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Inhibitory effect of munetone, an isoflavonoid, on 12-*O*-tetradecanoylphorbol 13-acetate-induced ornithine decarboxylase activity

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Abstract

Starting with an extract derived from the bark of *Mundulea sericea* Willd. (Leguminosae) that was active in the process of inhibiting 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase activity (ODC) in cultured mouse epidermal ME 308 cells, the isoflavonoid munetone was isolated and identified as an active principle (IC_{50} = 46 ng/ml). Topical application of munetone (0.04–5 μmol) to the skin of CD-1 mice 2 h prior to treatment with TPA (10 nmol) resulted in dose-dependent inhibition of epidermal ODC activity. In addition, munetone inhibited TPA-independent c-Myc-induced ODC activity with cultured BALB/c c-MycER cells, as well as 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced preneoplastic lesion formation in a mouse mammary gland organ culture (MMOC) system. These data suggest the potential of munetone to serve as a cancer chemopreventive agent by virtue of blocking the process of tumor promotion. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Munetone; 12-*O*-Tetradecanoylphorbol 13-acetate; Ornithine decarboxylase; c-MycER; Mammary organ culture; Cancer chemoprevention

1. Introduction

The leguminous shrub *Mundulea sericea* Willd. (syn. *M. suberosa*) (Leguminosae) is a plant that grows in tropical Africa and parts of India [1]. The bark, leaves and roots of this plant have been used for diverse purposes [2,3]. The isolation of several compounds from this plant has been reported earlier [4–6]. In our search for naturally occurring cancer chemopreventive agents, we have reported the isolation of various active compounds, including the rotenoids deguelin and tephrosin, from an ethyl acetate-soluble extract of the bark [7]. These agents were

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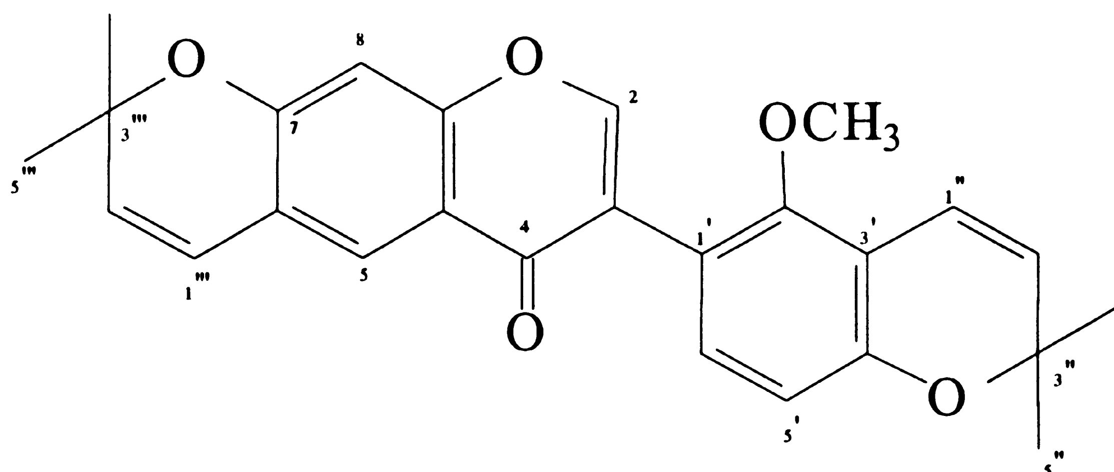

Fig. 1. Chemical structure of munetone.

potent inhibitors of phorbol ester-induced ornithine decarboxylase (ODC) activity in cell culture, and effective cancer chemopreventive agents in animal models [8–10]. In addition to the rotenoids, however, the extract of *M. sericea* bark was found to contain munetone as an active principle capable of inhibiting TPA-induced ODC activity in cell culture [11]. Since ODC activity and polyamine levels are related to the process of tumorigenesis [12,13], modulators are of interest as potential cancer chemotherapeutic or chemopreventive agents. Munetone was previously isolated from the root bark of *M. suberosa* Benth in 0.3% yield as the principal crystalline product with significant piscicidal activity [14,15], but the potential of this highly substituted isoflavonoid to modulate ODC has not been described. In cell culture and mouse skin, we currently report the potential of munetone to function in this capacity.

