A NOVEL AKT INHIBITOR PREVENTS UVB-INDUCED SIGNALING IN HUMAN SKIN CANCERS

By

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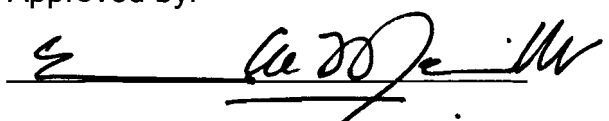

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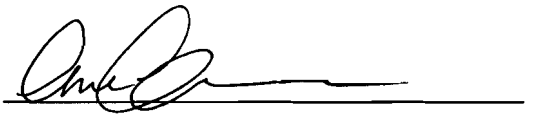
SIGNED: 

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ABSTRACT

There are over 1 million cases of skin cancer diagnosed yearly in the United States and an overwhelming 10,000 deaths associated with skin cancers. Of these deaths, about 8,000 are due to melanoma skin cancers, while the remaining 2,000 are due to non-melanoma skin cancers (NMSC). Particularly, NMSCs are a significant and increasing health problem in the U.S. Moreover, development of NMSC is primarily associated with chronic exposure to UV light. Recent advances in our understanding of the molecular basis of carcinogenesis have lead to the identification of potential molecular targets that might be used to prevent the progression of early skin cancer. AKT (protein kinase B), a plekstrin homology (PH) lipid binding domain and a serine/threonine kinase, is a key component of the phophatidylinositol-3-kinase (PtdIns3-K) cell survival signaling pathway, which is activated in skin cancers.

In this study, we describe the effects of a novel inhibitor of AKT (PH4) that binds to its PH domain, thus preventing the binding to PtIns-(3,4,5)P_3 in the plasma membrane and subsequent activation. We demonstrate that PH4 is able to prevent UVB- induced AKT activation and expression in HaCaT keratinocytes. Furthermore, the compound is lipophilic and readily penetrates the skin in mice when applied topically, and significantly decreases AKT levels in mice. Ongoing animal studies in the SKH-1 model will determine whether PH4 prevents UVB-induced skin carcinogenesis.

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BACKGROUND AND SIGNIFICANCE

Non-melanoma skin cancers

Skin cancers are comprised of two categories, non-melanoma skin cancers and melanoma skin cancers. Non-melanoma skin cancers (NMSC) are the most common and comprise of two forms: basal cell carcinomas (BCC) and squamous cell carcinoma (SCC). Although less prevalent, melanomas are the most serious types of skin cancers because they have a propensity of rapidly metatastasizing through the body. In the United States, BCC is the most common form of skin cancer, representing ~30% of all new skin cancer cases, with ~1 million cases diagnosed annually [8] and accounts for more than 90% of all skin cancer in the U.S. While these cancers hardly metastasize to other parts of the body, they can cause damage to surrounding tissues. Risk factors associated with the development of basal cell carcinomas include exposure to sun, light colored skin, and age. Generally, people who have fair or light skin and are older have higher rates of BCC. Subsequently, the face is the most common site for basal cell carcinoma. However, about 20% of these skin cancers occur in areas with low sun exposure such as the chest, arms, legs, back and scalp. Similar to the risk factors of BCC, risk factors associated with non-melenoma skin cancers include exposure to sun, age and exposure to ultraviolet radiation and therapeutic radiation. Research has indicated that the incidence of BCC appears to be increasing [9,10]. While BCC is less common than SCC, SCC have a >10-fold higher risk of metastasis and mortality [11]. Surgical treatment of SCC and BCC is frequently disfiguring, and local incidence recurrence is common. Furthermore, actinic keratosis (AK) also known as “solar keratosis” is a common solar-induced neoplasm confined to the epidermis and may develop as a result of long-term exposure to the sun. There are an estimated 5.2 million visits annually in America for actinic keratoses. AK is the initial manifestation in the spectrum of clinical and histological

abnormalities that may progress to invasive SCC. It is therefore evident that BCC and SCC represent severe health problems.

Targeted non-melanoma skin cancer chemoprevention

Cancer chemoprevention strategies are designed to prevent or delay the occurrence of cancer in high-risk populations, such as those with pre-malignant lesions, previous resected cancers or exposure to high levels of environmental carcinogens. These strategies include the use of natural or pharmacological agents, dietary intervention or life change. Pharmacological agents include plant based chemicals (phytochemicals) whose mechanism of action for the most part is unknown or rationally developed agents used to treat advanced cancer [12, 13] . Although diet and lifestyle changes can reduce the incidence of cancer in different human populations, for individuals with defined pre-neoplastic lesions or even with early cancer, the use of molecular targeted agent offers a alternative if the potential benefits outweigh the risk of toxicity. For such individuals chemoprevention and therapy can be regarded as a continuum, with the same molecularly targeted agents being used in chemoprevention and in later therapy. Chemoprevention will always require that the agents have low toxicity because of their likely long-term use. Validation of the molecular target approach for chemoprevention can be found in the development of agents with clinically proven preventive activity such as nordihydroguaiaretic (NDGA) [14] and perifosine [15], both inhibitors for AKT or celecoxib, a COX2 inhibitor [16]. Developing new chemopreventive agents is extremely costly. For this reason candidate chemopreventive agents have to be rigorously selected [12, 13] while following a certain criteria. Primarily, their molecular target should have a clear role in human tumorigenesis. Second, the agent needs to have minimal toxicity associated with long-term use. Third, it should also have demonstrable chemopreventive activity in genetically defined or

transgenic animal models of tumor progression that are to be targeted clinically. And finally, in order to cut down the risk and time of development, there should be a clear intellectual property position (specific binding properties) and clinical evidence of tolerability, low toxicity and target activity in humans. Clearly, it is very difficult to find an agent that meets all of these requirements. The agent we propose to study as a skin cancer chemopreventive agent is **PH4**. This agent has been developed in Dr. Meuillet's labs in collaboration with Dr. Powis at MD Anderson Cancer Center, as a novel AKT pleckstrin homology (PH) domain inhibitor and meets the criteria mentioned above. *First,* its molecular target AKT is important for tumorigenesis, showing increased expression in many early human tumors where it has been linked to decreased patient survival. *Second,* we present unpublished results that in the mouse **PH4** administered systemically, produces a marked decrease in the size of solid tumors while also preventing UVB induced AKT activation and expression in HaCaT keratinocytes. Third, **PH4** is well tolerated and given systemically to mice has very low toxicity. Finally, the compound is lipophilic with a logP of 7.54 and readily penetrates the skin when applied topically to the skin of mice significantly decreasing AKT levels. The parent compound from which PH4 was derived was identified during a focused *in silico* screen and modified to increase its bioavailability and solubility.

Background on the PH-domain inhibitors:

Most attempts to develop AKT inhibitors have focused on compounds that bind to the kinase ATP-binding pocket. Due to the similarity of the ATP pocket among serine/threonine kinases, particularly ACG family kinases to which AKT belongs, achieving target specificity has been extremely difficult. All the reported AKT ATP-pocket inhibitors also inhibit PKA which may

account for the relatively high toxicity of this type of inhibitor observed in animals and in patients (13). Previous studies involving D-3-deoxyphosphatidylinositol ether lipid (DPIEL) have provided proof-of-principle for using PH domains as drug targets (25). DPIEL exhibits a high binding affinity and selectivity for the PH domain of AKT (25). In addition, prior studies have shown that DPIEL does not inhibit other PH domain-containing proteins including PDK1, IRS-1, mSOS, and βARK (25). However, DPIEL is not an effective drug candidate because of its pharmacokinetic and pharmacodynamic properties (26).

