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CYTOSTATIC AND CYTOTOXIC PROPERTIES OF THE MARINE PRODUCT BISTRATENE A AND ANALYSIS OF THE ROLE OF PROTEIN KINASE C IN ITS MODE OF ACTION

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Abstract—Bistratene A is a polyether which was isolated from the marine ascidian *Lissoclinum bistratum* Sluiter. The hypothesis has been tested that the cytostatic effect of bistratene A is mediated by modulation of protein kinase C (PKC). Human-derived A549 lung and MCF-7 breast adenocarcinoma cells are extremely sensitive to growth inhibition induced by activators of PKC. Therefore, the effect of bistratene A on these cell lines was compared with that of the known PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The ability of bistratene A to modulate PKC activity in cellular cytosol was assessed to determine the involvement of PKC in the induction of cytostasis. Bistratene A inhibited the growth of both cell lines and initial seeding density determined its cytostatic potency. IC_{50} values were between 1.0 and 2.9 nM. Bistratene A also had a profound effect on the colony forming ability of A549 cells, preventing clonal growth at 5 nM. Using the incorporation of [^{3}H]thymidine into cells to assess DNA synthetic activity and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay to define cytotoxicity, the compound was found to have both cytostatic and cytotoxic properties. Bistratene A decomposed by 50% after only 2.8 hr in cell culture medium. TPA induced rapid motility and the formation of a network of branched colonies in both cell lines grown on Matrigel, whereas bistratene A did not cause the same effect. Cell cytosol was analysed for phorbol ester binding sites after treatment with bistratene A or TPA. Incubation with TPA (10 nM) caused a reduction in binding sites to 57% of binding in control cells after 30 min and to 35% after 24 hr. Bistratene A did not cause a significant change in binding sites. Assays of PKC activity in cellular cytosol revealed that bistratene A was unable to activate or inhibit the enzyme at concentrations of up to 10 μM. The results suggest that bistratene A is an exquisitely potent cytostatic agent in the two cell lines studied, but modulation of PKC is not involved in the mode of action by which it elicits this effect.

The evaluation of animals and plants as sources of cytostatic agents is an important part of the search for novel anticancer agents. Screening of natural products has yielded the promising antineoplastic agent taxol [1]. A particular focus of many current drug discovery programmes is the exploration of compounds with novel modes of action. This emphasis renders the current clinical trial of the antiproliferative agent bryostatin 1, extracted from the sea moss *Bugula neritina*, especially intriguing, as the only biochemical property so far discovered for this agent is its ability to activate protein kinase C (PKC‡) [2].

Bistratene A, also known as bistramide A, is another antiproliferative agent of marine origin. It is a polyether (Fig. 1) [3], which was isolated from the ascidian *Lissoclinum bistratum* [4, 5]. Bistratene A possesses cytotoxic properties in a variety of cell lines [4, 6–8]. Of particular interest is its ability to induce the incomplete differentiation of HL60 leukaemia cells along the monocyte/macrophage pathway. Bistratene A is able to activate the β isozyme of PKC *in vitro* but it is unclear whether there is a causal relationship between this property and its ability to induce cell differentiation [8].

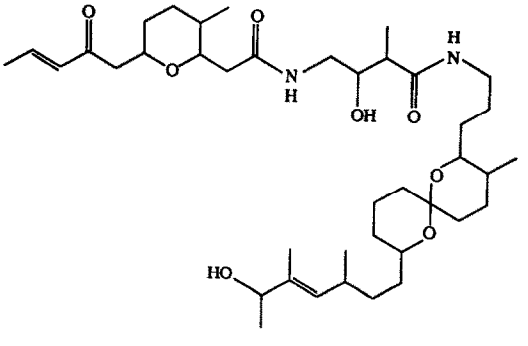

Fig. 1. Structure of bistratene A.

In the work reported here, the biological properties

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‡ Abbreviations: CFE, colony forming efficiency; DMSO, dimethylsulphoxide; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

of bistratene A were investigated to explore the relationship between its effects on growth and on PKC. To this end, bistratene A was incubated with two cell lines in which activators of PKC have been shown to cause cytostasis: human-derived A549 lung and MCF-7 breast adenocarcinoma cells [9, 10]. The hypothesis was tested that bistratene A mimics the effects of PKC activators such as the tumour-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [9, 10] and the experimental drugs bryostatins 1 and 2 [11, 12] in these cell lines. Experiments were conducted in which the effects of these agents on cell growth characteristics were compared. Furthermore, the effect of drug treatment on the behaviour and morphology of cells grown on the basement membrane substitute Matrigel was assessed. TPA has been shown to induce the formation of a network of branched colonies in certain cell lines grown on this matrix [13]. Finally, we analysed the effect of bistratene A on the levels and location of cellular PKC using assays for PKC activity and cytosolic phorbol ester binding sites to assess whether bistratene A causes cytostasis via modulation of PKC.

MATERIALS AND METHODS

Chemicals. TPA and other reagents were purchased from the Sigma Chemical Co. (Poole, U.K.) or Gibco BRL (Paisley, U.K.). Matrigel was obtained from Collaborative Research Inc. Biomedical Products Division (Bedford, MA, U.S.A). Bistratene A was isolated from *L. bistratum* collected at Heron Island reef, Australia, as described previously [5]. Stock solutions of bistratene A and TPA were prepared in dimethylsulphoxide (DMSO) and stored at −20°. The final concentration of DMSO did not exceed 0.5% in medium, which had no effect on cell growth.

Measurement of cell growth and clonogenicity. A549 lung and MCF-7 breast human adenocarcinoma cells originated from the American Type Culture Collection (Rockville, MD, U.S.A). A549 cells were cultured in Ham's F12 medium as described previously [9], and MCF-7 cells were cultured in minimal essential medium (Eagle's modification) with additional pyruvate (1 mM) and non-essential amino acids. Both media were supplemented with 10% foetal calf serum and glutamine (2 mM).

