Effects of Inhibitors of Protein Kinase C in Drug Sensitive and Multidrug Resistant MCF-7 Breast Carcinoma Cells

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For my Parents

With Much Love and Thanks

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CHAPTER 1

INTRODUCTION

1.1. Cancer and Cellular Signalling

Cancer can be considered a disease of abnormal cell growth mediated by altered intracellular signalling. Aberrant expression of cellular protooncogenes or tumour suppressor genes leads to the genesis of cancer. Further development to fully transformed phenotype and the maintenance of malignancy requires additional genetic alterations, thereby allowing the cancer cell to bypass normal regulatory mechanisms for cell proliferation. A major function of oncogenes is to code for proteins that are components of growth factor-activated intracellular signalling pathways (Patterson, 1992; Powis 1991). The proliferation and differentiation of normal cells is tightly regulated by a balance between the action of growth-stimulatory protooncogenes and the growth-inhibitory tumour suppressor genes. When an oncogene is overexpressed or constitutively active through mutation, or when a tumour suppressor gene is inactivated, the balance is upset and the cell receives continuous mitogenic stimulation and unrestrained growth is inevitable.

The conventional approach to cancer treatment involves the use of reagents which impair the dividing cell by interfering with the biochemical mechanics involved in cell division. These include DNA intercalating agents, DNA cross-linking agents (e.g. alkylating agents), topoisomerase inhibitors, cytoskeleton disrupting agents (e.g. vinblastine) or cytoskeleton rectifying agents (e.g. taxol). These cytotoxic drugs are effective but dose-limiting toxicity to normal tissues, such as the haematopoietic system, is a major obstacle. The combination of radiotherapy, surgery and chemotherapy offers the best treatment and have been successful against certain forms of cancer such as Hodgkin's lymphoma and bladder cancer (Levitzki, 1994). However, cancer mortality in the Western hemisphere has shown no significant decline in the past 45 years, so there is clearly a need for novel types of cancer drugs and components of signalling pathways provide attractive new targets.

The fact that neoplastic cells use the same signalling pathways as normal cells raises the problem of the degree of selectivity that can be obtained. The signalling pathways identified to be amplified pathways in certain malignancies make the cancer cell highly dependent on these pathways and therefore more vulnerable. Thus, it is likely that, cancer cells and abnormally proliferating cells will be affected more severely than normal cells. Normal cells in which the signalling network is balanced can compensate

for a sudden inhibition of some of its elements. Due to the degeneracy of growth factor signalling a cell can apparently survive the complete inhibition of a signalling pathway with other pathways taking over some, or all of the normal functions of the inhibited pathway. The results of a large number of animal gene knockout studies suggest that degeneracy is a general feature of growth factor signalling (Brookfield, 1992; Hooper, 1992). In order to inhibit an oncogene-activated signalling pathway it is not necessary to target the oncoprotein itself. Another component of the pathway, usually an enzyme, can be a surrogate target which may be more amenable to selective inhibition by a drug.

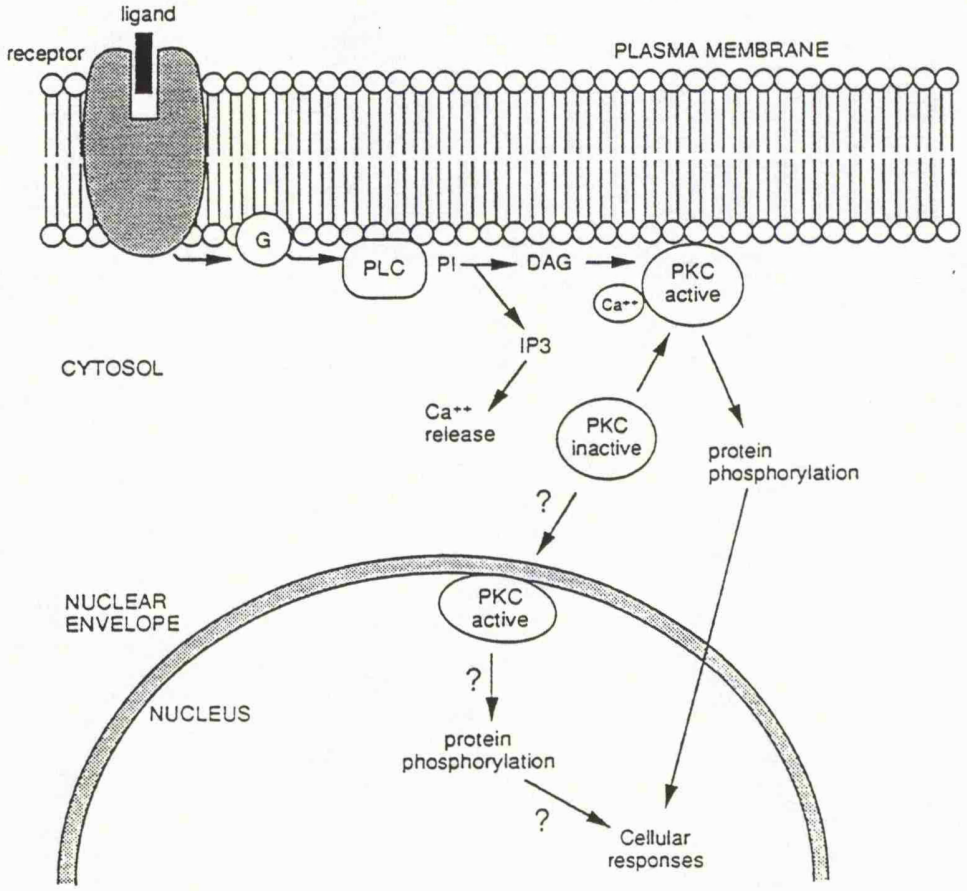
1.2. Protein Kinase C

Protein kinase C (PKC) was initially discovered and characterised by Nishizuka and co-workers in 1977 (Takai *et al*, 1977). Since then PKC has attracted enormous attention and a wealth of information has accumulated which is summarised in several reviews (Nishizuka, 1986, 1988, 1992; Parker *et al*, 1989; Bell and Burns, 1991; Azzi *et al*, 1992; Bell *et al*, 1992; Huang and Huang, 1993; Hug and Sarre, 1993; Liu, 1996). Two major discoveries in the early 1980's established the importance of PKC in signal transduction and tumour promotion. First that basal PKC activity was stimulated by diacylglycerol (DAG) (Kishimoto *et al*, 1980). Second that PKC was activated by, and was the major intracellular receptor for, tumour promoting phorbol esters (Castagna *et al*, 1982).

1.2.1. The PKC Pathway

The PKC pathway is outlined in figure 1.1. Ligand binding to specific cell surface receptors results in activation of phospholipase C (PLC) through a G-protein-coupled pathway. The resulting hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) generates inositol trisphosphate (IP_3) in the cytosol and DAG in the plasma membrane. IP_3 interacts with its receptor on a calciosome to cause the release of intracellular calcium stores and a rise in intracellular calcium (Ferris *et al*, 1989). The calcium may aid in recruiting cytosolic PKC to the membrane (translocation) where it remains inactive but primed for action. On the membrane PKC interacts with 4 phospholipid molecules and with one DAG to become fully activated and ready to phosphorylate

Figure 1.1 The PKC Pathway



Schematic representation of the activation of PKC in response to ligand binding at the cell surface. Taken from Olson *et al*, 1993.

cellular proteins (Bell, 1986). Ligand binding may also result in the activation of phospholipase A_2 (PLA_2) which causes the release of arachidonic acid. The activation of PKC by arachidonic acid is independent of phospholipid and so can occur in the cytosol. It has been suggested that soluble fatty acids activate soluble PKC to phosphorylate a different set of substrates (Khan *et al*, 1992). Figure 1.1 is a simplified representation and does not show the high degree of crosstalk that goes on between all of the signalling pathways.

Tumour promoting phorbol esters, such as TPA, possess a molecular structure, part of which is reminiscent of DAG. These agents can therefore substitute for DAG and

activate PKC. Since the phorbol esters are slowly metabolised, however, they persist in tissues for much longer times than DAG and cause prolonged PKC activation.

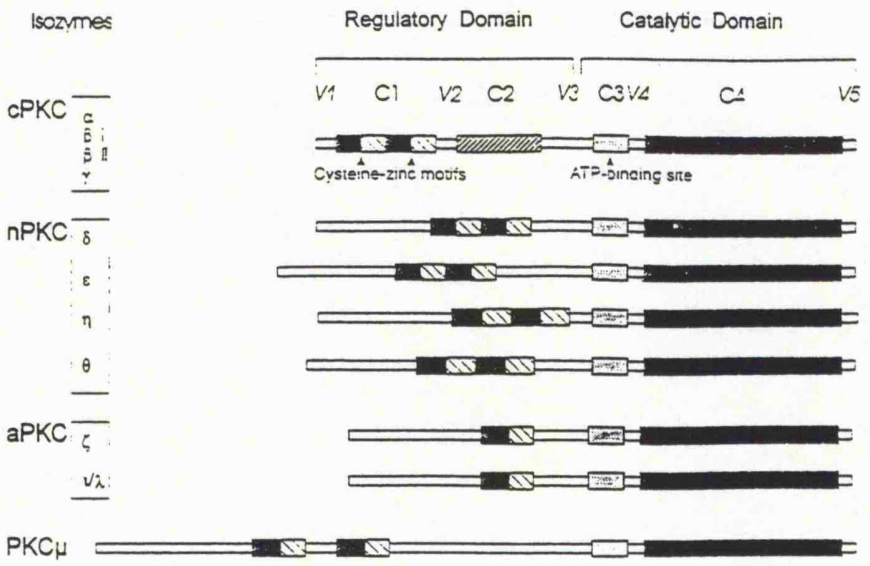
1.2.2. The PKC Family

Molecular cloning has led to the identification of twelve PKC isoenzymes (to date) and established that PKC is a multigene family. The primary amino acid sequence of PKC can be divided into four conserved functional domains (C1-C4) which are individually separated by variable domains (V1-V5) (figure 1.2). These regions form catalytic and regulatory domains joined by a hinge region (V3) which contains cleavage sites for proteases. All isoenzymes contain the catalytic domain which has an acidic sequence in C4 that functions as the substrate recognition site (House and Kemp, 1987). In addition, an ATP binding site is found within C3.

Structural differences in regulatory domain and different activation conditions have allowed division of PKC isoenzymes into four major categories: the Ca^{2+} dependent or conventional PKC (cPKC: α, βI, βII and γ); Ca^{2+} independent or novel PKC (nPKC: δ, ε, η and θ); atypical PKC (aPKC: ζ, ι and λ) and PKC-μ (which takes an intermediate position between the subgroups of nPKC and aPKC). The structural differences and tissue distribution of these proteins are illustrated in figure 1.2 and table 1.1.

The C1 domain contains a tandem repeat of cysteine rich sequences except in aPKCs where only one motif is present. Each motif binds two zinc ions and the zinc atom co-ordinates with one histidine nitrogen and three cysteine sulphur atoms to stabilise a particular conformation (Hubbard *et al*, 1991). Phorbol esters bind to this region (Burns and Bell, 1991). Newton, (1995) suggested that in the inactive state there are hydrophobic interactions between catalytic and regulatory domain. On phorbol ester binding these interactions are replaced by hydrophobic interactions with the membrane thus causing a major conformational change in the hinge region separating the catalytic from the regulatory domain. The aPKCs have only one cysteine rich region and thus do not bind DAG or phorbol esters. The spacing of the two regions also seems important as PKC-μ possesses a long spacing of 74 amino acids between the two motifs, compared to 15-22 amino acids in cPKCs and nPKCs, and shows no efficient phorbol ester binding (Johannes *et al*, 1994). Preceding cysteine rich motifs there is a pseudosubstrate sequence of 13-30 residues which resembles a phosphorylation site.

Figure 1.2 The PKC Family



Domain structure of PKC isoenzymes are shown in four groups cPKC, nPKC, aPKC and PKC-μ. The conserved (C1-C4) and variable (V1-V5) regions are depicted in the regulatory and catalytic domains. The cysteine rich motifs and ATP binding site are indicated. Taken from Liu, 1996.

Table 1.1 Tissue Distribution of PKC Isoenzymes

PKC ISOENZYME	TISSUE DISTRIBUTION
cPKC	
α	Ubiquitous
βI	Most tissues
βII	Most tissues
γ	Neural
nPKC	
δ	Ubiquitous
ε	Neural, immune, epithelium, heart
η	Neural, epithelium
θ	Ovary, skeletal muscle, platelets
aPKC	
ζ	Most tissues
ι	Ubiquitous
PKC	
μ	Unknown

However, the serine or threonine residue found in the substrate motif is changed to alanine in all PKC isoenzymes. Thus this motif cannot be phosphorylated but acts as a regulatory feature by blocking the catalytic site when inactive (Soderling, 1990).

The nPKCs and aPKCs lack the C2 region seen in the cPKCs and it was thought that this region contained a calcium binding site. However, Sossin and Schwartz (1993) pointed out that nPKCs contain an amino-terminal domain with striking similarity to the C2 consensus sequence. Recently Sprang and co-workers solved the crystal structure of C2 domain in a transmembrane protein, synaptotagmin (Sutton *et al*, 1995) and this has helped to solve the mystery behind the C2 domain in calcium independent nPKCs. The C2 key contains five aspartate residues that form calcium binding site. nPKCs have all the conserved residues of the C2 key except only two of five aspartates are present and arginine is present in position of one of them. Newton, (1995) suggested that the arginine forms an electrostatic interaction with aspartate thus mimicking the effect of calcium. Occupancy of this calcium site in cPKCs may convert them to a conformation that nPKCs already have.

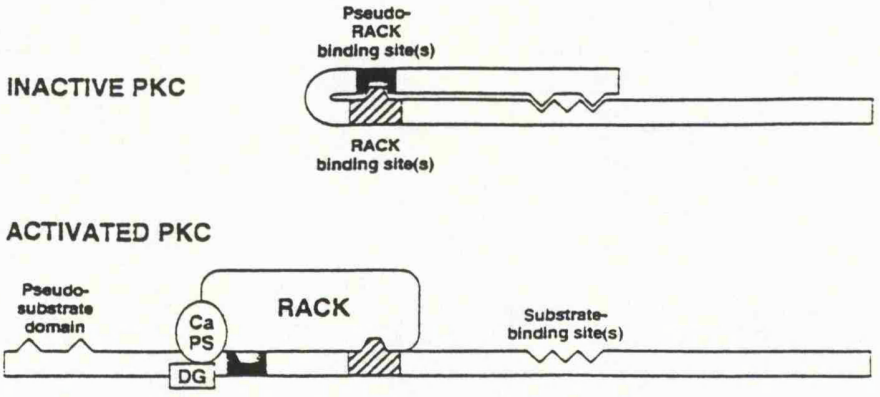
The existence of PKC heterogeneity poses the question as to why so many isoenzymes exist and what the functional relevance of each of them could be. The high degree of conservation of the individual PKC isoenzymes over various mammalian species indicates that maintaining the heterogeneity is somehow "worthwhile", and suggests that each could have a specific relevance for the organism. A variety of stable cell lines have been generated that overexpress PKC isoenzymes, allowing the examination of their effects on biological processes. In general, the effects observed depend very much on the cell lines and PKC isoenzyme used. Overexpression of murine PKC-δ in NIH 3T3 cells resulted in slower growth rate, decreased cell density whereas the overexpression of PKC-ε in the same cell line caused increased growth rate, cell density and tumourigenicity (Mischak *et al*, 1993). Overexpression of human PKC-α in NIH 3T3 cells resulted in a slightly transformed phenotype (Finkenzeller *et al*, 1992) and rat PKC-γ overexpression may (Persons *et al*, 1988) or may not (Cuadrado *et al*, 1990) result in transformation. Other approaches, such as, the use of dominant negative mutants of PKC isoenzymes or gene knock-out experiments may provide a better insight into the role of the individual isoenzymes.

1.2.3. Regulation of PKC

The regulation of PKC activity has been investigated for many years. Biophysical studies of the interactions between PKC and its lipid support involves the isolation of the enzyme. Such invasive techniques use divalent cation chelators which can solubilise certain membrane associated proteins (Wallach and Zingler, 1974) and ultracentrifugation which is capable of extracting extrinsic membrane proteins that are not tightly bound (Devauex and Seigneuret, 1985). PKC was considered to be a cytosolic enzyme that translocated to the membrane upon activation. It is now unclear whether the soluble form of PKC is truly cytosolic. The native enzyme may be loosely attached to the cytoplasmic side of plasma membrane in an inactive state (Wolf *et al*, 1985) but found in the cytosolic fraction due to the extraction procedure.

As mentioned in the previous section the C1 region of the regulatory domain contains a pseudosubstrate sequence (House and Kemp, 1987). In the unstimulated state the regulatory domain of the enzyme interacts with catalytic domain through the pseudosubstrate sequence. This prevents access of the substrate to the catalytic site (Soderling, 1990). Three molecular interactions are necessary for PKC activation: calcium binding to "soluble" PKC inducing translocation to the membrane; the interaction between calcium-PKC and the acidic phospholipid, phosphatidylserine (PS); and the action of DAG on this complex (Zidovetzki and Lester, 1992). It is not clear whether calcium binds to the lipid (Hui *et al*, 1983), acts as a bridge between enzyme and phospholipid (Bell, 1986) or binds to and subsequently stabilises the lipid-protein complex (Bazzi and Nelsestuen, 1991). The binding of the cofactors by whatever method induces a conformational change which releases the pseudosubstrate sequence thereby exposing the substrate binding site such that catalysis can take place (Orr *et al*, 1992). PKC activation involves the insertion of the enzyme into lipid membrane (Nelsestuen and Bazzi, 1991). Thus, the consequence of activation is the redistribution of the enzyme to membrane compartments, a process known as translocation (Kraft and Anderson, 1983). Substrate proteins associated with phospholipid membrane can then be phosphorylated on serine or threonine residues by PKC (Edashige *et al*, 1992). Figure 1.3 shows a proposed method of activation of PKC.

Figure 1.3 Activation of PKC



Schematic representation of inactive and active forms of PKC. The RACK binding site and substrate binding site are shown. Taken from Mochly-Rosen *et al*, 1995.

The phenomenon of translocation may be influenced by cellular proteins called receptors for activated C kinase (RACKs). These were first described by Mochly-Rosen *et al* (1991). It is now known that RACKs only bind activated PKC since the binding site only becomes exposed when the enzyme has bound to its cofactors. The C2 domain contains at least part of the binding site for RACKs (Mochly-Rosen *et al*, 1995). It has been suggested that RACKs may have specificity for PKC isoenzymes (Dekker and Parker, 1994).

Mature PKC is itself phosphorylated at 2 distinct sites. There is a transphosphorylation on the regulatory domain that renders the kinase catalytically competent and an autophosphorylation on the catalytic domain that may be important for the localisation of the enzyme (Dutil *et al*, 1994). PKC activity is terminated by proteolytic cleavage in the hinge region of the enzyme by calpain (Kishimoto *et al*, 1989). It has been proposed that the conformational change induced by the binding of activators to PKC increases the affinity of the enzyme for calpain (Savart *et al*, 1992). The family of 14-3-3 proteins regulate or mediate the interaction between protein kinases and other signal transduction proteins (Aitken *et al*, 1995). The tissue distribution of 14-3-3 almost parallels the known distribution of PKC (Ichimura *et al*, 1991). However, there have been conflicting results over the role of 14-3-3 as a regulator of PKC. Some have found them to be inhibitors of PKC activity, for example, KCIP-1 interacted with the DAG binding site (Toker *et al*, 1990) whilst others have found that 14-3-3 activates PKC (Isobe *et al*, 1992). Nevertheless, interaction of 14-3-3 isoforms with PKC is

established and it may be that the interaction of different isoforms with PKC is responsible for the different results obtained.

1.2.4. PKC as a Therapeutic Target

PKC has attracted attention as a target for cancer drug development for a number of reasons. It is the primary receptor for the tumour-promoting phorbol esters (Niedel *et al*, 1983; Sando and Young, 1983). Chronic exposure to phorbol esters is required for tumour promotion and this leads to depletion of PKC. Thus, it has been postulated that down regulation of PKC is necessary to relieve negative growth regulation of PKC and to promote tumourigenesis (Nishizuka, 1986). However, some PKC inhibitors, including staurosporine, inhibit tumour promotion (Yoshizawa *et al*, 1990; Borek *et al*, 1991). Therefore, the question remains whether sustained activation or down regulation of PKC is necessary for tumour promotion.

PKC can exert both positive and negative regulation on cell growth. Overexpression of PKC-α in a variety of cell types does not cause transformation (Borner *et al*, 1991; Eldar *et al*, 1990), while overexpression of PKC-βI may (Housey *et al*, 1988), or may not (Hsieh *et al*, 1989; Krauss *et al*, 1989) cause cell transformation. Overexpression of PKC-ε in NIH 3T3 cells causes their transformation (Mischak *et al*, 1993). Although there are clearly differences in pattern of expression, and the functions of PKC isoenzymes between normal and transformed cells, there is so far no consistent pattern to the distribution of the PKC isoenzymes in tumour cells that might provide a selective target for drug development.

PKC activity is altered in certain malignancies. Breast tumours contain much higher levels of PKC compared to normal breast tissue from the same patients (O'Brian *et al*, 1989a). Human malignant glioma cells have been shown to express greater PKC activity than non-neoplastic astrocytes and glial cells (Couldwell *et al*, 1991). However, 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 and carcinoma of the colon compared with normal mucosa (Guillem *et al*, 1987; Kopp *et al*, 1991; Kusunoki *et al*, 1992). The metastatic potential of leukaemic cells also correlates with PKC activity; the more aggressive cells contain higher PKC activity (Aflalo *et al*, 1992). Differential expression of PKC isoenzymes have been

reported in certain cancer cells. PKC-β is specifically elevated in thyroid cancer tissues compared to normal thyroid gland (Hagiwara *et al*, 1990).

Oncogene products may alter PKC isoenzyme expression. Transformation of rat embryo fibroblasts and liver epithelial cells by *ras* caused an increase in PKCs -α and -δ and a decrease in PKC-ε (Borner *et al*, 1990, 1992a, b). PKC can also activate nuclear protooncogenes and enhance transcription. PKC phosphorylates I-κB resulting in the release of NF-κB which then enters the nucleus, binds to DNA and triggers transcription (Ghosh and Baltimore, 1990).

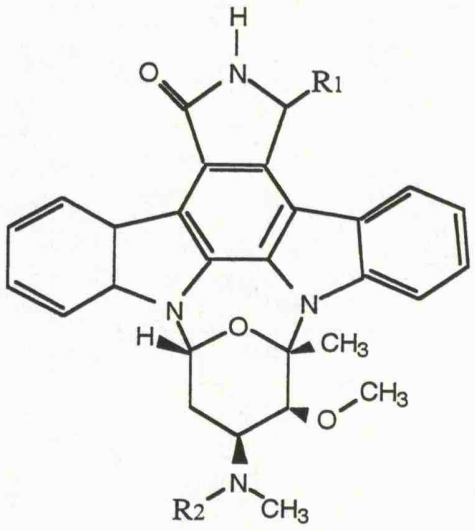
For all these reasons modulators of PKC, activators and inhibitors, have been investigated as potential anti-cancer agents. In the following section I will concentrate on the nine inhibitors used in this study. For further information on the range of PKC inhibitors reviews such as those by Gordge and Ryves, (1994) and Basu, (1993) have been published.

1.3. Inhibitors of PKC

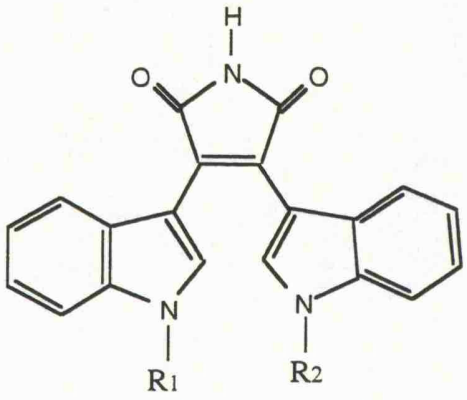
Nine PKC inhibitors were used in this study (figure 1.4), the indolocarbazoles staurosporine, UCN-01 and CGP 41251, the bisindolylmaleimides RO 31 8220 and GFX 109203X and calphostin C, NPC 15437, H-7 and miltefosine.

Staurosporine is a microbial alkaloid from *Streptomyces.* It is an extremely potent inhibitor of PKC with an IC_{50} value in the nM range (Tamaoki *et al*, 1986). However it also inhibits cAMP-dependent protein kinase (PKA) (Tamaoki *et al*, 1986) and p60 v-src tyrosine protein kinases (TPK) (Nakano *et al*, 1987) in this same range. Thus it cannot be considered a specific PKC inhibitor. Its potent anti-proliferative activity is attributed to its inhibition of diverse protein kinases. Although staurosporine is highly toxic *in vivo*, it shows anti-tumour activity at tolerated doses (Meyer *et al*, 1989). Staurosporine has served as the lead compound for the development of analogues that have greater selectivity for PKC. UCN-01 (UCN), also isolated from *Streptomyces,* is 7-hydroxystaurosporine. This agent demonstrates more selectivity for PKC inhibition in vitro (IC_{50} for PKC, 4.1nM; PKA, 42nM and TPK, 45nM) without loss of potency (Takahashi *et al*, 1990). The cytotoxicity of UCN is much lower compared to staurosporine. UCN, but not staurosporine, shows *in vivo* anti-tumour activity against

Figure 1.4 Inhibitors of PKC

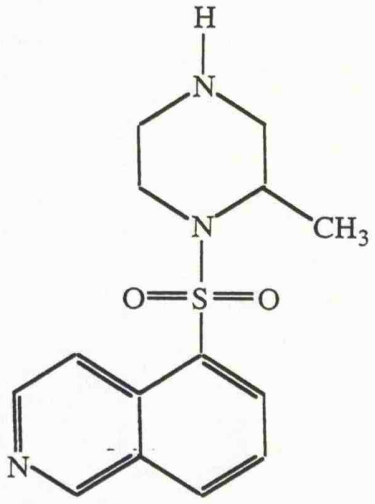


Stau: R_1, R_2 = H
UCN: R_1 = OH, R_2 = H
CGP: R_1 = H, R_2 = benzoyl

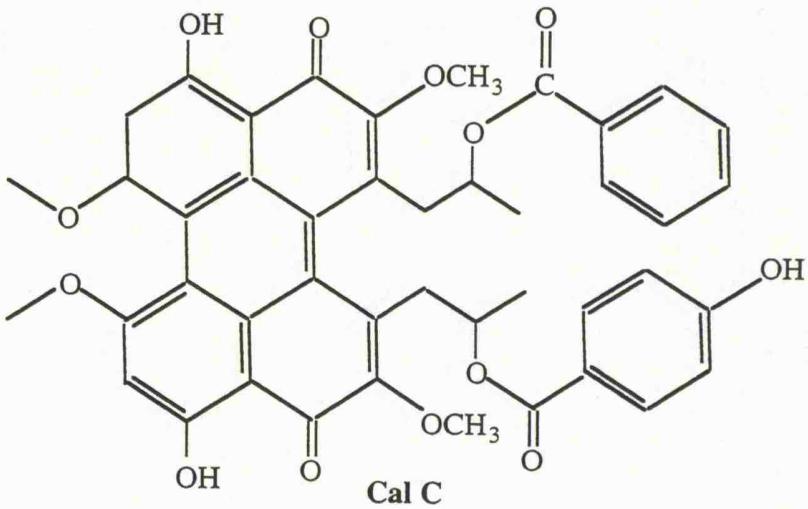


RO: $R_1 = CH_3$ $R_2 = CH_2(CH_2)_2S\text{-}C(=NH)\text{-}NH_2$

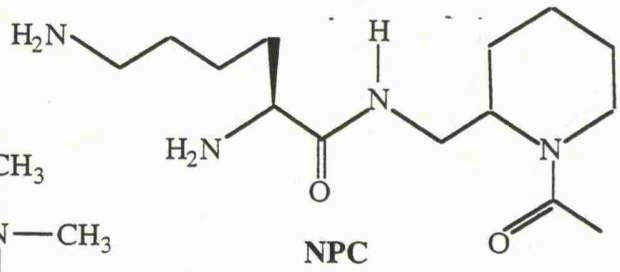
GF: R_1 = H, $R_2 = CH_2(CH_2)_2\text{-}N(CH_3)_2$



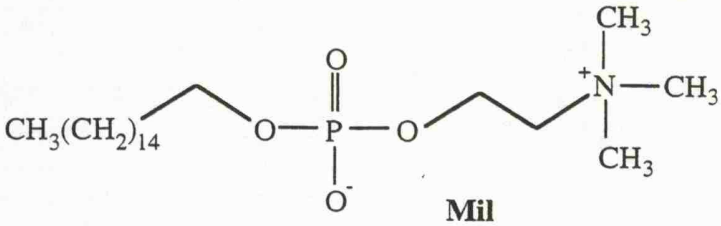
H-7



Cal C



NPC



Mil

The PKC inhibitors used in this study.

three human xenografts and two murine tumour models (Akinaga *et al*, 1991). They also demonstrated in this study that UCN is 5-10 times less toxic in mice than staurosporine, when compared at their maximum tolerated doses. UCN discriminates between PKC subfamilies, the calculated K_i values for cPKCs, nPKCs and aPKC-ζ are approximately 1 nM, 20 nM and 3.8 μM respectively (Mizuno *et al*, 1995). CGP 41251 (CGP) is *N*-benzoyl staurosporine. It has less potency but shows much more selectivity to PKC compared to staurosporine (Meyer *et al*, 1989). Meyer *et al* also showed that CGP and staurosporine exhibit significant anti-tumour activity against T-24 xenografts in athymic nude mice. The increased specificity to PKC was accompanied by increased tolerability and enhanced anti-tumour activity at equally tolerated doses. More recently using human tumour xenografts CGP has been shown to inhibit the growth of gastric cancer, colorectal cancer, breast cancer and lung cancer (Ikegami *et al*, 1995). CGP has also been shown to possess chemosensitising properties, and it reverses the multidrug resistance phenotype (Utz *et al*, 1994). This compound is currently undergoing clinical trials in the UK. Staurosporine, UCN and CGP are all indolocarbazoles and interact with the catalytic domain of PKC.

Another group of PKC inhibitors are the bisindolylmaleimides which include RO 318220 (RO) (Davis *et al*, 1989) and GF 109203X (GFX) (Toullec *et al*, 1991). The structure of these inhibitors is based on that of staurosporine, however, the geometry of the molecule has been changed by removing a bond in the aglycone and an introduction of an extra carboxyl group into the lactone ring. RO has improved selectivity for PKC over staurosporine (Dieter and Fitzke, 1991). RO inhibits the different PKC isoenzymes similarly (Wilkinson *et al*, 1993). GFX also shows selectivity for PKC and inhibits via binding in the ATP binding site (Toullec *et al*, 1991). Martiny-Baron *et al*, (1993) showed that GFX was less effective in inhibiting the calcium independent isoenzymes. It seems surprising that RO and GFX display selectivity for PKC despite strong homology between the ATP binding sites of the different protein kinases. However, the affinity of an inhibitor towards its binding site is likely to depend on the overall tertiary structure of the enzyme rather than the simple amino acid sequence of the binding site.

The isoquinolinesulphonamide, H-7, has been used extensively as a PKC inhibitor. This agent inhibits PKC competitively with respect to ATP, i.e., interacting with the

catalytic domain (Hidaka *et al*, 1984). This compound is far less potent than the staurosporine analogues inhibiting PKC in the μM range. The sole use of this agent as a PKC inhibitor is problematical since it also inhibits the cyclic nucleotide dependent kinases. The specificity of H-7 is further complicated by observations that it is less able to inhibit endogenous protein phosphorylation *in vivo* than when histone is employed as a substrate *in vitro* (Oudinet *et al*, 1992).

Calphostin C, isolated from *Cladosporium cladosporioides*, is a potent and specific inhibitor of PKC. It is 1000 times more inhibitory to PKC than other protein kinases and inhibits all isoenzymes to the same degree (Kobayashi *et al*, 1989). It interacts with the common regulatory region in all isoenzymes. Gopalakrishna *et al*, (1992) found that isolated PKC was irreversibly inactivated by a brief incubation with calphostin C in the presence of light and oxygen. It was suggested that calphostin C binds to PKC and undergoes photo-oxidation thus inducing a site specific modification. Thus this agent acts differently to the reversible inhibitors so far described. Recent reports have suggested that the target of calphostin C *in vivo* is not PKC (Gamou and Shimizu, 1994a; 1994b). The authors suggest that calphostin C actually activates a signalling pathway since the agent stimulates EGF receptor phosphorylation and induces the expression of early response genes.

The last two inhibitors used in this study, NPC 15437 (NPC) and miltefosine, both act at the regulatory domain and both inhibit PKC in the μM range. NPC is a specific PKC inhibitor with no inhibition of other protein kinases observed (Sullivan *et al*, 1991a). NPC interacts with the N-terminal region of the C1 domain. Sullivan *et al*, (1991b) suggested that such an interaction may block the ability of DAG / phorbol ester to alter the conformation of PKC in order to activate it. Miltefosine is an alkylphosphocholine which possesses good anti-tumour activity in dimethylbenzanthracene induced rat mammary tumours (Hilgard *et al*, 1988). However, the activity of miltefosine is extremely selective and other tumour types were insensitive to its action. Geilen *et al*, (1991) showed that miltefosine inhibits PKC and suggested that this was one possible mechanism of the anti-neoplastic action of this agent. Miltefosine has been under going clinical trials but the results so far are not as conclusive as those gained in animal models (Verweij *et al*, 1993a; 1993b).

1.4. Multidrug Resistance

Tumours can either present as intrinsically resistant or develop (acquire) resistance to chemotherapeutic agents used in the clinic. The resistance observed can be against a certain class of agent or pleiotropic i.e. against unrelated groups of compounds. Both types of resistance constitute a fundamental problem in the treatment of malignancies.

1.4.1. Cytotoxic Drug Resistance

The specificity of resistance to cytotoxic agents depends on the genetic changes that lead to resistance. Changes in target enzymes or uptake transporters for the drug give a resistance specific to a small group of structurally related analogues. In contrast, changes in cellular detoxification pathways, such as glutathione, result in resistance restricted to groups of drugs that have similar reactive species (Hamilton *et al*, 1985). Anthracyclines, such as adriamycin, daunomycin and etoposide, exert their cytotoxic effect by inhibiting the DNA strand rejoining activity of topoisomerase II (topo II) and thus induce double stand breaks in the genome (Tewey *et al*, 1984). P388 leukaemia cells resistant to adriamycin have been shown to have decreased levels of topo II (Deffie *et al*, 1989). Feldhoff *et al*, (1994) described a mutation in the topo II gene that changes the subcellular localisation of the enzyme so that it is found principally in the cytoplasm. Mutations that change the properties of the enzyme itself have also been described (Chan *et al*, 1993). Alkylating agents, such as cisplatin and melphalan, generate highly reactive electrophilic intermediates that add alkyl groups to DNA. Cells resistant to these agents have been reported to have increased levels of glutathione, (Hamilton *et al*, 1985), that scavenges the active species. Some cells have an increased ability to conjugate glutathione to the drugs via enhanced glutathione-S-transferase activity (Robson *et al*, 1987). Other resistance mechanisms exist, such as, increased levels dihydrofolate reductase activity in cells resistant to antifolates (Friedkin *et al*, 1962) and the deletion of deoxycytidine kinase in cells resistant to cytidine analogues (Chu and Fischer, 1965).

1.4.2. Multidrug Resistance

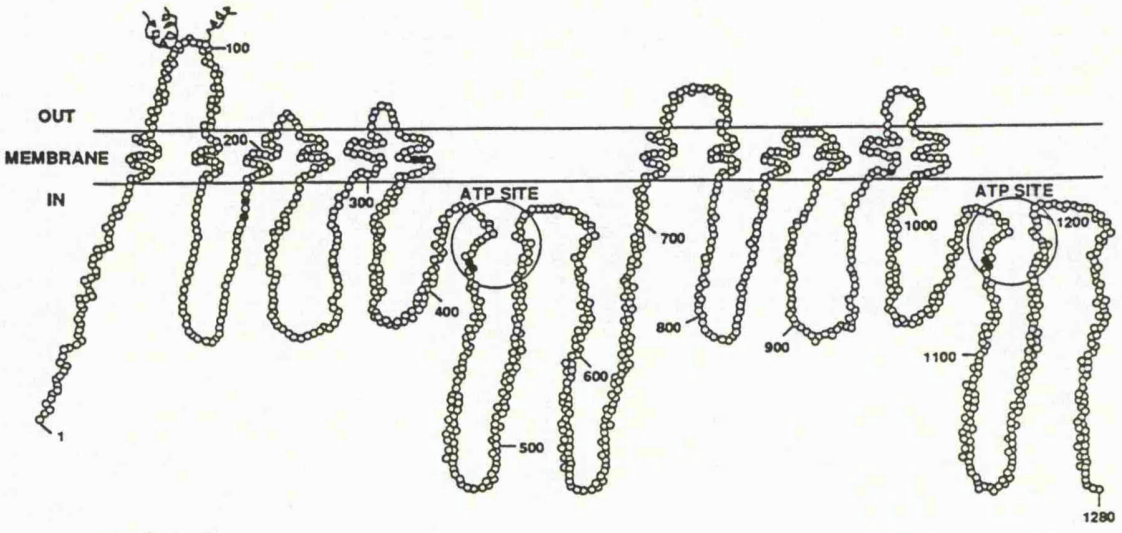
Multidrug resistance (MDR) is defined as the resistance of tumour cells to several structurally and functionally unrelated anti-cancer drugs. In vitro this resistance can

arise even after exposure to a single agent. This form of resistance is a major obstacle to successful chemotherapy. Classical MDR is mediated by P-glycoprotein (P-gp) which functions as an active drug efflux pump and extrudes drug reducing the intracellular concentration to below its therapeutic level (Gottesman and Pastan 1993). Thus the most consistent finding in MDR is reduced intracellular drug accumulation. Cancers can be insensitive to a wide variety of cytotoxic drugs but do not express P-gp. This phenotype was termed atypical MDR (Danks *et al*, 1987). A candidate gene for one such phenotype has been cloned and termed the multidrug resistance associated protein (MRP) (Cole *et al*, 1992). This protein is also a membrane pump. More recently a 110 kD protein has been identified in an MDR lung cancer cell line and has been named lung resistance related protein (LRP) (Scheper *et al*, 1993). It has been found to be overexpressed in non-P-gp MDR tumour cell lines. The deduced LRP amino acid sequence shows 88 % identity with a rat major vault protein and thus may also mediate drug resistance via a transport process (Scheffer *et al*, 1995).

1.4.3. P-glycoprotein

Humans have two MDR genes, *MDR1* and *MDR2*, which share 76 % homology. P-gp is the 170 kD protein product of the *MDR1* gene. Transfection of the *MDR1* cDNA into drug sensitive cells conferred upon them a resistance phenotype, demonstrating that the presence of P-gp alone is sufficient to bestow a classical MDR phenotype (Ueda *et al*, 1987). However, transfection of *MDR2* into cytotoxic sensitive cell lines did not result in the MDR phenotype (Schinkel et al, 1991). *MDR1* encodes a protein of 1280 amino acids (Chen *et al*, 1986) which consists of two halves that share 43 % homology. Each half has been shown to form six transmembrane domains and a highly conserved ATP-binding cassette motif. An N-terminal extracellular loop contains a number of glycosylation sites and the two halves are joined by a linker region (figure 1.5). P-gp has been found to have remarkable homology to a family of bacterial transport proteins that utilise ATP to transport metabolites and toxins across the membrane. It is possible that P-gp functions physiologically in this way to efflux endogenous substrates / toxins out of the cell. This theory is compatible with the tissue distribution of P-gp. It is located on the lumenal membranes of hepatocytes lining the bile canaliculae in the liver and the apical brush border of proximal tubular epithelia in the kidneys.

Figure 1.5 Human P-Glycoprotein



Model of human P-glycoprotein derived from sequence analysis. The filled-in balls are amino acid residues in which mutations have been shown to alter drug transport specificity. Taken from Gottesman and Pastan, 1993.

In the intestinal tract, it is found in the mucosal surface of the jejunum and the colon (Thiebaut *et al*, 1987), and the lumenal side of specialised capillary endothelial cells in the brain and testis (Thiebaut *et al*, 1989; Cordan-Cardo *et al*, 1989). Thus, it is proposed that physiologically P-gp has a protective role, excreting toxins from the blood into the urine, thus preventing natural plant toxins in the food entering the blood from the gut and preventing toxins from penetrating the brain. The physiological role of the murine equivalent of human *MDR2* is in the secretion of phospholipids into the bile (Smit *et al*, 1993).

P-gp is glycosylated and phosphorylated. Schinkel *et al* (1993) demonstrated that the absence of glycosylation did not alter the level or pattern of cross resistance. However, the absence of glycosylation sites reduced the efficiency with which drug resistant clones could be generated. They suggested that glycosylation contributed to the proper routing or stability of P-gp but not to drug transport. The major sites of phosphorylation are clustered within the linker region of P-gp (Chambers *et al*, 1993; 1994). These sites are serine residues and are thought to be phosphorylated by PKC. Recent mutational studies where the serine residues were replaced with alanines have shown that phosphorylation of P-gp is not necessary for its function as a drug

transporter (Germann *et al*, 1996). The authors suggest that phosphorylation may be essential for the transport of physiological substrates.

P-gp can recognise and efflux structurally diverse compounds. The major characteristics are: hydrophobic ring structure; variability in size, presence of a basic amino group and a high potency to partition within membranes (Ford *et al*, 1989; Ramu and Ramu, 1989; Pearce *et al*, 1989; Zamora *et al*, 1988). Pawagi *et al*, (1994) proposed that aromatic amino acid residues in the transmembrane region of P-gp provide a surface which is compatible to the geometry of the drug and thus may promote the passage of a variety of ring containing compounds. The authors also suggest that binding may induce conformational changes within the protein which result in the drug being transported.

1.4.4. MDR1

Most resistant cell lines have been established by a classic step-wise selection protocol where the drug is increased in small, sublethal increments. In the early stages of the development of resistance an increase in *MDR1* gene transcription appears to be responsible. However, at the higher levels of resistance development is paralleled by the appearance of cytogenetically detectable chromosomal anomalies resulting from gene amplification (Shen *et al*, 1986). The chromosomal abnormalities commonly associated with gene amplification are homogeneously staining regions (HSRs), abnormal banding regions (ABRs) or double minutes (DMs) (Biedler and Riehm, 1970; Riordan *et al*, 1985; Fojo *et al*, 1985). Frequently the degree of MDR correlates to the length of the HSR, or number of DMs observed. In the absence of the selecting drug the cells lose these features and become concomitantly sensitive to the same spectrum of drugs to which they originally expressed resistance (Dahloff *et al*, 1984; Meyers *et al*, 1985; Fojo *et al*, 1985). Such phenotypic reversion is a genotype that is characteristic of unstable forms of gene amplifications such as DMs and HSRs. Using *MDR1* DNA as a probe it was found that the gene amplification structures in many of the MDR cell lines contained amplified *MDR* genes (Riordan *et al*, 1985; Jongsma *et al*, 1987). Kohno *et al*, (1989) demonstrated that *MDR1* promoter could be activated directly on the addition of anti-cancer agents including vincristine, daunomycin and adriamycin. Thus the acquired expression of the *MDR1* gene may be an adaptation of the tumour cell to the presence of the chemotherapeutic agent.

Not much is known about the transcriptional regulation of *MDR1*. The promoter of the *MDR1* gene was shown to be a target for the c-Ha-Ras-1 oncogene and the p53 tumour suppressor gene products, both of which are associated with tumour progression (Chin *et al*, 1992) and the transcription factor SP1 has been shown to activate the *MDR1* promoter (Cornwell and Smith, 1993a). Uchiumi *et al*, (1993), demonstrated that the phosphorylation of transacting factors may modulate *MDR1* activity. Two signalling pathways have also been implicated in the activation of *MDR1*, one involving c-Raf kinase (Cornwell and Smith, 1993b) and the other being a PKA dependent pathway (Glazer and Rohlff, 1994). More recently the TPA activation of the *MDR1* promoter has been shown to be mediated by the transcription factor, EGR1, in hematopoietic cells (McCoy *et al*, 1995).

1.4.5. Clinical Significance of MDR

MDR1 has been implicated as a key determinant of response of some forms of cancer to chemotherapy. Studies of human tumour samples have suggested a correlation of *MDR1* expression to both intrinsic and acquired drug resistance. Fojo *et al*, (1987a; 1987b) and Goldstein *et al*, (1989) found high *MDR1* levels in a number of human tumour types that had not been previously exposed to chemotherapy. These tumours included colon cancer, renal cell carcinoma, hepatoma, chronic leukaemias and meningomas. These tumours are considered to intrinsically express the *MDR1* gene since they develop from cells that normally express the *MDR1* gene. Thus these tumour types characteristically display poor response rates to chemotherapy. Evidence for the development of acquired resistance comes from studies that have shown an increase in *MDR1* expression at relapse after chemotherapy (Lum *et al*, 1993). The frequency of P-gp positive tumours increases from the time of diagnosis to time of relapse, for example, for breast cancer frequency rises from 0-10 % (diagnosis) to 30-50 % (relapse) (Lum *et al*, 1993). Although increased transcription of the human *MDR1* gene in cultured cell lines often results from gene amplification, the *in vivo* amplification of the *MDR1* gene in cancer is rarely reported, possibly because it occurs infrequently or it is difficult to detect. Due to the heterogeneity of tumour cell populations, if only a small percentage of the cells harbour amplified *MDR1* genes it would be very difficult to detect. The amplification of *MDR1* in clinical samples of

mammary carcinoma has been reported (Lonn *et al*, 1992; Lonn *et al*, 1993). In these studies the cells were obtained by fine needle biopsies.

The prevalence of P-gp expression in tumours typically exhibiting an intrinsic resistance, the development of increased expression at the time of relapse after initial chemotherapy and the negative prognostic influence of P-gp expression in response and survival for certain tumour types have provided a strong rationale for the clinical study and development of MDR modulators. The non-specific transport role of P-gp has allowed identification of a wide array of chemical compounds with P-gp inhibitory activity in cell culture systems (Lum *et al*, 1993). On the basis of the findings by Tsuruo *et al*, (1982), who demonstrated that verapamil could reverse cellular resistance to vincristine and adriamycin in *MDR1* positive cells, initial clinical trials aimed at circumventing drug resistance mainly used verapamil as the MDR modulator. However, this agent causes cardiac toxicity and this has proved to be dose limiting (Ozols *et al*, 1987; Cairo *et al*, 1989). An analogue of verapamil, dexverapamil, has been developed that has potentially less cardiac toxicity with a similar potential to inhibit drug efflux. Other agents have been used in clinical trials as modulators of MDR and include cyclosporine (Yahanda *et al*, 1992; Bartlett *et al*, 1994) and tamoxifen (Trump *et al*, 1992; Millward *et al*, 1992).

1.5. PKC and MDR

There is very strong evidence to suggest a link between PKC and MDR and that PKC regulates P-gp function.

1.5.1. Increased PKC Activity in MDR Cells

There are multiple reports of increased PKC activity present in drug selected MDR cell lines in comparison to drug sensitive parental cell lines. These include adriamycin resistant breast cancer cells (MCF-7/DoxR) (Fine *et al*, 1988), promyelocytic leukaemia cells (HL-60/ADR) (Aquino *et al*, 1990), Sarcoma 180 cells (Posada *et al*, 1989), murine fibrosarcoma (UV-2237M-ADR^{R}) (Ward and O'Brian, 1991) and vincristine resistant murine erythroleukaemia cells (Sparatore *et al*, 1990). PKC activity levels are not only elevated in the cytosolic compartment but may be elevated in the nuclear compartment as well in at least one MCF-7 MDR cell line (Lee *et al*,

1992). Murine sarcoma S180 cells exposed to adriamycin for one hour were found to have markedly increased PKC activity and content, which suggested that PKC activation and elevation may be associated with early events in the selection of MDR tumour cells (Posada *et al*, 1989). Further evidence for this idea comes from the observation that the level of PKC activity directly correlates with the MDR phenotype in a series of murine fibrosarcoma cell line with different degrees of MDR (O'Brian *et al*, 1989b). These studies, however, do not provide direct proof that PKC directly modulates P-gp or affects the MDR phenotype.

1.5.2. Effect of PKC Activators on MDR

Treatment of MDR cells with PKC activators has been reported to increase the phosphorylation of P-gp in erythroleukaemia K562/ADR (Hamada *et al*, 1987), MCF-7/ADR (Fine *et al*, 1988) or KB-V1 (Chambers *et al*, 1990) cells. Phorbol esters have also been shown to decrease drug accumulation and drug sensitivity (Fine *et al*, 1988; Dong *et al*, 1991). TPA has been shown to induce *MDR1* expression in normal human lymphocytes (Chaudhary and Roninson, 1992).

1.5.3. Effect of PKC Inhibitors on MDR

PKC inhibitors have been shown to reverse the MDR phenotype. Staurosporine, a potent general kinase inhibitor, increases vincristine accumulation in MDR 2780AD, K562/ADR and KBC-4 cells (Sato *et al*, 1990). The authors postulated that staurosporine may be a substrate of P-gp because it inhibited the binding of vincristine to MDR cells. Staurosporine also caused a concentration dependent decrease in P-gp phosphorylation and a concomitant increase in daunomycin accumulation in vincristine resistant HL-60 cells (Ma *et al*, 1991). Calphostin C, a potent PKC specific inhibitor, modulates P-gp transport by binding to the protein (Bates *et al*, 1993). Thus, in studies which used PKC inhibitors that also bind to P-gp (such as staurosporine and calphostin C), the possibility exists that inhibition of P-gp function results from either inhibition of drug binding or inhibition of P-gp phosphorylation mediated by PKC. Sachs *et al*, (1995) have recently shown that safingol, a specific PKC inhibitor, reverses the MDR phenotype without altering P-gp drug binding suggesting that PKC is important for P-gp function.

1.5.4. PKC Isoenzymes in MDR

There are a number of studies which have demonstrated a specific pattern of PKC isoenzyme overexpression in MDR selected cell lines. Most of these reports have shown PKC-α to be overexpressed. A direct demonstration of a role for PKC in inducing MDR phenotype came from a study by Yu *et al*, (1991). This work showed that transfection of MCF-7 cells with *MDR1* alone did not contribute to a high degree of resistance to adriamycin. However, if PKC-α was also transfected a high degree of resistance to adriamycin and vinblastine was induced. Transfection of PKC-α on its own did not confer a MDR phenotype. Further evidence indicating that PKC-α is a positive regulator of P-gp function in breast cancer cells was suggested by the finding that expression of PKC-α antisense cDNA decreased PKC activity, P-gp phosphorylation and the MDR phenotype in drug resistant cells (Ahmad and Glazer, 1993). More recently an N-myristoylated peptide that contained a sequence corresponding to the pseudosubstrate region of PKC-α partially reversed the MDR in MCF-7-MDR cells through inhibition of PKC-α (Gupta *et al*, 1996). Although PKC-α is the most commonly overexpressed PKC isoenzyme it is not clearly established which isoenzyme is the most important in MDR. The phenomenon appears to be cell-type specific. PKCs -α and -θ have been shown to be elevated in non-drug selected resistant cell lines (Zhang *et al*, 1996). A recent study has looked at the expression of PKC isoenzymes and MDR associated genes in primary and relapsed state acute myelogeneous leukaemias (AML) (Beck *et al*, 1996). They found significantly enhanced MRP, GST-π and PKC-θ expression in relapsed state AML compared to primary AML. By far the strongest increase, though, was measured for *MDR1*. PKC-α and topoisomerase I gene expression showed some tendency to higher levels in relapsed states of AML.

1.5.5. Phosphorylation of P-glycoprotein

P-gp is a substrate for a number of protein kinases other than PKC, including PKA (Mellado and Horwitz, 1987), PK-1 (Staats *et al*, 1990) and an unknown kinase which is GTP regulated but not affected by inhibitors of cAMP, cGMP or PKC (Lelong *et al*, 1994). The amino acid sites of P-gp phosphorylation stimulated by phorbol ester *in*

situ mimic the sites phosphorylated in isolated P-gp vesicles by PKC, which suggests that the same sites are phosphorylated on P-gp by PKC *in vitro* and *in situ* (Chambers *et al*, 1990). The amino acid sites phosphorylated by PKC are all serine residues which are present in the linker region (Chambers *et al*, 1993). It is theorised that phosphorylation of P-gp may regulate its function but this has yet to be definitely proven and recent reports have suggested that PKC phosphorylation of P-gp is not necessary for its function (see discussion chapters 5 and 6).

There is ample evidence to suggest that PKC overexpression by one or more of its isoenzymes is associated with the MDR phenotype. This appears to be cell type dependent as well as drug dependent in that adriamycin induced MDR seems to be more commonly associated with an overexpression of PKC-α isoenzyme. Other issues complicating this process are the possible involvement of other kinases which have not yet been fully explored.

1.6. Aims of this Study

Breast cancer is the leading cause of death among women in the Western world, where, despite radical surgery, about 35 % of affected women die of it. Cancer can be considered a disease of abnormal cell growth mediated by altered intracellular signalling. The components of signalling pathways provide attractive targets for the development of new types of anti-cancer drugs. Attention has been drawn to PKC as a target and PKC inhibitors as potential anti-cancer agents for the reasons already discussed. The overall aim of this project was to define the role of PKC in the growth of MCF-7 breast carcinoma cells and in the development and maintenance of MDR in MCF-7/ADR cells. It is necessary to understand these mechanisms before PKC can be fully exploited as a novel target for anti-cancer treatment.

One of the aims of this project was to determine whether the growth inhibitory properties of nine PKC inhibitors was a direct consequence of their inhibition of the enzyme. MCF-7 human breast carcinoma cells were used as a model system.

PKC, when active, is located at the membrane (Kraft and Anderson, 1983). Most studies of PKC have used the soluble form of the enzyme and then added PS, calcium

and an activator to try and reconstitute the natural cellular environment. This method is not ideal although widely used. It has been suggested that PKC may be loosely attached to the membrane (Wolf *et al*, 1985) and that the isolation procedure of the enzyme strips it away. Thus we decided to look at isolated PKC that had been activated *in situ* i.e. under more natural conditions. A suitable separation method was developed and the hypothesis was tested that PKC inhibitors inhibit cytosolic and membrane derived PKC similarly.

There is evidence to suggest that P-gp might play a role in resistance to cytotoxic drugs used in breast cancer treatment (Sanfilippo *et al*, 1991; Keith *et al*, 1990; Verelle *et al*, 1991). Following chemotherapy virtually all breast tumours are P-gp positive regardless of initial P-gp status (Glazer and Rohlff, 1994). As already discussed MDR and P-gp are a major clinical problem. It is important to discover how the MDR phenotype is induced and how P-gp is regulated. Available evidence suggests that PKC is important in this process. One of the aims of this study was to investigate further the link between PKC and MDR using a MDR breast cell line and the parental drug sensitive cells. The isoenzyme profiles of both cell lines were investigated at the protein and mRNA levels. A revertant cell line was then used to determine how tightly PKC and MDR were linked.

The importance of PKC in the maintenance of MDR would suggest that inhibition of the enzyme could be beneficial. The hypothesis was tested that PKC inhibitors would modulate the MDR phenotype seen in the MDR breast cells. Thus the effects of five potent PKC inhibitors on *MDR1* and P-gp expression were investigated.

CHAPTER 2

MATERIALS

2.1. Chemicals and Reagents

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

Affiniti Research Products Ltd., Nottingham, Notts.
Monoclonal anti-PKC epsilon and anti-PKC theta antibodies.

Amersham International Plc., Little Chalfont, Bucks.
Molecular weight markers - rainbow coloured protein 14.3-200 kD, anti-mouse and anti-rabbit horseradish peroxidase linked whole antibodies, anti-rabbit Texas Red linked whole antibody, ECL Western blotting detection reagents, ECL hyperfilm, Hybond-N+ membrane, PKC Biotrak enzyme assay system, Sequenase version 2.0 DNA sequencing kit, [α-^{32}P]dCTP, [γ-^{32}P]ATP, [α-^{35}S]dATP, [γ-^{33}P]ATP, multiprime DNA labelling system.

Antigen Bioclear UK Ltd., Devizes, Wilts.
Polyclonal anti-PKC epsilon antibody (C-15).

Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts.
TEMED, ammonium persulphate, 30% acrylamide/bis solution 37.5:1, Bio-Rad protein assay dye reagent, prestained SDS-PAGE standards high range, AG501-x8 mixed bed resin, Poly-Prep chromatography columns.

Boehringer Mannheim, Lewes, East Sussex.
DNA molecular weight markers II.

Calbiochem-Novabiochem Co., Nottingham, Notts.
Calphostin C, bisindolylmaleimide (GF 109203X).

Cambridge Bioscience, Cambridge.
Polyclonal anti-mdr antibody (Ab-1), chromaspin 10 columns.

Coulter Electronics, Luton, Beds.
Isoton II diluent.

Fisons Scientific Equipment Plc., Loughborough, Leics.
Absolute ethanol, methanol, hydrochloric acid, phosphoric acid, glacial acetic acid, Whatman 3MM paper.

Gibco BRL, Glasgow, Scotland.
Polyclonal anti-PKC zeta antibody, RPMI 1640 medium, foetal calf serum, L-glutamine 200mM, Ca^{2+}/Mg^{2+}-free PBS (10x), Pen/Strep (100x), trypsin/EDTA (10x), Trizol reagent, Taq polymerase, agarose (electrophoresis grade), 100bp DNA ladder, 0.24-9.5kb RNA ladder, EcoR1, M-MLV reverse transcriptase.

ICN Flow, High Wycombe, Bucks.
Guanidine thiocyanate (research grade).

Invitrogen BV, NV Leek, The Netherlands.
TA cloning kit.

KJP, Milton Keynes, Beds.
Polaroid type 53 & 55 film.

Melford Laboratories Ltd., Chelsworth, Suffolk.
5-Bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal).

Oxoid (UNIPATH Ltd.), Basingstoke, Hamps.
PBS Dulbecco 'A' tablets.

Packard, Pangbourne, Berks.
Ultima Gold scintillation fluid.

Pharmacia Biotech., St. Albans, Herts.
NAP-5 columns, dNTP set 100mM solutions, pd(N)6 random primers.

Photosol Ltd., Basildon, Essex.
RG X-ray fixer and developer.

Promega, Southampton,
Wizard Minipreps DNA purification system, AMV reverse transcriptase, RNasin, T4 polynucleotide kinase.

Qiagen, Dorking, Surrey.
QIA quick-spin PCR purification kit, Blood and Cell Culture DNA Midi Kit.

Signet Laboratories Inc., Dedham, MA.
C219 monoclonal antibody to P-glycoprotein.

Tissue Culture Services, Boltoph Claydon, Bucks.
Monoclonal anti-PKC alpha antibody.

Other chemicals and reagents were purchased from **Sigma Chemical Co., Poole, Dorset.**

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

UCN-01 ; Kyowa Hakko Kogyo Co., Tokyo, Japan.
RO 31-8220 ; Roche Research Centre, Welwyn Garden City,
CGP 41251 ; Ciba Geigy, Basel, Switzerland.
NPC 15437 ; Nova Pharmaceutical Co., Baltimore, Maryland.
Miltefosine ; Asta Pharma, Frankfurt, Germany.

2.2. Solutions and Buffers

2.2.1. Cell Culture

Medium for MCF-7 Cells

MEM media

10 % foetal calf serum

100 iu/ml Penicillin

100 µg/ml Streptomycin

1 mM Pyruvate

2 mM Glutamine

Non-essential Amino Acids

Medium for WT & MCF-7/Adr Cells

RPMI 1640 media

10 % heat inactivated foetal calf serum

100 iu/ml Penicillin

100 µg/ml Streptomycin

2 mM Glutamine

Medium for A549 Cells

Hams F12 media

10 % foetal calf serum

100 iu /ml Penicillin

100 µg /ml Streptomycin

2 mM Glutamine

Trypsin/EDTA Solution (1 x)

10 ml Trypsin/EDTA 10 x solution

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

80 ml Sterile water

The solution was stored at 4°C.

PBS

1 PBS tablet

100 ml Distilled water

The solution was autoclaved and stored at 4°C

Drug Solutions

Stock solutions of all drugs were prepared in DMSO except for NPC 15437, which was dissolved in water, and stored at -20°C in aliquots. These solutions were diluted in DMSO/water to the appropriate concentration when required.

2.2.2. Cell Fractionation

W3 Wash Buffer

	final concentration
2.422 g Tris base	20 mM
8.766 g NaCl	150 mM
0.902 g glucose	5 mM

to 1 l water

The pH was adjusted to 7.4 with concentrated HCl and stored at 4°C. Prior to use, protease inhibitors leupeptin and aprotinin were added to give a final concentration of 2 μg /ml.

H8 Buffer

	final concentration
200 μl Tris-HCl 1.0 M, pH 7.5	20 mM
200 μl EDTA 100 mM, pH 7.5	2 mM
200 μl EGTA 100 mM, pH 7.5	2 mM
4 μl β-mercaptoethanol	6 mM
10 μl leupeptin 2 mg /ml	2 μg /ml
10 μl aprotinin 2 mg /ml	2 μg /ml

to 10 ml with distilled water

The buffer was prepared on the day of use. Stock solutions of Tris-HCl 1.0 M, pH 7.5 and EDTA and EGTA 100 mM, pH 7.5 were prepared and stored at 4°C.

Wash Buffer G

	final concentration
9.318 g KCl	125 mM
3.634 g Tris base	30 mM
1.072 g MgAc	5 mM

1.902 g EGTA	5 mM
3.14 ml β-mercaptoethanol	45 mM

to1 l with distilled water

The pH was adjusted to 7.5 with HCl and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 2 μg /ml.

Swelling Buffer

	final concentration
373.0 mg KCl	10 mM
1.817 g Tris base	30 mM
536.0 mg MgAc	5 mM
951.0 mg EGTA	5 mM
1.57 ml β-mercaptoethanol	45 mM
783.0 mg benzamidine	10 mM

to 500 ml with distilled water

The pH was adjusted to 7.5 with HCl and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 15 μg /ml.

Swelling Buffer with 25 % Glycerol

This was prepared as for swelling buffer, but 25 ml distilled water was replaced with 25 ml glycerol prior to pH adjustment. The protease inhibitors were added as for swelling buffer.

Swelling Buffer, 25 % Glycerol and 0.1 % Triton X100

5 ml swelling buffer with 25 % glycerol

25 μl Triton X100 20 % (w/v) in distilled water.

The protease inhibitors were added as for swelling buffer.

Swelling Buffer and 1% Nonidet P40

2 ml swelling buffer + leupeptin and aprotinin

20 mg Nonidet P40.

Sonication Buffer

	final concentration
121.1 mg Tris base	10 mM
584.4 mg NaCl	100 mM
40.7 mg $MgCl_2$	2 mM

190.2 mg EGTA	5 mM
314 µl β-mercaptoethanol	45 mM
1 g Nonidet P40	1 % (w/v)

to 100 ml with distilled water

The pH was adjusted to 8.0 with NaOH and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 15 µg /ml.

Lysis Buffer

	final concentration
47.6 mg $MgCl_2$	5 mM
8.4 mg $NaHCO_3$	1 mM
1.7 mg PMSF	100 µM

to 100 ml with distilled water

The pH was adjusted to 7.5 and stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 15 µg /ml.

Homogenisation Buffer

	final concentration
605.5 mg Tris base	50 mM
186.1 mg EDTA	5 mM
380.4 mg EGTA	10 mM
17.4 mg PMSF	1 mM
156.6 mg benzamidine	10 mM
54.0 µl β-mercaptoethanol	7.7 mM

to 100 ml with distilled water

The pH was adjusted to 7.5 and stored at 4°C. Protease inhibitors were added prior to use to give final concentrations of 15 µg /ml.

Homogenisation Buffer with 0.1 % Triton X100

10 ml Homogenisation buffer + leupeptin and aprotinin

50 µl Triton X100 20 % (w/v) in distilled water.

Homogenisation Buffer with 0.1 % Brij 58

10 ml Homogenisation buffer + leupeptin and aprotinin

150 µl Brij 58 6.67 % (w/v) in distilled water.

Tris Buffer

	final concentration
0.61 g Tris base	0.1 M
2.5 g SDS	5 % (w/v)
2.5 ml β-mercaptoethanol	5 % (v/v)

to 50 ml with distilled water

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

2.2.3. SDS/PAGE and Western Blot Analysis

Sample Buffer for PKC Analysis

	final concentration
10 ml Tris-HCl 500 mM, pH 6.8	125 mM
8 ml glycerol	20 % (v/v)
16 ml SDS 10 % (w/v)	4 % (w/v)
2 ml bromophenol blue (1 mg /ml)	50 μg /ml
1.23 g DTT	200 mM

to 40 ml with distilled water

The buffer was prepared omitting DTT and stored at room temperature. DTT was added prior to use.

Sample Buffer for P-gp Analysis

	final concentration
10 ml Tris-HCl 500 mM, pH 6.8	250 mM
7.2 ml glycerol 60 % (v/v)	21.6 %
0.8 ml bromophenol blue 5 % (w/v)	0.2 %
2 g SDS	10.0 % (w/v)

The buffer was stored at room temperature. 10 % β-mercaptoethanol (v/v) was added prior to use.

Separating Gel for PKC Analysis

	final concentration
5.0 ml Tris-HCl 1.5 M, pH 8.8	375 mM
0.2 ml SDS 10 % (w/v)	0.1 % (w/v)
5.336 ml 30 % acrylamide/bis 37.5:1	8 % (w/v)

9.362 ml distilled water

100 µl ammonium persulphate 10 % (w/v) 0.05 % (w/v)

7.5 µl TEMED

Separating gel was prepared shortly before pouring. Ammonium persulphate catalyses polymerisation and TEMED accelerates the reaction and so these two reagents were added last. This is sufficient for 4 mini gels.

Separating Gel for P-gp Analysis

This was prepared as for PKC gel except a 7.5 % acrylamide/bis final concentration was achieved. Thus 5 ml 30 % acrylamide/bis was used with 9.7 ml water.

Stacking Gel for PKC Analysis

	final concentration
2.5 ml Tris-HCl 0.5 M, pH 6.8	125 mM
0.1 ml SDS 10 % (w/v)	0.1 % (w/v)
1.62 ml 30 % acrylamide/bis 37.5:1	5 % (w/v)
5.73 ml distilled water	
50 µl ammonium persulphate 10 % (w/v)	0.05 % (w/v)
5 µl TEMED	

The stacking gel was prepared prior to use. Ammonium persulphate and TEMED were added last. This is sufficient for 4 mini gels.

Stacking Gel for P-gp Analysis

This was prepared as for PKC analysis except a 4.4 % acrylamide/bis final concentration was achieved. So 1.47 ml 30 % acrylamide/bis was added with 5.88 ml water.

Running Buffer

	final concentration
15.15 g Tris base	25 mM
72 g glycine	192 mM
5 g SDS	0.1 % (w/v)
to 5 l with distilled water	

The buffer was stored at 4°C.

Electrode Solution

	final concentration
23.24 g Tris base	48 mM
11.72 g glycine	39 mM
1.5 g SDS	0.0375 % (w/v)
800 ml methanol	20 % (v/v)

to 4 l with distilled water

The solution was stored at 4°C.

Transfer Solution

	final concentration
6.06 g Tris base	25 mM
28.83 g glycine	192 mM
400 ml methanol	20 % (v/v)

to 2 l with distilled water

The solution was stored at 4°C.

TBS-T 0.3 %

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

to 4l with distilled water

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

TBS-T 0.1 %

	final concentration
2.42 g Tris base	20 mM
8 g NaCl	137 mM
1 g Tween 20	0.1 % (w/v)

to 1 l with distilled water

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

Stripping Buffer (Western)

	final concentration
3.78 g Tris base	62.5 mM

10 g SDS	2 % (w/v)
3.49 ml β-mercaptoethanol	100 mM

to 500 ml with distilled water

The pH was adjusted to 6.7 and stored at room temperature.

2.2.4. RNA Isolation

DEPC Treated Water

1 l of pure water was treated with 0.5 ml DEPC.

It was shaken, left overnight and autoclaved twice to remove the DEPC to leave ultra clean, RNAse free water.

0.1 M Tris-HCl, pH 7.5

12.7 g Tris HCl

2.36 g Tris base

to 1 l with DEPC treated water.

This gives a buffer of pH 7.5. DEPC reacts rapidly with amines and so cannot be used to treat solutions containing buffers such as Tris.

4 M Guanidine Thiocyanate

236.32 g guanidine thiocyanate

to 500 ml with 0.1 M Tris-HCl, pH 7.5.

This was stored at room temperature.

0.1 M EDTA, pH 7.5

7.44 g EDTA

to 200 ml with pure water

The pH was adjusted to 7.5.

5.7 M Caesium Chloride

95.99 g CsCl

to 100 ml with 0.1 M EDTA, pH 7.5.

The solution was treated with 50 µl DEPC and stirred overnight. After autoclaving the volume was adjusted if necessary. Density should be 1.7g/l. Solution was stored at room temperature.

20 % SLS

20 g SLS

to 100 ml with DEPC treated water.

2 x Column Wash

	final concentration
2.94 g sodium citrate	0.1 M
5.84 g NaCl	1.0 M
74.44 mg EDTA	2.0 mM
0.2 g Na lauroylsarcosinate	0.2 % (w/v)

to 100 ml with DEPC treated water.

Solution was stored at room temperature.

Elution Buffer

	final concentration
121.2 mg Trizma hydrochloride	10 mM
27.8 mg Trizma base	10 mM
37.22 mg EDTA	1 mM
50 mg SDS	0.05 % (w/v)

to 100 ml with DEPC treated water

This gives a solution of pH 7.6 and was stored at room temperature.

2.2.5. Primer Extraction

2 M Sodium Acetate, pH 7.0

16.41 g NaAc

to100 ml with pure water

The pH was adjusted to 7.0. The solution was then treated with 50 µl DEPC, mixed and autoclaved.

2.2.6. Gel Analysis of DNA

50 x L Buffer

	final concentration
109.03 g Tris base	1.8 M
103.5 g NaH_2PO_4	1.5 M

7.31 g EDTA	50 mM

to 500 ml with pure water

Agarose Gel

	final concentration
0.8 g ultra pure agarose	1 % (w/v)
1.6 ml 50 x L buffer	1 x

78.4 ml pure water

Solution was heated in a microwave for approximately 1 min., shaking frequently. The solution was boiled until all of the agarose had dissolved. It was allowed to cool to approx. 50°C before pouring.

Loading Buffer

	final concentration
1 ml 0.5 M EDTA	0.1 M
2.5 g sucrose	50 % (w/v)
few grains bromophenol blue	approx. 0.2 % (w/v)

to 5 ml with pure water

Solution was filter sterilised and stored at 4°C.

Ethidium Bromide Solution

A solution of 5 µg/ml in pure water was used for staining gels. Ethidium bromide is very hazardous and should be handled with extreme care. The solution was protected from light and stored at room temperature.

2.2.7. Cloning of Probes

Luria-Bertani (LB) Broth

	final concentration
10 g bacto-tryptone	1 % (w/v)
5 g bacto-yeast extract	0.5 % (w/v)
10 g NaCl	1 % (w/v)

to 1 l with distilled water

The pH was adjusted to 7.0 with NaOH. The medium was autoclaved and ampicillin added, once it had cooled to 55°C, to give a final concentration of 50 µg/ml.

LB Agar

LB medium was prepared and 15 g/l agar was added before autoclaving.

X-Gal Stock Solution

400 mg 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) was dissolved in 10 ml dimethylformamide to give a 40 mg/ml stock solution. Solution was protected from light and stored at -20°C.

2.2.8. Sequencing Double-Stranded DNA template

3 M NaAc, pH 5.2

40.83 g NaAc in 100 ml pure water. The pH was adjusted to 5.2 using glacial acetic acid. Once autoclaved the solution was stored at room temperature.

5 X TBE

	final concentration
54 g Tris base	446 mM
27.5 g boric acid	445 mM
4.65 g EDTA	12 mM
to 1 l with pure water	

Denaturing Gel

	final concentration
15 ml 30 % acrylamide/bis 37.5:1	6 %
15 ml 5 x TBE	1 x
31.5 g urea	7 M
450 µl 10 % (w/v) ammonium persulphate	0.06 %
75 µl TEMED	
to 75 ml with pure water	

The urea was dissolved in the acrylamide and TBE and the volume made up to 75 ml with water. The solution was placed on ice to cool before the addition of ammonium persulphate and TEMED just before pouring.

2.2.9. Southern Blot Analysis

5 M Sodium Chloride

146.1 g NaCl

to 500 ml with pure water

0.2 M HCl (solution I)

5 ml concentrated HCl

245 ml pure water

12.5 M Sodium Hydroxide

50 g NaOH

to 100 ml with pure water

0.5 M NaOH, 1.5 M NaCl (solution II)

75 ml 5 M NaCl

10 ml 12.5 M NaOH

165 ml pure water

3 M Tris-HCl, pH 7.5

72.68 g Tris base

to 200 ml with pure water

Solution may have to be heated in order to dissolve the Tris. When cool pH was adjusted to 7.5.

1 M Tris-HCl, pH 7.5, 1.5 M NaCl (solution III)

75 ml 5 M NaCl

83.3 ml 3 M Tris-HCl, pH 7.5

91.7 ml pure water

20 x SSC (solution IV)

	final concentration
175.3 g NaCl	3 M
88.2 g sodium citrate	0.3 M

to 1 l with pure water

Prehybridisation Solution (Southern)

	final concentration
7.5 ml 20 x SSC	6 x
1.25 ml 5 % marvel	0.25 % (w/v)

16.25 ml pure water

Hybridisation Solution (Southern)

As prehybridisation solution with the addition of 10 % (w/v) dextran sulphate and 270-540 µl labelled probe.

The labelled probe was boiled for 2 min. and placed on ice before addition.

0.1 x SSC, 0.1 % SDS

	final concentration
1 ml 20 x SSC	0.1 x
1 ml 20 % SDS (w/v)	0.1 %
198 ml distilled water	

0.4 M Sodium Hydroxide

6.4 ml 12.5 M NaOH

to 200 ml with pure water

0.1 x SSC, 0.1 % SDS, 0.2 M Tris-HCl, pH 7.5

	final concentration
1 ml 20 x SSC	0.1 x
1 ml 20 % SDS (w/v)	0.1 %
13.3 ml 3 M Tris-HCl, pH 7.5	0.2 M
184.7 ml pure water	

2.2.10. Gel Analysis of RNA

10 x MOPS Buffer

	final concentration
20.93 g MOPS	0.2 M
20.51 g NaAc	0.5 M
1.86 g EDTA	10 mM
to 500 ml with DEPC treated water	

The pH was adjusted to 7.0 with NaOH. The solution was then autoclaved.

Formaldehyde Agarose Gel

	final concentration
0.8 g ultra pure agarose	1 %
8 ml 10 x MOPS buffer	1 x

14.4 ml formaldehyde (37 %) 6.66 %

57.6 ml DEPC treated water

The agarose and water were boiled in a DEPC treated conical flask. The other reagents were added carefully to avoid introducing air bubbles once the agarose had cooled to 60°C. The gel was poured in a fume hood since formaldehyde fumes are toxic.

Deionised Formamide

Formamide frequently contains ionic contaminants some of which can hydrolyse RNA. It is therefore essential that they are removed by deionisation. Formamide was added to mixed-bed resin (Bio-Rad) (5 g resin /100 ml formamide) and stirred for more than 1 hour. Formamide was filtered from the resin, aliquoted and stored at -20°C.

Sample Loading Buffer (RNA)

100 µl deionised formamide

30 µl formaldehyde (37 %)

20 µl 10 x MOPS buffer

0.9 µl EtBr (10 mg/ml)

This was made prior to use.

2.2.11. Northern Blot Analysis

50 x Denhardts Solution (DHT)

	final concentration
1 g Ficoll	1 %
1 g PVP	1 %
1 g BSA	1 %

to 100 ml with DEPC treated water

1 M Sodium Phosphate

120 g Na_3PO_4

to 1 l with DEPC treated water

Prehybridisation Solution (Northern)

	final concentration
12.5 ml deionised formamide	50 %

2.5 ml 50x DHT	5 x
7.5 ml 20x SSC	6 x
1.25 ml 1M Na_2PO_4, pH 6.8	50 mM
1 ml pure water	
250 µl sonicated salmon sperm DNA (10 mg /ml)	100 µg /ml

The salmon sperm DNA was boiled for 5 min and then placed on ice before addition.

Hybridisation Solution (Northern)

As for prehybridisation buffer with the addition of 10 % (w/v) dextran sulphate and 10^6 dpm/ml of labelled probe. Typically 10 ml hybridisation buffer was used per blot. Both the salmon sperm DNA and labelled probe were boiled and placed on ice before addition.

2 x SSC, 0.1 % SDS

	final concentration
25 ml 20x SSC	2 x
1.25 ml 20% SDS (w/v)	0.1 %
223.75 ml pure water	

2.2.12. Semi-Quantitative RT/PCR

Acrylamide Gel

	final concentration
6.7 ml 30 % bis / acrylamide	8 %
5.0 ml 5 x TBE	1 x
175 µl 10% ammonium persulphate	0.07 %
13.125 ml distilled water	

TEMED (8.75 µl) was added just prior to the gel being poured.

2.2.13. Genomic DNA Analysis

TE, pH 8.0

	final concentration
88.8 mg Trizma hydrochloride	10 mM

53.0 mg Trizma base	10 mM
37.22 mg EDTA	1 mM

to 100 ml with pure water

pH of solution was corrected to 8.0 using HCl.

2.2.14. Immunoprecipitation

Immunoprecipitation Buffer

	final concentration
0.25 ml 1 M Tris-HCl, pH 7.4	10 mM
1.25 ml 3 M NaCl	150 mM
2.5 ml 50 mM EDTA	5 mM
2.5 ml glycerol	10 % (v/v)
1.25 ml Triton X-100 (20 % w/v)	1 % (w/v)

to 25 ml with distilled water

Solution was stored at 4°C. Leupeptin and aprotinin were added prior to use to give final concentrations of 15 μg /ml.

Sepharose Beads

0.1 g protein A sepharose beads were suspended in 1ml immunoprecipitation buffer and stored at 4°C. Prior to use the beads were vortexed to mix thoroughly.

CHAPTER 3

METHODS

3.1. Cell Culture

3.1.1. Routine Cell Maintenance

Cell culture procedures were carried out aseptically in a class II laminar flow cabinet. Cells were maintained in a Sanyo CO_2 auto-zero incubator at 37°C with 5 % CO_2. MCF-7 and A549 cells were obtained from the European Collection of Animal Cell Cultures (ECACC), Porton Down, Wilts. MCF-7 cells stably resistant to adriamycin (MCF-7/Adr) and the parental drug sensitive counterparts (WT MCF-7) were acquired from Dr. Kenneth Cowan, National Cancer Institute, Bethesda, Md. (Batist *et al*, 1986). Cells were passaged routinely when they approached confluence to maintain logarithmic growth. Once resurrected from the cell bank, cells were allowed to go through at least 4 passages before being used for experiments and they were not used after passage 30. MCF-7 cells were maintained in Minimum Essential Medium (MEM), MCF-7/Adr and WT cells were grown in RPMI 1640 medium and A549 cells in Hams F12 medium (section 2.2.1.). Cells were washed with phosphate buffered saline (PBS) and 1 x trypsin/EDTA solution was used for the detachment of monolayers from culture flasks. During subculturing cells were reseeded using 5 % of the original culture for WT and A549 cells and 12 % for MCF-7/Adr and MCF-7 cells. MCF-7/Adr cells were maintained in 500 nM adriamycin to retain the high level of resistance.

3.1.2. Cell storage in Liquid Nitrogen

Cells were trypsinised and pelleted using a Haraeus Minifuge T at 400 g for 5 min. Cells were resuspended in FCS with 10 % dimethylsulphoxide (DMSO) to a density of $1x10^6$ cells / ml. Aliquots of 1 ml were frozen slowly to -80°C overnight and then immersed in liquid nitrogen. Cells were resurrected from the cell bank by rapid thawing to 37°C and each vial was transferred to a flask containing 30 ml culture medium.

3.1.3. Studies of Cell Growth

Cells were seeded at $2x10^4$ in 35 mm diameter wells in 3 ml medium. Cells (2 wells) were counted using a Coulter Counter (model ZM, settings: current 100, attenuation 16, T_l 26, T_u 99.9) every day. Doubling time was calculated from plotting cell number against time. Medium was replenished every two days.

3.1.4. Inhibition of Cell Growth

To investigate the effects of PKC inhibitors on cell growth, cells were seeded as above and allowed to attach for 4 h. A concentration range of an inhibitor was added into duplicate wells. Control cells were incubated with an equivalent volume of DMSO (0.3 % v/v) which had no effect on cell growth. Cells were left for 4 doubling times, medium and drug being replenished halfway through the incubation. Cells were then trypsinised and counted. Degree of growth inhibition was calculated as a percentage of drug free control. The mean concentration causing 50 % growth inhibition (IC_{50}) was determined for each drug from three separate experiments.

3.2. Fractionation of Cells

3.2.1. Preparation of Cytosolic Fraction of Cells for PKC Assay

Cells were cultured on 150 mm diameter petri dishes. On approaching confluence a cytosolic fraction was prepared. Plates were placed on ice and washed three times with 10 ml of ice-cold W3 wash buffer (section 2.2.2.). Plates were allowed to drain in order to remove all of the wash buffer. The cells were scraped into 0.2 ml of ice-cold H8 buffer (section 2.2.2.). Cells lysate was disrupted by sonication on ice using 3 blasts of a Branson sonifier 250 (10 % duty cycle, control output 2). The cytosolic fraction was separated from the particulate fraction using a Beckman L7 ultracentrifuge operating at 100,000 g (40,000 rpm) for 30 min at 4°C. The resulting supernatant was the cytosolic fraction which was kept on ice.

3.2.2. Preparation of Membrane Fraction of Cells for PKC Assay

Cells were cultured on petri dishes as above but before removal they were incubated with 10 μM phorbol dibutyrate (PDBu) for 30 min to redistribute the PKC to the particulate fraction. In order to establish the best protocol for obtaining the membrane fraction for PKC analysis 5 methods were tried.

Method 1

All steps were carried out on ice. The fraction was prepared according to a method modified from that described by Greif *et al*, 1992. Plates were washed three times with

ice-cold wash buffer G (section 2.2.2.). The buffer was removed and the cells were scraped into 0.2 ml of swelling buffer (section 2.2.2.). After 10 min the swollen cells were homogenised using a PTFE pestle (Jencon, Leighton Buzzard, Beds.) and a Citenco homogeniser (Fisons, Loughborough, Leics.). After 6 strokes (speed level 3) the homogenate was looked at under a microscope to ensure that all cells were disrupted but the nuclei were whole. The homogenate was laid over an equal volume of swelling buffer with 25 % glycerol (section 2.2.2.) in an eppendorf tube and centrifuged in an Haraeus Minifuge T at 600 g for 5 min at 4°C to remove the nuclei and unbroken cells. The opaque upper level contained the cytosolic and membrane fractions. This was centrifuged at 100,000 g for 30 min at 4°C. The supernatant was the cytosolic fraction which was discarded and the pellet was the membrane fraction. This was resuspended in 0.5 ml H8 buffer and sonicated (10 blasts, output level 2).

Method 2

All steps were carried out on ice. The fraction was prepared according to the method described by Chakravarthy *et al*, 1991. Plates of cells were washed three times with ice-cold PBS. Cells were scraped into 0.2 ml lysis buffer (section 2.2.2.) and left on ice for 5 min to allow cells to swell. Cells were disrupted by sonication (2 blasts, output level 2) and spun at 200 g for 5 min at 4°C in Minifuge T to remove nuclei and unbroken cells. Supernatant was centrifuged at 100,000 g for 10 min at 4°C. The membrane pellet was resuspended in 0.5 ml H8 buffer.

Method 3

The fraction was prepared using the method described by Carlson *et al*, 1993. Plates of cells were washed three times with ice-cold PBS. Cells were scraped into 0.2 ml homogenisation buffer (section 2.2.2.) and sonicated (2 blasts, output level 2). The nuclei and unbroken cells were removed by a low speed spin (200 g, 5 min, 4°C). The resulting supernatant was spun for 30 min at 100,000 g (4°C). The pellet was washed with homogenisation buffer and resuspended in homogenisation buffer containing 0.1 % Triton X-100 by sonication (2 blasts, output level 2). Following a 1 h incubation on ice the fraction was spun again at 100,000 g as before. The supernatant was the detergent-soluble membrane fraction.

Method 4

Rush *et al*, 1992 showed that under certain conditions Triton X-100 can inhibit protein kinases and solubilise undefined inhibitory factors associated with cellular membranes.

They showed that using Brij 58 instead of Triton could overcome this problem. Thus the membrane fraction was prepared as described under method 3 and Brij 58 (0.1 % w/v) was used instead of Triton X-100.

Method 5

This method combined methods 1 and 4. The membrane fraction was prepared as in method 1 but the pellet was resuspended in homogenisation buffer containing 0.1 % Brij 58 by sonication (3 blasts, output level 2) and left on ice for 1 h. The fraction was then spun at 100,000 g again for 30 min and the supernatant was used for the subsequent assays.

3.2.3. Preparation of Cytosolic, Membrane and Nuclear Fractions for Western Blot Analysis

Cells were cultured on 150 mm diameter petri dishes, 2 plates per treatment. Fractions were prepared initially as described in method 1 above. After the low speed spin the nuclear pellet was resuspended in 1 ml swelling buffer with 25 % glycerol and 0.1 % Triton X-100 (w/v) and pelleted by centrifugation at top speed in MSE microfuge for 2 min at 4°C. This procedure was repeated using swelling buffer with 25 % glycerol. The resulting pellet was resuspended in 0.2 ml sonication buffer (section 2.2.2.) and sonicated (10 blasts, output level 2). The cytosol and membrane fractions were separated by ultracentrifugation as previously described. The supernatant was the cytosolic fraction. The pellet was washed with swelling buffer and resuspended in 0.2 ml swelling buffer containing 1 % Nonidet P40 by sonication. This was the membrane fraction. All three fractions were kept on ice and the protein content of each fraction was determined using the Bradford assay (Bradford, 1976).

3.2.4. Preparation of Membrane Fraction for P-glycoprotein Determination

The membrane fraction was prepared as in section 3.2.3. with slight modifications. The membrane pellet obtained after ultracentrifugation was resuspended in 0.2 ml Tris buffer (section 2.2.2.) by sonication (10 blasts, output level 2). The protein content of the fraction was determined as before.

3.3. Western Blot Analysis

Western blot analysis involves separating proteins using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferring them to nitrocellulose electrophoretically and then using specific antibodies to detect the protein of interest.

3.3.1. Detection of PKC Isoenzymes

Cytosolic, membrane and nuclear fractions were prepared as described in section 3.2.3. The fractions were diluted 1:1 in sample buffer (section 2.2.3.) and boiled for 5 min. An aliquot (30 μg) of each sample was loaded onto a 5 % stacking gel and 8% separating gel with dimensions of 10 x 8 x 0.075 cm (section 2.2.3.). Proteins were resolved using a constant current (42 mA for 2 gels, 50 mA for 4 gels) on a Mighty Small gel apparatus (Hoefer Instruments). Electrophoretic progress was followed using rainbow molecular weight markers, 14.3-200 kD as recommended by the manufacturers (Amersham). The gels were immersed in electrode solution (section 2.2.3.) for 15 min. The separated proteins were transferred onto nitrocellulose (100 V, 4°C, 90 min) using Bio-Rad Mini Trans-Blot equipment. Blots were placed in 10 % non-fat dried milk in TBS-T 0.1 % (section 2.2.3.) and shaken overnight at 4°C to block non-specific binding. All the following procedures were carried out at room temperature on a shaker. The blots were washed in TBS-T 0.3 % (section 2.2.3.), 2 x 1 min and 3 x 5 min. Blots were exposed to PKC antibodies, 1 h for PKCs -α (TCS), -ε and -θ (Affiniti) or 2 h for PKC-ζ (Gibco). All antibodies were diluted in 1 % milk in TBS-T 0.1 %. The monoclonal mouse derived antibodies to PKC-α, -ε and -θ were incubated with the blot at 0.075, 1 and 2 μg / ml respectively and the polyclonal rabbit derived anti-PKC-ζ at a concentration of 2 μg / ml. After incubation the blots were washed in TBS-T 0.3 % as before and incubated with the appropriate horseradish peroxidase linked anti-mouse or anti-rabbit IgG secondary antibody (Amersham) for 1h using a 1 in 500 dilution. Blots were then washed as before and detection was by enhanced chemiluminescence (ECL) using reagents and hyperfilm from Amersham. The horseradish peroxidase acts as a catalyst for the oxidation of a luminol substrate which subsequently emits small but sustained quantities of light. This chemiluminescence is specifically enhanced allowing an image to be recorded on photosensitive film. Blots were exposed to film for 1 sec -5

min and developed using a X-ograph compact x-2 developer with Kodak GBX developer and fixer. Occasionally blots were stripped by a 30 min incubation with stripping buffer (section 2.2.3.) at 50°C. After washing in TBS-T 0.3 % (2 x 1 min and 2 x 10 min) they were blocked overnight in 10 % milk and reprobed the following day as previously described. Bands were quantified using laser densitometry (Molecular Dynamics Inc.).

3.3.2. Detection of P-Glycoprotein

The membrane samples was diluted 1:1 in sample buffer (section 2.2.3.). An aliquot (10 µg) of each sample was loaded onto a 4.4 % stacking gel and 7.5 % separating gel (section 2.2.3.). Proteins were resolved as described in section 3.3.1. Electrophoretic progress was monitored using pre-stained markers 48-199 kD (Bio-Rad). Gels were immersed in transfer buffer (section 2.2.3.) for 15 min. The separated proteins were transferred to nitrocellulose overnight (100 mA, 4°C) using Midget Multiblot equipment (Hoefer). The following day blots were blocked in 10 % milk (in TBS-T 0.1 %) for 2 h at room temperature. The blots were then washed with TBS-T 0.3 % as described in section 3.3.1. The monoclonal antibody to P-glycoprotein (P-gp), C219 (Signet), was diluted in 1 % milk in TBS-T 0.1 % to give a final concentration of 20 ng / ml. The antibody was incubated with the blot for 2 h at room temperature. The subsequent washing, secondary antibody (anti-mouse) incubation and detection was as described in the previous section.

3.4. Protein Kinase C Assays

PKC assays were carried out in the presence of cofactors, PS and TPA (activator of PKC) in the form of micelles, Ca^{2+} and Mg^{2+} using a kit from Amersham. PKC activity was measured via the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a specific peptide substrate following the protocol supplied by the manufacturer.

3.4.1. Determination of Optimal Conditions for PKC Activity

The kit component containing 8 mol % micelles and TPA (38.4 µM) was modified such that activity of PKC could be determined in the absence of PS and TPA. Micelles

were made as follows: 63.5 µl PS (10.4 mg / ml) was dried under a stream of nitrogen and vortexed with 0.2 ml Triton X-100 3 % (w/v). After a 10 min incubation at 27°C 0.8 ml distilled water was added. 100 mM Tris HCl, pH 7.5 (1 ml) was added to give the final assay component concentrations of 420 µM PS, 0.3 % Triton X-100 and 50 mM Tris HCl, pH 7.5. TPA could then be added to this mixture if necessary. As each component of the kit was left out of the assay H8 was added in its place to keep the reaction volume the same.

3.4.2. Enzyme Inhibition Assays

Both the cytosolic and membrane fractions were diluted 5-20 x using H8 buffer (section 2.2.2.) in order to dilute out endogenous inhibitors which could mask the effects of the drugs under investigation. PKC activity of each dilution was determined using 25 µl aliquots. Every time fresh cytosol or membrane was prepared dilutions were checked in order to ensure that in the dilution used for the subsequent assays all the endogenous inhibitors had been diluted out. To assess the PKC inhibitory effects of the drugs, a range of concentrations was added to the cytosol or membrane fraction before the assay and the degree of inhibition was determined as a percentage of drug free control. Radioactivity was counted using a Packard Tricarb 1500 scintillation counter.

3.5. RNA Isolation

3.5.1. Preparation of Total RNA (Caesium Chloride Method)

Cells were cultured on 3 x 150 mm diameter petri dishes. An aliquot (1 ml) 4 M guanidine thiocyanate (GITC) (section 2.2.4.) and 266.7 µl β-mercaptoethanol were pipetted into a glass universal. Cells were washed with PBS and 1 ml 4 M GITC was added to each plate. The cells were scraped and added to the universal giving a total of 4 ml. The final dilution of β-mercaptoethanol was 1 in 5. The cell lysate was homogenised using an ultra-turrax hand held homogeniser (Fisons) for 2 min. 100 µl 20 % sodium lauroyl sarcosinate ($1/40^{th}$ volume) (section 2.2.4.) was added and mixed gently. The homogenate was layered on top of 4.5 ml 5.7 M caesium chloride (section 2.2.4.) in polyallomer tubes (14 x 95 mm, Beckman). Balanced tubes were centrifuged

using a swing out rotor (SW40) at 10 000 g overnight at 20°C. The supernatant was removed carefully and the residual liquid was allowed to drain onto some tissue. The upper section of the tube was cut off leaving a piece approximately 1 cm long. The RNA was on the bottom of the tube. The pellet was resuspended in 0.5 ml ice cold 70 % ethanol (in DEPC treated water, section 2.2.4.) and transferred to an Eppendorf tube. The step was repeated with another 0.5 ml to ensure all the RNA was recovered. The tube was centrifuged for 15 min using a microfuge at top speed. The ethanol was removed very carefully and the pellet was allowed to air dry. The pellet was resuspended in 200 µl DEPC treated water and the optical density (OD) at 260 and 280 nm was measured using a Perkin Elmer λ2S spectrophotometer. The samples were stored at -80°C.

1. RNA mg / ml = OD 260 nm x 40* x dilution factor

   * for RNA an OD of 1 = 40 µg

   for DNA an OD of 1 = 50 µg (ssDNA = 33 µg)

3.5.2. Preparation of Total RNA (Trizol® Method)

The Trizol® (Gibco BRL) method of isolating RNA was used instead of the caesium chloride method due to the fact that less cells were needed and the method itself only took a few hours. Trizol® reagent, a solution of phenol and GITC, maintains the integrity of the RNA while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and organic phase. The RNA remains in the aqueous phase and is recovered by precipitation with isopropanol. The RNA was prepared as in the protocol supplied by Gibco BRL. Cells were seeded in 6 well plates (3 wells per cell line / treatment). The final RNA pellet was allowed to air dry briefly and resuspended in 5 µl water. The sample was then transferred to the next tube and the pellet was dissolved in the same 5 µl. This was repeated for the third tube to get a concentrated RNA sample, the OD of which was then measured and the amount of RNA determined using equation 1 (section 3.5.1.). The samples were stored at -20°C.

3.5.3. Preparation of PolyA$^+$ RNA

A typical mammalian cell contains approximately 10^{-11} g RNA of which 1-5 % is polyA$^+$ RNA or mRNA. This mRNA can be separated from the majority of RNA found in a cell by binding to oligo(dT) cellulose. PolyA$^+$ RNA was prepared from total RNA (caesium chloride method). Poly-Prep chromatography columns (Bio-Rad) were packed with 50-100 mg oligo(dT) cellulose. A column was prepared by the washing through of 5 ml 0.1 M NaOH (DEPC treated), 10 ml DEPC treated water and 5 ml 1 x column wash (section 2.2.4.). RNA sample was heated to 65°C for 5 min and an equal volume of 2 x column wash was added prior to cooling on ice. After 5 min the sample was added to column and the eluate collected. The eluate was heated to 65°C for 5 min and then reapplied to the column. Column was washed with 10 ml 2 x column wash and polyA$^+$ RNA was eluted with 3.5 ml elution buffer (section 2.2.4.). 3 M NaAc, pH 5.2 (350 µl) and absolute ethanol (8.75 ml) were added and the sample stored at -20°C overnight. mRNA was spun down for 30 min at 4°C (10 900 g) and most of the ethanol removed. Pellet was resuspended in remaining ethanol (approximately 1 ml) and transferred to an Eppendorf tube. mRNA was spun down using top speed of microfuge for 15 min and resuspended in small volume of treated water. OD of mRNA was determined and sample stored at -20°C. The column used for polyA$^+$ RNA isolation could be stored after the washing through with 10 ml DEPC treated water and 5 ml 0.1 M NaOH. A small amount of NaOH was sealed in the tube prior to storage at -20°C.

3.6. Probes for Northern Blot Analysis

Probes suitable for determining the message levels of all the different PKC isoenzymes are not commercially available. Using the technique of reverse transcription polymerase chain reaction (RT/PCR) probes for PKCs -α, -ε and -θ were generated. RT uses RNA to make cDNA and then PCR uses the cDNA as a template resulting in numerous copies of a specific sequence of DNA.

3.6.1. Primers

In order to amplify a specific sequence of DNA primers need to be designed to dictate the start and end points of transcription. The published sequences of human PKCs -α and -θ and rat PKC -ε were obtained from the database GENBANK. The three sequences were then matched against each other to identify areas of homology. To ensure the development of specific probes the primers must be against sequences specific for that particular isoenzyme. The computer program, PRIMER, was then used to identify suitable primers for each sequence. The primers used were those that were in areas specific to each isoenzyme. Figure 3.1 shows the sequences against which the primers were designed for each isoenzyme and table 3.1 shows the oligonucleotides that were made.

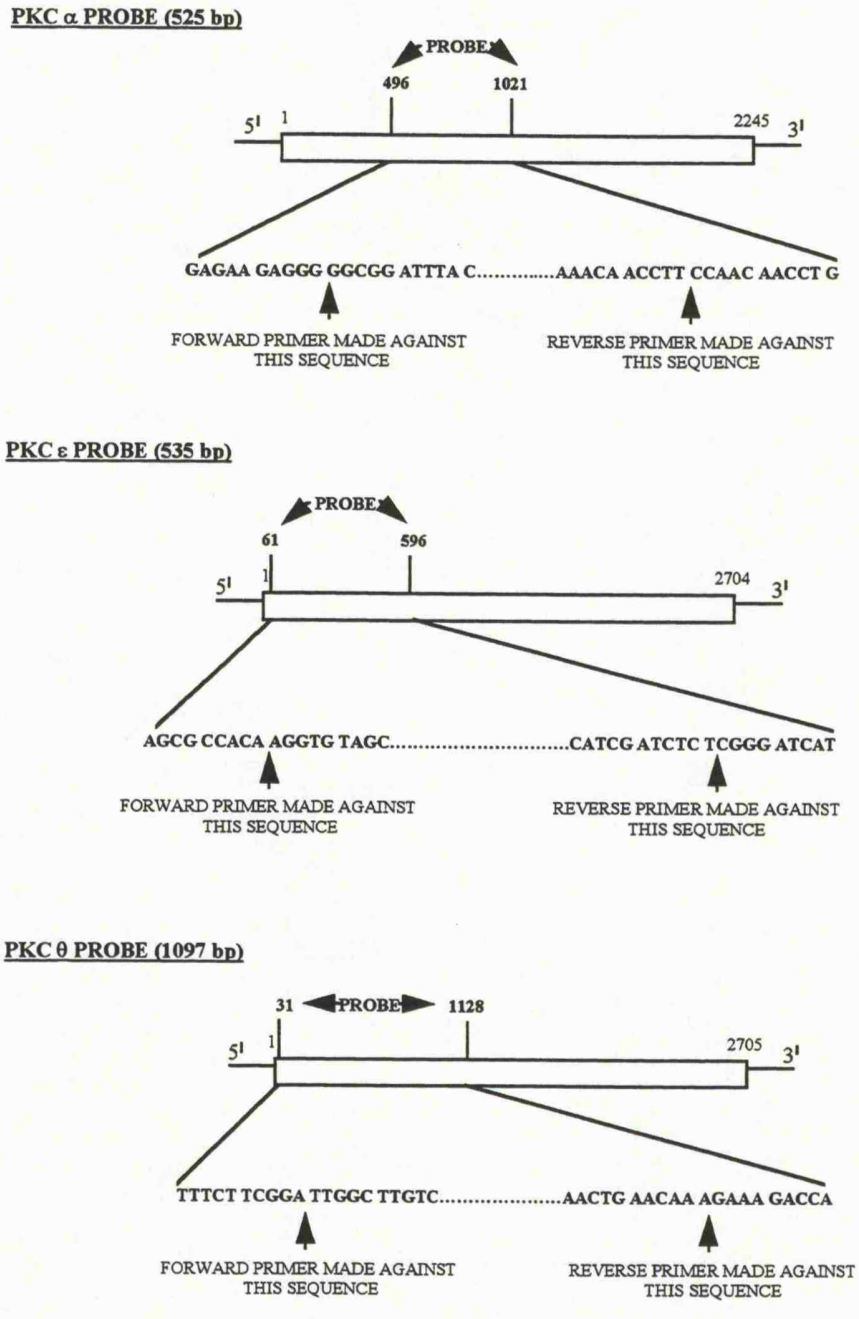
3.6.2. Primer Extraction

The oligonucleotides were synthesised in an ammonia solution and needed to be extracted. An aliquot (200 µl) oligonucleotide was mixed with 20 µl sodium acetate (2 M, pH 7.0, section 2.2.5.) and three volumes of absolute ethanol. The solution was precipitated on dry ice for 20 min and then centrifuged at top speed in a microfuge for 15 min. The pellet was washed 4-5 x with 1 ml 80 % ethanol, and then allowed to air dry. The pellet was resuspended in 50 µl water and the OD at 260nm measured.

3.6.3. Reverse Transcription / Polymerase Chain Reaction (RT/PCR)

Reverse transcription (RT) uses RNA as a template to make cDNA. DNA is made in the 5' to 3' direction. Thus the primer used in the RT reaction needs to be able to bind to the 3' end of the RNA sequence (i.e. the reverse primers), see figure 3.1. RNA from different cells was used to make the three different probes as cells that normally express that isoenzyme were needed for the template. RNA from A549 human lung carcinoma cells was used to make the probes for PKCs -α and -θ and RNAs from rat brain and WT MCF-7 human breast carcinoma cells were used to make the probe for PKC-ε. Both rat and human tissue were used for PKC-ε in order to check that the primers made against the rat sequence also bound to the human sequence. The reactions were carried out in sterile microtubes and all water was DEPC treated.

Figure 3.1 PKC Isoenzyme Primers and Probes



Sequences against which the PKC isoenzyme specific primers were synthesised resulting in the probe lengths indicated.

Table 3.1 Oligonucleotides Designed to Act as Primers

PKC ISOENZYME	FORWARD PRIMER	REVERSE PRIMER
α	GAGAAGAGGGGGCGGATTTAC	AAGGTTGTTGGAAGGTTGTTT
ε	AGCGCCACAAGGTGTAGC	ATGATCCCGAGAGATCGATG
θ	TTTCTTCGGATTGGCTTGTC	TGGTCTTTCTTTGTTCAGTT

Oligonucleotides designed as forward and reverse primers for RT/PCR reactions to synthesise PKC isoenzyme specific probes

An aliquot (20 μg) of total RNA and 10 pmoles of reverse primer were incubated at 60°C for 10 min and then at room temperature for 10 min. This step allowed the primer to anneal to the RNA. The extension mixture was then added, RNasin (1 U / μl), $MgCl_2$ (2.5 mM), PCR buffer (1 x), dNTPs (1 mM), AMV RT (0.75 U / μl) and water to a final reaction volume of 60 μl. The mix was incubated at 42°C for 1 h. A blank containing water instead of RNA was also used to ensure no contamination.

An aliquot (20 μl) from the RT reaction was used in the PCR reaction. To this forward and reverse primers (10 pmoles of each), PCR buffer (1 x), $MgCl_2$ (2.5 mM), Taq Polymerase (0.025 u / μl) and water to a final reaction volume of 100 μl was added. A blank containing 20 μl of water in place of the RT reaction mix and 1 mM dNTPs was also included. Thus there was a blank from the RT reaction and a separate blank for the PCR reaction. Mineral oil (50 μl) was laid on the top of the reaction mixtures to prevent any evaporation. A DNA thermal cycler 480 PCR machine (Perkin Elmer) was used with the following parameters, 94°C 1 min (denaturation temperature), 50°C 1 min (annealing temperature which is lower than the optimal annealing temperatures of the primers), 72°C 2.5 min (extension temperature) for 30 cycles. After 30 cycles the samples were cooled to and maintained at 4°C. Samples were stored at 4°C.

3.6.4. Gel Analysis of PCR Products

PCR products were checked by running out on a 1 % agarose gel containing 1 x L buffer (section 2.2.6.). 1 x L buffer was used as the running buffer. An aliquot (5 μl) PCR product was mixed with 1.5 μl loading buffer (section 2.2.6.) and run at 80 V for approximately 2 h or until the bromophenol blue had run ¾ of the way. Bromophenol blue runs at about the same rate as 300 bp DNA. A 100 bp ladder was also run so that the size of the PCR products could be estimated. The gel was stained with an ethidium bromide solution (section 2.2.6.) for 10 min and destained for 15 min. The gel was then examined under an UV transilluminator and a photograph taken.

3.6.5. Purification of PCR Products

PCR products were isolated from reaction mix using a kit from Qiagen. Basically the DNA was bound to a glass bead resin at high salt concentration and added to a

microspin column. The DNA was eluted from the column with water and stored at 4°C.

3.6.6. Agar Plate Preparation

Sterile L agar (section 2.2.7.) was melted and allowed to cool to 55°C. Ampicillin was added to a final concentration of 50 μg /ml. Agar was poured onto 9 cm petri dishes and stored at 4°C. Prior to use 40 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) (20 mg /ml) was spread onto the plates and allowed to dry. Plates then have to be protected from light. The PCR product will insert into the part of the vector that contains the lac gene (see figure 3.2). Colonies that do not contain an insert will be able to utilise the X-gal, thus forming blue colonies, since they have an intact lac gene. Colonies containing an insert have their lac gene disrupted, cannot use the X-gal and so form white colonies.

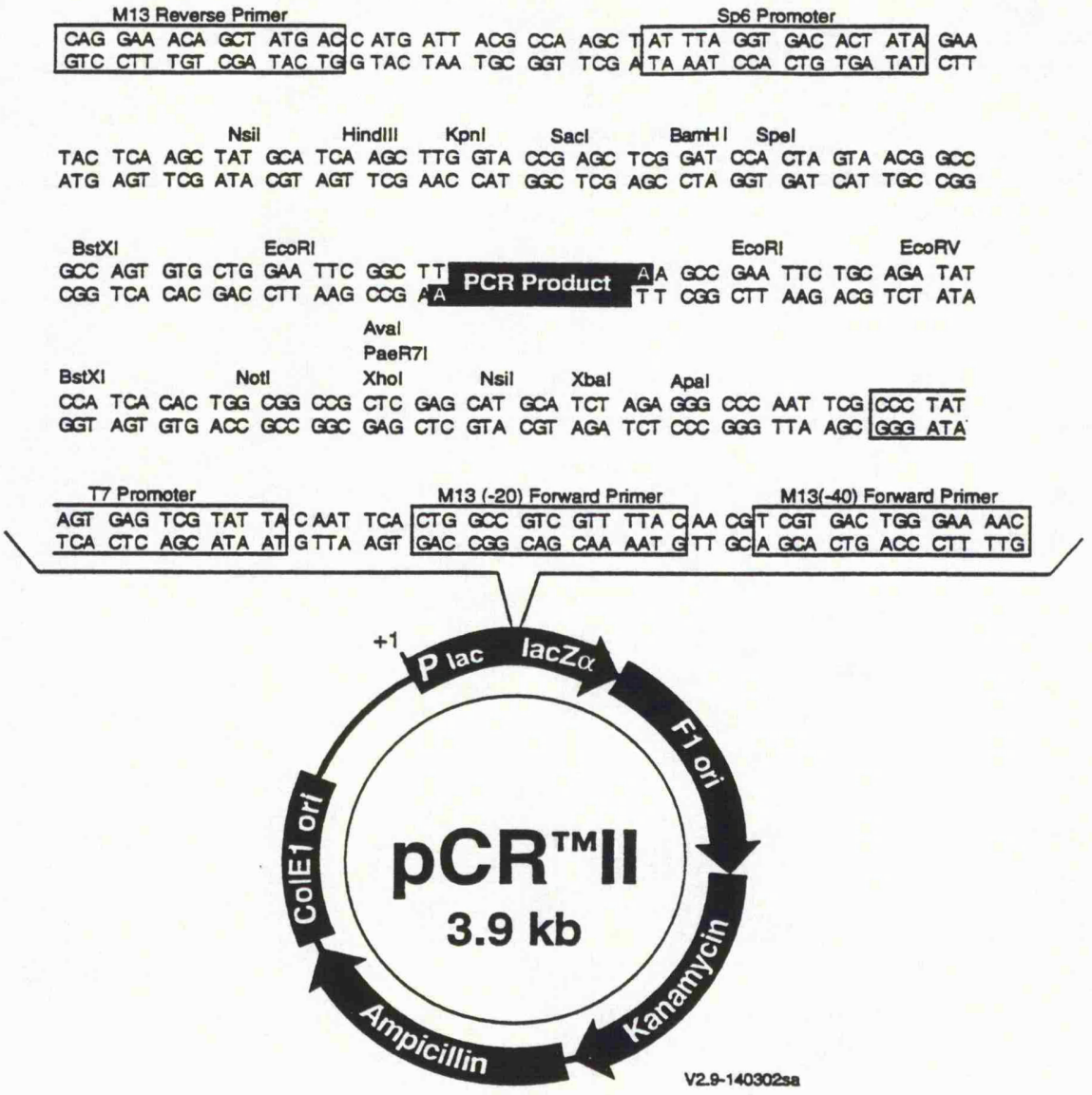
3.6.7. Cloning

The three probes were cloned into pCR™ II vector (figure 3.2) using the TA cloning® kit (Invitrogen). This kit provides a one-step cloning strategy for direct insertion of a PCR product into a plasmid vector. Taq polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine to the 3' ends of the PCR products. The linearised vector supplied in the kit has single 3' deoxythymidine residues. This allows the PCR products to ligate efficiently with the vector. PCR products were ligated at a 1:1 (vector:insert) ratio and a 1:3 ratio and the protocol supplied by the manufacturer was followed. 20 white colonies were picked for each PCR product. Each colony was grown in 10 ml LB broth (section 2.2.7.) containing 50 μg/ml ampicillin by incubating in a shaking incubator at 37°C. Glycerol stocks were prepared for each colony (0.85 ml cells + 0.15 ml sterile glycerol) and stored at -80°C.

3.6.8. Isolation of Plasmid DNA

Wizard minipreps (Promega) were used to isolate the plasmid DNA. Broth containing colonies was centrifuged at 300 g for 5 min to pellet the cells. Basically the cells were lysed and then added to a silica based resin. This was pipetted onto a column and the plasmid DNA was eluted off the column with water and stored at -20°C.

Figure 3.2 Map of pCR™II Vector



Map of linearised vector pCR™ II. The sequence of the multiple cloning site is shown with a PCR product inserted. The inserted PCR product is flanked on each side by EcoR1 sites. Taken from TA Cloning ® Kit Manual (Invitrogen).

To ensure the vector contained the correct insert digestion and sequencing experiments were carried out on isolated vectors for each PCR product.

3.6.9. Digestion of Vectors

As figure 3.2 shows the pCR II vector has two EcoR1 sites either side of the insert. Using computer software (Map program in Winconstin Package) it was determined that none of the PCR products contained an EcoR1 cut site. Thus by cutting the vector with EcoR1 the size of the insert can be calculated by running out on an agarose gel. An aliquot (250 ng) isolated plasmid DNA was incubated with 1 µl EcoR1 (Gibco BRL), 1 µl enzyme buffer and water to a final volume of 10 µl at 37°C for 1 h. Samples of digested and undigested vector were run out on a 1 % agarose gel as in section 3.6.4. alongside Type II DNA markers (Boehringer Mannheim) and 100 bp ladder. The gel was stained with ethidium bromide and visualised under UV light.

