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Role of PKC-mediated phosphorylation on P53 Localization and Function in Neuroblastoma

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ROLE OF PKC-MEDIATED PHOSPHORYLATION ON P53 LOCALIZATION AND FUNCTION IN NEUROBLASTOMA

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the Master of Science

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ABSTRACT

Neuroblastoma (NB) is the most common solid tumour in paediatrics, arising from primitive neural crest cells. The tumour suppressor protein, p53 is inactivated in NB through aberrant cytoplasmic localization, thus contributing to its tumourigenicity and multidrug resistance. Regulation of the p53 response pathway occurs through phosphorylation, however there may be dysregulation of p53 as NB contains abnormally high expression of PKCs. We investigated the role of PKC-mediated phosphorylation as a mechanism responsible for the p53 cytoplasmic sequestration in NB cell lines, IMR-32 and SHSY5Y. A pharmacological approach utilizing protein kinase inhibitors including H7, Bisindolyamide I (BisI), and Gö6976 were tested on their ability to relocalize p53 and reintroduce function. All the inhibitors were able to relocalize p53, however, only the general kinase and PKC inhibitors H7 and BisI, respectively were able to induce a decrease in cell proliferation and cell cycle accumulation as demonstrated by flow cytometric analysis. The conventional PKC isoform inhibitor Gö6976 had no effect on p53 function albeit able to relocalize p53. To further substantiate the effects with H7 and BisI as being p53-dependant, increased expression of Bax and p21 proteins served as a hallmark of p53 function. Antibody-mediated inhibition of PKC allowed us to identify the PKC isozymes involved in p53 regulation. Inhibition of PKCα resulted in accumulation and nuclear relocalization of p53. Furthermore substantiating p53 as a PKC substrate *in vivo*, serine residue phosphorylation of p53 decreased upon PKCα inhibition. We found that two independent PKC phosphorylation events and isoforms are responsible for regulation of p53 relocalization and activation. Specifically for NB, PKC inhibition can initiate and active the p53 response pathway. Electrospray ionization was used to monitor p53 peptide phosphorylation *in vitro*. Spectra revealed that phosphorylation variants ranging from mono- to tri-phosphorylated peptides can be detected. Thus, ESI was verified as an effective method of monitoring *in vitro* phosphorylation of p53 peptides.

DEDICATION

To my family.

ACKNOWLEDGEMENTS

Many thanks to all those you have helped me complete this journey!

Thank you to my supervisors, Dr. Douglas Franks and Dr. Zemin Yao for your invaluable guidance and the many lessons learned. Thank you to the members of my Thesis Advisory Committee, Dr. Micheline Paulin-Levasseur and Dr. David Barnes for your advice and many helpful comments. Many "*merci beaucoup*" to Dr Jenny Phipps for the many years of friendship and always helping me strive for the best. Special thank you to Dr. Saba Siddiqi for your wise words and kind heart. To Dr. Jiahua Chen, thank you for the countless hours laughing in front of the flow cytometer. Thank you Dr. Mitra Sadegihipour for teaching me the art of mass spectroscopy and to laugh often and much. Thank you to Dr. Dawn Song for your generosity and many stories. Much heart-filled thank you to Selma Hassan and Mary Rao, your support during the last few months were a blessing. A big thank you to all the members of PharmaGap Inc., it has been a pleasure to work with each and every one of you!

When I think about everything that we've went through,
maybe it's not the destination that matters,
maybe it's the journey. . .
To the journey.

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LIST OF ABBREVIATIONS

ATM	-	Ataxia-telangectasia mutated kinase
ATP	-	Adenosine Triphosphate
ATR	-	A-T related kinase
BCIP	-	5-bromo-4-chloro-3-indolylphosphate
BisI	-	Bisindolylmaleimide I
BSA	-	Bovine Serum Albumin
CKI	-	Casein Kinase I
CKII	-	Casein Kinase II
DFX	-	deferoxamine mesylate
DiI	-	1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanineperchlorate
DMEM	-	Dulbecco's Modified Eagle's Medium
DNA-PK	-	dsDNA-activated protein kinase
DSS	-	Disuccinimidyl suberate
EDTA	-	Ethylenediaminetetraacetic acid
EGTA	-	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetraacetic acid
ESI	-	Electrospray Ionization
FBS	-	Fetal Bovine Serum
GR	-	glucocorticoid receptor
HDM2	-	human homologue of mouse double minute protein 2
HPLC	-	High Performance Liquid Chromatography
IgG	-	Immunoglobulin G
MARCKS	-	myristoylated alanine-rich C-kinase substrate
NB	-	Neuroblastoma
NBT	-	Nitro Blue Tetrazolium
PAGE	-	Polyacrylamide Gel Electrophoresis
PP1	-	protein phosphatase 1
PP2A	-	protein phosphatase 2A
SDS	-	Sodium Dodecyl Sulfate
PBS	-	Phosphate-Buffered Saline
PI	-	Propidium Iodide
PKC	-	Protein Kinase C
TPA	-	12-*O*-tetradecanoyl phorbol 13-acetate

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1.0 INTRODUCTION

Cellular safeguards are in place to survey and maintain the integrity of the genome, so that mutations and aberrations are not passed on to the progeny. These are critical to ensure the continuation of life but also destruction, if an insurmountable amount of damage accrues. Numerous cellular signals converge on these safeguards and are assimilated to give an appropriate response. Such safeguards have an additional role as tumour-suppressor proteins, which act as 'cellular brakes' to control cell proliferation and keep cell numbers in check. When the safeguards are bypassed or inactivated, cell growth is left unchecked and uncontrolled—a defining characteristic of cancer.

At the heart of many stress signaling pathways is the p53 protein. Inactivation of the p53 protein is at the crux of the majority of cancers; thus, indicative of the importance of this tumour suppressor protein; p53 is no longer able to function as a cellular brake and the cancer cells are in continuous acceleration. Understanding the mechanisms of p53 inactivation has been a focal point of many studies. Unfortunately, as many signals are integrated by p53, many factors can contribute to its inactivation. However, the study of p53 should not seem daunting as insights will aid in the development of chemotherapeutic approaches with a glimmering hope of reclaiming the p53 protein and taming the cancerous beast.

1.1 p53, The Tumour Supressor Protein

p53 is a tumour suppressor protein more affectionately known as the "guardian of the genome." No other protein has engendered as much study since its discovery. It was initially characterized as a SV40 large T binding protein and believed to be an oncogenic protein (Lane and Crawford, 1979; Linzer and Levine, 1979; Kress *et al.*, 1979). The original perception of p53 has changed and evolved to its present status as a pivotal protein in the decision of cellular fate, underlying the fine balance between life and death. The importance of p53 has become increasingly clear as it is the most frequently altered protein in cancer.

1.1.1 p53 Structure and Function

p53 is a transcription factor involved in mediating appropriate responses to cellular stress, such as cell cycle arrest, DNA repair, and apoptosis. The ability of p53 to coordinate a variety of cellular cues and responses is intrinsically linked to its structure. p53 is a 53 kDa nuclear phosphoprotein consisting of 393 amino acids. It is divided into four main functional domains, the N-terminal transactivation domain, central sequence-specific DNA binding domain, and the C-terminal, which contains both the oligomerization and regulatory domains (Figure 1).

The first 42 amino acids include the transcriptional activation domain and the HDM2-binding site. The N-terminus allows p53 to recruit the basal transcriptional machinery to regulate expression of target genes. Residues F19, L22, and W23 have been demonstrated to be required for transcriptional activation of p53 *in vivo* (Lin *et al.*, 1995). The HDM2 protein (human homologue of MDM2, mouse double minute protein)

negatively regulates p53 function and levels by preventing its interaction with the transcriptional machinery and targeting p53 for ubiquitin-mediated degradation (Oliner *et al.*, 1993; Fuchs *et al.*, 1998). Residues L22 and W23 have also been shown to be involved in p53 binding to HDM2 (Lin *et al.*, 1994).

The central domain of p53 (residues 102 to 292) enables the protein to recognize and bind to consensus DNA sequences, PuPuPuC(A/T)(T/A)GPyPyPy-0 to 13 bp-PuPuPuC(A/T)(T/A)GPyPyPy, as identified by El-Deiry *et al.* (1992). 90% of p53 mutations reside within the central region which results in the loss of transcriptional activation function by disrupting p53-DNA contacts or the structural conformation of the p53 protein (Cho *et al.*, 1994).

The C-terminal is home to the tetramerization and regulatory domains. The tetramerization domain (residues 324-355) allows p53 to oligomerize into its functional tetramer unit. The nuclear-cytoplasmic shuttling of p53 is controlled by its nuclear localization signals (NLS) and nuclear export signal (NES). Encompassed within the tetramerization domain is the highly conserved major leucine-rich NES consisting of residues 340-351. Another NES is located in the N-terminal at residues 11-24. There are three NLS: the most active and highly conserved NLSI is located at residues 316-324, while NLS II and III are found at residues 370-376 and 380-386, respectively. The NLS and NES sequences bind to nuclear import and export receptors that allow p53 passage through the nuclear pore complex (Görlich & Mattaj, 1996; Nigg, 1997; Weis, 1998). The utmost end of the C-terminal of p53 can negatively regulate p53-DNA binding activity. The regulatory domain (residues 363-393) acts as a 'hinge' which can

allosterically regulate DNA-binding by interacting with the central sequence-specific DNA binding domain, locking p53 in an inactive form (Hupp *et al.*, 1992).

1.1.2 The p53 Response Pathway

p53 is a short-lived protein with a half-life of approximately 30 minutes. The p53 response pathway can be triggered by many stresses including oncogene expression, DNA damage (i.e. DNA strand breaks, DNA lesions), hypoxia, nucleotide pool depletion, heat shock, and viral infection (Zindy *et al.*, 1998; Palmero *et al.*, 1998; Nelson and Kastan, 1994; Maltzman and Czyzyk, 1984; Fritsche *et al.*, 1993; Graeber *et al.*, 1994; Linke *et al.*, 1996; Matsumoto *et al.*, 1994; de Stanchina *et al.*, 1998; Debbas and White, 1993). p53 can then activate a series of genes in accordance with the stimuli, involved in cell cycle arrest, DNA repair, or apoptosis (Figure 2). Prior to mounting a p53 response to cellular stimuli, several key steps need to occur to p53: stabilization with subsequent accumulation, nuclear relocalization, and activation (Figure 3).

Figure 1. Domain structure of the p53 protein.

The p53 protein consists of 393 amino acids. The N-terminal contains the transactivation domain (residues 1-42) which allows p53 to interact with the transcriptional machinery and a binding site for HDM2, a regulator of p53 stability. The core region is comprised of the sequence-specific DNA binding domain (residues 102-292). The C-terminal contains the tetramerization domain (residues 324-355) which shares common residues with the nuclear export signal (NES) (residues 340-351) and the negative regulatory domain (residues 363-393). The nuclear localization signals (NLS) are located at amino acids 300-323 (NLSI), 370-376 (NLSII), and 380-386 (NLSIII) (not shown). (Adapted from Stommel 1999.)

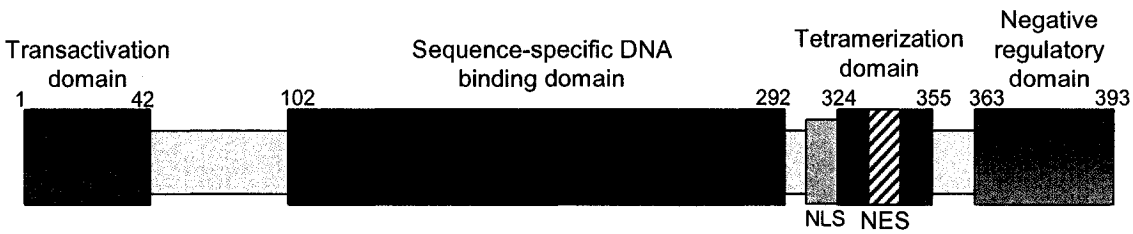

Transactivation domain
1
42
Sequence-specific DNA binding domain
102
292
Tetramerization domain
324
355
Negative regulatory domain
363
393
NLS
NES

Figure 2. The p53 response pathway.

p53 is the central plexus of many converging signals from DNA damaging agents, oncogenes, and other cellular stresses. Latent p53 is activated by post-translation modifications and elicits an appropriate response ranging from transrepression and transactivation of genes involved in cell cycle arrest, DNA repair, and apoptosis. (Adapted from Leblanc and May 2002.)

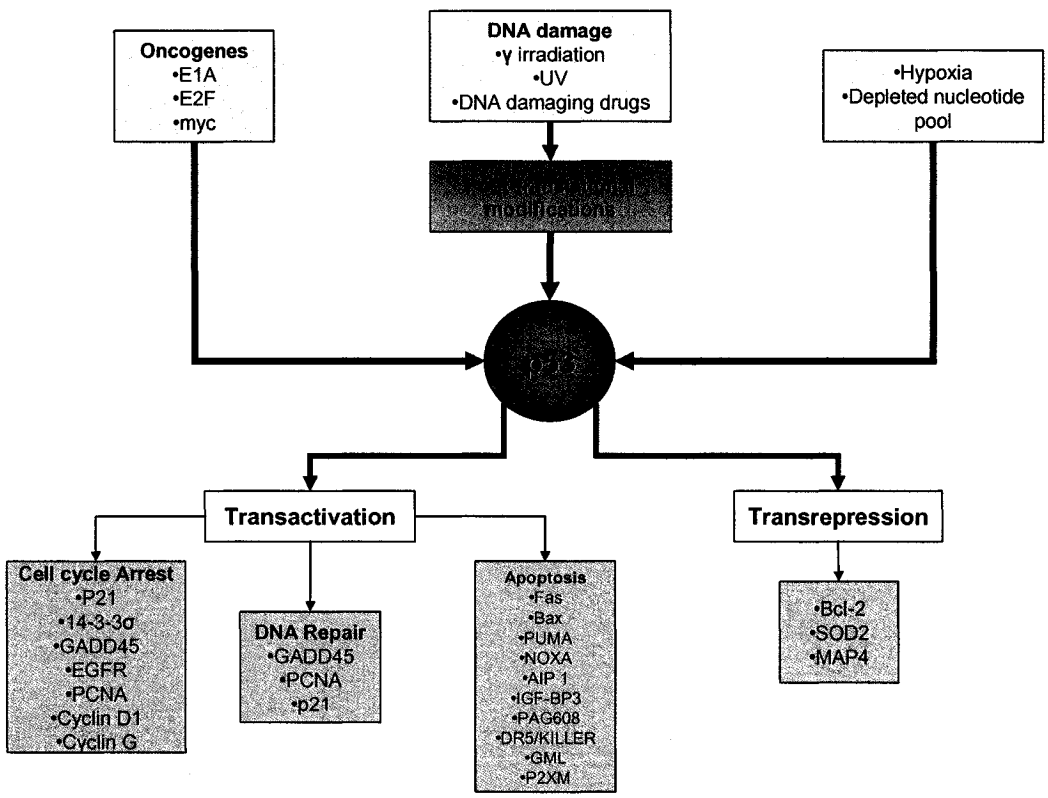
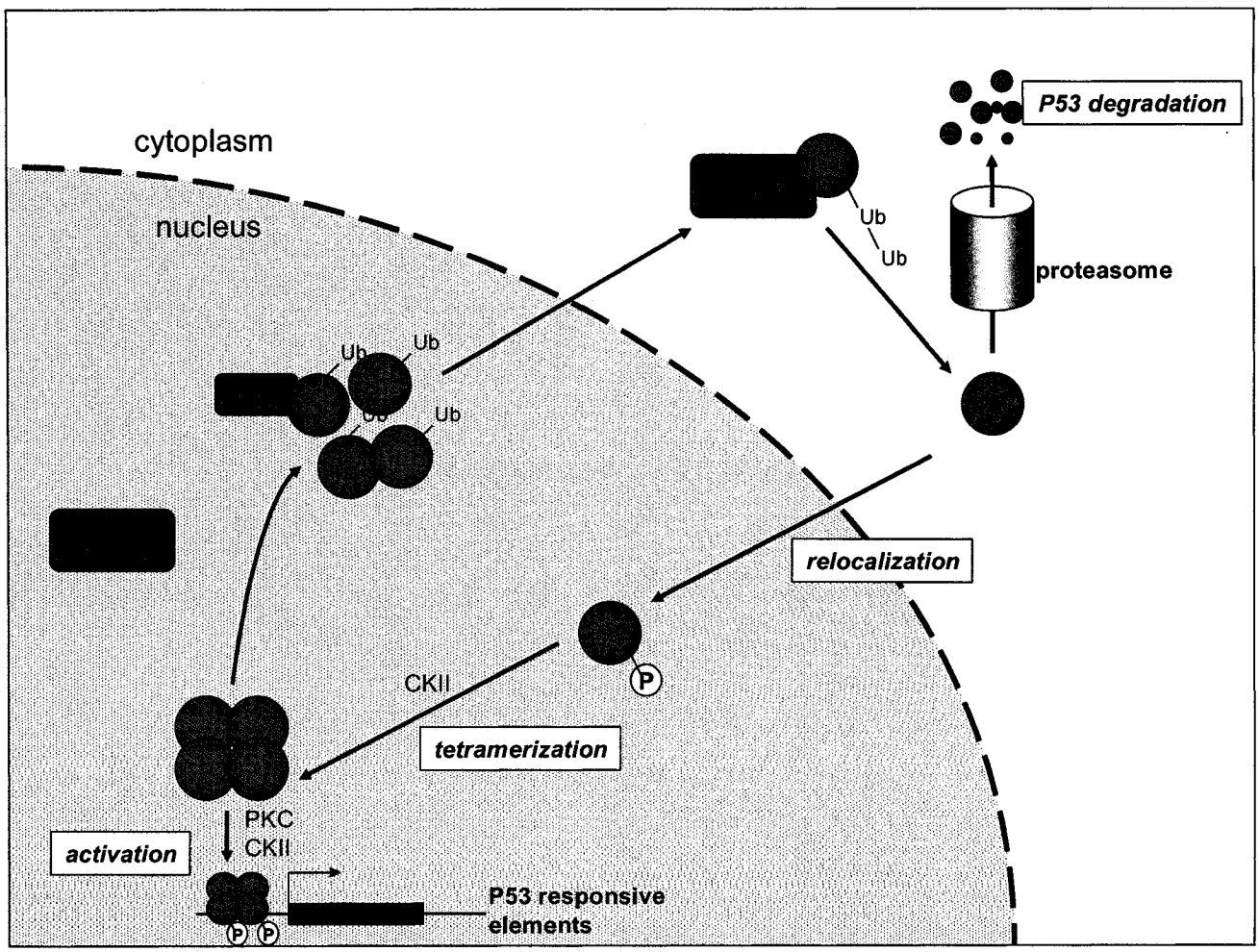

Oncogenes
•E1A
•E2F
•myc
DNA damage
•γ irradiation
•UV
•DNA damaging drugs
•Hypoxia
•Depleted nucleotide pool
modifications
Transactivation
Transrepression
Cell cycle Arrest
•P21
•14-3-3σ
•GADD45
•EGFR
•PCNA
•Cyclin D1
•Cyclin G
DNA Repair
•GADD45
•PCNA
•p21
Apoptosis
•Fas
•Bax
•PUMA
•NOXA
•AIP 1
•IGF-BP3
•PAG608
•DR5/KILLER
•GML
•P2XM
•Bcl-2
•SOD2
•MAP4

Figure 3. Nucleocytoplasmic shuttling of the p53 protein.

In unstressed cells, proteasomal degradation maintains p53 at very low levels. Upon cellular stress, phosphorylation stabilizes and activates p53, for example: phosphorylation of N-terminal p53 sites inhibits p53-HDM2 interaction (not shown) and serine 392 phosphorylation stimulates nuclear accumulation and localization by favouring tetramerization. Activation of p53 requires phosphorylation events within the C-terminal of p53 mediated by CKII and PKC with subsequent transcription of p53 responsive elements involved in cell cycle arrest, DNA repair, and apoptosis depending on the cellular stress. P53 is also regulated by interaction with its negative regulator, HDM2, which has E3-ubiquitin ligase activity. Ubiquitination of p53 promotes dissociation of the p53 tetramer, nuclear export, and proteolytic degradation of p53. (Adapted from Ljungman 2000.)


cytoplasm
nucleus
P53 degradation
Ub
Ub
proteasome
Ub
Ub
Ub
relocalization
P
CKII
tetramerization
PKC
CKII
activation
P53 responsive
elements
P
P

1.2 Regulation of the p53 Response Pathway

1.2.1 General Aspects of p53 regulation

p53 is regulated in a tightly coordinated dance to maintain expression levels, alter cellular sublocalization, and control activation.

HDM2 and p53 are involved in an auto-regulatory feedback loop. p53 activates many target genes, including the *hdm2* gene (Perry *et al.*, 1993; Barak *et al.*, 1993). In turn, HDM2 targets p53 for degradation. In unstressed cells, HDM2 suppresses p53 activity and maintains basal levels. Firstly, HDM2 shares the same binding domain as the transcription factor TFIID within p53. Upon HDM2-p53 binding, the p53 transactivation domain is buried within a hydrophobic cleft of HDM2 which prevents interaction with the transcriptional machinery and inhibits p53's ability to transactivate target genes (Kussie *et al.*, 1996). Secondly, the HDM2-p53 complex induces nuclear export of p53. The RING finger domain of HDM2 contains E3-ubiquitin ligase activity and can ubiquitinate lysine residues in the C-terminal of p53. As a result, the p53 tetramer subunits are disjoined, which exposes the NES allowing interaction with the nuclear export machinery (Lohrum *et al.*, 2001). Lastly, ubiquitination of p53 by HDM2 is not only the preparatory step towards nuclear export but also targets p53 for proteasome-mediated degradation in the cytoplasm (Honda *et al.*, 1997; Haupt *et al.*, 1997; Tao and Levine 1999).

Nucleocytoplasmic shuttling of the p53 protein occurs in a cell-cycle-dependant manner as p53 translocates to the nucleus during the G1/S and G2/M stage of the cell cycle to mediate the cell cycle checkpoints (Shaulsky *et al.*, 1990; Moll *et al.*, 1996; Taylor and Stark, 2001). The dynamic localization of the p53 protein is attributed to its

NLS and NES sequences. The proximity of the NLS and the containment of the NES within the tetramerization domain suggest that oligomerization may regulate p53 localization (Stommel *et al.*, 1999). The formation of p53 tetramers via the tetramerization domains of monomers masks the NES and prevents interaction with components of the nuclear export machinery. Thus, p53 is retained in the nucleus.

Other studies suggest that 'cytoplasmic tethers' are involved in the regulation of p53 cellular localization. An hsp70 family member, Mot2 was identified which interacts with the C-terminal of p53 and inhibits its nuclear translocation, thereby inactivating p53 transactivation in mot-2 transfected NIH3T3 cells (Wadhwa, *et al.*, 1998 and 2002). The anti-apoptotic protein, Bcl-2 in conjunction with c-myc, has been shown to inhibit p53 function by cytoplasmic localization (Ryan *et al.*, 1994; Beham *et al.*, 1997). However, the mechanism has not been clearly elucidated. Nikolaev *et al.* (2003) identified another p53 cytoplasmic anchor, Parc (p53-associated, Parkin-like cytoplasmic protein) which retains p53 in the cytoplasm of unstressed cells. In cooperation with S100B, cPKC can also regulate p53 nuclear accumulation and induce the G1 checkpoint (Scotto *et al.*, 1999).

p53 can exhibit self-regulation of its activation through its C-terminal negative regulatory domain. Binding of the regulatory domain to the central DNA-binding domain locks p53 in a latent form. Phosphorylation or acetylation of the C-terminal has been demonstrated to relieve this allosteric inhibition and activate sequence-specific DNA binding (Hupp *et al.*, 1992).

1.2.2 Regulation of p53 by Phosphorylation

Rapid regulation of signaling proteins is accomplished through post-translational modifications (phosphorylation, acetylation, and sumoylation). In response to cellular stress, p53 is subject to rapid changes in regards to stabilization and activation. There are at least 18 reported post-translationally modified sites in p53 (Appella & Anderson, 2000). Multiple sites in p53 are subject to phosphorylation depending upon the type of stress and the required response (Figure 4).

Stabilization of the p53 protein can occur by abrogation of HDM-2 binding via phosphorylation of residues in the binding interface of the two proteins (Shieh *et al.*, 1997). Serine 15, serine 20, and threonine 18 fall within or around the HDM-2 binding site of p53 and are modified in response to DNA damage. In response to ionization radiation, p53 is stabilized by phosphorylation of serine 15 of p53 by ATM (Ataxia-telangectasia mutated) kinase (Banin *et al.*, 1998; Canman *et al.*, 1998). Serine 20 and threonine 18 are phosphorylated by Chk2 and Casein Kinase I (CKI) respectively (Hirao *et al.*, 2000; Sakaguchi *et al.*, 2000). Serine 15 is also a substrate for dsDNA-activated protein kinase (DNA-PK) (Shieh *et al.*, 1997; Lees-Miller *et al.*, 1992) and is phosphorylated in response to genotoxic agents, such as cisplatin, arsenite, and an hypoxia mimicry, deferoxamine mesylate (DFX) (Persons *et al.*, 2000; Yih *et al.*, 2000; Ashcroft *et al.*, 2000). Upon exposure to UV light, A-T related (ATR) kinase phosphorylates p53 at serine 15 and 37 (Tibbetts *et al.*, 1999). To elicit a p53 response, a series of overlapping phosphorylation events are required while a single phosphorylation event may not be sufficient to modulate p53.

p53 exists in latent and active conformations (Wölcke *et al.*, 2003). A conformational switch needs to occur in order to relieve allosteric inhibition of the p53 DNA-binding activity by the interaction of the C-terminal domain (Hupp *et al.*, 1992). Phosphorylation sites exist in the C-terminal of p53. Both phosphorylation and dephosphorylation of this region are involved in the regulation of p53 tumour suppressor function. PKC phosphorylation of serine 378 within the C-terminal regulatory domain has been shown to activate p53 sequence-specific DNA binding (Takenaka *et al.*, 1995). Phosphorylation of serine 392, which occurs after UV and ionizing radiation *in vitro* and *in vivo* (Blaydes & Hupp, 1998; Wallace *et al.*, 2001) favours p53 tetramerization and subsequently activates p53 transcriptional activity (Sakaguchi *et al.*, 1997).

1.2.2.1 Phosphorylation of p53 by PKC

p53 has been characterized as a PKC substrate (Baudier *et al.*, 1992) with the PKC phosphorylation sites, consisting of serine residues 372, 376, and 378, falling within the C-terminal domain. While the precise PKC isoforms responsible for p53 phosphorylation have yet to be identified, Delphin *et al.* (1997) implicate the conventional Ca^{2+} dependent PKC isoforms in the phosphorylation of p53 *in vitro*.

The functional significance of the phosphorylation sites have not been fully elucidated. The involvement of C-terminal phosphorylation in p53-DNA binding and function has been a subject of argument. Sequence-specific DNA binding *in vitro* is stimulated by phosphorylation of serines 315, 378, and 392 (Hupp *et al.*, 1992). Casein Kinase II (CKII) phosphorylation of the C-terminal of bacterial expressed p53 has been shown to activate cryptic DNA binding activity (Hupp *et al.*, 1992). Alanine mutation of

serine 392 inhibited the cell proliferation suppression ability of p53 (Milne *et al.*, 1992). Hupp *et* al. (1994) also demonstrated activation of p53 DNA-binding activity *in vitro* by phosphorylation of serine 392 by CKII and PKC phosphorylation of serines 376 and 378. These studies provide evidence of the positive regulation of p53 function by phosphorylation. In contrast, abolishment of the serine 392 phosphorylation site was reported to have no effect on p53 function and tetramerization (Fiscella *et al.*, 1994; Roley & Milner 1994). More insight has been shed on the involvement of PKC-mediated phosphorylation on the regulation of p53. As modeled with synthetic p53 peptides, Hoffmann *et al.* (1998) demonstrated that phosphorylation at PKC or CKII sites, serine 378 and serine 392, respectively, decreased non-sequence-specific DNA binding. Following the decrease of non-sequence specific binding, p53 sequence-specific DNA binding function is activated by PKC mediated phosphorylation of serine 378 and reversed by protein phosphatase 1 (PP1) and 2A (PP2A) (Takenaka *et al.*, 1995). PKC activation of p53 DNA-binding activity is also abolished by CKII phosphorylation of serine 392 *in vitro* (Pospíšilová *et al.*, 2004). The phosphorylation status of the PKC sites in p53 is variable, as serine 378 is phosphorylated in irradiated cells, while serine 376 is dephosphorylated in response to genotoxic stress (Waterman *et al.*, 1998). These studies suggest that despite the similar effects of phosphorylation *in vitro*, the functional consequences *in vivo* vary drastically.

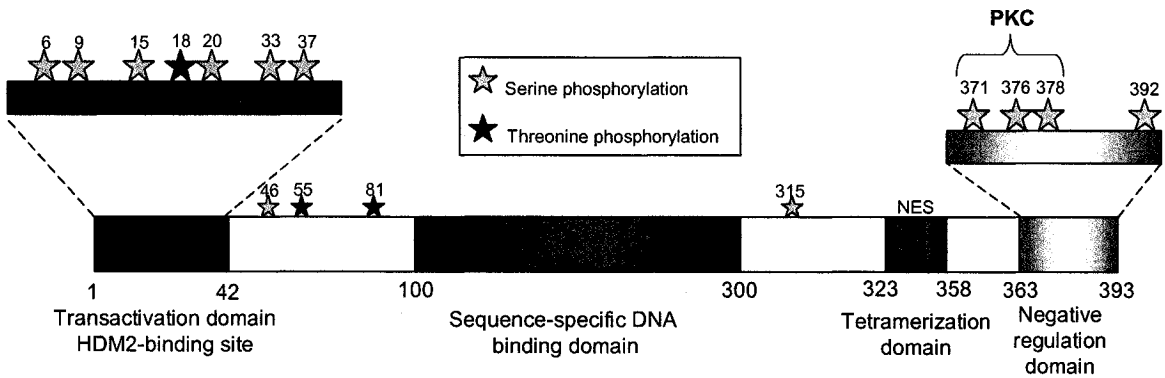
Whether PKC activation or inhibition is necessary for p53 regulation is also controversial. Many of the aforementioned studies indicate that PKC mediated phosphorylation of p53 plays a positive role in the activation of p53 function. This is further supported by Scotto *et al.*, (1999) who found that cPKC activation mediates

nuclear translation and accumulation of p53 in S100B-MEF cells. On the other hand, inhibition of constitutive p53 phosphorylation by the PKC inhibitor, H7, stimulates accumulation of active p53 in mouse (12)1/CA cells (Chernov *et al.*, 2001). Also, p53 ubiquitination and degradation is increased by PKC mediated phosphorylation of the C-terminal of p53 (*ibid*). Furthermore, ceramide-induced apoptosis occurs via inactivation of PKCα in Molt-4 cells (Lee *et al.*, 1996).

