

Protein Kinase C δ Is a Therapeutic Target in Malignant Melanoma with NRAS Mutation

Asami Takashima,[†] Brandon English,^{||} Zhihong Chen,[†] Juxiang Cao,[§] Rutao Cui,^{†,§} Robert M. Williams,^{||,‡} and Douglas V. Faller*,^{†,‡}

[†]Cancer Center, [‡]Departments of Medicine, Biochemistry, Pediatrics, Microbiology, Pathology and Laboratory Medicine, and

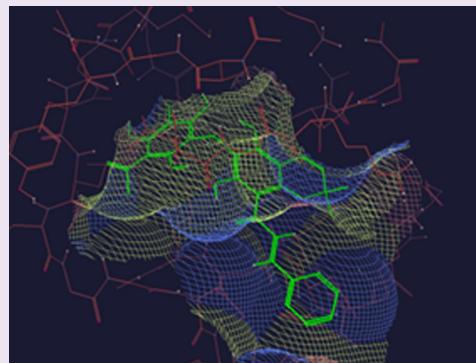
[§]Department of Dermatology, Boston University School of Medicine, 72 E Concord Street, Boston, Massachusetts 02118, United States

^{||}Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523, United States

[‡]University of Colorado Cancer Center, Aurora, Colorado 80045, United States

S Supporting Information

ABSTRACT: NRAS is the second most frequently mutated gene in melanoma. Previous reports have demonstrated the sensitivity of cancer cell lines carrying KRAS mutations to apoptosis initiated by inhibition of protein kinase C δ (PKC δ). Here, we report that PKC δ inhibition is cytotoxic in melanomas with primary NRAS mutations. Novel small-molecule inhibitors of PKC δ were designed as chimeric hybrids of two naturally occurring PKC δ inhibitors, staurosporine and rottlerin. The specific hypothesis interrogated and validated is that combining two domains of two naturally occurring PKC δ inhibitors into a chimeric or hybrid structure retains biochemical and biological activity and improves PKC δ isozyme selectivity. We have devised a potentially general synthetic protocol to make these chimeric species using Molander trifluoroborate coupling chemistry. Inhibition of PKC δ , by siRNA or small molecule inhibitors, suppressed the growth of multiple melanoma cell lines carrying NRAS mutations, mediated *via* caspase-dependent apoptosis. Following PKC δ inhibition, the stress-responsive JNK pathway was activated, leading to the activation of H2AX. Consistent with recent reports on the apoptotic role of phospho-H2AX, knockdown of H2AX prior to PKC δ inhibition mitigated the induction of caspase-dependent apoptosis. Furthermore, PKC δ inhibition effectively induced cytotoxicity in BRAF mutant melanoma cell lines that had evolved resistance to a BRAF inhibitor, suggesting the potential clinical application of targeting PKC δ in patients who have relapsed following treatment with BRAF inhibitors. Taken together, the present work demonstrates that inhibition of PKC δ by novel small molecule inhibitors causes caspase-dependent apoptosis mediated *via* the JNK-H2AX pathway in melanomas with NRAS mutations or BRAF inhibitor resistance.



Although melanomas account for less than 5% of skin cancer cases, they were responsible for more than 75% of estimated skin cancer deaths in 2012, and the incidence rate has been increasing for the last 30 years.¹ While chemotherapeutic treatments have improved response rates in metastatic melanoma, there has been no significant impact on survival for decades.¹

Melanoma is highly dependent upon the RAS/RAF/MEK/ERK pathway, one of the three major mitogen-activated protein kinase (MAPK) pathways. The components of this pathway, therefore, can serve as the targets of drugs for late-stage melanomas. BRAF (one of the three RAF isoforms) is the most commonly mutated gene in melanoma (45–55% of melanoma cases), while mutations in NRAS (one of the three RAS isoforms) are observed in 15–30% of melanoma cases.^{2,3} The BRAF inhibitor PLX4032 (vemurafenib) shows high activity in patients with BRAF-V600E mutation; however, responders eventually and inevitably became resistant to this drug and relapsed.⁴ One of the proposed mechanisms of acquired

resistance to vemurafenib is reactivation of MEK/ERK signaling independently of BRAF, the suppression of which had been the goal of PLX4032 action, through a variety of compensatory alterations.^{5,6} In contrast to BRAF, the oncogenic RAS/GAP switch is an exceedingly difficult target for rational drug discovery and is now widely considered “undruggable”.^{3,7,8} An “indirect” approach, targeting a survival pathway required by tumor cells bearing an activated RAS allele, may represent an alternative strategy for NRAS mutant melanomas.

We previously demonstrated that cancer cells carrying oncogenic KRAS mutations undergo apoptosis when protein kinase C δ (PKC δ) activity is inhibited by means of a chemical inhibitor, RNA interference, or a dominant-negative var-

Received: November 11, 2013

Accepted: February 7, 2014

Published: February 7, 2014

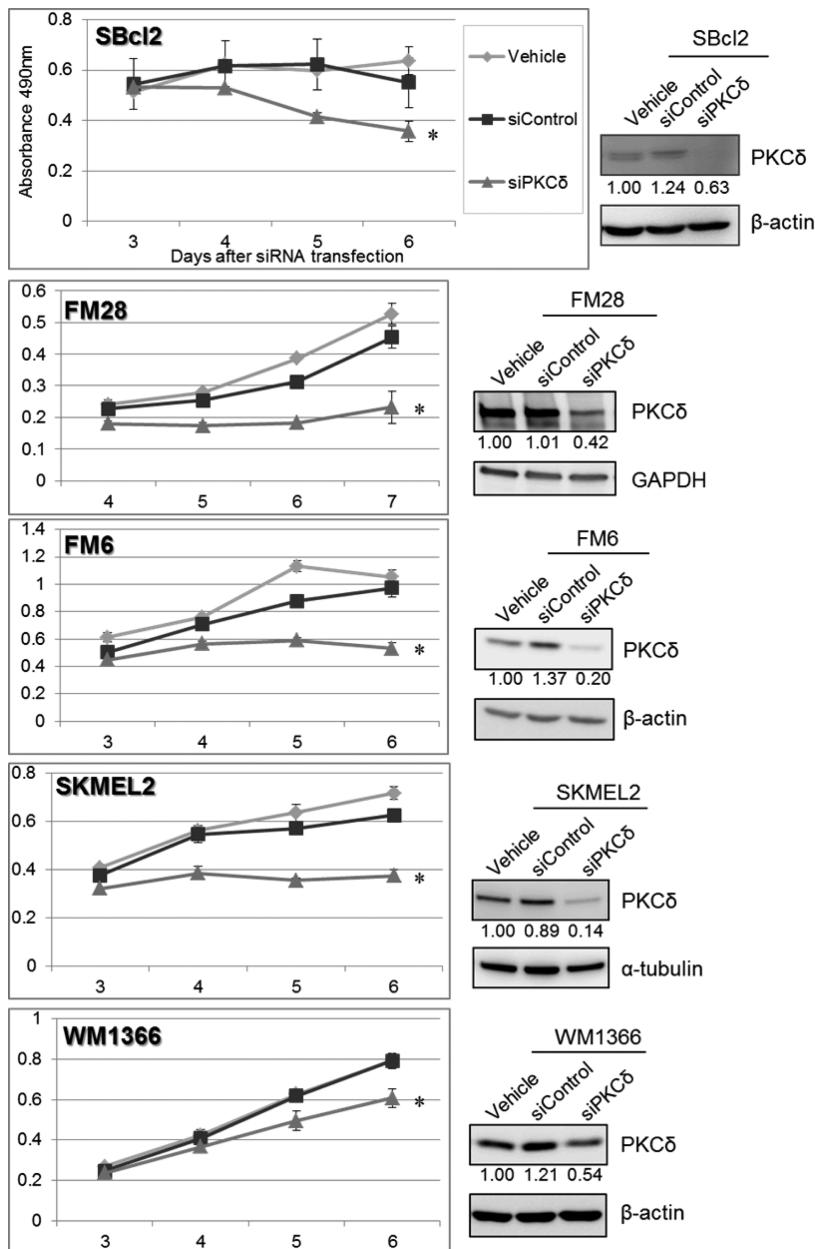
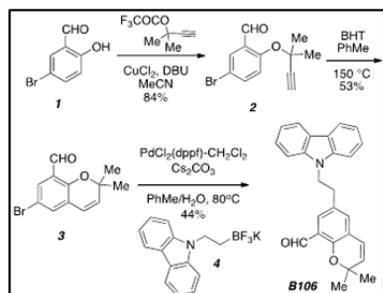
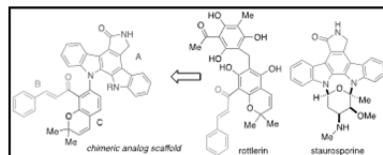
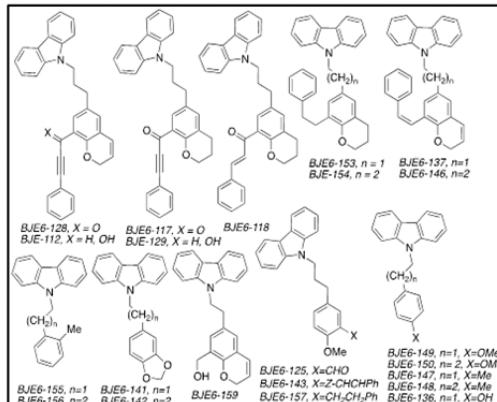
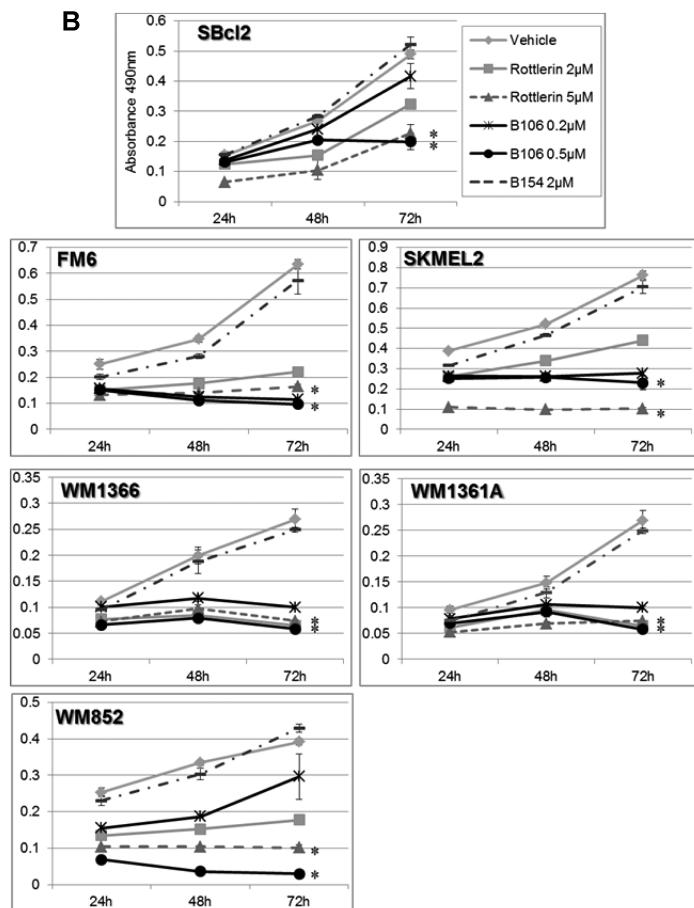


