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Role of Protein Kinase C-iota in Neuroblastoma and the Effect of ICA-1, a Novel Protein Kinase C-iota Inhibitor on the Proliferation and Apoptosis of Neuroblastoma Cells

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Role of Protein Kinase C-iota in Neuroblastoma and the Effect of ICA-1, a Novel Protein Kinase C-iota Inhibitor on the Proliferation and Apoptosis of Neuroblastoma Cells

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

I would like to dedicate my dissertation to my parents, Prasannan and Vijaya Pillai. It is their love, support and values that enabled me to overcome all obstacles and achieve my goals.

A special thanks to my wife and best friend, Shraddha Desai for standing beside me through all the challenges of graduate school.

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LIST OF ABBREVIATIONS

ICA-1: [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate

ATM: Aurothiomalate

PKC: Protein kinase C

CAK: Cyclin dependent kinase activating kinase

Cdk: Cyclin dependent kinase

MBP: Myelin basic protein

Caspases: Cysteine aspartate proteases

PARP: Poly (ADP-ribose) polymerase

siRNA: Short interfering RNA

DAPI: 4′, 6-diamidino-2-phenylindole

TUNEL: 3′ terminal deoxynucleotidyltransferase (TdT)- mediated dUTP nick end Labeling

EDTA: Ethylenediaminetetraacetic acid

DPBS: Dulbecco’s phosphate buffered saline without Mg$^{2+}$ and Ca$^{2+}$
ABSTRACT

Protein Kinase C-iota (PKC-ι), an atypical protein kinase C isoform manifests its potential as an oncogene by targeting various aspects of cancer cells such as growth, invasion and survival. PKC-ι confers resistance to drug-induced apoptosis in cancer cells. The acquisition of drug resistance is a major obstacle to good prognosis in neuroblastoma. The focus of the dissertation was three-fold: First to study the role of PKC-ι in the proliferation of neuroblastoma. Secondly, to identify the efficacy of [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate (ICA-1) as a novel PKC-ι inhibitor in neuroblastoma cell proliferation and apoptosis. Finally, to analyze whether PKC-ι could self-regulate its expression. Cyclin dependent kinase 7 (Cdk7) phosphorylates cyclin dependent kinases (cdks) and promotes cell proliferation. Our data shows that PKC-ι is an in-vitro Cdk7 kinase and that neuroblastoma cells proliferate via a PKC-ι/Cdk7/cdk2 cell signaling pathway. ICA-1 specifically inhibits the activity of PKC-ι but not that of PKC-zeta (PKC-ζ), the closely related atypical PKC family member. The IC₅₀ for the kinase activity assay was approximately 0.1µM which is 1000 times less than that of aurothiomalate, a known PKC-ι inhibitor. The phosphorylation of Cdk7 by PKC-ι was potently inhibited by ICA-1. ICA-1 mediates its antiproliferative effects on neuroblastoma cells by inhibiting the PKC-ι/Cdk7/cdk2 signaling pathway. ICA-1 (0.1µM) inhibited the in-vitro proliferation of BE(2)-C neuroblastoma cells by 58%.
Additionally, ICA-1 also induced apoptosis in neuroblastoma cells. Interestingly, ICA-1 did not affect the proliferation of normal neuronal cells suggesting its potential as chemotherapeutic with low toxicity. Hence, our results emphasize the potential of ICA-1 as a novel PKC-ι inhibitor and chemotherapeutic agent for neuroblastoma.

Bcr-Abl has been shown to regulate the activation of the transcription factor ELK-1 which in turn regulates the expression of PKC-ι. Alternatively, we hypothesize that PKC-ι can self regulate its expression by indirectly regulating the activity of Elk-1 in an ERK1 dependent manner. Our preliminary data shows that there was robust increase in the expression as well as association of PKC-ι and Elk-1 in actively proliferating neuroblastoma cells suggesting a potential role of PKC-ι in regulating the activity of Elk-1. Analysis of the subcellular fractions also presented a similar increase in the association between PKC-ι and Elk-1 in the nuclear fraction of actively proliferating cells as compared to cytoplasm. Interestingly, the nuclear expression of PKC-ι was also found to be higher in these cells, suggesting that PKC-ι translocated to the nucleus in actively proliferating cells and regulated the transcriptional activity of Elk-1. However, our data from in-vitro kinase activity demonstrated that PKC-ι was not an Elk-1 kinase but that it increased the phosphorylation of Elk-1 in the presence of ERK1, an upstream kinase of Elk-1 in the Bcr-Abl mediated regulatory pathway of PKC-ι. This suggested that ERK1 was integral to the self-regulatory activity of PKC-ι. In conclusion, we hypothesize that the self-regulatory mechanism of PKC-ι is initiated by the translocation PKC-ι into the nucleus where it activates ERK1.
This promotes the activation of its downstream target Elk-1 which subsequently upregulates the expression of PKC-ι
CHAPTER 1

PROTEIN KINASE C

1.1 Introduction

Cells are normally subjected to strict regulatory mechanisms that limit cell division. Protein kinases and protein phosphorylation control the entry and passage of cells through the cell cycle. Cancer is the outcome of abnormalities in these mechanisms that lead to unchecked cell division. The protein kinase C (PKC) family of Ser/Thr kinases is involved in transmembrane signal transduction pathways triggered by various extra and intracellular stimuli [1]. They were first discovered in bovine cerebellum by Yasutomi Nishizuka and colleagues in the 1970s at Kobe University, Japan [2]. They are involved in the control of various cellular responses like proliferation, migration, apoptosis and survival of cells [3-5]. The name PKC was coined by Nishizuka and coworkers as this enzyme was found to be activated by the Ca\(^{2+}\) dependent protease, Calpain [6]. Research following this has shown that PKCs not only require calcium for its activation but also co-factors like the lipid metabolite diacylglycerol (DAG) and phosphotidylserine (PS) [7-9].

1.2 PKC isozymes

The PKC family consists of thirteen isozymes that can be classified into different groups on the basis of their co-factor requirements [10, 11]. Group I is the conventional PKCs (cPKC) which includes the isoforms alpha (α), beta I (βI),
beta II (βII) and gamma (γ). They are also called classical isozymes as they are
dependent on calcium, DAG and phospholipids for their activation. Group II is
the novel PKCs (nPKC) and is comprised of delta (δ), epsilon (ε), eta (η) and
theta (θ). They are termed novel because they are calcium independent but
dependent on DAG and phospholipids. The atypical PKC isozymes form the
third group, so called because of their independence of calcium and DAG for
their activation. This group includes PKC zeta (ζ) and PKC iota (ι) in humans/
lambda (λ) in mouse. An additional family member, Protein Kinase D, mu (μ) is
introduced into the PKC family and is sometimes also referred to as an atypical
isozymes [12]. Another distant related family member includes PKC-related
kinases (PRK 1-3), known as PKN [13]

1.3 Structure of Protein kinase C

Protein kinase C family members have highly conserved carboxyl-terminal
catalytic domain. It is due to the structural similarity in the catalytic domain (also
known as the kinase domain), that all PKC isozymes fall under one parent family.
All the PKC isozymes differ from each other on the basis of their amino-terminal
regulatory domain. This domain is important for secondary messenger binding,
recruiting the enzyme to the membrane and protein-protein interactions [7]. The
first isozymes cloned were shown to possess four conserved sequences (C1-
C4), five variable sequences (V1-V5) and a pseudosubstrate region [14, 15].
The regulatory and the kinase domain are linked to each other by a hinge region
(V3) which is sensitive to cleavage by proteases. (Figure. 1).
1.3.1 The regulatory domain: The pseudosubstrate domain is located at the N-terminal adjacent to the C1 domain. This region has a peptide-sequence similar to that of a substrate but lacks alanine in the phospho-acceptor position. In the inactive form of PKCs, the pseudosubstrate prevents complete activation of PKC by blocking the substrate binding pocket [16, 17]. The pseudosubstrate is released from kinase domain, following the activation of PKCs [18, 19].

The C1 domain is the membrane binding domain common in all the PKC family members [20]. cPKCs and nPKCs are comprised of two tandem repeats of cysteine-rich sequence termed as C1A and C1B respectively [21]. They are involved in the binding of secondary lipid messenger diacylglycerol and phorbol esters as well as membrane lipids [22, 23]. Atypical PKCs have only one cysteine rich C1 domain. They lack the DAG and phorbol ester binding residues [20].

The C2 domain in cPKCs promotes their binding to anionic phospholipids in a Ca$^{2+}$-dependent manner [24, 25]. nPKCs have a C2-like domain incapable of binding to Ca$^{2+}$ due to absence of essential residues [26, 27]. However, the C2-like domain assists in protein-protein interactions. A similar mechanism is also observed in PRKs [13, 28]. aPKCs lack the C2 domain but comprise of an additional PB1 domain which is known to function in protein-protein interaction [29].

1.3.2 The catalytic domain: The catalytic domain is 40% homologous to the kinase domain of protein kinase A (PKA) and protein kinase B (AKT). C3 and C4 motifs form the conserved sequences in this kinase core. C3 domain
comprises the ATP binding site and C4 possesses the substrate binding sequence responsible for signal transduction [30].

1.4 Protein kinase C regulation

There are several important mechanisms that regulate complete maturation and activation of PKCs. Primarily, the protein is phosphorylated at specific residues which release PKCs to the cytosol rendering it catalytically competent. This initiates the translocation of the protein to the membrane in the presence of second messengers DAG, Ca^{2+}, PS and phorbol esters. This is then followed by removal of pseudosubstrate exposing the substrate binding pocket.

1.4.1 Regulation by Phosphorylation: The activation of PKC involves a cascade of three synchronized phosphorylations [31, 32] (Figure 2.2). The first step is phosphorylation at the activation loop triggered by phosphoinositide-dependent kinase -1 (PDK-1) that is upstream of PKC [33-38]. Phosphorylation at the activation domain rapidly initiates a chain reaction that involves autophosphorylation at the turn motif that further promotes the autophosphorylation at hydrophobic motif [34, 39]. The autophosphorylation at the turn motif is important in the maturation of PKC. It protects the catalytically activated form of PKCs from thermal degradation as well as proteolysis [40, 41]. Apart from being responsible for PKC activation, the turn motif is also important for protein-protein interaction as well as for initiating the mechanism of docking the enzyme to the membrane. The autophosphorylation at hydrophobic motif [34, 39] marks the third and final step towards complete maturation of protein kinase C. This serves to stabilize the protein from proteolytic degradation [42].
1.4.2 Regulation by secondary lipid messengers: Once phosphorylated, PKC is localized to the cytosol but is still inactive due to the presence of pseudosubstrate at its substrate binding pocket. Upon extracellular stimulation, phospholipase C stimulates the breakdown of phosphatidylinositol into phosphatidylinositol 3- kinase (PI3k) and DAG followed by release of Ca\(^{2+}\) from the internal stores. Ca\(^{2+}\) then binds to the C2 domain of cPKC inducing partial interaction with anionic phospholipids at the membrane. This induces the protein to bind DAG via its C1 domain. This high affinity interaction causes conformational changes in the protein, releasing the auto-inhibitory pseudosubstrate [43]. nPKCs do not have the Ca\(^{2+}\) binding C2 domain and hence depend on its C1 domain (particularly C1B) to build its strong interaction with the membrane. aPKCs on the other hand do not respond to either DAG or Ca\(^{2+}\).

