Antitumor-promoting activity of oligomeric proanthocyanidins in mouse epidermis *in vivo*

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Abstract. The flavanoid catechin and heterogenous samples of oligomeric proanthocyanidins extracted from various sources were compared for their ability to inhibit the biochemical and biological effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) in mouse epidermis *in vivo*. Topical applications of catechin fail to alter the hydroperoxide response to TPA but inhibit the induction of ornithine decarboxylase (ODC) activity and, to a lesser degree, the stimulation of RNA, protein, and DNA synthesis caused by this tumor promoter. Under similar conditions, condensed tannins (CTs) from guamuchil, loblolly pine, and southern red oak barks inhibit to various degrees all these biochemical markers of TPA promotion. The most effective antioxidant, loblolly pine bark CT, also inhibits TPA-induced ODC activity and macromolecule synthesis to a much greater degree than catechin or the other CTs tested. Pecan nut pith CT, however, has no inhibitory activity in this system. Pretreatments with 4 and 12 mg of loblolly pine bark CT remarkably inhibit the incidence and yield of skin tumors promoted by TPA in initiated mice, whereas similar doses of catechin are ineffective. Loblolly pine bark CT inhibits the 2nd rather than the 1st stage of tumor promotion. In contrast to their monomer units, therefore, some naturally occurring polyflavanoids have antioxidant activities and may be valuable against tumor propagation but their efficacy may vary considerably depending on their origin and structure.

Introduction

Multistage skin carcinogenesis is a sequence of tumor initiation, stage 1 (conversion) and stage 2 (propagation) promotion, and progression (1,2). Because the mutagenic events of tumor initiation are irreversible, one important approach to the prevention of cancers is to identify natural products that can inhibit the reversible propagation stage of tumorigenesis. Epidermal ODC induction, hydroperoxide (HPx) production, and RNA, protein and DNA synthesis are biochemical markers of skin tumor promotion by TPA that can be used to screen potential anti-tumor promoters worth testing in the classic 2-step model of skin carcinogenesis (1-5).

Hydrolyzable tannins (HTs) and CTs are polyphenolic compounds widely distributed in the plant kingdom and believed to provide a chemical defense against predation and ultraviolet (UV) radiation (6,7). The HTs have a sugar core with pendant esterified gallic acid (GA) (gallotannins) or hexahydroxydiphenic acid (ellagitannins) constituents and may possess a variable number of depsidically linked galloyl units in a polygalloyl chain. Upon acid hydrolysis, gallotannins and ellagitannins respectively yield GA and ellagic acid (EA). The CTs, or more correctly proanthocyanidins or polyflavanoids, derive from the condensation of flavan-3,4-diol (Fig. 1). The CTs have no sugar, do not readily break down, and almost invariably contain one or several types of flavan-3-ol monomer units, such as (+)-catechin, (+)-gallocatechin, (-)-epicatechin, and (-)-epigallocatechin (Fig. 1). These CT monomer units are flavanoids consisting of 2 aromatic rings A and B joined through a pyran ring C (Fig. 1). Ten classes of CTs are distinguished on the basis of the hydroxylation patterns of the A- and B-rings. In oligomeric and polymeric CTs, a variable number of these skeletons are bound together by interflavanoid linkages occurring at one or more sites (Fig. 1). Oligomers and polymers of catechin and epicatechin linked from the C-4 of one flavanoid unit to the C-8 or C-6 of an adjacent flavanoid unit are known as the procyanidins, the most common type of proanthocyanidins found in nature. While the C-4→C8 type of interflavanoid linkage predominates (Fig. 1), it is not, however, exclusive (6,7).

EA and gallotannins extracted from various sources, such as tannic acid (TA), decrease mutation, tumor initiation, and complete carcinogenesis and have recently been shown in our laboratory to inhibit the tumor-promoting activity of TPA in mouse skin (for review see refs. 8-11). The present investigation was undertaken to determine whether

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heterogenous CT samples can also inhibit the biochemical and biological effects of TPA in mouse epidermis *in vivo*. Our objective was to establish whether various oligomeric proanthocyanidins extracted from specific sources had different antitumor-promoting effects and were more effective than their flavan-3-ol monomeric units in inhibiting the tumor-promoting activity of TPA.

Materials and methods

Treatment of mice. Female CF-1 mice from Sasco Inc. (Omaha, NE), 7 weeks old, were housed and maintained, and their dorsal skins were shaved before experimentation (4). The solutions of TPA, mezerein (both from LC Services Corp., Woburn, MA), and 7,12-dimethylbenz[a]anthracene (DMBA) (Eastman Kodak Co, Rochester, NY) were prepared in acetone and delivered to the shaved backs of mice in a volume of 0.2 ml. Catechin (Sigma Chemical Co., St. Louis, MO) was dissolved in acetone. Oligomeric CTs were isolated from extracts of guamuchil bark (*Pithecellobium dulce*), loblolly pine bark (*Pinus taeda*), pecan nut pith (*Carya illinoiensis*), and southern red oak inner bark (*Quercus falcata*) and dissolved in a mixture of water:ethanol:acetone (18:18:64). Voucher specimens are deposited with the Southern Forest Experiment Station. All doses of flavanoids were applied topically in 0.4-ml aliquots 20 min before, and to the same area of skin as, each application of tumor promoter. Controls were treated with acetone and vehicle only and in every experiment all mice received the same volume of solvent.

