Two novel atypical PKC inhibitors; ACPD and DNDA effectively mitigate cell proliferation and epithelial to mesenchymal transition of metastatic melanoma while inducing apoptosis

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Abstract. Atypical protein kinase Cs (αPKC) are involved in cell cycle progression, tumorigenesis, cell survival and migration in many cancers. We believe that αPKCs play an important role in cell motility of melanoma by regulating cell signaling pathways and inducing epithelial to mesenchymal transition (EMT). ACPD and DNDA can be effectively used as potential therapeutic drugs for melanoma by inhibiting αPKCs.

Introduction

Melanoma is a type of cancer which occurs in melanocytes. Melanocytes are responsible for the production of the pigment melanin and are derived from the neural crest. Approximately 90% of melanoma occurs in skin (cutaneous melanoma), but it also rarely arises from the mucosal surfaces or areas which neural cells migrate. Examples are the eye, intestine and mouth. Malignant melanoma is very common among Caucasians (1). A total of 76,380 new cases of melanoma and 10,130 related deaths were expected in 2016 in the USA. Surgical resection, applications of immunotherapy, biologic therapy, radiation therapy, or chemotherapy may improve survival (2,3).

PKC belongs to the protein kinase enzyme family which post-translationally modify other proteins by phosphorylation of hydroxyl groups of serine and threonine amino acid residues, so PKCs tend to be involved in many signal transduction cascades. There are 15 PKC isozymes identified in humans; they are divided as classical, novel and atypical (αPKCs). αPKCs contain two structurally and functionally distinct isoforms: PKC-ι and PKC-ζ which are involved in cell cycle progression, tumorigenesis, survival and migration in many cancers (4-7). Lung cancer cell proliferation is highly dependent on the PKC-ι level through activation of the ERK1 pathway (6). Overexpression of PKC-ι plays an important role in the leukemia chemoresistance (7). PKC-ι is also involved in glioma cell proliferation; it regulates the phosphorylation of cyclin dependent kinase activating kinase/cyclin dependent kinase 7 pathway (8,9). Non-small lung cancer cell proliferation is highly dependent on PKC-ι (10). αPKCs are involved in TGF-β induced EMT by phosphorylating Par6 (11). We believe that both αPKCs are involved in EMT process of melanoma cells by regulating the formation of vimentin intermediate filament (VIF) assembly. EMT is an important event of tumor progression where the apicobasolaterally polarized...
cohesive epithelial cells of epithelium detach from its base- ment and gain the ability of mesenchymal cells, which move independently. EMT is characterized by the loss of E-cadherin and gain of vimentin. During such morphological changes of cells in EMT, cytoskeletal intermediate filaments undergo a massive composition change by initiating the expression of vimentin. Even though the role of vimentin in EMT is not fully understood, some literature strongly supports the relevance of VIF assembly in EMT as being part of tumorigenesis (12,13).

Selzer et al (14) has reported a comprehensive comparison of PKC isofrom expression between normal melanocytes, transformed melanocytes and melanoma cell lines. PKC-ι may play a role in cellular malignancy as shown by its association with the transformed phenotype of human melanomas in vivo and in vitro. PKC-ι and PKC-ζ are overexpressed in both transformed and malignant melanoma. In comparison, PKC-ζ was detected in low levels in normal melanocytes while PKC-ι was not detected. We also report that PKC-ι and PKC-ζ are overexpressed in SK-MEL-2 malignant melanoma cell line compared to undetectable levels of PKC-ι and low levels of PKC-ζ in PCS-200-013 normal melanocytes (15).

In the present study, we have investigated the effects of the novel aPKC specific inhibitors ACPD and DNDA on the proliferation, apoptosis, migration and invasion of two normal melanocyte cell lines (PCS-200-013 and MEL-F-NEO) and two malignant melanoma cell lines (SK-MEL-2 and MeWo). In this study, we showed that both inhibitors can decrease the levels of total and phosphorylated PKC-ζ and PKC-ι levels. We also found that both inhibitors can decrease the levels of both phosphorylated and total vimentin and increase E-cadherin levels which are associated with EMT. Treatment with inhibitors altered the levels of CD44, β-catenin, NF-κB p65, Par6, RhoA, AKT and PTEN whose roles are established in either cell survival or metastasis (16-18). Furthermore, we established that melanoma cells proliferate via aPKC/AKT/NF-κB mediated pathway while inducing the EMT via PKC-ι/Par6/RhoA pathway. We report that PKC-ι activates vimentin by phosphorylation. We also report that treatment with ACPD/DNDA induced apoptosis as shown by increasing caspase-3 levels, decreasing Bcl-2 levels and changes in Poly(ADP-ribose) polymerase (PARP) levels in addition to cell proliferation and downregulation of EMT in melanoma cells.

