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Regulation of Ornithine Decarboxylase Induction by Deguelin, a Natural Product Cancer Chemopreventive Agent[1]

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ABSTRACT

Deguelin, a plant-derived rotenoid, mediates potent chemopreventive responses through transcriptional regulation of phorbol ester-induced ornithine decarboxylase (ODC) activity. To explore the mechanism of this effect, the activity of this compound was evaluated with a number of model systems. Using cultured mouse epidermal 308 cells, the steady-state levels of both 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ODC mRNA and c-*fos* were decreased by treatment with deguelin. ODC activity was also inhibited by bullatacin and various antimitotic agents (podophyllotoxin, vinblastine, and colchicine), but only deguelin and bullatacin were active as inhibitors of ODC levels in a TPA-independent c-Myc-mediated induction system using cultured BALB/c c-MycER cells. These results suggest that antimicrotubule effects, as mediated by rotenone, for example, are not responsible for inhibitory activity facilitated by deguelin. This was confirmed by use of an *in vitro* model of tubulin polymerization in which deguelin and a variety of other rotenoids were investigated and found to be inactive. As anticipated, however, NADH dehydrogenase was inhibited by these rotenoids. Moreover, inhibition of this enzyme correlated with a rapid depletion of ATP levels and potential to inhibit either TPA- or c-Myc-induced ODC activity. It therefore seems that deguelin-mediated interference with transient requirements for elevated energy can inhibit the induction of ODC activity and thereby yield a cancer chemopreventive response.

INTRODUCTION

ODC[4] (EC 4.1.1.17) catalyzes the decarboxylation of ornithine to yield putrescine, which is converted to the higher polyamines spermidine and spermine. Because this is the only pathway in mammalian cells to provide putrescine, ODC is the first and often rate-limiting enzyme in polyamine biosynthesis (1). The activity of ODC is highly regulated and can be induced by a variety of growth-promoting stimuli including growth factors, steroid hormones, cyclic AMP-elevating agents, and tumor promoters such as TPA (2). ODC has a rapid turnover with a half-life of about 20 min and is regulated at transcriptional, posttranscriptional, translational, and posttranslational levels. ODC activity and the resulting polyamines are essential for cellular proliferation, and induction of ODC activity has been associated with tumor promotion (3). In addition, overexpression of the human *ODC* gene was found to lead to enhanced papilloma formation in response to skin tumor promotion in transgenic mice (4, 5). On the basis of these characteristics, ODC is considered an attractive target for both cancer chemotherapy and chemoprevention (6).

Cancer chemoprevention involves the use of natural or synthetic compounds or dietary components to delay, inhibit, or reverse the development of cancer in normal or preneoplastic tissue (7). This strategy of cancer prevention is partially based on epidemiological observations that suggest a high intake of fruits and vegetables is linked to a reduced cancer risk (8, 9). In our program for the procurement of novel plant-derived chemopreventive agents (10), we have used inhibition of TPA-induced ODC activity in mouse 308 cell culture as one monitor for chemopreventive activity. This led to the identification of rotenoids as extremely potent inhibitors of TPA-induced ODC activity in cell culture and as a new class of potential cancer chemopreventive agents (11, 12). Rotenoid-mediated inhibition of TPA-induced ODC activity was regulated at the transcriptional level, and deguelin (Fig. 1), one of the most potent rotenoid inhibitors, decreased ODC mRNA steady-state levels in a time- and dose-dependent manner (12). In an initial assessment of chemopreventive activity, deguelin was found to inhibit DMBA-induced preneoplastic lesion formation in MMOC (12). In addition, deguelin significantly reduced the incidence and multiplicity of DMBA/TPA-induced papillomas in the two-stage mouse skin model and of *N*-methylnitrosourea-induced mammary tumors in rats (13, 14). In the mouse skin model, deguelin-mediated inhibition of tumorigenesis correlates with inhibition of TPA-induced ODC activity (14, 15). The mechanism has not yet been investigated in the rat mammary tumorigenesis model, but it is possible that ODC also plays a role in this process.

Because deguelin was able to inhibit TPA-induced ODC activity by decreasing ODC mRNA steady-state levels without affecting the activity of PKC (12), a key enzyme in signal transduction (16), it was speculated that the pathway between PKC activation and events leading to the transient up-regulation of immediate early (c-*jun* and c-*fos*) and early (c-*myc* and *ODC*) genes could be the target of rotenoid-mediated inhibition. Therefore, we were interested in determining whether deguelin-mediated effects were limited to ODC induction or whether deguelin could influence the expression or *trans*-activating effects of proto-oncogenes, including c-*fos* and c-*myc*, located further upstream in the signal transduction cascade (17–19). It was also recalled that rotenone (Fig. 1) and rotenoid-containing plant extracts (*e.g.*, *Derris*, *Lonchocarpus*, or *Tephrosia* species) have long been used as insecticides and fish poisons in South America and East Africa (20). These toxic effects have been attributed to inhibition of mitochondrial NADH dehydrogenase (EC 1.6.99.3), complex 1 of the respiratory chain (21), which results in depletion of intracellular ATP (22). In addition, rotenone inhibits microtubule assembly and can arrest cells in mitosis by binding directly to tubulin in a manner analogous to that of colchicine (23).

