Rotenoids mediate potent cancer chemopreventive activity through transcriptional regulation of ornithine decarboxylase

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For the discovery of new cancer chemopreventive agents, we have studied the potential of plant extracts to inhibit phorbol ester-induced ornithine decarboxylase (ODC) activity in cell culture. Four active rotenoids were obtained from the African plant *Mundulea sericea* (Leguminosae). These isolates were highly potent when evaluated for inhibition of chemically induced preneoplastic lesions in mammary organ culture and inhibition of papillomas in the two-stage mouse skin model, and they appear to function by a unique mechanism at the level of ODC messenger RNA expression. Based on our findings, rotenoids can be regarded as promising new chemopreventive or anticancer agents.

Cancer chemoprevention involves the use of chemicals or dietary compounds to block or inhibit the development of cancer in normal or preneoplastic tissue[1]. This may be accomplished by a variety of naturally occurring compounds acting by distinct mechanisms[2–5]. Antioxidants like the vitamins C or E act as scavengers of reactive oxygen free radicals. *Allium* species such as garlic or onions and cruciferous vegetables like broccoli and cabbage often contain inducers of drug-metabolizing and detoxifying enzymes. Some compounds including the vitamins A or D induce cell differentiation, a process of cell maturation that stimulates immortal cancer cells to revert to normal cells. Another group of chemopreventive substances, anti-tumour promoters, inhibit events that occur after normal cells that have been initiated by a carcinogen come in contact with tumour-promoting agents such as phorbol esters (for example, 12-*O*-tetradecanoylphorbol-13-acetate, TPA). Repeated contact with the tumour promoter would ultimately result in the development of cancer[6].

Accordingly, for the discovery of cancer chemopreventive agents, we have monitored the ability of plant extracts to inhibit a variety of *in vitro* biomarkers[7,8], including inhibition of phorbol ester-induced ornithine decarboxylase (ODC) activity in cell culture. ODC is a key enzyme in the biosynthesis of polyamines and is highly inducible by growth-promoting stimuli including growth factors, steroid hormones, cAMP-elevating agents and tumour promoters[9,10]. ODC activity is finely tuned by a number of factors including the expression, stability and transcription rate of ODC messenger RNA, the stability and translation rate of the ODC enzyme, and post–translational modifications[11]. Since both ODC enzyme activity and the resulting polyamines are essential for cellular proliferation of normal mammalian cells and, on the other hand, are overexpressed in various cancer cells[12], it can be deduced that agents that inhibit polyamine synthesis may be good candidates for use in cancer chemotherapy and chemoprevention. Indeed, as an example, α-difluoromethylornithine (DFMO, Ornidyl®), a highly effective substrate-analog suicide inhibitor of ODC, is currently under investigation as a cancer chemopreventive agent in phase II clinical trials[4,5].

Because of these considerations, our group has been actively searching for new inhibitors of ODC and evaluating their potential to serve as chemopreventive agents. The screening of approximately 350 plant extracts for inhibition of TPA-induced ODC activity led to the identification of rotenoids as a highly active group of ODC inhibitors[13]. In the present study, the effects of rotenoids on the expression of TPA-induced ODC messenger RNA (mRNA) expression were analysed in a dose- and time-dependent manner. Additionally, we evaluated rotenoids for chemopreventive activities, that is, inhibition of chemically induced preneoplastic lesions in mammary organ culture and inhibition of papillomas in the two-stage mouse skin tumorigenesis model. The present report clearly demonstrates that rotenoids are promising anti-tumour or chemopreventive agents with activities equivalent or superior to those of previously known chemopreventive compounds and that the inhibition of

phorbol ester-induced ODC activity at the level of transcription is responsible for this activity.

Rotenoids as inhibitors of TPA-induced ODC activity

As a part of our program for the discovery of natural cancer chemopreventive agents from plants, we have assessed the potential of approximately 350 plant extracts to inhibit TPA-induced ODC activity in cultured mouse 308 cells, and have identified 17 active leads. The most potent extract, with an IC_{50} value (half-maximal inhibitory concentration) of 0.02 μg ml^{-1}, was derived from the African plant *Mundulea sericea* (Leguminosae). Subsequently, bioassay-guided fractionation of this extract using the above-mentioned screening assay as a monitor to follow activity led to the isolation of a variety of highly active compounds including the rotenoids deguelin (1), tephrosin (2), (–)-13α-hydroxytephrosin (3) and (–)-13α-hydroxydeguelin (4) (ref. 13). These four compounds were identified as potent inhibitors of TPA-induced ODC activity with IC_{50} values ranging from 0.0007 to 0.05 μM (Table 1).

