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THE BISTRATENES: NEW CYTOTOXIC MARINE MACROLIDES WHICH INDUCE SOME PROPERTIES INDICATIVE OF DIFFERENTIATION IN HL-60 CELLS

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Abstract—The biological effects of cytotoxic macrolide polyethers, the bistratenes, isolated from the ascidian *Lissoclinum bistratum*, have been examined. Bistratene A was toxic to HL-60 human promyelocytic leukemia cells with an IC_{50} value of 424 nM. At lower concentrations (10–100 nM), bistratene A induced the incomplete differentiation of these cells along the monocyte/macrophage pathway. These effects were not due to inhibition of DNA synthesis. Bistratene B had similar effects to bistratene A. At micromolar concentrations these compounds enhance the phospholipid—dependent activity of type II protein kinase C from bovine spleen. The bistratenes provide new probes for studying the molecular mechanisms governing cell growth and differentiation.

Human myeloid leukemia cells can differentiate into more mature cells in response to a variety of signals. The HL-60 cell line, derived from a patient with acute myeloblastic leukemia, has become an attractive model for studies of human myeloid cell differentiation [1, 2]. The cells, which resemble promyelocytes, can be induced to differentiate terminally *in vitro* into granulocyte-like cells by dimethylsulfoxide and retinoic acid, or into monocyte/macrophage-like cells by compounds such as the phorbol esters, 1,25-dihydroxyvitamin D_3 and sodium butyrate. The mechanisms by which these agents induce differentiation have remained largely obscure. The phorbol esters e.g. (TPA) mimic diacylglycerols in enhancing the calcium- and phospholipid-dependent activity of protein kinase C (PKC**) [3], and it is possible that the PKC-catalysed phosphorylation of specific proteins leads to the macrophage-like phenotypic changes induced by the phorbol esters. However, considerable controversy surrounds this hypothesis since some diacylglycerol analogs fail to induce differentiation of HL-60 cells [4–7]. Bryostatin 1, a marine macrocyclic lactone isolated from the bryozoan *Bugula neritina* [8], also enhances the calcium- and phospholipid-dependent PKC activity [9], but its effects on HL-60 cells are concentration-dependent. At 10 nM, bryostatin 1 induces monocytic differentiation, but at 100 nM, it inhibits the phorbol ester-induced differentiation [10]. In this paper we describe the properties of new marine cytotoxins, the bistratenes, recently isolated from the colonial ascidian *Lissoclinum bistratum* [11]. Bistratene A has also been given the name bistramide A [12]. These compounds possess antitumour activity *in vitro*, and cause certain changes indicative of the induction of differentiation of HL-60 cells. Evidence presented in this paper demonstrates that the bistratenes also enhance the phospholipid-dependent activity of PKC although at much higher concentrations than are required to produce differentiation.

MATERIALS AND METHODS

Materials and chemicals. Bistratenes A and B were isolated from *L. bistratum* collected at Heron Island Reef, Australia, as previously described [11]. The purity of bistratene B used in PKC assays was verified by reverse phase and normal phase thin layer chromatography. TPA, MTT (3,[4.5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide), phosphatidylserine, oleic acid, histone H1, and 1,2-diolein were purchased from the Sigma Chemical Co. (St Louis, MO). [γ-^{32}P]ATP was obtained from Bresatec, (Adelaide, Australia).

A monoclonal antibody against OKM-1 (a macrophage–granulocyte cell surface glycoprotein) was obtained from Ortho Diagnostics (Raritan, NJ). Phycoerythrin labelled antibodies to Leu-M3 (CDW14), the granulocyte marker Leu-11a (CD16), Leu-7 (HNK-1) and HLA-DR were purchased from Becton Dickinson (Paramus, NJ).

Cell culture and cytotoxicity determination. HL-60 cells were kindly provided by Prof. P. J. Smith, University of Queensland. The cells were cultured in RPMI 1640 medium containing 10% foetal calf serum. The cytotoxicity of the bistratenes to HL-60 cells was determined using a colorimetric assay based

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** Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; MTT, (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; IC_{50}, concentration of toxin giving 50% inhibition of cell growth; IL-1, interleukin-1; NSE, nonspecific esterase.

