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Development of TPA-induced Ornithine Decarboxylase (ODC) Inhibitors from Plants as Cancer Chemopreventive Agents

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Abstract – Chemical carcinogenesis is associated with the increase of intracellular polyamine levels, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse epidermal ODC activity are essential to skin tumor promotion by TPA. Therefore, for the discovery of new cancer chemopreventive agents, we have evaluated about 73 kinds of natural products to study inhibitory effects against ODC activity induced by TPA in T24 cell culture system. The total methanol extracts of plants fractionated into three layers (hexane, ethyl acetate and water layer) were tested and the hexane fraction of *Angelica gigas* (root bark, IC_{50}: 7.4 μg/ml) and the ethyl acetate fraction of *Corydalis ternata* (root, IC_{50}: 7.5 μg/ml) were the most effective on the inhibition of TPA-induced ODC activity. These active fractions are under investigation with further sequential fractionation using column chromatography.

Key words – Ornithine decarboxylase, cancer chemoprevention, TPA, T24 cell, *Angelica gigas, Corydalis ternata.*

Introduction

Ornithine decarboxylase (ODC) is the first enzyme in the polyamine biosynthesis pathway (Pegg and McCann, 1982). In mammalian cells, it provides the only route for synthesis of putrescine, which is then converted into the higher polyamines such as spermidine and spermine (McCann and Pegg, 1992). Polyamines play essential roles in the proliferation and development of mammalian cells and participate in the macromolecular synthesis (Pegg and McCann, 1982; Russell, 1985). ODC activity in quiescent cells is extremely low level and is increased within a few hours in response to many different stimuli, including growth factors, hormones, and tumor promoters (McCann and Pegg, 1992; Janne, *et al.*, 1978). It has been reported that ODC is elevated in human cutaneous epitheliomas compared with normal tissue (Scalabrino *et al.*, 1980), and the ODC activity of normal-appearing colonic mucosa from patients with familial polyposis was significantly higher than that of normal controls (Luk and Baylin, 1984). The degree of induction of ODC activity correlates with the potency of tumor promoters in the mouse skin model and with other organs (O'Brien, 1976; Boutwell and Verma, 1979). Recent studies have demonstrated that constitutive elevation of ODC occurs in both experimental models of carcinogenesis (Gilmour *et al.*, 1986; O'Brien *et al.*, 1987) and the human squamous carcinogenesis (Hietala *et al.*, 1988) during the skin progression. The pos-

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sible mechanism for this differential regulation of ODC in normal versus tumor tissue is the presence of altered form of ODC in chemically-transformed tissue which may escape normal cellular responses (Hietala *et al.*, 1988). Therefore, it was proposed that the elevation of excessive ODC levels may be used as a marker for the screening of inhibitors of tumor promotion, which can be specific inhibitors of ODC or indirectly reduce ODC induction (Pegg *et al.*, 1995). As an example, α-difluoromethl- yornithin (DFMO) is a specific enzyme-activated irreversible inhibitor of ODC and it has been widely studied for DFMO to inhibit variety of transplantable and chemically induced tumors *in vivo* (Rozhin *et al.*, 1984; Kingsnorth *et al.*, 1983; Marx *et al.*, 1987). The incidence of colon tumors in rats (Rozhin *et al.*, 1984) and mice (Kingsnorth *et al.*, 1983) after intrarectal instillation of a carcinogen is significantly reduced when DFMO is administrated. Therefore, as a strategy for the development of useful agents for cancer treatment or prevention, we evaluated natural products to find ODC inhibi- tors with higher potency and efficacy than DFMO, which has been by far the best known and most widely studied ODC inhibitor (Pegg *et al.*, 1995; Creaven *et al.*, 1993; Pendyala *et al.*, 1993).

Experimental

General chemicals – L-[1-^{14}C]ornithine (47.4 mCi/mmol, 100 μCi/ml) was purchased from Dupont company, USA. Pyridoxal phosphate (PLP), dithiothreitol (DTT) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chem. Co., USA. Scintillation cocktail (Polysafe) was purchased from Wallac Oy, Finland.

Plant materials – Medicinal plants were purchased from herb markets in Seoul, and voucher specimens have been deposited at Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Each of the dried herbs was sliced, and extracted 3 times with 100% methanol at room temperature. The methanol extracts were concentrated under reduced pressure below 40 ℃, and then the concentrated methanol extracts were partitioned into n-hexane, ethyl acetate, and water fractions.

Cell line and cell culture – T24 cells, a transitional epithelial cell line derived from a human bladder carcinoma, were purchased from American Type Culture Collection. They were maintained in McCoy's 5a medium (Sigma Chem. Co. USA) supplemented with 10% fetal bovine serum (Gibco BRL, USA) at 37 ℃ in a humidified atmosphere (5% CO_2).

