

Perifosine-mediated Akt inhibition in neuroendocrine tumor cells: role of specific Akt isoforms

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Abstract

The majority of neuroendocrine tumors (NETs) of the gastroenteropancreatic system show aberrant Akt activity. Several inhibitors of the phosphoinositide 3-kinase (PI(3)K)–Akt–mTOR signaling pathway are currently being evaluated in clinical phase II and III studies for the treatment of NETs with promising results. However, the molecular mechanisms and particularly the role of different Akt isoforms in NET signaling are not fully understood. In this study, we examine the effect of Akt inhibition on NET cells of heterogeneous origin. We show that the Akt inhibitor perifosine effectively inhibits Akt phosphorylation and cell viability in human pancreatic (BON1), bronchus (NCI-H727), and midgut (GOT1) NET cells. Perifosine treatment suppressed the phosphorylation of Akt downstream targets such as GSK3 α/β , MDM2, and p70S6K and induced apoptosis. To further investigate the role of individual Akt isoforms for NET cell function, we specifically blocked Akt1, Akt2, and Akt3 via siRNA transfection. In contrast to Akt2 knockdown, knockdown of Akt isoforms 1 and 3 decreased phosphorylation levels of GSK3 α/β , MDM2, and p70S6K and suppressed NET cell viability and colony-forming capacity. The inhibitory effect of simultaneous downregulation of Akt1 and Akt3 on tumor cell viability was significantly stronger than that caused by downregulation of all Akt isoforms, suggesting a particular role for Akt1 and Akt3 in NET signaling. Akt3 siRNA-induced apoptosis while all three isoform-specific siRNAs impaired BON1 cell invasion. Together, our data demonstrate potent antitumor effects of the pan-Akt inhibitor perifosine on NET cells *in vitro* and suggest that selective targeting of Akt1 and/or Akt3 might improve the therapeutic potential of Akt inhibition in NET disease.

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Introduction

Constitutive activation of the phosphoinositide 3-kinase (PI(3)K)–Akt–mTOR pathway is a common mechanism, by which tumor cells promote proliferation and cell survival. Estimates suggest that activating mutations in one or another PI(3)K–Akt–mTOR pathway component account for up to 30% of all human cancers and this has been associated with poor prognosis (Luo *et al.* 2003, Sawyers 2006). Lost expression of the PI(3)K inhibitor PTEN and aberrant activity of Akt and mTOR

have been observed in different neuroendocrine tumors (NETs) of heterogeneous origin (Wang *et al.* 2002, Shah *et al.* 2006, Kasajima *et al.* 2011). Akt is the primary effector of PI(3)K-generated phosphatidylinositol (3,4,5)-trisphosphate, thus being a principle mediator of growth factor-induced signal transduction. Through coordinated phosphorylation of downstream targets, Akt impinges on numerous cellular processes including proliferation, metabolism, survival, and migration.

Recent pharmaceutical efforts have resulted in the development of drugs targeting the PI(3)K–Akt–mTOR pathway. In two large placebo-controlled clinical phase III studies, the mTOR inhibitor everolimus has improved progression-free survival in patients with NETs of the pancreas as well as in patients with NETs of different origin suffering from carcinoid syndrome (Pavel *et al.* 2011, Yao *et al.* 2011). Perifosine is an oral alkylphospholipid, which interferes with the recruitment of Akt to the plasma membrane and inhibits the phosphorylation of Akt and multiple of its downstream targets including MDM2, GSK3 α/β , and p70S6K (Momota *et al.* 2005, Rahmani *et al.* 2005, Hideshima *et al.* 2006). Perifosine causes growth inhibition in various tumor cell lines and was also shown to sensitize tumor cells to conventional therapeutic antitumor agents and radiation (Momota *et al.* 2005, Rahmani *et al.* 2005, Vink *et al.* 2006). As mTOR inhibition may result in the activation of upstream PI(3)K/Akt neuroendocrine signaling (Zitzmann *et al.* 2010), direct Akt inhibition with perifosine could suppress this compensatory mechanism.

In mammals, Akt exists in three closely related isoforms with distinct expression patterns. While Akt1 is ubiquitously expressed, Akt2 expression varies between different organs, with high expression levels in insulin-responsive tissues such as muscle and liver. In contrast, Akt3 expression is more restricted to neuronal tissue (Stambolic & Woodgett 2006, Mendoza & Blenis 2007). Although redundant functions between the isoforms exist, recent investigations have demonstrated unique cell-type specific functions for each. Mice lacking Akt1 have small body size, mice lacking Akt2 show a diabetic phenotype, and mice lacking Akt3 have reduced brain size (Cho *et al.* 2001a,b, Easton *et al.* 2005, Tschopp *et al.* 2005). In melanoma cells, Akt3 was found to be the principal Akt isoform involved in tumor development, while in malignant glioma, Akt2 was shown to promote cell migration, invasion, and viability (Stahl *et al.* 2004, Zhang *et al.* 2009, Mure *et al.* 2010). In breast cancer, Akt1, though playing an important role in tumor induction, inhibits metastasis while Akt2 exhibits pro-migratory effects and facilitates proliferation and cell survival (Hutchinson *et al.* 2004, Dillon *et al.* 2009). Furthermore, in thyroid cancer and non-small cell lung cancer, tumor progression is predominantly regulated by Akt1 (Lee *et al.* 2011, Saji *et al.* 2011).

Here, we report inhibitory effects of the Akt inhibitor perifosine and isoform-specific siRNA against Akt1 and Akt3 on NET cell activity.

