Chapter

Atypical Protein Kinase Cs in Melanoma Progression

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Abstract

Melanoma is one of the fastest growing types of cancer worldwide in terms of incidence. To date, reports show over 92,000 new cases in the United States in 2018. Previously, we introduced protein kinase C-iota (PKC-ι) as an oncogene in melanoma. PKC-ι promotes survival and cancer progression along with PKC-zeta(ζ). In addition, we reported that PKC-ι induced metastasis of melanoma cells by increasing Vimentin dynamics. Our previous results showed that PKC-ι inhibition downregulated epithelial-mesenchymal transition (EMT), while inducing apoptosis. In this chapter, we summarized these findings which were based on the *in-vitro* applications of five specific atypical PKC (aPKC) inhibitors. In addition, the underlying mechanisms of the transcriptional regulation of PRKCI gene expression in melanoma is also discussed. Results demonstrated that c-Jun promotes PRKCI expression along with Interleukin (IL)-6/8. Furthermore, forkhead box protein O1 (FOXO1) acts as a downregulator of PRKCI expression upon stimulation of IL-17E and intercellular adhesion molecule 1 (ICAM-1) in melanoma cells. Overall, the chapter summarizes the importance of PKC-ι/ζ in the progression of melanoma and discusses the cellular signaling pathways that are altered upon inhibitor applications. Finally, we established that aPKCs are effective novel biomarkers for use in the design of novel targeted therapeutics for melanoma.

Keywords: PKC-iota (ι), PKC-zeta (ζ), metastasis, FOXO1, c-Jun

1. Introduction

The protein kinase C (PKC) is a family of Ser/Thr kinases which are involved in transmembrane signal transduction pathways triggered by various extra and intracellular stimuli [1]. Over time, more information has become available since the 1st discovery of PKCs in 1970s. Activation of PKCs may depend on calcium ions and cofactors like the lipid metabolite diacylglycerol (DAG) and phosphatidylserine (PS) [2, 3]. The PKC family consists of fifteen isozymes which are grouped into three on the basis of their co-factor requirements [4, 5]. First group is the conventional PKCs (cPKC) which includes the isoforms alpha (α), beta I (βI), beta II (βII) and gamma (γ) and they require calcium ions, DAG and phospholipids for the activation. Second group is the novel PKCs (nPKC) and it includes delta (δ), epsilon (ε), eta (η) and theta (θ). These are calcium ion independent but dependent on DAG and phospholipids. The aPKC isozymes are the third group, which are independent of Calcium and DAG for their activation. PKC-ζ and PKC-ι in humans

(lambda (λ) is the mouse homologs of iota) are the three aPKCs. Protein kinase D, mu (μ) and some PKC-related kinases (PRK1, PRK2 and PRK3), known as PKN are also considered as PKCs [6].

PKCs have extremely conserved carboxyl-terminal catalytic domain (kinase domain) and PKC isozymes differ from each other on the basis of their amino-terminal (N-terminal) regulatory domain. The N-terminal domain is very important for secondary messenger binding, recruiting the enzyme to the membrane and protein-protein interactions [2]. The pseudosubstrate (PS) domain is located at the N-terminal. PS has a peptide-sequence similar to that of a substrate but lacks alanine in the phosphoacceptor position. In the inactive form of PKCs, the PS prevents complete activation of PKC by blocking the substrate binding pocket [7]. The PS is released upon activation [8, 9]. The activation of PKCs typically involves a cascade of three coordinated phosphorylation events [10, 11]. First, phosphorylation takes place at the activation loop triggered by phosphoinositide-dependent kinase-1 (PDK-1) [12–14]. This initiates a chain reaction that involves autophosphorylation at the turn motif that further stimulates the autophosphorylation at hydrophobic motif of N-terminal [13]. The autophosphorylation at hydrophobic motif is the third and concluding step of the activation.

Atypical PKCs contains two structurally and functionally distinct isozymes in human, PKC-ι and PKC-ζ. The amino acid sequences in both PKC-ι and PKC-ζ are very similar to each other [15, 16]. PKC-ι is encoded by the PRKCI gene and PKC-ζ is encoded by the PRKCZ gene. They are believed to be involved in cell cycle progression, tumorigenesis, cell survival and cell migration of carcinoma cells. Additionally, aPKCs play important roles in insulin-stimulated glucose transport [16, 17]. PKC-ι specifically has a strong influence on cell cycle progression. It is also involved in changing cell polarity during cell division [17]. Lung cancer cell proliferation is highly dependent on the PKC-ι level since it increases tumor cell proliferation by activating the ERK1 pathway [15]. PKC-ι and PKC-ζ are expressed in both transformed and malignant melanoma [18]. Overexpression of PKC-ι plays an important role in the chemoresistance of leukemia [19]. PKC-ι is involved in glioma cell proliferation by regulating by phosphorylation of cyclin-dependent kinase activating kinase/cyclin-dependent kinase 7 pathway [20, 21]. A very important study by Selzer et al., investigated the presence of 11 PKC isoforms in 8 different melanoma metastases, 3 normal melanocyte cell lines and 3 spontaneously transformed melanocytes along with several melanoma tumor samples. PKC-ζ and PKC-ι

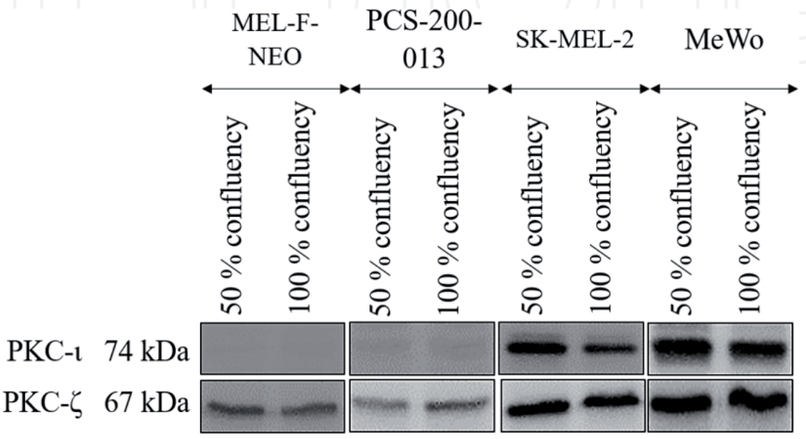

Figure 1.
aPKC expression comparison of normal melanocytes and melanoma cell lines. The expression of PKC-ι and PKC-ζ was reported at approximately 50 and 100% confluency for PCS-200-013 and MEL-F-NEO normal melanocytes against SK-MEL-2 and MeWo metastatic melanoma cells. Western blots were conducted with 50 μg of total proteins loaded in each lane and the complete procedure was adapted from Ratnayake et al. [67].

