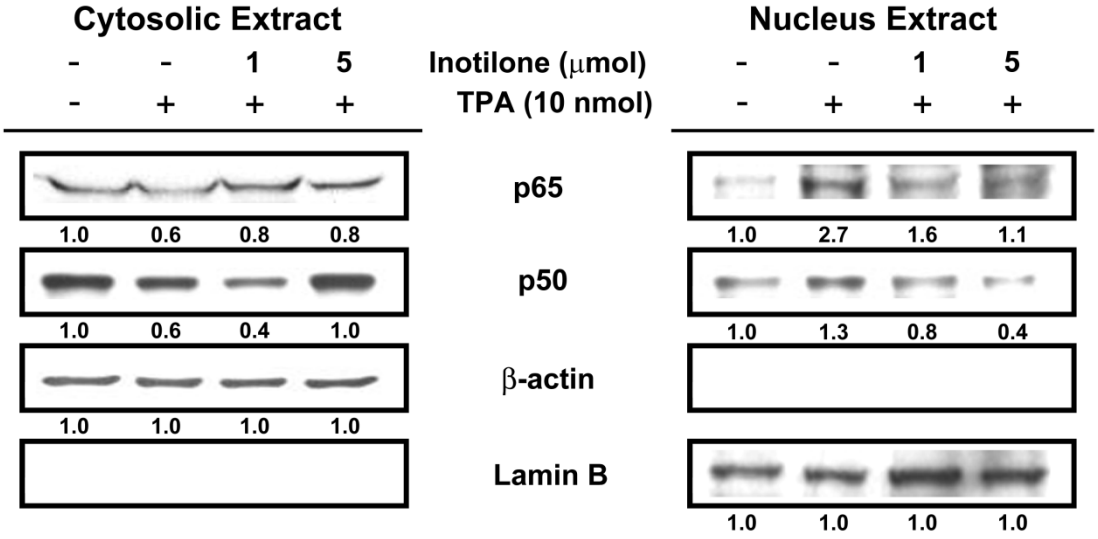


Figure 4.5 Effects of inotilone on TPA-induced activation and nuclear translocation of NF-κB.
Animals were treated as described in material and methods and sacrificed after 1 h of TPA treatment. (A) Total extract were analyzed for the phosphorylated and total IκBα. (B) Cytosolic and nuclear fractions 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.

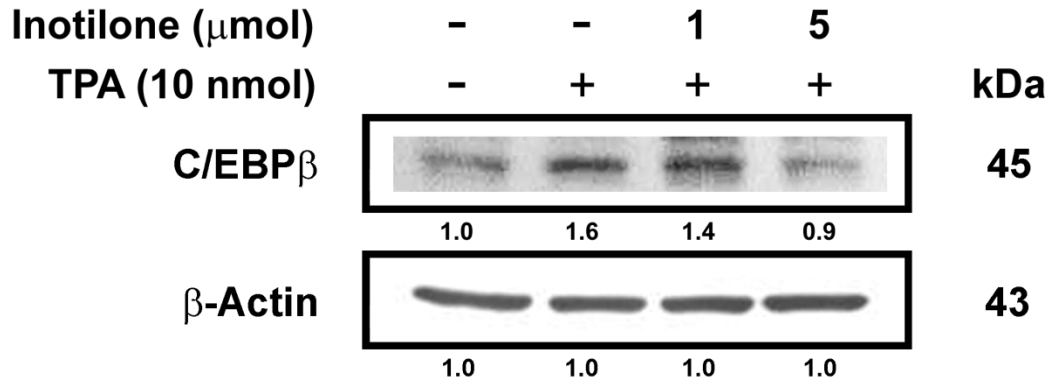


Figure 4.6 Effect of inotilone on the expression of C/EBPβ in TPA-treated mouse skin.
Animals were treated as described in material and methods. The epidermal lysates wre analyzed for C/EBPβ expression by western blotting.

4.3.3. 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 PI3K/Akt signaling pathways are involved in the activation of NF-κB in TPA-treated mouse skin. All of these kinases need to be phosphorylated to become activated. Therefore, the effects of inotilone on TPA-induced phosphorylation of p38, ERK, PI3K, and Akt were studied by western blotting. As shown in Figure 4.7, p38 and ERK MAPK in mouse skin were phosphorylated in response to TPA treatment. Pretreatment with inotilone could down-regulate TPA-induced phosphorylation of p38 and 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 which were blocked by pretreatment with inotilone.