Materials and methods

Cell culture and stable transfection

Human pancreatic neuroendocrine BON1 tumor cells were kindly provided by R Göke (Marburg, Germany) and were cultured in DMEM/F12 (1:1) medium (Gibco/Invitrogen). Stable BON1 clones were established as described previously (Vlotides *et al.* 2004). Human insulinoma CM cells and human midgut carcinoid GOT1 cells were kindly provided by P Pozzilli (Rome, Italy) and Ola Nilsson (Göteborg, Sweden) respectively. Human bronchopulmonary neuroendocrine NCI-H727 tumor cells were purchased from ATCC (Manassas, VA, USA). CM, GOT1, and NCI-H727 cells were cultured in RPMI-1640 medium (PAA, Pasching, Austria). All media were supplemented with 10% FCS (Biochrom, Berlin, Germany), 1% penicillin/streptomycin (Gibco), and 0.4% amphotericin B (Biochrom). GOT1 culture medium was additionally supplemented with 0.135 IU/ml insulin (B Braun, Melsungen, Germany) and 5 μ g/ml apo-transferrin (Sigma–Aldrich). All cells were cultured at 37 °C in a 5% CO₂ atmosphere.

Reagents

Perifosine was kindly provided by Aeterna Zentaris (Frankfurt, Germany).

Transfection of siRNA

All siRNA oligonucleotides, including the nontargeting siRNA, were purchased from Ambion, Inc. (Austin, TX, USA). The siRNA sequences used in this study are as follows: 5'-UUAUGCCGAUCGC-GUCACAUU-3' (B-GAL); 5'-GGGCACUUUCGG-CAAGGUGtt-3' (Akt1); 5'-GGAUGAAGUCGCUC-ACACAtt-3' (Akt2); and 5'-GGACCGCACAC-GUUUCUAUtt-3' (Akt3). Cells were transfected in antibiotic- and FCS-free medium using DharmaFECT 2 (BON1 and CM) and Dharmafect 3 (NCI-H727) according to the manufacturer's instructions (Dharmacon, Lafayette, CO, USA). Twenty-four hours after transfection, FCS was added in a final concentration of 10%.

Assessment of cell viability

Metabolic activity was measured using Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Following 3 h of incubation with Cell Titer 96 solution, absorbance at 492 nm was determined using an ELISA plate reader.

Colony-forming assay

Anchorage-independent growth was examined in a methylcellulose-based clonogenic assay. Cells were trypsinized and resuspended in DMEM/Ham's-F12 medium containing 0.9% methylcellulose (Fluka, Deisenhofen, Germany) and 10% FCS. Aliquots of 1 ml containing 500 cells were plated into 35 mm bacteriological Petri dishes (Greiner, Nürtingen, Germany) in the presence of insulin-like growth factor 1 (100 ng/ml). Developing colonies containing more than 50 cells were counted under an inverted microscope after 14–16 days.

Invasion assay

Invasion of the tumor cells was assessed according to their migration through a 12 μ m pore size polycarbonate filter (Neuro Probe, Inc., Gaithersburg, MD, USA) coated with Matrigel (1 mg/ml; Collaborative

Biomedical Products, Bedford, MA, USA), which was placed between the two compartments of an invasion chamber (Neuro Probe, Inc.). The lower compartment was filled with DMEM/Ham's-F12 medium containing 10% FCS. Single-cell suspensions (1×10^5 cells/well in a volume of 100 μ l) of BON1 were added on top of the filter in the upper compartment. After 16 h of incubation, the filters were removed, and the cells were fixed and stained with toluidine blue. Cells on the upper side of the filter were removed with a cotton swab (except for control wells for the determination of total cell fraction). Filters were cut out, placed in 200 μ l of 1% SDS solution, and incubated for 60 min at 37 $^{\circ}$ C to fully solubilize the toluidine blue stain. The absorbance of the dye was determined at 620 nm. The fraction of invaded cells was calculated as follows: % invasion = number of invaded cells/total cell number \times 100.

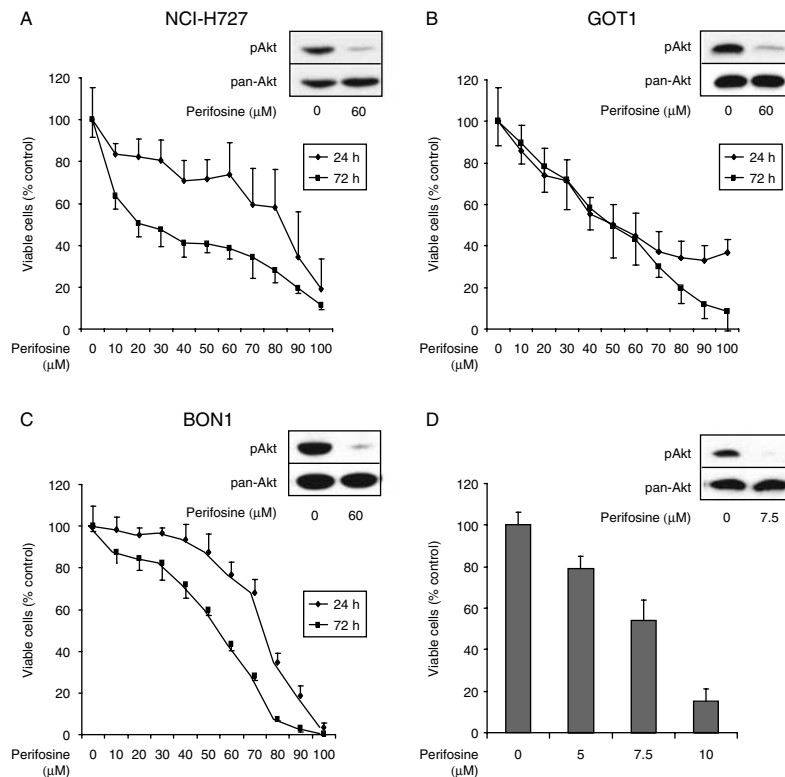


Figure 1 Perifosine-mediated inhibition of Akt phosphorylation and NET cell viability. NCI-H727 (A), GOT1 (B), and BON1 cells (C) were incubated with indicated concentrations of perifosine for 24 and 72 h in medium containing 10% FCS. Additionally, BON1 cells were incubated with indicated concentrations of perifosine for 72 h in medium containing 1% FCS (D). Cell viability was measured with Cell Titer 96 kit (Promega). Demonstrated are the mean values \pm s.d. of two independently performed experiments in sextuplicates ($n=12$). All values were significantly different from control ($P<0.05$) with the exception of values obtained by 24 h incubation of BON1 cells in medium containing 10% FCS and perifosine concentrations below 50 μ M. NCI-H727 (A), GOT1 (B), and BON1 cells (C and D) were incubated with indicated concentrations of perifosine for 2 h in medium containing 10% FCS (A, B and C) or 1% FCS (D). Protein expression levels of phosphorylated and total Akt were examined using western blot analysis. One representative blot out of three performed experiments is shown.