3.6.10. Sequencing

To confirm that the correct PCR product had ligated into the vector the insert for each sample was sequenced. Samples were prepared in the following way. Plasmid DNA (1-2 ρmoles) was diluted with water to give a working volume of 18 µl. An aliquot (2 µl) 2 M NaOH was added and incubated at room temperature for 5 min. The solution was neutralised with the addition of 2 µl 3 M NaAc (pH 5.2, section 2.2.8.). DNA was precipitated by the addition of 2.5 volumes 100% ethanol and left on dry ice for 15 min. The sample was spun at top speed in a microcentrifuge for 15 min. The pellet was washed with 200 µl 70 % ethanol and centrifuged as before for 2 min. The pellet was air dried and resuspended in 8 µl water. As figure 3.2 shows the M13 reverse primer can be used for sequencing the insert. M13 reverse primer (5 ρmole) was added and the final preparation of the samples was accomplished using a Sequanase kit (Amersham) and $\alpha[^{35}S]$dATP using the protocol supplied. Basically DNA synthesis is initiated where the primer anneals to the template. The synthesis reaction is terminated by the incorporation of nucleotide analogues that will not support the continued DNA elongation. The dideoxynucleoside triphosphates (ddNTP) lack the 3' OH group necessary for the elongation. When mixtures of dNTPs and one of the four ddNTPs are used the enzyme catalysed polymerisation is terminated in a fraction of the population

of chains at each site where a ddNTP can be incorporated. Four separate reactions with different ddNTPs give complete sequence information. A radioactively labelled nucleotide is also included in the synthesis so labelled chains of various lengths can be visualised by autoradiography after separation by high resolution electrophoresis. The samples were stored at -20°C.

A 6 % acrylamide, 7 M urea denaturing gel was prepared (section 2.2.8.) and poured to give a gel of dimensions 30 x 40 cm. Sharkstooth (14 cm) combs were put in upside down and the gel was left to polymerase for about 2 h. The gel was placed in model S2 sequencing gel electrophoresis apparatus (Gibco BRL). 1 x TBE buffer (section 2.2.8.) was used as the running buffer. The gel was pre-electrophoresed for 30 min at 60 W, max. current, max. volts using a LKB power pack (Hoefer). Samples were denatured at 75°C for 2 min. Combs were put in such that the teeth were just in the gel. An aliquot (2.5 µl) each sample was added in the order GATC and run for 2 h after which time another 2.5 µl each sample from sequencing reactions was added to different lanes and run for a further 1.5 h. The gel was fixed in 10 % methanol, 10 % acetic acid for 15 min. It was then transferred to 3 MM paper and dried 80°C for approximately 30 min. Once dry, the gel was exposed to film overnight in an autorad cassette at room temperature and then developed. The sequence was read using a light box.

3.7. Southern Blot Analysis

Southern Blot analysis was also used as a final method to ensure that the sequences obtained for the three probes were correct. In Southern blotting DNA is electrophoresed, transferred to a membrane and analysed by specific hybridisation.

3.7.1. Probes

Before the PCR products could be checked using this technique specific probes for each of the three sequences had to be made. Oligonucleotides (20mers) were designed for each PCR product which were specific to a sequence within each product. For alpha bases 871-890 were used thus the oligonucleotide made was 5' GAAGGTGAGTACTACAACGT 3'. For the epsilon sequence bases 431-450 were used, 5' GAGCTGGCTGTCTTTCACGA 3' and for the theta sequence bases 375-394

were used, 5' TGCAAGATACTTTCTGGAAA 3'. These oligonucleotides were end labelled with $\gamma[^{32}P]$ and used for hybridisation.

3.7.2. Southern Blotting

An aliquot (5 µl) each PCR product was run out on a 2 % agarose gel as in section 3.6.4. The DNA was transferred onto a positively charged nylon membrane (Hybond N^+, Amersham) using a VacuGene blotter (Pharmacia). The membrane was cut to the same size as the gel minus the wells. A mask was placed over the membrane with the hole being slightly smaller resulting in an overlap. The gel was then placed on the membrane. A steady vacuum of 50 mbars was obtained. Solution I (section 2.2.9.) was pipetted onto the gel and left for 20 min. This stage is the depurination step. Solution I was replaced with 30 ml solution II (section 2.2.9.) and left for 20 min. This is the denaturation step. Solution II was replaced with 30 ml solution III (section 2.2.9.) and left for 20 min. (neutralisation step). Solution III was removed and the blotting chamber was flooded with solution IV (20 x SSC, section 2.2.9.) to allow transfer for about 90 min. The blot was washed briefly in 2 x SSC to remove any agarose. The blot (DNA side up) was placed on a pad 3 MM paper soaked in 0.4 M NaOH for 20 min to fix the DNA to the membrane. After a brief wash with 5 x SSC the blot was wrapped in Saran Wrap and stored at 4°C until ready for hybridisation. The gel was stained with ethidium bromide as before to ensure that the DNA had transferred.

3.7.3. N-Labelling of Probes

The oligonucleotides that were being used as specific probes against the sequences for PKCs -α, -ε and -θ had to be labelled with $\gamma[^{32}P]$. Polynucleotide kinase (PNK) catalyses the transfer of a phosphate group to the 5' OH end of the oligonucleotide. The technique is called N-labelling. An aliquot (10 ρmoles) of oligo was mixed with 5 u T4 (PNK), 1 x PNK buffer, 3 µl $\gamma[^{32}P]$ ATP and water to a final volume of 10 µl. Reaction mix was incubated at 37°C for 10 min. The labelled oligo was extracted from the reaction mix using sephadex G-25 column (NAP-5 columns, Pharmacia). Water (170 µl) was added to the reaction mix which was then pipetted onto the column, the eluate was collected as the first fraction. Nine more fractions were collected by the addition of 9 x 180 µl water to the column and collecting the eluate each time. Each

fraction was counted using a Bench count (Scotlab). The hottest fraction was the labelled oligo and was used as the probe for hybridisation.

3.7.4. Hybridisation

Prehybridisation solution was prepared as in section 2.2.9. Blots were prehybridised in Hybridiser HB-1D ovens (Techne) for approximately 4 h at 42°C. Hybridisation solution was prepared (section 2.2.9.) minus probe and added to the blot. The probe was then added down the side of the tube away from the blot using a Gilson and mixed gently with the solution. Blots were hybridised overnight at 42°C. Hybridisation solution was removed and the blot was washed as follows, 25 ml 2 x SSC rinse, 50 ml 2 x SSC 10 min, 50 ml 2 x SSC 10 min and 50 ml 0.1 x SSC, 0.1 % SDS 10 min. All washes were at 42°C. Blot was checked with Geiger counter and if still very hot was rewashed. Blots were wrapped in Saran Wrap, exposed to film in an autorad cassette and placed at -80°C.

3.7.5. Stripping Southern Blots

Southern blots could be stripped and reprobed. Blot was incubated with 50 ml 0.4 M NaOH for 30 min at 45°C and then 50 ml 0.1 x SSC, 0.1 % SDS, 0.2 M Tris/HCl pH 7.5 (section 2.2.9.) for a further 15 min. After a quick wash with 2 x SSC the blot was exposed to film to ensure the probe had been removed. The blot could then be stored at 4°C or hybridised with another probe.

3.8. Northern Blot Analysis

Northern blotting is the electrophoresis and transfer of RNA to a membrane followed by hybridisation to a specific probe. It provides a measure of the levels of an RNA species within a cell.

3.8.1. Sample Preparation and Gel Analysis

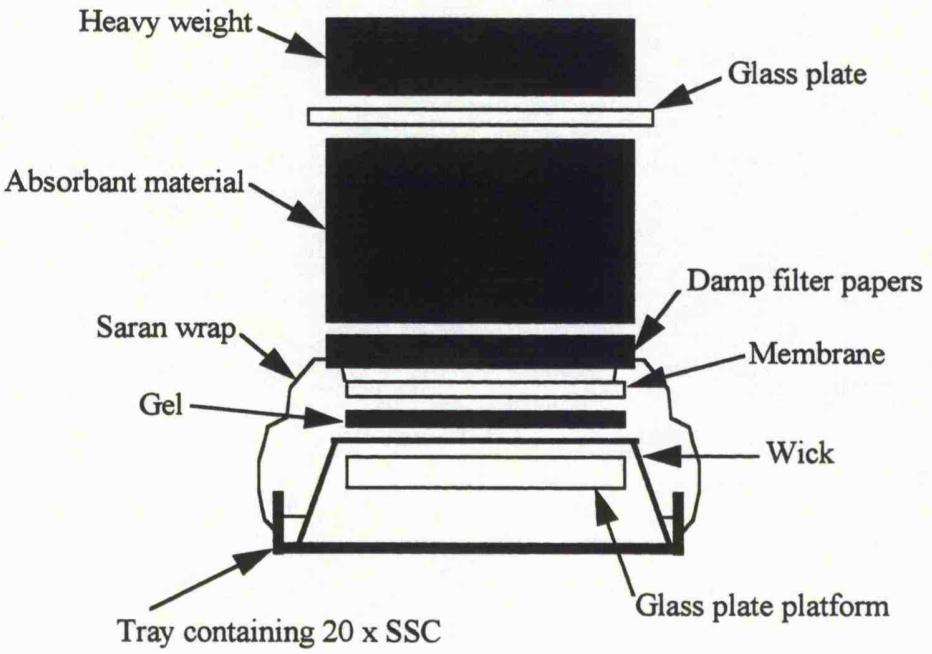
During gel electrophoresis RNA molecules must be completely unfolded by the addition of a denaturing agent. Formaldehyde denatures RNA by reacting with amine groups preventing the formation of G-C and A-T base pairs. A formaldehyde agarose gel was prepared as in section 2.2.10. and left to set for at least 2 h. The gel was

poured in a fume hood since formaldehyde fumes are toxic and highly irritating. RNA was isolated as described in section 3.5. Sample loading buffer (section 2.2.10.) was prepared prior to use and 3 volumes were added to 1 volume of RNA sample which makes it dense enough to be loaded into the well of a submarine gel. RNA samples, including a suitable RNA ladder, were completely denatured before loading by incubation at 65°C for 5 min. Both the formamide and heating disrupts hydrogen bonds and allows the formaldehyde to react with the bases. 1 x MOPS (section 2.2.10.) was used as the running buffer. Initially gels were run at 80 V for 2-3 h. This however was rapid and caused the RNA to run down the sides of the lane. Gels were subsequently run at 20 V overnight using gel apparatus that allowed the circulation of the running buffer by the connection of a peristaltic pump. This prevented the formation of a pH gradient across the gel. At the end of the run the gel was briefly visualised under UV to ensure that the samples had run. The 28S and 18S rRNA molecules were clearly visible. The RNA was then blotted onto a membrane.

3.8.2. Northern Blotting

A capillary blot was set up as in figure 3.3. 20 x SSC was used as the transfer buffer. The wick was made up of 2 pieces of 3 MM paper with each end dipping into the transfer solution. Gel was placed on the wick with wells facing down and air bubbles were gently removed. Membrane was placed on top of gel carefully to avoid air bubbles. Once the membrane has been placed onto the surface of the gel it cannot be moved as some RNA will be transferred immediately. Gel and membrane was surrounded with cling film to avoid short circuits i.e. the transfer solution must flow through the gel and membrane. The rest of the apparatus was assembled as in figure 3.3 and the blot was left for 24 h. The blot was dismantled as far as the cling film. The wick was picked up, turned over and placed membrane side down onto dry 3 MM paper. The wick was removed and the positions of the wells marked onto the membrane. The gel was peeled off and the blot was examined under UV as the ethidium bromide would have transferred with the RNA. The RNA ladder was marked onto the blot. The blot was washed in 5 x SSC for 5 min to remove any agarose and left to dry at room temperature for 30 min to fix the RNA to the membrane.

Figure 3.3 Capillary Blotting Apparatus



Capillary blot apparatus used for Southern and northern blot analysis.

The membrane was then baked at 80°C for 90 min to remove any residual formaldehyde from the RNA as this would impair the efficiency of the hybridisation. The blot could then be stored at 4°C until ready for hybridisation.

3.8.3. Random Labelling of Probes

Feinberg and Vogelstein (1983) introduced the use of random sequence hexanucleotides to prime DNA synthesis on denatured template DNA at numerous sites along its length. The 'Klenow' fragment of DNA polymerase 1 from *E Coli* is used for this reaction. A multiprime DNA labelling kit (Amersham) was used to label 100 ng of probe. The labelled DNA was extracted using NAP-5 columns as described in section 3.7.3. and stored at -20°C.

3.8.4. Hybridisation

Prehybridisation solution was prepared as in section 2.2.11. and the blot was prehybridised at 48°C for approximately 4 h. Hybridisation solution was prepared minus the probe and added to the blot. The denatured probe was added down the side of the tube away from the blot using a Gilson and carefully mixed with the solution. The blot was hybridised for approximately 20 h and then washed as follows, 2 washes of 2 x SSC, 0.1 % SDS for 30 min and 2 washes of 0.1 x SSC, 0.1 % SDS, 1 for 30 min and 1 for 1 h. All washes were at 55°C. The blot was then wrapped in Saran Wrap and either exposed to film at -80°C or exposed to a phosphorimager screen at room temperature overnight.

3.8.5. Stripping Northern Blots

A 0.5 % (w/v) SDS solution was boiled, poured onto the blot and allowed to cool to room temperature. The blot was checked for radioactivity and either stored at 4°C or hybridised with another probe.

3.9. Semi-Quantitative RT/PCR

RT/PCR has been shown to be several thousand fold more sensitive than the traditional RNA blot techniques. The technique involves translation of RNA to DNA followed by amplification using PCR. During PCR the amount of product initially increases

exponentially, but then the rate slows and finally plateaus. The goal of semi-quantitative PCR is to deduce the relative starting levels of target molecules among several samples. The incorporation of labelled primers into PCR products is measured by resolving products by gel electrophoresis. A titration analysis is performed by making a dilution series of RNA, amplifying by PCR and quantifying the signals produced. A log log plot of band density against amount RNA produces a straight line for each sample. Thus a value on X axis can be chosen and the corresponding values on the Y axis can be determined for both curves. The difference between the two values is equivalent to the relative difference in the initial number of RNA molecules for the two samples.

The sense primer for PKC-ε was found not to bind to the human sequence (see results, section 5.3) and so a new sense primer was designed, CTTCTTAAGATCAAAATCTG, which would give a PCR product of 384 bp. The primer was against bases 212-231 of human PKC-ε sequence.

The relative levels of PKCs -α, -ε and -θ mRNA in WT and MCF-7/Adr cells were determined. A RT reaction master mix was prepared as follows: 100 µl PCR buffer (10 x), 50 µl $MgCl_2$ (50 mM), 10 µl dATP, dTTP, dCTP and dGTP (all 100 mM), 9.9 µl hexamers (1515 ρmoles/ µl), 10 µl DTT (100 mM) and 515.1 µl DEPC treated water. RT reaction mix (7.25 µl per reaction) was added to a range of RNA dilutions (volume 2 µl, 2 tubes per dilution) along with 0.5 µl MMLV-RT and 0.25 µl RNAsin. A layer of mineral oil was added on top. RT reaction was carried out in DNA thermal cycler 480 PCR machine (Perkin Elmer) for 10 min 23°C, 45 min 42°C and 10 min 99°C. Reactions were kept at 4°C until PCR reaction.

One of the primers necessary for the PCR reaction was labelled with $\gamma[^{33}P]$. Labelling reaction was set up as follows: 5 µl $\gamma[^{33}P]$, 1 µl 50 ρmoles/ µl primer, 2 µl T4 kinase, 1 µl water and 1 µl 10 x kinase buffer. After 30 min at 37°C reaction was stopped with the addition of 1 µl 0.5 M EDTA and volume made up to 25 µl. Primer was purified through a Clontech chromaspin 10 column by spinning at 700 g for 3 min and stored at 4°C. DNA ladder (1 kb, Gibco BRL) was also labelled using 1 µl in the reaction and making up to 50 µl before purification.

PCR reaction master mix was made as follows: 100 µl PCR buffer, 50 µl $MgCl_2$, 100 µl sense and antisense primers (both 50 ρmoles/ µl) and 610 µl water. PCR reaction

mix (9.6 µl) was added to each RT reaction along with 0.2 µl Taq polymerase and 0.2 µl labelled primer. Reactions were spun down in a microfuge and put in DNA thermal cycler for 28 cycles of 94°C 1 min, 50°C 1 min and 72°C 2.5 min, the samples were then brought down to 4°C.

Loading buffer (section 2.2.6.) was added to each reaction tube (10 µl) and the samples spun in a microfuge. DNA ladder (10 µl) was mixed with 5 µl loading buffer. Samples (15 µl) and DNA ladder (5 µl) were added to 20 x 20 cm 8 % polyacrylamide gel (section 2.2.12.) and electrophoresed at 200 V for approximately 2 h using vertical gel electrophoresis apparatus model V15.17 and 20 tooth 1.5 mm thick comb (Gibco BRL). TBE (1 x, section 2.2.8.) was used as the running buffer. Duplicate samples were run on separate gels. Gels were transferred to Whatman 3MM paper, dried for approximately 30 min at 80°C and then exposed to phosphorimager cassette overnight at room temperature or film for 2-3 days at -80°C. The band volume of the PCR product was determined using Molecular Dynamics phosphorimager and software and plotted against RNA concentration in RT reaction.

3.10. Gene Amplification

Genomic DNA was isolated from 2 x 10^7 cells using a Blood and Cell Culture Midiprep kit from Qiagen. The protocol supplied was followed and DNA pellet was resuspended in 400 µl TE pH 8.0 (section 2.2.13.). The samples were shaken overnight but the pellets had not completely dissolved so samples were vortexed briefly and placed at 60°C for 1 h. Once the pellets had dissolved the OD of the samples were measured. Tips used for the transfer of DNA should have the ends cut to create a larger bore so as to prevent any shearing during pipetting. An aliquot of genomic DNA (20 µg) was taken and volume made up to 270 µl with TE pH 8.0. 10 x enzyme buffer (supplied with the restriction enzyme) was then added (30 µl) giving a total volume of 300 µl. Samples were left on ice for 2-3 h, contents were stirred 2-3 times with a narrow gel tip. Mixing by pipetting up and down was avoided as this would shear the DNA. Two lots of EcoR1 were added to the samples to a final concentration of 5 u / µg. Half was added, stirred gently and incubated at 37°C and the second half was added 30 min later. After stirring the samples were left at 37°C overnight.

Samples were heated to 70°C for 15 min to deactivate the enzyme. Half the sample was removed (i.e. 10 μg DNA) and reprecipitated by the addition of 15 μl 2M NaAc, pH 7.0 (section 2.2.5.) and 495 μl absolute ethanol. Samples were left on dry ice for 1 h and DNA was then pelleted by top speed in a microfuge for 20 min. DNA was resuspended in 15 μl TE, pH 8.0 (section 2.2.13.) and 5 μl loading buffer was added (section 2.2.6.). DNA was run out on a 0.8% agarose gel along with a 1 kb DNA ladder. Gel was run overnight at 20 V using 1 x L buffer as the running buffer.
Next day gel was washed as follows: 0.25 M HCl for 7 min, rinse with water, 0.5 M NaOH, 1.5 M NaCl for 30 min, rinse with water, 0.5 M Tris / HCl pH 7.4, 3 M NaCl for 30 min and a final rinse with water. A capillary blot was set up as in section 3.8.2. and left for 24 h. DNA was fixed to the membrane as described in section 3.7.2 and hybridised with labelled probe (section 3.7.4.). Detection was by autoradiography.

3.11. Immunoprecipitation

An antibody directed against one protein antigen in a mixture of proteins is used to isolate the specific antigen from the mixture. The antibody is then attached to a solid phase particle such as protein A sepharose beads which have specific affinity for the Fc portion of immunoglobulin molecules. Unbound molecules are separated by washing and the protein is then released from the antibody.
A membrane pellet was prepared as in section 3.2.3. from 2 plates of cells. The pellet obtained after ultracentrifugation was resuspended in 0.5 ml immunoprecipitation buffer (section 2.2.14.) and transferred to an Eppendorf tube. The protein content of the sample was determined as in section 3.2.3. The sample was incubated with 1.5 μg C219 P-gp specific antibody at 4°C overnight rotating end over end. 50 μl 10 % protein sepharose beads (section 2.2.14.) was added and incubated for at least 1 h at 4°C rotating end over end. Beads were pelleted by pulse centrifugation using a microcentrifuge and washed with 5 x 1 ml aliquots of immunoprecipitation buffer. The supernatant was removed each time using a needle and syringe. The beads were resuspended in 50 μl P-gp sample buffer (section 2.2.3.). Sample was sonicated in a sonicating water bath to remove the protein from the beads. The beads were then spun down. Samples were loaded and run out in a 8 % acrylamide gel (section 2.2.3.) as in

section 3.3.1. and blotted onto nitrocellulose as in section 3.3.2. Proteins were detected using the appropriate antibodies as described in section 3.3.1/2.

3.12. Indirect Immunofluorescence

Antibodies can be used to determine the distribution of an antigen within a cell. A label is attached to a specific antibody and the position of the label in the cell can be identified with a suitable microscope and used to infer the position of the antigen. In indirect immunofluorescence the label is attached to a secondary antibody.

Dilutions of both primary and secondary antibodies were checked in order to minimise non-specific and background binding. The anti-P-gp monoclonal antibody C219 (Signet Laboratories Inc.) and anti-PKC-ε polyclonal antibody C-15 (Santa Cruz) were used at concentrations of 1 and 10 μg / μl respectively. A FITC linked anti-mouse secondary antibody (Sigma) was then used against C219 and a Texas Red linked anti-rabbit secondary antibody (Amersham) against C-15. Both were used at a 1 in 10 dilution.

MCF-7/Adr cells were grown on sterile coverslips in 100 mm petri dishes until confluent. All stages were carried out at room temperature unless otherwise stated. Coverslips were placed in petri dishes on filter paper soaked in PBS. Cells were washed twice with PBS (2 min per wash) and fixed in 3% formaldehyde (in PBS) for 10 min. Cells were washed again and then permeabilised in 0.05 % Triton X-100 (in PBS) for 10 min. Cells were washed with PBS 4 times (2 x 1 min and 2 x 2 min). Primary antibody was made up in 20 % blocking serum and 60 μl added to each coverslip, 60 μl of PBS was added to the control and incubated overnight. For the dual label the first primary antibody was incubated with the cells for approximately 6 h, cells were washed and the second primary antibody was then left on cells overnight. Excess antibody was removed by a quick wash with PBS followed by 2 washes of 2 min and a final wash of 30 min. During this incubation the secondary antibody was diluted in 20 % blocking serum and spun for 10 min at top speed in microfuge. After removal of PBS 60 μl secondary antibody was added to each coverslip including the control and incubated for 1 h at 37°C. For the dual label experiments the two secondary antibodies were incubated separately with the cells, 1 h with each. Excess

antibody was removed by a quick wash with PBS followed by 2 x 2 min and 3 x 10 min washes. Coverslips were mounted on slides using antiphotobleaching mountant (BDH) and examined using confocal microscope.

RESULTS

CHAPTER 4

RELATIONSHIP BETWEEN CELL GROWTH INHIBITION AND PKC INHIBITION

4.1. Introduction

PKC plays a pivotal role in cell proliferation and aberrant expression of PKC has been linked with the development of malignancies. The aim of this work was to investigate the link between PKC inhibition and cell growth arrest. Nine PKC inhibitors of differential potency and selectivity for PKC were studied. They were assessed for their ability to interfere with cell proliferation and activity of PKC isolated from the cytosol of MCF-7 cells. Native PKC remains loosely attached with the cytoplasmic side of the plasma membrane (Wolf *et al*, 1985). In this inactive state the enzyme is readily recovered as a soluble cytoplasmic protein. Upon activation the enzyme becomes an integral membrane protein and so can be recovered in the particulate or membrane fraction of the cell. In most studies of PKC inhibition cytosolic enzyme preparations have been used, however, it seems that it is membrane PKC which is of functional importance in the cell. Thus the effect of the inhibitors on membrane derived PKC was investigated to determine whether the agents inhibited cytosolic and membrane PKC similarly.

4.2. Cytostatic Effects of PKC Inhibitors on MCF-7 Cells

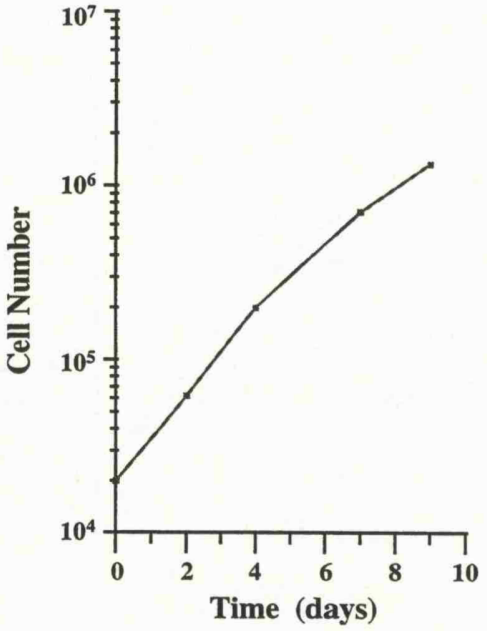
4.2.1. Determination of Doubling Time

The doubling time of the MCF-7 cells was determined as described in section 3.1.3. Data representative of three experiments is shown in figure 4.1. The doubling time of these cells was 35 ± 7 h.

4.2.2. Inhibition of Cell Growth by PKC Inhibitors

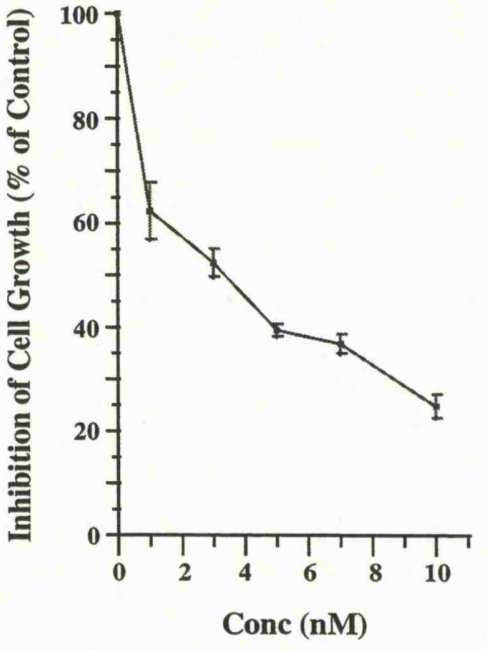
The cytostatic effects of the compounds, staurosporine, UCN-01 , GF 109203X (GFX), CGP 41251 (CGP), RO 31 8220 (RO), calphostin C, NPC 15-437 (NPC), H-7 and miltefosine (for structures see figure 1.4) were assessed according to the method described in section 3.1.4. Cell numbers were plotted against concentration as shown in figure 4.2. The IC_{50}s were determined from the graph and the mean value was calculated for each agent from three separate experiments (table 4.1).

Figure 4.1. MCF-7 Cell Growth Curve



Cells were seeded at $2x10^4$ in 35mm diameter wells. Cells were counted at the indicated times. The doubling time is calculated from the straight part of the graph.

Figure 4.2. Effect of Staurosporine on the Growth of MCF-7 Cells



Cells were seeded at $2x10^4$ in 35mm diameter wells in the presence of the indicated concentrations of staurosporine (2 wells per concentration). After 4 doubling times the cells were counted and inhibition was calculated as a percentage of drug free control. The IC_{50} was determined from the graph.

Table 4.1. Inhibition of Growth of MCF-7 Cells by Kinase Inhibitors

COMPOUND	GROWTH INHIBITION IC_{50} (μM)
Calphostin C	0.00092 ± 0.00011
Staurosporine	0.0032 ± 0.0001
UCN-01	0.0175 ± 0.0010
CGP 41251	0.097 ± 0.012
RO 31-8220	0.897 ± 0.013
NPC 15 437	3.70 ± 0.10
GF 109203X	7.30 ± 0.90
H-7	21.30 ± 1.50
Miltefosine	44.00 ± 0.50

Cells were seeded at $2x10^4$ in 35mm Diameter wells (2 wells per concentration) and counted following exposure to drugs for 4 doubling times. Drug and media were replenished once. Values are the mean ± s.d. of three separate experiments.

The most potent inhibitor of cell growth was calphostin C (IC_{50} 0.92nM). This agent is rather different to the others in the study as it is an inactivator of PKC, exerting its effect by the generation of singlet oxygen species in a light dependent manner (Gopalakrishna *et al.*, 1992). Staurosporine and its analogues were also potent growth inhibitors with IC_{50}s in the nM range, except GFX which showed an IC_{50} of 7.3 μM. The remaining inhibitors, NPC, H-7 and miltefosine, all demonstrated IC_{50}s in the μM range.

4.3. Inhibitory Potency of PKC Inhibitors Against Cytosolic PKC

4.3.1. Determination of Cytosolic PKC Activity

Cytosolic PKC was isolated from MCF-7 cells as described in section 3.2.1. Preliminary experiments were performed to optimise the assay by serial dilution of the cytosol. This was necessary in order to eliminate any interference by endogenous PKC inhibitors. Serial dilution eventually caused a proportionate decrease in PKC activity indicating that the inhibitors had been essentially removed. This procedure was performed for each separate cytosolic isolation to ensure that the endogenous inhibitors did not interfere and that the inhibition observed by the agents under investigation was a true reflection of their potency.

cPKCs require cofactors, such as phospholipid and calcium, and an activator, such as TPA, in order to function. The kit used for assessing PKC activity from Amersham contains all these factors. In order to determine the conditions for optimal cytosolic enzyme activity each of these factors was left out in turn and the resulting activity measured (see section 3.4.1.). The results are shown in table 4.2.

The results show that PKC activity was greatly reduced when any of the cofactors were removed from the incubate. If TPA, the activator, was left out, activity was approximately 20 % of maximal. If phosphatidylserine (PS) was missing from the incubate, activity was approximately 40 % of maximal. Thus greater activity was seen when TPA was present and maximal activity could not be achieved without PS. PKC activity was 83 % of maximal when only calcium was left out of the incubate.

Table 4.2. Conditions Required for Optimal Activation of Cytosolic PKC

FRACTION	CYTOSOLIC PKC ACTIVITY (% OF MAXIMAL)
+TPA/Ca^{2+}/PS	100
+TPA	38
+Ca^{2+}	23
+PS	19
+TPA/Ca^{2+}	45
+TPA/PS	83
+Ca^{2+}/PS	21

Cytosolic PKC assays were conducted with the cofactors indicated. Results are expressed as a percentage of maximal cytosolic PKC activity obtained by using the kit containing all the cofactors as directed by the manufacturer.

4.3.2. Inhibition of Cytosolic PKC

The PKC inhibitors that were used in the growth studies were assessed for their inhibitory potency against cytosolic PKC activity (see section 3.4.2.). A representative result for staurosporine is shown in figure 4.3. Mean IC_{50} values for each agent were calculated from three separate experiments and the results are shown in table 4.3.

Staurosporine and its analogues were all potent inhibitors of cytosolic PKC activity with IC_{50} values between 16 and 50 nM. All these agents inhibit via the catalytic domain of the PKC molecule. The agents that act via the regulatory domain, calphostin C, NPC, H-7 and miltefosine, were less potent, with IC_{50} values in the μM range.

4.4. Inhibitory Potency of PKC Inhibitors Against Membrane PKC

4.4.1. Translocation of PKC from the Cytosolic to the Membrane Fraction

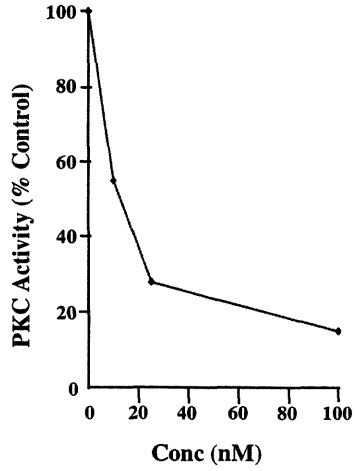
PKC is not detectable in the particulate fraction of naïve MCF-7 cells (Stanwell *et al.*, 1994). Phorbol esters such as phorbol dibutyrate (PDBu) translocate PKC to the membrane fraction of cells (Kraft and Anderson, 1983). Cells were treated with PDBu and then fractionated as described in section 3.2.3. PKC was determined by western blot analysis (see section 3.3.1.). PDBu treatment effectively translocated the PKC from the cytosolic to the membrane fraction, as shown in figure 4.4.

4.4.2. Methods for the Preparation of Membrane PKC

Various methods have been used to prepare the membrane fraction (Greif *et al.*, 1992; Chakravarthy *et al.*, 1991; Carlson *et al* ., 1993; Rush *et al.*, 1994). In order to assess which method would provide a fraction suitable for inhibition assays the methods was compared. In preliminary experiments A549 human lung carcinoma cells were used, as they contain more PKC activity than the MCF-7 cells.

Membrane fractions were prepared from naïve cells and from cells treated with 10 μM PDBu by the four methods described in section 3.2.2. Each fraction was diluted and assayed for PKC activity. The results shown in figure 4.5 were obtained from similarly diluted fractions. At this dilution most of the endogenous inhibitors present had been diluted out.

Figure 4.3. Inhibition of Cytosolic PKC by Staurosporine



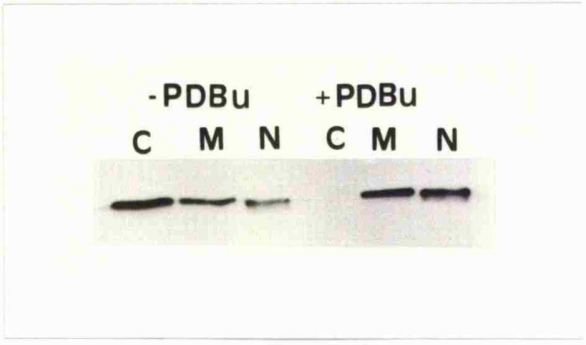
Cytosolic PKC assays were conducted in the presence of the indicated concentrations of staurosporine. Activity, as a percentage of drug free control, is representative of three experiments.

Table 4.3. Inhibition of Cytosolic PKC by Kinase Inhibitors

COMPOUND	CYTOSOLIC PKC INHIBITION IC_{50} (μM)
Staurosporine	0.0163 ± 0.0045
UCN-01	0.0178 ± 0.0020
GF 109302X	0.0205 ± 0.0043
CGP 41251	0.0442 ± 0.0060
RO 31-8220	0.4800 ± 0.0058
Calphostin C	1.09 ± 0.18
NPC 15 437	43.30 ± 6.90
H-7	54.30 ± 7.50
Miltefosine	577.00 ± 29.00

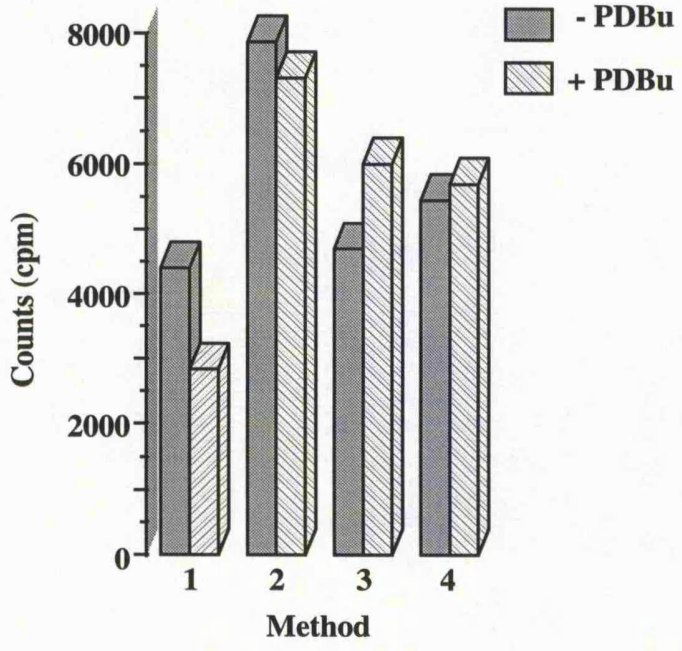
Cytosolic PKC activity was measured using a kit as directed by the manufacturer. Values are the mean ± s.d. of 3-5 separate experiments.

Figure 4.4. Effect of PDBu on the Localisation of PKC-ε



Western blot analysis of PKC-ε in cytosolic (C), membrane (M) and nuclear (N) fractions of naïve MCF-7 cells and of cells treated with 10μM PDBu for 30 min.

Figure 4.5. PKC Activity in the Membrane Fraction of A549 Cells Following Preparation by Four Different Methods.



The membrane fraction was prepared from A549 cells with or without PDBu (10μM) for 30 min by methods 1-4 as described in section 3.2.2. A 1 in 5 dilution of each fraction was assayed for activity using the kit as directed by the manufacturer, and the counts observed are plotted. Solid bars show the activity in untreated cells, hatched bars in cells after PDBu treatment.

Pre-treatment with PDBu did not increase the activity seen in the membrane fractions obtained from methods 1 and 2, and only slightly increased the activity in the fractions obtained from methods 3 and 4. This was surprising since western blot analysis had shown that the PKC was translocated to the membrane by PDBu. Greatest activity was observed in the fraction derived from method 2. This protocol was used to prepare membrane from PDBu treated MCF-7 cells. The resulting activity was sufficient to allow study of the inhibitors, however, the endogenous inhibitors could not be diluted out completely. Thus this method was not used.

Method 4 showed slightly greater activity in the membrane of treated cells and the detergent step may have aided with the removal of endogenous inhibitors. Fractions were prepared from naïve and PDBu treated MCF-7 cells using method 4, and assayed for activity (table 4.4). Endogenous inhibitors could be removed from this preparation, but PDBu treatment did not increase PKC activity in the membrane fraction. The pellet obtained from the detergent step was resuspended and assayed for activity. The pellet showed very high activity which was still present, even when the substrate was omitted from the incubate (table 4.4), suggesting that it was non-specific activity. The detergent step in method 4 not only extracted the endogenous inhibitors but also removed a lot of non-specific activity.

An initial step in method 4 is the sonication of the cells. This harsh treatment of the cells could have disrupted the binding of translocated PKC, causing its return to the cytosolic fraction. This reversal of translocation could explain why PDBu treatment did not increase enzyme activity in the membrane fraction. Thus methods 1 and 4 were combined to yield method 5, i.e. cells were homogenised rather than sonicated (section 3.2.2.). It was hoped that homogenisation would not disrupt the binding of the translocated PKC. Membrane fractions were prepared with or without 10μM PDBu treatment and with or without Brij 58 treatment using method 5. Non-specific activity was assessed by assaying samples in the absence of the substrate.

PDBu treatment increased the activity seen in the fraction by 50% and Brij 58 treatment decreased the amount of non-specific binding by 50% (table 4.5). When treatments were combined, the endogenous inhibitors could be diluted out and the non-specific activity was reduced to only 10 %.

Table 4.4. Membrane PKC Activity of MCF-7 Cells Following Separation by Method 4

SAMPLE	ACTIVITY	1 IN 5 DILUTION	1 IN 10 DILUTION
Naïve cells	4943.00	2743.25	1797.75
Treated cells	4143.25	2969.25	1905.00
Pellet	17829.70	nd	8483.25

nd, not determined. The membrane fraction was prepared from MCF-7 cells with or without PDBu (10μM) for 30 min by method 4 (see section 3.2.2.) and assayed for activity.

Table 4.5. Membrane PKC Activity of MCF-7 Cells Following Separation by Method 5

TREATMENT	ACTIVITY		% NON-SPECIFIC ACTIVITY
	1 IN 10 DILUTION	1 IN 20 DILUTION	
None	1439.00	730.00	50
+ Brij 58	556.00	288.25	26
+ PDBu	2389.75	1219.00	20
+ PDBu + Brij 58	1030.50	571.75	10

The membrane fraction was prepared from MCF-7 cells with or without PDBu (10μM) for 30 min and with or without Brij 58 (0.1%) treatments by method 5 (see section 3.2.2.) and assayed for PKC activity. Non-specific activity was assessed by determining PKC activity in the absence of substrate.

The resulting activity, however, yielded low counts of only just above 1000, so the fraction was prepared using a larger number of cells (approximately 10^7). Enzyme activity after a 1 in 5 dilution, which removed endogenous inhibitors, was sufficient to investigate the effects of PKC inhibitors.

As for cytosolic PKC the conditions required for optimal activation of membrane PKC were investigated and the results are shown in table 4.6. Unlike cytosolic PKC, the activity of the membrane enzyme was high even when the cofactors were left out of the incubate. When TPA was absent, PKC activity was approximately 80 % of maximal in contrast to 20 % of maximal seen with cytosolic PKC. If PS was missing from the incubate, activity was 85 %, and maximal activity was seen when only calcium was omitted. Thus the cofactors necessary for activation of cytosolic PKC were not required for the activity of membrane PKC.

4.4.3. Inhibition of Membrane PKC

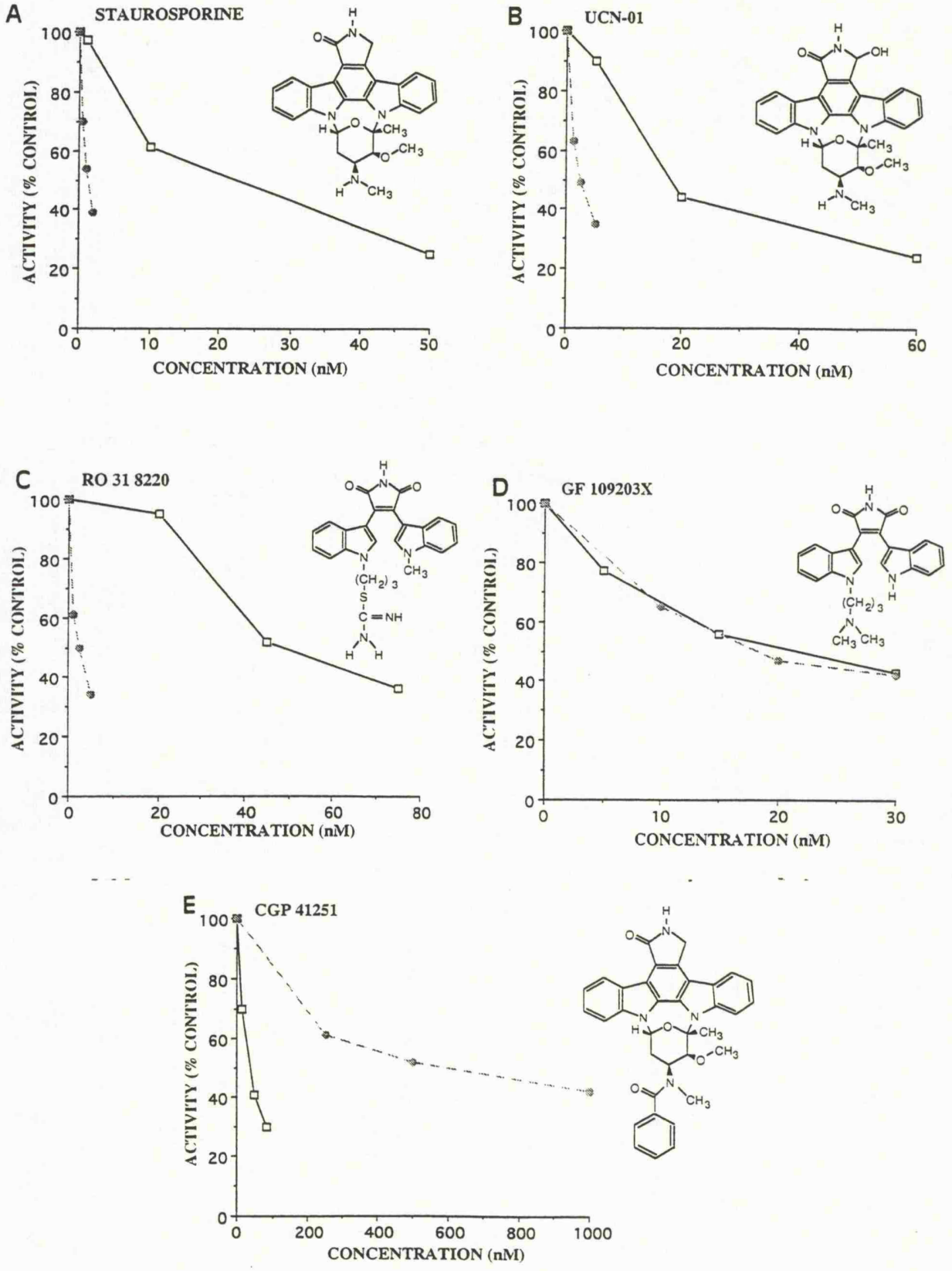
The inhibitory potencies of the PKC inhibitors were determined using membrane PKC. Graphs of percentage inhibition against concentration of agent for cytosolic and membrane PKC are shown in figure 4.6. The IC_{50} values and the fold difference between inhibition of cytosolic and membrane PKC are shown for each compound in table 4.7. There were remarkable differences between the PKC preparations in their susceptibility towards the inhibitory potential of the nine compounds tested. Only one compound, GFX, furnished the same IC_{50} value, irrespective of enzyme source. The most dramatic discrepancies in inhibitory potency between cytosolic and membrane derived PKC were seen with RO, staurosporine, CGP and calphostin C. RO and staurosporine inhibited membrane PKC 22 and 12 times, respectively, more effectively than cytosolic PKC. In contrast, CGP and calphostin C inhibited cytosolic PKC 14 and 28 times, respectively, better than membrane derived enzyme.

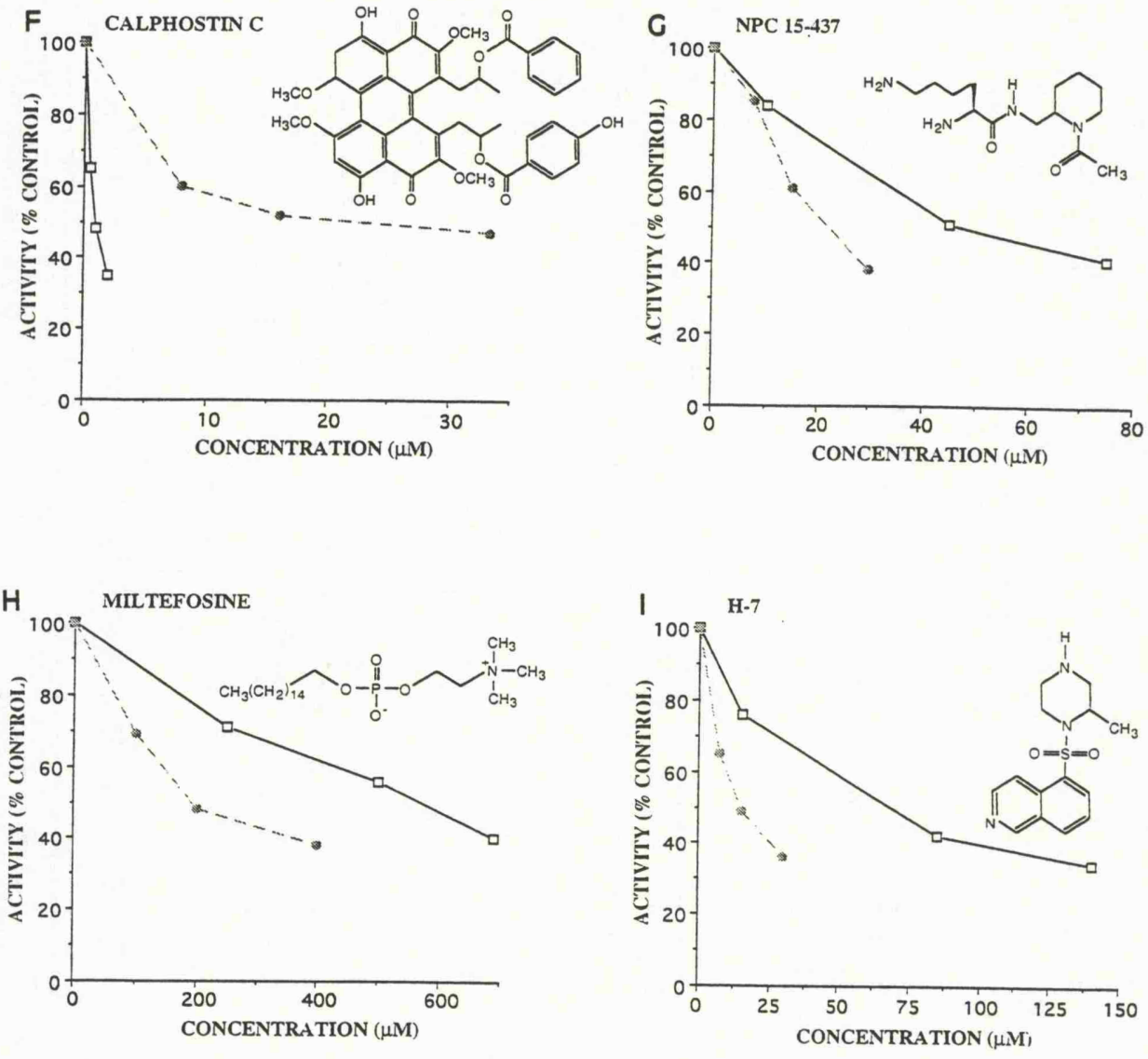
Table 4.6. Conditions Required for Optimal Activation of Membrane PKC

FRACTION	MEMBRANE PKC ACTIVITY (% OF MAXIMAL)
+TPA/Ca^{2+}/PS	100
+TPA	86
+Ca^{2+}	80
+PS	79
+TPA/Ca^{2+}	85
+TPA/PS	98
+Ca^{2+}/PS	85

Membrane PKC assays were conducted with the cofactors indicated. Results are expressed as a percentage of maximal membrane PKC activity obtained by using the kit containing all the cofactors as directed by the manufacturer.

Figure 4.6. Inhibition of Cytosolic and Membrane PKC by Kinase Inhibitors





Inhibition of cytosolic PKC (open squares) and membrane PKC (closed circles) from MCF-7 cells by kinase inhibitors. Inhibition was calculated as percentage of drug free control. The graphs show the result of one experiment representative of three.

Table 4.7. Inhibition of Membrane PKC by Kinase Inhibitors

COMPOUND	MEMBRANE PKC INHIBITION IC_{50}/ μM	RATIO [a]
Staurosporine	0.0013 ± 0.0001	12.3
RO 31-8220	0.0022 ± 0.0002	21.6
UCN-01	0.0032 ± 0.0009	5.5
GF 109203X	0.021 ± 0.004	1.0
CGP 41251	0.61 ± 0.14	0.07
H-7	15.20 ± 0.60	3.5
NPC 15 437	17.90 ± 2.80	2.4
Miltefosine	183.70 ± 17.00	3.1
Calphostin C	30.00 ± 6.20	0.04

[a] IC_{50} cytosolic PKC/ IC_{50} membrane PKC. Membrane PKC activity was measured using a kit as directed by the manufacturer. Values shown are in order of decreasing potency and are the mean ± s.d. of 3-5 separate experiments.

4.5. Discussion

One of the aims of this project was to investigate the link between PKC inhibition and cell growth arrest. Concentrations of staurosporine required for inhibition of growth are lower than those at which it inhibits PKC. This may reflect that staurosporine is not a specific PKC inhibitor and inhibits other kinases, such as, PKA and TPK at similar concentrations (Tamaoki and Nakano, 1990). Thus staurosporine may well interrupt a number of signalling pathways with the overall effect being potent cytostasis. The staurosporine analogues, which are all more selective for PKC show an opposite effect, in that they inhibit PKC more potently than cell growth. UCN-01 displayed identical IC_{50}s. Calphostin C had a very potent effect on growth with an IC_{50} of 0.9nM, but this potency is not reflected in its ability to inhibit cytosolic PKC *in vitro* (IC_{50} 1.09μM). NPC 15-437, H-7 and miltefosine all inhibit cell growth more potently than PKC, but compared to the staurosporine analogues they are a lot weaker with IC_{50}s > 15μM.

Taking the results together, although PKC inhibitors do cause cytostasis, there was no correlation between PKC inhibition and cell growth arrest. Inhibitors acting at the catalytic site appear to be better PKC inhibitors than those acting at the regulatory domain. It must be remembered, however, that PKC activity is generally determined using PKC that has been separated from its intracellular environment. Lipid and calcium cofactors are then used to try and reconstitute the endogenous environment. Thus the conditions under which PKC activity is determined *in vitro* may be very different to the situation *in situ*.

In naïve MCF-7 cells most of the PKC is readily recovered as a soluble protein, as adjudged by western blot analysis (Stanwell *et al*, 1994). PKC is extracted using buffers containing high concentrations of divalent cation chelators which are thought to have mild detergent properties capable of stripping/solubilising specific membrane proteins (Wallach and Zingler, 1974). Inactive PKC may not be located in the cytosol but may be loosely attached to the membrane (Wolf *et al*, 1985). This binding may be disrupted upon separation. Thus the term "cytosolic PKC" should be interpreted with caution. Upon activation PKC penetrates the bilayer and forms a stable bond with the membrane. The activators are degraded, but the inserted PKC remains active until it is

degraded in the process of protein turnover (Bazzi and Nelsestuen, 1988). So in the search for PKC inhibitors which may have clinical significance as drugs it may be more useful to look for potent inhibitors of membrane, rather than cytosolic PKC.

The conditions necessary for optimal activation of the cytosolic and membrane derived enzyme were investigated. PKC isolated from the cytosol of resting cells needed PS and an activator for activity, but calcium was not absolutely required. The predominant isoenzyme expressed in MCF-7 cells is calcium independent PKC-ε. Thus this result is not unexpected. In *in vitro* assays, the substrate used influences cofactor requirements (Bazzi and Nelsestuen, 1987; Liyanage *et al*, 1992). The substrate supplied with the kit could also result in calcium independent activity. The conditions required for optimal activity of the membrane derived PKC were different. Even when TPA was omitted from the incubate 80 % maximal activity was still observed. This activity increased when only calcium and/or PS were left out. These results are very similar to those obtained by Bazzi and Nelsestuen (1988) who observed that membrane inserted PKC was completely independent of calcium or added phospholipids and was only modestly influenced by PDBu. Chakravarthy *et al* (1991) also showed that PKC remained active in its native membrane associated state and did not require exogenous TPA or PS to function optimally. Prior to isolation, membrane PKC was translocated by PDBu, which would also have activated the enzyme. Activation is thought to involve a conformational change resulting in the insertion of the protein into the membrane and opening up of the catalytic site. Thus the isolated protein will be in its conformationally active shape and the cofactors which are required for this activation stage will then be superfluous. Whereas cytosolic PKC needs the cofactors for activation resulting in its insertion into the PS added, isolated membrane PKC is constitutively active.

There are many different ways of separating the particulate fraction of cells described in the literature. Four different methods were assessed for their suitability to provide membrane PKC. The initial problem which had to be overcome was the presence of endogenous inhibitors within the fraction. These factors need to be diluted out so as not to mask the effects of the additional inhibitors being studied. The methods described in the literature do not address this problem and it was found that some of the protocols resulted in fractions unsuitable for further study. Homogenisation was found to be a better method of cell disruption than sonication since sonication seemed

to disrupt the binding of translocated PKC to the membrane resulting in the loss of the enzyme. The results show that detergent solubilisation is also needed to remove non-specific activity and thus give a more accurate determination of PKC activity. Rush *et al* (1992) showed that Brij 58 was a better detergent to use than Triton X100 based on the number of endogenous inhibitors solubilised together with PKC. Thus the method eventually used to separate out the particulate fraction involved homogenisation as the cell disruption step and solubilisation with Brij 58 as the detergent step. This protocol yielded a fraction that showed less than 10 % non-specific activity and contained endogenous inhibitors that could be diluted out. The non-specific activity observed might be due to ATP binding to components within the fraction which are then removed on solubilisation or to other kinases phosphorylating proteins within the fraction.

Few investigations have addressed differences in susceptibility towards pharmacological inhibitors between cytosolic and membrane derived PKC. The ether lipid Et18-OMe, an experimental anti-tumour drug, was found to inhibit cytosolic PKC but behaved as an activator in preparations in which membrane PKC was used (Heesbeen *et al*, 1991). Most of the agents investigated in this study, including staurosporine, UCN and RO inhibited membrane PKC more potently than the cytosolic enzyme. Some intriguing differences were observed between the compounds. The bisindolylmaleimide GFX was an equipotent inhibitor of cytosolic and membrane PKC. CGP, which is very similar in structure to staurosporine, was over 8 times more potent at inhibiting cytosolic rather than membrane PKC. Could CGP have a different mechanism of action than the other staurosporine analogues? CGP may inhibit the activation step of the enzyme thus preventing its insertion into the membrane. CGP could then inhibit cytosolic PKC but not membrane PKC because the step at which the agent acts will have been passed. The enzyme has already been activated. Another explanation could be that CGP can bind more effectively to the inactive enzyme. Its large structure may result in it not being able to bind as efficiently to the active conformation. To test whether this was an isoenzyme specific phenomenon one could investigate the ability of the agent to inhibit, for example, membrane PKC-α as compared to cytosolic -α.