Inhibition of ODC mRNA expression

Studies to determine the mechanism of inhibition of TPA-induced ODC activity mediated by rotenoids included the analysis of TPA-induced ODC mRNA expression in mouse 308 cells treated with deguelin and tephrosin. Northern blot hybridization and slot-blot experiments revealed dose-dependent effects on the expression of ODC mRNA concomitantly with the inhibition of ODC enzyme activity (Fig. 1*a*, *b*). The effects of deguelin and tephrosin on the kinetics of the induction of ODC mRNA expression and ODC enzyme activity are shown (Fig. 2*a*, *b*). TPA-induced ODC mRNA expression was demonstrated to precede TPA–induced ODC activity. The treatment with deguelin or tephrosin (0.1 μM) reduced the mRNA expression to approximately 25% of the maximum response at 4 hours (TPA control). Over a period of 14 hours, the inhibition of ODC induction correlated with inhibition of the induction of ODC mRNA. A similar inhibitory profile of TPA-induced ODC activity and mRNA expression was reported for retinoic acid[14]. However, when tested for inhibition of TPA-induced ODC activity in mouse 308 cells, 13-*cis*-retinoic acid was three orders of magnitude less potent than deguelin (Table 1).

Effects on protein kinase C and HL-60 cell differentiation

Since protein kinase C (PKC), a key enzyme in signal transduction, is known to play a role in the induction of ODC activity and ODC mRNA (ref. 15), we determined the ability of the four rotenoids to affect this enzyme. Similar to retinoic acid, these compounds did not possess any affinity for the regulatory or catalytic domain of PKC as analysed by *in vitro* binding studies with radiolabelled phorbol-12,13-dibutyrate (PDBu) or staurosporine. Consequently, rotenoids had no stimulatory or inhibitory effect on PKC enzyme activity (Table 1). However, unlike retinoic acid, rotenoids had no influence on HL-60 cell differentiation as determined by nitroblue tetrazolium (NBT) reduction as a cellular marker (Table 1), and therefore seem not to interact with retinoic acid receptors. Additionally, we were not able to demonstrate inhibition of cyclo-oxygenase activity or induction of phase II enzymes like NAD(P)H:quinone oxidoreductase as an indication of alternative mechanisms of chemopreventive activity (data not shown).

Inhibition of preneoplastic lesions in mammary organ culture

The biological activity of the four isolated rotenoids was further investigated in a mouse mammary gland organ culture system. This *in vitro* system is known to demonstrate good correlation with animal studies in the detection of chemopreventive agents. At a test concentration of 10 μg ml^{-1}, all four compounds inhibited the formation of preneoplastic lesions as depicted in Table 1. The highest activity was demonstrated by deguelin which inhibited 100% of mammary lesion development. The effect of deguelin was dose-depen-

Table 1 Biological activities of *Mundulea sericea* bark ethyl acetate extract and four rotenoids (1–4) in comparison with 13-*cis*-retinoic acid

	TPA-induced ODC activity IC_{50}	PKC binding assay IC_{50}		PKC enzyme activity[a]	HL-60 cell differentiation		Mouse mammary gland organ culture[b]	
		PDBu	Staurosporine		ED_{50}	%Viability	% Incidence	%Inhibition
Mundulea sericea extract[c]	0.02	>10	>200	n.d.	>4	95	6.7(1/15)	91.3
Deguelin (1) R_1 = OH R_2 = H	0.0007	>500	>500	no effect	>10	93	0(0/15)	100.0
Tephrosin (2) R_1 = OH R_2 = OH	0.005	>500	>500	no effect	>10	95	6.7 (1/15)	89.0
(–)-13α- Hydroxytephrosin (3) R_1 = OH R_2 = OH	0.05	>500	>500	n.d.	>10	95	26.7 (4/15)	55.5
(–)-13α- Hydroxydeguelin (4) R_1 = H R_2 = OH	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	d	d
all-*trans*-Retinoic acid	2.8	223	n.d.	no effect	0.6	96	d	d

[a]n.d., not determined. No observed activation or inhibition (100% control activity) at a concentration of 25 μM was defined as 'no effect'.
[b]*M. sericea* extract and compounds 1–4 were tested at a concentration of 10 μg ml^{-1}.
[c]Ethyl acetate extract of *M. sericea* bark, prepared as previously described[14].
[d]13-*cis*-Retinoic acid and all-*trans*-retinoic acid were not included in this experimental design. Dose-dependent effects of all-*trans*-retinoic acid are shown in Fig. 3.

dent within the concentration range of 0.0013 to 12.7 μM. As shown in Fig. 3, deguelin proved to be as effective as all-*trans*-retinoic acid, which previously was shown to be one of the most effective agents in this test system.

