THE ROLE OF PROTEIN KINASE C MODULATION IN THE ANTIPROLIFERATIVE EFFECTS OF BISTRATENE A, BRYOSTATIN 1 AND PHORBOL ESTERS.

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The role of protein kinase C (PKC) modulation in the antiproliferative effects of bistratene A, bryostatin 1 and phorbol esters.

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PKC-mediated signalling pathways are important in cell growth and differentiation, and aberrations in these pathways are implicated in tumourigenesis. The objective of this project was to clarify the link between cell growth inhibition and PKC modulation.The PKC activators bryostatin 1 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) inhibited growth in A549 and MCF-7 adenocarcinoma cells with great potency, and induced HL-60 leukaemia cell differentiation. Bistratene A affected these cells similarly. Experiments were conducted to test the hypotheses that bistratene A exerts its effects via PKC modulation and that characteristics of cytostasis induced by bryostatin 1 and TPA depend upon PKC isozyme-specific events.

After incubation of A549 cells with TPA or bistratene A, 2D phosphoprotein electrophoretograms revealed three proteins phosphorylated by both agents. However, bistratene A was unable to induce the formation of cellular networks on the basement membrane substitute Matrigel, and staurosporine was unable to reverse bistratene A-induced [^{3}H]thymidine uptake inhibition, unlike TPA. Bistratene A did not induce PKC translocation or downregulation, activate or inhibit A549 and MCF-7 cell cytosolic PKC or compete for phorbol ester receptors. Western blot analysis and hydroxylapatite chromatography identified PKC α, ε and ζ in these cells. Bistratene A was unable to activate any of these isoforms.Therefore the agent does not exert its antiproliferative effects by modulation of PKC activity.

The abilities of bryostatin 1 and TPA (10nM-1μM) to induce PKC isoform translocation and downregulation were compared with antiproliferative effects. Both agents induced dose-dependent downregulation and translocation of PKC α and ε to particulate and nuclear cell fractions. PKC ζ was translocated to the particulate fraction by both agents in MCF-7 cells. The similarity of PKC isoform redistribution by these agents did not explain their divergent effects on cell growth, and the role of nuclear translocation of PKC in cytostasis was not confirmed by these studies. Alternative factors governing the characteristics of growth inhibition induced by these agents are discussed.

Keywords: growth inhibition, translocation, downregulation, A549 cells, MCF-7 cells.

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Section 1. Introduction

Section 1. Introduction.

1.1. Strategies for Treatment of Cancer.

Cancer is a common disease, affecting 1 in 3 people and accounting for 1 in 4 deaths in the UK. It arises from the abnormal and uncontrolled division of cells which subsequently invade and destroy surrounding tissues. Metastatic spread may occur, in which cancer cells relocate to new sites in the body to develop secondary tumours, with further disruption of normal tissue functions.

Treatment of this disease depends upon the type of tumour, its site, and the extent of spread. A multidisciplinary approach is usually taken, combining the three treatment modalities of chemotherapy, radiotherapy and surgery. Chemotherapy is usually a combination of several agents with differing modes of action to circumvent drug resistance and tumour heterogeneity. Agents currently in use are toxic to cells or arrest cellular proliferation: mechanisms of action include alkylation, crosslinking or intercalation of DNA and its subsequent inactivation, interference with nucleotide synthesis and hampering of mitotic spindle formation. Unfortunately normal proliferating cells such as those of haemopoietic or gastrointestinal origin are also targeted, leading to toxic side effects. Nevertheless, this approach has led to excellent cure rates in a subset of malignancies, particularly in children. The majority of solid tumours in adults, which are often slow-growing, remain refractory and there is therefore an urgent need for more effective and selective agents.

In the last decade, research has focused on understanding the aetiology of cancer. Only by unravelling the cause of each type of neoplasm will it be possible to design agents to overcome specific aberrations. There is now a greater understanding of genetic changes which induce cancer ; in the future this may lead to successful treatments via gene therapy (reviewed by Guttierrez *et al*, 1992). Sites that are more accessable for pharmacological intervention have been revealed by a knowledge of changes in cellular protein function during carcinogenesis, downstream of genetic events.

1.2. Cellular Signalling in Cancer.

Cancer can be viewed as a disease of the signalling system which controls cell proliferation and differentiation (Karin, 1992). In normal cells, signals are precipititated by growth factors, cytokines and hormones which regulate cell proliferation and differentiation by modulating transcription factor activity in the nucleus. This regulation is achieved via a complex network of protein interactions in which the transfer of phosphate groups from one molecule to another plays a major role (Hunter, 1990). The immutable stimulation or repression of members of a signal transduction pathway can lead to permanent cascade activation. This event can occur by a gain-of-function mutation or overexpression of proto-oncogenes, which are genes involved with positive growth regulation in normal cells, to form oncogenes. Oncogene products act as autocrine growth factors or downstream in the transduction cascade as receptors, regulatory proteins, kinases and transcription factors (Powis, 1991). A loss-of-function mutation of tumour suppressor genes, or lack of expression of their products (Marshall, 1991) and growth inhibitors such as transforming growth factor β (TGFβ) (Barnard *et al*, 1990), which are involved with negative growth control, also results in cascade activation (Fig.1). Further activation occurs by the inadvertant stimulation of aberrant pathways by endogenous hormones, dietary and other epigenetic factors (Weinstein, 1991). Subsequent erroneous phosphorylation or dephosphorylation events lead to changes in protein function throughout the cell. Changes include alterations in enzyme activity (Vmax) or affinity for substrates (km), changes in receptor-ligand binding, protein solubility or subcellular location, susceptibility of proteins to proteolysis, and modulation of protein-protein and protein-DNA interactions (Lord *et al*, 1988). Ultimately these changes in protein function lead to cell transformation.

1.3. Protein Kinase C (PKC).

Figure 1 is a simplified illustration of the complexity of signal transduction, but shows that there are innumerable sites for intervention with potential anticancer agents. It is hoped that the targetting of drugs towards key signalling proteins will allow normal cells to function

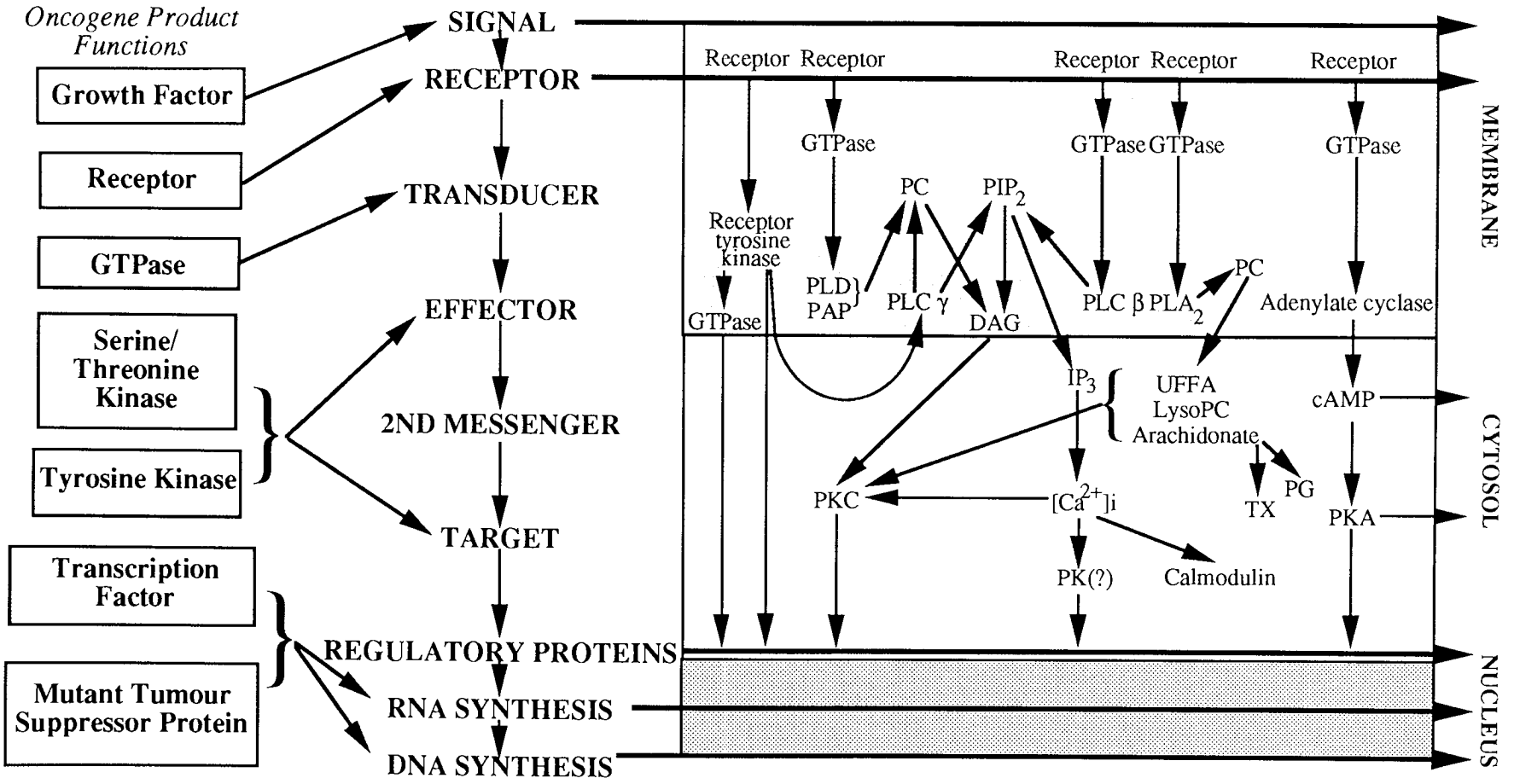


Figure 1. Cellular Signal Transduction Pathways used by Extracellular Signals and Oncogene Products.
The growth and differentiation of normal cells is controlled by the interaction of extracellular signals such as hormones and growth factors with receptors in the cell membrane. This interaction is translated into a response via a network of signalling pathways, some of which are illustrated above. GTPases are GTP-binding and hydrolysing enzymes, including heterotrimeric G proteins and monomeric 20-25kDa proteins of the ras superfamily. Light and dark shaded areas represent cell membrane and nucleus respectively. During tumourigenesis, oncogenes and mutant tumour suppressor genes are generated. Oncogene products gain the functions shown on the left. Mutation of tumour suppressor genes leads to a loss of function of the protein product. Abbreviations are listed in section 7.1. Modified from Powis, 1991.

via alternative signalling pathways whilst attenuating the hypereactive pathway eliciting uncontrolled cell growth after transformation, thus providing a degree of selectivity. Protein kinase C (PKC) has thus been identified as a suitable target for drug development associated with cell signalling.

1.3.1. PKC Isoforms.

PKC is a family of at least 10 isoforms of serine/threonine-specific kinases with common structural features that are grouped into conventional PKCs (cPKCs) and novel PKCs (nPKCs). cPKCs include the α (type III), β (type II), and γ (type I) isoforms with molecular weights of approximately 80kDa, which were first identified by enzyme purification and screening of a bovine brain cDNA library (Coussens *et al*, 1986). Low stringency screening has since identified a host of other isoforms termed nPKCs, including the δ, ε, ζ, η, θ and λ isoforms (Stabel and Parker, 1991, Osada *et al*, 1992, Nishizuka, 1992). Further PKC isoforms are generated from the alternative splicing of genes for PKC β to give PKC β_1 and β_2 (Coussens *et al*, 1987, Ono *et al*, 1987); proteins with differing molecular weights are generated as variants of PKC ε (Ono et al, 1988, Schaap *et al*, 1990, Baxter *et al*, 1992) and ζ (Ono *et al*, 1988, Masmoudi *et al*, 1989, Wetsel *et al*, 1992).

1.3.2. PKC Structure and Specific Binding Sites.

PKC isoforms are single polypeptides with 4 conserved regions of homology and 5 variable regions with low sequence similarities (Fig.2). Structural features have been reviewed in detail by Stabel and Parker, 1991. Two functional domains have been identified, the regulatory and kinase domains. A pseudosubstrate prototope sequence within the regulatory domain regulates enzyme activity by reversibly blocking the catalytic site and access of substrate (Pears and Parker, 1991). This sequence varies for each isoform, but consistently has positively charged basic residues such as arginine surrounding an alanine residue. Substitution of the alanine for serine in synthetic peptides of this region results in the generation of a PKC substrate (House and Kemp, 1987).

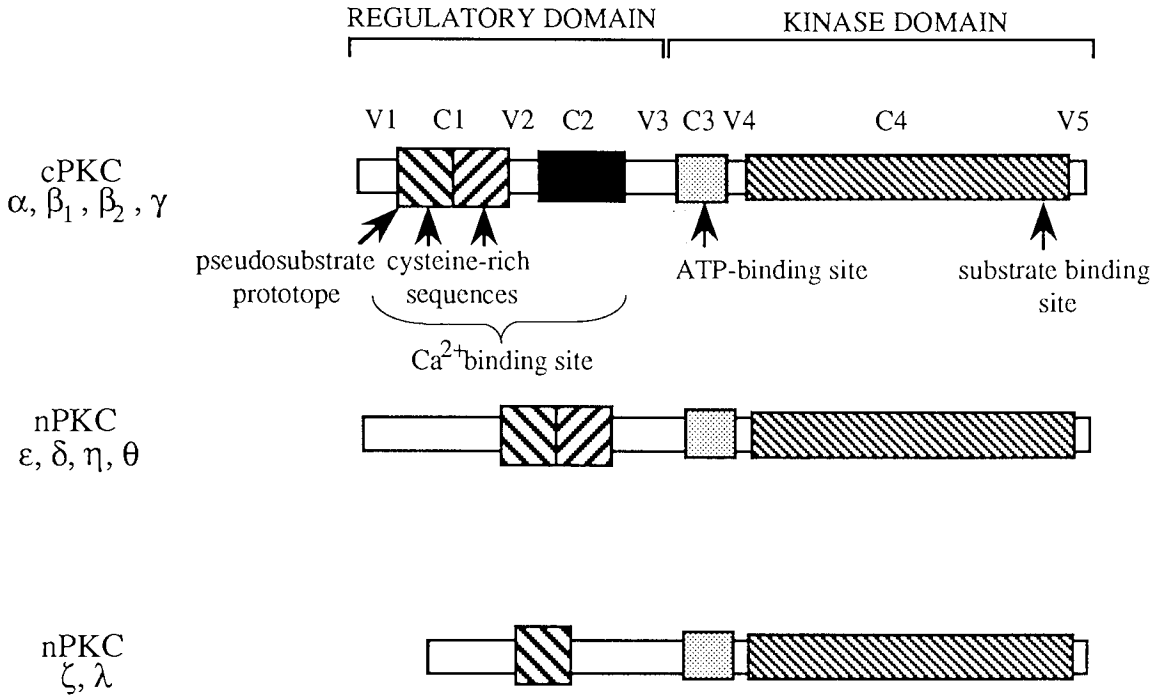


Figure 2. Schematic Representation of PKC Isoform Primary Structures
PKC is divided into 2 functional domains: the amino-terminal regulatory domain and the carboxy-terminal kinase domain. The latter contains ATP- and substrate-binding sequences, which are indicated by arrows. The regulatory domain contains the pseudosubstrate prototope, a receptor for DAG/phorbol esters/bryostatins and a binding site for phospholipids, all of which are within the C1 region, and a Ca^{2+} binding site within cPKCs for which the C2 region is essential (see section 1.3.2.). C1 to C4 and V1 to V5 indicate conserved and variable regions respectively. Homologous regions are emphasized by shading.

The pseudosubstrate prototope is adjacent to two cysteine-rich repeat sequences which bind zinc with high affinity (Quest *et al*, 1992) and resemble DNA binding 'zinc finger' motifs of transcription factors (Burns and Bell, 1991). PKC ζ contains a single motif (Fig.2). This area of the C1 region contains a receptor which binds diacylglycerol (DAG) as the endogenous ligand (see Fig.1 for signal transduction pathways for DAG generation). It is also a receptor for bryostatins and phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Fig.4b and c). The C1 region is also responsible for the binding of phospholipids (Ono *et al*, 1989a, Quest *et al*, 1992). These molecules act as PKC activators or cofactors by inducing a reversible conformational change that displaces the auto-inhibitory pseudosubstrate domain from the enzyme active site (Orr *et*

al, 1992). Unlike other isoforms, PKC ζ is unable to bind phorbol esters (Ono *et al*, 1989) and is not activated by them (Nakanishi and Exton, 1992), yet is activated by phospholipids such as phosphatidylinositol 3,4,5-trisphosphate (PIP_3) and phosphatidylserine (PS) (Nakanishi *et al*, 1993). The absence of a phorbol ester binding site from the ζ isoform is not due to the possession of only one cysteine-rich repeat as another protein with only one repeat, n-chimaerin, is able to bind phorbol esters with high affinity (Ahmed *et al*, 1990). It is probably due to differences in the amino acid sequences in between cysteine residues in the C1 region (Quest *et al*, 1992).

The C2 region is conserved in all cPKCs and is absent from nPKCs (Fig.2). This region does not contain any recognised calcium binding sequences, but until recently was thought to be the calcium binding domain of PKC as cPKCs require calcium for phorbol ester binding, histone kinase activity and movement of the enzyme to new cellular locations (translocation), but nPKCs (without the C2 domain) are not regulated by calcium under any circumstances (Ono *et al*, 1989, Schaap *et al*, 1990a, Mizuno *et al*, 1991). Recent evidence suggests that the C2 domain is not the major site for calcium binding, but is essential for the formation of a PKC tertiary conformation, in conjunction with the C1 domain and PS, which selectively binds Ca^{2+} (Weinstein *et al*, 1993).

PKC uses ATP as a phosphate donor, and a specific ATP binding site is located in the kinase domain, together with a substrate binding domain (Fig.2).

1.3.3. Modulation of PKC Activity.

PKC has a number of specific binding sites for cofactors and activators as described above and enzyme activity is regulated by their availability within the cell. Enzyme activity is also modulated allosterically by a host of other factors. Unsaturated free fatty acids such as arachidonate activate the enzyme, with differential activation of individual isozymes depending on the presence of other cofactors (Kitagawa *et al*, 1991, Khan *et al*, 1993). Isozyme-specific activation has also been discovered for certain phorbol esters, such as 12-deoxyphorbol-13-*O*-phenylacetate-20-acetate (DOPPA) which only activates PKC β_1

(Ryves *et al*, 1991). Although cPKCs contain the C2 region which is necessary for Ca^{2+}-binding, their activity is independent of the presence of calcium in certain circumstances. In *in vitro* assays, the substrate used influences cofactor requirements (Bazzi and Nelsestuen 1987, Liyanage *et al*, 1992). Substrates have been placed into three groups depending on this phenomenon ; group A includes substrates which interact directly with the enzyme active site and require no cofactors, eg protamine sulphate, group B substrates require acidic phospholipids but not Ca^{2+}, eg myelin basic protein, and group C refers to substrates which require the presence of phospholipids and Ca^{2+} for phosphorylation to take place, eg histone. Similarly, substrates may influence cofactor requirements *in vivo*. Further enzyme control is achieved by phosphorylation of PKC by itself or by other kinases (Pelech *et al*, 1991, Pears *et al*, 1992, Zhang *et al*, 1993) and by the presence of endogenous inhibitor (Pearson *et el*, 1990, Dong *et al*, 1990, Balazovich *et al*, 1992) and activator proteins (Goueli, 1991).

Modulation of kinase activity is also achieved by changes in quantity of enzyme present at specific locations within the cell. PKC relocates to new sites such as the cell membrane, nucleus and cytoskeleton upon activation or inhibition, a process known as translocation (Epand *et al*, 1990, Kiley *et al*, 1991, 1992). Intracellular sites have different phospholipid compositions and potential for DAG generation which could differentially modulate kinase activity. Translocation to a host of new cellular locations, particularly the nucleus, could also contribute to the production of a greater diversity of specific signals via protein phosphorylation at that particular site. Isoforms of PKC are translocated differentially by different modes of stimulation of the PKC signal transduction pathway (Crabos *et al*, 1992, Farese *et al*, 1992). The enzyme is also regulated quantitatively by post-activation degradation by calpains and other proteases, a process known as downregulation that results in loss of enzyme from the cell (Adams *et al*, 1989). This process is also heterogeneous for different isoforms (Kishimoto *et al*, 1989, Kiley *et al*, 1990a). The expression of mRNA for specific PKC isoforms is also regulated by activation of PKC in an autoregulatory fashion (McSwine-Kennick *et al*, 1991).

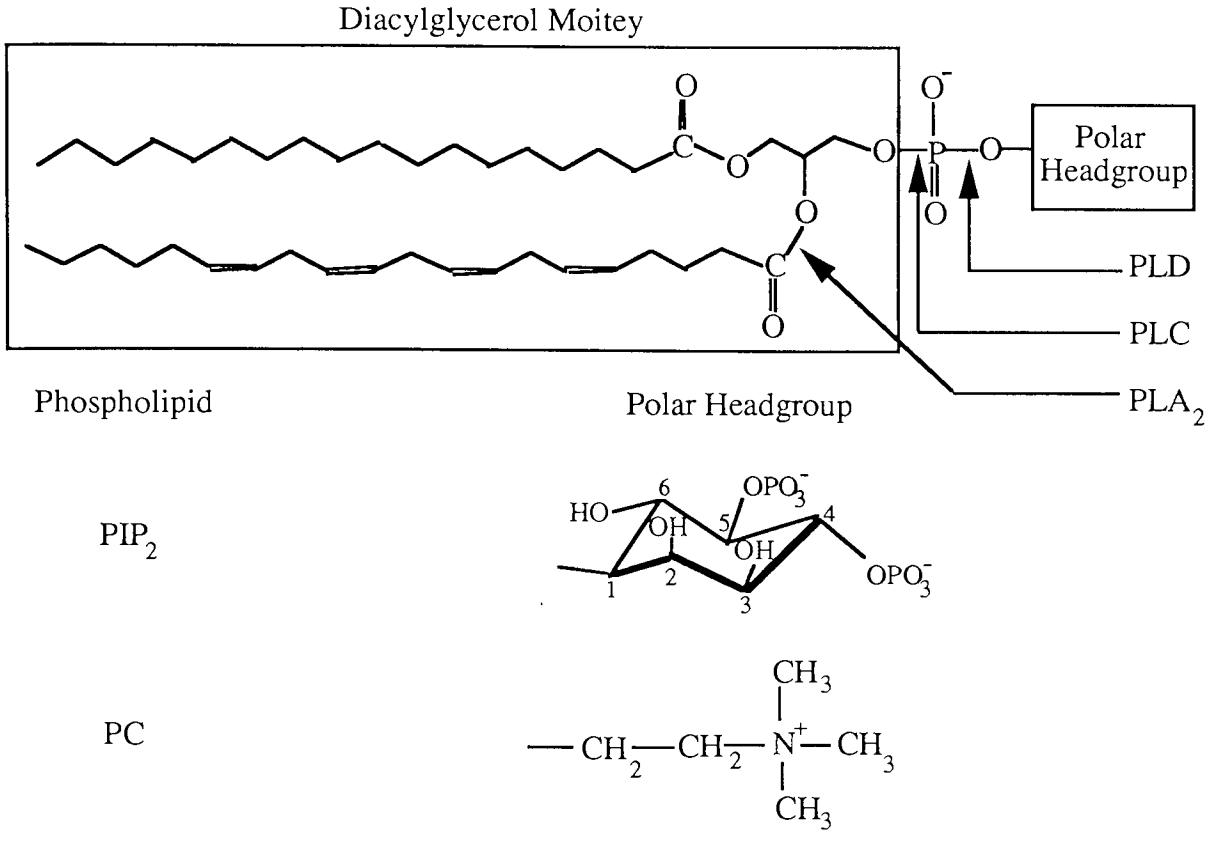


Figure 3. Generation of Signalling Molecules from Hydrolysis of Phospholipids by Phospholipases.
The signalling molecule DAG is produced via the hydrolysis of phospholipids such as the 1-stearoyl-2-arachidonoyl phospholipid depicted above. Many other molecular species of phospholipid are present intracellularly, with other combinations of fatty acids, and headgroups such as $IP_{[4,5]_2}$, choline, ethanolamine, serine and inositol. Only PLC isoforms catalyse the hydrolysis of PIP_2, which generates IP_3 in addition to DAG. Removal of the polar headgroup choline from PC by PLD is followed by further degradation of the remaining phosphatidic acid to DAG by PAP. Hydrolysis of phospholipid by PLA_2 gives rise to free fatty acids and lysoPC. For further details, see section 1.3.3. Modified from Liscovitch, 1992.

Within any particular cell type, the PKC signal transduction pathway is stimulated differentially. Cells possess a unique range of receptors on their cell surface, some of which activate isoforms of phospholipases A_2, C and D upon receptor-ligand interaction. Seven mammalian isoforms of phospholipase C (PLC) have been discovered to date, which are grouped into β, γ and δ subtypes (Jhon *et al*, 1993). These PLC isoforms catalyse the hydrolysis of phosphatidyinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3), which stimulates Ca^{2+} release from intracellular stores, and DAG. Phospholipase A_2 (PLA_2) generates the release of lysophosphatidylcholine (lysoPC) and unsaturated fatty acids such as arachidonic acid from phospholipids.

Phospholipase D (PLD) and phosphatidic acid phosphohydrolase (PAP) act in concert to generate DAG from phosphatidylcholine (PC) ; PLC can also produce DAG from this source (Fig.3). All these products of phospholipid hydrolysis activate PKC (Fig.1). PKC also regulates its own activity by phosphorylating these phospholipases and modulating their catalytic activity. Phosphorylation of PLC β and γ by PKC inhibits their function, whereas the enzyme stimulates catalysis by PLA_2 and D. It has been suggested that early effects of PKC activation, such as secretion, result from the rapid release of DAG and IP_3 by hydrolysis of PIP_2 by PLC and more protracted events such as proliferation and differentiation require sustained PKC activity via PC hydrolysis by PLA_2, C and D (reviewed by Nishizuka, 1992 and Liscovitch, 1992). Within any particular cell type, there is a unique distribution of isozyme subtypes (Wetsel *et al*, 1992). c- and nPKCs have recently been shown to phosphorylate different substrates *in vitro* (Liyanage *et al*, 1992) and within the cell (Nishikawa *et al*, 1992), providing further routes for signal diversity. Therefore cell-specific signals stimulate PKC activity *via* receptor interactions and a series of cell type-specific biological responses are produced.

The control of intracellular PKC activity is clearly complex. It is dependent upon the interplay between factors which modulate the activity of the enzyme itself or the relative amounts of its isoforms at subcellular locations, and by its position in the network of signalling pathways within a particular cell type .

1.3.4. Intracellular Substrates for PKC.

Cellular substrates proposed for PKC are diverse and are often proteins which are expressed differentially in cell types. Some of the substrates that have been identified are components of other signalling pathways, leading to cross-talk between signalling cascades *via* PKC-induced protein phosphorylation. PKC phosphorylates the epidermal growth factor (EGF) receptor, resulting in a rapid decrease in high affinity binding of EGF and inhibition of ligand-induced tyrosine phosphorylation (Iwashita and Kobayashi, 1992). Proteins which act downstream from receptors are also substrates for PKC in several signalling pathways, such as adenylate cyclase (Yoshimasa *et al*, 1987), and thus PKC may be involved with cross-talk with the protein kinase A (PKA) signalling pathway

(Fig.1). PKC also modulates other kinases through indirect mechanisms which are incompletely understood at present, such as mitogen activated protein kinase (MAP kinase) which is phosphorylated on tyrosine residues after treatment of cells with TPA. The product of the c-*raf* proto-oncogene, the serine/threonine specific Raf-1 kinase is phosphorylated indirectly on serine residues downstream of PKC activation (reviewed by Kozma and Thomas, 1992). PKC activity is influenced by signals arising from many pathways, discussed in section 1.3.3 ; the phosphorylation of multiple signalling molecules is also a means by which PKC can be integrated into a network of other pathways to generate a myriad of biological responses.

PKC also phosphorylates a number of substrates which play a role in gene expression and nuclear events during cell division. A common response to PKC activators such as TPA is the induction of genes harbouring a TPA response element (TRE). TRE is a short sequence of DNA (5'TGACTCA 3') that is recognised by AP-1 transcription factors, which are dimeric complexes of members of the Fos and Jun protein families. Fos is phosphorylated by PKC and this event may alter the activity of AP-1 (Abate *et al*, 1991). Control of the DNA binding and transcription-inducing ability of AP-1 hetero- or homo-dimers of Jun proteins is multifactorial but phosphorylation of two residues at the N-terminal end of Jun and dephosphorylation of three residues proximal to the DNA binding domain of these proteins appears to be involved with an increase in activity of some AP-1 dimers. PKC activation may stimulate Jun by phosphorylation of the two N-terminal amino acid residues directly or *via* increasing MAP kinase activity. PKC activation may also induce the dephosphorylation of the three residues which impart inhibitory effects on DNA binding *via* glycogen synthase kinase 3β (GSK-3β) phosphorylation and inactivation (Goode *et al*, 1992, Franklin *et al*, 1992, Baker *et al*, 1992). Activation of AP-1 and other genetic regulators such as NF-κB *via* PKC-mediated phosphorylation leads to the expression of a series of genes that encode proteins which act as transcription factors, enzymes, cytokines, and extracellular matrix proteins (Ogita *et al*, 1990, Kujubu *et al*, 1991). The aberrant functioning of transcription factors is an initiating event leading to transformation (section 1.3.5.). PKC is also able to phosphorylate several proteins which are involved with maintenance of the nuclear matrix and DNA topography : histone H3 is a nuclear target for

PKC in fibroblasts (Mahadevan *et al*, 1991) and topoisomerase I is activated by PKC-mediated phosphorylation (Pommier *et al*, 1990). Lamin B_2 is a substrate for PKC located at the nuclear envelope (Hocevar and Fields, 1990, Kasahara *et al*, 1991). Lamins are involved with the structural integrity of the nucleus and phosphorylation of these molecules may be involved with regulation of nuclear structure during cell division.

Many of the substrates proposed for PKC are involved with cytoskeletal rearrangement, including tau and MAP-2, which are neuronal microtubule-associated proteins (Correas *et al*, 1992), vinculin (Kawamoto and Hidaka, 1984) GAP-43 (Gordon-Weeks, 1989), adducin (Joshi *et al*, 1991) and the myristoylated alanine-rich C kinase substrate (MARCKS) protein family (Aderem, 1992). The MARCKS protein itself is thought to be a specific substrate for PKC and is ubiquitously expressed in cells. In its dephosphorylated state, MARCKS binds to F-actin with high affinity and induces its aggregation. Phosphorylation by PKC prevents F-actin aggregation and induces redistribution of the protein from its membrane-bound form to the cytosol. This process is also under the control of calcium-calmodulin, which binds to MARCKS reversibly in a Ca^{2+}-dependent fashion. The presence of calcium-calmodulin prevents PKC-dependent phosphorylation of MARCKS and inhibits the actin crosslinking activity of dephosphorylated MARCKS. Rearrangement of the cytoskeleton is implicated as one of the cellular processes regulating motility, secretion, morphological changes and transformation (Hartwig *et al*, 1992, Aderem, 1992).

1.3.5. Rationale for PKC as a Target for Anticancer Agents.

There is clear evidence that PKC functions incorrectly in cancer, leading to uncontrolled proliferation and also influencing metastatic spread and the development of a cytotoxic drug-resistant phenotype. This can occur by a fault in the enzyme itself or by constitutive activation/depression of the PKC signalling pathway by aberrant components up- or downstream from PKC.

PKC itself may be an oncogene product. A mutated form of the α isoform of this protein was found to be responsible for transformation in a UV-induced mouse fibrosarcoma

(Megidish and Mazurek, 1989), but this has since been disputed (Borner *et al*, 1991); mutagenic activation of PKC seems to be a rare occurrance in human tumours (Hunter, 1991). It is possible that overexpression of PKC plays a part in tumourigenesis. Total PKC activity is elevated in breast tumours compared to adjacent normal tissue (O'Brian *et al*, 1989) and in human malignant glioma cells compared to non-neoplastic astrocytes and glial cells (Couldwell *et al*, 1991). PKC α is overexpressed in C3H/10T^1/2 cells transformed with aflatoxin (Dunn *et al*, 1992). Fibroblasts that are transfected with and overexpress PKC α, β_1 or γ exhibit various growth abnormalities indicative of incomplete transformation (Housey *et al*, 1988, Persons *et al*, 1988, Finkenzeller *et al*, 1992). R6 fibroblasts overexpressing PKC β_1 show an exaggerated proliferative response to low concentrations of serum and a variety of growth factors (Hoshina *et al*, 1990) but require cooperativity with v-Ha-*ras,* v-*myc* or v-*fos* oncogenes to form highly tumourigenic cells (Hsiao *et al*, 1989, Weinstein, 1991). However, overexpression of PKC ε in these cells and also in NIH 3T3 fibroblasts is sufficient to induce tumour formation in nude mice, whereas overexpression of PKC δ in NIH 3T3 cells causes inhibition of cell growth, suggesting that PKC isoforms have differing effects on tumourigenicity within a particular cell type (Cacace *et al*, 1993, Mischak *et al*, 1993). Changes in quantity of individual PKC isozymes have also been noted in a number of neoplastic cells compared to their normal counterparts (Benzil *et al*, 1992, Yamanishi *et al*, 1991, Hagiwara *et al*, 1990, Dlugosz *et al*, 1992), suggesting that changes in PKC isoform expression may indeed be involved with the genesis of individual tumours.

In the colon, it has been suggested that PKC acts as a tumour suppressor protein and its loss leads to the development of colorectal carcinoma (Choi *et al*, 1990). Several independent studies have found a reduction in total PKC activity in adenoma or carcinoma of the colon compared with normal mucosa (Guillem *et al*, 1987, Kopp *et al*, 1991, Kusunoki *et al*, 1992) and overexpression of PKC β_1 in HT29 colon cancer cells inhibited growth and decreased tumourigenicity (Choi *et al*, 1990). Recent experiments using PKC α antisense oligonucleotides to block the synthesis of this isoform have shown that PKC

α is necessary for signal transduction pathways generated by the inhibitory growth factor $TGF\beta_1$ in colon cancer cells and hence loss of PKC from these cells may reverse the negative control of cell proliferation imposed by this growth factor (Huang and Chakrabarty, 1993). Activation of PKC with TPA in chinese hamster ovary cells that overexpress the δ, but not the α, β_2 or ζ isoforms inhibited cell growth and progression through the cell cycle, suggesting a negative role for the δ isoform in growth regulation of this cell type, as with NIH 3T3 fibroblasts (Watanabe *et al*, 1992). It is conceivable that loss of PKC δ may contribute to transformation in these cells.

Compelling evidence for the constitutive activation or depression of PKC signalling pathways in cancer was initially obtained in the mouse skin tumour model, in which carcinogenesis was identified as a two-stage process of initiation followed by promotion. It was observed that low doses of an initiating carcinogen failed to cause tumourigenesis unless succeeded by multiple treatments with a tumour promoter, which alone had no effect. One class of agents which act as potent tumour promoters are the PKC-activating phorbol esters, such as TPA (Fig.4b). Tumourigenesis is thought to occur via cooperation between Ha-*ras* oncogenes mutated during initiation, and PKC activation with subsequent protein phosphorylation during promotion (Hunter, 1991). Evidence of synergy between oncogene expression and PKC overexpression in fibroblast cells described above tends to corroborate this hypothesis, as does the fact that murine keratinocytes transfected with v-Ha-*ras* have highly elevated levels of DAG and form papillomas when grafted onto nude mice ; the latter effect can be blocked by treatment of cells with PKC inhibitors (Dlugosz *et al*, 1993). Further support for cooperativity between PKC and oncogenes arises from the numerous studies in which proto-oncogene products such as the p53 protein, c-Fos and c-Jun were overexpressed (Unlap *et al*, 1992, Goode *et al*, 1990), or their function was modified by phosphorylation *via* PKC activation by TPA (Franklin *et al*, 1992, Abate *et al*, 1991, Baudier *et al*, 1992). c-Jun can only cooperate with Ha-*ras* in oncogenic transformation when it is N-terminally phosphorylated (Smeal *et al*, 1991). Recent evidence suggests that PKC isozyme-specific events may also be involved, with the

selective irreversible loss of PKC α and β_2 upon tumour formation in the mouse skin model and retention of the δ, ε and ζ isoforms, suggesting that activation of certain isozymes in concert with the downregulation of others could provide a permissive environment for proliferation (Mills *et al*, 1992).

Metastasis is a complex multistep process in which in addition to loss of growth control, cells acquire an imbalanced regulation of motility and proteolysis, allowing cells to leave the primary tumour, invade local host tissue, enter the circulation, arrest at a different vascular bed, extravasate into the target organ, then proliferate and expand as a secondary tumour. Current research is beginning to unravel these processes at a molecular level (reviewed by Liotta *et al*, 1991, Ruoslahti, 1992). A comparison of metastatic cells *vs* non-metastatic cells of the same lineage has shown that membrane association or levels of PKC correlate with metastatic aggressiveness, suggesting a role for PKC in the regulation of this process (Gopalakrishna *et al*, 1988, Isakov *et al*, 1991). Studies using various PKC inhibitors have also provided evidence of a role for PKC in some stages of metastasis such as invasion (section 1.4.1.). The involvement of PKC in invasion may be an isozyme-specific event as only invasive gastric carcinoma cells express the β_1 isoform of PKC, and overexpression of this isoform in non-invasive cells induced invasiveness (Schwartz *et al*, 1993).

The development of resistance to cytotoxic agents is a major problem to successful chemotherapy. Several mechanisms of resistance have been identified, including alterations in drug metabolism, target proteins, cellular repair mechanisms, and carrier-mediated drug uptake. A phenomenon in which resistance of a cell type to agents such as anthracyclines, vinca alkaloids and podophyllotoxins occurs simultaneously is known as multidrug resistance (MDR). This phenotype occurs due to amplification of the *mdr1* gene and overexpression of its product, P-glycoprotein. This protein acts as a drug efflux pump and prevents accumulation of cytotoxic agents in the cell (Nooter and Herweijer, 1991). P-glycoprotein is a more efficient pump when phosphorylated and it is a substrate for PKC (Chambers *et al*, 1990). Therefore MDR may be amplified by overexpression of PKC.

This effect may be isozyme-specific, as multidrug-resistant MCF-7 cells overexpress PKC α but exhibit a decrease in expression of PKC ε and δ (Blobe *et al*, 1993). Similarly, overexpression of PKC α (Yu *et al*, 1991), β_1 (Fan *et al*, 1992), but not γ (Ahmad *et al*, 1992) conferred increased MDR suggesting that this is indeed a PKC isozyme-specific event.

1.4. PKC Modulators as Antitumour Agents.

PKC is at the convergence of a number of signalling pathways (Fig.1) and hence is ideally situated for pharmacological manipulation whether tumourigenesis is initiated by aberrations of the enzyme itself or by other factors impinging upon it. Because the predominant causative signalling factors for tumourigenesis seem to be cell-type specific, it is difficult to assess how PKC should be modified to generate beneficial effects for cancer treatment ; the relative importance of brief or prolonged activation, isozyme-specific effects and the consequences of PKC translocation and downregulation are difficult to interpret, and thus agents have been developed which modulate the enzyme by either activation or inhibition. Several agents which inhibit or stimulate PKC activity are discussed below.

1.4.1. PKC Inhibitors.

A host of compounds have recently been developed which act in a variety of different ways to inhibit PKC. One of the prototypes of this group of compounds is staurosporine (Fig.4d), a potent inhibitor which interacts with the kinase domain of the enzyme but also targets other kinases such as PKA and tyrosine kinases with almost equal potency (Tamaoki and Nakano, 1990). Staurosporine analogues such as the 7-hydroxy derivative UCN-01 (Tamaoki and Nakano, 1990) and the benzoyl derivative CGP 41251 (Meyer *et al*, 1989) display greater selectivity for PKC over other kinases. Another selective PKC inhibitory agent, calphostin C, was isolated from the fungus *Cladosporium cladosporioides* (Kobayashi *et al*, 1989) and it inactivates PKC irreversibly by oxidation in the presence of light (Gopalakrishna *et al*, 1992). The ether lipid hexadecylphosphocholine (miltefosine) interacts with the regulatory domain of PKC (Geilen *et al*, 1991).

These are promising agents for the treatment of cancer. Miltefosine is currently in clinical trial but its antitumour effect is probably only mediated partially by PKC inhibition (Verweij *et al*, 1992). Staurosporine is cytotoxic to many cell lines (Tamaoki *et al*, 1986). It is able to inhibit invasion of EJ bladder carcinoma cells *in vitro* suggesting antimetastatic activity (Schwartz *et al*, 1990). However, in the CD-1 mouse model, it behaves as a weak tumour promoter (Yoshikawa *et al*, 1990), and paradoxically augments phorbol ester effects (Jiang *et al*, 1992) or acts as a PKC activator (Dlugosz and Yuspa, 1991) in certain systems. Disappointingly, staurosporine has little effect on tumour models *in vivo*, unlike the specific PKC inhibitors UCN-01 (Akinaga *et al*, 1991) and CGP 41251 (Meyer *et al*, 1989). There is evidence of a role for PKC in metastasis (section 1.3.5.). The effect of staurosporine on invasion is probably due to inhibition of PKC rather than other kinases as other more specific PKC inhibitory agents are also antimetastatic; calphostin C is able to inhibit melanoma cell adhesion and lung colonization (Liu *et al*, 1992). PKC inhibitors have also been shown to reverse the multidrug resistant phenotype (Kulkarni *et al*, 1992). Therefore these agents have potential for the treatment of cancer due to their antiproliferative, differentiative and antimetastatic effects and possibly as modifiers of the MDR phenotype. Inhibitors that are specific for PKC have a wider therapeutic window and are less toxic than broad spectrum kinase inhibitors (Meyer *et al*, 1989) and in the near future these agents will undoubtedly prove their worth for the treatment of cancer in clinical trials.

1.4.2. Phorbol Esters.

PKC-activating phorbol esters such as TPA and phorbol-12,13-dibutyrate (PDBu) (Fig.4b) exert a plethora of biological effects in different cell types. They are known to possess tumour-promoting activity (section 1.3.5.) and initiate mitogenesis in cells, particularly those originating from skin such as fibroblasts and melanocytes (Rozengurt *et al*, 1984, Brooks *et al*, 1993).

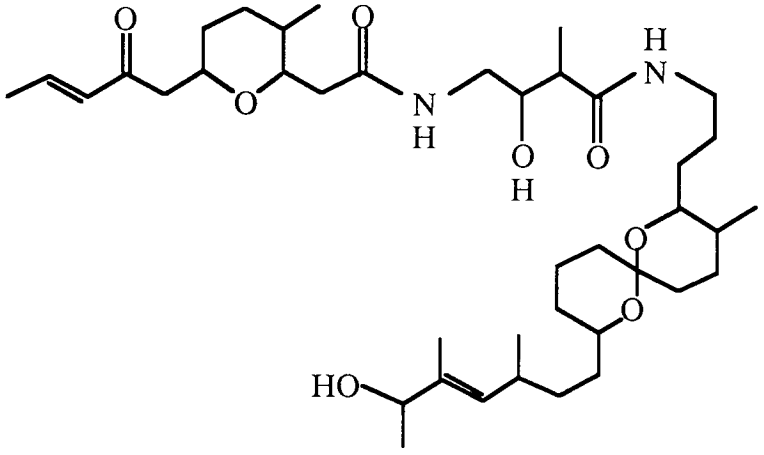
In contrast, TPA also induces cell maturation processes. It initiates the differentiation of many transformed cell types; it precipitates the maturation of HL-60 and U937 (Forsbeck *et al*, 1985) leukaemia cells to monocytes/macrophages, SH-SY5Y neuroblastoma cells to mature ganglion cells (Spinelli *et al*, 1982), and RD rhabdomyosarcoma cells (Aguanno *et*

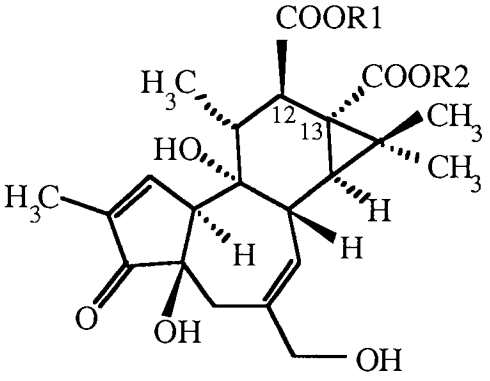
al, 1990) SW 48 colon cancer cells (Baron *et al*, 1990) and the lung squamous carcinoma cell lines NX002, CX140 and CX143 (Rabiasz *et al*, 1992) to more differentiated phenotypes. Differentiation is accompanied by growth inhibition in these cells; in MCF-7 human breast adenocarcinoma cells, TPA induces morphological changes indicative of differentiation and induces growth inhibition but the effect is reversible after removal of the agent (Valette *et al*, 1987) or irreversible, depending on laboratory (Roos *et al*, 1986). After treatment for 10-12 days, TPA is cytotoxic to these cells (Osborne *et al*, 1981).

TPA can also induce growth arrest without initiating differentiation. TPA exerts non-toxic antiproliferative effects on B16 murine melanoma cells (Coppock *et al*, 1990), A431 human epidermal cells (Smith *et al*, 1983) and HeLa cells (Suss *et al*, 1972). In A549 human lung carcinoma cells, TPA exerts a cytostatic effect within 12 h of application, but after 4-6 days, cell growth resumes in the continued presence of the agent (Gescher and Reed, 1985). Similar reversible effects on growth occur in SVK14 transformed human foreskin keratinocytes (McKay *et al*, 1983) and Hep G2 hepatoma cells (Duronio *et al*, 1990). TPA is capable of exerting stimulatory and inhibitory effects on growth in lymphocytes, depending on whether the agent is added acutely, stimulating interleukin-2 (IL-2) production and proliferation, or for prolonged periods, causing downregulation of PKC, inhibiting IL-2 production and proliferation (Grove and Mastro, 1991).

Although phorbol esters inhibit growth and initiate differentiation in some cell types, the tumour-promoting activity of these compounds obviously precludes their use as anticancer agents. However, they are useful tools to study the role of PKC in cell growth and maturation, and how PKC should be manipulated to generate beneficial effects for cancer treatment.

Figure 4. (a) Bistratene A



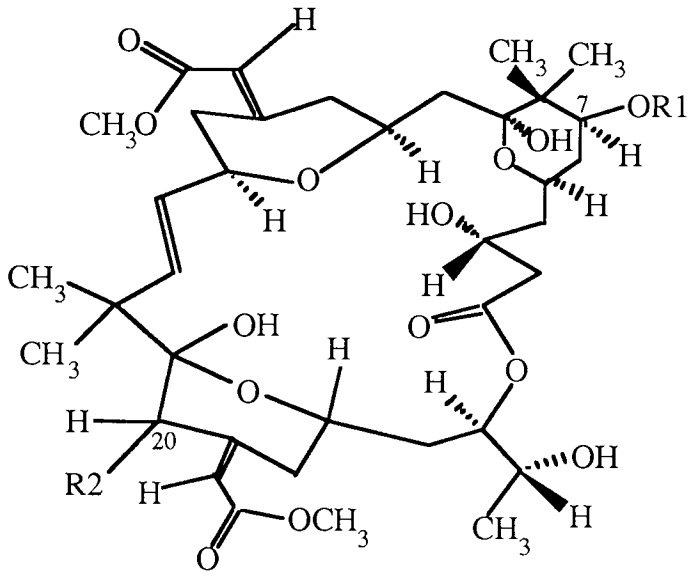


(b) Phorbol Esters

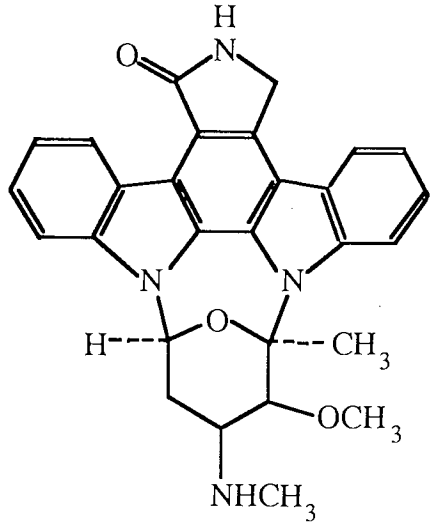
	R1	R2
TPA :	$(CH_2)_{12}CH_3$	CH_3
PDBu :	$(CH_2)_2CH_3$	$(CH_2)_2CH_3$

(c) Bryostatins

	R1	R2
Bryostatin 1 :	$COCH_3$	$OCO(CH)_4(CH_2)_2CH_3$
Bryostatin 2 :	H	$OCO(CH)_4(CH_2)_2CH_3$



(d) Staurosporine



1.4.3. Diacylglycerols (DAGs).

DAGs are the physiological ligands for PKC activation (section 1.3.2.) and can be generated intracellularly by the stimulation of numerous signalling pathways (Figs.1 and 3, reviewed by Nishizuka, 1992). Exogenously added DAGs are able to mimic some but not all of the biological effects initiated by phorbol esters. 1,2-sn-Dioctanoylglycerol is a second stage tumour promoter (Verma, 1988) and DAGs are mitogenic to Swiss 3T3 fibroblasts (Rozengurt *et al*, 1984). They do not stimulate growth in Mel-ab murine melanocytes unless applied at hourly intervals (Brooks *et al*, 1993), or A65T leukaemia cells, even when applied at bihourly intervals unlike TPA (Yamamoto *et al*, 1988). They are unable to mimic the differentiating ability of TPA in U937 monoblastoid cells (Ways *et al*, 1987) but reports are conflicting regarding HL-60 leukaemia cell differentiation : Ebeling *et al*, 1985 described differentiation by both agents, but other workers have been unable to induce differentiation with DAGs, even with bihourly applications, without the presence of the calcium ionophore A23187 to provide a stronger signal (Morin *et al*, 1987). DAGs mimic phorbol esters by initiating growth arrest in MCF-7 cells (Issandou *et al*, 1988) but are unable to inhibit the growth of A549 cells unless toxic concentrations are applied (Laughton *et al*, 1989). DAGs have a much lower affinity for PKC than phorbol esters and are rapidly metabolised intracellularly by kinases and lipases, thus DAG-mediated activation is transitory (Welsh and Cabot, 1987). This fact probably accounts for some of the differences between DAGs and TPA.

1.4.4. Bryostatins.

The bryostatins are a group of macrocyclic lactones derived from the marine organism *Bugula neritina* (Pettit *et al*, 1982, Fig.4c). Bryostatin 1 is the most studied of 17 derivatives ; it is equipotent with TPA at activating PKC *in vitro* (Kraft *et al,* 1986, Sako *et al*, 1988, Isakov *et al*, 1993) and interacts with the same receptor within the PKC regulatory domain (section 1.3.2., DeVries *et al*, 1988).

Bryostatins elicit a wide range of biological responses in cells. Like phorbol esters, these agents are mitogenic to Swiss 3T3 cells (Smith *et al*, 1985) and activate and induce proliferation of T cells, B cells and neutrophils (Berkow *et al*, 1985, Hess *et al*, 1988, Drexler *et al*, 1990). Bryostatin 1 is able to induce maturation of a variety of fresh and immortal leukaemic cells (Kraft *et al*, 1989, Jones *et al*, 1990, Lilly *et al*, 1990) and is antiproliferative to many cell lines including melanoma and renal tumour cells (Pettit *et al*, 1982).

Unlike phorbol esters, bryostatin 1 is ineffective as a complete tumour promotor and actually antagonises the tumour-promoting activity of TPA (Hennings *et al*, 1987). It is unable or is only partially able to elicit differentiation of a number of cell lines which respond to TPA, such as HL-60 cells (Kraft *et al*, 1986, 1989) and normal human epidermal keratinocytes (Jetten *et al*, 1989) and it antagonises the effects of TPA on these cells. Similarly, bryostatin 1 inhibits the growth of A549 (Dale and Gescher, 1989a) and MCF-7 cells (Kennedy *et al*, 1992) much more transiently than TPA (section 1.4.2.), and it actually reverses the growth inhibitory effects induced by TPA in these cells.

Bryostatin 1 is an effective antineoplastic agent *in vivo* using animal models for leukaemia, (Pettit *et al*, 1982), reticulum cell sarcoma, B-cell lymphoma, ovarian carcinoma and melanoma (Hornung *et al*, 1992) and it inhibits B16 melanoma metastasis (Schuchter *et al*, 1991). Because of its beneficial immunomodulating effects, the ability to inhibit tumour cell growth and/or initiate cell maturation *in vitro* and *in vivo*, coupled with a lack of tumour-promoting activity, bryostatin 1 is an excellent candidate for the treatment of cancer. Consequently, it is currently undergoing clinical trials for this indication. Preclinical toxicology studies indicated a murine LD_{10} value of 29μg/kg and bryostatin 1

entered phase 1 clinical trials at only 5μg/m^2, which was given at biweekly intervals as an infusion over one hour. The dose was increased incrementally up to 65μg/m^2 in new patients recruited into the trial. Flu-like symptoms were a common side effect, but myalgia was the dose-limiting toxic effect in this study. Bryostatin 1 is now in phase 2 trials, indicated for lymphoma, leukaemia, melanoma and hypernephroma at 35-50μg/m^2 given every two weeks or 25-35μg/m^2 weekly (Prendiville *et al*, 1993).

1.5. Bistratene A.

Bistratene A (also termed bistramide A) was originally described as a macrocyclic polyether but its structure has since been revised to that shown in Fig.4a (Foster *et al*, 1992). Like the bryostatins, it originates from a marine organism (Fig.5) and modulates the growth and maturation of cells in culture. It is a potent inhibitor of growth in HL-60, P388 and L1210 leukaemia cells (Watters *et al*, 1990, Gouiffes *et al*, 1988, Foster *et al*, 1992), T24 bladder carcinoma cells (Degnan *et al*, 1989), and KB cells (Gouiffes *et al*, 1988) with IC_{50} values within the nanomolar range. It is also cytostatic in two non-tumourigenic cell lines, with an IC_{50} of 22nM in normal umbilical cord endothelial cells (Gouiffes *et al*, 1988) and 99nM in MRC5CV1 fibroblasts (Degnan *et al*, 1989).

Bistratene A initiates differentiation in HL-60 cells towards monocytes/macrophages like TPA and bryostatins (section 1.4.2. and 1.4.4.) (Watters *et al*, 1990) and it induces a more differentiated phenotype in NSCLCN6 human epidermal lung cancer cells (Roussakis *et al*, 1991). The mechanism by which bistratene A mediates these effects is unknown but it has been suggested to involve modulation of PKC: the agent is able to partially activate purified PKC β from bovine spleen at 5-10μM concentrations (Watters *et al*, 1990).