Determination of ODC activity. The epidermal preparations from 2 mice were pooled in 3 ml of 25 mM Tris-HCl buffer, pH 7.6, containing 4 mM dithiothreitol, 1 mM EDTA, and 0.2 mM pyridoxal 5'-phosphate, homogenized, centrifuged, and ODC activity was determined in 0.1-ml aliquots of the clear soluble supernatants by measuring the release of $^{14}CO_2$ from L[1-^{14}C]ornithine-HCl (55 mCi/mmol; American Radiolabeled chemicals, St. Louis, MO) (8). The protein concentration of the epidermal samples was assayed with Bio-Rad dye reagent (Bio-Rad Laboratories, Richmond, CA).

Determination of HPx production. The epidermal preparations from 2 mice were pooled in 12.5 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 118 mM NaCl, 5.36 mM KCl, 1 mM $CaCl_2$, 0.84 mM $MgSO_4$ and 5 mM dextrose, homogenized, filtered through 3 layers of surgical gauze and centrifuged at 30,000 x g for 30 min. Aliquots of these final supernatants were incubated in the presence or absence of 5 mM NaN_3 for 3 h at 37°C and the HPx contents of the epidermal samples were assayed spectrophotometrically by a modification of the ferrithiocyanate method (3,4,9). The absorption of the red ferrithiocyanate complex formed in the presence of peroxide was measured against a reagent blank at 480 nm and the levels of HPx were quantitated with reference to calibration curves prepared under similar conditions with standards of H_2O_2 ranging from 10 to 200 μM. The background levels of HPx in control epidermal samples incubated without NaN_3 were subtracted from each value.

Determination of macromolecule synthesis. The rates of incorporation of [5-^{3}H(N)]cytidine (22 Ci/mmol; Moravek Biochemicals Inc., Brea, CA) into epidermal RNA, [4,5-^{3}H]leucine (100 Ci/mmol; Moravek) into epidermal protein, and [methyl-^{3}H]thymidine (51 Ci/mmol; Amersham Corp., Arlington Heights, IL) into epidermal DNA were determined in groups of mice respectively sacrificed 6, 12, or 16 h after TPA treatment (5). The mice received an i.p. injection of 30 μCi of either ^{3}H-cytidine, ^{3}H-leucine, or ^{3}H-thymidine 40 min before the indicated times. Control mice treated only with acetone and vehicle were sacrificed after the same 40-min periods of pulse-labelling. Epidermal homogenates were prepared from 2 mice, the macromolecules were precipitated by acidification with $HClO_4$, and the acid-insoluble pellets were washed essentially as described previously (5). RNA, protein, and DNA were hydrolyzed from the precipitate with 3 ml of either 0.3 N KOH for 3 h at 37°C, 0.5 N NaOH for 30 min at 80°C, or 0.5 N $HClO_4$ for 15 min at 90°C, respectively. The radioactivity incorporated in each sample was estimated by liquid scintillation counting in 0.2-ml aliquots of the above hydrolysates. The RNA and DNA contents of the samples were respectively determined by the orcinol reaction and the diphenylamine procedure (5). Data of all biochemical experiments were analyzed using Student's t-test with the level of significance set at $P<0.05$. Basal levels (controls) were subtracted from TPA-stimulated levels (TPA) to calculate the magnitudes of the ODC, HPx, RNA, protein, and DNA responses to TPA (% of TPA effect) in Tables I-III.

Tumor induction experiments. In the initiation-complete tumor promotion protocol (1,2), skin tumors were initiated in all female CF-1 mice by a single topical application of 25.6 μg of DMBA. Two weeks later, all mice were promoted twice a week (on days 1 and 4) for 20 weeks with 5.2 μg of TPA. In the initiation-2-stage promotion protocol (1,2), skin tumors were initiated in all female SENCAR mice (from Harlan Sprague Dawley Inc., Indianapolis, IN) by a single application of 6.4 μg of DMBA. Two weeks later, all mice were promoted twice a week for 2 weeks with 2.6 μg of TPA (stage 1) and then twice a week for 18 weeks with 2.8 μg of mezerein (stage 2). Various doses of catechin or loblolly pine bark CT were applied 20 min before either each TPA treatment in complete and stage 1 tumor promotion or each mezerein treatment in stage 2 tumor promotion. Initially, there were 32 mice in each treatment group. The incidence and yield of skin tumors were respectively recorded weekly and once every 2 weeks. Statistics for the differences between the means of papillomas (PAs)/mouse were performed using Student's t-test, whereas differences between PA incidences were compared using the X^2 statistic. The level of significance was set in both cases at $P<0.05$.