Cells (2×10^4) were routinely seeded in Nunclon six-well multidishes (3 mL medium, 3.5-cm diameter). Medium was aspirated and routinely replenished with or without drug every other day. Cells were counted with a Coulter Counter (model ZM) on the 5th day of incubation. The IC_{50} values quoted are the concentrations which caused inhibition of growth by 50%. The colony forming ability of cells was estimated using dishes coated with 1 mL gelatine solution (2%). This solution was removed and the well was washed with 2 mL phosphate-buffered saline (PBS). Cells (10^2 or 10^3/well) were seeded in 3 mL medium and bistratene A was added 24 hr later. Medium was replenished at 3–4 day intervals and cells were incubated for 10 days. Clones were fixed with 70% industrial methylated spirit, washed with PBS and stained with aqueous crystal violet 1%. The colony forming efficiency (CFE) of untreated A549 cells was 34.8 ± 9.7% at a seeding density of 10^2 and 29.4 ± 7.4% at 10^3 (mean ± SD, N = 18).

Incorporation of [^{3}H]thymidine into cells was measured as described previously [11]. Cells were seeded at 1.5×10^5/well (1.6×10^4/cm^2) and allowed to attach and settle for 20 hr. Bistratene A was then added. At regular time points up to 72 hr, cells were washed and incubated for 1 hr with [^{3}H]-thymidine.

Stability of bistratene A in cell culture medium. Bistratene A (200 nM) was incubated in Ham's F12 medium at 37° for varying time intervals up to 30 hr. This pre-treated medium was then incubated with A549 cells which had been seeded at 1.5×10^5/well 20 hr previously. After 12 hr, incorporation of [^{3}H]-thymidine into cells was assessed. A calibration curve of bistratene A concentration vs amount of [^{3}H]thymidine incorporated after incubation with cells for 12 hr was constructed for each experiment. The growth inhibitory potency of the bistratene A solution was converted to an equivalent bistratene A concentration from the calibration curve.

Cytotoxicity assays. Cells seeded at 4.4×10^3/well (0.6 cm diameter, 1.6×10^4 cells/cm^2) were allowed to attach and settle for 20 hr. They were then exposed to bistratene A for 24 hr. Cytotoxicity was assessed by the assay which employs the dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann [14]. Cells were incubated for 4 hr with 200 μL medium containing MTT (5 mg/mL). Cell monolayers were washed twice with PBS 200 μL and the MTT reduction product formazan was dissolved in buffered DMSO 200 μL (one part buffer with glycine 0.1 M, NaCl 0.1 M, pH 10.5, to eight parts DMSO) using a plate shaker for 20 min. Formazan absorption was measured at 550 nm using an Anthos 2001 plate reader. LC_{50} values are the concentrations which caused cytotoxicity in 50% of cells.

Morphology of cells on Matrigel. Liquid Matrigel (0.2 mL) was added to wells (1.6 cm diameter) and allowed to polymerize for 20 min at 37°. A suspension of 5×10^4 cells (2 mL) was added to each well. Prior to the addition of agent, plates were incubated for 1 hr to allow cell attachment. Wells were photographed after 48 hr.

Measurement of phorbol ester binding and PKC activity in cell cytosol. Cellular cytosol was prepared and phorbol ester binding sites were assayed using mixed micelles as described by Bradshaw *et al.* [15]. Tritiated phorbol-12,13-dibutyrate was employed as phorbol ester receptor ligand. The assay is based on the procedure of Hannun and Bell [16]. Cytosolic PKC activity was measured via the incorporation of the γ phosphate moiety of [^{32}P]ATP into a PKC-specific peptide using a kit from Amersham International (Amersham, U.K.) Activation was assessed in the presence of calcium (1 mM) and phosphatidylserine/Triton X-100 mixed micelles (8 mol%). The kit component containing TPA (3.2 μM) and mixed micelles was modified to exclude TPA. Agents were then added to give final assay mixture concentrations of 10 nM–10 μM bistratene A or 3.2 nM–3.2 μM TPA. Results are expressed as

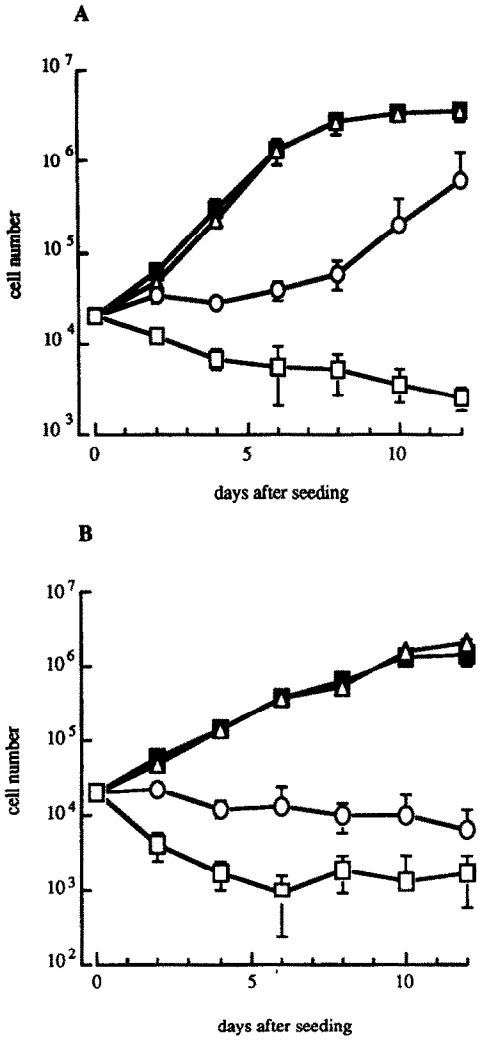

Fig. 2. Effect of bistratene A at 1 (triangles), 10 (circles) 100 (open squares) nM on the growth of (A) A549 cells and (B) MCF-7 cells. Closed squares indicate growth of control cells. Values are the means ± SD of three experiments, each conducted in triplicate.