Figure 1. Downregulation of PKC δ suppresses cell survival in melanoma cell lines with NRAS mutation. siRNA targeting PKC δ ("siPKC δ ") or nontargeting siRNA ("siControl") were transfected into SBcl2 and FM28 (50 nM), SKMEL2 (10 nM), and FM6 and WM1366 (5 nM), after establishing cell line-specific optimal transfection conditions. As a vehicle control, cells were treated in parallel with transfection reagent alone ("vehicle"). MTS assays were performed at 3 or 4 days after siRNA transfection. Each point represents the average of triplicates, and error bars indicate the standard deviations. p values (*) were calculated between vehicle control and siPKC δ on the last assay day ($p < 0.006$). Downregulation of PKC δ protein on the first assay day was assessed by immunoblot analysis. The relative band intensity of PKC δ is indicated below the image (normalized to loading controls, β -actin, α -tubulin, or GAPDH).

iant.^{9–12} Other groups also subsequently validated PKC δ as a target in cancer cells of multiple types with aberrant activation of KRAS signaling.^{13,14}

PKC δ belongs to the PKC family of serine/threonine protein kinases, which are involved in diverse cellular functions, such as proliferation, tumor promotion, differentiation, and apoptotic cell death.¹⁵ The PKC family is categorized into three subfamilies based on structural, functional, and biochemical differences and activators: the classical/conventional PKCs (α , β I, β II, γ), the novel PKCs (δ , ϵ , θ , μ), and the atypical PKCs (ζ , λ). The novel PKCs, including PKC δ , are characteristically activated by diacylglycerol (DAG) and are independent of the

need for the secondary messenger Ca^{2+} . PKC δ functions as either a pro-apoptotic or an antiapoptotic/pro-survival regulator depending upon cellular context, such as the specific stimulus or its subcellular localization.¹⁵ PKC δ is implicated as an early regulator in certain antiapoptotic/pro-survival signaling cascades through induction or suppression of downstream substrates, including ERK, AKT, and NF- κ B. Other context-dependent effectors of PKC δ include JNK, glycogen synthase kinase-3 (GSK3), FLICE-like inhibitory protein (FLIP), cIAP2, and p21 $^{\text{cip1/WAF1}}$. A role for PKC δ as an antiapoptotic/pro-survival regulator has been reported in various types of cancer cells, including non-small cell lung cancer, pancreatic, and colon

A Panel 1

Panel 2
**Scheme 1**
B
**Figure 2.** continued

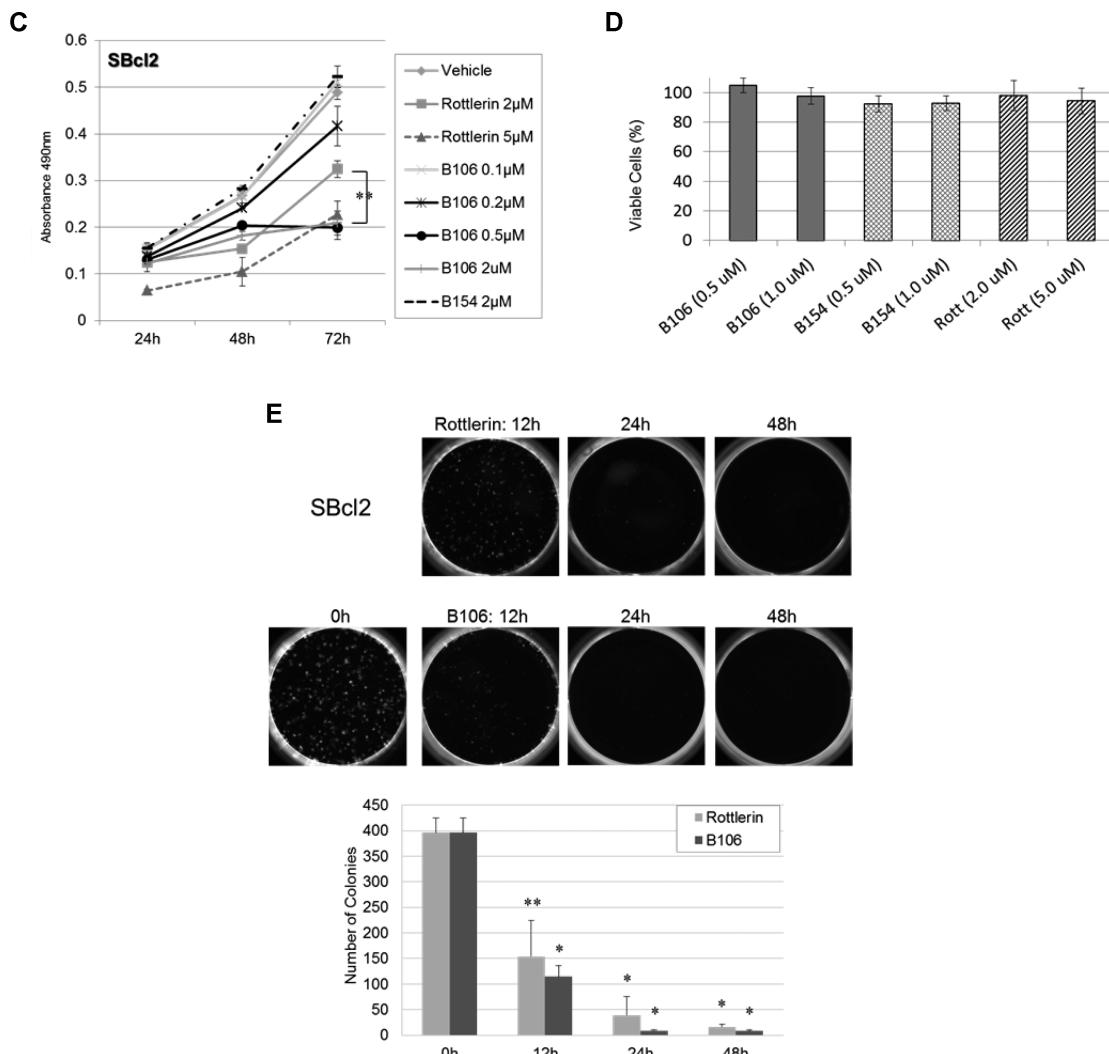


Figure 2. PKC δ inhibitors suppress survival in melanoma cell lines with NRAS mutations. (A) Structure and synthesis of PKC δ inhibitors. Panel 1: Design of mallotoxin/rottlerin-staurosporine hybrids. Scheme 1: Synthesis of B106. Panel 2: 3rd generation compounds. (B) PKC δ inhibitors suppress cell survival in melanoma cell lines with NRAS mutations. SBcl2, FM6, SKMEL2, WM1366, WM1361A, and WM852 cells were exposed to rottlerin (2 or 5 μ M) or B106 (0.2 or 0.5 μ M) for 24, 48, or 72 h, and MTS assays were performed at each time point. DMSO and B154 (2 μ M) served as a vehicle control and a negative compound control, respectively. Each point represents the average of triplicates and error bars indicate the standard deviations. *p* values (*) were calculated between DMSO (vehicle control) and rottlerin 5 μ M or DMSO and B106 0.5 μ M in each cell line at 72 h ($p < 0.0002$). (C) Titration of PKC δ inhibitor treatment. The expanded doses of B106 (0.1 μ M and 2 μ M) in the MTS assay in SBcl2 in Figure 2A are shown. ** indicates a *p* value < 0.5 between treatment of 2 μ M of rottlerin and B106. (D) Effects of PKC δ inhibitors on primary human melanocytes. Cell survival of human primary melanocytes exposed to the indicated concentrations of the compounds for 72 h (relative to DMSO-treated controls; mean \pm SD, $n = 3$). (E) PKC δ inhibitors induce irreversible effects on cell growth. SBcl2 cells were treated with rottlerin or B106 at 1 μ M for 0, 12, 24, or 48 h. After these exposure times, the same number of viable cells from each treatment condition was replated at low cell density and cells were cultured in medium without inhibitors for 8 days. Cell colonies were counted. Each point represents the average of triplicates and error bars indicate the standard deviations. *p* values: ** $p < 0.01$, * $p < 0.001$ compared to time 0 h.

