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14. ABSTRACT

Prostate carcinogenesis is closely linked to aberrant activation of Ras or Ras signaling pathways (*e.g.*, Raf-MEK, or PI_3K pathways). The incidence of activating PI_3K mutations in early and advanced prostate cancer, or loss of PTEN is very high. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway requires PKCδ. Unlike the classical PKC isozymes, PKCδ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKCδ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. In this work, we have validated the *hypothesis* that inhibition or down-regulation of PKCδ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors. We have developed PKCδ–specific small molecule inhibitors and demonstrated their anti-tumor activity against prostate cancer in vitro and in vivo.

15. SUBJECT TERMS
Prostate cancer; Ras; small molecule inhibitors; drug development

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INTRODUCTION:

Although activating point mutations of Ras in prostate cancer, are not common, prostate carcinogenesis, in particular, is closely linked to aberrant activation of Ras or Ras signaling pathways (*e.g.*, Raf-MEK, or PI_3K pathways). The incidence of activating PI_3K mutations, or loss of PTEN, is very high in early and advanced prostate cancer. Increased expression of the Ras/Raf/MEK/ERK pathway has been associated with advanced prostate cancer, hormonal independence and a poor prognosis. Strategies have been devised to target various stages of Ras signaling, ranging from inhibiting protein expression *via* antisense oligonucleotides, to blocking post-translational modification with farnesyltransferase inhibitors, to inhibiting downstream effectors. Unfortunately, these have shown minimal if any activity in prostate carcinoma in clinical trials, or have been limited by toxicity. 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 circumvents this limitation. We have demonstrated that, when aberrantly activated, Ras is lethal to the cell unless a survival pathway also initiated by Ras is active. This survival pathway requires PKCδ.[6,8,9] Unlike the classical PKC isozymes, PKCδ is not required for cell survival, and its inhibition or down-regulation in normal cells and tissues has no significant adverse effects. Inhibition of PKCδ in human and murine cells containing an activated Ras protein, however, initiates rapid and profound apoptosis. This molecular approach, targeting tumor cells containing a mutated oncogenic protein (and sparing normal cells), by altering a second protein or its activity, is sometimes termed "synthetic lethality."[11,12] Analogously, 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 PKCδ in human and murine models of prostate cancer with aberrant activation of Ras signaling will cause targeted cytotoxicity in these tumors. The Specific Aims of this Idea Proposal have been: i.) Testing the hypothesis that inhibition or down-regulation of PKCδ in human prostate cancer cell lines with dysregulation of Ras pathways selectively induces apoptosis. Using molecular modeling, 36 analogs of the initial lead PKCδ inhibitor were predicted to have more specificity and higher potency that the current lead compound. new analogs were synthesized and tested for activity and isozyme specificity *in vitro* and in tissue culture. The best one or two analogs were then tested in a head-to-head comparison with the prior lead compound to identify an optimal PKCδ inhibitor. A fourth generation of PKCδ inhibitors with superior drug-like properties were synthesized in the last year of the award, and tested against the third generation; ii.) Determine whether constitutive activation of *selected Ras effector pathways alone* (PI_3K Pathway, *via* the commonly-occurring loss of PTEN or activating mutations in PIK_3CA [p110α]; or constitutive, aberrant activation of the MEK-ERK signaling pathway) is sufficient to make prostate cancer cells susceptible to apoptosis after PKCδ inhibition. iii.) Test the ability of PKCδ inhibitors to induce selective cytotoxicity in human *prostate cancer stem cells*. iv.) Test this targeted approach in *in vivo* models of human prostate carcinoma. A xenograft model was employed, utilizing an activating Ras-mutant human prostate carcinoma cell line and a human prostate carcinoma cell line with aberrantly-activated Raf-signaling.

Innovation: Ras signaling is an attractive target for therapy of prostate cancer, 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 Ras or Ras effectors – their absolute requirement for a survival pathway mediated by PKCδ. In contrast, normal cells and tissues do not require PKCδ.

Impact: Current therapies for prostate cancer are inadequate, and aberrant activation of Ras or Ras pathways are common. A novel therapeutic modality selectively targeting prostate cancers with activation of Ras or Ras pathways will make a significant impact on the way prostate cancer is treated.

BODY:

TASK 1: Testing human prostate cancer cell lines for sensitivity to PKCδ inhibition

Status: COMPLETED

Methods: Assess the sensitivity of human prostate cancer cells with known activating mutations in H-Ras and wild-type Ras alleles to (non-Ras-mutated) prostate epithelial cells

Task 1a) Using siRNA to suppress PKCδ
Task 1b) Using new, specific small-molecule PKCδ inhibitors.

- Verify their PKCδ inhibitory activity and isozyme-specificity will be verified *in vitro* using purified PKC isozymes
- Testing their ability to induce apoptosis in prostate cancer cell lines, and selection of the most potent and PKCδ isozyme-selective for *in vivo* testing.

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

Results:

Task 1a: **siRNA –** To demonstrate the specificity of this targeted approach, we first used PKCδ-specific lenti-viral based shRNA to efficiently knockdown PKCδ protein in a human prostate cancer cell line, DU145. We developed lentiviruses containing shRNA directed against PKCδ, or a scrambled shRNA. Viruses were titered to determine moi for use. They were then used to infect prostate cancer cell lines. Even within 24 h, we observed very significant cytotoxicity, as assessed by LDH release. Parallel studies using these lentiviral vectors in other cell lines, including normal prostate cancer cells, were performed to validate PKCδ as a target for prostate tumor cells (these findings were presented in prior progress report).

Task 1b. We have describe the development of new specific PKCδ inhibitory molecules, and then show the results of the testing of these compounds on prostate cancer cell lines.

Pharmacophore Modeling and Development of new PKCδ Inhibitors: Highly isotype-specific PKCδ-inhibitory small molecules had not been identified by others to date. With our discovery and genetic validation that PKCδ 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 PKCδ-inhibitors, using mallotoxin/rottlerin [Lead Compound 1 (**LC-1**)] as a prototype structure for a moderately PKCδ-specific inhibitor (IC_{50}=5μM), and incorporated protein structural data for PKCθ, 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.[4] It inhibits purified PKCδ at an IC_{50} of 3-5 μM *in*

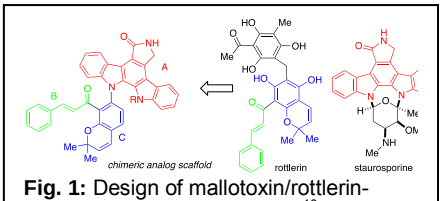
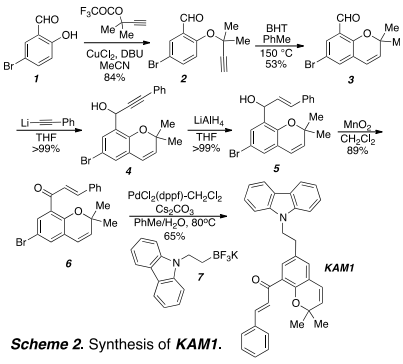


Fig. 1: Design of mallotoxin/rottlerin-staurosporine hybrids. (From [10])



Scheme 2. Synthesis of ***KAM1***.

The readily available aldehyde **10** is converted into the propargylether **2** and subjected to a Claisen rearrangement to install the pyran ring system characteristic of Rottlerin. Next, lithium acetylide addition to **3** provides **4**, which was subsequently reduced to the E-olefin **5** in excellent yield. Manganese dioxide oxidation of the secondary alcohol furnishes ketone **6**. Coupling of the Molander-type [3-5] trifluoroborate **7** (the "staurosporine" component) provided KAM1 in 65% yield. (from [6,7])

vitro, and inhibits PKCδ in cultured cells with an IC_{50} of 5 μM *in vivo* (but at 0.5 μM with exposure for >24 hrs, because of down-regulation of the PKCδ protein[5]). It is relatively selective for PKCδ over PKCα (PKCα IC_{50}: PKCδ IC_{50} is approximately 30:1). Furthermore, as we have published, this compound not only directly inhibits purified PKCδ, but also, over longer periods of exposure, significantly down-regulates PKCδ protein specifically, while having no effect on the levels of other PKC isozymes.[5] Thus, this compound inhibits PKCδ 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 μg/20 g) in mice did not produce any overt toxicity in our xenograft studies or others.[4] 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 PKCδ-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 PKCδ, its off-target effects, including inhibition of Cam Kinase III, MAPKAP-K2, and PRAK1 at IC_{50}s of <10 μM; its non-PKC-mediated effects on mitochondrial uncoupling and modulation of death receptor pathways;[8,9] and the lack of composition-of-matter IP around it, which would preclude eventual clinical development by big pharma.]

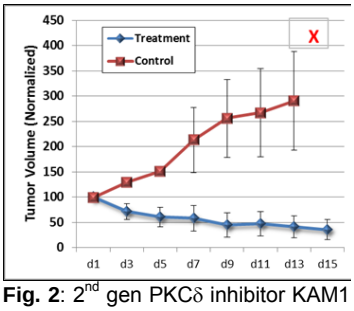


Fig. 2: 2nd gen PKCδ inhibitor KAM1 reduces tumor burden in a xenograft model of a human cancer with mut-KRAS (H460). Treatment was begun at a large tumor volume of 1 cm. 20ug/kg; IP 5/7 days. Measurement of 3 dimensions. Error Bars=SD P<0.01 for drug *vs* control by day 3. "**X**" = end of study.

We developed a pharmacophore model using a prototype chimeric structure based on mallotoxin/rottlerin) and a class of general protein kinase C inhibitors (the natural product staurosporine), and incorporating protein structural data for "novel" class PKCs. We designed and synthesized a 2nd generation set of analogs. In this 2nd generation of PKCδ inhibitors, the "head" group (A) has been made to resemble that of staurosporine and other bisindoyl maleimide kinase inhibitors, with domains B (cinnamate side chain) and C (benzopyran) conserved from the rottlerin scaffold to preserve isozyme specificity (**Fig. 1**). The chromene portion of mallotoxin/rottlerin was combined with the carbazole portion of staurosporine to produce **KAM1** (**Scheme 2**)[6] KAM1 had an IC_{50} of 4 μM for PKCδ (similar to mallotoxin), and better isozyme selectivity (IC_{50} of >120 μM for PKCδ (**Table 1**).[6] KAM1 showed anti-tumor cell activity *in vitro* and *in vivo* at concentrations comparable to mallotoxinin cell types including RAS-mut human neuroendocrine tumors, pancreatic cancers and lung cancer cells, as well as prostate cancers (**Fig. 2**) (see prior progress report).[6]

On the basis of SAR analysis of 2nd gen molecules like KAM1, we then generated 36 new 3rd gen compounds (please see Takashima, 2014, appended),[10] using the synthesis strategy shown in Scheme 2). The PKCδ inhibitory activity and isozyme-specificity of these 36 3rd gen analogs was assayed *in vitro,* using recombinant PKC isozymes, prior to comparative testing on

pancreatic cancer cell lines. These derivatives showed a broad range of PKCδ-inhibitory activity, ranging from IC_{50} of >40 μM (*e.g.,* B154, which we use as an "inactive" control in many of our assays) to IC_{50} of <0.05 μM (*e.g.*, B157 and B106, our "lead" 3rd generation compound) (**Fig. 3** and **Table 1**).[10] B106 produced substantial cytotoxicity against multiple human tumor lines at nM concentrations (10-40 times lower than mallotoxin or KAM1). Both mallotoxin and B106 dramatically inhibited clonogenic capacity of RAS-mut tumor cell lines after as little as 12 h exposure (see below, **Fig. 8**).[6,10] B106 was 1000-fold more selective for PKCδ versus PKCα [6,10] (Specificity for PKCδ over classical PKC isozymes, like PKCα is important: inhibition of PKCα is generally toxic to <u>all</u> cells, normal and malignant, and would make our agent non-"tumor-targeted." We are therefore seeking to maximize <u>PKCδ-isozyme-specificity</u> for the inhibitors to retain the tumor-targeted cytotoxic properties. We will eventually test selected inhibitors against an entire panel of recombinant PKC isozymes, including the classical, novel and atypical classes. (Please see below for "kinome analysis" of B106.) However, B106 and the other most-active 3rd gen compounds are not yet optimized and display limited solubility with resultant poor bioavailability *in vivo*.

We used the characterization of the 36 3rd gen compounds to design a 4th generation of PKCδ inhibitors, using pharmacophore modeling and SAR. A **major goal** of this 4th generation synthesis was to *increase the drug-like properties of the drug candidate molecules* (3rd generation molecules have not been optimized).Their activity will be described below.

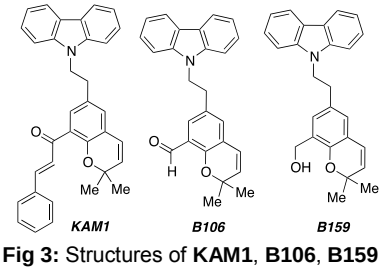


Fig 3: Structures of **KAM1**, **B106**, **B159**

The PKCδ 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.

Method: These assays utilize fluorogenic FRET detection (Z-lyte, R&D Systems) technology and peptide substrates, are robust and validated, and were used to screen the 2nd and 3rd and 4th generation PKCδ inhibitors we have synthesized.

Results:

1. PKCδ Activity Assays of 3rd and 4th Generation Compounds

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

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

Interpretation: Certain of the 3rd generation compounds showed substantially greater PKCδ–inhibitory activity and specificity than LC-1 or 2nd generation compounds. For example, one such novel compound ("B106") was <u>much more potent</u> than LC-1 (see prior progress reports), producing substantial cytotoxicity against Ras-mutant tumor lines at concentrations ~40 times lower than LC-1. This compound was <u>also active *in vivo*</u>, 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), with improved drug-like properties, has a PKCδ IC_{50} in the range of <u>0.05 μM</u> (compared to 3 μM for LC-1), is <u>1000-fold more inhibitory</u>

against PKCδ than PKCα *in vitro,* and produces cytotoxic activity against Ras-mutant cells at nM concentrations. (Specificity for PKCδ over classical PKC isozymes, like PKCα is important: inhibition of PKCα is generally toxic to <u>all</u> cells, normal and malignant, and would make our agent non-"tumor-targeted.") We seek to maximize PKCδ-<u>isozyme-specificity</u> for the inhibitors to retain the tumor-targeted cytotoxic properties. (We will eventually test selected inhibitors against an entire panel of recombinant PKC isozymes, including the classical, novel and atypical classes, but that is beyond the scope of this project.)

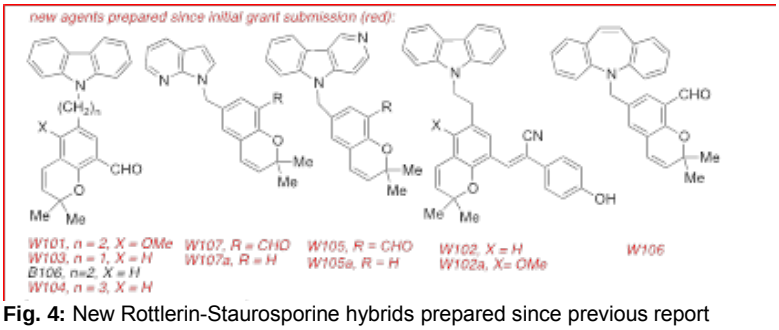


Fig. 4: New Rottlerin-Staurosporine hybrids prepared since previous report

B106 and the other most-active 3rd gen compounds were not optimized and displayed limited solubility with resultant poor bioavailability *in vivo*.

We used the characterization of the 36 3rd gen compounds to design a 4th generation of PKCδ inhibitors, using pharmacophore modeling and SAR. A **major goal** of this 4th generation synthesis has been to *increase the drug-like properties of the drug candidate molecules* (3rd generation molecules had not been optimized). In the past year, we have prepared an additional nine mallotoxin-staurosporine hybrids which are still being evaluated (see below). All of these compounds contain additional polar groups that improved water-solubility relative to their predecessor congeners (*e.g.,* **Figs. 4&5**). Space precludes a detailed description of the new synthetic technology we developed to access these structures.

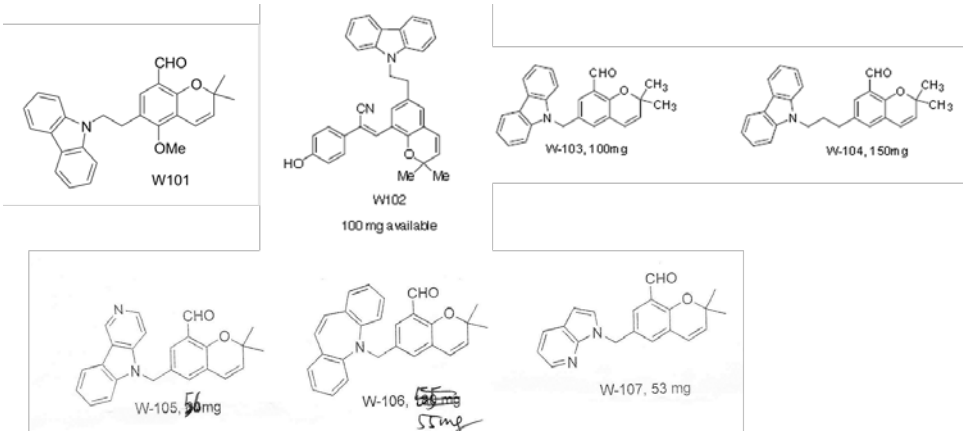


Fig. 5: 4th Generation Compounds

The synthesis of our latest hybrid molecules **W101** and **W102** with potentially improved solubility properties are shown in **Scheme 3**. These compounds and 5 others in the series have just been synthesized (**Fig. 4-5**). W101 has an IC_{50} on NRAS-mutant melanoma cells comparable to B106 (0.5 μM at 48 hrs) with markedly improved solubility. **Potential pitfalls.** As in any synthesis endeavor, empirical derivation of difficult coupling steps and related transformations inevitably appear. We have already investigated many coupling strategies and have found that the Molander borate technique and direct displacements of unhindered halides (Rottlerin half) have been quite successful. If the direct coupling of the fully substituted species such as **4** (Scheme 3) prove problematic, we will extend the number of CH_2 units from the aromatic ring, making the subsequent coupling less sensitive to steric effects.

Table 1 compares the 4 generations of PKCδ-inhibitory compounds tested to date.

Generation	PKCd IC_{50}	PKCa IC_{50}	PKCd IC_{50}/ PKCa IC_{50}
1	3 mM	75 mM	28-fold
2	2 mM	157 mM	56-fold
3	0.05 mM	50 mM	1000-fold
4	0.015 mM	>50 mM	2000-fold

Table 1: Comparative PKCδ inhibitory activity of 4 generations of novel compounds.

Interpretation:

The 3rd and 4th generations of PKCδ inhibitors (specifically B106 and W101, respectively) show activity against PKCδ at sub-μM levels, with good selectivity with respect to PKCα.

Future Plans relevant to this Aim: We are now using pharmacophore modeling and SAR from the 3rd gen molecules like B106 to design a 5th generation of PKCδ inhibitors. A major goal of this 5th generation synthesis will be to further increase the drug-like properties of the drug candidate molecules, as the 3rd generation molecules have not yet been optimized for drug-like properties (*e.g.,* water solubility; stability; ease of formulation; oral-bioavailability [not a "no-go" criterion]; Lipinski's rule of 5;[13-15] LogP<5, and the 4th generation molecules are not yet sufficiently water soluble for optimal *in vivo* use. Our lead compound 3rd gen compound, B106, has a MiLogP=6.259).

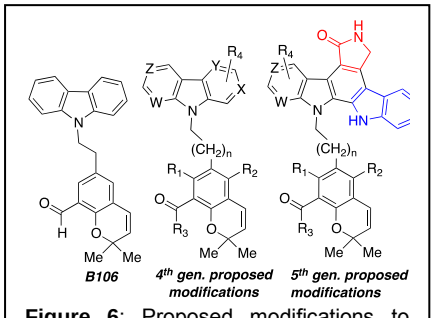
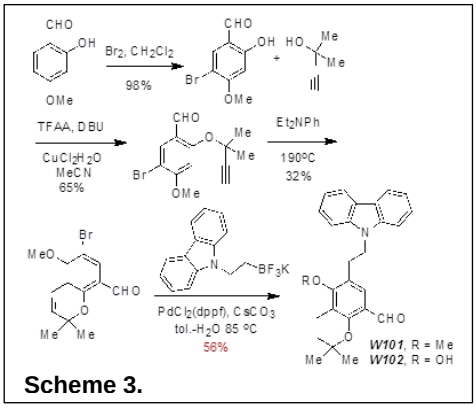


Figure 6: Proposed modifications to B106 to explore Structure-Activity relationships and improve pharmaceutical properties. R_1; R_2 = H or OH; n= 0,1,2,3; W, X, Y, Z, = N or CH; R_3 = H, cinnamoyl, other R; R_4 = H; OH; NH_2

Synthesis Plans. Scheme 3 illustrates the new synthetic technology and strategy that we have just initiated developing for the incorporation of more polar functionality within the structural framework of our initial lead compound, B106. Target series **6** (Scheme 3) contains the complete Rottlerin lower half structural core. Aldehyde species **3** will be a key intermediate from which the tethering length of CH_2 units linking the two chimeric halves, can be modified by using Wittig and Molander borate coupling methodologies with which the Williams lab has considerable expertise. Structural modifications to the core B106 lead compound will be made to improve their solubility and metabolic stability (**Fig. 6**) using the synthetic approaches noted in **Scheme 2**. We will start by simply adding polar groups to the B106 scaffold (**Fig. 6**), which is so far the most promising analog. Thus, as shown in **Fig. 6**, 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 (**Scheme 2**).



Scheme 3.

Based on the functional group modifications we are currently examining to increase water-solubility in just the 4th generation structural framework illustrated in Fig. 17, 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).[16] Further characterization of the pharmaceutical properties of these analogs will be carried out following evaluation of enzyme selectivity.

3. Testing of 3rd Generation PKCδ Inhibitor Compounds in Prostate Cancer Cell lines

Materials and Methods:

- Cells were grown on 60 mm tissue culture dishes, seeded to 1 x 10^5 cells per well:
- Cells were allowed to grow 24 h at 37oC and 5% CO_2.
- 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 h, 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 a prostate cancer cell line with an activating Ras mutation. The compounds were prepared in stock solutions. Results from representative cytotoxicity assays were shown in the prior Progress Report. We reported 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 prostate cancer cell lines with activation of Ras signaling pathways, and compared to LC1 (rottlerin) or vehicle.

Approach:

MTS Assay: DU145, LNCaP, PC-3, pZ-HPV-7 + 1, 2, 5, 10, 20 or 40uM Rottlerin x 96hrs. Treated on 3rd day after plated cells.
DU145, LNCaP, PC-3 (Prostate Cancer Cell Line), pZ-HPV-7 (Immortalized Prostate Cell Line) & Celprogen's Breast Cancer Stem Cells + 1, 2, 5, 10, 20 or 40uM Rottlerin x 24-96hrs.

Objective:

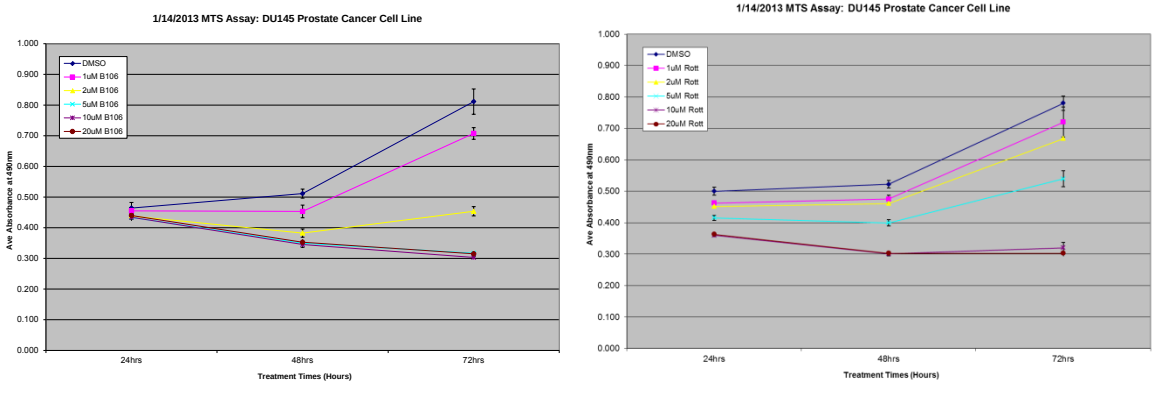
- To observe prostate cancer cell lines, immortalized prostate cells and Celprogen's Breast Cancer Stem Cells treated with 1, 2, 5, 10, 20 or 40 uM Rottlerin, PKC inhibitor. DU145 prostate cancer cells were the positive control for the effects of Rottlerin.

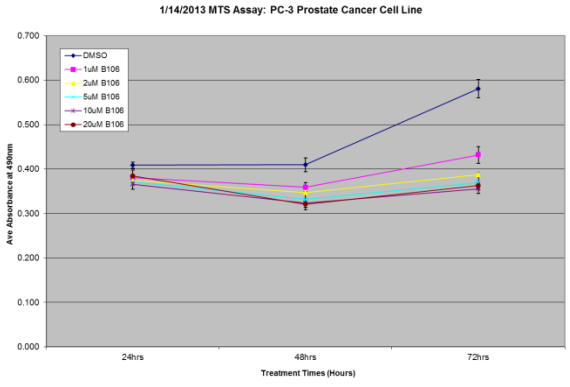
Materials and Methods

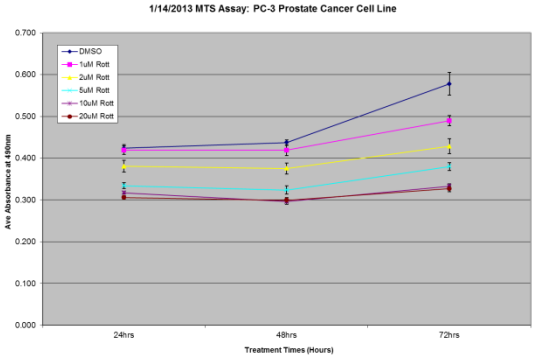
- Day 0: Cell plating day
  - Cells were plated at 2000 cells per well in 96 well plates. Quadruplicate samples were plated and grown at 37oC in 5% CO_2. Cells were allowed to grow for three days.
  - DU145 prostate cancer cells: 10%FBS (Invitrogen); Dulbecco's Modification of Earle's Media (MediaTech); 2mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; Passage 10.
  - LNCaP prostate cancer cells: 10% FBS (Invitrogen); RPMI 1640 (MediaTech); 2mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200 ug Streptomycin/ml (Invitrogen)); Passage 4.
  - PC-3 prostate cancer cells: 10% FBS (Invitrogen); Dulbecco's Modification of Earle's Media (MediaTech); 2 mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; Passage 10.
  - pZ-HPV-7 immortalized prostate cells: Keratinocyte Serum Free Media; 2 mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200 ug Streptomycin/ml (Invitrogen)); 0.015M HEPES; with trypsin inhibitor use for trypsin neutralization; Passage 7.
  - Breast Cancer Stem Cells (Celprogen): Celprogen's media with serum for Breast Cancer Stem Cells; Passage 7. Plated on Celprogen's Breast Cancer Stem Cell extracellular matrix.
- 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.
- 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.

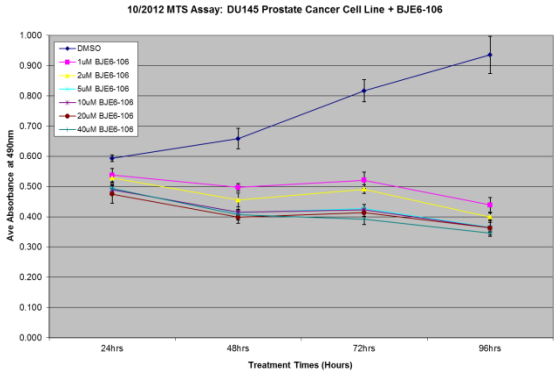
- MTS Assay was performed at each time point (CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega)) as described by manufacturer.
  - 20 ul of the assay buffer was added to each well. Cells were incubated for one hour at 37oC in 5% CO_2 humidified atmosphere.
  - The absorbance at 490 nm was read on the Molecular Devices, SpectraMax 190 plate reader.

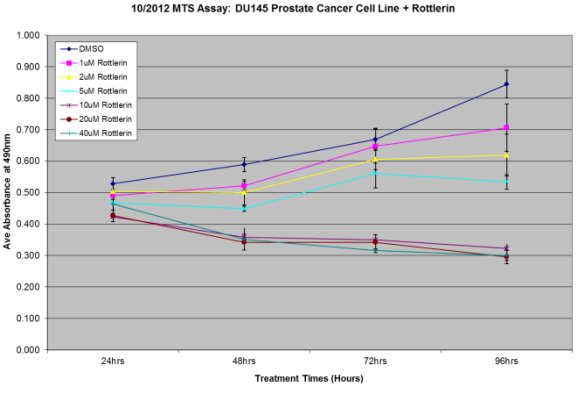
Figure 7: Relative Sensitivity of PrCa cell lines to 1st and 3rd generation PKCδ inhibitors.

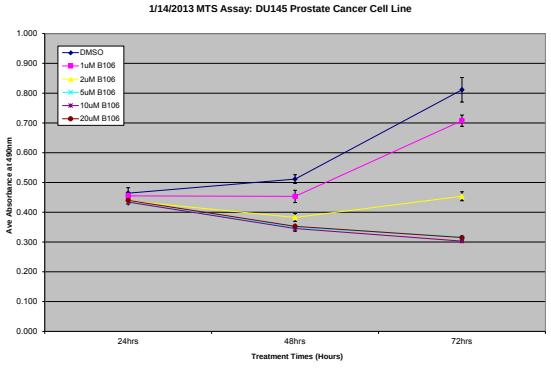


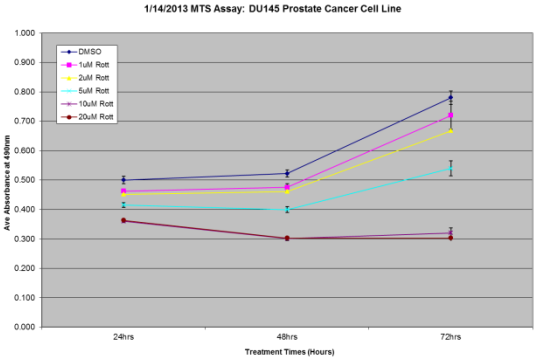

1/14/2013 MTS Assay: PC-3 Prostate Cancer Cell Line
DMSO
1uM B106
2uM B106
5uM B106
10uM B106
20uM B106
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

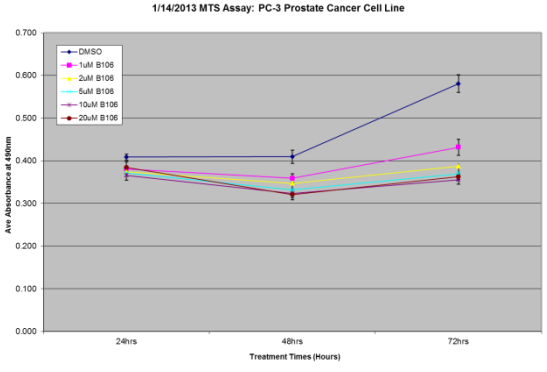

1/14/2013 MTS Assay: PC-3 Prostate Cancer Cell Line
DMSO
1uM Rott
2uM Rott
5uM Rott
10uM Rott
20uM Rott
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

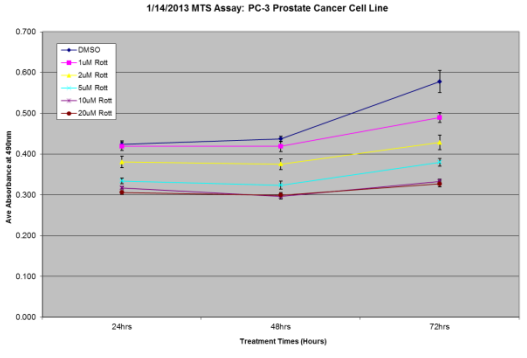

10/2012 MTS Assay: DU145 Prostate Cancer Cell Line + BJE6-106
DMSO
1uM BJE6-106
2uM BJE6-106
5uM BJE6-106
10uM BJE6-106
20uM BJE6-106
40uM BJE6-106
Ave Absorbance at 490nm
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
96hrs
Treatment Times (Hours)

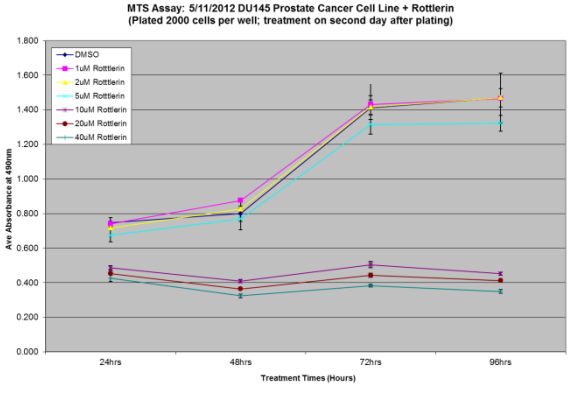

10/2012 MTS Assay: DU145 Prostate Cancer Cell Line + Rottlerin
DMSO
1uM Rottlerin
2uM Rottlerin
5uM Rottlerin
10uM Rottlerin
20uM Rottlerin
40uM Rottlerin
Ave Absorbance at 490nm
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
96hrs
Treatment Times (Hours)

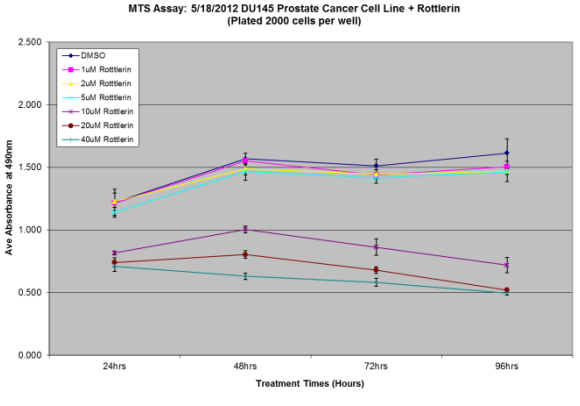












Interpretation:

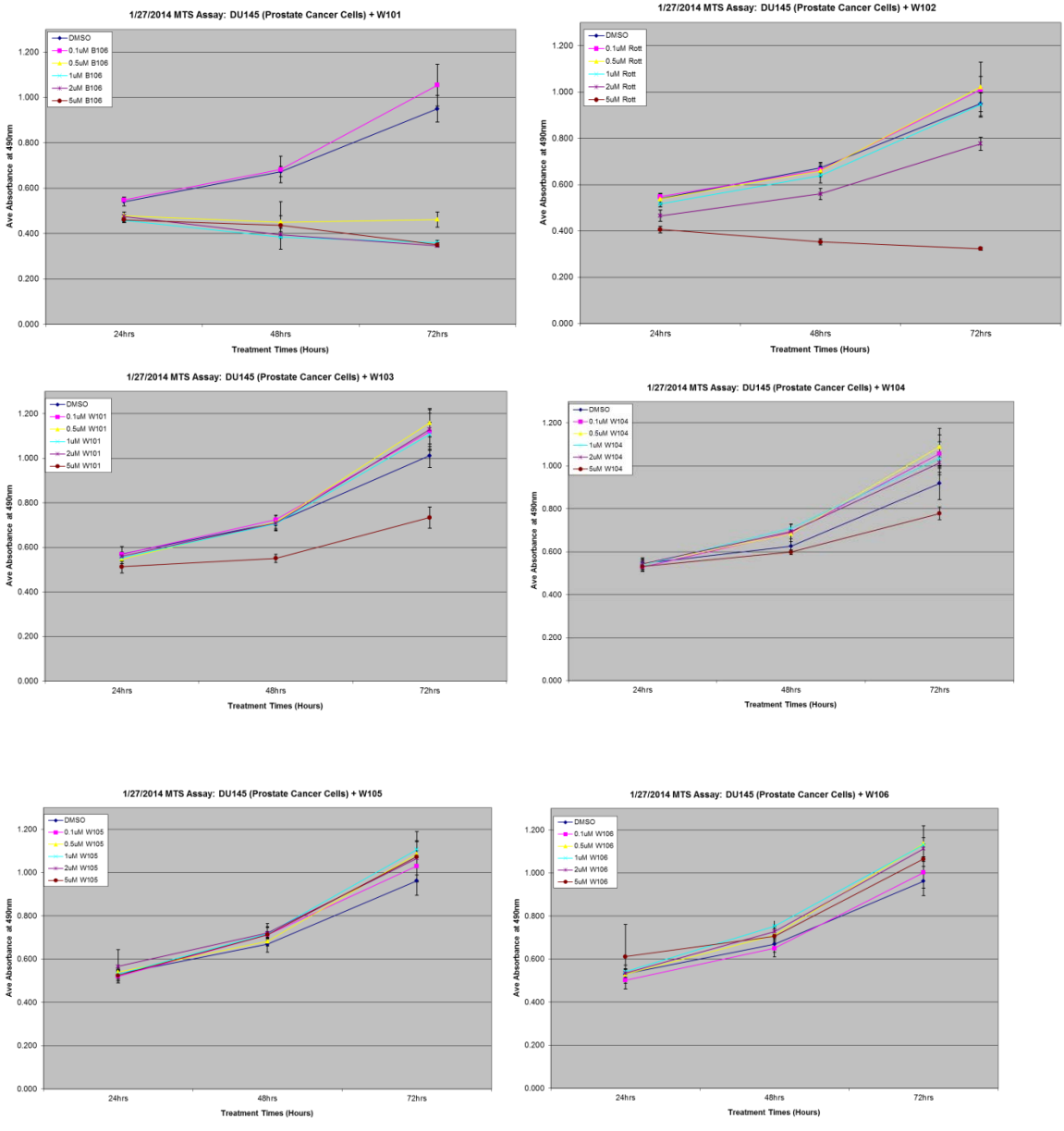
The third generation PKCδ inhibitor B106 was consistently and substantially more potent at inducing cytotoxic/cytostatic effects on all the prostate cancer cell lines with activation of Ras signaling examined than the 1st generation inhibitor (rottlerin, LC-I) (**Fig. 7**). IC_{50} for B106 was consistently < 1uM, whereas IC_{50} for rottlerin ranged from 6-10 uM.

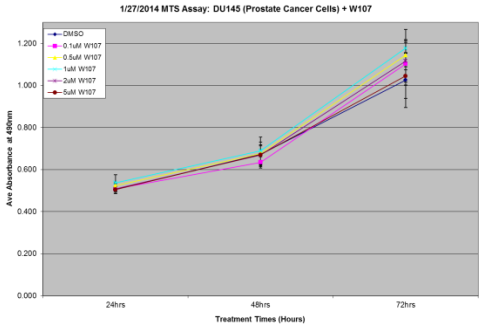
4. Testing of 3rd Generation PKCδ Inhibitor Compounds in Prostate Cancer Cell lines

B106 and the other most-active 3rd gen compounds were not optimized and displayed limited solubility with resultant poor bioavailability *in vivo*. New 4th gen compounds were synthesized, tested for PKCδ inhibitory activity, and then assayed on human prostate cancer cells, using the methodology described in section 3 above.

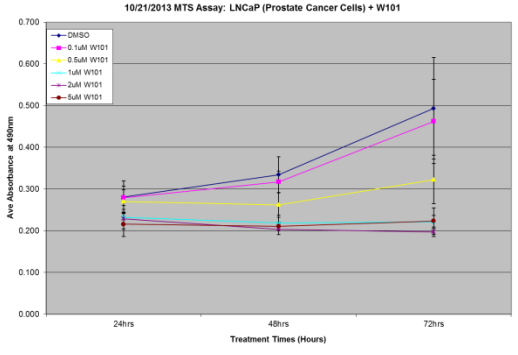
Figure 8: Relative Sensitivity of PrCa cell lines to 4th generation PKCδ inhibitors.