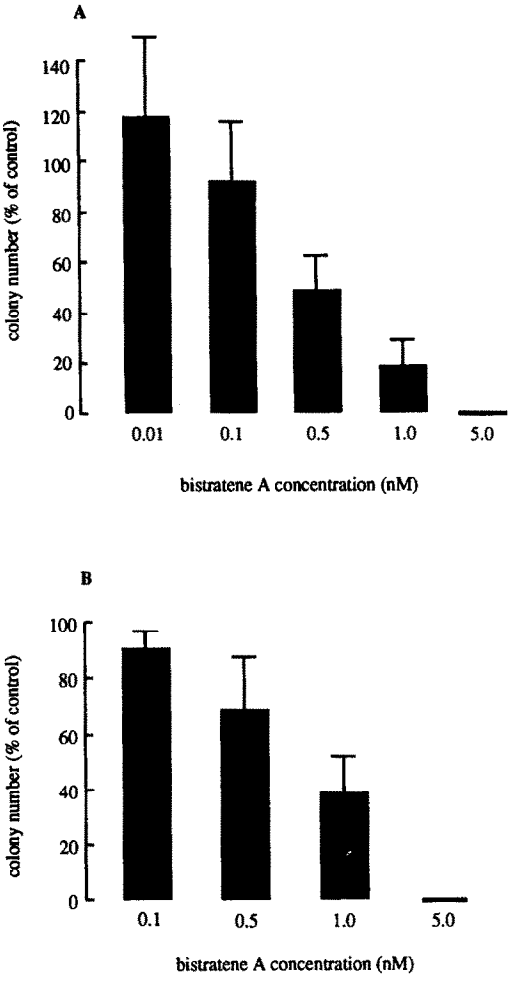

Fig. 3. Effect of bistratene A on the clonogenicity of A549 cells. Clonogenic assays were performed using (A) 10^2 and (B) 10^3 cells/well. Colonies were counted 10 days after seeding. Results are expressed as a percentage of clonogenic growth of control cells. For details of culture conditions, see Materials and Methods. Values are the means ± SD of three experiments, using six wells for each concentration.

a percentage of maximal PKC activity, defined as the PKC activity obtained by adding TPA 3.2 μM. Inhibition of PKC was determined with the same kit without modifications. Cytosolic PKC was activated maximally using TPA 3.2 μM and bistratene A (10 nM–1 μM) was added to the reaction mixture to assess its ability to inhibit PKC activity.

RESULTS

Cells were incubated with bistratene A and the effect of the agent on cell growth was observed for up to 12 days. In both cell lines, bistratene A concentrations of 10 nM and above inhibited cell growth, whereas 1 nM was innocuous (Fig. 2). The growth profile observed with 10 nM bistratene A in A549 cells (Fig. 2A) indicates that the cells restart growth after 6–8 days in culture in spite of repeated addition of the agent with each medium change. This phenomenon was not observed with MCF-7 cells (Fig. 2B). The IC_{50} derived from the plots of cell number versus bistratene A concentration yielded 2.3 ± 0.1 nM for A549 and 2.9 ± 0.4 nM for MCF-7 cells (mean ± SD, N = 3–4). These values were obtained with an original seeding density of 2×10^4/3.5-cm well. At lower seeding densities cells were even more sensitive to bistratene A: the IC_{50} was reduced to 1.0 ± 0.1 nM in A549 cells seeded at 5×10^3. The ability of bistratene A to affect cells seeded at very low density was determined in a clonogenicity assay. The CFE of A549 cells was found to be compromised by bistratene A: colony growth was progressively inhibited with increasing bistratene A concentration and colonies were not able to grow in the presence of 5 nM of the agent (Fig. 3). The intensity of the anticlonogenic effect

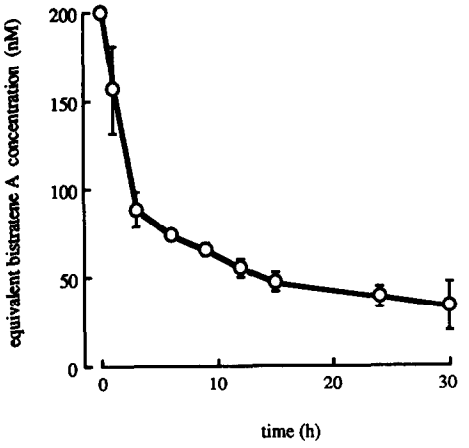

Fig. 4. Stability of bistratene A at 37° in culture medium. Values are the means ± SD of three or four experiments, each conducted in duplicate.

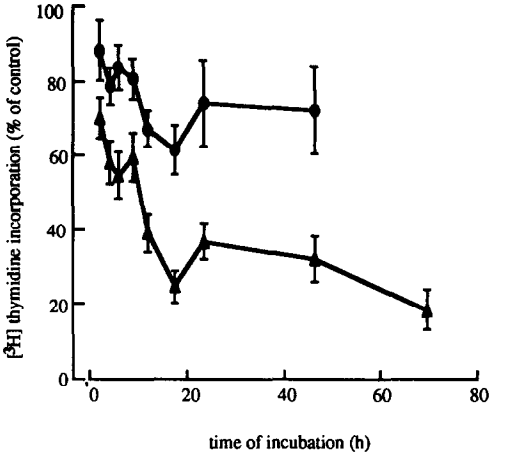

Fig. 5. Time course of effect of bistratene A at 15 (circles) and 50 (triangles) nM on the incorporation of [^{3}H]thymidine into A549 cells. At the indicated time points, cells were washed and incubated with [^{3}H]thymidine for 1 hr. Values are the means ± SD of three experiments, each conducted in triplicate.

was also influenced by the initial cell seeding density, as the reduction in CFE was more pronounced when cells were seeded at 10^2 (Fig. 3A) rather than at 10^3/well (Fig. 3B).

