Arch Pharm Res Vol 22, No 6, 559-564, 1999

Evaluation of the Potential of Cancer Chemopreventive Activity Mediated by Inhibition of 12-*O*-tetradecanoyl Phorbol 13-acetate-induced Ornithine Decarboxylase Activity

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(Received September 9,1999)

In order to discover new cancer chemopreventive agents from natural or synthetic products, a structurally diverse class of chemopreventive agents was evaluated using *in vitro* biomarker of inhibition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity in cultured mouse epidermal 308 (ME 308) cells. As a result, apigenin, benzylisothiocyanate, curcumin, diallyl disulfide, *N*-(4-hydroxyphenyl)retinamide (4-HPR), menadione, miconazole, nordihydroguaiaretic acid (NDGA) and phenethyl isothiocyanate showed potent inhibitory effects in this process. A chemically diverse group of compounds was included in the evaluation, such as flavonoids, retinoids, isothiocyanates, sulfur-containing compounds and phenolic antioxidant compounds. These data are suggestive to understand the cancer chemopreventive potential mediated by these substances.

Key words: Ornithine decarboxylase activity, Cancer chemopreventive agents.

INTRODUCTION

Cancer chemoprevention is considered as one of the most promising approaches to modulate cancer incidence and mortality. A variety of chemical entities (over 600) have been identified with potential chemopreventive properties from epidemiological surveys, experimental preclinical and clinical observations, and structural homology with known chemopreventive agents (Sharma *et al.*, 1994; Smith and Gupta, 1996). In a continuous searching for procurement of novel cancer chemopreventive agents, we have been evaluated the potential from natural and synthetic products using *in vitro* biomarker assay including inhibition of phorbol ester-induced ornithine decarboxylase activity in cell culture.

Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in polyamine bisynthesis and is responsible for converting L-ornithine to putrescine (Shantz *et al.*, 1996). In mammalian cells, ODC provides the only route for the production of putrescine which is then further converted by the action of other enzymes into the polyamines spermidine and spermine (Williams-Ashman and Canellakis, 1979). ODC activity and the resulting polyamines have been demonstrated to be essential for the process of cellular proliferation (Pegg, 1988; Metcalf *et al.*, 1978; Pohjanpelto *et al.*, 1985). In accordance with this essential role, ODC activity is greatly and rapidly induced in response to growth-promoting stimuli (Kahana and Nathans, 1984; Katz and Kahana, 1987; Tobias and Kahana, 1993). Many studies have suggested that overexpression of ODC plays an important role in tumor development. ODC activity is increased in cells that have been exposed to chemical carcinogens or tumor promoters (Pegg, 1988; Gilmour *et al.*, 1992), and tumor formation can be reduced on exposure to irreversible inhibitors of ODC, such as α-difluoromethylornithine (DFMO) (McCann and Pegg, 1992). Use of a number of animal models has shown that various types of cancers, e.g., skin, breast, colon, urinary bladder and intestinal, can be inhibited, dramatically in some cases, by DFMO (Thompson and Roman, 1986; Verma and Boutwell, 1987; Verma, 1990).

Accordingly, based on these considerations, in order to discover new cancer chemopreventive agents related to the modulation of tumor promotion stage, we have

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evaluated the potential of a diverse class of natural and synthetic compounds to inhibit phorbol ester-induced ODC activity with cultured mouse epidermal 308 cells.

MATERIALS AND METHODS

Chemical

L-[1-^{14}C]Ornithine (56 mCi/mmole, 100 μCi/ml) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Pyridoxal phosphate, dithiothreitol, chloramine T, trichloroacetic acid (TCA), ornithine, and NADH 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 (NAA) (10 mM, 100 ×), trypsin-EDTA solution (1 ×), antibiotic-antimycotic solution (PSF), minimal essential medium with Eagles salt (MEME) were from GIBCO-BRL (Grand Island, NY). Plant-derived compounds were primarily supplied from the phytochemistry component of the Program Project of the Program for Collaborative Research in the Pharmaceutical Sciences, Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, and used for biological tests. All other chemicals were purchased from commercial sources and were of the highest purity available.