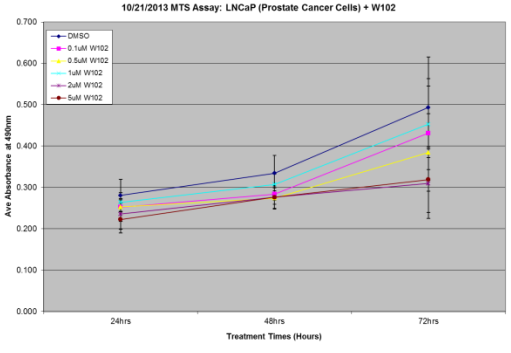
DU145

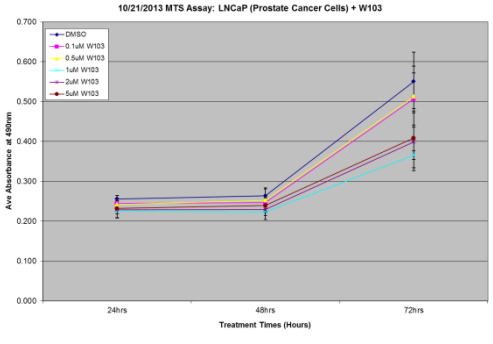


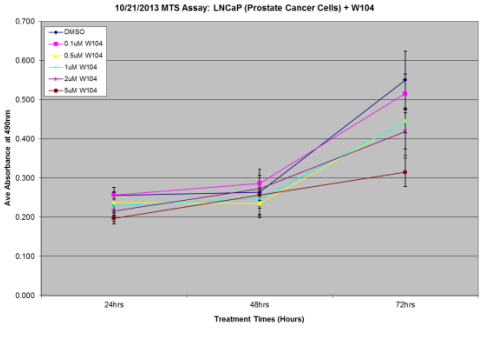

1/27/2014 MTS Assay: DU145 (Prostate Cancer Cells) + W107
DMSO
0.1uM W107
0.5uM W107
1uM W107
2uM W107
5uM W107
Ave Absorbance at 490nm
1.200
1.000
0.800
0.600
0.400
0.200
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

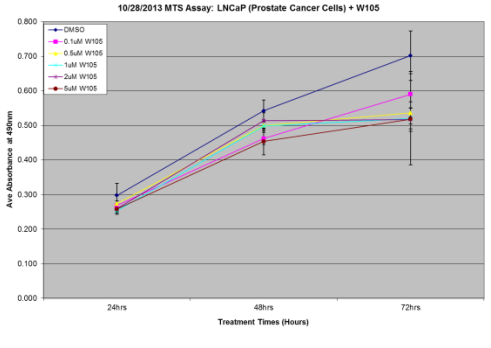
LNCaP

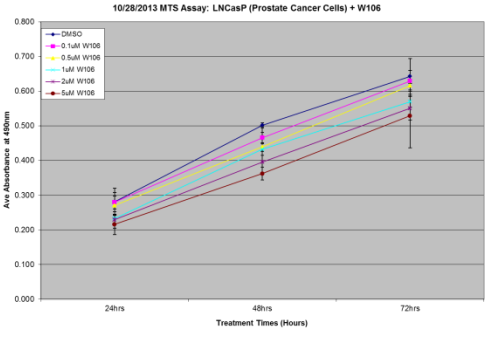

10/21/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W101
DMSO
0.1uM W101
0.5uM W101
1uM W101
2uM W101
5uM W101
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

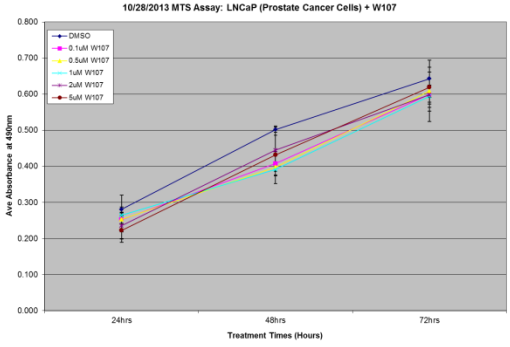

10/21/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W102
DMSO
0.1uM W102
0.5uM W102
1uM W102
2uM W102
5uM W102
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)


10/21/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W103
DMSO
0.1uM W103
0.5uM W103
1uM W103
2uM W103
5uM W103
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

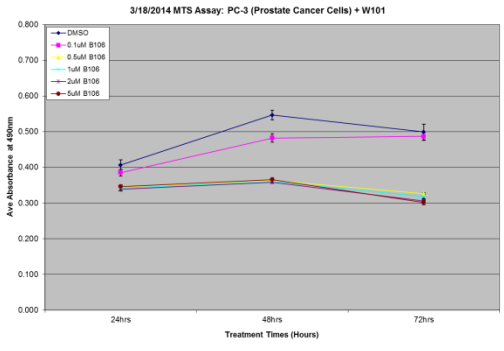

10/21/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W104
DMSO
0.1uM W104
0.5uM W104
1uM W104
2uM W104
5uM W104
Ave Absorbance at 490nm
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

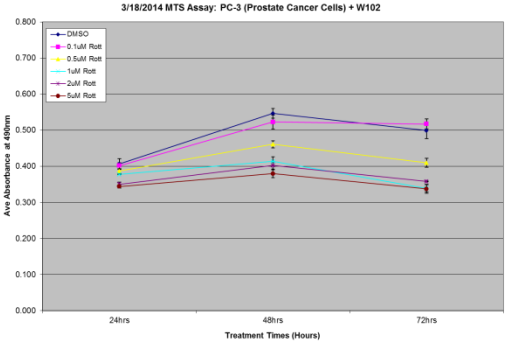

10/28/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W105
DMSO
0.1uM W105
0.5uM W105
1uM W105
2uM W105
5uM W105
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

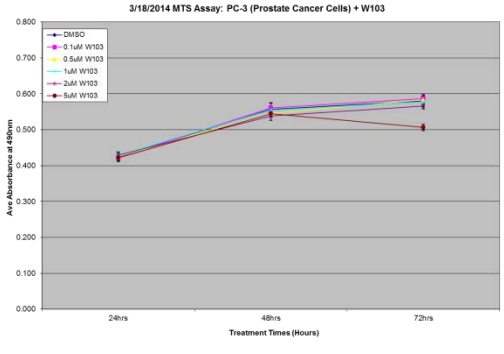

10/28/2013 MTS Assay: LNCasP (Prostate Cancer Cells) + W106
DMSO
0.1uM W106
0.5uM W106
1uM W106
2uM W106
5uM W106
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

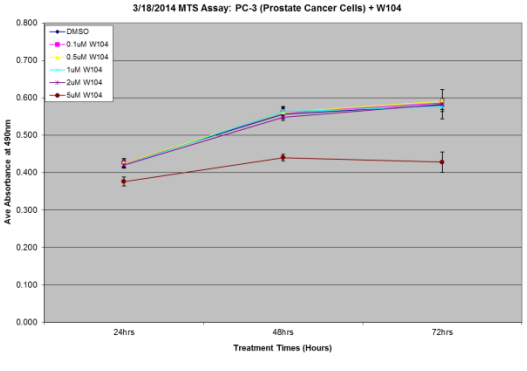

10/28/2013 MTS Assay: LNCaP (Prostate Cancer Cells) + W107
DMSO
0.1uM W107
0.5uM W107
1uM W107
2uM W107
5uM W107
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

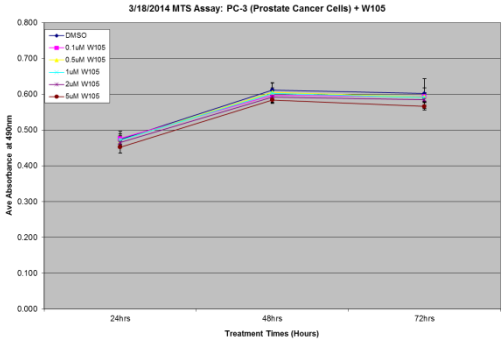
PC3

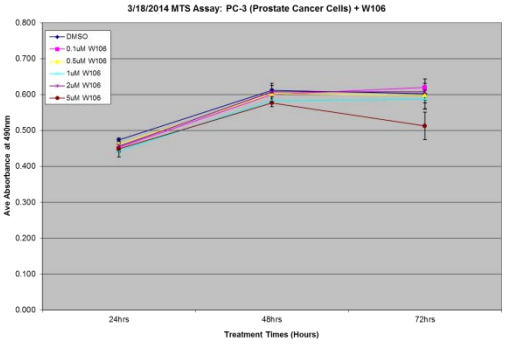

3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W101
DMSO
0.1uM B106
0.5uM B106
1uM B106
2uM B106
5uM B106
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)

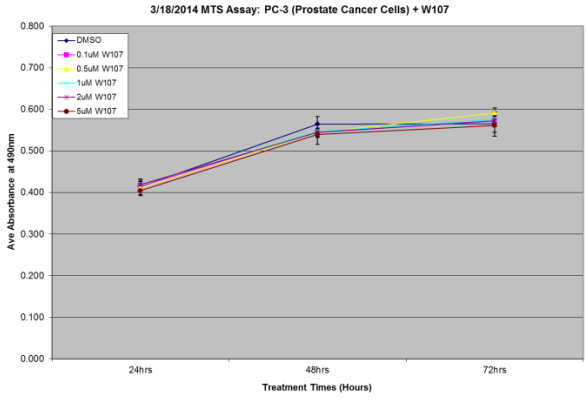

3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W102
DMSO
0.1uM Rott
0.5uM Rott
1uM Rott
2uM Rott
5uM Rott
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)


3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W103
DMSO
0.1uM W103
0.5uM W103
1uM W103
2uM W103
5uM W103
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)


3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W104
DMSO
0.1uM W104
0.5uM W104
1uM W104
2uM W104
5uM W104
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)


3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W105
DMSO
0.1uM W105
0.5uM W105
1uM W105
2uM W105
5uM W105
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)


3/18/2014 MTS Assay: PC-3 (Prostate Cancer Cells) + W106
DMSO
0.1uM W106
0.5uM W106
1uM W106
2uM W106
5uM W106
Ave Absorbance at 490nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
24hrs
48hrs
72hrs
Treatment Times (Hours)



Interpretation:

The 4th generation PKCδ inhibitors W101 and W102 were consistently and substantially active at inducing cytotoxic/cytostatic effects on all the prostate cancer cell lines with activation of Ras signaling examined than was the 1st generation inhibitor (rottlerin, LC-I) and 2nd generation inhibitors (**Fig. 8**). The IC_{50} for W101 was consistently <1 μM, comparable to B106 (3rd generation), whereas the IC_{50} for rottlerin ranged from 6-10 μM.

Effects on Immortalized Prostate Epithelial Cells

The effects of the 3rd generation PKCδ inhibitors on <u>non-tumor cells</u> was examined. The pZ-HPV-7 cell line was derived from primary human prostate epithelial cells by transformation with human papilloma virus. While not tumorigenic, they do exhibit some properties of transformed cells.

***Approach*:**

MTS Assay: pZ-HPV-7 cells (Immortalized Prostate Cell Line) were treated with Rottlerin or B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.

***Results*:** The pZ-HPV-7 cells were sensitive to PKCδ inhibition to some extent (**Fig. 9**). Whether this was caused by the transformation with HPV is not clear.

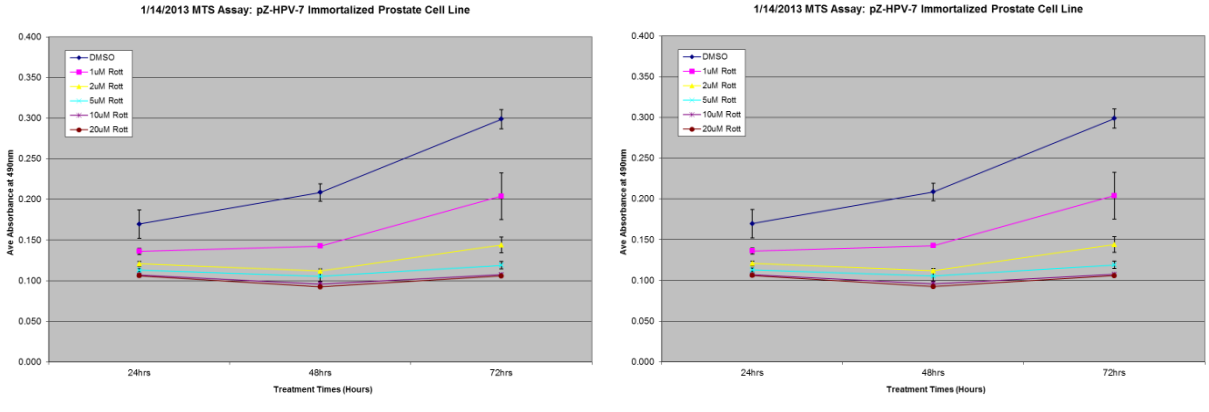


Figure 9: Relative Sensitivity of Normal Prostate cell lines to 1st and 3rd generation PKCδ inhibitors.

We decided therefore to examine the sensitivity of another cell line derived from a hormone-responsive epithelial tissue, which had not been immortalized using viral vectors (MCF 10A.

***Approach*:**

MCF 10A cells were treated with Rottlerin or B106 at the indicated concentrations. MTS assay was then carried out at 24, 48 and 72 h to quantitate cell growth (**Fig. 10**).

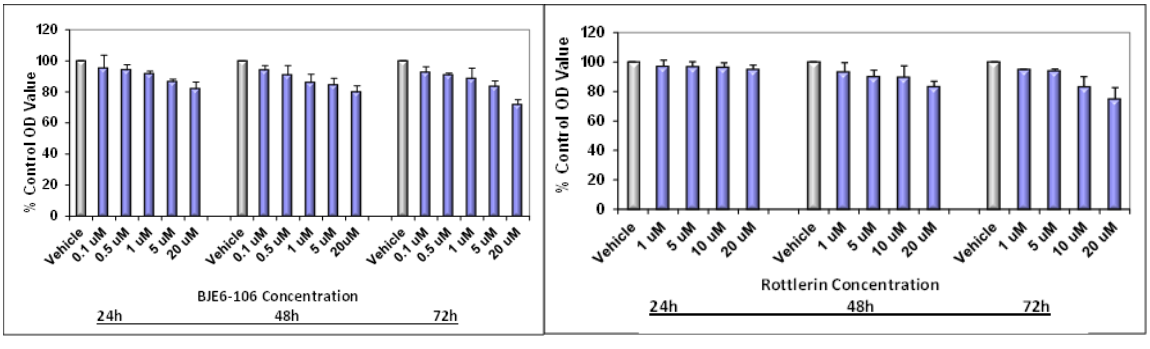
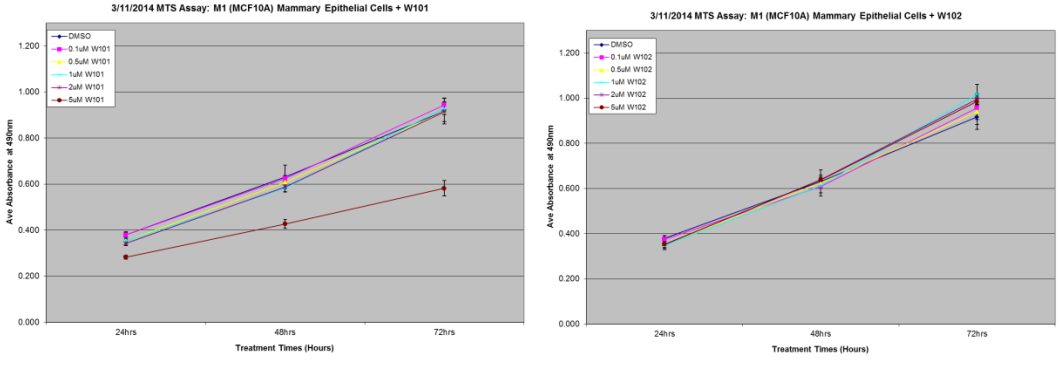


Figure 10

MCF 10A cells were treated with the 4th generation inhibitors at the indicated concentrations. MTS assay was then carried out at 24, 48 and 72 h to quantitate cell growth (**Fig. 11**).



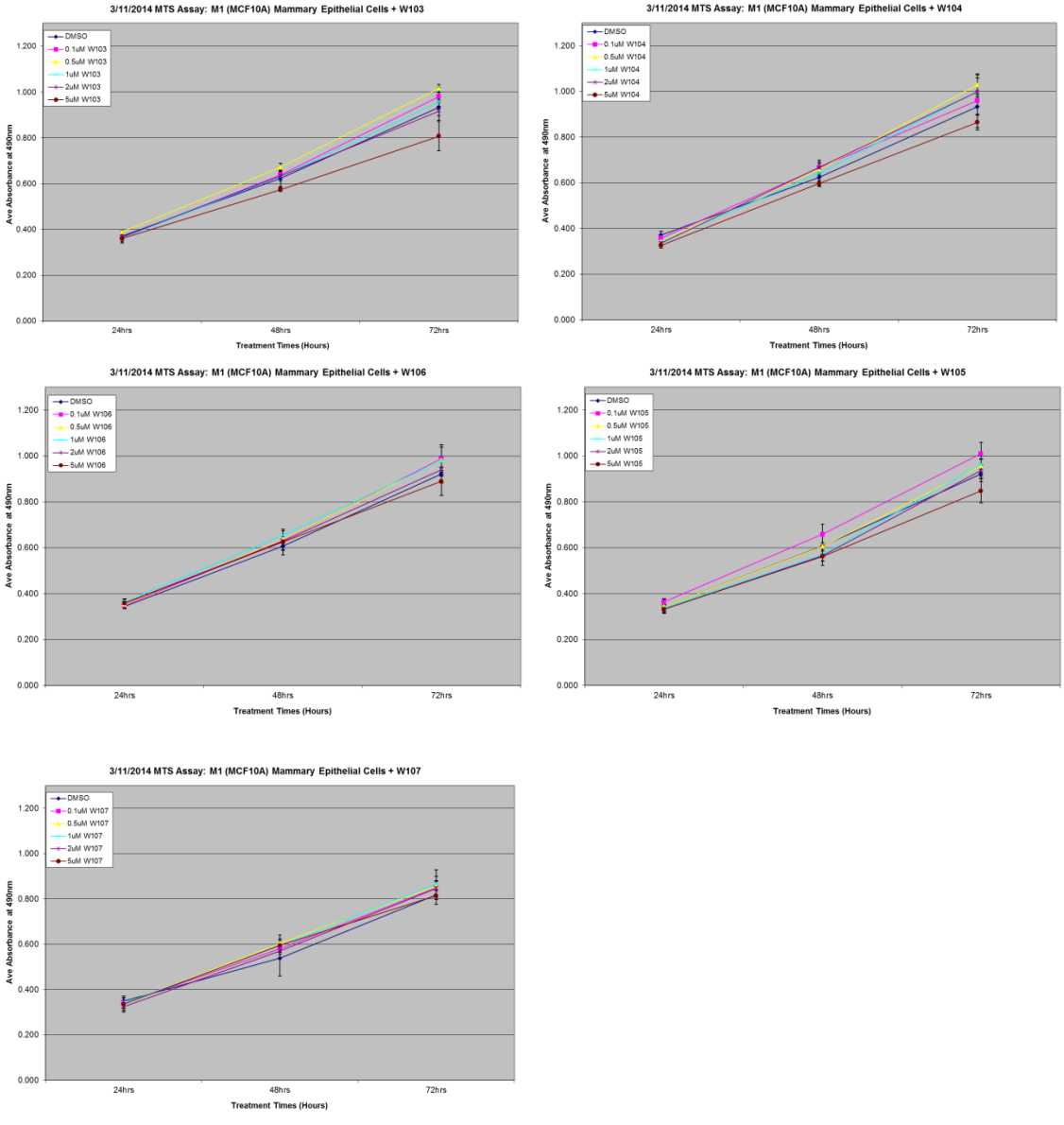


Figure 11

***Results*:** The "normal" human epithelial cell line MCF 10A was relatively insensitive to 1st and 3rd and certain 4th generation PKCδ inhibitors (W101, W102) (**Fig. 10-11)**. For MCF 10A, IC_{50} for B106 was consistently >> 20 μM, for W101 and W102 was greater than 5 μM and for rottlerin was also >> 20 uM at all timepoints.

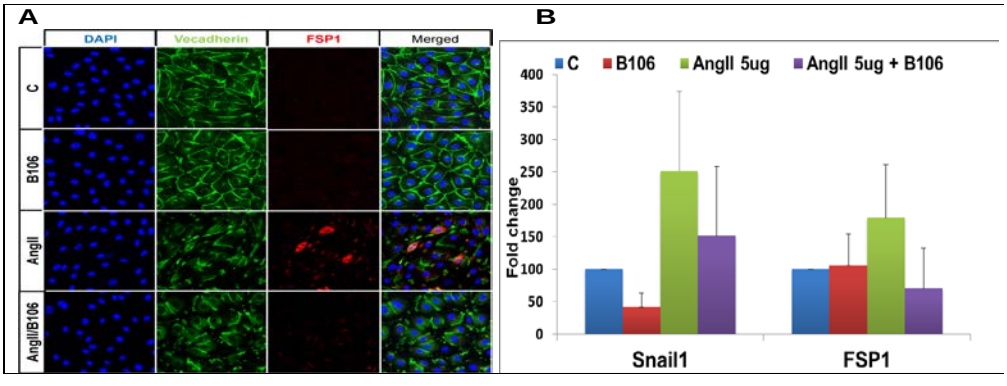
As an even more stringent assessment of the effects of 3rd gen PKCδ inhibitors on normal tissue, primary human microvascular endothelial cells were exposed to the compound in culture.

***Approach*:**

In this particular experiment, primary human microvascular endothelial cells were exposed to vehicle, B106, angiotensin (and inducer of endo to mesenchymal transition, EndoMT), or angiotensin + B106**.** Effects on morphology and gene induction were analyzed.

Figure 12: Ang II stimulation of EndoMT in human dermal microvascular endothelial cells (HDMVECs) is partially abrogated by B106.

A B



A: Cells were stimulated with 5µg/mL of Ang II for 24 hours. Note deregulation of Ve-cad (green) and upregulation of Fsp1 (red) in Ang II treated cells. B106 (5 µM) partially restored membrane distribution of Ve-cad and prevented Fsp1 upregulation

B: qPCR analyses of the indicated genes in control and Ang II-stimulated cells in the presence or absence of B106. Experiment was repeated three times. Bar graph shows Mean±SD.

Results: This 3rd generation PKCδ inhibitor did not cause cytotoxicity in normal human endothelial cells a concentration of 5 μM, as assessed by morphology (**Fig. 12A)** and induction of mesenchymal genes (**Fig. 12B)**.

As the most stringent assessment of the effects of 3rd gen PKCδ inhibitors on normal tissue, B106 was infused into the dermis of mice over 4 days.

Approach:

In this particular experiment, B106 (5µM/day/mouse) was administered alone or together with the Ang II (an inducer of skin fibrosis) via the Alzet osmotic pump.

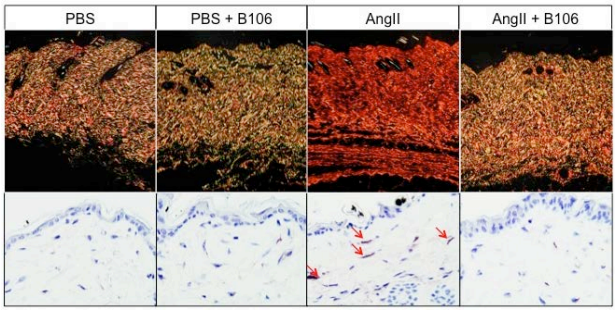


Fig. 13. B106 inhibits collagen deposition and myofibroblast accumulation in the Ang II treated mice. Top panels, representative histochemical staining with picrosirius red in the indicated conditions. Polarized images were obtained using the Olympus Bx41 microscope equipped with a digital camera. Bottom panels, αSMA-positive cells are indicated by arrows.

***Results*:** Treatment with B106 alone had a minimal effect on skin collagen content as assessed by picrosirius red staining (**Fig. 13**). Collagen deposition was markedly increased in the skin of Ang II treated mice as illustrated by the yellow and red birefringenence characteristic of the thicker, more densely packed collagen fibers and was visibly reduced by the addition of B106 with weaker greenish birefringenece representing thinner, more loosely packed fibers 80. Treatment with Ang II + B106 showed reduced number of CD163+ macrophages (not shown) as well as myofibroblasts, when compared to Ang II treatment alone (**Fig. 13**).

Importantly, systemic administration of B106 in this model did not result in clinically apparent toxicity in these mice (as would be expected from the finding that PKCδ null mice grow and develop normally, and are fertile), indicating there is a therapeutic window for PKCδ inhibition.

***Interpretation*:** B106, a potent and selective 3rd generation PKCδ inhibitor, is not toxic to normal cells either in culture or and *in vivo* at therapeutic concentrations.

PKCδ inhibitors have Anti-fibrotic/ anti-tumor stroma Activity:

In a new and important discovery, we have demonstrated that our PKCδ inhibitors block the fibrosis and tumor stroma formation induced by tumor-secreted TGFβ.

The critical role of the tumor "stroma" in cancer development has been increasingly recognized. Tumor initiation alone, triggered by mutations in proto-oncogenes and/or tumor suppressor genes, is insufficient for the development of cancers. Tumor promotion depends upon interaction between initiated cells and the microenvironment. The tumor stroma is dependent upon TGFβ elucidated by the tumor/stroma. Infiltrating tumor cells educate the host stroma of the target organ to support metastasis initiation.[17] Cancer-associated fibroblasts (CAFs) are recruited by cancer cell-secreted factors, such as TGFβ.[18-21] Through self-sustaining signaling of TGFβ, nearby fibroblasts transdifferentiate into myofibroblasts during tumor progression.[22,23] Fibrosis/desmoplasia characterizes tumor stroma, and TGFβ is a crucial inducer of α-SMA (smooth muscle actin)-positive CAFs[24] and lysl oxidase (LOX).[18,25,26] Fibrosis/desmoplasia also has consequences for the efficiency of drug delivery to the tumor, as drugs cannot penetrate tissue under positive interstitial fluid pressure (IFP).[27,28] TGFβ also induces a biologic program termed endothelial-to-mesenchymal transition (**endoMT**), which is important in initiating and sustaining tumor progression and angiogenesis.[18,29]

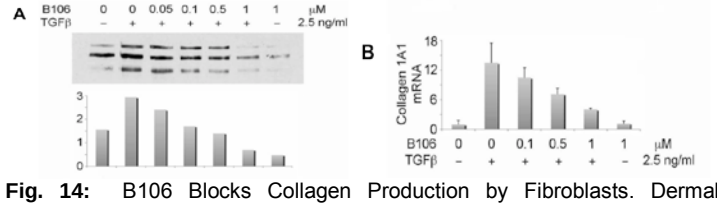


Fig. 14: B106 Blocks Collagen Production by Fibroblasts. Dermal fibroblasts were stimulated with TGFβ for 24 h in the presence or absence of the indicated concentration of PKC´ Inhibitor B106. (**A**) Secretion of collagen protein was examined by IB. (**B**) COL1A1 mRNA was analyzed by qPCR. SSc fibroblasts (unstimulated) were treated with the indicated doses of B106. Bar graphs represent Mean ± SD from two independent experiments.

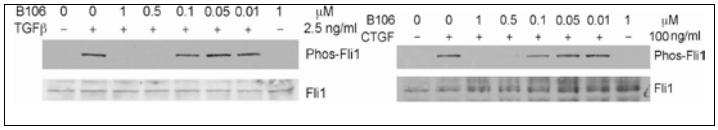


Fig. 15: B106 blocks Fli1 Phosphorylation in response to TGFβ or CTGF. Dermal fibroblasts were stimulated with TGF² (left) or CTGF (right) for 2 h. Fli1 phosphorylation was assessed by IB with the phospho-Fli1-specific Ab.

We have shown that PKCδ inhibitor B106 decreases type I collagen production by TGFβ-stimulated normal dermal fibroblasts (**Fig. 14;** Trojanowska, in preparation). TGFβ stimulates collagen production by activating PKCδ, which in turn kinases the collagen-gene regulating transcription factor **Fli1**.[30,31] We show here that B106 decreases Fli1

phosphorylation in response to either TGFβ or CTGF (connective tissue growth factor) (**Fig. 15;** Trojanowska, in preparation), thus validating "on-target activity" in cells. (Note that both TGFβ and CTGF induce the desmoplastic stroma which characterizes pancreatic adenocarcinoma[32] and melanoma.) These data together indicate that inhibition of PKCδ by our novel compounds blocks TGFβ- or CTGF-mediated fibrotic activity.

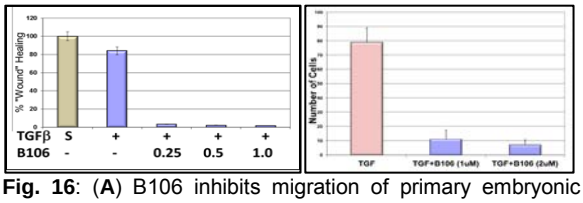


Fig. 16: (**A**) B106 inhibits migration of primary embryonic fibroblasts in response to TGFβ. The migration of cells into a scratch was monitored in the presence of 10% serum, TGFβ 5 ng/ml or TGFβ plus the indicated concentrations of B106 (μM). 22 h time-point shown here. (**B**) B106 inhibits transwell migration of primary embryonic fibroblasts in response to TGFβ. MEF cells plus vehicle or the indicated concentrations of B106 were placed into the upper chamber of a Boyden chamber, and TGFβ 5 ng/ml into the lower chamber. Migrating cells at 2 h were scored and quantitated.

- PKCδ-inhibitors prevent TGFβ-induced migration of fibroblasts: TGFβ-induction of tumor stroma cells/CAFs includes recruitment of mesenchymal cells by increasing their motility. B106 (and mallotoxin/rottlerin, not shown) inhibits the TGFβ-induced migration of primary mesenchymal cells on tissue culture plastic (**Fig. 16A**), and effectively inhibits transwell migration in response to TGFβ (**Fig. 16B**), at nM concentrations.

Thus, by blocking PKCδ, our new compounds not only directly induce cytotoxicity to the tumor cells, but have the potential to block the formation of tumor stroma, by blocking TGFβ-induced collagen formation, by blocking TGFβ-driven Endo-MT and also by preventing the formation of myofibroblasts by TGFβ.

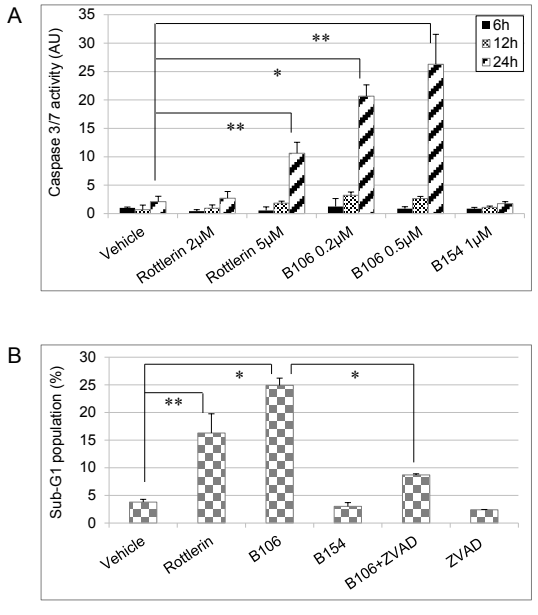


Figure 17: Inhibition of PKC´ induces caspase-dependent apoptosis. (A) Effector caspase 3/7 activation by PKC´ inhibition. Tumor cells were exposed to rottlerin (2 or 5μM) or B106 (0.2 or 0.5μM) for 6, 12 or 24 hours and caspase 3/7 activity was measured by luminogenic assay at each time point. DMSO and B154 (1μM) 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´ inhibition. SBcl2 cells were treated with rottlerin (5μM), B106 (0.5μM) alone, or B106 (0.5μM) plus pan-caspase inhibitor Z-VAD-FMK (100μM) 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 (0.5μM) 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: ** $p < 0.04$, * $p < 0.004$.

Mechanisms of Anti-tumor Action of PKCδ Inhibitors

(this data was published in Takashima, et al, 2014, [2] and will be only briefly described in this report).

Inhibition of PKCδ activity triggers caspase-dependent apoptosis. We next determined how PKCδ 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 PKCδ inhibition, the activity of effector caspases 3 and 7 was assessed in cells treated with PKCδ inhibitors. Twenty-four hours of exposure to rottlerin (5 μM) or B106 (0.2 and 0.5 M)

significantly increased the activity of caspase 3/7 in tumor cells compared to vehicle (DMSO) (**Figure 17A**). The effect of B106 on caspase 3/7 activation was greater than that of 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. 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 PKCδ inhibition.

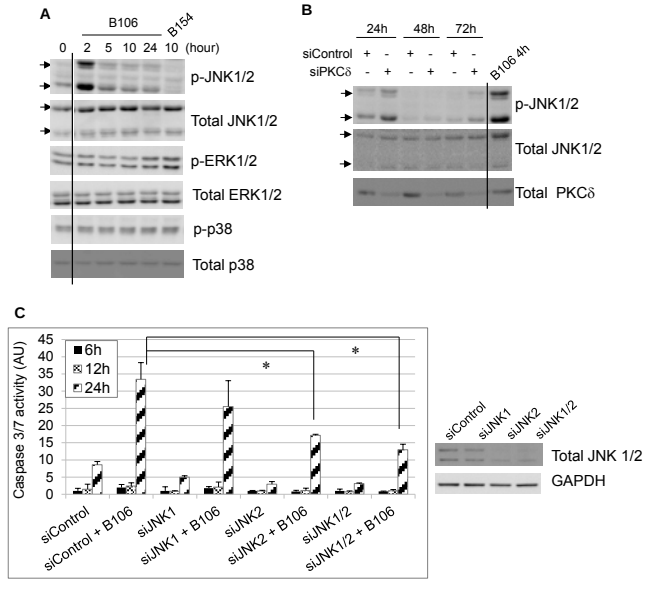


Figure 18: PKCδ inhibition triggers an apoptotic response through activation of JNK. (A, B) PKCδ inhibition activates JNK. Tumor cells were exposed to B106 (1μM) or negative control compound B154 (1μM) for indicated times (A) or transfected with siRNA targeting PKCδ ("siPKCδ ") or non-targeting siRNA ("siControl") at 5nM for the indicated times (B). Protein lysates were subjected to immunoblot analysis for levels of phosphorylated or total MAPK proteins. Phosphorylation sites: p-JNK1/2 (T183/Y185), p-ERK1/2 (T202/T204), p-p38 (T180/Y182). **(C) Activation of caspase 3/7 is mitigated by knockdown of JNK prior to B106 treatment.** Tumor cells were transfected with siRNA targeting JNK1 or JNK2 alone (5nM), or the combination of JNK1 and JNK2 siRNA (5nM each), or non-targeting siRNA (10nM) for 72 hours, and subsequently treated with B106 (0.5μM) or vehicle (DMSO) for 6, 12 and 24 h. Caspase 3/7 activity was measured by luminogenic assay at each time point. The average values of triplicates were normalized to that of the vehicle-treated sample at 6 hours between the pairs of the same siRNA. Error bars indicate the standard deviations. P values: * $p < 0.005$. Downregulation of JNK1/2 proteins were confirmed by immunoblot analysis. Cells were lysed after 72 h of siRNA transfection. Each of the two bands detected in immunoblotting with JNK1/2 antibodies represent assembly of different splicing variants from both JNK1 and 2 isoforms. Levels of GAPDH served as a loading control.

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 PKCδ 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 μM and even higher after treatment with B106 at 0.5 μM, whereas B154, a negative-control compound for B106, lacking PKCδ-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 PKCδ activity (**Figure 17B**). To determine whether activation of caspases by PKCδ inhibitors was necessary for the observed apoptosis, the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[Omethyl]-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 μM) prevented B106-induced caspase 3 cleavage in immunoblot analysis (data not shown). B106-induced DNA fragmentation was significantly abrogated when tumor cells were pretreated with Z-VAD-FMK (100 μM) (**Figure 17B**). 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 PKCδ inhibition attenuates tumor cell growth by inducing caspase-dependent apoptosis in RAS-mutant tumor cells.

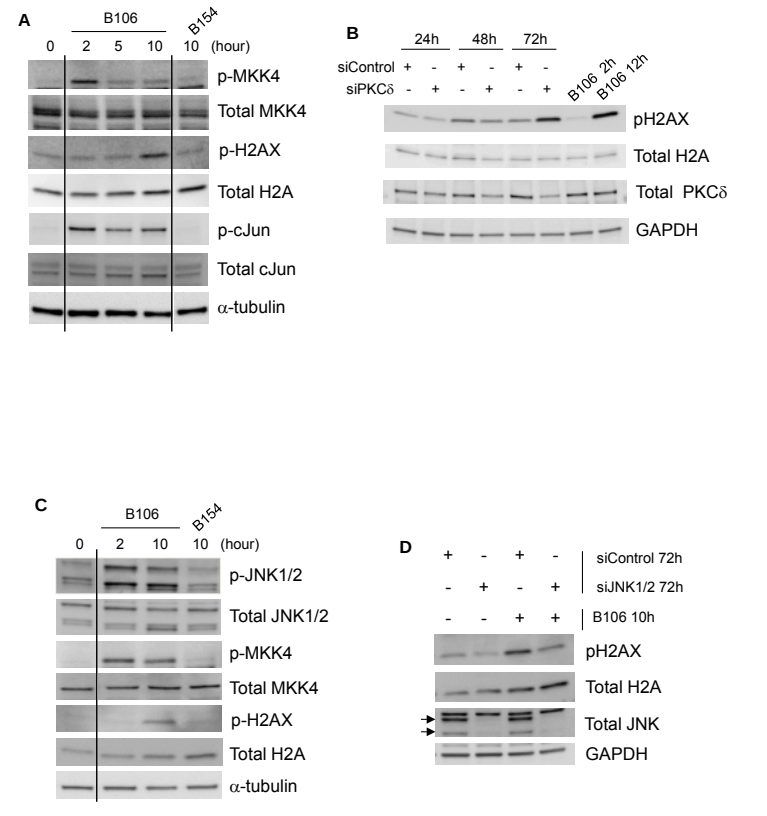


Figure 19: PKCδ inhibition activates the MKK4-JNK-H2AX pathway. (A) Activation of upstream and downstream components of the JNK pathway by B106. Tumor cells were treated with B106 or negative control compound B154 at 1μM for the indicated times. Protein lysates were subjected to immunoblot analysis for phosphorylated or total levels of the upstream (MKK4, MKK7) and the downstream (H2AX, cJun) components of JNK signaling. Levels of ±-tubulin served as a loading control. Phosphorylation sites: p-MKK4 (S257), p-MKK7 (S271/T275), p-H2AX (S139), p-cJun (S63). **(B) Selective downregulation of PKCδ results in phosphorylation of H2AX.** SBcl2 cells were transfected with siRNA targeting PKCδ ("siPKCδ") or non-targeting ("siControl") at 50nM for the indicated times. Protein lysates were subjected to immunoblot analysis for phosphorylation and total expression levels of H2AX and PKCδ protein. Levels of GAPDH served as a loading control. (C) PKCδ inhibition activates MKK4-JNK-H2AX pathway in WM1366. WM1366 cells were exposed to B106 (1μM) or negative control compound B154 (1μM) for indicated times. Protein lysates were subjected to immunoblot analysis for levels of phosphorylated or total MAPK proteins. **(D)** PKCδ **inhibition activates H2AX through JNK.** Tumor cells were transfected with siRNA targeting JNK1 and JNK2 together (5nM each) or non-targeting siRNA (10nM) for 72 hours and subsequently treated with B106 (0.5μM) or vehicle (DMSO) for 10 hours. Protein lysates were subjected to immunoblot analysis for phosphorylation and total expression levels of H2AX and JNK. Levels of GAPDH served as a loading control. Arrows indicate JNK1/2.

PKCδ inhibition triggers apoptotic response via the stress-responsive JNK pathway. To identify which intracellular signaling pathway PKCδ inhibition employs to induce cytotoxicity, the activation status of known downstream targets of PKCδ was examined after PKCδ inhibition, including MAPKs (ERK, p38 and JNK), AKT, NFκB pathway, cyclin-dependent kinase inhibitors, p53, IAPs, GSK3β or c-Abl. Inhibition of PKCδ activity in tumor cells by B106 induced phosphorylation (activation) of JNK1/2 (T183/Y185) most strongly after two h of exposure, with phosphorylation diminishing subsequently (**Figure 18A**). In contrast, phosphorylation of the closely-related MAPKs p38 and ERK was not affected by PKCδ inhibitors (**Figure 18A**). Consistent with these observations generated using chemical inhibitors, selective downregulation of PKCδ by transfection of PKCδ-specific siRNA induced phosphorylation of JNK1/2 at 24 h (when effects of siRNA on PKCδ levels were first observed), whereas transfection of negative-control non-targeting siRNA did not affect JNK1/2 phosphorylation (**Figure 18B**). Transfection of PKCδ-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.[33,34] Because of the data demonstrating that PKCδ inhibition causes caspase-dependent apoptosis (Figure 17) and JNK activation (Figures 18A

&B), the effect of inhibition of the JNK pathway during B106 treatment was explored to determine if there is a functional relationship. Tumor 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 h, and then exposed to B106 or DMSO (vehicle) for 6, 12 or 24 h, followed by measurement of caspase activity (**Figure 18C**). Analysis at 24 h 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 (the two bands detected in immunoblotting with JNK1/2 antibodies represent different splicing variants of both JNK1 and 2 isoforms). These data indicated that JNK is a necessary mediator of the apoptotic response induced by PKCδ inhibition.

PKCδ inhibition activates the MKK4-JNK-H2AX pathway. We tested for involvement of known upstream and downstream effectors of the JNK pathway following PKCδ inhibition. The MAPKK kinases MKK4 and MKK7 lie one tier above JNK. MKK4 was activated by B106 (as assessed by activating phosphorylation (**Figure 19A**), 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 PKCδ inhibitors (**Figure 19A**). Furthermore, activation of H2AX (histone H2A variant X), another downstream effector of JNK associated with its apoptotic actions,[35] was noted at later time points in response to B106 treatment (**Figure 19A**). B106 consistently induced H2AX phosphorylation as early as 10 h (times later than 24 hr were not studied because significant cytotoxicity is occurring after this time). The effect of PKCδ inhibition on H2AX activation was further confirmed by selective downregulation of PKCδ with siRNA. Phosphorylation of H2AX was observed at 72 hr after PKCδ siRNA transfection, but not in the cells transfected with negative-control siRNA (**Figure 19B**). This temporal course was consistent with the observation above of H2AX phosphorylation at a later time after the initiation of the MKK4/JNK cascade activation seen with PKCδ inhibitor treatment (**Figure 19A**). To ensure that activation of JNK pathway by B106 is not a cell-type-specific response, these pathway effectors were examined in another RAS-mutant tumor cell line WM1366. PKCδ inhibition by B106 treatment similarly induced phosphorylation of MKK4, JNK and H2AX in WM1366 cells (**Figure 19C**).

Because JNK affects diverse downstream effectors, we next determined whether JNK activation caused by PKCδ inhibition is directly linked to B106-induced H2AX activation. Cells were transfected with either negative-control siRNA or JNK1/2-specific siRNA for 72 h and then exposed to vehicle or B106 for another 24 h. 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 19D**). B106 exposure robustly induced phosphorylation of H2AX in control siRNA-treated cells (Lane 3, **Figure 19D)** as expected; in comparison, prior downregulation of JNK1/2 protein by siRNA attenuated B106-induced H2AX phosphorylation (Lane 4, **Figure 19D**). These findings confirmed that JNK lies upstream of H2AX, because H2AX is not activated in response to PKCδ inhibitors in the absence of JNK, supporting a model in which inhibition of PKCδ by B106 causes JNK/H2AX pathway signaling.

