Rotenoid-mediated cancer chemopreventive activity

Sang Kook Lee, Ph.D.

Ewha Womans University

Cancer Chemoprevention

Use of chemicals or dietary compounds to block or inhibit the development of cancer in normal or preneoplastic tissue

Biomarker Assay

Inhibition of phorbol ester (TPA)-induced ornithine decarboxylase (ODC) activity

Rationale

- ODC enzyme activity and the resulting polyamines are essential for cellular proliferation of normal mammalian cells
- Overexpressed in various cancer cells
- Agents that inhibit polyamine synthesis may be good candidate
- DFMO (α-difluoromethylornithine) is under phase II clinical trials

Levels of ODC activity in tumors

Tissue	Enzyme specific activity (nmol CO_2/mg protein/30min)
Normal epidermis	0.020
Papilloma	0.549
Carcinoma	2.798

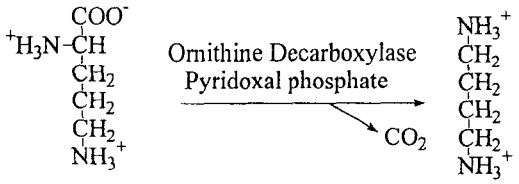
O'Brien, T.G. Cancer Res., 36, 2644, 1976

Ornithine Decarboxylase (ODC)

- the first and rate-limiting enzyme of polyamine biosynthesis
- catalyzes ornithine to putrescine, the precursor for the polyamines, which are necessary for cell growth and differentiation
- rapidly and transiently induction of ODC mRNA and activity following mitogenic stimulation; growth factors or tumor promoters
- ODC activity and polyamine levels are substantially elevated in transformed cells and tumors
- overexpression of ODC may be transgenic

Regulation of ODC enzyme activity

- The expression, stability and transcriptional rate of ODC mRNA
- The stability and translational rate of the ODC enzyme
- Post-translational modifications



The Basis of the Assay

- TPA can highly induce ODC activity
- Based on the inhibition of TPA-induced ODC activity by test materials
- Measuring the release of $[^{14}C]CO_2$ from L-[1-^{14}C]ornithine

Experiments

TPA-induced ODC activity in mouse epidermal cell culture

1. Cell culture

   Mouse epidermal cell (ME 308) was routinely cultured in S-MEM containining 5% d-FBS

2. Cell plating

   Initially 2 x 10^5 cells/ml/well in 24-well plate was plating and incubated for 18 h

3. Test compounds and TPA solution (200 nM) were added and incubated for further 6 h

4. Plates were washed with PBS (x 3) and frozen at -80°C

5. Determination of ODC enzyme activity by measuring the release of [^{14}C]CO_2 from L-[1-^{14}C]ornithine

   Substrate and cofactors: L-[1-^{14}C]ornithine, sodium phosphate buffer, EDTA, DTT, pyridoxal phosphate and cold L-ornithine

6. Reaction mixtures were added to each well

7. The release of $^{14}CO_2$ was captured by paper disk which was moistened with 1 N NaOH solution during incubation of plates at 37°C for 1 h.

8. The radioactivity was measured in paper disks

9. Protein content in each well was determined

Data analysis

- ODC activity expression: nmol $^{14}CO_2$/mg protein/h
- Relative percentage to the sample treated with TPA after subtracting the DMSO-treated group
- IC_{50} value is determined by dose-response curve