1.4.3 Regulation by anchoring proteins: The distribution and localization of PKCs also regulate their overall biological functioning. Proteins from the family of RACKs (receptors for activated C- kinase) bind different PKCs helping in their correct cellular localization and also increase their catalytic activity [44]. Another group of proteins called STICKs (substrates that interact with C-kinase) interact with immature, phosphorylated PKC bringing it close to PKC cofactors and release it when phosphorylated [45].
Figure 1.1 Structure of Protein Kinase C. The PKCs have highly conserved C-terminal catalytic domain. The three autophosphorylation sites are activation loop (A), turn motif (T) and hydrophobic motif (H). The PKCs differ in the regulatory domains. Conventional PKCs have C2 (Ca$^{2+}$-binding domain), C1A (DAG-binding) domain, C1B (PS-binding) domain. The novel PKCs have C-domain similar to conventional PKCs. The C2 domain is modified and incapable of binding to Ca$^{2+}$, thus called C2-like. The C1 domain of atypical PKCs bind to only PS. Instead of the C2 domain, they have the PB1 domain that is involved in protein-protein interaction. All PKCs have a pseudosubstrate domain in next to the N-terminal end. Adapted from [46].
1.4.4 Protein kinase C Deregulation: Activation of protein kinase C is followed by its deactivation step. Binding of co-factors to PKCs at membrane makes them catalytically competent. PKCs undergo conformational change removing the pseudosubstrate. Association of DAG to PKC is transient. At this point, the protein due to its open confirmation becomes highly sensitive to proteases like calpain and dephosphorylation by phosphatases like PP2A [47]. Ultimately, protein kinase C is subjected to degradation via ubiquitin-proteosome pathway [48, 49].
Figure 1.2 Activation of PKCs. The first step in the activation of PKCs is the phosphorylating of activation loop (A) by PDK-1 at the C-terminal. This triggers the rapid autophosphorylations at the turn motif (T) and hydrophobic motif (H), respectively. This promotes the release of the PKC into the cytosol where it is maintained in the inactive form by binding of the pseudosubstrate (PS) to the substrate binding domain. The subsequent steps in the activation of PKCs are triggered by signals that promote the lipid hydrolysis from the membrane and release cofactors such as Ca\(^{2+}\) and DAG. These events promote the translocation of the PKCs to the membrane where the co-factors bind to the regulatory domains of PKCs. Ca\(^{2+}\) binds to the C2 domain (orange) and DAG binds to C1 domain (blue). The binding of the co-factors generate the energy required to release the pseudosubstrate form the substrate binding site and promote the downstream signaling. Adapted from [46]
2.1 Introduction

Neuroblastoma is the most common extra-cranial tumor diagnosed in children. Its origin lays in the sympathetic nervous system, that is, the network of nerves transmitting neuronal messages originating in the brain to the various parts of the body. These highly lethal tumors comprise approximately 10% of all childhood cancers with a fatality rate of more than 15% [50]. As per the statistics of the American Cancer Society, approximately 650 new cases of neuroblastoma are diagnosed each year in the United States. Spontaneous regression of the tumor or maturity into a benign ganglieneuroma is a characteristic feature of this cancer [51]. The etiology of this disease hitherto remains obscure. The intensity of neuroblastoma lies in its primary target, infants and small children. The prognosis in case of infants is much better as compared to children over a year. In the latter case, metastasis is often observed at the time of diagnosis and the subsequent recovery of the patient is rare [52]. Despite significant educational efforts, improved diagnostic techniques, and rigorous therapies, neuroblastoma control remains static. Thus, justifying the importance of increased research to find a potential cure for this disease.

The distribution of neuroblastoma is represented by Figure 2.1. The primary site of neuroblastoma occurrence is the adrenal gland [53].
Figure 2.1A Distribution of neuroblastoma in patients <1 year. The chart represents the distribution of neuroblastoma by primary site in patients <1 years at diagnosis, among all races and both sexes. Adapted from [53].
Figure 2.1B Distribution of neuroblastoma in patients >1 year. The chart represents the distribution of neuroblastoma by primary site in patients >1 years at diagnosis, among all races and both sexes. Adapted from [53].
The diagnosis, staging and treatment of neuroblastoma have been explained based on the International criteria for neuroblastoma diagnosis, staging and treatment [54, 55].

2.1.1 Diagnosis

- X-rays
- Bone scans
- Computerized tomography (CT) scans
- Magnetic resonance imaging (MRI) scans

2.1.2 Staging: Stage I represents tumor that is localized. It can be totally removed with surgery. The symptoms of this stage are that the tumor is confined within a particular body compartment such as abdomen, pelvis or neck.

Stage IIA represents a tumor which is still localized. However, the surgical removal is not very easy. The symptoms are identical to Stage I.

Stage IIB is where the tumor remains localized similar to Stage IIA and the surgical removal of tumor is not easy. A major difference in Stage IIA and IIB is that in the latter, nearby lymph nodes show signs of tumor.

Stage III represents an advanced stage. By this time the surgical removal of the tumor is not possible. Lymph nodes may or may not contain cancer cells. The tumor might have begun to metastasize.

Stage IV is represented by metastasis.

Stage IVS is a specialized stage associated with infants <1 year old.
Patients with this stage have a good chance of recovery. Surprisingly, at this stage, neuroblastoma sometimes goes away on its own and may not require treatment.

2.1.3 Treatment: Surgery is usually the first choice of treatment; however, it is dependent on the location. In case where the tumor is found to be attached to nearby vital organs like the lungs or the spinal cord, then surgery is considered too risky.

Chemotherapy is the treatment option when surgery is risky. This may be used to promote the shrinking of the tumor to facilitate the surgical removal. This treatment modality is generally used before surgery and before bone marrow stem cell transplant.

Radiation therapy is used in the event of failure of surgery and chemotherapy, the next course of action is radiation therapy. High-risk neuroblastoma patients may receive radiation therapy after chemotherapy and surgery to prevent cancer from recurring.

Stem cell transplant is primarily used to replenish healthy cells. This is accompanied by high doses of chemotherapy to kill any remaining cancer cells.

2.1.4 PKC-ιota: PKC-ι was first identified in insulin-secreting rat cells RINm5F by Selbie et al. in 1993 [56]. It is 72% homologous to the other atypical family member PKC-ζ. However, it is less than 54% homologous to other PKC isoforms [56]. The role of PKC in carcinogenesis came into the limelight with the discovery of PKC as a receptor for the tumor-promoting phorbol esters in the early 1980s [9, 57]. However, PKC-ι is the only PKC isoform that has been
successfully proven to be an oncogene [58, 59]. Among the PKCs, PKC-ι is the 
most common genomic amplicon as identified by comparative genomic 
hybridization [60]. The expression of PKC-ι has been reported to be elevated in 
many cancers such as lung [61], pancreas [62], gastric [63], esophagus [64], 
hepatocyte [65], breast [66], ovarian [67-69], prostate [70] and brain [71]. It 
promotes cell survival in non small cell lung cancer [72] and prostate cancer [73]. 
PKC-ι has been known to induce resistance to drug mediated apoptosis in 
chronic myelogenous leukemia cells [74].

PKCs have been shown to promote multi-drug resistance in 
neuroblastoma cells which has been a potent problem in the prognosis of 
neuroblastoma [75, 76]. Work from our lab has shown the importance of PKC-ι 
in glioma [71] and prostate cancer [73], thus, we were interested in analyzing the 
role of PKC-ι in neuroblastoma.
Figure 2.2 Significance of PKC-ι as an oncogene. Adapted from [59, 77]. The significance of PKC-ι lies in the fact that it can mediate numerous oncogenic signaling mechanisms in cells that promote invasion, proliferation and cell survival. PKC-ι can be activated by a plethora of upstream targets such as dietary fats, EGF (epidermal growth factor), smoke carcinogens, Bcr-Abl and Ras.
2.2 Results

2.2.1 PKC-ι is overexpressed in neuroblastoma tissues: In order to analyze the role of PKC-ι in neuroblastoma we first determined the expression of PKC-ι in neuroblastoma tissues. Our preliminary work performed on neuroblastoma tissues showed a 2.5-fold increase in PKC-ι in adrenal neuroblastoma compared to normal adrenal biopsies ($P = 0.003$, Figure 2.3 A, B). β-actin loading controls demonstrated that approximately equal amounts of protein were loaded onto the SDS-PAGE.

2.2.2. PKC-ι is overexpressed in actively proliferating neuroblastoma cells: Furthermore, we tested the expression of PKC-ι in proliferating BE(2)-C cells. The PKC-ι expression levels were higher in 50% confluent cells (actively proliferating) than 100% confluent cells (contact inhibited and serum starved). Additionally, the expression of PKC-ζ, a closely related member of the atypical PKC family was analyzed. There was no change in the expression of PKC-ζ in response to contact inhibition. Caspase-3 expression levels were analysed to ensure that the decrease in the expression of PKC-ι was not an outcome of cells undergoing apoptosis (Figure 2.3 C, D). These results suggest a potential role of PKC-ι in the proliferation of BE(2)-C cells.

2.2.3. Proliferation of neuroblastoma cells is PKC-ι dependent: The potential role of PKC-ι in the proliferation in BE(2)-C cells was confirmed via gene silencing using PKC-ι siRNA. PKC-ι siRNA (150 nM) significantly reduced the cell proliferation by 53% at 48 hours ($P=0.002$) and by 65% at 72 hours ($P<0.001$) (Figure 2.3 E). The expression of levels of PKC-ι was also reduces
significantly \((P=0.04)\) (Figure 2.3F). Expression levels of PKC-\(\zeta\) remained invariant (Figure 2.3F). These results indicate that PKC-\(\iota\) plays an important role in the proliferation of neuroblastoma cells.