Suppression of skin tumorigenesis induced by DMBA

In addition to the studies conducted with mouse mammary glands, the effect of a *Mundulea sericea* acetone-soluble extract was analysed in the two-stage mouse skin carcinogenesis test using dimethylbenz[*a*]anthracene (DMBA) as an initiator and TPA as a promoter. During a 105-day study, 95% tumour incidence was observed in DMBA/TPA-treated mice (Table 2). Application of the plant extract 1 hour before the TPA treatment reduced the tumour incidence to 15%. The number of skin tumours increased linearly in the DMBA/TPA-treated group to 595 (29.8 per animal) after the 105-day period, whereas in the TPA/DMBA/extract-treated group, tumour formation was nearly 100% suppressed, and a total of only four tumours were observed. A two-stage mouse skin study recently conducted in our laboratory with deguelin confirms the potent anti-tumour-promoting activities of the plant extract. Application of 84 nmol of deguelin twice a week for 15 weeks resulted in a 98% inhibition of DMBA/TPA-induced tumour formation, whereas a 10-fold higher dose prevented tumour formation completely. The results of Konoshima *et al.*[16] are consistent with these data.

Discussion

The identification of new chemical entities for prevention of cancer, as well as elucidation of their mechanisms of action, is now one of the most promising areas in cancer research. In this report, we have described the identification of rotenoids as potent cancer chemopreventive agents. Rotenoids are commonly found in plants of the Leguminosae and are structurally related to rotenone, which is a widely used plant-derived insecticide and a fish poison[17,18]. Rotenone, an effective inhibitor of $NADH_2$ dehydrogenase[19] and tubulin self-assembly[20], is considered to be relatively harmless for mammals, especially after oral administration, and rapidly decomposes in light and air. In contrast to some natural products presently used in cancer chemotherapy, such as taxol, various rotenoids can be obtained easily using commercially available rotenone as starting material. For example, we have employed a four-step synthesis to provide gram quantities of deguelin with properties identical to the plant-derived compound[21]. Because of the remarkable chemopreventive activity mediated by this compound in mammary organ culture as well as in the two-stage mouse skin model, additional studies are now under way to assess the potential of deguelin to prevent mammary carcinogenesis.

A key factor in judging the potential value of a cancer chemopreventive agent is its mechanism of action. It is well-established that induction of ornithine decarboxylase is associated with tumour promotion[6,22], therefore, compounds capable of inhibiting this crucial step of tumour promotion must be considered as potential chemopreventive agents. Previously identified natural chemopreventive compounds, including retinoic acid and vitamin A[14]; curcumin[23], the yellow pigment in curry; and 18β-glycyrrhetinic acid[4], derived from a constituent of licorice root; act at least partially by this mechanism. Using the inhibition of phorbol ester-induced ODC activity as a primary screen, analysis of plant extracts for this activity led to the identification of deguelin, tephrosin and two novel 13-OH derivatives as highly effective ODC inhibitors. To further elucidate the mechanism of rotenoid-mediated activity, we determined the influence of rotenoid treatment on TPA-induced ODC mRNA expression by northern blot analysis and demonstrated dose- and time-dependent inhibition of ODC mRNA expression and ODC activity. These findings strongly suggest that suppression of transcription mediated by rotenoids is responsible for transcriptional regulation of TPA-induced ODC mRNA levels and, consequently, ODC activity. In further mechanistic investigations including the analysis of effects on PKC binding and activity, HL-60 cell differentiation, cyclooxygenase activity as a monitor for alterations of arachidonic acid metabolism, and NAD(P)H:quinone oxidoreductase as an inducible phase II enzyme involved in drug-metabolism and detoxifica-

