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Reactivation of AKT signaling following treatment of cancer cells with PI3K inhibitors attenuates their antitumor effects

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ABSTRACT

Targeting the phosphatidylinositol-3-kinase (PI3K) is a promising approach in cancer therapy. In particular, PI3K blockade leads to the inhibition of AKT, a major downstream effector responsible for the oncogenic activity of PI3K. However, we report here that small molecule inhibitors of PI3K only transiently block AKT signaling. Indeed, treatment of cancer cells with PI3K inhibitors results in a rapid inhibition of AKT phosphorylation and signaling which is followed by the reactivation of AKT signaling after 48 h as observed by Western blot. Reactivation of AKT signaling occurs despite effective inhibition of PI3K activity by PI3K inhibitors. In addition, wortmannin, a broad range PI3K inhibitor, did not block AKT reactivation suggesting that AKT signals independently of PI3K. In a therapeutical perspective, combining AKT and PI3K inhibitors exhibit stronger anti-proliferative and pro-apoptotic effects compared to AKT or PI3K inhibitorsalone. Similarly, in a tumor xenograft mouse model, concomitant PI3K and AKT blockade results in stronger anti-cancer activity compared with either blockade alone. This study shows that PI3K inhibitors only transiently inhibit AKT which limits their antitumor activities. It also provides the proof of concept to combine PI3K inhibitors with AKT inhibitors in cancer therapy.

1. Introduction

Activation of the phosphatidylinositol-3-kinase (PI3K) is frequently observed in a variety of human cancers [1–3]. Amplification of *PI3K* gene or activating mutations of the catalytic subunit of PI3K as well as loss of the phosphatase and tensin homolog (PTEN) tumor suppressor, which negatively regulates PI3K pathway, have all been described in cancer. In addition, PI3K is also activated by oncogenes such as RAS and tyrosine kinases like EGFR, MET and KIT. Following activation, PI3K promotes cancer cell growth and survival and therefore represents a promising target in cancer therapy [4]. Hence, several PI3K inhibitors have been developed and have demonstrated anti-cancer efficacy in pre-clinical models [3,5]. Initial clinical trials have however shown that the anticancer efficacy of PI3K inhibitors is weaker than expected and have suggested that PI3K inhibitors provide limited benefits when used as single agents. In fact, like for other targeted therapies, cancer cells adapt to PI3K inhibition and develop several resistance mechanisms to compensate for the inhibition of PI3K [6,7]. For example, PI3K inhibition leads to the upregulation of receptor tyrosine kinases or the activation of the RAS/RAF/ERK signaling pathway through the blockade of negative feedback loops which eventually reduces the anti-cancer activity of PI3K inhibitors [8,9]. Therefore, it is important to identify the resistance mechanisms used by cancer cells to overcome PI3K inhibition in order to design new therapeutic strategies aiming to potentiate the efficacy of PI3K inhibitors.

AKT, a member of the AGC serine-threonine kinase family, is a major downstream effector involved in the oncogenic activity of PI3K [10]. Generation of phosphatidylinositol-3,4,5 triphosphate by PI3K leads to the recruitment of AKT to the plasma membrane where it gets activated through phosphorylation at T308 site by phosphoinositide-dependent kinase-1 [11]. In addition, full activation of AKT also requires phosphorylation of S473 by mTORC2 [12]. In turn, activated AKT influences the activity of downstream effectors such as forkhead transcription factor family members, Bad, the apoptosis signaling kinase-1 which overall results in cell proliferation and survival [10].

In this study, we show that the inhibition of AKT by PI3K inhibitors in cancer cells is transient. Indeed, chronic exposure of cancer cells to chemical inhibitors of PI3K, despite effectively blocking PI3K activity, did not persistently block AKT phosphorylation and activation. We further show that the use of PI3K inhibitors in combination with AKT inhibitors exhibit stronger anti-cancer efficacy *in vitro* and *in vivo* compared to single therapy. Therefore, our results provide a rationale to combine PI3K and AKT inhibitors in cancer therapy.