Mechanism of action of compound PH4.

Data obtained in Dr. Meuillet and Dr. Powis's labs suggest that **PH4** is an active inhibitor of AKT function. Table 2 shows its biochemical properties, and Figs. 3*A* and *B* summarize the effects of **PH4** in BxPC-3 cells on AKT function (IC_{50} = 8.6 ± 0.8 μmol/L) and on its downstream targets. The compound was able to reduce the phosphorylation of AKT mainly on its Ser^{473} residue and less strongly on Thr^{308} residue without affecting total AKT protein expression while GSK3β and p70S6K were inhibited in a dose-dependent manner by compound **PH4**. The phosphorylation of PDK1 on Ser^{241} [71] was slightly affected by compound **PH4** at higher concentrations. Again, these data were in agreement with the SPR results and confirmed the possible selectivity of **PH4** for AKT at low concentrations. In order to define the mechanism of action of **PH4**, the fluorescent analog **PH4-NBD** was used [72] .The addition of the fluorescent NBD moiety did not alter the binding of PH4-NBD to the protein (Fig. 1*D*) and **PH4-NBD** inhibited AKT phosphorylation in a fashion similar to **PH4** in BxPC-3 cells (Fig. 3*C*). Finally, using confocal microscopy, compound **PH4-NBD** was found to be mainly located in the cytosol and/or lipid vesicles, potentially trapping AKT in the cytosol (Fig. 3*C*).

***In vivo* activities of the lead compound PH4**

Preliminary studies showed no toxicity of single doses up to 250 mg/kg, which was the maximum dose for compound PH4 that could be conveniently administered i.p. Anti-tumor activity was measured against BxPC-3 pancreatic cancer xenografts in *scid* mice with compound PH4 administered at a dose of 125 mg/kg i.p., twice a day for 5 d. Compound PH4 showed significant anti-tumor activity with cessation of tumor growth and even regression during the course of treatment. Tumor growth resumed at its original rate when the drug was removed.

Ultra Violet B-induced skin carcinogenesis

Studies have demonstrated that solar ultraviolet (UV) radiation, specifically its UVB (290–320 nm) component, is the major cause of skin cancer [17]. Additionally, UVB exposure is known to elicit a variety of adverse effects, such as erythema, sunburn cells, inflammation, hyperplasia, hyperpigmentation, immunosuppression, premature skin aging, and photocarcinogenesis [18-20]. The adverse effects induced by UV radiation are collectively known as the *UV response* [21, 22]. UVB radiation is a complete carcinogen, containing tumor-initiating and tumor-promoting potential [23]. Moreover, UV radiation has been implicated in the development of both BCC and SCC. Chemical and UVB radiation-induced carcinogenesis leading to SCCs in murine and humans is a multistep process and has been divided into defined stages of initiation, promotion and progression (Figure 2) [24]. UVB radiation is absorbed by DNA which causes damage primarily at sites of adjacent pyrimidines dimers [21, 24, 25]. As is seen in Figure 2, generation of DNA photoproducts has been shown to initiate tumorigenesis through exposure to UV irradiation. While mechanism(s) of the UVB-induced initiation and promotion have been studied (Figure 2), they are not fully understood. Various factors play major roles in clonal expansion into visible skin tumors, such as generation of reactive oxygen species causing oxidative stress and activation of multiple signaling cascades including the synthesis of prostaglandins (PG) [26-28].

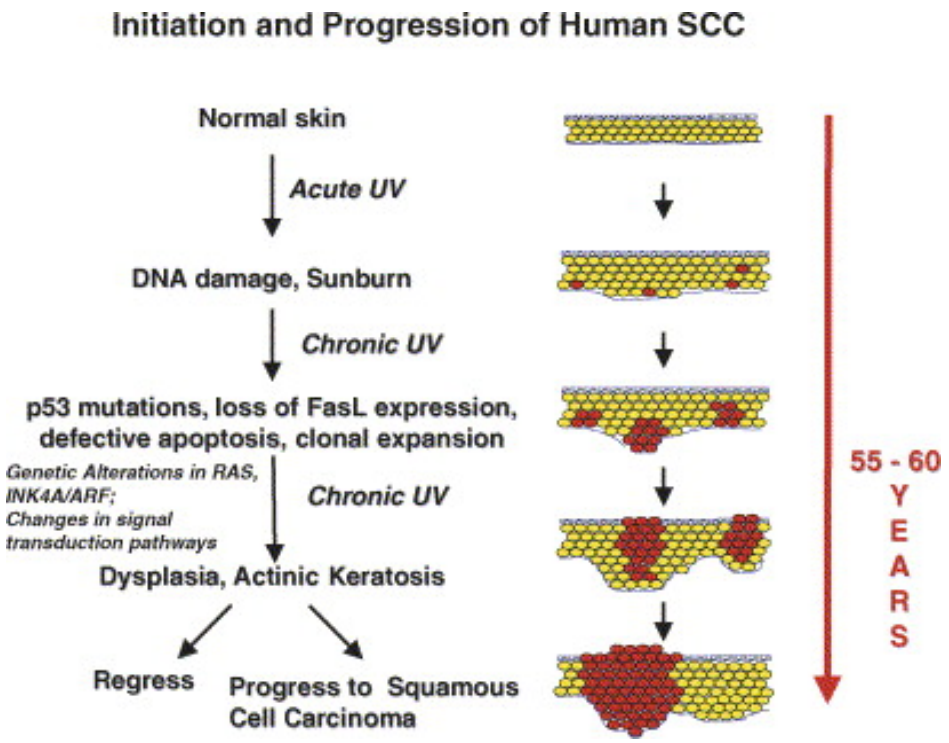


Figure 1: A model for UV-induced initiation and progression of SCC. UVB radiation induces DNA damage and apoptosis in epidermal keratinocytes. Generation of DNA photoproducts by UVB and defects in DNA repair and replication leads to accumulation of mutations in p53 tumor suppressor gene in keratinocytes, loss of Fas Ligand expression and apoptosis resistance. Upon repeated exposure to UV, p53-mutant cells undergo clonal expansion and accumulate mutations in other key genes. Some clones develop into preneoplastic lesions (actinic keratosis), and some of them progress into SCC [18].