As knowledge of the chemical properties of bistratene A is scarce, a study was designed to determine its stability under conditions used here routinely for cell culture. Drug was incubated with medium for time intervals of up to 30 hr before addition to A549 cells and measurement of [^{3}H]-thymidine incorporation (Fig. 4). After only 2.8 ± 0.4 hr, the effectiveness of bistratene A to inhibit [^{3}H]thymidine uptake was reduced by 50%.

The instability of bistratene A at 37° suggests that its potent effect on cell growth occurs probably within a short time span. In order to ascertain the time course of events which lead to bistratene-induced growth inhibition more precisely, its ability to interfere with the incorporation of [^{3}H]thymidine into cells was studied. The growth arresting effect of bistratene A manifested itself within 3 hr of incubation (Fig. 5).

A549 cells underwent considerable morphological changes on exposure to bistratene A (Fig. 6A). After exposure to the drug for 48 hr, cells were spread out and developed cytoplasmic extensions and vacuoles. Half of the cells appeared to be multinucleate (Fig. 6B). This finding is in agreement with the induction of polyploidy by bistratene A in NSCLCN6 L16 non-small cell lung cancer cells [17]. To determine if these changes in cell morphology were indicative of, or accompanied, cytotoxicity, the ability of bistratene A to interfere with cellular redox potential as indicated by the MTT assay was investigated. At concentrations exceeding 10 nM, bistratene A decreased cellular viability. The LC_{50} derived from this assay was 198 nM.

Some cell lines undergo unusual morphological changes on exposure to PKC activators when grown on the basement membrane substitute Matrigel [13]. Therefore, A549 cells were cultured on Matrigel and the effect of bistratene A on cell morphology was compared to that of TPA. Cells adopted a spheroid shape and tended to clump (Fig. 7A). Addition of TPA (10 nM) induced rapid cell motility and the formation of chains of cells within 4 hr. Subsequently, cells associated to form a network (Fig. 7B). Essentially the same effect was observed in MCF-7 cells grown on this matrix (results not shown). In contrast, bistratene A (10 and 100 nM) did not cause any significant alteration in cell morphology compared to control cells (Fig. 7C, D).

A549 cell cytosol was used as a source of PKC to assess the direct effect of bistratene A on this enzyme. PKC is the major phorbol ester receptor and therefore the assay for phorbol ester binding reflects levels of PKC [16]. Cytosolic phorbol ester binding sites were reduced by 43 ± 12% (mean ± SD, N = 3) of control values in A549 cells which were treated for 30 min with 10 nM TPA. Numbers of binding sites decreased further after 24 hr (results not shown). Cells continually passaged in the presence of phorbol-12,13-dibutyrate for at least 9 weeks also had a reduced number of phorbol ester binding sites. In contrast, bistratene A (10–100 nM) did not significantly alter phorbol ester binding, even after prolonged exposure to the agent. The effect of bistratene A on PKC activity in the cytosol of A549 cells was compared to that of TPA. Under the conditions of the assay, TPA activated the enzyme maximally at 32 nM, but bistratene A (10 nM–10 μM) did not augment PKC activity. Similarly, the agent did not affect PKC from MCF-7 cell cytosol. Bistratene A was unable to inhibit PKC from either A549 or MCF-7 cells (Table 1).

