1 Running Head: anti-inflammatory activities of inotilone

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3 Anti-inflammatory activities of inotilone from *Phellinus linteus* through 4 the inhibition of MMP-9, NF-κB, and MAPK activation *in vitro* and *in* 5 *vivo*

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1 **Abstract**

2 Inotilone was isolated from *Phellinus linteus*. The anti-inflammatory effects of
3 inotilone was studies by using lipopolysaccharide (LPS)-stimulated mouse macrophage
4 RAW264.7 cells and λ-carrageenan (Carr)-induced hind mouse paw edema model.
5 Inotilone was tested for its ability to reduce nitric oxide (NO) production, and the
6 inducible nitric oxide synthase (iNOS) expression. Inotilone was tested in the inhibitor of
7 mitogen activated protein kinase (MAPK) [extracellular signal-regulated protein kinase
8 (ERK), c-Jun NH_2-terminal kinase (JNK), p38], and nuclear factor-κB (NF-κB),
9 matrix-metalloproteinase (MMP)-9 protein expressions in LPS-stimulated RAW264.7
10 cells. When RAW264.7 macrophages were treated with inotilone together with LPS, a
11 significant concentration-dependent inhibition of NO production was detected. Western
12 blotting revealed that inotilone blocked the protein expression of iNOS, NF-κB, and
13 MMP-9 in LPS-stimulated RAW264.7 macrophages, significantly. Inotilone also
14 inhibited LPS-induced ERK, JNK, and p38 phosphorylation. *In vivo* test, inotilone
15 decreased the paw edema at the 4th and the 5th h after Carr administration, and it increased
16 the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase
17 (GPx). We also demonstrated inotilone significantly attenuated the malondialdehyde
18 (MDA) level in the edema paw at the 5th h after Carr injection. Inotilone decreased the
19 NO and tumor necrosis factor (TNF-α) levels on serum at the 5th h after Carr injection.
20 Western blotting revealed that inotilone decreased Carr-induced iNOS, cyclooxygenase-2
21 (COX-2), NF-κB, and MMP-9 expressions at the 5th h in the edema paw. An
22 intraperitoneal (*i.p.*) injection treatment with inotilone diminished neutrophil infiltration
23 into sites of inflammation as did indomethacin (Indo). The anti-inflammatory activities of

inotilone might be related to decrease the levels of MDA, iNOS, COX-2, NF-κB, and MMP-9 and increase the activities of CAT, SOD, and GPx in the paw edema through the suppression of TNF-α and NO. This study presents the potential utilization of inotilone, as a lead for the development of anti-inflammatory drug.

KEY WORDS: Inotilone; anti-inflammation; NO; TNF-α; MMP-9; NF-κB;

1 **Introduction**

2 Inflammation, a physiological response to infection or injury, plays a critical role in
3 chronic diseases, including asthma, rheumatoid arthritis, atherosclerosis, and Alzheimer's
4 disease, and it plays a role in various human cancers [1]. Among its mediators, inducible
5 nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are important enzymes that
6 regulate inflammatory processes [2]. In addition, one of the major factors involved in the
7 inflammation response is induced by lipopolysaccharide (LPS) and various inflammatory
8 mediator cytokines such as interferon, interleukins, and tumor necrosis factor (TNF)-α [3].
9 Many researchers reported that inflammatory effect induced by λ-carrageenan (Carr)
10 could be associated with free radical formation. Free radical, prostaglandin and NO will
11 be released when administrating with Carr for 1~5 h. The edema effect was raised to
12 maximum at the 3th h and its malondialdehyde (MDA) production was due to free radical
13 attack plasma membrane [4]. Therefore, in this paper, we examined the
14 anti-inflammatory effects of inotilone on LPS-induced RAW264.7 cells and Carr-induced
15 paw edema in mice.

16 The generation of reactive oxygen species (ROS) has been shown to modulate both
17 the expression and activity of MMPs [5]. ROS also increases the expression of MMPs via
18 cell signaling pathways, such as the mitogen activated protein kinase (MAPK) pathways
19 that are regulated by redox-sensitive phosphatases [6]. MAPK pathways are the
20 evolutionarily conserved kinase module that links extracellular signals to the machinery
21 controlling fundamental cellular processes such as growth, proliferation, differentiation,
22 and cell death [7]. An important amount of evidence has indicated that macrophages
23 under certain stimuli induce matrix metalloproteinase 9 (MMP-9) expression and protein

secretion through the activation of extracellular signal-regulated protein kinase (ERK) and nuclear factor-κB (NF-κB) signaling pathways [5]. MMP-9 expression in macrophages, mediates cell migration and proliferation by promoting extracellular matrix remodeling [8].

Phellinus linteus (Berk. & M.A. Curt.) (PL) is a mushroom that belongs to the genus *Phellinus* and it is commonly called "Sangwhang" in Taiwan. It is popular in oriental countries and has been traditionally used as food and medicine. PL contains many bioactive compounds, and it is known to prevent various diseases, such as cancer, ulcer, bacterial and viral infections and diabetes [9]. Recently, PL has been exhibited various biological activities, including anti-oxidative, anti-inflammatory, cytotoxic, anti-platelet aggregation, anti-diabetic, anti-dementia, and anti-viral effects [10]. PL has been isolated several aromatic compounds from the cultured mycelia such as hydroxybenzaldehyde, caffeic acid, hispolon, hispidin, and inotilone [11]. We recently reported that inotilone had previously been shown to possess anti-inflammatory [12], and α-glucosidase and aldose reductase inhibitory activities [9]. This study examined the anti-inflammatory effects of inotilone by using LPS-stimulated RAW264.7 cell *in vitro* and Carr-induced mouse paw edema model *in vivo*. The study also evaluated the effect of inotilone on MMP-9 expression associated NF-κB and MAPK signaling pathways to reveal molecular mechanism.