The identity of the PKC isoforms involved in p53 regulation has not been fully defined. Several studies highlight PKCα as a regulator of p53 and the events converging towards apoptosis (Lee *et al.*, 1996; Hasan *et al.*, 1999). PKCδ inhibition has been shown to suppress p53 expression via inhibition of p53 synthesis and not through increased p53 protein degradation (Abbas *et al.*, 2004). In contrast, DNA-damage and inhibition of cisplatin-mediated activation of PKCδ is necessary for p53 stabilization in HeLa cells (Johnson *et al.*, 2002). Subunits of PKCζ have been shown to phosphorylate p53 *in vitro* (Youmell *et al.*, 1998).

Figure 4. Phosphorylation sites within the p53 protein.

The p53 protein is subject to a series of phosphorylation events localized primarily in the N- and C-termini. Phosphorylation sites in p53 targeted by PKC (serine 371, 376, and 378) are indicated. Adapted from Leblanc and May 2002.


PKC
6 9 15 18 20 33 37
371 376 378
392
Serine phosphorylation
Threonine phosphorylation
46 55 81
315
NES
1 42 100 300 323 358 363 393
Transactivation domain
HDM2-binding site
Sequence-specific DNA
binding domain
Tetramerization
domain
Negative
regulation
domain

1.3 p53 and its Role in Cancer

The p53 gene was the first tumour suppressor gene to be identified. Though first described in 1979 with its humble beginnings as an oncogene, it is now one of the most significantly altered genes in cancer. In over half of cancers, p53 is non-functional. Inactivation of p53 can occur directly through mutational inactivation or through mutational-independent products which bind and interact with p53.

1.3.1 Mechanisms of p53 inactivation

1.3.1.1 Mutational Inactivation of p53

The p53 gene is located on the small arm of chromosome 17 and is comprised of 11 exons (Slee *et al.*, 2004). Over 90% of p53 mutations reside in the central DNA sequence-specific binding domain (residues 102-292), thus underlying the importance of p53 as a transcription factor. There are two types of mutations that disrupt p53-DNA binding, in residues that: (1) make direct contact with DNA and (2) are required for the structural integrity of the β sheet and loop-sheet helix motif of this domain (Levine, 1997). Residues R248 and R273 are the most frequently mutated residues which contact DNA. Commonly mutated residues required for the structural integrity are R175, G245, R249, and R282. Alterations of the above mentioned residues both result in the loss of p53 transcriptional activity observed in many cancers, including breast, colon, lung, brain, and pancreas (Vogelstein *et al.*, 2000).

1.3.1.2 Mutation-Independent Inactivation Mechanisms of p53

Interestingly, p53 can still maintain a wild-type sequence and yet be rendered non-functional through mutation-independent mechanisms such as nuclear exclusion or cytoplasmic sequestration. In order to mediate the p53 response pathway, it is necessary for p53 to translocate into the nucleus and interact with specific DNA sequences and the transcriptional machinery. Disruption of the nuclear-cytoplasmic shuttling of the p53 protein has been observed in several types of cancers, including breast cancer, colorectal carcinoma, retinoblastoma, and neuroblastoma (NB) (Moll *et al.*, 1992; Sun *et al.*, 1992; Schlamp *et al.*, 2000; Moll *et al.*, 1996). Nuclear exclusion of p53 has been attributed to (1) overexpression of the HDM2 protein, (2) interaction with viral proteins, (3) cytoplasmic anchoring proteins, and (4) hyperactive nuclear export machinery.

Overexpression of the HDM2 protein results in the continual nuclear export and degradation of the p53 protein. The HDM2 gene is amplified in many cancers, including leukemia, sarcomas, and gliomas (Bueso-Ramos *et al.*, 1993; Ladanyi *et al.*, 1993; Reifenberger *et al.*, 1993).

p53 can also be rendered inactive by interaction with viral proteins from human papillomavirus (HPV), SV40, and cytomegalovirus (CMV). The development of cervical cancer is associated with the presence of HPV. The E6 oncoprotein of HPV-18 binds to p53, targeting it for nuclear export and degradation in cytoplasmic proteasomes (Scheffner *et* al., 1990; Freedman & Levine, 1998). Other viral oncoproteins from DNA tumour viruses have also been shown to form complexes with p53 in transformed cells, such as SV40 large T binding protein, adenovirus E1B, Epstein-Barr virus nuclear antigen, and CMV IE84 protein (Lane & Crawford, 1979; Linzer & Levine, 1979; Kress *et al.*, 1979; Sarnow *et*

al., 1982; Szekely *et al.*, 1993; Speir *et al.*, 1994). CMV can sequester p53 in the cytoplasm by inhibiting p53 NLS function (Kovacs *et al.*, 1996; Wang *et al.*, 2001).

Cytoplasmic anchors may physically tether p53 in the cytoplasm and prevent its nuclear translocation. Parc (p53-associated parkin-like cytoplasmic protein) has been identified as a cytoplasmic anchor for p53 and subsequently, acts as a regulator of p53 subcellular localization and function (Nikolaev *et al.*, 2003). It is linked to p53 cytoplasmic sequestration in NB as it is overexpressed in NB cell lines and found to be primarily bound to cytoplasmic p53.

Dysregulation in the nuclear transport machinery resulting in hyperactive export could be another mechanism conferring cytoplasmic localization of p53. For example, Stommel *et al.* (1999) demonstrated that the appearance of static p53 cytoplasmic sequestration is a result of an imbalance in nuclear import and export in NB.

1.4 Neuroblastoma (NB)

NB is the most common form of solid tumour in paediatrics. It arises from primitive sympathetic neural precursors in the embryonal neural crest. Often, it manifests itself in the adrenal medulla gland or within other sites of sympathetic nervous tissue, such as the paraspinal sympathetic ganglia (Triche *et al.*, 1986; Brodeur, 2003). NB is one of the rare cancers that can regress spontaneously. However, children more than one year of age oftentimes have advanced disease progression upon diagnosis with poor prognosis. Despite aggressive therapy, the disease continues to evolve and metastasis occurs with no avail. Genetic abnormalities, such as ploidy status, oncogene amplification, and allelic loss have been shown to correlate with therapy response and

outcome. For example, favourable tumours are characterized with near-triploidy (Kanako *et al.*, 1987). In contrast, tumour with deletions of the short arm of chromosome 1 or allelic loss of 11q, and/or amplification of the N-MYC protocogene have an unfavourable outcome (Brodeur *et* al., 1981; Brinkschmidt *et al.*, 1997; Seeger *et al.*, 1985).

NB tumours are comprised of a heterogeneous subpopulation of cell types (Bernal *et al.*, 1983; Ross *et al.*, 1983). The N-type cells resemble their neuroblastic origins and contain a small cell body with short neuritic processes. The epitheliod-like S-type cells possibly originate from Schwann/melanoblast progenitors (Ciccarone *et al.*, 1989). A third NB cell type, the I-type cells exists which shares morphology and characteristics of both N- and S- cell types. I-type cells have been demonstrated to be an intermediate between N- and S- types and are able to transdifferentiate toward either cell type (Ross *et al.*, 1994).

1.4.1 p53 and Neuroblastoma

In the majority of cancers, p53 is subject to mutational inactivation. Curiously in NB, there is an absence of any p53 gene mutations as demonstrated in NB tumour cell lines and tumour specimens (Komuro *et al.*, 1993; Vogan *et al.*, 1993). Rather the p53 protein is rendered non-functional due to aberrant cytoplasmic localization (Moll *et al.*, 1995 and 1996) and thus is believed to contribute to tumourigenicity and multidrug resistance (Keshelava *et al.*, 2000 and 2001).

NB elegantly demonstrates cytoplasmic localization of p53. Though there are differences in subcellular p53 localization between the NB types and in undifferentiated and differentiated NBs (Isaacs *et al.*, 1998; Moll *et al.*, 1995). p53 function is abolished

in N-type cells due to cytoplasmic sequestration. In contrast, p53 is capable of nuclear translocation after DNA damage in S-type cells.

The mechanism(s) responsible for the abnormal p53 cytoplasmic sublocalization in NB has yet to be identified. p53 cytoplasmic localization is dependant on nuclear export and cytoplasmic retention. Nuclear export can be mediated by HDM2. A case of HDM2 amplification in a neuroblastoma tumour at relapse and the derived cell lines has been reported (Corvi *et al.*, 1995). In more detail, HDM2 mediated nuclear exclusion of p53 in NB has been supported by Rodriguez-Lopez *et al.* (2001) who demonstrate attenuated etoposide-mediated p53 dependant apoptosis in SH-EP1 cells which express 10-fold higher levels of HDM2 as compared to SHSY5Y cells. In addition, p53 cytoplasmic localization could also be due to hyperactive nuclear export (Stommel *et al.*, 1999). Cytoplasmic retention can also contribute to p53 cytoplasmic localization by preventing its transport into the nucleus. In NB, the glucocorticoid receptor (GR) has been shown to retain p53 in the cytoplasm and GR antagonists reintroduce p53 nuclear accumulation (Sengupta *et al.*, 2000). An anchoring protein, such as Parc, has been identified to be overexpressed in NB cell lines. Nikolaev *et al.* (2003) report that a large fraction of cytoplasmic p53 is bound to Parc, and RNAi suppression of Parc expression induces nuclear accumulation of active p53 and resensitization of NB to DNA-damaging agents. The mechanisms responsible for p53 cytoplasmic localization are varied and it is quite possible that dysregulation of several of the mechanisms contributes to the aberrant cytoplasmic localization observed in NB, and other carcinomas.

1.5 PKC and Cancer

1.5.1 Protein Kinase C (PKC)

Protein kinase C (PKC) comprises a family of serine/threonine kinases categorized into three groups, classical (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical isoforms (ζ, ι, λ), as based on domain structure and activator requirements (Mellor & Parker, 1998). The classical isoforms are activated by phorbol esters and are calcium sensitive, while the novel isoforms are only activated by phorbol esters. The activity of the atypical isoforms is independent of both calcium and phorbol esters. PKC is involved in various signal transduction pathways and is a critical regulator of cell proliferation, differentiation, transformation, and apoptosis (Hug & Sarre, 1993; Blumberg *et al.*, 1994; Gutcher *et al.*, 2003). The exact role of PKC in the regulation of cellular function is very complex and cell type-dependant. Each PKC isoform may have a specialized role and be influenced by the cellular background. For example, overexpression of PKCβI in HT-29 cells caused growth inhibition and tumour suppression (Choi *et al.*, 1990). In contrast, Courage *et al.* (1995) demonstrated that staurosporine-induced inhibition of PKC caused growth arrest. Considering the differential effects and distribution of the PKC isoforms, it will be necessary to define the PKC isoforms, in particular pertaining to NB for this study.

1.5.2 PKC and Neuroblastoma

Neuroblastoma (NB) cells express PKC isoforms, PKCα, βI, βII, δ, ε (Zeidman *et al.*, 1999a ; Lahn *et al.*, 2004). Activation of PKC with the phorbol ester, TPA under serum-free conditions has been shown to promote cell survival (Zeidman *et al.*, 1999a). Treatment of the NB cell line, SHSY5Y with TPA at a concentration and exposure conditions that down-regulates PKC leads to differentiation of cells to a neuronal phenotype as identified by neurite extension and expression of neuronal markers (Heikkila *et* al., 1989; Påhlman *et al.*, 1981; Parrow *et al.*, 1992). Neurite extension can also be triggered by PKC inhibitors H7, Gö6976, and staurosporine (Heikkila *et* al., 1989; Zeidman *et al.*, 1999a; Shea & Beermann, 1991). Studies have shown that PKCβI has a positive effect on growth and proliferation and that PKCβ inhibition by LY379196 attenuates multidrug resistance of NB cells (Svensson *et al.*, 2000; Svensson & Larsson, 2003). Of the novel isoforms, PKCε has been identified as a regulator of neurite outgrowth during NB cell differentiation (Fagerstrom *et al.*, 1996; Zeidman *et al.*, 1999b & 2002). Abnormal overexpression of PKCs has been observed in many cancers, including NB (Lahn *et al.*, 2004) and in biopsies (Phipps lab, unpublished). Activation of PKCα has been shown to result in increased tumour cell proliferation, loss of differentiation ability, and inhibition of apoptosis in NB (Lahn *et al.*, 2004; Leli *et al.*, 1992). It is possible that many characteristic features of NB, e.g. multi-drug resistance, uncontrolled cell proliferation, and inhibition of apoptosis can be attributed to PKC overexpression. However, the physiological substrate of PKC activity has not been ascertained in NB, PKC could be acting upon (1) signal transduction proteins (growth factor receptors, Ras, Raf, GAP, and MAPK cascade), (2) channel pumps, or (3)

transcription factors (Myc, Fos, and p53) (Kikkawa & Nishizuka, 1986; Magnelli & Chiarugi, 1997). In fact, all PKCs show activity on the same substrates. The specificity is assured by the sublocalization of the different isoforms and their phenotypic expression.

1.5.3 PKC and p53 in Neuroblastoma

p53 is heavily influenced by phosphorylation events as such, alterations in PKC activity will, in turn, have an effect on the p53 response pathway.

Specifically studies demonstrate that PKC has a role in p53-dependant apoptosis in NB (Ronca *et al.*, 1997). SHSY5Y cells exposed to H7 increased the half-life of the p53 protein and induced nuclear accumulation and apoptosis as characterized by DNA fragmentation and chromatin condensation. This study suggests that the cytotoxic effect of H7 occurs via a p53-dependant pathway.

Currently, the link between p53 regulation and PKC in neuroblastoma has yet to be fully evaluated. There are no studies which suggest dysregulation of PKC-mediated phosphorylation of p53 as a contributing factor to the inactivation of the p53 in NB.

1.6 RATIONALE AND OBJECTIVES

Inactivation of the p53 protein is a key alteration in many cancers. The majority of cancers contain inactivating mutations in the p53 protein. Curiously in some cancers, such as NB, p53 retains its wild-type sequence though is inactivated through cytoplasmic sequestration. Unfortunately, the underlying mechanisms responsible for p53 cytoplasmic localization in NB are poorly defined and have been previously attributed to binding partners and cytoplasmic tethers. The regulatory mechanism of p53 stabilization,

accumulation, localization and activation is deeply rooted in the phosphorylation status of p53. It is currently unknown how phosphorylation of the p53 protein contributes to the p53 phenotype in NB.

The goal of this study is to investigate the role of PKC-mediated phosphorylation on the regulation of p53 localization and function in NB, in an attempt to implicate dysregulation of post-translational modifications, i.e. phosphorylation by PKC on the aberrant cytoplasmic p53 localization in NB. We utilize a pharmacological approach using selective protein kinase inhibitors and characterize the effect on p53 localization and function in human NB cells following exposure to the inhibitors.

The objectives of this study can be summarized as follows:

1. to characterize the effect of PKC inhibitors on p53 localization and accumulation in human NB cells;
2. to correlate alterations in cell proliferation, cell cycle, and p53-responsive elements with reintroduction of p53 in the nucleus by PKC inhibition;
3. to identify the PKC isoforms responsible for modulating p53 in NB; and
4. to explore the potential of electrospray ionization as a possible technique for the study of phosphorylated p53.

2.0 MATERIALS AND METHODS

2.1 Cell Lines and Culture Conditions

Two human neuroblastoma cells lines, IMR-32 and SHSY5Y were cultured at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI respectively. DMEM was supplemented with supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (50 U/mL), streptomycin (50 ug/mL), nucleosides, nonessential amino acids (0.1 mM), L-glutamine (2 mM), sodium pyruvate (1 mM), and gentamicin (20 ug/mL). RPMI was supplemented with 10% (v/v) FBS, penicillin (50 U/mL), and streptomycin (50 ug/mL). Cells were grown in $75cm^2$ flasks and routinely trypsanized every third day. Medium was removed from the flask and the cell monolayers were washed with Ca^{2+} and Mg^{2+} free-phosphate buffered saline (PBS). 0.15% Trypsin-EDTA was added for 3 minutes to allow disociation of cells. The cells were resuspended in medium and centrifuged at 1000 rpm at 4°C for 5 minutes. The supernatant was discarded and the cells were resuspended in fresh medium. The cells were counted on a hemocytometer in the presence of trypan blue.

2.2 Cell Treatments

Cells were treated with protein kinase inhibitiors at a final concentration of 75 uM H7 (Sigma), 5 uM Bisindolylmaleimide I (BisI) (LC Laboratories), or 200 nM Gö6976 (Calbiochem). In addition, cells were treated with the SuperTIP peptide, PPLSMPRFMDYWEGLNENG (100 uM final concentration). SuperTIP was synthesized in our laboratory via the solid phase synthesis method, purified by HPLC, and analyzed

by mass spectroscopy. The regular medium, DMEM or RPMI was replaced with medium supplemented with one of the aforementioned compounds. Cells were incubated at 37°C for either 24, 48, or 72 hours.

2.3 Influx Pinocytic Cell-Loading

To specifically inhibit activity of the PKC isoforms, antibodies against the various PKC isoforms were introduced into the cells using the Influx™ Pinocytic Cell-Loading Reagent (Molecular Probes) as described in the manufacturer's protocol. Cells were trypsanized as usual and 1 x 10^6 cells were incubated with 20μL of prewarmed solution of PKC antibody and Hypertonic Loading medium (sucrose, polyethylene glycol, 20% FBS, 0.01 M HEPES (pH 7.4) in serum-free culture medium) at a 1:5 dilution for 10 minutes at 37°C. To the cell suspension, 1mL of Hypotonic lysis medium was added and then an additional 2 mL was added. Cells were further incubated for 1.5 minutes at 37°C and pelleted by centrifugation for 1 minute at 2000 rpm. Supernatant was removed and cells were resuspended in growth medium. Cells were counted and seeded on coverslips or in petri dishes.

2.4 Antibodies

Antibody	Supplier	Application Dilution		
		Immunocytochemistry	Immunoblotting	Flow Cytometry
p53(C-19) goat	Santa Cruz	1:250	1:1000	1:500
p53(FL-393) rabbit	Santa Cruz	1:250	-	-
p21(C-20) rabbit	Santa Cruz	1:500	1:1000	1:200
bax (N-20) rabbit	Santa Cruz	1:500	1:1000	1:200
cPKCα (C-20) rabbit	Santa Cruz	1:500	1:1000	-
PKCβI (C-16) rabbit	Santa Cruz	1:500	1:1000	-
PKCβII (C-18) rabbit	Santa Cruz	1:500	1:1000	-
PKCγ (C-19) rabbit	Santa Cruz	1:500	1:1000	-
PKCδ (C-20)rabbit	Santa Cruz	1:500	1:1000	-
nPKCε (C-15) goat	Santa Cruz	1:500	1:1000	-
nPKCζ (C-20) goat	Santa Cruz	1:500	1:1000	-
PKCμ (C-20) rabbit	Santa Cruz	1:500	1:1000	-
phosphoMARCKS rabbit	Signal Transduction Inc.	-	-	1:500
phosphoserine mouse	Sigma	-	1:500	-
β-tubulin (H-235) rabbit	Santa Cruz	-	1:1000	-
AlexaFluor 488nm goat anti-rabbit IgG	Molecular Probes	1:1000	-	1:1000
AlexaFluor 488nm donkey anti-goat IgG	Molecular Probes	1:1000	-	1:1000
Goat anti-rabbit alkaline phosphatase IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc.	-	1:5000	-
Donkey anti-goat alkaline phosphatase IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc.	-	1:5000	-
Goat anti-mouse alkaline phosphatase IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc.	-	1:5000	-

2.5 Immunocytochemistry

Cells grown on coverslips were rinsed three times with PBS containing Ca^{2+} and Mg^{2+}, fixed with 4% paraformaldehyde for 30 minutes, and permeabilized with 0.1% Triton X-100 in PBS for 10 minutes. Coverslips were washed three times with PBS. Non-specific sites were blocked with a 1% BSA in PBS for 1 hour prior to addition of primary antibody solution made up in 1% BSA. Coverslips were incubated with primary antibody solution for 1 hour at room temperature or overnight at 4°C with gentle agitation. Coverslips were washed three times with PBS and incubated with a 1:1000 dilution

secondary antibody solution prepared in 1% BSA in PBS for 1 hour. Coverslips were washed three times with PBS. In addition, nuclei were stained with 5 μg/mL Hoechst 33258 in PBS for 10 minutes. Coverslips were mounted onto glass slides for observation. Images were taken on a Nikon Olympus Microscope equipped with Epifluoresence. IMT2-DMG and IMT2-DMV filters were utilized at an emission of 515 nm, excitation of 405-425 nm and excitation of 405 nm and 455 nm, emission greater than 455 nm respectively. Images were analyzed using ImagePro Software and pseudocoloured green while hoechst images were pseudocoloured red.

2.6 Flow Cytometry

Flow cytometric analysis was performed on a Beckman Coulter Cytomics FC 500. Modfit software was utilized for data analysis.

2.6.1 Cell Proliferation—DiI Staining

To monitor cell proliferation, cells were stained with a lipophillic fluorescent marker, DiI (Molecular Probes) prior to exposure to protein kinase inhibitors. Cells were trypsanized according to normal protocols, pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C, and washed with DMEM containing no FBS (DMEM, 0% FBS). 1 x 10^6 cells were resuspended in 2mL DMEM, 0% FBS containing 4 uL of DiI solution and incubated for 20 minutes at room temperature. Solution was agitated every 5 minutes. Cells were pelleted by centrifugation and washed twice with DMEM, 0% FBS and resuspended in growth medium containing FBS. Cell concentration was determined for subsequent seeding and treatment. At various time intervals, cells were harvested by

trypsinization, washed with PBS containing no Ca^{2+} and Mg^{2+}, and resuspended in an appropriate amount of 1% BSA in PBS for subsequent analysis by flow cytometry.

2.6.2 Cell Cycle Analysis—Propidium Iodide Staining

1 x 10^6 cells were seeded in 10 cm Petri dishes. After exposure to the various protein kinase inhibitors, cells were collected by trypsinization and pelleted by centrifugation at 1000 rpm for 5 minutes at 4°C. Pellet was washed two times with PBS containing no Mg^{2+} and Ca^{2+}. For fixation, cells were resuspended in 2 mL of 4% paraformaldehyde in PBS and incubated on ice for 20 minutes. Cells were washed two times with PBS with centrifugation at 2000 rpm for 5 minutes at 4°C between each wash. For cellular permeabilization, 2 mL of ice cold ethanol was added and cells were incubated for 10 minutes on ice. At this point, cells were either stored at -20°C for future analysis or protocol was followed as described. Cells were washed two times with 1% BSA in PBS with centrifugation at 2000 rpm for 5 minutes at 4°C between each wash. RNA was digested by incubation of cells in 1 mL of 10 ug/mL RNase in 1% BSA in PBS for 30 minutes at 37°C. DNA was stained upon addition of propidium iodide for a final concentration of 50 ug/mL for 15 minutes at 37°C. Afterwards, cells were pelleted by centrifugation and washed once with PBS. Sample was resuspended in appropriate amount of PBS for subsequent analysis by flow cytometry.

2.6.3 Protein Expression Levels—Fluorescence Measurement

1 x 10^6 cells were seeded in 10 cm Petri dishes. After exposure to the various protein kinase inhibitors, cells were harvested by trysanization, and washed with PBS

containing no Ca^{2+} and Mg^{2+}. Cells were resuspended in 5 mL of 4% paraformaldehyde in PBS containing no Ca^{2+} and Mg^{2+} and incubated on ice for 15 minutes. After fixation, cells were pelleted by centrifugation (2000 rpm, 5 minutes, 4°C), washed once with PBS, and resuspended in 2 mL of 70% ethanol. At this point, samples were stored at -20°C until all timepoints were collected. Cells were rehydrated by addition of 2 mL of 1% BSA in PBS and incubated on ice for 5 minutes. Cells were pelleted and incubated in PBS for 5 minutes. Non-specific binding was blocked by incubation with 1 mL of 1% BSA and goat/donkey serum (depending on secondary antibody species) in PBS for 30 minutes at room temperature with inversion every 10 minutes. 1 ml of the appropriate primary antibody at the desired concentration made up in blocking solution was added to each sample and further incubated for 1 hour at room temperature with inversion every 15 minutes. Samples were washed once with PBS before addition of 1 ml of secondary antibody solution prepared in blocking solution and incubated at room temperature for 1 hour with inversion every 15 minutes. Cells were washed twice with PBS and resuspended in an appropriate amount of blocking solution for flow cytometric analysis.

2.6.4 Isolation and Immunostaining of Nuclei for Flow Cytometry

1 x 10^6 cells grown in 10 cm Petri dishes were washed twice with cold PBS containing Ca^{2+} and Mg^{2+}. 1 mL of hypotonic buffer (10 mM HEPES (pH 8.0), 0.5mM KCl, 2 mM $MgCl_2$) was added to each Petri and placed on ice for 10 minutes. Samples were collected and vortexed for 5 minutes. Nuclei were pelleted by centrifugation at 2500 rpm for 5 minutes at 4°C. Nuclei were fixed in 1% (w/v) paraformaldehyde prepared in XBE2 buffer (10 mM HEPES (pH 7.7), 100 mM KCl, 0.1 mM $CaCl_2$, 2 mM $MgCl_2$, 5

mM EGTA, 50 mM sucrose) for 15 minutes at room temperature and washed twice with XBE2 buffer with centrifugation at 1500 rpm for 5 minutes at 4°C between each wash. 2 mL of ice cold 70% ethanol was added to each sample and incubated on ice for 10 minutes. Samples were stored at -20°C until all timepoints were collected or the protocol was followed as described. Nuclei were washed once with XBE2 buffer prior to incubation with goat p53(C-19) IgG at a dilution of 1:200 prepared in 1% BSA in PBS for 1 hour at room temperature. Nuclei were washed again and incubated with the secondary antibody, AlexaFluor 488nm anti-goat IgG at a dilution of 1:500 prepared in 1% BSA in PBS for 1 hour at room temperature. Samples were washed twice and resuspended in an appropriate amount of PBS for subsequent analysis by flow cytometry.

2.7 Protein Extractions

2.7.1 Total Protein Extraction—RIPA

Cells grown in 10 cm petri dishes were washed with PBS containing no Ca^{2+} and Mg^{2+} and lysed with 350-500 uL of RIPA buffer (1% Nonidet-P40, 0.5% sodium deoxycholate, 0.1% SDS in PBS without Ca^{2+} and Mg^{2+}) containing 100 ug/mL PMSF, 1 mM sodium orthovanadate, 1ug/mL aprotonin, and 9.9 ug/mL leupeptin. Cells were scraped into eppendorfs and passed through a 26 gauge needle 10 times to shear DNA. Samples were incubated on ice for 30 minutes and centrifuged for 30 minutes at 14 000 rpm at 4°C in a Eppendorf Centrifuge 5415C. Supernatant was collected and stored at -70°C.

2.7.2 PKC Membrane Fractionation

Cells grown in 20-cm Petri dishes were lysed with hypotonic lysis buffer (1 mM $NaHCO_3$ and 5 mM $MgCl_2$) and incubated on ice for 5 minutes. Cells were then scraped and vortexed at room temperature for 2 minutes. To sediment the nuclei and unlysed cells, samples were centrifuged for 5 minutes at 2500 rpm, 4°C. Supernatant was collected and subjected to further centrifugation for 10 minutes at 100 000g, 4°C. Cytosolic supernatant fraction was collected and membrane pellet was resuspended in 2X assay buffer (50mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 uM $CaCl_2$, 200 uM $NaVO_4$, 2 mM NaF, 200 uM PMSF, 200 uM $Na_4P_2O_7$). Samples were stored at -70°C.

2.7.3 Protein Estimation

Protein concentration was determined by addition of 10 uL of protein sample to 1990 uL water, and 200 uL of BioRad protein assay reagent solution, and measuring the absorbance at 596 nm on an HP Dioarray Spectrophotometer. Protein concentration was calculated according to the values of a BSA standard curve.

2.8 Immunoprecipitation

500 μg of protein was incubated with 10 uL p53(FL-393) antibody (200 ug/mL) for 1 hour at 4°C on a shaking table. 20uL protein A/G plus-agarose IgG was added and incubated overnight at 4°C. Pellet was washed with 4 times with RIPA buffer, resuspended in 25 uL 1X electrophoresis sample buffer and boiled for 90 seconds. 25 uL of the immunoprecipitated protein was loaded onto a 10% SDS-PAGE gel.

2.9 Immunoblotting

Normalized proteins were loaded onto a 10% SDS-poly acrylamide gel, electrophoresed at 127 mV (25 mA) and electrophoretically transferred for 1 hour at a fixed voltage of 100 V onto a nitrocellulose membrane (Biorad). The primary antibodies were used at a 1:1000 dilution. The blots were incubated with the primary antibody for 1 hour at room temperature or overnight at 4°C. A 1:5000 dilution of rabbit anti-goat alkaline phosphatase conjugated IgG (Jackson) was placed on the blots for 1 hour and exposed to the alkaline phosphatase substrate, 0.01% (w/v) 5-bromo-4-chloro-3-indolylphosphate (BCIP) and 0.02% (w/v) nitro blue tetrazolium (NBT) (Sigma) in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) at 37°C. The reaction was stopped by rinsing with 5% acetic acid.

2.10 Total Protein Staining

2.10.1 Ponceau S

Following Western blotting, the membranes were stained in a Ponceau S solution (0.1% (w/v) in 5% acetic acid) (Sigma) for 10 minutes. The membranes were rinsed in several changes of water until background was removed and protein bands were apparent. Membranes were documented and remaining Ponceau S stain was removed by washing in several changes of water. Membranes were then probed with an appropriate antibody.