Activation of PI3K/Akt signaling pathway in skin cancer

Multiple signaling pathways are activated upon UVB stimulation but, for purposes of this paper, the pathway of focus is the phosphatidylinositol-3-kinase (PI3K) / AKT pathway. Figure 2 summarizes published data regarding PI3K/AKT pathway activation via exposure to UVB

using transgenic mice and cultured cells. Treamtent with PI3K inhibitor, LY294002 following AKT activation with DMBA shows significant inhibition of tumor promoter, IGF-1 in epidermis of BK5-1 transgenic mice [29]. Furthermore, topical treatment with PI3K inhibitor in UVB induced SKH-1 mice exhibit lower levels of AKT, counteracting UVB induced AP-1 and Cox-2 expression [30]. UVB light also induces increased phosphorylation of CREB at Ser^{129} through activation of the PI3K/AKT pathway, resulting in increased binding of CREB to the promoter regions of the c-FOS and COX2 genes and subsequently, increased transcription. This data presents evidence indicating that UVB activation of AKT pathway results in expression of AP-1 and COX-2, rendering the pathway important in skin cancer. Using PH4, our novel AKT inhibitor, we will measure pGSK3β, pAKT, and pFOXO3a_expression in cultured HaCaT and ras transformed HaCaT II,4 skin cancer cells and in scid and SKH-1 mouse models of skin cancer development. AKT/PKB is a Ser/Thr kinase that has been shown to be over-expressed in many cancers. The activation of downstream effectors of AKT are associated with the specific phosphorylation of serine 473. Furthermore, the phosphorylation of AKT Serine 473 residue has been implicated with improper and poor prognosis in skin cancers [31]. Both isoforms of AKT, AKT1 and AKT2 are found in epidermal keratinocytes, [32] where studies show that AKT activity is associated with differentiation [32-34]. Further experiments show that mice lacking AKT isoforms, that is, AKT1-null nor AKT2-null mice, display overt epidermal defects [26, 27]. However, the AKT1 and AKT2 double-null mice die shortly after birth [35], implicating the importance of AKT in carrying out normal functioning in an organism. Confirming the results seen in double null mice, it was shown that AKT-1 and AKT-2 behave redundantly within the epidermis and that loss of both isoforms in double knockout mice results in retarded keratinocyte proliferation and severly damaged hair follicle development [28] in the skin. The AKT1 isoform

has predominantly been expressed in skin and consequently provides a much larger contribution to skin development than AKT2. In contrast, AKT2 is expressed at low levels in the epidermis [36] and is largely expressed in brown adipose tissue where its contribution to adipogenesis in vitro is prevalent. Finally, continual expression of AKT1 can result in epithelial-mesenchymal transition and increaded invasiveness in SCC cell lines [37]. Furthermore, in adding to the specific localization and expression of AKT1 isoforms, AKT1 knockout mice express increase spontaneous apoptosis [38] while double knockout mice for apoptosis show no increase in spontaneous apoptosis [35]. The PI3K/Akt pathway was suggested to be a more critical ras effector function for skin carcinogenesis than the raf-Erk1/2 pathway [39]. Up-regulation of AKT activity may lead to suppression of ERK1/2 through the inhibition of the raf kinase [40]. PTEN, the dual protein and lipid phosphatase negatively regulate the PI3K/Akt pathway (Figure 2). However, somatic mutations in Pten have not been reported in human squamous cell carcinomas [41] and deletion of 10q23, where Pten is located, is also an infrequent event [42].

Finally, loss of function of the tumour suppressor PTEN, which results in activation of the PI3K/AKT pathway, occurs in 10–30% of melanomas, frequently concurrent with activating BRAF mutations [43-45]. A study using the Cre-LoxP system to generate a keratinocyte-specific null mutation of PTEN in mice, demonstrated the important regulatory role for PTEN in the normal development and oncogenesis in the skin [46]. Interestingly, AKT3 was shown to preferentially be activated in 43-67% of sporadic melanomas [47]. Thus, we propose to use AKT constructs in HaCaTII,4 (ras-transformed keratinocytes) to test the effects of **PH4**.

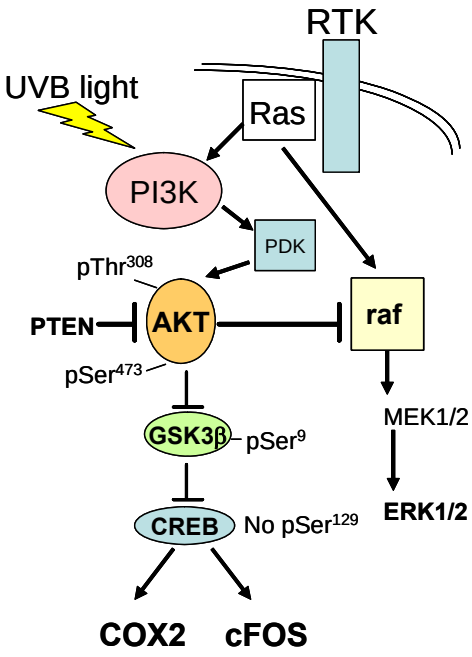


Figure 2: UVB-induced PI3K-AKT signaling *(adapted from Bowden [48]).*

Significance of targeting Akt for the prevention of skin cancer

Pro- and anti- apoptotic pathways play a large path in the survival of keratinocytes after UV irradiation. Human skin consists of regenerative basal cells and non-proliferative suprabasal cells that form the protective barrier. Since human skin is chronically exposed to UV light, protection of suprabasal cells against apoptosis is critical for maintenance of epidermal integrity and barrier function. However, protection of basal keratinocytes against apoptosis needs to be maintained at a fine balance. Preserving the pool of proliferative cells, may, in effect, foster survival of cells with existing oncogenic mutations, thereby promoting skin carcinogenesis. Selective targeting of the PI3K/Akt pathway should increase the sensitivity of keratinocytes to apoptotic signals, and thereby might be an effective strategy to prevent skin cancer. While the use of sunblocks and sunscreens are important for the primary prevention of skin cancer, they are not effective for many reasons. Therefore, new secondary prevention strategies are needed.

Effective secondary prevention of skin cancer will most easily be achieved through chemopreventative compounds that can be applied to high risk individuals prior to or after sun exposure, or to individuals with actinic keratosis (AK). Inhibition of AKT signaling has been associated with the biological actions of a number of chemopreventive compounds including curcumin [49], selenium [50], and the flavonoids quercetin [51], genistein [52], apigenin [53], and silibinin [54]. Nordihydroguaiaretic acid (NDGA, masoprocol, Actinex) is an inhibitor of UVB-light-induced PI3K/AKT pathway and NDGA has been shown to have chemopreventive activity, inhibiting inhibits DMBA-initiated, TPA-promoted formation of mouse-skin tumors [55]. However, NDGA treatment is highly irritant to the skin and its effects are not selective for PI3K/AKT. Treatment with the flavonoid silibinin also inhibits UVB-induced AKT phosphorylation [56] although it also has many other targets [57]. As noted neither of these compounds are selective for PI3K/Akt, they are relatively toxic and were not specifically developed as molecular targeted inhibitors. We have identified, developed and characterized a novel specific and non-toxic inhibitor of AKT, **PH4**, which is able to prevent UVB-induced AKT activation in human keratinocytes. Moreover, the compound is lipophilic and decreases AKT activity and expression when administered topically on the skin of mice. We propose to test the effects of this inhibitor in a prevention model of skin cancer.

As was stated earlier, skin cancer is a severe problem in the United States, taking 10,000 lives yearly. The promising results of PH4 make it an ideal compound, competitively binding to the PH domain and inhibiting its phosphorylation. In conjunction with the development of PH4, the goal of this experiment is to determine the expression and characterization of PH4 *in vitro* and *in vivo* using appropriate cell lines and mouse models. The author's hypothesis is that PH4 can be used as a chemopreventative measure for skin cancers by reducing the activation of AKT.