Results

The chain extender and terminal units of oligomeric CTs consist of similar monomers, such as catechin or epicatechin or their pyrogallol B-ring analogues (Fig. 1). Oligomeric CTs, however, have variable numbers of units/molecules. The induction of epidermal ODC activity observed 5 h after the last of 2 applications of TPA at a 72-h interval is much

Table I. Comparison of the inhibitory effects of various doses of monomeric and oligomeric flavanoids on TPA-induced ODC activity in mouse epidermis *in vivo*.

Treatment[a] (dose/application)	ODC activity at 5 h[b]		
	nmol CO_2/h/ mg protein[c]	% of control	% of TPA
Control	0.32 ± 0.03	100	
TPA (5.2 μg)	10.84 ± 0.88	3387	100
+ Catechin (1.33 mg)	9.72 ± 1.36[d]	3039	89
+Loblolly pine bark CT (1.33 mg)	8.53 ± 0.54[e]	2664	78
+Catechin (4 mg)	7.05 ± 0.85	2204	64
+Loblolly pine bark CT (4 mg)	5.85 ± 0.52[f]	1829	53
+Catechin (12 mg)	5.23 ± 0.70	1635	47
+Loblolly pine bark CT (12 mg)	3.14 ± 0.26[g]	981	27
+Guamuchil bark CT (12 mg)	9.44 ± 0.56[h]	2950	87
+Pecan nut pith CT (12 mg)	10.32 ± 0.86[d]	3226	95
+Southern red oak inner bark CT (12 mg)	7.57 ± 0.85	2365	69

[a]Catechin and CTs applied 20 min before each TPA treatment; [b]After 2 TPA treatments at a 72-h interval; [c]Means ± SD (n=6); [d]Not significantly different from TPA; [e]P<0.0005 and P<0.05, respectively smaller than TPA and TPA + catechin (1.33 mg); [f]P<0.025, significantly smaller than TPA + catechin (4 mg); [g]P<0.0005, significantly smaller than TPA + catechin (12 mg); [h]P<0.01, significantly smaller than TPA.

Table II. Comparison of the inhibitory effects of various doses of monomeric and oligomeric flavanoids on TPA-stimulated HPx production in mouse epidermis *in vivo*.

Treatment[a] (dose/application)	HPx production at 16 h[b]		
	nmol H_2O_2/3 h/ mg protein[c]	% of control	% of TPA
Control	10.1 ± 0.7	100	
TPA (5.2 μg)	32.0 ± 0.8	317	100
+ Catechin (1.33 mg)	33.9 ± 2.3[d]	335	109
+ Loblolly pine bark CT (1.33 mg)	26.3 ± 0.8[e]	261	74
+ Catechin (4 mg)	30.8 ± 1.0[d]	305	94
+ Loblolly pine bark CT (4 mg)	21.0 ± 1.6	208	50
+ Catechin (12 mg)	32.6 ± 1.6[d]	323	103
+ Loblolly pine bark CT (12 mg)	16.2 ± 1.8	160	28
+Guamuchil bark CT (12 mg)	26.1 ± 1.6[f]	258	73
+Pecan nut pith CT (12 mg)	34.3 ± 2.5[d]	340	
+Southern red oak inner bark CT (12 mg)	28.0 ± 1.9[g]	277	

[a]Catechin and CTs applied 20 min before each TPA treatment; [b]After 2 TPA treatments at a 48-h interval; [c]Means ± SD (n=4); [d]Not significantly different from TPA; [e]P< 0.0005, [f]P<0.005 and [g]P<0.01, significantly smaller than TPA.

greater than after a single of these treatments (8). Pretreatments with 12 mg of catechin or loblolly pine bark CT respectively inhibit TPA-induced ODC activity by 53 and 73% (Table I). Similar treatments with oligomeric CTs from southern red oak and guamuchil barks inhibit the ODC response to TPA to a lesser degree than the monomeric flavanoid. Pecan nut pith CT has no inhibitory effect (Table I). The inhibitory effect of loblolly pine bark CT is dose dependent and consistently greater than that of catechin, which disappears at a dose of 1.33 mg (Table I).

The increased HPx-producing activity of the TPA-treated epidermis has been characterized and its relevance to the mechanism of skin tumor promotion has been discussed (2-4,9). The stimulation of epidermal HPx production is near-maximal 16 h after the last of 2 applications of TPA at a 48-h interval (9). In contrast to its inhibition of TPA-induced ODC activity (Table I), catechin fails to alter the HPx response to TPA (Table II). But loblolly pine bark CT (12 mg) inhibits TPA-stimulated HPx production by 72%. Again, the CTs extracted from the barks of guamuchil or southern red oak are less effective than loblolly pine bark CT and the sample of pecan nut pith CT lacks inhibitory activity (Table II). The antioxidant activity of loblolly pine bark CT is dose dependent and persists at 1.33 mg (Table II).