cancers.^{16–20} Interestingly, these types of cancers are correlated with high rates of activating mutations in KRAS genes.^{7,8} Importantly, unlike many other PKC isozymes, PKC δ is not required for the survival of normal cells and tissues, and PKC δ -null mice are viable, fertile, and develop normally.²¹

Our previous studies demonstrating the synthetic lethal activity of PKC δ inhibition in pancreatic, lung, neuroendocrine, and breast cancers, and cancer stem-like cells (CSCs) with KRAS mutations^{9–12} suggested the potential of targeting PKC δ in melanomas with an activating NRAS mutation. In this study, we demonstrate that inhibition of PKC δ by siRNA or novel chemical compounds suppresses the growth of melanoma lines with NRAS mutations through induction of caspase-dependent

apoptosis. A novel PKC δ inhibitor developed through pharmacophore modeling exerted cytotoxic activity on NRAS mutant tumors at concentrations 1 log lower than commercially available PKC δ inhibitors. This cytotoxicity was mediated by activation of stress-responsive JNK-H2AX pathway, which involves a novel function of phospho-H2AX in mediating the apoptotic response. Furthermore, this study also showed that PKC δ inhibition can effectively inhibit the growth of PLX4032-resistant melanoma cells with BRAF mutations, demonstrating the potential of an approach targeting PKC δ in the substantial fraction of patients with melanoma who currently have only limited treatment options.

Table 1. Comparison of Properties of PKC δ Inhibitors^a

| cmpds. | generation | PKC δ IC ₅₀ | PKC α IC ₅₀ | PKC δ /PKC α selectivity | "Ras-specific" cytotoxicity |
|-----------|------------|-------------------------------|-------------------------------|--|-----------------------------|
| Rottlerin | 1st | 3–5 μ M | 75 μ M | 28-fold | 3–5 μ M |
| KAM1 | 2nd | 3 μ M | 157 μ M | 56-fold | 3 μ M |
| B106 | 3rd | 0.05 μ M | 50 μ M | 1000-fold | 0.5 μ M |
| B154 | 3rd | >40 μ M | >100 μ M | | none |

^aIn vitro kinase assays demonstrated that third generation PKC δ inhibitor B106 is more potent and more selective for PKC δ over PKC α than rottlerin/mallotoxin or the 2nd generation PKC δ inhibitor KAM1. B154 is used as an inactive (negative control) compound.

RESULTS AND DISCUSSION

PKC δ Is a Potential Therapeutic Target in Melanoma with NRAS Mutation. To validate the potential of this approach targeting PKC δ in melanomas with NRAS mutations, we first examined the effect of PKC δ -selective inhibition on cell growth by specifically and selectively knocking down PKC δ protein expression in multiple melanoma cell lines harboring NRAS mutations, using siRNA. The specificity of the PKC δ -specific siRNAs employed herein for PKC δ among all the other PKC isoforms has been previously demonstrated.^{9–11} Even partial knockdown of PKC δ protein significantly inhibited the proliferation of multiple melanoma cell types with NRAS mutations, including SBcl2, FM28, FM6, and SKMEL2 cells (Figure 1). Interestingly, the degree of protein knockdown did not appear to be the sole factor in determining the degree of growth inhibitory effect by siRNA transfection; some cell lines were more susceptible than others to cell growth inhibition resulting from PKC δ downregulation. No viable cells with chronic suppression of PKC δ could ever be isolated, consistent with our previous demonstration of a requirement for PKC δ activity for the viability in cells bearing mutationally activated RAS.

These cell survival assays verified that PKC δ is essential for survival of NRAS mutant melanoma cells.

Development of Novel PKC δ Inhibitor BJE6-106 (B106). Potent small molecule inhibitors of PKC δ have not previously been available. Broad (pan) inhibitors of PKC isozymes are generally toxic, as certain PKC isozymes are required for normal physiological functions, and inhibition of such isozymes by a nonselective PKC δ inhibitor can damage normal cells.^{22,23} We therefore pursued development of a more potent PKC δ inhibitor with higher PKC δ selectivity in order to explore the therapeutic potential of this approach of targeting PKC δ .

We initially generated a pharmacophore model based on molecular interactions of small molecules with "novel" class PKC isozymes. In the initial pharmacophore model for PKC δ inhibitors, mallotoxin/rottlerin, a naturally occurring product, with moderate aqueous solubility, and oral bioavailability,²⁴ was used as a prototype structure for a molecule with PKC δ -inhibitory activity (IC₅₀ = 5 μ M). Protein structural data for PKC θ , another "novel" PKC isozyme, which is also inhibited by mallotoxin/rottlerin, was incorporated (Supporting Information). Mallotoxin/rottlerin is relatively selective for PKC δ over PKC α (PKC δ IC₅₀:PKC α IC₅₀ is approximately 30:1). We and others have also shown that mallotoxin/rottlerin, at the concentrations employed herein, is not cytostatic or cytotoxic to normal primary cells or cell lines and is well-tolerated when administered orally or intraperitoneally to mice.^{9–12,24} This favorable toxicity profile, combined with its *in vivo* efficacy, made mallotoxin/rottlerin attractive as a starting point for modification and drug development. We further developed the pharmacophore model using a prototype chimeric structure

based on mallotoxin/rottlerin and a more general class of protein kinase C inhibitors (the natural product staurosporine), and incorporating protein structural data for "novel" class PKCs. The strategy was to retain most of the "bottom" part of mallotoxin/rottlerin (Figure 2A, panel 1), which is assumed to give mallotoxin/rottlerin its PKC δ specificity, but to vary the "head group", which is assumed to bind to the hinge region of the kinase active site. Numerous "head groups" from known potent kinase inhibitors were tested in the PKC δ model.¹¹ The criteria for selection was that the resulting molecule should form favorable interactions with the hinge region while the "bottom part" retained interactions with the binding site similar to that of staurosporine (from the X-ray crystallographic studies) and mallotoxin/rottlerin (from docking studies into PKC δ). In these second generation of PKC δ inhibitors, the "head" group was made to resemble that of staurosporine, a potent general PKC inhibitor, and other bisindoyl maleimide kinase inhibitors, with domains B (cinnamate side chain) and C (benzopyran) conserved from the mallotoxin/rottlerin scaffold to preserve isozyme specificity. The chromene portion of mallotoxin was combined with the carbazole portion of staurosporine to produce chimeric molecule including KAM1.¹¹ KAM1 was indeed active and more PKC δ -specific than rottlerin/mallotoxin and showed activity against cancer cells with activation of RAS or RAS signaling, including human neuroendocrine tumors, pancreatic cancers, and H460 lung cancer cells.¹¹ KAM1 had an IC₅₀ of 3 μ M for PKC δ (similar to mallotoxin/rottlerin) and better isozyme selectivity (IC₅₀ > 150 μ M for PKC α) (Table 1).¹¹

On the basis of structure–activity relationship (SAR) analysis of KAM1 and other second generation compounds, we then generated 36 new third generation compounds (Figure 2A, panel 2). These derivatives showed a broad range of PKC δ -inhibitory activity, ranging from IC₅₀ > 40 μ M to IC₅₀ < 0.05 μ M (Supporting Information Table 1). BJE6-106 (B106) (Figure 2A, Scheme 1), our current lead third generation compound, has an IC₅₀ for PKC δ of <0.05 μ M and targeted selectivity over classical PKC isozymes (a 1000-fold PKC δ selectivity over PKC α) (Table 1). BJE6-154 (B154) was among the least potent of the 36 compounds studied (PKC δ IC₅₀ > 40 μ M) and was used as a negative-control compound with minimal inhibitory activity against PKC δ .

