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14. ABSTRACT Activating mutations in N-RAS are found in 33% of primary melanomas, and are correlated with sun exposure and nodular lesions. Potential therapeutic strategies have been devised in the past to "directly" target RAS. Unfortunately, these have shown minimal if any activity in melanoma in clinical trials, because wild-type Ras and its downstream effectors are required for many critical cellular functions in normal cells, the therapeutic window for inhibiting Ras directly may be too narrow to exploit. Our novel alternative strategy has the potential to circumvent this limitation. Aberrantly activated, K-RAS or Ha-RAS are lethal to a tumor cell unless a survival pathway requiring PKCd is also active. Inhibition of PKCd in human and murine cells containing an activated K- or Ha-RAS protein initiates rapid and profound apoptosis. The dependency of tumor cells upon the activity of a non-oncogenic protein is sometimes termed "non-oncogene addiction." Hypothesis: inhibition or down-regulation of PKCd in human and murine models of melanoma with aberrant activation of N-RAS signaling will cause targeted cytotoxicity in these tumors. The Specific Aims/Study Design of this Discovery Proposal are: Test the hypothesis that inhibition or down-regulation of PKCd in human melanoma cell lines with NRAS mutations selectively induces apoptosis; 2) Determine whether aberrant activation of pathways downstream of RAS (in the setting of wild-type RAS alleles) will similarly sensitize human melanoma cells to PKCd inhibition; 3) Test this targeted approach in in vivo models of human melanoma. Impact: A novel therapeutic modality selectively targeting melanomas with activation of N-RAS would make a significant impact on the way melanoma is treated.					
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INTRODUCTION:

In the US, the risk of invasive melanoma has increased almost tenfold in the last 50 years. Patient survival from metastatic disease is only 15%, and the prognosis is extremely poor. (The excitement generated by the initial unprecedented clinical activity of BRAF inhibitors in melanoma has now been tempered by the realization that the tumor responses are unfortunately temporary, with a median time to progression of approximately seven months.) The RAS family members (which are proto-oncogene GTPases serving as critical signal transducers) are frequently mutated in melanomas. Activating mutations in N-RAS are found in 33% of primary melanoma tumors, and are correlated with sun exposure and nodular lesions. (Please excuse our overly-simplistic interpretation of the large literature supporting our proposal, and for not citing this supporting literature, but the strict page and reference constraints imposed by the guidelines make this impossible.) Because aberrant activation of N-RAS is so common in melanoma, RAS is an attractive target for a melanoma therapeutic. Indeed, strategies have been devised to target RAS “directly,” but, unfortunately, these approaches have shown minimal if any activity in melanoma -- because wild-type RAS and its downstream effectors are required for many critical cellular functions in normal cells, the therapeutic window for inhibiting RAS activity “directly” may be too narrow to exploit effectively.

- Our novel, alternative strategy has the potential to circumvent this limitation. We have demonstrated that aberrant activation of K-RAS or Ha-RAS is lethal to a tumor cell unless a specific survival pathway (also initiated by RAS) is also active.¹⁻⁵ This survival pathway specifically requires PKC δ .¹⁻³ Unlike the classical PKC isozymes, PKC δ is not required for survival of normal cells, and its inhibition or down-regulation in normal cells, tissues, and mice has no significant adverse effects *in vitro* or *in vivo*.¹⁻³ (PKC δ -null mice are healthy.) Inhibition of PKC δ in human and murine tumor cells with mutated K-RAS or H-RAS, however, initiates rapid and profound apoptosis. This molecular approach, targeting tumor cells containing a mutated oncogenic protein (and sparing normal cells) by modulating a second protein or its activity, is sometimes termed “synthetic lethality.” Analogously, the dependency of tumor cells upon the activity of a non-oncogenic protein, in this case PKC δ , is sometimes termed “non-oncogene addiction.” **First Hypothesis:** Inhibition or down-regulation of PKC δ in human and murine models of melanoma with mutational activation of N-RAS will cause targeted cytotoxicity in these tumors. (We have previously demonstrated the sensitivity of human tumor cells with mutational activation of K- or HRAS proteins to PKC δ inhibition. NRAS, however, has some significant differences compared to K- and HRAS, and whether mutated N-RAS would efficiently sensitize melanoma cells to apoptosis after PKC δ inhibition is unknown.)

- Importantly, our prior work has also suggested that aberrant activation of downstream RAS-effector pathways, even in the setting of normal RAS proteins, can sensitize cells to PKC δ inhibition.¹ BRAF (a downstream effector of RAS comprising an early component of the RAS/RAF/MEK/ERK signaling pathway) is activated by mutation (BRAV600E) in 50–70% of melanomas. We predict that this activation of a RAS effector pathway by BRAF would make this subset of melanoma cells dependent upon PKC δ activity. Another major effector pathway leading from RAS is the RAS/PI₃K/AKT signaling pathway. The tumor suppressor PTEN negatively-regulates the PI₃K pathway. Loss PTEN activity, leading to aberrant PI₃K/AKT activation, occurs in up to 40% of melanomas. **Second Hypothesis:** Aberrant activation of RAS downstream effector pathways, by the ^{V600E}BRAF mutation or PTEN loss, will sensitize melanoma cells to PKC δ inhibitors, even in the setting of wild-type N-RAS proteins.

Resistance to the new highly-active BRAF-inhibitor drugs for melanoma unfortunately invariably arises in treated patients, generally through alternative routes leading to aberrant activation of RAS/RAF/MEK signaling. Resistance most commonly results from the new development of activating mutations of NRAS, or activation of receptor tyrosine kinases (RTKs), like the PDGF-R β , which then cause aberrant activation of (normal) RAS proteins. Interestingly, our published studies have indicated that cell survival during pathological, chronic activation of even normal cellular RAS proteins requires PKC δ -dependent survival pathways.¹ Chronic activation of the PDGF-R β should cause chronic aberrant activation of the normal cellular Ras proteins, mimicking a mutated RAS state. Our targeted approach may therefore have anti-tumor activity in melanomas that have relapsed after an initial response to the new BRAF inhibitors. **Third Hypothesis:** Melanomas which have become resistant to BRAF-inhibitor drugs [by virtue of new mutations in NRAS or chronic activation of upstream RAS activators (PDGF-R β)] will be sensitive to PKC δ inhibition.

Innovation: N-Ras signaling is an attractive target for therapy of melanoma, but approaches aimed at Ras itself, or its critical signaling pathways, which are required in normal tissues, have had limited success. This “non-oncogene addiction” approach, however, exploits a weakness of tumor cells with aberrant activation of N-Ras or N-Ras effectors – their absolute requirement for a survival pathway mediated by PKC δ . In contrast, normal cells and tissues do not require PKC δ .

Impact: Current therapies for advanced melanoma are inadequate, and aberrant activation of N-Ras or Ras pathways is common in melanoma. A novel therapeutic modality selectively targeting melanomas with activation of Ras or Ras pathways would make a significant impact on the way melanoma is treated.

BODY:

General Materials and Methods

Reagents

BJE6-106 and BJE6-154 were synthesized, the details of which will be reported elsewhere. Rottlerin, PLX4032 (vemurafenib), propidium iodide, and RNase A were purchased from Axxora (San Diego, CA), LC Labs (Woburn, MA), Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA), respectively. Z-VAD-FMK was purchased from R&D Systems (Minneapolis, MN) and Enzo Life Sciences (Farmingdale, NY). Antibodies against phospho-SAPK/JNK (Thr183/Tyr185) (#4668), SAPK/JNK (#9252), phospho-Histone H2A.X (Ser 139) (#2577), Histone H2A (#2578), phospho-SEK1/MKK4) (#4514), SEK1/MKK4 (#9152), phospho-MKK7 (Ser271/Thr275) (#4171), MKK7 (#4172), phospho-c-Jun (Ser63) (#9261), c-Jun (#9165), phospho- ERK1/2 (Thr202/Tyr204) (#4370), phospho-p38 (Thr180/Tyr182) (#4511) and p38 (#9212) were purchased from Cell Signaling Technologies (Danvers, MA). Antibodies against ERK1 (K-23) and PKC δ (#610398) were purchased from Santa Cruz Biotechnology (Dallas, TX) and BD Biosciences (San Jose, CA), respectively. Antibodies against $\pm$ -Tubulin (#T6074), ²-Actin (#A1978) and GAPDH (#G8795) were purchased from Sigma-Aldrich. ON-TARGETplus SMART pool siRNA against JNK1 (L-003514), JNK2 (L-003505), H2AX (L-011682) and Non-targeting siRNA #1 (D-001810-01) were purchased from

Dharmacon (Lafayette, CO). Silencer Select siRNA against PKC δ (PRKCD) was purchased from Life Technologies (Carlsbad, CA).

Cell culture, siRNA transfection, plasmid stable transfection & PLX4032-resistant sub cell lines

SBcl2 was generously provided by the Department of Dermatology, Boston University School of Medicine (Boston, MA). A375 and SKMEL5 were generously provided by Dr. Remco Spanjaard (Boston University School of Medicine, Boston, MA). WM1366, WM1361A, WM852, FM28, FM6, SKMEL2 and SKMEL28 were generously provided by Dr. Anurag Singh (Boston University School of Medicine, Boston, MA). RAS or BRAF mutations were verified by sequencing. SBcl2 and A375 and its derivative lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. SKMEL5 was maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. All media were additionally supplemented with L-glutamine 2mM, penicillin 100 units/ml and streptomycin 100 μ g/ml. siRNA transfection was performed by reverse transcription using Lipofectamine RNAiMax (Invitrogen [Carlsbad, CA]) according to the product protocol, and media was changed the following day of transfection. PLX4032-resistant cell sublines were established according to the method described.¹ Briefly, A375 and SKMEL5 cells were plated at low cell density and treated with PLX4032 at 1 μ M or 0.5 μ M, respectively. The concentration of PLX4032 was gradually increased up to 4 μ M (A375) or 2 μ M (SKMEL5) over the course of a 3-4 week period, and clonal colonies were picked. Derived sublines of A375 and SKMEL5 were maintained in PLX4032-containing medium at 1-2 μ M (A375) or 0.5 μ M (SKMEL5).

Cell proliferation & Caspase assays

Cell proliferation assays (MTS assay) and caspase assays were performed with CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit and Caspase-Glo 3/7 Assay Systems (Promega [Madison, WI]) according to the manufacturers' protocols. Briefly, for the assays employing inhibitors, cells were plated in a 96-well plate (500-4000 cells per well depending on the cell lines and duration of the experiment), exposed to inhibitors 24 hours later and cultured for the durations indicated in the individual figure legends. For the assays employing siRNA, cells were plated the day of siRNA transfection, cultured for the duration indicated in the figure legends, and if indicated, treated with inhibitors. After the indicated treatment times, assay reagent was added and cell plates were incubated for 1 hour at 37°C (MTS assay) or 30 minutes at RT (caspase assay). Absorbance at 490nm (MTS assay) or luminescence (caspase assay) were measured using microplate readers for quantification.

Clonogenic colony assay

Cells were treated with drugs for the time indicated in the figure, and then the same number of viable cells from each treatment was replated at low cell density and cultured in medium without inhibitors for 8 days, at which time colony formation was quantitated. Cell colonies were stained with ethidium bromide for visualization on an ImageQuant LAS 4000 (GE Healthcare [Little Chalfont, United Kingdom]) and colonies enumerated.

DNA fragmentation assays

Cells were harvested and fixed in 1 ml of a 35% ethanol/DMEM solution at 4°C for 30 min. Cells were then stained with a solution containing 25µg/ml of propidium iodide/ml and 50µg/ml of RNase A in PBS and incubated in the dark at 37°C for 30 min for flow cytometric analysis. The proportion of cells in the sub-G1 population, which contain a DNA content of less than 2N (fragmented DNA), was measured as an indicator of apoptosis.