ODC assay – The enzyme assay is based on the previous method with some modification (Gerhauser, *et al.*, 1995). Briefly, confluent cells were washed 3 times with Ca^{2+}, Mg^{2+}-free Dulbecco's phosphate buffered saline (PBS, pH 7.4), and then digested with trypsin-EDTA for 20 min. The cells treated with trypsin-EDTA were plated at a density of 1×10^5 cells/ml/well in 24-well tissue culture plates and test samples were added in 24-well plates. After 18 hr incubation at 37°C in a humidified atmosphere (5% CO_2), 20 μl of TPA (final 200 nM) were added. After an additional 6 hr incubation, the cells were washed 3 times with PBS and placed in a freezer (-80 ℃) until the ODC assay was performed, usually within 3 days.

ODC activity was measured by the release of [^{14}C]CO_2 from L-[1-^{14}C]ornithine. The cells were taken through two cycles of thawing and freezing by briefly warming (37 ℃) the bottoms of the culture plates in water bath (2 min) and then placing them in a freezer (-80 ℃) for 30 min. The enzyme reaction was started by adding a substrate and cofactor mixture [0.5 μl of undiluted L-[1-^{14}C]ornithine (47.7 mCi/mmol, 0.05 μCi/ml) in reaction mixture containing 15 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 2.5 mM dithiothrei-

tol, 0.04 mM pyridoxal phosphate, total volume 200 μl]. The plate was covered with a sheet of punched parafilm and placed over each hole with a piece of filter paper (Whatman P5) and then 30 μl of 1 N NaOH was dropped onto a filter paper. The plate was covered with another sheet of parafilm and shaken for 1 hr at 37℃. The reaction was stopped by the injection of 5 N trichloracetic acid (50 μl) into each well and the punched holes were sealed with tape to prevent the leakage of CO_2 gas. The plate was shaken for an additional 1 hr at 37℃. The filter papers were then transferred to micro centrifuge tubes and the radioactivy of samples were measured with liquid scintillation counter (1450 MicroBeta Plus, Pharmarcia, Ltd., USA).

Protein determination – Chloramine T (50 μl) was added to each well to destroy the dithiothreitol (30 min incubation at room temperature), followed by NaOH (50 μl, 5.7 N) to make the solution alkaline and to solubilize the protein (30 min incubation at room temperature). The protein concentration was then determined essentially by Lowry procedure (Lowry *et al.*, 1951) with bovine serum albumin as standard.

Results and Discussion

It has been reported that the increase of intracellular polyamine levels and ODC activity is associated with chemical carcinogenesis and TPA-induced mouse epidermal ODC activity with the resultant accumulation of putrescine is essential to skin tumor promotion by TPA (O'Brien, 1976). Therefore, this pathway has become a target for the development of agents capable of inhibiting carcinogenesis and tumor growth. It was reported that natural chemopreventive compounds, including retinoic acid and vitamin A (Verma, 1985), curcumin (Lu *et al*, 1993), in curry, and 18β-glycyrrhetinic acid derived from a constituent of licorice root have chemoprevetive activities. There is a report that fruit extracts (lowbush blueberry, cranberry, and lingonberry) of *Vaccinium* species inhibit ODC activity and these activities could be related to the degree of polymerization of proanthocyanidins which exhibit the potential to suppress the promotion stage of chemically-induced carcinogenesis (Bomster, 1996). According to the other report, rotenoids derived from African plant *Mundulea sericea* potently inhibited ODC activity induced by TPA and these compounds mediate potent cancer chemopreventive activities through transcriptional regulation of ODC in chemically induced mammary organ culture and two-stage mouse skin model systems. (Gerhauser *et al*, 1995). We have scre- ened about 73 kinds of natural products to find inhibitory principles against the induction of ODC by the tumor promoter such as TPA.

Table 1. The inhibitory effects of plant extracts on the TPA-induced ODC activity in T24 cells

Scientific name/Family	Plant parts	ODC activity (IC_{50}, μg/ml)		
		Hexane fraction	Ethyl acetate fraction	Water fraction
Aconitum kusnezoffii/Ranunculaceae	Rt	>20	>20	>20
Acorus gramineus/Araceae	Wp	>20	>20	>20
Akebia quinata/Lardizabalaceae	Tu	12.5	15.9	>20
Albizzia julibrissin/Leguminosae	Bk	>20	>20	18.9
Amomum cardamomum/Zingiberaceae	Fr	>20	>20	>20
Angelica dahurica/Umbelliferae	Rt	>20	>20	>20
Angelica gigas/Umbelliferae	Rt	7.4	16.9	>20
Angelica tenuissima/Umbelliferae	Rt	>20	>20	>20
Aralia continentalis/Araliaceae	Rt	9.9	>20	>20

Table 1. Continued.