Thus, to gain further insight into the molecular mechanism of deguelin-mediated inhibition of ODC induction, we have investigated rotenoid-mediated effects on the cytoskeleton and microtubule assembly in relation to known antimitotic drugs, such as colchicine, vin-

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[4] The abbreviations used are: ODC, ornithine decarboxylase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMBA, dimethylbenz(*a*)anthracene; MMOC, mouse mammary organ culture; PKC, protein kinase C; SMP, submitochondrial particle.

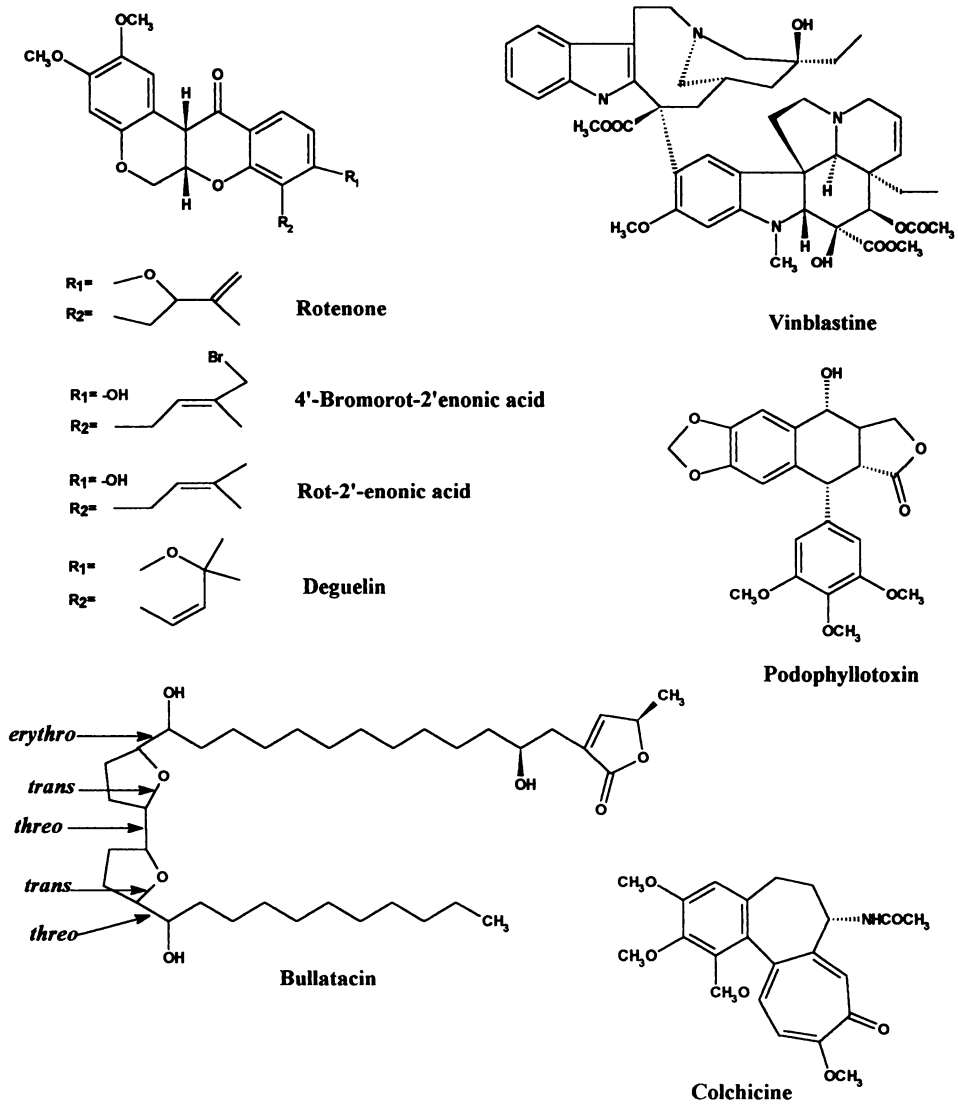

Fig. 1. Chemical structures of rotenoids (rotenone, deguelin, 4'-bromorot-2'-enonic acid, and rot-2'-enonic acid), antimitotic compounds (vinblastine, podophyllotoxin, and colchicine) and the NADH dehydrogenase inhibitor bullatacin.

blastine, and podophyllotoxin (Fig. 1). In addition, because the activation of signal transduction by mitogens results in a variety of immediate responses with a transiently elevated requirement for energy equivalents, it was reasoned that inhibition of NADH dehydrogenase could deplete ATP and thereby prevent ODC induction by TPA. ATP depletion could generally interfere with events initiated and induced by the application of TPA, such as the activation and translocation of PKC (24), or, more specifically, because ATP is required as a substrate for phosphorylation reactions, a decrease in intracellular ATP concentration could reduce signal transduction capacity through interference with protein phosphorylation. We currently report a good correlation between inhibition of ODC induction and inhibition of NADH dehydrogenase and the resulting depletion of ATP.