2. Materials and methods

2.1. Chemicals

L-[1-^{14}C]Ornithine (56 mCi/mmol, 100 μCi/ml) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Pyridoxal phosphate, dithiothreitol, chloramine T, trichloroacetic acid and ornithine were purchased from Sigma Chemical Co. (St. Louis, MO). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was obtained from ChemSyn Laboratories (Lenexa, KS). Dialyzed fetal bovine serum (d-FBS) was purchased from Hyclone Laboratories, Inc. (Logan, UT). Minimal essential medium without Ca^{2+} and Mg^{2+} (S-MEM), non-essential amino acid solution (10 mM, 100×), trypsin–EDTA solution (1×) and antibiotic–antimycotic solution (PSF) were from GIBCO-BRL (Grand Island, NY).

2.2. Munetone

Munetone was isolated from the bark of *M. sericea*. Briefly, the bark (5 kg) was extracted with methanol (40 l), defatted with petroleum ether (b.p. 60–90°C; 500 ml × 3), suspended in 150 ml of H_2O, and partitioned with ethyl acetate (3 × 400 ml) to afford 83 g of an ethyl acetate residue. Further purification was performed by silica gel column chromatography using petroleum ether and increasing amounts of $CHCl_3$ (0 to 100%), and then $CHCl_3$ with an increasing amounts of methanol (5–100%) as eluents. Through repeated column chromatography of the subfractions, munetone (200 mg, 0.004% yield) (Fig. 1) was isolated with the following physical and chemical characteristics: colorless powder from methanol; m.p. 188°C (lit. 192–193°C [15]); UV λ

max (MeOH) (log ϵ) 229 (4.5), 263 (4.5), 328 (4.0), 337 (4.0) nm; IR νmax (neat) 2980, 1645, 1614, 1475, 1473, 1361, 1111 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.43 (6H, s, 2 × Me-3$''$), 1.48 (6H, s, 2 × Me-3$'''$), 3.59 (3H, s, OCH_3-2$'$), 5.64 (1H, d, J = 10.0 Hz, H-2$''$), 5.74 (1H, d, J = 10.0 Hz, H-2$'''$), 6.45 (1H, d, J = 10.0 Hz, H-1$''$), 6.61 (1H, d, J = 10.0 Hz, H-1$'''$), 6.64 (1H, d, J = 8.0 Hz, H-5$'$), 7.13 (1H, d, J = 8.0 Hz, H-6$'$), 7. 88 (1H, s, H-5), 7.91 (1H, s, H-2); ^{13}C-NMR ($CDCl_3$) δ 27.85 (q, CH_3-3$''$, CH_3-3$'''$), 28.37 (q, CH_3-3$''$, CH_3-3$'''$), 61.75 (q, OCH_3-2$'$), 75.86 (s, C-3$''$), 77.76 (s, C-3$'''$), 103.92 (d, C-8), 112.28 (d, C-5$'$), 114.58 (s, C-3$'$), 117.04 (s, C-10), 117.12 (d, C-1$''$), 118.54 (s, C-3), 119.66 (s, C-6), 121.24 (d, C-1$'''$), 121.47 (s, C-1$'$), 123.45 (d, C-5), 130.15 (d, C-2$''$), 131.61 (d, C-2$'''$, C-6$'$), 153.74 (d, C-2), 154.06 (s, C-4$'$), 154.34 (s, C-2$'$), 157.44 (s, C-7), 157.87 (s, C-9), 175.82 (s, C-4); EIMS *m/z* (rel. int. %) 416 $[M]^+$ (19), 403 (4), 402 (27), 401 (100), 386 (2), 385 (8), 372 (2), 371 (8), 193 (22), 187 (6), 172 (8), 155 (5), 77 (7). Munetone is readily soluble in benzene, chloroform and ethyl acetate; fairly soluble in ether and acetone; sparingly soluble in methanol and ethanol; insoluble in petroleum ether [14].