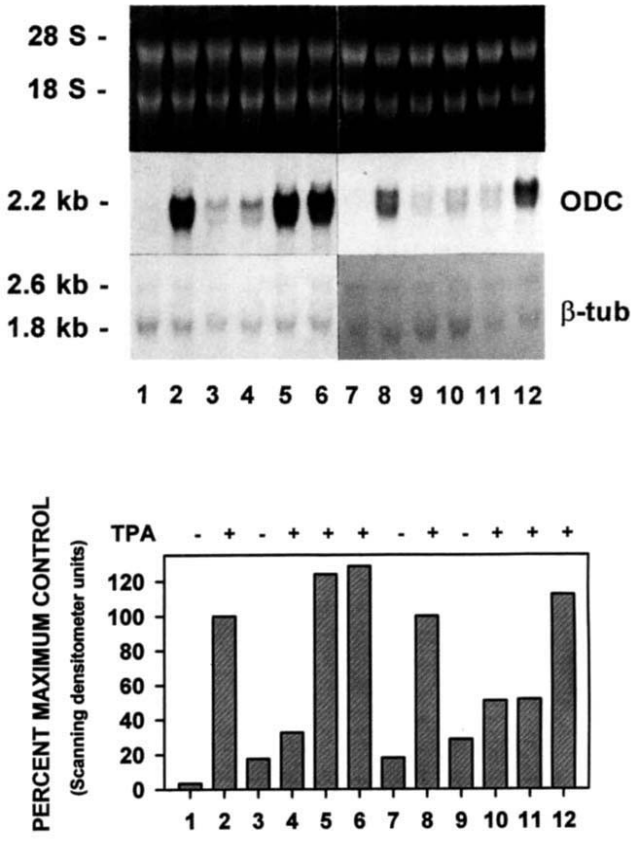


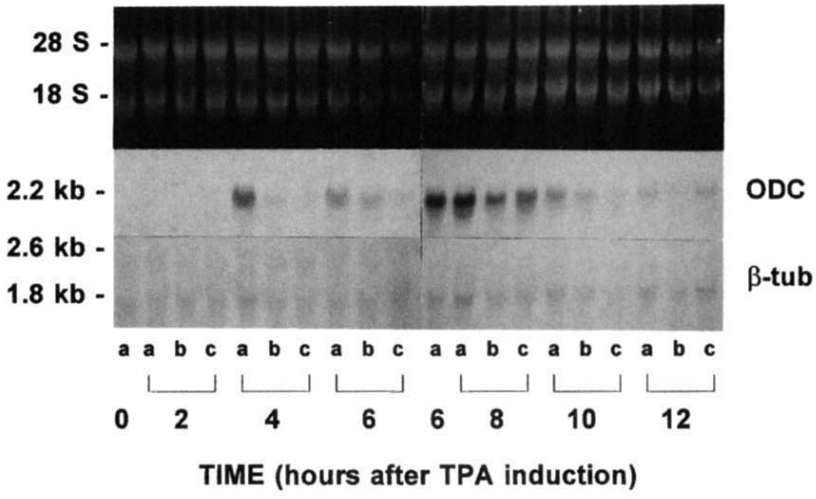
Fig. 1 *a*, Northern blot hybridization of RNA samples from cultured mouse 308 cells with ODC (middle) and β-tubulin (lower) probes. Upper: Ethidium bromide-stained gel. Cells were treated with varying concentrations of deguelin or tephrosin and 200 nM TPA as indicated for 6 h. Lane 1: DMSO control, 2: DMSO + TPA, 3: 0.04 μM deguelin, 4: 0.04 μM deguelin + TPA, 5: 0.02 μM deguelin + TPA, 6: 0.004 μM deguelin + TPA, 7: DMSO, 8: DMSO + TPA, 9: 0.004 μM tephrosin, 10: 0.01 μM tephrosin + TPA, 11: 0.004 μM tephrosin + TPA, 12: 0.0008 μM tephrosin + TPA. *b*, Densitometric scan of a slot-blot analysis of the RNA samples described in Fig. 1*a* (20 μg total RNA per slot) probed with an 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.

tion, no additional targets of rotenoid action were revealed. However, recent northern blot analyses have shown dose-dependent inhibition of TPA-induced c-*fos* and c-*myc* mRNA expression in mouse 308 cells (Gerhäuser *et al.*, in preparation), and this mechanism is capable of downregulating ODC enzyme activity[9,24,25]. Interestingly, curcumin has been reported recently to inhibit TPA-induced proto-oncogene mRNA expression in mouse skin[26], so similar mechanisms of chemopreventive activity may apply.

In summary, this report clearly demonstrates that rotenoids are promising anti-tumour or chemopreventive agents, and that inhibition of phorbol ester-induced ODC activity at the level of transcription is responsible for this activity. Because of these potent activities and unique mode of action, studies are now under way to determine the potential of deguelin to inhibit DMBA-induced mammary tumours in rats. It is hoped that this class of compounds will ultimately be useful in defining the armamentarium of drugs currently under development for the prevention of human cancer.