Method

Chemicals

LPS (endotoxin from *Escherichia coli*, serotype 0127:B8), Carr (Type IV), Indo,

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) and other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA). TNF-α was purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, anti-β-actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly-(vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA). The antibody against MMP-9, NF-κB, ERK, JNK, and p38 proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA).

Isolation and characterization of inotilone from fruiting body of PL

The fruiting body of PL (about 1.0 kg, air dry weight) was powdered, and extracted with 6 L 95% EtOH at room temperature (3 times, 72 h each). Extracts were filtered and combined together, and then evaporated at 40 ℃ (N-11, Eyela, Japan) to dryness under reduced pressure to give a dark brown residue (40 g). The yield obtained for PL is about 4 %. The crude extract was suspended in H_2O (1 L), and then partitioned with 1 L *n*-hexane (× 2), 1 L EtOAc (× 2) and 1 L *n*-butanol (× 2), successively.

Inotilone was purified from the EtOAc soluble portion (8 g) by a bioassay-guid separation. A portion of the active EtOAc fraction was subjected to silica gel chromatography using stepwise $CHCl_3$-MeOH (9:1, 8:2, 1:1 *v/v*) as eluent. Final purification was achieved by preparative HPLC (Spherisorb ODS-2 RP18, 5 µm (Promochem), 250×25 mm, acetonitrie-H_2O (83: 17 *v/v*), at a flow rate of 10 mL/min and UV detection at 375nm). The identification of inotilone was performed by comparing their physical spectral data with literature values [9].

1 Inotilone : ^{1}H NMR (400 MHz, DMSO) δ 2.55 (s, 3 H, CH_3), 5.80 (s, 1H, CH), 6.49
2 (s, 1 H, CH), 6.80 (d, 1 H, J = 8.4 Hz, ArH), 7.16 (dd , 1 H, J = 8.4, 2.0 Hz, ArH), 7.34 (d,
3 1 H, J = 2.0 Hz, ArH); ^{13}C NMR (100 MHz, DMSO) δ 15.9, 105.7, 112.3, 116.2, 118.2,
4 123.1, 125.0, 144.6, 145.7, 148.4, 180.9, 187.0.
5

6 Animals

7 Imprinting control region (ICR; 6-8 weeks male) mice were obtained from the
8 BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant
9 temperature of 22 ±1℃, and relative humidity of 55 ± 5 % with 12 h dark-light cycle for
10 at least 2 weeks before the experiment. They were given food and water *ad libitum*. This
11 animal study was approved by the Institutional Animal Care and Use Committee (IACUC)
12 of the China Medical University, Taiwan, and all animal procedures were performed
13 according to the IACUC policy. And the recommendations of the Committee for
14 Research and Ethical Issues of the International Association for the Study of Pain (IASP)
15 Ethical Guidelines (Committee for Research and Ethical Issues of the IASP, 1983) were
16 adhered in these studies. In particular, the duration of the experiments was as short as
17 possible and the number of animals was kept to a minimum.

18 After a 2-week adaptation period, male ICR mice (18-25 g) were randomly assigned
19 to four groups (n=6) of the animals in the study. The control group received normal saline
20 (intraperitoneal; *i.p.*). The other three groups included a Carr-treated, a positive control
21 (Carr + Indo) and inotilone administered groups (Carr + inotilone).
22

23 Cell culture

1 A murine macrophage cell line RAW264.7 (BCRC No. 60001) was purchased from
2 the Bioresources Collection and Research Center (BCRC) of the Food Industry Research
3 and Development Institute (Hsinchu, Taiwan). Cells were cultured in plastic dishes
4 containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA)
5 supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO_2 incubator (5%
6 CO_2 in air) at 37℃ and subcultured every 3 days at a dilution of 1:5 using 0.05%
7 trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).
8

9 Cell viability

10 Cells (2 x 10^5) were cultured in 96-well plate containing DMEM supplemented with
11 10% FBS for 1 day to become nearly confluent. Then cells were cultured with inotilone
12 in the presence of 100 ng/mL LPS for 24 h or 1 h. After that, the cells were washed twice
13 with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37℃ testing for cell
14 viability. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was
15 added. After 30-min incubation, absorbance at 570 nm was read by using a microplate
16 reader (Molecular Devices, Orleans Drive, Sunnyvale, CA).
17

18 Measurement of Nitric oxide/Nitrite

19 NO production was indirectly assessed by measuring the nitrite levels in the cultured
20 media and serum determined by a colorimetric method based on the Griess reaction [13].
21 The cells were incubated with inotilone (0, 1.56, 3.12, 6.25, 12.5, and 25 μM) in the
22 presence of LPS (100 ng/mL) at 37 ℃ for 24 h. Then, cells were dispensed into 96-well

1 plates, and 100 μL of each supernatant was mixed with the same volume of Griess
2 reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5%
3 phosphoric acid) and incubated at room temperature for 10 min, the absorbance was
4 measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale,
5 CA). Serum samples were diluted four times with distilled water and deproteinized by
6 adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After
7 centrifugation at 10,000×*g* for 5 min at room temperature, 100 μL supernatant was
8 applied to a microtiter plate well, followed by 100 μL of Griess reagent. After 10 min of
9 color development at room temperature, the absorbance was measured at 540 nm with a
10 Micro-Reader. By using sodium nitrite to generate a standard curve, the concentration of
11 nitrite was measured form absorbance at 540 nm.