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Table 1. Differentiation of HL-60 cells

Marker	None (Control)	Inducing agent Bistratene A (100 nM)	TPA (5 nM)
		% Positive	
Adhesion	0	100*	76[18]
Nonspecific Esterase	0	0	72[27]
Phagocytosis	0	0	57[18]
IL-1 production	0	0	ND 87[28]
OKM-1 antigen	11	79†	(10nM)
DR-Antigen	0	0	ND
Leu-M3 (CD11)	10	70†	ND
Leu-7 (HNK-1)	0	0	ND
Leu-11a (CD16)	0	0	ND

* All values were determined after 48 hr except for Leu-M3 which was determined at 96 hr.

† The concentration required to produce half-maximal expression was less than 50 nM for both OKM-1 and Leu-M3.

ND, not determined.

on conversion of MTT to a blue formazan product by live mitochondria [13, 14].

Assays for monocyte/macrophage markers. Adherence was assessed by visual examination after gently shaking the cultures and aspirating non-adherent cells. Cytocentrifuge smears were assayed for nonspecific esterase (NSE) 48 hr after treatment with bistratene A [15]. Phagocytosis was determined by incubating differentiated HL-60 cells with 0.8μ polystyrol–latex beads (Boehringer, Mannheim, F.R.G.).

Supernatants from bistratene-treated cultures were assayed for the presence of interleukin-1 (IL-1), by an ELISA assay using rabbit anti IL-1 as first antibody and an active-site directed anti IL-1 monoclonal antibody (mAb) as second antibody. Both antibodies were kindly provided by Dr A. C. Allison (Syntex, California). The presence of surface antigens HLA-DR, OKM-1, Leu-M3, Leu-7 and Leu-11a was determined by Fluorescence Activated Cell Sorting using the appropriate fluorescently labelled mAbs, and in the case of OKM-1, fluorescently labelled goat antimouse IgG as secondary antibody.

Purification and assay of PKC. PKC was isolated from bovine spleen (1000 g) by sequential chromatographies on DEAE-cellulose, phenyl-Sepharose, threonine-Sepharose and hydroxylapatite. Details of the method will be described in a forthcoming paper. The final preparation yielded 4.3 mg of type II PKC and 2.0 mg of type III PKC in electrophoretically pure form. Assays of enzyme activity were carried out essentially as described by Le Peuch *et al.* [16], using lysine-rich histone as substrate, and measuring the rate of incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (50 μM, 4 × 10^6 dpm/assay) into histone (1 mg/mL). All assays contained 10 mM $MgCl_2$ and 5 mM dithiothreitol. The concentrations of calcium ions, phosphatidylserine, diolein, TPA and bistratene included in the assays are given in Table 2. Phosphatidylserine was added as a sonicated vesicle preparation. Other compounds were added as solutions in either ethanol or methanol. The concentration of organic solvent, which was always less than 2% (v/v), was shown to have no effect on the enzyme activity.

Table 2. Effect of bistratene B on Type II protein kinase C activity

[Phosphatidyl-Serine] (μg/mL)	[TPA] (nM)	[Bistratene B] (μM)	Enzyme activity (%) ± 1 SD*
—	—	10	2 ± 1†
—	—	—	2 ± 1†
10	—	—	16 ± 4†
10	—	10	32 ± 2†
10	16	—	81 ± 1‡

* Activities are expressed as a percentage of the maximal activity measured in the presence of 10 μg/mL phosphatidylserine and 9.7 μM diolein. All assays were carried out in the presence of 10 μM calcium ions.

† The mean ± 1 SD of three determinations.

‡ The mean ± 1 SD of two determinations.

Assay of DNA synthesis inhibition. HL-60 cells were aliquoted into 96-well microtiter plates at 3 × 10^4 cells/100 μL, and treated with either Bistratene A (100 ng/mL, 140 nM) or cytosine arabinoside (0.5 μg/mL). Control cells received no drugs. At 1,2,4,6 and 8 hr after addition of the compounds, a 30 min pulse of [*methyl*-^{3}H]thymidine (Radiochemical Centre, Amersham, U.K., 1 μCi/well) was carried out. The incubation was terminated by freezing and the acid-precipitable DNA was subsequently harvested and counted using an LKB-Betaplate (1205) Harvester. Six replicates were performed at each point.