MATERIALS AND METHODS

Chemicals. Podophyllotoxin, vinblastine, colchicine, and TPA were purchased from Sigma Chemical Co. (St. Louis, MO). Rotenone, used as starting material for the synthesis of 4'-bromorot-2'-enonic acid, rot-2'-enonic acid, and deguelin (25), was obtained from Aldrich (Milwaukee, WI). Bullatacin, an annonaceous acetogenin (26), was a gift from J. L. McLaughlin (Purdue University, West Lafayette, IN). Structures of these test compounds are shown in Fig. 1. All cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY). L-[1-^{14}C]Ornithine (56 mCi/mmol, 100 μCi/ml) was purchased from Moravek Biochemicals, Inc. (Brea, CA). All materials, equipment, and biotinylated marker proteins for gel electrophoresis were from Bio-Rad (Hercules, CA). Cst-1 polyclonal antibody (797.3; a polyclonal antibody reactive with Src/Fyn/Yes) was a gift of S. A. Courtneidge (Sugen Inc. Research, Redwood City, CA).

TPA-induced c-*fos* mRNA Expression in Mouse 308 Cell Culture. Mouse 308 cell culture and the isolation and analysis of mRNA, using Northern blotting techniques to determine the influence of deguelin on TPA-induced c-*fos* mRNA steady-state levels, were performed as described previously (12, 27). In time course experiments, c-*fos* mRNA was induced by the addition of TPA (final concentration, 200 nM) at 180, 135, 90, 45, and 15 min, respectively, before the isolation of total RNA at time 0. Deguelin (0.04 μg/ml) and TPA were added simultaneously. The expression of c-*fos* mRNA was detected by hybridization with a cDNA probe for v-*fos* (1.0 kb; Oncor, Gaithersburg, MD) that was ^{32}P-labeled using the Prime-a-gene labeling system (Promega, Madison, WI). For dose-response analyses, deguelin was applied simultaneously with TPA (200 nM) in a concentration range of 0.0016–0.20 μg/ml. mRNA was isolated after an incubation period of 45 min and analyzed by slot-blot experiments [Bio-Rad slot-blot apparatus using Zeta Probe GT nylon membranes (Bio-rad)] according to the manufacturer's instructions. For quantitation of mRNA steady-state levels, densitometric scanning of autoradiographs was performed using the Ambis optical imaging system of Ambis 100 including Ambis Core Software 4.0 (Scanalytics, Billerica, MA). mRNA levels were expressed relative to the maximum control value.

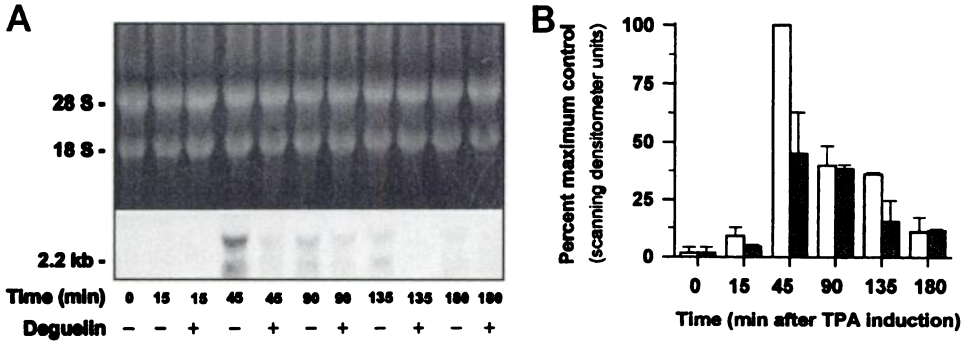

Fig. 2. *A*, Northern blot analysis of TPA-induced c-*fos* mRNA steady-state levels in mouse 308 cells after treatment with 0.04 μg/ml deguelin for the indicated time periods (20 μg of total RNA/lane). *Upper panel*, ethidium bromide-stained gel; *lower panel*, hybridization with a ^{32}P-labeled v-*fos* cDNA probe. Deguelin treatment is indicated by a +, whereas control cells (−) were treated with DMSO only. RNA isolation and fractionation, blotting, and hybridization conditions are described in "Materials and Methods." After washing, hybridized membranes were exposed to X-ray film for 5 days. *B*, densitometric scan of c-*fos* mRNA steady-state levels. Autoradiographs were scanned to quantify changes in c-*fos* mRNA levels in relation to the maximum control (100%). Results of two independent experiments were summarized and expressed as mean values ± SD. □, control cells; ▨, deguelin-treated cells.