Immunoblotting

Whole cell lysates were prepared in a buffer containing 20mM Hepes (pH 7.4), 10% glycerol, 2mM EDTA, 2mM EGTA, 50mM ²-glycerophosphate and 1% Triton-X100, 1mM dithiothreitol (DTT) and 1mM sodium vanadate, supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (100X) (Thermo Scientific [Waltham, MA]). Lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked at RT for 1-1.5h with 5% BSA or 5% non-fat dry milk in TBS-T (10mM Tris [pH 7.5], 100mM NaCl, 0.1% Tween 20) and probed with the appropriate primary antibodies (1:500-1:10,000) overnight. After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000-1:10,000) and visualized using the ECL system (GE Healthcare) on an ImageQuant LAS 4000.

Quantitative real-time PCR

RNA was extracted with RNeasy Mini kit purchased (Qiagen [Venlo, Netherlands]) according to the manufacturer's protocol. 1µg of RNA was used to synthesize cDNA in a 20µl reaction volume employing SuperScript III First-Strand Synthesis System (Invitrogen) or QuaniTect Reverse Transcription Kit (Qiagen) according to the product protocol. Quantitative real-time PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems (now under Life Technologies)) according to the manufacturer's protocol. Briefly, cDNA was diluted to a final concentration of 25ng per reaction, added to a primer set (5µM) and SYBR Green PCR Master Mix to a final volume of reaction mixture of 20µl, and run on an Applied Biosystems 7500 Fast Real-Time PCR system using the following thermal cycling protocol: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The relative amount of an mRNA of interest was calculated by normalizing the Ct value of the mRNA to the Ct of the internal control (²-actin). Primer sequences were: H2AX Forward: 5'-CAACAAGAAGACGCGAATCA-3', H2AX Reverse: 5'-CGGGCCCTCTTAGTACTCCT-3', ²-actin Forward: 5'-GCTCGTCGTCGACAACGGCTC-3', ²-actin Reverse: 5'-CAAACATGATCTGGGTCATCTTCTC-3'

TASK 1: Testing N-RAS-mutant human melanoma cells for apoptosis after inhibition or down-regulation of PKC δ :

Status: IN PROGRESS

Methods: *Methods:* Inhibition of PKC δ by siRNA, demonstration of knockdown by assessment of PKC δ protein levels, and assay of relative cell numbers at 48 or 72 hrs, compared to control siRNA. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay for enumeration of cells after treatment. PI staining with flow cytometry or LDH (Lactate Dehydrogenase) release assay will be used to document apoptosis in at least one responsive cell line from each group.

Cell lines to be tested include (depending upon their susceptibility to siRNA transfection):

- at least 3 of the following mutant N-RAS cell lines: SKMEL2, SBCI2, FM-28, SKMEL30, IPC-298
- at least 2 of the following wild-type N-RAS melanoma cell lines: A375, A2058, and SKMEL5

Assays: MTS assay for enumeration of cells at 48 and 72 hrs after treatment. LDH release assays or flow cytometry assays to assess cytotoxicity

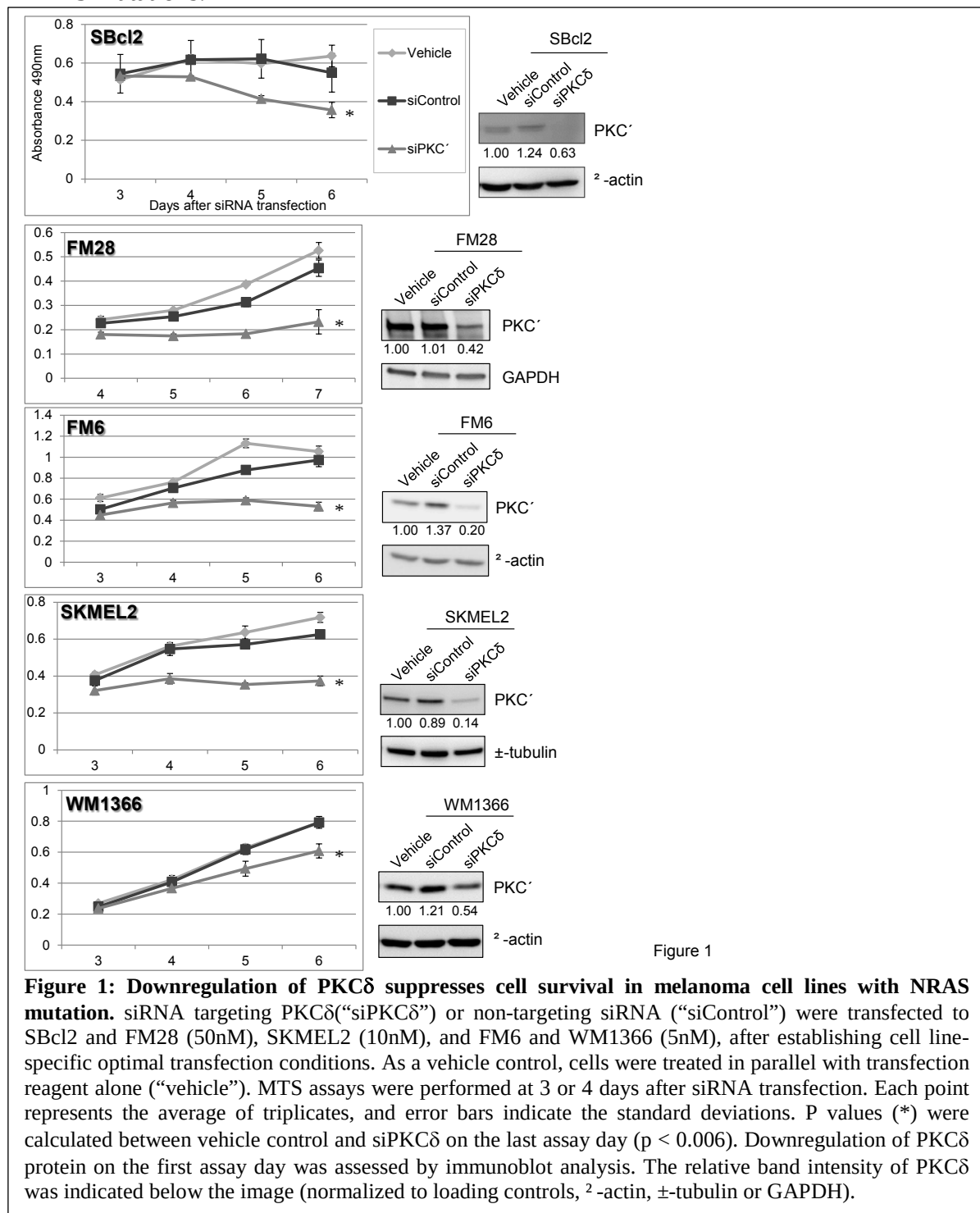
Results:

siRNA – The rationale for PKC δ as a target for a NRAS-specific therapy is supported by prior reports: 1) PKC δ inhibition preferentially inhibits the growth of pancreatic cancer cells with KRAS mutations (which are also prominent in many types of cancers with particularly high prevalence and mortality rates), and similarly inhibits cells into which activated KRAS or HRAS have been ectopically introduced, as well as a variety of other tumor cell lines with RAS mutations.²⁻⁵ PKC δ is not required for the proliferation or survival of normal cells or organisms.³⁻⁵

To validate the potential of this synthetic lethal approach targeting PKC δ in melanomas with NRAS mutations, we first examined the effect of PKC δ -selective inhibition on cell growth by knocking down PKC δ protein expression in multiple melanoma cell lines harboring NRAS mutations, using siRNA. MTS assays were conducted daily starting 3 or 4 days after siRNA transfection to quantitate the number of viable cells. Transfection reagent alone (without siRNA) served as a vehicle control. Even partial knockdown of PKC δ protein significantly inhibited proliferation of multiple melanoma cell types with NRAS mutations, including SBcl2, FM28, FM6 and SKMEL2 cells (**Figure 1**). Although transfection with negative-control siRNA produced slight cytotoxicity in some cell lines, the resulting proliferation curves did not differ significantly from those of vehicle control in these cell lines. Interestingly, the degree of protein knockdown, quantified by densitometric analysis, did not appear to be the sole factor in determining the degree of growth inhibitory effect by siRNA transfection, as these parameters did not always correlate among the cell lines. It is likely that some cell lines are more susceptible than others to cell growth inhibition resulting from PKC δ downregulation.

These cell survival assays verified that PKC δ can be a potential therapeutic target for melanomas

with NRAS mutations.



Small Molecule inhibitors. We describe the development of new specific PKC δ inhibitory molecules, and then show the results of the testing of these compounds on prostate cancer cell lines.

Pharmacophore Modeling and Development of new PKC δ Inhibitors: Highly isotype-specific PKC δ -inhibitory small molecules had not been identified by others to date. With our discovery

and genetic validation that PKC δ is the specific target molecule for this Ras-targeted approach, we generated a pharmacophore model based on molecular interactions with “novel” class PKC isozymes. We established an initial pharmacophore model for PKC δ inhibitors, using mallotoxin/rottlerin [Lead Compound 1 (**LC-1**)] as a prototype structure for a moderately PKC δ -specific inhibitor (IC₅₀=5 μ M), and incorporated protein structural data for PKC ϵ , another member of the “novel” group of PKC enzymes, which is also inhibited by mallotoxin. LC-1 is a naturally-occurring product, with moderate aqueous solubility, and oral bioavailability. It inhibits purified PKC δ at an IC₅₀ of 3-5 μ M *in vitro*, and inhibits PKC δ in cultured cells with an IC₅₀ of 5 μ M *in vivo* (but at 0.5 μ M with exposure for >24 hrs, because of down-regulation of the PKC δ protein). It is relatively selective for PKC δ over PKC α (PKC δ IC₅₀:PKC α IC₅₀ is approximately 30:1). Furthermore, as we have published, this compound not only directly inhibits purified PKC δ , but also, over longer periods of exposure, significantly down-regulates PKC δ protein specifically, while having no effect on the levels of other PKC isozymes.⁵ Thus, this compound inhibits PKC δ at two levels. We have demonstrated “Ras-specific” activity of this compound in a number of publications and assays (see above). Daily *i.p.* doses of up to 40 mg/kg (800 μ g/20 g) in mice do not produce any overt toxicity in our xenograft studies or others. Stability: Informal stability testing demonstrates >95% stability as a powder at room temp for >6 months. Toxicology: Pilot and published toxicity data indicate that the compound has a low toxicity profile (lowest lethal dose = 750 mg/kg, rat oral); 120 mg/kg (oral 6-day rat study) is the lowest toxic dose.^{6,7} This relative safety, combined with its *in vivo* efficacy, makes Lead Compound I attractive as a starting point for modification and drug development. We have demonstrated that better therapeutic candidates can be developed from it. The rationale for the development of new inhibitors was to improve the PKC δ -selectivity and potency. [Potential limitations on LC-1 itself as a therapeutic agent (despite its *in vivo* safety and activity) include its lack of high specificity for PKC δ ; its off-target effects, including inhibition of Cam Kinase III, MAPKAP-K2, and PRAK1 at IC₅₀s of <10 μ M; its non-PKC-mediated effects on mitochondrial uncoupling and modulation of death receptor pathways; and the lack of composition-of-matter IP around it, which would preclude eventual clinical development by big pharma.]

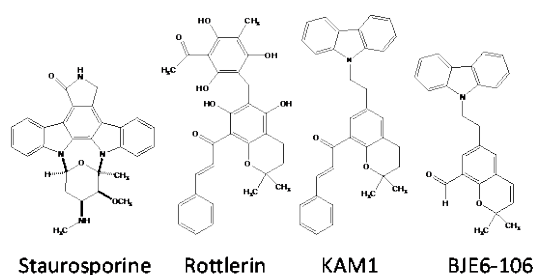
We designed and synthesized a 2nd generation set of analogs. In Analogs 1 and 2, the “head” group (A) was been made to resemble that of staurosporine, a potent general PKC inhibitor and other bisindoyl maleimide kinase inhibitors, with domains B and C conserved to preserve isozyme specificity. Ease of synthesis was a major factor in the design of this head group. Analogs 3 to 5 have “head groups” from other known kinase inhibitors: 1) Analog 3 from the crystal structure of an inhibitor bound to CDK2 (pdb code: 1FVT); 2) Analog 4 based on purine, found in a number of different potent kinase inhibitors; and 3) Analog 5 from a potent inhibitor of aurora kinase (pdb code 2F4J). The first 2nd generation chimeric molecule, KAM1, was indeed active, and more PKC δ -specific (see **Table 2**, below), and showed activity against cancer cells with activation of Ras or Ras signaling. Another 2nd generation compound we generated (CGX, with a very different composition but which fit the pharmacophore model) has demonstrated activity against multiple human cancer cell lines with activated K- or H-Ras alleles *in vitro* and *in vivo* in animal models. On the basis of SAR analysis of KAM1, we have this year generated 36 new 3rd generation compounds.

