March 2020

The Role of aPKCs and aPKC Inhibitors in Cell Proliferation and Invasion in Breast and Ovarian Cancer

Tracess B. Smalley

University of South Florida

Follow this and additional works at: https://scholarcommons.usf.edu/etd

Part of the Biochemistry Commons, Biology Commons, and the Cell Biology Commons

Scholar Commons Citation
Smalley, Tracess B., "The Role of aPKCs and aPKC Inhibitors in Cell Proliferation and Invasion in Breast and Ovarian Cancer" (2020). Graduate Theses and Dissertations. https://scholarcommons.usf.edu/etd/8300

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
The Role of aPKCs and aPKC Inhibitors in Cell Proliferation and Invasion in Breast and Ovarian Cancer

by

Tracess B. Smalley

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy Chemistry Department of Chemistry College of Arts and Sciences University of South Florida

Co-Major Professor: Mildred Acevedo-Duncan, Ph.D.
Co-Major Professor: Wayne Guida, Ph.D.
David Merkler, Ph.D.
Meera Nanjundan, Ph.D.
Theresa Evans-Nyugen, Ph.D.

Date of Approval:
March 6th, 2020

Keywords: PKC-zeta, invasion, breast cancer, ovarian cancer, clear cell ovarian carcinoma, Ect2, Rac1, RhoA

Copyright © 2020, Tracess B. Smalley
Dedication

I would like to dedicate my dissertation to my dear parents, Will and Kay Pahl and Eileen Caldwell, and my extremely supportive husband Jarred Smalley. Only with their love, prayers, values and support was I able to overcome the difficulties and hurdles of my graduate studies and achieve my goals.

Thank you to Mildred Acevedo-Duncan who saw a young scientist in me when I did not always see one myself.

I want to thank all of my close friends and family who gave me a push and a coffee when I needed one and a bed to sleep in when I needed some rest.

I want to express gratitude to the many teachers in my life that pressed me to be better and believed in me.
Table of Contents:

List of Figures ................................................................................................................................ iv

List of Tables ................................................................................................................................ vii

List of Abbreviations ........................................................................................................................ viii

Abstract ............................................................................................................................................. xi

Chapter 1: Protein Kinase C ............................................................................................................1
  1.1 Introduction ..............................................................................................................................1
  1.2 PKC isoforms, activation and domains .............................................................................1
  1.3 Atypical PKC as novel targets for cancer therapy .........................................................4
    1.3.1 PKC inhibitors ...........................................................................................................6
  1.4 With a closer look a PKC-ζ ..........................................................................................7
    1.4.1 PKC-ζ in invasion pathways ..................................................................................8

Chapter 2: Role of PKC-ζ in Breast Cancer ................................................................................12
  2.1 Introduction .........................................................................................................................12
  2.2 Results ................................................................................................................................15
    2.2.1 PKC-ζ is overexpressed in malignant breast tissues ...........................................15
    2.2.2 PKC-ζ protein levels are higher in invading breast cancer subtypes ...............18
    2.2.3 PKC-ζ and E-Cadherin levels in tissue specimens .........................................19
    2.2.4 PRKCZ gene silencing decreases the invasion of breast cells .....................21
  2.3 Acknowledgments .............................................................................................................23
  2.4 Funding ..............................................................................................................................24
  2.5 Availability of data and materials .................................................................................24
  2.6 Author’s contribution ......................................................................................................24
  2.7 Competing interests .......................................................................................................24

Chapter 3: ζ-Stat as a Novel Inhibitor in Ovarian Cancer .............................................................25
  3.1 Introduction .........................................................................................................................25
  3.2 Results ................................................................................................................................28
    3.2.1 PIK3CA and ARID1A are in the top mutated genes in all ovarian
tissue types and in CCOC .................................................................28

3.2.2. Select inhibitor effects on PKC-ζ and PKC-ι protein expression ........30
3.2.3. In-silico results and model validation ........................................31
3.2.3.1. Model viability .................................................................31
3.2.3.2. Docking results ..............................................................34
3.2.3.3. Virtual target screening results ........................................36
3.2.4. Inhibition of cell viability .......................................................37
3.2.5. Analysis of PKC-ζ/Ect2/Rac1/RhoA pathway ............................38
3.2.6. Analysis of ζ-Stat in TOV21G tumor xenografts .........................42
3.2.7. ζ-Stat interrupts the PKC-ζ/Ect2 via PKC-ζ protein decrease ..........43

3.3. Acknowledgments ........................................................................44
3.4. Funding ......................................................................................44
3.5. Availability of data and materials .................................................44
3.6. Conflict of interest .....................................................................45
3.7. Author contributions ..................................................................45
3.8. Supplemental figures ..................................................................46

Chapter 4: Discussion and Future Directions ........................................53

4.1. Roles of PKC-ζ in breast cancer ..................................................53
4.1.1. PKC-ζ expression was higher in invasive tissue subtypes ............53
4.1.2. PKC-ζ expression was higher in ductal carcinomas when compared
to lobular carcinomas .......................................................................54
4.1.3. PKC-ζ knockdown decreased cellular migratory behaviors ..........54
4.2. ζ-Stat as a novel inhibitor in ovarian cancer .................................56
4.2.1. Targeting aPKCs in CCOC ..................................................56
4.2.2. aPKCs are involved in the localization of Ect2 .........................58
4.2.3. ζ-Stat has therapeutic potential in CCOC ...............................58
4.3. Future directions .........................................................................60

Chapter 5: Materials and Methods .............................................................61

5.1. Analysis of PKC-zeta protein levels in normal and malignant breast tissue
subtypes ..............................................................................................61
5.1.1. Specimen collection and tissue fractionation ..............................61
5.1.2. Cell culture ..........................................................................62
5.1.3. Western Blot analysis ............................................................62
5.1.4. Densitometry ........................................................................63
5.1.5. Immunohistochemistry .........................................................63
5.1.6. Knockdown of PKC-ζ for invasion pathway analysis .................64
5.1.7. Cell invasion assay by crystal violet staining of invaded cells .......65
5.1.8. Phalloidin staining of F-actin ...............................................65
5.1.9. Statistical analysis .................................................................66
5.2. The atypical Protein Kinase C small molecule inhibitor ζ-Stat, and its effects
on invasion through decreases in PKC-ζ protein expression .............66
5.2.1. Antibodies and reagents ..................................................................................................... 66
5.2.2. Analysis of somatic gene mutations for ovarian cancer, CCOC and CCOC cell lines ................................................................. 67
5.2.3. Computational analysis of aPKCS ................................................................................. 68
  5.2.3.1. Protein preparation .................................................................................. 68
  5.2.3.2. Molecular dynamics ............................................................................ 69
  5.2.3.3. Consensus docking ............................................................................. 70
  5.2.3.4. Rigid receptor docking (RRD) .................................................. 71
  5.2.3.5. Induced fit docking (IFD) ................................................................ 71
  5.2.3.6. Quantum polarized ligand docking (QPLD) .................................. 72
  5.2.3.7. Molecular mechanics and generalized born surface area (MM/GBSA) ................................................................. 73
  5.2.3.8. Virtual target screening .................................................................... 74
5.2.4. Cell culture .................................................................................................................. 74
5.2.5. Atypical PKC expression during rapid growth and cell cycle arrest ....... 75
5.2.6. Preliminary screening of TOV21G and ES-2 with ICA-1S and ζ-Stat ...... 75
5.2.7. Cell viability assay .............................................................................................. 75
5.2.8. Cell lysate collection ......................................................................................... 76
5.2.9. Western Blot analysis ......................................................................................... 76
5.2.10. Endpoint PCR ................................................................................................. 77
5.2.11. Rac1 activation assay ....................................................................................... 77
5.2.12. Preparation of cytoplasmic and nuclear extracts ........................................ 78
5.2.13. Fluorescent microscopy .................................................................................... 78
5.2.14. Invasion assay ................................................................................................. 79
5.2.15. ζ-Stat in-vivo .............................................................................................. 79
5.2.16. In-vivo tumor fractionation ......................................................................... 80
5.2.17. Statistical analysis ............................................................................................. 80

References .......................................................................................................................... 82

Appendix A ......................................................................................................................... 93
List of Figures

Figure 1.1  Backbones of phorbol esters, DAG and phosphatidylserine. ..............................2
Figure 1.2  PKC enzyme activation and domains. .................................................................3
Figure 1.3  Atypical PKC are downstream of PI3K pathway: ..............................................8
Figure 1.4  String interaction network of cellular polarity complex via Rho GTPase interaction with aPKC/Par/Par3 ..............................................................10
Figure 1.5  Ect2 functions and activation of the Rho GTPases Cdc42, RhoA and Rac1.........11
Figure 2.1  Western Blot and immunohistochemistry analysis of PKC-ζ expression in normal and malignant tissues.................................................................19
Figure 2.2  The non-linear relationship between E-Cadherin expression and PKC-ζ expression and the E-Cadherin ratio measured by tumor nuclear grade in IDC.................................................................20
Figure 2.3  PKC-ζ knockdown retards invasion of MDA-MB-231 breast cancer cells ........22
Figure 2.4  Organization of F-actin upon the knock down of PKC-ζ. ..................................23
Figure 3.1  Atypical PKC inhibitors.....................................................................................27
Figure 3.2  PKC-ζ and PKC-ι protein expression in rapidly growing and serum starved cells and the effects of ICA-1S and ζ-Stat on PKC-ζ and PKC-ι protein expression in SHT290, TOV21G and ES-2 CCOC cell lines ..................31
Figure 3.3  Computational modeling results .................................................................33
Figure 3.4  Cell proliferation and viability of SHT290, TOV21G and ES-2 CCOC cell lines ..................................................................................................................38
Figure 3.5  Effects of ζ-Stat on Ect2 localization and RhoA protein and genomic expression ..........................................................39

Figure 3.6  Localization of Ect2 .................................................................................................................................40

Figure 3.7  PKC-ζ regulates invasion and migration of TOV21G ovarian cancer ..........................41

Figure 3.8  Effects of ζ-Stat on in-vivo xenografts with athymic nude mouse models ..........43

Figure 3.9  ζ-Stat decreases the amount of PKC-ζ, therefore decreasing the scaffold of Ect2 in the cytoplasm ..........................................................44

Figure S3.1  RMSD (Bottom) of backbone atoms and Radius of Gyration (Rg, Top) of all atoms graphs for PKC-ι Molecular Dynamics simulation .........................46

Figure S3.2  RMSF (Root Mean Square Fluctuation) plot of PKC-ι Molecular Dynamics simulation .................................................................47

Figure S3.3  RMSD (Bottom) of backbone atoms and Radius of Gyration (Rg, Top) of all atoms graphs for PKC-ζ Molecular Dynamics simulation .........................48

Figure S3.4  RMSF (Root Mean Square Fluctuation) plot of PKC-ζ Molecular Dynamics simulation .................................................................49

Figure S3.5  Raw Western blot images for TOV21G and SHT290 Figure 3.2A ..................50

Figure S3.6  Raw Western blot images for ES-2 Figure 3.2A ..................................................50

Figure S3.7  Raw Western blot images for TOV21G Figure 3.2B ..................................................51

Figure S3.8  Raw Western blot images for ES-2 Figure 3.2B ..................................................51

Figure S3.9  Raw Western blot images for TOV21G Rac1 PD utilizing GST PAK-PBD Figure 3.7D .................................................................52

Figure S3.10  Raw Western blot images for TOV21G RhoA and PKC-ζ protein decrease ....52

Figure 4.1  PIK3CA and ARID1A mutations in clear cell ovarian carcinoma ..................57
List of Tables

Table 1.1  Sequence identities match between PKC-ι and PKC isoforms in First zinc finger and catalytic domain.................................................................5

Table 2.1  The selection of breast specimens with pathological characteristics..................14

Table 2.2  The status of PKC-ζ in malignant and healthy breast tissue............................16

Table 2.3  Chi-square statistical analysis results of normal and malignant breast tissue. ......17

Table 2.4  Top mutated genes in all breast cancer types...................................................21

Table 3.1  Six most common gene mutations in all ovarian cancers.................................29

Table 3.2. Somatic mutations in CCOC and CCOC cell lines............................................30
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>Serine Threonine Kinase 1</td>
</tr>
<tr>
<td>ALKP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical PKC</td>
</tr>
<tr>
<td>ARID1A</td>
<td>AT-Rich Interaction Domain 1A</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5’-triphosphate</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BME</td>
<td>Basement membrane extracts</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-Raf proto-oncogene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>CCOC</td>
<td>Clear cell ovarian carcinoma</td>
</tr>
<tr>
<td>CDC42</td>
<td>Gene name for Cdc42</td>
</tr>
<tr>
<td>Cdc42</td>
<td>Cell division control protein 42 homolog</td>
</tr>
<tr>
<td>CDH1</td>
<td>Gene name for E-cadherin</td>
</tr>
<tr>
<td>COSMIC</td>
<td>Catalogue of Somatic Mutations in Cancer database</td>
</tr>
<tr>
<td>CRIB</td>
<td>Cdc42/Rac interactive binding</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in-situ</td>
</tr>
<tr>
<td>Ect2</td>
<td>Epithelial cell-transforming Sequence 2</td>
</tr>
<tr>
<td>ECT2</td>
<td>Gene name for Ect2</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ES-2</td>
<td>The ES-2 cell line was established from a surgical tumor specimen taken from a 47 year old black woman. The tumor was described as a poorly differentiated ovarian clear cell carcinoma. Initially, the cells were grown in soft agar. The cells exhibit low to moderate resistance to a number of chemotherapeutic agents including doxorubicin, cisplatin, carmustine, etoposide and cyanomorpholinodoxorubicin (MRA-CN). ES-2 cells express low levels of P glycoprotein.</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
</tbody>
</table>
FBS Fetal bovine serum
FOXL2 Forkhead box protein L2
GBSA Generalized born surface area
GDP Guanosine diphosphate
GEF Guanine exchange factor
GLU Glucose
GTP Guanosine triphosphate
HGSOC High grade serous ovarian carcinomas
ICA-1S 5-amino-1-((1R,2S,3R,4R)-2,3-dihydroxy-4-(hydroxymethyl)cyclopentyl)-1H-imidazole-4-carboxamide
ICA-1T Phosphorylated form of ICA-1S
IDC Invasive ductal carcinoma
IFD Induced fit docking
IKK Inhibitor of Kappa B Kinase
ILC Invasive lobular carcinoma
IP Immunoprecipitation
IκB Inhibitors of NF-kB
IκKαβ IκB kinase
IKARAS Kirsten Ras oncogene homolog
LCIS Lobular carcinoma in-situ
LN Lymph node
MD Molecular dynamics
MDA-MB-231 The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories. MB stands for Metastasis Breast Cancer, MDA stands for M.D. Anderson (were cell line was derived).
MM Molecular mechanics
mTOR Mechanistic Target Of Rapamycin Kinase
NA Not available
NFκB Nuclear factor kappa-light-chain-enhancer of activated B
NG Nuclear grade
NS Non-serous
PAK p21 activating kinase
Par3 Partitioning defective 3
Par6 Partitioning defective 6
PARP Poly(ADP-ribose) polymerase
PB1 Phox and Bem1 domain
PCR Polymerase chain reaction
PDB Protein data bank
PDZ Acronym for Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1)
PI3K Phosphoinositide 3-kinase
PIK3CA Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PKC Protein kinase c
SHT-290 Cell line SHT290 derived by introduction of the human telomerase reverse transcriptase (hTERT) into normal human endometrial stromal cells. Cells function like normal endometrial stromal according to criteria of proliferation, karyotype, cellular localization of cytoskeletal markers and nuclear staining, and basal gene expression based on microarray analysis. The development and characterization of these cells was reported in a publication. These cells function like normal human endometrial stromal cells in their support of normal endometrial epithelial cell function in vitro that mimics the functions of the epithelial cells in vivo.