Determination of TPA-induced ODC Activity in Cultured Mouse 308 Cells. Culture of mouse 308 cells, treatment of cells with inhibitors added in serial dilutions in DMSO (0.5% final DMSO concentration), and determination of ODC activity were performed as described previously (12). Protein content of cell lysates using BSA as a standard was measured according to Bradford (28) and used to calculate ODC specific activity (pmol of $^{14}CO_2$/mg of protein/h). Results were expressed as a percentage in comparison with a control sample treated with DMSO and TPA. IC_{50} values were generated from the results of at least five serial dilutions of inhibitors tested in duplicate.

Determination of c-Myc-induced ODC Activity. BALB/c c-MycER cells, a gift from A. J. Wagner and N. Hay (University of Chicago, Chicago, IL) were routinely cultured in DMEM containing 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B (Life Technologies, Inc.), and 500 μg/ml G418 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 were added in DMSO (10 μl), in duplicate, simultaneously with 1 μM β-estradiol (in 10 μl of DMSO; final DMSO concentration, 0.2%). After an incubation time of 6 h, plates were washed three times with PBS [Ca^{2+}- and Mg^{2+}-free (pH 7.4)] and stored at −85°C until tested.

The assay for ODC activity was performed in 24-well plates as described previously (12), with minor modifications. Cells were thawed at 37°C for 2 min and scraped into 180 μl of an incubation mixture containing 50 μl of 0.2 M sodium phosphate buffer (pH 7.5), 16 μl of 12.5 mM EDTA, 10 μl of 50 mM DTT, 4 μl of 5 mM pyridoxal 5-phosphate, and 100 μl of H_2O. The reaction was started by adding 200 nCi of L-[1-^{14}C]ornithine in 20 μl of H_2O. After an incubation period of 2 h, the reaction was terminated by adding 50 μl of 5.0 M aqueous trichloroacetic acid. The plates were incubated for an additional hour at 37°C, and liberated $^{14}CO_2$ was captured and estimated by liquid scintillation counting (12). Precipitated protein was solubilized by the addition of 5.7 M sodium hydroxide solution. The protein content was determined according to Bradford (28) using BSA as a standard protein. Specific activity was calculated as pmol of $^{14}CO_2$/mg of protein/h.

Determination of NADH Dehydrogenase Activity in Rat Liver SMPs. SMPs were prepared from frozen female Sprague Dawley rat liver (Pel-Freez, Rogers, AR) according to Adachi *et al.* (29), with minor modifications. Crushed frozen liver was homogenized in solution A [0.5 mM Tris-HCl buffer (pH 7.4), 250 mM sucrose, and 0.1 mM EDTA; 3 ml/g tissue]. The pH value of the homogenate was carefully maintained at 7.4 during homogenization. Centrifugation steps during purification were performed at 20,000 × *g* rather than at 7,000 × *g*. Activity was determined by measuring oxygen consumption using a Clark electrode. The reaction mixture (final volume, 600 μl) contained 50 μM potassium phosphate buffer (pH 7.4), 10 μl of 100 mM NADH, and 100–150 μg of SPMs in 50 μM potassium phosphate (pH 7.4) containing 50% glycerol. Oxygen consumption was monitored at 37°C for 5–10 min. Inhibitors (in 10 μl of DMSO) were tested at five serial dilutions in duplicate.

Determination of ATP Levels. Mouse 308 cells were cultured as described previously (12). For the determination of intracellular ATP, cells were plated in 100-mm culture dishes. When confluence was reached, cells were treated with serial dilutions of test compounds (in DMSO, 0.1% final concentration) for 1 h. Subsequently, cells were washed three times with PBS and precipitated with 0.5 ml of ice-cold 6% aqueous trichloroacetic acid. The precipitates were collected in microcentrifuge tubes, lysed by three repetitive freeze-thaw cycles, and centrifuged (5 min at 12,000 × *g*). ATP was measured spectrophotometrically based on the reactions described by Adams (30) using a diagnostics kit (Sigma Chemical Co.). A standard curve with ATP (disodium salt, Sigma Chemical Co.) in a concentration range of 0–100 μg/ml (0–181 μM) was used to calculate the ATP content of cell supernatants.

Inhibition of Tubulin Polymerization. For this assay, 1.0 mg/ml (10 μM) purified bovine brain tubulin was induced to assemble by the addition of 0.8 M glutamate + 0.4 mM GTP. Tubulin and varying drug concentrations were preincubated for 15 min at 30°C in the absence of GTP. The samples were placed on ice, GTP was added, and the samples were transferred to 0°C cuvettes (Pelletier-type temperature controller). Baselines were established in the spectrophotometer (350 nm), and the temperature was increased over about 60 s to 30°C. The drug IC_{50} value is defined as the interpolated concentration (from the experimental values) inhibiting the extent of assembly at 20 min by 50%. Compounds were dissolved in DMSO, and the solvent was present in the reaction mixtures at a final concentration of 4% (31).