12

13 Determination of MMP-9 by Zymography

14 MMP in the medium released from RAW264.7 cells was assayed using gelatin
15 zymography (7.5% zymogram gelatin gels) according to the methods reported by Liao et
16 al. (2006) [14] with some modification. Briefly, the culture medium was electrophoresed
17 (120 V for 90 min) in a 10% SDS-PAGE gel containing 0.1% gelatin. The gel was then
18 washed at room temperature in a solution containing 2.5% (*v/v*) Triton X-100 with two
19 changes and subsequently transferred to a reaction buffer for enzymatic reaction
20 containing 1% NaN_3, 10 mM $CaCl_2$ and 40 mM Tris–HCl, pH 8.0, at 37 °C with shaking
21 overnight (for 12-15 h). Finally, the MMP gel was stained for 30 min with 0.25% (*w/v*)

1 Coomassie blue in 10% acetic acid (*v/v*) and 20% methanol (*v/v*) and destained in 10%
2 acetic acid (*v/v*) and 20% methanol (*v/v*).

3

4 ## Carr-induced Edema

5 The Carr-induced hind paw edema model was used for determination of
6 anti-inflammatory activity [15]. Animals were *i.p.* treated with inotilone (1.25, 2.50, and
7 5 mg/kg), Indo (10 mg/kg) or normal saline, 30 min prior to injection of 1% Carr (50 μL)
8 in the plantar side of right hind paws of the mice. The paw volume was measured
9 immediately after Carr injection and at 1, 2, 3, 4, and 5 h intervals after the administration
10 of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese,
11 Italy). The degree of swelling induced was evaluated by the ratio a/b, where was the
12 volume of the right hind paw after Carr treatment, and b was the volume of the right hind
13 paw before Carr treatment. Indo was used as a positive control. After 5 hrs, the animals
14 were sacrificed and the Carr-induced edema feet were dissected and stored at -80 ℃.
15 Also, blood were withdrawn and kept at -80 ℃.

16 In the secondary experiment, the right hind paw tissue and paw edema tissue took at
17 the 5th h. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately
18 placed in cold normal saline four times their volume and homogenized at 4 ℃. Then the
19 homogenate was centrifuged at 12,000×*g* for 5 min. The supernatant was obtained and
20 stored at −20 ℃ for MDA assays. The whole paw edema tissue was rinsed in ice-cold
21 normal saline, and immediately placed in cold normal saline one time their volume and
22 homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000×*g* for 5 min. The
23 supernatant was obtained and stored at −20 ℃ for the antioxidant enzymes (CAT, SOD

and GPx) activity assays. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

MDA Assay

MDA from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substances (TBARS) method [16]. Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

Measurement of Serum TNF-α by an Enzyme-Linked Immunosorbent Assay (ELISA)

Serum levels of TNF-α were determined using a commercially available ELISA kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF-α was determined from a standard curve. The concentrations were expressed as pg/mL.

Antioxidant Enzyme Activity Measurements

The following biochemical parameters were analyzed to check the antioxidant enzyme activity of inotilone in the paw edema by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome *c* reduction [17]. The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was

1 defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by
2 50%. Total CAT activity was based on that of Aebi [18]. In brief, the reduction of 10 mM
3 H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance
4 at 240 nm. The activity was calculated using a molar absorption coefficient, and the
5 enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per
6 milligram protein per minute. Total GPx activity in cytosol was determined according to
7 Paglia and Valentine's method [19]. The enzyme solution was added to a mixture
8 containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the
9 absorbance at 340 nm was measured. The activity was calculated by using a calibration
10 curve of GPx established from bovine whole blood. A linear relationship between the
11 activity (unit /mL) of GPx and the reduction of NADPH absorbance at 340 nm was found
12 and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram
13 protein per minute.
14

15 Protein Lysate Preparation and Western blot Analysis

16 The stimulated murine macrophage cell line RAW264.7 cells were washed with PBS
17 and lysed in an ice-cold lysis buffer [10% glycerol, 1% Triton X-100, 1mM Na_3VO_4,
18 1mM EGTA, 10mM NaF, 1mM $Na_4P_2O_7$, 20 mM Tris buffer (pH 7.9), 100 mM
19 β-glycerophosphate, 137 mM NaCl, 5 mM EDTA, and one protease inhibitor cocktail
20 tablet (Roche, Indianapolis, IN, USA)] on ice for 1 h, followed by centrifugation at
21 $12{,}000\times g$ for 30 min at 4℃. Soft tissues were removed from individual mice paws and
22 homogenized in a solution containing 10 mM CHAPS, 1 mM phenylmethylsulphonyl
23 fluoride (PMSF), 5 μg/mL, aprotinin, 1 μM pepstatin and 10 μM leupeptin. The