TOV21G Grade 3, stage III, primary malignant adenocarcinoma; clear cell carcinoma. This cell line was initiated in October of 1991 from a patient of French-Canadian descent with no family history of ovarian cancer.
Abstract

Research has demonstrated that the atypical protein kinase C-zeta (PKC-ζ) is a component of many dysregulated pathways in breast and ovarian cancer, including cellular proliferation, survival, and cell cycle upregulation. Breast and ovarian cancers affect women every day and are second and fifth leading cause of cancer death. Women who seek treatments are commonly met with invasive surgeries or chemotherapy. Protein kinase C (PKC) is a family of serine and threonine phosphorylating kinases that have been shown to modulate and transduce signaling cascades that play roles in the development and survival of cancers. Atypical PKC (aPKC), have been heavily suggested to participate in phosphoinositide 3-kinase (PI3K) misregulated cancers (such as breast and ovarian) and inhibition of these pathway branches have demonstrated decreases in malignant behaviors. In our studies, we investigated (1) PKC-ζ protein in breast tissue to evaluate its potential as a biomarker for breast cancer invasion and (2) the effects of the atypical PKC-ζ inhibitor ζ-Stat on ovarian cell lines (TOV21G and ES-2) to determine the outcomes on proliferation and cellular invasion. In breast cancer tissues, we showed that an overexpression of PKC-ζ protein can be indicative of carcinogenesis. We also evaluated the invasive behavior of MDA-MB-231 breast cancer cells upon the knockdown of PKC-ζ with a Transwell invasion assay and an immunofluorescent probe for filamentous actin (F-actin) organization. In ovarian cancer, we utilized clear cell ovarian carcinoma cells (CCOC) because this subtype of ovarian cancer represents 5% of incidence, presents unique pathological features, has poor prognosis and has a high reoccurrence rate after treatment. Overall, our results showed that PKC-ζ is linked to
proliferation and invasion in both breast and ovarian cancer. These results illustrate that PKC-ζ plays a role in invasion pathways through the Ras-related C3 botulinum toxin substrate 1 (Rac1) and Ras homolog gene family member A (RhoA). Additionally, our results showed that TOV21G tumor growth in athymic female mice was decreased when treated with the PKC-ζ inhibitor, ζ-Stat. These data suggest that PKC-ζ is a biomarker and a novel target in the carcinogenesis of breast and CCOC and its inhibition by way of ζ-Stat decreased the rate of proliferation, tumor growth and expression of invasive protein pathways.
Chapter 1: Protein Kinase C

1.1. Introduction

There are many routes to cancer development and evolution. Healthy functioning intracellular signaling proteins make cellular homeostasis and regulation possible. However, when these signaling cascades become irregular, small changes can occur in the cellular environment. Over time, these aberrations can accumulate and transform a functioning “normal” cell into a tumorigenic cell.

In the early 1980s, protein kinase C became a focus of interest in tumorigenesis. Phorbol esters had long been of interest in cancer development. Phorbol esters were shown to promote tumorigenesis in mice via prolonged topical treatment to the skin; however the proteins that relayed the messages from these natural products remained aloof. The Protein Kinase C (PKC) enzymes were first identified by Nishizuka and collaborators; it was five years later that the proteins were discovered to be activated by phorbol esters which. This discovery launched a drug discovery movement with the PKC family in its scope.

1.2. PKC isoforms, activation mechanisms and domains

PKCs are an enzymatic family of proteins that have been found to be a component in cancer progression. These proteins are phosphorylated on serine and threonine residues and are generally
activated by secondary messengers such as diacylglycerol (DAG), calcium (Ca2+) and phorbol esters. PKCs are also activated when they localize to the cellular membrane and interact with phosphatidylserine (PS). Phorbol esters are an analog of diacylglycerols, which also have similar structures to phospholipids such as phosphatidylycerin (Figure 1.1).

![Phorbol ester, Diacylglycerol, Phosphatidylserine](image)

**Figure 1.1** Backbones of phorbol esters, DAG and phosphatidylserine.

Activation of these proteins varies based on isoform specific domains. There are three isoform classifications within the PKC family: conventional PKC-α, PKC-βI and PKC-βII (splice variant) and PKC-γ; novel PKC-δ, PKC-ε, PKC-η and PKC-θ; and the atypical PKC-ζ and PKC-ι/λ. DAG, calcium and PS, activates the conventional PKC isoforms while the novel PKC isoforms are only
activated by DAG and PS and are calcium insensitive (Figure 1.2). The atypical PKC are both DAG and calcium insensitive but can be activated by PS as well as Partitioning Defective 6 (Par6) complexing \(^4\). The unique regulation of the different classifications of this protein family is determined on the presence or absence of domains and motifs.

![Figure 1.2 PKC enzyme activation and domains. The three subgroups of the PKC family are conventional, novel and atypical. While all three groups have a regulatory and catalytic domain, there is great variation between the domains and hinge regions homology. Phospholipids, calcium and DAG are the secondary messengers that activate these proteins \(^5,6\). The V1-5 represent the variable regions of the proteins. Note: Data for this figure was taken from references 5 and 6.](image)

The diverse domains of the PKC family of enzymes perform many tasks in regulation, cellular localization and phosphorylation. The C1 and C2 domains (regulatory) position the PKC on the inner leaflet of the plasma membrane and are the portion of the protein where DAG and calcium activate the protein, respectively \(^4\). Phorbol esters create a response by involvement with the twin conserved zinc fingers C1A and C1B \(^4\). Adenosine triphosphate (ATP) binds to the C3 domain,
and the catalytic kinase site has three phosphorylation sites known as the activation loop residue, turn motif and hydrophobic site.

Furthermore, the different regions of PKC proteins are what give it unique characteristics and behavior. The pseudo-substrate site (ψ-substrate), which is a part of the regulatory domain, mimics a substrate consensus sequence for PKC except instead of having a serine/threonine to phosphorylate, it has an alanine (Figure 1.2). This site binds to the catalytic kinase domain of the protein, folding the protein in half and causing its deactivation. The V3 region is the hinge at which the protein folds when inactivated or when the pseudo-substrate is bond to the catalytic domain. The C1 domain is cysteine rich, has two repeats and participates in protein-protein interaction (possibly with G proteins). The C2 domain mediates intermolecular interactions, is a β sandwich (two sheets of four components) and binds negatively charged phospholipids, such as PS. The C3 and C4 domain are the ATP and substrate binding sites as well as the kinase activity site. The V5 region is highly flexible and contains an important posttranslational phosphorylation site within the PKC and is needed to make the protein mature.

1.3. Atypical PKC as novel targets for cancer therapy

The PKC family members have been found to regulate many malignant pathways and play a significant role in cancer development. In particular, the atypical isoforms (PKC-ι and PKC-ζ) have been suggested to participate in oncogenesis at varying levels and tissue specificity. In addition, the aPKCs have been linked to cancer progression in non-small cell lung, epithelial ovarian, breast, prostate and colon carcinomas. Interestingly, many PKC isoforms are located on different chromosomes, apart from PKC-ι and PKC-δ (located on the long arm of chromosome...
three). This trend holds true as far as the atypical which are located on separate chromosomes (PKC-ι on 3q26 and PKC-ζ on 1p36) and have been noted to perform non-redundant tasks. A comparison of sequence identities between the most conserved amino acid domains of PKC isoforms illustrates the closest homology of PKC-ι and PKC-ζ (77% in the first zinc finger and 84% in the catalytic domain) (Table 1.1) 17.

Table 1.1 Sequence identities match between PKC-ι and PKC isoforms in first zinc finger (C1α) and catalytic domain. The table represents a homology comparison of the two most conserved domains in the PKC isoforms with PKC-ι. The genomic location of PKC isoforms was found with the GeneCards Human Gene Database 17,18. Note: Data for this table was used from references 17 and 18.

<table>
<thead>
<tr>
<th>PKC isoform</th>
<th>First zinc finger</th>
<th>Catalytic domain</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>ι</td>
<td>100</td>
<td>100</td>
<td>3q26.2</td>
</tr>
<tr>
<td>α</td>
<td>40</td>
<td>50</td>
<td>17q24.2</td>
</tr>
<tr>
<td>β</td>
<td>40</td>
<td>52</td>
<td>16p12.2</td>
</tr>
<tr>
<td>γ</td>
<td>39</td>
<td>48</td>
<td>19q13.42</td>
</tr>
<tr>
<td>δ</td>
<td>32</td>
<td>48</td>
<td>3p21.1</td>
</tr>
<tr>
<td>θ</td>
<td>34</td>
<td>44</td>
<td>10p15.1</td>
</tr>
<tr>
<td>ε</td>
<td>39</td>
<td>53</td>
<td>2p21</td>
</tr>
<tr>
<td>η</td>
<td>38</td>
<td>51</td>
<td>14q23.1</td>
</tr>
<tr>
<td>ζ</td>
<td>77</td>
<td>84</td>
<td>1p36.33</td>
</tr>
</tbody>
</table>

Although the genes PRKCI and PRKCZ (PKC-ι and PKC-ζ) are not commonly mutated in cancer, they have been found to be overexpressed. The long arm of the third chromosome has been found to be a frequent amplicon and driver in epithelial malignant tissue growth 19. Genes that are found overexpressed on the 3q26 portion of chromosome three are ECT2, SOX2, PRKCI and PIK3CA 19. In an investigation by Fields et al., the authors found that cancers affected by the copy number gains of the 3q26 amplicon were most abundant in lung squamous, esophagus, ovarian, cervix and head and neck squamous cell carcinoma 19. Furthermore, PKC-ι overexpression has
been observed in several cancers including melanoma, neuroblastoma and prostate \textsuperscript{11,20,21}. This overexpression of aPKC is another reason that research has searched for inhibitors specific to PKC.

1.3.1. PKC inhibitors

For the last 20 years, PKC-specific inhibitors has been a goal of academia and industrial research. Three modes of inhibition have made great strides and include (a) competitive small molecule inhibitors for the ATP site, (b) activators that mimic phorbol esters that bind to the C1 domain and (c) peptides that disrupt protein-protein interactions \textsuperscript{3}. The pan-PKC inhibitor Staurosporine is the most studied ATP binding small molecule and it binds all isozymes (due to high homology in the ATP site) \textsuperscript{3}. In addition, a naturally occurring macro lactone called Bryostatin has shown anti-cancer properties and clinical relevance \textsuperscript{3,22}. Bryostatin is known to mimic DAG binding in the C1 domain of PKC isoforms and reduce proliferation \textsuperscript{3,22}. However, the results from clinical trials for PKC inhibitors have been mixed and challenging \textsuperscript{3}. New inhibitor development for this protein family is essential for the molecular targeting of PKC, cancer treatment and chemosensitization.

With an emphasis on aPKC small molecule inhibitors, ICA-1 and ζ-Stat have been studied. ICA-1 was first published in cancer treatment in 2011 by Pillai \textit{et al.} These investigators determined through \textit{in-silico} molecular docking that ICA-1 was specific to PKC-ι and bound to the amino acids 469-475 (glutamine, isoleucine, arginine, isoleucine, proline, arginine and serine) \textsuperscript{21}. Further studies have shown that ICA-1 decreased the proliferation and invasion of neuroblastoma, glioblastoma, melanoma, prostate and colorectal cells \textsuperscript{10,20,21,23,24}. Shortly after ICA-1 emerged, ζ-Stat soon came into view as a PKC-ζ specific inhibitor and showed great
potential in disrupting aberrant PKC pathways \cite{10,20}. Furthermore, studies of the combination of these small molecule inhibitors with FDA approved drugs have begun to surface and show excellent promise. The combination of 5-fluorouracil (chemotherapeutic drug) and ζ-Stat significantly reduced the growth and migration of colorectal cells and caused a synergistic effect for DNA fragmentation \cite{25}. The drugs ICA-1 and ζ-Stat should continue to be developed due to their continued relevance to cancer treatment.

1.4. With a closer look a PKC-ζ

Although PKC-ι has been greatly studied and is characterized as an oncogene, the roles PKC-ζ are still under dispute. Research has shown that PKC-ζ behaves as proapoptotic tumor suppressor as well as an oncogenic driver \cite{4,26}. As mentioned before, PKC-ι is on the common epithelial amplicon 3q26; however, PKC-ζ is on the short arm of the first chromosome. An investigation by Ragnarsson et al. showed that in 683 human solids tumors from different locations in the body, 369 (54\%) demonstrated a loss of heterozygosity in the distal portion of chromosome 1p \cite{27}. These high percentages of loss of heterozygosity were seen in the 1p36, 1p35-p34, 1p32 and 1p31 regions \cite{27}. A loss in heterozygosity is common in cancer development and means that there is a somatic loss of a wild-type allele for a tumor suppressor gene \cite{28}. These data indicate that the genomic location of PRKCZ (1p36.33) may make this gene susceptible to loss of heterozygosity and the loss of its tumor suppressor function, therefore transforming this protein into an oncogene.

Moreover, PKC-ζ (and PKC-ι) has been shown to be down stream of the phosphoinositide 3-kinase (PI3K) signaling pathway. This pathway is one of the most altered pathways in human cancer and is the focus of many targeted inhibitors \cite{29}. PKC-ζ resides in a branch of the PI3K
pathway that controls signaling cascades related to apoptosis, motility and proliferation (Figure 1.3). Previous studies have shown that the inhibition of PKC-ζ activity or PRKCZ gene knockdown caused significant decreases in cell proliferation, movement and death\textsuperscript{10,30,31}. In support, research performed by Islam et al. demonstrated that the treatment of colorectal cells with the PKC-ζ novel inhibitor ζ-Stat significantly decreased the wound healing and cell viability\textsuperscript{10}. Research continues to reiterate the importance of targeting PKC-ζ to shut down these transduction pathways and decrease a tumor’s overall malignancy.

![Figure 1.3](image)

**Figure 1.3** - Atypical PKC are downstream of PI3K pathway. The atypical PKC are downstream of the PI3K signaling cascade and can regulate phenotypic responses such as invasion, tumor growth and survival.

### 1.4.1. PKC-ζ in invasion pathways

Additionally, PKC-ζ has been implicated in migration and invasion specific pathways. PKC-ζ has been noted to regulate cell invasion through indirect actin reorganization and RhoA family activation. Rho family GTPases (i.e. Rac1, Cdc42 (Cell division control protein 42 homolog), RhoA) are activated through the Par6/Par3/aPKC complex. Par6 and Par3 (Partitioning defective 3) are scaffolding proteins that complex with one another due to the presence of their PDZ domain\textsuperscript{32}. This complex is further known to interact with the aPKC through the unique PB1 (Phox and
Bem1) domain. The CRIB (Cdc42/Rac interactive binding) domain (located in proximity of the Par6 PB1 domain) further interacts with GTP (guanosine triphosphate) bound Rho GTPases such as Cdc42 and Rac1. Analysis by the STRING network database strengthens this argument. This database simplifies access to protein-protein interactions (either direct or indirect) by collecting high-throughput experimental data, mining databases and literature and utilizing predictions from genomic context analysis. According to this database, the assessment of this web of proteins demonstrated (a) a high potential of association for RHOA (RhoA), RAC1 (Rac1), CDC42 (Cdc42), PRKCI (PKC-ι), PRKCZ (PKC-ζ), PARD6A (Par6) and PARD3 (Par3) and (b) that these proteins play a role in cellular polarity and junction assembly (Figure 1.4).

Cell polarity is essential in invasion and migration. Some normal functions of cell migration are wound healing and embryotic development; however abnormal behavior in these biological processes can cause tumor metastasis. The two types of migration are defined as (a) random migration where cells migrate in all directions and (b) directed migration where cells migrate in a given direction. In both instances, the front and rear axis of the cell are polarized by actin reorganization and polymerization. Cdc42, Rac and RhoA regulate this cytoskeleton rearrangement and can make actin filament-based structures assemble and disassemble (including filopodia, invadopodia and lamellipodia). These Rho GTPases are activated by the guanine exchange factor (GEF) epithelial cell transforming 2 protein (Ect2). This process involves the Ect2 protein exchanging the GDP (guanosine diphosphate) for a GTP in the Rho GTPases. Once activated, the conformational changes (between inactive and activate states) in the Rho proteins allows for the binding of the CRIB domain to further signaling molecules, such as p21 activating kinase (PAK) and Par6.
Figure 1.4 String interaction network of cellular polarity complex via Rho GTPase interaction with aPKC/Par/Par3. This is a depiction of an interaction network derived from STRING v11. The inputted protein names were RHOA (RhoA), RAC1 (Rac1), CDC42 (Cdc42), PRKCI (PKC-ι), PRKCZ (PKC-ζ), PARD6A (Par6) and PARD3 (Par3). This interaction network had 7 nodes, 21 edges, 6 average node degrees and a PPI enrichment p-value of $2.22 \times 10^{-15}$. This means that the inputted proteins have more predicted interactions among themselves than what would be expected for a random set of proteins of similar size, drawn from the genome. Such an enrichment indicates that the proteins are at least partially biologically connected, as a group. Analyses of (a) establishment or maintenance of cell polarity (red nodes) and (b) cell junction assembly (blue nodes) were also performed with a false discovery rate (FDR) of $2.80 \times 10^{-9}$ and $1.28 \times 10^{-7}$, respectively. Note: Data for this figure was taken from reference 34.

Additionally, Ect2 plays different roles in normal and malignant cells. Ect2 has many normal functions, including cytokinesis, where it is found in the nucleus. However, in transformed malignant cells, Ect2 is mislocalized and is commonly found in the cytosol where it participates in migratory pathways (Figure 1.5)\textsuperscript{15,41}. The investigators Fields et al. found that Ect2 was an oncogene in human cancer and participated in the Par6/Par3/aPKC polarity complex\textsuperscript{42–44}. The mislocalization of Ect2 via the Par6/aPKC scaffold allowed for increased Ect2 activation of cytosolic Rac1\textsuperscript{44}. In Ect2-deficient non-small cell lung cancer cells (by RNAi), the authors found that Rac1 activation, anchorage-independent growth and invasion were all inhibited\textsuperscript{44}. Their
findings suggest that oncogenic Ect2 inhibition via the disruption of the Par6/Par3/aPKC complex may decrease the aggressive behavior of cancer cells and decrease the chances of later stage metastases.

