Antitumorigenic Activities of Chalcones (II). Photo-isomerization of Chalcones and the Correlation with Their Biological Activities

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Some chalcones are known to be phototransformed in a solution from *trans* into *cis* isomers. 3-Hydroxy-3′-methylchalcone (3′Me-3-C) has been found to be isomerized from *trans* into *cis* by irradiation of daylight in the methanolic solution. The presence of a hydroxyl in the 2′- or 4-position in the *trans*-chalcone structure prevents phototransformation into *cis* isomers. The feasibility of phototransformation of chalcones was discussed using UV-spectral analysis. The phototransformed *cis*-3′Me-3-C showed more potent antitumorigenic activity than the original *trans* form.

The generally recognized parallelism between antitumorigenic and antiinflammatory activities was not observed in *trans* and *cis* 3′-Me- and 4′-Me-3C, which are antitumorigenic but inactive in 12-*O*-tetradecanoylphorbol 13-acetate (TPA)- and arachidonic acid (AA)-induced mouse ear edema. However, inhibitory activity against ornithine-decarboxylase (ODC) was commonly observed in both naturally occurring and synthetic antitumorigenic chalcones.

Key words chalcone derivative; Photo-isomerization; Antitumor promoting activity

3-Hydroxy-3′-methylchalcone (3′Me-3-C), whose antitumorigenic activity was reported in the previous report,[1-3] is isomerized from the original *trans* (cisoid *E*) into *cis* (cisoid *Z*) form by the irradiation of daylight into the methanolic solution.

The photo-isomerization of chalcones was reported by several researchers,[4-6] but none of them discussed the structural correlation between their feasibility of phototransformation and their biological activities, with which the present study is mainly concerned.

MATERIALS AND METHODS

Chemicals Chalcone (Benzalacetophenone) was purchased from Nakalai Tesque, Inc. (Japan). 3-Hydroxy-3′-methylchalcone (*trans* form) and 3-hydroxy-4′-methylchalcone (*trans* form) were synthesized by the method described in our previous paper.[3]

cis-Benzalacetophenone was obtained according to the method of Lutz and Jordan.[4]

cis 3-Hydroxy-3′-methylchalcone (*cis*-3′Me-3-C) and *cis* 3-hydroxy-4′-methylchalcone (*cis*-4′Me-3-C) were prepared as follows: Ordinary (*trans*) 3′Me-3-C or 4′Me-3-C (500 mg) was dissolved in MeOH (1 l) and exposed to daylight for 1 week. The reaction mixture was concentrated, and *trans* and *cis* isomers were separated using a preparative HPLC.

HPLC condition: Column, Senshu Pak. ODS 5251-N (20 mm × 200 mm); solvent, MeOH : H_2O = 7 : 3; flow rate, 10 ml/min; detector, UV (set at 254 nm). Sample charge volume: 25 mg.

t_R (min): *trans* 3′Me-3-C = 11.5, *trans* 4′Me-3-C = 11.2.

t_R (min): *cis* 3′Me-3-C = 8.0, *cis* 4′Me-3-C = 7.8.

Recrystallization was accomplished by dissolving *cis* 3′Me-3-C or *cis* 4′Me-3-C in *n*-pentane at room temperature and by cooling the mixture at −20 °C until the completion of crystallization.

cis 3′Me-3-C, colorless prism, mp 65—66 °C, UV λ_{max}^{EtOH} nm (log ε): 207 (4.59), 254 (4.19), 297 (3.92); HRMS (*m/z*) *Anal.* Calcd for $C_{16}H_{14}O_2$ (M^+): 238.0994, Found: 238.0993.

cis 4′Me-3-C, colorless prism, mp 84—85 °C, UV λ_{max}^{EtOH} nm (log ε): 207 (4.48), 262 (4.15); HRMS (*m/z*) *Anal.* Calcd for $C_{16}H_{14}O_2$ (M^+): 238.0994, Found: 238.1002.

12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Sigma. Radioactive inorganic phosphate (^{32}Pi, carrier-free) was obtained from Japan Radioisotope Association. [6,7-^{3}H]Estradiol ([^{3}H]-E_2) was obtained from Amersham, Tokyo, Japan.

Cell Culture HeLa cells (human cervical cancer cells) were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum. HGC-27 cells (human gastric cancer cells) were cultured in Dulbecco's modified EMEM supplemented with 10% fetal bovine serum. Cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C.