The PKC δ inhibitory activity and isozyme-specificity of the 36 3rd generation analogs was assayed *in vitro*, using recombinant PKC isozymes, prior to comparative testing on prostate cancer cell lines.

Compound	PKC δ IC ₅₀	PKC α IC ₅₀	Cytotoxicity relative to Rottlerin			
			DU145	HRasG12N	NP-PCSC	P-PCSC
Rottlerin	4.4		xx	xxx	xxxx	xx
KAM-1	5.6					
B058	5.8		o	o	o	x
B071	3		o	o	o	o
B095	8		x	o	o	xx
B097	9.5		xx	o	o	o
B106	0.08	200	xxx	xxx	xxxx	
B108	3		o	o	o	o
B109	0.09	800	o	o	o	o
B111	2.5	100	x	o	o	
B112	6		xxx	xx	xx	xx
B117	4.5		o	o	o	o
B118	? 10		o	o	o	o
B121	4		o	o	o	o
B125	0.25	800	x	o	o	x
B128	6.5		xxx	o	o	xx
B129	6.5		x	o	o	x
B130	4		o	o	o	o
B131	10		o	o	o	o
B136	14		x	o	o	o
B137	3		o	o	o	o
B141	4.8		x	o	o	o
B142	3.5		o	o	o	o
B143	6		o	o	o	o
B146	5		o	o	o	o
B147	5.8		x	xx		x
B148	4		o	o	o	o
B149	0.31	>1000	x	xx	xx	o
B150	0.625	800	o	o	o	o
B151	< 0.05	>1000	o	o	o	o
B152	< 0.05	>1000	o	o	o	o
B153	3		o	o	o	o
B154	23		o	o	o	o
B155	3		o	o	o	o
B156	7.5		o	o	o	o
B157	5		o	o	o	o
B158	4.5		o	o	o	o
B159	7		xxxx	xx	xx	xx

Table 1: Summary PKC δ and PKC α inhibitory activity of 36 3rd generation compounds, expressed as IC₅₀. Summary of relative cytotoxicity on multiple prostate cancer cell lines, relative to rottlerin.

PKC δ inhibitors



Generation	PKC δ IC ₅₀	PKC α IC ₅₀	PKC δ /PKC α Selectivity Ratio
1	3 μ M	75 μ M	28-fold
2	2 μ M	157 μ M	56-fold
3	0.05 μ M	50 μ M	1000-fold

Table 2

than PKC α *in vitro*, and produces cytotoxic activity against Ras-mutant cells at nM concentrations. (Specificity for PKC δ over classical PKC isozymes, like PKC α , is important: inhibition of PKC α is generally toxic to all cells, normal and malignant, and would make our agent non-“tumor-targeted.”) We are therefore seeking to maximize PKC δ -isozyme-specificity for the inhibitors to retain the tumor-targeted cytotoxic properties. We will eventually test

Method: These assays utilize fluorogenic FRET detection (Z-lyte, R&D Systems) technology and peptide substrates, are robust and validated, and have been used to screen the 2nd and 3rd generation PKC δ inhibitors we have synthesized.

Results:

1. PKC δ Activity Assays of 3rd Generation Compounds

Recombinant PKC δ enzyme and FRET substrate. Compounds were tested at 5, 10 and 50 μ M. and results were shown in prior progress report. The selectivity of the inhibitors for PKC δ were assessed by comparison with PKC α -inhibitory activity, using recombinant PKC α enzyme and FRET substrate.

The information from the enzymatic activity/inhibitor assays above were compiled into a summary table (**Table 1**) for purposes of comparison.

Interpretation: Certain of the 3rd generation compounds show substantially greater PKC δ inhibitory activity and specificity than LC-1

or 2nd generation compounds. For example, one such novel compound (“B106”) is much more potent than LC-1 (**Table 1**), producing substantial cytotoxicity against Ras-mutant tumor lines at concentrations ~40 times lower than LC-1. This compound is also active in vivo, in a Ras-mutant cell xenograft assay. Both LC-1 and B106 dramatically inhibited clonogenic capacity of Ras-mutant tumor cell lines after as little as 12 h exposure. A newer derivative of this particular compound (CGD63), not yet optimized with respect to drug-like properties, has a PKC δ IC₅₀ in the range of 0.05 μ M (compared to 3 μ M for LC-1), is 1000-fold more inhibitory against PKC δ

selected inhibitors against an entire panel of recombinant PKC isozymes, including the classical, novel and atypical classes.

Table 2 compares the 3 generations of PKC δ -inhibitory compounds tested to date.

3. Testing of 3rd Generation PKC δ Inhibitor Compounds in Melanoma Cell lines

Materials and Methods:

- Cells were grown on 60 mm tissue culture dishes, seeded to 1×10^5 cells per well:
- Cells were allowed to grow 24 hrs at 37°C and 5% CO₂.
- On treatment day, media was removed from each plate and replaced with either vehicle or test compound in growth media
 - ♦ DMSO (vehicle for compounds)
 - ♦ Compounds tested at various concentrations
- At 48 or 72 hrs, cells were harvested, and viable cell mass quantitated via MTT or MTS assay.

We initially tested the entire panel of 36 3rd generation compounds against human cancer cell lines with an activating Ras mutation. The compounds were prepared in stock solutions. We found that certain 3rd generation compounds (106, 147, 149, 112 and 159) showed toxicity against this cell line comparable to LC-1 or greater than LC-1. Compound 106 (“B106”) consistently showed the most consistent and highest activity and was chosen as the lead compound for the subsequent studies.

B106 was tested at multiple concentrations against a panel of human melanoma cell lines with activation of Ras signaling pathways, and compared to LC1 (roscovitine) or vehicle.

Approach:

MTS Assay: SBcl2, FM6, WM1366, SKMEL2, WM1361A, and WM852 + vehicle (DMSO), or 2 or 5 μ M Rottlerin or 0.2 or 0.5 μ M B106 or 5 μ M B154 (negative control compound) x 96hrs. Treated on 3rd day after plated cells.

Objective:

- To quantitate effects of small molecule PKC δ inhibitors on melanoma cell lines.

Materials and Methods

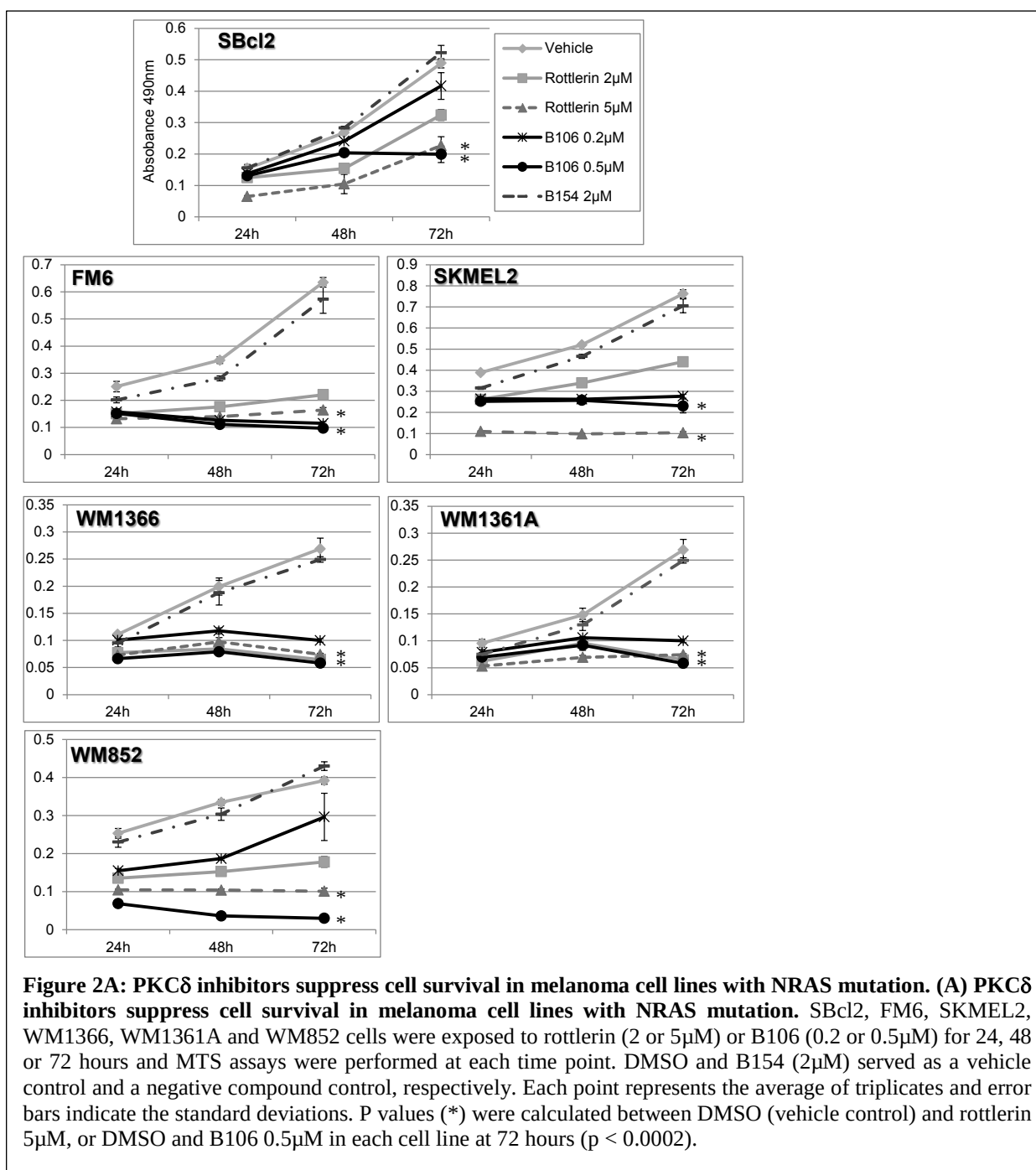
- Day 0: Cell plating day
 - Cells were plated at 2000 cells per well in 96 well plates. Quadruplicate samples were plated and grown at 37°C in 5% CO₂. Cells were allowed to grow for three days.
 - melanoma cells: 10%FBS (Invitrogen); Dulbecco’s Modification of Earle’s Media (MediaTech); 2mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; Passage 10.
- Day 3: Treatment day
 - Media was removed from each well and replaced with 0.1ml of treatment

prepared in fresh growth media and filter sterilized:

- DMSO (Fisherbrand), vehicle.
 - Rottlerin (EMD Chemical), 40 mM stock in DMSO, aliquoted, not re-frozen.
 - B106, 40 mM stock in DMSO, aliquoted, not re-frozen.
 - B154, 40 mM stock in DMSO, aliquoted, not re-frozen.
- Day 4 (24 hr tmt), Day 5 (48 hr tmt), Day 6 (72 hr tmt), Day 7 (96 hr tmt):
 - Observations were made on the confluency of treated cells compared with vehicle treatment.
 - MTS Assay was performed at each time point (CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega)) as described by manufacturer.
 - 20 ul of the assay buffer was added to each well. Cells were incubated for one hour at 37°C in 5% CO₂ humidified atmosphere.
 - The absorbance at 490 nm was read on the Molecular Devices, SpectraMax 190 plate reader.