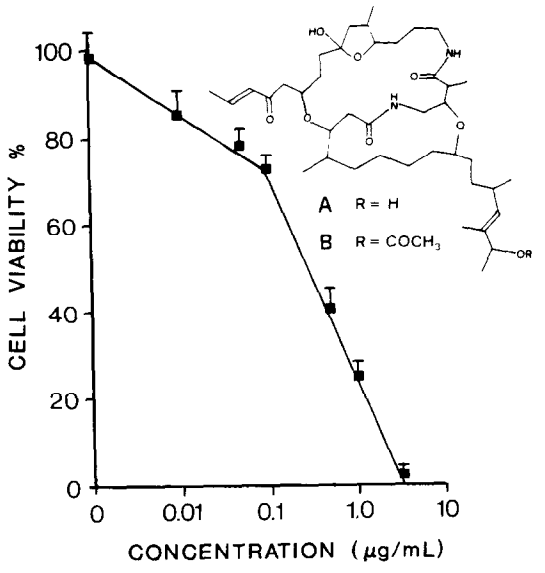

Fig. 1. Cytotoxicity of bistratene A towards HL-60 cells *in vitro*. Cells were plated at a density of 10^5/mL and bistratene A added at the indicated concentrations. After 48 hr the viability of the cells was determined colorimetrically using MTT. The experiment was performed three times with eight assays at each concentration. The error bars show the standard deviation.

RESULTS

HL-60 cell differentiation

Previous results from our laboratories have demonstrated that the marine macrolides bistratenes A and B (Fig. 1) are cytotoxic to both normal and transformed human cells (T24 bladder carcinoma) with similar IC_{50} values, 0.07 μg/mL (99 nM) and 0.09 μg/mL (84 nM), respectively [11]. When HL-60, promyelocytic human leukaemia cells, were treated with these compounds, cytotoxicity was observed at high concentrations, but at lower concentrations dramatic changes in the morphology of these cells were observed, indicative of differentiation. The effect of increasing concentrations of bistratene A on the viability of HL-60 cells is shown in Fig. 1. The IC_{50} value with these cells was 424 nM. At concentrations below this value HL-60 cells undergo differentiation. After 2 hr of continuous exposure to bistratene A (100 nM), HL-60 cells adhered to the culture plate, and acquired a spindle-shaped morphology and prominent pseudopodia (Fig. 2b). The cells remained mostly isolated from one another and with time there was an increase in cell size (Fig. 2c). TPA-treated cells were more strongly adherent and showed greater clumping (Fig. 2d). The morphology of bistratene-treated HL-60 cells is suggestive of cells in the process of differentiation along the monocyte/macrophage pathway, in that prominent pseudopodia are present. The nature of the differentiated cells was investigated in greater detail using a variety of cell surface and other differentiation markers (Table 1). These cells show some of the characteristics of macrophages, for example adherence to plastic and expression of OKM-1 and Leu-M3 antigens, but do not appear to be activated since they lack HLA-DR expression and nonspecific esterase activity, do not phagocytose and fail to produce IL-1.

DNA synthesis

It has been shown that inhibitors of DNA synthesis and other antimetabolites are weak inducers of HL-60 differentiation [17], thus it was important to determine whether the bistratenes exert their effects through inhibition of DNA synthesis. As a measure of DNA synthesis, incorporation of [*methyl*-^{3}H]thymidine into DNA was measured at various times after addition of 140 nM bistratene A to HL-60 cells. For comparison, cells were also exposed to 0.5 μg/mL of the DNA synthesis inhibitor cytosine arabinoside. As seen in Fig. 3, bistratene A had no inhibitory effect on DNA synthesis during the 8 hr period studied.