^{32}Pi-incorporation into Phospholipids of Culture Cells ^{32}Pi-incorporation into phospholipids of HeLa cells was assayed according to the method described previously.[7]

Cell-proliferation Assay HGC-cells (4×10^4 cells/2 ml of medium) were subcultured in 35 mm diameter Petri dishes. Chalcone derivatives were dissolved in dimethyl sulfoxide and mixed with ethanol for sterilization for 16 h. Ten μl of the sterilized solution of the compounds was added to the culture medium and the control culture was treated with vehicle alone. All cells were incubated and counted as described previously.[2]

Type-II Estrogen-receptor-binding Experiment Type-II estrogen-receptor-binding was assayed according to the method described previously.[2]

Assay for AA- and TPA-induced Mouse Ear Edema Six-week-old male ddY mice weighing 30—35 g (Japan SLC,

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Shizuoka) were used for the experiments. The animals were kept in an environmentally controlled room (24±1 °C, 55±10% humidity) and allowed free access to food and water. Induction of mouse ear edema was based on the method of Inoue *et al.*[8,9] for AA and for TPA. AA and TPA were dissolved in acetone at a concentration of 100 mg/ml and 100 μg/ml, respectively. Each mouse was then treated with 20 μl of acetone solution (AA: 2 mg per ear, TPA: 2 μg per ear) on both surfaces of an ear. Test compounds were dissolved in acetone for topical application and were given 30 min before the irritant treatment. Mice in the control group received the vehicle only. Ear thickness was measured 1 h and 5 h after AA and TPA treatment with dial calipers (Ozaki Factory, Tokyo) measuring to an accuracy of 0.01 mm. Estimation of the effect of test compounds on edema was expressed as an inhibition percent compared with the control.

Measurement of Ornithine Decarboxylase (ODC) Activity in Mouse Skin Backs of mice (ICR, female, 7 weeks old) were shaved with a hair remover, then treated with TPA (17 nmol in 200 μl of acetone per mouse)±test sample (1.7 μmol/mouse) administered by painting. After incubation for 4 h, the skin preparation was sonicated in 1 ml of 50 mM phosphate buffer (pH 7.2) containing 5 mM dithiothreitol, 0.1 mM EDTA and 0.04 mM pyridoxal phosphate.

A supernatant obtained by centrifugation of the treated skin preparation was assayed for protein concentration and ODC activity. The ODC assay was carried out as follows:

Fifty μl of the supernatant was mixed with 100 μl of a mixture (48 mM phosphate buffer (pH 7.2) containing 0.25 mM dithiothreitol, 1 mM EDTA, 0.01 mM pyridoxal phosphate and 0.45 mM [^{3}H] ornithine). After incubation for 1 h at 37 °C, the reaction was stopped by placing the reaction mixture on ice for 10 min. An aliquot (100 μl) was applied to Whatman p 80 paper (2.3 cm diameter disk, a strong cation-exchanger for selective binding of putrescine). The paper was washed with 0.1 N NH_4OH (1 l) twice. After drying, the radioactivity was counted.

RESULTS AND DISCUSSION

Chemistry It has been found that 3-hydroxy-3′-methylchalcone (3′Me-3-C) and 3-hydroxy-4′-methylchalcone (4′Me-3-C), whose antitumorigenic activity was reported in our previous report,[3] are isomerized from the original *trans* (cisoid *E*) into *cis* (cisoid *Z*) form by irradiation of daylight in a methanolic solution (Fig. 1).

However, chalcones isolated from licorice, such as licochalcone A, echinatin, isoliquiritigenin and almost all naturally occurring chalcones, are not isomerized from the *trans* into the *cis* form by daylight irradiation through a window (data not shown). Examining more than 40 simple *trans* chalcone derivatives which were synthesized for the antitumorigenic experiments,[3] we found that those possessing a hydroxyl group at the 2′- or 4-position resist photo-conversion. The samples were dissolved in MeOH at a concentration of 0.5 mg/ml and injected into HPLC immediately, or after standing for 1 d to 1 week, to observe whether a new peak representing a *cis* isomer appears in the HPLC.

The isomerization of photo-transformable chalcones from the *trans* into the *cis* form in the methanolic solution by irradiation of daylight lasted 1—3 d. The experiment was performed by the side of a window at an average temperature of 24 °C from July 14 to 16, 1992, but direct sunshine was avoided. On day 1, about 65% of *trans* 3′Me-3-C (0.2 mg/ml) was converted into the *cis* isomer. On day 2, the photo-conversion reached an equilibrium at the average ratio of *trans* and *cis* of 15 : 85.

trans-cis Chalcone isomers are separable by HPLC, and the isomers were identified by UV and NMR spectra. An obvious hypsochromic shift of the UV-absorption maximum was observed by the conversion of *trans* into *cis* isomers.