Collectively, these data suggest that PKCδ 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 PKCδ inhibition. Although phosphorylation of H2AX is best known as a consequence of DNA double-stranded breaks in the DNA damage response, recent studies have demonstrated that phosphorylation of H2AX resulting from JNK activation actively mediates the induction of

apoptosis.[36] Our findings of PKCδ inhibition-induced activation of the JNK/H2AX pathway and caspase-dependent apoptosis raised the possibility that inhibition of PKCδ activity caused caspase-dependent apoptosis through activation of the JNK/H2AX pathway. Accordingly, the direct involvement of H2AX in apoptotic response to PKCδ inhibition was examined. SBcl2 cells were transfected with siRNA targeting H2AX, or non-targeting 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 compared to the cells pre-treated with control siRNA (**Figure 20A**).

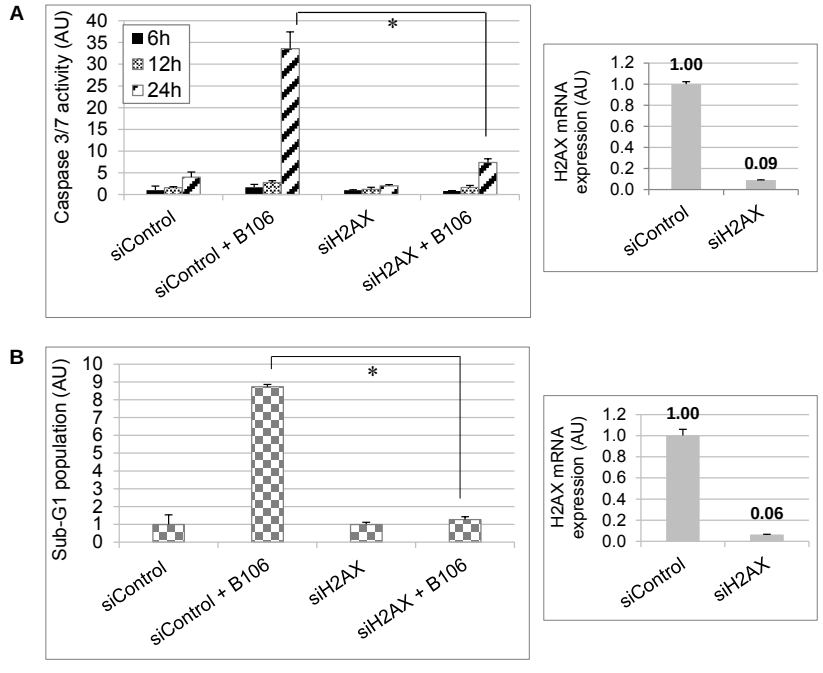


Figure 20: H2AX is a critical apoptotic regulator in apoptosis induced by PKCδ inhibition. (A) Activation of caspases 3/7 is mitigated by knockdown of H2AX prior to B106 treatment. Tumor cells were transfected with siRNA targeting H2AX or non-targeting siRNA at 5nM for 72 h, and subsequently treated with B106 (0.5μM) or vehicle for 6, 12 or 24 h. Caspase 3/7 activity was measured by luminogenic assay at each time point. The average values of triplicates were normalized to that of the vehicle-treated sample at 6 h between the pairs of the same siRNA. Error bars indicate the standard deviations. P values: * $p < 0.005$. Downregulation of H2AX was confirmed by quantitative PCR. The amount of mRNA was measured instead of protein due to difficulty to detect H2AX in an immunoblotting analysis. mRNA was extracted 72 h after siRNA transfection. **(B) Induction of DNA fragmentation is mitigated by knockdown of H2AX prior to B106 treatment.** Tumor cells were transfected with siRNA targeting H2AX, or non-targeting siRNA, at 5nM for 72 h, and subsequently exposed to B106 (0.5μM) or vehicle for 24 h. The proportion of sub-G1 population was measured by flow cytometric analysis following propidium-iodide staining of DNA. The average values of duplicate were normalized to that of the vehicle-treated sample between the pairs of the same siRNA. Error bars indicate the standard deviations. P value: * $p < 0.0004$. Downregulation of H2AX was confirmed by quantitative PCR. The amount of mRNA was measured instead of protein due to difficulty to detect H2AX in an immunoblotting analysis. mRNA was extracted after 96 h of siRNA transfection.

To explore a direct link between H2AX and the execution of apoptosis, PKCδ inhibition-induced DNA fragmentation was examined in the presence or absence of H2AX. Similar to the experiment in Figure 20A, SBcl2 cells were transfected with either negative-control siRNA or siRNA targeting H2AX for 72 h, and then subjected to PKCδ inhibition by B106 treatment for the next 24 hr. DNA fragmentation was assessed by flow cytometric analysis following propidium iodide staining of DNA. PKCδ inhibition by B106 treatment increased DNA fragmentation 8.5-fold in the cells transfected with negative control siRNA (**Figure 20B**). In contrast, PKCδ inhibition by B106 treatment failed to induce DNA fragmentation in the absence of H2AX, induced by transfection of siRNA targeting H2AX (**Figure 20B**). 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 20B**). Collectively, these results suggest that

inhibition of PKC by B106 treatment triggers caspase-dependent apoptosis through activation of the JNK-H2AX stress-responsive signaling pathway.

Task 1c) Determine the duration of PKCδ inhibition required to irreversibly initiate the apoptotic process.

Method/Assays:

1. Washout Studies: Exposure to inhibitors of PKCδ for different intervals of time, followed by washout, and assay of cell number over time. In this representative study, the DU145 cell line was used, and LC-1 (rottlerin) was used as the inhibitor (data shown in prior progress report).

2. Clonogenic Assays: Human prostate cancer stem cells in culture were exposed to a small-molecule inhibitor of PKCδ for 6, 18, 24, or 48 h, then the inhibitor was washed out and a clonogenic assay carried out. Colonies formed were enumerated. Treatment times indicate the duration of exposure to the inhibitor prior to replating (**Fig. 21**).

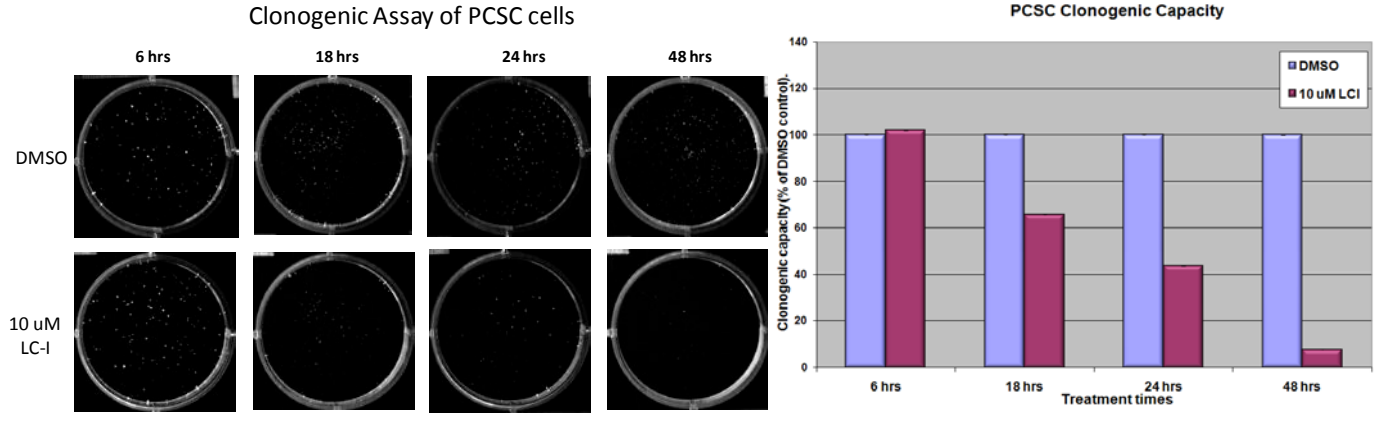


Fig. 21: Clonogenicity Assay using human Prostate Cancer Stem Cells (PCSC). Human prostate cancer stem cells in culture were exposed to a small-molecule inhibitor of PKCδ for 6, 18, 24, or 48 h, then the inhibitor was washed out and a clonogenic assay carried out. Treatment times indicate the duration of exposure to the inhibitor. Error bars (very small and difficult to see) indicate SEM. $p < 0.05$ for the 18, 24 and 48 h exposures compared to DMSO control.

***Interpretation*:** Cytotoxic effects on prostate cancer cells are observed after exposure to 1st generation PKCδ inhibitors for a period of 6 h. Longer periods of exposure produced progressively more toxicity. Replacing with fresh PKCδ inhibitors does not enhance the cytotoxic effect.

***Follow-up Studies*:** These studies were repeated using a 3rd generation inhibitor, B106. Results confirmed that irreversible cytotoxic effects occur within 6 hr of exposure. The data is presented below in the section on Cancer Stem Cells (p. 32).

TASK 2: Determine whether constitutive activation of *selected Ras effector pathways alone* is sufficient to make human prostate cancer cells susceptible to apoptosis after PKCδ inhibition: (utilizing prostate cancer cells with aberrant activation of the PI_3K pathway or aberrant activation of the Ras-MEK-ERK pathway.

Status: COMPLETE

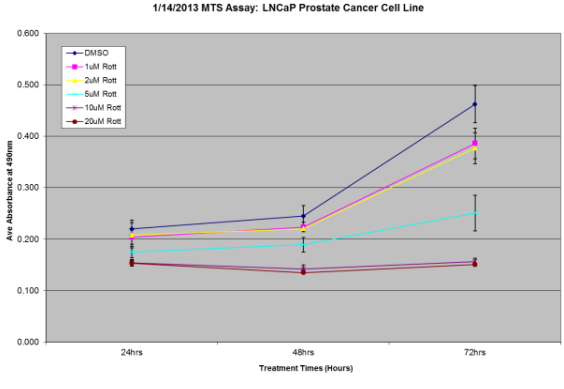
Task 2a) PI_3K Pathway: utilizing the LNCaP line with the commonly-occurring loss of PTEN [*e.g.,* LNCap] [11]

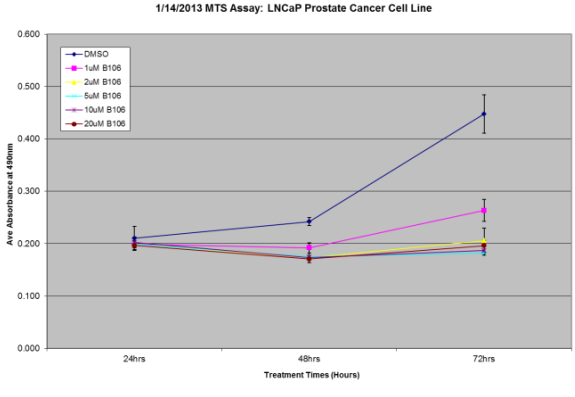
Progress:

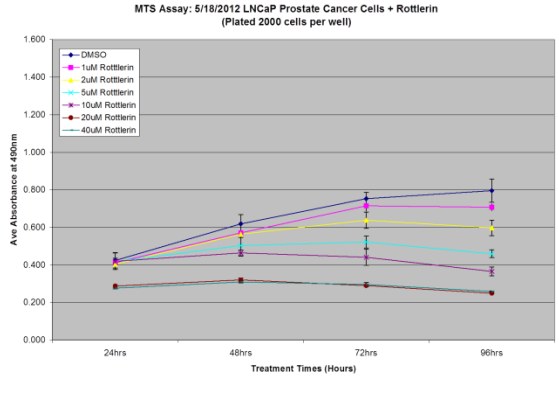
We have tested our lead 4th generation inhibitor (W101), the most active 3rd generation inhibitor (B106), and our first generation compound (LC-I/rottlerin) and against a prostate cancer cell line with activation of PI_3K pathway (LNCaP). Examples of these studies are shown below (**Fig. 22**).

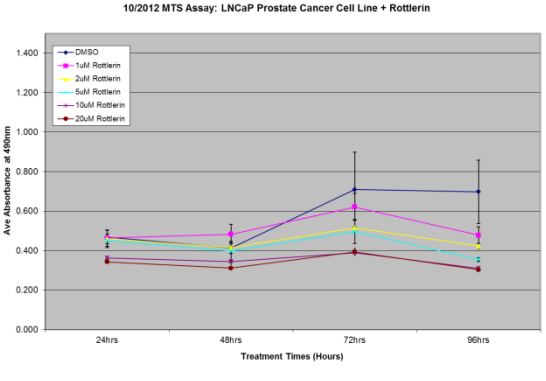
Approach:

MTS Assay: LNCaP cells were treated with W101, Rottlerin or B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.









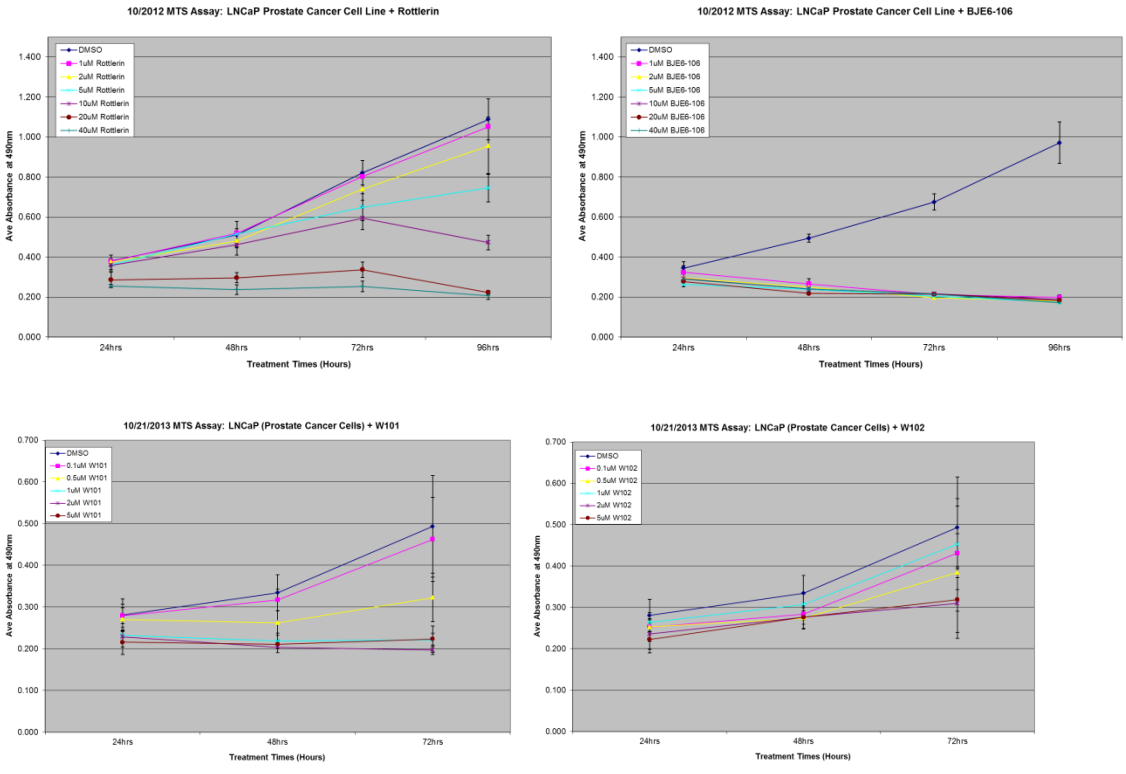


Figure 22

Results: IC_{50} for W101 and B106 were consistently < 1uM, whereas IC_{50} for rottlerin ranged from 6-10 uM (**Fig. 22**).

Interpretation: The fourth generation PKCδ inhibitor W101, and the third generation PKCδ inhibitor B106 were consistently and substantially more potent at inducing cytotoxic/cytostatic effects against this PTEN-mutant (PI_3K pathway-activated) prostate cancer cell line greater than LC-1/rottlerin.

Task 2b) MEK-ERK Pathway: Human prostate cancer cell line CWR22Rv1 has constitutive, aberrant activation of the MEK-ERK signaling pathway, with wild type PTEN and PI_3K signaling

Progress: The analysis of effects of PKCδ inhibitors on CWR22Rv1 is complete. This line was be tested for susceptibility to PKCδ inhibition by 3rd and 4th generation small molecule inhibitors, with assay of cell numbers at specific intervals by MTT assay (**Fig. 23).**

Interpretation: The fourth generation PKCδ inhibitor W101, and the third generation PKCδ inhibitor B106 were consistently and substantially more potent at inducing cytotoxic/cytostatic effects against this prostate cancer cell line with aberrant activation of the MEK-ERK signaling pathway than LC-1/rottlerin.

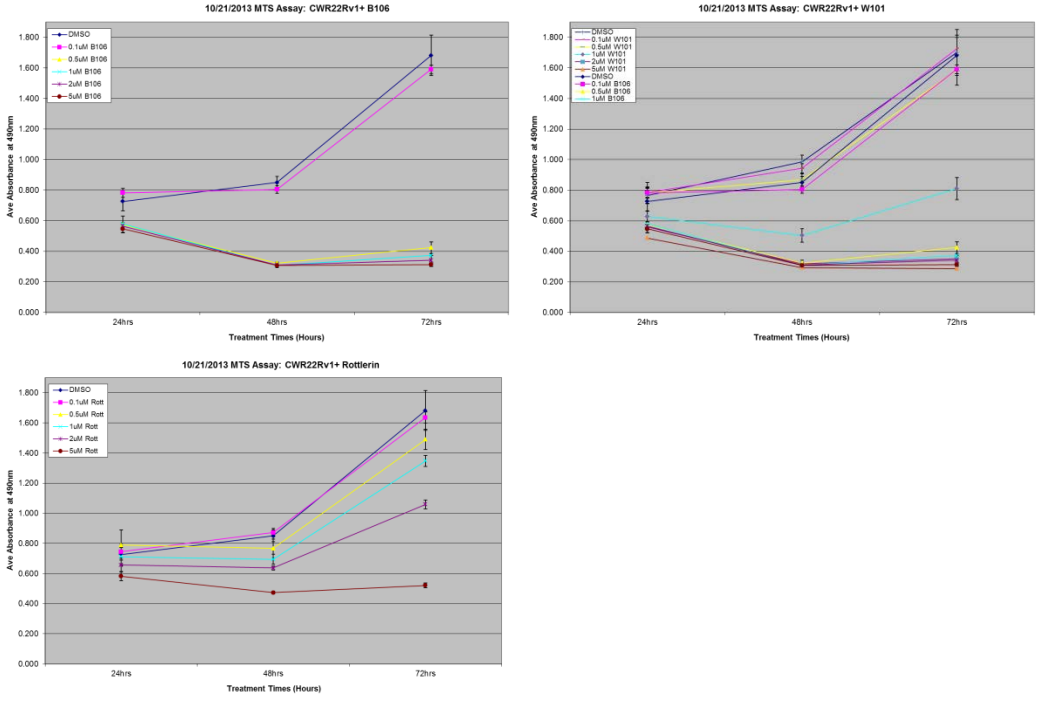


Figure 23

We have also demonstrated that activation of the MEK-ERK pathway is sufficient to sensitize cells to PKCδ inhibitors. In these studies we transformed the "normal" human epithelial cell line MCF 10A to constitutively activate MEK-ERK (MCF 10C, or "M3").

***Methods*:** Cells were treated with vehicle or various concentrations of PKCδ inhibitors and MTS assays to assess cell number were done at 24, 48 and 72 h of exposure.

***Results*:** MCF 10A cells are relatively insensitive to PKCδ inhibition, whereas the MCF 10C (M3) cells have become very susceptible (**Fig. 24**) (published in Chen, et al, 2014 [1]).

For MCF 10A, IC_{50} for B106 was consistently >> 20 uM, and IC_{50} for rottlerin was also >> 20 uM at all timepoints. In contrast, for MCF 10C, at 72 hrs, IC_{50} for B106 was ~ 0.1 uM, and IC_{50} for rottlerin was also ~ 1.0 uM.

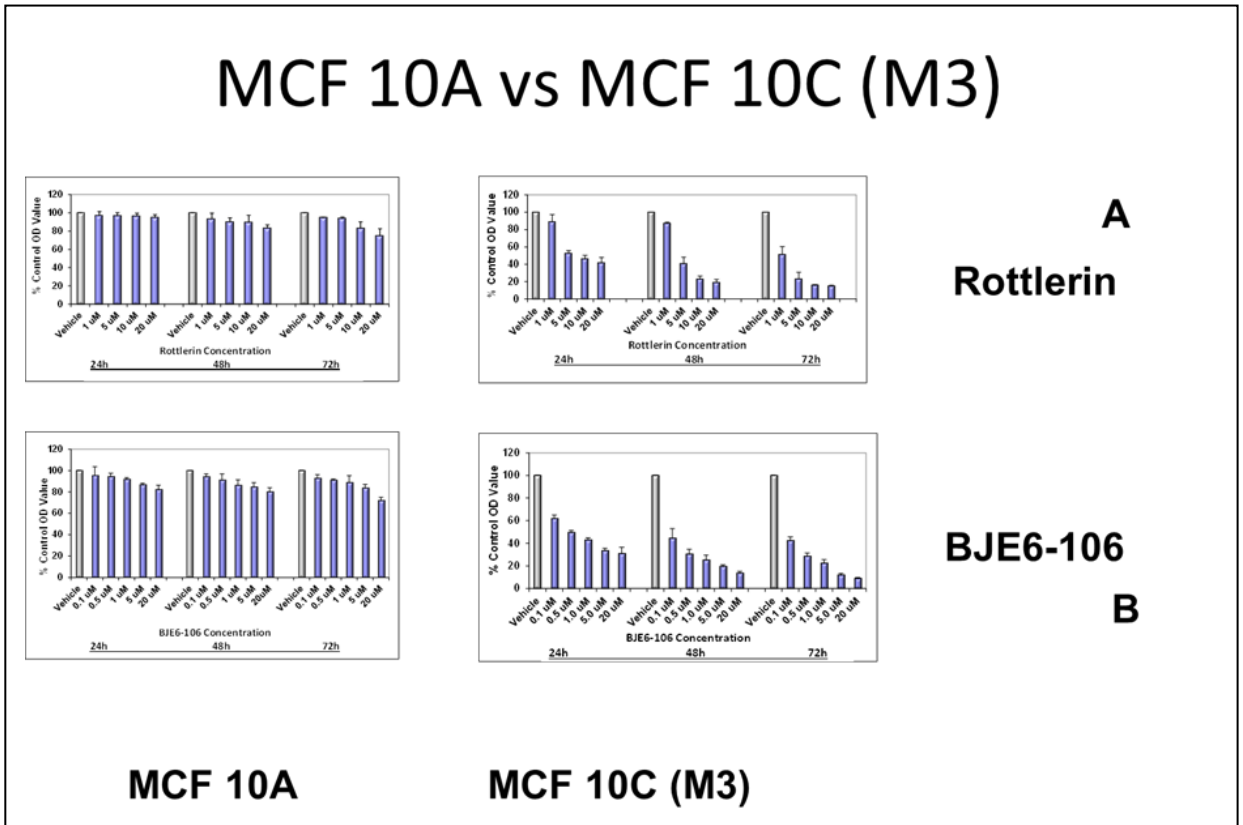
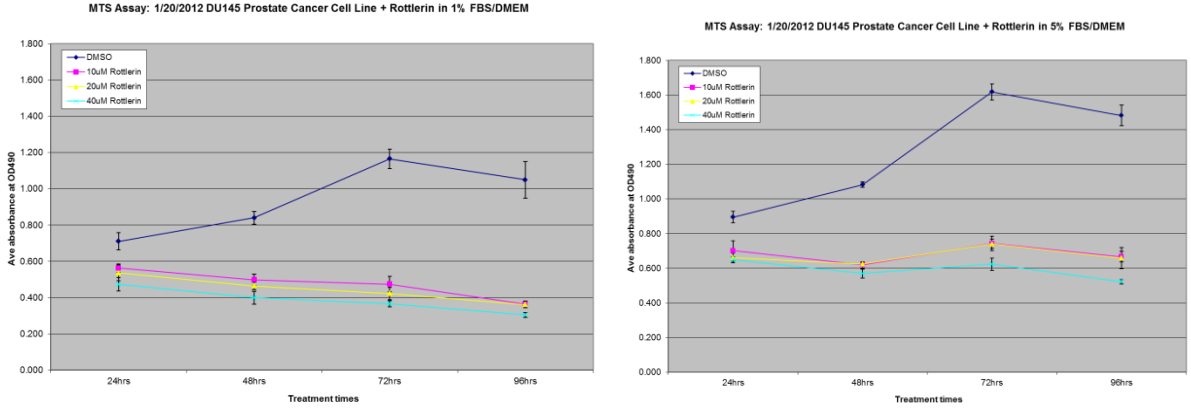


Fig. 24: (published in Chen, et al, 2014 [1,2]).

Interpretation: Constitutive activation of MEK/ERK is sufficient to renders cells sensitive to PKCδ inhibitors.

Whether cancer cells need to be proliferating to become susceptible to PKCδ inhibition was studied in a system in which proliferation was slowed by serum deprivation.

Approach: Testing effects of growth rate on sensitivity to PKCδ inhibitors over the 96 h of treatment. Cells were grown in 1%, 5% or 10% serum conditions, and exposed to rottlerin or vehicle. MTS assay was performed at 24, 49, 72 and 96 h.



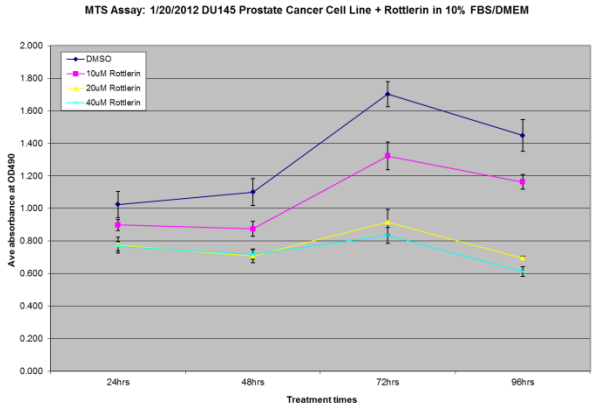


Fig 25. DU145 grown in reduced serum.

***Results*:** Cell proliferation was slowed by PKCδ inhibitors even more efficiently when cells were proliferating slowly (1% or 5% serum), compared to normal culture growth conditions (10% serum) (**Fig. 25**).

Interpretation: Rate of proliferation does not alter susceptibility of human prostate tumor cells to PKCδ inhibitors.

TASK 3: Test the ability of PKCδ inhibitors to induce selective cytotoxicity in human prostate cancer stem cells.

Human prostate cancer stem cells (PrCSC: CD44+, CD133+, SSEA3/4+, Oct4+, alkaline phosphatase+, aldehyde dehydrogenase+, and telomerase+) were purchased from Celprogen (San Pedro, CA), cultured under conditions which maintain their undifferentiated state, and tested for their susceptibility to PKCδ knockdown by siRNA, or PKCδ inhibition by a small molecule inhibitor, with assay of cell numbers at 48 hrs by MTT assay.

Status: COMPLETED

Progress:

We first demonstrated that prostate cancer stem cells (CSC) are susceptible to PKCδ suppression using siRNA (shown in prior progress report) (**Fig. 26**).

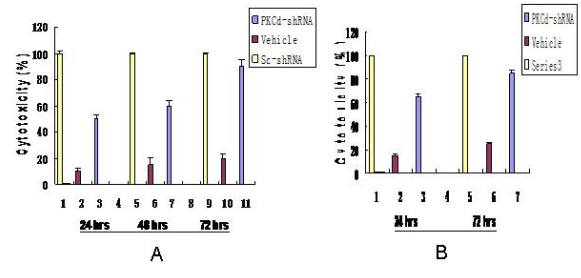


Figure 2. Cytotoxic effects of PKC δ-knockdown by shRNA-lentivirus on human pancreatic and prostate cancer stem cells. Pancreatic CSC **(A)** and prostate CSC **(B)** were grown and infected with PKC δ-shRNA lentivirus or scrambled shRNA lentivirus (Vector) or vehicle controls (Vehicle). Cell cytotoxicity was evaluated by LDH-release assay and showed that LDH release/cytotoxicity were detected within 24 hours of exposure to the PKC δ shRNA, and this release increased as a function of time in both cancer stem cells.

Fig. 26

We then tested LC-1 and 3rd generation PKCδ-inhibitory compounds on prostate cancer CSCs.

Much of this work on Prostate Cancer Stem Cells has been published (Chen, et al, 2014 [1]), so we will primarily summarize the results and refer to the appended publication.

***Methods*:** Primary human prostate cancer stem cells were treated with vehicle or various concentrations of PKCδ inhibitors and MTS assays to assess cell number were done at 24, 48 and 72 h of exposure. Examples of such studies are shown below (**Fig. 27-28).**

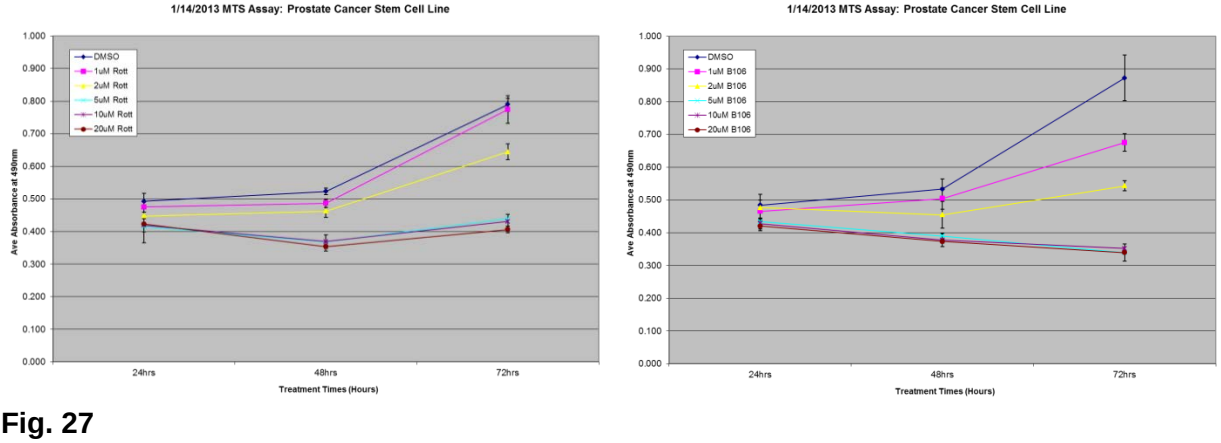


Fig. 27

***Results*:** Primary human prostate cancer stem cells were sensitive to PKCδ inhibition (**Fig. 27-29**).

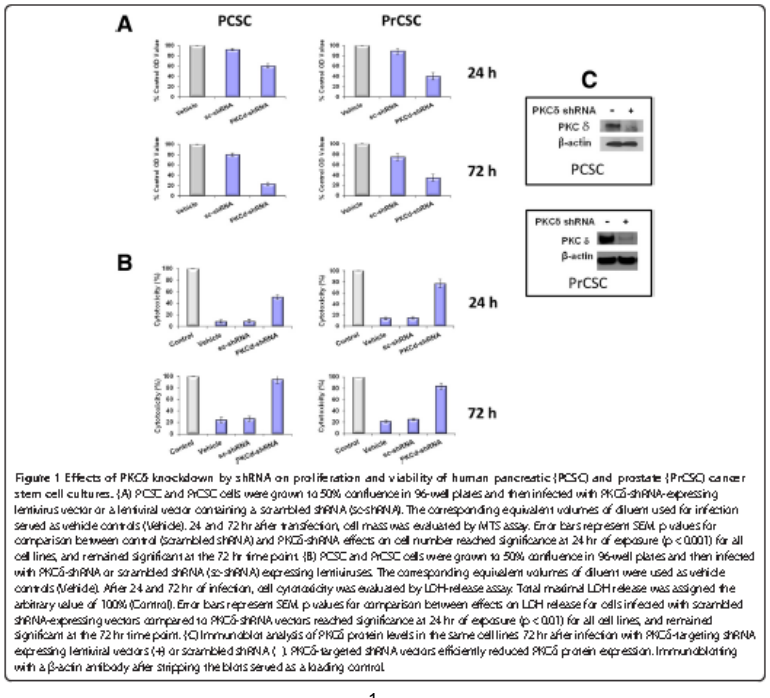


Figure 1 Effects of PKCδ knockdown by shRNA on proliferation and viability of human pancreatic (PCSC) and prostate (PrCSC) cancer stem cell cultures. (A) PCSC and PrCSC cells were grown to 50% confluence in 96-well plates and then infected with PKCδ-shRNA-expressing lentivirus vector or a lentiviral vector containing a scrambled shRNA (sc-shRNA). The corresponding equivalent volumes of diluent used for infection served as vehicle controls (Vehicle). 24 and 72 hr after transfection, cell mass was evaluated by MTS assay. Error bars represent SEM. p values for comparison between control (scrambled shRNA) and PKCδ-shRNA effects on cell number reached significance at 24 hr of exposure ($p < 0.001$) for all cell lines, and remained significant at the 72 hr time point. (B) PCSC and PrCSC cells were grown to 50% confluence in 96-well plates and then infected with PKCδ-shRNA or scrambled shRNA (sc-shRNA) expressing lentiviruses. The corresponding equivalent volumes of diluent were used as vehicle controls (Vehicle). After 24 and 72 hr of infection, cell cytotoxicity was evaluated by LDH-release assay. Total maximal LDH release was assigned the arbitrary value of 100% (Control). Error bars represent SEM. p values for comparison between effects on LDH release for cells infected with scrambled shRNA-expressing vectors compared to PKCδ-shRNA vectors reached significance at 24 hr of exposure ($p < 0.01$) for all cell lines, and remained significant at the 72 hr time point. (C) Immunoblot analysis of PKCδ protein levels in the same cell lines 72 hr after infection with PKCδ-targeting shRNA expressing lentiviral vectors (+) or scrambled shRNA (-). PKCδ-targeted shRNA vectors efficiently reduced PKCδ protein expression. Immunoblotting with a β-actin antibody after stripping the blots served as a loading control.

Fig. 28: From Chen, et al, 2014 [1]

The sensitivity of human cancer stem cell cultures to inhibition of PKCδ was first examined using shRNA methodology to specifically and selectively knockdown transcripts for the isozyme. Cell cultures derived from a primary human pancreatic adenocarcinoma (PCSC) and from a primary human prostate adenocarcinoma (PrCSC), isolated by tumor spheroid formation, were studied. These cells were characterized as "stem-like" by a number of criteria. The PCSC and the PrCSC cultures were $CD44^+$, $CD133^+$, $Nanog^+$, $Sox2^+$, aldehyde $dehydrogenase^+$, and $telomerase^+$. The PCSC cultures were also $Nestin^+$. Both cell types were tumorigenic at <1000 cells in xenograft assays in SCID mice, and also formed tumor spheroids at high efficiency. Lentiviral vectors expressing a PKCδ-specific shRNA (PKCδ-shRNA) or a scrambled shRNA (sc-shRNA) were used to deplete PKCδ levels in the cells. Knockdown of PKCδ by shRNA was growth-inhibitory in both the human prostate (PrCSC) and pancreatic (PCSC) cancer stem cells, with significant effects observed at early as 24 hr after infection, and progressing up to 72 hr (**Fig. 28A**). The non-targeted lentiviral vector (sc-shRNA) generated modest but reproducible effects on cell growth over time, as we have observed in prior reports.[8,9,37] Cytotoxic effects of PKCδ depletion on the PCSC and PrCSC cultures were assessed by quantitating release of cellular LDH. Significant cytotoxicity was elicited by the PKCδ-specific shRNA as early as 24 hr after infection, with LDH release approaching the maximum possible levels by 72 hr. The effects of the scrambled shRNA on LDH release did not differ from those of the infection vehicle alone at any time point (**Fig. 28B**). Efficient knockdown of the PKCδ isozyme was verified by immunoblotting (**Fig. 28C**).

Exposure of PCSC and PrCSC cultures to rottlerin produced a significant dose-dependent inhibition of proliferation as early as 24 hr after exposure (**Fig. 29A**). Similarly, rottlerin induced cytotoxicity in both CSC cultures in a dose-dependent fashion, as assessed by LDH release (**Fig. 29B**). The duration of PKCδ inhibition required to irreversibly prevent CSC proliferation was next assessed. Exposure to rottlerin efficiently decreased the clonogenic capacity of PCSC. Eighteen hr of exposure to rottlerin, followed by washout, was sufficient to decrease the clonogenic capacity of PCSC by 40%, and increasing the duration of the exposure to 48 hr reduced the clonogenic potential by more than 90% (**Fig. 29C**). KAM1 induced cytotoxicity as assessed by LDH release in a dose-dependent fashion in both PCSC and PrCSC cultures at concentrations as low as 2.5 μM (PCSC) and 5 μM (PrCSC) (**Fig. 29E**).

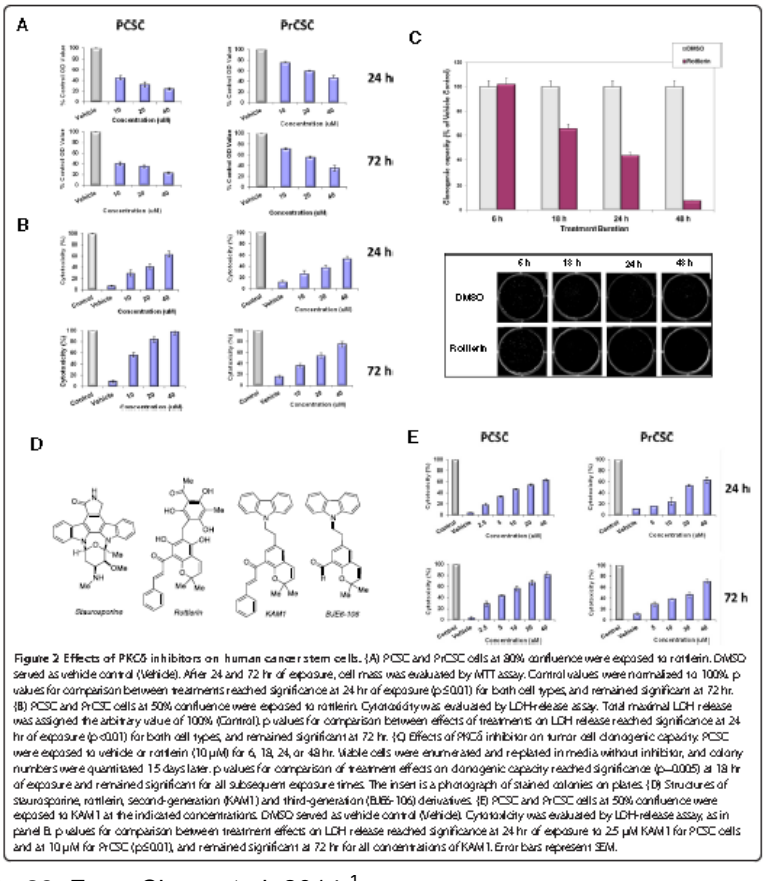
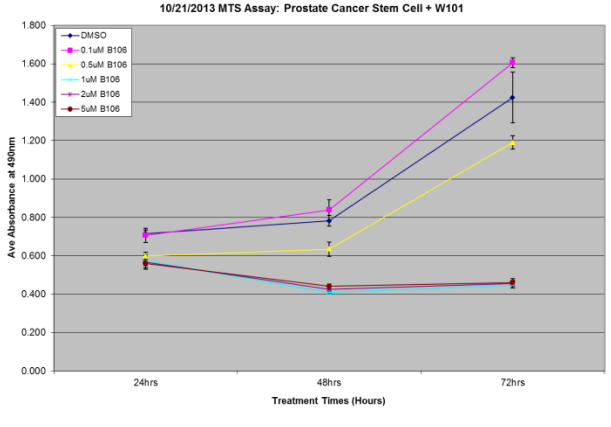
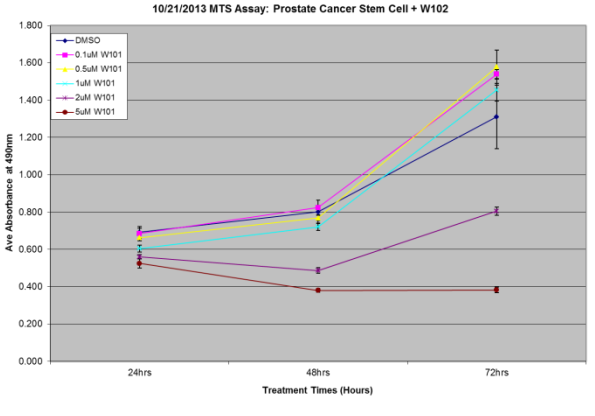


Figure 2 Effects of PKCδ inhibitors on human cancer stem cells. (A) PCSC and PrCSC cells at 80% confluence were exposed to rottlerin. DMSO served as vehicle control (Vehicle). After 24 and 72 hr of exposure, cell mass was evaluated by MTT assay. Control values were normalized to 100% p values for comparison between treatments reached significance at 24 hr of exposure ($p \leq 0.01$) for both cell types, and remained significant at 72 hr. (B) PCSC and PrCSC cells at 50% confluence were exposed to rottlerin. Cytotoxicity was evaluated by LDH-release assay. Total maximal LDH release was assigned the arbitrary value of 100% (Control). p values for comparison between effects of treatments on LDH release reached significance at 24 hr of exposure ($p < 0.01$) for both cell types, and remained significant at 72 hr. (C) Effects of PKCδ inhibitor on tumor cell clonogenic capacity. PCSC were exposed to vehicle or rottlerin (10 μM) for 6, 18, 24, or 48 hr. Viable cells were enumerated and replated in media without inhibitor, and colony numbers were quantitated 15 days later. p values for comparison of treatment effects on clonogenic capacity reached significance ($p = 0.005$) at 18 hr of exposure and remained significant for all subsequent exposure times. The insert is a photograph of stained colonies on plates. (D) Structures of staurosporine, rottlerin, second-generation (KAM1) and third-generation (BJE6-106) derivatives. (E) PCSC and PrCSC cells at 50% confluence were exposed to KAM1 at the indicated concentrations. DMSO served as vehicle control (Vehicle). Cytotoxicity was evaluated by LDH-release assay, as in panel B. p values for comparison between treatment effects on LDH release reached significance at 24 hr of exposure to 2.5 μM KAM1 for PCSC cells and at 10 μM for PrCSC ($p \leq 0.01$), and remained significant at 72 hr for all concentrations of KAM1. Error bars represent SEM.

