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Induction of Plasminogen Activator in Cultured Cells by Macrocyclic Plant Diterpene Esters and Other Agents Related to Tumor Promotion[1]

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ABSTRACT

In vitro **systems that are responsive to tumor-promoting agents may facilitate the identification of such agents and the analysis of their mode of action. We have previously reported that the potent tumor promoter phorbol-12-myristate-13-acetate induces the synthesis of the enzyme plasminogen activator in cultured chick embryo fibroblasts. We have, therefore, tested various compounds for their ability to induce plasminogen activator in chicken embryo fibroblasts. Among these, phorbol esters and other macrocyclic diterpene esters isolated from species of the families Euphorbiaceae and Thymelaeaceae were potent inducers of plasminogen activator. These compounds maximally induced enzyme to the same levels, although they differed in their relative molar potencies. Structural requirements for *in vitro* activity paralleled the requirements for activity *in vivo*. These results indicate that induction of plasminogen activator is a useful marker for the biologically active macrocyclic diterpene esters. On the other hand, tumor-promoting agents such as anthralin, cantharidin, Tween 60, and tobacco leaf extract failed to induce plasminogen activator.**

INTRODUCTION

Tumor promoters have been defined as agents that are not in themselves carcinogenic but that significantly increase tumor incidence in animals pretreated with carcinogens. The existence of tumor promoters was first rigorously demonstrated by Berenblum and Shubik (3), who used the 2-stage carcinogenesis protocol for the identification of promoting agents. The overall contribution of tumor-promoting substances of natural or industrial origin to the incidence or rate of progression of human cancer is unknown, but epidemiological evidence and data from animal studies suggest their potential relevance in several forms of carcinoma (12, 20, 29). An understanding of this phenomenon and the identification of etiological agents is therefore of considerable importance.

The phorbol esters, macrocyclic diterpene esters found in the seed oil of *Croton tiglium*, a leafy shrub of the family Euphorbiaceae, are the most potent known promoting agents (10). In addition to their tumor-promoting activity in mouse skin, nanomolar concentrations of phorbol esters affect cultured cells, altering growth properties and morphology (8), increasing phospholipid synthesis (26), inducing plasminogen activator (30, 31) and ornithine decarboxylase (33), and inhibiting cellular differentiation (6, 21, 32). The availability of *in vitro* systems responsive to the phorbol esters provides an opportunity to analyze the biochemical origin of activity of these compounds under well-defined conditions. In addition, *in vitro* systems may facilitate the assay of suspected promoting agents. With these ends in mind, we have tested various compounds for their ability to induce plasminogen activator in cultured cells. Three categories of compounds were examined: (*a*) phorbol, its esters and derivatives with known tumor-promoting activity; (*b*) macrocyclic plant diterpene esters from the families Thymelaeaceae and Euphorbiaceae, which are structurally similar to the phorbol esters and have been identified as antileukemic principles (14-17); and (*c*) tumor promoters and cocarcinogens of diverse origin.

MATERIALS AND METHODS

Phorbol, PMA,[2] 4-*O*-methyl-PMA, phorbol-12,13-didecanoate, and 4α-PDD were obtained from Consolidated Midland, Brewster, N. Y.; Tween 60 and cantharidin were from Sigma Chemical Co., St. Louis, Mo.; nicotine was from Aldrich Chemical Co., Milwaukee, Wis.; anthralin was the generous gift of Dr. Sydney Belman; cigarette smoke condensate, fractions, and reconstituted smoke condensate prepared according to the method of Swain *et al.* (27) were generous gifts of Dr. William Benedict; barium hydroxide extracts of tobacco (Pall Mall) were prepared according to published methods (5); asbestos fibers (amosite, crocidolite, and chrysotile) were in accord with IUCC standards and were the generous gift of Dr. A. Langer; ingenol-3,20-dibenzoate, phorbol-12-tiglate-13-decanoate, G-1, gnidimacrin-20-palmitate, G-20, gnidilatin-20-palmitate, G-5, and gnilatimacrin-20-palmitate were generous gifts of Dr. Morris Kupchan.