DISCUSSION

The growth of A549 and MCF-7 cells is exquisitely

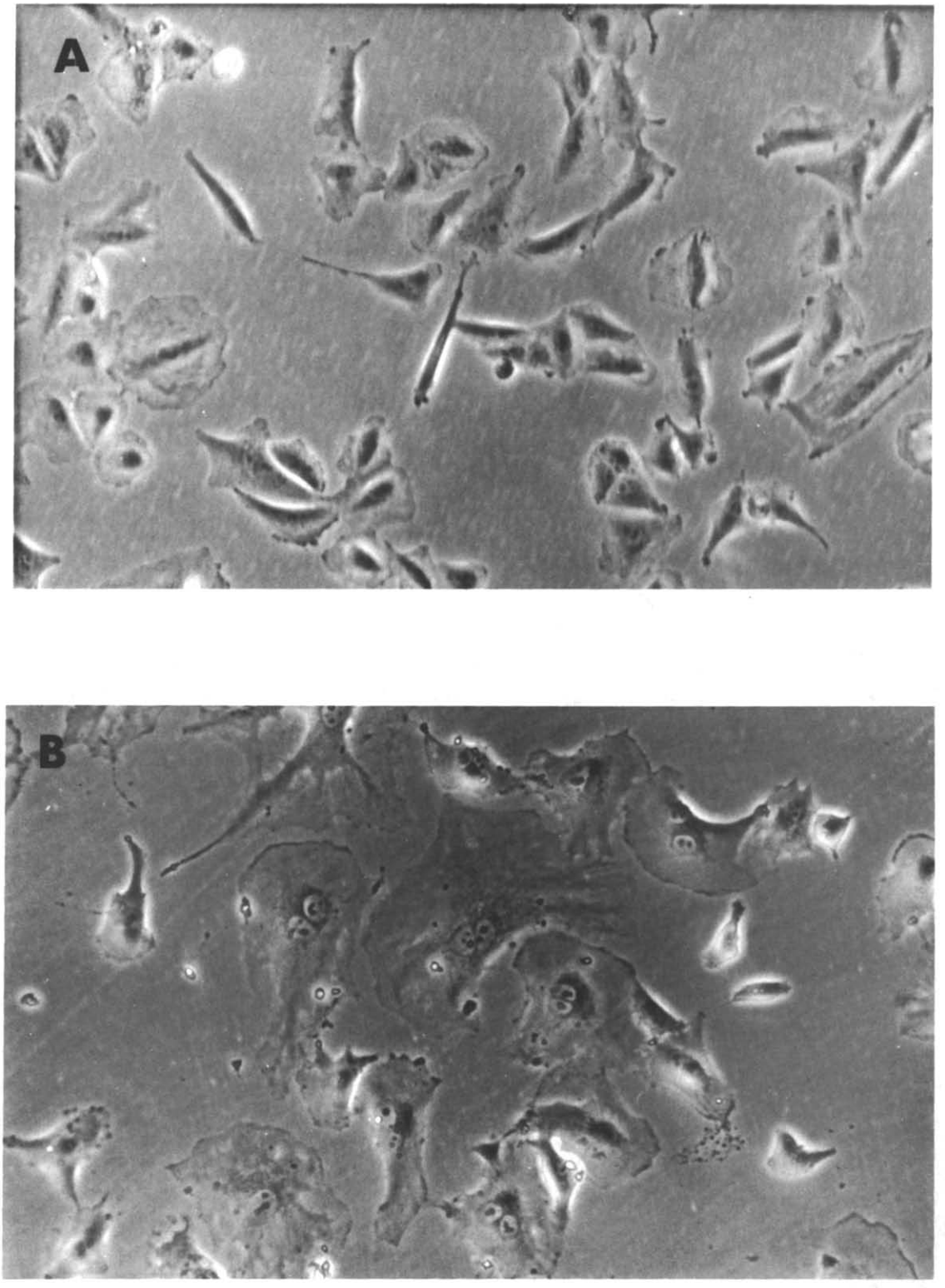

Fig. 6. Effect of bistratene A on the morphology of A549 cells. (A) Control cells; (B) cells exposed to bistratene A (10 nM) for 48 hr. Cells were seeded at 2×10^4/3.5-cm diameter well and bistratene A was added after 4 hr.

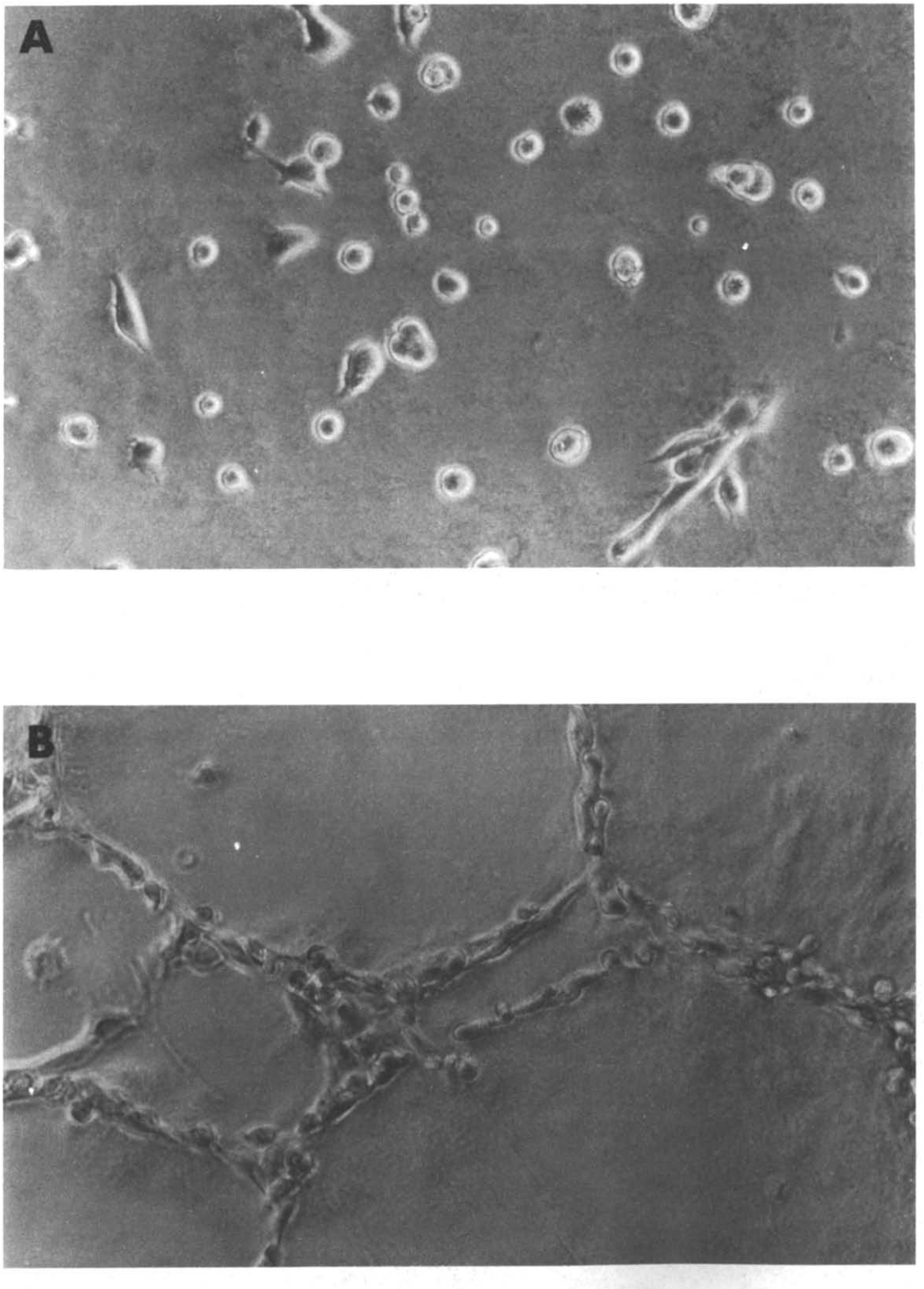

Fig. 7. Morphological changes in A549 cells grown on Matrigel. (A) Cells without drug; (B) cells with TPA 10 nM; (C) with bistratene A 10 nM and (D) with bistratene A 100 nM. Photographs are representative of three experiments, each conducted in duplicate.