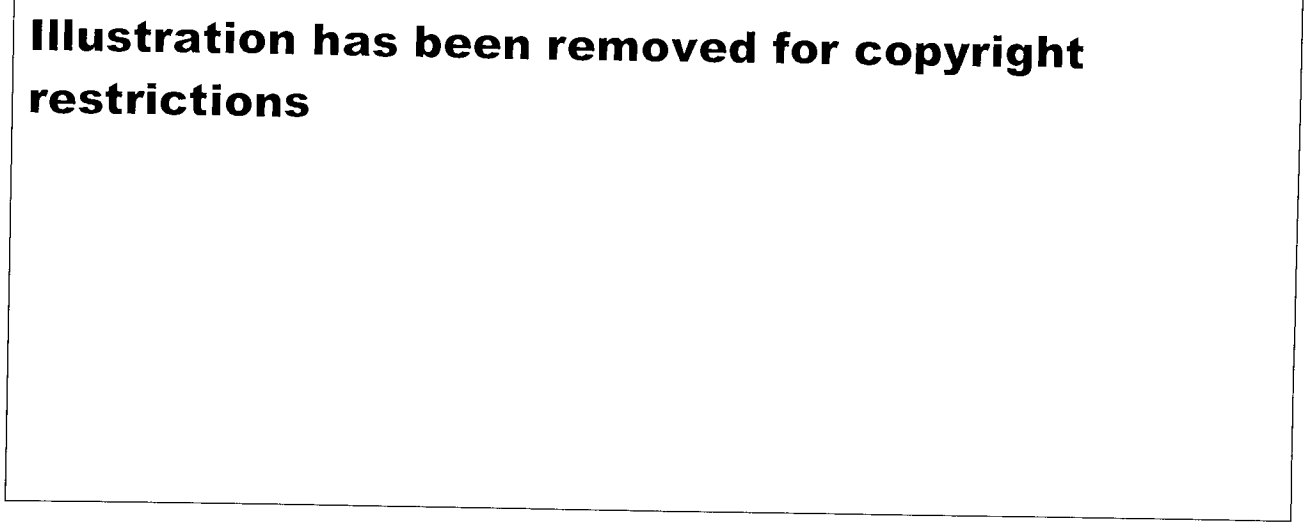


Figure 5. Marine source of Bistratene A.
Bistratene A was obtained from extraction of the marine ascidian *Lissoclinum bistratum* Sluiter, pictured above in its coral reef habitat. The extraction procedure involves homogenisation in methanol/toluene (3:1), extraction into 1M sodium nitrate solution, extraction with chloroform, evaporation and finally preparative reverse-phase HPLC, giving a yield of 0.56g/kg wet weight (Degnan *et al*, 1989).

1.6. Objectives of this Study.

The improved understanding of cellular signalling pathways gained in the last two decades has uncovered numerous new cellular targets for anticancer agents. Due to its pivotal role in cell proliferation, differentiation and metastasis, PKC is currently under scrutiny as a potential enzyme in the signal transduction cascade whose modulation may prevent cancer cell growth.

The primary goal in our laboratory is to develop new chemotherapeutic strategies for the treatment of cancer and unravel their modes of action. The agents described in section 1.4 are potent modulators of cell growth and differentiation and exert their effects through PKC. Bistratene A may also exert its effects via interaction with this enzyme or its signalling pathway. Numerous studies have compared the diversity of biological effects of PKC modulators, attempting to find the determining factors for antiproliferative,

differentiative and antimetastatic properties which would be beneficial for the treatment of cancer. Hypotheses that have been proposed include transient or prolonged enzyme activation, translocation or downregulation, phosphorylation of specific protein substrates and isozyme-specific effects.

Previous work in this laboratory has focused on the growth inhibitory effects of PKC modulators and their precise mechanism of action. In A549 lung carcinoma cells, phorbol esters and bryostatins are exquisitely potent inhibitors of DNA synthesis but cell growth resumes in the continued presence of these agents within hours for bryostatins and after several days for TPA (sections 1.4.2. and 1.4.4.). A similar scenario occurs in MCF-7 breast adenocarcinoma cells, in which bryostatin has very little effect on cell growth but TPA is potently cytostatic (sections 1.4.2. and 1.4.4.). Initiation of growth arrest was found to be associated with activation of PKC in A549 cells (Bradshaw *et al*, 1992). However, the brevity of action of bryostatins and prolonged duration of action of TPA cannot be explained by their effects on PKC activity alone. It has been surmised that discrepancies in cellular responses are caused by the differential abilities of these agents to selectively activate, translocate or downregulate certain isoforms of PKC.

The overall objective of the experiments described here was to clarify the link between growth arrest and PKC modulation. The agents upon which these investigations were focused were bryostatin 1, bistratene A and TPA. Concerning bryostatin 1 and TPA, the hypotheses were tested that the growth inhibitory responses of A549 and MCF-7 cells to these agents are related to cellular PKC isotype content, and that differences in responses to the two agents are associated with activation, translocation or downregulation of specific isoforms. To test these hypotheses, the effect of bryostatin 1 on cell growth was reinvestigated and compared with that of TPA. Using similar culture conditions as those used for growth studies, the PKC isoform profile of A549 and MCF-7 cells was established by Western blot analysis and changes in isoform location and levels were assessed in cellular fractions after exposure to the two agents. A particular emphasis was placed on assessing translocation of PKC isoforms to the cell nucleus, as this event could have implications for the expression of genes pivotal for the regulation of proliferation (Clemens *et al*, 1992). It was hoped that this work would clarify more precisely how

modulation of PKC activity attenuates cancer cell growth.

Regarding bistratene A, the hypothesis was tested that this agent exerts its cytostatic and cytotoxic effects *via* PKC activation. By studying effects on growth in A549 and MCF-7 cells, the relationship between the biological effects of bistratene A and modulation of PKC was assessed. In particular, the hypothesis was tested that bistratene A mimics the effects of TPA and bryostatin on growth in these cells, and reversal of these effects can be induced with the kinase inhibitor staurosporine. PKC is involved with the development of metastasis (section 1.3.5.) and it was pertinent to evaluate bistratene A for antimetastatic effects. Growth characteristics of cells grown on the basement membrane substitute Matrigel have been suggested to correlate with metastatic potential (Albini *et al*, 1987); thus the effects of bistratene A, bryostatin and TPA on A549 and MCF-7 cells were compared when grown on this substrate. To further assess effects on metastasis, the ability of these agents to prevent invasion through membranes coated with Matrigel was also investigated.

In order to explore the effect of bistratene A on PKC, a series of experiments was carried out which assess modulation of the enzyme: protein phosphorylation changes in A549 cells were determined, and cellular locational changes of PKC were examined via Western blotting and the mixed micelle assay for phorbol ester binding after incubation with TPA and bistratene A. The latter assay was also used to determine competition for phorbol ester receptors between PDBu and bistratene A. Finally, the ability of bistratene A to activate or inhibit A549 and MCF-7 cell cytosolic PKC and specific PKC isoforms was tested directly using assays for PKC activity.

Section 2. Materials

Section 2. Materials.

2.1. Chemicals and Reagents.

The following chemicals and reagents were obtained from the sources indicated in italics:

Amersham International Plc., Amersham, Bucks.

Monoclonal antibody to PKC α and β isozymes (mouse derived), detection kit for Western blotting (colour reaction with mouse-derived antibodies), ECL detection kit for Western blotting, Hybond C nitrocellulose, Hyperfilm ECL, Hyperfilm MP, PKC assay kit, rainbow coloured and [^{14}C]-labelled protein molecular wt. markers (14.3-200kDa), [^{3}H]thy (5Ci/mmol.).

Amicon Corporation, Danvers, Mass., USA.

Diaflow ultrafiltration membranes (YM30).

BDH Chemicals Ltd., Poole, Dorset.

$CaCl_2$ solution (1M), EDTA, EGTA, hydroxylapatite for nucleic acid research, $MgCl_2$, magnesium acetate, β-mercaptoethanol, KH_2PO_4, K_2HPO_4, phenol red, TCA.

Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts.

Acrylamide, ammonium persulphate, Bio-Rad protein assay dye reagent, N,N'methylene bisacrylamide, nitrocellulose, PDA, TEMED.

Collaborative Research Inc. Biomedical Products Division, Bedford, Mass. USA.

Matrigel.

Coulter electronics, Luton, UK.

Isoton II (azide free).

Fisons Plc., Loughborough, Leics.

DMSO, glacial acetic acid, hydrochloric acid, methanol, phosphoric acid.

Gibco BRL, Paisley, Glasgow, Scotland.

Polyclonal antibodies to PKC α, β, γ, δ, ε and ζ isoforms (rabbit-derived), tissue culture grade: DMEM medium, foetal calf serum, L-glutamine 200mM, Ham's F12 nutrient

medium, newborn calf serum, Ca^{2+}- and Mg^{2+}- free PBS (10x), Pen/Strep (100x), RPMI 1640 medium, trypsin/EDTA (10x).

Hoefer Scientific Instruments (UK), Newcastle under Lyme, Staffs.

Nitrocellulose, cellulose acetate sheets for gel drying, mylar sheets.

IBF Biotechnics, Villeneuve la Garenne, France.

AcA Ultrogel 202.

ICN Flow, High Wycombe, Bucks.

Monoclonal antibodies to PKC α, β and γ (mouse derived) from the Seikagaku Corporation, Japan, [γ-^{32}P]ATP (>4000Ci/mmol), $^{32}P_i$ (PO_4^{3-} in 0.02M HCl, 285 Ci/mg P_i), tissue culture grade: MEM (Eagle with Earle's salts and sodium bicarbonate 850mg/l), sodium pyruvate 100mM.

Imperial Laboratories (Europe) Ltd., Andover, Hants.

Foetal calf serum.

LKB Pharmacia, Milton Keynes.

Ampholines pH 4-6 and 3.5-10, Optiphase Hisafe II scintillation fluid, cellulose acetate membrane for Western blotting.

Millipore UK Ltd., Harrow, Middlesex.

0.2μm filters, Ultrafree CL low binding cellulose membranes (10kD mw limit).

New England Nuclear, Du Pont (UK) Ltd., Southampton, Hants.

[^{3}H]PDBu (10-20 Ci/mmol).

Oxoid Laboratories Ltd., Basingstoke, Hants.

PBS tablets.

Tissue Culture Services, Botolph Clayton, Herts.

Monoclonal mouse-derived antibody to PKC α, targetted to the catalytic domain.

Whatman Labsales Ltd., Croydon, Surrey.

DE52 anion exchanger, P81, 3mmChr and 17 Chr chromatography paper, filter paper.

Other chemicals and reagents were purchased from the Sigma Chemical Company, Poole, Dorset.

The following substances were generous gifts provided by the sources indicated:

Bistratene A ; Dr. Diane Watters, Queensland Institute of Medical Research, Brisbane, Australia.

Bryostatins 1 and 2 ; Dr. G.R. Pettit, Arizona State University, Tempe, Arizona, USA.

Polyclonal antibody to PKC ε, Dr. P. Parker, ICRF, Lincoln's Inn's Fields, London.

Polyclonal antibody to PKC η (rabbit derived) ; Dr.Shin-ichi Osada, Yokohama City University School of Medicine, Yokohama, Japan.

Polyclonal antibody to the MARCKS protein ; Dr. P.J. Blackshear, Howard Hughes Medical Institute Laboratories, Duke University, Durham, NC, USA.

2.2. Solutions, Buffers and Gels.

2.2.1. Cell Culture.

Drug Treatments.

Bistratene A, phorbol esters, bryostatins and staurosporine were dissolved in DMSO at concentrations of 1-10mM. Bistratene A was stored at -70°C and the other compounds at -20°C in aliquots, which were diluted in DMSO to the appropriate concentration when required.

Concentrated Versene Stock Solution (10x)

20 PBS tablets

742mg EDTA

100mg phenol red

to 200ml with distilled H_2O.

The pH was adjusted to 7.4 with 1M NaOH. The solution was then filtered, autoclaved and stored at 4°C.

Trypsin/Versene Solution.

10ml versene stock solution (10x)

10ml trypsin/EDTA 10x solution

80ml sterile distilled H_2O.

The solution was stored at 4°C.

Trypsin/EDTA Solution

10ml trypsin/EDTA 10x solution

10ml Ca^{2+}- and Mg^{2+}- free PBS 10x solution

80ml sterile distilled H_2O.

The solution was stored at 4°C.

PBS

10 PBS tablets

1 litre distilled H_2O.

The solution was autoclaved if necessary and stored at 4°C.

Acid Fixative

10ml glacial acetic acid

50ml methanol

40ml distilled H_2O.

2.2.2. Cell Fractionation Buffers.

Concentrated W3 Wash Buffer (10x)

12.114g Tris base

43.83g NaCl

4.505g glucose

to 500ml with distilled H_2O.

The pH of the solution was adjusted to 7.4 with concentrated HCl and was stored at 4°C. Prior to use, the concentrated W3 stock solution was diluted 10x with distilled H_2O. The protease inhibitors leupeptin and aprotinin (frozen aliquots of 2mg/ml in distilled H_2O) were added using 1µl per ml W3. Final concentrations of buffer components were thus: 20mM Tris-HCl, 150mM NaCl, 5mM glucose, 2µg/ml leupeptin, 2µg/ml aprotinin.

H8 Buffer.

	final concentration
200µl Tris-HCl 1.0M, pH 7.5	20mM
200µl EDTA 100mM, pH 7.5	2mM
200µl EGTA 100mM, pH 7.5	2mM

4µl β-mercaptoethanol	6mM
10µl leupeptin (2mg/ml)	2µg/ml
10µl aprotinin (2mg/ml)	2µg/ml

to 10ml with distilled H_2O.

The buffer was prepared on the day of use. Stock solutions of Tris-HCl 1M, pH 7.5, EDTA and EGTA 100mM, pH 7.5, were prepared and routinely stored at 4°C.

PKC Assay Cell Fractionation Buffer

	final concentration
5ml Tris-HCl 100mM, pH 7.5	50mM
500µl EDTA 100mM, pH 7.5	5mM
1ml EGTA 100mM, pH 7.5	10mM
30mg β-mercaptoethanol	0.3% (w/v)
100µl benzamidine 1.0M	10mM
20µl PMSF in ethanol (25mg/ml)	50µg/ml
10µl aprotinin (2mg/ml)	2µg/ml
10µl leupeptin (2mg/ml)	2µg/ml

distilled H_2O to 10ml.

The buffer was prepared shortly before use and stored on ice.

Wash Buffer G.

	final concentration
4.659g KCl	125mM
1.817g Tris base	30mM
536.2mg magnesium acetate	5mM
951.0mg EGTA	5mM
1.57ml β-mercaptoethanol	45mM
500µl aprotinin (2mg/ml)	2µg/ml
500µl leupeptin (2mg/ml)	2µg/ml

to 500ml with distilled H_2O.

Buffer was prepared minus leupeptin and aprotinin and was adjusted to pH 7.5 with HCl, then stored at 4°C. Protease inhibitors were added on the day of use from frozen aliquots.

Swelling Buffer.

	final concentration
74.6mg KCl	10mM
363.3mg Tris base	30mM
107.2mg magnesium acetate	5mM
190.2mg EGTA	5mM
314µl β-mercaptoethanol	45mM
156.6mg benzamidine	10mM
1.36mg aprotinin	13.6µg/ml
750µl leupeptin (2mg/ml)	15µg/ml

to 100ml with distilled H_2O.

The buffer was prepared omitting leupeptin and the pH adjusted to 7.5 using HCl. It was stored at 4°C. Leupeptin was added on the day of use.

Swelling Buffer with 25% Glycerol.

This was prepared as for swelling buffer, but 25ml distilled H_2O was replaced with 25ml glycerol prior to pH adjustment.

Swelling Buffer, 25% Glycerol and 0.1% Triton X100.

2ml swelling buffer with 25% glycerol

10µl Triton X100 20% (w/v) in distilled H_2O.

Buffer for Sonication of Nuclei.

	final concentration
121.1mg Tris base	10mM
584.4mg NaCl	100mM
40.7mg $MgCl_2$	2mM
190.2mg EGTA	5mM
314µl β–mercaptoethanol	45mM
1.36mg aprotinin	13.6µg/ml
750µl leupeptin (2mg/ml)	15µg/ml
1g Nonidet P40	1% (w/v)

to 100ml with distilled H_2O.

The buffer was prepared omitting leupeptin and the pH adjusted to 8.0 with 1M NaOH. It

was stored at 4°C. Leupeptin was added using 2mg/ml frozen stock aliquots just prior to use.

2.2.3. MTT Assay.

Glycine Buffer.

	final concentration
375.4mg glycine	100mM
292.2mg NaCl	100mM

to 50ml with distilled H_2O.

The pH was adjusted to 10.5 with 10M NaOH and stored at 4°C.

2.2.4. Mixed Micelle Assay for Phorbol Ester Binding.

Buffer A.

	final concentration
50ml Tris-HCl 200mM, pH 7.5	20mM
100µl $CaCl_2$ 1.0M	200µM
375µl Triton X100 20% (w/v)	0.015% (w/v)

to 500ml with distilled H_2O.

The buffer was stored at 4°C.

Buffer B.

	final concentration
50ml Tris-HCl 200mM, pH 7.5	20mM
100mg sodium azide	0.02% (w/v)

to 500ml with distilled H_2O.

The buffer was stored at 4°C.

2.2.5. Buffers and Gels for SDS/PAGE and Western Blots.

Sample Buffer.

	final concentration
10ml Tris-HCl 500mM, pH 6.8	125mM
8ml glycerol	20% (v/v)

16ml SDS 10% (w/v)	4% (w/v)
2ml bromophenol blue (1mg/ml)	50µg/ml
1.23g DTT	200mM

to 40ml with distilled H_2O.

The buffer was prepared omitting DTT and stored at room temperature. DTT was added just before use.

Acrylamide/Bis 30%/0.8%.

	final concentration
60g acrylamide	30% (w/v)
1.6g bis	0.8% (w/v)

to 200ml with distilled H_2O.

The solution was stored in darkness at 4°C for up to 1 month before use.

Separating Gel.

	final concentration
5ml Tris-HCl 1.5M, pH 8.8	375mM
200µl SDS 10% (w/v)	0.1% (w/v)
5.336ml acrylamide/bis 30%/0.8%	8% (w/v)

9.364ml distilled H_2O

100µl ammonium persulphate 10% (w/v)

7.5µl TEMED.

Ammonium persulphate 10% solution was made and stored at 4°C for up to a week prior to use. The separating gel was prepared shortly before pouring between the gel plates. Ammonium persulphate catalyses polymerization and TEMED accelerates the reaction and therefore these two agents were added last. This is sufficient for 1 large or 4 mini gels of 0.75mm width.

Stacking Gel Tris/SDS Buffer.

	final concentration
6.06g Tris base	500mM
400mg SDS	0.4% (w/v)

to 100ml with distilled H_2O.

The buffer was adjusted to pH 6.8 with HCl and stored at 4°C.

Stacking Gel.

	final concentration
2.5ml stacking gel tris/SDS buffer	125mM Tris/0.1% SDS
1.65ml acrylamide/bis 30%/0.8%	5% (w/v)
5.75ml distilled H_2O	
100µl ammonium persulphate 10% (w/v)	
7µl TEMED.	

The stacking gel was prepared when required. Ammonium persulphate and TEMED were added last. This is sufficient for 1 large or 4 mini gels.

Running Buffer

15.15g Tris base

72g glycine

5g SDS

to 5 litres with distilled H_2O.

The buffer was stored at 4°C.

Electrode Solution.

	final concentration
11.72g glycine	39mM
23.24g Tris base	48mM
1.5g SDS	0.0375% (w/v)
800ml methanol	20% (v/v)
to 4 litres with distilled H_2O.	
The solution was stored at 4°C.	

TBS.

	final concentration
9.68g Tris base	20mM
32g NaCl	137mM
to 4 litres with distilled H_2O.	

The pH was adjusted to 7.6 with concentrated HCl and the buffer stored at 4°C.

TBS-T.

A series of buffers containing 0.1, 0.3 and 0.5% (w/v) Tween 20 in TBS were prepared. These buffers were designated TBS-T 0.1%, TBS-T 0.3% and TBS-T 0.5% respectively. They were stored at 4°C.

High Salt TBS-T 0.3%

	final concentration
9.68g Tris base	20mM
116.9g NaCl	500mM
12g Tween 20	0.3% (w/v)

to 4 litres with distilled H_2O.

The pH was adjusted to 7.6 with concentrated HCl and the buffer was stored at 4°C.

Stripping Buffer.

	final concentration
3.78g Tris base	62.5mM
10g SDS	2% (w/v)
3.49ml β-mercaptoethanol	100mM

to 500ml with distilled H_2O.

The pH was adjusted to 6.7 and stored at 4°C.

2.2.6.Two-Dimensional Gel Electrophoresis Buffers and Gels.

USB.

	final concentration
5.405g urea	9M
200mg CHAPS	2% (w/v)
120mg DTT	1.2% (w/v)
500µl Ampholines pH 3.5-10	2% (w/v)
400µl Nonidet P40	4% (v/v)

to 10ml with distilled H_2O.

The buffer was prepared at room temperature, without the application of heat, as increased temperatures favour the formation of cyanate ions from urea which may react with proteins. The buffer was frozen at -20°C in 500µl aliquots for up to 1 month.

PDA Solution.

169mg PDA

18.93ml acrylamide/bis 30%/0.8%.

The solution was stored at 4°C.

Tube Gel.

	final concentration
6.187g urea	9M
1.5ml PDA solution	4% (w/v)
150mg CHAPS	1.3% (w/v)
188µl Ampholines pH 4-6	0.7% (w/v)
375µl Ampholines pH 3.5-10	1.3% (w/v)
75µl Nonidet P40	0.7% (v/v)

4.5ml distilled H_2O

37.5µl ammonium persulphate 10% (w/v)

7.5µl TEMED.

Initially, the urea was dissolved at room temperature in 4ml distilled H_2O and the CHAPS in 0.5ml distilled H_2O. All constituents except ammonium persulphate and TEMED were then combined and degassed for 2 minutes. The latter two agents were added immediately before gel formation after degassing. This is sufficient for 15 large tube gels of 1.5mm width.

2nd Dimension Gradient Gel.

	12% acrylamide	8% acrylamide
glycerol	1.75ml	———
Tris-HCl 1.5M, pH 8.8	2.92ml	5.83ml
SDS 10% (w/v)	117µl	233µl
acrylamide/bis 30%/0.8%	4.53ml	6.04ml
distilled H_2O	2.29ml	11.14ml
ammonium persulphate 10% (w/v)	29.17µl	58.3µl
TEMED.	7.25µl	14.5µl

Components of the 8 and 12% gels were mixed individually, adding the ammonium persulphate and TEMED last, before pumping through a gradient maker (see methods).

This is sufficient for one 16x16x 0.15cm gel.

Stacking Gel for 2nd Dimension .

	final concentration
2.5ml Tris-HCl 500mM, pH 6.8	125mM
1.14ml acrylamide/bis 30%/0.8%	3.5%
100µl SDS 10% (w/v)	0.1%
6.26ml distilled H_2O	
25µl ammonium persulphate 10% (w/v)	
10µl TEMED.	

The components of the stacking gel were combined. Ammonium persulphate and TEMED were added immediately before pouring.

Equilibration Buffer.

50ml Tris-HCl 125mM, pH 6.8

5ml glycerol

2g SDS

30mg bromophenol blue.

The buffer was stored at room temperature.

2nd Dimension Running Buffer.

30g Tris base

140g glycine

5g SDS

to 5 litres with distilled H_2O.

This buffer was stored at 4°C.

2.2.7. Buffers for Isolation of PKC Isoforms.

DE52 Salt Free Buffer.

	final concentration
1.21g Tris base	20mM
93mg EGTA	500µM
95mg EDTA	500µM
349µl β-mercaptoethanol	10mM

10ml glycerol 2% (v/v)

to 500ml with distilled H_2O.

The buffer was adjusted to pH 7.5 with HCl and stored at 4°C.

DE52 Salt Buffer.

This buffer is the same as the DE52 salt free buffer with the addition of 800mM NaCl (23.38g in 500ml).

20mM Phosphate Buffer.

Acid		Base	
1.361g KH_2PO_4	20mM	1.742g K_2HPO_4	20mM
50ml glycerol	10% (v/v)	50ml glycerol	10% (v/v)
to 500ml with distilled H_2O.		to 500ml with distilled H_2O.	

Acid was gradually added to 500ml base until the pH was 7.8. Agents were added to 500ml of this buffer as follows :

	final concentration
500ml K/HPO_4 20mM, pH 7.8	20mM
93mg EGTA	500µM
95mg EDTA	500µM
349µl β-mercaptoethanol.	10mM

The final pH of this solution was 7.5. The buffer was stored at 4°C.

300mM Phosphate Buffer.

Acid		Base	
20.413g KH_2PO_4	300mM	26.127g K_2HPO_4	300mM
50ml glycerol	10% (v/v)	50ml glycerol	10% (v/v)
to 500ml with distilled H_2O.		to 500ml with distilled H_2O.	

Acid was gradually added to 500ml base until the pH was 7.5. Agents were added to 500ml of this buffer as follows :

	final concentration
500ml K/HPO_4 300mM, pH 7.5	300mM
93mg EGTA	500µM
95mM EDTA	500µM
349µl β-mercaptoethanol	10mM.

The buffer was stored at 4°C.

2.2.8. Solutions for Alternative PKC Assay.

Solution C.

	final concentration
400µl Tris-HCl 1.0M, pH 7.5	40mM
4.0mg histone type IIIS	400µg/ml
21.4mg magnesium acetate	10mM
6µl $CaCl_2$ 1.0M	600µM
40µl PS vesicles (4mg/ml)	16µg/ml

to 10ml with distilled H_2O.

PS vesicles (4mg/ml) were prepared by drying 400µl of PS solution (10mg/ml in 95% chloroform and 5% methanol) under a stream of nitrogen in a glass tube. Ice-cold Tris-HCl 40mM, pH 7.5 (1ml) was added and the mixture was sonicated on ice using 10 second bursts of an MSE sonicator (amplitude 26 microns) for 3-5 mins until a creamy appearance was noted. This preparation was stored for up to 2 weeks prior to the assay at -196°C. Solution P was as above, with the exclusion of PS vesicles and the replacement of $CaCl_2$ with 3.8mg EGTA (1mM final concentration). Solution T was the same as C with the addition of 5µl TPA (200µM) and solution B was the same as solution C with the addition of 5µl bistratene A (200µM) (100nM final concentrations).

Section 3. Methods.

Section 3. Methods.

3.1.Cell Culture.

3.1.1. Routine Cell Maintenance.

All cell culture procedures were carried out in laminar flow cabinets using aseptic technique. Cells were maintained in Heraeus CO_2 auto-zero or Leec incubators at 37°C with 5% CO_2. All cells were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Wilts, except for MCF-7 cells stably resistant to Adriamycin (MCF-7Adr cells), which were obtained from Dr. T. Bradshaw at Nottingham University. Medium was replenished every 2-3 days and cells were passaged when approaching confluence (monolayers) or when they reached a density of $8x10^5$ per ml (suspension cultures). Cells were used in experiments between months two and six after resurrection from the cell bank. A549 cells were routinely grown in Ham's F12 medium supplemented with 10% foetal calf serum (FCS), penicillin 100iu/ml, streptomycin 100µg/ml (pen/strep) and glutamine (2mM). Trypsin/versene solution was used for the detachment of monolayers from culture vessels. During subculturing, cells were reseeded using 5-10% of the original culture. The medium recommended for MCF-7 cells by the suppliers was Minimum Essential Medium (MEM) Eagle with glutamine (2mM) and 10% FCS, but cells exhibited poor growth in this medium. Therefore modifications to MEM were evaluated, using different percentages of FCS (5 or 20%) and the addition of sodium pyruvate as an energy source (Nutt *et al*, 1991). RPMI 1640 medium supplemented with 2g/l sodium bicarbonate, 2mM glutamine, 1µM insulin, and 5% FCS (Valette *et al*, 1987) was also evaluated. Finally, optimal growth was achieved using MEM Eagle (with Earle's salts and 850mg/l sodium bicarbonate) with 10% FCS, glutamine (2mM), sodium pyruvate (1mM) and a non-essential amino acid supplement (Sigma). MCF-7Adr cells were maintained in RPMI 1640 medium with 10% heat-inactivated FCS and glutamine (2mM). NIH 3T3 cells were grown in Dulbecco's Modified Eagles Medium (DMEM) with 10% newborn calf serum, glutamine (2mM) and sodium pyruvate (1mM). Monolayers of MCF-7 and NIH 3T3 cells required washing with Ca^{2+}- and Mg^{2+}- free phosphate-

buffered saline (PBS) before detachment from culture vessels with trypsin/EDTA. Both cell lines were subcultured using 10-20% of the original culture. HL-60 cells were grown in RPMI 1640 medium with 10% FCS, pen/strep and glutamine (2mM) and were subcultured with 30% of the original culture.

3.1.2 Cell Storage in Liquid Nitrogen.

Cells were trypsinised and pelleted using a Heraeus Labofuge 6000 at 1000rpm for 5 min. Cells were resuspended at a cell density of $1x10^6$ cells/ml in FCS with 10% dimethylsulphoxide (DMSO), with the exception of HL-60 cells, which were resuspended in FCS with 10% glycerol. This was due to their propensity to differentiate when using 10% DMSO as cryopreservative. Aliquots of cell suspension (1ml) were placed in cryogenic vials and frozen slowly to -80°C for 4h before immersion in liquid nitrogen. Cells were resurrected from the cell bank by rapid thawing to 37°C and immediate introduction of each vial of cell suspension into 40ml culture medium.

3.1.3. Studies of Cell Growth.

HL-60 cells were grown in T25 flasks seeded at $1x10^5$ cells/ml. Bistratene A or an equivalent volume of DMSO vehicle (0.1% (v/v)) was added to flasks. The vehicle had no effect on cell growth or differentiation at this concentration. Cells were analysed for morphology changes and were counted using a Coulter Counter after 2 days (Model ZM-settings : current 240, attenuation 8, T_l 20, T_u 99.9). Growth was expressed as a percentage of the increase in cell number of control flasks.

A549 and MCF-7 cells were seeded at $2x10^4$ or $5x10^3$ in 35mm diameter wells in 3ml medium. After 4 h of incubation, agents under investigation were added. Control cells were incubated with an equivalent volume of DMSO vehicle (0.3% (v/v)) which had no effect on cell growth. Medium and agents were replenished every two days. Cells were counted using a Coulter Counter on alternate days for 12 days (Model ZM settings for A549 cells : current 130, attenuation 16, T_l 12.0, T_u 99.9, and settings for MCF-7 cells : current 190, attenuation 32, T_l 13.0, T_u 99.9).

IC_{50} values were determined by counting the cell number after 5 days of exposure to bistratene A, feeding cells and reapplying drug on day 3. Growth was expressed as a percentage of controls. The IC_{50} is the concentration of bistratene A at which there is a 50% reduction in growth compared to vehicle-treated control cells.

3.1.4. Clonogenic Assays.

Wells (35mm) were initially coated with gelatin by application of 1ml of a 2% aqueous solution, followed by aspiration and finally washing the thin layer with 2ml PBS. Medium (3ml) containing 10^2, $3x10^2$, or 10^3 A549 cells was then introduced, avoiding gelatin coat disturbance. Bistratene A was added after 24 h. Medium and bistratene A were replenished every 3-4 days. Clones were stained by fixing with 70% industrial methylated spirits (IMS) for 15 min, washing with PBS, staining for 3 min with crystal violet 1% in aqueous solution, then plunging wells carefully into a large volume of water until excess crystal violet was removed. Colonies were counted with the naked eye after 10 or 20 days of incubation. Preliminary experiments indicated that the colony forming efficiency (CFE) of A549 cells remained unchanged from 10 to 20 days. The CFE of untreated cells was 34.8 ± 9.7% for a seeding density of 10^2, 43.6 ± 10.1% when seeded at $3x10^2$, and 29.4 ± 7.4% when seeded at 10^3.

3.1.5. Inhibition of Incorporation of [^{3}H]thy by Bistratene A.

Cells were seeded at $1.5x10^5$ per 35mm diameter well ($1.6x10^4$ cells per cm^2) and allowed to attach and settle for 20 h. Bistratene A was then added. In preliminary experiments, after 24 h, cells were washed with medium (1ml) and incubated for 1 h with 1ml medium containing 5-[methyl ^{3}H]-thymidine ([^{3}H]thy) (1μCi/ml) as described by Dale and Gescher, 1989a. Monolayers were then placed on ice and washed 6 times with ice-cold PBS to remove excess radiolabelled medium. Cells were fixed with 1ml acid fixative (section 2.2.1.) for > 30 mins at 4°C, washed twice with ice-cold PBS and solubilised into 2x500μl sodium dodecylsulphate (SDS) 1% (w/v) aqueous solution. Radioactivity was counted using a Packard Tricarb CA2000 scintillation counter after the addition of 10ml Optiphase Hisafe II scintillant to each sample. Cell counts were conducted in parallel and

results expressed as a percentage of control [^{3}H]thy incorporation per 10^5 cells. These initial 24 h incubation period experiments indicated that inhibition of incorporation of [^{3}H]thy occurred with concentrations of bistratene A above 10nM at this seeding density. Concentrations of 15, 50 and 200nM were chosen for an analysis of the time course of growth inhibitory events, to reflect the spectrum from partial to almost complete inhibition of [^{3}H]thy incorporation. The [^{3}H]thy uptake of cells was measured at regular intervals for a period of 72 h after the addition of bistratene A.

3.2. Stability of Bistratene A.

3.2.1. Stability in Cell Culture Medium at 37°C.

Bistratene A (200nM) was maintained at 37°C in Ham's F12 medium with 10% FCS, pen/strep and glutamine 2mM, for time intervals up to 30h. This pre-treated medium was then incubated with A549 cells which had been seeded at $1.5x10^5$ in 35mm diameter wells 20h previously. After 12h, incorporation of [^{3}H]thy was assessed. Simultaneously, incorporation of [^{3}H]thy into A549 cells after a 12h exposure to bistratene A (20-200nM concentrations) was assessed. A calibration curve of bistratene A concentration *vs* [^{3}H]thy incorporation was constructed. The growth-inhibitory potency of pre-incubated bistratene A was expressed as an equivalent bistratene A concentration from this curve.

3.2.2. Stability to Repeated Freezing and Thawing.

Samples of 60μM bistratene A in DMSO (20μl) were frozen to -20°C and thawed to room temperature before refreezing for 30 min, up to 7 times. Aliquots (10μl) of these samples were then added to the medium (3ml) of A549 cells which were seeded at $1.5x10^5$ in 35mm diameter wells 20h previously, giving a concentration of 200nM. After 12h, incorporation of [^{3}H]thy was assessed. A calibration curve comparing bistratene A concentration to [^{3}H]thy incorporation after a 12h incubation was constructed. The growth inhibitory potency of the frozen and thawed samples was converted to an equivalent bistratene A concentration from the calibration curve.

3.3. Cytotoxicity Assays.

3.3.1. LDH Assay.

Cytotoxicity was assessed using the release of lactate dehydrogenase (LDH) from cells as a measure of cytotoxicity (Laughton *et al*, 1989). A549 cells were seeded at a density of $1.5x10^5$ per 35mm well ($1.6x10^4$ per cm^2). After 20 h, medium was removed and replaced with 1.5ml medium containing 1% FCS and bistratene A. Cells were incubated for 24 h. FCS contains reduced nicotinamide adenine dinucleotide (NADH) oxidizing enzymes which interfere with the assay and hence a reduction from the usual 10% was required. After the drug treatment period, medium was collected and stored on ice until assayed for LDH as originally described by Leathwood and Plummer, 1969, using spectrophotometry. The assay utilizes catalysis of the following reaction by LDH:

$$NADH + H^+ + \text{pyruvate} \longrightarrow NAD^+ + \text{lactate}$$

PBS (2.4ml), 3.6mM NADH (100µl) and sample (400µl) were heated to 37°C in a cuvette before initiating the reaction with the addition of 30mM sodium pyruvate (100µl). The rate of decrease in UV absorbance at 340nm, reflecting the rate of oxidation of NADH to NAD^+, was measured over 10 min using a Beckman DU70 spectrophotometer. Maximal LDH release was measured using 400µl samples obtained from cells grown in 3ml medium with 1% FCS, to which 160µl of 20% Triton X100 was added for 5 mins, causing complete cell lysis. Cell counts were performed before and after drug treatment and results were expressed as a percentage of maximal LDH release per 10^5 cells, subtracting the release of LDH from naive cells.

3.3.2. MTT Assay.

Dehydrogenase enzymes within active mitochondria convert the pale yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to a dark blue formazan product. A reduction in synthesis of this compound indicates decreased cell viability or a reduction in cell number and therefore reflects cytotoxicity (Mosmann, 1983, Denizot *et al*, 1986, Holt *et al*, 1988). A549 cells were seeded in 96 well plates at $4.4x10^3$ per 6mm diameter well ($1.6x10^4$ cells per cm^2). After 20 h, bistratene A was added and

cells were exposed to the agent for 24 h. Fresh medium (200µl) containing MTT (5mg/ml) was then incubated with the cells for 4 h. Monolayers were then washed twice with PBS (200µl) and formazan crystals were dissolved in buffered DMSO (1 part glycine buffer (section 2.2.3.) to 8 parts DMSO) using a plate shaker for 20 mins. Formazan production was measured quantitatively using UV absorbtion at 550nm on an Anthos 2001 plate reader.

3.4. Invasion Assays.

3.4.1. Morphology of Cells on Matrigel.

Liquid Matrigel (200µl, 3.7mg protein/ml) was added to 16mm diameter wells and allowed to polymerise for 20 mins at 37°C. Medium containing $5x10^4$ A549 or MCF-7 cells (2ml) was added to each Matrigel-coated well and to a series of uncoated control wells. Plates were incubated for 1 h to allow the attachment of cells before the addition of agents. Cells were photographed after 4, 24 or 48 h.

3.4.2. Assay of Cellular Migration through Matrigel.

An *in vitro* invasion assay was carried out as described by Fridman *et al*, 1990. Polycarbonate filters (8µM pore size Nucleopore) were coated with Matrigel (25µg protein, 6.8µl) diluted with DMEM to 20µl and were incubated at 37°C for 1 min to ensure polymerisation. Filters were placed between the two compartments of a modified Boyden chamber. The lower compartment contained conditioned medium from NIH 3T3 cells containing chemoattractants. In the upper compartment, A549 cells were seeded at $2x10^5$ in 1ml DMEM with 10% FCS, pen/strep and glutamine (2mM). Chemotaxis control experiments were carried out using uncoated filters to confirm the tendency of cells to migrate towards the compartment containing chemoattractants. Drug was added to media in both compartments at equimolar concentration before introduction into the Boyden chamber. Chambers were incubated at 37°C for 8 h. The filters were then removed and fixed with 70% IMS before staining with crystal violet 1% as in section 3.1.4. Cells on the shiny upper side of the filter were removed using a cotton bud, and cells on the lower side were counted in a fixed field using microscopy. The number of cells which had migrated to

the underside of the membrane per unit area in drug-treated chambers was compared to controls.

3.5. Fractionation of Cells.

3.5.1. Preparation of Cytosolic and Particulate Fractions of Cells (Method A).

Cells were cultured on 140mm diameter petri dishes and at the appropriate time point, medium was discarded, plates were placed on ice and monolayers were washed three times with 10ml ice-cold wash buffer W3 (section 2.2.2). All W3 was removed and monolayers were scraped off each plate into two 250µl portions of ice-cold H8 buffer (section 2.2.2.). Cells were disrupted by sonication on ice using three 10 second bursts on an MSE sonicator (amplitude 26 microns). A Pegasus 65 Ultracentrifuge was used to separate the cytosol from the particulate fraction, operating at 100,000g for 30 min at 4°C. Supernatant was removed and held on ice as the cytosolic fraction and the cell pellet was redispersed in H8 Buffer containing 1% (w/v) Nonidet P40 (500µl). This was sonicated and centrifuged again as above and the resulting supernatant was designated the detergent-soluble particulate fraction.

3.5.2. Preparation of Cytosolic, Particulate and Nuclear Fractions of Cells (Method B).

Cells were cultured on 140mm diameter petri dishes (minimum of 2 plates per drug treatment) and at the appropriate time point, medium was discarded, plates were placed on ice and monolayers were washed three times with 10ml ice-cold wash buffer G (section 2.2.2.). All procedures were carried out on ice thereafter, according to a method modified from that described by Grief *et al*, 1992. Each stage of the separation was verified by staining a portion of homogenate with crystal violet 1% (w/v) in PBS, which localises in cell nuclei, and observing on a slide using phase contrast microscopy. Wash buffer G was removed and cells were scraped off each plate into 250µl swelling buffer (section 2.2.2.). Cells were allowed to swell in the buffer for 10 min before homogenising in a glass tube with PTFE pestle (Jencon, Leighton Buzzard, Beds) using a Tri-R Stir-R homogeniser, model S63C. A549 cells were homogenised at setting 3.5 for 25 strokes and MCF-7 cells at setting 3.0 for 20 strokes. The homogenate was then carefully laid over an equal volume

of swelling buffer with 25% glycerol (section 2.2.2.) in an Eppendorf 1.5ml tube and centrifuged at 400g for 5 min at 4°C in a Heraeus Minifuge T. The pellet obtained contained nuclei and was resuspended in 1ml swelling buffer and 25% glycerol with 0.1% (w/v) Triton X100. Nuclei were then pelleted by centrifugation at 13,500rpm for 2 min at 4°C, resuspended in swelling buffer with 25% glycerol and pelleted again. Finally, the nuclei were resuspended in buffer for the sonication of nuclei (section 2.2.2., 150µl per petri dish) and were sonicated for 3 x 10 seconds at 26 microns amplitude. The opaque upper layer from the 400g spin contained cytosolic and particulate fractions. This was centrifuged at 100,000g for 30 min at 4°C. The supernatant was designated the cytosolic fraction and the pellet was dispersed in swelling buffer containing 1% (w/v) Nonidet P40. Membranes and particulate matter were then disrupted by sonication for 3 x 10 seconds at 26 microns amplitude on ice, finally yielding the particulate fraction of cells.

3.6. Phorbol Ester Receptor Binding Studies.

3.6.1. Assessment of Phorbol Ester Binding in Cell Cytosol after Treatment with Bistratene A and Phorbol Esters.

Changes in the number of phorbol ester receptors in the cytosol of drug-treated cells were determined using the mixed micelle assay of Hannun and Bell, 1986, 1987. Crude cytosolic extracts of A549 cells seeded at $2x10^6$ in 140mm plates were prepared (method A, section 3.5.1.) after incubation for (i) 20 h and drug treatment for 30 mins or 24 h, (ii) 50 h in the presence of drug. The latter treatment was used on cells which were continuously cultured in the presence of bistratene A 15nM or PDBu 100nM for a minimum of 3 months. Micelles containing 20 mol % PS, 80 mol % Triton X100 were prepared by drying 142.4µl PS (10mg/ml in 95% chloroform/5% methanol) under a stream of nitrogen in a glass tube. Triton X100 3% w/v (150µl) was then added. This was mixed and vortexed for 2 mins, then incubated at 27°C for 10 mins. Buffer M containing the following components was prepared : Tris-HCl 200mM pH 7.5 (150µl), $CaCl_2$ 22mM (150µl), mixed micelles (150µl). The binding reaction was initiated at room temperature by combining 15µl buffer M, 25µl [20-^{3}H(N)]-phorbol-12,13-dibutyrate ([^{3}H]PDBu) (100nM, 13.2Ci/mmol) and 10µl cytosolic fraction (reaction mixture concentrations of

[^{3}H]PDBu 50nM, $CaCl_2$ 2.2mM, Tris-HCl pH 7.5 20mM, PS 20 mol % in micelles, 1.2mM). Non-specific binding was determined by adding nonlabelled PDBu (50μM) to the reaction mixture. Bound [^{3}H]PDBu (phorbol ester receptor fraction) was separated by gel filtration on a 2ml column of Ultrogel AcA 202 in siliconised pasteur pipettes using 950μl Buffer A (section 2.2.4.) to elute. Columns were washed with 5ml Buffer A after each elution and stored at 4°C in Buffer B (section 2.2.4.). Radioactivity of the eluate was counted in 10ml Optiphase Hisafe II scintillant using a Packard Tricarb CA 2000 scintillation counter. Cytosolic protein (mg/ml) was measured using the Bradford method (1976), with Bio-Rad protein dye reagent and results were expressed as disintegrations per minute (DPM) per mg cytosolic protein as a percentage of controls. Non-specific binding was less than 15% of total binding.

3.6.2. Competition for Phorbol Ester Binding Sites by Bistratene A.

Experiments were carried out to assess competition for phorbol ester receptor sites between [^{3}H]PDBu and TPA or bistratene A. Cytosolic extract of naive A549 cells was used as a source of phorbol ester receptors and bistratene A or TPA (10 and 100nM) were added to the reaction mixture. Non specific binding was determined as above and again the phorbol ester receptor fraction was collected by gel filtration and radioactivity was determined. Non-specific binding was less than 15 % of total binding.

3.6.3. Preparation of Leucocyte Fractions from Whole Blood for Assessment of Phorbol Ester Binding.

An analysis of the phorbol ester receptor capacity of human leucocytes was performed. Whole blood was subjected to double gradient density centrifugation to separate granulocytes from mononuclear cells/platelets. Histopaque 1077 (3ml) was carefully layered onto 3ml Histopaque 1119 in a 15ml centrifuge tube. A fresh sample of blood was anticoagulated using lithium heparin and 6ml was layered on the Histopaque 1077 to form a triple layer (Fig.6a). Tubes were centrifuged at 700g for 30 min. This had to be performed at room temperature to prevent cell clumping. Two opaque white layers of cells could be clearly seen (Fig.6b). Cell layers were aspirated into separate tubes and initially PBS was added to the cells and used for washing as described by the manufacturers of

Histopaque at room temperature. Low phorbol ester binding was obtained using PBS as buffer, even if kept on ice. Therefore PBS was replaced with H8 buffer (section 2.2.2.) which contains protease inhibitors and divalent cation chelators to prevent PKC breakdown. Erythrocyte contaminants also underwent lysis in H8, unlike PBS, and thus could be removed from the leucocyte fractions by repeated washing. Ice-cold H8 (10ml) was added to leucocyte fractions and all other stages were carried out at 4°C or on ice. Cells were pelleted from the H8 buffer by centrifugation at 1700rpm for 5 mins, then were resuspended and pelleted in a further 10ml. This H8 wash was repeated again to ensure the complete removal of Histopaque and erythrocytes. Cells were then resuspended in 500µl H8. Cell cytosolic fractions were prepared (Method A, section 3.5.1.) and assessed for phorbol ester binding sites (section 3.6.1).

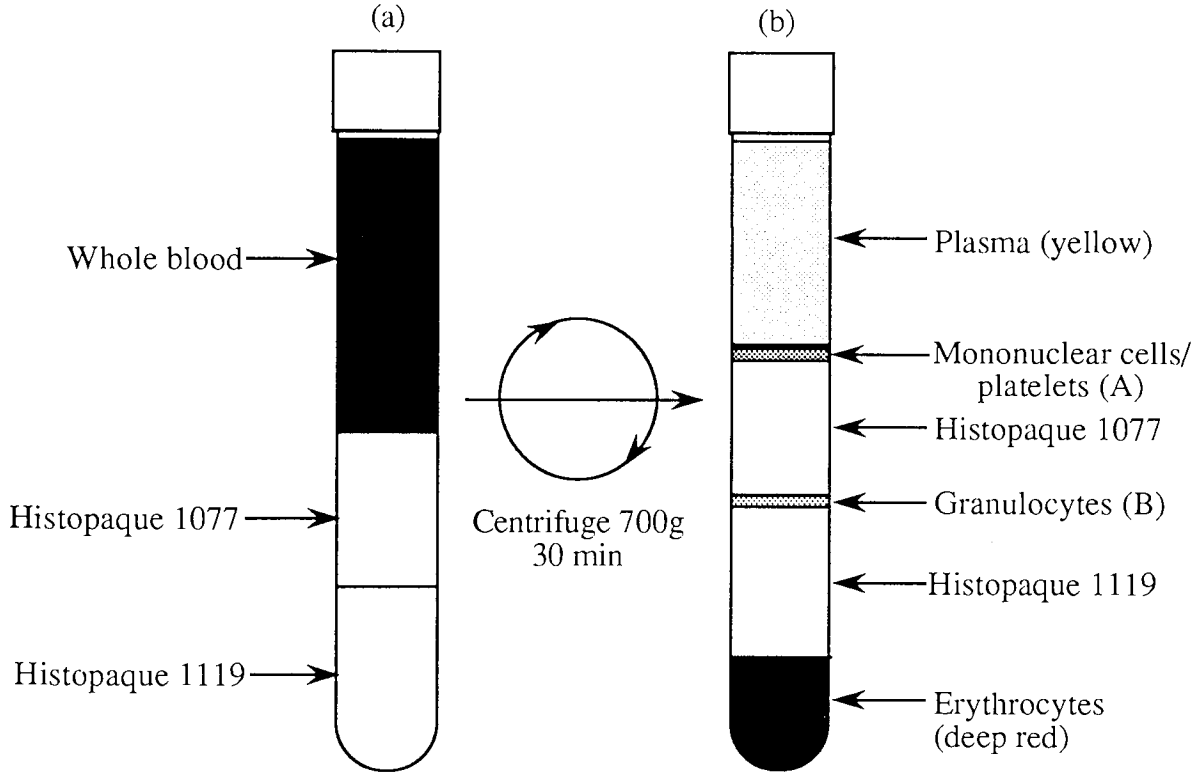


Figure 6. Double Density Gradient Centrifugation of Whole Blood for the Separation of White Blood Cell Fractions.
Anticoagulated whole blood (6ml) was layered onto Histopaque 1119 (3ml) and Histopaque 1077 (3ml) in a 15ml clear plastic centrifuge tube as above (a). Tubes were centrifuged at 700g for 30 min at room temperature. After centrifugation (b), cell layers A and B were collected by aspiration and placed in separate tubes.

3.7 Western Blotting.

Western blotting is a technique in which proteins are separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferred to a nitrocellulose sheet electrophoretically and proteins are detected using specific antibodies.

3.7.1. Detection of PKC α/β in Cells after Exposure to Bistratene A and Phorbol Esters.

A549 and MCF-7 cells were cultured as in section 3.6.1. Cytosolic and particulate fractions were prepared (section 3.5.1. Method A) and their protein concentrations were determined (Bradford, 1976). Cell fractions were mixed 1:1 with sample buffer (section 2.2.5) and the mixtures were immersed in a boiling water bath for 5 mins. Samples (50μl) were loaded onto a 5% stacking and 8% separating gel with dimensions of 16x16x0.075 cm (section 2.2.5.). Proteins were resolved by SDS/PAGE (Laemmli, 1970) at 10°C with a constant current of 25mA per gel. Electrophoretic progress was followed using coloured rainbow molecular weight markers (14.3-200kD). Mouse immunoglobulin was used as a positive control for the blotting process. Separated proteins were transferred to a nitrocellulose sheet after saturation of all components with electrode solution (section 2.2.5.) at a constant current of 0.8mA/cm^2 for 1 h (Towbin et al, 1979) using an LKB Novablot kit with Multiphore II base. Non-specific binding was blocked by immersion of blots at 4°C overnight in Tris-buffered saline with Tween 20 (TBS-T) 0.1% and 5% dried milk (section 2.2.5.). All other procedures were carried out at room temperature on a shaker. The blot was blocked for a further h in TBS-T 0.5% and 5% dried milk, then rinsed twice and washed three times for 5 mins in TBS-T 0.3%. PKC was visualised by firstly incubating the blots for 1 h with a monoclonal antibody to PKC α and β (Amersham), diluted 1 in 500 in TBS-T 0.1% with 1% dried milk. The immunodetection procedure was completed using a blotting detection kit for mouse antibodies (Amersham), which involved incubation for 20 mins with an anti-mouse immunoglobulin G (IgG) biotinylated 2° antibody (1 in 500 dilution in TBS-T 0.1%/1% milk), followed by incubation with a streptavidin-alkaline phosphatase (ALP) conjugate and finally the addition of substrates for ALP. PKC was visualised as a purple band on the nitrocellulose.

3.7.2. Detection of PKC Isozymes.

A series of PKC isozyme-specific antibodies were used to detect and localise isozymes present in A549 and MCF-7 cells. Enhanced chemiluminescence (ECL) was used as a detection method instead of the colour reaction with ALP described above (see section 4.3.4).

Cellular cytosolic, particulate and nuclear fractions were prepared as in section 3.5.2. Samples were prepared in sample buffer (section 3.7.1.). Proteins were resolved on 5% stacking/8% separating gels (section 2.2.5.), using constant current (16mA per gel) on a Bio-Rad Mini Protean II or Hoefer Mighty Small gel apparatus. Each lane was loaded with 30μg protein. After immersing gels in electrode solution for 15 mins, separated proteins were transferred to nitrocellulose using Bio-Rad Mini Trans-Blot equipment, as directed by the manufacturer, at 100 V for 90 min. Non-specific binding was blocked by shaking blots in TBS-T 0.1%/10% milk overnight at 4°C, and gels were exposed to a range of antibodies to PKC isoforms at room temperature for 3 h. All antibodies were diluted in TBS-T 0.1%/1% milk. Monoclonal mouse-derived antibodies to PKC α, β and γ (Seikagaku) and polyclonal rabbit-derived antibodies to PKC α, ε and ζ (Gibco) were incubated with the blot at 1μg/ml whereas antibodies to PKC β, γ and δ (Gibco) were used at 2μg/ml. A monoclonal antibody detecting the catalytic domain of PKC α (TCS) was incubated with blots using a dilution of 0.075μg/ml. Rabbit sera containing antibodies to PKC η (Dr. S. Osada) and ε (Dr. P. Parker) were used at dilutions of 1:1000 and 1:3000 respectively. Occasionally, antibody solutions were reused for subsequent blot detection. The Gibco antibodies were subject to profuse non-specific binding. Attempts to eradicate this problem included increasing the overnight blocking conditions from 5 to 10% milk or 5% bovine serum albumin (BSA), and raising Tween 20 concentrations in TBS-T from 0.1 to 0.3 or 0.5% (w/v). These changes were unsuccessful, but using 500mM rather than 137mM NaCl in the TBS-T 0.3% washes (section 2.2.5.) between each stage of detection did improve the clarity of results. The detection of PKC ε was refractory to these technique refinements, probably due to the low level of isozyme present. However the specific PKC

ε band could be identified with certainty with the use of the peptide from which the antibody was raised. All Gibco antibodies were supplied with their complementary peptide. This peptide was used to block the band detected for PKC to allow differentiation between specific and non-specific binding. It was diluted using a ratio of 1 part peptide to 2 parts complementary antibody by weight and incubated for 3 h to compare with results using the antibody alone. After washing blots with high salt TBS-T 0.3% as in 3.7.1., the appropriate horseradish peroxidase linked anti-mouse or anti-rabbit IgG 2° antibody (Sigma) was shaken with the blot for 1 h using a 1 in 500 dilution in TBS-T 0.1%/1% milk. The PKC α specific monoclonal antibody (TCS) gave a clearer signal with less non-specific binding using a 2° antibody obtained from Amersham. Blots were then washed again with high salt TBS-T 0.3% and detected using reagents for ECL and Hyperfilm ECL (Amersham). Blots were exposed to film for 5 sec-5 min, and were then developed with Kodak GBX developer and fixer. Blots were occasionally stripped by incubating at 50°C for 30 min in stripping buffer (section 2.2.5.), washed as previously, blocked overnight and reprobed the following day.