Inhibition of PKC δ Activity Induces Cell Growth Inhibition in Melanoma Cell Lines with NRAS Mutations. To investigate the effect of PKC δ inhibition by small molecule compounds on tumor cell growth, tumor cell survival was assessed in the presence of mallotoxin/rottlerin or B106 using a panel of melanoma cell lines with Q61 NRAS mutations, including SBcl2, FM6, SKMEL2, WM1366, WM1361A, and WM852 (Figure 2B, Table 2). Cells were exposed to rottlerin (2 or 5 μ M) or B106 (0.2 or 0.5 μ M) and viable cells were quantitated at 24, 48, and 72 h after treatment. Rottlerin consistently inhibited proliferation of all cell lines at 5 μ M, and

Table 2. Confirmed NRAS Q61 Mutations of the Cell Lines

| cell line | allele | amino acid | type |
|-----------|--------|------------|--------------|
| SBcl2 | C181A | Q61K | homozygous |
| FM6 | C181A | Q61K | heterozygous |
| FM28 | C181A | Q61K | homozygous |
| SKMEL2 | A182G | Q61R | heterozygous |
| WM-1361A | A182G | Q61R | heterozygous |
| WM-1366 | A182T | Q61L | heterozygous |
| WM852 | A182G | Q61R | homozygous |

intermediate inhibitory effects were observed at 2 μM . The third generation PKC δ inhibitor B106 effectively inhibited growth of all cell lines tested at 0.5 μM , and at 0.2 μM in some cell lines, which is at least 10 times lower than the concentration of rottlerin required to exert the same magnitude of cytotoxic effect. Both inhibitors demonstrated dose-dependent cytotoxic effects, and B106 at 0.5 μM was significantly more active than rottlerin at 2 μM (Figure 2C). Exposure to B154 at 2 μM produced a proliferation curve similar to vehicle (DMSO) treatment in all cell lines, consistent with our hypothesis that the cell growth inhibition induced by B106 resulted from the inhibition of PKC δ activity. Furthermore, B106 produced no statistically significant effects on the proliferation of primary human melanocytes at concentrations of 0.5 and 1.0 μM , indicating the tumor-specific effect of B106 (Figure 2D).

To assess the irreversible damage done to the cells by PKC δ inhibition in a different manner, clonogenic colony assays were performed using SBcl2 melanoma cells to determine the kinetics of the action of PKC δ inhibitors on the growth and proliferative characteristics of the cells. In contrast to a proliferation assay, which examines potentially temporary and reversible effects on proliferation and survival, clonogenic assays assess irreversible effects of a compound on cell viability and proliferative capacity. Cells were exposed to mallotoxin/rottlerin or B106 for 12, 24, or 48 h and then replated in medium without inhibitors, and the difference in colony-forming ability of cultures was assessed. Both mallotoxin/rottlerin and B106 treatment significantly decreased the number of colonies formed in SBcl2 cells after as little as 12 h of treatment, and approximately 40-fold reduction in the number of colonies was observed with 48 h of drug exposure (Figure 2E). These results demonstrate an irreversible cytotoxic effect of these PKC δ inhibitors on tumor cell growth, even after limited and transient exposure to the compounds.

Collectively, these results supported PKC δ as a potential therapeutic target in melanomas with NRAS mutation. The new PKC δ inhibitor B106 demonstrated activity at nanomolar concentrations, and may serve as a lead compound for future modifications.

Inhibition of PKC δ Activity Triggers Caspase-Dependent Apoptosis. We next determined how PKC δ inhibition results in suppression of tumor cell growth in melanoma. Activated caspase 3 and caspase 7, the ultimate executioners of apoptosis, trigger proteolytic cleavage of crucial key apoptotic proteins, which in turn leads to late apoptotic events, including DNA fragmentation. The activity of effector caspases 3 and 7 was assessed in cells treated with PKC δ inhibitors. Twenty-four hours of exposure to rottlerin (5 μM) or B106 (0.2 and 0.5 μM) significantly increased the activity of caspase 3/7 in SBcl2 cells compared to vehicle (DMSO) (Figure 3A). The effect of B106 on caspase 3/7 activation was greater than that of

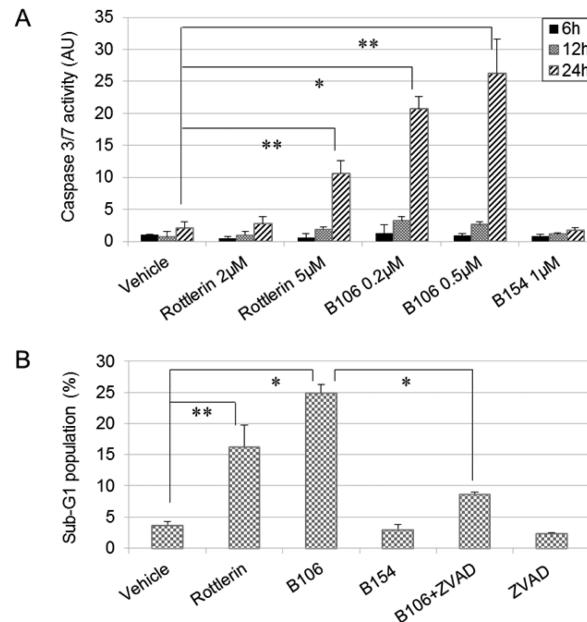


Figure 3. Inhibition of PKC δ induces caspase-dependent apoptosis. (A) Effector caspase 3/7 activation by PKC δ inhibition. SBcl2 cells were exposed to rottlerin (2 or 5 μM) or B106 (0.2 or 0.5 μM) for 6, 12, or 24 h and caspase 3/7 activity was measured. DMSO and B154 (1 μM) served as a vehicle control and a negative compound control, respectively. The average values of triplicates were normalized to those of vehicle-treated sample at 6 h. Error bars indicate the standard deviations. p values: ** $p < 0.003$, * $p < 0.0002$. (B) DNA fragmentation induced by PKC δ inhibition. SBcl2 cells were treated with rottlerin (5 μM), B106 (0.5 μM) alone, or B106 (0.5 μM) plus the pan-caspase inhibitor Z-VAD-FMK (100 μM) together for 24 h. The proportion of sub-G1 population was measured by flow cytometry. Values represent the average of duplicates and error bars indicate the standard deviations. p values: ** $p < 0.04$, * $p < 0.004$.

rottlerin: a 10-fold increase at 0.2 μM and a 12.5-fold increase at 0.5 μM of B106, in contrast to a 5-fold increase by rottlerin at 5 μM . These findings indicated the potential involvement of caspase 3/7-mediated apoptosis in response to PKC δ inhibition.

As evidence of apoptosis, induction of DNA fragmentation, a hallmark of late events in the sequence of the apoptotic process, in the presence or absence of PKC δ inhibitors was assessed by flow cytometric analysis. The proportion of cells containing a DNA content of less than $2n$ (fragmented DNA), categorized as the “sub-G1” population and considered in the late apoptotic phase, was significantly higher after treatment with rottlerin at 5 μM and even higher after treatment with B106 at 0.5 μM , whereas B154, a negative-control compound for B106, lacking PKC δ -inhibitory activity, produced no more fragmented DNA than did vehicle control (DMSO), suggesting the effect of B106 on DNA fragmentation was related to inhibition of PKC δ activity (Figure 3B). To determine whether activation of caspases by PKC δ inhibitors was necessary for the observed apoptosis, the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) was employed. Pretreatment of cells with Z-VAD-FMK (50 μM) prevented B106-induced caspase 3 cleavage in immunoblot analysis (data not shown). B106-induced DNA fragmentation was significantly abrogated when SBcl2 cells were pretreated with Z-VAD-FMK (100 μM) (Figure 3B). Taken together, these data suggest that PKC δ inhibition attenuates tumor cell