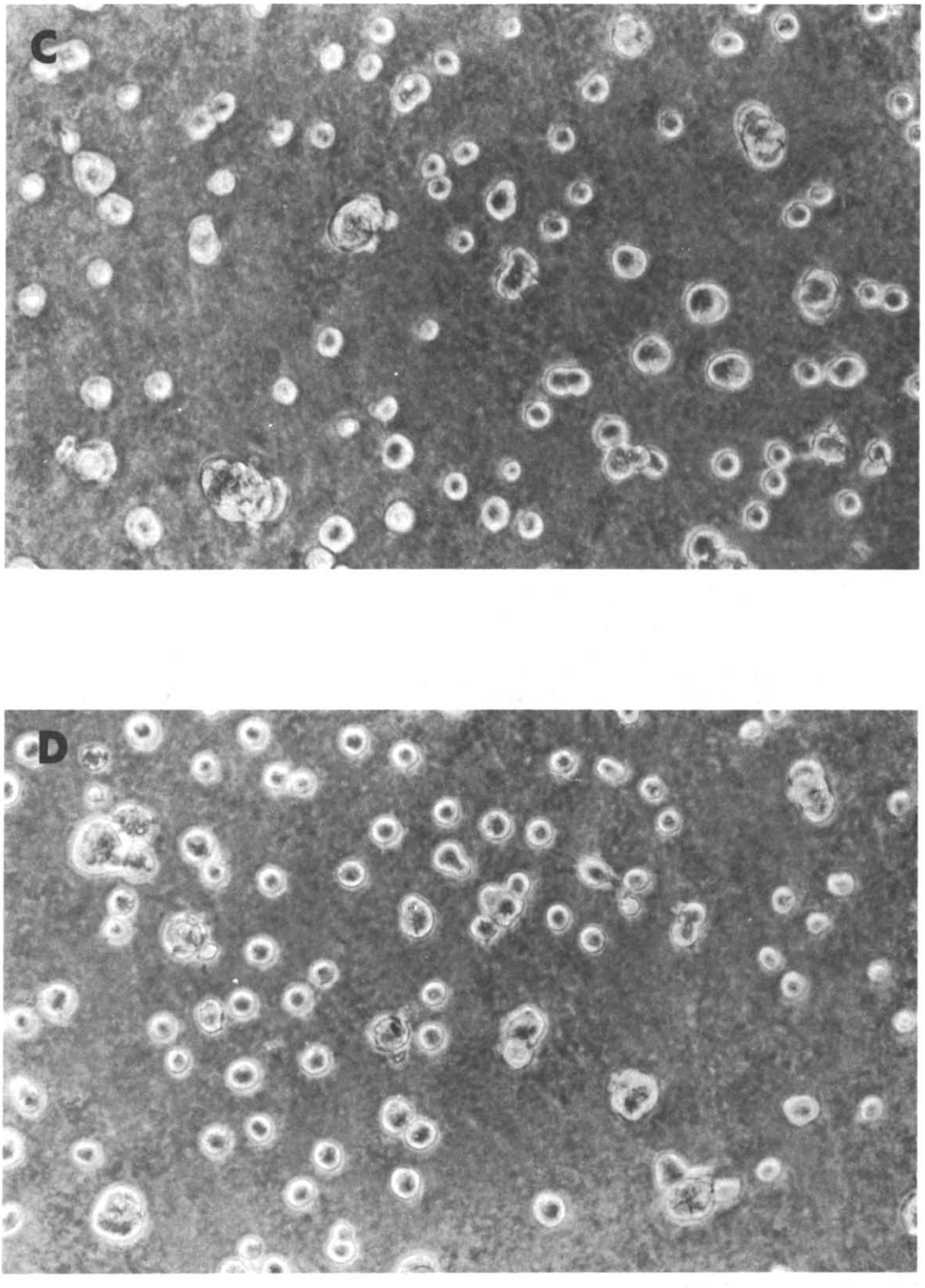

Fig. 7—*continued*.

Table 1. Effect of bistratene A on activity of PKC from A549 or MCF-7 cells stimulated maximally by TPA (3.2 μM)

Bistratene A concentration (nM)	PKC activity (% of activity without drug)	
	A549 cells	MCF-7 cells
10	97.3 (6.5)	101.8 (3.8)
100	98.2 (4.7)	96.9 (4.5)
1000	101.9 (5.2)	96.3 (2.3)

Conditions of PKC isolation and incubation are described in Materials and Methods.

* Values are the means (±SD) of three experiments, each conducted in triplicate.

sensitive towards bistratene A. Its growth-inhibitory potency, as reflected by an IC_{50} value of 1.0–2.9 nM, is lower than the IC_{50} values established in previous studies ranging from 20 nM in P388 murine leukaemia [6] to 424 nM in HL60 cells [8]. The clonogenicity of A549 cells was more potently affected by bistratene A than the growth of cells in high density culture and IC_{50} values were dependent on initial cell seeding density. The finding that cell number is a crucial determinant of intensity of effect of bistratene A may be due to either metabolic inactivation or cellular production of a factor which abrogates bistratene-induced cytostasis. Bistratene A was found to be relatively unstable in physiological medium. Measurement of its cytotoxic potential using the MTT assay confirmed that at concentrations of above 10 nM it caused irreparable damage to A549 cells within 24 hr.