were found in all transformed melanocytes and melanoma metastases samples in very high levels. PKC-ζ was also found in normal melanocytes in low levels. **Figure 1** demonstrates a comparison of the aPKC expression in two normal melanocyte cell lines (PCS-200-013 and MEL-F-NEO) against two melanoma cell lines (SK-MEL-2 and MeWo) which were used for our studies in Acevedo-Duncan's laboratory at the University of South Florida. As demonstrated in **Figure 1**, normal melanocytes did not show detectable levels of PKC-ι compared to the larger expression observed in SK-MEL-2 and MeWo cell lines. Moreover, PKC-ζ expression was very low in both normal melanocyte cell lines compared to heightened expression in melanoma cells. These results supported the expression patterns demonstrated by patient samples as described in Selzer et al. [18]. All these results indicate a strong relationship between aPKCs and melanoma progression. Here, we discuss our key findings of our recent research on melanoma owing to its relationship with aPKCs in a detailed manner.

2. Atypical PKCs promote cell differentiation, survival of melanoma cells via NF-κB and PI3K/AKT pathways

Nuclear factor kappa-light-chain-enhancer of activated B (NF-κB) and phosphatidylinositol 3-kinase and protein kinase B (PI3K/AKT) pathways are often hyper-activated in many different cancers in order to promote cellular differentiation, growth and survival. Overexpression of aPKCs is often associated with anti-apoptotic effects in many cancers. We have published outcomes of *in-vitro* treatments of aPKC specific inhibitors in which, treatments decreased melanoma cell population markedly compared to normal melanocytes [22–25]. These results confirm that melanoma cellular functions are highly dependent on aPKCs, but normal melanocytes do not depend on aPKCs.

Our recent publications describe the *in-vitro* effects of five aPKC inhibitors on melanoma cell lines compared to normal melanocytes [22, 23]. 2-Acetyl-1, 3-cyclopentanedione (ACPD) and 3,4-diaminonaphthalene-2,7-disulfonic acid (DNDA) are specific to both PKC-ι and PKC-ζ while [4-(5-amino-4-carbamoylimidazol-1-yl)-2,3-dihydroxycyclopentyl] methyl dihydrogen phosphate (ICA-1T) along with its nucleoside analog 5-amino-1-((1R,2S,3S,4R)-2,3-dihydroxy-4-methylcyclopentyl)-1H-imidazole-4-carboxamide (ICA-1S) which are specific to PKC-ι and 8-hydroxy-1,3,6-naphthalenetrisulfonic acid (ζ-Stat) is specific to PKC-ζ. These compounds were identified from the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) database using molecular docking simulations. "AutoDockTools" and "AutoDock Vina" programs were used for the docking simulation by selecting structural pockets in PKC-ι and PKC-ζ which were compatible with small drug like molecules. Sixteen different pockets were identified on PKC-ι and PKC-ζ structures using "fpocket," a very fast open source protein pocket (cavity) detection system based on Voronoi Tessellation. We confirmed the presence of a potentially druggable allosteric site in the structure of PKC-ι using solved crystal structure of PKC-ι. The pocket located in C-lobe of the kinase domain, is framed by solvent exposed residues of helices αF-αI and the activation segment. PKC-ι inhibitors were predicted to interact with this site with moderate affinity based on molecular docking. Combinations of drugs targeting the ATP binding site and allosteric sites would be expected to more effectively inhibit cancer cell growth [23]. More details about other aPKC inhibitors form different research groups can be found in the latter portion of the chapter.

All five inhibitors were cytostatic to malignant cells rather than cytotoxic. Cells underwent growth arrest before apoptotic stimulation. Regardless, ICA-1S and ICA-1T showed a minor toxicity towards malignant melanoma cells, suggesting

that all inhibitors were effective against malignant cells without harming normal cells. This is an indication that melanoma cells heavily rely on aPKCs to remain viable which was observed in some other cancers [19, 20, 26, 27]. These previous reports show that overexpression of aPKCs have an anti-apoptotic effect [15, 19–21, 28, 29]. Our two previous publications on the applications of aPKC specific inhibitors report apoptosis analysis on MeWo and SK-MEL-2 cells. The data confirmed that inhibition of aPKCs lead to induce apoptosis [22, 23]. Increase in Caspase-3, increase in poly ADP ribose polymerase (PARP) cleavage, and a decrease in B-cell lymphoma-2 (Bcl-2) all indicate apoptosis stimulation [30–33]. All five inhibitors have demonstrated similar pattern on these markers. But, increase in Caspase-3 levels is not always a direct indication of inducing the apoptosis due to the tight binding of cleaved Caspase-3 with X-linked inhibitor of apoptosis protein (XIAP). XIAP undergoes auto-ubiquitylation, but this process delays apoptosis until all XIAP is removed [34]. On the other hand, PARP is a known downstream target for Caspase-3, therefore we have also tested direct PARP and cleaved PARP levels upon inhibitor treatments. PARP cleavage increases upon inducing the apoptosis [35]. Bcl-2 inhibits Caspase activity by preventing Cytochrome c release from the mitochondria and/or by binding to the apoptosis-activating factor (APAF-1). In our studies, PKC-ι and PKC-ζ inhibition decreased Bcl-2 levels which depicted an increase in apoptotic activity in both SK-MEL-2 and MeWo cell lines. These data confirms that aPKCs have an anti-apoptotic effect in the tested melanoma cells.