CEF and HeLa cells were grown as monolayer cultures in Eagle's minimal essential medium supplemented with 5% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) in a humidified incubator at 37° under an atmosphere of 5% CO_2. CEF were prepared by standard procedures from 9- to-12-day chick embryos (23) and were used between the third and the ninth passage.

Plasminogen activator was assayed by published methods (31). The assay is based on quantitation of solubilization of ^{125}I-labeled fibrin due to proteolytic digestion in the

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[2] The abbreviations used are: PMA, phorbol-12-myristate-13-acetate (also called 12-*O*-tetradecanoylphorbol-13-acetate); 4-*O*-methyl-PMA, 4-*O*-methylphorbol-12-myristate-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; G-1, gnidimacrin; G-20, gnidilatin; G-5, gnilatimacrin; CEF, chicken embryo fibroblasts.

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presence of plasminogen and sample. For the experiments described here, the sample was a lysate of cells prepared as described previously (31). Plasminogen was prepared from outdated human plasma (7), and ^{125}I-labeled fibrin-coated dishes were prepared as described by Unkeless *et al.* (28).

For the purpose of assaying the induction of plasminogen activator by various compounds, CEF from third- to eighth-passage cultures were exposed to test agents for either 12 hr in the absence of serum or 24 hr in the presence of serum. At the end of this time, cell lysates were prepared as previously described (31) and were stored frozen until assayed for plasminogen activator. Induction of plasminogen activator by phorbol esters is slower in the presence of serum that in its absence but reaches the same level of induction under both conditions in 24 and 12 hr, respectively (unpublished work). More importantly, the presence or absence of serum does not affect the dose-response curve of CEF to the phorbol esters.

RESULTS

Table 1 summarizes the response of CEF to phorbol, its esters and derivatives, and to a series of macrocyclic plant diterpene esters isolated from members of the families Euphorbiaceae and Thymelaeaceae, which have been characterized as antileukemic agents. Cells were exposed to varying concentrations of each agent for 12 hr, and the amount of activity in cellular lysates was determined. In Chart 1, typical dose-response curves are plotted for a few of the compounds studied. With the most potent compounds, induction occurs in the nanomolar range. Maximum-fold induction observed is within a factor of 2 for each active compound, and the level of induction plateaus for all compounds studied at sufficiently high concentrations. The level of induction observed in CEF treated with these compounds does not represent the maximum level of synthesis of which these cells are capable. When CEF infected with Rous sarcoma virus are treated with PMA, they produce plasminogen activator levels 10 to 40 times higher than when uninfected cells are similarly treated (30). From data such as those shown in Chart 1, a dose that produced 50% of the maximum effect can be estimated for each compound, and these values are indicated in Table 1. Of the compounds tested, only 4α-PDD (up to 1.4 × 10^{-6} M) and phorbol (up to 2.5 × 10^{-5} M) were completely inactive.