Protein extraction, immunoprecipitation, and western blotting

Protein extraction and western blotting were performed as described previously in detail (Zitzmann et al. 2006). Primary antibodies used were pAkt (Ser473; #4060), pan-Akt (#2920), Akt1 (#2967), Akt2 (#2964), pERK1/2 (#9101), ERK1/2 (#9102), pJNK (#9251), JNK (#9252), pp70S6K (#9234), p70S6K (#9202), Bcl-XL (BCL2L1) (#2762), pGSK3 α/β (#9331), GSK3 β (#9315), and pMDM2 (#3521; Cell Signaling, Danvers, MA, USA); BCL2 (610539; BD, Franklin Lakes, NJ, USA), Akt2 (sc-5270; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Akt3 (MAB1463; R&D Systems, Minneapolis, MN, USA), and β -actin (ab20272; Abcam).

Quantification of DNA fragmentation and cell cycle analysis

The rate of apoptotic cell death was quantified by determining DNA fragmentation according to Nicoletti et al. (1991). Briefly, cells were incubated for 24 h in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μ g/ml propidium iodide) and analyzed by flow cytometry on a FACSCalibur analyzer (BD) using CellQuest Software (BD). Nuclei to the left of the G₁-peak containing hypodiploid DNA were considered apoptotic.

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Perifosine decreases the viability of NET cells

To determine the antitumor potential of Akt inhibition in NET disease, human pancreatic neuroendocrine BON1, bronchus carcinoid NCI-H727, and midgut carcinoid GOT1 tumor cells were incubated with increasing concentrations of the pan-Akt inhibitor perifosine for 24 and 72 h respectively. As shown in Fig. 1A, B and C, perifosine dose dependently decreased the viability of all examined NET cells. Significant effects were observed in all NET cell lines starting at concentrations as low as 10 μ M (NCI-H727 and GOT1 cells after incubation for 24 h, all NET cells after incubation for 72 h) and peaked at the highest perifosine dose tested (100 μ M). Inhibition of neuroendocrine cell viability by perifosine was associated with decreased Akt phosphorylation as demonstrated

by western blot (Fig. 1A, B and C). The inhibitory effect of perifosine was potently enhanced under low serum conditions (1% FCS; Fig. 1D); therefore, serum-reduced medium (1% FCS) was used for further experiments.

Perifosine induces apoptosis

In order to examine the mechanism of inhibition of cell viability in NET cells by perifosine, BON1 cells were treated for 48 h with increasing concentrations of

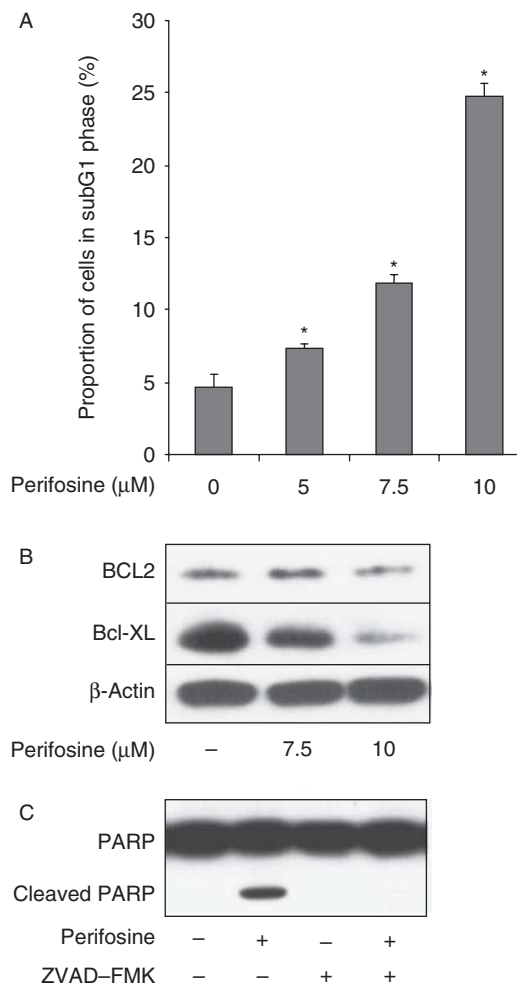


Figure 2 Perifosine-mediated induction of apoptosis. BON1 cells were treated with indicated concentrations of perifosine for 24 h. (A) The proportion of cells in subG1 was examined by flow cytometry. Shown are the mean values \pm s.d. of one representative experiment in triplicates ($n=3$) out of the three performed. (B) BON1 cells were treated with indicated concentrations of perifosine for 8 h. The expression of BCL2 and Bcl-XL was evaluated by western blot analysis. (C) BON1 cells were treated with perifosine (7.5 μ M) alone or in combination with ZVAD-FMK (40 μ M). Total compared to cleaved PARP protein expression levels were examined using western blot analysis. For B and C, one representative blot out of three performed experiments is shown, * $P < 0.05$ vs control.