Materials and methods

Cell Culture

HaCaT and HaCaT II,4 *ras* transformed cells were obtained from the Bowden lab at the Arizona Cancer Center, were maintained in Dulbeco's modified Eagle Medium (DMEM) and cultured at 37 °C and 5% CO_2. All media were purchased from Mediatech (Herndon, VA), supplemented with 10% FBS from Gemini Bio-Products (Sacramento, CA) and 1x Penicillin-Streptomycin-Glutamine from Gibco (Grand Island, NY).

Reagents and Treatments

HaCat and HaCat II,4 cells were serum starved for 12-14 hours at 70 to 80% confluency overnight prior to treatment. After being serum starved, cells were treated with increasing micromolar concentrations (of 0μM, 1μM, 5μM, 10μM, and 20μM) of the AKT PH4 inhibitor and incubated at 37°C and 5% CO_2 for four hours. After incubation with the PH4 inhibitor, cells were exposed to UVB and incubated again under the same conditions for one hour.

Western Blot Analysis

Following PH4 treatment and UVB exposure, cells were harvested in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 20% SDS) supplemented with Protease Inhibitor Cocktail, 0.4 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM sodium phosphate. All protease and phosphatase inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). The protein concentrations of the cell lysates were quantified using Protein Assay Reagent from Bio-Rad (Hercules, CA). Equal amounts of proteins (35 μg) were loaded onto 10% NuPage Bis-Tris gels from Invitrogen (Carlsbad, CA). Following transfer, PVDF membranes were blocked with 5% non-fat dry milk dissolved in PBS-Tween. After blocking, membranes were incubated

with primary and secondary antibodies as listed in **Table 1**. Proteins were visualized by ECL reagents from Perkin-Elmer (Boston, MA) and exposed to HyBlot CL films from Denville Scientific (Metuchen, NJ). Densitometric quantification of the bands was conducted using the Image J software.

Antibodies

All primary antibodies used for Western Blot analysis and their incubation conditions are summarized in **Table 1** below. Mouse and rabbit secondary antibodies coupled to horseradish peroxidase were obtained from GE HealthCare (Piscataway, NJ). Western Blot membranes were incubated with the appropriate secondary antibody at 1:10,000 dilution in 1% milk in PBS-Tween. Secondary fluorescent antibodies used for the immunofluorescence studies were obtained from Molecular Probes (Eugene, OR).

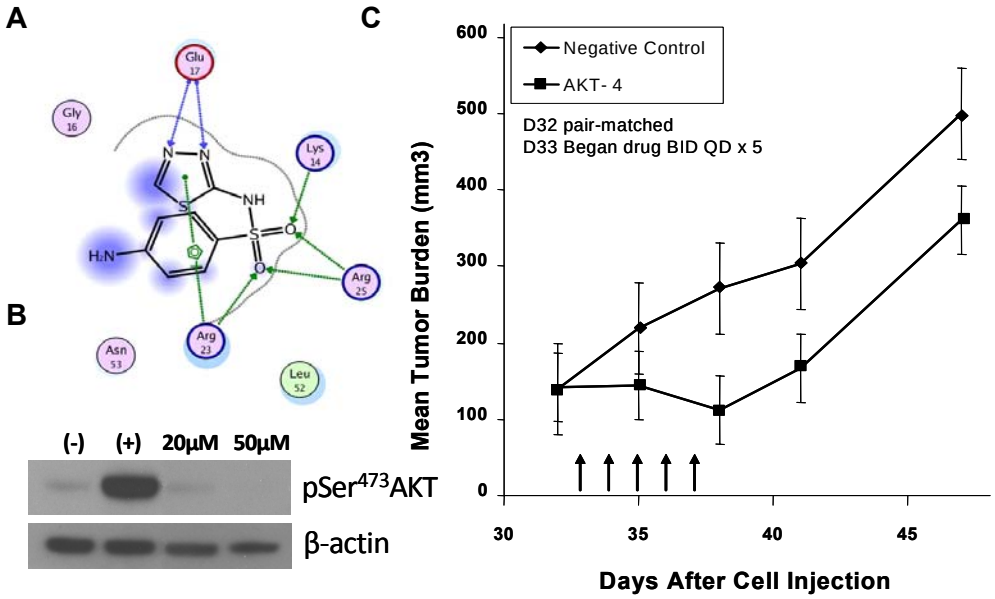
Table 2: Antibodies used in Western Blot analysis

Protein	1° Ab Dilution	2° Ab	Source
PARP	1:1000 in 5% Milk	Rabbit	Cell Signaling (Danvers, MA)
pS473-Akt	1:1000 in 5% BSA	Rabbit	Cell Signaling (Danvers, MA)
Total Akt	1:1000 in 5% BSA	Rabbit	Cell Signaling (Danvers, MA)
p-Erk-1/2	1:1000 in 5% BSA	Rabbit	Cell Signaling (Danvers, MA)
ERK-2	1:1000 in 1% Milk	Mouse	Santa Cruz (Santa Cruz, CA)
pGSK3-β	1:1000 in 5% BSA	Rabbit	Cell Signaling (Danvers, MA)
GSK3- β	1:1000 in 5% BSA	Rabbit	Cell Signaling (Danvers, MA)
pFOX03a	1:1000 in 5% BSA	Mouse	Cell Signaling (Danvers, MA)
FOX03a	1:1000 in 5% BSA	Mouse	Cell Signaling (Danvers, MA)
β-actin	1:1000 in 1% Milk	Mouse	Sigma Aldrich (St. Louis, MO)

Results Section

This section provides the data in systematic manner, while we attempt to provide results that support our hypothesis: PH4 inhibits AKT activation occuring after UVB exposure. In order to test our hypothesis we have consistently used two skin cell lines HaCaT and HaCaTII,4, where HaCaT II,4 are ras transformed and are more representative of an enviornment with pre-existing mutations as in cancer.

Figure 3: Characterization of PH4



A, Shows the molecular docking mode of PH4 in AKT-PH domain. The Amino acids involved in the binding of the compounds are labeled in pink. Note the importance of the sulfonamide function with the two arginines and the lysine. **B,** Typical Western blot obtained with BxPC3 pancreatic cancer cell lysates. Cells were treated with 20 or 50 **μ**M of **PH4** for 3 hours. Cells were then stimulated with EGF (+) (50ng/ml, 20min.) and pAKT was evaluated using specific antibodies. Note a total decrease of pAKT following 20μM **PH4. C,** Antitumor activity of **PH4** was evaluated in Scid mice with 150 mm^3 subcutaneous BxPC3 pancreatic cancer xenografts that were treated with (▲) vehicle or with (■) **PH4** 125 mg/kg in 0.1% Tween 20 in water by intraperitoneal injection twice a day for 5 days, as shown by the arrows. Values are the mean of 8 mice per group and bars are SE. * $p < 0.05$ compared to vehicle control.