3.8 Two-Dimensional Gel Electrophoresis (Phosphoprotein Maps).

2-D gel electrophoresis is a technique which allows excellent resolution of proteins. Initial separation is by isoelectric focusing (IEF) and is dependent on protein charge, followed by polyacrylamide gel separation via molecular weight according to the method of O'Farrell, 1975. Proteins were detected by silver staining after 2D gel electrophoresis or cells were incubated with $^{32}P_i$ and spots were detected by autoradiography, yielding phosphoprotein maps.

Plates (90mm diameter) were seeded with $7x10^5$ A549 cells and were incubated for 24 h. Medium was then replaced with 0.1mCi/ml $^{32}P_i$ in 4ml medium and this was incubated with the cells until equilibrium phosphate uptake at 6 h. Agents were added to the cells for a further 2 h. Dishes were then placed on ice, medium was aspirated and monolayers were washed with 2x10ml ice-cold PBS. Cells were scraped from plates into 1ml PBS, pelleted

at 13,500rpm for 2 mins and the pellet was solubilised in urea sample buffer (USB) (section 2.2.6.) for 30 mins. Samples were frozen overnight or used immediately. After a 2 min spin at 13,500rpm to pellet any insoluble cell debris, samples were assessed for protein concentrations using a method modified from that of Bradford by mixing 650µl Bio-Rad protein dye reagent concentrate, 2.035ml distilled H_2O, 7.5µl 0.1M HCl and 6µl sample. Absorbtion at 595 nm was read and compared to a calibration curve constructed using BSA as a standard. Tube gels for IEF were prepared (section 2.2.6.). Glass gel tubes (1.5mm diameter) were routinely soaked in chromic acid, washed with water, rinsed in methanol and dried in an oven to remove all traces of acrylamide. The tubes were filled with gel mix by a water displacement method to ensure uniform tube gel length. Glass tubes were tied together with their ends aligned, placed in a small beaker and tube gel mix was added. The beaker was then carefully filled with water to overlay the tube gel mix and the whole assembly was plunged into a 16cm depth of water for 1 h. Excess gel was removed from the end of the tubes with a scalpel and gels could be stored in clingfilm at 4°C for up to a week. Second dimension polyacrylamide non-linear gradient gels were prepared using the apparatus in Fig.7 (see section 2.2.6. for gel formulae). The 12% gel was poured into the mixing chamber and the 8% gel into the reservoir chamber. Gel mix was pumped between glass plates using a peristaltic pump at a rate of 5ml/min. The apparatus was flushed through with water after each gel and the gel was overlaid with water and allowed to set before adding a stacking gel (0.5cm depth, section 2.2.6.). Gradient gels using mixes of a third 12%, two thirds 8% acrylamide allowed the separation and visualisation of proteins with molecular weights within the range of 15-100kDa. Tube gels were placed in a Bio-Rad model 175 tube cell with the upper chamber containing 500ml NaOH (20mM) and the lower chamber 3 litres H_3PO_4 (10mM). Tube gels were pre-focused using a Bio-Rad 1000/500 power supply for 1 h at 200V to ensure pH gradient formation. Samples (50µg protein) were then loaded onto the tube gels and focused at 700V for 15 h, then at 1kV for 1 h. Gels were extruded from their glass tubes using water pressure with a syringe onto the top of the 2nd dimension stacking gel. Equilibration buffer (70µl)(section 2.2.6.) was loaded along the gel length and all air pockets between stacking gel and tube gel were removed. Remaining space between the gel plates was filled with running buffer and molecular weight markers (^{14}C-labelled) were

loaded onto the stacking gel in the gap between the tube gel and 2nd dimension gel spacer. Gels were subjected to PAGE using a Bio-Rad Protean II gel system at 40mA per gel, cooling with tap water. Gels were dried between two cellulose acetate sheets using a Hoefer Easy Breeze Gel Drier on low setting overnight, then were placed in Sigma lightning autoradiography cassettes with Hyperfilm MP for 6 days. Film was developed using Kodak GBX developer and fixer. Samples were made using cells treated with the same drugs but without $^{32}P_i$. Gels obtained using these samples were silver stained with a Bio-Rad silver staining kit using the manufacturers protocol but with 6x20 min washes after the oxidant step. The pH gradient of the tube gels after IEF was assessed by (i) using IEF markers (Sigma) (ii) cutting focused tube gels into 10 equal portions, sonicating the gel segments in 3ml distilled H_2O and measuring pH at room temperature.

Autoradiographic visualisation of phosphoproteins produced a blackened background haze emanating from the top basic corner of the second dimension gel, which obscured protein spots. This was due to large molecular weight phosphorylated molecules being unable to enter gels successfully, causing blackening. Possible causative factors included the incorporation of $^{32}P_i$ into nucleic acids, aggregation of protein, nucleic acids and Ampholines, or incomplete solubilisation of protein. Incubation of samples with nucleases or complete removal of nucleic acids by selective precipitation or extraction may be used to overcome these problems (Dunbar, 1988, Hames *et al*, 1990), but each of these methods inevitably causes some loss of protein or the appearance of artifactual spots. To retain all proteins but enhance their separation, the volume of USB was increased to enhance solubilisation of cell proteins, which partially resolved the blackening. Phosphoproteins of low molecular weight were also obscured at the bottom of the second dimension slab gel by the dye front which appeared as a black line several mm in width due to the presence of small phosphorylated entities and free [γ-^{32}P]ATP/$^{32}P_i$. Protein spots were visualised more clearly between the two areas with black backgrounds by altering the second dimension slab gel recipe. A variety of uniform and gradient gels were assessed using 8-12% acrylamide. The recipe used ($^1/_3$ 12%, $^2/_3$ 8% acrylamide gradient gel) provided gels with the clearest display of proteins within the required mw range of 15-100kDa.

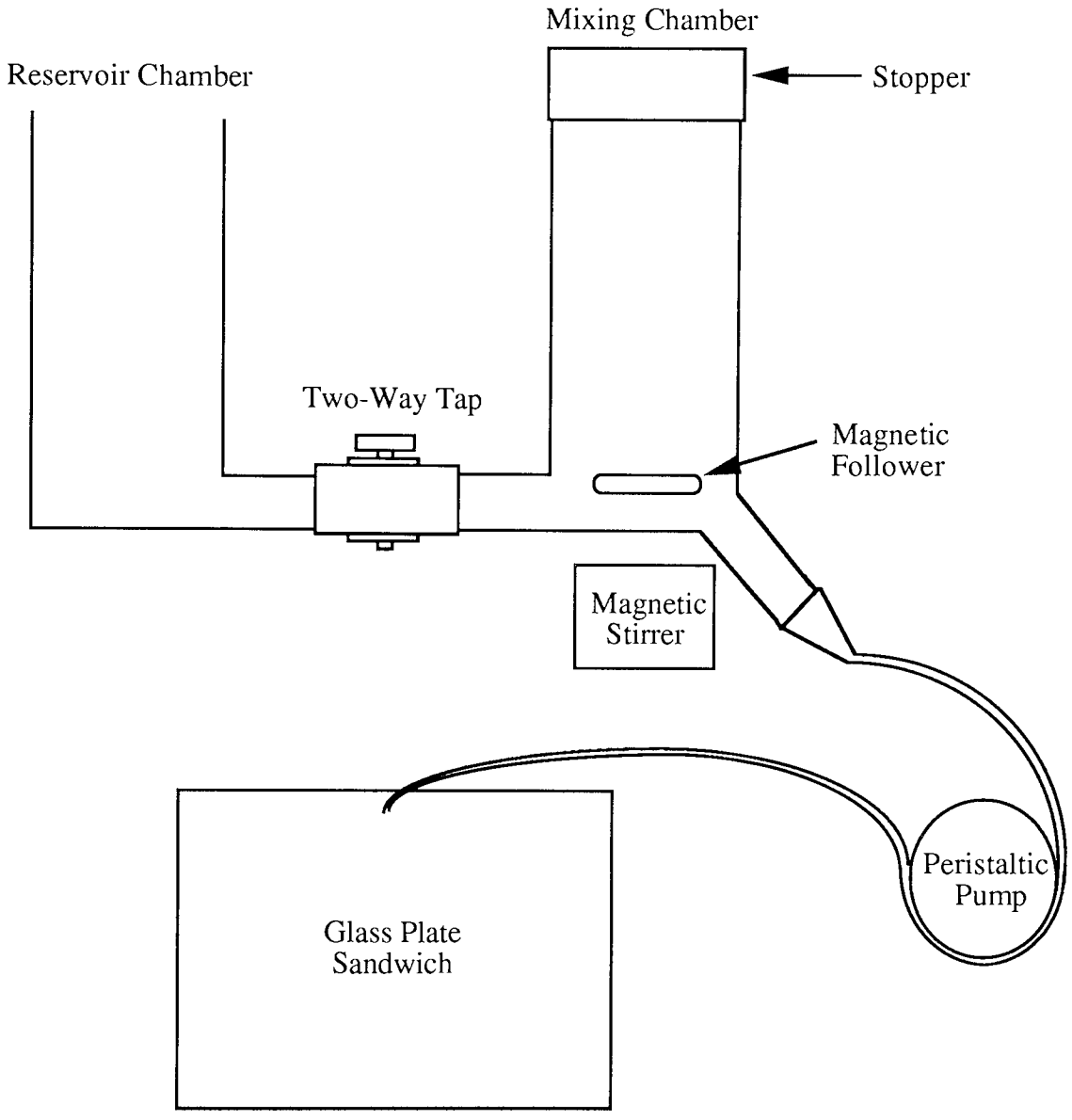


Figure 7. Apparatus for the Formation of Gradient Gels.
Exponentially decreasing gradient polyacrylamide gels (concave gradient gels) were produced using the above apparatus. The gradient maker was supported 2cm above a magnetic stirrer using a clamp stand. The two gradient maker chambers were interconnected via a two-way tap. Tygon tubing led from the mixing chamber through a peristaltic pump to the centre of glass gel plates and was held in place with tape throughout gel formation. The gel mix containing glycerol (to ensure gradient stability and prevent cracking of gels during drying) and the highest % acrylamide was poured into the mixing chamber with the tap closed. The stopper was inserted and excess pressure was reduced to atmospheric using a needle as a valve, which was then removed. The low % acrylamide mix was poured into the reservoir chamber, the pump and magnetic stirrer turned on and the tap was opened to commence gel formation. The gel mix volume remained constant in the mixing chamber as gel from the reservoir chamber gradually moved in by air displacement. Linear gradients can be produced with the same equipment minus the stopper, using equal volumes of solutions in the two chambers, as in section 3.10.1.

3.9. Protein Kinase C Assays.

PKC assays were carried out in the presence of the cofactors PS in the form of micelles (Amersham kit) or vesicles (alternative assay), Ca^{2+} and Mg^{2+}. PKC activity was evaluated by determining the amount of γ-phosphate of [γ-^{32}P]ATP incorporated into a peptide substrate (Amersham) or histone type IIIS. Activity was assessed in the presence of TPA, bryostatins 1 and 2 and bistratene A.

3.9.1. Activation of PKC in Cell Cytosol by Bistratene A or TPA.

The cytosolic fraction of cells (section 3.5.1. Method A) was used as a source of PKC to assess activation by bistratene A. A549 cell cytosol was diluted with H8 buffer 20-40x and MCF-7 cytosol was diluted 10x in order to dilutionally inactivate endogenous factors such as PKC inhibitors, ATPases or lipases which may interfere with the assay (Table 2). The kit component containing 8 mol % micelles and TPA (38.4μM) was modified to exclude TPA. Micelles were made as in section 3.6.1. Initially, 63.5μl PS (10.4mg/ml) was dried under a stream of N_2, then was vortexed and incubated with 200μl Triton X100 3% (w/v). Distilled water (800μl) and 1ml Tris-HCl 100mM pH 7.5 were added, giving assay component concentrations of 420μM PS, 0.3% (w/v) Triton X100 and 50mM Tris HCl pH 7.5. Drug was then added to give final assay mix concentrations of bistratene A 10nM-10μM or TPA 3.2nM-3.2μM. Results were expressed as a % of maximal PKC activity per mg cytosolic protein, which was the activity obtained by adding TPA 3.2μM to reconstituted micelles.

3.9.2. Alternative PKC Assay.

Solutions C, P, T and B were prepared as in section 2.2.8. and stored on ice. Aliquots of alternate hydroxylapatite fractions (10μl) were pipetted into the curved-base wells of a flexible plastic microplate. Solution C, P, T or B (50μl) was added to each hydroxylapatite fraction and the plates were stored on ice until 2 mins before commencement of the reaction, then were transferred to a water bath at 30°C. To start the reaction, 40μl of a solution containing 5.68μCi/ml [γ-^{32}P]ATP and 25μM cold ATP was added to each well

with an 8-channel pipette. Final assay concentrations were 20mM Tris-HCl pH 7.5, 200µg/ml histone IIIS, 5mM magnesium acetate, 300µM calcium chloride (except in reaction mixtures containing solution P where it was replaced with 500µM EGTA), 8µg/ml PS in vesicles (absent from solution P reaction mixtures), 10µM cold ATP and 2.27µCi/ml [γ-^{32}P]ATP. Additionally, reaction mixtures containing solution T or B contained 50nM TPA or bistratene A respectively. After 10 min at 30°C, the reaction was terminated with 12µl trichloroacetic acid (TCA) (100% (w/v)). Plates were maintained at 4°C for > 10 mins to allow the precipitation of histone, and were then covered with plate-sealing tape and centrifuged at 1000rpm for 10 mins. The reaction mixture supernatant was carefully removed from each well and replaced with 10% (w/v) TCA (200µl). Plates were again centrifuged to pellet acid-precipitable material and the pellet washing process was repeated once more. After removal of the final acid wash, plates were cut into individual microwells and each well was placed in a Packard Pony scintillation vial. Radioactivity was determined without the addition of scintillation fluid by a 1 min Cerenkov count on a Packard Tricarb CA 2000 scintillation counter. Alternatively, after termination of the reaction, 80µl of reaction mix was placed onto 2cm squares of Whatman P81 paper and allowed to soak in for 1 min. Papers were then washed for 3x10 mins in 10% (w/v) TCA (10ml per paper), and were then rinsed in methanol, dried and placed in Packard Pony scintillation vials with 5ml Optiphase Hisafe II scintillant before counting.

3.9.3. Inhibition of PKC by Bistratene A.

Using the Amersham kit, cytosolic PKC (10-40 x dilution-see section 3.9.1) was maximally activated in the presence of 3.2µM TPA. Bistratene A (10nM-1µM) was added to the reaction mix to assess inhibition of maximal activity.

3.10. Separation of PKC Isoforms.

PKC isozymes from A549 and HL-60 cell cytosolic and particulate fractions were separated using DE52 anion exchange chromatography with linear salt gradient followed by separation on hydroxylapatite using a linear phosphate gradient.

3.10.1. Separation of PKC Isoforms on DE52 and Hydroxylapatite Columns.

The cytosolic and particulate fractions from 20 subconfluent 140mm plates of A549 cells or 10^8 HL-60 cells were obtained as in section 3.5.1. All stages thereafter were carried out at 4°C. Cell fractions were loaded onto a 2cm length, 1cm diameter column of DE52 anion exchanger which had been previously washed with 20ml salt free buffer (section 2.2.7.) at 1ml/min using a peristaltic pump. The loaded column was washed with 5ml salt free buffer, then a gradient maker (Fig.7) was connected to the pump and the column outlet to a fraction collector. The gradient maker reservoir was filled with 20.4ml salt buffer and the mixing chamber (with magnetic stirrer) with 20.4ml salt free buffer. Simultaneously the valve between the two chambers was opened, the pump was switched on (1ml/min) and the fraction collector was started (1min/fraction). A PKC assay was carried out using the unmodified Amersham kit (section 3.9) on alternate fractions. Active fractions were pooled and loaded onto an 8cmx1cm diameter hydroxylapatite column which had been previously washed with 30ml 20mM phosphate buffer. The loaded column was washed with a further 30ml 20mM phosphate buffer (0.4ml/min) under pressure from the peristaltic pump. The column outlet was then connected to a fraction collector and a gradient maker was attached to the peristaltic pump. The gradient maker mixing chamber was filled with 42ml 20mM phosphate buffer and the reservoir with 42ml 300mM phosphate buffer. Simultaneously, the tap between the two gradient maker chambers was opened, the pump was switched on (0.4ml/min) and the fraction collector was started (2.5min/fraction). Eighty four fractions were collected and the phosphate gradient was assessed using atomic absorbance of potassium for each fraction. Alternate fractions were assayed for PKC activity using the Amersham kit or the alternative PKC assay. The assays were modified to assess activity in the presence of bistratene A instead of TPA (section 3.9.1.) and TPA was also substituted with bryostatins 1 and 2 in the Amersham assay. The ability to phosphorylate the substrate histone type IIIS instead of the peptide substrate using the Amersham assay kit was also examined by replacing the peptide kit component with histone type IIIS 7.2mg/ml in 50mM Tris HCl pH 7.5, giving a final assay concentration of 600µg/ml.

3.10.2. Concentration of Fractions Containing PKC.

Sequences of fractions containing a peak of PKC activity were pooled and proteins were

concentrated by initially using an Amicon concentrator (model 8050) on ice, which forces solutions through membranes under N_2 pressure. Membrane YM30, retaining substances of mw 30kDa and higher, was used to reduce the sample volume to 500µl. Ice-cold distilled H_2O (20ml) was then added to the sample and forced through the concentrator under pressure for removal of K^+ ions by desalting. This process was repeated and the resultant desalted concentrated protein solution (500µl) was then placed in the upper chamber of an Ultrafree CL low binding cellulose membrane filter unit centrifuge tube (Millipore). A Beckman 21 Ultracentrifuge, operating at 5000g for 1h at 4°C, was used to further concentrate the sample down to approximately 200µl. Protein concentrations were measured (Bradford, 1976). The sample was then diluted with glycerol 1: 1 and stored at -20°C or diluted 1 : 1 with sample buffer (section 2.2.5.) and placed in a boiling water bath for 5 mins. Western blotting was used to determine the PKC isozyme of each peak from the hydroxylapatite separation.

3.11. Effect of Bistratene A on Tyrosine Kinase Activity.

Epidermal growth factor (EGF) receptors obtained from A431 cells were used as a source of tyrosine kinase. The inhibitory or activatory effect of bistratene A on enzyme activity was studied by measuring the catalytic transfer of the γ-phosphate of [γ-^{32}P]ATP to the specific substrate poly[GAY].

Medium was aspirated from a confluent flask (175cm^2) of A431 cells and the monolayer was washed with PBS, then scraped into 10ml of ice-cold PBS. The cell suspension was centrifuged at 800g for 5 min at 4°C, then resuspended and incubated on ice for 30 min in 1ml lysis buffer (5mM Tris-HCl, 2mM $MgCl_2$, 2mM EGTA, 200µg/ml soybean trypsin inhibitor, 500µg/ml phenylmethylsulphonyl fluoride (PMSF), pH 7.4). Cells were homogenised using 3-5 strokes of a Potter homogeniser and the lysate was then centrifuged at 5000g for 5 min. The supernatant was taken and centrifuged again at 100,000g for 1 h at 4°C. The pellet from the second spin was resuspended and incubated on ice for 30 min in 1ml solubilisation buffer (20mM Hepes, 20% (w/v) glycerol, 1% (w/v) Triton X100, 0.1% BSA, 0.05% sodium azide, pH 7.4). This semi-purified EGF

receptor tyrosine kinase preparation was stored in aliquots at -70°C until required. Tyrosine kinase activity was evaluated using an assay in the presence of EGF to assess inhibition of tyrosine kinase by bistratene A (1-1000nM) or vehicle (DMSO 1%). Activation of tyrosine kinase by bistratene A was assessed in the absence of EGF using the same assay conditions. (Reproduced with the kind permission of Dr. Ian Dale, Xenova, Slough).

Section 4. Results.

Section 4. Results.

4.1. Effects of TPA and Bryostatin 1 on the Growth of A549 and MCF-7 Cells.

The effect of TPA and bryostatin 1 on the growth of A549 cells has been described (section 1.4.2. and 1.4.4.). Growth inhibition by TPA was confirmed with cells seeded at two different densities with concentrations ranging from 10-1000nM (Fig.8). Total cytostasis was induced for a minimum of 24 h with this range of concentrations (measuring [^{3}H]thy incorporation-Dr. I. Dale, unpublished observation), and antiproliferative effects were progressively diminished at concentrations below 10nM (Gescher and Reed, 1985). In the continued presence of TPA, cells recommenced growth after 1-6 days. This resumption of growth occurred in a paradoxical fashion with the lowest concentration (10nM) exerting the most prolonged period of cytostasis. The antiproliferative effect of TPA was also dependent upon initial cell seeding density. When cells were seeded at $2x10^4$ in 35mm diameter wells ($2x10^3$ per cm^2), 10nM TPA prevented growth for 6 days (Fig.8a), whereas when cells were seeded at $1.25x10^5$ ($1.3x10^4$ per cm^2), cells began to grow after only 4 days (Fig.8b). Cells grown continuously in TPA for a minimum of 3 months were less sensitive to the growth inhibitory effects of this compound than naive cells, as described previously by Gescher and Reed, 1985 (Fig.8c). Morphological changes induced by TPA were consistent with effects reported by Dale and Gescher, 1989a. Cells rounded up within 2 h of incubation with the agent (Fig.11d); similar effects were seen with bryostatins 1 and 2, but were less prolonged. The effect of bryostatins on A549 cell growth has been described in detail elsewhere (section 1.4.4.).

MCF-7 cells responded somewhat differently to these agents. Reports in the literature suggest that TPA is either a cytostatic, cytotoxic or maturation-inducing agent, depending on laboratory (section 1.4.2). The results presented here indicate that TPA is able to induce all of these effects; as with A549 cells, the intensity of effect is inversely related to dose above 10nM (Fig.9) and is dependent upon initial cell seeding density. The

antiproliferative effect of TPA was less pronounced when cells were seeded at higher density (Fig.9a *vs* Fig.9b). As reported elsewhere, TPA had a prolonged antiproliferative effect on MCF-7 cells. After exposure to the agent for more than 6 days, TPA (10-100nM) was cytotoxic, as shown by a reduction in cell number. However, at 1μM, cells were able to overcome these effects; at low seeding density cells remained in cytostasis, and at high density, cells continued to proliferate after 6 days. Conversely, bryostatin 1 had little effect on the growth of MCF-7 cells. When cells were seeded at $2x10^4$ (Fig.10a) or $1.25x10^5$ (results not shown) in 35mm dishes, growth inhibition was insignificant if evaluated by cell counting. However, when incorporation of [^{3}H]thy was assessed in cells seeded at $1.25x10^5$ per 35mm well, a significant ($p \leq 0.001$) inhibition of thymidine uptake of 1 h duration was detected, which was of similar intensity (25%) for bryostatin 1 concentrations from 10nM to 1μM (Fig.10b).

MCF-7 cells are typical polygonal epithelial cells (Fig.12a). Their morphology was radically changed after treatment with TPA (10nM-1μM). After 24 h, cells rounded up and developed cytoplasmic processes (Fig.12di). After 3 days, cells enlarged, spread out and numerous perinuclear and cytoplasmic granules appeared, suggesting cellular maturation (Fig.12dii and diii), as described in detail by Valette *et al,* 1987, and Osborne *et al* , 1981. However, after treatment with bryostatin 1(10nM-1μM), morphology of the cells was unchanged from vehicle-treated control cells.

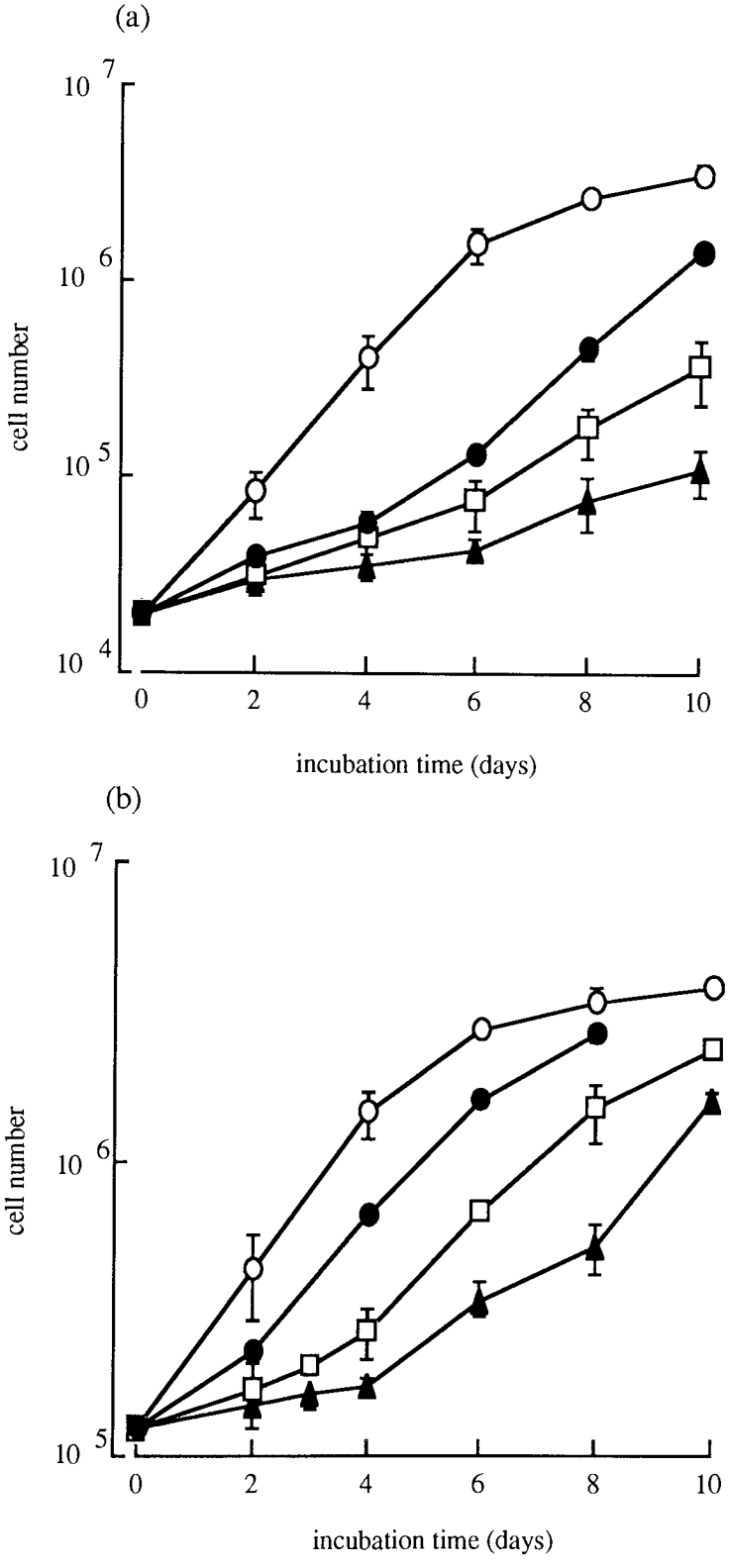
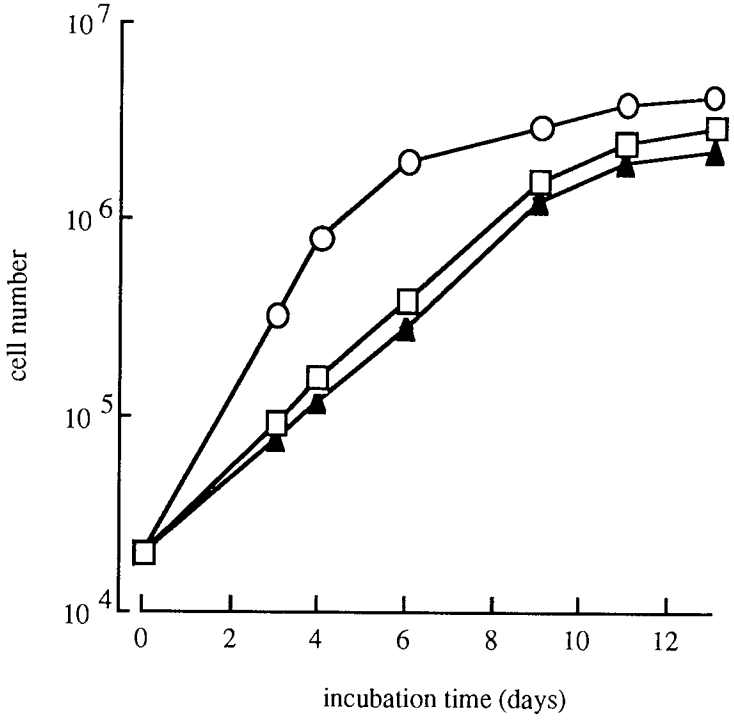


Figure 8. Effect of TPA on the Growth of A549 Cells.
Cells were seeded at (a,c) $2x10^4$ and (b) $1.25x10^5$ in 35mm diameter dishes. Cells were grown in the presence of 10nM (triangles), 100nM (squares) and 1μM (closed circles) TPA. Fig. 8c overleaf shows the growth of cells which were passaged in the presence of TPA 10nM(triangles) or 100nM (squares) for > 3 months, then were exposed to the agent at the same concentration for 13 days. Vehicle-treated control cells are represented by open circles. Results are the mean ± SD of 3 experiments, each conducted in triplicate.

(c)



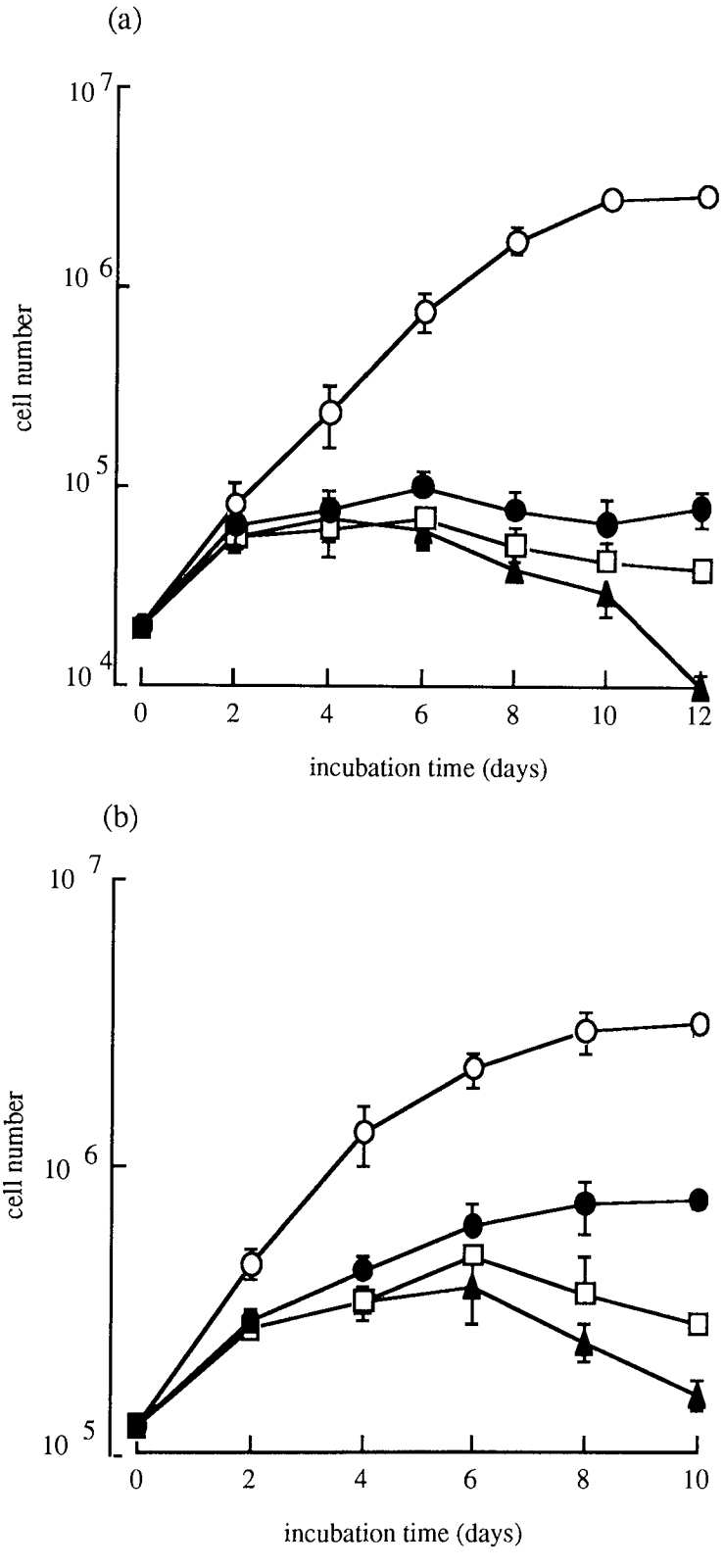


Figure 9. Effect of TPA on the Growth of MCF-7 Cells.
Cells were seeded at (a) $2x10^4$ and (b)$1.25x10^5$ in 35mm diameter wells. Cells were grown in the presence of 10nM (triangles), 100nM (squares), or 1μM (closed circles) TPA for 10-12 days.Vehicle-treated control cells are shown by open circles. Results are the mean ± SD of 3 experiments, each conducted in triplicate.

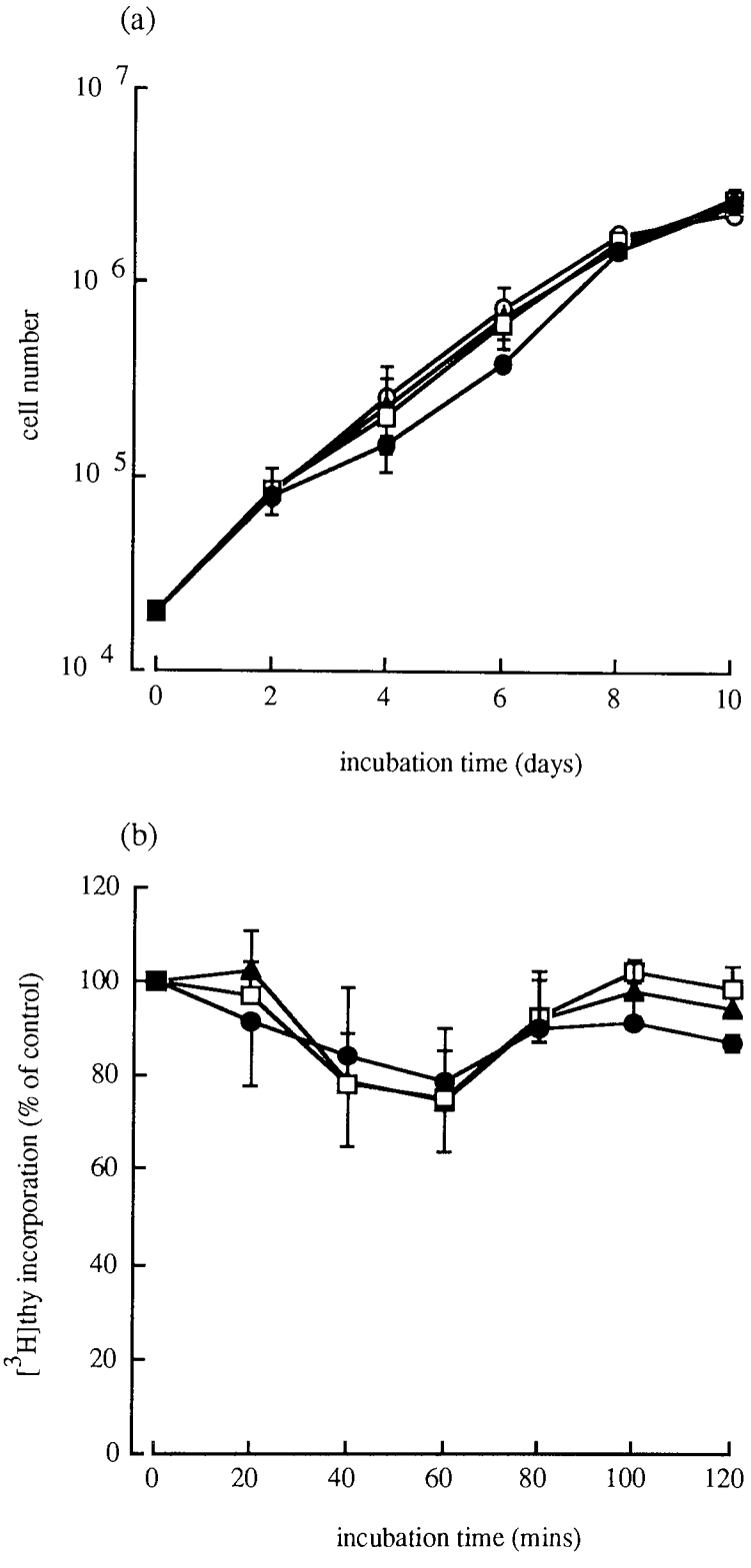
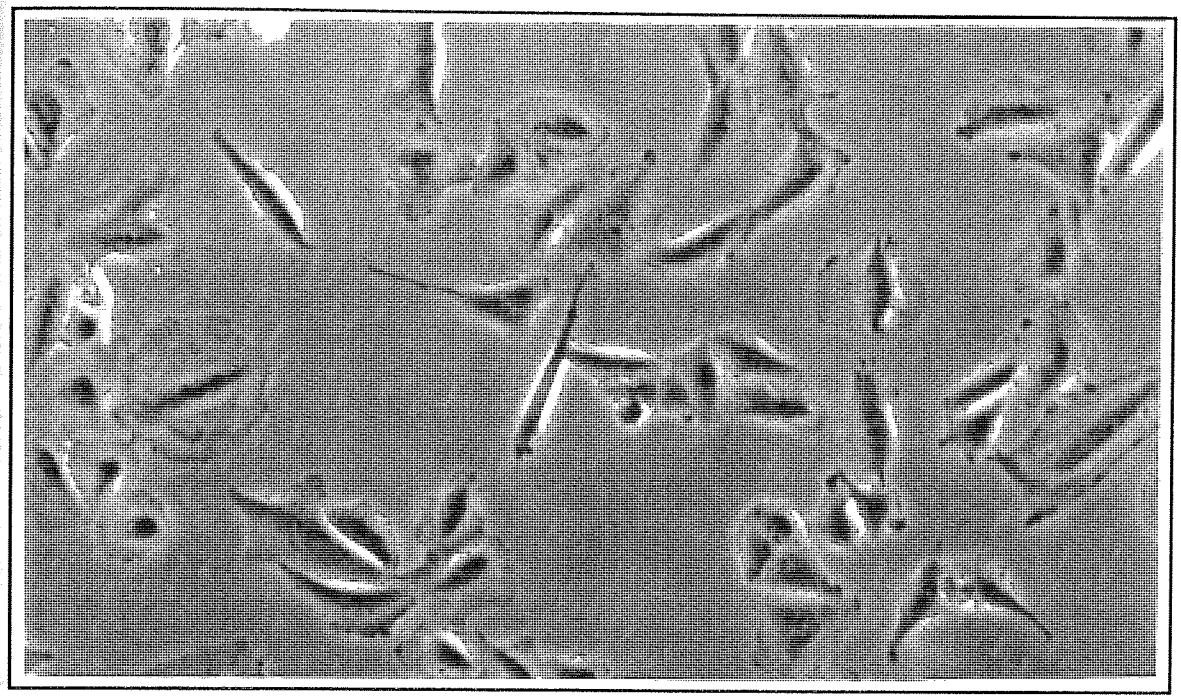


Figure 10. Effect of Bryostatin 1 on the Growth of MCF-7 Cells.
Cells were seeded at (a) $2x10^4$ (b) $1.25x10^5$ in 35mm diameter wells and were allowed to attach to plastic for 24 h before the addition of 10nM (triangles), 100nM (squares) or 1μM (closed circles) bryostatin 1. Vehicle-treated control cells are depicted in (a) by open circles. Cell numbers were counted on alternate days in (a) and [^{3}H]thy uptake was assessed in (b) over a 2 h time period. Results are the mean of 2 (a) or 3 (b) experiments, each conducted in triplicate.

(ai)



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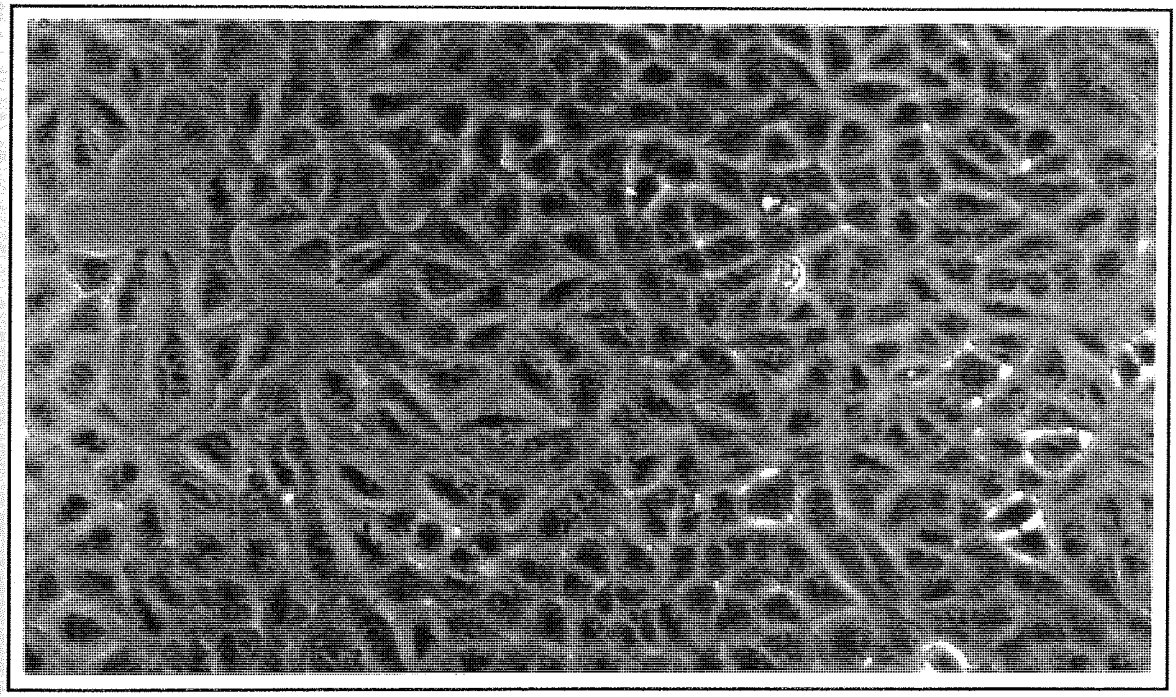
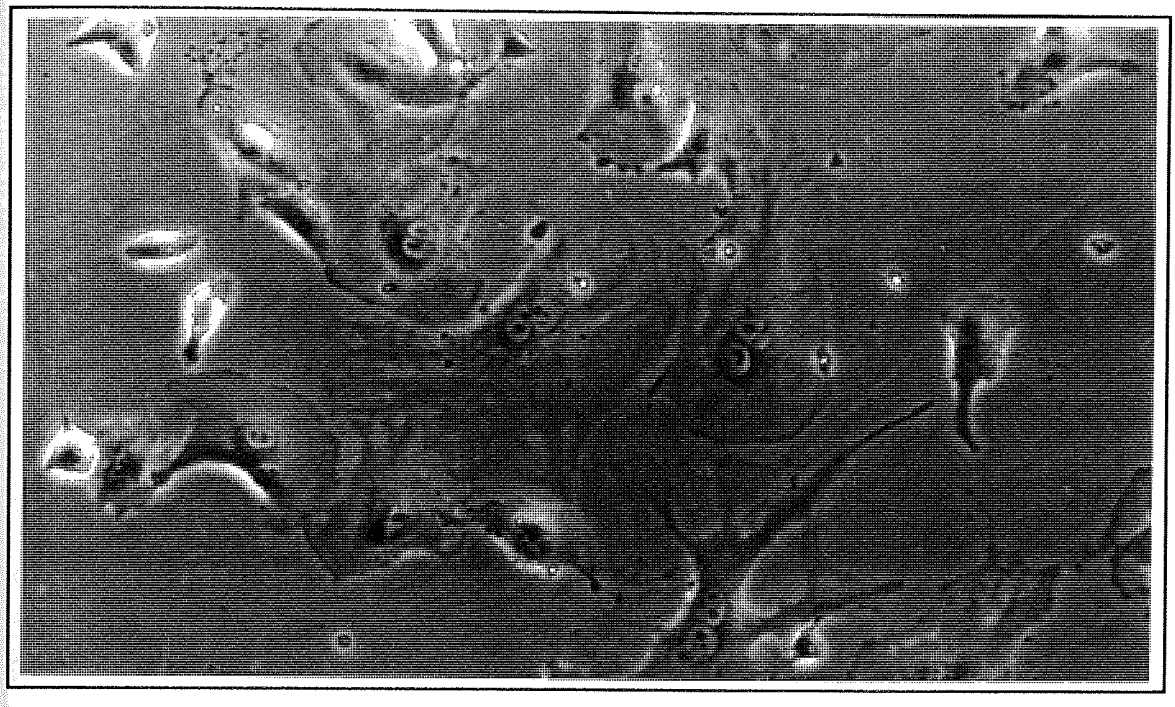


Figure 11. Morphology of A549 Cells after Treatment with Bistratene A and TPA. A549 cells were seeded at $2x10^4$ per 35mm diameter well and (a) DMSO vehicle, (b)10nM or (c)100nM bistratene A and (d) 10nM TPA was added 4 h later. Cells were photographed after 48 h, or 8 days (aii and bii only). Photographs were taken with an Olympus OM2 SP camera on an Olympus CK2 microscope. Magnification is 243x actual size, as measured using a stage micrometer.

(bi)



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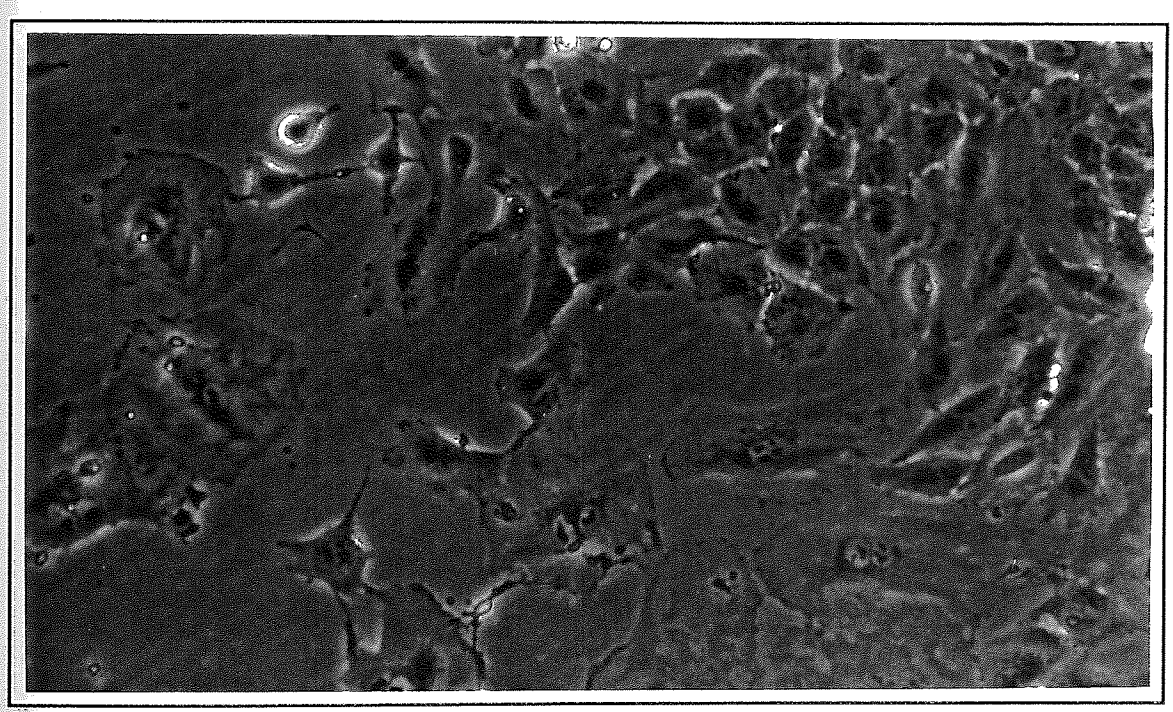
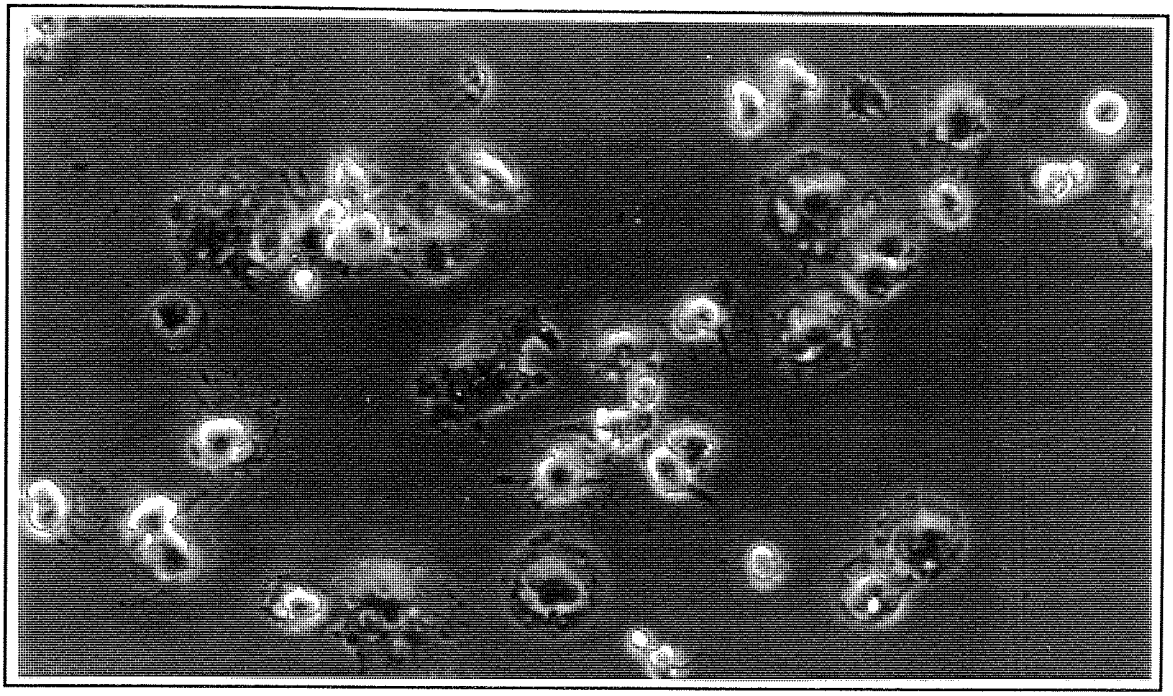


(c)



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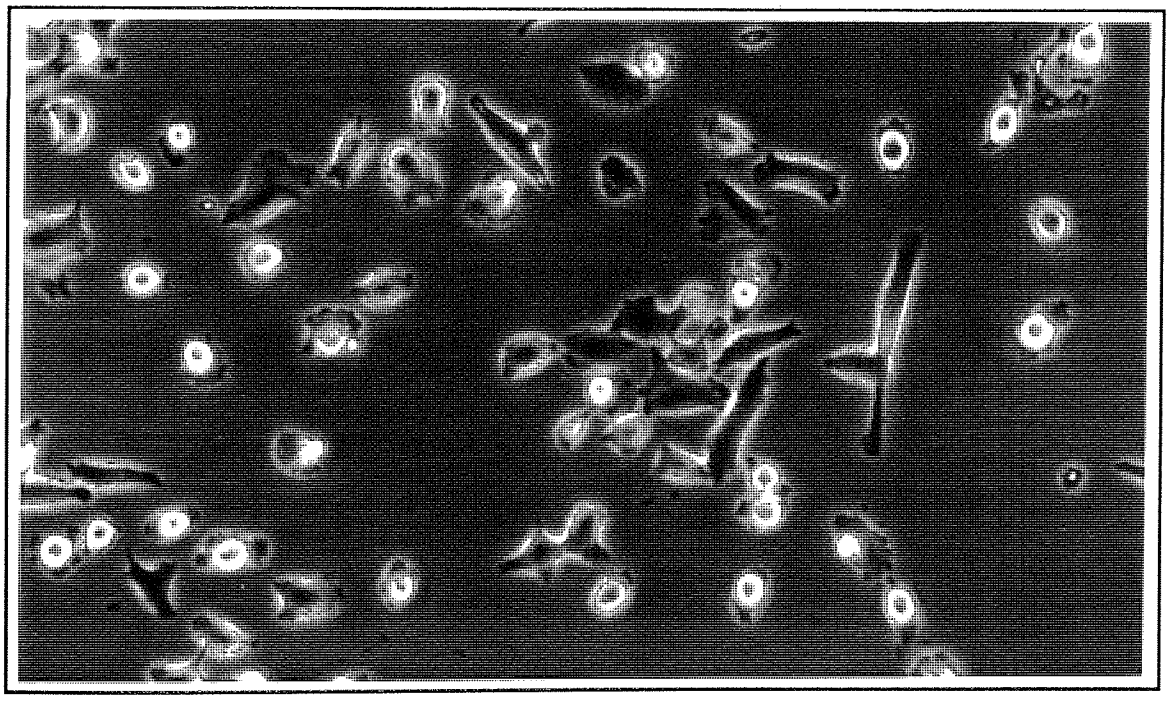

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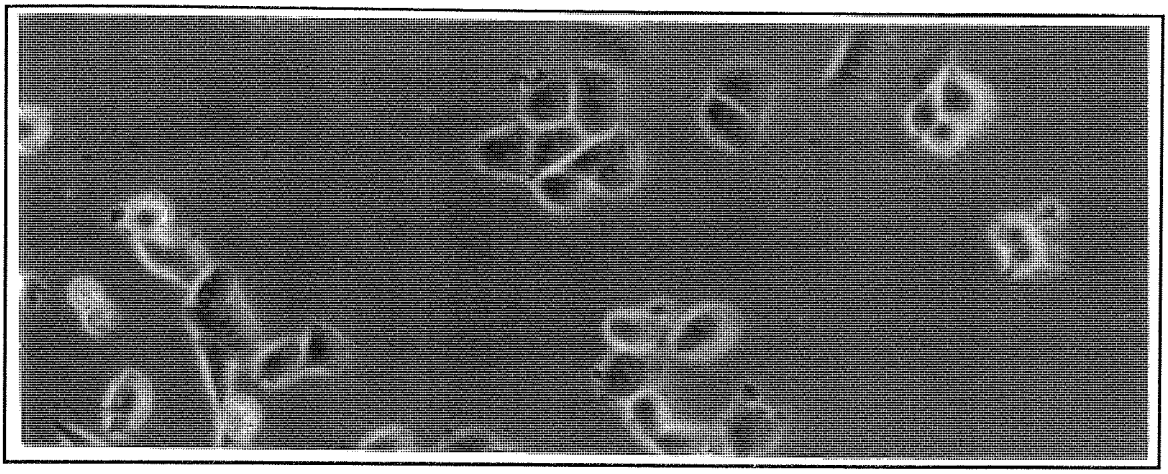

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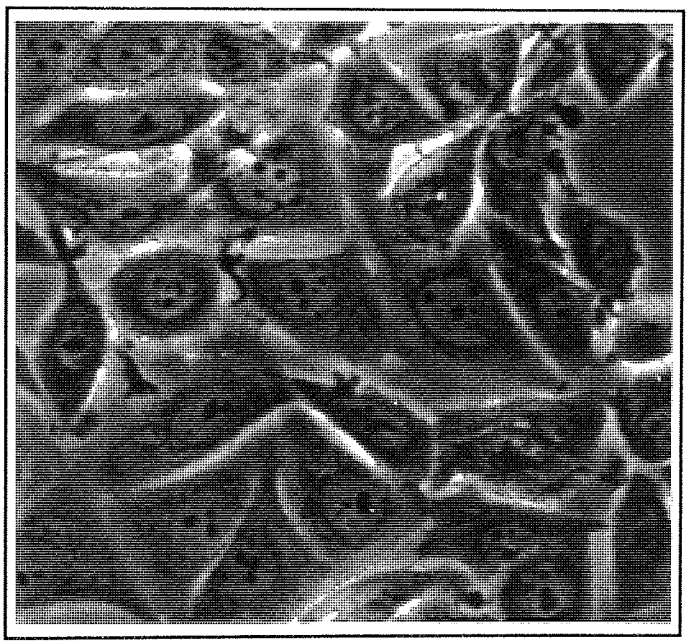
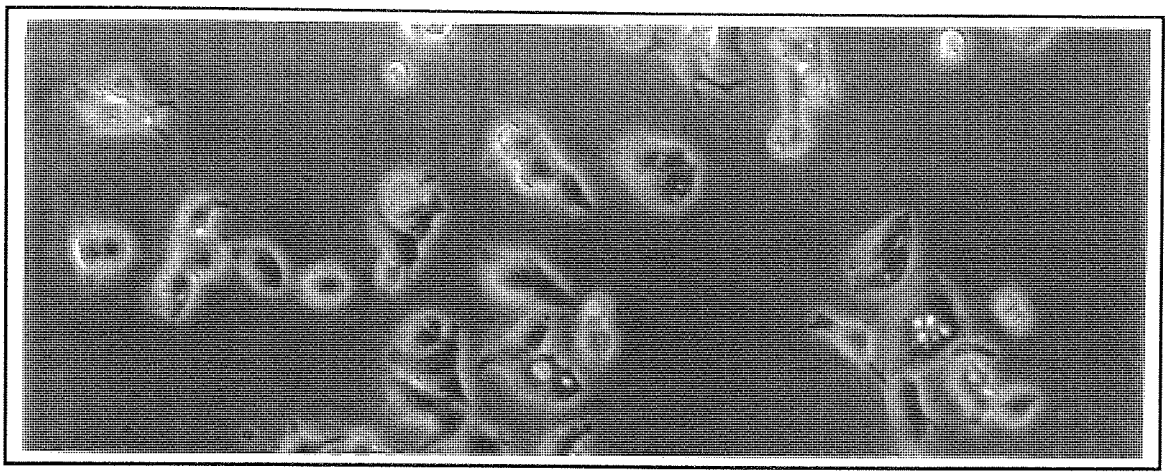


Figure 12. Morphology of MCF-7 Cells after Treatment with Bistratene A or TPA. Cells were seeded at $2x10^4$ in 35mm diameter dishes and DMSO vehicle (a), 10nM (b) or 100nM (c) bistratene A, and 10nM TPA (d) was added after 4 h. Phase contrast micrographs of cells were taken after 24 h (ai and di only), or 96 h using an Olympus OM2 SP camera on an Olympus CK2 microscope. Magnification is 485x (aii and diii only) or 243x actual size, as measured using a stage micrometer.