Fig 29. From Chen, et al, 2014 [1]

***Methods*:** Primary human prostate cancer stem cells were also treated with vehicle or various concentrations of 4th generation PKCδ inhibitors and MTS assays to assess cell number were done at 24, 48 and 72 h of exposure. Examples of such studies are shown below (**Fig. 29).**





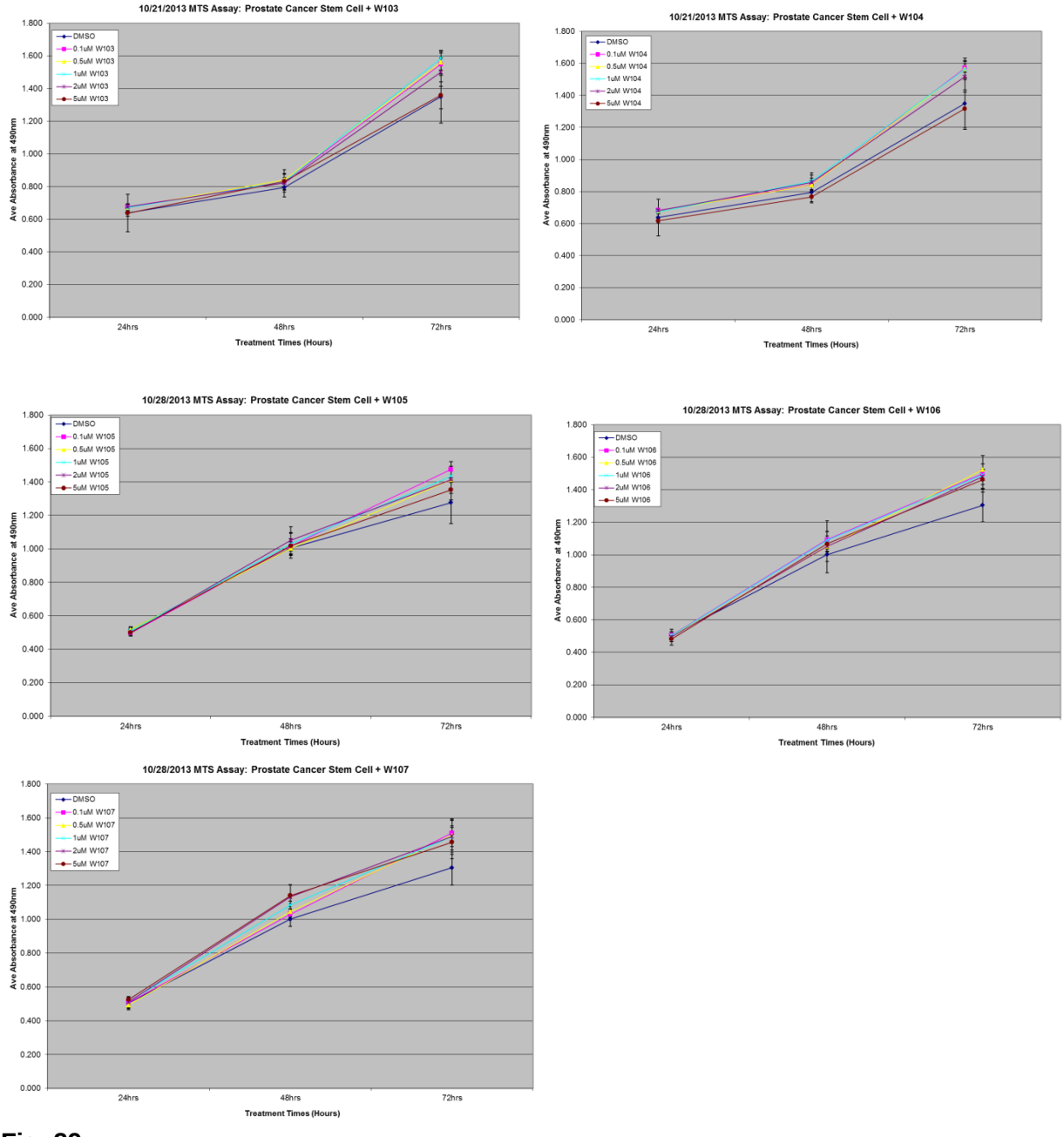


Fig. 29

***Results*:** Primary human prostate cancer stem cells are sensitive to 4th generation PKCδ inhibitor compounds W101 and W102. (**Fig. 28**). IC_{50} for W101 was ~ 1 μM.

Cancer Stem Cells are thought to be characterized by a slow proliferation rate, or dormancy in many tumors. Whether prostate cancer stem cells need to be proliferating to become susceptible to PKCδ inhibition was studied in a system in which proliferation was slowed by serum deprivation.

***Approach*:** Testing effects of growth rate of prostate cancer stem cells on sensitivity to PKCδ inhibitors over the 96 h of treatment. Cells were grown in 1%, or 5% serum conditions, and exposed to rottlerin or vehicle. MTS assay was performed at 24, 49, 72 and 96 h.

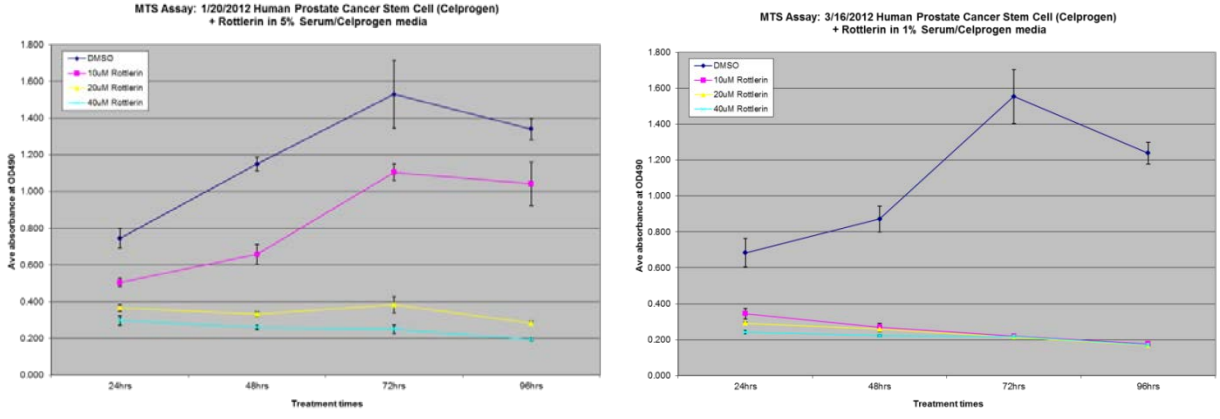


Fig 30. Human Prostate Cancer stem cells grown in reduced serum.

***Results*:** Cytotoxicity was induced by PKCδ inhibitors efficiently when cells were proliferating slowly (1% or 5% serum), compared to normal culture growth conditions (10% serum) (**Fig. 30**).

Interpretation: Rate of proliferation does not alter susceptibility of human prostate cancer stem cells to PKCδ inhibitors.

CSC exhibit a spectrum of biological/functional, biochemical, and molecular features that are consistent with a stem-like phenotype, including growth as non-adherent spheres (clonogenic potential), superior (tumorigenic) ability to form a new tumor in an *in vivo* xenograft assays, unlimited self-renewal, and the capacity for multipotency and lineage-specific differentiation.[38-42] In particular, CSCs are able to form colonies from a single cell more efficiently than their progeny[43] and to grow as spheres (tumor spheres) in non-adherent, serum-free culture conditions.[44] Sphere formation in non-adherent cultures has been used as a surrogate *in vitro* method for detecting CSCs from primary human tumors,[45-49] and to purify a subpopulation of CSC-like cells from tumor cell cultures.

Our first objective was to determine if human prostate cancer cell lines can form tumorspheres.

***Methods*:** Stem Cell Technologies Technical Bulletin, Tumorsphere Culture of Human Breast Cancer Cell Lines

1. Plated cell lines in complete MammoCult Media (Stem Cell Technologies) in 6well Ultra Low Attachment plates (Corning) at 20,000 cells/well.
a. DU145, 10%FBS/DMEM (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 10.
b. LNCaP, 10%FBS/RPMI 1640 (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 11.
c. PC-3, 10%FBS/DMEM (Life Technologies/MediaTech) complete with Pen/Strep (MediaTech), L-Glutamine (MediaTech), HEPES (Fisher), passage 7.

d. pZ-HPV-7, Keratinocyte-serum free media, passage 8.
e. Representative photos were taken at 3 days (**Fig. 30**) and 6 days (**Fig. 31**).
2. Quantitation method by transferring 6 well TS and media to microcentrifuge tube; rapid spin down; aspirate media; gently resuspend TS with 1ml pipette; transferred to 96well non-sterile, assay plate; drew cross hairs under well to section quadrants for counting.

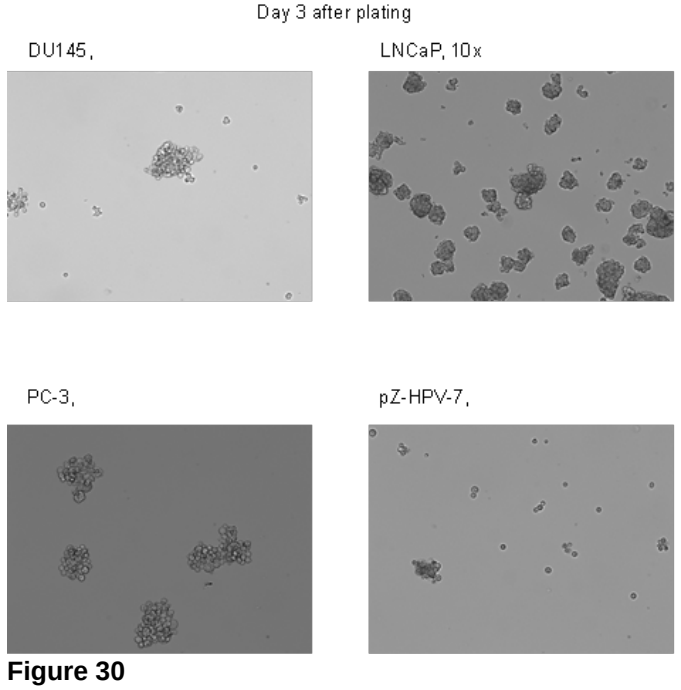


Figure 30

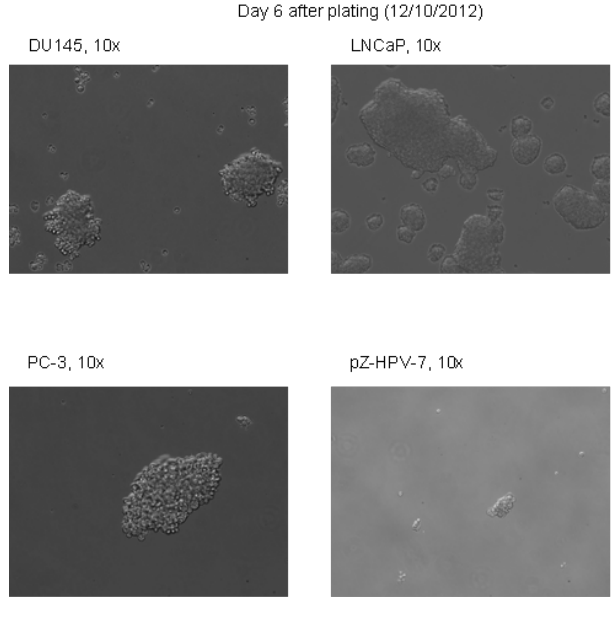


Figure 31

Results: The human prostate tumor cell lines (DU-145, PC3, LNCaP) efficiently formed spheres at a frequency of about 2-5% of input cells (**Figs. 30-31**). The non-tumorigenic pZ-HPV-7 line did not.

We next determined whether 1st and 3rd generation PKCδ inhibitors could inhibit the formation of tumor spheres by the CSC subpopulation.

Methods: Prostate cancer cells were plated and grown in the presence of rottlerin or B106 at the indicated concentrations, and sphere numbers quantitated.

Results: B106 at 1 and 5 uM, and Rottlerin at 10 uM were very efficient at inhibiting the formation of tumorspheres (**Fig. 32**). B106 at 0.5-1.0 μM and rottlerin at 10 μM efficiently inhibited the growth of tumor spheroids generated from the prostate cancer cell lines (DU145, >98% inhibition, $p<0.001$; and PC3, >96% inhibition, $p < 0.001$). Chen, et al, 2014 [1]

Interpretation: PKCδ inhibitors efficiently inhibit the ability of prostate CSC from proliferating and forming tumors.

TASK 4: Test this Ras-targeted approach in an *in vivo* model of human prostate carcinoma.

Progress: Not yet successfully completed.

This task has been initiated, but technical problems have prevented its successful completion to date. 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 prostate carcinoma. A xenograft model has been employed, utilizing a human prostate carcinoma cell line with aberrantly-activated Ras-signaling (PC3). Two cohorts of 8 immunodeficient (nu/nu) mice each were implanted with a xenograft, one treated with vehicle control (100% DMSO) and one given the B106 PKCδ inhibitor at the MTD, given i.p. in 100% DMSO. Tumor growth was serially quantitated.

Results: There was no statistically-significant effect of B106 on tumor growth compared to vehicle (DMSO) controls (**Fig. 32**).

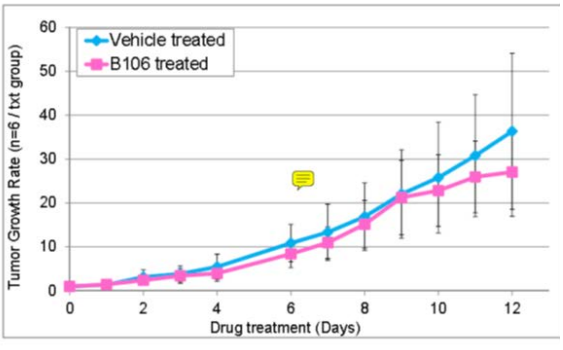


Fig. 32

Interpretation: This result was disappointing, given the high potency of B106 against prostate tumor cell lines in tissue culture. We believe at this time however that this result is due to poor bioavailability of the drug in this experiment. B106 is so hydrophobic that it must be given in 100% DMSO, and precipitates whenever any aqueous solvent is added, including ethanol. The drug appeared to precipitate when given i.p., and therefore little if any reached the circulation or tumor.

We have pursued two different routes to improve the bioavailability of the drug: 1) using microparticle encapsidation techniques; 2) modifications to improve hydrophilicity.

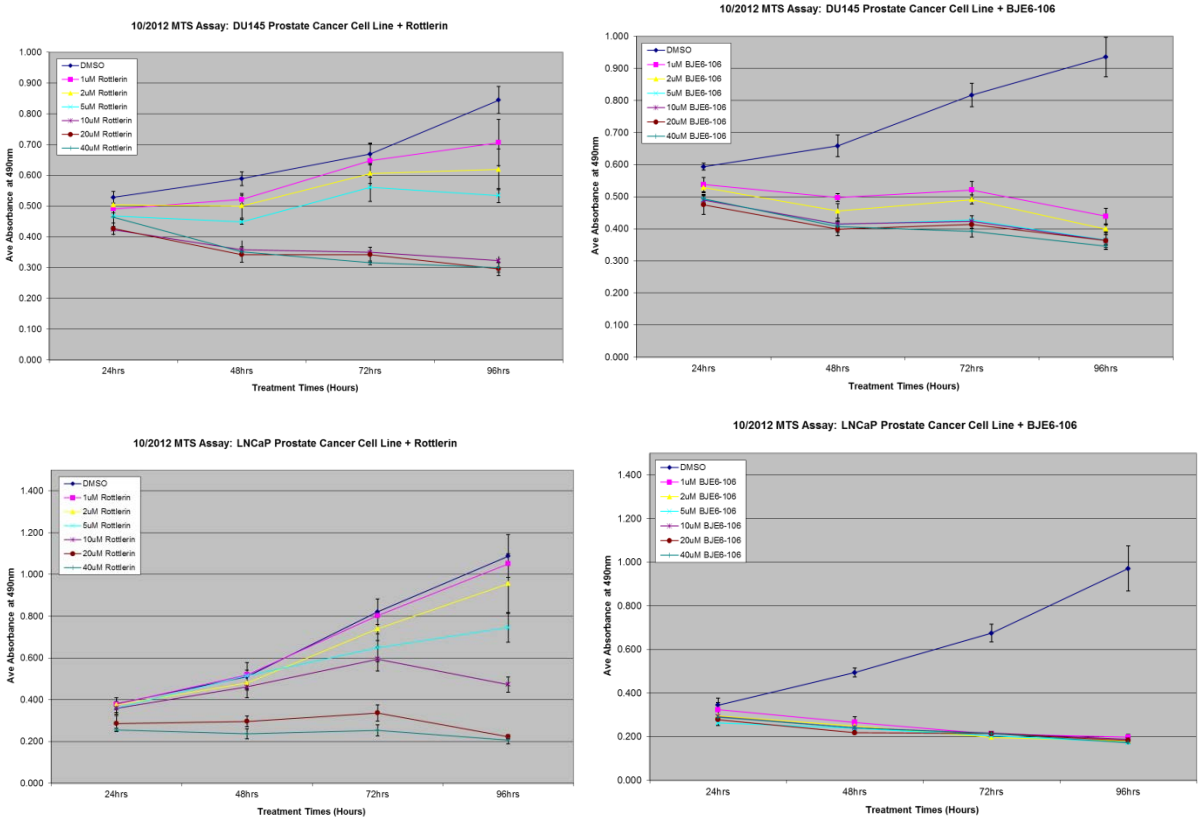
Microparticle Encapsidation:

Approach:

We have used proprietary liposome technology to bind the hydrophobic B106 into a lipid matrix.

MTS Assay: DU145 or LNCaP cells were treated with Rottlerin or liposomal B106 at the indicated concentrations for 96 hrs. MTS assay was then carried to quantitate cell growth.

Fig. 33. New formulation. MTT assay



Results: The LNCaP and DU145 cells were sensitive to rottlerin, as expected, and to the liposomal B106, with IC_{50}s in the range we found for the non-liposomal B106. IC_{50} for liposomal B106 was consistently < 1uM, whereas IC_{50} for rottlerin ranged from 6-10 uM (**Fig. 33**).

Interpretation:

Liposomal encapsulated B106 remains cytotoxic to prostate cancer cells in culture. This type of "packaging" may be useful for *in vivo* delivery of the drug. However, manufacturing enough of the encapsulated drug to give to an animal is not possible. The encapsulated material is not stable and must be prepared fresh before each injection, making animal studies impractical.

We have also tried emulsifying B106 in peanut oil, without any effect in animal studies.

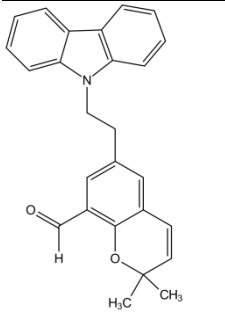
Furthermore, we have solubilized B106 in the vehicle Cremaphor, but found that the cremaphor vehicle alone was so toxic when given in the volumes needed to solubilize the drug, that xenograft experiments could not be carried out.

Chemical modifications of B106 to improve drug-like properties.

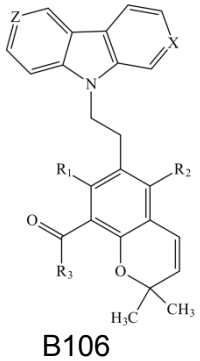
We have characterized our 4th generation inhibitors in vitro and in tissue culture, and have selected W101 as the most potent. W101 is somewhat more soluble than B106. We are currently manufacturing an ultrapure lot to use in vivo in xenograft studies. Furthermore, have

designed a generation of molecules beyond the 4th generation (W compounds) with modifications made to improve their solubility and metabolic stability. The proposed modifications are shown in **Figure 34**. These will be synthesized by our medicinal chemist collaborators using the synthetic approaches noted in **Figure 35**. We will start by simply adding more polar groups to the B106 scaffold, which is thus far the most promising analog. Thus, as shown in Figure 19, $R_1$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. Space does not permit a detailed description of the synthetic plan but it can be said that these new 5th generation analogs do not pose a significant synthetic challenge and are well within the expertise of our consult, Professor Robert Williams, and should be amenable to the basic synthetic chemistry platform that was developed to make KAM1 (Figure 35). Single substitutions will be evaluated at first, and then combinations of substitutions on the B106 core will be prepared. A total of 8-12 compounds 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.

Figure 34. Proposed modifications to B106 to Explore Structure Activity Relationships and Improve Pharmaceutical Properties

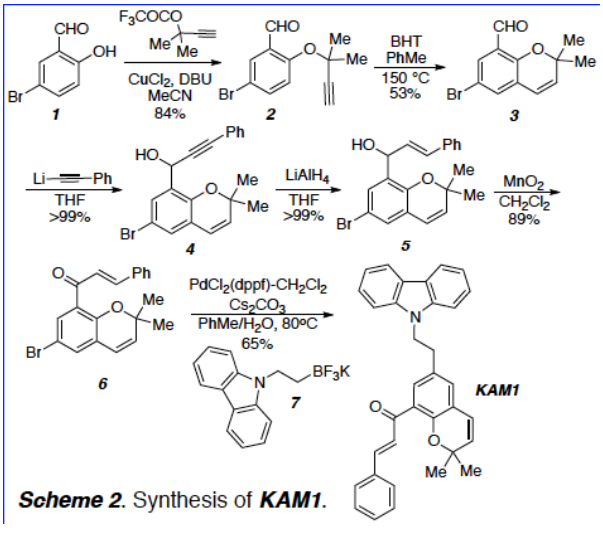


R_1; R_2 = H or OH
X; Z = N or CH
R_3 = H, cinnamoyl, other R



B106 Proposed Modifications

Figure 35. Synthetic scheme used for KAM1



Scheme 2. Synthesis of ***KAM1***.

The potency and selectivity of the new analogs will initially be evaluated for PKCδ and PKCδ inhibitory activity using recombinant PKCα or PKCδ (Invitrogen) and the K-lyte Kinase Assays (Invitrogen) with a 'PKC kinase-specific' peptide substrate, as we have described.[1,2] For molecules that look promising (*e.g.,* low nM potency and >1000x selectivity vs. PKCα, additional closely related kinases will be evaluated including PKCβI, PKCβII PKCγ, PKCε, PKCη PKCθ, PKCζ and PKCι. The molecules with the optimal potency and selectivity will be further characterized. Criteria for advancement include at least 1000 fold selectivity versus PKCα,

which is important in many cellular processes and is a fundamental regulator of cardiac contractility and Ca^{2+} 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 (PKCβI, PKCβII PKCγ, PKCε, PKCη PKCθ, PKCζ and PKCι). 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 PKCγ (important in neuronal function) and PKCε (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 PKCα, 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 and 4th 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.

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

- Demonstrated the sensitivity human prostate cancers to PKCδ inhibition
- Showed activity of specific PKCδ inhibition against human prostate cancer *stem* cells
- Designed and synthesized 36 new 3rd gen compounds as PKCδ inhibitors
- Tested the activity of these 36 new 3rd gen compounds against PKCδ and PKCα
- Established MTD for our lead 3rd gen compound
- Determined the duration of exposure to PKCδ inhibitor drug necessary to achieve maximal cytotoxicity
- Developed a 4th generation of PKCδ inhibitors through pharmacophore modeling and SAR
- Demonstrated that our lead 3rd and 4th generation compounds (B106 and W101) have 5-10 greater potency in inducing cytotoxicity against a panel of human prostate cancer cells than LC-1.
- Demonstrated that our lead 3rd generation compounds (B106 and W101) are relatively non-toxic to "normal" human epithelial and primary human endothelial cells in culture.
- Demonstrated that our lead 3rd generation compound (B106) is not toxic when infused directly into the skin of a mouse over 7 days.
- Demonstrated that targeted inhibition of PKCδ by our 3rd and 4th generation compounds blocks TGFβ-induced fibrosis.
- Demonstrated that targeted inhibition of PKCδ by our 3rd and 4th generation compounds blocks TGFβ-induced migration.
- Demonstrated that targeted inhibition of PKCδ by our 3rd and 4th generation compounds blocks TGFβ-induced EndoMT.
- Demonstrated that targeted inhibition of PKCδ by our 3rd and 4th generation compounds blocks TGFβ-induced myofibroblast formation.
- Demonstrated that rapid cell proliferation is not necessary for tumor susceptibility to PKCδ inhibitors.
- Demonstrated that our lead 3rd and 4th generation compounds (B106 and W101) have 5-10 greater potency than LC-1 in inducing cytotoxicity against a variety of prostate cancer stem cells (CSCs).
- Demonstrated that our lead 3rd generation compound (B106) can be packaged into liposomes without loss of cytotoxic activity.
- Designed a strategy for synthesis of more hydrophilic analogs of our lead 3rd and 4th generation compounds.

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

Publications:

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

Takashima, A., Chen, Z., 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. 2014. ACS Chemical Biology, *in press*.

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. 2014. BMC Cancer, 14: 90-98.

Patent Applications:

Application No.: 12/282,432
METHOD FOR TREATING CANCERS WITH INCREASED RAS SIGNALING

Application No.: No. PCT/US2013/60683
PKC DELTA INHIBITORS FOR USE AS THERAPEUTICS

CONCLUSION:

In our three years of work, we have made substantial progress. We have succeeded in demonstrating that multiple types of human prostate cancer cells are susceptible to PKCδ inhibition, using siRNA as a "specificity" test, and multiple structurally-distinct small molecule PKCδ inhibitors. These findings validate PKCδ as a target in prostate cancer, and provide proof-of-principle for the use of PKCδ inhibitors as potential therapeutics. Furthermore, we have shown the utility of PKCδ inhibition as a strategy for the elimination of prostate cancer stem cells. We have refined the initial PKCδ inhibitor lead compound now through 4 generations, producing small molecules of increasing potency and PKCδ specificity. This generation is being be optimized for "drug-like" properties, to facilitate moving into *in vivo* testing of tumor xenografts.

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

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APPENDICES: 3 publications

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

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SUPPORTING DATA: included above

EXPERT OPINION

Targeting the RAS oncogene

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Introduction: The Ras proteins (K-Ras, N-Ras, and H-Ras) are GTPases that function as molecular switches for a variety of critical cellular activities and their function is tightly and temporally regulated in normal cells. Oncogenic mutations in the RAS genes, which create constitutively-active Ras proteins, can result in uncontrolled proliferation or survival in tumor cells.

Areas covered: The paper discusses three therapeutic approaches targeting the Ras pathway in cancer: i) Ras itself, ii) Ras downstream pathways, and iii) synthetic lethality. The most adopted approach is targeting Ras downstream signaling, and specifically the PI_3K-AKT-mTOR and Raf-MEK pathways, as they are frequently major oncogenic drivers in cancers with high Ras signaling. Although direct targeting of Ras has not been successful clinically, newer approaches being investigated in preclinical studies, such as RNA interference-based and synthetic lethal approaches, promise great potential for clinical application.

Expert opinion: The challenges of current and emerging therapeutics include the lack of "tumor specificity" and their limitation to those cancers which are "dependent" on aberrant Ras signaling for survival. While the newer approaches have the potential to overcome these limitations, they also highlight the importance of robust preclinical studies and bidirectional translational research for successful clinical development of Ras-related targeted therapies.

Keywords: AKT, MEK, mTOR, nononcogene addiction, PI3K, Raf, Ras, RNAi-based therapy, synthetic lethality, targeted therapy

Expert Opin. Ther. Targets (2013) 17(5):507-531

1. Introduction

The Ras proteins (H-Ras, K-Ras, and N-Ras) are GTPases which regulate signal transduction underlying diverse cellular activities, including proliferation, survival, growth, migration, differentiation or cytoskeletal dynamism. GTP-bound ("on-state") Ras proteins convert extracellular stimuli into intracellular signaling cascades, which eventually evoke changes in cellular activities; this signaling ceases when Ras-bound GTP is hydrolyzed to GDP as the result of another signaling cascade. Thus, in normal cells, Ras proteins function as molecular switches for critical changes in cellular activities, such as cell proliferation and survival, and their proper and tight regulation is indispensable to maintain the homeostasis of cells and, ultimately, the entire organism.

Conversely, uncontrolled activity of the Ras proteins, or the molecular components of their downstream pathways, can result in serious consequences, including cancers and other diseases. Indeed, approximately 30% of human tumors are estimated to harbor activating mutations in one of the three Ras isoforms: KRAS, NRAS, and HRAS [1]. KRAS is most frequently mutated among three isoforms in malignancies; its mutation rate in all tumors is estimated to be 25 – 30% [1]. KRAS mutation is especially prominent in colorectal carcinoma (40 – 45% mutation rate), non-small cell lung cancer (NSCLC) (16 – 40%) and pancreatic ductal carcinoma (69 – 95%) [1]. In contrast, activating mutations of NRAS and HRAS are less common (8% and 3% mutation rate, respectively). Malignant melanomas

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Article highlights.

- The Ras GTPase family proteins regulate critical cellular activities including cell proliferation, differentiation, and survival. Oncogenic mutations of RAS are prominent in many types of cancers with particularly high prevalence and mortality rates. The Ras proteins or the components of their downstream signaling pathways have been studied for pharmacological intervention of aberrant Ras signaling in cancer cells as an anticancer therapy.
- Direct targeting of the Ras proteins has been challenging. For example, the FTIs/GGTIs have failed in part due to their lack of target (Ras protein) specificity, and antisense oligonucleotides to Ras have lacked sufficient clinical activity. Newer approaches utilizing RNA interference technology, currently in preclinical studies, have the potential for future clinical application.
- Among the multiple Ras downstream pathways, the Raf-MEK-ERK and PI_3K-AKT-mTOR pathways have been the major focus of drug discovery/development for inhibition of Ras signaling. There are four FDA-approved drugs (Raf inhibitors and mTOR inhibitors), and some promising investigational drugs which are in the late clinical trial phases in this category. The inhibitors in these classes are utilized in those cancer types which are characterized by the existence of aberrantly high Ras signaling.
- One major obstacle to the application of Ras-effector inhibitors is the emergence of drug resistance. Some drugs demonstrate remarkable clinical activity initially in treatment, but tumors eventually and inevitably relapse due to the development of resistance to these drugs. Accumulating evidence suggests that employing combination therapy in the first line of treatment for a simultaneous inhibition of multiple Ras downstream pathways may prevent cancer cells from switching to alternative survival pathways and escaping.
- The synthetic lethal approach identifies synthetic lethal interactors of Ras proteins, whose inhibition is toxic only to those tumor cells with aberrant Ras pathway activity. Although this approach remains in the preclinical phase, it presents the potential to provide treatment options for the cancer types with activating mutations of RAS or high Ras activity which are not addressed by current Ras-targeted therapies.

This box summarizes key points contained in the article.

predominantly harbor NRAS mutations (20 – 30% prevalence) [1]. The activating oncogenic mutations most commonly occur in codons 12, 13 and 61, in the GTPase catalytic domains, identically among the three isoforms. Eighty percent of KRAS mutations are observed in codon 12, whereas NRAS mutations preferentially involve codon 61 (60%) compared to codon 12 (35%) [2]. HRAS mutations are divided almost equally among codon 12 (50%) and codon 61 (40%) [2]. Regardless of isoform type or codon location, all these activating mutations render Ras proteins resistant to GTP hydrolysis (and consequent Ras inactivation) stimulated by GTPase-activating proteins (GAPs). These constitutively-activated oncogenic Ras mutant proteins, therefore, initiate intracellular signaling cascades without the input of extracellular stimuli, resulting in uncontrolled cell proliferation and abnormal cell survival.

2. Ras proteins

Due to the space limitations, this section is focused on the basic background of Ras protein biology and biochemistry, particularly related to the therapeutic interventions to be discussed later. For further details on the biology and biochemistry of the Ras proteins, their activation by upstream signaling pathways, and their downstream signaling pathways, readers should refer to the excellent reviews listed in Refs. [2-7].

2.1 Structure

The two major structural components in Ras proteins are the catalytic domain, called the G domain, and the C-terminal hypervariable region (HVR). The catalytic G domain, which is highly homologous among the three isoforms, contains the phosphate-binding loop (P-loop) and two parts of the nucleotide-binding switch regions (Switch I and Switch II) [2]. All of the frequently mutated amino acid residues (Gly12, Gly13, and Gln61) are located within these motifs, which are critical for Ras catalytic activity. The HVR is the site of post-translational modifications that are required for Ras proteins to be translocated to the plasma membrane. The HVRs of the three isoforms share only 15% homology, and this divergence is proposed to contribute to the functional differences among the isoforms, although has not yet been definitively linked to function [8]. Each Ras isoform undergoes a slightly different post-translational modification process due to the sequence variation in the HVRs, which thereby defines what set of mediator enzymes are allowed to access to the HVR.

To become functionally active, newly-synthesized Ras proteins are subjected to a series of post-translational modifications [9]. After translation in the cytosol, Ras proteins are farnesylated on the cysteine within the "CAAX box" motif, the C-terminal region in the HVRs. This brings immature Ras proteins to the ER, where the CAAX box is truncated by proteolysis and methylated. The final modification, palmitoylation, matures Ras proteins for translocation to the plasma membrane. As Ras cannot be activated without membrane translocation, farnesylation is essential for Ras function and has been intensively studied as a target for potential pharmacological interventions. Studies using farnesyltransferase (FTase) inhibitors (FTIs), however, revealed that K-Ras and N-Ras alternatively can be geranylgeranylated, which is equally capable of facilitating translocation of Ras proteins to the membrane when farnesylation is inhibited by FTIs [10].

Ras proteins anchor in the cytoplasmic membrane via the HVR once they reach the membrane. In some cases, the Ras proteins are bound by Ras-escort proteins in the HVR. These proteins include galactin-1 and galactin-3, which have strong binding affinity to GTP-H-Ras and GTP-K-Ras,

respectively [11]. Ras-escort proteins stabilize the Ras proteins in the GTP-bound (active) state [6]. Disruption of the interaction between these escort proteins and Ras has been exploited as a strategy to modulate aberrant Ras signaling.

2.2 Function

The importance of K-Ras expression during development is illustrated by the embryonic lethality of K-Ras knockout mice, as a result of liver defects and anemia [3]. In contrast, mice with HRAS or NRAS knockouts are completely viable without any obvious phenotypes [3]. Although mouse models do not entirely mimic human tumorigenesis, transgenic and knock-in mouse models provide proof of the physiological contribution of oncogenic Ras proteins to tumorigenesis. Expression of oncogenic H-Ras or K-Ras under tissue-specific promoters induces various types of malignancies in multiple transgenic mouse models [4]. For example, one conditional K-Ras G12D knock-in model produced lung tumors after activation of the oncogenic KRAS gene.

2.3 Proteins controlling Ras

As cell proliferation signaling should be initiated only when it is required for growth, development, or tissue repair, predominantly via an extracellular stimulus (e.g., through receptor tyrosine kinases [RTKs], steroid hormone receptors or G-protein-coupled receptors), the activity of the Ras proteins is normally tightly and temporally controlled in normal cells. For example, upon the arrival of ligands/growth factors to RTKs, the receptors homo- or hetero-dimerize, autophosphorylate each other on specific tyrosine residues and recruit adaptor proteins (e.g., Grb2 or Shc) to their SH2 domain (s), which leads to recruitment of guanine nucleotide exchange factors (GEFs) to the plasma membrane (**Figure 1**). Ras becomes activated when a GEF stimulates dissociation of GDP, allowing rapid replacement by the more abundant GTP. Conformational changes caused by binding of GTP increase the binding affinity of the Ras proteins to their downstream effectors, such as the Raf family proteins or the phosphatidylinositol 3 kinases (PI_3K), which in turn activate a series of kinase chain reactions. Activated Ras is eventually inactivated by hydrolysis of the bound GTP, which is accelerated by GAPs. Because the exchange of GDP and GTP is an extremely slow process in both directions under physiological conditions without catalysis by GAPs and GEFs, the balance between GAP and GEF activities is a crucial regulatory mechanism for Ras activation status (for review of GAPs and GEFs, see [12]).

The aberrant activity of any of the molecules involved in Ras activation can be oncogenic. RTK family members, including epidermal growth factor receptors (EGFRs), HER2/ERBB2 or insulin-like growth factor 1 receptor (IGF1R) are frequently hyperactivated due to overexpression, genetic mutation and/or gene amplification in many types of cancers including lung, colon, breast, ovarian and stomach carcinomas [13].

2.4 Downstream effectors of Ras

The proximal downstream Ras effectors are defined as proteins which have a strong affinity to GTP-Ras, are thereby activated, and initiate a subsequent cascade of signaling [5]. Ras effectors share a characteristic Ras-binding domain (the Ras core effector domain). Among more than 10 reported Ras effectors, Raf and PI_3K and their downstream pathways have been most extensively studied, because of their importance both in the normal physiological setting and in tumorigenesis. Thus, these pathways have been the primary targets of cancer drug discovery and development.

The Raf-MEK-ERK pathway comprises the major part of the mitogen-activated protein kinase (MAPK) pathway system, and the Raf kinases are on the MAPK-kinase-kinase (MAPKKK) tier. The Raf family consists of three isoforms: A-Raf, B-Raf, and C-Raf/Raf-1. B-Raf is the strongest MEK kinase and A-Raf is the weakest MEK activator. A-Raf preferentially activates MEK1, while B-Raf and C-Raf activate both MEK1/2 with equal efficiency [6]. Activation of MEK1/2 by Raf family leads to the activation of the MAPK, ERK. The BRAF gene is mutated in 66% of melanomas and 12% of colorectal cancers, whereas mutations of C-Raf, A-Raf or MEK1/2 are rarely found in any cancer [6,14]. Regardless of the location of the mutation or aberrant activation in cascade, abnormalities in this pathway lead to elevation of phospho (activated)-ERK1/2, as observed in numerous human cancers. Activated ERKs are translocated to the nucleus and activate transcription factors whose target genes include regulators of cell proliferation or cell cycle regulation, or, in some cases, negative feedback regulators of the Raf-MEK-ERK pathway (**Figure 1**).

PI_3Ks convert phosphatidylinositol (4,5)-bisphosphate (PIP-2) to phosphatidylinositol (3,4,5)-triphosphate (PIP-3) by phosphorylation. Although there are three classes of PI_3Ks, class I PI_3Ks have been most studied and are almost exclusively the target of pharmacological PI_3K inhibitors of all classes. PI_3Ks are heterodimeric proteins consisting of one catalytic subunit (isoforms: p110α/PIK3CA, p110β/PIK3CB, p110δ/PIK3CD) and one regulatory subunit. PDK1 is recruited to the membrane by PIP-3, is activated, and phosphorylates AKT at Thr308. There are three AKT isoforms (AKT1/2/3). As AKTs exert either survival or apoptotic signaling, depending on the cellular context, the downstream substrates of AKTs include a wide range of proteins, such as apoptotic regulators (e.g., BAD), transcription factors (e.g., FOXO), and other kinases (e.g., glycogen synthase kinase-3β [GSK3β], tuberous sclerosis 2 [TSC2]) [7]. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase comprised of two types of multikinase complexes. mTOR complex 1 (mTORC1), regulated by TSC2, phosphorylates ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Consequently, mTORC1 plays an important role in translational initiation. mTOR complex 2 (mTORC2) not only lies downstream of AKTs, but also contributes to the activation of

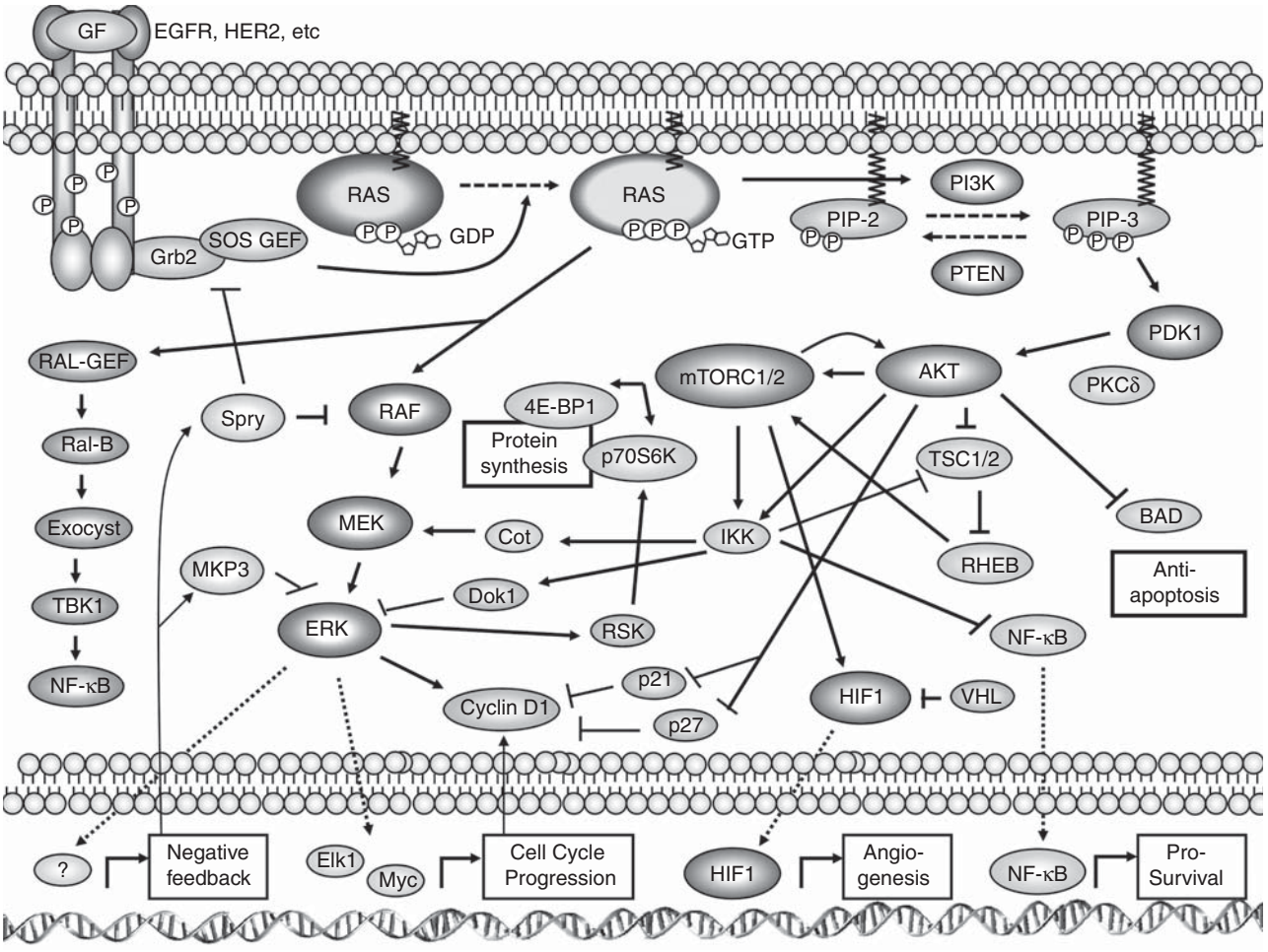

Figure 1. Ras signaling pathways.