Materials and methods

Materials. ACPD (product no. R426911) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and DNDA was obtained from the National Institute of Health (NIH; Bethesda, MD, USA). They were dissolved in sterile distilled water (vehicle) before use. Antibodies were purchased as follows. PKC-ι (cat. no. 610175) and PKC-ζ (cat. no. sc-17781), NF-κB p65 (cat. no. sc-372-G) and caspase-3 (cat. no. sc-7272) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho PKC-ζ (Thr 410) (cat. no. PA5-17837), phospho PKC-ι (Thr 555) (cat. no. 44-968G), E-cadherin (cat. no. 701134), vimentin (cat. no. MA3-745) and myelin basic protein (MBP) (cat. no. PA1-10008) from Thermo Fisher Scientific (Waltham, MA, USA), phospho-vimentin (Ser39) (cat. no. 13614S), PTEN (cat. no. 9559S), phospho PTEN (Ser380) (cat. no. 9551), AKT (cat. no. 4691X), phospho AKT (Ser473) (cat. no. 4059S), PARP (cat. no. 9532) and cleaved-PARP (cat. no. 9185) from Cell Signaling Technology (Danvers, MA, USA). β-actin-peroxidase (cat. no. A3854) from Sigma-Aldrich, CD44 (cat. no. ab97478), RhoA (cat. no. ab54835) and β-catenin (cat. no. ab16051) from Abcam (Cambridge, UK). Phospho-MBP (Thr 125) (cat. no. 05-429) from EMD Millipore (Billerica, MA, USA). Enhanced chemiluminescence solution (product no. 34080) was purchased from Pierce (Rockford, IL, USA).

Dulbecco's phosphate-buffered saline without Mg2+ and Ca2+ (DPBS) (product no. D8537) and Trypsin-EDTA (ethylene-diaminetetraacetic acid) solution (product no. T4049) were purchased from Sigma-Aldrich. WST-1 reagent for cell proliferation (cat. no. 1164807001) was purchased from Roche Diagnostics (Mannheim, Germany). Basement membrane extraction (BME) and Calcein-AM solutions were purchased from Trevigen (Gaithersburg, MD, USA) and Molecular Probes (Eugene, OR, USA), respectively. Human small interfering RNA (siRNA) for PKC-ι (cat. no. SR303741) and for PKC-ζ (cat. no. 303747) were purchased from Origene Technologies Inc. (Rockville, MD, USA). Human recombinant proteins PKC-ι (PV3183), PKC-ζ (P2273) and MBP (MBS17422) were purchased from Thermo Fisher Scientific and MyBioSource (San Diego, CA, USA), respectively.

Database preparation and molecular docking. Database preparation was performed using the National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) and molecular docking was performed using ‘AutoDockTools’ and ‘AutoDock Vina’ programs by selecting structural pockets in PKC-ι and PKC-ζ which were compatible with small drug like molecules. PKC-ι and PKC-ζ structural pockets were identified based on ‘fpocket’, a very fast open source protein pocket (cavity) detection system based on Voronoi Tessellation. The detailed procedure was performed as described in Pillai et al (19).

Cell culture. PCS-200-013, SK-MEL-2 and MeWo cell lines were purchased from the American Type Tissue Culture Collection (ATCC; Rockville, MD, USA) and MEL-F-NEO cell line was purchased from Zen-Bio, Inc. (Research Triangle Park, NC, USA). Furthermore, cells were cultured at 37°C and 5% CO2.

Dermal cell basal medium (PCS-200-030) with melanocyte growth kit (PCS-200-042) were used for PCS-200-013 and melanocyte growth medium (MEL-2) were used for MEL-F-NEO cell culturing according to the respective instruction manual. Eagle's minimum essential media (EMEM) (90% v/v) with fetal bovine serum (FBS) (10% v/v) and penicillin (5 µg/ml) were used for SK-MEL-2 and MeWo cell culturing. All cell lines were seeded and grown as monolayers in T25 or T75 flasks.

PKC activity assay. PKC activity assay was conducted by monitoring the phosphorylation of myelin basic protein (MBP) (0.025 mg/ml), a known substrate for PKCs. The detailed procedure was performed as described in the study by Pillai et al (19) for both ACPD and DNDA on recombinant PKC-ι and PKC-ζ (0.01 µg/ml) using a series of inhibitor concentrations (0-10 µM). Samples then fractionated by SDS-PAGE...
and immunoblotted. Kinase activity was calculated based on the densitometry values of western blots (WB).