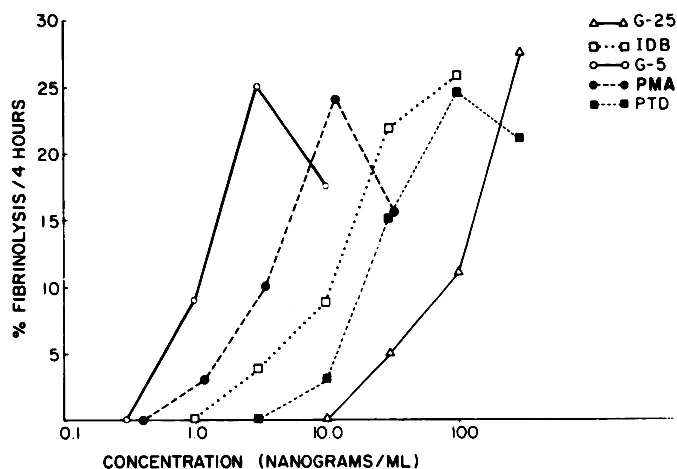

Chart 1. The induction of plasminogen activator in CEF by varying concentrations of macrocyclic plant diterpene esters. Subconfluent plates of CEF were exposed to the indicated agents for 12 hr in the absence of serum. Cellular lysates were prepared and plasminogen activator levels were measured as percentage of fibrinolysis, as indicated in the text. *IDB*, ingenol dibenzoate; *PTD*, phorbol-12-tiglate-13-decanoate; *G-25*, gnidimacrin-20-palmitate.

Table 1

Induction of plasminogen activator by phorbol, phorbol esters, and other macrocyclic plant diterpenes

CEF were exposed to agents over a broad range of concentrations, and the amount of plasminogen activator in cell lysates was determined as described (31). ED_{50} was determined as the dose producing 50% of maximal induction. See text for details and references.

Compound	ED_{50} (× 10^{-9} M)	Biological activity
Phorbol	>26,000	a
PMA	7.8	b
4-*O*-Methyl PMA	150	c
Phorbol-12,13-didecanoate	7.1	b
4α-PDD	>1,400	c
Phorbol-12-tiglate, 13-decanoate	43	d
Ingenol-3,20-dibenzoate	18	d
Mezerein	1.5	d
G-1	1.2	d
Gnidimacrin-20-palmitate (G-25)	100	d
G-5	3.1	d
Gnilatimacrin-20-palmitate (G-18)	56	d
G-20	3.0	d
Gnidilatin-20-palmitate (G-21)	56	d

[a] Inactive as tumor promoter.
[b] Potent tumor promoter.
[c] Reportedly inactive as tumor promoter.
[d] Antileukemic.

The structures of the compounds tested are shown in Chart 2. Three different macrocyclic ring structures (phorbol, mezerein, and ingenol) have activity, and all share a common 7-membered ring in a fixed orientation to a 5-membered ring. Mezerein, G-1, G-20, and G-5 are as potent or more so than is PMA. When the C_{20} alcohols of the gnidia compounds (G-1, G-20, and G-5 are esterified (gnidimacrin-20-palmitate, gnidilatin-20-palmitate, gnilatimacrin-20-palmitate), molar potency is reduced 20- to 100-fold.

Listed in Table 2 are miscellaneous agents that we tested for induction of activity in CEF or HeLa. Cultures of CEF or HeLa were treated with the indicated agent for 24 hr in the presence of serum and plasminogen activator levels determined in cellular lysates. For each experiment, cultures treated with 5 × 10^{-8} M PMA were included as positive controls. The tumor-promoting agents anthralin (4), cantharidin (13), and Tween 60 (25) were tested as were the asbestos fibers amosite, crocidolite, and chrysotile, which may act as cocarcinogens (24) and are reported to induce plasminogen activator in mouse peritoneal macrophages (9). These compounds did not induce plasminogen activator in CEF or HeLa. Extracts of tobacco leaf and cigarette smoke condensate have been repeatedly shown to possess tumor-promoting activity in mouse skin (5, 29). In addition, both tobacco extracts and smoke condensate contain macrocyclic diterpenes, similar in structure to phorbol (22). For these reasons, we tested a barium hydroxide extract of tobacco with demonstrated promoting activity (5), an ex-