2.3. Assessment of TPA-induced ornithine decarboxylase (ODC) activity with mouse epidermal ME 308 cells

Mouse epidermal 308 cells were cultured in S-MEM medium containing non-essential amino acids (1×), d-FBS (5%), Ca^{2+} (0.05 mM), and antibiotic–antimycotics (1×) at 37°C in a 5% CO_2 atmosphere. For determination of TPA-induced ODC activity, cells were distributed to 24-well plates at an initial density of 2×10^5 cells/ml/well. After an 18 h preincubation, test materials dissolved in DMSO were added in duplicate (5 µl, 0.5% final DMSO concentration) before the induction of ODC activity with TPA (200 nM). After an additional incubation period of 6 h, plates were washed twice with PBS and stored at (80°C until analyzed. ODC activity was assayed directly in the 24-well plates as described previously [8]. In brief, frozen cells were lysed by quickly thawing the bottom of the culture plates in a warm water bath (37°C, 2 min). A substrate and cofactor mixture (200 µl containing 2 µl of L-[1-^{14}C]ornithine (200 nCi, 56 mCi/mmol, 100 µCi/ml), 50 µl of sodium phosphate buffer (0.2 M, pH 7.2), 16 µl of EDTA (12.5 mM), 10 µl of dithiothreitol (50 mM), 4 µl of pyridoxal phosphate (5 mM in 10 mM NaOH) and 118 µl of unlabelled L-ornithine (78 µg/ml)) were added to each well. Released [^{14}C]CO_2 was captured by paper disks which were moistened with 30 µl of 1 N NaOH during incubation of plates at 37°C for 1 h while shaking. The amount of radioactivity captured on NaOH-impregnated filter disks was determined by scintillation counting using a Wallac 1450 MicroBeta® liquid scintillation counter. Protein was determined by the Bio-Rad method using bovine serum albumin as a standard. Results were calculated as nmol [^{14}C]CO_2/h per mg protein and expressed as a percentage in comparison with controls treated with DMSO or TPA.

2.4. Determination of c-Myc-induced ODC activity in cultured c-MycER cells

BALB/c c-MycER cells (a gift of A.J. Wagner and N. Hay, University of Chicago, IL) were routinely cultured in DMEM containing 100 units/ml penicillin G solution, 100 units/ml streptomycin sulfate, 250 ng/ml amphotericin B (GIBCO-BRL, Grand Island, NY) and 500 µg/ml G418 sulfate (Geneticin®, GIBCO-BRL), supplemented with 10% hormone-free fetal bovine serum at 37°C in a 5% CO_2 atmosphere. For the determination of ODC activity, cells were plated in 100-mm culture dishes in phenol red-free DMEM without G418 and grown to subconfluence. After 3 days of serum starvation (0.5% serum), test compounds (10 µl) were added in DMSO, in duplicate, simultaneously with 1 µM β-estradiol (in 10 µl DMSO, 0.2% final DMSO concentration). After an incubation time of 6 h, plates were washed three times with PBS (Dulbecco's phosphate-buffered saline, Ca^{2+}- and Mg^{2+}-free, pH 7.4) and stored at (85°C until analyzed. The assay was performed in 24-well plates as described previously with minor modifications [10]. Cells were thawed at 37°C for 2 min and scraped into 180 µl of a reaction mixture containing 50 µl 0.2 M sodium phosphate buffer, pH 7.5, 16 µl 12.5 mM EDTA, 10 µl 50 mM dithiothreitol, 4 µl 5 mM pyridoxal 5-phosphate and 100 µl H_2O. The reaction was initiated by adding 200 nCi L-[1-^{14}C]ornithine in 20 µl H_2O. After an incubation period of 2 h, the reaction was terminated by adding

