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In Vitro and *In Vivo* Activity of 12-*O*-3-*N*-Dansylamino TPA

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Abstract—*Dansyl–TPA, a fluorescent TPA analogue, which is a label with a high affinity for* C3H/10T1/2 *cells and induces* ^{3}H-*choline release from these cells (Tran* et al. Nouv. J. Chim 1984, **8**, 751–757), *has been studied for* in vitro *promoting activity in the same cell line initiated by a carcinogen MNNG and* in vivo *short-term mouse skin tests.* In vitro, *dansyl–TPA expresses transforming effect in its own (without MNNG pretreatment) as well as increases the production of transformed foci in MNNG-treated cells. In* in vivo *skin tests, dansyl–TPA displays lower effects than TPA on mouse skin. These results indicate a low promoting potential of dansyl–TPA.*

INTRODUCTION

IT IS WELL known that in mouse skin, the phorbol ester 12-0-tetradecanoyl phorbol 13-acetate (TPA) is a potent tumor promoter as well as a potent inflammatory and hyperplastic agent [1, 2]. It has also been clearly demonstrated that *in vitro*, TPA has promoting effects in several tissue culture systems [3–8]. Simultaneously, in mouse skin and in cultured cells, TPA also induces a variety of biochemical and biological responses including partial induction of transformed phenotype, modulation of differentiation and alteration of membrane functions [9–12].

To analyze the mechanism of action of TPA, we have synthesized a fluorescent TPA, 12-0-3-N-dansylamino TPA referred to as dansyl–TPA [13, 14]. The newly synthesized compound behaves like TPA in cell culture: e.g. it displaces the binding of ^{3}H-PDBu to 10T1/2 cells and increases ^{3}H-choline release from these cells [14]. Dansyl–TPA also activates the soluble mouse brain protein kinase C in a manner similar to TPA [15]. Our labeling experiments on living cells demonstrated that dansyl–TPA molecules readily enter the cells and are distributed within intracellular organelles [16].

In order to further characterize the biological properties of dansyl–TPA we examine, in the present paper, the promoting effect of the fluorescent dansyl–TPA in C3H/10T1/2 cells previously initiated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). To check for potential promoting potency, the appearance of type II and type III foci was scored. In addition, the effects of dansyl–TPA on mouse epidermis and sebaceous glands were investigated. The effects of dansyl–TPA were compared with those of TPA in 10T1/2 cells and in mouse skin.

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MATERIALS AND METHODS

Chemicals

Dansyl–TPA [14] was diluted in acetone for *in vivo* studies or in culture medium from a stock solution in EtOH, and routinely checked by thin-layer chromatography in $CHCl_3$/acetone (7 : 3). Dansyl–tetradecanoate (dansyl–tetr) [14] was used in the same conditions as dansyl–TPA.

TPA and MNNG were purchased respectively from P. Borchert (Eden Prairie, U.S.A.) and from Fluka (Buchs, Switzerland).

Cell culture

C3H/10T1/2 cells were obtained from the laboratory of the late Dr. C. Heidelberger (University of Southern California Comprehensive Cancer Center, Los Angeles, CA) and routinely grown using basal minimal Eagle's medium (Flow) supplemented with 10% inactivated fetal calf serum (FCS).

Animals

For *in vivo* studies, 45-day-old female Swiss mice from a specific-pathogen-free breeding house

(Institut de Recherches Scientifiques sur le Cancer, Villejuif, France) were used.

Transformation of C3H/10T1/2 *cells*

The transformation assay was carried out according to the protocol of Boreiko *et al.* [17]. One day after plating 2000 cells/60 mm dish, the cells were treated for 4 hr with a phosphate-buffered saline solution containing MNNG dissolved in acetone (final acetone concentration 0.5%), which was then replaced by complete Eagle's basal medium supplemented with 10% FCS. Three days later dansyl–TPA or TPA dissolved in ethanol were added at each medium change. The number of dishes containing foci type II and III was scored 40 days after plating. Dansyl–tetradecanoate was used as a negative control.

Sebaceous gland suppression and epidermal hyperplasia induction in mouse skin

The *in vivo* short-term test was carried out according to Lazar *et al.* [18]. 45-day-old Swiss mice received three dorsal applications on days 1, 3 and 5 of dansyl–TPA or TPA dissolved in acetone. The controls were treated with acetone. The treated skin areas were removed on day 8. The number of sebaceous glands and the thickness of the epidermis were measured by standard procedures [18]. The experimental results are given relative to the controls as previously reported [19].