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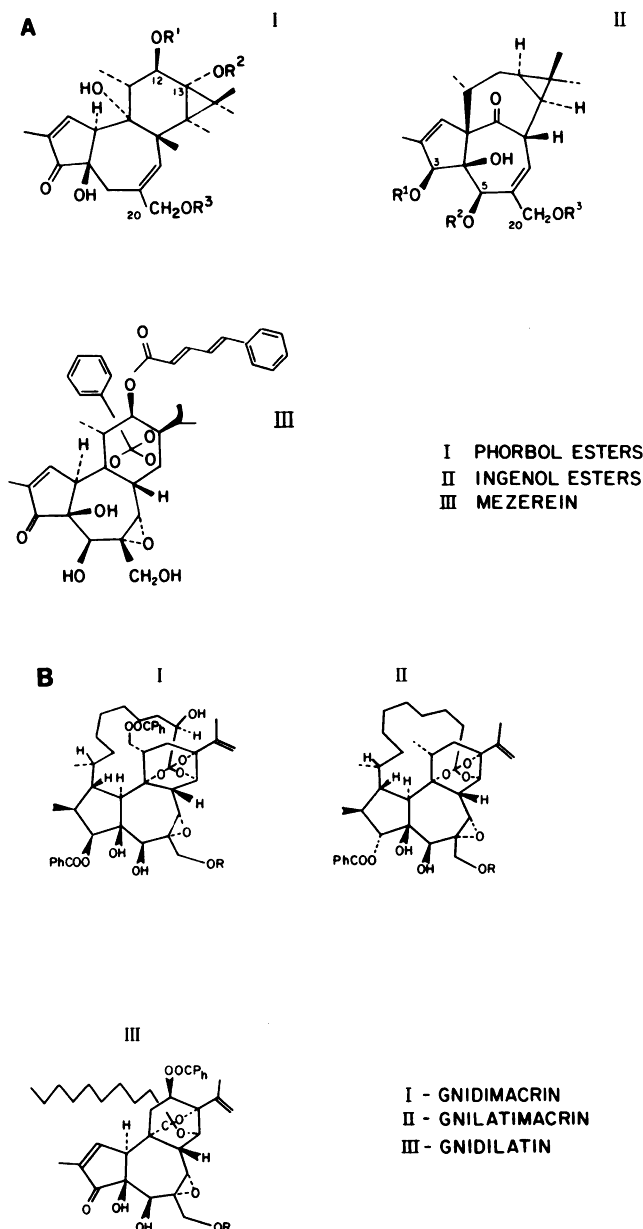

Chart 2. Structures of some macrocyclic plant diterpenes derived (*A* and *B*) from the families Euphorbiaceae (*AI* and *AII*) and Thymelaeaceae (*AIII* and *B*).

tract of cigarette smoke condensate, and various chemical fractions of the latter according to the scheme of Swain *et al.* (27) for the ability to induce plasminogen activator in either CEF of HeLa. None of the extracts of fractions thereof exhibited biological activity characteristic of the phorbol esters.

DISCUSSION

Plasminogen activator is a serine protease that specifically cleaves plasminogen to plasmin, the major fibrinolytic factor in blood (28). Measurable levels of plasminogen activator are found in a variety of body tissues as well as in cell and organ cultures (1). In a previous paper (31), we reported on the induction of the enzyme in cultured CEF and HeLa cells by the potent tumor promoter phorbol-12-myristate-13-acetate. We report here the results of tests of additional phorbol esters and derivatives, promoters of diverse origins, and related agents.

Table 2

Agents that fail to induce plasminogen activator in CEF or HeLa

Cells were exposed to agents for 24 hr in the presence of medium with serum, cell lysates were prepared, and plasminogen activator was assayed as described.

Agent	Dose (μg/ml)	Cell	Induction[a]
PMA	0.03	CEF	+[b]
		HeLa	+
Anthralin	1-30	CEF	NS
		HeLa	NS
Cantharidin	10-100	CEF	NS
	1-30	HeLa	NS
Tween 60	10-1000	CEF	NS
		HeLa	NS
Asbestos			
Amosite	0.1-10	CEF	NS
Crocidolite	0.1-10	CEF	NS
Chrysotile	0.1-10	CEF	NS
Tobacco, $Ba(OH_2)$ extract	30-1000	HeLa	NS
Cigarette smoke condensate, dimethyl sulfoxide extract	25-75	CEF	NS
		HeLa	NS
Cigarette smoke condensate fractions[c]	5-20	CEF	NS
		HeLa	NS
Cigarette smoke reconstitute[d]	3-30	CEF	NS
		HeLa	NS
Nicotine	100-1000	CEF	NS

[a] In each experiment, cultures of CEF and HeLa were exposed to PMA (30 ng/ml) as a positive control for induction.

