Manuscript Title

A novel inhibitor of AKT1-PDPK1 interaction efficiently suppresses the activity of AKT pathway and restricts tumor growth in vivo.

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Running Title

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Abstract

The serine/threonine kinase AKT/PKB has a critical role in the regulation of cell proliferation. Since AKT signaling is deregulated in numerous human malignancies it has become an attractive anticancer drug target. A number of small-molecule AKT kinase inhibitors have been developed, however, severe side effects have prevented their use in clinical trials. To find inhibitors of AKT1 signaling with principally novel mechanism of action we carried out a live cell-based screen for small-molecule inhibitors of physical interaction between AKT1 and its primary activator PDPK1. The screen revealed one molecule – NSC156529, which downregulated AKT1 signaling, efficiently decreased the proliferation of human cancer cells in vitro and substantially inhibited the growth of prostate tumor xenografts in vivo. Interestingly, the treated tumor xenografts exhibited higher expression level of normal prostate differentiation markers but did not show augmented cell death suggesting that the identified compound primarily enhances the differentiation of malignant cells towards normal prostate epithelium and thus poses as an attractive lead compound for developing novel antitumor agents with less cytotoxic side-effects.

Introduction

The serine/threonine kinase AKT (also known as protein kinase B, PKB) belongs to the AGC family of kinases and has a central role in regulating the survival and proliferation of normal and malignant cells (1, 2). Abnormal activation of the AKT pathway has been commonly described in prostate, breast, liver and colorectal carcinomas (3-7). Furthermore, the constitutive activation of the PI3K/AKT pathway confers resistance to many chemotherapeutic drugs and is a poor prognostic marker for a number of cancer types (8), thus, targeting AKT pathway is a promising strategy in tumor therapy.

Correspondingly, a variety of AKT inhibitors have been developed to date (9). Most of these are the inhibitors of kinase activity and by binding to its kinase active site act as ATP-competitors. Since the ATP binding pocket of AKT/PKB, PKA and PKC is highly homologous, these inhibitors have an activity towards PKA and PKC (10). Phosphatidylinositol (PI) analogs block PI(3,4,5)P3 binding to AKT, prevent its translocation to plasma membrane and subsequent activation (11). The PI analogs are more selective to AKT but they have a potential to interfere with the function of other pleckstrin homology (PH) domain-containing proteins (9). In addition, a few inhibitors of PDPK1, the critical upstream activator of AKT, have been developed, which suppress the kinase activity of PDPK1 either as ATP competitors or by allosteric mechanisms (12, 13). Nevertheless, the data regarding their usability in clinical setting is scarce.

Protein-protein interactions (PPIs) are key events in vast majority of molecular signaling pathways and are thus attractive targets for intervention with small-molecular compounds. Despite of the challenges that the researchers face when designing the PPI inhibitors the number of small molecules that specifically interfere with PPIs, which are crucial for the integrity of the pathway of interest, is constantly growing (14). Although there exist a number

of small-molecule inhibitors of the AKT pathway, to our best knowledge no true PPI inhibitors that target this pathway have been identified so far. However, the compounds, which lock the intramolecular interaction between the kinase active site and PH domain of the AKT molecule in the inactive state can be viewed as a specific subclass of allosteric PPIs (15, 16). At least one such compound has entered successfully into the clinical trials underlining the potential of the small molecules with PPI inhibitory properties in tumor therapy (17).

To identify AKT pathway inhibitors with principally novel mechanism of action we decided to target the critical step in AKT pathway - the physical interaction of PDPK1 and AKT1, which results in phosphorylation of AKT at T308 and its activation (18). For the detection of the PDPK1-AKT1 interaction we took advantage of the protein complementation assay based on *Renilla* luciferase (*R*luc) (19). First, two specially crafted halves of *R*luc, which do not possess the luciferase activity, are fused to the interaction partners. Next, the fusion proteins are expressed in cells and in the case these proteins interact the *R*luc fragments are brought in close proximity, which results in reconstitution of the enzyme activity (Figure 1A). Due to the reversible nature of the interaction between the *R*luc fragments the inhibition of protein-protein interaction unfolds the functional enzyme and destroys the *R*luc activity. Using a small-molecule library screen in live cells, we identified one compound - NSC156529 - that interacted preferentially with PDPK1, inhibited AKT1 phosphorylation and suppressed AKT-mediated signal transduction to several AKT1 substrates. Furthermore, the discovered compound efficiently decreased the proliferation of human cancer cells in vitro and inhibited tumor growth in a prostate tumor xenograft model in vivo. Interestingly, in addition to the reduction in the mitotic activity in NSC156529-treated tumors the tumor cells exhibited a substantial increase in the expression of prostate differentiation markers suggesting that the inhibition of tumor growth was achieved at least in part through enhanced differentiation of tumor cells towards prostate epithelium.

Materials and Methods

Cell cultures and transfections

Low-passage cultures (p3-8) of cell-lines (source; date received) were used in this study: human non-small-cell lung carcinoma cells H1299 (American Type Culture Collection (ATCC), 03.2004), human embryonic kidney cells HEK293 (ATCC; 02.1993, human hepatoma cells Hep3B (ATCC; 2011), PC-3 (ATCC; 05.2006), K07074 mouse primary liver tumor cell line (established in-house; 09.2013). H1299, Hep3B, HEK293 and K07074 cells were cultivated in IMDM medium (Lonza). The normal primary human fibroblasts and osteoblasts (a kind gift from Aare Märtson and Siim Suutre (Clinic of Traumatology and Orthopedics, Tartu University Clinics)) were cultivated in IMDM medium, for osteoblasts IMDM was supplemented with 100µg/ml ascorbic acid (Sigma). Human prostate cancer cells PC-3 were grown in RPMI medium (Gibco). All media were supplemented with 10% (v/v) fetal bovine serum, 50units/ml penicillin, 50µg/ml streptomycin and 1mM L-glutamine. Cells were maintained at 37°C in a humidified atmosphere supplemented with 5% (v/v) CO_2.

The identity of cell lines was routinely verified using species-specific primers and karyotyping.

High throughput screen

NCI Diversity Set I (National Cancer Institute) containing 2000 small-molecular compounds was used to perform the screen. H1299 cells were transfected either with 6µg pCNEO empty vector, 3µg of p53-F1, HDM2-F2, Akt1-F1, Pdpk1-F2 or 2µg of full length *Renilla* luciferase expression vector (pRluc). 48 hours after the transfection cells were incubated with DMSO, GSK 2334470, Nutlin-3 or with compounds originating from the chemical library for 2 hours at concentration 10µM. Next, luciferase activity and cell number were measured using ViviRen Live Cell Substrate (Promega), CellTiter-Glo® Reagent (Promega) and TECAN

InfiniteM200 PRO plate reader according to the manufacturer's instructions. The luciferase reads were normalized to the cell viability counts and the average of 2 experiments for each data point was calculated. The testing of 74 initial hits was performed in 4 replicates as described above. Additionally H1299 cells transfected with pRLuc were used to evaluate the effect of the compounds on *Renilla* luciferase.

To assess the quality of the screen setup the Z-factor was measured using the constructs encoding the p53-F1 and HDM2-F2 fusion proteins and the inhibitor of p53-HDM2 interaction - Nutlin-3 (10μM). The calculated value of the Z-factor for *R*luc PCA was 0.5 demonstrating its suitability for detecting the inhibition of the protein-protein interactions. Retrospectively calculated Z-factor for AKT1-PDPK1 interaction when using 10μM AKT-PDPK1 inhibitor NSC156529 was 0.75.

***In situ* proximity ligation assay (PLA)**

In situ proximity ligation assay (PLA) allows a specific and sensitive detection of protein-protein interactions. PLA is based on the immunodetection of the interaction partners with specific primary and oligo-tagged secondary antibodies. If the proteins of interest interact the *in situ* PCR generates specific DNA sequence, which is detected by the hybridization with fluorescently labeled oligos (20).

PC-3 cells were plated on cover slips, incubated with 20μM NSC156529, 20μM PDPK1 inhibitor or 20μM DMSO for 4 hours and fixed with 4% paraformaldehyde (AppliChem) for 10 min at RT. Subsequently, cells were permeabilized with methanol for 10 min at -20 °C, followed by washing for 2x2 min in PBS, 0.05% Tween-20 at RT. Slides were blocked with in situ blocking buffer for 1 hour at RT, followed by the incubation with primary antibodies recognizing AKT1 (anti-mouse, 1:100; Cell Signaling) and PDPK1 (anti-rabbit, 1:100; Cell Signaling) overnight at 4°C. The negative control was performed using only one primary

antibody. After washing the slides in Washing Buffer A for 2x5 min, the PLA probes were incubated in 40 µl in situ blocking buffer for 1 hour at +37°C. Next, the cells were washed 2x5 min in Washing Buffer A followed by the incubation in 40 µl ligation solution for 1 hour at +37°C. The slides were washed 2x2 min in Wash Buffer A and incubated in 40µl Amplification-Polymerase solution for 100 min at +37°C. The slides were washed in 1x Wash Buffer B for 2x10 min and in 0,01x Wash Buffer B for 1 min. The cells were left to dry, then mounted with Mounting Medium, incubated for 15 minutes and imaged. The images were acquired with Olympus BX-71 fluorescent microscope using bundled Cell-R software. 5 different fields per each slide were acquired and analyzed using Imaris®7.6.3 software (Bitplane AG, Switzerland). The number of PLA signals per cell was counted defining a true signal as a local intensity maximum above a background threshold. The same input parameters were used throughout all experiments. All the experiments were repeated at least 3 times.