4E-BP1: Eukaryotic initiation factor 4E-binding protein 1; Dok1: Docking protein 1; EGFR: Epidermal growth factor receptor; Elk1: E twenty-six (ETS)-like transcription factor 1; GEF: Guanine nucleotide exchange factor; GF: Growth factor; HER2: Human epidermal growth factor receptor 2; HIF1: Hypoxia-inducible factor 1; IKK: Inhibitors of NF-κB (IκB) kinase; MKP3: Mitogen-activated protein kinase (MAPK) phosphatase 3; mTORC: Mammalian target of rapamycin complex; NF-κB: Nuclear Factor-κ (kappa) B; p70S6K: p70 ribosomal S6 kinase; PI3K: Phosphatidylinositol 3 kinase; PDK1: Phosphoinositide-dependent kinase-1; PKCδ: Protein kinase C δ (delta); PIP-2: Phosphatidylinositol (4,5)-bisphosphate; PIP-3: Phosphatidylinositol (3; 4,5)-triphosphate; PTEN: Phosphatase and tensin homolog; Spry: Sprouty (protein family); TSC: Tuberous sclerosis complex; RHEB: Ras homolog enriched in brain; RSK: Ribosomal S6 kinase; TBK1: TANK-binding kinase 1; VHL: Von Hippel–Lindau tumor suppressor.

AKTs by phosphorylating AKTs on Ser473 following initial Thr308 phosphorylation by PDK1 (**Figure 1**) [15].

2.5 The components of Ras signaling provide potential therapeutic targets

Because of its central role in intracellular signal transduction, malignant transformation and progression (including proliferation, migration, morphological changes, and epithelial-mesenchymal transition [EMT]), Ras proteins have been a focus of research in cancer drug discovery and development. "Oncogene addiction" describes a model in which cancer cells are highly dependent on the activity of a single oncogene (despite many other genetic abnormalities) for continued tumor cell proliferation and survival. KRAS "addiction" is among the best known examples [16]. However, it is clear that the presence of a mutated Ras allele in a given tumor does not predict "oncogene addiction." Indeed, tumor types which are uniformly addicted to a single, specific oncogene (i.e., BCR-ABL in chronic myelogenous leukemia), appear to be the exceptions rather than the rule. While targeting Ras proteins or mutant forms of Ras proteins directly became the early strategy, a number of issues have confounded this approach, and the Ras proteins themselves are no longer considered feasible pharmaceutical targets, as will be discussed later. The current most widely-adopted strategy is to target instead the components of Ras downstream signaling pathways, such as the Raf-MEK-ERK or PI_3K-AKT-mTOR pathways. There have already been some notable clinical successes stemming from this approach, and many other drug candidates with better drug properties

and target specificity are under clinical investigation. A newer approach, sometimes termed "synthetic lethality," is to selectively attack cancer cells by targeting another protein, which is independent of the Ras signaling pathway, but upon which cells with mutant Ras expression (tumor cells) are dependent. This state is also sometimes termed "nononcogene addiction." In this approach, the activated, mutated Ras signaling is utilized as a cancer cell marker rather than drug target.

3. Targeting Ras directly

Unlike the case for many kinase inhibitors, targeting the catalytic domain of the Ras proteins is technically challenging, due to the structural characteristics of GTPases [8]. This limitation redirected efforts to directly target Ras proteins into two alternative strategies: i) preventing the expression of Ras proteins; or, ii) blocking the localization of Ras proteins to the plasma membrane where Ras proteins are activated and then function as a molecular switch.

3.1 Inhibiting Ras expression

The first approach utilizes the gene silencing techniques that prevent mRNAs of Ras proteins from being translated. Gene silencing technology utilizes two different methodologies: antisense oligodeoxynucleotides (ODNs), or RNA interference (RNAi). ISIS2503, an antisense ODN against H-Ras, produced selective suppression of H-Ras mRNA and protein in cell culture systems, and showed antitumor activities in mouse xenograft models including a pancreatic carcinoma system [17,18]. In a Phase I trial, ISIS2503 was well tolerated with relatively minor adverse events, although no consistent reduction in H-Ras mRNA levels was observed in patients' peripheral blood lymphocytes [18]. Single-agent Phase II trials in the patients with advanced colorectal cancer, pancreatic cancer and NSCLC did not address clinical activities [19-21]. Phase II trials of ISIS2503 in combination with gemcitabine in advanced pancreatic cancer, with docetaxel in previously treated advanced NSCLC, and with paclitaxel in metastatic breast cancer, failed to demonstrate a significant improvement in response rate and survival rate, or tumor regression, compared to conventional treatment alone [22-24]. The failure of ISIS2503 in human trials can be explained by insufficient recognition of the importance of the genetic background in the diseases targeted. The development of ISIS2503 was based on preclinical studies in which ISIS2503, but not a K-Ras-specific ODN, exhibited antitumor activity, although HRAS was infrequently mutated in the cancer types that were targeted in these clinical trials [17]. The much more frequently mutated K-Ras has also been targeted for potential clinical application; however, the effect of K-Ras antisense ODN on tumor cell growth inhibition appears to be more variable, and unpredictably dependent on cell or ODN types [17,25,26].

An advantage of the more recent RNAi technology is the extraordinary specificity against the target sequence, enabling selective silencing of an oncogenic Ras with a single point mutation, so that treatment could spare normal cells expressing a wild-type Ras (**Figure 2**). Several groups reported that selective knockdown of mutant K-Ras or H-Ras via small interfering RNA (siRNA) induced significant growth inhibition in cell lines of pancreatic cancers, lung cancers, colorectal cancers, and ovarian cancers, and, more encouragingly, in animal models [27-32]. Although RNAi-based therapy has not progressed to human testing in malignant conditions, it appears to have better clinical potential in comparison to antisense ODNs, based on the predicted *in vivo* knockdown efficacy and applicability for K-Ras targeting. Nonetheless, there are very significant challenges in terms of delivery of the RNAi to the local tumor environment. High molecular-weight molecules/drugs like nucleic acids are generally more difficult to deliver effectively, and exogenous RNA could become the target of neutralization by the immune system. Furthermore, some studies have demonstrated that silencing a Ras gene/protein alone may not be sufficient to kill all tumors containing activated Ras, but rather only those tumors in which the activated Ras is critical for the survival of the tumor ("Ras-dependent" tumors). This consideration has led to the alternative concept of exploiting the finding that tumor cells harboring oncogenic RAS mutations may become dependent on other nononcogenic proteins for survival ("nononcogene dependency"). Inhibition or knockdown of this nononcogenic protein can then efficiently induce selective cytotoxicity in the Ras-mutant tumor cells, while sparing normal cells ("synthetic lethality," to be discussed later).

3.2 Inhibiting Ras protein processing/localization

Aside from preventing the translation of Ras proteins by RNAi, an alternative approach to targeting Ras involves the prevention of newly-translated Ras proteins from being activated, by inhibiting the post-translational modification that is necessary to translocate Ras to the plasma membrane, using FTIs. Preclinical studies demonstrated the potency of FTIs, showing efficacy against H-Ras and K-Ras substrates, and tumor growth inhibition *in vitro* and *in vivo* in a number of cancer cell line models [33-35]. To date, two FTIs, tipifarnib and lonafarnib, advanced to Phase III trials; but with little success so far. Phase II trials with tipifarnib produced no responses in the most of the cancers evaluated including metastatic pancreatic cancer, NSCLC, and advanced colon cancer, but some activity in advanced breast cancer and myelodysplastic syndrome (MDS) [36-41]. Multiple Phase III trials of tipifarnib monotherapy in acute myeloid leukemia (AML) and in refractory advanced colorectal cancer, and in combination with gemcitabine in advanced pancreatic cancer, did not produce improvements in overall survival [42-44]. A Phase II/III trial of the combination of tipifarnib and gemtuzumab ozogamicin has been initiated in AML and high-risk MDS; however, the current status of the trial is unknown

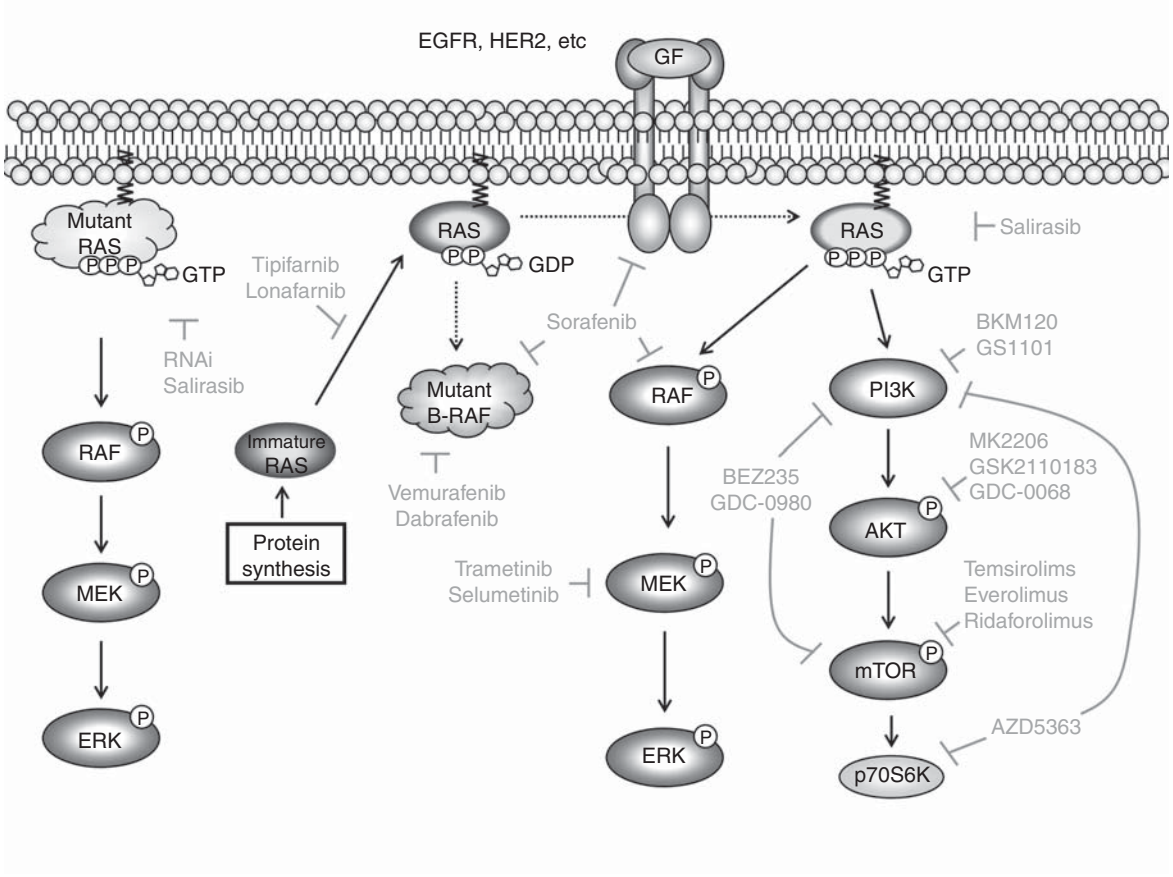

Figure 2. Targeting Ras signaling. Green arrows denote what targets the agents/methodologies described in the text.
EGFR: Epidermal growth factor receptor.

due to withdrawal of gemtuzumab ozogamicin from the market. A Phase III trial of lonafarnib in combination therapy with carboplatin/paclitaxel failed to improve overall survival in advanced or metastatic NSCLC [45].

The disappointing clinical outcomes in most clinical trials testing FTIs were hinted at in some preclinical studies. For example, cell lines with no RAS mutations were also susceptible to FTIs both in the *in vitro* and *in vivo* xenograft models [33,46], suggesting that the drugs did not selectively target oncogenic Ras. Additionally, while H-Ras is an exclusive target of FTases, K-Ras and N-Ras become geranylgeranylated alternatively in the presence of FTIs, so that they are still translocated to the plasma membrane for full activation [10]. Accordingly, it was then proposed that the combinational use of FTIs and geranylgeranyltransferase (GGTase) inhibitors (GGTIs) would be required to suppress K-Ras activity, although such combinations might produce undesired toxicity to normal cells, by inhibition of processing of critical molecules other than Ras that require these modifications for activation [47]. In addition, the lack of validated biomarkers to measure any inhibitory effect of the drugs on FTase activity in clinical trials made it more challenging to assess whether pharmacodynamic goals were being achieved with the dosing regimens utilized. Finally, although FTIs were initially developed as Ras-specific inhibitors in the preclinical setting, FTIs and GGTIs appear to also act via unidentified "off-target" pathways and can no longer be considered as Ras-specific inhibitors.

In contrast to FTIs and GGTIs that are intended to inhibit the membrane recruitment of Ras proteins, the intended action of salirasib (s-trans, trans-farnesylthiosalicylic acid), a Ras farnesylcysteine mimetic, is to dislodge oncogenic Ras proteins, or physiologically-activated Ras proteins, from the plasma membrane by competing with Ras for binding to the Ras-escort proteins galectin 1 and galectin 3 (**Figure 2**) [11]. Salirasib was shown to inhibit activation of the Raf-MEK-ERK pathway, and inhibit tumor growth, in both the *in vitro* and *in vivo* models of pancreatic, lung, colorectal, and hepatocellular carcinomas HCC and brain tumors [48-51]. Salirasib was well tolerated as both a single agent or in combination with gemcitabine: 79% of patients showed no drug-related toxicity greater than grade 1 [52,53]. Approximately 40% down-regulation of K-Ras expression was observed in paired biopsies from accessible tumors in

two subjects [52]. So far, Phase I and II trials of salirasib as a single agent, or in combination with gemcitabine, in metastatic pancreatic adenocarcinoma and lung adenocarcinoma have been completed. Although have been reported (e.g., stable disease, 1-year survival rate) [52,53], extensive further clinical testing will be required to determine if there is a significant impact on tumor response and survival, as well as reliable proof of target modulation.

4. Targeting Ras effectors

While many investigational drugs targeting Ras effectors remain in early phase trials (**Table 1**), four such drugs have reached the market to date: mTOR inhibitors (temsirolimus and everolimus) and RAF inhibitors (sorafenib and vemurafenib). In general, there appears to be two approaches in the current Ras-effector drug developmental strategies: i) focusing on particular types of disease areas by targeting one or a few isozyme(s) in the same kinase class; and ii) extending the target disease areas by expanding into diseases which share a similar genetic background or activation of similar signaling pathways, using a multikinase inhibitor.

4.1 Targeting the PI_3K-AKT-mTOR pathway

The PI_3K-AKT-mTOR pathway is well characterized for its role in cellular survival signal transduction. Physiologically, the AKT pathway promotes cell survival by inhibiting pro-apoptotic regulators, facilitating p53 degradation, modulating the activity of cell-cycle regulators and regulating cell mass [54]. The involvement of the PI_3K-AKT-mTOR pathway in cancer is indicated by the frequency of aberrantly high activity of the pathway in various types of cancers, in addition to the very common findings of genetic alterations in pathway components, such as oncogenic mutations of PIK3CA and AKT1, or loss of function of PETN [54]. The PI_3K-AKT-mTOR pathway also plays an important role in promoting tumor angiogenesis via transcriptional activation of vascular endothelial growth factor (VEGF) through mTOR, leading to the stimulation of endothelial cell survival, growth and proliferation [55].

4.1.1 *PI_3K inhibitors*

There are many investigational drugs in this class currently undergoing early clinical trials, and two drugs have advanced into Phase III trials to date. BKM120 is an oral pan-Class I PI_3K inhibitor that also inhibits the constitutively-activated mutant PIK3CA [56]. Interestingly, PIK3CA-mutant cell lines were more sensitive to BKM120 than PIK3CA wild-type lines, which might support the potential of this drug in malignancies, considering that alteration or aberrant activation of the PI_3K pathway is seen many types of cancers [56]. Preclinical *in vivo* studies demonstrated strong antitumor and antiangiogenic activities [56]. In the first-in-human Phase I trial, BKM120 was well tolerated [57]. Consistent with other PI_3K pathway inhibitors, dose-limiting toxicity included hyperglycemia (which would be expected given the established involvement of the PI_3K pathway in insulin signaling), mood alteration (likely due to the effects of PI_3K inhibition in the CNS) and skin rash [57]. Early antitumor activity was demonstrated: one patient with triple-negative breast cancer and a KRAS mutation achieved a partial response and seven patients remained on-study for more than 8 months [57]. Ongoing Phase III trials are being conducted with BKM120 as a single agent, or in combination with fulvestrant, in patients with previously-treated locally-advanced or metastatic breast cancer (estrogen receptor [ER]-positive, HER2-negative). In the Phase I trial, pharmacodynamic analysis demonstrated dose-dependent inhibition of the PI_3K pathway by BKM120, and a possible correlation with outcome was suggested [57]. Currently, several Phase II trials are being conducted to test this correlation. Combination of BKM120 with letrozole was also well tolerated in ER+/HER2- metastatic breast cancers, and combination therapies with many other chemotherapeutic agents in various types of cancers are now being tested, predominantly in Phase I trials.

GS1101 (formerly CAL-101) was strategically developed as an isoform-specific inhibitor of $PI_3K\delta$, which is exclusively expressed in leukocytes. The preclinical studies verified: i) expression of $PI_3K\delta$ in B cells collected from chronic lymphocytic leukemia (CLL) patients; ii) elevated activation of PI_3K in peripheral B cells from CLL patients, compared to B cells from healthy volunteers; and, iii) great sensitivity to GS1101 in peripheral leukemia cells from CLL patients compared to normal peripheral blood mononuclear cells [58,59]. *In vitro* activity of GS1101 was also demonstrated against Hodgkin lymphoma, multiple myeloma (MM), and mantle cell lymphoma cells [60-62]. Phase I trials of single agent or combinatorial use with other agents showed acceptable toxicity, reduction of AKT phosphorylation, and some clinical activity, such as reduction in lymphadenopathy and high rates of tumor regression in the majority of participating patients [63-67]. Based on these results, four Phase III trials of GS1101 are ongoing, either as a single agent or in combination with rituximab, ofatumumab or bendamustine in CLL patients. Additional Phase I or II studies in different types of hematological malignancies are also underway. Interestingly, recent reports of *in vitro* studies in glioblastoma suggested the potential application of GS1101 beyond hematological cancers [68,69].

4.1.2 *AKT inhibitors*

Compared to PI_3K inhibitors, there are fewer AKT inhibitors in human testing. The most advanced is MK2206, which is currently being investigated in Phase II trials. MK2206 is an oral allosteric AKT inhibitor that prevents translocation of AKT proteins to the plasma membrane and subsequent activation, by binding AKT proteins and inducing a conformational change. The inhibitory action of MK2206 is highly specific for AKT1 and AKT2 [70]. *In vitro* studies indicated

Table 1. Investigational drugs targeting Ras effectors.

Name	Targets	Currently most advanced trial phase (combination)	Cancer types	Clinicaltrials.gov identification	Refs.
BKM120	Class I PI_3K	III (single), III (+ Fulvestrant)	Previously-treated ER+/HER2- breast cancer	NCT01610284, NCT01633060	[56,57]
GS1101, CAL-101	$PI_3K\delta$	III (single), III (+ Rituximab), III (+ Bendamustine/Rituximab), III (+ Ofatumumab)	CLL	NCT01539291, NCT01539512, NCT01569295, NCT01659021	[58,64-67]
GDC-0941	$PI_3K\alpha/\delta$	II (+ Fulvestant), II (+ Carboplatin/ Paclitaxel, + Carboplatin/Paclitaxel/ Bevacizumab)	AI-refractory advanced or metastatic breast cancer, Advanced or recurrent NSCLC	NCT01437566, NCT01493843	[144-146]
PX-866	$PI_3K\alpha/\gamma/\delta$	II (single), II (single)	Recurrent or metastatic castration resistant prostate cancer, Glioblastoma multiforme	NCT01331083, NCT01259869	[147,148]
BAY80-6946	Class I PI_3K	II (single)	Relapsed, indolent or aggressive non-Hodgkin's lymphomas	NCT01660451	[149]
XL147	Class I PI_3K	II (single), I/II (+ Letrozole)	Advanced or recurrent endometrial cancer, Breast cancer	NCT01013324, NCT01082068	[150,151]
BYL719	$PI_3K\alpha$	I/II (+Cetuximab), Ib (+MEK162)	Recurrent or metastatic head and neck squamous cell carcinoma, Advanced solid tumors	NCT01602315, NCT01449058	[152]
INK1117	$PI_3K\alpha$	I (single)	Metastatic solid tumors	NCT01449370	
IPI-145	$PI_3K\gamma/\delta$	I (single)	Advanced hematological malignancies	NCT01476657	
ZSTK474	Class I PI_3K	I (single)	Advanced solid malignancies	NCT01280487	[153]
AMG319	$PI_3K\delta$	I (single)	Relapsed or refractory lymphoid malignancies	NCT01300026	
MK2206	AKT1/2	II (single), II (single), II (single), II (single), II (single), II (single), II (+Erlotinib Hydrochloride), II (single), II (single), II (+AZD6244), II (single)	Refractory RCC, Refractory HCC, Refractory advanced gastric or gastroesophageal junction cancer, Platinum-refractory ovarian/ fallopian tube/peritoneal cancer, Advanced PIK3CA(mu)/PTEN loss breast cancer, Relapsed refractory advanced NSCLC, Relapsed refractory acute myelogenous leukemia, Recurrent or advanced PIK3CA(mu) endometrial carcinoma, Recurrent or metastatic nasopharyngeal carcinoma, Advanced colorectal carcinoma, Relapsed or refractory DLBCL	NCT01239342, NCT01239355, NCT01260701, NCT01283035, NCT01277757, NCT01294306, NCT01253447, NCT01307631, NCT01349933, NCT01333475, NCT01481129	[70-73]

AI: Aromatase inhibitor; CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; ER: Estrogen receptor; HCC: Hepatocellular carcinoma; mu: Mutant; MCT-1: Multiple copies in T-cell lymphoma-1; NSCLC: Non-small cell lung cancer; pNET: Pancreatic neuroendocrine tumor; PTEN: Phosphatase and tensin homolog; RCC: Renal cell carcinoma.

Table 1. Investigational drugs targeting Ras effectors (continued).

Name	Targets	Currently most advanced trial phase (combination)	Cancer types	Clinicaltrials.gov identification	Refs.
GSK2110183	AKT1/2/3	II (single), II (+ Ofatumumab)	Hematological malignancies, CLL	NCT01531894, NCT01532700	[74]
GDC-0068	AKT1/2/3	I (+ GDC-0973), I (+ GSK1120212)	Advanced solid tumors, Locally advanced or metastatic solid tumors	NCT01562275, NCT01138085	[75]
AZD5363	AKT1/2/3, p70S6K, PKA, 14 other AGCs	I (single), I (single), I (+paclitaxel)	Advanced solid malignancy/ advanced or metastatic PIK3CA(mu) breast cancer, Advanced solid malignancy, Breast cancer	NCT01226316, NCT01353781, NCT01625286	[76,77]
ARQ092	AKT1/2/3	I (single)	Advanced solid tumors	NCT01473095	[154]
GSK2141795	AKT1/2/3	I (+ GSK1120212)	Pancreatic cancer/ endometrial cancer/ colorectal cancer	NCT01138085	[155]
Ridaforolimus	mTORC1	III (single), II (+ Exemestane)	Advanced bone and soft tissue sarcomas, Post-menopausal breast cancer	NCT00538239, NCT01605396	[82,83]
CC-223	mTORC1/2	I/II (single), I (+ Erlotinib or oral Azacitidine)	Refractory advanced tumors, NSCLC	NCT01177397, NCT01545947	[156]
ME-344	mTORC1/2	I (single)	Refractory solid tumors	NCT01544322	
AZD2014	mTORC1/2	I (single), I (+ Fulvestrant)	Advanced solid malignancies, Advanced metastatic breast cancer	NCT01026402, NCT01597388	[157]
OSI-027	mTORC1/2	I (single)	Solid tumors or lymphoma	NCT00698243	[158]
BEZ235	Class I PI_3K, mTORC1/2	II (single), II (single)	Advanced pNET, pNET after failure of mTOR Inhibitor therapy	NCT01628913, NCT01658436	[85-91]
GDC-0980	Class I PI_3K, mTORC1/2	II (+ Fulvestrant), II (single), II (+ Abiraterone Acetate)	AI-refractory advanced or metastatic breast cancer, Recurrent or persistent endometrial carcinoma, Castration-resistant prostate cancer previously- treated with docetaxel	NCT01437566, NCT01455493, NCT01485861	[92,93]
PF-04691502	Class I PI_3K, mTORC1/2	II (+ PF-05212384) , II (+ Letrozole), II (+ Exemestane)	Recurrent endometrial cancer, Early breast cancer, Advanced breast cancer	NCT01420081, NCT01430585, NCT01658176	[159]
PKI-587, PF-05212384	Class I PI_3K, mTORC1/2	II (single)	Recurrent endometrial cancer	NCT01420081	[160]
XL765, SAR245409	Class I PI_3K, mTORC1/2	II (single), I/II (+ Letrozole)	Relapsed or refractory lymphoma or leukemia subtypes, Breast cancer	NCT01403636, NCT01082068	[161,162]
GSK2126458	Class I PI_3K, mTORC1/2	I (+ GSK1120212)	Advanced solid tumor	NCT01248858	[163]
DS-7423	Class I PI_3K, mTORC1/2	I (single)	Advanced solid malignant tumors	NCT01364844	

AI: Aromatase inhibitor; CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; ER: Estrogen receptor; HCC: Hepatocellular carcinoma; mu: Mutant; MCT-1: Multiple copies in T-cell lymphoma-1; NSCLC: Non-small cell lung cancer; pNET: Pancreatic neuroendocrine tumor; PTEN: Phosphatase and tensin homolog; RCC: Renal cell carcinoma.

Table 1. Investigational drugs targeting Ras effectors (continued).

Name	Targets	Currently most advanced trial phase (combination)	Cancer types	Clinicaltrials.gov identification	Refs.
PWT33597-101	$PI_3K\alpha$, mTORC1/2	I (single)	Advanced malignancies	NCT01407380	[164]
SF1126 (LY294002 pro-drug)	Class I PI_3K, mTORC1/2	I (single)	Advanced or metastatic solid tumors cancer	NCT00907205	[165]
Dabrafenib	B-Raf(mu)	III (+ Trametinib), III (+ Trametinib)	BRAF(mu) melanoma, Unresectable or metastatic BRAFV600E/K(mu) melanoma	NCT01584648, NCT01597908	[105]
Regorafenib	B-Raf(mu), KIT(mu), RET (mu), VEGFR1/2/3, PDGFRβ, FGFR1	III (single), II (+ FOLFIRI)	Refractory metastatic colorectal cancer, Metastatic colorectal cancer	NCT01584830, NCT01298570	[166,167]
LGX818	B-Raf(mu)	I/II (single), I (single)	BRAF-dependent advanced solid tumors, Advanced or metastatic BRAF(mu) melanoma	NCT01543698, NCT01436656	[168]
RAF265	B-Raf, VEGFR2	I (single), I (+ MEK162)	Locally advanced or metastatic melanoma, Advanced solid tumors with RAS(mu) or +BRAFV600E(mu)	NCT00304525, NCT01352273	[169,170]
RO5212054	B-Raf(mu)	I (single)	Advanced solid tumors with BRAFV600(mu)	NCT01143753	
ARQ736	B-Raf(mu)	I (single)	Advanced solid tumors with NRAS (mu) or BRAFV600E(mu)	NCT01225536	
Trametinib	MEK1/2	III (+ Dabrafenib), III (+ Dabrafenib)	Unresectable or metastatic BRAF V600E/K(mu) melanoma	NCT01597908, NCT01584648	[115-118]
Selumetinib	MEK1/2	I/II (+ Sorafenib), II (single), II (+ Temsirolimus), II (+ Erlotinib Hydrochloride), II (+ Erlotinib Hydrochloride), II (single), II (+ MK2206), II (+ MK2206), II (+ MK2206),	Advanced HCC, Metastatic uveal melanoma, Metastatic, recurrent, or locally advanced unresectable soft tissue sarcomas, Locally advanced or metastatic pancreatic adenocarcinoma, KRAS(WT/mu) advanced NSCLC, MCT-1 Related relapsed or refractory DLBCL, Advanced colorectal carcinoma, Relapsed BRAF(mu) melanoma, previously treated metastatic pancreatic cancer	NCT01029418, NCT01143402, NCT01206140, NCT01222689, NCT01239290, NCT01278615, NCT01333475, NCT01519427, NCT01658943	[14,119-122]
MEK162	MEK1/2	II (single), Ib (+ BYL719), Ib/II (+ LGX818), I (single), I (+ Paclitaxel)	Advanced melanoma, Advanced solid tumors, BRAF dependent advanced solid tumors, Advanced	NCT01320085, NCT01449058, NCT01543698, NCT01469130, NCT01649336	[171]

AI: Aromatase inhibitor; CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; ER: Estrogen receptor; HCC: Hepatocellular carcinoma; mu: Mutant; MCT-1: Multiple copies in T-cell lymphoma-1; NSCLC: Non-small cell lung cancer; pNET: Pancreatic neuroendocrine tumor; PTEN: Phosphatase and tensin homolog; RCC: Renal cell carcinoma.

Table 1. Investigational drugs targeting Ras effectors (continued).

Name	Targets	Currently most advanced trial phase (combination)	Cancer types	Clinicaltrials.gov identification	Refs.
			solid tumors, Epithelial ovarian, fallopian Tube or peritoneal cancer		
Pimasertib, AS703026	MEK1/2	I/II (+ Gemcitabine), I (+ Temsirolimus), I (single)	Hematological malignancies/ acute myeloid leukemia, Pancreatic cancer, Locally advanced or metastatic solid tumors	NCT00957580, NCT01378377, NCT01390818	[172,173]
Refametinib	MEK1/2	I/II (+ Gemcitabine), Ib (+ BAY80-6946)	Advanced pancreatic cancer, Advanced cancer	NCT01251640, NCT01392521	[174,175]
WX-554	MEK1/2	I/II (single)	Solid tumors	NCT01581060	[176]
AS703988	MEK1/2	I (single)	Solid tumors	NCT01453387	
GDC-0973	MEK1/2	I (+ GDC-0941), I (+ Vemurafenib), I (+ GDC-0068)	Locally advanced or metastatic solid tumors, Metastatic BRAF(mu) melanoma, Locally advanced or metastatic solid tumors	NCT00996892, NCT01271803, NCT01562275	[145]
GDC-0623	MEK	I (single)	Locally advanced or metastatic solid tumors	NCT01106599	
RO4987655	MEK1	I (single)	Advanced solid tumors,	NCT00817518	[177]
TAK-733	MEK1/2	I (single), I (+ Alisertib)	Advanced nonhematological malignancies	NCT00948467, NCT01613261	[178]
E6201	MEK1, MEKK1	I (single)	Solid tumors	NCT00794781	[179]

AI: Aromatase inhibitor; CLL: Chronic lymphocytic leukemia; DLBCL: Diffuse large B-cell lymphoma; ER: Estrogen receptor; HCC: Hepatocellular carcinoma; mu: Mutant; MCT-1: Multiple copies in T-cell lymphoma-1; NSCLC: Non-small cell lung cancer; pNET: Pancreatic neuroendocrine tumor; PTEN: Phosphatase and tensin homolog; RCC: Renal cell carcinoma.

anti-proliferative activity in tumor cells with activation of HER2, with mutations of PTEN or PIK3CA, or with AKT2 amplification, the types of genetic alterations that could provoke constitutive activation of the AKT signaling pathway [71]. Consistent with our understanding that aberrant AKT activation commonly serves as one mechanism of cancer drug resistance, *in vivo* models showed improved responses to chemotherapeutic agents when MK2206 was added to the regimen (erlotinib, carboplatin and gemcitabine in a NSCLC model, lapatinib in breast and ovarian cancer models, docetaxel in a prostate cancer model) [72]. MK2206 was well tolerated in a Phase I trial, and dose-limiting toxicities included skin rash, nausea, pruritus, diarrhea and hyperglycemia [70]. Reduction in phosphorylation of AKT (Ser473) in all tumor biopsies validated the pharmacodynamic endpoint [70]. One patient with advanced pancreatic adenocarcinoma previously resistant to four regimens of chemotherapy experienced 23% reduction in tumor size, while two other patients with advanced pancreatic neuroendocrine tumors displayed minor reduction in tumor size [70]. Stable disease was observed in three patients for 4 months or longer, and in another three patients for 6 months or longer [70]. Concurrent treatment with MK2206 and trastuzumab in HER2-positive tumors produced one complete remission in a breast cancer patient and 16% of patients experienced stable disease for at least 4 months [73]. Currently, additional Phase II trials are underway. Trial regimens include MK2206 as a single agent, in combination therapy, in previously-treated patients, or in patients with PIK3CA mutations or PTEN loss.

GSK2110183, an oral ATP-competitive inhibitor of all three isoforms of AKT, has also advanced to Phase II trials. Preliminary results from the first-in-human Phase I study, focusing primarily on MM, in which the PI_3K/AKT pathway is constitutively activated, exhibited good tolerability and clinical activity as monotherapy in heavily-pretreated MM patients [74]. Another ATP-competitive inhibitor of AKT1/2/3, GDC-0068, was shown to effectively block phosphorylation of downstream targets of AKT in cell culture systems, and this was confirmed in *in vivo* xenograft models in a dose-dependent manner [75]. Antitumor activity was reported in the same *in vivo* model, which had aberrantly activated PI_3K-AKT-mTOR signaling [75]. GDC-0068 recently completed a single agent safety and dose-determination Phase I trial and is now undergoing Phase I combination trials.

In contrast to the high target specificity of the ATP-competitive inhibitors described above, the AKT inhibitor AZD5363 was found to possess inhibitory activity against AKT isoforms, p70S6K and PKA, as well as 14 other AGC family kinases in *in vitro* kinase assays (**Figure 2**). Cell lines carrying wild-type RAS together with either an activating mutation of PIK3CA or PTEN mutation/loss were particularly sensitive to AZD5363 [76]. In an *in vivo* HER2-positive breast cancer model with trastuzumab resistance, AZD5363 displayed antitumor activity as monotherapy and this antitumor activity was enhanced by combination with docetaxel, lapatinib, or trastuzumab [76]. Furthermore, addition of AZD5363 to trastuzumab resensitized HER2-positive tumors with PIK3CA mutations to the treatment [77]. These preclinical studies suggested that the activity of AZD5363 can be maximized when it is used against tumors with a particular genetic profile. AZD5363 is now undergoing several Phase I trials as monotherapy or combinatorial therapy.

4.1.3 *mTOR inhibitors*

Renal cell carcinomas (RCC) typically express high level of the transcription factor hypoxia-inducible factor 1 (HIF1), which is one substrate of mTOR. Uncontrolled transcription of pro-angiogenic factors regulated by HIF1, including VEGF, contributes to tumor angiogenesis in RCC [55]. RCC, therefore, presents a potential therapeutic opportunity for the early mTOR inhibitors temsirolimus and everolimus, which bind to a component of mTORC1 and prevent initiation of the mTOR signaling cascade. Preclinical studies demonstrated these inhibitors repressed the growth of a wide range of cancer cell lines, accompanied by decreased activities of downstream markers of mTOR signaling [78,79]. Interestingly, antitumor activity was observed in some tumor models *in vivo* even when the cell lines themselves were insensitive to the drug *in vitro*, suggesting that indirect effects may have contributed to the *in vivo* antitumor activity, such as attenuation of tumor angiogenesis by antiangiogenic factors downstream of mTOR signaling [78]. In the registration Phase III trial of single-agent temsirolimus compared to interferon α, temsirolimus improved overall survival in patients with advanced RCC, and most adverse events were manageable [80]. Similarly, everolimus prolonged progression-free survival (PFS) over the placebo group (4.9 months vs 1.9 months) in a Phase III trial of patients of advanced RCC previously treated with sunitinib or sorafenib, leading to its approval in this disease, although overall survival was not different between everolimus-treated patients and placebo group [81]. Serious adverse events included infections (10%), dyspnea (7%), and fatigue (5%) [81]. Everolimus was later approved for three more indications: subependymal giant cell astrocytoma, metastatic pancreatic neuroendocrine tumor (14% of these cancers have a genetic mutation in the mTOR pathway), and ER-positive/HER2-negative advanced breast cancer.

Ridaforolimus is an investigational oral agent under development for maintenance therapy for patients with metastatic soft tissue or bone sarcoma who have stable disease or better after four or more cycles of chemotherapy. The rationale for the application of ridaforolimus to sarcoma is twofold: i) the mTOR pathway is involved in the development of mesenchymal cells, from which sarcomas arise; ii) mTOR inhibition decreases the expression level of EWS fusion proteins, the product of gene fusion between EWS and transcription factor genes, which is a key event in the development of Ewing sarcoma [82]. Clinical trials were conducted in breast cancer, endometrial cancer, hematological malignancies, sarcoma

and solid tumors in Phases I or II. Generally, ridaforolimus showed good tolerability, predictable and manageable adverse events and an indication of mTOR pathway inhibition in patient samples [83]. Ridaforolimus demonstrated more promising clinical activity in sarcomas in Phase I and II studies compared to the Phase II trials with everolimus and temsirolimus [82]. Based on the Phase II observation of prolonged PFS in advanced sarcoma patients, the application to a maintenance regimen was pursued in a Phase III trial in patients with advanced bone and soft tissue sarcomas who had at least stable disease following prior chemotherapy. Median PFS and 6-month PFS rates were 17.7 weeks and 34% in the ridaforolimus group and 14.6 weeks and 23% in the placebo group [82]. No statistical improvement in overall survival was reported. In June 2012, the US Food and Drug Administration (FDA) rejected the approval of New Drug Application for ridaforolimus in its present form and required additional clinical trial(s) for further assessment of safety and efficacy [84]. A currently ongoing Phase II trial is evaluating effects of the combination therapy of ridaforolimus and exemestane in comparison to single-agent treatment with ridaforolimus, dalotuzumab or exemestane on PFS in postmenopausal, ER-positive breast cancer patients. Multiple Phase I trials of ridaforolimus in combination with other agents in various types of cancers are underway.

Unlike these first-generation mTOR inhibitors, which are collectively called rapalogs (rapamycin analogs), new-generation inhibitors currently in early phase trials are predominantly mTORC1/2 dual inhibitors. As dual inhibition of mTORC1 and mTORC2 presumably leads to the complete inhibition of the mTOR pathway, better antitumor clinical activity is expected.

4.1.4 *PI_3K-mTOR dual inhibitors*

Since mTOR possesses a motif that structurally resembles the catalytic domain of PI_3K, some inhibitors that were designed to target PI_3K or mTOR have a dual-inhibitory effect on both kinases. BEZ235 inhibits class I PI_3Ks and mTORC1/2 (**Figure 2**). Preclinical studies demonstrated growth-inhibitory activity in breast cancer cells with HER2 amplification, glioma cells, lung and ovarian cancer cells, all of which are characterized by aberrant activation of the PI_3K-AKT-mTOR pathway [85-88]. Interestingly, cell lines harboring KRAS or BRAF mutations, or EGFR amplification, all which would lead to PI_3K-AKT activation, were less sensitive to BEZ235 in breast cancer models, while ovarian cancer cell lines with activating PI_3K mutations or PTEN loss were more sensitive to the same drug [85,87]. The first-in-human Phase I trial produced partial responses in patients with lung cancer and ER-positive breast cancers, and 24% of patients had stable disease over 4 months [89]. BEZ235 is now being tested in Phase I and Ib/II trials. Preliminary results reported that BEZ235 in combination with trastuzumab showed acceptable safety in patients with PI_3K- or PTEN-altered, HER2-positive metastatic breast cancer and BEZ235 as a single agent given twice-daily produced some evidence of clinical activity (stable disease in two colorectal and one endometrial cancer) [90,91].

