Effect of Chemical Constituents from Plants on 12-*O*-Tetradecanoylphorbol-13-acetate-Induced Inflammation in Mice[1)]

Ken YASUKAWA,*[,a] Michio TAKIDO,[a] Mieko TAKEUCHI[b] and Shigeki NAKAGAWA[c]

Department of Pharmacy, College of Science & Technology, Nihon University,[a] Kanda Surugadai, Chiyoda-ku, Tokyo 101, Japan, National Cancer Center Research Institute,[b] Tsukiji, Chuo-ku, Tokyo 104, Japan and Department of Biochemistry, Nihon University School of Medicine,[c] Ohyaguchikami-machi, Itabashi-ku, Tokyo 173, Japan. *Received October 20, 1988*

The induction of edema in the mouse ear has been established as a reliable *in vivo* assay for tumor promoters. Therefore, inhibitors of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema are most likely to be inhibitors of skin tumor promotion. Besides the application for this assay for the screening of compounds, it also allows comparison of the activities of groups of related compounds such as flavonoids. Results obtained in this way showed that the double bond at C-2 and C-3 of the flavonoid structure is a prerequisite for anti-tumor-promoting activity, and indicated that activity in this screening assay for inhibitors of TPA-induced ear edema reflects the anti-tumor-promoting effect in two-stage carcinogenesis.

Keywords anti-tumor promoter; 12-*O*-tetradecanoylphorbol-13-acetate; flavonoid; anti-inflammation; plant constituent

Introduction

The concept of two-stage carcinogenesis, consisting of initiation and promotion, was first proposed by Beremblum.[2)] The former stage involves irreversible damage to deoxyribonucleic acid whereas the latter is a longer process associated with reversible and irreversible changes following initiation. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA), a typical tumor promoter, has various biological and biochemical effects on susceptible tissues.[3,4)] Studies on phorbol esters have shown that the potency of irritant activity is well correlated with tumor-promoting activity.[5,6)] Inflammation is induced by tumor promoters,[7)] as shown by histological examination of inflammatory response to TPA in mouse ear by Young *et al.*[8)] Various inhibitors of skin tumor promotion, quercetin,[9)] oleanolic acid, ursolic acid,[10)] glycyrrhetic acid[11)] and caffeine,[12)] inhibited TPA-induced inflammation in mouse skin. A comparison of the results obtained using anti-tumor promoters with those[9-12)] from two-stage carcinogenesis induced by 7,12-dimethylbenz[*a*]anthracene and TPA revealed good agreement. Various flavonoids inhibited TPA-induced inflammation in mouse skin, and the double bond at C-2 and C-3 of the flavonoid structure was proved to be a prerequisite for anti-tumor-promoting activity.

Materials and Methods

Chemicals TPA was purchased from Chemicals for Cancer Research Inc., Minnesota, U.S.A. Dimethylsulfoxide was from E. Merck, Darmstadt, West Germany. Acetone and chloroform were purchased from Tokyo Kasei Kogyo Co., Ltd., Japan.

Animals Female ICR mice were obtained from Shizuoka Laboratory Animal Center, Shizuoka, Japan, and housed in an air-conditioned room (22—23 °C) lit from 08:00 to 20:00. Food and water were available *ad*

TABLE I. Inhibitory Effects of Various Compounds on TPA-Induced Inflammation

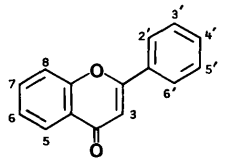
Compound	Inhib.[a)]	Source	Compound	Inhib.[a)]	Source
Paeonol	25	Com [Ald.]	Apigenin	75[c)]	Com [Ald.]
Arbutin	16	Com [T.K.]	Quercetin	50[c)]	Com [T.K.]
Salicin	14	Com [Roth]	Rutin	43[c)]	S.j.
Protocatechuic acid	4	Com [T.K.]	Flavanone	4	Com [T.K.]
(+)-Catechin	1	Com [T.K.]	Naringenin	4	Com [Roth]
Gallic acid	20[b)]	Com [T.K.]	Naringin	1	Com [T.K.]
Ellagic acid	7	Com [T.K.]	Eriodictyol	2	Com [Roth]
Cinnamaldehyde	0	Com [Sig.]	Taxifolin	22[c)]	Com [Sar.]
Eugenol	25[b)]	f.O.	Daizdin	−10	Com [Roth]
Torosachrysone	11	C.t.	Citral	26[c)]	f.O.
Magnolol	13	Com [Nak.]	Linalool	30[c)]	f.O.
Emodin	23	R.R.	Limonene	19	f.O.
Rhein	42[c)]	R.R.	Honokiol	38[c)]	Com [Nak.]
Sennoside-A	−11	Com [Roth]	Menthol	8	f.O.
Umbelliferone	15[b)]	H.m.	Sitosterol	40[c)]	Com [Gas.]
Esculetin	5	Com [Roth]	Squarene	2	Com [T.K.]
Esculin	8	Com [Roth]	Oleanolic acid	73[c)]	Com [Sar.]
Phyllodulcin	26[c)]	H.m.	Ursolic acid	90[c)]	Com [Sar.]
α-Tocopherol	50[c)]	Com [T.K.]	Glycyrrhetic acid	97[c)]	Com [Nak.]
Rubrofusarin	−24	C.o.	Glycyrrhizin	44[c)]	f.M.
Khellin	18	Com [Roth]	Caffeine	66[c)]	Com [Ald.]