PI3K/AKT mediated NF-κB activation is a major anti-apoptotic pathway, wherein aPKCs play a role in releasing NF-κB complex to translocate to the nucleus and promote cell survival. Win et al. reported that PKC-ζ actively upregulates the activation of NF-κB nuclei translocation thereby inducing cancer cell survival in prostate cancer cells [36, 37]. In addition, PI3K stimulates IκB kinase (IKKα/β) through activation of AKT by phosphorylation at S473 or S463, which ultimately stimulates translocation of NF-κB complex into the nucleus, heightening cell survival [38]. Phosphatase and tensin homolog (PTEN) regulates the levels of PI3K. Phosphorylation at S380 leads to the inactivation of PTEN, thereby increasing the levels of PI3K followed by enhancement in phosphorylated AKT (S473/S463). Our data indicates that inhibition of PKC-ι and PKC-ζ expressively decreased the levels of phosphorylated PTEN and phosphorylated AKT [23]. This specifies that PKC-ζ and PKC-ι may upregulate the PI3K/AKT pathway to induce cellular survival of melanoma cells. Additionally, we tested the levels of NF-κB translocation by separating the nuclear extracts from the cell lysates and found that NF-κB levels in the nuclei decreased upon aPKC inhibition. This suggested that translocation of activated NF-κB into nuclei was blocked as a result of inhibition of aPKCs. Furthermore, we also found that aPKC inhibition increased the levels of inhibitor of kappa B (IκB) while decreasing the levels of phosphorylated IκB (S32) and phosphorylated IKKα/β (S176/180), confirming that both PKC-ι and PKC-ζ play a role in phosphorylation of IKKα/β and IκB: increased levels of IκB therefore remain bound to NF-κB complex and prevent the translocation to the nucleus to promote cell survival (**Figure 2**). As summarized in **Figure 2**, our data also demonstrate the effects of TNF-α stimulation on the expression of aPKCs [23]. TNF-α is a cytokine, involved in the early phase of acute inflammation by activating NF-κB. TNF-α stimulation significantly increased NF-κB levels in both cytosol and nuclei. Increased NF-κB production promotes increases in total and phosphorylated aPKCs and increased the levels of Bcl-2, which enhanced melanoma cell survival. We observed amplified levels of IκB and NF-κB, which together enhanced the phosphorylation of IκB due to the augmented levels of aPKCs [23]. On the other hand, PI3K/AKT signaling can be diminished by inhibiting aPKCs via downregulation of NF-κB. These results confirm that both PKC-ζ and PKC-ι are rooted in cellular survival via NF-κB and PI3K/AKT pathways.

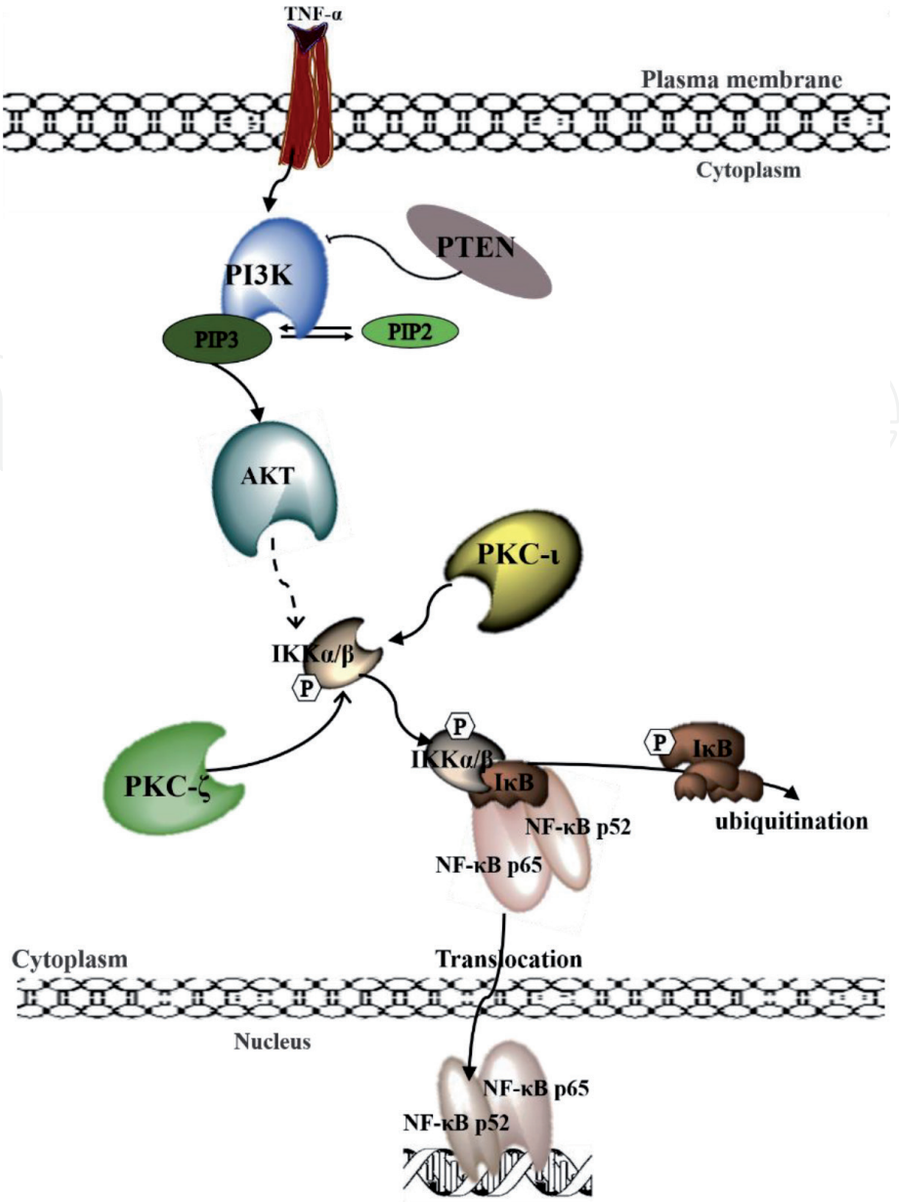

Figure 2.
A schematic summary of the involvement of PKC-ι and PKC-ζ in melanoma progression via NF-κB and PI3K/AKT pathways. Upon extracellular stimulation with TNF-α, activation of AKT through PIP_3 takes place as a result of inactivation of PTEN. Activated AKT pathway can lead to cell survival, rapid proliferation and differentiation which are critical parts of melanoma progression. AKT could indirectly stimulate NF-κB pathway along with PKC-ι and PKC-ζ in which they play a stimulatory role on IKK-α/β in order to promote the releasing the NF-κB complex from IκB to translocate into nucleus.

3. PKC-ι promotes metastasis by promoting epithelial-mesenchymal transition (EMT) and activating Vimentin

Throughout EMT, epithelial cells lose apical-basal polarity, remodel the extra cellular matrix (ECM), rearrange the cytoskeleton, drive changes in signaling programs that control the cell shape maintenance and adapt gene expression to obtain mesenchymal phenotype, which is invasive and increases individual cell motility [39]. EMT's key features comprise downregulation of E-cadherin to destabilize tight junctions between cells and upregulation of genes such as Vimentin that may assist mesenchymal phenotype.