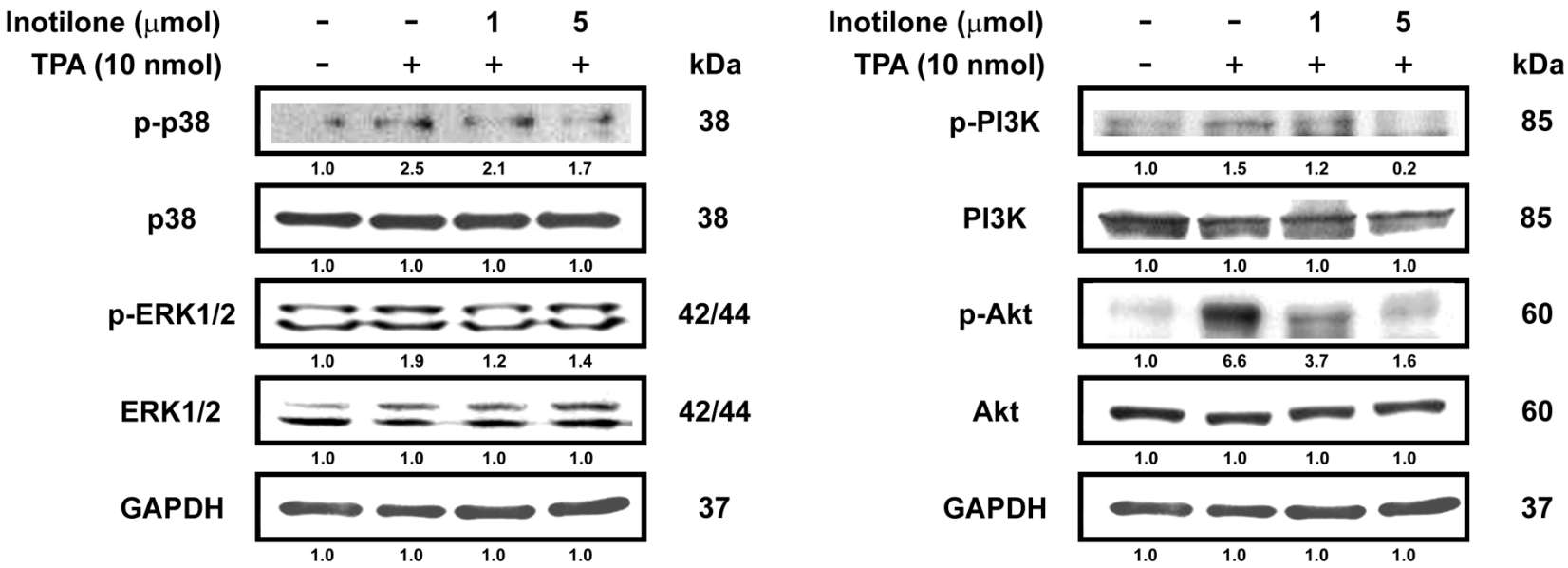


Figure 4.7 Effects of inotilone on TPA-induced activation of ERK, p38 MAPK and PI3K/Akt signaling pathways. Animals were treated as described in material and methods and sacrificed after 1 h after TPA treatment. The epidermal lysates were analyzed for the phosphorylated and total p38, ERK, PI3K and Akt.

4.3.4. Effects of Inotilone on Epidermal Hyperplasia and Infiltration of Leukocytes Induced by TPA In Mouse Skin

The anti-inflammatory activitis of inotilone were also evaluated by its effects on TPA-mediated induction of epidermal hyperplasia and leukocytes infiltration. As summarized in Table 4.2, 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 leukocyte infiltration were also presented in Table 4.2. A greater number of leukocytes in dermis were observed upon TPA treatment as compared to the control animals. After 24 h of TPA treatment, the number of infiltrated leukocytes were increased from $23.0 \pm 4.8/mm^2$ to $77.7 \pm 8.7/mm^2$ ($P < 0.001$). Pretreatment the skin with 1 and 5 μmol inotilone inhibited the filtration by 71 % and 91 %, respectively.

Table 4.2 Inhibitory effects of inotilone on TPA-induced skin thickness and infiltrated leukocytes in mouse skin

Group	Epidermal thickness (μm)	Infiltrated leukocytes (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.

4.3.5. Suppression of DMBA-initiated and TPA-promoted Mouse Skin Tumorigenesis

The anti-tumor promotion activity of inotilone was evaluated on TPA-induced tumor formation in DMBA-initiated mouse skin. At the end of the experiment, no statistically significant difference in body or organ weight between the mice treated with and without inotilone indicating that the topical application of inotilone did not cause any toxicity (Table 4.3). As presented in Table 4.4, 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 20^{th} 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 4.5). The size distribution of papillomas of TPA treatment group was 86 % at 1-3 mm^2, 10 % at 3-5 mm^2, and 4 % 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 (4 %) and more with

size < 3 mm^2 (96 %) as compared with TPA treatment group. Papillomas with size > 5 mm^2 were completely inhibited.

Table 4.3 Body and organ weight of ICR female mice after 20-week treatment.