Compounds were applied 30 min before TPA; ear thickness was determined at 8 h after TPA treatment. *a*) Inhibition ratio at 2 mg/ear. *b*) $p<0.05$, *c*) $p<0.01$ by Student's *t* test as compared to the control group. Abbreviations: C.o., *Cassia obtusifolia*; C.t., *C. torosa*; Com [Ald.], commercial reagent [Aldrich]; Com [Gas.], commercial reagent [Gasukuro Kogyo]; Com [Nak.], commercial reagent [Nakarai]; Com [Roth], commercial reagent [Carl Roth]; Com [Sar.], commercial reagent [Sarget]; Com [Sig.], commercial reagent [Sigma]; Com [T.K.], commercial reagent [Tokyo Kasei]; f.M., from Minophagen; f.O., from Ogawa & Co., Ltd.; H.m., *Hydrangea macrophylla* var. *thunbergii*; R.R., Rhei Rhizoma; S.j., *Sophora japonica*.

libitum.

Assay of TPA-Induced Inflammation TPA (1 μg) dissolved in acetone (20 μl) was applied to the right ear only of ICR mice by means of a micropipette. A volume of 10 μl was delivered to both the inner and outer surfaces of the ear. A sample or its vehicle, dimethylsulfoxide–acetone (1 : 9) or chloroform (20 μl), as a control, was applied topically about 30 min before each TPA treatment. The edema was measured 8 h after TPA treatment. Application of an active sample (2, 0.4, 0.1 or 0.02 mg/ear) inhibited TPA-induced inflammation in a dose-dependent manner. For ear thickness determinations, a pocket thickness gauge with a range of 0—

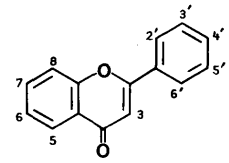
TABLE II. Inhibitory Effect of Flavone Derivatives on TPA-Induced Inflammation



Compound No.	Substituents									ED_{50}[a]	Inhib.[b]	Source
	C-5	C-6	C-7	C-8	C-2′	C-3′	C-4′	C-5′	C-6′			
F-1	H	H	H	H	H	H	H	H	H	—	17	Com [T.K.]
F-2	OH	H	H	H	H	H	H	H	H	—	32[c]	Com [Sar.]
F-3	H	OH	H	H	H	H	H	H	H	—	19	Com [Sar.]
F-4	H	H	OH	H	H	H	H	H	H	—	36[c]	Com [Sar.]
F-5	H	OMe	H	H	H	H	H	H	H	6.0	80[d]	Com [Sar.]
F-6	OH	H	OH	H	H	H	H	H	H	—	42[d]	Com [Sar.]
F-7	OMe	H	OMe	H	H	H	H	H	H	—	75[d]	Com [Sar.]
F-8	OH	OH	OH	H	H	H	H	H	H	—	42[d]	S.b.
F-9	OMe	OMe	OMe	H	H	H	H	H	H	—	28	Syn.
F-10	OH	H	OH	OMe	H	H	H	H	H	7.0	49[d]	S.b.
F-11	OH	H	OH	H	H	H	OH	H	H	6.3	75[d]	Com [Ald.]
F-12	OH	Glc	OH	Ara	H	H	OH	H	H	—	21	D.s.
F-13	OH	H	OH	H	H	OH	OH	H	H	—	15	Com [Roth]
F-14	OH	H	OR_1	H	H	OH	OH	H	H	—	31	Com [Roth]
F-15	OH	H	OH	H	H	OH	OMe	H	H	—	23	Com [Roth]
F-16	OH	H	OR_2	H	H	OH	OMe	H	H	—	20	Com [Ald.]
F-17	OH	OMe	OH	OMe	OH	H	H	H	OMe	—	43[d]	S.b.