Methods

Determination of TPA-induced ODC activity. Mouse epidermal 308 cells (supplied by Stuart H. Yuspa) were cultured in S-MEM medium (Gibco BRL, Grand Island, New York) containing non-essential amino acids (1×, Sigma), dialysed fetal bovine serum (5%) (Hyclone, Logan, Utah), Ca^{2+} (0.05 mM), 100 units/ml penicillin G sodium, 100 units ml^{-1} streptomycin sulfate and 250 ng ml^{-1} amphotericin B (Gibco BRL) at 37 °C in a 5% CO_2 atmosphere. For determination of TPA-induced ODC activity, cells were plated at an initial density of 2×10^5 cells per well per milliliter in 24–well plates. After an 18 hour pre-incubation, 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 final concentration). After an additional incubation time of 6 h, plates were washed twice with PBS and kept at –85 °C until tested. ODC activity was assayed directly in 24–well plates as described by Lichti and Gottesman[27] by measuring the release of $[^{14}C]CO_2$ from L-[1–^{14}C]ornithine HCl (200 nCi final concentration, 56 mCi/mmol, Moravek Biochemicals, Inc., Brea, California) in the presence of 290 µM nonradioactive ornithine HCl. The amount of radioactivity captured in NaOH-impregnated filter discs was determined by scintillation counting in 24-well plates using a Wallac 1450 MicroBeta® liquid scintillation counter (Wallac, Inc., Gaithersburg, Maryland). Protein was determined according to the Lowry procedure[28]. Interfering dithiothreitol contained in the reaction mixture was destroyed by adding Chloramine T (50 µl, 8 mg ml^{-1}) to each well (30-min incubation at RT), followed by NaOH (50 µl, 5.7 M) to solubilize the protein. The protein was measured in 96-well plates using an aliquot of the reaction mixture and bovine serum albumin (Sigma) as a standard. The optical density was measured at 660 nm using a BT2000 Microkinetic Reader (Fisher Biotech). The results were calculated as nmol $[^{14}C]CO_2$ h^{-1} per mg protein and expressed as a percentage in comparison with a control treated with DMSO and TPA (no inhibition of induction). Dose–response curves were prepared, and the results were expressed as IC_{50} values in micromolar concentrations (except for the ethyl acetate extract of *M. sericea* bark, which is given in micrograms per millilitre.



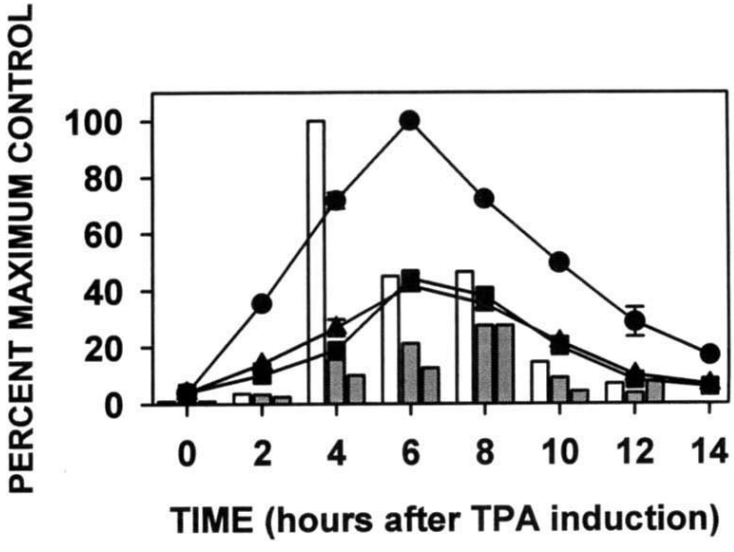


Fig. 2 *a*, Northern blot hybridization of RNA samples from cultured mouse 308 cells treated with DMSO (a, 0.5% final concentration), deguelin (b, 0.1 µM) or tephrosin (c, 0.1 µM) and 200 nM TPA for 0, 2, 4, 6, 8, 10, and 12 h with ODC (middle) and β-tubulin (lower) probes. Upper: Ethidium bromide-stained gel. *b*, Densitometric scan of autoradiographs of the gels shown in Fig. 2*a* in comparison with the ODC enzymatic activity. Differences in the intensity of the two separate gels were normalized for the DMSO/TPA-treated control (6-h incubation), which was separated on both gels. ODC mRNA expression (scanning densitometer units) and ODC enzyme activity (nmol $^{14}CO_2$ h^{-1} mg^{-1} protein) are expressed as percent in comparison with the maximum control value (4 h and 6 h, respectively). Bar graph: TPA-induced ODC mRNA expression: □, DMSO control; □, deguelin; □, tephrosin. Line graph: TPA-induced ODC enzyme activity: •, DMSO control; △, deguelin; ■, tephrosin.