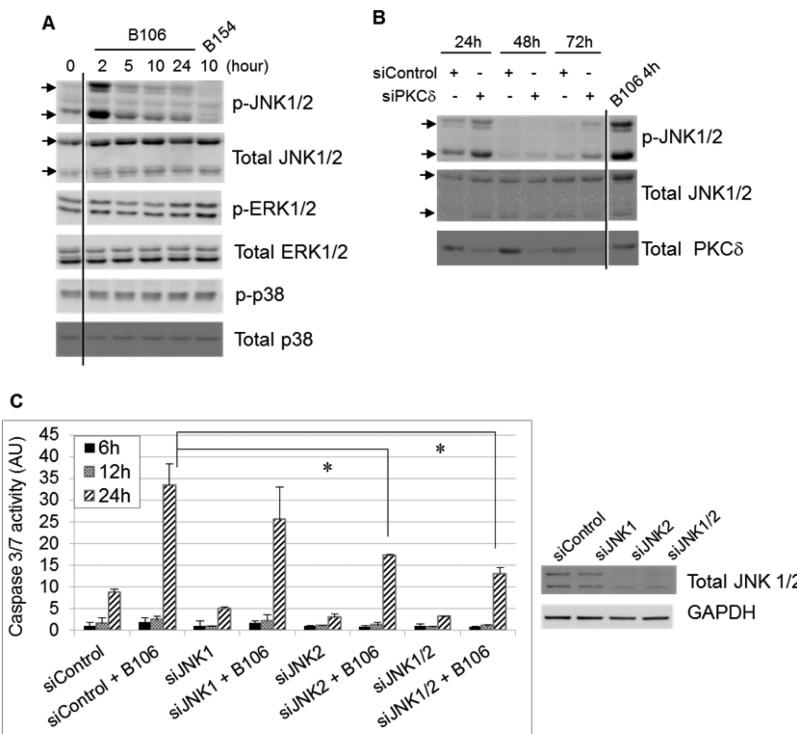


Figure 4. PKC δ inhibition triggers an apoptotic response through activation of JNK. PKC δ inhibition activates JNK. (A, B) SBcl2 cells were exposed to B106 (1 μ M) or the negative control compound B154 (1 μ M) for indicated times (A) or transfected with siRNA targeting PKC δ (“siPKC δ ”) or nontargeting siRNA (“siControl”) at 5 nM for the indicated times (B). Protein lysates were subjected to immunoblot analysis for levels of phosphorylated or total MAPK proteins. (C) Activation of caspase 3/7 is mitigated by knockdown of JNK prior to B106 treatment. SBcl2 cells were transfected with siRNA targeting JNK1 or JNK2 alone (5 nM), or the combination of JNK1 and JNK2 siRNA (5 nM each), or nontargeting siRNA (10 nM) for 72 h, and subsequently exposed to B106 (0.5 μ M) or vehicle (DMSO) for 6, 12, and 24 h. Caspase 3/7 activity was measured. The average values of triplicates were normalized to those of the vehicle-treated sample at 6 h between the pairs exposed to the same siRNA. Error bars indicate the standard deviations. p values: * p < 0.005. Downregulation of JNK1/2 proteins were confirmed by immunoblot analysis at 72 h. In panels A and B, certain lanes not relevant to this discussion were excised, as indicated by the vertical lines.

growth by inducing caspase-dependent apoptosis in NRAS mutant melanoma cells.

PKC δ Inhibition Triggers Apoptotic Response via the Stress-Responsive JNK Pathway. To identify which intracellular signaling pathway PKC δ inhibition employs to induce cytotoxicity, the activation status of known downstream targets of PKC δ was examined after PKC δ inhibition, including MAPKs (ERK, p38, and JNK), AKT, NF κ B pathway, cyclin-dependent kinase inhibitors, p53, IAPs, GSK3 β , or c-Abl. Inhibition of PKC δ activity in SBcl2 cells by B106 induced phosphorylation (activation) of JNK1/2 (T183/Y185) most strongly after 2 h of exposure (Figure 4A). In contrast, phosphorylation of the closely related MAPKs p38 and ERK was not affected by PKC δ inhibitors (Figure 4A). Consistent with these observations generated using chemical inhibitors, selective downregulation of PKC δ by transfection of PKC δ -specific siRNA induced phosphorylation of JNK1/2 at 24 h, (when effects of siRNA on PKC δ levels were first observed) (Figure 4B). Transfection of PKC δ -specific or negative control siRNA did not affect phosphorylation levels of ERK or p38.

Among its pleiotropic cellular activities, JNK is an effector in certain apoptotic responses, and some chemotherapeutic agents, including paclitaxel, cisplatin and doxorubicin, employ the JNK pathway for their cytotoxic activity.^{25,26} Because of the data demonstrating that PKC δ inhibition causes caspase-dependent apoptosis (Figure 3) and JNK activation (Figures 4A and B), the effect of inhibition of the JNK pathway during B106 treatment was explored to determine if there is a

functional relationship. SBcl2 cells were transfected with nonspecific siRNA or siRNA specific for JNK1 or JNK2 alone, or cotransfected with JNK1- plus JNK2-specific siRNA for 72 h, and then exposed to B106 or DMSO (vehicle) for 6, 12, or 24 h, followed by measurement of caspase activity (Figure 4C). Analysis at 24 h after B106 treatment showed that knockdown of JNK2 alone, and coknockdown of JNK1 and 2, mitigated B106-induced caspase 3/7 activation in rough proportion to the knockdown efficiency of JNK1/2 proteins. These data indicated that JNK is a necessary mediator of the apoptotic response induced by PKC δ inhibition.

PKC δ Inhibition Activates the MKK4-JNK-H2AX Pathway. We tested for involvement of known upstream and downstream effectors of the JNK pathway following PKC δ inhibition. The MAPKK kinases MKK4 and MKK7 lie one tier above JNK. MKK4 was activated by B106 (Figure 5A), whereas MKK7 was not phosphorylated in response to B106 (data not shown). Activation of the canonical JNK substrate, c-Jun, was also observed in response to B106 exposure, confirming the activation of the JNK pathway by PKC δ inhibitors (Figure 5A). Furthermore, activation of H2AX (histone H2A variant X), another downstream effector of JNK associated with its apoptotic actions,²⁷ was noted at later time points in response to B106 treatment (Figure 5A). B106 consistently induced H2AX phosphorylation as early as after 10 h of exposure. The effect of PKC δ inhibition on H2AX activation was further confirmed by selective downregulation of PKC δ with siRNA. Phosphorylation of H2AX was observed at 72 h after PKC δ

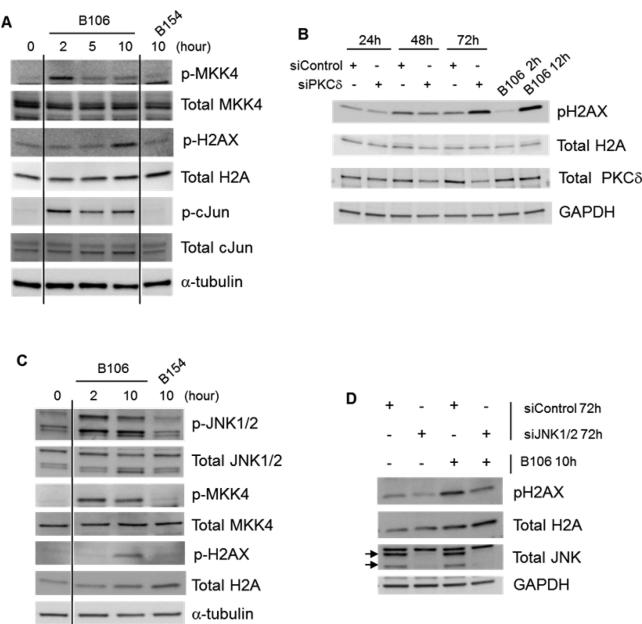


Figure 5. PKC δ inhibition activates the MKK4-JNK-H2AX pathway. (A) Activation of upstream and downstream components of the JNK pathway by B106. SBcl2 cells were exposed to B106 or the negative control compound B154 at 1 μ M for the indicated times. Protein lysates were subjected to immunoblot analysis. (B) Selective downregulation of PKC δ results in phosphorylation of H2AX. SBcl2 cells were transfected with siRNA targeting PKC δ (“siPKC δ ”) or nontargeting (“siControl”) at 50 nM for the indicated times. Protein lysates were subjected to immunoblot analysis. In panels A and B, certain lanes not relevant to this discussion were excised, as indicated by the vertical lines. (C) PKC δ inhibition activates H2AX through JNK. SBcl2 cells were transfected with siRNA targeting JNK1 and JNK2 together (5 nM each) or nontargeting siRNA (10 nM) for 72 h and subsequently exposed to B106 (0.5 μ M) or vehicle (DMSO) for 10 h. Protein lysates were subjected to immunoblot analysis. Arrows indicate JNK1/2.

siRNA transfection (Figure 5B). PKC δ inhibition by B106 treatment similarly induced phosphorylation of MKK4, JNK and H2AX in NRAS mutant melanoma WM1366 cells (Figure 5C).

Because JNK affects diverse downstream effectors, we next determined whether JNK activation caused by PKC δ inhibition is directly linked to B106-induced H2AX activation. Knockdown of JNK1/2 itself slightly reduced basal phospho-H2AX (pH2AX) expression, indicating that basal phosphorylation of H2AX is regulated by JNK (Lane 2, Figure 5D). B106 exposure robustly induced phosphorylation of H2AX in control siRNA-treated cells (Lane 3, Figure 5D); in comparison, prior downregulation of JNK1/2 protein by siRNA attenuated B106-induced H2AX phosphorylation (Lane 4, Figure 5D). Collectively, these data suggest that PKC δ inhibition directly or indirectly activates MKK4 in cells containing mutated NRAS, which in turn activates JNK1/2 and subsequently H2AX.

H2AX is a Critical Regulator of Caspase-Dependent Apoptosis Induced in Response to PKC δ inhibition. Although phosphorylation of H2AX is best known as a consequence of DNA double-stranded breaks in the DNA-damage response, facilitating repair,^{28–30} recent studies have demonstrated that phosphorylation of H2AX at Ser 139 resulting from JNK activation actively mediates the induction of apoptosis by inducing DNA fragmentation in UV- or

chemotherapy-damaged cells.^{31–34} Accordingly, the direct involvement of H2AX in apoptotic response to PKC δ inhibition was examined. SBcl2 cells were transfected with siRNA targeting H2AX, or nontargeting siRNA, for 72 h and then exposed to B106 for 6, 12, or 24 h, with subsequent assay of caspase 3/7 activation. Downregulation of H2AX prior to B106 treatment greatly decreased the level of caspase 3/7 activation at 24 h of B106 exposure (Figure 6A).