Compounds were applied 30 min before TPA; ear thickness were determined at 8 h after TPA treatment. *a*) μmol. *b*) Inhibition ratio at 2 mg/ear. *c*) $p<0.05$, *d*) $p<0.01$ by Student's *t* test as compared to the control group. Abbreviation: Com [Ald.], commercial reagent [Aldrich]; Com [Roth], commercial reagent [Carl Roth]; Com [Sar.], commercial reagent [Sarget]; Com [T.K.], commercial reagent [Tokyo Kasei]; D.s., *Desmodium styracifolium*; S.b., *Scutellaria baicalensis*; Ara, arabinose; Glc, glucose; R_1, glucose; R_2, rutinose.

TABLE III. Inhibitory Effect of Flavonol Derivatives on TPA-Induced Inflammation



Compound No.	Substituents										ED_{50}[a]	Inhib.[b]	Source
	C-3	C-5	C-6	C-7	C-8	C-2′	C-3′	C-4′	C-5′	C-6′			
F-18	OH	H	H	H	H	H	H	H	H	H	—	34[c]	Com [T.K.]
F-19	OH	OH	H	OH	H	H	H	H	H	H	—	40[c]	Com [Roth]
F-20	OH	OH	H	OH	H	H	H	OH	H	H	4.1	73[d]	Com [T.K.]
F-21	OH	OH	H	OH	H	H	H	OMe	H	H	3.8	70[d]	Com [Roth]
F-22	OMe	OH	H	OH	H	H	H	OMe	H	H	1.8	90[d]	Com [T.K.]
F-23	OH	OH	H	OMe	H	H	H	OMe	H	H	4.4	70[d]	Com [Roth]
F-24	OMe	OH	H	OMe	H	H	H	OMe	H	H	5.3	62[d]	Com [Roth]
F-25	OH	OH	H	OR_1	H	H	H	OH	H	H	—	48[d]	Com [Roth]
F-26	OR_2	OH	H	H	H	H	H	OH	H	H	2.8	52[d]	L.m.
F-27	OH	H	H	OH	H	H	OH	OH	H	H	—	24[c]	Com [Roth]
F-28	OH	OH	H	OH	H	OH	H	OH	H	H	—	39[d]	Com [Roth]
F-29	OH	OH	H	OH	H	H	OH	OH	H	H	6.6	50[d]	Com [T.K.]
F-30	OR_3	OH	H	OH	H	H	OH	OH	H	H	—	43[d]	S.j.
F-31	OH	OH	H	OH	H	H	OH	OH	OH	H	—	37[d]	M.r.
F-32	OR_4	OH	H	OH	H	H	OH	OH	OH	H	3.4	53[d]	M.r.
F-33	OMe	OH	OMe	OMe	H	H	OH	OH	H	H	—	32[d]	V.r.

Compounds were applied 30 min before TPA; ear thickness were determined at 8 h after TPA treatment. *a*) μmol. *b*) Inhibition ratio at 2 mg/ear. *c*) $p<0.05$, *d*) $p<0.01$ by Student's *t* test as compared to the control group. Abbreviations: Com [Roth], commercial reagent [Carl Roth]; Com [T.K.], commercial reagent [Tokyo Kasei]; L.m., *Lysimachia mauritiana*; M.r., *Myrica rubra*; S.j., *Sophora japonica*; V.r., *Vitex rotundifolia*; R_1, neohesperidose; R_2, 2-rhamnosylrobinobiose; R_3, rutinose; R_4, rhamnose.

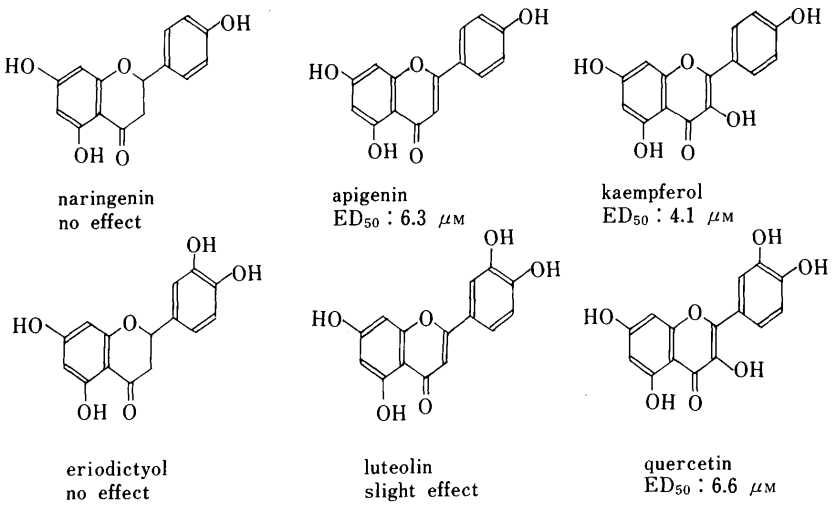


Fig. 1. Relative Inhibitory Activities of Flavanone, Flavone and Flavonol Derivatives on TPA-Induced Inflammation