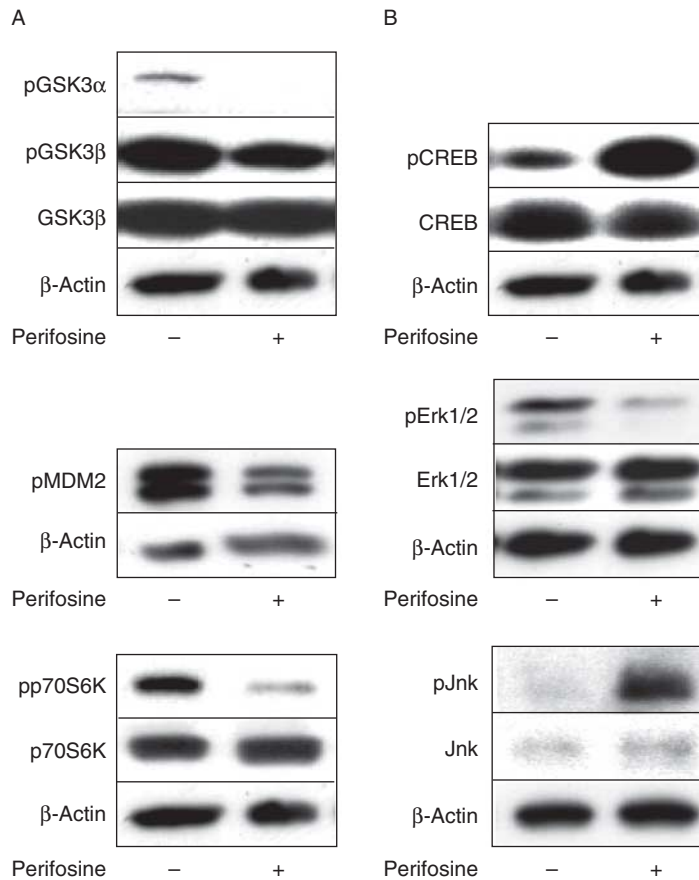


Figure 3 Perifosine-mediated inhibition of Akt downstream signaling. BON1 cells were treated with 7.5 μ M perifosine for 8 h. Protein expression levels of pGSK3 α/β , GSK3 α/β , pMDM2, pp70S6K, p70S6K, pCREB, CREB, pERK1/2, ERK1/2, pJNK, Jnk, and β -actin loading control were examined using western blot analysis. For each protein, one representative blot out of three performed experiments is shown.

perifosine and cell cycle analysis was performed. Perifosine treatment did not significantly alter the cell cycle phase distribution of BON1 cells (data not shown) but dose dependently increased the number of subG1 events (Fig. 2A). Significant effects were observed at a concentration as low as 5 μ M and peaked at the highest concentration tested. Consistent to the data obtained by FACS analysis, perifosine decreased the expression of the anti-apoptotic proteins BCL2 and Bcl-XL (Fig. 2B). Specific induction of apoptosis by perifosine was confirmed by cleavage of PARP, which was abrogated by cotreatment with the pan-caspase inhibitor ZVAD-FMK (40 μ M; Fig. 2C).

Perifosine-mediated inhibition of Akt downstream signaling

Akt affects survival signaling through coordinated phosphorylation of multiple proteins of the apoptotic and cell cycle regulatory machinery. We thus

examined the influence of perifosine on the phosphorylation status of several well-known Akt substrates. As measured by western blot, perifosine significantly decreased GSK3 α/β , p70S6K, and

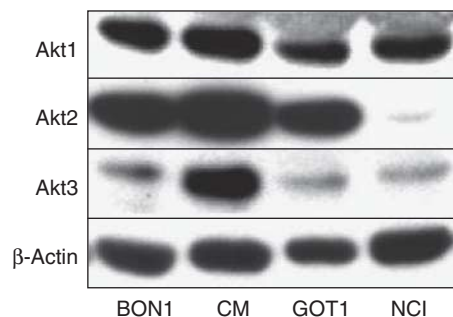


Figure 4 Expression patterns of Akt isoforms in neuroendocrine tumor cells. Basal expression levels of Akt1, Akt2, and Akt3 in human pancreatic BON1 and CM cells, midgut GOT1, and bronchus NCI-H727 cells were detected by western blot analysis.

MDM2 (Fig. 3A), while strongly inducing CREB phosphorylation (Fig. 3B). In order to identify whether CREB phosphorylation in response to perifosine is due to compensatory activation of MAPK survival signaling, we examined the phosphorylation status of ERK1/2 and JNK after 2 h of incubation with perifosine. While significantly decreasing the level of phosphorylated ERK1/2, perifosine treatment potently activated JNK signaling (Fig. 3B).

Expression patterns of Akt isoforms in NET cells

In order to elucidate the role of the different Akt isoforms in NET disease, we first examined the specificity of commercially obtained antibodies against Akt1 (Cell Signaling, Cat. #2967), Akt2 (Santa Cruz, Cat. #sc-5270 and Cell Signaling, Cat. #2964), and Akt3 (R&D, Cat. #MAB1463). As determined by two-dimensional gel electrophoresis, Akt1, Akt2, and Akt3 slightly differ in size (Supplementary Figure 1, see section on supplementary data given at the end of this article). Akt isoforms from whole cell lysates were separated by electrophoresis on a 8% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane, followed by western blot analysis with each Akt isoform-specific antibody. Anti-Akt1 and -Akt2 from Cell Signaling and anti-Akt3 from R&D showed no cross-reactivity (Supplementary Figure 2, see section on supplementary data given at the end of this article) between different Akt isoforms and were used for further experiments.

We next determined basal expression levels of Akt1, Akt2, and Akt3 in four NET cell lines of heterogeneous origin. The expression pattern of Akt1, Akt2, and Akt3 strongly varies between NET cells of bronchial (NCI-H727), midgut (GOT1), and pancreatic (CM and BON1) origin (Fig. 4). All the four NET cell lines expressed significant amounts of Akt1. NCI-H727 cells expressed lower levels of Akt2 compared with the other NET cells, while highest Akt3 expression levels were detected in pancreatic CM cells (Fig. 4).