Abbreviations: PI3K, phosphatidylinositol-3-kinase; PTEN, phosphatase and tensin homolog.

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2. Methods

2.1. Cell, reagents, antibodies

All cell lines were obtained from the American Type Culture Collection and maintained in DMEM medium supplemented with 10% fetal calf serum at 37 °C and 5% CO_2. NVP-BEZ235 and perifosine were obtained from LC laboratories, wortmannin and AKTi-1/2 were from Calbiochem. Antibodies directed against phospho-AKT (Ser473), phospho-AKT (Thr308), AKT, phospho-S6 ribosomal protein (Ser235/236), S6 ribosomal protein, phospho-GSK-3β (Ser9), phospho-FOXO1/3a (Thr24/Thr32) and cleaved caspase-3 were from Cell Signaling. Antibody against phospho-PRAS40 was from Millipore. Antibody against CD31 was purchased from BD Biosciences.

2.2. Western blot analysis

Cells were grown in 6-well plates (3×10^5 cells/well) and treated as indicated. Cells were washed in ice-cold PBS and lysed in RIPA buffer supplemented with protease inhibitor cocktail and sodium orthovanadate (Santa Cruz Biotechnologies). Lysates were cleared by centrifugation and resultant supernatants were tested for protein concentrations using BCA assay (Pierce). Equal amounts of protein (20 μg) were separated on 4–12% polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences) and immunoblotted with primary antibodies followed by infrared secondary antibodies. Bands from immunoreactive proteins were visualized by an Odyssey infrared imaging system.

2.3. Cell count

LS174T, 786-0 or MDA-MB231 were plated on 6-well plates (Costar) at a density of 10^4 cells/well. Twelve hours later, cells were treated with PX-866 (500 nM), NVP-BEZ235 (1 μM), AKTi-1/2 (20 μM) or a combination of PX-866 and AKTi-1/2 or NVP-BEZ235 and AKTi-1/2 or DMSO as a control for 48 h. Alternatively, perifosine (2 μM) was used instead of AKTi-1/2. Adherent cells were subsequently collected and trypan-blue negative cells were counted using a Neubauer hemocytometer.

2.4. Apoptosis assay

The Cell Death Detection ELISA^{plus} kit (Roche) was used to measure apoptosis. Cancer cells were seeded in 96-well plates at a density of 10^3 cells per well. Twelve hours later, cells were treated with NVP-BEZ235 (1 μM) or PX-866 (500 nM) either alone or in combination with Akti-1/2 (20 μM) or perifosine (2 μM). After 72 h of treatment cells were harvested and apoptosis was determined following the manufacturer's instructions. Results are represented as the mean enrichment factor (absorbance of the treated cells/absorbance of the control cells).

2.5. PI3K activity assay

Cancer cells were treated with NVP-BEZ235 (1 μM) or PX-866 (500 nM) or DMSO as a control for 3 or 48 h, subsequently washed twice in ice cold PBS and lysed in lysis buffer containing 1% Nonidet P-40, 137 mM NaCL, 20 mM Tris–HCl, pH 7.4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM sodium orthovanadate and 1 mM PMSF. Lysates were cleared by centrifugation and resultant supernatants were tested for protein concentrations using BCA assay (Pierce). An equal amount of protein was incubated with an anti-PI3K antibody (Millipore) at 4 °C for 1 h under constant rotation and immunocomplexes were subsequently captured with protein A agarose beads at 4 °C for an additional hour. Beads were washed three times in lysis buffer and three times in washing buffer containing 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA and 0.1 mM sodium orthovanadate. The activity of immunoprecipitated PI3K was determined using a PI3K activity ELISA according to the manufacturer's instructions (Echelon). The bars represent relative PI3K activity compared to untreated cells ± SD.