1 homogenates were centrifuged at 12,000×*g* for 20 min, and 30 μg of protein from the
2 supernatants was then separated on 10% sodium dodecylsulphate–polyacrylamide gel
3 (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. After transfer, the
4 membrane was blocked for 2 h at room temperature with 5% skim milk in Tris-buffered
5 saline-Tween (TBST; 20 mM Tris, 500 mM NaCl, pH 7.5, 0.1% Tween 20). The
6 membranes were then incubated with antibody in 5% skim milk in TBST for 2 h at room
7 temperature. The membranes were washed three times with TBST at room temperature
8 and then incubated with a 1 : 2000 dilution of anti-mouse IgG secondary antibody
9 conjugated to horseradish peroxidase (Sigma, St Louis, MO, U.S.A.) in 2.5% skim milk
10 in TBST for 1 h at room temperature. The membranes were washed three times and the
11 immunoreactive proteins were detected by enhanced chemiluminescence (ECL) by using
12 hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The
13 results of Western blot analysis were quantified by measuring the relative intensity
14 compared to the control using Kodak Molecular Imaging Software (Version 4.0.5,
15 Eastman Kodak Company, Rochester, NY) and represented in the relative intensities.
16

17 ## Histological Examination

18 For histological examination, biopsies of paws took 5 hrs following the intraplantar
19 injection of Carr. The tissue slices were fixed in Dietric solution (14.25% ethanol, 1.85%
20 formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded
21 ethanol and embedded in Paraplast (Sherwood Medical). Sections (7 μm thick) were
22 deparaffinized with xylene and stained with trichromic Van Gieson, and antigen retrieval
23 was performed with citrate buffer, then blocked with 5% normal goat serum in PBS and

1 incubated with rabbit anti-COX-2 and anti-iNOS in PBS with 5% normal goat serum.
2 The sections were incubated with biotinylated goat anti-rabbit IgG. After washing in PBS,
3 sections were processed with the Dako kit (Dako REALTM envision TM detection
4 system). Thus, some sections were stained with hematoxylin and eosin, while others were
5 processed for iNOS and COX-2 immunohistochmistry staining. All samples were
6 observed and photographed with BH2 Olympus microscopy. Every three to five tissue
7 slices were randomly chosen from Control, Carr, Indo and inotilone-treated (5 mg/kg)
8 groups [20].
9

10 ## Statistical Analysis

11 Experimental results were presented as the mean ± standard deviation (SD) of three
12 parallel measurements. IC_{50} values were estimated using a non-linear regression
13 algorithm (SigmaPlot 8.0; SPSS Inc. Chicago, IL). Data obtained from animal
14 experiments were expressed as mean standard error (± S.E.M.). Statistical evaluation was
15 carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple
16 range tests). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.
17

18 # Results

19 ## Isolation of inotilone from PL and its structural characterization

20 PL was isolated via extensive chromatographic purification of the ethyl
21 acetate-soluble fraction of the dried fruiting body. The chemical structure of the purified
22 yellow powder was elucidated by NMR spectroscopy and mass spectrometry studies and
23 it was identified as inotilone (Fig. 1A) [9].

Cell viability and effect of inotilone on LPS-induced NO production in macrophages

The effect of inotilone on RAW264.7 cell viability was determined by a MTT assay. Cells cultured with inotilone at the concentrations (0, 1.56, 3.12, 6.25, 12.5, and 25 μM) used in the presence of 100 ng/mL LPS for 24 h did not change cell viability (Fig. 1B). Inotilone did not interfere with the reaction between nitrite and Griess reagents at 25 μM (data not shown). Unstimulated macrophages, after 24 h of incubation in culture medium produced background levels of nitrite. When RAW264.7 macrophages were treated with different concentrations of inotilone (0, 1.56, 3.12, 6.25, 12.5, and 25 μM) together with LPS (100 ng/mL) for 24 h, a significant concentration-dependent inhibition of nitrite production was detected. There was either a significant decrease in the nitrite production of group treated with 3.12 μM inotilone ($p < 0.05$) or very or highly significant decrease of groups treated respectively with 6.25, 12.5 and 25 μM of inotilone when compared with the LPS-alone group ($p < 0.01$ or $p < 0.001$). The IC_{50} value for inhibition of nitrite production of inotilone was about 10.24 ± 0.35 μM (Fig. 1C).

Inhibition of LPS-induced iNOS and COX-2 protein by inotilone

In order to investigate whether the inhibition of NO production was due to a decreased iNOS and COX-2 protein level, the effect of inotilone on iNOS and COX-2 protein expression was studied by immunoblot. The results showed that incubation with

inotilone (0, 6.25, 12.5, and 25 μM) in the presence of LPS (100 ng/mL) for 24 hrs inhibited iNOS proteins expression in mouse macrophage RAW264.7 cells in a dose-dependent manner (Fig. 1D). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands were analyzed using Kodak Quantity software in three independent experiments and showed an average of 67.1 and 13.6% down-regulation of iNOS and COX-2 proteins after treatment with inotilone at 25 μM compared with the LPS-alone.