Inhibition of DMBA-induced Lesion Formation in Mouse Mammary Gland Organ Culture. The identification of potential inhibitors of DMBA-induced preneoplastic lesion formation in mammary organ culture was described previously (32). Briefly, thoracic pairs of mammary glands were incubated with growth-promoting hormones in the presence of 10 μg/ml or serial dilutions of test compounds for 10 days. The glands were incubated for an additional 14 days with insulin-containing medium. DMBA was included in the medium for 24 h on the third day of culture to induce preneoplastic mammary lesions. Glands were fixed in 10% formalin, stained with alum carmine, and evaluated for the presence of mammary lesions. The incidence of lesion formation (the percentage of glands with mammary lesions of the total number of glands/group) in the compound-treated groups was compared with that of DMBA control glands, and the percentage of inhibition was calculated.

RESULTS

TPA-induced c-*fos* mRNA Expression in Mouse 308 Cell Culture. The inhibitory effect of deguelin on ODC mRNA steady-state levels has been reported previously (12). Currently, we investigated the effect of deguelin on TPA-induced c-*fos* mRNA steady-state levels. As illustrated in Fig. 2*A*, using Northern blotting techniques, deguelin also was found to reduce the steady-state levels of phorbol ester-induced c-*fos* mRNA in a time-dependent manner. Maximum induction of c-*fos* mRNA was observed 45 min after the application of TPA. This maximum response was reduced ~50% by simultaneous treatment with deguelin (Fig. 2*B*). Similar inhibitory effects on TPA-induced up-regulation of the c-*fos* transcript were detected when

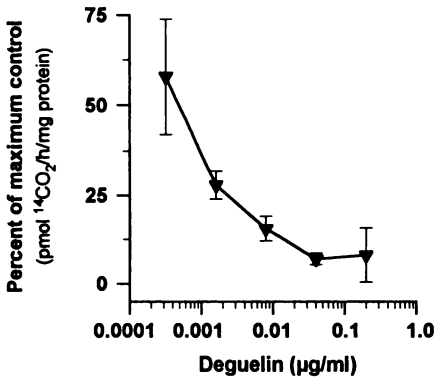

Fig. 3. Inhibition of c-Myc-induced ODC activity by deguelin. Cultured BALB/c c-MycER cells were serum-starved for 3 days, and ODC expression was induced by the addition of 1 μM β-estradiol. Deguelin (▼) in a concentration range of 0.00032–0.2 μg/ml was added simultaneously with β-estradiol, and the reaction was terminated after an incubation period of 6 h. ODC activity of deguelin-treated cells was analyzed and expressed relative to a solvent control (0.1%, DMSO final concentration). Experimental details are described in "Materials and Methods." Control values were as follows: basal level, 24.3 ± 0.6 pmol of $^{14}CO_2$/mg of protein/h; after a 6-h induction with β-estradiol, 91.5 ± 0.2 pmol of $^{14}CO_2$/mg of protein/h.

deguelin was added 4.5 or 26 h before the application of TPA (data not shown). Also, steady-state levels of TPA-induced c-*fos* mRNA observed after 45 min of induction were inhibited in a dose-dependent manner by deguelin in a concentration range of 0.0016–0.2 μg/ml (data not shown). The IC_{50} value for deguelin was ~0.044 μg/ml.

Inhibition of c-Myc-induced ODC Activity. To determine the effect of deguelin on ODC activity induced by c-Myc, evaluations were performed using BALB/c 3T3 cells expressing a c-Myc-estrogen receptor chimeric protein (BALB/c c-MycER) that were kindly provided by N. Hay and A. J. Wagner. 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 (12), dose-dependent inhibition of c-Myc-induced ODC activity was mediated by deguelin with an IC_{50} value of 0.0005 μg/ml (Fig. 3; Table 1).

Inhibition of ODC Induction by Inhibitors of NADH Dehydrogenase and Antimitotic Agents. Rotenone is a known inhibitor of NADH dehydrogenase and tubulin polymerization (21, 23). To determine whether these properties might contribute to the inhibition of ODC induction, a series of compounds (Fig. 1) was tested for potential to inhibit phorbol ester-induced ODC activity in cultured mouse 308 cells. As summarized in Table 1, bullatacin, another inhibitor of NADH dehydrogenase (33), and the antimitotic compounds colchicine, vinblastine, and podophyllotoxin (34) were potent inhibitors of TPA-induced ODC activity. IC_{50} values were all below 250 ng/ml, as were those of deguelin, rotenone, and 4′-bromorot-2′-enoic acid. In the case of podophyllotoxin, TPA-mediated induction of ODC mRNA was decreased in a time- and dose-dependent manner.[5]

We also investigated the potential of these compounds to inhibit c-Myc-induced ODC activity with the BALB/c c-MycER cells. In addition to deguelin, bullatacin and 4′-bromorot-2′-enoic acid, an intermediate in the synthesis of deguelin (25), strongly inhibited c-Myc-mediated ODC induction, with IC_{50} values of 0.0017 and 0.0056 μg/ml, respectively. In contrast, rotenone and the classic antimitotic agents failed to inhibit c-Myc-mediated ODC induction (Table 1), as did rot-2′-enonic acid.