Figure 3 summarizes the properties of PH4 in pancreatic BxPC-3 cancer cells. PH4 binds to the PH domain of AKT and displace its natural ligand PI3,4,5P_3 with a K_i of 2.4±0.6 μM using surface plamon resonnance (SPR) technology and inhibits AKT activity with an IC_{50} of 6.3±0.9 μM in pancreatic cancer cells. As is shown in Figure 4, PH4 exhibits good anti-tumor activity in BxPC3 pancreatic cancer xenograft model. The immunoblot staining shows an increased basal level of pAKT in BxPC3 cells after stimulation with EGF indicating the appropriate pathway is stimulated. More importantly, the western blot shows a significant decrease in levels of pAKT at 20μM and 50μM**.** Finally, the figure shows a statistically significant decrease in the mean tumor burden of *scid* mice treated i.p. with AKT-4, particularly between days 32-37.

Figure 4: PH4 induces apoptosis in HaCaT and HaCaT II,4 cells.

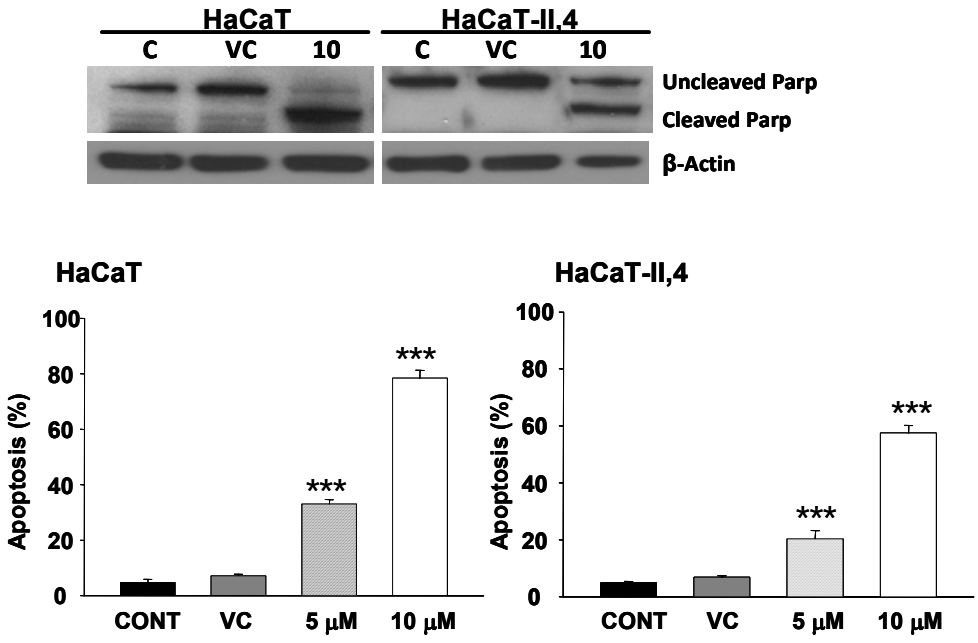


Figure 4: PH4 induces apoptosis in HaCaT and HaCaT-II,4 cells. A, Cells are incubated with DMSO vehicle control (VC) or 10 μM PH4 for 3 hours. PARP cleavage is noted in presence of **PH4** indicating apoptosis. **B**, apoptosis is measured by a morphological assay. Statistically significant increase in apoptosis is noted in both cell lines in presence of 5 or 10 μM of **PH4** ($p<0.001$).

Following the characterization of the compound, we wanted to look at the affects of PH4 on apoptosis in both cell lines. Both cell lines were treated under the conditions described in the methods with 10 μM (and 5μM) of PH4 and then probed using PARP antibody. As is seen by the immunoblots and confirmed by the morpholoigical assay, there was a statistically significant increase in PARP cleavage and a corresponding decrease in uncleaved PARP at 5μM and 10μM. Additionally, similar to the results seen in Figure 4, HaCaT II,4 cells are more resistant to apoptosis as is seen by the comparatively lower level of PARP cleavage to HaCaT cells. These results indicate that PH4 is inducing apoptosis in both cell lines after treatment with PH4 and UVB exposure.

Figure 5: PH4 locates in the cytosol, the perinuclear region and the plasma membrane of HaCaT cells

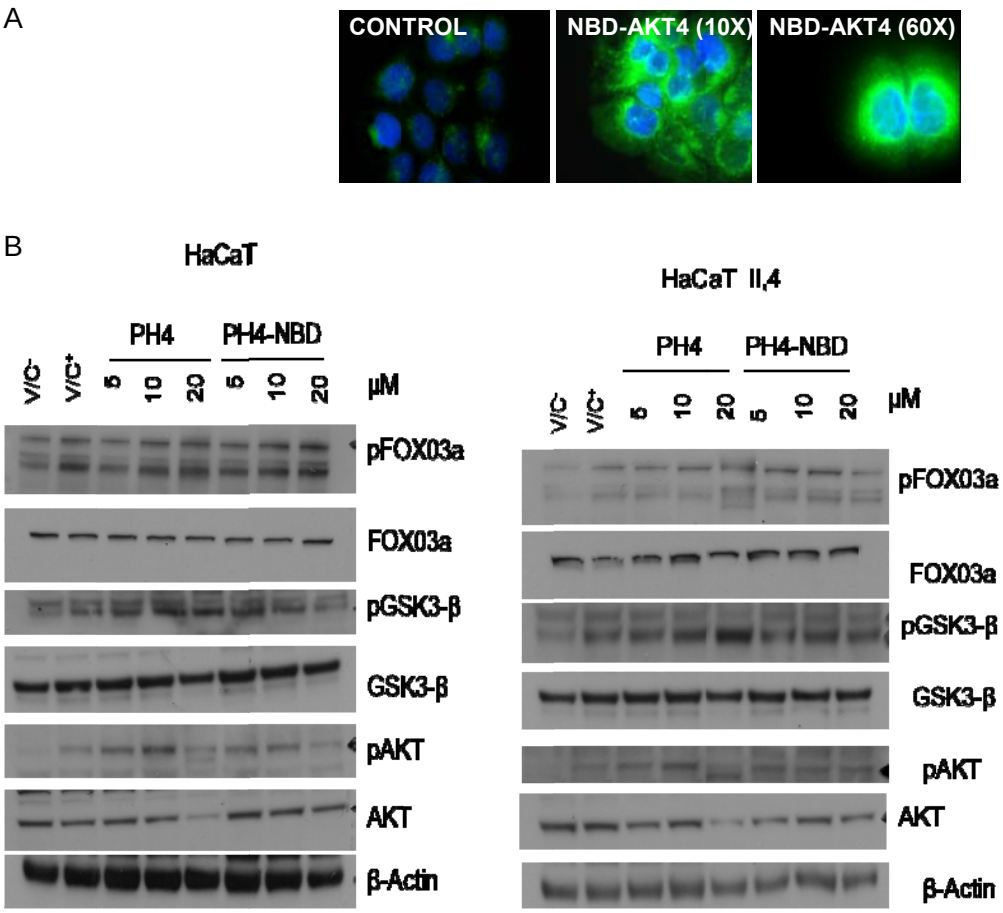


Figure 5: A, In order to investigate the mechanism of action for PH4, we have synthesized a fluorescent 7-nitroben-2-oxa-1,3-diazole (NBD) derivative of PH4, treated HaCaT cells for 3 hours with the compounds, fixed the cells and visualized them under a fluorescent microscope (FITC filters). The nuclear stain was obtained using DAPI. B, In order to assess the viability of NBD-PH4 as an appropriate tag, cells were treated with the immunoflouresent tagged PH4 under the same conditions as normal PH4 in both HaCaT and HaCaT II,4 cell lines. Cells were treated with the drug in a dose dependent manner , incubated for 4 hours and then exposed to UVB.