Northern blotting and slot-blot analysis. Mouse 308 cells (4×10^5cells in 75 cm^2 flasks) were cultured in 10 ml S-MEM medium as described above for 18 h before the induction of mRNA by addition of TPA. The compounds (50 µl, 0.5% final DMSO concentration) were added simultaneously with TPA (200 nM final concentration). The incubation was terminated after 6 h for dose–response analyses, or at the indicated time points in time-course experiments. Total RNA was isolated according to a guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi[29] using 2-ml RNAzol® (Tel-Test Inc., Friendswood, Texas) per 75 cm^2 flask. The RNA pellet obtained after isopropanol precipitation was washed once with 75% ethanol and dissolved in 25 µl 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The yield and purity was determined spectrophotometrically at 260/280 nm. For northern blot analysis, RNA samples (generally 20–30 µg total RNA) were prepared as described by Kroczek and Siebert[30] and fractionated electrophoretically in a Wide Mini-Sub electrophoresis system (Bio-Rad, Hercules, California) for 160 min at 75 V under denaturing conditions in 1.2% agarose gels containing 1.1% formaldehyde. RNA was denatured by soaking the gel for 15 min in 50 mM NaOH and transferred to nylon membranes (Zeta Probe GT, Bio-Rad) by vacuum-blotting using a Bio-Rad Model 785 Vacuum Blotter for 90 min with 20× SSC as the blotting buffer (1× SSC is 150 mM NaCl, 15 mM Na_3citrate, pH 7.0). The membranes were rinsed briefly in 2× SSC and RNA was fixed to the damp membranes by UV cross-linking using a GS Gene Linker (Bio-Rad). Slot-blot experiments using a Bio-Rad slot-blot apparatus and Zeta Probe GT nylon membranes (Bio-Rad) to quantify mRNA expression were performed according to the instruction manual. Membranes were prehybridized at 50 °C in 0.25 M sodium phosphate buffer (pH 7.2) containing 7% SDS for 10–60 min before the addition of a specific 41-mer oligonucleotide complementary to nt 1041–1082 of mouse mRNA for ODC (5′–TTA-CAA-GGA-TTT-GCA-TAG-ATA-ACC-CTC-TCT-GCA-GGC-ACC-C–3′), 5′ end-labeled with [γ-^{32}P]ATP (10 µM, 6000 Ci $mmol^{-1}$, 10 mCi ml^{-1}, Amersham) and T4 polynucleotide kinase (Promega). Generally, membranes were hybridized for 24 h. Membranes were rinsed two times with 20 mM sodium phosphate buffer (pH 7.2) containing 5% SDS at room temperature, followed by washing at 50 °C for 10–15 min using the same buffer. After exposure of hybridized membranes to X-ray film (Fuji RX) at −80 °C for 1–5 days using intensifying screens, membranes were stripped of the ODC probe in 0.1 × SSC, 0.5% SDS at 70 °C for 30–45 min and rehybridized at 65 °C with a 1.5-kb human β-tubulin cDNA probe (Dβ–1, Oncor, Gaithersburg, Maryland) which had been labeled with [α-^{32}P]dCTP (6,000 Ci $mmol^{-1}$, 10 mCi ml^{-1}, Amersham) by random primed labeling using the Prime-a-Gene® labeling system (Promega). For quantitation of mRNA expression, densitometric scanning of autoradiographs was performed on a GS 300 densitometer using GS 365 W software (Hoefer Scientific Instruments, San Francisco, California). All experiments were repeated at least twice.