Akt isoform-specific siRNA: effects on Akt downstream signaling

To assess the specific role of each Akt isoform for downstream signaling within the classical PI(3)K–Akt survival pathway, we used siRNAs to specifically knockdown Akt1, Akt2, and Akt3. Akt1 and Akt2 siRNA selectively and potently reduced the expression of the corresponding Akt isoform (Fig. 5A and B). As Akt3 is barely detectable in BON1 and NCI-H727 cells, the specificity of Akt3 siRNA was confirmed in

CM cells, which express relatively high basal levels of Akt3 (Fig. 5C).

We next examined the effect of Akt isoform-specific siRNA on the phosphorylation status of GSK3 α/β , p70S6K, and MDM2 in human pancreatic BON1 cells by western blot analysis. Akt1 and Akt3 but not Akt2 knockdown suppressed GSK3 α/β , p70S6K, and MDM2 phosphorylation (Fig. 6A). Furthermore, Akt1 and Akt3 but not Akt2 knockdown potently decreased

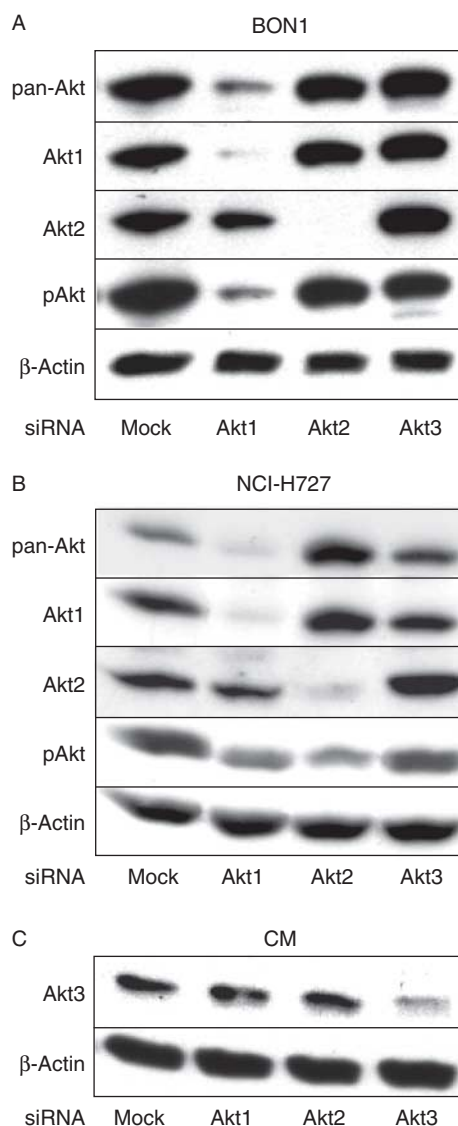


Figure 5 Akt isoform siRNA in neuroendocrine tumor cells. Human pancreatic BON1 (A), bronchus NCI-H727 (B), and pancreatic CM (C) cells were transfected with nontargeted *B-GAL* siRNA or siRNA against Akt1, Akt2, and Akt3. The effectiveness of the siRNAs was verified by western blot analysis of Akt1, Akt2, and Akt3 48 h after transfection. For each protein, one representative blot out of three performed experiments is shown.

the phosphorylation of ERK1/2 (Fig. 6B). To exclude the possibility that Akt1 and Akt3 compensate for each other to induce ERK1/2 signaling, BON1 cells were additionally double transfected with siRNA against Akt1 and Akt3. Significantly, the level of pERK1/2 was further decreased by simultaneous Akt1/Akt3 depletion (Fig. 6B).

Isoform-specific Akt siRNA: effects on cell viability, colony-forming capacity, and cell invasion in NET cells

To assess the effects of Akt1, Akt2, and Akt3 knockdown on NET cell viability, BON1 and NCI-H727 cells were transfected with nontargeted *B-GAL* siRNA or siRNA against Akt1, Akt2, or Akt3 and assayed for cell viability 72 and 144 h after transfection. In both the cell lines, downregulation of Akt1 and Akt3 significantly decreased cell viability (Fig. 7A and B). In contrast, transfection with Akt2-specific siRNA did not affect NCI-H727 cells but enhanced the viability of BON1 cells (Fig. 7A and B).

Similar to the observed effects on cell viability, the colony-forming capacity of Akt1 and Akt3 siRNA-transfected BON1 cells was substantially reduced while the transfection of Akt2 siRNA significantly increased the number of colonies 14 days after seeding (Fig. 7C).

Cell invasion of Akt siRNA-treated BON1 cells was assessed with the use of a specific cell invasion assay. siRNA transfection with any Akt isoform-specific siRNA decreased BON1 cell invasion by ~50% (Fig. 8A and B).

Akt2 siRNA attenuates the antitumor effect of pan-Akt inhibition

To compare the relative potency of Akt isoform-specific siRNA to pharmacological nonselective Akt inhibition, BON1 cells were transfected with nontargeted *B-GAL* siRNA, double transfected with siRNA against Akt1 and Akt3 or triple transfected with siRNA against Akt1, Akt2, and Akt3. The effectiveness of the double and triple transfection is shown in Fig. 9A. Consistent with the effects of Akt2 siRNA on cell viability in BON1 cells (Fig. 7A), double knockdown of Akt1 and Akt3 suppressed cell viability more effectively than triple knockdown of Akt1, Akt2, and Akt3 (Fig. 9B).