### 2.3. Summary

Previous work from our laboratory has found that PKC-\(\iota\) is overexpressed in actively proliferating prostate cancer and glioma cells [71, 73]. Similarly, we found higher expression of PKC-\(\iota\) in adrenal neuroblastoma tissue as well as neuroblastoma cells suggesting the involvement of PKC-\(\iota\) in the proliferation of neuroblastoma cells. Our data suggests that PKC-\(\iota\) is involved in the proliferation of neuroblastoma cells. Therefore, it is of interest to analyze whether there is a PKC-\(\iota\) mediated proliferation pathway in neuroblastoma.
Figure 2.3 A, B. PKC-ι is overexpressed in neuroblastoma tissues. (A) Normal and adrenal neuroblastoma tissue biopsies (60µg) were subjected to SDS-PAGE and Western blotting was performed with monoclonal antibodies against PKC-ι. (B) Densitometric analysis of representative immunoblots from 11 normal adrenal specimens and 11 adrenal malignant neuroblastomas are shown.
Figure 2.3 C, D PKC-ι is overexpressed in neuroblastoma cells. (C) Endogenous levels of PKC-ι in BE(2)-C cells were determined by Western blot analysis and quantified using densitometry, respectively. Equal amounts of cellular proteins (100µg) from actively proliferating (50%) and contact inhibited (100% S) cells were loaded in each well and immunoblotted for PKC-ι or PKC-ζ. Caspase-3 was analyzed to ensure that the effect on the levels of proteins was not due to apoptosis. β-actin levels were analyzed as a loading control (D) Represents densitometry for the expression of PKC-ι in BE(2)-C cells.
Figure 2.3 E, F Effect of PKC-ζ knockdown. BE(2)-C cells were treated with PKC-ζ siRNA (150nM) for 24-72 hours as described in Materials and Methods. At the indicated time points, viable cells were counted using Trypan blue exclusion assay. The data represents n=3 independent experiments (E). The same population of cells treated with either control siRNA or PKC-ζ siRNA was immunoblotted for PKC-ζ. The levels of PKC-ζ were analyzed to determine the specificity of PKC-ζ siRNA. β-actin levels were analyzed as a loading control. Western blot represents N=3 independent experiments (F).
CHAPTER 3
NEUROBLASTOMA CELLS PROLIFERATE VIA PKC-ι/Cdk7/cdk2
PROLIFERATION PATHWAY

3.1 Introduction

Cyclin dependent kinases (cdks) are serine/threonine protein kinases that are mainly involved in regulation of the cell cycle. The Cdk family is comprised of the following members:

Cdk1 binds to cyclin A to form the M-phase promoting factor (MPF) and initiates mitosis [78]. Cdk1/cyclin A complex may also play a role in preparation of the cells for mitosis [79].

Cdk2 coordinates the transition from Gap1 phase to DNA synthesis phase (G1→S). Cdk2/cyclin E complex triggers the initiation of DNA synthesis at the commencement of S-phase [78].

Interactions of Cdk1 and Cdk2 with cyclin B have been reported in G2 and M-phase of the cell cycle [80].

CDK3 is closely related to Cdc2 and Cdk2 and has been reported to play a role in progression through Gap1 phase [81-83].

Cdk4/cyclin D complex promotes the progression through the Gap1-phase. They regulate the cell cycle by suppressing the action of pRb protein. [84, 85].

Cdk5 is required during post-mitotic stages of neural differentiation [86, 87].
Cdk6/cyclin D complex promotes progression through the Gap1 phase. They regulate the cell cycle by suppressing the action of pRb protein.

Cdk4 and Cdk6 are termed as secondary Cdks since the cell cycle is not inhibited in the absence of these proteins [84, 85].

Cdk7 is involved in the regulation of the activity of the cdks. It is part of CAK (Cyclin dependent kinase activating kinase) complex [78, 88]. The only CAK identified in eukaryotes is Cdk7 [89].

Cdk8/cyclin C complex is involved in transcription by virtue of their association with RNA polymerase II [90-93].

Cdk9/cyclin T complex is involved in transcription [94].

3.1.1 Regulation of Cdks: The activation of cdks requires binding to cyclin as well as phosphorylation at conserved T residue by CAK [95, 96]. CAK is a trimeric complex composed of Cdk7, cyclin H and MAT1 [97-101]. CDK7 plays a dual role; first as CAK, it phosphorylates CDKs to promote cell-cycle and second as a component of the transcription factor TFIIH, it phosphorylates the C terminal domain (CTD) of Pol II [95]. Cdk7 regulates the cell-cycle by acting as an upstream regulator promoting a conformational change that enhances the activity of target cdks [102]. As part of the transcription machinery, CDK7 acts as a downstream effector kinase [103]. At mitosis, xeroderma pigmentosum disorder group D (XPD) promotes the sequestration of CAK from TFIIH releasing the trimetric CAK to act as a cell-cycle promoter [104]. The activation of Cdk7 is dependent on phosphorylation at two conserved sites. Phosphorylation at T170
on the T-loop induces stable dimerization with cyclin H while phosphorylation at S164 on the activation loop promotes stabilization of CAK complex [105].

Deactivation of cdks is mediated by Cdk inhibitory subunits (CKIs) or regulatory kinases that phosphorylate the cdk subunit at inhibitory sites near the N terminus [106, 107]. CKIs can be divided into two main families:

Cip/Kip forms the first group. It includes p21, p27, and p57: They inhibit cdk2- and cdk4/6-cyclin complexes involved in G1 and G1/S control.

Ink4 family consists p15, p16, p18, and p19: They specifically inhibit cdk4 and cdk6-cyclin D complexes.

Thus, CKIs proteins are important in regulating the cell-cycle in response to unfavorable environmental conditions or intracellular signals such as DNA damage [108, 109].

Previous work in our laboratory demonstrated crosstalk between PKC-ι and Cdk7 in the regulation of the glioma cell cycle and prostate cancer cell proliferation [73, 110].

From chapter 2 (Figure 2.3), we observed that PKC-ι is involved in the proliferation of neuroblastoma cells, therefore, we investigated whether the proliferation is via PKC-ι/Cdk7 pathway.
Figure 3.1 Cdns in cell-cycle. The figure represents major Cdns that participate in progression of cell cycle [78].
3.2 Results:

3.2.1 Cdk7 is a substrate for PKC-ι: Since, previous work from our laboratory demonstrated crosstalk between PKC-ι and Cdk7 [73, 110], we first determined the role of PKC-ι as an in-vitro kinase of Cdk7. Recombinant PKC-ι was incubated with recombinant Cdk7 in an in-vitro kinase activity assay as described under Materials and Methods. The data demonstrated that recombinant PKC-ι directly phosphorylated Cdk7 at T170 (Figure 3.2). A 60% increase in the phosphorylation of Cdk7 was observed ($P=0.02$).

3.2.2 BE(2)-C cells proliferate via the PKC-ι/Cdk7/cdk2 pathway: Next, we investigated whether PKC-ι plays a proliferative role in neuroblastoma through the Cdk7 pathway as shown in previous studies [110]. Our results show that PKC-ι directly associated with the phosphorylated forms of both Cdk7 (T170) and its downstream target cdk2 (T160) (Figure 3.3 A). There was a significant increase in the levels of pCdk7 in co-immunoprecipitated (both PKC-ι and Cdk7) samples compared to individual immunoprecipitation. When compared with PKC-ι alone, the increase was 37% ($P=0.05$) and with immunoprecipitation of Cdk7 alone it was 38% ($P=0.001$). The subsequent increase in the levels of pcdk2 (T160) in co-immunoprecipitation samples were found to be 65% higher compared to immunoprecipitation of PKC-ι alone ($P=0.01$) and 37% for Cdk7 ($P=0.001$). These results suggest that Cdk7 may be a direct downstream substrate for PKC-ι in BE(2)-C cells. To further analyze the role of PKC-ι as an in-vitro kinase of Cdk7, recombinant PKC-ι was incubated with immunoprecipitated Cdk7 in an in-vitro kinase activity assay as described
previously. Recombinant PKC-ι directly phosphorylated Cdk7 at T170 (Figure 3.3 B). A 70% increase in the phosphorylation of Cdk7 was observed ($P=0.02$). The property of PKC-ι to directly phosphorylate Cdk7 in BE(2)-C cells was confirmed by gene silencing using PKC-ι siRNA. Immunoblotting for pCdk7 displayed that knockdown of PKC-ι by RNA interference lead to a 60% reduction in the phosphorylation of Cdk7 ($P=0.03$) (Figure 3.3 C). These results suggest that the proliferation of the neuroblastoma cells maybe mediated by the crosstalk between PKC-ι and Cdk7.

![Figure 3.2 PKC-ι directly phosphorylates. Cdk7 (A) Active PKC-ι (0.5μg) and active Cdk7 (0.5μg) were subjected to an in-vitro kinase activity assay. The proteins were then separated by SDS-PAGE and Western blotted to determine the phosphorylation of Cdk7 at T170 and levels of pan-Cdk7. Data represents $N=3$ independent experiments.](image-url)
Figure 3.3 A, B BE(2)-C cells proliferate via the PKC-ι/Cdk7/cdk2 pathway. (A, B) Column 1 represents negative controls (-). The first negative control contains whole cell lysates (200μg) plus rabbit IgG whole molecule (50μl of 1:1 v/v) while the second negative control was comprised of whole cell lysates (200μg) plus rabbit IgG whole molecule (50μl) and normal rabbit IgG serum (5μg). (A) PKC-ι and Cdk7 were immunoprecipitated separately as well as co-immunoprecipitated from whole cell extracts (200μg) using specific antibodies. Western blot analysis was performed to determine pCdk7 at T170, pcdk2 at T160, pan-Cdk7, pan-cdk2 and β-actin. (B) Cdk7 (5μg) was immunoprecipitated from whole cell extracts (200μg), then subjected to an in-vitro kinase activity assay in the presence and absence of recombinant PKC-ι (0.5μg). The proteins were then separated by SDS-PAGE and Western blotted to determine the phosphorylation of Cdk7 at T170 and levels of pan-Cdk7.
3.3 Summary

Our data from in-vitro kinase activity assay demonstrates that PKC-ι is an in-vitro Cdk7 kinase. Immunoprecipitation studies suggests that there is crosstalk between PKC-ι and Cdk7 in neuroblastoma cell line BE(2)-C cells and that the cells might be proliferating by the PKC-ι/Cdk7/cdk2 pathway. The involvement of PKC-ι in the proliferation was confirmed via PKC-ι knockdown using siRNA which inhibited the phosphorylation of Cdk7. Therefore, our data suggests that neuroblastoma cells proliferate via PKC-ι/Cdk7/cdk2 pathway.
CHAPTER 4
ICA-1, A NOVEL PKC-ι INHIBITOR

4.1 Introduction

ICA-1 is a novel PKC-ι inhibitor. ICA-1 is the abbreviation for [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate. PKC-ι is the only PKC isoform that has been proven to be an oncogene [58, 59] PKC-ι mediates numerous signaling pathways that promote carcinogenesis (Figure 2.2). It promotes cell survival in prostate cancer [73] and and drug resistance in chronic myelogenous leukemia cells [74]. However, there has been limited success in developing PKC-ι inhibitors. Furthermore, PKC-ι and PKC-ζ are 84% homologous with respect to the amino acid sequences of their catalytic domain. This sequence homology is a major hindrance in successfully designing an inhibitor that is specific to only PKC-ι and shall not inhibit PKC-ζ.

Aurothiomalate (ATM) is only one FDA approved PKC-ι inhibitor [111], certifying the need to develop more potent PKC-ι inhibitors. ATM binds to the the PB1 domain of PKC-ι and inhibits the protein-protein interaction of PKC and its downstream target. However, since PKC-ι and PKC-ζ have highly conserved PB1 domain, ATM could induce non-specific inhibition of PKC-ζ. ICA-1, was designed to bind to the unique sequence of amino acids (469-475) since, both PKC-ι and PKC-ζ exhibited heterogeneity in this particular sequence [56].
In the current study, we demonstrated that ICA-1 specifically targets the activity of PKC-ι with an IC$_{50}$ of 10 nM in-vitro; however, it did not inhibit the activity of PKC-ζ. Comparative study of the effect of ATM on the activity of PKC-ι versus PKC-ζ showed that ATM inhibited the activity of both the isozymes. Therefore, our data supports the potential of ICA-1 as a novel chemotherapeutic agent for cancer research.