[5] C. Gerhäuser, E. J. Kennelly, H. H. S. Fong, A. D. Kinghorn, R. G. Mehta, R. C. Moon, and J. M. Pezzuto. Inhibition of phorbol ester-induced OCT activity and mRNA expression in cultured mouse 308 cells by podophyllotoxin and derivative isolated from *Amanoa oblongifolia* stembark, manuscript in preparation.

When further tested for potential to inhibit DMBA-induced lesion formation in the MMOC model of carcinogenesis, podophyllotoxin and colchicine completely blocked preneoplastic lesion formation at a concentration of 10 μg/ml, whereas vinblastine was inactive (0% inhibition) at this concentration. As summarized in Table 2, podophyllotoxin-mediated effects were dose dependent in a concentration range of 0.01–10 μM (0.0041–4.1 μg/ml), with an IC_{50} value of about 0.5 μM.

Potential of Test Agents to Inhibit Tubulin Polymerization. Under the reaction conditions used (see "Materials and Methods"), IC_{50} values for inhibition of tubulin polymerization were 1.7 μM for colchicine and 1.6 μM for podophyllotoxin (Table 1; Ref. 31). Rotenone and (12*S*)-hydroxyrotenone inhibited tubulin polymerization with IC_{50} values in the 2–3 μM range (data not shown). 4′-Bromorotenonic acid had modest inhibitory activity, with an IC_{50} value of about 25 μM. Deguelin and 11 other rotenoids examined [tephrosin; isorotenone; (±)-6a,12a-dehydrorotenone; 12,12a-dehydrorotenone; rot-2′-enonic acid; 4′-methoxyrotenonic acid; dihydro-12-deketorotenone; dihydrodeguelin; 2′-phenylseleno-dihydrodeguelin; (7*S*)-hydroxydeguelin; and 7a,13a-dehydrodeguelin] had negligible activity, with IC_{50} values of >40 μM (the highest concentration examined).

Inhibition of NADH Dehydrogenase. Studies were performed to determine whether differences in the inhibitory potencies of rotenone derivatives were due to differential effects on the mitochondrial

Table 1 *Inhibition of ATP formation, TPA-induced ODC activity in cultured mouse 308 cells, and c-Myc-induced ODC activity in cultured BALB/c MycER cells mediated by rotenoids, NADH inhibitors, and antimitotic agents*

Compound	TPA-induced ODC activity[a,b]	C-Myc-induced ODC activity[a,c]	ATP formation[a]	Tubulin polymerization[a]
Deguelin	0.010[d]	0.0005	0.0023	>15.9
Rotenone	0.005	<0.2[e]	0.0030	0.8
4′-Bromorot-2′-enonic acid	0.014	0.0056	0.0052	11.9
Rot-2′-enonic acid	>1.0	>0.2	0.94	>15.9
Bullatacin	0.00012	0.0017	<0.0016	ND[f]
Colchicine	0.235	>0.2[g]	>0.2[h]	0.68
Podophyllotoxin	0.026	>0.2[g]	>0.2[h]	0.66
Vinblastine	0.015	>0.2[g]	>0.2[h]	1.4

[a] Results are expressed as IC_{50} values in μg/ml.
[b] These data were derived from separate experiments. In the absence of test substances, the following control values were obtained: 216 ± 126 (−TPA) and 3876 ± 750 pmol of $^{14}CO_2$/mg of protein/h (+TPA) (n = 6). In each experiment, activity was increased >10-fold on treatment with TPA.
[c] These data were derived from separate experiments. In the absence of test substances, the following control values were obtained: 18.6 ± 8.6 (−β-estradiol) and 99.1 ± 45.5 pmol $^{14}CO_2$/mg of protein/h (+ β-estradiol); (n = 5). In each case, activity was increased >5-fold on treatment with β-estradiol. In addition to the compounds listed in the table, the following rotenoids were found to be inactive at a test concentration of 40 μM (~16 μg/ml): isorotenone; (±)-6a,12a-dehydrorotenone; 12,12a-dehydrorotenone; 4′-methoxyrot-2′-enonic acid; dihydro-12-deketorotenone; dihydrodeguelin; 2′-phenylseleno-dehydrodeguelin; (7*S*)-hydroxydeguelin; and 7a,13a-dehydrodeguelin.
[d] Mean value of IC_{50} obtained with various lots of synthetic deguelin. We have previously reported an IC_{50} value of 0.0003 μg/ml for plant-derived deguelin (12).
[e] IC_{50} was not determined; 77.5% inhibition was observed at a test concentration of 0.2 μg/ml.
[f] ND, not determined.
[g] ODC activity was elevated ~1.5-fold at a test concentration of 0.2 μg/ml.
[h] No effect on ATP levels at a test concentration of 0.2 μg/ml.