2.10.2 MemCode™ Reversible Protein Staining

Total protein was visualized using the MemCode™ Reversible Protein Stain Kit from Pierce according to manufacturer directions. Following Western blotting, membranes

were rinsed with ultrapure water and incubated with 25 mL MemCode™ Reversible Protein Stain for 30 seconds at room temperature. Background staining was removed by addition and subsequent decanting of 25 ml MemCode™ Destaining Reagent three times and with a final rinse incubated for 5 minutes. Membrane was rinsed with water four times and a final wash incubated for 5 minutes. Membranes were documented and stain was erased by incubation with 30 mL MemCode™ stain eraser for 5 minutes, followed by washing with water. Membranes were then probed with an appropriate antibody.

2.11 *In vitro* PKC Phosphorylation Assay

1 ug/uL of Peptide46, GSRAHSSHLKSKKGQSTSRHKK (Bachem), a p53 C-terminal peptide was incubated in reaction buffer (3.75 mM Tris-HCl (pH 7.0), 0.15 mM PMSF, 2.5 uM leupeptin, 0.023 uM aprotinin, 0.639 uM phosphotidylserine, 0.322 uM dioleoyl-*sn*-glycerol, 0.15 mM ATP, 0.3 mM $CaCl_2$, 0.45 mM $MgCl_2$) containing 0.36 ug/uL membrane fraction overnight at 37°C. To stop the reaction, samples were heated at 95°C for 5 minutes.

2.12 Electrophoretic Separation of Peptides on Agarose Gels

To the peptide sample from the kinase reaction, glycerol (7.4%) and bromophenol blue (0.2%) were added. Samples were loaded into a 0.8% agarose gel prepared in 50mM Tris-HCl (pH 8.0) and separated at 100V for 25 minutes in 50mM Tris-HCl buffer.

2.13 Coomassie Blue Staining of Agarose Gels

After separation of peptide by electrophoretic separation on agarose gels, the gel was incubated for 30 minutes in fixation solution (50% methanol, 12% glacial acetic acid, 5% glycerol) with constant agitation. Gel was dried in an forced air oven at 37°C for 90 minutes and was rinsed three times with distilled water for 20 minutes. Gel was placed overnight in staining solution (0.05% Coomassie brilliant blue R-250 (w/v), 10% acetic acid, 25% methanol). Background was destained by incubating gel in several changes of destaining solution (40% methanol, 7% acetic acid). Gel was mounted between pre-wetted cellophane sheets (BioRad) and allowed to dry.

2.14 Electrospray ionization (ESI) of Peptide46

After subjecting Peptide 46 to the *in vitro* PKC phosphorylation reaction, samples were cleared through a 0.22 μm filter (Millipore). Electrospray-ionization analysis was performed on a VQ Quatro electrospray mass spectrometer.

2.15 Data Analysis

Microsoft Excel 7.0 spreadsheet was used to analyze data and generate the graphs for the cellular and nuclear p53 accumulation, assessment of the antibody-mediated inhibition of PKC methodology, and G2-M/S ratios. Western blot membranes were scanned using HP Precisionscan Pro. The densitometric analysis of the membrane was performed using Gel-Pro Analyzer 3.1 software.

3.0 RESULTS

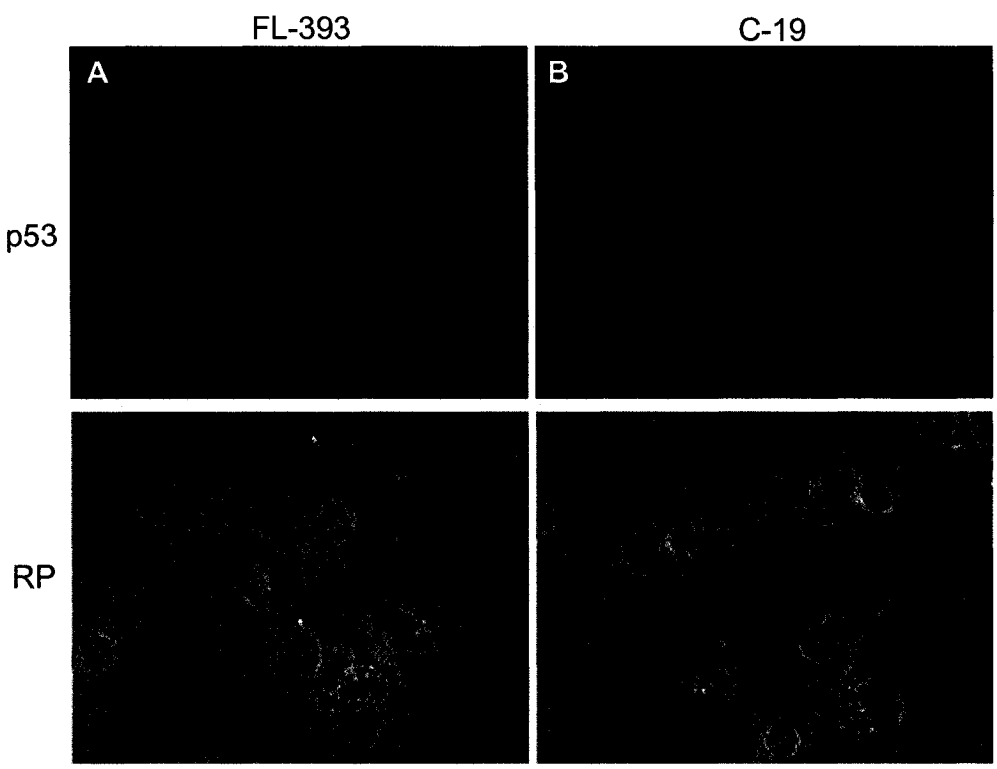
3.1 Characterization of p53 localization in neuroblastoma cell lines, IMR-32 and SHSY5Y

3.1.1 Choice of p53 Antibody

The sensitivity and specificity of an antibody are crucial characteristics for experimental interpretation, such as the evaluation of protein expression and localization. Danks *et al.* (1998) demonstrated that many commercially available p53 antibodies vary in their reactivity. Thus, the first step of this study was to identify a p53 antibody which would be best suited for characterization of p53 localization as demonstrated through immunocytochemistry. Two p53 antibodies available through Santa Cruz were evaluated, p53(FL-393) and p53(C-19). The FL-393 antibody preparation recognizes epitopes throughout the full length p53 protein (393 residues), whereas the C-19 p53 antibody has an epitope towards the C-terminal of p53. Even though both antibodies are directed to recognize p53, their reactivity differs in immunofluorescence staining (Figure 5). FL-393 exhibited strong nuclear staining of p53 in the human NB cell line, IMR-32; even though NB contain abberant cytoplasmic localization of p53 (Moll, *et al.*, 1995; Tweddle *et al.* 2001). On the other hand, p53 staining with the C-19 antibody was predominately cytoplasmic as was expected with NB cells. As a result, the p53(C-19) antibody was utilized for the duration of the study in order to maintain consistent and reliable data interpretation.

Figure 5. Comparison of reactivity of p53 antibodies, FL-393 and C-19.

The human neuroblastoma cell line, IMR-32 containing cytoplasmically localized p53 was immunostained with two different p53 antibodies (**A**) FL-393, an antibody raised against the whole length of the p53 protein (residues 1-393) and (**B**) C-19, an antibody with an epitope corresponding to the C-terminal of the p53 protein. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 and probed with the p53 IgGs and the appropriate secondary AlexaFluor 488nm IgG as described in the Materials and Methods section. Reverse phase images (RP) also included. Images were originally taken at 20x magnification.


FL-393
C-19
A
B
p53
RP

3.1.2 Neuroblastoma Cell Models

Two human NB cell lines were selected for this study, IMR-32 and SHSY5Y. Both of which exhibit cytoplasmic localization of p53, however each to varying degrees as assessed by p53 immunostaining (Figure 6). The p53 protein in IMR-32 is predominately cytoplasmic. Though in SHSY5Y, some nuclear p53 is present while the majority is sequestered in the cytoplasm. The observed subcellular localization of p53 in these NB cell lines is in agreement with previous studies (Issacs, *et al.*, 1998; Moll, *et al.*, 1995).

3.1.3 p53 Localization and Cell Culture Conditions

The influence of cell culture conditions, such as confluency and age after plating on p53 sublocalization was monitored by immunocytochemistry. IMR-32 cells were cultured at two cell densities, 2 x 10^4cells/4 cm^2 and 1 x 10^5cells/4 cm^2 (12-well plate) and monitored for 24 and 48 hours after plating (Figure 7). In the former condition, the cells were plated at a very low density such that cells were very sparse and intercellular contact was limited. In the latter condition, the cells were plated at a five-fold higher density in which there was contact between cells. At 24 hours after plating, p53 subcellular localization was predominately cytoplasmic at both plating densities. The cell cultures were then examined 48 hours after plating. Again, there was a change in the subcellular distribution of p53 after 48 hours with both cell densities. At the lower cell density (2 x 10^4cells/4 cm^2), there were a few cells that exhibited the characteristic p53 cytoplasmic sublocalization, however, another subset of cells exhibited both nuclear and cytoplasmic p53 sublocalization. When the higher cell density was maintained in culture

for 48 hours, there was an absence of cells that contained exclusively cytoplasmic sublocalization of p53. Instead, the cells exhibited both cytoplasmic and nuclear staining. These results suggest that culture age after plating can affect p53 subcellular localization and is further influenced by cell confluency in older cultures. Thus the cell densities for performing the experiments were carefully selected so that the effects of culture conditions on p53 were minimized.

3.2 PKC Isoform Expression in IMR-32 and SHSY5Y

Altered and abnormally high PKC expression levels has been observed in biopsies and several human tumours, including NB (Lahn *et al.*, 2004; Phipps laboratory data, unpublished 2002). Thus, part of this study has been dedicated to the characterization of the expression of PKC isozymes in the two human NB cell lines, IMR-32 and SHSY5Y. It is important to characterize the PKC isoforms in the cells because of differential expression of PKC isoforms and specific activity in various cell types. PKC expression was analyzed at the protein level by immunocytochemistry and immunoblotting. PKC isoforms α, βI, βII, δ, ε, ζ, and μ (PKD) are expressed in IMR-32 and SHSY5Y. The subcellular localization of each of the isoforms differs. The α-isozyme is clearly located in the cytoplasm and discrete anchorage points within the cellular membrane (Figure 8A). Despite their similarity, PKCβI and βII splice species have very distinct localizations; PKCβI is observed throughout the cell, while PKCβII is localized within the nucleus. Very low PKCγ immunoreactivity was detected with immunofluorescence and is possibly attributed to non-specific binding of the antibody as this isoform was not observed by Western blotting (Figure 8B). Of the novel isoforms, PKCδ expression is greater than that

of PKCε. The δ-isozyme is located throughout the cell with some perinuclear accumulation. PKCε is generally cytoplasmic with some nuclear localization. A similar localization is observed for the atypical PKC isoform, PKCζ. PKCμ or PKD is well expressed as monitored through western blotting (Figure 8B). A summary of the expression of the PKC isoforms in IMR-32 and SHSY5Y NB cell lines is outlined in Table 1.

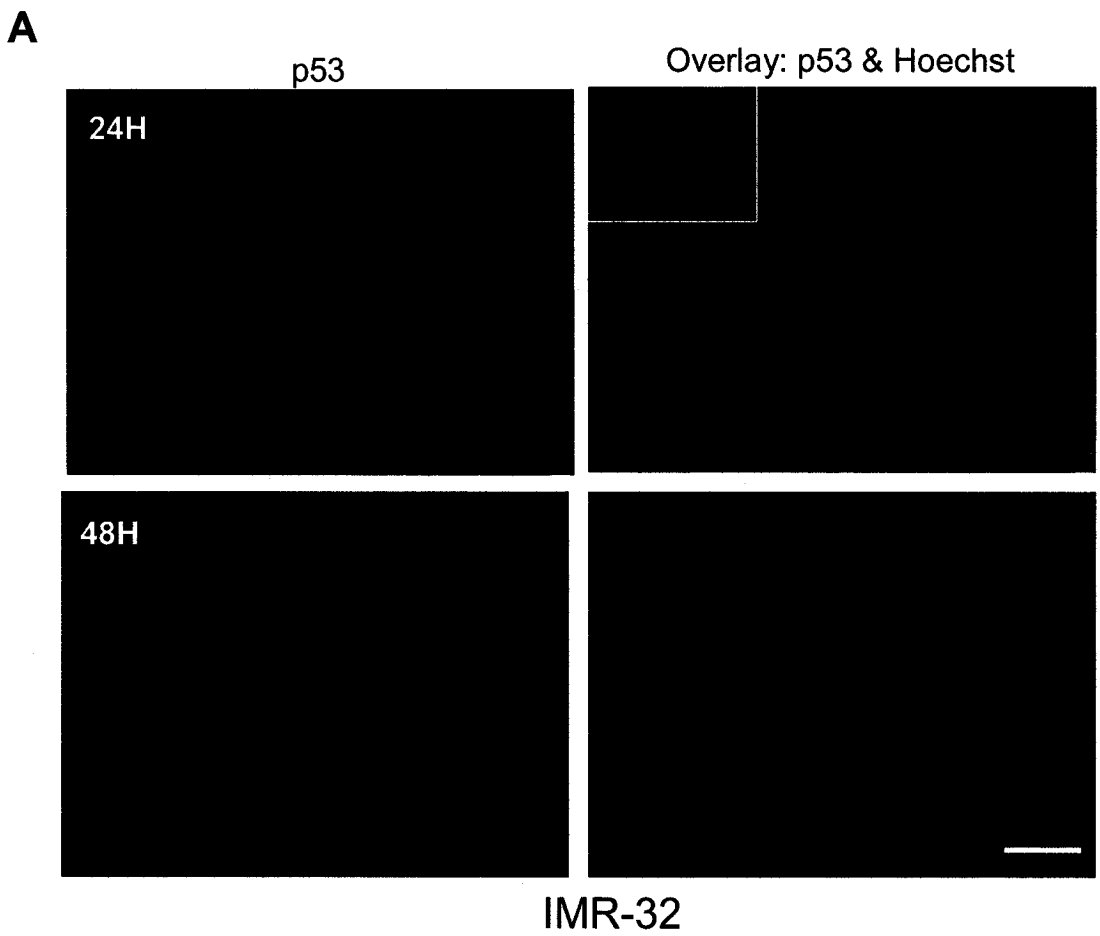
Table 1. Expression of PKC isoforms in N-type neuroblastoma cells, IMR-32 and SHSY5Y.

PKC Class	PKC Isoform	IMR-32	SHSY5Y
Classical	α	+++	+++
	βI	++	++
	βII	+	+
	γ	-	-
Novel	δ	++	++
	ε	++	++
	η	-	-
	θ	-	-
Atypical	ζ	+	+
	ι	ND	ND
	λ	ND	ND

+++: strong expression, ++: moderate expression, +: minimal expression, -: no expression, ND: not determined

Figure 6. Cytoplasmic localization of p53 in human neuroblastoma cell lines, IMR-32 and SHSY5Y.

(**A**) IMR-32 and (**B**) SHSY5Y cell lines were immunostained for p53, 24 and 48 hours after seeding. Cells were fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 as described in the Materials and Methods section. To identify p53 localization, cells were probed with goat p53 (C-19) IgG and AlexaFluor 488nm anti-goat IgG. Nuclei were visualized by staining with Hoechst dye and were merged with corresponding p53 images; *insert* is of nuclei staining before merge. Bar = 50μm


A
p53
Overlay: p53 & Hoechst
24H
48H
IMR-32

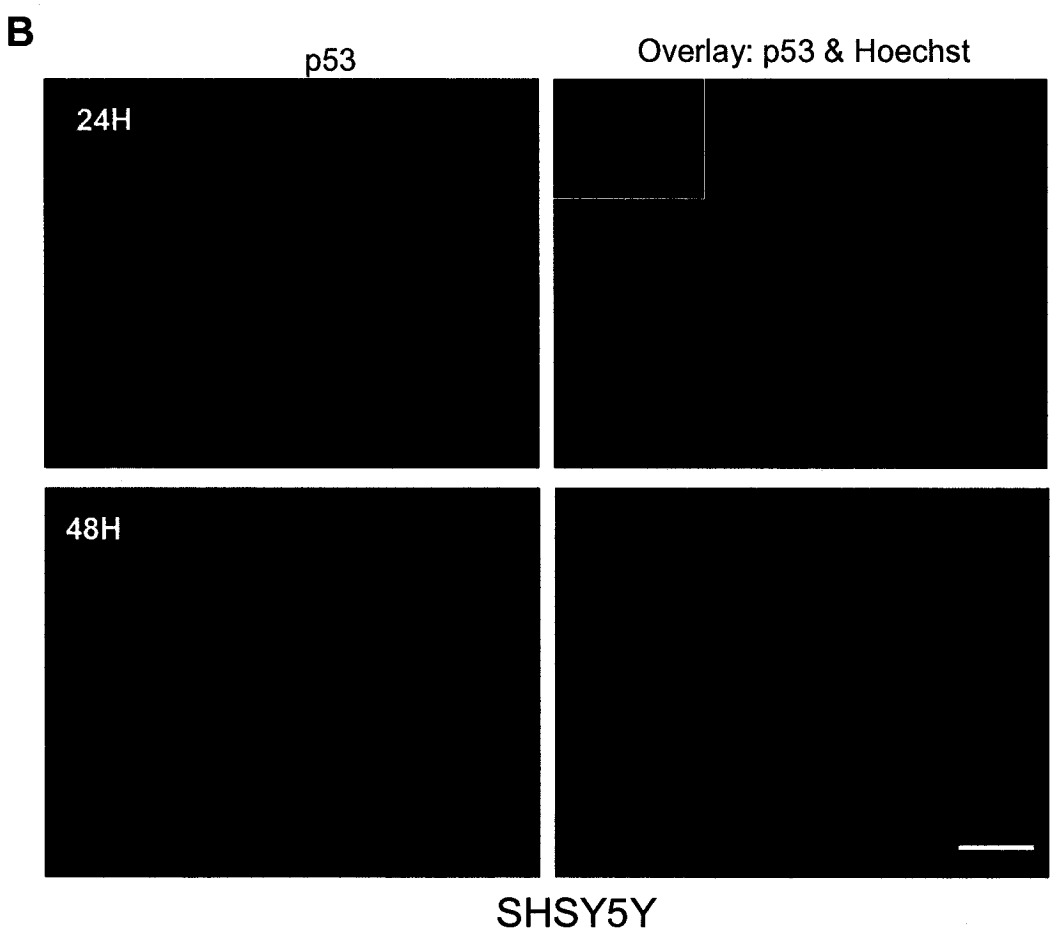

B
p53
Overlay: p53 & Hoechst
24H
48H
SHSY5Y

Figure 7. Cell culture conditions affect p53 localization.

IMR-32 cells were seeded at 2 x 10^4 and 1 x 10^5 cells per 4 cm^2 well of a 12-well plate to determine the effect of confluency and culture age on p53. At 24 and 48 hours after seeding, cells were immunostained for p53. Cells were fixed, permeabilized, and probed with goat p53 (C-19) IgG and AlexaFluor 488nm anti-goat IgG. Images were originally taken at 10x magnification.

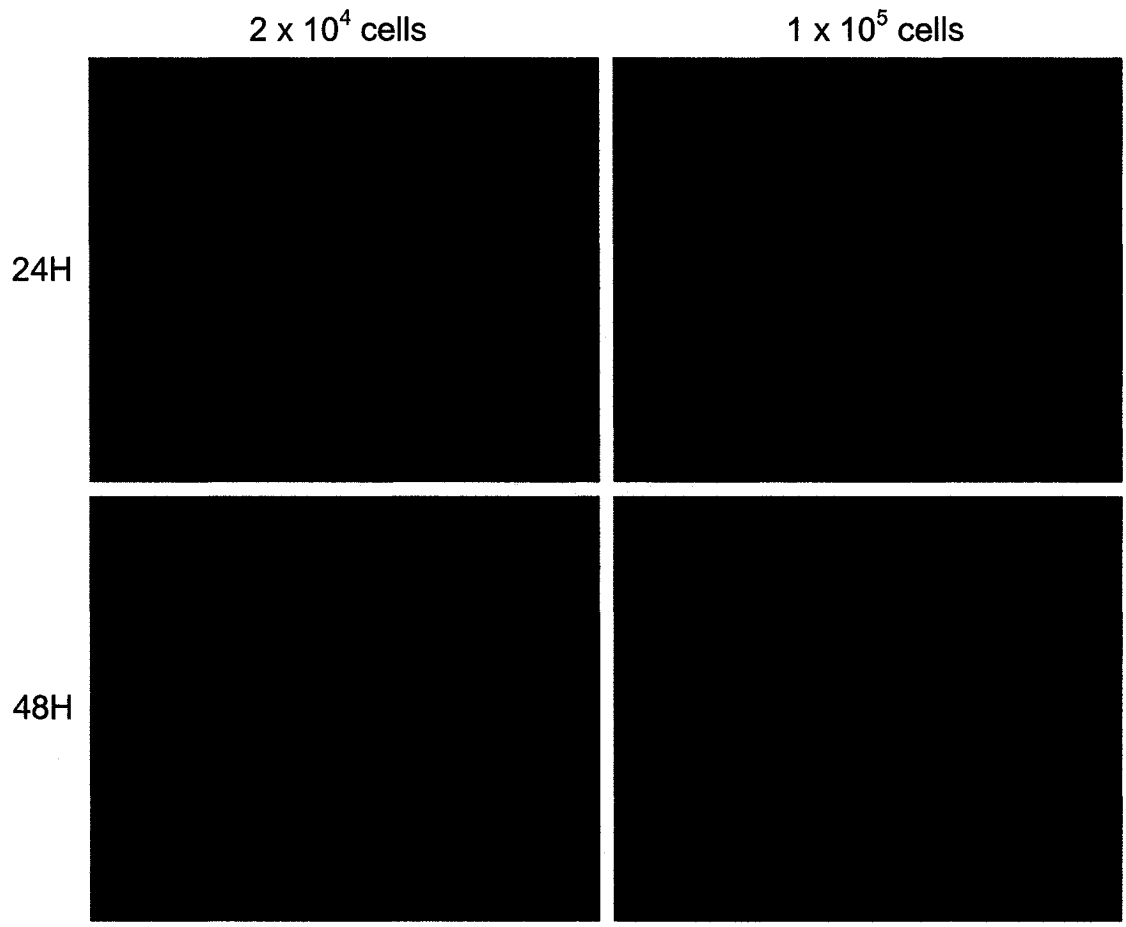
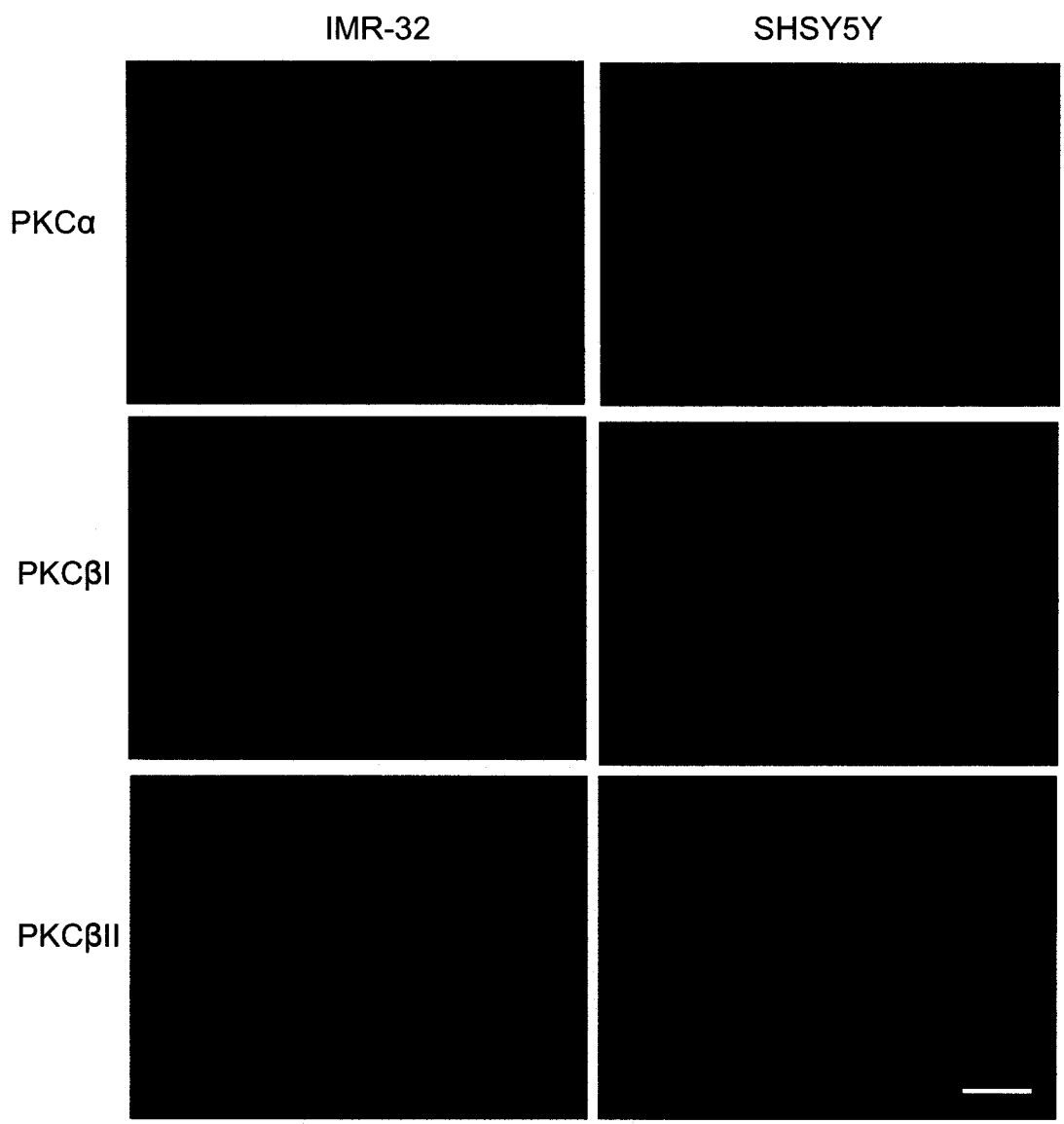

2 x 10^4 cells
1 x 10^5 cells
24H
48H

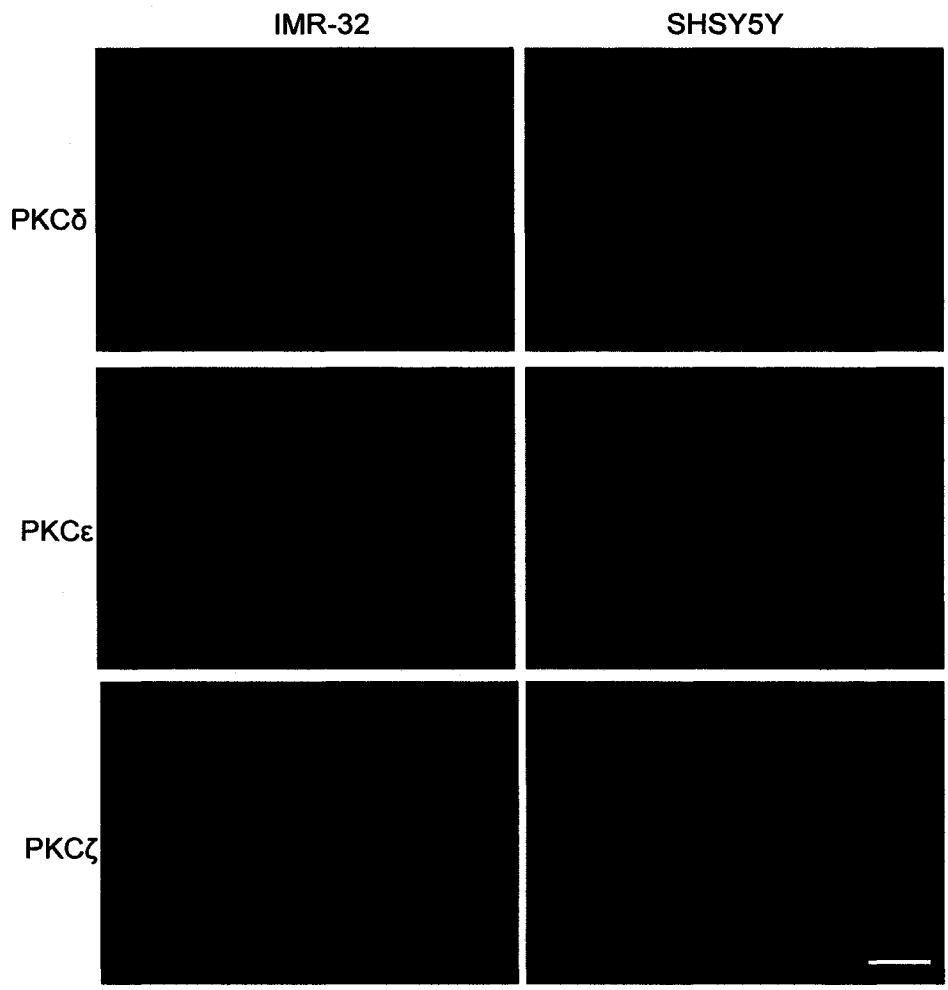
Figure 8. Characterization of PKC isoforms in neuroblastoma cell lines, IMR-32 and SHSY5Y.

(**A**) Indirect immunofluorescence staining of PKC isoforms, PKCα, βI, βII, δ, ε, and ζ in the human neuroblastoma cell lines, IMR-32 and SHSY5Y. PKC isoforms were detected by probing with relevant PKC antibodies and the corresponding Alexa Fluor 488nm secondary antibody. Bar = 50μm. The same fluorescence gating was maintained during image capturing.

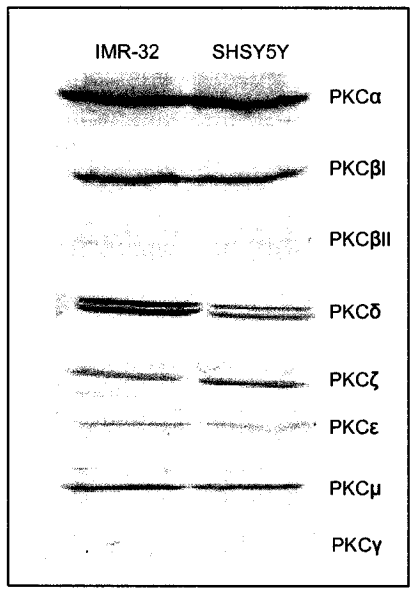
(**B**) Immunoblotting of PKC isoforms in neuroblastoma cell lines, IMR-32 and SHSY5Y from whole cell extracts. Proteins were electrophoretically separated on 10% SDS-PAGE and transferred to a nitrocellulose membrane, as described in the Materials and Methods section. The membrane was probed with PKC antibodies, anti-PKCα (C-20), anti-PKCβI (C-16), anti-PKCβII (C-18), anti-PKCδ (C-20), anti-PKCζ (C-20), anti-PKCε (C-15), anti-PKCμ (C-20), and anti-PKCγ (C-19).