Activation of PKC

The effect of bistratene B on the activity of type II PKC from bovine spleen was examined. The results are summarized in Table 2. In the presence of 10 μg/mL phosphatidylserine and 10 μM calcium ions, conditions under which the enzyme is not fully activated, bistratene B (10 μM) enhanced the activity two-fold. Under the same conditions, diolein (9.7 μM) and TPA (16 nM) enhance the activity by approximately six-fold and five-fold respectively. In the absence of added phospholipid no effect of bistratene B on enzyme activity was observed at concentrations up to 360 μM. We have observed similar effects of bistratene B, diolein and TPA on the activity of the enzyme in the presence of 600 μM oleic acid. Bistratene B enhanced the fatty acid-dependent activity of type II PKC by 4.2-fold, whereas diolein (9.7 μM) and TPA (16 nM) both enhanced the activity by approximately seven-fold. The concentration of bistratene B required for half-maximal enhancement of oleic acid-dependent activity was 5 μM, similar to the concentration required for half-maximal enhancement of phospholipid-dependent activity. The effects of bistratene A on PKC were comparable to those with bistratene B. While the activation of PKC at these high fatty acid concentrations is obviously not of physiological relevance, the results provide additional evidence to support the conclusion that bistratene B interacts with PKC in a manner similar to the interaction of diolein and TPA with the enzyme.

DISCUSSION

We have shown that novel marine cytotoxins, the bistratenes, can induce some changes indicative of differentiation of HL-60 cells at nanomolar concentrations. Although all of the bistratene-treated cells adhere to the culture dish they are not as strongly adherent as those treated with TPA and very little clumping is observed. They also differ from TPA-treated HL-60 cells as evidenced by lack of NSE activity and inability to phagocytose latex beads. Bryostatin induces a lower percentage of adherent cells than does TPA [18]. However, the differentiated cells express NSE and phagocytose,

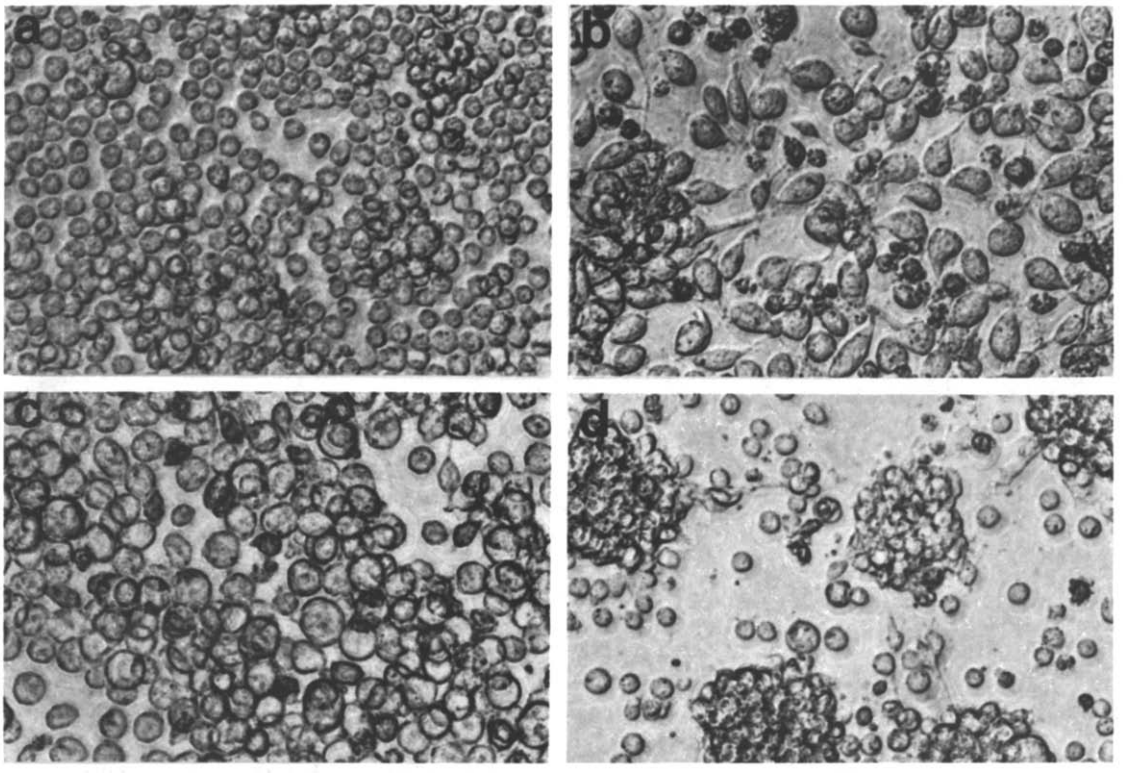

Fig. 2. Photomicrographs of HL-60 cells treated with differentiating agents. (a) Control; (b) 100 nM bistratene A, 48 hr; (c) 100 nM bistratene A, 96 hr; (d) 20 nM TPA, 48 hr. Magnification × 400.