Western Blot analysis

The lysates obtained from PC-3 cells treated with 20µM NSC156529, 20µM PDPK1 inhibitor and 20µM DMSO were separated by SDS-PAGE and blotted using antibodies listed in Supplementary Table S1. The signals were detected using Fujifilm LAS4000 image analysis system. The experiment was repeated three times.

In vitro cell growth analysis

24 hours after seeding the PC-3 cells were incubated with 10µM small molecular compounds, 10µM PDPK1 inhibitor and 10µM DMSO for 24, 48, 72 and/or 96 hours. Cell proliferation was assessed using the CellTiter-Glo luminescent cell viability assay (Promega) according to manufacturer´s protocol. Each experiment was carried out in triplicate and at least 3 independent experiments were performed.

Xenograft assays

Xenograft experiments were performed as a service by vivarium of the department of Gene Technology, Tallinn Technology University using proprietary protocols and cell line. Briefly, $5x10^6$ PC-3 human prostate carcinoma cells stably expressing EGFP were injected s.c. into the right flank of athymic Nude ($Foxn1^{nu/nu}$) female mice. Tumors were grown 17 days until they reached a median size of 29-32mm^3. For injections the compound was dissolved in vehicle (13,75% DMSO, 1,25% Tween80, 85% double distilled sterile water). Animals were randomly divided into 4 groups: group A (n=16) received 10mg/kg of compound, group B (n=11) received 5mg/kg of compound, group C (n=10) received 1mg/kg of compound and group D (n=16) was treated with vehicle only. Subcutaneous injections were performed approx. 1cm around the tumor every other day during 28-day treatment period. Tumor sizes were measured with external caliper and tumor volumes were calculated as follows: width x width x length/2 (21). Simultaneously, the tumor size was measured by in vivo fluorescence imaging using IVIS® Lumina I In Vivo Imaging System (Caliper Life Sciences, Perkin Elmer, USA) and Living Image 4.0 software (Caliper Life Sciences, USA). Data were collected at different wavelengths (excitation: 430nm and 465nm; emission filter: 515-575nm). During the measurements mice were anesthetized using a vapor Isoflurane inhalation narcosis system Gas Anesthesia System for IVIS (Perkin Elmer, USA). All animal experiments were approved by local ethics authorities.

Body weight and the general condition of mice were assessed at least three times a week. Blood samples were obtained from mice prior sacrifice. ALT and AST values were measured using standard laboratory routines at the Central Laboratory of the Tartu University Clinics. Finally, mice were sacrificed, tumors were removed and weighed, and tumor samples were embedded in OCT and stored at –80 °C for further use.

Microscale thermophoresis (MST) binding assay

MST enables the detection of interactions between various molecules regardless of their size or origin. Measuring the changes in fluorescence, which reflect the motility of fluorescently tagged molecules in a microscopic temperature gradient caused by binding an unlabeled interaction partner causes the shift in the motility of labeled molecules (22). Binding kinetics of AKT1-EYFP and PDPK1-EYFP to NSC156529 was measured by MST with a NanoTemperMonolith NT.115 instrument (NanoTemper Technologies). H1299 cells were transfected either with 5µg AKT1-EYFP or PDPK1-EYFP expressing vector. The resulting cell lysates were diluted in reaction buffer (50mM Tris–HCl pH7.5, 150mM NaCl, 10mM $MgCl_2$, 0,05% Tween20, 0,05% Tween80). Serial dilutions of NSC156529 were prepared with the identical buffer (concentrations 200µM – 6,1nM). For thermophoresis, each ligand dilution was mixed with one volume of labeled proteins. After 1 hour incubation at 4°C approximately 4µL of each solution was filled into Monolith NT standard treated capillaries (NanoTemper Technologies GmbH). Thermophoresis was measured using a Monolith NT.115 instrument (NanoTemper Technologies GmbH). The signal values were analyzed using NT.Analysis software version 1.5.41 (NanoTemper Technologies).

RT-qPCR

RNA was isolated from human tumor xenografts using TRIzol (GIBCO) according to manufacturer's instructions. 1µg of total RNA was reverse transcribed with a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's instructions. All qPCR reactions were carried out with an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) and the data acquired were analyzed with ABI SDS software (version 2.1 from Applied Biosystems). The primer sequences used are listed in Supplementary Table S2. Target cDNAs were normalized to endogenous mRNA levels of the housekeeping

reference gene *Hprt1*. The PCR reactions were performed at least 3 times for each sample. Statistical significance was assessed with Student t-test.

Immunofluorescence analysis

5µm thick sections were treated with 4% paraformaldehyde (PFA; Applichem) and 0,1% Triton-X-100 for 10-15 minutes at RT. Sections were blocked with 4% normal goat serum (NGS) and incubated with primary antibodies (See antibody list Supplementary Table S3) followed by incubation of fluorochrome-conjugated secondary antibodies (See Supplementary Table S3), both 1h at RT. The images were acquired with Olympus BX-71 fluorescent microscope using bundled Cell-R software.

See the Supplementary Information page for further details.

Results

Screening for the inhibitors of AKT1 and PDPK1 interaction

To identify the inhibitors of AKT1 and PDPK1 interaction we carried out a small chemical library screen using the NCI Diversity Set I small-molecular compound library consisting of 2000 compounds in live cells. We took advantage of the protein complementation assay (PCA) based on split *Renilla reniformis* luciferase (*R*luc) fragments (F1 and F2) fused to the proteins of interest (19) (Figure 1A).

The screening experiments were performed in H1299 cells, which are known to harbor an active AKT signaling (23, 24) and are well suited for experiments involving DNA transfection (Figure 1B). To rule out unspecific modulation of the luciferase readouts quadruple replicates of H1299 cells transfected with pCNEO cloning vector (see Supplementary information); H1299 cells transfected with p53-F1 and HDM2-F2 treated with either DMSO or Nutlin-3; and H1299 cells transfected with AKT1-F1, PDPK1-F2 treated with DMSO were used (Supplementary Table S4). To eliminate the error caused by the well-to-well variation in cell numbers the luciferase reads were normalized to the viability counts as described in "Materials and methods". In addition, the viability counts were used to identify and exclude toxic compounds. The cells treated with vehicle only (DMSO) were used to set the baseline values. The cells transfected with p53-F1 and HDM2-F2 fusions treated with Nutlin-3 were used as assay controls. At least 2-fold reduction in luciferase signal upon Nutlin-3 addition was considered to indicate a successful experiment.

The screen was performed in two steps. In the first step the 2000 chemicals were screened for their ability to inhibit the interaction of transfected AKT1-F1 and PDPK1-F2.

The overall selection criteria for selection of compounds were set as follows: 1) at least 50% reduction of normalized AKT1-F1 - PDPK1-F2 interaction values when compared to the

average of DMSO-treated AKT1-F1 and PDPK1-F2-transfected cells; 2) 25% or less reduction in cell viability counts when compared to the average of DMSO-treated controls; 3) less than 10% reduction in full-length *Rluc* signal when compared to the average reading of DMSO-treated full-length *Rluc*-transfected cells (Figure 1C).

We identified 74 chemicals out of 2000, which inhibited the AKT1-F1 - PDPK1-F2 interaction at least 50% or more. The toxicity criterion was not applied at this stage. However, 412 chemicals from 2000 elicited acute toxicity towards H1299 cells (downregulation of viability counts 25% or more). In the next step the identified 74 chemicals were retested for AKT1-PDPK1 PCA inhibition; in parallel, the inhibition of full-length *Rluc* and cell toxicity were evaluated. All 74 chemicals inhibited the AKT1-PDPK1 PCA, however, 29 chemicals of 74 concomitantly inhibited the activity of full-length *Rluc* and 13 chemicals were toxic for cells. 9 of 45 chemicals, which specifically inhibited the AKT1-PDPK1 PCA reduced the viability counts 25% or more and thus were excluded from further analysis. As a result, 36 chemicals were considered for the next evaluation step (Supplementary Table S5).

Evaluation of the compounds in respect of their ability to reduce the AKT1 phosphorylation at T308

The hallmark of AKT1 activation by PDPK1 is the phosphorylation of AKT1 at T308 (18, 25, 26). To evaluate the ability of the 36 chemicals identified in the previous step to inhibit the phosphorylation of T308 we performed a western blot analysis of H1299 cells treated with these chemicals (Figure 2A). To minimize possible indirect effects on the AKT1 phosphorylation the treatment was carried out for 4 hours. GSK 2334470, which directly inhibits the kinase properties of PDPK1, was used as a positive control. By using an antibody, which specifically recognized phospho-T308 on AKT1 we identified 12 compounds of 36, which inhibited AKT1 protein phosphorylation to a various degree. None of these compounds

changed significantly the basal levels of AKT1 and PDPK1 proteins. In parallel, the cultured cells treated with these compounds were carefully examined for signs of toxicity and 4 chemicals of 12, which did not cause notable changes in cell density and morphology during the 4-hour treatment, were chosen for further analysis (Figure 2A, B, Supplementary Figure S1 A-D).