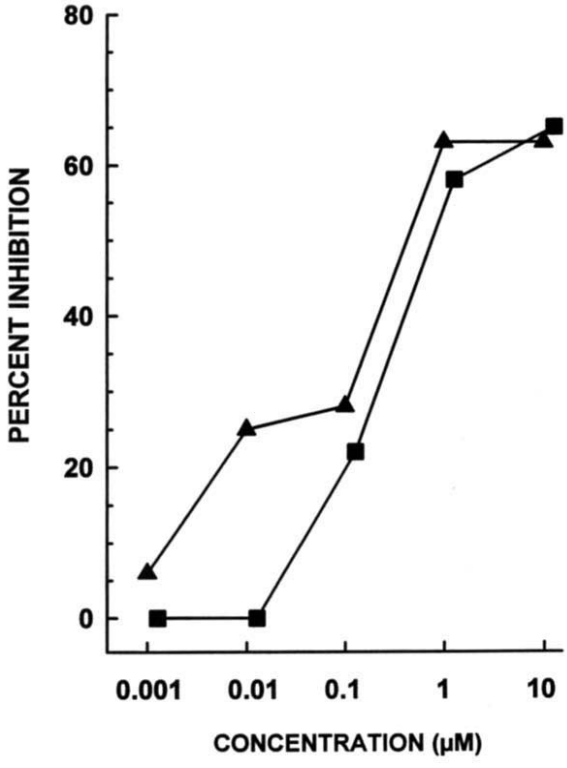


Fig. 3 Dose-dependent inhibition of DMBA-induced lesion formation in mouse mammary gland organ culture by deguelin (■) and all-*trans*-retinoic acid (△).

Protein kinase C-binding assay. The PKC-binding assay was performed in 96-well microtitre plates essentially as described by De Vries *et al.*[31], using calf brain homogenate as a source of soluble PKC. Each reaction mixture (200 µl) contained calf brain homogenate (25 µg protein per 200 µl per well) as a source of PKC, bovine serum albumin (200 µg per well), 20 mM Tris-HCl buffer (pH 7.4), and 1 µl of diluted [20-^{3}H(N)]phorbol-12,13-dibutyrate (PDBu) (20 Ci $mmol^{-1}$, 20 nCi, Du Pont/NEN, Wilmington, Delaware), diluted with ethanol, final concentration 5 nM) or 1 µl of diluted [^{3}H]staurosporine (160 Ci $mmol^{-1}$, 20 nCi, Du Pont/NEN, diluted with ethanol, final concentration 0.625 nM). Test substances (10 µl, dissolved in DMSO) were added to the reaction mixture to yield five concentrations in the range of 0.8–200 µg ml^{-1} (5% final DMSO concentration). Incubations were performed in duplicate for 1 hour at 37 °C. Unbound [^{3}H]PDBu or [^{3}H]staurosporine was removed by filtration with 50 mM Tris-HCl buffer (pH 7.4) through glass fiber filter mats (Type B, Wallac Inc., Gaithersburg, Maryland) using a Tomtec Mach III 96-well plate harvester (Tomtec, Orange, Connecticut). Radioactivity was determined by scintillation counting in a Wallac 1450 MicroBeta™ liquid scintillation counter (Wallac Inc.). The amount of [^{3}H]PDBu or [^{3}H]staurosporine bound in the presence of nonradioactive PDBu (4 µM) or staurosporine (2 µM) was used to measure nonspecific binding. Specific binding was calculated from the difference between total and nonspecific binding. When applicable, results were expressed as IC_{50} values as micromolar concentrations (except for the ethyl acetate extract of *M. sericea* bark, which is given as micrograms per millilitre.).

Protein kinase C enzyme assay. The purification of PKC-α from calf brain and the PKC assay were performed essentially as described by Da Silva *et al.*[32] and references cited therein. PKC enzyme activity was assayed by measuring the transfer of the γ-phosphate group of [γ-^{32}P]ATP into histone III-S. The reaction mixture (80 µl) consisted of 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.2 mg ml^{-1} histone III-S, 0.5 mg ml^{-1} phosphatidylserine in 3% Triton X-100, 10 µM [γ-^{32}P]ATP (6000 Ci $mmol^{-1}$, 10 mCi ml^{-1}, Amersham), and the test compounds in DMSO (5 µl, 5% final DMSO concentration). The reaction was ini-

Table 2 Effects of a *Mundulea sericea* extract in the two-stage mouse skin carcinogenesis assay

Group[a]	Treatment	DMBA/TPA	Tumour incidence	Tumour multiplicity	Mean body weight (g)	% Survival
1	Vehicle (Acetone)	+	95.0	29.8	31.1	100
2	*M. sericea* extract[b]	+	15.0[c]	0.2	31.2	100
3	*M. sericea* extract[b]	–	0	0	31.7	100
4	None	–	0	0	32.4	100

[a] $n = 20$ mice.
[b] Acetone-soluble portion of an ethyl acetate extract[14] of the bark of *M. sericea*.
[c] $P < 0.01$ when compared to group 1 via Fisher's exact test.

tiated by the addition of 20 µl enzyme solution. After an incubation time of 10 min at 30 °C, the reaction was terminated by spotting 85 ,µl of the reaction mixture onto Whatman P81 cation-exchange paper. Effects of test compounds on PKC activity were measured either after preactivation of PKC α by addition of PDBu (0.2 µg ml^{-1}) or without prior activation. Results were expressed as a percentage relative to the DMSO-treated group activated by PDBu after subtraction of the DMSO-treated non-activated control group. Dose–response relationships were obtained by testing serial dilutions in duplicate. If neither activation nor inhibition (100% control activity) was detected at a final concentration of 25 µM, the result was defined as 'no effect'.