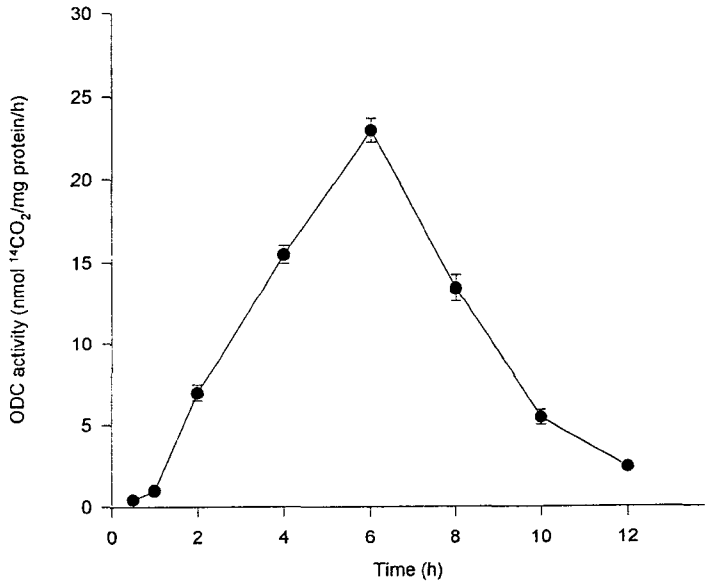


Fig. Time-course of TPA-induced ODC activity in mouse 308 cells.
The concentration of TPA used was 200 nM.

Screening of plant extracts

- ~ 1,000 plant extracts tested
- ~ 30 plant extracts: Active (IC_{50}: < 4.0 μg/ml)
- *Mundulea sericea* (Leguminosae): IC_{50}: 0.016 μg/ml

---Bioassay-guided fractionation---

Table. Plant extracts which showed potent TPA-induced ODC activities

Plant name, part used	Family	IC_{50} (μg/ml)	MMOC
Macleaya cordata (ST)	Papaveraceae	2.3	44.5
Mundulea sericea (QD)	Leguminosae	0.016	91.3
Tephrosia purpurea (AC)	Leguminosae	3.4	87.8
Zamia debilis (LF)	Cycadaceae	1.6	ND
Trichilia havanensis (RT)	Meliaceae	2.7	ND
Trichilia hirta (ST)	Meliaceae	1.5	ND
Agromuellera macrophylla (RM)	Euphorbiaceae	0.16	33.3
Tephrosia purpurea (FR)	Leguminosae	0.18	16.6
Zanthoxylum rhetsa (ST)	Rutaceae	1.0	20.0
Calophyllum inophyllum (LK)	Guttiferae	1.1	ND
Turpinia heterophylla (TW)	Staphyleaceae	1.2	0
Tephrosia purpurea (AC)	Leguminosae	1.2	16.0
Zanthoxylum fagara (LP)	Rutaceae	2.8	0
Aglaia ponapensis (LS)	Meliaceae	0.7	0
Thuja occidentalis (LP)	Cupressaceae	0.2	0
Maprounea africana (RT)	Euphorbiaceae	0.01	55
Euphorbia quinquiocostata (RM)	Euphorbiaceae	1.3	ND
Ostodes paniculata (LP)	Euphorbiaceae	0.5	50
Dirca occidentalis (LP)	Thymelaeaceae	0.1	0
Ostodes paniculata (QD)	Euphorbiaceae	0.9	50
Ostodes paniculata (LF)	Euphorbiaceae	0.5	50
Trichadenia zeylanica (SB)	Flacourtiaceae	2.2	12.5
Ostodes paniculata (FU)	Euphorbiaceae	0.4	25.0
Peddiea fischeri (RT)	Thymelaeaceae	2.2	71.0
Aquilaria malaccensis (SB)	Thymelaeaceae	0.9	0
Gnidia kraussiana (LE)	Thymelaeceae	0.07	14.0
Rhazya stricta (LF)	Apocynaceae	1.6	0
Mitragyna inermis (SB)	Rubiaceae	0.4	52
Aleurites fordii (SD)	Euphorbiaceae	0.3	14.0
Maprounea guianensis (SB)	Euphorbiaceae	0.6	83.0
Casimiroa edulis (SD)	Rutaceae	3.5	100

Mundulea sericea (Leguminosae)

- Widely distributed in Africa and parts of India
- Originally collected in Kenya in 1976
- No prior cancer chemopreventive studies
- Ethnomedically used for minor ailments

- Ethyl acetate extract of the bark of this plant: significantly inhibited TPA-induced ODC activity without affecting protein kinase C activity

- Dramatic inhibitory effect of mammary lesion formation in MMOC

Table. Inhibitory effects of TPA-induced ODC activity derived from *Mundulea sericea* (Leguminosae) extracts and compounds