2.6. Xenograft model

Animal experiments were in accordance with the Swiss federal animal regulations and approved by the local veterinary office. Female nude eight-weeks old mice were purchased from Charles River Laboratories. LS174T cells (3×10^6) were injected subcutaneously into the flank. Once the tumor xenografts reached 100 mm^3, mice were randomized into different groups ($n = 5$/group) and treated once daily with vehicle, NVP-BEZ235 (30 mg/kg/day), perifosine (30 mg/kg/day) or NVP-BEZ235 in combination with perifosine. NVP-BEZ235 was solubilized in one volume of N-methylpyrrolidone and further diluted in nine volumes of PEG 300. Perifosine was dissolved in 0.9% NaCl and was given by intraperitoneal injections. Tumor volumes were measured using caliper measurements every day and calculated with the formula $V = \pi/(6a2b)$ where a is the short axis and b the long axis of the tumor. Animals were sacrificed after 20 days of treatment and the tumors were excised and processed for Western blot and histological analysis.

2.7. Immunohistochemistry and immunofluorescence

Tumors were fixed in formalin and embedded in paraffin. PCNA (Abcam) and cleaved caspase-3 (Cell Signaling) stainings were performed according to the manufacturer's protocol. Color development was achieved using 3,3′ diaminobenzidine. For CD31 immunostainings, tumors were frozen in OCT compound on dry ice. Ten micrometer sections were cut on a cryostat and immunolabeling with an anti-CD31 antibody (BD biosciences) was performed as previously described [13].

2.8. Statistical analysis

Results are mean values ± standard error. Statistical analysis were performed using a Student t-test.

3. Results

3.1. PI3K inhibitors transiently inhibit AKT

To test the effects of PI3K inhibitors on AKT phosphorylation and AKT-induced signaling in cancer cells, LS174T cells were treated with PX-866 [14], BKM-120 [15], GDC-0941 [16], all selective inhibitors of PI3K or with NVP-BEZ235 [17], a dual PI3K/mTOR inhibitor. We used the following drug concentrations: 500 nM of PX-866, 5 μM of BKM-120, 1 μM of GDC-0941 or 1 μM of NVP-BEZ235 that were effective in blocking AKT T308 and AKT S473 phosphorylation (Suppl. Fig. 1). We found, by Western blot analysis, that all PI3K inhibitors tested blocked AKT T308 as well as AKT S473 phosphorylation within 3 h of treatment (Fig. 1). We further observed that the phosphorylation of AKT substrates such as GSK-3β, FOXO3a and PRAS40 was also reduced by PI3K inhibitors within 3 h of treatment (Fig. 1). However, these effects did not persist since the inhibition of AKT T308 and AKT S473 phosphorylation by selective PI3K inhibitors as well as AKT T308 by the dual