Figure 1.5 Ect2 functions and activation of the Rho GTPases Cdc42, RhoA and Rac1. In normal cells the GEF Ect2 plays a role in processes such as cytokinesis; however in transformed malignant cells, Ect2 has been shown to play roles in migration and invasion. Ect2 exchanges the GDP for a GTP in Rho GTPases such as RhoA, Cdc42 and Rac1.
2.1. Introduction

In the current research, cancer statistics show that invasive breast cancer is projected to have 234,190 new cases and deaths, for which 231,840 will be women. Approximately 40,290 women will die from invasive breast cancer in 2018. Although the percentage of mortalities has decreased over last few years, breast cancer still ranks as the second leading cause of cancer death in women; treatments usually entail invasive surgeries, including breast-conserving surgeries and mastectomies. From a clinical standpoint, invasive ductal carcinoma is the most common form of breast cancer, affecting 50-75% of breast cancer diagnoses. Breast cancer is a heterogeneous disease, and research has shown that the top mutated genes in breast carcinomas and carcinomas in-situ are PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), TP53 (Tumor Protein P53), and CDH1 (E-Cadherin). However, this investigation shows a link between PKC-ζ protein overexpression and breast cancer development, particularly in invasive behavior. For this reason, breast cancer biomarkers are of interest, as they may help to predict breast cancer incidents and contribute to better therapeutic regimens.
The complete understanding of histological and pathological features of the lobular and ductal carcinomas are far from full elucidation. In an investigation by Ruibal et al., the authors concluded that, in the absence of the axillary node, lobular carcinomas had a higher concentration of Breast cancer estrogen-inducible protein (pS2) than ductal carcinomas. Lobular carcinomas were also shown to have a higher frequency of diploidy, which suggests that lobular carcinomas are less aggressive and grow slower.

An overexpression of PKC-ζ protein promotes carcinogenesis by stimulating cancer cell proliferation through pathways such as the Nuclear Factor Kappa B (NF-κB), which plays an essential role in cancer initiation and progression. Previous studies suggest that PKC-ζ is a regulatory factor for the nuclear translocation of NF-κB that in turn represses E-Cadherin. Although the link between the loss of E-Cadherin and cancer prognosis remains ambiguous, recent findings showed that E-Cadherin possesses a vital tumor suppressive role. Moreover, some researchers have paralleled PKC-ζ to the phosphorylation of the Inhibitor of Kappa B Kinase (IKK) complex, which in turn phosphorylates the Inhibitor of Kappa B (IκB) and triggers IκB degradation. The degradation of IκB releases NF-κB, allowing its translocation from the cytosol into the nucleus, where it functions as a transcription factor. Furthermore, studies show that NF-κB also plays a role in epithelial to mesenchymal transition, a crucial carcinogenic event.

PKC-ζ has also been linked to metastatic behaviors of cancer cells. In a study by Islam et al., the Rac1/Pak1/β-Catenin signaling cascade in colorectal cancer cell lines was evaluated after the inhibition of PKC-ζ. The knockdown of PKC-ζ decreased the nuclear translocation of β-Catenin which ultimately lead to reduced colorectal cell proliferation and metastasis. These data were...
further supported by another investigation performed by Wu et al., which determined that inhibition of PKC-ζ in breast cancer cell lines decreased adhesion and actin polymerization. These studies advocate the theory that PKC-ζ is a critical component of the invasive behaviors of cancer cells.

Although PKC-ζ has been studied in invasive breast cancers (most commonly ductal), there have been no examinations of PKC-ζ expression in other types of breast cancer (such as carcinomas in-situ). An investigation by Lin et al. confirmed that atypical protein kinase C isoforms were elevated in breast cancer tissues (invasive ductal carcinoma, specifically) when compared to adjacent healthy breast tissue. Additionally, Schöndorf et al. determined that antineoplastic agents affected the activation of PKC in invasive ductal carcinoma breast cancer tumors. In our study, the focus was to further investigate the PKC-ζ expression profile in the four histological subtypes of breast cancer such as lobular carcinoma in-situ (LCIS), invasive lobular carcinoma (ILC), ductal carcinoma in-situ (DCIS) and invasive ductal carcinoma (IDC). We also evaluated the difference in the PKC-ζ expression among healthy, invasive and non-invasive tissues. Moreover, the invasive characteristics of MDA-MB-231 breast cancer cells were examined upon the inhibition of PKC-ζ. We found that PKC-ζ was overexpressed in IDC and ILC tissue specimens compared to other subtypes. In addition, the inhibition of PKC-ζ decreased the invasion of MDA-MB-231 breast cancer cells.

Table 2.1 The selection of breast specimens with pathological characteristics. Tissue specimen were selected as described in material and methods and further sorted by the presence of PKC-ζ protein. *LN=lymph node, ER= estrogen receptor, PR= progesterone receptor, LCIS= lobular carcinoma in-situ, ILC=invasive lobular carcinoma, IDC=invasive ductal carcinoma, DCIS=ductal carcinoma in-situ, NA=not available.
| NA | NA | NA | Normal | Black | no | 9  |
| NA | NA | NA | Normal | NA    | no | 4  |
| NA | NA | NA | Normal | White | no | 15 |
| NA | NA | NA | Normal | White | yes | 4  |
| NA | NA | NA | LCIS   | Black | no | 1  |
| +  | II/III | +  | +     | LCIS  | White | yes | 1  |
| NA | NA | NA | ILC    | Black | no | 1  |
| -  | II/III | NA | NA     | ILC   | NA | no | 1  |
| NA | NA | NA | ILC    | NA    | no | 1  |
| -  | II/III | +  | +     | ILC   | NA | no | 1  |
| NA | NA | NA | ILC    | NA    | no | 1  |
| -  | II/III | +  | +     | ILC   | White | no | 1  |
| NA | NA | NA | ILC    | NA    | no | 1  |
| +  | II/III | +  | +     | IDC   | Black | yes | 1  |
| NA | NA | NA | IDC    | NA    | no | 1  |
| -  | II/III | +  | +     | IDC   | NA | no | 1  |
| NA | NA | NA | IDC    | NA    | no | 1  |
| -  | II/III | NA | NA     | IDC   | NA | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | no | 1  |
| NA | NA | NA | IDC    | NA    | no | 1  |
| -  | III/III | NA | NA    | IDC   | NA | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | NA | yes | 2  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
| -  | III/III | NA | NA    | IDC   | White | yes | 1  |
| NA | NA | NA | IDC    | NA    | yes | 1  |
2.2. Results

2.2.1. PKC-ζ is overexpressed in malignant breast tissues

PKC-ζ protein expression was measured in breast samples with no tumor complication and malignant breast tissue samples by Western Blot analysis. Tissue specimen were selected as described in material and methods and further sorted by the presence of PKC-ζ protein (Table 2.1). Our investigation showed a correlation between the overexpression of PKC-ζ and malignant breast cancer tissue (Figure 2.1A, 2.1B; Table 2.3A). It was challenging to obtain DCIS ($N=6$) and LCIS ($N=3$) since these tissue types have lower occurrence rates; hence the results reflect a comparison of the two main subtypes: IDC, $N=29$, (50-75% diagnoses) and ILC, $N=13$ (10-15% diagnoses). The Westerns represent the majority of samples. Normal tissue only had 3 of 32 samples with expression of PKC-ζ and DCIS only had 1 of 6 samples with PKC-ζ present (Table 2.2).

Table 2.2 The status of PKC-ζ in malignant and healthy breast tissue. Healthy breast tissue was obtained from breast reductions or the area adjacent to a patient’s malignant tumors. Malignant tumors were either LCIS, ILC, DCIS, or IDC. The expression of PKC-ζ was evaluated as not present (ratio 0), weakly present (ratio < 0.01) and positively present (ratio > 0.01).

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Not present</th>
<th>Weakly present</th>
<th>Positively present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal tissue</td>
<td>29</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Lobular carcinoma in-situ</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>8</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Ductal carcinoma in-situ</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>6</td>
<td>5</td>
<td>18</td>
</tr>
</tbody>
</table>

Among the IDC subtypes of malignant breast tissue, over 74% of tissue samples overexpressed PKC-ζ protein. Less than 5% of healthy breast tissue samples exhibited PKC-ζ protein expression.
Although a paired Student's t-test \((p < 0.05)\) and one-way ANOVA \((p < 0.01)\) showed no significant correlation between the overexpression of PKC-ζ protein in malignant tissue (all four subtypes) and healthy tissue, it did demonstrate a significant correlation between healthy breast tissue and IDC (Tukey HSD p-value 0.0056404, \(p < 0.01\); Scheffé p-value 0.0121289, \(p < 0.05\); Bonferroni and Holm p-value 0.0063104, \(p < 0.01\)) (Figure 2.1B). According to the significance of the contingency table (Table 2.3A), there is an established association between the protein expression of PKC-ζ and sample type \((p < 0.00001)\). However, the PKC-ζ expression could not be statistically linked to the presence of ER (estrogen receptor) and nuclear grade (Table 2.3B, 2.3C). These data suggest that it is unlikely that healthy breast tissue samples overexpress PKC-ζ protein. Instead, PKC-ζ is overexpressed in malignant breast tissue samples.

\[
\begin{array}{lccccccc}
<table>
<thead>
<tr>
<th>\text{A}</th>
<th>\text{Normal}</th>
<th>\text{LCIS}</th>
<th>\text{ILC}</th>
<th>\text{DCIS}</th>
<th>\text{IDC}</th>
<th>\text{Row Totals}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-ζ present</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>23</td>
<td>33</td>
</tr>
<tr>
<td>PKC-ζ absent</td>
<td>29</td>
<td>2</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>Column Totals</td>
<td>32</td>
<td>3</td>
<td>13</td>
<td>6</td>
<td>29</td>
<td>83 (Grand Total)</td>
</tr>
</tbody>
</table>

**The chi-square statistic is 32.6715. The \(p\) value is < 0.00001. LCIS=lobular carcinoma in-situ, ILC=invasive lobular carcinoma, DCIS=ductal carcinoma in-situ, IDC=invasive ductal carcinoma, ER=estrogen receptor, NG1-3=nuclear grade 1-3.**

\[
\begin{array}{c|c|c|c|c|ccc}
<table>
<thead>
<tr>
<th>\text{B}</th>
<th>\text{ER+}</th>
<th>\text{ER-}</th>
<th>\text{Row Totals}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-ζ present</td>
<td>10</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>PKC-ζ absent</td>
<td>6</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td>Column Totals</td>
<td>16</td>
<td>9</td>
<td>25 (Grand Total)</td>
</tr>
</tbody>
</table>
\]
The chi-square statistic is 0.0434. The $p$-value is 0.834969. The result is not significant at $p < .10$. ER=estrogen receptor

<table>
<thead>
<tr>
<th></th>
<th>NG1</th>
<th>NG2</th>
<th>NG3</th>
<th>Row Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-ζ present</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>PKC-ζ absent</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td><strong>Column Totals</strong></td>
<td><strong>7</strong></td>
<td><strong>6</strong></td>
<td><strong>9</strong></td>
<td><strong>22 (Grand Total)</strong></td>
</tr>
</tbody>
</table>

The chi-square statistic is 0.2. The $p$-value is 0.90485. The result is not significant at $p < .10$. NG1-3=nuclear grade 1-3

2.2.2  PKC-ζ protein levels are higher in invading breast cancer subtypes

To compare the expression of PKC-ζ in non-invading tissues and invading tissues, Western Blot and immunohistochemistry were performed. Western Blot data showed that PKC-ζ protein expression was higher in IDC when compared to the ILC, LCIS and the DCIS (Figure 2.1A, 2.1B). According to immunohistochemistry findings, breast specimens were scored for the expression of PKC-ζ by the pathologist (Figure 2.1C). Normal tissue had no to moderate expression (score: 0,2), LCIS had abundant expression (score: 3,3), ILC had moderate to strong expression (score: 2,3+), DCIS had moderate to abundant expression (score: 2,3), and IDC had abundant to strong expression (score: 3,3+). The most robust expression was found to be in ILC and IDC represented by the 3+ score. However, ILC was found to have moderate to strong expression, whereas IDC was found to have abundant to strong expression. These findings suggest that PKC-ζ is found higher in invading tissues when compared to non-invading tissues and more so in IDC when compared to ILC. These data suggest that the stage between *in-situ* and invading malignancy can be correlated to an increased PKC-ζ protein expression.
Figure 2.1 Western Blot and immunohistochemistry analysis of PKC-ζ expression in normal and malignant tissues. Malignant tissues were evaluated for the expression of PKC-ζ. (A) We ran 20-30 µg of tissue lysate and probed with a PKC-ζ antibody. (B) The ratios of PKC-ζ to β-actin in healthy tissue and the four subtypes of malignant breast tissue were derived using the densitometry. The white bar (healthy) represents the control and the error bars represent the standard error. The standard deviation, a Student’s t-test ($p < 0.05$) and a one-way ANOVA was used to evaluate the Western Blot data as well with the post-hoc Tukey’s HSD test ($p < 0.01$), Scheffé multiple comparison ($p < 0.05$), Bonferroni ($p < 0.01$) and Holm ($p < 0.01$). Statistical tests showed no meaningful relationship between the healthy and LCIS, or the ILC and DCIS. The * represents a statistically relevant relationship between normal and IDC breast tissue. (C) FFPE samples were probed with PKC-ζ and stained for immunohistochemistry. The scoring was based on the abundance or lack thereof PKC-ζ expression: Score 0 no expression; Score 1 weak expression; Score 2 moderate expression; Score 3 abundant expression; Score 3 plus (+) strong expression. Image original magnification: 20X. LCIS= lobular carcinoma in-situ, ILC=invasive lobular carcinoma, IDC=invasive ductal carcinoma, DCIS=ductal carcinoma in-situ.

2.2.3 PKC-ζ and E-Cadherin levels in tissue specimens

Since the decreased expression of E-Cadherin is indicative of a more aggressive phenotype, the relationship between PKC-ζ and E-Cadherin protein levels was studied. Western Blot analysis was used in the context of probing for PKC-ζ and E-Cadherin in IDC breast tissues (Figure 2.2A). We randomly selected 8 samples out of the 29 IDC tissue specimen to illustrate the data. We could not establish a significant relationship between PKC-ζ expression and E-Cadherin expression in
IDC samples. Our linear regression test (Figure 2.2B) showed no significance ($p < 0.05$) between the expressions of PKC-ζ and E-Cadherin protein levels in 20 randomly selected IDC breast tissue samples. The $N$ value was too low to take into consideration for LCIS, ILC and DCIS. Normal breast tissues were not taken into consideration since the PKC-ζ expression was only found in 3 samples out of 32. However, our investigation established the relationship between E-Cadherin protein expression and nuclear grade diagnosis (Figure 2.2C). The Scarff-Bloom-Richardson scale data was derived from the pathology reports (summarized in Table 2.1). The results show that E-Cadherin protein expression had an inverse relationship to nuclear grade diagnosis. Even though the PRKCZ gene is not one of the top mutated genes in breast cancer (carcinomas and carcinomas in-situ), CDH1 is on the top of the list (Table 2.4) 47.

**Figure 2.2** The non-linear relationship between E-Cadherin expression and PKC-ζ expression and the E-Cadherin ratio measured by tumor nuclear grade in IDC. We investigated the expression of PKC-ζ in comparison to E-Cadherin expression in IDC diagnosed breast tissues by (A) Western Blot. The Western Blot represents 8 samples of IDC. (B) We normalized densities of PKC-ζ and E-Cadherin protein expression based on β-actin levels and plotted them on a scattered plot graph to test for a linear relationship (N=20). (C) The E-Cadherin ratios were further compared to nuclear grade diagnosis listed on the pathology report, according to the Scarff-Bloom-Richardson scale.

