Biochemical Pharmacology, Vol. 38, No. 17, pp. 2925–2927, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SHORT COMMUNICATIONS

The ability of diterpene esters with selective biological effects to activate protein kinase C and induce HL-60 cell differentiation

(*Received* 1 *November* 1988; *accepted* 24 *February* 1989)

The human promyelocytic HL-60 cell line may prove a useful tool in elucidating the role of protein kinase C (PKC) in the regulation of cell differentiation, since these cells can be induced to differentiate in culture into adherent monocytes by treatment with PKC activators, notably phorbol esters [1]. Paradoxically, phorbol esters more commonly produce a deregulation of growth control, promoting tumour development in mouse skin [2] and inducing lymphocyte proliferation *in vitro* [3, 4], for example. These apparently conflicting roles may be explained by the heterogeneous nature of PKC itself; the existence of separate isozymes [5, 6] and active proteolytic fragments [7], complex cofactor requirements and differing substrate affinities [8].

12-Tetradecanoylphorbol-13-acetate (TPA), the most commonly used diterpene ligand, has a broad spectrum of biological effects, precluding its use in the dissection of divergent roles for PKC within cells. In contrast, we have demonstrated the existence of compounds which are as potent as TPA in activating mammalian brain PKC *in vitro* [9] yet are not tumour promoting [10], and are less able to induce lymphocyte proliferation under certain conditions (Edwards *et al.*, unpublished results).

In this report, we compare the ability of these compounds to induce HL-60 cell differentiation.

Materials and methods

Cell source and routine maintenance. The cell line HL-60 [11] was obtained from the European Culture Collection (Porton Down, U.K.) and grown in RPMI 1640 medium, supplemented with 15% foetal calf serum, 2% glutamine and penicillin/streptomycin (50 U/ml/50 μg/ml, Gibco, Uxbridge, U.K.) at 37° in a humidified atmosphere of 95% air/5% CO_2. Cells were maintained at concentrations between 0.5 and 2.0 × 10^6 cells/ml by twice weekly passage of log-phase cultures. The doubling time of the cells was 1.5–2 days. Under these conditions, spontaneous differentiation (determined by adherence, morphology and formazan staining following incubation with nitroblue tetrazolium) was never greater than 1%.

Test compounds. Sources, structural details and selected biological activities of test compounds are summarized in Table 1. For assay, serial dilutions of test compounds at 1000-fold final assay concentration were made in acetone.

Assay of differentiation potential. On day 1, healthy log-phase cultures were pooled, washed by centrifugation at 1000 *g* for 10 min followed by resuspension, and diluted to a concentration of 0.5 × 10^6 cells/ml. Aliquots (7.5 ml) were dispensed into 25 cm^3 plastic tissue culture flasks (Flow Laboratories, Irvine, CA), and 7.5 μl of the appropriate dilution of the test compound added. In the controls the equivalent amount of pure acetone was added. The cultures were then incubated for 48 hr. On day 3, supernatants were decanted and the monolayer of adherent, differentiated cells washed with phosphate buffered saline (2 × 2 ml). Trypsin–EDTA solution was added (Gibco, 2 ml) and the monolayers incubated for 10 min, at which point microscopic examinations confirmed resuspension of the monolayer. Phosphate-buffered saline (6 ml) was added to each culture, and the number of cells present in both monolayer suspensions and supernatants determined using a Coulter counter (Coulter Electronics, Luton, U.K.).

Results and discussion

With the exception of resiniferatoxin, which was inactive at all concentrations tested (up to 200 nM), the diterpene esters examined were capable of inducing differentiation of HL-60 cells into adherent monocytes, with potencies in the nanomolar range (Fig. 1). An interesting feature of the response was the distinct inhibitory action on cell proliferation which occurred at concentrations well below that required to induce full differentiation (Table 2). In these experiments cell division stopped immediately and doubling did not occur after exposure to any of the compounds. It is noteworthy that resiniferatoxin also possessed this inhibitory activity, possibly indicating partial progression to the differentiated state. However, in view of the reduction in cell number observed, the contribution of cytotoxic effects cannot be excluded.

The relative potencies of the diterpenes correlated well with their known ability to activate protein kinase C from mammalian brain *in vitro* [9]. This is in contrast to the results observed for bryostatin, another activator of the enzyme, which was found to inhibit TPA-induced differentiation [15]. Structural requirements for induction of differentiation appear to be more stringent than those conferring irritancy (Table 1), in that a primary hydroxyl at C20 is necessary for full efficacy. However, the presence of a saturated side chain at positions 12 or 13, although increasing potency, is not an absolute requirement for this activity, as it is for complete tumour promoting ability. It has been observed that PKC activation by different

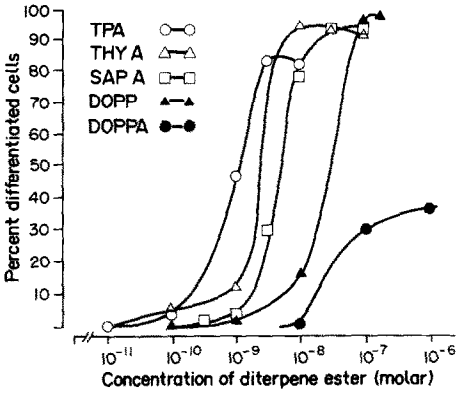

Fig. 1. Induction of HL-60 cell differentiation by diterpene esters. The data are the mean of 2–4 experiments. Resiniferatoxin was inactive at concentrations up to 200 nM. Abbreviations are explained in Table 1.