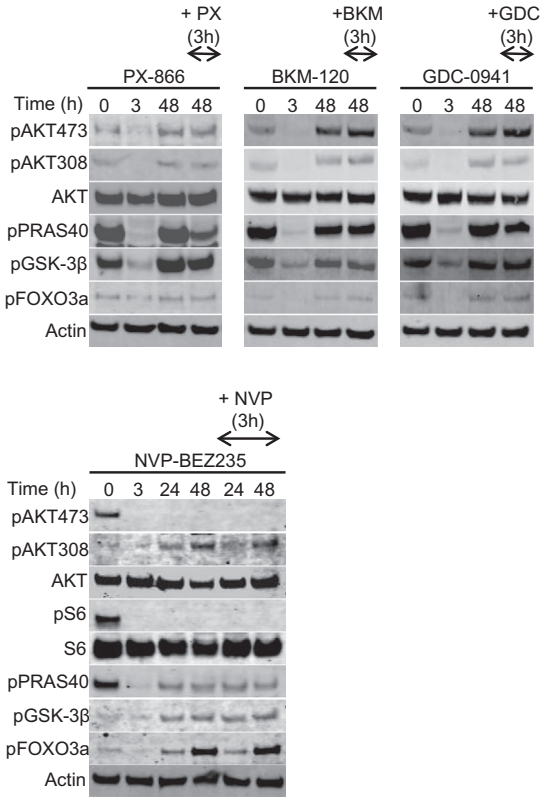

Fig. 1. PI3K inhibitors transiently block AKT and its effectors. LS174T cells were treated with 500 nM of PX-866, 5 μM of BKM-120, 1 μM of GDC-0941 or 1 μM of NVP-BEZ235 for the indicated times. In addition, cells were also retreated with 500 nM of PX-866 (PX 3 h), 5 μM of BKM-120 (BKM 3 h), 1 μM of GDC-0941(GDC 3 h) or 1 μM of NVP-BEZ235 (NVP 3 h) for 3 h, 48 h after the first treatments. Cells were harvested and lysates were immunoblotted with the indicated antibodies.

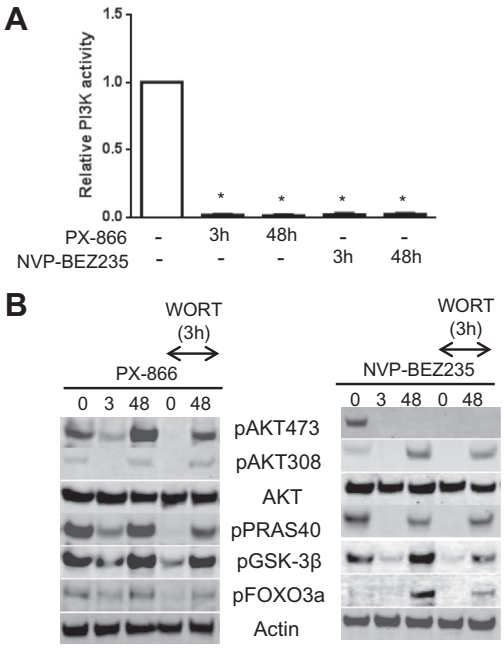

Fig. 2. AKT signals despite PI3K inhibition. (A) LS174T cells were treated with PX-866 (500 nM) or NVP-BEZ235 (1 μM) for 3 or 48 h. Cells were harvested and PI3K activity was monitored. Kinase activities are expressed as fold induction compared to untreated cells. $^{*}p < 0.05$ compared to untreated cells. (B) LS174T cells were treated with PX-866 (500 nM) or NVP-BEZ235 (1 μM) for the indicated times. After 48 h of treatment cells were treated with wortmannin (WORT 3 h) for 3 h. Cells were harvested and lysates were immunoblotted with the indicated antibodies.

PI3K/mTOR inhibitor was lost following prolonged treatment (48 h). In addition, the inhibition of GSK-3β, FOXO3a and PRAS40 phosphorylation by PI3K inhibitors was also markedly reduced after 48 h of treatment (Fig. 1). To rule out that the reactivation of AKT phosphorylation and AKT-mediated signaling was not due to a decrease of drug concentrations in the culture medium, fresh PI3K inhibitors were added to the culture for 3 h, 48 h after the initial exposure of cancer cells to PI3K inhibitors. Readdition of PI3K inhibitors did not reduce AKT T308 phosphorylation nor did they significantly affect the phosphorylation of GSK-3β, FOXO3a and PRAS40 (Fig. 1). These results were not restricted to LS174T cells since we made similar observations in 786-0 and MDA-MB-231 cancer cells (Suppl. Fig. 2). Of note however, we could not detect AKT T308 phosphorylation in MDA-MB-231 cells. Finally, to further test whether the reinduction of PRAS40, FOXO3a and GSK-3β phosphorylation resulted from the reactivation of AKT and not another kinase, cells were treated with PX-866 or NVP-BEZ235 for 48 h and subsequently treated for 3 h with a chemical inhibitor of AKT (AKTi-1/2, Ref. [18]). We found that the inhibition of AKT reduced the phosphorylation of PRAS40, FOXO3a and GSK-3β (Suppl. Fig. 3). Taken together, these data suggest that the inhibition of AKT by PI3K inhibitors is transient.