20
Table 2.4 Top mutated genes in all breast cancer types. This table was created after a review of the Catalogue of Somatic Mutations in Cancer database.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Product</th>
<th>Protein Function</th>
<th>Chromosome Location</th>
<th>Percent Mutation of Samples Tested (%)</th>
<th>Highest Percent Mutation (%)</th>
<th>Mutation Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIK3CA</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha</td>
<td>Phosphorylates certain signaling molecules</td>
<td>3q26.3</td>
<td>27</td>
<td>98.97</td>
<td>Substitution missense</td>
</tr>
<tr>
<td>TP53</td>
<td>p53</td>
<td>Tumor suppressor, regulates cell cycle</td>
<td>17p13.1</td>
<td>24</td>
<td>50.30, 25.20</td>
<td>Substitution missense, other</td>
</tr>
<tr>
<td>MED12</td>
<td>Mediator complex subunit 12</td>
<td>Initiation of transcription</td>
<td>Xq13</td>
<td>20</td>
<td>71.04</td>
<td>Substitution missense</td>
</tr>
<tr>
<td>CDH1</td>
<td>E-cadherin (Caderin 1)</td>
<td>Cell adhesion</td>
<td>16q22.1</td>
<td>11</td>
<td>28.07, 18.42, 17.54</td>
<td>Deletion frameshift, substitution nonsense, substitution nonsense</td>
</tr>
<tr>
<td>GATA3</td>
<td>GATA binding protein 3</td>
<td>Transcription factor</td>
<td>10p15</td>
<td>10</td>
<td>48.58, 16.60</td>
<td>Insertion frameshift, deletion frameshift</td>
</tr>
</tbody>
</table>

2.2.4 PRKCZ gene silencing decreases the invasion of breast cells

To investigate the effects of PKC-ζ inhibition on the invasive behavior of breast cancer cells, a Transwell invasion assay and immunostaining of F-actin were performed. When compared to the control, PKC-ζ knockdown decreased the invasion of breast cancer cells by 60% and was significant (Student’s t-test $p < 0.05$, one-way ANOVA Tukey HSD $p$-value 0.0029646, $p < 0.01$; Scheffé $p$-value 0.0049622, $p < 0.01$; Bonferroni $p$-value 0.0040067, $p < 0.01$ and Holm $p$-value 0.0040067, $p < 0.01$) (Figure 2.3A, 2.3B). In addition, the levels of two important components of metastatic pathways, Rac1 and RhoA, were also decreased dramatically (Figure 2.3C). Moreover,
MDA-MB-231 breast cancer cells were fixed and stained with phalloidin probe to visualize the impacts of PRKCZ gene silencing on F-actin organization. The silencing of PRKCZ caused the reorganization of F-actin around cell cytoskeleton (Figure 2.4). Taken together, these data advance the theory that PKC-ζ modulates the invasive behavior of breast cancer cells by the regulation of invasion through the Rac1/RhoA pathway and cytoskeleton filaments.

**Figure 2.3** PKC-ζ knockdown retards invasion of MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells were placed in the upper chamber of Transwell plate coated with 0.5x BME and serum containing media was placed in the lower chamber as a chemoattractant. Following transfection with siPRKCZ and scrambled RNA, cells that invaded through the BME and migrated into the lower chamber were stained with crystal violet and (A) observed under a microscope at 10X magnification (the scale bar represents 1mm). Untreated cells and cells treated with the transfection reagent (Si-Tran) were also used to establish the effect on invasion. (B) The cells were counted for each treatment image (N=3), averaged and analyzed with the standard deviation, a Student’s t-test (p <0.05) and a one-way ANOVA was also used to evaluate the data with the post-hoc Tukey’s HSD test (p < 0.01), Scheffé multiple comparison (p < 0.01), Bonferroni (p < 0.01) and Holm (p < 0.01). All statistical tests were used to determine the statistical significance of the invasion data. (C) The cells were further investigated by Western Blot to analyze the Rac1/RhoA pathway. This data was quantified and averaged for the graph with standard error of the mean represented.
Figure 2.4 Organization of F-actin upon the knockdown of PKC-ζ. MDA-MB-231 breast cancer cells were treated with SiTran, scrambled RNA and siPRKCZ for 24 hours. The cells that received no treatment were used as the control. These cells were then stained with 1X Phalloidin-iFluor 594 and counterstained with DAPI (4',6-diamidino-2-phenylindole). The scale bar represents 100 µm. Original magnification: 20X.

2.3. Acknowledgments

The authors would like to thank Noel Clark and Alexis Lopez at the Tissue Core, Moffitt Cancer Center (Tampa, FL, USA) for their assistance. Tissue samples were provided by the NCI Cooperative Human Tissue Network.
2.4 Funding

The grant code for funding of this project is the USF Foundations 42-0142.

2.5 Availability of data and materials

The datasets we used and/or analyzed during the current study are available from the corresponding authors upon reasonable request. The datasets we generated and/or analyzed during the current study are available from the COSMIC repository at https://cancer.sanger.ac.uk/cosmic.

2.6 Author’s contribution

TS contributions include tissue selection and fractionation, Western Blot analysis, densitometry analysis, statistical analyses, pathway analysis and writing. SI contributions include the invasion assay, phalloidin staining, and writing. CA contributions include preliminary Western Blot analysis. AA contributions to the paper include statistical analysis of E-Cadherin expression and nuclear grade. MD contributions to the paper include writing-review, editing, resources, supervision and funding acquisition. The authors read and approved the final manuscript.

2.7 Competing interests

The author declares that there are no conflicts of interest regarding the publication of this paper.
Chapter 3: ζ-Stat as a novel inhibitor in ovarian cancer

Note to the reader: This paper has been accepted for publication in Frontiers in Oncology: Women’s Cancer: DOI: 10.3389/fonc.2020.00209

3.1. Introduction

In 2019, it is estimated that approximately 1.7 million new incidences of cancer will be diagnosed and 600,000 cancer deaths will occur. Cancer has become the leading cause of death in 21 states, despite decreases in cancer death rates since 1991. Specifically, ovarian cancer is estimated to reach 22,530 diagnoses and cause 13,980 cancer deaths per year. This constitutes 5% of cancer deaths among women and is responsible for being the most lethal gynecological cancer diagnosis.

The most common ovarian cancer diagnosis is epithelial ovarian carcinomas (EOC), which constitutes 85-90% of diagnosis. Of this percent, clear cell ovarian carcinoma (CCOC) represents 5% of incidence and presents unique pathological features and a chemo resistant phenotype. CCOC is the third most common subtype of ovarian cancer, has a higher risk of reoccurrence, and lower survival rate. Furthermore, CCOC is characterized as a non-serous (NS) ovarian cancer and has been found to be more invasive than high grade serous ovarian carcinoma (HGSOC). It has been found that patients with NS tumors have poor prognosis. Consequently, CCOC is proposed to be associated with endometriosis and it has been suggested that the precancerous lesions from endometriosis can lead to CCOC (relative risk = 12.4). Endometrial
cancer and CCOC have been shown to have an overexpression of Protein kinase C (PKC) isoforms which play important roles in these cancers development and resistance. PKC is an enzymatic family of proteins that have been found to be a component in cancer progression. These proteins phosphorylate the serine and threonine residues of substrates and are generally activated by compounds such as diacylglycerol (DAG), calcium (Ca\(^{2+}\)) and phorbol esters. There are three classifications within the PKC family which include the conventional PKC-\(\alpha\), \(\beta\), \(\beta\text{II}\) (splice variant), \(\gamma\), the novel PKC-\(\delta\), \(\varepsilon\), \(\eta\), \(\theta\), and the atypical PKC-\(\zeta\), \(\iota/\lambda\).

The atypical PKC isoforms, PKC-\(\iota\) and PKC-\(\zeta\), have been suggested to participate in the increased proliferation of ovarian cancer. PKC-\(\iota\) has also been identified as a highly amplified gene in CCOC and is noted for its role in apical-basal polarity loss. In addition, due to mutations in the PIK3CA gene and inactivation of Phosphatase and Tensin Homolog (PTEN), the Phosphoinositide 3-Kinase (PI3K)/ Serine Threonine Kinase 1 (AKT)/ Mechanistic Target Of Rapamycin Kinase (mTOR) pathway has also been upregulated in CCOC. The upregulation of this pathway increases the expression of downstream survival targets (e.g. PKC-\(\zeta\)). PKC-\(\zeta\) has been shown to be involved in tumorigenesis, tissue invasion, and cancer progression through the modulation of cell migration machinery, such as Ras Homolog Family Member A (RhoA), Rac Family Small GTPase 1 (Rac1), and Epithelial Cell Transforming 2 (Ect2).

The ECT2 gene is highly amplified in CCOC and may increase migratory behavior. Ect2 is a Rho GTPase specific guanine nucleotide exchange factor (GEF) which activates this family of proteins by the addition of a phosphate group to Guanosine diphosphate (GDP). The overexpression of Ect2 protein promotes increased activation of the Rho GTPases, which in turn can facilitates invasion through cytoskeleton reorganization.
Previous studies have indicated that novel aPKC inhibitors ICA-1S and ζ-Stat (Figure 3.1) decreased the migratory behaviors of colorectal cancer cells and were selective for PKC-ι/λ and PKC-ζ, respectively \(^{10,20}\). These small molecule inhibitors were also shown to decrease cell viability in colorectal cancer and melanoma \(^{10,20}\).

\[
\begin{align*}
\text{ICA-1 Nucleoside analog (ICA-1S)} \\
5\text{-amino-1-((1R,2S,3R,4R)-2,3-dihydroxy-4-} \\
\text{(hydroxymethyl)cyclopentyl)-1H-} \\
\text{imidazole-4-carboxamide} \\
\text{MW: 256.26g/mol} \\
\end{align*}
\]

\[
\begin{align*}
8\text{-hydroxynaphthalene-1,3,6-} \\
\text{trisulfonic acid} \\
\text{MW: 384.34g/mol} \\
\end{align*}
\]

\[
\text{ζ-Stat}
\]

Figure 3.1 Atypical PKC inhibitors. The molecular structures and molecular weights of ICA-1S and ζ-Stat. ICA-1S was synthesized by United Chemistry Resources and ζ-Stat was distributed by the NCI.

Furthermore, computational molecular docking was performed on PKC-ι and a homology model of PKC-ζ (since there is no crystal structure available) with ICA-1S and ζ-Stat \(^{20}\). In this study, the authors suggested that ICA-1S bound to a potential allosteric pocket \(^{20}\). However, a more in-depth analysis of ζ-Stat is needed for subsequent studies. The further development of computational modeling is pivotal for drug discovery optimization and helps push these small molecule inhibitors towards a clinical setting. Computational studies can generate mechanistic
understandings of the activity these compounds present, can allow for inhibitor improvement, and can institute further signaling investigations.

It has been suggested that the distal downstream signal cascade of PI3K/aPKC pathway should be targeted due to the genotypic and phenotypic reliance of this pathway in CCOC for survival and invasion. The aims of this study were to further determine the binding mechanisms of ζ-Stat, expand on the tissue range of these compounds by investigating the effects in CCOC cell lines, investigate the therapeutic potential of ζ-Stat in CCOC, and to illustrate the disruption of invasion via the PKC-ζ signaling cascade.

3.2. Results

3.2.1. PIK3CA and ARID1A are in the top mutated genes in all ovarian tissue types and in CCOC

To understand the genetic landscape of ovarian cancer, we utilized the Catalogue of Somatic Mutations in Cancer bioinformatics database (COSMIC) 67. The results demonstrated that the top mutations in ovarian cancer overall are TP53 (p53), FOXL2 (Forkhead box protein L2), KRAS (Kirsten Ras oncogene homolog), PIK3CA (Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha), ARID1A (AT-rich interaction domain 1A) and BRAF (B-Raf proto-oncogene) (Table 3.1). The search was then refocused to only contain CCOC samples and the top two mutated genes found were PIK3CA (33%) and ARID1A (50%) (Table 3.2). These results suggest that one of the most common gene mutations in ovarian cancer and CCOC is PIK3CA, which is approximately 10% of mutated samples in all the ovarian tissue in the database. Due to
this, the downstream survival targets PKC-ζ and PKC-ι are likely to be overexpressed, amending the need for their explicit targeting.

Table 3.1 Six most common gene mutations in all ovarian cancers. The table below describes the six most common gene mutations in all ovarian cancers according to the Catalogue of Somatic Mutations in Cancer database (COSMIC). The gene name, protein product, general function, chromosomal location, percent of all samples with mutation, highest type of mutation and most common mutation type are listed.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein Product</th>
<th>Protein Function</th>
<th>Chromosomal Location (human)</th>
<th>Percent Mutation of Samples Tested</th>
<th>Highest Percent Mutation</th>
<th>Mutation Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP53</td>
<td>p53</td>
<td>Tumor suppressor, regulates cell cycle</td>
<td>17p13.1</td>
<td>46</td>
<td>55.89, 20.23</td>
<td>Substitution missense, other</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Forkhead box protein L2</td>
<td>Transcription factor</td>
<td>3q23</td>
<td>20</td>
<td>100</td>
<td>Substitution missense</td>
</tr>
<tr>
<td>KRAS</td>
<td>Kirsten Ras oncogene homolog, (KRAS proto-oncogene, GTPase)</td>
<td>Regulation of cell division</td>
<td>12p12.1</td>
<td>12</td>
<td>100</td>
<td>Substitution missense</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha</td>
<td>Phosphorylates certain signaling molecules</td>
<td>3q26.3</td>
<td>10</td>
<td>96.88</td>
<td>Substitution missense</td>
</tr>
<tr>
<td>ARID1A</td>
<td>AT-rich interaction domain 1A</td>
<td>Regulate transcription by altering chromatin structure</td>
<td>1p35.3</td>
<td>9</td>
<td>40, 38.26, 23.48</td>
<td>Substitution missense, deletion frame shift, insertion frame shift</td>
</tr>
<tr>
<td>BRAF</td>
<td>B-Raf proto-oncogene, serine/threonine kinase</td>
<td>This protein plays a role in regulating the MAP kinase/ERKs signaling pathway, which affects cell division, differentiation, and secretion.</td>
<td>7q34</td>
<td>7</td>
<td>97.63</td>
<td>Substitution missense</td>
</tr>
</tbody>
</table>
In addition, the PIK3CA mutation was used to select two cell lines that would be representative of this mutation in CCOC. Two commonly utilized cell lines, TOV21G and ES-2 were selected based on their genetic profile. While both cell lines possess a PIK3CA mutation, TOV21G has an ARID1A mutation and ES-2 has a TP53 mutation (Table 3.2).

Table 3.2 Somatic mutations in CCOC and CCOC cell lines. The table below describes the five most common gene mutations in CCOC according to the Catalogue of Somatic Mutations in Cancer database (COSMIC). The gene name and percent of samples with mutation are listed. The gene mutations for TOV21G CCOC cells and ES-2 CCOC cells are listed with specific mutation type.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Percent Mutation of Samples Tested</th>
<th>Somatic mutations in CCOC</th>
<th>Somatic mutations in cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARID1A</td>
<td>50</td>
<td>ARID1A</td>
<td>Y551fs<em>72,Q758fs</em>75, H1047Y</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>33</td>
<td>PIK3CA</td>
<td>S241F</td>
</tr>
<tr>
<td>TERT</td>
<td>17</td>
<td>K267fs<em>9, G143fs</em>4</td>
<td>G13C</td>
</tr>
<tr>
<td>TP53</td>
<td>11</td>
<td>PTEN</td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td>8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.2. Select inhibitor effects on PKC-ζ and PKC-ι protein expression

To determine which inhibitors affect PKC-ζ and PKC-ι protein expression in CCOC, Western Blots were employed with ICA-1S and ζ-Stat (Figure 3.1) as potential inhibitors. Initially, the expression of these aPKCs were investigated in rapidly growing cells (50%) and cell cycle arrested (serum free, SF) (Figure 3.2A). The density of each band was quantified using analytical software. The results showed that ICA-1S did not affect PKC-ζ or PKC-ι protein expression (Figure 3.2B); however, ζ-Stat substantially decreased the expression of PKC-ζ in TOV21G cells (p value 0.00225, F = 9.5709, t = -4.413) and ES-2 cells (not significant) but not PKC-ι protein expression.
These results suggest that ζ-Stat is selective to PKC-ζ decreased expression and could be used to interrupt the PKC-ζ pathways.

**Figure 3.2** PKC-ζ and PKC-ι protein expression in rapidly growing and serum starved cells and the effects of ICA-1S and ζ-Stat on PKC-ζ and PKC-ι protein expression in SHT290, TOV21G and ES-2 CCOC cell lines. (A) SHT290, TOV21G and ES-2 cell lines were harvested at 50% and 48 hrs after serum starvation (SF) (N=3). The membranes were probed with anti-PKC-ζ and PKC-ι to investigate protein expression. (B) TOV21G and ES-2 cell lines were treated with 3μM ζ-Stat for 24, 48 and 72 hrs. An untreated control and vehicle control (DMSO) are also illustrated. The immunoblots were probed with PKC-ζ, PKC-ι and β-actin (loading control) (N=3). (C) The raw data’s densitometry was quantified using ImageJ software and analyzed with a one-way ANOVA. Standard deviation is represented. (** p < 0.01).

### 3.2.3. *In-silico* results and model validation

#### 3.2.3.1. Model viability
RMSD and $R_g$ plots of PKC-ι (Figure S3.1) displayed asymptotic behavior beyond 7 ns. RMSD appeared to equilibrate near 2.3 Å, similar to the reported crystallographic resolution of 2 Å\textsuperscript{68}. Any additional fluctuation is likely from the disordered tail regions and missing residues 446 – 454 as evidenced from the PKC-ι RMSF plot (Figure S3.2). From these analyses, the MD model is considered viable due to the ability of the model to accurately maintain the lowest energy structure under significant perturbation.