Plant extract and compounds	ODC activity (IC_{50}: μg/ml)	MMOC (% inhibition)
Mundulea sericea	0.016	91.3
4-Hydroxylonchocarpin	0.74	
Munsericin*	1.02	
Lupinifolin	0.58	
Lupinifolinol	0.9	50
Mundulin	3.93	
Mundulinol	0.0033	8.7
Munetone	0.046	88
Mundulone	0.032	
Deguelin	0.0002	100
13α-Hydroxydeguelin*	0.0039	33.3
Tephrosin	0.0019	89
13α-Hydroxytephrosin*	0.021	55.5
Oleanolic alcohol	0.53	

Table. Biological activities of *Mundulea sericea* bark ethyl acetate extract and four rotenoids in comparison with retinoic acids

Test samples	TPA-induced ODC activity (IC_{50})	PKC binding assay (IC_{50})		PKC enzyme activity	HL-60 cell differentiation		Mouse mammary gland organ culture	
		PDBu	Staurosporine		ED50	% Viability	% Incidence	% Inhibition
Mundulea sericea extract	0.02	>10	>200	n.d	> 4	95	6.7 (1/15)	91.3
Deguelin	0.0007	>500	>500	no effect	>10	93	0 (0/15)	100.0
Tephrosin	0.005	>500	>500	no effect	>10	95	6.7 (1/15)	89.0
13α-OH tephrosin	0.05	>500	>500	n.d	>10	95	26.7 (4/15)	55.5
13α-OH deguelin	0.01	>500	>500	n.d	>10	95	40.0 (6/15)	33.3
13-*cis*-Retinoic acid	0.6	366	>500	no effect	0.8	90		
All-*trans*-Retinoic acid	2.8	223	n.d	no effect	0.6	96		

The Effect of Deguelin on TPA-induced ODC Activity

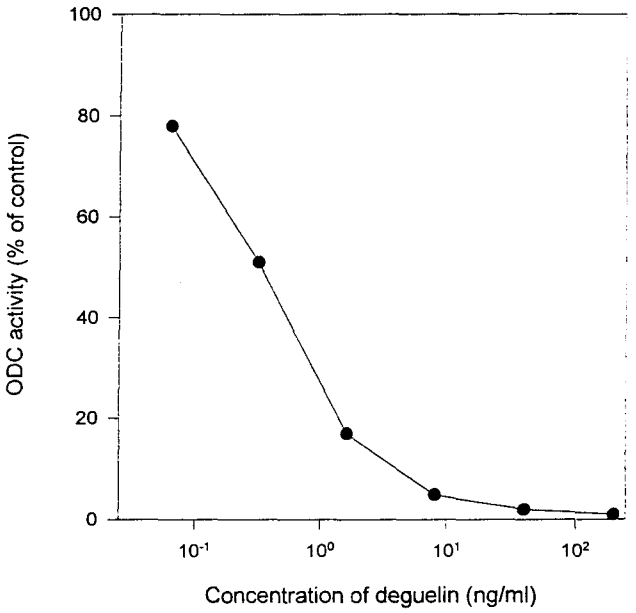


Fig. Dose-response inhibitory effect of deguelin on TPA-induced ODC activity in cultured ME 308 cells

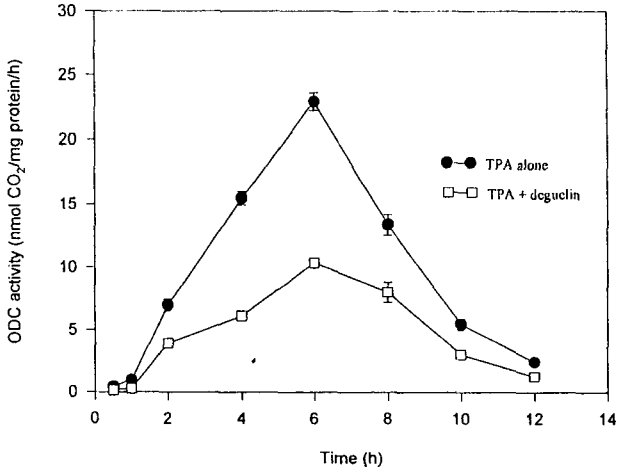


Fig. The effect of deguelin on TPA-induced ODC activity in time-courses.