4.2 Results

4.2.1 Binding of ICA-1 to a sequence of amino acids in the catalytic domain of PKC-ι: To establish the therapeutic potential of PKC-ι as a drug target we screened compounds that would bind to a unique sequence in the catalytic domain of PKC-ι and inhibit its activity. The sequence targeted comprised amino acid residues 469-475 (glutamine, isoleucine, arginine, isoleucine, proline, arginine and serine). Molecular docking demonstrated the binding of ICA-1 (Figure 4.1 A) to this sequence (Figure 4.1 B).

4.2.2 ICA-1 specifically inhibits the activity of PKC-ι: Since, PKC-ι and PKC-ζ are 84% homologous with respect to the amino acid sequences of their catalytic domain [56], our primary focus was to determine the specificity of ICA-1 by performing a kinase activity assay to analyze the effects of ICA-1 on the activity of PKC-ι and PKC-ζ. Recombinant active PKC-ι or PKC-ζ, was incubated with MBP (myelin basic protein), a known substrate for PKCs [112], in the presence or absence of ICA-1. ICA-1 significantly inhibited the activity of PKC-ι by 16% at 0.1 µM ($P= 0.03$), 25% at 1 µM ($P<0.05$) and 45% at 5µM ($P<0.002$) (Figure 4.2 A). However, ICA-1 did not have any significant effect on the activity
of PKC-ζ (Figure 4.2 C). Furthermore, we made a comparative study between the specificity of ICA-1 and ATM for PKC-ι and PKC-ζ using a PKC activity assay kit. ICA-1 significantly decreased the activity of PKC-ι (Figure 4.3 A) by 22.6% at 0.1 µM ($P=0.01$), 53.47% at 1 µM ($P=0.006$) and 80% at 5 µM ($P=0.002$) but not PKC-ζ (Figure 4.3 B) demonstrating the selectivity of ICA-1 for PKC-ι and not PKC-ζ. However, ATM at 100µM inhibited the activity of both PKC-ι by 67% ($P=0.02$) and PKC-ζ by 55 % ($P=0.01$) (Figure 4.3 C, D). Lower concentrations of ATM had no significant effect on the activity of either of the enzymes. The ICA-1 induced phosphotransferase inhibition was tested using a more sensitive γ-32P ATP method. The IC50 obtained was approximately 10 nM ($P=0.01$) (Figure 4.4). Collectively, these results show that ICA-1 is a specific PKC-ι inhibitor.

4.2.3 ICA-1 is a competitive inhibitor of PKC-ι: To test whether the inhibitory effect of ICA-1 on the activity of PKC-ι is competitive or non-competitive, we analyzed the effect of ICA-1 by varying the concentration of MBP (0.0025mg/ml - 0.02 mg/ml). Data from the Lineweaver- burke plot (Figure 4.5) show that the inhibitory effects of ICA-1 was inversely proportional to the substrate concentration suggesting that ICA-1 is a competitive inhibitor of PKC-ι.
Figure 4.1 Chemical structure and Molecular Docking of ICA-1. (A) The structure of ICA-1 [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl]methyl dihydrogen phosphate. It has a molecular weight of 336.18 g/mol and is soluble in water. (B) Molecular docking of ICA-1 on amino acid residues 469 - 475 of the catalytic domain of PKC-ι.
Figure 4.2 A, B, C, D. ICA-1 specifically inhibits the activity of PKC-ι.
ICA-1 specifically inhibits the activity of PKC-ι. Recombinant active PKC-ι (A) or PKC-ζ (C) (0.01µg/µl) was incubated with myelin basic protein (5µg) in the presence or absence of ICA-1 (0.1µM, 1µM and 5µM) in a kinase activity assay. (A, C) Show the Western blot analysis for the effect of ICA-1 on the activities of PKC-ι and PKC-ζ respectively. Western blots represents N=3 independent experiments. (B, D) represent the densitometry for effect of PKC-ι and PKC-ζ activity respectively, upon ICA-1 treatment.
Figure 4.3 A, B, C, D. Comparison between ICA-1 and ATM. Recombinant PKC-ι or PKC-ζ (0.01μg/μl) was treated with different concentrations of ICA-1 (A, B) or ATM (C, D) for 10 min at 4°C followed by incubation with phosphatidyserine and ATP for 30 min at 30°C. Data represents N=3 independent experiments.
Figure 4.4 IC₅₀ of ICA-1. Recombinant PKC-ι (0.01μg) was treated with different concentrations of ICA-1 for 10 min at 4°C followed by incubation with PIP₃ for 10 min at 30°C. Data represents N=2 independent experiments.
Figure 4.5 Competitive inhibition of PKC-ι activity by ICA-1. Recombinant active PKC-ι (0.01μg/μl) was incubated with varying concentrations of MBP (0.0025 mg/ml, 0.05 mg/ml, 0.010 mg/ml and 0.02 mg/ml) in the presence or absence of ICA-1 (1 μM) followed by detection by ELISA based assay. Data represents N=3 independent experiments.
4.3 Summary

ICA-1 specifically inhibits the activity of PKC-ι and did not have any significant effect on activity of PKC-ζ. Comparison with the FDA-approved PKC-ι inhibitor, ATM showed that ICA-1 is a more potent and specific PKC-ι inhibitor. The inhibitory action of ICA-1 is competitive. This is very important since, it suggests that the mode of action of ICA-1 may not involve ATP- competitive binding. Thus, our data suggests that ICA-1 is an interesting agent to be tested on neuroblastoma cell which proliferate via PKC-ι mediated pathway.
CHAPTER 5
ICA-1 INHIBITS PROLIFERATION AND INDUCES APOPTOSIS OF NEUROBLASTOMA CELLS

5.1 Introduction

Neuroblastoma is the most common extra cranial tumor diagnosed in children and in infants [113]. They are multidrug resistant tumors and show poor prognosis [114]. The primary target of the disease is infants and small children. The prognosis is much better in infants as compared to children over a year whereas in the latter recovery of the patient is rare [52]. Despite significant research efforts and advanced therapeutic regiments, neuroblastoma control continues to be a medical dilemma.

We demonstrate that the neuroblastoma cells proliferate via PKC-ι/Cdk7/cdk2 pathway. ICA-1 treatment resulted in inhibition of this pathway. Treatment with ICA-1 induced chromatin condensation as shown by 4’, 6-diamidino-2-phenylindole (DAPI) staining of the nucleus. ICA-1 also induced DNA fragmentation which was analyzed using 3’ terminal deoxynucleotidyltransferase (TdT)- mediated dUTP nick end-labeling (TUNEL) assay depicting the pro-apoptotic potential of ICA-1. Furthermore, ICA-1 promoted apoptosis of neuroblastoma cells via induction of Caspase-3 activation and subsequent cleavage of PARP.
5.2 Results

5.2.1 ICA-1 inhibited the proliferation of BE(2)-C neuroblastoma cells:
Since, our results (Figure 3.3) demonstrated the role of the PKC-ι/Cdk7/cdk2 pathway in the proliferation of neuroblastoma cell line BE(2)-C, we hypothesized that treatment with ICA-1 should inhibit the proliferation of these cells. We analyzed the effect of ICA-1 on normal neuronal cells LA1-5s and the neuroblastoma BE(2)-C cell line. Cell viability assays showed that ICA-1 had no significant effect on the proliferation of LA1-5s cells (Figure 5.1 A), but significantly inhibited the proliferation of the neuroblastoma cell line, BE(2)-C by 58% at 48 hours \((P=0.01)\) with an IC\(_{50}\) of 0.1µM (Figure 5.1 B). Furthermore, we made a comparative study between the effects of ICA-1 with that of ATM on the two cell lines. Interestingly, we found that ATM (100µM) inhibited the proliferation of BE(2)-C (Figure 5.2 A). However, this concentration also inhibited the proliferation of LA1-5s cells (Figure 5.2 B).

5.2.2 ICA-1 inhibited the in-vitro phosphorylation of Cdk7 by PKC-ι: Since, ICA-1 specifically inhibited PKC-ι activity and PKC-ι is an in-vitro Cdk7 kinase, we investigated the effect of ICA-1 on phosphorylation of Cdk7. ICA-1 inhibited the in-vitro phosphorylation of Cdk7 by recombinant PKC-ι (Figure 5.3).

5.2.3 ICA-1 inhibited the proliferation of BE(2)-C neuroblastoma cells by abrogating the PKC-ι/Cdk7/cdk2 pathway: In order to prove that the molecular mechanism of ICA-1 is inhibition of the PKC-ι/Cdk7/cdk2 pathway, we analyzed the effect of ICA-1 (0.1µM) in cell culture. BE(2)-C cells were treated with ICA-1 (0.1µM) for 48 hours with addition of drug every 24 hours. ICA-1 inhibited the
phosphorylation of Cdk7 by 55% \((P=0.001)\) which further down regulated the phosphorylation of cdk2 by 65% \((P=0.01)\) (Figure 5.4). Therefore, our data reveal the possible mechanism by which ICA-1 exhibits its anti-proliferative effect on BE(2)-C cells.

5.2.4 ICA-1 induces apoptosis in BE(2)-C cells: Since, ICA-1 inhibited the proliferation of neuroblastoma cells; we tested whether the drug could induce apoptosis in these cells. BE(2)-C cells treated with ICA-1 (0.1µM) exhibited chromatin condensation and nuclear fragmentation compared to control cells as displayed by nuclear staining with DAPI (Figure 5.5 A, B). DNA damage following ICA-1 treatment was also detected as shown by TUNEL assay. The increase in fluorescence in response to ICA-1 treatment represents the increase of free 3’ OH groups of cleaved DNA (Figure 5.5 C, D). Because ICA-1 induced apoptosis in neuroblastoma, we further determined whether ICA-1 also induces other apoptotic responses such as Caspase and PARP cleavage. Treatment of BE(2)-C with ICA-1 (0.1µM) induced cleavage of Caspase-3 as shown by Western blot analysis (Figure 5.5 E). PARP, a downstream target for Caspase-3 was also cleaved in response to ICA-1 treatment (Figure5.5 E). Thus, our data strongly suggests that ICA-1 induces apoptosis in BE(2)-C neuroblastoma cells.
Figure 5.1 A, B ICA-1 inhibits the cell proliferation of BE(2)-C neuroblastoma cells. LA1-5s and BE(2)-C cells ($5 \times 10^4$) were treated with ICA-1 (A, B) from 24-72 hours. At the indicated time points, viable cells were counted using Trypan blue exclusion assay. The control cells were incubated with equal volume of vehicle control (sterile water). Data represents $N=3$ independent experiments and the mean number of viable cells and standard error were plotted.
Figure 5.2 A, B ATM inhibits the cell proliferation of both normal neuronal and neuroblastoma cells. LA1-5s and BE(2)-C cells (5x10^4) were treated with ATM (A, B) from 24-72 hours. At the indicated time points, viable cells were counted using Trypan blue exclusion assay. The control cells were incubated with equal volume of vehicle control (DPBS). Data represents N=3 independent experiments and the mean number of viable cells and standard error were plotted.
Figure 5.3 A, B ICA-1 inhibited the in-vitro phosphorylation of Cdk7 by PKC-ι. (A) Recombinant active PKC-ι (0.5µg) and active Cdk7 (0.5µg) were subjected to an in vitro kinase activity assay in the presence of ICA-1 (0.1µM and 5µM). The expression of pCdk7 (T170) and pan-Cdk7 were determined by Western blot analysis. (B) Represents the densitometry for the effect of ICA-1 on the in-vitro phosphorylation of Cdk7 by PKC-ι. Data represents N=3 independent experiments.
Figure 5.4 A, B ICA-1 abrogated the PKC-ι/Cdk7/cdk2 pathway. (A) BE(2)-C cells treated with ICA-1 (0.1µM) for 48 hour were harvested and whole cell extracts were prepared. Equal protein (100µg) was loaded on SDS-PAGE followed by immunoblotting for pCdk7 (T170) and pcdk2 (T160) pan-Cdk7, pan-cdk2, PKC-ι and β-actin to examine the difference between control cells (-) and ICA-1 treated cells (+). (B) Represent the densitometry for the decrease in the pCdk7 and pcdk2 upon ICA-1 treatment. Data represents N=3 independent experiments.
Figure 5.5 A, B, C, D ICA-1 induces apoptosis in BE(2)-C cells. (A, C) depict cells treated with vehicle control. (B, D) depict cells treated with ICA-1 (0.1µM). (A, B) Cells were stained with DAPI to visualize the cellular nuclei. (C, D) Cells were subjected to TUNEL assay. Data represents N=3 independent experiments.
Figure 5.5 E ICA-1 induces apoptosis in BE(2)-C cells. (E) BE(2)-C cells were treated with either vehicle control or ICA-1 (0.1µM) for 24, 48 and 72 hours. At the indicated time points, whole cell extracts were prepared followed by SDS-PAGE and subsequently Western blotting for Caspase-3 activity and PARP cleavage. β-actin levels were used as loading control. Data represents N=3 independent experiments.
5.3. Summary