Vimentin is a very important structural protein which belongs to the family of type III intermediate filament proteins. Intermediate filaments (IFs) make up a vast network of interconnecting proteins between the plasma membrane and the

nuclear envelope and convey molecular and mechanical information between the cell surface and the nucleus. IF protein expression is cell type and tissue specific. Mesenchymal cells, fibroblasts, lymphocytes and most types of tumor cells express Vimentin [40, 41]. Vimentin is essential for organizing microfilament systems, changing cell polarity, and thereby changing cellular motility. Moreover, increased Vimentin expression during EMT is a hallmark of metastasis which plays a very important role in gaining rear-to-front polarity for transforming epithelial cells. In addition to EMT, Vimentin expression is observed in cell mechanisms involved in cellular development, immune response and wound healing [22, 23, 42].

Vimentin is activated via phosphorylation. Various kinases such as; RhoA kinase, protein kinase A, PKC, Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), cyclin-dependent kinase 1 (CDK1), RAC-alpha serine/threonine-protein kinase (AKT1) and RAF proto-oncogene serine/threonine-protein kinases (Raf-1-associated kinases) have been shown to play a role in regulation of Vimentin via phosphorylation. Studies show that amino acid sites S6, S7, S8, S33, S38 (same as S39 since some literature use M as the starting amino acid of Vimentin), S55 (or S56), S71, S72, and S82 (S83) amongst others, serve as specific phosphorylation sites on the head region of Vimentin [41, 43–50].

Our previous reports demonstrated the effects of aPKC inhibition on melanoma cell migration and invasion [22, 23]. Migration and invasion studies in cancer research are very important because the main cause of death in cancer patients is related to metastatic progression. For cancer cells to spread and distribute throughout the body, they must migrate and invade through ECM, undergo intravasation into blood stream and extravasation to form distant tumors [51]. ACPD and DNDA treated samples demonstrated a reduction of melanoma motility but it was not conclusive which aPKC is responsible for upregulating metastasis, since both ACPD and DNDA inhibit PKC-ι and PKC-ζ [22]. This was solved using specific PKC-ι inhibitors (ICA-1S and ICA-1T) and a PKC-ζ specific inhibitor ζ-Stat. Migration and invasion were markedly reduced for samples treated with ICA-1T and ICA-1S compared to ζ-Stat treated samples, suggesting that PKC-ι inhibition significantly diminishes melanoma cell migration and invasion suggesting that only PKC-ι is involved in EMT in melanoma [23]. aPKC/Par6 signaling is known to stimulate EMT upon activation of TGF-β receptors in lung cancer cells. TGF-β activated aPKC/Par6 stimulates degradation of RhoA which leads to the depolymerization of filamentous actin (F-actin) and loss of epithelial structural integrity resulting a reduction in cell-cell adhesion [52]. RhoA is a GTPase, which promotes actin stress fiber formation thereby maintains cell integrity. Furthermore, TGFβ upregulates Zinc finger protein SNAI1 (SNAIL1) and Paired related homeobox-1 (PRRX1) transcription factors that drive genetic reprogramming to facilitate EMT [53]. Cells lose E-cadherin while gaining Vimentin during this process. We have recently reported that inhibition of PKC-ι using ICA-1T and ICA-1S significantly increased the levels of E-cadherin and RhoA while decreasing total and phosphorylated Vimentin (S39) and Par6. None of these protein levels were significantly changed as a result of PKC-ζ inhibition. We also reported that TGFβ treatments increased the expression of PKC-ι, Vimentin, phosphorylated Vimentin and Par6 while decreasing E-cadherin and RhoA [23]. These results confirmed the involvement of PKC-ι in EMT stimulation. Immunoprecipitation of PKC-ι confirmed a strong association with Par6 in both melanoma cells which was confirmed with reverse-immunoprecipitation of Par6. Previously published reports state that both aPKCs associate with Par6 and phosphorylate at S345 [54]. Interestingly, only PKC-ι showed an association with Par6, which confirmed that PKC-ι is a major activator of EMT in melanoma. In addition, immunoprecipitation of PKC-ι and Vimentin strongly confirmed an association between PKC-ι and Vimentin [22]. *si*RNA knockdown of PKC-ι and

PKC-ζ, immunofluorescent staining and real time quantitative polymerase chain reaction (RT-qPCR) techniques were also used to study the association of Vimentin with PKC-ι. Our immunofluorescence staining revealed that the shape of melanoma cells significantly changed upon inhibition of PKC-ι. Both Vimentin and PKC-ι levels were relatively low in ICA-1T treated cells in comparison to their respective controls. In addition, invasive characteristics such as formation of lamellipodia, filopodia and invadopodia were distinctively visible in both controls, though they were not apparent in PKC-ι inhibited cells. Reduction of nuclei volume and cell size, also confirmed the growth retardation we observed in melanoma cells upon aPKC inhibitor treatments that had resulted in lesser growth in treated cells. As observed in qPCR experiments, treatments with PKC-ι specific inhibitors ICA-1T and ICA-1S, depicted a corresponding downregulation of PKC-ι suggested that PKC-ι plays a role in its own regulation [23]. This is further discussed in the next topic in Part 4.

Phosphorylation of Vimentin at S39 is required for its activation and inhibition of PKC-ι diminishes this activation process. The reduced levels of total Vimentin observed in Western blots for ICA-1T and ICA-1S treated cells indicate that without PKC-ι, unphosphorylated Vimentin undergoes rapid degradation. In addition to activating Vimentin, PKC-ι appears to play a role in regulating Vimentin expression in some carcinoma cells [55].