Results and Interpretation: 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 rottlerin or B106 using multiple melanoma cell lines with NRAS mutations, including SBcl2, FM6, SKMEL2, WM1366, WM1361A and WM852 (**Figure 2A**). 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 hours after treatment. Rottlerin consistently inhibited proliferation of all cell lines at 5 μ M, and intermediate inhibitory effects were observed at 2 μ M. The 3rd generation PKC δ inhibitor B106 effectively inhibited growth of all cell lines tested at 0.5 μ M, and at 0.2 μ M in some cell lines, which is at least ten times lower than the concentration of rottlerin required to exert the same magnitude of cytotoxic effect. Exposure to B154 at 2 μ M 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. These assays demonstrated the greater potency of B106 on tumor cell growth inhibition in comparison to rottlerin, with activity at nanomolar concentrations. A clonogenic colony assay was performed using SBcl2 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 of cells while being exposed to a compound, clonogenic assays assess irreversible effects on cell viability and proliferative capacity, which are likely more relevant to potential clinical application. Cells were exposed to rottlerin or B106 for 12, 24 or 48 hours and then re-plated in medium without inhibitors, and the difference in colony-forming ability of cultures was assessed. Both rottlerin and B106 treatment significantly decreased the number of colonies formed in SBcl2 cells after as little as 12 hours of treatment, and approximately 40-fold reduction in the number of colonies was observed with 48 hours of drug treatment (**Figure 2B**). These results demonstrate an irreversible cytotoxic effect of these PKC δ inhibitors on tumor cell growth.



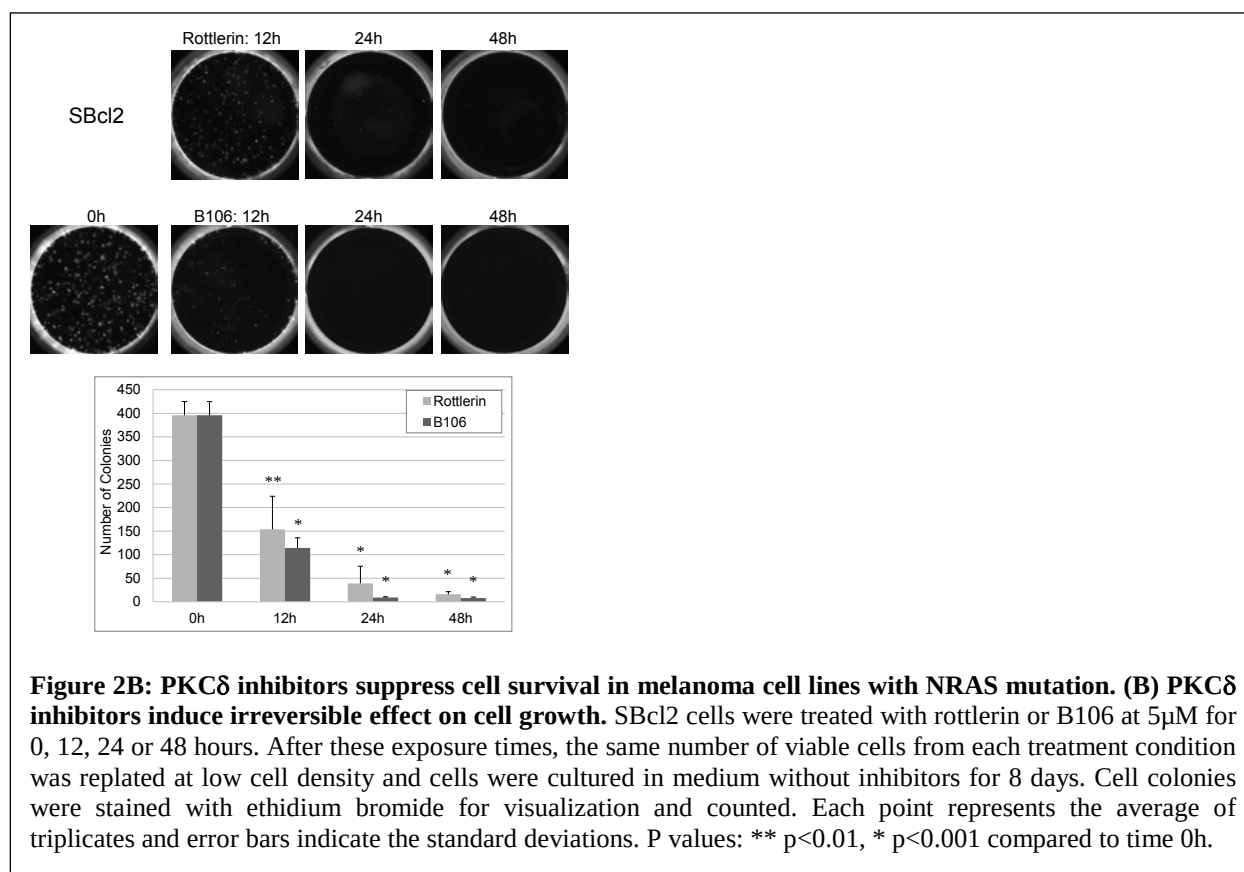
Cytotoxicity Assays

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. Apoptosis, which can be initiated by various stimuli, intrinsic or extrinsic inducers, is mediated in many cases by a proteolytic cascade of caspases, a family of cysteine proteases.

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. To explore the possible involvement of apoptosis in the cell growth inhibition induced by PKC δ inhibition, 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 μ M) or B106 (0.2 and 0.5 μ M) 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 rottlerin: a 10-fold increase at 0.2 μ M and a 12.5-fold increase at 0.5 μ M of B106, in contrast to a 5-fold increase by rottlerin at 5 μ M. The negative-control compound B154 did not induce the activity of caspase 3/7. 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 following propidium iodide staining of DNA. 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 μ M and even higher after treatment with B106 at 0.5 μ M, 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. Z-VAD-FMK irreversibly binds to the catalytic site of caspase proteases and prevents caspases from being cleaved and activated. Pre-treatment of cells with Z-VAD-FMK (50 μ M) 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 μ M) (**Figure 3B**). Exposure to Z-VAD-FMK alone produced only a similar fraction of sub-G1 cells as did vehicle or B154 treatment. Taken together, these data suggest that PKC δ inhibition attenuates tumor cell growth by inducing

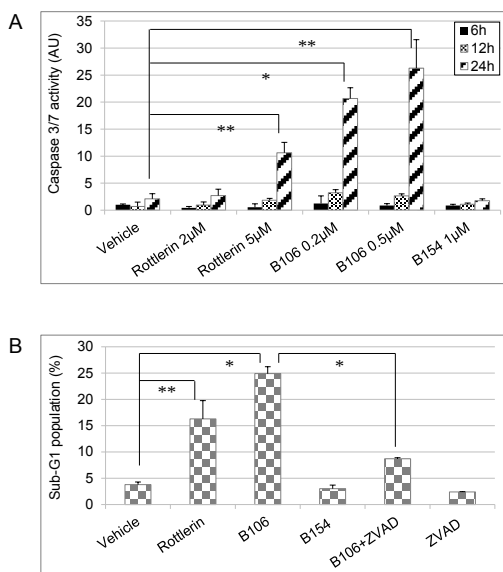


Figure 3: Inhibition of PKC δ induces caspase-dependent apoptosis. (A) Effector caspase 3/7 activation by PKC δ inhibition. SBcl2 cells were exposed to rottlerin (2 or 5 μ M) or B106 (0.2 or 0.5 μ M) for 6, 12 or 24 hours and caspase 3/7 activity was measured by luminogenic assay at each time point. DMSO and B154 (1 μ M) served as a vehicle control and a negative compound control, respectively. The average values of triplicate were normalized to that of vehicle-treated sample at 6 hours. 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 μ M), B106 (0.5 μ M) alone, or B106 (0.5 μ M) plus pan-caspase inhibitor Z-VAD-FMK (100 μ M) together for 24 hours. The proportion of sub-G1 population was measured by flow cytometric analysis following propidium-iodide staining of DNA. DMSO and B154 (0.5 μ M) served as a vehicle control and a negative compound control, respectively. Values represent the average of duplicates and error bars indicate the standard deviations. P values: ** $p < 0.04$, * $p < 0.004$.

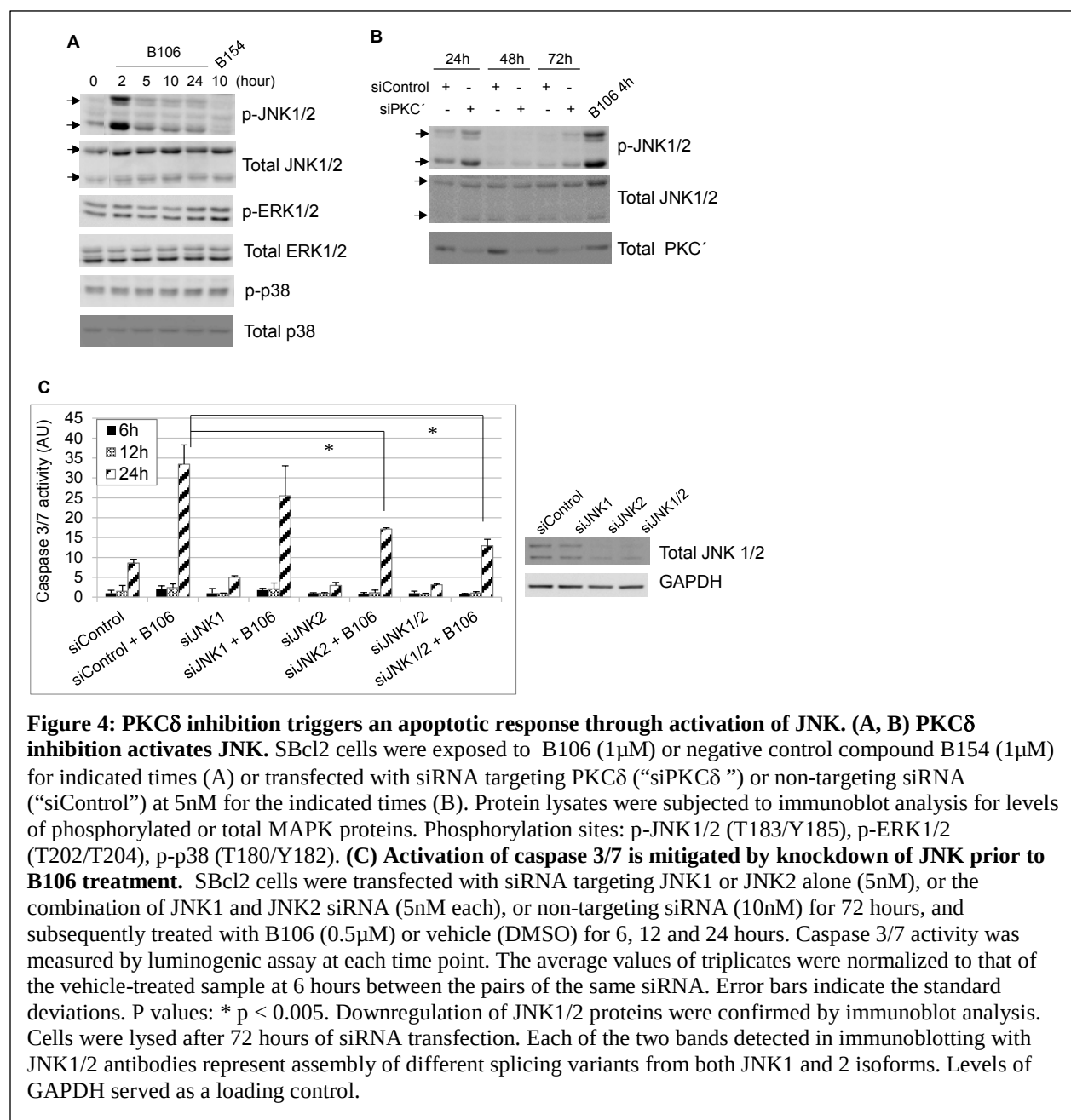
caspase-dependent apoptosis in NRAS-mutant melanoma cells.