In ^{1}H-NMR spectra, the *trans-cis* conversion was revealed by an upfield shift of the signals of olefinic protons at Hα and Hβ and by a decrease of their coupling constants[5] (Table 1). In ^{13}C-NMR spectra, the chemical shifts of carbonyl carbon and α-olefinic carbon to the higher fields and of the β-olefinic carbon signal downfield were observed by the *trans-cis* transformation (Table 2).

It is noted that the alcoholic solution of photo-untransformable chalcones gave a bathochromic UV-absorption at a higher wavelength region ($\lambda_{max} > 346$ nm) in comparison with that given by the phototransformable ones ($\lambda_{max} < 328$ nm), suggesting a dominant molecular planality in the former group of compounds (Table 3).

Biological Activities Effect of *trans*- and *cis*-Chalcone Derivatives on TPA-enhanced ^{32}Pi-Incorporation into Phospholipids of Cultured Cells: As shown in Table 4, *cis*-form chalcones showed more potent inhibitory activity against TPA-enhanced ^{32}Pi-incorporation into phospholipids of cultured cells than the *trans* isomer. This biological reaction is employed as an antitumorigenic screening test showing good parallelism with *in vivo* experiments.[3] Both isomers of 3′Me-3-C inhibited 100% at 5 μg/ml, while at a diluted concentration (3 μg/ml), *cis*-3′Me-3-C revealed higher inhibitory potency than the *trans*-isomer, 75.6 and 43.6%, respectively.

Effect of *trans*- and *cis*-3′Me-3-C on the Growth of

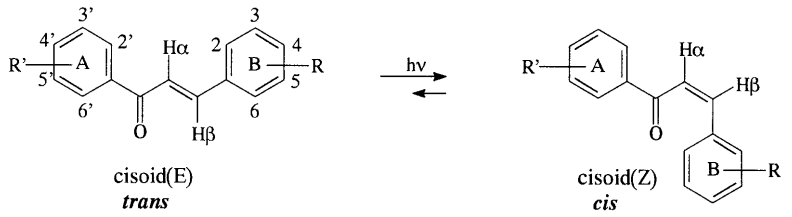

Fig. 1. Photoisomerization of Chalcones

Table 1. The ^{1}H-NMR Chemical Shifts (ppm) of *trans* and *cis* Chalcones

	3-Hydroxy-3′-methylchalcone		3-Hydroxy-4′-methylchalcone	
	trans	*cis*	*trans*	*cis*
H_α	7.83 (J=15.5 Hz)	6.72 (J=12.9 Hz)	7.82 (J=15.5 Hz)	6.69 (J=13.1 Hz)
H_β	7.64 (J=15.5 Hz)	6.92 (J=12.9 Hz)	7.63 (J=15.5 Hz)	6.90 (J=13.1 Hz)
Ar-OH	9.65	9.42	9.64	9.42
Ar-Me	2.42	2.35	2.40	2.35

Chemical shift (δ) in DMSO-d_6 at 400 MHz.

Table 2. The ^{13}C-NMR Chemical Shifts (ppm) of *trans* and *cis* Chalcones

No.	3-Hydroxy-3′-methylchalcone		3-Hydroxy-4′-methylchalcone	
	trans	*cis*	*trans*	*cis*
1	136.0	136.5	135.1	134.1
2	115.3	115.5	115.2	115.6
3	157.8	157.1	157.7	157.1
4	117.8	115.8	117.8	115.8
5	129.9	129.3	129.4	129.3
6	120.0	120.1	120.0	120.1
1′	138.2	138.3	143.6	144.0
2′	129.0	129.0	128.7	128.8
3′	137.8	136.7	129.4	129.4
4′	133.8	134.2	136.0	136.5
5′	128.8	128.8	129.4	129.4
6′	125.7	126.0	128.7	128.8
C=O	189.0	194.6	188.7	194.2
α	122.0	127.5	121.9	127.5
β	144.2	137.1	144.3	137.4
CH_3	20.9	20.9	21.2	21.2

Chemical shifts (δ) in DMSO-d_6 at 100 MHz.