(b)



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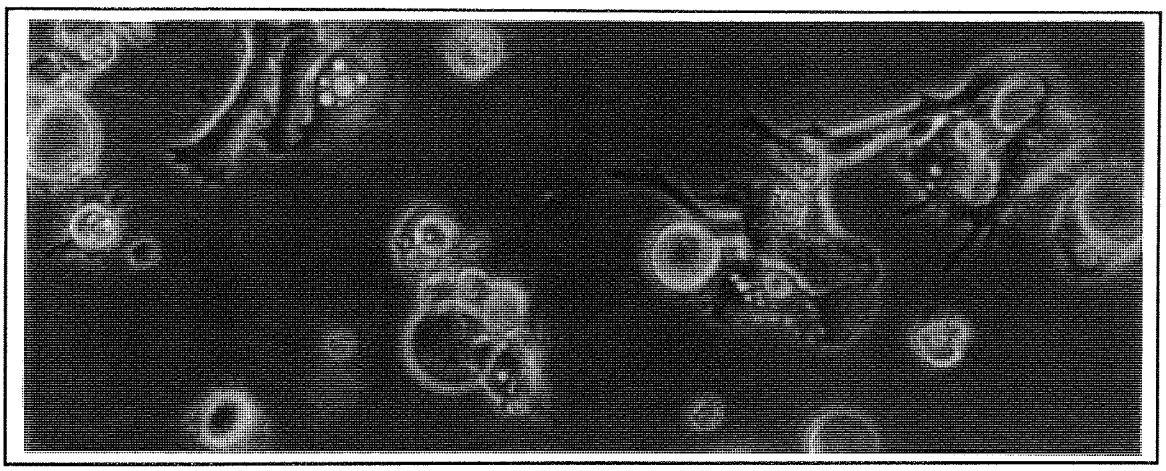

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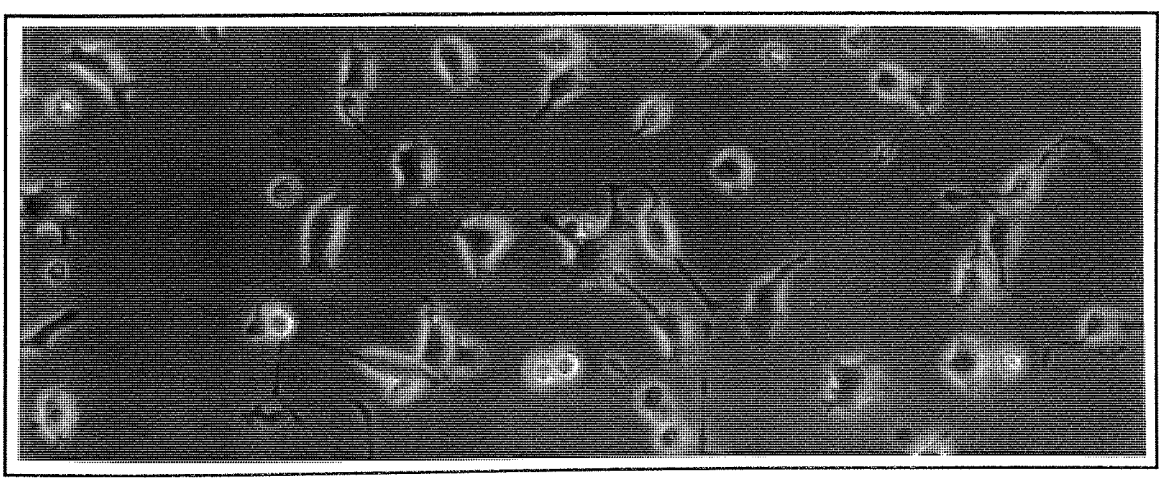

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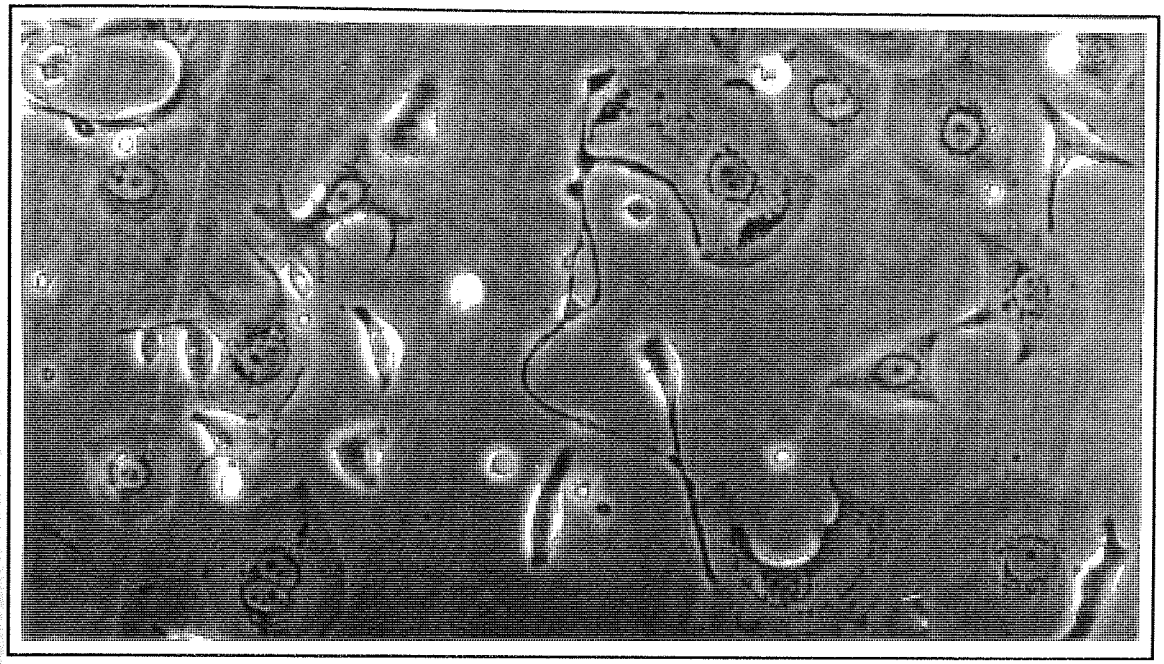

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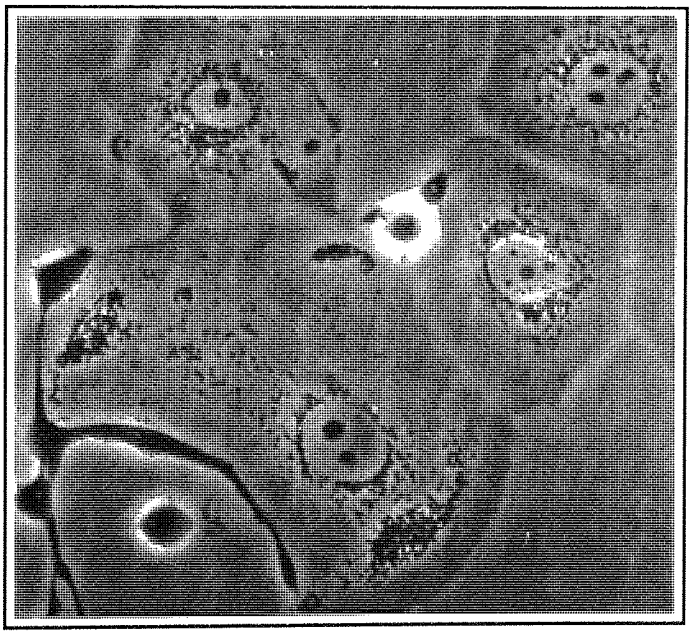


4.2. Effects of Bistratene A on HL-60, A549 and MCF-7 Cells.

In these experiments, the hypothesis was tested that bistratene A mimics the effects of phorbol esters and bryostatins in HL-60, A549 and MCF-7 cells.

4.2.1. Effect on Cellular Growth.

4.2.1.1. Effect of Bistratene A on HL-60 Promyelocytic Leukaemia Cells.

Bistratene A has been shown to inhibit growth and induce partial differentiation along the monocyte/macrophage pathway in HL-60 cells (Watters *et al*, 1990). On repeating this work in our laboratory, a similar pattern of effects was observed. Incubation with bistratene A (10nM) for 48 h (section 3.1.3) caused a 49.7 ± 11.0% (n=8) reduction in cell replication compared to untreated cells and 10% of cells were enlarged. At 100nM, there was complete cytostasis with growth reduced to 5.3 ± 3.6% (n=7) of controls. There were also marked changes in cell morphology. Cells were larger than controls with great variation in size. Some were ovoid with prominent pseudopodia. By day 10, cells had granular cytoplasm but changes were similar to those seen at 48 h in all other respects. Morphologial changes were akin to those observed by Watters at 100nM, but higher concentrations of bistratene A were required by their group to elicit a growth inhibitory response (IC_{50} of 424nM).

4.2.1.2. Effect of Bistratene A on Growth and Morphology of A549 and MCF-7 Cells.

A549 and MCF-7 cells were incubated with bistratene A for up to 12 days. In both cell lines, proliferation was not affected by 1nM bistratene A (Figs.13a and b). In MCF-7 cells the compound caused prolonged cytostasis at 10nM, and a reduction in cell number at 100nM, implying cytotoxicity (Fig.13b). In A549 cells, 10nM bistratene A caused complete cytostasis for 6 to 8 days (Fig.13a). After this time, cell growth resumed and eventually confluency was reached. At 100nM, cell numbers gradually reduced, suggesting cytotoxicity as with MCF-7 cells.

When bistratene A (10nM) was removed from the culture medium of A549 cells on day 6, the cells recovered more quickly than in its presence, but with 100nM, drug removal did

not reverse effects, indicating lethality at this concentration (Fig.14). Bistratene A induced the same effects at 15nM as at 10nM, but was found to cause a reduction in cell number and irreversible growth inhibition at $\geq$ 20nM in A549 cells using the growth conditions described above.

A549 cells were seeded at $2x10^4$ per 35mm dish and were incubated in the presence of PDBu (50nM) for up to 12 days. Like TPA and bistratene A, PDBu caused inhibition of growth in A549 cells (Fig.15). After 6 days, PDBu was removed and the cells were washed 4 times with medium before the addition of medium supplemented with bistratene A (15nM), PDBu (50nM) together with bistratene A (15nM), or vehicle alone. Removal of PDBu on day 6 allowed cells to proliferate exponentially like untreated control cells. The substitution of PDBu for bistratene A did not induce the cytostatic response of naive cells to bistratene A as shown in Fig.13a, but caused growth inhibition greater than that of PDBu. The two agents together induced cytotoxicity with a progressive reduction in cell number.

When A549 cells grown in the presence of TPA are trypsinised and reseeded, full sensitivity to TPA is restored. However, after continuous growth in the presence of TPA for several months, the cells lose their sensitivity towards growth inhibitory effects (Gescher and Reed, 1985, Fig.8c). The hypothesis was tested that A549 cells would respond similarly to bistratene A. Cells seeded at $5x10^4$ in T25 flasks ($2x10^3$ per cm^2), which had resumed growth after 15 days of exposure to 10nM bistratene A, were trypsinised and reseeded at the same density. Cells were found to possess full sensitivity towards the agent with characteristic morphological changes and cytostasis up to day 6, after which growth resumed as observed previously. Cells were seeded at the same density and were passaged continuously in the presence of bistratene A (15nM) for > 9 weeks. After long term exposure, confluency was reached more quickly than for naive cells treated with 15nM bistratene A seeded at the same density, and cells exhibited partial resistance to the agent (Fig.16).

The IC_{50} for bistratene A in A549 and MCF-7 cells was found to be very similar when the

cells are seeded at $2x10^4$ per 35mm well. IC_{50} values of 2.3 ± 0.1nM (n=9) and 2.9 ± 0.4nM (n=9) were obtained for A549 and MCF-7 cells, respectively (Fig.17). However, the IC_{50} in A549 cells seeded at a lower density ($5x10^3$ per 35mm well) was found to be significantly reduced (Student's unpaired t-test $p<0.001$) to 1.0 ± 0.1nM (n=12). When cells were seeded at $2x10^5$ in 35mm diameter wells, 10nM bistratene A had little effect on cell growth and morphology, suggesting density-dependent growth inhibitory properties of this compound.

In order to investigate the density dependency further and to assess the effects of bistratene A on sparsely seeded cells, clonogenic assays were performed. Bistratene A up to 0.1nM had no effect on clonal growth. Above this concentration, the CFE of A549 cells was progressively compromised with increasing bistratene A concentration (Fig.18a). Colonies were unable to grow with 5nM bistratene A. Cells seeded at 10^2 were more sensitive to the growth inhibitory effects of bistratene A than those seeded at higher densities, again illustrating the relationship between the potency of this compound and initial seeding density. Inhibition of colony growth could be partially reversed by incubation for a further 10 days at all seeding densities (Fig.18b). At 1nM bistratene A, there was a significant reversal of effect on CFE with all seeding densities (Student's paired t-test, $p=0.005$). This result suggests the development of resistant clones after prolonged treatment, as observed previously in 12-day growth studies. However, colonies were not able to overcome the effects of 5nM bistratene A, even after incubation for 20 days. The agent is probably cytotoxic at this concentration when applied to cells seeded at such low density.

A549 cells underwent striking morphology changes on exposure to bistratene A (Fig.11b and c). When seeded at $2x10^4$ in 35mm diameter wells, the agent (10nM) induced the flattening and spreading out of cells after 48 h. Cells were predominantly binucleate but occasionally up to six nuclei were observed within a single cell . This is in agreement with the findings of Roussakis *et al*, 1991, using a non-small cell lung cancer line. Upon recommencement of growth at day 8, morphology of cells reverted to normal (Fig.11bii). Cells at the same density treated with 100nM bistratene A reduced in size and became

rounded. Portions of cellular material adhering to the culture vessel surface were scattered radially from the cell of origin. Some cells appeared to fuse together in small groups (Fig.11c). At 100nM, cells were strongly adherent to plastic culture wells, requiring 5x trypsin for detachment from culture vessels. These changes were dissimilar to those observed with TPA, where at 1nM-1μM concentrations, TPA induced cells to become rounded and protrude from the culture vessel surface (Fig.11d). Little change in the morphology of MCF-7 cells was observed in the presence of 10nM bistratene A, except for an increase in vacoule formation (Fig.12b). At 100nM, MCF-7 cells exhibited signs of toxicity, with gross vacuolation, lysis and loss of cells from the monolayer (Fig.12c). Again, morphological changes were dissimilar to those induced by TPA, but bryostatins do not alter the morphology of MCF-7 cells (section 4.1).

4.2.1.3. Inhibition of Incorporation of [^{3}H]thy by Bistratene A.

This assay measures the uptake of [^{3}H]thy into cells and reflects their level of DNA synthetic activity. The time course of effect of bistratene A on cell growth was followed by monitoring DNA synthesis inhibition for 72 h after a single application of agent. Bistratene A reduced [^{3}H]thy incorporation within 1 h (Fig.19). At a concentration of 15nM, A549 cells were able to recover and proliferate after an initial 18 h period of growth retardation. At higher concentrations, inhibition reached a trough at 18 h and fell below 20% of control at 72 h. This point could not be measured using 15nM as cells had reached confluency and hence underwent contact inhibition. After 24 h, there was a decrease in [^{3}H]thy incorporation of 24.4 ± 12.1% at 15nM (n=9), 62.5 ± 5.0% at 50nM (n=9) and 87.9 ± 0.5% at 200nM (n=3).

4.2.1.4. Effect of Bistratene A on A549 Cell Growth with Changes in Frequency of Medium Replenishment.

The experiments described above show that the ability of A549 cells to overcome the antiproliferative effects of bistratene A is dependent on drug concentration and initial cell seeding density. Incorporation of [^{3}H]thy was reduced for only 18 h at 15nM but was irreversible at higher concentrations. The reversibility could be due to a number of factors,

including metabolism or chemical breakdown of bistratene A and its consequent loss, or possibly secretion of a factor or production of an enzyme by cells which is able to overcome the growth inhibition. In order to test these hypotheses, A549 cells seeded at $2x10^4$ in 35mm diameter wells were incubated with bistratene A (10nM) for 12 days as described previously. Agent and medium were replenished either every 24 h, or at 3 day intervals. Vehicle-treated control cells were also incubated using this regime. Vehicle-treated cells grown in medium which was changed every 3rd day were unable to reach the same density as those which underwent daily medium changes (Fig.20). Cells treated with bistratene A (10nM) with medium changed every 3 days gave results very similar to alternate day changes, undergoing cytostasis for 6 days followed by a renewed ability to proliferate (Fig.13a). Bistratene A was found to be profoundly and irreversibly cytostatic for up to 12 days with daily medium changes. This implies that bistratene A is being lost from the medium by metabolism or chemical degradation, or a factor which overcomes growth inhibition is being secreted and is removed by daily medium changes.

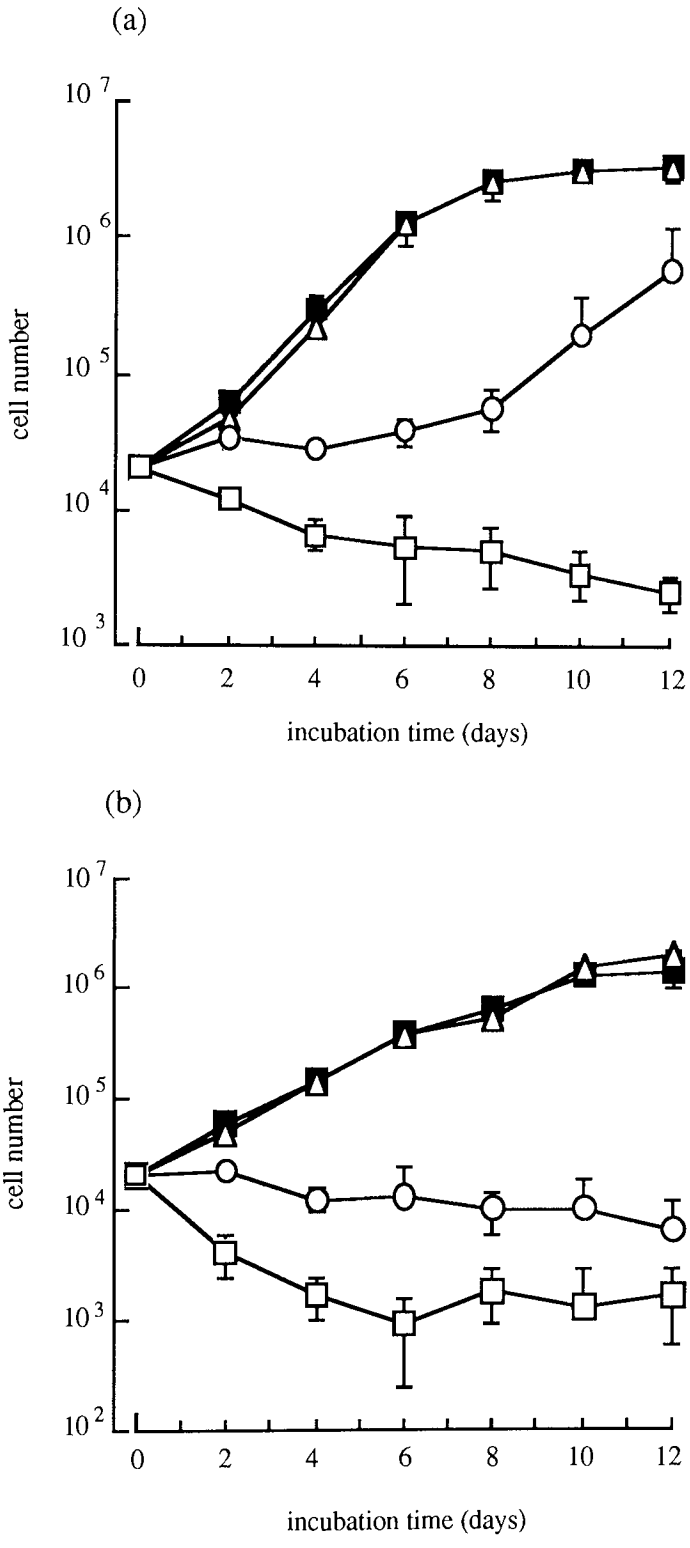


Figure 13. Effect of Bistratene A on the Growth of A549 and MCF-7 Cells.
A549 (a) and MCF-7 (b) cells were seeded at $2x10^4$ in 35mm diameter wells and were treated with 1nM (triangles), 10nM (circles), and 100nM (open squares) bistratene A. The growth of control cells is shown by closed squares. Values are the mean ± SD of at least 3 different experiments, each conducted in triplicate.

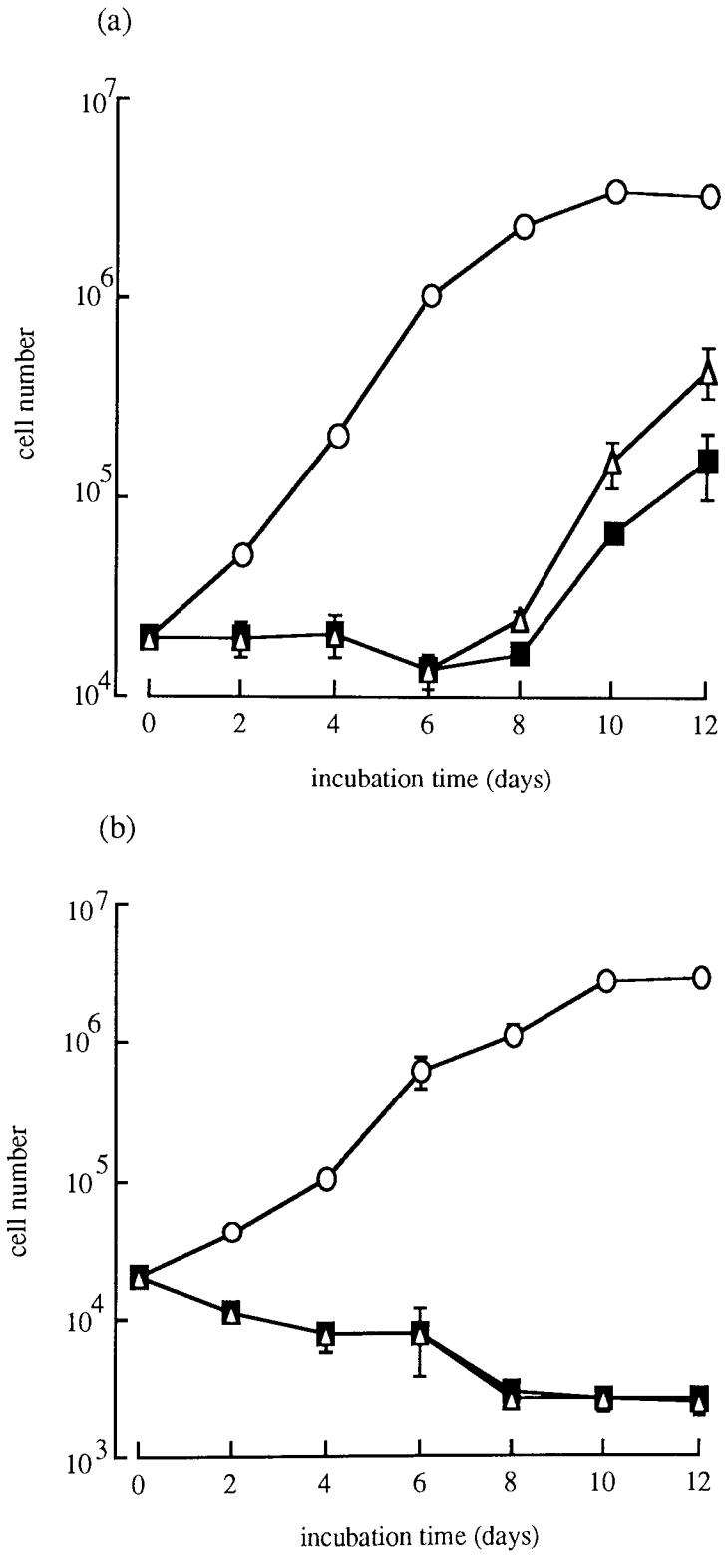


Figure 14. Ability of A549 Cells to Recover after Removal of Bistratene A on Day 6 . Cells were grown in the presence of DMSO vehicle alone (open circles) or in the presence of 10nM (a) or 100nM (b) bistratene A for 12 days (closed squares) or for 6 days, then in the presence of vehicle alone for a further 6 days (open triangles). Results are the mean ± SD of one experiment conducted in triplicate, representative of three.

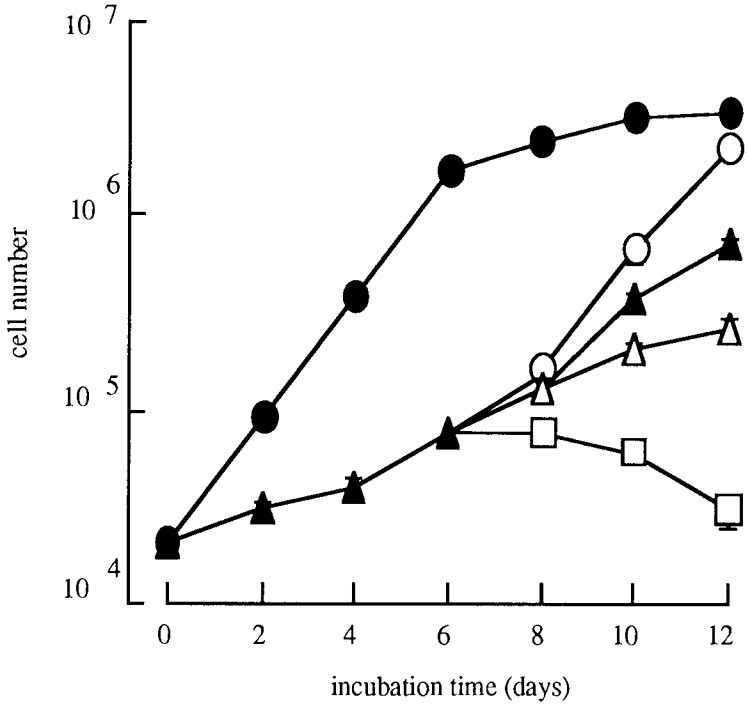


Figure 15. Growth of A549 Cells in the Presence of PDBu and its Replacement on Day 6 with Bistratene A .
Cells were seeded at $2x10^4$ in 35mm diameter wells and 50nM PDBu (closed triangles) or DMSO vehicle (closed circles) were added after 4 h. After 6 days of growth in the presence of PDBu, cells were washed 4x in medium and incubated for a further 6 days in the presence of (i) DMSO vehicle (open circles), (ii) PDBu 50nM (closed triangles), (iii) bistratene A 15nM (open triangles) and (iv) bistratene A 15nM and PDBu 50nM (open squares). Results are the mean ± SD of one experiment conducted in triplicate.

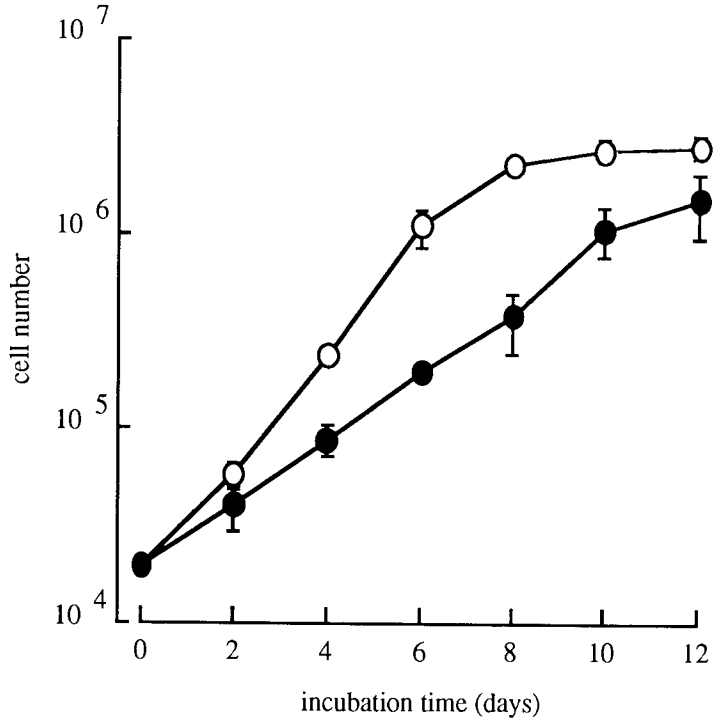


Figure 16. Development of Resistance to Bistratene A by A549 Cells.
Cells were routinely grown in the presence of 15nM bistratene A for > 3 months. Cells were then seeded at $2x10^4$ in 35mm diameter dishes and treated as previously with bistratene A 15nM (closed circles). Open circles denote vehicle-treated naive A549 cells. Results are the mean ± SD of 2 experiments, each conducted in triplicate.

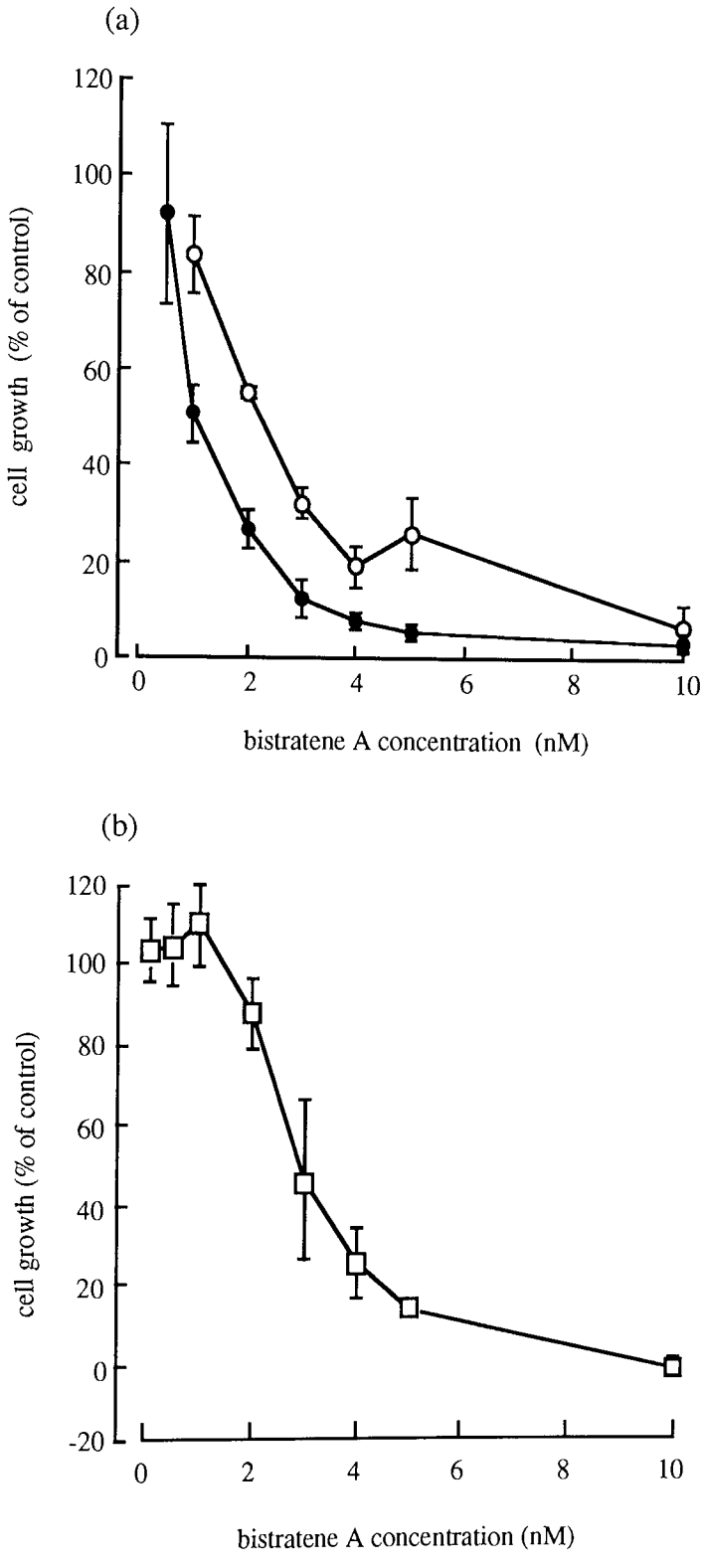


Figure 17. Effect of Different Concentrations of Bistratene A on the Growth of (a) A549 and (b) MCF-7 Cells and Determination of IC_{50}.
Cells were counted 5 days after seeding at $2x10^4$ ((a) open circles, (b) squares) or $5x10^3$ ((a) closed circles) per 35mm diameter well. Values are the mean ± SD of 3 or 4 experiments, each conducted in triplicate.

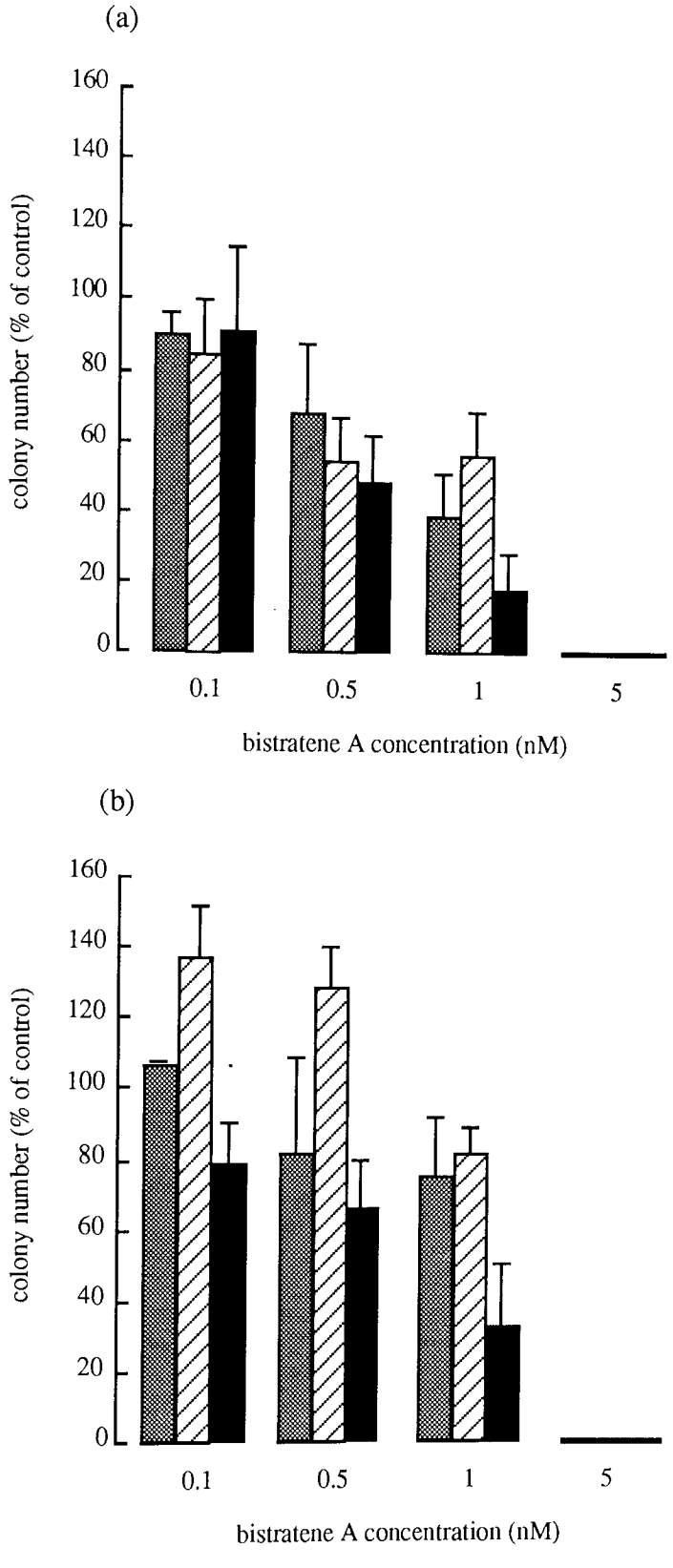


Figure 18. Effect of Bistratene A on the Colony Forming Efficiency (CFE) of A549 Cell Clones.
A549 cells were seeded at 10^3 (grey bars), $3x10^2$ (hatched bars) and 10^2 (black bars) in 35mm diameter wells. Colonies were counted 10 days (a) or 20 days (b) after seeding. Results are expressed as a % of clonogenic growth of untreated controls. Values are the mean ± SD of 2-4 experiments, using 6 wells for each concentration.

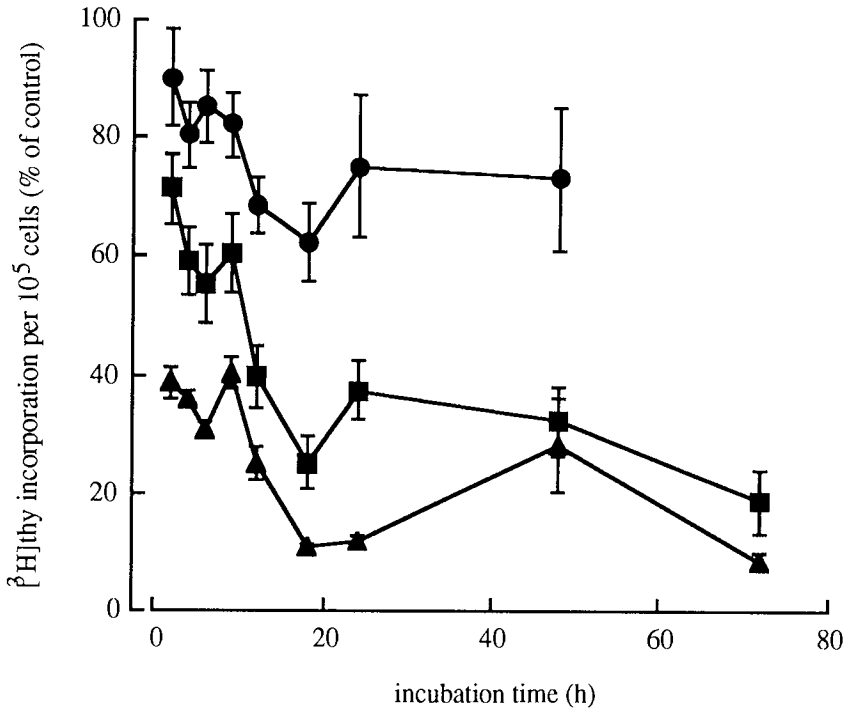


Figure 19. Time Course of Inhibition of Incorporation of [^{3}H]thy into A549 Cells after Treatment with Bistratene A.
The effect of 15 (circles), 50 (squares) and 200nM (triangles) bistratene A on [^{3}H]thy incorporation was assessed at the indicated time points. Values are the mean ± SD of 3 experiments, each conducted in triplicate for 15 and 50nM concentrations, and 1 experiment conducted in triplicate for 200nM.

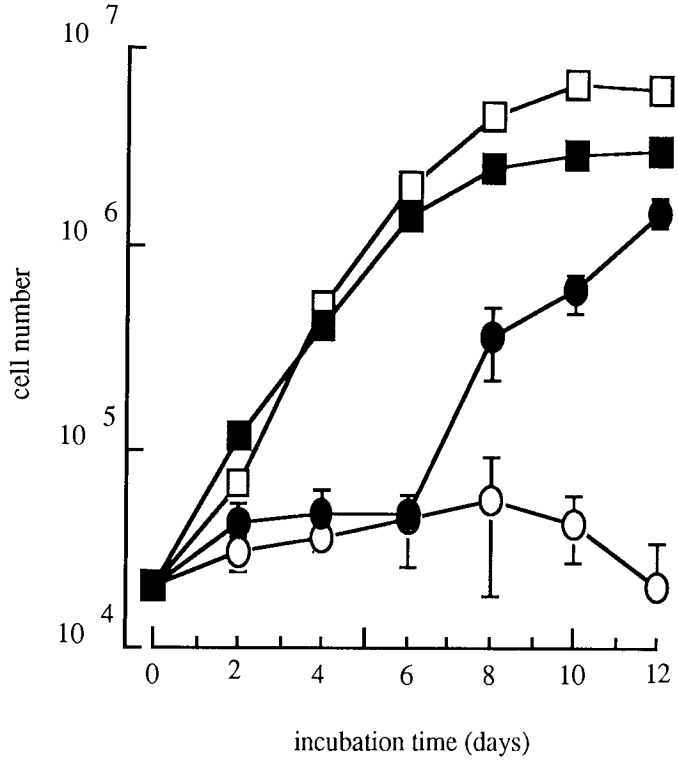


Figure 20. Differential Effects of Bistratene A on A549 Cell Growth with Changes in Frequency of Medium Replenishment.
Medium and bistratene A (10nM) were replenished daily (open circles and squares) or every 3 days (closed circles and squares). Squares indicate vehicle-treated control cells and circles indicate bistratene A-treated cells. Values are the mean ± SD of one experiment conducted in triplicate, representative of two.

4.2.2. Stability of Bistratene A.

To date there is little information on the chemical stability of bistratene A. The molecule does not easily lend itself to analysis as it does not absorb ultraviolet light (200-700nm-results not shown) and the search for suitable conditions for HPLC analysis has been unsuccessful (Watters, personal communication). Therefore stability studies were carried out using a bioassay. Bistratene A was incubated under conditions used for cell culture, or subjected to repeated freezing and thawing, then was incubated with A549 cells and [^{3}H]thy uptake was determined as a measure of the amount of agent remaining. This technique does not determine the actual amount of bistratene A as bioactive breakdown products may be generated, but will demonstrate loss of biological activity .

4.2.2.1. Stability of Bistratene A in Cell Culture Medium at 37°C.

When incubated in conditions routinely used for cell culture at 37°C, the growth inhibitory potency of bistratene A was reduced by 50% after 2.8 ± 0.4 h (Fig.21a). This loss of effectiveness was biphasic, with initial rapid removal from the system followed by a slower phase, which suggests accumulation of a less cytostatic product or multiple steps in the degradation process. This decomposition, possibly by hydrolysis and ring-opening or degradation by esterases or amidases present in serum (see Fig.4a for structure) is obviously a major factor governing the effectiveness and longevity of action of this agent in cell culture.

4.2.2.2. Stability of Bistratene A to Repeated Freezing and Thawing.

Stock solutions of bistratene A were routinely stored at -70°C. Solutions (in DMSO) used frequently were stored at -20°C for a few days in aliquots which could be re-used up to 5 times. The hypothesis was tested that degradation due to freeze-thawing may contribute to the reversal of growth inhibition seen during bistratene A treatment. It was found that freezing and thawing solutions did not cause instability and degradation of bistratene A (Fig.21b).

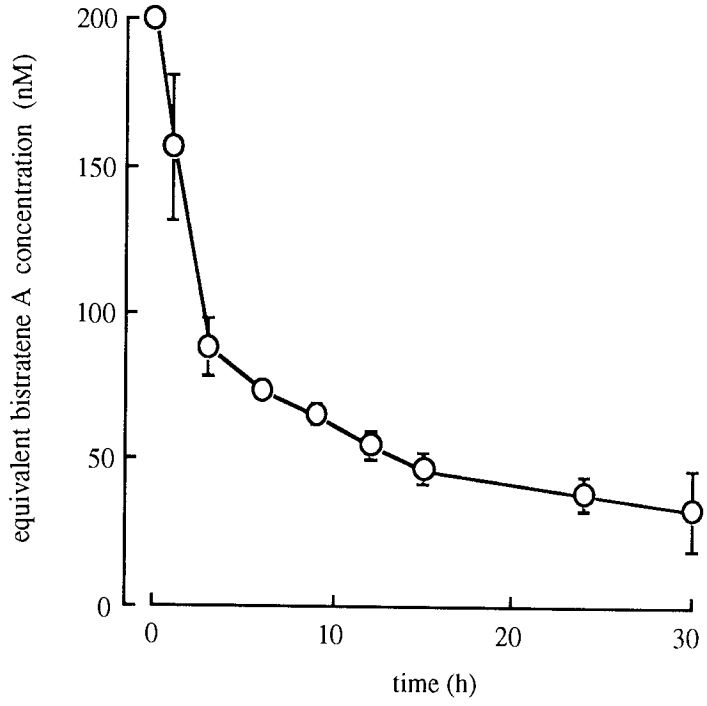


Figure 21a. Stability of Bistratene A in Medium at 37°C.
Values are the mean ± SD of 3 or 4 experiments, each conducted in duplicate.

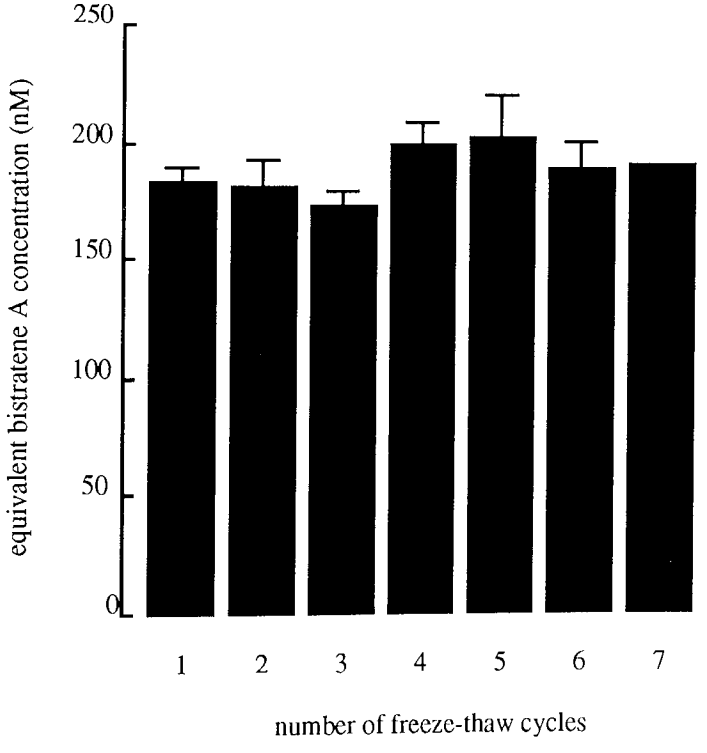


Figure 21b. Stability of Bistratene A to Freeze-Thaw Cycles
Values are representative of 2 experiments, each conducted in duplicate (mean ± SD).

4.2.3. Cytotoxicity of Bistratene A.

Bistratene A rapidly decomposed in conditions routinely used for cell culture, yet was still able to exert profound antiproliferative effects on cells. The LDH and MTT assays were used to determine more precisely the degree of cyotoxicity involved with this inhibition of cell growth after a 24 h incubation with a single application of agent. In both assays, cytotoxicity was not detected at 10nM and at concentrations less than 10nM, cell death was significantly less than that in untreated control cells (Student's unpaired t-test $p<0.025$). This phenomenon of reduced cell death/ enhanced proliferation by established toxic agents at very low doses has been reported by other workers (Vichi *et al*, 1989, Gummer *et al*, 1991).

Using the LDH assay on A549 cells, cytotoxicity increased in a dose dependent manner beyond 10nM concentrations, but appeared to plateau at 0.5-1μM with LDH release values of 33.1-35% of maximal respectively. Cytotoxicity at 50nM was 16.9± 1.9% (n=9) (Fig. 22a).

Cytotoxicity as measured by the MTT assay increased in a dose dependent manner at concentrations above 10nM. At 1μM, absorbance was only 18.8 ± 10.5% of control (n=32), which means a decrease in rate of MTT reduction by 81.2%. There was a decrease in formazan production of 32.5 ± 11.6% using 50nM bistratene A (n=32). An LC_{50} of 198nM was determined from this assay (Fig.22b).

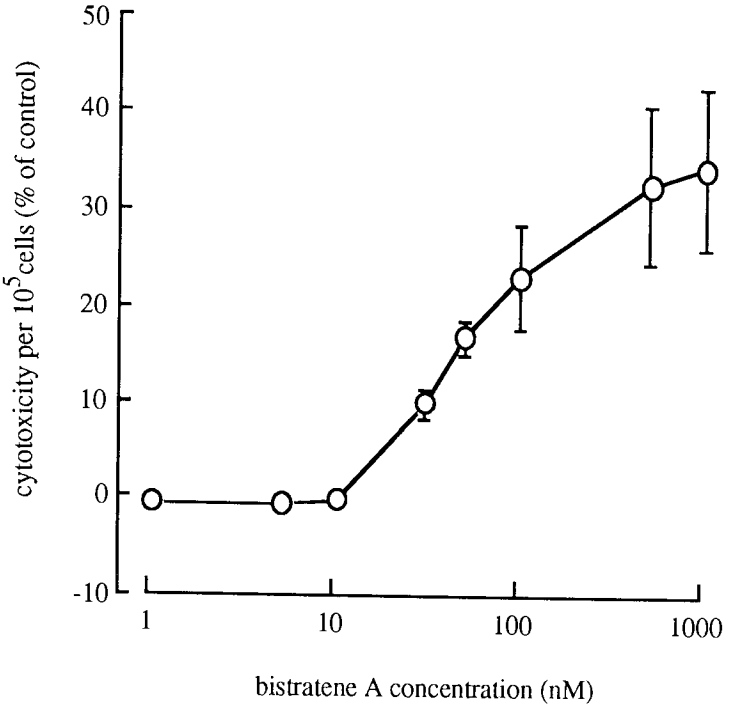


Figure 22a. LDH Assay for Cytotoxicity.
Cytotoxicity of bistratene A in A549 cells was measured using the amount of LDH released into medium after a 24 h drug treatment. Results are expressed per 10^5 cells as a % of the LDH release from Triton X100 treated control cells undergoing total lysis, minus the LDH released from untreated control cells by 'natural cell death'. Values are the mean ± SD of 3 experiments, each conducted in triplicate.

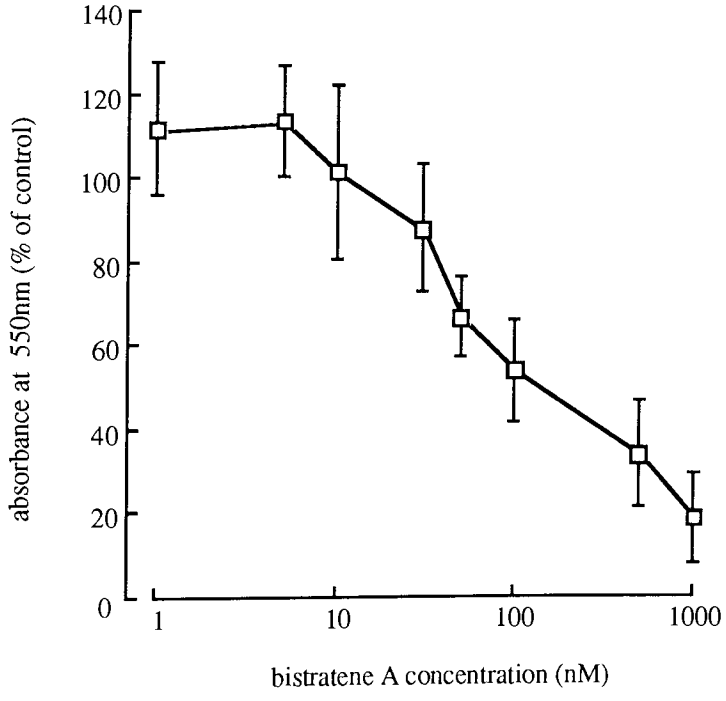
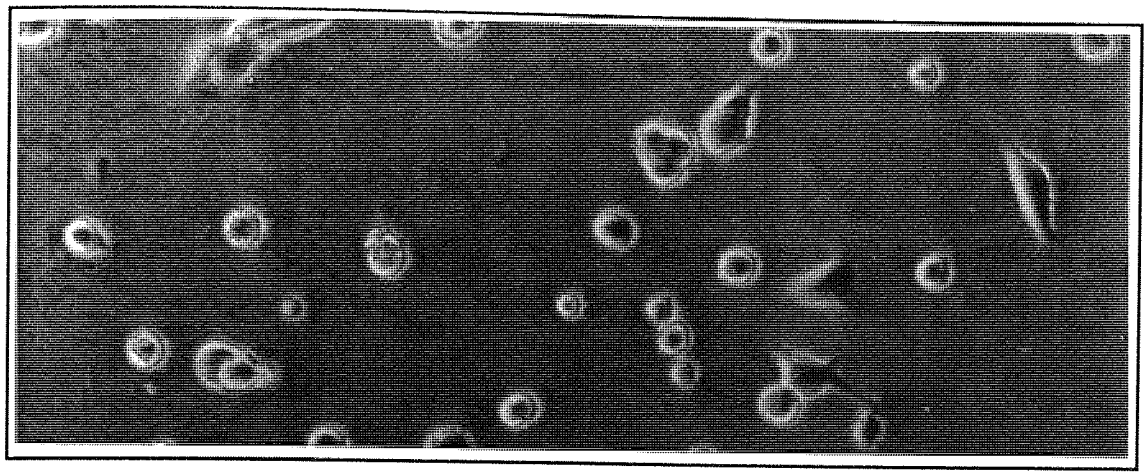


Figure 22b. MTT Assay for Cytotoxicity.
Cytotoxicity of bistratene A towards A549 cells was measured by the MTT assay. Formazan production was measured via UV absorbtion at 550nm. Results were expressed as a % of formazan production in cells without bistratene A. Values are the mean ± SD of 4 experiments, each using 8 microwells per concentration.