Sequential increases in epidermal RNA, protein, and DNA synthesis respectively occur 6, 12 and 16 h after TPA treatment (5). The alteration of DNA synthesis by TPA is characterized by an early 12-h period of inhibition followed by 2 peaks of maximal stimulation at about 16 and 32 h (4,5). Pretreatments with 12 mg of catechin or CT extracts from guamuchil and loblolly pine barks inhibit the stimulation of DNA synthesis observed 16 h after TPA by 30, 26 and 51%, respectively, whereas pecan nut pith CT and southern red oak inner bark CT are ineffective (Table III). Loblolly pine bark CT also decreases the inhibition and stimulation of DNA synthesis caused by TPA at 8 and 32 h, respectively (data not shown). Moreover, oligomeric CTs from loblolly pine bark inhibit TPA-stimulated RNA, protein, and DNA synthesis in a dose dependent manner and consistently more than the monomeric precursor of proanthocyanidin synthesis, catechin (Table III). Overall, the effects of epicatechin on TPA-induced ODC activity, HPx production, and macromolecule synthesis are identical with those of catechin and have been

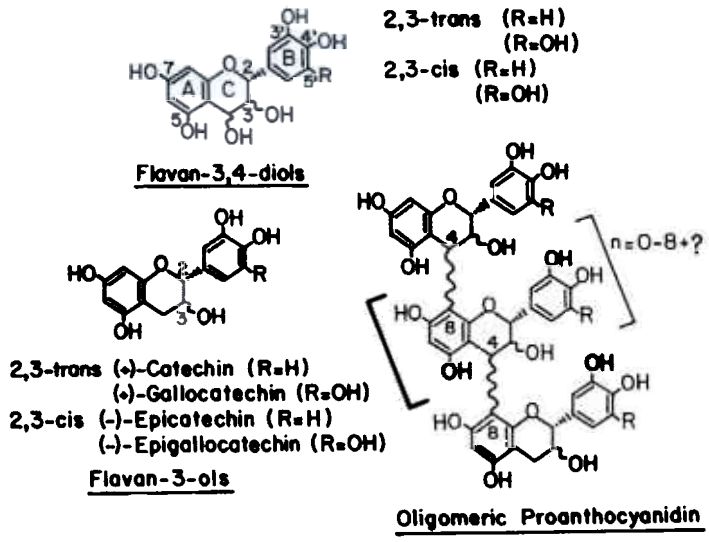

Figure 1. Chemical structures of oligomeric proanthocyanidins and their monomeric precursors.

Table III. Comparison of the inhibitory effects of various doses of monomeric and oligomeric flavanoids on TPA-stimulated macromolecule synthesis in mouse epidermis *in vivo*.

Treatment[a] (dose/application)	RNA synthesis at 6 h[b] cpm/μg RNA[d]	% of control	% of TPA	Protein synthesis at 12 h[b] cpm/μg protein[d]	% of control	% of TPA	DNA synthesis at 16 h[c] cpm/μg DNA[d]	% of control	% of TPA
Control	14.3 ± 1.9	100		2.5 ± 0.5	100		31.2 ± 3.1	100	
TPA (5.2 μg)	40.1 ± 2.8	280	100	8.8 ± 0.4	352	100	256.0 ± 14.6	821	100
+Catechin(4 mg)	34.5 ± 4.1[e]	241	78	8.0 ± 0.5[e]	319	87	215.8 ± 22.9[f]	692	82
+Loblolly pine bark CT (4mg)	27.1 ± 4.6[g]	189	50	6.6 ± 0.6[h]	263	65	168.8 ± 20.2[h]	541	61
+Catechin (12 mg)	32.5 ± 5.5	228	71	7.2 ± 1.1	287	74	188.1 ± 23.5	603	70
+Loblolly pine bark CT (12 mg)	24.4 ± 3.7[i]	171	39	5.7 ± 0.4[i]	228	51	142.3 ± 21.2[j]	456	49
+Guamuchil bark CT (12 mg)							196.9 ± 15.0	631	74
+Pecan nut pith CT (12 mg)							268.6 ± 43.0[k]	861	106
+Southern red oak inner bark CT (12 mg)							229.9 ± 27.4[k]	737	88

[a]Catechin and CTs applied 20 min before each TPA treatment; [b]After a single TPA treatment; [c]After 2 TPA treatments at a 72-h interval; [d]Means ± SD (n=4); [e]P<0.05 and [f]P<0.025, significantly smaller than TPA; [g]P<0.05 and [h]P<0.025, significantly smaller than TPA +catechin (4 mg); [i]P<0.05 and [j]P<0.025, significantly smaller than TPA + catechin (12 mg); [k]Not significantly different from TPA.

deleted from the results. As observed in our previous TA studies (8,9,11), loblolly pine bark CT post-treatments applied 20 min after the tumor promoter can also inhibit the ODC, HPx, and DNA markers of TPA promotion (data not shown), suggesting that the pretreatments of skin with oligomeric proanthocyanidins do not simply produce a barrier inhibiting the penetration of TPA and its interaction with target epidermal cells.

The abilities of loblolly pine bark CT and catechin to inhibit complete tumor promotion by TPA were compared at 4 and 12 mg, the doses used to characterize their effectiveness against the biochemical markers of tumor promotion. In spite of its lack of antioxidant activity, catechin was tested in tumorigenesis experiment to determine whether its weak inhibitions of the ODC and macromolecule responses to TPA were sufficient to significantly inhibit skin

Table IV. Comparison of the inhibitory effects of loblolly pine bark CT on the induction of mouse skin PAs by the complete and two-stage tumor promotion protocols.