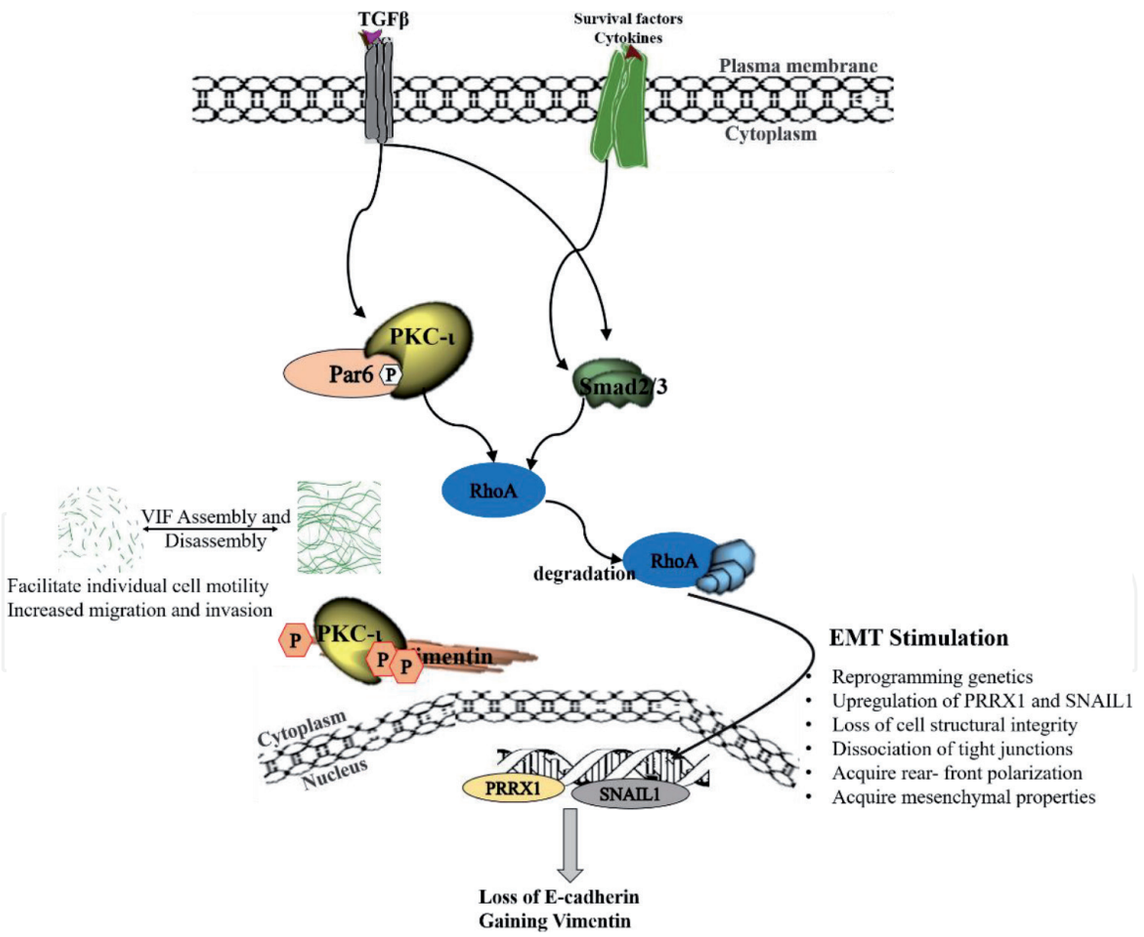

Figure 3.
A schematic summary of the involvement of PKC-ι in melanoma progression via activation of EMT and Vimentin signaling. Upon extracellular stimulation with TGFβ, PKC-ι associates and activates Par6, which stimulates the degradation of RhoA thereby upregulates EMT. SNAIL1 and PRRX1 are two very important transcription factors and they drive EMT process by upregulating Vimentin while downregulating E-cadherin. PKC-ι activates Vimentin by phosphorylation and this initiates disassembly of VIF and facilitates cellular motility. During this process, cadherin junctions are disrupted as a result of loss of E-cadherin and β-catenin is translocated to nucleus to upregulate the production of facilitating proteins such as CD44 which further stimulate migration and EMT. Activated Vimentin changes cell polarity to maintain the mesenchymal phenotype of melanoma cells in-vitro.

As summarized in **Figure 3**, based on our published reports, we believe that TGFβ stimulated PKC-ι/Par6/RhoA and Smad2/3 pathways to induce EMT in melanoma through transcriptional activities of SNAIL1 and PRRX1. Vimentin and PKC-ι activation are upregulated simultaneously to facilitate EMT in melanoma. PKC-ι activated Vimentin thereby regulates the dynamic changes in melanoma metastasis. Our results further confirms that PKC-ι inhibition using specific inhibitors such as ICA-1T and ICA-1S, not only reduce melanoma cell survival but also negatively affects the melanoma metastatic progression by downregulating EMT. Taken together, this novel concept can be used to develop more specific effective therapeutics for melanoma patients based on PKC-ι. PKC-ι can be used as a novel biomarker to mitigate melanoma metastasis using specific inhibitors.

4. Self-regulation of PKC-ι is a crucial mechanism making PKC-ι an important novel target in melanoma anti-cancer therapeutics

In our previous study, we identified PKC-ι as a major component responsible for inducing cell growth, differentiation, survival and EMT promotion in melanoma, as a result of PKC-ι specific inhibitor applications [22, 23]. In addition to these findings, we noted that the inhibition of PKC-ι leads to a decrease in its own expression of PRKCI gene. This indicates that PKC-ι plays a role in its expression in melanoma. The PRKCI gene is located on chromosome 3 (3q26.2), a region identified as an amplicon [56]. Our latest published results describe the transcriptional regulation of PRKCI with an insight view of cell signaling crosstalk in melanoma cells. FOXO1 and c-Jun were identified as possible transcription factors that can bind to the PRKCI promoter region through PROMO and Genomatix Matinspector. These two transcription factors (TFs) were systematically silenced to analyze the downstream effect on PKC-ι expression.

c-Jun is the first discovered oncogenic TF that is associated with metastatic breast cancer, non-small cell lung cancer and several other types of cancer [57–59]. We found a positive correlation between c-Jun with PKC-ι expression. Phosphorylation at S63 and S73 by JNKs (c-Jun N-terminal kinases) activates c-Jun, thereby increasing c-Jun targeted gene transcription. c-Jun stimulates the oncogenic transformation of 'ras' and 'fos' in several type of cancers [60]. FOXO1 is a well-known tumor suppressor and we found it suppresses the expression of oncogenic PKC-ι. FOXO1 also plays a key role in gluconeogenesis, insulin signaling and adipogenesis. AKT is known to deactivate FOXO1 by phosphorylating FOXO1 at T24, which drives FOXO1 nuclear exclusion, leading to ubiquitination [61, 62]. Therefore, the phosphorylation of FOXO1 is an indication of its downregulation. FOXO1 plays a crucial regulatory role in both the intrinsic and extrinsic pathways of apoptosis in many types of cancers, demonstrating an association between FOXO dysregulation and cancer progression [63, 64]. Additionally, upregulation of FOXO1 inhibits cancer cell proliferation, migration and tumorigenesis [65]. Notably, FOXO1 can also be downregulated by ERK1/2 and PKC-ι, in addition to AKT [66]. In our most recent study, we demonstrated that, due to PKC-ι inhibition, the availability of active phosphorylated PKC-ι decreases, making it ineffective at deactivating FOXO1 through phosphorylation at T24. Importantly, this is the first showing direct involvement of PKC-ι in its own expression regulation and PKC-ι inhibition that leads to continuous upregulation of FOXO1 [67].