The PKC inhibitory potential of NPC, H-7 and miltefosine was 2-3 times greater against membrane PKC as opposed to that in the cytosol, but the IC_{50}s observed were

greater than 15μM and therefore of lesser potential therapeutic relevance. The increased efficacy of these compounds against membrane PKC could be related to their interaction with the enzyme. Activated PKC is bound to the membrane associated with its various cofactors. In the isolation of cytosolic PKC the enzyme is separated from these factors which then have to be reintroduced in order to reconstitute active PKC. The added cofactors may not be exactly the same as the endogenous factors, for example, the phospholipid added is phosphatidylserine (PS), but in the cells the lipid may not be pure PS. Cytosolic PKC may not be able to insert into the added PS optimally so that the inhibitor binding sites may not be as accessible as with membrane PKC. Perhaps this model reflects more closely the *in situ* environment and the inhibitors may be able to bind more easily.

The inhibitory potential of calphostin C was dramatically decreased when PKC was in the membrane, with an IC_{50} 30 times greater than that for cytosolic PKC. Gopalakrishna *et al* (1992) reported that calphostin C-induced inactivation of PKC was rapid in the membrane fraction as compared to the cytosol. In that study intact cells were exposed to calphostin C, prior to the isolation of cytosolic and membrane fractions and measurement of PKC activity. An incubation time of less than 10 min with 0.5 μM calphostin C caused PKC inactivation by 50 %. I found that incubation with 30 μM calphostin C for 1 h inhibited PKC activity by approximately 50 %. The major difference between the two studies is the way that the cellular fractions are prepared. The fact that the compound is a far more potent inhibitor of cytosolic PKC rather than of membrane PKC, as outlined above, could be the consequence of the fact that the cytosolic fraction contains more of the factors and metabolic pathways that calphostin C may need for maximal inactivation. In Gopalakrishna's study calphostin C was incubated with intact cells, in which metabolic pathways and cofactors are present. It is, therefore, conceivable that the way in which calphostin C inactivates PKC involves more than just binding to the enzyme. Wang et al (1993) suggested that the generation of reactive oxygen and free radicals produced by calphostin C when illuminated may affect the DAG binding domain. The agent damages the activator binding site thus inactivating the enzyme. Thus in my study where the isolated enzyme has already been inactivated, the step at which the agent acts has been passed thus resulting in a decreased inhibitory potency. Recent work by Rotenberg et al

(1995) has shown that cofactors provide a template that is required for productive interaction of PKC and calphostin C. The isolated membrane PKC, in this study, is independent of its cofactors thus the template may not be produced. Thus, results from inhibition studies must be viewed with caution as the findings are likely to depend on the conditions used.

From this study it can be seen that a majority of PKC inhibitors inhibit membrane-bound PKC more potently than cytosolic PKC. Membrane derived PKC may be a better model for inhibition studies than the use of the cytosolic enzyme because it reflects, more faithfully, the homeostasis of activated PKC in intact cells.

CHAPTER 5

RELATIONSHIP BETWEEN PKC AND MDR

5.1. Introduction

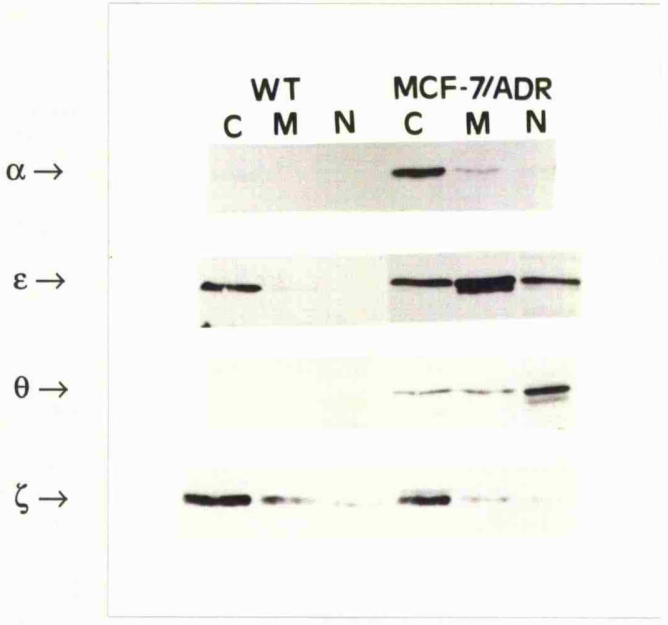
PKC has been implicated in the development and maintenance of MDR (see introduction). The aim of this study was to investigate the relationship further using wild type (WT) MCF-7 cells, their drug resistant counterparts MCF-7/Adr cells which express the MDR phenotype and revertant (REV) MCF-7/Adr cells that have lost resistance to adriamycin. MCF-7/Adr cells, selected by stepwise increase in adriamycin in the media, were originally obtained from Dr. K. Cowan, NIH, Bethesda, MD. (Batist *et al*, 1986). REV cells were obtained from the continuous passage of MCF-7/Adr cells in the absence of adriamycin.

5.2. PKC Isoenzyme Protein Expression in WT and MCF-7/Adr Cells

PKC isoenzymes expressed at the protein level by both cell lines were investigated using western blot analysis. Cells were separated into cytosolic, membrane and nuclear fractions (section 3.2.3.). Equal amounts of protein for each fraction were loaded on SDS/PAGE gels, blotted onto nitrocellulose and isoenzymes detected using monoclonal antibodies, except for PKC-ζ where a polyclonal antibody was used. (section 3.3.1.).

PKC-α was overexpressed in the cytosolic fraction of MCF-7/Adr cells (figure 5.1). A small amount of the isoenzyme could be detected in WT cells but it was at the detection limit. PKC-θ expression was induced in resistant cells and was detectable in all three fractions. It could not be found in WT cells. PKC-ε was detectable in both cell lines. Overall expression was slightly reduced in the resistant cells and the distribution pattern was different. In WT cells the isoenzyme was located primarily in the cytosolic fraction, but in MCF-7/Adr cells it was present in all three fractions. Significantly less PKC-ε was present in the cytosolic fraction with more being distributed to the membrane and nuclear fractions of MCF-7/Adr cells when compared to WT cells (figure 5.2). There was no significant difference in the expression of PKC-ζ between the two cell lines. Previous work in our laboratory has shown that PKCs -β, -δ and -γ are not detectable in MCF-7 cells (Stanwell *et al*, 1993). Table 5.1 summarises the overall expression of the four PKC isoenzymes detectable in WT and MCF-7/Adr cells.

Figure 5.1 PKC Isoenzymes Detectable at the Protein Level in WT and MCF-7/Adr Cells.



C Cytosolic fraction M Membrane fraction N Nuclear fraction

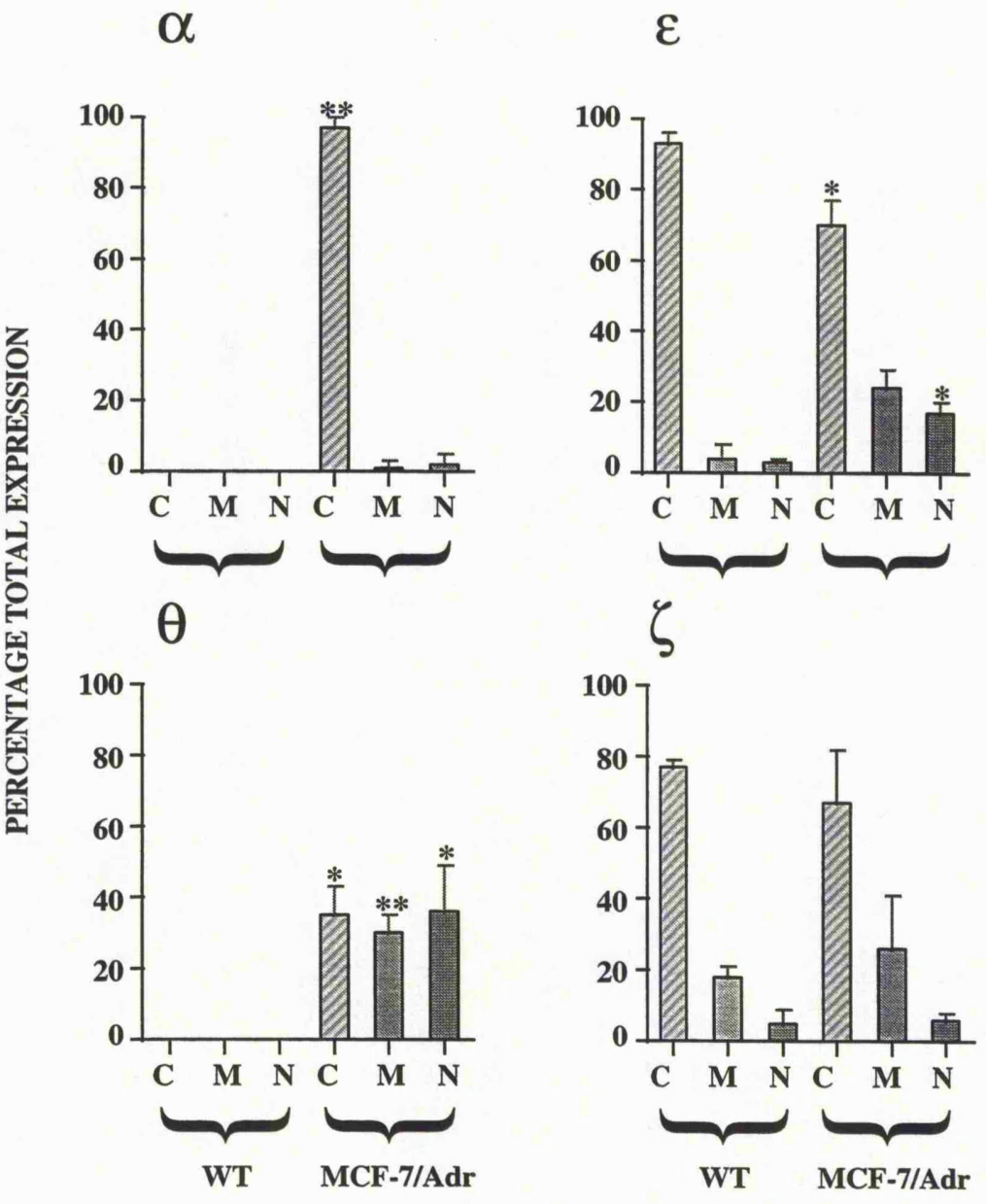
Cellular fractions were prepared and western blot analysis performed as described in section 3.3.1. Arrows indicate the position of PKC proteins. Blots shown are representative of three separate experiments.

Table 5.1 Overall Expression of PKC Isoenzymes at the Protein Level in WT and MCF-7/Adr Cells.

ISOENZYME	WT	MCF-7/Adr
α	+/-	++++
θ	-	++
ε	+++	++
ζ	++++	+++

Expression was quantified using laser densitometer (Molecular Dynamics).
+ Arbitrary measure based on consistent observations from three separate experiments.

Figure 5.2 PKC Isoenzyme Distribution in WT and MCF-7/Adr Cells



Distribution is expressed as percentage of total cellular expression for each individual cell type. Expression was determined by western blot analysis with the appropriate antibodies in the cytosolic (C), membrane (M) and nuclear (N) fractions. Values are the means ± s.d. of three experiments, statistical significance was determined (*$P \leq 0.05$, **$P \leq 0.001$) by analysis of variance.

To study the mRNA levels of the PKC isoenzymes, initially northern blot analysis was attempted. This proved unsatisfactory due to a lack of sensitivity and so RT/PCR was performed. In the following sections the development of the PKC probes and the subsequent RT/PCR are described.

5.3. Synthesis of PKC Isoenzyme Specific Probes

In order to substantiate the isoenzyme expression observed at the protein level, mRNA levels of the isoenzymes that had changed at the protein level, i.e. PKCs -α, -θ and -ε, were investigated in both cell lines by northern blot analysis. Initially probes specific to the different PKC isoenzymes had to be synthesised as they were not readily available. Specific primers were designed from the cDNA sequences of the three isoenzymes and used in RT/PCR reactions to produce DNA sequences of 500-1000 bp in length which could then be labelled and used as probes (section 3.6.).

5.3.1. Isolation of Total RNA

RNA templates for the RT/PCR reactions were provided by cells that normally express the desired isoenzyme. RNA from A549 cells was used with the primers for PKCs -α and -θ. The primers for PKC-ε were designed using the rat PKC-ε sequence and so RNA was isolated from rat brain for this isoenzyme. PKC-ε primers were also incubated with RNA from WT cells in the hope that the primers would also bind to the human sequence.

Total RNA was isolated from A549 and MCF-7 WT cells and from rat brain using the caesium chloride method (section 3.5.1.). The OD at 260 and 280 nm was measured for each sample and table 5.2 shows typical yields of total RNA. Total RNA was calculated using equation 1 (section 3.5.1.).

5.3.2. RT/PCR Using Specific Primers and Isolated RNA

Total RNA was amplified using the appropriate primers in a RT/PCR reaction (section 3.6.3.). RNA from A549 cells was incubated with primers for PKCs -α and -θ and RNA from rat brain and WT cells was incubated with primers for PKC-ε.

Table 5.2 Total RNA Yield After Caesium Chloride Extraction.

SAMPLE	ABSORBANCE 260	280	RATIO	TOTAL RNA (μg)
WT	0.1091	0.0713	1.5	174.6
A549	0.0796	0.0481	1.7	127.4
RAT BRAIN	0.0730	0.0446	1.6	116.8

Absorbances shown are for a 1 in 200 dilution of the isolated RNA and are representative of all isolations.
[a] Ratio is absorbance 260/280. Total amount of RNA is calculated using equation 1 (section 3.5.1.).

The PCR products were run out on agarose gel, stained with ethidium bromide and visualised using UV light (section 3.6.4.). Results are shown in figure 5.3.

Lane 3 shows a single product around 500 bp in length. The PKC-α product should be 525 bp. Thus the product from A549 RNA and PKC-α primers was of the correct length. Lanes 4 and 5 show clearly that there was no contamination of the reaction.

Lane 6 shows the products produced when A549 RNA was incubated with PKC-θ primers. Two bands were clearly visible, one of approximately 400 bp and one of around 1000 bp. The PKC-θ product should be 1098 bp. Thus the smaller band was non-specific. Lanes 7 and 8 show that there was no contamination.

Lane 9 shows that no product was formed from the incubation of WT RNA and PKC-ε primers but a product of around 550 bp was produced with rat brain RNA (lane 10). The PKC-ε product should be 535 bp long. The primers designed against the rat PKC-ε sequence only produced a product with rat brain RNA. Lanes 11 and 12 show the absence of any contamination.

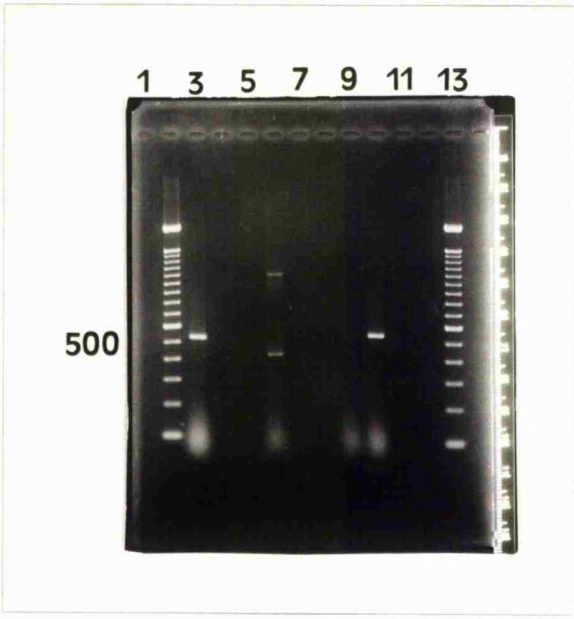
5.3.3. Cloning of PKC Isoenzyme Probes

Due to the presence of non-specific bands in the PKC-θ PCR reaction it was decided that the probes should be cloned into a vector. A clone that contained the correct probe sequence, i.e. that corresponding to 1098 bp, could then be isolated and used for the northern blot analysis. Although the PKC-ε primers did not produce a product with human RNA, the -ε probe should still bind to human RNA due to a high degree of homology between the rat and human sequences (>90 %). PCR products from the three isoenzyme specific primers were cloned into pCR^{TM}II vector using a TA cloning kit from Invitrogen (section 3.6.7.). Plasmid DNA was isolated from 5 or 6 of the 20 colonies picked for each PCR reaction using Wizard minipreps from Promega (section 3.6.8.).

5.3.4. Digestion of Vectors

In order to ensure that the DNA inserted into the vector was of the correct size an aliquot of plasmid was digested with EcoR1, which has cut sites either side of the insert, and electrophoresed (section 3.6.9.).

Figure 5.3 RT/PCR Products using Specific Primers for PKCs -α, -θ and -ε



RT/PCR was carried out as described in section 3.6.3. An aliquot of PCR product was run out on 1 % agarose gel (section 3.6.4.). The gel was stained with ethidium bromide and visualised using UV light. Lanes 2 & 13: 100 bp DNA ladder, lane 3: A549 RNA + PKC-α primers, lanes 4 & 5: RT and PCR blanks for PKC-α primers, lane 6: A549 RNA + PKC-θ primers, lanes 7 & 8: RT and PCR blanks for PKC-θ primers, lane 9: WT MCF-7 RNA + PKC-ε primers, lane 10: rat RNA + PKC-ε primers, lanes 11 & 12: RT and PCR blanks for PKC-ε primers.

Figure 5.4A shows the digestion products from 6 of the colonies picked after the cloning of the PKC-α PCR product. The lanes are in pairs of non-digested sample against digested sample. The non-digested sample ran as two bands. The top band of around 6 kb was the open circular form of the plasmid DNA whilst the lower band (≅ 2.6 kb) was the super-coiled form which migrated considerably faster than the open circles. After digestion the excised insert ran with an approximate size of 500 bp. The other band was the linear form of the plasmid DNA. It would seem that all 6 samples contained the same insert.

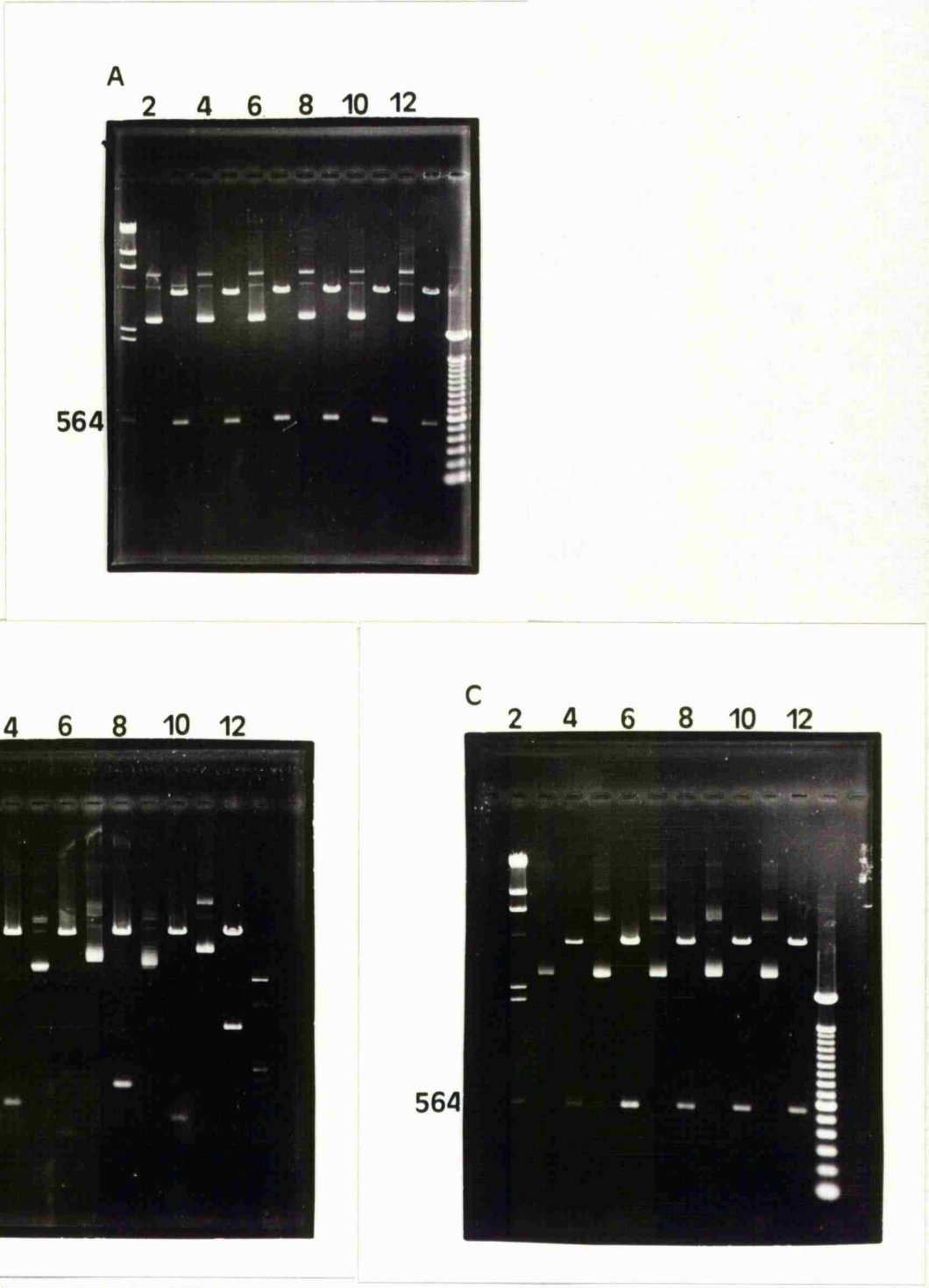
Figure 5.4B shows the digestion products after the cloning of PKC-θ PCR product. Only 1 of the 5 samples contained an insert of around 1000 bp (lane 12). The others had inserts of lower molecular weight of between 100 and 400 bp. Figure 5.3 showed that the RT/PCR reaction with the -θ primers produced non-specific bands which have then inserted into the vector resulting in clones containing the wrong insert.

Figure 5.4C shows the results from the digestion of 5 plasmid DNA samples from the cloning of PKC-ε PCR product. All 5 samples contained an insert of approximately 500 bp.

5.3.5. Sequencing of Cloned PCR Products

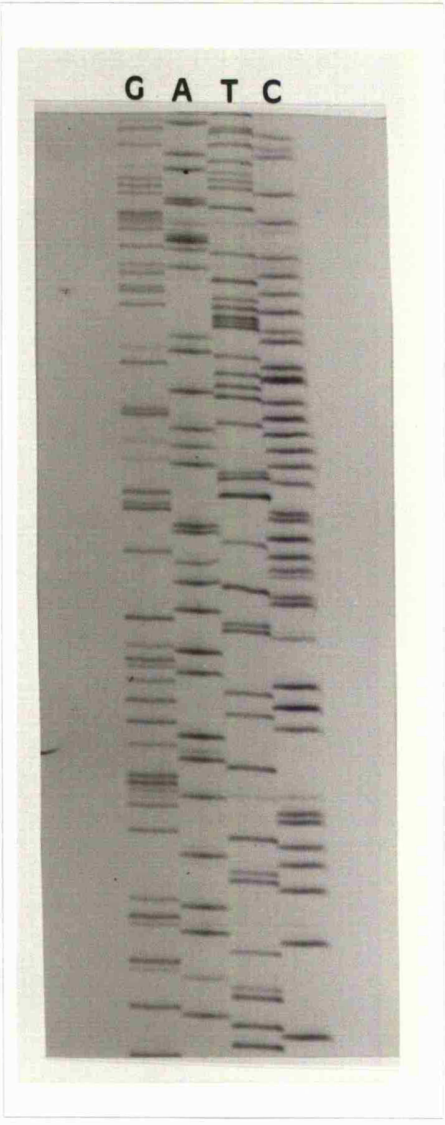
To confirm that the correct insert had ligated into the vector an insert for each PCR product was sequenced. For PKC-θ the sample containing the insert of approximately 1000 bp was sequenced and for PKCs -α and -ε one sample that showed an insert of the correct size were sequenced. The pCR™II vector has an initiation site for the M13 reverse primer near to where the ligation occurs and this is used for the sequencing reactions (section 3.6.10.). Figure 5.5 shows a typical result and as much of the sequence as possible was read off using a light box. The read sequences were then compared back to the sequences that the three sets of primers should have made. Figure 5.6 shows the read sequences for each of the three inserts with the underlined regions signifying areas of homology to the actual sequence. Figure 5.6A shows that the PKC-α insert matched the actual sequence by approximately 250 bp. PKC-ε insert matched the published sequence by approximately 270 bp and PKC-θ by 200 bp (figures 5.6B and C respectively). The three inserts matched the expected sequences.

Figure 5.4 Restriction Digestion of Plasmids Containing PKC probes for -α, -θ and -ε with EcoR1



Plasmids containing probes for PKCs -α, -θ and -ε were digested with ECO R1 and run out on 1 % agarose gel as described in section 3.6.9. and results are shown in figures A, B and C respectively. High molecular weight DNA and 100 bp DNA ladders were also run and some of the molecular weights are shown. The samples are in pairs of undigested plasmid and digested plasmid.

Figure 5.5 Sequencing Gel for PKC-ε



Sequencing was carried out as described in section 3.6.10. The figure shows part of the sequencing gel obtained for PKC-ε and is representative for those obtained for PKCs-α and -θ. The lanes are in the order GATC and the sequence was read using a light box..

Figure 5.6 Sequences of the PKC Isoenzyme Probes

A

771
↓
CAACAGTATTACAGTCCAGTCAG∧TT∧TTGTTCCTT∧CTGAA∧∧ATCCATAG
GGAAAGGAAACCTCAAACCCTCGA∧TGACTTCTACGGCCATGG∧CACCT∧C
CAAT∧TTCGAA∧GAATTGGTTCTTCTTCC∧CACAT∧ATGTTCGTAGGGGTA
GGCCTTCCCTGCTCCTTCCTTTGTACCTTGA∧TCCGTC∧TTTAAGCTCT∧TC∧
∧GGTTTGAACCGGGACGACCGTTGTTTCAGTAGTCAGGGAGACTTCTGTCC
TTTGCCGGAAGGTTGTTGGAATTCGGCTTAAGG
↑vector →
1020

B

334
↓
GCTGTCAGAGACTGGAGTTAGC∧GATGCA∧C∧TGT∧GCCTAGGCTT∧∧∧∧∧
GTGCGTTCGTC∧TCTGGTTGTCAGGCCGGACCGTGCTACT∧AAGCAGTGA∧
TACACACGTTACCGATCTTCTAGCTCGACCGACAGAAAGTGCTACGAG∧AT
AGCCGATGCTGCTGAAGCACCCGTTGACGTGGTAGGTCAAGCTCCTCGAC
GACGTCTTACCCTCGGCAGTGAAGCTCCTGACCTAACTAGACCTCGGTCCT
TCCT∧TTCAGATGCACTAGTAGCTAGAGAGCCCTA∧TATTGGGCTTAAG
↑vector→
596

C

923
↓
GATACTCCGTGAGTGTCAGCGACGA∧∧T∧T∧T∧TGAC∧∧GTC∧AGAAGTCTC
∧TCCAGGCCAA∧TTTAACCAGAGGGTACGAGGTAGTTTTTACTTCATTCCG
GCGGTACAAATGGCTGTGGCCCTTTTTCTCTCGGAGTCCC∧TAAAGGACCC
TCAGAGGCAAC∧TACTCCACCTA∧TTTACACGGTAGAAGGTCTTGGACTTG
ACTTGTTTCTTTCAGGTTTCGGCTTAAG
↑vector→
1128

Figures A, B and C show the sequences read from the gels for PKCs -α, -ε and -θ respectively. Areas of homology to the published sequences are underlined and the arrows indicate the bases where homology starts and the join between insert and vector. Bases in **bold** are incorrect (either a wrong or additional base to the published sequence) and ∧ represents a missing base.

Thus the PKC primers have bound specifically to RNA and resulted in DNA sequences that will be specific for each isoenzyme and thus suitable as probes for northern blot analysis.

5.3.6. Southern Blot Analysis of PCR Products

As a final confirmation that the sequences obtained for the three probes were correct Southern blot analysis was performed. Specific oligonucleotides (20mers) were designed for each PCR product (section 3.7.1.). These sequences were then N-labelled with $\gamma^{32}P$ using polynucleotide kinase (section 3.7.3.) and used as probes. Table 5.3 shows typical N-labelling results. The most radioactive fraction was used as the probe for hybridisation.

Products from the PCR reactions with PKCs -α, -ε and -θ primers were run out on a gel, Southern blotted as described in section 3.7.2. and then hybridised with one of the specific probes (section 3.7.4.). After autoradiography the blot was stripped (section 3.7.5.) and reprobed separately with the other two specific probes. Figures 5.7A, B and C show the results obtained after hybridisation with probes against PKC-α, -θ and -ε PCR products. Each probe bound specifically to a single PCR product.

Thus the PCR products for each of the three isoenzymes were successfully cloned, sequenced and verified by Southern blotting.

5.4. PKC Isoenzyme mRNA Expression in WT and MCF-7/Adr Cells

The probes produced in the above section were used in northern blot analysis to determine the PKC isoenzyme mRNA levels in WT and MCF-7/Adr cells.

5.4.1. Isolation of Total RNA

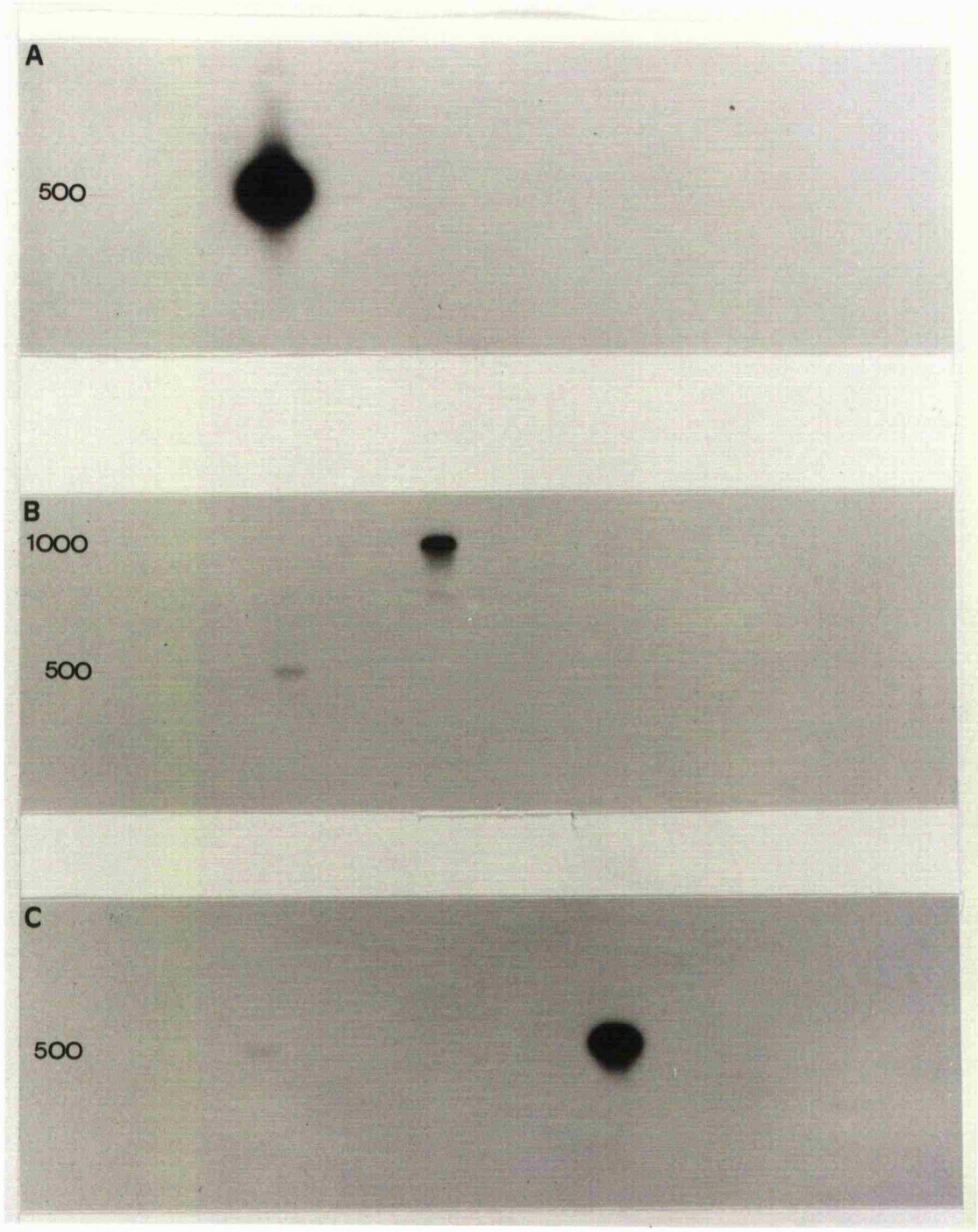
Total RNA was isolated from WT and MCF-7/Adr cells as described in section 3.5.2. Trizol® method was used in preference to the caesium chloride method because of the time difference in preparation. The one-step Trizol® isolation took approximately 3 h compared to 2 days. The OD at 260 and 280 nm was measured for both samples and table 5.4 shows typical yields of total RNA.

Table 5.3 N-labelling of Oligonucleotides

FRACTION	COUNTS / DPM
1	153
2	153
3	357
4	4,161,734
5	23,911,224
6	12,956,224
7	6,964,897
8	5,427,193
9	4,379,387
10	2,988,571

Oligonucleotides were N-labelled as described in section 3.7.3. The results shown are representative of all labelling reactions.

Figure 5.7 Southern Blot Analysis of PKC Specific Probes



Southern blot analysis was performed and hybridised with PKC specific probes using an aliquot of PCR product as described in section 3.7.2. The internal oligonucleotides specific for each PKC probe were N-labelled and hybridised with the blot (section 3.7.4.). Figures A, B and C show the autoradiographs obtained using oligonucleotides specific for PKC -α, -θ and -ε probes respectively. The blot was stripped between each hybridisation.

Table 5.4 Total RNA Yield After Trizol® Extraction

SAMPLE	ABSORBANCE 260	280	RATIO	TOTAL RNA (μg)
WT	0.5413	0.2916	1.9	60.62
MCF-7/Adr	0.4236	0.2310	1.8	47.44

Absorbances shown are for a 1 in 200 dilution of the isolated RNA and are representative of all isolations.
[a] Ratio is absorbance 260/280. Total amount of RNA is calculated using equation 1 (section 3.5.1.).

5.4.2. Northern Blot Analysis

RNA (10 μg) from WT and MCF-7/Adr cells was electrophoresed on an agarose gel and blotted onto a membrane as described in sections 3.8.1. and 3.8.2. Figure 5.8 shows a typical RNA gel prior to blotting. The 28S and 18S rRNA molecules are clearly visible. The blot was then hybridised with a labelled probe.

Plasmids containing each of the three probes were used as templates in PCR reactions with the isoenzyme specific primers in order to obtain enough product to label and use as a probe. The PCR products were purified (section 3.6.5.) and 100 ng each labelled with $\alpha^{32}P$. Table 5.5 shows typical results from random labelling of the probes. Each probe was hybridised with the membrane separately as described in section 3.8.4.

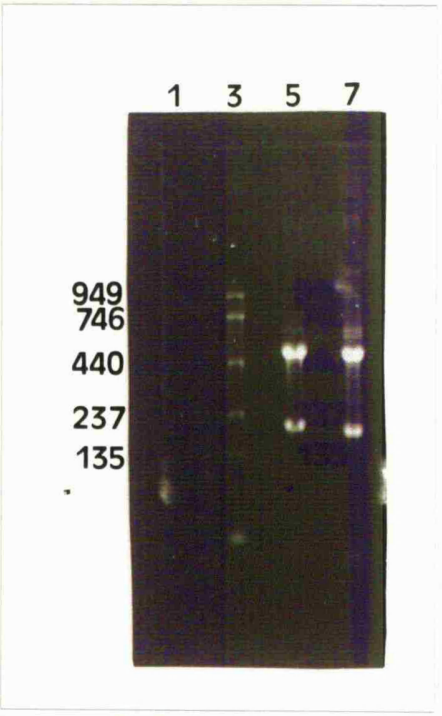
After hybridisation the blot was washed, exposed overnight and analysed using a phosphorimager (Molecular Dynamics). No bands were observed after hybridisation with any of the three PKC probes. Various parameters were changed such as amount probe added, hybridisation temperatures and washing stringency but still no bands could be detected. It was decided that the inability to detect hybridisation could have been due to a sensitivity problem and so the experiment was repeated using polyA$^+$ RNA (mRNA) instead of total RNA.

5.4.3. Northern Blot Analysis Using PolyA$^+$ RNA

PolyA$^+$ RNA was isolated from total RNA as described in section 3.5.3. PolyA$^+$ RNA yields were 3.2 μg for MCF-/Adr and 4.2 μg for WT cells from approximately 8 x 10^7 cells.

Triplicate lanes of PolyA$^+$ RNA (1 μg) from WT and MCF-7/Adr cells were run out on agarose gel and blotted onto a membrane. Some rRNA was carried over resulting in faint rRNA bands being observed. The membrane was cut into three and hybridised with each of the 3 PKC probes as before and exposed to phosphorimager. However, hybridisation was still not detectable even though approximately 5 - 10 times more mRNA was loaded onto the gel than in section 5.4.2. To confirm that the blotting and hybridisation procedure was working efficiently three blots were hybridised with a probe specific to GAPDH. Bands of 1.27 kb were observed for both cell lines on all three blots (figure 5.9).

Figure 5.8 Electrophoresis of Total RNA



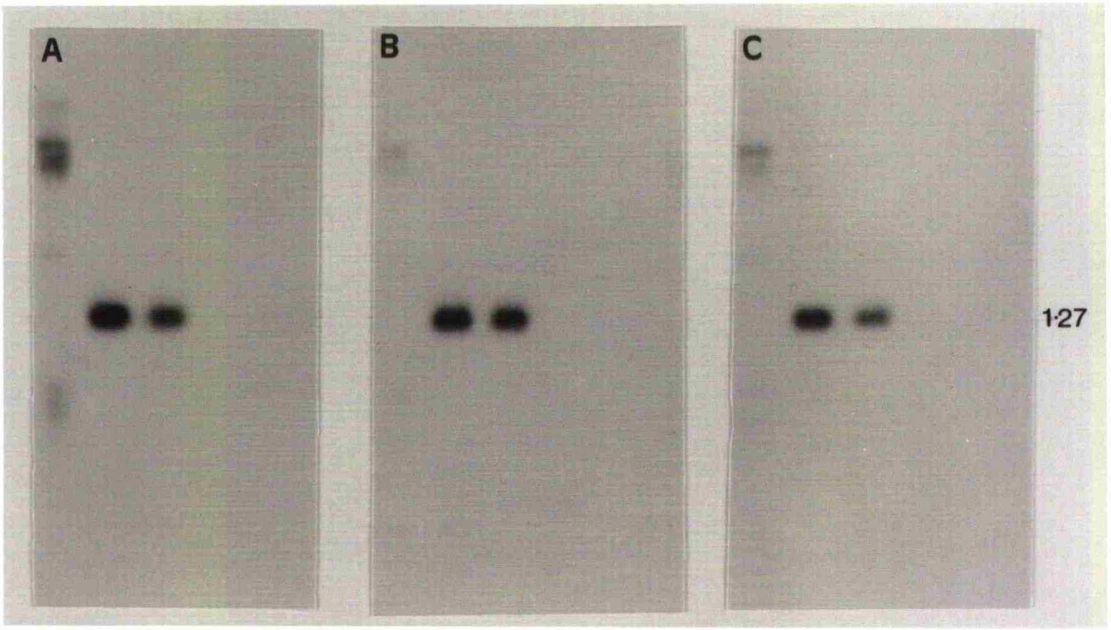
RNA was isolated and electrophoresed as described in sections 3.5.2. and 3.8.1. respectively. Lane 3 contains RNA ladder of the indicated molecular weights. Lanes 5 and 7 contain 10 µg total RNA from WT and MCF-7/Adr cells respectively. The gel has been visualised using UV light.

Table 5.5 Random Labelling of Probes

FRACTION	COUNTS / DPM
1	51
2	153
3	1,173
4	2,961,377
5	35,505,765
6	16,115,969
7	7,405,000
8	5,278,010
9	2,387,142
10	1,144,132

Primers were random labelled as described in section 3.8.3. The results shown are representative of all labelling reactions. The most radioactive fraction was used as the probe.

Figure 5.9 Northern Blot Analysis of PolyA$^+$ RNA with GAPDH Probe



PolyA$^+$ RNA was isolated from WT and MCF-7/Adr cells and 1 μg of each was electrophoresed in triplicate lanes as described in sections 3.5.3. and 3.8.1. respectively. The blots were hybridised with a labelled GAPDH probe and bands visualised by autoradiography. The three blots A, B and C had been hybridised previously with PKC -α, -ε and -θ probes respectively.

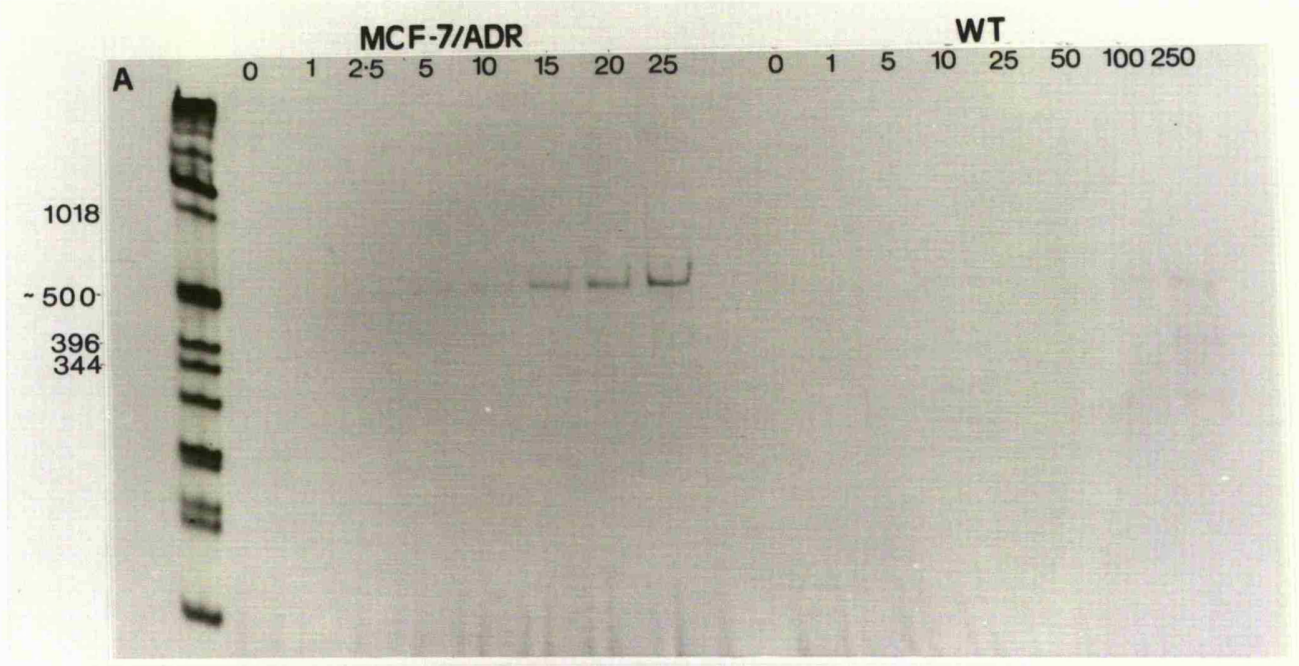
Northern blot analysis was not sensitive enough to determine the levels of PKC mRNA in the two cell lines. Thus, a more sensitive method, RT/PCR was used to quantify the PKC isoenzyme mRNA levels.

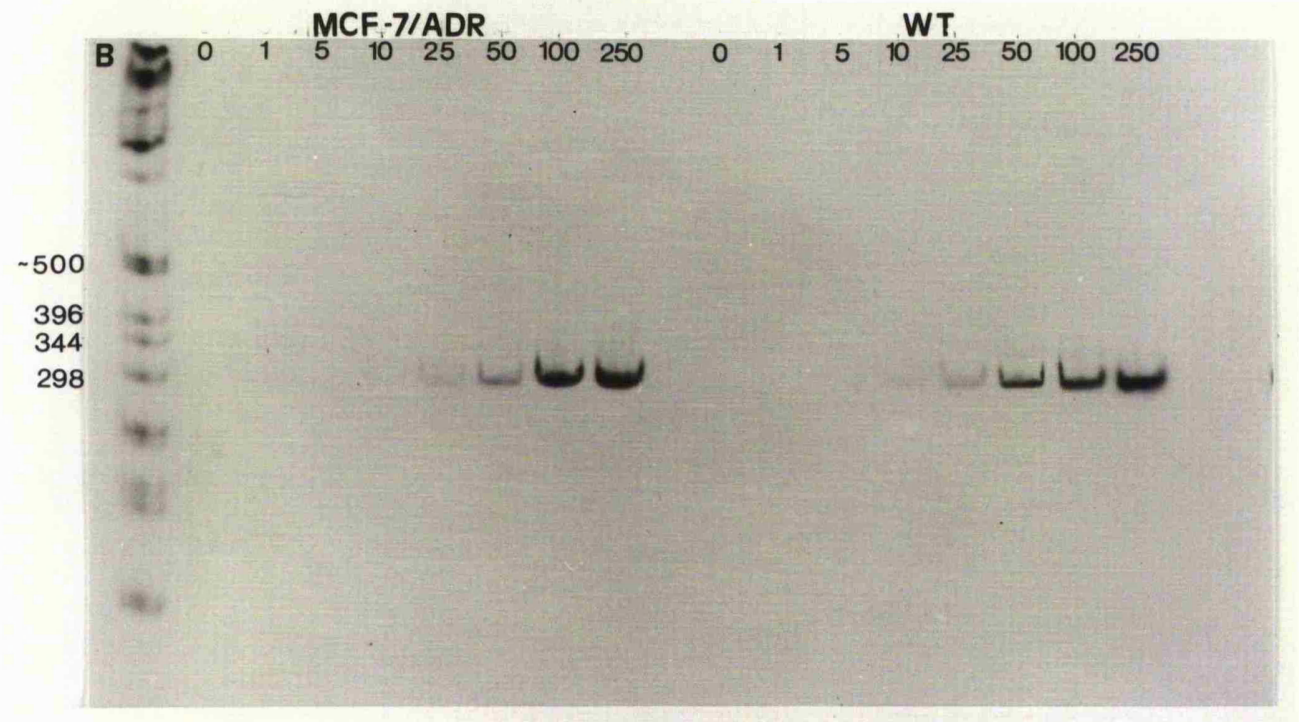
5.4.4. Semi-quantitative RT/PCR

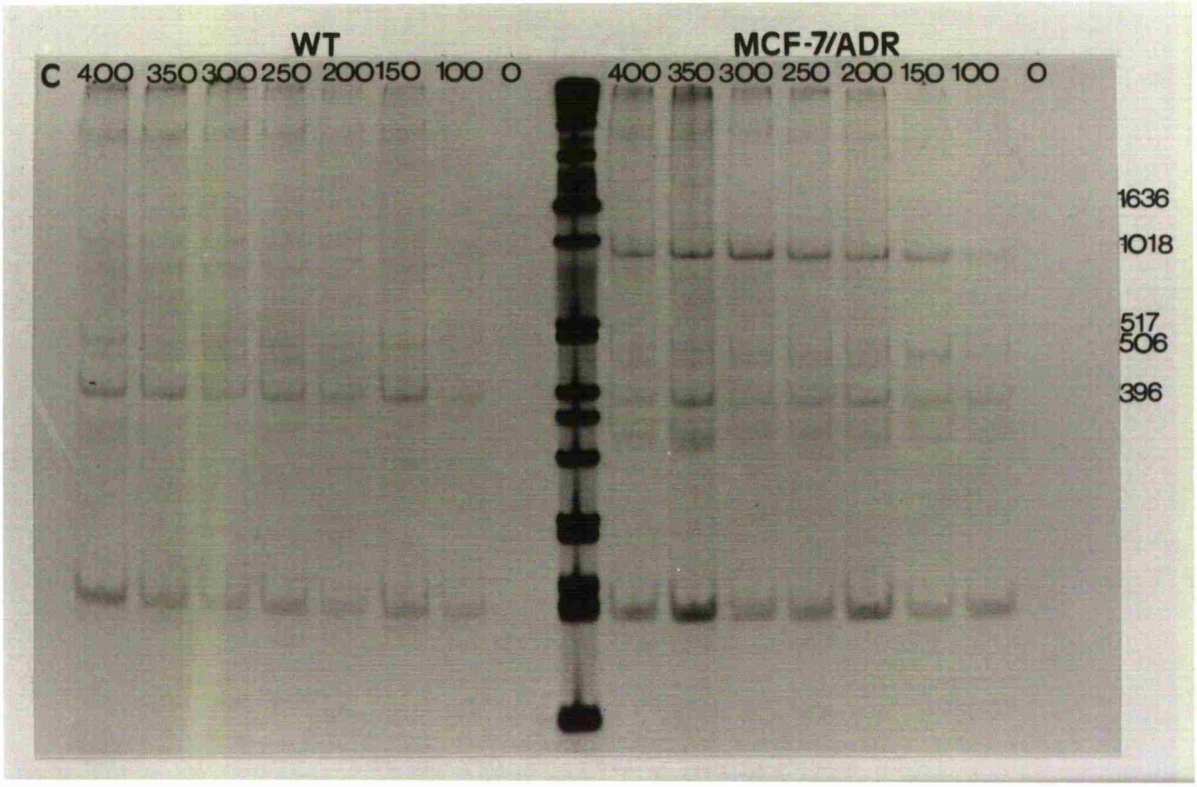
To overcome the sensitivity problem semi-quantitative RT/PCR was performed for the three PKC isoenzymes using total RNA from WT and MCF-7/Adr cells as described in section 3.9. Figures 5.10A, B and C show representative results obtained using primers for PKCs -α, -ε and -θ respectively. The bands observed were of the correct size for the primers: PKC-α primers resulted in a product of approximately 500 bp (should be 525 bp), PKC-ε approximately 320 bp (should be 384 bp) and PKC-θ approximately 1000 bp (should be 1097 bp). The log value of band volume, as determined by the phosphorimager, when plotted against log amount of RNA in the reaction should produce a straight line. Figure 5.11 shows the results from RT/PCR reaction using primers for PKC-α using RNA from WT and MCF-7/Adr cells. A fold difference in the amount of mRNA for PKC-α present in the two different cell lines can be calculated from the graph by determining the amount of RNA necessary to produce a band of the same density. The difference in mRNA for three PKC isoenzymes in the two cell lines was calculated. PKC-α mRNA was 10-12 fold greater in MCF-7/Adr cells as compared to WT cells. PKC-ε mRNA levels were approximately 2 fold higher in WT cells as compared to MCF-7/Adr cells. PKC-θ mRNA was at the detection limit in WT cells but was present in MCF-7/Adr cells.

In summary, in MCF-7/Adr cells PKC-θ mRNA expression was induced, PKC-α mRNA was increased significantly, either due to increase in transcription or to stabilisation of the message, and PKC-ε mRNA expression was lower. These results complement the changes observed for the protein products of these mRNAs.

Figure 5.10 RT/PCR of PKCs -α, -ε and -θ in WT and MCF-7/Adr Cells

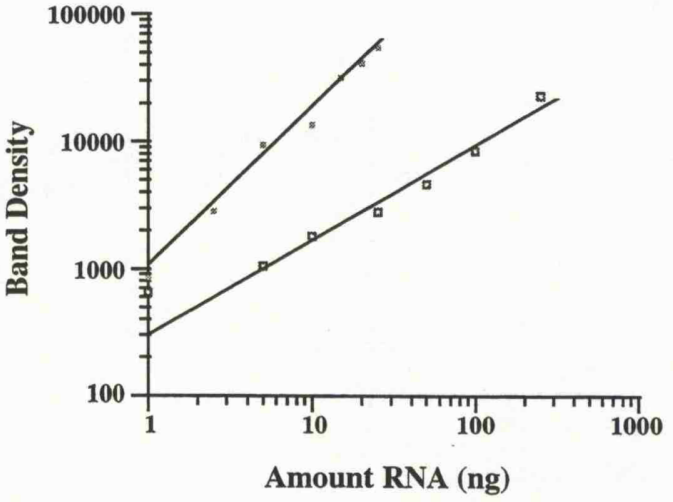






RT/PCR was performed as described in section 3.9. with the specific primers for PKCs -α, -ε and -θ. [^{33}P] labelled PCR products were run out and detected by exposure to film. Lanes indicate the amount of total RNA (ng) used in the reaction. Figures A, B and C show the results obtained with PKC-α, -ε and -θ primers respectively. A 1 kb DNA ladder with the molecular weights indicated was run.

Figure 5.11 Semi-quantitative RT/PCR for PKC-α in WT and MCF-7/Adr Cells



Semi-quantitative RT/PCR was performed and quantified as described in section 3.9. for PKC-α using RNA from WT (open squares) and MCF-7/Adr cells (closed squares).

5.5. PKC Isoenzyme and MDR1 Expression in Revertant MCF-7/Adr Cells

Cells that have been made resistant *in vitro* to cytotoxic agents may lose resistance if maintained in drug free medium for some time (Dahllof *et al*, 1984; Meyers *et al*, 1985; Fojo *et al*, 1985). The aim of this study was to see how PKC isoenzyme and *MDR1* expression changed during this period of resistance reversal.

5.5.1. Sensitivity Towards Adriamycin

MCF-7/Adr cells were grown in the absence of adriamycin and the resistance to adriamycin was monitored over time. Figure 5.12 shows how the sensitivity of the cells increased with time. Adriamycin IC_{50} for MCF-7/Adr cells was 2.02 ± 0.4 μM. After 10 weeks without drug the IC_{50} was 1.6 μM, 80 % that seen at week 0. The IC_{50} then decreased, at week 15 it was 0.86 μM and at 20 weeks it was 0.18 μM (9 % of week 0). At week 24 it was 12.5 nM approximately twice that of WT cells (5.0 nM).

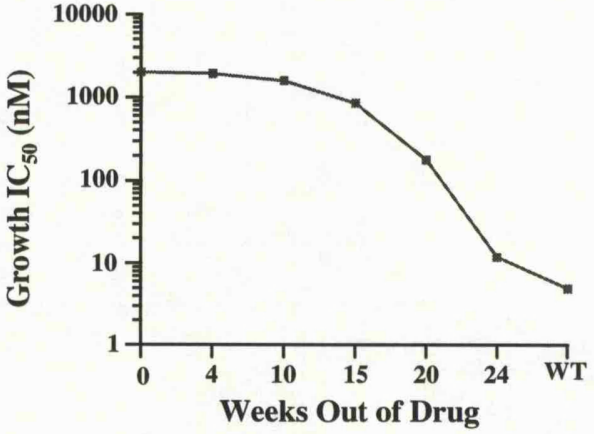
5.5.2. P-Glycoprotein Expression

P-glycoprotein expression was monitored over the same time course by western blot analysis (section 3.3.2.). Figure 5.13 shows how expression of the protein decreased with time. Expression remained high up to week 15 out of drug where the level was still 82 % of week 0. However, by week 20 expression had decreased to 18 % of week 0 and the protein could not be detected at week 24. P-glycoprotein was not detectable in the WT cells.

5.5.3. MDR1 Expression

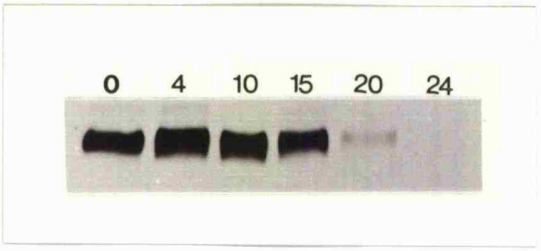
MDR1 expression was determined using northern blot analysis. Total RNA was isolated from the cells at each time point, 10 μg electrophoresed on an agarose gel and blotted onto a membrane. The probe used was specific to the 4.4 kb *MDR1* transcript. Figure 5.14 shows how the message level of *MDR1* decreased with time. There was a gradual decrease in expression with 63 % of week 0 detectable at week 10, 56 % at week 15 and 40 % at week 20. By week 24 expression was 1 % of week 0, comparable to that seen in WT cells. These results have been corrected for any loading differences using GAPDH expression for each sample.