ICA-1 specifically inhibited the proliferation of neuroblastoma cells and did not have a significant effect on the proliferation of normal neuronal cells. However, ATM inhibited the proliferation of both cells. Thus, our data supports that ICA-1 is less toxic than ATM. ICA-1 inhibited the capacity of PKC-ι as an *in-vitro* Cdk7 kinase as well as inhibited the PKC-ι/Cdk7/cdk2 pathway in BE(2)-C cells. The inhibition of proliferation was accompanied by induction of apoptosis.

Thus, ICA-1 inhibits the proliferation and induces apoptosis in neuroblastoma cells.
CHAPTER 6

SELF REGULATION OF PKC-ι

6.1 Introduction

PKC family of serine/threonine kinases are involved in many cellular responses such as proliferation, differentiation, motility, and survival. Different PKC isoforms have been reported to be involved in different types of cancers like brain, neuroblastoma, prostate, etc. [115]. However, the knowledge regarding the transcriptional and translational regulation of PKCs is still rudimentary. The promoters of several PKCs (α, β, γ) have been cloned till date, but few have been linked to cancer [116-120]. Among the PKC family, PKC-ι is the only isoform that has been proven to be an oncogene [58, 59]. It is the most common genomic amplicon as identified by comparative genomic hybridization [60]. The expression of PKC-ι has been reported to be elevated in many cancers such as lung [61], breast [66], ovarian [67-69], prostate [70], brain [71], etc. The expression of PKC-ι is regulated by Bcr-Abl [121].

6.1.1 Bcr-Abl: The translocation of the abl tyrosine kinase gene from the long arm of chromosome 9 to the breakpoint cluster region (bcr) of chromosome 22, the Philadelphia chromosome is the genetic cause of Chronic myelogenous leukemia (CML) [122]. Ninety five percent of CML patients test positive for the mutation in Philadelphia chromosome [123]. Numerous forms of bcr-abl result
from various breakpoints in the bcr genes [123]. The 210-kDa p210\textit{bcr-abl} gene is the most common form while 185-kDa p185\textit{bcr-abl} is diagnosed in 25% of CML patients [123]. Bcr-Abl regulates a plethora of signaling pathways, however, resistance to apoptosis associated with Bcr-Abl-is dependent on the Ras/MEK and PI 3-kinase pathways [124, 125] (Figure.6.1) Among the PKC family, PKCβII is essential for Bcr-Abl mediated CML cell proliferation whereas PKCι promotes Bcr-Abl-mediated resistance to drug-induced apoptosis [121, 126, 127].

\textit{Figure 6.1 Bcr-Abl activates both PI3K and MAPK pathways. Adapted from [124]}
Activation of the ELK-1 consensus sequence near the promoter region of PKC-ι is it the target of Bcr-Abl in the regulation of PKC-ι expression [121].

Regulation of PKC-ι by Bcr-Abl has been shown to regulate the activation of the transcription factor ELK-1 which is downstream of Ras/MEK pathway [121].

(Figure 6.2)

![Diagram of Bcr-Abl mediated regulation of PKC-ι expression](image)

*Figure 6.2 Bcr-Abl mediated regulation of PKC-ι expression. Adapted from [128]*
6.1.2 **Ets family:** Ets family of transcription factors play a vital role in coordinating cellular responses and gene expression [129]. A characteristic feature of the Ets family is a conserved winged helix-turn-helix DNA binding domain [130-132]. They are the downstream target of MAPK pathways [131]. The Ets family can be divided into two major groups [129].

The first group is comprised of Ets1, Ets2 and Pointed. These have a conserved C-terminal DNA binding domain and a N-terminal domain referred to as pointed domain which houses the single MAP phosphorylation site [133, 134].

The second group is the ternary complex factors (TCFs): These include Elk1, Sap1a, Sap1b, Fli1 and Net. They have a transactivation domain that can be phosphorylated by MAPK at multiple serine and threonine residues. [135, 136].

The Ets family mediate their transcriptional activity by binding to specific sequences called the Ras-response element (RRE) or serum-response elements (SRE) that are located near the promoter region of various genes [129].

6.1.3 **Elk-1:** Among the Ets family members, Elk-1 is a crucial molecule because it is activated by all three MAPK pathways, namely, p38, JNK and ERK [137-139]. JNK and ERK phosphorylate different residues in the C-domain of Elk-1 while the docking site for p38 is still unknown [140, 141].

Alternate to the Bcr-Abl mediated regulation of PKC-ι expression, we hypothesize that PKC-ι may self regulate its expression by directly associating with ERK and promoting its translocation to the nucleus where it activates the transcription factor Elk-1.
6.2 Results

6.2.1 Expression of PKC-ι and Elk-1 is higher in actively proliferating neuroblastoma cells: To analyze whether there is a correlation between the expression profile of PKC-ι and Elk-1 in neuroblastoma, we primarily determined the expression of PKC-ι and Elk-1 in neuroblastoma cells. The expression of PKC-ι was higher in actively proliferating malignant neuroblastoma BE(2)-C and LA-55n cells as compared to serum starved cells. However, the expression of PKC-ι in non-malignant neuroblastoma LA-15s cells was reverse, that is, PKC-ι expression in actively proliferating cells was less than serum starved cells. Next, we analyzed the expression of the transcription factor Elk-1 in these cells. The expression of Elk-1 corresponded with the expression profile of PKC-ι. In case of malignant neuroblastoma cells, Elk-1 expression was higher in actively proliferating cells while in non-malignant cells, the expression was higher in serum starved cells (Figure.6.3).

The data suggests that there is correlation between expression of PKC-ι and Elk-1 in neuroblastoma.

6.2.2 PKC-ι directly associated with Elk-1 in actively proliferating cells: Since, there was a correlation between expression of PKC-ι and Elk-1 in neuroblastoma, we further analyzed if there was an association between the two proteins. Immunoprecipitation studies showed that the association between PKC-ι and Elk-1 coincided with the expression pattern of the two proteins in neuroblastoma cells. The association was higher in actively proliferating malignant neuroblastoma BE(2)-C and LA-55n cells as compared to serum
starved cells. However, the expression of PKC-ι in non-malignant neuroblastoma LA-15s cells was reversed, the association was more robust in serum starved cells than actively proliferating cells (Figure.6.4).

Thus, our data suggests a crosstalk between PKC-ι and Elk-1.

6.2.3 Subcellular association of PKC-ι and Elk-1: Since, the association between PKC-ι and Elk-1 was higher in actively proliferating malignant neuroblastoma cells; we analyzed the subcellular association between the two proteins. Our hypothesis was that PKC-ι would associate with cytoplasmic Elk-1 and promote its nuclear translocation. The association of PKC-ι and Elk-1 was higher in the nuclear fraction of actively proliferating BE(2)-C neuroblastoma cells suggesting that PKC-ι may not influence the nuclear translocation of Elk-1 (Figure 6.5).

6.2.4 Sub-cellular expression of PKC-ι and Elk-1: Since the nuclear association between PKC-ι and Elk-1 was more robust than their cytoplasmic association, our new hypothesis was that PKC-ι could translocate into the nucleus and directly activate the transcription factor Elk-1. Thus, the rationale was that the nuclear expression of the two proteins should be higher in actively proliferating cells. Analysis of the subcellular fractions of actively proliferating LA1-55n and BE(2)-C malignant neuroblastoma cells demonstrated that the expression of PKC-ι was higher in the nucleus as compared to the cytoplasm. In the case of serum starved cell, the expression of PKC-ι was higher in the cytoplasm (Figure 6.6 A, B). This data suggested that in actively proliferating
neuroblastoma cells, PKC-ι translocates to the nucleus and directly activates the transcriptional activity of Elk-1.

6.2.5 PKC-ι indirectly phosphorylates Elk-1: In order to test our hypothesis that PKC-ι can directly activate Elk-1, we analyzed the capacity of PKC-ι as an Elk-1 kinase. Recombinant active PKC-ι did not directly phosphorylate recombinant active Elk-1. However, the phosphorylation of Elk-1 by ERK1 was increased in the presence of recombinant active PKC-ι (Figure 6.7). This suggested that PKC-ι could indirectly promote the phosphorylation of Elk-1 in ERK-1 dependent manner.

6.2.6 Association of PKC-ι and ERK1 in neuroblastoma cells: Since, PKC-ι promoted the in-vitro phosphorylation of Elk-1 in ERK-1 dependent manner we analyzed the association between PKC-ι and ERK1 in neuroblastoma cells. Immunoprecipitation studies showed that the association between PKC-ι and ERK1 coincided with the association of PKC-ι and Elk-1. The association was higher in actively proliferating malignant neuroblastoma LA1-55n cells as compared to serum starved cells (Figure.6.8). This data suggested that PKC-ι could regulate is expression by regulating the activity of ERK-1.

6.2.7 Subcellular expression ERK1 in neuroblastoma cells: Our data suggested that PKC-ι could regulate the activity of ERK1 and its downstream target Elk-1. Thus, the rationale was that there should be a correlation between the subcellular expression of the PKC-ι and ERK1. Analysis of the subcellular fractions of actively proliferating malignant neuroblastoma LA1-55n cells demonstrated that the expression of ERK1 was higher in the nucleus as
compared to the cytoplasm. In the case of serum starved cell, the expression of ERK1 was higher in the cytoplasm (Figure 6.9).