GDC-0980 also inhibits both class I PI_3Ks and mTORC1/2, as verified by inhibition of downstream components of the PI_3K-mTOR pathway (**Figure 2**) [92]. GDC-0980 inhibited proliferation of various cancer cell lines, producing G_1 cell cycle arrest, with the greatest activity seen in breast, prostate and lung cancer lines [92]. The observation that melanoma and pancreatic cancer cell lines were less susceptible to the inhibition of this pathway might be explained by the frequent mutation of KRAS or BRAF in these tumors, which could enhance drug resistance [92]. Inhibition of tumor growth was observed in animal xenograft studies, including models developed from cell lines harboring activated PI_3K or loss of PTEN [92]. Phase I trial results indicated tolerability and showed antitumor activity, including tumor regression in patients with mesothelioma, gastrointestinal stromal tumor and adrenal cell carcinoma [93].

4.2 Targeting the Raf-MEK pathway

The Raf-MEK pathway may be a particularly central component of Ras signaling to target for cancer therapeutics. Barbacid and others, using "Ras-less" cells, have demonstrated that the MAPK pathway is necessary and sufficient for proliferation and migration of normal cells, and that none of the other Ras effector pathways, including PI_3K, could substitute in this model [94]. Furthermore, in certain K-Ras-driven lung cancer models C-Raf, rather than A-Raf or B-Raf, is the critical Raf kinase mediating the oncogenic effect of K-Ras [95].

4.2.1 *Raf inhibitors*

In the search for potential therapeutics to block aberrant activation of the Raf-MEK-ERK pathway in cancer cells, pharmacological inhibitors of Raf kinases and MEK kinases have been most intensively pursued. Two Raf inhibitors have been approved by the FDA to date. Sorafenib was approved for the treatment of patients with advanced RCC and unresectable HCC. Although sorafenib was designed to target C-Raf, it also effectively inhibits wild-type B-Raf and the oncogenic B-Raf V600E mutant, as well as the VEGF receptor 1 (VEGFR1), VEGFR2, VEGFR3, and platelet-derived growth factor receptor-β (PDGFRβ) tyrosine kinases in biochemical assays *in vitro* (**Figure 2**) [96]. The inhibitory effect on the VEGFRs was presumed to contribute to the observed disruption of tumor microvasculature in the *in vivo* models [96]. Interestingly, a Phase I trial in RCC demonstrated that a reduction of vascular permeability correlated with better PFS [97]. A Phase III study resulted in prolonged PFS in the patients treated with sorafenib (5.5 months) in comparison to the placebo group (2.8 months) [98]. In the case of HCC, blockade of both Raf-MEK-ERK signal transduction and tumor angiogenesis is postulated to contribute to the antitumor activity. A Phase II trial showed correlation between the pharmacodynamic marker of decreased levels of

phospho-ERK expression and prolonged time to progression (TTP) [99]. Both median survival and TPP were nearly 3 months longer for HCC patients treated with sorafenib than for those given placebo in the Phase III monotherapy study [98]. Currently, more than 150 clinical trials in the different phases are being conducted with sorafenib in various cancers, in single or combination regimens.

The discovery of frequent BRAF mutations in a wide range of cancers attracted attention to B-Raf as a druggable target [100]. Theoretically, specifically targeting mutant B-Raf, the expression of which is confined to cancer cells, would enable tumor-selective drug activity, while sparing normal cells that carry wild-type B-Raf. Most investigational drugs currently in clinical trials are selective for the BRAF-V600E mutant, which is particularly common in melanoma (and in colorectal cancer at a lower frequency). The recently FDA-approved agent vemurafenib preferentially inhibits the V600E mutant form of B-Raf over wild-type (**Figure 2**). Inhibition of ERK phosphorylation, induction of cell cycle arrest and apoptosis were exclusively observed in BRAF-V600E-positive cells [101]. A Phase II trial in previously-treated melanoma patients with mutant B-Raf achieved a remarkable response rate of 53% and a median duration of response of 6.7 months [102,103]. A Phase III trial which compared the efficacy of vemurafenib to that of dacarbazine in the patients with previously-untreated BRAF-V600E-positive melanomas verified the higher response rate and improved rates of overall survival and PFS over the standard treatment group [104]. Vemurafenib was approved by FDA in 2011 for the treatment of patients with previously untreated metastatic or unresectable melanoma with the BRAF-V600E mutation, with concurrent approval of a BRAF-V600E mutation assay (companion diagnostic). Among the investigational drugs in this class, the most advanced at this time is dabrafenib, which has higher specificity against mutant B-Raf and a similar preclinical profile to vemurafenib (**Figure 2**) [105]. Encouraged by a Phase II trial that confirmed a 59% response rate to dabrafenib in melanoma, several Phase III trials are currently ongoing. Preliminary result from a randomized monotherapy trial reported improved median PFS over dacarbazine treatment [105].

While these mutant BRAF inhibitors produce improved overall survival in the patients with BRAF mutations compared to standard treatment options, a major challenge remains: essentially all patients treated with these drugs relapse due to the development of drug resistance, with the median TTP of 7 months for vemurafenib and 5 months for dabrafenib [102,105]. Several models for resistance have been proposed: i) reactivation of the MEK-ERK pathway bypassing BRAF (e.g., secondary mutations in NRAS, hyperactivation/overexpression of C-Raf, or activation of another MAPKK COT); or, ii) adaptive dependency on alternative pathways (hyperactivation/overexpression of RTKs, such as PDGFRβ or IGF1R, or the AKT pathway) (**Figure 3**) [106-109].

Interestingly, B-Raf mutant-selective inhibitors, collectively called class I B-Raf inhibitors, were reported to paradoxically activate the MEK-ERK pathway via C-Raf in a Ras activity-dependent manner in RAS-mutant cells, leading to accelerated cell proliferation [110-112]. Moreover, RAS mutations (predominantly HRAS) were detected in 60% of tumor samples taken from patients who developed secondary tumors (cutaneous squamous-cell carcinomas and keratoacanthomas) after treatment with class I B-Raf inhibitors [113]. In this study, HRAS mutation was demonstrated to be associated with accelerated cell proliferation due to increased MAPK pathway activity both *in vitro* and *in vivo* in response to exposure to B-Raf inhibitors. While the sequence of the events between the evolution of RAS mutations and the development of secondary tumors is still unclear, the mutational status of the RAS genes should be carefully monitored in patients who are treated with a class I B-Raf inhibitor over the course of the treatment.

4.2.2 *MEK inhibitors*

In contrast to the strategy underlying the development of B-Raf inhibitors to specifically target mutant oncogenic forms of the kinase, the MEK kinases are rarely mutated, and do not provide a tumor-specific target. Instead, tumor selectivity for the MEK inhibitors in development is sought by the choice of tumors to be targeted, i.e., cancer types in which Raf-MEK-ERK signaling is aberrantly activated relative to normal cells, and on which the tumor cells are dependent. This strategy was supported by early studies using the first-generation MEK inhibitor CI-1040, which showed activity in BRAF-mutant cell lines. This B-Raf mutant selectivity of CI-1040 was proposed to be MEK-dependent; mutation of BRAF was associated with enhanced and selective sensitivity to MEK inhibition, in comparison to cells harboring either a wild-type BRAF or a RAS mutation [114].

The most promising investigational MEK inhibitor is trametinib, which is now being investigated in several Phase III trials. Trametinib is a highly-selective allosteric inhibitor of MEK1/2. Inhibition of ERK phosphorylation as an indicator of proof-of-concept and cell growth inhibition were confirmed in multiple cell lines with activating mutations driving the MAPK pathway [115]. *In vivo* efficacy was also observed in models with activating mutations in BRAF or KRAS [115]. A Phase I study in melanoma patients indicated substantial clinical activity (the response rate) correlated with the BRAF mutational status [116]. Trametinib showed tolerability with manageable adverse events and a favorable pharmacokinetics and pharmacodynamics [117]. A Phase III trial in metastatic melanoma patients with BRAF mutations, in comparison to dacarbazine- or paclitaxel-treatment groups, achieved improved PFS and overall survival, in the similar manner to vemurafenib but with less significance [118]. Seventy-four percent of patients had some degree of tumor regression and 22% had sustained tumor regression [118]. Ongoing Phase III trials are exploring the safety and efficacy

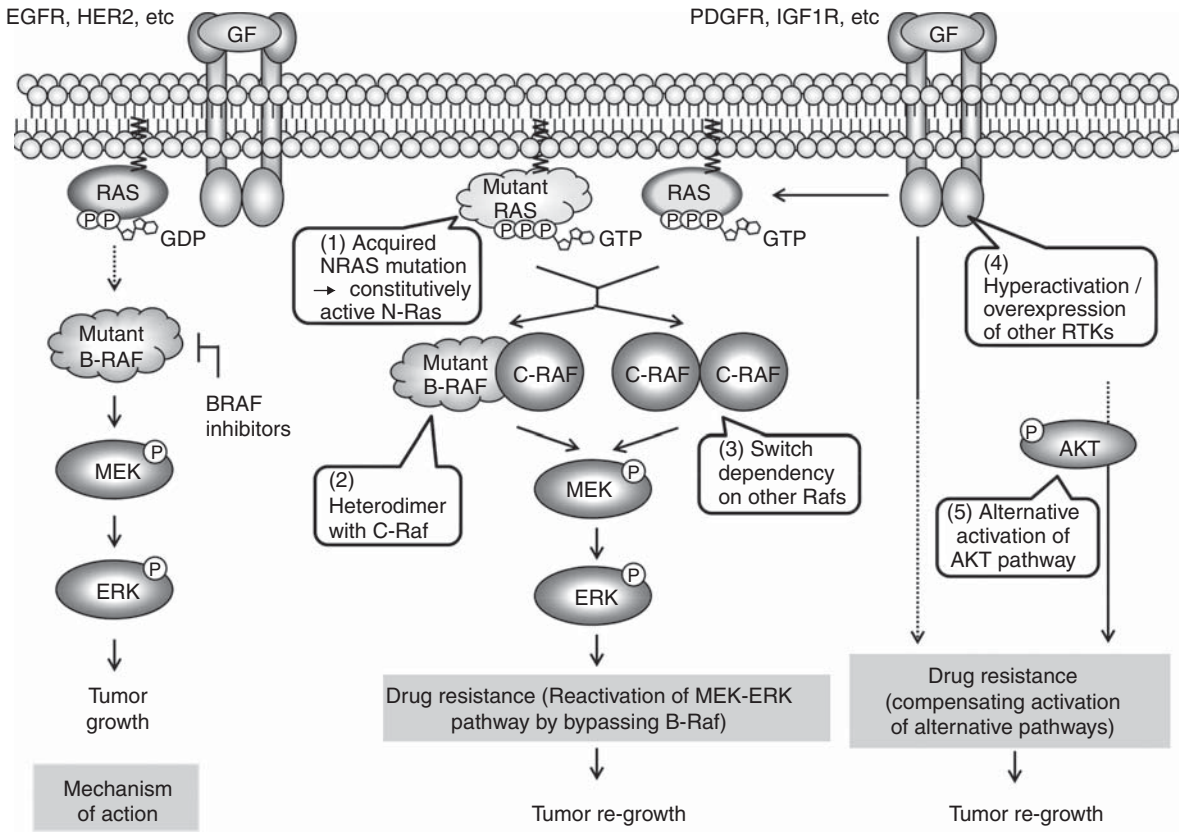

Figure 3. Mechanism of action and resistance of BRaf inhibitors. (Left) Mechanism of action: BRaf inhibitors prevent transmission of the MEK-ERK pathway specifically in tumorigenic cells carrying mutant BRaf which have low Ras activity. (Middle) Mechanism of resistance (reactivation of MEK-ERK pathway): Secondary mutation in NRAS converts normal NRas protein that is activated only on GTP-bound state into oncogenic Ras protein that is constitutively activated (1). High Ras activity induces heterodimerization of mutant BRaf with CRaf (2) or increases dependency on other Raf family members (3) in accordance with hyperactivation or overexpression of CRaf. These events reactivate MEK-ERK pathway by bypassing BRaf inhibition by the inhibitors. (Right) Mechanism of resistance (activation of alternative pathways): constant inhibition of BRaf increases the activity or expression of other RTKs; such as PDGFR or IGF1R (4) or the activity of AKT pathway (5). Cells adapt the condition of lack of MEK-ERK signaling by switching survival signaling dependency on alternative pathways.

EGFR: Epidermal growth factor receptor; GF: Growth factor; HER2: Human epidermal growth factor receptor 2; IGF1R: Insulin-like growth factor 1 receptor; PDGFR: Platelet-derived growth factor receptor-β.

of combination therapy of trametinib with the mutant-BRAF inhibitor dabrafenib.

Selumetinib is currently undergoing multiple trials in Phases I, I/II, and II. Preclinical studies demonstrated inhibition of proliferation in cell lines containing BRAF or RAS mutations of colon, pancreatic, breast cancer, and melanoma origin, while NSCLC cell lines with RAS mutations, and non-V600E-BRAF mutations, were not as sensitive as BRAF-V600E mutant cells [14,119]. *In vivo* activity in colorectal and pancreatic cancer models suggested the possibility of expanded indications beyond melanoma [14]. While selumetinib was well tolerated, with a manageable safety profile, monotherapy Phase II trials showed no clinical activity compared to conventional chemotherapies in HCC, advanced melanoma, or advanced pancreatic cancer [120-122]. The pharmacodynamic marker of reduction in ERK phosphorylation in selumetinib-treated patients was achieved, despite the lack of clinical response, suggesting this agent may provide additional activity if combined with a B-Raf inhibitor [120,121].

Interestingly, the earlier proposition that the mutational status of BRAF and RAS predicts the sensitivity of cells to MEK inhibitors was partially supported and partially refuted by a series of recent studies. A BRAF mutation was consistently an indicator of sensitivity over wild-type BRAF, whereas the correlation of RAS mutation with tumor sensitivity varied among studies using different compounds or cell lines [14,114,115,119]. One possible explanation for this

discrepancy is that BRAF mutation could affect MEK activity with less variation among different cell systems, as it is an immediate upstream effector of MEK. In contrast, RAS mutations might produce different outcomes among different cell lines, as Ras is involved in the genesis of many signaling pathways in addition to the Raf-MEK pathway. The direct coupling of B-Raf to MEK may make these tumor cells more likely to be dependent on MEK activity for proliferation, whereas activating Ras mutations may provide the cells with a number of proliferative signals, making them less likely to be dependent on MEK activation alone.

While the complexity of Ras downstream signaling allows cells to have flexible and timely positive- or negative-functional regulatory options in response to changing environmental signals, it also provides for redundancy among these pathways, so that cells can develop alternative mechanisms to compensate for any failure of the original signaling pathway. This is particularly the case in the setting of the "hyper-mutator phenotype," which characterizes malignancy. From the pharmacological point of view, this, therefore, presents a major challenge to targeting the Ras signaling pathways. The mechanisms underlying resistance to B-Raf inhibitors were discussed earlier. For MEK inhibitors, alternative activation of the PI_3K-AKT pathway or remodeling upstream signaling (Ras or Raf) to bypass MEK has been reported [123-125]. Paradoxically, the inhibitors that selectively attack single "cancer cell-specific markers" (e.g., BRAF-mutation, overexpression of $PI_3K\delta$) or "cancer cell-specific events" (e.g., hyperactivation of the Raf-MEK or PI_3K-AKT pathways) appear to provide the most facile opportunities for cancer cells to develop drug resistance despite the sometimes remarkable antitumor activities produced early in the course of the treatment. As a strategy to conquer this paradox, accumulating evidence suggests the necessity of combinational therapeutic approaches to block multiple pathways simultaneously [125-127].

5. Synthetic lethal approaches

Because activating mutations of Ras proteins are among the most frequent oncogenic events in human cancers, targeting mutated Ras should be a promising opportunity for a tumor-specific therapeutic approach. However, as described above, targeting Ras proteins themselves for anticancer therapy has been challenging for a number of reasons, and Ras proteins are now widely considered to be "undruggable" targets. Meanwhile, the recently-emerging (or rediscovered) strategies variously termed "synthetic lethality" and "nononcogene addiction" have produced a framework for the development of indirect approaches to targeting mutant Ras in cancer cells. Two genes are in a so-called "synthetic lethal" interaction if a mutation of either gene alone is compatible with viability but simultaneous mutations of both genes lead to cell death [128]. The concept of synthetic lethality is over 60 years old and has been used in yeast and drosophila, and more recently in human systems, to identify critical components of survival pathways, now including those survival pathways uniquely operative in cancers [129]. Thus, inhibition of a synthetic lethal interactor of Ras by chemotherapy theoretically kills only tumorigenic cells with a mutated RAS gene without affecting normal cells. Similarly, "nononcogene addiction" describes the situation in which transformation of a cell (whether by a known oncogene or unknown mechanisms) renders it dependent on a normally nonessential protein for survival [130]. That nonessential (nononcogenic) protein can then become the target of a therapeutic strategy, which should be cancer-specific and spare normal cells. These concepts have provided a new approach to target oncogenic Ras indirectly: that is, to discover synthetic lethal interactors, or critical "nononcogenes," which are more druggable than Ras, and then develop therapeutic methods to inhibit these interactors.

Several groups employed RNAi high-throughput screening to identify synthetic lethal interactors of Ras, in which genes whose knockdown specifically killed K-Ras-dependent cancer cells were sought [131-136]. One of these studies yielded TANK-binding kinase 1 (TBK1), a noncanonical IκB kinase that regulates the NFκB survival pathway, as a potential synthetic lethal partner of mutant K-Ras [131]. Follow-up analyses in individual cell lines of lung cancer with mutant K-Ras or wild-type K-Ras revealed that suppression of TBK1, or its reported upstream effector Ral-B, provoked apoptosis uniquely in K-Ras-dependent cancer cell lines through activation of the NFκB signaling pathway. This approach was further supported by the observation of elevated activity of Ras and the NFκB pathway in lung adenocarcinoma clinical samples with mutant K-Ras in comparison to wild-type K-Ras samples. A simultaneous report corroborated the requirement for the NFκB pathway in cancers with KRAS mutations in a mouse model [137].

In contrast, the discovery of serine/threonine kinase 33 (STK33) as a synthetic lethal interactor with Ras now appears to be incorrect. STK33 was identified from the screening of eight cell lines representing different types of K-Ras-dependent cancers [132]. STK33 belongs to the calcium/calmodulin-dependent kinase family but its physiological function is unknown. The initial report stated that STK33 activity was required for the survival of cancer cells with K-Ras dependency. However, a more recent study questioned this conclusion [133]. In this latter study, inhibition of STK33, whether by siRNA, dominant-negative mutant overexpression, or small molecule inhibitors, had no effect on the survival of KRAS mutant cells. Additionally, a synthetic lethal siRNA screening conducted in this study did not indicate STK33 as a synthetic lethal interactor.

In contrast to the above examples of RNAi-based discoveries of synthetic lethal or nononcogene addiction targets, the earlier identification of the protein kinase C delta (PKCδ) isozyme as a Ras synthetic lethal interactor originated from a focused study of Ras signaling pathways. PKCδ is a

serine/threonine kinase of the PKC family, novel class, and functions in a number of cellular activities including cell proliferation, survival or apoptosis [138]. However, PKCδ is not required for the proliferation of normal cells, and PKCδ-null animals develop normally and are fertile, suggesting the potential tumor specificity of a PKCδ-targeted approach [139]. PKCδ was validated as a target in cancer cells of multiple types with activation of H-Ras or K-Ras, using both genetic (siRNA, dominant-negative PKCδ) and small molecule inhibitors [140]. Inhibition of PKCδ induced apoptosis in pancreatic cancer cell lines with activating KRAS mutations at least in part through suppression of AKT signaling, and "Ras-dependency" in the tumors was not required for the cytotoxic effects [140,141]. More recently, tumors with aberrant activation of the PI_3K pathway in the setting of wild-type RAS alleles have also been shown to be dependent on PKCδ activity, potentially expanding the potential application of this approach beyond tumors with mutational activation of Ras [142]. Not-yet-published studies documenting the susceptibility of melanoma cells with NRAS mutations, and melanoma lines which have become resistant to B-Raf inhibitors, to PKCδ suppression or inhibition have stimulated the development of novel, more specific, and more potent, small molecule PKCδ inhibitors as potential therapeutics in tumors with aberrant Ras signaling [143].

Although none of the Ras synthetic lethal approaches have progressed to human trials, this concept proposes a potential and unique approach to cancer types with high RAS mutational frequencies: that is, it utilizes the mutant Ras proteins as markers to identify potentially susceptible tumors, rather than as pharmacological targets. Hypothetically, this approach allows a synthetic-lethal-partner-targeted therapy to confine its anti-proliferative activity only to tumorigenic cells with RAS mutations. The controversy that has been raised in the recent preclinical studies presented by different groups, however, represents a current obstacle in this research area. While these studies carefully screened a number of cell lines representing different types of cancers, context-dependent issues, such as variations in cell lines or RNAi libraries, or the complexities that arise from the combinations of these parameters, can complicate such open-ended screens, and tumor cell viability is not a molecularly-specific endpoint. Furthermore, it is noteworthy that the biological consequences of down-regulating a protein target by RNAi do not necessarily reflect the effects of a small molecule inhibitor bound to the target. An *in vitro* screen based primarily on RNAi should, therefore, be interpreted with caution if its goal is as proof-of-concept for the development of an inhibitor against the activity of the target molecule as a therapeutic (and this is indeed generally the ultimate plan for such RNAi screening programs). Comprehensive follow-up studies to understand signaling pathways in which the target is involved, its interaction with other proteins, and the fitness of an inhibitor of the target in the entire gamut of normal cellular activities or *in vivo* efficacy/toxicity are needed.

6. Conclusion

The Ras GTPases (K-Ras, N-Ras, and H-Ras) function as molecular switches for critical cellular activities, such as cell proliferation or growth, differentiation, and survival in normal cells and are tightly and temporally regulated by multiple signaling pathways. Pharmacological interventions in situations of uncontrolled Ras activity or downstream signaling, which is often prominent among the deadliest types of cancers, have been sought since the discovery of H-Ras as an oncogene in bladder cancers.

The currently most widely-employed approach to inhibiting Ras signaling is to target one or more components of the Ras downstream pathways, such as the two major Ras downstream signaling pathways: PI_3K-AKT-mTOR and Raf-MEK-ERK. Two Raf inhibitors and two mTOR inhibitors are currently approved and utilized in the clinic, and many investigational drugs with higher target specificity, better drug property and promising clinical activity are being investigated in clinical trials. Drug resistance has been a major issue in this category of drugs, however, suggesting the necessity of combination therapy to avoid the development of resistance and maximize clinical outcome in the use of these inhibitors.

The recently re-emerging concept "synthetic lethality" has provided a new therapeutic framework for targeted cancer therapy, which redefines the role of oncogenic Ras proteins as cancer cell "markers" rather than targets. This approach seeks to discover synthetic lethal interactors of Ras for pharmacological intervention, which should then selectively kill tumor cells harboring RAS mutations. Although therapies based on this strategy have not reached human testing, several synthetic lethal interactors have been proposed as targets, inhibitors identified, and their clinical potential is being investigated in preclinical settings.

7. Expert opinion

Since the discovery of the Ras proteins nearly half century ago, Ras has been intensively studied and has become one of the most well-understood oncoproteins. Oncogenic mutations of Ras proteins are found in up to 30% of all human tumors, and are particularly frequent in those types of cancers with the highest mortality rates, such as lung, colorectal and pancreatic cancers and melanomas. This makes the Ras proteins attractive pharmacological targets for cancer therapeutics. As effective direct inhibition of Ras activity was discovered to be unexpectedly challenging, the components of the Ras downstream signaling pathways have instead been exploited for inhibition by pharmacological agents. There has already been some notable successes employing this approach and additional promising investigational drugs are in clinical trials, some of which may emerge into the market over the next few years. It is important to note, however, that many of the approaches described above are not truly

"tumor-specific." Except for those agents which target only a mutated, oncogenic form of Ras or Ras effector (such as mutant Ras-specific siRNA, or the V600E mutant B-Raf inhibitors), all of these agents block those critical physiological Ras signaling pathways which are required for the viability of all cells, both normal and malignant. This crucial factor limits our ability to utilize them in the clinic at doses which would be more effective against the tumor, as normal cell function becomes increasingly compromised.

Other remaining challenges of Ras-effector inhibitors include drug resistance and unaddressed disease areas. The complexity and redundancy of Ras signaling pathways provide the tumor cells many opportunities for drug resistance and confine the target disease areas to those cancers with high dependency on these pathways, such as melanomas, RCC or HCC. Although the application of combination therapies in first-line regimens, to establish complete blockade of multiple Ras downstream pathways, might avoid or slow the establishment of drug resistance, it would still leave cancers with high RAS mutation rates but without Ras pathway-dependency uncovered.

The ultimate goal for Ras-related targeted therapy is to establish therapeutics that can overcome the current limitations described above: tumor-specificity and limited cancer indications. In this respect, the "synthetic lethal" approach raises the hope of generating antitumor activity in cancers with high RAS mutation rates regardless of Ras pathway dependency or independency. Because synthetic lethality utilizes aberrant Ras signaling as a "marker" for sensitivity rather than as a direct drug target, it is to be expected to be applicable to those types of cancers that cannot be effectively targeted by Ras-effector-inhibitor drugs (i.e., Ras-signaling-pathway-independent cancers) while sparing normal cells unaffected. This new research framework will be accelerated in the coming years aiming clinical application.

The earlier failure of strategies to develop FTIs as Ras-specific therapeutics teaches a crucial lesson in the development of targeted therapies. Thorough preclinical studies are essential for the efficient and successful clinical development of a targeted therapeutic. While it is difficult to fully verify and validate the mechanism of action and predict proof-of-concept prior to moving into the complex and confounding variables of a clinical study, good preclinical studies enable the establishment of methodologies to create multiple validated pharmacodynamic markers which inform clinical studies, whether successful or unsuccessful. Robust preclinical data also provide a framework for improving developmental strategies for later-phase trials, such as selection of target disease areas/patient populations, clinical endpoints and regimens.

Because Ras and its downstream signaling evoke various types of cellular responses, depending on signaling, cellular, and tissue context, the history of Ras therapeutic development highlights the importance of "bidirectional translational research" in the development of Ras-related targeted therapies. Translational research is defined as exploiting the effective transition of knowledge from the bench to the clinic to seek a better clinical outcome. Yet, the fitful progress and unexpected complexities in the clinical application of these new targeted agents also demand a return back to the bench with clinical data and samples, to develop new solutions or applications. As the recent clinical successes of Ras-effector inhibitors with high target specificity demonstrates, strong reciprocal interactions between the lab and the clinic, as well as between academia and industry, lead to greater and more rapid benefits for patients.

The future holds great promise for "Ras-targeted" therapeutic approaches. Some of the drugs targeting specific or multiple Ras-effectors in the late clinical phases show impressive activity in certain malignancies, and will likely reach the market after accelerated FDA approval. RNAi-based approaches targeted mutated RAS will be tested in the clinical, although many technical hurdles remain to be addressed. As Ras synthetic lethal interactor proteins are identified, and drugs to target them are developed, we will see a completely new type of anticancer agent/approach reach clinical testing, ideally one without toxicity to normal cells and tissues.

Declaration of interest

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

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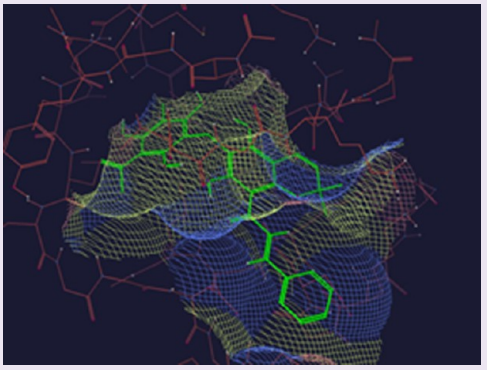
Supporting Information

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 Cδ (PKCδ). Here, we report that PKCδ inhibition is cytotoxic in melanomas with primary NRAS mutations. Novel small-molecule inhibitors of PKCδ were designed as chimeric hybrids of two naturally occurring PKCδ inhibitors, staurosporine and rottlerin. The specific hypothesis interrogated and validated is that combining two domains of two naturally occurring PKCδ inhibitors into a chimeric or hybrid structure retains biochemical and biological activity and improves PKCδ isozyme selectivity. We have devised a potentially general synthetic protocol to make these chimeric species using Molander trifluorborate coupling chemistry. Inhibition of PKCδ, by siRNA or small molecule inhibitors, suppressed the growth of multiple melanoma cell lines carrying NRAS mutations, mediated *via* caspase-dependent apoptosis. Following PKCδ 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 PKCδ inhibition mitigated the induction of caspase-dependent apoptosis. Furthermore, PKCδ 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 PKCδ in patients who have relapsed following treatment with BRAF inhibitors. Taken together, the present work demonstrates that inhibition of PKCδ 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 "undrugable".[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 (PKCδ) activity is inhibited by means of a chemical inhibitor, RNA interference, or a dominant-negative var-

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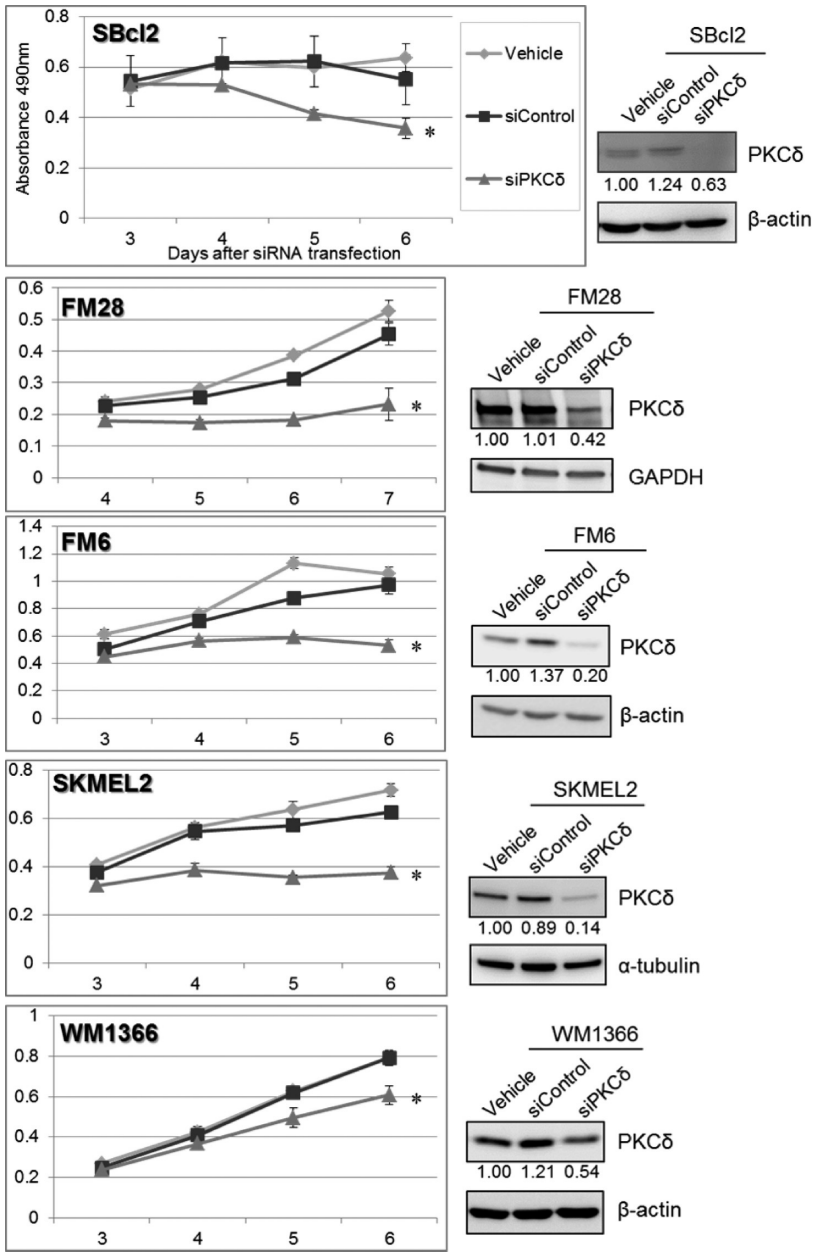

Figure 1. Downregulation of PKCδ suppresses cell survival in melanoma cell lines with NRAS mutation. siRNA targeting PKCδ ("siPKCδ") or nontargeting siRNA ("siControl") were transfected into SBcl2 and FM28 (50 nM), SKMEL2 (10 nM), and FM6 and WM1366 (5 nM), after establishing cell line-specific optimal 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 siPKCδ on the last assay day ($p < 0.006$). Downregulation of PKCδ protein on the first assay day was assessed by immunoblot analysis. The relative band intensity of PKCδ is indicated below the image (normalized to loading controls, β-actin, α-tubulin, or GAPDH).

iant.[9−12] Other groups also subsequently validated PKCδ as a target in cancer cells of multiple types with aberrant activation of KRAS signaling.[13,14]

PKCδ 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, βI, βII, γ), the novel PKCs (δ, ε, θ, μ), and the atypical PKCs (ζ, λ). The novel PKCs, including PKCδ, are characteristically activated by diacylglycerol (DAG) and are independent of the need for the secondary messenger Ca^{2+}. PKCδ 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] PKCδ 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-κB. Other context-dependent effectors of PKCδ include JNK, glycogen synthase kinase-3 (GSK3), FLICE-like inhibitory protein (FLIP), cIAP2, and $p21^{Cip1/WAF1}$. A role for PKCδ as an antiapoptotic/pro-survival regulator has been reported in various types of cancer cells, including non-small cell lung cancer, pancreatic, and colon

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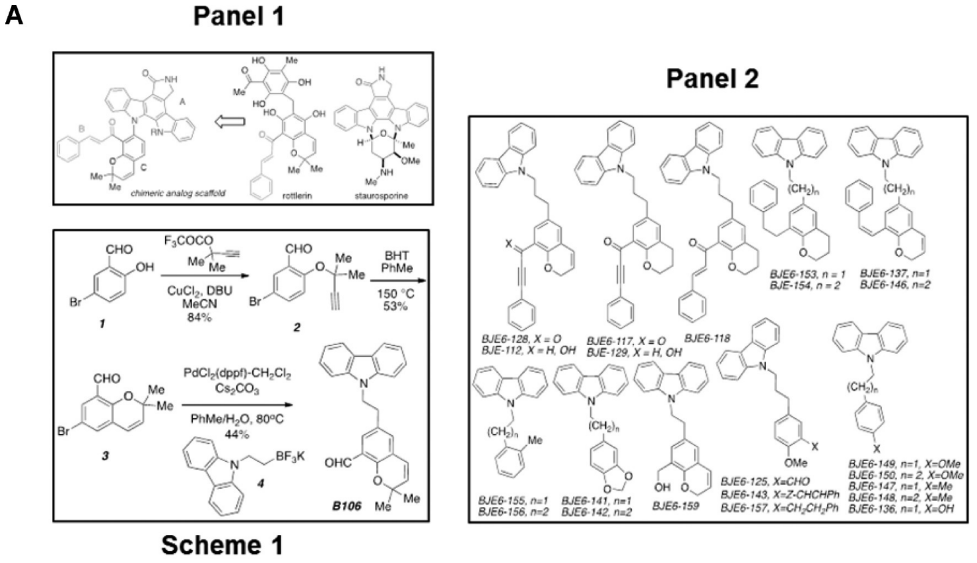

Scheme 1

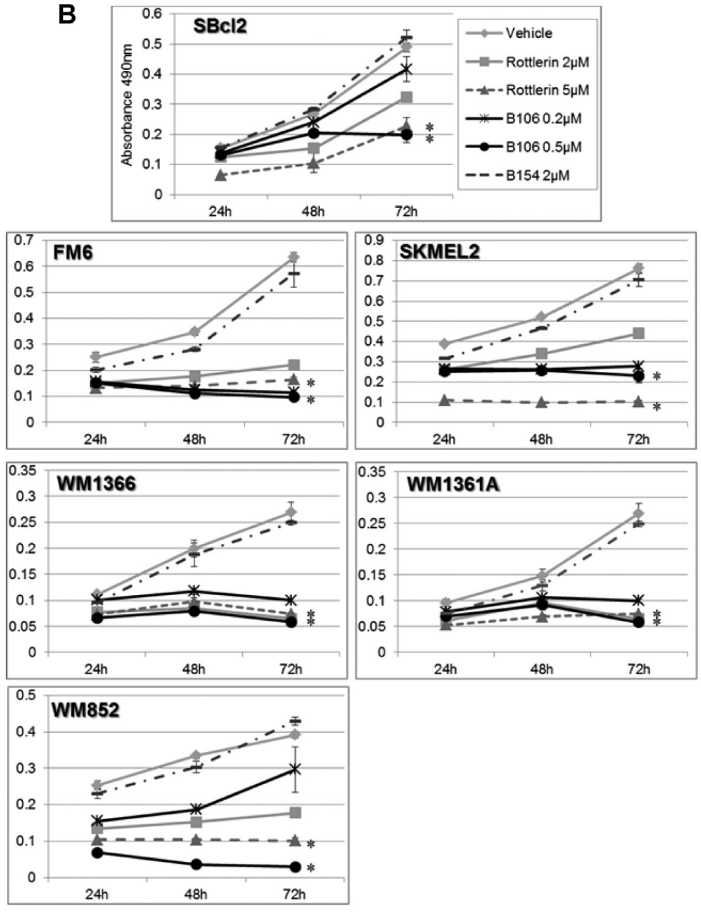

Figure 2. continued

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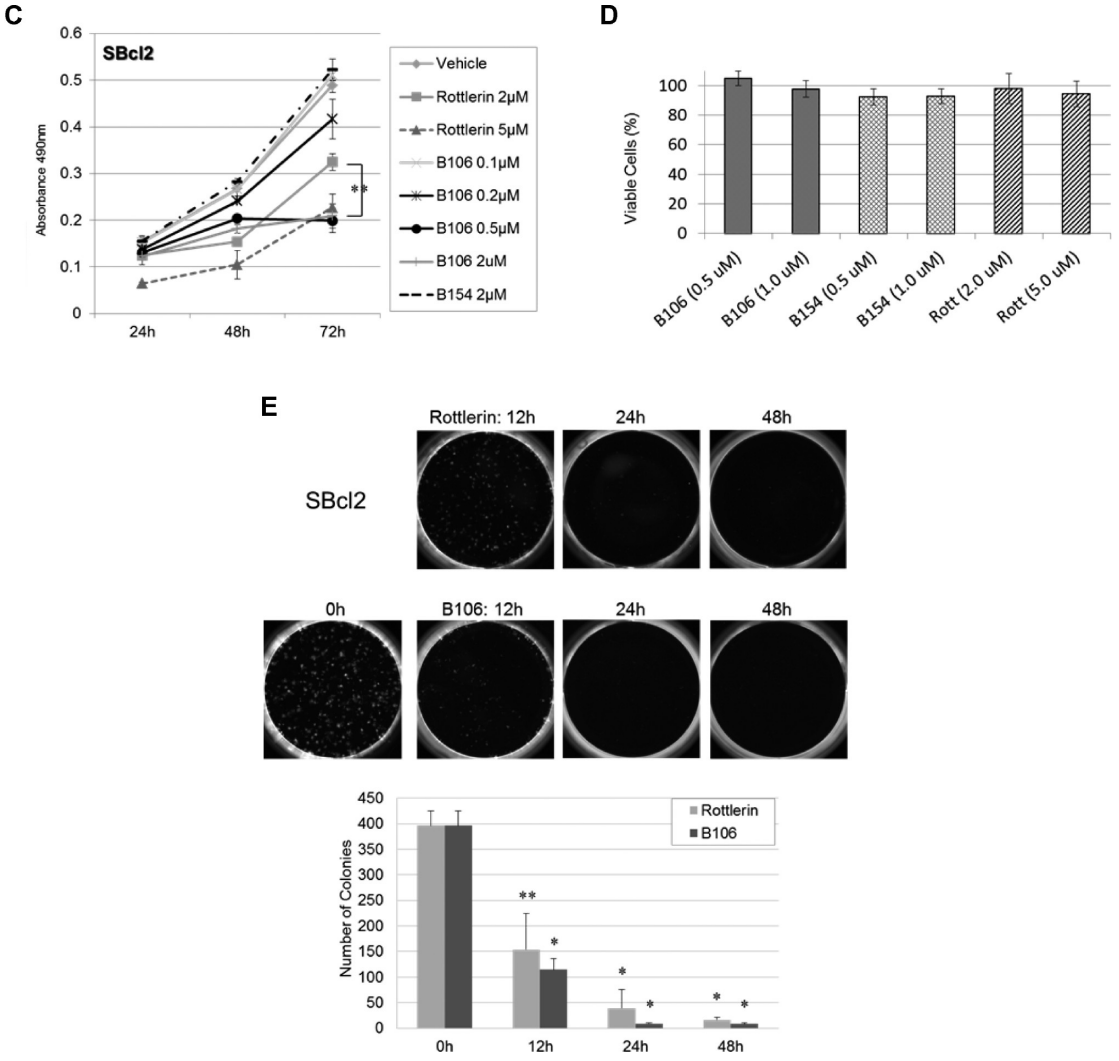

Figure 2. PKCδ inhibitors suppress survival in melanoma cell lines with NRAS mutations. (A) Structure and synthesis of PKCδ inhibitors. Panel 1: Design of mallotoxin/rottlerin-staurosporine hybrids. Scheme 1: Synthesis of B106. Panel 2: 3rd generation compounds. (B) PKCδ inhibitors suppress cell survival in melanoma cell lines with NRAS mutations. SBcl2, FM6, SKMEL2, WM1366, WM1361A, and WM852 cells were exposed to rottlerin (2 or 5 μM) or B106 (0.2 or 0.5 μM) for 24, 48, or 72 h, and MTS assays were performed at each time point. DMSO and B154 (2 μM) 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 μM or DMSO and B106 0.5 μM in each cell line at 72 h ($p < 0.0002$). (C) Titration of PKCδ inhibitor treatment. The expanded doses of B106 (0.1 μM and 2 μM) in the MTS assay in SBcl2 in Figure 2A are shown. ** indicates a p value < 0.5 between treatment of 2 μM of rottlerin and B106. (D) Effects of PKCδ 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 ± SD, $n = 3$). (E) PKCδ inhibitors induce irreversible effects on cell growth. SBcl2 cells were treated with rottlerin or B106 at 1 μM 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, PKCδ is not required for the survival of normal cells and tissues, and PKCδ-null mice are viable, fertile, and develop normally.[21]

Our previous studies demonstrating the synthetic lethal activity of PKCδ inhibition in pancreatic, lung, neuroendocrine, and breast cancers, and cancer stem-like cells (CSCs) with KRAS mutations[9−12] suggested the potential of targeting PKCδ in melanomas with an activating NRAS mutation. In this study, we demonstrate that inhibition of PKCδ by siRNA or novel chemical compounds suppresses the growth of melanoma lines with NRAS mutations through induction of caspase-dependent apoptosis. A novel PKCδ inhibitor developed through pharmacophore modeling exerted cytotoxic activity on NRAS mutant tumors at concentrations 1 log lower than commercially available PKCδ 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 PKCδ inhibition can effectively inhibit the growth of PLX4032-resistant melanoma cells with BRAF mutations, demonstrating the potential of an approach targeting PKCδ in the substantial fraction of patients with melanoma who currently have only limited treatment options.