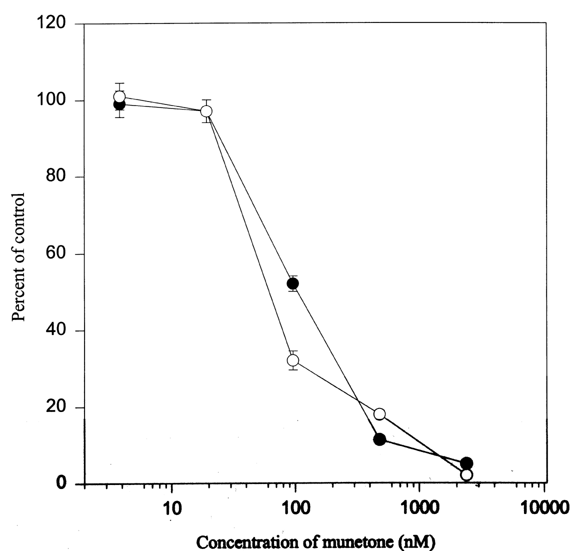

Fig. 2. Inhibition of TPA- and c-Myc-induced ODC activity with cultured cells. Mouse epidermal ME 308 cells (●) were simultaneously treated with TPA (200 nM) and the indicated concentrations of munetone for a period of 6 h. ODC activity was then determined as described in Section 2. ODC activity of TPA-treated solvent control was 1.50 ± 0.04 [^{14}C]CO_2 nmol/mg protein per h; without TPA treatment, ODC activity was 0.05 ± 0.004 [^{14}C]CO_2 nmol/mg protein per h. Data represent the average percent ± S.D. of the TPA-treated control group. Tests were performed in duplicate. Alternatively, cultured BALB/c c-MycER cells (○) were serum-starved for 3 days, and ODC expression was induced by the addition of 1 μM β-estradiol. The indicated concentrations of munetone were added simultaneously with β-estradiol, and the reaction was terminated after an incubation period of 6 h. ODC activity of munetone-treated cells was analyzed and expressed relative to a solvent control (0.1% DMSO, final concentration) as described in Section 2. ODC activity of β-estradiol-treated solvent control was 950.0 ± 50.0 [^{14}C]CO_2 pmol/mg protein/h; without β-estradiol treatment, ODC activity was 150.0 ± 20.0 [^{14}C]CO_2 pmol/mg protein/h. Data represent the average percent ± S.D. of the β-estradiol-treated control group. Tests were performed in duplicate.

50 μl 5.0 M trichloroacetic acid solution. The plates were incubated for an additional 1 h at 37°C. Liberated [^{14}C]CO_2 was measured by liquid scintillation counting. Precipitated protein was solubilized by addition of 5.7 M NaOH solution. The protein content was determined according to Bradford [16] using bovine serum albumin as a standard protein. The specific activity was calculated as pmol [^{14}C]CO_2/h per mg protein.

2.5. Assessment of TPA-induced ODC activity in mouse skin

Female CD-1 mice were obtained from Charles River Breeding Laboratories, Portage, MI, at 5–6 weeks of age, and held in quarantine for 1 week. Animals were randomized by weight into groups of four, and the hair on the dorsal surface was removed by shaving on the day of the experiment. Test compounds and TPA (ChemSyn Science Laboratories, Lenexa, KS) were dissolved in 0.2 ml of spectrograde acetone and applied to the shaved backs of the animals. After respective induction periods, mice were sacrificed by CO_2 narcosis followed by cervical dislocation. Skin samples were promptly removed, bisected, frozen in liquid N_2, and stored at (80°C until analyses were performed. For the determination of ODC activity [10], one half of each skin sample was homogenized for 15 s in 2 ml of ice-cold 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 mM pyridoxal phosphate (PLP) and 0.1 mM EDTA, and centrifuged at 15 000 rev./min for 30 min. In 24-well plates, 100 μl aliquots of the clear supernatants were mixed on ice with 115 μl of 40 mM Tris–HCl buffer, pH 7.5, containing 8 mM dithiothreitol, 0.64 mM pyridoxal phosphate and 0.8 mM EDTA. The reaction was initiated by the addition of 250 nCi L-[1-^{14}C]ornithine (56 mCi/mmol, 100 μCi/ml) in 1.94 mM L-ornithine (25 μl). After an incubation period of 1 h at 37°C, the reaction was stopped by addition of 50 μl of 5.7 N trichloroacetic acid solution and [^{14}C]CO_2 release was measured as described above. Protein content was determined using a Bio-Rad protein determination kit and used to calculate ODC specific activity (pmol [^{14}C]CO_2/mg protein per h). Bovine serum albumin was used as a standard protein.