Figure 5.12 Growth Inhibition of Adriamycin in Revertant MCF-7/Adr Cells



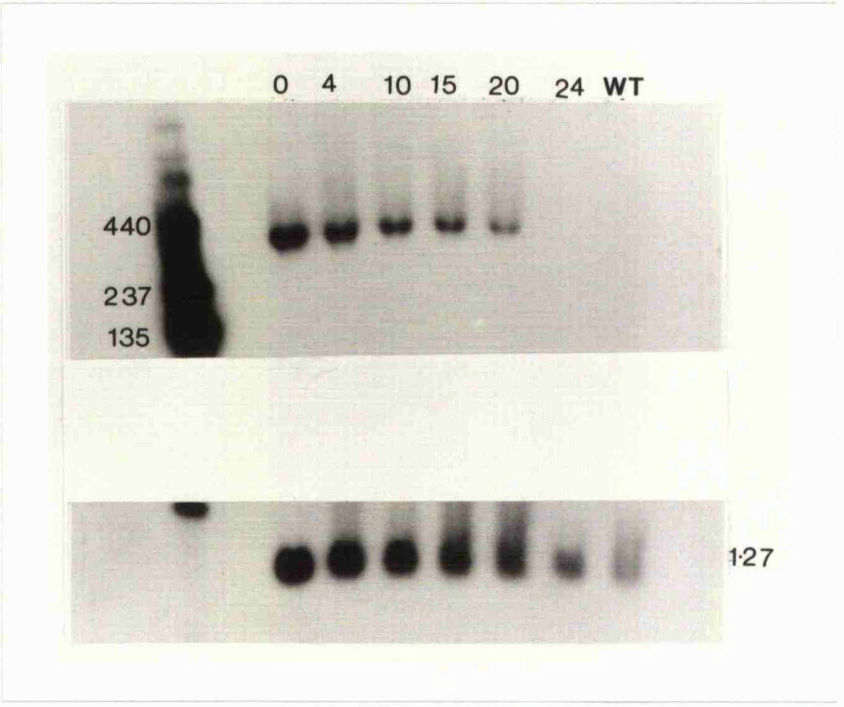
MCF-7/Adr cells from each time point were grown in the presence of adriamycin and an IC_{50} value determined as described in section 3.1.4. A growth curve with control MCF-7/Adr cells was performed at each time point.

Figure 5.13 P-Glycoprotein Expression in Revertant MCF-7/Adr Cells



Membrane samples were isolated from cells at the time points indicated as described in section 3.2.4. and run out on a gel. P-glycoprotein was detected by western blot analysis using a monoclonal antibody (section 3.3.2.).

Figure 5.14 MDR1 mRNA Levels in Revertant MCF-7/Adr Cells



Total RNA was isolated from cells at each time point and northern blot analysis performed as described in sections 3.5.2 and 3.8 respectively. Blot was hybridised with a probe specific to human *MDR1* and exposed to film (top panel). After stripping the blot was hybridised with a probe to GAPDH and the results are shown in the bottom panel.

5.5.4. PKC Isoenzyme Protein Expression

I have shown that MCF-7/Adr cells possess a different PKC isoenzyme profile compared to WT cells, with changes being seen in the expression of PKCs -α, -ε and -θ. Thus the expression of these three isoenzymes was determined in cells at each time point (figure 5.15). PKCs -α and -θ remained detectable at similar levels up to week 20 but expression was lost by week 24. PKC-ε was detectable in all three fractions of the cell up to week 20 but at week 24 it was only detectable in the cytosolic fraction. Thus the PKC isoenzyme profile of the cells was the same as in MCF-7/Adr cells up to week 20, but by week 24 it had reverted to that in WT cells.

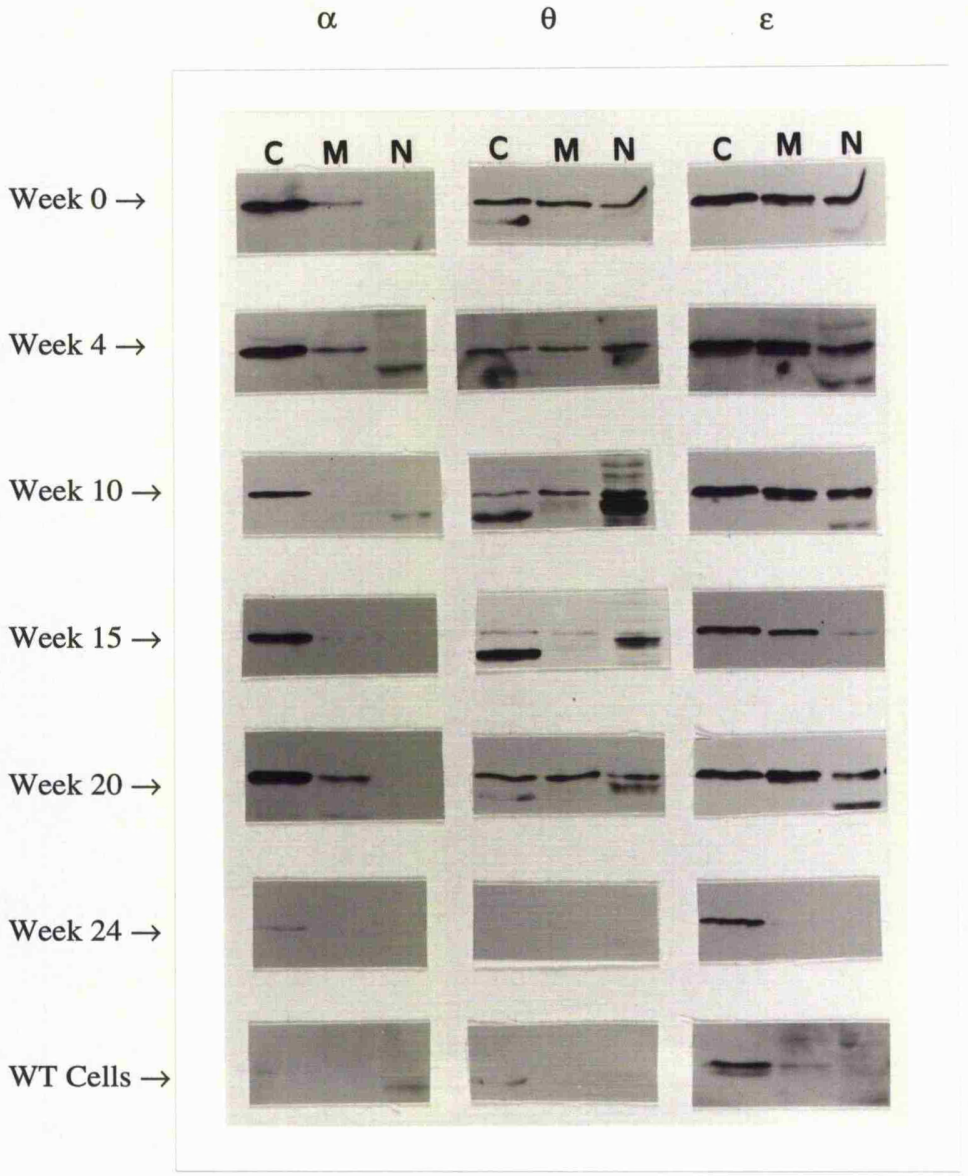
5.5.5. PKCs -α and -θ mRNA Expression

Results shown in section 5.4.4. showed that mRNA levels of PKCs -α and -θ were altered in MCF-7/Adr cells as compared to WT cells. The mRNA levels of these 2 isoenzymes was monitored in the revertant cells, and results obtained are shown in figure 5.16. The autorad shows that PKC-α mRNA level was high for up to 20 weeks and then decreased such that at week 24 it was at a similar level to that in WT cells. PKC-θ mRNA was detectable up to week 20, but it was very low by week 24. Thus these results mimic those seen at the protein level in that levels decreased between weeks 20 and 24.

5.5.6. MDR1 Gene Level

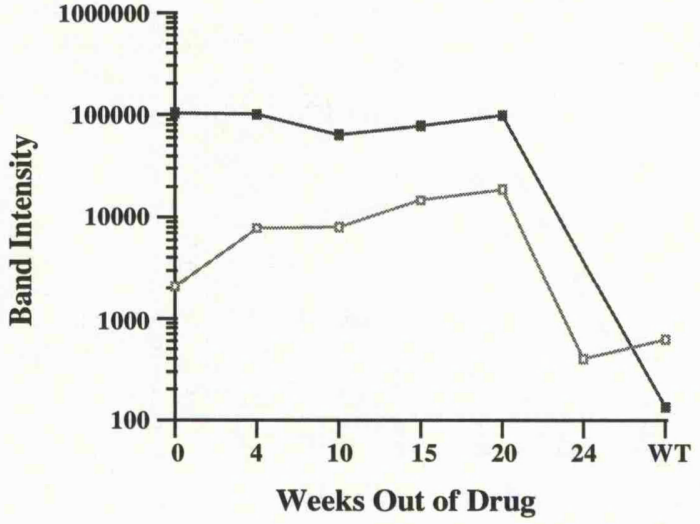
Cells that have been made resistant to cytotoxic agents *in vitro* and express the MDR phenotype often contain an amplification of the *MDR1* gene that encodes P-glycoprotein. The level of *MDR1* gene expression was determined in the revertant cells. Genomic DNA was isolated from cells at each time point, run out on a gel and Southern blotted as described in section 3.10. Figure 5.17 shows the gel prior to blotting. The lanes show a smear of DNA with some bands visible. The blot was hybridised with a labelled probe specific to *MDR1*, washed and exposed to film. Figure 5.18 shows the results obtained. Amplification of the gene was observed up to week 20 at similar levels but was lost by week 24 where levels were the same as in WT cells.

Figure 5.15 Protein Expression of PKCs -α, -ε and -θ in Revertant MCF-7/Adr Cells



Cells from each time point were fractionated and PKC isoenzymes detected by western blot analysis as described in sections 3.2.3. and 3.3.1. respectively.

Figure 5.16 mRNA Levels of PKCs -α and -θ in Revertant MCF-7/Adr Cells

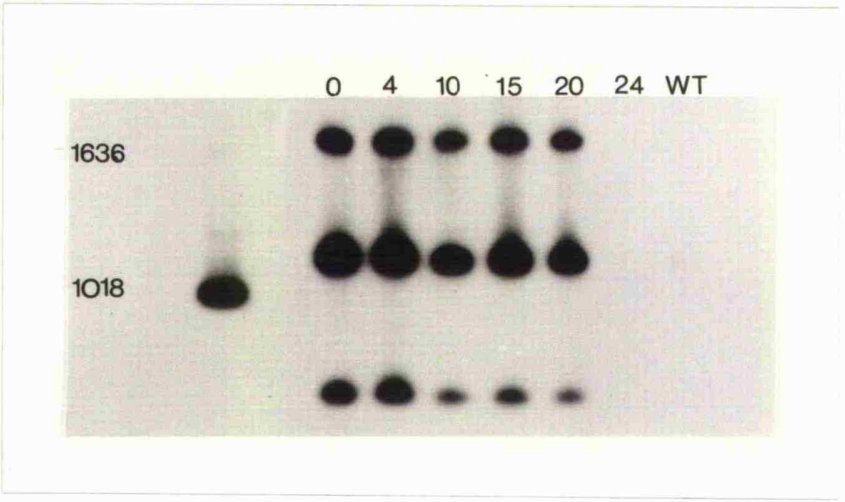


Semi-quantitative RT/PCR with PKC-α (closed squares) and -θ primers (open squares) was performed using 10 ng total RNA from cells at each time point as described in section 3.9. Labelled PCR products were quantified using phosphorimager and plotted against time out of drug.

Figure 5.17 Electrophoresis of Genomic DNA

Genomic DNA was isolated from cells at each time point and electrophoresed as described in section 3.10. Lane 2 contains 1 kb DNA ladder and lanes 4-10 contain DNA from the indicated time points. Gel was visualised under UV light having been stained with ethidium bromide.

Figure 5.18 MDR1 Gene Amplification in Revertant MCF-7/Adr Cells

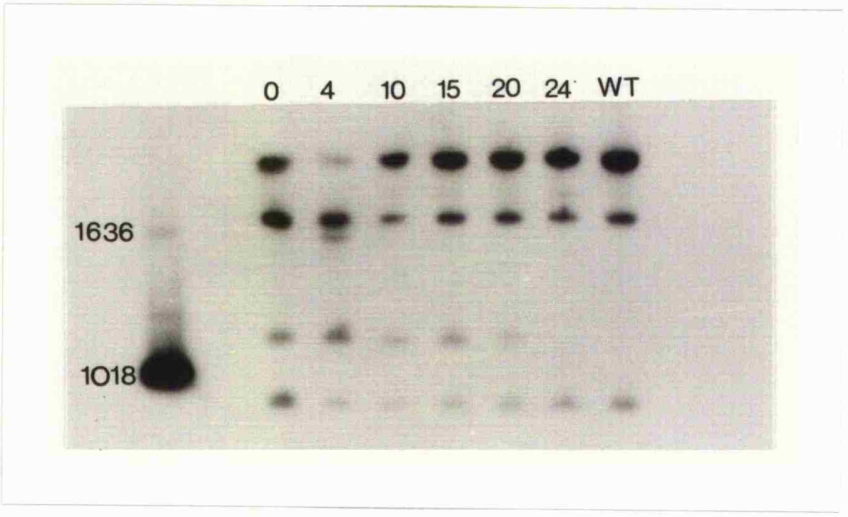


Genomic DNA gel was Southern blotted as described in section 3.10. and hybridised with a labelled probe specific to *MDR1*. The lanes contain DNA from the indicated time points. Bands were visualised by autoradiography.

5.5.7. PKC-α Gene Level

PKC-α mRNA levels increased 10-12 fold in resistant cells. This could have been due to a gene amplification event as for *MDR1*. To test this hypothesis the blot from 5.5.6 was stripped and rehybridised with a labelled probe specific for PKC-α. The level of PKC-α gene was the same for all samples (figure 5.19). No PKC-α amplification was detected in the resistant cells.

Figure 5.19 PKC-α Gene Level in Revertant MCF-7/Adr Cells



The blot from figure 5.18 was stripped (section 3.8.5) and reprobed with a probe specific to bases 496-1021 of PKC-α. The bands were visualised by autoradiography.

5.6. Discussion

Many research groups have studied PKC mRNA levels to compare them to the respective protein levels but PKC isoenzyme specific probes are not readily available. So primers were designed using cDNA sequences for human PKCs -α and -θ and rat PKC-ε that would make cDNA probes of suitable length for use in northern blot analysis. PCR products were not observed with the primers for PKC-ε when human RNA was used as a template, but a product was produced with rat RNA. The human sequence of PKC-ε (Basta *et al*, 1992) is 206 bp shorter than the rat sequence. The sense primer for PKC-ε was against part of the beginning of the rat sequence (bases 61-78). These bases are not in the human sequence. This explained the lack of PCR product with the human RNA. However, the rat and human sequences share 90 % homology and so the probe synthesised should hybridise with human PKC-ε.

The PCR products were labelled and used as probes to determine the levels of mRNA of PKCs -α, -ε and -θ in WT and MCF-7/Adr cells. The probes did not result in bands when total RNA was used. I tried to optimise the technique by using polyA$^+$ RNA but the probes still did not hybridise suggesting that the method was not sensitive enough for MCF-7/Adr cells. Most of the reports in the literature, where mRNA levels of PKC isoenzymes have been investigated, have used greater amounts of RNA. Some researchers have used between 20 and 30 μg of total RNA (Carlson *et al*, 1993; Dean *et al*, 1994) whereas others have used polyA$^+$ RNA (Blobe *et al*, 1993; Mishima *et al*, 1994; Doi *et al*, 1994). All of the studies have used probes in excess of 1 kb in length and some have used the whole cDNA sequence of the isoenzyme. Obviously different cells contain varying levels of the PKC isoenzyme mRNA and this will affect the sensitivity of the method. The amount of RNA used in this study was on the low side compared to many of the reports in the literature and the probes used were also a lot smaller. Due to the lack of sensitivity of this method for the WT and MCF-7/Adr cells the message levels of the PKC isoenzymes was determined by semi-quantitative RT/PCR.

Blobe *et al* (1993) reported that PKC-α mRNA could not be detected in WT cells but it was in MCF-7/Adr cells. Northern blot analysis was used in this investigation. Using the more sensitive method of RT/PCR it can be seen that WT cells do express PKC-α

mRNA but it is 10-12 fold lower than the levels seen in the resistant cells. PKC-ε mRNA levels were similar in both cell types. PKC-θ mRNA could not be detected in WT cells but it was expressed in MCF-7/Adr cells. The differences seen in mRNA levels between the two cell lines reflect the differences seen at the protein level.

There is strong evidence to suggest a link between PKC and MDR (see introduction). Elevated PKC-α expression is consistently seen in conjunction with P-glycoprotein (P-gp) expression (Posada *et al*, 1989; Ward and O'Brian, 1991), but my work has shown for the first time that expression of another isoenzyme, PKC-θ, is also induced in cultured resistant cells. Colleagues in the MRC unit have shown that another breast carcinoma cell line, MDA-MB-468, contains a small number of cells that express P-glycoprotein intrinsically. These cells, when isolated and compared to the majority of MDA-MB-468 population, were shown to have the same changes of PKC isoenzymes as the MCF-7 cells including the induction of PKC-θ expression in all three fractions (Zhang *et al*, 1996). A recent report by Beck *et al* (1996) has shown that there was a significant increase in *MDR1* and PKC-θ gene expression in patients with relapsed acute myelogeneous leukaemia. This is the first report of an association between MDR and PKC-θ in a clinical situation. PKC-θ has a unique tissue distribution being found normally in skeletal muscle, lymphoid organs and haemopoietic cell lines (Baier *et al*, 1994) so its expression in drug resistant breast cells is unclear. In MCF-7/Adr cells two nPKCs are expressed, PKCs -ε and -θ, and their distribution is the same in that they are found in all three fractions of the cell. Combined these data suggest an important role for nPKCs in the maintenance of the MDR phenotype. The expression of isoenzymes in the particulate fraction could represent a function linked with the activity of P-gp. Chambers *et al* (1990) demonstrated that PKC phosphorylated P-gp, and they showed later that phosphorylation occurred at three serine sites within the linker region of the P-gp molecule (Chambers *et al*, 1995). The membrane associated PKCs could be important for this phosphorylation.

In the absence of the drugs against which resistance was induced, many MDR cell lines regain sensitivity to the same spectrum of drugs to which they originally expressed resistance (Dahllof *et al*, 1984; Meyers *et al*, 1985; Fojo *et al*, 1985). This study has shown that to maintain a high level of resistance the selected MCF-7/Adr cells have to be grown in the presence of the drug. It has been shown that MDR cells have an

increased rate of transcription as well as a gene amplification (Morrow *et al*, 1992; Madden *et al*, 1993; Davies *et al*, 1996). When MCF-7/Adr cells were maintained in drug free medium the level of *MDR1* mRNA started to decrease almost immediately suggesting that the presence of the drug may be the cause of the increased transcription rate. After 4 weeks *MDR1* mRNA has decreased to 79 % of control and by week 15 it was 56 % without a change in gene amplification (figure 5.18). When *MDR1* mRNA had dropped to 40 % P-gp expression had decreased and the cells had become more sensitive to adriamycin. *MDR1* gene amplification is most commonly manifested as either intrachromosomal DNA structures such as homogeneous staining regions (HSRs) or extracellular circular DNAs also known as double minutes. These amplification structures are unstable; if the selection drug is withdrawn, the MDR-resistant phenotype is lost over time concomitantly with a decrease in the length of the HSR or in the number of DMs (Lothstein and Horwitz, 1986; Shen *et al*, 1986). In this study the loss of the *MDR1* gene amplification occurred very suddenly between 20 and 24 weeks out of drug. A time course for this phenomenon has not been recorded before.

There is good evidence that PKC-α may be involved in modulating MDR, but the exact mechanism for this effect has not been determined. Yu *et al* (1991) showed that transfection of MCF-7 cells with *MDR1* cDNA did not contribute to the high degree of resistance seen in cells selected for adriamycin resistance. However, transfection of PKC-α and *MDR1* conferred greater resistance whereas transfection of PKC-α on its own had little effect. Transfection of *MDR1* and PKC-γ did not result in high levels of resistance (Ahmad *et al*, 1992). Although there have been many reports of PKC-α overexpression in resistant cells, the possibility of PKC-α gene overexpression has not been investigated. The genes for MDR1 and PKC- α are on chromosomes 7 and 17 respectively. The co-amplification of two genes that lie on separate chromosomes would seem highly unlikely. This study clearly shows that the PKC- α gene is not amplified so the increase in expression is either due to an increase in transcription or to a stabilisation of mRNA. This study, however, does show that although PKC and MDR genes are not co-amplified they are tightly regulated. The PKC isoenzyme changes observed in MCF-7/Adr cells revert back to WT phenotype between weeks 20 and 24 i.e. at the same time that the *MDR1* gene amplification is lost. The MCF-7/Adr

cells used in this study can be separated into two sub-populations which contain different levels of *MDR1* gene amplification and P-gp (Davies *et al*, 1996). The two populations are termed LP-gp (low P-gp) and HP-gp (high P-gp). Although the degree of resistance is different the PKC isoenzyme levels are the same in both cell types. This suggests that the PKC changes are an early event in the development of resistance. It is conceivable that the PKC alterations are necessary before gene amplification can occur and consequently the actual levels of the isoenzymes are not important for the degree of resistance observed in MCF-7/Adr cells.

It was always thought that the link between PKC and MDR was that PKC was necessary for the phosphorylation and function of P-gp. However, recent reports bring this concept into doubt. Germann *et al* (1996) have shown that if the PKC phosphorylation sites of P-gp are mutated P-gp still functions as normal. Scala *et al* (1995) have shown that down-regulation of PKC using bryostatin decreased P-gp phosphorylation but did not affect P-gp function. Phosphorylation of P-gp may affect its affinity for certain drugs and change the drug resistance profile of MDR cells (Bates *et al*, 1992). Alternatively the velocity of drug transport may be affected (Aftab *et al*, 1994). The PKC isoenzymes expressed, especially those in the membrane, may be important for these events. PKC may be more directly involved in the activation of the *MDR1* gene whose promoter has AP-1 sites which can interact with PKC. Stress induced AP-1 binding activity was attenuated by a PKC inhibitor in MCF-7/Adr cells (Lee *et al*, 1995). Increased nuclear PKC-α expression has been reported in MCF-7/Adr cells (Lee et al, 1992) and could be important for the transcription of *MDR1* gene. PKC-θ is highly expressed in nuclear fraction of MCF-7/Adr cells. This isoenzyme has not been researched extensively in relation to MDR. PKC-θ could be just as important for the development of resistance as PKC-α appears to be. It is conceivable that PKC phosphorylates transcription factors necessary for *MDR1* activity. PKC has been shown to modulate programs of cell differentiation by inducing phosphorylation of cell specific transcription factors. Inhibition of myogenesis by activated PKC appears to be directed at the myogenic regulatory factors which mediate muscle specific gene activation by binding to target sequences in the control regions of muscle specific genes (Olson *et al*, 1990).

Overall this study has quantified the PKC changes between WT and MCF-7/Adr cells at the protein and mRNA level and shown the existence of a tight link between PKC and MDR expression in MCF-7/Adr cells. The results suggest that PKC is important for the transcription of *MDR1* gene and the function of P-gp. PKC-α is the isoenzyme most commonly overexpressed in MDR cells, however, PKC-θ has not been extensively studied. The fact that both these isoenzymes are overexpressed in MCF-7/Adr as compared to WT cells suggests that they are important for the MDR phenotype induced in the resistant cells.

CHAPTER 6

MODULATION OF MDR BY PKC INHIBITORS

6.1. Introduction

In the previous chapter I have described how the expression of PKC and MDR appear to be intricately linked and that the expression of the MDR phenotype in MCF-7/Adr cells may be dependent on PKC. If this is the case then inhibition of PKC may affect MDR in such a way that may prove beneficial in the clinical situation. Some PKC inhibitors have been shown to reverse the MDR phenotype (see introduction). The aim of this study was to investigate the effects of PKC inhibitors, staurosporine and its four analogues, in MCF-7/Adr cells.

6.2. Cytostatic Effects of Staurosporine and its Analogues on MCF-7/Adr Cells

Staurosporine and its analogues are potent inhibitors of the growth of WT MCF-7 cells (see chapter 4). In this study the same data was determined for MCF-7/Adr cells, and table 6.1 shows the IC_{50} values obtained for both cell lines. GFX was equipotent in both WT and MCF-7/Adr cells furnishing the same IC_{50} value, 7.3 and 8.8 μM respectively. RO was a much poorer growth inhibitor of MCF-7/Adr cells with an IC_{50} 12 times greater in the resistant cells (11.3 as compared to 0.9 μM in WT cells). UCN was also less potent in MCF-7/Adr cells with an IC_{50} ratio of 7. Staurosporine and CGP were 2-3 fold less potent in resistant cells.

These data indicate that the MCF-7/Adr cells show a high degree of resistance to RO which could have been due to the possibility that it was pumped out of the cells by P-gp. To test this hypothesis the growth studies were repeated but PKC inhibitors were co-incubated with 5 μM reserpine. Reserpine is itself an inhibitor of P-gp function and colleagues have shown that at 5 μM it completely blocks the efflux of rhodamine out of MCF-7/Adr cells (unpublished data). Therefore, if RO was being pumped by P-gp, reserpine should increase its cytostatic effect in the MCF-7/Adr cells. Reserpine (5 μM) did not affect the growth of the cells. Table 6.2 shows the IC_{50} values obtained for PKC inhibitors when resperine was also present. Inhibition of P-gp function caused the MCF-7/Adr cells to be much more sensitive to the cytostatic effects of adriamycin with the IC_{50} being 15 times smaller.

Table 6.1 Inhibition of Growth of WT and MCF-7/Adr Cells

	GROWTH INHIBITION IC_{50} (μM)		
COMPOUND	WT	MCF-7/Adr	RATIO [a]
Staurosporine	0.0032 ± 0.0001	0.0065 ± 0.0005	2.03 *
UCN-01	0.018 ± 0.001	0.123 ± 0.031	7.03 *
RO 31-8220	0.897 ± 0.013	11.30 ± 2.10	12.60 **
CGP 41251	0.097 ± 0.012	0.283 ± 0.015	2.91 *
GFX 109203	7.30 ± 0.90	8.80 ± 0.30	1.21

Results are the means and standard deviations of at least 3 separate experiments.
[a] Ratio is MCF-7/Adr IC_{50} / WT IC_{50} * $P < 0.005$ ** $P < 0.001$

Table 6.2 Effect of Reserpine on Growth Inhibition in MCF-7/Adr Cells

	GROWTH INHIBITION IC_{50} (μM)		
COMPOUND	MCF-7/Adr	+ RESERPINE	RATIO [a]
Adriamycin	1.55 ± 0.05	0.104 ± 0.013	14.90
Staurosporine	0.0065 ± 0.0005	0.0100 ± 0.0009	0.65
UCN-01	0.123 ± 0.031	0.035 ± 0.006	3.51
RO 31-8220	11.30 ± 2.10	1.76 ± 0.16	6.42 *
CGP 41251	0.283 ± 0.015	0.307 ± 0.025	0.92
GFX 109203	8.80 ± 0.30	5.90 ± 0.75	1.49

Results are the mean and s.d. of at least 3 separate experiments.
[a] Ratio is MCF-7/Adr IC_{50}/MCF-7 / MCF-7/Adr IC_{50} + reserpine (5 μM).
*$P < 0.005$ ** $P < 0.001$

The presence of reserpine did not affect the growth inhibitory properties of staurosporine, CGP or GFX, the IC_{50} values determined were approximately the same as those obtained in the absence of reserpine. Resperine did not increase the cytostatic properties of these agents which suggests that they are not affected by P-gp activity. However, UCN and RO were rendered more potent when resperine was present, UCN by a factor of 3 and RO by a factor of 6 indicating that they are transported by P-gp. These data indicate that not all PKC inhibitors have potential as MDR modulators as some of them appear to be substrates for P-gp *per se* and subsequently pumped out of the cell. As a consequence of these results the PKC inhibitors, staurosporine, CGP and GFX, which are not pumped by P-gp, were used to examine how inhibition of PKC affects P-gp expression.

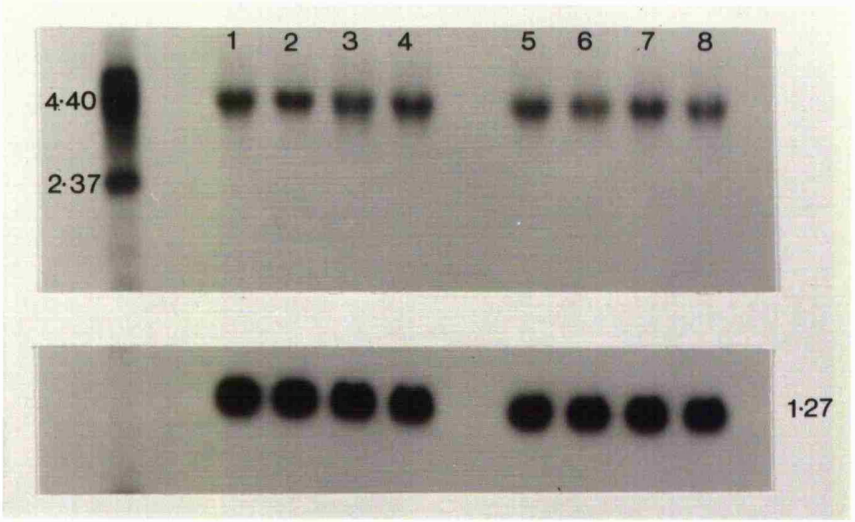
6.3. Effects of PKC Inhibitors on MDR1 Levels

PKC may be important for the transcription of *MDR1* as suggested in chapter 5. Thus the effect of PKC inhibition on *MDR1* levels was investigated. Cells were treated with staurosporine (10 nM), CGP (100 nM) and GFX (1 μM) for 24 and 48 h. Total RNA was isolated, as described in section 3.5.2., and *MDR1* levels determined by northern blot analysis (section 3.8.). Figure 6.1 shows a representative *MDR1* blot (top panel) with the relative GAPDH levels (bottom panel). Figure 6.2 shows the mean and standard deviations of the *MDR1* levels from three separate experiments. There was a significant 20 % reduction of *MDR1* expression after 24 h by staurosporine. There was no further decrease after 48 h. *MDR1* expression was not affected by treatment for 24 h with GFX or CGP. There was a slight decrease in *MDR1* after 48 h exposure, when levels fell to 91 and 89 % of control respectively.

6.4. Effects of PKC Inhibitors on P-Glycoprotein Expression

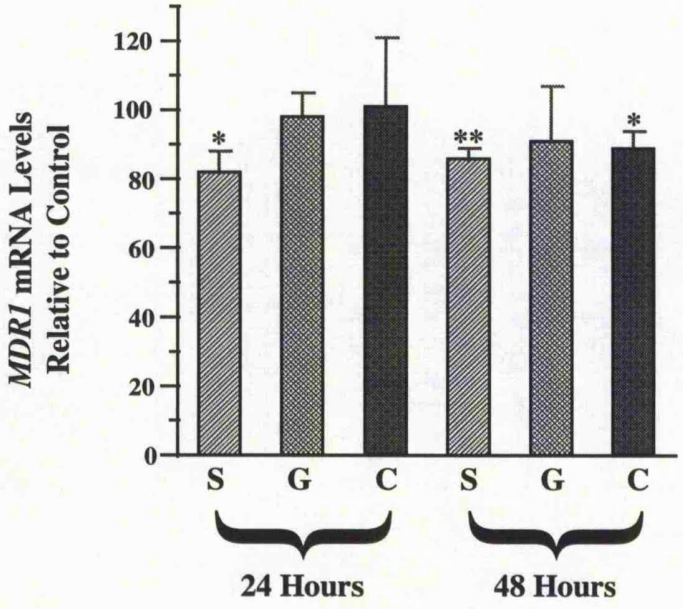
As *MDR1* mRNA levels were reduced by staurosporine, GFX and CGP P-gp protein levels were also determined in treated cells as described in section 3.3.2., to check for an analogous effect. The results from three separate experiments were quantified by laser densitometry and are shown in figure 6.3.

Figure 6.1 Effect of PKC Inhibitors on MDR1 mRNA Levels



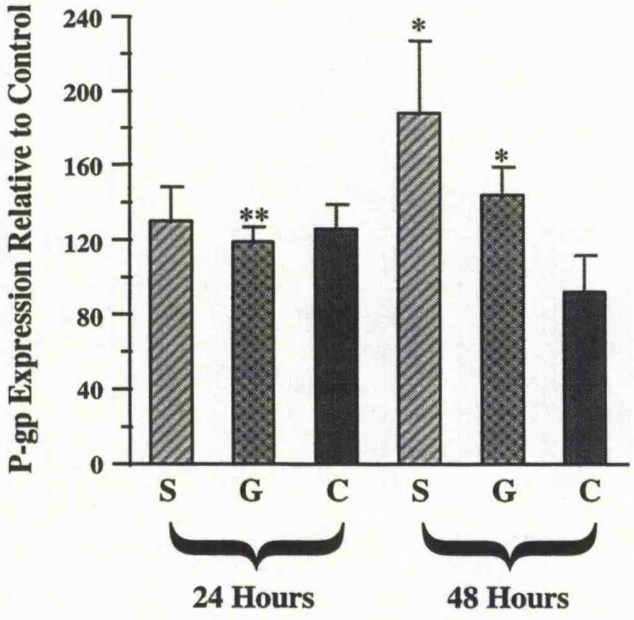
MDR1 mRNA levels were detected as described in section 3.8. and a representative autorad is shown in the top panel. Blot was also hybridised with GAPDH probe to check for equal gel loading and is shown in the bottom panel.

Figure 6.2 MDR1 mRNA Levels after Exposure to PKC Inhibitors



Cells were treated with 10 nM staurosporine (S), 1 μM GFX (G) and 100 nM CGP (C) for 24 and 48 h. Their effects on *MDR1* expression were quantified by laser densitometry as a percentage of control expression. The values shown are the mean and s.d. for 3 separate experiments. Control levels are set at 100 %.
Significance was determined by analysis of variance. * $P < 0.05$, ** $P < 0.005$

Figure 6.3 Effects of PKC Inhibitors on P-gp Expression



Cells were treated with 10 nM staurosporine (S), 1 μM GFX (G) and 100 nM CGP (C) for 24 and 48 h. Their effects on P-gp levels were quantified by laser densitometry as a percentage of control expression. The values shown are the mean and s.d. for 3 separate experiments. Control levels are set at 100 %.
Significance was determined by analysis of variance. * $P < 0.05$, ** $P < 0.005$

In contrast to the effects on *MDR1* mRNA all three compounds had increased P-gp protein levels after 24 h by around 30 %. However, after 48 h exposure P-gp protein levels in cells treated with CGP were the same as control, whilst staurosporine and GFX significantly increased detectable P-gp by 188 and 144 % respectively.
Thus staurosporine, GFX and to a lesser extent CGP either stabilised P-gp or caused an increase of *MDR1* translation as they did not increase *MDR1* mRNA levels.

6.5. Effects of PKC Inhibitors on PKC-ε

Staurosporine and GFX both cause the translocation of PKC-ε from the cytosol to the membrane in A549 cells at concentrations of 10 nM and 1 μM respectively. CGP does not affect the distribution of the isoenzyme (Courage *et al*, 1995). In MCF-7/Adr cells a proportion of PKC-ε is located in the membrane fraction (section 5.2). The effects of the three inhibitors on PKC-ε in MCF-7/Adr cells were investigated to determine whether the disparity seen with the 24 and 48 h treatments on P-gp levels were associated with their effects on PKC-ε.
Cells were treated for 24 and 48 h with staurosporine, GFX and CGP. Cells were fractionated, and PKC-ε detected and sub-cellular distribution quantified as described in section 3.3. The effects of the inhibitors on PKC-ε localisation are shown in table 6.3. After 24 h the distribution of PKC-ε in cells treated with CGP was unaltered. However, both staurosporine and GFX caused translocation from the cytosol to the membrane fraction such that the percentage of PKC-ε found in the membrane increased from 25 % (control) to 43 and 45 % respectively. After 48 h treatment staurosporine resulted in more translocation with 88 % of PKC-ε detectable now present in the membrane fraction. Likewise 48 h GFX exposure resulted in 57 % of total PKC-ε being located in the membrane. As at 24 h, CGP treated cells showed a similar distribution of isoenzyme to control cells, where 60 % of PKC-ε was detectable in the cytosolic fraction.

Table 6.3 Effect of PKC Inhibitors on Sub-Cellular Distribution of PKC-ε

COMPOUND	24 h C	24 h M	24 h N	48 h C	48 h M	48 h N
CONTROL	62 ± 13	25 ± 8	13 ± 6			
STAUROSPORINE	41	43	16	8	88	4
GFX 109203X	48	45	8	36	57	8
CGP 41251	60	29	12	60	36	5

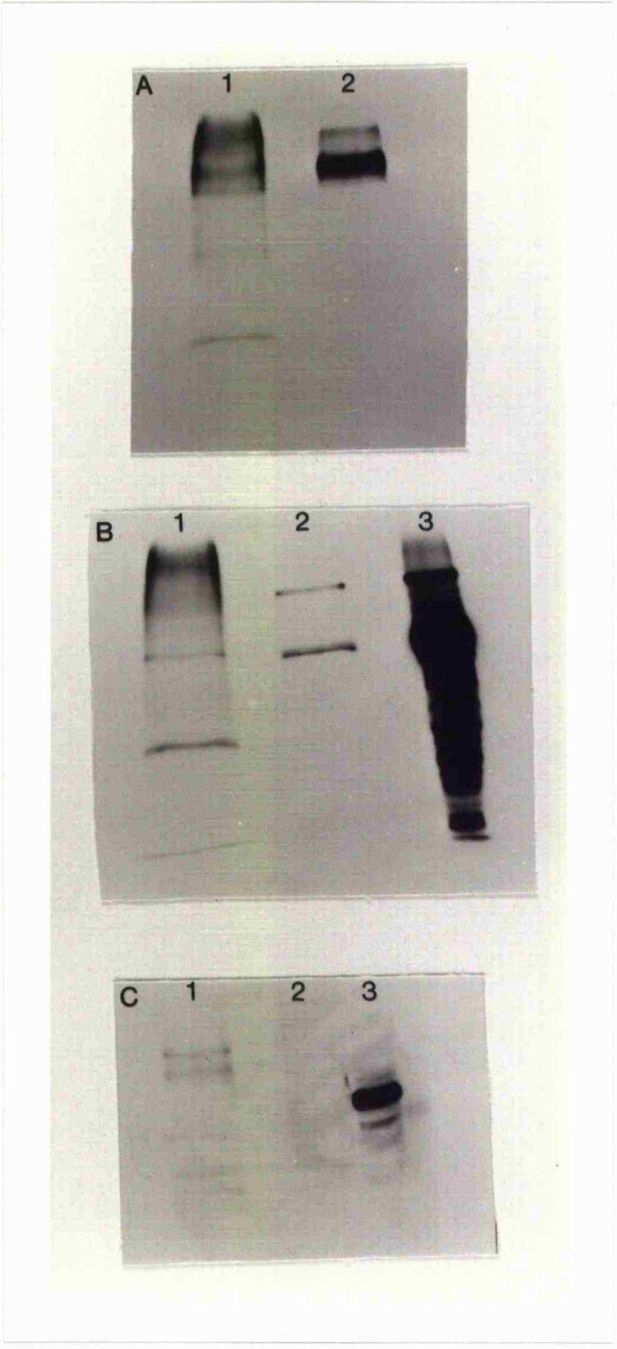
Cells were treated with 10 nM staurosporine, 1 μM GFX and 100 nM CGP for 24 and 48 h. Expression was determined by western blot analysis in cytosolic (C), membrane (M) and nuclear (N) fractions. Distribution is expressed as percentage of total cellular expression for each individual treatment. Values are the mean and s.d. of at least three experiments for control cells and the mean of two experiments for treated cells.

6.6. Immunoprecipitation of P-glycoprotein

The membrane localisation of PKC-ε suggests that it may be important for maintenance of expression or activity of P-gp in MCF-7/Adr cells. Ahmad *et al* (1994) demonstrated a co-localisation and affinity of PKC-α and P-gp using a baculovirus expression system. The aim of my study was to investigate whether the same phenomenon occurred with PKC-ε and P-gp in MCF-7/Adr cells. The membrane fraction of MCF-7/Adr cells was isolated, P-gp immunoprecipitated and analysed by western blotting (section 3.11.). The blot was probed with antibodies specific for P-gp and PKC-ε derived from a different animal species than the P-gp antibody used for the immunoprecipitation. This was to avoid any non-specific binding by the secondary antibody to any original antibody present in the immunoprecipitate. A representative blot is shown in figure 6.4A. The immunoprecipitated P-gp (lane 1) had a lower electrophoretic mobility than P-gp alone (lane 2) resulting in a larger diffuse band and suggesting a larger molecular weight. The immunoprecipitate contained a protein detectable by anti-PKC-ε antibody (lane 1, figure 6.4B) which was the same size as PKC-ε as shown by the rat brain control (lane 3). This band was also present in the control membrane sample run as a positive marker for P-gp (lane 2). This is not surprising as PKC-ε is detectable in membrane fraction of MCF-7/Adr cells. The band was specifically bound by PKC-ε antibody since it was not seen when the blot was probed with P-gp antibody. The immunoprecipitate was also probed with an antibody to PKC-α but a band of the correct size was not detected (figure 6.4C).

The results indicate that P-gp and PKC-ε co-immunoprecipitate suggesting that they are tightly associated within the membrane of MCF-7/Adr cells.

Figure 6.4 Immunoprecipitation of P-gp from MCF-7/Adr Cells



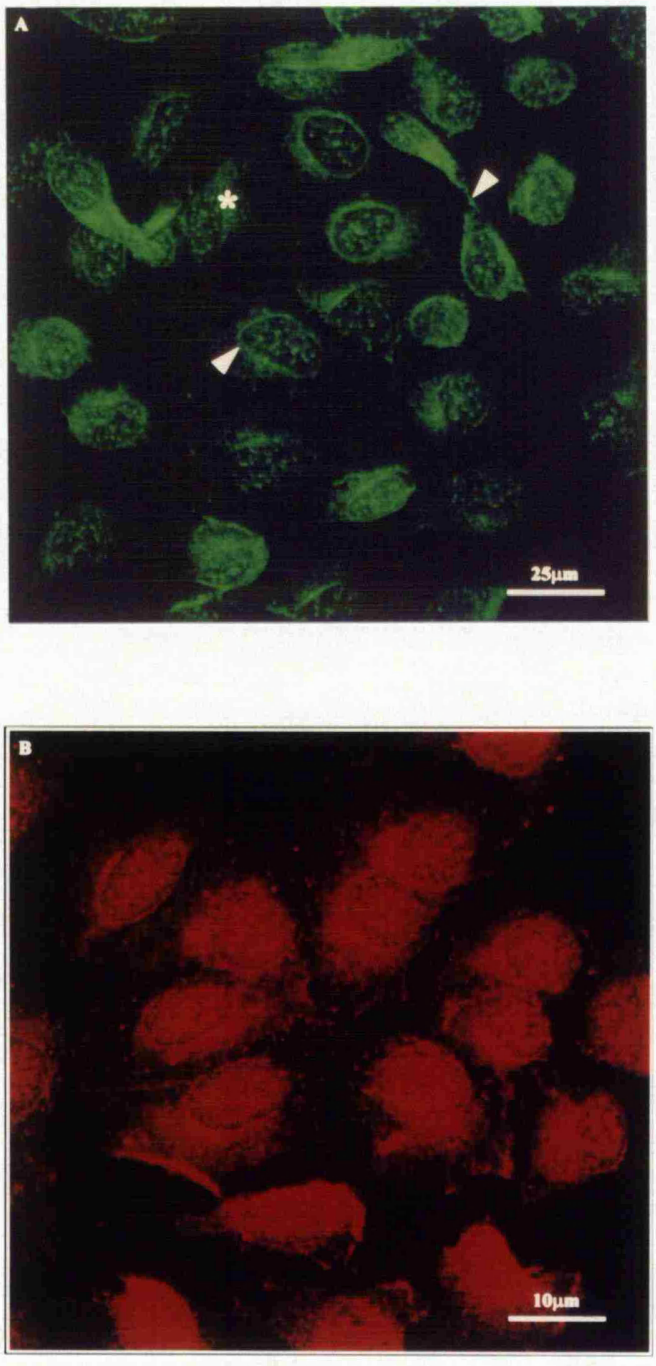
Immunoprecipitation was carried out as described 3.11. Proteins were then electrophoresed and western blotted. Lane 1: immunoprecipitate, lane 2: P-gp control, lane 3: PKC control. Blots were probed with antibodies specific for P-gp (A), PKC-ε (B) and PKC-α (C) . Positive controls were a membrane sample from MCF-7/Adr cells for P-gp and a rat brain sample for PKCs -ε and -α.

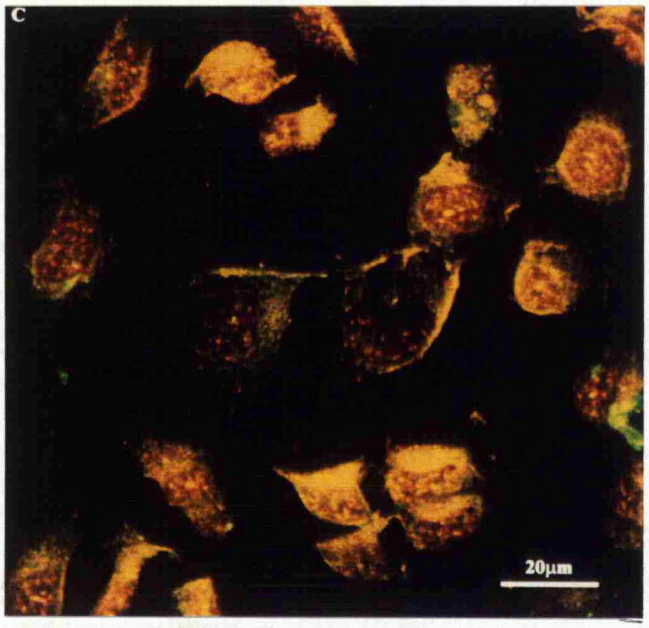
6.7. Indirect Immunofluorescence of P-glycoprotein and PKC-ε

To substantiate the results of the immunoprecipitation, indirect immunofluorescence using antibodies to P-gp and PKC-ε (section 3.12.) was performed to see whether the proteins were co-localised within the cell. Figure 6.5A shows the distribution of P-gp in MCF-7/Adr cells as green fluorescence. The protein was located primarily in the plasma and nuclear membranes, as indicated by the arrow heads, with some detectable in cytosol (indicated by a star). PKC-ε (red fluorescence) was detectable in all three fractions of the cell (figure 6.5B) as previously shown by western blotting (section 5.2.). The antibodies were then co-incubated and the results are shown in figure 6.5C. The red and green fluorescence indicate PKC-ε and P-gp respectively and yellow fluorescence occurs in areas where both antibodies have bound. There was an abundance of PKC-ε detectable in the nucleus. Co-localisation of both proteins occurred in plasma and nuclear membranes resulting in yellow fluorescence. No green fluorescence was observed on its own suggesting that where P-gp was located PKC-ε was also found. Figure 6.6 shows the cytofluorogram from the dual label experiment. Most of the fluorescence was yellow indicating both proteins were co-localised. There was some red fluorescence on its own which reflects the PKC-ε staining in the nuclei but very little green fluorescence on its own.

Thus in MCF-7/Adr cells P-gp and PKC-ε were co-localised consistent with the notion that PKC-ε is important for P-gp function.

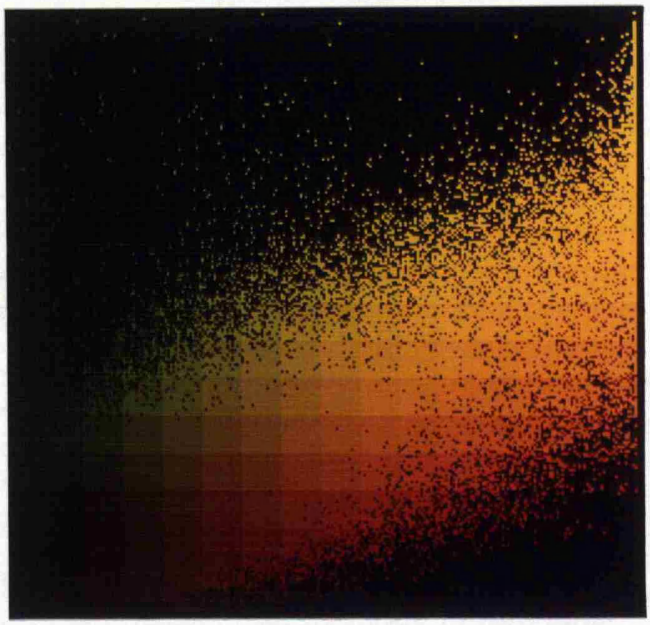
Figure 6.5 Indirect Immunofluorescence of P-gp and PKC-ε in MCF-7/Adr Cells





Indirect immunofluorescence was performed as described in section 3.12. Figures A and B show the localisation of P-gp and PKC-ε from single label experiments. Figure C shows the co-localisation of the two proteins from dual label experiment where red and green fluorescence indicate PKC-ε and P-gp respectively. Yellow fluorescence results from co-localisation of the proteins. Results are representative of 2 experiments.

Figure 6.6 Cytofluorogram from Indirect immunofluorescence (Dual Label)



The cytofluorogram shows the overall fluorescence from the view seen in figure 6.5C. The majority is yellow fluorescence indicating the co-localisation of the FITC and Texas Red linked secondary antibodies directed against PKC-ε and P-gp respectively.

6.8. Discussion

Staurosporine and its four analogues UCN, CGP, RO and GFX differ dramatically in their cytostatic potency against MCF-7/Adr cells. UCN and CGP were more potent growth inhibitors than the bisindolylmaleimides RO and GFX which reflects the sensitivity seen in WT cells (section 4.2.). Staurosporine was slightly less cytostatic in resistant cells when compared to WT cells (growth IC_{50} values of 6.5 and 3.2 nM respectively). Similarly CGP, (N-benzoyl staurosporine), retains the ability of staurosporine to overcome MDR. However UCN, (7-hydroxy staurosporine), was affected by cellular resistance with an IC_{50} value 7 times greater in MCF-7/Adr cells than in WT cells. The substituents on the indole nitrogens of the bisindolylmaleimides also determined whether the compounds were affected by cellular resistance mechanisms. MCF-7/Adr cells were as sensitive as WT cells towards GFX, but displayed markedly reduced sensitivity to RO. GFX bears a methyl group on one indole nitrogen and a N,N-dimethylaminopropyl moiety on the other, whereas RO carries a hydrogen on one and a N-propylisothiourea group on the other (see figure 1.4).

The resistance observed in MCF-7/Adr cells against the growth-inhibitory properties of RO and UCN is most likely mediated by P-gp. This inference can be drawn from the fact that sensitivity of the cells to the two drugs was significantly restored by inclusion of reserpine into cellular incubate. In the work of colleagues conducted in parallel to my project staurosporine and CGP have been shown to bind to P-gp (Budworth *et al*, 1996) but this work suggests that they are inefficiently transported. RO was a better substrate of P-gp than UCN and GFX had little affinity for, and was not transported by, P-gp. Another piece of evidence to suggest that the resistance to RO was caused by its transportation by P-gp has also been shown by colleagues. A549 cells made resistant to RO, by exposure to stepwise increases in concentration over 4 months, express P-gp, whereas WT A549 cells do not (unpublished data).

The PKC inhibitors used in this study share similar PKC-inhibitory potency (section 4.3) yet they differ dramatically in their abilities to reverse P-gp mediated drug resistance. This discrepancy suggests that inhibition of PKC activity by these compounds is not the major mechanism by which they modulate P-gp transport. There

are varying reports in the literature about the resistance reversal properties of PKC inhibitors. Most research indicates that the inhibitors have a direct effect by binding to P-gp rather than through the inhibition of PKC. This conclusion has been reached by different experimental approaches. Sato *et al*, (1990) showed that staurosporine inhibited the binding of vincristine to P-gp and Miyamoto *et al*, (1993) determined that the potency of staurosporine derivatives on drug accumulation was correlated with their inhibitory activity on drug efflux but was not correlated with their activity on protein kinases. CGP (150 nM) had an effect on rhodamine accumulation in a MDR lymphoblastoid cell line within 10 min and it was suggested that this rapid inhibition of efflux was due to a direct mechanism of action (Utz *et al*, 1994). However, Laredo *et al*, (1994) showed that staurosporine effects on rhodamine accumulation / efflux in resistant AML cells were caused by concentrations much lower than those required to displace the P-gp binding probe azidoprazosin (35 nM against >1 μM respectively). They concluded that staurosporine was acting through PKC inhibition rather than P-gp binding. Sachs *et al*, (1995) suggested that the reversal of resistance shown by the PKC inhibitor safingol in MCF-7/Adr cells was due to its kinase inhibitory effects rather then by a direct action on P-gp. Safingol inhibited vinblastine binding to P-gp when intact cells were used, but had no effect on isolated membranes. These discrepancies in the literature could be explained by the fact that different cell lines were used which would contain different amounts and isoenzymes of PKC and also varying levels of P-gp expression, all of which could affect the potency and mechanism of action of the inhibitors. However, my data indicates that the structure of an inhibitor determines whether it can bind and directly affect P-gp function or whether it acts through a signalling pathway.

The work in this study has shown that GFX was not affected by the resistance mechanisms operative in MCF-7/Adr cells and colleagues have shown that GFX did not affect rhodamine accumulation or efflux with concentrations up to 640 nM. Gekeler *et al*, (1995), have recently shown that GFX efficiently modulated MRP-associated MDR in HL60/AR and GLC4/ADR cell lines. The growth inhibitory properties of GFX were the same in wild type and resistant cells, consistent with my findings. They found that 8 μM GFX reversed the resistance of HL60/AR cell line to vincristine and at 10 μM there was strong inhibition of rhodamine efflux. The concentrations used in this study were higher than those investigated by colleagues

and it may be that if higher concentrations has been used with the MCF-7/Adr cells similar results may have been observed.

The MCF-7/Adr cells displayed a 2-3 fold resistance to all the agents, except GFX, even when P-gp function was inhibited by reserpine. Other cellular resistance mechanisms could be responsible for his phenomenon. Mechanisms other than P-gp associated MDR include alterations in the detoxification enzyme glutathione-S transferase and alterations in quantity and / or function of topoisomerase II (Hamilton *et al*, 1985; Deffie *et al*, 1989; Chan *et al*, 1993; see introduction).

Staurosporine significantly decreased *MDR1* mRNA levels by 20 % after 24 / 48 h treatment in MCF-7/Adr cells ($P = 0.002$). Sampson *et al*, (1993) have shown that exposure to staurosporine for 24 h resulted in a decrease of *MDR1* mRNA to 60 % of control in MDR KB-V1 cells. GFX and CGP had no effect on *MDR1* mRNA after 24 h but levels were reduced after 48 h by 10 %. Thus the PKC specific inhibitors affected *MDR1* mRNA levels but the non-specific kinase inhibitor, staurosporine, had a greater effect over a shorter time period. This would suggest that PKC may be involved in the regulation of *MDR1* but other kinases may also be important.

