Tumorigenesis in Mouse Skin: Inhibition by Synthetic Inhibitors of Proteases

Abstract. *Three synthetic inhibitors of proteases (tosyl lysine chloromethyl ketone, tosyl phenylalanine chloromethyl ketone, and tosyl arginine methyl ester) inhibit the tumorigenesis initiated in mouse skin by 7,12-dimethylbenz*(a)*anthracene and promoted by croton oil or its active principle, phorbol ester. These protease inhibitors, when applied directly to mouse skin, inhibit some of the irritant effects of the tumor promoter and are not toxic.*

Two distinct biological processes are involved in chemical tumorigenesis in mouse skin—initiation by primary carcinogens and promotion by cocarcinogens (*1*, *2*). The mechanisms of both processes are poorly understood. Initiators (primary carcinogens) require only a single application and may produce a somatic mutation or activate an oncogenic virus. These processes cannot be readily repaired or reversed once fixed in the genome. These proposed mechanisms of initiation are at least consonant with the observation that single initiating effects last virtually for the lifetime of the animal (*2*). The mechanism by which promoters (cocarcinogens) act is even less well understood. These agents require repeated application, and the response to them is modified by a number of exogenous factors such as caloric restriction (*3*), treatment with cortisone (*4*), or polyinosinic polycytidylic acid (*5*). We now show that specific inhibitors of proteases suppress promotion of tumor by croton oil or its purified active principle, phorbol myristate acetate (hereafter phorbol ester) (*2*). These results suggest that an endogenous protease may play a role in the mechanism of cocarcinogenesis.

We used the chloromethyl ketones of tosyl lysine (TLCK) and phenylalanine (TPCK), which inhibit trypsin and chymotrypsin, respectively, by forming a covalent adduct to histidine in the active site (*6*). In addition, both compounds reportedly inhibit the sulfhydryl protease, papain, by forming a covalent compound with the essential sulfhydryl group (*7*). We also used a competitive inhibitor for trypsin and papain, tosyl arginine methyl ester (TAME). These materials were applied to mouse skin along with the promoting agents, or separately; they reduced the immediate inflammatory effects of croton oil or phorbol ester, as well as tumor promotion by these compounds.

Tumorigenesis was initiated with 10 μg of 7,12-dimethylbenz(*a*)anthracene (DMBA) and promoted with 1 μg or 0.1 μg of phorbol ester (Schuchardt-U.S.A., Katonah, N.Y.) or 5 μg of croton oil in 10 μl of acetone or dimethyl sulfoxide (DMSO) applied three times weekly to the ears of mice. Each experiment and control group contained 21 Carworth CF-1 strain animals. The time of first appearance of grossly visible tumor was noted, but only tumors larger than 1 mm and persisting over 30 days were scored. In the first experiment, 1 μg of phorbol ester was used as the promoter, and the effect of 10 μg of TLCK applied with the promoter three times a week was studied (Fig. 1A). In the control group (initiated with DMBA and then treated with phorbol ester alone) 50 percent of the animals bore tumors 50 days after promotion was begun, 100 percent had tumors at 90 days. In the group treated with TLCK, 50 percent of the animals bore tumors at 100 days; there was only 20 percent increase over this value at 200 days. In the second experiment, 1 μg of TLCK was used as the inhibitor in conjunction with 0.1 μg of phorbol ester; the onset of tumors was delayed by 100 days (Fig. 1B). The tumor response of control mice in experiment 2 (treated with 0.1 μg phorbol ester three times a week) was virtually identical to that of test mice in the first experiment (treated with 1 μg of phorbol ester three times a week, with 10 μg of TLCK as inhibitor) (Fig. 1, A and B). The incidence of tumors and the time of occurrence of first tumors were virtually the same. Thus, it can be suggested that 10 μg of TLCK inhibited 90 percent of the promoting activity of 1 μg of phorbol ester in the first experiment.

In a third experiment, 5 μg of croton oil was used as the promoter three times weekly, and the effects of 1 μg of TLCK, TPCK, or TAME were tested. In this experiment, TPCK virtually suppressed all tumor formation for 200 days, whereas TLCK and TAME delayed the appearance of visible tumors and reduced the numbers of tumor-bearing animals to less than 50 percent of the controls over the same period (Table 1).

Irritation was scored for intensity of erythema 24 hours after application of the promoting agent by a method similar to that used by Hecker and associates in their bioassay of promoting agents in croton oil (*8*). All three inhibitors of protease reduced erythema when applied for a week or longer. Inhibition of irritation was also observed upon histological examination of leukocyte infiltration in the early period after one application of phorbol ester (1 μg). Resultant leukocyte invasion was significantly reduced by a single application of 10 μg of TPCK or TLCK applied in DMSO 5 minutes after phorbol ester was applied (Fig. 2). Inhibition of erythema and leukocyte invasion was most consistently observed when the dose of antiprotease was ten times greater (by weight) than that of phorbol ester.