Specific Akt3 siRNA induces apoptosis

To elucidate the mechanisms underlying the loss of NET cell viability after Akt1 and Akt3 deprivation, FACS analysis of BON1 cells transfected with nontargeted *B-GAL* siRNA or siRNA against Akt1, Akt2, or Akt3 was performed. As assessed by flow

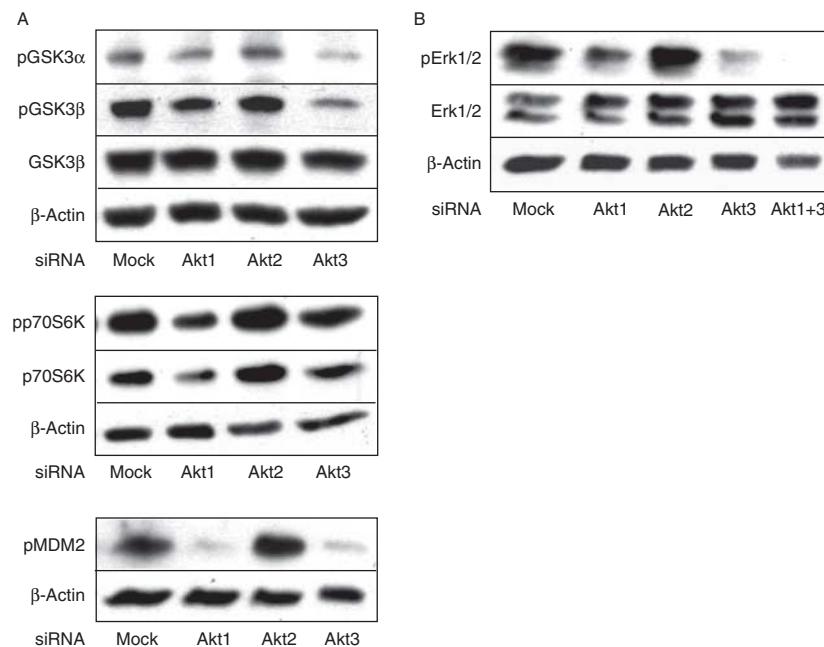
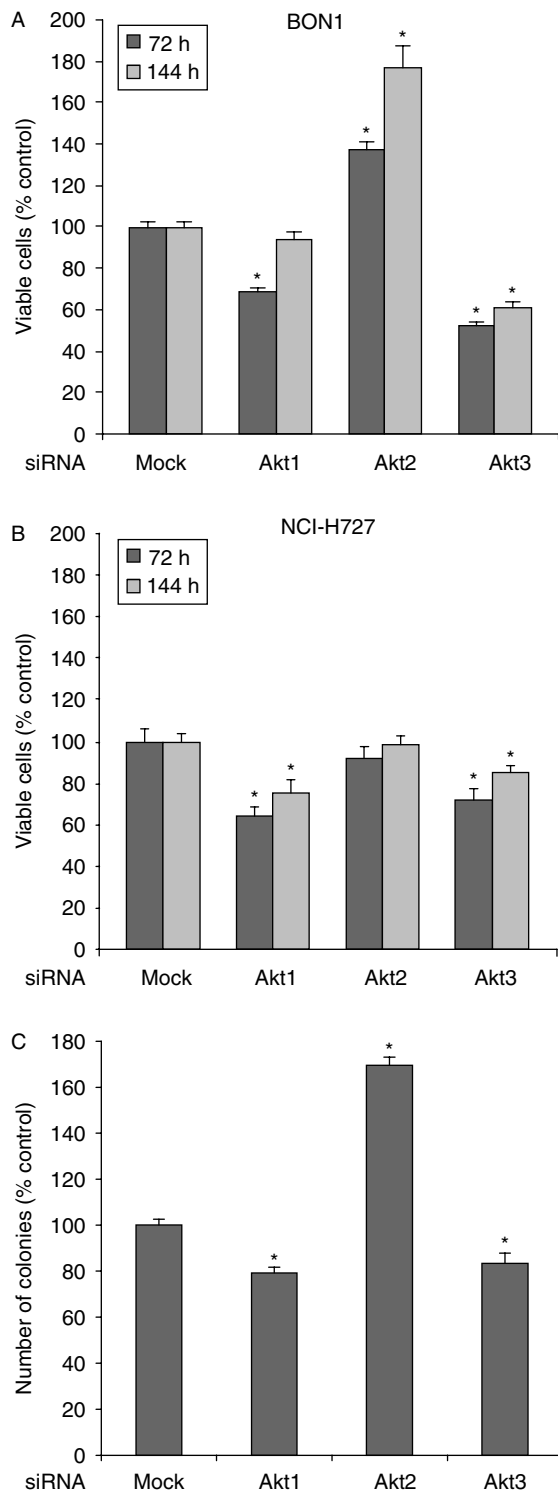


Figure 6 Effects of Akt siRNA on downstream signaling. Human pancreatic BON1 cells were mono-transfected with nontargeted *B-GAL* siRNA, siRNA against Akt1, Akt2, and Akt3 or double transfected with siRNA against Akt1 and Akt3. Forty-eight hours after transfection, the expression levels of pGSK3α/β, GSK3α/β, MDM2, pp70S6K, and p70S6K (A) as well as pERK1/2 and ERK1/2 (B) were evaluated by western blot analysis. For each protein, one representative blot out of three performed experiments is shown.

cytometry, downregulation of Akt3 significantly increased the fraction of cells with subG1 DNA content (Fig. 10A). Specific induction of apoptosis was confirmed by decreased expression of BCL2 and Bcl-XL in Akt3 siRNA-treated BON1 cells (Fig. 10B).



Discussion

The PI(3)K–Akt–mTOR signaling pathway is aberrantly active in most human cancers including NETs and offers an attractive therapeutic target. Two large placebo-controlled clinical phase III studies demonstrated efficacy of the mTOR inhibitor everolimus for the treatment of advanced pancreatic NETs and NETs of different origin suffering from carcinoid syndrome (Pavel *et al.* 2011, Yao *et al.* 2011). However, mTOR inhibition may lead to p70S6K-mediated feedback activation of PI3K/Akt signaling (O'Reilly *et al.* 2006). Direct Akt inhibition could override this compensatory mechanism, thus increasing the efficacy of pharmacological PI(3)K–Akt–mTOR pathway inhibition in NET disease.