NSC156529 inhibits the interaction of endogenous AKT1 and PDPK1 proteins

Next we sought to find out whether the chemicals selected in the previous steps could inhibit the interaction of endogenous AKT1 and PDPK1 proteins. For this we utilized *in situ* proximity ligation assay (*in situ* PLA) in PC-3 prostate cancer cells known to harbor an activated AKT signaling pathway (27). The PC-3 cells, seeded to glass slides, were incubated with the 4 chemicals selected in the previous step and DMSO for 4 hours. A number of AKT1-PDPK1 interaction sites were readily detected in the DMSO-treated PC-3 cells (Figure 2C, Supplementary Figure S2 A) whereas the count of AKT1-PDPK1 interaction sites was significally decreased in the cells treated with the NSC156529 (Figure 2C, Supplementary Figure S2 G) compared to the AKT2-PDPK1 or AKT3-PDPK1 interactions (Supplementary Figure S2 H-I) indicating the preferred inhibition of AKT1-PDPK1 interaction by NSC156529. The number of AKT1-PDPK1 interaction sites in cells treated with NSC5113, NSC15784 and NSC292596 (Supplementary Figure S2 D-F, J) remained at the same level as in the control cells. Cells treated with PDPK inhibitor and cells with no primary antibody incubation were used as controls (Supplementary Figure S2 B, C). Based on this experiment one compound - NSC156529 was selected for further analysis.

Next we treated H1299 cells transfected with AKT-F1 and PDPK-F2 with increasing concentrations of NSC156529. The results of luciferase assay showed that NSC156529 dose-dependently inhibited AKT1-PDPK1 interaction (Figure 3A) with calculated IC_{50}=3,862μM.

NSC156529 interacts preferentially with PDPK1

The experiments conducted so far showed that NSC156529 prevents the interaction between AKT1 and PDPK1. This might be caused by direct interaction of NSC156529 either with AKT1 or PDPK1. To test this hypothesis we studied the potential interaction between NSC156529 and EYFP-tagged AKT1 or PDPK1 in cell lysates using microscale thermophoresis (MST). For MST analysis either EYFP-tagged AKT1 or PDPK1 (AKT1-EYFP; PDPK1-EYFP) were transfected into H1299 cells, the cell lysates containing fluorescently labeled proteins were treated with serially diluted unlabeled NSC156529 (concentration range 200μM-6,1nM) and analyzed using a dedicated MST instrument as described in "Materials and Methods". While no stable binding of NSC156529 to AKT1-EYFP could be detected (Supplementary Figure S3 A) a binding event with the Kd of 981±131nM was determined for the NSC156529 and PDPK1-EYFP interaction (Supplementary Figure S3 B) suggesting that NSC156529 binds directly to PDPK1.

Two well-defined docking sites have been identified on PDPK1 molecule: the ATP pocket and PIF-pocket, which is required for the phosphorylation of selected PDPK1 targets (28). To examine the possibility that NSC156529 binds to these sites docking calculations using Glide (Glide, v6.2, Schrödinger, LLC, New York, NY, 2014) with standard settings were performed using the previously published PDPK1 crystal structure (PDB ID - 1H1W, (29)). The results of the calculations demonstrated no reliable binding of NSC156529 to these two sites, suggesting that NSC156529 most likely interacts with PDPK1 at other less structured locations (personal communication from Uko Maran and Alfonso T. García-Sosa).

NSC156529 inhibits the AKT1 signaling pathway

Previous experiments showed that NSC156529 inhibits the interaction between AKT1 and PDPK1 proteins and reduces phosphorylation of AKT1 protein at Thr308. To find out

whether the treatment with NSC156529 decreases also the phosphorylation of AKT1 downstream targets we studied the phosphorylation status of GSK3β, FOXO3a, BAD and procaspase 9 by Western Blot (30-36). PC-3 cells were incubated with NSC15652 and with DMSO or PDPK1 inhibitor as background and positive controls correspondingly for 4 hours. The incubation with NSC156529 resulted in a marked decrease in the phosphorylation of the studied AKT1 target proteins (Figure 3B). The intracellular amount of these proteins did not change with the exception of FOXO3a where both NSC156529 and, to a lesser extent, the PDPK1 inhibitor reduced the overall FOXO3a protein level. Taken together these results indicate that the treatment with NSC156529 results in the inhibition of the key biochemical activities of the AKT signaling pathway.

NSC156529 preferentially reduces the proliferation of cultured tumor cells

Our next goal was to determine the effect of NSC156529 on the growth of malignant and normal cells. PC-3 prostate tumor cells, normal primary human fibroblasts and osteoblasts were treated with increasing concentrations of NSC156529 for 96 hours. The treatment with DMSO was used as reference. We found that NSC156529 inhibits dose-dependently the growth of PC-3 cells, fibroblasts and osteoblasts (Figure 3C). Notably, the growth of PC-3 cells was inhibited to a larger extent than that of normal cells. To verify that our observations were not cell line specific, other four immortalized and tumor cell lines, Hek293, H1299, K07074 and Hep3B were treated with 10μM NSC156529, DMSO and PDPK1 inhibitor for 24, 48, 72 and 96 hours (Supplementary Figure S4 A-D). The experiments showed that NSC156529 inhibited strongly the growth of these cell lines by 96h. Notably, NSC156529 was more efficient in inhibiting the cell growth than PDPK1 inhibitor (Supplementary Figure S4 C, D).

NSC156529 reduces human tumor xenograft growth *in vivo*

To examine the ability of the NSC156529 to suppress tumor growth *in vivo*, we took advantage of a tumor xenograft model (Figure 4A). To establish tumors nude mice were injected subcutaneously with PC-3 prostate cancer cells, which constitutively expressed EGFP. When tumors achieved the median size of 29-32 mm^3 the mice were injected with NSC156529 subcutaneously three times a week at concentrations 1mg/kg, 5mg/kg, 10mg/kg or with vehicle only. The tumor size was measured externally by using a caliper and in parallel the number of tumor cells was monitored by using an in vivo imaging device as described in "Materials and Methods". During the 28-day treatment period, the suppression of tumor growth was observed for all concentrations used and NSC156529 inhibited growth of PC-3-derived tumors (Figure 4B). Measurements obtained with external caliper were consistent with GFP measurements showing unequivocally that NSC156529 reduces the tumor growth in vivo (Figure 4C).

During the treatment course, no adverse side effects, such as weight loss, ulcerations or general non-well-being of the animals were observed. To assess the toxicity of the compound the ALT and AST levels, indicative of liver damage were measured from sera collected from NSC156529- and vehicle-treated mice at the endpoint of the experiment (Supplementary Figure S5). No considerable increase in the ALT and AST values was detected showing that in our experimental system NSC156529 does not exert hepatotoxicity.

NSC156529 inhibits cell proliferation and induces the expression of differentiation markers in vivo

To shed light on the mechanism by which NSC156529 inhibits the tumor growth in nude mice, we first studied the activity of AKT pathway in the NSC156529-treated and control tumor xenografts by evaluating the phosphorylation status of T308 in AKT1 and BAD using

immunofluorescence analysis (Figure 5A, B). We could confirm the inhibition of the AKT pathway as the number of cells positive for the pAKT(T308) and the phosphorylated form of BAD protein were noticeably reduced in NSC156529-treated PC-3-GFP xenografts. To study the mitotic activity present in the control and NSC156529-treated xenografts we stained the tumor sections with an antibody recognizing phospho-histone H3 (pH3) - a marker for mitotic cells. The number of pH3-positive cell nuclei was decreased in NSC156529-treated xenografts indicating that at least in part the inhibition of cell proliferation accounts was causing the inhibition of tumor growth (Figure 5C, D).

A plausible mechanism by which NSC156529 could inhibit the tumor growth was the increase in the number of apoptotic cells in the treated tumors. To study this possibility we detected the presence of apoptosis markers - fragmented DNA and cleaved caspase-3 (CC3) in tumor xenograft cryosections. To detect the presence of DNA fragments we used terminal deoxynucleotidyl transferase nick end labeling (TUNEL) assay and to measure the amount CC3 we utilized a specific antibody recognizing this form of caspase 3. We could detect the presence of a number of apoptotic cells positive for TUNEL and CC3 already in the untreated tumors (Supplementary Figure S6 A, B). The presence of cells, which spontaneously undergo apoptosis, is a characteristic feature of malignant neoplasms. The treatment with NSC156529 did not significantly increase the number of cells positive for TUNEL nor CC3, which indicates that the induction of apoptosis did not have a significant role in the growth reduction of mouse tumor xenografts induced by NSC156529.

Another possible mechanism for the tumor growth reduction is the induction of differentiation. Since PC-3 cells display properties of poorly differentiated prostate cancer (37) we hypothesized that downregulation of AKT pathway induced the differentiation of grafted tumor cells. To test this possibility we studied the expression of cytokeratins 15, 17, 18 and 8 in tumor xenografts. Cytokeratins 15 and 17 (CK15/17) are the markers for human

prostate basal epithelial cells and cytokeratins 8 and 18 (CK8/18) label the differentiated luminal cells (38). We found that the expression of CK15/CK17 (Figure 6A, C, D) and CK8/CK18 (Figure 6B, E, F) was increased in NSC156529-treated PC-3-GFP xenografts indicating that NSC156529 limits the tumor growth at least in part by directing the cancer cells to differentiate.