Group	No. of mice	Body (g)	Liver (g)	Kidney (g)	Spleen (g)
(1) Acetone/Acetone	12	37.3 ± 5.8	2.18 ± 0.26	0.15 ± 0.02	0.57 ± 0.04
(2) Acetone/TPA	11	33.0 ± 3.1	2.10 ± 0.28	0.25 ± 0.09	0.57 ± 0.12
(3) Inotilone 1 μmol/ TPA	12	31.5 ± 2.6	1.93 ± 0.35	0.22 ± 0.15	0.56 ± 0.08
(4) Inotilone 5 μmol/TPA	12	33.6 ± 4.7	1.82 ± 0.30	0.16 ± 0.05	0.51 ± 0.08

Table 4.4 Anti-tumor promotion effects of inotilone on DMBA/TPA-induced skin tumorigenesis in ICR female mice.

Treatment	No. of mice	No. of tumor per mouse	% mice with tumors
(1) Acetone/Acetone	12	-	-
(2) Acetone/TPA	11	24 ± 1.4	100
(3) Inotilone 1 μmol/ TPA	12	13 ± 2.8[a]	83
(4) Inotilone 5 μmol/TPA	12	6 ± 2.1[b]	50

Statistical analysis was done by Student's t-test. [a] $P < 0.01$and [b] $P < 0.001$ were versus group 2.

Table 4.5 Effects of inotilone on the size distribution of papillomas induced by TPA in DMBA-initiated mouse skin.

Treatment	No. of mice	1 to < 3 mm^2 (%)	3 to < 5 mm^2 (%)	> 5 mm^2 (%)
Acetone/Acetone	12	-	-	-
Acetone/TPA	11	21 ± 3.6 (86 %)	2.5 ± 1.2 (10 %)	1 ± 0.1 (4 %)
Inotilone 1 μmol/ TPA	12	11 ± 2.8[a] (87 %)	1.3 ± 0.7 (10 %)	0.3 ± 0.1 (2 %)
Inotilone 5 μmol/TPA	12	6.5 ± 2.3[b] (96 %)	0.3 ± 0.1 (4 %)	-

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.

4.4. Discussion

Cancer chemoprevention is regarded as a promising alternative strategy to control cancer based on the concept that cancer development is a multi-step process and can be divided into three stages: initiation, promotion, and progression [50]. While the chemoprevention approach targeting at the initiation stage is most desirable, the interference in the promotion stage appears to be most appropriate and practical. The major reason for this is that tumor promotion is a reversible event, at least in early stages, and requires repeated and prolonged exposure of a promoting agent. 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 anti-tumor promoters as well. Inotilone, as discussed in Chapter 3, has shown to exhibits anti-inflammatory *in vitro*. In the present study, the effects of inotilone on TPA-mediated inflammatory and tumor-promoting responses in mouse skin were investigated.

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 application of inotilone before TPA treatment inhibited TPA-induced iNOS, COX-2, VEGF, and MMP-9 expression in a dose dependent manner (Figure 4.4). It has been reported that TPA induced NO, produced by iNOS, and VEGF production in human

polymorphonuclear leukocytes whereas over expressed VEGF led to the induction of vascular hyperpermeability. In addition, PGE_2 derived by COX-2 is a well known mediator to increase vascular permeability and cell proliferation. Although the effect of inotilone on vascular permeability was not evaluated, the reduction of epidermal hyperplasia and leukocyte infiltration by inotilone may be partly attributed to the suppression of iNOS, VEGF, and COX-2 production (Table 4.2 and Figure 4.4).

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 nuclear translocation of p50 and p65 subunits of NF-κB by suppressing the phosphorylation and subsequent proteolytic degradation of IκBα (Figure 4.5). 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 [58;100]. Topical application of inotilone inhibited TPA-induced activation of ERK1/2 and p38 MAPK as well as NF-κB suggesting that inotilone attenuated TPA-induced NF-κB activation possibly through inhibition of these upstream kinases. The inhibitory effect of inotilone on another signaling pathway, PI3K/Akt, was also observed. Activation of PI3K leads to phosphorylation of phosphatidylinositides, which then Akt, which plays a pivotal role in several cell signaling networks involved in carcinogenesis, including cell proliferation, differentiation, survival, invasion, and metastasis [101]. In addition, it has also been suggested that PI3K/Akt pathway can contributes 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, leading to eventual iNOS, COX-2, and other mediators suppression 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. Moreover, C/EBPβ appears to play a crucial role in promoting proliferation and is implicated in tumor development in several cell types/tissues. Increased expression of C/EBPβ is associated with human breast, colorectal and ovarian tumorigenesis. Zhu *et al*. have also shown that C/EBPβ-deficient mice are completely resistant to chemically induced skin carcinogenesis [102]. Unlike the *in vitro* study which 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. 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.

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 anti-tumor 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 ERK1/2, and p38 MAPK and PI3K/Akt signaling pathways.

In summary, the results have shown that pretreatment with inotilone inhibited TPA-mediated acute inflammation and tumor promotion 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, ERK1/2 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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