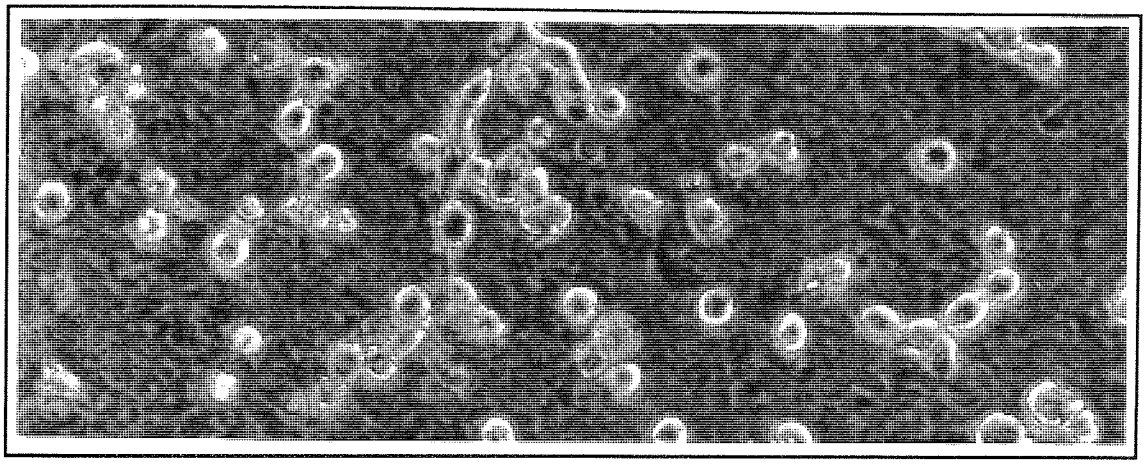
4.2.4. Effects of Bistratene A, TPA and Bryostatins 1 and 2 on Cellular Invasion.

A desirable property of prospective antitumour agents is the aptitude to suppress the formation of metastases. The ability of tumour cells to degrade components of basement membranes or connective tissue correlates positively with metastatic potential (Zetter, 1990). A technique to assess metastatic ability of cells exploiting this observation was devised by Albini *et al*, (1987). Cells were grown on a layer of the basement membrane substitute Matrigel. Morphological and behavioural changes to cells when grown on this matrix were suggested to indicate invasiveness and hence reflect metastatic aggressiveness. Cells were grown on Matrigel in the presence of bistratene A and the results compared to those seen with TPA and bryostatins 1 and 2 to explore further the ability of bistratene A to mimic cellular responses to these agents. A549 and MCF-7 cells adopted a spheroid shape when grown on Matrigel ; MCF-7 cells tended to form clusters (Figs. 23a and 24a). Addition of TPA (10nM) to A549 cells induced rapid motility and the formation of chains of cells within 4 h (Fig. 23di). Within 24-48 h, cells were rounded or had elongated processes, and associated into a polygonal network (Fig.23dii and iii). Bryostatin 1 (10nM) and 2 (100nM) were found to induce essentially the same dramatic morphological changes (results not shown), unlike bistratene A (10 and 100nM) which did not cause any significant changes to cells (Figs. 23b and c). MCF-7 cells were found to react in a similar way as A549 cells to the agents investigated on Matrigel, except that bistratene A actually reduced cell cluster formation, with cells being isolated on the matrix in the presence of the agent, and at 100nM, bistratene A exerted toxicity to these cells with cell lysis (Figs. 24b-d). As a corollary of the results of these studies, invasion assays were attempted using the ability of cells to pass through a filter coated with Matrigel in the presence or absence of drug to assess more accurately the induction of invasiveness by the agents. Unfortunately, difficulties in obtaining a uniform layer of Matrigel on the polycarbonate filters prevented the procurement of meaningful results. Experiments were intended to reveal differences between the number of A549 cells passing through filters after an 8 h incubation in the presence of bistratene A compared to TPA and the bryostatins. The number of cells per unit area on the underside of the filter would then be compared with untreated controls for an indication of the invasive potential after drug treatment.

(a)



(b)



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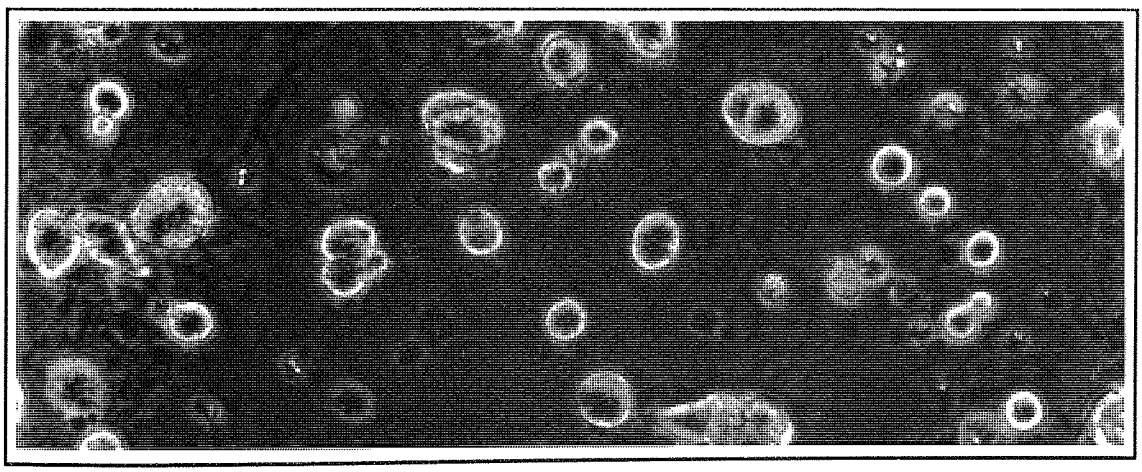
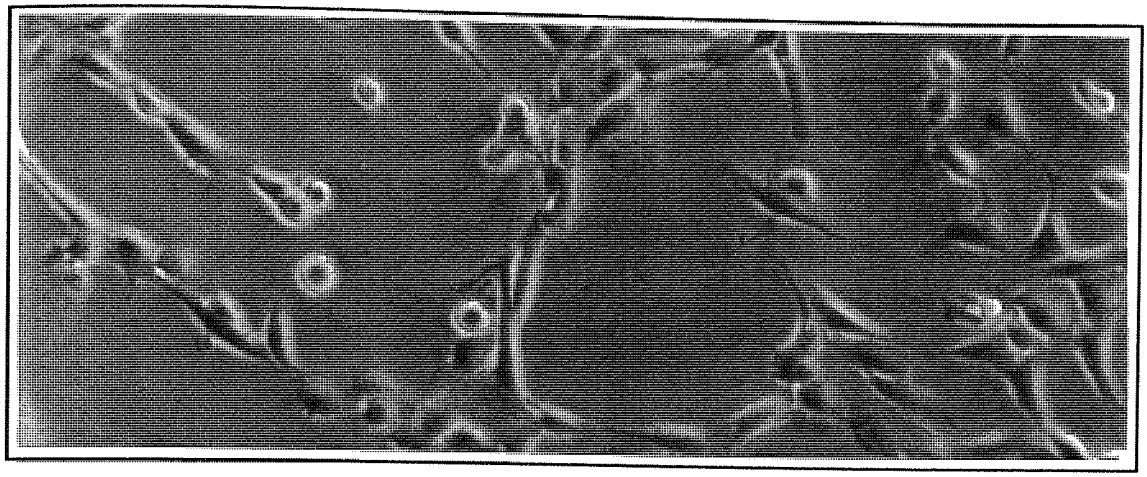


Figure 23. Morphology of A549 Cells when Grown on Matrigel-Effects of TPA, Bryostatins 1 and 2 and Bistratene A.
For culture conditions, see section 3.4.1. A549 cells were grown on Matrigel and were allowed to settle for 24 h, then exposed to DMSO vehicle (a), bistratene A 10nM (b) or 100nM (c) and TPA 10nM (d). For results with bryostatins, see section 4.2.4. Photographs were taken after 4 h (di only) or 48 h. Results were repeated on 3 separate occasions. Magnification is 485x (diii only) or 243x actual size.

(di)



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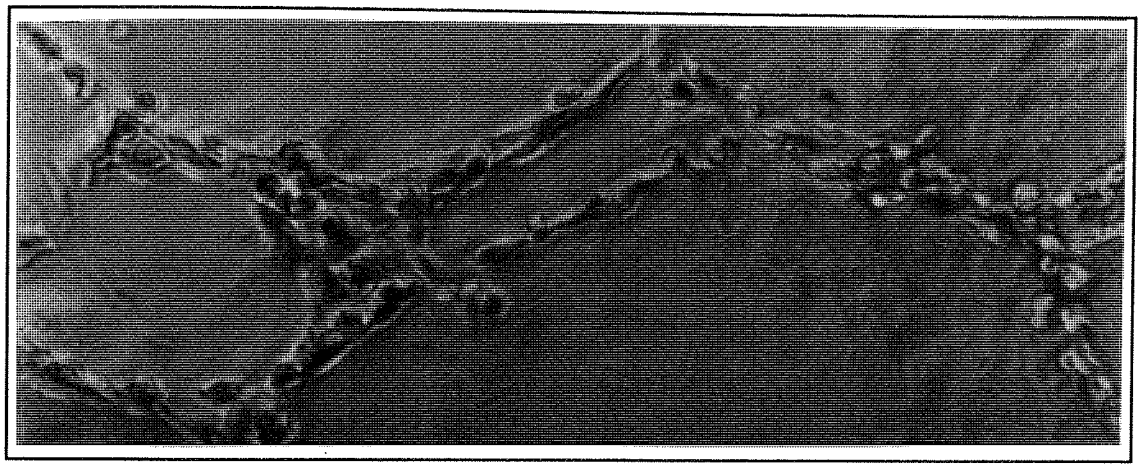
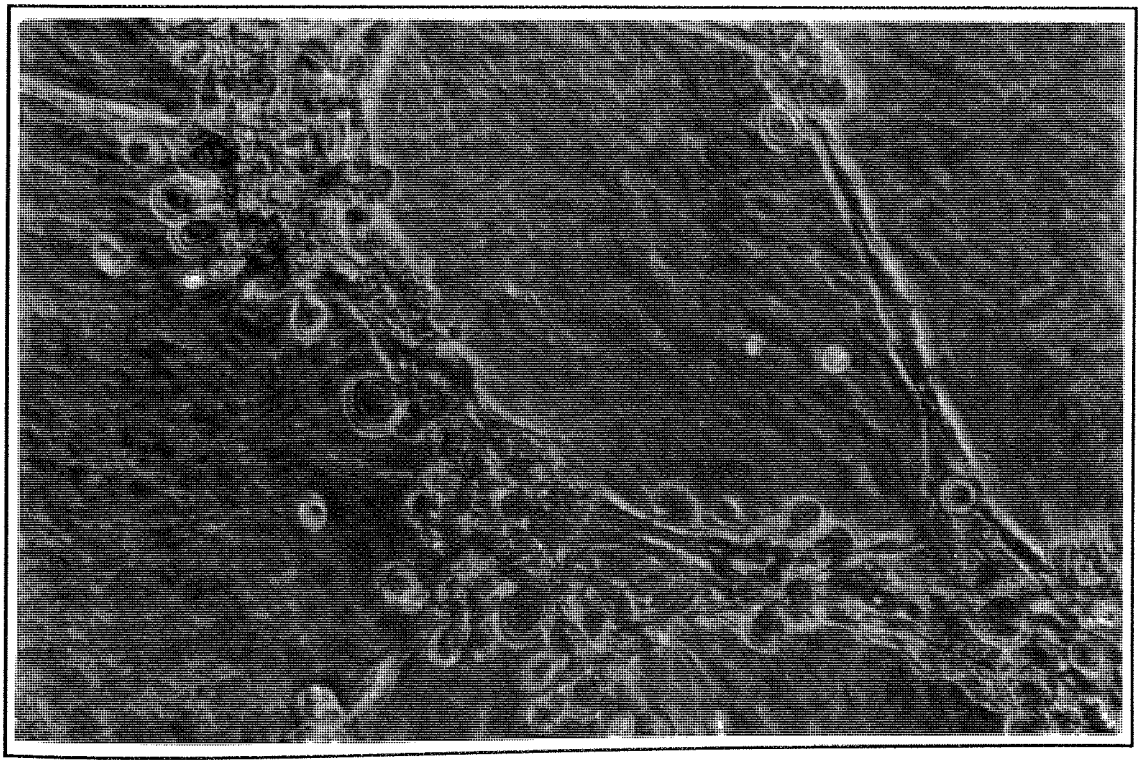
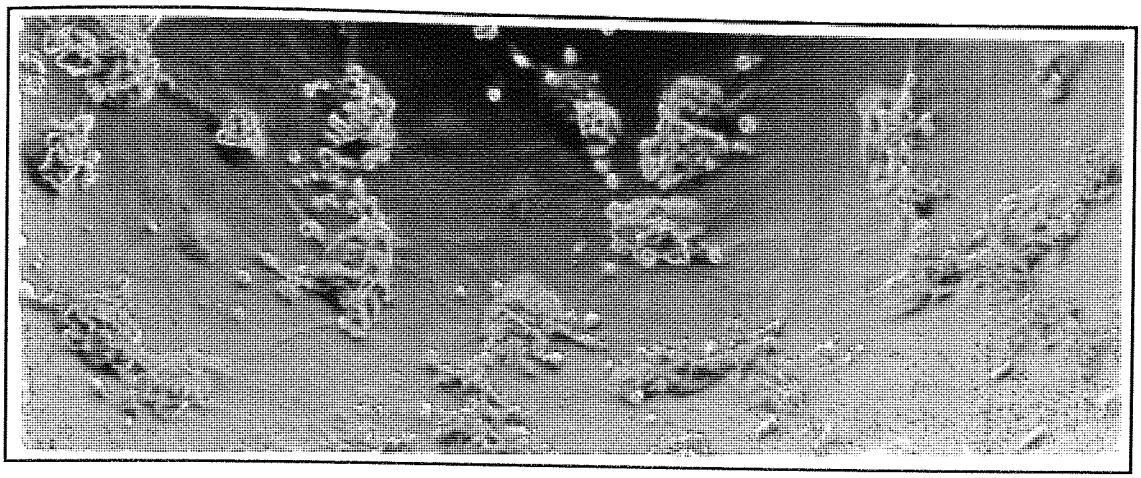


(diii)



(ai)



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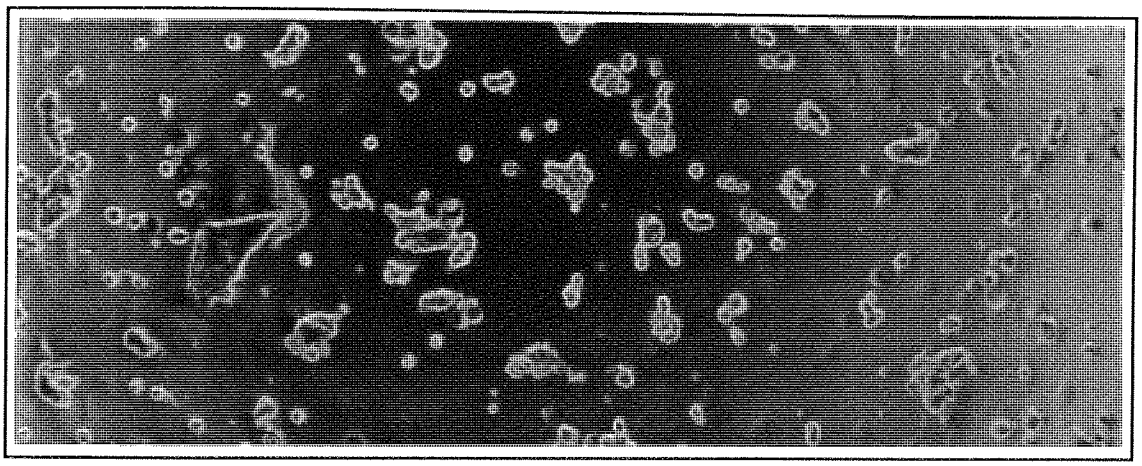

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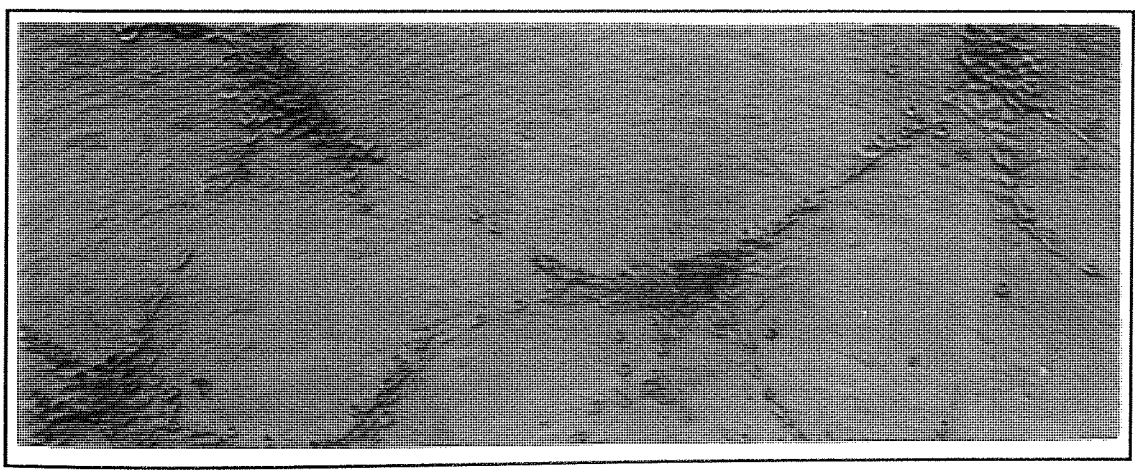
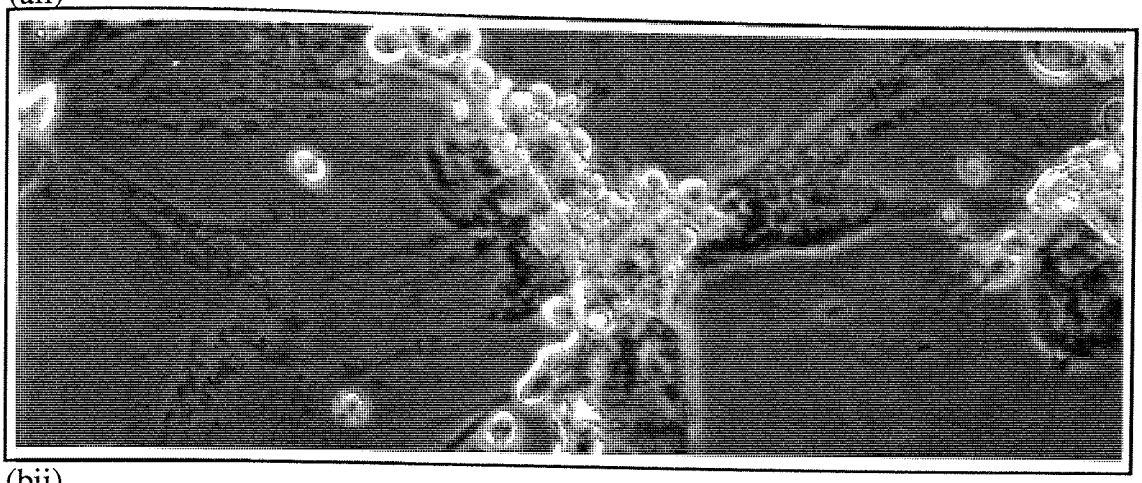
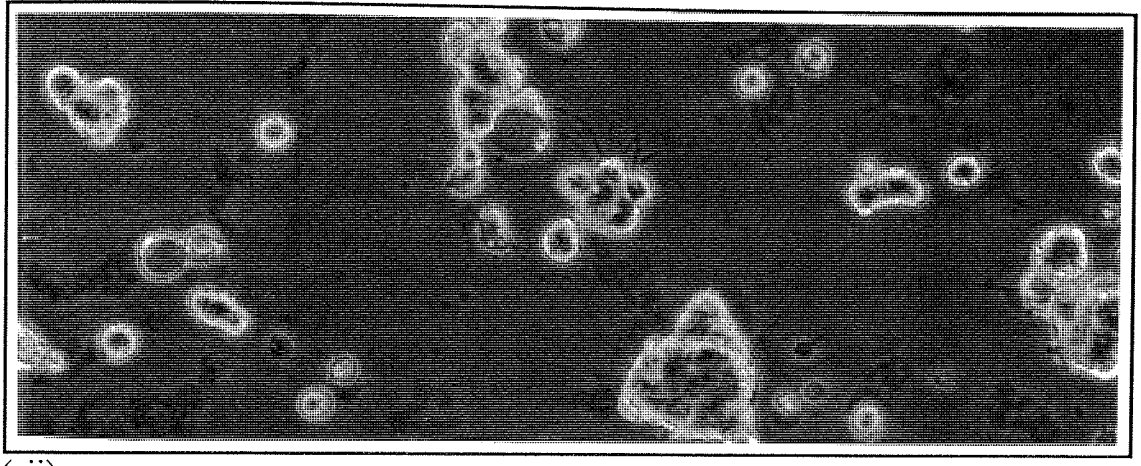


Figure 24. Morphology of MCF-7 Cells when Grown on Matrigel-Effects of TPA, Bryostatin 1 and 2 and Bistratene A.
For culture conditions, see section 3.4.1. MCF-7 cells were grown on a layer of Matrigel and were exposed to DMSO vehicle (a), bistratene A 10nM (b) or 100nM (c) and TPA 10nM (d). Results for bryostatins were the same as for (d). Photographs were taken after 48 h. Results were repeated on 2 separate occasions. Magnification is 97x actual size in i above, and 243x actual size in ii overleaf.

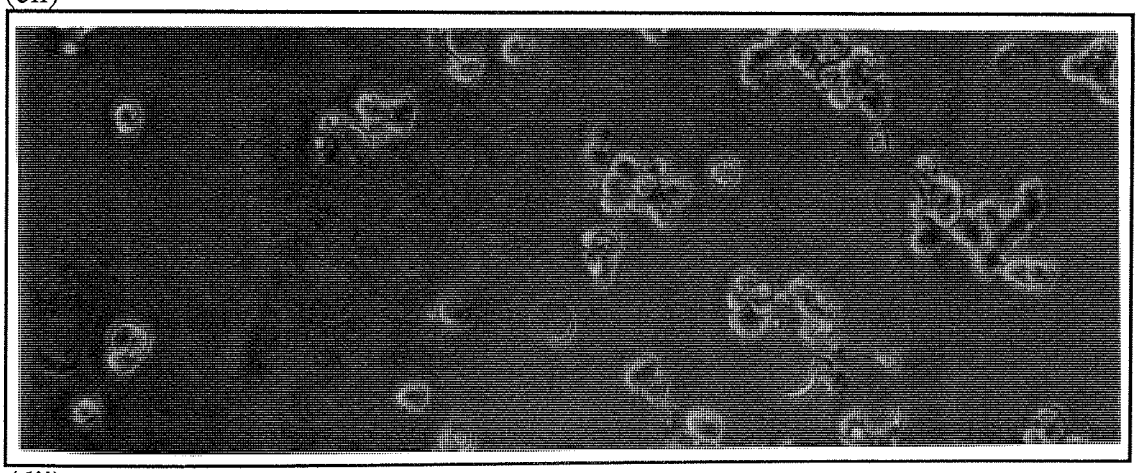
(aii)



(bii)



(cii)



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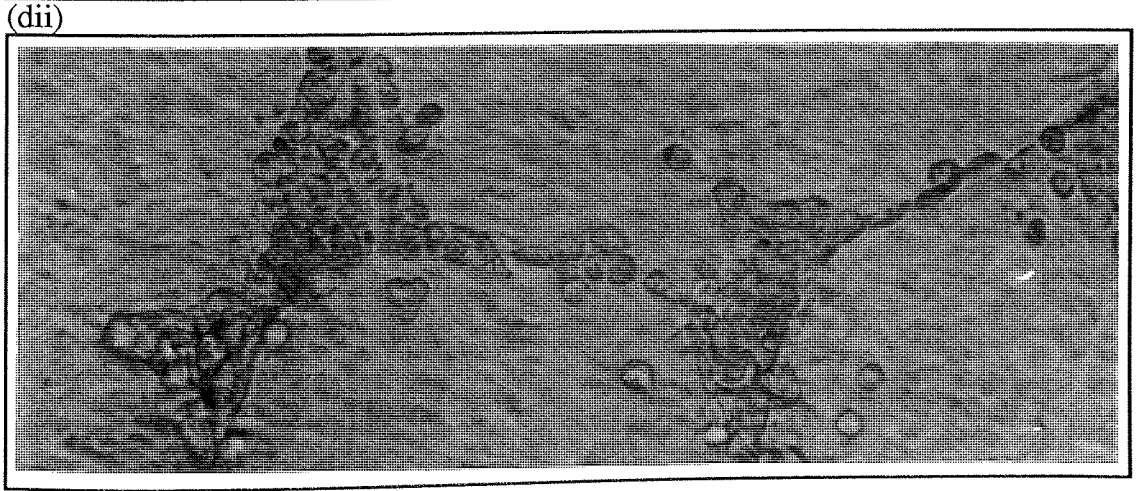


4.3. The Involvement of PKC in the Antiproliferative Effects of Bistratene A.

Bistratene A has been shown to share many of the growth inhibitory and differentiating properties of known PKC activators. To test for the involvement of PKC modulation in these cellular responses to the agent, a technique was required to assess effects on PKC within the cell. At the commencement of this series of experiments, a technique for the direct measurement of intracellular PKC activation by exogenous agents was not available. Therefore a combination of techniques was employed to provide indirect evidence of enzyme status within the cell. These included the reversal of bistratene A-induced growth inhibition by the addition of a PKC inhibitor and analysis of cellular protein phosphorylation by bistratene A compared with established PKC activators by 2D gel electrophoresis. Enzyme activation induces alterations in PKC location and quantity and the effects of bistratene A *vs* phorbol esters on these parameters were monitored by phorbol ester binding and Western blotting. Finally, the direct effect of bistratene A on isolated PKC (total cytosolic enzyme and isolated isozymes) was measured in an enzyme activity assay, again comparing results with those produced by bryostatins and phorbol esters.

More recently, methods for assessment of intracellular PKC activation have been reported in the literature. A cell permeabilisation technique was described by Alexander *et al*, 1990, although high basal phosphorylation precluded detection of low levels of PKC activation by agents in this assay (Basu *et al*, 1992) and permeabilisation may render cells responsive to agents which would otherwise be ineffective. A recently developed technique measures PKC in its membrane-associated state and differentiates between "primed" and "active" PKC (Chakravarthy *et al*, 1991). This method has the disadvantage of only detecting PKC at one cellular location, and would not detect PKC-activating agents which induce translocation to the nucleus or activate PKC in cell cytosol. However, it does provide a more physiological setting for PKC *in vitro* and could become an established technique pending the easy availability of the peptide used as substrate or the scrutiny of commonly used substrates under the same assay conditions. In hindsight, it would have been

interesting to attempt the assays cited above and compare results with those of the PKC assay used (section 4.3.5.) to provide a definitive answer on the modulation of PKC by bistratene A.

4.3.1. Reversal of Bistratene A-Induced Cytostasis by Staurosporine.

Staurosporine is a potent but not very selective inhibitor of PKC. To determine if the growth inhibitory effect of bistratene A could be reversed by staurosporine, cells were cultured and [^{3}H]thy incorporation measured at regular time intervals up to 12 h as in section 3.1.5. Cells were exposed to 100nM staurosporine, or 15, 50 and 200nM bistratene A in the presence and absence of 100nM staurosporine. A549 cell PKC is almost completely inhibited by this staurosporine concentration *in vitro* (Bradshaw *et al*, 1992). Results were expressed per 10^5 cells as a percentage of [^{3}H]thy uptake of untreated controls. Staurosporine alone was able to induce a small reduction in [^{3}H]thy uptake during this time period. The presence of staurosporine and bistratene A (15 and 50nM) had an additive effect on inhibition of [^{3}H]thy uptake from 2-4 h after dosing but thereafter inhibition of uptake was similar to that seen with bistratene A alone. (Figs. 25a and b). Using bistratene A concentrations of 200nM (Fig.25c), uptake was the same as bistratene A alone throughout the time course. No reversal of bistratene A-induced growth inhibition was detected using staurosporine as a PKC inhibitor.

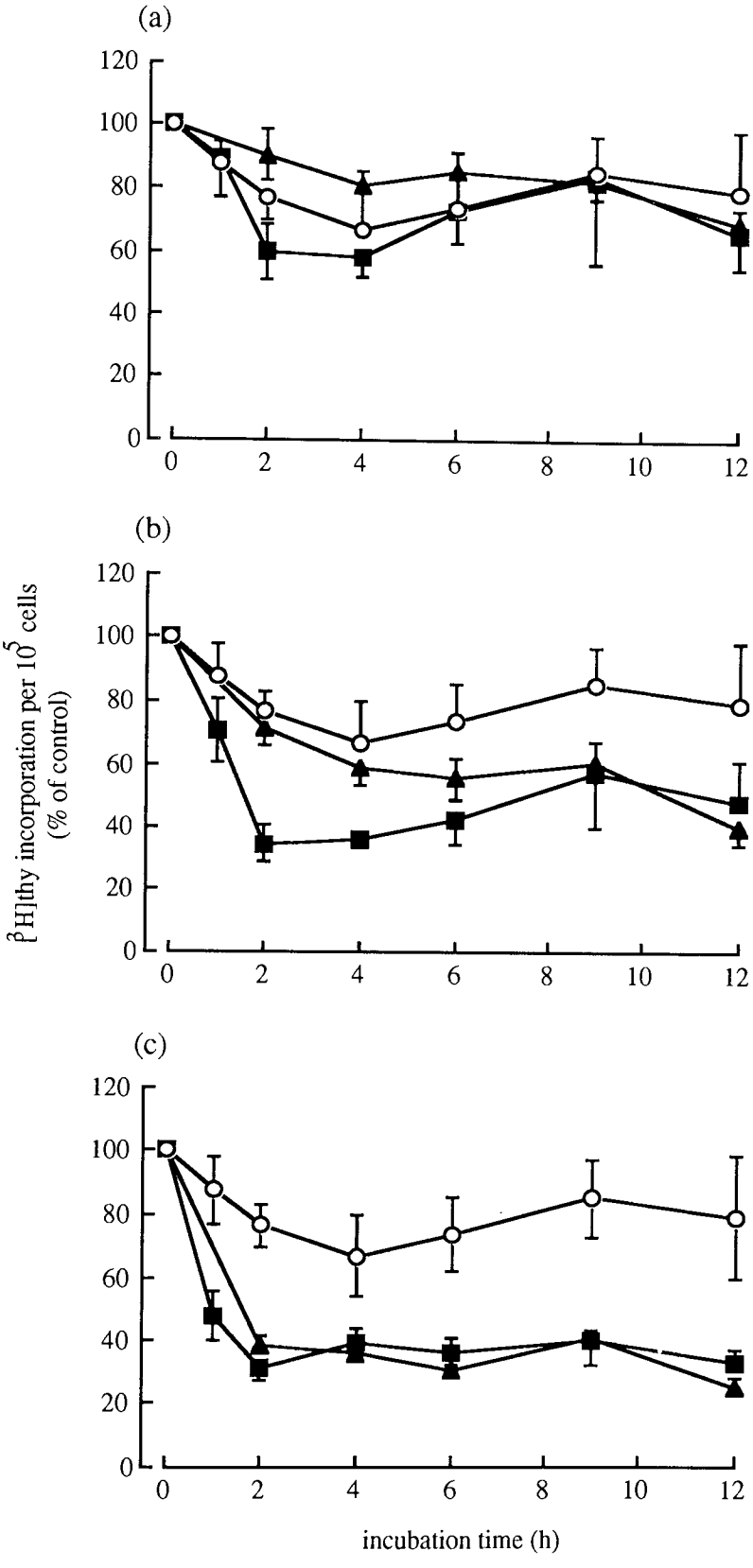


Figure 25. Influence of Staurosporine on the Inhibition of [^{3}H]thy Incorporation into A549 Cells by Bistratene A.

A549 cells were incubated for up to 12 h with (a) 15nM, (b) 50nM and (c) 200nM bistratene A, with (squares) or without (triangles) 100nM staurosporine. Circles depict treatment with staurosporine (100nM) alone. [^{3}H]thy uptake was measured at the indicated time points and results were expressed as a percentage of uptake by vehicle-treated control cells. Results are the mean ± SD of 3 separate experiments, each conducted in triplicate.

4.3.2. Two-Dimensional Gel Electrophoresis of A549 Cell Proteins Treated with Bistratene A or TPA.

2D gel electrophoresis was used to separate and detect phosphorylated proteins in whole cell samples after the attainment of equilibrium $^{32}P_i$ uptake and its intracellular conversion to [γ-^{32}P]ATP prior to incubation of cells with TPA, bryostatins or bistratene A. Phosphoprotein maps of cells treated with these agents were compared and consistent increases or decreases in phosphorylation were detected visually by two independent investigators viewing at least five gels per treatment.

4.3.2.1. Determination of Molecular Weight/pI of Proteins Detected on 2D Gels.

Rainbow coloured or ^{14}C-labelled molecular weight markers were used to assess the molecular weight of proteins in the 2nd dimension gel, but determination of phosphoprotein pI was more difficult to assess following separation with this urea-containing system (section 3.8). Two methods of pI determination were used but both techniques had the disadvantage of requiring a separate tube gel for pI measurement and therefore results were not exactly the same as the cell samples. Protein markers using proteins with known pI are available (Sigma). Results were impossible to interpret running the markers on separate tube gels, then staining with Coomassie Brilliant Blue G-250 as directed by the manufacturers using this system. Protein markers with low pI migrated towards the acidic end of the tube gel as expected, but there was no direct relationship between pI and protein migration. Amyloglucosidase from Aspergillus niger (pI 3.6) and carbonic anhydrase I from human erythrocytes (pI 6.6) consistently formed broad bands instead of sharp localisation at one site within the tube gel. Soybean trypsin inhibitor (pI 4.6) visualised as two bands after IEF and markers with pI values of 8.6 and 9.3 were not detected, indicating that the pH gradient did not reach 8.6 (Fig.26). These phenomena have been described by other workers (Pharmacia publications, Olsson *et al*, 1981, Rocha *et al*, 1991) and are thought to be caused by interaction of urea with the proteins, inducing migration to anomalous pI positions via conformational changes, protein carbamylation and effects of urea on carrier Ampholines and water. The degree of urea interference depends on the protein itself and therefore the use of protein markers in urea-containing

systems is not a good reflection of the pH gradient along tube gels. The effects of urea on proteins would obviously also apply to sample proteins and hence phosphoproteins detected may be spurious carbamoylation products of other phosphoproteins or may have migrated to a false pI depending on urea interactions. However, drug-treated cell samples run on urea-containing 2D gels are able to supply useful information regarding similarities or differences between agents. The pH at which a protein reaches steady state in 1st dimension gels with 9M urea (pI (urea)) can be compared to the results of other workers using the same method and its identity verified by other means.Values for pI (urea) were determined more reliably from the measurement of the pH gradient across tube gels by tube gel slicing and pH determination of each slice (section 3.8). Recently, 2D SDS-PAGE protein standards have been developed by Bio-Rad which are designed for use in systems containing urea and may be more suitable than the methods cited above.

4.3.2.2. Effect of TPA, Bryostatins and Bistratene A on the Phosphorylation of A549 Cell Proteins.

TPA enhanced the phosphorylation status of at least eight proteins with molecular weights of 19, 28, 50, 56, 63, 64, 64 and 87-97kDa (Fig.27b). In preliminary experiments, bryostatins 1 and 2 markedly increased the phosphorylation of the 28 and 87-97 kDa proteins described for TPA (results not shown: n=2). Bistratene A induced the phosphorylation of a minimum of five proteins with molecular weights of 18, 19, 50, 64 and 95 kDa (Fig.27c). The increased phosphorylation of three proteins was common to TPA and bistratene A : 19kDa, pI (urea) 4.5, 50kDa, pI (urea) 5.0, and 64kDa, pI (urea) 4.9. The 19kDa protein was more markedly phosphorylated by TPA than bistratene A. The most striking phosphorylation induced by bistratene A was of an 18kDa protein, pI (urea) 6.0, which was unaffected by TPA or the bryostatins.

4.3.2.3. Determination of the Identity of Proteins Phosphorylated by TPA and Bryostatins.

Proteins of 27-28kDa, pI 5-6 (urea) (Regazzi *et al*, 1988, Darbon *et al*, 1990) and 80-87kDa, pI 4.4-4.8 (urea) (Stumpo *et al*, 1989, James *et al*, 1989) have been reported to be specific substrates for PKC. TPA and bryostatins 1 and 2 but not bistratene A induced the

phosphorylation of two proteins of mw 28kDa, pI (urea) 5.6 and 87-97kDa, pI (urea) 4.3-4.4. To determine if the 87-97kDa protein was identical with the MARCKS protein, antibodies to MARCKS were obtained with the aim of Western blotting 2D gels to visualise the location of this protein and compare it to the location of PKC which is also 80kDa and undergoes autophosphorylation (Blackshear *et al*, 1986). However the pI of PKC is 5.8 in urea-containing systems (Blackshear *et al*, 1986) and therefore the two proteins are easily distinguished by 2D gel analysis. Initially, SDS/PAGE was carried out on A549 whole cell samples in (i) Laemmli sample buffer (section 2.2.5.) or (ii) Urea Sample Buffer (USB)(section 2.2.6.). Gels were subjected to Western blotting and detected with the MARCKS antibody at a 1: 1000 dilution (see section 3.7.2. for method). The antibody detected a protein at 80kDa in samples using Laemmli buffer but no protein was detected with samples in USB (Fig.28). Similar results were obtained using an antibody specific for PKC α/β (results not shown). Therefore it seems that these proteins, when solubilised in USB, are changed to the extent that specific antibodies are unable to recognise them. Therefore confirmation of the identity of MARCKS by Western blotting of 2D gels was not possible.

4.3.2.4. Inhibition of Protein Phosphorylation by Staurosporine.

Phosphoprotein maps were produced for cells treated for 2 h with the PKC inhibitor staurosporine (50nM) with and without 100nM TPA. Staurosporine alone did not cause the phosphorylation or dephosphorylation of A549 cell proteins, but prevented the changes induced by TPA except for proteins with molecular weights of 56 and 64kDa (Figs. 27d and e). Reversal of the phosphorylation of the 87-97kDa protein by staurosporine gives further credence to the claim that this protein is indeed the MARCKS protein.

4.3.2.5. Silver Staining of 2D Maps.

Samples were prepared as in section 3.8. using cells treated with vehicle, TPA (100nM) or bistratene A (100nM) for 2 h but with the omission of $^{32}P_i$ in the initial 6 h incubation. Gels were silver stained (section 3.8). The proteins phosphorylated by TPA and bistratene A were identified and compared to gels of untreated cells to determine whether the enhanced spot intensities were due to increased phosphorylation or increased protein

synthesis. By visually comparing gels (n=4), no change in amount of protein could be detected for proteins phosphorylated by either agent, including the 18kDa protein uniquely phosphorylated by bistratene A or for the 28kDa protein phosphorylated in the presence of TPA (Fig.29). Therefore the increase in spot intensity was due to protein phosphorylation. The 87-97kDa protein phosphorylated by TPA was detected by silver staining as a run of proteins with progressively decreasing electrophoretic mobility and acidic pI shift in the presence of vehicle, TPA and bistratene A. The run of phosphoprotein spots detected after exposure of cells to TPA are therefore not different phosphorylation species, but are more likely to be variants of the same phosphoprotein generated on 2D maps by protein-urea interactions.

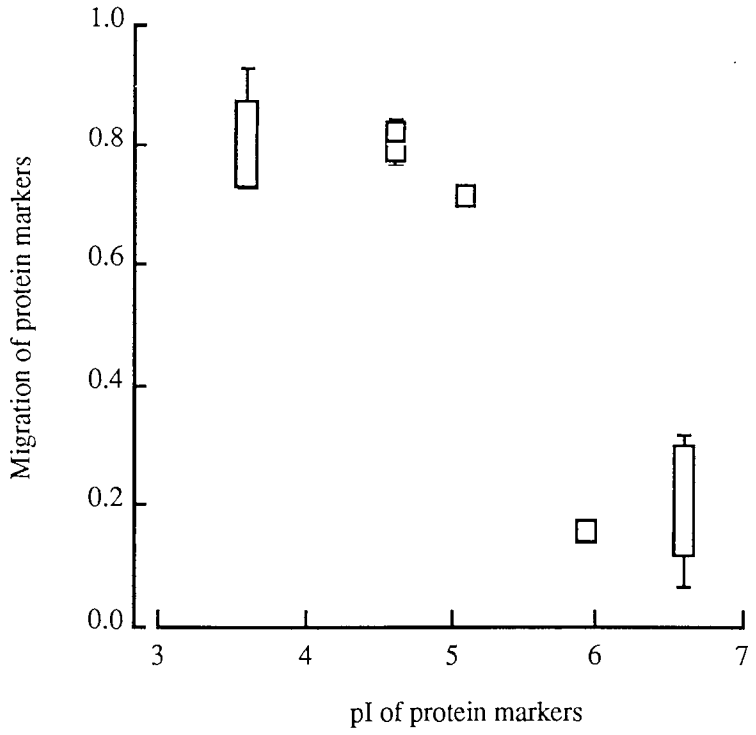


Figure 26. Migration of IEF Markers (Sigma) in Tube Gels vs pI in Aqueous Media
IEF markers were focused in tube gels containing 9M urea and their migration was determined by dividing the distance migrated by protein bands from the basic end of the tube gel by the tube gel length. Values are the mean ± SD of 4-8 separate IEF experiments.

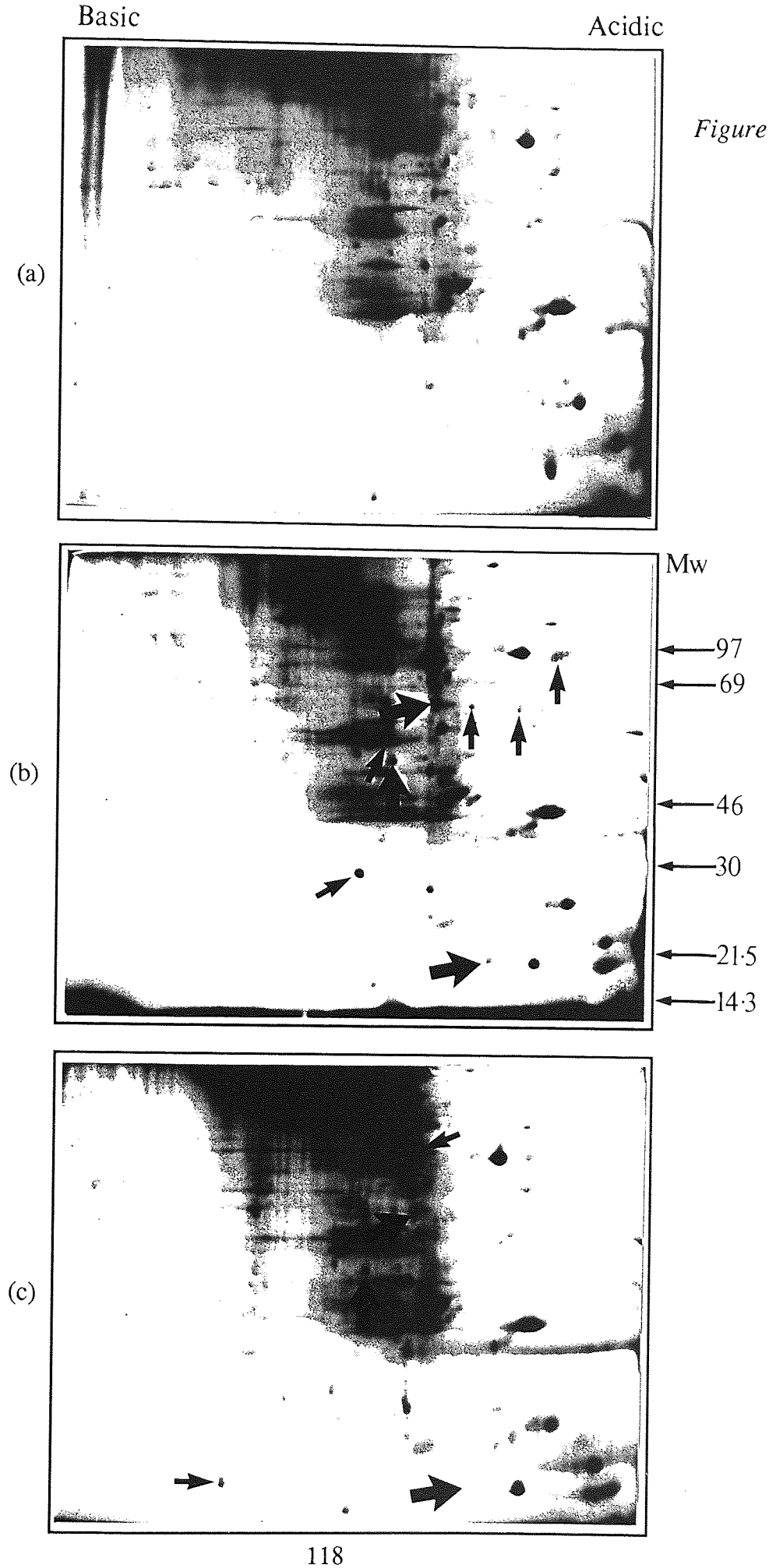


Figure 27.

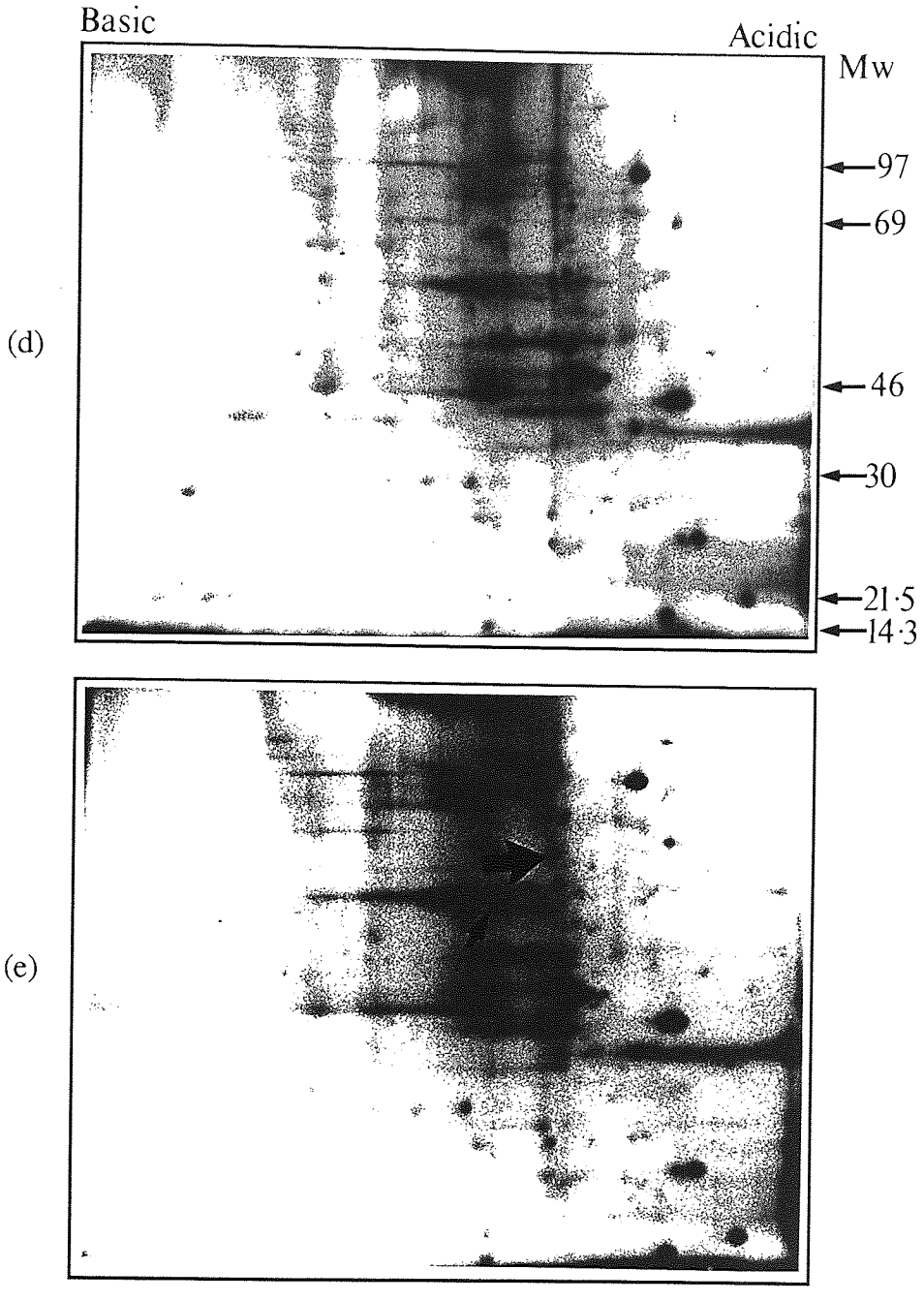


Figure 27. Effect of TPA, Bistratene A and Staurosporine on Phosphorylation of A549 Cell Proteins.
See section 3.8 for methods. Cells were treated for 2 h with (a) DMSO vehicle, (b) TPA 100nM, (c) bistratene A 100nM, (d) staurosporine 50nM and (e) staurosporine 50nM and TPA 100nM. Proteins with an increase in phosphorylation are marked with arrows: large arrows denote proteins which are phosphorylated by both TPA and bistratene A, and small arrows denote proteins only phosphorylated by the agent indicated. In (e), arrows denote proteins which are phosphorylated by TPA (100nM) alone, which are also phosphorylated when staurosporine and TPA are added together.

1 2 3

Figure 28. Detection of the MARCKS Protein in A549 Cells.
A549 cells were denatured in Laemmli sample buffer or USB. Cell protein (100µg) was loaded per lane and samples were subjected to SDS PAGE and Western blotting; the MARCKS protein was detected using a specific antibody (section 4.3.2.3.). The sample in lane 1 was prepared by scraping cells into ice-cold PBS, centrifuging at 13,500rpm and resuspending the pellet in Laemmli sample buffer (section 2.2.5.) diluted 1:1 with distilled H_2O. This was then placed in a boiling water bath for 3 mins. In lane 2, cells were scraped from culture dishes directly into H8 buffer (200µl), then 200µl sample buffer was added and this was boiled for 3 mins. Samples in lane 3 were prepared in the same manner as lane 1 except that the cell pellet was resuspended in USB and was allowed to solubilise for 30 mins.

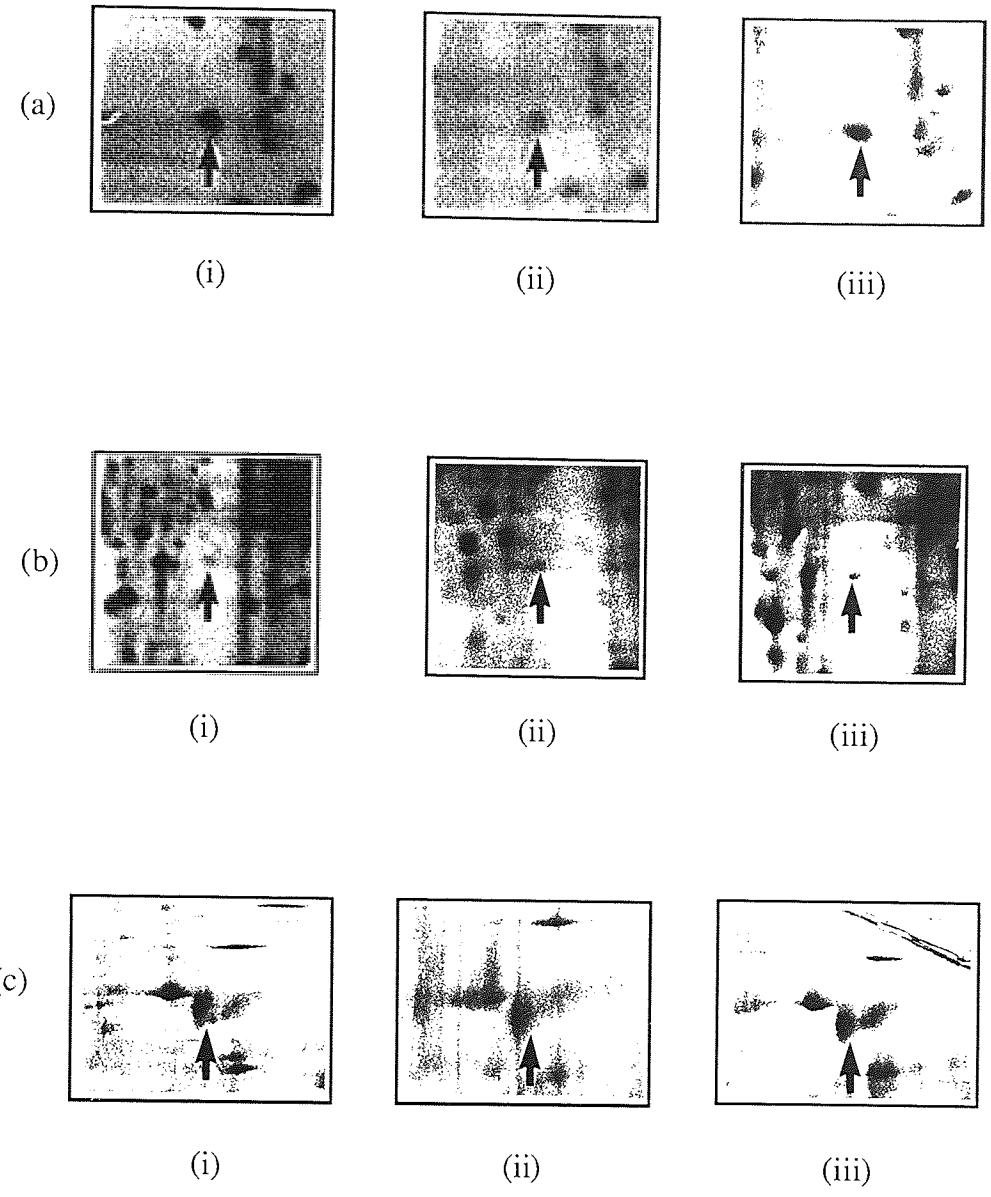


Figure 29. Effect of TPA and Bistratene A on Cell Protein Synthesis-Silver Staining of 2D Gels.
See section 3.8 for methods. Samples were prepared from cells which had been treated for 2 h with (i) DMSO vehicle, (ii) TPA 100nM and (iii) bistratene A 100nM. 2D gel electrophoresis of these samples followed by silver staining visualised cell proteins. Proteins with a mw of (a) 18kDa and pI(urea) 6.0, (b) 28kDa and pI(urea) 5.6, and (c) 87-97kDa and pI(urea) 4.3-4.4 are shown above for each drug treatment. Proteins of interest are marked with arrows.