**Figure 6.3 Expression of PKC-ι and Elk-1 is higher in actively proliferating neuroblastoma cells.** Endogenous levels of PKC-ι in malignant neuroblastoma cells, BE(2)-C and LA1-55n and non-malignant cells, LA1-5s were determined by Western blot analysis. Equal amounts of cellular proteins (100µg) from actively proliferating (50%) and contact inhibited (100% S) cells were loaded in each well and immunoblotted for PKC-ι and Elk-1. β-actin levels were analyzed as a loading control. Data is representative of N=2 independent experiments.
Figure 6.4 Association of PKC-ι and Elk-1 in neuroblastoma cells. PKC-ι (5µg) was immunoprecipitated from LA1-5s non-malignant neuroblastoma cells as well as BE(2)-C and LA1-55n malignant neuroblastoma cells. The samples were subjected to in-vitro kinase activity assay and were analyzed for pElk-1 and pan Elk-1 using Western blot analysis. Data is representative of N=2 independent experiments.

Figure 6.5 Association of PKC-ι and Elk-1 in subcellular fractions of neuroblastoma cells. PKC-ι (5µg) was immunoprecipitated from BE(2)-C malignant neuroblastoma cells. They samples were analyzed for pan Elk-1 using Western blot analysis. Data is representative of N=2 independent experiments.
Figure 6.6 A, B Subcellular expression of PKC-ι and Elk-1 in neuroblastoma cells. Endogenous levels of PKC-ι and Elk-1 in malignant neuroblastoma cells, LA1-55n and BE(2)-C cells were determined by Western blot analysis. Equal amounts of cellular proteins (30µg) from subcellular fractions of actively proliferating (50%) and contact inhibited (100% S) cells were loaded in each well and immunoblotted for PKC-ι and Elk-1. Histone H1 was used as a purity control for the experiment. Data is representative of N=2 independent experiments.
Figure 6.7 PKC-ι indirectly phosphorylates Elk-1. Recombinant active PKC-ι (0.5μg) and active Elk-1 (0.5μg) in the presence (column 3) and absence (column 1) of recombinant active ERK1 were subjected to an in-vitro kinase activity assay. ERK1 and Elk-1 (second column) was the positive control. The proteins were then separated by SDS-PAGE and Western blotted to determine the phosphorylation of Elk-1 at T170. Data represents N=2 independent experiments.

Figure 6.8 Association of PKC-ι and ERK1 in neuroblastoma cells. PKC-ι (5µg) was immunoprecipitated from LA1-5s non-malignant neuroblastoma cells as well as BE(2)-C and LA1-5s malignant neuroblastoma cells. The samples were subjected to in-vitro kinase activity assay and were analyzed for pERK1 and pan ERK1 using Western blot analysis. Data is representative of N=2 independent experiments.
Figure 6.9 Subcellular expression ERK1 in neuroblastoma cells. Endogenous levels of ERK1 in malignant neuroblastoma LA1-55n cells were determined by Western blot analysis. Equal amounts of cellular proteins (30µg) from subcellular fractions of actively proliferating (50%) and contact inhibited (100% S) cells were loaded in each well and immunoblotted for ERK1 and β-actin.
6.3 Summary

Based on our data, we hypothesize that in contrast to Bcr-Abl mediated regulation of PKC-ι, PKC-ι can directly regulate the transcriptional activity of Elk-1, thereby self-regulating its expression.

Our hypothesis can be summarized by the following diagram:

*Figure 6.10 Self-regulation of PKC-ι. We hypothesize that PKC-ι translocates into the nucleus in actively proliferating neuroblastoma cells where it activates ERK1. This promotes the activation of its downstream target Elk-1 which subsequently upregulates the expression of PKC-ι.*
CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

7.1 Discussion

Very little research has been conducted using PKC-ι specific inhibitors. To date, the only compounds identified as inhibitors of atypical PKC isoforms are aurothiomalate (ATM) and aurothioglucose. ATM, which is currently in phase I clinical trial evaluation for lung cancer (sponsored by NCI) has IC$_{50}$ ranging from ~0.3 µM – >100 µM [111]. However, ATM has not been shown to act specifically on PKC-ι versus ζ, and the mechanism of action for ATM is not fully understood, as shown by its off target effects. PKC-ι and PKC-ζ are 84% homologous with respect to the amino acid sequences of their catalytic domain [56] making it very difficult to synthesize an inhibitor that is specific. Nevertheless, in this study the sequence targeted for molecular docking was amino acid residues 469-475 (glutamine, isoleucine, arginine, isoleucine, proline, arginine and serine). The molecular docking method was based on an experimental design that included rapid functional testing of specificity between PKC isoforms as the primary step of in vitro screening. This important aspect of the approach eliminates candidates that bind the ATP binding pocket (which is conserved between PKC isoforms). Although it is straightforward to model numerous potential interactions by molecular docking in silico, such as docking molecules into the ATP binding site or other surfaces, our approach pinpointed the most diverse structural
elements in PKC-ι and purposefully evaluated specificity in vitro to avoid compounds that interact with structural features that are common to PKC isoforms such as the ATP binding pocket. This directed molecular docking approach has been successfully used in a number of studies [142]. The current study established the chemotherapeutic potential of the novel PKC-ι inhibitor, ICA-1. Comparative study of ICA-1 and ATM showed that ICA-1 specifically inhibited the activity of only PKC-ι, whereas ATM exhibited inhibitory effects on both PKC-ι and PKC-ζ, suggesting non-specificity of the drug. The IC$_{50}$ of ATM was 100µM which is 1000 times more than the IC$_{50}$ for ICA-1. Furthermore, the PIP$_3$ activation assay showed that ICA-1 specifically inhibited the activity of PKC-ι with an IC$_{50}$ of 0.01 µM. Since this assay measures the femtomoles of ($\gamma$-$^{32}$P) ATP transferred, it was possible to measure the efficacy of ICA-1 over a broad range of concentrations yielding the most sensitive data. However, we did not test the inhibitory effect of ATM using PIP$_3$ activity assay because lower concentrations of ATM did not exhibit any effects on the activity of either PKC-ι or PKC-ζ. The structure of ICA-1 is similar to FDA approved chemotherapeutic drug Fludarabine, administered as 5′-nucleotide monophosphate [143]. Thus, we speculate that ICA-1 may be transported into the cell by a mechanism that utilizes a nucleoside transporter similar to Fludarabine [144].

Previously, we found that PKC-ι is overexpressed in actively proliferating prostate cancer and glioma cells [71, 73]. Similarly, we found higher expression of PKC-ι in adrenal neuroblastoma tissue as well as neuroblastoma cells suggesting the involvement of PKC-ι in the proliferation of neuroblastoma cells.
Phenotypically, neuroblastoma cells can be divided into 3 distinct groups: sympathoadrenal neuroblasts (N-type that form slow growing tumors), non-neuronal precursor cells (S-type which are non-malignant) and I-type which are morphologically intermediate to that of N and S cells. I-type cells possess the biochemical properties of both N and S cells [145]. Thus, the rationale behind our choice of BE(2)-C cells (I-type) was to ensure that our results are representative of all the major neuroblastoma phenotypes. The S-type have been shown to be normal neuronal cells [146]. Thus, LA1-5s cells were used as control to analyze the toxicity of ICA-1.

To determine the mechanism underlying the anti-proliferative action of ICA-1, we first identified a signaling pathway by which PKC-i may promote constitutive proliferation of BE(2)-C cells. We focused on investigating the role of cdks since they are involved in regulation of cell cycle progression [78]. Our data suggest that PKC-i is an in-vitro Cdk7 kinase. Endogenously, PKC-i directly associated and phosphorylated both Cdk7 as well as its downstream target cdk2 suggesting that PKC-i is regulating or promoting neuroblastoma cell proliferation via the PKC-i/Cdk7/cdk2 pathway. Hence, this pathway was targeted to verify the efficacy of ICA-1.

We were not able to obtain the effects of ICA-1 on the cell cycle because of the BE(2)-C cells grow in clusters. The results obtained from cell cycle analysis by flow cytometry yielded a very high percentage of aggregates making it impossible to determine the significance of the data (n=8) (data not shown).
Collectively, our data exhibit the potential of ICA-1 as a novel PKC-ι inhibitor. The efficacy of ICA-1 at such low concentration (0.1µM) and its negligible effect on normal neuronal cells suggests its potential as a chemotherapeutic with little toxicity. Therefore, future studies will investigate the effects of ICA-1 on an in-vivo mouse model to establish the feasibility of advancing ICA-1 to clinical trials. Furthermore, PKC-ι expression should be useful as a biomarker for neuroblastoma when treating patients with anti-PKC-ι therapy (with ICA-1 or one of its derivatives) for personalized medicine.

Bcr-Abl has been shown to regulate the activation of the transcription factor ELK-1 which in turn regulates the expression of PKC-ι [121]. Alternatively, we hypothesize that PKC-ι self regulates its expression by indirectly regulating the activity of ELK-1. Our preliminary data shows that the expression of PKC-ι and Elk-1 were higher in actively proliferating LA1-5S (non-malignant), BE(2)-C and LA1-55n (malignant )neuroblastoma cells. Immunoprecipitation studies show that association of PKC-ι with phosphorylated Elk-1 was more robust in these cells suggesting a crosstalk between the two proteins. Analysis of the subcellular association of these two proteins revealed increased association between PKC-ι and Elk-1 in the nucleus fraction of actively proliferating BE(2)-C cells as compared to cytoplasm. Interestingly, the nuclear expression of PKC-ι was also found to be higher in these cells, sugesting that PKC-ι translocated to the nucleus in actively proliferating cells and regulated the transcriptional activity of Elk-1. Although, recombinant active PKC-ι did not phosphorylate recombinant active Elk-1, it increased the phosphorylation of Elk-1 in the presence of ERK1,
an upstream kinase of Elk-1 in the Bcr-Abl mediated PKC-ι regulatory pathway. Thus, our data suggested that ERK1 was integral to the self-regulatory activity of PKC-ι. Furthermore, expression of ERK1 was also found to be higher in the nuclear fraction of our actively proliferating model cell systems.