To date, no crystallographic structure exists of PKC-ζ. As such, a homology model was attempted using the Schrödinger program Prime\textsuperscript{69–71} with the human PKC-ζ sequence (UniProt Q05513) and the crystallized PKC-ι structures as templates. PKC-ι was chosen as the primary template due to its highest sequence identity (49.0%) and homology (53.8%) given from a BLAST search\textsuperscript{72} coupled with highest overall structural resolution. A pre-generated SWISS-MODEL\textsuperscript{73–75} homology model was used as a control for structural reference. The Prime model (Figure 3.3C) was identical to the SWISS-MODEL variant, so only the Prime model was used for further analysis. RMSD and $R_g$ plots of the PKC-ζ homology model (Figure S3.3) do show asymptotic behavior after 1.2 ns, however the RMSD equilibration is averaged to 4.4 Å and is clearly outside any acceptable resolution. The $R_g$ plot also shows a significant expansion indicative of unfolding with internal water infiltration. RMSF plots (Figure S3.4) also show substantial backbone movement beyond 2 Å at regions of PKC-ζ residues differing from PKC-ι with notable disruption of predicted secondary structure. These factors conclude that the homology model does not represent a physical low energy structure and therefore cannot be used for further modeling. Despite this, the ATP binding region is largely stable with RMSF well within reasonable values (Figure 3.3D). This allows inclusion of the PKC-ζ homology model ATP binding site with
docking studies to compare binding modes between PKC-ι and PKC-ζ ATP binding sites (Figure 3.3C – yellow region).
The Schrödinger program SiteMap was used for optimizing placement of grids used in the docking studies and to search for any other potential binding sites. A possible allosteric site for PKC-ι was identified by SiteMap near residues 397 – 400 in the activation segment of the PKC-ι c-lobe consisting of a pocket made by α-helices E and F and the interhelical loop between α-helices H and I (Figure 3.3C – magenta region). SiteMap also scores regions based on potential hydrogen bonding, hydrophobicity, and pocket volume. Scores of 0.8 or greater are considered the cutoff for distinguishing between drug-binding and non-drug-binding sites. The PKC-ι ATP site was scored at 1.004 and the potential allosteric site was scored at 0.779. The potential allosteric site score is notably within SiteMap calibration error and was still included in docking studies due to proximity of the pocket with the PKC-ι activation segment and for the possibility of induced fit effects opening the site.

3.2.3.2. Docking results

Minimal direct binding data exists for PKC-ι and PKC-ζ so a consensus docking approach was utilized to gauge the optimal docking algorithm for the ATP and potential allosteric site. The approach detailed utilizes five different computational methods of discerning theoretical binding affinities: two unique scoring functions (SP and XP) for ridged docking methods, IFD to account for potential induced fit effects, QPLD can resolve polarization effects through QM/MM techniques, and MM/GBSA is superior in clarifying penalties for solvent interactions. Employing these functions when little empirical evidence exists to correlate results helps identify weaknesses of each technique for a particular model. Error can also be statistically minimized, yielding a more accurate summary of ligand binding dispositions and affinities.
Staurosporine was used as a docking control for the ATP site since binding data exists for both PKC-ι (261 nM $K_i^{76}$, values converted from IC$_{50}$s) and PKC-ζ (131 nM $K_i^{77}$, values converted from IC$_{50}$s). ATP is not used as a docking control due to poor model forcefields and lack of direct binding data.

Docking results are summarized in Figure 3.3E, F, and G. Docking scores for staurosporine controls are well within reasonable agreement with literature values. Docking scores and poses for each molecule are nearly identical for the ATP site of PKC-ι and PKC-ζ and both prefer staurosporine by a significant margin (Figure 3.3E and F, green highlight). XP scoring consistently yielded scores in closest agreement to literature values and highest Pearson correlation to overall averages. IFD and QPLD have poorer correlation and control accuracy, suggesting a less pronounced influence of charge factors and induced fit effects since including polarization and site flexibility does not increase docking accuracy. MM/GBSA scores exhibit similarly reduced correlation and high variance, entailing that solvent effects are also not likely a major factor for binding; this is understandable given the pocket depth. These analyses signify that the hydrophobic centers of the site (Figure 3.3A) are the dominant factors in ligand binding with the ATP site for both models. As opposed to the pan-kinase inhibitor, staurosporine, ICA-1S and ζ-Stat display negligible binding with the ATP site.

The potential allosteric site was also studied (Figure 3.3C&G), but only for PKC-ι since the corresponding PKC-ζ allosteric site model could not be validated. As such, no conclusions should be drawn concerning possible interactions of these compounds with any potential allosteric sites on PKC-ζ (only the ATP site achieved an apparent suitable stability for docking studies). Of the three molecules, the potential allosteric site appears to prefer only ICA-1S with a theoretical $K_D$ of 1.4 μM. A recent study by Ratnayake et al. $^{20}$ measured myelin basic protein (MBP)
phosphorylation by PKC-ι and PKC-ζ in the presence of ICA-1S, ICA-1T (the phosphorylated version of ICA-1S), and ζ-Stat. Docking figures match expected activity of PKC-ι for ζ-Stat having negligible inhibition. ICA-1S activity as a PKC-ι inhibitor gives some support to the existence of the potential allosteric site, since modeling suggests that ICA-1S does not interact significantly with the ATP site, but does display binding at concentrations similar to experimental values for inducing inhibition. Unfortunately, the data for PKC-ζ is less clear. All the modeling can show is if the two compounds in question can effectively bind to the ATP site of PKC-ζ. This may indirectly imply an allosteric site exists for PKC-ζ if inhibition is experimentally observed and the compound in question does not appear to have favorable docking to the ATP site. For ICA-1S, there is negligible affinity for the ATP site and experiment reflects a lack of inhibition. Modeling also suggests an allosteric mechanism may be present for ζ-Stat as binding is also negligible for the ATP site. Experimental inhibition should be observed for any compounds that compete for the ATP site since there is no significant difference between the ATP sites of PKC-ι and PKC-ζ.

3.2.3.3. Virtual target screening results

VTS uses a large curated protein structure library to which molecules of interest are docked. Statistical calibrations and baselines are applied to average and relate docking scores with each individual and class of proteins. A kinase-enriched library (1,451 proteins, 464 transferases, and 65 unique kinases) was assigned for docking with the three compounds. A hit on a protein is classified as the potential (p value < 0.05) of the molecule of interest to bind to the specified protein with a theoretical K_D of 10 μM or less. This analysis can infer the specificity of a molecule for a particular class of proteins. The VTS results for each compound are listed in Figure 3.3H.
The staurosporine control gave an expected baseline commensurate of a pan-kinase inhibitor. It displayed low to moderate total protein activity with moderately high interaction with general transferases and hit a majority of kinases, alluding a clear preference for kinases. ICA-1S demonstrated a low total protein activity with a slight but pronounced increase in affinity for transferases and kinases. This suggests a possibility of seeing some expected broad kinase interference for ICA-1S. ζ-Stat, however, portrays significant specificity in VTS. It has similar low hit percentages for all protein classes, implying little to no expected kinase activity.

3.2.4. Inhibition of cell viability

The effects of ζ-Stat on CCOC cellular viability was investigated via WST-1 methodologies. The results revealed that 10μM ζ-Stat did not significantly effect SHT290 normal endometrial stromal cells, but did significantly decreased the viability by 37% in TOV21G cells ($p$ value 0.0436, $F = 4.2461$, $t = -3.058$) and by 57% in ES-2 cells ($p$ value 0.00363, $F = 7.2918$, $t = -4.220$) (Figure 3.4A-C). These results suggest that ζ-Stat decreases the viability of CCOC but has negligible effects on normal endometrial stromal cells.
Figure 3.4 Cell proliferation and viability of SHT290, TOV21G and ES-2 CCOC cell lines. (A) SHT290, (B) TOV21G and (C) ES-2 cells were treated for 0, 24, 48 and 72 hrs with 1μM, 3μM, 5μM and 10μM ζ-Stat. WST-1 assays were run after 72 hrs of ζ-Stat treatment (Vehicle control N=12, treatment N=3). A standard curve for each cell line is also represented. Standard deviation is represented. A one-way ANOVA was tested for the WST-1 assays. (* p < 0.05, ** p < 0.01).

3.2.5. Analysis of the PKC-ζ/Ect2/Rac1/RhoA pathway

To determine the downstream effects of ζ-Stat on invasion, immunofluorescence, Western Blots and semi-quantitative endpoint PCR techniques were utilized. Vehicle control and ζ-Stat treated TOV21G cells were probed with anti-Ect2 and imaged. The results showed that Ect2 was present in the filamentous extensions in control cell. After treatment, the polarity of the filamentous extensions decreased and Ect2 was found to be more abundant around the nucleus (Figure 3.5A-B). Western results demonstrated that 3μM of ζ-Stat decreased RhoA protein expression as well as mRNA expression (Figure 3.5C-D). These results suggest that the decrease in PKC-ζ protein expression reduces the expression of RhoA at the genomic level.
Figure 3.5 Effects of ζ-Stat on Ect2 localization and RhoA protein and genomic expression. (A) TOV21G cells were seeded on a four-chamber slide and treated for 72 hrs with 10μM ζ-Stat. The slide was fixed with formaldehyde and immunostained with anti-Ect2, phalldoidin dye, and DAPI. Original magnification is 40X and scale bar is 20μm. (B) Ect2 is visualized in the filamentous extension of the TOV21G cell. (C) A Western Blot for TOV21G and ES-2 cells treated for 72 hrs with vehicle control (DMSO) and 3μM ζ-Stat is represented with an immunoblot that was probed for PKC-ζ and RhoA (N=3). (D) TOV21G and ES-2 cells were treated for 72 hrs with 3μM ζ-Stat and harvested with RNAbee for mRNA semi-quantification. Endpoint PCR was run with RhoA and GAPDH primers (mRNA control) (N=3).

Furthermore, Ect2 localization was observed by immunofluorescence with and without treatment. The PCC showed that the control had a lower amount of Ect2 nuclear localization (0.57) in comparison to the treated (0.72) TOV21G cells (Figure 3.6A). In contrast, ES-2 cells had little effect as both the control and the treated cells had a PCC value of 0.69 (Figure 3.6B). In addition, the filamentous actin (F-actin) organization was investigated via phalloidin stain. In Figure 3.6C, the F-actin in the control for TOV21G showed filamentous extensions, whereas in the treated cells, the F-actin seemed to aggregate within the cell, rounding the edges. Although a nuclear
fractionation could not confirm the translocation of Ect2 into the nucleus is TOV21G cells (Figure 3.6 D), it did show that this translocation did occur in ES-2 cells. An explanation for this may be that the ζ-Stat is disrupting the cell structure causing the cells to become more globular and thereby making the cytosol surround the nucleus in a more significant manner.

Moreover, TOV21G cells were treated with 10μM ζ-Stat and seeded into 96 welled transwell plates. After 24hrs of treatment, the cells were fixed and stained to determine the effects ζ-Stat on invasion and migration. Our data showed that ζ-Stat drastically decreased invasion and migration.
when compared to the control (Figure 3.7A). After the images were quantified, the data revealed that the decrease in invasion and migration was statistically significant (Figure 3.7B&C; invasion $p$ value 0.002826, $t = 4.859$; migration $p$ value $< 0.001$, $t = 6.1887$). To further illustrate this point, Rac1 activation was investigated utilizing a GST pull down method. The negative and positive control display how well the assay data fits the intended model ($p$ value $< 0.001$, $t = 7.675$). Compared to the sample control, the amount of activated Rac1 pulled down from treated samples was only 37% ($p$ value 0.044, $t = -3.044$; Figure 3.7D). These data reinforce the theory that ζ-Stat decreases the invasion and migration of CCOC through a decreased activation of Rac1.

**Figure 3.7** PKC-ζ regulates invasion and migration of TOV21G ovarian cancer. (A) TOV21G cells were grown, serum starved for 48hrs, and placed in the upper chamber of transwell plate coated with 0.1x BME and serum (10%) containing media was placed in the lower chamber as a chemo attractant. Following treatment with 10μM ζ-stat for 24hrs, cells that invaded through BME and migrated into the lower chamber were stained with crystal violet and observed under microscope. Original magnification is 10x and scale bar represents 1mm. (B) Invasion (N=4) and (C) migration (N=4) fixed and stained cells on the lower chamber were quantified using ImageJ FIJI software, average and plotted. Standard deviation error bars are represented. (** $p < 0.01$, *** $p < 0.001$). (D) Activated Rac1 (GTP bound) was pulled down using GST tagged PAK-PBD and analyzed by Western Blot. Densitometry of activated Rac1 bands were plotted (N=4). Standard deviation error bars are represented.
3.2.6. Analysis of the $\zeta$-Stat in TOV21G tumor xenografts

To determine the effects of $\zeta$-Stat \textit{in-vivo}, we injected athymic nude female mice with TOV21G cells and sequentially treated mice for 35 days. At the endpoint of the experiment, the tumors were harvested, and the blood serum was screened for enzymes associated with kidney and liver failure, as well as glucose levels for screening diabetes. Our data exhibited statistically significant changes in tumor volume between vehicle control and treated mice (Figure 3.8A) starting on day 14 ($p$ value 0.006343, $t = 3.4389$) up until day 35 ($p$ value 0.001136, $t = 4.4827$). Results also demonstrated that $\zeta$-Stat decreased tumor growth by more than 50% by the endpoint of the experiment (Figure 3.8B-C) and decreased PKC-$\zeta$ and RhoA expression in tumors by more than 40%. The treatments did not lower the mouse population’s body weight (Figure 3.8D) and did not have a significant effect on the enzyme panel (Figure 3.8E-F). These preliminary results suggest that $\zeta$-Stat can be used for the treatment of CCOC and does not cause short-term toxicity.
Figure 3.8 Effects of ζ-Stat on in-vivo xenografts with athymic nude mouse models. (A) Six vehicle control mice and six treatment mice were injected with 1 x 10^6 cells/per mouse flank in 0.2mL of media of TOV21G CCOC cells. Vehicle control mice tumors were treated with 1x DPBS and treatment mice were treated with 20mg/kg of ζ-Stat. Tumors were measured every other day and plotted. An unpaired student T test was performed, and standard deviation error bars are presented (* p < 0.05, ** p < 0.01). (B) Tumors were harvested after 35 days of treatment and a picture was taken. Tumors were fractionated and protein lysates were run via Western blot. Analysis of the protein expression of PKC-ζ and RhoA were investigated. β-actin was used as a control. Densitometry of the results are represented in bar graph as expression percent of the control with standard deviation error bars. (C) Endpoint treated tumor volumes were graphed as a percent of the control (D) Mouse weight was recorded once per week. An unpaired student T test was performed, and standard deviation error bars are presented (no significant difference observed). (E-F) Endpoint blood serum was taken and screened for glucose (GLU), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase levels for toxicity. An unpaired student T test was performed, and standard deviation error bars are presented (no significant difference observed).

3.2.7. ζ-Stat interrupts the PKC-ζ/Ect2 via PKC-ζ protein decrease

Our predicted pathway models that PKC-ζ scaffolds Ect2 to the cellular membrane (Figure 3.9). This mis-localization of Ect2 permits the wild-type Ect2 more access to Rac1 and therefore increases its activation. Upon increased Rac1 activation, CCOC invasion is increased. PKC-ζ protein level decrease via ζ-Stat, releases Ect2 from the membrane scaffold, and re-localizes Ect2 to the nucleus, limiting its access to cytosolic Rac1 and decreasing Rac1 activation.
Figure 3.9 ζ-Stat decreases the amount of PKC-ζ, therefore decreasing the scaffold of Ect2 in the cytoplasm. The hypothesized pathway illustrates the scaffolding of Ect2 via PKC-ζ in the cytoplasm. Treatment with ζ-Stat decreases PKC-ζ expression thereby there is less PKC-ζ to bind Ect2, allowing Ect2 to re-localize to the nucleus. This decreases Ect2 cellular access to Rac1, reducing Rac1 activation and cell migration.

3.3. Acknowledgments
The authors would like to acknowledge DeVon DeLoach in the Department of Comparative Medicine at the University of South Florida for aiding with running our serum enzyme panel and Dr. Emily Miedel, VMD, DACLAM, Associate Director of Comparative Medicine at the University of South Florida for her expert feedback and analysis.

3.4. Funding
We acknowledge the generous financial contribution from the Celma Mastry Foundation.

3.5. Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request. The dataset generated and/or analyzed during the current study are available from COSMIC repository, www.COSMIC.Sanger.ac.UK.