Although PKC inhibitors caused a reduction in the levels of *MDR1* mRNA there was no concomitant decrease in P-gp protein levels. In fact P-gp expression increased such that after 48 h in the presence of staurosporine levels increased almost 2 fold (188 % of control). These data are in keeping with that of others. Sampson *et al*, (1993) noted that after 8 days with 10 nM staurosporine there was a slight increase in P-gp expression in MDR KB-V1 cells. Chaudhary and Roninson, (1992), reported that an overnight incubation with 100 nM staurosporine caused an increase in P-gp expression in human leukaemia cell lines. They also reported that other P-gp binding compounds, cyclosporin A and verapamil, increased P-gp expression, and they suggested that these agents and staurosporine may act through a common mechanism. If this phenomenon was a consequence of direct binding to P-gp then CGP should have had a similar effect whilst GFX should not because CGP binds to P-gp but GFX does not. Both agents caused an increase in detectable P-gp after 24 h treatment but after 48 h P-gp expression had returned to control levels in cells treated with CGP whereas cells treated with GFX had greater expression (140 % of control). Thus it would seem that the P-gp stabilisation was not due to the interaction between agent and protein.

Previous work has shown that both staurosporine and GFX affect the localisation of PKC-ε in A549 cells but CGP does not (Courage *et al*, 1995). The effects of these agents on PKC-ε in MCF-7/Adr cells was investigated. CGP did not affect PKC-ε distribution. Both staurosporine and GFX caused an approximately 20 % increase in the amount of PKC-ε detectable in the membrane fraction after 24 h. Staurosporine had a greater effect after 48 h with nearly 90 % of PKC-ε localised in the membrane, whereas GFX resulted in nearly 60 % membrane associated PKC-ε. P-gp levels showed a similar pattern, in that, expression was greater after 48 h treatment with staurosporine than at 24 h and that staurosporine had more of an effect than GFX. P-gp levels after 24 h CGP treatment were increased but had returned to control levels by 48 h. It is intriguing that all three compounds have an affect on P-gp expression initially but only staurosporine and GFX can sustain the effect. It is possible that the translocation of PKC-ε by staurosporine and GFX to the membrane could mean that more PKC-ε is in the vicinity of P-gp resulting in a stable complex. Smith *et al*, (1995), has suggested that phosphorylation may play a role in the regulation of P-gp expression. PKC-ε could be responsible for P-gp phosphorylation in MCF-7/Adr cells. PKC-ε co-immunoprecipitated with P-gp suggesting that PKC-ε may interact with P-gp in the membrane. PKC-α was not detectable in the immunoprecipitate. Ahmad *et al*, (1994), showed that P-gp and PKC-α were tightly associated in a baculovirus expression system and co-immunoprecipitated with antibodies against either P-gp or PKC-α. Aftab *et al*, (1994), studied the immunoprecipitation of P-gp using MCF-7/Adr cells. They reported that additional phosphoproteins co-precipitated with P-gp in the immune complexes. These additional bands were not present in immunoprecipitates from WT cells. The phosphorylation of the additional proteins was increased in the presence of activated PKC. The authors concluded that the proteins were either degradation products or proteins associated with P-gp that may play a role in its function. It is possible that in MCF-7/Adr cells PKC-ε, which is redistributed to the membrane fraction, is important for P-gp function or stabilisation. The apparent stabilisation of P-gp caused by staurosporine and GFX could be a result of their effects on PKC-ε. They could be translocating even more PKC-ε to the vicinity of P-gp resulting in a more stable complex.

To get a better idea of the *in situ* localisation of P-gp and PKC-ε, MCF-7/Adr cells were used in indirect immunofluorescence studies. Initially the location of the 2 proteins were investigated individually. P-gp was located on plasma and nuclear membranes. Baldini *et al*, (1995), reported increased levels P-gp in nuclei of U-2OS/Dx and in LOVO-R-100 MDR cell lines and suggested that the exclusion of adriamycin from the nucleus may be a major mechanism of resistance in these cells. PKC-ε was found in all compartments of the cell which is consistent with the results from western blot analysis on fractionated cells. Preliminary experiments with the dual label suggested that P-gp and PKC-ε were co-localised on the plasma and nuclear membranes. The cytofluorogram showed significant co-localisation with very little P-gp detectable on its own. There are no reports in the literature of similar studies.

The evidence that PKC is important for P-gp function was discussed in the previous chapter. Fine *et al*, (1996) showed that calcium independent and dependent isoenzymes phosphorylated specific serine sites within the P-gp linker region differentially. Thus it is possible that in MCF-7/Adr cells it is PKC-ε that is important for modulation of P-gp function. Scala *et al*, (1995), demonstrated that P-gp functioned as normal even when PKC-α was down regulated with bryostatin in MCF-7/Adr cells. However, they also mentioned that PKC-ε was not totally down regulated. In MCF-7/Adr cells a proportion of PKC-ε is expressed in the membrane and it has been reported that PKC-ε present in the membrane of mouse keratinocytes could not be down regulated by either TPA or bryostatin (Szallasi *et al*, 1994) and PKC-ε in the membranes of HIRC-B cells could not be down regulated by TPA (Zhao *et al*, 1994). Thus bryostatin did not down regulate PKC-ε which may be the isoenzyme that is important for P-gp function in MCF-7/Adr cells. Mutational analysis of phosphorylation sites in P-gp have indicated that phosphorylation may not be important for function (Germann *et al*, 1996). Fine *et al*, (1996) have suggested that cryptic phosphorylation sites may exist that only appear when substrate is bound to P-gp. Topographic changes may occur that then allow kinases to phosphorylate residues. Thus the importance of PKC mediated phosphorylation of P-gp cannot be ruled out.

In summary PKC inhibitors affect *MDR1* and P-gp expression differently in MCF-7/Adr cells. *MDR1* mRNA levels decrease possibly due to the inhibition of the phosphorylation of transcription factors responsible for maintaining the transcription of

the *MDR1* gene. However, the inhibitors seem to cause some sort of stabilisation of P-gp. It could be that it is the inhibitors effect on PKC-α that results in the decrease in *MDR1* levels but their effect on PKC-ε that causes the P-gp stabilisation. This would explain why all three inhibitors affect *MDR1* levels but only staurosporine and GFX affect P-gp. CGP is less effective at translocating PKC-ε than staurosporine and GFX. The immunofluorescence and immunoprecipitation studies suggest that in MCF-7/Adr cells PKC-ε and P-gp are co-localised and intrinsically associated. Thus it appears that there is an ill defined but essential link between PKC, *MDR1* and P-gp.

CHAPTER 7

GENERAL DISCUSSION

The overall aim of this project was to explore the role of PKC in the growth of human breast carcinoma (MCF-7) cells and in the development and maintenance of the MDR phenotype in drug resistant breast carcinoma (MCF-7/Adr) cells. To this end a range of PKC inhibitors were used in both cell lines. Staurosporine and its four analogues UCN, CGP , RO and GFX were studied extensively. Two of these compounds, UCN and CGP, are currently in clinical trial as anti-cancer agents, and so it is important to identify their mechanism of action.

PKC is a pivotal enzyme in the signalling process from extracellular stimuli to the nucleus. The high degree of crosstalk that links many of the signalling cascades implies that many enzymes are involved in the transmission of messages. However, this also means that inhibition of a single enzyme can perhaps be bypassed by the cell indicating the existence of redundancy in signal transduction. Redundancy of certain PKC isoenzymes with respect to cell proliferation might explain why there was no significant correlation between the extent of growth inhibition and PKC inhibition. GFX is arguably the most specific PKC inhibitor used in this study. Whilst it was found to be a very potent PKC inhibitor it was much weaker as a growth inhibitor than staurosporine. This highlights the fact that inhibition of one signal transduction step, even if this comprises an enzyme family, does not affect the growth of the cell to the same degree as the inhibition of several steps.

Due to the structural similarities between the staurosporine analogues it can be expected that they act in the same or similar ways. However, this study has clearly shown that this is not the case. The staurosporine analogues were all more potent inhibitors of membrane-derived than cytosolic PKC, except for CGP whose ability to inhibit membrane PKC was significantly diminished. The major difference between the two enzyme preparations was that the membrane-derived PKC was already in its active conformation. Activation of PKC involves two steps, the movement to the membrane and its insertion into the bilayer resulting in the opening up of the enzyme such that it takes on its active conformation. The pseudosubstrate sequence is no longer able to interact with the active site thus allowing the binding of a substrate. CGP may cause its PKC inhibitory effect by somehow interfering with the conformational change of the enzyme or it may be that it interacts with the cytosolic enzyme prior to activation and unable to bind as efficiently to the active conformation of the enzyme.

In the MCF-7/Adr cells two of the staurosporine analogues, RO and UCN, appeared to be substrates of P-gp which explains their reduced cytostatic/cytotoxic properties in the resistant compared to the WT cells. This is an important finding considering that UCN is in clinical trial and likely to be used in patients who have already had chemotherapy, as an initial treatment, and their tumours may express the MDR phenotype as a consequence. UCN may not be a suitable anti-cancer agent against tumours that are derived from tissues that express P-gp and are intrinsically resistant. Of the other three agents staurosporine and CGP, but not GFX, appear to bind to P-gp but are not transported. This is consistent with most of the evidence in the literature according to which PKC inhibitors that modulate MDR seem to do so by binding directly to P-gp and not by inhibiting PKC. Due to this property CGP is a potent MDR reverser (Utz *et al*, 1994) and a good potential anti-cancer agent. It possesses cytostatic as well as MDR modulating properties and could be suitable for treatment of a wide range of cancers. The results of the clinical trials are awaited with much anticipation.

The results obtained suggest that the cytostatic potencies of the PKC inhibitors are not a direct consequence of their inhibition of PKC, and that their MDR modulating properties vary considerably and, again, are probably not a direct result of PKC inhibition. Subtle differences in structure can produce significant changes in the properties of an agent.

This study has confirmed that there might be a tight link between PKC and MDR. The time course study of the cells as they lost their resistant phenotype would suggest that, in MCF-7/Adr cells, PKC-α is involved in MDR from an early stage of the development of resistance. The isoenzyme expression is upregulated at the same time as *MDR1* gene amplification occurs. Either PKC-α is necessary for the amplification step or it is needed for the increased transcription of *MDR1* that occurs because of the amplification. From recent reports in the literature it would seem that PKC-α may be more important in the actual transcription of the *MDR1* gene than in the regulation of P-gp function. Most researchers have concentrated on PKC-α in the investigation of MDR and may have overlooked other isoenzymes of importance. I have shown that PKC-ε is co-localised with P-gp in the plasma and nuclear membranes of MCF-7/Adr cells and this isoenzyme may be involved in the regulation of P-gp. Staurosporine and

GFX can both interact with PKC-ε causing its translocation (Courage et al, 1995) but CGP cannot. In this study staurosporine and GFX caused stabilisation of P-gp, but CGP did not. This discrepancy could be a result of differential interactions with PKC-ε. Thus different PKC isoenzymes may have different roles involved with the maintenance of MDR within drug resistant cells.

PKC and MDR are linked and more extensive studies of the roles of the different PKC isoenzymes within resistant cells in patients may provide a better understanding of the mechanisms involved. Perhaps PKC inhibitors may be utilised as MDR modulators in cases where resistance has been clearly linked to PKC.

In conclusion this study has shown that PKC inhibitors are not equally useful as potential anti-cancer agents or as MDR modulators. The agents studied here are undoubtedly valuable tools to help unravel the many roles of PKC within a cell. If specific PKC isoenzyme inhibitors could be developed then this complex picture could possibly become clearer. Perhaps then PKC could be fully exploited as a therapeutic target. Furthermore UCN and/or CGP may show promise as therapeutic agents for reasons not directly related to PKC. It is also conceivable that an agent which possesses a unique multi-kinase inhibitory pattern may be a good antineoplastic agent.

CHAPTER 8

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CHAPTER 9

APPENDICES

9.1. Abbreviations

ABR	abnormal banding region
AMV-RT	avian myeloblastosis virus - reverse transcriptase
aPKC	atypical protein kinase C
ATP	adenosine 5'trisphosphate
bis	N,N'methylene bisacrylamide
bp	base pairs
BSA	bovine serum albumin
cAMP	adenosine 3'5'-cyclic monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	guanosine 3'5'-cyclic monophosphate
CGP	CGP 41251 (N-benzoyl staurosporine)
cPKC	conventional protein kinase C
CTP	cytosine 5'trisphosphate
DAG	diacylglycerol
DEPC	diethylpyrocarbonate
DM	double minute
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(b-aminoethylether) N,N,N',N'-tetraacetic acid
EtBr	ethidium bromide
FCS	foetal calf serum
g	acceleration due to gravity
GFX	GF 109203X
GITC	guanidine thiocyanate
h	hours

H-7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine
HSR	homogeneous staining regions
IC_{50}	50% inhibitory concentration
IP_3	inositol 1,4,5-trisphosphate
kb	kilobase pairs
kD	kilodaltons
LRP	lung resistance protein
mA	milliamps
MCF-7/Adr	adriamycin resistant MCF-7 cells
MDR	multidrug resistance
MEM	minimal essential medium
min	minutes
MMLV-RT	moloney murine leukaemia virus - reverse transcriptase
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
MRP	multidrug-resistance related protein
nPKC	novel protein kinase C
NPC	2,6-diamino-N-([1-(1-oxotridecyl)-2-piperidinyl]methyl)hexanamide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDBu	phorbol-12,13-dibutyrate
P-gp	p-glycoprotein
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSF	phenylmethylsulphonyl fluoride
PNK	polynucleotide kinase
PS	phosphatidylserine

PVP	polyvinylpyrrolidone
RACKS	receptors for activated C kinase
RNA	ribonucleic acid
RO	RO 31 8220
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
s.d.	standard deviation
SDS	sodium dodecylsulphate
SLS	sodium laurylsarcossinate
TBS	Tris buffered saline
TEMED	N,N,N',N'-tetramethylethylenediamine
Topo II	topoisomerase II
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TPK	tyrosine protein kinase
UCN	UCN-01 (7-hydroxystaurosporine)
V	volts
WT	wild type MCF-7 cells
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactosidase

9.2. Communications

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Differential inhibition of cytosolic and membrane-derived protein kinase C activity by staurosporine and other kinase inhibitors

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Abstract **The hypothesis was tested that 9 kinase inhibitors with diverse specificities for protein kinase C (PKC), including staurosporine and four of its analogues, interfere differently with PKC derived from either the cytosolic or particulate fractions of MCF-7 breast carcinoma cells. GF 109203X inhibited the enzyme identically in either preparation. CGP 41251 and calphostin C inhibited cytosolic PKC more effectively than membrane-derived PKC with ratios of IC_{50} (cytosolic PKC) over IC_{50} (membrane-derived PKC) of 0.07 and 0.04, respectively. The other six agents inhibited membrane-derived PKC more potently than cytosolic enzyme. Staurosporine and RO 31 8220 exhibited IC_{50} ratios of 12.3 and 21.6, respectively. The results suggest that there are dramatic differences between kinase inhibitors in their divergent effects on cytosolic and membrane-derived PKC which should be borne in mind in the interpretation of their pharmacological properties.**

Key words: Protein kinase C inhibition; Staurosporine analogue; MCF-7 breast carcinoma cell

1. Introduction

Protein kinase C (PKC) is a family of enzymes which are pivotal constituents of signalling pathways and thus regulate cellular growth, differentiation and a variety of processes involved with homeostasis [1]. In its inactive state a major proportion of PKC can be recovered as a soluble enzyme in most cell types. However the activated apoenzyme is thought to be positioned at the surface of intracellular membranes to permit efficient phosphorylation of membrane proteins [2]. In most cells PKC activition elicits the rapid redistribution of enzyme from the cytosol to the membrane and nucleus [3]. Inhibitors of PKC have been frequently used as probes to clarify the role which the enzyme plays in regulating cell function [4]. They are also under consideration as potential therapeutic agents in the treatment of diseases such as asthma, dermatological disorders, rheumatoid athritis, cancer and AIDS [5]. Many of the agents which possess PKC-inhibitory activity, like the indolocarbazole staurosporine, isolated from *Streptomyces staurosporeus* [6], inhibit a variety of kinases indiscriminately and thus lack selectivity for PKC. However during the last few years analogues of staurosporine have been synthesised which retain, at least in part, the high kinase-inhibitory potency of the parent molecule, but possess much better selectivity for PKC [7–10]. Two such PKC-specific analogues, UCN-01 and CGP 41251, have been shown to possess antineoplastic activity in human tumour models grown in rodents [9, 11]. In most investigations of the ability of compounds to inhibit PKC, cytosolic enzyme preparations have been used. In the light of the fact that it is predominantly PKC in the membrane which is of functional importance, we tested the hypothesis that kinase inhibitors display different potencies against membrane-derived as compared to cytosolic enzyme. To that end nine well-characterised PKC inhibitors, including staurosporine and four analogues, were investigated with respect to their ability to inhibit PKC prepared from the cytosol or membrane of human-derived MCF-7 breast cancer cells. The susceptibility of these cells towards the growth-arresting ability of PKC inhibitors has recently been described [12].

2. Materials and methods

2.1. Chemicals and reagents

Calphostin C, H-7 and GF 109203X were purchased from Calbiochem-Novabiochem Co. (Nottingham, UK). UCN-01, RO 31 8220, CGP 41251, NPC 15-437 and miltefosine were gifts from Kyowa Hakko Kogyo Co. (Tokyo, Japan), Roche Research Centre (Welwyn Garden City, UK), Ciba Geigy (Basel, Switzerland), Nova Pharmaceutical Co. (Baltimore, MD, USA) and Asta Pharma (Frankfurt, Germany), respectively. All other chemicals and reagents including staurosporine were obtained from Sigma Chemical Co. (Poole, UK). Cell culture medium, and serum were purchased from Gibco BRL (Paisley, UK). Stock solutions of drugs were prepared in DMSO, except in the case of NPC 15-437 which was dissolved in water, and stored at −20°C. Monoclonal antibodies against PKC-α and -ε were purchased from Tissue Culture Services Ltd. (Botolph Claydon, UK) and Affiniti Ltd. (Nottingham, UK), respectively, and a polyclonal antibody against PKC-ζ was obtained from Gibco BRL (Paisley, UK).

2.2. Cell growth and preparation of cellular fractions

MCF-7 breast carcinoma cells from the European Collection of Animal Cell Cultures (Salisbury, UK) were maintained routinely in an atmosphere of O_2:CO_2 (95:5) in minimum essential medium (Eagles modified) with fetal calf serum (10%), pyruvate (1 mM), L-glutamine (2 mM), non-essential amino acids, penicillin (100 iu/ml) and streptomycin (100 μg/ml). Cells were cultured in petri dishes (140 mm diameter) until they reached confluency. Cell cytosol was prepared according to Dale et al. [13]. The particulate (membrane) fraction was obtained from cells which had been exposed to phorbol dibutyrate (10 μM) for 30 min prior to fractionation to achieve redistribution of PKC to the membrane. Several methods of membrane preparation were evaluated. The procedure ultimately chosen was essentially that described by Greif et al. [14], except that the membrane pellet, once collected, was suspended by sonication and solubilised in buffer containing Brij 58 (0.1%), as described by Rush et al. [15]. The suspension was left on ice for 1 h and recentrifuged. The resultant supernatant was investigated. In comparison to the other protocols tested this method was optimal in that it furnished a preparation which (i) allowed endogenous inhibitors to be diluted out effectively, (ii) contained only low 'non-specific' radioactivity (10% of total), which means activity not associated with PKC-specific substrate, and (iii) retained PKC activity associated with the membrane. Membrane-derived PKC activity was between 185 and

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Abbreviations: IC_{50}, concentration which inhibited enzyme activity by 50%; PKC, protein kinase C; aPKC atypical PKC; cPKC, conventional PKC; nPKC, novel PKC;

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198% of that seen in cells which had not been exposed to phorbol dibutyrate.

2.3. PKC activity assay

Cytosolic and membrane preparations were diluted by a factor of between 5 and 20, depending on preparation, with H8 buffer (20 mM Tris-HCl, 2 mM of both EDTA and EGTA, 6 mM β-mercaptoethanol, 2 μg/ml of both leupeptin and aprotinin, at pH 7.5) until endogenous inhibitors, which would have masked effects of the drugs under investigation, were diluted out. Aliquots (25 μl) of the diluted cytosol or particulate fraction were placed in microplate wells and PKC activity was determined using a kit from Amersham International plc (Aylesbury, UK). PKC was activated with 12-*O*-tetradecanoylphorbol-13- acetate and the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a PKC-specific peptide was measured. Radioactivity was counted on a Packard Tricarb 1500 scintillation counter. Drugs were added to the incubate and degree of inhibition of PKC activity was expressed as percentage of drug-free control. Whereas cytosolic PKC did not phosphorylate the PKC-specific substrate significantly in the absence of TPA, $Ca^{2+}$and phospholipid, membrane-derived enzyme exhibited already 85% of maximal achievable activity in the absence of TPA, and 90% without added Ca^{2+} and phospholipid.

2.4. Western blot analysis

Immunoblot analysis was performed as described previously [16]. The amount of protein loaded per lane was 20 μg. Detection was by enhanced chemiluminescence generated by oxidation of luminol in the presence of hydrogen peroxide using an ECL kit from Amersham International.

3. Results

In order to compare the effect of kinase inhibitors on PKC activity associated with cellular cytosol or membrane, cytosol was obtained from naive cells and membrane fraction from cells in which PKC had been redistributed to the membrane by exposure to phorbol dibutyrate for 30 min. Immunoblot analysis showed that cytosolic PKC consisted mainly of PKC-ε and -ζ with little PKC-α, which afforded a faint band not detectable by densitometry or on a photograph. PKC-ζ was also found in the membrane of untreated cells (Fig. 1). On treatment with phorbol dibutyrate nPKC-ε underwent translocation to the particulate fraction, whereas the phorbol ester-insensitive aPKC-ζ did not (Fig. 1). Fig. 2 shows that there were remarkable differences between the PKC preparations in their susceptibility towards the inhibitory potential of the nine compounds tested. Staurosporine was the most and miltefosine the least potent PKC inhibitor. Only one compound, GF 109203X, furnished the same IC_{50} value irrespective of enzyme source (Table 1). UCN-01, H-7, NPC 15–437 and miltefosine were all stronger inhibitors of membrane-derived than of cytosolic PKC. For these agents differences in IC_{50} values amounted to between 2.4- to 5.5-fold (Table 1). The most dramatic discrepancies in inhibitory potency between cytosolic and membrane-derived PKC were seen with RO 31 8220, staurosporine, CGP 41251 and calphostin C. RO 31 8220 and staurosporine inhibited membrane-derived enzyme 22 and 12 times, respectively, more effectively than cytosolic PKC, as judged by differences in IC_{50} values. In contrast, CGP 41521 and calphostin C inhibited cytosolic PKC 14 and 28 times, respectively, better than membrane-derived enzyme.

4. Discussion

The nine kinase inhibitors tested in this study are widely used as probes for the involvement of PKC in cellular processes, and

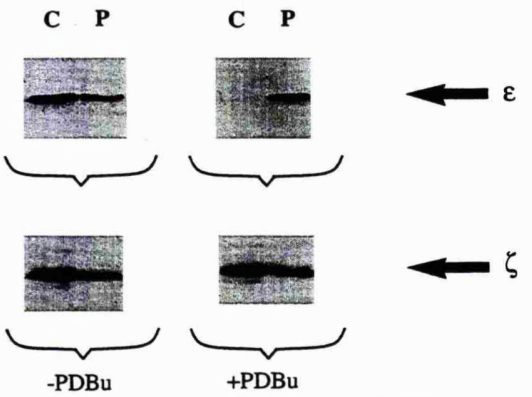

Fig. 1. Effect of exposure of MCF-7 cells to phorbol dibutyrate (PDBu) (10 μM) on localisation of PKC-ε and -ζ. Cytosolic (C) and particulate fractions (P) were prepared and analysed by Western blotting as described in section 2. Cells did not immunoreact with antibodies against PKC-β, -γ, -δ, or -θ, and cytosol reacted only faintly with a monoclonal anti-PKC-α antibody (results not shown).

two of them, UCN-01 and CGP 41251 are experimental antitumour agents [9,11]. The results presented above show, for the first time, that all but one differ markedly in their ability to modulate PKC activity depending on whether the enzyme was derived from the cytosol or the membrane subsequent to its redistribution there. PKC in the membrane is arguably more important as a functional kinase than cytosolic enzyme. The results presented above imply that potent kinase modulators can display as much as a twentyfold difference in inhibitory efficacy between cytosolic and membrane-derived PKC. This discrepancy may explain the frequently observed lack of correlation between pharmacological response and PKC inhibition. Particularly noteworthy is the increased inhibitory potency of RO 31 8220 and staurosporine against membrane-derived as compared to cytosolic enzyme, and the markedly decreased ability of CGP 41251 and calphostin C to inhibit membrane-derived in comparison to cytosolic PKC. Calphostin C does not only inhibit PKC, but also inactivates it irreversibly in a light-dependent fashion [17]. Gopalakrishna et al. showed that calphostin C exhibited higher potency against membrane-derived than cytosolic PKC in experiments in which cells were treated with the drug preceeding preparation of cellular subfractions and subsequent determination of enzyme activity [18]. This finding contrasts starkly with the result described above according to which calphostin C, when incubated with cellular subfractions rather than intact cells, inhibited cytosolic PKC much more effectively than membrane-derived enzyme. The disparity suggests that intact cells are able to provide cofactors and/or to catalyse calphostin C metabolism necessary for maximal inactivation of the enzyme, whereas isolated homogenate fractions lack this ability. Few investigations have addressed differences in susceptibility towards pharmacological inhibitors between cytosolic and membrane-derived PKC. The ether lipid 1-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphocholine, an experimental antitumour drug, was found to inhibit PKC when enzyme was derived from cellular cytosol, but paradoxically, to behave like a PKC activator in preparations in which the enzyme was obtained after its redistribution to the membrane [19].

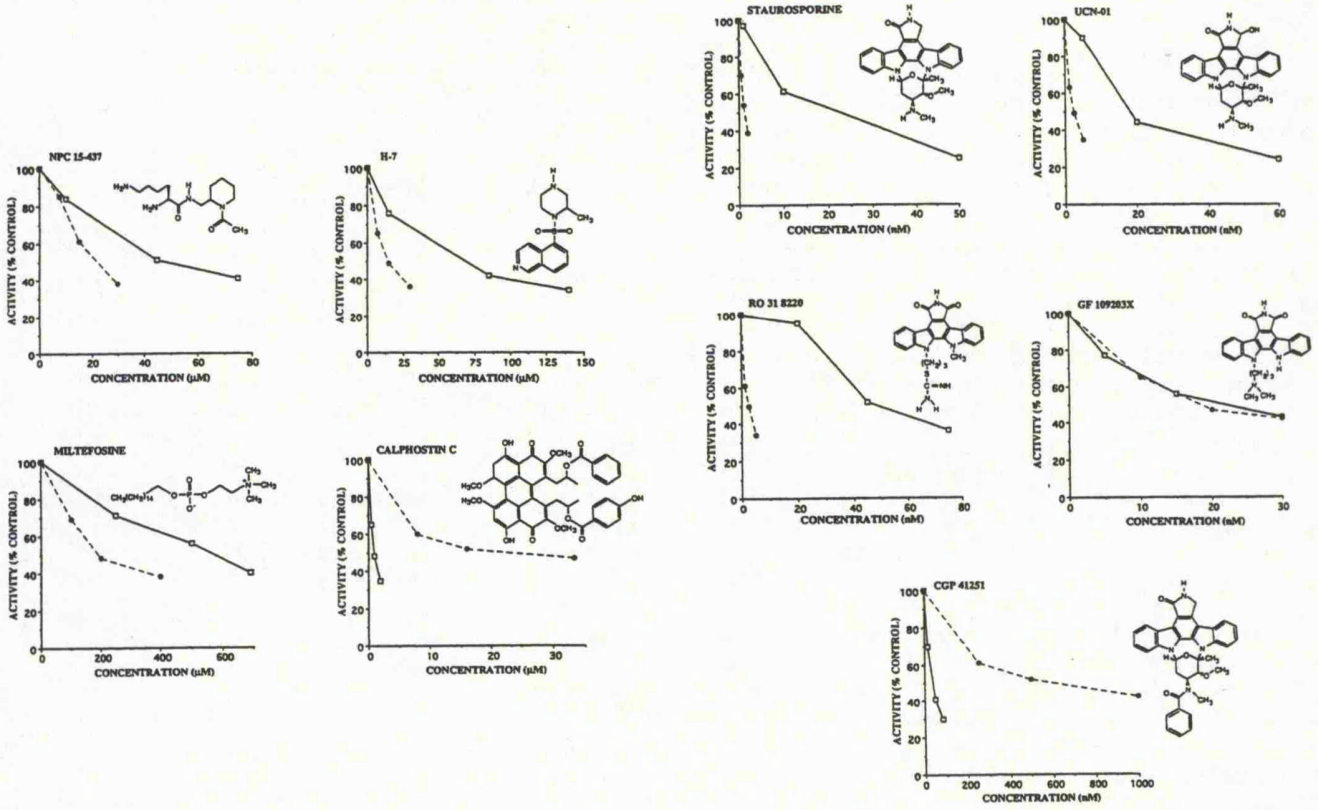

Fig. 2. Inhibition of cytosolic (open squares) and membrane-associated PKC (closed circles) from MCF-7 cells by staurosporine, UCN-01, RO 31 8220, GF 109203X, CGP 41251, NPC 15–437, H-7, miltefosine and calphostin C. In preliminary experiments a suitable concentration range was established for each compound and 3 appropriately spaced concentrations were chosen. The graphs show the result of one experiment representative of three.

In the light of this finding and the results described above for calphostin C, CGP 41251, staurosporine and RO 31 8220, it is conceivable that the selectivity of some kinase inhibitors for PKC, when it is derived from the membrane, over other kinases is very different from that based on values reported in the literature which were derived using cytosolic PKC.

Table 1
Inhibition of cytosolic and membrane PKC from MCF-7 cells by kinase inhibitors

Compound	IC_{50}[a] (μM)		
	Cytosolic PKC	Membrane PKC	Ratio[b]
Staurosporine	0.0163 ± 0.0045	0.0013 ± 0.0001	12.3
UCN-01	0.0178 ± 0.0020	0.0032 ± 0.0009	5.5
GF 109203X	0.021 ± 0.004	0.021 ± 0.004	1.0
CGP 41251	0.044 ± 0.006	0.61 ± 0.14	0.07
RO 31 8220	0.048 ± 0.006	0.0022 ± 0.0002	21.6
Calphostin C	1.09 ± 0.18	30.0 ± 6.2	0.04
NPC 15-437	43.3 ± 6.9	17.9 ± 2.8	2.4
H-7	54.3 ± 7.5	15.2 ± 0.6	3.5
Miltefosine	577 ± 29	184 ± 17	3.1

[a]Concentration at which enzyme activity is inhibited by 50%. Values are the mean ± S.D. of 3 experiments. PKC was measured using a PKC-specific peptide substrate as described in section 2.
[b]IC_{50} (cytosolic PKC)/IC_{50} (membrane-associated PKC).

Six of the agents studied here, H-7 [20], staurosporine [6] and its cogeners UCN-01 [7], RO 31 8220 [8], CGP 41251 [9] and GF 109203X [10], are thought to inhibit PKC at the catalytic site. Three of them, calphostin C [21], NPC 15–437 [22] and miltefosine [23] act at the regulatory domain of the enzyme. UCN-01, RO 31 8220, CGP 41251, GF 109203X, calphostin C and NPC 15–437 possess specificity for PKC, whereas H-7, staurosporine and miltefosine are non-selective kinase inhibitors. In the light of the results outlined above it is doubtful that the differences between the compounds are mechanistically linked to either PKC domain, at which the inhibitors interact with the enzyme, or degree of selectivity for PKC.

The MCF-7 cells used in this study express only PKC-ε and -ζ, apart form traces of PKC-α at the detection limit. Other PKC isoenzymes were not found in the cells [16]. On the assumption that there are no additional as yet undefined phorbol ester-responsive PKC isoenzymes in MCF-7 cells, the activity measured by the assay used in this study was due to PKC-ε in both the cytosolic and membrane preparations, in the latter after efficient redistribution by phorbol dibutyrate. In the absence of altered isoenzyme content, which difference between the two preparations might explain their differential sensitivity towards the kinase inhibitors? It is probably the fact that unlike cytosolic enzyme, membrane-derived PKC on isolation from phorbol ester-pretreated cells exists in a 'physiologically acti-

vated state'. It has been pointed out that membrane-derived PKC differs considerably in activation properties from cytosolic enzyme, even after the lipid environment has been similarly reconstituted in both [2]. Membrane-derived PKC seems to maintain its characteristic shape and properties in spite of the fact that its isolation procedure requires the use of detergent disrupting the intrinsic interactions between the protein and its lipid microenviroment.

It could be argued that the observations outlined above are applicable only to the particular cocktail and relative levels of PKC isoenzymes expressed in the MCF-7 cells used in this study. The results of a preliminary experiment conducted with human-derived A549 lung carcinoma cells render this possibility unlikely. These cells express predominantly PKC-α, together with PKC-ζ and -ε at lower levels [16]. The differences in potency of PKC inhibitors for cytosolic and membrane-derived PKC in these cells were very similar to those shown in Fig. 2 (results not shown).

Staurosporine at 10^{-9} M and its analogues UCN-01, RO 31 8220 and GF 109203X in the 10^{-7} to 10^{-6} M range cause the translocation of PKC-ε from the cytosol to the membrane and nucleus of A549 cells [12]. In contrast, CGP 41251 does not possess this property. It is intriguing that among the staurosporine analogues studied here CGP 41251 is the only one which inhibited membrane-derived PKC less potently than cytosolic enzyme. The two phenomena, ability to induce PKC-ε translocation on the one hand and high affinity for membrane PKC on the other, might be mechanistically related in this class of compound.

In summary, the results presented here show that kinase inhibitors can interfere with cytosolic and membrane-derived PKC to a markedly different degree. The high potency of staurosporine and RO 31 8220, and the weak efficacy of CGP 41251 and calphostin C against membrane-derived PKC should clearly be considered in the interpretation of their pharmacological effects.

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Comparison of ability of protein kinase C inhibitors to arrest cell growth and to alter cellular protein kinase C localisation

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Summary Inhibitors of protein kinase C (PKC) such as the staurosporine analogues UCN-01 and CGP 41251 possess antineoplastic properties, but the mechanism of their cytostatic action is not understood. We tested the hypothesis that the ability of these compounds to arrest growth is intrinsically linked with their propensity to inhibit PKC. Compounds with varying degrees of potency and specificity for PKC were investigated in A549 and MCF-7 carcinoma cells. When the log values of drug concentration which arrested cell growth by 50% (IC_{50}) were plotted against the logs of the IC_{50} values for inhibition of cytosolic PKC activity, two groups of compound could be distinguished. The group which comprised the more potent inhibitors of enzyme activity (calphostin C, staurosporine and its analogues UCN-01, RO 31-8220, CGP 41251) were the stronger growth inhibitors, whereas the weaker enzyme inhibitors (trimethylsphingosine, miltefosine, NPC-15437, H-7, H-7I) affected proliferation less potently. GF 109203X was exceptional in that it inhibited PKC with an IC_{50} in the 10^{-8} M range, yet was only weakly cytostatic. To substantiate the role of PKC in the growth inhibition caused by these agents, cells were depleted of PKC by incubation with bryostatin 1 (1 μM). The susceptibility of these enzyme-depleted cells towards growth arrest induced by staurosporine, RO 31-8220, UCN-01 or H-7 was studied. The drug concentrations which inhibited incorporation of [^{3}H]thymidine into PKC-depleted A549 cells by 50% were slightly, but not significantly, lower than those observed in control cells. These results suggest that PKC is unlikely to play a direct role in the arrest of the growth of A549 and MCF-7 cells mediated by these agents. Staurosporine is not only a strong inhibitor of PKC but also mimics activators of this enzyme in that it elicits the cellular redistribution of certain PKC isoenzymes. The ability of kinase inhibitors other than staurosporine to exert a similar effect was investigated. Calphostin C, H-7, H-7I, miltefosine, staurosporine, UCN-01, RO 31-8220, CGP 41251 or GF 109203X were incubated for 30 min with A549 cells in the absence or presence of the PKC activator 12-*O*-tetradecanoyl phorbol-13-acetate. The subcellular distribution of PKC-α-, -ε and -ζ was measured by Western blot analysis. None of the agents affected PKC-α or -ζ. UCN-01, RO 31-8220 and GF 109203X (0.1–1 μM) mimicked staurosporine by causing the cellular translocation of PKC-ε, whereas calphostin C, CGP 41251, H-7, H-7I or miltefosine did not alter the cellular localisation of this PKC isoenzyme. According to these results, translocation of certain PKC isoenzymes is not only a consequence of PKC activation but, in the case of selected staurosporine analogues, also of PKC inhibition.

Keywords: cytostasis; protein kinase C inhibitors; staurosporine analogues; adenocarcinoma cells

Protein kinases such as the serine- and threonine-specific enzyme family protein kinase C (PKC) are essential elements in the transduction of cellular signals elicted by growth factors and hormones, and they play a pivotal role in the regulation of cell differentiation and proliferation (Nishizuka, 1992). Not surprisingly therefore, they are prominent targets for the design of novel anti-cancer drugs. Pharmacological interference with PKC seems a logical therapeutic strategy because in certain experimental neoplasias altered PKC function or expression have been linked to malignant transformation (Cacace *et al.*, 1993; Mischak *et al.*, 1993). Modulators of PKC activity are currently under clinical evaluation, and the results of two phase I trials of the PKC activator bryostatin 1 have recently been published (Phillip *et al.*, 1993; Prendiville *et al.*, 1993). The dose-limiting toxicity of bryostatin 1 was myalgia, and in one of the trials the drug exhibited activity against malignant melanoma (Phillip *et al.*, 1993). Numerous inhibitors of PKC have been described, and most of them possess cytostatic and cytotoxic properties (Tamaoki and Nakano, 1990). They may also be useful in therapy as synergistic enhancers of the antitumour activity of conventional cytotoxic anti-cancer drugs (Posada *et al.*, 1989; Sato *et al.*, 1990; Utz *et al.*, 1994) and as inhibitors of tumour cell invasion (Schwartz *et al.*, 1993). Most of these molecules are only modestly selective inhibitors of PKC and affect a variety of kinases. For example, the microbial product staurosporine is a potent, but non-selective, kinase inhibitor. Two analogues with higher specificity for PKC, UCN-01 and CGP 41251, have shown significant antitumour activity *in vivo* (Meyer *et al.*, 1989; Akinaga *et al.*, 1991). Both of these drugs are in preparation for phase 1 clinical evaluation as anti-cancer drugs, the former in Japan and the United States and the latter in Europe. In the work described here the hypothesis was tested that inhibition of PKC is related to inhibition of cell growth. Several PKC inhibitors of diverse chemical structure (Figure 1) were assessed for their ability to interfere with PKC activity and proliferation in two human-derived carcinoma cell lines. Furthermore, their effect on the growth of cells which had lost PKC activity via enzyme down-regulation was studied.

Paradoxically staurosporine is not only an effective inhibitor of PKC but it also shares certain properties with activators of this enzyme (Dlugosz and Yuspa, 1991). In most cell types PKC is contained predominantly in the cytosol and its activation engenders alterations in the subcellular localisation of the enzyme. Activator-induced translocation of PKC is thought to position the enzyme in close proximity to its physiologically important substrates. For example, in human A549 lung and MCF-7 breast carcinoma cells the tumour promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and the experimental anti-tumour agent bryostatin 1 provoke the rapid redistribution of PKC from the cytosol to the particulate and nuclear fractions (Issandou *et al.*, 1988; Dale *et al.*, 1989). Intriguingly, staurosporine can also cause PKC translocation. In GH_4C_1 rat pituitary cells it has been shown to elicit the redistribution of the PKC isoenzymes ∂ and ε from the cytosol to the membrane (Kiley *et al.*, 1992). Furthermore, staurosporine has been found to augment the TPA-induced translocation of PKC-α to the membrane

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(Bradshaw *et al.*, 1992). In view of these findings we wished to elucidate whether the effect on the subcellular localisation of PKC is a general feature of PKC inhibitors or a specific property of staurosporine. To answer this question the effect of a series of diverse kinase inhibitors on the localisation of the PKC isoenzymes contained in A549 cells was investigated. The agents tested include H-7, its positional isomer H-7I and the staurosporine analogues UCN-01, CGP 41251, RO 31-8220 and GF 109203X, which are thought to inhibit PKC at the catalytic domain, and the regulatory domain inhibitors calphostin C and miltefosine. The overall aim of the study was to increase the understanding of the way in which different PKC inhibitors interact with tumour cells and to explore the role which this class of agent may play in cancer therapy.

Materials and methods

Drugs and reagents

RO 31-8220, CGP 41251, NPC 15437, trimethylsphingosine, UCN-01, miltefosine and bryostatin 1 were provided by Roche Research Centre (Welwyn Garden City, UK), Ciba Geigy (Basle, Switzerland), Nova Pharmaceutical (Baltimore, MD, USA), Biomembranes Institute (Seattle, WA, USA), Kyowa Hakko Kogyo (Tokyo, Japan), Asta Pharma (Frankfurt, Germany) and Cancer Research Institute, Arizona State University (Tempe, AR, USA) respectively. Calphostin C, H-7 and GF 109203X were acquired from Calbiochem-Novabiochem (Nottingham, UK). Other chemicals and reagents including staurosporine, H-7I and TPA were purchased from Sigma (Poole, UK) and ^{3}H-labelled thymidine ([^{3}H]Tdr) from Amersham International (Amersham, UK). Stock solutions of NPC 15437, trimethylsphingosine and H-7 were prepared in water, those of other kinase inhibitors, TPA and bryostatin in dimethyl sulphoxide (DMSO), and stored at -20°C. The final concentration of DMSO in the medium did not exceed 0.3%, which on its own did not affect cell growth. A monoclonal antibody against PKC-α was purchased from Tissue Culture Services Biologicals (Botolph Claydon, UK). The polyclonal antibody against PKC-ζ and its antigenic peptide was acquired from Gibco BRL (Paisley, UK) and a polyclonal antibody against PKC-ε was provided by Dr P Parker (ICRF Laboratories, London, UK).

Cell growth

Human A549 lung and MCF-7 breast carcinoma cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Cells (passage number 10–30) were cultured in an atmosphere of 5% carbon dioxide, the former in Ham's F-12 medium with penicillin/streptomycin, the latter in minimum essential medium (Eagle's modification) with additional pyruvate (1 mM) and non-essential amino acids. Both media were supplemented with 10% FCS (Imperial Laboratories Europe, Andover, UK, and Gibco) and glutamine (2 mM). Cells were subcultured routinely twice weekly to maintain logarithmic growth. For cell proliferation studies cells were seeded at a density of 1.3×10^4 cm^{-2} and incubated with 3 ml of medium including agents, which was replenished at intervals of 48 h (A549) or 72 h (MCF-7). Following incubation for 4 days (A549) or 6 days (MCF-7) with drugs, cell number was assessed using a Coulter Counter Model ZM. In order to achieve PKC depletion, cells seeded at $1-2 \times 10^5$ per well (3.5 cm diameter) were incubated for 24 h with bryostatin 1 (1 μM). Under these conditions growth inhibition caused by bryostatin 1 is negligible (Dale and Gescher, 1989; Stanwell *et al.*, 1994). Bryostatin was removed by extensive washing of the cells followed by a 2 h recovery period. In previous work using the A549 cell line this washing procedure has been shown to eliminate bryostatin-mediated effects. The cells were then incubated for a further 24 h with staurosporine, RO 31-8220, UCN-01 or H-7. In some experiments cells were incubated with inhibitor for 48 rather than 24 h, in this case bryostation was not removed and left in the incubate. After removal of agents inhibition of DNA synthesis was evaluated by measurement of [^{3}H]Tdr incorporation into cells as described previously (Dale and Gescher, 1989). Radioactivity was counted using a Packard 1500 Tricarb scintillation counter.

Measurement of PKC activity

Cytosol was prepared as described previously (Dale *et al.*, 1989), and aliquots were placed with kinase inhibitor at appropriate concentrations in microplate wells. PKC activity was measured via the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a PKC-specific peptide using a kit from Amersham International. The value obtained with this kit is the sum of activities of phospholipid-dependent, Ca^{2+}-dependent and -independent PKC isoenzymes which are activated by phorbol esters.

Preparation of cellular fractions and Western blot analysis

Cytosolic, particulate (which contains membranes, cytoskeleton and cell organelles) and nuclear fractions of cells were obtained essentially as described before (Greif *et al.*, 1992) with some modifications (Stanwell *et al.*, 1994). Protein content of the fractions was measured by the Bradford assay (Bradford, 1976). Western blot analysis was performed as described previously (Stanwell *et al.*, 1994). The amount of protein loaded per lane was 20 μg for detection of cPKC-α and 30 μg for nPKC-ε. Detection was by enhanced chemiluminescence generated by oxidation of luminol in the presence of hydrogen peroxide using an ECL kit from Amersham International. Immunoreactivity was quantitated using Molecular Dynamics Computing Densitometer, and the values shown in Figures 4 and 5 are expressed as a percentage of the sum of immunoreactive protein in all three fractions. The significance of differences in band intensity was evaluated by Student's *t*-test.

Results

Relationship between inhibition of PKC and inhibition of cell growth

Nine PKC inhibitors, staurosporine (Tamaoki *et al.*, 1986), UCN-01 (Takahashi *et al.*, 1987), RO 31-8220 (Davis *et al.*, 1992), CGP 41251 (Meyer *et al.*, 1989), GF 109203X (Toullec *et al.*, 1991), calphostin C (Kobayashi *et al.*, 1989), miltefosine (Überall *et al.*, 1991), NPC-15437 (Sullivan *et al.*, 1991) and H-7 (Hidaka *et al.*, 1984) (for structures see Figure 1), were investigated for their ability to inhibit cell growth and PKC activity isolated from the cytosol of two carcinoma cell lines. In the case of the A549 cells, two further agents, trimethylsphingosine (Endo *et al.*, 1991) and H-7I, a positional isomer of H-7, were included. Of these agents staurosporine and calphostin C inhibited growth most effectively in both cell types (Table I). Staurosporine and its analogues were the most potent inhibitors of PKC activity. H-7I was the least effective inhibitor of cell growth and PKC activity in A549 cells. When the logs of the IC_{50} values obtained for PKC inhibition were plotted against the logs of the IC_{50} values for growth arrest (Figure 2) two groups of compound could be distinguished. The group which comprised the more potent inhibitors of PKC activity, i.e. staurosporine, UCN-01, RO 31-8220, CGP 41251 and calphostin C, were also the stronger growth inhibitors with IC_{50} values of <1 μM. Of the stronger PKC inhibitors used here, GF 109203X behaved exceptionally, in that it interfered with cell growth only weakly, with an IC_{50} of >7 μM. Trimethylsphingosine, miltefosine, NPC-15437, H-7 and H-7I displayed IC_{50} values for PKC inhibition in the 10–100 μM range. They arrested growth at concentrations of >1 μM. When a line of best fit was computed from the logarithmic plot it was characterised by correlation coefficients of $r = 0.64$ for A549 and $r = 0.59$

for MCF-7 cells. These results suggest that the ability of these agents to arrest cell growth is not reflected by their capability to inhibit PKC. Nevertheless, this conclusion has to be interpreted with caution in view of differences in design between the two experiments, in that the effect of the compounds on proliferation was assessed in intact cells, whereas enzyme inhibition was measured in cytosolic extracts *in vitro*.

Growth arrest in PKC-depleted cells

To define more precisely the role which PKC might play in growth arrest caused by these agents, their effect on cells with down-regulated PKC was investigated. The hypothesis tested in this experiment was that, if inhibition of PKC mediates growth arrest, then PKC-depleted cells should be less susceptible towards the cytostatic potential of these agents than PKC-proficient naive cells. Cells were exposed for 24 h to bryostatin 1 at 1 μM, which caused the complete down-regulation of conventional (c) PKC-α and novel (n) PKC-ε as assessed by Western blot analysis (Stanwell *et al.*, 1994). Subsequently cells were washed and incubated with staurosporine, RO 31-8220, UCN-01 or H-7 for 24 h. Their proliferative competence was adjudged by measurement of incorporation of [^{3}H]Tdr into cells. PKC remained undetectable by immunoblotting for up to 30 h following the removal of bryostatin. Figure 3 shows that treatment of A549 cells with bryostatin did not decrease the degree to which DNA synthesis was inhibited by either the general kinase inhibitor staurosporine or the PKC-specific agent RO 31-8220. In fact, the bryostatin-treated cells were consistently, albeit weakly, more sensitive towards the effect of the kinase inhibitors than naive cells. The IC_{50} values obtained for staurosporine, RO 31-8220, UCN-01 and H-7 in PKC-depleted cells were 0.5 nM, 1.6 μM, 47 nM and 20 μM respectively. The corresponding values determined in these experiments in control cells were 1.2 nM, 1.9 μM, 68 nM and 32 μM respectively. The IC_{50} values determined by this method for the staurosporine analogues are about twice the IC_{50} values provided by cell counting (see Table I). This discrepancy is probably due to differences in procedures between the two experiments, particularly with regard to exposure time and seeding density. Cells responded similarly to GF 109203X, which, like RO 31-8220, is a PKC-specific bisindolylmaleimide analogue of staurosporine (Toullec *et al.*, 1991). Likewise, in parallel experiments PKC-depleted MCF-7 cells did not markedly differ from control MCF-7 cells in sensitivity towards kinase inhibitors (results not shown). In order to rule out the possibility that the incubation time was insufficient for the kinase inhibitors to exert maximal growth-inhibitory potency, the experiment was repeated and the cells were exposed to staurosporine or RO 31-8220 for 48 h rather than 24 h with bryostatin present in the incubate for the duration of the entire experiment. The IC_{50} values determined in these experiments are similar to those described above. Also, under these conditions the sensitivity of the PKC-depleted cells towards the growth-arresting potential of the kinase inhibitors was slightly increased compared with that of their PKC-proficient counterparts (results not shown), analogous to the observation made in the 24 h exposure study.

Effect of PKC inhibitors on PKC isoenzyme localisation

Cells were incubated for 30 min with staurosporine (1 nM to 1 μM), UCN-01, RO 31-8220, CGP 41251, GF 109203X (all 0.1 and 1 μM), calphostin C (0.5μM), miltefosine (200μM),

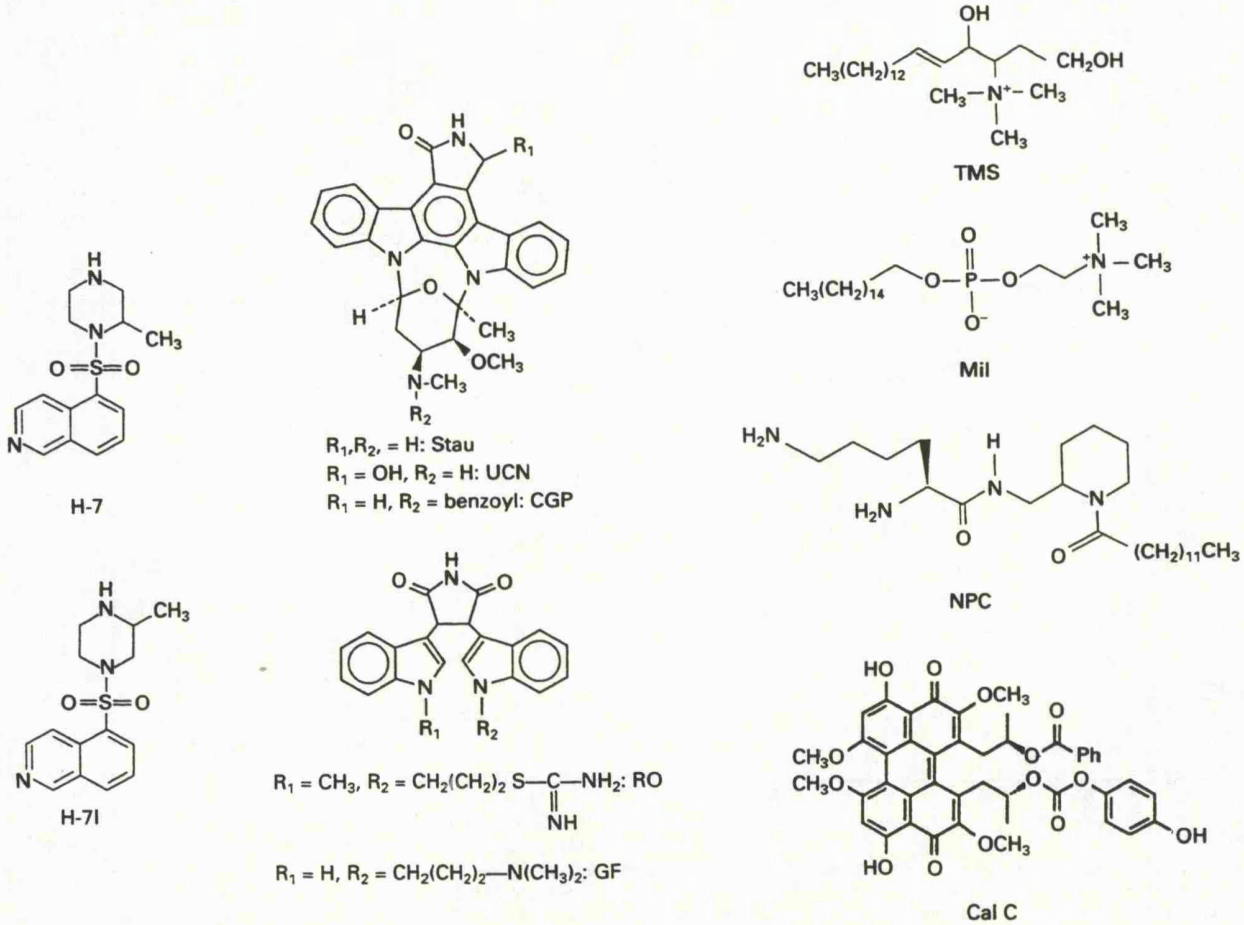

Figure 1 Structures of the compounds used in this study. Abbreviations are: Cal C, calphostin C; CGP, CGP 41251; GF 109203X; Mil, miltefosine; NPC, NPC 15437; RO, RO 31-8220; Stau, staurosporine; TMS, trimethylsphingosine; UCN, UCN-01.

H-7 or H7-I (both 0.5 mM) in the presence or absence of TPA (25 nM). Cellular fractions were prepared, and levels of PKC isoenzymes were determined by Western blot analysis using a monoclonal anti-PKC-α antibody and polyclonal antibodies against PKCs-ε and -ζ. Our previous studies have shown that these three PKC isoenzymes are abundantly expressed in A549 cells (Stanwell *et al.*, 1994).

Consistent with its ability to attenuate the TPA-mediated change in distribution of phorbol ester binding sites (Bradshaw *et al.*, 1992), staurosporine increased TPA-induced translocation of cPKC-α. Although the effect was not significant, as deduced from the densitometry readings, a definite trend was observed ($P = 0.08$) (Figure 4). One other agent, UCN-01 at 1 μM, behaved similarly. However, results with UCN-01 were inconsistent, as it augmented TPA in only three out of six blots. The staurosporine analogues CGP 41251, RO 31-8220 and GF 109203X, like calphostin C, miltefosine, H-7 and H-7I, failed to affect TPA-induced cPKC-α redistribution. None of the inhibitors changed TPA-induced relocalisation of nPKC-ε. However, when staurosporine, UCN-01, RO 31-8220 and GF 109203X were incubated with cells on their own, they caused the redistribution of nPKC-ε from the cytosol to the particulate and nuclear fractions (Figure 5). Staurosporine was the most potent

Table I Inhibition of PKC and growth of A549 and MCF-7 cells by kinase inhibitors

Compound	$IC_{50\ PKC}$[a] (μM) A549	$IC_{50\ PKC}$[a] (μM) MCF-7	$IC_{50\ Growth}$[b] (μM) A549	$IC_{50\ Growth}$[b] (μM) MCF-7
Ro 31-8220	0.023±0.004	0.048±0.0058	0.780±0.04	0.897±0.013
GF 109203X	0.026±0.004	0.021±0.004	7.6±0.5	7.3±0.9
UCN-01	0.033±0.007	0.0178±0.002	0.034±0.002	0.0175±0.001
Staurosporine	0.066±0.003	0.0163±0.0045	0.00065±0.00005	0.0032±0.0001
CGP 41251	0.079±0.007	0.044±0.006	0.082±0.01	0.097±0.012
Calphostin C	0.487±0.062	1.09±0.18	0.001±0.00013	0.00092±0.00011
TMS	38.00±2.00	ND	3.10±0.20	ND
NPC 15-437	39.00±5.00	43.30±6.90	4.30±0.10	3.70±0.10
H-7	55.00±5.00	54.30±7.50	29.00±2.00	21.30±1.50
Miltefosine	57.00±16.00	577.00±29.00	24.00±1.00	44.00±0.50
H-7I	99.00±0.30	ND	262.00±26.00	ND

[a]$IC_{50\ PKC}$, 50% inhibitory concentration for PKC activity. Cytosolic PKC was measured using a PKC-specific peptide substrate from Amersham International. [b]$IC_{50\ Growth}$, 50% inhibitory concentration for growth. Cells were counted following exposure to the drugs for 4 (A549) or 6 days (MCF-7). ND, not determined.