In Figure 5 we have investigated the mechanism of action of PH4-NBD, the immunoflouresent tag (NBD) indicated that PH4 localizes in three areas: the perinuclear region, the cytosol and the plasma membrane. Particularly, localization to the plasma membrane is highly expressed in Figure 8A, confirming the expected mechanism of AKT after stimulation. Additionally, PH4-NBD was probed for pAKT, AKT and downstream effectors (pGSK3-β, GSK3-β, pFOX03a, and FOX03a) in both cell lines. While AKT and pAKT showed decreased levels of pAKT in both cell lines, with and without the NBD tag, at 20μM, the downstream effectors showed conflicting results. This may be in part due to activation of other affected pathways.

Figure 6: PH4 inhibits UVB-induced AKT phosphorylation in HaCaT Cells.

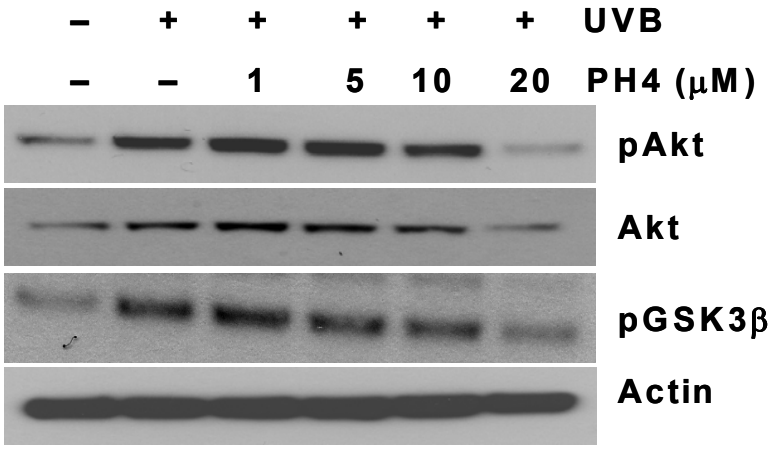


Figure 6: Typical representative western blot obtained from HaCaT cells that were pre-treated for 4 hours with increasing concentrations of **PH4** and then were UVB irradiated. Cells were lysed after 1hour. Similar results were obtained in HaCaT II,4, a cell lines that has been ras-transformed to make it tumorigenic in mice (Figure 10)

After assessing the preliminary work on PH4 inhibitor, determining its localization and its PARP cleavage , the effects of PH4 on UVB induced HaCaT and HaCaTII,4 cells was tested. Figure 6 demonstrates that PH4 inhibitor effectively decreases pAKT, AKT and GSK3β levels in HaCaT cell lines in a dose dependent manner. Particularly, note that that 10 and 20 μM of PH4 counteract UVB-induced AKT phosphorylation as well as one downstream target, pGSK3-β. Additionally, total AKT is also getting down-regulated in a dose dependent manner. The dose dependent decrease in GSK3β indicates that AKT inhibition subsequently reduces activation of its downstream effectors, subsequently confirming that the appropriate pathway is activated when pre-treated with PH4.

Figure 7: Inhibition of AKT by PH4 between 1-6 hours

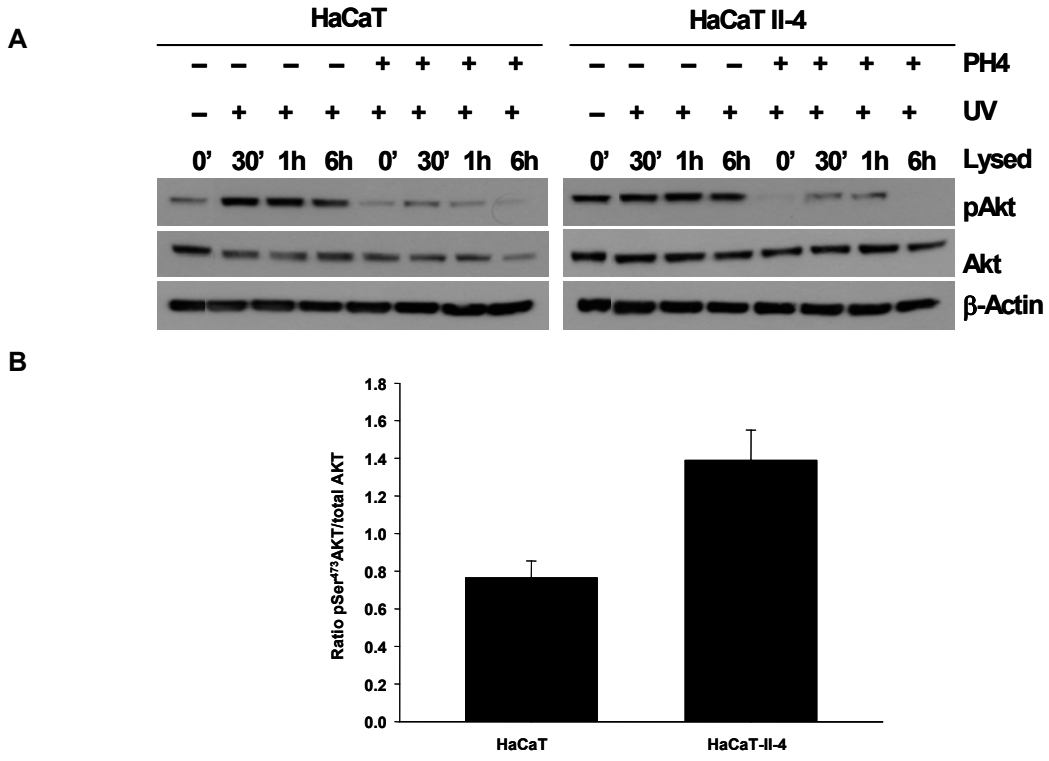


Figure 7: PH4 inhibits AKT activation overtime following UVB exposure. A) Cells were incubated with 10 μM PH4 for 4 hours and then exposed to UVB (250J/m^2) and lysed at the indicated times. B) High basal levels of pAKT in HaCaT II,4 as compared to HaCaT.

In Figure 7 HaCaT and HaCaT II,4 cells were treated as described in the methods section. The cells were incubated with PH4 for 4 hours (the time point established from Figure 5) and then exposed to UVB and lysed at varying time points 0', 30', 1h, and 6h. The results indicate that in both cell lines there is a significant decrease in pAKT levels between 1h-6h of treatment with PH4. Additionally, it can be seen that *ras* transformed HaCaT II,4 cells shows higher resistance to PH4 induced AKT expression due to pre-existing amplified AKT levels in the cells. B) Corresponding quantification of western blots indicate High basal levels of pAKT in HaCaT II,4 as compared to HaCaT, correlating with the immunoblot expression of AKT in both cell lines.