3.6. Conflict of interest

The authors declare that this study received funding from the Celma Mastry Ovarian Cancer Foundation. The funder was not involved in the study design, collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

3.7. Author contributions

TS contributions include cell culture, analysis of somatic gene mutations, cell viability assays, cell lysate collection, Western Blot analysis, semi-quantitative endpoint PCR, Rac1 activation assay, fluorescent microscopy, in-vivo experiments, and the statistical analysis of Western Blots, cell viability, particle counts for invasion and migration, tumor volume, mouse body weights, and enzyme panel. In addition, TS contributed to writing and experimental design. RM contributed by providing molecular dynamics simulations, homology modeling, molecular docking, virtual target screening, site screening, computational and statistical analysis, and writing support. RP contributed by assisting in-vivo experimentation. SMI contributed by performing the invasion and migration assay. RB contributed by performing Western blots. MD contributions to the paper include writing-review, editing, resources, supervision and funding acquisition. The authors read and approved the final manuscript.
3.8. Supplemental figures

**Figure S3.1** RMSD (Bottom) of backbone atoms and Radius of Gyration ($R_g$, Top) of all atoms graphs for PKC-1 Molecular Dynamics simulation. Equilibration appears to occur after 7 ns with an average RMSD of 2.3Å.
Figure S3.2 RMSF (Root Mean Square Fluctuation) plot of PKC-ι Molecular Dynamics simulation. Graph shows the RMSD per residue of α-carbon over length of simulation. Most displacements are under 2Å aside from tail regions and chain discontinuities from missing residues 446-454.
Figure S3.3 RMSD (Bottom) of backbone atoms and Radius of Gyration (Rg, Top) of all atoms graphs for PKC-ζ Molecular Dynamics simulation. Some asymptotic behavior does appear to occur, however, an average RMSD of 4.5Å and increasing $R_g$ implies unfolding and significant deviation from any structural energetic minimum.
**Figure S3.4** RMSF (Root Mean Square Fluctuation) plot of PKC-ζ Molecular Dynamics simulation. Graph shows the RMSD per residue of α-carbon over length of simulation. The ATP region, approximately residues 300-450, show displacements under 2Å. Most homologous residues show RMSD values well above 2Å.
Figure S3.5 Raw Western blot images for TOV21G and SHT290 Figure 3.2A. (A) Western blot of PKC-ι in TOV21G and SHT290 cells at 50% and serum free (SF). (B) Western blot of PKC-ζ in TOV21G and SHT290 cells at 50% and serum free (SF). (C) Western blot of β-actin in TOV21G and SHT290 cells at 50% and serum free (SF). Rows 3 and 4 were used for the TOV21G image and rows 7 and 8 were used for the SHT290 image.

Figure S3.6 Raw Western blot images for ES-2 Figure 3.2A. (A) Western blot of PKC-ι in ES-2 cells at 50% and serum free (SF). Bands are loaded in the opposite order. (B) Western blot of PKC-ζ in ES-2 cells at 50% and serum free (SF). Bands are loaded in the opposite order. (C) Western blot of β-actin in ES-2 cells at 50% and serum free (SF). Bands are loaded in the opposite order.
Figure S3.7 Raw Western blot images for TOV21G Figure 3.2B. (A) Western blot of PKC-ι and its corresponding β-actin in TOV21G cells. (B) Western blot of PKC-ζ and its corresponding β-actin in TOV21G cells.

Figure S3.8 Raw Western blot images for ES-2 Figure 3.2B. (A) Western blot of PKC-ι and its corresponding β-actin in ES-2 cells. (B) Western blot of PKC-ζ and its corresponding β-actin in ES-2 cells.
Figure S3.9 Raw Western blot images for TOV21G Rac1 PD utilizing GST PAK-PBD Figure 3.7D. (A) Western blot of Rac1 exposure time of 1 minute. Rows 1 and 2 are the positive and negative control, respectively. (B) Western blot of Rac1 exposure time of 2 minutes. The positive control is saturated (marked in pink). Rows 5 and 6 are the vehicle control and 10μM treatment for TOV21G cells.

Figure S3.10 Raw Western blot images for TOV21G RhoA and PKC-ζ protein decrease (A) Western blot of RhoA in TOV21G cells with vehicle control (Row 2) and 3μM treatment (Row 3). (B) Western blot of PKC-ζ in TOV21G cells with vehicle control (Row 2) and 3μM treatment (Row 3). (C) Western blot of β-actin in TOV21G cells with vehicle control (Row 2) and 3μM treatment (Row 3).
Chapter 4: Discussion and future directions

4.1 Role of PKC-ζ in breast cancer

4.1.1 PKC-ζ expression was higher in invasive tissue subtypes

Previously, Yin et al. showed that the expression of PKC-ζ was higher in invading breast tissues compared to samples uncomplicated with tumors and the highest PKC-ζ protein expression existed in stage III human breast ductal carcinomas. Likewise, Paul et al. concluded that the depletion of PKC-ζ reduced the invasive behaviors of MDA-MB-231 cells by upregulating epithelial markers such as Zonula occludens-1 (ZO-1) and E-Cadherin. They also found that PKC-ζ activation (phosphorylated PKC-ζ levels) was found higher in invasive tissues (i.e. IDC and metastatic tissues) when compared to non-invasive tissues (DCIS). PKC-ζ expression did not significantly change with the presence or absence of receptors (ER and HER2). Our Western Blots and Chi-squared analysis support these findings. They performed a PKC-ζ knockdown mouse study and found that the depletion of PKC-ζ leads to an approximately 50% reduction in primary tumor growth compared to the control within five weeks. Similarly, our Western Blot analysis and immunohistochemistry data of IDC and ILC suggest that PKC-ζ might also be a novel component in pathways that affect cancer cell invasion and metastasis.

PKC-ζ assists a transcription factor (NFκB-p65) that downregulates targets such as E-Cadherin and ZO-1. In addition, decreased E-Cadherin levels cause the cells to no longer adhere to the
extracellular matrix causing the cells to migrate, invade, or metastasize. Moreover, *H.L. Chua et al.* showed that NF-κB induced elevated expression of Zinc finger E-box binding homeobox 1 and 2 (ZEB-1 and ZEB-2) which ultimately repress the E-Cadherin levels. In our investigation, we could not establish a statistical relationship between PKC-ζ and E-Cadherin protein expression in IDC (Figure 2.2B) as per the linear regression analysis. However, previous studies illustrated an increase in E-Cadherin levels in PKC-ζ knockdown MDA-MB-231 cells.

### 4.1.2 PKC-ζ expression was higher in ductual carcinomas when compared to lobular carcinomas

In addition, our findings showed an increased expression of PKC-ζ in IDC tissues when compared to other subtypes (Figure 2.1A-C) which may be because of the difference in pathological features of ductal and lobular tumors. Ductal carcinomas are lined with a two-layered stratified cuboidal epithelium resting on the basement membrane. This cuboidal epithelium contains tight junctions where E-Cadherins are located and play a central role in cell-to-cell adhesion. In contrast, lobular carcinomas vary in terms of molecular and genetic aberrations compared to ductal carcinomas. Lobular carcinomas are epithelial-like, growing individually in sheets or in a single file. In our investigation, E-Cadherin levels were also compared to the nuclear grade listed on the pathology report (Figure 2.2C, Table 2.3C), which suggest the previous studies that described a decline in E-Cadherin as a part of the epithelial to mesenchymal transition leads to metastasis.
4.1.3 PKC-ζ knockdown decreased cellular migratory behaviors

During metastasis, directional cell movement involves five distinct steps: leading edge membrane protrusion, adhesion of the protrusion to the substrates, cell body translocation, de-adhesion of the tail from the substrate and trailing edge retraction \(^81\). These processes are mainly controlled by reorganization of the actin in the cell cytoskeleton which in turn are regulated by GTPases (Guanosine Triphosphatases)\(^82\). Among the GTPases, RhoA, Rac1, and Cdc42 are most commonly studied because of their crucial role in actin assembly and formation of metastatic structures of cells, such as filopodia, lamellipodia and stress fibers \(^36\). Rac1 and Cdc42 produce localized actin polymerization at the leading edge which pushes the membrane forward in slender like structure known as filopodia and sheet-like structure known as lamellipodia that ultimately generate locomotive force in migrating cells \(^83\). In contrast, RhoA promotes the assembly of contractile actomyosin filaments and acts on the rear end of the migrating cells, inducing tail detachment \(^34\). Thus, Rac1 and Cdc42 stimulate leading edge protrusion, and RhoA stimulates trailing edge retraction in metastatic cells. We found that the knockdown of PKC-ζ by siPRKCZ reduced the invasion of MDA-MB-231 breast cancer cells by 60% \((p < 0.05)\) when compared to control (Figure 2.3A, 2.3B). In addition, there was a decreased expression of both Rac1 and RhoA in siPRKCZ transfected MDA-MB-231 breast cancer cells compared to control (Figure 2.3C). Furthermore, our immunostaining analysis of F-actin illustrated that actin filaments were nicely organized around the cells with the inhibition of PKC-ζ (Figure 2.4). Hence, PKC-ζ may play an essential role in the invasion and migration of breast cancer cells by the regulation of RhoA and Rac1 pathways.

To conclude, our findings suggest that PKC-ζ is found to be most abundant in invading tissue subtypes and may be a functional component of invasion pathways such as Rac1 and RhoA. The
use of PKC-ζ-specific inhibitors could be used to correlate the decrease in expression or functionality of PKC-ζ with the decrease in invasive behavior in breast cancer.

4.2 ζ-Stat as a novel inhibitor in ovarian cancer

In this study, we discovered that the small molecule inhibitor, ζ-Stat, is a prospective drug candidate to investigate as a novel potential treatment for CCOC. We also investigated the PKC-ζ/Ect2/Rac1 activation pathway and found that ζ-Stat decreases the invasive behavior of CCOC by decreasing cytosolic Ect2 and Rac1 activation.

4.2.1 Targeting aPKCs in CCOC

To understand the therapeutic potential of a protein target, there must be an appreciation of the underlying genetic abnormalities specific to the cancer type. The TP53 gene (tumor suppressor p53) is the most commonly mutated gene in all ovarian cancers and is especially a prognostic marker of HGSOC \(^84\). In contrast, CCOC typically has a wild type TP53 and mutations in the tumor suppressor ARID1A \(^85\). Although PRKCZ and PRKCI are not in the top mutated genes in CCOC, PIK3CA is mutated (~30%) and is located on the third chromosome’s long arm. Interestingly, PIK3CA (3q26.32), ECT2 (3q26.31) and PRKCI (3q26.2) are all located on the long arm of chromosome three and in proximity. The ARID1A (1p36.11) is also located up stream of PRKCZ (1p36.33) on the short arm of the first chromosome. It has been noted that mutations and deficiencies in ARID1A have been shown to sensitize cancers to PARP and PI3K inhibitors (Figure 4.1) \(^86–88\).
PIK3CA and ARID1A mutations in clear cell ovarian carcinoma. PIK3CA and ARID1A are commonly mutated in CCOC. When ARID1A is not mutated, it indirectly inhibits and regulates the PI3K pathway. ARID1A mutations increase the upregulation of the PI3K pathway, typically through loss of function via truncation of protein translated.

Figure 4.1 PIK3CA and ARID1A mutations in clear cell ovarian carcinoma. PIK3CA and ARID1A are commonly mutated in CCOC. When ARID1A is not mutated, it indirectly inhibits and regulates the PI3K pathway. ARID1A mutations increase the upregulation of the PI3K pathway, typically through loss of function via truncation of protein translated.

Previous literature has suggested that the atypical PKCs and their pathways should be the focus of targeted treatment. One explanation for this is that the atypical PKC isoforms have been linked to signaling pathways needed for cancer survival and growth. A study performed by Yao et al. illustrated the dramatic changes to prostate cells malignancy upon PKC-ζ silencing. In our study, when CCOC (TOV21G and ES-2) cells grew rapidly and were cell cycle arrested (serum starved), the aPKCs were present in both conditions. However, the expressions of PKC-ζ and PKC-ι were found to be very low in the normal endometrial stromal cells. These findings may indicate that CCOC cells have a reliance on aPKC overexpression for cell viability. An interesting complication in other types of CCOC is that ζ-Stat does not specifically limit expression of PKC-ζ but also effects downstream targets in the PKC-ζ/Ect2/RhoA pathway. As shown in Figure 3.2, ζ-Stat had a negligible effect on PKC-ζ expression in ES-2 cells, however in Figure 5, there was a
decrease in RhoA protein and mRNA expression. This leads to the conclusion that ζ-Stat has
generalized effect on the pathway dependent on cell type. Furthermore, these data support that
PKC-ζ/Ect2/RhoA pathway contains relevant targets for CCOC due to the lack of overexpression
in normal tissue and the overexpression in cancerous cell lines.

Equally important, a previous study showed that the knock down of PKC-ζ using siRNA
decreased the expression of RhoA and Rac1. Furthermore, this study illustrated that the knock
down of PKC-ζ decreased the invasive behavior of breast cancer cells by more than 40%. Our data
supports previous data and reiterates the therapeutic potential for targeting PKC-ζ in CCOC.

4.2.2 aPKCs are involved in the localization of Ect2

The mis-localization and overexpression of Ect2 has been linked to aPKCs and Ect2 dependent
malignant transformation. Cytoplasmic Ect2 has more access to Rho GTPases and increases the
protein family’s activation. Liu et al. identified Ect2 as an activator of the Par6/Par3/aPKC polarity
complex and further showed that Ect2 stimulated PKC-ζ activity. Moreover, the oncogenic
activity of Ect2 was shown to be regulated by aPKC via the phosphorylation of the Thr 328 site
and a mutation in this site (T328A) rendered the Ect2 unable to interact with Par6/aPKC complex
or activate Rac1. Upon treatment with ζ-Stat, the decrease of PKC-ζ disturbs the localization of
Ect2 in TOV21G cells. This specific effect is not seen in ES-2 as correlations are equal before and
after ζ-Stat treatment. Therefore, it does not seem that PKC-ζ is the specific target but more of the

4.2.3 ζ-Stat has therapeutic potential in CCOC
A common treatment regime for ovarian cancer patients typically involves chemotherapy (paxitaxol), PARP (poly ADP ribose polymerase) inhibitors and bevacizumab (targets angiogenesis)\textsuperscript{62}. However, concerns for current treatments still involve toxicity, drug-resistance, reoccurrence and the side effects to the patients\textsuperscript{90}. Our data showed that mice treated with ζ-Stat did not have significant side effects when compared to mice treated with the vehicle control. In particular, the mice did not have significant fluctuations in body weight, or differences in the enzyme panel which screened for liver and kidney damage. Further, the viability of normal endometrial stromal cells did not change significantly upon treatment of 10μM ζ-Stat. All these data support the prospect that this compound may be a less toxic alternative maintenance drug for CCOC.

The presence of PKC-ζ and PKC-ι in proliferation, invasion and migration make this protein a unique target for therapies. In the previous literature, ζ-Stat was shown to decrease the invasion and migration of melanoma cells and increase apoptosis\textsuperscript{20}. It has also been suggested the ζ-Stat is selective to PKC-ζ in melanoma and colorectal cell lines\textsuperscript{10, 20, 91}. In support of the previous data, our data showed that ζ-Stat had a dramatic effect on CCOC invasion and migration.

However, new evidence supports the theory that ζ-Stat does not inhibit kinase activity of PKC-ζ through the ATP binding region. Our computational and \textit{in-vitro} data advocates that ζ-Stat decreases the expression of signaling proteins (PKC-ζ and RhoA). This phenotypic effect consistently modifies kinase interaction networks which results in decreases in cellular viability, invasion and tumor growth\textsuperscript{10, 20}. The effects that ζ-Stat elicited was decreased PKC-ζ protein, re-localized Ect2 to the nucleus and decreased the presence of activated Rac1. ζ-Stat specifically decreased the protein expression of PKC-ζ in comparison to PKC-ι, however this mechanism is still unknown. The data suggests that ζ-Stat may be generating an epigenetic effect, which in turn
regulates the expression of these proteins. It has been suggested that using aPKC inhibitors can epigenetically regulate the expression of aPKCs through transcription factors, such as FOX01.

Additionally, according to the computational data, ICA-1S exhibited poor binding with the PKC-ι and PKC-ζ ATP site and had a predicted moderate affinity with a possible PKC-ι allosteric site. This fits well with previously performed MBP phosphorylation experiments. Some expected kinase activity with mid-range binding promiscuity is anticipated for this molecule. However, this conclusion is contradicted by some evidence from the experiment, therefore it could simply be that the generated PKC-ζ model is insufficient for correlating physical data. ζ-Stat exhibits extremely poor binding with the ATP site and PKC-ι allosteric site. With no expected kinase activity and low binding promiscuity, any inhibition seen using ζ-Stat is likely specific for unique binding pockets.

To further the exploration of ζ-Stat being a potential therapeutic for CCOC, a mechanistic understanding of the direct protein binding is required for PKC-ζ and PKC-ι.