Scientific name/Family	Plant parts	ODC activity (IC_{50}, μg/ml)		
		Hexane fraction	Ethyl acetate fraction	Water fraction
Asparagus cochinchinensis / *Liliaceae*	Bk	>20	>20	>20
Aster tatricus/Compositae	Rt	>20	>20	>20
Benicasa cerifera/Cucurbitaeae	Fr	>20	>20	>20
Boswellia carteriz/Bruseraceae	Rr	>20	>20	>20
Bulpleurum falcatum/Umbelliferae	Rt	>20	>20	>20
Campanula takesimana/Campanulaceae	Wp	19.5	>20	>20
Chaenomeles sinensis/Rosaceae	Fr	>20	>20	>20
Citrus unshiu/Rutaceae	Fb	>20	>20	>20
Cnidium officinale/Umbelliferae	Rt	14.9	>20	>20
Codonopsis pilosula/Campanulaceae	Rt	>20	>20	>20
Commelina communis/Commelinaceae	Wp	9.7	>20	>20
Cornus officinalis/Cornaceae	Fr	>20	>20	>20
Corydalis ternata/Papaveraceae	Rt	>20	7.5	13.0
Crataegus pinnatifida/Rosaceae	Fr	>20	>20	>20
Cuscuta australia/Convolvulaceae	Sd	>20	>20	>20
Cynanchum wilfordi/Asclepiadaceae	Wp	>20	>20	>20
Cyperus rotundns/Cyperaceae	Rt	>20	16.6	>20
Dioscorea batata/Dioscoreaceae	Rb	>20	>20	>20
Epimedium koreanum/Berberidaceae	Lf, St	>20	>20	>20
Equisetum hyemale/Equisetaceae	Wp	>20	>20	>20
Foeniculum vulgare/Umbelliferae	Fr	>20	>20	>20
Fritillaria verticillata/Liliaceae	Wp	>20	>20	>20
Gardenia jasminoides/Rubiaceae	Fr	>20	>20	>20
Gastrodia elata/Orchidaceae	Rt	>20	>20	>20
Gentiana scabra/Loganiaceae	Rt	>20	>20	>20
Gleditschia japonica/Leguminosae	Fr	>20	>20	>20
Hydnocarpus anthelmintica/Flacourtiaceae	Sd	>20	>20	>20
Kalopanax pictum/Araliaceae	Bk	>20	>20	>20
Lemna paucicostata/Lemnaceae	Wp	>20	>20	>20
Ligusticum delavayi/Umbelliferae	Rt	>20	>20	>20
Lilium pumilum/Liliaceae	Rb	>20	>20	>20
Lonicera contusa/Caprifoliaceae	Fl	>20	>20	>20
Lonicera japonica/Caprifoliaceae	Fl	>20	>20	>20
Lycium chinense/Solanaceae	Fr	>20	>20	>20
Malva verticillata/Malvaceae	Sd	>20	>20	>20
Morus alba/Moraceae	Rt	>20	17.5	>20
Nelumbo nucifera/Nymphaeaceae	Fr	>20	>20	>20
Paeonia moutan/Ranunculaceae	Bk	>20	17.0	>20
Paeonia obovata/Ranunculaceae	Rt	>20	>20	>20
Panax ginseng/Araliaceae	Rt	9.7	>20	>20
Perilla sikokiana/Labiatae	Sd	>20	13.6	>20
Peucedanum japonicum/Umbelliferae	Rt	>20	>20	>20
Phlomis umbrosa/Labiatae	Rt	>20	>20	>20
Phytolacca esculenta/Phytolaccaceae	Rt	>20	>20	>20
Platycodon grandiflorum/Campanulaceae	Rt	>20	>20	>20
Pleuropterus cilinervis/Polygonaceae	Rt	>20	>20	>20
Poria cocos/Polyporaceae	Rb	9.6	>20	>20

Table 1. Continued.