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Table 1. Comparison of Properties of PKCδ Inhibitors[a]

cmpds.	generation	PKCδ IC_{50}	PKCα IC_{50}	PKCδ/PKCα selectivity	"Ras-specific" cytotoxicity
Rottlerin	1st	3−5 μM	75 μM	28-fold	3−5 μM
KAM1	2nd	3 μM	157 μM	56-fold	3 μM
B106	3rd	0.05 μM	50 μM	1000-fold	0.5 μM
B154	3rd	>40 μM	>100 μM		none

[a]*In vitro* kinase assays demonstrated that third generation PKCδ inhibitor B106 is more potent and more selective for PKCδ over PKCα than rottlerin/mallotoxin or the 2nd generation PKCδ inhibitor KAM1. B154 is used as an inactive (negative control) compound.

RESULTS AND DISCUSSION

PKCδ Is a Potential Therapeutic Target in Melanoma with NRAS Mutation. To validate the potential of this approach targeting PKCδ in melanomas with NRAS mutations, we first examined the effect of PKCδ-selective inhibition on cell growth by specifically and selectively knocking down PKCδ protein expression in multiple melanoma cell lines harboring NRAS mutations, using siRNA. The specificity of the PKCδ-specific siRNAs employed herein for PKCδ among all the other PKC isoforms has been previously demonstrated.[9−11] Even partial knockdown of PKCδ 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 PKCδ downregulation. No viable cells with chronic suppression of PKCδ could ever be isolated, consistent with our previous demonstration of a requirement for PKCδ activity for the viability in cells bearing mutationally activated RAS.

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

Development of Novel PKCδ Inhibitor BJE6-106 (B106). Potent small molecule inhibitors of PKCδ 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 PKCδ inhibitor can damage normal cells.[22,23] We therefore pursued development of a more potent PKCδ inhibitor with higher PKCδ selectivity in order to explore the therapeutic potential of this approach of targeting PKCδ.

We initially generated a pharmacophore model based on molecular interactions of small molecules with "novel" class PKC isozymes. In the initial pharmacophore model for PKCδ 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 PKCδ-inhibitory activity (IC_{50} = 5 μM). Protein structural data for PKCθ, another "novel" PKC isozyme, which is also inhibited by mallotoxin/rottlerin, was incorporated (Supporting Information). Mallotoxin/rottlerin is relatively selective for PKCδ over PKCα (PKCδ IC_{50}:PKCα IC_{50} 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 PKCδ 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 PKCδ 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 PKCδ). In these second generation of PKCδ 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 PKCδ-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 IC_{50} of 3 μM for PKCδ (similar to mallotoxin/rottlerin) and better isozyme selectivity (IC_{50} > 150 μM for PKCα) (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 PKCδ-inhibitory activity, ranging from IC_{50} > 40 μM to IC_{50} < 0.05 μM (Supporting Information Table 1). BJE6-106 (B106) (Figure 2A, Scheme 1), our current lead third generation compound, has an IC_{50} for PKCδ of <0.05 μM and targeted selectivity over classical PKC isozymes (a 1000-fold PKCδ selectivity over PKCα) (Table 1). BJE6-154 (B154) was among the least potent of the 36 compounds studied (PKCδ IC_{50} > 40 μM) and was used as a negative-control compound with minimal inhibitory activity against PKCδ.

Inhibition of PKCδ Activity Induces Cell Growth Inhibition in Melanoma Cell Lines with NRAS Mutations. To investigate the effect of PKCδ 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 μM) or B106 (0.2 or 0.5 μM) and viable cells were quantitated at 24, 48, and 72 h after treatment. Rottlerin consistently inhibited proliferation of all cell lines at 5 μM, and

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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 PKCδ 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 PKCδ 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 PKCδ inhibition in a different manner, clonogenic colony assays were performed using SBcl2 melanoma cells to determine the kinetics of the action of PKCδ 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 PKCδ inhibitors on tumor cell growth, even after limited and transient exposure to the compounds.

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

Inhibition of PKCδ Activity Triggers Caspase-Dependent Apoptosis. We next determined how PKCδ 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 PKCδ 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

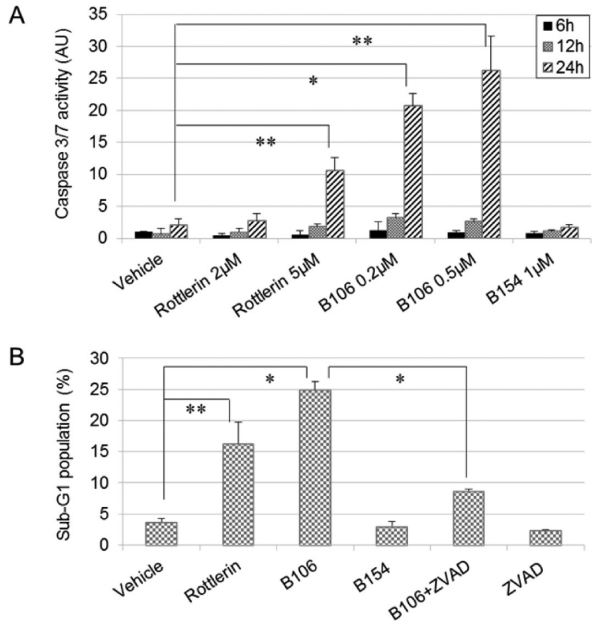

Figure 3. Inhibition of PKCδ induces caspase-dependent apoptosis. (A) Effector caspase 3/7 activation by PKCδ inhibition. SBcl2 cells were exposed to rottlerin (2 or 5 μM) or B106 (0.2 or 0.5 μM) for 6, 12, or 24 h and caspase 3/7 activity was measured. DMSO and B154 (1 μM) served as a vehicle control 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 PKCδ 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 PKCδ 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 PKCδ 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 PKCδ-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 PKCδ activity (Figure 3B). To determine whether activation of caspases by PKCδ inhibitors was necessary for the observed apoptosis, the pan-caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[Omethyl]-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 PKCδ inhibition attenuates tumor cell

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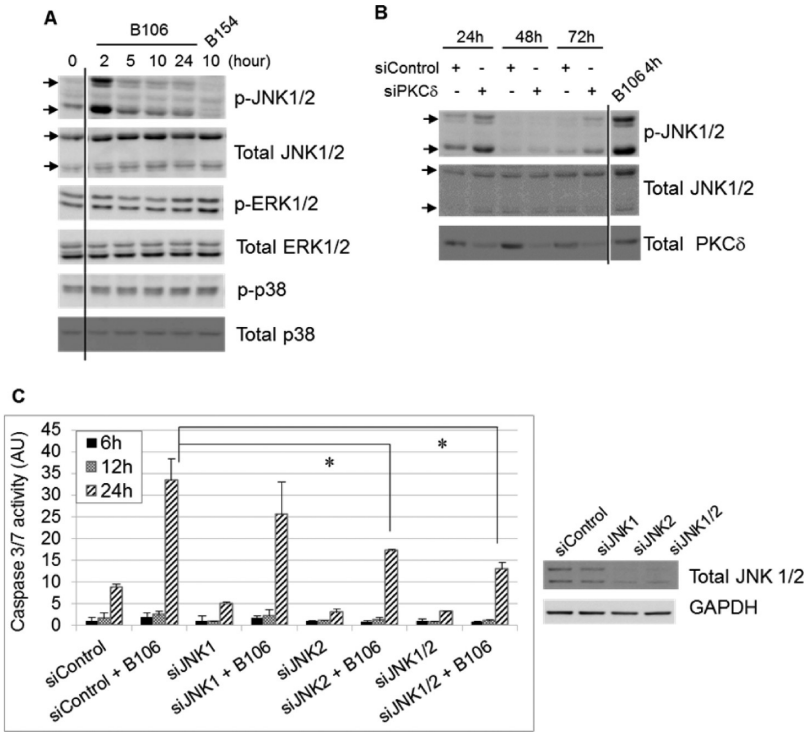

Figure 4. PKCδ inhibition triggers an apoptotic response through activation of JNK. PKCδ inhibition activates JNK. (A, B) SBcl2 cells were exposed to B106 (1 μM) or the negative control compound B154 (1 μM) for indicated times (A) or transfected with siRNA targeting PKCδ ("siPKCδ") or nontargeting siRNA ("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 μM) 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.

PKCδ Inhibition Triggers Apoptotic Response via the Stress-Responsive JNK Pathway. To identify which intracellular signaling pathway PKCδ inhibition employs to induce cytotoxicity, the activation status of known downstream targets of PKCδ was examined after PKCδ inhibition, including MAPKs (ERK, p38, and JNK), AKT, NFκB pathway, cyclin-dependent kinase inhibitors, p53, IAPs, GSK3β, or c-Abl. Inhibition of PKCδ 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 PKCδ inhibitors (Figure 4A). Consistent with these observations generated using chemical inhibitors, selective downregulation of PKCδ by transfection of PKCδ-specific siRNA induced phosphorylation of JNK1/2 at 24 h, (when effects of siRNA on PKCδ levels were first observed) (Figure 4B). Transfection of PKCδ-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 PKCδ 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 PKCδ inhibition.

PKCδ Inhibition Activates the MKK4-JNK-H2AX Pathway. We tested for involvement of known upstream and downstream effectors of the JNK pathway following PKCδ 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 PKCδ 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 PKCδ inhibition on H2AX activation was further confirmed by selective downregulation of PKCδ with siRNA. Phosphorylation of H2AX was observed at 72 h after PKCδ

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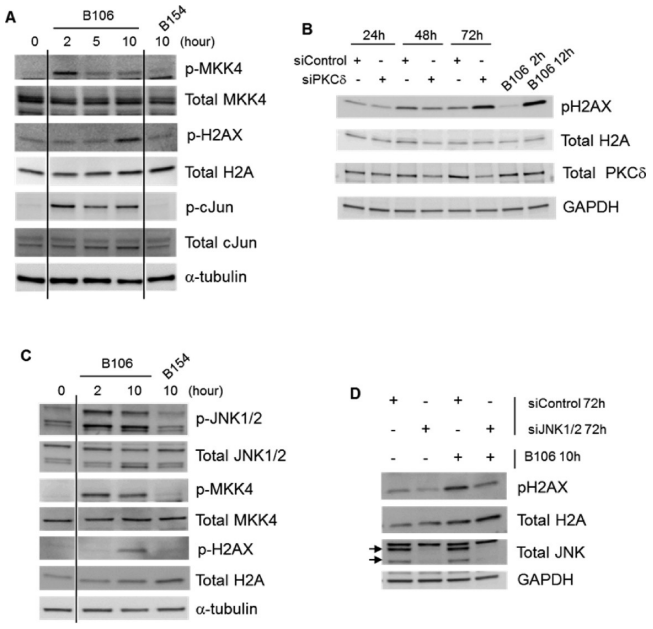

Figure 5. PKCδ inhibition activates the MKK4-JNK-H2AX pathway. (A) Activation of upstream and downstream components of the JNK pathway by B106. SBcl2 cells were exposed to B106 or the negative control compound B154 at 1 μM for the indicated times. Protein lysates were subjected to immunoblot analysis. (B) Selective downregulation of PKCδ results in phosphorylation of H2AX. SBcl2 cells were transfected with siRNA targeting PKCδ ("siPKCδ") 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) PKCδ 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 μM) or vehicle (DMSO) for 10 h. Protein lysates were subjected to immunoblot analysis. Arrows indicate JNK1/2.

siRNA transfection (Figure 5B). PKCδ 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 PKCδ 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 PKCδ 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 PKCδ 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 PKCδ 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).

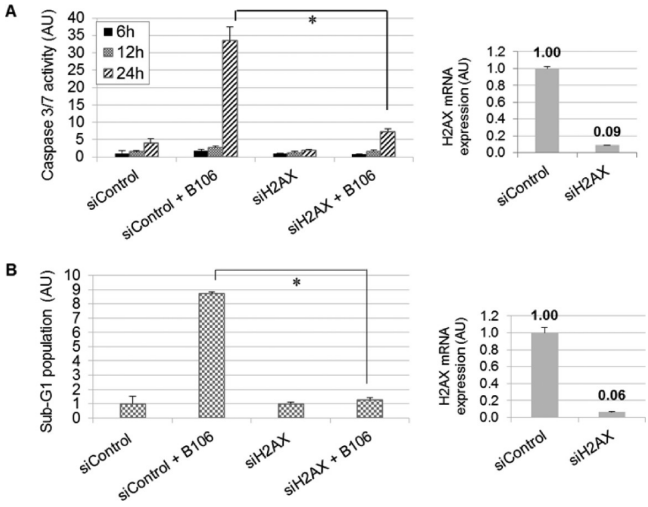

Figure 6. H2AX is a critical apoptotic regulator in apoptosis induced by PKCδ inhibition. (A) Activation of caspases 3/7 is mitigated by knockdown of H2AX prior to B106 treatment. SBcl2 cells were transfected with siRNA targeting H2AX or nontargeting siRNA at 5 nM for 72 h, and subsequently exposed to B106 (0.5 μM) 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 at72 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 μM) 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, PKCδ 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 PKCδ inhibition by exposure to B106 for 24 h. PKCδ inhibition by B106 treatment increased DNA fragmentation 8.5-fold in the cells transfected with negative control siRNA (Figure 6B). In contrast, PKCδ 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 PKCδ 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 PKCδ Inhibition. The inevitable

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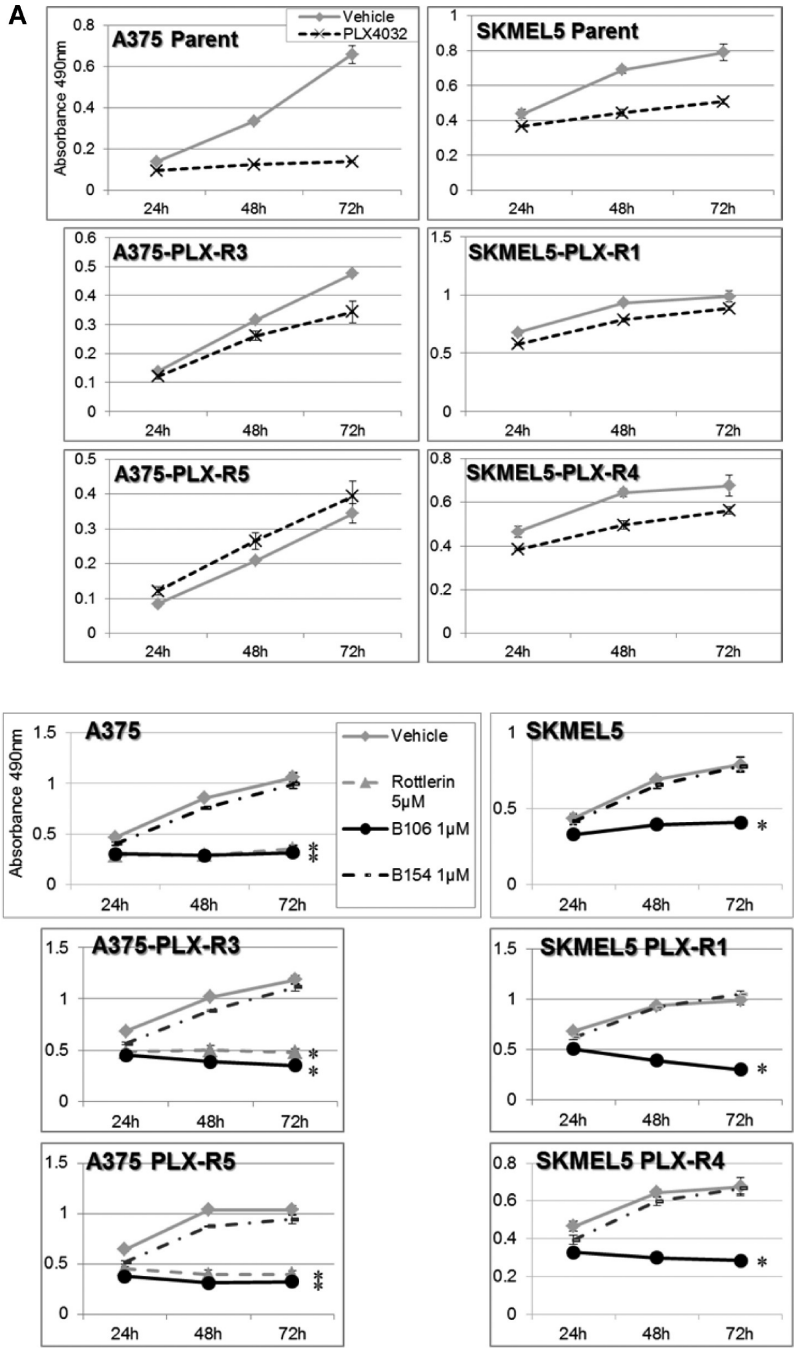

Figure 7. PKCδ inhibitors suppress growth of PLX4032-resistant BRAF mutant melanoma cells. (A) Establishment of PLX4032-resistant cell sublines. To establish PLX4032 resistant cell lines, two individual melanoma cell lines with BRAF mutations, A375 and SKMEL5, were continuously exposed to increasing concentrations of PLX4032 up to 10 μM (A375) and 2 μM (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 μM. (B) PKCδ 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 μM) or B106 (1 μM) for 24, 48, or 72 h, and MTS assays were performed at each time point. DMSO and B154 (1 μM) 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 μM, or DMSO and B106, 1 μM 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 PKCδ 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 PKCδ 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

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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 PKCδ inhibitors at concentrations comparable to the NRAS mutant melanoma lines (Figure 7B). The parental cell lines A375 and SKEML5 (both BRAF-V600E mutant) were also susceptible to PKCδ 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 PKCδ activity for survival.[9−12]

PKCδ 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 PKCδ 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 PKCδ and RAS, further work disclosed that aberrant activation of certain RAS effector pathways, PI_3K-AKT and CRAF-MEK, would also confer sensitivity to PKCδ inhibition.[9−12] Importantly, PKCδ was demonstrated to be nonessential for the survival and proliferation of normal cells and animals,[21] suggesting that a therapeutic approach targeting PKCδ would likely spare normal cells, but inhibit the proliferation of tumor cells whose survival depends on PKCδ activity. This report underlines the potential of PKCδ-targeted therapy as a cancer-specific therapy targeting melanoma with NRAS mutations. Cell proliferation and clonogenic assays demonstrated that inhibition of PKCδ 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 PKCδ inhibitors does not depend on a specific NRAS mutation for their activity. Similarly, PKCδ 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 PKCδ 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 PKCδ inhibition.[12]

The novel PKCδ inhibitor B106, which showed 1000-fold selectivity against PKCδ over PKCα 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 PKCδ 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 PKCδ, 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 PKCδ inhibition activates the JNK pathway through MMK4 to mediate caspase-dependent apoptosis. Consistent with our findings, a recent report demonstrated that knockdown of PKCδ 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] PKCδ inhibition evoked phosphorylation of H2AX subsequent to JNK activation, positioning H2AX phosphorylation downstream of JNK after PKCδ 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 PKCδ 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 PKCδ, 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 μM).[9−11] Herein, we show that human primary melanocytes are not affected by B106. In addition, continuous local infusion of B106 at 5 μM concentrations is not cytotoxic to dermal and subdermal tissues in mice. Derivatives of the third generation PKCδ 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 PKCδ 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. PKCδ-inhibitory activity data. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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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.

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RESEARCH ARTICLE Open Access

Protein kinase C-delta inactivation inhibits the proliferation and survival of cancer stem cells in culture and *in vivo*

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Abstract

Background: A subpopulation of tumor cells with distinct stem-like properties (cancer stem-like cells, CSCs) may be responsible for tumor initiation, invasive growth, and possibly dissemination to distant organ sites. CSCs exhibit a spectrum of biological, biochemical, and molecular features that are consistent with a stem-like phenotype, including growth as non-adherent spheres (clonogenic potential), ability to form a new tumor in xenograft assays, unlimited self-renewal, and the capacity for multipotency and lineage-specific differentiation. PKCδ is a novel class serine/threonine kinase of the PKC family, and functions in a number of cellular activities including cell proliferation, survival or apoptosis. PKCδ has previously been validated as a synthetic lethal target in cancer cells of multiple types with aberrant activation of Ras signaling, using both genetic (shRNA and dominant-negative PKCδ mutants) and small molecule inhibitors. In contrast, PKCδ is not required for the proliferation or survival of normal cells, suggesting the potential tumor-specificity of a PKCδ-targeted approach.

Methods: shRNA knockdown was used validate PKCδ as a target in primary cancer stem cell lines and stem-like cells derived from human tumor cell lines, including breast, pancreatic, prostate and melanoma tumor cells. Novel and potent small molecule PKCδ inhibitors were employed in assays monitoring apoptosis, proliferation and clonogenic capacity of these cancer stem-like populations. Significant differences among data sets were determined using two-tailed Student's t tests or ANOVA.

Results: We demonstrate that CSC-like populations derived from multiple types of human primary tumors, from human cancer cell lines, and from transformed human cells, require PKCδ activity and are susceptible to agents which deplete PKCδ protein or activity. Inhibition of PKCδ by specific genetic strategies (shRNA) or by novel small molecule inhibitors is growth inhibitory and cytotoxic to multiple types of human CSCs in culture. PKCδ inhibition efficiently prevents tumor sphere outgrowth from tumor cell cultures, with exposure times as short as six hours. Small-molecule PKCδ inhibitors also inhibit human CSC growth *in vivo* in a mouse xenograft model.

Conclusions: These findings suggest that the novel PKC isozyme PKCδ may represent a new molecular target for cancer stem cell populations.

Keywords: Protein Kinase C isozymes, Synthetic lethal interaction, Cancer-initiating cell, Xenograft tumor model

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Background

Much recent data supports the model that a subpopulation of tumor cells with distinct stem-like properties is responsible for tumor initiation, invasive growth, and possibly dissemination to distant organ sites [1-3]. This small subpopulation of cells can divide asymmetrically, producing an identical daughter cell and a more differentiated cell, which, during their subsequent divisions, generate the vast majority of tumor bulk [4,5]. A number of names have been used to identify this subpopulation, including "cancer progenitor cells," "cancer stem cell-like cells," and "cancer-initiating cells," but the term "cancer stem cell" (CSC) has received wide acceptance [6].

The first identification of CSCs in solid tumors was made in 2003, when CSCs were identified and isolated from breast cancers using CD44 and CD24 markers [7]. Subsequently, CSCs have been identified in a variety of solid tumors, including glioblastoma [8-10], osteosarcoma [11], chondrosarcoma [12], prostate cancer [13], ovarian cancer [14-18], gastric cancer [19], lung cancer [20,21], colon cancer [22-25], pancreatic cancer [26,27], melanoma [28-30], head and neck cancer [31], and others. CSCs isolated from these different tumor types share some common characteristics including drug resistance, ability to repopulate tumors, and asymmetric division.

CSC exhibit a spectrum of biological, biochemical, and molecular features that are consistent with a stem-like phenotype, including growth as non-adherent spheres (clonogenic potential), superior ability to form a new tumor in *in vivo* xenograft assays, unlimited self-renewal, and the capacity for multipotency and lineage-specific differentiation [1,32-35]. In particular, CSCs are able to form colonies from a single cell more efficiently than their progeny [36] and to grow as spheres in non-adherent, serum-free culture conditions [37]. Sphere formation in non-adherent cultures has been used as a surrogate *in vitro* method for detecting CSCs from primary human tumors [8,20,25,38,39]. CSC populations also variably exhibit "stem cell-like" markers, such as Nanog, Sox2, aldehyde-dehydrogenase positivity, and telomerase.

Chemoresistance is also considered a hallmark of CSCs [6,40]. They characteristically survive chemo- and radiotherapeutic interventions [41] and may thus be responsible for both tumor relapse and metastasis [42]. CSCs are often innately less sensitive to treatment than are the bulk of the tumor cells that they generate [43,44]. These features support the hypothesis that CSCs are the cell subpopulation that is most likely responsible for treatment failure and cancer recurrence [32].

Aberrant activation of Ras signaling, either through mutation of the Ras genes themselves, or through constitutive upstream or downstream signaling, is very common in solid tumors. We have previously identified the protein kinase C delta (PKCδ) isozyme as a Ras synthetic lethal interactor [45-48]. PKCδ is a serine/threonine kinase of the PKC family, a member of the novel class, and functions in a number of cellular activities including cell proliferation, survival or apoptosis [49]. However, PKCδ is not required for the proliferation of normal cells, and PKCδ-null animals develop normally and are fertile, suggesting the potential tumor-specificity of a PKCδ-targeted approach [50]. PKCδ was validated as a target in cancer cells of multiple types with aberrant activation of Ras signaling, using both genetic (siRNA and dominant-negative PKCδ) and small molecule inhibitors [45], by our group [45,47] and later by others [51,52]. "Ras-dependency" in these tumors was not required for these synthetic-lethal cytotoxic effects [45,46]. Tumors with aberrant activation of the PI_3K pathway or the Raf-MEK-ERK pathway in the setting of wild-type RAS alleles have also been shown to require PKCδ activity for proliferation or survival [47,48].

In this report, we demonstrate that CSC-like cell populations derived from multiple types of human primary tumors, from human cancer cell lines, and from transformed human cells require PKCδ activity and are susceptible to agents which deplete PKCδ protein or activity.

Methods

Cell culture

MCF10A and MCF10C breast cell lines were derived at the Barbara Ann Karmanos Cancer Institute (Detroit, MI) and maintained in DMEM-F/12 medium containing 5% heat-inactivated horse serum, 10 μg/mL insulin, 20 ng/mL epidermal growth factor, 0.1 μg/mL cholera enterotoxin, and 0.5 μg/mL hydrocortisone [53,54]. Breast cancer cell lines MCF7, Hs587T, and MDA231 were purchased from ATCC, and were propagated in 10% fetal bovine serum (Invitrogen, Grand Island, NY); Dulbecco's Modification of Earle's Media (Cellgro, Herndon, VA); 2 mM L-Glutamine (Invitrogen); 200 U Penicillin/ml; 200 μg Streptomycin/ml (Invitrogen).

Human breast cancer stem cells (BCSC: CD133+, CD44+, SSEA3/4+, Oct4+, Alkaline Phosphatase+, Aldehyde Dehydrogenase+, Telomerase+), pancreatic cancer stem cells (PCSC: $CD44^+$, $CD133^+$, $SSEA3/4^+$, $Oct4^+$, Alkaline $Phosphatase^+$, Aldehyde $Dehydrogenase^+$, $Telomerase^+$, and $Nestin^+$), and prostate cancer stem cells (PrCSC: $CD44^+$, $CD133^+$, $SSEA3/4^+$, $Oct4^+$, alkaline $phosphatase^+$, aldehyde $dehydrogenase^+$, and $telomerase^+$) were purchased from Celprogen (San Pedro, CA), and cultured using specialized media and tissue culture plastic and matrix, to preserve their CSC phenotype, according to the manufacturer's instructions.

Reagents

Rottlerin was purchased from (EMD Biosciences, San Diego, CA). The PKCδ inhibitor KAM1 was previously

described [47]. BJE6-106 was synthesized as described elsewhere [55]. Briefly, 9-(2-(trifluoro-λ^4-boranyl) ethyl)-9*H*-carbazole, potassium salt (Molander Salt 1), 6-bromo-2,2-dimethyl-2H-chromene-8-carbaldehyde, 64.0 mg (0.213 mmol, 1 equiv.), PdCl2(dppf)-CH2Cl2, and anhydrous Cs_2CO_3 were combined to form 6-(2-(9*H*-carbazol-9-yl)ethyl)-2,2-dimethyl-2*H*-chromene-8-carbaldehyde (BJE6-106).

Tumor sphere formation

Tumor self-renewing and anchorage-independent spheroids were obtained by culturing breast cancer cells MCF7, Hs587T and MDA231; melanoma cells SBcl2 and FM6; human breast cancer stem cells and pancreatic cancer stem cells in stem cell-selective conditions according to the manufacturer's instructions (StemCell Technologies, Tukwila, WA). Briefly, cancer and cancer stem cells were propagated in 6-well ultra-low adherent plates (Corning) in Complete MammoCult Medium (Human) by adding 50 mL of MammoCult Proliferation Supplements to 450 mL of MammoCult Basal Medium (StemCell Technologies). The following were added to obtain Complete MammoCult Medium: 4 ug/mL Heparin (StemCell Technologies), 0.48 μg/mL hydrocortisone (StemCell Technologies), 200 U penicillin/ml; and 200 μg streptomycin/ml (Invitrogen).

Flow cytometry

Cell staining for CD24 or CD44: MCF7 and MCF7 spheres, Hs587T and Hs587T spheres, MDA231 and MDA231 spheres, breast cancer stem cells and breast cancer stem cell spheres were collected and stained or dual-stained with Fluorescein isothiocyanate (FITC)-anti-CD24 and (PerCP-Cy)-anti-CD44 (BD Pharmingen, San Diego, CA) monoclonal antibody (mAbs) for 30 min on ice. The stained cancer cells and sphere populations were analyzed by FACSCAN analysis.

Clonogenic assays

100,000 cells were seeded on 100 mm dishes with 10 ml media per dish [47]. On day 4, cells were treated with a PKCδ inhibitor or vehicle control for either 6, 18, 24 or 48 hours. Cells were trypsinized; counted *via* the trypan blue exclusion method in order to determine the number of live cells in the sample, and 300 live cells were seeded in triplicate onto 6-well plates. Cells were monitored for appropriate colony size and re-fed every three to four days. At Day 15, cells were stained with ethidium bromide [56] and counted using UVP LabWorks software (Waltham, MA).

Cell proliferation assays

Cell proliferation was assessed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Roche, Mannheim, Germany). The number of viable cells growing in a single well on a 96-well microtiter plate was estimated by adding 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline [PBS]). After 4 h of incubation at 37°C, the stain was diluted with 100 μl of dimethyl sulfoxide. The optical densities were quantified at a test wavelength of 570 nm and a reference wavelength of 690 nm on a multiwell spectrophotometer. In some assays, MTS was used as substrate (Promega, Madison, WI), and the absorbance of the product was monitored at 490 nm. Cell enumeration was carried out using a hemocytometer, and viable cells identified by trypan blue exclusion.

PKC kinase activity assays

Assays were carried out using recombinant PKCδ or PKCα, (Invitrogen) and the Z-lyte Kinase Assays (Invitrogen) with a "PKC-kinase-specific" peptide substrate. FRET interactions produce a change in fluorescence (ex455/ex520) upon phosphorylation. The kit was used according to the manufacturer's instructions.

Cytotoxicity assay

LDH release was assessed by spectrophotometrically measuring the oxidation of NADH in both the cells and media. Cells were seeded in 24-well plates, and exposed to PKCδ inhibitors or vehicle. After different times of exposure, cytotoxicity was quantified by a standard measurement of LDH release with the use of the LDH assay kit (Roche Molecular Biochemicals) according to the manufacturer's protocol. Briefly, total culture medium was cleared by centrifugation. For assay of released LDH, supernatants were collected. To assess total LDH in cells, Triton X-100 was added to vehicle (control) wells to release intracellular LDH. LDH assay reagent was added to lysates or supernatants and incubated for up to 30 min at room temperature in dark, the reaction was stopped, and the absorbance was measured at 490 nm. The percentage of LDH release was then calculated as the LDH in the supernatants as a fraction of the total LDH.

Immunoblot analyses

Levels of proteins were measured and quantitated in cells as we have previously reported [45]. Harvested cells were disrupted in a buffer containing 20 mM Tris (pH 7.4), 0.5% NP-40, and 250 mM NaCl with protease and phosphatase inhibitors. Total protein (40 μg) was separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes or PVDF membranes. Membranes were blocked overnight and probed with affinity-purified antibodies against: PKCδ (BD Transduction Labs, San Jose, CA), or β-actin or α-tubulin (Sigma Aldrich, St. Louis, MO). Antibodies against human ERK, phospho-ERK1/2 (Thr202/Tyr204), AKT and phospho-AKT (Ser473), JNK and phospho-JNK (Thr183/Tyr185) were purchased from

Cell Signaling (Danvers, MA). After washing, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies and visualized using the Amersham enhanced chemiluminescence ECL system, and quantitated by digital densitometry.

Down-regulation of PKC by shRNA and lentiviral vectors

shRNA duplexes for PKCδ (shRNAs) were obtained from Qiagen (Valencia, Ca). The shRNA sequences for targeting PKCδ and the corresponding scrambled shRNAs used as negative controls were previously described [47]. The lentiviral vectors were previously described [46]. After infection of cells with the vectors, one aliquot was utilized in proliferation assays and a parallel aliquot was subjected to immunoblotting to assay the efficiency of the knockdown.

Xenograft studies

These studies were performed with the approval of the Institutional Animal Care and Use Committee of Boston University. Breast cancer stem cells (2×10^5) grown from a metastatic tumor were suspended in human breast cancer stem cell complete growth media (Celprogen, San Pedro, CA) and injected subcutaneous into the right flank of female J:NU mice (The Jackson Laboratory, ME) under anesthesia. After palpable tumors developed, the mice were divided into two groups of animals. The control group received daily intraperitoneal injections of vehicle (DMSO) while the treatment group received daily intraperitoneal injections of a PKCδ inhibitor (rottlerin 5,000 μg/kg) for 15 days. The length and width of tumors were measured with a vernier caliper and tumor volumes were calculated. Survival was calculated as the day tumors reached the maximum size allowed by the protocol (2 cm diameter).

Statistical analysis

Experiments were carried out in triplicate for all experimental conditions. Data are shown as mean ± SD. Where applicable, a two-tailed Student's t test or ANOVA was performed on the means of two sets of sample data and considered significant if $p \leq 0.05$.

Results

Inhibition of PKCδ is growth-inhibitory and cytotoxic in human prostate and pancreatic cancer stem cells

The sensitivity of human cancer stem cell cultures to inhibition of PKCδ was first examined using shRNA methodology to specifically and selectively knockdown transcripts for this PKC isozyme and thereby specifically validate PKCδ as a target in CSCs. Cell cultures derived from a primary human pancreatic adenocarcinoma (PCSC) and from a primary human prostate adenocarcinoma (PrCSC), isolated by phenotypic markers, were studied. These cells were characterized as "stem-like" by a number of criteria. The PCSC and the PrCSC cultures were $CD44^+$, $CD133^+$, $Nanog^+$, $Sox2^+$, aldehyde dehydrogenase$^+$, and telomerase$^+$. The PCSC cultures were also $Nestin^+$. Both cell types were tumorigenic at <1000 cells in xenograft assays in SCID mice, and also formed tumor spheroids at high efficiency. Lentiviral vectors expressing PKCδ-specific shRNAs (PKCδ-shRNA), which we have previously shown to be specific for the PKCδ isozyme among all the other PKC isozymes [45-47], were used to deplete PKCδ levels in the cells. A vector containing a scrambled shRNA (sc-shRNA) served as a control. Specific knockdown of PKCδ by shRNA was growth-inhibitory in both the human prostate (PrCSC) and pancreatic (PCSC) cancer stem cells, with significant effects observed at early as 24 hr after infection, and progressing up to 72 hr (Figure 1A). The non-targeted lentiviral vector (sc-shRNA) generated modest but reproducible effects on cell growth over time, as we have observed in prior reports [45-47]. Cytotoxic effects of PKCδ depletion on the PCSC and PrCSC cultures were assessed by quantitating release of cellular LDH. Significant cytotoxicity was elicited by the PKCδ-specific shRNA as early as 24 hr after infection, with LDH release approaching the maximum possible levels by 72 hr. The effects of the scrambled shRNA on LDH release did not differ from those of the infection vehicle alone at any time point (Figure 1B). Efficient knockdown of the PKCδ isozyme was verified by immunoblotting (Figure 1C).

While the specificity of shRNA is essential for validation of a target, small-molecule enzyme inhibitors are more likely than shRNA to translate towards clinical application. We therefore next examined the effects of existing and novel small molecule inhibitors of PKCδ. Rottlerin, a natural product, has been identified as a PKCδ inhibitor for many years [47], with an *in vitro* IC_{50} of approximately 5 μM in our kinase assays (Table 1), in good agreement with the literature [57,58] (although it also exerts inhibitory effects on certain non-PKC kinases at concentrations comparable to the IC_{50} for PKCδ [59]). We and others have shown that 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 (see also the studies on normal human breast epithelial cells and the *in vivo* studies later in this report) [45-47]. Exposure of PCSC and PrCSC cultures to rottlerin produced a significant dose-dependent inhibition of proliferation as early as 24 hr after exposure (Figure 2A). Similarly, rottlerin induced cytotoxicity in both CSC cultures in a dose-dependent fashion, as assessed by LDH release (Figure 2B). The duration of PKCδ inhibition required to irreversibly prevent CSC proliferation was next assessed. Exposure to rottlerin efficiently decreased the clonogenic capacity of PCSC. Eighteen hr of exposure to rottlerin, followed by washout,

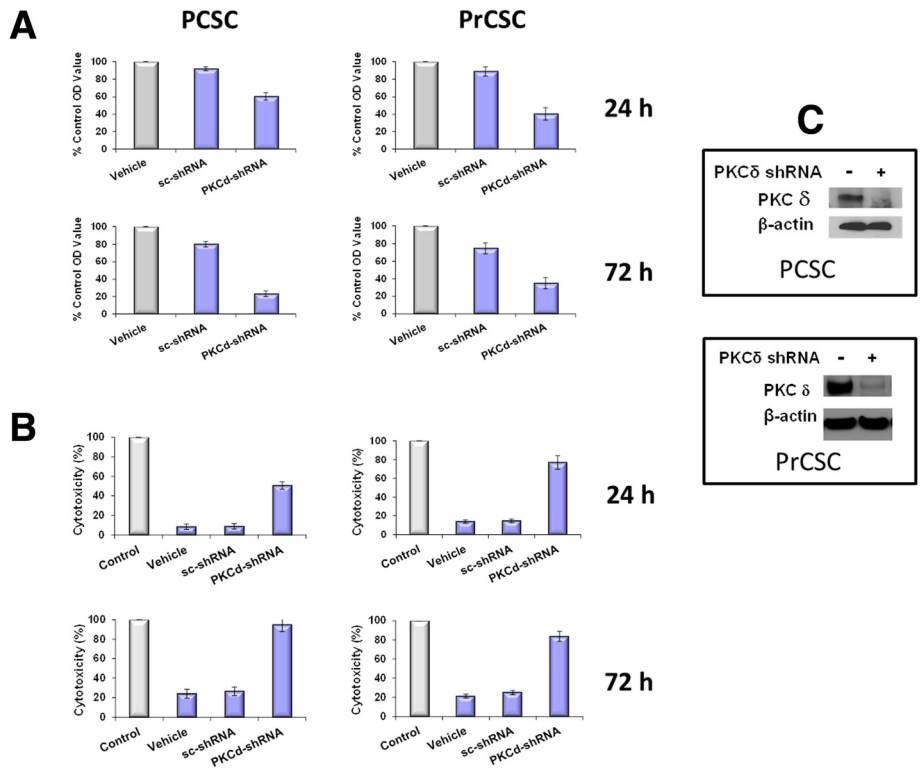

Figure 1 Effects of PKCδ knockdown by shRNA on proliferation and viability of human pancreatic (PCSC) and prostate (PrCSC) cancer stem cell cultures. (A) PCSC and PrCSC cells were grown to 50% confluence in 96-well plates and then infected with PKCδ-shRNA-expressing lentivirus vector or a lentiviral vector containing a scrambled shRNA (sc-shRNA). The corresponding equivalent volumes of diluent used for infection served as vehicle controls (Vehicle). 24 and 72 hr after transfection, cell mass was evaluated by MTS assay. Error bars represent SEM. p values for comparison between control (scrambled shRNA) and PKCδ-shRNA effects on cell number reached significance at 24 hr of exposure ($p < 0.001$) for all cell lines, and remained significant at the 72 hr time point. **(B)** PCSC and PrCSC cells were grown to 50% confluence in 96-well plates and then infected with PKCδ-shRNA or scrambled shRNA (sc-shRNA) expressing lentiviruses. The corresponding equivalent volumes of diluent were used as vehicle controls (Vehicle). After 24 and 72 hr of infection, cell cytotoxicity was evaluated by LDH-release assay. Total maximal LDH release was assigned the arbitrary value of 100% (Control). Error bars represent SEM. p values for comparison between effects on LDH release for cells infected with scrambled shRNA-expressing vectors compared to PKCδ-shRNA vectors reached significance at 24 hr of exposure ($p < 0.01$) for all cell lines, and remained significant at the 72 hr time point. **(C)** Immunoblot analysis of PKCδ protein levels in the same cell lines 72 hr after infection with PKCδ-targeting shRNA expressing lentiviral vectors (+) or scrambled shRNA (–). PKCδ-targeted shRNA vectors efficiently reduced PKCδ protein expression. Immunoblotting with a β-actin antibody after stripping the blots served as a loading control.

was sufficient to decrease the clonogenic capacity of PCSC by 40%, and increasing the duration of the exposure to 48 hr reduced the clonogenic potential by more than 90% (Figure 2C).