2.6. Inhibition of DMBA-induced lesion formation in mouse mammary gland organ culture

Four-week-old BALB/c female mice (Charles River) were pretreated for 9 days with 1 μg estradiol and 1 mg progesterone. The thoracic pairs of mammary glands were dissected on silk and transferred to 60-mm culture dishes containing 5 ml of Waymouth 751/1 MB medium supplemented with 100 units of streptomycin and penicillin and 35 μg/ml glutamine. The glands were incubated in a 95% O_2

Table 1
Dose-dependent inhibition of munetone on TPA-induced ODC activity in mouse skin[a]

Treatment	TPA	Test sample dose (μmol/mouse)	ODC activity	
(pmol $^{14}CO_2$/mg per h ± SD)	% Inhibition[b]			
Acetone	–	0	28.5 ± 8.5	–
Acetone	+	0	402.3 ± 89.3	–
Munetone	+	0.04	347.5 ± 28.3	14.6
Munetone	+	0.2	320.0 ± 58.0	22.0
Munetone	+	1.0	236.4 ± 63.9[c]	44.4
Munetone	+	5.0	149.6 ± 48.8[c]	67.6

[a] Female CD-1 mice ($n = 5$) were topically treated with test sample (200 μl acetone) 2 h prior to the induction of ODC by application of TPA (10 nmol in 200 μl acetone). Mice were killed 5 h after TPA treatment, dorsal skin was removed and ODC activity was determined.

[b] After normalization for the untreated acetone control, percent reduction of ODC activity was calculated in comparison with the TPA-treated acetone control.

[c] Mean value ($P < 0.05$) statistically different from TPA-treated control (Dunnett's test with $n = 5$).

and 5% CO_2 atmosphere in the presence of 5 μg insulin, 5 μg prolactin, 1 μg aldosterone, and 1 μg hydrocortisone per ml of medium at 37°C for 10 days. During this growth-promoting phase, glands were exposed to 2 μg/ml DMBA between 72 and 96 h. After the exposure to DMBA, the glands were rinsed in medium and transferred to new dishes with fresh medium. Following 10 days of growth-promoting phase, the glands were permitted to regress by withdrawing all the hormones except insulin for an additional 14 days. The test compounds (10 μg/ml) were added to the media during the first 10 days of growth-promoting phase. At the end of the studies, glands were fixed in 10% buffered formalin, stained with alum carmine, and the incidence of mammary lesions (glands with unregressed areas) was recorded. The incidence of lesion formation (the percentage of glands with mammary lesions) in the compound-treated groups was established in relation to that of DMBA-treated control glands [17].

3. Results

3.1. Effect of munetone on TPA-induced ODC activity in cultured ME 308 cells

The potential of munetone to inhibit TPA-induced ODC activity was evaluated with cultured mouse epidermal ME 308 cells. Munetone showed potent inhibition of TPA-induced ODC activity with an IC_{50} value of 46 ng/ml (Fig. 2).

3.2. Effect of munetone on c-Myc-induced ODC activity in cultured c-MycER cells

In order to determine the effect of munetone on ODC activity induced by c-Myc, evaluations were performed using BALB/c c-MycER cells expressing a c-Myc-estrogen receptor chimeric protein. In this cell line, exogenous β-estradiol interacts with the estrogen receptor and activates c-Myc. This activation is followed by an induction of ODC mRNA and enzyme activity [18]. In addition to potent inhibition of TPA-induced ODC activity, dose-dependent inhibition of c-Myc-induced ODC activity was mediated by munetone with an IC_{50} value of approximately 30 ng/ml (Fig. 2).