The inhibitors used may interfere with promotion by blocking proteolytic activity arising from interaction of the promoting agent with some tissue constituent. We were able to show that 24 hours after application of phorbol ester (1 μg) to the ears of STS mice (a tumor-sensitive strain) (*3*), the specific tissue activity against TAME, the synthetic substrate of trypsin, at neutral *p*H increases three- to fivefold over control values. The increased activity of the tissue homogenates could be completely inhibited by addition of 0.1*M* TLCK and partially by 0.1*M* TPCK (*9*). With respect to the source of protease, it has

Table 1. Inhibition of tumorigenesis by protease inhibitors. All animals were given 10 μg of DMBA as initiator and then 5.0 μg of croton oil in acetone, applied three times weekly as promoter. The protease inhibitors TLCK, TPCK, and TAME were applied in DMSO three times weekly in 1.0-μg doses, 1 to 2 hours after applications of croton oil. All treatments were applied to ear skin of mice (the controls received DMSO alone). The average times of appearance of tumors in all three experimental groups are significantly different from the controls at $P < .005$; T indicates the number of tumor-bearing mice; S indicates the number of survivors.

Weeks on promotion	Inhibitor treatment							
	Control		TPCK		TLCK		TAME	
	T	S	T	S	T	S	T	S
10	8	19	0	21	0	21	0	21
12	10	19	0	21	0	21	0	21
14	11	19	0	21	1	21	3	21
16	11	19	0	21	4	21	5	21
18	11	19	0	21	4	21	5	21
20	11	19	0	21	4	21	5	21
22	11	19	0	21	5	21	5	21
24	11	19	1	21	5	21	5	21
30	11	19	1	21	5	21	5	21

been noted that phorbol ester, croton oil, and Tween can release enzymes from rabbit liver lysosomes in vitro and that the effectiveness of these agents in this regard closely parallels their effectiveness as promoters of tumors (*10*).

On the other hand, Sivak *et al.* have failed to confirm this finding in mouse skin lysosomes (*11*), but isolation of undamaged lysosomes is notoriously difficult in this tissue. As a determination of protease we measured the esterase activity with the synthetic substrate TAME. This substrate is hydrolyzed by many proteases, for example, trypsin, plasmin, thrombin, and papain, and its hydrolysis to tosylarginine and methanol serves as a sensitive method for assay of protease (*12*). Mouse ears were frozen in liquid nitrogen, pulverized, extracted with neutral buffer, and sonically disrupted for 15 seconds. Extracts of the ears of mice treated with phorbol ester contained increased TAME esterase compared to untreated controls. It seems unlikely that the mere liberation of enzyme previously bound to lysosome was responsible for the change induced by phorbol ester. Rather it may be that protease activity is related to the inflammatory changes induced by phorbol ester in mouse skin. As in other tissues increases in hydrolytic activity are ascribed to the influx of hydrolase-rich phagocytic cells. In addition to the acute influx of leukocytes into the target tissue (Fig. 2B) an early and sustained increase in vascular permeability occurs after treatment with phorbol ester (*9*).