Effects of inotilone on the LPS-stimulated activation of mitogen-activated protein kinases (MAPKs)

MAPKs play critical roles in the regulation of cell growth and differentiation, and control cellular responses to cytokines and stresses. In particular, ERK, p38, and JNK are known to be important for the activation of NF-κB [20, 21]. To explore whether the inhibition of NF-κB activation by inotilone is mediated through the MAPK pathway, MAPK phosphorylation was examined by Western blot in RAW 264.7 cells pretreated with inotilone and then with LPS. As shown in Fig. 1E, inotilone suppressed the LPS-induced activation of ERK, JNK, and p38 MAPKs in a time-dependent manner. However, the expression of non-phosphorylated ERK, JNK, and P38 MAPKs was unaffected by LPS or LPS plus inotilone. These results suggest that phosphorylation of MAPKs may be involved in the inhibitory effect of inotilone on LPS-stimulated NF-κB binding in RAW 264.7 cells.

Inhibition of LPS-induced MMP and NF-κB proteins by inotilone

1 The effect of inotilone on MMP-9 activation was analyzed by gelatin zymography
2 and immunoblot. As shown in Fig. 2A, the results showed that the incubation with
3 inotilone (0, 6.25, 12.5, and 25 μM) in the presence of LPS for 24 h MMP-9 activation in
4 mouse macrophage RAW264.7 cells in a dose-dependent manner. The intensity of protein
5 bands were analyzed by using Kodak Quantity software in three independent experiments
6 and showed an average of 68.1% down-regulation of MMP-9 activation after the
7 treatment with inotilone at 25 μM compared with the LPS-alone. The effect of MMP-9
8 expression by inotilone in the presence of LPS (100 ng/mL) for 24 h was assessed by
9 Western blotting. The results showed the incubation with inotilone (0, 6.25, 12.5, and 25
10 μM) in the presence of LPS for 24 h inhibited MMP-9 proteins expression in mouse
11 macrophage RAW264.7 cells in a dose-dependent manner (Fig. 2B). The detection of
12 β-actin was also performed in the same blot as an internal control. The intensity of
13 protein bands was analyzed by using Kodak Quantity software in three independent
14 experiments and the result showed an average of 58.9% down-regulation of MMP-9
15 proteins, respectively, after the treatment with inotilone at 25 μM compared with the
16 LPS-alone.

17 The effect of NF-κB expression by inotilone in the presence of LPS for 1 h was
18 assessed by Western blotting. And the intensity of protein bands showed an average of
19 79.2% increase of NF-κB protein after treatment with inotilone at 25 μM compared with
20 the LPS-alone (Fig. 2C). Therefore, it can be concluded that inotilone is capable of
21 inhibiting iNOS expression in LPS induced RAW264.7 cells via attenuation of NF-κB
22 signaling by ERK, p38, and JNK.

23

1 Effects of inotilone on Carr-induced mouse paw edema

2 Because inotilone effectively inhibited iNOS inductions in macrophages, studies 3 were extended to determine whether inotilone affected acute phase inflammation in 4 animal models. In this study, we used Carr-induced edema because this model is widely 5 employed for screening the effects of anti-inflammatory drugs. Carr-induced paw edema 6 is shown in Fig. 3A. Inotilone (5 mg/kg) inhibited ($p < 0.001$) the development of paw 7 edema induced by Carr (10 mg/kg) at the 4th h and the 5th h after the treatment, 8 significantly. Inotilone at the concentration of 5 mg/kg, the levels of edema volume were 9 decreased to 56.2% of that observed in the Carr alone group. Indo (10 mg/kg) 10 significantly decreased the Carr induced paw edema at the 4th h and the 5th h after the 11 treatment ($p < 0.001$).

12

13 Effects of inotilone on the MDA level

14 The MDA level increased significantly in the edema paw at the 5th h after Carr 15 injection ($p < 0.001$). However, the MDA level was decreased significantly by treatment 16 with inotilone (5 mg/kg) ($p < 0.001$), as well as 10 mg/kg Indo (Fig. 3B). The inhibition 17 mice MDA levels compared with the Carr group are 18.3%, 39.7%, and 43.3%, 18 respectively.

19

20 Effects of inotilone on the TNF-α level

21 The TNF-*α* level increased significantly in serum at the 5th h post-Carr injection ($p <$ 22 0.001). However, inotilone (5 mg/kg) decreased the TNF-α level in serum at the 5th h 23 after Carr injection ($p < 0.01$), as well as 10 mg/kg Indo (Fig. 3C). In the range of 1.25-5

mg/kg, inotilone could inhibit the level of TNF-α to 10.6-40.3% of the observation in Carr group.

Effects of inotilone on the NO level

In Fig. 3D, the NO level increased significantly in the edema serum at the 5th h post-Carr injection ($p < 0.001$). Inotilone (5 mg/kg) significantly decreased the serum NO level ($p < 0.001$). Meanwhile, in the range of 1.25-5 mg/kg, inotilone could inhibit the level of nitrite to 26.2-59.7% of the observation in Carr group. The inhibitory potency was similar to that of Indo (10 mg/kg) at the 5th h after induction.

Effects of inotilone on activities of antioxidant enzymes

At the 5th h after the intrapaw injection of Carr, paw edema tissues were also analyzed for the biochemical parameters such as CAT, SOD, and GPx activities. Carr decreased the activities of CAT, SOD, and GPx in paw edema by 29.3%, 33.9%, and 32.1%, respectively, in comparison to control group. In the range of 1.25-5 mg/kg, inotilone could increase the activities of CAT to 114.3%-125.6%, SOD to 108.2%-139.6%, and GPX to 108.8%-123.5%, respectively, of that observed in Carr along group. Indo also exhibited increase effects in the activities of CAT (129.2%), SOD (140.7%), and GPx (124.1%) in comparison to Carr group (P<0.01) (Table 1). These data implied that the protective effects of inotilone might be attributed to its elevation in the antioxidant enzymes activities of Carr induced mice.