3.2. Reactivation of AKT occurs despite PI3K inhibition

We next investigated whether AKT reactivation could be due to a loss of PI3K inhibition by PI3K inhibitors. To test this, we monitored PI3K activity in LS174T cells treated with NVP-BEZ235 or PX-866 for 3 or 48 h. We found that NVP-BEZ235 or PX-866 significantly blocked PI3K activity after 3 or 48 h of treatment suggesting that AKT signaling occurred in LS174T cells despite PI3K inhibition (Fig. 2A). To further demonstrate that AKT reactivation is independent of PI3K, we treated LS174T cells with the PI3K inhibitor wortmannin [19], 48 h after the treatment with PX-866 or NVP-BEZ235. We found that whereas wortmannin blocked AKT phosphorylation and signaling in previously untreated cells, treatment of cancer cells with PX-866 or NVP-BEZ235 rendered AKT phosphorylation and signaling resistant to wortmannin (Fig. 2B).

3.3. In vitro anti-proliferative effects of PI3K inhibitors in combination with AKT inhibitors

Since exposure of cancer cells to PI3K inhibitors did not result in a persistent inhibition of AKT signaling, we next hypothesized that the inhibition of AKT would potentiate the anti-cancer efficacy of PI3K inhibitors. To test this, LS174T or 786-0 cells were treated with PX-866 or NVP-BEZ235 either alone or in combination with an AKT inhibitor. We used two different AKT inhibitors: AKTi-1/2 [18], an allosteric inhibitor of AKT isoforms 1 and 2, and perifosine [20], an alkylphospholipid that blocks the translocation of AKT to the plasma membrane. We observed that PX-866, NVP-BEZ235, AKTi-1/2 or perifosine significantly reduced the number of cancer cells after 48 h of treatment. Combining PX-866 with either AKTi-1/2 or perifosine further reduced the number of cancer cells compared to either treatment alone. Similar observations were made when NVP-BEZ235 was combined to AKTi-1/2 or perifosine (Fig. 3A). In addition, whereas individual treatments increased cancer cell apoptosis after 48 h, the combination of PI3K inhibitors with AKT inhibitors greatly increased the level of apoptosis (Fig. 3B).

3.4. Combining PI3K and AKT inhibitors exhibits stronger anti-cancer efficacy than either treatment alone

We next analyzed the relevance of our *in vitro* observations in tumor xenografts *in vivo*. To address this, nude mice bearing LS174T tumor xenografts were treated with NVP-BEZ235 once xenografts reached 100 mm^3. We first determined whether AKT signaling was inhibited following prolonged treatment with NVP-BEZ235. To test this, tumor xenografts were harvested 8 h after the administration of NVP-BEZ235 either after an initial administration or following 10 days of treatment. Western blot analysis of the tumor xenografts revealed that while a single administration of NVP-BEZ235 inhibited the phosphorylation of AKT and its downstream effectors FOXO3a, GSK-3β and PRAS40, it had no effect after 10 days of treatment. Only AKT S473 phosphorylation remained inhibited after 10 days suggesting that mTORC2 inhibition by NVP-BEZ235 persisted (Fig. 4A). Since NVP-BEZ235 did not inhibit AKT signaling, we next hypothesized that the anti-tumor activity of NVP-BEZ235 would be enhanced by the concomitant inhibition of AKT. To test this, nude mice bearing LS174T tumor xenografts were treated with NVP-BEZ235, perifosine, a combination of both or vehicles as a control. Tumor growth was monitored and compared between each treatment. We found that the growth of tumor xenografts was reduced in NVP-BEZ235 or perifosine treated mice compared to untreated mice. The anticancer efficacy of NVP-BEZ235 was greater than perifosine. In addition, the growth of tumor xenografts from mice treated with NVP-BEZ235 in combination with perifosine was also significantly reduced compared to either