Table 3. UV-Absorption of Photo-transformable and -untransformable Chalcones (in MeOH)

Compound	UV_{max} (nm)	
Photo-transformable:		
3-Hydroxy-3′-methylchalcone	258	305
3-Hydroxy-4′-methylchalcone	258	306
3-Hydroxy-2′-methylchalcone	247	298
4′-Hydroxy-4-methylchalcone	232	328
Chalcone	267	309
Photo-untransformable:		
2′-Hydroxychalcone	316	360
2′,4′,4-Trihydroxychalcone (Isoliquiritigenin)	239	370
4-Hydroxy-4′-methylchalcone	238	347
2′,3-Dihydroxy-5′-methylchalcone	311	362
4-Hydroxychalcone	247	348
4,4′-Dihydroxychalcone	237	348
4,4′-Dihydroxy-2-methoxychalcone (Echinatin)	309	369

Cultured Human Gastric Cancer Cells: Table 5 shows that *cis*-3′Me-3-C inhibited the growth of cultured human gastric cancer cells (HGC-27 cells) more potently than *trans*-3′Me-3-C.

Effect of 3′Me-3-C Isomers on [^{3}H]-Estradiol (E_2) Binding to Type II Estrogen Binding Sites (EBS): Type II EBS have been reported by Ranelletti *et al.*[10a-c] and Markaverich *et al.*[11a-c] to exist in many normal and malignant tissues and to be distant from the true estrogen receptor. A flavonoid-like endogeneous ligand, methyl *p*-hydroxyphenyllactate (MeHPLA) binds to type-II EBS and regulates normal and malignant cell proliferation. Esterase in malignant tissues hydrolyses MeHPLA into HPLA, which is readily metabolized and causes a lack of cell regulatory control associated with tumor cell proliferation. Some stable isosteric blockers of EBS, such as luteolin, quercetin and 4,4′-dihydroxy-chalcone, were shown to be effective against the proliferation of cultured human breast cancer cells (MCF-7).[11b] Previously, Satomi[2] reported that *trans*-3′Me-3-C competed with [^{3}H]-E_2 for binding to type II EBS in a dose-dependent manner. Therefore, we examined whether *cis*-3′Me-3-C interacts with type II EBS. The result showed that the *cis* isomer also competed with E_2 binding at type II EBS, but there was no remarkable difference in the activities between *trans* and *cis* isomers (Table 6).

Table 4. Effect of *trans* and *cis* Isomers of Chalcone Derivatives on TPA-Enhanced ^{32}Pi-Incorporation into Phospholipids of Culture Cells

Compound		Inhibition (%)	
		trans	*cis*
3-Hydroxy-3′-methylchalcone	5 μg/ml	100	100
	3 μg/ml	43.6	75.6
3-Hydroxy-4′-methylchalcone	5 μg/ml	97.6	100
Chalcone	5 μg/ml	49.3	85.2

HeLa cells were incubated with or without the test compound (5 μg/ml or 3 μg/ml), and after 1 h, ^{32}Pi (370 kBq per culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, and then the radioactivity incorporated into the phospholipid fraction was measured. Data, expressed as percentage of inhibition on TPA-enhanced ^{32}Pi-incorporation, are mean values of duplicate experiments.

Table 5. Effect of the Stereo-isomers of 3-Hydroxy-3′-methylchalcone on the Proliferation of HGC-27 Cells

Condition	Number of viable cells ($\times 10^5$ cells/dish)	Inhibition (%)
Control	7.90	
trans 3-Hydroxy-3′-methylchalcone	5.40	31.6
cis 3-Hydroxy-3′-methylchalcone	3.02	61.8

HGC-27 cells were inoculated at a density of 4×10^4 cells/dish, and 24 h later, treated with 0.5 μg/ml of the indicated compounds or vehicle as a control. The number of viable cells was counted by the Trypan blue exclusion method 3 d after treatment. Data are mean values of duplicate experiments.