Effects of inotilone on Carr-induced iNOS and COX-2 protein expression in

1 mouse paw edema

2 To investigate whether the inhibition of NO production was due to a decreased iNOS
3 and COX-2 protein level, the effect of inotilone on iNOS and COX-2 proteins expression
4 were studied by Western blot. The results showed that the injection of inotilone (5 mg/kg)
5 on Carr-induced for 5 h inhibited iNOS and COX-2 proteins expression in mouse paw
6 edema (Fig. 4A). The intensity of protein bands was analyzed by using Kodak Quantity
7 software in three independent experiments and showed an average of 76.7% and 87.2%
8 reduction of iNOS and COX-2 protein, respectively, after treatment with inotilone at 5
9 mg/kg compared with the Carr-induced alone. In addition, the protein expression showed
10 an average of 46.1% and 57.3% reduction of iNOS and COX-2 protein after the treatment
11 with Indo at 10.0 mg/kg compared with the Carr-induced alone. The down-regulation of
12 iNOS and COX-2 activity of the inotilone (5 mg/kg) was better than Indo (10.0 mg/kg).

13

14 Effects of inotilone on Carr-induced MMP-9 and NF-κB protein expressions
15 in mouse paw edema

16 The results showed that the injection of inotilone (5 mg/kg) on Carr-induced for 5 h
17 inhibited MMP-9 and NF-κB proteins expression in mouse paw edema (Fig. 4B and 4C).
18 The intensity of protein bands was analyzed by using Kodak Quantity software in three
19 independent experiments and the result of it showed an average of 69.3% reduction of
20 MMP-9 protein after the treatment with inotilone at 5 mg/kg compared with the
21 Carr-induced alone. In addition, the protein expression showed an average of 57.5%
22 reduction of MMP-9 protein after the treatment with Indo at 10.0 mg/kg compared with
23 the Carr-induced alone (Fig. 4B). And the intensity of protein bands showed an average

of 96.8% increase of NF-κB protein (p<0.001) (Fig. 4C).

Inotilone modulates the activation of MAPK pathways in mouse paw edema

The activation of MAPK pathways in particular the phosphorylation of ERK1/2, JNK, and p38 expression were investigated by Western blot in paw edema tissues homogenates at the 5th h after Carr injection. A significant increase in p-ERK1/2, p-JNK, and p-p38 levels was observed in Carr-treated mice (Fig. 4D). The treatment of mice with inotilone significantly reduced the level of p-ERK1/2, p-JNK, and p-p38 levels in mouse paw edema. On the contrary, inotilone treatment prevented the Carr-induced expression of these kinases. The intensity of protein bands were analyzed by using Kodak Quantity software in three independent experiments and showed an average of 59.8%, 61.7%, and 71.8% reduction of p-ERK1/2, p-JNK, and p-p38 proteins after the treatment with inotilone at 5 mg/kg compared with the Carr-induced alone (p<0.001) (Fig. 4D).

Histological examination

Paw biopsies of the control mice showed marked cellular infiltration in the connective tissue. The infiltrates accumulated in collagen fibers and intercellular spaces. Paw biopsies of mice treated with inotilone (5 mg/kg) showed a reduction in inflammatory responses induced by Carr. Histologically, inflammatory cells were reduced in number and confined to the surroundings of the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction in intercellular spaces. Moreover, the hypodermis connective tissues were not damaged (Fig. 5A). Neutrophils were increased with Carr treatment (Fig. 5B). Indo and inotilone (5

1 mg/kg) could decrease the neutrophils numbers as compared to the Carr-treated group
2 (Fig. 5C and 5D). No inflammation, tissue destruction, iNOS and COX-2
3 immunoreactive cells (Fig. 5E and 5I). At the 5th h after intraplantar Carr injection,
4 numerous iNOS and COX-2 immunoreactive cells were observed in the brown site of
5 paw tissue (Fig. 5F and 5J). Administration of Indo and inotilone (5 mg/kg) 30 min prior
6 to the Carr injection markedly reduced the increase in iNOS amd COX-2 immunoreactive
7 cells in paws (Fig. 5G, 5H, 5K, and 5L).
8

9 ## Discussion

10 Inflammation represents a highly coordinated set of events that allow tissues to
11 respond to injury, and it requires the participation of various cell types expressing and
12 reacting to diverse mediators in a sequential manner [22]. In the present study, we
13 demonstrated the anti-inflammatory activities of inotilone in both *in vitro* and *in vivo*
14 experimental systems, by using LPS-stimulated RAW264.7 macrophages and a mouse
15 model of topical inflammation respectively. The inhibitory activities against iNOS as
16 shown in *in vitro* assays appear to confer on inotilone a potent *in vivo* efficacy in mouse
17 suggesting its potential therapeutic usage as a novel topical anti-inflammatory source of
18 health food. The pathology of inflammation is initiated by complex processes triggered
19 by microbial pathogens such as LPS which is a prototypical endotoxin. LPS can directly
20 activate macrophages which trigger the production of inflammatory mediators, such as
21 NO, prostaglandin E2 (PGE_2), TNF-α and leukotrienes [23]. However, no report has been
22 issued on the anti-inflammatory effect of inotilone *in vivo* and the mode of action
23 involved. Thus, this study was aimed to evaluate the anti-inflammatory effect of inotilone

1 by screening the effects of inotilone on LPS-induced pro-inflammatory molecules *in vitro*
2 and on acute phase inflammation *in vivo*. And, we also evaluated the mechanism of
3 inotilone on MMP-9 and NF-κB expressions associated MAPK signaling pathways in the
4 anti-inflammation.