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 2 or c-Abl. Inhibition of PKC δ activity in SBcl2 cells by B106 induced phosphorylation (activation) of JNK1/2 (T183/Y185) most strongly after two hours of exposure, with phosphorylation diminishing subsequently (**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 hours (when effects of siRNA on PKC δ levels were first

observed), whereas transfection of negative-control non-targeting siRNA did not affect JNK1/2 phosphorylation (**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. Because of the data demonstrating that PKC δ inhibition

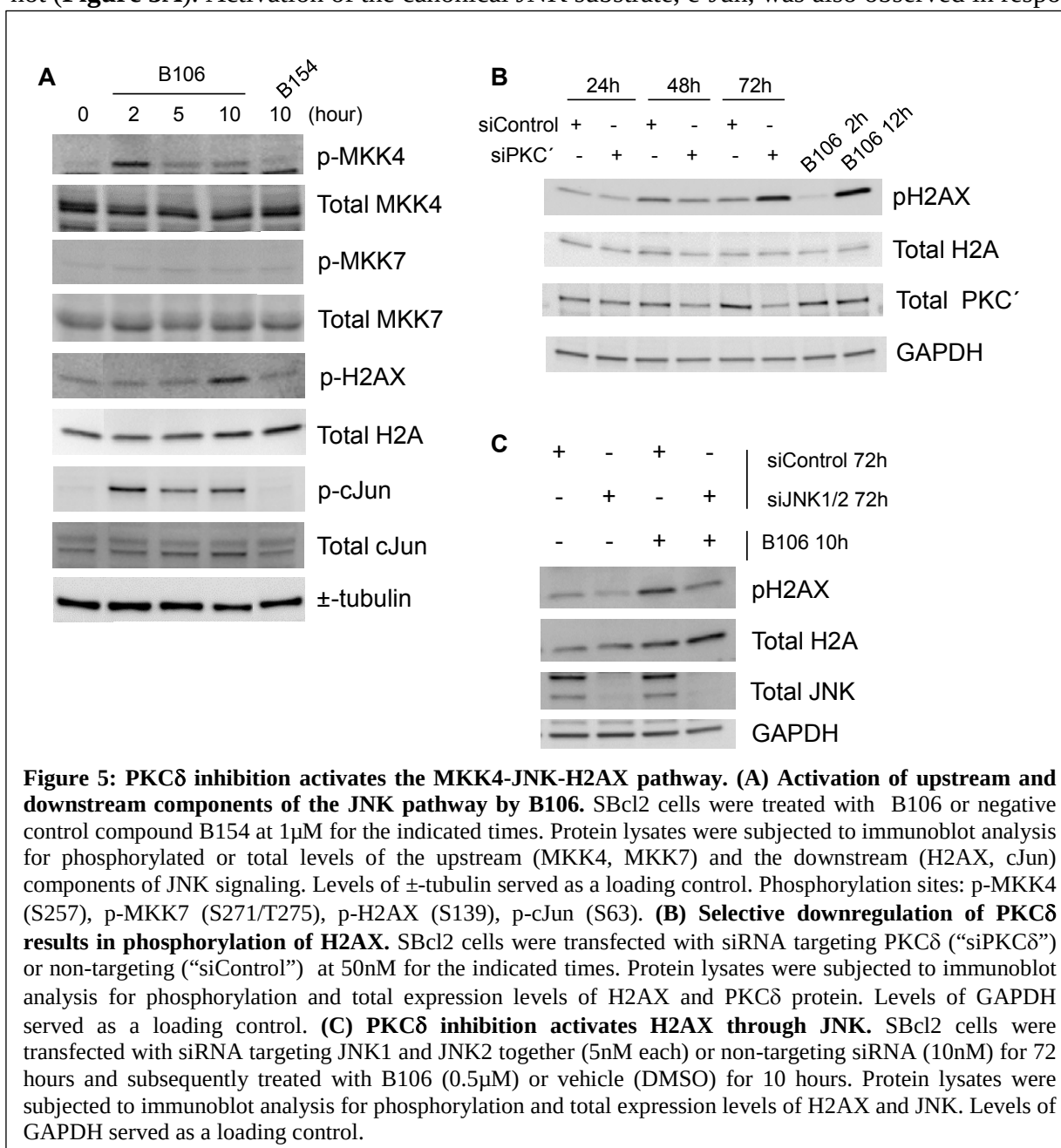


causes caspase-dependent apoptosis (**Figure 3**) and JNK activation (**Figures 4A and 4B**), the effect of inhibition of the JNK pathway during B106 treatment was explored to determine if there is a functional relationship. SBcl2 cells were transfected with non-specific siRNA or siRNA specific for JNK1 or JNK2 alone, or co-transfected with JNK1- plus JNK2-specific

siRNA for 72 hours, and then exposed to B106 or DMSO (vehicle) for 6, 12 or 24 hours, followed by measurement of caspase activity (**Figure 4C**). Analysis at 24 hours after B106 treatment showed that knockdown of JNK2 alone, and co-knockdown of JNK1 and 2, mitigated B106-induced caspase 3/7 activation in rough proportion to the knockdown efficiency of JNK1/2 proteins, as determined by immunoblot analysis. 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 (as assessed by activating phosphorylation) whereas MKK7 was not (**Figure 5A**). 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, was noted at later time points in response to B106 treatment (**Figure 5A**). B106 consistently induced H2AX phosphorylation as early as 10 hours (times later than 24 hours were not studied because significant cytotoxicity is occurring after this time). 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 hours after PKC δ siRNA transfection, but not in the cells transfected with negative-control siRNA (**Figure 5B**). This temporal course was consistent with the observation above of H2AX phosphorylation subsequent to the initiation of the MKK4/JNK cascade activation seen with PKC δ inhibitor treatment (**Figure 5A**). To ensure that activation of JNK pathway by B106 is not a cell-type-specific response, these pathway effectors were examined in another NRAS mutant melanoma cell lines WM1366. PKC δ inhibition by B106 treatment similarly induced phosphorylation of MKK4, JNK and H2AX in WM1366 cells (data not shown).

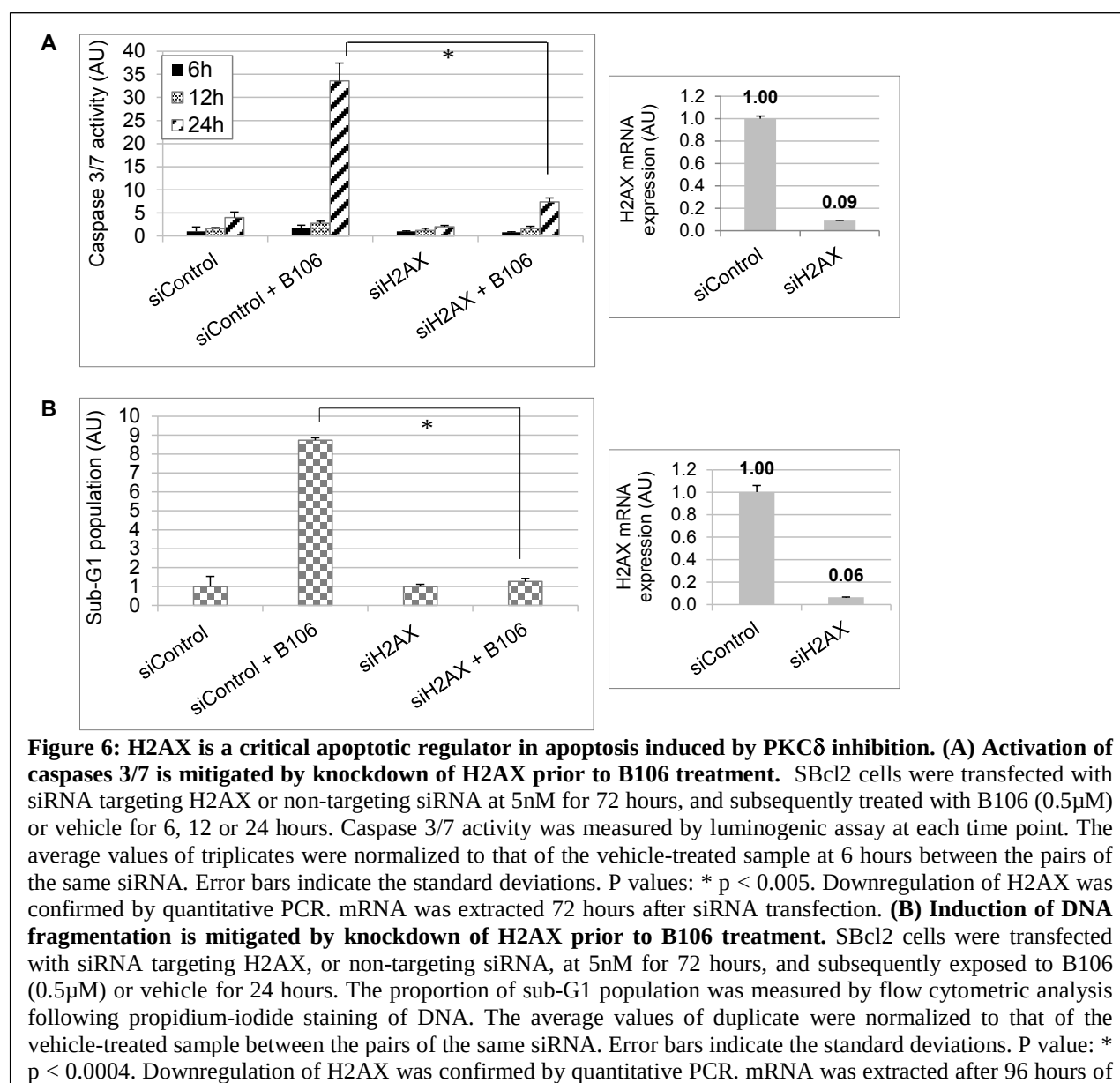
Because JNK affects diverse downstream effectors, we next determined whether JNK activation caused by PKC δ inhibition is directly linked to B106-induced H2AX activation. Cells were transfected with either negative-control siRNA or JNK1/2-specific siRNA for 72 hours and then exposed to vehicle or B106 for another 24 hours. 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 5C**). B106 exposure robustly induced phosphorylation of H2AX in control siRNA-treated cells (Lane 3, **Figure 5C**) as expected; in comparison, prior downregulation of JNK1/2 protein by siRNA attenuated B106-induced H2AX phosphorylation (Lane 4, **Figure 5C**). These findings confirmed that JNK lies upstream of H2AX, because H2AX is not activated in response to PKC δ inhibitors in the absence of JNK, supporting a model in which inhibition of PKC δ by B106 causes JNK/H2AX pathway signaling.

Collectively, these data suggest that PKC δ inhibition in cells containing mutated NRAS activates MKK4, directly or indirectly, 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-strand breaks in the DNA damage response, recent studies have demonstrated that phosphorylation of H2AX resulting from JNK activation actively mediates the induction of apoptosis.⁶ Our findings of PKC δ inhibition-induced activation of the JNK/H2AX pathway and caspase-dependent apoptosis raised the possibility that inhibition of PKC δ activity caused caspase-dependent apoptosis through activation of the JNK/H2AX pathway. Accordingly, the direct involvement of H2AX in apoptotic response to PKC δ inhibition was examined. SBcl2 cells were transfected with siRNA targeting H2AX, or non-targeting siRNA, for 72 hours and then exposed to B106 for 6, 12 or 24 hours, 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 hours of B106 exposure compared to the cells pre-treated with control siRNA (**Figure 6A**).

Subsequently, in order 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. Similar to the experiment in Figure 6A, SBcl2 cells were transfected with either negative-control siRNA or siRNA targeting H2AX for 72 hours, and then subjected to PKC δ inhibition by B106 treatment for the next 24 hours. DNA fragmentation was assessed by flow cytometric analysis following propidium iodide staining of DNA. 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, induced by transfection of siRNA targeting H2AX (**Figure 6B**). B106-induced DNA fragmentation in the cells with H2AX downregulation was significantly reduced compared to that in the cells with H2AX expression, 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.

Interpretation of TASK 1 results:

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 will serve as a lead compound for future modifications.

TASK 1 Plans for Next Period:

- We will assess the activities of our PKC δ inhibitors on non-transformed human cells, including primary human melanocytes and other epithelial cell types.
- We will assess the activities of our PKC δ inhibitors on melanoma cancer stem cells (CSCs).

TASK 2: Determine whether aberrant activation of pathways downstream of Ras (in the setting of wild-type Ras alleles) will similarly sensitize human melanoma cells to PKC δ inhibition.