A




IMR-32
SHSY5Y
PKCδ
PKCε
PKCζ

B



3.3 Involvement of PKC in Regulation of p53 Accumulation and Localization in NB

p53 cytoplasmic localization has been attributed to HDM2 overexpression and hyperactive nuclear export. Regulation of p53 can also be influenced by post-translation modifications, such as, phosphorylation. PKC phosphorylation sites in the C-terminal of p53 have been identified to be involved in the regulation of the p53 protein (Magnelli & Chiarugi 1997; Appella & Anderson 2001). Overexpression of PKCs is observed in NB; it is possible that PKC mediated phosphorylation of p53 is a causative factor to the abnormal cytoplasmic localization of p53 in NB. To implicate PKC in the regulation of p53 localization in NB and its contribution to the cytoplasmic p53 phenotype, a pharmacological approach was utilized.

3.3.1 Effect of Treatment with PKC Inhibitors on p53 Accumulation and Localization in NB

IMR-32 and SHSY5Y cells were treated with 75uM H7, 5uM BisI, and 200 nM Gö6976 for 24 hours and the effects on p53 were monitored by immunocytochemistry. As a positive control, cells were treated with the HDM2 binding peptide, SuperTIP (100uM). The negative regulator of p53, HDM2 inhibits p53 transcriptional activation and induces p53 degradation (Oliner *et al.*, 1993; Fuchs *et al.*, 1998). SuperTIP abrogates HDM2-p53 interaction, thereby preventing HDM-2 mediated degradation of p53 and leads to p53 stabilization and accumulation (Bötteger *et al.*, 1997).

Treatment of the NB cell lines with all the protein kinase inhibitors led to p53 accumulation and localization (Figure 9A & B). H7, a non-specific protein kinase inhibitor also caused p53 accumulation and relocalization. In addition, the cells

underwent morphological changes, such as neurite extension. At the specified concentration of 75uM, H7 is not known to be a selective kinase inhibitor (Hidaka *et al.*, 1984) and nevertheless, exhibits inhibition of other protein kinases. To corroborate the involvement of PKCs in p53 relocalization, the cells were exposed to bisindolylmaleimide I (BisI), a specific inhibitor of the PKC family. BisI treatment led to accumulation and changes in the subcellular localization of p53.

The PKC isoform(s) implicated in p53 regulation in NB has not been identified. Abnormally high levels of PKCα have been reported in human tumours and cancer cell lines, including NB (Lahn *et al.*, 2004). PKCα has been demonstrated to phosphorylate p53 *in vitro* (Youmell *et al.*, 1998), though controversy still remains as to whether this occurs *in vivo.* Thus, the cells were exposed to a conventional PKC inhibitor, Gö6976 in order to implicate the conventional PKC isoforms (α, βI/II, γ) in the regulation of p53 localization in NB. Inhibition of the cPKCs caused accumulation with changes in the subcellular localization of p53. The effect of the inhibitors on p53 in the two cell lines was generally the same. However, there were some slight differences in the alteration of subcellular p53 localization between the two cell lines. It was observed in IMR-32 cells treated with Gö6976 that p53 accumulated in the cytoplasm with some perinuclear localization. No perinuclear p53 localization was observed in SHSY5Y cells exposed to Gö6976, instead an increase of both cytoplasmic and nuclear p53 was observed.

The p53 accumulation observed through immunocytochemistry was further verified by quantification of p53 levels using flow cytometry. IMR-32 and SHSY5Y cells were treated with the protein kinase inhibitors for 24, 48, and 72 hours (Figure 10A, only IMR-32 results shown). A longer treatment period was utilized to determine the long

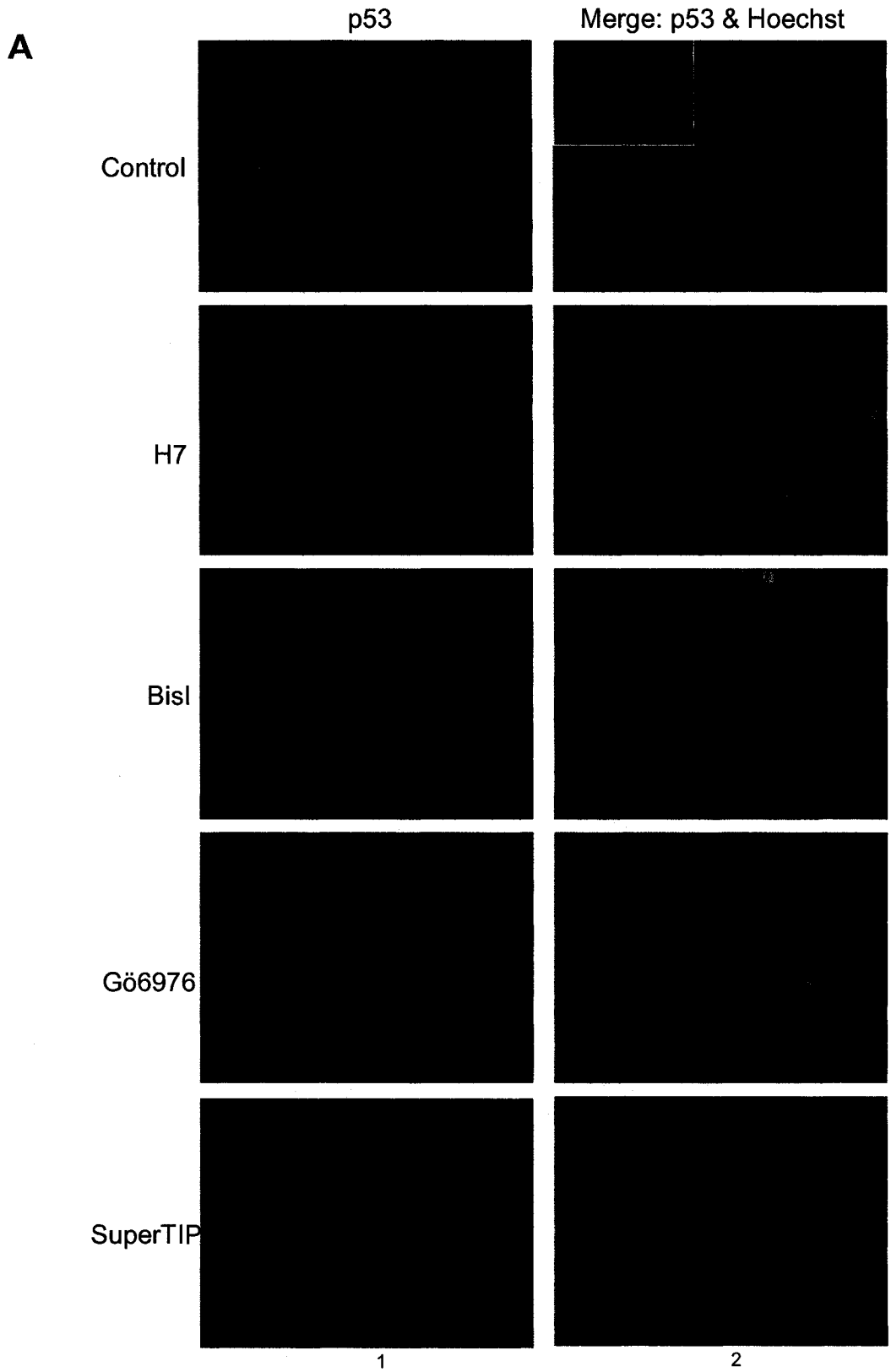
term effects of PKC inhibition on p53 expression. At the specified time intervals, the cells were harvested by trypsinization, fixed, permeabilized, and the presence of p53 was detected by probing with a p53 specific antibody and appropriate secondary antibody. Total cellular p53 levels increase upon protein kinase inhibition. At 24 hours, p53 levels are 117%, 157%, and 77% as compared to untreated IMR-32 cells for H7, BisI, and Gö6976 exposure, respectively. Protein kinase inhibition is the most dramatic at 48 hours duration with % p53 levels as compared to the control being 653%, 152%, and 106% for H7, BisI, and Gö6976 treatment, respectively. At 72 hours, the effect of the inhibitors on p53 accumulation begins to diminish.

As well as monitoring overall p53 levels, nuclear p53 levels were also quantified by flow cytometry. Nuclei were isolated by exposing cells to a hypotonic buffer and subjected to a similar protocol as whole cells for flow cytometry processing. As expected, the protein kinase inhibitors induced nuclear accumulation of p53 in IMR-32 cells (Figure 10B). Prominent increase in nuclear p53 is observed with all the inhibitors at 48 and 72 hours exposure.

Elevated p53 levels continued with prolonged H7 treatment in conjunction with nuclear accumulation of p53. After 24 hours of BisI treatment, it can be ascertained that there was an initial cytoplasmic p53 increase due to an elevation in overall p53 levels yet with no extensive change in nuclear p53 levels. Though with prolonged BisI exposure (48 and 72 hours), nuclear p53 accumulation occurred. A similar phenomenon was noted with Gö6976. Similar trends in regards to p53 accumulation were observed with the SHSY5Y cells (data not shown). These results implicate the PKC family in the regulation of p53 localization, more specifically, the conventional PKCs.

Figure 9. Exposure to PKC inhibitors induces p53 relocalization in neuroblastoma cells.

Subcellular localization of p53 in (**A**) IMR-32 and (**B**) SHSY5Y cells upon treatment with 75uM H7, 5uM BisI, and 200nM Gö6976 for 24 hours. For a positive control, cells were treated with the HDM-2 binding peptide, SuperTIP at 100uM. Localization of p53 was analyzed by immunofluorescence by probing with a goat anti-p53 antibody (C-19) and donkey anti-goat Alexa Fluor 488nm antibody (*panel 1*). Nuclei were visualized with Hoechst dye and images were merged with p53 fields (*panel 2*); *insert* is of nuclei staining before merge. Images originally taken at 20x magnification.


A
p53
Merge: p53 & Hoechst
Control
H7
Bisl
Gö6976
SuperTIP
1
2

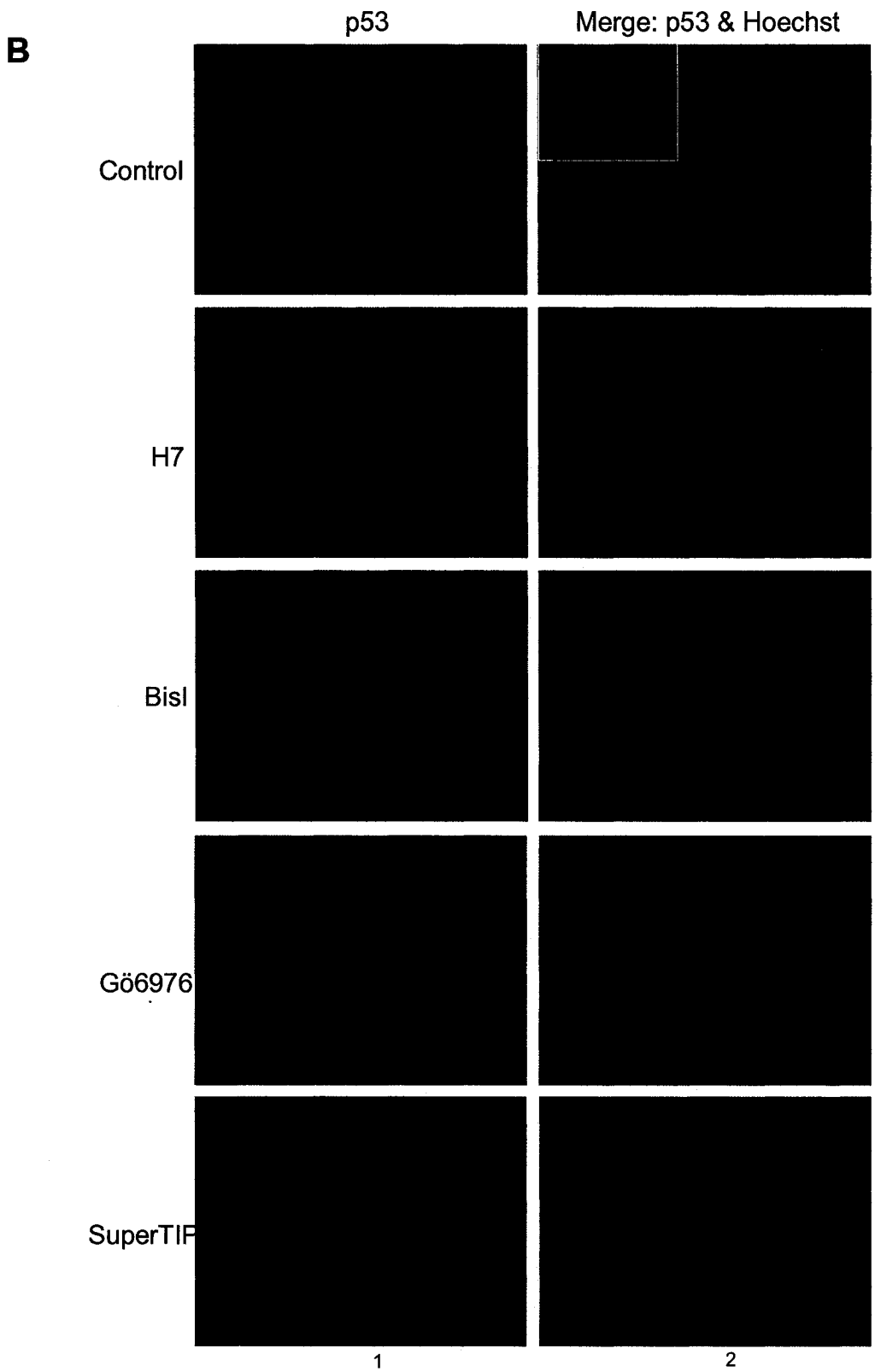
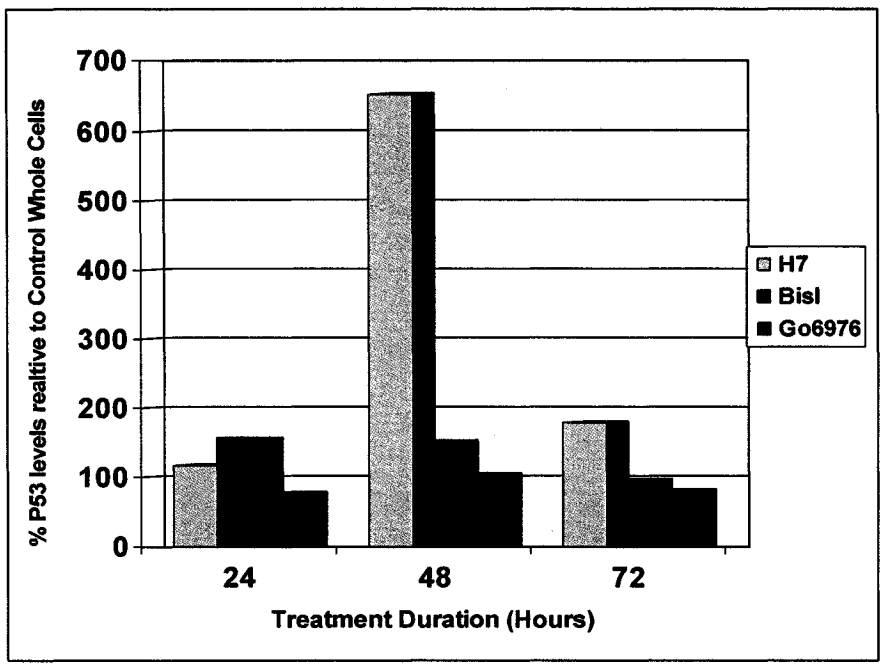

B
p53
Merge: p53 & Hoechst
Control
H7
Bisl
Gö6976
SuperTIP
1
2

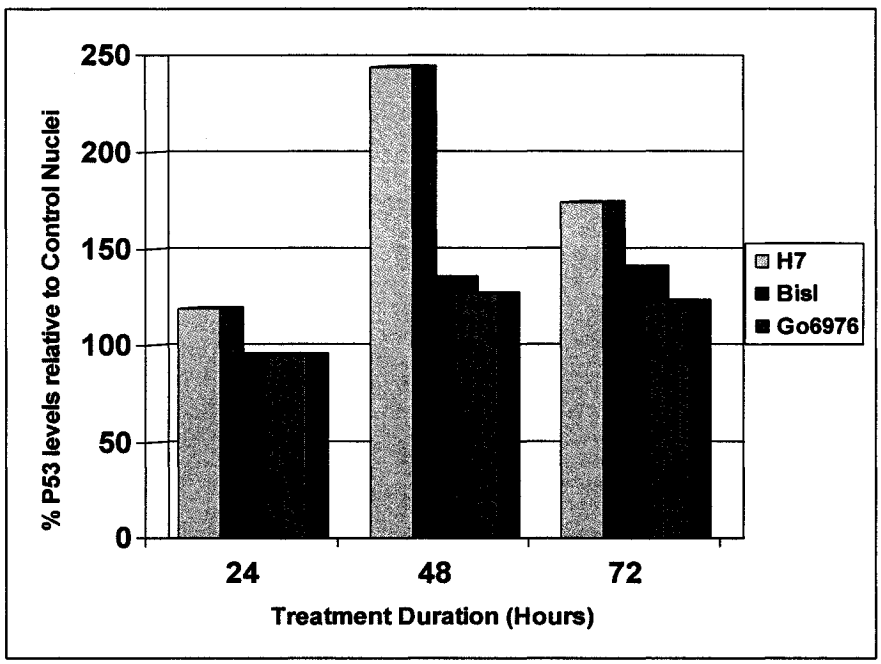
Figure 10. Inhibition of protein kinases induce p53 accumulation.

Quantification of p53 protein levels in (**A**) whole cells and (**B**) isolated nuclei of IMR-32 cells treated with 75uM H7, 5uM BisI, and 200nM Gö6976 for 24, 48, and 72 hours. Cells were harvested by trypsinization. Nuclei were isolated following exposure to a hypotonic buffer. p53 was detected in the samples by incubation with a p53 specific antibody, p53(C-19) IgG and the corresponding Alexa488nm IgG and subjected to flow cytometry analysis. Approximately 90% of the cells were labeled. p53 protein levels are represented as log values (FL1 log) of mean p53 fluorescence as identified from flow cytometry plots. p53 protein levels are represented as % of untreated control cells or isolated nuclei. Experiment was performed in triplicate and representative data shown.

A


700
600
500
400
300
200
100
0
% P53 levels realtive to Control Whole Cells
24
48
72
Treatment Duration (Hours)
H7
Bisl
Go6976

B


250
200
150
100
50
0
% P53 levels relative to Control Nuclei
24
48
72
Treatment Duration (Hours)
H7
Bisl
Go6976

3.3.2 Evaluation of Cell Loading Reagent and Antibody-Mediated Inhibition of PKC

Prior to the utilization of antibody-mediated inhibition of PKC to specifically target the PKC isoforms and monitor the effects on p53, the efficacy of the Influx™ Cell Loading Reagent for intracellular delivery of PKC IgG was assessed. Furthermore, the cellular presence of the PKC IgG was monitored in order to establish treatment durations in which the effects of antibody-mediated inhibition on p53 could be evaluated.

3.3.2.1 Longevity of PKC Antibody Incorporation in Cells

The persistence of the PKC antibody within the cells was initially established in order to define an observational window in which the effects on p53 could be monitored. Rabbit PKCα antibody was loaded into IMR-32 cells. A corresponding blank loading was also performed. At various time points, the cells were harvested, probed with a secondary antibody, AlexaFluor 488nm goat anti-rabbit, and subjected to flow cytometry analysis. Twenty four hours after loading, the PKC IgG was still persistent within the cells (Figure 11). However 3 hours later at 27 hours, the amount of PKC IgG label within the cell decreased and most significantly, fell to essentially control levels at 48 hours, which corresponds to non-specific binding.

The cell loading reagent is effective at introducing PKC antibodies into cells as their presence was also detected 24 hours later by immunocytochemistry (Figure 12). The presence of the PKC antibody within the cell is fundamental for the binding and inhibition of PKCs. Thus antibody-mediated inhibition of PKCs can only be monitored

over short time periods, approximately 24 hours. For longer time points, a different means of PKC inhibition would need to be utilized.

3.3.2.2 Efficacy of Antibody-Mediated Inhibition of PKC

Antibody-mediated inhibition has been utilized to target and inhibit a variety of antigens, e.g. HLA alloimmune response, HIV reverse transcriptase, HDM2, and PKC (Crow *et al.*, 2000; Wu *et al.*, 1993; Böttger *et al.*, 1997; Leli *et al.*, 1992).

The efficacy of the antibody-mediated PKC inhibition methodology was verified by monitoring phosphorylation of MARCKS peptide. MARCKS, myristoylated alanine-rich C-kinase substrate is a "specific PKC substrate whose phosphorylation has been used as a marker of PKC activation *in vivo*" (Aderem, 1992).

In this study, MARCKS peptide was loaded into the cells alone or in combination with the PKCα antibody. MARCKS phosphorylation was detected by probing with an anti-phosphoMARCKS IgG and fluorescence levels were quantified by flow cytometry. MARCKS phosphorylation was observed 2 and 24 hours after cell loading. Additionally, MARCKS-loaded cells were treated with chemical PKC inhibitors (BisI and Gö6976) to compare the efficacy of the antibody-mediated PKC inhibition. From the results, it appears that 2 hours after cell loading is not a sufficient duration for the antibody-mediated inhibition to be effective as the level of phosphoMARCKS with the PKCα antibody is comparable to the cells loaded with only the MARCKS peptide (Figure 13, 14). Whereas, the chemical inhibitors have already decreased PKC activity as there is less MARCKS phosphorylation being observed. Short term exposure to the phorbol ester, TPA stimulates PKC activity and chronic exposure down-regulates PKC activity

(Rodriguez-Pena & Rozengurt, 1984). At an exposure time of 2 hours, TPA is already behaving as a suppressor of PKC activity in the IMR-32 cell line. The level of phosphorylated MARCKS with TPA is less than that of cells with no additional treatment. A clear inhibition of PKCα activity is observed after 24 hours as the incidence of phosphorylated MARCKS is much lower than the MARCKS cells with no antibody. The chemical inhibitors are also effective at sustaining PKC inhibition over this duration as assessed by phosphoMARCKS levels. As with the 2 hour time point, TPA continues to inhibit PKC-mediated phosphorylation of the MARCKS peptide after 24 hours. These results indicate that antibody-mediated inhibition is effective at inhibiting PKC activity after 24 hours and similar to the inhibition exerted by the chemical inhibitor Gö6976.

3.3.3 Effect of Antibody-Mediated Inhibition of PKC on p53

Following the clear assessment of meaningful experimental conditions, PKC antibodies directed against various PKC isoforms were introduced into the NB cells using the Influx™ Molecular Probe Cell Loading Reagent as a means of specifically targeting and inhibiting the PKC isoforms. The potential effects on p53 localization were monitored by immunocytochemistry. This method was utilized as a means of identifying the PKC isoform(s) involved in p53 relocalization as there are no available specific PKC isoform chemical inhibitors.

The effect of antibody-mediated inhibition of PKCα on p53 was evaluated 24 hours after PKC IgG was introduced into the neuroblastoma cells. Inhibition of PKCα led to nuclear relocalization of p53 in IMR-32 cells (Figure 15A). In the cells loaded with an empty control (CT), p53 staining is diffuse and primarily cytoplasmic. After PKCα IgG

cell loading (MP + PKCα IgG), the subcellular localization of p53 is altered to a nuclear accumulation. The signal was not due to the immunoreactivity of the secondary antibody to the PKCα IgG as antibodies utilized for PKC inhibition and p53 detection were raised in different species and thus, specifically recognized by their corresponding secondary antibody. The controls of probing with the secondary alone were done separately.

The cellular accumulation of p53 was further assessed by Western blot analysis of cell lysates from PKC IgG-loaded cells (Figure 15B). It was confirmed that PKCα inhibition induces cellular accumulation of p53. Additionally, PKCβI inhibition was able to induce a slight p53 accumulation, though, not to the same magnitude as PKCα. p53 accumulation in the Western blots was quantified by measurement of the optical density of the p53 band and expressed as % p53 expression relative to the control (Figure 15C).

The effect of antibody-mediated inhibition of PKC on p53 was also studied by immunocytochemistry in the SHSY5Y cell line. The inhibition of PKCα and βI induced p53 relocalization and accumulation was observed in SHSY5Y (Figure 16), though it was not as pronounced as in IMR-32 cells. PKCα inhibition caused both a cytoplasmic and nuclear accumulation of p53 in SHSY5Y (Figure 16B), whereas in untreated cells, p53 is predominately localized in the cytoplasm at low levels (Figure 16A). Inhibition of PKCβI also affected p53 subcellular distribution in SHSY5Y cells as perinuclear localization of p53 was observed (Figure 16C).

Other PKC (PKC δ, ε, ζ) IgGs were studied for their possible implications in p53 relocalization and accumulation, however, no significant effects were observed (data not shown). The alterations in p53 as a result of antibody-mediated inhibition of the specific PKC isoforms are summarized in Table 2.

Table 2. Effect of antibody-mediated inhibition of PKC isoforms on p53 relocalization and accumulation in human neuroblastoma cell lines, IMR-32 and SHSY5Y.

Inhibition of PKC isoform	Cell Line	
	IMR-32	SHSY5Y
PKC α	+++	++
PKC β	+	+
PKC ε	-	-
PKC δ	-	-
PKC ζ	-	-

+++: strong accumulation and nuclear relocalization, ++: moderate accumulation and relocalization, +: minimal effect on p53 accumulation and relocalization, -: no effect observed

3.3.4 Characterization of Changes in p53 Phosphorylation Status upon Antibody-Mediated Inhibition of PKC

p53 has several serine residues in its C-terminal that can be targeted for phosphorylation by PKC. In an attempt to directly implicate PKC phosphorylation of p53 in the qualitative changes seen with antibody-mediated inhibition of PKCs, p53 phosphorylation status on serine residues was examined. Cells were untreated or loaded with PKC IgGs using Molecular Probes Influx™ Cell Loading Reagent. Total cellular protein was harvested 24 hours later and immunoprecipated for p53. Samples were resolved by SDS-PAGE and immunoblots were probed with an anti-phosphoserine antibody.

A significant difference in the levels of p53 phosphorylated on serine residues was observed upon inhibition of PKCα (Figure 17) in IMR-32 cells. The band intensities correspond to the level of serine phosphorylation of p53 which indicate that after antibody-mediated inhibition of PKCα, p53 serine residue phosphorylation decreases as compared to untreated cells. PKCβI was previously identified in this study as another isoform of having a possible involvement in p53 regulation. A slight decrease in serine residue phosphorylation of p53 is observed after antibody-mediated inhibition of PKCβI. However, the decrease in serine residue phosphorylation is more considerable with PKCα inhibition than with PKCβI.

This evidence strongly implicates the PKC isozyme-α in the regulation of p53 relocalization and accumulation.

Figure 11. PKCα IgG persists in IMR-32 cells up to 24 hours.

Flow cytometry quantification of PKCα IgG levels in IMR-32 cells after introduction of PKCα IgG (■, MP + PKCα IgG) or an empty control (♦, Control). The PKCα IgG was introduced in the cells using Molecular Probes Influx™ Pinocytic Cell Loading Reagent. Cells were loaded by exposure to a hypertonic medium in the presence or absence of PKCα IgG with brief transfer into a hypotonic medium. At various timepoints (0, 24, 27, and 48 hours) after introduction of the antibody, cells were harvested and processed for flow cytometry analysis. Presence of PKCα IgG was detected by probing cells with AlexaFluor 488nm goat anti-rabbit IgG. Graph values are represented as fluorescence log values (FL 1 log). Experiment was performed in triplicate and representative data shown.

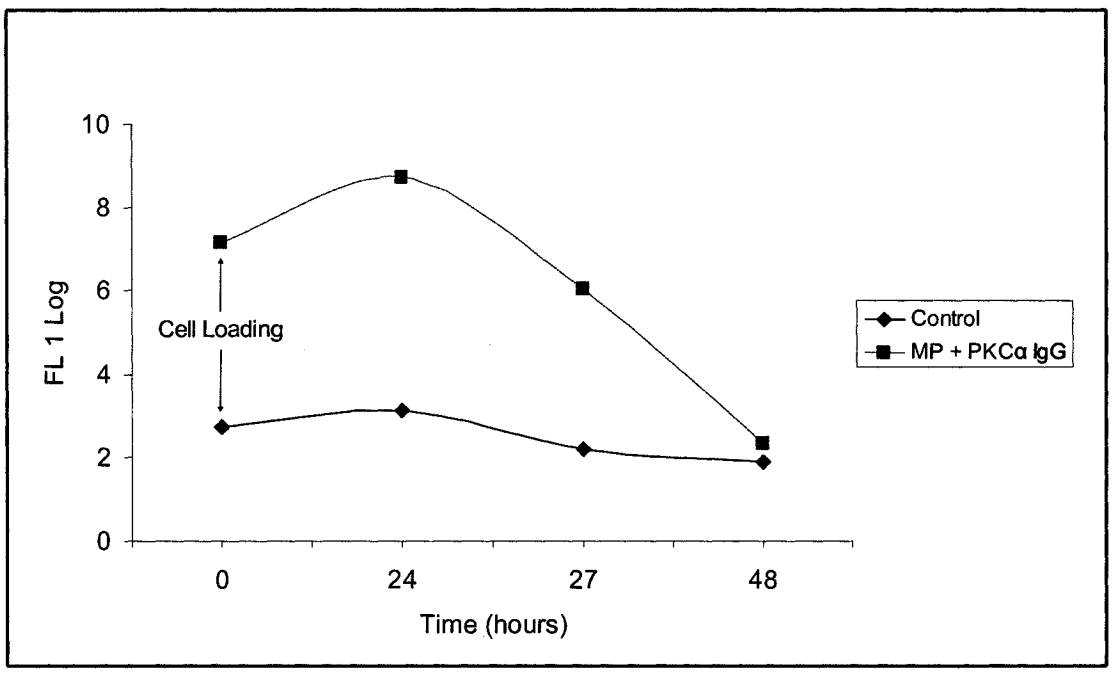

10
8
6
4
2
0
FL 1 Log
Cell Loading
0
24
27
48
Time (hours)
Control
MP + PKCα IgG

Figure 12. Indirect immunofluorescence of PKCα IgG introduced into IMR-32 cells.