As previously reported, we have sought to develop novel PKCδ-inhibitory molecules with greater specificity for PKCδ compared to essential PKC isozymes, such as PKCα, using pharmacophore modeling and structure-activity relationships (SAR) [47]. We designed and synthesized a set of analogs based on this strategy. In this 2nd generation of PKCδ inhibitors, the "head" group (carbazole portion) was made to resemble that of staurosporine, a potent general PKC inhibitor, and other bisindoyl maleimide kinase inhibitors, with two other domains (cinnamate side chain and benzopyran) conserved from the rottlerin scaffold to preserve isozyme specificity. The first such chimeric molecule reported, KAM1 (Figure 2D), was indeed active, like staurosporine, but was also more PKCδ-specific, and showed potent activity against Ras-mutant human cancer cells in culture and *in vivo* animal models, while not producing cytotoxicity in non-transformed cell lines [47]. KAM1 induced cytotoxicity as assessed by LDH release in a dose-dependent fashion in both PCSC and PrCSC cultures at concentrations as low as 2.5 μM (PCSC) and 5 μM (PrCSC) (Figure 2E).

Table 1 Comparison of three generations of PKCδ inhibitors

Generation	PKCδIC$_{50}$	PKCαIC$_{50}$	PKCδ/PKCα Selectivity ratio
1	3 μM	75 μM	28-fold
2	2 μM	157 μM	56-fold
3	0.05 μM	50 μM	1000-fold

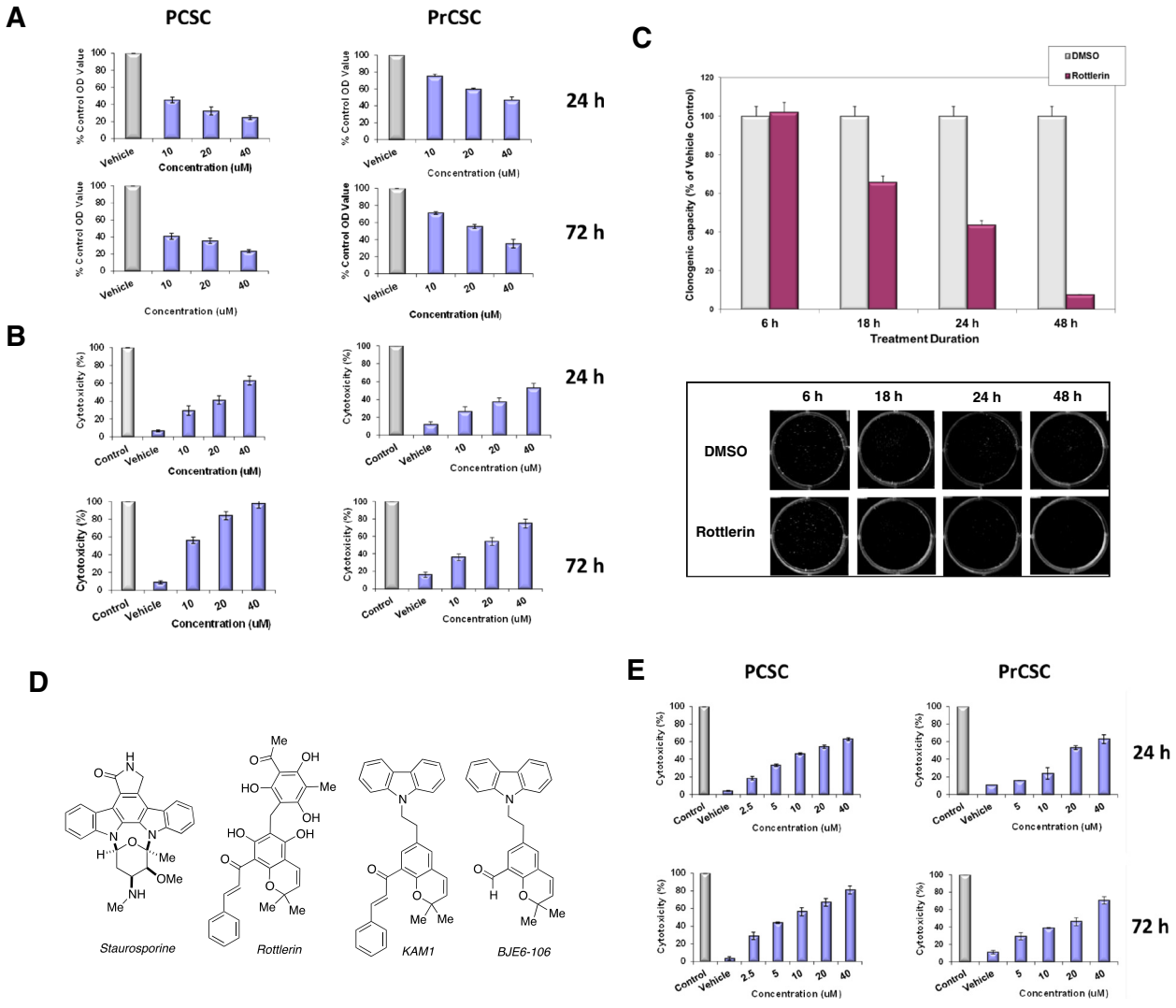

Figure 2 Effects of PKCδ inhibitors on human cancer stem cells. (A) PCSC and PrCSC cells at 80% confluence were exposed to rottlerin. DMSO served as vehicle control (Vehicle). After 24 and 72 hr of exposure, cell mass was evaluated by MTT assay. Control values were normalized to 100%. p values for comparison between treatments reached significance at 24 hr of exposure ($p \leq 0.01$) for both cell types, and remained significant at 72 hr. **(B)** PCSC and PrCSC cells at 50% confluence were exposed to rottlerin. Cytotoxicity was evaluated by LDH-release assay. Total maximal LDH release was assigned the arbitrary value of 100% (Control). p values for comparison between effects of treatments on LDH release reached significance at 24 hr of exposure ($p < 0.01$) for both cell types, and remained significant at 72 hr. **(C)** Effects of PKCδ inhibitor on tumor cell clonogenic capacity. PCSC were exposed to vehicle or rottlerin (10 μM) for 6, 18, 24, or 48 hr. Viable cells were enumerated and re-plated in media without inhibitor, and colony numbers were quantitated 15 days later. p values for comparison of treatment effects on clonogenic capacity reached significance ($p = 0.005$) at 18 hr of exposure and remained significant for all subsequent exposure times. The insert is a photograph of stained colonies on plates. **(D)** Structures of staurosporine, rottlerin, second-generation (KAM1) and third-generation (BJE6-106) derivatives. **(E)** PCSC and PrCSC cells at 50% confluence were exposed to KAM1 at the indicated concentrations. DMSO served as vehicle control (Vehicle). Cytotoxicity was evaluated by LDH-release assay, as in panel B. p values for comparison between treatment effects on LDH release reached significance at 24 hr of exposure to 2.5 μM KAM1 for PCSC cells and at 10 μM for PrCSC ($p \leq 0.01$), and remained significant at 72 hr for all concentrations of KAM1. Error bars represent SEM.

On the basis of SAR analyses of KAM1, we then designed thirty-six new 3rd-generation analogs. The synthetic chemistry platform that was used to prepare KAM1 was modified to synthesize these additional analogs, which were then tested for biochemical and cellular activity. The PKCδ-inhibitory activity and isozyme-specificity of this 3rd generation was quantitated *in vitro*. A number of these 3rd generation analogs demonstrated significant increases in potency and isozyme specificity over rottlerin (1st generation) and KAM1 (2nd generation). The new compound selected for study in this report, BJE6-106, is much more potent than rottlerin. BJE6-106 has an (*in vitro*) PKCδ IC_{50} in the range of 0.05 μM, compared to 3 μM for rottlerin (Table 1), is approximately 1000-fold more

inhibitory against PKCδ than PKCα *in vitro*, and produces cytotoxic activity against cells with aberrant Ras signaling at nM concentrations [55].

The activity of the 3rd generation PKCδ inhibitor BJE6-106 on the growth of PCSC cells in culture was compared to rottlerin. BJE6-106 inhibited the growth of PCSC cultures at concentrations as low as 0.1 μM, and had an (in culture) IC_{50} of approximately 0.5 μM at 48 hr (Figure 3). In contrast, rottlerin produced no significant inhibitory activity at 0.5 μM, and displayed an IC_{50} at 48 hr of approximately 3 μM. LDH release assays also showed greater than 10-fold increases in potency for BJE6-106 compared to rottlerin (data not shown).

Inhibition of PKCδ prevents tumor sphere formation

Sphere formation assays, which have been commonly used to identify and purify normal and malignant stem cells, were used to select a "CSC-like population" from established human breast cancer cell lines Hs578T, MDA231 and MCF7. A subpopulation of these cell lines could grow as non-adherent spheres and be continuously propagated in a defined serum-free medium *in vitro*. Flow cytometry and immunofluorescence analysis indicated that sphere-derived cells from cell lines contained a much larger proportion of cells expressing CD44, a candidate surface marker of breast cancer stem cells, and/or a smaller proportion of cells expressing the non-stem cell marker CD24, compared with adherent cells (Figure 4A). The frequency of spheroid formation relative to input cell number was low for the tumor cell lines (≤2-3%), as expected. In contrast, spheroid formation from the cultures of primary PCSC or primary breast cancer stem cells (BCSC) was much more efficient (45% and 53%, respectively). As expected, the CD24/CD44 profiles of cells in the spheres derived from the primary PCSC and BCSC did not differ from the adherent cells (not shown).

Addition of rottlerin or BJE6-106 to the culture medium very efficiently inhibited the formation of spheroids from all of these cell types (Figure 4B), demonstrating cytostatic or cytotoxic activity on tumor cells having a CSC-like phenotype. Interestingly, the actions of these compounds appeared to be even more potent on the CSC subpopulation in the MCF7 cell line than on the adherent "parental" cells (although different assays are being compared). When the MCF7 adherent population, containing predominantly non-CSC, was exposed

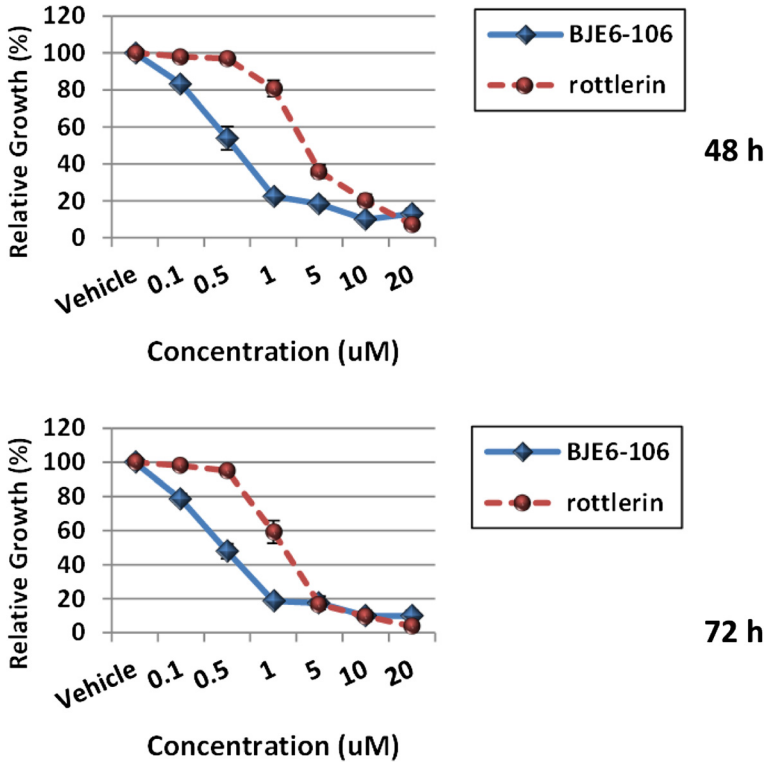

Figure 3 Effects of a 3rd generation small molecule PKCδ inhibitor on human pancreatic cancer stem cell cultures. PCSC cells were grown to 80% confluence in 96-well plates and then exposed to BJE6-106 at concentrations ranging from 0.1 to 20 μM, or to rottlerin at concentrations ranging from 1 to 20 μM. The corresponding equivalent volume of solvent (DMSO) was used as a vehicle control (Vehicle). After 48 and 72 hr of exposure, cell mass was evaluated by MTT assay. Control values were normalized to 100%. Error bars represent SEM. p values for comparison between vehicle and rottlerin effects on cell number at 48 hr reached significance at 1 μM, and for BJE6-106 at 0.1 μM ($p \leq 0.02$), and remained significant at the 72 hr time point.

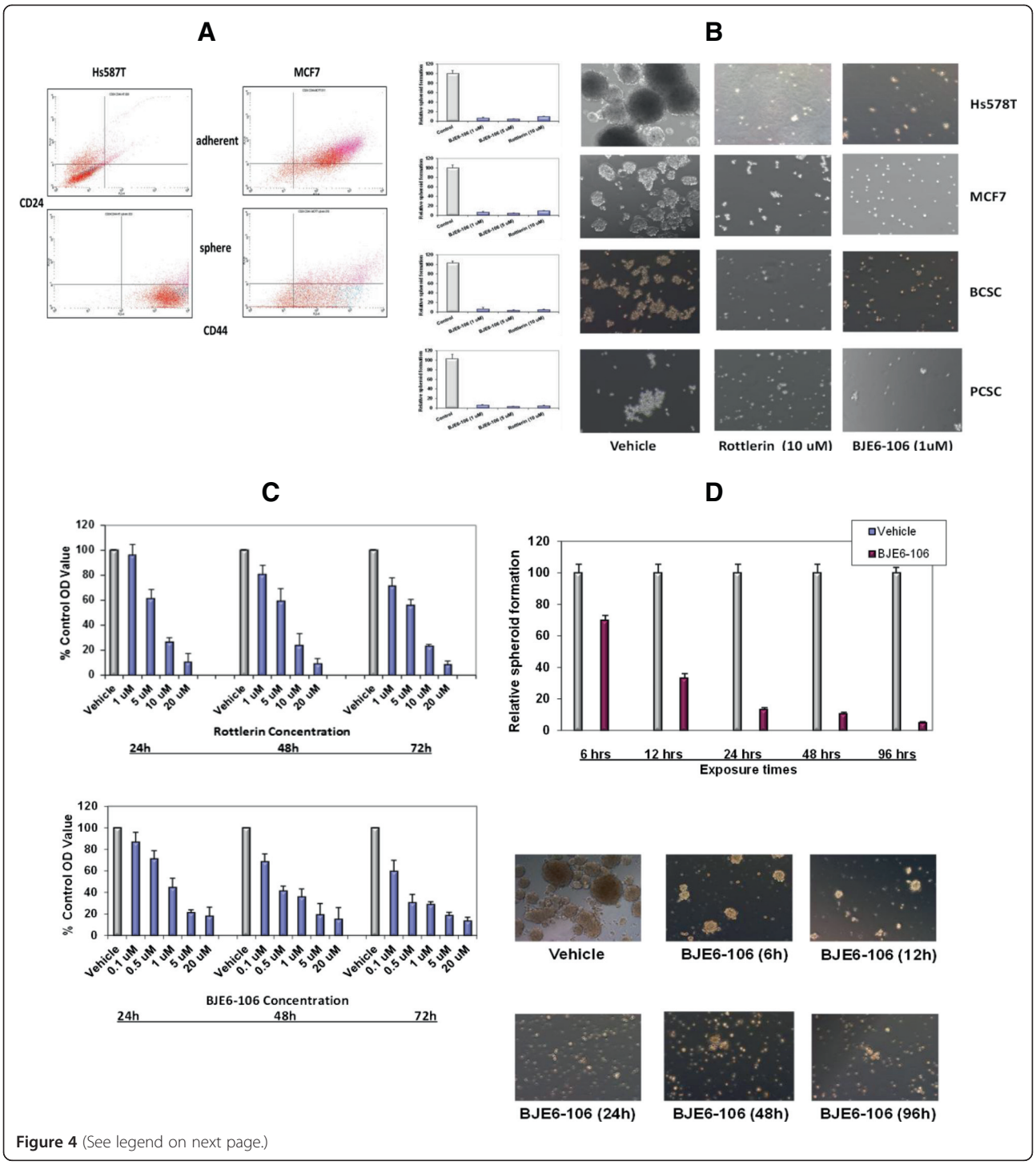

Figure 4 (See legend on next page.)

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Figure 4 Effects of PKCδ inhibitors on human tumor cell spheroid formation. (A) Hs578T and MCF7 were plated under adherent or non-adherent conditions. Tumor spheroids and adherent cells were collected at 96 hr, stained for CD24 and CD44, and analyzed by flow cytometry. **(B)** Hs578T, MCF7, breast cancer stem cells (BCSC) and pancreatic cancer stem cells (PCSC) were plated in tumor spheroid media, in the presence of rottlerin, BJE6-106, or DMSO (Control). Tumor spheroids were enumerated at 96 hr, and normalized to the number of spheroids in the control cultures (assigned an arbitrary value of 100%). p values for comparison between vehicle and rottlerin or BJE6-106 effects were significant ($p \leq 0.001$). Photographs are of representative areas of the culture plates. **(C)** MCF7 cells were exposed BJE6-106 or to rottlerin at the indicated concentrations. The corresponding equivalent volume of solvent (DMSO) was used as a vehicle control (Vehicle). After 24, 48 and 72 hr of exposure, cell mass was evaluated by MTT assay. Control values were normalized to 100%. p values for comparison between vehicle and rottlerin effects on cell number at 24 hr reached significance at 5 μM, and for BJE6-106 at 0.5 μM ($p \leq 0.02$), and were significant for all concentrations tested at 48 and 72 hr time points. **(D)** Hs578T cells were exposed to vehicle or BJE6-106 (1 μM) for 6, 12, 24, 48 or 96 hr. Viable cells were enumerated and re-plated in media without BJE6-206, and spheroid numbers were quantitated 96 hr later. p values for comparison between vehicle and BJE6-106 effects on spheroid number were significant after 6 hr of exposure ($p \leq 0.02$), and remained significant at all time points thereafter. Error bars represent SEM.

to rottlerin or BJE6-106, concentrations in excess of 10 μM and 1 μM, respectively, were required to repress growth by more than 80% (Figure 4C). In contrast, growth of MCF7 spheroids was inhibited greater than 90% by rottlerin at 10 μM and BJE6-106 at 1 μM. Washout studies using spheroid formation demonstrated that as little as 6 hr of exposure to BJE6-106 at 1 μM significantly repressed spheroid formation of Hs578T cells, with near maximum inhibition achieved by 24 hr of exposure (Figure 4D).

In parallel studies, BJE6-106 at 0.5-1.0 μM and rottlerin at 10 μM also efficiently inhibited the growth of tumor spheroids generated from two human melanoma cell lines (SBcl2, >99.5% inhibition, $p < 0.001$; FN5, >99.5% inhibition, $p < 0.001$), two human pancreatic cancer cell lines (MiaPaCa2, >97% inhibition, $p < 0.001$; Panc1, >99% inhibition, $p < 0.001$); and two prostate cancer cell lines (DU145, >98% inhibition, $p < 0.001$; PC3, >96% inhibition, $p < 0.001$).

A CSC-like phenotype can be induced during epithelial-mesenchymal transition (EMT) in transformed cell lines. Transformation of the "normal" human mammary epithelial cell line MCF 10A and selection for a tumorigenic, metastatic phenotype *in vivo* produced the derivative line MCF 10C [53,54], which exhibits an EMT phenotype [60]. Cells of this malignant derivative also became ALDH + [61]. Transformation of these cells rendered them sensitive to rottlerin (Figure 5A) and to BJE6-106 (Figure 5B), compared to the parental MCF 10A line. The IC_{50} of rottlerin and BJE6-106 for the MCF 10C derivative was approximately 1 μM and 0.1 μM, respectively, at 72 hr, whereas the IC_{50} for the parental MCF 10A cells were >20 μM.

The MCF 10C derivative also acquired the ability to efficiently form non-adherent spheroids (Figure 5C), in contrast to the parental MCF 10A cells. Growth of these spheroids was efficiently inhibited by exposure to rottlerin at 10 μM or to BJE6-106 at 1 μM (Figure 5D and E).

The relative lack of toxicity of PKCδ inhibition on the non-transformed, "normal" breast epithelial MCF 10A cells is noteworthy, and further supports the established non-essential role of this isozyme in normal cells and tissues. In other work, we have demonstrated that normal mouse embryo fibroblasts and human primary fibroblasts and epithelial cells and microvascular endothelial cells and primary melanocytes survive and proliferate in the setting of PKCδ knockdown or in concentrations of PKCδ inhibitors which are lethal to tumor cell lines with aberrant Ras signaling ([45-47,55]; Trojanowska et al., in preparation).

Inhibition of PKCδ inhibits CSC tumor xenograft growth

Another property of CSCs is their high tumorigenic potential. We therefore next sought to determine if PKCδ inhibition would inhibit the growth of CSCs *in vivo*. While the 3rd generation PKCδ inhibitory compounds such as BJE6-106 are more potent and more cytotoxic to tumor cells and CSCs than previous generations, they have not been optimized for drug-like properties and are highly hydrophobic and poorly bioavailable, making efficient delivery of this generation of compounds *in vivo* unreliable. We therefore tested a prior-generation PKCδ inhibitor, rottlerin, which is readily bioavailable, in a tumor model. The human breast cancer stem cell (BCSC) cultures efficiently formed tumors as xenografts in nude mice. In comparison to vehicle control, rottlerin delivered intraperitoneally 5 days out of 7 effectively inhibited the growth of the xenografts, even producing tumor regression (Figure 6A). Survival was calculated on the day when tumor size reached the predetermined limit volume in the animals. The survival of the treated cohort extended long beyond the treatment interval, with some animals remaining tumor-free even at day 300 (Figure 6B).

We have previously demonstrated that depletion of PKCδ is selectively toxic for cells with aberrant activation of Ras or Ras signaling pathways. Of the cell lines and CSC studied in this report, only a minority bore activating mutations of Ras itself (the pancreatic cancer cells are K-Ras mutant, and the melanoma cells are N-Ras mutant). MCF7 and the primary prostate and breast cancer stem cells, for example, had normal Ras

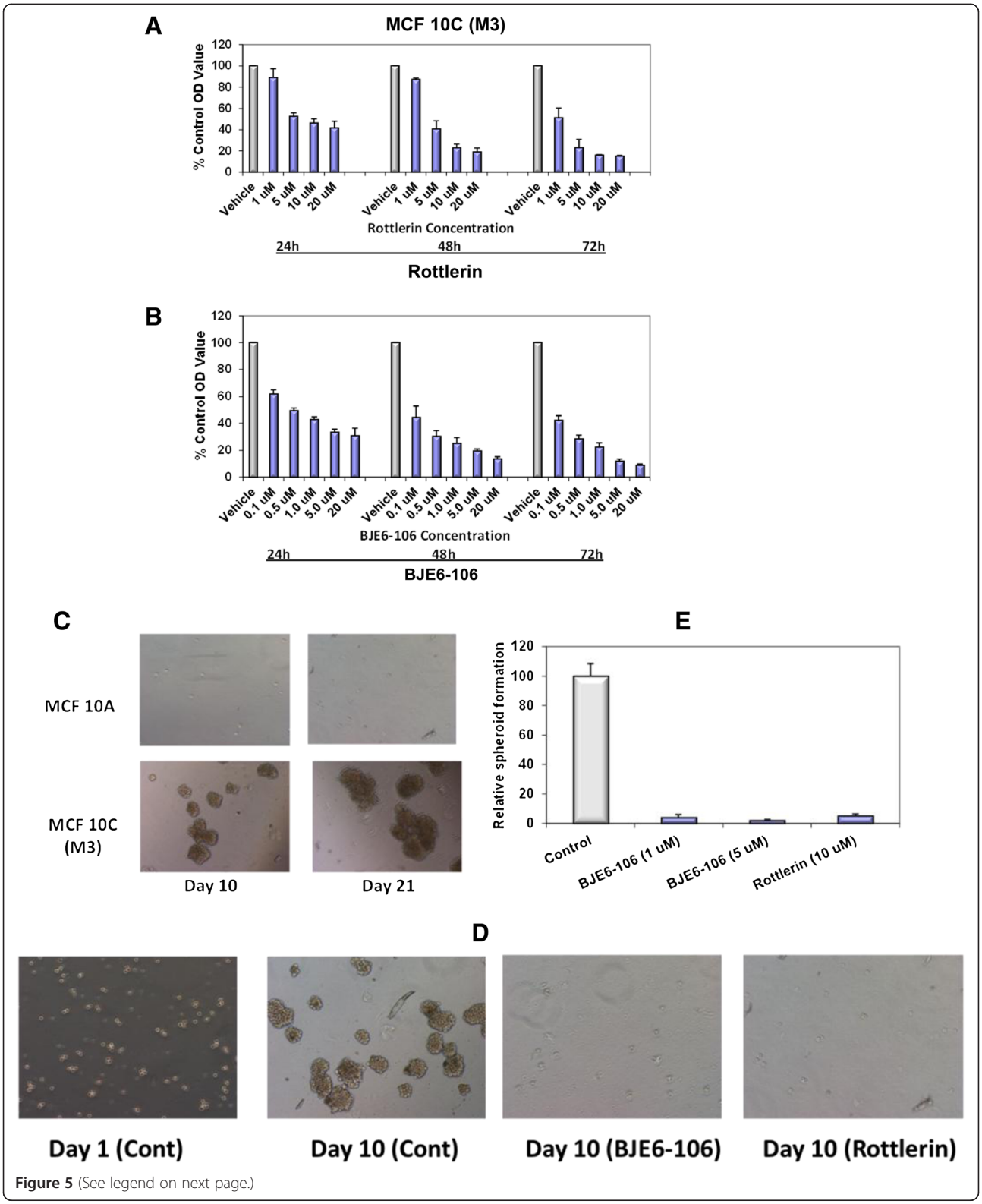

Figure 5 (See legend on next page.)

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Figure 5 Effects of PKCδ inhibitors on growth and spheroid formation in non-transformed and transformed human breast epithelial cells. MCF 10A cells and cells from the derived tumorigenic line MCF 10C (also called M3), were grown to 80% confluence in 96-well plates and then exposed to rottlerin at concentrations ranging from 1 to 20 μM **(A)** or to BJE6-106 at concentrations ranging from 0.1 to 20 μM **(B)**. The corresponding equivalent volume of solvent (DMSO) was used as a vehicle control (Vehicle). After 24, 48 and 72 hr of exposure, cell mass was evaluated by MTT assay. Control (vehicle) values were normalized to 100%. Error bars represent SEM. p values for comparison between vehicle and PKCδ inhibitors on MCF 10A cell number only reached significance ($p < 0.05$) at 48 hr at 20 μM for rottlerin, and at 1 μM for BJE6-106. In contrast, significant effects of the inhibitors on the MCF 10C cells were observed as early as 24 hr for rottlerin (at 5 μM) and for BJE6-106 (at 0.1 μM). **(C)** MCF 10A and MCF 10C cells were plated at 10,000 cells per well in tumor spheroid media, and spheroid formation was assessed at days 10 and 21. Representative photographs are shown. **(D)** MCF 10C cells were plated at 10,000 cells per well in tumor spheroid media, in the presence of rottlerin (5 μM), or BJE6-106 (1 μM or 5 μM), or DMSO vehicle (Control). Tumor spheroids were enumerated at 10 days. Representative photographs are shown. **(E)** Spheroid numbers were normalized to the number of spheroids in the control cultures (assigned an arbitrary value of 100%) and plotted. Error bars represent SEM. p values for comparison between vehicle and rottlerin or BJE6-106 effects on spheroid number were significant ($p < 0.001$).

alleles. Analysis of Ras signaling pathways of cells derived from the CSCs, however, showed relative increases of pERK or pAKT, compared to the respective parental (adherent, non-spheroid) cells (Figure 7). These findings indicate relative activation of the MEK/ERK pathway in BCSC, MCF7 and Hs578T CSCs, and relative activation of the PI_3K-AKT pathway in MDA231 CSCs.

Discussion

Small populations of cancer cells within multiple types of solid tumors have been identified based on cell surface marker expression and other phenotypic and functional characteristics. These subpopulations of tumor cells have often demonstrated a >100-fold increase in tumorigenic potential, compared to the remainder of the cells in the tumor. Furthermore, tumors that form from these cancer stem cells are indistinguishable from the human tumors in which they originate, indicating that the tumor-initiating cells are stem cell-like in their ability to self-renew and give rise to a heterogeneous cell population. Much recent data suggests that elimination of these cancer stem cells, which are typically resistant to conventional

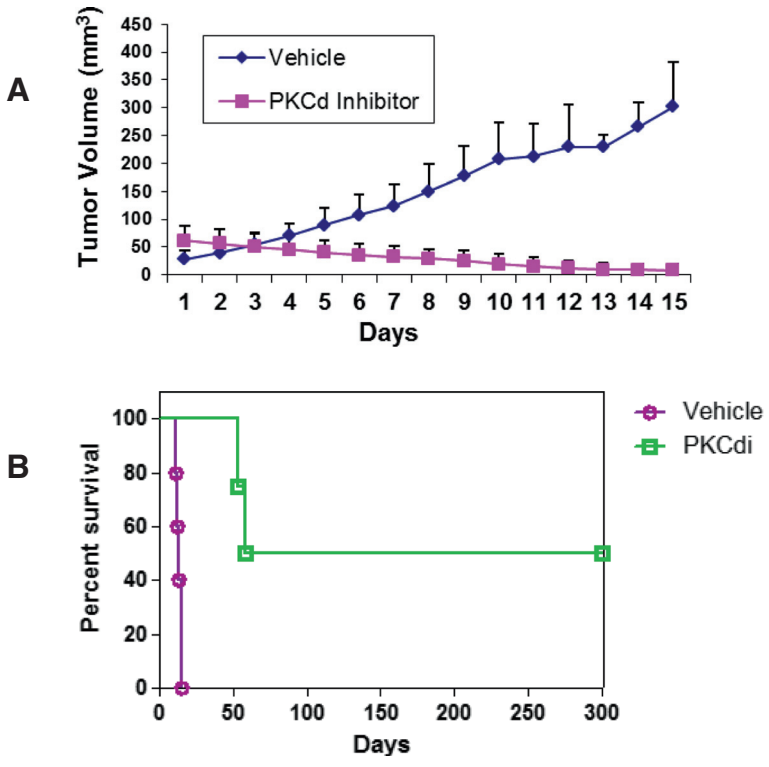

Figure 6 Effects of PKCδ inhibitor on tumor growth and survival in a xenograft human breast cancer stem cell model. Breast cancer stem cell xenografts were established and animals were treated with vehicle or rottlerin for 15 days, as described in Methods. **(A)** Tumor volumes plotted over time, until tumors in all the control animals reached the maximum volume allowed by the protocol (approximately 15 days). **(B)** Kaplan-Meier plot of survival of vehicle control or rottlerin (PKCδi)-treated animals, with monitoring continuing after cessation of treatment at day 15.

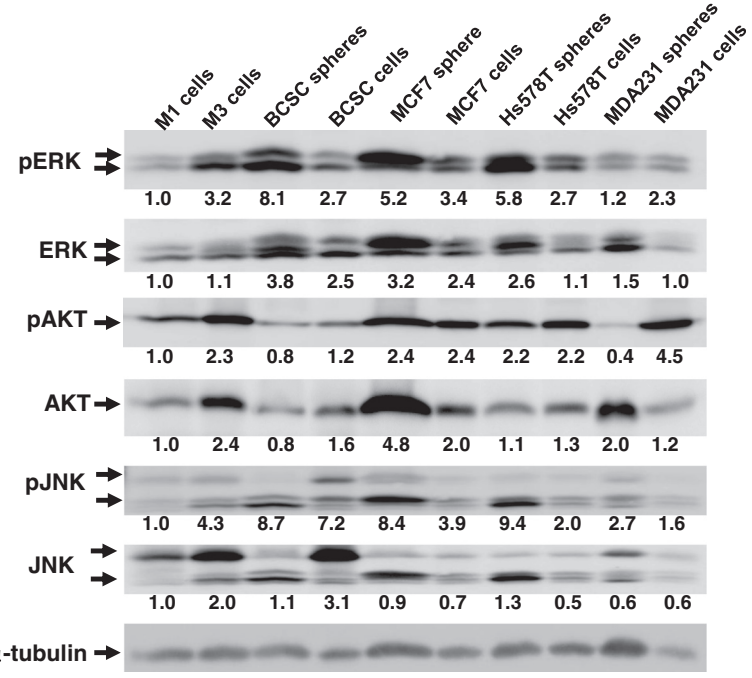

Figure 7 Immunoblot analysis of Ras-signaling pathways in tumor cells, cell lines, or spheres. Hs578T, MCF7, MDA231 and breast cancer stem cells (BCSC) were plated in tumor spheroid media under adherent or non-adherent conditions. Tumor spheroids and adherent cells were collected at 96 hr, and lysed. MCF 10A (M1) and MCF 10C (M3) lysates were also prepared. The lysates were separated by electrophoresis and immunoblotted with antibodies against ERK, pEKR, AKT, pAKT, JNK, pJNK. Immunoblotting of α-tubulin serves as a loading control. For quantitation, the digital intensity of the bands was first normalized to α-tubulin in each lane, and then expressed relative to the signal for the MCF 10A (M1) cell line. Values are shown under each band.

therapies, represents the most formidable barrier to curing solid tumors [1,4,5,32,33,35]. CSCs, or subclones thereof, are the most likely perpetrators of invasion and metastasis [6,62].

Recent findings have shown the existence of activated and quiescent repertoires of stem cells in established tumor cell lines as well as primary tumor cell isolates, and their ability to interchange between these conditions [37]. Sphere-forming assays (SFA) are believed to evaluate the differentiation and self-renewal capabilities of a tumor cell population by assessing the potential of a tumor cell to behave like a stem cell, and are widely used in stem cell studies [37]. Sphere-forming assays have been commonly used to retrospectively identify normal and cancer stem cells, and measure stem cell/early progenitor activity in multiple types of solid cancers [38,63,64]. Increased expression of "stemness-related genes" [65] was observed when comparing solid tumor cell lines grown as 3D spheroids to monolayers.

Our identification of PKCδ as a critical mediator of survival in multiple types of solid tumors, including prostate, breast, lung, pancreatic, neuroendocrine and melanomas [45-48] raised the possibility that CSC populations might be similarly dependent upon the activity of this enzyme. The effects of PKCδ inhibition on CSCs, however, had not been previously explored.

We first validated PKCδ as a target in diverse CSCs by demonstrating here that specific and selective down-regulation of PKCδ by shRNA was sufficient to prevent the growth of human breast, pancreatic and prostate cancer stem-like cell cultures, and to induce cytotoxicity.

Potential therapeutic translation of this synthetic lethal approach required the development of small molecule probes. As no PKCδ-selective inhibitors had been developed to date, we initially used pharmacophore modeling and docking of rottlerin, a well-established but not highly-specific inhibitor of PKCδ, into the crystal structure of PKCθ, to identify regions of the molecule important for PKCδ-selectivity. The initial new molecule showing activity against PKCδ (KAM1) was formed by combining structural elements of the broad spectrum protein kinase inhibitor staurosporine and rottlerin. The chromene portion of rottlerin was combined with the carbazole portion of staurosporine to produce KAM1 [47]. KAM1 was further modified to develop 36 new analogs, including BJE6-106, which inhibits PKCδ with an IC_{50} value of 50 nM and is approximately 1000-fold selective versus PKCα. Specificity for PKCδ over "classical" PKC isoforms, like

PKCα, is important, as inhibition of PKCα is generally toxic to all cells, normal and malignant, and would render these inhibitors non-"tumor-targeted". We have shown that B106 exerts potent cytotoxic activity against N-Ras-mutant human melanomas and B-Raf-mutant melanoma lines that have developed resistance to B-Raf inhibitors by aberrant activation of alternative Ras signaling pathways [48,55].

We demonstrate here that first, second and third generation PKCδ inhibitors (exemplified by rottlerin, KAM1 and BJE6-106, respectively), inhibit the growth of human cancer stem-like cell cultures isolated from tumors, as well as CSC-like cells derived from cell lines by spheroid formation on non-adherent surfaces. Our prior studies would have predicted that the CSC isolates or spheroids derived from cell lines that contained activating mutations of N-Ras or K-Ras would likely be susceptible to PKCδ suppression (*e.g.*, the K-Ras mutant pancreatic carcinomas and the N-Ras mutant melanomas). The reason for the susceptibility of the stem-like tumor cells containing wt-Ras alleles, however, was not immediately apparent. One reason for their susceptibility is likely to be upregulation of Ras effector pathways (MEK-ERK or PI_3K/AKT signaling) in CSC spheres derived from cell lines, compared to the non-CSC parental cultures. We have reported previously that isolated activation of the MEK-ERK effector pathway or the PI_3K/AKT effector pathway was sufficient to make cells dependent upon PKCδ for survival [45-47]. The finding of higher levels of Ras effector pathway activation in the CSC sphere subpopulation compared to the parental cells may also explain why in at least one instance (MCF7) the sphere-forming CSC cells were substantially more susceptible to PKCδ inhibition than non-CSC cells population. Interestingly, a recent report has identified a requirement for PKCδ in erbB2-driven proliferation of breast cancer cells [66], and erbB2 drives aberrant Ras pathway signaling. Furthermore, activation of MAPK pathways in basal-like breast cancers has been reported to promote a cancer stem cell-like phenotype [67], and activation of Ras/MAPK signaling was reported to protect breast cancer stem cells from certain stem-cell targeted drugs [68]. Collectively, these reports, together with our findings, suggest that a PKCδ-targeted approach to breast cancer stem cell populations, which exploits a synthetic lethal interaction with aberrant Ras signaling, may be particularly effective.

Inhibitory effects of PKCδ suppression on the IL6-Stat3 axis, which is critical for CSC genesis or maintenance in a number of tumor cells types [69-71], may also contribute to the actions of PKCδ inhibition on CSC growth and survival, and will be reported separately.

Epithelial-to-mesenchymal transition (EMT), induced either by paracrine signaling from cancer-associated fibroblasts (CAFs) or neighboring tumor cells, has been associated with the acquisition of a stem cell phenotype [72]. In culture, when immortalized normal or transformed human mammary epithelial cells (HMECs) are stimulated to undergo an epithelial-to-mesenchymal transition (EMT), the transition confers stem-like cell properties upon normal or transformed epithelial cells in culture, partly because the cells acquire a CD44+/CD24 (low) phenotype, similar to breast cancer stem cells.

The idea that cancer cells might reversibly transition between epigenetically-defined tumorigenic and non-tumorigenic states is of interest in part because mechanisms that generate reversible heterogeneity can confer resistance to therapies [73,74]. We took advantage of a previously-established cell line model system for breast cancer EMT, which consists of a parental spontaneously-immortalized mammary epithelial cell line, MCF 10A (M1), and one of its derivatives, MCF 10C (M3), derived from a xenograft in nude mice that progressed to carcinoma [53,54]. These cell lines were previously reported to exhibit distinct tumorigenic properties when re-implanted in nude mice; MCF 10A is non-tumorigenic, while MCF 10C forms low-grade, well-differentiated carcinomas [53,54,60]. Furthermore, MCF 10C has acquired phenotypic changes consistent with mesenchymal morphology and gene and protein expression patterns characteristic of EMT, including expression of mesenchymal markers (fibronectin, vimentin, and N-cadherin) with concomitant downregulation of E-cadherin, β-catenin, and γ-catenin. MCF 10C also expresses high levels of Nanog, and Sox4, which are markers of cancer stem cells [61]. We found that the mesenchymal, CSC-like MCF 10C subline was much more sensitive to PKCδ inhibitors than the epithelial-like "normal" MCF 10A cells from which they were derived. Furthermore, the MCF 10C line acquired the capacity to efficiently form spheroids when grown in non-adherent conditions, and this tumor spheroid formation was inhibited by inhibition of PKCδ activity.

Conclusions

Collectively, these findings suggest that human cancer stem-like cells isolated from diverse sources and tumor types require PKCδ activity for their growth or maintenance *in vitro* and *in vivo*, making this isozyme a novel tumor-specific target. Taken together with the previous demonstration by our group and others of the cytotoxic effects of PKCδ inhibition on the non-CSC population of many tumor cell types, PKCδ inhibitors hold the promise of eliminating both the majority non-CSC population and the latent and resistant CSC population comprising human tumors.

Abbreviations

BCSC: Primary human breast adenocarcinoma stem cells; CSC: Cancer stem-like cell; MAPK: MAP kinase; PCSC: Primary human pancreatic adenocarcinoma stem

cells; PKCδ: Protein kinase C delta; PKCα: Protein kinase C alpha; PrCSC: Primary human prostate adenocarcinoma stem cells; shRNA: Short hairpin RNA.

Competing interests
DVF and RMW have applied for a patent on certain of the PKC-delta inhibitory compounds described in this report. The other authors have no competing interests to disclose.

Authors' contributions
ZC and LWF carried out the molecular and biochemical studies, and participated in the preparation of the manuscript. RMW and DVF designed the novel inhibitory compounds. RMW synthesized the compounds and participated in the preparation of the manuscript. DVF conceived the study, and participated in its design and coordination and drafted the manuscript. All authors read and approved the final manuscript.

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