Inhibition of expression of PKC-ι and PKC-ζ with siRNA. SK-MEL-2 and MeWo cells (4x10⁴) were cultured in T25 flasks and treated with either siRNA (20 nM) for PKC-ι or PKC-ζ or scrambled siRNA after 24 h post-plating time and incubated for 48 h. Detailed procedure was performed as described in the study by Win and Acevedo-Duncan (20).

Inhibitor dose response curves for cell viability. PCS-200-013, MEL-F-NEO, SK-MEL-2 and MeWo cells (4x10⁴) were cultured in T25 flasks and treated with either an equal volume of sterile water (vehicle control) or ACPD or DNDA (0.1-3.5 µM). Additional doses of sterile water or ACPD or DNDA were supplied every 24 h during a 3-day incubation period and cells were subsequently lifted using Trypsin-EDTA solution (1.5 ml/flask) and neutralized with the equal volume of media. Subsequently, live cells were counted using the Scepter, an automated cell counter from Millipore (Billerica, MA, USA) at 24-h intervals. Cell counts obtained from Scepter were compared with the counts obtained from the Cellometer Vision from Nexcelom Bioscience (Lawrence, MA, USA).

WST-1 assay for cell viability and cytotoxicity. Approximately 4x10⁴ cells/well (PCS-200-013, MEL-F-NEO, SK-MEL-2 and MeWo) were cultured in a 96-well plate. After 24-h post-plating time, fresh media were supplied (200 µl/well) and treated with either an equal volume of sterile water (vehicle control) or with the half maximal inhibitory concentration (IC₅₀) of ACPD (2.5 µM) or DNDA (2.5 µM). This IC₅₀ was obtained based on the cell viability counts in the previous experiments. Additional doses were supplied every 24 h during a 3-day incubation period. At the end of the 3-day treatment, media were removed and fresh media (100 µl) were added with 4-[3-(4-iodophenyl)-2-(4-nitropheny))-2h-5-tetrazolio]-1,3-benzene disulfonate (WST-1) reagent (10 µl) to each well. The absorbance was measured at 450 nm for every 1 h up to 6-10 h using the Synergy HT microplate reader from BioTek Instruments Inc. (Winooski, VT, USA).

Assays for cell migration and invasion

Wound healing assay. The detailed procedure was performed for SK-MEL-2 and MeWo cells as described by O’Connell et al (21). Cells were treated with either sterile water or ACPD or DNDA to achieve the final concentration of 2.5 µM and plates were incubated at 37˚C and 5% CO₂ for 4 days. Detailed procedure was performed as described in the study by Win et al (22) and samples were then fractionated by SDS-PAGE and immunoblotted.

Basement membrane extract (BME) invasion assay (Boyden chamber assay). This in vitro invasion assay was performed for SK-MEL-2 and MeWo cells as described by O’Connell et al (21). BME (0.5X) was used instead of Matrigel. Crystal violet (0.5%) was used to stain the cells adhered to the bottom of the lower chamber in order to visualize the inhibition of invasion. Images of the stained cells were taken from Motic AE31E microscope (x40 magnification).

Immunoprecipitation and western blot analysis. Approximately 1x10⁵ cells (SK-MEL-2 and MeWo) were cultured in T75 flasks and 24 h post-plating, fresh media were supplied and cells were treated with either an equal volume of sterile water or ACPD or DNDA (2.5 µM). Additional doses were supplied every 24 h during a 3-day incubation period. Cells were then lifted using Trypsin and cell lysate were collected either with cell lysis buffer (cat. no. C7027; Invitrogen) or IP lysis buffer (cat. no. 87788; Thermo Fisher Scientific). The WB and immunoprecipitation were performed as described in the study by Win et al (22) and samples were then fractionated by SDS-PAGE and immunoblotted.

Densityometry. The intensity of each WB band was measured using ‘AlphaView’ software for ‘Fluorchem’ systems developed by ProteinSimple (San Jose, CA, USA) in which the background intensity was subtracted from the intensity of each band to obtain the corrected intensity of the proteins.

Statistical analysis. All data are presented as mean ± SD. Statistical analysis was performed with one or two-way ANOVA followed by Tukey’s HSD test as multiple comparisons tests using the ‘VassarStats’ web tool for statistical analysis. P≤0.05 or P≤0.01 indicated statistical significance.