The aim of this study was to determine whether the characteristics of growth arrest caused by bistratene A in these cells are comparable to those exerted by known PKC activators. The concentrations of bistratene A necessary to inhibit the growth of A549 cells are comparable to those at which TPA interferes with the proliferation of these cells [9]. Bistratene A at 10 nM concentrations inhibited the growth of A549 cells for 6–8 days, after which proliferation resumed, even though cells were re-exposed to the drug with each change of medium. This decrease in sensitivity towards the drug with time was not observed in MCF-7 cells. Similarly, TPA (10 nM) induced cytostasis in A549 cells for 6 days prior to uninhibited proliferation [9], whereas in MCF-7 cells cytostasis was irreversible at this TPA concentration [10]. Inhibition of A549 cell growth by PKC activators is mediated by cytostatic rather than cytotoxic mechanisms [9, 11]. It was therefore of interest to find out if the effect of bistratene A was a cytostatic rather than a cytotoxic one. The results presented above suggest that bistratene A has both cytostatic and cytotoxic properties. There is a 100–200-fold discrepancy between IC_{50} and LC_{50} values, although experiments are not directly comparable as they were conducted with differing cell densities and varying incubation times. The ability of bistratene A (50 nM) to inhibit incorporation of [^{3}H]thymidine was greater than its cytotoxic potency after incubation for 24 hr at equivalent cell densities. These observations suggest that bistratene A, like TPA, is able to cause growth arrest at low concentrations without damaging cells irreversibly. Taken together, the data is in accordance with the notion that these agents activate the same biochemical mechanisms to bring about growth arrest. The accepted mechanism responsible for the cytostatic action of TPA is activation of PKC [18]. Thus, PKC could be tendered as a target via which bistratene A exerts its action. This suggestion is particularly judicious in view of the finding that bistratene A has been reported to activate isolated PKC-β, albeit only at micromolar concentrations [8].

It has been suggested that branched colony formation of cells grown on Matrigel is reflective of an invasive and metastatic phenotype [19]. PKC activation by TPA was shown to change cell motility and collagenase IV production in certain malignant fibroblast cells and thus to increase their invasive properties [13], and inhibition of PKC activity by staurosporine had an anti-invasive effect on human bladder carcinoma cells [20]. It has to be stressed that invasion is only one of the many stages involved in the complex process of metastasis, but PKC modulation is clearly pivotal in the development of metastasis [21, 22]. We report here for the first time that TPA induces the formation of branched colonies in A549 cells on Matrigel. The dramatic morphological changes seen on Matrigel have to be interpreted with caution as they are not necessarily related to metastic potential and may be cell type specific, as Noel *et al.* [23] found that normal mesangial cells and keratinocytes were also able to form a network and invade Matrigel. These authors also failed to find a correlation between cellular organization on Matrigel in isolated clusters, or in a network, and the ability to invade the Matrigel matrix. A549 and MCF-7 cells are known to be metastic *in vivo* and did not form branched colonies on Matrigel, yet did so when treated with TPA. This ability to increase motility and form a network may therefore be related to specific protein phosphorylations which are modulated by PKC. The lack of effect of bistratene A in inducing these changes suggests that this agent does not mimic phorbol esters by activating PKC in intact cells.

Further experiments using PKC from the cytosol of A549 cells support the notion that bistratene A does not affect PKC directly. Unlike TPA, it did not elicit either the redistribution of phorbol ester receptor sites from the cytosol to the membrane or subsequent downregulation. Likewise, at concentrations up to 10 μM, bistratene A did not cause activation of PKC. This finding using crude cytosolic extracts is in contrast to the results of Watters *et al.* [8], who found that bistratene A (10 μM) was able to activate purified PKC-β submaximally in the presence of phosphatidylserine or oleic acid. A549 cells contain mainly PKC-α [24] and PKC-ζ (Stanwell and Gescher, unpublished), and MCF-7 cells have been reported to contain a variety of isozymes [25, 26]. Preliminary experiments suggest that bistratene A is unable to activate any of these PKC isozymes. Our results outlined above also show that bistratene A was unable to inhibit PKC under

conditions in which staurosporine caused inhibition with an IC_{50} of 6.1 nM [15].

In summary, bistratene A is able to elicit potent cytostasis and cytotoxicity in two cell lines, the growth of which is exquisitely sensitive to PKC modulation. However, it seems unlikely that the agent inhibits cell growth in these cells via activation or inhibition of PKC. Bistratene A appears to exert its effects by subtle interference with a cellular signal transduction mechanism which does not involve PKC.