Figure 8: Effects of PH4 in the mouse skin on total AKT

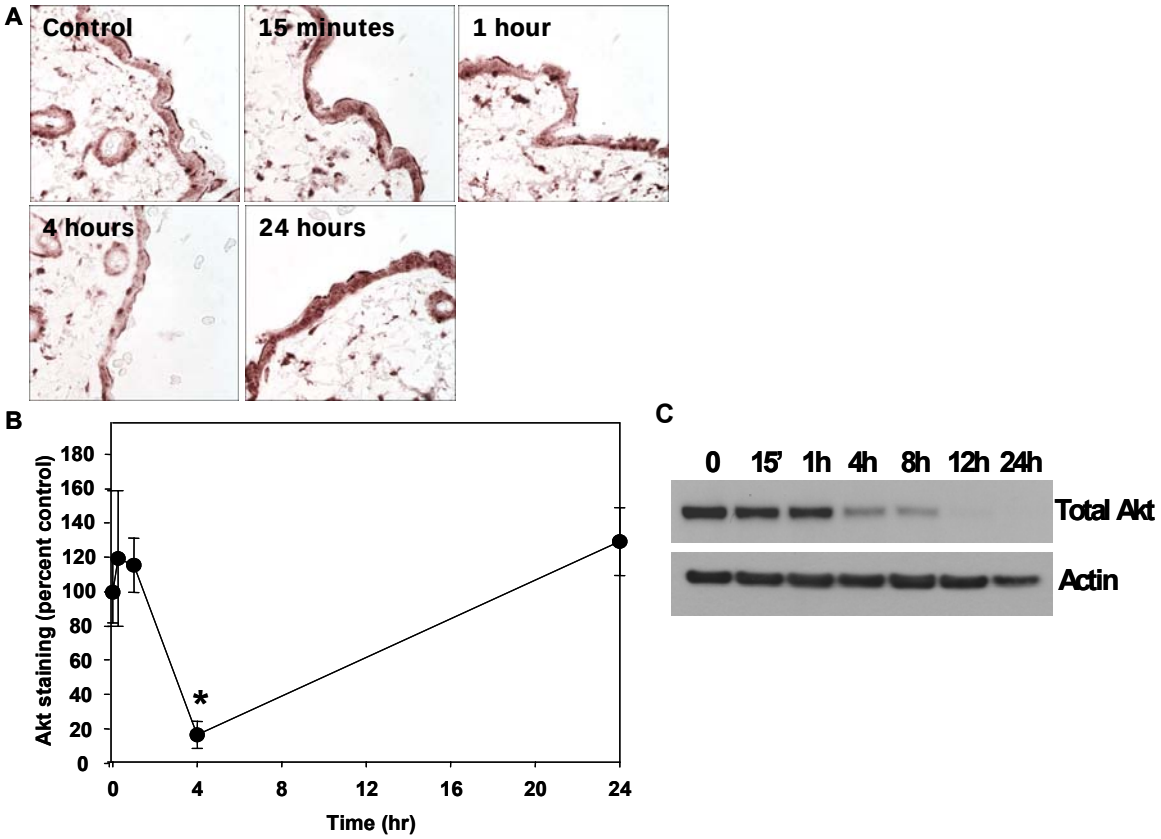


Figure 8**: Effects of PH4 in the mouse skin on total AKT. A,** Immunohistochemistry for AKT in the mouse skin. Topical application of **PH4** 20 mg/ml in 0.1 ml acetone, in *Scid* mice. Skin biopsies were obtained at the times

shown and stained for AKT. **B,** Quantification of AKT staining. N = 3. Staining measured by quantitative immunohistochemistry with correction for non specific background staining. p-Akt was not detectable in dermal layer. * for $p < 0.05$. **C,** Time course for PH4 (10 μM) on total AKT1 expression. HaCaT cells were incubated with the compound during the indicated period of time and then lysed.

After development and characterization of drug the in-vivo activity of PH4 on the mice was examined. Due to the hydorophobicity of **PH4** (logP is 7.54) and its aliphatic tail, PH4 can easily cross the plasma membrane when applied to murine models. In order to test the activity of **PH4** on the skin, the compound was applied topically in acetone to the skin of *Scid* mice. Skin biopsies were taken and immunohistochemistry for pAKT and AKT was performed on the sections. As can be seen in Figure 8A, immunohistochemistry of the skin biopsies show a decreased expression of pAKT (brown staining) at four hours. The qualitative immunohistochemistry results were quantified in Figure 8B where the percent control of AKT staining showed a statistically significant decrease in pAKT levels at 4 hours following a return to basal levels of AKT at 24 hours. The return to basal levels indicates that PH4 does not have long term effects on total AKT levels, increasing its marketability. Finally, Figure 8C summarizes the time course for AKT expression *in vitro* with an immunoblot following **PH4** application.

Finally, we have initiated a pilot study using the SKH-1 hairless mouse model for skin cancer (**Figure 9**). Here cells were obtained from the skin of SKH-1 mice, sonicated and protein quantified for immunoblotting. Particularly, note that 1 hour treatment of the mice prior UVB with PH4 is efficient in decreasing pAKT and AKT as well as pERK in SKH-1 mice. This study is ongoing (to end June 2009) and provides *in vivo* data that correlates with the *in vitro* data of all the HaCaT and HaCaT II,4 cell models.

Figure 9: PH4 inhibits UVB- induced AKT activation in skin of SKH-1 mice

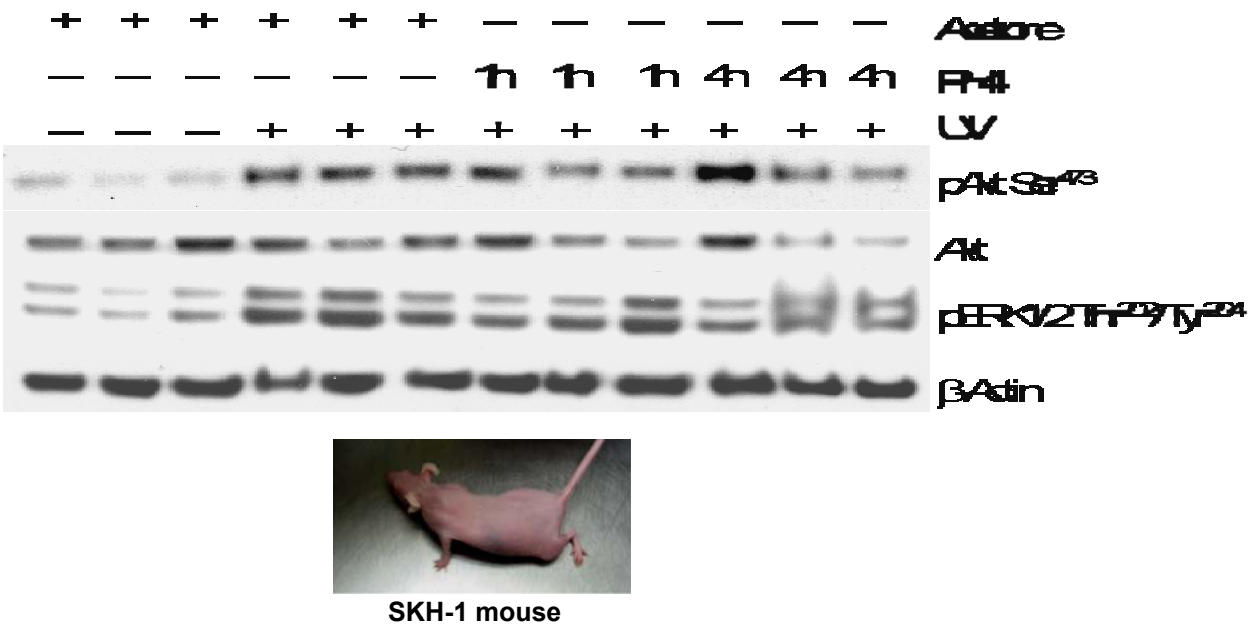


Figure 9: SKH-1 mice were treated and their skin analyzed for pAKT, pERK , AKT and actin using western blotting. Four groups of SKH-1 mice were tested: Group1: acetone (0.2ml); Group 2: UVB+ acetone (10mg/ml; 0.2ml acetone); Group 3: PH4 (10mg/ml; 0.2ml acetone) *1 hour* prior to UVB; Group 4: PH4 (10mg/ml; 0.2ml acetone) *4 hour* prior to UVB