Results

Specific binding of ACPD and DNDA to aPKCs. To establish the therapeutic potential of aPKCs, ACPD (Fig. 1A) and DNDA (Fig. 1B) were identified based on molecular docking (MD). Approximately 3x10⁷ drug like organic compounds (molecular weight <500 g/mol) in NCI/DTP, were screened by positioning them in the structural pockets of PKC-ι and PKC-ζ and then scored based on predicted polar and non-polar interactions. ACPD was found to interact with amino acid residues Gln 469, Ile 470, Lys 485 and Leu 488 of the catalytic domain of PKC-ι (Fig. 1C) and Arg 265, Pro 267, Asp 269 and Lys 290 of the catalytic domain of PKC-ζ (Fig. 1D). DNDA interacts with amino acid residues of Asp 339, Asp 382, Leu 385 and Thr 393 of the catalytic domain of PKC-ι (Fig. 1E) and Asp 337, 395 of the catalytic domain of PKC-ζ (Fig. 1F). Approximately -7 kcal/mol docking score was obtained for ACPD and DNDA separately for PKC-ι and PKC-ζ for 4 different pockets. Sixteen pockets were identified and tested for both PKC-ι and PKC-ζ separately and all the pockets that scored above -6.5 kcal/mol were rejected to identify these specific binding sites of the inhibitors. The results here suggest that both ACPD and DNDA interact with PKC-ι and PKC-ζ in a fairly equal manner.

Specific kinase activities of ACPD and DNDA. Determination of specific activity of inhibitors was essential since over 70% similarity is observed in the primary structures of PKC-ι and PKC-ζ catalytic domains (5,23,24). Specificity of ACPD was previously reported as it inhibits both PKC-ι and PKC-ζ without affecting other PKC isoforms (25). Additionally, ACPD does not inhibit other kinases such as AMPK, Akt2, FGFR1/2/3/4, mTOR, GSK3β, IRAK1/4, JAK1/2, MEK1, ERK1/2, JNK1/2, P38A, Src, ROCK2 and PI3K (26,27). This confirms our finding of ACPD in molecular docking experi-
ments that it did not show significant binding to other kinases apart from PKC-\(\iota\) and PKC-\(\zeta\). We conducted kinase activity assay (in vitro) of ACPD and DNDA in order to confirm our virtual screening data. Kinase activities of ACPD and DNDA (Fig. 1G) were determined for a series of concentrations (0.1-10 \(\mu\)M) using recombinant active PKC-\(\iota\) or PKC-\(\zeta\) in the presence of MBP which is a well-known substrate for PKCs (28). Both compounds demonstrated significant inhibitions for both PKC-\(\iota\) and PKC-\(\zeta\) under all tested concentrations.

Both compounds showed maximum inhibition for their 10 \(\mu\)M solutions as ACPD on PKC-\(\iota\) as 44% (\(P \leq 0.05\)), ACPD on PKC-\(\zeta\) as 41% (\(P \leq 0.05\)), DNDA on PKC-\(\iota\) as 38% (\(P \leq 0.05\)) and DNDA on PKC-\(\zeta\) as 29% (\(P \leq 0.05\)). This confirms that DNDA also shows specific inhibition on PKC-\(\iota\) and PKC-\(\zeta\) in addition to ACPD. These kinase activity data confirm our virtual screening data.

Inhibitor dose-response curves. Dose curves for ACPD and DNDA were obtained to investigate the effects on cell proliferation of normal and malignant cell lines over a wide range of concentration. ACPD and DNDA were treated with either equal volume of sterile water (control) or ACPD or DNDA (0.1-3.5 \(\mu\)M). Additional doses of sterile water or ACPD or DNDA were supplied every 24 h during a 3-day incubation period. Subsequently, cells were lifted and counted. Cell count for PCS-200-013 and MEL-F-NEO cells were obtained for 3 days due to longer doubling time. The two malignant cell lines (SK-MEL-2 and MeWo) were quantified by counting the viable cells at 24-h intervals. N=3 experiments were performed for each cell line and mean ± SD are plotted. Statistical significance is indicated by asterisks as *\(P<0.05\) and **\(P<0.01\).
inhibition for MEL-F-NEO normal melanocyte cells (Fig. 2B). Both inhibitors significantly decreased cell proliferation of SK-MEL-2 and MeWo upon increasing the concentrations. ACPD decreased proliferation by 20% for 1.5 µM (P≤0.01), 48% for 2.5 µM (P≤0.01) and 51% for 3.5 µM (P≤0.01) (Fig. 2C) and DNDA decreased 24% for 1.5 µM (P≤0.01), 52% for 2.5 µM (P≤0.01) and 57% for 3.5 µM (P≤0.01) (Fig. 2D) in the SK-MEL-2 cell line. ACPD decreased proliferation by 41% for 1.5 µM (P≤0.01), 54% for 2.5 µM (P≤0.01) and 58% for 3.5 µM (P≤0.01) (Fig. 2E) and DNDA decreased proliferation by 41% for 1.5 µM (P≤0.01), 46% for 2.5 µM (P≤0.01) and 48% for 3.5 µM (P≤0.01) (Fig. 2F) in the MeWo cell line. These results suggest that both inhibitors can effectively decrease the cell population while not having a significant effect on normal melanocytes. Based on these results the IC_{50} of ACPD and DNDA for both drugs were found to be ~2.5 µM and this concentration was used in later experiments.