Discussion

The purpose of the present study was to discover small molecular compounds that would utilize a principally novel mechanism to inhibit the signal transduction along the PI3K/AKT pathway. We chose to target the interaction between AKT1 and PDPK1 - a critical step in the AKT signaling cascade. To reach our goal we took advantage of the reversible complementation of two *Renilla* luciferase fragments, which enabled us to detect the interaction of two proteins of interest in live cells (19). The screening and following experiments identified one chemical - NSC156529 - that reduced the interaction of overexpressed and endogenous AKT1 and PDPK1 proteins. Concomitantly, this compound reduced AKT1 phosphorylation and inhibited tumor cell growth in vitro and in vivo tumor xenograft model. Although the detailed molecular mechanism of AKT1 inactivation by NSC156529 remains to be elucidated our results suggest that it interfered with the AKT1-PDPK1 interaction by direct binding to PDPK1.

Prostate cancer is the second leading cause of death among men in Western world. Although this disease can have a relatively benign course as well-differentiated forms of this tumor remain indolent and are never lethal, the undifferentiated prostate cancer is a highly malignant disease, which requires radical intervention (39). Thus, the discovery of novel substances that would direct the tumor cells to differentiate rather than induce cell death is an emerging possibility for cancer treatment. As an example of a differentiation-based tumor therapy is the

addition of retinoids in the acute promyelocytic leukemia (APML) treatment scheme, which dramatically improves patient survival (40). Furthermore, conventional chemotherapy is often associated with the development of drug resistance and systemic toxicity, thereby limiting therapeutic effectiveness (41). For this reason, using a combined therapy scheme where one component would be compound, which induces tumor cell differentiation would facilitate the reduction of drug dosage, limit the occurrence of side effects and reduce the occurrence of drug resistance (42, 43). It is known that malignant prostate cancer cells and cell lines including PC-3 harbor an increased level of AKT signaling (27). Consequently, our finding that NSC156529-treated xenografts expressed increased levels of differentiation markers suggests that manipulating the activity of AKT pathway, at least at the level of AKT1-PDPK1 interaction, might open up a new option for tumor treatment via inducing cell differentiation.

Hyperactivated AKT signaling pathway has also been found in many human tumor types (6, 44-46). In line with this, our in vitro cell growth analysis confirmed that the cell proliferation inhibiting effect of NSC156529 extended to the cell lines of variable origin, thus the potential use of this chemical should not be limited with prostate cancer only. Indeed, data mining revealed that NSC156529 inhibited the growth of B16 melanoma cells and leukemia cell lines L1210 and P388 in tumor xenograft assays conducted by NCI within the framework of Developmental Therapeutics Program (47).

Although NSC156529 inhibited effectively the growth of normal human fibroblasts and osteoblasts *in vitro*, the compound was well-tolerated *in vivo* when using subcutaneous administration route. Since systemic administration of this compound to the blood stream was not tested the relatively low general toxicity observed during our studies might be caused by a reduced exposure of the host organism to NSC156259. When the compound was administered intraperitoneally severe symptoms of irritation were observed warranting for potential local toxicity on mucous membranes and endothelium. Interestingly, we did not notice any severe

symptoms of irritation at the injection sites in skin. Further toxicity studies should resolve this discrepancy.

Taken together, we have demonstrated that the small molecular compound NSC156529 is a potent inhibitor of AKT1 signaling pathway in tumor cells. NSC156529 reduces the AKT activity, inhibits the activation of AKT target proteins that are involved in regulating cell survival, proliferation and metabolism and inhibits the growth of tumor cells in vitro and in vivo. Notably, the treatment with NSC156529 increases the differentiation status of cancer cells, which presents this compound as a promising lead for the development of a novel class of tumor therapeutics.

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References

1. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005;24(50):7455-64.
2. Bononi A, Agnoletto C, De Marchi E, Marchi S, Patergnani S, Bonora M, et al. Protein kinases and phosphatases in the control of cell fate. Enzyme research. 2011;2011:329098.
3. Cheung M, Testa JR. Diverse mechanisms of AKT pathway activation in human malignancy. Current cancer drug targets. 2013;13(3):234-44.
4. Shi W, Zhang X, Pintilie M, Ma N, Miller N, Banerjee D, et al. Dysregulated PTEN-PKB and negative receptor status in human breast cancer. International journal of cancer Journal international du cancer. 2003;104(2):195-203.
5. Roy HK, Olusola BF, Clemens DL, Karolski WJ, Ratashak A, Lynch HT, et al. AKT proto-oncogene overexpression is an early event during sporadic colon carcinogenesis. Carcinogenesis. 2002;23(1):201-5.
6. Le Page C, Koumakpayi IH, Alam-Fahmy M, Mes-Masson AM, Saad F. Expression and localisation of Akt-1, Akt-2 and Akt-3 correlate with clinical outcome of prostate cancer patients. British journal of cancer. 2006;94(12):1906-12.
7. Itoh N, Semba S, Ito M, Takeda H, Kawata S, Yamakawa M. Phosphorylation of Akt/PKB is required for suppression of cancer cell apoptosis and tumor progression in human colorectal carcinoma. Cancer. 2002;94(12):3127-34.
8. Huang WC, Hung MC. Induction of Akt activity by chemotherapy confers acquired resistance. Journal of the Formosan Medical Association = Taiwan yi zhi. 2009;108(3):180-94.
9. Lindsley CW. The Akt/PKB family of protein kinases: a review of small molecule inhibitors and progress towards target validation: a 2009 update. Current topics in medicinal chemistry. 2010;10(4):458-77.
10. Li Q, Zhu GD. Targeting serine/threonine protein kinase B/Akt and cell-cycle checkpoint kinases for treating cancer. Current topics in medicinal chemistry. 2002;2(9):939-71.
11. Gills JJ, Dennis PA. The development of phosphatidylinositol ether lipid analogues as inhibitors of the serine/threonine kinase, Akt. Expert opinion on investigational drugs. 2004;13(7):787-97.
12. Bobkova EV, Weber MJ, Xu Z, Zhang YL, Jung J, Blume-Jensen P, et al. Discovery of PDK1 kinase inhibitors with a novel mechanism of action by ultrahigh throughput screening. J Biol Chem. 2010;285(24):18838-46.
13. Nagashima K, Shumway SD, Sathyanarayanan S, Chen AH, Dolinski B, Xu Y, et al. Genetic and pharmacological inhibition of PDK1 in cancer cells: characterization of a selective allosteric kinase inhibitor. J Biol Chem. 2011;286(8):6433-48.
14. Jin L, Wang W, Fang G. Targeting protein-protein interaction by small molecules. Annu Rev Pharmacol Toxicol. 2014;54:435-56.
15. Lindsley CW, Zhao Z, Leister WH, Robinson RG, Barnett SF, Defeo-Jones D, et al. Allosteric Akt (PKB) inhibitors: discovery and SAR of isozyme selective inhibitors. Bioorg Med Chem Lett. 2005;15(3):761-4.
16. Zhao Z, Robinson RG, Barnett SF, Defeo-Jones D, Jones RE, Hartman GD, et al. Development of potent, allosteric dual Akt1 and Akt2 inhibitors with improved physical properties and cell activity. Bioorg Med Chem Lett. 2008;18(1):49-53.
17. Hirai H, Sootome H, Nakatsuru Y, Miyama K, Taguchi S, Tsujioka K, et al. MK-2206, an allosteric Akt inhibitor, enhances antitumor efficacy by standard chemotherapeutic agents or molecular targeted drugs in vitro and in vivo. Mol Cancer Ther. 2010;9(7):1956-67.
18. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. The EMBO journal. 1996;15(23):6541-51.