Table 2 *Effect of podophyllotoxin on the development of DMBA-induced preneoplastic lesion formation observed with MMOC*

Treatment group	Concentration (μM)	% Incidence	% Inhibition
DMBA control	None	66.7 (10/15)	ND[a]
Podophyllotoxin	100	20 (3/15)	70[b]
	10	26.7 (4/15)	60
	1	26.7 (4/15)	60
	0.1	46.7 (7/15)	30
	0.01	60 (9/15)	10

[a] ND, not determined.
[b] Signs of toxicity were visible.

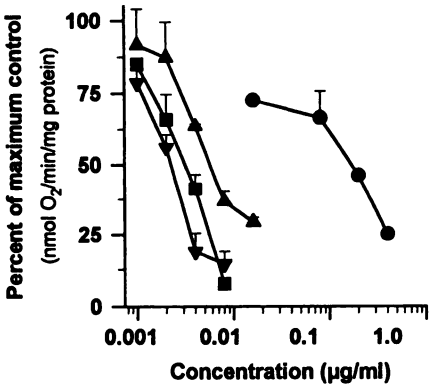

Fig. 4. Dose-dependent inhibition of mitochondrial respiration by deguelin (▼), rotenone (■), 4′-bromorot-2′-enonic acid (▲), and rot-2′-enonic acid (●). Rat liver SMPs were used as a source of NADH dehydrogenase. Oxygen consumption (mean ± SD of duplicate determinations in nmol of O_2/mg of protein/min) was monitored at 37°C for at least 5 min using a Clark electrode and compared to a solvent control value. Experimental details are described in "Materials and Methods."

respiratory chain; potential to inhibit NADH dehydrogenase activity was assessed using rat liver SMPs. As illustrated in Fig. 4, rotenone, deguelin, and 4′-bromorot-2′-enonic acid inhibited oxygen consumption in a dose-dependent manner, with IC_{50} values in the range of 0.002–0.006 μg/ml. Rot-2′-enonic acid was significantly less active.

Depletion of ATP in Cultured Mouse 308 Cells. NADH dehydrogenase and the respiratory chain contribute to intracellular ATP production. Therefore, studies were performed to determine whether the observed inhibition of NADH dehydrogenase would result in depletion of ATP. Mouse 308 cells were treated with inhibitors for 1 h. As summarized in Fig. 5 and Table 1, treatment with rotenone, deguelin, 4′-bromorot-2′-enonic acid, and bullatacin led to a rapid depletion of intracellular ATP levels with IC_{50} values <10 ng/ml. Rot-2′-enonic acid was significantly less active in this process, as were colchicine, podophyllotoxin, and vinblastine, with IC_{50} values of >0.2 μg/ml.

DISCUSSION

Deguelin, a plant-derived rotenoid, was identified as a promising cancer chemopreventive agent with significant activity in the two-stage mouse skin carcinogenesis model and in the *N*-methylnitrosourea-induced rat mammary tumor model (12, 14). It is reasonable to suggest that chemopreventive activity is mediated by transcriptional regulation of tumor promoter-induced ODC activity (12, 14, 15), at least in part, and the molecular mechanism of this process is of interest. As an initial approach, we asked whether deguelin-mediated transcriptional regulation was limited to ODC, or whether deguelin could affect the transcription of genes located further up-stream in the signal transduction cascade. Using Northern blotting techniques, deguelin was observed to reduce steady-state levels of the proto-oncogene c-*fos* in response to TPA stimulation in a time- and dose-dependent manner. c-Fos could contribute to intranuclear transcriptional control of ODC via interaction of the Fos/Jun (Ap1) complex with Ap1 binding sites of the ODC promoter (17). In addition to the Ap1 complex, the proto-oncogene product c-Myc *trans*-activates transcription of the *ODC* gene (18, 35), so we assessed the potential of deguelin to prevent TPA-independent c-Myc-mediated induction of ODC enzymatic activity. Using cultured BALB/c c-MycER cells expressing a c-Myc estrogen receptor fusion protein (c-MycER), we showed that activation of ODC activity was strongly inhibited by deguelin.

Effects of this type could be mediated by microtubule-dependent mechanisms. For example, induction of mouse epidermal ODC by TPA was inhibited by prior application of colchicine, and this effect was reversed by N^6-2′-*O*-dibutyryl 3′,5′-cyclic AMP (36, 37). It was therefore concluded that microtubule-containing colchicine-sensitive structures might be related to events of the signal transduction cascade initiated by application of a tumor promoter like TPA. Of relevance to the current investigation, it is well-established that rotenone can serve as an antimitotic agent with a mechanism of action similar to that of colchicine (38). Brinkley *et al.* (39) and Marshall and Himes (23) described in detail the inhibition of spindle microtubule assembly by rotenone. Consequently, we were interested in determining whether this property might contribute to a reduction in tumor promoter-induced ODC activity. Accordingly, the antimitotic agents podophyllotoxin, vinblastine, and colchicine, as well as rotenone itself, were tested for potential to inhibit TPA-induced ODC activity and found to be active.