[b] +, greater than 10% fibrinolysis in 3 hr; NS, no significant activity above untreated cultures (less than 1% fibrinolysis in 3 hr).

[c] All 12 fractions of cigarette smoke condensate were tested according to the fractionation scheme of Swain *et al.* (27).

[d] Fractions were pooled (27).

The potent tumor-promoting phorbol esters PMA and phorbol-12,13-didecanoate were both potent inducers of plasminogen activator while phorbol was completely inactive (2). 4-*O*-methyl-PMA, which has been reported to be inactive as a tumor promoter in mouse (18), was active in our system, although only at relatively high concentrations. It is thus possible that this compound was not tested for tumor promotion at sufficiently high doses. Consistent with our finding is the report that 4-*O*-methyl-PMA induces ornithine decarboxylase in cultures of mouse epidermal cells (33). 4α-PDD is claimed to be inactive as a tumor promoter (E. Hecker, personal communication), yet Mondal *et al.* (19) find that it promotes chemical transformation of mouse 10T 1/2 cells *in vitro*. We find 4α-PDD to be inactive as an inducer of plasminogen activator in CEF. We feel that the discrepancies in the *in vitro* and *in vivo* systems merit further investigation.

In addition to the above compounds, we have tested

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macrocyclic diterpene esters extracted from plants of the families Euphorbiaceae and Thymelaeaceae which are structurally related to the phorbol esters and have antileukemic activity (14-17). These compounds were all potent inducers of plasminogen activator, demonstrating that induction in cultured CEF can be used as a specific indicator for the biologically active macrocyclic plant diterpene esters. The active compounds have highly lipophilic fatty acid esters or orthoesters and a C_{20} primary alcohol. When the C_{20} alcohol of the gnidia compounds is esterified, the compounds become 20- to 100-fold less active on a molar basis. Loss of tumor-promoting activity also occurs on esterification of the C_{20} alcohol of the phorbol esters (10). Structural requirements for *in vitro* activity parallel, at least in part, the requirements for activity *in vivo*. Macrocyclic diterpene esters of the gnidia class have not in general been studied for promoting activity. Mezerein, which we have found to be more potent than PMA in the induction of plasminogen activator in CEF, does promote in mouse skin, although weakly (11).

Cocarcinogens and promoting agents thought to be unrelated to the phorbol esters were tested for the ability to induce plasminogen activator. Anthralin, cantharidin, and Tween 60 do not induce. Asbestos fibers, which have been reported to induce plasminogen activator synthesis in mouse peritoneal macrophage (9), do not induce plasminogen activator in CEF. Cigarette smoke condensates and extracts of tobacco, which are tumor promoting in mouse skin (5, 29), similarly fail to induce the enzyme in CEF or HeLa. Induction of plasminogen activator *in vitro* thus does not reflect the full complexity of events governing promotion. The reasons for this may be varied: the particular cell lines used in this study may not be sensitive to the agents tested due to metabolism or failure to metabolize or due to the absence of an appropriate receptor; in complex mixtures, such as tobacco smoke condensates, the active agent may be present but below the threshold of detection, or inhibiting agents may also be present; and finally, it is possible that the promoting activities of anthralin, cantharidin, Tween 60, and tobacco extracts are mediated by different mechanisms than that of the phorbol esters. Since tumor promotion may require a complex interaction between multiple cell types and multiple responses, it may not be possible for any one *in vitro* assay system to predict the promoting activity of all classes of suspected promoting agents. The great variety of agents that evoke somewhat different responses, however, should facilitate an understanding of the events that comprise tumor promotion.

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