As we discussed earlier in Part 2, our previous data showed that PKC-ι inhibition significantly downregulated the PI3K/AKT1 pathway, thereby suppressing the activation of AKT [22, 23]. Downregulation of NF-κB due to PKC-ι inhibition, result in downregulation of AKT. Our latest data shows that it increases total FOXO1 level,

while reducing its phosphorylated levels [67]. This confirms that NF-κB downregulation upregulates FOXO1 activity as a result of PKC-ι specific inhibition. Elevated FOXO1 negatively influenced PKC-ι expression and phosphorylation at T555. This further confirms our previous observations with PKC-ι inhibition with ICA-1T and ICA-1S, where total PKC-ι, phosphorylated PKC-ι, NF-κB activation and activated AKT (S473) were significantly reduced [23]. These results could be due to the tight regulation of PKC-ι expression by FOXO1, which retards PRKCI from transcription. Such results confirmed that FOXO1 is a major regulator which suppresses the expression of PRKCI. Interestingly, c-Jun and phosphorylated c-Jun (S63) levels were not significantly altered as a result of NF-κB *si*RNA knockdown. This suggests that NF-κB diminution does not affect PKC-ι expression over c-Jun. Instead, c-Jun is known to protect cancer cells from apoptosis by cooperating with NF-κB signaling to facilitate survival upon TNF-α stimulation [68]. These overall effects have been summarized in **Figure 4**. We have previously shown how TNF-α upregulates NF-κB and AKT pathways along with PKC-ι expression in these two melanoma cell lines

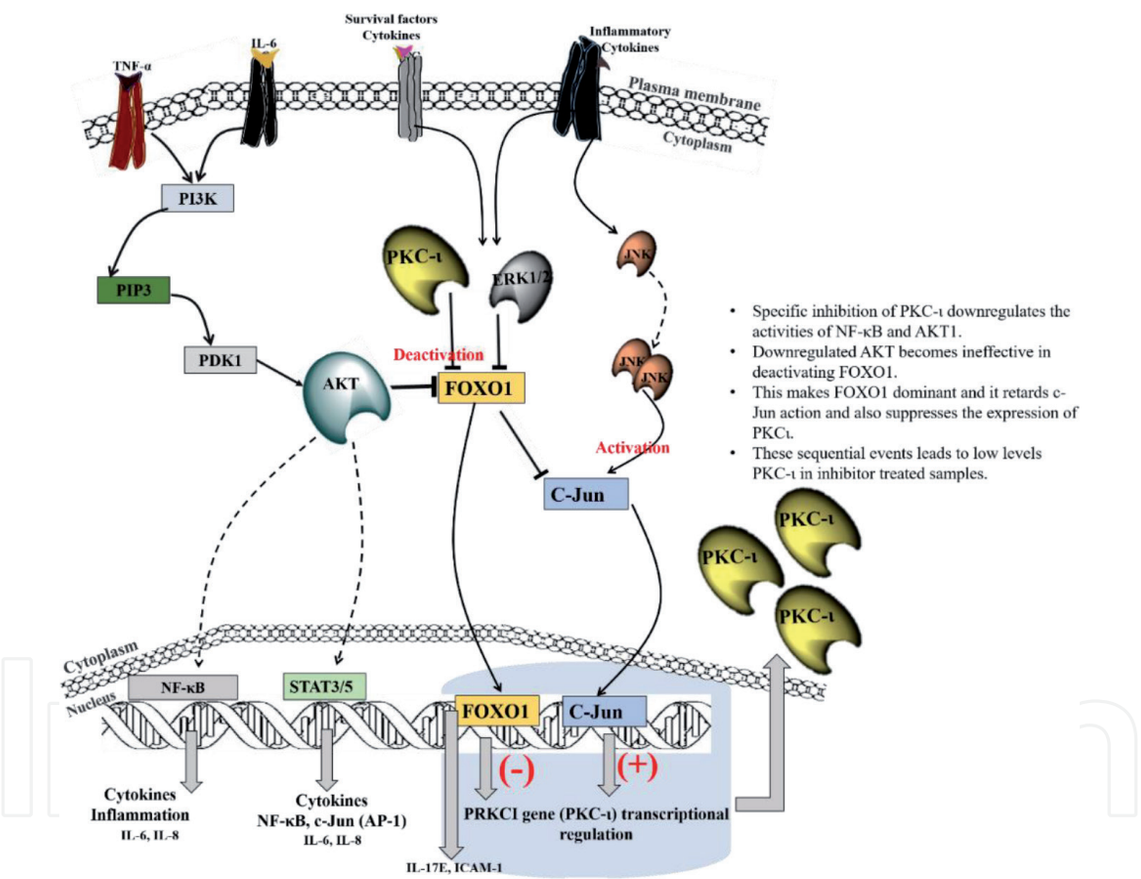

Figure 4.
A schematic summary of the regulation of the expression of PKC-ι in melanoma. This model depicts the interactions between NF-κB, PI3K/AKT/FOXO1, JNK/c-Jun and STAT3/5 signaling pathways during the PKC-ι regulation. PKC-ι plays an important role in the regulation of its own expression in an intricate signaling web through c-Jun and FOXO1. PKC-ι is overexpressed in melanoma cells due to elevated transcriptional activity of c-Jun with the aid of PI3K/AKT, NF-κB, STAT3/5 signaling. The specific inhibition of PKC-ι initiates a disruption to rapid PKC-ι expression cycle in melanoma where the reduced activity of PKC-ι downregulates the NF-κB pathway and its transcriptional activity, which in turn diminishes the expression of IL-6/8. As a result of this AKT activity reduction, FOXO1 gets upregulated. FOXO1 turns out to be the most important TF regulating PKC-ι expression after the disruption initiated as a result of PKC-ι inhibition. Dominant FOXO1 negatively regulates the expression of PKC-ι and also diminishes the JNK activity to retard its activation of c-Jun. we found c-Jun as the transcription component which upregulates PKC-ι expression. The downregulation of IL-6 and IL-8 expression leads to the lessened STAT3/5 signaling, which causes c-Jun transcriptional reduction. This whole process continues and leads to the further downregulation of NF-κB, AKT and JNK/c-Jun while upregulating FOXO1, which leads to the continuation of the attenuation of PKC-ι expression. As a result, the total PKC-ι level decreases in melanoma cells.