Wound healing assay. Wound healing assay was performed to investigate the effect of ACPD and DNDA on malignant melanoma cell migration in vitro. Wound healing assay is commonly used to investigate in vitro migration of cancer cells (30). Photographs for each cell line are compared as ‘day 0’ (starting point) and ‘day 3’ or ‘day 4’ for both malignant cell lines (Fig. 4A). Cells treated with ACPD 2.5 µM and DNDA 2.5 µM were compared with their respective controls. The areas of the scratch (wound) were calculated and compared to determine the statistical significance (Fig. 4B). We found that both inhibitors significantly reduce the wound closure (P≤0.01) of both cell lines compared to respective controls. Results suggested that both drugs are equally effective in reducing cell migration in vitro.

BME invasion assay. This invasion assay was performed to investigate the effect of ACPD and DNDA on malignant melanoma cell invasion in vitro. Although it is similar to Boyden chamber assay, it avoids scraping off the Matrigel and staining to assess the number of migrated cells through the filter. Hence, the method carries less human error compared to a conventional Boyden chamber assay (21). Migrated cells were stained with a fluorescent marker; Calcein-AM. Live cells cleave the ester (AM) of the molecule in order to produce fluorescence. Thus, the amount of fluorescence accumulate in the bottom chamber is proportional to the number of invaded melanoma cells.
The relative fluorescent units (excitation at 485 and emission at 528 nm) after 2 h of exposure were reported for inhibitor treatments for both SK-MEL-2 and MeWo cell lines compared to controls (Fig. 4C). Invasion was significantly reduced (P≤0.05) by 24 and 21% in ACPD (2.5 µM) treated SK-MEL-2 and MeWo cells compared to its control. In DNDA (2.5 µM) treated samples, the invasion was significantly reduced (P≤0.05) by 32% in both SK-MEL-2 and MeWo cell lines compared to its control. Invaded cells on the bottom chamber were stained with crystal violet and images were taken in randomly chosen fields as the visual representation of the invasion assay (Fig. 4D).

Effect of ACPD and DNDA on αPKC levels in malignant melanoma. Before analyzing the levels of αPKCs in inhibitor treated melanoma cell lines using WB, we compared the protein levels of αPKCs, E-cadherin, vimentin and Bcl-2 in MEL-F-NEO normal melanocytes at 100% confluency and 50% confluency. PKC-ι and vimentin were not detected at either confluency level in MEL-F-NEO cells. PKC-ζ, E-cadherin and Bcl-2 levels were found to be higher in 50% confluency level compared to 100% confluency (Fig. 5A). β-actin was used as the internal control to ensure that equal amounts of proteins were loaded in each lane in the SDS-PAGE.

WB were performed to investigate the effect of ACPD and DNDA on the expression of αPKCs on malignant melanoma (Fig. 5B). ACPD significantly reduced the PKC-ι level by 43 and by 31% of pPKC-ι in SK-MEL-2 cell line and by 46% of PKC-ι and 26% of pPKC-ι in MeWo cells. DNDA significantly decreased the levels of PKC-ι by 52%, pPKC-ι by 33% in SK-MEL-2 and by 27% of PKC-ι and pPKC-ι by 20% in MeWo cells. Also, ACPD significantly reduced the PKC-ζ level by 42% and by 29% of pPKC-ζ in SK-MEL-2 cell line and by 32% of PKC-ζ and 23% of pPKC-ζ in MeWo cells. DNDA significantly reduced the levels of PKC-ζ by 33%, pPKC-ζ by only 17% in SK-MEL-2 and by 60% of PKC-ζ and pPKC-ζ by 29% in MeWo. All values (percent) were calculated and compared to their respective control in WB (Fig. 5B; densitometry analysis) and significance was indicated as P≤0.05. β-actin was used as the internal control to ensure that equal amounts of proteins were loaded in each lane in the SDS-PAGE.
Effect of ACPD and DNDA on apoptosis of malignant melanoma. Since both inhibitors significantly inhibit melanoma cell proliferation, we tested the potential of the inhibitors on inducing apoptosis (Fig. 5C). Caspase-3 levels significantly increased by 26 and 17% in ACPD treated SK-MEL-2 and MeWo cells, respectively. Caspase-3 levels significantly increased by 32 and 39% in DNDA treated SK-MEL-2 and MeWo cells, respectively. PARP levels significantly decreased by 33 and by 24% in ACPD treated SK-MEL-2 and MeWo cells, respectively, while cleaved-PARP levels significantly increased by 14 and 18%, respectively. In DNDA treated samples, PARP levels significantly decreased by 12 and by 9% in SK-MEL-2 and MeWo cells, respectively, while cleaved-PARP levels significantly increased by 16 and 10%, respectively. Similarly, Bcl-2 levels significantly decreased by 13 and by 25% in ACPD treated SK-MEL-2 and MeWo cells, respectively, while in DNDA treated cells Bcl-2 levels decreased by 7 and by 32% in SK-MEL-2 and MeWo cells, respectively. All values (percent) were calculated compared to their respective control in WB (Fig. 5C; densitometry analysis).
and significance are indicated as $P \leq 0.05$. β-actin was used as the internal control to ensure that equal amounts of proteins were loaded in each lane in the SDS-PAGE.