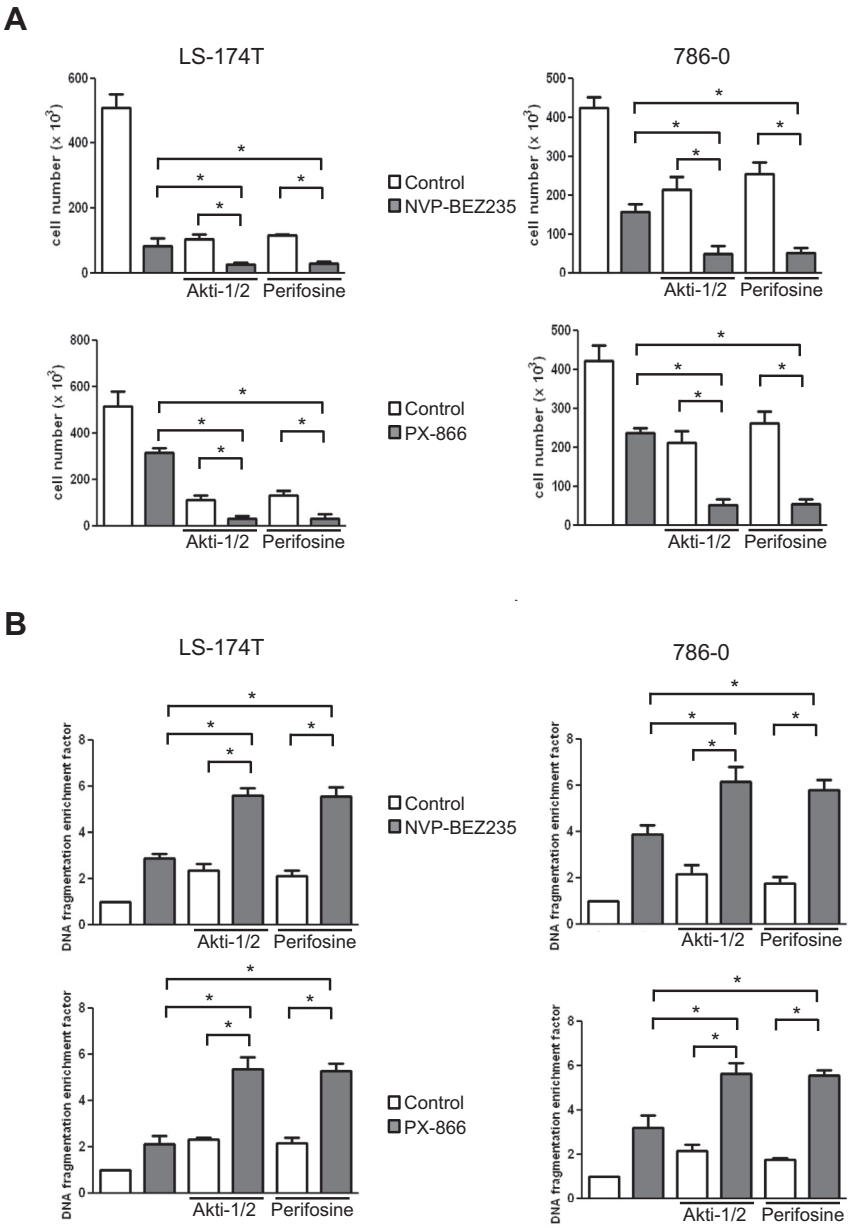

Fig. 3. Blocking AKT potentiates the anti-cancer efficacy of PI3K inhibitors *in vitro*. (A) 10^4 LS174T (left panels) or 786-0 (right panels) cells were plated on 6-well plates and treated with NVP-BEZ235 (1 μM, upper panels) or PX-866 (500 nM, lower panels) either alone or in combination with AKTi-1/2 (20 μM) or perifosine (2 μM). After 72 h of treatment cell were harvested and counted. Columns, mean cell count of three independent experiments; Bars, SD. (B) 10^3 LS174T or 786-0 were plated on 96-well plates and treated as in A. Cells were harvested and apoptosis was measured by quantifying DNA fragmentation. Columns, mean apoptosis enrichment factor; Bars, SD. $^*p < 0.05$ as specified by brackets.