5 LPS-induced macrophage activation increased the production of pro-inflammatory
6 cytokines, NO by iNOS and PGE_2 by COX-2, which are the main cytotoxic and
7 pro-apoptotic mechanisms participating in the innate response in many mammals [24].
8 Therefore, LPS which stimulated macrophages can be effectively used as a model to
9 study inflammation and potential anti-inflammatory mediators with their action
10 mechanisms. Although iNOS plays a pivotal role in immunity against infectious agents
11 by producing an excess amount of NO, this enzyme has come into the spotlight for its
12 detrimental roles in inflammation-related diseases [25]. *In vitro* models such as
13 macrophage cells or other cell lines are useful materials with a steady high-level
14 production of NO. The mechanisms by which inotilone inhibits macrophage functions
15 have not been elucidated. Examination of the cytotoxicity of inotilone in RAW264.7
16 macrophages using MTT assay has indicated that inotilone even at 25 μM did not affect
17 the viability of RAW264.7 cells. Inotilone inhibited iNOS expression in LPS-stimulated
18 macrophages and subsequently inhibited the NO production, whereas it decreased the
19 enzyme activity of COX-2 instead of its expression to reduce PGE_2 production [12]. In
20 addition, Carr-induced inflammatory response has been linked to neutrophil infiltration
21 release NO as well as that of PGE_2. Results *in vitro* showed that inotilone suppressed
22 LPS-induced production of NO, and the protein expression of iNOS and COX-2.
23 The similar results for inotilone inhibits LPS-induced NO and PGE_2 production through

1 modulating iNOS expression and COX-2 enzyme activity [12].

2 Inhibiting NF-κB and MAPK pathways have been suggested as the two major
3 mechanisms underlying the attenuation of LPS-induced inflammatory cytokine
4 production. NF-κB plays a crucial role as the transcription factor in regulating many of
5 the pro-inflammatory cytokine genes. LPS stimulation elicits a cascade leading to the
6 activation of NF-κB [7]. The MAPKs play a critical role in the regulation of cell growth
7 and differentiation and in the control of cellular responses to cytokines and stressors.
8 Moreover, MAPKs are involved in the LPS-induced signalling pathway by which iNOS
9 is expressed [26]. In the present study, we have demonstrated that the phosphorylation of
10 MAPKs can be induced by LPS. The treatment with inotilone was found to significantly
11 inhibit LPS-induced JNK, ERK, and p38 phosphorylation at 5, 10, 15, 30, and 60 min.
12 Therefore, this suggests that JNK, ERK, and p38 are involved in the inhibition by
13 inotilone of LPS-stimulated NF-κB binding in RAW 264.7 cells. Ajizian et al. (1999)
14 suggested that activation of ERK is thought to be involved in LPS-induced macrophage
15 responses [27]; in addition, JNK and p38 are activated by LPS stimulation and they have
16 been postulated to play important roles in controlling iNOS gene expression [28]. In this
17 study, we found that the treatment of inotilone blocked the activation of ERK1/2, JNK,
18 and p38 MAPK, suggesting that inotilone suppresses LPS-induced NF-κB translocation
19 by inhibiting the activation of these intracellular signaling cascades and it decreases the
20 protein level of iNOS.

21 MMPs are involved in several pathological processes including cancers and
22 inflammation. Among the MMPs, MMP-9 is secreted by macrophages regulates
23 leukocyte migration in inflammatory diseases [5]. MMP-9 regulation involves

1 transcriptional regulation, post-translational cleavage, and antagonism by physiological
2 inhibitors [29]. In transcriptional regulation, MMP-9 expression is controlled by
3 transcriptional factors including activator protein-1 (AP-1) and NF-κB, which bind to the
4 corresponding binding sites in the MMP-9 promoter region [30]. In various kinds of cells,
5 different stimuli induce MMP-9 expression through activation of the MEK-ERK or
6 phosphoinositide 3-kinase (PI3K)-Akt signaling pathways, which subsequently activate
7 AP-1 and NF-κB [31]. Also, p38 MAPK up-regulates MMP-9 expression in Raw 264.7
8 cells stimulated with LPS [32]. However, the upstream regulatory pathways that control
9 the expression and secretion of MMP-9 are very complex and not well understood.
10 Inotilone also decreased the phosphorylation of Akt and PI3K expression [12]. Our
11 results are that inotilone inhibited the activities and the expressions of MMP-9 through
12 decreasing of ERK signaling pathway which subsequently decrease NF-κB expression.
13 Carr-induced paw edema is a well-established model of edema formation which is
14 commonly used for the screening of anti-inflammatory drugs. The intraplantar injection
15 of Carr-induces inflammatory responses, including increases in paw volume and
16 neutrophil infiltration [33]. Recent studies have shown that Carr-induced peripheral
17 release of NO as well as that of PGE_2 [25]. NO plays a major role in edema formation in
18 inflammatory responses and tissue injury and Carr-induced the release of TNF-α level in
19 the tissue [23]. Our results revealed that inotilone and Indo significantly inhibited the
20 development of edema the 4th h and the 5th h after treatment. It was found that the
21 injection of Carr into the mice paw induces the liberation of bradykinin, which later
22 induces the biosynthesis of prostaglandin and other autacoids, which are responsible for
23 the formation of the inflammatory exudates [20]. Our Carr-induced mice paw edema