**Effect of ACPD and DNDA on signaling pathways and EMT.** As shown in Fig. 6, we also investigated the effects of ACPD and DNDA on EMT by studying the signaling cascades crucial for cancer cell proliferation, survival, migration and invasion. The purpose of the analysis was to obtain a better understanding of how aPKCs are involved in the progression of melanoma. We tested the effects of ACPD and DNDA on proteins β-catenin, CD44, vimentin, Par6, PTEN, RhoA, E-cadherin, phosphorAKT and NF-κB p65 to determine how Wnt signaling, NF-κB signaling and AKT signaling are affected by aPKC inhibitors during EMT stimulation.

β-catenin significantly decreased by 39 and 26% in ACPD treated SK-MEL-2 and MeWo cells, respectively compared to 23 and 31% downregulation in DNDA treated samples. CD44 also decreased significantly by 19 and 34% in ACPD treated SK-MEL-2 and MeWo cells, in comparison to 27 and 43% downregulation in DNDA treated samples. Vimentin levels significantly decreased by 51 and 38% in ACPD treated SK-MEL-2 and MeWo cells, in comparison to 27 and 43% downregulation in DNDA treated samples. Vimentin levels significantly decreased by 51 and 38% in ACPD treated SK-MEL-2 and MeWo cells, respectively compared to 23 and 31% downregulation in DNDA treated samples. E-cadherin levels increased significantly by 18 and 35% in ACPD treated SK-MEL-2 and MeWo cells, while there were 28 and 29% increases in DNDA treated samples. Notably, NF-κB p65 levels significantly increased by 31 and 69% in ACPD treated SK-MEL-2 and MeWo cells, and there were 49 and 89% increases in DNDA treated samples. Phospho vimentin (S473) levels significantly decreased by 82 and 67% in ACPD treated SK-MEL-2 and MeWo cells, respectively, compared to 57 and 41% decreases in DNDA treated samples. Par6 levels significantly decreased by 83 and 74% in ACPD treated SK-MEL-2 and MeWo cells, compared to 79 and 58% decreases in DNDA treated samples. PTEN levels significantly increased by 44 and 55% in ACPD treated SK-MEL-2 and MeWo cells, respectively, compared to 68 and 48% increases in DNDA treated samples. RhoA levels significantly increased by 87 and 70% in ACPD treated SK-MEL-2 and MeWo cells, respectively, compared to 80 and 66% increases in DNDA treated samples. All values (percent) were calculated compared to their respective controls in WB (Fig. 6; densitometry analysis) and significance was indicated as $P \leq 0.05$. β-actin was used as the internal control to ensure that equal amounts of proteins were loaded in each lane in the SDS-PAGE.

**siRNA treatments for PKC-ι and PKC-ζ.** Both melanoma cell lines were treated with siRNA for PKC-ι and PKC-ζ to knock down the expression of said proteins and subsequently investigated the levels of protein expression for the proteins tested (Fig. 7). Scrambled siRNA was also used in addition to the control and there was no significant difference observed between the control and scrambled siRNA treatments for the said proteins.
siRNA treatments of PKC-ι resulted in significant decrease in PKC-ι level by 87 and 64% in SK-MEL-2 and MeWo cell lines, respectively. PKC-ζ decreased by 11 and 0% which is not significant, while Bcl-2 significantly decreased by 68 and 84%, vimentin decreased by 73 and 67%, phospho vimentin (S39) significantly decreased by 92 and 81%, E-cadherin...
increased by 59 and 46%, RhoA increased by 33 and 26%, Par6 decreased by 42 and 55%, PTEN significantly increased by 94 and 88%, phospho AKT (S473) decreased by 22 and 31% and NF-κB increased by 26 and 40% in PKC-ι siRNA treated SK-MEL-2 and MeWo cells, respectively. siRNA treatments of PKC-ζ resulted in significant decrease in PKC-ζ level by 83 and 76% in SK-MEL-2 and MeWo cell lines, respectively. PKC-ι decreased by 16 and 7% which is not significant, Bel-2 significantly decreased by 71 and 62%, vimentin decreased only by 9 and 13%, which is not significant, phospho vimentin (S39) only decreased by 10 and 17%, E-cadherin significantly increased by 20 and 14%, Par6 decreased by 11 and 9%, PTEN increased by 39 and 41%, phospho AKT (S473) decreased by 29 and 28% and NF-κB increased by 37 and 35% in PKC-ι siRNA treated SK-MEL-2 and MeWo cells, respectively. RhoA levels of PKC-ζ siRNA treated samples did not show a significant difference to its control. All significance values are indicated as P≤0.05.