RESULTS

Transformation of C3H/10T1/2 *cells*

In order to test the ability of dansyl–TPA and dansyl–tetradecanoate to transform C3H/10T1/2 cells, we performed three initiation-promotion experiments by comparison with the effects of TPA. Table 1 summarizes the results obtained from the three experiments. Transformation data are given as the number of dishes with transformed foci over the total number of dishes and also expresses the percentage of dishes with transformed foci. Dishes were scored for the presence of type II and/or type III transformed foci (Figs. 1 and 2), both of which have tumorigenic potency as determined by the criteria of Reznikoff *et al.* [20].

As reported in Table 1, dansyl–TPA at the dose of 0.1 μg/ml is not toxic to the cells, which is in agreement with the standard promotion assay *in vitro*. However, at 0.25 μg/ml it displays a toxic effect. On the other hand, dansyl–TPA at 0.1 and 0.25 μg/ml shows a transforming effect by itself. When the cells were initiated with MNNG at a dose of 0.3 μg/ml and then treated with dansyl–TPA at 0.1 and 0.25 μg/ml, no increase in the percentage of dishes containing foci was observed. In the same way, no significant change in the number of foci was either observed when cells were initiated with MNNG at the dose of 0.5 μg/ml and treated with 0.25 μg/ml of dansyl–TPA. However, when the cells were initiated with a higher concentration of MNNG (0.5 μg/ml) and treated with 0.1 μg/ml of dansyl–TPA, an enhancement of foci production is observed when compared to the cells treated with dansyl–TPA alone. This increase in the number of foci suggests a promoting effect of dansyl–TPA.

At the high dose of dansyl–TPA (0.25 μg/ml), a decrease in the percentage of dishes with foci in comparison with the promoting low dose (0.1 μg/ml) in both groups of MNNG-treated cells (0.3 and 0.5 μg/ml) is observed. This may probably be due to the toxic effect of dansyl–TPA at 0.25 μg/ml, which might hide the promoting dose-effect.

In contrast, no transformed foci were observed when 10T1/2 cells were initiated with MNNG at the doses of 0.3 and 0.5 μg/ml and then treated with dansyl-tetradecanoate at the dose of 0.25 μg/ml.

Short-term skin tests

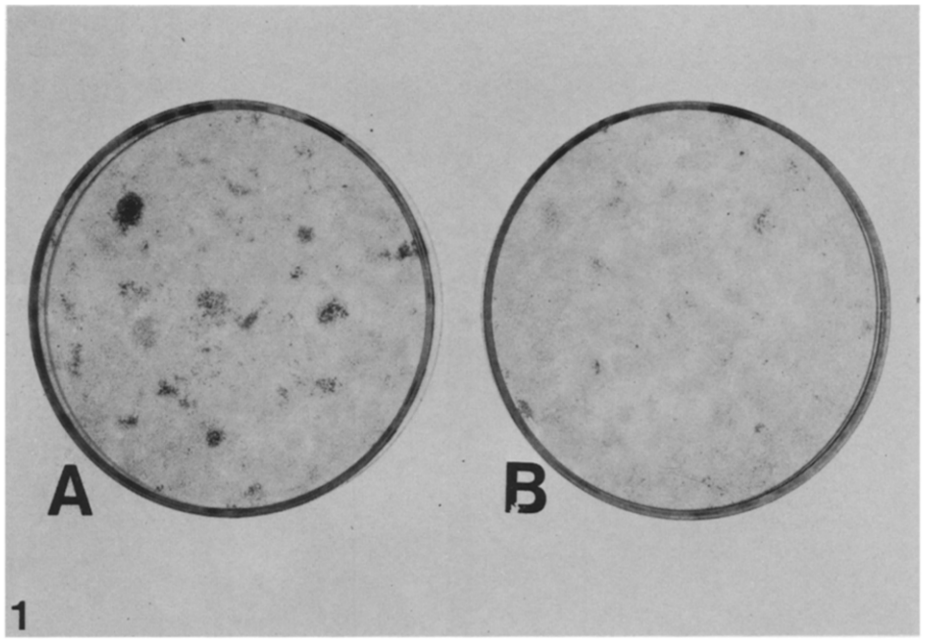
Two *in vivo* experiments on mouse skin were performed with dansyl–TPA at doses ranging from 10 to 40 μg/ml and compared to TPA at the dose of 20 μg/ml (Table 2).

As reported in Table 2 for hyperplasia tests, the activity of dansyl–TPA compared to the acetone control shows an increase with increasing concentrations. Although it remains less active than TPA, a slight response of dansyl–TPA is obtained at the dose of 40 μg/ml. This effect is significantly greater than the control ($P \leq 0.001$).