4.3.3. Phorbol Ester Receptor Binding Studies.

PKC is the major phorbol ester receptor (Ashendel, 1985, Blumberg, 1988) and therefore the degree of phorbol ester binding in a sample reflects the amount of PKC present. The mixed micelle assay for phorbol ester binding (Hannun and Bell, 1986, 1987) was used to quantify binding using [^{3}H]PDBu as ligand.

4.3.3.1. The Influence of Bistratene A and Phorbol Esters on A549 Cell Cytosolic Receptor Number.

Incubation of cells with agents which activate PKC induces a shift in the cellular location of the enzyme from the cytosol to membrane or nucleus (Kraft and Anderson, 1983, Wolf *et al*, 1985, Hocevar and Fields, 1991) and ultimately its degradation by proteases (Young *et al*, 1987, Nishizuka, 1988). These processes, known as translocation and downregulation respectively, were demonstrated to occur after incubation of A549 cells with the PKC activator TPA (10nM) with subsequent loss of PKC from the cytosol (Dale *et al*, 1989). To evaluate the ability of bistratene A to translocate PKC, A549 cell cytosolic phorbol ester binding was determined after incubation in the presence of agents for 30 mins, 24 h, or for a prolonged period (>9 weeks). Cytosolic fractions were prepared and analysed for phorbol ester receptor binding per mg protein. Results were expressed as a percentage of untreated control cell binding. After incubation with 10nM TPA for 30 min there was a significant reduction in [^{3}H]PDBu binding to 56.6 ± 11.6% of control values which was further reduced to 34.8 ± 19.0% after 24 h. In contrast, bistratene A (10 and 100nM) did not induce a notable change in phorbol ester binding (Figs.30a and b). Cells which had grown continuously in PDBu (100nM) exhibited a reduction in phorbol ester binding to 43.6 ± 8.1% of controls. There was a small but significant ($p<0.001$) increase in phorbol ester binding to 117.9 ± 13% control values in cells exposed to bistratene A (15nM) for prolonged periods (Fig.30c).

4.3.3.2. Competition for Phorbol Ester Receptors Between Bistratene A and [^{3}H]PDBu.

Direct competition between [^{3}H]PDBu and bistratene A for phorbol ester binding sites was assessed by an attempt to displace [^{3}H]PDBu from its cytosolic receptors by the

addition of bistratene A to the mixed micelle assay reaction mix. Bistratene A did not appear to compete for receptors as addition of the agent at 10 and 100nM concentrations did not reduce the amount of bound [^{3}H]PDBu detected in A549 cell cytosol, unlike TPA (Table 1).

4.3.3.3. Phorbol Ester Binding Capacity of Human Leucocytes.

A phase 1 clinical trial of bryostatin 1 as an anticancer agent was recently completed at the Christie Hospital, Manchester and the John Radcliffe Hospital, Oxford. The initial test dose used was calculated from the LD_{10} in mice to be 5μg/m^2, which was raised to 65μg/m^2, given over 1 h every two weeks (Prendiville *et al*, 1993). At these low dosages, plasma levels of the agent were not detectable using the techniques of HPLC and mass spectrometry for pharmacokinetic analysis. Therefore, a simple way of following the longevity of bryostatin in the circulation was sought. It was suggested that measurement of changes in phorbol ester binding would reflect the effects of the drug and act as a biological marker. The mixed micelle assay for phorbol ester binding could be used to gain such information economically and a large number of samples could be processed simultaneously. Blood is a very variable substance in cancer patients due to the disease itself and previous treatment causing selective leucopenia. Ideally, phorbol ester receptor binding changes should be monitored in a single cell population but in practice rapidity and ease of sample preparation precluded complex separation techniques. White blood cells were chosen for study as they have easily detectable levels of phorbol ester binding (Nishizuka, 1989) unlike erythrocytes (Speizer *et al*, 1987). Double density gradient centrifugation was used to partially purify cell fractions. Results were expressed per mg of cellular protein to take variable leucopenia into account. An assessment of leucocyte phorbol ester binding was carried out with the aim of (i) optimizing the separation technique to retain as much PKC in the sample as possible after removal from the patient, (ii) determining which leucocytes to use in the trial by ascertaining which type had the highest phorbol ester binding in normal subjects and (iii) finding out whether it was possible to store samples for 2 days with the aim of posting samples to Aston for testing. Details of optimal assay conditions are in section 3.6.3. Using fresh blood from volunteers, it was found that the cell fraction containing platelets and mononuclear cells (T

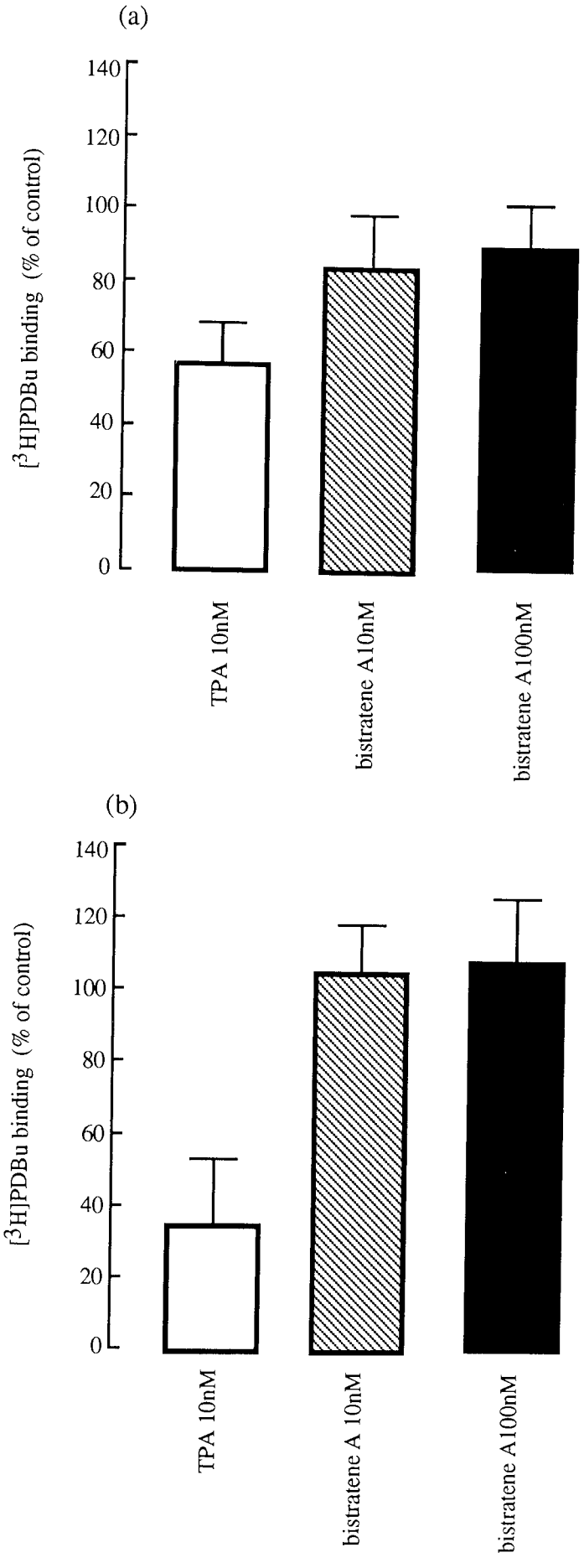
and B cells) had the highest phorbol ester binding, which was 7-fold the value for granulocytes per mg protein. Mononuclear cells/platelets (layer A, Fig.6) bound 460.9 ± 35.5 picomoles [^{3}H]PDBu per mg protein, whereas granulocytes (layer B, Fig.6) bound only 65.8 ± 11.4 picomoles. Results are the mean ± SD of 2 experiments, each conducted in triplicate. After the initial separation of leucocytes, patient samples were stored at -70°C for 2 days to simulate postal transfer on dry ice. The alternative of posting at room temperature was discounted due to PKC instability. Phorbol ester binding of mononuclear cells was reduced by 25.4% after 48 h at -70°C (n=3). This degradation was unacceptably high and sample postage was ruled out.

Table 1. Competition for Binding to Phorbol Ester Receptors Between [^{3}H]PDBu and Bistratene A or TPA.

Agent added	Binding to phorbol ester receptors (% of untreated controls)
Bistratene A 10nM	104.5 ± 5.9*
Bistratene A 100nM	104.0 ± 8.6
TPA 10nM	98.5 ± 8.4
TPA 100nM	58.3 ± 4.8

*Values are the mean ± SD of 3 experiments, each conducted in triplicate.

Figure 30.


(a)
[3H]PDBu binding (% of control)
140
120
100
80
60
40
20
0
TPA 10nM
bistratene A10nM
bistratene A100nM
(b)
[3H]PDBu binding (% of control)
140
120
100
80
60
40
20
0
TPA 10nM
bistratene A 10nM
bistratene A100nM

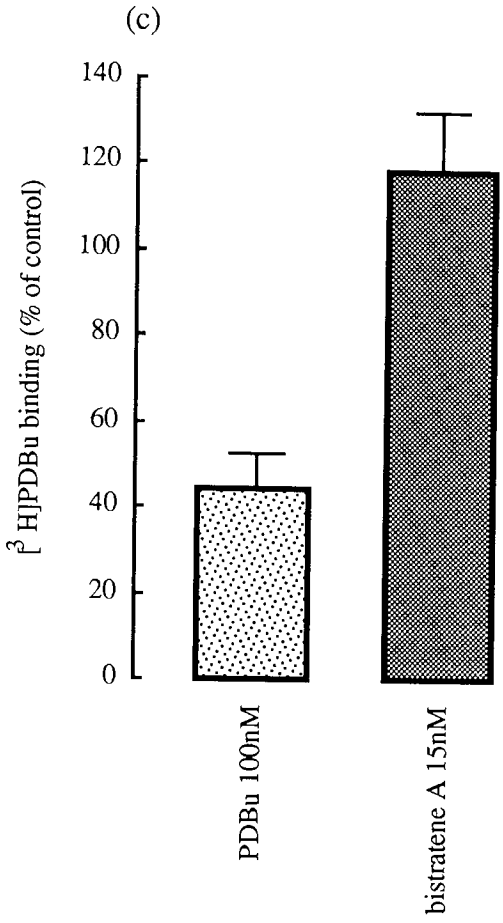


Figure 30. Phorbol Ester Binding Capacity of Cytosol of A549 Cells Grown in the Presence of Bistratene A or Phorbol Esters.
Cells were incubated in the presence of bistratene A, TPA or PDBu for (a) 30 mins, (b) 24 h, or (c) 9 weeks at the indicated concentrations. Phorbol ester binding was determined using the mixed micelle assay of Hannun and Bell, 1987. Results are the mean ± SD of at least 3 experiments, each conducted in triplicate.

4.3.4. Treatment of Cells with Bistratene A or Phorbol Esters; Detection of PKC α/β in Cell Fractions by Western Blotting.

Phorbol ester binding experiments indicated that PKC was lost from the cytosolic fraction by exposing cells to phorbol esters, but not bistratene A. Western blotting of cell cytosol and particulate fractions was performed to differentiate between location changes and downregulation of the enzyme and to assess changes in PKC located in the membrane which was not measured using the mixed micelle assay. A549 and MCF-7 cells contain PKC α. MCF-7 cells have also been reported to contain PKC β and possibly other isozymes (section 5.3). Therefore a monoclonal antibody specific for PKC α and β (Amersham) was used to detect PKC in cytosol and detergent-soluble particulate fractions of these cells after growth in the presence of agents (section 3.7.1.). Gels in Fig.31 except for Fig.31bii were loaded using 50μl of sample per lane during PAGE, and the protein in samples was determined according to the method of Bradford, 1976. Band intensity was measured using an LKB 2202 Ultroscan laser densitometer with five readings per band. Results were calculated by multiplying intensity by the width of each band and dividing by sample protein concentration, expressing this value as a percentage of total band intensity of vehicle-treated control cells (Fig.32). A549 cells contained substantially more PKC α/β than MCF-7 cells (Fig.31 *cf* Fig.33). In both cell types, TPA (10nM) caused the translocation of PKC from cytosol to membrane. This occurred after 30 min in A549 cells (Fig.31a) and after 24 h in MCF-7 cells (Fig.33). Translocation of PKC α/β was not detected in MCF-7 cells after incubation with TPA (10nM) for 30 mins (results not shown), but in later experiments, PKC α translocated partially to the particulate fraction in MCF-7 cells within 30 min, remaining at that cellular location for at least 24 h (Figs.45 and 47). The lack of translocation detected in these early experiments after a 30 min incubation with TPA (10nM) may be due to the lack of sensitivity of the alkaline phosphatase colour reaction detection system. Protein is less concentrated in cell membrane fractions than in cytosolic fractions, and therefore a 50μl loading volume would contain less protein, which may also be contributory to this discrepancy. Unfortunately, levels of PKC detected in MCF-7 cells were too low for analysis of blots by laser

densitometry and therefore results could not be determined for equal protein loads for each lane to assess the degree of downregulation after a 24 h exposure to TPA (10nM). Downregulation ensued after 24 h in the presence of TPA in A549 cells, with a loss of translocated PKC from the particulate fraction and PKC reduced to 39 ± 6% of control in the cytosol (Figs. 31bi and 32bi). PKC was undetectable in fractions from A549 cells grown continuously in PDBu (Fig.31c). In contrast to these effects of phorbol esters, bistratene A had little effect on the distribution or downregulation of PKC α or β in these cell lines, even after growth in the continuous presence of the agent (Figs. 31-33).

Because of the growth inhibition induced by TPA and bistratene A, cellular extracts were prepared from fewer cells after incubation for 24 h with these agents, and less protein was loaded per lane using 50μl of sample. Therefore visual analysis of blots was not possible and densitometric scanning had to be performed to validate results (compare Fig.31bi with 32bi). Experiments confirmed that laser densitometry of blots loaded with 50μl sample per lane, expressing band intensity per unit protein, gave qualitatively similar results to those obtained by loading each lane with a volume of sample containing the same amount of protein (Fig.31bi compared with Fig.31bii and 32bii). Loading equivalent protein onto each lane for PAGE gave results which were easier to interpret visually. Also, densitometric scanning of feint bands on nitrocellulose was not possible due to the high irregular background of nitrocellulose itself and problems with fibres therein. Thereafter, all Western blotting was performed loading equal amounts of protein per lane. Lack of sensitivity of laser densitometry was circumvented by using ECL for detection of proteins as results are obtained as black autoradiographic bands on clear film which is an inherently more sensitive technique than the colour reaction on nitrocellulose and eradicates background problems of measuring band intensity through a nitrocellulose sheet.

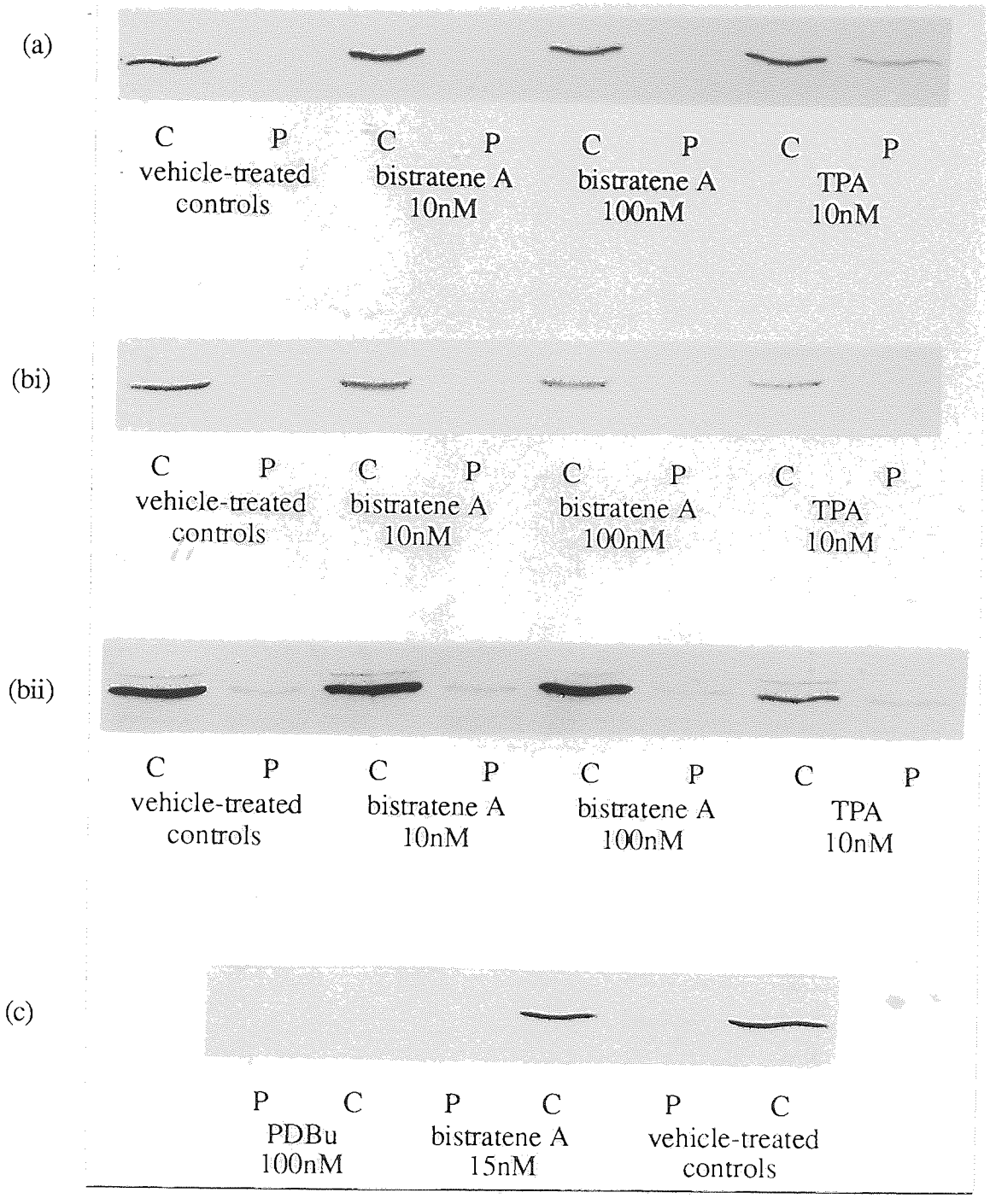


Figure 31. Western Blots Showing PKC α or β in Cytosolic and Particulate Fractions of A549 Cells Grown in the Presence of Bistratene A or Phorbol Esters.
Cells were exposed to agents for (a) 30 mins, (b) 24 h, and (c) a minimum of 9 weeks. Lanes were loaded with 50µl (a,bi and c) or 50µg protein (bii). C refers to samples prepared from cell cytosol and P to the detergent-soluble particulate fraction. PKC (α and β isozymes) was detected by a colour reaction on nitrocellulose itself (See section 3.7.1. for details of methodology).

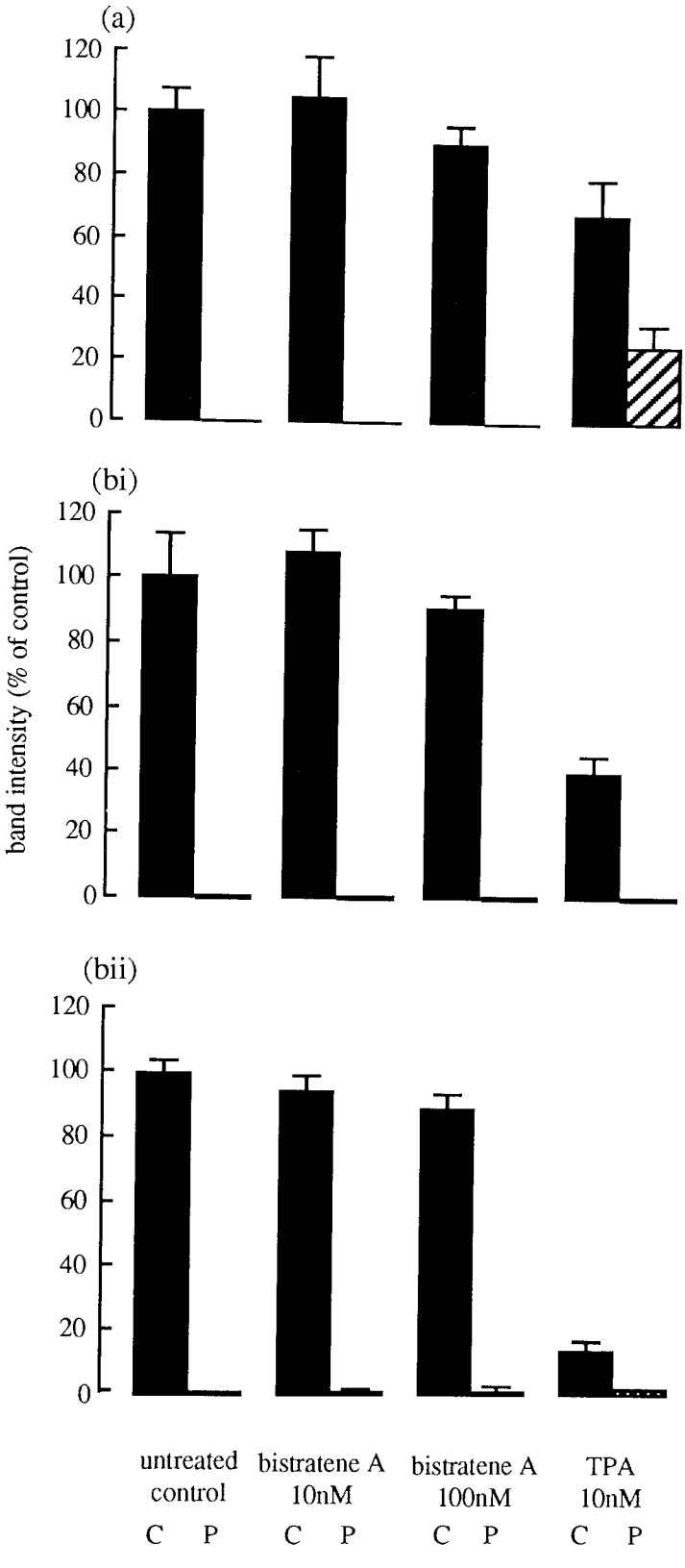


Figure 32. Laser Densitometry of Representative Blots Showing Effect of Bistratene A or Phorbol Esters on PKC Quantity and Distribution in A549 Cells.
Western blots from Figs. 31a and b were analysed by laser densitometry, taking 5 readings per band. Results were corrected by multiplying values by band width and dividing by protein measurements to give band intensity per unit protein. Results were then expressed as the mean percentage (± SD) of total PKC detected in vehicle-treated control samples.

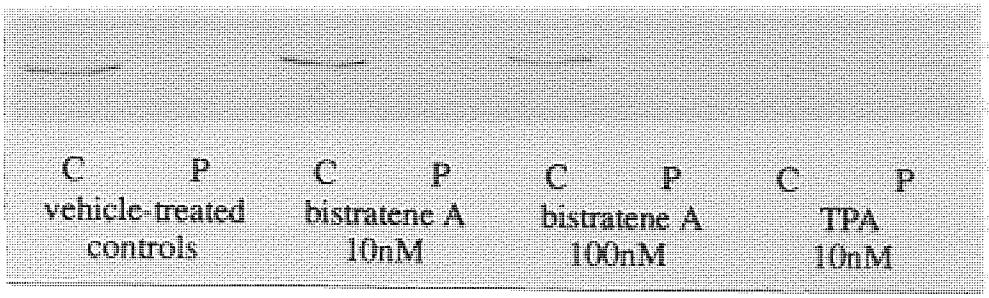


Figure 33. Western Blot Showing PKC α and/or β in Cytosolic and Particulate Fractions of MCF-7 Cells Treated with Bistratene A or TPA.
Cells were exposed to agents for 24 h. Samples were prepared from cell cytosol (C) and the detergent-soluble particulate fraction (P). Each lane was loaded with 50µl of sample. PKC (α and β isozymes) were detected by a colour reaction on nitrocellulose itself (See section 3.7.1. for methodology).

4.3.5. Modulation of PKC in vitro by Bistratene A, Phorbol Esters and Bryostatins.

The ability to induce translocation and downregulation of PKC is not a foolproof marker for PKC activation. Certain derivatives of the tumour promoter lyngbyatoxin A are able to activate PKC *in vitro* but do not induce its downregulation in intact cells (Basu *et al*, 1992), although the ability of these compounds to penetrate cell membranes was not proven unambiguously by the authors and this result may be due to low lipophilicity and lack of drug uptake preventing intracellular PKC downregulation. Transient PKC activation without downregulation has been described for diacylglycerols (Issandou *et al*, 1988, 1989). There are also reports of enhancement of phorbol ester-induced translocation by PKC inhibitors (Wolf *et al*, 1988, Bradshaw *et al*, 1992) and the compound thapsigargin does not modulate PKC but does induce its translocation (Kiley *et al*, 1992). Therefore experiments were conducted to assess the ability of bistratene A to activate PKC directly.

4.3.5.1. Assessment of PKC from A549 and MCF-7 Cell Cytosol-Cofactor Requirements and Specificity of Substrate.

A commercially available PKC assay kit was used for activation studies (section 3.9.1.). Preliminary experiments were performed to optimize the assay by serial dilution of cytosol with H8 buffer (section 2.2.2.) to eliminate interference by endogenous PKC inhibitors, ATPases and lipases. A much greater quantity of PKC activity was detected in A549 cells than MCF-7 cells. In both cell lines, serial dilution eventually caused a proportionate reduction in PKC activity, indicating that interfering cytosolic enzymes and inhibitors had been inactivated by dilution, as measured by incorporation of [γ-^{32}P]ATP into the peptide substrate (Table 2). Dilutions which were linearly related to PKC activity were used in further experiments (see section 3.9.1.).

A series of experiments were then devised to confirm the specificity of the peptide substrate for PKC by analysis of cofactor requirements (Table 3). Cytosolic PKC isotypes present in A549 and MCF-7 cells exhibited divergent spectra of cofactor requirements. In A549 cells, very little PKC was activated without TPA in the presence or absence of PS. The addition of TPA to the reaction mixture in the absence of PS increased the

incorporation of phosphate into the substrate, but to a lesser extent than in its presence (Table 3). Maximal PKC activity could not be attained using 3.2μM TPA without PS. In MCF-7 cell cytosol, there was substantial peptide phosphorylation in the absence of all cofactors, which was enhanced by the presence of PS and Ca^{2+} without TPA (Table 3). It was not clear whether this phosphorylation was caused by active PKC; certain isoforms may be influenced by the substrate to catalyse phosphate transfer in the presence of only PS and Ca^{2+}, or without activators or cofactors, the substrate behaving as a group A type (see section 1.3.3.). It was also possible that other activated kinases were phosphorylating the peptide substrate. The latter hypothesis is unlikely as the substrate is shown by the manufacturers to be PKC-specific.

The omission of Ca^{2+} from the reaction mixture and its replacement with 1mM EGTA unveiled surprising results. It was found that the PKC in A549 and MCF-7 cell cytosol did not require the presence of calcium ions for full activation (Table 3). This result was unexpected as A549 cells are known to contain PKC α (Hirai *et al*, 1989) and Western blots had previously detected PKC α or β (section 4.3.4.). The activity of α and β PKC is dependent upon calcium (Nishizuka, 1988). Therefore it was postulated that other calcium-independent isozymes may be present which would interfere with the assay, or PKC could be inexplicably degrading to PKM, which is fully active in the absence of cofactors. In order to test the latter hypothesis, PKC was isolated in PKC assay cell fractionation buffer (section 2.2.2.) which contains more protease inhibitors and β-mercaptoethanol than H8 and is recommended by Amersham. PKC activity was only $8.9 \pm 0.7\%$ (n=4) of the value for cytosol extracted into H8 using this alternative buffer. The reasons for this loss of activity are unclear but suggest an inhibitory function of PMSF or benzamidine on PKC. Clearly these results do not refute the use of H8 buffer but as H8 had been successfully used to extract whole PKC detectable by Western blots it was thought unlikely that PKM production was the explanation for the calcium-independent nature of PKC and this hypothesis was not pursued further.

The substrate used in PKC assays *in vitro* has an impact on cofactor requirements of the enzyme (section 1.3.3.). It was therefore possible that the peptide substrate used was of the group B variety, and even the so-called "calcium-dependent" PKC isozymes (α, β and γ) would have no requirement for Ca^{2+} in its presence. To test this hypothesis, A549 cell cytosol was subjected to DE52 and hydroxylapatite column chromatography (see sections 3.10 and 4.3.5.4. for technique and results) for separation of PKC isoforms. Activity of peaks was assessed in the presence of TPA 3.2μM and PS, but in the absence of Ca^{2+}, which was replaced with 1mM EGTA. Both peaks, including the peak corresponding to PKC α, were fully activated without Ca^{2+} (Fig.36c) and therefore it is likely that Amersham's peptide substrate is indeed a group B substrate. Identical ED_{50} values for TPA in the presence and absence of calcium using purified PKC α in this assay would have confirmed this hypothesis definitively.

4.3.5.2. Activation of PKC from Cytosolic Cell Fraction by Bistratene A or TPA .

A549 and MCF-7 cell cytosol was isolated (section 3.5.1. method A) and used as a source of PKC to test the ability of bistratene A and TPA to activate the enzyme. A549 cell cytosolic PKC was not activated in the presence of Ca^{2+} and PS alone. The addition of 3.2nM TPA increased PKC activity to 62.7 ± 13.9% of maximal which increased to 100% at 32nM. Maximal PKC activity in the presence of TPA was 14-fold the activity seen in its absence. In contrast bistratene A did not activate PKC with concentrations up to 10μM (Fig.34a). As discussed in section 4.3.5.1., kinases in MCF-7 cell cytosol showed substantial peptide phosphorylation in the absence of PS and TPA (Table 3). A significant increase in kinase activity occurred with the addition of 32nM TPA to the reaction mixture ($p<0.025$). This was further enhanced to maximal levels with a concentration of 3.2μM, producing a 1.6-fold increase in activity compared with activity in the presence of Ca^{2+} and PS alone. Again, bistratene A (10nM-1μM) did not augment substrate peptide phosphorylation in the absence (results not shown) or presence of PS and Ca^{2+} (Fig.34b).

4.3.5.3. Inhibition of PKC by Bistratene A.

Certain PKC inhibitors have been shown to induce some cellular properties reminiscent of PKC activators. The PKC inhibitor staurosporine elicited a differentiation response with the formation of cornified envelopes in normal mouse keratinocytes similar to that seen after treatment with TPA and the effects of both agents could be reversed by bryostatin 1 (Dlugosz *et al*, 1991). Ornithine decarboxylase activity in mouse epidermis is induced by both TPA and staurosporine and the two agents act synergistically on this response (Jiang *et al*, 1992). HL-60 leukaemia cells can be induced to differentiate along the monocyte/macrophage pathway by TPA (Rovera *et al*, 1979) and also by the PKC inhibitor K252a (Taoka *et al*, 1990), and proliferation of the A549 cell line is inhibited by PKC activators (Gescher and Reed, 1985) and inhibitors (Bradshaw *et al*, 1992). It was therefore of interest to assess the ability of bistratene A to inhibit PKC activity. PKC from A549 and MCF-7 cell cytosol was not inhibited by bistratene A from 10nM-1μM (Table 4) using assay conditions in which staurosporine was able to inhibit PKC with an IC_{50} of 6.1nM (Bradshaw *et al*, 1992).

4.3.5.4. Separation of PKC Isozymes in A549 Cell Cytosolic and Particulate Fractions.

A549 cells have been reported to contain predominantly PKC α and possibly other isozymes (Hirai *et al*, 1989). Activation of minor amounts of other PKC isotypes could play an important part in their growth inhibitory response to bistratene A. A549 cell cytosol was rich in PKC activity (Table 2), necessitating 20-40x dilution before analysis of the ability of agents to activate the enzyme. Thus the effect of bistratene A on isoforms other than PKC α could have been overlooked using the cytosolic fraction directly in the PKC assay (Fig.34a), minor isoform activation being masked by the overwhelming intensity of PKC α activation. It is also possible that bistratene A activates PKC isoforms in cellular locations other than the cytosol. To test these hypotheses, PKC isoforms were isolated by DE52 and hydroxylapatite column chromatography (section 3.10) from cell cytosol and membrane preparations. PKC activity was analysed in each fraction from the DE52 column using the Amersham kit with TPA 3.2μM, PS and Ca^{2+}. Enzyme was eluted as one major peak, which merged with a smaller peak at higher salt concentrations, in

cytosolic and particulate fractions (Fig.35). Using Amersham's specific peptide as substrate, activity was found to be greater than that seen with histone IIIS. Later work revealed that different batches of histone had widely different phosphorylation capacities, and therefore samples of histone were batch-tested and the most effective batch was used thereafter. The minor high salt peak contained a kinase, possibly PKM, which was substantially active in the absence of all cofactors using both substrates (Figs.35a and b). However this peak may be PKC as the ζ isoform phosphorylates histone H1 in the absence of cofactors (Ono *et al*, 1989) and nPKCs are eluted at higher salt concentrations using DE52 anion exchange than cPKCs (Wetsel *et al*, 1992). Therefore, enzyme-containing fractions which generated both peaks were pooled and subjected to hydroxylapatite chromatography using a phosphate gradient. Chromatograms were compared to those observed with a human brain preparation (Fig.39). Cytosolic and particulate fractions exhibited the same pattern of peaks with very little activity in the particulate fraction (Fig.36). As expected, using the Amersham kit for PKC activity in the presence of 3.2μM TPA, the major peak was detected in the α (type III) position, at 120-170mM phosphate. The identity of this peak was confirmed by pooling these fractions, concentrating the peak (section 3.10.2.) and immunodetection of PKC α using isozyme-specific antibodies after Western blotting (see insert, Fig.36a). A smaller peak was also found in the β (type II) position at 60-90mM phosphate using the Amersham peptide as substrate, but it was not detected with histone IIIS (Fig.36a). Both peaks were also observed when TPA was substituted for bryostatin 1 (3μM) and the Amersham peptide was used as substrate (Fig.36b).

4.3.5.5. Effect of Bistratene A on PKC Isozyme Activity in A549 Cells.

The ability of bistratene A (50nM and 3μM) to activate PKC in fractions obtained from hydroxylapatite column chromatography was tested using the Amesham kit with peptide (Fig.37a) and histone IIIS as substrate (Fig.37b). All background phosphorylation activity due to PS or Ca^{2+} was removed to focus on activity induced by TPA or bistratene A alone. It was found that bistratene A did not induce an increase in PKC activity of either peak

using the Amersham kit at either concentration. TPA only augmented activity in the major peak (Fig.37a). However, in the laboratory of Dr. Janet Lord at Birmingham University using an alternative assay with histone IIIS as substrate and PS in the form of vesicles, not micelles, two new peaks were detected with bistratene A (50nM) (Fig.40b). I was unable to repeat this result in our laboratory, nor could this result be repeated using the Amersham kit modified to use histone IIIS as phosphoacceptor (Fig.37b).

4.3.5.6. Effect of Bistratene A on PKC Isozyme Activity in HL-60 Cells.

DE52 and hydroxylapatite column chromatography were used to separate the PKC isozymes in HL-60 leukaemia cell cytosol. PKC assays (Amersham) were conducted on alternate DE52 fractions, again detecting one large peak of activity followed by a minor peak, as in A549 cells. The PKC activity of fractions obtained from hydroxylapatite column chromatography was evaluated using the Amersham kit with micellar PS and the alternative assay using vesicular PS in the presence of TPA. Two major peaks were detected in the α and β positions with the Amersham assay (Fig.38a). Using the alternative assay, three major and three minor peaks were detected (Fig.39). TPA was replaced with 3μM and/or 50nM bistratene A in both assays. Using the modified Amersham assay, with peptide or histone IIIS as substrate, TPA induced a substantial increase in activity of both peaks. Conversely, bistratene A caused no increase in PKC activity above levels produced in the presence of PS and Ca^{2+} alone (Fig.38b and c). Using the alternative assay, bistratene A induced the emergence of two peaks (Fig.40a, courtesy of Dr. Janet Lord). The histone phosphotransferase activity of both peaks was dependent upon bistratene A concentration, but kinase activity progressively decreased when high concentrations of agent were employed (Fig.41, courtesy of Dr. J. Lord).

4.3.5.7. Western Blot Analysis of PKC Isozymes Present in A549 and MCF-7 Cells.

Experiments described in section 4.3.4. demonstrated that A549 and MCF-7 cells contain PKC α and/or β. A large peak was detected in the α and a smaller peak in the β position of the fractions of A549 cell cytosol eluted from the hydroxylapatite column when a PKC-specific peptide was used as substrate, but only one peak was observed in the α position

when histone IIIS was employed (Fig.36a). The nPKC isoform ε elutes off hydroxylapatite in the same region as the β isoform (Konno et al, 1989, Powell et al, 1992) and PKC δ and ζ elute between the α and β isoforms, peaking at 110 and 100mM potassium phosphate respectively (Ogita *et al*, 1992, Mizuno *et al*, 1991, Nakanishi and Exton, 1992). Therefore the identity of the minor peak from the hydroxylapatite separation was uncertain. Different assays detected different peaks of activity from the hydroxylapatite column using TPA and bistratene A as activators and hence the inconsistency of results demanded a definitive method for resolution of PKC subspecies present in A549 and MCF-7 cells. The isoform content of each cell type was analysed using a range of isozyme-specific antibodies on cytosolic, particulate and nuclear cell fractions (section 3.7.2.). As expected, A549 and MCF-7 cells contained PKC α, which was more abundant in A549 cells per mg protein and was predominantly located in the cytosol of both cell lines (Fig.42). PKC β and γ were not detected in either cell type when probed with monoclonal (Seikagaku) and polyclonal (Gibco) antibodies. Similarly, PKC δ and η were not detected. Rat lung cytosolic/particulate and nuclear fractions were used as positive controls for PKC η as low levels are present in brain but it is abundant in skin and lung (Osada *et al*, 1990). PKC η (L) was present almost exclusively within the nuclei of lung tissue, as has been reported for skin cells (Greif *et al*, 1992). It was not detected in the lung-derived A549 cell line which is consistent with the fact that these cells are derived from alveolar type II cells and PKC η has been found only in bronchial epithelia, not alveolar cells of lung (S. Osada, personal communication). MCF-7 and A549 cells expressed PKC ε and ζ , predominantly in cytosol. Both isozymes were more abundant in MCF-7 cells. Hydroxylapatite column chromatography of A549 cell cytosol suggested that PKC α was the only isozyme present in large quantity. The position of the minor peak (Powell *et al*, 1992) and the ability of enzymes therein to phosphorylate the peptide substrate but not histone (Fig.36a, Schaap *et al*, 1989) is consistent with the notion that

this peak is PKC ε. PKC ζ was not isolated by this separation technique.

The antibody for PKC ζ immunodetected two bands of 80 and 70kDa molecular weight in both cell types, both of which could be blocked completely by incubation of antibody with the complementary peptide from which it was raised (see section 3.7.2. for methodology). To ascertain whether the antibody was binding specifically to the other major isoform with a molecular weight of 80kDa in these cells, PKC α, or whether both bands were PKC ζ but were different splice variants (see section 1.3.1), two experiments were devised. Firstly, a crossover experiment was performed, in which an attempt was made to block the band detected by the PKC α antibody with the PKC ζ antigenic peptide, and to block the bands detected by the PKC ζ antibody with the PKC α antigenic peptide. Blots were stripped and reprobed so that exactly the same bands could be compared. It was found that the two peptides were not interchangeable in their blocking capacity . The PKC ζ antigenic peptide did not block the detection of PKC α. Nor did the PKC α antigenic peptide block either band detected using the PKC ζ antibody (Fig.43a). This implied that the two antibodies did not recognise the same site on PKC α to cause cross-reactivity. The PKC ζ antibody could bind to a site common to both isozymes that is different to the unique site recognised by the PKC α antibody.

A second experiment was conducted, looking at the ability of the ζ and α antibodies to recognise PKC present in A549 cell cytosol and mouse brain, and PKC α semi-purified and concentrated from hydroxylapatite column chromatography fractions (section 4.3.5.4.). After subjecting samples to SDS/PAGE and blotting proteins onto nitrocellulose, they were probed for PKC α, then stripped and reprobed for PKC ζ to assess cross-reactivity (Fig.43b). Results confirmed that PKC α was indeed the upper

80kDa band detected with the ζ-specific antibody. Recently it has been confirmed that only the lower band detected by the Gibco antibody is PKC ζ (Tsutsumi *et al*, 1993).

Table 2. Effect of Dilution on PKC Activity in Cell Cytosol.

Dilution factor	Picomoles of phosphate transferred to peptide per min	
	A549 Cells	MCF-7 Cells
1	3.15 ± 0.11*	0.97 ± 0.01*
5	11.37 ± 0.18	ND
10	12.36 ± 0.34	1.57 ± 0.0
15	8.88 ± 0.74	ND
20	7.73 ± 1.29	0.80 ± 0.09
40	4.63 ± 0.53	0.47 ± 0.03
100	ND	0.22 ± 0.0

*Undiluted cytosol had a protein concentration of 1.5-2.5mg/ml, which was standardised to 1mg/ml for comparison between the two cell lines. PKC activity was determined using the Amersham kit with peptide substrate and reaction in the presence of PS, $Ca^{2+}$and 3.2μM TPA. Values are the mean ± SD of 2 or 3 determinations from one experiment, representative of 3.

Table 3. PKC Activity in Cell Cytosol-Dependence on TPA and the Cofactors Ca^{2+} and PS.

TPA	-	-	3.2nM	3.2μM	3.2μM	3.2μM	3.2μM
PS	-	+ (reconstituted)	-	-	+ (reconstituted)	+	+
Ca^{2+}	+	+	+	+	+	+	-
A549	7.9 ± 4.9	6.6 ± 2.4	28.5*	63.3*	94.5 ± 4.6	100 ± 6.4	110.8 ± 12.2
MCF-7	44.7 ± 14.2	60.8 ± 11.3	ND	ND	95.3 ± 5.1	100 ± 3.9	90.6*

PKC assays were conducted with (+) or without (-) PS and TPA at the indicated concentrations, in the presence of calcium acetate 1mM. The assay component containing mixed micelles of PS and Triton X 100 was reconstituted as in section 3.9.1. and the addition of TPA 3.2μM provided an assay mix which which was not significantly different to that made by the manufacturers (see above : $p<0.05$). In some experiments, the assay component containing calcium acetate was excluded from the reaction mixture and replaced with 12mM EGTA, giving a final reaction mix concentration of 1mM EGTA. Results were expressed as a percentage of maximal PKC activity obtained by using the kit with 3.2μM TPA as directed by the manufacturers. Values are the mean ± SD of 2 or 3 experiments, each conducted in triplicate : *values were obtained from a single determination.

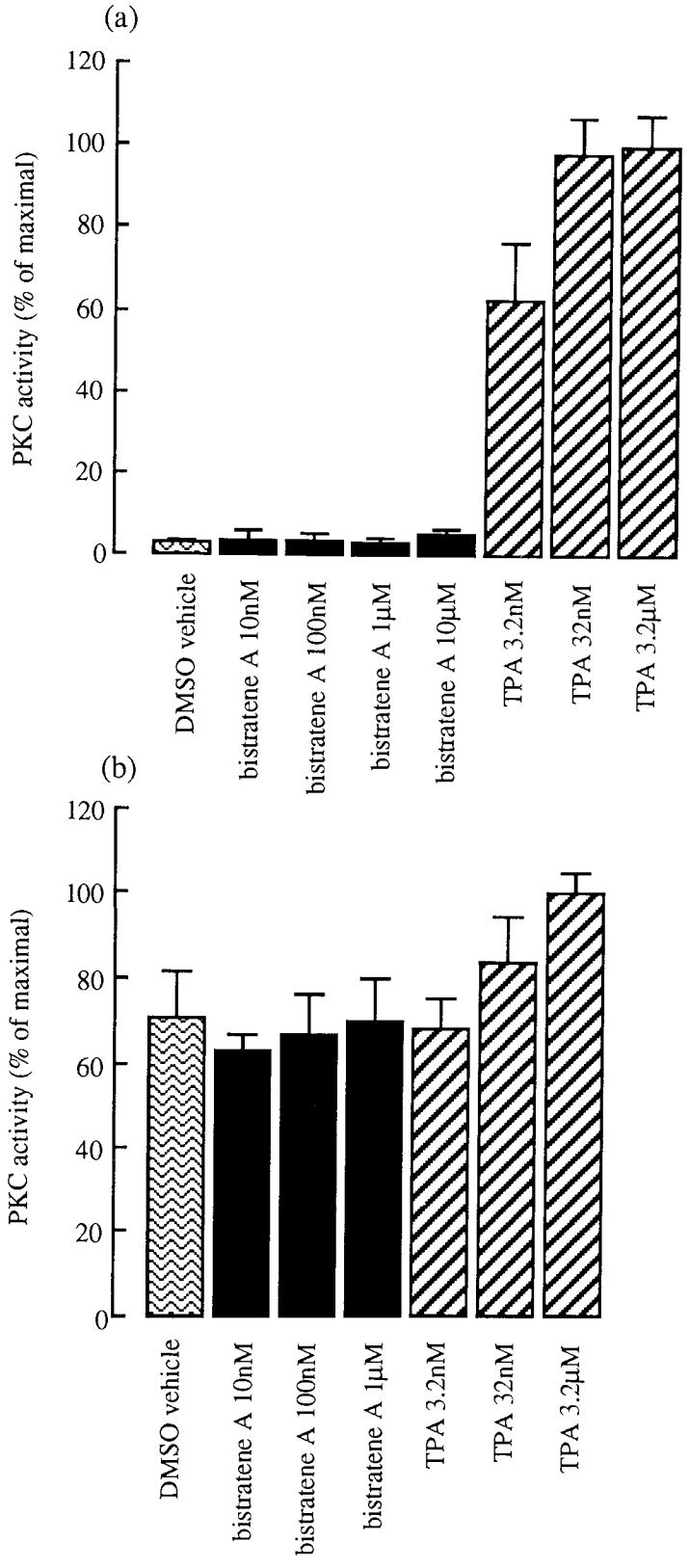


Figure 34. Activation of PKC from Cell Cytosol by Bistratene A and TPA .
The effect of bistratene A and TPA on PKC activity in (a) A549 and (b) MCF-7 cell cytosol was assessed in the presence of phosphatidylserine (PS) and calcium acetate (1mM). Results are expressed as a percentage of maximal PKC activity obtained using reconstituted micelles (section 3.9.1.) and 3.2μM TPA for stimulation. Values are the mean ± SD of 2 (figure 34b) or 3 (figure 34a) experiments, each conducted in triplicate.

Table 4. Effect of Bistratene A on Activity of PKC from A549 or MCF-7 Cells Stimulated Maximally by TPA (3.2μM) †

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 section 3.9.1.
*Values are the mean ± SD of 3 experiments, each conducted in triplicate.

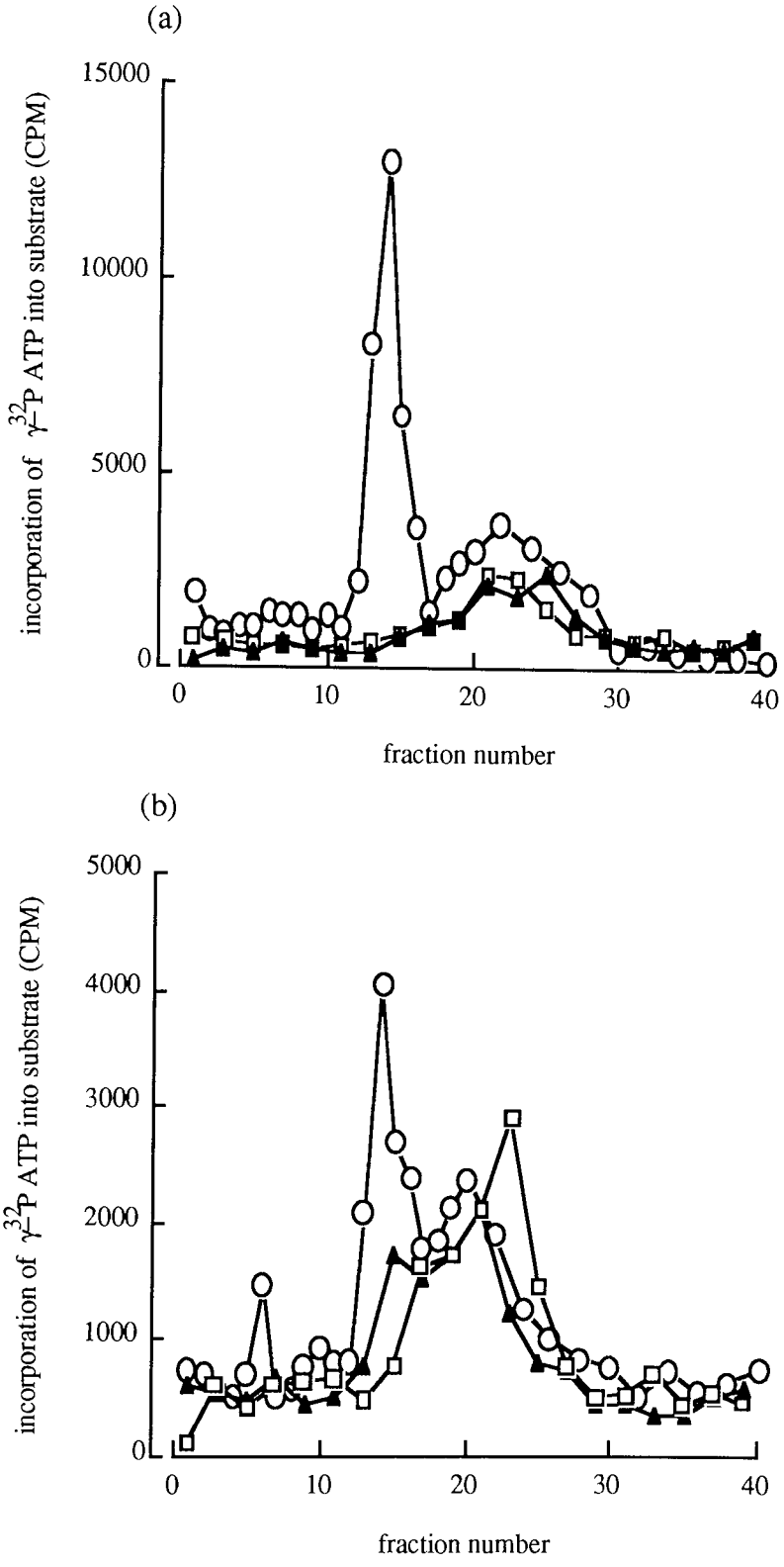


Figure 35. Separation of PKC on DE52 Anionic Exchange Resin.
A549 cell cytosol was prepared from 20 sub-confluent 14cm diameter dishes (section 3.5.1.) and passed through a DE52 column with NaCl gradient (0-800mM). Each of 40 fractions was tested for PKC activity using the Amersham kit with (a) peptide and (b) histone IIIS as substrate in the presence of TPA(3.2μM), PS and Ca^{2+} (open circles), in the presence of PS and Ca^{2+} alone (triangles) or in the absence of all cofactors (squares). Results are representative of 5 separate experiments.