4.3 Future directions

In future research, PKC-ζ should continue to be a focus and target for molecular therapies, especially in breast and ovarian cancers. The small molecule inhibitors ICA-1 and ζ-Stat should be further investigated for its pre-clinical significance in-vivo and absorption, distribution, metabolism and excretion studies. In addition, to further validate the in-silico results, direct binding studies of these small molecule inhibitors should be performed with PKC-ι and PKC-ζ protein to have a better mechanistic understanding of where and how the small molecules are binding.
5.1. Analysis of PKC-zeta protein levels in normal and malignant breast tissue subtypes

5.1.1. Specimen collection and tissue fractionation

The NCI-supported Cooperative Human Tissue Network (Birmingham, Alabama) collected and provided the breast tissue samples (normal, LCIS, ILC, DCIS, and IDC). The specimens were collected and snap-frozen in liquid nitrogen or placed in dry ice and stored in a liquid nitrogen vapor phase freezer (-196 °C), where the tissues stayed until shipment. The tissues were shipped in dry ice. Formalin-fixed paraffin-embedded tissues (FFPE) were also provided for immunohistochemistry staining. Tissues were selected based on their histological features (normal, LCIS, ILC, DCIS, and IDC). The mean age of patient samples collected was 51 years and the collection period of the samples was 2001-2015. Normal tissues were selected from breast reduction patients with no previous diagnosis of cancer or the area adjacent to a patient’s malignant tumors. Patients with DCIS and LCIS were selected based on the lack of invasive tissue adjacent to the extraction site; some tissues were taken from patients with invasive tissues in the opposite breast.

The tissue specimens were then resuspended and sonicated for 3 x 5 sec cycles on ice in 1 mL of homogenization buffer (Pierce® Immuno Precipitation Lysis Buffer, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol, Thermo Fisher Scientific, MA), followed by, centrifugation at 12,879 x g for 15 mins at 4°C to obtain cell extracts. Subsequently,
250 µL of albumin removal resin (Pierce™ Albumin Depletion Kit, Thermo Fisher Scientific, Waltham, MA, USA) was added to the lysate and left at 4°C overnight. The samples were further centrifuged at 12,879 x g for 15 mins at 4°C, the supernatant was subsequently collected, and protein content was measured according to Pierce® 660 nm Protein Assay reagent protocol (Thermo Fisher Scientific, Waltham, MA, USA) and NanoDrop 2000.

5.1.2. Cell culture

The metastatic breast cancer cell line, MDA-MB-231 was obtained from American Type Tissue Culture Collection (ATCC, Manassas, VA, USA). The MDA-MB-231 cells were sub-cultured and maintained in T75 flasks containing Dulbecco’s Modified Eagle’s Minimum media (DMEM), supplemented with 10% FBS (Fetal Bovine Serum) and 1% antibiotics (Penicillin 10 U/ml and streptomycin 10 mg/ml). Cells were incubated at 37°C and 5% CO₂. Cells were used at 70-80% confluency for the experiments.

5.1.3. Western Blot analysis

Like tissue lysates, MDA-MB-231 cell extracts were also prepared after the addition of cell lysis buffer, sonication, and centrifugation (at 12,879 x g for 15 minutes at 4°C). For Western Blot analysis, an equal amount (20-30µg) of protein from tissue and cell lysates were loaded in 10% polyacrylamide gels and separated by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transblotted onto a 0.4µM nitrocellulose membrane. Subsequently, the membranes were incubated with 5% milk blocking solution, followed by, primary solution of anti-PKC-ζ (SC-17781, 1:1000, Santa Cruz Biotechnology,
Dallas, TX; and 9372s, 1:1000, Cell Signaling Technology, Danvers, MA), anti-E-Cadherin (701134, 1:1000, Invitrogen, Carlsbad, CA), anti-RhoA (ab54835, 1:4000, Abcam, Cambridge, UK) and anti-Rac1 (4651s, 1:1000, Cell Signaling Technology, Danvers, MA) in 5% BSA (Bovine serum albumin). Finally, the membranes were incubated with secondary antibodies. All the secondary antibodies (anti-rabbit and anti-mouse) were obtained from Bio-Rad Laboratories (#170-6515 and #170-6516, 1:2000, Hercules, CA). The immunoreactive bands were then visualized by chemiluminescence reaction, according to the manufacturer’s instructions (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA). A monoclonal antibody to β-actin (SC-1616, Santa Cruz Biotechnology, Dallas, TX) was used as a loading control.

5.1.4. Densitometry

The densitometry was performed using Image J (NIH) software by the subtraction of background noise from the density of each band to derive the corrected intensity. All samples were normalized based on the intensity of β-actin bands on each blot.

5.1.5. Immunohistochemistry

FFPE tissues received from CHTN were sent to the Tissue Core at Moffitt Cancer Center, Tampa, FL. Two different patient tissues were selected for each subtype (normal, LCIS, ILC, DCIS, and IDC) based on the criteria mentioned previously, with a total N=10. Briefly, tissues were stained with Haemotoxylin and Eosin (H&E), and PQC (pathology quality control) were performed by Tissue Core pathologist to confirm breast tissue subtype and diagnosis. The slides
were prepared and stained with antibodies for PKC-ζ (ab59364, 1:1000, rabbit polyclonal, Abcam, Cambridge, UK). A uterine carcinoma specimen was selected as a positive control based on antibody data sheet and the Human Protein Atlas recommendations. Slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) as per the manufacturer's protocol with proprietary reagents. Briefly, slides were deparaffinized on the automated system with EZ Prep solution (Ventana). The heat-induced antigen retrieval method (CC1 standard) was used with PKC-ζ (Ventana). The primary rabbit antibody that reacts to human was used at a 1:1000 concentration in Dako antibody diluent (Carpenteria, CA) and incubated for 32 mins. The Ventana OmniMap Anti-Rabbit Secondary Antibody was used for 16 mins. The detection system used was the Ventana ChromoMap kit and slides were then counterstained with Hematoxylin. Slides were subsequently dehydrated and cover-slipped as per normal laboratory protocol. The Moffitt Tissue Core pathologist selected the optimal condition, titration, and incubation time to be used on control and the breast selected slides. Subsequently, the pathologist evaluated the slides using the combinative semi quantitative scores (score 0-3). Images were taken on a light microscope Olympus BX51.

5.1.6. Knockdown of PKC-ζ for invasion pathway analysis

Human breast cancer cells MDA-MB-231 were grown in 100mm plates and transfected with 20nM of scrambled RNA and siPRKCZ (5’-GCAUGAUGACGAGGAUAUUGACUGG-3’, SR303747A, Origene, Rockville, MD, USA) for 48 hours. Cells were lysed as previously described and the lysates were run on Western Blots.
5.1.7. Cell invasion assay by crystal violet staining of invaded cells

Cells were serum starved for 24 hours, followed by detachment and plating into the upper chamber of a 96 wells 8µm Transwell permeable support (Corning Inc., Corning, NY), coated with 0.5x basement membrane extract (BME) (Corning Inc., Corning, NY) for the evaluation of invasion. Serum (10%) containing media was loaded into the receiver plate (lower chamber) as a chemoattractant. MDA-MB-231 cells at the upper chamber were transfected with 20nM siPRKCZ for 24 hours. Four experimental treatment groups for the cells were performed: control (non-treated), Si-Tran (transfection reagent), scrambled siRNA (random RNA) and siRNA for PRKCZ (for knockdown of PKC-ζ protein expression). The cells were treated with the transfection reagent (Si-Tran) and universal scrambled RNA to establish the effect of targeted small interfering RNA (siPRKCZ) only. The invasive cells that passed into the lower chamber were then fixed with 4% paraformaldehyde, stained with 2% crystal violet in 2% ethanol, washed with distilled water and photographs were captured after drying using a light microscope Motic AE31E.

5.1.8. Phalloidin staining of F-actin

Human breast cancer cells MDA-MB-231 (1 x 10^4 cells) were grown in 2 wells chamber slides, followed by transfection with 20nM universal scrambled RNA and siPRKCZ for 24 hours. In addition, cells were also evaluated with the transfection reagent and without any treatment to establish the targeted effect of PKC-ζ knockdown. Fixation was performed with 4% paraformaldehyde. F-actin was subsequently stained with 1X Phalloidin-iFluor 594 (Abcam, Cambridge, UK) in 1% bovine serum albumin (BSA)-phosphate buffered saline (PBS) solution for an hour at room temperature. Cells were washed, counterstained with the nuclear stain 4’,6-
diamidino-2-phenylindole (DAPI) (Invitrogen Inc., Carlsbad, CA) and examined under Nikon MICROPHOT-FX fluorescence microscope (Ex/Em = 590/618). Photographs were captured using ProgRes®Capture 2.9.0.1.

5.1.9. Statistical analysis

The statistical significance of the Western Blot data was evaluated by a Student’s t-test (normal \( N=32 \); LCIS \( N=3 \); ILC \( N=13 \); DCIS \( N=6 \); IDC \( N=29 \); overall \( N=83 \) at \( p < 0.05 \); standard error represented) and the linear regression test (\( N=20 \); \( R^2 \) value) with GraphPad software\(^4\). A one-way ANOVA was also used to evaluate the Western Blot data as well with the post-hoc Tukey’s HSD test (\( p < 0.01 \)), Scheffé multiple comparison (\( p < 0.05 \)), Bonferroni (\( p < 0.01 \)) and Holm (\( p < 0.01 \)). The contingency table Chi-squared statistical analysis (normal \( N=32 \); LCIS \( N=3 \); ILC \( N=13 \); DCIS \( N=6 \); IDC \( N=29 \); overall \( N=83 \) at \( p < 0.00001 \)) for the expression of PKC-ζ and breast subtype was performed using the Chi-Squared Test Calculator from Social Science Statistics \(^5\). Clinical parameters such as estrogen receptor expression and Scarff-Bloom-Richardson grade (presented in the pathology reports) were also investigated with this statistical software (\( N=30 \) and \( N=22 \), respectively). A one-way ANOVA was used to analyze the number of cells invaded after crystal violet staining; the statistical test was significant at a \( p \) value of < 0.05.

5.2. The atypical Protein Kinase C small molecule inhibitor ζ-Stat, and its effects on invasion through decreases in PKC-ζ protein expression

5.2.1. Antibodies and reagents
The small molecule inhibitors, ζ-Stat and ICA-1S, were obtained from the National Institute of Health (NIH) branch National Cancer Institute (NCI) and United Chem Resources in Birmingham Alabama, respectively. The sources of cell lines, reagents and antibodies were: TOV21G and ES-2 CCOC cell lines (American Type Culture Collection, USA); SHT290 normal endometrial stromal cell line (Kerafast, USA); MCDB 121, Media 199, F12K, penicillin and streptomycin, trypsin, Dulbecco’s phosphate buffered saline (DPBS) and Mito + (Corning, USA); McCoy’s media (HyClone, USA); Opti-MEM I (Gibco, USA); Fetal bovine serum (FBS, Atlanta Biologicals, USA); human insulin (MP Biomedicals, LLC, France); dimethyl sulfoxide (DMSO, Sigma Aldrich, USA); Water-Soluble Tetrazolium (WST-1, Roche, USA); Halt protease and phosphatase inhibitors cocktail and Protein A/G magnetic beads (Thermo Scientific, USA); anti-PKC-ζ (9372s, 1:1000, Cell Signaling, USA); anti- PKC-ι (610178 1:1000, BD, USA); anti-β-actin (A3854, 1:40000, Sigma Aldrich, USA); anti-RhoA (ab54835,1:4000, Abcam, USA); anti-Ect2 (07-1364, 1:1000, Millipore, USA); anti-β-tubulin (5346t, 1:1000, Cell Signaling, USA); anti-PARP (9532s, 1:1000, Cell Signaling, USA); Donkey anti-rabbit IgG Alexa-488 (A21206, 1:500, Invitrogen, USA); Goat anti-rabbit (170-6515, 1:2000, Bio-Rad Laboratories, USA); Goat anti-mouse (170-6516, 1:2000, Bio-Rad Laboratories, USA); Activated Rac1 pulldown kit (BK035, Cytoskeleton, USA); 96-well transwell insert and basement membrane extract (BME; both Corning Inc., Corning, NY, USA); RNA bee (Amsbio, United Kingdom); Qiagen RT Kit (205113, Qiagen, Germany); RhoA PCR primers (HP100025, Sino Biological, USA); Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) PCR primers (Eurofins, USA); NE-PER Nuclear and Cytoplasmic Extraction Reagents (78835, Thermo Fisher Scientific, USA).

5.2.2. Analysis of somatic gene mutations for ovarian cancer, CCOC and CCOC cell lines
The selection of CCOC cell lines employed the COSMIC database. Initially, the search was focused on all ovarian subtypes in the database. The search was then re-focused on clear cell carcinomas. Furthermore, the COSMIC cell line project was utilized for the analysis of mutations in TOV21G and ES-2 cells.

5.2.3. Computational analysis of aPKCs

5.2.3.1. Protein preparation

Protein model systems for PKC-ι and PKC-ζ were prepared using the Schrodinger software suite. Protein structure coordinates were downloaded from the Protein Data Bank (PDB). The PKC-ι model was generated from the PDB 3A8W entry co-crystallized with adenosine triphosphate (ATP). The apo structure, PDB 38AX, was used to cross reference conformational states from Molecular Dynamics (MD) simulations. No crystal structure currently exists of PKC-ζ, necessitating the need for homology modeling to attempt to produce a potentially viable docking model for PKC-ζ. Two PKC-ζ models were built, one utilizing SWISS-MODEL and another employing the Prime homology program using the human PKC-ζ sequence (UniProt Q05513) and the crystallized PKC-ι structures as templates. PDB systems were prepared with the Protein Preparation Wizard (PrepWizard) in Maestro. Cofactors used in crystallization (such as sulfate or phosphate ions), ligands, and additional protein dimers were deleted. Bond orders were then assigned, including disulfide bridges, and original hydrogens were deleted and later replaced to reduce bad contacts and other crystal artifacts before protonation and hydrogen bond optimization. All waters were retained for assisting in the determination of side chain protonation states and initial hydrogen bond optimization. Missing side chains were added and optimized using.
Prime. Hydrogen atoms were then added to the protein, remaining cofactors, and to any added structural waters. The program PROPKA \textsuperscript{103} was used for the prediction of protein ionization states at 7.4 pH and ProtAssign was used for hydrogen bond optimization. After automatic hydrogen assignment, visual inspection was used to flip residues and change protonation states at protein-protein interfaces if and when appropriate.

5.2.3.2. Molecular dynamics

MD simulations were performed with the Desmond MD program \textsuperscript{104-107}. A cubic simulation box was created extending at least 10Å from the protein with imposed periodic boundary conditions. TIP3P waters \textsuperscript{108} were added to solvate the simulation box and was then electrically neutralized by introducing sodium ions. The OPLS-3 all-atom force field \textsuperscript{109} was then applied to all atoms. The SHAKE algorithm \textsuperscript{110} was used to constrain all bonds in the system and the REference System Propagator Algorithm (RESPA) \textsuperscript{111} with an integration time step of 2 fs was employed. The Particle Mesh Ewald (PME) algorithm was used to calculate long-range electrostatics with a real-space cutoff of 13 Å. Van der Waals interactions were cutoff at 16 Å. The systems were simulated in an NPT ensemble using the Nose–Hoover temperature coupling scheme \textsuperscript{112} at a temperature of 310 K and a constant pressure of 1 atm using the Martyna-Tuckerman-Tobias-Klein (MTTK) barostat \textsuperscript{113,114}.

All systems were energy minimized with a truncated newtonian conjugate gradient (TNCG) method \textsuperscript{115} followed by multiple restrained minimizations to randomize systems before equilibration and final simulation. Heavy atoms of the protein were held fixed during heating for an initial 12 ps NVT ensemble simulation at 10 K with the Berendsen thermostat \textsuperscript{116}. This was
followed by simulations at 1 atm in the NPT ensemble for 12 ps at 10 K and 24 ps at 310 K. Unrestrained equilibration MD was then performed for 24 ps at 310 K and 1 atm. Finally, unconstrained production MD was performed on PKC-ι and PKC-ζ systems for 250 ns. Energies were recorded every 2 ps and trajectory frames were recorded every 5 ps.

Final system equilibration was determined by the observation of asymptotic behavior of the potential energy, Root Mean Square Deviation (RMSD), and Radius of Gyration (Rg) profiles and visual inspection of trajectories guided by Root Mean Square Fluctuation (RMSF) profiles (Supplementary Material).

5.2.3.3. Consensus docking

After equilibration was determined, a hierarchical average linkage clustering method based on RMSD was utilized to determine an average representative structure for the equilibrated PKC-ι system. The program PROPKA was then implemented again on the equilibrated structure to test consistency of side chain protonation states at 7.4 pH. The representative structure was then used for consensus docking incorporating five diverse and complimentary docking methods described below. By applying these varied energy scoring methods, the weaknesses of each method can be identified for a particular model and error statistically minimized, yielding a more accurate summary of ligand binding dispositions and affinities.

As a check for the placement of the grids used in the docking studies and for further analysis of the binding cavities for the ATP binding site and the potential allosteric site, Schrödinger's SiteMap program \textsuperscript{117–119} was employed. SiteMap searches the protein structure for likely binding
sites and highlights regions within the binding site suitable for occupancy by hydrophobic groups, hydrogen-bond donors, acceptors, or metal-binding functionality of the ligand.

The ligands ICA-1S, ζ-Stat, and ATP were prepared using the program LigPrep and the OPLS-3 all-atom force field was applied to all ligand atoms.