Reverse phase and immunocytochemistry images of IMR-32 cells 24 hours after introduction of PKCα IgG (MP + PKCα IgG) or a blank control (Control) using the Molecular Probes Influx™ Pinocytic Cell Loading Reagent. Cells were loaded by exposure to a hypertonic medium in the presence or absence of PKCα IgG with further incubation in a hypotonic medium. Twenty four hours after introduction of the antibody, cells were fixed, permeabilized, and the presence of PKCα IgG (rabbit) was detected by probing cells with AlexaFluor 488nm goat anti-rabbit IgG. Bar = 25 μm.

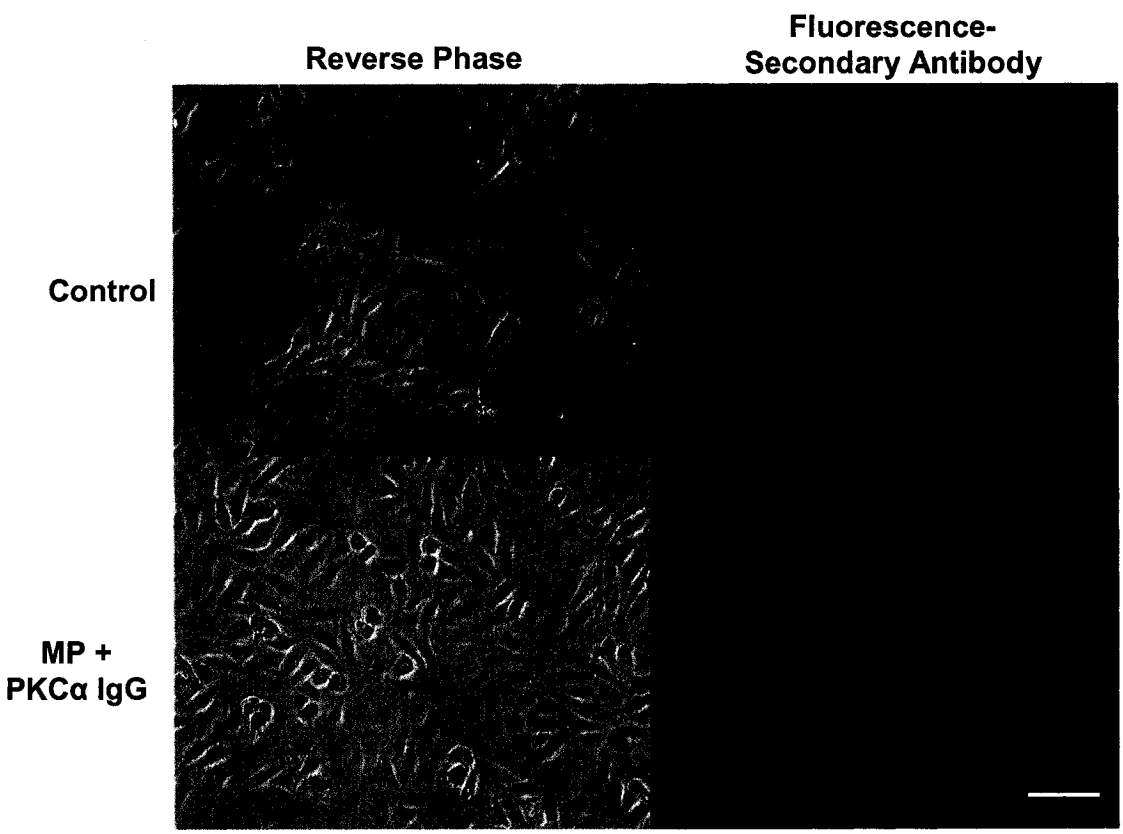

Reverse Phase
Fluorescence-
Secondary Antibody
Control
MP +
PKCα IgG

Figure 13. PKCα IgG and PKC inhibitors, BisI and Gö6976 decrease phosphorylation of MARCKS peptide *in vivo*.

Quantification of PhosphoMARCKS levels in IMR-32 cells by flow cytometry. MARCKS peptide, a protein kinase substrate and PKCα IgGs were introduced into IMR-32 cells using Molecular Probes Influx™ Pinocytic Cell Loading Reagent. Cells were loaded by exposure to a hypertonic medium containing the peptide alone or the peptide and PKCα IgG with brief exposure to a hypotonic medium. Cells were then placed in normal growth medium or supplemented with 1 uM TPA, 5uM BisI, or 200nM Gö6976. After 2 and 24 hours, cells were harvested by trypsinization and processed for flow cytometry analysis. Phosphorylation of the MARCKS peptide was detected by probing cells with phosphoMARCKS IgG and AlexaFluor 488nm goat anti-rabbit IgG. Mean phosphoMARCKS levels are represented as log values (FL 1 log) as identified from the flow cytometry plots. Experiment was performed in triplicate and representative data shown.

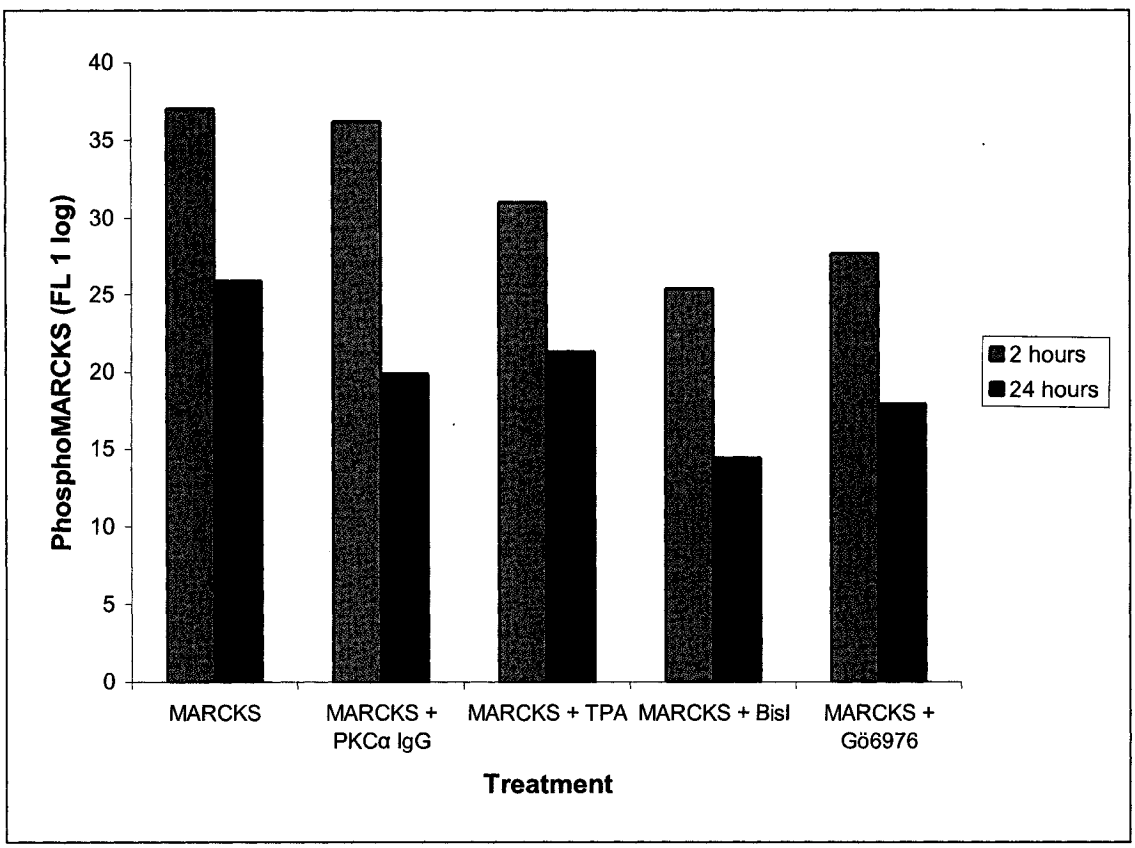

40
35
30
25
20
15
10
5
0
PhosphoMARCKS (FL 1 log)
2 hours
24 hours
MARCKS
MARCKS + PKCα IgG
MARCKS + TPA
MARCKS + BisI
MARCKS + Gö6976
Treatment

Figure 14. PKCα and PKCδ antibodies decrease phosphorylation of MARCKS peptide *in vivo*.

Quantification of PhosphoMARCKS levels in IMR-32 cells by flow cytometry. MARCKS peptide, a protein kinase substrate and various PKC IgGs (α, ε, δ, ζ) were introduced into IMR-32 cells using Molecular Probes Influx™ Pinocytic Cell Loading Reagent. Cells were loaded by exposure to a hypertonic medium containing the peptide alone or the peptide and PKC IgG with brief exposure to a hypotonic medium. After 24 hours, cells were harvested by trypsinization and processed for flow cytometry analysis. Phosphorylation of the MARCKS peptide was detected by probing cells with phosphoMARCKS IgG and AlexaFluor 488nm goat anti-rabbit IgG. Mean phosphoMARCKS levels are representated as log values (FL 1 log) as identified from the flow cytometry plots. Fluorescence associated with non-specific binding of the secondary antibody was subtracted from each sample ($fluorescence_{phosphoMARCKS} - fluorescence_{secondary\ antibody\ only}$). Experiment was performed in triplicate and representative data shown.

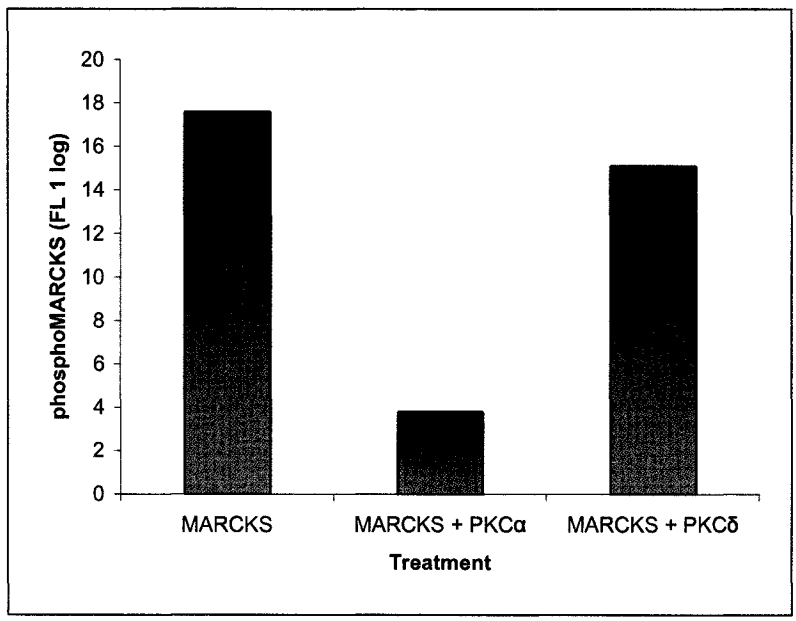

20
18
16
14
12
10
8
6
4
2
0
phosphoMARCKS (FL 1 log)
MARCKS
MARCKS + PKCα
MARCKS + PKCδ
Treatment

Figure 15. Antibody-mediated inhibition of PKCα induces p53 accumulation in IMR-32 cells.

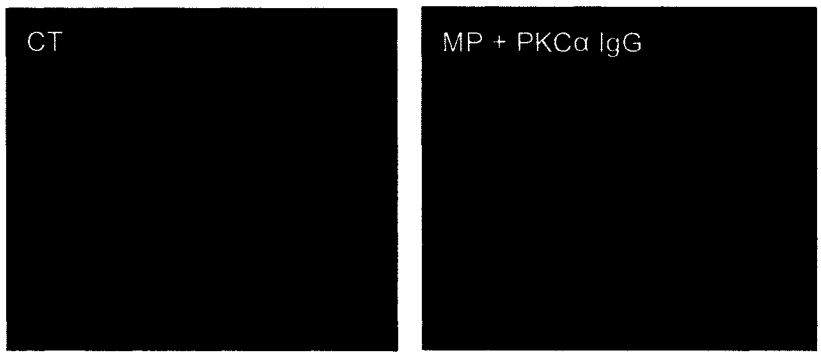
(**A**) Immunocytochemistry staining of p53 in IMR-32 cells loaded with an empty control (CT) or PKCα IgG (MP + PKCα IgG) using Molecular Probes Influx™ Cell Loading Reagent. p53 relocalization and accumulation was observed in cells 24 hours after PKCα IgG loading.

(**B**) p53 Western blot of IMR-32 cells loaded with an empty control (CT), PKCα IgG (MP + PKCα IgG), or PKCβI IgG (MP + PKCβI IgG) using Molecular Probes Influx™ Cell Loading Reagent.

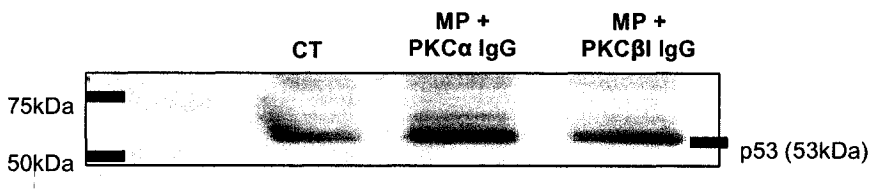
Twenty-four hours after cell loading, total cell lysates were subjected to p53 immunoprecipitation. Proteins were separated on a 10% SDS-PAGE gel, immunoblotted onto nitrocellulose membrane, detected for p53 and visualized as detailed in the Materials and Methods.

(**C**) Densitometric analysis of p53 expression after cell loading with PKC IgGs. Data are expressed as the percentage intensity of the control band and are the mean ± SD of three separate experiments. Level of significance: $P<0.05$ (*) compared with control cells according to the Student's t-test.

A


CT
MP + PKCα IgG

B


CT
MP + PKCα IgG
MP + PKCβI IgG
75kDa
50kDa
p53 (53kDa)

C

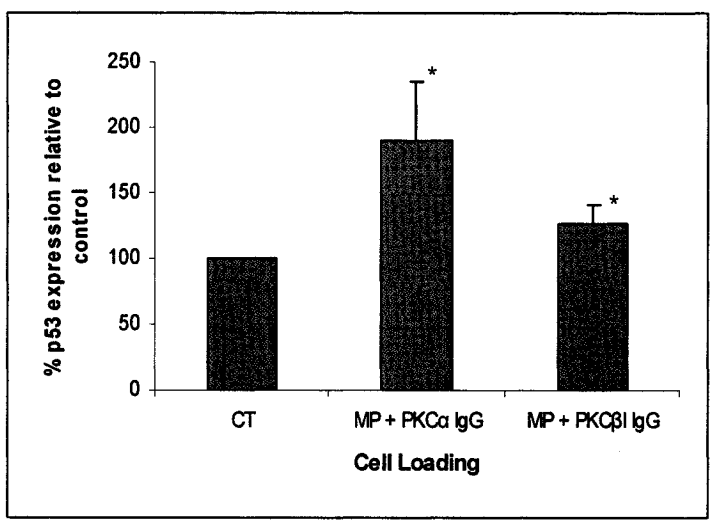

% p53 expression relative to control
250
200
150
100
50
0
*
*
CT
MP + PKCα IgG
MP + PKCβI IgG
Cell Loading

Figure 16. Inhibition of PKCα and βI induces p53 accumulation and relocalization in SHSY5Y cells.

SHSY5Y cells were loaded with either (**A**) an empty control, (**B**) PKCα IgG, or (**C**) PKCβI IgG using Molecular Probes Influx™ Cell Loading Reagent. Cells were loaded by exposure to a hypertonic medium in the presence or absence of PKC IgG with exposure to hypotonic medium. After 24 hours, cells were subjected to immunofluorescence staining. p53 was detected by probing with a goat anti-p53 antibody (C-19) and donkey anti-goat Alexa Fluor 488nm antibody. Nuclei were visualized with Hoechst dye and merged with corresponding p53 images. Bar = 50 μm.

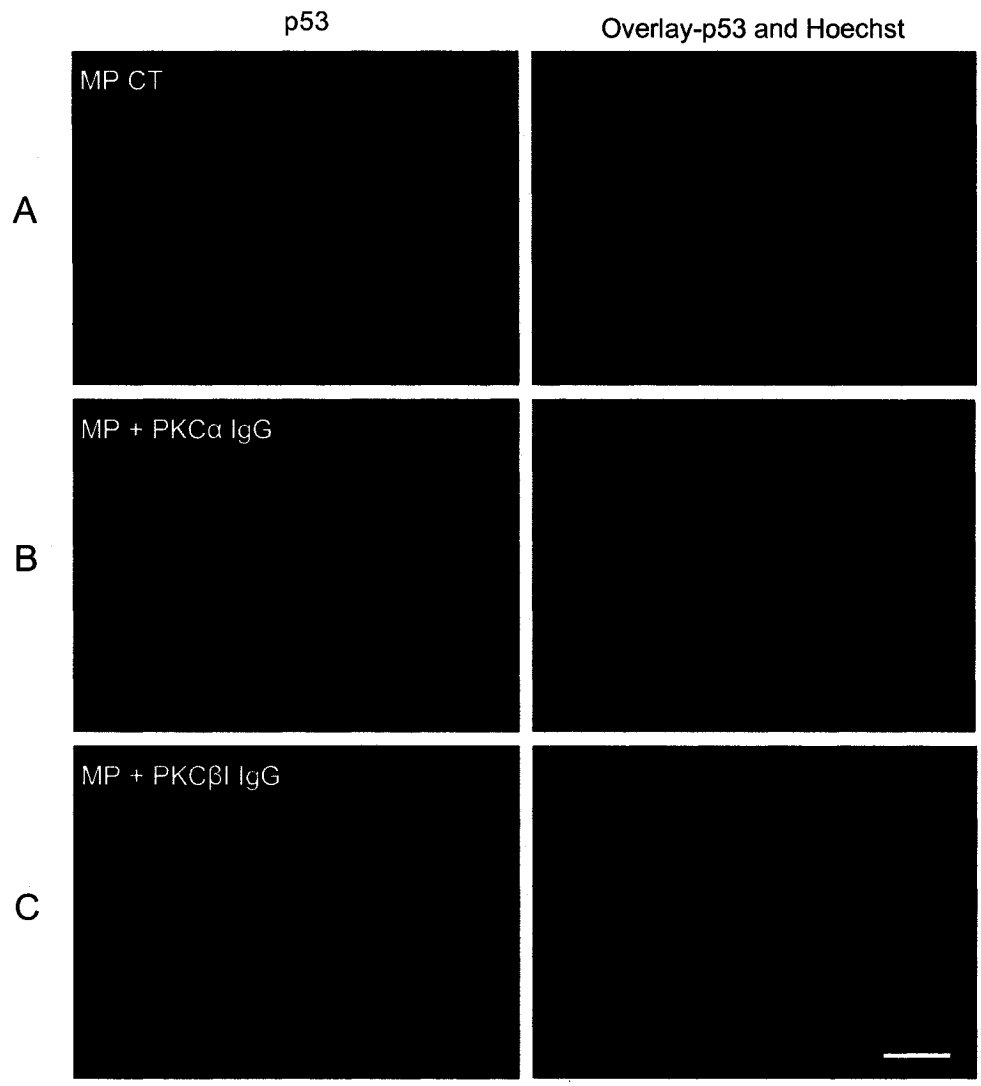
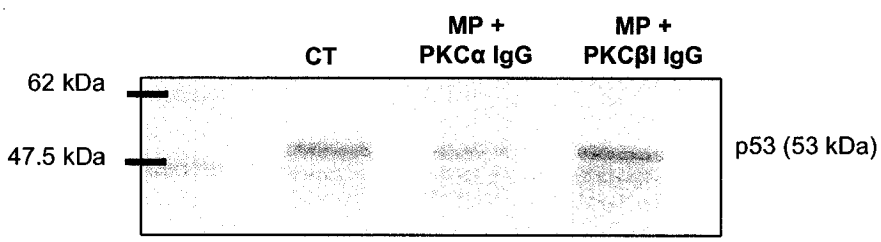

p53
Overlay-p53 and Hoechst
A
MP CT
B
MP + PKCα IgG
C
MP + PKCβI IgG

Figure 17. Phosphoserine analysis of p53 from neuroblastoma cells, IMR-32 loaded with PKCα or PKCβI IgGs.

p53 was immunoprecipitated with anti-p53 (FL-393) IgG following total protein extraction from control cells (CT) and cells loaded with PKCα IgG (MP + PKC α IgG) or PKCβI IgG (MP + PKCβI IgG) 24 hours prior using the Molecular Probes Influx™ Cell Loading Reagent. Proteins were resolved by SDS-PAGE on a 10% gel. Western blots were probed with an anti-phosphoserine antibody. Molecular weight standard is indicated on the left of the blot. Molecular masses are expressed in kilodaltons (kDa).


CT
MP +
PKCα IgG
MP +
PKCβI IgG
62 kDa
47.5 kDa
p53 (53 kDa)

3.4 Involvement of PKC in the Regulation of p53 Function in NB

P53 plays a pivotal role in assimilating cellular cues and evoking a proper response of signal transduction pathways involved in cell growth, cell cycle arrest, and apoptosis. In NB, p53 is cytoplasmically sequestered though there is controversy as to whether the p53 signalling pathway is intact. Thus after restoration of nuclear p53 accumulation by PKC inhibition, the effect on NB proliferation and p53 functionality was assessed.

3.4.1 Effect of PKC Inhibitors on Cell Proliferation in IMR-32 and SHSY5Y Cells

To determine if cell proliferation is altered when p53 is relocalized to the nucleus upon treatment with protein kinase inhibitors, cell proliferation was monitored by staining live cells with a fluorescent membrane linker, DiI and further analysis was conducted by flow cytometry. DiI is an amphipathic carbocyanine membrane probe comprised of a fluorophore and lipophilic aliphatic tail which can insert into the cellular membrane (Molecular Probes). At each cellular division, the resulting daughter cells retain only half the amount of DiI as compared to the mother cell. The DiI levels retained in each successive generation will be halved as compared to the preceding generation. Thus, the resulting number of cell generations can be determined by measuring the relative fluorescence intensity of the individual cells in a population by flow cytometry analysis. The results are calculated as the percentage of cells of a given fluorescence intensity within the whole population.

The NB cell lines, IMR-32 and SHSY5Y were treated with the protein kinase inhibitors for 24, 48, and 72 hours. According to the cell proliferation profiles (Figure 18) and distribution of cells in each generation (Tables 3A & B), the inhibitors H7 and BisI decrease cell proliferation. On the other hand, Gö6976 and SuperTIP, though able to induce p53 relocalization had no effect on cell proliferation as the cell generation profiles are comparable to control cells.

After 24 hours of treatment, H7 is able to retard IMR-32 cell proliferation as 50.65% of the parental cells have not been recruited to the second generation; whereas in the control cells, only 4.83% of the parental generation remains. The majority of the control cells have already reached the third generation (61%) after 24 hours; this generation does not appear in H7-treated IMR-32 cells until 72 hours. The decrease in cell proliferation upon H7 treatment is further substantiated by an observed decrease in the cell proliferation indices and increase in non-proliferation indices as compared to the control (Table 4A & B).

BisI is also able to decrease IMR-32 cell proliferation. After 24 hours of treatment, a non-proliferation index of 0.54 is observed for BisI while control cells have a value of 0.06 (Table 4A). Treatment with BisI produces cell proliferation profiles with the majority of cells lagging one generation behind the controls (Figure 18A and Table 3A). After 24 hours, 68.50% of the BisI cell population is in generation two while the untreated cell population is comprised of 61.04% in generation three. This trend continues through to 48 and 72 hours.

Similar trends were also observed with the SHSY5Y cell line. Only exposure to the general kinase and PKC inhibitors, H7 and BisI respectively, decreased the rate of

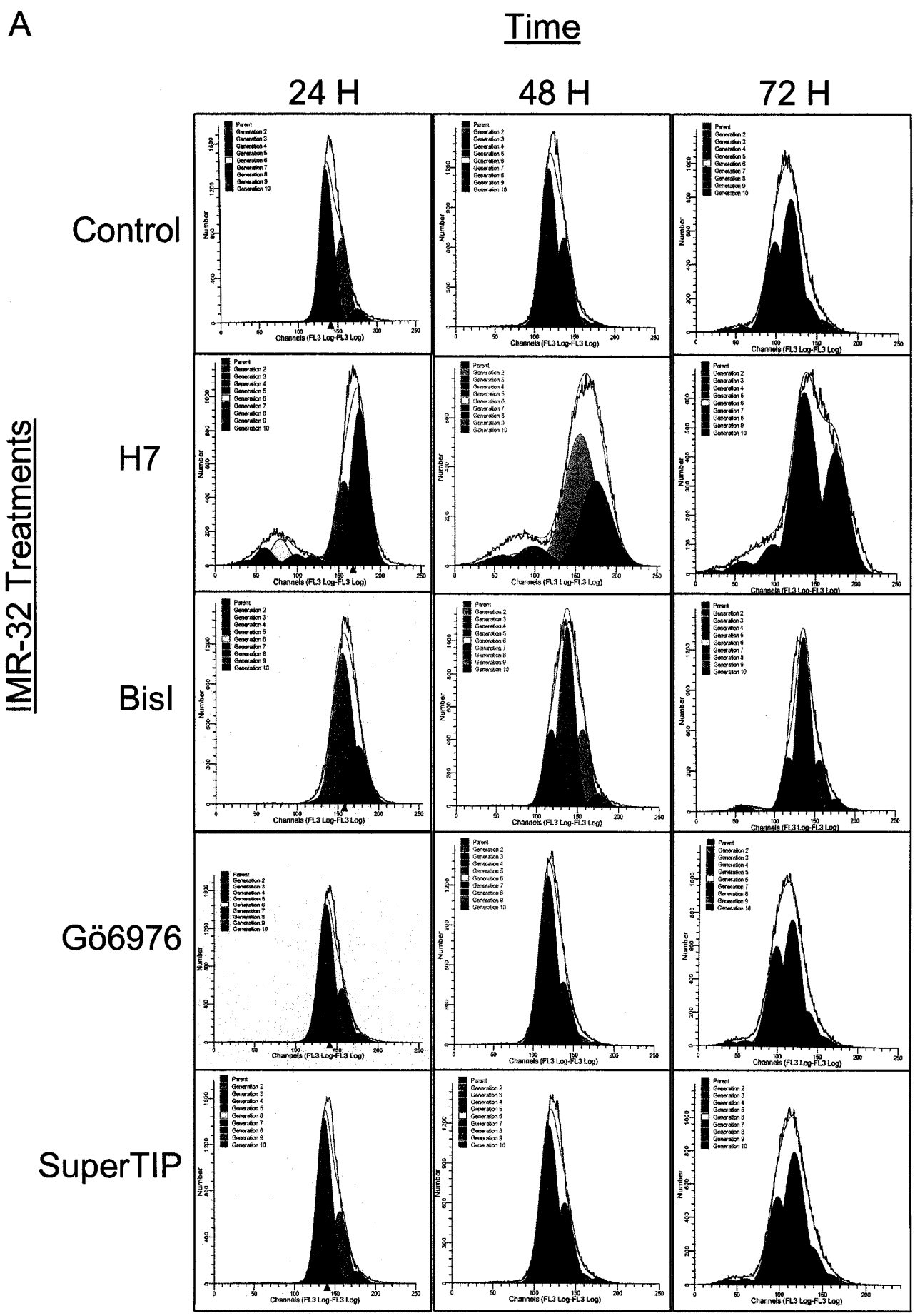
proliferation in SHSY5Y cells (Figure 18B), despite the ability of Gö6976 and SuperTIP to relocalize p53 (Figure 9B). Cell population distribution of the various generations is detailed in Table 4B. H7 drastically reduces the proliferation of SHSY5Y cells. After 24 hours treatment with H-7, 95.5% of the parental cells remain, in comparison to the untreated control cells, 96.3% of the cell population is comprised of cells in the second generation (Table 4B). This trend continues with H7 at 48 and 72 hours exposure, the majority of the cell population with treatment is always one generation behind untreated cells. Bis I exposure also decreases the rate of cell proliferation as noted by the altered cell generation distribution (Table 4B), though not to the same extent as H7. A decrease in the proliferation index is observed at 24, 48, and 72 hours for H7 (1.04, 1.93, 2.89) and BisI (1.47, 3.56, 4.52) as compared to control cells (1.95, 3.91, 5.22) (Table 4B) with a concomitant increase in the nonproliferating fraction of cells.

In summary, the non-specific protein kinase C inhibitor, H7 was able to retard the growth of both NB cells lines to a much greater extent than BisI; whereas, the conventional PKC inhibitor, Gö6976 and SuperTIP peptide had no effect on cell proliferation.

Figure 18. H7 and BisI exposure decreases rate of proliferation of IMR-32 and SHSY5Y cells.

Cell generation profiles of (**A**) IMR-32 and (**B**) SHSY5Y cells exposed to 75uM H7, 5uM BisI, 200nM Gö6976, and 100uM SuperTIP for 24, 48, and 72 hours. Prior to treatment, cells were stained with DiI and cultured for 24 hours. At the various time intervals, cells were harvested by trypsinization, and analyzed by flow cytometry. ModFit software was used to generate the cell proliferation profiles. Generations are depicted by the following colours: ■parental, ■generation 2, ■generation 3, ■generation 4, and ■generation 5.