19. Stefan E, Aquin S, Berger N, Landry CR, Nyfeler B, Bouvier M, et al. Quantification of dynamic protein complexes using Renilla luciferase fragment complementation applied to protein kinase A activities in vivo. Proc Natl Acad Sci U S A. 2007;104(43):16916-21.
20. Gajadhar A, Guha A. A proximity ligation assay using transiently transfected, epitope-tagged proteins: application for in situ detection of dimerized receptor tyrosine kinases. BioTechniques. 2010;48(2):145-52.
21. Euhus DM, Hudd C, LaRegina MC, Johnson FE. Tumor measurement in the nude mouse. Journal of surgical oncology. 1986;31(4):229-34.
22. Jerabek-Willemsen M, Wienken CJ, Braun D, Baaske P, Duhr S. Molecular interaction studies using microscale thermophoresis. Assay and drug development technologies. 2011;9(4):342-53.
23. Hernandez VJ, Weng J, Ly P, Pompey S, Dong H, Mishra L, et al. Cavin-3 dictates the balance between ERK and Akt signaling. eLife. 2013;2:e00905.
24. Grabinski N, Bartkowiak K, Grupp K, Brandt B, Pantel K, Jücker M. Distinct functional roles of Akt isoforms for proliferation, survival, migration and EGF-mediated signalling in lung cancer derived disseminated tumor cells. Cellular Signalling. 2011;23(12):1952-60.
25. Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, et al. Role of translocation in the activation and function of protein kinase B. The Journal of biological chemistry. 1997;272(50):31515-24.
26. Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, et al. Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene. 1998;17(3):313-25.
27. Won KJ, Kim BK, Han G, Lee K, Jung YJ, Kim HM, et al. NSC126188 induces apoptosis of prostate cancer PC-3 cells through inhibition of Akt membrane translocation, FoxO3a activation, and RhoB transcription. Apoptosis : an international journal on programmed cell death. 2014;19(1):179-90.
28. Biondi RM, Komander D, Thomas CC, Lizcano JM, Deak M, Alessi DR, et al. High resolution crystal structure of the human PDK1 catalytic domain defines the regulatory phosphopeptide docking site. EMBO J. 2002;21(16):4219-28.
29. Biondi RM, Nebreda AR. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. Biochem J. 2003;372(Pt 1):1-13.
30. Downward J. PI 3-kinase, Akt and cell survival. Seminars in cell & developmental biology. 2004;15(2):177-82.
31. Rena G, Guo S, Cichy SC, Unterman TG, Cohen P. Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. The Journal of biological chemistry. 1999;274(24):17179-83.
32. Biggs WH, 3rd, Meisenhelder J, Hunter T, Cavenee WK, Arden KC. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(13):7421-6.
33. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science (New York, NY). 1998;282(5392):1318-21.
34. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995;378(6559):785-9.
35. Forde JE, Dale TC. Glycogen synthase kinase 3: a key regulator of cellular fate. Cellular and molecular life sciences : CMLS. 2007;64(15):1930-44.
36. Bijur GN, Jope RS. Rapid accumulation of Akt in mitochondria following phosphatidylinositol 3-kinase activation. Journal of neurochemistry. 2003;87(6):1427-35.
37. Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). Investigative urology. 1979;17(1):16-23.
38. Hudson DL, Guy AT, Fry P, O'Hare MJ, Watt FM, Masters JR. Epithelial cell differentiation pathways in the human prostate: identification of intermediate phenotypes by keratin expression. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society. 2001;49(2):271-8.

39. Rodney S, Shah TT, Patel HR, Arya M. Key papers in prostate cancer. Expert Rev Anticancer Ther. 2014;14(11):1379-84.
40. Cruz FD, Matushansky I. Solid tumor differentiation therapy - is it possible? Oncotarget. 2012;3(5):559-67.
41. Leszczyniecka M, Roberts T, Dent P, Grant S, Fisher PB. Differentiation therapy of human cancer: basic science and clinical applications. Pharmacology & therapeutics. 2001;90(2-3):105-56.
42. Faivre S, Djelloul S, Raymond E. New paradigms in anticancer therapy: targeting multiple signaling pathways with kinase inhibitors. Seminars in oncology. 2006;33(4):407-20.
43. Bozic I, Reiter JG, Allen B, Antal T, Chatterjee K, Shah P, et al. Evolutionary dynamics of cancer in response to targeted combination therapy. eLife. 2013;2:e00747.
44. Paweletz CP, Charboneau L, Bichsel VE, Simone NL, Chen T, Gillespie JW, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene. 2001;20(16):1981-9.
45. Malik SN, Brattain M, Ghosh PM, Troyer DA, Prihoda T, Bedolla R, et al. Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8(4):1168-71.
46. Xin L, Teitell MA, Lawson DA, Kwon A, Mellinghoff IK, Witte ON. Progression of prostate cancer by synergy of AKT with genotropic and nongenotropic actions of the androgen receptor. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(20):7789-94.
47. Developmental Therapeutics Program NCI/NIH. National Cancer Institute. 1955.

Figure legends

Figure 1. Screen setup and screening procedure for inhibitors of AKT1 and PDPK1 interaction. *A*. A schematic representation of the PCA strategy using *R*luc fragments to study the interaction between AKT1 and PDPK1 proteins in vivo. *B.* Workflow of small molecular library screening. *C*. Selection procedure of AKT1-PDPK1 inhibitors.

Figure 2. AKT1-PDPK1 interaction inhibitor selection based on pAKT(T308) level and in situ PLA method. *A*. The phosphorylation of AKT1 protein at Thr308 following treatment with selected chemicals. *B.* Formulas and the NSC numbers of the 4 chemicals selected for further analysis. *C*. NSC156529 inhibits AKT1-PDPK1 interaction in PC-3 cells as detected by *in situ* PLA. Results are represented as quantitative analysis of the number of PLA signals per cell.

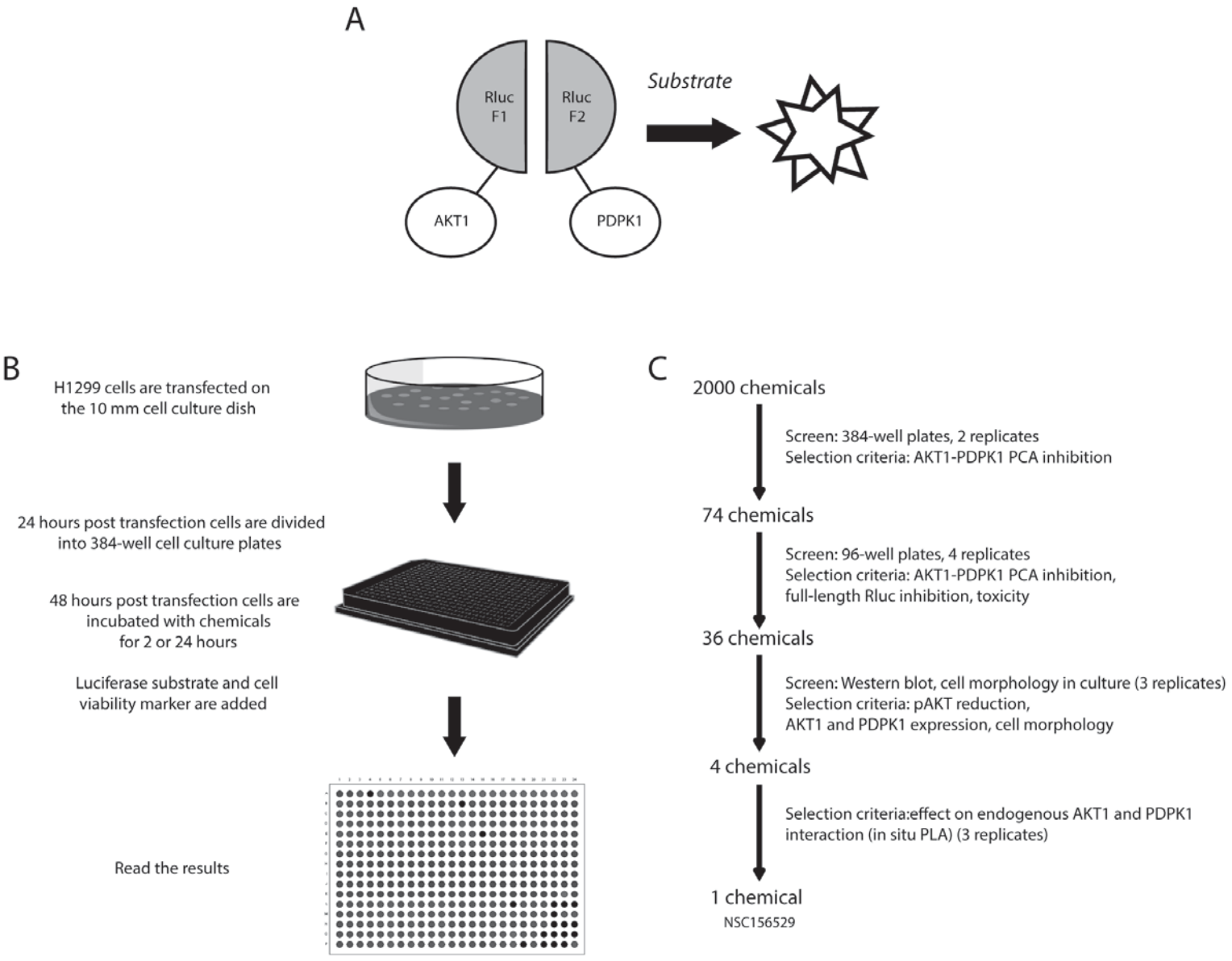
Figure 3. NSC156529 reduces the phosphorylation of AKT1 target proteins in PC-3 cells and inhibits the growth of tumor cells in vitro. *A*. NSC156529 inhibits dose-dependently AKT1-PDPK1 interaction. *B.* Western blot analysis of AKT1, pAKT1(T308), BAD, pBAD(S136), FOXO3a, pFOXO3a(T32), pFOXO(S253), GSKβ, pGSKβ(S9) and phosphorylated procaspase (S196) proteins in PC-3 cells. Actin was used as a loading control. All samples derive from the same experiment and plots were processed in parallel. *C.* Luminescent cell viability assay of PC-3 cells, normal human fibroblasts and osteoblasts treated with indicated concentrations of DMSO or NSC156529 for 96 hours.

Figure 4. NSC156529 inhibits tumor cell growth in human xenograft model in vivo. *A*. A schematic representation of the tumor xenograft experiment. *B, C.* Tumor sizes were measured with external caliper (*B*) and in parallel with in vivo fluorescence imaging (*C*). The growth of NSC156529-treated tumors was arrested in all concentrations used ($p = 0.0002$, Mann-Whitney U test, data from fluorescence imaging).