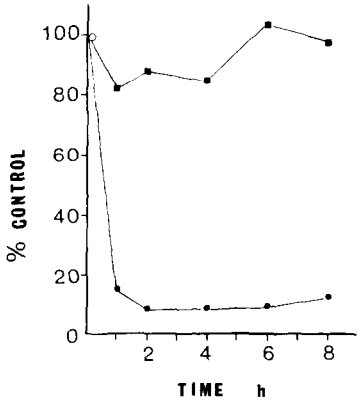

Fig. 3. Effect of bistratene A and cytosine arabinoside on DNA synthesis in HL-60 cells. Cells were plated into microtiter plates at a density of $3 \times 10^4/100\ \mu L$ and treated for the indicated times with either 100 ng/mL bistratene A or 0.5 μg/mL cytosine arabinoside. A 30 min pulse of [*methyl*-^{3}H]thymidine (1 μCi/well) was added at the end of the incubation period and the acid-precipitable DNA was collected onto filters and processed for liquid scintillation counting. Six replicates were performed at each time point and results are expressed as a percentage of control (no drug treatment). The points represent the average of two experiments. Bistratene A (■) and cytosine arabinoside (●).

indicating similarities between the mature phenotypes induced by these two agents.

The results of the present study also show that the bistratenes enhance the phospholipid-dependent activity of type II PKC. While the enhancement by bistratene B is not as dramatic as that seen in the presence of diolein or TPA, the results provide preliminary evidence that the bistratenes exhibit some similarity to these compounds in their effect on the enzyme. The ability of bistratene B, diolein and TPA to enhance fatty acid-dependent PKC activity supports this conclusion. It is clear that the concentrations of the bistratenes needed for maximal activation of type II PKC in the *in vitro* assay systems (~ 10 μM) are much higher than those required to elicit differentiation in HL-60 cells (10–100 nM) and it is unlikely on this basis, that the activation of type II PKC is the primary mechanism by which the bistratenes induce differentiation. It will be of interest however to characterize further the interaction of the bistratenes with other PKC isoenzymes, since it has been shown that bryostatin displaces TPA less efficiently from type II than from types I and III PKC [19], and in addition, TPA promotes the differential translocation of PKC isoenzymes [20, 21]. It is still uncertain whether enhancement of PKC activity is a sufficient stimulus to induce leukemic cell dif-

ferentiation [4–7] and there is a growing body of evidence which suggests that there are PCK-independent processes involved in the differentiation induced by the phorbol esters and bryostatins [21–23]. There is also a distinct lack of correlation between the translocation of PKC and other biological effects mediated by this enzyme [24].

Preliminary experiments using Indo-1 loaded mouse thymocytes [25] have shown that bistratene A does not cause a change in the intracellular free calcium concentration and is therefore not a calcium 'ionophore' (D. Watters and R. Tellam, unpublished observations). This study has also shown that bistratene A has no significant effect on DNA synthesis over an 8 hr period and since morphological changes indicative of differentiation are evident as early as 1–2 hr after addition of bistratene, it is clear that inhibition of DNA synthesis is not the mechanism by which these compounds act on HL-60 cells. It remains to be determined whether the primary mode of action of the bistratenes involves PKC or a PKC-independent process.

In summary we have identified a new class of compounds, the bistratenes, which induce the incomplete differentiation of HL-60 cells. Roussakis *et al.* [26] have recently reported that bistramide (bistratene) causes the morphological differentiation of human lung carcinoma cells. The bistratenes are readily isolated in significant quantities and provide useful new probes for the study of molecular mechanisms governing cell growth regulation and differentiation.

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