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REFERENCES

1. Rowinsky EK and Donehower RC, Taxol: twenty years later, the story unfolds. *J Natl Cancer Inst* **83**: 1778–1781, 1991.
2. Pettit GR, The bryostatins. In: *Progress in the Chemistry of Organic Natural Products* (Eds. Hertz W, Kirby GW, Steglich W and Tamm C), pp. 154–195. Springer Verlag, New York, 1991.
3. Foster MP, Mayne CL, Dunkel R, Pugmire RJ, Grant DM, Kornprobst JM, Verbist JF, Biard JF and Ireland CM, Revised structure of bistramide A (bistratene A): application of a new program for the automated analysis of 2D INADEQUATE spectra. *J Am Chem Soc* **114**: 1110–1111, 1992.
4. Gouiffes D, Moreau S, Helbecque N, Bernier JL, Henichart JP, Barbin Y, Laurent D and Verbist JF, Proton nuclear magnetic study of bistramide A, a new cytotoxic drug isolated from *Lissoclinum bistratum* Sluiter. *Tetrahedron* **44**: 451–459, 1988.
5. Degnam BM, Hawkins CJ, Lavin MF, McCaffrey EJ, Parry DL and Watters DJ, Novel cytotoxic compounds from the ascidian *Lissoclinum bistratum*. *J Med Chem* **32**: 1354–1359, 1989.
6. Gouiffes D, Juge M, Grimaud N, Welin L, Sauviat MP, Barbin Y, Laurent D, Roussakis C, Henichart JP and Verbist JF, Bistramide A, a new toxin from the urochordata *Lissoclinum bistratum* Sluiter: isolation and preliminary characterization. *Toxicon* **26**: 1129–1136, 1988.
7. Roussakis C, Gratas C, Chinou I, Audouin F, Robillard N, Gouiffes D and Verbist JF, Activite differenciatrice de deux substances naturelles: le Cu_1 et le bistramide vis-a-vis des NSCLC *in vitro*. *Bull Cancer* **76**: 478–479, 1989.
8. Watters D, Marshall K, Hamilton S, Michael J, McArthur M, Seymour G, Hawkins C, Gardiner R and Lavin M, The bistratenes: new cytotoxic marine macrolides which induce some properties of indicative of differentiation in HL60 cells. *Biochem Pharmacol* **39**: 1609–1614, 1990.
9. Gescher A and Reed DJ, Characterization of the growth inhibition induced by tumor-promoting phorbol esters and of their receptor binding in A549 human lung carcinoma cells. *Cancer Res* **45**: 4315–4321, 1985.
10. Valette A, Gas N, Jozan S, Roubinet F, Dupont MA and Bayard F, Influence of 12-*O*-tetradecanoylphorbol-13-acetate on proliferation and maturation of human breast carcinoma cells (MCF-7): relationship to cell cycle events. *Cancer Res* **47**: 1615–1620, 1987.
11. Dale IL and Gescher A, Effects of activators of protein kinase C including bryostatins 1 and 2, on the growth of A549 human lung carcinoma cells. *Int J Cancer* **43**; 158–163, 1989.
12. Nutt JE, Harris AL and Lunec J, Phorbol ester and bryostatin effects on growth and the expression of oestrogen-responsive and TGF-β1 genes in breast tumour cells. *Br J Cancer* **64**: 671–676, 1991.
13. Fridman R, Lacal JC, Reich R, Bonfil DR and Ahn CH, Differential effects of phorbol ester on the *in vitro* invasiveness of malignant and non-malignant fibroblast cells lines. *J Cell Physiol* **142**: 55–60, 1990.
14. Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1983.
15. Bradshaw TD, Gescher A and Pettit GR, Modulation by staurosporine of phorbol-ester-induced effects on growth and protein kinase C localization in A549 human lung carcinoma cells. *Int J Cancer* **51**: 144–148, 1992.
16. Hannun YA and Bell RM, Mixed micellar assay for phorbol ester binding. *Methods Enzymol* **141**: 287–293, 1987.
17. Roussakis C, Robillard N, Riou D, Biard JF, Pradal G, Piloquet P, Debitus C and Verbist JF, Effects of bistramide A on a non-small-cell bronchial carcinoma line. *Cancer Chemother Pharmacol* **28**: 283–292, 1991.
18. Dale IL, Bradshaw TD, Gescher A and Pettit GR, Comparison of the effects of bryostatins 1 and 2 and 12-*O*-tetradecanoylphorbol-13-acetate on protein kinase C activity in A549 human lung carcinoma cells. *Cancer Res* **49**: 3242–3245, 1989.
19. Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM and McEwan RN, A rapid *in vitro* assay for quantitating the invasive potential of tumor cells. *Cancer Res* **47**: 3239–3245, 1987.
20. Schwartz GK, Redwood SM, Ohnuma T, Holland JF, Droller MJ and Liu BCS, Inhibition of invasion of invasive human bladder carcinoma cells by protein kinase C inhibitor staurosporine. *J Natl Cancer Inst* **82**: 1753–1756, 1990.
21. Gopalakrishna R and Barsky SH, Tumor promoter-induced membrane bound protein kinase C regulates hematogenous metastasis. *Proc Natl Acad Sci USA* **85**: 612–616, 1988.
22. Korczak B, Whale C and Kerbel RS, Possible involvement of Ca^{2+} mobilization and protein kinase C activation in the induction of spontaneous metastasis by mouse mammary adenocarcinoma cells. *Cancer Res* **49**: 2597–2602, 1989.
23. Noel AC, Calle A, Emonard HP, Nusgens BV, Simar L, Foidart J, Lapiere CM and Foidart JM, Invasion of reconstituted basement membrane matrix is not correlated to the malignant metastatic cell phenotype. *Cancer Res* **51**: 405–414, 1991.
24. Hirai M, Gamou S, Kobayashi M and Shimizu N, Lung cancer cells often express high levels of protein kinase C activity. *Jpn J Cancer Res* **80**: 204–208, 1989.
25. Bignon E, Ogita K, Kishimoto A and Nishizuka Y, Protein kinase C subspecies in estrogen receptor-positive and -negative human breast cancer cell lines. *Biochem Biophys Res Commun* **171**: 1071–1078, 1990.
26. Ahn CH, Choi WC, Kong JY and Fine RL, Detection of six immunoreactive protein kinase C isozymes in human breast MCF-7 and multidrug resistant MCF-7/DOX cells and effects of phorbol ester treatment. *Proc Am Assoc Cancer Res* **33**: 455, 1992.