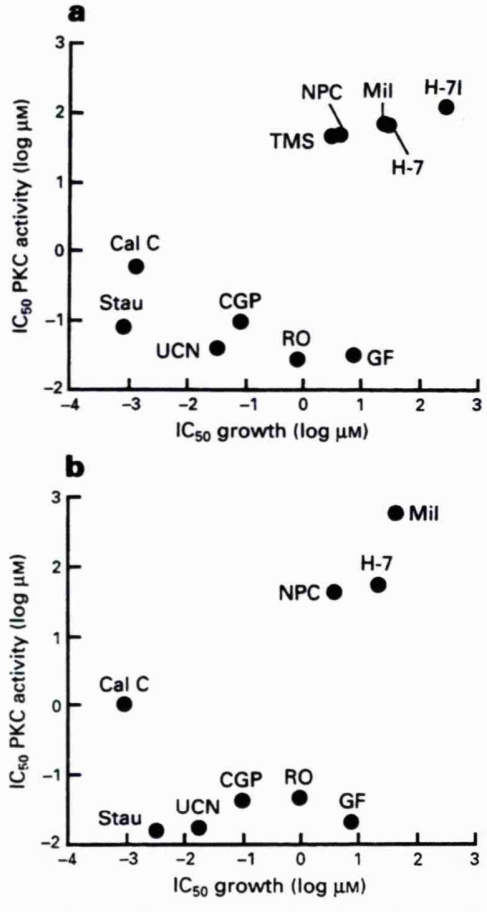

Figure 2 Relationship between the ability of compounds to arrest cell growth and to inhibit cytosolic PKC activity in A549 (**a**) and MCF-7 cells (**b**). Points are the mean of three experiments, each conducted in duplicate. For details of growth conditions and PKC assay see the Materials and methods section. Abbreviations are as explained in the legend to Figure 1.

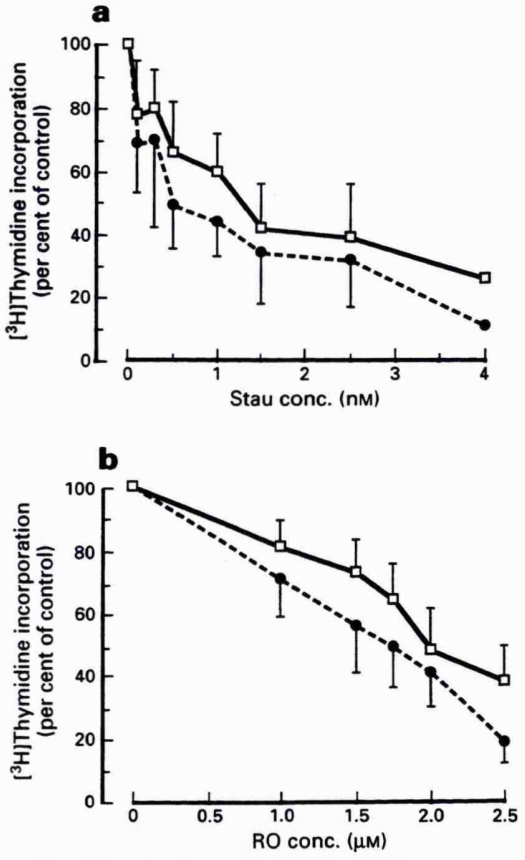

Figure 3 Effect of staurosporine (**a**) and RO 31-8220 (**b**) on incorporation of [^{3}H]Tdr into naive A549 cells (□) and PKC-depleted A549 cells (●). cPKC-α and nPKC-ε were completely down-regulated by incubation with bryostatin 1 (1 μM) for 24 h. Subsequently cells were exposed to kinase inhibitors for a further 24 h period. Results are the mean ± s.d. of three experiments, each conducted in duplicate. For details of the assay see the Materials and methods section.

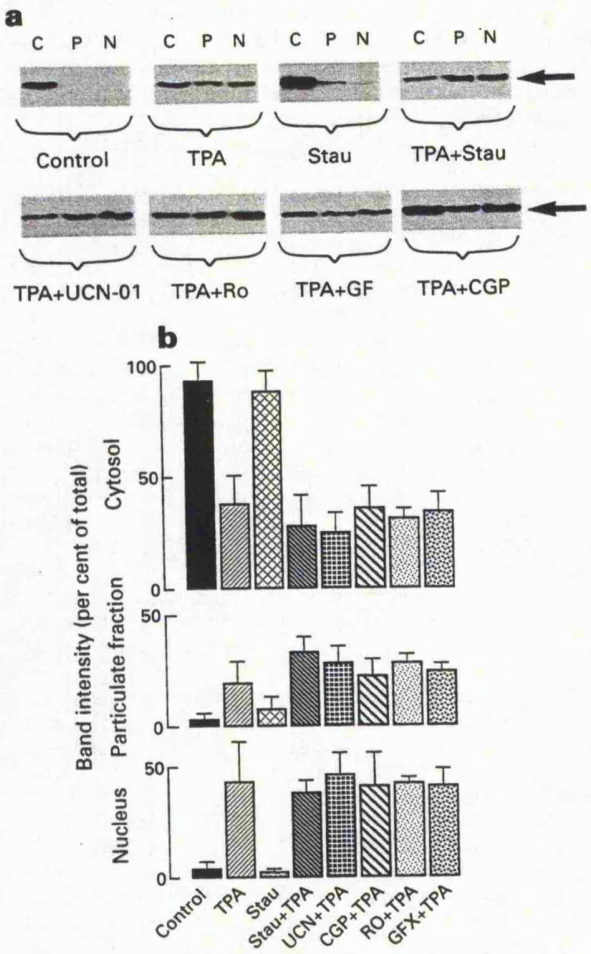

Figure 4 Western blot analysis of cPKC-α (**a**) and quantitation by laser densitometry of cPKC-α protein (**b**) in cytosolic (C), particulate (P) and nuclear (N) fractions from naive A549 cells (control) and cells which have been incubated for 30 min with TPA (25 nM) or staurosporine (1 μM) alone, or TPA together with staurosporine, UCN-01, RO 31-8220, GF 109203X or CGP 41251 (all 1 μM). The blot obtained with staurosporine alone is representative of those obtained with the other inhibitors in the absence of TPA (results not shown). Arrow indicates position of cPKC-α protein. For details of the assay see Materials and methods section. For abbreviations see legend to Figure 1. Values in **b**, which are expressed as percentage of the sum of immunoreactive protein in all three fractions, are the mean ± s.d. of three separate blots. cPKC-α levels in subfractions of cells incubated with TPA and either calphostin C (0.5 μM), H-7, H-7I (both 500 μM) or miltefosine (200 μM) were not different from those shown with TPA alone (results not shown).

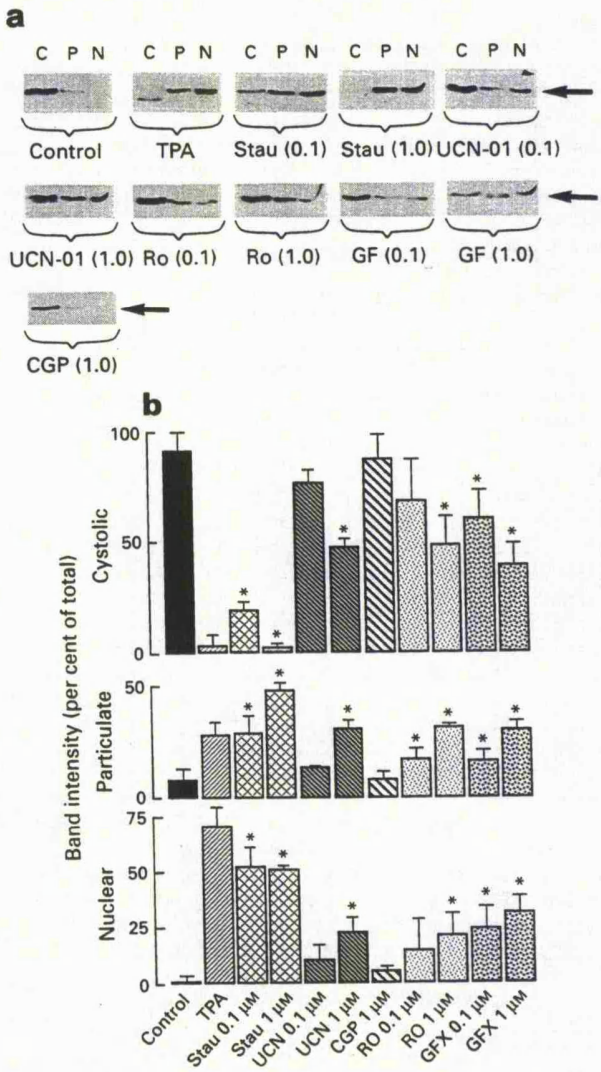

Figure 5 Western blot analysis of nPKC-ε (**a**) and quantitation by laser densitometry of nPKC-ε protein (**b**) in cellular fractions of naive A549 cells and of cells which have been incubated for 30 min with TPA (25 nM), staurosporine, UCN-01, RO 31-8220, GF 109203X (each 0.1 or 1.0 μM), or CGP 41251 (1 μM). Cells were separated into cytosolic (C), particulate (P) and nuclear (N) fractions. Arrow indicates positions of nPKC-ε. Values in **b**, which are expressed as percentage of the sum of immunoreactive protein in all three fractions, are the mean ± s.d. of 3–6 separate blots. Stars indicate that the difference between control and exposed cells is significant ($P<0.05$). nPKC-ε levels in subfractions of cells which were incubated with calphostin C (0.5 μM), H-7, H-7I (500 μM each) or miltefosine (200 μM) were not different from those observed in control cells (results not shown). For details of the assay see the Materials and methods section. For abbreviations see legend to Figure 1.

translocating agent, with a slight effect observed at 1 and 10 nM (result not shown). For all four agents the effect was noticeable at 0.1 μM and strong at 1 μM. Thus, except in the case of staurosporine, the concentrations required to elicit nPKC-ε translocation were considerably greater than those which caused PKC inhibition (see Table I). CGP 41251, calphostin C, miltefosine, H-7 or H7-I did not alter nPKC-ε localisation. The intracellular distribution of the third PKC isoenzyme expressed in A549 cells, PKC-ζ (Stanwell *et al.*, 1994), was not affected by any of the inhibitors.

Discussion

The finding that PKC inhibitors possess antiproliferative properties can be rationalised in terms of the fact that the enzyme is a pivotal regulator of cell growth and differentiation. Thus its inhibition is likely to cause interruption of signal transduction pathways vital for cellular survival. Nevertheless the experimental basis for the notion that inhibition of PKC activity is causally associated with ability to arrest growth has been tenuous, particularly in view of the fact that most so-called 'PKC inhibitors' possess only modest specificity for PKC, or none at all. Some evidence for such a link was provied by experiments on quercetin, staurosporine (Hofmann *et al.*, 1988) and the alkyllysophospholipid BM 41440 (Grunicke *et al.*, 1989) in 3T3 fibroblasts, even though these compounds are clearly promiscuous in their ability to inhibit kinases. In these cells the dose–effect relationship with regard to inhibition of PKC was found to be similar to the dose–response curves for depression of replication.

In the study presented above two separate experimental approaches were adopted, and the results do not support the notion that there is a mechanistic link between inhibition of

PKC and growth arrest. Firstly, there was no relationship between the IC_{50} values for inhibition of growth and of kinase activity. The pattern discernible in Figure 2 probably mirrors gross differences between agents in their affinity for kinases in general, not only for PKC. Secondly, PKC-depleted cells were not less, but more, sensitive towards the growth-inhibitory effect of the kinase inhibitors compared with naive cells. If growth arrest caused by PKC inhibitors was indeed mediated by the interruption of signal transduction mechanisms operating via PKC one would expect proliferation of PKC-depleted cells to be less affected by these agents than that of PKC-proficient cells. This puzzling finding can perhaps be explained by the increased intracellular availability of kinase inhibitors enabling them to interact with other targets when PKC-α and -ε are eliminated as binding sites. An alternative explanation relates to the fact that cellular targets of bryostatin 1 other than PKC have yet to be defined. Bryostatin might up-regulate certain kinases, which consequently can be more effectively inhibited in the presence of staurosporine or its analogues. It is now recognised that results obtained using agents such as bryostatin to down-regulate PKC have to be interpreted with prudence as the initial enzyme activation caused by them elicits numerous intracellular events before loss of PKC. Irrespective of the mechanism involved, our results hint at the possibility that the combination of kinase inhibitors with PKC-down-regulating agents such as bryostatin 1 may be of therapeutic benefit.

The cells used in this study express cPKC-α, nPKC-ε and PKC-ζ (Stanwell *et al.*, 1994). Of these enzymes cPKC-α and nPKC-ε were efficiently down-regulated by exposure to bryostatin, whereas the ζ-isoenzyme is resistant to down-regulation by this agent (Stanwell *et al.*, 1994). Therefore one could surmise that it is inhibition of PKC-ζ which might mediate the growth arrest caused by these agents irrespective of cellular PKC-α and -ε levels. This hypothesis seems especially compelling in the light of the recent suggestion that PKC-ζ plays a critical role in the control of mitogenic signal-induced proliferative cascades downstream of $p21^{ras}$ (Berra *et al.*, 1993). However, PKC-ζ is unlikely to be involved with the mechanism of growth inhibition as it seems to be virtually unaffected by analogues of staurosporine, at least in intact cells (Martiny-Baron *et al.*, 1993). It is also noteworthy that the effect of PKC down-regulation was essentially equal in the two cell lines regardless of the fact that relative levels of PKC-α and -ε differ considerably between them (Stanwell *et al.*, 1994). As PKC is apparently not a primary mediator of the cytostatic properties of kinase inhibitors in A549 and MCF-7 cells, other kinases may be involved. One candidate is $p34^{cdc2}$ kinase, which is inhibited by staurosporine almost as strongly as PKC (Gadbois *et al.*, 1992). Overall, the observations described above cast doubt on the notion that PKC-selective inhibitors are superior in their antineoplastic properties to inhibitors of other kinases, an inference which should be borne in mind in the search for novel kinase inhibitors as anti-cancer drugs.

Staurosporine possesses a puzzling mixture of phorbol ester agonistic and antagonistic properties (Dlugosz and Yuspa, 1991). It inhibits PKC enzyme activity potently, but also causes the subcellular translocation of nPKCs (Kiley *et al.*, 1992) and augments TPA-induced redistribution of phorbol ester binding sites (Bradshaw *et al.*, 1992). The results described above demonstrate for the first time an intriguing difference between staurosporine and its analogues. UCN-01, RO 31-8220 and GF 109203X mimicked staurosporine by eliciting translocation of nPKC-ε from the cytosol to the membrane and nucleus, whereas CGP 41521 and the PKC inhibitors structurally unrelated to staurosporine did not. None of the agents enhanced TPA-induced cPKC-α redistribution significantly. Restriction of nPKC-ε redistributory activity to staurosporine and selected cogeners suggests that this phenomenon is mechanistically associated with interaction at the catalytic domain of the enzyme, where these agents are thought to inhibit PKC (Tamaoki *et al.*, 1986; Takahashi *et al.*, 1987; Toullec *et al.*, 1991). In contrast, calphostin C (Kobayashi *et al.*, 1989) and miltefosine (Überall *et al.*, 1991) act at the regulatory site. The finding that H-7, which also acts at the catalytic site (Hidaka *et al.*, 1984), did not alter nPKC-ε localisation may be a corollary of its lower affinity for the enzyme compared with that of staurosporine, UCN-01, RO 31-8220 and GF 109203X.

It seems pertinent to compare the concentrations required to elicit nPKC-ε redistribution with those at which these compounds precipitate other biological effects, such as inhibition of PKC activity *in vitro* and arrest of cell growth. The concentrations of UCN-01, RO 31-8220 and GF 109203X which changed nPKC-ε localisation significantly (0.1 μM) exceed their IC_{50} values for PKC inhibition by factors of 3, 4 and 4 respectively. This difference suggests that PKC isoenzyme translocation, which is known to be a consequence of enzyme activation, may also be caused by potent enzyme inhibition. But then it is difficult to explain why CGP 41251, a strong PKC inhibitor, was incapable of causing PKC translocation. Furthermore, in the case of staurosporine the lowest nPKC-ε-redistributory concentrations (1 and 10 nM) were only weakly inhibitory in the PKC activity assay. The concentrations of staurosporine and UCN-01 required for translocation are 2- to 3-fold higher than the IC_{50} values at which they inhibited the growth of A549 cells. Yet in the case of RO 31-8220 and GF 109203X concentrations with antiproliferative efficacy are bound to elicit appreciable translocation of nPKC-ε.

One of the aims of this study was to explore any relationship between structure and activity among staurosporine and four of its analogues with respect to their effects on cell growth and on PKC. In both cell types the order of growth-inhibitory potency was staurosporine > UCN-01 > CGP 41251 > RO 31 8220 > GF 109203X. The order of their enzyme-inhibitory efficacy was RO 31-8220 = GF 109203X = UCN-01 > staurosporine = CGP 41251 with PKC obtained from A549 cells, and staurosporine = UCN-01 = GF 109203X > RO 31 8220 = CGP 41251 with PKC from MCF-7 cells. Intriguingly, CGP 41251 is a strong inhibitor of both cell growth and PKC activity, but incapable of eliciting nPKC-ε translocation. In contrast, UCN-01 interfered strongly with growth and PKC activity and also altered nPKC-ε localisation, whereas RO 31-8220 and GF 109203X are efficacious enzyme inhibitors and caused nPKC-ε redistribution, yet they are rather poor inhibitors of cell growth. These differences in potency allow tentative interpretation of the complicated structure–activity relationships which govern the abilities of these compounds to affect PKC and cell growth. The indolocarbazole staurosporine inhibited PKC and growth with high potency. These properties are not adversely affected by the addition of a hydroxy group at carbon 7 of the aglycone part (UCN-01), or of a benzoyl moiety on the nitrogen in position 4′ of the glycone part of the staurosporine molecule (CGP 41251) (see Figure 1). Yet the presence of *N*-benzoyl abolishes its nPKC-ε translocatory efficacy. The change from the indolocarbazole to the bisindolylmaleimide structural skeleton, that is from staurosporine to RO 31-8220 and GF 109203X, retains the ability to inhibit PKC and to translocate nPKC-ε, but diminishes strongly the growth-inhibitory potential of the molecule.

In conclusion, this study suggests that PKC does not play a direct role in the mechanisms by which PKC inhibitors arrest the growth of A549 and MCF-7 carcinoma cells. Furthermore, it seems that for a PKC inhibitor to affect subcellular enzyme localisation it has to be an analogue of staurosporine which possesses a high affinity for PKC. Further studies on these compounds will help to understand the mechanism by which they interfere with cell growth and perhaps point to other constituents of signal-transducing kinase cascades as suitable targets in the development of novel drugs.

Abbreviations: IC_{50}, 50% inhibitory concentration; cPKC, conventional (Ca^{2+}-dependent) protein kinase C; nPKC, novel (Ca^{2+}-independent) protein kinase C; [^{3}H]Tdr, [methyl-[^{3}H]thymidine; TPA., 12-*O*-tetradecanoyl phorbor-13-acetate.

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Short Communication

COMPARISON BETWEEN INHIBITION OF PROTEIN KINASE C AND ANTAGONISM OF CALMODULIN BY TAMOXIFEN ANALOGUES

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Abstract—A variety of analogues of tamoxifen were tested for inhibition of protein kinase C (PKC) activity in MCF-7 breast cancer cells. These results were compared with the calmodulin antagonism exhibited by the analogues as measured by inhibition of calmodulin-dependent cyclic AMP phosphodiesterase. The same structural features that enhanced PKC inhibition also led to an increase in calmodulin antagonism, namely 4-iodination and elongation of the basic side-chain. The most potent analogue has a 4-iodine substituent and eight carbon atoms in its basic side-chain with IC_{50} values of 38 μM for PKC inhibition and 0.3 μM for calmodulin antagonism, which compares with 92 and 6.8 μM, respectively, for tamoxifen. Some selectivity was achieved with a ring-fused analogue that retained the potency of tamoxifen as a PKC inhibitor, but lacked calmodulin antagonism.

Key words: anti-oestrogens; triphenylethylenes; cAMP phosphodiesterase; protein kinase C

Tamoxifen is used widely for the treatment of advanced breast cancer [1]. Besides its action as an anti-oestrogen, tamoxifen has effects on other important components of intracellular signalling pathways, such as antagonism of calmodulin action [2–4] and inhibition of the enzyme PKC¶ [5, 6, 7]. In order to examine the significance of these activities to the antitumour action of the drug, we have reported structural requirements necessary for the interaction with calmodulin, using analogues of tamoxifen [8, 9].

Amongst the 4-substituted analogues, the potency as antagonists of the calmodulin-dependent activity of the cAMP-PDE enzyme increased with lipophilicity and the cytotoxicity towards MCF-7 cells correlated with calmodulin antagonism [8], as has been suggested by others [3]. Tamoxifen was demonstrated to inhibit the PKC enzyme from rat and human tissues with IC_{50} values from 30 to 100 μM, [5, 7, 10] while its metabolites, 4-hydroxytamoxifen and *N*-desmethyltamoxifen, gave values of 25 and 8 μM respectively [6]. A report using MCF-7 cells as a source of the enzyme gave IC_{50} values between 50 and 250 μM for tamoxifen depending on the concentration of phosphatidylserine used in the assay [11]. However, the role, if any, of PKC inhibition in the cytotoxicity displayed by anti-oestrogens against MCF-7 cells remains unclear [11, 12].

Therefore, for this present study, we have compared the calmodulin antagonism exhibited by some tamoxifen analogues with their ability to inhibit PKC activity in the cytosolic fraction of MCF-7 cells. These cells have been reported to contain the α-, γ-, δ-, ε- and ζ-isoenzymes of PKC [13] even though their expression differs depending on cell source. We found the wild type MCF-7 cells to contain abundant amounts of PKC-ε with lesser amounts of PKC-α and PKC-ζ [14]. The assay used here to measure PKC activity utilizes TPA as an enzyme activator. TPA does not activate ζ, so the results obtained with the wild type cytosolic fraction are due to the combined activity of the α- and ε-isoenzymes. The Adriamycin-resistant MCF-7 cells have a different isoenzyme profile with overexpression of the α-isoenzyme [15]. Therefore, the differential expression of PKC-α and PKC-ε in the two cell types would permit detection of any selectivity between the two isoenzymes in susceptibility towards inhibition by the tamoxifen analogues.

Materials and Methods

Chemicals. Tamoxifen was obtained from Aldrich Chemical Co. (U.K.). The analogues were synthesized by the following literature procedures: (**2**) ref. [16]; (**3**) and (**4**) ref. [17]; (**5**), (**6**) and (**7**) ref. [19] and (**10**) ref. [20]. The structures are shown in Fig. 1. The PKC assay kit and [8-^{3}H]cyclic AMP (26 Ci/mmol) were purchased from Amersham International plc (Amersham, U.K.). All other chemicals were purchased from Sigma (Poole, U.K.). Stock solutions of all compounds were prepared in DMSO and stored at −20°.

Determination of PKC activity and calmodulin antagonism. The culture conditions for the wild type and adriamycin-resistant MCF-7 cells were as described [14]. Cells were cultured in Petri dishes (140 mm diameter) and the cytosolic fraction was prepared when they approached confluence. Briefly, plates were placed on ice and washed three times with 10 mL of ice-cold wash buffer (20 mM Tris-HCl, 150 mM glucose, 2 μg/mL leupeptin, 2 μg/mL aprotinin, adjusted to pH 7.4). Cells were scraped into 0.25 mL of ice-cold H8 buffer (20 mM Tris-HCl, 2 mM

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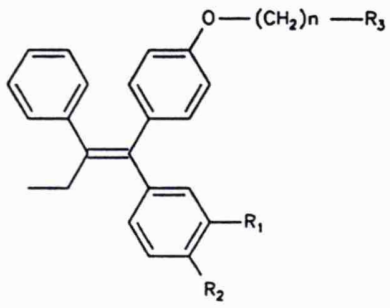
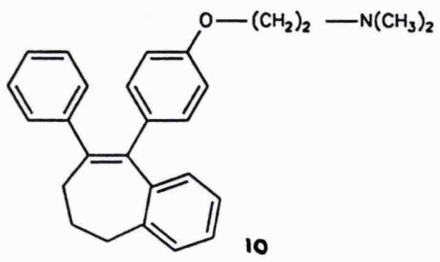
¶ Abbreviations: PKC, protein kinase C; cAMP-PDE, cyclic adenosine monophosphate phosphodiesterase





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Compound	n	R_1	R_2	R_3
tamoxifen **1**	2	H	H	$N(CH_3)_2$
droloxifene **2**	2	OH	H	$N(CH_3)_2$
idoxifene **3**	2	H	I	$N(CH_2)_4$
4	2	H	I	$N(CH_3)_2$
5	4	H	I	$N(CH_3)_2$
6	8	H	I	$N(CH_3)_2$
7	2	H	-C≡CH	$N(CH_3)_2$
8	2	H	H	-N NH
9	2	H	H	$NHCH_3$

Fig. 1. Structures of tamoxifen analogues.

EDTA, 2 mM EGTA, 6 mM β-mercaptoethanol, 2 μg/mL leupeptin, 2 μM/mL aprotinin adjusted to pH 7.5) and were disrupted by sonication using a Branson sonifier 250. The cytosolic fraction was separated from the particulate fraction by ultracentrifugation (100 000 *g* for 30 min, 4°). The cytosolic fraction was diluted by a factor of 5–15 using H8 buffer in order to dilute out endogenous peptide inhibitors which interfere with maximal PKC activity as described in the protocol for the Amersham International assay kit. Aliquots (25 μL) of the dilutions were placed in microplate wells and PKC activity was measured by the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a PKC specific peptide. The kit contains TPA as enzyme activator at a final concentration of 13 μM. Tamoxifen and its analogues at various concentrations were added to aliquots of the cytosolic fraction and the degree of inhibition of PKC activity was calculated as a percentage of enzyme activity in drug-free incubates. Calmodulin-dependent cyclic AMP phosphodiesterase was assayed as previously described [8, 21]. The enzyme was measured using [8-^{3}H] cyclic AMP as substrate and 5′-nucleotidase from snake venom to convert tritiated AMP into adenosine. Batch elution with Dowex anion exchange resin enabled the product nucleosides to be separated from the substrate. The basal activity (calmodulin independent) of the phosphodiesterase was determined by adding 1 mM EGTA to the assay medium.

Results and Discussion

The IC_{50} values for inhibition by the anti-oestrogens of the PKC activity in wild type MCF-7 cells are shown in Table 1. The cytosol of these cells contains a combination of the α- and ε-isoenzymes. Compound **1–4** and **10** were also assayed against the PKC activity, which consists predominantly of the α-isoenzyme, in the cytosol of Adriamycin-resistant MCF-7 cells. These compounds produced IC_{50} values similar in potency to those observed with the wild type cells (data not shown). Therefore, these results are consistent with the notion that the anti-oestrogens lack selectivity towards the various isoforms as has been demonstrated previously [22]. For convenience, the other derivatives were assayed against PKC activity of the wild type cells only. Tamoxifen, **1**, inhibited PKC with an IC_{50} value of 92 μM which is in good agreement with published values [5, 7]. Its major metabolite, *N*-desmethyltamoxifen, with an IC_{50} of 45 μM is a more potent inhibitor as demonstrated by others [6]. This may be due to the *N*-desmethyl group acting as a hydrogen bond donor, which can not occur with the dimethyl substituent. Support for this comes from the increased potency towards PKC exhibited by piperazinotamoxifen, **8**, another analogue with a N–H bond suitable for hydrogen bonding. It is doubtful that the concentrations of tamoxifen and its metabolites attained *in vivo* are sufficiently high to suppress

Table 1. Inhibition of PKC and calmodulin antagonism by tamoxifen analogues*

Compound	PKC inhibition wild type MCF-7 (IC_{50} μM)	Calmodulin antagonism inhibition of cAMP-PDE (IC_{50} μM)†
1	92.3 ± 6.7	6.8 ± 1.0‡
2	95.3 ± 4.2	12.9 ± 2.6
3	70.7 ± 7.1	1.5 ± 0.2
4	48.3 ± 8.3	2.3 ± 0.7‡
5	38.8 ± 1.4	1.3 ± 0.7
6	38.0 ± 1.5	0.3 ± 0.2
7	74.0 ± 14.4	4.6 ± 2.0
8	52.0 ± 5.6	> 50‡
9	45.7 ± 3.8	4.9 ± 1.0
10	90.0 ± 3.0	> 50§

* Each IC_{50} value is the mean of three experiments ±SD.

† None of the compounds inhibited the basal activity of cAMP-PDE when assayed at final concentrations of 10 and 20 μM.

‡ IC_{50} values as reported in ref. [8].

§ IC_{50} value as reported in ref. [29].

PKC activity markedly. In patients, serum concentrations of tamoxifen and *N*-desmethyltamoxifen are around 0.5 μM and intratumoural levels were shown to be 5–10 times higher [23–25]. The lipophilic nature of these compounds may enable them to accumulate within cellular compartments and reach higher concentrations. Droloxifene (3-hydroxytamoxifen) and idoxifene(pyrrolidino-4-iodotamoxifen) are two anti-oestrogens that are in clinical trials [26, 27] and have similar activity against PKC in MCF-7 cells as tamoxifen. However, 4-iodotamoxifen (**4**) showed more potent inhibition with an IC_{50} of 48 μM. A further increase in potency was produced by elongation of the basic side-chain to give the four and eight carbon derivatives, **5** and **6**. Therefore, increasing the lipophilic nature of these anti-oestrogens increases the inhibitory activity towards PKC. This supports the observations that tamoxifen analogues inhibit PKC via interactions with phospholipids [5, 7, 12] which, in turn, blocks the membrane binding of these enzymes [28].

Interestingly, some of the structural features of these compounds which increase inhibition of PKC also attenuate their antagonism of calmodulin function. The 4-iodinated derivative **4**, was approximately three-fold more potent a calmodulin antagonist than tamoxifen and extension of the basic side-chain to give **5** and **6** also produces increases in potency. Indeed, it has been suggested that because tamoxifen as well as other calmodulin antagonists, such as chlorpromazine and trifluoperazine, inhibit PKC activity there must be some homology between the regulatory domain of PKC and calmodulin [5]. However, within this series of triphenylethylene anti-oestrogens we can define the structural features that confer selectivity for calmodulin antagonism. For example, piperazinotamoxifen, **8**, has increased potency as a PKC inhibitor compared with tamoxifen, but it has lost the antagonism of calmodulin function, with no inhibition observed up to a concentration of 50 μM. In addition, replacement of the dimethylamino group, **4**, by pyrrolidino, **3**, caused a modest increase in calmodulin antagonism, but decreased PKC inhibition. These results show that the basicity of the side-chain substituent of tamoxifen is essential for calmodulin antagonism. This is supported by studies undertaken by Bouhoute and Leclercq who demonstrated that the basic side-chain is directly involved in the antagonistic activity of tamoxifen towards the association of the oestrogen receptor with calmodulin coupled to Sepharose [4]. Compound **10**, which has the same RBA to the oestrogen receptor as tamoxifen [29], has a similar potency as a PKC inhibitor, but lacks the calmodulin antagonism. Its structure differs from tamoxifen only in that the ethyl group is constrained into a fused ring conformation [30]. Therefore, a study of the properties of this ring-fused analogue and related derivatives might shed further light on the mechanism of action of tamoxifen as a chemotherapeutic agent.

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Regulation of P-glycoprotein *1* and *2* gene expression and protein activity in two MCF-7/Dox cell line subclones

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Summary The MCF-7 doxorubicin-resistant cell line MCF-7/Dox has been used extensively for studies of the multidrug resistance phenomenon. Using fluorescence-activated cell sorting (FACS), these cells were separated into two populations on the basis of rhodamine 123 (R123) accumulation. We designated these as low P-glycoprotein (LP-gp) and high P-gp (HP-gp) cells on the basis of their P-gp content. Using the reverse transcriptase-polymersase chain reaction technique controlled by homologous internal standards, we analysed levels of *MDR1* and *MDR2* mRNA in each cell type. LP-gp and HP-gp cells had *MDR1* mRNA levels of 2.17 ± 0.17 and 6.65 ± 2.29 amol ng^{-1} total RNA respectively, compared with 0.00088 ± 0.00005 amol ng^{-1} in wild-type MCF-7 cells (MCF-7/WT). MCF-7/WT cells additionally contained 0.023 ± 0.016 amol ng^{-1} of *MDR2* mRNA, which was unchanged in LP-gp cells, but lower than in HP-gp cells, which contained 0.42 ± 0.08 amol ng^{-1}. Both LP-gp and HP-gp cells contained increased copies of the *MDR1* gene. However, the degree of gene amplification did not correlate with the changes in *MDR1* mRNA levels, indicating further regulatory levels of gene expression. The level of P-gp detected by MRK16 correlated with R123 accumulation. HP-gp cells expressed a 10-fold higher level of P-gp1 than LP-gp cells. However, there was only a 3-fold increase in *MDR1* mRNA level in HP-gp cells compared with LP-gp cells. These data suggest that some regulation of P-gp1 expression also occurred at the post-translational level. Phosphorylation of P-gp by protein kinase C (PKC)-α is necessary for its activity. Our analysis of PKC-α, θ and ε isozyme levels, and subcellular distribution, shows a co-regulation of expression with P-gp, suggesting a necessary role for PKC in P-gp regulation.

Keywords: multidrug resistance; gene expression; MCF-7; flow cytometry; protein kinase C

Multidrug resistance (MDR) caused by overexpression of the membrane ATPase pump P-glycoprotein (P-gp) can be induced in cells in culture by exposure to cytotoxic agents at progressively increasing concentrations (Riordan and Ling, 1979; Kartner *et al.*, 1983*a,b*; Chan *et al.*, 1988). *In vitro* levels of resistance to the inducing agent of greater than 1000-fold compared with wild-type cells have been achieved (Beck *et al.*, 1979; Shen *et al.*, 1986*a*). P-gps are coded for by the MDR gene family, the number of members of which varies between species. Humans possess two MDR genes, *MDR1* and *MDR2* (Chin *et al.*, 1989; Ng *et al.*, 1989). Of these, only *MDR1* codes for a P-gp (P-gp 1) that can confer a drug resistance phenotype when transfected into drug-sensitive cells (Ueda *et al.*, 1987; Choi *et al.*, 1991). Multidrug-resistant cells, particularly those which display high levels of resistance, often possess an increased copy number of the *MDR1* gene (Batist *et al.*, 1986; Fairchild *et al.*, 1987; Endicott and Ling, 1989; Van der Bliek and Borst, 1989). However, in clinical samples resistance levels due to P-gp expression appear to be lower than those induced *in vitro*, and gene amplification has rarely been observed (Efferth and Osieka, 1993). Additionally, cells selected *in vitro* for lower levels of drug resistance do not show *MDR1* gene amplification (Shen *et al.*, 1986*b*; Kohno *et al.*, 1994).

One enzyme system that seems to be an important post-translational regulator of P-gp activity via phosphorylation is protein kinase C (PKC). The phosphorylation state and activity of P-gp can be augmented by PKC activators, such as tumour-promoting phorbol esters (Chambers *et al.*, 1990, 1992; Ma *et al.*, 1991) and inhibited by agents such as staurosporine (Chambers *et al.*, 1990, 1992; Chaudhary and Roninson, 1992; Bates *et al.*, 1993). Of the PKC isozymes, only PKC-α has to date been identified as important in the post-translational regulation of P-gp activity (Yu *et al.*, 1991; Ahmad and Glazer, 1993; Ahmad *et al.*, 1994). We analysed PKC expression levels and subcellular distribution to evaluate possible candidates that may have a role to play in phosphorylation and regulation of P-gp.

Doxorubicin-resistant MCF-7 cells (MCF-7/Dox) have been used frequently as a model for the study of MDR resistance and its modulation. These cells were initially derived by exposure of the MCF-7 breast carcinoma cell line to increasing concentrations of doxorubicin (Batist *et al.*, 1986). MCF-7/Dox cells show a 192-fold increase in resistance towards doxorubicin and cross-resistance to a variety of other drugs such as actinomycin D and vinblastine (Batist *et al.*, 1986). The resistance phenotype is stable for 3 months when the cells are maintained in drug-free medium. It is not clear how P-gp-mediated drug resistance in these cells is regulated at the genetic level and which molecular events are required to maintain it. The cells are characterised by a number of biochemical changes when compared with their wild-type counterparts, in particular elevated expression of P-gp, amplification of the *MDR* gene (Cowan *et al.*, 1986; Fairchild *et al.*, 1987), augmented expression of glutathione *S*-transferase (Batist *et al.*, 1986), and increased expression of PKC (Fine *et al.*, 1988).

During a study to validate P-gp activity using rhodamine-123 (R123) accumulation and efflux we detected the presence of two cell populations in our culture of MCF-7/Dox cells with differing rates of R123 efflux. We sorted these cells into two lines with different P-gp levels and used them to explore mechanisms of *MDR1* and *2* gene regulation and the involvement of PKC isoenzymes in the regulation of P-gp activity.

Materials and methods

Cell culture and maintenance

MCF-7/WT and MCF-7/Dox cells were gifts from J Carmichael, City Hospital, Nottingham, and were originally obtained from K Cowan, NIH, Bethesda, MD, USA. The cells were maintained in phenol red-free RPMI-1640 (Gibco/BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 $\mu g\ ml^{-1}$ gentamycin.

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Flow cytometric analysis of rhodamine (R123) accumulation and retention

Cells (1×10^6) were allowed to attach to 55 mm Petri dishes (Falcon) for 1.5 h in 3 ml of complete medium. Following cell attachment the medium was replaced with 5 ml of serum-free medium and cells were cultured for 10 or 20 min with 1.66 μM R123. After this incubation period cells were washed with phosphate-buffered saline (PBS) and either harvested or treated with 5 ml of fresh medium and maintained in culture for 1.5 h to allow for dye efflux to occur before cell harvest. Cells were detached by treatment with trypsin-EDTA at 4°C, washed and resuspended in 1 ml of ice-cold PBS, and 10 μg ml^{-1} propidium iodide (PI) was added. Flow cytometric analysis of the cells was carried out using a Becton Dickinson FACScan flow cytometer with excitation wavelength set at 488 nm. R123 fluorescence was collected after passage through a 515–545 nm bandpass filter, and PI fluorescence was collected after passing through a 546–606 nm bandpass filter. Single and multiparameter measurements of R123 forward angle, side angle scatter, R123 and PI fluorescence were collected for 10 000 events. Fluorescence data were collected on a four decade log scale and analysed using Lysis 2 software. Only R123 fluorescence from viable cells (i.e. PI-excluding cells) were evaluated.

The effects of P-gp inhibitors on R123 accumulation and efflux were studied by co-incubating cells with R123 and inhibitor (5 μM) for 10 min. The efflux of R123 over a 1.5 h period was determined by incubating the cells in R123-free medium but containing the inhibitor (5 μM).

Simultaneous detection of R123 accumulation and P-gp levels

Cells (1×10^6) were allowed to accumulate R123 for 20 min as described above, then harvested, resuspended in 250 μl PBS containing 10% normal goat serum (PBSG) and incubated with 10 μg of MRK16 antibody (Kamiya Biochemical Company, Thousand Oaks, CA, USA) at 4°C for 30 min. After washing with ice-cold PBS the cells were incubated with R-phycoerythrin (RPE)-conjugated F(ab')$_2$ fragment of affinity-purified goat anti-mouse IgG (1:10 dilution) (Dako) in PBSG for 30 min at 4°C, before being washed with 2 ml of PBS and resuspended in 1 ml PBSG before flow cytometric analysis. RPE fluorescence was collected after passage through a 464–606 nm bandpass filter. Duplicate analyses were performed in which the RPE-conjugated antibody was replaced by PI (10 μg ml^{-1}) so that dead cells could be eliminated from the subsequent analysis.

Flow cytometric sorting of cells

Cells (4×10^7) were allowed to take up R123 for 20 min before being harvested and resuspended in 4 ml of PBS at 4°C. The cells were sorted into high and low R123 accumulation populations using a FACS Vantage flow cytometer. The cells were sorted at about 2000 per second which resulted in an accumulation of 0.9×10^6 of both 'high' and 'low' R123 fluorescing cells during a 40 min sorting period. Only two populations were detectable by R123 accumulation in the MCF-7/Dox cell line. They were present at a ratio of approximately 6:4 (low–high R123 fluorescence). Cells were sedimented by centrifugation and cultured. After 2 h the culture medium was replaced.

Analysis of gene amplification

DNA was extracted from each of the cell lines by lysis and phenol–chloroform extraction. Three digests of the DNA were made with *Eco*RI, *Hind*III and *Nco*I and 10 μg of each digest electrophoresed on a 1% non-denaturing agarose gel. After electrophoresis the DNA was transferred to Hybond N (Amersham) by capillary transfer as previously described (Sambrook *et al.*, 1989). The DNA was cross-linked to the nylon by UV light (Stratagene UV crosslinker, 120 000 μJ) and hybridised to probes pHDR5A (Ueda *et al.*, 1987) and *MDR2pvu*II (Currier *et al.*, 1992), which detected *MDR1/2* and *MDR2* gene fragments respectively. Hybridisation was performed as previously described for the use of these probes in Northern analysis for RNA expression (Gant *et al.*, 1991, 1992). The blots were washed with 0.1 × SSC/0.1%SDS for 1 h at 42°C. Gels were visualised and quantitated using one specific band for *MDR1* and *MDR2* as described below for quantitation of the RT–PCR gels.

Determination of cytotoxicity

Cells were seeded at 2×10^4 per 35-mm-diameter dish in 3 ml of medium. After a 4 h incubation period to allow cell attachment various concentrations of doxorubicin were added. The toxicity of each concentration was determined in duplicate dishes. The cells were left for 96 h (four doubling times) and medium and doxorubicin was replenished at 48 h. The cells were trypsinised and counted using a Coulter Counter model ZM (Coulter Electronics, Luton, UK). Growth inhibition was calculated as a percentage of drug-free control. The concentration of doxorubicin causing a 50% growth reduction (IC_{50}) was determined for each cell line.

RT–PCR analysis of gene expression

RT–PCR was carried out using internal RNA standards. The internal standard was a modified sequence of the cellular RNA being amplified and which contained the same primer sites. The modification made the internal standard sequence slightly longer by duplication of a piece of sequence between the primer sites. Details of primer construction are given below. In the analysis between 20 and 300 ng of cellular RNA was mixed with between 0.005 and 20 pg of the internal standard RNA in a final reaction volume of 10 μl containing 20 mM Tris-HCl, pH 8.4, and 50 mM potassium chloride, Mg^{2+} 2.5 mM, RNAsin (Promega) 1 U μl^{-1}, MMLV-reverse transcriptase (Gibco/BRL) 10 U μl^{-1}, dNTP 1 mM, hexamers (Pharmacia) approximately 15 pmol μl^{-1} and dithiothreitol 1 mM. Hexamers were annealed at 23°C for 10 min, products extended at 42°C for 45 min and the reaction terminated by heating to 99°C before being quick chilled to 4°C (Futscher *et al.*, 1993). The PCR stage was carried out by the addition of reagents (made as a master mix) to concentrations of 20 mMTris-HCl, pH 8.4, 50 mM potassium chloride, 2.5 mM Mg^{2+}, 1 pmol μl^{-1} sense and antisense primer (see below for primers) of which 0.01 pmol μl^{-1} sense primer was end labelled with ^{33}P, 0.05% w-1 detergent (Gibcol/BRL) and 2.5 U *Taq* DNA polymerase in a final volume of 20 μl. The nucleotides were derived entirely from the original reverse transcriptase reaction and so were at a final concentration of 0.5 mM for each nucleotide. PCR was carried out for 28 cycles at an annealing temperature of 55°C for *MDR1* and 59°C for *MDR2*. Denaturation was at 95°C for 1 min in each cycle except the first in which it was extended to 5 min. The extension time was 2 min (72°C) in each cycle except the last in which it was extended to 5 min. Annealing was for 2 min in each cycle. For each set of reactions a negative control was run that did not contain RNA. PCR products were not detectable in this reaction.

After PCR a 5 μl aliquot of each reaction was analysed on an 8% non-denaturing gel. After drying, the gel was exposed in a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA, USA). Expression of each RNA was analysed by volume analysis with a local background using Image Quant 3.3 software (Molecular Dynamics).

To calculate absolute RNA concentrations five reactions for each RNA sample were performed using the same amount of cellular RNA in each, but an increasing amount of the internal standard RNA. The ratio of the PCR band volume from cellular RNA over band volume due to internal standard RNA was plotted as a double log$_e$ plot against the amount of the internal standard. The amount of internal standard and cellular RNA are equal when the ratio of these

bands is 1 ($\log_e = 0$). Care was taken to ensure that the experimental data spanned a ratio of 1 so that linearity to a ratio of one was not assumed. For each sample three analyses were performed. Kinetics and reaction characteristics are published in Zhang *et al.* (1996).

Construction of the MDR internal standard RNAs

The piece of the *MDR1* gene chosen for RT–PCR assay was the region between bases 1991 (numbered from the adenosine of the translation initiation codon) and 2416. The region was amplified using primers 5′-AAAAAGATCAACTCGTAG-GAGTG-3′ (sense strand) and 5′-GCACAAAATACAC-CAACAA-3′ (antisense strand). The internal standard was constructed by duplication of the region between base 2040 (*Hind*III site) and 2085 by PCR of this region using the sense primer described above and an antisense primer spanning bases 2065 to 2085 which had a *Hind*III site added at the 5′ end. Following PCR the product was cut with *Hind*III and inserted into the *Hind*III site of the assay sequence. Thus the internal standard was 51 bp longer than the assay region allowing for inclusion of a second *Hind*III site. The whole construct was contained in the pGem T vector (Promega) and sequenced using a primer to the SP6 promoter region. The plasmid was linearised using *Pst*I before transcription of sense RNA from the T7 promoter.

Construction of the *MDR2* internal standard control was very similar to that of *MDR1*. The area chosen for RT–PCR assay spanned bases 1343 (numbered from the adenosine of the translation initiation codon) and 1570. The region was amplified using primers 5′-TGATGAGGGCACAATTAA-CA-3′ (sense) and 5′-GTGTCAAATTTCTGTGGAAT-3′ (antisense). The internal standard was made by duplication of the sequence between bases 1408 and 1494 (*Nco*I site) using the antisense primer above and a primer between bases 1408 and 1427 which had an *Nco*I site added to the 5′ end. The PCR product was cut with *Nco*I and inserted into the analysis region at the *Nco*I site. The whole construct was contained in the pGEM4Z plasmid (Promega). The insert was sequenced using a primer to the SP6 promoter and linearised using *Pst*I before transcription of sense RNA from the SP6 promoter.

Following transcription the size and integrity of the RNA was checked on a denaturing agarose gel and concentration assessed by determining optical absorbance at 260 nm. The molecular weight of the construct was determined, taking into account the additional sequence derived from the vector. The RNA was aliquoted and stored at −80°C before use.

Western blot analysis of PKC isozymes

Cells were grown on 140 mm Petri dishes. When they approached confluence cytosolic, particulate (membrane) and nuclear fractions were prepared as previously described (Greif *et al.*, 1992) with some modifications (Stanwell *et al.*, 1994). The protein content of each sample was determined by the method of Bradford, (1976).

Western blot analysis was performed as described previously (Stanwell *et al.*, 1994) loading 20 μg of protein per lane. Monoclonal antibodies to PKC-α (TCS, Botolph Claydon, UK) PKC-ε and -θ (Afinitti, Nottingham, UK) and a polyclonal antibody to PKC-ζ (Gibco, Paisley, UK) were used. Detection was by enhanced chemiluminescence using an ECL kit (Amersham, UK). Immunoreactivity was quantified using a Molecular Dynamics computing densitometer and Image Quant software.

Results

Identification of two subclones by R123 efflux

Accumulation and efflux of R123 was compared in MCF-7/WT and MCF-7/Dox cells. Whereas MCF-7/WT cells

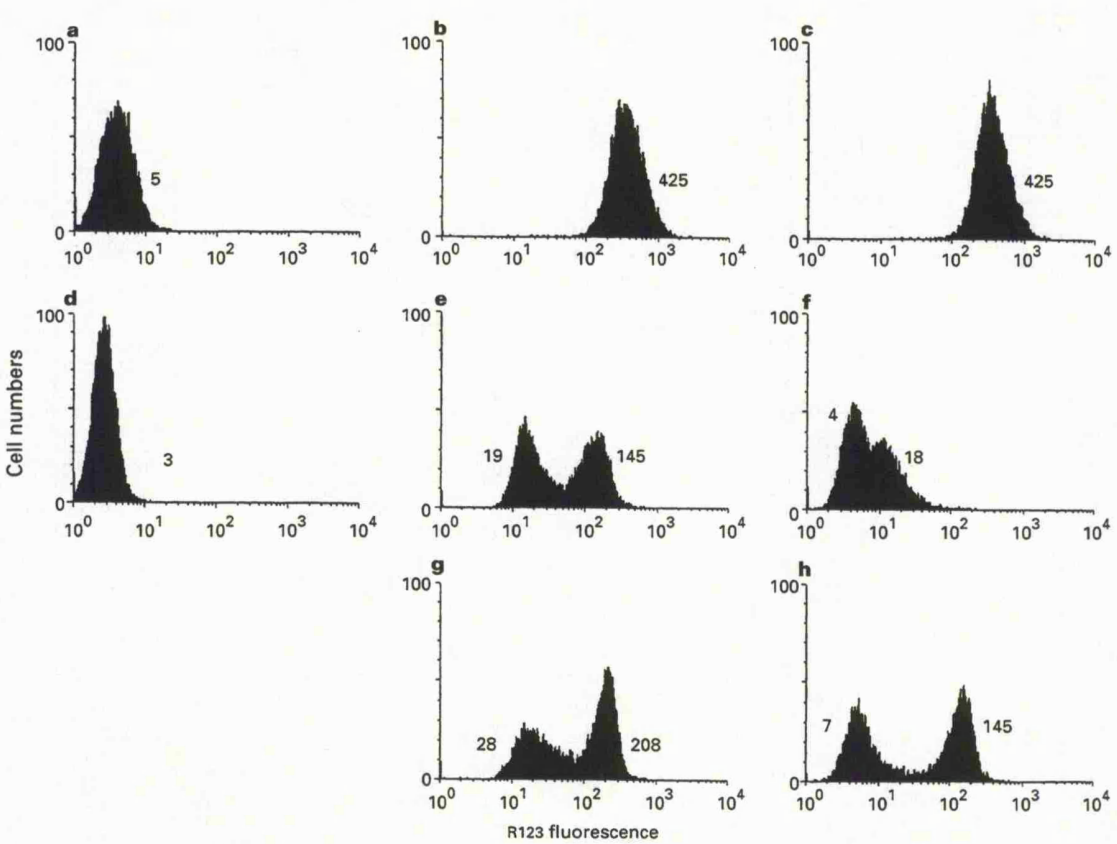

Figure 1 R123 uptake and retention in MCF-7/WT (**a**–**c**) or MCF-7/Dox cells (**d**–**f**), and effect of verapamil (**g**, **h**). Cells were incubated without (**a**, **d**) or with R123 for 10 min without (**b**, **e**) or with (**c**, **f**) a subsequent 1.5 h incubation period in the absence of the dye. MCF-7/Dox cells were exposed to R123 and verapamil 5 μM without (**g**) or with (**h**) a subsequent 1.5 h dye retention period in the continued presence of verapamil. Each value represents the mean fluorescence of the cell population.

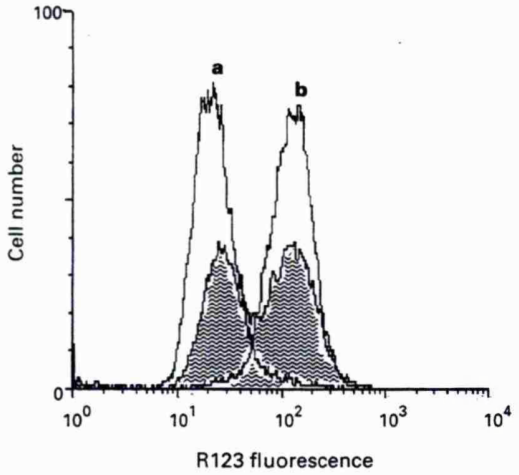

Figure 2 R123 accumulation after exposure for 10 min to the dye of MCF-7/Dox cells (hatched area) or in the sorted 'low' (**a**) or 'high' (**b**) R123 fluorescence cells.

accumulated and retained R123 efficiently accumulation was drastically reduced and efflux enhanced in MCF-7/Dox cells (Figure 1). In our MCF-7/Dox cells two populations were evident, both with a higher R123 efflux rate than that observed in the parent MCF-7/WT cells. The cells in the two populations were present at an approximately 6:4 (low–high R123 fluorescence) ratio, and together constituted the entire MCF-7/Dox population. When verapamil was present more R123 was accumulated and R123 efflux was substantially reduced. A similar result was obtained with reserpine, except that this inhibitor was more potent than verapamil (data not shown).

Cell sorting and culture on the basis of R123 efflux

The cells were sorted on the basis of their ability to efflux R123, and established in culture. After sorting two cell populations were obtained each of which was homogeneous for R123 accumulation and efflux (Figure 2). To ensure that the two separated cell lines were from the same source their DNA was profiled and fingerprinted. The results of the analysis (not shown) show that the low and high fluorescence cells were indistinguishable on the basis of their DNA.

Analysis of P-gp levels using antibodies and FACS analysis

With the MRK-16 antibody (Figure 3) two cell populations were evident, confirming that the MCF-7/Dox cell line is composed of two populations with differing P-gp levels. Correlation of P-gp content in MCF-7/Dox cells, detected by

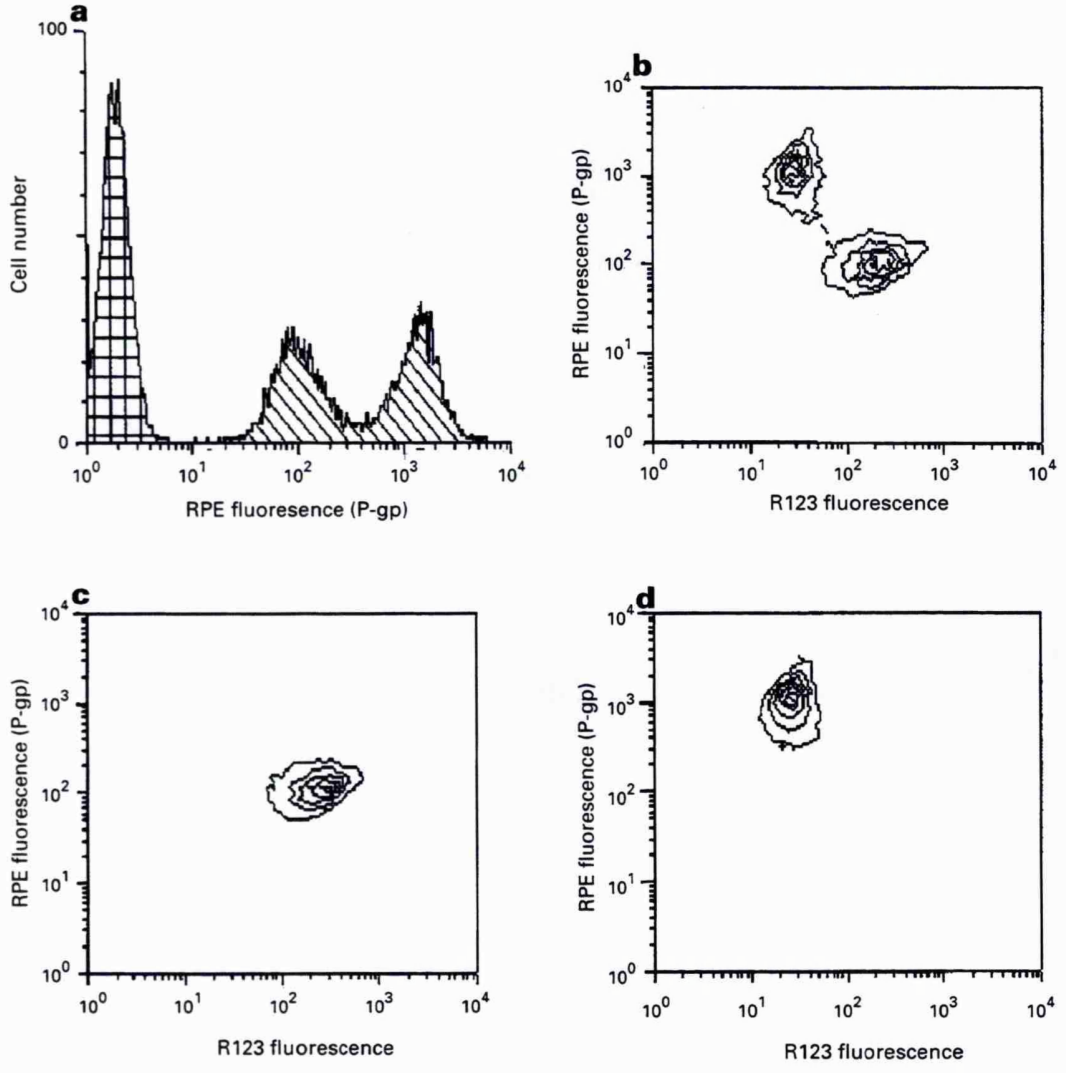

Figure 3 P-gp content in MCF-7/Dox (**a** and **b**), LP-gp (**c**) and HP-gp cells (**d**). Cells were stained with MRK-16 after incubation with R123. Cross-hatched peaks show fluorescence in the absence, striped peaks in the presence of antibody. Cell incubations, immunodetection and flow cytometry were carried out as described under Materials and methods.