Table 1. Chemical and biological properties of diterpene esters

Source*	Compound trivial name	Chemical name	Relative irritancy†	Tumour promotion potential‡	PKC activation (Relative potency§)
1	TPA	12-*O*-tetradecanoyl phorbol-13-acetate	1.0	High	1.0
2	DOPP	12-Deoxyphorbol-13-phenyl-acetate	0.2	None	0.25
2	DOPPA	12-Deoxyphorbol-13-phenyl-acetate-20-acetate	0.2	None	<0.01
3	Sapintoxin A (SAP A)	12-*O*-[2-methylaminobenzoyl]-4-deoxyphorbol-13-acetate	0.3	None	0.5
3	Thymeleatoxin A (THY A)	9,13,14-Orthobenzoyl-6,7-epoxy-5-hydroxy-resiniferonol-12-cinnamate	0.2	Weak	0.8
3	Resiniferatoxin (RSX)	9,13,14-Orthophenylacetyl-resiniferonal-20-[3-methoxy-4-hydroxy]phenylacetate	35	None	<0.01

Compound sources: (1) Sigma Chemical Co., Poole, Dorset, U.K.; (2) Scientific Marketing Associates, London, U.K.; (3) isolated by F. J. Evans and co-workers, Department of Pharmacognosy, The School of Pharmacy, London, U.K.
† From Refs 12–14. Relative ability to induce mouse ear erythema.
‡ From Refs 10 and 16 and unpublished results.
§ From Ref. 9. This is quoted as PKC activation relative to TPA.

Table 2. Growth inhibition of HL-60 cell cultures by diterpene esters

Compound	Final cell no. ($\times 10^5$ ml^{-1})
Control	7.5
TPA	4.1
THY A	2.1
SAP A	2.5
RSX	1.3

The compounds were tested at a concentration of 0.1 nM. The experiments were carried out a total of four times and ±SE was less than 5% in all cultures.

diterpenes displays a variable cofactor requirement; for example, the non-promoting compound Sapintoxin A may be converted to a complete promoter by concomitant administration of calcium ionophore at non-promoting doses [16]. Distinct forms of PKC show variation in Ca^{2+} requirements and it has been suggested that this may be responsible for some of the selective actions of these diterpene esters [9]. The fact that the three major isozymes of PKC are known to be present in HL-60 cells [17] may be responsible for the lack of selectivity observed in this system.

In this report, we have examined the ability of a range of diterpene esters with selective biological actions (e.g. irritant but not tumour promoting) to induce HL-60 cell differentiation *in vitro*. In general, potency of differentiation induction correlated well with ability to activate PKC from mammalian brain, but not with either irritancy or tumour promotion. All compounds also exhibited an inhibitory effect on cell growth which was not necessarily associated with progression to the fully differentiated state. On a structural basis, diterpene esters appear more selective in their ability to induce differentiation than to produce erythema, but requirements are not as stringent as those conferring tumour promoting potential.

Acknowledgements—This work was supported by a project grant from the Science and Engineering Research Council (to FJE and AA).

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Biochemical Pharmacology, Vol. 38, No. 17, pp. 2927–2930, 1989.
Printed in Great Britain.

0006–2952/89 $3.00 + 0.00

Human ileum phenol sulfotransferase

(*Received* 1 *December* 1988; *accepted* 11 *March* 1989)

Sulfation is an important pathway for the biotransformation of phenolic drugs and amine neurotransmitters in humans and other animal species. The gastrointestinal tract represents the first line of defence against noxious substances present in the diet or administered orally. A normal human diet may contain up to 600 mg of phenolic compounds daily [1]. Powell *et al.* [1] demonstrated that only conjugated phenol is present in portal blood, 72% as phenyl sulfate.

Phenol sulfotransferase (PST) which catalyses sulfation is a cytosolic enzyme. At least two different molecular forms of the enzyme were found in human blood platelet, brain, lung and liver [2–6]. These forms differ in their substrate specificity, thermal stability and sensitivity to inhibitors. The thermostable form (TS) catalyses the sulfation of micromolar concentrations of exogenous phenols, the thermolabile form (TL) catalyses the sulfation of catecholamines and millimolar concentrations of phenols [2–4]. However, the TS form present in human liver can also sulfate dopamine at high concentrations [6]. On the other hand, bovine small intestine PST [7], as well as rat stomach [8] and small intestine enzymes (Barańczyk-Kuźma *et al.*, unpublished), do not use endogenous catecholamines as substrates.

Experiments presented in this communication concern the human ileum PST and were performed to determine whether the properties of human small intestine phenol sulfotransferase are similar to those of human lung [4, 9] and other human tissues enzymes [2, 3, 6], or to PSTs from the gastrointestinal tract of other species [7, 8].

Methods and results

Materials. Reagents: [^{35}S]PAPS (sp. act. 1.1–2.8 Ci/mmole) was obtained from New England Nuclear (Boston, MA) and stored at −20°. Phenol and 2-naphthol were products of Merck (Darmstadt, F.R.G.); other acceptor substrates were purchased from Sigma Chemical Co. (Poole, U.K.). Sephadex G-100 and Dextran 2000 were from Pharmacia (Uppsala, Sweden), and acrylamide and methylenebisacrylamide were from Fluka (Buchs, Switzerland). Marker proteins: bovine serum albumin, chicken ovalbumin, aldolase, horse myoglobin (Sigma) were used. All other chemicals were of analytical grade.

Tissues. Human small intestine (ileum part) was obtained at the time of surgery of 56–68 year old patients (four female and five male) undergoing clinically indicated partial ileum resection for the removal of the colon carcinoma. The fresh tissues with no pathological changes observed by histological methods were used for PST extraction.

PST extraction. PST was extracted from the mucosa or from enterocytes isolated according to Weiser [10]. The fresh mucosa or enterocytes were homogenized in 3 vol. of