Association of PKC-ι and vimentin. We immunoprecipitated (IP) PKC-ι and PKC-ζ separately and WB experiments were conducted independently for E-cadherin, CD44, vimentin and NF-κB p65. PKC-ζ IP samples did not show any association with any mentioned proteins. Only vimentin immunoblot showed an association with PKC-ι IP samples (Fig. 8). This result suggests that PKC-ι associate with vimentin. To confirm this association, vimentin was immunoprecipitated, developed for said proteins and only PKC-ι was associated with vimentin.

Discussion

PKC-ι and PKC-ζ both have a wide range of effects and are overexpressed in many human cancers (4-7,31-33). Common upstream elements can activate both, but they each perform their own functions (34-37). The domains between PKC-ι and PKC-ζ are largely conserved; given nearly 70% of the similarity, it was important to identify inhibitors specific to aPKCs, and at the same time determine how much each was inhibited. A previous study showed the ACPD did not affect many upstream elements of the aPKC activation pathways (25-27). The computational screening and kinase activity assay data show that ACPD and DNDA are specific inhibitors of aPKCs. ACPD showed the same effect as an inhibitor for both PKC-ζ and PKC-ι in a relative sense. DNDA showed a better action on PKC-ι than PKC-ζ (Fig. 1G).

Determination of cell viability and cytotoxicity of malignant and normal cells after inhibitor treatments was necessary, considering our ultimate intent was to determine if the inhibitors had potential as therapeutic drugs. As observed in WST-1 experiments (Fig. 3A and D), neither
inhibitor had any significant effect on the normal melanocyte cell viability. This in turn suggests that neither inhibitor proved toxic to the normal cells. Both ACPD and DNDA are cytostatic, rather than cytotoxic. Regardless, DNDA showed a mild toxicity towards both malignant melanoma cell lines, suggesting both inhibitors are effective against malignant cells without harming normal cells. This confirms the previous data, which suggested that malignant cells rely on aPKCs to remain viable (8,7,38,39). As observed in the WB on normal melanocytes (Fig. 5A), normal melanocytes produced no detectable levels of PKC-ζ, but they produced PKC-ι. Malignant melanoma cells (Fig. 5B) overexpressed both PKC-ι and PKC-ζ. The total and phosphorylated levels of PKC-ι and PKC-ζ significantly reduced upon treatments with both ACPD and DNDA. Similarly, melanoma cell proliferation was markedly reduced (Fig. 2C-F) upon treatments while not observably affecting cell proliferation on normal melanocyte cells (Fig. 2A and B). This confirms that melanoma cellular functions are highly dependent on aPKCs, but normal melanocytes do not depend on aPKCs.

Previous studies have shown that overexpression of aPKCs have an anti-apoptotic effect in many cancers (4-9,12). We could determine whether treatment with the inhibitors could induce apoptosis, we tested apoptotic markers through a WB (Fig. 5C). Increase in caspase-3, increase in PARP cleavage, and decrease in Bcl-2 all indicate apoptosis stimulation (40-43). Increase in caspase-3 levels is not always a direct indication of induction of apoptosis due to the tight binding of cleaved caspase-3 with X-linked inhibitor of apoptosis protein (XIAP). XIAP undergoes auto-ubiquitilation, but this process delays apoptosis until all XIAP is removed (44). Owing to this, we also tested direct PARP and cleaved PARP levels because PARP is a known downstream target for caspase-3. It cleaves more upon inducing apoptosis (45). We also tested Bcl-2 levels since it inhibits caspase activity by preventing cytochrome c release from the mitochondria and/or by binding to the apoptosis-activating factor (APAF-1). ACPD and DNDA treatments each depicted an increase in apoptotic activity in both SK-MEL-2 and MeWo cell lines. The data confirm some treatments each depicted an increase in apoptotic activity in both SK-MEL-2 and MeWo cell lines, suggesting both inhibitors are effective against malignant cells without harming normal cells. This confirms the previous data, which suggested that malignant cells rely on aPKCs to remain viable (8,7,38,39). As observed in the WB on normal melanocytes (Fig. 5A), normal melanocytes produced no detectable levels of PKC-ι, but they produced PKC-ζ. Malignant melanoma cells (Fig. 5B) overexpressed both PKC-ι and PKC-ζ. The total and phosphorylated levels of PKC-ι and PKC-ζ significantly reduced upon treatments with both ACPD and DNDA. Similarly, melanoma cell proliferation was markedly reduced (Fig. 2C-F) upon treatments while not observably affecting cell proliferation on normal melanocyte cells (Fig. 2A and B). This confirms that melanoma cellular functions are highly dependent on aPKCs, but normal melanocytes do not depend on aPKCs.