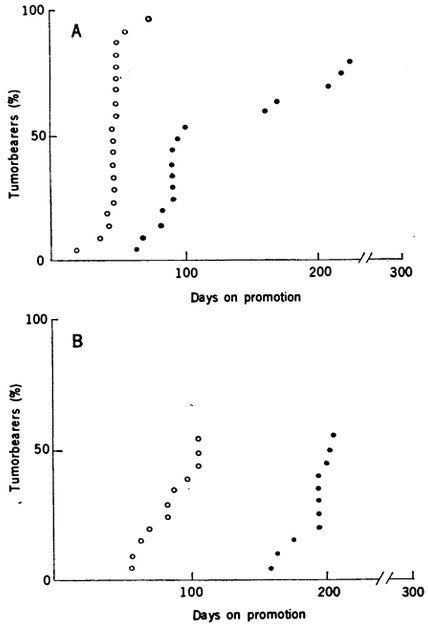

Fig. 1. Time patterns of appearance of skin tumors showing TLCK inhibition. Each point represents a single, tumor-bearing animal and is plotted according to the time of appearance of a grossly visible tumor. (A) Experiment 1. All animals were given an initiating treatment of 10 μg of DMBA in acetone. Three days after initiation, three applications per week of 1.0 μg of phorbol ester in acetone was begun. (○) Control animals; (●) animals that received 10.0 μg of TLCK (tosyl lysine chloromethyl ketone) together with phorbol ester. Average time of tumor appearance in controls was 51.6 days; in animals receiving TLCK it was increased to 122.4 days, a difference significant at $P < .1$. The average number of tumors per animal was 2.57 for controls and 1.90 for animals receiving TLCK. This difference was significant at $P < .05$ by a Student's *t*-test. (B) Experiment 2. All animals were treated as in experiment 1, except that doses of phorbol ester and TLCK were reduced tenfold. (○) Controls; (●) animals receiving TLCK. Average time of tumor appearance in controls was 83.9 days; in animals receiving TLCK the average time was 189.6 days. Average numbers of tumors per animal were 0.71 and 0.52 for control and treated groups, respectively, not a significant difference. The difference in average time of appearance is significant at $P < .005$.

Possible mechanisms by which proteolytic enzymes, appearing in response to promoters of tumors, may act to enhance expression of carcinogenic transformation could include gene activation by removal of repressor substances (histones?) (*10*). A similar mechanism has been proposed for a tissue-bound TAME esterase during fertilization in sea urchin ova (*13*). Minute concentrations of TPCK and TLCK inhibit lymphocyte mitosis induced by phytohemagglutinin, perhaps by a similar block of gene derepression (*14*). The efficacy of inhibition of promotion by these protease inhibitors appears to be greater than that reported for injections of polyinosinic polycytidylic acid (*5*). In addition, the protease inhibitors did not exhibit toxicity over 45 weeks of treatment. Rubin's (*15*) identification of a factor causing overgrowth of Rous sarcoma in tissue

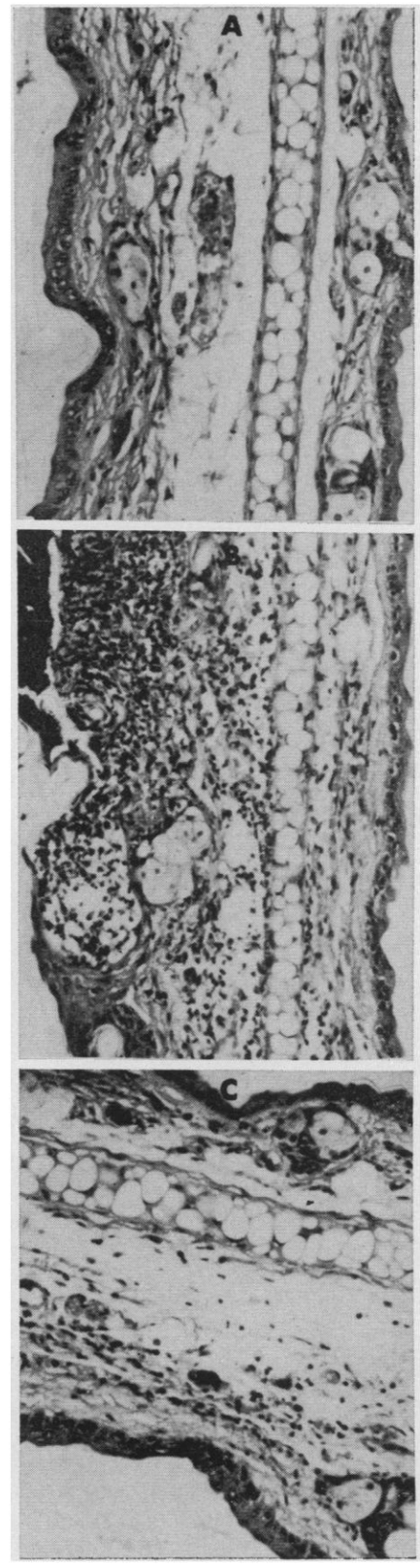

Fig. 2. Twenty-four-hour leukocytic infiltration in CF-1 strain mouse ears; hematoxylin and eosin (× 182). Animals were treated with (A) acetone followed by DMSO (control); (B) 1 μg of phorbol ester in acetone followed by DMSO; (C) 1 μg of phorbol ester in acetone followed by 10 μg of TPCK in DMSO.

culture which could be replaced by the proteolytic enzyme trypsin points to the important role of proteases in growth.