Group	Tumor promotion treatment (2x/wk)[a] (dose/application)	wk of 1st PA	Observations at 20 wks				
			wt/ mouse (g)	% of survival	% of mice with PAs	PAs/mouse No. means ± SD	PAs/mouse % of groups 1 or 6
Complete promotion protocol with CF-1 mice (20 wks)							
1.TPA (5.2 μg)		6	33.7	96.9	96.8	11.29 ± 2.42	100
2.+ Catechin (4 mg)		6	32.7	100.0	96.9[b]	9.34 ± 2.74[b]	83
3.+ Loblolly pine bark CT (4 mg)		7	32.8	96.9	54.8	2.58 ± 1.63	
4.+ Catechin (12 mg)		6	33.4	100.0	90.6[b]	8.66 ± 1.98[b]	
5.+ Loblolly pine bark CT (12 mg)		12	33.0	100.0	6.5	0.10 ± 0.12	
Two-stage promotion protocol with SENCAR mice							
Stage 1 (4x/2 wks)	Stage 2 (36x/18 wks)						
6.TPA (2.6 μg)	Mezerein (2.8 μg)	6	38.6	100.0	100.0	8.29 ± 1.44	100
7.+Loblolly bark CT (12 mg)	Mezerein	6	38.7	100.0	100.0	7.57 ± 2.27[b]	91
8. TPA		6	39.5	100.0	33.3	17 ± 0.55	14

11,13). The antitumor-promoting activity of these naturally occurring polyphenols, however, is unlikely to be explained on the basis of their tanning activity. Monomers and dimers do not possess significant tanning activity. In general, tanning ability appears at the trimeric level and increases in parallel with the MW of HTs and CTs (6,7,12). But the ellagi- and gallotannin monomers EA and GA have significant antitumor-promoting effects in spite of their lack of tanning activity (8-11,13). Moreover, semisynthetic monomeric flavanoid derivatives, such as catechin dialkyl ketals and epicatechin-4-alkysulphides, are antioxidants and elicit much better inhibitory effects on TPA-induced ODC activity and macromolecule synthesis than catechin or epicatechin, even though they have no more tanning potential than their parent molecules (14). Conversely, chestnut wood ellagitannin (*Castanea dentata*) (15) and pecan nut pith CT fail to inhibit some of the biochemical responses to TPA in spite of their tanning activity.

Tumor promoters enhance the generation of reactive O_2 species (ROS) by inflammatory and epidermal cells and decrease their degradation (2,3,16-19). The prolonged HPx response to TPA is consistent with the decreased capacity for H_2O_2 detoxification by catalase throughout the epidermis (3,9,20). The kinetics of TPA-induced xanthine oxidase (XO) activity and HPx production are identical *in vivo*, suggesting a causal relationship between these 2 events (3,9,21). Even though catechin inhibits to some degree the ODC and macromolecule markers of TPA promotion, the lack of antitumor-promoting activity of this CT monomer is likely to be due to its inability to mimic the antioxidant effects of oligomeric CTs and inhibit the prolonged HPx response linked to skin tumor promotion. Conversely, loblolly pine bark CT may be an effective anti-tumor promoter because of its potent antioxidant activity and stronger inhibitory effects on ODC induction and macromolecule synthesis. Tannins, which have a high reducing power and form complexes with various metal ions and cofactors, may chelate Fe and inhibit the Fe-catalyzed reactions generating free radicals (6,7,12). The time course of TPA-stimulated HPx production after oligomeric proanthocyanidin pretreatment (data not shown) is similar to that previously observed after TA pretreatment, suggesting that HTs and CTs may both inhibit the TPA-activated enzymes generating ROS and scavenge the ROS produced (9).

The ability of catechin to inhibit TPA-induced ODC activity without altering TPA-stimulated HPx production confirms the dissociation observed between these 2 responses, which are essential but not sufficient for skin tumor promotion (3,4). Polyphenolic antioxidants, therefore, do not inhibit the HPx response to TPA because of their potency against ODC induction, or vice versa. Complementary effects resulting from ODC induction, DNA synthesis, and cell proliferation may be required to fully maintain hyperplasia and achieve tumor promotion (4). The effectiveness of CTs and HTs as inhibitors of tumor promotion is likely to reflect the combination of their inhibitory effects against the 3 ODC, HPx, and DNA markers of TPA promotion (10,15). ODC induction is an excellent biochemical marker of stage 2 promotion (1). In general, different classes of tumor promoters induce XO activity and HPx production in relation with their activities in complete or stage 2 promotion (3,9,21). Moreover, antioxidant treatments

are also more effective against stage 2 tumor promotion (2). Since loblolly pine bark CT is a powerful antioxidant that fails to inhibit the 1st stage of tumor promotion, our results suggest that i) ROS are unlikely to play a major role in stage 1 promotion, and ii) the effectiveness of loblolly pine bark CT against complete skin tumor promotion may be linked to its ability to inhibit the stage 2 rather than the stage 1 tumor-promoting effects of TPA. Tannins may inhibit proteolytic reactions by forming soluble and insoluble complexes with enzymes and/or their substrates (22). But loblolly pine bark CT is unlikely to inhibit the activities of proteolytic enzymes involved in the mechanism of skin tumor promotion by TPA, since protease inhibitors inhibit stage-1 tumor promotion by TPA but neither stage-2 tumor promotion by mezerein nor the ODC, XO, HPx, and hyperplastic responses to TPA in mouse epidermis *in vivo* (1,23,24).