TPA (200 nM) and deguelin (0.02 μg/ml) were used in this experiment.

Table. Influence of *Mundulea* extract on mouse skin tumorigenesis in female CD-1 mice

Group (20 mice)	Treatment	DMBA/TPA	Tumor Incidence	Tumor Multiplicity	Mean Body Weight	Survival (%)
1	Vehicle (Acetone)	+	95.0	29.8	31.1	100
2	*Mundulea* extract	+	15.0[a]	0.2	31.2	100
3	*Mundulea* extract	-	0	0	31.7	100
4	None	-	0	0	32.4	100

[a] $p < 0.01$ when compared to Group 1 via Fisher's Exact Test.

Photograph

Fig. The Effect of *Mundulea sericea* extract on the total number of observed tumors in female CD-1 mice receiving DMBA/TPA topically

Mechanism study

- Transcriptional regulation of ODC mRNA expression
- TPA-independent pathway in ODC activity:
  c-Myc-induced ODC activity in Balb/c c-MycER cells
- NADH dehydrogenase activity
- Change of energy balance: ATP depletion
- Modulation of protein phosphorylation
- Tubulin polymerization

Transcriptional Regulation of ODC mRNA expression

Inhibition of ODC mRNA expression:

Assay system: Northern blot analysis in cultured ME 308 cells

TPA (200 nM) induces ODC mRNA expression

The Effects of deguelin and rotenoids on ODC mRNA expression were determined

Results: Photograph 1

Fig. Northern blot hybridization of RNA samples from cultured ME 308 cells with ODC and β-tubulin probes.

Cells were treated with varying concentrations of deguelin and tephrosin and TPA as indicated for 6 h.

RNA samples were hybridized with ^{32}P-labelled ODC-specific oligonucleotide

Intensities were normalized for signals obtained after reprobing with a β -tubulin probe and are shown in comparison with the TPA-treated DMSO control

Photograph 2

Fig. Northern blot hybridization of RNA samples from cultured ME 308 cells treated with DMSO, deguelin or tephrosin and TPA for an indicated times with ODC and β-tubulin probes.

Photograph 3

Fig. Densitometric scan of autoradiographs of the gels shown in photograph 2 in comparison with the ODC enzymatic activity.

ODC mRNA expression (scanning densitometric units) and ODC enzyme activity are expressed as percent in comparison with the maximum control value (4 h and 6 h, respectively)

TPA-independent c-Myc-induced ODC activity

Assay System: Balb/c c-MycER cell culture

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Estradiol (E2) induces chimeric protein c-MycER

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c-MycER induces ODC gene expression and ODC enzyme activity

The Effect of deguelin on c-Myc-induced ODC activity

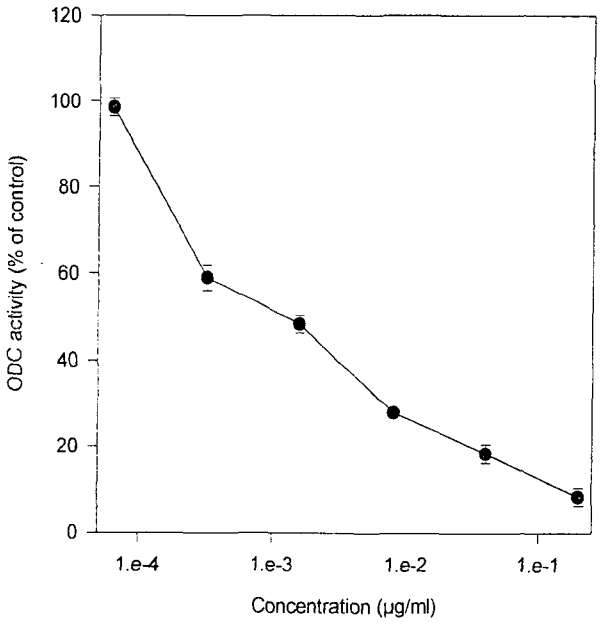
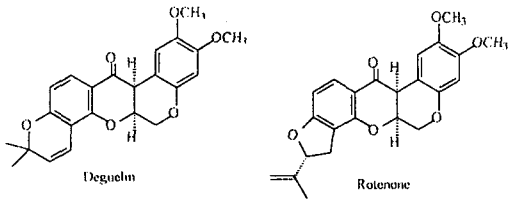