In the sebaceous gland tests, it is found that, unlike TPA, dansyl–TPA does not induce any decrease in the number of sebaceous glands even at the maximal dose of 40 μg/ml (Table 2). On the contrary, a slight increase observed in the number of sebaceous glands from 6.5 to 19.2% can be attributed to an hypertrophia of the glands.

DISCUSSION

As it appears from Table 1, the results of the present study show that dansyl–TPA enhances the transformation of cells in a two-stage process of carcinogenesis in the same manner as TPA. However, as indicated in Table 1, when applied alone dansyl–TPA appears capable of transforming the cells by itself. This effect cannot however be attributed to the dansyl group since the long chain dansyl–tetradecanoate non-associated with the phorbol moiety displays non-transforming activity. Nevertheless, one cannot exclude the probability that a combined effect between the dansyl and the phorbol moiety may play an important role in increasing the activity of dansyl–TPA. Indeed, as



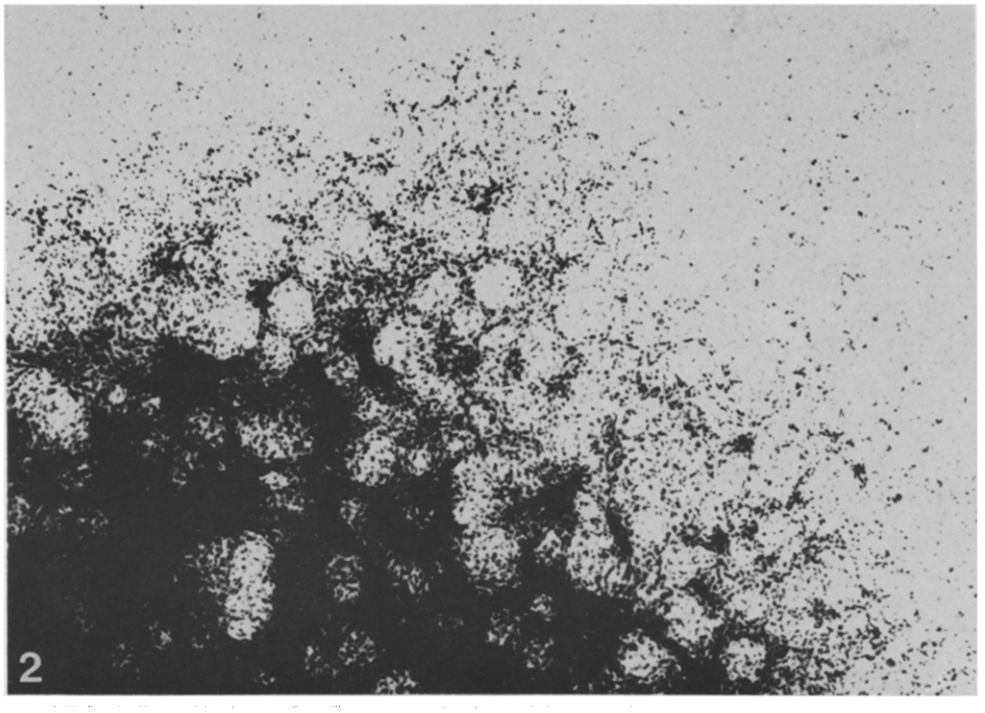


Fig. 1. *Cultures of C3H/*101*T/2 cells fixed and stained* 40 days *after plating. A: transformed foci; cells initiated with MNNG* (0.5 μg/ml), *and then treated with dansyl–TPA* (0.1 μg/ml) *at each medium change. B: acetone control.* × 0.75.

Fig. 2. *Details from focus showing the morphology of type* III *focus.* × 0.48.

Table 1. *Transformation of* C3H/101T1/2 *cells produced by MNNG, dansyl–TPA and TPA treatment*

Treatment* for 4 hr (μg/ml)	Treatment† in each medium change (μg/ml)	Relative cloning efficiency (%)‡	No. of dishes with type II and III foci/Total no. of dishes	% with type II and III foci
Ethanol (0.5%)	–	100	0/17	0
MNNG (0.3)	–	22.9	0/25	0
MNNG (0.3)	TPA (0,25)	13.7	5/18	27.8
–	TPA (0,25)	100	0/16	0
MNNG (0.3)	dansyl–tetr (0,25)	36.7	0/9	0
MNNG (0.3)	dansyl–TPA (0,1)	28.9	10/29	34.5
MNNG (0.3)	dansyl–TPA (0,25)	25.7	6/25	24
MNNG (0.5)	–	15.1	0/10	0
MNNG (0.5)	dansyl–tetr (0.25)	5.9	0/8	0
MNNG (0.5)	dansyl–TPA (0.1)	5.9	5/9	55.6
MNNG (0.5)	dansyl–TPA (0.25)	9.1	3/10	30
–	dansyl–tetr (0.25)	91.9	0/12	0
–	dansyl–TPA (0.1)§	91.9	2/6	33.3
–	dansyl–TPA (0.25)§	36.7	4/12	33.3