In conclusion, we have developed a novel inhibitor of AKT (**PH4**) that binds to the pleckstrin homology domain of AKT thus preventing its activation. Interestingly, **PH4** also reduced AKT expression in HaCaT cells after a period of 1 to 6 hours of treatment with the compound (Western blot). This observation was also correlated in mice where the total AKT protein was also reduced as demonstrated in the skin biopsies (immunohistochemistry for total AKT). **PH4** exhibits anti-tumor activity in various cancer cells xenografts models. We have found that **PH4** is able to prevent UVB-induced AKT activation and expression in HaCaT keratinocytes cells (Western blots). Moreover, the compound is lipophilic and readily penetrates the skin when added topically and significantly decreases AKT levels in SKH-1 mouse skin (immunohistochemistry and Western blots). It was also determined in a pilot study that 1 hour

prior UVB treatment, **PH4** is efficient in reducing pAKT in the skin of SKH-1 mice. A study is ongoing in SKH-1 mice using this protocol in collaboration with Dr. Bowden [5].

Discussion

The development of an AKT inhibitor is of great interest due to its dominant role as a cellular inhibitor of apoptosis. AKT activation is initiated by membrane translocation, occurring after cellular stimulation (i.e. UVB or EGF) and phosphorylation at serine^{473} and binding of the PH domain to PtdIns(3,4,5)P_3 (PIP_3) generated by the activation of the PI3K pathway. AKT plays a vital role a cell survival pathway and as such, has been shown to be implicated in numerous cancers including prostate, breast and skin cancers. Particularly, this paper has focused on the characterization and expression of a newly developed novel pleckstrin homology (PH) domain inhibitor of AKT, PH4. These data provide preliminary studies for the development and testing of a novel PH inhibitor, PH4. Additionally, with the accumulation of this data and the ongoing *in vitro* studies of SHK-1 mice, we will attempt to begin clinical trials (phase 0) in the near future.

Of the data presented above, this section will only address the following figures: Figure 4, Figure 5B, Figure 6 and Figure 9. To begin, Figure 4 was an immunoblot representation of PARP cleavage following PH4 treatment and incubation for 4 hours and UVB stimulation. At both 5 and 10 μM there was a significant decrease of uncleaved PARP in conjunction with an increased activation of cleaved PARP, indicating that PH4 induces apoptosis in a dose dependent manner by competitively binding to the PH domain of AKT.

In order to characterize the mechanism of PH4 we tagged PH4 with an NBD derivative that is capable of flouresence using DAPI. The localization of PH4 was then qualitatively

expressed in the cytosol region, the perinuclear region and the plasma membrane, with a large amount of activation being at the plasma membrane. Subsequent studies on the viability of the PH4 derivative were made to determine whether PH4-NBD derivative showed the same AKT stimulation as with PH4. The Western blots for both cell lines show a decreased expression of pAKT and AKT in PH4 and PH4-NBD activation at 20μM. However, pFOX03a and pGSK3-β did not show similar results. Where in other immunoblots and experiments pGSK3-β showed similar expression to pAKT, in Figure 5B, expression actually appears to increase at 20μM. Similar results are seen for FOX03a. Consequently, FOX03a may have a valid explanation. FOX03a is a Forkhead family transcription factor, whose activation (phosphorylation) translocates the protein to the cystosol. If PH4 is inhibiting AKT activation by competitively binding to the PH domain, it is effectively inhibiting FKHR which is a path that leads to apoptosis. Therefore, it may be said that this series of downstream events may actually be increasing the amount of pFOX03a in the cytosol in a dose dependent manner. These results are thus seen in the immunoblots of Figure 5B.

Finally, Figures 6 and 9 two of the most pivotal experiments in this paper because they provide both *in vitro* and *in vivo* data that suggests PH4 administered prior to UVB treatment significantly decreases pAKT , total AKT, and pGSK3-β levels which is consistent with the expected literature on AKT. Specifically, in the HaCaT and ras-transformed HaCaT II,4 pAKT levels are significantly decreased at 20μM while in the SKH-1 mice studies, a dose of 10mg/ml one hour prior to UVB exposure is sufficient in producing decreased levels of pAKT.

In summary this paper has shown the following: PH4 is a novel AKT inhibitor that binds to the pleckstrin homology domain of AKT, preventing its activation. It functions *in vitro* by inhibiting UVB-induced AKT activation in two skin cell models, HaCaT and ras- transformed

HaCaT II, 4 cell lines. Particularly, this activation is reduced at a specific time point between 1-6 hours of treatment with the compound. As is shown by the synthesis of an NBD derivative of PH4, AKT localizes in the perinuclear region, the cytosol and, most importantly the plasma membrane following AKT stimulation. Additionally, PH4 induces PARP cleavage and apoptosis in HaCaT and HaCaT II,4 in a dose dependent manner. With regards to *in vivo* data, PH4 is lipophilic with a logP of 7.54 and readily penetrates the skin when added topically, significantly decreasing AKT levels in mice. Preliminary *in vivo* studies in SKH-1 mice show that 1 hour prior to UVB exposure PH4 is effective in reducing pAKT . Ongoing studied in SKH-1 mice will determined whether PH4 prevents UVB-induced skin carcinogenesis.

Future Studies:

There are many questions that remain to be answered in this research that would serve to make it more comprehensive. The most important question that needs to be answered is whether PH4 can also work as a cancer treatment rather than just a chemopreventative agent, ideal for at risk populations. Currently all the data obtained indicates lower levels of AKT and pAKT when PH4 is administered prior to treatment. It would be interesting to see what effects PH4 has on existing skin tumor cells if applied after UVB exposure.

Additionally, it may be necessary to test PH4 effects in different cell lines to see if the cellular response to AKT is the same in varying tissues or if it is acting differently in skin tissues. Subsequently it may also be interesting to investigate whether there are cell specific AKT isoforms. While the literature states that the AKT1 isoform is most likely expressed in keratinocytes, it may be of value to determine if there is specificity in PH4 binding to any one type of isoform given its accurate tissue identification.

Finally, the most important future study is the ongoing SKH-1 mice study (Figure 9). This study will provide *in vivo* data that corresponds to the *in vitro* data seen in most of the western blots, particularly corresponding to data seen in Figure 6.

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