[23]. However, the data from the current study suggest that the TNF-α downstream target is mainly FOXO1, where it 'switches off' through the phosphorylation of elevated AKT. The inhibition of PKC-ι diminishes this AKT activation, thereby upregulating FOXO1 activity [67].

On the other hand, *si*RNA treatments for of c-Jun and FOXO1 revealed that c-Jun also plays a role in PKC-ι expression, apart from FOXO1. Enzyme-linked immunosorbent assay (ELISA) experiments were conducted to investigate cell signaling crosstalks. These findings demonstrated links between PKC-ι expression with the cytokines, interleukin (IL)-6, IL-8, IL-17E and ICAM-1, along with some other key cellular signaling points. Phosphorylation at S536 on the NF-κB p65 transactivation domain is an indication of dimerization of NF-κB subunits. ELISA results revealed a more than two fold increase of NF-κB p65 (S536) in PKC-ι inhibited samples. According to Ratnayake et al., PKC-ι inhibition downregulates NF-κB translocation to the nucleus therefore phospho-NF-κB levels increase in order to diminish the effect of PKC-ι inhibition. However, elevated FOXO1 does not allow NF-κB to annex the control since it is missing the essential assistance needed from PKC-ι due to its inhibition from ICA-1T and ICA-1S inhibitors [67] (**Figure 4**). Abnormal STAT3/5 activity has been shown to be connected to multiple types of cancer [69–72]. Cytokines such as IL-6 and IL-5, upregulate STAT signaling, thereby induces cell survival in many types of cancer [69, 70, 73]. Importantly, upregulated STAT3 increases the transcription of c-Jun [69, 74]. Our ELISA results indicated that STAT3 and STAT5 activities were retarded due to PKC-ι inhibition, signifying c-Jun diminution. Hornsveld et al., and few other reports have provided connections between the JNK pathway and FOXO1, explaining its tumor suppressing features by weakening JNK activity [75, 76]. However, JNK activates c-Jun. Our latest Western blot and real time qPCR analysis demonstrated that c-Jun depletion lessened PKC-ι expression, which suggested that c-Jun acts as an activator of PKC-ι expression. This confirms that both FOXO1 and c-Jun are involved in regulating PKC-ι expression. The results suggest that FOXO1 plays a major role over c-Jun only upon PKC-ι inhibition, possibly through multiple mechanisms, such as the reduction of JNK signaling, retarding PKC-ι expression and cell cycle arrest. FOXO1 induces cell cycle arrest by promoting the transcription of cell cycle kinase inhibitors or cyclin-dependent kinase inhibitor (CKI). p21 and p27 are two well-known downstream CKIs induced by FOXOs [66, 75]. Especially, FOXO1 is also believed to induce anoikis, which is apoptosis that occurs when cells detach from the extracellular matrix. Our ELISA results revealed significantly higher levels of p21 in PKC-ι inhibited cells, suggesting that the inhibition of PKC-ι induces cell cycle arrest through FOXO1 [67]. This also explains why apoptosis was stimulated in melanoma cells as a result of inhibition of PKC-ι in addition to downregulation of PI3K/AKT and NF-κB pathways. Overall, FOXO1 is very important in enhancing anti-tumor activities upon PKC-ι inhibition and it plays the central role of oncogenic PKC-ι depletion.

The next three paragraphs focus on more details concerning cytokine expression changes observed as a result of PKC-ι inhibition [67]. IL-6, IL-8, IL-17E and ICAM-1 expression were significantly altered in melanoma cells upon PKC-ι knockdown [67]. As shown by the results of both Western blot and RT-qPCR analyses, the protein levels of IL-6 and IL-8 (as well as their mRNA levels) decreased, while the levels of IL-17E and ICAM-1 increased significantly upon PKC-ι knockdown by *si*RNA [67]. This suggests that c-Jun and FOXO1 driven PKC-ι expression is involved in autocrine signaling. The micro-environment of a tumor, and in particular melanoma, is regularly exposed to numerous inflammatory factors and immune cells. The effect of these factors function to either promote chronic inflammation or engage in antitumor activity [77]. Cytokines are examples of these inflammatory factors; they play an essential role in regulating the tumor microenvironments [78].

They are vital in order to promote or dysregulate tumor progression and metastasis. Chemokine C-X-C motif ligand-1 (CXCL)-1, CXCL-12, IL-18, CXCL-10, IL-6 and IL-8 are known to promote cancer metastasis. Interestingly, CXCL-1, CXCL-10, CXCL-12 and IL-18 levels were not significantly altered due to PKC-ι depletion in melanoma cells.

IL-6 contributes to the degradation of IκB-α, leading to the upregulation of NF-κB translocation. We have previously discussed that PKC-ι stimulates NF-κB translocation through IκB-α degradation [23]. The translocation of NF-κB to the nucleus induces cell survival through the transcription of various survival factors as well as other pro-survival cytokines [69, 73, 79]. IL-8 plays a role in regulating polymorphonuclear neutrophil mobilization. In melanoma, IL-8 has been attributed to extravasation, a key step in metastasis. Studies have shown that the expression of IL-8 in melanoma is regulated via NF-κB. When NF-κB is translocated to the nucleus, IL-8 expression increases, leading to the promotion of a more favorable microenvironment for metastasis [80, 81]. Our results indicated that both IL-6 and IL-8 expression levels decreased upon diminution of PKC-ι [67].

Some cytokines promote anti-tumor activity by exploiting an immune response. ICAM-1 plays a key role in the immune response, including antigen recognition and lymphocyte activation [82, 83]. ICAM-1 is known for the inhibition of tumor progression through the inhibition of the PI3K/AKT pathway. Tumor cells are exposed to cytotoxic T-lymphocytes as a result of ICAM-1 [83]. According to ovarian cancer clinical data, inhibition of ICAM-1 expression is associated with an increased risk of metastasis for the patients within the first 5 years from the point of diagnosis [82, 83]. IL-17E (IL-25) is another anti-tumor cytokine belongs to a family of cytokines known as IL-17. Treatment with recombinant active IL-17E has been shown to decrease tumor growth of melanoma and pancreatic cancer [84, 85]. The upregulation of IL-17E is linked to the increased expression of TH17 cells. T cells, such as TH17 have been implicated in the inhibition of tumor-infiltrating effector T cells. The exact mechanism of IL-17E function in the anti-tumor effect has not been studied well enough [86]. Particularly, our most recent results indicated that ICAM-1 and IL-17E protein levels and mRNA expression increased upon PKC-ι knockdown by *si*RNA [67]. This strongly supports that anti-tumor signaling is upregulated upon the knockdown or inhibition of oncogenic PKC-ι via an autocrine manner through IL-17E and ICAM-1. Moreover, the results suggest that IL-17E and ICAM-1 play an important down-regulatory role in the regulation of PKC-ι expression along with FOXO1, opposite to IL-6/8 assisted c-Jun [67].