Figure 5. NCS156529 inhibits AKT signaling and decreases the mitotic activity of the cells in tumor xenografts. PC-3 xenograft cryosections were subjected to immunofluorescence analysis using specific primary antibodies to pAKT(T308) (*A*) and pBAD(Ser136) (*B*), EGFP expression served for the identification of grafted PC-3 cells. The tumor sections were stained with phospho-histone H3 (pH3) antibody (*C)*. Quantitation of pH3-positive cell numbers (*D*).

Figure 6. NSC156529 induces tumor cell differentiation in PC-3 tumor xenografts. The PC-3 xenograft cryosections were immunostained with antibodies recognizing CK15, CK17 (*A*) and CK8, CK18 (*B*). The expression of cytokeratins 15 and 17 (CK15/17) (*C, D*) and cytokeratins 8 and 18 (CK8/18) (*E, F*) was measured by qPCR and normalized to Hprt1. The mean of three experiments is shown.

Figure 1



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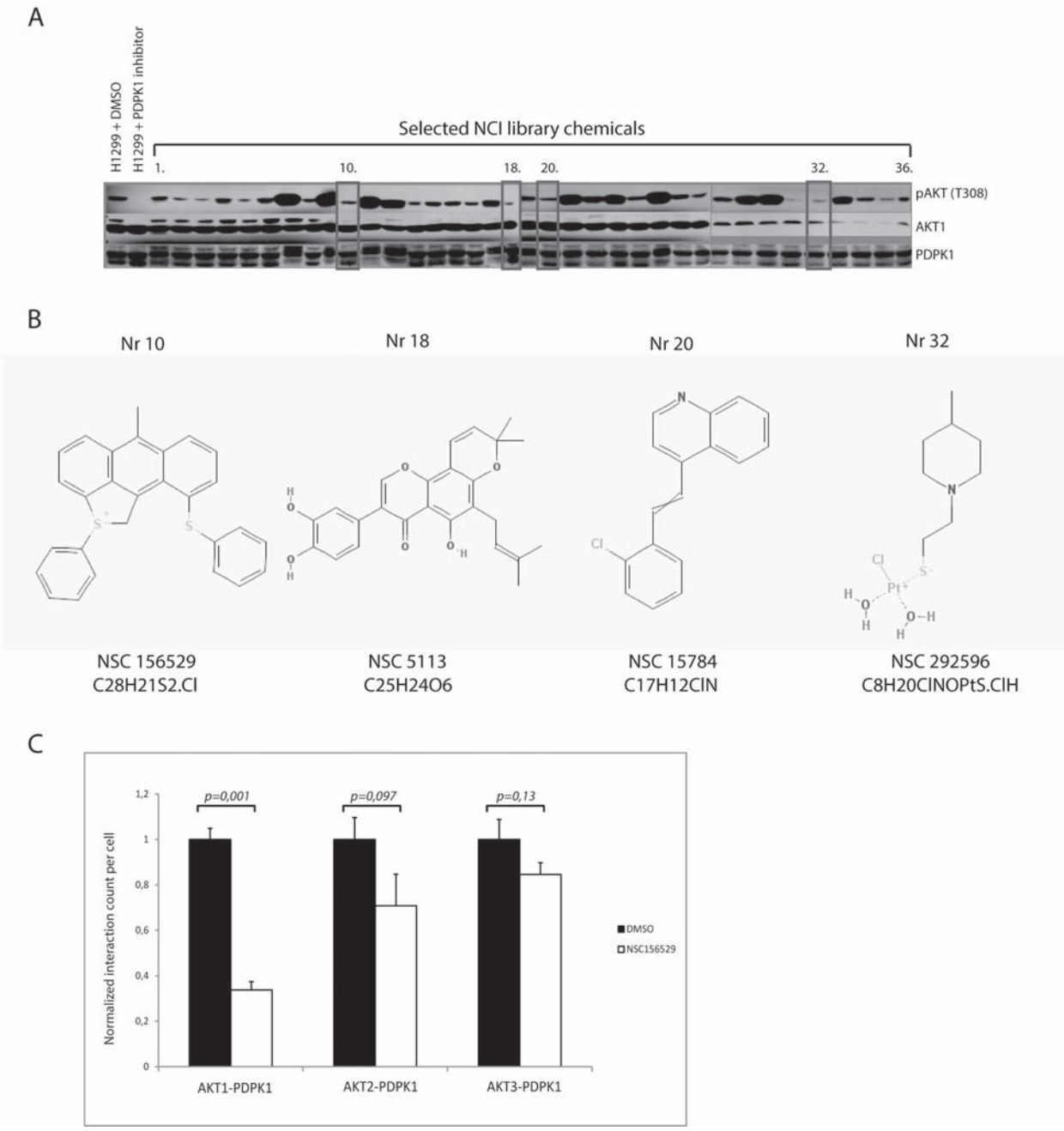
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 2



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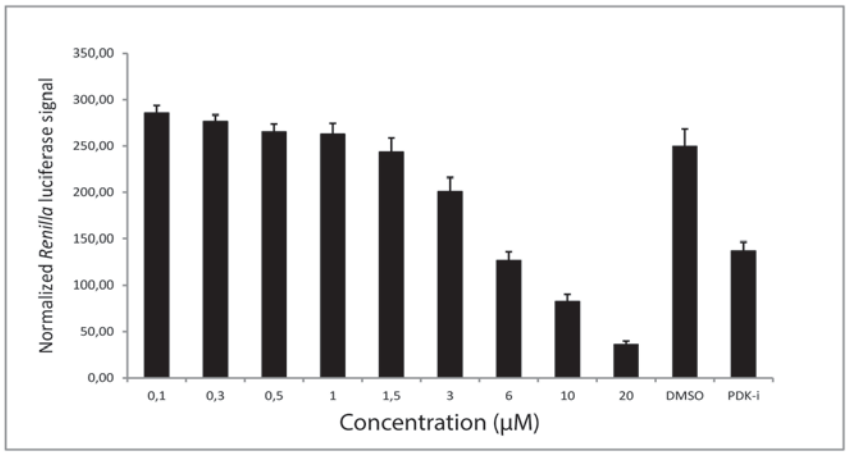

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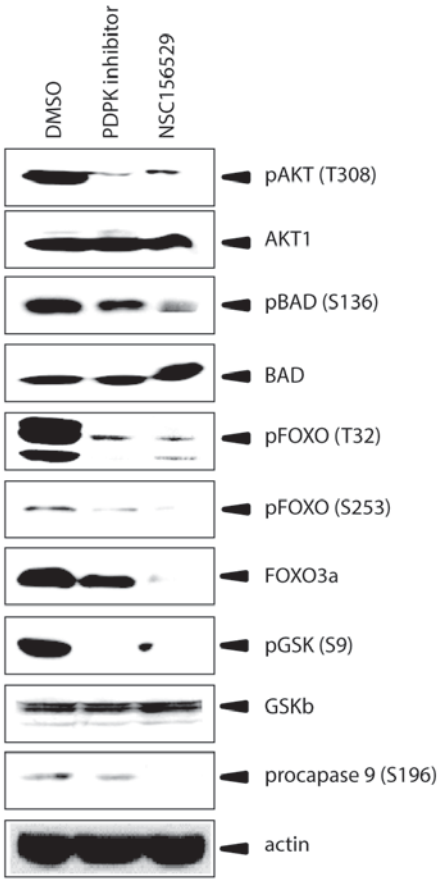

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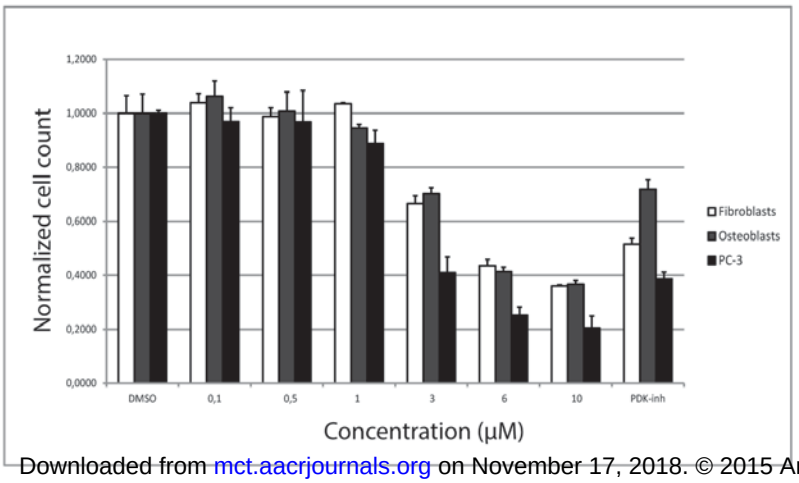