A


Time
24 H
48 H
72 H
IMR-32 Treatments
Control
H7
BisI
Gö6976
SuperTIP
Parent
Generation 2
Generation 3
Generation 4
Generation 5
Generation 6
Generation 7
Generation 8
Generation 9
Generation 10
Number
Channels (FL3 Log-FL3 Log)

B

Time

24 H | 48 H | 72 H

SHSY5Y Treatments

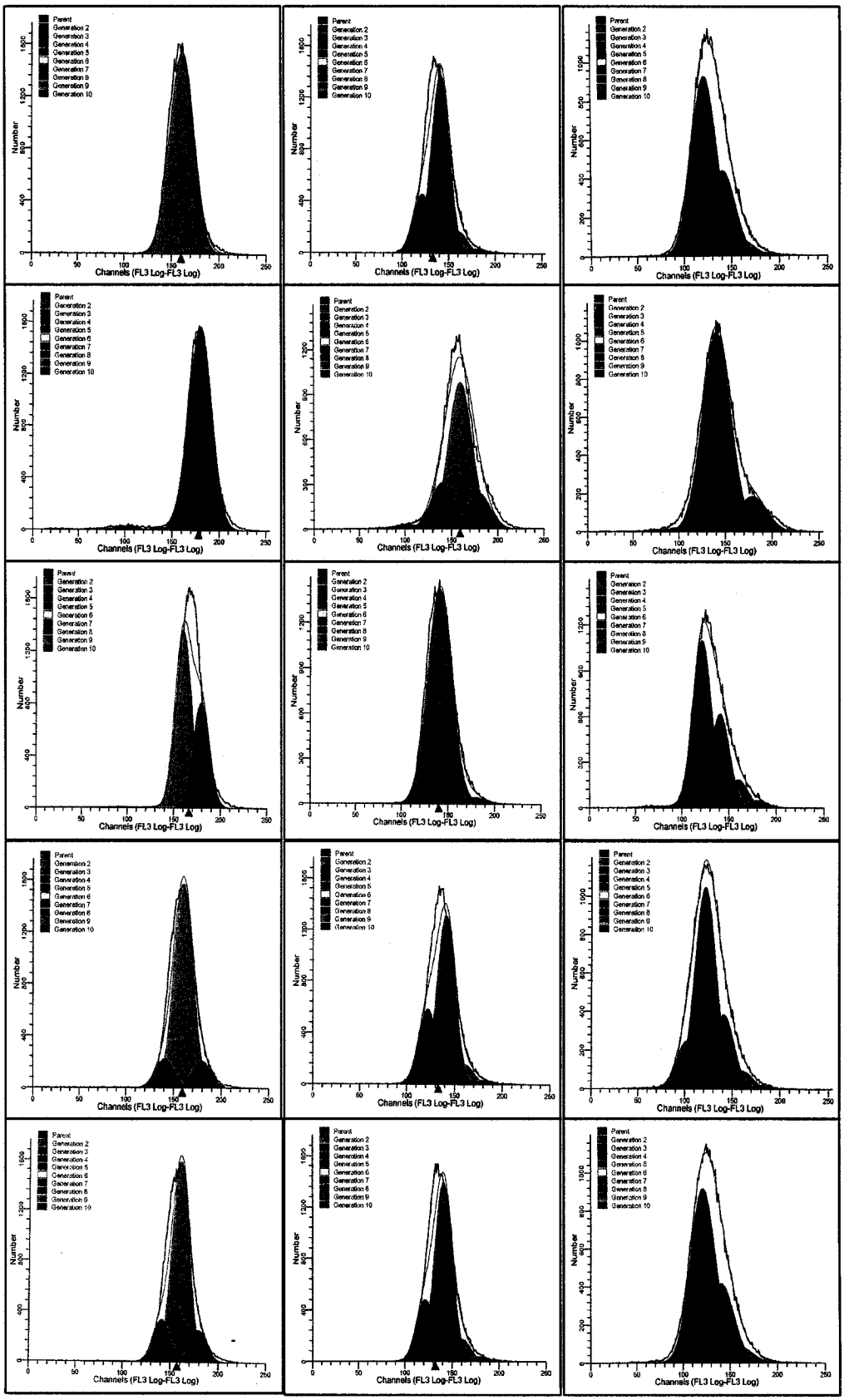
Control

H7

BisI

Gö6976

SuperTIP



IMR-32		Parental	Gen 2	Gen 3	Gen 4	Gen 5
24 H	CT	4.83	33.33	61.04	0.01	0.19
	H7	50.65	27.18	0.00	2.83	3.52
	Bis	26.22	68.50	4.08	0.67	0.00
	Gö6976	4.39	26.58	68.33	0.12	0.11
	SuperTIP	4.78	28.57	66.01	0.14	0.06
48H	CT	1.67	3.91	33.40	59.12	1.06
	H7	32.49	50.38	0.00	0.00	7.56
	Bis	3.71	22.15	51.58	22.02	0.00
	Gö6976	1.34	3.86	25.33	67.59	0.85
	SuperTIP	1.85	3.93	31.17	61.11	0.87
72H	CT	0.72	4.58	12.29	46.67	31.75
	H7	31.71	6.93	46.73	0.00	7.52
	Bis	3.90	16.89	57.73	17.93	0.00
	Gö6976	0.64	3.62	12.26	45.53	34.41
	SuperTIP	0.77	3.87	13.39	46.58	30.84

Table 3A. Effect of PKC inhibitors on the cell generation distribution of IMR-32 cells. Values are displayed as percentages of the total cell population. The generation containing the largest cell percentage is highlighted in yellow. DiI stained IMR-32 cells were treated with 100uM H7, 5uM BisI, 200nM Gö6976, and 100uM SuperTIP for 24, 48, and 72 hours. At the various timepoints, the cells were harvested by trypsinization. The DiI content of the cells was established by flow cytometry with use of the ModFit software for data deconvolution.

SHSY5Y		Parental	Gen 2	Gen 3	Gen 4	Gen 5
24 H	CT	3.03	96.31	0.09	0.00	0.26
	H7	95.50	0.00	1.25	0.53	1.58
	Bis	37.07	61.90	0.00	0.29	0.18
	Gö6976	10.38	77.97	11.09	0.00	0.28
	SuperTIP	11.60	72.54	15.36	0.00	0.23
48H	CT	1.80	8.14	67.52	22.29	0.00
	H7	15.53	62.36	19.89	0.00	1.94
	Bis	2.75	4.27	92.64	0.00	0.00
	Gö6976	1.60	7.47	62.74	27.92	0.00
	SuperTIP	1.83	8.39	66.53	23.07	0.00
72H	CT	1.27	5.43	29.18	61.75	2.14
	H7	14.16	0.00	79.26	4.32	1.69
	Bis	2.56	9.47	31.39	55.73	0.01
	Gö6976	1.07	5.23	21.32	58.05	13.88
	SuperTIP	1.17	5.72	28.12	61.87	2.48

Table 3B. Effect of PKC inhibitors on the cell generation distribution of SHSY5Y cells. Values are displayed as percentages of the total cell population. The generation containing the largest cell percentage is highlighted in yellow. DiI stained SHSY5Y cells were treated with 100uM H7, 5uM BisI, 200nM Gö6976, and 100uM SuperTIP for 24, 48, and 72 hours. At the various timepoints, the cells were harvested by trypsinization. The DiI content of the cells was established by flow cytometry with use of the ModFit software for data deconvolution.

Cell Line: IMR-32		Control	H7	BisI	Gö6976	SuperTIP
Proliferation Index	24H	2.72	1.53	1.62	2.87	2.81
	48H	5.14	1.71	3.29	5.52	5.17
	72H	7.16	2.11	3.44	7.56	7.20
Nonproliferation Index	24H	0.78	0.78	0.43	0.13	0.13
	48H	0.56	0.56	0.12	0.07	0.10
	72H	0.67	0.67	0.13	0.05	0.06

Table 4A. Effect of PKC inhibitors on proliferation and nonproliferation indices of IMR-32 cells. The proliferation index is the average number of cell divisions the cell population has undergone. DiI stained IMR-32 cells were treated with 100uM H7, 5uM BisI, 200nM Gö6976, and 100uM SuperTIP for 24, 48, and 72 hours. At the various timepoints, the cells were harvested by trypsinization. DiI content of the cells was established by flow cytometry with use of the ModFit software for data deconvolution.

Cell Line: SHSY5Y		Control	H7	BisI	Gö6976	SuperTIP
Proliferation Index	24H	1.95	1.04	1.47	1.92	1.93
	48H	3.91	1.93	3.56	4.08	3.91
	72H	5.22	2.89	4.52	5.83	5.27
Nonproliferation Index	24H	0.06	0.99	0.54	0.20	0.22
	48H	0.07	0.30	0.10	0.07	0.07
	72H	0.07	0.41	0.12	0.06	0.06

Table 4B. Effect of PKC inhibitors on proliferation and nonproliferation indices of SHSY5Y cells. The proliferation index is the average number of cell divisions the cell population has undergone. DiI stained SHSY5Y cells were treated with 100uM H7, 5uM BisI, 200nM Gö6976, and 100uM SuperTIP for 24, 48, and 72 hours. At the various timepoints, the cells were harvested by trypsinization. DiI content of the cells was established by flow cytometry with use of the ModFit software for data deconvolution.

3.4.2 Effect of PKC Inhibitors on Cell Cycle Progression in IMR-32 and SHSY5Y Cells

In the previous section, it was observed that exposure to PKC inhibitors decreases cellular proliferation of IMR-32 and SHSY5Y cells. The following experiments detail the identification of the cell cycle phase(s) in which the effects of relocalized p53 by PKC inhibition are being manifested.

To determine the effect of the PKC inhibitors, H7 and BisI on cell cycle progression and cell cycle accumulations in IMR-32 and SHSY5Y cells, the cells were stained with propidium iodide (PI) and subjected to flow cytometric analysis.

From the IMR-32 cell cycle profiles (Figure 19A), H7 initially caused an S-phase accumulation in the first 24 hours as compared to the control; 62% versus 38% respectively. However, further H7 exposure induced an accumulation of cells in the G2-M phases and concomitant decrease in cells in the S-phase as compared to the control after 24 hours exposure. An increased G2-M/S ratio was observed in IMR-32 cells in the presence of H7 as compared to the control (Figure 20A); H7 and untreated (in parentheses) G2-M/S ratios were 0.53 (0.07) and 1.23 (0.45) after 48 and 72 hours treatment, respectively. Overall, the effects of H7 treatment on the IMR-32 cells can be characterized as initially inducing a cell cycle accumulation in S-phase, with a G2-M phase accumulation with prolonged treatment which corresponds to a decrease in the kinetics of DNA synthesis ending in the G2 block.

Though not as pronounced as H7, BisI does induce IMR-32 cell cycle accumulations. After 24 hours, BisI caused an initial G2-M accumulation as compared to the control; 12% versus 8%. This G2-M accumulation persists into 48 hours though

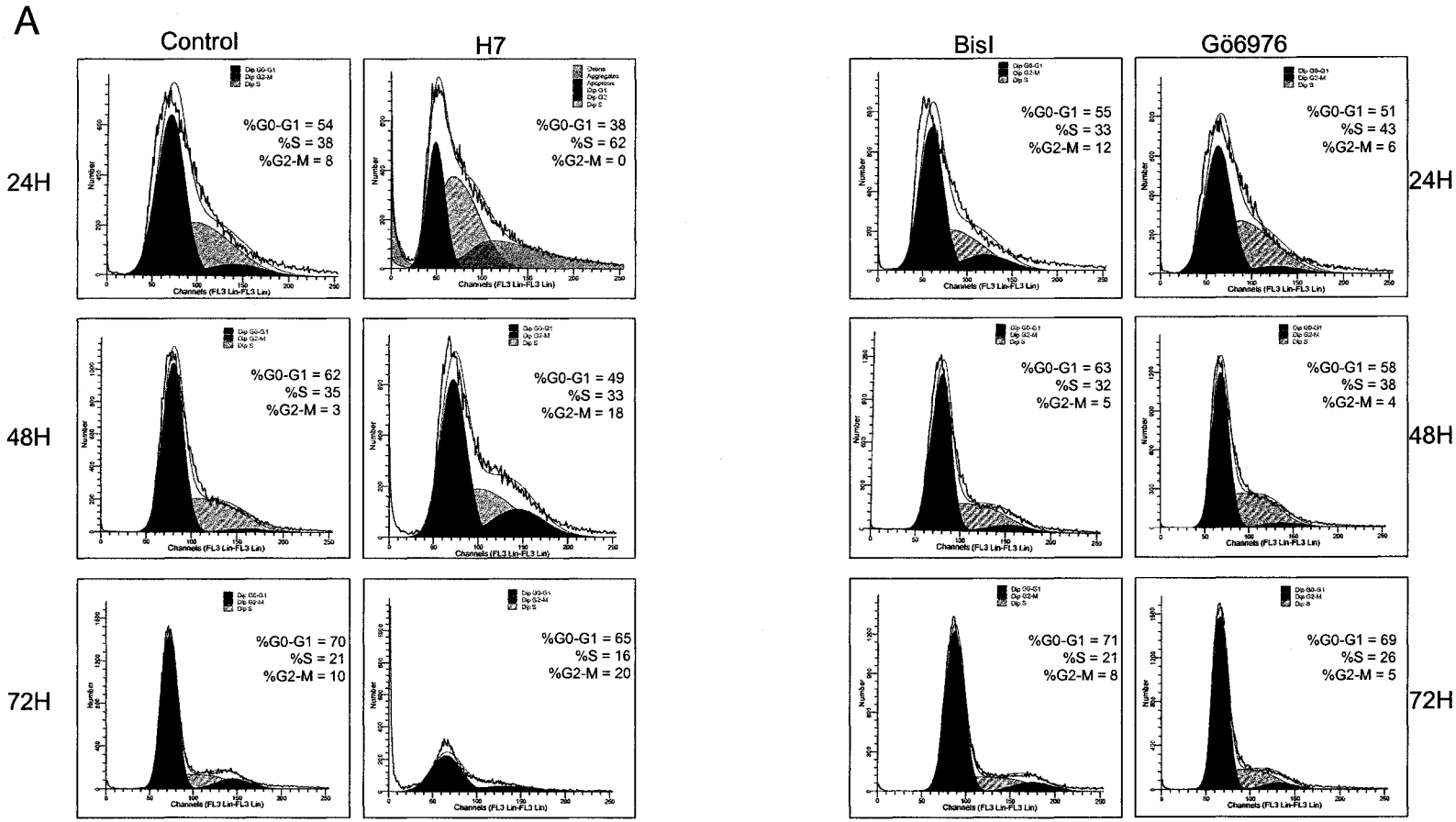
disappears afterwards. The effects of BisI are not as extensive as that of H7, and thus may only demonstrate a less persistent PKC inhibition.

The cell cycle distribution of SHSY5Y cells is slightly different after treatment with the protein kinase inhibitors. Within the first 48 hours of H7 exposure, an accumulation of cells is observed in the G2-M phases of the cell cycle as compared to the control cells (Figure 19B), 9% versus 3% after 24 hours and 11% versus 9% at 48 hours, respectively. In contrast to IMR-32 cells, changes in the percentages of SHSY5Y cells in the S-phase are not observed until 72 hours exposure. At this time point, a clear accumulation of H7 treated SHSY5Y cells was observed in the S-phase as compared to the control with values of 26% and 11% respectively. This trend continues into 96 hours of treatment with almost a two-fold increase in the percentage of cells in the S phase with H7 treatment as compared to the control, 24% versus 14% (not shown). Overall, the response of the SHSY5Y cells to H7 is initially observed as a G2-M phase accumulation, with a S-phase accumulation with prolonged exposure.

A complete cell cycle arrest is not observed with the protein kinase inhibitors as the cells still progress through the cell cycle albeit at a slower rate. This is supported by the cell proliferation profiles from the previous section that demonstrate that there is not a cessation of cell growth rather proliferation occurring at a decreased rate (Figure 18).

Figure 19. Alteration in cell cycle distribution in neuroblastoma cell lines after exposure to protein kinase inhibitors.

Cell cycle profiles of (**A**) IMR-32 and (**B**) SHSY5Y cells exposed to 75uM H7, 5uM BisI, and 200nM Gö6976 for 24, 48, and 72 hours. At the various time intervals, cells were harvested by trypsanization, incubated with RNaseA, and DNA was stained with propidium iodide. Samples were analyzed by flow cytometry. ModFit software was used to generate the cell cycle profiles. Percentages of cell population in G1-G0, S, and G2-M phases are as indicated. Experiment performed in triplicate and representative data shown.


A
Control
H7
Bisl
Gö6976
24H
48H
72H
%G0-G1 = 54
%S = 38
%G2-M = 8
%G0-G1 = 38
%S = 62
%G2-M = 0
%G0-G1 = 55
%S = 33
%G2-M = 12
%G0-G1 = 51
%S = 43
%G2-M = 6
%G0-G1 = 62
%S = 35
%G2-M = 3
%G0-G1 = 49
%S = 33
%G2-M = 18
%G0-G1 = 63
%S = 32
%G2-M = 5
%G0-G1 = 58
%S = 38
%G2-M = 4
%G0-G1 = 70
%S = 21
%G2-M = 10
%G0-G1 = 65
%S = 16
%G2-M = 20
%G0-G1 = 71
%S = 21
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%S = 26
%G2-M = 5

B

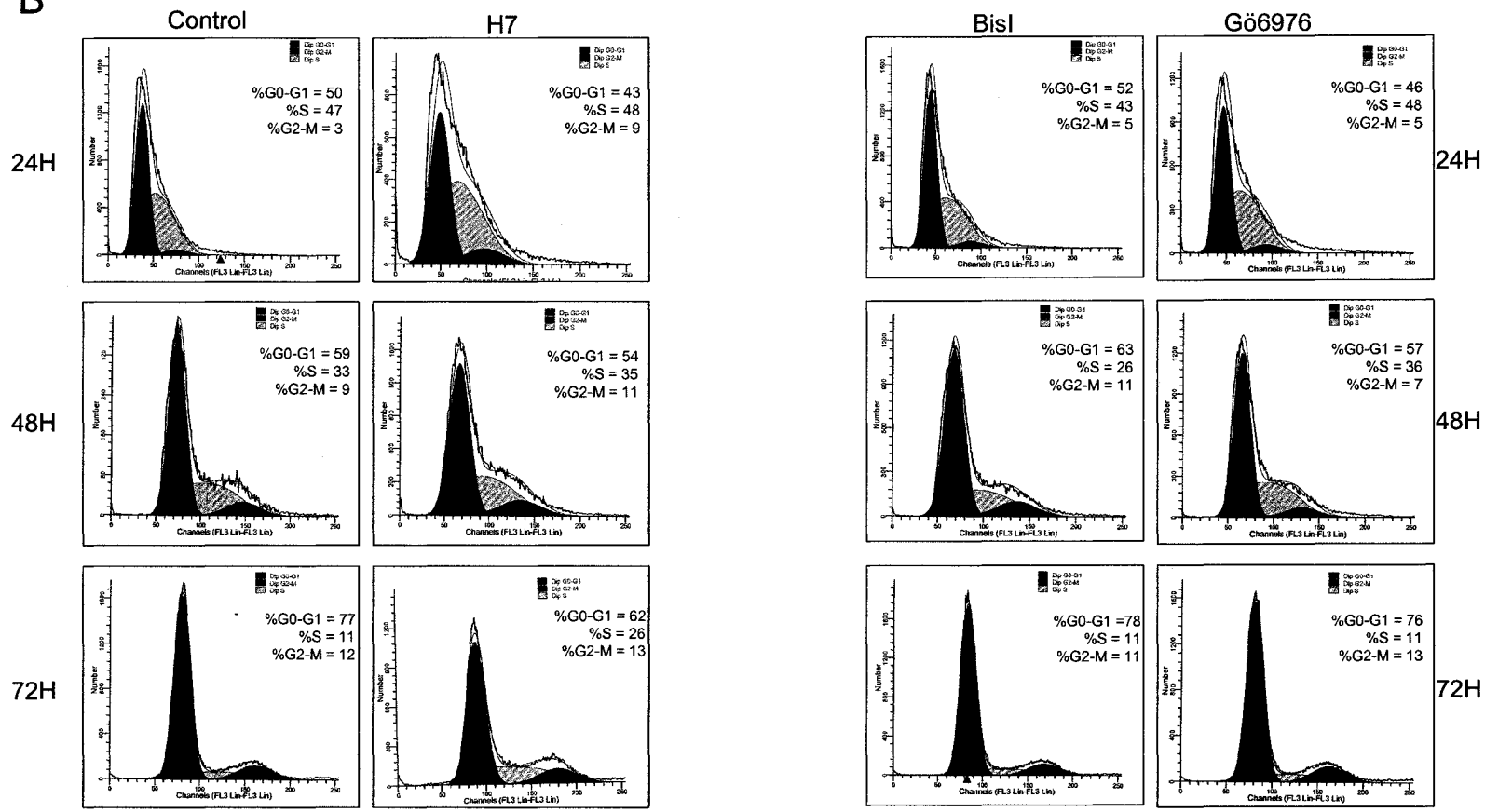
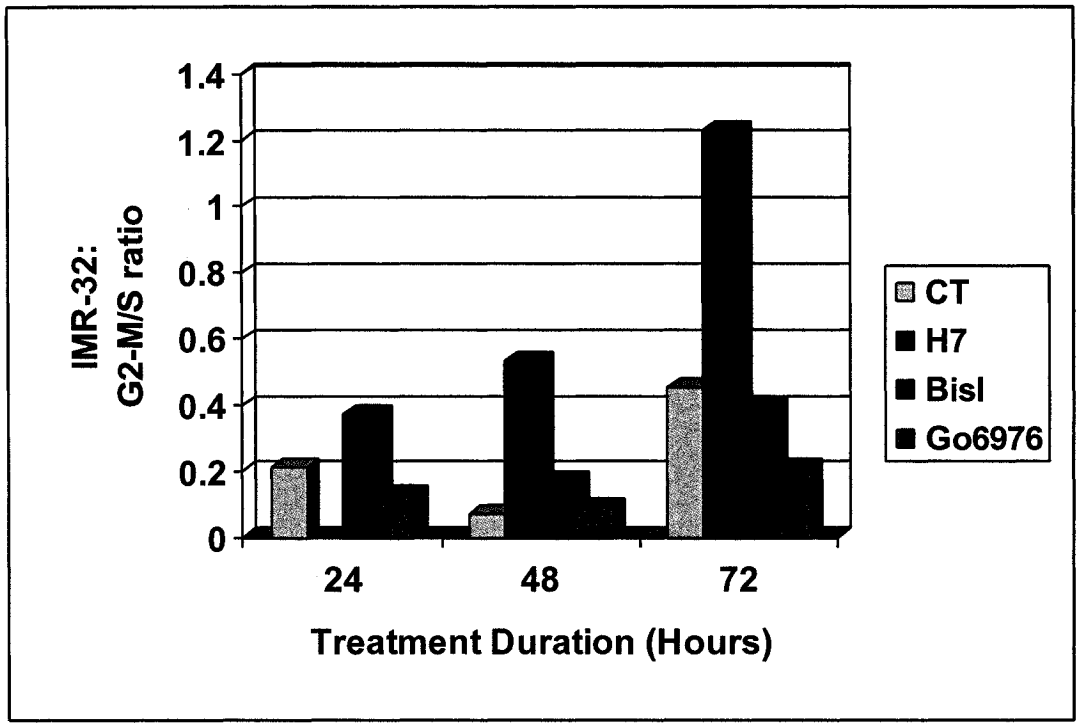

Control
H7
Bisl
Gö6976
24H
48H
72H
%G0-G1 = 50
%S = 47
%G2-M = 3
%G0-G1 = 43
%S = 48
%G2-M = 9
%G0-G1 = 52
%S = 43
%G2-M = 5
%G0-G1 = 46
%S = 48
%G2-M = 5
%G0-G1 = 59
%S = 33
%G2-M = 9
%G0-G1 = 54
%S = 35
%G2-M = 11
%G0-G1 = 63
%S = 26
%G2-M = 11
%G0-G1 = 57
%S = 36
%G2-M = 7
%G0-G1 = 77
%S = 11
%G2-M = 12
%G0-G1 = 62
%S = 26
%G2-M = 13
%G0-G1 =78
%S = 11
%G2-M = 11
%G0-G1 = 76
%S = 11
%G2-M = 13

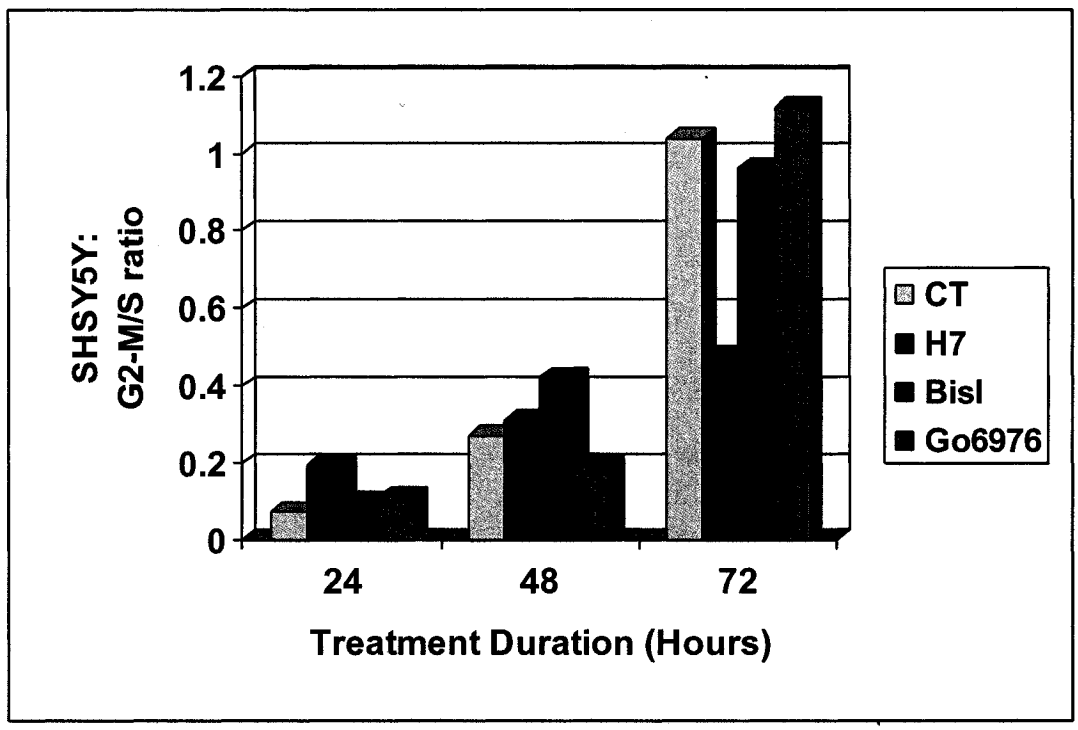
Figure 20. Changes in G2-M/S ratio after protein kinase inhibitor exposure in neuroblastoma cells.

Bars represent G2-M/S ratios of (**A**) IMR-32 and (**B**) SHSY5Y cells in untreated control cells (CT) and treatment with 75uM H7, 5uM BisI, and 200nM Gö6976 for 24, 48, and 72 hours. Cells percentages from propidium iodide-cell cycle analysis profiles (as illustrated in figure 19) were utilized for ratio calculations.

A


IMR-32:
G2-M/S ratio
1.4
1.2
1
0.8
0.6
0.4
0.2
0
24
48
72
Treatment Duration (Hours)
CT
H7
Bisl
Go6976

B


SHSY5Y:
G2-M/S ratio
1.2
1
0.8
0.6
0.4
0.2
0
24
48
72
Treatment Duration (Hours)
CT
H7
Bisl
Go6976

3.5 Effect of p53 Relocalization on Expression of p53 Responsive Elements, p21 and Bax

p53 has the capacity to bind to specific DNA sequences through its central DNA binding domain and has been identified as a transcription factor responsible for the induction of the expression of gene proteins involved in DNA repair, cell cycle arrest, and apoptosis (Levine, 1997). Cell cycle progression is regulated through heterodimeric protein kinases, which are comprised of the regulatory subunit, cyclins and cyclin-dependant kinases (CdKs), the catalytic subunit (Sherr, 1993). A p53-responsive gene, *p*21 is responsible for cell cycle arrest at G1 and G2 phases. p21 blocks cell proliferation by binding and inhibiting cyclin activity, thus, allowing DNA damages to be repaired. If DNA damages cannot be repaired, p53 will elicit a cellular response for the expression of apoptotic genes; one such gene product is *bax*. Bax is a pro-apoptotic mitochondrial protein involved in the release of cytochrome C and cascade activation of caspases during apoptosis (Korsmeyer *et al.*, 2000; Schuler *et al.*, 2000).

Upon relocalization of p53 to the nucleus with PKC inhibitors, H7 and BisI, cell proliferation and cell cycle are altered. In comparison, Gö6976 had no effect on either of the measured parameters (Figure 18, 19, 20). These observations raise the question: are the changes associated with cell proliferation and the cell cycle attributed to p53 activation? To test whether p53 is functional upon relocalization, the protein expression of p53-responsive elements, p21 and Bax were monitored by flow cytometry in the NB cells after treatment with the protein kinase inhibitors.

Expression of p21 is elevated upon exposure of H7 in IMR-32 (Figure 21A). The level of p21 expression is 71% and 97% fold higher with H7 than untreated cells after 48 and 72 hours respectively. This is consistent with the observed G2-M phase accumulation

associated with H7 treatment (Figure 19). Treatment with the PKC inhibitor, BisI slightly increased p21 expression at 48 and 72 hours as compared to untreated IMR-32 cells. The maximal effect of BisI on p21 expression was observed after 48 hours treatment; p21 levels were 29% greater than the control. On the contrary, Gö6976 had no effect on p21 levels as expression was comparable to steady-state levels.

p53 functionality was studied further by monitoring the alterations in the p53 responsive pro-apoptotic product, Bax. Elevation in Bax protein levels associated with H7 exposure is time-dependent. At 48 and 72 hours of H7 treatment, Bax levels were 9% and 27% higher with H7 than that from control cells (Figure 21B). In contrast, BisI and Gö6976 had no significant effect on Bax levels.

p53 is not only able to induce expression of pro-apoptotic proteins but also suppresses the expression of anti-apoptotic proteins (Ho & Benchimol, 2003). p53 functionality was further assessed by evaluation of the levels of the anti-apoptotic protein, Bcl-2 by Western blotting. Upon H7 treatment, Bcl-2 levels are lower than for the control (Figure 22) in both NB cell lines. No significant changes were noted for BisI and Gö6976.