In contrast to conventional Akt inhibitors, which target the ubiquitous ATP-binding pocket, perifosine binds the lipid-binding PH domain, thus interfering with the recruitment and activation of Akt more specifically. It is the first oral alkylphospholipid exerting promising growth inhibitory effects in a number of different tumor cell lines (Gills & Dennis 2009). However, its activity on NET cells has not been investigated yet. In this study, we demonstrate that perifosine potently inhibits Akt phosphorylation and cell viability in three human NET cell lines of pancreatic, small intestine, and bronchus origin. Perifosine-mediated suppression of cell viability was associated with the induction of apoptosis, as confirmed by an increased fraction of cells with subG1 content and PARP cleavage. Consistent with the previous studies on other tumor entities, perifosine significantly reduced the phosphorylation levels of the known Akt downstream targets GSK3 α/β , MDM2, and p70S6K. In addition, perifosine also inhibited RAF–MEK–ERK1/2 signaling, a critical pathway for NET cell viability (Zitzmann *et al.* 2010). Recently, it has been shown that like Akt, ERK1/2 may also be upregulated in response to mTOR inhibition, thus potentially limiting the antitumor efficacy of mTOR inhibitors (Carracedo *et al.* 2008, Zitzmann *et al.* 2010, Svejda *et al.* 2011). Compared with mTOR inhibitors,

Figure 7 Effects of specific Akt isoform siRNA on NET cell viability and colony-forming capacity. Cell viability in siRNA-treated human pancreatic BON1 (A) and bronchus NCI-H727 (B) NET cells was measured using Cell Titer 96 kit (Promega) 72 and 144 h after transfection. Demonstrated are the mean values \pm s.d. of three independently performed experiments in sextuplicate ($n=18$). The colony-forming capacity of BON1 cells was determined by enumeration of the formed colonies 14 days after plating (C). Demonstrated are the mean values \pm s.d. of one representative experiment in triplicates ($n=3$) out of three performed experiments. * $P<0.05$ vs mock-transfected control.

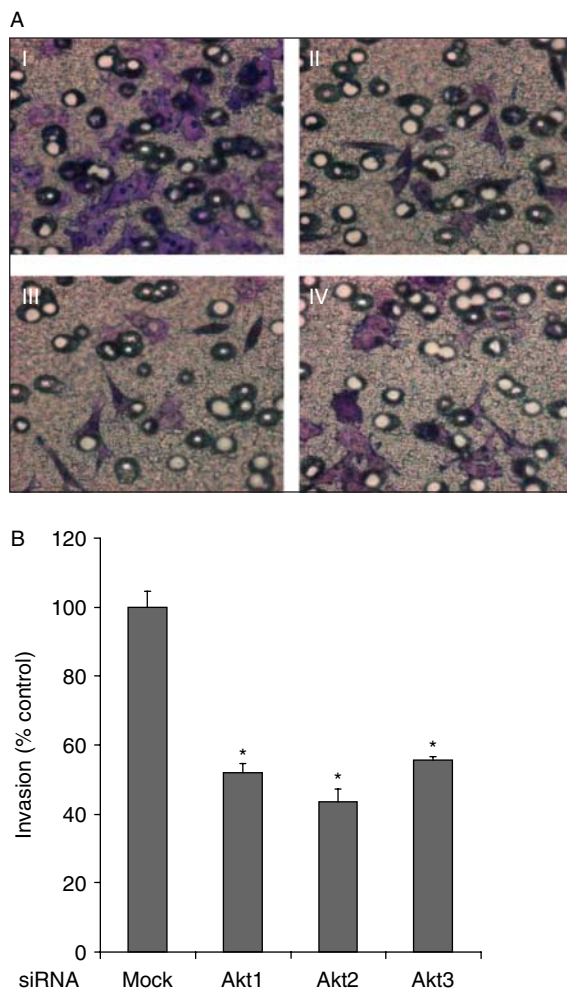


Figure 8 Effects of specific Akt isoform siRNA on neuroendocrine tumor cell invasion. BON1 cells were transfected with nontargeted *B-GAL* (I) siRNA or siRNA against Akt1 (II), Akt2 (III) or Akt3 (IV). Forty-eight hours after transfection, the cells were loaded into invasion chambers and after 16 h of incubation, light microscopic pictures of the invaded cells were taken (A) before their total numbers were evaluated as described in the Materials and methods section (B). Shown are the mean values \pm s.d. of three independently performed experiments in triplicates ($n=9$). * $P<0.05$ vs mock-transfected control.

direct Akt inhibition with perifosine appears to have a more broad inhibitory effect on the PI(3)K–Akt–mTOR pathway without compensatory activation of PI(3)K and ERK1/2 signaling. On the other hand, perifosine induced CREB and JNK phosphorylation, suggesting a specific and nontoxic inhibitory effect on Akt and ERK signaling.

Several clinical phase I/II studies have explored the effects of perifosine alone and in different combination therapy approaches (Ernst *et al.* 2005, Argiris *et al.* 2006, Gills & Dennis 2009, Ghobrial *et al.* 2010). Although high plasma concentrations up to 16.2 μ M

have been reached, tumor response rates to perifosine as a single agent have been modest (Gills & Dennis 2009). In contrast, preliminary reports of combination therapy with perifosine plus capecitabine in colon cancer and perifosine plus bortezomib and dexamethasone in multiple myeloma seem to be promising (Bendell *et al.* 2011, Richardson *et al.* 2011). However, to our knowledge, no clinical data on the effects of perifosine in NETs are yet available.