MRK-16 antibody, with their R123 accumulation showed that the low R123 fluorescence cells had the higher P-gp content. Likewise, the high R123 fluorescence cells expressed the lower P-gp level. Thus, R123 accumulation was inversely correlated with P-gp content (Figure 3). Furthermore, the sorted high R123 fluorescence cell line expressed the lower P-gp levels (Figure 3) and will therefore be referred to in the following text as LP-gp (low P-gp) cells. The sorted R123 low fluorescence cells expressed higher P-gp levels and will therefore be referred to as HP-gp (high P-gp) cells. Both for MRK-16 binding and R123 accumulation there was a 10-fold difference between LP-gp and HP-gp cells.

Sensitivity of cells to doxorubicin cytotoxicity

Sensitivity of the cells against doxorubicin as reflected by IC_{50} values decreased in the order MCF-7/WT > LP-gp > MCF-7/Dox > HP-gp (Table I).

Gene amplification

MDR gene amplification was analysed in all four MCF-7 cell types (Figure 4). In LP-gp, MCF-7/Dox and HP-gp cells, we found *MDR1* gene amplifications of 9-, 40- and 78-fold over the MCF-7/WT cells respectively. For *MDR2* we found similar amplification values of 8-, 36- and 65-fold respectively.

Table I Doxorubicin cytotoxicity in MCF-7/Dox cell subclones

MCF-7 cell clone	*IC_{50}[a] (μM)*
MCF7-WT	0.04 ± 0.01
LP-gp	0.61 ± 0.13
MCF-7/Dox	1.55 ± 0.05
HP-gp	1.99 ± 0.12

[a] Cells (2×10^4) were exposed to various doxorubicin concentrations for 96 h with the medium being replenished at 48 h. After 96 h the cells were detached by trypsinisation and counted using a Coulter counter. Inhibition of growth was calculated as a percentage of drug free control.

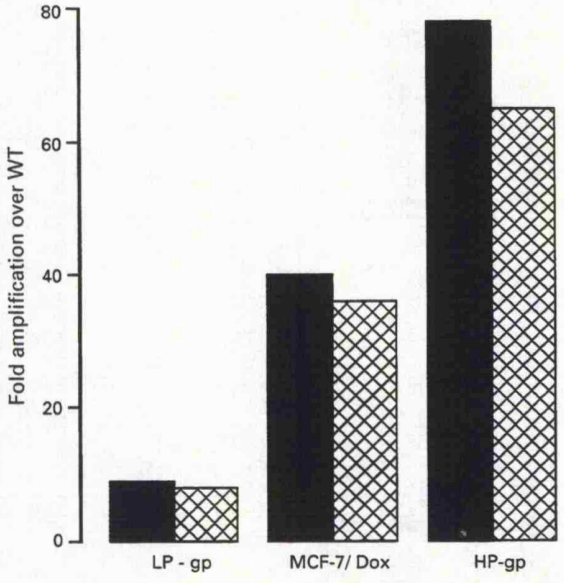

Figure 4 Amplification of the *MDR1* (■) and *MDR2* (▩) genes in MCF-7/Dox, LP-gp and HP-gp cells over MCF-7/WT cells. Using the pHDR5A probe, which detects the *MDR1* and *MDR2* genes, or the *MDR2pvu*II probe, which detects only *MDR2*, copy numbers of the *MDR1* and *2* genes in each cell type were determined as described under Materials and methods. The results were quantitated using volume analysis on a phosphorimager. Equal DNA loading was assessed by hybridisation to a probe for the GAPDH gene which spanned bases 2 to 1023 of the GAPDH cDNA (Fort *et al.*, 1985).

Analysis of cellular mRNA levels

Using RT–PCR with primers specific for the *MDR1* and *MDR2* genes and RNA internal standard controls it was possible to detect and quantitate *MDR1* and *MDR2* mRNA in all four MCF-7 cell types. In the MCF-7/WT cells the *MDR1* mRNA level was low at 0.00088 ± 0.00005 amol ng^{-1} RNA compared with the *MDR2* mRNA level, which was 0.023 ± 0.016 amol ng^{-1} RNA (Figure 5). *MDR2* gene expression occurs in many normal tissues (Brown *et al.*, 1993; Lee *et al.*, 1993). Therefore it was not surprising that *MDR2* expression was higher than that of *MDR1* in the

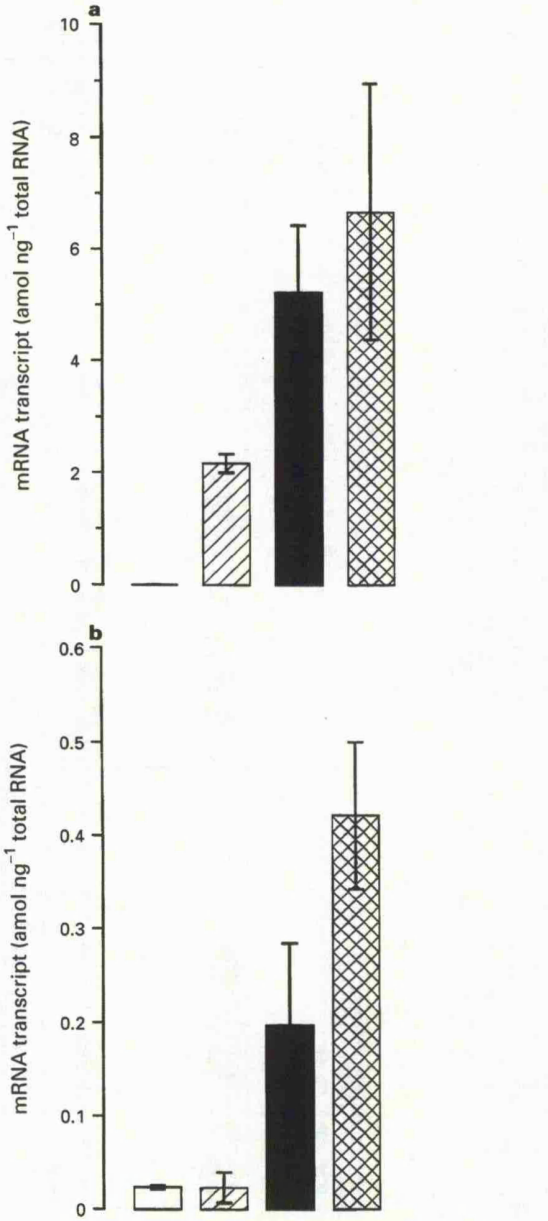

Figure 5 mRNA levels of *MDR1* (**a**) and *MDR2* (**b**) in MCF-7/WT (□), LP-gp (▨), MCF-7/Dox (■), and HP-gp (▩) cells. Levels of mRNA for *MDR1* and *MDR2* were determined using internally controlled RT–PCR, as described in Materials and methods. Results are the means ± s.d. of three determinations, except for *MDR1* in MCF-7/WT cells, which is the mean of two determinations.

MCF-7/WT cells. In LP-gp cells *MDR1* mRNA levels were 2.17 ± 0.17 amol ng^{-1} RNA, which constitutes a 2500-fold increase over the MCF-7/WT cells. HP-gp cells contained 6.65 ± 2.29 amol ng^{-1} RNA of *MDR1* mRNA, a 7556-fold increase. In HP-gp cells *MDR2* mRNA levels were 0.42 ± 0.08 amol ng^{-1} RNA, 18 times the level observed in MCF-7/WT and LP-gp cells. MCF-7/Dox cells expressed levels of *MDR1* and *MDR2* mRNA that were intermediate between the levels of LP-gp and HP-gp cells, consistent with the ratio of LP-gp and HP-gp subpopulations in the parent MCF-7/Dox cell line.

Analysis of PKC isozymes

As PKC-α has been shown to play a role in the regulation of P-gp activity (Yu *et al.*, 1991; Ahmad and Glazer, 1993; Ahmad *et al.*, 1994) levels and subcellular distribution of PKC isozymes were examined. All three MCF-7 doxorubicin-resistant cell lines expressed PKC-α, -ζ, -ε and -θ (Figure 6 and Table II). Other PKC isozymes could not be detected by Western blot analysis. Total PKC expression was similar between LP-gp and HP-gp cells, but the isozyme distribution pattern in these cells differed substantially from that seen in

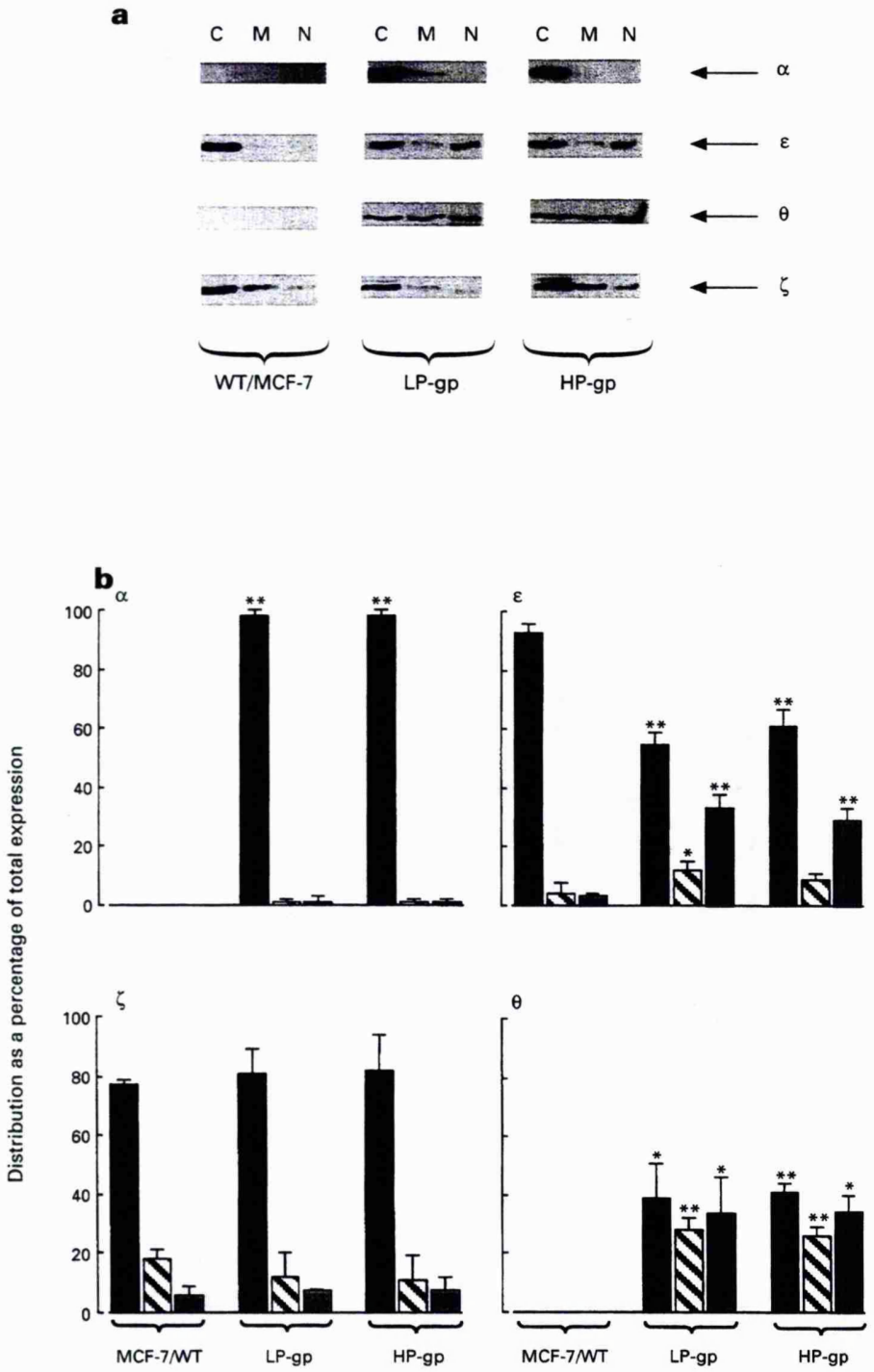

Figure 6 Western blot analysis (**a**) and distribution (**b**) of PKC isozymes in MCF-7/WT, LP-gp and HP-gp cells. Distribution is expressed as a percentage of total cellular expression for each individual cell type. Expression was determined by Western blot analysis with the appropriate antibodies in the cytosolic (■), membrane (▨) and nuclear fractions (▩). Data for the MCF-7/Dox cells were very similar to those in LP-gp/HP-gp cells, and are not shown. Values are the means ± s.d. of three experiments, statistical significance was determined (*$P \leqslant 0.05$, **$P \leqslant 0.001$) by analysis of variance.

Table II PKC expression in MCF-7/WT cells and their drug-resistant counterparts

PKC	MCF-7/WT[a]	MCF-7/Dox	LP-gp	HP-gp
α	+[b]	++++	++++	++++
θ	–	+++	+++	+++
ε	++++	+	+	+
ξ	++++	++	++	++

[a] Cells were harvested at near confluence and subcellular fractions were isolated. Analysis of protein levels in the cytosolic, membrane and nuclear fractions was by Western blotting with specific antibodies as described in Materials and methods. Antibody binding was detected using enhanced chemiluminescence and quantitated using a Molecular Dynamics densitometer. [b]Arbitrary measure based on consistent observations from three separate experiments. A representative blot is shown in Figure 6.

MCF-7/WT cells (Figure 6). Expression of PKC-α was undetectable in MCF-7/WT cells but it was found at identical levels in LP-gp and HP-gp cells. Most of the enzyme was localised in the cytosol, whereas 1% was associated with each of the membrane and nuclear compartments. Overall expression of PKC-ε and PKC-ζ in both LP-gp and HP-gp cells was decreased compared with MCF-7/WT cells (Table II). This decrease was mainly accounted for by a loss of cytosolic enzyme. However, in LP-gp and HP-gp cells PKC-ε was located to a greater extent in the nuclear and membrane fractions compared with MCF-7/WT cells. PKC-θ was not detectable in MCF-7/WT cells, but abundant in the resistant cells. In this case isozyme distribution was relatively homogeneous between the subcellular fractions.

Discussion

The multidrug-resistant MCF-7/Dox cell line was originally derived in 1986 and has been widely used for studies of P-gp (Batist *et al.*, 1986). The results presented above show that our MCF-7/Dox cells comprised two subclones that were separable according to their ability to efflux R123. The presence of two clones in the MCF-7/Dox cells has been shown previously by MRK-16 antibody binding, but was not discussed (Yu *et al.*, 1991). In contrast another very recent analysis with MRK16 found only one cell clone (Molinari *et al.*, 1994).

In the work outlined above we present six separate pieces of evidence that suggest differences in P-gp expression levels between LP-gp and HP-gp cells that constitute the MCF-7/Dox cell line: (i) they accumulate and retain R123 differently; (ii) they demonstrate different levels of immunoreactivity with the anti-P-gp1 antibody MRK-16; (iii) they exhibit different degrees of *MDR1* and *MDR2* gene amplification; (iv) they display different sensitivity towards doxorubicin; (v) they possess different levels of *MDR1* and *MDR2* mRNA; and (vi) they are differentially susceptible towards verapamil.

MDR1 mRNA levels were 2500- and 7556-fold greater in the LP-gp and HP-gp cells respectively, than in MCF-7/WT cells. The difference in *MDR1* mRNA levels between LP-gp and HP-gp cells was therefore only 3-fold. However, both R123 accumulation and MRK16 binding indicate a difference of approximately one order of magnitude in the amount of P-gp1 protein between LP-gp and HP-gp cells. Therefore a post-translational regulatory step for P-gp1 stability may be operative in HP-gp cells. Both *MDR1* and *2* genes were amplified in LP-gp cells about 9- and 8-fold respectively, over MCF-7/WT cells. In LP-gp cells this amplification translated into an increase of *MDR1* mRNA level of 2500, whereas *MDR2* mRNA was not increased. This discrepancy indicates that a regulatory step was involved in the control of the *MDR1* gene in which the rate of gene transcription or mRNA half-life was increased about 250-fold over MCF-7/WT cells to result in the observed mRNA level. The result with *MDR2* is more difficult to analyse, given the possibility that the gene may not have been amplified intact. In the case of HP-gp cells the picture was similar. *MDR1* and *MDR2* gene amplification in HP-gp cells was 78- and 65-fold respectively, over MCF-7/WT cells. However, *MDR1* and *MDR2* mRNA levels were increased 7556- and 18-fold respectively. In analogy to LP-gp cells this difference indicates an increase in *MDR1* gene transcription rate or mRNA half-life of about 100-fold over MCF-7/WT cells. An increase in *MDR1* transcription rate has been previously observed in MCF-7/Dox, doxorubicin-resistant ovarian 2780 and human colon carcinoma SW620 cells (Morrow *et al.*, 1992; Madden *et al.*, 1993). These data suggest that regulation of mRNA level in the LP-gp and HP-gp cells may occur to a greater degree at the transcriptional rather than post-transcriptional level.

Correlation of the results obtained by fluorescence analysis using the MRK-16 antibody with those observed for R123 accumulation suggests that R123 efflux was mediated completely via P-gp. Furthermore, as MRK-16 is specific for the P-gp1 isoform (Schinkel *et al.*, 1991, 1993), we conclude that R123 efflux is driven by P-gp1 and not P-gp2, the mRNA for which was increased in HP-gp but not LP-gp cells. This result is consistent with that previously obtained by Ludescher *et al.* (1993) in B-cell chronic lymphocytic leukaemia. All MCF-7-derived cell clones had detectable *MDR2* mRNA levels. The correlation between R123 accumulation and P-gp1 levels as detected by MRK 16 in LP-gp and HP-gp cells indicates that there is no additional post-translational regulation of P-gp protein activity in HP-gp in comparison with LP-gp cells.

As cPKC-α has been shown to play an important role in the post-translational regulation of P-gp activity (Yu *et al.*, 1991; Ahmad and Glazer, 1993; Ahmad *et al.*, 1994), we analysed the level and distribution of PKC isozymes. In LP-gp and HP-gp cells overall levels of PKC isozymes -α, -ε, -ζ and -θ, were similar. However, compared with MCF-7/WT cells the resistant cells expressed higher levels of cPKC-α and nPKC-θ and lower levels of nPKC-ε and aPKC-ζ. The subcellular distribution of nPKC-ε differed significantly between LP-gp, HP-gp and MCF-7/WT cells. The resistant cells possessed more nPKC-ε in the membrane, and especially the nucleus, than wild-type cells. The fact that this distribution pattern locates nPKC-ε more efficiently in close vicinity to P-gp is commensurate with a role for this enzyme in the post-translational regulation of P-gp1 activity. This finding is consistent with the report of Slapak *et al.*, 1993, who observed a decrease in overall PKC activity in the cytosol of HL-60 cells with acquired vinblastine resistance and an increase in enzyme content in the membrane fractions compared with HL-60 WT cells. The results presented above show for the first time that PKC-θ is present in MCF-7/Dox cells and its subclones. Its absence in MCF-7/WT cells and expression in all three cell compartments, particularly in the membrane and nuclear fractions of LP-gp and HP-gp cells, render it another candidate for post-translational regulation of P-gp activity.

In conclusion we derived two cell lines from the widely used MCF-7/Dox cell line, of the same origin, which differed in their expression of both *MDR1* and *MDR2* genes. This difference translated into an altered level of P-gp1 expressed in the plasma membrane and a corresponding difference in the ability of each cell line to efflux R123. In both LP-gp and HP-gp cells the change in *MDR2* mRNA over MCF-7/WT cells was smaller than the increase in *MDR2* gene copy number indicating a decreased transcription rate or mRNA stability or defective amplicon. The opposite was the case for the *MDR1* gene in which the increase in *MDR1* mRNA level was greater than that which could be accounted for by increased gene copy number. This result indicates an increased rate of *MDR1* gene transcription or mRNA stability. Comparison of MRK-16 binding with R123 retention in each cell type is consistent with the hypothesis that R123 efflux occurs only through P-gp1. Comparison of P-gp protein level and mRNA levels between LP-gp and HP-gp cells suggested that in addition to the transcriptional/post transcriptional regulation of *MDR1* expression some post-translational increase in protein stability occurred.

However, the identical 10-fold difference in R123 accumulation and P-gp levels between LP-gp and HP-gp cells indicated no change in post-translational regulation of protein activity. Analysis of PKC isozymes indicated a dramatic increase in nPKC-α and nPKC-θ levels in both LP-gp and HP-gp cells over MCF-7/WT cells. In comparison with MCF-7/WT cells nPKC-ε expression was reduced overall in the resistant cell types, however the enzyme was distributed differently. More nPKC-ε was located in the membrane and nucleus, which might indicate an important role for nPKC-ε in the regulation of P-gp1 activity.

Overall, the results highlight the complicated and multifaceted nature of regulation of the multidrug resistance phenotype in MCF-7/Dox cells. A better appreciation of how it is controlled will help to devise more efficacious therapies to overcome P-gp-mediated drug resistance.

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Comparison of staurosporine and four analogues: their effects on growth, rhodamine 123 retention and binding to P-glycoprotein in multidrug-resistant MCF-7/Adr cells

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Summary The potent kinase inhibitor staurosporine and its protein kinase C (PKC)-selective analogue CGP 41251 are known to sensitise cells with the multidrug resistance (MDR) phenotype mediated by P-glycoprotein (P-gp) to cytotoxic agents. Here four PKC-selective staurosporine cogeners, CGP 41251, UCN-01, RO 31 8220 and GF 109203X, were compared with staurosporine in terms of their MDR-reversing properties and their susceptibility towards P-gp-mediated drug efflux from MCF-7/Adr cells. Staurosporine was the most potent and the bisindolylmaleimides RO 31 8220 and GF 109203X the least potent cytostatic agents. When compared with MCF-7 wild-type cells, MCF-7/Adr cells were resistant towards the growth-arresting properties of RO 31 8220 and UCN-01, with resistance ratios of 12.6 and 7.0 respectively. This resistance could be substantially reduced by inclusion of the P-gp inhibitor reserpine. The ratios for GF 109203X, staurosporine and CGP 41251 were 1.2, 2.0 and 2.9 respectively, and they were hardly affected by reserpine. These results suggest that RO 31 8220 and UCN-01 are avidly transported by P-gp but that the other compounds are not. Staurosporine and CGP 41251 at 10 and 20 nM, respectively, decreased efflux of the P-gp probe rhodamine 123 (R123) from MCF-7/Adr cells, whereas RO 31 8220 and GF 109203X at 640 nM were inactive. CGP 41251 was the most effective and GF 109203X the least effective inhibitor of equilibrium binding of [^{3}H]vinblastine to its specific binding sites, probably P-gp, in MCF-7/Adr cells. Overall, the results imply that for this class of compound the structural properties that determine susceptibility towards P-gp-mediated substrate transport are complex. Comparison with ability to inhibit PKC suggests that the kinase inhibitors affect P-gp directly and not via inhibition of PKC. Among these compounds CGP 41251 was a very potent MDR-reversing agent with high affinity for P-gp and least affected by P-gp-mediated resistance, rendering it an attractive drug candidate for clinical development.

Keywords: CGP 41251; MCF-7/Adr cells; multidrug resistance; protein kinase C; staurosporine

Recent advances in the understanding of cellular signal transduction pathways have led to the discovery of potent inhibitors of protein kinases with cytostatic and chemosensitising properties (Powis, 1992). Prominent among these molecules is the indolocarbazole alkaloid staurosporine (Figure 1), a natural product first isolated from *Streptomyces staurosporeus* (Omura *et al.*, 1977). Staurosporine has been the lead molecule for the synthesis of a variety of novel kinase inhibitors, for example 7-hydroxystaurosporine (UCN-01) and 4'-*N*-benzoyl staurosporine (CGP 41251), both of which possess anti-cancer activity in rodents (Akinaga *et al.*, 1991; Meyer *et al.*, 1989) (Figure 1). Replacement of the indolocarbazole structure with the related bisindolylmaleimide system (Figure 1) furnished a series of staurosporine cogeners exemplified by RO 31 8220 (Davis *et al.*, 1992) and GF 109203X (Toullec *et al.*, 1991). A feature shared by UCN-01, CGP 41251, RO 31 8220 and GF 109203X is that they inhibit protein kinase C (PKC) selectively, whereas the parent staurosporine molecule inhibits a variety of kinases indiscriminately. Staurosporine (Sato *et al.*, 1990; Sampson *et al.*, 1993) and CGP 41251 (Utz *et al.*, 1994) have been shown to sensitise cells with P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) to cytotoxic agents. Therefore this type of kinase inhibitor might be attractive as a chemotherapeutic agent because of its MDR-reversing activity in combination with cytostatic properties. UCN-01 and CGP 41251 are currently in preparation for phase I clinical evaluation as anti-cancer drugs.

The mechanisms by which staurosporine and its analogues mediate their cytostatic and MDR reversing effects are unclear. It is conceivable that strong, but non-selective, kinase-inhibitory potency is a major determinant of activity exerted by this type of agent. Alternatively high selectivity for PKC, albeit coupled with weaker inhibitory potency, might be required for optimal pharmacological efficacy. It is also possible that cytostasis and MDR reversal are mediated by mechanisms unrelated to kinase inhibition. We reported recently that RO 31 8220 and GF 109203X are much less potent inhibitors of the growth of A549 and MCF-7 cells than staurosporine, UCN-01 and CGP 41251, whereas all five compounds were strong inhibitors of the PKC contained in these cells, with IC_{50} values of below 100 nM (Courage *et al.*, 1995). In the present study these five compounds are compared as modifiers of P-gp-mediated MDR. Their abilities to modulate the following three properties involving P-gp were investigated in the human breast cancer-derived multidrug-resistant MCF-7/Adr cell line: (1) growth of these cells in contrast to that of their wild-type counterparts; (2) binding of [^{3}H]vinblastine to P-gp; and (3) cellular accumulation and efflux of rhodamine 123 (R123) and [^{3}H]vinblastine. R123 has been shown to be a suitable fluorescent probe for P-gp-mediated transport (Neyfakh, 1988; Efferth *et al.*, 1989; Hofmann *et al.*, 1992). The overall objective of the work was to explore the mechanisms of cytostasis and resistance reversal caused by kinase inhibitors in a MDR cell line, with the ultimate aim to assess the potential use of such compounds in cancer therapy.

Materials and methods

Chemicals, reagents and cells

UCN-01, RO 31 8220 and CGP 41251 were gifts from Kyowa Hakko Kogyo Co. (Tokyo, Japan), Roche Research Centre (Welwyn Garden City, UK) and Ciba Geigy (Basle, Switzerland) respectively. GF 109203X was purchased from Calbiochem-Novabiochem Co. (Nottingham, UK).

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[^{3}H]Vinblastine (11 or 21 Ci mmol^{-1}) was obtained from Amersham (Amersham, UK). Other chemicals and reagents including staurosporine were obtained from Sigma Chemical Co. (Poole, UK). Cell culture medium and serum were purchased from Gibco BRL (Paisley, UK). Stock solutions of drugs were prepared in dimethyl sulphoxide (DMSO) and stored at −20°C. MCF-7 breast carcinoma cells and their counterparts that have acquired stable resistance to doxorubicin by exposure to the drug (MCF-7/Adr) were a gift from J Carmichael (University of Nottingham, UK) and originally derived in the laboratory of K Cowan (NCI, Bethesda, MD, USA). MCF-7 cells were also obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), and these were indistinguishable from the above MCF-7 cells. Cells were maintained routinely in an atmosphere of oxygen–carbon dioxide (95:5), MCF-7 cells in minimum essential medium (Eagle's modified) with fetal calf serum (FCS) (10%), pyruvate (1 mM) and non-essential amino acids, MCF-7/Adr cells in RPMI-1640 medium with heat-inactivated FCS (10%). Cultures of both cell types contained L-glutamine (2 mM), penicillin (100 IU ml^{-1}) and streptomycin (100 ug ml^{-1}). Cells were subcultured when they were confluent.

Effect of staurosporine analogues on cell growth

Cells (2×10^4) were seeded in wells (35 mm diameter) with 3 ml of medium. After a 4 h attachment period compounds at various concentrations were added. Cells were left for 4 days (four doubling times) with medium containing the staurosporine analogue being replenished on day 2. Cells were trypsinised and counted using a model ZM Coulter counter. Incubations were conducted in duplicate. Growth inhibition was expressed as percentage of cell number in drug-free control incubates. In some experiments the effect of the staurosporine analogues on cell growth was observed in the presence of 5 μM reserpine, which reverses MDR. At this concentration reserpine did not interfere with proliferation. IC_{50} values were derived from three separate experiments each performed in duplicate. The resistance ratios shown in Table I are the ratios of IC_{50} in MCF-7/Adr over IC_{50} in MCF-7, or IC_{50} in MCF-7/Adr in the absence of reserpine over IC_{50} in its presence.

Flow cytometric analysis of R123 accumulation and efflux

Cells were maintained in medium without phenol red but with FCS (10%) and gentamicin (50 μg ml^{-1}). Cells (10^6) were allowed to attach for 1.5 h in dishes (55 mm diameter, Falcon) with 3 ml of medium. Medium was replaced with serum-free medium (5 ml) containing staurosporine analogue (10–640 nM) and R123 (1.66 μM), and cells were incubated for 20 min. Thereafter cells were either washed with phosphate-buffered saline (PBS) (pH 7.4) and detached for flow cytometric analysis of R123 accumulation, or processed to determine R123 efflux as follows: cells were treated with fresh serum-free medium (5 ml) without R123 but with staurosporine analogue, and maintained for a further 20 min, during which dye efflux occurred. Cells were detached by treatment with trypsin-EDTA, washed with ice-cold PBS and resuspended in PBS (1 ml), to which propidium iodide (10 μg) was added. Flow cytometric analysis was carried out on a Becton Dickinson FACScan flow cytometer with the excitation wavelength set at 488 nm. Fluorescence emission caused by R123 or propidium iodide was measured after passage through bandpass filters spanning 515–545 or 564–

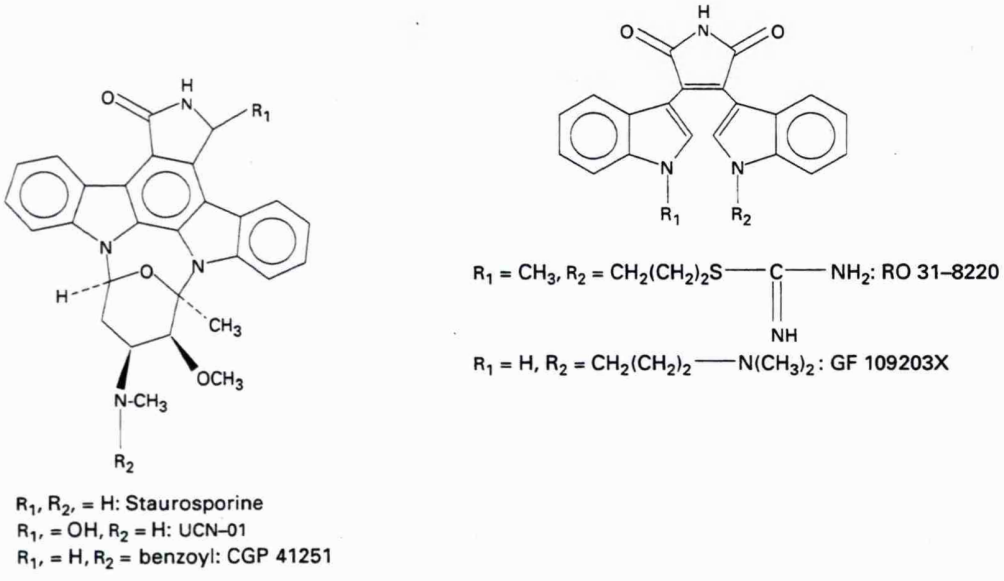

Figure 1 Structures of staurosporine and its four analogues used in this study.

Table I Inhibition of growth of MCF-7 and MCF-7/Adr cells by staurosporine analogues and effect of reserpine

Compound	IC_{50}[a] (μM) MCF-7	IC_{50}[a] (μM) MCF-7/Adr	Ratio (MCF-7/Adr MCF-7)	IC_{50} (μM) MCF-7/Adr + reserpine[b]	Ratio (−Reserpine + reserpine)
Staurosporine	0.0032 ± 0.0001[c]	0.0065 ± 0.0005*[d]	2.0	0.010 ± 0.001	0.7
UCN-01	0.0175 ± 0.001	0.123 ± 0.031*[d]	7.0	0.035 ± 0.006*[e]	3.5
RO 31 8220	0.897 ± 0.013	11.3 ± 2.1***[d]	12.6	1.76 ± 0.16**[e]	6.4
CGP 41215	0.097 ± 0.012	0.283 ± 0.015*[d]	2.9	0.31 ± 0.03	0.9
GF 109203X	7.3 ± 0.9	8.8 ± 0.3	1.5	5.90 ± 0.75	1.5

[a]Concentration which caused inhibition of cell growth by 50%. [b]The IC_{50} of doxorubicin in MCF-7/Adr cells was 1.6 ± 0.1 μM, in the presence of reserpine it was 0.10 ± 0.01, which gave a ratio of 16. [c]Mean ± s.d. of three experiments. [d]Difference to MCF-7 cells. [e]Difference to MCF-7/Adr without reserpine. *$P < 0.005$. **$P < 0.001$. ***$P < 0.0001$.

606 nm respectively. Single- and multi-parameter data of forward angle scatter, side scatter, R123 fluorescence and propidium iodide fluorescence were collected for 10^4 cells. Fluorescence was measured on a four-decade log scale. The data were analysed using Lysis 2 software. Only R123 fluorescence of viable, propidium iodide-excluding cells was analysed.

[^{3}H]Vinblastine accumulation in cells

MCF-7 and MCF-7/Adr cells were grown to confluency in 12-well plates. Medium was replaced with Hepes-buffered RPMI (pH 7.4) supplemented with magnesium chloride (5 mM), glucose (5 mM) and staurosporine analogue (300 nM). [^{3}H]Vinblastine accumulation was estimated as described previously (Ferry *et al.*, 1995). The experiment was started by addition of [^{3}H]vinblastine (1–2 nM) to the incubate. [^{3}H]Vinblastine accumulation reached steady state after 20 min and remained stable for at least 2 h. Cells were incubated at 37°C for 1 h, after which the medium was aspirated and replaced with scintillant (1 ml). Cell-free control incubates showed that absorption of [^{3}H]vinblastine to the well constituted less than 5% of [^{3}H]vinblastine retained by cells.

[^{3}H]Vinblastine binding to P-gp

[^{3}H]Vinblastine binding to P-gp was assessed as described by Ferry *et al.* (1992). For each membrane preparation 10^9 cells were homogenised in 40 ml ice-cold Tris buffer [Tris-HCl 50 mM, phenylmethylsulphonyl fluoride (PMSF) 0.1 mM, pH 7.4] to which 0.1 mM EDTA had been added. The homogenate was centrifuged at 3500 *g* for 10 min; the supernatant was recentrifuged at 40 000 *g* for 20 min, the resultant pellet was resuspended in Tris buffer (10 ml). This membrane pellet was enriched 3.1-fold in saturable [^{3}H]vinblastine binding relative to whole homogenate. Membranes (5–15 μg protein) were incubated for 90 min with [^{3}H]vinblastine (5–10 nM) and staurosporine analogues at various concentrations in Tris buffer (0.25 ml) at 23°C. Bound [^{3}H]vinblastine was separated from free drug by rapid filtration through Whatman GF/C filters prewetted with buffer containing 0.1% bovine serum albumin (BSA). Filters were washed twice with 5 ml of ice-cold Tris-HCl containing magnesium chloride (both 20 mM). Assays were performed in duplicate. Filters were dried and retained radioactivity was quantitated by liquid scintillation counting. Filter blank absorption under these conditions accounted for <0.5% of total filtered radioactivity. These conditions yielded 5000–10 000 d.p.m. total binding and 2000–5000 d.p.m. non-specific binding, defined by 3 μM unlabelled vinblastine. Specific vinblastine binding was only observed in MCF-7/Adr cells and not in their drug-sensitive counterparts (Ferry *et al.*, 1995). Binding data were modelled by non-linear regression using the AR module of BMDP (BMDP Statistical Software, USA) and Kaliedagraph (Ablebeck Software, USA). [^{3}H]Vinblastine binding inhibition curves were analysed by non-linear curve fitting without transformation of the data using d.p.m.

Results

Inhibition of cell growth

In order to explore whether the resistance mechanisms operative in multidrug-resistant MCF-7/Adr cells affect the

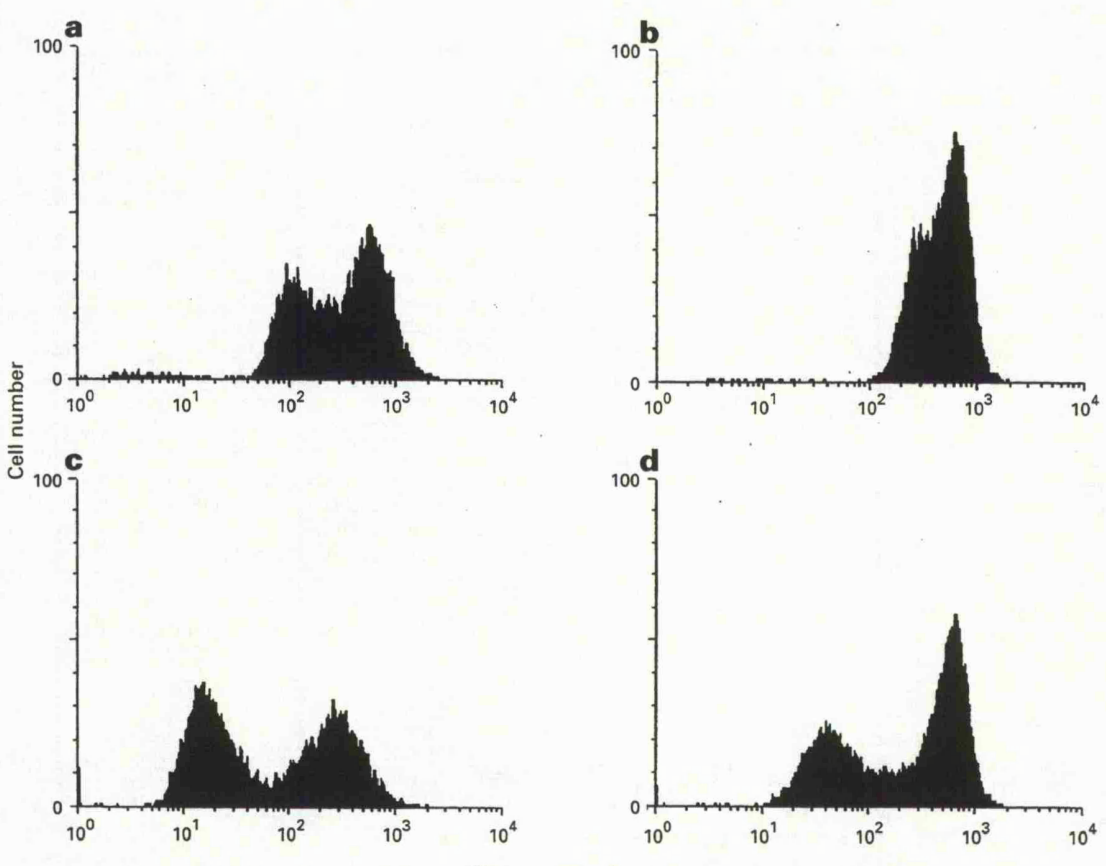

Figure 2 Effect of CGP 41251 on accumulation (**a** and **b**) and efflux (**c** and **d**) of R123 in MCF-7/Adr cells. Fluorescence histograms were obtained in cells treated without (**a** and **c**) or with (**b** and **d**) CGP 41251 (80 nM) for 20 min together with R123 (**a** and **b**) or for 20 min after initial loading with, and removal of, the dye (**c** and **d**).

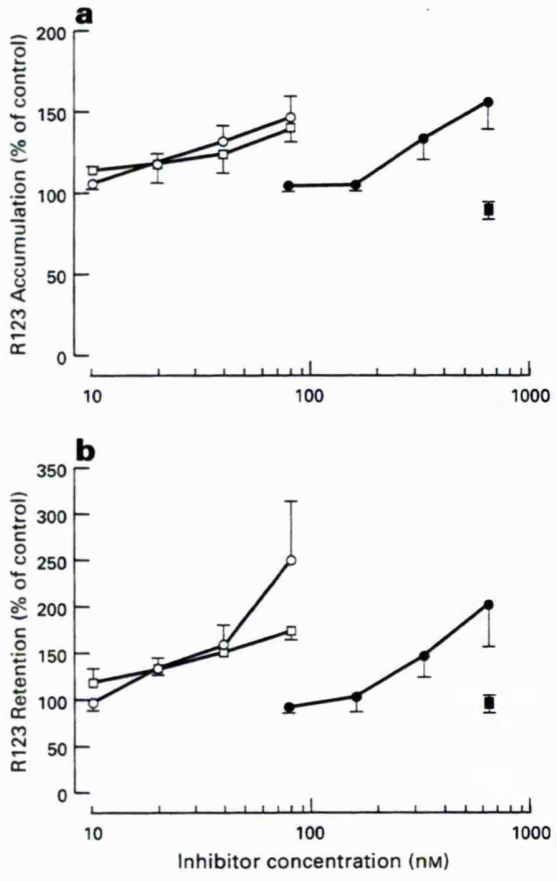

Figure 3 Comparison of the effects of staurosporine (□), CGP 41251 (○), UCN-01 (●), RO 31 8220 and GF 109203X (■) on accumulation (**a**) and efflux (**b**) of R123 in MCF-7/Adr cells. Fluorescence histograms were obtained in cells treated without or with agent for 20 min together with the dye (**a**) or for 20 min after initial loading with, and removal of, the dye (**b**). Data are presented as percentage change in comparison with control cells and they constitute the mean ± s.d. of three experiments. Values for cells with CGP 41251, staurosporine and UCN-01 were significantly different from control cells ($P < 0.05$, Student's *t*-test) in the case of all points except those for staurosporine at 10 and 20 nM in **a**, and 10 nM in **b**, and for UCN-01 at 80 and 160 nM in **b**.

antiproliferative potential of staurosporine and its four analogues, compounds were incubated with cells and growth inhibition was compared with that seen in wild-type MCF-7 cells. The IC_{50} values derived from the growth curves are shown in Table I. Staurosporine was the most potent cytostatic agent in both cell types. The rank order for growth inhibition was staurosporine > UCN-01 > CGP 41521 > RO 31 8220 > GF 109203X in wild-type cells and staurosporine > UCN-01 > CGP 41521 > GF 109203X > RO 31 8220 in drug-resistant cells. MCF-7/Adr cells displayed considerable resistance towards RO 31 8220 and UCN-01, with resistance ratios of 12.6 and 7.0 respectively. The cells were not resistant towards GF 109203X.

Modulation of growth inhibition by reserpine

Reserpine is an effective modifier of P-gp-mediated cytotoxic drug resistance (Pearce *et al.*, 1989). In order to establish the role of P-gp in the resistance of MCF-7/Adr cells towards the staurosporine analogues, cells were grown in the presence of analogue and reserpine. In preliminary experiments reserpine (5 μM) was found to increase the sensitivity towards doxorubicin 14.3-fold. Table I shows that reserpine sensitised cells most effectively against RO 31 8220 and UCN-01. Ratios of IC_{50}s observed in cells without reserpine compared with cells in its presence were 6.4 and 3.5 for RO 31 8220 and UCN-01 respectively. In contrast, reserpine did not change the sensitivity of MCF-7/Adr cells towards CGP 41521 and had only a marginal effect on the cytostasis mediated by staurosporine and GF 109203X. Furthermore, reserpine did not affect the sensitivity of wild-type MCF-7 cells towards RO 31 8220 (result not shown).

Modulation of R123 accumulation and efflux and [^{3}H]vinblastine accumulation

For investigation of the effect of staurosporine and its analogues on R123 accumulation and efflux, MCF-7/Adr cells were incubated with R123 and kinase inhibitors and analysed by flow cytometry. Flow cytometric analysis detected two fluorescent populations in the control MCF-7/Adr cells (Figure 2). These two subpopulations differ in P-gp expression in that the cells with low R123 fluorescence possess more P-gp activity than those which display high dye (Davies *et al.*, 1996). Figure 2 demonstrates the extent to which CGP 41251 at 80 nM increased the accumulation of R123 and decreased its efflux. In order to quantitate the effects of the kinase inhibitors, combined mean fluorescence values of both cell subpopulations in control and treated cells were compared. Figure 3 summarises the effects on R123 accumulation and efflux. R123 levels were significantly increased by 10 nM staurosporine and 20 nM CGP 41251. At 80 nM the increase over control levels in accumulation and retention was 40 ± 8% and 75 ± 10% respectively for staurosporine, and 47 ± 12% and 150 ± 62% respectively for CGP 41521. Significant effects of UCN-01 were seen at 80 nM on R123 accumulation and at 320 nM on dye efflux, whereas RO 31 8220 and GF 109203X did not alter either R123 accumulation or efflux at 640 nM. Therefore, the rank order of potency with respect to modulation of cellular R123 levels by the kinase inhibitors is CGP 41251 = staurosporine > UCN-01 > RO 31 8220 = GF 109203X.

In order to confirm the results observed with R123 the effect of the staurosporine analogues (300 nM) on the accumulation of [^{3}H]vinblastine into cells was studied. Accumulation of [^{3}H]vinblastine into MCF-7/Adr and MCF-7 cells was rapid, reaching steady state by 30 min (Hofmann *et al.*, 1992). Consistent with the data described for the experiments with R123 above, CGP 41251 increased [^{3}H]vinblastine accumulation in MCF-7/Adr cells from 1280 ± 60 d.p.m. in control cells to 3200 ± 160 d.p.m. (mean ± s.d. of 3 experiments). UCN-01 and GF 109203X were less potent, raising [^{3}H]vinblastine accumulation to 2110 ± 90 and 1790 ± 85 d.p.m. respectively. RO 31 8220 had no effect. None of these agents at 300 nM affected [^{3}H]vinblastine accumulation in wild-type MCF-7 cells.

Inhibition of binding of [^{3}H]vinblastine to P-gp

MCF-7/Adr cells overexpress P-gp (Fairchild *et al.*, 1987). Surface membrane preparations containing P-gp were obtained from MCF-7/Adr cells and the ability of staurosporine and its analogues to inhibit the binding of [^{3}H]vinblastine to P-gp was explored. Table II shows that CGP 41521 was the most potent and GF 109203X the least effective inhibitor of vinblastine binding, with K_i values of 32

Table II Inhibition of binding of [^{3}H]vinblastine to P-gp

Compound	*K_i (nM)*
CGP 41251	32 ± 11[a] (6)[b]
Staurosporine	145 ± 20 (4)
RO 31 8220	210 ± 145 (3)
UCN-01	293 ± 120 (3)
GF 109203X	780 ± 190 (4)

[a]Mean ± s.e.m. [b]Number of experiments in parenthesis.

and 780 nM respectively. The rank order of inhibitory potency was CGP 41251 > staurosporine > RO 31 8220 > UCN-01 > GF 109203X. Staurosporine and its analogues are thought to bind to the ATP binding site of PKC. Therefore we explored the possibility that addition of ATP (1 mM) causes a shift in the binding inhibition curve of CGP 41251 to P-gp. ATP did not have an effect, which suggests that the drug did not bind to the ATP-binding domain of P-gp (result not shown). The assay probes for specific vinblastine binding sites, probably P-gp (Ferry *et al.*, 1995), but it has to be stressed that their identity is under discussion and they may include sites other than P-gp.

Discussion

The results outlined above highlight considerable differences in cytostatic and MDR reversing ability between kinase inhibitors of the staurosporine type. In Table III these differences are summarised and juxtaposed with the PKC-inhibitory properties of these compounds. Our results show that staurosporine, UCN-01, CGP 41251, RO 31 8220 and GF 109203X differ 1300-fold in their cytostatic potency against MCF-7/Adr cells, which contrasts markedly with their abilities to inhibit PKC. In the case of PKC derived from the cytosol of MCF-7 cells, IC_{50} values for enzyme inhibition ranged only from 16 nM for staurosporine to 48 nM for RO 31 8220 (Courage *et al.*, 1995).

The resistance observed in MCF-7/Adr cells against the growth-inhibitory properties of RO 31 8220 and UCN-01 is probably mediated by P-gp. This inference can be drawn from the fact that sensitivity of the cells to the two drugs was partially but significantly restored by inclusion of the P-gp inhibitor reserpine into the cellular incubate. That reserpine was unable to reverse resistance fully indicates that it might have a similar binding affinity for P-gp as RO 31 8220 and UCN-01. Alternatively, 5 μM reserpine might have been insufficient to reverse resistance completely. Of the analogues studied, staurosporine and CGP 41251 were the most potent modulators of cellular R123 efflux and of vinblastine binding. However, their cytostatic potential was affected only a little by the presence of P-gp in the resistant cells. These results suggest that, even although both agents have a high binding affinity for P-gp, they are inefficiently transported by it. In contrast, UCN-01 is probably efficiently transported by P-gp as borne out by the resistance of MCF-7/Adr cells to it. Yet UCN-01 has a low binding affinity for P-gp, allowing reserpine to compete effectively for binding to P-gp and to increase its cytotoxic potential in MCF-7/Adr cells. RO 31 8220 displayed properties similar to those of UCN-01, except that it was transported even more efficiently by P-gp than UCN-01 as adjudged by the strong resistance which MCF-7/Adr cells displayed against it. GF 109203X was neither subject to P-gp-mediated resistance nor able to influence R123 efflux. It was also the weakest inhibitor of [^{3}H]vinblastine binding to P-gp among this series of compounds. Therefore GF 109203X seems to lack affinity for P-gp and susceptibility for transport by P-gp. Interestingly, GF 109203X has recently been shown to be an efficient modulator of drug resistance mediated via the MDR-related protein (Gekeler *et al.*, 1995). One of the conclusions which can be drawn from the differences between these compounds (see Table III) is that the MDR-reversing ability of staurosporine analogues appears to be linked to the indolocarbazole structure. Consistent with this conclusion are the results of a preliminary study in which the abilities of CGP 41251 and RO 31 8220 to sensitise MCF-7/Adr cells against doxorubicin were compared. The former at 80 nM increased the sensitivity of cells towards doxorubicin by a factor of 2, but the latter at 2 μM had no effect (J Budworth and A Gescher, unpublished).

How can the differences between the compounds in cytostatic potency and susceptibility towards the effect of reserpine be integrated with those of the short-term assays of R123 accumulation and efflux and [^{3}H]vinblastine binding (Table III)? Of the five compounds investigated, CGP 41251 has the highest affinity to the vinblastine binding site and is hardly subject to resistance or modulation by reserpine. In the light of the high affinity of CGP 41251 for P-gp, these findings may reflect its long dwell time on its P-gp binding site, suggesting that the pump transports the drug slowly. For GF 109203X occupancy of P-gp is likely to be low because of its low affinity. UCN-01 and RO 31 8220, inhibitors with medium affinity for P-gp, can occupy the pump and turnover is high. RO 31 8220 was the most inconsistent of the five molecules in that it did not modulate R123 efflux or [^{3}H]vinblastine accumulation, yet was subject to P-gp-mediated resistance. The reasons for the observed differences are undoubtedly complex. One variable which has not been considered in the interpretation outlined above is the possible effect of these kinase inhibitors on *mdr*1 mRNA levels. Transcription of the *mdr*1 gene is stimulated through a c-raf kinase pathway, which operates downstream from ras (Cornwall and Smith, 1993). Staurosporine has recently been reported to decrease *mdr*1 expression (Bhat *et al.*, 1994; L McKinley and TW Gant, unpublished). Thus we cannot exclude the possibility that the differences in growth-inhibitory properties between MCF-7 and MCF-7/Adr cells described above are a consequence of altered *mdr*1 expression, perhaps via kinase inhibition. Alternatively, in the growth inhibition experiments described above, blockade of P-gp at sub-growth inhibitory concentrations may have elicited accumulation of an endogenous P-gp substrate that in turn increased P-gp expression, thus attenuating the effects of the compounds via a feedback mechanism. These possibilities are currently under investigation.

One of the aims of this study was to probe for the link between PKC inhibition and ability to inhibit P-gp. There seems to be a close functional association between MDR and PKC (O'Brian *et al.*, 1989; Chambers *et al.*, 1990), but the mechanisms involved are unclear. Here we describe staurosporine as a potent growth inhibitor of MCF-7/Adr cells and a good MDR-reversing agent. Staurosporine is one of the most effective PKC inhibitors thus far discovered (Tamaoki and Nakano, 1990), but arguably the least selective of the five agents under investigation here. The PKC-specific staurosporine cogeners used in this study share similar PKC-inhibitory potency (Courage *et al.*, 1995). Yet the results described above suggest that these staurosporine analogues differ dramatically in both ability to reverse P-gp-mediated drug resistance and susceptibility towards transport by P-gp. This discrepancy suggests that inhibition of total

Table III Summary of effects of staurosporine analogues

Compound	Effect on R123 efflux	Resistance in MCF-7/Adr cells	MDR reversal by reserpine	Inhibition of [^{3}H]vinblastine binding	PKC inhibition: Potency	PKC inhibition: Specificity
Staurosporine	+[a]	–	–	(+)	+	–
UCN-01	(+)	+	+	(+)	+	+
CGP 41215	+	–	–	+	+	+
RO 31 8820	–	+	+	(+)	+	+
GF 109203X	–	–	–	–	+	+

[a] +, potent effect; (+), weak effect; –, no effect.

PKC activity by these compounds is not a major mechanism by which they modulate P-gp transport. A similar inference has been drawn previously for staurosporine in other cell types (Miyamato *et al.*, 1993; Wasukawa *et al.*, 1993).

In conclusion, the results presented here indicate that selectivity for PKC is probably not a prerequisite for a staurosporine analogue to possess MDR reversing properties in cells which overexpress P-gp. These molecules differ substantially in ability to bind to and to be transported by P-gp. Among the five kinase inhibitors investigated the indolocarbazoles CGP 412521 and staurosporine have the highest affinity for P-gp but are transported only slowly. Thus they possess the most suitable properties as MDR-reversing agents. Of the two compounds, CGP 41251 is the more attractive drug candidate for potential clinical use, because it lacks the toxicity associated with staurosporine (Meyer *et al.*, 1989).

Abbreviations

BSA, bovine serum albumin; FCS, fetal calf serum; IC_{50}, concentration which inhibits growth by 50%; MDR, multidrug resistance; P-gp, P-glycoprotein; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; R123, rhodamine 123

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