In conclusion, based on the published results from Acevedo-Duncan's laboratory and other available information, it is suggested that PKC-ι itself plays an important role in its expression in a complex signaling web through the transcriptional activation/deactivation of c-Jun and FOXO1. The retarded activity of PKC-ι due to application of specific inhibitors such as ICA-1S and ICA-1T, causes a down-regulation of the NF-κB pathway and its transcriptional activity, which reduces the expression/production of IL-6 and IL-8. In addition, as a result, the activity of AKT decreases, upregulation of FOXO1 activity takes place. FOXO1 is the most important TF regulating PKC-ι expression and IL-17E and ICAM-1 cytokines seem to play a stimulatory role for FOXO1 to attenuate PKC-ι. FOXO1 negatively regulates the expression of PKC-ι, diminishing JNK activity which leads to retard c-Jun activation. IL-6 and IL-8 expression are downregulated via PKC-ι-mediated NF-κB transcriptional activity reduction. IL-6/8 attenuation leads to STAT3/5 signaling downregulation, further reducing c-Jun expression. This whole process continues and leads to the further downregulation of NF-κB, c-Jun and upregulation of FOXO1, which leads to the continuation of the depletion of PKC-ι expression. As a result of this sequence of events, the total PKC-ι level decreases in melanoma cells,

which initiated as a result of PKC-ι inhibition using specific inhibitors. These results indicate that PKC-ι is being regulated in a rather complex manner, which involves itself as a key component. PKC-ι specific inhibition using ICA-1S and ICA-1T leads to a decrease in its own production, and during this process, PKC-ι inhibition also triggers multiple anti-tumor/pro-apoptotic signaling. This makes PKC-ι one of the central key points of interest to specifically target and diminish as a means of treating melanoma. The results also strongly suggest that PKC-ι is a prime novel biomarker that can be targeted to design and develop personalized and targeted therapeutics for melanoma.

5. State of atypical PKC inhibitors

We have discussed the effects of five aPKC specific inhibitors throughout this chapter. The structures of these compounds are shown in **Figure 5**.

Atypical PKCs were first considered as a novel therapeutic target by Stallings-Mann et al. in 2006. They screened aurothiomalate as a potent inhibitor of the interaction between PB1 domain of PKC-ι and Par6 [87]. Half maximal inhibitory concentration (IC50) of aurothiomalate ranged from 300 nM to 100 μM and indicated that some cell lines are insensitive (i.e. H460 and A549 lung cancer cells) to the inhibitor [87].

Blázquez et al. tested calphostin C and chelerythrine against West Nile virus (WNV) which significantly inhibit WNV multiplication in cell culture without affecting cell viability. They report that PKCs have also been implicated in different steps during viral replication. Calphostin C and chelerythrine two wide range PKC inhibitors that target all three PKC classes. Results indicate that atypical PKCs are involved in WNV multiplication process which can be effectively retard using said inhibitors [88].

Kim et al. reported the application of Echinochrome A as an inducer of cardiomyocyte differentiation from mouse embryonic stem cells. Echinochrome A was

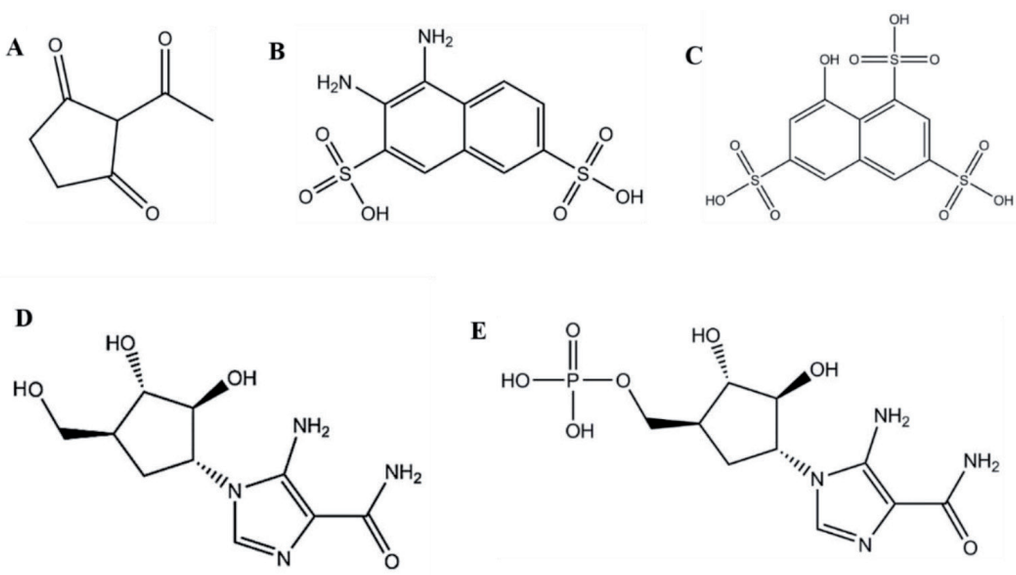

Figure 5.
Structures of the aPKC specific inhibitors (ACPD, DNDA, ζ-Stat, ICA-1S and ICA-1T). chemical structures of ACPD (a) and DNDA are specific to both PKC-ι and PKC-ζ, ζ-Stat (C) is specific to PKC-ζ while ICA-1S (D) and ICA-1T (E) are specific to PKC-ι. molecular weights (MW) of ACPD (140.14 g/mol), DNDA (318.32 g/mol), ζ-Stat (MW = 384.34 g/mol), ICA-1S (MW = 256.26 g/mol) and ICA-1T (MW = 336.24 g/mol), respectively.

extracted from sea urchins. They investigated the potential use of Echinochrome A as an aPKC specific inhibitor and found that IC_{50} for PKC-ι is 107 μM under *in-vitro* kinase assay conditions. Molecular docking simulation results suggested a direct binding of Echinochrome A with PKC-ι [89].

An important study by Kwiatkowski et al. identified an azaindole-based scaffold for the development of more potent and specific PKC-ι inhibitors. They described fragmented based approach an introduced a new class of potential aPKC inhibitors based on azaindole [90].

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Conflict of interests

The authors declare that they have no competing interests.

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