Akt exists in three different isoforms. All Akt isoforms have been shown to contribute to proliferation

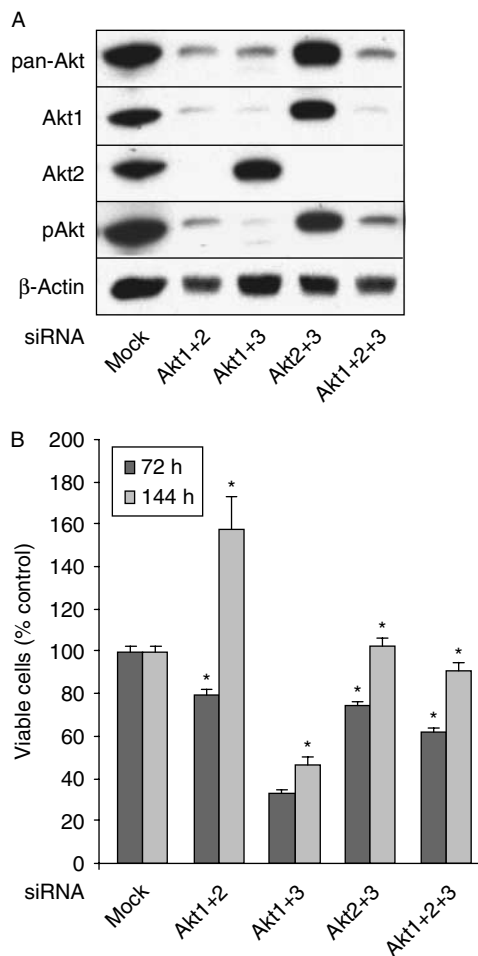


Figure 9 Akt2 siRNA attenuates the antitumor effect of pan-Akt inhibition in BON1 cells. (A and B) Human pancreatic BON1 cells were transfected with nontargeted *B-GAL* siRNA, double transfected with siRNA against Akt1 and Akt3, or triple-transfected with siRNA against Akt1, Akt2, and Akt3. The effectiveness of the double and triple transfection was verified by western blot analysis 48 h after transfection (A). One representative blot out of three performed experiments is shown. Cell viability was measured using Cell Titer 96 kit (Promega) 72 and 144 h after transfection (B). Demonstrated are the mean values \pm s.d. of three independently performed experiments in sextuplicate ($n=18$). * $P<0.05$ vs untreated control.

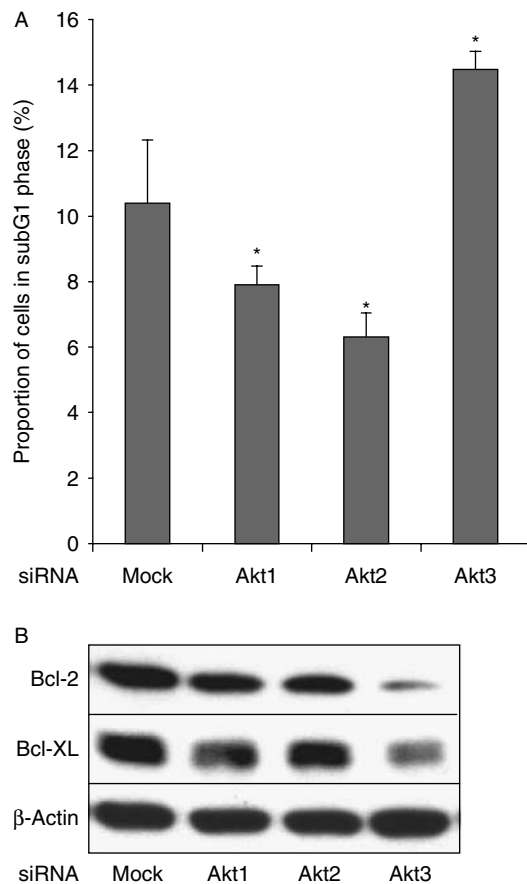


Figure 10 Specific Akt3 siRNA induces apoptosis in BON1 cells. BON1 cells were transfected with nontargeted *B-GAL* siRNA or siRNA against Akt1, Akt2, or Akt3. Forty-eight hours after transfection, the proportion of cells in subG1 phase was examined by flow cytometry (A). Shown are the mean values \pm s.d. of one representative experiment in triplicates ($n=3$) out of three experiments performed. * $P<0.05$ vs mock-transfected control. The expression of BCL2 and Bcl-XL was evaluated by western blot analysis (B). One representative blot out of three performed experiments is shown.

and cell survival, their relative importance seems to be cell-type specific (Koseoglu *et al.* 2007). In order to explore the mechanisms of Akt isoform-mediated regulation of NET cell activity, pancreatic BON1 and bronchus NCI-H727 cells were transfected with isoform-specific Akt siRNAs. Only downregulation of Akt1 and Akt3 decreased phosphorylation of the classical Akt downstream targets GSK3 α/β , MDM2, and p70S6K and suppressed cell viability and colony-forming capacity, suggesting a particular role for these Akt isoforms in NET activity. Like perifosine, downregulation of Akt1 and Akt3 significantly decreased ERK1/2 phosphorylation, indicating that these Akt isoforms positively control ERK1/2 activity. Furthermore, Akt3 ablation strongly induced apoptosis in

BON1 cells, indicating that this isoform is particularly relevant to NET cell survival.

To further evaluate the therapeutic potential of isoform-specific Akt inhibition in NET disease, double- and triple-transfection studies were performed. Significantly, the antitumor effect of simultaneous downregulation of Akt1 and Akt3 was much stronger than that caused by simultaneous downregulation of all Akt isoforms, demonstrating the potential advantage of selective Akt inhibition over pan-Akt inhibition in our NET cell model.

Together, our results demonstrate potent inhibitory effects of the pharmacological Akt inhibitor perifosine in NET cells. The lack of compensatory ERK or Akt activation as has been observed for mTOR inhibitors might offer therapeutic benefit. We could also demonstrate that particularly Akt1 and Akt3 seem to be important for NET cell viability while Akt2 may have antitumor activity. Thus, selective targeting of Akt1 and/or Akt3 might be even more effective in NET disease.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-12-0074>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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