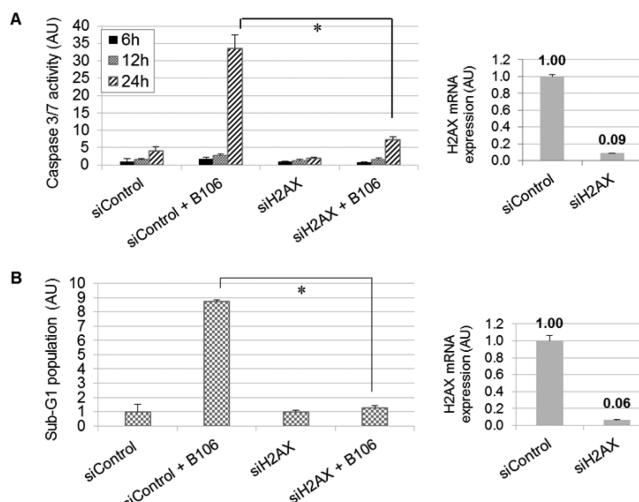


Figure 6. H2AX is a critical apoptotic regulator in apoptosis induced by PKC δ inhibition. (A) Activation of caspases 3/7 is mitigated by knockdown of H2AX prior to B106 treatment. SBcl2 cells were transfected with siRNA targeting H2AX or nontargeting siRNA at 5 nM for 72 h, and subsequently exposed to B106 (0.5 μ M) or vehicle for 6, 12, or 24 h. Caspase 3/7 activity was measured. The average values of triplicates were normalized to those of the vehicle-treated sample at 6 h between the pairs exposed to the same siRNA. Error bars indicate the standard deviations. p values: * p < 0.005. Downregulation of H2AX at 72 h was confirmed by quantitative PCR. (B) Induction of DNA fragmentation is mitigated by knockdown of H2AX prior to B106 treatment. SBcl2 cells were transfected with siRNA targeting H2AX, or nontargeting siRNA, at 5 nM for 72 h, and subsequently exposed to B106 (0.5 μ M) or vehicle for 24 h. The proportion of sub-G1 population was measured by flow cytometry. The average values of duplicates were normalized to those of the vehicle-treated samples between the pairs exposed to the same siRNA. Error bars indicate the standard deviations. p value: * p < 0.0004. Downregulation of H2AX at 96 h was confirmed by quantitative PCR.

To explore a direct link between H2AX and the execution of apoptosis, PKC δ inhibition-induced DNA fragmentation was examined in the presence or absence of H2AX. SBcl2 cells were transfected with either negative-control siRNA or siRNA targeting H2AX for 72 h, and then subjected to PKC δ inhibition by exposure to B106 for 24 h. PKC δ inhibition by B106 treatment increased DNA fragmentation 8.5-fold in the cells transfected with negative control siRNA (Figure 6B). In contrast, PKC δ inhibition by B106 treatment failed to induce DNA fragmentation in the absence of H2AX (Figure 6B), indicating that H2AX is necessary for B106-induced apoptosis (Figure 6B). Collectively, these results suggest that inhibition of PKC δ by B106 treatment triggers caspase-dependent apoptosis through activation of the JNK-H2AX stress-responsive signaling pathway.

BRAF Inhibitor-Resistant BRAF Mutant Melanoma Lines Are Susceptible to PKC δ Inhibition. The inevitable

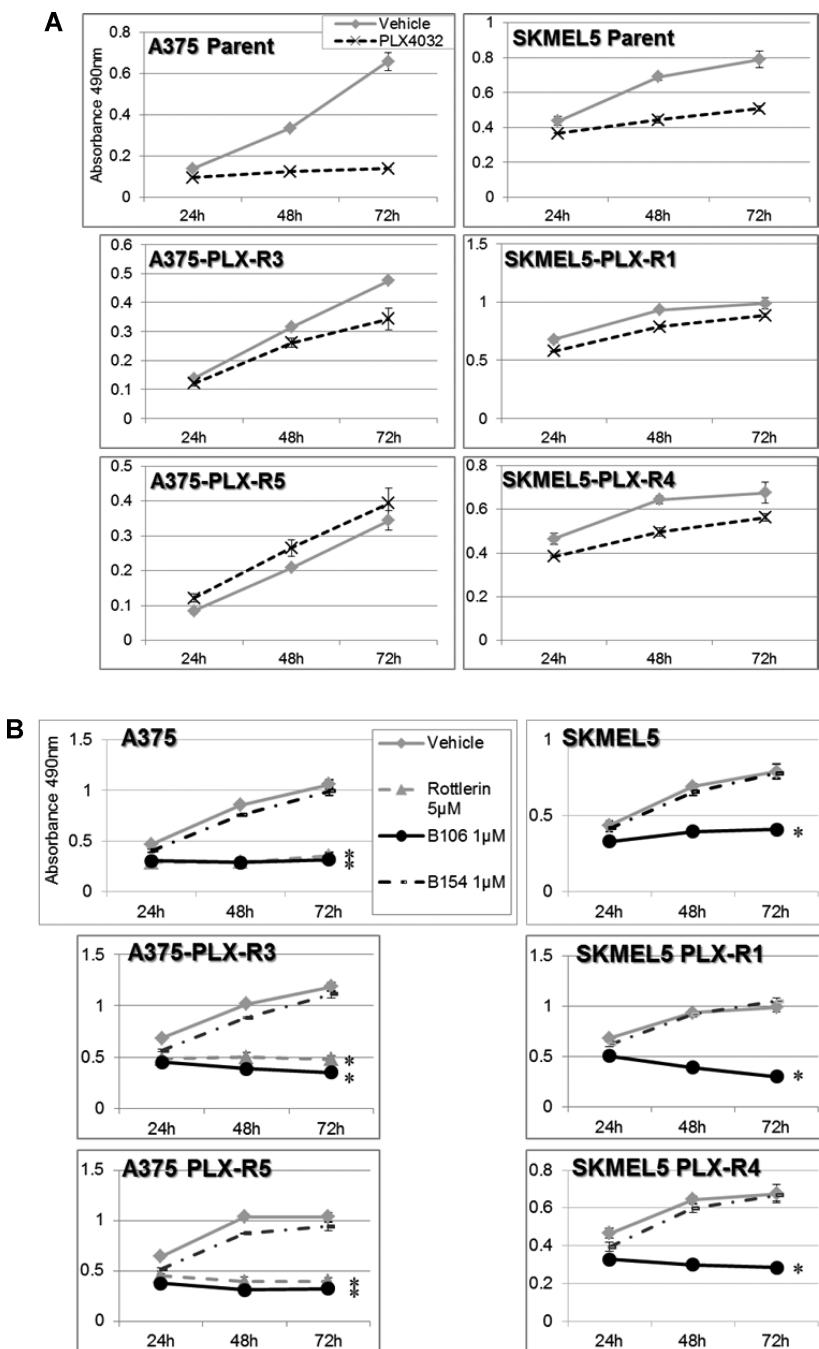


Figure 7. PKC δ inhibitors suppress growth of PLX4032-resistant BRAF mutant melanoma cells. (A) Establishment of PLX4032-resistant cell sublines. To establish PLX4032 resistant cell lines, two individual melanoma cell lines with BRAF mutations, A375 and SKMEL5, were continuously exposed to increasing concentrations of PLX4032 up to 10 μ M (A375) and 2 μ M (SKMEL5). To confirm resistance to PLX4032, the viability of PLX4032-resistant cells and their parental cells was measured by MTS assay during treatment with PLX4032 at 1 μ M. (B) PKC δ inhibitors suppress survival of PLX4032-resistant cells. Two PLX4032-resistant cell sublines derived from A375 (left) and SKMEL5 (right) cells were exposed to rottlerin (5 μ M) or B106 (1 μ M) for 24, 48, or 72 h, and MTS assays were performed at each time point. DMSO and B154 (1 μ M) served as a vehicle control and a negative compound control, respectively. Each point represents the average of triplicates and error bars indicate the standard deviations. p values (*) were calculated between DMSO (vehicle control) and rottlerin 5 μ M, or DMSO and B106, 1 μ M in each cell line at 72 h ($p < 0.0002$).

development of resistance to the BRAF inhibitor PLX4032 (vemurafenib) in melanomas bearing BRAF mutations remains an ongoing clinical challenge. Several proposed models of PLX4032 resistance involve reactivation of RAS-MEK/ERK mitogenic pathway, induced, for example, by the secondary mutations of NRAS at position 61, or activation of alternative pathways leading to reactivation of ERK signaling, such as