In conclusion, we propose a novel self-regulatory mechanism of PKC-ι in neuroblastoma cells. We hypothesize that the self-regulation of PKC-ι commences with the translocation of PKC-ι into the nucleus where it promotes the activation of ERK1. This culminates in increased activation of Elk-1, a downstream target of ERK1 and subsequent upregulation in the expression of PKC-ι. Our hypothesis can be summarized as PKC-ι/ ERK1/ Elk-1/ PKC-ι pathway (Figure 6.10)

7.2 Suggestions for future studies

- Determine the potency of ICA-1 in-vivo
- Study the effect of ICA-1 on other cancers models such as prostate, breast cancer, ovarian cancer, etc.
- Analyze the pharmacodynamics and pharmacokinetics of ICA-1
- Examine the effect of ICA-1 on other Protein kinase C isozymes
- Evaluate the effect of ICA-1 on other protein kinases
- Investigate the effect of other protein kinase C in neuroblastoma
- Analyze the role of PKC-ι in neuroblastoma cell survival
- Investigate the effect of ICA-1 on the self regulation of PKC-ι
- Study the effect of PKC-ι on the regulation of other members of the Ets family.
CHAPTER 8
MATERIALS AND METHODS

8.1 Reagents

ICA-1 or [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl]methyl dihydrogen phosphate was synthesized and purchased from Southern Research Institute (Birmingham, AL). It was dissolved in sterile distilled water (vehicle) before use. Recombinant PKC-ι and recombinant PKC-ζ active enzymes were purchased from Millipore (Temecula, CA). Myelin basic protein (MBP) was purchased from Sigma (St. Louis, MO). PKC-ι antibody was purchased from BD Biosciences (San Jose, CA). The antibodies to phospho-MBP (pMBP) (Thr 98), MBP, PKC-ζ, pElk-1 were purchased from Millipore. PKC activity assay kit was purchased from Assay Designs (Ann Arbor, MI). For the $^{32}$P labeling, recombinant PKC-ι from BioVision (Mountain View, CA), PIP$_3$ [Phosphatidylinositol (3,4,5)-trisphosphate] from Avanti Polar Lipids (Alabaster, Al), ($\gamma$-$^{32}$P)ATP from NEN Life Science Products Inc. (Boston, MA) and PKC-ε Pseudosubstrate from (Invitrogen Inc.) were used. The antibodies to Cdk7, phospho-cdk2 (pckd2, Thr 160), Caspase-3, Elk-1, ERK-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). pCdk7 (Thr 170) antibody was purchased from Abcam (Cambridge, MA). The antibodies purchased from Cell Signaling were: PARP and cleaved PARP. Enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate) was
purchased from Pierce (Rockford, IL). Dulbecco’s phosphate buffered saline without Mg\(^{2+}\) and Ca\(^{2+}\) (DPBS) and Trypsin-EDTA (ethylenediaminetetraacetic acid) solution was purchased from Fisher Scientific (Norcross, GA).

8.2 Cell culture

Phenotypically, neuroblastoma cells can be divided into 3 distinct groups: sympathoadrenal neuroblasts (N-type that form slow growing tumors), non-neuronal precursor cells (S-type which are non-malignant) and I-type which are morphologically intermediate to that of N and S cells. I-type cells possess the biochemical properties of both N and S cells [145]. The S-type have been shown to be normal neuronal cells [146]. Thus, LA1-5s cells were used as control to analyze the toxicity of ICA-1.

BE(2)-C human neuroblastoma cells, were obtained from the American Tissue Culture Collection (Rockville, MD). These cells are clones of the SK-N-BE(2) human neuroblastoma cell line [147]. Phenotypically, they are classified as I-type neuroblastoma cells [145]. LA1-5s and LA1-55n cells were obtained from Dr. Robert Ross’ laboratory in Fordham University, NY. Cells (2x 10\(^6\)) were grown as a monolayer in 75 cm\(^2\) tissue culture flasks and maintained at 37°C in a humidified atmosphere comprised of 5% CO\(_2\). The complete growth medium was 89% by volume of 1:1 mixture of Eagle's Minimum Essential Medium from ATCC and Ham’s F12 Medium from Invitrogen corporation (Carlsbad, CA). Additionally, the media was supplemented with 10% fetal bovine serum from ATCC and 1% antibiotics (Penicillin 10 U/ml and streptomycin 10µg/ml from Fisher Scientific, Norcross, GA.)
8.3 Atypical PKC profiling

Preliminary work was performed on neuroblastoma tissues obtained from the Nationwide Children’s Hospital Biopathology Center. The expression of PKC-ι in adrenal neuroblastoma \((n=11)\) was compared to normal adrenal biopsies \((n=11)\). Furthermore, actively proliferating BE(2)-C cells (50% confluent) were harvested and cell lysates were prepared as described in the ‘Immunoprecipitation and Western Blot analysis’ section. Another set of cells were grown until contact inhibition was achieved (100% confluent). Subsequently, the cells were washed twice with DPBS, followed by addition of starved media (absence of fetal bovine serum) for an additional 48 hours. Cells were then harvested and cell lysates were prepared. Proteins (100µg) from both sets of cell lysates were separated by SDS-PAGE and immunoblotted for PKC-ι and PKC-ζ with specific antibodies.

8.4 Cell fractionation and Western Blot Analysis

Cells were placed on ice to terminate the incubation. The cells were washed twice with DPBS and then scraped at 4 °C, resuspended and sonicated in 300µl of homogenization buffer (50 mM HEPES at pH 7.5), 150 mM NaCl, 1 mM EDTA and 2 mM EGTA [glycol-bis(2-aminoethylether)-N, N, N’, N’-tetraacetic acid], 0.5% Triton-X 100, 1 mM sodium orthovanadate, 0.5 M NaF, 0.2 M PMSF (phenylmethylsulphonylfluoride), 1mM DTT (dithiothreitol) and 0.15 U/ml aprotinin [148]. The cell suspension was centrifuged at 16,000 g for 30 minutes to obtain cell extracts. The protein content was measured according to Bradford [149]. Protein samples were separated by 12% on sodium dodecyl sulfate-
polyacrylamide gel electrophoresis (SDS-PAGE) (1.5mm thick gel) using Protean II xi 16cm gel loading system (BioRad) and electrophoresed at 160volts and subsequently transblotted by electroblotting with transfer buffer 100 ml of 10 x tris glycine (0.25 M tris and 1.92 M glycine) and 20% methanol in 1 L of distilled water and electroblotted for one hour at 24 volts. For Western blot analysis, each blot was blocked for 1 hour with 5% (w/v) fat-free dry milk in tris-buffered saline with 0.05% tween-20 (TBST) solutions at room temperature. Protein bands were probed with primary antibody in 5% milk in blocking buffer at 4°C overnight. The primary antibodies dilutions were 1:1000-1:5000. Membranes were subsequently washed three times for 15 minutes with TBST. Secondary antibodies such as horseradish-peroxidase-conjugate anti-mouse, anti-rabbit or anti-goat were diluted 1:1000 in 5% milk TBST. The membranes were incubated with secondary antibody (1:5000) at room temperature for 1 hour. Immunoreactive bands were visualized with SuperSignal West Pico Chemiluminescent substrate.

8.5 Densitometry

The intensity of each band was measured using the Quantity one, 1-D analysis software (BioRad Laboratories). In order to derive the correct intensity of each band, the background intensity was subtracted from the band intensity. Mean absorbance of three independent studies were compared by means of Student’s t-test to determine the statistical significance of the data.
8.6 Inhibition of PKC-ι with siRNA

The siRNA was a pool of 3 target-specific 19-25 nucleotides siRNAs designed to knock down PKC-iota gene expression. Each vial contains 3.3 nmol of lyophilized siRNA, sufficient for a 10 μM solution once resuspended using company protocol. Their mRNA sequences were:

663: 5'-CAAGCCAAGCGUUUCAACA-3'
   5'-UGUUGAAACGCUUGCUUG- 3'
729: 5'-GGAACGAUUGGGGUUGUCAU-3'
   5'-AUGACAAACCCAAUCGUCC-3'
2137: 5'-CCCAAUAUCUUCUCUUGUA-3'
   5'-UACAAGAGAAGAUAUUGGG-3'

These were designed by Santa Cruz Biotechnology. BE(2)-C cells (5 x 10^4) were plated and grown in 25 cm^2 tissue culture plates. Twenty four hour post-plating cells were transfected with either control siRNA sequences (containing scrambled sequence which does not lead to specific degradation of any known cellular mRNA; sequence not revealed by Santa Cruz Biotechnology) or PKC-ι gene silencing sequences (150nM). Cell viability was determined over a time period of 24, 48 and 72 hour. Cell viability was performed as using Trypan Blue Exclusion assay. To analyze the effect of siRNA on PKC-ι expression, cells (2x10^5) were treated with PKC-ι siRNA for 48 hours and then total lysate were prepared. The protein (100μg) was separated by SDS-PAGE followed by Western blot to determine the expression of PKC-ι and PKC-ζ. To determine the effect of PKC-ι siRNA on the phosphorylation of Cdk7, the protein was
immunoprecipitated using Cdk7 antibody (5µg) and subjected to Western blot analysis.

8.7 In-vitro kinase activity assay to analyze the phosphorylation of Cdk7 by PKC-ι

The recombinant active PKC-ι (0.5µg) and Cdk7 (0.5µg) purchased from Millipore (Temecula, CA) were suspended in 200µl of PKC kinase buffer [150] to test the ability of PKC-ι as an in-vitro Cdk7 kinase. The reaction was terminated after incubation for 10 min at 30°C by placing the samples in ice and adding sample loading buffer. Samples were then fractionated by SDS-PAGE and immunoblotted via Western Blot analysis.

8.8 Immunoprecipitation

Cells were placed on ice to terminate the incubation. The cells were washed twice with DPBS and then scraped at 4°C, resuspended and sonicated in 300µl of homogenization buffer (50 mM HEPES at pH 7.5), 150 mM NaCl, 1 mM EDTA and 2 mM EGTA [glycol-bis(2-aminoethylether)-N, N', N'-tetraacetic acid], 0.5% Triton-X 100, 1 mM sodium orthovanadate, 0.5 M NaF, 0.2 M PMSF (phenylmethysulphonyl-fluoride), 1mM DTT (dithiothreitol) and 0.15 U/ml aprotinin [148]. The cell suspension was centrifuged at 16,000 g for 30 minutes to obtain cell extracts. The protein content was measured according to Bradford [149]. Approximately 200µg of protein was immunoprecipitated with affinity purified antibodies (5µg). Cell lysates were first subjected to pre-clearance by incubation with agarose beads for 30 minutes at 4°C. The cell lysates were subsequently incubated with 5µg of either PKC-ι or Cdk7 antibody or both in case
of co-immunoprecipitation. The immune complexes were rocked overnight at 4°C and further incubated at 4°C for 2 hours with IgG-agarose beads specific to the strain of primary antibody. Post incubation, the beads were washed three times with 1mL of complete lysis buffer. Samples were then separated on 12% SDS-PAGE and transblotted onto a nitrocellulose membrane. The proteins were detected by Western blot analysis.

8.9 Database preparation

The National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) maintains a repository of 139,644 samples (the plated compound set). The three-dimensional coordinates for the NCI/DTP plated compound set in the MDL sd format was converted to the mol2 format by the program SDF2MOL2 (UCSF). Partial atomic charges for the ligands were calculated using SYBDB (UCSF) and added to the plated compound set mol2 file. This was performed by David Ostrov, Ph. D. at the University of Florida.

8.10 Molecular docking

Molecular docking was done by selecting specific structural pockets in PKC-ι protein that may be suitable for interactions with drug-like small molecules. Molecular docking simulations where each one of approximately 300,000 small molecules (MW < 500) is positioned in the selected structural pocket and scored based on predicted polar (e.g., H bond) and non-polar (e.g. Van der Waal’s) interactions. The 30 top scoring compounds for each selected structural pocket were obtained for use in functional assays. The grid based scoring method sums the attractive and repulsive forces between atoms in each docked small molecule.
and the site selected for molecular docking. Interactions between charges on atoms in ICA-1 with amino acids are computed as an electrostatic score. The electrostatic score and van der Waals scores are summed to generate an overall energy score which was used to rank the highest scoring compounds.