*Twenty-four hours after plating.
†Three days after the first 24-hr treatment.
‡The absolute cloning efficiency of controls vary from 21.7 to 29%.
§Scored in two experiments.

Table 2. *Effect of dansyl-TPA and TPA on epidermal thickness and sebaceous glands in mouse skin*

Compounds	Dose* (μg/application)	No. of mice	Epidermal thickness†	Hyperplasia (% of control acetone)	Sebaceous glands‡
No treatment	–	21	20.55 ± 0.60§	–	13.24 ± 0.69
Acetone	0.05 ml	22	19.55 ± 0.52	–	14.18 ± 0.68
dansyl–TPA	10	10	20.00 ± 0.76	102.3	15.10 ± 1.69
	20	10	22.90 ± 1.20	117.2	15.40 ± 1.49
	40	10	23.80 ± 1.33	122.0	16.90 ± 1.49
TPA	20	22	50.09 ± 1.97	256.2	9.09 ± 0.89

*Total dose applied in 3 applications, each of which contained the test compound as a solution in 0.05 ml acetone, and covering 8 cm^2 of skin.
†In arbitrary units as defined in ref. [19].
‡In 12 microscopic fields as described in ref. [18].
§Mean ± S.E.

shown by us [14], when compared to TPA dansyl–TPA displays higher binding affinity as well as higher activity in choline release in 10T1/2 cells. Such an increase in the activity of the TPA analogues has also been observed with photoaffinity phorbol esters [21, 22].

Conversely, in mouse skin dansyl–TPA shows no effect on sebaceous gland test and a low effect in hyperplasia tests. This might suggest that dansyl–TPA readily penetrates the epidermis and the dermis where it is rapidly degraded, or that the introduction of a dansyl group into TPA results in a low skin irritant activity of the analogue. In fact, we do not observe the classical irritative reaction induced by TPA: oedema, polymorphic cell infiltration. It has been generally demonstrated that

irritancy is one of the properties of tumor promoters, even if it is not a sufficient condition for promoting activity [23]. Further *in vivo* experiments are needed in order to elucidate the respective effects of dansyl–TPA observed on mouse skin and in cell culture. A similar behaviour has been previously observed with 4α-phorbo didecanoate (4α-PDD) by Mondal *et al.* [8]. The analogue 4α-PDD produces an enhancement of methylcholanthrene (MCA) transformation while it is reported that this compound is only a weak skin irritant and a non-promoter in mouse skin ([24]; I. Chouroulinkov, unpublished results).

As shown in this paper and from our previous results [14], dansyl–TPA is a compound with a high affinity for 10T1/2 fibroblasts and increases ^{3}H-choline release from these cells [14] as well as proving active in promoting the transformation of the same cell line. Thus, in this respect, dansyl–TPA can be employed as a probe for TPA. We have demonstrated that dansyl–TPA enters the cell. The cytoplasmic distribution of the marker is not entirely random; rather, the marker may distribute in various organelles and the nuclear membrane [15, 16]. In addition, we have noted that the marker remains unchanged as long as 48 hr [15]. Studies on the action of TPA and related phorbol ester tumor promoters in cell culture systems have demonstrated that these agents can exert highly pleiotropic effects on the growth, function, and differentiation of a variety of cell types [5, 11, 12]. The pleiotropic responses of TPA, it has been suggested, might be mediated through high-affinity binding sites at the cell surface [25–27], which mainly appears to be associated with protein kinase C [28]. However, the cascade of molecular and cellular events that lead to the cell transformation process remain to be elucidated. In view of the search of molecular and cellular targets of phorbol esters and because it is a relatively stable marker within C3H/10T1/2 cells, dansyl–TPA is at present a pertinent tool and will facilitate studies of its biological action relevant to the mechanism of the promoting process. These studies should be extended to other cell lines either fibroblastic or epithelial.

Note added in proof: Recent results on fluorescent derivatives of phorbol esters different from ours have been described by Liskamp *et al.* [29].

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