Determination of TPA-induced ornithine decarboxylase (ODC) activity in cultured mouse 308 cells

The mouse epidermal 308 cells, originally established from Balb/c mouse skin initiated with DMBA (Strickland *et al.*, 1988), were obtained from Dr. Stuart H. Yuspa, National Institute of Health, Bethesda, MD and cultured in S-MEM medium containing non-essential amino acids (1 ×), dialyzed fetal bovine serum (5%), Ca^{2+} (0.05 mM) and antibiotic-antimycotic (penicillin-streptomycin-amphotericin B) (1%) at 37°C in a 5% CO_2 atmosphere. When the cultures were confluent, they were washed with Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS), and fresh medium was added. After an additional 24 hr, cells were treated with trypsin-EDTA (1 ×) for 20 min and plated at an initial density of 2×10^5 cells/ml/well in 24-well tissue culture plates. After an 18 hr incubation, test samples (5 μl, dissolved in DMSO) were added, in duplicate, simultaneously with 20 μl of TPA solution (final 200 nM). After an additional 6 hr incubation, the cells were washed twice with Ca^{2+}, Mg^{2+}-free PBS (pH 7.4) and frozen immediately by placing the plates in a freezer at -80°C until the assay for ODC activity was performed. ODC activity was assayed directly in 24-well plates by measuring the release of [^{14}C]CO_2 from L-[1-^{14}C]ornithine. Frozen cells were lysed by briefly 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, from Moravek), 50 μl of sodium phosphate buffer 0.2 M in phosphate, pH 7.2], 16 μl of EDTA (12.5 mM adjusted to pH 8.0 with NaOH), 10 μl of dithiothreitol (DTT, 50 mM in water), 4 μl of pyridoxal phosphate (PLP, 5 mM in 10 mM NaOH) and 118 μl of cold L-ornithine (78 μg/ml, final 365 μM)] were added to each well. The release of [^{14}C]CO_2 gas was captured by paper disks which were moistened with 30 μl of 1 N NaOH during incubation of plates at 37°C for 1 hr while shaking. The amount of radioactivity captured by the paper disks was then measured with scintillation cocktail. The protein concentration in each well was determined essentially by the Lowry procedure (Lowry *et al.*, 1951). The optical density was read at 660 nm in 96-well plates using BT2000 Microkinetic Reader (Fisher Biotech). Bovine serum albumin was used as a standard. The results of assay were calculated as nmol $^{14}CO_2$/mg protein/hr and expressed as a percentage in comparison with a control treated with DMSO and TPA. Dose-response curves were prepared and the results were expressed as IC_{50} values (Lee *et al.*, 1999).

RESULTS

Time-Course Study of TPA-Induced ODC Activity

In order to determine the optimal induction time for ODC activity, mouse epidermal cells were treated with TPA (200 nM) for various time periods. As shown in Fig 1, ODC activity increased for up to 6 h, and this was followed by a decrease in the induction of enzyme

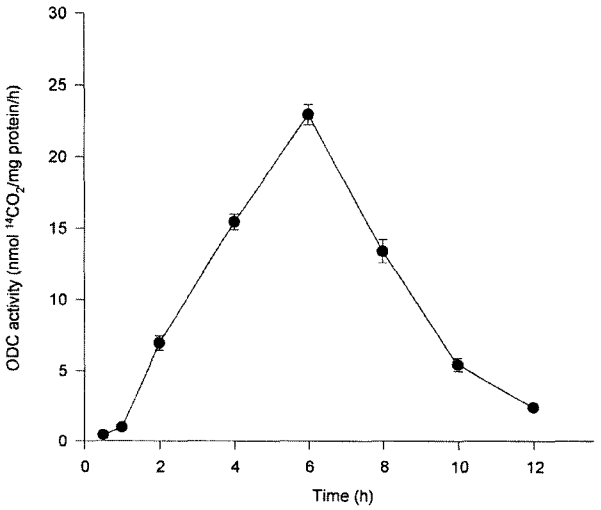

Fig. 1. Time-course of TPA-induced ODC activity in mouse 308 cells. The final concentration of TPA used was 200 nM.