Results revealed that upon knocking down PKC-ι, total and phosphorylated vimentin levels significantly decreased by 73 and 93% for SK-MEL-2 cells, as well as 67 and 81% for MeWo cells. The effect of PKC-ζ knockdown on vimentin is negligible compared to the large effect we observed in PKC-ι knockdown. Our results suggest that both vimentin and PKC-ι work together changing the polarity in cancer cell migration. Vimentin activates upon the binding of PKC-ι and phosphorylates at Ser39. It has been previously shown that Par6 can be phosphorylated by aPKCs upon activation of TGF-β receptors, and activated Par6 stimulates EMT in A549 adenocarcinoma cells (11,49). TGF-β activation stimulates degradation of RhoA and cells lose E-cadherin while increasing vimentin. Both inhibitor treatments increased the levels of E-cadherin and RhoA, indicating the inhibition of PKC-ι or PKC-ζ or both can lead to complete stop or reversal of melanoma EMT (Fig. 6). To confirm the results, we tested the levels of Par6 and RhoA and E-cadherin levels in siRNA treated cells (Fig. 7). Results revealed that PKC-ι knockdown increased both E-cadherin and RhoA effectively compared to the PKC-ζ knockdown. In PKC-ζ siRNA treatments, the RhoA effect is only negligible, though its effect on E-cadherin is less when one compares it to the PKC-ι knockdown. This suggests that only PKC-ι is responsible for stimulating EMT. TGF-β stimulation also activates the Wnt/β-catenin pathway; in that case, stabilized β-catenin translocates to the nucleus and inhibits metastasis suppressors in melanoma (16). Previous research supports our observations here that negative regulation of EMT is observed upon inhibition of aPKCs by ACPD and DNDA. It has been previously shown that activated Vimentin inhibits PTEN by increasing the phosphorylation of PTEN to enhance PI3K/AKT activity which leads to cell differentiation and survival of osteoblasts (50). This process can also inhibit apoptosis via the NF-κB pathway (51). Increases in PTEN levels in both ACPD and DNDA treated samples and siRNA treated samples for PKC-ι and PKC-ζ strongly support this statement. Notably, increased levels of PTEN in PKC-ζ treated siRNA samples suggested the involvement of PKC-ζ.

It has been published that PKC-ζ stimulates IKK α/β, which

ultimately stimulates translocation of NF-κB complex into the nucleus resulting in increased cell survival (52). Notably, we found increases in NF-κB p65 levels upon inhibitor treatments, but also upon both siRNA treatments. This may seem illogical, but previous studies have shown that expression of NF-κB p65 has a corresponding increase in IkB, resulting in an auto-regulatory loop (52). It is possible that the upstream signaling for NF-κB translocation is not being inhibited by negative feedback since translocation has been inhibited downstream, resulting in more NF-κB production. NF-κB is still inhibited from translocating to the nucleus. This causes cell mediated apoptosis and cell survival rate reductions. In summary (Fig. 9), our results suggest that PKC-ι is responsible for inducing EMT, while PKC-ζ is mainly involved in NF-κB signaling to promote cell growth and survival. In addition, PKC-ι is involved in stimulating AKT signaling by inhibiting PTEN via vimentin activation. Activated vimentin induces the phosphorylation of PTEN.

In conclusion, our results suggest that ACPD and DNDA are effective aPKC inhibitors in melanoma cells, and do not affect normal melanocytes. ACPD and DNDA can reduce the cell proliferation, migration and invasion, while inducing apoptosis. Results also show a direct relationship between vimentin, PKC-ι and EMT. aPKC inhibitors (ACPD and DNDA) can reduce EMT progression, or possibly reverse it by specifically inhibiting PKC-ι. Finally, collected evidence suggest ACPD and DNDA could be potential therapeutic drugs for malignant melanoma.

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