Chronic CT or HT treatments do not alter the body weight, rate of survival, and skin morphology of the mice in our long-term tumor experiments, suggesting that cytotoxicity is unlikely to explain their antitumor-promoting activity (10,11). Moreover, the ability of loblolly pine bark CT to maintain the incidence and yield of skin tumors inhibited after 20 weeks of complete or 2-stage tumor promotion suggests that polyflavanoids decrease the tumor-promoting activities of TPA and mezerein and do not simply delay or slow down the rate of PA development. As previously observed with TA (8), the inhibition of TPA-induced ODC activity by oligomeric proanthocyanidins is reversible and cannot be explained on the basis of cytotoxicity, pH fluctuation, or traces of polyphenols directly interacting with components of the enzyme assay, suggesting that both HTs and CTs interfere with the action of TPA and/or the molecular pathways regulating enzyme activities (8). Similarly, both HTs and CTs fail to alter TPA-induced DNA synthesis at 16 h when they are applied 15 h after the tumor promoter, i.e. 20 min before the 40-min period of pulse-labeling with ^{3}H-thymidine (11,14,15). Thus, tannins inhibit the mechanism by which TPA stimulates DNA synthesis without directly interfering with the incorporation of precursors into this nucleic acid. The inhibitory effects of tannins *in vivo* are not simply due to nonspecific protein complexation and enzyme inactivation since these compounds can stimulate or facilitate the activities of epidermal enzymes involved in xenobiotic detoxification and antioxidation, such as glutathione S-transferases (for review see ref. 9).

Comparative studies with monomeric, dimeric, and trimeric procyanidins purified from Douglas-fir bark CT (*Pseudotsuga menziesii*) suggest that the antioxidant activity of CTs may increase with the number of monomer units attached by interflavanoid linkage in their molecules (25). The functionality of the A-ring is related to resorcinol, phloroglucinol, or pyrogallol whereas that of the B-ring is related to phenol, catechol, or pyrogallol (6,7,12). The reactivity of the A-ring is of primary importance to the formation of interflavanoid bonds and high MW polymerized structures that may complex proteins and inactivate enzymes. Both OH groups of A- and B-rings may synergistically interact with proteins (7,26). The metal-chelating, antioxidant, and free radical-scavenging activities of CTs may be associated with the reducibility of the B-ring, which is proportional to the increasing number of OH substituents in phenol, catechol, or pyrogallol. Indeed, the antioxidant and antiviral activities of CTs increase with the number of pyrogallol moieties in the molecule and the degree of polymerization (6,7,12).

Epigallocatechin gallate (EGCG), a CT monomer with a GA substituent (6,7,12), is the main polyphenolic constituent of green tea (*Camellia sinensis*) and inhibits the specific binding of ^{3}H-TPA to mouse skin, the number of phorbol ester receptors, the activation of protein kinase C (PKC) by teleocidin, TPA-induced ODC activity, H_2O_2 formation and oxidative DNA damage, and skin tumor promotion by TPA, teleocidin, or UV-B radiation in DMBA-initiated mice (27-31). However, catechin does not inhibit tumor promotion or the activation of PKC by teleocidin (32). And in contrast to *n*-propyl gallate, catechin and epicatechin fail to inhibit, or inhibit to a lesser degree, TPA-induced epidermal lipoxygenase and ODC activities, DNA synthesis, and skin tumor promotion (8-10,33). Since a single flavanoid skeleton has no or very little antitumor-promoting effects as compared to GA and its derivatives (8-10,13-15), the GA moiety of EGCG may be essential for the antioxidant, anti-inflammatory, and antitumor-promoting activities of this flavan-3-ol monomeric unit (34). The galloylation of flavanoids strongly influences their degree of astringency and protein binding affinity (35). Addition of GA groups as esters at C-3 of the pyran ring increases the cytotoxicity and antiviral activity of various CTs (7,12). Moreover, the activation of human leukocytes and HL-60 cells by EGCG may be attributable to the special location of the GA group relative to that of the B-ring in the flavanoid skeleton (36). Direct interactions between procyanidins and proteins have been shown to increase with the number of GA substituents and with the degree of polymerization (37). The antioxidant properties of procyanidins are also influenced by galloylation and the position of the GA substituents (Gali HU *et al*: Proc Am Assoc Cancer Res 34: 180, 1993). Incidentally, the non-TPA-type tumor promoter thapsigargin, which neither binds to the phorbol ester receptor nor induces PKC activity, triggers to various degrees the biochemical markers of TPA promotion and these thapsigargin effects can also be inhibited by tannins (24). Since the biochemical markers of tumor promotion may be mediated by PKC-dependent and -independent pathways depending on the type of triggering agent, CTs and HTs are unlikely to inhibit the effects of TPA- and non-TPA-type tumor promoters solely because they decrease binding to the phorbol ester receptor or PKC activation and down-regulation.