Scientific name/Family	Plant parts	ODC activity (IC_{50}, μg/ml)		
		Hexane fraction	Ethyl acetate fraction	Water fraction
Poncirus trifoliata/Rutaceae	Fb	9.4	>20	>20
Prunus armeniaca/Hamamelidaceal	Sd	>20	>20	>20
Pterocarpus santalinus/Leguminosae	St	19.8	>20	>20
Pulsatilla chinensis/Ranunculaceae	Rt	>20	9.9	>20
Rehmania glutinosa/Scrophulariaceae	Rt	>20	>20	>20
Rubus coreanus/Hamamelidaceae	Fr	14.4	>20	>20
Rubus crataegifolius/Rosaceae	Rb, St	>20	19.5	>20
Sambucus williamsii/Caprifolicaeae	Ap	>20	>20	>20
Scrophularia buergeriana/Scrophulariaceae	Rt	>20	>20	>20
Siegesbeckia pubescens/Compositae	Wp	>20	>20	>20
Smilax china/Liliaceae	Rt	>20	>20	>20
Spiraea saicifolia/Rosaceae	Rb	19.5	>20	>20
Strychnos ignatii/Loganiaceae	Sd	>20	>20	>20
Typha orientalis/Typhaceae	Fl	>20	>20	>20
Xanthium strumarium/Compositae	Fr	13.9	>20	>20
Zanthoxylum piperitum/Rutaceae	Rb, St	16.4	9.7	>20
Zingiber officinale/Zingiberaceae	Rt	17.4	14.1	>20
α-Difluoromethylornithine (positive control)		2.9		

Abbreviations: aerial parts (Ap), bark (Bk), flower (Fl), fruits (Fr), fruit bark (Fb), leaf (Lf), root (Rt), root bark (Rb), ruber resin (Rr), seeds (Sd), stem (St), tuber (Tu), and whole plant (Wp). IC_{50} is the concentration of test sample required to inhibit TPA-induced ODC activity by 50%.

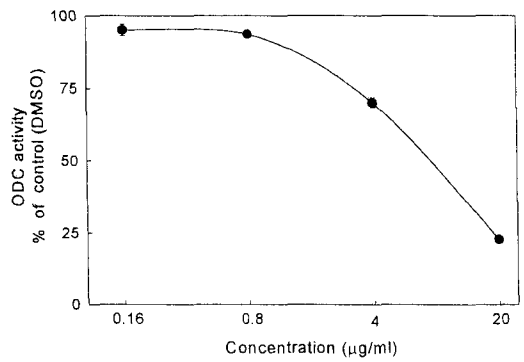

Fig. 1. Inhibition of TPA-induced ODC activity in T 24 cells by the hexane fraction of *Angelica gigas* (root). The results are expressed as the relative enzyme activities as compared to control and values are the means of two individual determinants; *bars*, SD.

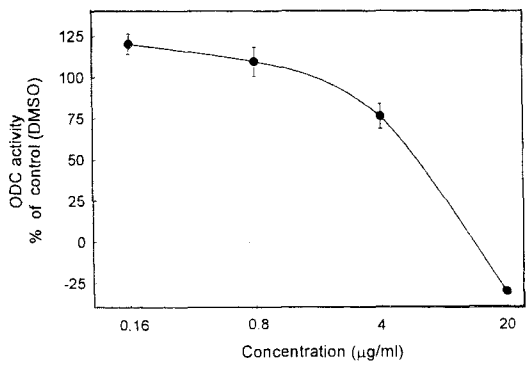

Fig. 2. Inhibition of TPA-induced ODC activity in T24 cells by the ethyl acetate fraction of *Corydalis ternate* (root). The results are expressed as the relative enzyme activities as compared to control and values are the means of two individual determinants; *bars*, SD.

The results were indicated in Table 1. The effective extracts against TPA-induced ODC activity were the hexane fractions of *Angelica gigas* (root, IC_{50}: 7.4 μg/ml), *Poncirus trifoliata* (fruit bark, IC_{50}: 9.4 μg/ml), *Poria cocos* (root bark, IC_{50}: 9.6 μg/ml), *Panax ginseng* (root bark, IC_{50}: 9.7 μg/ml), *Commelina communis* (whole plant, IC_{50}: 9.7 μg/ml), *Aralia continentalis* (root, IC_{50}: 9.9 μg/ml) and the ethyl acetate fractions of *Corydalis ternata* (root, IC_{50}: 7.5 μg/ml), *Zanthoxylum piperitum* (root bark and stem, IC_{50}: 9.7 μg/

ml), *Pulsatilla chinensis* (root bark, IC_{50}: 9.9 μg/ml). The hexane fraction of *Angelica gigas* and the ethyl acetate fraction of *Corydalis ternata* dose-dependently inhibited TPA-induced ODC activity, as shown in Fig. 1 and 2. α-Difluoromethylornithine (DFMO) which is known as an irreversible inhibitor of ODC was used as positive control and represented IC_{50} value of 2.9 μg/ml. Therefore, active plant extracts found in this assay system are under investigation with activity-guided fractionation methods to find active principles, which might be used as cancer chemopreventive agents with safety and potency.

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