Fig. Dose-response inhibitory effect of deguelin on c-Myc-induced ODC activity in cultured Balb/c MycER cells.

Cells were incubated for 3 days under serum starvation (0.5%) and then ODC activity was induced by addition of E2 (1 μM) for 6 h. Deguelin was added simultaneously with E2 and then further incubated for 6 h.

NADH-dehydrogenase activity

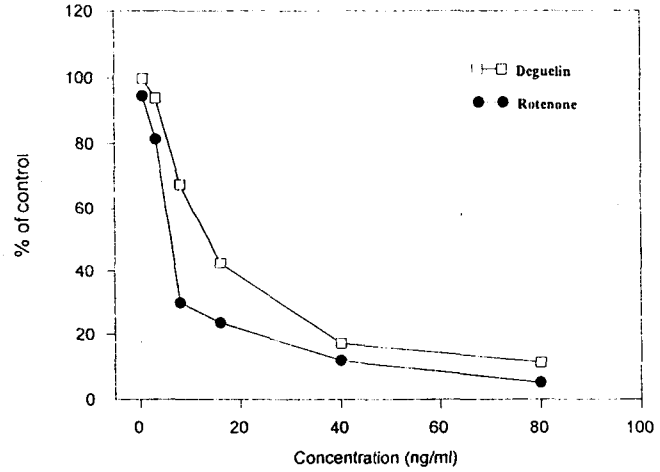


Assay system: Rat liver submitochondrial particles (SMP) as enzyme sources

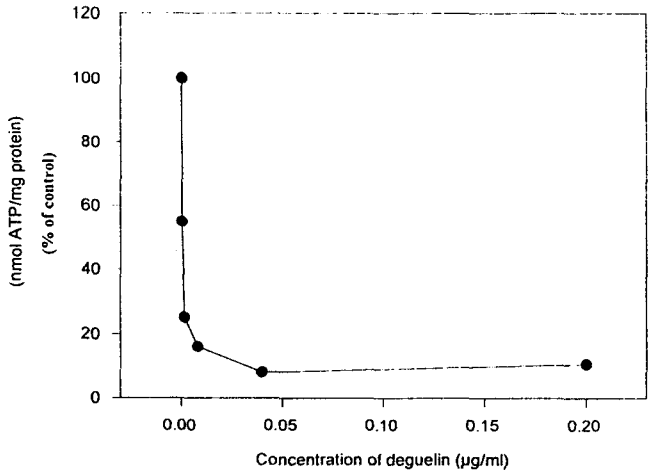
Measuring the absorbance change of NADH at 340 nm using spectrophotometer

Reaction mixture containing sodium phosphate buffer, 10 mM NADH and SMP (100 μg): O.D. change was monitored for 5 min.

The Inhibitory effects of deguelin and rotenone on NADH-dehydrogenase activity



Inhibitory effect of deguelin on ATP formation in ME 308 cells



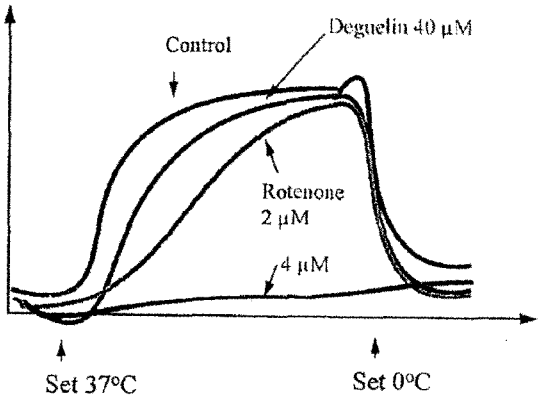
Modulation of Protein phosphorylation

Assay system: Western blot analysis of phosphorylated proteins in cultured ME 308 cells