Table I. Inhibition of TPA-induced ODC activity by cancer chemopreventive agents.[a]

Compound tested	$IC_{50}(\mu M)$[b]
Acetaminophen	> 50
N-Acetyl-L-cysteine	> 10
Allylmethyl disulfide	> 50
Apigenin	3.2
D-Arginine	> 50
Aspirin	> 50
BASF 47343 (4-HPR derivative)	4.3
BASF 48750	> 10
Benzene brassinin	> 50
Benzyl isothiocyanate (BITC)	4.0
Betulinic acid	> 10
Blumenol A	30.7
Brassinin	> 50
Brusatol	> 0.02
Calcium-D-glucarate	> 50
Capsaicin	> 10
Carbenoxolone	17.6
L-Carnosine	> 50
β-Carotenr	> 50
(+)-Catechin	>10
Chlorophyllin	> 10
Cineol	> 50
Coumestrol	> 50
Cryptoporic acid D	> 2[c]
Cryptoporic acid E	> 2[c]
Curcumin	4.0
Cyclobrassinin	> 50
Cysteamine	> 50
Daidzein	> 50
Dehydrocostus lactone	> 10
Dehydroepiandrosterone (DHEA)	> 10
Diallyl sulfide (DAS)	3.5
Dibromoacetophenone	> 50
Difluoromethyl ornithine (DFMO)	20
Ellagic acid	> 10
(-)-Epigallocatechin-3-O-gallate (EGCG)	> 50
Esculetin	> 50
Etoperidone.HCl	> 10
Ferulic acid	> 10
Fisetin	> 10
6,4-Dihydroxyflavone	> 50
Gallic acid	> 50
Genkwanin	> 50
18b-Glycyrrhetinic acid	> 50
6-Hydroxy-7,9-octadecadiynoic acid ethyl ether	> 50
N-(4-Hydroxyphenyl)retinamide (4-HPR)	5.0
Ibuprofen	> 50
Indole-3-carbinol	> 10
Indoline-brassinin	> 50
Indomethacin	> 50
Levamisole	> 50
D-Limonene	> 50
Linoleic acid	> 50
Luteolin	> 10
D-Mannitol	> 50
Meclofenamate, sodium	> 50
Melatonin	> 50
Menadione (Vitamin K_3)	5.0
DL-Methionine	> 10
p-Methoxyphenol	> 50
Methylene blue	> 50
Methylbrassinin	> 50
Miconazole	4.1
Morin	> 50
Morusin	6.7
Molybdate, sodium	> 50
Nicotinic acid (Vitamin B_3)	> 50
Nordihydroguaiaretic acid (NDGA)	6.3
N-[2-cyclohexyloxyl-4-nitrophenyl]-methanesulfonamide (NS-398)	> 50
Nonivamide (NVA)	> 10
9-Octadecanoic acid	> 50
Oxothiazolidone	> 50
Palmitoleic acid	> 50
[8]-Paradol	> 50
Phenylethyl isothiocyanate (PEITC)	6.1
Phytic acid	> 50
Piroxicam	> 50
Propylene glycol	> 50
Quercetin	> 10
trans-Resveratrol	> 10
13-*cis*-Retinoic acid	1.2
All-*trans*-Retinoic acid	2.5
Retinol	> 10
Retinol acetate	> 10
Rhodamine B	> 50
Selenite, sodium	> 50
Silymarin	> 50
β-Sitosterol	> 50
Spirobrassinin	> 50
Sulforaphane	6.8
Sulforaphane-brassinin side chain	> 10
Sulindac	> 50
Suramin	> 50
Tamoxifen	> 10
Tetracycline	> 50

Table I. (Continued)

Compound tested	IC_{50}(μM)
Thiolutin	>0.08
α-Tocopherol acetate	> 50
Ursolic acid	> 10
Vitamin D_3	> 50
Verapamil	12.0
Yadanziolide C	> 10

[a]Inhibition of TPA-induced ODC activity was determined in cultured ME 308 cells.
[b]Compounds were first screened at a concentration of 50 μM. IC_{50} values were then determined with active compounds by generating dose-response curves.
[c]Toxic effects at > 2 μg/ml.
Activity criteria: IC_{50} (μM) < 50.0

activity. Based on these results, a 6 h treatment period was used for the evaluation of test compounds.

Evaluation of the Potential of Cancer Chemopreventive Agents to Inhibit TPA-Induced ODC Activity

A diverse class of cancer chemopreventive agents was evaluated for potential to inhibit TPA-induced ODC activity. As summarized in Table I, apigenin, benzyl isothiocyanate, curcumin, diallyl disulfide, *N*-(4-hydroxyphenyl) retinamide (4-HPR), menadione, miconazole, nordihydroguaiaretic acid (NDGA) and phenethyl isothiocyanate showed potent inhibitory effects on TPA-induced ODC activity in cultured ME 308 cells. A chemically diverse group of compounds was included in the evaluation, such as flavonoids, retinoids, isothicyanates, sulfur-containing compounds and phenolic antioxidant compounds.