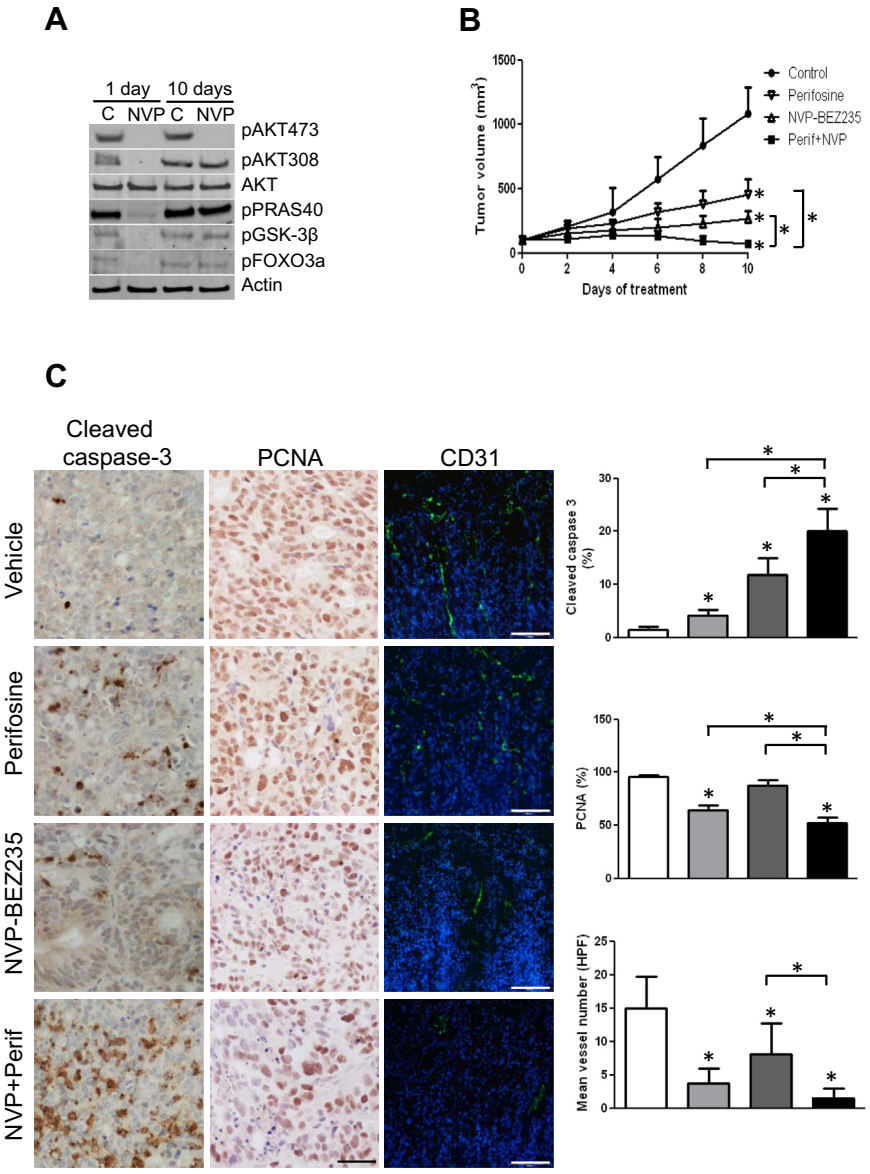

Fig. 4. Anti-tumor activity of NVP-BEZ235 in combination with perifosine. (A) Mice bearing LS174T tumors were treated once the tumors reached 100 mm^3 with vehicle or NVP-BEZ235 (30 mg/kg/day) for 1 or 10 days. Following sacrifice, tumors were harvested and tumor lysates were immunoblotted with the indicated antibodies. (B) Mice bearing LS174T tumors were randomized once the tumors reached 100 mm^3 to vehicle, NVP-BEZ235 (30 mg/kg/day), perifosine (30 mg/kg/day) or the combination for 10 days. Results are represented as mean tumor volume ± SD ($n = 5$ mice/group). $^*p < 0.05$ compared to control or as specified by brackets. (C) Representative photomicrographs showing immunohistochemical and immunofluorescence stainings of tumor xenografts harvested after 10 days of treatment using anti-cleaved caspase-3, anti-PCNA and anti-CD31 antibodies (magnification 400×). The graphs show the average percentage of cleaved caspase-3 or PCNA-positive cells as well as the average number of CD31-positive vessels from 20 high power fields per group. $^*p < 0.05$ compared to control or as specified by brackets.

treatment alone (Fig. 4B). Immunohistochemical analysis of the tumors revealed that NVP-BEZ235 produced a predominantly cytostatic effect with modest induction of apoptosis. In contrast, perifosine significantly increased apoptosis with minimal effects on tumor cell proliferation. Both, NVP-BEZ235 and perifosine decreased tumor angiogenesis. More importantly, combination therapy resulted in both decreased tumor cell proliferation and increased tumor cell apoptosis compared to monotherapy. Similar observations were made for tumor angiogenesis (Fig. 4C).

4. Discussion

PI3K controls tumor growth and therefore represents a promising target in cancer therapy. However, so far clinical trials have shown that PI3K have limited benefits in cancer patients. Several resistance mechanisms to PI3K inhibitors have been characterized including activation of alternative proliferative and survival pathways. In our study, we further show that PI3K inhibitors only transiently block AKT signaling which counteract their anti-cancer efficacy. Indeed, whereas acute treatment of cancer cells with PI3K inhibitors effectively blocks