IGF1R or AKT.⁶ Our previous studies have demonstrated the effectiveness of PKC δ inhibitors in the cells with the aberrant CRAF-ERK activation even in the absence of mutations in RAS oncogenes.^{9–12} We therefore investigated whether PKC δ inhibition could be similarly effective in those BRAF mutant melanoma cells that have become refractory to a BRAF inhibitor (PLX4032). We generated BRAF-V600E mutant

melanoma cell sublines resistant to PLX4032 by continuously exposing A375 and SKMEL5 cells to PLX4032, with gradually increasing concentrations of the drug over weeks. Resistance to PLX4032 was verified by comparing their sensitivity to the drug with that of their parental cells (Figure 7A). PLX-R derivative lines from both A375 and SKMEL5 grew in the presence of concentrations of PLX4032 which were cytotoxic to the parental cells. Sequencing revealed that these resistant cell lines retained wild-type NRAS alleles at position 61. The resistant cell sublines derived from both the A375 or SKMEL5 parent lines acquired distinct aberrant alterations in RAS pathway signaling that may be responsible for their resistance (increased activation of ERK1,2 in the resistant A375 lines, and increased CRAF in the resistant SKMEL5 lines). All of these PLX4032-resistant lines were susceptible to cytotoxicity induced by PKC δ inhibitors at concentrations comparable to the NRAS mutant melanoma lines (Figure 7B). The parental cell lines A375 and SKMEL5 (both BRAF-V600E mutant) were also susceptible to PKC δ inhibition (Figure 7B); this finding is consistent with our previous report that cells with aberrant activation/mutation of RAF signaling, and consequent activation of this RAS effector pathway (even in the presence of normal RAS alleles) require PKC δ activity for survival.^{9–12}

PKC δ as a Therapeutic Target in Melanomas with NRAS Mutations or BRAF Inhibitor Resistance. Somatic point mutations of RAS genes at codons 12, 13, and 61 are the most common dominant oncogenic lesions in human cancer,^{2,3} making aberrant RAS signaling an important therapeutic target. Inhibition of PKC δ preferentially inhibits the growth of cancer cell lines with genomic mutations in KRAS or HRAS genes, or oncogenic activation of KRAS proteins.^{9–12,35,36} While initially characterized as a specific synthetic lethal interaction between PKC δ and RAS, further work disclosed that aberrant activation of certain RAS effector pathways, PI₃K-AKT and CRAF-MEK, would also confer sensitivity to PKC δ inhibition.^{9–12} Importantly, PKC δ was demonstrated to be nonessential for the survival and proliferation of normal cells and animals,²¹ suggesting that a therapeutic approach targeting PKC δ would likely spare normal cells, but inhibit the proliferation of tumor cells whose survival depends on PKC δ activity. This report underlines the potential of PKC δ -targeted therapy as a cancer-specific therapy targeting melanoma with NRAS mutations. Cell proliferation and clonogenic assays demonstrated that inhibition of PKC δ suppressed cell growth in multiple melanoma cell lines with NRAS mutations, as well as in PLX4032-resistant cell lines. The cell lines with NRAS mutation that were used in this study had different amino acid substitutions of NRAS codon 61, suggesting the effect of PKC δ inhibitors does not depend on a specific NRAS mutation for their activity. Similarly, PKC δ inhibition was effective in the PLX4032-resistant cell lines tested herein, regardless of the differences in their apparent resistance mechanisms, further supporting the potential of this approach. Constitutive MEK/ERK signaling appears to mediate the majority of acquired resistance to BRAF inhibitors,⁶ and we have previously reported that aberrant activation of the MEK/ERK arm of the RAS signaling pathway is sufficient to render cells susceptible to PKC δ inhibition, even in the absence of activating mutations of RAS alleles.^{9–11} Furthermore, we have recently demonstrated that cancer “stem-like” cells (CSCs) derived from a variety of human tumors, including melanomas, are susceptible to PKC δ inhibition.¹²

The novel PKC δ inhibitor B106, which showed 1000-fold selectivity against PKC δ over PKC α in preliminary *in vitro* kinase assays, was active at nanomolar concentrations, 10 times lower than for rottlerin. These results in cell culture systems suggest the potential of the newest PKC δ inhibitors as targeted agents, although the *in vivo* efficacy of B106 is yet to be determined. The hydrophobicity of B106 molecule and its rapid metabolism, requiring continuous infusion to generate a pharmacodynamic signal, makes it unsuitable for testing in tumor xenograft models.

Induction of apoptosis is one of the most desirable mechanisms for cytotoxic therapeutic action. The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), a downstream targets of PKC δ , is activated in response to cellular stresses, including genotoxic stresses.³⁷ Many chemotherapeutic agents employ the JNK pathway for their cytotoxic activity.^{38,39} This study demonstrates that PKC δ inhibition activates the JNK pathway through MMK4 to mediate caspase-dependent apoptosis. Consistent with our findings, a recent report demonstrated that knockdown of PKC δ induced apoptosis with elevated phosphorylation of JNK in NIH-3T3 cells stably transfected with HRAS.³⁵ Among the known downstream effectors of JNK, a series of recent reports proposed an active role for phospho-H2AX in apoptosis.^{31–34} PKC δ inhibition evoked phosphorylation of H2AX subsequent to JNK activation, positioning H2AX phosphorylation downstream of JNK after PKC δ inhibition. Collectively, these results demonstrate the importance of H2AX as an active apoptotic mediator, providing functional evidence showing it to be a necessary component of apoptosis initiated by PKC δ inhibition.

The concept of targeting cancer therapeutics toward specific mutations or aberrations in tumor cells that are not found in normal tissues has the potential advantages of high selectivity for the tumor and correspondingly low secondary toxicities. We have previously demonstrated that knockdown of PKC δ , or its inhibition by previous generations of small molecules, was not toxic to nontransformed primary murine and human cell lines, primary human endothelial cells, or to tumor lines without aberrant activation of the RAS signaling pathway, at concentrations which are profoundly cytotoxic to melanoma lines bearing NRAS mutations (0.5–2.5 μ M).^{9–11} Herein, we show that human primary melanocytes are not affected by B106. In addition, continuous local infusion of B106 at 5 μ M concentrations is not cytotoxic to dermal and subdermal tissues in mice. Derivatives of the third generation PKC δ inhibitor B106 are being generated, using structure function analysis of the 36 compounds in that cohort and medicinal chemistry to enhance drug-like properties, to facilitate future *in vivo* studies. Collectively, our studies suggest that PKC δ suppression may offer a promising tumor-specific option for a subpopulation of melanomas for which we have currently a limited number of effective therapeutics.

ASSOCIATED CONTENT

Supporting Information

Synthesis data. PKC δ -inhibitory activity data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Phone: 617-638-4173. Fax: 617-638-4176. E-mail: dfaller@bu.edu.

Notes

The authors declare the following competing financial interest(s): D.V.F. and R.M.W. have applied for a patent covering some of the structures disclosed in this report. The other authors declare no other conflicts of interest.

ACKNOWLEDGMENTS

We thank R. Spanjaard and A. Singh (Boston University School of Medicine, Boston, MA) for generously providing cell lines. This study was supported by the Melanoma Research Alliance, National Cancer Institute grants CA112102, CA164245, and CA141908; Department of Defense grants PC100093 and CA110396; a research award from the Scleroderma Foundation, the Raymond and Beverly Sackler Fund for the Arts and Sciences, and the Karin Grunebaum Cancer Research Foundation (D.V.F.); and the Colorado State University Cancer Super Cluster and Phoenicia Biosciences, Inc. (R.M.W.).