DISCUSSION

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines, by catalyzing the decarboxylation of ornithine to putrescine, and is highly inducible by growth promoting stimuli such as growth factors, hormones, and tumor promoters (McCann and Pegg, 1992; OBrien *et al.*, 1997; Peña *et al.*, 1993). ODC activity is controlled by a number of factors including the expression, stability and transcriptional rate of ODC mRNA, the stability and translation rate of the ODC enzyme, and post-translational modifications (van Daalen Waters *et al.*, 1989). Polyamines are also considered to play essential roles in normal cell proliferation and differentiation, but are over-expressed in various transformed and tumor cells (Auvinen *et al.*, 1992). Since ODC is the key component that regulates the cellular concentration of polyamines, it can be reasoned that agents that can inhibit ODC and block polyamine synthesis may be good candidates for chemoprevention (Kelloff *et al.*, 1994; Szarka *et al.*, 1994). Based on these considerations, we have been searching for new ODC inhibitors and evaluating their potential to serve as chemopreventive agents from approximately 90 compounds derived from natural and synthetic substances.

As a result, several compounds were shown to be active in this assay system (Table I). Retinoids are considered as potent chemopreventive agents with multiple mechanisms. Verma *et al.* showed that inhibition of retinoids on tumor formation in two-stage carcinogenesis is highly correlated with inhibitory effects on TPA-induced ODC activity (Verma *et al.*, 1977; Verma *et al.*, 1979). Several flavonoids including apigenin also showed inhibitory effects on phorbol ester-induced ODC activity in animal models (Nakadate *et al.*, 1984; Wei *et al.*, 1990). The isothiocyanate class of compounds induce detoxification enzymes (Phase II enzyme) and function as cancer chemopreventive agents. In this ODC-induction system, several compounds of isothiocyanate class such as benzyl isothiocyanate, phenylethyl isothiocyanate and sulforaphane also exhibited some degree of activity. Nordihydroguaiaretic acid (NDGA) has been reported as a lipoxygenase inhibitor and also an antitumor promoter in the DMBA-TPA two stage skin carcinogenesis and MNU-induced mammary carcinogenesis animal models (Nakadate *et al.*, 1982; McCormick and Spicer, 1987; Mehta and Moon, 1991). Inhibitory effects of NDGA on TPA-induced ODC activity could also be a mechanism contributing to the cancer chemopreventive activity of this agent. Curcumin is a promising cancer chemopreventive agents with antioxidant activity, inhibitory effects in the two-stage carcinogenesis mouse skin model, and modulatory effects on arachidonic acid metabolism (Srimal and Dhankan, 1973; Sharma, 1976; Huang *et al.*, 1988; Huang *et al.*, 1995; Nagabhushan and Bhide, 1992). Huang *et al.* also reported inhibition of TPA-induced ODC activity by curcumin in mouse (Huang *et al.*, 1992). Diallyl sulfide (DAS) [$(CH_2{=}CH\ CH_2)_2\ S$], a component of garlic oil, has been considered as a potential chemopreventive agent. DAS showed potent inhibitory activity toward rat microsomal nitro-soamine metabolism and CYP450 IIE1-mediated enzyme activity (Brady *et al.*, 1988). Further, DAS exhibited inhibitory activity on 1,2-dimethylhydrazine - induced colon and liver cancer in animal models (Wargovich and Goldberg, 1985; Hayes *et al.*, 1987; Wargovich, 1987). DAS-mediated inhibitory effects on TPA-induced ODC activity in cultured mouse cells might be an additional mechanism by which DAS serves as a chemopreventive agent. Thus, the inhibitory effects of test compounds in cell culture can be correlated with *in vivo* animal models and thus the TPA-induced ODC activity assay system appears to be a useful short-term biomarker assay for screening and evaluation of test agents.

Taken together, the results of this study hopefully will contribute to an understanding of the mechanism of cancer chemopreventive activity mediated by active substances in this process.

ACKNOWLEDGEMENTS

This work was supported in part by program project P01 CA48112 awarded by the National Cancer Institute, NIH, Bethesda, MD, U.S.A.

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