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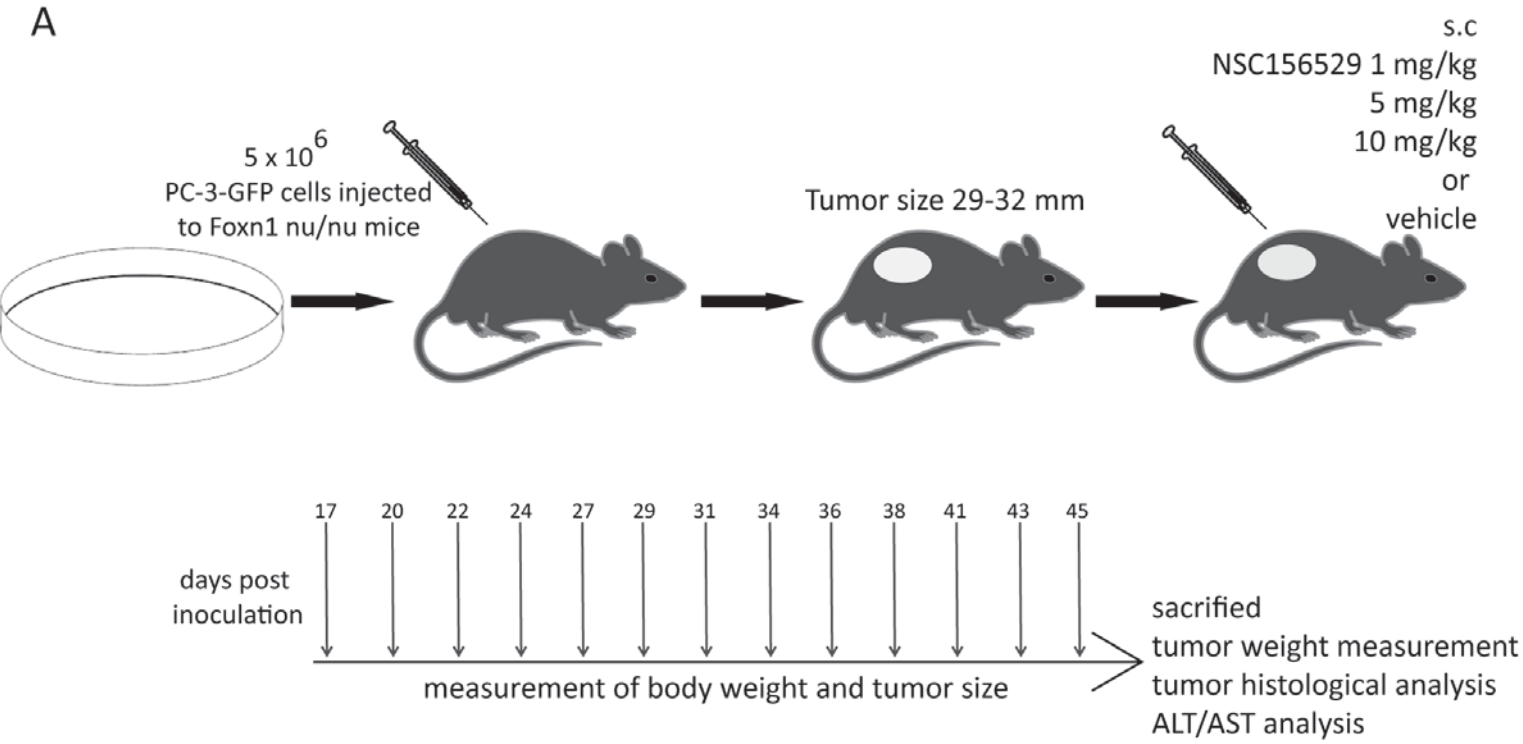
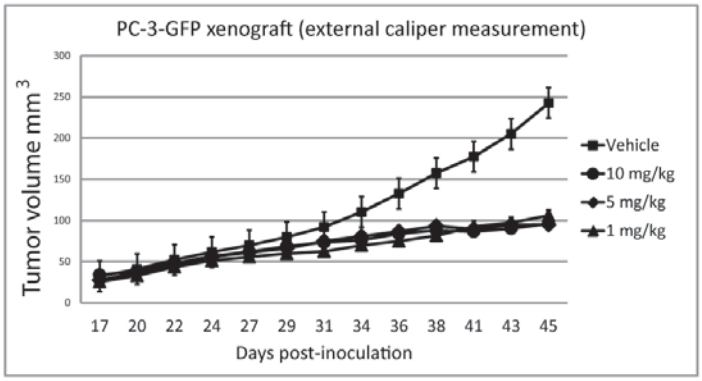
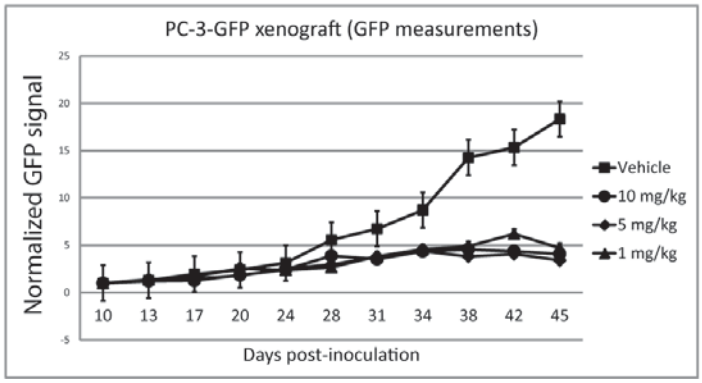
Figure 4







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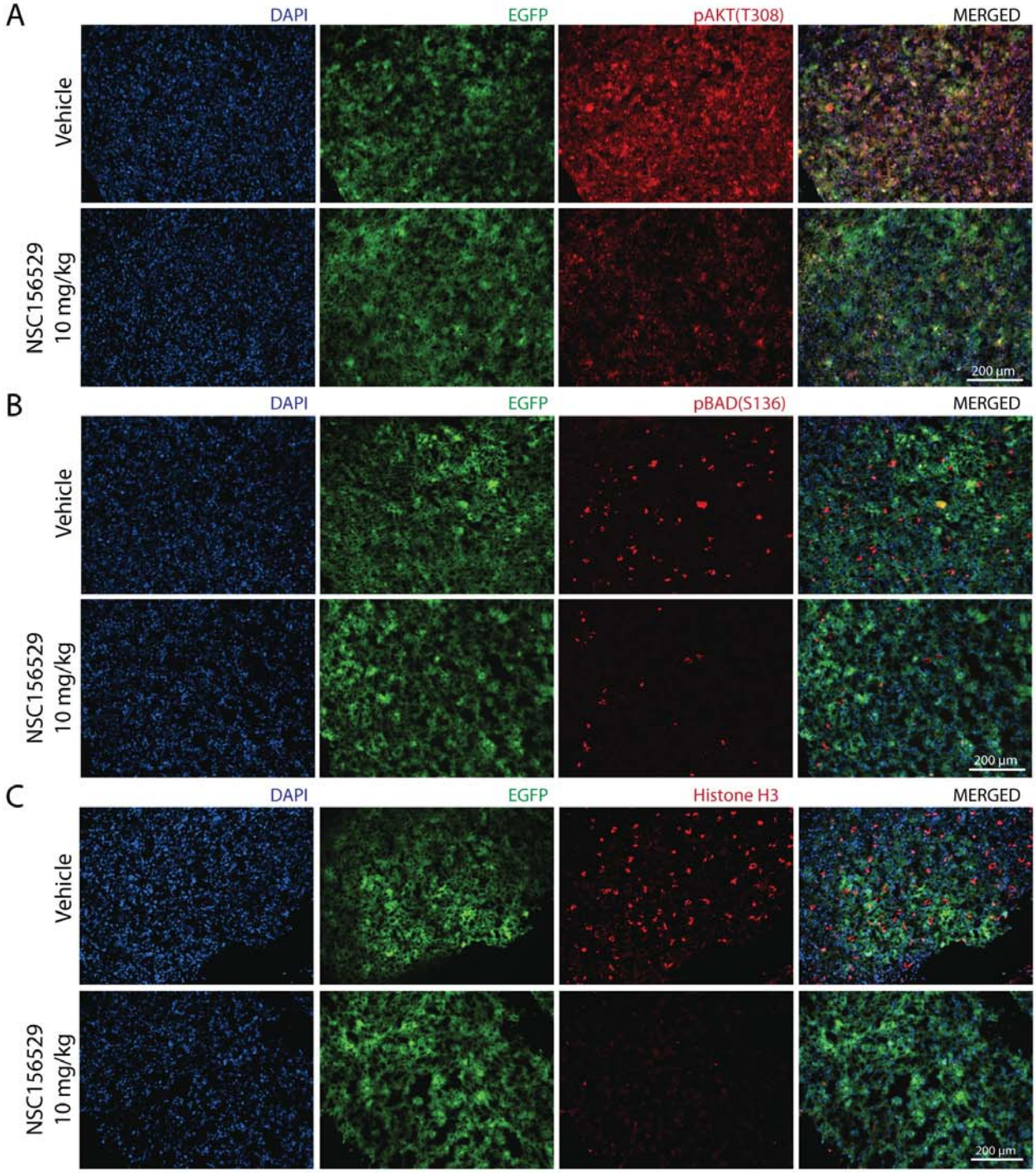