2a) BRAF V600E melanoma cells which have developed resistance to BRAF inhibitors
Cell lines to be tested include:

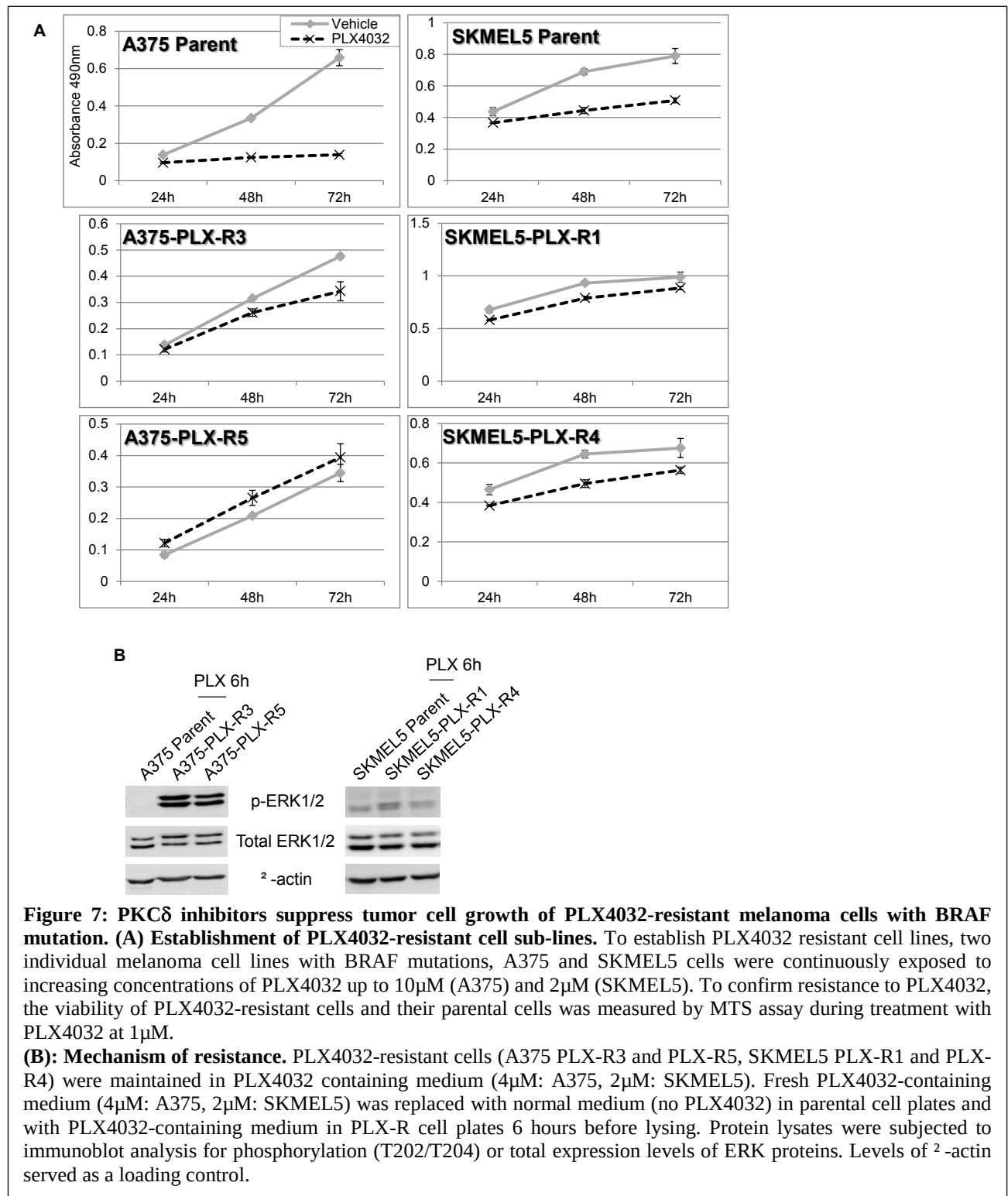
- A375 cell line and derivative lines selected for resistance to BRAF inhibitor
- SKMEL5 cell line and derivative lines selected for resistance to BRAF inhibitor

Methods: These lines were tested for susceptibility to PKC δ inhibition by at least 2 small molecule inhibitors of PKC δ (*e.g.*, “B106” and “B154”) with assay of cell numbers at 24, 48, and 72 hrs by MTS assay. PI staining with flow cytometry or LDH (Lactate Dehydrogenase) release assay will be used to document apoptosis.

Results and Interpretation

BRAF inhibitor-resistant BRAF mutant melanoma lines are susceptible to PKC δ inhibition

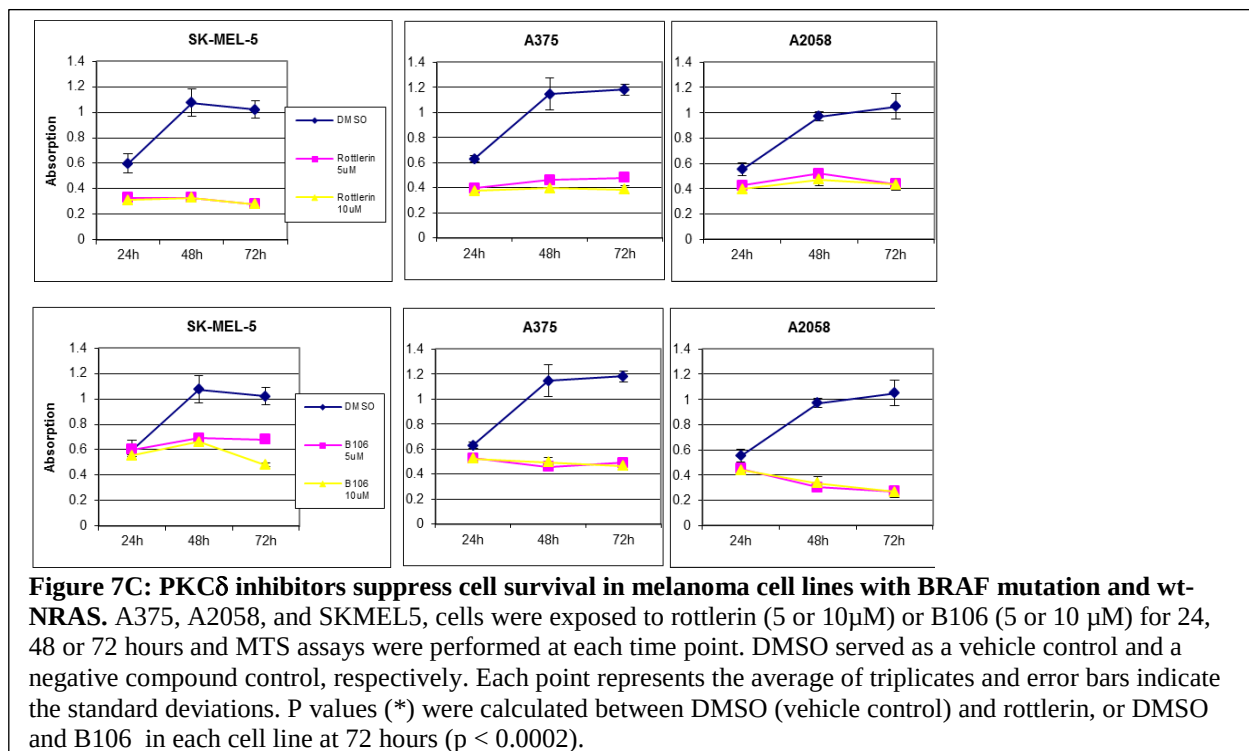
In the 50-70% of melanomas bearing BRAF mutations, the BRAF inhibitor PLX4032 (vemurafenib) produces dramatic clinical responses, but the inevitable development of resistance to the drug remains an ongoing challenge. One of the proposed models of PLX4032 resistance involves secondary mutations of NRAS, or alternative mechanisms of activation of the RAS-MEK/ERK mitogenic pathway.⁷ Because our studies have demonstrated the effectiveness of PKC δ inhibitors in NRAS mutant cells, we sought to investigate whether PKC δ inhibition could be similarly effective in those BRAF mutant melanoma cells that have become refractory to a BRAF inhibitor (PLX4032). To test this hypothesis, we first generated BRAF V600E mutant melanoma cell sub-lines resistant to PLX4032 by continuously exposing A375 and SKMEL5 cells to PLX4032, with gradually increasing concentrations of the drug over weeks. The morphology of PLX4032-resistant cells (referred as “PLX-R”) was flatter, and more enlarged and spindle-looking compared to their parental cells in both the A375-PLX-Rs and SKMEL5-PLX-Rs derivatives (data not shown). Resistance of PLX-Rs 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 of NRAS revealed that none of these resistant cell lines had acquired activating NRAS mutations at position 61. Immunoblot analyses demonstrated that the resistant cell sublines from either the A375 or SKMEL5 parent lines apparently have distinct mechanisms responsible for their resistance. A375-PLX-R sublines exhibited higher phosphorylation levels of ERK in the presence of PLX4032 compared to the

parent A375 cells, in which ERK phosphorylation was completely suppressed in the presence of PLX4032 (**Figure 7B**), indicating that the A375-PLX-R cells became resistant to PLX4032 treatment by bypassing BRAF inhibition by the drug, leading to reactivation of ERK pathway.

Conversely, SKMEL5-PLX-Rs remained responsive to ERK-MAPK pathway inhibition by PLX4032 (**Figure 7B**), and yet were capable of surviving under the drug-treatment conditions, suggesting that these cells employ an alternative pathway for survival to compensate for mutant BRAF/ERK inhibition by the drug. Despite the differences in apparent mechanisms of resistance to PLX4032 treatment, all of these resistant lines were susceptible to cytotoxicity induced by PKC δ inhibitors at concentrations comparable to the NRAS-mutant melanoma lines, compared



to treatment with vehicle (DMSO) or the negative-control compound B154 (**Figure 7C**).

Interpretation of TASK 2 results:

Collectively, these results supported PKC δ as a potential therapeutic target in melanomas with BRAF mutations and BRAF mutations resistant to BRAF inhibitors.

TASK 2 Plans for Next Period:

- We will assess the activities of our PKC δ inhibitors on cells into which oncogenic BRAF has been introduced.
- Sequence the BRAF inhibitor resistant derivatives and determine if they are NRAS mutant.

TASK 3: Test this RAS-targeted approach in *in vivo* models of human melanoma.

These studies will extend the *in vitro* studies above into proof-of-principle *in vivo* experimental models.

3a) High-purity synthesis of quantities of selected new PKC δ inhibitors sufficient for *in vivo* studies.

Status: Completed for B106

3b) Obtain ACURO approval for *in vivo* studies

Status: Completed

3c) Establish a xenograft model with the N-RAS-mutant SBCl2 cell line, and test at least two PKC δ inhibitors (*e.g.*, “B106” and a derivative of B106) for anti-tumor activity, at one or more doses, depending upon tumor responses or safety of the drug. Estimate 7 cohorts of 15 animals plus 5 controls = 110 animals

Status: Initiated, see below

3d) Establish a xenograft model with the BRAF-mutant A375 cell line, and test the lead PKC δ inhibitor (*e.g.*, “B106”) for anti-tumor activity, at one or more doses depending upon tumor responses or safety of the drug. Estimate 3 cohorts of 15 animals plus 5 controls = 50 animals

Status: Not yet initiated. Awaiting a more bioavailable inhibitor

3e) Establish a xenograft model with the BRAF-inhibitor-resistant A375 cell lines, and test the lead PKC δ inhibitor (*e.g.*, “B106”) for anti-tumor activity, at one or more doses. Estimate 4 cohorts of 15 animals = 60 animals

Status: Not yet initiated. Awaiting a more bioavailable inhibitor

Methods: Models to be employed are xenograft models, utilizing the N-RAS-mutant SBCl2 human melanoma line, BRAF-mutant A375 cell line and a drug-resistant BRAF-mutant A375 line derived in Aim/Task 2 above. Drugs to be tested, or vehicle, will be administered *i.p.* daily, and tumor size quantitated over time.