1 model enabled us to demonstrate the ability of inotilone to inhibit edema induced by
2 acute inflammation. These results in conjunction with the marked inhibition of
3 LPS-induced NO and TNF-α productions by inotilone in macrophages imply that the
4 anti-edema effects of inotilone might result from its inhibition of NO and TNF-α
5 syntheses in the peripheral tissues. The proinflammatory cytokines such as TNF-α and
6 IL-1β are small secreted proteins, which mediate and regulate inflammation. TNF-α
7 induces a number of physiological effects including septic shock, inflammation, and
8 cytotoxicity [13]. Also, TNF-α is a mediator of Carr-induced inflammatory incapacitation,
9 and it is able to induce the further release of kinins and leukotrienes, which is suggested
10 to have an important role in the maintenance of long-lasting nociceptive response [3]. In
11 this study, we found that inotilone decreased the TNF-α level after the Carr injection.
12 The Carr-induced inflammatory response has been linked to neutrophils infiltration
13 and the production of neutrophils-derived free radicals, as well as the release of other
14 neutrophils-derived mediators [4]. Many researchers demonstrated that inflammatory
15 effect induced by Carr is associated with free radical. Free radical, prostaglandin and NO
16 will be released when administrating with Carr for 1-6 h [3]. The reaction of NO with
17 superoxide anion forms peroxynitrite, a potent cytotoxic oxidant
18 eliciting lipid peroxidation and cellular damage. MDA, an indicator of lipid peroxidation,
19 and antioxidant enzymes (CAT, SOD, and GPx) were also measured for evaluating the
20 ability to scavenge radicals. Thus, inflammatory effect would result in the accumulation
21 of MDA [34]. In this study, there were significantly decreases in MDA level with
22 inotilone treatment. Furthermore, there was significantly increase in CAT, SOD, and GPx
23 activities with inotilone treatment. We assume the suppression of MDA production is

1 probably due to the increases of CAT, SOD, and GPx activities.

2 The MAPk family plays important roles in regulation of cell proliferation and cell
3 death in response to various cellular stresses. During Carr-treated mice, oxidative stress
4 and inflammatory cytokines activated MAP kinase kinases, leading to phosphorylation of
5 ERK1/2, JNK, and p38 [35]. In the present study, we have observed an increase of
6 phosphorylated MAPKs in the paw edema tissues at the 5th h after Carr which is
7 significantly reduced by the treatment with inotilone. Therefore, inotilone might alter
8 NADPH oxidase activity through the inhibition of MAPK phosphorylation. Recent study
9 also showed that the mechanism of action of inflammation involved the inhibition of the
10 NADPH-oxidase-dependent superoxide production, the reduction of the intracellular
11 GSH/GSSG ratio and prevention of the activation of the nuclear transcription factor
12 NF-κB, which is an important mediator of inflammation [36].

13 Natural products are a valuable source of novel bioactive secondary metabolites.
14 Various bioassays exist in which the anti-inflammatory activity of these products can be
15 evaluated, having demonstrated that inotilone possesses anti-inflammatory activity *in*
16 *vitro* and *in vivo* model of inflammation. In this model of *in vivo* acute inflammation,
17 TNF-α and NO release in mice serum dropped markedly upon pretreatment with
18 inotilone. It has been demonstrated that several natural product compounds which fall
19 under the class of phenolic compounds act as strong inhibitors of NF-κB activation [37].
20 Inotilone also inhibited MMP-9 induction *via* suppression of NF-κB activity and MAPKs
21 phosphorylation. LPS has been reported to up-regulate MMP-9 production in
22 macrophages and neutrophils, astrocytes, and mast cells indicating the possible
23 involvement of this enzyme in mediating the local infiltration of these inflammatory cells

1 [38]. From the present results, it was indicated that inotilone may regulate the above
2 mentioned inflammatory responses through both inactivation of NF-κB and MAPKs.
3 In conclusion, inotilone suppresses LPS-induced MMP-9 expression by inhibiting
4 the activation of NF-κB via ERK, p38, and JNK signaling pathways in RAW 264.7 cells.
5 This is the first study showing that inotilone inhibits LPS-stimulated RAW 264.7 cells
6 through specific inhibition of NF-κB-dependent MMP-9 expression via ERK, p38, and
7 JNK signaling pathways (Fig. 6). And it is associated with the increase in the activities of
8 antioxidant enzymes (CAT, SOD, and GPx) and inhibit of iNOS, COX-2, MMP-9,
9 NF-κB, and MAPK expressions *in vivo*. These results suggest that inotilone represents a
10 potential anti-inflammatory agent and this new beneficial effect may expand future
11 researches on anti-inflammatory properties of inotilone *in vitro* and *in vivo*.
12

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19

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