AKT phosphorylation and signaling, chronic exposure results in the reactivation of AKT signaling (Fig. 1). We obtained similar results with four different inhibitors of PI3K, therefore minimizing the possibility that our observations were due to off target effects of the drugs. In addition, it was previously reported that another PI3K inhibitor (LY294002) transiently reduced AKT phosphorylation in 786-0 and Caki-1 renal cancer cells with a progressive return to baseline levels after 48 h of treatment [21]. Similarly, treatment of cancer cells with the dual

PI3K/mTOR inhibitor NVP-BEZ235 only transiently blocks AKT phosphorylation [22,23]. Although these studies did not address the effects of PI3K inhibitors on the downstream effectors of AKT, they confirm that the inhibition of AKT phosphorylation by PI3K inhibitors is transient.

Emerging evidence also shows that several molecular mechanisms can induce AKT signaling independently of PI3K in cancer cells [24]. Indeed, whereas PI3K is well characterized as playing a major role in AKT activation, other kinases have been identified to activate AKT directly without requiring PI3K participation. For example, AckI directly regulates AKT recruitment to the plasma membrane by inducing AKT Tyr176 phosphorylation and the association of AKT with phosphatidic acid [25]. In addition Src [26], PTK6 [27], IKBKE [28], TBK1 [29] and DNA-PK [30] have all been shown to directly activate AKT independently of PI3K thus underlying the variety of kinases that cancer cells might use to retain AKT activity following PI3K inhibition.

In summary, although PI3K has been well characterized to play a major role in AKT activation, our study shows that treating cancer cells with PI3K inhibitors does not result in sustained inhibition of AKT phosphorylation and signaling. Our study further demonstrates that AKT reactivation following treatment of cancer cells with PI3K inhibitors limits their anticancer efficacy. Our findings therefore suggest to carefully monitor AKT activity following treatment of cancer cells with PI3K inhibitors and to combine AKT and PI3K inhibitors to achieve optimal anti-cancer activity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.014.

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