HL-60 cell differentiation assays. HL-60 (human promyelocytic leukemia) cells were maintained in RPMI medium (Gibco BRL) supplemented with 5% heat-inactivated calf serum, 100 units ml^{-1} of penicillin G sodium and 100 µg ml^{-1} of streptomycin sulfate (Gibco BRL) at 37 °C in a humidified atmosphere at 5% CO_2 in air. To test the potency of agents to induce cell differentiation, HL-60 cells (2.4×10^5 cells per 2 ml) were preincubated for 18 h in 24-well plates. Test compounds dissolved in DMSO (2 µl, 0.1% final DMSO concentration) were then added. Control cultures were treated with the same amount of DMSO. After four days of incubation, reduction of NBT, nonspecific/specific acid esterase (NSE/SE) activity, and [^{3}H]thymidine incorporation were analysed as described recently by Chen *et al.*[33] and references cited therein. To determine effects on NBT reduction, a 1:1 mixture of a cell suspension (1×10^6 cells) and a freshly prepared TPA/NBT solution (phosphate buffered saline solution containing 2 mg ml^{-1} of NBT and 5 µg ml^{-1} of TPA) was incubated for 1 h at 37 °C. Positive cells were able to reduce NBT yielding intracellular black-blue formazan deposits, and this was determined by microscopic examination. The results were expressed as a percentage of NBT-positive cells. ED_{50} values (half-maximal effective dose) as µM (except for *M. sericea* extract, which is given in micrograms per millilitre) were calculated from the results of serial dilutions tested in duplicate. Percent viability was determined by trypan blue dye exclusion. Rotenoids had no effect on NSE/SE activity and [^{3}H]thymidine incorporation at a final concentration of up to 10 µM (data not shown).

Inhibition of DMBA-induced lesion formation in mammary organ culture. Mammary glands from 4-week-old Balb/c mice were incubated in organ culture[34] with insulin (5 µg ml^{-1}), prolactin (5 µg ml^{-1}), aldosterone (1 µg ml^{-1}), and hydrocortisone (1 µg ml^{-1}) for 10 days (15 glands per group). The test compounds at a concentration of 10 µg ml^{-1} (corresponding to a final rotenoid concentration of approximately 25 µM) were present in the medium during this initial growth period. DMBA was added to the medium for 24 hours on day 3 of the culture (2 µg ml^{-1}). After a growth-promoting period of 10 days, the glands were incubated with insulin alone for an additional 14 days. This allowed the mammary gland structure to regress back to ductal morphology. Glands were fixed in 10% buffered formalin and stained with alum carmine. Percent incidence of mammary lesions (glands with unregressed areas) was determined. The data from compound-treated groups were compared to control groups with a mammary lesion incidence of 60–100%. In each case, no toxicity was observed. Dose-dependent effects of deguelin (0.0013 to 12.7 µM) and all-*trans*-retinoic acid (0.001 to 10 µM) were analysed following the same protocol. Signs of toxicity were observed in glands treated with all-*trans*-retinoic acid at a concentration of 10 µM, whereas no toxicity was observed with deguelin at the highest concentration tested (12.7 µM).

Two-stage skin carcinogenesis test[35]. Four groups of 20 female CD-1 mice (4–5 weeks old) were initiated by applying 50 µg DMBA in 0.2 ml acetone as a single application on the shaved backs. For tumour promotion, the mice were treated with 2.5 µg TPA in 0.2 ml of acetone twice weekly for 15 weeks. An acetone-soluble portion of an extractive of *Mundulea sericea* bark prepared using ethyl acetate[13] (dissolved in 0.2 ml acetone) was applied to the backs of mice at a concentration of 1% twice weekly for 15 weeks, one hour before TPA treatment. Animals were weighed daily and observed for tumour development. Deguelin was tested at doses of 84 and 840 nmol in 0.2 ml acetone.

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