In summary, BisI was only able to alter expression of p21 with no effect on the levels of pro- and anti-apoptotic proteins, Bax and Bcl-2 respectively (Figure 21 & 22). At a concentration of 5 uM BisI, no apoptotic cells were observed. However, the H7 treatment induced apoptosis. The inhibition of PKCs by BisI is effective at altering cell proliferation and the cell cycle without causing cell death. In comparison to the general kinase inhibitor, H7 caused cell cycle accumulations and increased the level of the pro-

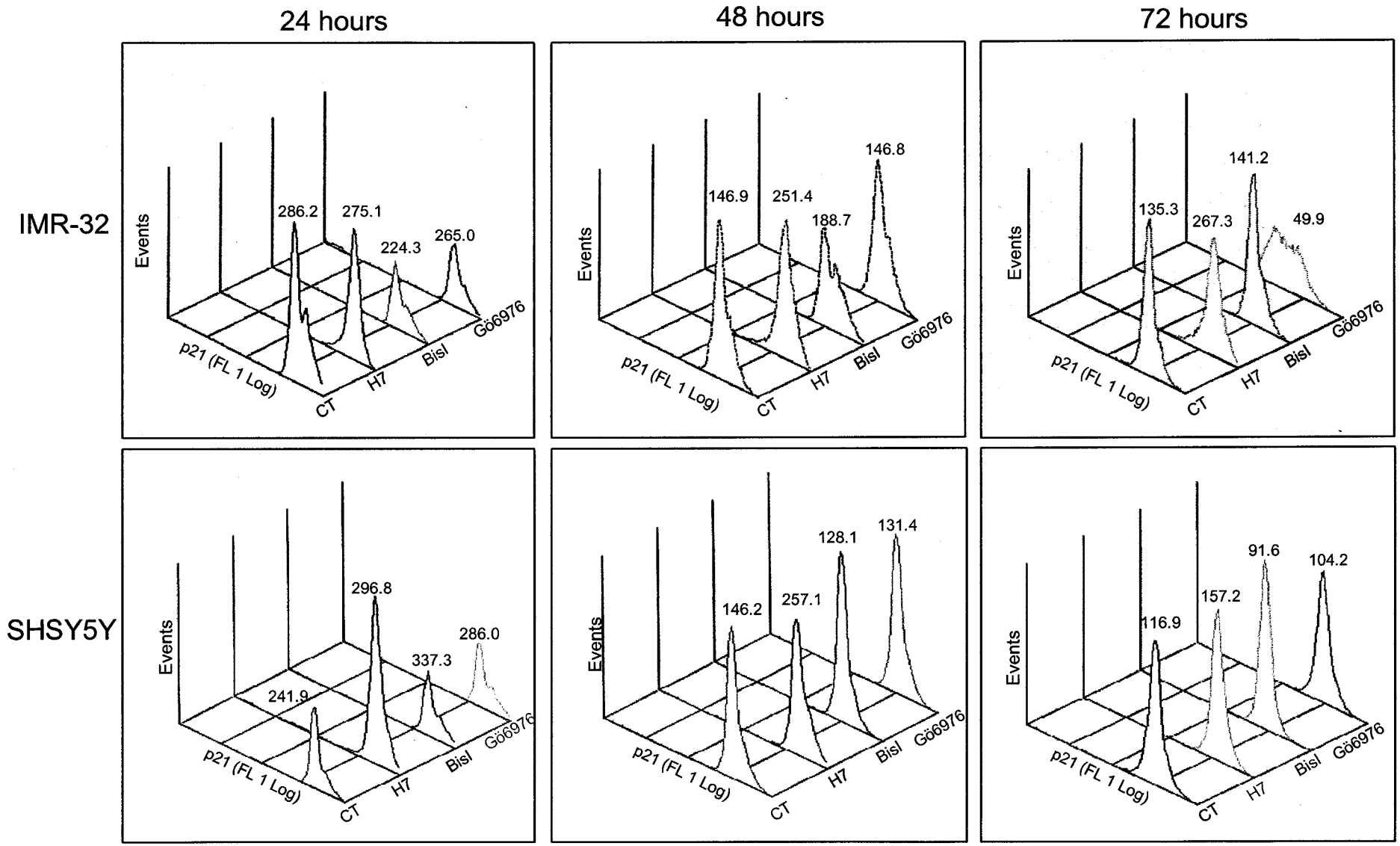
apoptotic protein, Bax with an associated decrease in the level of the anti-apoptotic protein, Bcl-2 (Figure 19-22).

The changes observed in cell proliferation, the cell cycle, and p53 responsive elements indicate that the P53 pathway is functional when p53 is relocalized to the nucleus with the inhibitors, H7 and BisI. However despite the ability to relocalize p53 with cPKC inhibition by Gö6976, the p53 response pathway was not activated. Thus, indicating that a cPKC isoform is not involved in inactivating the p53 pathway.

Figure 21. Exposure to H7 and BisI alters expression of p53-responsive elements, p21 and bax in neuroblastoma.

(**A**) p21 and (**B**) Bax protein levels were measured in IMR-32 *(top row)* and SHSY5Y *(bottom row)* cells were treated with 75 uM H7, 5 uM BisI, and 200 nM Gö6976 for 24, 48, and 72 hours. At the indicated times, cells were harvested by trypsinization and processed for flow cytometric analysis. Levels of p21 and bax protein expression were assessed by probing with p21(C-20) IgG and Bax (N-20) IgG and the corresponding secondary AlexaFluor 488nm antibodies. Mean fluorescence levels are indicated for each treatment. Experiment performed in triplicate and representative data shown.

A


24 hours
48 hours
72 hours
IMR-32
SHSY5Y
Events
p21 (FL 1 Log)
CT
H7
BisI
Gö6976
286.2
275.1
224.3
265.0
146.9
251.4
188.7
146.8
135.3
267.3
49.9
141.2
296.8
241.9
337.3
286.0
146.2
257.1
128.1
131.4
116.9
157.2
91.6
104.2

B

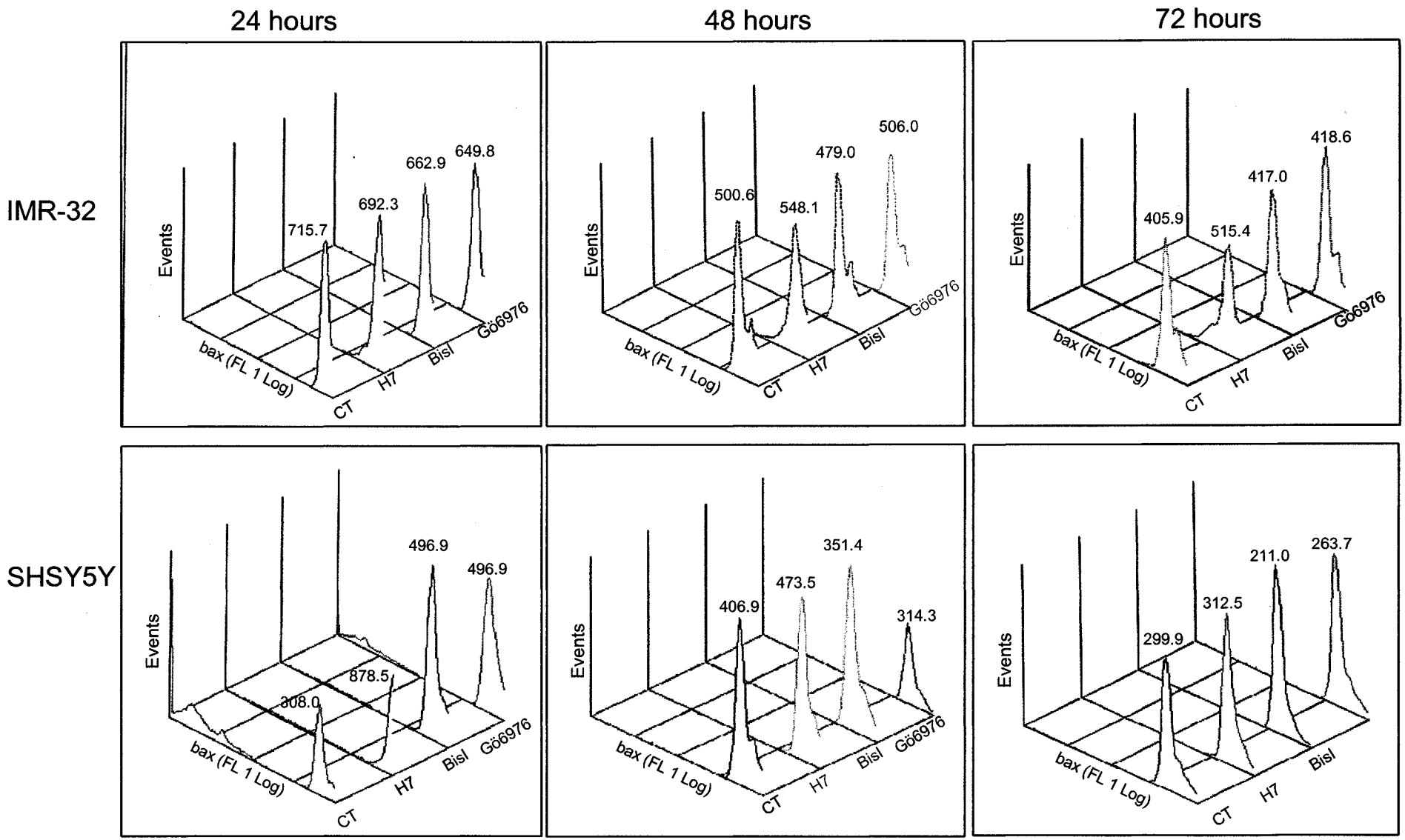
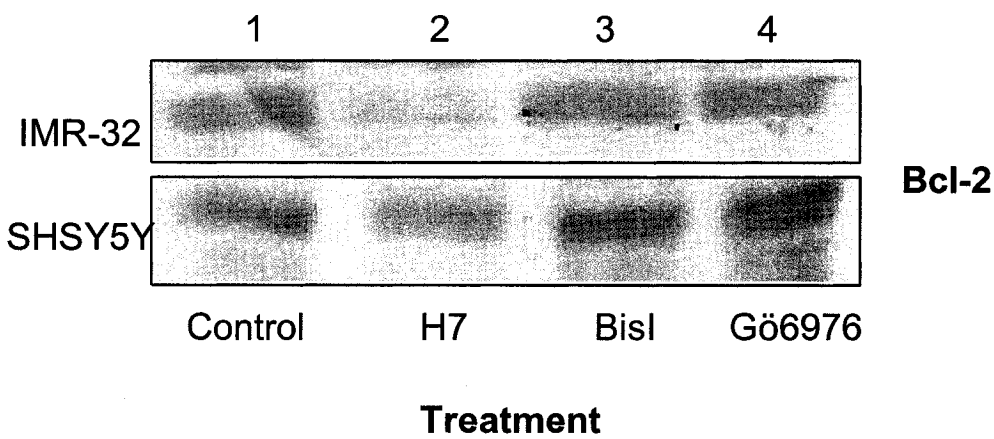

24 hours
48 hours
72 hours
IMR-32
SHSY5Y
Events
bax (FL 1 Log)
CT
H7
Bisl
Gö6976
715.7
692.3
662.9
649.8
500.6
548.1
479.0
506.0
405.9
515.4
417.0
418.6
308.0
878.5
496.9
496.9
406.9
473.5
351.4
314.3
299.9
312.5
211.0
263.7

Figure 22. H7 decreases bcl-2 protein levels in neuroblastoma cells, IMR-32 and SHSY5Y.

IMR-32 and SHSY5Y were exposed to various protein kinase inhibitors, 75 uM H7, 5 uM BisI and 200 nM Gö6976 for 24 hours. Whole protein lysates were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. Bcl-2 protein levels were assessed by immunoblot analysis by probing with bcl-2 (N-19) IgG.


1
2
3
4
IMR-32
SHSY5Y
Bcl-2
Control
H7
Bisl
Gö6976
Treatment

3.6 Identification of PKC Isoforms Responsible for p53 Phosphorylation-- Monitoring Phosphorylation by Electrospray Ionization

3.6.1 *In vitro* PKC Phosphorylation of C-terminal p53 Peptide

As a strategy to identify the PKC isoforms responsible for p53 phosphorylation, an *in vitro* PKC phosphorylation reaction with a p53 peptide substrate comprised of residues 361 to 382 was utilized. Peptide phosphorylation was analyzed by electrospray ionization mass spectroscopy (ESI). The peptide commercially available under the name, Peptide 46 encompasses the three putative PKC phosphorylation sites, serine 371, serine 376, and serine 378. Peptide 46 has an observed molecular weight of 2432.9 Daltons (Figure 23A). Five major peaks, *m/z* 348.3, 406.6, 487.5, 609.0, and 812.1 were observed in the positive electrospray spectrum (Figure 23B) which corresponds to charge states of +7, 6, 5, 4, 3 respectively.

Phosphorylation of the peptide will be detected by a shift in the major peaks in relation to the number of phosphorylated residues (*m* +*n80/z* with n = number of phosphorylated sites). A primary priority was to determine if Peptide 46 could be a PKC substrate *in vitro*. In the kinase reaction, the peptide was incubated with a mixture of PKCs obtained through a membrane fractionation procedure (refer to Materials and Methods) and phosphorylation was detected by ESI.

A list of the calculated m/z peaks corresponding to the non-phosphorylated and phosphorylated versions of Peptide 46 is outlined in Table 5.

Table 5. Expected m/z peaks of non-phosphorylated and phosphorylated Peptide 46 in positive electrospray spectrum. Peptide 46 and its phosphorylated variants are designated A through D with their calculated molecular weights in Daltons (M). Major peaks, m/z are indicated numerically ranging from charge states of + 1 through 6.

			m/z of positive charge states					
			1	2	3	4	5	6
	Number of phosphorylations	M	(M+1)/1	(M+2)/2	(M+3)/3	(M+4)/4	(M+5)/5	(M+6)/6
A	0	2432.7	2433.7	1217.4	811.9	609.2	487.5	406.5
B	1	2512.7	2513.7	1257.4	838.6	629.2	503.5	419.8
C	2	2592.7	2593.7	1297.4	865.2	649.2	519.5	433.1
D	3	2672.7	2673.7	1337.4	891.9	669.2	535.5	446.5

m/z peaks corresponding to mono-, di-, and tri- phosphorylated forms of the peptide were observed with charge states of 6, 5, 4, and 3 in the positive ESI spectrum (Figure 24).

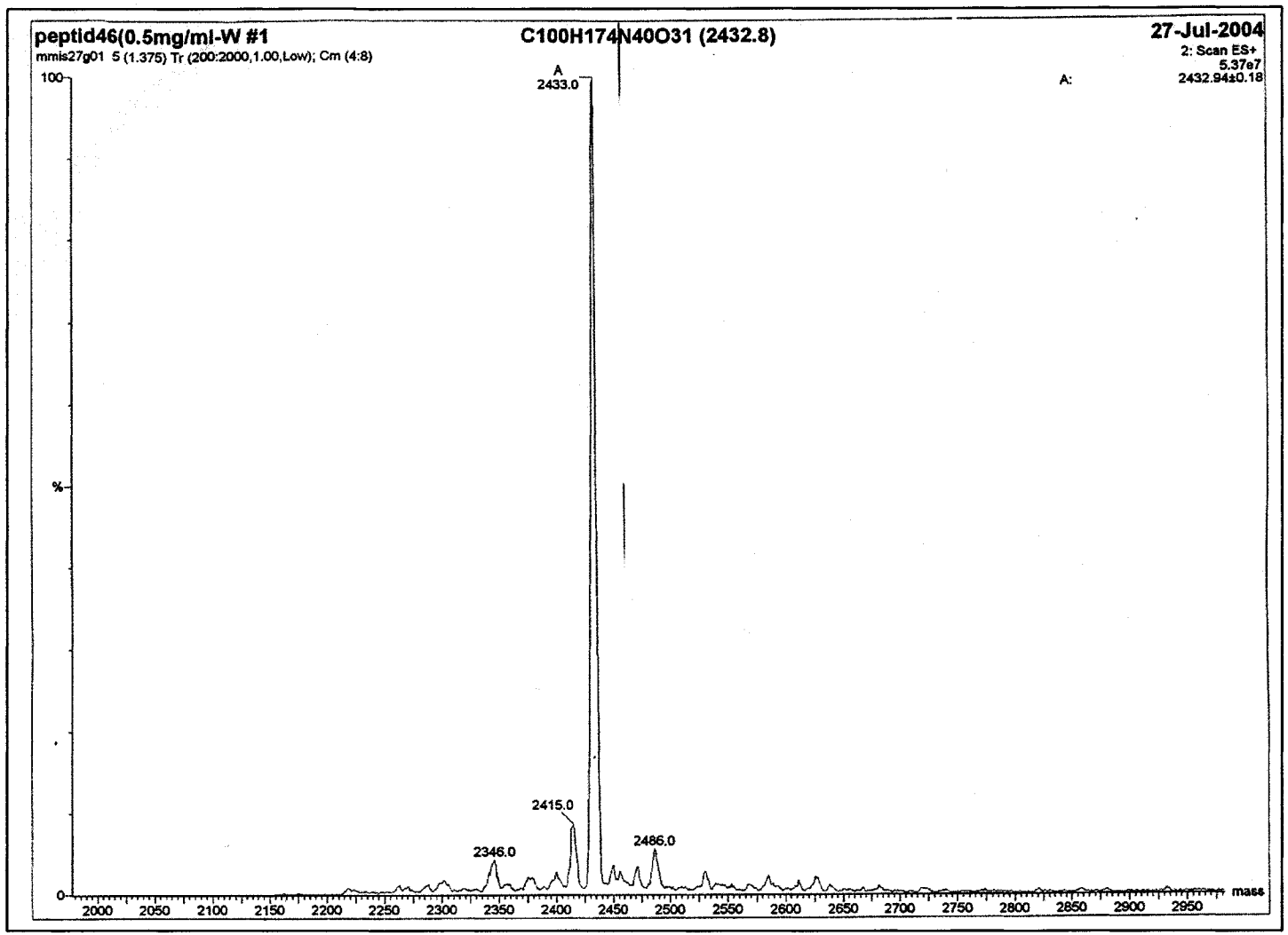
To further verify phosphorylation of the p53 peptide, the sample peptide was separated by agarose gel electrophoresis and visualized with Coomassie blue staining. The non-phosphorylated peptide, Peptide 46 has a net +7 charge. Upon peptide phosphorylation, a phosphate group will be introduced and a charge of -3 will be imparted on the peptide. Thus the net charge of the peptide will be less positive depending on the number of phosphorylation events occurring on the peptide. Resolution will occur on an agarose gel to allow for separation on the basis of net charge. The non-phosphorylated peptide has a more positive net charge and will migrate to the cathode (-), whereas less positive peptides will not be as strongly attracted and result in limited migration towards the cathode. Phosphorylated variations of the p53 peptide were observed as migration of the peptide was retarded in comparison to the peptide incubated in the absence of the enriched PKC membrane fraction (Figure 25).

In an attempt to identify the phosphorylation status and which p53 residues (serine 372, 376, 378) are being altered after PKC inhibition, nuclear extracts of cells treated with the PKC inhibitors were separated by SDS-PAGE and the band corresponding to p53 was excised, subjected to in-gel trypsin digestion, and analyzed by LC-MS in collaboration with Dr. John Kelly. However, conclusive results were not obtained (data not shown).

Figure 23. Mass spectrometric analysis of C-terminal p53 peptide, Peptide46 GSRAHSSHLKSKKGQSTSRHKK.

(**A**) Deconvoluted mass spectrum of Peptide46 with an observed molecular mass of 2432.9 daltons. (**B**) Positive-ion electrospray mass spectrum of Peptide46 with m/z ion peaks annotated (A3 to A7).

A


peptid46(0.5mg/ml-W #1
mmis27g01 5 (1.375) Tr (200:2000,1.00,Low); Cm (4:8)
C100H174N40O31 (2432.8)
27-Jul-2004
2: Scan ES+
5.37e7
2432.94±0.18
A:
A
2433.0
2415.0
2346.0
2486.0
100
%
0
mass
2000 2050 2100 2150 2200 2250 2300 2350 2400 2450 2500 2550 2600 2650 2700 2750 2800 2850 2900 2950

B

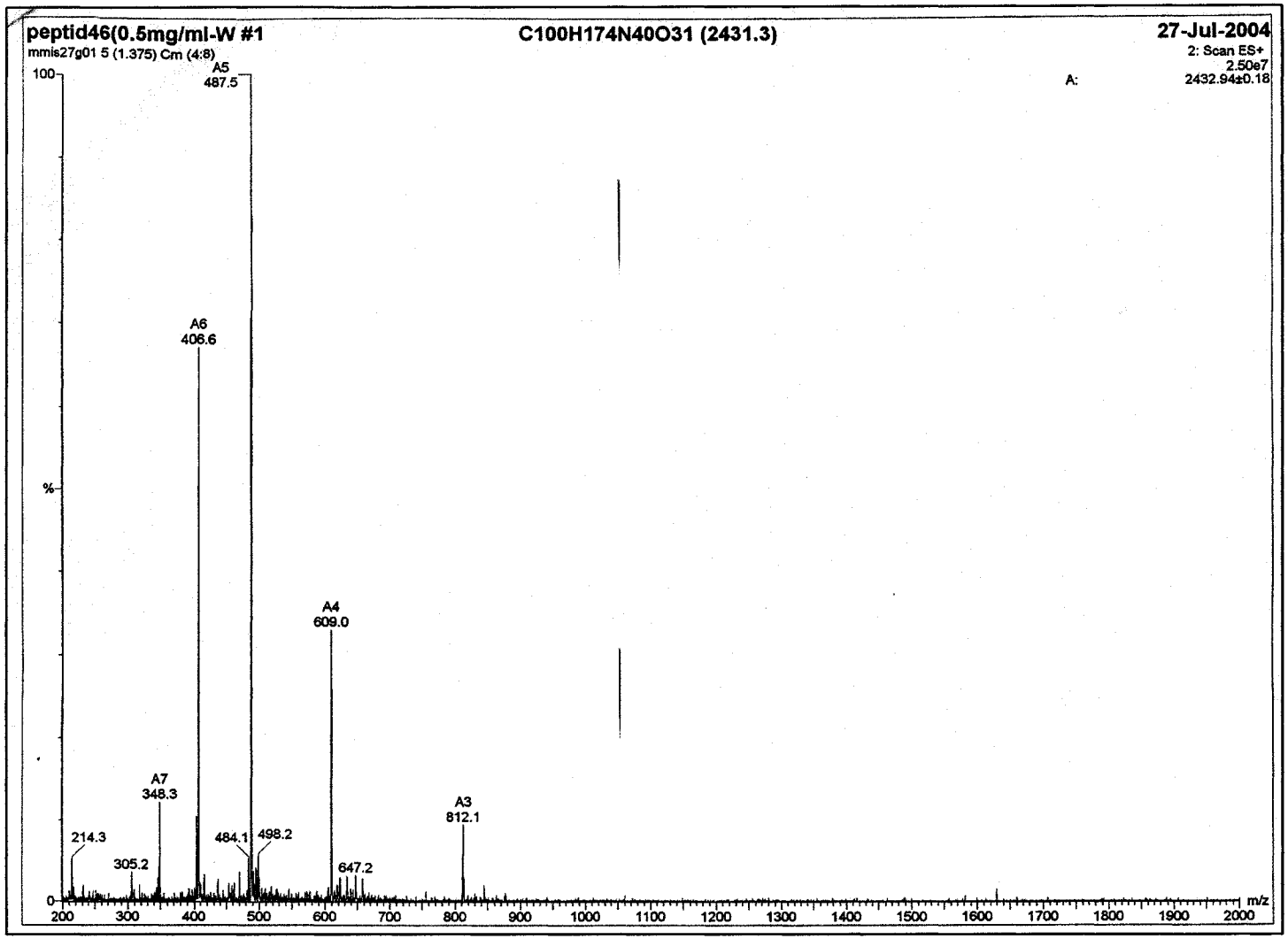

peptid46(0.5mg/ml-W #1
mmis27g01 5 (1.375) Cm (4:8)
C100H174N40O31 (2431.3)
27-Jul-2004
2: Scan ES+
2.50e7
A:
2432.94±0.18
100
%
0
A5
487.5
A6
406.6
A4
609.0
A7
348.3
A3
812.1
214.3
305.2
484.1
498.2
647.2
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
m/z

Figure 24. Electrospray analysis of C-terminal p53 peptide, Peptide46 after *in vitro* PKC kinase reaction.

Positive-ion electrospray mass spectrum of Peptide46 and phosphorylated varients with m/z ion peaks (3 to 6) annotated by the following colours: **(A)** □ non-phosphorylated peptide, **(B)** monophosphorylated peptide, **(C)** ■ diphosphorylated peptide, and **(D)** ■ triphosphorylated peptide.

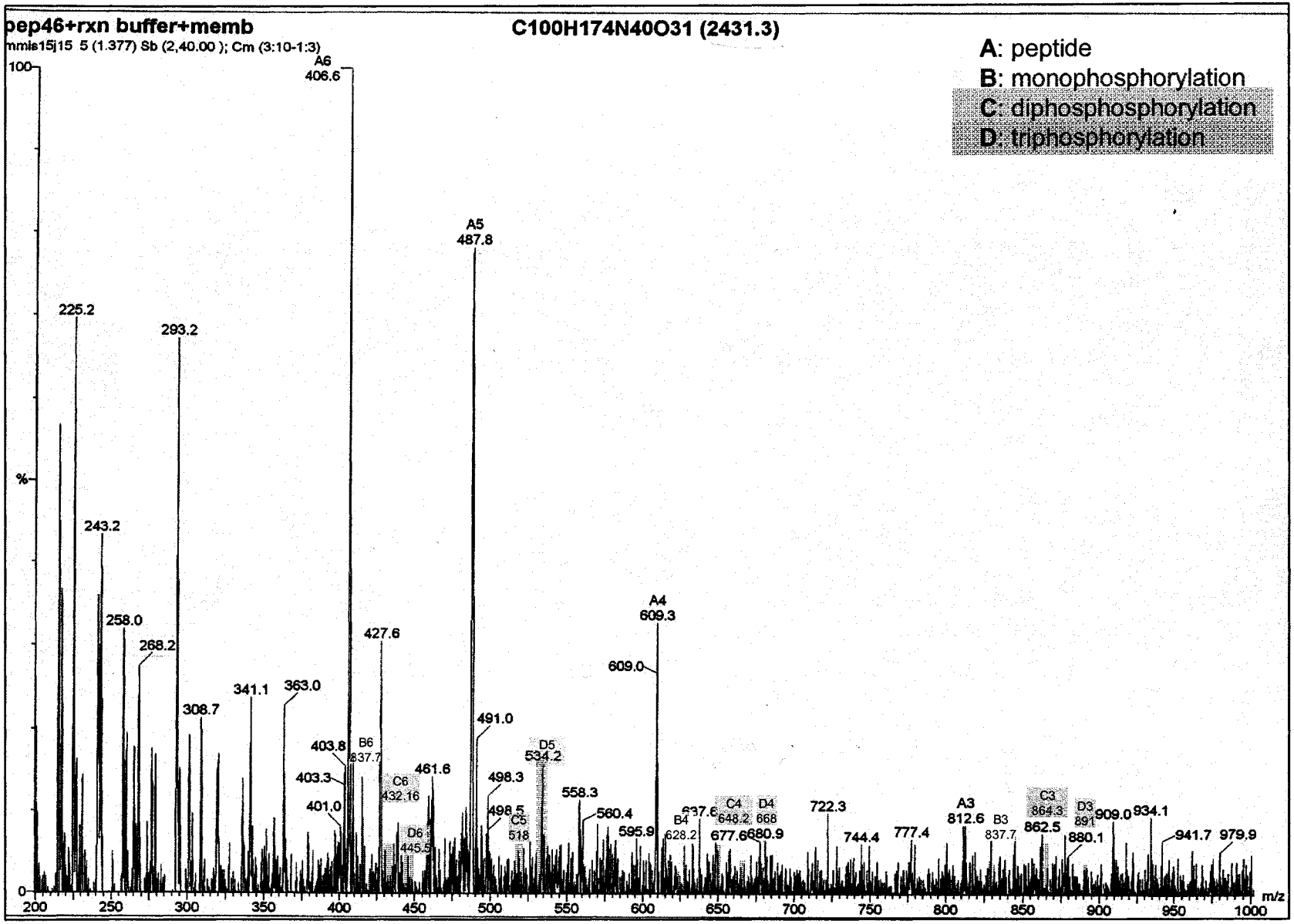
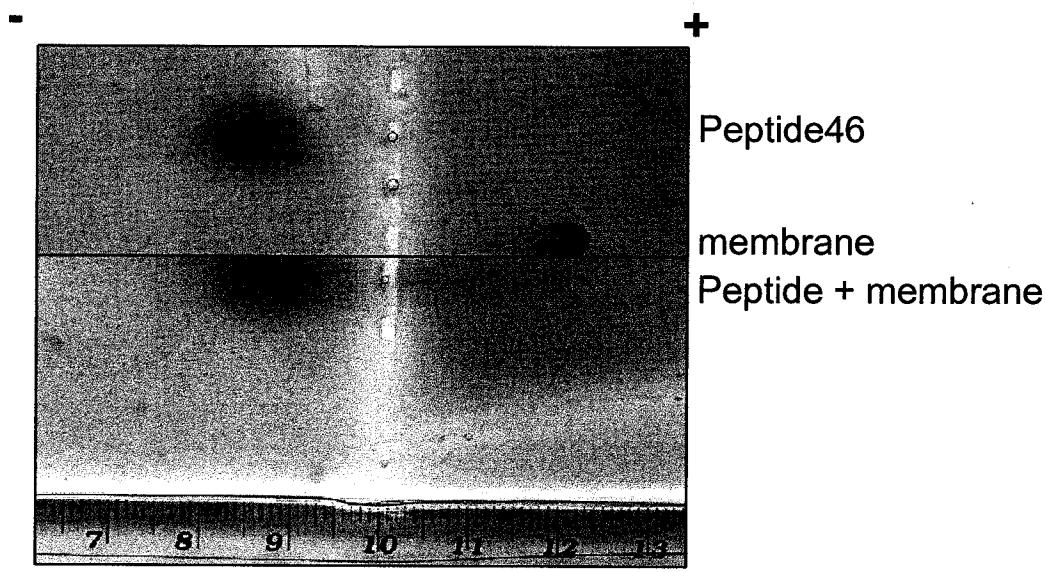

pep46+rxn buffer+memb
nmis15j15 5 (1.377) Sb (2,40.00); Cm (3:10-1:3)
C100H174N40O31 (2431.3)
A: peptide
B: monophosphorylation
C: diphosphophosphorylation
D: triphosphorylation
100
%
0
m/z
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
A6
406.6
A5
487.8
A4
609.3
225.2
293.2
243.2
258.0
268.2
308.7
341.1
363.0
427.6
609.0
491.0
403.8
B6
837.7
403.3
401.0
461.6
C6
432.16
498.3
498.5
C5
518
D5
534.2
558.3
560.4
595.9
B4
628.2
637.6
677.6
C4
648.2
D4
668
680.9
722.3
744.4
777.4
A3
812.6
B3
837.7
C3
864.3
862.5
D3
891
880.1
909.0
934.1
941.7
979.9
D6
445.5

Figure 25. Electrophoretic Separation of phosphorylated and nonphosphorylated Peptide46 on agarose gel.