Effect of Chalcone Derivatives on AA- and TPA-Induced Mouse Ear Edema: A topical application of AA to mouse ear caused an edema response by 1 h after treatment, whereas the edema response to TPA reached a maximum at 5 h after treatment. It is well-known that products of the arachidonate pathway are involved in the development of both models.[9,12-14] Compounds with 5-lipoxygenase inhibitory activity are more effective against AA-edema, and those compounds with cyclooxygenase inhibitory activity are more effective against TPA-edema.[14] It has generally been recognized that antitumorigenically active compounds are also anti-

Table 6. Effect of Pretreatment with the Stereo-isomers of 3-Hydroxy-3′-methylchalcone on [^{3}H]-E_2 Binding to Type-II EBS

Condition	[^{3}H]-E_2 binding, dpm/10^4 cells	(% of control)
Control	1581	
trans 3-Hydroxy-3′-methylchalcone	1124	71.1
cis 3-Hydroxy-3′-methylchalcone	1066	67.4

HGC-27 cells were pre-treated with 3 μM *trans* or *cis* 3-Hydroxy-3′-methylchalcone (molar ratio to [^{3}H]-E_2 was 100 : 1) for 24 h. After the removal of each drug, a binding assay was carried out as described previously.[2] Data are the mean values of duplicate experiments.

Table 7. Effect of Chalcone Derivatives on Mouse Ear Edema Induced by TPA and AA

Compounds	Inhibition (%) TPA edema	AA edema
Licochalcone A	90±1***	30±4**
trans 3-Hydroxy-3′-methylchalcone	6±5	9±4
cis 3-Hydroxy-3′-methylchalcone	2±4	NT
trans 3-Hydroxy-4′-methylchalcone	23±10	6±4
cis 3-Hydroxy-4′-methylchalcone	3±2	NT
2′,4-Dihydroxychalcone	22±10	−8±3
2′,4′-Dihydroxychalcone	41±5***	8±4
2′,4′,4-Trihydroxychalcone (Isoliquiritigenin)	38±2***	−3±3

Compounds (0.5 mg/ear) were applied topically 30 min before TPA (2 μg/ear) and AA (2 mg/ear) treatment. Values are expressed as the mean±S.E.M. of 6—7 animals. Statistical significance from the control at $**p<0.01$ and $***p<0.001$. NT; not tested.

inflammatorily effective *in vivo* in AA- and TPA-induced models.[15,16] In the present study, however, anti-tumorigenically active chalcones have been divided into anti-inflammatorily effective and ineffective groups. Thus, licochalcone A, a leukotriene inhibitor[15] and echinatin are anti-inflammatorily effective,[16] whereas *trans*- and *cis*-3′Me-3-C and 4′Me-3-C are ineffective, even in the TPA-induced inflammation model. This might be practicable as a mechanism of cancer chemoprevention (Table 7).

Inhibition of ODC Activity: It has been well recognized that physiological polyamines are capable of interaction with DNA, and that ODC is acting as a key enzyme of polyamine metabolism.[17] Inhibitors of ODC, such as α-difluoromethylornithine (DFMO), are effective in cancer chemoprevention.[18] DFMO is also used to cure mice infected with *Trypanozoma brucei brucei*, which is related to the parasite that causes human sleeping sickness.[19] Tannic acid is also effective against TPA-induced ODC activity, which may be related to the antitumorigenic activity of hydrolysable tannins.[20] The rotenoid and chalcones isolated from *Mundulea sericea*, a leguminose-ous plant found in central and southern tropical Africa and parts of India, were reported to inhibit TPA-induced ODC-activity in relation to their cancer chemopreventive effects.[21] Thus, our chalcone preparations were studied in terms of TPA-induced ODC activity (Table 8). In this case 3′Me-3-C and 4′Me-3-C, along with licochalcone A and isoliquiritigenin, also showed inhibitory activities on ODC. The photo-transformed *cis*-3′Me-3-C had a more potent effect against ODC than the original *trans* isomer. Inhibitory activities on ODC have generally been observed in those antitumorigenically active compounds tested so far in the present study, which suggests a significant role of ODC inhibition in the mechanism of cancer chemo-prevention of this series of chalcone derivatives.

Table 8. Effect of Chalcones on the Activity of TPA-induced Ornithine Decarboxylase (ODC)

Compound (1.7 μmol/mouse)	ODC-Activity (nmol putrescine/mg protein/h)	Inhibition %
Acetone	2.09	
Control	45.26	
Licochalcone A	26.88	42.6
trans 3-Hydroxy-3′-methylchalcone	30.78	33.5
cis 3-Hydroxy-3′-methylchalcone	22.46	52.8
trans 3-Hydroxy-4′-methylchalcone	29.81	35.8
2′,4′,4-Trihydroxychalcone (Isoliquiritigenin)	27.26	41.7

TPA: 17 nmol. Data are values of representative experiments, and proved to be reproducible.

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