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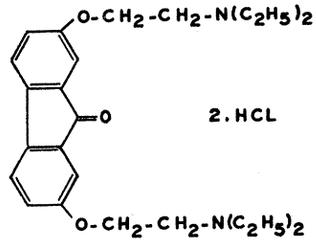
Tilorone Hydrochloride: An Orally Active Antiviral Agent

Abstract. *Tilorone hydrochloride, 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one dihydrochloride, given orally to mice before they are infected is active against at least nine viruses of both RNA and DNA groups. The compound is effective when given prophylactically; the optimum time of treatment depends on the route of infection.*

Tilorone hydrochloride, the orange, water-soluble dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]fluoren-9-one, is a broad-spectrum, orally active antiviral agent.



This compound is active against diverse pathologic conditions. The compound was administered by gavage in aqueous 0.15 percent hydroxyethyl cellulose to CF-1 male mice (15 to 18 g). The animals, in groups of 10 to 30, were observed for 10 days after they were inoculated with virus. Best results were observed against Semliki Forest virus, which is an RNA virus of the arbovirus group; treated mice were completely protected from lethal infections by virus preparations inoculated subcutaneously into the groin (0.1 ml). Although few treated mice survived the severe challenge of vesicular stomatitis virus inoculated intracranially (0.03 ml in the temporal region), the survival time was increased. Deaths from subcutaneously inoculated picornaviruses, and encephalomyocarditis and Mengo viruses can be largely averted by one prophylactic oral dose of the compound.

Infections caused by intraperitoneal challenges (0.1 ml) of the DNA virus herpes simplex were significantly tempered in mice treated orally with tilorone, especially if treatment was begun at least 24 hours before inoculation and continued daily for 7 days. Respiratory infections from intranasal instillation (0.05 ml) of the RNA myxoviruses influenza B (Massachusetts), influenza A/equine-1 (Prague), and influenza A_2 (Jap/305) appear to be least responsive to treatment with tilorone although the survival times of treated mice were extended.

Lung consolidation provoked by influenza A_2 (Jap/305) was significantly diminished in mice treated orally with 250 mg/kg 24 hours before intranasal challenge. However, lung lesions induced either by influenza A_0 (PR_8) virus or lipopolysaccharide did not respond to oral treatment. Furthermore, the course of infection in treated mice inoculated intranasally with lethal doses of influenza A_0 (PR_8) remained unaltered.

Tail lesions induced by subcutaneous inoculation of vaccinia (*1*), a DNA virus, were conspicuously reduced in mice treated daily for 7 days beginning 1 day before challenge. The tails were inoculated subcutaneously (0.1 ml) with 5 ID_{50} of virus (infective dose for 50 percent of the mice). The severity of the lesions was rated subjectively 8 days after infection. A numerical scoring system, that ranged from no lesion (0) to a maximum condition (3), was used. Lesion scores from groups of 20 mice were averaged. Tilorone was more active than *N*-methylisatin-β-thiosemicarbazone under these test conditions. The average tail-lesion score for nontreated mice was 2.00. Mice treated with 250, 100, and 50 mg/kg had average tail-lesion scores of 0.20, 0.74, and 1.40, respectively. Mice treated orally with 250 mg of *N*-methylisatin-β-thiosemicarbazone per kilogram of body weight had an average tail-lesion score of 0.90.

Against encephalomyocarditis virus, activity was demonstrated when tilorone was given orally 48 hours before challenge (Table 2). However, optimum activity was observed when the compound was given 24 hours before virus inoculation. Doses given 3 hours before or after inoculation were weakly active,

Table 1. Oral activity of tilorone hydrochloride (250 mg/kg per dose) against lethal virus infections in mice. The compound was administered before infection at the times indicated. Deaths were recorded over a 10-day period. Survivors were given a score of 11 in calculations of the mean day of death. Groups of 10 to 30 mice were used. Abbreviations: SC, subcutaneous; IC, intracranial; IN, intranasal; IP, intraperitoneal; SF, Semliki Forest; VSV, vesicular stomatitis; EMC, encephalomyocarditis; HS, herpes simplex; T, treated; C, control.

Virus	Route	LD_{50}	Tilorone (hr)	Survivors (%)		Day of death (mean)		*P**
				T	C	T	C	
SF	SC	32	24	100	0	11.0	6.5	<.001
VSV	IC	252	48, 24	20	0	7.2	4.0	<.001
EMC	SC	18	22	80	0	10.1	4.7	<.001
Mengo	SC	39	24	80	5	10.2	5.6	<.001
Influenza	IN							
B (Mass.)		21	24	50	10	9.8	8.0	.01
A/Equine-1		21	24	40	10	9.5	7.8	.01
A_2 (Jap/305)		4	24	30	10	8.9	7.1	.01
HS	IP	63	24, 2†	45	0	10.0	8.5	<.001

* Probability value (Student's *t*-test; 2-tailed). † Plus once a day for 6 days after inoculation.