TPA induces protein phosphorylation

The Effects of deguelin on TPA-induced protein phosphorylation were determined by western blot analysis

Assay for tubulin polymerization



Time-course study of TPA on induction of ODC activity

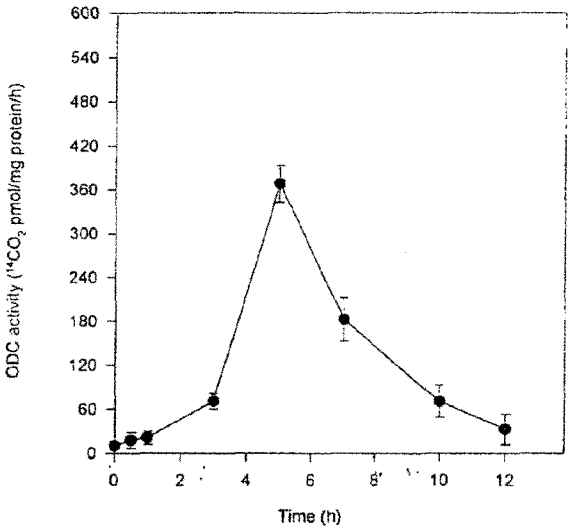


Fig Time-course study of TPA on induction of ODC activity in mouse skin model.

TPA (10 nmol) was applied to CD-1 mice skin for the indicated times and then skin was removed, bisected. One half of each skin was used for determination of ODC activity.

The Effect of deguelin on TPA-induced ODC activity in CD-1 mice skin

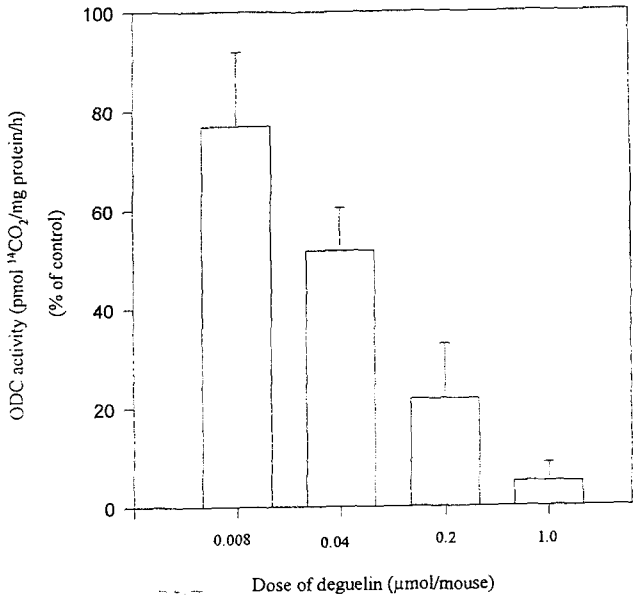


Fig. Dose-response effect of deguelin on TPA-induced ODC activity in CD-1 mice skin.

Test compounds (dissolved in 200 μl of acetone) were dosaged prior to 2 h applification of TPA solution (10 nmol in 200 μl acetone). After 5 h exposure, mouse skin was removed and bisected. One half of skin was analyzed for ODC activity. Four mice per group were used for this assay.

In vivo animal carcinogenesis model

1. Evaluation of deguelin on two-stage DMAB/TPA skin carcinogenesis model with CD-1 mice

2. Evaluation of deguelin on two-stage MNU mammary carcinogenesis model with Sparague-Dawley rats

Conclusion

Rotenoids :

- are promising antitumor or cancer chemopreventive agents
- inhibit TPA-induced ODC activity at the ODC mRNA transcriptional level
- inhibit c-Myc-induced ODC activity in the upstream regulation
- deplete ATP formation by inhibition of NADH dehydrogenase activity
- inhibit signal transduction
- inhibit potential tumor formation in animal models