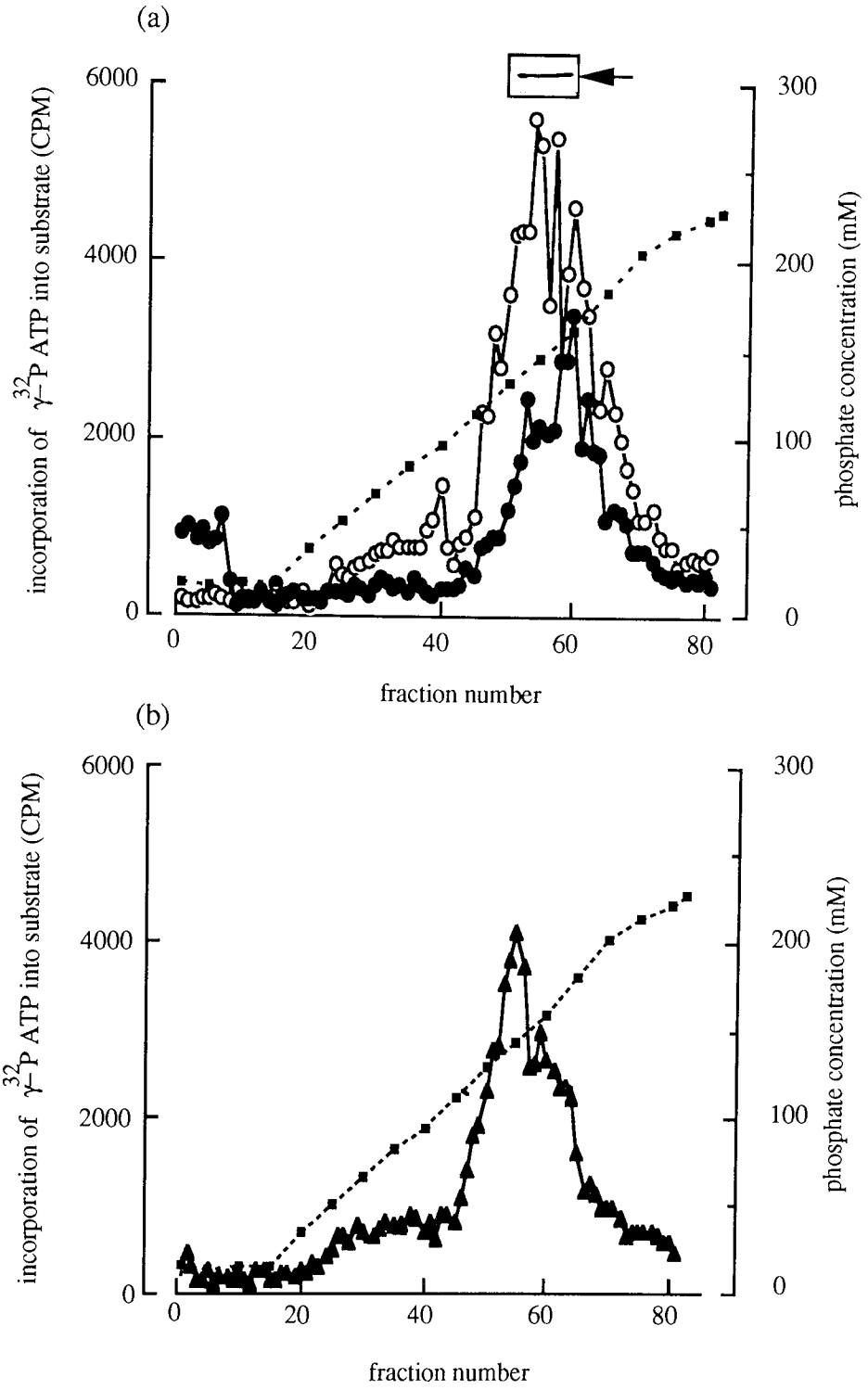
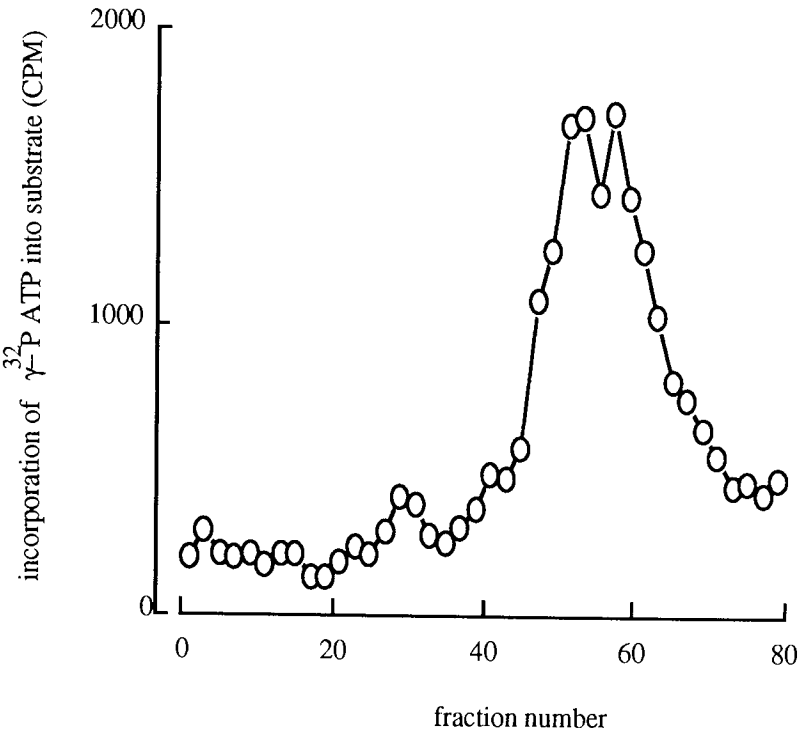
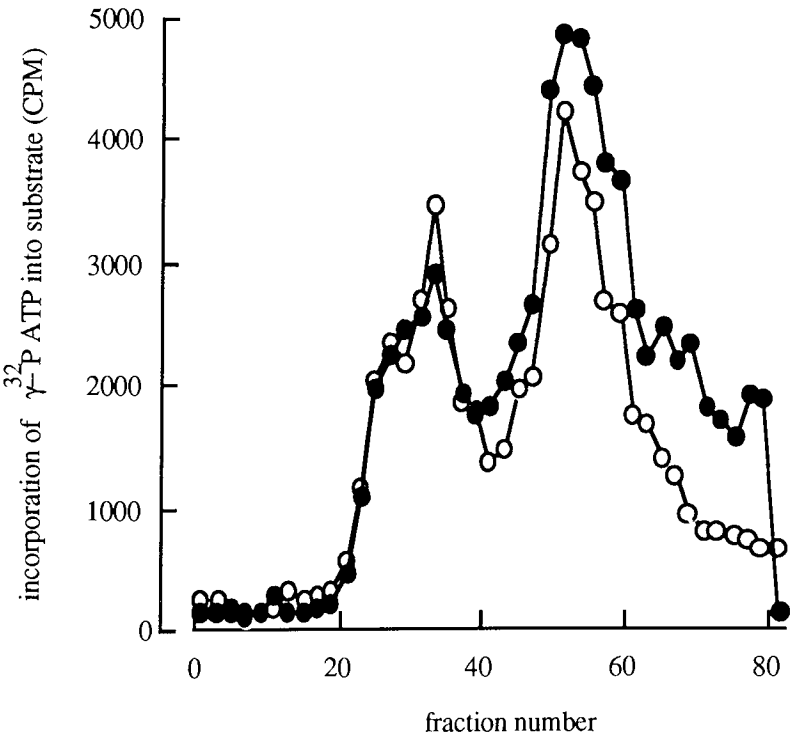


Figure 36. Separation of A549 Cell PKC Isozymes on Hydroxylapatite.
Cell cytosol was eluted from a hydroxylapatite column with a phosphate gradient and fractions were assessed for PKC activity using the Amersham kit (section 3.10)((a), (b), (d)). Overleaf, (c) depicts the activity of fractions obtained from the elution of detergent-soluble membrane proteins of A549 cells. Activity in the presence of TPA (3.2μM), PS and $Ca^{2+}$is represented by open circles throughout. Activity of fractions with histone IIIS (600μg/ml) as substrate instead of the Amersham peptide is shown in (a) (closed circles). In (b), TPA was replaced with 3μM bryostatin 1(triangles) and in (d), using cell cytosol from a separate experiment, activity was assessed in the absence of Ca^{2+}(closed circles). The phosphate gradient is represented by a dotted line. Results are representative of 2-5 determinations for each assay.

(c)


2000
1000
0
incorporation of $^{32}\gamma$-P ATP into substrate (CPM)
0
20
40
60
80
fraction number

(d)


5000
4000
3000
2000
1000
0
incorporation of $^{32}\gamma$-P ATP into substrate (CPM)
0
20
40
60
80
fraction number

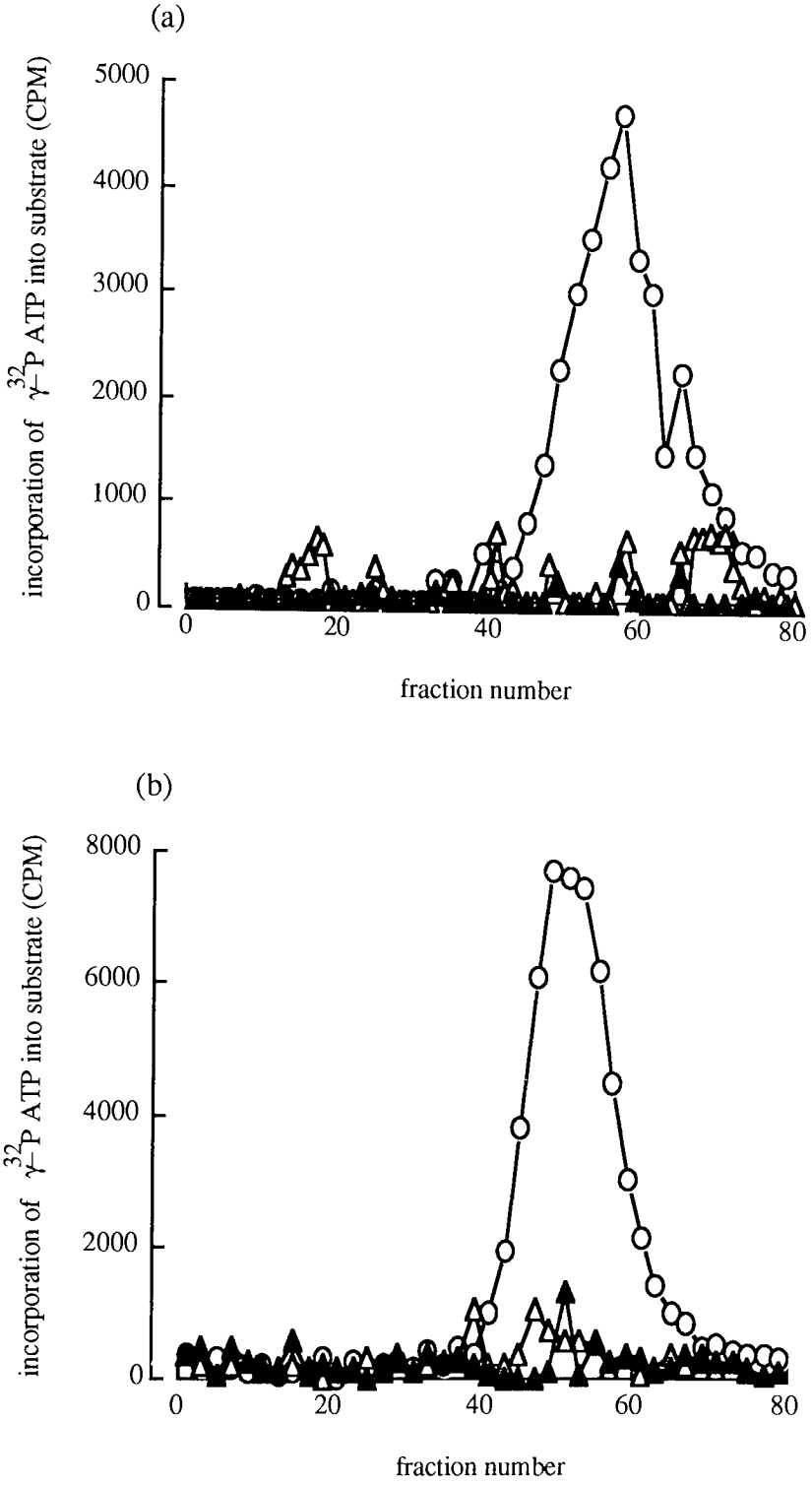


Figure 37. Activation of PKC Isozymes in A549 Cells by Bistratene A.
A549 cell cytosol was eluted from a hydroxylapatite column with a phosphate gradient (section 3.10.). PKC activity was assessed in alternate fractions using Amersham's assay with (a) peptide or (b) histone IIIS as substrate. Activity was tested in the presence of 3.2μM TPA, Ca^{2+} and PS (circles). TPA was removed from the assay and replaced with 3μM (open triangles) or 50nM (closed triangles) bistratene A. Activity in the presence of Ca^{2+} and PS was subtracted from all values to show changes in activity initiated by TPA or bistratene A alone. Results are representative of 3 separate experiments.

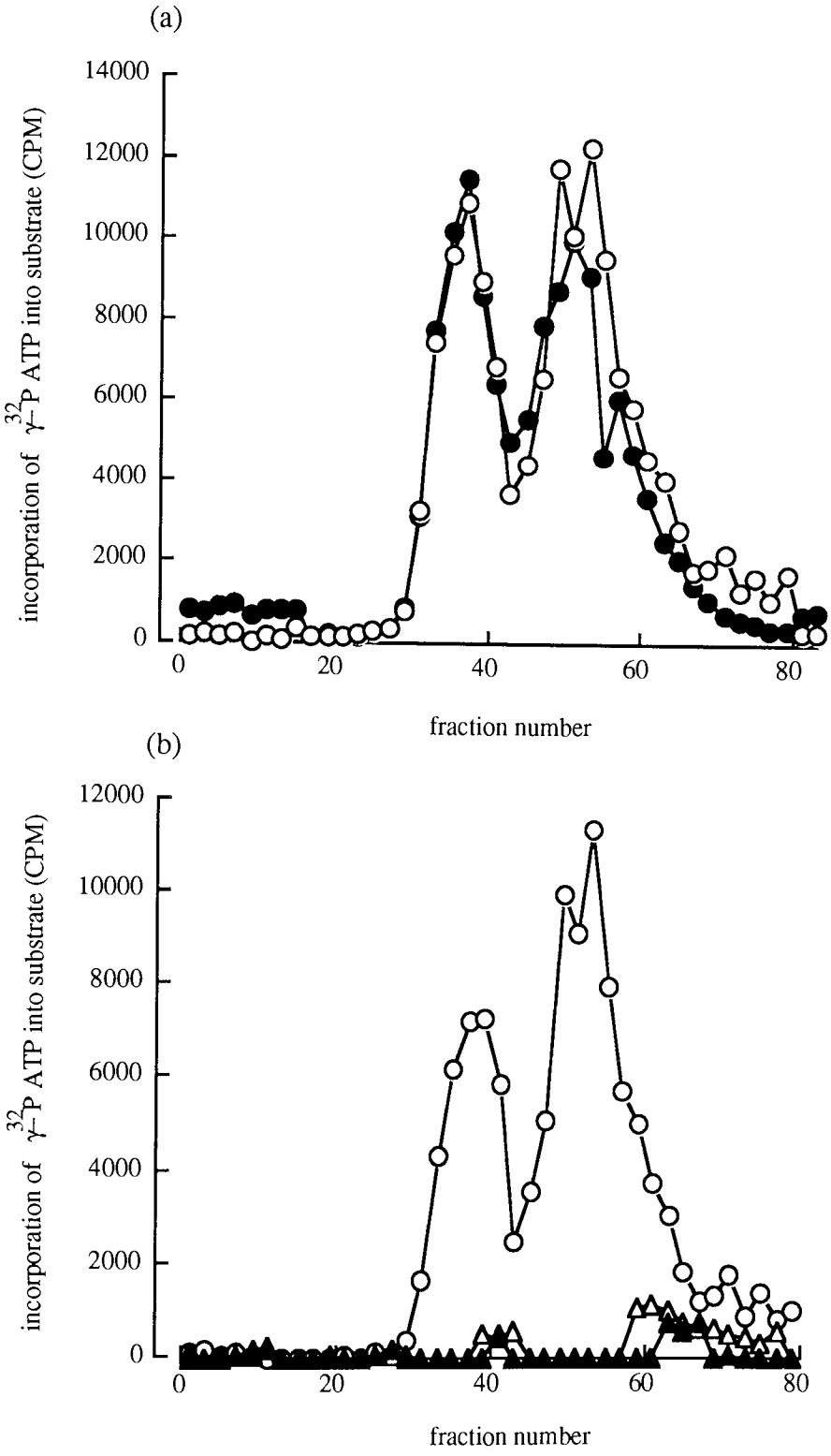
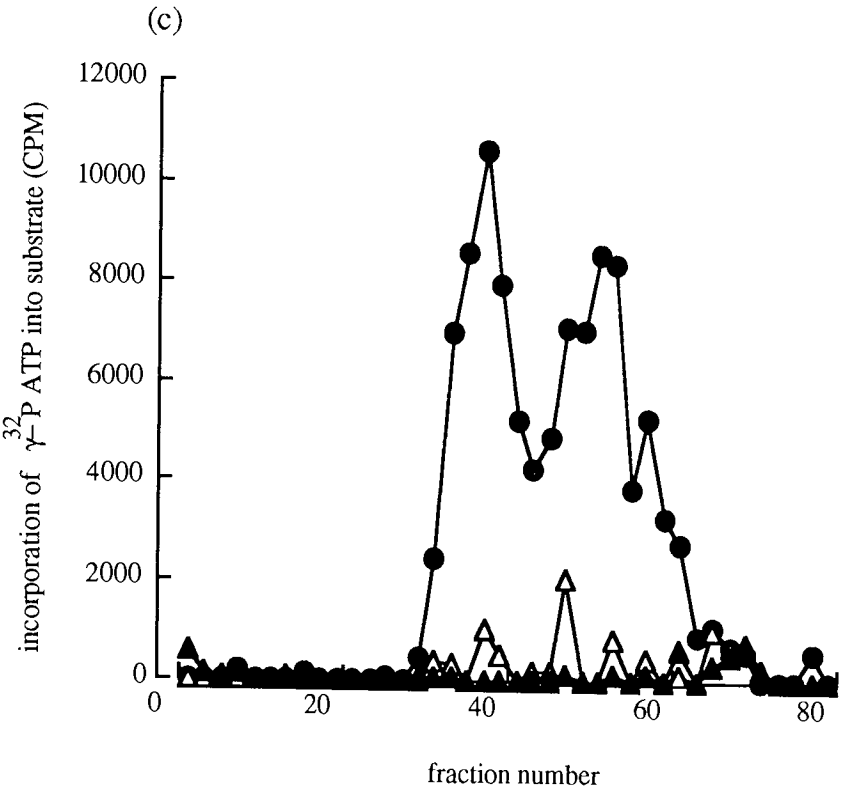


Figure 38. Activation of PKC Isozymes by Bistratene A in HL-60 Cells.
HL-60 cell cytosol was eluted from hydroxylapatite with a phosphate gradient (section 3.10.). PKC activity was assessed in alternate fractions using Amersham's specific peptide substrate (open circles) or histone IIIS (closed circles) in the presence of TPA (3.2μM), PS and Ca^{2+}(a). Activity induced by bistratene A 50nM(closed triangles) or 3μM (open triangles) was assessed using the peptide (b) or histone IIIS (c, overleaf) as substrates. In (b) and (c), activity in the presence of $Ca^{2+}$and PS was subtracted from all values to show changes in activity initiated by TPA or bistratene A alone. Results are representative of 2 separate experiments.


(c)
12000
10000
8000
6000
4000
2000
0
0
20
40
60
80
incorporation of $^{32}\gamma$-P ATP into substrate (CPM)
fraction number

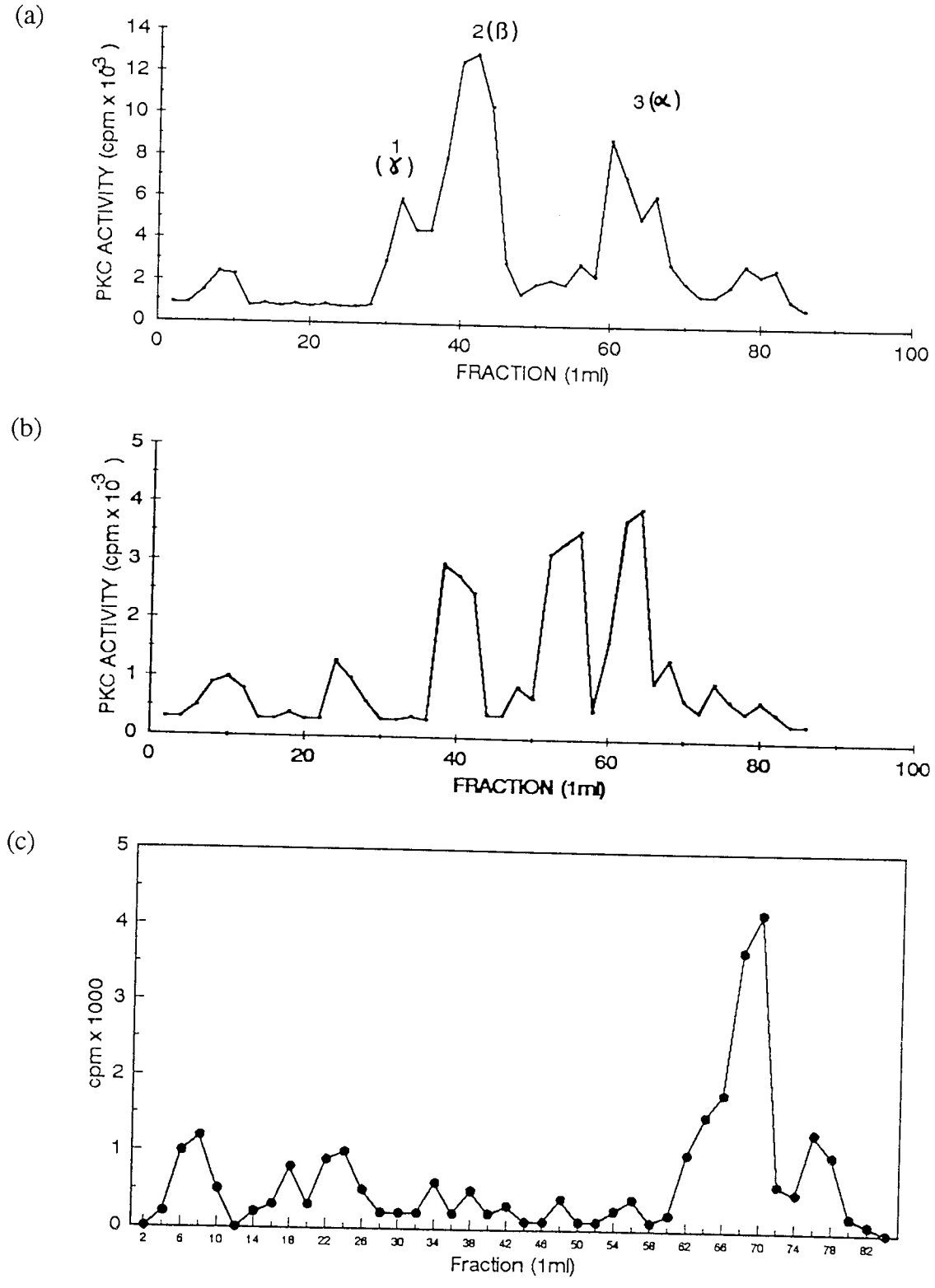
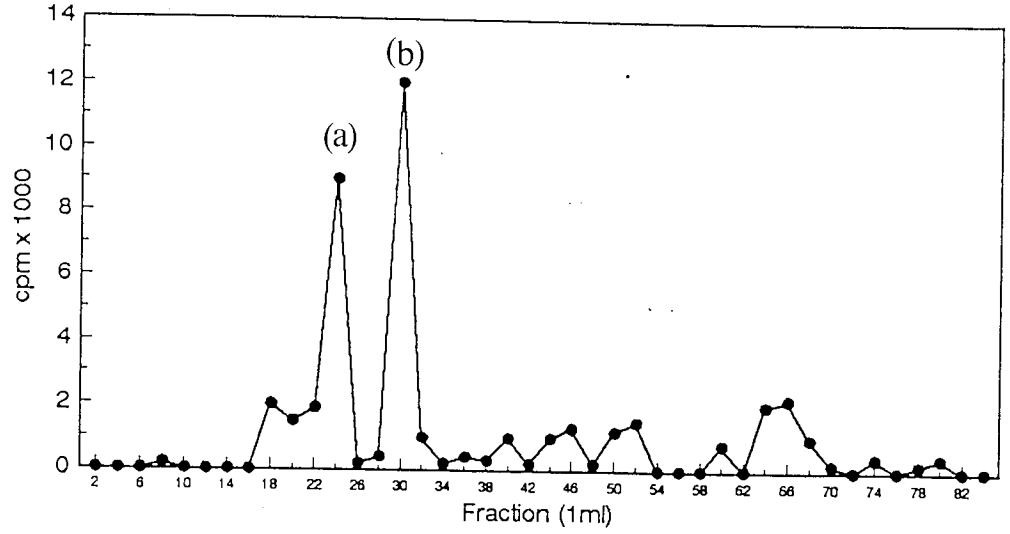


Figure 39. Separation of PKC Isoforms from Human Foetal Brain, HL-60 and A549 Cells (courtesy of Dr. J. Lord).

Cytosol from (a) human foetal brain tissue, (b) HL-60 cells and (c) A549 cells was separated by hydroxylapatite column chromatography (section 3.10). Alternate fractions obtained from the column were tested for PKC activity using the alternative assay, with TPA (50nM), Ca^{2+} and PS in vesicles. Precipitated histone IIIS was centrifuged and washed in the final stage of the assay (section 3.9.2.). Activity in the presence of Ca^{2+} and PS alone is subtracted from results.

(a)



(b)

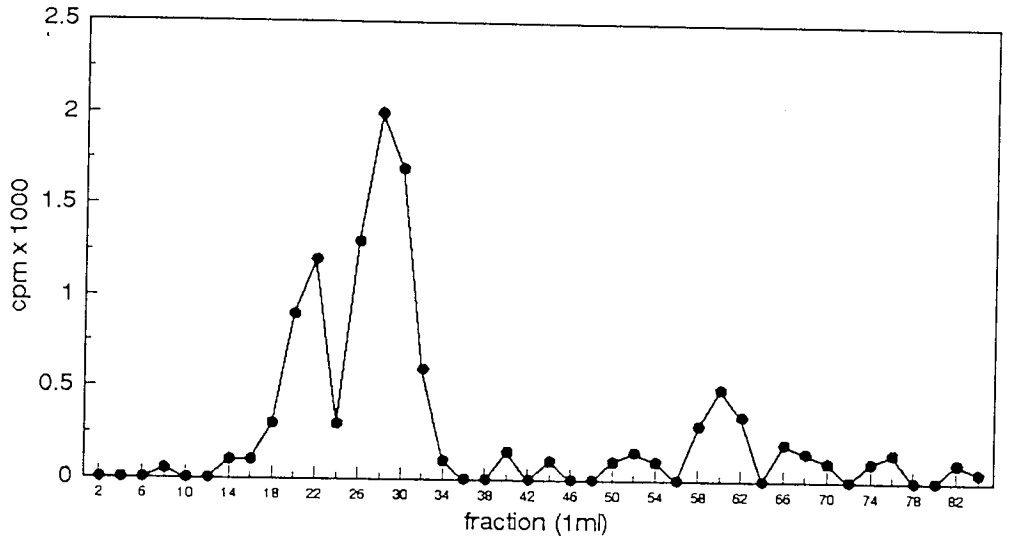


Figure 40. Activation of Kinases Within Hydroxylapatite Fractions Obtained from HL-60 and A549 Cell Cytosol by Bistratene A (courtesy of Dr. J. Lord).
Alternate fractions from hydroxylapatite column chromatography of HL-60 (a) and A549 cell cytosol (b) were tested for PKC activity using the alternative assay (section 3.9.2.) in the presence of bistratene A (50nM), Ca^{2+} and PS in vesicles. Activity in the presence of PS and Ca^{2+} alone was subtracted from results.

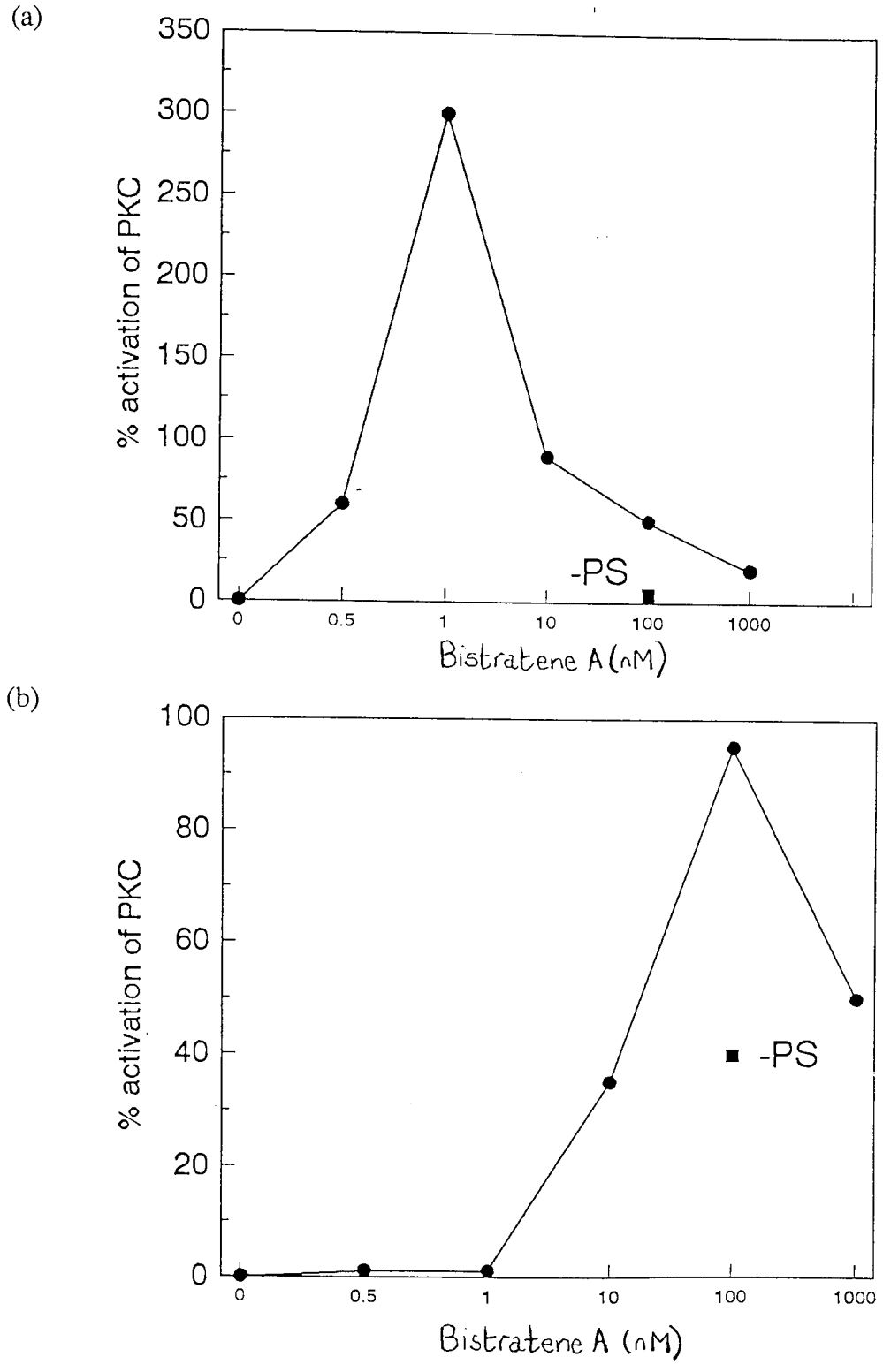


Figure 41. Dose Dependency of Activation of HL-60 Cell Kinases by Bistratene A (courtesy of Dr. J. Lord).
Dose-dependency of histone phosphotransferase activity was assessed for peaks (a) and (b) induced by bistratene A in fractions from the hydroxylapatite chromatographic separation of HL-60 cytosol (Fig.40a). Activity was determined using the alternative PKC assay (section 3.9.2.) in the presence of Ca^{2+}, PS in vesicles and bistratene A (0.5-1000nM). Activity in the absence of PS with 100nM bistratene A was also determined.

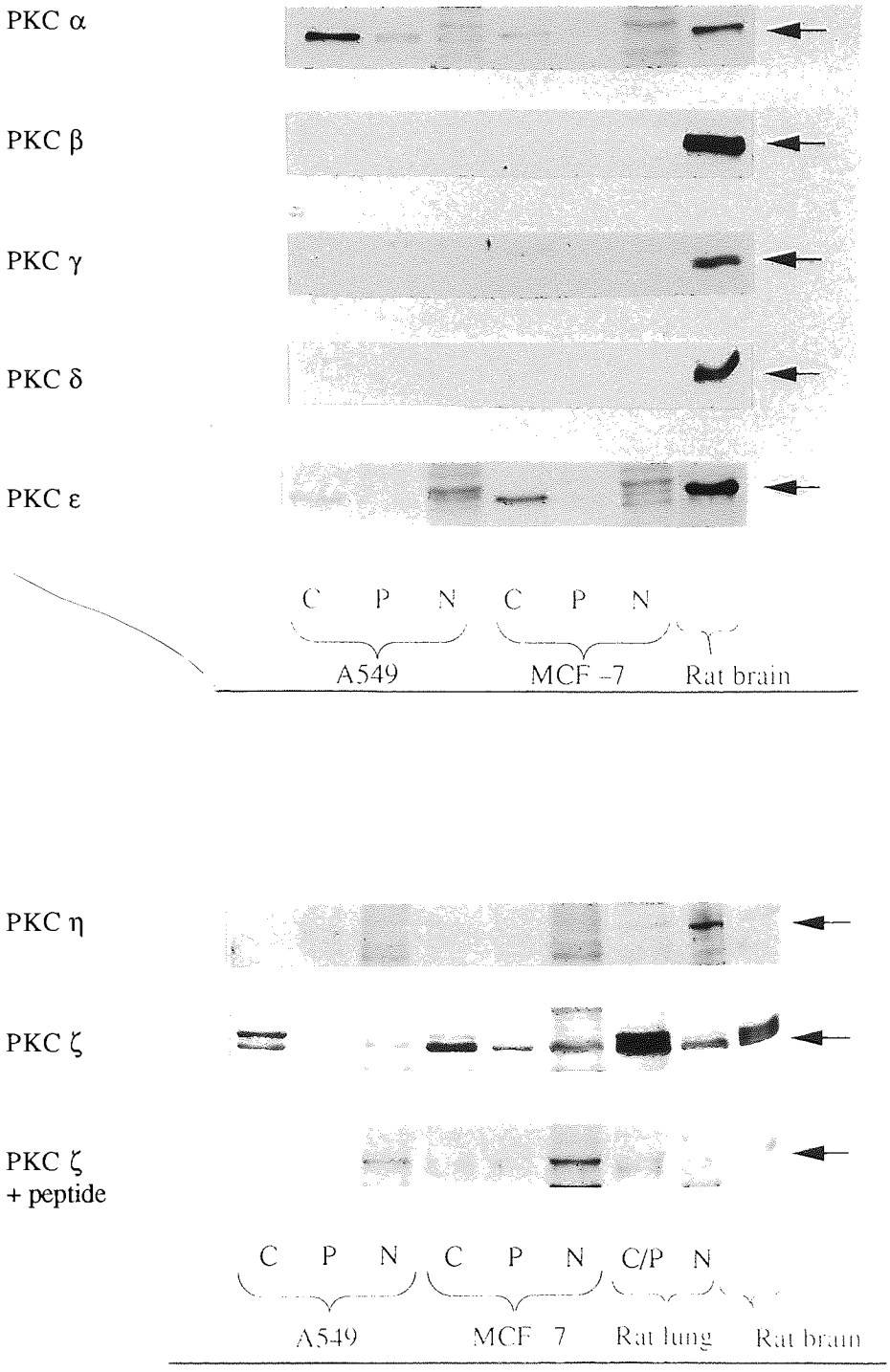


Figure 42. Expression of PKC Isozymes in Cytosolic, Particulate and Nuclear Fractions of A549 and MCF-7 Cells Detected by Western Blotting.
Cells were separated into cytosolic (C), particulate (P) and nuclear (N) fractions which were subjected to SDS/PAGE and Western blotting. PKC isozymes were detected with specific antibodies (section 3.7.2.). Rat brain and lung tissues were used as positive controls. Antibodies obtained from Gibco were supplied with the peptide from which they were raised. Co-incubation of peptide and antibody resulted in the blockage of PKC-specific bands detected. Results of co-incubation experiments are shown for PKC ζ .

Figure 43. Identification of PKC Isoforms Detected by an Antibody to PKC ζ. Analysis of Possible Cross-Reactivity of Antibody with PKC α.

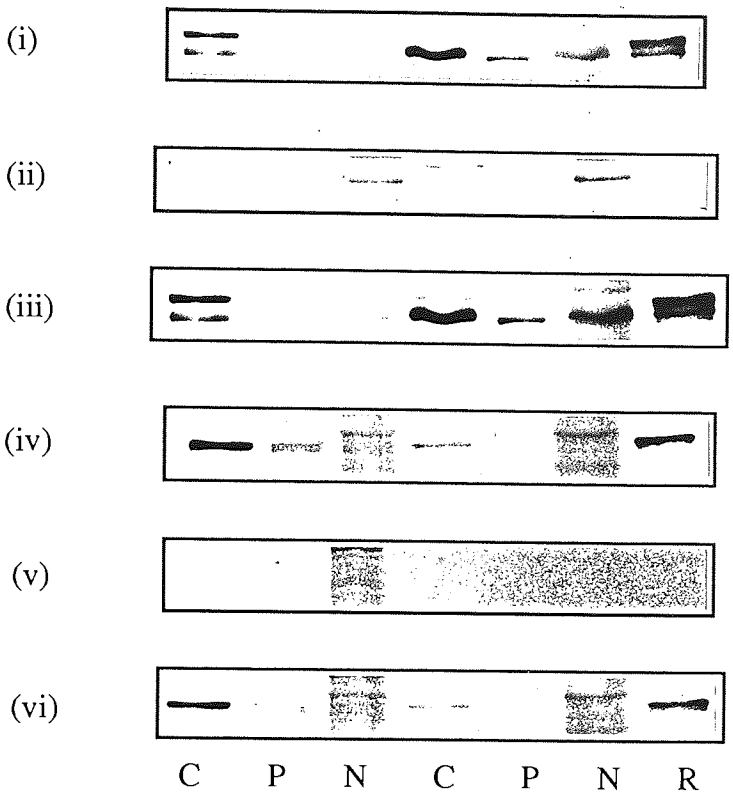


Figure 43a.
Samples of cytosolic (C), particulate (P) and nuclear (N) fractions of A549 and MCF-7 cells and rat brain extract (R) were subjected to SDS PAGE and Western blotting. Isozymes were detected with specific antibodies (Gibco) to PKC ζ (i) and α (iv). Non-specific binding was differentiated from specific binding by incubating blots with antibody to PKC ζ (ii) and α (v) with the complementary antigenic peptides from which they were raised. Cross-reactivity between the binding sites of the α and ζ–specific antibodies was tested by incubating the antibody to PKC ζ with the PKC α peptide (iii) and the antibody to PKC α with the PKC ζ peptide (vi). Results are representative of two determinations.

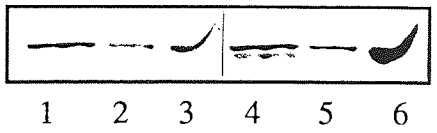


Figure 43b.
Samples of A549 cell cytosol (lanes 1 and 4), PKC α semi-purified from hydroxylapatite column chromatography (section 3.10.2.) (lanes 2 and 5), and rat brain tissue (lanes 3 and 6) were prepared and subjected to SDS/PAGE and Western blotting. Lanes 1 to 3 were probed with a monoclonal antibody to PKC α (TCS) and lanes 4 to 6 were probed with an antibody to PKC ζ (Gibco). Results are representative of two experiments.

4.4. Effect of Bistratene A on Tyrosine Kinase Activity.

Bistratene A was found to be ineffective at inhibiting or activating PKC, yet was capable of initiating the intracellular phosphorylation of proteins (section 4.3.2.). The possibility of involvement of another kinase in these effects was tested. Cells contain a host of tyrosine kinases, which are mostly membrane-bound receptors or proteins with src homology (Hunter, 1990, 1991). An assay using the EGF receptor as a source of tyrosine kinase was used. A549 and MCF-7 cells both contain the EGF receptor (Gardner *et al*, 1990, Koga *et al*, 1990). Bistratene A was unable to activate or substantially inhibit the enzyme up to a concentration of 1μM (Tables 5 and 6).

Table 5. Inhibition of EGF Receptor Tyrosine Kinase Activity by Bistratene A.

Bistratene A concentration (nM)	% inhibition of tyrosine kinase activity
1	-2
10	1
100	2
1000	6

Values are the mean of 3 determinations from one experiment. Reproduced with the permission of Dr. Ian Dale, Xenova, Slough.

Table 6. Activation of EGF Receptor Tyrosine Kinase Activity by Bistratene A.

Bistratene A concentration (nM)	EGF (10nM)	Tyrosine kinase activity CPM ± SD
0	+	8634 ± 566
0	-	531 ± 38
1000	+	8037 ± 665
1000	-	476 ± 110

Values are the mean of 3 determinations from one experiment. Reproduced with the permission of Dr. Ian Dale, Xenova, Slough.

4.5. Relationship Between Growth Inhibition Induced by PKC Activators and Modulation of PKC Isozymes.

It has been shown that bistratene A was able to inhibit cell growth in A549 and MCF-7 cells in a similar manner to TPA. Unlike TPA, bistratene A did not activate PKC (section 4.3.). Bryostatin 1 and TPA are able to activate PKC *in vitro* with equal efficacy and potency (Sako *et al*, 1988) yet their effects on the growth of our cell lines are subtly different (section 4.1.). The exact mechanisms leading to inhibition of growth by these agents are still unclear, and research has so far been unable to explain differences between the effects of these two agents fully. One possibility is that TPA and bryostatins modulate locational changes in PKC isozymes differently. Differential translocation of PKC isozymes to the cell nucleus may lead to differential expression of certain genes related to cellular growth. Therefore experiments were conducted to visualise the location of each isozyme present in A549 and MCF-7 cells after treatment with both agents by Western blotting. Results were assessed in conjunction with growth inhibitory studies at the same cell seeding density (section 4.1.) to test the hypotheses that (i) inhibition of growth by PKC activators is influenced by isozyme-specific translocation and/or downregulation, (ii) differences in growth inhibition induced by TPA and bryostatin 1 are due to differences in translocation and/or downregulation of specific PKC isozymes.

4.5.1. Translocation of PKC Isozymes in A549 and MCF-7 Cells After Treatment with TPA or Bryostatin 1 for 30 Mins.

Figs.44 and 45 illustrate that in A549 and MCF-7 cells, both agents induced translocation of PKC α and ε to the particulate fraction and cell nucleus in a dose-dependent fashion from 10nM to 1μM concentrations. There was complete mobilisation of PKC ε from the cytosol after a 30 min exposure to either agent (10nM-1μM) in both cell types. Translocation of this isozyme to the nucleus from the particulate fraction was augmented with increasing concentration. In contrast, translocation of PKC α from the cytosol and its relocation to particulate and nuclear fractions was more gradual with increasing concentration of agent. Complete translocation of

PKC α from cytosol required 1μM concentrations of either agent in A549 cells. Bryostatin 1 was slightly less potent than TPA at initiating translocation of this isozyme in A549 cells, and the difference between the two agents was more marked in MCF-7 cells. Nevertheless, bryostatin 1 induced the translocation of PKC α in MCF-7 cells to the particulate and nuclear fractions in a dose-dependent fashion, but the isozyme was still predominantly located in cytosol after a 30 min exposure to 100nM bryostatin 1, unlike the response to 100nM TPA. PKC α was detected exclusively in the particulate and nuclear fractions of A549 and MCF-7 cells using 1μM concentrations of either agent. PKC ζ was little affected by these agents in A549 cells, being translocated to the nuclear and particulate fractions by ≤ 10% at all concentrations of TPA and bryostatin as measured by laser densitometry. In MCF-7 cells, nuclear localisation of PKC ζ was also increased by <10% with both agents, but this isozyme was noticeably translocated to the particulate fraction (Table 7).

Table 7. Laser Densitometric Scanning of Blots. Detection of PKC ζ in MCF-7 Cell Fractions after Treatment with TPA for Bryostatin 1 for 30 mins.

Concentration of agent (nM)	PKC in cellular fractions (%)					
	C	P	N	C	P	N
0	* 84	6	10	90	10	0
10	72	20	8	64	33	3
100	54	28	18	56	32	12
1000	54	35	11	60	40	0
		TPA			Bryostatin 1	

*Values were determined by laser densitometry and are the mean of 3 readings of each band from one blot which was representative of 3. C, P and N refer to cell cytosolic, particulate and nuclear fractions respectively.

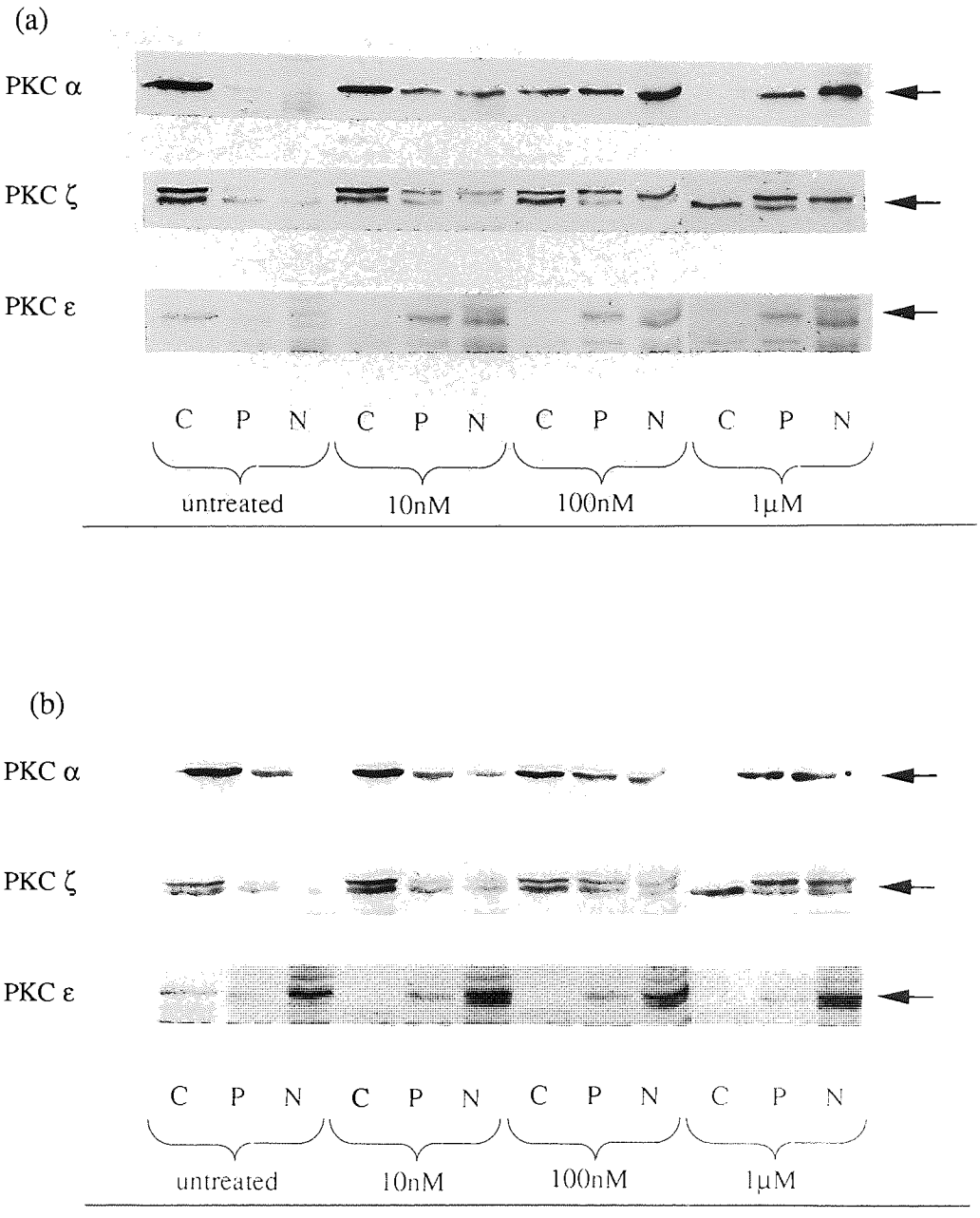


Figure 44 . Location of PKC Isozymes in A549 Cell Fractions after Incubation (30 min) with TPA and Bryostatin 1
Cells were seeded at $2x10^6$ in 14cm diameter dishes ($1.3x10^4$ per cm^2), incubated for 24 h, then exposed to TPA (a) or bryostatin 1(b) for 30 mins. Cells were then separated into cytosol (C), particulate fraction (P), and nuclei (N). PKC isozymes were detected in each fraction by Western blotting using 30μg protein per lane (see section 3.7.2. for methods). Results are representative of 2-3 determinations.

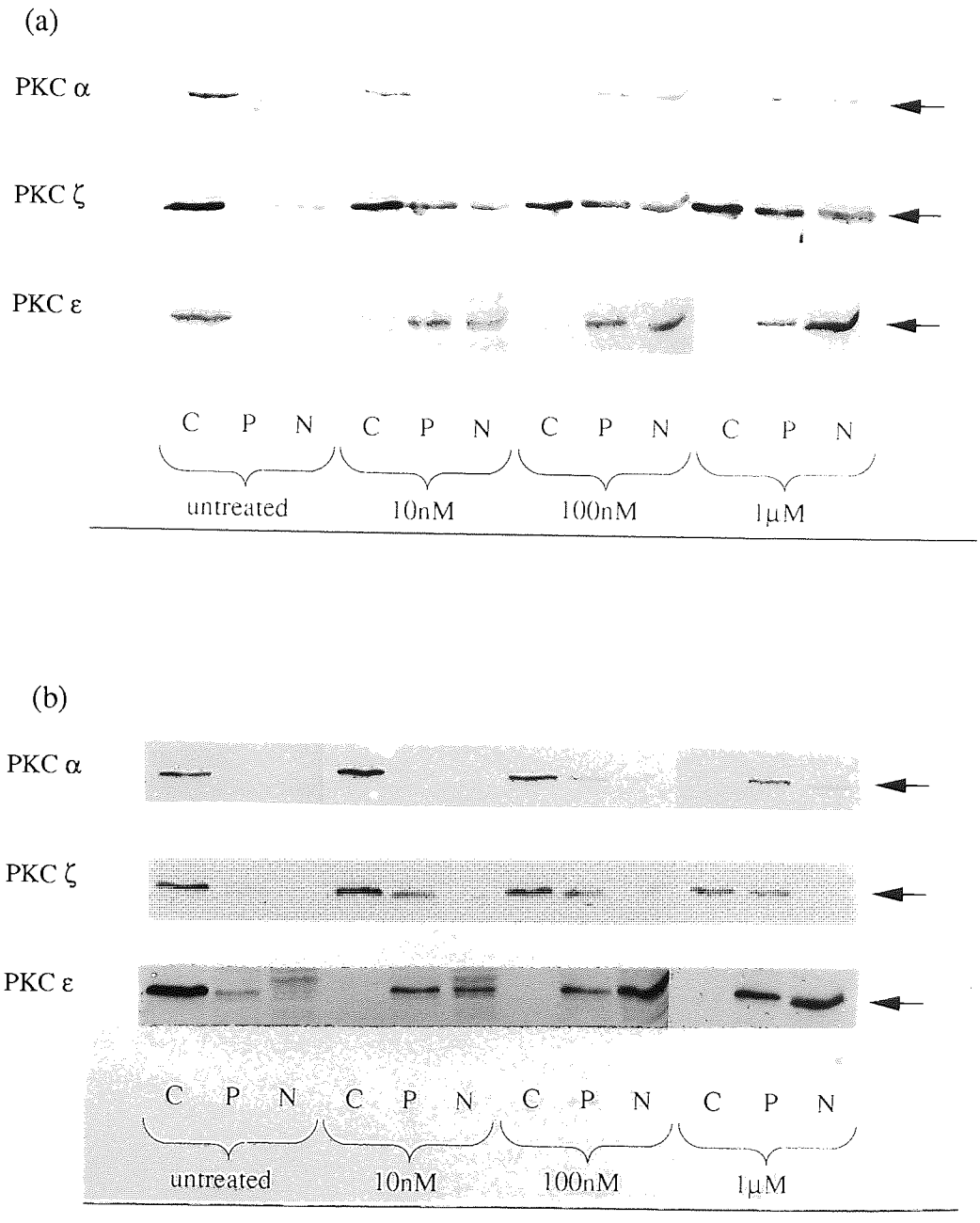


Figure 45. Location of PKC Isozymes in MCF-7 Cell Fractions after Incubation (30 min) with TPA or Bryostatin 1
Cells were seeded at $2x10^6$ in 14cm diameter dishes ($1.3x10^4$ per cm^2), incubated for 24 h, then exposed to TPA (a) or bryostatin 1(b) for 30 mins. Cells were then separated into cytosol (C), particulate fraction (P), and nuclei (N). PKC isozymes were detected in each fraction by Western blotting using 30μg protein per lane (see section 3.7.2. for methods). Results are representative of 2-3 determinations.

4.5.2. Effects on PKC Isozymes in A549 and MCF-7 Cells After Treatment with TPA or Bryostatin 1 for Prolonged Periods.

A549 cells were treated with bryostatin 1 for 18 h, the nadir of growth inhibition with 10nM concentrations. Western blot analysis revealed that PKC ε was completely downregulated by the agent (10nM-1μM) (Fig.46) and after 48 h, when proliferation has recommenced in the continuous presence of the agent, this isoform was still absent from cells (results not shown). PKC ζ was unaffected by bryostatin 1 at 18 h and 48 h. PKC α (3 ± 1%) was detectable after treatment with 10nM concentrations for 18 h and was present in trace amounts after 48 h. Bryostatin 1 (100nM-1μM) induced complete downregulation of PKC α at 18 and 48 h.

TPA affected the isozymes in essentially the same manner, but was slightly less potent than bryostatin 1 at initiating the downregulation of PKC α. After 24 h, there was a complete loss of PKC ε, no effect on PKC ζ and total loss of PKC α except for cells treated with 10nM concentrations, in which 15 ± 3% of levels of the α isoform detected in vehicle-treated cells remained in the cytosol (Fig.46). After 5 days, cell growth had resumed in the continuous presence of TPA 10nM-1μM (Fig.8b). After this time, PKC ε and ζ levels were unchanged from those seen at 24 h, but PKC α was downregulated further to 5 ± 2% of vehicle-treated control cells with 10nM TPA. When cells were continuously passaged for >9 weeks in 10nM or 100nM TPA, PKC ζ was not downregulated, but PKC α and ε were undetectable (results not shown).

MCF-7 cells exhibited a similar pattern of downregulation after treatment for 24 h with these agents (Fig.47). Both agents (10nM-1μM) induced complete downregulation of PKC ε, and as with A549 cells, bryostatin 1 was able to induce the downregulation of PKC α more potently than TPA, although both agents were less effective in MCF-7 cells

than A549 cells. At 100nM concentrations, PKC α was still detectable using TPA but not bryostatin 1. As in A549 cells, PKC ζ was not downregulated using either agent after prolonged exposure.

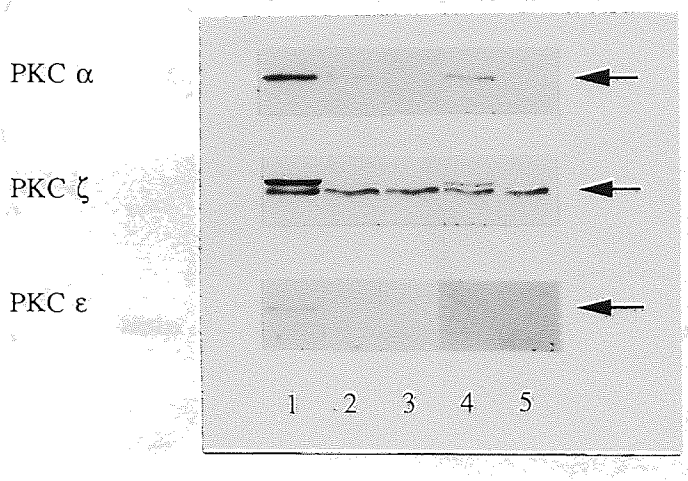


Figure 46. PKC Isozymes in A549 Cell Cytosol after Prolonged Incubation with TPA or Bryostatin 1.

Cells were seeded at $2x10^6$ ($1.3x10^4$ per cm^2), incubated for 24 h, then were exposed to DMSO vehicle (lane 1), bryostatin 1 10nM (lane 2) and 100nM (lane 3) for 18 h, or TPA 10nM (lane 4) and 100nM (lane 5) for 24 h. Cells were separated into cytosolic, particulate and nuclear fractions and subjected to SDS/PAGE and Western blotting (30μg protein per lane). Isozymes were detected using specific antibodies (see section 3.7.2. for methods). PKC isozymes detected in A549 cell cytosol are shown above. The particulate and nuclear fractions did not contain any immunoreactable PKC after treatment with either agent (results not shown). Results are representative of 3 determinations.