3.3. Effect of munetone on TPA-induced ODC activity in mouse skin

As shown in Table 1, TPA (10 nmol) treatment enhanced ODC activity in the mouse skin. Munetone application (0.04–5.0 μmol) inhibited this induction in a dose-dependent manner.

3.4. Effect of munetone on DMBA-induced preneoplastic lesion formation in mouse mammary gland organ culture

The biological activity of munetone on DMBA-induced preneoplastic lesion formation in mouse mammary gland organ culture was investigated. The activity of chemopreventive agents in this system is known to demonstrate a good correlation with in vivo

animal models. As a result, using 15 glands per group, munetone showed 88% inhibition of DMBA-induced preneoplastic lesion formation. The incidence of mammary lesion formation was 1/15 (6.7%) for munetone-treated glands (10 μg/ml) as compared to 9/15 (60%) for glands exposed to DMBA in the absence of munetone, showing a significant difference between the groups ($P = 0.007$; χ^2 analysis using Yates correction). No toxicity was found at the test concentration.

4. Discussion

Cancer chemoprevention is one strategy for reducing cancer mortality. It involves the use of natural or synthetic substances or dietary components to inhibit, delay, or reverse the development of cancer in normal or preneoplastic tissue before malignancy [19,20]. A variety of natural product derived chemical constituents mediate cancer chemopreventive activity [11,21,22]. In our program designed for the procurement of novel plant-derived chemopreventive agents, inhibition of TPA-induced ODC activity in cultured mouse epidermal ME 308 cells has been used as a monitor for bioassay-guided fractionation.

Ornithine decarboxylase (ODC) catalyzes the decarboxylation of ornithine to form putrescine; it is highly inducible by growth-promoting stimuli such as growth factors, hormones, and tumor promoters [12,13]. Activity is controlled by a number of factors including the expression, stability and transcriptional rate of ODC mRNA, the stability and translational rate of ODC enzyme, and post-translational modifications [23]. Polyamines also play an essential role in normal cellular proliferation and differentiation, but are over-expressed in various transformed and tumor cells [24]. Since ODC is a key factor in regulating the cellular concentration of polyamines, it can be reasoned that agents capable of inhibiting ODC activity and blocking polyamine synthesis may be good candidates for chemoprevention [25,26]. Indeed, α-difluoromethylornithine (α-DFMO), an irreversible inhibitor of ODC, mediates chemopreventive activity [27]. Based on these considerations, in searching for new ODC inhibitors, we have demonstrated that rotenoids are potent inhibitors of TPA-induced ODC activity and effective chemopreventive agents [7–10]. Utilizing the same starting material (*M. sericea*), we now report the isolation and identification of an additional active principle, the highly substituted isoflavonoid munetone. This substance showed potent inhibition of TPA-induced ODC activity in cell culture. In addition, based on the finding that the proto-oncogene product c-Myc *trans*-activates transcription of the *ODC* gene [18,28], we assessed the potential of munetone to suppress TPA-independent c-Myc-mediated induction of ODC activity. Using cultured BALB/c c-MycER cells expressing a c-Myc estrogen receptor fusion protein (c-MycER), munetone showed strong inhibition of c-Myc-induced ODC activity. Based on these data, a study was conducted with CD-1 mice, and it was found that application of munetone to the skin significantly suppressed TPA-induced ODC activity in a dose-dependent manner. Similar to deguelin [8,10], data obtained with these model systems suggest munetone functions through suppression of TPA-induced ODC gene expression. Overall, it is reasonable to suggest that munetone may potentially serve as a chemopreventive agent. Synthetic schemes are currently under development that should provide sufficient quantities of material for full-term chemoprevention studies involving animal models.

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