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References

1. Slaga TJ: Multistage skin tumor promotion and specificity of inhibition. In: Mechanisms of Tumor Promotion. Slaga TG (ed.) CRC Press, Boca Raton, FL, Vol. 2: pp189-196, 1984.
2. Perchellet JP and Perchellet EM: Antioxidants and multistage carcinogenesis in mouse skin. Free Radical Biol Med 7: 377-408, 1989.
3. Perchellet EM and Perchellet JP: Characterization of the hydroperoxide response observed in mouse skin treated with tumor promoters *in vivo*. Cancer Res 49: 6193-6201, 1989.
4. Perchellet EM, Jones D and Perchellet JP: Ability of the Ca^{2+} ionophores A23187 and ionomycin to mimic some of the effects of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate on hydroperoxide production, ornithine decarboxylase activity, and DNA synthesis in mouse epidermis *in vivo*. Cancer Res 50: 5806-5812, 1990.
5. Baird WM, Sedgwick JA and Boutwell RK: Effects of phorbol and four diesters of phorbol on the incorporation of tritiated precursors into DNA, RNA, and protein in mouse epidermis. Cancer Res 31: 1434-1439, 1971.
6. Haslam E: Chemistry and Pharmacology of Natural Products; Plant Polyphenols: Vegetable Tannins Revisited. Cambridge University Press, Cambridge, 1989.
7. Hemingway RW and Karchesy JJ: Chemistry and Significance of Condensed Tannins. Plenum Press, New York, NY, 1989.
8. Gali HU, Perchellet EM and Perchellet JP: Inhibition of tumor promoter-induced ornithine decarboxylase activity by tannic acid and other polyphenols in mouse epidermis *in vivo*. Cancer Res 51: 2820-2825, 1991.
9. Gali HU, Perchellet EM, Klish DS, Johnson JM and Perchellet JP: Hydrolyzable tannins: potent inhibitors of hydroperoxide production and tumor promotion in mouse skin treated with 12-O-tetradecanoylphorbol-13-acetate *in vivo*. Int J Cancer 51: 425-432, 1992.
10. Gali HU, Perchellet EM, Klish DS, Johnson JM and Perchellet JP: Antitumor-promoting activities of hydrolyzable tannins in mouse skin. Carcinogenesis 13: 715-718, 1992.
11. Gali HU, Perchellet EM, Gao XM, Bottari V and Perchellet JP: Antitumor-promoting effects of gallotannins extracted from various sources in mouse skin *in vivo*. Anticancer Res 13: 915-922, 1993.
12. Hemingway RW and Laks PE: Plant polyphenols: Synthesis, Properties, Significance. Plenum Press, New York, NY, 1992.
13. Perchellet JP, Gali HU, Perchellet EM, Klish DS and Armbrust AD: Antitumor-promoting activities of tannic acid, ellagic acid, and several gallic acid derivatives in mouse skin. In: Plant Polyphenols: Synthesis, Properties, Significance. Hemingway, RW and Laks PE (eds). Plenum Press, New York, NY, pp783-801, 1992.
14. Gali HU, Perchellet EM, Gao XM, Laks PE and Perchellet JP: Inhibitory effects of semisynthetic flavonoid derivatives on the biochemical markers of tumor promotion in mouse epidermis *in vivo*. Cancer Lett 72: 149-156, 1993.
15. Perchellet JP, Gali HU, Perchellet EM, Laks PE, Bottari V, Hemingway RW and Scalbert A: Antitumor-promoting effects of gallotannins, ellagitannins and flavonoids in mouse skin *in vivo*. In: Food Phytochemicals for Cancer Prevention. Huang M-T, Osawa T, Ho C-T and Rosen RT (eds.) American Chemical Society, Washington, DC, ACS Symposium Series 546, Vol. I: pp303-327, 1994.
16. Perchellet EM, Abney NL and Perchellet JP: Stimulation of hydroperoxide generation in mouse skins treated with tumor-promoting or carcinogenic agents *in vivo* and *in vitro*. Cancer Lett 42: 169-177, 1988.
17. Robertson FM, Beavis AJ, Oberyszyn TM, O'Connell SM, Dodikos A, Laskin DL, Laskin JD and Reiners JJ: Production of hydrogen peroxide by murine epidermal keratinocytes following treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. Cancer Res 50: 6062-6067, 1990.
18. Sirak AA, Beavis AJ and Robertson FM: Enhanced hydroperoxide production by peripheral blood leukocytes following exposure of murine epidermis to 12-O-tetradecanoylphorbol-13-acetate. Carcinogenesis 12: 91-95, 1991.
19. Frenkel K and Gleichauf C: Hydrogen peroxide formation by cells treated with a tumor promoter. Free Rad Res Commn 12-13: 783-794, 1991.