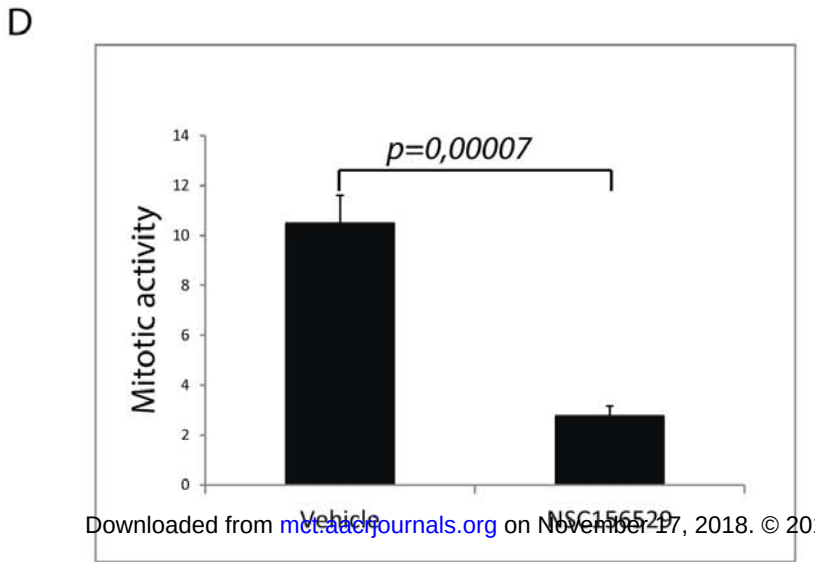

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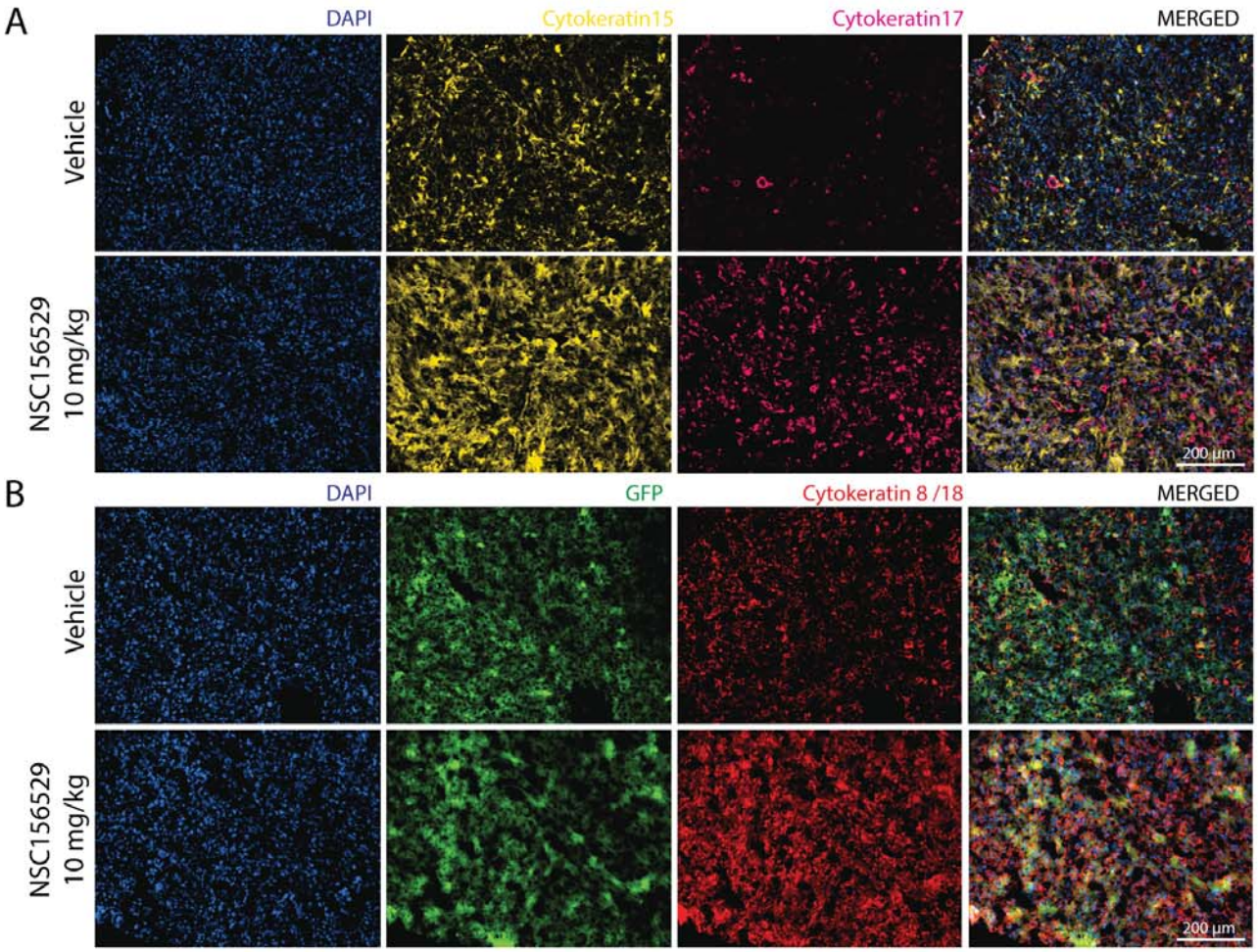

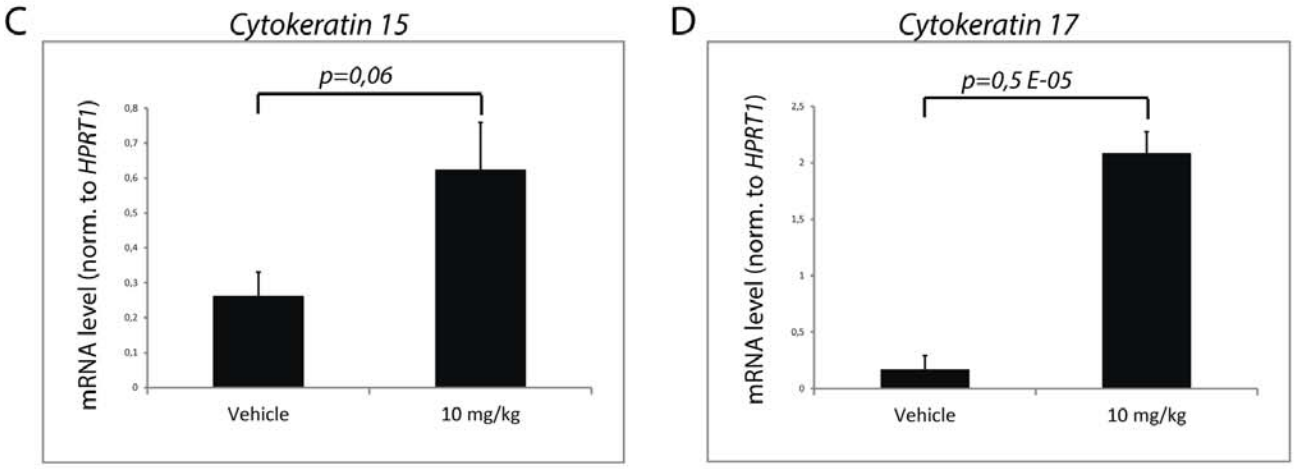

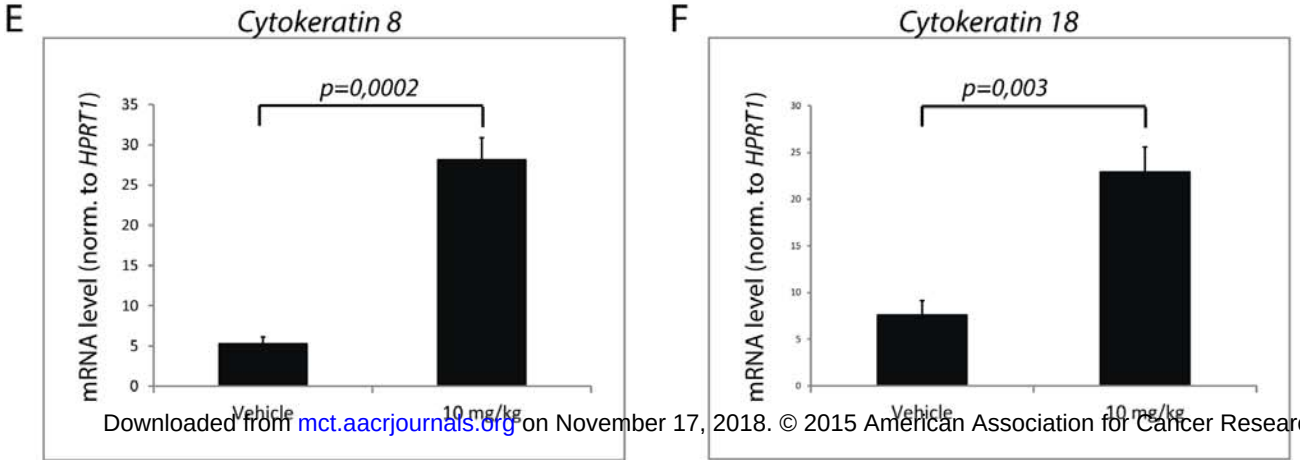

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Molecular Cancer Therapeutics

A novel inhibitor of AKT1-PDPK1 interaction efficiently suppresses the activity of AKT pathway and restricts tumor growth in vivo

Kristina Mäemets-Allas, Janeli Viil and Viljar Jaks

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