9 mm, graduated at 0.01-mm intervals and modified so that the contact surface area was increased, thus reducing the tension, was applied to the tip of the ear. Each value was the mean of individual determinations from 5 mice, and ED_{50} values were determined by graphic interpolation of data for at least 4 dose levels.

Results and Discussion

Various chemical constituents from plants were tested for their ability to reduce the intensity of TPA-induced ear edema (Table I). The tumor promoter inhibitors, quercetin,[9] morin,[9] oleanolic acid, ursolic acid,[10] glycyrrhetic acid[11] and caffeine,[12] efficiently suppressed the TPA-induced ear edema. The lipoxgenase inhibitors, quercetin, kaempferol and baicalein, inhibited ear edema development more markedly than the cyclooxygenase inhibitor, paeonol, which had a more moderate effect. The flavonoids showed inhibition of TPA-induced ear edema (Tables II and III). Of the various flavonoids, the same flavones and flavonols were proved to inhibit inflammation, whereas flavanones and (+)-catechin had no effect. Apigenin and kaempferol types were more effective than the other types. These compounds have oxygen functions at the 5, 7 and 4′ positions of the flavone structure. In flavonols and flavones of the same type, the flavonol derivatives showed greater inhibition than flavone derivatives (Fig. 1). On the basis of the inhibitory activities of flavonoid derivatives, a double bond at C-2 and C-3 of the flavonoid structure is considered to be a prerequisite for anti-tumor-promoting activity. The results of this screening assay using TPA-induced ear edema were in accord with those[9-12] on anti-tumor promoter activity in two-stage carcinogenesis. Since flavonoids and triterpenes are widely distributed in the plant kingdom in fruits and vegetables, they are likely to be of importance for the prevention of cancer.

Acknowledgement This study was supported in part by Grants-in-Aid for Scientific Research, Nihon University.

References

1) A part of this work was presented at the 2nd International Symposium on Plant Flavonoids in Biology and Medicine, Strasbourg, September 1987; V. Cody, E. Middleton, Jr., J. B. Harborne and A. Beretz (eds.), "Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties," by Alan R. Liss, Inc., New York, 1988, p. 247.
2) I. Beremblum, *Cancer Res.*, **1**, 807 (1941).
3) E. Hecker, "Carcinogenesis," Vol. 2, ed. by T. J. Slaga, A. Sivak and R. K. Boutwell, Raven Press, New York, 1978, p. 11.
4) R. K. Boutwell, "Carcinogenesis," Vol. 2, ed. by T. J. Slaga, A. Sivak and R. K. Boutwell, Raven Press, New York, 1978, p. 49.
5) E. Hecker, *Cancer Res.*, **28**, 2338 (1968).
6) B. L. VanDuuren, *Prog. Exp. Tumor Res.*, **11**, 31 (1969).
7) M. Gschwendt, W. Kittstein, G. Furstenberger and F. Marks, *Cancer Lett.*, **25**, 177 (1984).
8) J. M. Young, B. M. Wagner and D. A. Spires, *J. Invest. Dermatol.*, **80**, 48 (1983).
9) R. Kato, T. Nakadate, S. Yamamoto and T. Sugimura, *Carcinogenesis*, **4**, 1301 (1983); T. Nakadate, S. Yamamoto, E. Aizu and R. Kato, *Gann*, **75**, 214 (1984).
10) H. Tokuda, H. Ohigashi, K. Koshimizu and Y. Ito, *Cancer Lett.*, **33**, 279 (1986).
11) H. Nishino, K. Yoshioka, A. Iwashima, H. Takigawa, S. Konishi, H. Okamoto, H. Okabe, S. Shibata, H. Fujiki and T. Sugimura, *Jpn. Cancer Res.*, **77**, 33 (1986).
12) K. Yasukawa, M. Takido, M. Takeuchi and S. Nakagawa, *Yakugaku Zasshi*, **108**, 794 (1988).