Docking calculations were performed with the October 15, 2002 version of DOCKv5.1.0. The coordinates for the crystal structure PKC, PDB code 1ZRZ, were used. The molecular surface of the structure was explored using sets of spheres to describe potential binding pockets. The spheres literally fill in the available pocket spaces where a ligand might be able to form a complex. DOCK uses the spheres as guides to search for orientations of each molecule that fit into the selected sites. The sites selected for molecular docking were defined using the SPHGEN/SITESELECTOR programs and filtered through the CLUSTER program. The SPHGEN program generates an unbiased grid of points that reflect the actual shape of the selected site. The CLUSTER program groups the selected spheres to define the points that are used by DOCK to match (superimpose) potential ligand atoms with spheres. Twenty two spheres were used to define the PKC-ι site for molecular docking. Each compound in the NCI/DTP repository was positioned in the selected site in 1000 different orientations. Intermolecular AMBER energy scoring (vdw + columbic), contact scoring and bump filtering implemented in DOCKv5.1.0. PYMOL were used to generate molecular graphic images. All molecular docking jobs were performed on a high performance supercomputer at the University of Florida running linux. This was performed by David Ostrov, Ph. D. at the University of Florida.
8.11 Phosphorylation of MBP by recombinant active PKC-ι or recombinant active PKC-ζ enzymes

The PKC-ι or PKC-ζ (0.01µg/µl) was incubated with different concentrations of ICA-1 (0.1 µM, 1 µM or 5 µM) for 10 min at 4°C. This was followed by incubation at 30°C in 200µl of PKC kinase buffer [150] to test the ability of these PKC isoforms to phosphorylate a final concentration of 0.025 mg/ml MBP, a known substrate for PKCs [112]. The reaction was terminated after 10 min by placing the samples in ice and adding sample loading buffer. Samples were then fractionated by SDS-PAGE and immunoblotted.

8.12 PKC activity assay kit

The selectivity of ICA-1 and ATM for PKC-ι versus PKC-ζ were compared using a PKC activity assay kit (Assay Designs). The recombinant active PKC-ι (0.01µg) or PKC-ζ (0.01µg) were incubated with different concentrations of ICA-1 (0.1 µM, 1 µM or 5 µM) or ATM (0.01µM, 0.1µM, 1µM or 100µM) for 10 min at 4°C followed by incubation with phosphatidylinerine (5µg) and ATP (0.0025 mg/ml) for 30 min at 30°C. The remaining steps were per the manufacturer’s protocol.

8.13 32P labeling

Briefly, 0.01µg of recombinant active PKC-ι was treated with different concentrations of ICA-1 for 10 min at 4°C before adding 10 fM PIP₃, phosphatidylinerine (4µg), (γ⁻³²P)ATP (50 µmol/L) and as substrate, serine analog of the PKC-ε pseudosubstrate (40 µmol/L). This mixture was suspended in 100µl
of reaction mixture composed of Tris-HCl (50 mmol/L at pH 7.5), (sodium orthovandate) Na₃VO₄ (100µmol/L), (sodium phosphate) Na₂P₂O₇ (100µmol/L), (sodium fluoride)NaF (1 mmol/L), (phenylmethanesulfonylfluoride) PMSF (100µmol/L) and (magnesium chloride) MgCl₂ (5 mmol/L) followed by incubation for 10 min at 30°C. After incubation, the ³²P labeled substrate was trapped on P-81 filter paper and counted in a liquid scintillation counter.

8.14 Competitive inhibitory assay

The assay was performed using an ELISA plate which was coated by incubating with 1µg of phospho-MBP (pMBP) (T98) overnight at 4°C. The antibody dilution buffer used was 3% BSA (Bovine serum albumin). Twenty-four hours post-incubation, the wells were washed 3-4 times with DPBS buffer and blocked with 200µl of antibody dilution buffer at room temperature for 2 hour.

Recombinant active PKC-ι (0.01µg/µl) was incubated with or without ICA-1 (1 µM) for 10 min at 4°C prior to addition of 50µl of PKC kinase buffer [150] to test the inhibitory effects of ICA-1 on PKC-ι activity utilizing varying concentrations of MBP (0.0025 mg/ml, 0.05 mg/ml, 0.010 mg/ml and 0.02 mg/ml). This reaction was performed in separated tubes and terminated after 10 min incubation at 30°C by placing the samples in ice. The reaction cocktail was subsequently transferred to the ELISA plate (primary antibody bound) and incubated at room temperature for 2 hours. The wells were washed 3-4 times with DPBS buffer and incubated with secondary antibody at room temperature for 1 hour. Subsequently, wells were washed 3-4 times with DPBS buffer and incubated with tetramethylbenzidine (TMB) reagent for 1 hour at room
temperature. Acid stop solution was added to each well and the absorption was measured at 450nm.

8.15 In-vitro kinase activity assay to analyze the effect of ICA-1 on the phosphorylation of recombinant Cdk7 by recombinant PKC-ι

The recombinant active PKC-ι (0.5μg) and Cdk7 (0.5μg) were purchased from Millipore (Temecula, CA) and were suspended in 200μl of PKC kinase buffer [150] to test the ability of these PKC-ι as an in-vitro Cdk7 kinase. The same reaction was repeated in the presence of ICA-1 drug (0.1uM, 1µM and 5uM). In both the experiments, the reaction was terminated after incubation for 10 min at 30°C by placing the samples in ice and adding sample loading buffer. Samples were then fractionated.

8.16 Cell viability assay

LA1-5s and BE(2)-C cells (5x 10⁴) were cultured in 25 cm² tissue culture flasks. Twenty-four hours post plating, fresh media was supplied and cells were incubated with either equal volume of sterile water (vehicle control) or ICA-1 (0.04μM to 0.1μM). Following the initial exposure to ICA-1, additional doses of sterile water or ICA-1 were supplied every 24 hour during the three day incubation period. In order to analyze the effect of ATM (aurothiomalate) on cell viability, LA1-5s and BE(2)-C cells were treated with DPBS (vehicle control) or ATM (0.01µM - 100µM). Cell viability was determined at 24, 48 and 72 hours. Cell viability was quantified using a Trypan blue exclusion assay.
8.17 Analysis of apoptosis

The first method used was DAPI staining. BE(2)-C cells were grown in glass chamber slide (BD Bioscience) and treated with ICA-1 as in the cell viability assay. Forty eight hours post-treatment, cells were washed three times with cold 1x DPBS, fixed with 1:1 methanol and acetone (5 minutes at -20°C) and washed again 3 times with 1x DPBS. Cell nuclei were visualized in mounting medium containing DAPI (blue, Vector Laboratories, Inc) using a Nikon Eclipse TE2000-U microscope. Pictures were captured by NIS-Elements F Version 2.10.

The second method used was TUNEL Assay. DNA fragmentation was visualized by means of fluorescence staining using a TUNEL apoptosis detection kit according to the manufactures instructions. BE(2)-C cells were grown and treated with ICA-1 as explained for DAPI staining. At 48 hours, the cells were fixed using 4% paraformaldehyde in 0.1 M NaH₂PO₄, pH 7.4, followed by incubation for 60 minutes in a reaction mixture composed of biotin-dUTP and terminal deoxynucleotidyl transferase. The sample was then incubated with fluorescein isothiocyanate-avidin (FITC-avidin) for 30 minutes. The positively stained cells were observed and photographed for DAPI staining.

Finally, the apoptosis was determined by analysis of Caspase-3 and PARP cleavage. BE(2)-C cells were cultured in 100 mm tissue culture flasks and treated with ICA-1 as in the cell viability assay. Protein (200µg) was separated by SDS-PAGE and immunoblotted.
8.18 Preparation of cytoplasmic and nuclear extracts

Cells were washed twice with cold PBS and resuspended in hypotonic buffer. The buffer is comprised of 50 mM HEPES (pH 7.4), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 2.5 lg/m leupeptin, 0.15 U/ml aprotinin. The lysates were then centrifuged at 30 min 4000g for 30 min at 4 °C. The cytoplasmic fraction (supernatant) was separated and collected. The nuclear fraction (pellet) was suspended in a Buffer containing 400 mM KCl and 0.5% Triton X-100. The lysates were then centrifuged at 14,000g for 30 min at 4°C and the supernatant containing nuclear extracts was collected.

8.19 In-vitro kinase activity assay to analyze the phosphorylation of endogenous Elk-1 by PKC-ι

PKC-ι (0.5μg) was immunoprecipitated from cell lysates of BE(2)-C malignant neuroblastoma cells and T98G malignant glioma cells. And suspended in 200μl of PKC kinase buffer [150] to test the ability of PKC-ι to phosphorylate Elk-1. The reaction was terminated after incubation for 10 min at 30°C by placing the samples in ice and adding sample loading buffer. Samples were then fractionated by SDS-PAGE and immunoblotted via Western Blot analysis.

8.20 In-vitro kinase activity assay to analyze whether PKC-ι directly phosphorylates recombinant Elk-1

Recombinant active PKC-ι (0.5μg) and recombinant active Elk-1 (0.5μg) were subjected to an in-vitro kinase activity assay in the presence and absence of recombinant active ERK1. The phosphorylation of Elk-1 by ERK1 was used as the positive control. The proteins were then separated by SDS-PAGE and
Western blotted to determine the phosphorylation of Elk-1. Data represents $N=2$ independent experiments.
REFERENCES


4. Stensman H LC: *Protein kinase C epsilon is important for migration of neuroblastoma cells.* *BMC Cancer* 2008, 8.


42. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR: **Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1)


56. Selbie LA, Schmitz-Peiffer C, Sheng Y, Biden TJ: **Molecular cloning and characterization of PKC iota, an atypical isoform of protein kinase C**


73. Win HY A-DM: **Role of protein kinase C-iota in transformed nonmalignant RWPE-1 cells and androgen-independent prostate carcinoma DU-145 cells.** *Cell Prolif* 2009, **42**:182-194.


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Prajit Prasannan Pillai received his Bachelor of Science in Biochemistry from University of Mumbai in April 2004. Thereafter, he received a Master of Science in Biochemistry from University of Mumbai in May 2006. He was accepted to the doctoral program in the Department of Chemistry at University of South Florida in August 2006. He pursued his doctoral research under the guidance of his major professor Mildred Acevedo-Duncan Ph. D. Prajit has a strong background in Cancer Biology, signal transduction mechanism and small molecule inhibitors. He discovered the novel PKC-ι/Cdk7/cdk2 proliferation pathway in neuroblastoma. He was integral in designing and implementing strategies to verify the specificity of ICA-1, a novel PKC-ι inhibitor. Prajit has 5 years of teaching experience at the Department of Chemistry at University of South Florida. Prajit has several publications in peer-reviewed scientific journals. Prajit has been the recipient of the Frank L and Helen M Tharp scholarship granted by University of South Florida. He has presented his research at various national and international conferences such as American Association of Cancer Research organization’s annual meetings and Florida Annual Meeting and Exposition organized by American Chemical Society.