REFERENCES

- (1) Siegel, R., Naishadham, D., and Jemal, A. (2013) Cancer statistics, 2013. *Ca—Cancer J. Clin.* 63, 11–30.
- (2) Inamdar, G. S., Madhunapantula, S. V., and Robertson, G. P. (2010) Targeting the MAPK pathway in melanoma: Why some approaches succeed and others fail. *Biochem. Pharmacol.* 80, 624–637.
- (3) Takashima, A., and Faller, D. V. (2013) Targeting the RAS oncogene. *Expert. Opin. Ther. Targets* 17, 507–531.
- (4) Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebbe, C., Jouary, T., Schadendorf, D., Ribas, A., O'Day, S. J., Sosman, J. A., Kirkwood, J. M., Eggermont, A. M., Dreno, B., Nolop, K., Li, J., Nelson, B., Hou, J., Lee, R. J., Flaherty, K. T., and McArthur, G. A. (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N. Engl. J. Med.* 364, 2507–2516.
- (5) Nazarian, R., Shi, H., Wang, Q., Kong, X., Koya, R. C., Lee, H., Chen, Z., Lee, M. K., Attar, N., Sazegar, H., Chodon, T., Nelson, S. F., McArthur, G., Sosman, J. A., Ribas, A., and Lo, R. S. (2010) Melanomas acquire resistance to B-RAF(V600E) inhibition by RTK or N-RAS upregulation. *Nature* 468, 973–977.
- (6) Trunzer, K., Pavlick, A. C., Schuchter, L., Gonzalez, R., McArthur, G. A., Hutson, T. E., Moschos, S. J., Flaherty, K. T., Kim, K. B., Weber, J. S., Hersey, P., Long, G. V., Lawrence, D., Ott, P. A., Amaravadi, R. K., Lewis, K. D., Puzanov, I., Lo, R. S., Koehler, A., Kockx, M., Spleiss, O., Schell-Steven, A., Gilbert, H. N., Cockey, L., Bollag, G., Lee, R. J., Joe, A. K., Sosman, J. A., and Ribas, A. (2013) Pharmacodynamic effects and mechanisms of resistance to vemurafenib in patients with metastatic melanoma. *J. Clin. Oncol.* 31, 1767–1774.
- (7) Schubbert, S., Shannon, K., and Bollag, G. (2007) Hyperactive Ras in developmental disorders and cancer. *Nat. Rev. Cancer* 7, 295–308.
- (8) Malumbres, M., and Barbacid, M. (2003) RAS oncogenes: The first 30 years. *Nat. Rev. Cancer* 3, 459–465.
- (9) Xia, S., Forman, L. W., and Faller, D. V. (2007) Protein kinase C δ is required for survival of cells expressing activated p21RAS. *J. Biol. Chem.* 282, 13199–13210.
- (10) Xia, S., Chen, Z., Forman, L. W., and Faller, D. V. (2009) PKC δ survival signaling in cells containing an activated p21Ras protein requires PDK1. *Cell Signal.* 21, 502–508.
- (11) Chen, Z., Forman, L. W., Miller, K. A., English, B., Takashima, A., Bohacek, R. A., Williams, R. M., and Faller, D. V. (2011) The proliferation and survival of human neuroendocrine tumors is dependent upon protein kinase C δ . *Endocr. Relat. Cancer* 18, 759–771.
- (12) Chen, Z., Forman, L. W., Williams, R. M., and Faller, D. V. (2014) Protein kinase C δ inactivation inhibits the proliferation and survival of cancer stem cells in culture and *in vivo*. *BMC Cancer*, in press.
- (13) Zhu, T., Chen, L., Du, W., Tsuji, T., and Chen, C. (2010) Synthetic lethality induced by loss of PKC δ and mutated Ras. *Genes Cancer* 1, 142–151.
- (14) Symonds, J. M., Ohm, A. M., Carter, C. J., Heasley, L. E., Boyle, T. A., Franklin, W. A., and Reyland, M. E. (2011) Protein kinase C δ is a downstream effector of oncogenic K-Ras in lung tumors. *Cancer Res.* 71, 2087–2097.
- (15) Basu, A., and Pal, D. (2010) Two faces of protein kinase C δ : The contrasting roles of PKC δ in cell survival and cell death. *Sci. World J.* 10 (2272–84), 2272–2284.
- (16) Lonne, G. K., Masoumi, K. C., Lennartsson, J., and Larsson, C. (2009) Protein kinase C δ supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway. *J. Biol. Chem.* 284, 33456–33465.
- (17) Wang, Q., Wang, X., Zhou, Y., and Evers, B. M. (2006) PKC δ -mediated regulation of FLIP expression in human colon cancer cells. *Int. J. Cancer* 118, 326–334.
- (18) Baudot, A. D., Jeandel, P. Y., Mouska, X., Maurer, U., Tartare-Deckert, S., Raynaud, S. D., Cassuto, J. P., Ticchioni, M., and Deckert, M. (2009) The tyrosine kinase Syk regulates the survival of chronic lymphocytic leukemia B cells through PKC δ and proteasome-dependent regulation of Mcl-1 expression. *Oncogene* 28, 3261–3273.
- (19) Clark, A. S., West, K. A., Blumberg, P. M., and Dennis, P. A. (2003) Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells: PKC δ promotes cellular survival and chemotherapeutic resistance. *Cancer Res.* 63, 780–786.
- (20) Mauro, L. V., Grossoni, V. C., Urtreger, A. J., Yang, C., Colombo, L. L., Morandi, A., Pallotta, M. G., Kazanietz, M. G., Bal de Kier Joffe, E. D., and Puricelli, L. L. (2010) PKC δ promotes tumoral progression of human ductal pancreatic cancer. *Pancreas* 39, e31–e41.
- (21) Leitges, M., Mayr, M., Braun, U., Mayr, U., Li, C., Pfister, G., Ghaffari-Tabrizi, N., Baier, G., Hu, Y., and Xu, Q. (2001) Exacerbated vein graft arteriosclerosis in protein kinase C δ -null mice. *J. Clin. Invest.* 108, 1505–1512.
- (22) Soloff, R. S., Katayama, C., Lin, M. Y., Feramisco, J. R., and Hedrick, S. M. (2004) Targeted deletion of protein kinase C λ reveals a distribution of functions between the two atypical protein kinase C isoforms. *J. Immunol.* 173, 3250–3260.
- (23) Mochly-Rosen, D., Das, K., and Grimes, K. V. (2012) Protein kinase C, an elusive therapeutic target? *Nat. Rev. Drug Discov.* 11, 937–957.
- (24) Chou, W. H., Choi, D. S., Zhang, H., Mu, D., McMahon, T., Kharazia, V. N., Lowell, C. A., Ferriero, D. M., and Messing, R. O. (2004) Neutrophil protein kinase C δ as a mediator of stroke-reperfusion injury. *J. Clin. Invest.* 114, 49–56.
- (25) Hatzivassiliou, G., Song, K., Yen, I., Brandhuber, B. J., Anderson, D. J., Alvarado, R., Ludlam, M. J., Stokoe, D., Gloor, S. L., Vigers, G., Morales, T., Aliagas, I., Liu, B., Sideris, S., Hoeflich, K. P., Jaiswal, B. S., Seshagiri, S., Koeppen, H., Belvin, M., Friedman, L. S., and Malek, S. (2010) RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature* 464, 431–435.
- (26) Heidorn, S. J., Milagre, C., Whittaker, S., Nourry, A., Niculescu-Duvas, I., Dhomen, N., Hussain, J., Reis-Filho, J. S., Springer, C. J., Pritchard, C., and Marais, R. (2010) Kinase-dead BRAF and oncogenic RAS cooperate to drive tumor progression through CRAF. *Cell* 140, 209–221.
- (27) Lu, C., Zhu, F., Cho, Y. Y., Tang, F., Zykova, T., Ma, W. Y., Bode, A. M., and Dong, Z. (2006) Cell apoptosis: Requirement of H2AX in DNA ladder formation but not for the activation of caspase-3. *Mol. Cell* 23, 121–132.
- (28) Yuan, J., Adamski, R., and Chen, J. (2010) Focus on histone variant H2AX: To be or not to be. *FEBS Lett.* 584, 3717–3724.
- (29) Rogakou, E. P., Nieves-Neira, W., Boon, C., Pommier, Y., and Bonner, W. M. (2000) Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139. *J. Biol. Chem.* 275, 9390–9395.
- (30) Mukherjee, B., Kessinger, C., Kobayashi, J., Chen, B. P., Chen, D. J., Chatterjee, A., and Burma, S. (2006) DNA-PK phosphorylates

histone H2AX during apoptotic DNA fragmentation in mammalian cells. *DNA Repair (Amst)* 5, 575–590.

(31) Kaplan, F. M., Shao, Y., Mayberry, M. M., and Aplin, A. E. (2011) Hyperactivation of MEK-ERK1/2 signaling and resistance to apoptosis induced by the oncogenic B-RAF inhibitor, PLX4720, in mutant N-RAS melanoma cells. *Oncogene* 30, 366–371.

(32) Jane, E. P., and Pollack, I. F. (2010) Enzastaurin induces H2AX phosphorylation to regulate apoptosis via MAPK signalling in malignant glioma cells. *Eur. J. Cancer* 46, 412–419.

(33) Liu, Y., Tseng, M., Perdreau, S. A., Rossi, F., Antonescu, C., Besmer, P., Fletcher, J. A., Duensing, S., and Duensing, A. (2007) Histone H2AX is a mediator of gastrointestinal stromal tumor cell apoptosis following treatment with imatinib mesylate. *Cancer Res.* 67, 2685–2692.

(34) Wen, W., Zhu, F., Zhang, J., Keum, Y. S., Zykova, T., Yao, K., Peng, C., Zheng, D., Cho, Y. Y., Ma, W. Y., Bode, A. M., and Dong, Z. (2010) MST1 promotes apoptosis through phosphorylation of histone H2AX. *J. Biol. Chem.* 285, 39108–39116.

(35) Zhu, T., Chen, L., Du, W., Tsuji, T., and Chen, C. (2010) Synthetic lethality induced by loss of PKC δ and mutated Ras. *Genes Cancer* 1, 142–151.

(36) Symonds, J. M., Ohm, A. M., Carter, C. J., Heasley, L. E., Boyle, T. A., Franklin, W. A., and Reyland, M. E. (2011) Protein kinase C δ is a downstream effector of oncogenic K-Ras in lung tumors. *Cancer Res.* 71, 2087–2097.

(37) Johnson, G. L., and Nakamura, K. (2007) The c-jun kinase/stress-activated pathway: Regulation, function, and role in human disease. *Biochim. Biophys. Acta* 1773, 1341–1348.

(38) Lee, L. F., Li, G., Templeton, D. J., and Ting, J. P. (1998) Paclitaxel (Taxol)-induced gene expression and cell death are both mediated by the activation of c-Jun NH2-terminal kinase (JNK/SAPK). *J. Biol. Chem.* 273, 28253–28260.

(39) Koyama, T., Mikami, T., Koyama, T., Imakiire, A., Yamamoto, K., Toyota, H., and Mizuguchi, J. (2006) Apoptosis induced by chemotherapeutic agents involves c-Jun N-terminal kinase activation in sarcoma cell lines. *J. Orthop. Res.* 24, 1153–1162.