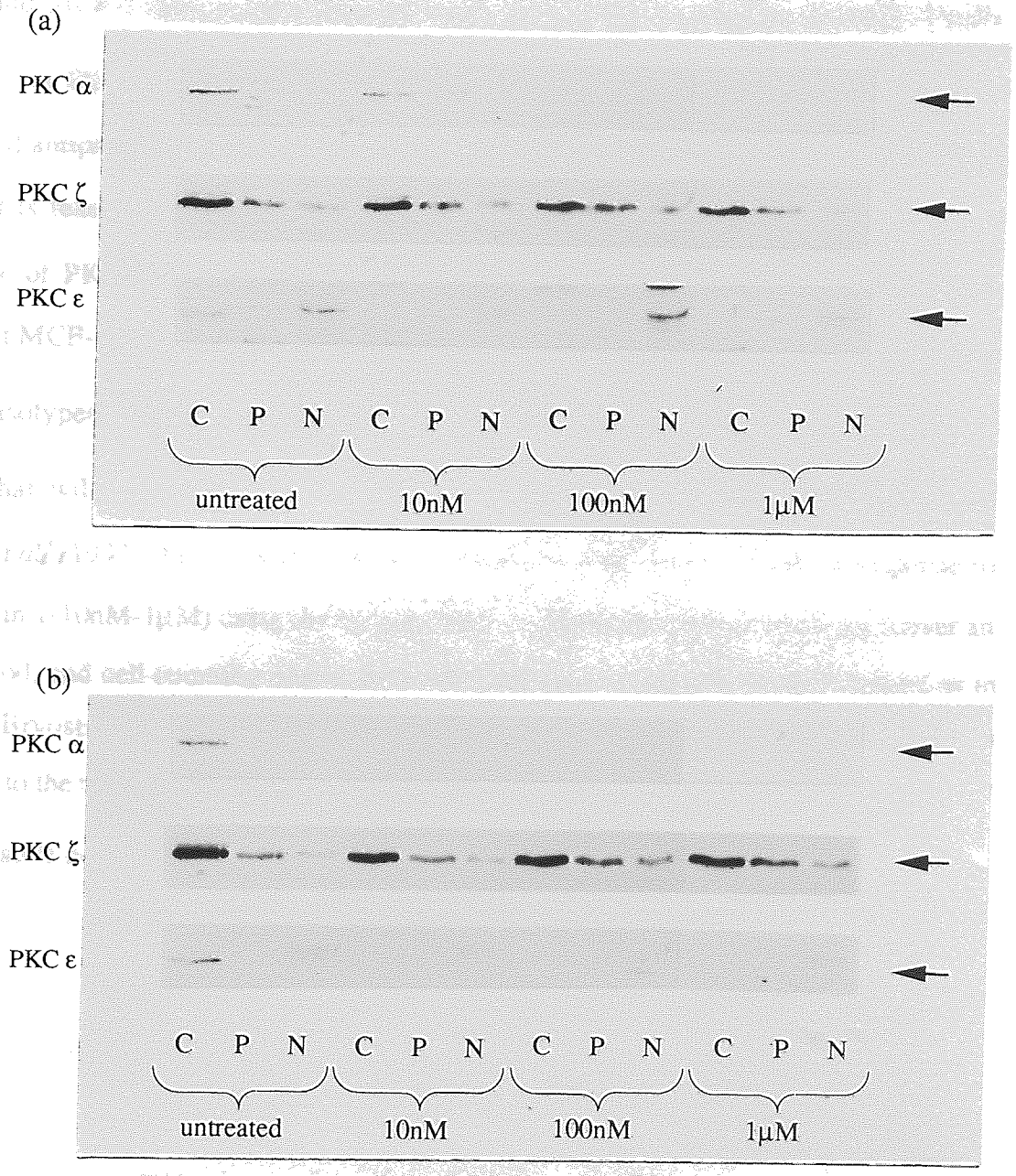


Figure 47. Location of PKC Isozymes in MCF-7 Cell Fractions after Prolonged Incubation with TPA or Bryostatin 1.
Cells were seeded at $2x10^6$ in 14cm diameter dishes ($1.3x10^4$ per cm^2), incubated for 24 h, then were exposed to TPA (a) or bryostatin 1(b) for 24 h. Cells were then separated into cytosol (C), particulate fraction (P), and nuclei (N). PKC isozymes were detected in each fraction by Western blotting using 30μg protein per lane (see section 3.7.2. for methods). Results are representative of 3 determinations.

4.5.3. Effect of Bryostatin 1 on the Growth of MCF-7 Cells Overexpressing PKC α.

A549 and MCF-7 cells contain PKC α, ε and ζ in different proportions; MCF-7 cells contain less PKC α and more PKC ε and ζ (section 4.3.5.7.). Bryostatin 1 evokes more profound antiproliferative effects on A549 cells than on MCF-7 cells (sections 1.4.4. and 4.1.). It is feasible that bryostatin 1 requires the presence of high quantities of the α isozyme of PKC to inhibit cell growth. To test this hypothesis, a stably Adriamycin-resistant MCF-7 cell clone was acquired (MCF-7Adr) and tested for content of PKC α, ε and ζ isotypes. The cells were found to overexpress PKC α and contained less PKC ε and ζ than wild-type MCF-7 cells (Fig.48), which is in agreement with the findings of Blobe *et al*, (1993). MCF-7Adr cells were assessed for an antiproliferative response to bryostatin 1(10nM-1μM) using the measurement of [^{3}H]thy incorporation every h over an 8 h period, and cell counting over 6 days, with the same conditions for cell culture as in Fig.10. Bryostatin 1 had no effect on cell proliferation measured by either technique, in contrast to the transient inhibition of [^{3}H]thy incorporation exchibited by wild-type MCF-7 cells (results not shown).

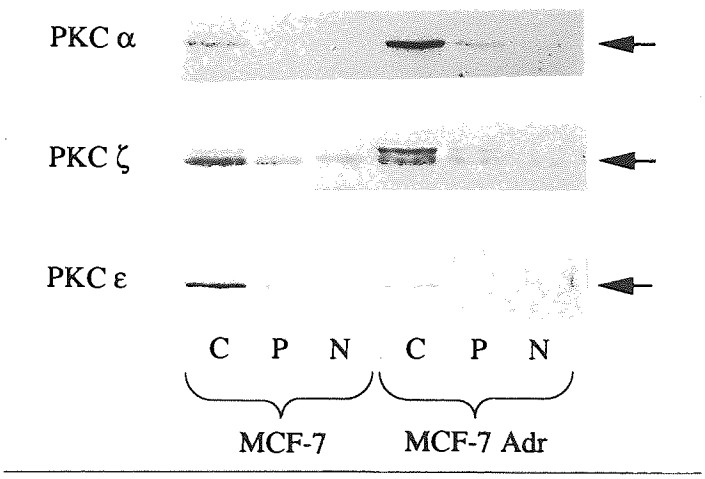


Figure 48. Expression of PKC Isozymes in MCF-7 Adr Cell Fractions.
Cells were separated into cytosolic (C), particulate (P) and nuclear fractions (N). PKC isozymes were detected in each fraction using specific antibodies after SDS/PAGE and Western blotting of samples (30μg protein per lane) (section 3.7.2.). Results are representative of 3 determinations.

Section 5. Discussion

Section 5. Discussion.

The overall aim of this project was to elucidate the relationship between inhibition of cell growth and PKC modulation. As outlined previously, the A549 and MCF-7 cell lines were employed for study as these cells are extremely sensitive to growth inhibition induced by PKC modulators and previous work in this laboratory characterising the effects of PKC activators such as phorbol esters and bryostatins on A549 cells provided the background upon which the work described here is based. In particular, experiments were conducted to (i) determine whether bistratene A exerts its effect on cell growth via PKC modulation in order to assess whether it can be used as a tool to study PKC, and (ii) ascertain whether effects on cell growth initiated by bryostatin 1 and TPA are regulated by PKC isozyme-specific events.

5.1. Effect of TPA and Bryostatin 1 on A549 and MCF-7 Cells.

Initially a reinvestigation of effects of TPA and bryostatin 1 on cell growth was undertaken in order to confirm results of previous authors, and provide the basis for further work using the same cell culture conditions. TPA induced changes in morphology and growth arrest in A549 and MCF-7 cells; findings were in agreement with effects reported previously (section 1.4.2.). In addition, it was found that the intensity of the growth inhibitory effect was dependent upon initial cell seeding density, and duration of cytostasis was inversely related to concentration of TPA above 10nM in both cell types (section 4.1.). Bryostatin 1 had a transient growth inhibitory effect on A549 cells (Dale and Gescher, 1989a), the intensity and duration of which was also inversely related to concentration above 10nM, suggesting a similar mode of action for these two agents. However, unlike TPA, bryostatin 1 had little effect on MCF-7 cell growth (section 4.1.).

5.2. Effect of Bistratene A on Cell Growth and PKC.

As observed in other cell types (section 1.5), bistratene A was potently cytostatic in A549, MCF-7 (section 4.2.1.2.) and HL-60 cells (section 4.2.1.1.), and induced changes in the morphology of HL-60 cells consistent with differentiation towards monocytes/

macrophages, as reported by Watters *et al*, (1990). Experiments were conducted to determine whether the characteristics of the growth arrest initiated by bistratene A were comparable to those elicited by PKC activators. Morphological changes in A549 and MCF-7 cells were unlike those seen with the phorbol ester TPA, but in many other ways the growth inhibitory characteristics of these two agents were similar (section 4.2.1.2.). In both cell types, the concentration of bistratene A necessary to inhibit cell growth was of the same order as that of TPA (Gescher and Reed, 1985, Osborne *et al*, 1981), with IC_{50} values of 1.0-2.9nM. Like TPA (section 4.1.), growth inhibition by bistratene A was dependent upon seeding density, which was exemplified by the potency of bistratene A as an inhibitor of A549 cell clonal growth compared to cells seeded at higher density, and the dependency of IC_{50} values on initial cell seeding density. Bistratene A (10nM) elicited cytostasis for 6-8 days in A549 cells, after which proliferation resumed in the continued presence of the agent. An investigation of the reversibility of growth arrest in these cells revealed that this effect could be overcome by more frequent replacement of medium and agent (section 4.2.1.4.). A major factor determining the effectiveness of bistratene A is its low chemical stability at 37°C in culture medium (section 4.2.2.). Therefore the agent is more potently cytostatic if continually replaced and in hindsight, it would have been pertinent to examine the effects of the agent more closely with frequent additions of agent. In MCF-7 cells, bistratene A (10nM) evoked prolonged cytostasis with a small reduction in cell number (Fig.13b). Under the same conditions, TPA (10nM) elicited similar effects on A549 and MCF-7 cells, inducing cytostasis for 6 days in A549 cells, followed by recommencement of cell growth, and prolonged cytostasis with a reduction in cell number from day 10 onwards in MCF-7 cells, suggesting cytotoxic effects after prolonged exposure (Figs.8a and 9a). After incubation with TPA and bistratene A, trypsinisation and reseeding of cells restored full sensitivity towards the agent, and continuous culture of A549 cells in the presence of either compound gave rise to a phenotype which was temporarily less sensitive to growth inhibitory effects (section 4.1 and 4.2.1.2.). The phorbol ester PDBu is also growth inhibitory to A549 cells and after incubation for 6 days in the presence of the agent, it was substituted for bistratene A. Growth inhibition was greater than that of PDBu for the following 6 days, but less than that of naive cells to bistratene A. This could have been due to bistratene A having the same mechanism of action as PDBu to induce resistance to growth inhibition after 6 days. A more likely

explanation is that cell numbers were greater after 6 days and therefore growth inhibition was less pronounced, as the effects of bistratene A were found to be density dependent.

In A549 cells, inhibition of cell growth by PKC-activating phorbol esters is primarily due to cytostasis rather than cytotoxicity. Paradoxically, the duration of cytostasis decreases with increasing phorbol ester concentration in A549 and MCF-7 cells. It was therefore of interest to assess whether bistratene A was cytostatic or cytotoxic. Bistratene A (100nM) caused a progressive reduction in cell number in both cell lines which could not be reversed in the case of A549 cells upon removal of the agent after 6 days (Fig.14), suggesting cytotoxicity. However, there is a 100-200 fold discrepancy between IC_{50} and LC_{50} values in A549 cells, although these values were obtained from experiments with different cell densities and incubation times and are therefore not directly comparable. Under the same culture conditions, bistratene A (50nM) was able to inhibit [^{3}H]thy uptake in cells (section 4.2.1.3.) more potently than expected on the basis of its cytotoxic potency (section 4.2.3.), suggesting that the agent is primarily cytostatic. Bistratene A becomes progressively more cytotoxic with increasing concentration, unlike TPA, and also with increasing incubation time above the concentration at which cells can recover from growth arrest.

It has been suggested that the formation of branched colonies of cells on the basement membrane substitute Matrigel is indicative of an invasive and metastatic phenotype (Albini *et al*, 1987). PKC activation by TPA can increase branched colony formation of WI-38 fibroblasts by enhancing cell motility and collagenase IV production when the cells are grown on this substrate, giving rise to more invasive cells. Conversely, HT1080 fibrosarcoma cells are less invasive after treatment with TPA (Fridman *et al*, 1990). Inhibition of PKC by staurosporine in bladder and gastric carcinoma cells reduces their ability to invade Matrigel (Schwartz *et al*, 1990, 1993a). Clearly, PKC has an important role in the control of the invasion process using Matrigel as an *in vitro* model for basement membranes, and invasion is one of the crucial steps in metastasis development (section 1.3.5.). However, studies using Matrigel may not reflect the invasiveness of cells *in vivo*: Noel *et al*, (1991) compared the invasiveness and morphology of a large series of normal

and malignant cells on this matrix, and could not establish a correlation between the organisation of cells cultured on Matrigel, whether isolated, in clusters, or in a network, the invasion of Matrigel, and invasiveness *in vivo*. In fact, A549 and MCF-7 cells did not form branched colonies of cells on Matrigel (section 4.2.4.), although both cell types are metastatic *in vivo* (Wang *et al*, 1992, Thompson *et al*, 1993). TPA and bryostatin 1 and 2 induced the formation of A549 and MCF-7 cell networks on this matrix. The ability to increase motility and undergo drastic morphological changes may therefore be a consequence of PKC-induced phosphorylation of cytoskeletal regulatory proteins, such as the actin-binding MARCKS protein (section 1.3.4.) or possibly phosphorylation of integrins, which are membrane glycoproteins which bind to components of basement membrane matrices (Ruoslahti 1992). However, some PKC activators are not able to induce this effect : the synthetic DAG diolein was unable to mimic TPA in stimulating WI-38 cells to invade Matrigel, but was able to partially inhibit the invasion of HT1080 cells like TPA (Fridman *et al*, 1990). Bistratene A was unable to induce the formation of cellular networks in either cell type, and it actually reduced cluster formation in MCF-7 cells on Matrigel, cells being isolated on Matrigel in the presence of this agent (section 4.2.4.).

Overall, the results demonstrate that bistratene A is a potent inhibitor of cell growth in A549 and MCF-7 cells, with certain similarities and some differences in the characteristics of this effect compared with TPA and the bryostatins.

Using two-dimensional gel electrophoresis, proteins phosphorylated when A549 cells were grown in the presence of TPA were determined and compared to changes in phosphoprotein status induced by the presence of bistratene A (section 4.3.2.). TPA induced the phosphorylation of at least eight proteins, three of which were also phosphorylated by bistratene A. The proteins affected by TPA were not phosphorylated when the cells were incubated in the presence of TPA and staurosporine, except for two proteins with molecular weights of 56 and 64kDa (section 4.3.2.4.), further emphasising the role of PKC in these phosphorylation events. On the basis of their pI(urea) and molecular weight for identification, two of the proteins heavily phosphorylated by TPA and the bryostatins, but not by bistratene A or when cells were co-incubated with TPA and staurosporine, could be the 28kDa stress protein (Regazzi *et al*, 1988, Darbon *et al*, 1990),

and the 87kDa MARCKS protein (Aderem, 1992), which are thought to be specific PKC substrates. Unfortunately, confirmation of the identity of MARCKS was not possible by detection of protein spots on 2D gels by Western blot analysis with a specific antibody. Semi-purification of this protein from cell samples by boiling to precipitate heat-labile proteins whilst retaining MARCKS in the soluble fraction or by immunoprecipitation (Blackshear *et al*, 1986), followed by 2D gel electrophoresis and detection of phosphoproteins, would perhaps have identified this protein definitively.

Bistratene A also induced the phosphorylation of two proteins which were not affected by TPA or the bryostatins. The protein most strikingly phosphorylated in the presence of bistratene A but not the other agents had a molecular weight of 19kDa and a pI (urea) of 6.0. This 2D gel position is the same as that of the recently documented phosphoprotein stathmin (also known as p19, Op18, prosolin, pp17, pp20-23), which is thought to be a relay protein linking signal transduction pathways during cell growth and differentiation. Stathmin is ubiquitously expressed in cells, and is phosphorylated by activation of the cAMP-dependent protein kinase (PKA). However, it is also phosphorylated after treatment of cells with TPA (Sobel, 1991) and hence the phosphoprotein detected in A549 cells is unlikely to be stathmin. Changes in protein phosphorylation after exposure of HL-60 cells to bistratene A were compared to changes induced by TPA and bryostatin 5 (Watters *et al*, 1992). Bistratene A induced the phosphorylation of a 25kDa basic protein, and a 20kDa acidic protein, pI(urea) 6.7, both of which were unaffected by TPA or bryostatin 5. The latter protein was phosphorylated on tyrosine and serine residues, demonstrating that the protein in HL-60 cells is not stathmin, as stathmin does not contain tyrosine residues. Proteins with increasing phosphorylation status migrate to positions with lower pI values during 2D gel electrophoresis and therefore it is feasible that the 20kDa protein phosphorylated in HL-60 cells in response to bistratene A is identical with the 19kDa protein in A549 cells. This protein is phosphorylated within 5 min of application of bistratene A to HL-60 cells, and the degree of phosphorylation is nearly maximal at 10nM, a concentration at which physiological effects occur (Watters *et al*, 1992). Phosphorylation of this protein may be a crucial signalling event in triggering the effects of bistratene A on cell growth and differentiation.

Bistratene A does not inhibit phosphatases 1 or 2A (Watters, personal communication) and does not activate or inhibit the EGF receptor tyrosine kinase (section 4.4.). It is possible that the agent modulates the activity of serine/threonine kinases such as PKC isozymes. Alternatively, it might interact with components of a signalling pathway which could then modulate kinase activity indirectly, as discussed in section 1.3.4. Considering the similar biological effects of bistratene A and TPA in A549, MCF-7 and HL-60 cells, and the common phosphorylation of at least three proteins by both agents in A549 cells, the hypothesis was tested that bistratene A modulates the activity of PKC, possibly with specificity for particular isoforms.

Determination of loss of phorbol ester binding sites from A549 cell cytosol, and analysis of PKC α/β by Western blotting demonstrated that unlike TPA, bistratene A was unable to induce the translocation of PKC within 30 mins of exposure of A549 and MCF-7 cells to the agent. Bistratene A was also unable to induce downregulation of PKC, compete with [^{3}H]PDBu for phorbol ester receptors (sections 4.3.3. and 4.3.4.) or inhibit PKC under conditions in which staurosporine inhibited the enzyme potently with an IC_{50} of 6.1nM (Bradshaw *et al*, 1992). Nor did it activate PKC in cell cytosol at concentrations of up to 10μM. The latter finding is in contrast to results of Watters *et al*, (1990), which suggested that bistratene A (5-10μM) activated PKC β, albeit submaximally, in the presence of PS or oleic acid, but not in their absence. The reasons for this discrepancy are unclear, but it could be related to isozyme specificity or assay conditions. In the PKC assay used by Watters *et al*, histone was the substrate and PS was incorporated into vesicles, whereas in the assay employed in this laboratory, a PKC-specific peptide was the substrate and PS was present in micelles (section 4.3.5.).

To assess whether a PKC isoform present in minor amounts in cells was activated by bistratene A, isoforms of A549 and HL-60 cells were separated using DE52 and hydroxylapatite column chromatography. In A549 cells, the major peak of activity was the α isoform of PKC, in agreement with Hirai *et al*, (1989), and the minor peak was most likely PKC ε (section 4.3.5.4. and 4.3.5.7.). Bistratene A (50nM and 3μM) was unable to

activate PKC present in any fractions from the separation of A549 cells on hydroxylapatite. However, in the laboratory of Dr. Janet Lord at Birmingham University, similarly using hydroxylapatite chromatography to obtain kinase-containing fractions, two new peaks of activity were obtained in the presence of bistratene A (50nM) (section 4.3.5.5.). This effect could not be repeated in our laboratory using the same assay reaction mixture as Dr. Lord or when using a PKC assay kit with histone IIIS or a PKC-specific peptide as substrate. A possible reason for this discrepancy may be that hydroxylapatite columns were used up to three times in our laboratory, then fresh columns were prepared, whereas Dr. Lord routinely used columns up to ten times, which perhaps led to modification of binding properties of the columns and therefore separation of different kinases. Another possibility is that artifactual peaks were generated by employing a PKC assay which separates phosphorylated histone by acid precipitation and centrifugation rather than using binding to phosphocellulose paper ; the former method is prone to greater background variability and was used by Dr. Lord but not in our laboratory, although the dose dependency of bistratene A-induced enzyme activity tends to refute this hypothesis.

Two major peaks of PKC activity were detected in naive HL-60 cells, in agreement with Beh *et al*, (1989), McSwine-Kennick *et al*, (1991) and Tanaka *et al*, (1992). Dr. Lord was able to detect 3 major and 3 minor peaks of activity using the same cells. Several authors have also detected three major peaks of PKC activity in HL-60 cells from hydroxylapatite column chromatographic separations (Sawamura *et al*, 1989, Nishikawa *et al*, 1990). The presence of the extra peak is not due to the use of different substrates for the assay, as Dr. Lord used histone IIIS as substrate, and in our laboratory, a PKC-specific substrate and histone IIIS both generated two peaks. The use of PS in vesicles rather than micelles, or the absence of DTT from the assay mixture may have allowed the phosphorylation of histone by other related kinases and the appearance of more peaks of activity. It is also feasible that a third major peak of PKC activity could have been masked in the assay used in this laboratory due to the intensity of substrate phosphorylation and merging of two peaks. The major PKC subspecies isolated from HL-60 cells are PKC α and β (Tanaka *et al*, 1992). Recently, PKC θ has been found to be a major PKC isoform in cells of haemopoietic origin (Baier *et al*, 1993). This isoform may be present in HL-60 cells and

could account for the other peak detected by some workers using hydroxylapatite column chromatography in this cell type.

In HL-60 cells, bistratene A (50nM and 3μM) was unable to induce the phosphorylation of a PKC-specific substrate or histone IIIS by enzymes in any of the fractions eluted from the hydroxylapatite column. Again, in the laboratory of Dr. Lord, as was the case with A549 cells, two new peaks of kinase activity were generated by the presence of bistratene A (50nM). Both peaks exhibited a biphasic activation response to bistratene A, being activated with increasing concentrations to a maximal level, then to a lesser extent with increasing concentrations thereafter (section 4.3.5.6.).

PKC isozymes present in A549 and MCF-7 cells were characterized by Western blot analysis of cellular fractions to be PKC α, ε and ζ (section 4.3.5.7.). PKC α and ε separated by hydroxylapatite column chromatography from A549 cell cytosol were not activated by bistratene A. PKC ζ could not be isolated using this separation technique, possibly because of loss on purification, lack of detection as a poor substrate for this isozyme was used, or because the actual cellular levels of PKC ζ are very low. The antibody used to detect this isozyme by Western blot analysis might bind avidly to PKC ζ and thus create a strong autoradiographic signal although the enzyme is present in minute amounts. In MCF-7 cell cytosol, the ζ and ε isoforms were more prevalent and there was less PKC α than in A549 cells (section 4.3.5.7.). PKC assays of MCF-7 cell cytosol demonstrated that the PKC-specific peptide substrate was substantially phosphorylated (45± 14%) in the absence of cofactors, and activity was enhanced further to 61± 11% of maximal after addition of PS and Ca^{2+} to the reaction mixture. PS is able to activate PKC ζ maximally (Nakanishi *et al*, 1993) and it is feasible that the PKC-specific substrate may induce some activity in certain isoforms in the absence of any cofactors (see section 4.3.5.1.). Therefore, the baseline activity in MCF-7 cell cytosol in the absence of TPA could be due to PKC ζ. Bistratene A was unable to activate MCF-7 cell cytosolic PKC in

the presence or absence of PS and Ca^{2+} (section 4.3.5.2.), suggesting an inability to activate PKC ζ.

In conclusion, bistratene A is a potent growth inhibitory and cytotoxic agent in A549 and MCF-7 cells, and it is able to induce the cytostasis and partial differentiation of HL-60 cells in a similar manner to TPA and the bryostatins. It is also able to induce the phosphorylation of several cellular proteins the same as TPA. However, it is unlikely that bistratene A inhibits cell growth *via* modulation of isozymes of PKC, or by indirectly influencing PKC activity as the enzyme is not translocated or downregulated by the agent and staurosporine was unable to reverse its growth inhibitory effects in cells. Bistratene A might turn out to be a useful agent for studying signalling processes involved with cell growth and differentiation, but cannot be used as a tool for studying the role of PKC in these events. It would be intriguing to discover the signalling pathway *via* which bistratene A exerts its potent effects on cell growth and differentiation and this is currently being pursued further by Dr. Watters and her collaborators.

5.3. The Significance of Translocation and Downregulation of PKC Isozymes in Growth Inhibition Induced by Agents which Activate PKC.

Bistratene A was able to initiate growth arrest in A549 and MCF-7 cells with great potency without influencing PKC activity. TPA and bryostatins also inhibited proliferation potently in these cells and activation of PKC is a property common to both of these agents. TPA and bryostatin 1 are equipotent activators of crude PKC preparations *in vitro*, yet the intensity and duration of their effects on cells are markedly different, suggesting that their biological effects are also influenced by other factors. A549 and MCF-7 cells responded to both agents by a reduction in proliferative capacity. In both cell types, bryostatin 1 had a more transient effect than TPA, particularly in MCF-7 cells (sections 1.4.2., 1.4.4. and 4.1.).

In several studies a definite causal relationship has been discovered to exist between biological effects and PKC activation, downregulation or translocation. The relative

importance of these events seems to be dependent on cell type. The proliferative response of T cells to TPA correlated well with interleukin-2 (IL-2) production which was generated after PKC activation, and the loss of PKC from the cell *via* downregulation produced a reduction in IL-2 levels and a consequent reduction in proliferative capacity (Grove and Mastro, 1991). Melanocytes are quiescent in routine cell culture conditions, but the presence of TPA induced proliferation. In melanocytes of human origin, low concentrations of TPA (16nM) triggered maximal rates of cell growth. The agent provided sustained PKC activation and induced secretion of an autocrine growth factor by these cells (Akita *et al*, 1992), whereas melanocytes derived from a murine source required 200nM TPA for proliferation, and downregulation of PKC correlated with proliferation (Brooks *et al*, 1991). Hep G2 hepatoma cells responded to phorbol esters with inhibition of [^{3}H]thy uptake of 18-24 h duration which reverted to normal levels thereafter in the continued presence of the agent (Duronio *et al*, 1990), similar to the effect of bryostatin 1 on A549 cells. These authors detected only partial downregulation of cellular PKC during inhibition of DNA synthesis and also after the resumption of cell division to the same rate as control cells, suggesting that Hep G2 cells can escape the growth inhibitory effects of phorbol esters when PKC activity is reduced below a certain critical level within the cell.

The divergent biological effects of TPA and bryostatins have been attributed to differential effects on PKC translocation or downregulation. In Jurkat leukaemic T cells and peripheral blood T lymphocytes, bryostatin 5 initiated more profound downregulation of PKC than TPA and the ability of TPA but not bryostatin to induce T cell proliferation was attributed to this property (Isakov *et al*, 1993). Divergent effects on cell growth and differentiation between phorbol esters and bryostatin 1 in HL-60 leukaemia cells were reported to be due to differential translocation to the nucleus. PDBu initiated differentiation and translocation of PKC to the plasma membrane, whereas bryostatin 1 antagonized this effect, was incapable of differentiating these cells, and induced translocation of PKC to the nuclear envelope with subsequent phosphorylation of lamin B (Fields *et al*, 1988). Therefore in certain cell types, translocation or downregulation of PKC certainly seems to be intrinsically linked to the differing biological activity of these two agents.

The divergent antiproliferative effects of bryostatin 1 and TPA on A549 and MCF-7 cells could also be influenced by isozyme-specific events. The hypotheses were tested that : (i) differences in the response of A549 *vs* MCF-7 cells to these agents are related to the presence of different isozymes in these cell types, (ii) selective redistribution to different subcellular locations, particularly to the nucleus, or downregulation of specific PKC isozymes elicited by TPA and bryostatins are prerequisites for growth inhibition induced by these agents, and (iii) differences in effects of the two agents are a function of their differential effects on specific PKC isozymes.

A549 cells have previously been reported to contain PKC α (Hirai *et al*, 1989) and ε (Kim *et al*, 1992). MCF-7 cells were reported to contain PKC α, β, γ, δ, ε and ζ (Ahn et al, 1992), PKC α and β and an unidentified type of PKC (Bignon *et al*, 1990), the α, β_2, δ, ε and ζ isoforms (Blobe *et al*, 1993) or the α and γ (referred to as τ) isoforms but no PKC β_2 (Kennedy *et al*, 1992). This disagreement demanded a careful assessment of isozymes present in both cell types in our laboratory. Western blot analysis of A549 and MCF-7 cell cytosolic, particulate and nuclear fractions revealed that both cell types contained the same PKC isozymes located predominantly in the cytosol, although they were present in different quantities. PKC α, ε and ζ were detected (section 4.3.5.7.). Due to lack of specific antibodies, it was not possible to assess the expression of PKC θ and λ in these cells.

Very little PKC α was detected in MCF-7 cells, and these cells exhibited a 25% reduction in [^{3}H]thy uptake of only 1 h duration after treatment with bryostatin 1, unlike A549 cells in which the α isoform was abundant and [^{3}H]thy incorporation was inhibited for up to 18 h. Bryostatin 1 was shown to activate crude preparations of PKC with equal potency to TPA (Isakov *et al*, 1993, Sako *et al*, 1988), and PKC α and ε isolated from A549 cells were activated fully by TPA and bryostatin 1 when isoforms were separated by

hydroxylapatite column chromatography, but this effect was only examined with 3μM concentrations (section 4.3.5.4.). It is feasible that bryostatin 1 activates one particular isozyme more effectively at growth inhibitory concentrations. PKC α was isolated using hydroxylapatite column chromatography and concentrated (section 3.10.2.), but unfortunately, too little PKC ε was obtained for semi-purification using this method for a comparative assessment of isoform activation by bryostatin 1.

A number of elegant techniques have been devised recently which will be useful in assessing the relative importance of the activation of individual PKC isozymes in a physiological response. These include inhibition of the function of specific PKC isoforms using PKC inhibitors with a degree of selectivity such as GF 109203X, which only inhibits the activity of cPKCs but not nPKCs (Toullec *et al*, 1991), by cell permeabilisation and intracellular delivery of isozyme-specific antibodies (Leli *et al*, 1992) or by introducing antisense oligonucleotides to inhibit isozyme expression (Dominguez *et al*, 1992, Baxter *et al*, 1992a). The opposite approach uses activation of a specific isoform using PKC activating phorbol esters with a degree of specificity such as thymeleatoxin and resiniferatoxin, which activate cPKCs but not PKC δ or ε (Ryves *et al*, 1991) or insertion of PKC isoforms into cells to analyse their contribution to a particular biological effect : techniques include overexpression of a range of PKC isoforms in one cell type (Watanabe *et al*, 1992), and cell permeabilisation and washing to remove endogenous PKC, then insertion of a series of exogenous purified PKC isozymes (Ozawa *et al*, 1993). The use of a combination of these techniques could clarify further the role of activation of specific PKC isoforms in growth arrest.

The relative quantity of the α isoform in A549 and MCF-7 cells may determine susceptibility towards bryostatin-induced growth arrest. The fact that A549 cells which have undergone prolonged exposure to PDBu, with consequent loss of PKC from the cell, are unresponsive to inhibition of [^{3}H]thy uptake induced by bryostatin 1 gives credence to this suggestion (T.D. Bradshaw and A. Gescher, unpublished observation). To test this hypothesis further, an MCF-7 cell variant which was Adriamycin-resistant (MCF-7Adr)

was acquired as overexpression of the α isoform of PKC is documented in these cells (Blobe *et al*, 1993). An examination of the PKC isoforms present in these cells showed that they did indeed overexpress PKC α and also contained less PKC ε and ζ than wild-type MCF-7 cells. Bryostatin 1 (10nM-1μM) failed to inhibit [^{3}H]thy incorporation or growth of these cells (section 4.5.3.). Therefore our hypothesis was refuted by this experiment. However, this conclusion may be untenable as MCF-7Adr cells may be very efficient at preventing the intracellular accumulation of bryostatin 1 as this agent is a naturally-derived macromolecule and may be ejected from the cell by a P-glycoprotein-dependent process similarly to Adriamycin (section 1.3.5.). This hypothesis could be tested by measuring uptake of radiolabelled bryostatin by wild-type and Adriamycin-resistant MCF-7 cells.

It is unlikely that levels of PKC ε influence the ability of bryostatin 1 to exert greater antiproliferative effects in A549 cells compared to MCF-7 cells as this isozyme is more prevalent in MCF-7 cells. It is also unlikely that the relative amount of PKC ζ in each cell type has a bearing on the difference in intensity of antiproliferative affect as this isoform does not bind and is not activated by phorbol esters, which are thought to share their binding site with bryostatin 1 (section 1.3.2.). After a 30 min exposure to bryostatin 1 or TPA, PKC ζ was redistributed from the cytosol to the particulate fraction in MCF-7 cells in a dose-dependent fashion (section 4.5.1.). This could have consequences for activation of this isozyme as it is activated fully in the presence of PS (Nakanishi *et al*, 1993), which is a component of cellular membranes and therefore translocation would bring PKC ζ in close proximity with its activator. However, this effect does not seem to correlate with intensity or duration of antiproliferative effect as both agents initiated this effect in MCF-7 cells, with divergent sequalae on growth, and neither agent induced >10% translocation in A549 cells.

An assessment of subcellular redistribution of the α and ε isoforms of PKC was also made

after 30 min exposure to TPA and bryostatin 1 in an attempt to link effects on cell growth with modulation of PKC. The ε isoform, when localised at the nucleus, was detected as a protein of slightly lower molecular weight on SDS polyacrylamide gels. This may have been caused by interaction of PKC with other nuclear components, leading to structural changes and consequent increased electrophoretic mobility. Often a decrease in electrophoretic mobility of PKC is encountered after translocation to the particulate fraction of cells, due to autophosphorylation (Kiley *et al*, 1992, Pears *et al*, 1992). This phenomenon was not detected with PKC α or ε derived from A549 and MCF-7 cells.

TPA and bryostatin 1 exerted very similar effects on translocation of the α and ε isoforms of PKC in A549 and MCF-7 cells. PKC α was translocated less readily from cytosol than the ε isoform, especially in MCF-7 cells. The mechanism which triggers association of PKC isoforms with the nucleus and components of the particulate fraction such as the cytoskeleton and cell membrane is at present unclear. One possibility is that proteins at these locations may act as molecular tethers for PKC. Several candidate proteins have been tentatively identified within the particulate fraction of heart and brain tissue, termed RACKS (receptors for activated C kinase). These proteins bind to the C2 domain of PKC (and hence are only receptors for cPKCs) in a Ca^{2+}, PS and DAG-dependent manner (Mochly-Rosen *et al*, 1991, 1992). The necessity for activated PKC in the process of translocation is unclear. The PKC β_1-specific activator DOPPA and the α, β and γ isoform-specific activator thymeleatoxin were able to initiate translocation of the α, β, δ and ε isoforms (Roivainen and Messing, 1993). Translocation of PKC ζ in certain cell types and absence of this effect in others, as seen here in A549 and MCF-7 cells and reported for other cell types (Ways *et al*, 1992, Borner *et al*, 1992) after treatment with phorbol esters, which do not bind or activate this isozyme, is also inconsistent with the hypothesis that receptors bind to activated PKC. In fact, the PKC inhibitor staurosporine was capable of translocating PKC ε and δ (Kiley *et al*, 1992) and enhanced phorbol ester-

induced translocation of PKC α (Bradshaw *et al*, 1992). The calcium ATPase inhibitor thapsigargin, which promotes intracellular calcium release, is also able to induce the translocation PKC ε and δ, although these isozymes do not bind and are insensitive to Ca^{2+} (Kiley *et al*, 1992). These workers suggested that translocation of c- and nPKCs may be mediated *via* different PKC binding proteins, with the phosphorylation status of these proteins being crucial in the translocation process. Conformational changes to PKC isoforms in response to the binding of activators or inhibitors may also influence this process. Therefore enhanced translocation of PKC ε compared with the α isoform and translocation of PKC ζ in MCF-7 cells but not in A549 cells may be a consequence of the presence of different binding proteins for these isoforms. The higher affinity of phorbol esters for PKC ε than PKC α (Marquez *et al*, 1992) may also influence differential translocation of these two isoforms.

Bryostatin 1 was a slightly less potent agent than TPA at initiating translocation of PKC α, particularly in MCF-7 cells, but as with TPA, it was able to initiate enzyme redistribution from cytosol to the particulate and nuclear fractions of both cell types. Both agents induced the translocation of PKC ε with equal potency and efficacy (section 4.5.1.).

An attempt to compare the effects of the two agents on cell growth with ability to induce translocation of individual isozymes showed little correlation between these events. In A549 cells, 1μM concentrations of bryostatin 1 initiated a 60% inhibition of DNA synthesis after 6 h that reverted to control levels thereafter, whereas TPA (1μM) induced complete cytostasis which was of at least 24 h duration, as measured by [^{3}H]thy incorporation into cells (I. Dale, unpublished results). Both agents induced translocation of PKC α and ε to the particulate and nuclear fractions at this concentration, TPA inducing the redistribution of slightly more PKC α to the nucleus. At lower concentrations, effects on A549 cell growth were more profound, total cytostasis occurring within 12 h at 10nM

with both agents (I. Dale, unpublished), yet only a little PKC α was detected in the particulate and nuclear fractions after 30 min exposure to agents.

Likewise in MCF-7 cells, both agents induced a dose-dependent translocation of PKC α and ε to the particulate and nuclear subcellular fractions. Translocation of PKC α by bryostatin 1 was less pronounced than that initiated by TPA, particularly to the nuclear fraction, but the effect of bryostatin 1 on cell proliferation was very weak and did not increase with dose escalation, in contrast to the complete cytostasis initiated by TPA (10nM-1μM) in this cell type (section 4.1.).

The translocation of PKC ε and ζ in both cell types induced by TPA and bryostatin 1 seemed to be independent of their effects on cell proliferation, and differential translocation of PKC α by these agents was insufficient as a single factor to account for their widely differing effects on cell growth. However, in A549 cells, high concentrations of bryostatin 1 and TPA evoked more rapid antiproliferative effects than low concentrations and enhanced translocation to the nucleus may therefore be important in the early events leading to cytostasis. The translocation of PKC to the cell nucleus would be a logical site at which to phosphorylate proteins important for cellular proliferation (section 1.3.4.). In MCF-7 cells, results suggest that localisation of PKC at the nucleus is not important in the growth inhibitory response to PKC activators because of the differential effects of the two agents on cell growth. An alternative explanation is that once at the nucleus, PKC is insufficiently activated by bryostatin 1 to phosphorylate nuclear proteins essential for a growth inhibitory response, or only transiently phosphorylates proteins, providing inadequate signalling events to trigger growth arrest. The dramatic effect of TPA on this cell type has been suggested to be caused by induction of expression of $TGF\beta_1$. This inhibitory growth factor is normally expressed in an active form at low levels in MCF-7 cells ; TPA enhances the expression of $TGF\beta_1$ in both active and latent forms in MCF-7 cells but not in an MCF-7 cell clone which is resistant to TPA-induced growth inhibition (Guerrin *et al*, 1992). Furthermore, bryostatin 1 does not induce the expression of

$TGF\beta_1$ in MCF-7 cells (Nutt *et al*, 1991) and hence this differential effect on $TGF\beta_1$ expression may account for the difference in growth inhibitory effect of the two agents and explains, at least in part, why TPA inhibits cell growth. However, exogenously applied $TGF\beta_1$ has little effect on the growth of late passage MCF-7 cells, which were used in these studies, due to loss of TGFβ receptors (Zugmaier *et al*, 1989) but TPA has been shown to increase receptor numbers in other cell types with consequent growth inhibition (Sing *et al*, 1990, Takaishi *et al*, 1990) and this effect, coupled with enhanced $TGF\beta_1$ expression, may explain the growth inhibition induced by TPA in MCF-7 cells. The $TGF\beta_1$ gene contains two TPA response elements. These are base pair sequences recognised by the AP-1 transcription factor. The phosphorylation status of AP-1 is altered by PKC, which induces transcription factor activation (section 1.3.4.) and therefore it seems likely that expression of $TGF\beta_1$ mRNA by TPA results from PKC activation in the nucleus. In MCF-7 cells, very little nuclear PKC was detected until translocated to that site from the cytosol. It is unclear whether the enzyme is able to penetrate nuclear pores to reside on the inner nuclear envelope or within the nuclear matrix, which would be necessary for AP-1 activation (Irvine and Divecha, 1992). This hypothesis was not addressed in the studies presented here, as whole nuclei were analysed by Western blotting. If this is the case and changes in phosphorylation status of AP-1 by PKC is the initiating factor in $TGF\beta_1$ mRNA synthesis, then translocation to this site would be a prerequisite for MCF-7 cell growth inhibition. However, the inability of bryostatin 1 to induce $TGF\beta_1$ mRNA expression is not explained by this hypothesis as the agent was able to induce translocation of PKC α to nuclei. It also translocates PKC ε to nuclei like TPA but this isoform is incapable of phosphorylating GSK-3β and may be incapable of activating AP-1 by this mechanism (see section 1.3.4., Goode *et al*, 1992), although overexpression experiments in 3Y1 fibroblasts suggest that activation of both the α and ε isoforms initiates the expression of TRE-containing genes (Hata *et al* ,1993). It is also possible that the induction of

$TGF\beta_1$ expression by TPA may occur indirectly *via* signalling events downstream from the phosphorylation of proteins distant from the nucleus by PKC and nuclear translocation may be irrelevant to this process.

In A549 cells, expression of $TGF\beta_1$ does not account for the growth inhibitory effects of TPA or bryostatin 1 as the cells secrete a latent form of TGFβ and these agents do not modulate the generation of the active form or the production of TGFβ receptors in these cells (Bradshaw and Gescher, unpublished observations).

A549 and MCF-7 cell PKC ε was completely downregulated after incubation for 18-24 h with TPA and bryostatin 1 (10nM-1μM), and this isozyme was not reexpressed in cells incubated with either agent for more prolonged periods of time. The downregulation of this isozyme did not correlate with the intensity or duration of antiproliferative effects or with the resumption of cell growth in either cell type. PKC ζ was not downregulated in response to either agent, and after 24 h in the continuous presence of agent in MCF-7 cells, the isozyme redistributed to the same subcellular locations as in untreated control cells, residing predominantly in the cytosol. Regarding PKC α, bryostatin 1 was a slightly more potent agent at initiating downregulation in both cell types (section 4.5.2.). Downregulation is the degradation and loss of PKC from the cell initiated by calpain and other proteases (section 1.3.3.). The process was initially thought to be initiated by autophosphorylation of PKC (Ohno *et al*, 1990), but this hypothesis has since been disproved (Pears and Parker, 1991a, Freisewinkel *et al*, 1991). Recent evidence suggests that TPA induces downregulation by causing a conformational change in PKC which increases the affinity of the kinase for calpain (Savart *et al*, 1992). Differential downregulation of PKC isoforms in response to phorbol esters is a well-documented phenomenon (section 1.3.3.) and occurs because of differential rates of degradation of different isozymes by various proteases, and possibly as a consequence of translocation to subcellular sites at which these proteases reside (Pontremoli *et al*, 1990). It would be interesting to examine whether the greater potency of bryostatin 1 at initiating

downregulation of PKC α is due to the agent inducing an increase in affinity of calpains for this isozyme more potently than TPA, or whether other proteases or factors are involved. This slight difference in downregulation of PKC α in relation to effects of the two agents on growth seems to be of little consequence. In A549 cells, TPA (10nM) induced downregulation to 15% of initial levels after 24 h, and the isozyme was not detected using 100nM and 1μM concentrations after this time (section 4.5.2.). In fact, TPA (100nM) induced a total loss of TPA-inducible cellular PKC activity after 4 h, and after 1 h with 300nM concentrations (Ian Dale, unpublished observation). Cytostasis was maintained for 4, 2 and 1 days with 10, 100 and 1000nM TPA, respectively. After 5 days, when cell growth had resumed (section 4.1.), PKC α was detected at 5% of the level in untreated cells after exposure to 10nM TPA, but was still undetectable in cells treated with higher concentrations of the agent. After continuous growth in the presence of TPA (10 and 100nM), no PKC α or ε were detected. Bryostatin 1 induced a greater downregulatory effect on PKC α in A549 cells, with only 3% remaining after 18 h using 10nM concentrations, and a trace could still be detected after 48 h, when DNA synthesis has returned to its usual rate in these cells. A reduction in the quantity of PKC α present in A549 cells to levels below a certain threshold may contribute to the duration of growth inhibition as with Hep G2 cells (Duronio *et al*, 1990), but this is obviously not the major factor involved. Studies on the time course of loss of total cellular PKC activity and phorbol ester binding sites from A549 cells after exposure to TPA and bryostatin are in agreement with this conclusion (Dale *et al*, 1989). High concentrations of TPA induce the phosphorylation of more cellular proteins than low concentrations (Lord *et al*, 1988a) and one could speculate that cellular signalling cascades which override those which induce the growth inhibitory effect of TPA are triggered with greater efficacy by high dose treatment with this agent, inducing a more rapid resumption of growth.

In MCF-7 cells, differential downregulation of PKC α by TPA and bryostatin 1 does not seem to be a determinant of induction or longevity of cytostasis, as the isozyme was detected after 24 h, albeit at low levels, after treatment with bryostatin 1 (10nM) but not at

higher concentrations, yet antiproliferative effects were only of 1 h duration at all concentrations of this agent. TPA (10nM) did not substantially downregulate PKC α in these cells after 24 h ; at 1μM concentrations the complete loss of this isozyme was apparent, yet both concentrations effectively induced cytostasis. Furthermore, 1,2-dioctanoyl-*sn*-glycerol, a cell permeant DAG, was able to induce potent growth inhibition and cellular maturation similar to that seen with TPA in this cell type with 8 hourly additions of agent, yet it was unable to induce downregulation of PKC in these cells (Issandou *et al*, 1988). As with A549 cells, reduced longevity of effect of TPA-induced cytostasis using high concentrations may be due to the extent of phosphorylation of proteins which are part of a rescue signal transduction pathway, antagonising the growth inhibitory effects which are dominant when using 10nM concentrations of TPA.

Our results in MCF-7 cells are in conflict with those of Kennedy *et al* (1992) using the same cell type. These authors found PKC γ in MCF-7 cells which was unresponsive to TPA- or bryostatin-induced translocation, and PKC α was detected predominantly in the detergent-soluble particulate (cell membrane) fraction ; after incubation of MCF-7 cells with agents for 30 min, translocation of this isoform to the cell membrane was further enhanced by TPA (100nM) but this isozyme was totally downregulated by bryostatin 1 (100nM). This discrepancy may have been related to the fact that cells were incubated overnight in serum-free medium before commencing studies on isoform distribution, yet the authors correlated their findings with effects of these agents on the growth of cells in the presence of serum. They concluded that differences in antiproliferative effects of bryostatin 1 and TPA were induced by translocation of PKC α by TPA and its downregulation by bryostatin 1.

Our findings on translocation and downregulation of PKC α and ε in response to TPA and bryostatin 1 are generally in agreement with the findings of Jalava *et al*, (1993), using SH-SY5Y neuroblastoma cells. As seen with A549 and MCF-7 cells, they found that PKC ε was translocated and downregulated with similar kinetics by both agents, but the α

isoform was differentially modulated, being more rapidly downregulated by bryostatin 1. The authors showed that phosphorylation of the MARCKS protein by phorbol esters was much more prolonged than that induced by bryostatin 1 and suggested that the differential effects of the agents on PKC α partially explained their opposite effects on cell differentiation. Ng and Guy (1992) compared the biological effects of TPA with those of bryostatin 1 in U937 and primary B lymphocytes and found that after treatment of cells with bryostatin 1, the agent was slightly less potent at initiating translocation of PKC to the plasma membrane than TPA, but this effect was insufficient to explain the divergent effects of these two agents on cell growth and differentiation. Therefore, as with A549 and MCF-7 cells differential translocation and downregulation of PKC in these cell types may be contributory factors, but are insufficient to fully explain the different biological effects of bryostatin 1 and TPA .

The use of a range of growth inhibitory concentrations of agents gave results adequate to relate growth inhibition with PKC isoform translocation and downregulation, but an examination of the time course of the translocation process may have provided more definitive evidence. However, insufficient time was available to attempt this task. An alternative technique for analysis of PKC isozyme distribution is by immunofluorescent staining of cells with specific antibodies to PKC isoforms linked to fluorescein-conjugated secondary antibodies, and analysis by confocal microscopy (Murti *et al*, 1992). Unfortunately, only PKC α could be investigated easily using this technique as the commercially-available PKC ε antibody exhibited profuse non-specific binding, particularly to nuclear material, and the PKC ζ-specific antibody also bound to PKC α and possibly other isoforms. This technique has the advantage of visualising isoforms at precise subcellular locations and would have been an excellent method for analysing A549 and MCF-7 cells after treatment with TPA or bryostatin and may have revealed translocation of PKC isoforms to unique sites after stimulation with these agents which may not have been revealed using crude cell fractionation and Western blotting. The two techniques in combination would have provided definitive evidence of the role of PKC isoform translocation and downregulation in the growth inhibitory response of A549 and

MCF-7 cells to TPA and bryostatin 1.

Bryostatins bind to PKC with great avidity and dissociate from PKC much more slowly than phorbol esters *in vitro*, possibly giving these agents the properties of "partial activators" or "partial inhibitors" (Lewin *et al*, 1992). It is likely that the differential effects of these two agents upon binding to PKC is the major contributive factor accounting for their divergent antiproliferative effects on A549 and MCF-7 cells, and the "partial inhibitor" effects of bryostatins would also explain the reversal of TPA-induced growth inhibition by these agents in both of these cell types (Dale *et al*, 1989, Kennedy *et al*, 1992). The employment of PKC assay conditions with Triton X 100/PS mixed micelles rather than PSin vesicles causes a reduction in the affinity of both agents for PKC, but the reduction in binding of bryostatin is of a much greater magnitude (Lewin *et al*, 1992). The fact that different *in vitro* lipid environments also influence the degree of difference in binding of the two agents also suggests that the similarity of biological effects of these two agents in one cell type and dissimilarity in another, as with A549 and MCF-7 cells respectively, could be determined by the lipid composition of different cell types. Bryostatin 1 may be antiproliferative to A549 cells but not to MCF-7 cells as a consequence of this fact and also because of the low levels of PKC α in MCF-7 cells. These events together might prevent sufficient phosphorylation of MCF-7 cell proteins, perhaps located within the cell nucleus, that are essential for a growth inhibitory response.

5.4. Conclusions.

In summary, A549 and MCF-7 cell proliferation is inhibited potently by agents which activate PKC, such as phorbol esters and bryostatins. Growth is also arrested by bistratene A. The latter agent induces intracellular protein phosphorylation, suggesting interference with cellular signalling pathways, but it does not exert its effect by activation of PKC. The antiproliferative effects of phorbol esters and bryostatins are intrinsically linked to their ability to activate PKC but the intensity and duration of biological effect does not seem to be associated with their respective abilities to translocate or downregulate specific PKC isoforms. A more likely explanation for differences in their effects is their differing

binding to PKC with consequences for duration of phosphorylation of substrate proteins. Translocation of PKC α and/or ε to the cell nucleus may be an essential prerequisite for initiation of growth inhibition but the presence of PKC at this site is not a determinant of the intensity or duration of this effect. PKC ζ does not appear to have a role in the growth inhibitory response of A549 and MCF-7 cells to these agents. A closer analysis of the extent and duration of phosphorylation of PKC substrate proteins and their location and function might give a better understanding of the mechanisms by which TPA and bryostatin 1 exert antiproliferative effects on cells. Assessment of the PKC isoforms involved with these phosphorylation events and their position within the complex network of cell signalling pathways might eventually help our understanding of which of these processes are important for inhibition of growth in cells, and therefore suggest more specific sites for intervention by agents used for the treatment of cancer.

Section 6. References.

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Section 7. Appendices.

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7.1. Abbreviations.

ATP	adenosine 5'triphosphate
[γ-^{32}P]ATP	adenosine 5'-[γ-^{32}P]triphosphate
BSA	bovine serum albumin
bis	N,N'methylene bisacrylamide
cAMP	adenosine 3'5'-cyclic monophosphate
cDNA	complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulphonate
Ci	curies
CPM	counts per minute
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagles Medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DOPPA	12-deoxyphorbol-13-*O*-phenylacetate-20-acetate
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid
FCS	foetal calf serum
g.	acceleration due to gravity
G protein	guanine nucleotide binding protein
GSK 3β	glycogen synthase kinase 3β
h.	hours
H_2O	water
[^{3}H]PDBu	[20-^{3}H(N)]-phorbol-12,13-dibutyrate
[^{3}H]thy	5-[methyl ^{3}H]-thymidine

IC_{50}	concentration which inhibits cell growth by 50%
IEF	isoelectric focusing
IgG	immunoglobulin G
IL-2	interleukin-2
IMS	industrial methylated spirit
IP_3	inositol 1,4,5-trisphosphate
kDa	kilodaltons
LDH	lactate dehydrogenase
mA	milliamps
MAP kinase	mitogen activated protein kinase (also known as MAP2 kinase (microtubule associated protein 2 kinase), ERK(extracellular signal-regulated kinase) , MBP kinase (myelin basic protein kinase))
MARCKS	myristoylated alanine-rich C-kinase substrate
MEM	minimal essential medium
min.	minutes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	nicotinamide adenine dinucleotide (reduced form)
PAGE	polyacrylamide gel electrophoresis
PAP	phosphatidic acid phosphohydrolase
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDA	piperazine diacrylamide
PDBu	phorbol-12,13-dibutyrate
Pen/Strep	penicillin 100iu/ml and streptomycin 100µg/ml
PG	prostaglandins
P_i	phosphate (PO_4^{3-})
pI	isoelectric point (aqueous)
pI(urea)	isoelectric point (urea)
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PK	protein kinase

PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulphonyl fluoride
PS	phosphatidylserine
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecylsulphate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with Tween 20
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF β	transforming growth factor β
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TX	thromboxane
UFFA	unsaturated free fatty acids
USB	urea sample buffer
V	volts

Other abbreviations are SI notation.

7.2. Publications

Stanwell C., Gescher A., Pettit G.R. The role of protein kinase C isoenzymes in the growth inhibition caused by bryostatin 1 in human A549 lung and MCF-7 breast carcinoma cells. (submitted)

Stanwell C., Gescher A., Watters D. Cytostatic and cytotoxic properties of the marine product bistratene A and analysis of the role of protein kinase C in its mode of action. *Biochem. Pharmacol.*, **45**, 9, 1753-1761 (1993)

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