This task has been initiated. We initially established the MTD for B106, our lead compound at this time, then tested it against a xenograft model.

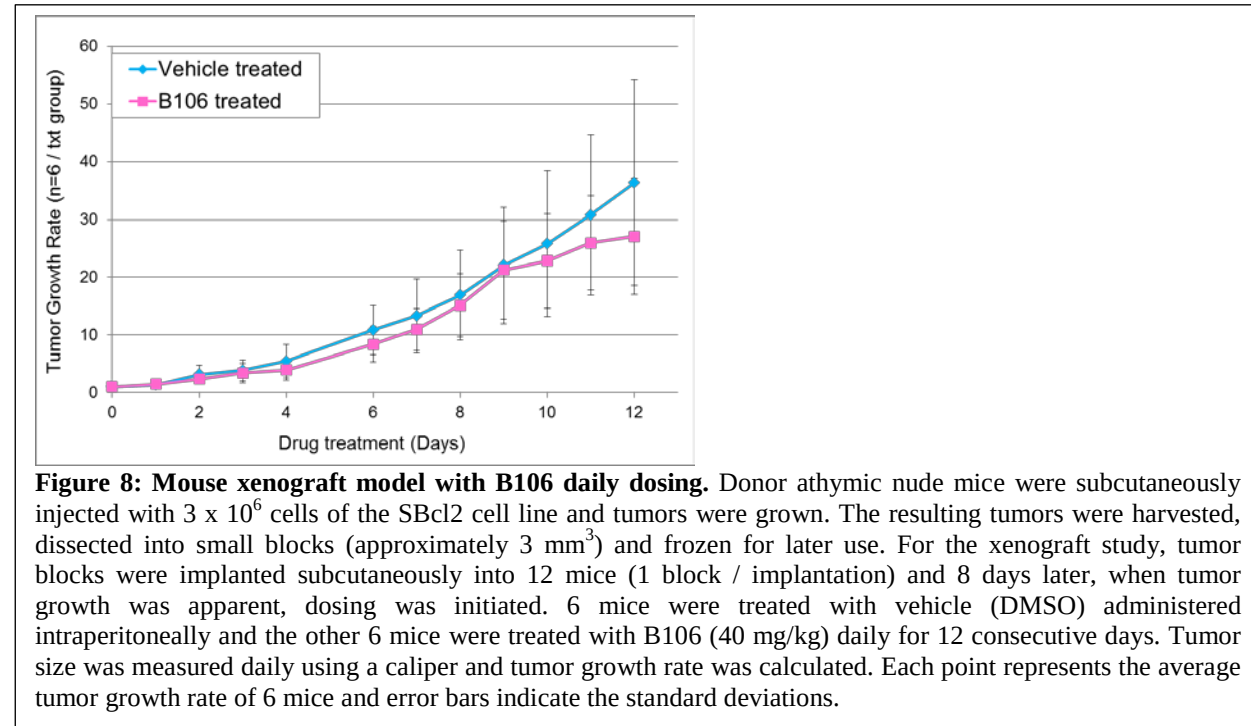
Methods: Test this targeted approach in *in vivo* models of human melanoma. A xenograft model has been employed, utilizing a human melanoma cell line with aberrantly-activated N-Ras-signaling (SBcl2). Prior to the experiment to test B106 for anti-tumor efficacy in mice, tumors were grown and harvested from donor animals for future implantation. Athymic nude mice (CrI:NU(NCr)-Foxn1nu homozygous, Charles River) were injected subcutaneously with 3×10^6

cells of the SBcl2 cell line and tumors were grown. Approximately 3 months later, these tumors were harvested, dissected into small blocks (approximately 3 mm³) and frozen in 10% DMSO/medium. For the xenograft study, tumor blocks were implanted subcutaneously into 12 mice and 8 days later, when tumor growth was apparent, dosing was started. 6 mice were administered vehicle (DMSO) intraperitoneally and the other 6 mice were administered B106 (40mg/kg) daily for 12 consecutive days. Tumor size was documented daily and tumor growth rate was calculated.

Results:

Based on the potent anti-tumor activity of B106 in the melanoma cell culture systems, we proceeded to test the efficacy of B106 *in vivo* in a mouse xenograft tumor model. Tumor blocks of SBcl2 cells were implanted into 12 mice and dosing was started 8 days after tumor implantation; 6 mice received B106 intraperitoneally at 40 mg/kg and the other 6 mice were given DMSO, the vehicle solvent of B106, daily for 12 consecutive days. Tumor size was measured daily and tumor growth rate was calculated. Treatment of B106 did not produce any difference in tumor growth compared to the DMSO-treated group (**Figure 8**).

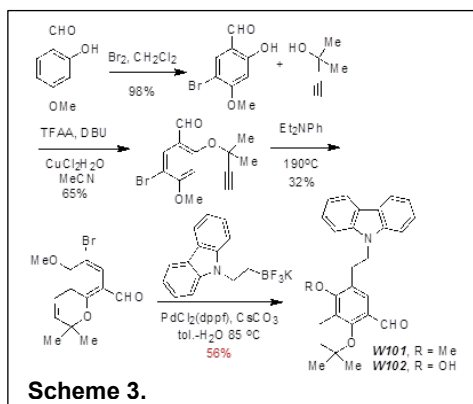
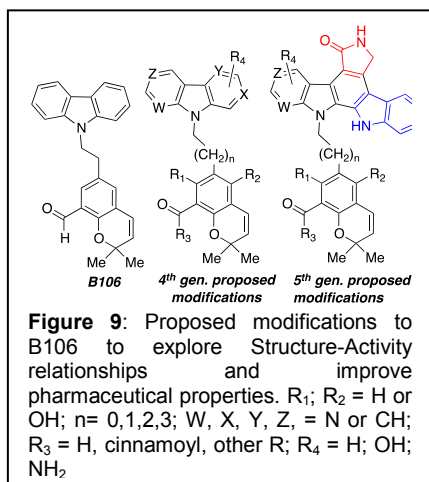
Interpretation: There was no statistically-significant effect of B106 on tumor growth compared to vehicle (DMSO) controls (**Fig. 8**). There are, however, two concerns remaining at this point regarding pharmacokinetics of B106. First, it is unclear whether the drug entered into the systemic circulation and delivered to the local areas, as B106 is extremely hydrophobic and could not be diluted with hydrophilic solvent. Secondary, there is no information regarding how fast the drug is metabolized at this time. A new generation of compounds with hydrophilic modification of the B106 structure is currently being synthesized (see below). When any of these



compounds is revealed to be as potent as B106 in the cell culture system, its *in vivo* efficacy and pharmacokinetics will be tested.

We are therefore pursuing a route to improve the bioavailability of the drug *via* modifications to improve hydrophilicity.

TASK 3 Plans for Next Period: Chemical modifications of B106 to improve drug-like properties.



Structural modifications to the core B106 lead compound will be made to improve their solubility and metabolic stability (**Fig. 9**) using the synthetic approaches noted in **Scheme 2**. We will start by simply adding polar groups to the B106 scaffold, which is thus far the most promising analog. Thus, as shown in **Fig. 9**, R_1 and R_2 , which are hydroxyl groups in rottlerin and are hydrogen atoms in B106, will be sequentially substituted with OH groups which should improve water solubility. In addition, we plan to perform an isosteric replacement of the aromatic CH groups (X and Z) with basic nitrogen atoms which will be protonated at physiological pH providing for additional water solubility and perhaps improved potency. These new 4th generation analogs do not

pose a significant synthetic challenge and are well within the expertise of the Williams lab, and should be amenable to the basic synthetic chemistry platform that was developed to make KAM1. Based on the functional group modifications we are currently examining to increase water-solubility in just the 4th generation structural framework illustrated in Figure 9, the number of possible analogs will be expand considerably. For example, just taking the minimal set of possible unique structures embodied by the 4th generation species, there are at least 810 possible structural embodiments. We do not propose to make all of these combinations, but rather, will

endeavor to substitute the more polar functional residues such as the hydroxyl residues at R_1 and R_2 and the nitrogen atoms into the “staurosporine” heterocycle to develop an SAR around the most promising new analogs. Single substitutions will be evaluated initially, and then combinations of substitutions on the B106 core will be prepared. The 5th generation species will be evaluated to sequentially introduce the fused pyrrolidinone (red) and then fused indole moieties (blue) of the staurosporine cap group. Here again, the number of possible unique structural possibilities rapidly expands covering hundreds and possibly thousands of compounds. As in any synthetic investigation, we shall begin with those heterocycle and polar group substitutions that are the most readily accessible, test these substances as they are prepared to guide the design and synthesis of subsequent analogs. A total of 50-100 compounds per year will be synthesized and evaluated. They will be evaluated for solubility and octanol:water partitioning coefficient (logP). Further characterization of the pharmaceutical properties of these analogs will be carried out following evaluation of enzyme selectivity. The synthesis of our latest hybrid molecules **W101** and **W102** with potentially improved solubility properties are shown

below in **Scheme 3**. These compounds have just been synthesized as this progress report was submitted and are being evaluated.

The potency and selectivity of the new analogs will initially be evaluated for PKC δ and PKC α inhibitory activity using recombinant PKC α or PKC δ (Invitrogen) and the Omnia Kinase Assays (Invitrogen) with a 'PKC kinase-specific' peptide substrate. Incorporation of a chelation-enhanced fluorophore results in an increase in fluorescence ($\lambda_{\text{ex}}360/\lambda_{\text{em}}485$) upon phosphorylation. The kits will be used according to the manufacturer's instructions. For molecules that look promising (*e.g.*, low nM potency and >1000x selectivity vs. PKC α , additional closely related kinases will be evaluated including PKC β I, PKC β II, PKC γ , PKC ϵ , PKC η , PKC θ , PKC ζ and PKC ι . The molecules with the optimal potency and selectivity will be further characterized. Criteria for advancement include at least 1000 fold selectivity versus PKC α , which is important in many cellular processes and is a fundamental regulator of cardiac contractility and Ca²⁺ handling in myocytes, improved solubility of at least 10 ng/mL and octanol:water partitioning coefficient (logP) in the 1.5 – 3.5 range. Compounds that exhibit these characteristics will then be evaluated for their selectivity against other PKC family members. As B106 has already been shown to be safe when administered to mice at therapeutically active doses, this compound will be profiled against the other PKC family members (PKC β I, PKC β II, PKC γ , PKC ϵ , PKC η , PKC θ , PKC ζ and PKC ι). The profile of B106 will act as a template against which other compounds will be compared. Regardless of those results, compounds will be sought with at least 100-fold selectivity against the most biologically important other protein kinase C family members, including PKC γ (important in neuronal function) and PKC ϵ (important in apoptosis, cardioprotection from ischemia, heat shock response, as well as insulin exocytosis). Inhibitory activity against the other PKC family members will also be evaluated to fully profile the compounds.

Compounds with at least 1000 fold selectivity against PKC α , a logP of 1.5– 3.5 and solubility of at least 10 mg/mL will be further characterized for their biological activity. The ability of selected inhibitors to induce cytotoxicity in human prostate cancer cells will be assessed as we did for the 3rd generation compounds (Tasks I and II above). These compounds will also be evaluated for cytotoxicity on normal human endothelial cells (human umbilical vein endothelial cells, available from ATCC) using dye exclusion as well as other primary normal human cells, as described in Task I above.

TASK 4 Data Analysis and Preparation of Report

Status: Completed for Year 1.

Discussion of Findings to Date.

Current obstacles in melanoma therapy

Approval of the BRAF inhibitor PLX4032 (vemurafenib), one of the first targeted therapies in melanoma, represented a major advance in the treatment of this disease. BRAF-V600E-mutant tumors, the target of vemurafenib, account for 50-70% of melanoma cases.⁸ Despite the remarkably high response rate and tumor regression in responders, however, the tumors eventually and inevitably relapse due to the development of resistance to this class of drugs. Ironically, the complexity and redundancy of RAS/MAPK signaling pathways provide the tumor cells many opportunities for drug resistance, which rapidly develops. There are no targeted therapies available for melanomas harboring NRAS mutations, the second most frequent genotype after BRAF mutations. The MEK inhibitors currently in clinical trials are not likely to become an important option in NRAS-mutant melanomas due to their unexpectedly preferential activity against BRAF-mutant melanomas.⁹ Moreover, there are potential risks with respect to the use of BRAF inhibitor in RAS-mutant melanomas,¹⁰⁻¹³ including their potential to cause secondary tumors with RAS mutations,^{14,15} further highlighting the need to explore therapeutic options other than BRAF inhibitors. Melanomas with NRAS mutations and BRAF-mutant melanomas resistant to BRAF inhibitors therefore represent unmet medical needs, and constitute a large proportion of the melanoma cases.