We then tested this series of antimitotic compounds with the BALB/c c-MycER cell line to determine whether c-Myc and *trans*-activation of the ODC promoter could be regarded as potential targets. This notion is consistent with recent findings indicating that the amino-terminal domain of c-Myc associates with α-tubulin and microtubules (40). However, as summarized in Table 1, the antimitotic compounds, including rotenone, were not inhibitors of c-Myc-induced ODC activity, but they actually increased basal and induced ODC activity by ~1.5-fold at a test concentration of 0.2 μg/ml. Similar results were obtained by Rumsby and Puck (41) when the potential of colchicine to inhibit ODC activity induced by medium change after serum starvation was compared in normal fibroblasts and transformed HeLa cells. On the other hand, potent inhibition of ODC activity was observed when BALB/c c-MycER cells were treated with deguelin.

As a final assessment of the role of antimicrotubule effects in deguelin-mediated inhibition of ODC induction, a variety of rotenoids were evaluated as potential inhibitors of tubulin polymerization using an *in vitro* test system. Rotenone and 4′-bromorot-2′-enonic acid were active in this process, yielding IC_{50} values of about 2.5 μM (1.0 μg/ml) and 25 μM (11.9 μg/ml), respectively, but deguelin and a variety of other rotenoids were inactive, mediating (at most) minimal effects at a concentration of 40 μM (15.9 μg/ml). Because deguelin, in contrast to the antimitotic compounds, was equally active in inhibiting both TPA- and c-Myc-induced ODC activity, we conclude that inhibition of microtubule assembly does not contribute to the mechanism of inhibition of ODC induction.

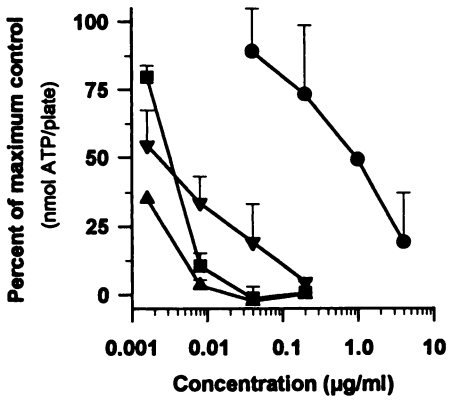

Fig. 5. Depletion of ATP levels in mouse 308 cells. Cultured mouse 308 cells were treated with four serial dilutions of bullatacin (▲), rotenone (■), deguelin (▼), and rot-2′-enonic acid (●) in a concentration range of 0.0016–4 μg/ml. ATP levels of compound-treated cells were analyzed and compared to a solvent control (0.1%, DMSO final concentration) to calculate the percentage of ATP in comparison to the solvent control. Experimental details are described in "Materials and Methods." ATP levels of the solvent control were in the range of 39 ± 3 to 52 ± 2 nmol of ATP/confluent plate in 10 independent experiments.

Another well-established activity of rotenoids is potential to serve as insecticides (20). This effect has been attributed to potent inhibition of mitochondrial NADH dehydrogenase (42). The respiratory chain NADH dehydrogenase, also known as complex I, links the electron transfer from NADH to ubiquinone, translocating protons across the mitochondrial inner membrane and leading to the production of about one-third of cellular ATP (33). In addition to rotenone, a variety of natural products have been identified as inhibitors of complex I. These include tannins and related compounds (43), annonaceous acetogenins (44), and antioxidants such as butylated hydroxyanisole (45) and nordihydroguaiaretic acid (46, 47). Interestingly, both butylated hydroxyanisole and nordihydroguaiaretic acid, besides serving as strong free radical scavengers, were reported previously to inhibit TPA-induced ODC activity and two-stage mouse skin tumorigenesis (48–50).

Our observations concerning rotenoid-mediated inhibition of NADH dehydrogenase and the depletion of ATP in mouse 308 cells could be anticipated on the basis of previous reports that have described the action of this compound class. Most importantly, however, rot-2′-enonic acid, which was significantly less active in affecting NADH dehydrogenase and ATP production relative to deguelin and rotenone, was more than 100-fold less active in inhibiting TPA-induced ODC activity. Moreover, bullatacin, a well-known inhibitor of NADH dehydrogenase, was identified as an extremely potent inhibitor of ODC induction by both TPA and c-Myc. Also, bullatacin was a potent inhibitor of cellular ATP formation. The positive correlation of these activities provides strong evidence that a reduction of energy equivalents by nontoxic concentrations of inhibitors is sufficient to block signal transduction triggered by phorbol esters. Ultimately, this may prove to be an important mechanism in preventing tumor promotion.

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