5.2.3.4. Rigid receptor docking (RRD)

Rigid docking simulations were performed by Glide. Glide uses a GlideScore fitness function based on Chemscore for estimating binding affinity, but includes a steric-clash term, adds buried polar terms to penalize electrostatic mismatches, and modifies other secondary terms. Docking simulations used both the standard precision (SP) and extra precision (XP) methods. XP mode is a refinement algorithm enforced only on good ligand poses. Sampling is based on an anchor and refined growth strategy and the scoring function includes a more complete treatment of some of the SP energetic terms, such as the solvation and hydrophobic terms. Docking grids were defined by a rectangular ligand atom inclusion outer box of 22Å and ligand centroid constraint inner box of 10Å in the x, y, and z directions originating from the binding cavity centroid defined by SiteMap for the proposed allosteric site and by the original co-crystallized ATP ligand centroid for the ATP binding site.

5.2.3.5. Induced fit docking (IFD)

The IFD methodology incorporates both the docking program Glide to account for ligand flexibility and the Refinement module in the Prime program to account for receptor
flexibility. The Schrödinger IFD protocol attempts to model induced-fit effects from alterations in binding site conformation due to ligand binding in order to increase accuracy of binding affinity estimates and prediction of possible binding modes.

The position of the cubic docking grid for the ATP binding site was centered on the original co-crystallized ligand centroid and from the binding cavity centroid defined by SiteMap for the proposed allosteric site with a box size of 29 Å for both. A constrained minimization of the receptor was performed with an RMSD cutoff of 0.18 Å. An initial softened potential Glide docking of the ligand set was then implemented with the standard precision (SP) mode and a van der Waals scaling factor of 0.5 was applied to the non-polar atoms of the receptor and ligands. The resulting top 20 poses of the ligands were used to sample protein plasticity by conformational searches and minimizations of binding pocket residues within 6 Å of any ligand pose for all complexes obtained. The new receptor conformations were then redocked using complexes within 30 kcal/mol from the best scoring structure. Glide docking parameters for this step were reset to the default hard potential function with a van der Waals scaling of 1.0 and SP mode.

The estimated binding affinity of each complex was reported in the GlideScore and used to compare differences between each ligand while the Emodel score is used to inter-compare poses of the ligands. Emodel places more significance on weighting force field components (electrostatic and van der Waals energies), making it better for comparing conformers as opposed to comparing chemically-distinct species.
5.2.3.6. Quantum polarized ligand docking (QPLD)

To account for ligand polarization upon binding, Quantum Mechanics/Molecular Mechanics (QM/MM) docking was performed by the Schrödinger QM-Polarized Ligand Docking Protocol (QPLD)\textsuperscript{129-132}. The protocol first employs RRD using Glide in SP mode. In this step, the top five poses of each ligand in the initial RRD were used. Potential ligand polarization induced by the protein were then calculated with QSite\textsuperscript{131,133,134} at the B3LYP/6-31G* level. The ligand force fields were then reconstructed with QM/MM modified charges, redocked, and five poses of each ligand were saved for evaluation.

5.2.3.7. Molecular mechanics and generalized born surface area (MM/GBSA)

The MM/GBSA method combines molecular mechanics energy terms and implicit solvation models to calculate the binding-free energy based on docking complexes. The protocol, implemented by the Prime MM-GBSA module, calculates optimized free energies for the free protein and free ligand and references them with the original bound complex energy\textsuperscript{135}. Polar contributions are calculated using the Generalized Born (GB) model\textsuperscript{136}, an implicit solvent model is based on a variable dielectric surface Generalized Born (VD-SGB) approach, where the variable dielectric value for each residue was fit to a large number of side-chain and loop predictions while the non-polar energy is estimated using the solvent accessible surface area (SASA)\textsuperscript{137}. The simulation was performed based on receptor–ligand complex structures obtained from induced fit docking. The obtained ligand poses were minimized using the local optimization feature in Prime, whereas the energies of complex were calculated with the OPLS-3 force field and Generalized-Born/Surface Area continuum solvent model\textsuperscript{138}. During the simulation process, the ligand strain
energy is also considered. A known issue with MM/GBSA is that scores do not accurately reproduce absolute physical binding affinities but display great efficacy at ranking compounds in a relative manner \(^{139-142}\). We developed a correlation function using a single-layer logistic regression to rescale MM/GBSA scores based on the other docking score algorithms. This retains the ranking accuracy of MM/GBSA and allows us to proportion the results in a minimally biased and physically relevant manner.

5.2.3.8. Virtual target screening (VTS)

VTS is a system designed to virtually screen a molecule of interest to a large library of protein structures. The current protein library consists of 1,451 structures with a concentration of kinases. The system is calibrated with a set of small drug-like molecules are docked against each structure in the protein library to produce benchmark statistics. VTS was employed as a theoretical assay of potential kinase activity and gauge of potential biological promiscuity. The calibration procedure allows the analysis to accurately predict inhibitor–kinase binding affinities when \(K_d < 10 \, \mu\text{M}\) (defining a hit) and \(K_d \geq 10 \, \mu\text{M}\) are both considered (72% accuracy in the best case) \(^{143}\). Therefore, the VTS system is able to robustly discriminate protein binders from nonbinders and give some inclination as to potential binding promiscuity of the molecule of interest with respect to the protein group tested.

5.2.4 Cell culture
The CCOC cells lines TOV21G and ES-2 were cultured in MCDB 131: Media 199(1:1 ratio) and McCoy’s medium, respectively, supplemented with 10% FBS, 100 units/mL of penicillin and 100μg/mL of streptomycin. The immortalized normal human endometrial stromal cell line, SHT290, was maintained in F12K: Media 199 (1:1 ratio) and supplemented with 5% FBS, 0.1% Mito+, 2μg/mL of human insulin, 100 units/mL of penicillin and 100μg/mL of streptomycin. All cell cultures represented were passaged less than 10 times. Cell cultures were maintained in an incubator at 37 °C and 5% CO2 atmosphere.

5.2.5 Atypical PKC expression during rapid growth and cell cycle arrested

SHT290, TOV21G and ES-2 cells were seeded into 100mm plates and grown to 50% confluency. Rapidly growing cells were harvested at 50% confluence and the cell cycle arrested cells were serum starved for an additional 48 hrs in reduced serum medium Opti-MEM I (N=3).

5.2.6 Preliminary screening of TOV21G and ES-2 with ICA-1S and ζ-Stat

TOV21G and ES-2 cells were seeded into 100mm plates and grown to 50% confluency. Control cells were treated with equal amounts of DMSO (vehicle) and treatment cells were treated with 3μM concentrations of ICA-1S and ζ-Stat for 24, 48 and 72 hrs (N=3). Cells were collected at 24, 48 and 72 hrs and run on a Western blot.

5.2.7 Cell viability assay
Cells were seeded in to 96-well plate at 800 cells per well with 200µL of media. Cells were treated with different concentrations of DMSO (vehicle to match treatment, \(N=12\)) and \(\zeta\)-Stat (1µM, 3µM, 5µM and 10µM, \(N=3\)). After 72 hrs of treatment, the cell viability was analyzed using WST-1 at wavelengths 450 and 630 nm. The plates were read on a BioTek SynergyHT microplate reader. Standard curves for each cell line was generated based on the number of cells added and the absorbance recorded.

5.2.8 Cell lysate collection

Media was extracted from the vessel and 250µL of lysis buffer [Pierce® Immuno Precipitation Lysis Buffer, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol] with protease and phosphatase inhibitors was added to the plates. Cells were scraped and collected from the vessel (on ice) and the suspension was sonicated for 3 x 5 s cycles on ice. The samples were centrifuged at 4°C at 12,000 x g for 15 ms. The supernatant (cell lysate) was removed from the cellular membrane pellet and placed in a secondary micro centrifuge tube. Protein content was measured per Bradford Assay Reagent on a BioTek SynergyHT microplate reader at 595nm.

5.2.9 Western Blot Analysis

Cell lysates containing equal amounts of protein (20-40µg) were loaded in each lane and run on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted to a 0.45µm nitrocellulose membrane. Cell lysates were probed with the primary antibodies against PKC-\(\zeta\), PKC-\(\iota\), RhoA and \(\beta\)-actin (for loading control) and re-probed with secondary antibodies
for development. Immunoreacted bands were visualized by enhanced chemiluminescence per the manufacturer’s instructions [Thermo Scientific™ SuperSignal™ West Pico PLUS Chemiluminescent Substrate]. Band densitometry was performed with ImageJ FIJI software and normalized densities were derived by the ratio of the protein of interest over the control (β-actin).

5.2.10 Endpoint PCR

The mRNA from TOV21G and ES-2 cells treated with vehicle control and 3μM of ζ-Stat was isolated via the manufacture’s protocol for RNAbee. The mRNA was then quantified using the BioTek Synergy HT Take5 plate and cDNA was synthesized using the Qiagen reverse transcription (RT) kit per the manufacturer’s protocol. Endpoint polymerase chain reaction (PCR) was performed with primers for RhoA and the housekeeping gene GAPDH (forward 5’CCA-CCC-ATG-GCA-AAT-TCC-ATG-GCA-3’ and reverse 5’TCT-AGA-CGG-CAG-GTC-AGG-TCC-ACC-3’). PCR products were analyzed on an agarose gel (N=3).

5.2.11 Rac1 activation assay

Cells were cultured in 150mm plates and lysed as previously described. The cells were treated with DMSO (control) and 10μM of ζ-Stat for 72 hrs and harvested (N=4). Glutathione S-transferase (GST) was tagged to the protein binding domain (PBD) of p21 activated kinase (PAK). A positive control and negative control were performed to determine assay efficiency. Briefly, 500μg of protein were balanced in 200μL of cell lysis for each sample. The positive control
received 200µM of non-hydrolyzable guanosine 5'-O-[gamma-thio] triphosphate (GTPγS) and the negative control received 200µM of guanosine diphosphate (GDP). These samples were incubated at room temperature (RT) for 15 ms. All samples (positive, negative, DMSO control and treatment) were incubated with GST-tagged PAK-PBD agarose beads for 1 hr 4°C. These samples were pelleted at 5,000 x g (at 4°C) and washed with Wash Buffer. The pelleted beads were re-suspended with 20µL of 2X Laemmli sample buffer and boiled at 95°C for 2 ms.

5.2.12 Preparation of cytoplasmic and nuclear extracts

TOV21G and ES-2 cells were seeded in 100mm tissue culture plates (1.5x10^5). Cells were treated for 72 hours with 10μM ζ-Stat (DMSO control) and harvested with trypsin. The instructions provided by the manufacturer were followed to fractionate the cytoplasmic and nuclear portions. The extracts were analyzed via immunobLOTS and translocation of Ect2 was investigated.

5.2.13 Fluorescent microscopy

TOV21G cells were seeded into 4 chambered slides at a 500 cells per well concentration and after 24 hrs, were treated with a vehicle control (DMSO) and 10μM of ζ-Stat every 24 hrs for 72 hrs. Cells were then fixed with 4% paraformaldehyde for 15 ms and immunostained with Ect2 antibody at 4°C overnight with light agitation. The slides were incubated with Alexa 488 rabbit secondary antibody for 1 hr at room temperature RT. Subsequently, the slides were stained with Phalloidin conjugated to Texas red dye for 30 ms at RT, mounted with solution containing 4',6-
diamidino-2-phenylindole (DAPI) and imaged on an Olympus BX53 Digital Upright Fluorescent Microscope.

5.2.14 Invasion assay

For the evaluation of invasion, cells were serum starved for 48 hrs, followed by detachment and plating into the upper chamber of a 96-well (8 µm) transwell permeable support, coated with 0.1X BME. Serum (10%) containing media was loaded into the lower chamber as a chemoattractant. Subsequently, TOV21G cells at the upper chamber were treated with 10µM of ζ-stat for 24 hr (N=4). Two experimental treatment groups for the cells were performed: Control (DMSO vehicle) and treatment. The invasive cells that passed into the lower chamber were then fixed with 4% paraformaldehyde, stained with 2% crystal violet in 2% ethanol, washed with distilled water and photographs were captured after drying using a light microscope Motic AE31E. For migration, a similar protocol was followed except without coating the transwell insert with BME. The assay was quantified with ImageJ FIJI software.

5.2.15 ζ-Stat in-vivo

The following experiments outline the investigations of ζ-Stat in TOV21G clear cell carcinoma ovarian xenografts. We have an Institutional Animal Care and Use Committee (IACUC) approved by Adrienne Booker for the discussed studies. The study involved 12 athymic female nude mice weighing between 20-25g and >10 weeks of age. The 12 mice were divided into two groups after TOV21G cells were implanted (1 x 10⁶ cells/per mouse flank in 0.2mL of media). The first group
was the vehicle control group (N=6), which received 100 µL of 1x DPBS. The second group (N=6) was injected with 100 µL of 20mg/kg of ζ-Stat dissolved in 1x DPBS. The tumor volume was calculated using the formula: length x width x width x \( \frac{1}{2} \). Three days after the implantation of the cells, tumors were treated as of day 0. The treatments were administered every other day subcutaneously intra-tumor and around the tumor site for 35 days.

At the end point of the experiment, tumors and heart serum were harvested. Tumors were imaged and measured, and blood serum was analyzed for enzymatic levels of glucose (GLU), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALKP) at the Moffitt Research Facility. Briefly, blood chemistry analysis was performed by initially collecting whole blood in a serum separator tube, which then sat for 20 ms at RT before centrifugation. Once the blood was centrifuged the serum was separated and placed in a specialized sample cup made for the IDEXX CatalystDx. The cup containing the serum and the desired chemistry slides were then placed into the CatalystDx for analysis.

5.2.16 In-vivo tumor fractionation

Tumors were re-suspended and sonicated in 700 µL of homogenization buffer [Pierce® Immuno Precipitation Lysis Buffer, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol]. The suspension was sonicated for 3 x 5 sec cycles on ice. The tumor suspensions were centrifuged at 12,000 x g for 15 mins to obtain cell extracts. The supernatant was removed and protein content was measured using Bradford reagents.
5.2.17 Statistical analysis

R studio software was used for statistical analyses. A one-way ANOVA with a Tukey’s multiple comparisons test was performed for Western Blot analyses and cell viability. A two-tailed unpaired student T-test was utilized for the statistical significance of the particle counts for cell migration and invasion, day to day tumor volume, mouse body weight and individual enzyme levels. The Pearson’s Correlation Coefficient (PCC) was utilized for co-localization and was analyzed using ImageJ FIJI software, using the Coloc2 plugin.
References


47. COSMIC. Catalogue of Somatic Mutations in Cancer. (2016).


74. Bordoli, L., Kiefer, F., Arnold, K., Benkert, P., Battey, J. & Schwede, T. Protein structure


94. GraphPad Software. GraphPad QuickCalc: linear regression calculator. (2018).


Appendix A

Publishers granted permissions to reproduce those contents and the emails and online copyright agreements.

---

Spandidos Publications Esub no. : 203654

1 message

ol@osats.spandidos-publications.com <ol@osats.spandidos-publications.com>  
Reply-To: ol@spandidos-publications.com  
To: maavedo@mail.usf.edu  
Cc: tracessp@mail.usf.edu  

Tue, Feb 4, 2020 at 4:47 AM

Dear Dr Mildred Acevedo-duncan,

Our reference: OL-1818-203654

Title: Analysis of PKC-zeta protein levels in normal and malignant breast tissue subtypes

By: Tracey Smalley

Thank you for your email.

The paper should be cited appropriately in your dissertation.

Yours sincerely,

Spandidos Publications
2.1. Open access and copyright

All Frontiers articles from July 2012 onwards are published with open access under the CC-BY Creative Commons attribution license (the current version is CC-BY, version 4.0 http://creativecommons.org/licenses/by/4.0/). This means that the author(s) retain copyright, but the content is free to download, distribute and adapt for commercial or non-commercial purposes, given appropriate attribution to the original article.

Upon submission, author(s) grant Frontiers an exclusive license to publish, including to display, store, copy and reuse the content. The CC-BY Creative Commons attribution license enables anyone to use the publication freely, given appropriate attribution to the author(s) and citing Frontiers as the original publisher. The CC-BY Creative Commons attribution license does not apply to third-party materials that display a copyright notice to prohibit copying. Unless the third-party content is also subject to a CC-BY Creative Commons attribution license, or an equally permissive license, the author(s) must comply with any third-party copyright notices.

Preprint Policy

Frontiers supports preprint policy encourages full open access at all stages of a research paper, to share and generate the knowledge researchers need to support their work. Authors publishing in Frontiers journals may share their work ahead of submission to a peer-reviewed journal, as well as during the Frontiers review process, on repositories or preprint servers (such as ArXiv, PeerJ Preprints, OSF and others), provided that the server imposes no restrictions upon the author’s full copyright and re-use rights. Also note that any manuscript files shared after submission to Frontiers journals, during the review process, must not contain the Frontiers logo or branding.