20. Reiners JJ, Thai G, Rupp T and Cantu AR: Assessment of the antioxidant/prooxidant status of murine skin following topical treatment with 12-O-tetradecanoylphorbol-13-acetate and throughout the ontogeny of skin cancer. Part I: quantitation of superoxide dismutase, catalase, glutathione peroxidase and xanthine oxidase. Carcinogenesis 12: 2337-2343, 1991.
21. Reiners JJ, Pence BC, Barcus MCS and Cantu AR: 12-O-tetradecanoylphorbol-13-acetate-dependent induction of xanthine dehydrogenase and conversion to xanthine oxidase in murine epidermis. Cancer Res 47: 1775-1779, 1987.
22. Mole S and Waterman PG: Tannic acid and proteolytic enzymes: enzyme inhibition or substrate deprivation? Phytochemistry 26: 99-102, 1987.
23. Pence BC and Reiners JJ: Murine epidermal xanthine oxidase activity: correlation with degree of hyperplasia induced by tumor promoters. Cancer Res 47: 6388-6392, 1987.
24. Perchellet EM, Gali HU, Gao XM and Perchellet JP: Ability of the nonphorbol ester-type tumor promoter thapsigargin to mimic the stimulatory effects of 12-O-tetradecanoylphorbol-13-acetate on ornithine decarboxylase activity, hydroperoxide production, and macromolecule synthesis in mouse epidermis *in vivo*. Int J Cancer 55: 1036-1043, 1993.
25. Gali HU, Perchellet EM, Gao XM, Karchesy JJ and Perchellet JP: Comparison of the inhibitory effects of monomeric, dimeric, and trimeric procyanidins on the biochemical markers of skin tumor promotion in mouse epidermis *in vivo*. Planta Med (In press).
26. Kawamoto H, Nakatsubo F and Murakami K: Synthesis of condensed tannin derivatives and their protein-precipitating capacity. J Wood Chem Technol 10: 59-74, 1990.
27. Yoshizawa S, Horiuchi T, Fujiki H, Yoshida T, Okuda T and Sugimura T: Antitumor promoting activity of (-)-epigallocatechin gallate, the main constituent of 'tannin' in green tea. Phytotherapy Res 1: 44-47, 1987.
28. Huang M-T, Ho C-T, Wang ZY, Ferraro T, Finnegan-Olive T, Lou Y-R, Mitchell JM, Laskin JD, Newmark H, Yang CS and Conney AH: Inhibitory effect of topical application of a green tea polyphenol fraction on tumor initiation and promotion in mouse skin. Carcinogenesis 13: 947-954, 1992.
29. Wang Z-W, Huang M-T, Ferraro T, Wong C-Q, Lou Y-R, Reuhl K, Iatropoulos M, Yang CS and Conney AH: Inhibitory effect of green tea in the drinking water on tumorigenesis by ultraviolet light and 12-O-tetradecanoylphorbol-13-acetate in the skin of SKH-1 mice. Cancer Res 52: 1162-1170, 1992.
30. Agarwal R, Katiyar SK, Zaidi SIA and Mukhtar H: Inhibition of skin tumor promoter-caused induction of epidermal ornithine decarboxylase in SENCAR mice by polyphenolic fraction isolated from green tea and its individual epicatechin derivatives. Cancer Res 52: 3582-3588, 1992.
31. Katiyar SK, Agarwal R, Wood GS and Mukhtar H: Inhibition of 12-O-tetradecanoylphorbol-13-acetate-caused tumor promotion in 7,12-dimethylbenz[a]anthracene-initiated SENCAR mouse skin by a polyphenolic fraction isolated from green tea. Cancer Res 52: 6890-6897, 1992.
32. Horiuchi T, Fujiki H, Hakii H, Suganuma M, Yamashita K and Sugimura T: Modulation of phorbol ester receptors in mouse skin by application of quercetin. Jpn J Cancer Res 77: 526-531, 1986.
33. Nakadate T, Yamamoto S, Aizu S and Kato R: Effects of flavonoids and antioxidants on 12-O-tetradecanoylphorbol-13-acetate-caused epidermal ornithine decarboxylase induction and tumor promotion in relation to lipoxygenase inhibition by these compounds. Gann 75: 214-222, 1984.
34. Kroes BH, van den Berg AJJ, Quarles van Ufford HC, Van Dijk H and Lebadie RP: Anti-inflammatory activity of gallic acid. Planta Med 58: 499-504, 1992.
35. Okuda T, Mori K and Hatano T: Relationship of the structures of tannins to the binding activities with hemoglobin and methylene blue. Chem Pharm Bull 33: 1424-1433, 1985.
36. Sakagami H, Hatano T, Yoshida T, Tanuma S-I, Hata N, Misawa Y, Ishii N, Tsutsumi T and Okuda T: Stimulation of granulocytic cell iodination by tannins and related compounds. Anticancer Res 10: 1523-1532, 1990.
37. Cheynier V, Rigaud J and de Silva JMR: Structure of procyanidin oligomers isolated from grape seeds in relation to some of their chemical properties. In: Plant Polyphenols: Synthesis, Properties, Significance. Hemingway RW and Laks PE (eds.) Plenum Press, New York, NY, pp281-294, 1990.