A C-terminal p53 peptide, Peptide46 was subjected to *in vitro* PKC kinase reaction. Peptide46 was incubated in the absence and presence of an enriched PKC membrane fraction at 37°C. Samples were loaded and separated on a 0.8% agarose gel at 100V for 25 minutes. The peptide was visualized by subjecting the gel to Coomassie blue staining.


-
+
Peptide46
membrane
Peptide + membrane
7
8
9
10
11
12
13

4.0 DISCUSSION

The p53 protein has been the topic of much discussion upon its initial discovery in 1979. This tumour suppressor protein has been deemed pivotal in the integration of cellular signals and the initiation of appropriate responses to stress but also in cell cycle regulation under normal conditions. It is especially important in the maintenance of the integrity of the genome and regulation of cell proliferation. Though inactivation of the p53 protein results in uncontrolled proliferation—a defining feature of cancer. In the majority of cancers, p53 is inactivated, thus underlying its importance in the surveillance of cellular activity.

Many p53 inactivations are attributed to mutations within the central sequence-specific DNA binding domain. Interestingly, some cancers, including NB retain wild-type p53; however, it is inactivated by a non-mutational mechanism, cytoplasmic sequestration. The underlying cause for the aberrant cytoplasmic localization is unknown. In normal cells, p53 is regulated by post-translational modifications thus altering stabilization, localization and function. Of particular interest for this study are the PKC phosphorylation sites within the C-terminal of p53. They are attributed to the regulation of p53 DNA-binding activity. Dysregulation of the post-translational modifications of p53, especially phosphorylation could result in p53 inactivation. Overexpression of PKCs has been observed in many malignancies of the nervous system, including NB (Lahn *et al.*, 2004; Phipps laboratory, unpublished data). It is possible that the overexpression of PKCs in NB could exacerbate its normal control over p53 and impart an abnormal p53 sublocalization with subsequent inactivation of the p53 response pathway.

PKC-mediated phosphorylation on p53 regulation offers a new contributing mechanism to cytoplasmic localization in NB. The purpose of this study was to examine the role of PKC-mediated phosphorylation on the regulation of p53 localization and function in NB with identification of the possible PKC isoforms involved.

4.1 Characterization of NB cell lines, IMR-32 and SHSY5Y

Since NB is comprised of various cell types of neuroblastic, Schwann-like and intermediate characteristics, only one cell type was chosen for the purpose of this study. Representatives of N-type NB cells were chosen for this study, IMR-32 and SHSY5Y cell lines which exhibit impaired p53 function due to cytoplasmic sequestration (Issacs *et al.*, 1998). The subcellular distribution of p53 in both cell lines is predominately cytoplasmic; however there were slight differences between each cell line as determined by immunostaining (Figure 6). In IMR-32 cells, p53 is clearly localized within the cytoplasm. In contrast to SHSY5Y cells, some p53 staining was observed in the nucleus with the majority in the cytoplasm. Differences in the subcellular p53 localization between these cell lines could be attributed to N-*myc* amplification. IMR-32 cells are the more aggressive of the two cells lines as it contains a 25-fold amplification of the N-*myc* gene. It is not clear why N-*myc* amplification is associated with advanced stages of NB and poor prognosis. Recent evidence has indicated that the p53 regulator, *hdm*2 is a transcriptional target of N-myc (Slack *et al.*, 2005). Perhaps, N-*myc* amplification observed in some NB transactivates HDM2 with an increase in baseline levels; contributing to the subsequent inhibition of the p53 protein and inactivation of the response pathway, thus leading to deficient cell cycle arrest and apoptosis. Elevated

HDM2 levels observed in IMR-32 cells attributed to N-*myc* amplification could in turn, be the basis for the differences in p53 sublocalization as compared to the SHSY5Y cell line. This could possibly warrant future investigation.

Phosphorylation of the p53 protein is also involved in its regulation. The kinase chosen for this study was the PKC family. The role of PKC signal transduction is complex and influenced by cell-type. Thus it was important to detail the PKC isoform expression and distribution between the two NB cell lines, IMR-32 and SHSY5Y. The expression of PKC isoforms α, βI, βII, δ, ε, ζ were observed in both the IMR-32 and SHSY5Y cells (Figure 8). PKC isoforms θ and η were not included as they are not expressed in IMR-32 and SHSY5Y cells as previously reported by Zeidman *et al.* (1999a). Expression of PKCζ in IMR-32 cells has not been previously evaluated; it was demonstrated that there is low expression of PKCζ in IMR-32 cells. The absence of the PKCγ isoform in IMR-32 and SHSY5Y cells was confirmed by evaluation with Western blotting (Figure 8B) and in accordance with Zeidman *et al.* (1999a). A complete detail of the PKC isoforms in the NB cell lines, IMR-32 and SHSY5Y has been compiled (Table 1). Acquiring the PKC isoforms expression profile provided insight into the possible isoforms targets for this study.

4.2 Effect of PKC inhibition on p53 localization in NB

There is controversy regarding the effects of PKC-mediated phosphorylation on p53, also the identity of the isoforms involved and whether PKC activation or inhibition is necessary to induce the p53 response pathway. Even p53 phosphorylation experiments tend to result in different functional consequences both *in vitro* and *in vivo*. This study

would not only identify PKC-mediated phosphorylation of p53 as a possible mechanism conferring abnormal p53 cytoplasmic sublocalization in NB but shed insight into the aforementioned concerns. Since an overexpression of PKCs in observed in NB, we believed PKC inhibition may possibly alleviate its influence on p53. Hence, the effect of PKC inhibition on p53 localization in NB was assessed by treating IMR-32 and SHSY5Y cells with protein kinase inhibitors of varying specificity and monitoring p53 localization by immunostaining (Figure 9). Exposure to all the inhibitors altered p53 subcellular localization. Treatment with a general kinase inhibitor, H7 caused both morphological and immunocytochemical changes in p53 accumulation and localization. The more specific inhibitors, BisI and Gö6976, inhibitors of the PKC family and conventional PKCs (cPKC), respectively were also able to induce p53 accumulation and relocalization in the NB cell lines. Interestingly, the observations contrasted with a previous report that Gö6976 prevented nuclear accumulation of p53 in S100B-MEF cells (Scotto *et al.*, 1999). The variations observed between studies clearly demonstrate the diverse response of the PKC isoforms in different cellular backgrounds.

From the experimental results, it can be ascertained that regulation of p53 localization in NB involves the cPKC isoforms, either PKCα, βI or βII as nuclear accumulation is observed upon cPKC inhibition with Gö6976 (Figure 9). The possible involvement of the conventional isoform, PKCγ has already been negated as this isoform is not expressed in the NB cells (Figure 8, Table 1). The role of cPKC in p53 localization may not be exclusive to NB. These observations could possibly transcend beyond cancer and into the normal regulation of p53 as Chernov *et al.* (1998) also demonstrated p53 accumulation with H7 and BisI exposure in mouse and human cells. The effects of PKC

involvement in p53 regulation in NB may be exacerbated due to abnormal PKC overexpression in this disease.

The PKC isoforms involved in regulation of p53 localization have not yet been identified. As a means of specifically inhibiting the PKC isoforms, antibody-mediated inhibition was utilized as there are no commercially available specific PKC isoform inhibitors with the exception of rottlerin and LY333531 that show preferred selectivity to PKCδ and PKCβI/II respectively at particular concentrations. However, the specificity of these inhibitors has been subject to uncertainty as Davies *et al.* (2000) provide evidence that rotterlin is unable to demonstrate specific PKC inhibitory activity as initially reported by Gschwendt *et al.* (1994). Specificity analysis and co-crystallization studies by Komander *et al.* (2004) reveal that the PKCβ specific inhibitor, LY333531 also inhibits PDK1. Thus in light of this evidence, these inhibitors were not utilized for this study and instead our experimental design focussed on antibody-mediated inhibition of PKC.

Antibody-mediated inhibition of PKCα caused p53 nuclear localization and accumulation (Figure 15, 16). The greatest effect was observed with PKCα inhibition, any changes in p53 upon PKCβI inhibition are minor and perhaps insignificant. This could be possibly attributed to the similarity of the PKCα and β isoforms. PKC δ, ε, and ζ are not involved in p53 subcellular localization in NB as inhibition of these isoforms exerted no changes (Table 2). Our results strongly indicate that PKCα is responsible for regulation of p53 localization in NB. These results correlate with cotransfection studies that propose PKCα to be involved in the regulation of p53 (Youmell *et al.*, 1998).

4.3 Effect of PKC inhibition on p53 functionality in NB

There is debate as to whether the p53 signalling pathway is intact despite cytoplasmic localization in NB. Some studies indicate that cytoplasmic sequestration of p53 impairs its function resulting in a defective G1 checkpoint after DNA damage (Issacs *et al.*, 1998; Moll *et al.*, 1996; Tweedle *et al.*, 2001). On the other hand, other studies demonstrate that in spite of cytoplasmic localization p53 function is not compromised and that the DNA damage G1 checkpoint and apoptosis can still occur (Goldman *et al.*, 1996; Ronca *et al.*, 1997). Thus, this project examined if p53 was functional after nuclear relocalization mediated by PKC inhibition in NB.

The influence of relocalized p53 was associated with changes in cell proliferation, cell cycle and p53-reponsive gene products were monitored. It was identified that despite p53 relocalization upon cPKC inhibition by Gö6976 exposure, cell proliferation was unaltered (Figure 18). For a p53 response to be mounted, key distinct events are required: p53 stabilization/accumulation, translocation, and activation. Our results indicate that cPKCs are involved in the initial preparatory steps but are not sufficient to activate p53 in NB. This is consistent with the model that p53 stabilization and activation are regulated by distinct phosphorylation events (Chernov *et al.*, 1998). From the experimental data, there was no perceivable difference in p53-dependant cellular responses upon relocalization of p53 with Gö6976. On the other hand, Zeidman *et al.* (1999a) demonstrated efficacy of Gö6976 to inhibit proliferation and induce apoptosis at micromolar concentrations (2 uM) in SHSY5Y cells. Our experiments utilized nanomolar concentrations (200 nM) of Gö6976 which may not be sufficiently potent. Gö6976 is known to be a specific cPKC inhibitor; however, Kohn *et al.* (2003) has shed insight into

the mechanism of action of Gö6976. Interestingly, Gö6976 may not be an exclusive cPKC inhibitor but is also able to inhibit Chk1 and possibly, Chk2 kinase activity. It is possible that the altered cellular effects observed by Zeidman *et al.* (1999a) at a micromolar concentration of Gö6976 are not exclusively due to cPKC inhibition but attributed to Chk1/2 inhibition. The specificity of the inhibitors utilized in our study is a limiting factor as they may not be exclusively selective for PKC. Dose-response experiments with protein kinase inhibitors to determine the effective IC_{50} concentrations at which inhibition of PKC and other protein kinases would need to be assessed to ensure specificity and reliability.

p53 stabilization by abolishment of HDM2 interaction by means of the SuperTIP peptide was able to induce p53 accumulation and relocalization (Figure 9). The increasing levels of p53 would eventually saturate the nuclear export machinery and/or cytoplasmic tethers leading to an influx of p53 into the nucleus. However, simply altering the subcellular localization of p53 by disruption of p53-HDM2 binding did not alter proliferation of the NB cells (Figure 18). In contrast, Böttger *et al.* (1997) observed that the SuperTIP peptide can induce p53 accumulation as well as p53 activation. These conflicting results may be attributed to the different cell models utilized in the studies. Böttger *et al.* (1997) utilized a rat thyroid epithelial cell line VRn.6 which overexpress HDM2. It has been demonstrated that overexpression of HDM2 results in protection from apoptosis (Haupt *et al.*, 1996). In this study, human NB cell lines, IMR-32 and SHSY5Y were of the utmost interest. The mechanism(s) contributing to p53 non-functionality in NB is complex. However in IMR-32 and SHSY5Y cells, HDM2 is not overexpressed (Zaika *et* al., 1999; Rodriguez-Lopez *et al.* 2001) and thus, may not be a major

contributing factor to the abnormal cytoplasmic localization and inactivation of p53 in NB. Most likely, p53 is in a latent state and despite accumulation and nuclear relocalization by abrogation of p53-HDM2 binding via SuperTIP, an additional trigger is required for p53 to be in an active form.

Interestingly when the entire PKC family is inhibited by exposure to BisI, a specific inhibitor of the PKC family, a decrease in cell proliferation is then observed with a concomitant cell cycle accumulation, and increase in p21 expression (Figure 18, 19, 20, 21). A dual response is possibly occurring with BisI treatment; firstly, p53 relocalization upon cPKC inhibition as we demonstrated and secondly, p53 activation as atypical and novel PKC isoforms are also being targeted for inhibition. This experimental data strongly supports Chernov *et al.* (1998) report of two distinct phosphorylation events regulating p53 stabilization and activation.

The identity of the PKC isoforms involved in the regulation of the p53 transduction pathway still remains to be uncovered. To specifically identify the PKC isoform(s) involved in p53 regulation, an antibody mediated inhibition of PKCs was utilized. The experimental data identifies PKCα as the isozyme involved in p53 mis-localization as PKCα inhibition resulted in p53 nuclear localization and accumulation as detected by immunocytochemistry and Western blotting (Figure 15, 16). Due to the narrow observational window, approximately 24 hours after antibody delivery (Figure 11), this method of PKC inhibition could not be utilized in the p53 functionality experiments. Decreased antibody presence can be most likely attributed to degradation of the antibody and cell division. In addition, *de novo* synthesis and activation of PKCα upon membrane transfer from the cytoplasmic pool could overcome antibody-mediated inhibition of PKC

as the cellular reserve of PKC antibody is finite and non-renewing. Thus, for future experiments involving monitoring the prolonged effect of PKCα inhibition on p53 functionality, another method of targeting the PKC isoforms will need to be utilized such as phosphorothiorate antisense RNA that has been used successfully in the Phipps laboratory (Patent File: 1360-103 DR) and showed growth rate proliferation decrease in IMR-32 cells or small interfering RNA (siRNA) (Sajan *et al.*, 2006).

H7 has been previously reported to induce p53 accumulation and is a known inducer of p53-dependant apoptosis (Chernov *et al.*, 1998; Ronca *et al.*, 1997). The experimental data corresponds well with these previous observations as changes in p53 sublocalization and levels increased upon H7 exposure in both of the NB cell lines (Figure 9, 10). While the p53 sublocalization data is in agreement with the aforementioned studies, there is some discrepancy with our experimental data and the report that induction of p53 by PKC inhibition does not activate transcription from p53-dependant promotors (Chernov *et al.*, 1998). As a hallmark of p53 function, changes in protein expression of p53 responsive elements, p21 and Bax were monitored. Flow cytometry analyses demonstrated an increase in p21 and Bax protein levels after PKC inhibition upon exposure to H7 and BisI (Figure 21).

It is controversial whether PKC activation or inhibition is required for p53 relocalization and activation. Some studies are in favour of PKC inhibition (Chernov *et al.*, 1998; Ronca *et al.*, 1997), while others support PKC activation (Youmell *et al.*, 1998; Scotto *et al.*, 1999). This may be due to opposite effect exerted by different PKC isozymes. This study demonstrates that PKC inhibition is required for p53 relocalization and activation, particularly in NB.

4.4 p53 phosphorylation

Post-translational modifications of proteins, in particular, phosphorylation on serine, threonine, and tyrosine residues are an important regulatory mechanism of cellular signalling (Hunter, 1995). Identification of the phosphorylation sites is essential in understanding the importance of these sites and how phosphorylation status alters protein function. Initial efforts and the majority of studies in protein phosphorylation studies involve the use of radioisotopic labelling with ^{32}P (Wettenhall, *et al.*, 1991; Yan *et al.*, 1998). Though sensitive, many are averse to the use of radioactivity. Other strategies for identifying protein phosphorylation include: 2D-gel electrophoresis, site-directed mutagenesis, phosphoamino antibodies, and *in vitro* kinase assays (Larsen *et al.*, 2001; Arad-Dann *et al.*, 1993; Zhao *et al.*, 1989). Despite these methods, phosphoprotein studies have been difficult due to the low stoichiometry of phosphorylated proteins and the limitations of available biological sample. Analysis techniques require selective isolation of phosphoproteins and/or very sensitive methods for detection, such as, mass spectroscopy (Zappacosta *et al.*, 2002; Qin and Chait, 1997).

PKC phosphorylation sites have been identified in the C-terminal of p53 protein corresponding to serine residues 372, 376, and 378 (Baudier *et al.*, 1992). p53 has been identified to be phosphorylated by PKC *in vitro*; however, there is controversy as to whether p53 is a PKC substrate *in vivo*. Antibody-mediated inhibition of PKCα correlated with a decrease in serine phosphorylation of the p53 protein (Figure 17). The experimental data indicates that p53 is a PKC substrate *in vivo*. There are no available antibodies with epitopes against this region of the p53 protein. As this is a narrow region of the protein, no specific antibody could be raised that would be able to distinguish

phosphorylated and non-phosphorylated versions of each of the residues; thus, making this methodology to discriminate the phosphorylation status of the C-terminal of p53 a non-viable option. Our efforts shifted towards mass spectroscopy.

Our experimental data demonstrates that p53 can be phosphorylated by PKC *in vitro*. We have identified Electrospray Ionization (ESI) as a novel method to identify phosphorylated peptides. A p53 C-terminal peptide, Peptide46 was subjected to an *in vitro* PKC kinase reaction with a PKC membrane fraction and the peptide phosphorylation status was monitored by mass spectroscopy using ESI (Figure 24). Shifts in the major m/z peaks corresponding to phosphorylated variations of the peptide were observed. These results demonstrate that the C-terminal of p53 is a PKC substrate *in vitro* and that ESI is a viable method to evaluate peptide phosphorylation. Further sample optimization will also need to be implemented to increase the signal of the phosphopeptides by phosphopeptide enrichment and sample desalting and purification. Electrophoretic mobility analysis by agarose gel verified the presence of the phosphorylated variants of the p53 peptide (Figure 25). While the migration of Peptide46 is retarded upon phosphorylation, the different phosphorylation variants (mono-, di-, tri-phosphorylation) are not individually identifiable due to the resolution limitations of the agarose gel. For future studies, other means of separation will need to be utilized such as 2D-gel electrophoresis or HPLC.

To fully classify the PKC isoforms which phosphorylate the C-terminal of p53, the kinase reaction would need to be performed with isolates of the specific purified PKC isoforms available commercially. In this study, we have characterized the expression of the PKC isoforms in NB,

thus, not all the PKC isoforms would need to be investigated in the future. Experimentation with PKC isozymes, α, δ, ε, and ζ will perhaps prove the most fruitful.

5.0 CONCLUSIONS AND CLOSING REMARKS

In conclusion, these experiments substantiate that PKC-mediated phosphorylation of p53 contributes to the aberrant p53 cytoplasmic localization and inactivation in NB. This mechanism is characterized by two distinct independent phosphorylation events mediated by separate PKC isoforms. Dissection of the protein kinases identified that PKC inhibition can initiate and activate the p53 response pathway in NB. This goes against the notion that PKC activity is required for p53 inactivation observed in other cell types. It is clear that the various PKC isoforms and subsequent phosphorylation events exhibit diverse responses in different cellular backgrounds. Classification of the PKC isoforms expressed in the human NB cell lines, IMR-32 and SHSY5Y further delineated the candidate PKC isoforms involved in p53 regulation, specifically for NB.

Based on these results, a model of the involvement of PKC in the regulation of p53 localization and activation emerges in which PKCα is involved in p53 localization/accumulation, whereas another PKC isoform, possibly PKC δ, ε, or ζ is responsible for regulation of p53 activation.

The ability to efficiently and specifically observe p53 phosphorylation will be essential in future studies. We identify ESI as an alternative method to monitoring *in vitro* phosphorylation of p53 peptides.

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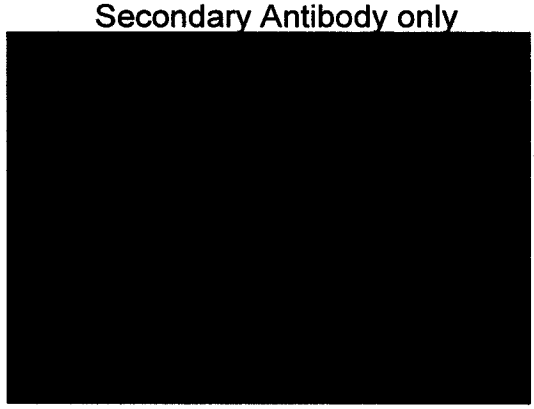
APPENDIX

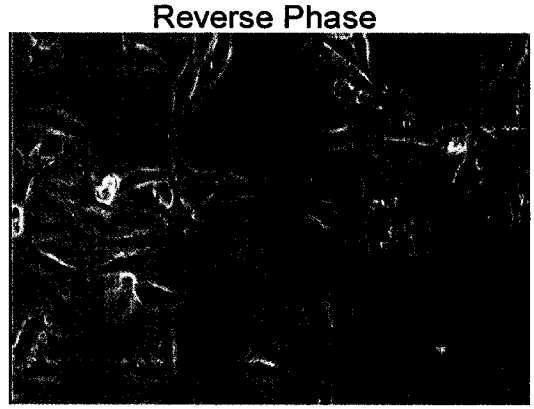
Figure 26. Nonspecific binding of secondary antibody, AlexaFluor 488nm.

IMR-32 cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and probed with the secondary antibody, AlexaFluor 488nm antirabbit in the absence of any primary antibody. Reverse phase images also shown. Images were originally taken at 10x magnification.

Secondary Antibody only



Reverse Phase



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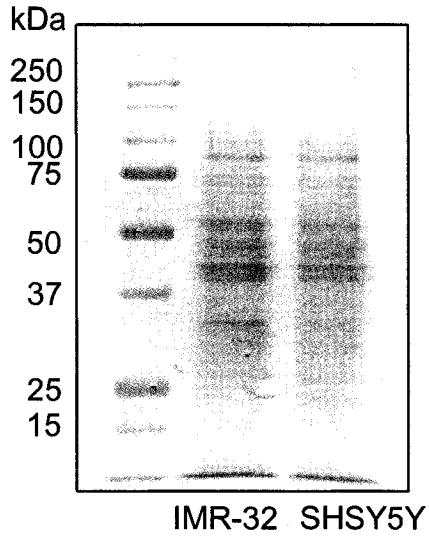
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37
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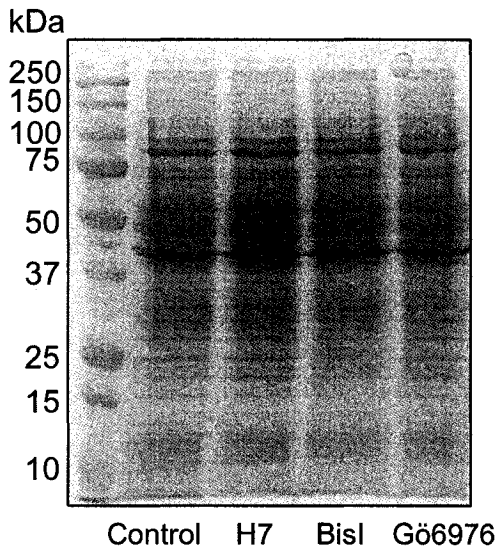
IMR-32
SHSY5Y

B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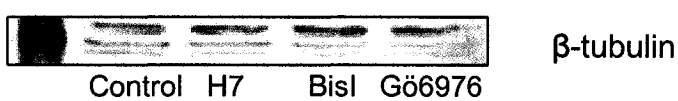
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Bisl
Gö6976
β-tubulin

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Education

2006-ongoing **MD, Doctorate of Medicine**
University of Toronto, Faculty of Medicine

2003-2006 **M.Sc. Biochemistry 'Role of PKC-mediated phosphorylation on p53 localization and function in neuroblastoma'**
University of Ottawa, Department of Biochemistry, Microbiology & Immunology
PharmaGap Inc.
Supervised by Dr Jenny Phipps and Dr Douglas Franks
Co-Supervised by Dr Zemin Yao

1999-2003 **B.Sc. Honours in Biochemistry**
University of Ottawa
Supervised by Dr Jenny Phipps
4th year dissertation – 'Restoration of p53 localization in neuroblastoma using synthetic peptides'

Research Interests

My principle research interests lie in the field of cancer research, specifically the aberrant cytoplasmic localization of the tumour suppressor protein, p53 in neuroblastoma (NB). My previous research included utilizing a peptide-based therapeutic approach to restore normal p53 sublocalization. During this time, the presence of a p53 isoform was identified in NB, which provided the basis of my future research. I am currently investigating the role of PKC-mediated phosphorylation of p53 in alteration of p53 localization and functionality. My future research plans are to identify the physiological role of the lower molecular weight p53 isoform, p47, with characterization of p47 expression in tumour biopsies and cancer cell lines.

Research Experience

Summer 2003-2006 **Student Researcher and Master's Research**
This is a collaborative industry-led project by Dr Jenny Phipps at PharmaGap Inc.

Monitored the effect of PKC inhibition on p53 expression and the p53-response pathway using flow cytometry, immunocytochemistry, PCR, and Western blotting.

Identified the PKC isoform responsible for altering p53 localization in NB.

Investigated the C-terminal phosphorylation status of p53 in NB using mass spectroscopy techniques.

Achieved creation of a model system to study and characterize the physiological role of p47. Cloning techniques were mastered to generate p47 tetracycline-inducible cell lines.

Alongside to my graduate research, I have been a mentor to summer and high school students.

Summer 2002-April 2003

Student Researcher and Honour's Research
This was a collaborative project with Professor Leda Raptis at Queen's University, whom provided the necessary EpiZap technology. The results have lead to further research currently being undertaken by her scientific team.

Established the use of the EpiZap micro-electroporation technology as an effective means of introduction of peptides into adherent cells.

Identified peptide mimicries able to restore normal p53 sublocalization in neuroblastoma and sensitivity to chemotoxic agents.

Summer 2001

Student Researcher, National Research Council, Ottawa
Dr Danielle Carrier and Dr Jenny Phipps

Successfully screened a panel of compounds for cytotoxicity and effect of Gap Junction Communiation (GJIC) on rat glial and human neuroblastoma cell lines.

Determined the optimal concentration and exposure length of effectors for assessment of GJIC using Lucifer dye migration assay.

Data collected aided Master's student, Anna Jasenska.

Teaching Experience

2005

Teacher Assistant
BCH 4125 Cellular Regulation and Control, Dr Steffany Bennett
Reviewed grant proposals (English and French) and assigned a score according to a CIHR rating scale.

Described evaluation of the proposed experiments and written grant for each student in a primary grant reviewer feedback comment sheet.

2004 **Teacher Assistant**
BCH 4125 Cellular Regulation and Control, Dr Douglas Franks
Checked and graded final examinations.

Presentations

2005 **- Ottawa Regional Cancer Centre (ORCC) Seminar**

2004 **- BMI Graduate Students Poster Day** (presented orally)
Abstract and poster is attached.
Nguyen, L., Franks, D., and Phipps, J. Regulation of p53 localization and function by PKC-mediated phosphorylation in neuroblastoma.

2003 **- Biochemistry Honour's Research Poster Day** (presented orally)
- University of Ottawa Biochemistry Student Seminar Series
Nguyen, L. and Phipps, J. Mini p53 and me: Restoration of p53 localization in neuroblastoma using synthetic peptides.

Publications

In preparation **Nguyen, L.**, Franks, D., and Phipps, J. PKCα isoform regulates p53 localization not function in neuroblastoma.

Nguyen, L., Chen, J., and Phipps, J. A lower molecular weight p53 isoform, p47 mediates cytoplasmic localization of p53.

Awards

2005	Canderel Défi/FCAR Centre Grant, McGill University
2003-2005	NSERC Industrial Postgraduate Scholarship, University of Ottawa
2003-2005	University of Ottawa National Excellence Scholarship
2003	Ontario Graduate Scholarship, University of Ottawa
Summer 2003	NSERC Undergraduate Research Award
Summer 2002	NSERC Undergraduate Research Award
2002	Ottawa Hospital Certificate of Merit
Summer 2001	NSERC Undergraduate Research Award
1999-2003	University of Ottawa Dean's Honour List
1999-2003	University of Ottawa Admissions Scholarship

Extracurricular Activities

Interpersonal **Patient Services**, Ottawa General Hospital, 2002-2005
Choir, Soprano Singer, St. Joseph's Church, Ottawa, 2003-2006

Languages English: fluent, French and Vietnamese: working knowledge
French Language Practice, NRC Practice Campaign, 2004-2006

Sports **Fencing**, member of the University of Ottawa Excalibur Fencing Club, 2005-2006

Softball, member of the NRC SIMS Softball League, 2003-present

References

Available upon request

Figure 27. Loading Controls

(A) A representative total protein stain of IMR-32 and SHSY5Y total protein extracts using Ponceau S for loading control of PKC isoforms expression.

Total protein was extracted from cultured NB cell lines IMR-32 and SHSY5Y as described in the Materials and Methods section. 50ug of protein per cell line was resolved by SDS-PAGE on a 10% gel. Western blots were stained with Ponceau S.

(B) A representative total protein stain for total cell lysates of IMR-32 cells treated with protein kinase inhibitors using MemCode™ Reversible Protein Stain.

Cultured IMR-32 cells were treated with 75uM H7, 5uM BisI, and 200nM Gö6976 for 24 hours and total protein was extracted as described in the Materials and Methods section. 50ug of protein was resolved by SDS-PAGE on a 10% gel. Western blots were stained with MemCode™ Reversible Protein Stain to visualize total proteins.

(C) A representative β-tubulin loading control of total cell lysates of SHSY5Y cells treated with protein kinase inhibitors.

Cultured SHSY5Y cells were treated with 75 uM H7, 5uM BisI, and 200nM Gö6976 for 24 hours and total protein was extracted as described in the Materials and Methods section. 50ug of protein was resolved by SDS-PAGE on a 10% gel. Western blots were probed with an anti-β-tubulin antibody.