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,^{8,9} making aberrant Ras signaling an important therapeutic target in these cancers. The rationale for a PKC δ -targeted therapy against tumors with aberrant RAS signaling is based on prior findings by our group and later others. Inhibition of PKC δ was shown to preferentially inhibit the growth of cancer cell lines with genomic mutations in *KRAS* or *HRAS* genes, oncogenic activation of *KRAS* proteins, or aberrant activation of RAS signaling pathways.^{3,4,16-18} In addition, PKC δ was demonstrated to be non-essential for the survival and proliferation of normal cells and animals,¹⁹ suggesting that a therapeutic approach targeting PKC δ would likely spare normal cells, but inhibit proliferation of tumor cells whose survival depends on PKC δ activity: that is, provide a tumor-specific therapeutic approach. Furthermore, one of the proposed mechanisms of BRAF inhibitor-resistance is the evolution of secondary mutations in *NRAS*,¹ suggesting a potential link between BRAF-resistant melanoma and RAS mutations. Taken together, these reports above underline the potential of PKC δ -targeted therapy as a cancer-specific therapy targeting tumors with NRAS mutations in melanoma. Accordingly, we sought to determine if tumors with oncogenic mutations in NRAS, specifically melanomas with activating mutations of NRAS at position 61, would be dependent upon PKC δ for survival.

The present study supports the potential of PKC δ as a therapeutic target in melanomas. 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, the most frequently mutated codon in NRAS (data not shown), suggesting the effect of PKC δ inhibitors does not depend on a specific NRAS mutation for their activity (unlike BRAF inhibitors, some of which are selective to only the most common

mutation, V600E). 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. Sequencing of the NRAS genes in these resistant sublines revealed no activating mutations at position 61, despite the evidence of constitutive MEK/ERK pathway signaling. Constitutive MEK/ERK signaling (even in the absence of new activating mutations in NRAS) appears to mediate the majority of acquired resistance to BRAF inhibitors.⁷ [A comprehensive analysis of resistance mechanism in these derived sublines is being reported separately.] We have previously reported that aberrant activation of the MEK/ERK arm of the RAS signaling pathway, by introduction of a constitutively-activated CRAF gene, is sufficient to render cells susceptible to PKC δ inhibition, even in the absence of activating mutations of RAS alleles.³

The novel PKC δ inhibitor B106, which showed 1000-fold selectivity against PKC δ over PKC α in preliminary *in vitro* kinase assay, was active at nanomolar concentrations, ten 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 the B106 molecule makes it unsuitable for testing in tumor xenograft models.)

PKC δ inhibition induces caspase-dependent apoptosis via the JNK-H2AX pathway

Induction of apoptosis is one of the most desirable mechanisms for cytotoxic therapeutic action, as theoretically it selectively kills cells in which an apoptotic signal is introduced without adversely affecting surrounding cells and hence limits inflammation or tissue scarring. One of the downstream targets of PKC δ , stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) is activated in response to cellular stresses, including genotoxic stresses [*e.g.*, ionizing radiation (IR), ultraviolet radiation (UV)], chemotherapeutic agents, or reactive oxygen species (ROS)] and is involved in a variety of cellular activities, such as cell proliferation, differentiation, inflammatory response, DNA repair, motility, metabolism and apoptosis.²⁰ The JNK pathway comprises one of the three major MAPK pathways. The JNK family is on the MAPK tier and consists of three isozymes: JNK1, JNK2 and JNK3. JNK1 and JNK2 are ubiquitously expressed while JNK3 is restricted to brain, heart and testis.²⁰ Alternative splicing yields multiple splicing variants of all three isozymes: each of JNK1 and 2 genes contributes both a short form (46kDa) and a long form (54kDa), and a minor 52kDa form is believed to derive mostly to JNK3.²⁰ Upon activation of the JNK pathway, both the 46kDa and 54kDa forms become phosphorylated at Thr183/Tyr185 by two upstream MAPKKs, SEK1/MKK4 and MKK7. MKK4 appears to preferentially phosphorylate Tyr185 and MKK7 favors Thr183.²¹ While MKK7 is a JNK-selective MAPKK, MKK4 is capable of activating both JNK and the other closely related MAPK, p38. (39) The pro-apoptotic JNK pathway, initially discovered as a UV radiation-responsive signaling kinase cascade (39), induces apoptosis in part through regulating Bcl2 family members in the cytosol. In response to apoptotic signals, JNK activates the pro-apoptotic Bcl-2 family proteins Bax and Bad, possibly in part through regulating BH3-only members of the Bcl-2 family including Bim, Bid or Bif, 14-3-3 or the FOXO transcription factor.²² Alternatively, JNK also activates transcription factors including the components of activator protein 1 (AP-1), a transcription factor complex containing c-Jun, JunD or ATF2. In turn, AP-1 activates transcription of pro-apoptotic facilitators, such as TNF $\pm$ or Fas-L (death receptor ligands) or Bak (a pro-apoptotic Bcl2 member).²³ Supporting the critical role of JNK in apoptosis, mouse embryonic fibroblasts with JNK1/2 knocked-down were resistant to apoptosis induced by various apoptotic stimuli.²⁴ Many chemotherapeutic agents, including paclitaxel,

cisplatin or doxorubicin, employ the JNK pathway for their cytotoxic activity.^{25,26} This study demonstrated that PKC δ inhibition activated the JNK pathway through MMK4 to mediate caspase-dependent apoptosis. This was somewhat unexpected, as MKK7 activation was anticipated because of its more selective ability to activate JNK relative to p38. Activation of JNK was consistently induced by PKC δ inhibition, either *via* siRNA or small molecule inhibitors such as B106, but the closely-related MAPKs p38 and ERK (also potential targets of PKC δ in different settings) were not activated. Furthermore, the induced absence of JNK1/2 expression in the cells significantly mitigated caspase activity induced by PKC δ inhibition. These results demonstrate the functional importance and necessity of the activation of this pathway in PKC δ inhibition-induced caspase-dependent apoptosis in NRAS mutant melanoma cells. 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.¹⁶

Among the known downstream effectors of JNK, a series of recent reports proposed an active role for phospho-H2AX in apoptosis. H2AX (histone H2A variant X) is a minor subtype of the H2A family. Approximately 10% of histone H2A proteins are H2AX in normal fibroblasts, although the proportion varies in different tissues (ranging up to 20%).²⁷ H2AX in its phosphorylated form (phospho-H2AX, also called γ H2AX) is primarily known for its role in DNA double strand break (DSB) repair, and has long been utilized as a marker of DSB. Upon DSB formation, PI₃K-like kinases [ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK)], are activated and phosphorylate H2AX at Ser 139.²⁸ Phosphorylated H2AX is one of the first proteins recruited to DSB sites during the DNA damage response, and facilitates the access of repair proteins to the site.²⁸

In the context of apoptosis, earlier studies reported that H2AX phosphorylation can occur as a consequence of apoptosis. DSBs generated from DNA fragmentation during the apoptotic process were reported to produce phosphorylated H2AX.²⁹ DNA-PK and ATM were shown to be responsible for the phosphorylation of H2AX in this process.³⁰ However, a series of recent reports have instead proposed a more active role of phospho-H2AX in induction of apoptosis. In this model, phosphorylation of H2AX at Ser 139 provokes apoptosis by inducing DNA fragmentation in UV-damaged cells.⁶ Supporting this model, several studies reported phospho-H2AX-mediated apoptosis, some of which are involved in apoptosis induced by chemotherapeutic agents.³¹⁻³³ PKC δ inhibition by B106 treatment evoked phosphorylation of H2AX subsequent to JNK activation. Specific knockdown of PKC δ using siRNA verified the role of PKC δ : JNK phosphorylation occurred by 24 hours after siRNA transfection and H2AX phosphorylation was observed subsequently. The phosphorylation of H2AX induced by B106 treatment was mitigated in the absence of JNK expression, positioning H2AX phosphorylation downstream of JNK after PKC δ inhibition. Caspase activation caused by PKC δ inhibition was significantly decreased in the enforced absence of H2AX expression. Furthermore, as a direct readout of apoptosis, DNA fragmentation was almost completely abrogated by knockdown of H2AX prior to 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 towards specific mutations or abnormalities in tumor cells which 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 non-transformed murine and human cell lines, or to tumor lines without aberrant activation of the RAS signaling pathway.^{5,34,35} In unpublished work, we have shown that B106 does not inhibit the growth of primary murine mesenchymal cells, primary human endothelial cells and human epithelial cells at concentrations which are profoundly cytotoxic to melanoma lines bearing NRAS mutations (0.5-2.5 μ M) (Chen, et al, in preparation). In addition, continuous local infusion of B106 at 5 μ M concentrations is not cytotoxic to dermal and subdermal tissues in mice (Trojanowska and Faller, in preparation). Derivatives of the 3rd 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, these studies suggest that PKC δ suppression may represent a tumor-targeted approach to a subpopulation of melanomas for which there is currently no targeted treatment.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Demonstrated the sensitivity human melanomas to PKC δ inhibition
- Designed and synthesized 36 new compounds as PKC δ inhibitors
- Tested the activity of these 36 new compounds against PKC δ and PKC α
- Tested the activity of these 36 new compounds against human melanoma cells
- Established MTD for our lead compound
- Determined the duration of exposure to PKC δ inhibitor drug necessary to achieve maximal cytotoxicity
- Demonstrated that our lead 3rd generation compound (B106) has 5-10 greater potency in inducing cytotoxicity against a panel of human melanoma cells than LC-1.
- Demonstrated that our lead 3rd generation compound (B106) has 5-10 greater potency than LC-1 in inducing cytotoxicity against BRAF mutant melanomas resistant to BRAF inhibitors.
- Designed a strategy for synthesis of more hydrophilic analogs of our 3rd generation lead compound.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Takashima, A. and Faller, D.V. Targeting the RAS oncogene. *Expert Opinion on Therapeutic Targets*, 2013, 17(5):507-31.

Takashima A, Williams RM, Faller DV. Targeting N-Ras in malignant melanoma. *Proceedings of the Annual Meeting of the AACR* (2013) Oral Presentation.

Takashima, A., English, B., Williams, R.A., Faller, D.V. Protein kinase C δ is a therapeutic target in malignant melanoma with NRas mutation or BRAf inhibitor-resistance. (2013) Under review.

Chen, Z., Forman, L.W., Williams, R.M. Faller, D.V. Protein Kinase C-delta Inactivation Inhibits the Proliferation and Survival of Cancer Stem Cells in Culture and *in vivo*. (2013) Under review.

CONCLUSION:

In our first year of work, we have made substantial progress. We have succeeded in demonstrating that multiple types of NRAS mutant human melanoma cells are susceptible to PKC δ inhibition, using siRNA as a “specificity” test, and multiple structurally-distinct small molecule PKC δ inhibitors. These findings validate PKC δ as a target in melanoma, and provide proof-of-principle for the use of PKC δ inhibitors as potential therapeutics. Furthermore, we have shown the utility of PKC δ inhibition as a strategy for the elimination of BRAF-mutant melanomas which are resistant to BRAF inhibitors. We have refined the initial PKC δ inhibitor lead compound now through 2 generations, producing small molecules of increasing potency and PKC δ specificity. Our next generation will be optimized for “drug-like” properties, to facilitate moving into *in vivo* testing of tumor xenografts.

This *in vivo* testing in animal models has not yet proven successful due to the chemical properties of the lead 3rd generation molecule, but refinement of the lead compound is moving forward. Results of such studies will demonstrate the efficacy of this approach, provide informal toxicology, and informal PK.

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APPENDICES: None

SUPPORTING DATA: included above